Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Departamento de Química Orgánica e Inorgánica
Programa de doctorado: Síntesis y Reactividad Química

Development of enzyme-catalysed resolution, desymmetrisation and cascade processes. Use of lipases, alcohol dehydrogenases and transaminases in stereoselective synthesis

Doctoral Thesis

Ángela Mourelle Insua

2019

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Memoria presentada para optar al Grado de Doctor en Química con Mención Internacional

Dissertation to apply for the Degree of Doctor in Chemistry with International Doctor Mention

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Diseño de procesos de resolución, desimetrización y en cascada catalizados por enzimas. Empleo de lipasas, alcohol deshidrogenasas y transaminasas en síntesis estereoselectiva	Inglés: Development of enzyme-catalysed resolution, desymmetrisation and cascade processes. Use of lipases, alcohol dehydrogenases and transaminases in stereoselective synthesis

2.- Autor	
Nombre: Ángela Mourelle Insua	DNI/Pasaporte/NIE: -B
Programa de Doctorado: Síntesis y reactividad química	
Órgano responsable: Departamento de Química Orgánica e Inorgánica	

RESUMEN (en español)

Esta Tesis Doctoral se engloba dentro del campo de la Biocatálisis, disciplina que estudia el empleo de enzimas como catalizadores de reacciones químicas que generalmente se desarrollan con una gran eficiencia atómica, bajo condiciones de reacción suaves y con una alta (estereo)selectividad. Todo ello, permite el desarrollo de rutas sintéticas más económicas y que generan menos deshechos que los procesos químicos convencionales, lo que se ha traducido en que la Biocatálisis haya ido ganando importancia en la industria como una herramienta sostenible para la preparación de compuestos de interés en muy diversos sectores como el farmacéutico, agrícola o textil, entre otros.

Esta Tesis Doctoral consta de una introducción general y seis capítulos distribuidos en dos bloques en los que se emplean distintas clases de enzimas con la intención de desarrollar procesos altamente estereoselectivos aprovechando la quiralidad intrínseca que presentan los biocatalizadores.

En la Introducción, se adentra al lector en el campo de la Biocatálisis, definiendo las diferentes clases de enzimas existentes, y haciendo especial hincapié en los biocatalizadores empleados en el desarrollo de este trabajo como son las lipasas, las alcohol deshidrogenasas y las transaminasas, así como los diferentes medios de reacción en los que pueden llevarse a cabo las biotransformaciones.

En los Capítulos 1-3 (Bloque I), se ha abordado la síntesis de compuestos nitrogenados ópticamente activos. Para ello, se han utilizado dos tipos de enzimas: las lipasas, pertenecientes a la clase de las hidrolasas, y las transaminasas, que son un tipo de transferasas.

En el Capítulo 1, se ha empleado la lipasa de *Candida antarctica* de tipo B (CAL-B) para poner a punto un proceso de resolución cinética de aminas racémicas que son precursores de derivados de benzoxazina y que habían sido sintetizadas previamente. Así, se consiguió llevar a cabo la síntesis enantioselectiva de dichas aminas y de las correspondientes amidas. Estos resultados han sido publicados en *"Stereoselective Access to 1-[2-Bromo(het)aryloxy]propan-2-amines Using Transaminases and Lipases; Development of a Chemoenzymatic Strategy Toward a Levofloxacin Precursor"* (*J. Org. Chem.* **2016**, *81*, 9765-9774).

En el Capítulo 2, se ha hecho uso de diversas transaminasas para llevar a cabo la biotransaminación de γ- y δ-cetoésteres y sintetizar los correspondientes aminoésteres, inestables en el medio de reacción y que ciclan espontáneamente dando lugar a interesantes lactamas ópticamente activas. Los resultados obtenidos tras la puesta a punto de este proceso en cascada se recogieron en el artículo titulado *"Conversion of γ- and δ-Keto Esters into Optically Active Lactams. Transaminases in Cascade Processes"* (*Adv. Synth. Catal.* **2018**, *360*,

686-695).

En el Capítulo 3 se han utilizado transaminasas en la resolución cinética dinámica de una serie de α-alquil-β-cetoamidas, previamente sintetizadas mediante métodos químicos convencionales. Tras un intenso proceso de optimización, se han conseguido obtener las correspondientes β-aminoamidas sustituidas en posición α de manera diastereo- y enantioenriquecida. Un resumen de estos resultados ha sido recientemente enviado a la revista *Catalysis Science & Technology* para su posible publicación con el artículo titulado "*Efficient Synthesis of α-Alkyl-β-Amino Amides by Transaminase-Mediated Dynamic Kinetic Resolutions*" (ID del manuscrito: CY-ART-05-2019-001004).

En el Bloque II (Capítulos 4-6) se han empleado alcohol deshidrogenasas como catalizadores en procesos de resolución cinética dinámica y desimetrización. Además, se ha llevado a cabo un estudio de su actividad empleando como cosolvente una mezcla eutéctica compuesta por glucosa y cloruro de colina.

En el Capítulo 4, se han estudiado una serie de β-cetoamidas sustituidas en posición α como sustratos de diferentes alcohol deshidrogenasas sobreexpresadas en *Escherichia coli*. Así, se han sintetizado las correspondientes β-hidroxiamidas de manera enantio- y diastereoselectiva. Los resultados obtenidos mediante este procedimiento han sido recientemente aceptados para su publicación en *"Synthesis of α-Alkyl-β-Hydroxy Amides through Biocatalytic Dynamic Kinetic Resolution Employing Alcohol Dehydrogenases"* (*Adv. Synth. Catal.* **2019**, DOI: 10.1002/adsc.201900317).

En el Capítulo 5 se ha puesto a punto un método de desimetrización de 1,4-dicetonas utilizando la alcohol deshidrogenasa proveniente de *Ralstonia* species. Tras optimizar distintos parámetros de reacción como el cosolvente empleado, la temperatura y el tiempo de reacción, se han conseguido obtener los correspondientes (1*S*,4*S*)-dioles con buenos rendimientos y selectividades, publicando los resultados en *"Stereoselective Enzymatic Reduction of 1,4-Diaryl-1,4-Diones to the Corresponding Diols Employing Alcohol Dehydrogenases"* (*Catalysts* **2018**, *8*, 150).

Finalmente, en el Capítulo 6 se ha estudiado el comportamiento de diversas alcohol deshidrogenasas en disolventes compuestos por medios acuosos y una mezcla eutéctica que contiene glucosa y cloruro de colina. La novedad de este sistema radica en el empleo de esta mezcla eutéctica como cosolvente y como fuente de glucosa con el fin de regenerar el cofactor necesario para el funcionamiento de los enzimas redox. Los resultados obtenidos se han recogido en el artículo "*A Designer Natural Deep Eutectic Solvent to Recycle the Cofactor in Alcohol Dehydrogenase-Catalysed Processes*", recientemente aceptado en *Green Chem.* **2019**, DOI:10.1039/C9GC00318E.

RESUMEN (en Inglés)

This Doctoral Thesis is included in the field of Biocatalysis, a discipline that studies the use of enzymes as catalysts for chemical reactions that generally are developed with great atomic efficiency, under mild reaction conditions and with high (stereo)selectivity. This allows the development of feasible synthetic routes, more economical and that generate less waste than conventional chemical processes. Therefore, Biocatalysis has gained great importance in the industrial sector as a sustainable tool for the preparation of interesting chemical compounds with multiple applications in the pharmaceutical, agricultural or textile departments, among others.

This Doctoral Thesis has been divided in a general introduction and six chapters distributed in two blocks depending on the use of different enzyme classes, with the intention of developing highly stereoselective processes taking advantage of the intrinsic chirality displayed by biocatalysts.

In the Preface, the reader is introduced in the field of Biocatalysis, defining the different classes of existing enzymes, and with special emphasis on the biocatalysts used in the development of this work such lipases, alcohol dehydrogenases and transaminases, as well as different reaction media in which biotransformations can be carried out.

In Chapters 1-3 (Part I), the synthesis of optically active nitrogenated compounds has been addressed. For this, two enzyme classes have been used, which are lipases belonging to the hydrolases family, and transaminases that are a type of transferases.

In Chapter 1, *Candida antarctica* lipase type B (CAL-B) has been used for the kinetic resolution of racemic amines that are precursors of benzoxazine derivatives, which were previously chemically synthesised. Thus, it was possible to carry out the enantioselective synthesis of the mentioned amines and the corresponding amides. These results have been published in "*Stereoselective Access to 1-[2-Bromo(het)aryloxy]propan-2-amines Using Transaminases and Lipases; Development of a Chemoenzymatic Strategy Toward a Levofloxacin Precursor*" (*J. Org. Chem.* **2016**, *81*, 9765-9774).

In Chapter 2, various transaminases have been used to carry out the biotransamination of γ- and δ-keto esters to synthesise the corresponding amino esters, unstable in the reaction medium, which have spontaneously cyclised giving rise to interesting optically active lactams. The results obtained after the development of this cascade process were collected in the article entitled "*Conversion of γ- and δ-Keto Esters into Optically Active Lactams. Transaminases in Cascade Processes*" (*Adv. Synth. Catal.* **2018**, *360*, 686-695).

In Chapter 3, transaminases have been used in the dynamic kinetic resolution of a series of α-alkyl-β-keto amides, previously synthesised by conventional chemical methods. After an exhaustive optimisation process, the corresponding α-substituted β-amino amides were synthesised in a diastereo- and enantioenriched manner. A summary of these results has been recently sent to the journal *Catalysis Science & Technology* for possible publication as an article entitled "*Efficient Synthesis of α-Alkyl-β-Amino Amides by Transaminase-Mediated Dynamic Kinetic Resolutions*" (Manuscript ID: CY-ART-05-2019-001004).

In Part II (chapters 4-6), alcohol dehydrogenases have been used as catalysts in dynamic kinetic resolution and desymmetrisation processes. In addition, their activities have been studied using a eutectic mixture composed of glucose and choline chloride as cosolvent.

In Chapter 4, a series of β-substituted α-keto amides were tested as substrates of different alcohol dehydrogenases overexpressed in *Escherichia coli*. Thus, the corresponding β-hydroxy amides were synthesised in an enantio- and diastereoselective manner. The results obtained in this project have recently been accepted for publication in "*Synthesis of α-Alkyl-β-Hydroxy Amides through Biocatalytic Dynamic Kinetic Resolution Employing Alcohol Dehydrogenases*" (*Adv. Synth. Catal.* **2019**, DOI: 10.1002 / adsc.201900317).

In Chapter 5, the desymmetrisation of 1,4-diketones using alcohol dehydrogenase from *Ralstonia* species has been developed. After optimising reaction parameters such as the cosolvent type, temperature and reaction time, it was possible to obtain the corresponding (1*S*,4*S*)-diols with good yields and selectivities, publishing the results in "*Stereoselective Enzymatic Reduction of 1,4-Diaryl-1,4-Diones to the Corresponding Diols Employing Alcohol Dehydrogenases*" (*Catalysts* **2018**, *8*, 150).

Finally, Chapter 6 has been devoted to study the behaviour of various alcohol dehydrogenases in solvents composed by aqueous media and a eutectic mixture containing glucose and choline chloride. The novelty of this system lies in the use of this eutectic mixture as both cosolvent and as source of glucose to regenerate the cofactor necessary for the correct outcome of these transformations. A summary of these results has been published as a full paper entitled "*A Designer Natural Deep Eutectic Solvent to Recycle the Cofactor in Alcohol Dehydrogenase-Catalysed Processes*" recently accepted in *Green Chem.* **2019**, DOI:10.1039/C9GC00318E.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN SÍNTESIS Y REACTIVIDAD QUÍMICA

Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

FORMULARIO RESUMEN DE TESIS POR COMPENDIO

1.- Datos personales solicitante	
Apellidos: Mourelle Insua	Nombre: Ángela

Curso de inicio de los estudios de doctorado	2014/ 2015

	SI	**NO**
Acompaña acreditación por el Director de la Tesis de la aportación significativa del doctorando	X	
Acompaña memoria que incluye		
Introducción justificativa de la unidad temática y objetivos	X	
Copia completa de los trabajos *	X	
Resultados/discusión y conclusiones	X	
Informe con el factor de impacto de la publicaciones	X	
Se acompaña aceptación de todos y cada uno de los coautores a presentar el trabajo como tesis por compendio	X	
Se acompaña renuncia de todos y cada uno de los coautores a presentar el trabajo como parte de otra tesis de compendio		X

* Ha de constar el nombre y adscripción del autor y de todos los coautores asi como la referencia completa de la revista o editorial en la que los trabajos hayan sido publicados o aceptados en cuyo caso se aportará justificante de la aceptación por parte de la revista o editorial

FOR-MAT-VOA-033

Artículos, Capítulos, Trabajos

Trabajo, Artículo 1

Titulo (o título abreviado)	Stereoselective Access to 1-[2-Bromo(het)aryloxy]propan-2-amines Using Transaminases and Lipases; Devlopment of a Chemoenzymatic Strategy Toward a Levofloxacin Precursor
Fecha de publicación	23 de Septiembre de 2016
Fecha de aceptación	23 de Septiembre de 2016
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	*J. Org. Chem.* **2016**, *81*, 9765-9774
Factor de impacto	4.335 (2016)

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	María López-Iglesias
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Vicente Gotor
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Vicente Gotor-Fernández

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Trabajo, Artículo 2

Titulo (o título abreviado)	Conversion of γ- and δ-Keto Esters into Optically Active Lactams. Transaminases in Cascade Processes
Fecha de publicación	27 de diciembre de 2017
Fecha de aceptación	10 de diciembre de 2017
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	*Adv. Synth. Catal.* **2018**, *360*, 686-695
Factor de impacto	5.123 (2017)

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Luiz Arthur Zampieri
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Iván Lavandera
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Vicente Gotor-Fernández

Trabajo, Artículo 3

Titulo (o título abreviado)	Stereoselective Enzymatic Reduction of 1,4-Diaryl-1,4-Diones to the Corresponding Diols Employing Alcohol Dehydrogenases
Fecha de publicación	6 de abril de 2018
Fecha de aceptación	3 de abril de 2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	*Catalysts* **2018**, *8*, 150
Factor de impacto	3.465 (2017)

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Gonzalo de Gonzalo
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Iván Lavandera
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	Vicente Gotor-Fernández

Trabajo, Artículo 4

Titulo (o título abreviado)	Synthesis of α-Alkyl-β-Hydroxy Amides through Biocatalytic Dynamic Kinetic Resolution Employing Alcohol Dehydrogenases
Fecha de publicación	4 de abril de 2019
Fecha de aceptación	4 de abril de 2019
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	*Adv. Synth. Catal.* **2019**, DOI: 10.1002/adsc.201900317
Factor de impacto	5.123 (2017)

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Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Vicente Gotor-Fernández
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Trabajo, Artículo 5

Titulo (o título abreviado)	A designer natural deep eutectic solvent to recycle the cofactor in alcohol dehydrogenase-catalysed processes
Fecha de publicación	
Fecha de aceptación	13 de mayo de 2019
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	*Green Chem*. **2019**, DOI: 10.1039/C9GC00318E
Factor de impacto	8.586 (2017)

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Iván Lavandera
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Vicente Gotor-Fernández

Trabajo, Artículo 6

Titulo (o título abreviado)	"Efficient Synthesis of α-Alkyl-β-Amino Amides by Transaminase-Mediated Dynamic Kinetic Resolutions", enviado a la revista *Catalysis Science & Technology*
Fecha de publicación	
Fecha de aceptación	
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	
Factor de impacto	5.365 (2017)

Coautor2 x Doctor □ No doctor . Indique nombre y apellidos	Daniel Méndez-Sánchez
Coautor3 x Doctor □ No doctor . Indique nombre y apellidos	Iustina Slabu
Coautor4 x Doctor □ No doctor . Indique nombre y apellidos	James L. Galman
Coautor5 x Doctor □ No doctor . Indique nombre y apellidos	Nicholas J. Turner
Coautor6 x Doctor □ No doctor . Indique nombre y apellidos	Vicente Gotor-Fernández
Coautor7 x Doctor □ No doctor . Indique nombre y apellidos	Iván Lavandera

En caso de compendio de un número de artículos superior a seis, se incorporarán hojas suplementarias conforme a este modelo

Org. Chem. **2016**, *81*, 9765-9774). Esta revista contaba en el año 2016 con un **índice de impacto de 4.335**, ocupando la **posición 8 de 59** en el campo de "Química Orgánica" según el Journal Citation Reports. Por tanto, se encuentra en el **primer cuartil** según el Journal Citation Reports (JCR).

El Capítulo 2 versa sobre el uso de transaminasas para la biotransaminación de γ- y δ-cetoésteres con el fin de sintetizar lactamas ópticamente activas. Los resultados obtenidos tras la puesta a punto de este proceso en cascada se recogieron en el artículo titulado *"Conversion of γ- and δ-Keto Esters into Optically Active Lactams. Transaminases in Cascade Processes"* (*Adv. Synth. Catal.* **2018**, *360*, 686-695). Esta revista contaba en el año 2017 con un **índice de impacto de 5.123**, ocupando la **posición 3 de 71** dentro de la categoría "Química Aplicada", por tanto, dentro del **primer decil** según el JCR.

En el Capítulo 3 se han sintetizado una serie de α-alquil-β-cetoamidas para estudiar posteriormente su resolución cinética dinámica empleando transaminasas, con el fin de obtener β-aminoamidas α-sustituidas de manera diastereo- y enantioenriquecida. Con los resultados obtenidos se ha redactado el artículo titulado "*Transaminases as Efficient Catalysts in Dynamic Transformations: Synthesis of α-Alkyl-β-Amino Amides*" que ha sido recientemente enviado a la revista Catalysis Science & Tecnology con un **índice de impacto de 5.365** en 2017, ocupando la **posición 32 de 146** dentro de la categoría "Química Física", por tanto, dentro del **primer cuartil** según el JCR.

El Capítulo 4 describe la síntesis de una serie de β-cetoamidas α-sustituidas y su biorreducción empleando alcohol deshidrogenasas para obtener las correspondientes β-hidroxiamidas de manera enantio- y diasteroselectiva. Los resultados obtenidos han sido recientemente aceptados para su publicación en *"Synthesis of α-Alkyl-β-Hydroxy Amides through Biocatalytic Dynamic Kinetic Resolution Employing Alcohol Dehydrogenases"* (*Adv. Synth. Catal.* **2019**, DOI: 10.1002/adsc.201900317). Esta revista contaba en el año 2017 con un **índice de impacto de 5.123**, ocupando la **posición 3 de 71** dentro de la categoría "Química Aplicada", por tanto, dentro del **primer decil** según el JCR.

El Capítulo 5 ha servido para llevar a cabo la desimetrización de una familia de 1,4-dicetonas utilizando la alcohol deshidrogenasa proveniente de *Ralstonia* species, dando lugar a una serie de (1*S*,4*S*)-dioles con buenos rendimientos y selectividades. Los resultados más destacados se han publicado en *"Stereoselective Enzymatic Reduction of 1,4-diaryl-1,4-diones to the Corresponding Diols Employing Alcohol Dehydrogenases"* (*Catalysts* **2018**, *8*, 150). Esta revista contaba en el año 2017 con un **índice de impacto de 3.465**, ocupando la **posición 55 de 146** dentro de la categoría "Química Física", por tanto, dentro de la **primera mediana** según el JCR.

Finalmente, el Capítulo 6 describe la biorreducción de una serie de cetonas empleando alcohol deshidrogenas en disolventes compuestos por medios acuosos y una mezcla eutéctica que contiene glucosa y cloruro de colina, sirviendo a su vez este como sistema para reciclar el cofactor nicotinamídico. Los resultados obtenidos se han enviado a la revista *Green Chemistry* donde se ha obtenido la aceptación provisional del artículo "*A designer natural deep eutectic solvent to recycle the cofactor in alcohol dehydrogenase-catalysed processes*" (GC-COM-01-2019-000318.R1). Esta revista contaba en el año 2017 con un **índice de impacto de 8.586**, ocupando la **posición:**

- **21 de 170** dentro de la categoría "Química Multidisciplinar", por tanto, dentro del **primer cuartil** según el JCR.
- **2 de 33** dentro de la categoría "Ciencia y Tecnología Verde y Sostenible", por tanto, dentro del **primer decil** según el JCR.

En Oviedo a 22 de mayo de 2019

Directores de la Tesis Doctoral

Fdo.: Vicente Gotor Fernández

Fdo. Iván Lavandera García

Abbreviations and acronyms

[α]	Specific rotation
µL	Microliter(s)
Ac	Acetyl
AD-TA	Transaminase from *Alkaligenes denitrificans*
ADH	Alcohol dehydrogenase
ADH-A	Alcohol dehydrogenase from *Rhodococcus ruber*
ADH-T	Alcohol dehydrogenase from *Thermoanaerobacter* species
AH	*Alcaligenes* species
AlaDH	Alanine dehydrogenase
AmDH	Amine dehydrogenase
AO	Amine oxidase
ArR-TA	Transaminase from *Arthrobacter* species
ArS-TA	Transaminase from *Arthrobacter citreus*
Asp	Aspartate
ATA	Amine transaminase
BmTA	Transaminase from *Bacillus megaterium*
Bn	Benzyl
^{t}Bu	*tert*-Butyl
BY	Baker's yeast
c	Conversion
^{o}C	Celsius degrees
CAL-A	*Candida antarctica* lipase type A
CAL-B	*Candida antarctica* lipase type B
CAR	Carboxylic acid reductase
Cat.	Catalytic
CATH	Catalytic asymmetric transfer hydrogenation
CGRP	Calcitonin gene-related peptide
CHBE	Ethyl 4-chloro-3-hydroxybutyrate
ChCl	Choline chloride
COBE	Ethyl 4-chloro-3-oxobutyrate
CR	Carbonyl reductase
CRL	*Candida rugosa* lipase
Cv-TA	Transaminase from *Chromobacterium violaceum*

Abbreviations and acronyms

DCM	Dichloromethane
de	Diastereomeric excess
DEAD	Diethyl azadicarboxylate
DEPA	Diethyl phosphoramidate
DEPT	Distortionless enhancement by polarization transfer
DES	Deep eutectic solvent
DIBOA	2,4-Dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one
DIMBOA	2,4-Dihydroxy-7-methoxy-(2*H*)-1,4-benzoxazin-3(4*H*)-one
DKR	Dynamic kinetic resolution
DMSO	Dimethyl sulfoxide
DPP-4	Dipeptidyl peptidase IV
dr	Diastereomeric ratio
DYRKR	Dynamic reductive kinetic resolution
E	Enantioselectivity
EC	Enzyme Commission
ee	Enantiomeric excess
EG	Ethylene glycol
equiv	Equivalent(s)
Et	Ethyl
EtOAc	Ethyl acetate
EtOH	Ethanol
FDH	Formate dehydrogenase
GC	Gas chromatography
GDH	Glucose dehydrogenase
Glu	Glucose
Gly	Glycerol
h	Hour(s)
HBA	Hydrogen bond acceptor
HBD	Hydrogen bond donor
Hex	Hexane
His	Histidine
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry

IL	Ionic liquid
IPA	Isopropylamine
IR	Infrared spectroscopy
IRED	Imine reductase
KR	Kinetic resolution
KRED	Ketoreductase
LAPs	Liver acetone powders
LbADH	Alcohol dehydrogenase from *Lactobacillus brevis*
LDH	Lactate dehydrogenase
M	Molar
MAO	Monoamine oxidase
Me	Methyl
MeCN	Acetonitrile
MeOH	Methanol
mg	Miligram(s)
mL	Mililiter(s)
mM	Milimolar
mmol	Milimol(s)
MOPE	1-(4-Methoxyphenyl)ethan-1-ol
MTBE	*tert*-Butyl methyl ether
NADES	Natural deep eutectic solvent
NADH	Nicotinamide-adenine dinucleotide
NADPH	Nicotinamide-adenine dinucleotide phosphate
NBS	*N*-Bromosuccinimide
NMR	Nuclear magnetic resonance
Pc-*spuC*	Transaminase from *Pseudomonas chlororaphis* subsp. *aureofaciens* containing *spuC* gene
PDC	Pyruvate decarboxylase
PFEI	*Pseudomonas fluorescens* esterase I
Pf-*spuC*	Transaminase from *Pseudomonas fluorescens* containing *spuC* gene
PLE	Pig liver esterase
PLP	Pyridoxal 5'-phosphate
PMP	Pyridoxamine 5'-phosphate
PPL	Porcine pancreatic lipase

Abbreviations and acronyms

Pp-*spuC*	Transaminase from *Pseudomonas putida* containing *spuC* gene
n-Pr	*n*-Propyl
^{i}PrOH	Propan-2-ol
^{n}PrOH	*n*-Propanol
PSL	*Pseudomonas* (or *Burkholderia*) *cepacia* lipase
RasADH	Alcohol dehydrogenase from *Ralstonia* species
RedAm	Reductive aminase
RML	*Rhizomucor miehei* lipase
rpm	Revolutions per minute
r.t.	Room temperature
SCF	Supercritical fluid
sCO_2	Supercritical carbon dioxide
Ser	Serine
SyADH	Alcohol dehydrogenase from *Sphingobium yanoikuyae*
t	Time
T	Temperature
TA	Transaminase
TeSADH	Alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*
THF	Tetrahydrofuran
TI_1	Tetrahedral intermediate I
TI_2	Tetrahedral intermediate II
Tris·HCl	Tris(hydroxymethyl)aminomethane hydrochloride
U	Urea
Vf-TA	Transaminase from *Vibrio fluvialis*
v/v	Volume/volume ratio
w/w	Weight/weight ratio

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Preface

0.1. Sustainable chemistry and Biocatalysis

In the last decades, Chemistry in particular and Science in general have been redefined from the traditional concept of efficiency focus on chemical yield to new values related to Sustainable Chemistry or Green Chemistry.[1] This means that, nowadays, chemists are focused on developing processes that minimise the environmental hazards of chemical processes and their products. The 12 principles of Green Chemistry published by Anastas and Warner in 1998[2] and condensed by Tang and co-workers under the word "productively" (Table 0.1),[3] brought together all requirements that a sustainable process must include. Today, catalysis seems to be the best solution to the problem. In this way, instead of, *e.g.*, traditional reducing, oxidising agents and other reactants that must be employed in stoichiometric amounts, catalysts can be added in smaller amounts to the reaction media.

Table 0.1. Principles of Green Chemistry condensed under the word productively.

Principles of Green Chemistry	
P	*Prevent waste*
R	*Renewable materials*
O	*Omit derivatisation steps*
D	*Degradable chemical products*
U	*Use safe synthetic methods*
C	*Catalytic reagents*
T	*Temperature, pressure ambient*
I	*In-process monitoring*
V	*Very few auxiliary substances*
E	*E-factor, maximise feed in product*
L	*Low toxicity of chemical products*
Y	*Yes, it is safe*

When referring to catalysis, the use of enzymes is worth mentioning. The application of biocatalysts date back to the Ancient Egypt but it took many centuries for Biocatalysis to take part in chemical industrial processes. Probably, the turning point was defined by the research developed by Zaks and Klibanov

[1] (a) R. A. Sheldon, J. M. Woodley, *Chem. Rev.* **2018**, *118*, 801-838; (b) V. Gotor-Fernández, M. J. Hernáiz Gómez-Dégano, *An. Quim.* **2017**, *113*, 27-35.
[2] P. T. Anastas, J. C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, New York (USA), **1998**, p. 30.
[3] S. L. Y. Tang, R. L. Smith, M. Poliakoff, *Green Chem.* **2005**, *7*, 761-762.

showing that enzymes were capable of working in organic solvents.[4] From this point on, chemists together with engineers, biologists and biotechnologists have created a new whole horizon for enzymes to produce efficient and *greener* processes.

The bond that brings together the two parts of this doctoral thesis is Biocatalysis and its utilisation to create new straightforward and/or more efficient chemical methodologies. For this reason, the next pages have been conceived to immerse the reader in this field focusing on the use of different enzyme classes in organic synthesis by making use of a variety of reaction media moving from water as its natural environment to organic and neoteric solvents.

0.2. Biocatalysis in organic synthesis

0.2.1. Enzymes as catalysts

Enzymes can be defined as Nature's catalysts. That suggests they are the natural catalysts as they are biodegradable proteins formed by the combination of the 22 proteinogenic amino acids. They are essentially non-hazardous catalysts which usually lead to the formation of the desired product in high yields. The fact that they are very specific means that their catalysed processes usually do not need the involvement of protection and deprotection steps, therefore occurring without the generation of much waste. Additionally, biotransformations take place under mild reaction conditions (low temperature and pressure and neutral pH), thus lowering the formation of undesired products.

The active site of enzymes contains the amino acids that take part in the catalysis and that are responsible for one of the most remarkable characteristics of enzymes: their selectivity. Biocatalysts have the ability to distinguish between different functional groups with similar reactivity, the so-called chemoselectivity. They are regioselective catalysts, which means that they are able to differentiate two or more identical functional groups that differ in the region of the compound they are situated in. Finally, they present stereoselectivity or the capability of distinguishing between different chirality elements of the same molecule. In this

[4] (a) G. Kirchner, M. P. Scollar, A. M. Klibanov, *J. Am. Chem. Soc*. **1985**, *107*, 7072-7076; (b) A. Zaks, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 3192-3196.

way, a prochiral substrate can be transformed into an enantiopure product or kinetic resolutions can be performed starting from a racemic substrate.

The importance of enantioselectivity in a chemical process is well-known for chemists in academia and industry as each enantiomer of a racemic mixture can produce different biological effects in a living organism. There are several examples of chiral molecules employed as drugs in which one enantiomer possesses the pharmaceutical properties and the other one has harmful effects for patients.[5] The most common and tragic example is "Thalidomide".[6] This drug was sold as a racemate by Chemie Grünenthal, a German company, in the 1960s. In that moment, the teratogenic effect of the (*S*)-enantiomer was not known. That lack of awareness provoked that several infants were born with severe birth defects after their mothers used this drug for morning sickness.

For the moment, only the advantages of using enzymes as catalysts have been introduced but, of course, there are still some important drawbacks for a chemist to use biocatalysts.[7] First of all, enzymes exist in just one enantiomeric form so it is very difficult to invert the selectivity by choosing the "other enantiomer" of the catalyst. Besides, they usually work under specific mild reaction conditions (with the exception of some extremophile organisms) and it is difficult to modify those conditions maintaining the enzyme activity. In addition, they are susceptible to suffer from substrate or product inhibition and sometimes they need expensive cofactors to display their activity. However, different techniques as enzyme engineering, enzyme immobilisation or the development of efficient regeneration cofactor systems are playing an important role to prevent these limitations.

0.2.2. Enzyme classification

Nowadays, thousands of enzymes have been isolated and characterised, so along the years a need to classify them into classes was demanded. The Enzyme Commission (EC) has classified the enzymes into six classes according to the type

[5] E. Thall, *J. Chem. Educ.* **1996**, *73*, 481-484.
[6] J. E. Ridings, *Methods Mol. Biol.* **2013**, *947*, 575-586.
[7] K. Faber, *Biotransformations in Organic Chemistry*, 6th Ed., Springer, Berlin (Germany), **2011**.

of reaction they catalyse (Table 0.2). Each group is designated by a four-digit code which comes before the acronym of the Enzyme Commission.[8]

It is important to point out that, even though each enzyme in Nature catalyses a particular reaction, the concept of enzyme promiscuity has gathered strength in the last decades.[9] Some biocatalysts are able to display unexpected activities: (i) working under reaction conditions different from their natural ones (condition promiscuity); (ii) accepting unexpected substrates (substrate promiscuity); (iii) or catalysing different chemical transformations (catalytic promiscuity).[10] Over the last decades, many research groups have channelled their efforts into the discovery of new enzymatic activities.[11]

Table 0.2. Classification of enzymes depending on the main reaction they catalyse.

Oxidoreductases	**EC.1**	Oxidation, reduction and oxygenation processes
Transferases	**EC.2**	Transfer of acyl, phosphoryl, glycosyl and amino groups
Hydrolases	**EC.3**	Hydrolysis of esters, peptides and glycosides
Lyases	**EC.4**	Addition of molecules to C=C, C=N and C=O bonds
Isomerases	**EC.5**	Olefin isomerisation and racemisation processes
Ligases	**EC.6**	Formation of C–O, C–N, C–S and C–P bonds

In this doctoral thesis, enzymes from groups EC.1, EC.2 and EC.3 are employed. In particular, we will make use of alcohol dehydrogenases (ADHs), which are oxidoreductases (EC.1); transaminases (TAs), which are transferases (EC.2); and lipases, which are hydrolases (EC.3). Below, the most relevant characteristics of all these enzymes will be discussed.

[8] K. Drauz, H. Gröger, O. May, *Enzyme Catalysis in Organic Synthesis*, Wiley-VCH, Weinheim (Germany), **2012**.
[9] Recent reviews: (a) B. P. Dwivedee, S. Soni, M. Sharma, J. Bhaumik, J. K. Laha, U. C. Banerjee, *ChemistrySelect* **2018**, *3*, 2441-2466; (b) S. D. Copley, *Curr. Opin, Struct. Biol.* **2017**, *47*, 167-175; (c) R. D. Gupta, *Sustain. Chem. Process.* **2016**, *4*, 2; (d) M. López-Iglesias, V. Gotor-Fernández, *Chem. Rec.* **2015**, *15*, 743-759.
[10] K. Hult, P. Berglund, *Trends Biotechnol.* **2007**, *25*, 231-238.
[11] (a) J. Wang, X. Shen, J. Wang, Y. Yang, Q. Yuan, Y. Yan, *ACS Synth. Biol.* **2018**, *7*, 1238-1243; (b) M. A. Emmanuel, N. R. Greenberg, D. G. Oblinsky, T. K. Hyster, *Nature* **2016**, *540*, 414-417; (c) A. Cuetos, M. García-Ramos, E. Fischereder, A. Díaz-Rodríguez, G. Grogan, V. Gotor, W. Kroutil, I. Lavandera, *Angew. Chem. Int. Ed.* **2016**, *55*, 3144-3147.

0.2.2.1. Alcohol dehydrogenases

Alcohol dehydrogenases (ADHs), also known as carbonyl reductases (CRs) or ketoreductases (KREDs), are oxidoreductases that catalyse the reversible reduction of ketones and aldehydes to their corresponding alcohols.[12] For this purpose, they need a nicotinamide cofactor (Figure 0.1) as source of hydride, either nicotinamide-adenine dinucleotide (NADH) or nicotinamide-adenine dinucleotide phosphate (NADPH). These cofactors are relatively unstable compounds and expensive, which implies that their use in stoichiometric amounts is not viable. For this reason, a cofactor regeneration system is needed in order to eliminate this drawback and create efficient and economically viable enzymatic processes, especially when working at large scale.[13]

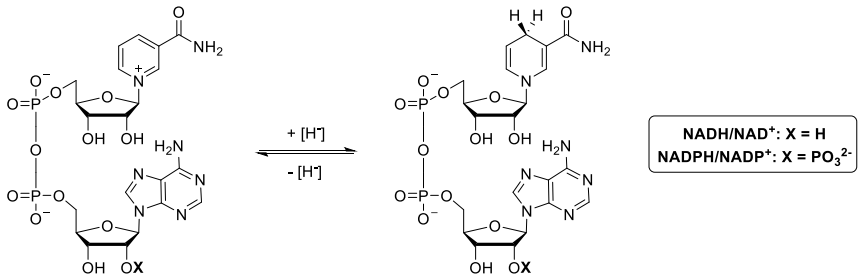


Figure 0.1. Phosphorylated ($NADP^+$) and non-phosphorylated (NAD^+) cofactors needed by ketoreductases and their reduced versions (NADPH and NADH).

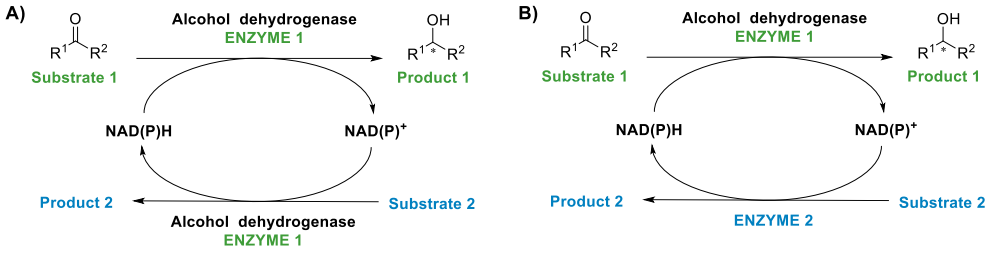
Several methodologies as photochemical and electrochemical techniques or using other catalysts (metals or enzymes) have been described to recycle the cofactor. However, the coupled-substrate and the coupled-enzyme systems are the most popular (Scheme 0.1). The first one is the simplest one (Scheme 0.1.A) as a single enzyme (the ADH) performs the substrate transformation but also the cofactor recycling. Isopropanol is typically employed as co-substrate as it is an inexpensive commercial alcohol which is transformed into acetone.

---

[12] (a) N. J. Turner, L. Humphreys, *Biocatalysis in Organic Synthesis: The Retrosynthesis Approach*, Royal Society of Chemistry, Cambridge (UK), **2018**; (b) W. Hummel, *Adv. Biochem. Eng. Biotechnol.* **1997**, *58*, 145-184; (c) W. Kroutil, H. Mang, K. Edegger, K. Faber, *Curr. Opin. Chem. Biol.* **2004**, *8*, 120-126.

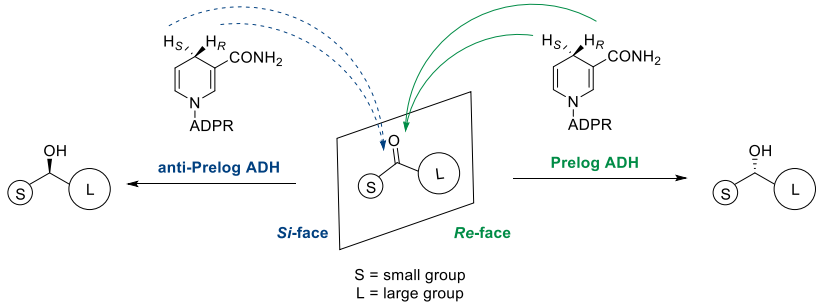
[13] (a) A. Weckbecker, H. Gröger, W. Hummel, *Adv. Biochem. Eng. Biotechnol.* **2010**, *120*, 195-242; (b) H. Wu, C. Tian, X. Song, C. Liu, D. Yang, Z. Jiang, *Green Chem.* **2013**, *15*, 1773-1789; (c) W. Hummel, H. Gröger, *J. Biotechnol.* **2014**, *191*, 22-31.

Scheme 0.1. Common regeneration systems to regenerate the cofactor needed in alcohol dehydrogenase-mediated transformations. A) Coupled-substrate system. B) Coupled-enzyme system.



In the coupled-enzyme approach (Scheme 0.1.B), a second enzyme and a second substrate are used to regenerate the cofactor. Well-known examples are those systems which employ a formate dehydrogenase (FDH) or a glucose dehydrogenase (GDH) as second enzyme. This approach is particularly advantageous as the second reaction is quasi-irreversible: formate is transformed into carbon dioxide by the FDH and glucose is converted into gluconic acid by the GDH.

Scheme 0.2. Prelog's rule to predict the stereopreference of an ADH in the transfer of a hydride from the cofactor to the carbonyl group.



One of the most remarkable characteristics of ADHs is their stereoselectivity (Scheme 0.2). They can specifically catalyse the attack of the hydride from NAD(P)H to one face of the prochiral ketone. They can also abstract the hydride at α-position of just one of the two enantiomers of a racemic alcohol. This selectivity is usually in accordance to "Prelog's rule", published by Prof. Prelog in 1964.[14] This way, a Prelog ADH (more commonly found) transfers the hydride from the

[14] V. Prelog, *Pure Appl. Chem.* **1964**, *9*, 119-130.

cofactor to the *Re* face of the carbonyl group and an anti-Prelog ADH transfers the hydride to the *Si* face.

ADHs are typically classified in three different groups: long chain ADHs (from 600 to 750 amino acids), medium chain ADHs (around 350 amino acids) and short chain ADHs (around 250 amino acids). Medium and short chain ADHs are the most employed in redox processes and their reaction mechanisms have been deeply studied.[15] The most important medium chain ADHs are Zn^{2+}-dependent enzymes which are formed by two different domains: a dinucleotide binding domain and a substrate binding domain where the active site is situated The cofactor is placed in the dinucleotide binding domain while there is a Zn^{2+} ion situated in the substrate binding domain. The fact that this Zn^{2+} is tightly attached to the active site amino acids has been proved by adding different chelators. In any case the deactivation of the enzyme was observed which actually highlight the strength of that binding.[16] Zinc is coordinated to two cysteine residues, one histidine residue and a molecule of water. This way, a tetracoordinated zinc intermediate is formed (Figure 0.2.A). There are two different hypotheses for the evolution of this species to the product of the enzymatic reaction: the first one involves the water molecule shift by means of the hydroxyl group of the alcohol (Figure 0.2.B); the second one suggests the formation of a pentacoordinated zinc species (Figure 0.2.C).

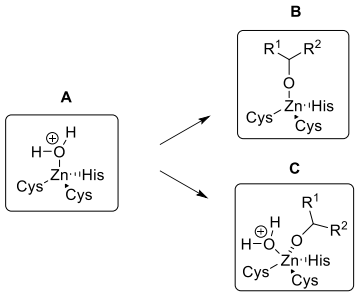
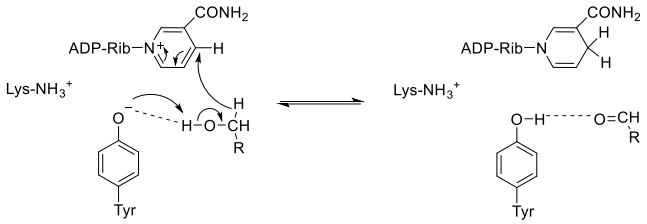


Figure 0.2. Zinc coordination proposed for different intermediates in the biotransformations catalysed by Zn-dependent ADHs.

[15] P. A. Frey, A. D. Hegeman, *Enzymatic Reaction Mechanisms*, Oxford University Press, New York (USA), **2007**.
[16] (a) B. Kosjek, W. Stampfer, M. Pogorevc, W. Goessler, K. Faber, W. Kroutil, *Biotechnol. Bioeng.* **2004**, *82*, 55-62; (b) O. Danielsson, S. Atrian, T. Luque, L. Hjelmqvist, R. González-Duarte, H. Jörnvall, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 4980-4984.

Short chain ADHs are the most common ones and their mechanism is related to the tyrosine residue situated in the active site of these enzymes. That residue is protonated when the alcohol is oxidised and it is deprotonated when a prochiral ketone is reduced into the corresponding alcohol (Scheme 0.3). Short chain ADHs are not Zn-dependent enzymes but they usually require Mg^{2+} ions, which are needed to stabilise the quaternary structure of the protein. The removal of these cations by the addition of chelators leads to the deactivation of the ADH.[17]

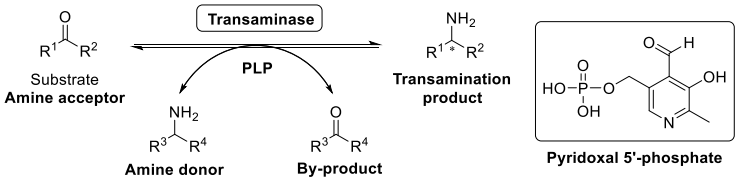
Scheme 0.3. Participation of a tyrosine in the mechanism of a biotransformation mediated by a short chain ADH.[17]



0.2.2.2. Transaminases

Transaminases (TAs) or aminotransferases are transferases that catalyse the reversible transfer of an amino group between an amine donor (typically an α-amino acid) and an amine acceptor (keto acids, ketones or aldehydes), using pyridoxal 5'-phosphate (PLP) as cofactor (Scheme 0.4).[18] First proof of an enzyme catalysing a transamination process was found by Needham in 1930.[19] Since them, a huge variety of transaminases have been discovered and studied.

Scheme 0.4. Reversible amine transfer from an amine donor to an amine acceptor catalysed by transaminases.



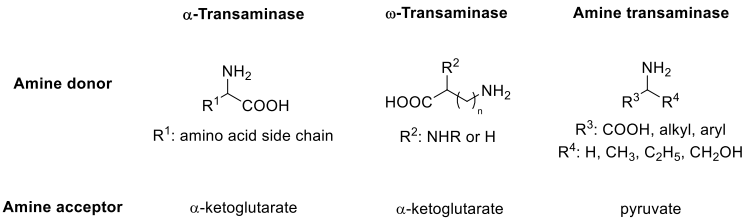
[17] N. H. Schlieben, K. Niefind, J. Müller, B. Riebel, W. Hummel, D. Schomburg, *J. Mol. Biol.* **2005**, *349*, 801-813.

[18] Recent reviews: (a) S. A. Kelly, S. Pohle, S. Wharry, S. Mix, C. C. R. Allen, T. S. Moody, B. F. Gilmore, *Chem. Rev.* **2018**, *118*, 349-367; (b) E. E. Ferrandi, D. Monti, *World J. Microbiol. Biotechnol.* **2018**, *34*, 13; (c) I. Slabu, J. L. Galman, R. C. Lloyd, N. J. Turner, *ACS Catal.* **2017**, *7*, 8263-8284; (d) F. Guo, P. Berglund, *Green Chem.* **2017**, *19*, 333-360.

[19] D. M. Needham, *Biochem. J.* **1930**, *24*, 208-227.

Based on the position of the transferred amino group, transaminases have been classified in three main groups (Scheme 0.5): α-transaminases (α-TAs), ω-transaminases (ω-TAs) and amine transaminases (ATAs). α-Transaminases are very specific; they only accept substrates containing groups which are located at α-position to the amino group. ω-TAs transfer terminal amino groups which are located further away from the carboxylic moiety. Finally, amine transaminases do not require the presence of a carboxylic group. That implies they can accept aldehydes and ketones as substrates. For that reason, ATAs are the most important transaminases applied to organic synthesis as they can give access to chiral amines from prochiral ketones.

Scheme 0.5. Classification of transaminases according to the position of the transferred amino group.

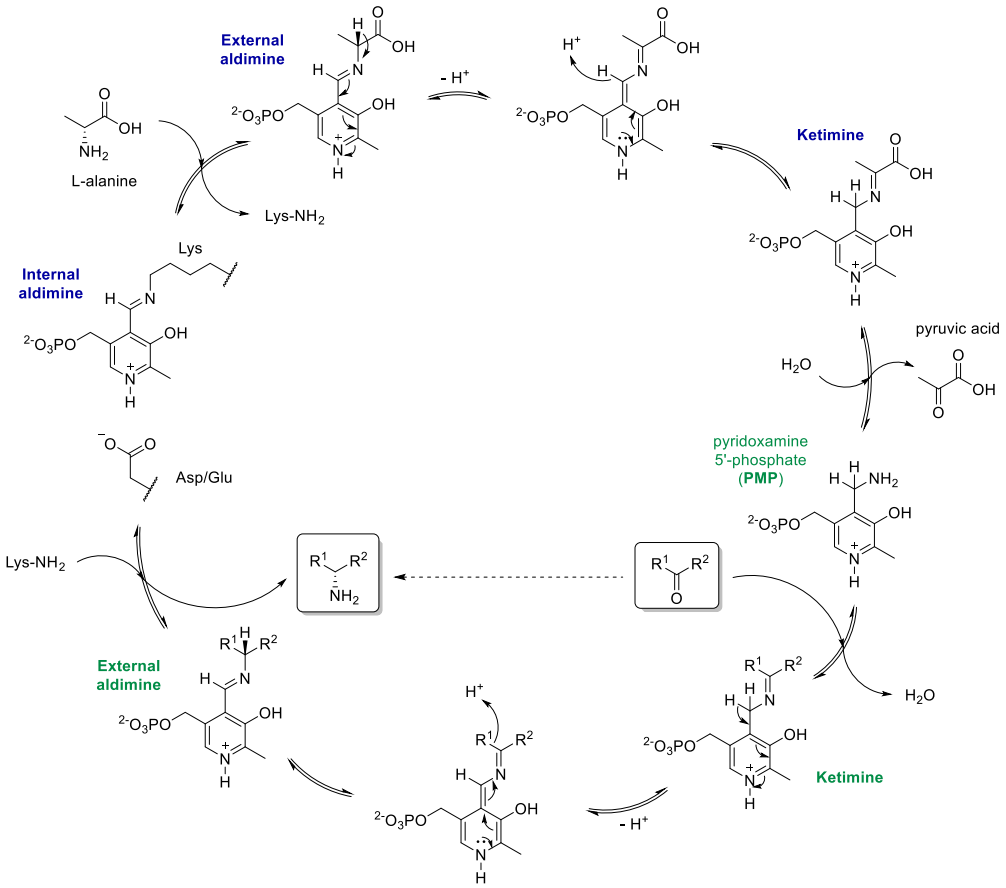


As said before, TAs require the use of PLP as cofactor. This molecule is the same one for all transaminases described before and it is essential for the transfer of the amino group between the amine donor and the amine acceptor. The aspartate aminotransferase reaction mechanism has been deeply studied (Scheme 0.6)[20] and it is considered to be similar for all transaminases. It is completely reversible and it could be divided in two different parts. In the first one (blue in Scheme 0.6), the PLP is bound to a lysine which is situated in the active site of the TA. They form an internal aldimine which is activated by an aspartate or a glutamate group and it is capable of reacting with the amine donor (L-alanine) to form an external aldimine. The double bond of the aldimine isomerises to produce a ketimine mediated by the catalytic lysine, which undergoes hydrolysis to obtain the pyridoxamine 5'-phosphate (PMP) and the coproduct of the transamination (pyruvic acid). In the second step (green in Scheme 0.6), the PMP reacts with an amine acceptor, a molecule containing a carbonyl group (for example, a ketone). Again, a ketimine, which is lately transformed into an external aldimine, is formed.

[20] (a) H. Mizuguchi, H. Hayashi, K. Okada, I. Miyahara, K. Hirotsu, H. Kagamiyama, *Biochemistry* **2001**, *40*, 353-360; (b) J. M. Goldberg, J. F. Kirsh, *Biochemistry* **1996**, *35*, 5280-5291.

Finally, the interaction with the lysine leads to the production of the biotransamination product (a chiral amine) and the regeneration of the cofactor that can participate in a new catalytic cycle.

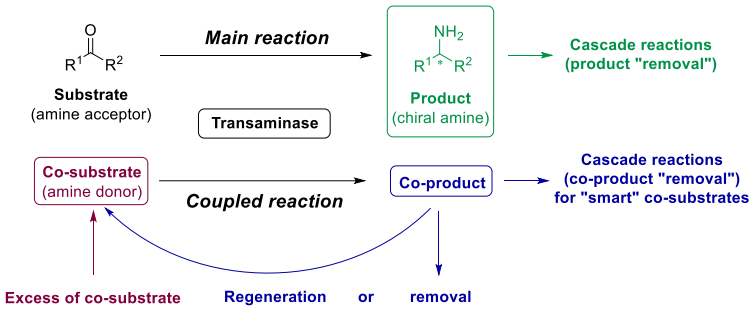
Scheme 0.6. Catalytic cycle for a transaminase-mediated reaction.



Chiral amines can be obtained using transaminases through a kinetic resolution (KR) starting from the racemic amine, or alternatively *via* asymmetric amination of a prochiral ketone. In KR, one of the amine enantiomers reacts to form the corresponding ketone, so the unreacting amine enantiomer can be ideally obtained in enantiopure form when 50% conversion is reached. In terms of applicability, it is more interesting to synthesise a chiral amine from a prochiral ketone as it is possible to get 100% conversion. Actually, unless the ketone is not easily available, the asymmetric biotransamination of prochiral ketones is the

method of choice. Nevertheless, there is an important drawback associated to this methodology, which is the unfavourable thermodynamic equilibrium. This problem is especially remarkable when using ATAs and it forces the utilisation of a system to shift the equilibrium to the formation of the chiral amine. To solve this issue, different approaches have been used (Scheme 0.7).[18,21]

Scheme 0.7. Shifting the equilibrium towards the formation of chiral amines in TA-catalysed processes.



Amine transaminases natural amine donor is usually L-alanine, which is transformed into pyruvic acid. Consequently, one of the most typically used methodologies to shift the equilibrium consists in the removal of this coproduct or its regeneration back to alanine (Scheme 0.8). In this manner, the lactate dehydrogenase (LDH) system is one of the most popular methods (Scheme 0.8.A). By using this methodology, the pyruvic acid is transformed into lactate by LDH. For this purpose, it is necessary the addition of nicotinamide cofactor $NAD(P)^+$/NAD(P)H and a third enzyme (typically glucose dehydrogenase or formate dehydrogenase) to regenerate it. Since this method was first reported by Shin and Kim,[22] several authors have reported the synthesis of interesting amines by using this methodology.[23]

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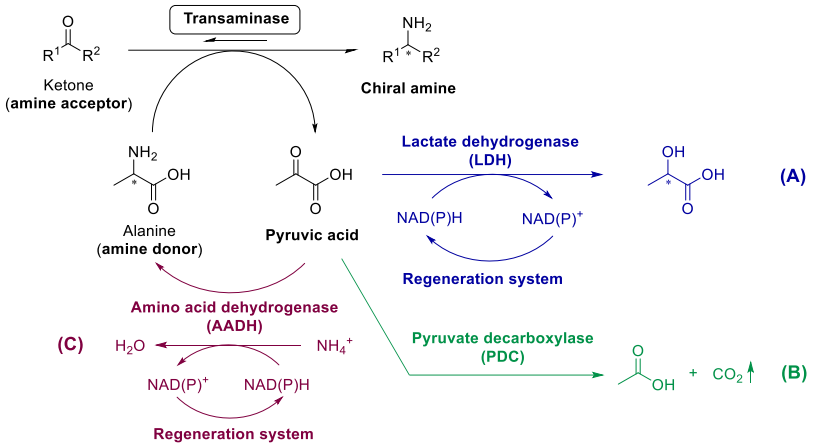
[21] P. Tufvesson, J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto, J. M. Woodley, *Biotechnol. Bioeng.* **2011**, *108*, 1479-1493.
[22] J. S. Shin, B. G. Kim, *Biotechnol. Bioeng.* **1999**, *62*, 206-211.
[23] (a) N. Ritcher, J. E. Farnberger, D. Pressnitz, H. Lechner, F. Zepeck, W. Kroutil, *Green Chem.* **2015**, *17*, 2952-2958; (b) D. Pressnitz, C. S. Fuchs, J. H. Sattler, T. Knaus, P. Macheroux, F. G. Mutti, W. Kroutil, *ACS Catal.* **2013**, *3*, 555-559; (c) M. Fuchs, D. Koszelewski, K. Tauber, J. Sattler, W. Banko, A. K. Holzer, M. Pickl, W. Kroutil, K. Faber, *Tetrahedron* **2012**, *68*, 7691-7694; (d) F. G. Mutti, C. S. Fuchs, D. Pressnitz, N. G. Turrini, J. H. Sattler, A. Lerchner, A. Skerra, W. Kroutil, *Eur. J. Org. Chem.* **2012**, 1003-1007; (e) S. Schätzle, F. Steffen-Munsberg, A. Thontowi, M. Höhne, K. Robins, U. T. Bornscheuer, *Adv. Synth. Catal.* **2011**, *353*, 2439-2445; (f) F. G. Mutti, C. S. Fuchs, D. Pressnitz, J. H. Sattler, W. Kroutil, *Adv. Synth. Catal.* **2011**, *353*, 3227-3233; (g) D. Koszelewski, M.

Scheme 0.8. Strategies for pyruvic acid removal or alanine regeneration.



A second approach to eliminate the pyruvic acid is the pyruvate decarboxylase (PDC) system (Scheme 0.8.B).[24] By adding the PDC, the pyruvate is degraded into acetaldehyde and carbon dioxide. Apparently, this system is a more straightforward approach than the LDH one as the formation of CO_2 makes the reaction *quasi*-irreversible and it is not necessary the addition of any nicotinamide cofactor. However, some transaminases can react with the acetaldehyde leading to the formation of ethylamine. Consequently, larger amounts of the amine donor are required.

As said before, it is also possible to regenerate the amine donor (alanine). In this way, amino acid dehydrogenases can be used (Scheme 0.8.C). The most commonly used one is the alanine dehydrogenase which is capable of transforming the pyruvic acid back to alanine by adding a source of ammonium, $NAD(P)^+$/NAD(P)H and a regeneration system of the reduced nicotinamide cofactor. As alanine is being regenerated, it is possible to add it in catalytic amounts reducing the inhibition problems related to high concentrations of the amine donor.[23g,23h,25]

Göritzer, D. Clay, B. Seisser, W. Kroutil, *ChemCatChem.* **2010**, *2*, 73-77; (h) M. D. Truppo, J. D. Rozzell, J. C. Moore, N. J. Turner, *Org. Biomol. Chem.* **2009**, *7*, 395-398; (i) D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell, W. Kroutil, *Adv. Synth. Catal.* **2008**, *350*, 2761-2766.

[24] M. Höhne, S. Kühl, K. Robins, U. T. Bornscheuer, *ChemBioChem* **2008**, *9*, 363-365.

[25] (a) M. D. Truppo, N. J. Turner, J. D. Rozzel, *Chem. Commun.* **2009**, 2127-2129; (b) D. Koszelewski, I. Lavandera, D. Clay, G. M. Guebitz, D. Rozzell, W. Kroutil, *Angew. Chem. Int. Ed.* **2008**, *47*, 9337-9340.

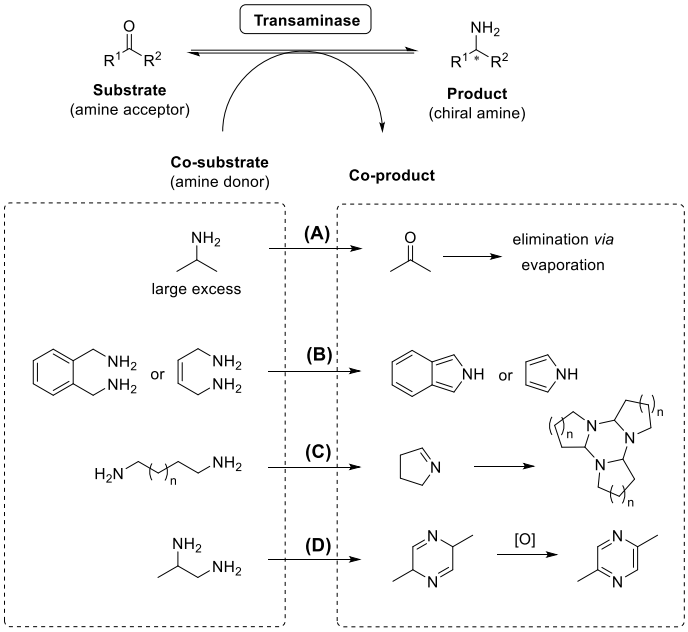
The addition of other amines as source of the amino group has been deeply studied in the last decade (Scheme 0.9). Isopropylamine is a promising cosubstrate as it is an economically affordable achiral amine which can work with (*R*)- and (*S*)-selective TAs and leads to the formation of volatile acetone (Scheme 0.9.A). The main drawback is that it is necessary to use it in large amounts (≥50 equivalents) in order to shift the equilibrium and obtain high conversions of the amine product. This experimental issue leads to the fact that not many transaminases are capable of accepting isopropylamine as amine donor under these conditions.[26] Another possibility is the evaporation of the acetone co-product due to its low boiling point.[27] In this way, some enzyme engineering studies have been done in order to improve the substrate acceptance and the enzyme stability.[28]

Apart from isopropylamine, elegant studies have been done using sacrificial cosubstrates, also known as "smart" cosubstrates. Several methodologies have been published applying this concept,[29] but diamines have caused such an interest.[30] In 2014, *ortho*-xylene diamine was tested as amino donor due to the ability of the resulting isoindole product to polymerise.[30a] A similar approach is the one developed in our research group in 2016 using the *cis*-but-2-ene-1,4-diamine (Scheme 0.9.B).[30b] Again, the resulting product undergoes cyclisation, aromatisation and a polymerisation process to produce a dark-coloured polymer. Alternatively, aliphatic 1,ω-polyamines seem to be a good choice as they are readily available and pretty affordable. In this way, it has been demonstrated that some transaminases can accept mono- and polyamines as amino donor. The co-product cyclises and the imine intermediate trimerises (Scheme 0.9.C).[30c] Similar

[26] (a) R. Abu, J. M. Woodley, *ChemCatChem* **2015**, *7*, 3094-3105; (b) E. S. Park, M. S. Malik, J. Y. Dong, J. S. Shin, *ChemCatChem* **2013**, *5*, 1734-1738; (c) K. Fesko, K. Steiner, R. Breinbauer, H. Schwab, M. Schürmann, G. A. Strohmeier, *J. Mol. Catal. B: Enzym.* **2013**, *96*, 103-110; (d) K. E. Cassimjee, C. Branneby, V. Abedi, A. Wells, P. Berglund, *Chem. Commun.* **2010**, *46*, 5569-5571.
[27] P. Tufvesson, C. Bach, J. M. Woodley, *Biotechnol. Bioeng.* **2014**, *111*, 309-319.
[28] (a) C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman, G. J. Hughes, *Science* **2010**, *329*, 305-309; (b) M. Höhne, S. Schätzle, H. Jochens, K. Robins, U. T. Bornscheuer, *Nat. Chem. Biol.* **2010**, *6*, 807-813.
[29] (a) H. Bea, H. Park, S. Lee, H. Yun, *Chem. Commun.* **2011**, *47*, 5894-5896; (b) T. Li, A. B. Kootstra, I. G. Fotheringham, *Org. Process. Res. Dev.* **2002**, *6*, 533-538; (c) B. Wang, H. Land, P. Berglund, *Chem. Commun.* **2013**, *49*, 161-163.
[30] (a) A. P. Green, N. J. Turner, E. O'Reilly, *Angew. Chem. Int. Ed.* **2014**, *53*, 10714-10717; (b) L. Martínez-Montero, V. Gotor, V. Gotor-Fernández, I. Lavandera, *Adv. Synth. Catal.* **2016**, *358*, 1618-1624; (c) I. Slabu, J. L. Galman, N. J. Weise, R. C. Lloyd, N. J. Turner, *ChemCatChem* **2016**, *8*, 1038-1042; (d) S. E. Payer, J. H. Schrittwieser, W. Kroutil, *Eur. J. Org. Chem.* **2017**, 2553-2559.

results were recently obtained using vicinal 1,2-diamines (Scheme 0.9.D), which dimerise and then spontaneously oxidise to form a pyrazine.[30d]

Scheme 0.9. Equilibrium displacement using isopropylamine or "smart" cosubstrates.



Finally, it is possible to shift the equilibrium by productive cascade reactions involving the major product (the chiral amine). However, this type of transaminase-catalysed processes will be further discussed in Chapter 2.

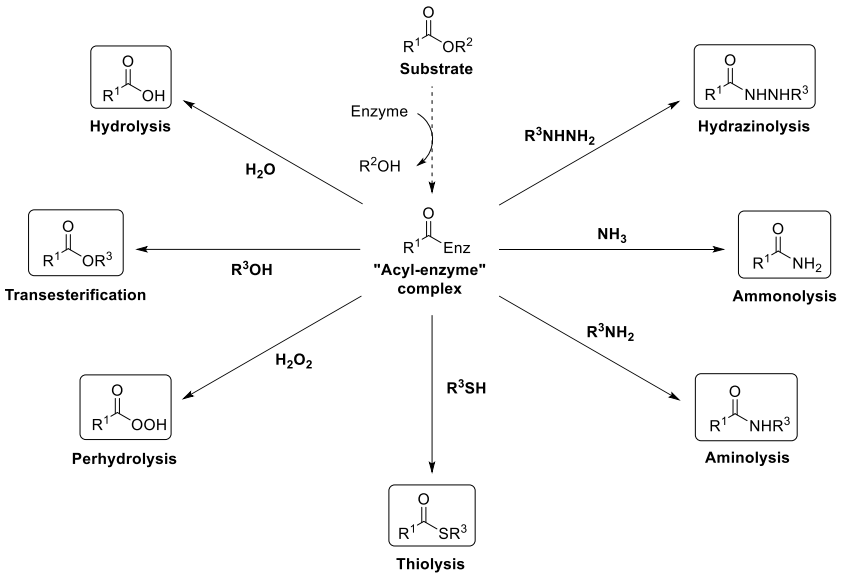
0.2.2.3. Lipases

Lipases are serine hydrolases which is one of the largest known enzyme classes. In nature, they catalyse the hydrolysis of lipids but they are able to accept a wide range of substrates. Besides, they present some characteristics that have turned them into one of the most exploited classes of enzymes. Firstly, they display high stereo-, chemo- and regioselectivity. Second, they are very stable enzymes that can work in organic media and at high temperatures. Thirdly, they do not need the addition of any cofactor for a proper action.[31]

[31] U.T. Bornscheuer, R. J. Kazlauskas, *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*, 2nd Ed., Wiley-VCH, Weinheim (Germany), **2006**.

The main difference between lipases and other hydrolases is that their catalytic activity significantly increases when it is situated in a lipid/water interface. This phenomenon is known as "interfacial activation" and it implies that the enzyme can take on different conformations and, consequently, accept different substrates.[32] Therefore, it is possible to replace their natural nucleophile, water, for a wide range of compounds such alcohols, peroxides, ammonia, amines, hydrazines or thiols.[33] Consequently, lipases can catalyse many different organic synthetic transformations that are represented in Scheme 0.10.

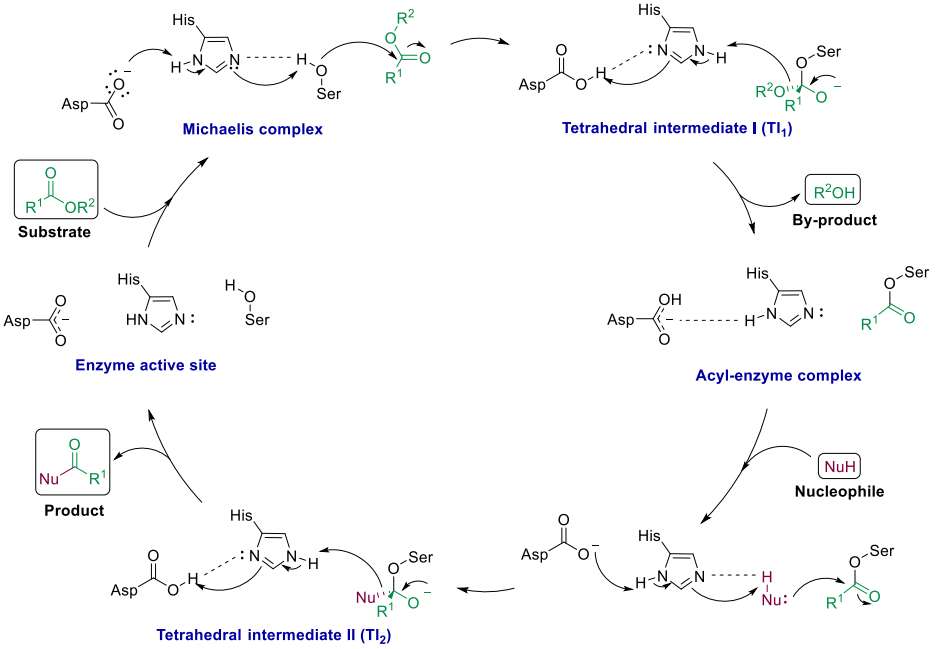
Scheme 0.10. Lipase-catalysed transformations.



The mechanism of action of lipases has been deeply studied[34] (Scheme 0.11) and it is associated to a catalytic triad formed by residues of aspartate (Asp), histidine (His) and serine (Ser) which are placed in the active site of the enzyme. There are two essential steps in the mechanism which involve the formation of tetrahedral intermediates: the tetrahedral intermediate I (TI_1) and the tetrahedral intermediate II (TI_2).

[32] (a) R. Verger, *Trends Biotechnol.* **1997**, *15*, 32-38; (b) R. D. Schmid, R. Verger, *Angew. Chem. Int. Ed.* **1998**, *37*, 1608-1633.
[33] (a) V. Gotor-Fernández, V. Gotor, in *Asymmetric Organic Synthesis with Enzymes*, (Eds. E. García-Urdiales, I. Alfonso, V. Gotor), Wiley-VCH, Weinheim (Germany), **2008**; (b) V. Gotor, *Bioorg. Med. Chem.* **1999**, *7*, 2189-2197.
[34] F. van Rantwijk, R. A. Sheldon, *Tetrahedron* **2004**, *60*, 501-519.

Scheme 0.11. Serine hydrolases mechanism.



First of all, the substrate and the enzyme join together in a non-covalent manner forming an enzyme-substrate complex which is known as Michaelis complex. This occurs as the aspartate located in the catalytic triad is capable of deprotonating the serine by using the histidine as electron mediator. Therefore, the serine hydroxyl group reacts with the carbonyl group of the substrate forming the TI_1. Later on, this intermediate evolves into the acyl-enzyme complex. Again, aspartate and histidine have an unquestionable influence in the following transformation. At this point, the nucleophile added into the reaction media reacts with the acyl-enzyme complex to produce the TI_2 that undergoes a subsequent transformation, allowing the regeneration of the enzyme and the release of the product. It is important to point out that the enantioselectivity of the process is typically attributed to the TI_2 as the active site presents two different pockets: one assigned to the nucleophile and the other one used for the acyl group.

Currently, the most commonly used lipases are the lipases from *Candida antarctica* type A and B (CAL-A and CAL-B) and from *Pseudomonas* (renamed as *Burkholderia*) *cepacia* (PSL). In this doctoral thesis, we will take full advantage of

the CAL-B catalytic action. For this reason, next some of the most outstanding characteristics of this enzyme will be discussed.

0.2.2.3.1. *Candida antarctica* lipase type B (CAL-B)

CAL-B[35] comes from *Candida antarctica* yeast, which was found in the Antarctica during a research focused on the discovery of extremophile enzymes. It is a globular protein composed by a polypeptide chain of 317 amino acids forming a 3D structure that is known as α/β-hydrolase fold (Figure 0.3.A). CAL-B contains two different channels in its active site where the substrates are placed. Those channels are placed almost parallel to each other, and spread out from the serine contained in the active site to the surface of the protein. They are partially separated by two hydrophobic isoleucine chains. Apart from those channels, CAL-B includes a movable α-helix motif which allows the solvent to enter the active site.

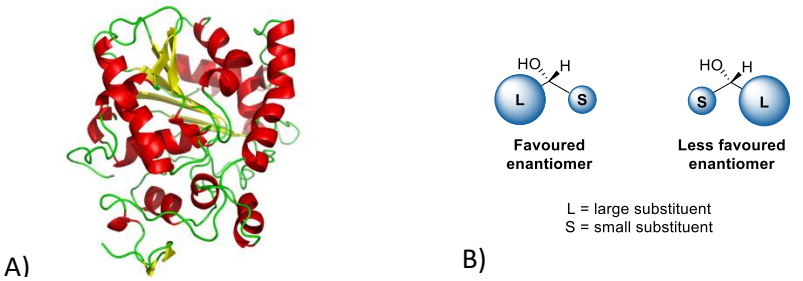


Figure 0.3. A) CAL-B 3D structure. B) Kazlauskas' rule to predict CAL-B enantioselectivity.

The CAL-B enantiopreference is traditionally explained using the wide-known semi-empirical Kazlauskas' rule.[36] It was originally employed for secondary alcohols and it can be applied to all lipases. Despite it is not an absolute method, it has been highly employed to designate the absolute configuration of enzymatic kinetic resolution products. This rule is based on the size of the substituents at the stereogenic centre and it predicts which enantiomer leads to a faster reaction. Thus, when a substrate has a large-sized substituent (L) and a small-sized substituent (S), it is accepted that the favourite enantiomer will be placed in the enzyme in a particular way: B will be accommodated in the bigger pocket while S will be

[35] S. Lutz, *Tetrahedron: Asymmetry* **2004**, *15*, 2743-2748.
[36] R. J. Kazlauskas, A. N. E. Weissfloch, A. T. Rappaport, L. A. Cuccia, *J. Org. Chem.* **1991**, *56*, 2656-2665.

placed in the smaller one (Figure 0.3.B). The disadvantaged enantiomer is placed in the other way around. That is why the most different in size the substituents are, the most selective the biotransformation is.

0.2.3. Reaction media

Water is the natural solvent for enzymes. However, there are some drawbacks associated to its utilisation. For that reason, the employment of non-aqueous media in biotransformations has been deeply studied in the last decades. Nowadays, biocatalysts can be used in water, organic solvents or neoteric solvents.

In this doctoral thesis we have made use of organic solvents (Chapter 1) and water (Chapters 2-5) as reaction media but we have also introduced a new reaction media formed by mixtures of buffer and a deep eutectic solvent (Chapter 6). Therefore, some of the most relevant advantages and disadvantages of each reaction medium will be further discussed.

0.2.3.1. Aqueous or organic systems

As said before, water is the natural solvent for enzymes. For this reason, it is the most frequently used for biocatalytic purposes. This fact has been generally considered an advantage as water is accepted to be a green solvent.[37] However, contaminated water cannot be released into the environment without previous treatment so that statement is true just for pure water.[38]

Apart from that, using water as a reaction media has some drawbacks associated to substrate solubility and the isolation of the corresponding product. Sadly, the first issue is typically solved by using low reagent concentrations which are incompatible for industrial purposes. Even so, the addition of a small amount of an organic solvent can sometimes allow for using higher substrate concentrations. The most commonly used cosolvents are alcohols as methanol (MeOH), ethanol (EtOH) or *n*-propanol (^{n}PrOH) and polar solvents as dimethyl sulfoxide (DMSO), 1,4-dioxane or tetrahydrofuran (THF). They can be typically used up to 10% v/v without loss of enzyme activity.[1b] Moreover, some reactions as, *e.g.*

[37] (a) R. A. Sheldon, *Green Chem.* **2005**, *7*, 267-278; (b) M.-O. Simon, C.-J. Li, *Chem. Soc. Rev.* **2012**, *41*, 1415-1427.
[38] Y. Ni, D. Holtmann, F. Hollmann, *ChemCatChem* **2014**, *4*, 930-943.

transesterification or perhydrolysis (see Scheme 0.10), cannot be performed in water due to competing product hydrolysis.

Some of these drawbacks can be solved by using an organic solvent as reaction medium. When Zaks and Klibanov[39] first reported that some enzymes were active in organic solvents, the era of "non-aqueous Biocatalysis" was born.[40] Since then, several research groups have gone in depth in the study of enzymes which are capable of working in organic solvents. Hydrolytic enzymes have led to satisfactorily results as they can catalyse a huge variety of transformations in organic media.[41] In contrast, there are not many examples of non-hydrolytic enzymes that are capable of working in non-aqueous media. For instance, Kroutil and co-workers reported the application of a solvent-resistant alcohol dehydrogenase from *Rhodococcus ruber* (ADH-A) overexpressed in *E. coli* in the bioreduction of a wide panel of ketones.[42] Similarly, Hibino and Ohtake described the use of a recombinant ADH from *Rhodococcus rhodochrous* in *E. coli* capable of working in solvent-free organic media.[43]

Despite all the advantages related to the use of conventional organic solvents instead of water as reaction media, there are still some important drawbacks that need to be highlighted: (i) many of those solvents are non-environmentally friendly, (ii) normally enzyme activity dramatically drops in the presence of organic solvents even at low concentrations of the organic media. These disadvantages encouraged scientists to look for new solvent classes, which could be compatible with biocatalysts.

0.2.3.2. Neoteric solvents

The word neoteric means new or modern. If we take a moment to think about new or modern solvents we will quickly think about those solvents which can be obtained for renewable and/or natural sources and which show low toxicity and volatility, this is, environmentally friendly solvents. In this way, supercritical

[39] A. Zaks, A. M. Klibanov, *Science* **1984**, *224*, 1249-1251.
[40] (a) R. A. Sheldon, *Chem. Eur. J.* **2016**, *22*, 12984-12999; (b) *Organic Synthesis with Enzymes in Non-Aqueous Media*, Eds. G. Carrea, S. Riva, Wiley-VCH, Weinheim (Germany), **2008**.
[41] A. M. Klibanov, *Acc. Chem. Res.* **1990**, *23*, 114-120.
[42] G. de Gonzalo, I. Lavandera, K. Faber, W. Kroutil, *Org. Lett.* **2007**, *9*, 2163-2166.
[43] A. Hibino, H. Ohtake, *Process Biochem.* **2013**, *48*, 838-843.

fluids, ionic liquids and deep eutectic solvents have been used in the field of Biocatalysis.

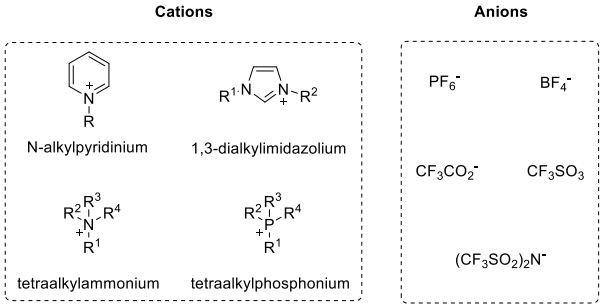
A supercritical fluid (SCF) is any substance above its critical point but below the pressure required to condense into a solid. They behave as liquids and show characteristics relative to both gas and liquid. Supercritical carbon dioxide (sCO_2) is typically the SCF of choice because it is nontoxic, non-flammable, readily available and inexpensive. Moreover, its critical point (31 °C and 74 bar) is perfectly compatible with the conditions required by enzymes. First reports using SCF for Biocatalysis date back from 1985 when Wilke and co-workers[44] and Krukonis and co-workers[45] found an alkaline phosphatase and a polyphenol oxidase, respectively, that were active in a supercritical CO_2 system. Since then, it is possible to find many publications in which different enzymes are used in sCO_2-buffer systems.[46]

Ionic liquids (ILs) are fluids composed entirely of ions which are liquid below 100 °C (Scheme 0.12). They are typically formed by an organic cation (containing an imidazolium or pyridinium core) and an anion that has a strongly delocalised negative charge (*e.g.* BF_4, PF_6, etc.). Owing to their remarkable properties, they have been widely proposed as potential alternatives to volatile organic solvents. The capability of dissolving a huge variety of highly polar organic and inorganic compounds, the stability at high temperatures, the low vapour pressure, the non-flammability and the possibility of recycling them are probably the most important properties that they possess. Furthermore, they have revealed as solvents that do not lead to protein inactivation and they can even enlarge the biocatalyst selectivity.[47] For all these reasons, the utilisation of ILs in Biocatalysis has become very popular in the last decades since the first three examples in this field were

[44] T. W. Randolph, H. W. Blanch, J. M. Prausnitz, C. R. Wilke, *Biotechnol. Lett* **1985**, *7*, 325-328.
[45] D. A. Hammond, M. Karel, A. M. Klibanov, V. J. Krukonis, *Appl. Biochem. Biotechnol.* **1985**, *11*, 393-400.
[46] (a) A. L. B. Dias, P. dos Santos, J. Martínez, *J. CO_2 Util.* **2018**, *23*, 159-178; (b) H. N. Hoang, Y. Nagashima, S. Mori, H. Kagechika, T. Matsuda, *Tetrahedron* **2017**, *73*, 2984-2989; (c) H. Monhemi, M. R. Housaindokht, *J. Supercrit. Fluids* **2016**, *117*, 147-163; (d) H. N. Hoang, T. Matsuda, *Tetrahedron Lett.* **2015**, *56*, 639-641; (e) T. Matsuda, *J. Biosci. Bioeng.* **2013**, *3*, 233-241.
[47] (a) M. Moniruzzaman, N. Kamiya, M. Goto, *Org. Biomol. Chem.* **2010**, *8*, 2887-2899; (b) P. Lozano, E. Alvarez, J. M. Bernal, S. Nieto, C. Gómez, G. Sanchez-Gomez, *Curr. Green Chem.* **2017**, *4*, 116-129.

reported in 2000.[48] Along these years, several biocatalysts such as hydrolases, oxidoreductases, lyases and whole cells have been assayed using ILs as solvents or cosolvents.[49] However, lipases have been by far the most investigated enzymes in this field starting from Sheldon and co-workers publication in which CAL-B catalysed alcoholysis, ammonolysis and perhydrolysis reactions in combination with ILs as reaction media.[48c]

Scheme 0.12. Examples of commonly used cations and anions to form ionic liquids.

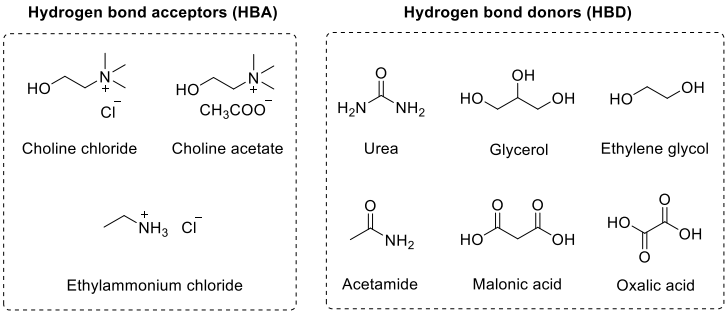


In spite of the huge applicability that ILs have shown as solvents or cosolvents in biotransformations, some of them have revealed to be actually toxic[50] and poorly biodegradable.[51] This fact has provided an excellent opportunity for deep eutectic solvents (DESs) to be the focus of interest in the last years as environmentally benign alternatives to traditional organic solvents.[52] A deep eutectic solvent is a mixture of components which interact *via* intermolecular forces. Depending on the nature of the components used, there are different classes of DESs. Among them, those components can be a quaternary ammonium salt (*e.g.*, choline chloride)

[48] (a) G. S. Cull, J. D. Holbrey, V. Vargas-Mora, K. R. Seddon, G. J. Lye, *Biotechnol. Bioeng.* **2000**, *69*, 227-233; (b) M. Erbeldinger, A. J. Mesiano, A. Russell, *Biotechnol. Prog.* **2000**, *16*, 1129-1131; (c) R. M. Lau, F. van Rantwijk, K. R. Seddon, R. A. Sheldon, *Org. Lett.* **2000**, *2*, 4189-4191.
[49] (a) T. Itoh, *Chem. Rev.* **2017**, *117*, 10567-10607; (b) C. E. Paul, I. Lavandera, V. Gotor-Fernandez, V. Gotor, *Top. Catal.* **2014**, *57*, 332-338; (c) C. E. Paul, V. Gotor-Fernández, I. Lavandera, J. Montejo-Bernardo, S. García-Granda, V. Gotor, *RSC Adv.* **2012**, *2*, 6455-6463; (d) C. Rodríguez, G. de Gonzalo, M. W. Fraaije, V. Gotor, *Green Chem.* **2010**, *12*, 2255-2260; (e) F. van Rantwijk, R. A. Sheldon, *Chem. Rev.* **2007**, *107*, 2757-2785; (f) G. de Gonzalo, I. Lavandera, K. Durchschein, D. Wurm, K. Faber, W. Kroutil, *Tetrahedron: Asymmetry* **2007**, *18*, 2541-2546.
[50] H. Monhemi, M. R. Housaindokht, A. A. Moosavi-Movahedi, M. R. Bozorgmehr, *Phys. Chem. Chem. Phys.* **2014**, *16*, 14882-14893.
[51] A. Jordan, N. Gathergood, *Chem. Soc. Rev.* **2015**, *44*, 8200-8237.
[52] Some recent reviews: (a) E. L. Smith, A. P. Abbott, K. S. Ryder, *Chem. Rev.* **2014**, *114*, 11060-11082; (b) D. A. Alonso, A. Baeza, R. Chinchilla, G. Guillena, I. M. Pastor, D. J. Ramón, *Eur. J. Org. Chem.* **2016**, 612-632; (c) P. Xu, G.-W. Zheng, M.-H. Zong, N. Li, W.-Y. Lou, *Bioresour. Bioprocess.* **2017**, *4*, 34.

acting as a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) molecule and, on account of their proportion, the resulting DES has a melting point that is lower that of each individual component (Scheme 0.13). Due to the origin of the components of this kind of DES, formed by primary metabolites such as amino acids, sugars, polyols, choline and organic acids, they have been described as natural deep eutectic solvent (NADES).[53]

Scheme 0.13. Representative examples of some DES components.



Even though the eutectic phenomena has been part of our daily life for centuries represented by honey or syrup, the first time the term "deep eutectic solvent" was employed date just from 2004.[54] In that moment of time, Abbott and co-workers reported a large drop in the melting point of a choline chloride and urea mixture. Since then, the research related to the application of DESs as alternative to conventional organic solvents has been increasing not only in the field of Biocatalysis but in many other fields as material synthesis or extraction procedures (Figure 0.4). But, which are the advantages of DESs over ILs and why are they becoming so popular? Basically, DESs have similar properties than ILs (low volatility, low flammability and low melting point), but they have two main advantages over their ancestors: their easy preparation and their lower cost.[52c] A DES mixture is prepared by mixing the individual components under constant stirring at moderate temperature. This is a simple strategy compared to the preparation of ILs which involves the synthesis of an intermediate salt and the following anion exchange to produce the corresponding IL. Additionally, the most

[53] Y. H. Choi, J. van Spronsen, Y. T. Dai, M. Verberne, F. Hollmann, I. Arends, G. J. Witkamp, R. Verpoorte, *Plant Physiol.* **2011**, *156*, 1701-1705.
[54] A. P. Abbott, D. Boothby, G. Capper, D. L. Davies, R. K. Rasheed, *J. Am. Chem. Soc.* **2004**, *126*, 9142-9147.

commonly used (NA)DESs are made from natural materials, which exist in large amounts and are relatively inexpensive to produce.

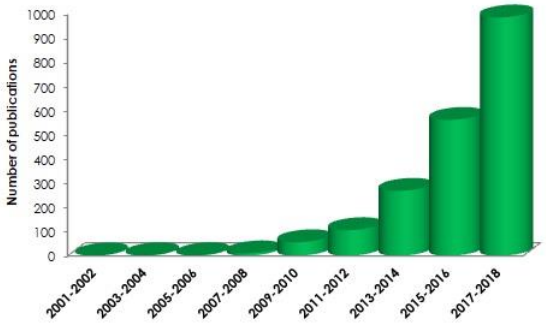


Figure 0.4. Number of publications on deep eutectic solvents from 2001 to 2018 (searched by SciFinder on October 23, 2018).

For all these reasons, the boom of deep eutectic mixtures to be used as solvents or cosolvents in biocatalytic routes has been evident in recent years. Even though the application of DESs in transformations mediated by enzymes will be further discussed in Chapter 6, it is important to point out in this Introduction that several types of biocatalysts have been explored along the last decade. Kazlauskas and co-workers were the first team to report enzymatic-catalysed biotransformations in DES in 2008.[55] During their research, four lipases were investigated towards transesterification processes. Since then, several hydrolases such as proteases[56] or epoxide hydrolases[57] but, especially, lipases[58] have been tested including DESs in the reaction media. Other enzymes such as peroxidases[59]

[55] J. T. Gorke, F. Srienc, R. J. Kazlauskas, *Chem. Commun.* **2008**, 1235-1237.
[56] (a) S.-L. Cao, H. Xu, X.-H. Li, W.-Y. Lou, M.-H. Zong, *ACS Sustainable Chem. Eng.* **2015**, *3*, 1589-1599; (b) Z. Maugeri, W. Leitner, P. Domínguez de María, *Eur. J. Org. Chem.* **2013**, 4223-4228.
[57] D. Lindberg, M. de la Fuente Revenga, M. Widersten, *J. Biotechnol.* **2010**, *147*, 169-171.
[58] (a) S. Siebenhaller, C. Muhle-Goll, B. Luy, F. Kirschhöfer, G. Brenner-Weiss, E. Hiller, M. Günther, S. Rupp, S. Zibek, C. Syldatk, *J. Mol. Catal. B: Enzym.* **2016**, *133*, 281-287; (b) D. González-Martínez, V. Gotor, V. Gotor-Fernández, *Eur. J. Org. Chem.* **2016**, 1513-1519; (c) A. Petrenz, P. Domínguez de María, A. Ramanathan, U. Hanefeld, M. B. Ansorge-Schumacher, S. Kara, *J. Mol. Catal. B: Enzym.* **2015**, *114*, 42-49; (d) C. R. Müller, I. Meiners, P. Domínguez de María, *RSC Adv.* **2014**, *4*, 46097-46101.
[59] B.-P. Wu, Q. Wen, H. Xu, Z. Yang, *J. Mol. Catal. B: Enzym.* **2014**, *101*, 101-107.

and benzaldehyde lyases[60] have also been assayed. In the same way, whole-cell biotransformations have been explored using DESs as part of the reaction media.[61]

[60] Z. Maugeri, P. Domínguez de María, *J. Mol. Catal. B: Enzym.* **2014**, *107*, 120-123.
[61] (a) J. Paris, N. Ríos-Lombardía, F. Morís, H. Gröger, J. González-Sabín, *ChemCatChem* **2018**, *10*, 4417-4423; (b) C. R. Müller, I. Lavandera, V. Gotor-Fernández, P. Domínguez de María, *ChemCatChem* **2015**, *7*, 2654-2659.

Objectives

As introduced in the Preface of this Doctoral Thesis, biocatalytic processes are characterised by the high selectivity displayed by enzymes, and their occurrence with exquisite atom efficiency under mild reaction conditions. Thus, the main objective of this thesis is the study of different biocatalyst classes (lipases and transaminases in Part I and alcohol dehydrogenases in Part II) in order to perform sustainable transformations including acylation, transamination and reduction reactions.

With that purpose, a general strategy has been followed in all the chapters consisting in a preliminary enzymatic screening in order to find suitable efficient and selective enzymes for a defined transformation, and then searching for the optimal reaction conditions that allow the development of reproducible and effective methodologies. Later on, important efforts have been made in the look for scaling up reactions for the already developed protocols, confirming in this manner the applicability of the new procedures.

In Chapters 1 to 5, the development of different (chemo)enzymatic protocols have been addressed in order to synthesise optically pure compounds: 1-[2-bromo(het)aryloxy]propan-2-amines (Chapter 1), γ- and δ-lactams (Chapter 2), α-alkyl-β-amino amides (Chapter 3), α-alkyl-β-hydroxy amides (Chapter 4) and 1,4-diaryl-1,4-diols (Chapter 5), which can be used as valuable chiral building blocks in organic synthesis.

Additionally, in Chapter 6 we have focused on two of the main difficulties concerning the use of alcohol dehydrogenases: the recyclability of the cofactor and the substrate solubility. In this way, a general procedure in which a designer natural deep eutectic solvent can be used as both cosolvent and cosubstrate in ADH-catalysed bioreductions has been developed.

Part I

Biocatalysis in the synthesis of nitrogenated compounds

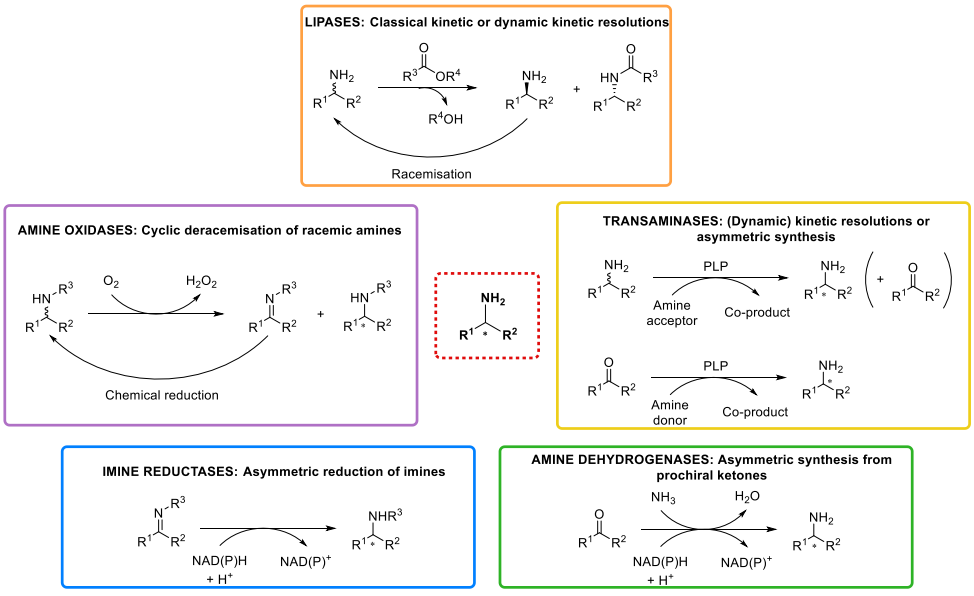
Chapter 1

Chemoenzymatic synthesis of enantioenriched 1-[2-bromo(het)aryloxy]propan-2-amines as valuable precursors of benzoxazine derivatives

1.1. Bibliographic background

The production of chiral amines has been a challenge for organic chemists as they play a key role in the pharmaceutical, agrochemical and chemical industries for the production of valuable products. They are typically used as synthons for the preparation of biologically active molecules but they also have important applications as resolving agents, chiral auxiliaries and organocatalytic reagents.[62] In industry, high chemical and enantiomeric purities (>99% *ee*) are often needed. In this way, Biocatalysis is an important tool to be considered as it provides access to a huge variety of chiral amines taking advantage of different (chemo)enzymatic strategies (Scheme 1.1).[63]

Scheme 1.1. Enzymatic strategies towards the synthesis of chiral amines.



Traditionally, classical kinetic and dynamic kinetic resolutions of racemic amines catalysed by lipases have been developed for the production of enantiopure

[62] T. C. Nugent. *Chiral Amine Synthesis: Methods, Developments and Applications*, Wiley-VCH: Weinheim (Germany), **2010**.

[63] (a) M. D. Patil, G. Grogan, A. S. Bommarius, H. Yun, *ACS Catal*, **2018**, *8*, 10985-11015; (b) S. P. France, G. A. Aleku, M. Sharma, J. Mangas-Sánchez, R. M. Howard, J. Steflik, R. Kumar, R. W. Adams, I. Slabu, R. Crook, G. Grogan, T. W. Wallace, N. J. Turner, *Angew. Chem. Int. Ed.* **2017**, *56*, 15589-15593; (c) H. Kohls, F. Steffen-Munsberg, M. Höhne, *Curr. Opin. Chem. Biol.* **2014**, *19*, 180-192; (d) D. Ghislieri, N. J. Turner, *Top. Catal.* **2014**, *57*, 284-300; (e) M. Höhne, U. T. Bornscheuer, *ChemCatChem* **2009**, *1*, 42-51.

amines,[64] while the combination of amino oxidases (AOs) and chemical reagents has allowed their cyclic deracemisation in combination with chemical reducing agents.[65] More recently, the capability of transaminases (TAs) to perform classical kinetic and dynamic kinetic resolutions of racemic amines as well as the asymmetric synthesis of chiral amines from prochiral ketones has been exploited.[18] Furthermore, imine reductases (IREDs) and reductive aminases (RedAms) have demonstrated that they play an important role in the asymmetric reduction of imines or the reductive amination of carbonylic compounds,[66] while amine dehydrogenases (AmDHs) have shown up as efficient catalysts in the synthesis of chiral amines from prochiral ketones.[67]

1.1.1. 1-Aryloxypropan-2-amines as important building blocks

Optically active 1-aryloxypropan-2-amines are particularly important nitrogenated compounds (Figure 1.1).

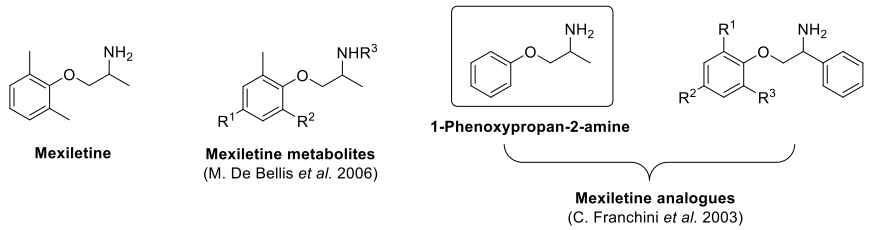


Figure 1.1. Structure of mexiletine and its metabolites and analogues.

1-(2,6-Dimethylphenoxy)propan-2-amine, also named mexiletine, is a compound that has stood out in this family of amines due to its properties as

[64] (a) P. Hoyos, V. Pace, A. R. Alcántara, *Adv. Synth. Catal.* **2012**, *354*, 2585-2611; (b) Y. Kim, J. Park, M.-J. Kim, *ChemCatChem* **2011**, *3*, 271-277; (c) J. H. Lee, K. Han, M.-J. Kim, J. Park, *Eur. J. Org. Chem.* **2010**, *6*, 999-1015; (d) A. Ghanem, *Tetrahedron* **2007**, *63*, 1721-1754; (e) V. Gotor-Fernández, R. Brieva, V. Gotor, *J. Mol. Catal. B: Enzym.* **2006**, *3-4*, 111-120; (f) A. Ghanem, H. Y. Aboul-Enein, *Tetrahedron: Asymmetry* **2004**, *15*, 3331-3351.

[65] (a) R. Carr, M. Alexeeva, M. J. Dawson, V. Gotor-Fernández, C. E. Humphrey, N. J. Turner, *ChemBioChem* **2005**, *6*, 637-639; (b) R. Carr, M. Alexeeva, A. Enright, T. S. C. Eve, M. J. Dawson, N. J. Turner, *Angew. Chem. Int. Ed.* **2003**, *42*, 4807-4810.

[66] (a) J. Mangas-Sánchez, S. P. France, S. L. Montgomery, G. A. Aleku, H. Man, M. Sharma, J. I. Ramsden, G. Grogan, N. J. Turner, *Curr. Opin. Chem. Biol.* **2017**, *37*, 19-25; (b) G. Grogan, N. J. Turner, *Chem. Eur. J.* **2016**, *22*, 1900-1907.

[67] (a) T. Knaus, W. Böhmer, F. G. Mutti, *Green Chem.* **2017**, *19*, 453-463; (b) M. J. Abrahamson, E. Vázquez-Figueroa, N. B. Woodall, J. C. Moore, A. S. Bommarius, *Angew. Chem. Int. Ed.* **2012**, *51*, 3969-3972; (c) N. Itoh, C. Yachi, T. Kudome, *J. Mol. Catal. B: Enzym.* **2000**, *10*, 281-290.

antiarrhythmic agent.[68] For this reason, mexiletine and its analogues have attracted great attention for clinical purposes.[69] In therapy, mexiletine has been commercialised as a racemate. However, in vivo and in vitro studies have revealed that the (–)-(*R*)-enantiomer is more active than the (+)-(*S*)-mexiletine.[70] Hence developing asymmetric approaches to obtain these derivatives is highly appealing. In the field of Biocatalysis, several methodologies have been described in order to produce (*S*)-mexiletine and its analogue 1-phenoxypropan-2-amine. The first approach dates back from 2002 when our research group described the production of enantiopure (+)-(*S*)-mexiletine and the enantiopure acetylated (*R*)-enantiomer through stereoselective acetylation of the racemate using CAL-B.[71] Since then, several approaches involving the utilisation of transaminases,[23d,23f,25b,26c,72] amine dehydrogenases,[73] as biocatalysts have been developed to obtain optically active mexiletine or its analogue 1-phenoxypropan-2-amine. Remarkably, Jamison and co-workers reported the continuous flow synthesis of (–)-(*R*)-mexiletine and (*R*)-1-phenoxypropan-2-amine using immobilised *E. coli* cells containing the (*R*)-transaminase from *Arthrobacter* sp.[26c] Also worthy to be commented is the approach developed by Turner and co-workers in which racemic alcohols were transformed into enantiopure amines through a redox self-sufficient enzymatic cascade mediated by an alcohol dehydrogenase and an amine dehydrogenase (Scheme 1.2).[73d] By using this methodology, (*R*)-1-phenoxypropan-2-amine among other chiral amines was produced with high conversions and excellent enantioselectivity starting from the corresponding racemic alcohols. More recently, this approach has been developed in a more fashionable way by using an ADH and an AmDH co-immobilised on controlled porosity glass Fe^{III} ion-affinity beads.[73a]

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[68] (a) A. Catalano, A. Carocci, M. S. Sinicropi, *Curr. Med. Chem.* **2015**, *22*, 1400-1413; (b) L. Labbé, J. Turgeon, *Clin. Pharmacokinet.* **1999**, *37*, 361-384.

[69] (a) M. De Bellis, A. De Luca, F. Rana, M. M. Cavalluzzi, A. Catalano, G. Lentini, C. Franchini, V. Tortorella, D. C. Camerino, *Br. J. Pharmacol.* **2006**, *149*, 300-310; (b) C. Franchini, A. Carocci, A. Catalano, M. M. Cavalluzzi, F. Corbo, G. Lentini, A. Scilimati, P. Tortorella, D. C. Camerino, A. De Luca, *J. Med. Chem.* **2003**, *46*, 5238-5248.

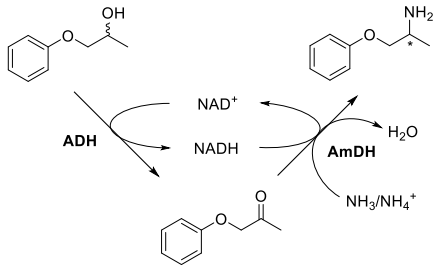
[70] (a) J. Turgeon, A. C. G. Uprichard, P. M. Bélanger, D. W. C. Harron, O. Grech-Bélanger, *J. Pharm. Pharmacol.* **1991**, *43*, 630-635; (b) R. J. Hill, H. J. Duff, R. S. Sheldon, *Mol. Pharmacol.* **1988**, *34*, 659-663.

[71] J. González-Sabín, V. Gotor, F. Rebolledo, *Tetrahedron: Asymmetry* **2002**, *13*, 1315-1320.

[72] (a) L. H. Andrade, W. Kroutil, T. F. Jamison, *Org. Lett.* **2014**, *16*, 6092-6095; (b) F. G. Mutti, W. Kroutil, *Adv. Synth. Catal.* **2012**, *354*, 3409-3413.

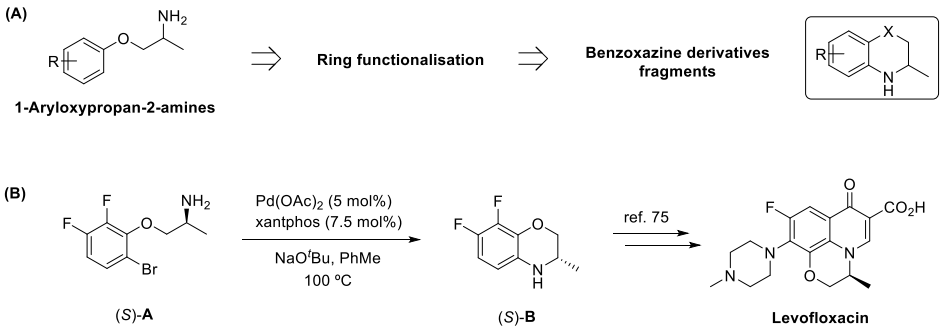
[73] (a) W. Böhmer, T. Knaus, F. G. Mutti, *ChemCatChem* **2018**, *10*, 731-735; (b) A. Pushpanath, E. Siirola, A. Bornadel, D. Woodlock, U. Schell, *ACS Catal.* **2017**, *5*, 3204-3209; (c) M. P. Thompson, N. J. Turner, *ChemCatChem* **2017**, *9*, 3833-3836; (d) F. G. Mutti, T. Knaus, N. S. Scrutton, M. Breuer, N. J. Turner, *Science* **2015**, 349, 1525-1529.

Scheme 1.2. Redox self-sufficient enzymatic cascade mediated by an ADH and an AmDH.



The introduction of additional functionalities in the aromatic ring provides new opportunities in medicinal and synthetic chemistry (Scheme 1.3.A). For example, the syntheses of several benzoxazine precursors have been described starting from substituted 1-aryloxypropan-2-amines such as (*S*)-1-(6-bromo-2,3-difluorophenoxy)propan-2-amine (Scheme 1.3.B).[74] This amine can subsequently be transformed into the biologically active antibiotic levofloxacin.[75]

Scheme 1.3. (A) Ring-substituted 1-aryloxypropan-2-amines in the synthesis of benzoxazine precursors. **(B)** Synthesis of Levofloxacin precursor, (*S*)-**B** from (*S*)-1-(6-bromo-2,3-difluorophenoxy)propan-2-amine, (*S*)-**A**.



In the next section, the importance of benzoxazine derivatives will be explained besides some enzymatic methodologies to access 3,4-dihydro-2*H*-1,4-benzoxazines.

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[74] (a) M. Kumar Parai, G. Panda, *Tetrahedron Lett.* **2009**, *50*, 4703-4705; (b) J. F. Bower, P. Szeto, T. Gallagher, *Org. Lett.* **2007**, *17*, 3283-3286.

[75] (a) L. A. Mitscher, P. N. Sharma, D. T. W. Chu, L. L. Shen, A. G. J. Pernet, *J. Med. Chem.* **1987**, *30*, 2283-2286; (b) K. Sakano, S. Yokohama, I. Hayakawa, S. Atarashi, S. Kadoya, *Agric. Biol. Chem.* **1987**, *51*, 1265-1270; (c) S. Atarashi, S. Yokohama, K. Yamazaki, K. Sakano, M. Imamura, I. Hayakwa, *Chem. Pharm. Bull.* **1987**, *35*, 1896-1902; (d) I. Hayakawa, S. Atarashi, M. Imamura, S. Yokohama, N. Higashihashi, K. Sakano, M. Ohshima, US patent, 5,053,407, **1986**.

1.1.2. Synthesis of enantioenriched 3,4-dihydro-2*H*-1,4-benzoxazines

The skeleton of benzoxazine has been intensively studied for organic chemists as an important heterocyclic system for building natural and designed synthetic compounds.[76] The two first benzoxazine derivatives isolated were 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA, Figure 1.2) and 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA, Figure 1.2).

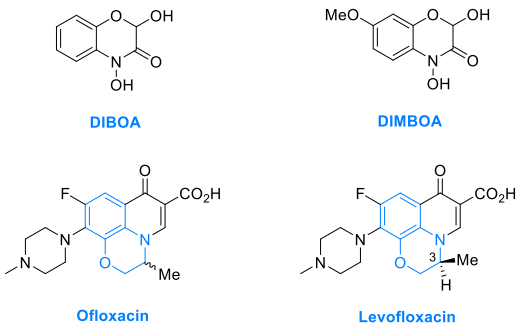

Figure 1.2. DIBOA, DIMBOA, ofloxacin and levofloxacin structures.

Since then, 1,4-benzoxazine scaffold has been frequently used as suitable skeleton for the design of biologically active molecules. From this large family of compounds, 3,4-dihydro derivatives are particularly interesting as many usable compounds, ranging from herbicides and fungicides to therapeutic drugs, contain that scaffold.[77] In fact, some benzoxazine derivatives have already beaten the clinical research phase and they have been introduced into the market, while others are still been tested as feasible drugs.[78] A benzoxazine derivative which has already been commercialised is ofloxacin (Figure 1.2), a broad-spectrum antibiotic which is utilised in the treatment of infections caused by gram-positive and gram-negative bacteria. The (*S*)-enantiomer, levofloxacin, which has already been introduced in the previous section (Scheme 1.3), is responsible for the biological activity of the drug being from 8 to 128 times more active than the (*R*)-enantiomer.[79] This is such an example of the importance of enantiopurity when referring to drugs.

[76] (a) Y. Sugimoto, T. Otani, S. Oie, K. Wierzba, Y. Yamada, *J. Antibiot.* **1990**, *43*, 417-421; (b) Y. S. Shen, X. Y. Ming, B. Yu, T. Otani, H. Saito, Y. Yamada, *J. Antibiot.* **1989**, *42*, 1294-1298.
[77] N. Siddiqui, R. Ali, M. S. Alam, W. Ahsan, *J. Chem. Pharm. Res.* **2010**, *4*, 309-316.
[78] J. Ilaš, P. Š. Anderluh, M. S. Dolenc, D. Kikelj, *Tetrahedron* **2005**, *61*, 7325-7348.
[79] B. Achari, S. B. Mandal, P. K. Dutta, C. Chowdhury, *Synlett* **2004**, 2449-2467.

As said before, the synthesis of 1,4-benzoxazines and their derivatives has been intensively studied. However, in this introduction we will focus on those methodologies developed towards the production of optically active 3,4-dihydro-2*H*-1,4-benzoxazines as they can be access from 1-aryloxypropan-2-amines. In this way, many chemical procedures such as the asymmetric hydrogenation of imine precursors have been described to achieve enantioenriched 1,4-benzoxazines.[80] An appropriate alternative to asymmetric catalysis is the kinetic resolution of racemic mixtures of the corresponding 1,4-benzoxazine derivatives. In this research field, the work developed by Krasnov and co-workers must be emphasised as they have tested several chiral acyl chlorides as resolving acylating agents in non-enzymatic kinetic resolutions.[81]

In the field of Biocatalysis, enzymes have been scarcely used in the synthesis of chiral 1,4-benzoxazines. This is probably surprising as several hydrolytic enzymes have accessed chiral heterocyclic molecules before.[82] One of the few examples that can be found in the literature is the hydrolysis of dimethyl 2-methyl-2-(2-nitrophenoxy)malonates mediated by pig liver esterase (PLE).[83] By using a phosphate buffer pH 7 and DMF as cosolvent in a 4:1 v/v ratio, the (*R*)-monomethyl 2-methyl-2-(2-nitrophenoxy)malonates were obtained with moderate to high selectivity (73-88% *ee*). Afterwards, both enantiomers of methyl 2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylates, 2-carboxylic acids or 2-carboxamides were synthesised after a series of chemical transformations (Scheme 1.4).

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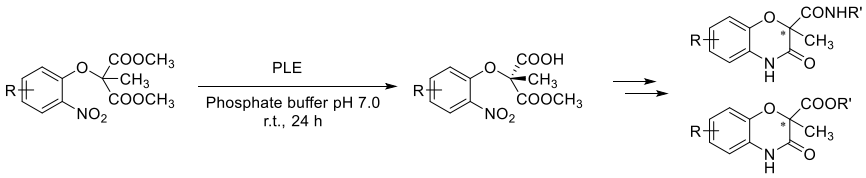
[80] (a) Y. Zhang, R. Zhao, R. L.-Y. Bao, L. Shi, *Eur. J. Org. Chem.* **2015**, *15*, 3344-3351; (b) X. W. Liu, C. Wang, Y. Yan, Y. Q. Wang, J. Sun, *J. Org. Chem.* **2013**, *78*, 6276-6280; (c) K. Gao, C. B. Yu, D. S. Wang, Y. G. Zhou, *Adv. Synth. Catal.* **2012**, *354*, 483-488; (d) D. S. Kundu, J. Schmidt, C. Bleschke, A. Thomas, S. Blechert, *Angew. Chem. Int. Ed.* **2012**, *51*, 5456-5459; (e) J. G. de Vries, N. Mršić, *Catal. Sci. Technol.* **2011**, *1*, 727-735; (f) M. Rueping, M. Stoeckel, E. Sugiono, T. Theissmann, *Tetrahedron* **2010**, *66*, 6565-6568.

[81] (a) D. A. Gruzdev, E. N. Chulakkov, G. L. Levit, M. A. Ezhikova, M. I. Kodess, V. P. Krasnov, *Tetrahedron: Asymmetry* **2012**, *23*, 1640-1646; (b) V. P. Krasnov, G. L. Levit, M. A. Korolyova, I. M. Bukrina, L. S. Sadretdinova, I. N. Andreeva, V. N. Charushin, O. N. Chupakhin, *Russ. Chem. Bull. Int. Ed.* **2004**, *53*, 1253-1256; (c) V. P. Krasnov, G. L. Levit, M. I. Kodess, V. N. Charushin, O. N. Chupakhin, *Tetrahedron: Asymmetry* **2004**, *15*, 859-862; (d) V. P. Krasnov, G. L. Levit, I. M. Bukrina, I. N. Andreeva, L. S. Sadretdinova, M. A. Korolyova, M. I. Kodess, V. N. Charushin, O. N. Chupakhin, *Tetrahedron: Asymmetry* **2003**, *14*, 1985-1988; (e) V. N. Charushin, V. P. Krasnov, G. L. Levit, M. A. Korolyova, M. I. Kodess, O. N. Chupakhin, M. H. Kim, H. S. Lee, Y. J. Park, K. C. Kim, *Tetrahedron: Asymmetry* **1999**, *10*, 2691-2702.

[82] E. Busto, V. Gotor-Fernández, V. Gotor, *Chem. Rev.* **2011**, *111*, 3998-4035.

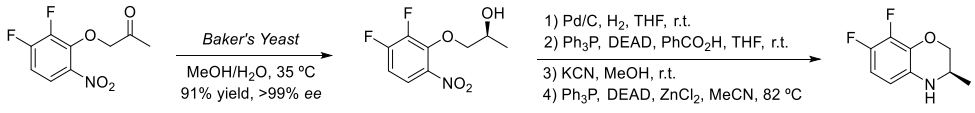
[83] M. Breznik, V. Hrast, A. Mrcina, D. Kikelj, *Tetrahedron: Asymmetry* **1999**, *10*, 153-167.

Scheme 1.4. PLE-catalysed hydrolysis of dimethyl 2-methyl-2-(2-nitrophenoxy)malonates and application to benzoxazine derivatives synthesis.



In addition to the previous approach, other enzymatic pathways have been developed towards the synthesis of levofloxacin. For instance, the bioreduction of a prochiral nitro ketone into the corresponding enantiopure nitro alcohol using Baker's yeast (BY) as biocatalyst has been reported (Scheme 1.5).[84] Another protocol involves the synthesis of levofloxacin with moderate enantioselectivity using immobilised PLE as biocatalyst and the ofloxacin butyl ester as substrate.[85]

Scheme 1.5. Chemoenzymatic synthesis of levofloxacin by using Baker's yeast.



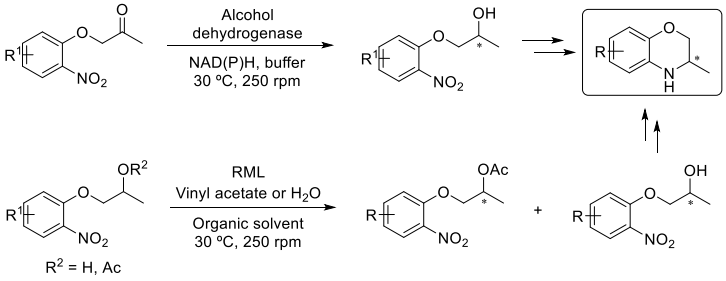
Besides, in 2015 Krasnov and co-workers accessed (*S*)-7,8-difluoro-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine by the enantioselective microbial hydrolysis of the corresponding racemic acetamide in the presence of *Rhodococcus erythropolis* 25 and *Microbacterium paraoxydans* 20-11c sp. bacteria.[86]

Furthermore, a chemoenzymatic approach towards the asymmetric synthesis of 1,4-benzoxazines including the preparation of a levofloxacin precursor has been recently described in our research group.[87] In this work, alcohol dehydrogenases and lipases have been utilised to access both enantiomers of different 3-methyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine (Scheme 1.6). The bioreduction of 1-(2-nitrophenoxy)propan-2-ones led to the production of the (*S*)-alcohols when using the ADH from *Rhodococcus ruber* (ADH-A) and to the synthesis of the (*R*)-

[84] S. B. Kang, E. J. Ahn, Y. Kim, *Tetrahedron Lett.* **1996**, *37*, 9317-9320.
[85] (a) S. Y. Lee, B. H. Min, S. H. Hwang, Y. M. Koo, C. K. Lee, S. W. Song, S. Y. Oh, S. M. Lim, S. L. Kim, D. I. Kim, *Biotechnol. Bioprocess Eng.* **2001**, *6*, 179-182; (b) S. Y. Lee, B. H. Min, S. H. Hwang, Y. M. Koo, C. K. Lee, S. W. Song, S. Y. Oh, S. M. Lim, S. L. Kim, D. I. Kim, *Biotechnol. Lett.* **2001**, *23*, 1033-1037.
[86] E. N. Chulakov, G. L. Levit, A. A. Turnashov, N. P. Lugovskaya, N. B. Remezovskaya, A. Yu. Maksimov, V. A. Demakov, V. P. Krasnov, *Russ. Chem. Bull. Int. Ed.* **2015**, *5*, 1097-1099.
[87] M. López-Iglesias, E. Busto, V. Gotor, V. Gotor-Fernández, *J. Org. Chem.* **2015**, *80*, 3815-3824.

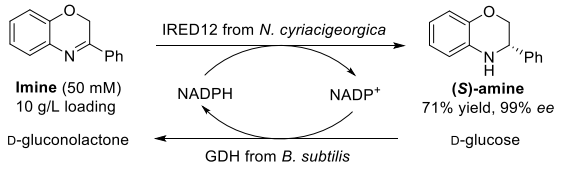
alcohols when commercial ADH evo-1.1.200 was used as biocatalyst. Additionally, kinetic resolution processes were developed using the lipase from *Rhizomucor miehei* (RML). This way, (*S*)-alcohols were obtained through acetylation processes while the hydrolysis of the racemic acetates led to the synthesis of (*R*)-alcohols.

Scheme 1.6. Chemoenzymatic process to access chiral 1,4-benzoxazine derivatives.



The last biocatalytic approach towards the synthesis of optically active 3,4-dihydro-2*H*-1,4-benzoxazine has been recently published by Gröger and co-workers. [88] In this approach, the stereoselective bioreduction of 2*H*-1,4-benzoxazines has been developed using imine reductases (IREDs) as biocatalysts. This way, the corresponding enantioenriched amines were obtained (up to 99% *ee*). Additionally, the efficiency of the method was demonstrated by scaling up the process up to a substrate loading of 10 g/L in the presence of just 0.1 g/mmol lyophilised IRED. After 26 hours, the amine was obtained in 71% yield and excellent enantioselectivity (Scheme 1.7).

Scheme 1.7. Representative example of the bioreduction of 2*H*-benzoxazines using IREDs as catalysts.



Due to the scarcity of biocatalytic pathways towards the synthesis of benzoxazine derivatives, a new chemoenzymatic approach to access enantioenriched 1-[2-bromo(het)aryloxy]-propan-2-amines through kinetic

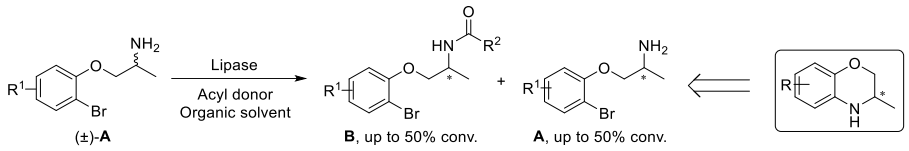
[88] N. Zumbrägel, P. Machui, J. Nonnhoff, H. Gröger, *J. Org. Chem.* **2019**, *84*, 1440-1447.

resolution processes mediated by lipase from *Candida antarctica* type B (CAL-B) will be described in this chapter.

1.2. Discussion

The lipase-catalysed resolution of racemic 1-[2-bromo(het)aryloxy]propan-2-amines has been undertaken for the production of the enantiopure derivatives which can be employed as important synthetic building blocks for the synthesis of, *e.g.* enantiopure 3,4-dihydro-2*H*-1,4-benzoxazines (Scheme 1.8). For this purpose, a chemical route to synthesise the racemic amines, (±)-**A**, was developed. Later on, these compounds were used as substrates for a lipase-mediated kinetic resolution protocol. This way, the enantioenriched amides, **B**, and the remaining chiral amines, **A**, could be produced.

Scheme 1.8. Lipase-catalysed kinetic resolution of a series of 1-[2-bromo(het)aryloxy]propan-2-amines as valuable precursors of benzoxazine derivatives.



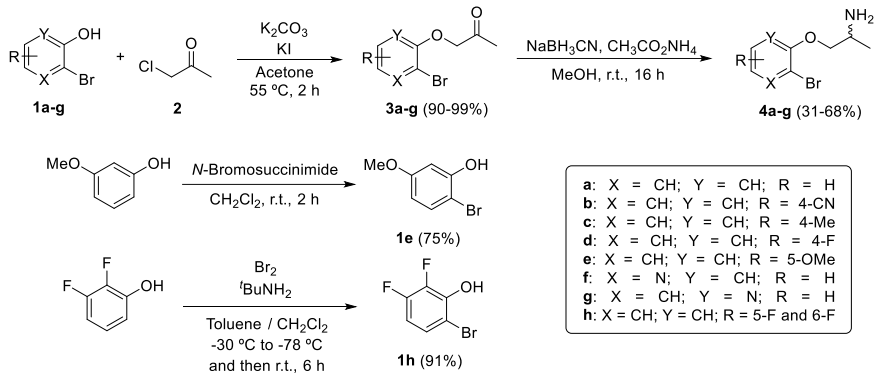
1.2.1. Chemical approach to obtain racemic 1-[2-bromo(het)aryloxy]propan-2-amines, (±)-4a-h

The racemic amines **4a-h** were synthesised using as starting material the corresponding commercially available 2-bromophenols **1a-d** and pyridinols **1f** and **1g** (Scheme 1.9). A selective bromination of 3-methoxyphenol using *N*-bromosuccinimide (NBS) in dry dichlorometane (DCM) was needed to access 2-bromo-5-methoxyphenol **1e**.[89] In a similar approach, a selective bromination of 2,3-difluorophenol was performed to synthesise 2-bromo-5,6-difluorophenol (**1h**). In this particular case, the procedure using NBS as bromination agent led to a mixture of polybrominated products. For that reason, equimolecular amount of bromine and 2 equiv of *tert*-butylamine in toluene were added at low temperature to afford **1h** after purification by column chromatography.[74b] Afterwards, substrates **1a-h** were alkylated using 1 equiv of chloroacetone (**2**), 2 equiv of potassium carbonate and catalytic amounts of potassium iodide. Acetone was employed as solvent and the reaction mixture was refluxed for 2 hours to achieve the ketones **3a-h** in high to quantitative yields. These ketones were used as starting material in the synthesis of the racemic amines. Thus, **4a-h** were obtained through reductive

[89] M. O. Kitching, T. E. Hurst, V. Snieckus, *Angew. Chem. Int. Ed.* **2012**, *51*, 2925-2929.

amination using 2 equiv of sodium cyanoborohydride and a large excess of ammonium acetate in dry methanol. After 16 hours and purification by column chromatography on silica gel, the amines were afforded in low to moderate yields. Longer reaction times were tested in order to improve these results as well as palladium-catalysed reductive aminations in the presence of ammonium formate. Sadly, these approaches did not lead to better results.

Scheme 1.9. Chemical synthesis of the racemic amines **4a-h** from the corresponding 2-bromophenols **1a-e**,**h** and pyridinols **1f** and **1g**.



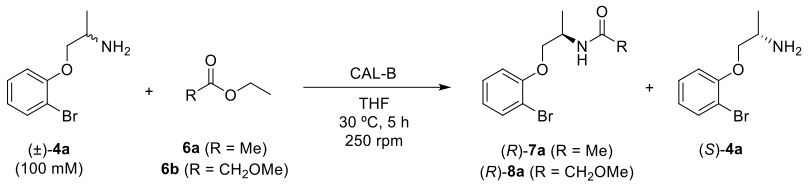
1.2.2. Enzymatic acylation of 1-[2-bromophenoxy]propan-2-amine, (±)-4a

Once the amines (±)-**4a-h** were synthesised, the hydrolase-catalysed kinetic resolution of these racemic substrates was attempted. Lipase-catalysed acylation of racemic amines and alcohols using esters as acyl donors is a common strategy for the resolution of these compounds.[90] Furthermore, lipase from *Candida antarctica* type B (CAL-B) has been successfully used in our research group in the kinetic resolution of primary amines.[71,91] For these reasons, CAL-B was the enzyme of choice for our study and non-activated esters such as ethyl acetate (**6a**) and ethyl methoxyacetate (**6b**) were initially tested as acyl donors. The less hindered racemic amine, 1-(2-bromophenoxy)propan-2-amine (**4a**, 100 mM) was chosen as model substrate (Table 1.1) employing dry tetrahydrofuran (THF) as solvent. CAL-B was added in ratio 1:1 w/w regarding the substrate, 3 equiv of the acyl donors were

[90] (a) D. Méndez-Sánchez, M. López-Iglesias, V. Gotor-Fernández, *Curr. Org. Chem.* **2016**, *11*, 1186-1203; (b) M. Paravidino, U. Hanefeld, *Green Chem.* **2011**, *13*, 2651-2657.
[91] (a) M. Rodríguez-Mata, V. Gotor-Fernández, J. González-Sabín, F. Rebolledo, V. Gotor, *Org. Biomol. Chem.* **2011**, *9*, 2274-2278; (b) O. Torre, E. Busto, V. Gotor-Fernández, V. Gotor, *Adv. Synth. Catal.* **2007**, *349*, 1481-1488.

initially assayed and the biotransformations were incubated at 30 ºC and 250 rpm. After 5 hours, we observed similar selectivity but higher reactivity when using **6b** as acyl donor (entries 1 and 2).

Table 1.1. Kinetic resolution of racemic amine **4a** in dry THF using CAL-B as biocatalyst and ethyl acetate (**6a**) or ethyl methoxyacetate (**6b**) as acyl donors.[a]

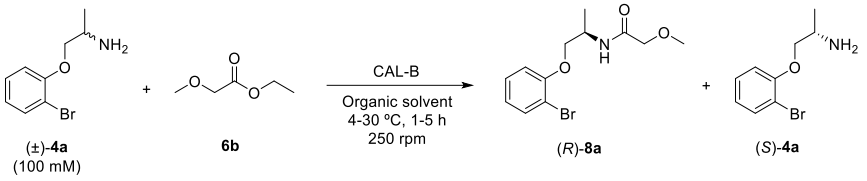


Entry	Acyl donor	t (h)	ee_p (%)[c]	ee_s (%)[d]	c (%)[d]	E[e]
1	**6a**	5	95	38	28	57
2	**6b**	5	83	99	54	56

[a] Reaction conditions: **4a** (100 mM in THF), CAL-B (ratio 1:1 amine:enzyme in weight), 3 equiv **6a-b**, 30 ºC, 5 h at 250 rpm. [b] Ratio amine **4a**:CAL-B (w/w). [c] Enantiomeric excess values were determined by HPLC. [d] Conversion: $c = ee_s/(ee_s + ee_p)$. [e] Enantioselectivity: $E = \ln[(1 - c)(1 - ee_p)]/\ln[(1 - c)(1 + ee_p)]$.

At this point, we decided to continue the study of different reaction parameters using ethyl methoxyacetate as acyl donor, although using a less amount to stop the reactions closer to 50% conversion (Table 1.2). First of all, the lipase-catalysed acylation was carried out using a lower amount of ethyl methoxyacetate, this is, 1 or 2 equiv (entries 2 and 3), leading to conversion values close to 50% and very high selectivity in short reaction times. This way, when using 2 equiv of **6b**, the enantiopure methoxyacetamide (*R*)-**8a** and the enantioenriched (*S*)-**4a** were obtained after just 1.5 hours. Later on, the influence of different solvents, temperatures and amounts of enzyme was studied. Hence, *tert*-butyl methyl ether (MTBE) was also assayed as solvent (entry 4), finding that the reaction occurred very quickly but with less selectivity than in THF. Additionally, the temperature was decreased down to 4 and 20 ºC (entries 5-7), achieving in both cases the (*R*)-amide and the (*S*)-amine with high selectivity. Nevertheless, reducing the loading of enzyme did not reveal better results (entries 8 and 9).

Table 1.2. Optimisation of reaction parameters of the KR of amine (±)-**4a** using CAL-B as biocatalyst and ethyl methoxyacetate (**6b**) as acyl donor.[a]

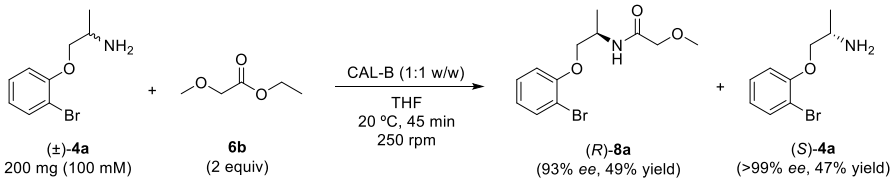


Entry	CAL-B[b]	Solvent	**6b** equiv	T (°C)	t (h)	ee_p (%)[c]	ee_s (%)[c]	c (%)[d]	E[e]
1	1:1	THF	3	30	5	83	99	54	56
2	1:1	THF	1	30	2.5	95	95	50	154
3	1:1	THF	2	30	1.5	93	>99	52	156
4	1:1	MTBE	1	30	1.5	69	>99	59	72
5	1:1	THF	2	4	1	97	92	49	>200
6	1:1	THF	2	4	2	96	99	51	>200
7	1:1	THF	2	20	1	95	99	51	>200
8	0.5:1	THF	2	20	1	97	86	47	195
9	0.5:1	THF	2	20	2	96	96	50	193

[a] Reaction conditions: **4a** (100 mM in THF or MTBE), CAL-B (ratio w/w), **6b** (1-3 equiv), 4-30 ºC, 1-5 h at 250 rpm. [b] Ratio amine **4a**:CAL-B (w/w). [c] Enantiomeric excess values were determined by HPLC. [d] Conversion: $c = ee_s/(ee_s + ee_p)$. [e] Enantioselectivity: $E = \ln[(1 - c)(1 - ee_p)]/\ln[(1 - c)(1 + ee_p)]$.

After all these experiments, 100 mM substrate concentration in THF, 2 equiv of **6b**, CAL-B as biocatalyst in a ratio substrate/enzyme 1:1 (w/w) and 20 ºC (entry 7) were chosen as adequate reaction conditions and they were applied in the kinetic resolution of 200 mg of (±)-**4a** (Scheme 1.10). After 45 minutes, it was possible to achieve 51% conversion with excellent selectivity. Purification by column chromatography led to the isolation of optically active methoxyacetamide (*R*)-**8a** (93% *ee*, 49% isolated yield) and enantiopure amine (*S*)-**4a** (>99% *ee*, 47% isolated yield), proving the reproducibility and applicability of this protocol.

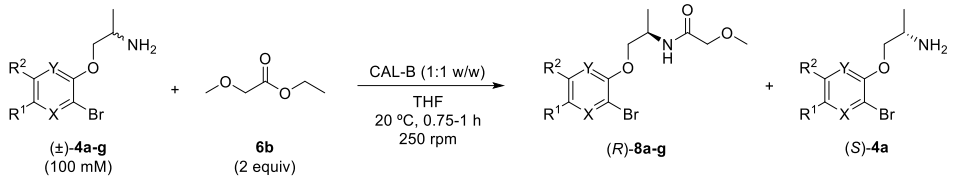
Scheme 1.10. Scale-up of the kinetic resolution of racemic amine **4a** mediated by CAL-B.



1.2.3. Kinetic resolution of racemic 1-[2-bromo(het)aryloxy]propan-2-amines, (±)-4b-h

After developing the lipase-catalysed kinetic resolution of (±)-**4a** and in order to increase the scope of substrates, the same reaction conditions optimised for the model substrate (Table 1.2, entry 7), were applied in the kinetic resolution of racemic amines **4b-h** (Table 1.3). In this way, the protocol was extended to a panel of substrates differing in the pattern substitution in the phenyl ring (**4b-e**,**h**) and also to pyridine derivatives (**4f** and **4g**).

Table 1.3. Lipase-catalysed kinetic resolution of 1-[2-bromo(het)aryloxy]propan-2-amines (**4a-g**) using CAL-B and **6b** in dry THF.[a]



Entry	Amine	R^1	R^2	X	Y	t (h)	ee_p (%)[b]	ee_s (%)[b]	*c* (%)[c]	*E*[d]
1	**4a**	H	H	CH	CH	1	95	99	51	>200
2	**4b**	CN	H	CH	CH	1	93	90	50	91
3	**4c**	Me	H	CH	CH	1	97	>99	51	>200
4	**4d**	F	H	CH	CH	1	95	>99	51	>200
5	**4e**	H	OMe	CH	CH	0.75	88	>99	53	115
6	**4e**	H	OMe	CH	CH	1	79	>99	56	62
7	**4f**	H	H	N	CH	0.75	99	>99	50	>200
8	**4f**	H	H	N	CH	1	97	>99	51	>200
9	**4g**	H	H	CH	N	0.75	98	>99	50	>200
10	**4g**	H	H	CH	N	1	93	>99	52	>200

[a] Reaction conditions: **4a-g** (100 mM in THF), CAL-B (ratio 1:1 in weight), 2 equiv of **6b**, 20 ºC, 0.75-1 h at 250 rpm. [b] Enantiomeric excess values were determined by HPLC. [c] Conversion: $c = ee_s/(ee_s + ee_p)$. [d] Enantioselectivity: $E = \ln[(1 - c)(1 - ee_p)]/\ln[(1 - c)(1 + ee_p)]$.

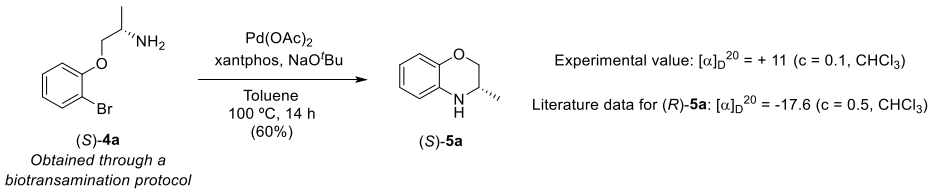
Amine (±)-**4b** containing a cyano group at position 4 of the phenyl ring (entry 2), led to worse results in terms of selectivity to those obtained with the model substrate (**4a**, entry 1). However, substrates (±)-**4c** and **4d** led to excellent enantioselectivity of the corresponding methoxyacetamides, (*R*)-**8c** and (*R*)-**8d** and very high *ee* values of the remaining amines, (*S*)-**4c** and (*S*)-**4d** (entries 3 and 4). The presence of a methoxy motif at position 5 of the phenyl group, (±)-**4e**, speeded up the acylation process and it was possible to obtain both enantioenriched amine and amide after just 45 minutes (entry 5). The same effect was observed with the

pyridine derivatives, **4f** and **4g** (entries 7 and 9). Longer reaction times for these three substrates led to a loss of selectivity (entries 6 and 10). Unfortunately, amine (±)-**4h**, which can subsequently be transformed into the levofloxacin precursor (Scheme 1.3.B),[75] revealed to be unstable in the reaction media, so the attempts to resolve it were unsuccessful.

1.2.4. Assignment of the absolute configuration

The absolute configuration of amines **4a-g** and the corresponding methoxyacetamides **8a-g** had to be established. In this way, amine (*S*)-**4a** was previously synthesised through a transaminase-catalysed approach and its configuration elucidated by its transformation into (*S*)-3-methyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine, (*S*)-**5a** (Scheme 1.11). [92] The benzoxazine derivative precursor (*R*)-**5a** had already been reported in our research group,[87] so the absolute configuration of (*S*)-**4a** was demonstrated by comparing the optical rotation of the formed (*S*)-**5a** with the previously described. The assignment of the absolute configuration of the amine **4a** and the corresponding amide **8a** obtained through our lipase-catalysed protocol was established by measuring the optical rotation of **4a** and comparing that value to the one obtained *via* transamination. This way, it was concluded that CAL-B led to the formation of (*S*)-**4a** and (*R*)-**8a**. Later on, the optical rotation values for the remaining amines **4b-g** and the amides **8b-g** were measured and, again, compared to the ones obtained through the biotransamination experiments, concluding that the lipase-catalysed protocol led in all cases to the production of the (*R*)-amides, affording the (*S*)-amines untouched. This statement is in agreement with the expected configurations considering the Kazlauskas' rule which has been explained in this Thesis preface.

Scheme 1.11. Assignment of the absolute configuration of enantiopure **4a** obtained through a biotransamination protocol.

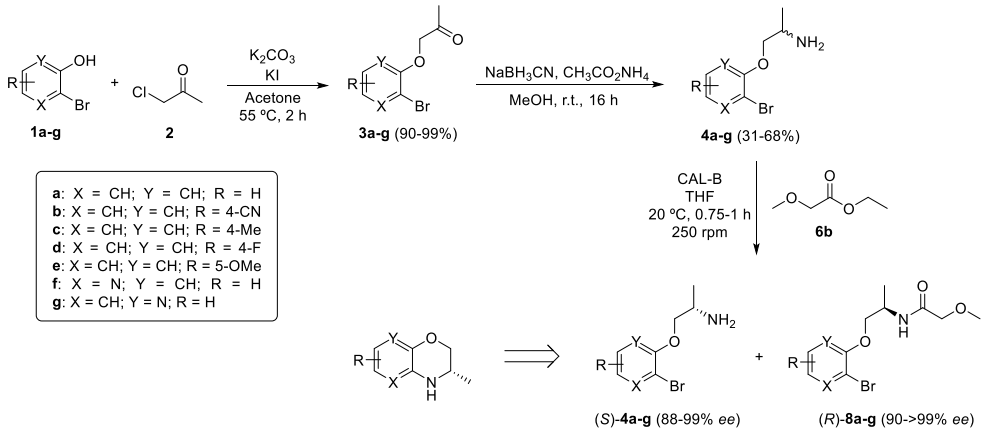


[92] Ph D Thesis, María López Iglesias, "Synthesis of nitrogenated heterocycles through combination of chemical and enzymatic strategies. Application of Biocatalysis in non-conventional processes", University of Oviedo, **2015**.

1.2.5. Final remarks

To sum up, we have described the chemoenzymatic synthesis of optically active 1-[2-bromo(het)aryloxy]propan-2-amines using the lipase from *Candida antarctica* type B as biocatalyst in the stereoselective reaction step (Scheme 1.12). Reaction conditions such as the best acyl donor, the amount of enzyme, the solvent of choice, the temperature and the reaction time were optimised using 1-(2-bromophenoxy)propan-2-amine as model substrate. Later on, CAL-B in combination with 2 equiv of ethyl methoxyacetate allowed the kinetic resolution of a large family of 1-[2-bromo(het)aryloxy]propan-2-amines, affording conversion values close to the ideal 50% and high to excellent enantioselectivities for both (*S*)-amines and (*R*)-amides in short reaction times. The enantioenriched **4a-g** amines are valuable precursors of benzoxazine derivatives.

Scheme 1.12. Chemoenzymatic approach to stereoselectively access (*S*)-1-[2-bromo(het)aryloxy]propan-2-amines **4a-g** and the corresponding (*R*)-amides **8a-g**.



1.3. Experimental section

1.3.1. General information

Chemical reagents were purchased from Acros Organics and Sigma-Aldrich, and used without further purification. Dry solvents were distilled over an adequate desiccant under nitrogen: dichloromethane and methanol over CaH_2, while for toluene and tetrahydrofuran the sodium/benzophenone system was used. *Candida antarctica* lipase type B (CAL-B, Novozyme 435, 7300 PLU/g) was kindly donated by Novozymes.

1.3.2. Synthesis of 2-bromophenols 1e and 1h

*Synthesis of 2-bromo-5-methoxyphenol (**1e**)*[89]

N-Bromosuccinimide (811 mg, 4.55 mmol) was added in one portion to a solution of 3-methoxyphenol (500 µL, 4.55 mmol) in dry CH_2Cl_2 (90 mL) under inert atmosphere. The reaction mixture was stirred at room temperature for 2 h and then washed with water (30 mL). The organic layer was dried over Na_2SO_4, subjected to filtration and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (10% Et_2O/hexane), to afford the bromophenol **1e** as a colourless oil (693 mg, 75% yield).

*Synthesis of 6-bromo-2,3-difluorophenol (**1h**)*[74b]

Bromine (119 µL, 2.31 mmol) was added dropwise over 3 min to a cooled (–30 ºC) solution of $^tBuNH_2$ (485 µL, 4.61 mmol) in dry toluene (5.8 mL) until formation of a yellow solution. The mixture was cooled to –78 ºC and after 10 min, a solution of 2,3-difluorophenol (300 mg, 2.31 mmol) in dry CH_2Cl_2 (0.6 mL) was added dropwise over 5 min. The mixture was allowed to warm slowly to room temperature over 4 h, stirring the resulting mixture for additional 1.5 h at this temperature. The mixture was diluted with EtOAc (10 mL) and washed with an aqueous HCl 1 M solution (2 x 10 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The reaction crude was purified by column chromatography on silica gel (15% Et_2O/hexane), to afford the bromophenol **1h** as a colourless oil (439 mg, 91% yield).

1.3. Experimental section

1.3.3. Synthesis of 1-[2-bromo(het)aryloxy]propan-2-ones 3a-h

Chloroacetone (**2**, 82 µL, 1.03 mmol) was added to a mixture of potassium carbonate (238 mg, 1.72 mmol), potassium iodide (41 mg, 0.25 mmol) and the corresponding bromophenol **1a-h** (0.86 mmol) in acetone (3 mL) at room temperature. The mixture was stirred and heated at 55 ºC for 2 h. After this time, the solution was added to water (5 mL) and the product was extracted with Et_2O (4 x 10 mL). The organic layers were combined, washed with water (20 mL), dried over Na_2SO_4, filtered and concentrated in vacuo, isolating the bromoacetophenones **3a-h** with high purity without further purification (90-99% yield).

1.3.4. Synthesis of racemic 1-[2-bromo(het)aryloxy]propan-2-amines 4a-h

To a solution of the corresponding ketone **3a-h** (0.43 mmol) in dry MeOH (1.4 mL), ammonium acetate (335 mg, 4.34 mmol) and sodium cyanoborohydride (55 mg, 0.87 mmol) were successively added under inert atmosphere. The mixture was stirred at room temperature for 16 h. After this time, water (15 mL) was added to quench the reaction. The solution was carefully acidified with a few drops of a concentrated aqueous HCl solution and washed with Et_2O (3 x 15 mL), discarding the combined organic layer. The aqueous phase was basified with 2-3 pellets of NaOH and extracted with Et_2O (3 x 20 mL), combining the organic layers, which were dried over Na_2SO_4, filtered and concentrated in vacuo. The resulting reaction crude was purified by column chromatography on silica gel (10% MeOH/CH_2Cl_2) to afford the racemic amines **4a-h** (31-68% yield).

1.3.5. Synthesis of racemic 1-[2-bromo(het)aryloxy]propan-2-methoxyacetamides 8a-h

4-Dimethylaminopyridine (1.5 mg, 0.012 mmol), triethylamine (25 µL, 0.18 mmol) and methoxyacetyl chloride (11 µL, 0.12 mmol) were successively added to a solution of the corresponding racemic amine **4a-h** (0.06 mmol) in dry CH_2Cl_2 (2.1 mL). The reaction was stirred at room temperature for 1 h and after this time the solvent was removed by distillation under reduced pressure. The crude was purified by column chromatography on silica gel (5% MeOH/CH_2Cl_2) to afford the corresponding methoxyacetamides **8a-h** (85-99% yield).

1.3.6. General procedure for the lipase-catalysed kinetic resolution of racemic amines 4a-h

Ethyl methoxyacetate (**6b**, 23.5 µL, 0.20 mmol) and CAL-B (ratio 1:1 in weight amine/enzyme) were added to a suspension containing the corresponding racemic amine **4a-h** (0.10 mmol) in dry THF (0.1 M, 1 mL) under inert atmosphere. The reaction was shaken at 20 ºC and 250 rpm for the necessary time (0.75-1 h) to achieve a conversion closed to 50% (see Table 1.3), monitoring the biotransformation by HPLC analysis. The enzyme was filtered off, washed with CH_2Cl_2 (3 x 5 mL) and the solvent was evaporated under reduced pressure. The crude reaction was purified by column chromatography on silica gel (eluent gradient 5-10% MeOH/CH_2Cl_2) to afford the corresponding optically active methoxyacetamides (*R*)-**8a-g** (88-99% *ee*) and amines (*S*)-**4a-g** (90-99% *ee*).

1.3.7. Semi-preparative kinetic resolution of 1-(2-bromophenoxy)propan-2-amine (4a)

Ethyl methoxyacetate (**6b**, 204.0 µL, 1.74 mmol) and CAL-B (200 mg, ratio 1:1 in weight amine/enzyme) were added to a suspension containing the racemic amine **4a** (200 mg, 0.87 mmol) in dry THF (0.1 M, 8.7 mL) under inert atmosphere. The reaction was shaken at 20 ºC and 250 rpm for a 45 min to achieve a 51% conversion with excellent selectivity. The enzyme was filtered off, washed with CH_2Cl_2 (3 x 15 mL) and the solvent evaporated under reduced pressure. The crude reaction was purified by column chromatography on silica gel (eluent gradient 5-10% MeOH/CH_2Cl_2) to afford the corresponding optically active methoxyacetamide (*R*)-**8a** (93% *ee*, 49% isolated yield) and amine (*S*)-**4a** (>99% *ee*, 47% isolated yield).

Publication 1

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Stereoselective Access to 1-[2-Bromo(het)aryloxy]propan-2-amines Using Transaminases and Lipases; Development of a Chemoenzymatic Strategy Toward a Levofloxacin Precursor

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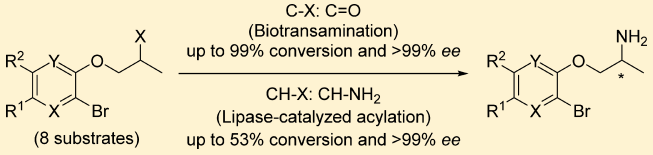
Supporting Information

ABSTRACT: Two independent enzymatic strategies have been developed toward the synthesis of enantioenriched 1-[2-bromo(het)aryloxy]propan-2-amines. With that purpose a series of racemic amines and prochiral ketones have been synthesized from commercially available 2-bromophenols or brominated pyridine derivatives bearing different pattern substitutions in the aromatic ring. Biotransamination experiments have been studied using ketones as starting materials, yielding both the (*R*)- and (*S*)-amine enantiomers with high selectivity (91−99% *ee*) depending on the transaminase source. In a complementary approach, the classical kinetic resolutions of the racemic amines have been investigated using *Candida antarctica* lipase type B as biocatalyst. Ethyl methoxyacetate was found as a suitable acyl donor leading to the corresponding (*S*)-amines (90−99% *ee*) and (*R*)-amides (88−99% *ee*) with high selectivity in most of the cases. A preparative biotransamination process has been developed for the synthesis of (2*S*)-1-(6-bromo-2,3-difluorophenoxy)propan-2-amine in 61% isolated yield after 24 h, a valuable precursor of the antimicrobial agent Levofloxacin.



INTRODUCTION

Chiral amines are attractive building blocks for the synthesis of biologically active and high added-value products with interest in different chemical industrial sectors.[1] Remarkably, the use of biotransformations provides nowadays a plethora of possibilities for the design of stereoselective routes toward enantiopure amines and their derivatives.[2] Optically active 1-aryloxy-propan-2-amines (Scheme 1, R^2= NH_2) are particularly attractive nitrogenous compounds, the absolute configuration of their chiral center having a remarkable importance in their biological profiles.[3] From this family, 1-(2,6-dimethylphenoxy)propan-2-amine, also called as mexiletine, has attracted great attention for clinical purposes due to its properties as antiarrhythmic agent.[4] In past years, the versatility of biocatalytic reactions have been demonstrated toward the asymmetric synthesis of the non substituted 1-phenoxy-propan-2-amine by means of lipase-catalyzed resolutions,[5] and more recently biotransamination reactions from the corresponding propanones.[6] Remarkably, Turner and co-workers reported the conversion of racemic alcohols into enantiopure amines through a redox self-sufficient enzymatic cascade using an alcohol dehydrogenase and an amine dehydrogenase, yielding among other chiral amines, the enantiopure (*R*)-1-phenoxy-propan-2-amine in 84% conversion.[7]

The introduction of additional functionalities provides new opportunities in medicinal and synthetic chemistry. In this context, we have recently described the versatility of 1-(2-nitroaryloxy)propan-2-ones for the synthesis of benzoxazine fragments (Scheme 1), including the preparation of (*S*)-(−)-7,8-difluoro-3-methyl-3,4-dihydro-2*H*-benzo[*b*][1,4]-oxazine, a key precursor of the antimicrobial agent Levofloxacin (Figure 1).[8] On the one hand, lipase from *Rhizomucor miehei* was able to catalyze with excellent selectivity the resolution of racemic alcohols and acetates through acylation and hydrolysis reactions, respectively. On the other hand, various alcohol dehydrogenases (ADHs) led to the production of both (*R*) and (*S*)-1-(2-nitroaryloxy)propan-2-ols with different pattern substitution in the aromatic ring using the evo-1.1.200 ADH and the ADH from *Rhodococcus ruber* (ADH-A), respectively.

Herein, we have carried out a chemoenzymatic route toward benzoxazine precursors, identifying 1-[2-bromo(het)aryloxy]-propan-2-ones as key compounds. First, biotransamination experiments have been investigated for the production of the corresponding amine enantiomers depending on the transaminase source. Alternatively, the reductive amination of the ketones can provide access to the corresponding racemic amines, which will be used as starting materials in lipase-catalyzed resolutions. The presence of the amino and bromo substitutions in the aromatic ring opens up the possibility of developing metal-catalyzed intramolecular cyclization for the production of optically active benzoxazine derivatives such as (*S*)-(−)-7,8-difluoro-3-methyl-3,4-dihydro-2*H*-benzo[*b*][1,4]-oxazine, a valuable precursor of Levofloxacin.

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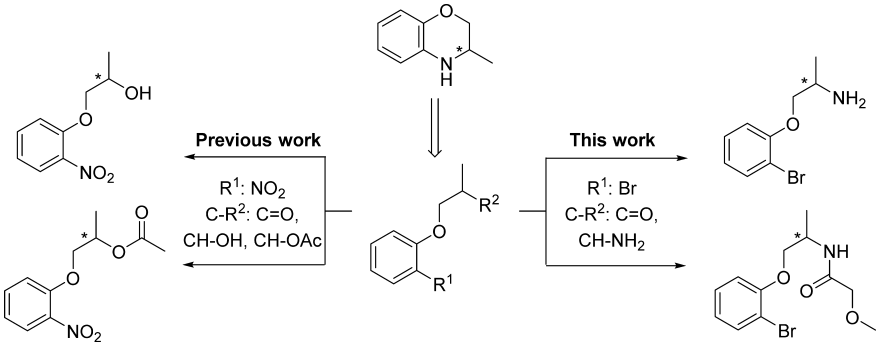
Scheme 1. Chemoenzymatic Routes Toward Valuable Benzoxazine Precursors from 1-(2-Nitrophenoxy)propan-2-ones (left) and 1-(2-Bromophenoxy)propan-2-ones (right)



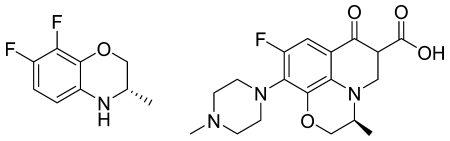


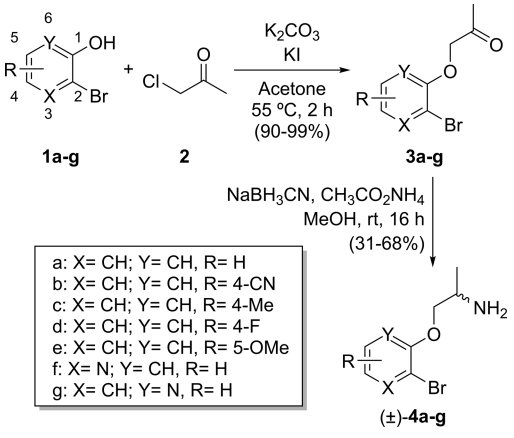
Figure 1. Structures of (*S*)-(−)-7,8-difluoro-3-methyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine (left) and the antimicrobial agent Levofloxacin (right).

RESULTS AND DISCUSSION

Two independent strategies were undertaken for the production of enantiopure 1-[2-bromo(het)aryloxy]propan-2-amines: (a) the transaminase-catalyzed amination of the corresponding 1-[2-nitro(het)aryloxy]propan-2-ones; and (b) the lipase-catalyzed resolution of the racemic amines. Bearing this in mind, a general and convergent route to prepare the prochiral ketones **3a−g** and racemic amines **4a−g** was performed starting from the corresponding commercially available 2-bromophenols **1a−d** and pyridinols **1f** and **1g** (Scheme 2), requiring a previous selective bromination of 3-methoxyphenol at the C-2 position with *N*-bromosuccinimide in the case of the non commercially available methoxy derivative **1e**.[9]

Substrates **1a−g** were alkylated using an equimolecular amount of chloroacetone (**2**) in the presence of 2 equiv of potassium carbonate and catalytic amounts of potassium iodide in refluxing acetone, obtaining after 2 h the ketones **3a−g** in high to quantitative yields. Prochiral ketones **3a−g** served as substrates for biotransamination reactions, but also as starting materials for the synthesis of the racemic amines through reductive amination using 2 equiv of sodium cyanoborohydride in combination with a large excess of ammonium acetate in methanol. Thus, racemic amines **4a−g** were obtained after 16 h at room temperature in low to moderate yields after purification by column chromatography on silica gel. The use of longer reaction times or alternatively the palladium-catalyzed reductive aminations in the presence of ammonium formate did not provide better results.

Scheme 2. Chemical Synthesis of Prochiral Ketones 3a−g and Racemic Amines 4a−g



Biotransamination experiments were initially explored as they allow the synthesis of (*S*)- or (*R*)-amines depending on the enzyme selectivity. In the last decades transaminases (TAs) have emerged as powerful enzymes for the synthesis of chiral amines starting from racemic amines, but more importantly from prochiral ketones as they provide access to a desired amine enantiomer in theoretically 100% yield.[10] The most structurally simple ketone was selected as the model compound for an initial enzyme screening, this is 1-(2-nitrophenoxy)-propan-2-one (**3a**) using a 50 mM substrate concentration. Different amine donors (L/D-alanine or isopropylamine) and cofactor recycling systems were used depending on the transaminase acceptance, which are described in the Experimental Section and Supporting Information. The temperature was kept at 30 °C and the reactions were shaken at 250 rpm in a phosphate buffer 100 mM pH 7.5. Based on our previous experience with the commercially available transaminases,[11] a 2.5% v/v of DMSO was added in the biotransaminations with these TAs to favor the solubility of the ketone **3a** in the reaction medium.

A set of 37 commercially available transaminases and 4 enzymes overexpressed in *Escherichia coli* were tested, including the (*S*)-selective *Arthrobacter citreus*[12] and *Chromobacterium violaceum* TAs,[13] and the (*R*)-selective *Arthrobacter* species[14] and *Aspergillus terreus* TAs.[15] The best results are shown in Table 1, while the results with the total 41 transaminases appear in the Supporting Information (Table S1). From the entire transaminase set, only 12 displayed complete selectivity toward the formation of single enantiomers, 10 for the (*S*)-**4a** and 2 for its antipode (Table S1). From all these selective enzymes, 10 of them led to the amine in more than 40% conversion (Table 1). Remarkably, the (*S*)-selective enzymes ATA-200 and ATA-256 gave the (*S*)-**4a** with excellent conversions (entries 2 and 5, > 96% conversion), obtaining the maximum value in the (*R*)-**4a** formation with the TA from *Aspergillus terreus* (At, 70% conversion, entry 10). For the At TA, an optimization study of the reaction conditions was

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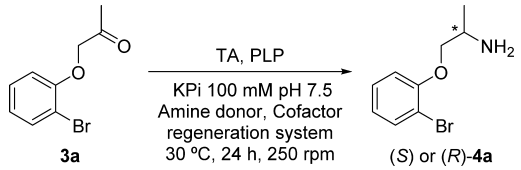
Table 1. Biotransamination of Ketone 3a in Phosphate Buffer 100 mM pH 7.5 after 24 h at 30 °C



entry	TA	amine donor[a]	c (%)[b]	ee (%)[b]
1	TA-P1-G06[c]	IPA	67	> 99 (*S*)
2	ATA-200[c]	IPA	97	> 99 (*S*)
3	ATA-251[c]	IPA	66	> 99 (*S*)
4	AT1-254[c]	IPA	89	> 99 (*S*)
5	ATA-256[c]	IPA	99	> 99 (*S*)
6	ATA-260[c]	IPA	53	> 99 (*S*)
7	ATA-P1-B04[c]	IPA	94	> 99 (*S*)
8	Cv	L-Ala	77	> 99 (*S*)
9	ArR sp.	D-Ala	45	> 99 (*R*)
10	At	D-Ala	70	> 99 (*R*)
11	At[c]	D-Ala	65	> 99 (*R*)
12	At[d]	D-Ala	96	> 99 (*R*)

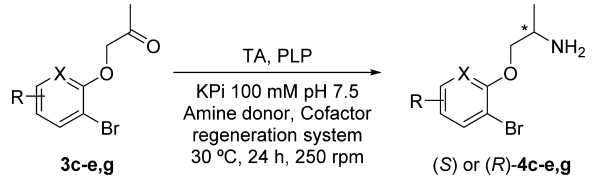
[a]Isopropylamine (IPA) or alanine (L- or D-Ala) was used as amine donors. [b]Conversion and enantiomeric excess values were calculated by GC analysis after derivatization of the amines in the reaction crude with Ac_2O. Absolute configurations appear in parentheses. [c]DMSO was added as cosolvent (2.5% v/v). [d]Double amount of enzyme was used (see Experimental Section).

performed, which showed that the use of DMSO as cosolvent led to a slightly lower conversion (65%, entry 11), while a significant improvement was achieved when doubling the amount of enzyme (96%, entry 12).

The scale-up of the process was performed for 100 mg of ketone **3a** employing the enzyme ATA-256 as biocatalyst, obtaining the enantiopure amine (*S*)-**4a** in 60% isolated yield after a purification by column chromatography (Scheme 3). The absolute configuration of the resulting enantiopure amine (*S*)-**4a** was assigned by its transformation into the 3-methyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine using palladium(II) acetate and xantphos, yielding the benzoxazine (*S*)-**5** in 66% isolated yield without loss of the optical purity. Its optical rotation value was compared with the one previously described in the literature,[16] assigning the (*S*)-configuration for the amine **4a** obtained through the ATA-256 biotransformation.

With these results in hand, the transaminase-catalyzed reactions were performed over the aryloxypropanones **3b**–**e** and hetaryloxypropanones **3f** and **3g** (Table S2). It must be highlighted that a low reactivity was observed with all the TAs tested when ketones **3b** bearing a cyano functionality in the C-4 position and the pyridine derivative **3f** were tested as substrates (<45% conversion). In addition, the formation of side-reaction products was detected in some cases when performing the biotransamination over **3b**. The non heteroaromatic ketones bearing the methyl and the fluoro substitutions on the C-4 position (**3c** and **3d**) led to the best selectivities (Table 2,

Table 2. Biotransamination of Ketone 3c–e,g (50 mM) in Phosphate Buffer 100 mM pH 7.5 after 24 h at 30 °C[a]



entry	substrate	R	X	TA	c (%)[a]	ee (%)[a]
1	**3c**	4-Me	CH	ATA-200[b]	77	> 99 (*S*)
2	**3c**	4-Me	CH	ATA-254[b]	77	> 99 (*S*)
3	**3c**	4-Me	CH	ATA-256[b]	68	> 99 (*S*)
4	**3c**	4-Me	CH	ATA-P1-B04[b]	64	> 99 (*S*)
5	**3c**	4-Me	CH	At	91	> 99 (*R*)
6	**3d**	4-F	CH	ATA-200[b]	76	> 99 (*S*)
7	**3d**	4-F	CH	ATA-254[b]	87	> 99 (*S*)
8	**3d**	4-F	CH	ATA-256[b]	98	> 99 (*S*)
9	**3d**	4-F	CH	ATA-P1-B04[b]	95	> 99 (*S*)
10	**3d**	4-F	CH	At	57	> 99 (*R*)
11	**3e**	5-OMe	CH	ATA-251[b]	97	93 (*S*)
12	**3e**	5-OMe	CH	ATA-256[b]	87	93 (*S*)
13	**3e**	5-OMe	CH	ATA-P1-B04[b]	72	89 (*S*)
14	**3e**	5-OMe	CH	TA-P1-G06[b]	> 99	91 (*S*)
15	**3g**	H	N	ATA-251[b]	93	95 (*S*)
16	**3g**	H	N	ATA-254[b]	88	97 (*S*)
17	**3g**	H	N	TA-P1-G06[b]	97	97 (*S*)

[a]Conversion and enantiomeric excess values were calculated by GC analysis after derivatization of the amines in the reaction crude with Ac_2O. Absolute configurations appear in parentheses. [b]DMSO was added as cosolvent (2.5% v/v).

entries 1–10), obtaining both amine enantiomers depending on the transaminase source. From the set of commercially available transaminases the best results were found with the ATA-200, ATA-254, ATA-256, and ATA-P1-B04, which led to the (*S*)-amines **4c** and **4d**, the fluoro substituted one leading in some cases to almost quantitative conversions (entries 8 and 9). In fact, the 100-mg preparative biotransamination of ketone **3d** with the ATA-256 led to the enantiopure (*S*)-**4d** in 66% isolated yield and very high purity. For the 5-methoxy derivative **3e**, good to quantitative conversions were achieved although none of the enzymes provided access to the (*S*)-**4e** in enantiopure form (entries 11–14). This amine was obtained in 91% *ee* and 74% isolated yield in the preparative biotransformation when using the TA-P1-G06. Finally, the pyridine ketone **3g** led to the (*S*)-**4g** in 95–97% *ee* and high to excellent conversion values (88–97%, entries 15–17). Unfortunately,

Scheme 3. Chemoenzymatic Synthesis of (*S*)-3-Methyl-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine (5) for the Absolute Configuration Assignment of the Amine 4a

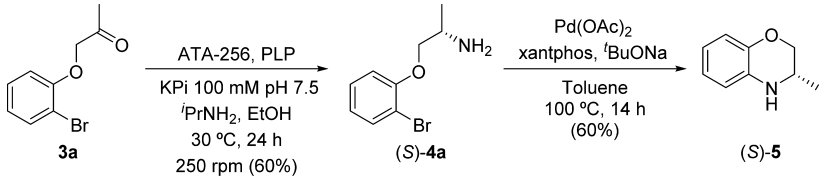


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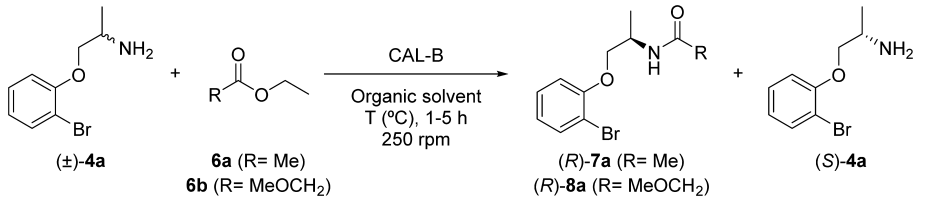
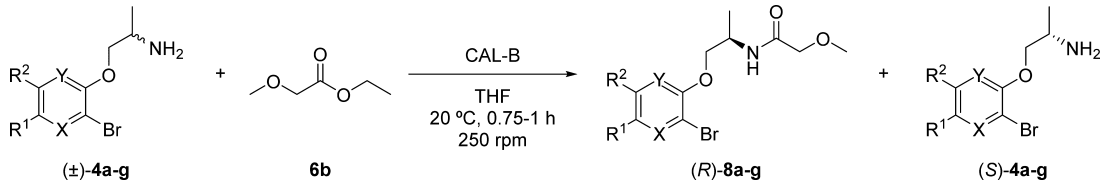
Table 3. Lipase-Catalyzed Kinetic Resolution of 1-(2-Bromophenoxy)propan-2-amine (4a) Using CAL-B[a]



entry	CAL-B[b]	solvent	ester[c]	T (°C)	t (h)	ee_p (%)[d]	ee_s (%)[d]	c (%)[e]	E[f]
1	1:1	THF	**6a** (3 equiv)	30	5	95	38	28	57
2	1:1	THF	**6b** (3 equiv)	30	5	83	99	54	56
3	1:1	THF	**6b** (1 equiv)	30	2.5	95	95	50	154
4	1:1	THF	**6b** (2 equiv)	30	1.5	93	> 99	52	156
5	1:1	MTBE	**6b** (2 equiv)	30	1.5	69	> 99	59	72
6	1:1	THF	**6b** (2 equiv)	4	1	97	92	49	> 200
7	1:1	THF	**6b** (2 equiv)	4	2	96	99	51	> 200
8	1:1	THF	**6b** (2 equiv)	20	1	95	99	51	> 200
9	0.5:1	THF	**6b** (2 equiv)	20	1	97	86	47	195
10	0.5:1	THF	**6b** (2 equiv)	20	2	96	96	50	193

[a]Reaction conditions: **4a** (100 mM in THF or MTBE), CAL-B (ratio w/w), **6a**–**b** (1–3 equiv), 4–30 °C, 1–5 h at 250 rpm. [b]Ratio amine **4a**: CAL-B (w/w). [c]Equivalents of ester in parentheses. [d]Determined by HPLC. Isolated yields in parentheses. [e]$c = ee_s/(ee_s + ee_p)$. [f]$E = \ln[(1 - c)(1 - ee_p)]/\ln[(1 - c)(1 + ee_p)]$.[21]

Table 4. Lipase-Catalyzed Kinetic Resolution of 1-[2-Bromo(het)aryloxy]propan-2-amines (4a–g) Using CAL-B and Ethyl Methoxyacetate (6b) in Dry THF[a]



entry	amine	R^1	R^2	X	Y	t (h)	ee_p (%)[b]	ee_s (%)[b]	c (%)[c]	E[d]
1	**4a**	H	H	CH	CH	1	95	99	51	> 200
2	**4b**	CN	H	CH	CH	1	93	90	50	91
3	**4c**	Me	H	CH	CH	1	97	> 99	51	> 200
4	**4d**	F	H	CH	CH	1	95	> 99	51	> 200
5	**4e**	H	OMe	CH	CH	0.75	88	> 99	53	115
6	**4f**	H	H	N	CH	0.75	99	> 99	50	> 200
7	**4g**	H	H	CH	N	0.75	98	> 99	50	> 200

[a]Reaction conditions: **4a**–**g** (100 mM in THF), CAL-B (ratio 1:1 in weight), **6b** (2 equiv), 20 °C, 0.75–1 h at 250 rpm. [b]Determined by HPLC. Isolated yields in parentheses. [c]$c = ee_s/(ee_s + ee_p)$. [d]$E = \ln[(1 - c)(1 - ee_p)]/\ln[(1 - c)(1 + ee_p)]$.[21]

none of the commercially available TAs gave good activities for the preparation of the (*R*)-amines, the synthesis of (*R*)-**4c** and (*R*)-**4d** in enantiopure form being achieved with 91% and 57% conversion, respectively, when using the At-TA (entries 5 and 10).

In the search of an alternatively methodology for the synthesis of enantiopure amines, the hydrolase-catalyzed kinetic resolution of the racemic amines **4a**–**g** was attempted. Lipase-catalyzed acylation is a common strategy for the resolution of racemic amines and alcohols under mild reaction conditions,[17] mainly using esters as acyl donors.[18] So at this point, an initial screening of the reaction conditions was performed with the less hindered substrate, 1-(2-bromophenoxy)propan-2-amine (**4a**) as the model substrate (Table 3). *Candida antarctica* lipase type B (CAL-B)[19] was the enzyme of choice based on its capability to selectively produce nitrogenous compounds, and tetrahydrofuran was used as solvent since it allows a complete solubility of the amine **4a** in 100 mM concentration. Two non activated esters, such as ethyl acetate (**6a**) and ethyl methoxyacetate (**6b**), were initially assayed as acyl donors, which resulted in a similar selectivity but higher reactivity for **6b** after 5 h (entries 1 and 2). A notable decrease of the enantioselectivity was observed over the time when using **6b**, so the biotransformations were carried out with lower amount of the ester (1 or 2 equiv, entries 3 and 4), which led to conversions close to the ideal 50% and very high selectivity in short reaction times. Similarly, the reaction with methyl *tert*-butyl ether (MTBE) as solvent occurred very quickly but with less selectivity in comparison with THF. At this point, the reaction temperature was decreased, leading to both the (*R*)-amide and the (*S*)-amine with excellent selectivity at both 4 and 20 °C (entries 6–8), while reducing the loading of the enzyme did not provide additional benefits (entries 9 and 10). The reaction at 20 °C and 2 equiv of **6b** (entry 8) was performed at a 200 mg-scale, finding similar results in a highly stereoselective process that allowed the recovery of the (*R*)-amide **8a** and the remaining amine (*S*)-**4a** in 49% and 47% isolated yield, respectively (see Experimental Section).

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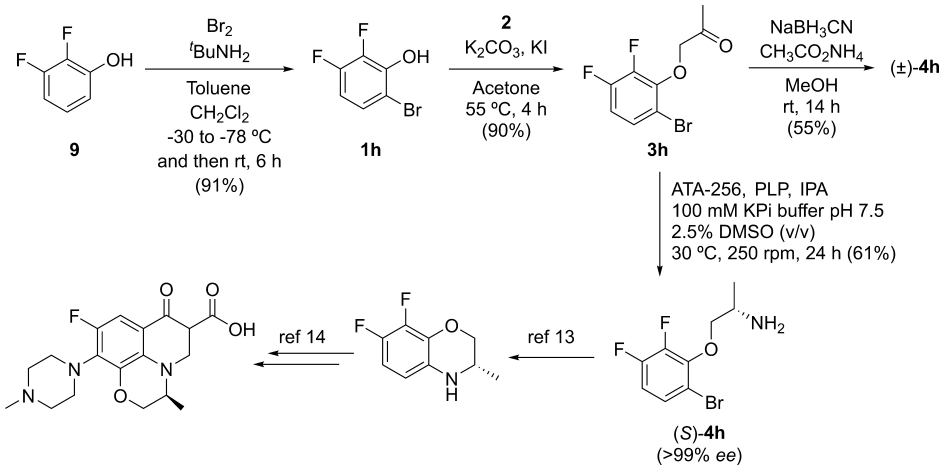
Scheme 4. Chemoenzymatic Synthesis of the Levofloxacin Precursor (*S*)-4h



The assignments of the absolute configurations for the optically active amide **8a** and the remaining amine **4a** were performed by measuring the optical rotation of **4a**, which was compared with the one obtained through transaminase-catalyzed reactions. It was concluded that the lipase-catalyzed reaction led to the (*R*)-**8a** and the (*S*)-**4a**, which is also in agreement with the expected configurations considering the Kazaluskas' rule.[20]

Once adequate reaction conditions were found for the resolution of the racemic amine **4a**, these are 100 mM substrate concentration in THF, 2 equiv of **6b**, CAL-B as enzyme in ratio 1:1 (w/w) with respect to the substrate, and 20 °C, the kinetic resolution was extended to a significant panel of amines bearing different pattern substitutions in the phenyl ring (**4b**–**e**) but also including pyridine derivatives, such as **4f** and **4g**. The results are shown in Table 4 and in all cases close to 50% conversion values were reached (additional information is given in Table S4). The corresponding (*R*)-amides **8a**–**g** and the remaining amines (*S*)-amines **4a**–**g** were isolated with very high to excellent selectivities in just 45 min for the most reactive substrates, such as the one with the methoxy substitution in the C-5 position and the pyridine derivatives (entries 4–7). Interestingly, the lipase-catalyzed approach provide an efficient stereoselective access to all the tested amines, allowing the isolation of enantioenriched **4b** and **4f** that were not obtained through transaminase-catalyzed reactions. The optical rotation values for the remaining amines were measured and later compared with the ones obtained through biotransamination experiments with transaminases of known stereospecificity, concluding that the CAL-B catalyzed acylations led to the (*R*)-methoxyacetamides **8a**–**g** and the (*S*)-amines **4a**–**g**.

Once that the versatility of CAL-B and transaminases was demonstrated in the synthesis of valuable optically active 1-[2-bromo(het)aryloxy]propan-2-amines, we expanded the possibilities of this chemoenzymatic strategy toward the synthesis of (*S*)-**4h**. This amine can be effectively cyclized to the corresponding benzoxazine precursor,[22] which is a valuable precursor of the antimicrobial agent Levofloxacin.[23] The synthetic pathway is depicted in Scheme 4 starting from the commercially available 2,3-difluorophenol (**9**). First, a selective bromination of the aromatic ring was performed using 2 equiv of *tert*-butylamine and equimolecular amount of bromine in toluene at low temperature, yielding after column chromatography the 2-bromo-5,6-difluorophenol (**1h**) in 91% isolated yield.[22a] This procedure improves the results using NBS, which led to a mixture of polybrominated products. Next, the *O*-alkylation proceeded smoothly after 4 h providing the ketone **3h** in 90% yield, which was subjected to the reductive amination reaction with sodium cyanoborohydride and ammonium acetate to yield the racemic amine **4h**. On the one hand, an initial attempt of the stereoselective lipase-catalyzed acylation was performed, but this amine resulted to be quite unstable in the reaction medium, both in the absence or presence of the enzyme. On the other hand, from a set of transaminases (see Table S3) the ATA-256 provided the best results yielding the Levofloxacin precursor (*S*)-**4h** in 99% conversion and 61% isolated yield after purification by column chromatography on silica gel.

CONCLUSIONS

The chemoenzymatic synthesis of optically active 1-[2-bromo(het)aryloxy]propan-2-amines, which are valuable precursors of benzoxazine derivatives have been explored by using transaminases or *Candida antarctica* lipase type B for the stereoselective reaction step. Starting from commercially available 2-bromophenols or pyridine derivatives, prochiral ketones have been obtained through a simple alkylation reaction in good yields. The chemical reductive amination have provided access to the corresponding racemic amines, using both ketones and amines for extensive biocatalytic reaction studies. Transaminases have catalyzed the amination of ketones with especially good results in terms of conversion and selectivity when (*S*)-selective enzymes were considered. In a complementary approach starting from the corresponding racemic amines, their lipase-catalyzed resolution was studied, finding high to excellent selectivity values for the formation of the remaining (*S*)-amines and the (*R*)-methoxyacetamides in close to 50% conversion value.

The applicability of this synthetic strategy has been demonstrated in the production of a valuable precursor of the antimicrobial agent Levofloxacin. Therefore, the (*S*)-1-(6-bromo-2,3-difluorophenoxy)propan-2-amine has been prepared with complete selectivity and a global 50% yield, starting from 2,3-difluorophenol through a three-step sequence that involves a selective bromination reaction, alkykation step, and final biotransamination process using the commercially available ATA-256 enzyme.

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EXPERIMENTAL SECTION

Synthesis of 2-Bromo-5-Methoxyphenol (1e).[9] *N*-bromosuccinimide (811 mg, 4.55 mmol) was added in one portion to a solution of 3-methoxyphenol (500 μL, 4.55 mmol) in dry CH_2Cl_2 (90 mL) under inert atmosphere. The reaction mixture was stirred at room temperature for 2 h and then washed with water (30 mL). The organic layer was dried over Na_2SO_4, subjected to filtration, and concentrated in vacuo. The crude product was purified by column chromatography on silica gel (10% Et_2O/hexane), to afford the bromophenol **1e** as a colorless oil (693 mg, 75%). Physical and spectral data were found to be consistent with those previously reported in the literature.[9]

General Procedure for the Synthesis of Ketones 3a−g. Chloroacetone (**2**, 82 μL, 1.03 mmol) was added to a mixture of potassium carbonate (238 mg, 1.72 mmol), potassium iodide (41 mg, 0.25 mmol), and the corresponding bromophenol **1a−g** (0.86 mmol) in acetone (3 mL) at room temperature, and the mixture was stirred and heated at 55 °C for 2 h. After this time, the solution was added to water (5 mL) and the product was extracted with Et_2O (4 × 10 mL). The organic layers were combined, washed with water (20 mL), dried over Na_2SO_4, and concentrated in vacuo, isolating the bromacetophenones **3a−g** with high purity without further purification (90−99%).

*1-(2-Bromophenoxy)propan-2-one (**3a**).* White solid (195 mg, 99% yield). R_f (40% EtOAc/hexane): 0.61. mp: 67−68 °C. IR (NaCl): ν 3450, 3066, 3007, 2919, 1732, 1586, 1575, 1478, 1444, 1431, 1360, 969, 932, 863, 830 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.37 (s, 3H), 4.54 (s, 2H), 6.77 (dd, $^3J_{HH}$ = 8.2 Hz, $^4J_{HH}$ = 1.3 Hz, 1H), 6.89 (td, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.3 Hz, 1H), 7.25 (ddd, $^3J_{HH}$ = 8.4, 7.5 Hz, $^4J_{HH}$ = 1.6 Hz, 1H), 7.58 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.6 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 27.0 (CH_3), 73.8 (CH_2), 112.1 (C), 113.1 (CH), 122.9 (CH), 128.6 (CH), 133.7 (CH), 154.1 (C), 205.5 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_9H_9BrNaO_2)^+$ $(M+Na)^+$ 250.9678; found 250.9691.

*3-Bromo-4-(2-oxopropoxy)benzonitrile (**3b**).* Yellowish solid (216 mg, 99% yield). R_f (20% Et_2O/hexane): 0.33. mp: 109−110 °C. IR (KBr): ν 3364, 3054, 2987, 2306, 1731, 1601, 1440, 1377, 1322, 923, 896 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.39 (s, 3H), 4.66 (s, 2H), 6.81 (d, $^3J_{HH}$ = 8.5 Hz, 1H), 7.60 (dd, $^3J_{HH}$ = 8.5 Hz, $^4J_{HH}$ = 2.0 Hz, 1H), 7.88 (d, $^4J_{HH}$ = 2.0 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 26.9 (CH_3), 73.4 (CH_2), 106.4 (C), 112.7 (CH), 112.8 (C), 117.4 (C), 133.1 (CH), 137.1 (CH), 157.6 (C), 203.4 (C) ppm. HRMS (ESI$^+$, m/z): calcd for $(C_{10}H_8BrNNaO_2)^+$ $(M+Na)^+$ 275.9631; found 275.9625; calculated for $(C_{10}H_9BrNO_2)^+$ $(M+H)^+$ 253.9811; found 253.9813.

*1-(2-Bromo-4-methylphenoxy)propan-2-one (**3c**).* Yellowish solid (207 mg, 99% yield). R_f (20% Et_2O/hexane): 0.26. mp: 47−49 °C. IR (KBr): ν 3055, 2925, 1724, 1606, 1496, 1359, 1290, 947, 882 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.28 (s, 3H), 2.36 (s, 3H), 4.51 (s, 2H), 6.66 (d, $^3J_{HH}$ = 8.3 Hz, 1H), 7.03 (dd, $^3J_{HH}$ = 8.3 Hz, $^4J_{HH}$ = 2.0 Hz, 1H), 7.39 (d, $^4J_{HH}$ = 2.0 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.3 (CH_3), 27.1 (CH_3), 74.1 (CH_2), 112.0 (C), 113.2 (CH), 129.1 (CH), 132.9 (C), 134.2 (CH), 152.2 (C), 206.1 (C) ppm. HRMS (ESI$^+$, m/z): calcd for $(C_{10}H_{11}BrNaO_2)^+$ $(M+Na)^+$ 264.9835; found 264.9827.

*1-(2-Bromo-4-fluorophenoxy)propan-2-one (**3d**).* White solid (195 mg, 92% yield). R_f (20% Et_2O/hexane): 0.26. mp: 67−68 °C. IR (KBr): ν 3401, 3055, 2306, 1730, 1722, 1594, 1489, 1361, 947, 866 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.36 (s, 3H), 4.52 (s, 2H), 6.75 (dd, $^3J_{HH}$ = 9.0 Hz, $^4J_{FH}$ = 4.6 Hz, 1H), 6.98 (ddd, $^3J_{HH}$ = 9.0 Hz, $^3J_{FH}$ = 7.7 Hz, $^4J_{HH}$ = 3.0 Hz, 1H), 7.34 (dd, $^3J_{FH}$ = 7.7 Hz, $^4J_{HH}$ = 3.0, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 27.0 (CH_3), 74.5 (CH_2), 112.6 (d, $^3J_{FC}$ = 9.9 Hz, C), 114.2 (d, $^3J_{FC}$ = 8.6 Hz, CH), 115.0 (d, $^2J_{FC}$ = 22.9 Hz, CH), 121.0 (d, $^2J_{FC}$ = 25.8 Hz, CH), 151.0 (d, $^4J_{FC}$ = 2.6 Hz, C), 157.4 (d, $^1J_{FC}$ = 244.5 Hz, C), 205.3 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_9H_8BrFNaO_2)^+$ $(M+Na)^+$ 268.9584; found 268.9578.

*1-(2-Bromo-5-methoxyphenoxy)propan-2-one (**3e**).* White solid (205 mg, 92% yield). R_f (50% EtOAc/hexane): 0.68. mp: 62−64 °C. IR (KBr): ν 2840, 1734, 1722, 1586, 1488, 1421, 1360, 1307, 1201, 1169, 1067, 1025 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.32 (s, 3H), 3.73 (s, 3H), 4.47 (s, 2H), 6.30 (d, $^4J_{HH}$ = 2.7 Hz, 1H), 6.41 (dd, $^3J_{HH}$ = 8.7 Hz, $^4J_{HH}$ = 2.7 Hz, 1H), 7.39 (d, $^3J_{HH}$ = 8.7 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 26.9 (CH_3), 55.6 (CH_3), 73.6 (CH_2), 101.1 (CH), 102.7 (C), 107.2 (CH), 133.5 (CH), 154.8 (C), 160.2 (C), 205.4 (C) ppm. HRMS (ESI$^+$, m/z): calcd for $(C_{10}H_{11}BrNaO_3)^+$ $(M+Na)^+$ 280.9784, found: 280.9792.

*1-[(2-Bromopyridin-3-yl)oxy]propan-2-one (**3f**).* White solid (178 mg, 90% yield). R_f (20% Et_2O/hex): 0.38. mp: 73−74 °C. IR (KBr): ν 3054, 2986, 1738, 1565, 1415, 1362, 967, 896, 793, 749, 704 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.40 (s, 3H), 4.60 (s, 2H), 7.05 (dd, $^3J_{HH}$ = 8.1 Hz, $^4J_{HH}$ = 1.6 Hz, 1H), 7.23 (dd, $^3J_{HH}$ = 8.1 Hz, $^3J_{HH}$ = 4.7 Hz, 1H), 8.06 (dd, $^3J_{HH}$ = 4.7 Hz, $^4J_{HH}$ = 1.6 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 26.9 (CH_3), 73.5 (CH_2), 120.0 (CH), 123.4 (CH), 133.0 (C), 142.4 (CH), 151.3 (C), 204.1 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_8H_9BrNO_2)^+$ $(M+H)^+$ 229.9811; found 229.9812; calculated for $(C_8H_8BrNNaO_2)^+$ $(M+Na)^+$ 251.9631; found 251.9624.

*1-[(3-Bromopyridin-2-yl)oxy]propan-2-one (**3g**).* Brown solid (192 mg, 97% yield). R_f (20% Et_2O/hex): 0.20. mp: 101−103 °C. IR (KBr): ν 3055, 2987, 1738, 1656, 1603, 1527, 1422, 1406, 1371, 970, 856 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.31 (s, 3H), 4.76 (s, 2H), 6.14 (t, $^3J_{HH}$ = 7.0 Hz, 1H), 7.18 (dd, $^3J_{HH}$ = 7.0, $^4J_{HH}$ = 1.9 Hz, 1H), 7.78 (dd, $^3J_{HH}$ = 7.0, $^4J_{HH}$ = 1.9 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 27.7 (CH_3), 58.9 (CH_2), 106.1 (CH), 116.4 (C), 137.6 (CH), 142.2 (CH), 158.8 (C), 200.1 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_8H_8BrNNaO_2)^+$ $(M+Na)^+$ 251.9631; found 251.9630.

General Procedure for the Synthesis of Racemic 1-[2-Bromo(het)aryloxy]propan-2-amines 4a−g. To a solution of the corresponding ketone **3a−g** (0.43 mmol) in dry MeOH (1.4 mL), ammonium acetate (335 mg, 4.34 mmol) and sodium cyanoborohydride (55 mg, 0.87 mmol) were successively added under inert atmosphere. The mixture was stirred at room temperature for 16 h, and after this time H_2O (15 mL) was added to quench the reaction. The solution was acidified with a few drops of concentrated aqueous HCl and extracted with Et_2O (3 × 15 mL). The organic layers were discarded and the aqueous phase basified with 2−3 pellets of NaOH, and extracted with Et_2O (3 × 20 mL). The organic layers were combined, dried over Na_2SO_4, and concentrated in vacuo. The reaction crude was purified by column chromatography on silica gel (10% MeOH/CH_2Cl_2), to afford the racemic amines (31−68%).

*1-(2-Bromophenoxy)propan-2-amine (**4a**).* Yellowish oil (36 mg, 36% yield). R_f (10% MeOH/CH_2Cl_2): 0.28. IR (NaCl): ν 3356, 2965, 2929, 2227, 1658, 1614, 1597, 1487, 1369, 1295, 975, 855, 730 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.22 (d, $^3J_{HH}$ = 6.6 Hz, 3H), 2.25 (brs, 2H), 3.22−3.57 (m, 1H), 3.74 (dd, $^2J_{HH}$ = 8.9 Hz, $^3J_{HH}$ = 7.4 Hz, 1H), 3.96 (dd, $^2J_{HH}$ = 8.9 Hz, $^3J_{HH}$ = 4.0 Hz, 1H), 6.75−6.92 (m, 2H), 7.24 (td, $^3J_{HH}$ = 8.3 Hz, $^4J_{HH}$ = 1.6 Hz, 1H), 7.53 (dd, $^3J_{HH}$ = 7.9 Hz, $^4J_{HH}$ = 1.6 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 19.4 (CH_3), 46.5 (CH), 75.2 (CH_2), 112.3 (C), 113.4 (CH), 122.1 (CH), 128.5 (CH), 133.3 (CH), 155.1 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_9H_{13}BrNO)^+$ $(M+H)^+$ 230.0175; found 230.0178.

*4-(2-Aminopropoxy)-3-bromobenzonitrile (**4b**).* Yellowish oil (44 mg, 40% yield). R_f (10% MeOH/CH_2Cl_2): 0.33. IR (NaCl): ν 3364, 3358, 3104, 2227, 1750, 1596, 1494, 1461, 1398, 1397, 886, 816, 751, 715, 672 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.24 (d, $^2J_{HH}$ = 6.6 Hz, 3H), 2.12 (brs, 2H), 3.42−3.53 (m, 1H), 3.82 (dd, $^2J_{HH}$ = 8.8, $^3J_{HH}$ = 7.3 Hz, 1H), 4.01 (dd, $^2J_{HH}$ = 8.8, $^3J_{HH}$ = 4.1 Hz, 1H), 6.93 (d, $^3J_{HH}$ = 8.6 Hz, 1H), 7.58 (dd, $^3J_{HH}$ = 8.5, $^4J_{HH}$ = 2.1 Hz, 1H), 7.83 (d, $^4J_{HH}$ = 2.1 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 19.5 (CH_3), 46.1 (CH), 75.5 (CH_2), 105.3 (C), 112.7 (C), 112.8 (CH), 117.7 (C), 133.1 (CH), 136.6 (CH), 158.6 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{10}H_{12}BrN_2O)^+$ $(M+H)^+$ 255.0128; found 255.0139.

*1-(2-Bromo-4-methylphenoxy)propan-2-amine (**4c**).* Yellowish oil (33 mg, 31% yield). R_f (10% MeOH/CH_2Cl_2): 0.35. IR (NaCl): ν 3355, 2925, 2932, 1663, 1486, 1373, 1287, 933, 796, 782, 685 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.22 (d, $^3J_{HH}$ = 6.6 Hz, 3H), 2.27 (s, 3H), 2.60 (brs, 2H), 3.37−3.48 (m, 1H), 3.72 (dd, $^2J_{HH}$ = 8.9, $^3J_{HH}$

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= 7.4 Hz, 1H), 3.94 (dd, $^2J_{HH}$ = 8.9, $^3J_{HH}$ = 4.0 Hz, 1H), 6.78 (d, $^3J_{HH}$ = 8.4 Hz, 1H), 7.03 (dd, $^3J_{HH}$ = 8.4, $^4J_{HH}$ = 2.1 Hz, 1H), 7.35 (d, $^4J_{HH}$ = 2.1 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 19.3 (CH_3), 20.2 (CH_3), 46.4 (CH), 75.3 (CH_2), 112.0 (C), 113.5 (CH), 128.9 (CH), 131.8 (C), 133.6 (CH), 152.9 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{10}H_{15}BrNO)^+$ $(M+H)^+$ 244.0332; found 244.0322.

*1-(2-Bromo-4-fluorophenoxy)propan-2-amine (**4d**).* Yellowish oil (42 mg, 39% yield). R_f (10% MeOH/CH_2Cl_2): 0.23. IR (NaCl): ν 3410, 3012, 2979, 1687, 1433, 1326, 1290, 947, 867, 753, 701 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.22 (d, $^3J_{HH}$ = 6.6 Hz, 3H), 2.43 (brs, 2H), 3.33−3.53 (m, 1H), 3.71 (dd, $^2J_{HH}$ = 8.8, $^3J_{HH}$ = 7.4 Hz, 1H), 3.93 (dd, $^2J_{HH}$ = 8.8, $^3J_{HH}$ = 4.0 Hz, 1H), 6.83 (dd, $^3J_{HH}$ = 9.1, $^4J_{HF}$ = 4.8 Hz, 1H), 6.93−7.00 (m, 1H), 7.28 (dd, $^3J_{HF}$ = 7.8, $^4J_{HH}$ = 3.0 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 19.2 (CH_3), 46.4 (CH), 75.8 (CH_2), 112.4 (d, $^3J_{FC}$ = 9.9 Hz, C), 114.1 (d, $^3J_{FC}$ = 8.5 Hz, CH), 114.8 (d, $^2J_{FC}$ = 22.6 Hz, CH), 120.3 (d, $^2J_{FC}$ = 25.8 Hz, CH), 151.7 (d, $^4J_{FC}$ = 2.4 Hz, C), 156.7 (d, $^1J_{FC}$ = 243.3 Hz, C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_9H_{12}BrFNO)^+$ $(M+H)^+$ 248.0081; found 248.0085.

*1-(2-Bromo-5-methoxyphenoxy)propan-2-amine (**4e**).* Colorless oil (74 mg, 66% yield). R_f (50% EtOAc/hexane): 0.38. IR (NaCl): ν 3368, 2330, 2174, 1591, 1489, 1467, 1306, 1283, 1203, 1170, 1061, 1023, 828 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.24 (d, $^3J_{HH}$ = 6.6 Hz, 3H), 2.68 (brs, 2H), 3.39−3.52 (m, 1H), 3.70−3.79 (m, 1H) overlapped signal with 3.77 (s, 3H), 3.95 (dd, $^2J_{HH}$ = 8.9 Hz, $^3J_{HH}$ = 4.0 Hz, 1H), 6.39 (dd, $^3J_{HH}$ = 8.7 Hz, $^4J_{HH}$ = 2.7 Hz, 1H), 6.49 (d, $^4J_{HH}$ = 2.7 Hz, 1H), 7.38 (d, $^3J_{HH}$ = 8.7 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 15.3 (CH_3), 48.4 (CH), 55.9 (CH_3), 69.9 (CH_2), 102.1 (CH), 103.1 (C), 108.8 (CH), 133.3 (CH), 154.5 (C), 160.3 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{10}H_{15}BrNO_2)^+$ $(M+H)^+$ 260.0281, found: 260.0275.

*1-[(2-Bromopyridin-3-yl)oxy]propan-2-amine (**4f**).* Yellowish oil (32 mg, 32% yield). R_f (10% MeOH/CH_2Cl_2): 0.28. IR (NaCl): ν 3330, 3321, 2960, 2929, 2227, 1645, 1596, 1563, 1538, 1494, 1447, 1416, 849, 795, 726, 680 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.19 (d, $^3J_{HH}$ = 6.5 Hz, 3H), 1.78 (brs, 2H), 3.35−3.48 (m, 1H), 3.72 (dd, $^2J_{HH}$ = 8.6, $^3J_{HH}$ = 7.5 Hz, 1H), 3.93 (dd, $^2J_{HH}$ = 8.6, $^3J_{HH}$ = 4.1 Hz, 1H), 7.11 (dd, $^3J_{HH}$ = 8.1, $^4J_{HH}$ = 1.6 Hz, 1H), 7.18 (dd, $^3J_{HH}$ = 8.1, $^3J_{HH}$ = 4.6 Hz, 1H), 7.96 (dd, $^3J_{HH}$ = 4.6, $^4J_{HH}$ = 1.6 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 19.8 (CH_3), 46.2 (CH), 75.7 (CH_2), 119.9 (CH), 123.5 (CH), 133.2 (C), 141.5 (CH), 152.3 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_8H_{12}BrN_2O)^+$ $(M+H)^+$ 231.0128; found 231.0123.

*1-[(3-Bromopyridin-2-yl)oxy]propan-2-amine (**4g**).* Yellowish oil (67 mg, 68% yield). R_f (10% MeOH/CH_2Cl_2): 0.21. IR (NaCl): ν 3419, 3093, 2345, 2177, 1706, 1648, 1583, 1530, 1427, 1396, 1327, 976, 866, 855 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.16 (d, $^3J_{HH}$ = 6.5 Hz, 3H), 2.15 (brs, 2H), 3.39−3.52 (m, 1H), 3.70 (dd, $^2J_{HH}$ = 12.9 Hz, $^3J_{HH}$ = 8.1 Hz, 1H), 4.08 (dd, $^2J_{HH}$ = 12.9 Hz, $^3J_{HH}$ = 4.7 Hz, 1H), 6.08 (t, $^3J_{HH}$ = 7.0 Hz, 1H), 7.37 (dd, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.9 Hz 1H), 7.74 (dd, $^3J_{HH}$ = 7.0 Hz, $^4J_{HH}$ = 1.9 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 20.9 (CH_3), 45.8 (CH), 58.4 (CH_2), 105.8 (CH), 116.7 (C), 138.1 (CH), 141.7 (CH), 159.3 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_8H_{11}BrN_2NaO)^+$ $(M+Na)^+$ 252.9947; found 252.9945.

*Synthesis of Racemic N-[1-(2-bromophenoxy)propan-2-yl]-acetamide (**7a**).* 4-Dimethylaminopyridine (8.0 mg, 0.066 mmol), triethylamine (72.2 μL, 0.983 mmol), and acetic anhydride (72.2 μL, 0.655 mmol) were successively added to a solution of the racemic amine **4a** (75 mg, 0.328 mmol) in dry CH_2Cl_2 (2 mL). The reaction was stirred at room temperature for 1 h and after this time the solvent was removed by distillation under reduced pressure. The crude was purified by column chromatography on silica gel (5% MeOH/CH_2Cl_2), to afford the racemic acetamide **7a** as a white solid (86 mg, 96%). R_f (10% MeOH/CH_2Cl_2): 0.65. mp: 67−68 °C. IR (KBr): ν 3053, 2987, 1768, 1662, 1608, 1498, 1267, 914, 896, 761, 721 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.37 (d, $^3J_{HH}$ = 6.9 Hz, 3H), 2.01 (s, 3H), 3.97−4.07 (m, 2H), 4.35−4.47 (m, 1H), 6.13 (d, 1H, $^3J_{HH}$ = 7.7 Hz), 6.82−6.93 (m, 2H), 7.26 (ddd, $^3J_{HH}$ = 8.2, $^3J_{HH}$ = 7.4, $^4J_{HH}$ = 1.6 Hz, 1H), 7.53 (dd, $^3J_{HH}$ = 7.8, $^4J_{HH}$ = 1.6 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.0 (CH_3), 23.8 (CH_3), 45.0 (CH), 72.1 (CH_2), 112.7 (C), 114.0 (CH), 127.8 (CH), 129.1 (CH), 133.7 (CH), 155.3 (C), 170.4 (C) ppm. HRMS (ESI$^+$, m/z): calcd for $(C_{11}H_{15}BrNO_2)^+$ $(M+H)^+$: 272.0281 found: 272.0287; calcd for $(C_{11}H_{14}BrNNaO_2)^+$ $(M+Na)^+$: 294.0100 found: 294.0103.

General Procedure for the Synthesis of Racemic Methoxyacetamides 8a−g. 4-Dimethylaminopyridine (1.5 mg, 0.012 mmol), triethylamine (25 μL, 0.18 mmol), and methoxyacetyl chloride (11 μL, 0.12 mmol) were successively added to a solution of the corresponding racemic amine **4a−g** (0.06 mmol) in dry CH_2Cl_2 (2.1 mL). The reaction was stirred at room temperature for 1 h and after this time the solvent was removed by distillation under reduced pressure. The crude was purified by column chromatography on silica gel (5% MeOH/CH_2Cl_2), to afford the corresponding methoxyacetamide **8a−g** (85−99%).

*N-[1-(2-Bromophenoxy)propan-2-yl]-2-methoxyacetamide (**8a**).* White solid (18 mg, 98% yield). R_f (10% MeOH/CH_2Cl_2): 0.79. mp: 52−53 °C. IR (KBr): ν 3409, 3054, 2983, 2937, 2829, 1678, 1586, 1574, 1483, 1266, 986, 932, 896, 738, 704, 555 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.32−1.60 (m, 3H), 3.42 (s, 3H), 3.89−3.92 (m, 2H), 4.03−4.05 (m, 2H), 4.43−4.51 (m, 1H), 6.77−6.93 (m, 3H), 7.18−7.33 (m, 1H), 7.45−7.59 (m, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.1 (CH_3), 44.4 (CH), 59.6 (CH_3), 72.0 (CH_2), 72.3 (CH_2), 112.8 (C), 113.8 (CH), 122.7 (CH), 129.0 (CH), 133.7 (CH), 155.2 (C), 169.5 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{12}H_{17}BrNO_3)^+$ $(M+H)^+$ 302.0386; found 302.0381; calculated for $(C_{12}H_{16}BrNNaO_3)^+$ $(M+Na)^+$ 324.0206; found 324.0204.

*N-[1-(2-Bromo-4-cyanophenoxy)propan-2-yl]-2-methoxyacetamide (**8b**).* White solid (17 mg, 87% yield). R_f (10% MeOH/CH_2Cl_2): 0.63. mp: 102−103 °C. IR (KBr): ν 3417, 3012, 2182, 1696, 1669, 1605, 1510, 1456, 1298, 1267, 965, 868, 852 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.42 (d, $^3J_{HH}$ = 6.9 Hz, 3H), 3.43 (s, 3H), 3.85−3.97 (m, 2H), 4.07−4.16 (m, 2H), 4.45−4.53 (m, 1H), 6.75 (d, 1H, $^3J_{HH}$ = 6.9 Hz), 6.97 (d, $^3J_{HH}$ = 8.5 Hz, 1H), 7.60 (dd, $^3J_{HH}$ = 8.5, $^4J_{HH}$ = 2.1 Hz, 1H), 7.85 (d, $^4J_{HH}$ = 2.1 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 17.8 (CH_3), 43.7 (CH), 59.2 (CH_3), 71.7 (CH_2), 71.8 (CH_2), 105.6 (C), 112.9 (C), 113.0 (CH), 117.7 (C), 133.2 (CH), 136.7 (CH), 158.4 (C), 169.3 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{13}H_{16}BrN_2O_3)^+$ $(M+H)^+$ 327.0339; found 327.0335; calculated for $(C_{13}H_{15}BrN_2NaO_3)^+$ $(M+Na)^+$ 349.0158; found 349.0164.

*N-[1-(2-Bromo-4-methylphenoxy)propan-2-yl]-2-methoxyacetamide (**8c**).* White solid (16 mg, 85% yield). R_f (10% MeOH/CH_2Cl_2): 0.67. mp: 64−65 °C. IR (KBr): ν 3419, 3093, 2345, 2177, 1698, 1623, 1597, 1419, 1265, 896, 751, 706 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.41 (d, $^3J_{HH}$ = 6.8 Hz, 3H), 2.29 (s, 3H), 3.43 (s, 3H), 3.85−3.97 (m, 2H), 4.00−4.04 (m, 2H), 4.58−4.35 (m, 1H), 6.80 (d, $^3J_{HH}$ = 8.3 Hz, 1H), 6.95 (d, 1H, $^3J_{HH}$ = 7.9 Hz), 7.00−7.13 (m, 1H), 7.37 (d, $^4J_{HH}$ = 2.0 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 17.7 (CH_3), 20.2 (CH_3), 44.0 (CH), 59.3 (CH_3), 71.8 (CH_2), 71.9 (CH_2), 112.1 (C), 113.5 (CH), 128.9 (CH), 132.1 (C), 133.7 (CH), 152.8 (C), 169.1 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{13}H_{19}BrNO_3)^+$ $(M+H)^+$ 316.0543, found: 316.0532; calculated for $(C_{13}H_{18}BrNNaO_3)^+$ $(M+Na)^+$ 338.0362, found 338.0360.

*N-[1-(2-Bromo-4-fluorophenoxy)propan-2-yl]-2-methoxyacetamide (**8d**).* White solid (19 mg, 99% yield). R_f (10% MeOH/CH_2Cl_2): 0.64. mp: 80−81 °C. IR (KBr): ν 3409, 3054, 2986, 1733, 1678, 1527, 1492, 1265, 985, 909, 896, 865, 748, 705 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.41 (d, $^3J_{HH}$ = 6.8 Hz, 3H), 3.43 (s, 3H), 3.91 (m, 2H), 4.01 (m, 2H), 4.38−4.54 (m, 1H), 6.86 (dd, $^3J_{HH}$ = 9.1, $^4J_{HF}$ = 4.7 Hz, 1H), 6.99 (m, $^3J_{HH}$ = 9.1, $^3J_{HF}$ = 7.8, $^4J_{HH}$ = 3.0 Hz, 1H), 7.26−7.34 (m, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 17.6 (CH_3), 44.0 (CH), 59.2 (CH_3), 71.9 (CH_2), 72.4 (CH_2), 112.5 (d, $^3J_{FC}$ = 9.5 Hz, C), 114.0 (d, $^3J_{FC}$ = 8.5 Hz, CH), 114.8 (d, $^2J_{FC}$ = 22.6 Hz, CH), 120.4 (d, $^2J_{FC}$ = 26.3 Hz, CH), 151.5 (C), 156.8 (d, $^1J_{FC}$ = 243.8 Hz, C), 169.2 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{12}H_{16}BrFNO_3)^+$ $(M+H)^+$ 320.0292, found 320.0287; calculated for $(C_{12}H_{15}BrFNNaO_3)^+$ $(M+Na)^+$ 342.0112, found 342.0117.

*N-[1-(2-Bromo-5-methoxyphenoxy)propan-2-yl]-2-methoxyacetamide (**8e**).* White solid (17 mg, 87% yield). R_f (10% MeOH/

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CH_2Cl_2): 0.71. mp: 67−68 °C. IR (KBr): ν 3420, 2934, 2401, 2111, 1670, 1639, 1589, 1423, 1257, 1023, 934, 829 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.41 (d, $^3J_{HH}$= 6.8 Hz, 3H), 3.43 (s, 3H), 3.79 (s, 3H), 3.91 (d, $^3J_{HH}$ = 5.6 Hz, 2H), 4.02−4.12 (m, 2H), 4.36−4.61 (m, 1H), 6.43 (dd, $^3J_{HH}$ = 8.6 Hz, $^4J_{HH}$ = 2.7, 1H), 6.50 (d, $^4J_{HH}$ = 2.7 Hz, 1H), 6.91 (d, 1H, $^3J_{HH}$ = 7.3 Hz), 7.41 (d, $^4J_{HH}$ = 8.6 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 17.7 (CH_3), 43.9 (CH), 55.6 (CH_3), 59.3 (CH_3), 71.8 (CH_2), 71.9 (CH_2), 100.9 (CH), 103.0 (C), 106.9 (CH), 133.1 (CH), 155.5 (C), 160.2 (C), 169.2 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{13}H_{18}BrNNaO_4)^+$ $(M+Na)^+$ 354.0311, found 354.0316.

*N-{1-[(2-Bromopyridin-3-yl)oxy]propan-2-yl}-2-methoxyacetamide (**8f**).* White solid (18 mg, 99% yield). R_f (10% MeOH/CH_2Cl_2): 0.57. mp: 70−80 °C. IR (KBr): ν 3400, 2933, 1667, 1651, 1563, 1530, 1447, 1418, 1294, 796, 727 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.43 (d, $^3J_{HH}$ = 6.8 Hz, 3H), 3.44 (s, 3H), 3.85−3.97 (m, 2H), 4.03−4.12 (m, 2H), 4.44−4.54 (m, 1H), 6.85 (d, 1H, $^3J_{HH}$ = 7.9 Hz), 7.17−7.25 (m, 2H, H_4), 8.02 (dd, $^3J_{HH}$ = 4.4, $^4J_{HH}$ = 1.8 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 17.6 (CH_3), 43.7 (CH), 59.3 (CH_3), 71.6 (CH_2), 71.9 (CH_2), 119.9 (CH), 123.5 (CH), 133.2 (C), 141.7 (CH), 152.0 (C), 169.3 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{11}H_{15}BrN_2NaO_3)^+$ $(M+Na)^+$ 325.0158, found: 325.0145.

*N-{1-[(2-Bromopyridin-3-yl)oxy]propan-2-yl}-2-methoxyacetamide (**8g**).* White solid (16 mg, 89% yield). R_f (10% MeOH/CH_2Cl_2): 0.65. mp: 138−139 °C. IR (KBr): ν 3478, 3319, 2922, 1649, 1596, 1537, 1494, 1378, 1297, 975, 848, 760, 657 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.30 (d, $^3J_{HH}$ = 6.2 Hz, 3H), 3.39 (s, 3H), 3.86−3.96 (m, 2H), 3.97−4.08 (m, 2H), 4.21−4.39 (m, 1H), 6.10 (t, $^3J_{HH}$ = 7.2 Hz, 1H), 6.95 (d, 1H, $^3J_{HH}$ = 6.9 Hz), 7.25−7.37 (m, 1H), 7.73 (dd, $^3J_{HH}$ = 7.2, $^4J_{HH}$ = 1.9 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 18.1 (CH_3), 45.9 (CH), 53.9 (CH_2), 59.4 (CH_3), 71.8 (CH_2), 106.2 (CH), 116.6 (C), 137.3 (CH), 141.8 (CH), 159.6 (C), 169.9 (C) ppm. HRMS (ESI$^+$, m/z): calculated for $(C_{11}H_{16}BrN_2O_3)^+$ $(M+H)^+$ 303.0339, found 303.0342; calculated for $(C_{11}H_{15}BrN_2NaO_3)^+$ $(M+Na)^+$ 325.0158, found: 325.0165.

General Procedure for the Enzymatic Kinetic Resolution by Acylation of Racemic Amines 4a−g. Ethyl methoxyacetate (**6b**, 23.5 μL, 0.20 mmol) and CAL-B (ratio 1:1 in weight amine/enzyme) were added to a suspension containing the corresponding racemic amine **4a−g** (0.10 mmol) in dry THF (0.1 M, 1 mL) under inert atmosphere. The reaction was shaken at 20 °C and 250 rpm for the necessary time (0.75−1 h) to achieve a good kinetic resolution (see Tables 3 and 4). The reaction was followed by HPLC analysis until around 50% conversion was reached. The enzyme was filtered off, washed with CH_2Cl_2 (3 × 5 mL), and the solvent was evaporated under reduced pressure. The crude reaction was purified by column chromatography on silica gel (eluent gradient 5−10% MeOH/CH_2Cl_2), to afford the corresponding optically active methoxyacetamides (*R*)-**8a−g** (88−99% *ee*) and amines (*S*)-**4a−g** (90 → 99% *ee*).

Optical rotation values for the (*R*)-methoxyacetamides **8a−g**: $[\alpha]_D^{20}$ + 35.4 (*c* 1, EtOH) for (*R*)-**8a** in 93% *ee*; $[\alpha]_D^{20}$ + 36.4 (*c* 1, EtOH) for (*R*)-**8b** in 93% *ee*; $[\alpha]_D^{20}$ + 31.8 (*c* 1, EtOH) for (*R*)-**8c** in 96% *ee*; $[\alpha]_D^{20}$ + 25.3 (*c* 0.5, EtOH) for (*R*)-**8d** in 93% *ee*; $[\alpha]_D^{20}$ + 12.6 (*c* 1, EtOH) for (*R*)-**8e** in 88% *ee*; $[\alpha]_D^{20}$ + 19.8 (*c* 1, EtOH) for (*R*)-**8f** in 99% *ee*; $[\alpha]_D^{20}$ − 50.6 (*c* 1, EtOH) for (*R*)-**8g** in 83% *ee*.

Optical rotation values for the (*S*)-amines **4a−g**: $[\alpha]_D^{20}$ + 6.4 (*c* 0.5, EtOH) for (*S*)-**4a** in >99% *ee*; $[\alpha]_D^{20}$ − 30.5 (*c* 0.5, EtOH) for (*S*)-**8b** in 90% *ee* obtained by chemical derivatization of (*S*)-**4b**; $[\alpha]_D^{20}$ + 6.4 (*c* 1, EtOH) for (*S*)-**4c** in >99% *ee*; $[\alpha]_D^{20}$ + 2.5 (*c* 1, EtOH) for (*S*)-**4d** in >99% *ee*; $[\alpha]_D^{20}$ + 13.6 (*c* 1, EtOH) for (*S*)-**4e** in 91% *ee*; $[\alpha]_D^{20}$ + 4.2 (*c* 1, EtOH) for (*S*)-**4f** in >99% *ee*; $[\alpha]_D^{20}$ + 31.3 (*c* 0.5, EtOH) for (*S*)-**4g** in >99% *ee*.

Scale-Up of the Enzymatic Kinetic Resolution by Acylation of Racemic Amine 4a. Ethyl methoxyacetate (**6b**, 204.0 μL, 1.74 mmol) and CAL-B (200 mg, ratio 1:1 in weight amine/enzyme) were added to a suspension containing the corresponding racemic amine **4a** (200 mg, 0.87 mmol) in dry THF (0.1 M, 8.7 mL) under inert atmosphere. The reaction was shaken at 20 °C and 250 rpm for 45 min to achieve a 51% conversion with excellent selectivity. The enzyme was filtered off, washed with CH_2Cl_2 (3 × 15 mL) and the solvent evaporated under reduced pressure. The crude reaction was purified by column chromatography on silica gel (eluent gradient 5−10% MeOH/CH_2Cl_2), to afford the corresponding optically active methoxyacetamide (*R*)-**8a** (93% *ee*, 49% isolated yield) and amine (*S*)-**4a** (>99% *ee*, 47% isolated yield).

General Procedure for the Biotransamination of Ketones 3a−h Employing Isopropylamine as Amino Donor. A solution of ketone **3a−h** (0.025 mmol, 50 mM) in DMSO (12.5 μL) was added to a suspension of a commercial TA (2 mg) in phosphate buffer 100 mM pH 7.5 (500 μL) containing PLP (1 mM) and isopropylamine (1 M). The mixture was shaken at 30 °C and 250 rpm for 24 h. Then, the reaction was quenched by adding an aqueous NaOH 4 M solution (400 μL) and extracted with EtOAc (3 × 500 μL). The organic phases were combined and dried over Na_2SO_4. Reaction crude was analyzed through GC to determine conversion values, and later the enantiomeric excess after an *in situ* derivatization. For the methoxy derivative **4e**, HPLC was employed to determine de *ee* value of the enantioenriched amine.

General Procedure for the Biotransamination of Ketones 3a,c,d Employing Alanine as Amino Donor and Alanine Dehydrogenase as Regeneration System. To a suspension of ketones **3a−d** (0.05 mmol, 50 mM) in a 100 mM phosphate buffer pH 7 (440 μL) were successively added ammonium formate (100 μL of 1.5 M solution in a 100 mM phosphate buffer pH 7; final concentration 150 mM), alanine (250 μL of 1 M solution in phosphate buffer 100 mM pH 7; final concentration 250 mM), NAD^+ (100 μL of 10 mM solution in a 100 mM phosphate buffer pH 7; final concentration 1 mM), PLP (100 μL of 10 mM solution in a 100 mM phosphate buffer pH 7; final concentration 1 mM), lyophilized cells of *E. coli* containing overexpressed transaminases (20 mg), formate dehydrogenase (FDH, 2.6 mg, 11 U), and alanine dehydrogenase (AlaDH, 10 μL, 11 U). D- or L-alanine were used as amine donor depending on the (*R*) or (*S*)-transaminase selectivity, respectively. The resulting mixture was shaken at 30 °C and 250 rpm for 24 h. After this time the reaction was quenched by adding aqueous NaOH 4 M (400 μL), extracted with ethyl acetate (3 × 500 μL), and organic phases were combined and dried with Na_2SO_4. Reaction crude was analyzed through GC to determine conversion values and, after an *in situ* derivatization, the enantiomeric excess was calculated.

*Synthesis of 6-Bromo-2,3-difluorophenol (**1h**).*[22a] Bromine (119 μL, 2.31 mmol) was added dropwise over 3 min to a cooled (−30 °C) solution of $^tBuNH_2$ (485 μL, 4.61 mmol) in dry toluene (5.8 mL) until formation of a yellow solution. The mixture was cooled to −78 °C and after 10 min, a solution of 2,3-difluorophenol (**9**, 300 mg, 2.31 mmol) in dry CH_2Cl_2 (0.6 mL) was added dropwise over 5 min. The mixture was allowed to warm slowly to room temperature over 4 h, stirring the resulting mixture for additional 1.5 h at this temperature. The mixture was diluted with EtOAc (10 mL) and washed with an aqueous HCl 1 M solution (2 × 10 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The reaction crude was purified by column chromatography on silica gel (15% Et_2O/hexane), to afford the bromophenol **1h** as a colorless oil (439 mg, 91%). Physical and spectral data were found to be consistent with those previously reported in the literature.[22a]

*Synthesis of 1-(6-Bromo-2,3-difluorophenoxy)propan-2-one (**3h**).* Chloroacetone (82 μL, 1.03 mmol) was added to a mixture of potassium carbonate (238 mg, 1.72 mmol), potassium iodide (41 mg, 0.25 mmol), and the bromophenol **1h** (111.9 mg, 0.86 mmol) in acetone (3 mL) at room temperature, and the mixture was stirred and heated at 55 °C for 2 h. After this time the solution was added to water (5 mL) and the product was extracted with Et_2O (4 × 10 mL). The organic layers were combined, washed with water (20 mL), dried over Na_2SO_4, and concentrated in vacuo, isolating the bromacetophenone **3h** as an orangish oil (205 mg, 90%). R_f (20% EtOAc/hexane): 0.44. IR (NaCl): ν 3461, 3094, 2921, 1741, 1724, 1612, 1588, 1485, 1462, 1429, 1360, 1297, 1208, 1179, 1058, 1019, 991, 975, 881, 801 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.35 (s, 3H), 4.64 (d, $^5J_{FH}$ = 0.9 Hz, 2H), 6.74−6.92 (m, 1H), 7.26 (ddd, $^3J_{HH}$ = 9.2 Hz, $^4J_{FH}$ = 5.4, $^5J_{FH}$ = 2.4 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 26.7 (CH_3), 77.5 (d, $^4J_{FC}$ = 4.8 Hz, CH_2), 111.1 (d, $^3J_{FC}$ = 3.6 Hz, C), 112.9 (d, $^2J_{FC}$

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= 18.4 Hz, CH), 127.2 (dd, $^3J_{FC}$ = 7.4 Hz, $^4J_{FC}$ = 4.2 Hz, CH), 144.5 (dd, $^1J_{FC}$ = 251.8 Hz, $^2J_{FC}$ = 14.8 Hz, C), 144.8 (d, $^2J_{FC}$ = 7.3 Hz, C), 150.8 (dd, $^1J_{FC}$ = 250.3 Hz, $^2J_{FC}$ = 11.5 Hz, C), 204.1 (C) ppm. HRMS (ESI$^+$, *m*/*z*): calcd for $(C_9H_7BrF_2NaO_2)^+$ $(M+Na)^+$ 286.9490, found: 286.9480.

*Synthesis of 1-(6-Bromo-2,3-difluorophenoxy)propan-2-amine (**4h**).* To a solution of the ketone **3h** (80.0 mg, 0.43 mmol) in dry MeOH (1.4 mL), ammonium acetate (335 mg, 4.34 mmol) and sodium cyanoborohydride (55 mg, 0.87 mmol) were successively added under inert atmosphere. The mixture was stirred at room temperature for 14 h. The reaction crude was purified by column chromatography on silica gel (10% MeOH/CH_2Cl_2), to afford the racemic **4h** as a colorless oil (63 mg, 55%). R_f (10% MeOH/CH_2Cl_2): 0.41. IR (NaCl): ν 3243, 3091, 2335, 1613, 1489, 1457, 1294, 1209, 1055 cm^{-1}. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.26 (d, $^3J_{HH}$ = 6.6 Hz, 3H), 3.28 (brs, 2H), 3.42−3.57 (m, 1H), 3.90−4.02 (m, 1H), 4.16 (ddd, $^2J_{HH}$ = 9.3 Hz, $^3J_{HH}$ = 3.9 Hz, $^5J_{FH}$ = 1.3 Hz, 1H), 6.82 (dt, $^3J_{FH}$ = 9.2 Hz, $^3J_{HH}$ = 7.6, $^4J_{FH}$ = 7.6 Hz, 1H), 7.25 (ddd, $^3J_{HH}$ = 7.6 Hz, $^4J_{FH}$ = 4.9, $^5J_{HF}$ = 2.5 Hz, 1H) ppm. ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 15.1 (CH_3), 48.7 (CH), 74.3 (d, $^4J_{FC}$ = 4.6 Hz, CH_2), 111.7 (d, $^3J_{FC}$ = 3.8 Hz, C), 113.8 (d, $^2J_{FC}$ = 18.4 Hz, CH), 127.2 (dd, $^3J_{FC}$ = 7.4 Hz, $^4J_{FC}$ = 4.2 Hz, CH), 144.3 (dd, $^2J_{FC}$ = 10.1 Hz, $^3J_{FC}$ = 1.8 Hz, C), 145.0 (dd, $^1J_{FC}$ = 252.5 Hz, $^2J_{FC}$ = 14.5 Hz, C), 150.7 (dd, $^1J_{FC}$ = 250.8 Hz, $^2J_{FC}$ = 11.3 Hz, C) ppm. HRMS (ESI$^+$, *m*/*z*): calcd for $(C_9H_{11}BrF_2NO)^+$ $(M+H)^+$ 265.9987, found: 265.9989.

*Preparative Biotransamination of Ketones **3a,d,e,h**.* In a Falcon tube, the TA (35 mg, ATA-256 for ketones **3a,d,h** and TA-P1-G06 for **3e**) was suspended in a phosphate buffer 100 mM pH 7.5 (8.8 mL) containing PLP (1 mM) and isopropylamine (1 M). Then, a solution of ketones **3a**, **3d**, **3e**, or **3h** (0.44 mmol, 50 mM) in EtOH (220 μL) was added. The mixture was shaken at 30 °C and 250 rpm for 24 h. Then, the reaction was quenched by adding an aqueous NaOH 4 M solution until pH ≈ 10 (~2 mL) and extracted with EtOAc (3 × 15 mL). The organic phases were combined, dried over Na_2SO_4, filtered and the solvent was removed under reduced pressure. The reaction crude was purified by column chromatography (MeOH/CH_2Cl_2 mixtures), yielding the amines (*S*)-**4a,d,e,h** in moderate to good yields (60−74%) and good to excellent enantiomeric excess (91 → 99% *ee*). $[\alpha]_D^{20}$= +4.8 (*c* 0.4, EtOH) [for (*S*)-**4h** in >99% *ee*].

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01828.

Enzyme activity screenings, biotransformation reaction course, analytical separations, and ^{1}H, ^{13}C and DEPT NMR spectra for described organic compounds (PDF)

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Notes

The authors declare no competing financial interest.

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(16) Experimental value for (*S*)-**5**: $[\alpha]_D^{20}$ + 11 (c = 0.1, $CHCl_3$); literature data $[\alpha]_D^{20}$ −17.6 (c 0.5, $CHCl_3$) in ref 8.

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DOI: 10.1021/acs.joc.6b01828
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Chapter 2

Synthesis of optically active γ- and δ-lactams through transaminase-catalysed cascade transformations

2.1. Bibliographic background

Lactams are important nitrogenated heterocycles in organic chemistry due to their remarkable biological activities and their important applications in medicine and pharmacology, also serving as valuable intermediates for more complex structures such as synthetic polymers.[93] β-Lactam (azetidin-2-one) ring has been particularly studied since Sir Alexander Fleming discovered the first antibiotic of the human history, penicillin, in 1928.

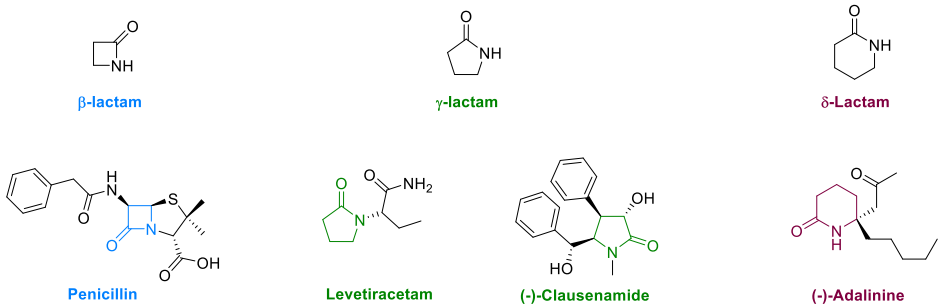


Figure 2.1. General structure of β-, γ- and δ-lactams. Examples of biologically active molecules containing lactam rings.

Penicillin, as many other pharmaceuticals, includes a β-lactam core (Figure 2.1).[94] However, the need of moving away from β-lactams in order to avoid the increasing bacterial resistance towards this traditional core, have opened the door to γ-lactams (pyrrolidin-2-ones) and δ-lactams (piperidin-2-ones), which are present in many biologically active compounds.[95] For instance, in the family of γ-lactams, levetiracetam is one of the main anticonvulsant drugs used to treat epilepsy[96] and (–)-clausenamide is such a promising drug candidate for treatment of neurodegenerative diseases such as Alzheimer's disease.[97] Additionally, (–)-

[93] M. A. Ogliaruso, J. F. Wolfe, *Synthesis of Lactones and Lactams*, John Wiley & Sons, Inc., West Sussex (United Kingdom), **1993**.
[94] (a) R. I. Aminov, *Environ. Microbiol.* **2009**, *11*, 2970-2988; (b) S. M. Drawz, R. A. Bonomo, *Clin. Microbiol. Rev.* **2010**, *1*, 160-201; (c) P. Pérez-Faginas, M. T. Aranda, M. T. García-López, A. Francesch, C. Cuevas, R. González-Muniz, *Eur. J. Med. Chem.* **2011**, *46*, 5108-5119; (d) C. R. Pitts, T. Lectka, *Chem. Rev.* **2014**, *114*, 7930-7953.
[95] (a) E. Martínez de Marigorta, J. M. de los Santos, A. M. Ochoa de Retana, J. Vicario, F. Palacios, *Synthesis* **2018**, *50*, 4539-4554; (b) W. Delong, W. Lanying, W. Yongling, S. Shuang, F. Juntao, Z. Xing, *Eur. J. Med. Chem.* **2017**, *130*, 286-307; (c) J. Caruano, G. G. Muccioli, R. Robiette, *Org. Biomol. Chem.* **2016**, *14*, 10134-10156.
[96] B. Abou-Khalil, *Neuropsychiatr. Dis. Treat.* **2008**, *4*, 507-523.
[97] S.-F. Chu, J.-T. Zhang, *Acta Pharm. Sin. B* **2014**, *4*, 417-423.

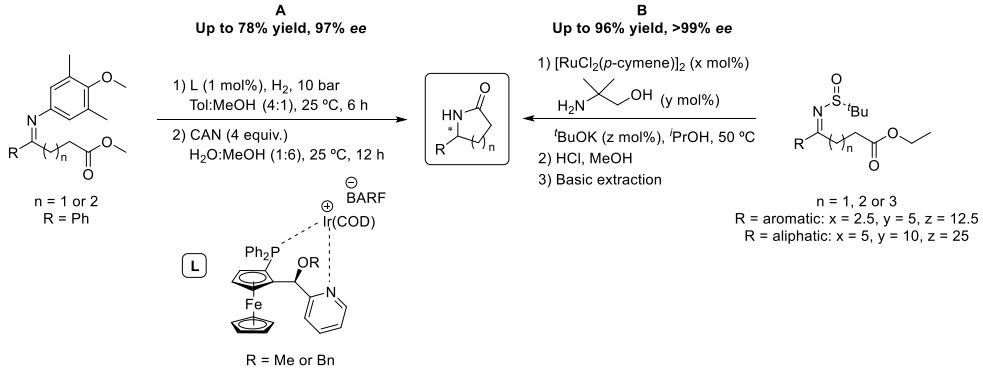
adalinine, containing the δ-lactam scaffold, is a coccinellid alkaloid isolated from ladybird *Adalia bipunctata*.[98]

2.1.1. Synthesis of chiral γ- and δ-lactams

The importance of chirality in these compounds is reflected in Figure 2.1, where it is possible to observe that all the biologically active lactams shown there are chiral molecules. In order to produce chiral γ- and δ-lactams, several chemical and enzymatic methodologies have been developed in recent years.

The synthesis of the γ-lactam core has been specially studied, and some interesting reviews have been published in last years. For instance, in 2014 Gagosz and co-workers published a compilation of metal-catalysed approaches to obtain γ-lactams, some of them introducing chirality in the final molecule.[99] Two years later, Robiette and co-workers discussed different synthetic approaches to this interesting scaffold, including cyclisation approaches, annulation protocols and redox methodologies but also considering natural sources to obtain γ-lactams.[95c] More recently, Palacios and co-workers reviewed multicomponent reactions to produce γ-lactams.[95a]

Scheme 2.1. Synthesis of chiral γ- and δ-lactams using iridium and ruthenium catalysts.



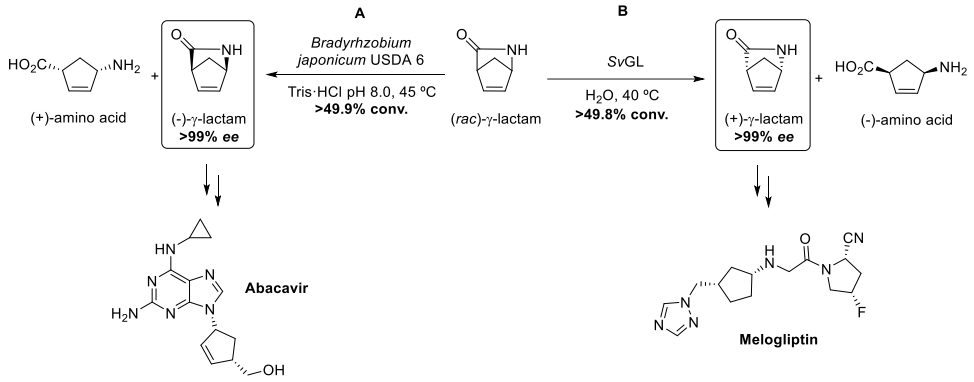
Furthermore, some asymmetric procedures have been described to achieve both γ- and δ-lactams. For instance, an iridium-catalysed procedure to produce molecules containing chiral pyrrolidin-2-one or piperidin-2-one scaffolds was

[98] (a) N. Yamazaki, T. Ito, C. Kibayashi, *Tetrahedron Lett.* **1999**, *40*, 739-742; (b) G. Lognay, J. L. Hemptinne, F. Y. Chan, C. H. Gaspar, M. Marlier, *J. Nat. Prod.* **1996**, *59*, 510-511.
[99] L.-W. Ye, C. Shu, F. Gagosz, *Org. Biomol. Chem.* **2014**, *12*, 1833-1845.

developed by Cheemala and Knochel (Scheme 2.1.A).[100] A similar approach using a ruthenium catalyst led also to the formation of enantioenriched ε-lactams (Scheme 2.1.B).[101] In both cases, the imino esters used as starting material underwent an asymmetric hydrogenation to produce the corresponding amines that were able to spontaneously cyclise into the desired lactams after a deprotection step.

Apart from the numerous chemical approaches, several enzymatic strategies towards the synthesis of chiral γ- and δ-lactams have been reported until now implying the use of different enzyme classes. For instance, γ-lactamases have been applied to the production of the Vince lactam, namely 2-azabicyclo[2.2.1]hept-5-en-3-one (Scheme 2.2).

Scheme 2.2. Synthesis of (–)- and (+)-γ-lactam through a lactamase-catalysed ring-opening of the racemic γ-lactam.[102,103]



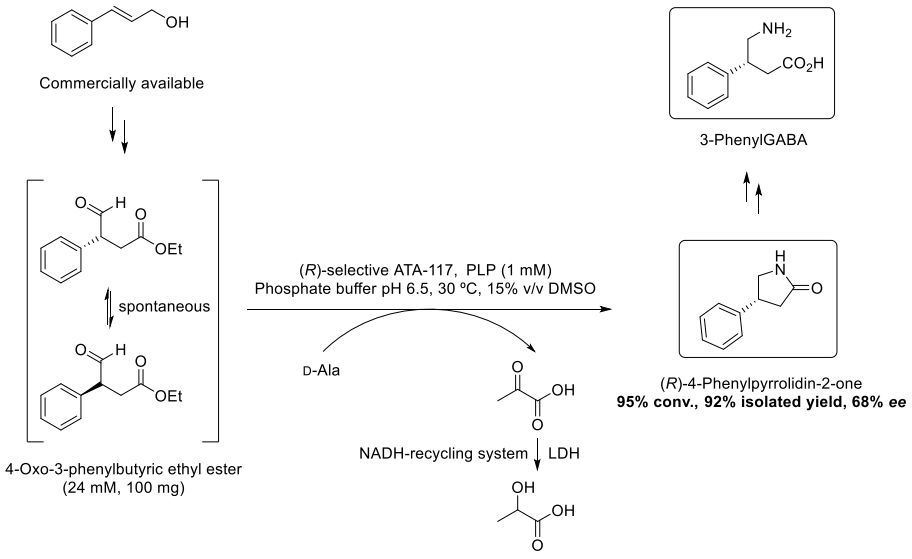
This γ-lactam is particularly interesting as the (–)-enantiomer is the precursor of Abacavir and the (+)-enantiomer is a key intermediate in the synthesis of Melogliptin. Both Abacavir and Melogliptin possess antibiotic and antiviral activities and their synthesis have been reported through the ring-opening of lactams catalysed by lactamases. On the one hand, the employment of (+)-γ-lactamase from *Bradyrhzobium japonicum* USDA 6 led to the (+)-amino acid and the remaining (–)-γ-lactam with excellent enantioselectivity and 50% conversion (Scheme 2.2.A).[102] On the other hand, a complementary approach using a promiscuous chloroperoxidase from *Streptomyces viridochromogenes* DSM 40736

[100] M. N. Cheemala, P. Knochel, *Org. Lett.* **2007**, *16*, 3089-3092.
[101] D. Guijarro, Ó. Pablo, M. Yus, *J. Org. Chem.* **2013**, *78*, 3647-3654.
[102] S. Gao, S. Zhu, R. Huang, Y. Lu, G. Zheng, *Bioorg. Med. Chem. Lett.* **2015**, *25*, 3878-3881.

(*Sv*GL), which showed (–)-γ-lactamase activity, made possible the preparation of the (–)-amino acid and the remaining (+)-γ-lactam with excellent selectivity after reaching 50% conversion (Scheme 2.2.B).[103]

Other hydrolases such as esterases and lipases have also allowed the production of γ- or δ-lactams, respectively. In this way, Hidalgo and co-workers introduced a point mutation in the wild type *Pseudomonas fluorescens* esterase I (PFEI), obtaining an improvement of 200-fold in the (–)-γ-lactamase activity for the kinetic resolution of the Vince lactam.[104] Furthermore, Ostaszewski and co-workers reported the kinetic resolution of racemic 2-acetyl-4-aryl-1,4-dihydro-2*H*-isoquinolin-3-ones containing a δ-lactam core. As catalyst for this reaction, lipases such as *Pseudomonas cepacia* lipase (PSL) in organic medium (MTBE and Et_2O) or liver acetone powders (LAPs) in aqueous medium were employed, obtaining the enantiopure unprotected (*R*)-lactams and the remaining protected (*S*)-lactams.[105]

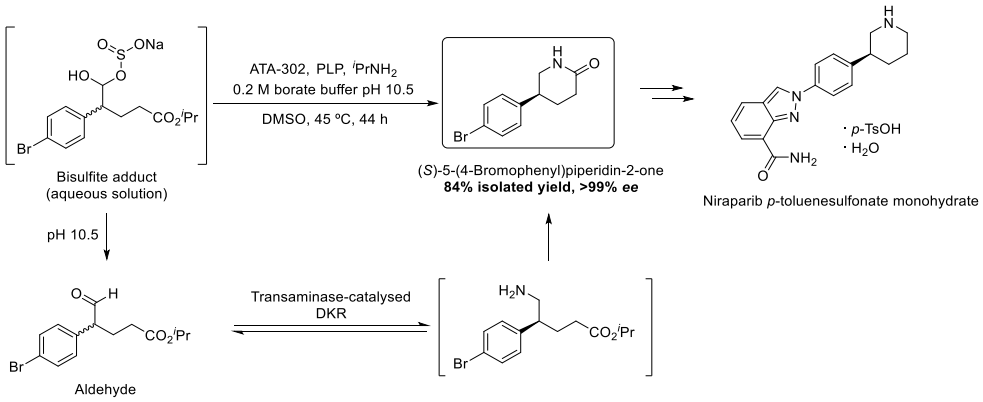
Scheme 2.3. Synthesis of (*R*)-4-phenylpyrrolidin-2-one through a transaminase-catalysed dynamic kinetic resolution.



[103] J.-G. Yin, Y. Gong, X.-Y. Zhang, G. W. Zheng, J.-H. Xu, *Catal. Sci. Tecnhol.* **2016**, *16*, 6305-6310.
[104] L. L. Torres, A. Schließmann, M. Schmidt, N. Silva-Martin, J. A. Hermoso, J. Berenguer, U. T. Bornscheuer, A. Hidalgo, *Org. Biomol. Chem.* **2012**, *17*, 3388-3392.
[105] D. Koszelewski, M. Cwiklak, R. Ostaszewski, *Tetrahedron: Asymmetry* **2012**, *23*, 1256-1261.

All the enzymatic pathways explained until now showed a serious drawback from a synthetic point of view as only 50% conversion of the desired product can be achieved, which is the inherent limitation when developing kinetic resolutions. A good alternative to get higher conversions (up to 100% conv.) consists in making use of transaminases as biocatalysts. For instance, Kroutil and co-workers reported the synthesis of both enantiomers of 4-phenylpyrrolidin-2-one through the transaminase-catalysed dynamic kinetic resolution of the 4-oxo-3-phenylbutyric acid ethyl ester.[106] Here, a remarkable achievement was the successful synthesis of the (*R*)-enantiomer as it could be transformed into the biologically active 3-phenylGABA. This enantiomer was produced in high yields and moderate enantioselectivity using the commercial transaminase ATA-117 and D-alanine as amine donor in combination with the lactate dehydrogenase system (Scheme 2.3). In this manner, one of the aldehyde enantiomers could be preferentially transformed into the corresponding amine that cyclised in the reaction media to produce the desired γ-lactam with moderate selectivity.

Scheme 2.4. Synthesis of enantiopure (*S*)-5-(4-bromophenyl)piperidin-2-one through a transaminase-catalysed dynamic kinetic resolution.



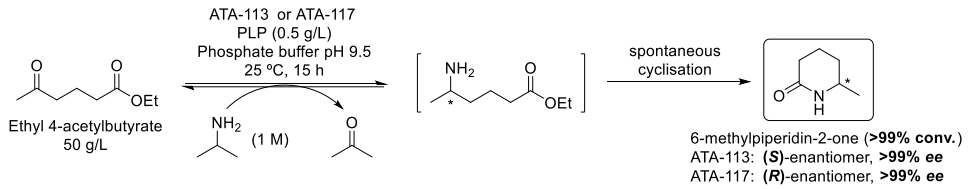
A similar approach was developed by Chung *et al.* in 2013 for the chemoenzymatic synthesis of the orally active poly(ADP-ribose)polymerase inhibitor Niraparib (Scheme 2.4).[107] Using this methodology, the enantioselective step was the formation of the δ-lactam through a transaminase-catalysed process in which an aldehyde was transformed into the corresponding amine which underwent

[106] D. Koszelewski, D. Clay, K. Faber, W. Kroutil, *J. Mol. Catal. B: Enzym.* **2009**, *60*, 191-194.
[107] C. K. Chung, P. G. Bulger, B. Kosjek, K. M. Belyk, N. Rivera, M. E. Scott, G. R. Humphrey, J. Limanto, D. C. Bachert, K. M. Emerson, *Org. Process Res. Dev.* **2014**, *18*, 215-227.

spontaneous cyclisation in the reaction media, leading to the formation of the desired δ-lactam with high yield and excellent stereoselectivity. The aldehyde was not directly added as it was found to suffer from oxidative degradation in the reaction medium. Instead, a bisulfite adduct was employed as starting material releasing the parent aldehyde *in situ* under the basic conditions of the transaminase-catalysed protocol.

Finally, the asymmetric synthesis of 6-methylpiperidin-2-one was achieved by Turner and co-workers starting from a 1,5-keto ester, ethyl 4-acetylbutyrate (Scheme 2.5).[108] Herein, the starting material was not an aldehyde but a ketone which meant that depending on the selectivity of the transaminases, the (*S*)- or the (*R*)-enantiomer of the amino ester can be obtained in theoretically 100% conversion. In this way, (*S*)-selective ATA-113 and (*R*)-selective ATA-117 allowed the production of both enantiomers of the δ-lactam with full conversion and excellent enantioselectivity after the spontaneous cyclisation of the amino ester in the reaction media.

Scheme 2.5. Transamination of ethyl 4-acetylbutyrate followed by spontaneous cyclisation to the desired 6-methylpiperidin-2-one.



At this point, three different transaminase-catalysed methodologies to produce enantioenriched γ- and δ-lactams have been introduced. In all cases, a cascade reaction takes place. First of all, an amino ester is synthesised and afterwards this chiral intermediate is transformed into the corresponding lactam in a spontaneous way. Transaminase-catalysed cascade transformations are not only fancy processes but they have also some advantages that will be discussed from now on.

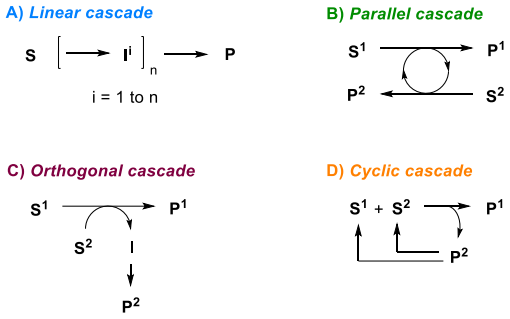
2.1.2. Transaminases as biocatalysts in cascade transformations

Enzymatic cascades represent a major opportunity to develop smart synthesis strategies offering several advantages as shorten reaction routes, avoidance of unstable or toxic intermediates, minimisation of the use of organic solvents,

[108] M. D. Truppo, J. D. Rozzell, N. J. Turner, *Org. Process Res. Dev.* **2010**, *14*, 234-237.

improvement in the atom efficiency or even control of unfavourable reaction equilibrium.[109] The concept of "cascade" is related to those processes in which several independent reactions take place simultaneously or in a consecutive manner. According to that statement, cascade transformations can be classified into four groups (Scheme 2.6).[110]

Scheme 2.6. Classification of cascade processes into: **A)** linear cascade; **B)** parallel cascade; **C)** orthogonal cascade; **D)** cyclic cascade.



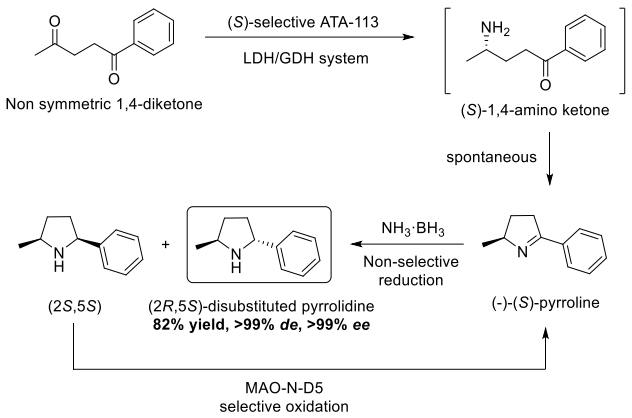
Biocatalysed linear cascades (Scheme 2.6.A) are the most common ones consisting of consecutive transformations, which can occur in a multienzymatic fashion or can be triggered by a chemical reaction followed by a biotransformation or vice versa. In fact, in this chapter we will develop a cascade composed by a biocatalysed reaction followed by a spontaneous chemical transformation. Parallel cascades (Scheme 2.6.B) involve two coupled-processes so the product(s) formation take place simultaneously through the recycling of, for example, a cofactor.[111] In an orthogonal cascade (Scheme 2.6.C) a co-substrate (S^2) is transformed into an intermediate (I) which is further converted into the co-product (P^2). For instance, the lactate dehydrogenase, the pyruvate decarboxylase and the amino acid dehydrogenase systems (Scheme 0.8) are orthogonal cascade processes. In the same way, the use of smart co-substrates that after the reaction with the transaminases undergo spontaneous transformations (Scheme 0.9) is also an example of this kind of process. Finally, cyclic cascades (Scheme 2.6.D) are

[109] M. D. Patil, G. Grogan, A. Bommarius, H. Yun, *Catalysts* **2018**, *8*, 254.
[110] R. C. Simon, N. Richter, E. Busto, W. Kroutil, *ACS Catal.* **2014**, *4*, 129-143.
[111] A. Rioz-Martínez, F. R. Bisogno, C. Rodríguez, G. de Gonzalo, I. Lavandera, D. E. Torres Pazmiño, M. W. Fraaije, V. Gotor, *Org. Biomol. Chem.* **2010**, *8*, 1431-1437.

typically used for stereoinversion processes.[112] The main characteristic of these cascades is that the product P^2 is converted back to one or both substrates.

Due to their relevance for the development of our research, we will focus on transaminase-catalysed linear cascades. In particular, those in which an enzymatic step is followed by the spontaneous cyclisation of the intermediate. In this way, several methodologies have been developed.

Scheme 2.7. Regio- and stereoselective transaminase/monoamine oxidase cascade to access chiral 2,5-disubstituted pyrrolidines.



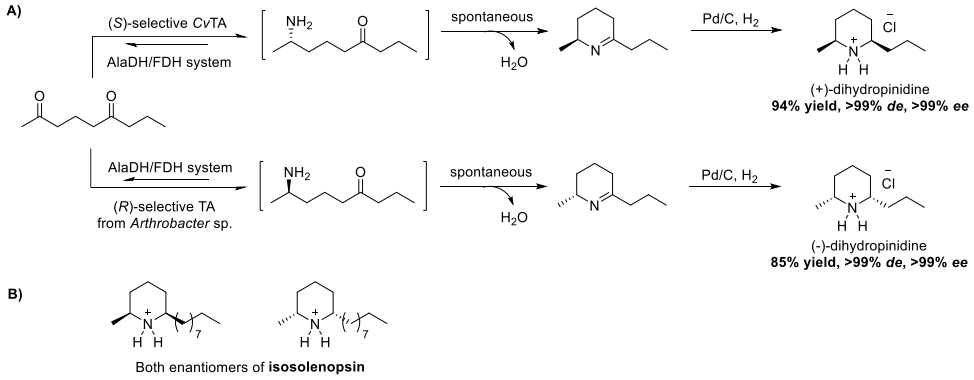
A very interesting example is the one published by Turner and co-workers in 2014,[113] disclosing a regio- and stereoselective cascade process to access chiral 2,5-disubstituted pyrrolidines (Scheme 2.7). In the first step, an asymmetric 1,4-diketone underwent a transamination process to produce the (*S*)-amino ketone which spontaneously cyclised into the (–)-(*S*)-5-methyl-2-phenyl-1-pyrroline. Later on, the pyrroline underwent a non-selective reduction into the corresponding pyrrolidine. The capability of the monoamine oxidase (MAO-N-D5) to convert the (2*S*,5*S*)-pyrrolidine back into the pyrroline through a selective oxidation, allowed the accumulation of the optically pure (2*R*,5*S*)-2-methyl-5-phenylpyrrolidine which was obtained in 82% isolated yield and excellent diastereomeric ratio.

Kroutil and co-workers have also provided interesting examples in this field. In 2012, Simon *et al.* reported the regio- and stereoselective monoamination of 1,5-

[112] C. C. Gruber, I. Lavandera, K. Faber, W. Kroutil, *Adv. Synth. Catal.* **2006**, *348*, 1789-1805.
[113] E. O'Reilly, C. Iglesias, D. Ghislieri, J. Hopwood, J. L. Galman, R. C. Lloyd, N. J. Turner, *Angew. Chem. Int. Ed.* **2014**, *53*, 2447-2450.

diketones into 2,6-disubstituted piperidines,[114] achieving the chemoenzymatic synthesis of the insect antifeedant (+)-dihydropinidine and its enantiomer (Scheme 2.8.A). The first step consisted in the transamination of nonane-2,6-dione into the corresponding amino ketone, which underwent spontaneous cyclisation into 2-methyl-6-propyl-2,3,4,5-tetrahydropyridine. The (*S*)-enantiomer was formed by using the transaminase from *Chromobacterium violaceum* while its antipode (*R*)-enantiomer came from the reaction of the substrate with the transaminase from *Arthrobacter* sp. Both enantiomers can be transformed into the 2,6-disubstituted piperidines after a diastereoselective hydrogenation step. Later on, the same strategy was applied to the synthesis of both enantiomers of the alkaloid isosolenopsin (Scheme 2.8.B).[115]

Scheme 2.8. A) Regio- and stereoselective chemoenzymatic approach to (+)-dihydropinidine and its enantiomer. **B)** Isosolenopsin enantiomers obtained through the same chemoenzymatic cascade.

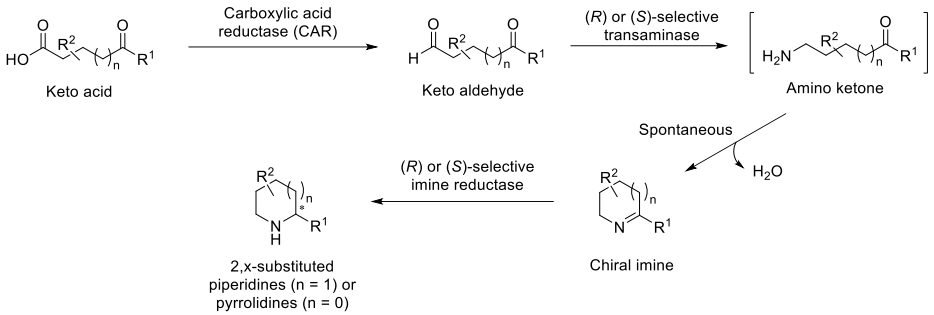


Last but not least, Turner and co-workers have developed a similar approach to obtain mono- and disubstituted piperidines and pyrrolidines making use of a multienzymatic cascade protocol (Scheme 2.9).[116] In the first step, a carboxylic acid reductase (CAR) transformed a keto acid into the corresponding keto aldehyde. A second step involved the utilisation of transaminases which allowed the formation of a chiral amino ketone that spontaneously cyclised into the corresponding chiral imine. The final step was the asymmetric reduction of that

[114] R. C. Simon, B. Grischek, F. Zepeck, A. Steinreiber, F. Belaj, W. Kroutil, *Angew. Chem. Int. Ed.* **2012**, *51*, 6713-6716.
[115] R. C. Simon, C. S. Fuchs, H. Lechner, F. Zepeck, W. Kroutil, *Eur. J. Org. Chem.* **2013**, 3397-3402.
[116] S. P. France, S. Hussain, A. M. Hill, L. J. Hepworth, R. M. Howard, K. R. Mulholland, S. L. Flitsch, N. J. Turner, *ACS Catal.* **2016**, *6*, 3753-3759.

imine mediated by an imine reductase (IRED). Depending on the TAs and IREDs selectivities, the four diastereoisomers of the 2,x-(di)substituted piperidines or pyrrolidines were susceptible to be obtained.

Scheme 2.9. Multienzymatic approach to chiral mono- or disubstituted piperidines and pyrrolidines.



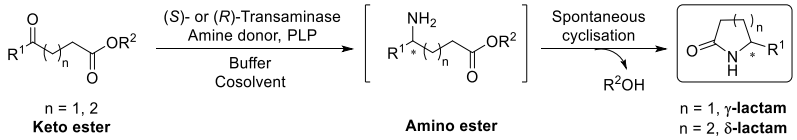
All the transaminase-catalysed strategies that have been introduced until now have something in common. In all cases the transamination product, either an amino ketone or an amino ester, is removed from the reaction media due to its spontaneous transformation into a lactam or a cyclic imine. This fact can be considered as an intrinsic way of shifting the thermodynamically disadvantaged equilibrium to the formation of the amine intermediate.

Herein, we will present a transaminase-catalysed cascade protocol to produce a set of chiral γ- and δ-lactams. Different 1,4- and 1,5-keto esters will be employed as starting material to synthesise the corresponding 1,4- and 1,5-amino esters which will spontaneously cyclise to obtain the desired lactams. Several reaction parameters, including the influence of the substrate concentration, temperature, reaction time and the amount of amine donor needed to shift the equilibrium will be deeply studied.

2.2. Discussion

A general one-pot two-step synthetic pathway for the asymmetric preparation of γ- and δ-lactams has been developed. It is based on the biotransamination of easily accessible γ- and δ-keto esters to form 1,4- and 1,5-chiral amino esters, respectively, which are unstable in the reaction media and undergo a spontaneous cyclisation to achieve the final lactams (Scheme 2.10). An exhaustive screening of transaminases and optimisation of reaction conditions has been performed in order to achieve both enantiomers of the lactams in a high-yielding and stereoselective manner.

Scheme 2.10. One-pot two-step transaminase-catalysed cascade protocol to transform γ- and δ-keto esters into optically active lactams.



2.2.1. Preliminary screening of transaminases using ethyl levulinate (1a) as model substrate. Influence of the leaving group and the length of the alkyl chain

Levulinic acid derivatives are easily accessible from lignocellulosic biomass and in some cases they are commercially available.[117] For this reason, ethyl levulinate (**1a**) was chosen as model substrate for this research project. A wide panel of commercial transaminases was tested towards the biotransformation of **1a**. Additionally, freeze-dried cell powder of *E. coli* containing transaminases such as the (*S*)-selective TAs from *Chromobacterium violaceum* (Cv-TA)[118] and a variant from *Arthrobacter citreus* (ArS-TA),[119] or the (*R*)-selective ones from *Arthrobacter* species (ArR-TA)[120] and its mutant (ArRmut11-TA)[28a] were also tested. All of them, commercial and made-in-house TAs were known to accept isopropylamine

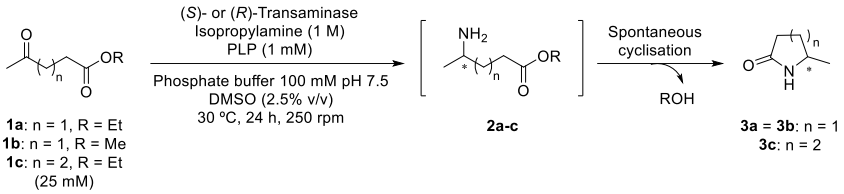
[117] (a) J. D. Vidal, M. J. Climent, P. Concepcion, A. Corma, S. Iborra, M. J. Sabater, *ACS Catal.* **2015**, *5*, 5812-5821; (b) A. S. Touchy, S. M. A. H. Siddiki, K. Kon, K.-I. Shimizu, *ACS Catal.* **2014**, *4*, 3045-3050.
[118] U. Kaulmann, K. Smithies, M. E. B. Smith, H. C. Hailes, J. M. Ward, *Enzyme Microb. Technol.* **2007**, *41*, 628-637.
[119] S. Pannuri, S. V. Kamat, A. R. M. Garcia, Cambrex North Brunswick Inc., PCT Int. Appl. WO 063336A2 20060615, **2006**.
[120] Y. Yamada, A. Iwasaki, N. Kizaki, K. Matsumoto, Y. Ikenaka, M. Ogura, J. Hasegawa, Kaneka Corporation, PCT Int. Appl. WO 9848030A1 19981029, **1998**.

(IPA) as amine donor. Based on our experience in biotransamination reactions, initially a 25 mM substrate concentration was considered and 2.5% of DMSO was used as cosolvent in combination with phosphate buffer 100 mM pH 7.5 containing 1 mM concentration of the cofactor, the pyridoxal 5'-phosphate (PLP). In order to drive the equilibrium towards the formation of the amino ester and the subsequent lactam, a large excess of IPA was added (1 M concentration, 40 equiv). All the reactions were incubated at 30 ºC for 24 hours. After that time, only the formation of the optically active 5-methylpyrrolidin-2-one (**3a**) was observed, denoting the spontaneous intramolecular cyclisation in the reaction media of ethyl 4-aminopentanoate (**2a**), which was not observed in any case. The best results are summarised in Table 2.1 (entries 1-5), finding that the best (*R*)-TA was TA-P2-B01 from Codexis Inc. (entry 2, 87% conv. and >99% *ee*) while the best (*S*)-TA was the made-in-house ArS-TA (entry 5, 97% conv. and >99% *ee*). Hence both enantiomers of γ-lactam **3a** were achieved with high conversions and excellent *ee* values.

As a continuation of the preliminary screening, we also studied the influence of the ester leaving group. For that purpose, we made use of methyl levulinate (**1b**) as substrate which also allowed the synthesis of **3a**, which is the same lactam that was obtained from **1a** as the only difference is the alkoxy leaving group of the amino ester intermediate. The best results have also been summarised in Table 2.1 (entries 6 to 11), showing conversions over 80% in all cases and excellent selectivities towards the formation of both enantiomers. In general, similar results were found for both substrates but in some cases TAs revealed higher activity when the methyl ester was used as substrate; compare for instance the results obtained using TA-P2-A07 as biocatalyst (entries 1 and 7).

Later on, the influence of the alkyl chain length was also studied. At this point, ethyl 5-oxohexanoate (**1c**), which led to the formation of the δ-lactam 6-methylpiperidin-2-one (**3c**), was the substrate of choice. As for the previous substrates, both enantiomers of the lactam were achieved with high conversions and excellent *ee* values (Table 2.1, entries 12-18), not observing the 1,5-amino ester intermediate in any case. The best results for this substrate were those obtained with the made-in-house (*S*)-transaminase from *Arthrobacter citreus* (entry 18, 98% conv. and >99% *ee*).

Table 2.1. Asymmetric transformation of keto esters **1a-c** into γ-lactam **3a** and δ-lactam **3c**.[a]



Entry	Keto ester	n	R	Transaminase	*c* (%)[b]	*ee* (%)[c]
1	**1a**	1	Et	TA-P2-A07	41	>99 (*R*)
2	**1a**	1	Et	TA-P2-B01	87	>99 (*R*)
3	**1a**	1	Et	ATA-237	92	>99 (*S*)
4	**1a**	1	Et	TA-P1-B04	89	>99 (*S*)
5	**1a**	1	Et	ArS-TA	97	>99 (*S*)
6	**1b**	1	Me	ATA-412	84	>99 (*R*)
7	**1b**	1	Me	TA-P2-A07	81	>99 (*R*)
8	**1b**	1	Me	TA-P2-B01	80	>99 (*R*)
9	**1b**	1	Me	TA-P1-A06	90	>99 (*S*)
10	**1b**	1	Me	TA-P1-G06	91	>99 (*S*)
11	**1b**	1	Me	ArS-TA	>99	>99 (*S*)
12	**1c**	2	Et	ATA-013	89	96 (*R*)
13	**1c**	2	Et	ATA-033	87	97 (*R*)
14	**1c**	2	Et	TA-P2-B01	86	97 (*R*)
15	**1c**	2	Et	ATA-200	86	>99 (*S*)
16	**1c**	2	Et	TA-P1-A06	88	>99 (*S*)
17	**1c**	2	Et	TA-P1-G06	96	99 (*S*)
18	**1c**	2	Et	ArS-TA	98	>99 (*S*)

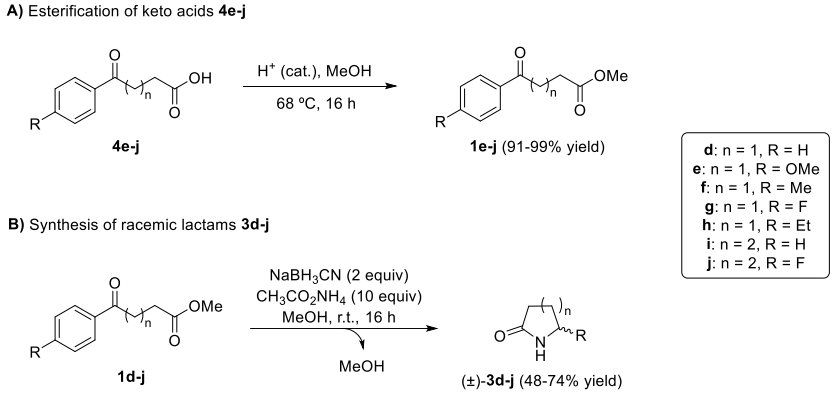
[a] Reaction conditions: Substrates **1a-c** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 (500 µL final volume), 30 °C, 24 h, 250 rpm. [b] Conversion values determined by GC. [c] Determined by HPLC using chiral columns. Absolute configurations of the corresponding lactams appear in brackets.

2.2.2. Chemical approach to methyl 4-aryl-4-oxobutanoates (1d-h), methyl 5-aryl-5-oxobutanoates (1i and 1j) and the corresponding racemic lactams (3d-j)

Looking for further exploitation of the synthetic approach, we decided to broaden the substrate scope over methyl 4-aryl-4-oxobutanoates (**1d-h**) and methyl 5-aryl-5-oxobutanoates (**1i** and **1j**). Even though only the methyl 4-oxo-4-phenylbutanoate (**1d**) was commercially available, methyl esters **1e-j** were easily synthesised from the corresponding keto acids **4e-j** (Scheme 2.11.A). After an esterification reaction using catalytic amounts of concentrated H_2SO_4 aqueous solution in refluxing methanol, the desired esters were obtained in excellent yields (91-99%). Later on, the racemic lactams (**3d-j**) were synthesised through a

reductive amination protocol using 2 equiv of sodium cyanoborohydride ($NaBH_3CN$) and 10 equiv of ammonium acetate in dry methanol (Scheme 2.11.B). After 16 hours, the reaction mixture was purified by column chromatography on silica gel, affording the lactams (±)-**3d-j** in moderate yields (48-74%).

Scheme 2.11. A) Synthesis of methyl 4-aryl-4-oxobutanoates (**1e-h**) and methyl 5-aryl-5-oxopentanoates (**1i** and **1j**). **B)** Synthesis of racemic 5-arylpyrrolidin-2-ones (**3d-h**) and 6-arylpiperidin-2-ones (**3i** and **3j**).



2.2.3. Broadening the enzymatic substrate scope. Synthesis of optically pure 5-arylpyrrolidin-2-ones (3d-h) and 6-arylpiperidin-2-ones (3i and 3j)

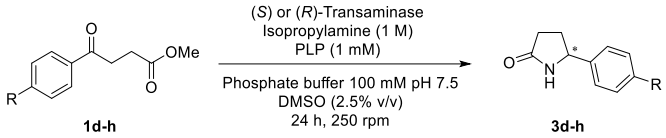
γ-Keto ester **1d** was the first one to be tested (Table 2.2, entries 1-6). Initially, the same reaction conditions employed for keto esters **1a-c** were tested: 25 mM substrate concentration, 2.5% of DMSO as cosolvent in a phosphate buffer 100 mM pH 7.5, 1 mM concentration of the cofactor and a large excess of IPA (1 M). After 24 hours at 30 °C the best results using this substrate were those obtained with ATA-303 and ATA-237, leading only to a 59% conversion of the enantiopure (*R*)-**3d** (entry 1) and to a 45% conversion of the (*S*)-**3d** (entry 4, 98% *ee*), respectively.

In the search of higher conversions, we decided to study the effect that the substrate concentration and temperature had in the activity of these transaminases. For this purpose, substrate concentration was decreased from 25 mM to 15 mM (entries 2 and 5) finding better results in terms of conversions for both transaminases (78 and 57%, respectively). Later on, the temperature was increased up to 45 °C maintaining a 15 mM substrate concentration (entries 3 and 6).

Remarkably, under these reaction conditions ATA-303 led to the formation of enantiopure (*R*)-5-phenylpyrrolidin-2-one with 97% conversion (entry 3).

At this point, 15 mM substrate concentration and 45 °C were chosen as optimal reaction conditions for this set of substrates and the biotransformation of keto esters **1e-h** was attempted (entries 7-19). In all cases, both enantiomers of the corresponding γ-lactams were obtained with high conversions (81-99%) and complete selectivities.

Table 2.2. Stereoselective transamination of γ-keto esters **1d-h** to achieve chiral 5-arylpyrrolidin-2-ones **3d-h**.[a]



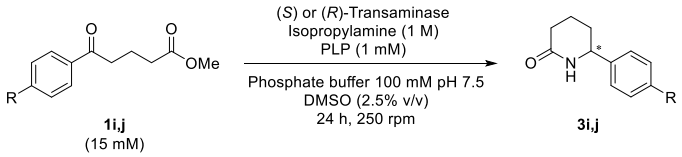
Entry	R (Keto ester)	[Keto ester] (mM)	T (°C)	Transaminase	*c* (%)[b]	*ee* (%)[c]
1	H (**1d**)	25	30	ATA-303	59	>99 (*R*)
2	H (**1d**)	15	30	ATA-303	78	>99 (*R*)
3	H (**1d**)	15	45	ATA-303	97	>99 (*R*)
4	H (**1d**)	25	30	ATA-237	45	98 (*S*)
5	H (**1d**)	15	30	ATA-237	57	>99 (*S*)
6	H (**1d**)	15	45	ATA-237	57	93 (*S*)
7	OMe (**1e**)	15	45	ATA-025	94	>99 (*R*)
8	OMe (**1e**)	15	45	ATA-033	95	>99 (*R*)
9	OMe (**1e**)	15	45	ATA-234	91	>99 (*S*)
10	Me (**1f**)	15	45	ATA-025	98	>99 (*R*)
11	Me (**1f**)	15	45	ATA-033	98	>99 (*R*)
12	Me (**1f**)	15	45	ATA-234	89	>99 (*S*)
13	F (**1g**)	15	45	ATA-024	99	>99 (*R*)
14	F (**1g**)	15	45	ATA-025	98	>99 (*R*)
15	F (**1g**)	15	45	ATA-415	99	>99 (*R*)
16	F (**1g**)	15	45	ATA-234	81	>99 (*S*)
17	Et (**1h**)	15	45	ATA-025	83	>99 (*R*)
18	Et (**1h**)	15	45	ATA-033	86	>99 (*R*)
19	Et (**1h**)	15	45	ATA-234	82	>99 (*S*)

[a] Reaction conditions: Substrates **1d-h** (15-25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 (500 µL final volume), 30-45 °C, 24 h, 250 rpm. [b] Conversion values determined by GC. [c] Determined by HPLC using chiral columns. Absolute configurations of the corresponding lactams appear in brackets.

Next, methyl 5-aryl-5-oxopentanoates **1i** and **1j** were tested as substrates in order to synthesise the corresponding δ-lactams **3i** and **3j** in an asymmetric fashion. Then, 15 mM substrate concentration was initially used, incubating the

biotransformations at 30 ºC (Table 2.3, odd entries). Good results were obtained in the synthesis of (*R*)-**3i** when using (*R*)-selective ATA-415 (entry 3), while (*S*)-selective ATA-237 led to both (*S*)-**3i** and (*S*)-**3j** with high conversions and *ee* values (entries 5 and 9). An increase in the temperature (even entries) did not always lead to better results. For instance, a drop in the ATA-415 reactivity was observed (entry 4). However, ATA-237 displayed the highest activities (94-96% conversion) with high stereocontrol (93-95% *ee*) at 45 °C (entries 6 and 10).

Table 2.3. Stereoselective transaminase-catalysed transformation of δ-keto esters **1i** and **1j** into 6-arylpiperidin-2-ones **3i** and **3j**.



Entry	R (Keto ester)	T (°C)	Transaminase	*c* (%)[b]	*ee* (%)[c]
1	H (**1i**)	30	ATA-033	20	>99 (*R*)
2	H (**1i**)	45	ATA-033	28	>99 (*R*)
3	H (**1i**)	30	ATA-415	71	>99 (*R*)
4	H (**1i**)	45	ATA-415	47	>99 (*R*)
5	H (**1i**)	30	ATA-237	94	94 (*S*)
6	H (**1i**)	45	ATA-237	94	95 (*S*)
7	F (**1j**)	30	ATA-025	43	>99 (*R*)
8	F (**1j**)	45	ATA-025	58	>99 (*R*)
9	F (**1j**)	30	ATA-237	90	92 (*S*)
10	F (**1j**)	45	ATA-237	96	93 (*S*)

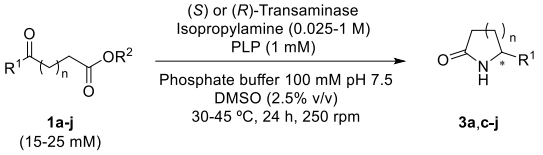
[a] Reaction conditions: Substrates **1i** or **1j** (15 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 (500 µL final volume), 30-45 °C, 24 h, 250 rpm. [b] Conversion values determined by GC. [c] Determined by HPLC using chiral columns. Absolute configurations of the corresponding lactams appear in brackets.

2.2.4. Driving the equilibrium towards the amine formation. Isopropylamine concentration

It has already been discussed the importance of shifting the equilibrium towards the amine product in transaminase-catalysed transformations. Until now, a large excess of the amine donor (isopropylamine, IPA, 40-67 equiv) has been employed to guarantee the cascade efficiency. However, as commented in the bibliographic background, the transaminase product is removed from the reaction medium in this type of cascade transformation. This means that the reversibility of the equilibrium should be noticeably diminished. For that reason, we considered

the use of lower amounts of IPA to provide a more atom efficient procedure (Table 2.4).

Table 2.4. Influence of the isopropylamine concentration in the biotransformation of keto esters **1a-j** into the optically active lactams **3a**,**c-j**.[a]



Entry	[Keto ester]	[^{i}PrNH$_2$] (mM)	T (°C)	TA	*c* (%)[b]	*ee* (%)[c]
1	**1b** (25 mM)	1000	30	TA-P1-G06	91	>99 (*S*)
2	**1b** (25 mM)	500	30	TA-P1-G06	91	>99 (*S*)
3	**1b** (25 mM)	100	30	TA-P1-G06	89	>99 (*S*)
4	**1b** (25 mM)	50	30	TA-P1-G06	80	>99 (*S*)
5	**1b** (25 mM)	25	30	TA-P1-G06	71	>99 (*S*)
6	**1a** (25 mM)	1000	30	ATA-237	92	>99 (*S*)
7	**1a** (25 mM)	100	30	ATA-237	85	>99 (*S*)
8	**1c** (25 mM)	1000	30	ArS-TA	98	>99 (*S*)
9	**1c** (25 mM)	100	30	ArS-TA	84	>99 (*S*)
10	**1d** (15 mM)	1000	45	ATA-303	97	>99 (*R*)
11	**1d** (15 mM)	100	45	ATA-303	89	>99 (*R*)
12	**1e** (15 mM)	1000	45	ATA-025	94	>99 (*R*)
13	**1e** (15 mM)	100	45	ATA-025	91	>99 (*R*)
14	**1f** (15 mM)	1000	45	ATA-025	98	>99 (*R*)
15	**1f** (15 mM)	100	45	ATA-025	93	>99 (*R*)
16	**1g** (15 mM)	1000	45	ATA-025	98	>99 (*R*)
17	**1g** (15 mM)	100	45	ATA-025	94	>99 (*R*)
18	**1h** (15 mM)	1000	45	ATA-025	83	>99 (*R*)
19	**1h** (15 mM)	100	45	ATA-025	35	>99 (*R*)
20	**1i** (15 mM)	1000	45	ATA-237	94	94 (*S*)
21	**1i** (15 mM)	100	45	ATA-237	95	89 (*S*)
22	**1j** (15 mM)	1000	45	ATA-237	96	96 (*S*)
23	**1j** (15 mM)	100	45	ATA-234	97	90 (*S*)

[a] Reaction conditions: Substrates **1a-j** (15-25 mM), transaminase (2 mg), isopropylamine (0.025-1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 (500 µL final volume), 30-45 °C, 24 h, 250 rpm. [b] Conversion values determined by GC. [c] Determined by HPLC using chiral columns. Absolute configurations of the corresponding lactams appear in brackets.

Methyl levulinate (**1b**, 25 mM) was chosen as model substrate and the TA-P1-G06-catalysed cascade transformation into γ-lactam (*S*)-**3a** was set up (entries 1-5), using from an equimolar amount (25 mM) to a 40-molar excess of isopropylamine (1 M). Remarkably, enantiopure **3a** was achieved after 24 hours with similar conversion values between 0.1-1 M IPA concentrations (entries 1-3, 89-91% conv).

When just 50 mM concentration of isopropylamine was added into the reaction media, a significant drop in the conversion was observed (entry 4, 80% conversion). Nevertheless, it is important to point out that a high conversion was still observed using a 25 mM of IPA concentration (entry 5, 71% conversion). Keeping these results in mind, the other keto esters **1a**,**c-j** were tested at 100 mM IPA concentration (entries 6-23). Slightly lower conversions were found compared to those obtained adding a larger excess of IPA (1 M) with the exception of methyl 4-(4-ethylphenyl)-4-oxobutanoate (**1h**) transamination. In this particular case, a remarkable negative effect in the efficiency of the process was observed (entry 19, 35% conversion).

2.2.5. Semi-preparative transaminase-catalysed cascade transformations

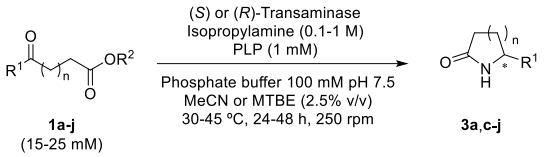
The last step in our research was to demonstrate the applicability of the process by setting up semi-preparative biotransformations using from 30 to 100 mg of substrates **1a-j** (Table 2.5).

All reactions were carried out under the optimised reaction conditions. In order to achieve high conversions, 1 M isopropylamine was added in all cases. Additionally, cosolvents with lower boiling points than DMSO were used in order to simplify the work-up protocols. In this way, control reactions were set up using acetonitrile (MeCN) and *tert*-butyl methyl ether (MTBE), finding MeCN a good cosolvent for commercial transaminases and MTBE for ArS. Alkyl keto esters **1a-c** were used at 25 mM substrate concentration and 30 °C while for aryl keto esters **1d-j** substrate concentration was reduced down to 15 mM and biotransformations were incubated at 45 °C. Under these conditions, the desired lactams were afforded in good to high isolated yields (66-89%) and excellent selectivities (94->99% *ee*).

In order to confirm their absolute configurations, optical rotation values were measured for all the isolated optically active lactams and the obtained values were in accordance with those reported in the literature.[121]

[121] (a) (*S*)-5-Methylpyrrolidin-2-one (**3a**): $[\alpha]_D^{25} = -10.0$ (c = 1.0, EtOH). Described in the literature: $[\alpha]_D^{25} = -21.3$ (c = 0.9, water) for the (*S*)-enantiomer. J. M. García, M. A. Maestro, M. Oiarbide, J. M. Odriozola, J. Razkin, C. Palomo, *Org. Lett.* **2009**, *11*, 3826-3829; (b) (*S*)-6-Methylpiperidin-2-one (**3c**): $[\alpha]_D^{22} = +23.7$ (c = 1.0, EtOH). Described in the literature: $[\alpha]_D^{20} = -24.0$ (c = 0.4, CH_2Cl_2) for the (*R*)-enantiomer. M. Amat, N. Llor, J. Hidalgo, C. Escolano, J. Bosch, *J. Org. Chem.* **2003**, *68*, 1919-1928; (c) (*R*)-5-Phenylpyrrolidin-2-one (**3d**): $[\alpha]_D^{20} = +42.2$ (c = 1.0, EtOH). Described in the literature [100]: $[\alpha]_D^{20} = +41.0$ (c = 0.4, CH_2Cl_2) for the (*R*)-enantiomer; (d) (*R*)-5-(4-Methoxyphenyl)pyrrolidin-2-one (**3e**): $[\alpha]_D^{20} = +28.1$ (c = 1.0, EtOH). Described in the literature

Table 2.5. Semi-preparative synthesis of optically pure γ-lactams **3a**,**d-h** and δ-lactams **3c**,**i**,**j** through a transaminase-catalysed cascade protocol.[a]



Entry	[**1a-j**] (mM)	[IPA] (M)	T (°C)	Transaminase	Cosolvent	t (h)	*c* (%)[b]	*ee* (%) [c]
1	25 (**1a**)	1	30	ATA-237	MeCN	24	97 (75)	>99 (*S*)
2	25 (**1b**)	1	30	TA-P1-G06	MeCN	48	88 (80)	>99 (*S*)
3	25 (**1c**)	1	30	ArS-TA	MTBE	24	98 (89)	>99 (*S*)
4	15 (**1d**)	1	45	ATA-303	MeCN	24	97 (88)	>99 (*R*)
5	15 (**1e**)	1	45	ATA-025	MeCN	48	82 (74)	>99 (*R*)
6	15 (**1e**)	0.1	45	ATA-025	MeCN	48	89 (80)	>99 (*R*)
7	15 (**1f**)	1	45	ATA-025	MeCN	48	91 (86)	>99 (*R*)
8	15 (**1g**)	1	45	ATA-025	MeCN	48	80 (75)	>99 (*R*)
9	15 (**1h**)	1	45	ATA-025	MeCN	48	89 (81)	>99 (*R*)
10	15 (**1i**)	1	45	ATA-237	MeCN	24	77 (70)	94 (*S*)
11	15 (**1j**)	1	45	ATA-237	MeCN	24	70 (66)	96 (*S*)
12	15 (**1j**)	0.1	45	ATA-237	MeCN	24	93 (90)	91 (*S*)

[a] Biotransaminations of **1a-c** into **3a** and **3c** under the following conditions: 25 mM **1a-c**, 1 M isopropylamine, 2.5% v/v MeCN (**1a**,**b**) or MTBE (**1c**), phosphate buffer 100 mM pH 7.5, 30 °C, 24 or 48 h, 250 rpm. Biotransaminations of **1d-j** into **3d-j** under the following conditions: 15 mM keto ester, 1 M isopropylamine, 2.5% v/v MeCN, phosphate buffer 100 mM pH 7.5, 45 °C, 24 h, 250 rpm. [b] Conversion values determined by GC. Isolated yields appear in brackets [c] Determined by HPLC using chiral columns. Absolute configurations of the corresponding lactams appear in brackets.

Furthermore, two preparative biotransformations were set up with a 10-fold reduced amount of IPA (entries 6 and 12) finding high conversions (89-93%) and excellent selectivities (91->99% *ee*). These results showed that the use of lower IPA concentration (100 mM) could also lead to remarkable results in this kind of process.

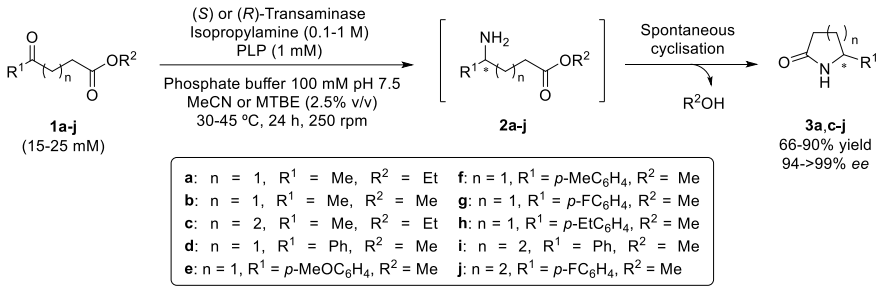
2.2.6. Final remarks

To sum up, the asymmetric synthesis of a wide panel of γ- and δ-lactams has been described through a simple and straightforward one-pot two-step

[101]: $[\alpha]_D^{20}$ = +40.5 (c = 0.7, CH_2Cl_2) for the (*R*)-enantiomer; (e) (*S*)-6-Phenylpiperidin-2-one (**3i**): $[\alpha]_D^{20}$ = –48.6 (c = 1.0, EtOH). Described in the literature: $[\alpha]_D^{20}$ = –58.0 (c = 0.54, $CHCl_3$) for the (*S*)-enantiomer. R. Sallio, S. Lebrun, F. Agbossou-Niedercorn, C. Michon, E. Deniau, *Tetrahedron: Asymmetry* **2012**, *23*, 998-1004; (f) (*S*)-6-(4-Fluorophenyl)piperidin-2-one (**3j**): $[\alpha]_D^{20}$ = –47.2 (c = 0.60, EtOH). Described in the literature [121e]: $[\alpha]_D^{20}$ = –47.2 (c = 0.60, $CHCl_3$) for the (*S*)-enantiomer.

transaminase-catalysed protocol (Scheme 2.12). In the first step, different keto esters were transformed into the corresponding chiral amino esters. In the second step, those intermediates underwent a spontaneous intramolecular cyclisation in the reaction medium to achieve the enantioenriched lactams. 10 substrates with different substitution pattern were tested towards this transaminase-catalysed cascade transformation, isolating 6 γ-lactams and 3 δ-lactams in moderate to good yields (66-90%) and high optical purity (94->99% *ee*).[122] Additionally, the thermodynamically favoured cyclisation process makes possible to reduce the amine donor equivalents needed in the transamination process without significant drop in the conversion values.

Scheme 2.12. Transaminase-catalysed cascade process to access optically pure γ- and δ-lactams.



[122] It is worth mentioning that substrates **1a** and **1b** led to the same lactam **3a**.

2.3. Experimental section

2.3.1. General information

Chemical reagents were purchased from commercial sources (Sigma-Aldrich, Acros Organics and Fluka) and used as received except dry methanol that was previously distilled under nitrogen using calcium hydride as desiccant.

Codex Transaminase ATA Screening Kit (ATASK-000250) and pyridoxal 5'-phosphate (PLP) were purchased from Codexis. Transaminases from *Chromobacterium violaceum* (Cv-TA, internal plasmid number pET20)[118] and *Arthrobacter* sp. [ArS-TA (pEG29),[119] ArR-TA (pEG23),[120] and ArRmut11-TA (pEG90)[28a]] overexpressed on *E. coli* cells were provided by Prof. Wolfgang Kroutil (University of Graz).

2.3.2. Synthesis of γ-keto esters 1e-h and δ-keto esters 1i and 1j

To a solution of the corresponding keto acid **4e-j** (1.6 mmol) in MeOH (8 mL, 0.2 M), a few drops of concentrated sulphuric acid were added at room temperature. The mixture was stirred and heated at 68 ºC overnight. After this time, water (10 mL) was added. The mixture was neutralised with an aqueous NaOH 2 M solution and the product was extracted with CH_2Cl_2 (3 x 15 mL). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated under reduced pressure, isolating the corresponding keto esters **1e-h**,**i**,**j** in excellent purity (91-99% yield).

2.3.3. Chemical synthesis of racemic γ-lactams 3d-h and δ-lactams 3i and 3j

Ammonium acetate (200 mg, 2.6 mmol) and sodium cyanoborohydride (33 mg, 0.52 mmol) were successively added to a solution of the corresponding keto ester **1d-j** (0.26 mmol) in dry MeOH (1.0 mL) under inert atmosphere. The mixture was stirred at room temperature during 16 h and, after this time, water (5 mL) was added to quench the reaction. The solution was basified until pH around 11 by adding a saturated aqueous Na_2CO_3 solution. Then the mixture was extracted with Et_2O (3 x 10 mL) and the organic layers were combined, dried over Na_2SO_4, filtered and concentrated under reduced pressure. The product was purified by

chromatography column on silica gel (2% MeOH/CH_2Cl_2), affording the racemic lactams **3d-j** (48-78% yield).

2.3.4. Transamination of γ- and δ-keto esters 1a-j using commercially available transaminases

In a 1.5 mL Eppendorf tube, transaminase (2 mg) and the corresponding keto ester (**1a-j**, 15 or 25 mM) were added in phosphate buffer 100 mM pH 7.5 (500 µL, 1 mM PLP, 1 M isopropylamine), using DMSO (12.5 µL) as cosolvent. The reaction was shaken at 30 or 45 ºC and 250 rpm for 24 h and stopped by the addition of a saturated aqueous Na_2CO_3 solution (200 µL). Then the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into lactams **3a**,**c-j** were determined by GC and *ee* values measured by HPLC.

2.3.5. Biotransamination of γ- and δ-keto esters 1a-j using transaminases overexpressed in *E. coli*

In a 1.5 mL Eppendorf tube, dry cells of *E. coli* overexpressing the transaminase (10 mg) and keto ester **1a-j** (25 mM) were added in phosphate buffer 100 mM pH 7.5 (500 µL, 1 mM PLP, 1 M isopropylamine) using DMSO (12.5 µL) as cosolvent. The reaction was shaken at 30 ºC and 250 rpm for 24 h and stopped by the addition of a saturated aqueous Na_2CO_3 solution (200 µL). Then the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into lactams **3a**,**c-j** were determined by GC and *ee* values were measured by HPLC.

2.3.6. Preparative biotransformation of γ-keto esters 1a-c into optically active lactams

In an Erlenmeyer flask, the transaminase (30 mg, ATA-237 for **1a**, TA-P1-G06 for **1b** or ArS-TA for **1c**) and keto ester (**1a-c**, 30 mg, 25 mM) were added in phosphate buffer 100 mM pH 7.5 (1 mM PLP, 1 M isopropylamine) and cosolvent (2.5% v/v MeCN for **1a** and **1b** and 2.5% v/v MTBE for **1c**). The reaction was shaken at 30 ºC and 250 rpm for 24 h (**1a** and **1c**) or 48 h (**1b**) and then stopped by the addition of a saturated aqueous Na_2CO_3 solution until pH 10-11. Then, the mixture was extracted with EtOAc (5 x 15 mL), the organic layer separated by

centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography ($MeOH/CH_2Cl_2$ mixtures), isolating the enantiopure lactams (*S*)-**3a** and **3c** in good yields (75-89%).

2.3.7. Semi-preparative transamination of γ-keto esters 1d and 1e and δ-keto esters 1i and 1j

In an Erlenmeyer flask, the TA (30 mg, ATA-303 for keto ester **1d**, ATA-025 for keto ester **1e** and ATA-237 for keto esters **1i** and **1j**) and keto ester (**1d**, **1e**, **1i** and **1j**, 30 mg, 15 mM) were added in phosphate buffer 100 mM pH 7.5 (1 mM PLP, 0.1 or 1 M isopropylamine) and MeCN (2.5% v/v). The reaction was shaken at 45 ºC and 250 rpm for 24 h (**1d**, **1i** and **1j**) or 48 h (**1e**) and then stopped by adding a saturated aqueous Na_2CO_3 solution until pH 10-11. Then the mixture was extracted with EtOAc (5 x 15 mL), the organic layer separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$ mixtures), yielding the lactams (*R*)-**3d** and **3e** and (*S*)-**3i** and **3j** in moderate to good yields (66-90%) and good to excellent enantiomeric excess (91->99%).

2.3.8. Preparative transamination of γ-keto esters 1f-h

In an Erlenmeyer flask, ATA-025 (100 mg) and keto ester (**1f-h**, 100 mg, 15 mM) were added in phosphate buffer 100 mM pH 7.5 (1 mM PLP, 0.1 or 1 M isopropylamine) and MeCN (2.5% v/v). The reaction was shaken at 45 ºC for 48 h and then stopped by adding a saturated aqueous Na_2CO_3 solution until pH 10-11. Then, the mixture was extracted with EtOAc (5 x 15 mL), the organic layer separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography on silica gel ($MeOH/CH_2Cl_2$ mixtures), yielding lactams (*R*)-**3f-h** in good yields (75-86%) and excellent enantiomeric excess (>99%).

Publication 2

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Conversion of γ- and δ-Keto Esters into Optically Active Lactams. Transaminases in Cascade Processes

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Dedicated to Prof. Vicente Gotor on occasion of his 70th birthday

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Abstract: A one-pot two-step enzymatic strategy has been designed for the production of optically active γ- and δ-lactams in aqueous medium under mild conditions. The approach is based on the biotransamination of ethyl or methyl keto esters bearing different alkyl or aryl substitution patterns at α-position to the ketone functionality. In this manner, the keto esters were transformed into the corresponding amino esters with excellent conversions, which underwent spontaneous cyclisation in the reaction medium without addition of external reagents. Depending on the transaminase selectivity, both lactam enantiomers can be obtained, so initial enzyme screenings were performed using commercially available and *made in house* enzymes. Reaction conditions were optimised focusing on the substrate concentration, temperature and ratio of amine donor *vs* acceptor. Thus, ten γ- and δ-lactams were obtained in good to high isolated yields (70–90%) and excellent selectivities (94–99%) after one or two days at 30 or 45 °C.

Keywords: Cascade reaction; Intramolecular cyclisation; Lactams; Stereoselective synthesis; Transaminase

1 Introduction

Lactams are pivotal compounds in organic chemistry since they are present in numerous bioactive products, also serving as valuable intermediates for more complex structures including synthetic polymers.[1] Apart from β-lactams (azetidin-2-ones, Figure 1) that constitute the most important class of currently approved antibiotics,[2] the syntheses of γ-lactams (pyrrolidin-2-ones) and δ-lactams (piperidin-2-ones) have attracted considerable attention in recent years,[3] due to the presence of these nitrogen containing heterocycles in demanding biologically active compounds.

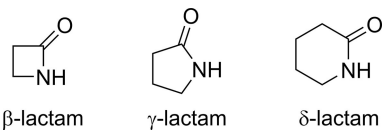
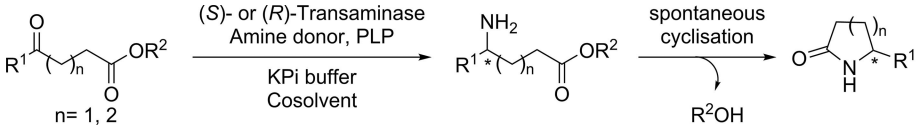


Figure 1. General structure of β-, γ- and δ-lactams.

Biocatalysis provides access to optically pure products under mild reaction conditions, including the stereoselective synthesis of lactams.[4] Kinetic resolutions of racemic lactams *via* hydrolase-catalysed ring opening or N-acylation reactions, have gained great attention in the last decades using lactamases, lipases or esterases.[5] However, most of the examples are focused on the preparation of optically active β-lactams, and are limited to 50% yield due to the inherent limitations of classical kinetic resolutions.

Interestingly, lipases have served for the production of lactams through intramolecular cyclisation of carboxylic acid derivatives intermediates, although these approaches have been disclosed in a non-selective fashion.[6] Transaminase-catalysed processes have also been reported for the production of lactams



Scheme 1. General transformation of γ- and δ-keto esters into optically active lactams mediated by transaminases.

in a highly selective manner.[7] This strategy is based on the biotransamination of aldo or keto esters and subsequent intramolecular cyclisation of the so-obtained optically active amino esters. In this manner, the chemical equilibrium is shifted to the formation of the desired lactams. In this scenario, the usefulness of commercially available transaminases has been reported for the conversion of ethyl 4-oxo-3-phenylbutyrate into (*R*)-4-phenylpyrrolidin-2-one,[7a] isopropyl 4-(4-bromophenyl)-5-oxopentanoate into (*S*)-5-(4-bromophenyl)piperidin-2-one,[7c] and ethyl 4-acetylbutyrate in both (*S*)- and (*R*)-6-methylpiperidin-2-one.[7b] More recently a novel reductive aminase from *Aspergillus oryzae* has been engineered and applied in the reductive amination of ethyl levulinate using three aliphatic amines, allowing the asymmetric synthesis of optically active N-substituted lactams.[8]

Herein, we propose a general strategy for the asymmetric preparation of γ- and δ-lactams based on the biotransamination of easily accessible keto esters, followed by intramolecular cyclisation in the own reaction medium (Scheme 1). Screening and optimisation of the reaction conditions will be disclosed in order to give access to both enantiomers of a series of lactams in a one-pot two-step synthetic approach. The selection of the proper transaminase will be a key issue, affecting the reaction conditions such as pH, substrate and amine donor concentrations and temperature, to develop high-yielding and stereoselective protocols.

2 Results and Discussion

Levulinic acid derivatives are considered valuable lactam precursors due to their simple preparation from lignocellulosic biomass and in some cases commercial availability.[9] For that reason ethyl levulinate (**1a**) was selected as model substrate for this biotransamination screening. A 25 mM substrate concentration was initially considered in order to test 26 commercial transaminases, all of them accepting isopropylamine (IPA) as amine donor. Initially, IPA was used in a large excess (1 M concentration), aiming to drive the equilibrium towards the formation of the amino ester **2a**, which will later spontaneously cyclise into the lactam **3a**.

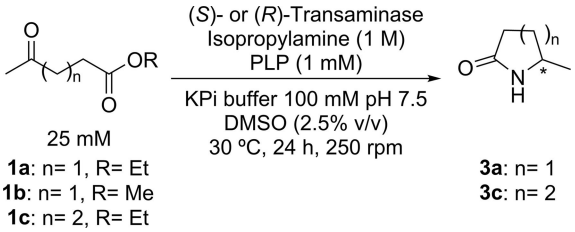
For an exhaustive screening study, more data can be found in Table S3 in the Supporting Information. Based on previous studies,[10] 2.5% of DMSO was used as cosolvent in combination with phosphate buffer 100 mM pH 7.5. The reactions were incubated for 24 h at 30 °C, observing the solely formation of optically active 5-methylpyrrolidin-2-one (**3a**), obtained after spontaneous intramolecular cyclisation in the reaction medium of ethyl 4-aminopentanoate (**2a**). The best results have been summarised in Table 1 (entries 1–5), finding the best conditions for TA-P2-B01 (87% conversion, >99% *ee* (*R*), entry 2) and ATA-237 (92% conversion, >99% *ee* (*S*), entry 3) in the complementary synthesis of both **3a** lactam antipodes. In addition, transaminases such the (*S*)-selective ones from *Chromobacterium violaceum* (Cv-TA)[11] and a variant from *Arthrobacter citreus* (ArS-TA),[12] and the (*R*)-selective TAs from *Arthrobacter* species (ArR-TA)[13] and an evolved mutant (ArRmut11-TA),[14] were also tested as freeze-dried cell powder of *E. coli* containing the corresponding overexpressed enzyme. Particularly, an excellent activity and selectivity for ArS-TA was achieved towards the formation of (*S*)-**3a** (97% conversion, >99% *ee*, entry 5).

We also studied the effect of the leaving group, performing biotransamination experiments over methyl levulinate (**1b**, R = Me), which affords an alternative access to **3a** (entries 6–11). Conversions over 80% were reached leading to both lactam enantiomers with excellent selectivity, finding a much higher reactivity for some enzymes such as TA-P2-A07 when considering the methyl instead of the ethyl ester as starting material (compare entries 1 and 7). For a full comparison of the transaminase activity between substrates **1a** and **1b**, see Tables S3 and S4 in the Supporting Information. In summary, hits for both substrates **1a**,**b** were found, although in general better conversions were achieved with the methyl keto ester derivative.

Looking for a further exploitation of the synthetic approach, the study of a substrate with an increased alkyl chain length between both carbonyl groups was considered. Thus, both enantiomers of δ-lactam 6-methylpiperidin-2-one were straightforward obtained from ethyl 5-oxohexanoate (**3c**) in a highly selective manner (>88% conversion and >96% *ee*), using different enzymatic preparations (see entries 12–18 and also Tables S3 and S4 in the Supporting Information).

Next, the enzymatic study over methyl 4-aryl-4-oxobutanonates was envisaged, firstly starting from commercially available methyl 4-oxo-4-phenylbuta-

Table 1. Stereoselective transformation of keto esters **1a–c** into lactams **3a** and **3c**.



Entry	Keto ester	n	R	Transaminase	*c* [%][a]	*ee* [%][b]
1	**1a**	1	Et	TA-P2-A07	41	>99 (*R*)
2	**1a**	1	Et	TA-P2-B01	87	>99 (*R*)
3	**1a**	1	Et	ATA-237	92	>99 (*S*)
4	**1a**	1	Et	TA-P1-B04	89	>99 (*S*)
5	**1a**	1	Et	ArS-TA	97	>99 (*S*)
6	**1b**	1	Me	ATA-412	84	>99 (*R*)
7	**1b**	1	Me	TA-P2-A07	81	>99 (*R*)
8	**1b**	1	Me	TA-P2-B01	80	>99 (*R*)
9	**1b**	1	Me	TA-P1-A06	90	>99 (*S*)
10	**1b**	1	Me	TA-P1-G06	91	>99 (*S*)
11	**1b**	1	Me	ArS-TA	>99	>99 (*S*)
12	**1c**	2	Et	ATA-013	89	96 (*R*)
13	**1c**	2	Et	ATA-033	87	97 (*R*)
14	**1c**	2	Et	TA-P2-B01	86	97 (*R*)
15	**1c**	2	Et	ATA-200	86	>99 (*S*)
16	**1c**	2	Et	TA-P1-A06	88	>99 (*S*)
17	**1c**	2	Et	TA-P1-G06	96	99 (*S*)
18	**1c**	2	Et	ArS-TA	98	>99 (*S*)

[a] Conversion values determined by GC (see Supporting Information).
[b] Determined by HPLC using chiral columns (see Supporting Information). Absolute configurations of the corresponding lactams **3a** and **3c** appear in brackets.

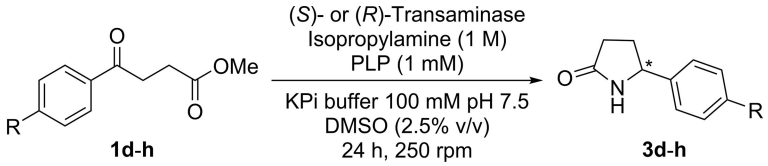
noate (**1d**), and later extending the study to compounds bearing different substitution patterns at the *para*-position of the aromatic ring (OMe, Me, F and Et). These keto esters **1e–h** were obtained through esterification of the corresponding carboxylic acids with refluxing methanol in acidic media (98–99%). Subsequent reductive amination using sodium cyanoborohydride and ammonium acetate at room temperature led to the synthesis of the racemic lactams **3d–h** (59–74%), used as references for the enzymatic transformations.

γ-Keto ester **1d** was initially studied and the best results are summarised in Table 2. In most of the cases, a lower reactivity was detected in comparison with alkylated esters **1a–c** (see Tables S3–S5 in the Supporting Information for additional data). In fact, conversions lower than 60% were attained when working at 25 mM substrate concentration (entries 1 and 4), even with the best candidates. In order to reach higher conversions maintaining the excellent selectivity towards the synthesis of **3d**, two reaction parameters were analysed: the substrate concentration (15 mM, entries 2 and 5) and the temperature (45 °C, entries 3 and 6). Remarkably, in the case of ATA-303 it was possible to obtain enantiopure (*R*)-**3d** in 97% conversion at 15 mM concentration and 45 °C.

Under these conditions, keto esters **1e–h** (entries 7–19) were transformed, yielding in all cases the lactams in enantiomerically pure form and in 81–99% conversion, depending on the enzyme (see Tables S6–S9 in the Supporting Information for extensive screenings). Interestingly, ATA-025 and ATA-033 were found as very robust enzymes for the formation of the (*R*)-enantiomers, while ATA-234 allowed the formation of their antipodes in a complete selective manner, and much higher conversions in comparison with the one achieved with the non-substituted keto ester **1d** (56% conversion and 97% *ee*, see Table S5 in the Supporting Information).

As a continuation, 5-aryl-5-oxopentanoates **1i** and **1j** were selected in order to obtain the corresponding 6-arylpiperidin-2-ones **3i** and **3j**. Firstly, the chemical syntheses of both keto esters and the corresponding lactams were carried out, following identical procedures than the ones described for **1e–h** and **3d–h**. In general, biotransamination experiments led to lower conversion values in comparison with the results obtained with the homologue butanoates **1d** and **1g**

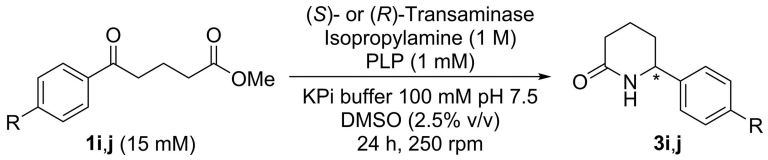
Table 2. Stereoselective transformation of γ-keto esters **1d–h** into 5-arylpyrrolidin-2-ones **3d–h**.



Entry	R (Keto ester)	[Keto ester] [mM]	T [°C]	Transaminase	*c* [%][a]	*ee* [%][b]
1	H (**1d**)	25	30	ATA-303	59	>99 (*R*)
2	H (**1d**)	15	30	ATA-303	78	>99 (*R*)
3	H (**1d**)	15	45	ATA-303	97	>99 (*R*)
4	H (**1d**)	25	30	ATA-237	45	98 (*S*)
5	H (**1d**)	15	30	ATA-237	57	>99 (*S*)
6	H (**1d**)	15	45	ATA-237	57	93 (*S*)
7	OMe (**1e**)	15	45	ATA-025	94	>99 (*R*)
8	OMe (**1e**)	15	45	ATA-033	95	>99 (*R*)
9	OMe (**1e**)	15	45	ATA-234	91	>99 (*S*)
10	Me (**1f**)	15	45	ATA-025	98	>99 (*R*)
11	Me (**1f**)	15	45	ATA-033	98	>99 (*R*)
12	Me (**1f**)	15	45	ATA-234	89	>99 (*S*)
13	F (**1g**)	15	45	ATA-024	99	>99 (*R*)
14	F (**1g**)	15	45	ATA-025	98	>99 (*R*)
15	F (**1g**)	15	45	ATA-415	99	>99 (*R*)
16	F (**1g**)	15	45	ATA-234	81	>99 (*S*)
17	Et (**1h**)	15	45	ATA-025	83	>99 (*R*)
18	Et (**1h**)	15	45	ATA-033	86	>99 (*R*)
19	Et (**1h**)	15	45	ATA-234	82	>99 (*S*)

[a] Conversion values determined by GC (see Supporting Information).
[b] Determined by HPLC using chiral columns (see Supporting Information). Absolute configurations of the corresponding lactams **3d–h** appear in brackets.

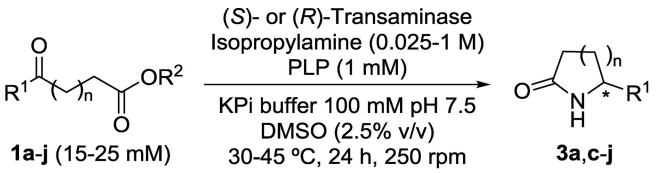
Table 3. Stereoselective transformation of δ-keto esters **1i,j** into 6-arylpiperidin-2-ones **3i,j**.



Entry	Keto ester	R	T [°C]	Transaminase	*c* [%][a]	*ee* [%][b]
1	**1i**	H	30	ATA-033	20	>99 (*R*)
2	**1i**	H	45	ATA-033	28	>99 (*R*)
3	**1i**	H	30	ATA-415	71	>99 (*R*)
4	**1i**	H	45	ATA-415	47	>99 (*R*)
5	**1i**	H	30	ATA-237	94	94 (*S*)
6	**1i**	H	45	ATA-237	94	95 (*S*)
7	**1j**	F	30	ATA-025	43	>99 (*R*)
8	**1j**	F	45	ATA-025	58	>99 (*R*)
9	**1j**	F	30	ATA-237	90	92 (*S*)
10	**1j**	F	45	ATA-237	96	93 (*S*)

[a] Conversion values determined by GC (see Supporting Information).
[b] Determined by HPLC using chiral columns (see Supporting Information). Absolute configurations of the corresponding lactams **3i** and **3j** appear in brackets.

(Table 3 and see additional data in Tables S10 and S11 in the Supporting Information). An increase in the temperature led to a conversion improvement for the (*R*)-selective ATA-033 and ATA-025 (entries 1–2 and 7–8), although this failed for ATA-415, which led to the best conversion value towards enantiopure (*R*)-**3i**



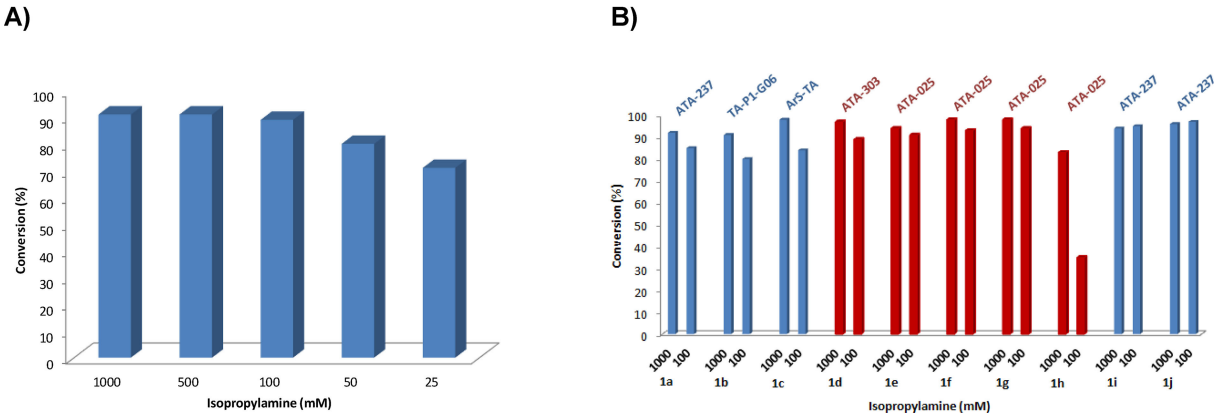


Figure 2. Influence of the isopropylamine concentration in the TA-catalysed transformation of keto esters **1a–j** into lactams **3a,c–j**. Blue colour denotes the production of (*S*)-amines (89->99% *ee*), while red bars are used to indicate (*R*)-amines (> 99% *ee*). Figure 2A **(left):** Biotransamination of keto ester **1b** into lactam **3a** using TA-P1-G06 under the following conditions: 25 mM **1b**, 25–1000 mM $^{i}PrNH_2$, 30 °C, 24 h and 250 rpm. Figure 2B **(right):** Biotransamination of keto esters **1a–j** into lactams **3a,c–j** under the following conditions: for **1a–c**, 25 mM keto ester, 0.1 or 1 M $^{i}PrNH_2$, 30 °C, 24 h and 250 rpm; for **1d–j**, 15 mM keto ester, 0.1 or 1 M $^{i}PrNH_2$, 45 °C, 24 h and 250 rpm.

at 30 °C (entries 3 and 4). (*S*)-Selective ATA-237 displayed the highest activities (94–96% conversion) acting with a high stereocontrol (93–95% *ee*, entries 6 and 10) at 45 °C.

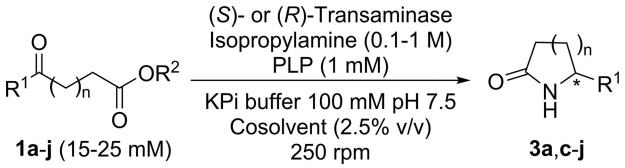
Driving the equilibrium towards the formation of amine products is a key issue in transaminase-catalysed reactions, as the reductive amination process is thermodynamically hampered; therefore, a large molar excess of the amine donor is required. In the last years, the search for novel smart co-substrates or the *in situ* removal of the formed co-products have been applied.[15] The advantage of the present strategy is the formation of an amine intermediate that cyclises in the same reaction medium forming the final lactam, so the reversibility of the equilibrium should be dramatically diminished. For that reason, we envisaged the use of lower contents of isopropylamine and the results have been summarised in Figure 2 (for numerical data see Table S12).

Methyl levulinate (**1b**, 25 mM) was selected as model substrate, and the biotransformations with TA-P1-G06 at 30 °C were carried out using from an equimolar amount to a 40-molar excess of IPA (Figure 2A). After 24 h, similar conversions (89–91%) were found between 0.1–1 M IPA concentrations, observing a significant decrease at 50 mM IPA concentration (80%). Even at equimolar amount, a high conversion was still observed (71%). Then, the transaminase-catalysed reactions with all the previously tested substrates (Figure 2B) were carried out at 0.1 and 1 M IPA concentrations, finding in general slightly lower conversions at lower IPA contents, although a clear negative effect was only remarkable for the case of **1h**.

Finally, preparative biotransformations (up to 100-mg scale) were carried out under the optimised reaction conditions using 1 M of IPA in order to assure very high conversions (Table 4), yielding in all cases the desired lactams with good to high isolated yields (66–89%) and in general excellent selectivities (94–>99% *ee*). Cosolvents with a lower boiling point than DMSO were used, such as MeCN for commercially available TAs, and MTBE for ArS-TA, searching for high-yielding processes towards the final products with easier work-up protocols. Optical rotation values were measured for all the isolated lactam products, and their values compared with reported data in order to assign their absolute configurations.[16–20]

Keto esters **1a–c** bearing an alkyl substitution attached to the ketone functionality were used at 25 mM substrate concentration and 30 °C, while for aromatic compounds **1d–j** the substrate concentration was reduced to 15 mM, and a higher temperature (45 °C) was required to reach conversions over 70%. As previously observed in Figure 2, the use of a lower

Table 4. Stereoselective transformations of keto esters **1b–j** into lactams **3a,c–j** in preparative scale.



Entry	[Keto ester]	[^{i}PrNH$_2$] [M]	T [°C]	Transaminase	Cosolvent	t [h]	*c* [%][a]	*ee* [%][b]
1	25 (**1a**)	1	30	ATA-237	MeCN	24	97 (75)	>99 (*S*)
2	25 (**1b**)	1	30	TA-P1-G06	MeCN	48	88 (80)	>99 (*S*)
3	25 (**1c**)	1	30	ArS-TA	MTBE	24	98 (89)	>99 (*S*)
4	15 (**1d**)	1	45	ATA-303	MeCN	24	97 (88)	>99 (*R*)
5	15 (**1e**)	1	45	ATA-025	MeCN	48	82 (74)	>99 (*R*)
6	15 (**1e**)	0.1	45	ATA-025	MeCN	48	89 (80)	>99 (*R*)
7	15 (**1f**)	1	45	ATA-025	MeCN	48	91 (86)	>99 (*R*)
8	15 (**1g**)	1	45	ATA-025	MeCN	48	80 (75)	>99 (*R*)
9	15 (**1h**)	1	45	ATA-025	MeCN	48	89 (81)	>99 (*R*)
10	15 (**1i**)	1	45	ATA-237	MeCN	24	77 (70)	94 (*S*)
11	15 (**1j**)	1	45	ATA-237	MeCN	24	70 (66)	96 (*S*)
12	15 (**1j**)	0.1	45	ATA-237	MeCN	24	93 (90)	91 (*S*)

[a] Conversion values determined by GC (see Supporting Information). Isolated yields appear in brackets.
[b] Determined by HPLC using chiral columns (see Supporting Information). Absolute configurations of the corresponding lactams **3a**,**c–j** appear in brackets.

amount of IPA (100 mM) could also lead to practical processes (entries 6 and 12). Thus, (*S*)- and (*R*)-selective TAs were chosen for preparative biotransformations and the amount of IPA was 10-fold reduced, obtaining high conversions (89–93%) and good to excellent selectivities (91–>99% *ee*) for substrates **1e** and **1j**.

3 Conclusions

A simple and straightforward one-pot two-step process has been described for the transformation of different γ- and δ-keto esters into the corresponding optically active lactams. The strategy is based on a selective biotransamination reaction that allows the formation of the amino ester intermediates, which undergo spontaneous intramolecular cyclisation in the own aqueous medium without the addition of external reagents. Overall, this is a general process as it was extended to 10 substrates with different substitution patterns. Amino ester intermediates were not detected in any case, facilitating the isolation of the final optically active lactam products.

Chiral 6-substituted piperidin-2-ones and 5-substituted pyrrolidin-2-ones were obtained with high optical purity (94–>99% *ee*) and generally in good to very high yields (66–90%) for selected preparative biotransformations, the choice of the transaminase allowing the preparation of the desired lactam enantiomer under mild reaction conditions. Substrate concentration and temperature resulted to be key issues for driving the reactions to high conversions, being possible the reduction of the amine donor equivalents in selected cases without a significant drop of the conversion values due to the thermodynamically favoured intramolecular cyclisation process.

Experimental Section

General Methods

Codex Transaminase ATA Screening Kit (ATASK-000250) and pyridoxal 5′-phosphate (PLP) were purchased from Codexis. Transaminases from *Chromobacterium violaceum* (Cv-TA, internal plasmid number pET20) and *Arthrobacter sp.* [ArR-TA (pEG23), ArS-TA (pEG29) and ArRmut11-TA (pEG90)] overexpressed on *E. coli cells* were provided by Prof. Wolfgang Kroutil (University of Graz).[13c] All other reagents were obtained from commercial sources (Sigma–Aldrich, Acros, and Fluka) and used as received except dry methanol that was previously distilled under nitrogen using calcium hydride as desiccant.

NMR spectra were recorded on a Bruker AV300 MHz spectrometer. All chemical shifts (δ) are given in parts per million (ppm) and referenced to the residual solvent signal as internal standard. IR spectra were recorded on a Bruker ALPHA spectrophotometer on NaCl pellets or KBr pellets. ν_{max} values are given for the main absorption bands. High resolution mass spectra (HRMS) were obtained in a Micro Tof Q spectrometer using ESI^+ or ESI^-. Measurement of the optical rotation values was carried out at 590 nm on a PerkinElmer 241 polarimeter.

Gas chromatography (GC) and high performance liquid chromatography (HPLC) analyses were performed for conversion and enantiomeric excess measurements (see the

Supporting Information). GC analyses were performed on an Agilent HP7820 GC chromatograph equipped with a FID detector. HPLC analyses were carried out in a Hewlett Packard 1100 chromatograph UV detector at 210 nm. Thin-layer chromatography (TLC) was conducted with Merck Silica Gel 60 F254 precoated plates and visualised with UV and potassium permanganate stain. Column chromatography was performed using Merck Silica Gel 60 (230–400 mesh).

General procedure for the chemical synthesis of γ-keto esters 1e–h and δ-keto esters 1i and 1j. To a solution of the corresponding keto acid **4e–j** (1.6 mmol) in MeOH (8 mL, 0.2 M), a few drops of concentrated sulfuric acid were added at room temperature. The mixture was stirred and heated at 68 °C overnight. After this time, H_2O (10 mL) was added. The mixture was neutralised with an aqueous NaOH 2 M solution and the product was extracted with CH_2Cl_2 (3× 15 mL). The organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure, isolating the corresponding keto esters **1e–h,i,j** in excellent purity, which were later used without further purification (91–99%).

Methyl 4-(4-methoxyphenyl)-4-oxobutanoate (1e). Yellowish solid (323 mg, 99% yield). R_f (50% EtOAc/Hexane): 0.4. Mp: 46–47 °C. IR (KBr): ν 3058, 2954, 2918, 2844, 1737, 1602, 1439, 1318, 1030, 737 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.73 (t, $^3J_{HH}$ = 6.7 Hz, 2H), 3.25 (t, $^3J_{HH}$ = 6.7 Hz, 2H), 3.68 (s, 3H), 3.84 (s, 3H) 6.92 (d, $^3J_{HH}$ = 8.9 Hz, 2H), 7.94 (d, $^3J_{HH}$ = 8.9 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 28.0 (CH_2), 32.9 (CH_2), 51.7 (CH_3), 55.4 (CH_3), 113.7 (2 CH), 129.5 (C), 130.2 (2 CH), 163.5 (C), 173.4 (C), 196.5 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{12}H_{14}NaO_4)^+$ $(M+Na)^+$ 245.0784; found 245.0780.

Methyl 4-(4-methylphenyl)-4-oxobutanoate (1f). Yellowish solid (322 mg, 99% yield). R_f (50% EtOAc/Hexane): 0.63. Mp: 51–52 °C. IR (KBr): ν 3057, 2990, 2953, 2921, 2850, 1737, 1608, 1438, 1307, 1029, 738 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.43 (s, 3H), 2.78 (t, $^3J_{HH}$ = 6.7 Hz, 2H), 3.32 (t, $^3J_{HH}$ = 6.7 Hz, 2H), 3.72 (s, 3H), 7.28 (d, $^3J_{HH}$ = 7.8 Hz, 2H), 7.90 (d, $^3J_{HH}$ = 8.2 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 21.6 (CH_3), 28.0 (CH_2), 33.2 (CH_2), 51.8 (CH_3), 128.1 (2 CH), 129.2 (2 CH), 134.0 (C), 144.0 (C), 173.4 (C), 197.6 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{12}H_{14}NaO_3)^+$ $(M+Na)^+$ 229.0835; found 229.0831.

Methyl 4-(4-fluorophenyl)-4-oxobutanoate (1g). White solid (210 mg, 98% yield). R_f (50% EtOAc/Hexane): 0.37. Mp: 51–52 °C. IR (KBr): ν 3064, 2954, 2918, 2850, 1738, 1599, 1439, 1412, 1268, 1157, 737 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.76 (t, $^3J_{HH}$ = 6.6 Hz, 2H), 3.29 (t, $^3J_{HH}$ = 6.6 Hz, 2H), 3.70 (s, 3H), 7.13 (t, $^3J_{HH}$ = 8.6 Hz, 2H), 8.01 (dd, $^3J_{HH}$ = 8.9 Hz, $^3J_{HF}$ = 5.4 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 27.9 (CH_2), 33.2 (CH_2), 51.8 (CH_3), 115.6 (d, $^2J_{CF}$ = 21.9 Hz, 2 CH), 130.6 (d, $^3J_{CF}$ = 9.3 Hz, 2 CH), 132.9 (d, $^4J_{CF}$ = 2.8 Hz, C), 165 (d, $^1J_{CF}$ = 254.8 Hz, C), 173.2 (C), 196.4 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{11}H_{11}FNaO_3)^+$ $(M+Na)^+$ 233.0584; found 233.0583.

Methyl 4-(4-ethylphenyl)-4-oxobutanoate (1h). Yellowish oil (528 mg, 98% yield). R_f (50% EtOAc/Hexane): 0.5. IR (NaCl): ν 3057, 2969, 2920, 2877, 2850, 1737, 1607, 1439, 1415, 1266, 1169, 738 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.26 (t, $^3J_{HH}$ = 7.6 Hz, 3H), 2.67–2.78 (m, 4H), 3.30 (t, $^3J_{HH}$ = 6.7 Hz, 2H), 3.70 (s, 3H), 7.28 (d, $^3J_{HH}$ = 8.3 Hz, 2H), 7.91 (d, $^3J_{HH}$ = 8.3 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 15.1 (CH_3), 28.0 (CH_2), 28.9 (CH_2), 33.2 (CH_2), 51.7 (CH_3), 128.0 (2 CH), 128.2 (2 CH), 134.2 (C), 150.1 (C), 173.4 (C), 197.6 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{13}H_{16}NaO_3)^+$ $(M+Na)^+$ 243.0992; found 243.0990.

Methyl 5-oxo-phenylpentanoate (1i). Yellowish oil (489 mg, 92% yield). R_f (50% EtOAc/Hexane): 0.57. IR (KBr): ν 3057, 2986, 2953, 2848, 1735, 1449, 1419, 1372, 1266, 1179, 704, 692 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.08 (quin, $^3J_{HH}$ = 7.1 Hz, 2H), 2.44 (t, $^3J_{HH}$ = 7.2 Hz, 2H), 3.04 (t, $^3J_{HH}$ = 7.1 Hz, 2H), 3.66 (s, 3H), 7.44 (m, 2H), 7.54 (m, 1H), 7.95 (dd, $^3J_{HH}$ = 8.4 Hz, $^4J_{HH}$ = 1.4 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 19.3 (CH_2), 33.0 (CH_2), 37.4 (CH_2), 51.5 (CH_3), 127.9 (2 CH), 128.5 (2 CH), 133.0 (CH), 136.7 (C), 173.6 (C), 199.3 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{12}H_{14}NaO_3)^+$ $(M+Na)^+$ 229.0835; found 229.0835.

Methyl 5-(4-fluorophenyl)-5-oxopentanoate (1j). White solid (504 mg, 91% yield). R_f (50% EtOAc/Hexane): 0.33. Mp: 46–47 °C. IR (KBr): ν 3059, 2953, 2849, 1735, 1598, 1410, 1371, 1267, 1157, 738 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 2.06 (quin, $^3J_{HH}$ = 7.1 Hz, 2H), 2.46 (t, $^3J_{HH}$ = 7.1 Hz, 2H), 3.04 (t, $^3J_{HH}$ = 7.1 Hz, 2H), 3.60 (s, 3H), 7.14 (t, $^3J_{HH}$ = 8.6 Hz, 2H), 8.00 (dd, $^3J_{HH}$ = 8.9 Hz, $^3J_{HF}$ = 5.4 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 19.3 (CH_2), 33.0 (CH_2), 37.3 (CH_2), 51.6 (CH_3), 115.6 (d, $^2J_{CF}$ = 21.8 Hz, 2 CH), 130.6 (d, $^3J_{CF}$ = 9.4 Hz, 2 CH), 133.2 (d, $^4J_{CF}$ = 3.3 Hz, C), 165.0 (d, $^1J_{CF}$ = 254.9 Hz, C), 173.6 (C), 197.7 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{12}H_{13}FNaO_3)^+$ $(M+Na)^+$ 247.0741; found 247.0742.

General procedure for the chemical synthesis of racemic γ-lactams 3d–h and δ-lactams 3i and 3j. In order to develop robust analytical methods for monitoring the biotransaminations, the synthesis of racemic lactams was previously chemically performed in the following manner: Ammonium acetate (200 mg, 2.6 mmol) and sodium cyanoborohydride (33 mg, 0.52 mmol) were successively added to a solution of the corresponding keto ester **1d–j** (0.26 mmol) in dry MeOH (1.0 mL) under inert atmosphere. The mixture was stirred at room temperature during 16 h and, after this time, H_2O (5 mL) was added to quench the reaction. The solution was basified until pH around 11 by adding a saturated aqueous solution of Na_2CO_3. Then the mixture was extracted with Et_2O (3×10 mL) and the organic layers were combined, dried over Na_2SO_4 and concentrated under reduced pressure. The product was purified by chromatography column on silica gel (2% MeOH/CH_2Cl_2), affording the racemic lactams **3d–j** (48–74%).

5-Phenylpyrrolidin-2-one (3d). White solid (29 mg, 74% yield). R_f (5% MeOH/CH_2Cl_2): 0.38. Mp: 107–108 °C. IR (KBr): ν 3055, 2959, 2926, 2872, 2858, 1687, 1266, 1157, 740, 705 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.94–2.06 (m, 1H), 2.38–2.52 (m, 2H), 2.54–2.66 (m, 1H), 4.78 (t, $^3J_{HH}$ = 7.1 Hz, 1H), 6.01 (br s, 1H), 7.28–7.42 (m, 5H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 30.2 (CH_2), 31.4 (CH_2), 58.0 (CH), 125.6 (2 CH), 127.9 (CH), 128.9 (2 CH), 142.3 (C), 178.4 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{10}H_{11}NNaO)^+$ $(M+Na)^+$ 184.0733; found 184.0735.

5-(4-Methoxyphenyl)pyrrolidin-2-one (3e). Yellowish solid (26 mg, 60% yield). R_f (5% MeOH/CH_2Cl_2): 0.43. Mp: 115–116 °C. IR (KBr): ν 3426, 3055, 2986, 1696, 1265, 1035, 739 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.96 (m, 1H), 2.39–2.56 (m, 3H), 3.80 (s, 3H), 4.71 (t, $^3J_{HH}$ = 7.0 Hz, 1H), 6.44 (br s, 1H), 6.89 (d, $^3J_{HH}$ = 8.7 Hz, 2H), 7.21 (d, $^3J_{HH}$ = 8.7 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 30.4 (CH_2), 31.5 (CH_2), 55.3 (CH_3), 57.6 (CH), 114.2 (2 CH), 126.8 (2 CH), 134.4 (C), 159.2 (C), 178.4 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{11}H_{13}NNaO_2)^+$ $(M+Na)^+$ 214.0838; found 214.0836.

5-(4-Methylphenyl)pyrrolidin-2-one (3f). White solid (31 mg, 73% yield). R_f (5% MeOH/CH_2Cl_2): 0.5. Mp: 89–90 °C. IR (KBr): ν 3425, 3054, 2985, 2922, 2852, 1695, 1266, 1156, 738, 705 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.93–2.06 (m, 1H), 2.38 (s, 3H), 2.45 (m, 2H), 2.51–2.62 (m, 1H), 4.73 (t, $^3J_{HH}$ = 7.1 Hz, 1H), 6.23 (br s, 1H), 7.14–7.28 (m, 4H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 21.0 (CH_3), 30.3 (CH_2), 31.4 (CH_2), 57.8 (CH), 126.2 (2 CH), 129.2 (2 CH), 137.7 (C), 139.4 (C), 178.4 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{11}H_{13}NNaO)^+$ $(M+Na)^+$ 198.0889; found 198.0888.

5-(4-Fluorophenyl)pyrrolidin-2-one (3g). White solid (25 mg, 59% yield). R_f (5% MeOH/CH_2Cl_2): 0.5. Mp: 138–139 °C. IR (KBr): ν 3425, 3055, 2986, 2924, 1698, 1606, 1512, 1422, 1265, 738 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.88–2.00 (m, 1H), 2.41–2.52 (m, 2H), 2.58 (m, 1H), 4.78 (t, $^3J_{HH}$ = 7.1 Hz, 1H), 7.05 (t, $^3J_{HH}$ = 8.6 Hz, 2H), 7.08 (s, 1H), 7.29 (dd, $^3J_{HH}$ = 8.7 Hz, $^4J_{HF}$ = 5.2 Hz, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 30.3 (CH_2), 31.4 (CH_2), 57.4 (CH), 115.7 (d, $^2J_{CF}$ = 21.6 Hz, 2 CH), 127.3 (d, $^3J_{CF}$ = 8.1 Hz, 2 CH), 130.7 (d, $^4J_{CF}$ = 3.1 Hz, C), 162.0 (d, $^1J_{CF}$ = 246.1 Hz, C), 178.3 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{10}H_{10}FNNaO)^+$ $(M+Na)^+$ 202.0639; found 202.0635.

5-(4-Ethylphenyl)pyrrolidin-2-one (3h). Yellowish solid (30 mg, 69% yield). R_f (5% MeOH/CH_2Cl_2): 0.55. Mp: 82–83 °C. IR (KBr): ν 3425, 3054, 2984, 2970, 2931, 1696, 1422, 1265, 739 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.24 (t, $^3J_{HH}$ = 7.6 Hz, 3H), 1.99 (m, 1H), 2.37–2.47 (m, 2H), 2.48–2.60 (m, 1H), 2.66 (q, $^3J_{HH}$ = 7.6 Hz, 2H), 4.74 (t, $^3J_{HH}$ = 7.1 Hz, 1H), 6.05 (br s, 1H), 7.24 (m, 4H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 15.5 (CH_3), 28.4 (CH_2), 30.3 (CH_2), 31.4 (CH_2), 57.9 (CH), 125.6 (2 CH), 128.3 (2 CH), 139.6 (C), 144.1 (C), 178.3 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{12}H_{15}NNaO)^+$ $(M+Na)^+$ 212.1046; found 212.1044.

6-Phenylpiperidin-2-one (3i). White solid (12 mg, 48% yield). R_f (5% MeOH/CH_2Cl_2): 0.46. Mp: 112–113 °C. IR (KBr): ν 3389, 3054, 2985, 2957, 293, 2853, 1657, 1265, 738, 704 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.63–1.74 (m, 1H), 1.76–1.86 (m, 1H), 1.87–1.99 (m, 1H), 2.03–2.17 (m, 1H), 2.37–2.52 (m, 2H), 4.57 (dd, $^3J_{HH}$ = 8.8, 4.6 Hz, 1H), 6.02 (s, 1H), 7.28–7.43 (m, 5H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 19.7 (CH_2), 31.3 (CH_2), 32.2 (CH_2), 57.8 (CH), 126.0 (2 CH), 127.9 (CH), 128.8 (2 CH), 142.4 (C), 172.3 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{11}H_{13}NNaO)^+$ $(M+Na)^+$ 198.0889; found 198.0887.

6-(4-Fluorophenyl)piperidin-2-one (3j). White solid (11 mg, 50% yield). R_f (5% MeOH/CH_2Cl_2): 0.54. Mp: 99–100 °C. IR (KBr): ν 3389, 3055, 2986, 2930, 1659, 1265, 739 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.59–1.70 (m, 1H), 1.71–1.86 (m, 1H), 1.87–1.98 (m, 1H), 2.11 (m, 1H), 2.37–2.56 (m, 2H), 4.56 (dd, $^3J_{HH}$ = 8.9, 4.5 Hz, 1H), 6.02 (br s, 1H), 7.08 (t, $^3J_{HH}$ = 8.6 Hz, 2H), 7.29 (m, 2H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 19.8 (CH_2), 31.2 (CH_2), 32.3 (CH_2), 57.2 (CH), 115.7 (d, $^2J_{CF}$ = 21.6 Hz, 2 CH), 127.7 (d, $^3J_{CF}$ = 8.2 Hz, 2 CH), 138.2 (d, $^4J_{CF}$ = 2.9 Hz, C), 164.0 (d, $^1J_{CF}$ = 246.8 Hz, C), 172.2 (C). HRMS (ESI^+, *m/z*): calcd for $(C_{11}H_{12}FNNaO)^+$ $(M+Na)^+$ 216.0795; found 216.0793.

General protocol for the transformation of γ- and δ-keto esters 1a–j into optically active lactams using commercially available transaminases. In a 1.5 mL Eppendorf tube, transaminase (2 mg) and the corresponding keto ester (**1a–j**, 15 or 25 mM) were added in a phosphate buffer 100 mM pH 7.5 (500 μL, 1 mM PLP, 1 M isopropylamine), using DMSO (12.5 μL) as cosolvent. The reaction was shaken at 30 or 45 °C and 250 rpm for 24 h and stopped by the addition of an aqueous Na_2CO_3 saturated solution (200 μL). Then the mixture was extracted with EtOAc (2 × 500 μL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into lactams **3a-j** were determined by GC and *ee* values measured by HPLC.

General method for the transformation of γ- and δ-keto esters 1a–j into optically active lactams using transaminases overexpressed in *E. coli*. In a 1.5 mL Eppendorf tube, dry cells of *E. coli* overexpressing the transaminase (10 mg) and γ-keto ester (**1a–j**, 25 mM) were added in a phosphate buffer 100 mM pH 7.5 (500 μL, 1 mM PLP, 1 M isopropylamine) using DMSO (12.5 μL) as cosolvent. The reaction was shaken at 30 °C and 250 rpm for 24 h and stopped by the addition of an aqueous Na_2CO_3 saturated solution (200 μL). Then the mixture was extracted with EtOAc (2 × 500 μL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into lactams **3a-j** were determined by GC and *ee* values measured by HPLC.

Preparative biotransformation of γ-keto esters 1a–c into optically active lactams. In an Erlenmeyer flask, the transaminase (30 mg, ATA-237 for **1a**, TA-P1-G06 for **1b** or ArS-TA for **1c**) and keto ester (**1a–c**, 30 mg, 25 mM) were added in a phosphate buffer 100 mM pH 7.5 (1 mM PLP, 1 M isopropylamine) and cosolvent (2.5% v/v MeCN for **1a–b** and 2.5% v/v MTBE for **1c**). The reaction was shaken at 30 °C and 250 rpm for 24 h (**1a** and **1c**) or 48 h (**1b**) and then stopped by the addition of an aqueous Na_2CO_3 saturated solution until pH 10–11. Then, the mixture was extracted with EtOAc (5 × 15 mL), the organic layer separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography (MeOH/CH_2Cl_2 mixtures), isolating the enantiopure lactams (*S*)-**3a**,**c** in good yields (75–89%, respectively).

(*S*)-**5-Methylpyrrolidin-2-one** [(*S*)-**3a**]: Yellowish solid (19 mg, 80% yield). R_f (5% MeOH/CH_2Cl_2): 0.57. Mp: 43–44 °C. IR (NaCl): ν 3227, 3054, 2971, 2930, 1694, 1462, 1265 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.17 (d, $^3J_{HH}$ = 6.3 Hz, 3H), 1.46–1.74 (m, 1H), 2.14–2.25 (m, 1H), 2.25–2.34 (m, 2H), 3.56–3.90 (m, 1H), 7.37 (s, 1H). ^{13}C NMR

(300.13 MHz, $CDCl_3$): δ 19.8 (CH_2), 22.7 (CH_3), 30.4 (CH_2), 30.9 (CH_2), 48.7 (CH), 172.5 (C). HRMS (ESI^+, m/z): calcd for for $(C_5H_9NNaO)^+$ $(M+Na)^+$ 122.0576; found 122.0574.

(*S*)-**6-Methylpiperidin-2-one** [(*S*)-**3c**]: White solid (19 mg, 89% yield). R_f (5% MeOH/CH_2Cl_2): 0.51. Mp: 87–88 °C. IR (NaCl): ν 3392, 3054, 2972, 2935, 1659, 1468, 1265 cm^{-1}. ^1H NMR (300.13 MHz, $CDCl_3$): δ 1.18 (d, $^3J_{HH}=6.4$ Hz, 3H), 1.26–1.36 (m, 1H), 1.52–1.79 (m, 1H), 1.75–1.98 (m, 2H), 2.09–2.45 (m, 2H), 3.44–3.55 (m, 1H), 6.69 (s, 1H). ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 22.1 (CH_3), 29.0 (CH_2), 30.7 (CH_2), 50.2 (CH), 178.7 (C). HRMS (ESI^+, m/z): calcd for $(C_6H_{11}NNaO)^+$ $(M+Na)^+$ 136.0732; found 136.0730.

Preparative biotransformation of γ-keto esters 1d and 1e and δ-keto esters 1i and 1j into optically active lactams. In an Erlenmeyer flask, the TA (30 mg, ATA-303 for keto ester **1d**, ATA-025 for keto ester **1e** and ATA-237 for keto esters **1i** and **1j**) and keto ester (**1d**, **1e**, **1i** and **1j**, 30 mg, 15 mM) were added in phosphate buffer 100 mM pH 7.5 (1 mM PLP, 0.1 or 1 M isopropylamine) and MeCN (2.5% v/v). The reaction was shaken at 45 °C and 250 rpm for 24 h (**1d**, **1i** and **1j**) or 48 h (**1e**) and then stopped by adding an aqueous saturated solution of Na_2CO_3 until pH 10–11. Then the mixture was extracted with ethyl acetate (5×15 mL), the organic layer separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography (MeOH/CH_2Cl_2 mixtures), yielding the lactams (*R*)-**3d** and **3e** and (*S*)-**3i** and **3j** in moderate to good yields (66–90%) and good to excellent enantiomeric excess (91->99%).

Preparative biotransformation of γ-keto esters 1f–h into optically active lactams. In an Erlenmeyer flask, ATA-025 (100 mg) and keto ester (**1f–h**, 100 mg, 15 mM) were added in phosphate buffer 100 mM pH 7.5 (1 mM PLP, 0.1 or 1 M isopropylamine) and MeCN (2.5% v/v). The reaction was shaken at 45 °C for 48 h and then stopped by adding a saturated solution of Na_2CO_3 until pH 10–11. Then, the mixture was extracted with ethyl acetate (5×15 mL), the organic layer separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography (MeOH/CH_2Cl_2 mixtures), yielding lactams (*R*)-**3f–h** in good yields (75–86%) and excellent enantiomeric excess (>99%). Optical rotation values for (*R*)-lactams **3f–h**: $[\alpha]_D^{25}=+24.5$ (c=1.0, EtOH) for (*R*)-**3f** in >99% *ee*; $[\alpha]_D^{25}=+21.1$ (c=1.0, EtOH) for (*R*)-**3g** in >99% *ee*; $[\alpha]_D^{25}=+44.1$ (c=1.0, EtOH) for (*R*)-**3h** in >99% *ee*.

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[19] (*R*)-5-(4-Methoxyphenyl)pyrrolidin-2-one (**3e**): $[\alpha]_D^{20} = +28.1$ (c = 1.0, EtOH). Described in the literature: $[\alpha]_D^{20} = +40.5$ (c = 0.7, CH_2Cl_2) for the (*R*)-enantiomer. D. Guijarro, O. Pablo, M. Yus, *J. Org. Chem.* **2013**, *78*, 3647–3654.

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Chapter 3

Transaminases in dynamic kinetic protocols. Chemoenzymatic synthesis of enantio- and diastereoenriched α-alkyl β-amino amides

3.1. Bibliographic background

The importance of chiral β-amino amides (or β^3-amino amides) lies in their potential biological activities. For instance, sitagliptin[123] (Figure 3.1) containing the β-amino amide core is used for type 2 diabetes treatment as it exhibits dipeptidyl peptidase IV (DPP-4) inhibitory activity. Furthermore, α-substituted β-amino amides (or $\beta^{2,3}$-amino amides) can also display many interesting biological activities. For example, bestatin[124] (Figure 3.1) and KNI-272[125] (Figure 3.1) have been reported as aminopeptidase and HIV protease inhibitors, respectively.

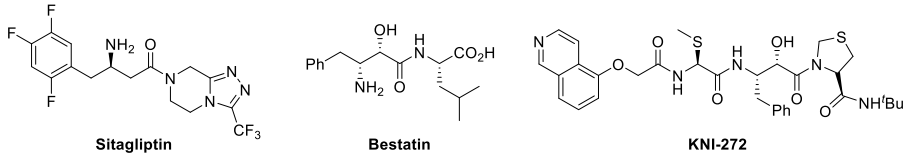

Figure 3.1. Biologically active compounds containing the (α-substituted) β-amino amide core.

Moreover, β-amino amides are synthetically useful compounds as they can be employed to produce valuable 1,3-diamines through reductive processes, which for instance can be used as ligands in metal-catalysed reactions.[126]

3.1.1. Asymmetric synthesis of (α-substituted) β-amino amides

Due to the importance of these compounds, several chemical approaches have been described in order to synthesise chiral β-amino amides (Scheme 3.1):

(a) The Mannich reaction has been the most recurrently studied.[127] In order to induce chirality, authors have employed enantiopure imines[127c] or

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amides[127d,l,m] as starting material in the presence of a strong base at very low temperatures. Additionally, good diastereoselectivities have been typically found when using bases with sterically hindered ligands[127b,k] or metals as samarium[127j] or cobalt.[127i] Furthermore, it is possible to get access to α-substituted β-keto amides in high diastereomeric ratios and excellent enantiomeric excess starting from 7-azaindoline amides by using the Barton's base in the presence of a copper catalyst and a chiral ligand.[127a,d,f-h]

(b) The reductive amination of β-keto amides.[128]

(c) The aza-Michael addition of α,β-unsaturated amides.[129]

(d) The reduction[130] or hydrogenation[131] of enamine precursors.

(e) Other methods involving multicomponent transformations[132] or the reduction of oxime precursors.[133]

Furthermore, some enzymatic methodologies have provided access to this kind of compounds with high *ee* values (Scheme 3.1):

(f) Whole-cells showing nitrile hydratase activity have been capable of selectively hydrolysing racemic β-amino nitriles through kinetic resolution processes.[134]

J. Chen, B. Sun, N. Kumagai, M. Shibasaki, *Org. Lett.* **2016**, *18*, 2391-2394; (g) L. Brewitz, F. Arteaga Arteaga, L. Yin, K. Alagiri, N. Kumagai, M. Shibasaki, *J. Am. Chem. Soc.* **2015**, *137*, 15929-15939; (h) L. Yin, L. Brewitz, N. Kumagai, M. Shibasaki, *J. Am. Chem. Soc.* **2014**, *136*, 17958-17961; (i) O. Prieto, H. W. Lam, *Org. Biomol. Chem.* **2008**, *6*, 55-57; (j) J. M. Concellón, H. Rodríguez-Solla, C. Concellón, C. Simal, N. Alvaredo, *Synlett* **2010**, 2199-2121; (k) S. Saito, T. Tsubogo, S. Kobayashi, *Chem. Commun.* **2007**, 1236-1237; (l) A. Iza, J. L. Vicario, L. Carrillo, D. Badía, *Synthesis* **2006**, 4065-4074; (m) J. L. Vicario, D. Badía, L. Carrillo, *J. Org. Chem.* **2001**, *66*, 9030-9032.

[128] (a) Y. Lou, Y. Hu, J. Lu, F. Guan, G. Gong, Q. Yin, X. Zhang, *Angew. Chem. Int. Ed.* **2018**, *57*, 14193-14197; (b) C. G. Goodman, J. S. Johnson, *J. Am. Chem. Soc.* **2015**, *137*, 14574-14577; (c) D. Mtat, R. Touati, B. B. Hassine, *Tetrahedron Lett.* **2014**, *55*, 6354-6358; (d) D. Steinhuebel, Y. Sun, K. Matsumura, N. Sayo, T. Saito, *J. Am. Chem. Soc.* **2009**, *131*, 11316-11317.

[129] (a) J. Etxebarria, J. L. Vicario, D. Badía, L. Carrillo, N. Ruiz, *J. Org. Chem.* **2005**, *70*, 8790-8800; (b) S. G. Davies, A. J. Edwards, I. A. S. Walters, *Recl. Trav. Chim. Pays-Bas* **1995**, *114*, 175-183.

[130] S. Quiao, J. Wu, J. Mo, P. T. Spiegener, B. N. Zhao, B. Jiang, G. Li, *Synlett* **2017**, *28*, 2483-2488.

[131] (a) S. Mallik, V. Bhajammanavar, I. Ramakrishna, M. Baidya, *Org. Lett.* **2017**, *19*, 3843-3846; (b) K. B. Hansen, Y. Hsiao, F. Xu, N. Rivera, A. Clausen, M. Kubryk, S. Krska, T. Rosner, B. Simmons, J. Balsells, N. Ikemoto, Y. Sun, F. Spindler, C. Malan, E. J. J. Grabowski, J. D. Armstrong III, *J. Am. Chem. Soc.* **2009**, *131*, 8798-8804.

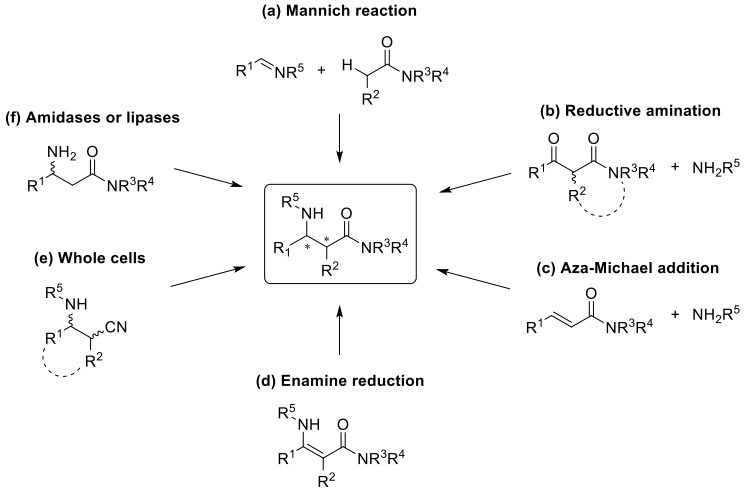
[132] (a) B. Huang, L. Zeng, Y. Shen, S. Cui, *Angew. Chem. Int. Ed.* **2017**, *56*, 4565-4568; (b) J. E. Semple, T. D. Owens, K. Nguyen, O. E. Levy, *Org. Lett.* **2000**, *2*, 2769-2772.

[133] M. Vishe, J. N. Johnston, *Chem. Sci.* **2019**, *10*, 1138-1143.

[134] (a) O. K. L. Rapheeha, M. P. Roux-van der Merwe, J. Badenhorst, V. Chhiba, M. L. Bode, K. Mathiba, D. Brady, *J. Appl. Microbiol.* **2017**, *122*, 686-697; (b) V. Chhiba, M. L. Bode, K. Mathiba,

(g) Other enzymatic methodologies include the lipase-catalysed aminolysis of racemic β-lactams[135] or the acylation of β-amino amides,[136] and the hydrolysis of racemic β-amino amides using aminopeptidases[137] or amidases.[138]

Scheme 3.1. Synthetic approaches to access chiral (α-substituted) β-amino amides.



All these enzymatic pathways have a main drawback: conversions were limited to a maximum of 50%. To overcome this issue, desymmetrisation of prochiral compounds[139] or dynamic kinetic resolutions (DKRs)[64c,140] of racemic derivatives can be employed as efficient synthetic tools.

3.1.2. Transaminases towards the synthesis of chiral amines

There are several transaminase-catalysed methodologies that allow the synthesis of chiral amines (Scheme 3.2).[109,141] When starting from a racemic amine,

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W. Kwezi, D. Brady, *J. Mol. Catal. B: Enzym.* **2012**, *76*, 68-74; (c) D.-Y. Ma, D.-X. Wang, J. Pan, Z.-T. Huang, M.-X. Wang, *J. Org. Chem.* **2008**, *73*, 4087-4091; (d) M. Winkler, L. Martínková, A. C. Knall, S. Krahulec, N. Klempier, *Tetrahedron* **2005**, *61*, 4249-4260; (e) M. Preiml, H. Hönig, N. Klempier, *J. Mol. Catal. B: Enzym.* **2004**, *29*, 115-121.

[135] X.-G. Li, M. Lähitie, L. T. Kanerva, *Tetrahedron: Asymmetry* **2008**, *19*, 1857-1861.

[136] E. Choi, Y. Kim, Y. Ahn, J. Park, M.-J. Kim, *Tetrahedron: Asymmetry* **2013**, *24*, 1449-1452.

[137] T. Heck, D. Seebach, S. Osswald, M. K. J. ter Wiel, H.-P. E. Kohler, B. Geueke, *ChemBioChem* **2009**, *10*, 1558-1561.

[138] Z. Xue, Y. Chao, D. Wang, M. Wang, S. Qian, *J. Ind. Microbiol. Biotechnol.* **2011**, *38*, 1931-1938.

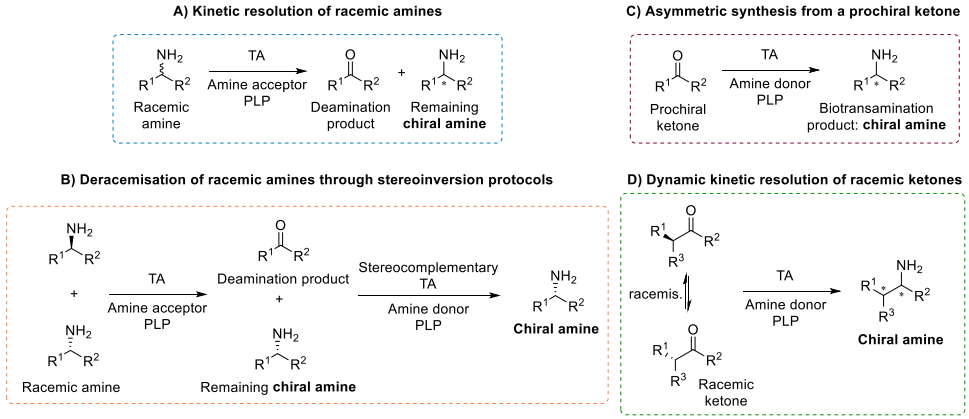
[139] E. García-Urdiales, I. Alfonso, V. Gotor, *Chem. Rev.* **2011**, *111*, PR110-PR180.

[140] A. Díaz-Rodríguez, I. Lavandera, V. Gotor, *Curr. Green Chem.* **2015**, 6965-6982.

[141] S. Mathew, H. Yun, *ACS Catal.* **2012**, *2*, 993-1001.

it is possible to develop a kinetic resolution *via* deamination (Scheme 3.2.A) or the deracemisation of those amines through stereoinversion protocols (Scheme 3.2.B). Alternatively, if a prochiral ketone is employed as starting material, the asymmetric synthesis can lead to optically active amines (Scheme 3.2.C) while the dynamic kinetic resolution of racemic ketones can be developed *via* biotransamination (Scheme 3.2.D).

Scheme 3.2. Asymmetric synthesis of amines catalysed by transaminases. **A)** Kinetic resolution of a racemic amine. **B)** Deracemisation of a racemic amine through a stereoinversion protocol. **C)** Asymmetric synthesis from a prochiral ketone. **D)** Dynamic kinetic resolution of a racemic ketone.



The kinetic resolution of a racemic amine leads to the formation of a ketone, which is the deaminated product, and the remaining chiral amine up to a 50% conversion. For this purpose, stoichiometric equivalents of an amine acceptor (*i.e.* pyruvate) are needed as the equilibrium favours the product formation. Transaminase-catalysed KRs just involve the utilisation of one enzyme (the TA) and the reaction rate is usually much faster compared with the asymmetric synthesis.[142] However, there are some disadvantages related to these processes such as the low atom efficiency by means of conversion values and the product and substrate inhibition which makes the reaction unsuitable at high concentrations.[63e,143] Even though several methodologies have been developed to

[142] M. S. Malik, E.-S. Park, J.-S. Shin, *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1163-1171.
[143] J.-S. Shin, B.-G. Kim, *Biotechnol. Bioeng.* **1997**, *55*, 348.

solve drawbacks related to KRs,[144] protocols leading to theoretically full conversions always seem to be more attractive.

On the one hand, deracemisation processes can also lead to chiral amines in theoretically full conversion. In this manner, a racemic amine can be used as starting material obtaining as the sole product one of the enantiomers of that racemic mixture by using two stereocomplementary transaminases. This is known as a deracemisation process by enantioselective stereoinversion.[108,110] In a first step, a TA leads to the formation of a ketone as a result of the selective deamination process of one of the enantiomers of the racemic amine while the other one remains untouched. In the second step, the asymmetric transamination of the ketone by an enantiocomplementary TA takes place. This way, an enantiopure amine can be obtained in theoretically full conversion if the cascade is efficient.[18c]

On the other hand, the asymmetric synthesis of chiral amines catalysed by TAs can be accomplished. It consists in the biotransamination of a prochiral ketone to produce one enantiomer of the desired amine. The main advantages of these protocols are that they are quantitative (up to 100% yield) and that the selectivity of the desired amine does not depend on the conversion value but only on the TA. Nevertheless, to achieve high conversions it is compulsory to drive the unfavourable thermodynamic equilibrium to the formation of the amine as it was already discussed in the Preface and in the Chapter 2 of this Doctoral Thesis. In spite of this main drawback, the fact that direct synthesis is highly demanded in industry,[145] makes asymmetric synthesis much more desirable than kinetic resolutions.

Finally, dynamic kinetic resolution (DKR) processes can also lead to chiral amines with theoretically full conversion starting from a racemic carbonyl compound. This way, the possibility of achieving high conversion values rely on the capability of carbonyl precursors to racemise.[18c] This methodology involves the employment of just one transaminase that is able to perform the kinetic resolution of a racemic ketone into the corresponding optically active amine, while the non-reacting enantiomer of the ketone suffers from a rapid racemisation process.

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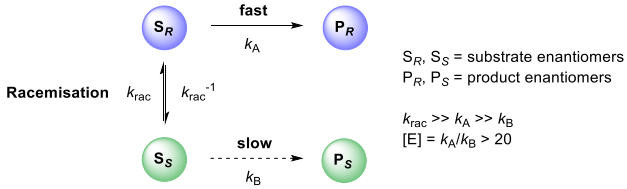
[144] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, *Trends Biotechnol.* **2010**, *28*, 324-332.
[145] D. J. Constable, P. J. Dunn, J. D. Hayler, G. R. Humphrey, J. L. Leazer, Jr., R. J. Linderman, K. Lorenz, J. Manley, B. A. Pearlman, A. Wells, A. Zaks, T. Y. Zhang, *Green Chem.* **2007**, *5*, 411-420.

DKR have been deeply explored in hydrolase-catalysed processes in combination with a metal-based racemisation.[64c,146] However, other enzymes such as transaminases have been scarcely reported as catalysts in DKRs. In the next section we will explain the kinetics involved in this kind of process and we will sum up the examples of transaminase-mediated DKRs that can be found in the bibliography.

3.1.3. Dynamic kinetic resolutions. Transaminase-catalysed DKRs

A DKR consists in two processes taking place at the same time. On the one hand, the kinetic resolution of a racemic mixture and, on the other hand, the non-reacting substrate undergoes a racemisation process (Scheme 3.3).

Scheme 3.3. DKR of a racemic mixture.



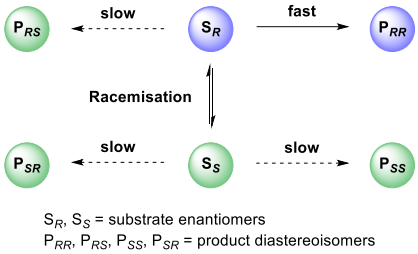
For an efficient DKR to take place, some requirements must be fulfilled.[139,147] First, the racemisation process must be at least ten times faster than the enzyme-catalysed transformation of the slow-reacting enantiomer (k_{rac} >> k_A >> k_B). Second, in order to achieve the products with high optical purity, it is necessary a very selective kinetic resolution. This means that the enantiomeric ratio (k_A/k_B) must be at least 20 ([E] ≥ 20). Finally, the kinetic resolution and the racemisation need to be compatible under the same reaction conditions. When all these conditions are fulfilled, the racemic substrate can be completely converted into a unique enantiopure product in theoretically 100% yield. The substrate racemisation can be performed by a chemocatalyst such as a metal catalyst,[148] a biocatalyst[149] or it can take place spontaneously in the reaction media.[150] Importantly, when a

[146] (a) A. S. de Miranda, L. S. M. Miranda, R. O. M. A. de Souza, *Biotechnol. Adv.* **2015**, *33*, 372-393; (b) H. Pellissier, *Tetrahedron* **2011**, *67*, 3769-3802.
[147] B. Martín-Matute, J.-E. Bäckvall, *Curr. Opin. Chem. Biol.* **2007**, *11*, 226-232.
[148] O. Långvik, T. Saloranta, D. Y. Murzin, R. Leino, *ChemCatChem* **2015**, *7*, 4004-4015.
[149] B. Schnell, K. Faber, W. Kroutil, *Adv. Synth. Catal.* **2003**, *345*, 653-666.
[150] C. Rodríguez, G. De Gonzalo, A. Rioz-Martínez, D. E. Torres Pazmiño, M. W. Fraaije, V. Gotor, *Org. Biomol. Chem.* **2010**, *5*, 1121-1125.

chemocatalyst is used to carry out the racemisation, it is necessary that the transition metal does not react with the product.

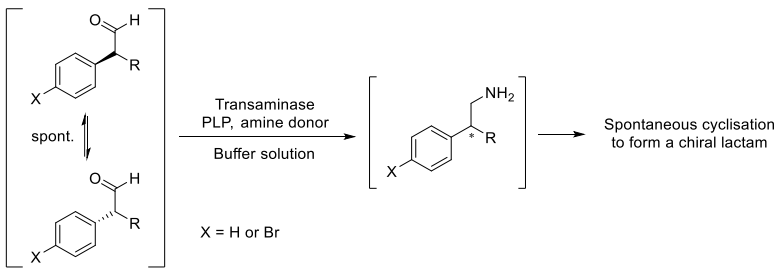
It is important to point out that a DKR can lead to the asymmetric synthesis of one enantiomer but it can also produce a diastereoisomer when the reaction occurs along with the creation of a new stereogenic centre (Scheme 3.4).[146b]

Scheme 3.4. Synthesis of diastereoisomers *via* DKR.



As described in the previous section, transaminase-catalysed DKRs have been scarcely reported. The first example of a DKR leading to the formation of just one enantiomer, date back to 2009. It was developed by Kroutil and co-workers and it has already been introduced in the previous chapter (Scheme 2.3).[106] A similar approach to that one which has also been discussed in Chapter 2 is the one reported by Emerson and co-workers (Scheme 2.4).[107] In both cases, a racemic aldehyde is transformed into the corresponding enantiopure amine which undergoes spontaneous cyclisation to produce a chiral lactam (Scheme 3.5).

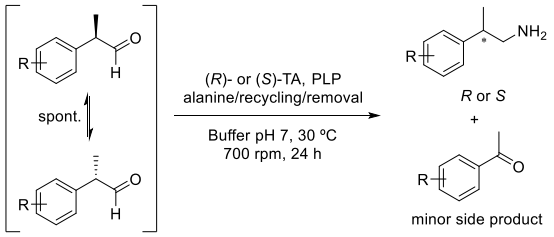
Scheme 3.5. Overview of the transaminase-catalysed DKRs introduced in Schemes 2.3 and 2.4.



Two other similar approaches are found in the bibliography. The first one consists in the biotransamination of 2-arylpropanal derivatives that suffer from a DKR process to achieve the corresponding chiral 2-arylpropan-1-amines (Scheme

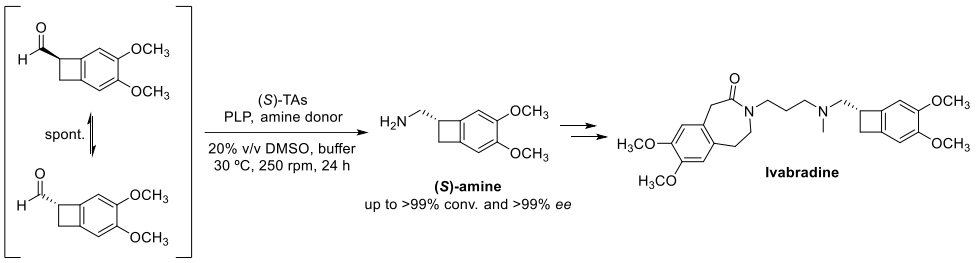
3.6).[151] The non-substituted (R = H) substrate led to the formation of (*R*)- and (*S*)-amines with moderate conversions and high selectivities (83% and 92% *ee*, respectively). Later on, substrates bearing methyl and methoxy groups at *para*-, *meta*- and *ortho*-positions in the phenyl ring were considered, finding that it was possible to prepare both amine enantiomers with high selectivity for most of the studied cases. The formation of the corresponding acetophenones as side products was observed in a small proportion in many cases.

Scheme 3.6. DKR of 2-arylpropanal derivatives to produce β-chiral 2-arylpropan-1-amines.



The second example was reported by González-Sabín and co-workers in the search of straightforward asymmetric routes towards the heart-rate reducing agent Ivabradine.[152] One of those routes consists in the DKR of a racemic aldehyde to synthesise an α-chiral amine (Scheme 3.7). Even though the desired enantiomer is the (*S*)-amine, also the (*R*)-isomer could be obtained with full conversion and high enantioselectivity (up to 88% *ee*).

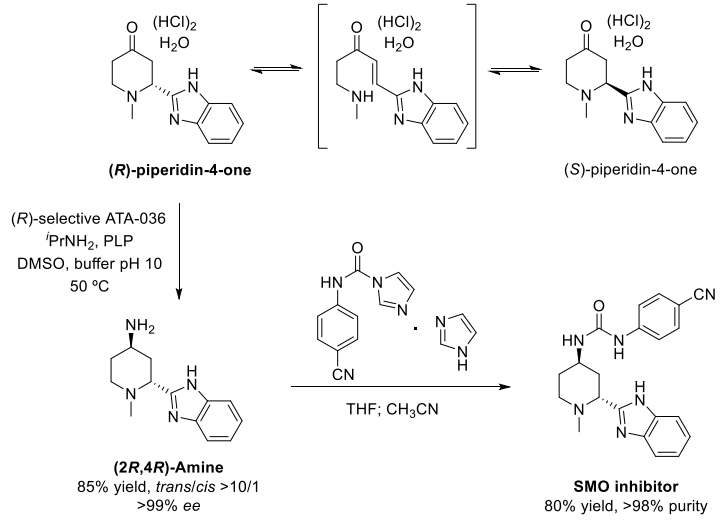
Scheme 3.7. Transaminase-catalysed DKR to access a valuable chiral precursor of the heart-rate reducing agent Ivabradine.



[151] C. S. Fuchs, M. Hollauf, M. Meissner, R. C. Simon, T. Besset, J. N. H. Reek, W. Riethorst, F. Zepeck, W. Kroutil, *Adv. Synth. Catal.* **2014**, *356*, 2257-2265.
[152] S. Pedragosa-Moreau, A. Le Flohic, V. Thienpondt, F. Lefoulon, A.-Marie Petit, N. Ríos-Lombardía, F. Morís, J. González-Sabín, *Adv. Synth. Catal.* **2017**, *359*, 485-493.

Aside from these procedures to produce just one enantiomer from a racemic mixture, some DKRs towards the synthesis of diastereoisomers have already been developed using transaminases. In 2014, Peng *et al.* reported the asymmetric synthesis of a smoothened receptor (SMO) inhibitor (Scheme 3.8).[153] As starting material, a piperidin-4-one was employed, finding that this substrate was able to racemise in a mixture of DMSO and buffer at pH 10. Although the authors did not find direct evidence, the racemisation mechanism was proposed to follow a reversible retro-aza-Michael/aza-Michael transformation. The transamination process was accomplished using commercial (*R*)-selective ATA-036 as biocatalyst and a temperature of 50 °C for 50-60 h. Under those conditions, the desired (2*R*,4*R*)-amine was obtained in 85% yield, a diastereomeric ratio of >10:1 (*trans*/*cis*) and >99% *ee*. Once the amine was synthesised, the desired product could be formed in 80% yield and >98% purity by adding a slight excess of an *N*-carbamoylimidazole derivative directly in the aqueous medium.

Scheme 3.8. Enzymatic synthesis of a diastereoenriched amine as precursor of a SMO inhibitor through a TA-catalysed DKR.

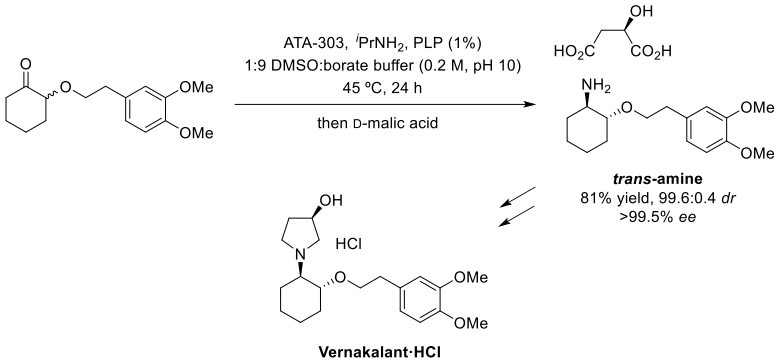


The same year, Truppo and co-workers reported the chemoenzymatic synthesis of the antiarrhythmic agent Vernakalant.[154] Again, the enzymatic step

[153] Z. Peng, J. W. Wong, E. C. Hansen, A. L. A. Puchlopek-Dermenci, H. J. Clarke, *Org. Lett.* **2014**, *16*, 860-863.
[154] J. Limanto, E. R. Ashley, J. Yin, G. L. Beutner, B. T. Grau, A. M. Kassim, M. M. Kim, A. Klapars, Z. Liu, H. R. Strotman, M. D. Truppo, *Org. Lett.* **2014**, *16*, 2716-2719.

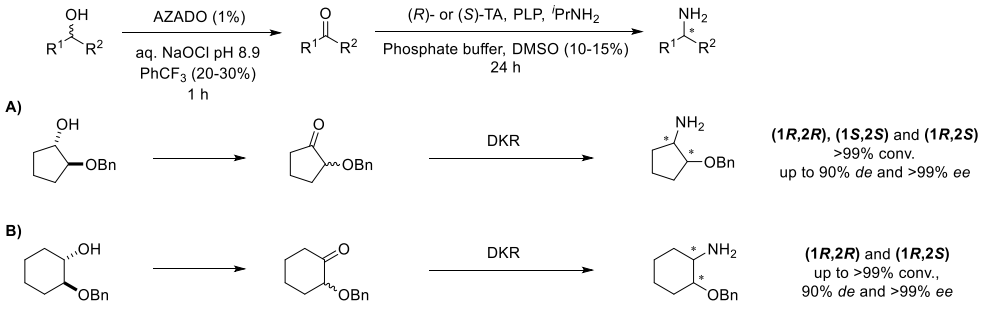
was responsible for the desired chirality in the final molecule. However, in this case a cyclohexanone derivative was used as biotransamination substrate obtaining the corresponding *trans*-amine as the crystalline D-maleate salt using an evolved enzyme (ATA-303, Scheme 3.9).

Scheme 3.9. TA-catalysed DKR of a cyclohexanone derivative to obtain a diastereo- and enantiopure valuable precursor of Vernakalant.



Later on, in 2017 our research group developed a hybrid organo- and biocatalytic system to transform racemic alcohols into the corresponding amines in a diastereo- and enantioselective way (Scheme 3.10).[155]

Scheme 3.10. Catalytic amination of racemic alcohols to form optically active amines through a one-pot two-step chemoenzymatic protocol.



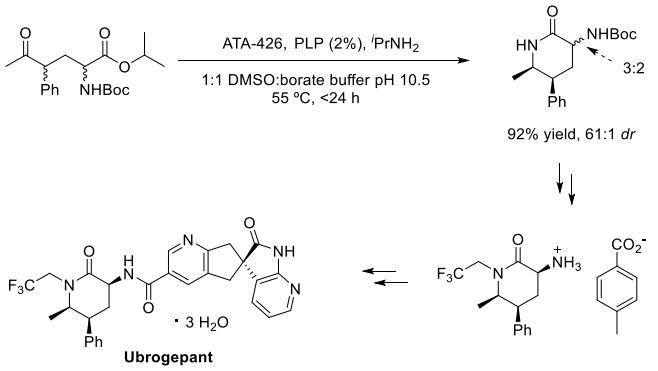
First of all, a racemic alcohol was oxidised into the corresponding ketone through a catalytic non-selective process using the AZADO/NaOCl oxidation system, which is capable of operating at basic pH aqueous medium. Those reaction conditions allowed the development of a one-pot two-step protocols in which the

[155] E. Liardo, N. Ríos-Lombardía, F. Morís, F. Rebolledo, J. González-Sabín, *ACS Catal.* **2017**, *7*, 4768-4774.

second step corresponds to the biotransamination of the ketone into the corresponding enantiopure amine. For the particular cases of (±)-*trans*-2-(benzyloxy)cyclopentanol (Scheme 3.10.A) and (±)-*trans*-2-(benzyloxy)cyclohexanol (Scheme 3.10.B), the ketones resulting from the oxidation step were racemates, so consequently a DKR took place and, depending on the TA, it was possible to achieve three out of the four possible diastereoisomers of 2-(benzyloxy)cyclopentanamine and two diastereoisomers of 2-(benzyloxy)cyclohexanamine.

The same year, Yasuda *et al.* reported the synthesis of Ubrogepant, a calcitonin gene-related peptide (CGRP) receptor antagonist (Scheme 3.11). For this purpose, two key fragments had to be synthesised.[156] One of them was a chiral lactam that could be achieved by a transaminase-mediated DKR which simultaneously set two stereocenters.

Scheme 3.11. Chemoenzymatic approach towards Ubrogepant.

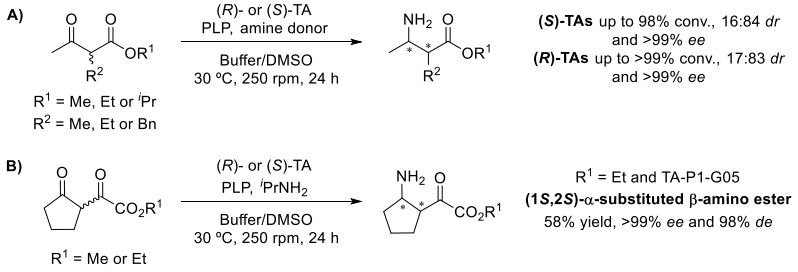


Finally, a transaminase-catalysed DKR which is closely related to the research described in this chapter is the one reported in our research group in 2013.[157] Thus, a DKR protocol was developed for the synthesis of a wide panel of diastereo- and enantioenriched α-alkyl-β-amino esters starting from the corresponding racemic β-keto esters (Scheme 3.12). The influences of the pattern substitution related to the α-alkyl chain (R^2) and ester alkyl moiety (R^1) were analysed (Scheme 3.12.A). In addition, two cyclic compounds were also employed as starting materials for the

[156] N. Yasuda, E. Cleator, B. Kosjek, J. Yin, B. Xiang, F. Chen, S.-C. Kuo, K. Belyk, P. R. Mullens, A. Goodyear, J. S. Edwards, B. Bishop, S. Ceglia, J. Belardi, L. Tan, Z. J. Song, L. DiMichele, R. Reamer, F. L. Cabirol, W. L. Tang, G. Liu, *Org. Process Res. Dev.* **2017**, *21*, 1851-1858.
[157] A. Cuetos, I. Lavandera, V. Gotor, *Chem. Commun.* **2013**, *49*, 10688-10690.

DKR processes, finding that the best results were those obtained in the biotransamination of ethyl 2-oxocyclopentanecarboxylate using the commercial TA-P1-G05 (Scheme 3.12.B). In total, 11 different α-substituted β-keto esters were tested as substrates finding high conversion and *ee* values and, in general, moderate *de* values.

Scheme 3.12. Synthesis of α-substituted β-amino esters through a DKR protocol.

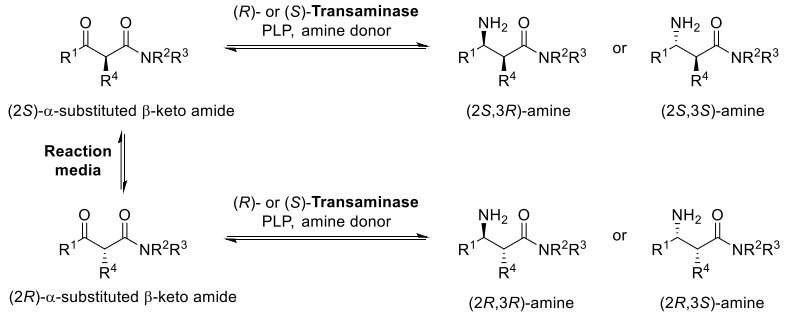


Due to the shortage of TA-catalysed DKRs that can be found in the bibliography and our group experience working with α-substituted β-keto esters as substrates in this kind of processes, we decided to develop a TA-mediated protocol to produce different diastereo- and enantioenriched α-alkyl-β-amino amides through DKR transformations. Several reaction parameters including the source and amount of the TA, pH and temperature were studied in order to disclose an efficient and general asymmetric methodology.

3.2. Discussion

Herein, we introduce a general chemoenzymatic pathway to obtain diastereo- and enantioenriched α-substituted β-amino amides, based on the transaminase-mediated DKR of a set of racemic α-alkyl-β-keto amides which were previously synthesised (Scheme 3.13). For this purpose, a chemical route towards these substrates was developed. Afterwards, the suitability of these compounds for TA-catalysed DKR transformations as well as several reaction parameters were deeply studied. Depending on the transaminase selectivity, all four diastereoisomers could be susceptible to be achieved after the biotransamination protocols.

Scheme 3.13. Synthesis of diastereo- and enantioenriched α-substituted β-amino amides through transaminase-mediated DKRs.



3.2.1. Synthesis of α-alkyl-β-keto amides 3a-h and the corresponding racemic amino amides 4a-h

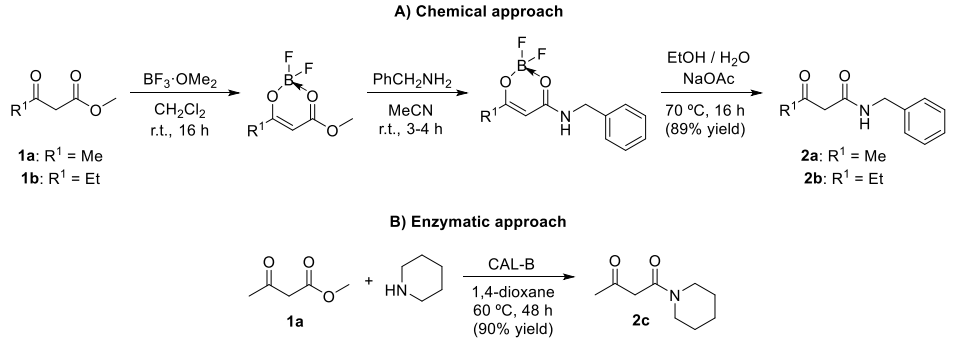
Initially, a wide panel of α-substituted β-keto amides were synthesised bearing different substitution patterns at the α- (R^1) and γ-position (R^4), as well as in the nitrogen substitution (R^2 and R^3). As starting materials, commercially available β-keto esters **1a** and **1b** were used and two different chemical methodologies applied in order to obtain the corresponding β-keto amides **2a-c** (Scheme 3.14).

For this purpose, two different methodologies have been employed. First of all, we made use of an enzymatic protocol that had been developed in our research group in 1993.[158] This procedure consists in a lipase-catalysed aminolysis with 1.1 equiv of piperidine using CAL-B as biocatalyst in 1,4-dioxane (0.1 M). It led to the synthesis of the β-keto amide **2c** in high yield after 48 h reaction at 60 ºC and a

[158] M. J. García, F. Rebolledo, V. Gotor, *Tetrahedron Lett.* **1993**, *38*, 6141-6142.

purification by column chromatography (Scheme 3.14.B). However, it only led to the formation of **2a** and **2b** in less than 20% yield. This fact encouraged us to try a three-step chemical strategy developed by Polanc and co-workers.[159] In a first step, the β-keto ester was treated with 2 equiv of boron trifluoride methyl etherate in dry dichloromethane (0.2 M) to form a 1,3,2-dioxaborinane. This compound underwent a nucleophilic substitution reaction using 1.3 equiv of the corresponding amine (benzylamine) in acetonitrile (0.2 M) under mild reaction conditions to obtain the amido complex. The last step consisted in the deprotection of the β-keto amide using 5 equiv of sodium acetate in a 1:1 mixture of ethanol and water at 70 °C. Thus, β-keto amides **2a** and **2b** were obtained in high yields (89%) after purification by column chromatography (Scheme 3.14.A).

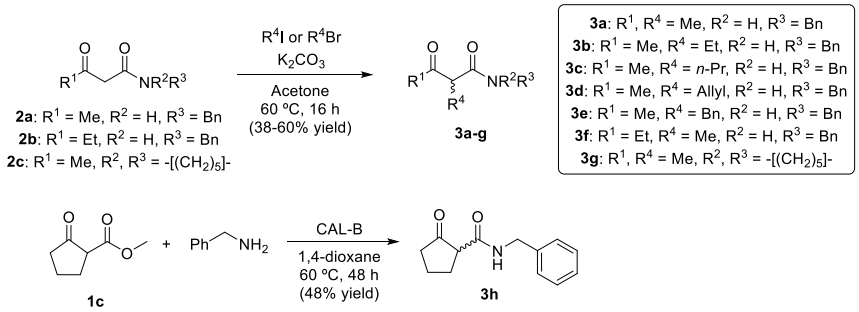
Scheme 3.14. Synthesis of β-keto amides **2a-c**.



Later on, those β-keto amides were α-alkylated by treatment with 1.3 equiv of different alkyl halides (R^4I and R^4Br) in the presence of 1.3 equiv of potassium carbonate as base and using acetone as solvent (Scheme 3.15). After overnight reaction at 60 °C, α-alkylated β-keto amides **3a-g** were obtained in low to moderate yields after purification by column chromatography (38-60%). The cyclic substrate **3h** was straightforward synthesised in low yield (48%) from the corresponding commercially available racemic methyl 2-oxocyclopentanecarboxylate (**1c**) using a CAL-B mediated aminolysis with benzylamine as nucleophile.

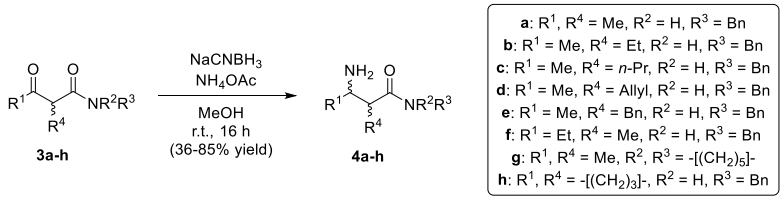
[159] B. Štefane, S. Polanc, *Tetrahedron* **2007**, *63*, 10902-10913.

Scheme 3.15. Synthesis of α-alkylated β-keto amides **3a-h**.



With the racemic substrates (**3a-h**) in hand, it was necessary to synthesise the racemic amines **4a-h** that would serve as standards to develop GC and HPLC analysis methods. With that purpose, the reductive aminations of the α-substituted β-keto amides **3a-h** were attempted using 2 equiv of $NaCNBH_3$ as reduction agent and 10 equiv of ammonium acetate as nitrogen source in dry methanol (Scheme 3.16). Mixtures of *syn*- and *anti*-diastereoisomers of α-substituted β-amino amides **4a-h** were obtained in low to moderate yields (36-85%).

Scheme 3.16. Synthesis of the racemic mixtures of *syn*- and *anti*-diastereoisomers of α-substituted β-amino amides **4a-h**.

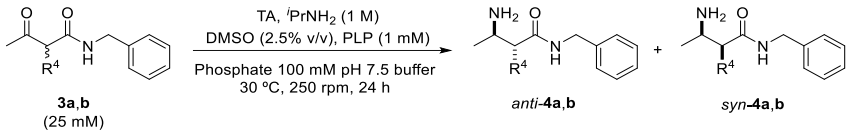


3.2.2. Biotransamination of α-methylated β-keto amide 3a and α-ethylated substrate 3b. Influence of temperature, amount of TA and pH in the DKR process

α-Methylated β-keto amide **3a** was chosen as model substrate for our study and 28 commercial transaminases from Codexis Inc. were tested. Initially 25 mM concentration of **3a** was used, selecting DMSO as cosolvent (2.5% v/v) and phosphate buffer 100 mM pH 7.5 containing PLP (1 mM) as reaction media. In all cases, isopropylamine (IPA, $^{i}PrNH_2$) was added in a large excess (1 M) as amine donor in order to shift the equilibrium towards the amine formation. All biotransformations were incubated at 30 °C and 250 rpm for 24 h finding good

conversions, excellent enantioselectivity and moderate to low diastereoselectivity with some (*S*)- and (*R*)-transaminases (Table 3.1, odd entries).

Table 3.1. Transamination of substrates **3a** and **3b** bearing a methyl or an ethyl group, respectively, at the α-position.[a]



Entry	Substrate	Transaminase	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	**3a**	ATA-237 (*S*)	95	33:67	>99	>99
2	**3b**	ATA-237 (*S*)	93	69:31	50	50
3	**3a**	ATA-251 (*S*)	98	65:35	>99	>99
4	**3b**	ATA-251 (*S*)	98	80:20	21	21
5	**3a**	ATA-254 (*S*)	89	85:15	>99	>99
6	**3b**	ATA-254 (*S*)	93	82:18	4	4
7	**3a**	ATA-260 (*S*)	96	31:69	>99	>99
8	**3b**	ATA-260 (*S*)	97	81:19	26	28
9	**3a**	ATA-013 (*R*)	98	62:38	>99	>99
10	**3b**	ATA-013 (*R*)	96	80:20	>99	>99
11	**3a**	ATA-024 (*R*)	97	50:50	>99	>99
12	**3b**	ATA-024 (*R*)	97	78:22	>99	>99
13	**3a**	ATA-025 (*R*)	98	52:48	>99	>99
14	**3b**	ATA-025 (*R*)	97	78:22	>99	>99
15	**3a**	ATA-033 (*R*)	98	53:47	>99	>99
16	**3b**	ATA-033 (*R*)	97	78:22	>99	>99
17	**3a**	ATA-415 (*R*)	97	60:40	>99	>99
18	**3b**	ATA-415 (*R*)	96	80:20	>99	>99

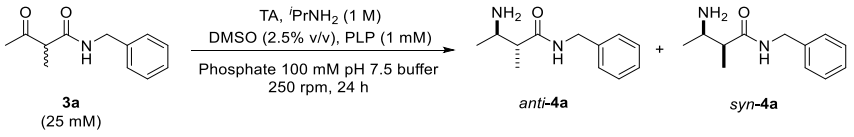
[a] Reaction conditions: Substrates **3a** or **3b** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 (500 µL final volume), 30 °C, 24 h and 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns.

These poor diastereomeric ratios could be due to a slow racemisation. However, before testing other reaction conditions, we decided to study the influence of the alkyl group at the α-position. For this purpose, the α-ethylated substrate **3b** was employed as starting material for the transamination using all the transaminases working on the model substrate (Table 3.1, even entries). After analysing the reaction mixtures, we found out that (*S*)-TAs lost their selectivity with this substrate, going from excellent *ee* values when using **3a** to moderate or low enantiomeric values with **3b** (entries 2, 4, 6 and 8). Nevertheless, (*R*)-transaminases still provided good results in terms of conversion and

enantioselectivity values (entries 10, 12, 14, 16 and 18). Furthermore, they usually led to better diastereomeric ratios (up to 82:18 *anti*:*syn*) than those obtained with the model substrate.

At this point, we decided to focus our attention in the development of a more efficient DKR procedure (Table 3.2). For this purpose, the model substrate **3a** was employed as starting material, while the (*S*)-selective ATA-251 and the (*R*)-selective ATA-013 were chosen as biocatalysts. Hence, we considered the possibility of slowing down the enzymatic transamination process by decreasing the temperature or adding less amount of enzyme. On the one hand, the use of a lower temperature (down to 10 ºC) led to worse conversion values and similar diastereomeric ratios with ATA-251 (entry 2) and a dramatic loss of activity with ATA-013 (entry 5). On the other hand, when lower amounts of transaminase were used (1 mg), slightly lower conversions and similar diastereoselectivity were attained with both enzymes (entries 3 and 6).

Table 3.2. Influence of temperature and amount of transaminase in the DKR of model substrate **3a**.[a]



Entry	Transaminase	T (°C)	TA (mg)	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	ATA-251 (*S*)	30	2	98	62:38	>99	>99
2	ATA-251 (*S*)	10	2	65	63:37	>99	>99
3	ATA-251 (*S*)	30	1	94	65:35	>99	>99
4	ATA-013 (*R*)	30	2	98	65:35	>99	>99
5	ATA-013 (*R*)	10	2	11	n.d.	n.d.	n.d.
6	ATA-013 (*R*)	30	1	74	70:30	>99	>99

[a] Reaction conditions: Substrate **3a** (25 mM), transaminase (1-2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 (500 µL final volume), 10-30 °C, 24 h and 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns. n.d.: not determined.

Later on, we decided to explore the influence of the pH in order to speed up the racemisation process by increasing the pH up to 10 (Figure 3.2.A). After 24 h similar results were found with (*S*)-transaminases but a significant improvement in the process was found using (*R*)-transaminases. Thus, at pH 10, (*R*)-TAs were

capable of producing the *anti*-amine with full conversions, excellent *ee* values and improved *dr* going from ratios close to 50:50 up to 80:20 *anti*:*syn*. Encouraged by these results, the biotransaminations of **3b** were set up at pH 10 (Figure 3.2.B). Sadly, even though the DKR led to full conversions and excellent *ee* values, no better diastereomeric ratios towards the formation of *anti*-**4b** were found, as they remained untouched in some cases (ATA-024 and ATA-025) or were much lower in other cases (ATA-013, ATA-033 and ATA-415).

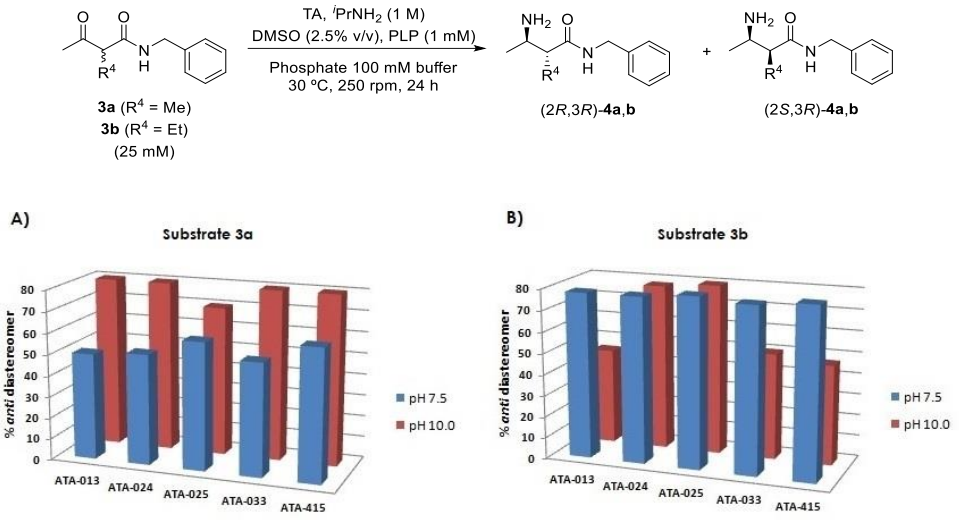


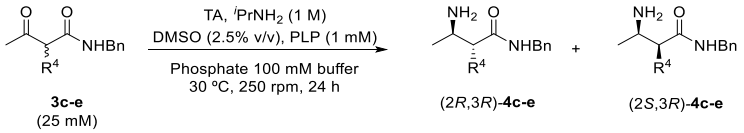
Figure 3.2. Influence of the pH in the DKR of α-alkylated β-keto amides **3a** and **3b** mediated by (*R*)-transaminases. Blue colour is used to indicate the percentage of *anti*-amine (>99% *ee* in all cases) at pH 7.5, while red bars denote the percentage of *anti*-diastereoisomer (>99% *ee* in all cases) at pH 10.0. **A)** Biotransamination of **3a**. **B)** Biotransamination of **3b**.

3.2.3. Extending the substrate scope towards other α-substituted β-keto amides. Biotransamination of substrates 3c-e

Next, substrates **3c-e** bearing different substitution patterns at the α-position to the ketone (R^4) were tested at pH 7.5 and pH 10. The best results provided by (*R*)-transaminases ATA-013 and ATA-025 have been summarised in Table 3.3. Substrates **3c** and **3d**, bearing a *n*-propyl and an allyl rest at the α-position, led to similar results than those obtained in the biotransamination of **3b**. This means that at pH 7.5 good conversions, moderate diastereomeric ratios towards the formation of the *anti*-amines (**4c** and **4d**) and excellent *ee* values of both diastereoisomers

were found (entries 1, 3, 5 and 7). Furthermore, when the pH was basified up to pH 10, the biotransformation led to the formation of the amines with full conversions and excellent enantioselectivity and, in these cases, with slightly better diastereoselectivity (entries 2, 4, 6 and 8). Also, the reactivity of **3e** bearing the bulkiest substituent (a benzyl group) was studied, finding an inversion in the diastereoselectivity as the *syn*-diastereoisomer was the major one. At pH 7.5, high conversions, moderate diastereomeric ratios and excellent *ee* values were observed (entries 9 and 11). At pH 10, a significant drop in the ATA-013 activity was found (from 90 to 75% conv., entries 9 and 10) while ATA-025 results were similar to those obtained at pH 7.5 (entries 11 and 12).

Table 3.3. Transaminase-mediated DKR of α-substituted β-keto amides **3c-e** at pH 7.5 and pH 10.[a]



Entry	R^4	TA	pH	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	*n*-Pr (**3c**)	ATA-013	7.5	67	83:17	>99	>99
2	*n*-Pr (**3c**)	ATA-013	10	>99	87:13	>99	>99
3	*n*-Pr (**3c**)	ATA-025	7.5	97	82:18	>99	>99
4	*n*-Pr (**3c**)	ATA-025	10	>99	85:15	>99	>99
5	Allyl (**3d**)	ATA-013	7.5	97	74:26	>99	>99
6	Allyl (**3d**)	ATA-013	10	>99	80:20	>99	>99
7	Allyl (**3d**)	ATA-025	7.5	97	70:30	>99	>99
8	Allyl (**3d**)	ATA-025	10	97	75:25	>99	>99
9	Bn (**3e**)	ATA-013	7.5	90	22:78	>99	>99
10	Bn (**3e**)	ATA-013	10	75	30:70	>99	>99
11	Bn (**3e**)	ATA-025	7.5	99	21:79	>99	>99
12	Bn (**3e**)	ATA-025	10	99	20:80	>99	>99

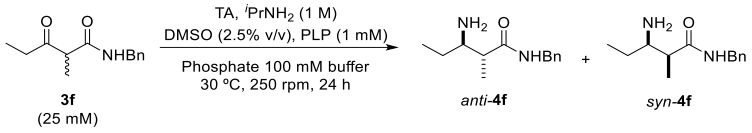
[a] Reaction conditions: Substrates **3c-e** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5-10 (500 µL final volume), 30 °C, 24 h, 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns.

3.2.4. Influence of R^1 and the amide moiety. Biotransamination of acyclic substrates 3f and 3g and cyclic substrate 3h

Looking for a further exploitation of this synthetic approach, the biotransamination of substrates differing in the length of the R^1 chain (**3f**) and in

the amide moiety (**3g**) was attempted. Additionally, a cyclic substrate (**3h**) was also considered as starting material.

Table 3.4. Synthesis of diastereoenriched and enantiopure **4f**.[a]



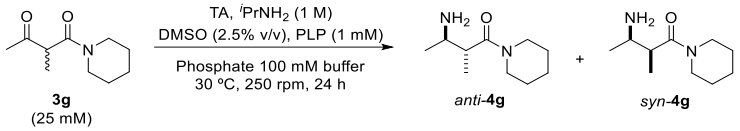
Entry	TA	pH	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	ATA-234 (*S*)	7.5	15	99:1	>99	>99
2	ATA-013 (*R*)	7.5	38	82:18	>99	>99
3	ATA-013 (*R*)	10	22	80:20	>99	>99
4	ATA-025 (*R*)	7.5	85	78:22	>99	>99
5	ATA-025 (*R*)	10	91	73:27	>99	>99

[a] Reaction conditions: Substrate **3f** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5-10 (500 µL final volume), 30 °C, 24 h, 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns.

First of all, substrate **3f** bearing an ethyl group in R^1 position was used as starting material for the biotransamination process (Table 3.4). In this case, only one (*S*)-TA displayed significant activity with this substrate (ATA-234, entry 1) and, even though it revealed very good diastereoselectivity towards the synthesis of the *anti*-diastereoisomer and excellent *ee* values, the conversion was very low (15% conv.). Furthermore, (*R*)-transaminase ATA-013 was still capable of synthesising the corresponding amine **4f** but the conversion dropped down to 38% conv. at pH 7.5 and 22% conv. at pH 10 (entries 2 and 3). However, ATA-025 remained as a robust biocatalyst providing high conversions, moderate diastereomeric ratios and excellent enantioselectivity (entries 4 and 5).

The influence of the amide moiety was studied by introducing a piperidine group (Table 3.5). The biotransamination of **3g** was efficient with a few (*S*)-TAs as happened with the model substrate **3a** (entries 1 and 2), while (*R*)-TAs led to conversion values and diastereomeric ratios comparable to those achieved with substrates **3b-d** (entries 3-6). Excellent enantioselectivity was observed in all cases. Furthermore, an increase in the pH when using (*R*)-TAs (entries 4 and 6) led to better *dr*, especially when ATA-025 was the TA of choice (91:9 *anti*:*syn*).

Table 3.5. Biotransformation of racemic keto amide **3g** into the corresponding amine **4g**.[a]

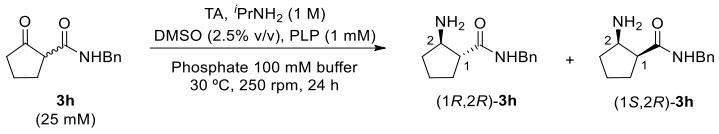


Entry	TA	pH	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	ATA-251 (*S*)	7.5	84	32:68	>99	>99
2	ATA-260 (*S*)	7.5	77	14:86	>99	>99
3	ATA-013 (*R*)	7.5	74	62:38	>99	>99
4	ATA-013 (*R*)	10	70	88:12	>99	>99
5	ATA-025 (*R*)	7.5	95	64:36	>99	>99
6	ATA-025 (*R*)	10	>99	91:9	>99	>99

[a] Reaction conditions: Substrates **3g** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5-10 (500 µL final volume), 30 °C, 24 h, 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns.

Finally, encouraged by previous studies where using a cyclic β-keto ester as starting material had led to high diastereomeric ratios,[157] we decided to use the cyclic β-keto amide **3h** as substrate in our DKR process (Table 3.6).

Table 3.6. Transaminase-catalysed DKR of cyclic β-keto amide **3h**.[a]



Entry	TA	pH	*c* (%)[b]	Ratio *trans*:*cis*[c]	*ee trans* (%)[c]	*ee cis* (%)[c]
1	ATA-013	7.5	57	78:22	>99	>99
2	ATA-013	10	65[d]	n.d.	n.d.	n.d.
3	ATA-025	7.5	89	80:20	>99	>99
4	ATA-025	10	97	75:25	>99	>99

[a] Reaction conditions: Substrate **3h** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5-10 (500 µL final volume), 30 °C, 24 h, 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns. [d] By-product determined. n.d.: not determined.

In this case, only *R*-transaminases achieved the biotransamination of **3h** leading to moderate conversion values when using ATA-013 (entry 1) and high conversions with ATA-025 (entry 3). Moderate *dr* and excellent *ee* values were found in both cases. When increasing the pH up to 10, a side-product corresponding to the enamine intermediate obtained after the addition of one molecule of isopropylamine was observed for the ATA-013 (entry 2)

transformation, while ATA-025 (entry 4) led to excellent conversion and *ee* values, and similar diastereoselectivity than at pH 7.5.

3.2.5. Looking for an efficient (*S*)-transaminase

At this point, it seemed evident that only (*R*)-TAs were capable of accepting all our substrates to perform efficient DKRs. For this reason, we decided to explore the activity of some made-in-house transaminases. In this way, TAs containing the *spuC* gene from *Pseudomonas* species, in particular *P. putida* (Pp-*spuC*), *P. chlororaphis* subsp. *aureofaciens* (Pc-*spuC*) and *P. fluorescens* (Pf-*spuC*),[160] and transaminases from *Vibrio fluvialis* (Vf),[22] from *Chromobacterium violaceum* (Cv),[118] from *Alkaligenes denitrificans* (AD),[161] from *Bacillus megaterium* (BmTA)[23g,162] and a high overproducing transaminases variant BmTA S119G[163] were tested towards α-substituted β-keto amides **3a-h** finding that only Bm-TA and BmTA S119G were capable of transforming our substrates (Table 3.7).

The desired amines **3a-d**,**h** were successfully synthesised by both (*S*)-transaminases. In all cases, moderate to high conversions and excellent *ee* values were found, BmTA and BmTA S119G leading to the formation of *syn*-**4a** with moderate to diastereomeric ratios (entries 1 and 2). However, with the rest of the substrates, the BmTA led to the formation of the *anti*-diastereomer as major product with moderate *dr* (entries 3, 5, 7 and 15) while BmTA S119G revealed to prefer the opposite diastereomer, leading to the formation of *syn*-**4b-d**,**h** with low to moderate diastereoselectivity (entries 4, 6, 8 and 16). Unfortunately, we found out that only substrates bearing a methyl group in the R^1 position were good substrates for these enzyme preparations. This means that substrate **3f** was not accepted by BmTA and BmTA S119G (entries 11 and 12). Similarly, to **3f**, the α-benzylated substrate (**3e**, entries 9 and 10) was not a good substrate for these enzymes. BmTA was still capable of producing the corresponding amine with 15% conv. and moderate *dr* towards the formation of *anti*-**4e** but BmTA S119G completely lost its activity. Similar results were found with the substrate bearing a

[160] J. L. Galman, I. Slabu, N. J. Weise, C. Iglesias, F. Parmeggiani, R. C. Lloyd, N. J. Turner, *Green Chem.* **2017**, *2*, 361-366.
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piperidine group in the amide moiety (**3g**, entries 13 and 14), this is BmTA revealed low conversion values and BmTA S119G was not capable of producing **4g**.

Table 3.7. Synthesis of α-substituted β-amino amides **4a-h** through a DKR catalysed by (*S*)-selective transaminases BmTA and BmTA S119G.[a]

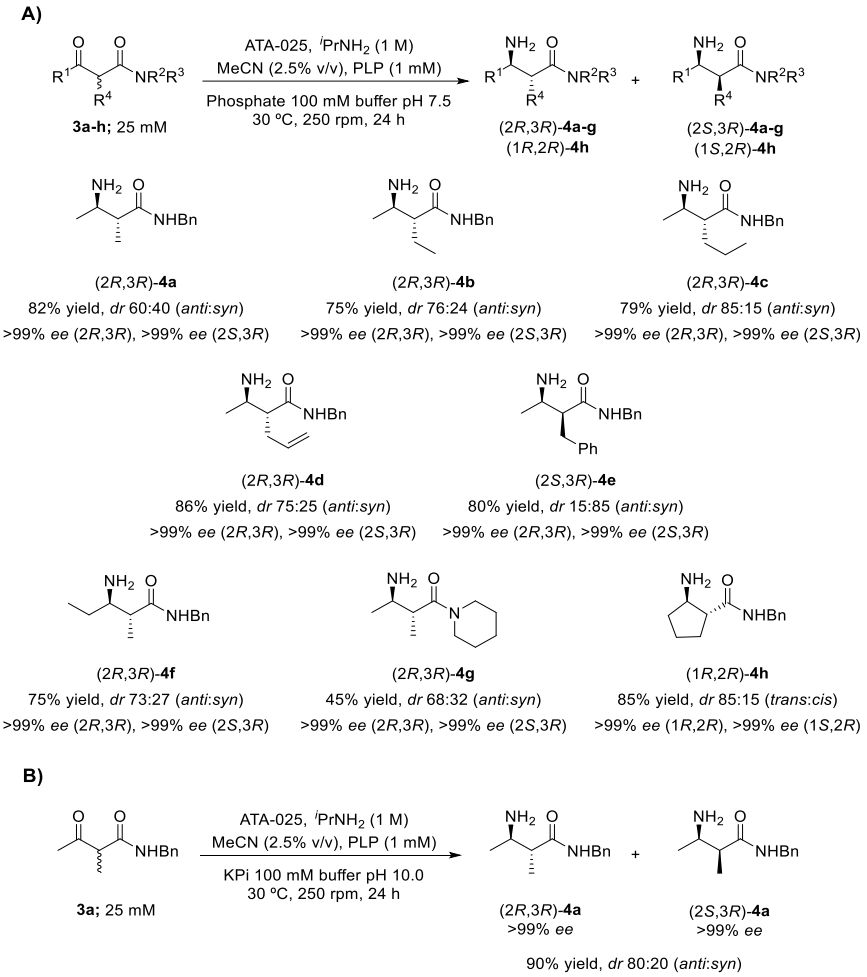
Entry	Substrate	TA	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	**3a**	BmTA	91	35:65	>99	>99
2	**3a**	BmTA S119G	60	30:70	>99	>99
3	**3b**	BmTA	70	60:40	>99	>99
4	**3b**	BmTA S119G	57	45:55	>99	>99
5	**3c**	BmTA	76	70:30	>99	>99
6	**3c**	BmTA S119G	49	41:59	>99	>99
7	**3d**	BmTA	78	60:40	>99	>99
8	**3d**	BmTA S119G	58	42:58	>99	>99
9	**3e**	BmTA	15	75:25	>99	>99
10	**3e**	BmTA S119G	<1	n.d.	n.d.	n.d.
11	**3f**	BmTA	<1	n.d.	n.d.	n.d.
12	**3f**	BmTA S119G	<1	n.d.	n.d.	n.d.
13	**3g**	BmTA	30	45:55	>99	>99
14	**3g**	BmTA S119G	<1	n.d.	n.d.	n.d.
15	**3h**	BmTA	92	80:20	>99	>99
16	**3h**	BmTA S119G	79	30:70	>99	>99

[a] Reaction conditions: Substrates **3a-h** (5 mM), transaminase (2 mg/mL), isopropylamine (50 equiv), PLP (1 mM), DMSO (1% v/v, 5 µL), HEPES buffer 50 mM pH 9.0 (500 µL final volume), 37 °C, 24 h, 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns. n.d.: not determined.

3.2.6. Semi-preparative biotransformations

The last step in our research was to demonstrate the applicability and the reproducibility of this procedure by setting up semi-preparative biotransformations. At the same time, this would allow us to isolate and fully characterise the new diastereoenriched and enantiopure α-substituted β-amino amides **4a-h**. For this purpose, the robust ATA-025 was chosen as biocatalyst and DMSO was swapped for acetonitrile (MeCN) as cosolvent in order to facilitate the work-up protocol.

Scheme 3.17. Semi-preparative biotransformations of α-substituted β-keto amides **3a-h** into the corresponding diastereoenriched and enantiopure amines **4a-h**. **A)** Semi-preparative DKRs of **3a-h** at pH 7.5. **B)** Synthesis of α-methylated β-amino amide **4a** at pH 10.

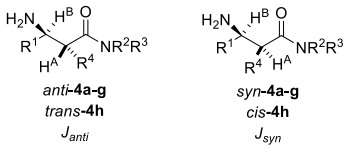


DKRs of all substrates were carried out under the optimised reaction conditions at pH 7.5 (Scheme 3.17.A). Diastereoenriched and enantiopure amines **4a-h** were obtained after acid-base extractions in moderate to high yields (45-86%) from 100 mg of the corresponding racemic α-substituted β-keto amides **3a-h**. The *anti*-diastereoisomer was always obtained as major product with the exception of the α-benzylated β-amino amide **4e**, finding low to moderate diastereomeric ratios (from 60:40 to 85:15) and excellent enantioselectivity in all cases (>99% *ee* for both diastereoisomers). Additionally, the biotransformation of **3a** into the corresponding α-methylated β-amino amide **4a** was performed at pH 10 (Scheme

3.17.B). In this case, the *anti*-amine was obtained as major diastereoisomer in moderate *dr* (80:20 *anti*:*syn*), high isolated yield (90%) and excellent *ee* values for both diastereoisomers (>99% *ee*).

All the amines were fully characterised by ^{1}H, ^{13}C and DEPT-NMR experiments, IR spectroscopy and high resolution mass spectrometry (HR-MS). Furthermore, homodecoupling NMR experiments were performed and the coupling constants between H^A and H^B calculated in order to determine which diastereoisomer was the major one (Table 3.8). In all cases, with the exception of the α-benzylated β-amino amide **4e**, the *anti*-diastereoisomer was found to be the major one.

Table 3.8. Coupling constant values between diastereomeric protons H^A and H^B determined by homodecoupling NMR experiments.



	4a	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**	**4h**
J_{anti} (Hz)	5.6	5.6	5.5	5.3	5.7	5.6	8.0	5.9
J_{syn} (Hz	3.9	4.8	4.7	4.2	4.8	3.4	5.5	3.6

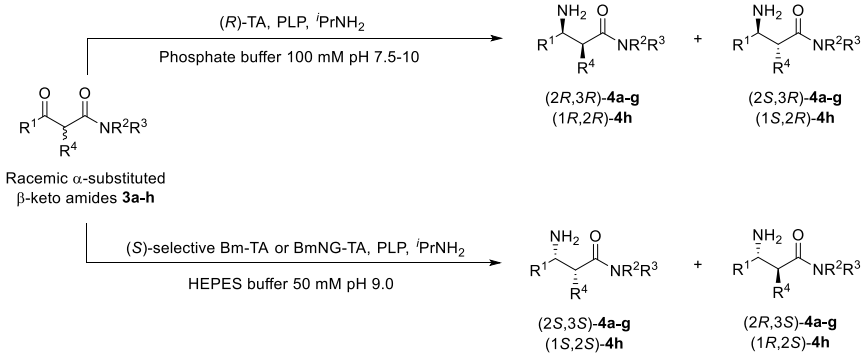
3.2.7. Final remarks

The DKR of racemic α-substituted β-keto amides **3a-h** bearing different pattern substitutions at the α- and γ-position and in the amide moiety has been developed using commercial (*R*)-transaminases and made-in-house (*S*)-TAs (Scheme 3.18). (*R*)-Transaminases displayed better performance as they recognised all the keto amides **3a-h** at the same time that were able to work at remarkable substrate concentrations (25 mM in comparison with the 5 mM used for their complementary made-in-house (*S*)-TAs), attaining in general high conversions, moderate diastereomeric ratios and excellent *ee* values by choosing the appropriate pH for each particular case.

Furthermore, semi-preparative biotransformations using ATA-025 were set up, obtaining results comparable to those obtained at small-scale. This fact proves the reproducibility and applicability of the method and it allows the

characterisation of a wide panel of new diastereomeric mixtures of enantiopure α-alkyl-β-amino amides.

Scheme 3.18. Overview of the transaminase-mediated DKR of α-substituted β-keto amides.



3.3. Experimental section

3.3.1. General information

Chemical reagents were purchased from different commercial sources and used without further purification. The solvents employed, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), diethyl ether (Et_2O), hexane (Hex), dimethylsulfoxide (DMSO), acetonitrile (MeCN) and isopropanol (^{i}PrOH) were employed without previous drying. 1,4-Dioxane, methanol (MeOH) and acetone were distilled over an adequate desiccant under nitrogen; this was sodium, calcium hydride and calcium sulphate, respectively.

Commercial transaminases were purchased from Codexis, and transaminases from *Bacillus megaterium*[162] and the high overproducing transaminases variant BmTA S119G[163] overexpressed on *E. coli* cells were obtained as previously described in the bibliography.

3.3.2. Synthesis of β-keto amides 2a-c and 3h

3.3.2.1. Chemical approach[159]

Boron trifluoride etherate (227 mg, 2 mmol) was added to a solution of the corresponding β-keto ester **1a** or **1b** (1 mmol) in dichloromethane (5 mL, 0.2 M) at room temperature. The reaction was stirred for 24 h, and then the solvent was distilled under reduced pressure. The resulting 2,2-difluoro-4-alkoxy-1,3,2-dioxaborinanes were dissolved in acetonitrile (5 mL, 0.2 M) and benzylamine (147 µL, 1.3 mmol) was added at room temperature. The reaction mixture was stirred at room temperature for 3 h. After that time, MeCN was evaporated in the rotary evaporator. Sodium acetate (410 mg, 5 mmol), ethanol (5 mL) and water (5 mL) were added to the resulting 2,2-difluoro-4-alkylamino-1,3,2-dioxaborinane and the mixture was heated at reflux overnight. Ethanol was then removed under reduced pressure and the residue extracted with EtOAc (3 x 20 mL), washed with water (2 x 10 mL), dried over sodium sulphate, filtered and evaporated to dryness. The products were purified by column chromatography on silica gel (30% EtOAc/Hex) to afford the β-keto amides **2a** and **2b** as yellowish solids (89% yield).

3.3.2.2. Enzymatic approach[158]

Piperidine or benzylamine (1.1 mmol) was added to a suspension containing the β-keto ester **1a** or **1c** (1 mmol, 0.1 M), dry 1,4-dioxane (10 mL) and CAL-B (50 mg) in an Erlenmeyer flask under inert atmosphere. The reaction was shaken at 30 ºC and 250 rpm for 48 h. The enzyme was filtered off, washed with CH_2Cl_2 (3 x 10 mL) and the solvent evaporated under reduced pressure. The crude reaction was purified by column chromatography on silica gel (30% EtOAc/Hex), affording the corresponding β-keto amides **2c** and **3h** as yellowish oils (91 and 48% yield, respectively).

3.3.3. Synthesis of racemic α-substituted β-keto amides 3a-g

Potassium carbonate (180 mg, 1.3 mmol) was added to a solution of the corresponding β-keto amide **2a-c** (1 mmol, 0.5 M) in dry acetone (2 mL) under argon atmosphere. The mixture was stirred for 5-10 min and, after that, the corresponding alkyl iodide or alkyl bromide (1.3 mmol) was added. The reaction was stirred at reflux temperature overnight. The residue was extracted with CH_2Cl_2 (3 x 10 mL) and washed with water (2 x 5 mL). Afterwards, the organic layers were collected and dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. The product was purified by column chromatography using silica gel (60% EtOAc/Hex) to afford the α-substituted β-keto amides **3a-g** (38-60% yield).

3.3.4. Synthesis of racemic α-substituted β-amino amides 4a-h

Ammonium acetate (770 mg, 10 mmol) and sodium cyanoborohydride (128 mg, 2 mmol) were successively added to a solution of the corresponding keto amide **3a-h** (1 mmol, 0.3 M) in dry MeOH under inert atmosphere. The mixture was stirred at room temperature during 16 h and, after this time, H_2O (5 mL) was added to quench the reaction. The solution was acidified using an aqueous solution HCl 3 M up to pH ~ 3 and extracted with Et_2O (4 x 10 mL). The resulting aqueous layer was basified by adding 2-3 pellets of NaOH up to pH ~ 13 and extracted with Et_2O (4 x 10 mL). The organic layers were combined, dried over Na_2SO_4, filtered and the solvent distilled under reduce pressure, obtaining the corresponding amines **4a-h** (36-70%).

3.3.5. Biotransamination of α-substituted β-keto amides 3a-h using commercial transaminases

In a 1.5 mL Eppendorf tube, transaminase (2 mg) and α-substituted β-keto amides (**3a-h**, 25 mM) were added in phosphate buffer 100 mM pH 7.5 or 10.0 (final volume: 500 µL, 1 mM PLP, 1 M isopropylamine), and DMSO (12.5 µL, 2.5% v/v). The reaction was shaken at 10 or 30 °C and 250 rpm for 24 h and stopped by the addition of an aqueous saturated solution of Na_2CO_3 (200 µL). Then the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions of α-substituted β-amino amides **4a-h** were determined by GC and *ee* and *dr* were measured by HPLC.

3.3.6. Biotransamination of α-substituted β-keto amides 3a-h using transaminases from *Bacillus megaterium* and from *Bacillus megaterium* S119G

In a 1.5 mL Eppendorf tube, transaminase (2 mg) and α-substituted β-keto amides (**3a-h**, 5 mM) were added in HEPES buffer 50 mM pH 9.0 (500 µL, 1 mM PLP, 250 mM isopropylamine), and DMSO (5.0 µL). The reaction was shaken at 37 °C and 250 rpm for 24 h and stopped by the addition of an aqueous saturated solution of Na_2CO_3 (200 µL). Then the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions of α-substituted β-amino amides **4a-h** were determined by GC and *ee* and *dr* were measured by HPLC.

3.3.7. Semi-preparative biotransformation of α-substituted β-keto amides 3a-h into α-substituted β-amino amides 4a-h using commercial transaminase ATA-025

In an Erlenmeyer flask, ATA-025 (75 mg) and α-substituted β-keto amide (100 mg, 25 mM) were added in phosphate buffer 100 mM (1 mM PLP, 250 mM isopropylamine, pH 7.5 for keto amides **3a-h** and pH 10.0 for keto amide **3a**) and MeCN (2.5% v/v). The reaction was shaken at 30 °C and 250 rpm for 24 h and then stopped by adding a saturated aqueous solution of Na_2CO_3 until pH 10-11. Then, the mixture was extracted with EtOAc (3 x 15 mL), the organic layer separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4 and conversions were measured by GC analysis and *ee* and *dr* were measured by HPLC. In order to obtain the pure amines, the reaction crude was

acidified using HCl up to pH ~ 3 and extracted with Et_2O (4 x 10 mL). The aqueous layer was basified by adding 2-3 pellets of NaOH up to pH ~ 13 and extracted with Et_2O (4 x 10 mL). The organic layers were combined and dried over Na_2SO_4 and the solvent was eliminated under reduced pressure, obtaining the amines **4a-h** in moderate to high yields (45-90%).

Publication 3

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ARTICLE

Efficient synthesis of α-alkyl-β-amino amides by transaminase-mediated dynamic kinetic resolutions

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The biocatalytic stereocontrolled synthesis of various acyclic *anti*- or *syn*-α-alkyl-β-amino amides through a dynamic kinetic resolution strategy is demonstrated. A series of commercially available and in-house transaminases (TAs) were employed to perform the transamination over a series of previously chemically synthesized racemic α-alkyl-β-keto amides. Among them, commercial (*R*)-selective TAs showed the best activities and selectivities, usually giving access preferentially to the *anti*-diastereoisomers with low to high diastereomeric ratios (up to 96%) and excellent enantiomeric excess (>99%). The stereoselective biotransamination experiments were successfully demonstrated at semipreparative (25 mM, 100 mg substrate), leading the corresponding optically active α-alkyl-β-amino amides in 45-90% isolated yield after a simple liquid-liquid extraction protocol

Introduction

Chiral β-amino amides (also denoted as β^3-amino amides) are valuable compounds due to their potential biological activities. Among them, it can be highlighted those that exhibits dipeptidyl peptidase IV (DPP-4) inhibitory activity such as sitagliptin[1] (**A**, Fig. 1A), which are employed for type-II diabetes treatment. Since sitagliptin was discovered, other related β-amino amide analogs have been synthesized and biologically tested,[2] affording very promising results (*e.g.* compound **B**,[2e] Fig. 1A). Moreover, if they are substituted at α-position (denoted as $\beta^{2,3}$-amino amides), many interesting biological activities can be displayed, such as bestatin[3] (**C**, Fig. 1A), an aminopeptidase inhibitor, or KNI-272 (**D**,[4] Fig. 1A), an HIV protease inhibitor. Also, β-amino amides are synthetically useful precursors of valuable 1,3-diamines through reductive processes, which have been applied as ligands in metal-catalyzed reactions.[5]

Among the different synthetic approaches (Fig. 1B) that have been described to get access to enantio- or diastereoenriched β^3- or $\beta^{2,3}$-amino amides, the Mannich transformation has been the most recurrently applied.[6] To induce chirality, enantiopure imines[6k] or amides[6a,b,i] have been utilized as precursors in the presence of a strong base at very low temperatures (<–55 °C). Also, the employment of bases with sterically hindered ligands[6c,l] or metals such as samarium[6d] or cobalt[6e] gave straightforward access to β-amino amides with usually good diastereoselectivities. A very interesting methodology has been developed by Kumagai, Shibasaki and co-workers, where different β-amino amides bearing substitutions at α position such as trifluoromethyl,[6f] fluorine,[6g] methyl,[6h] chlorine,[6j] or benzyloxy[6m] were synthesized with high diastereomeric ratios (*dr*) and excellent enantiomeric excess (*ee*) starting from 7-azaindoline amides in the presence of a copper catalyst with a chiral ligand and using the Barton's base. Other more specific synthetic methods involve the aza-Michael addition on α,β-unsaturated amides,[7] multicomponent transformations,[8] the hydrogenation[9a,b] or reduction[9c] of enamines, the reductive amination of β-keto amides,[10] or the reduction of oxime precursors.[11]

Remarkably, enzymatic methodologies (Fig. 1B) have also led to β-amino amides with excellent selectivities under mild reaction conditions.[12] For instance, racemic β-amino nitriles have been selectively hydrolyzed *via* kinetic resolution (KR) using whole-cells displaying nitrile hydratase activity.[13] *Rhodococcus* species were found as the best candidates, and through this method the corresponding β-amino amides could be synthesized together with the corresponding β-amino acids due to the concomitant amidase activity found in the enzymatic preparation. Other enzymatic approaches to get access to these compounds have been the lipase-catalyzed aminolysis of racemic β-lactams[14] or the acylation of β-amino amides,[15] and the hydrolysis of racemic β-amino amides using aminopeptidases[16] or amidases.[17] All these protocols presented the limitation of a 50% conversion towards the final products, which is inherent to KRs. In order to overcome this drawback, desymmetrization of prochiral compounds[18] or dynamic kinetic resolutions (DKRs)[19] of racemic derivatives can be employed as efficient synthetic tools.

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Electronic Supplementary Information (ESI) available: experimental procedures, compound characterization, enzymatic screenings and optimizations of the reaction conditions, analytics, HPLC chromatograms, and NMR spectra are described. See DOI: 10.1039/x0xx00000x

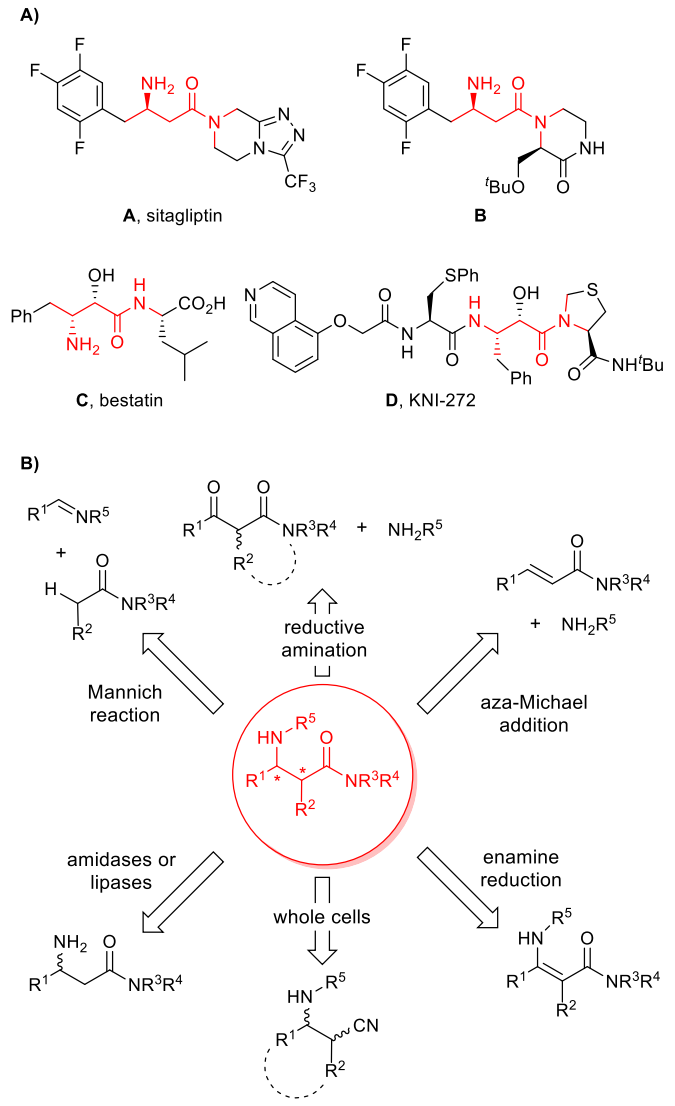


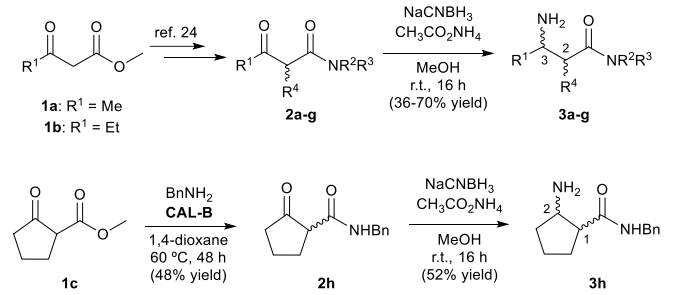
Fig. 1. A) Biologically active compounds **A**-**D** containing the β^3- or $\beta^{2,3}$-amino amide core. B) Chemical and enzymatic approaches to obtain chiral (α-substituted) β-amino amides.

Transaminases (TAs, also known as aminotransferases),[20] belong to a group of enzymes that have been intensively applied in the last decade towards the stereoselective synthesis of amines. They catalyze the reversible transfer of an amino group between an amine donor (typically an α-amino acid) and an amine acceptor (keto acids, ketones or aldehydes), using pyridoxal 5'-phosphate (PLP) as a cofactor. Amongst the different transformations that can mediate, the asymmetrization of carbonyl compounds can be underlined. An outstanding example of the relevance of these biocatalysts at industrial scale is the development of a variant from *Arthrobacter* sp. TA to obtain sitagliptin from the ketone precursor at high substrate concentration.[21] Since Kroutil and co-workers showed the first example,[22a] TAs have been successfully applied in DKR processes.[22b-i] Researchers have usually taken advantage of the high acidity of the α-proton in the carbonylic derivatives, so the low reacting enantiomer can be easily racemized even at neutral pH. In one of our groups a DKR protocol was designed for the synthesis of a wide panel of diastereo- and enantioenriched α-alkyl-β-amino esters starting from the corresponding racemic β-keto esters employing TAs.[23] In general, high conversion and *ee* values were found, however, moderate *dr* values (<60%) were observed. Following this study and due to the lack of biocatalytic methodologies to synthesize $\beta^{2,3}$-amino amides, we decided to develop a TA-mediated protocol to produce different diastereo- and enantioenriched α-alkyl-β-amino amides through DKR transformations. Several reaction parameters including the source and amount of TA, pH and temperature have been studied in order to disclose an efficient and general asymmetric protocol.

Results and discussion

Initially, a wide panel of α-substituted β-keto amides were synthesized bearing different substitution patterns at the γ- (R^1) and α-position (R^4), as well as in the amide protecting group (R^2 and R^3).[24] As starting materials, commercially available β-keto esters **1a** and **1b** were used and a chemical[25] or an enzymatic[26] methodology was applied in order to obtain the corresponding β-keto amides **2a-g** (Scheme 1). The cyclic substrate **2h** was straightforward synthesized in moderate yield (48%) from the corresponding commercially available racemic methyl 2-oxocyclopentanecarboxylate (**1c**) using a CAL-B mediated aminolysis with benzylamine as nucleophile.

Once the substrates were obtained, it was necessary to synthesise the racemic amines **3a**-**h** that would serve as standards to develop GC and HPLC analysis methods (see ESI). With that purpose, the reductive aminations of the α-substituted β-keto amides **2a**-**h** were attempted using 2 equiv of $NaBH_3CN$ as reduction agent and 10 equiv of ammonium acetate as nitrogen source in dry methanol (Scheme 1). Mixtures of *syn*- and *anti*-diastereoisomers of the α-substituted β-amino amides **3a**-**h** were obtained with low to high yields (36-70%).



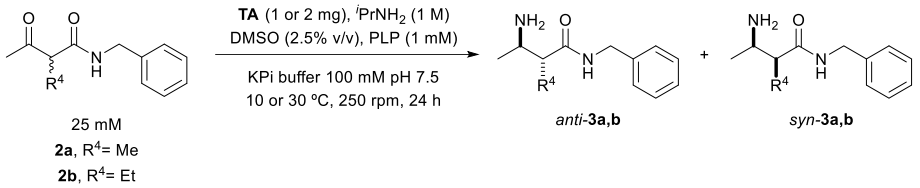
2a, **3a**: R^1, R^4 = Me, R^2 = H, R^3 = Bn; **2b**, **3b**: R^1 = Me, R^4 = Et, R^2 = H, R^3 = Bn
2c, **3c**: R^1 = Me, R^4 = *n*-Pr, R^2 = H, R^3 = Bn; **2d**, **3d**: R^1 = Me, R^4 = Allyl, R^2 = H, R^3 = Bn
2e, **3e**: R^1 = Me, R^2 = H, R^3, R^4 = Bn; **2f**, **3f**: R^1 = Et, R^4 = Me, R^2 = H, R^3 = Bn
2g, 3g: R^1, R^4 = Me, R^2, R^3 = -[$(CH_2)_5$]-

Scheme 1 Synthesis of racemic α-substituted β-keto amides and β-amino amides.

N-Benzyl-2-methyl-3-oxobutanamide (**2a**) was chosen as model substrate for our study and 28 commercial transaminases from Codexis Inc. were tested (2 mg). Based on previous studies using transaminases,[27] 25 mM concentration of **2a** was used, selecting DMSO as cosolvent (2.5% v/v) for solubilizing the substrate, and phosphate buffer 100 mM pH 7.5 (final volume: 500 µL) containing PLP (1 mM) as reaction media. A large excess (1 M) of isopropylamine (^{i}PrNH$_2$) was added as amine donor in order to shift the equilibrium towards the amine formation. All biotransformations were incubated at 30 °C and 250 rpm for 24 hours finding good conversions, excellent enantioselectivities and low to moderate diastereoselectivities with some (*S*)- and (*R*)-transaminases (Table 1 and Section 4.1 in the ESI). As can be seen, in most cases the *anti* isomer of **3a** was obtained (an exception is shown in entry 1 with ATA-260). These usually poor diastereomeric ratios could be due to a slow racemization rate. In order to develop a more efficient DKR procedure, the possibility of slowing down the enzymatic transamination process was attempted by decreasing the temperature or adding less amount of enzyme with the (*S*)-selective ATA-251 (entries 4 and 5) and the (*R*)-selective ATA-013 (entries 7 and 8). On the one hand, a lower temperature (10 °C) led to diminished conversion values and similar diastereomeric ratios with ATA-251 (entry 4) and a dramatic loss of activity with ATA-013 (entry 7). On the other hand, when a lower loading of the TA was used (1 mg), slightly lower conversions and similar diastereoselectivities were attained with both enzymes (entries 5 and 8).

Since the use of lower amounts of enzyme and cooler temperatures did not show better results, the application of typical TA reaction conditions to afford the DKR on other substrates was next envisaged. This way, the influence of the alkyl group at the α-position could be studied. For this purpose, *N*-benzyl-2-ethyl-3-oxobutanamide (**2b**) was employed as starting material for the transamination using all the transaminases working on the model substrate (Table 1 and Section 4.2 in the ESI). After analyzing the reaction mixtures, we found out that (*S*)-TAs lost their selectivity with this substrate except in the case of ATA-234, which afforded *anti*-(2*S*,3*S*)-**3b** with very high *dr* and *ee* values (entry 9). Nevertheless, (*R*)-transaminases still provided good results in terms of conversion and enantioselectivity values (entry 10). Furthermore, higher diastereomeric ratios (up to 80:20 *anti*:*syn*) were obtained in comparison with those achieved with the model substrate **2a**.

Table 1 Transamination of substrates **2a** and **2b** under dynamic conditions using commercial TAs[a]



Entry	Substrate	Transaminase	T (°C)	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	**2a**	ATA-260 (*S*)	30	96	31:69	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
2	**2a**	ATA-254 (*S*)	30	89	85:15	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
3	**2a**	ATA-251 (*S*)	30	98	65:35	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
4	**2a**	ATA-251 (*S*)	10	65	63:37	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
5	**2a**	ATA-251 (*S*)[d]	30	94	65:35	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
6	**2a**	ATA-013 (*R*)	30	98	62:38	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
7	**2a**	ATA-013 (*R*)	10	11	n.d.	n.d.	n.d
8	**2a**	ATA-013 (*R*)[d]	30	74	70:30	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
9	**2b**	ATA-234 (*S*)	30	64	97:3	95 (2*S*,3*S*)	n.d.
10	**2b**	ATA-013 (*R*)	30	96	80:20	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)

[a] Reaction conditions: Substrates **2a** or **2b** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 (final volume: 500 µL), 10 or 30 °C, 24 h and 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns. The major diastereoisomer is shown in parentheses. [d] Only 1 mg of transaminase was used. n.d. not determined.

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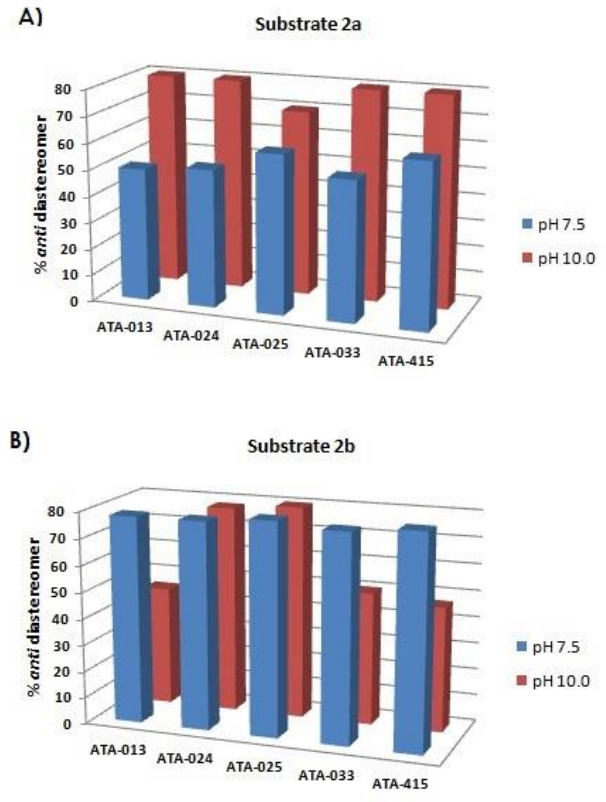

Fig. 2. Influence of the pH in the DKR of α-alkylated β-keto amides **2a** and **2b** mediated by (*R*)-transaminases. Blue color is used to indicate the percentage of *anti*-(2*R*,3*R*)-**3a**,**b** (>95% conversion and >99% *ee* in all cases) at pH 7.5, while red bars denotes the percentage of *anti*-(2*R*,3*R*)-**3a**,**b** (>97% conversion and >99% *ee* in all cases) at pH 10.0. A) Biotransaminations with **2a**. B) Biotransaminations with **2b**.

Later on, the influence of the pH was analyzed in order to speed up the racemization process by increasing the pH up to 10 (Sections 6.1 and 6.2 in the ESI). After 24 hours, no improvement was found with the (*S*)-TAs for substrate **2a** but significantly better results were achieved in this DKR process using the (*R*)-TAs (Fig. 2A). Thus, at pH 10, these transaminases were capable of producing *anti*-(2*R*,3*R*)-**3a** with full conversions, excellent *ee* values and improved *dr* going from ratios close to 50:50 *anti*:*syn* up to 80:20 *anti*:*syn*. Encouraged by these results, the biotransaminations of **2b** into **3b** were set up at pH 10 (Fig. 2B). However, even though the DKR led to full conversions and excellent *ee* values, no better diastereomeric ratios towards the formation of *anti*-(2*R*,3*R*)-**3b** were found, as they remained untouched in some cases (ATA-024 and ATA-025) and were much lower in other cases (ATA-013, ATA-033 and ATA-415). Therefore, it can be concluded that pH can largely affect the selectivity of these processes, but that it must be checked carefully for each substrate as it was not observed a clear trend.

Afterwards, additional substrates **2c**-**e** bearing different substitution patterns at the α-position to the amide (R^4= *n*-Pr, allyl, and benzyl, respectively) were tested at pH 7.5 and pH 10 (Table 2 and Sections 4.3-4.5 and 6.3-6.5 in the ESI). The best results were provided by (*R*)-transaminases ATA-013, ATA-024 and ATA-033. Substrates **2c** and **2d**, bearing a *n*-propyl and an allyl rest at the α-position, respectively, led to similar results than those obtained in the biotransamination of **2b** (R^4= Et). At pH 7.5 good conversions, moderate diastereomeric ratios towards the formation of the *anti*-amines (**3c** and **3d**) and excellent *ee* values for both diastereoisomers were found (entries 1 and 3). Furthermore, when the pH was basified up to pH 10, the biotransformations led to the formation of the amines with full conversions and excellent enantioselectivities and similar *dr* (entries 2 and 4). Next, the reactivity of **2e** bearing the bulkiest substituent (a benzyl group) was studied, finding an inversion in the diastereoselectivity as the *syn*-diastereoisomer was the major one. At pH 7.5, high conversions, moderate diastereomeric ratios and excellent *ee* values were observed (*e.g.*, ATA-033, entry 5). At pH 10, a significant improvement in the diastereoselectivity was observed for this biocatalyst, as can be seen in entry 6.

Then, *N*-benzyl-2-methyl-3-oxopentanamide (**2f**) bearing an ethyl group in R^1 position was used as starting material for the biotransamination process (Table 2 and Sections 4.6 and 6.6 in the ESI). In this case, only one (*S*)-TA displayed significant activity with this substrate (ATA-234, entry 7) and, even though, it revealed very good diastereoselectivity and excellent *ee* values towards the synthesis of the *anti*-(2*S*,3*S*)-diastereoisomer, the conversion was very low (15% conv.). Furthermore, several (*R*)-transaminases were capable of synthesizing the corresponding amine **3f**, being ATA-024 especially good providing high conversions, moderate diastereomeric ratios and excellent enantioselectivity (entries 8 and 9). The influence of the amide moiety was studied by introducing a piperidine group (Table 2 and Sections 4.7 and 6.7 in the ESI). The biotransamination of **2g** revealed to be efficient with (*S*)-TAs as occurred with the model substrate **2a**, obtaining very promising results with ATA-256 (entry 10), which afforded selectively *syn*-(2*R*,3*S*)-**3g**. On the other hand, several (*R*)-TAs led to conversion values and diastereomeric ratios comparable to those achieved with substrates **2b**-**d**. Excellent enantioselectivities were observed in all cases. Furthermore, an increase in the pH up to 10 led to better *dr*, especially when ATA-025 was the TA of choice (entries 11 and 12).

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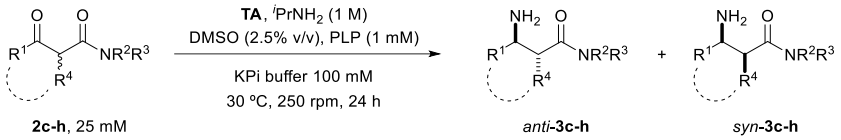
Table 2 Transaminase-mediated DKR of α-substituted β-keto amides **2c-h** at pH 7.5 and pH 10[a]



Entry	Substrate	TA	pH	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	**2c**	ATA-024	7.5	97	89:11	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
2	**2c**	ATA-024	10	>99	88:12	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
3	**2d**	ATA-013	7.5	97	74:26	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
4	**2d**	ATA-013	10	>99	80:20	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
5	**2e**	ATA-033	7.5	99	28:72	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
6	**2e**	ATA-033	10	99	13:87	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
7	**2f**	ATA-234	7.5	15	99:1	>99 (2*S*,3*S*)	n.d.
8	**2f**	ATA-024	7.5	84	79:21	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
9	**2f**	ATA-024	10	97	72:28	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
10	**2g**	ATA-256	7.5	85	2:98	n.d.	>99 (2*R*,3*S*)
11	**2g**	ATA-025	7.5	95	64:36	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
12	**2g**	ATA-025	10	>99	91:9	>99 (2*R*,3*R*)	>99 (2*S*,3*R*)
13[d]	**2h**	ATA-415	7.5	82	90:10	>99 (1*R*,2*R*)	>99 (1*S*,2*R*)
14[d]	**2h**	ATA-415	10	97	92:8	>99 (1*R*,2*R*)	>99 (1*S*,2*R*)

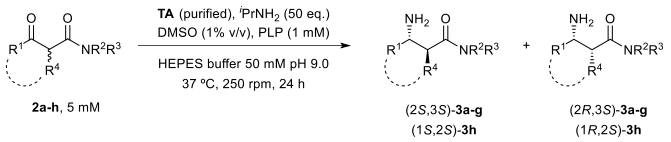
[a] Reaction conditions: Substrates **2c-h** (25 mM), transaminase (2 mg), isopropylamine (1 M), PLP (1 mM), DMSO (2.5% v/v, 12.5 µL), phosphate buffer 100 mM pH 7.5 or 10 (final volume: 500 µL), 30 °C, 24 h, 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns. The major diastereoisomer is shown in parentheses. [d] Note that for this substrate the chiral centers are in positions 1 and 2, as shown in Scheme 1. n.d. not determined.

Finally, encouraged for previous studies where excellent diastereomeric ratios were attained by using a cyclic β-keto ester as starting material,[23] the biotransamination of cyclic β-keto amide **2h** was ambitioned through this DKR strategy (Table 2 and Sections 4.8 and 6.8 in the ESI). In this case, only (*R*)-TAs seemed to be effective biocatalysts, leading ATA-415 to the best results (entries 13 and 14). Especially at pH 10, excellent conversions, *ee* values and high *dr* (92:8) towards the formation of (1*R*,2*R*)-**3h** was attained. It was interesting to observe that ATA-013 could catalyze at pH 10 the formation of a side-product corresponding to the enamine intermediate obtained after the addition of one molecule of isopropylamine to the β-keto amide (65%).

All the amines were fully characterized by ^{1}H, ^{13}C and DEPT-NMR experiments, IR spectroscopy and high resolution mass spectrometry (HR-MS). Furthermore, homodecoupling NMR experiments were performed and the coupling constants between H_2 (H_1 for **3h**) and H_3 (H_2 for **3h**) calculated in order to determine which diastereoisomer was the major one (Section 8 in the ESI). In all cases, with the exception of the α-benzylated β-amino amide **3e**, the *anti*-diastereoisomer was found to be preferentially formed, which is consistent with the previous

results obtained with the commercial enzymes when α-alkyl-β-hydroxy amides were synthesized.[23]

Table 3 Synthesis of α-substituted β-amino amides **2a**-**h** through a DKR process catalyzed by (*S*)-selective transaminases *Bm*TA and *Bm*TA S119G[a]



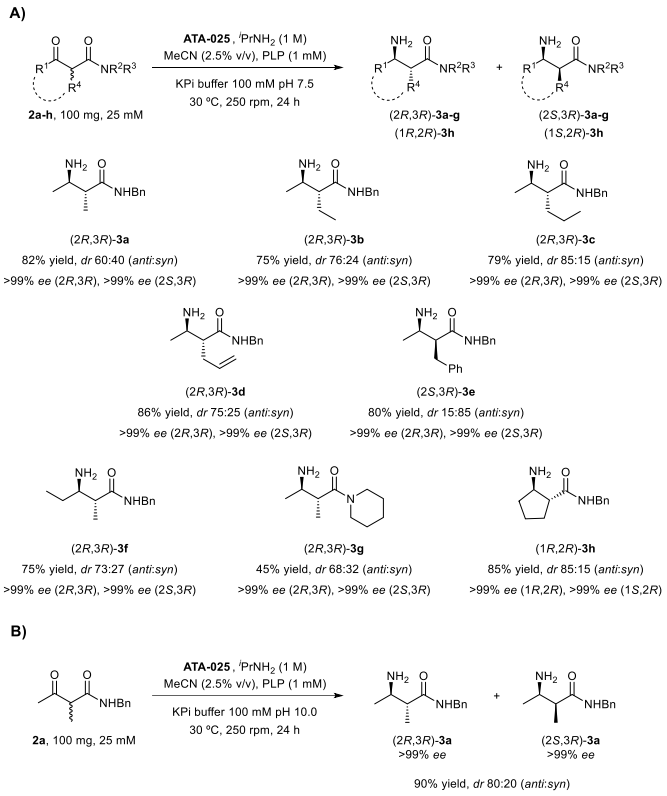
Entry	Substrate	TA	*c* (%)[b]	Ratio *anti*:*syn*[c]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	**2a**	*Bm*	91	35:65	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
2	**2a**	*Bm* S119G	60	30:70	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
3	**2b**	*Bm*	70	60:40	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
4	**2b**	*Bm* S119G	57	45:55	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
5	**2c**	*Bm*	76	70:30	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
6	**2c**	*Bm* S119G	49	41:59	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
7	**2d**	*Bm*	78	60:40	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
8	**2d**	*Bm* S119G	58	42:58	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
9	**2e**	*Bm*	15	75:25	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
10	**2e**	*Bm* S119G	<1	n.d.	n.d.	n.d.
11	**2f**	*Bm*	<1	n.d.	n.d.	n.d.
12	**2f**	*Bm* S119G	<1	n.d.	n.d.	n.d.
13	**2g**	*Bm*	30	45:55	>99 (2*S*,3*S*)	>99 (2*R*,3*S*)
14	**2g**	*Bm* S119G	<1	n.d.	n.d.	n.d.
15[d]	**2h**	*Bm*	92	80:20	>99 (1*S*,2*S*)	>99 (1*R*,2*S*)
16[d]	**2h**	*Bm* S119G	79	30:70	>99 (1*S*,2*S*)	>99 (1*R*,2*S*)

[a] Reaction conditions: Substrates **2a**-**h** (5 mM), transaminase (2 mg/mL), isopropylamine (50 equiv), PLP (1 mM), DMSO (1% v/v, 5 µL), HEPES 50 mM pH 9.0 (500 µL final volume), 37 °C, 24 h, 250 rpm. [b] Conversion values were determined by GC analysis. [c] Selectivities (*dr* and *ee*) were determined by HPLC analysis using chiral columns. The major diastereoisomer is shown in parentheses. [d] Note that for this substrate the chiral centers are in positions 1 and 2, as shown in Scheme 1. n.d. not determined.

At this point, it seemed evident that only (*R*)-TAs were capable of accepting all this panel of substrates to perform efficient DKRs. For this reason, we decided to explore the activity of some in-house transaminases. TAs containing the *spuC* gene from *Pseudomonas* species, in particular *P. putida* (*Pp* spuC), *P. chlororaphis* subsp. *aureofaciens* (*Pc* spuC) and *P. fluorescens* (*Pf* spuC),[28] and transaminases from *Vibrio fluvialis* (*Vf*),[29] *Chromobacterium violaceum* (*Cv*),[30] *Alcaligenes denitrificans* (*Ad*),[31] *Bacillus megaterium* (*Bm*)[32] and our high overproducing transaminase variant *Bm* S119G[33] were tested towards α-substituted β-keto amides **2a**-**h** finding that only *Bm*TA and *Bm*TA S119G were capable of reacting with the selected substrates (Table 3).

The desired enantiopure amines **3a**-**d**,**h** were successfully synthesized by both (*S*)-transaminases. In all cases, moderate to high conversions and excellent *ee* values were found, leading *Bm*TA and *Bm*TA S119G to the formation of *syn*-(2*R*,3*S*)-**3a** with low diastereomeric ratios (entries 1 and 2). However, with the rest of the substrates, *Bm*TA led to the preferential formation of the *anti*-diastereoisomer as the major product with low to moderate *dr* (entries 3, 5, 7 and 15), while *Bm*TA S119G afforded the opposite diastereoisomer, leading to the formation of *syn*-**3b**-**d**,**h** with low to moderate diastereoselectivities (entries 4, 6, 8 and 16). Unfortunately, the α-benzylated keto amide (**2e**, entries 9 and 10) was not a good substrate for these enzymes, meanwhile the substrate **2f** was not accepted by either *Bm*TA or *Bm*TA S119G (entries 11 and 12). *Bm*TA was still capable of producing *anti*-(2*S*,3*S*)-**3e** with 15% conversion and moderate *dr* but *Bm*TA S119G completely lost its activity. Similar results were found with the substrate bearing a piperidine group in the amide moiety (**2g**), the *Bm*TA displaying low activity (entry 13), while *Bm*TA S119G was not capable of producing **3g** in any extension (entry 14).

In order to demonstrate the applicability and reproducibility of this procedure, semipreparative biotransformations were set up. Hence, this would allow us to isolate and fully characterized the new diastereoenriched and enantiopure α-substituted β-amino amides **3a**-**h**. For this purpose, the robust ATA-025 was chosen as biocatalyst and DMSO was replaced with acetonitrile (MeCN) as cosolvent in order to facilitate the work-up protocols. DKRs of all substrates were carried out under the optimized reaction conditions at pH 7.5 (Scheme 2A).[34] The final products were obtained after acid-base extractions in moderate to high isolated yields (45-86%) starting from 100 mg of the corresponding racemic α-substituted β-keto amides **2a**-**h**. The *anti*-diastereoisomer was always obtained as the major product with the exception of α-benzylated β-amino amide **3e**, finding low to moderate diastereomeric ratios (from 60:40 to 85:15) and excellent enantioselectivities in all cases (>99% *ee* for both diastereoisomers). Additionally, the biotransformation of **2a** into the corresponding α-methylated β-amino amide **3a** was performed at pH 10 (Scheme 2B). In this case, the *anti*-amino amide was obtained as the major diastereoisomer in moderate *dr* (80:20 *anti*:*syn*), high isolated yield (90%) and excellent *ee* values for both diastereoisomers (>99% *ee*).



Scheme 2 Semipreparative scale biotransformations of α-substituted β-keto amides **2a-h** into the corresponding diastereoenriched and enantiopure amines **3a-h**. A) At pH 7.5. B) Synthesis of α-methylated β-amino amide **3a** at pH 10. The major diastereoisomer is drawn.

Conclusions

β-Amino amides are recurring structures that can be found in many biologically active compounds. While various efficient chemical methodologies have been described to obtain these derivatives in enantioenriched form, the introduction of a second chiral center at α-position difficult the selective synthesis of the amino amides due the occurrence of four possible product diastereoisomers. This is especially challenging for the case of acyclic α-alkyl-β-amino amides, where effective chemical or enzymatic methods have still not been found. Due to the exceptional relevance that transaminases are acquiring in the last decade to catalyze aminations of carbonyl compounds under very simple and mild conditions, herein we propose a straightforward manner to synthesize these compounds *via* DKR of a series of easily accessible racemic α-alkyl-β-keto amides bearing different substituents at both α- and γ-positions, and also in the amide moiety using commercial and in-house TAs.

After reaction optimization, various commercial (*R*)-transaminases displayed interesting activities as they recognized the different keto amides **2a-h** working at a remarkable substrate concentration (25 mM), attaining in general high conversions, moderate diastereomeric ratios and excellent *ee* values by choosing the appropriate pH for each particular case. In general, *anti*-isomers were preferentially obtained except for the case of the α-benzylated compound, where the *syn*-(2*S*,3*R*)-**3e** isomer was preferentially achieved. Remarkably, in-house (*S*)-selective TAs from *Bacillus megaterium* (*Bm*TA and a mutant *Bm*TA S119G) were also able to transform some of these substrates, affording the opposite enantiomers *anti*-(2*S*,3*S*)- and *syn*-(2*R*,3*S*)-**3a-d** or *trans*-(1*S*,2*S*)- and *cis*-(1*R*,2*S*)-**3h** with low diastereomeric ratios.

Furthermore, semipreparative biotransformations using commercially available ATA-025 were set up, obtaining results comparable to those obtained at small-scale. This fact proved the reproducibility and applicability of the method, facilitating the characterization of a wide panel of new diastereoenriched α-substituted β-amino amides.

Conflicts of interest

There are no conflicts to declare.

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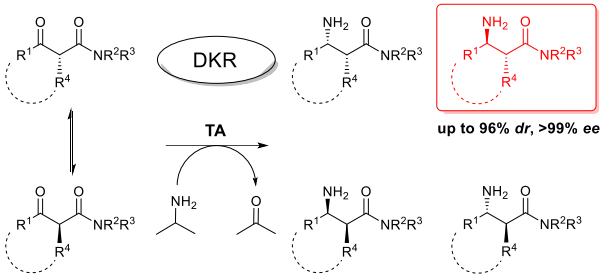
34 General protocol for the semipreparative biotransamination of α-substituted β-keto amides **2a**-**h** using the commercial transaminase ATA-025. In an Erlenmeyer, ATA-025 (75 mg) and α-substituted β-keto amide (100 mg, 25 mM) were added in phosphate buffer 100 mM (1 mM PLP, 250 mM isopropylamine, pH 7.5 for keto amides **2a-h** and pH 10.0 for keto amide **2a**) and MeCN (2.5% v/v). The reaction was shaken at 30 °C and 250 rpm for 24 h and then stopped by adding a saturated aqueous solution of Na_2CO_3 until pH 10-11. Then,

the mixture was extracted with EtOAc (3 x 15 mL), the organic layer separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4 and conversions were measured by GC analysis. In order to obtain the pure amines, the crude reaction was acidified using HCl up to pH ~ 3 and extracted with Et_2O (4 x 10 mL). The aqueous layer was basified by adding 2-3 pellets of NaOH up to pH ~ 13 and extracted with Et_2O (4 x 10 mL). The organic layers were combined and dried over Na_2SO_4 and the solvent was eliminated under reduced pressure, obtaining the amines **3a-h** in moderate to high yields (45-90%).

Table of Contents Entry



A transaminase-catalyzed dynamic kinetic resolution is described for the stereoselective synthesis of a series of α-alkyl-β-amino amides

Part II

Alcohol dehydrogenases applied to the synthesis of chiral hydroxy derivatives

Chapter 4

Ketoreductases in dynamic kinetic protocols. Chemoenzymatic synthesis of enantio- and diastereoenriched α-alkyl-β-hydroxy amides

4.1. Bibliographic background

Redox processes are among the most important transformations in organic chemistry. Particularly, carbonyl reductions have attracted special attention in both industry and academia as they can provide a wide range of valuable products.[164] In this way, alcohol dehydrogenases (ADHs or KREDs), also called ketoreductases or carbonyl reductases, could be the catalysts of choice. As introduced in the preface of this doctoral thesis, ADHs are oxidoreductases that catalyse the interconversion between alcohols and aldehydes or ketones.[12]

These redox enzymes have been deeply studied, being their high stereoselectivity towards the production of chiral alcohols the most remarkable characteristic.[14] However, three major drawbacks have limited their application in asymmetric synthesis at large scale: the low solubility of substrates in aqueous media, the need of an expensive nicotinamide cofactor and the strict substrate specificity.[165] On the one hand, the use of organic solvents, ionic liquids or supercritical CO_2 in mono- and biphasic reactions has helped to overcome the solubility problem. Also, the development of efficient and relatively cheap cofactor recycling systems,[13] as shown in the introduction of this Thesis, has largely minimised the quantity needed of NAD(P). Finally, the discovery of new (mutated) ADHs has helped to widen substrate the scope of these enzymes. Those efforts have turned into many ADH-catalysed processes which allow the production of chiral pharmaceuticals and fine chemicals.[166]

4.1.1. Alcohol dehydrogenases towards the synthesis of chiral alcohols

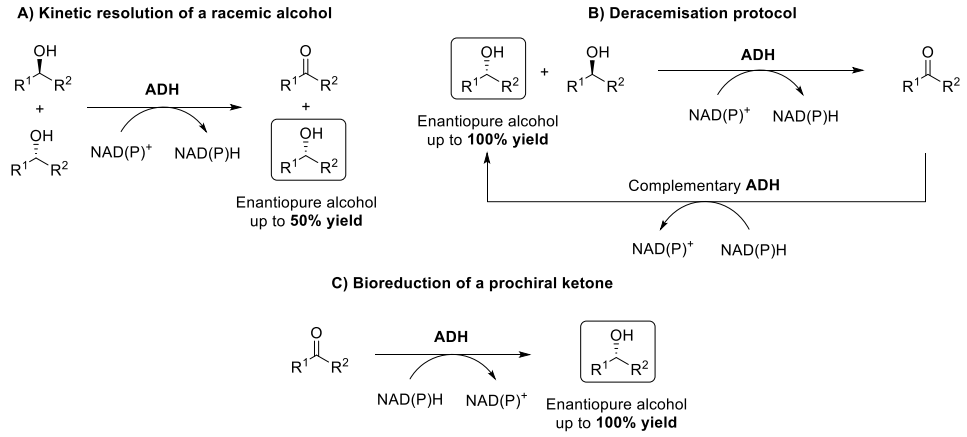
As said before, ADHs catalyse the interconversion between hydroxyl and carbonyl groups. This means that they can be used as biocatalysts in the kinetic resolution of racemic alcohols or in the reduction of prochiral ketones.

As already commented before, in a kinetic resolution protocol one of the enantiomers of a racemate is transformed into the product much faster than the other one. In this manner, the non-reacting enantiomer can be obtained in high *ee* but in just a 50% theoretical yield (Scheme 4.1.A). In order to achieve a single

[164] J. Magano, J. R. Dunetz, *Org. Process Res. Dev.* **2012**, *16*, 1156-1184.
[165] M. M. Musa, R. S. Phillips, *Catal. Sci. Technol.* **2011**, *1*, 1311-1323.
[166] Y.-G. Zheng, H.-H. Yin, D.-F. Yu, X. Cheng, X.-L. Tang, X.-J. Zhang, Y.-P. Xue, Y.-J. Wang, Z.-Q. Liu, *Appl. Microbiol. Biotechnol.* **2017**, *101*, 987-1001.

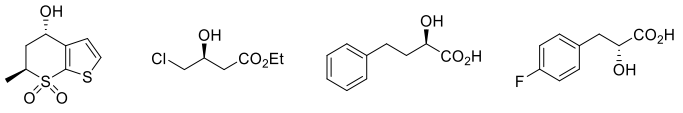
enantiomer in higher yields (up to 100%), deracemisation protocols can be developed using complementary ADHs (Scheme 4.1.B). Thus, the enantioselective oxidation of one enantiomer leads to the formation of the corresponding ketone which undergoes a stereoselective reduction to produce the other enantiomer through a stereoinversion protocol.[167]

Scheme 4.1. ADH-catalysed synthesis of chiral alcohols.



Another approach towards the synthesis of enantiopure alcohols in theoretically 100% yield, consists in the bioreduction of a prochiral ketone (Scheme 4.1.C). In this way, many protocols have been developed using whole cells or isolated ketoreductases to produce chiral alcohols from the corresponding carbonylic compounds.[168] Additionally, several chemical and biotechnological companies have developed industrial-scale bioreduction processes to produce enantiopure alcohols, which are important intermediates for agro-, pharma- and fine chemical applications (Scheme 4.2).

Scheme 4.2. Examples of optically pure alcohols obtained at industrial scale through ADH-catalysed bioreduction of the corresponding ketones.

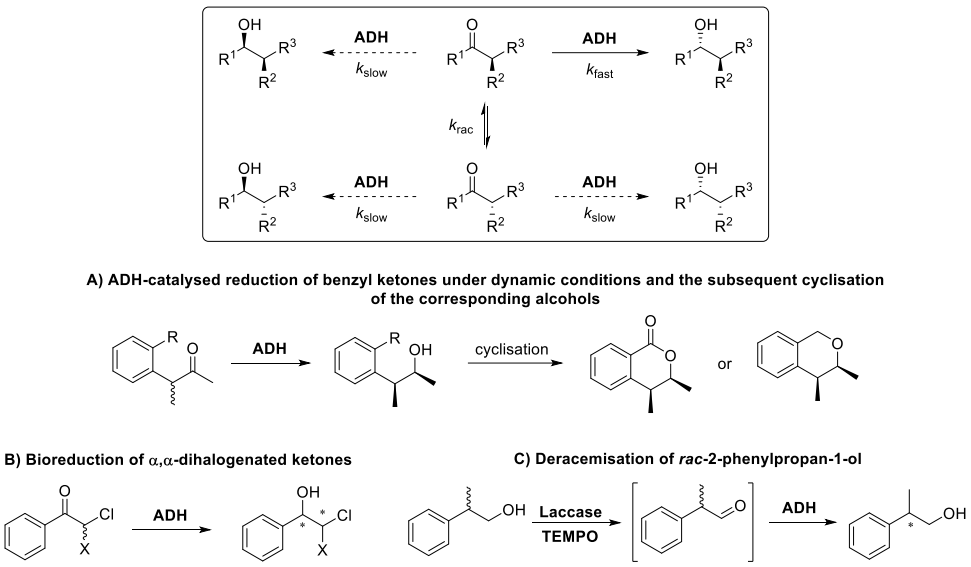


[167] W. Kroutil, H. Mang, K. Edegger, K. Faber, *Adv. Synth. Catal.* **2004**, *346*, 125-142.
[168] F. Hollmann, I. W. C. E. Arends, D. Holtmann, *Green Chem.* **2011**, *13*, 2285-2313.

4.1.2. ADH-catalysed DKRs. Dynamic reductive kinetic resolution

As explained in Chapter 3 (Section 3.1.3), a DKR consists in the kinetic resolution of a racemic mixture taking place at the same time that the racemisation of the non-reacting substrate. ADHs have been used applied to the reduction of prochiral compounds under dynamic conditions (Scheme 4.3), also known as dynamic reductive kinetic resolution (DYRKR).[169]

Scheme 4.3. General overview of an ADH-catalysed DYRKR and some recent examples developed by Gotor and co-workers.



Some examples of ADH-catalysed DKR processes have been developed in our research group. For instance, the DYRKR of benzyl ketones to produce heterocyclic scaffolds after intramolecular cyclisation of the corresponding alcohol intermediates has been studied (Scheme 4.3.A).[170] In a different research, ADHs were employed to reduce racemic α,α-dihalogenated ketones through a dynamic

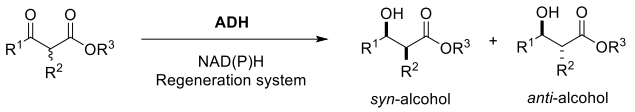
[169] In the case of a ketone with a chiral centre, it will be possible to obtain selectively one stereoisomer among the four possible isomers. However, in the case of an aldehyde with a chiral centre present in the molecule, the maximum number of stereoisomers will be two. For more information, see: G. A. Applegate, D. B. Berkowitz, *Adv. Synth. Catal.* **2015**, *357*, 1619-1632.

[170] (a) D. Méndez-Sánchez, J. Mangas-Sánchez, E. Busto, V. Gotor, V. Gotor-Fernández, *Adv. Synth. Catal*. **2016**, *358*, 122-131; (b) J. Mangas-Sánchez, E. Busto, V. Gotor, V. Gotor-Fernández, *Org. Lett*. **2013**, *15*, 3872-3875.

protocol (Scheme 4.3.B).[171] Furthermore, a smart approach towards the deracemisation of racemic 2-phenylpropan-1-ol was developed (Scheme 4.3.C).[172] This transformation was achieved through a one-pot two-step process involving the laccase/TEMPO-mediated non-selective oxidation of the alcohol and the bioreduction of the corresponding aldehyde under dynamic conditions.

Until now, we have introduced some examples using benzyl ketones, acetophenones and 2-phenylpropanal as substrates. However, ADHs have been typically used in the DYRKR of β-keto ester systems.[169] For instance, Gotor and co-workers reported a dynamic protocol that can be considered as the precursor of the research that we will introduce in this chapter (Scheme 4.4).[173] Thus, several α-substituted β-keto esters were tested as substrates in the ADH-catalysed DKR to produce enantio- and diastereoenriched α-substituted β-hydroxy esters. In this chapter, we will introduce a similar synthetic approach towards the production of enantio- and diastereoenriched α-substituted β-hydroxy amides.

Scheme 4.4. ADH-catalysed dynamic kinetic resolution of α-substituted β-keto esters.



4.1.3. β-Hydroxy amides. Asymmetric approaches towards the synthesis of enantio- and diastereoenriched α-substituted β-keto amides

Chiral β-hydroxy amides are important derivatives in organic chemistry as they have multiple applications (Scheme 4.5). For instance, they are present in the core of antitumor molecules such as bengamides[174] and they have been used as asymmetric ligands in key organic transformations as well.[175] Furthermore, they

[171] K. Kędziora, F. R. Bisogno, I. Lavandera, V. Gotor-Fernández, J. Montejo-Bernardo, S. García-Granda, W. Kroutil, V. Gotor, *ChemCatChem* **2014**, *6*, 1066-1072.
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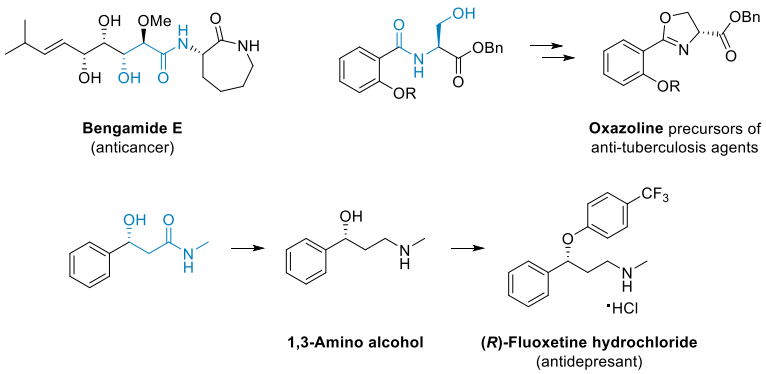
are important building blocks in the synthesis of compounds such as oxazolines,[176] oxazoles,[177] pyrrolidines,[178] azetidines[179] and 1,3-amino alcohols.[180]

Scheme 4.5. Some important applications of β-hydroxy amide derivatives.



Due to the importance of these compounds, several chemical approaches have been developed towards the synthesis of chiral β-hydroxy amides (Scheme 4.6):

(a) The aldol condensation between an amide and an aldehyde[181] or an acylsilane,[182] sometimes starting from chiral amides.

(b) The hydrogenation of β-keto amides using ruthenium[183] or iridium[184] complexes.

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[176] (a) M. Brandstätter, F. Roth, N. W. Luedtke, *J. Org. Chem.* **2015**, *80*, 40-51; (b) B. Li, S.-Q. Wang, B. Liu, B.-F. Shi, *Org. Lett.* **2015**, *17*, 1200-1203; (c) G. C. Moraski, M. Chang, A. Villegas-Estrada, S. G. Franzblau, U. Möllmann, M. J. Miller, *Eur. J. Med. Chem.* **2010**, *45*, 1703-1716.
[177] G. C. Moraski, S. G. Franzblau, M. J. Miller, *Heterocycles* **2010**, *80*, 977-988.
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[181] (a) G. Lemonnier, T. Poisson, S. Couve-Bonnaire, P. Jubault, X. Pannecoucke, *Eur. J. Org. Chem.* **2013**, 3278-3289; (b) M. Ocejo, L. Carrillo, J. L. Vicario, D. Badía, E. Reyes, *J. Org. Chem.* **2011**, *76*, 460-470; (c) M. Rodríguez, J. L. Vicario, D. Badía, L. Carrillo, *Org. Biomol. Chem.* **2005**, *3*, 2026-2030; (d) J. L. Vicario, M. Rodriguez, D. Badía, L. Carrillo, E. Reyes, *Org. Lett.* **2004**, *6*, 3171-3174.
[182] (a) R. B. Lettan II, C. V. Galliford, C. C. Woodward, K. A. Scheidt, *J. Am. Chem. Soc.* **2009**, *131*, 8805-8814; (b) R. B. Lettan II, T. E. Reynolds, C. V. Galliford, K. A. Scheidt, *J. Am. Chem. Soc.* **2006**, *128*, 15566-15567.
[183] (a) W. Li, W. Fan, X. Ma, X. Tao, X. Li, X. Xie, Z. Zhang, *Chem. Commun.* **2012**, *48*, 8976-8978; (b) W. Li, X. Ma, W. Fan, X. Tao, X. Li, X. Xie, Z. Zhang, *Org. Lett.* **2011**, *13*, 3876-3879; (c) R. Touati, T. Gmiza, S. Jeulin, C. Deport, V. Ratovelomanana-Vidal, B. B. Hassine, J.-P. Genet, *Synlett* **2005**, 2478-2482.

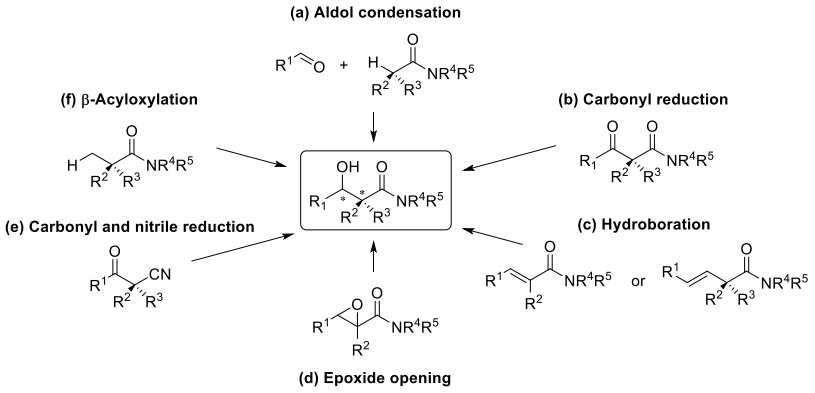
(c) The hydroboration process of unsaturated amides using rhodium[185] or copper[186] complexes as catalysts.

(d) The regioselective opening of α,β-epoxy amides with sodium bis(2-methoxyethoxy)aluminium hydride.[180]

(e) The catalytic reduction of β-keto nitriles mediated by a ruthenium catalyst.[187]

(f) The β-acyloxylation of a primary sp^3 C-H bond from simple amides catalysed by palladium.[188]

Scheme 4.6. Chemical protocols towards the synthesis of chiral (α-substituted) β-keto amides.



The synthesis of chiral α-substituted β-hydroxy amides is more challenging due to the formation of four possible diastereoisomers. However, some protocols have been developed to achieve these compounds in a high selective manner. For example, aldol condensations at low temperatures have demonstrated to be a powerful tool in order to achieve α-substituted β-hydroxy amides in high yields and excellent selectivity.[189] Additionally, the opening of chiral epoxides have also led

[184] (a) Y. Hu, X. Yin, Z. Chen, X.-Q. Dong, X. Zhang, *Org. Chem. Front.* **2018**, *5*, 2000-2003; (b) J.-H. Xie, X.-Y. Liu, X.-H. Yang, J.-B. Xie, L.-X. Wang, Q.-L. Zhou, *Angew. Chem. Int. Ed.* **2012**, *51*, 201-203.

[185] (a) S. M. Smith, J. M. Takacs, *Org. Lett.* **2010**, *12*, 4612-4615; (b) S. M. Smith, J. M. Takacs, *J. Am. Chem. Soc.* **2010**, *132*, 1740-1741; (c) S. M. Smith, N. C. Thacker, J. M. Takacs, *J. Am. Chem. Soc.* **2008**, *130*, 3734-3735.

[186] H. Chea, H.-S. Sim, J. Yun, *Adv. Synth. Catal.* **2009**, *351*, 855-858.

[187] R. González-Fernández, P. Crochet, V. Cadierno, *Org. Lett.* **2016**, *18*, 6164-6167.

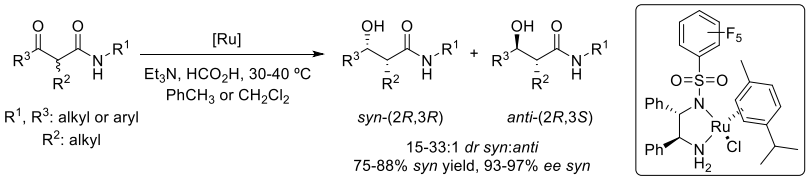
[188] L. Zhou, W. Lu, *Org. Lett.* **2014**, *16*, 508-511.

[189] (a) T. Takeuchi, N. Kumagai, M. Shibasaki, *J. Org. Chem.* **2018**, *83*, 5851-5858; (b) Z. Liu, T. Takeuchi, R. Pluta, F. Arteaga Arteaga, N. Kumagai, M. Shibasaki, *Org. Lett.* **2017**, *19*, 710-713; (c) A. Matsuzawa, H. Noda, N. Kumagai, M. Shibasaki, *J. Org. Chem.* **2017**, *82*, 8304-8308; (d) K.

to very interesting results.[174,190] Nevertheless, these two methods present some drawbacks: the aldol condensations must be accomplished at very low temperatures and the epoxides must be already enantiopure.

The simplest and more direct synthetic pathway is the reduction of racemic amides under dynamic conditions. Thus, the dynamic kinetic resolution of α-substituted β-keto amides can be performed due to the high acidity of the α-hydrogen. Different chemical agents have been employed to perform efficient DKRs. For instance, boranes[191] and hydrides[192] have allowed the synthesis of racemic β-hydroxy amides with high diastereomeric excess. Ruthenium[178,193] and iridium[194] complexes have achieved not only high diastereoselectivity but also excellent enantioselectivity towards the DKR of α-substituted β-keto lactams. Additionally, a ruthenium complex has appeared as an efficient hydrogenation catalyst for the formation of acyclic α-alkylated β-hydroxy amides with good diastereo- and enantioselectivity (Scheme 4.7).[195]

Scheme 4.7. Asymmetric hydrogen transfer reaction of α-alkyl-β-keto amides mediated by a ruthenium complex.



Biocatalysis has provided a few methodologies towards the synthesis of chiral β-hidroxy amides. For instance, in the 90's Gotor and co-workers reported the aminolysis of racemic β-hydroxy esters catalysed by the lipase B from *Candida*

Weidner, Z. Sun, N. Kumagai, M. Shibasaki, *Angew. Chem. Int. Ed.* **2015**, *54*, 6236-6240; (e) K. Weidner, N. Kumagai, M. Shibasaki, *Angew. Chem. Int. Ed.* **2014**, *53*, 6150-6154; (f) B. R. Kusuma, G. E. L. Brandt, B. S. J. Blagg, *Org. Lett.* **2012**, *14*, 6242-6245; (g) J. L. Vicario, D. Badía, E. Domínguez, M. Rodríguez, L. Carrillo, *J. Org. Chem.* **2000**, *65*, 3754-3760.

[190] L. Martín-Ortiz, S. Chammaa, M. S. Pino-González, A. Sánchez-Ruiz, M. García-Castro, C. Assiego, F. Sarabia, *Tetrahedron Lett.* **2004**, *45*, 9069-9072.

[191] (a) G. Bartoli, M. Bosco, E. Marcantoni, M. Massaccesi, S. Rinaldi, L. Sambri, *Synthesis* **2004**, 3092-3096; (b) G. Bartoli, M. Bosco, R. Dalpozzo, E. Marcantoni, M. Massaccesi, S. Rinaldi, L. Sambri, *Tetrahedron Lett.* **2001**, *42*, 8811-8815.

[192] G. Bartoli, M. Bosco, E. Marcantoni, P. Melchiorre, S. Rinaldi, L. Sambri, *Synthesis* **2004**, 73-76.

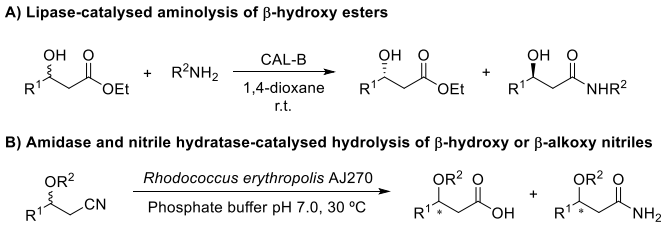
[193] D. Lynch, R. E. Deasy, L.-A. Clarke, C. N. Slattery, U. B. Rao Khandavilli, S. E. Lawrence, A. R. Maguire, N. A. Magnus, H. A. Moynihan, *Org. Lett.* **2016**, *18*, 4978-4981.

[194] D.-H. Bao, X.-S. Gu, J.-H. Xie, Q.-L. Zhou, *Org. Lett.* **2017**, *19*, 118-121.

[195] J. Limanto, S. W. Krska, B. T. Dorner, E. Vazquez, N. Yoshikawa, L. Tan, *Org. Lett.* **2010**, *3*, 512-515.

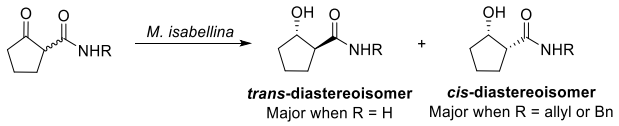
antarctica (Scheme 4.8.A).[196] The enantioenriched β-hydroxy esters and β-hydroxy amides obtained from the resolution protocol, were lately transformed into 1,3-amino alcohols. Additionally, different enantioenriched β-hydroxy amides were obtained from β-hydroxy or β-*O*-protected nitriles by the nitrile hydratase/amidase performance of *Rhodococcus erythropolis* whole cells (Scheme 4.8.B). Both protocols have provided low yields and good to excellent enantioselectivities.[134c,197]

Scheme 4.8. Enzymatic strategies towards the synthesis of enantioenriched β-hydroxy or β-alkoxy amides.



Furthermore, the reduction of β-keto amides using yeasts has provided the corresponding hydroxy amides in low yields and selectivities,[198] while the bioreduction of the same substrates catalysed by the fungus *Mortierella isabellina* delivered the products in high yields and typically high enantioselectivity.[199] Additionally, this fungus was employed in the bioreduction of 2-oxocyclopentanecarboxamides, obtaining the corresponding β-hydroxy amides in high yields, good to moderate diastereoselectivity and excellent enantioselectivity towards the formation of the mayor diastereoisomer (Scheme 4.9).[200]

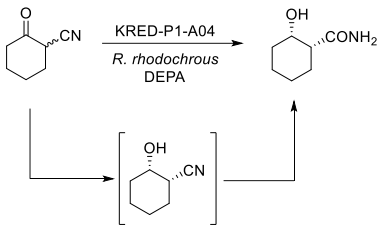
Scheme 4.9. Fungus-catalysed bioreduction of racemic 2-oxocyclopentanecarboxamides.



[196] (a) M. J. García, F. Rebolledo, V. Gotor, *Tetrahedron: Asymmetry* **1993**, *10*, 2199-2210; (b) M. J. García, F. Rebolledo, V. Gotor, *Tetrahedron: Asymmetry* **1992**, *12*, 1519-1522.
[197] D.-Y. Ma, D.-X. Wang, J. Pan, Z.-T. Huang, M.-X. Wang, *Tetrahedron: Asymmetry* **2008**, *19*, 322-329.
[198] (a) R. E. Saxon, H. Leisch, T. Hudlicky, *Tetrahedron: Asymmetry* **2008**, *19*, 672-681; (b) N. Athanasiou, A. J. Smallridge, M. A. Trewhella, *J. Mol. Catal. B: Enzym.* **2001**, *11*, 893-896; (c) T. Hudlicky, G. Gillman, C. Andersen, *Tetrahedron: Asymmetry* **1992**, *2*, 281-286.
[199] M. Quirós, F. Rebolledo, R. Liz, V. Gotor, *Tetrahedron: Asymmetry* **1997**, *18*, 3035-3038.
[200] M. Quirós, F. Rebolledo, V. Gotor, *Tetrahedron: Asymmetry* **1999**, *10*, 473-486.

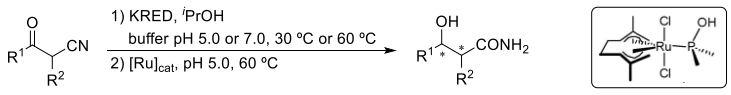
Very recently, the synthesis of (1*R*,2*S*)-2-hydroxycyclohexanecarboxamide has been described through a biocascade involving the ADH-catalysed bioreduction of the corresponding β-keto nitrile and the subsequent transformation of the β-hydroxy nitrile intermediate into the β-hydroxy amide by using whole cells of *Rhodococcus rhodochrous* in the presence of diethyl phosphoramidate (DEPA) as amidase inhibitor, which allows to stop the reaction with the amide formation and avoiding its further hydrolysis to the corresponding carboxylic acid (Scheme 4.10).[201]

Scheme 4.10. Biocascade towards the synthesis of (1*R*,2*S*)-2-hydroxycyclohexanecarboxamide.



Finally, a similar approach was developed using alcohol dehydrogenases in combination with a ruthenium catalyst (Scheme 4.11).[202] Thus, the sequential protocol allowed the synthesis of (α-substituted) β-hydroxy nitriles that, in a second step, were transformed into the corresponding (α-substituted) β-hydroxy amides. This process led to the formation of the desired amides in high yields and excellent enantio- and/or diastereoselectivity.

Scheme 4.11. Sequential chemoenzymatic protocol towards the synthesis of enantio- and/or diastereoenriched (α-substituted) β-hydroxy amides.



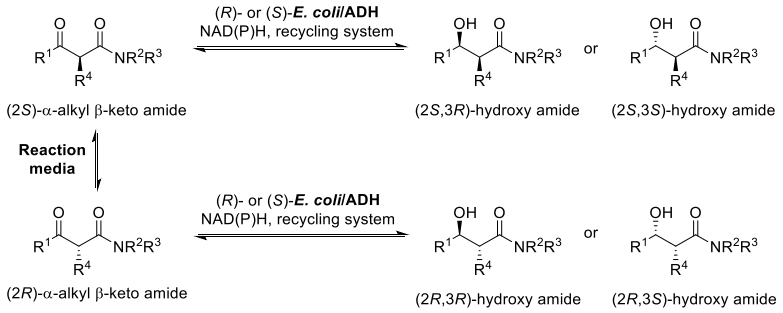
Due to the lack of general enzymatic methods to produce optically active β-hydroxy amides, we decided to take advantage of the applicability of ADHs to carry out DYRKR of (α-substituted) β-keto esters to try to expand their synthetic potential to the dynamic bioreduction of (α-substituted) β-keto amides under mild reaction conditions.

[201] E. Liardo, N. Ríos-Lombardía, F. Morís, J. González-Sabín, F. Rebolledo, *Org. Lett.* **2016**, *18*, 3366-3369.
[202] E. Liardo, R. González-Fernández, N. Ríos-Lombardía, F. Morís, J. García-Álvarez, V. Cadierno, P. Crochet, F. Rebolledo, J. González-Sabín, *ChemCatChem* **2018**, *10*, 4676-4682.

4.2. Discussion

The chemoenzymatic synthesis of diastereo- and enantioenriched α-alkyl-β-hydroxy amides has been developed using alcohol dehydrogenases from different bacteria sources overexpressed in *E. coli via* dynamic reduction. For this purpose, the chemical synthesis of a wide panel of α-alkyl-β-keto amides was firstly developed. Later on, the suitability of these compounds as substrates for the ADH-catalysed DKR transformations was studied. As shown in Scheme 4.12, all four diastereoisomers could be achieved depending on the ketoreductase selectivity.

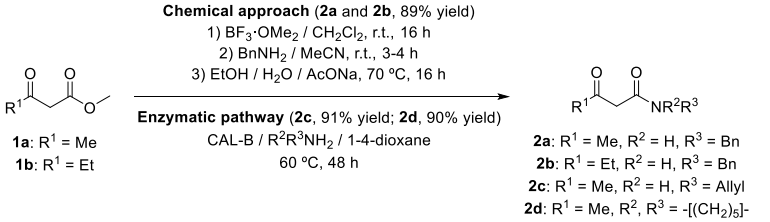
Scheme 4.12. ADH-catalysed DYRKR to obtain diastereo- and enantioenriched α-alkyl-β-hydroxy amides.



4.2.1. Synthesis of β-keto amides 2a-d, α-alkyl-β-keto amides 3a-h and the corresponding racemic hydroxy amides 4a-d and 5a-h

The synthesis of β-keto amides differing in the amide moiety and the substitution pattern at the α-position was the starting point in our research. For this purpose, we made use of the chemical strategies already described in Chapter 3 (Schemes 3.14 and 3.15).

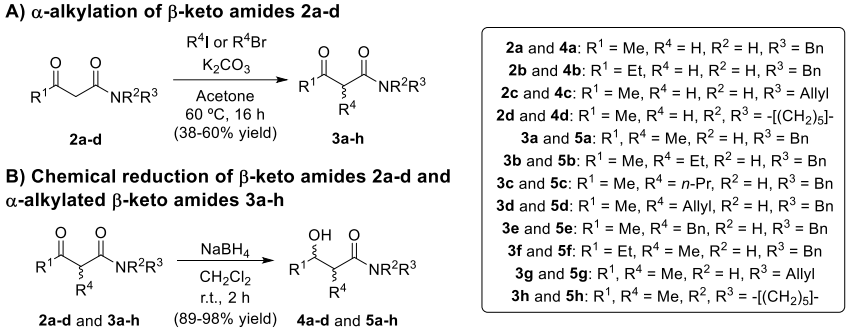
Scheme 4.13. Synthesis of β-keto amides **2a-d** using the commercially available β-keto esters **1a** and **1b** as starting materials.



Therefore, β-keto amides **2a-d** were synthesised through a chemical[159] or an enzymatic approach[158] using the commercially available β-keto esters **1a** and **1b** as starting materials (Scheme 4.13). Thus, all β-keto amides were obtained in high yields after purification by column chromatography (89-91%).

Later on, the β-keto amides were alkylated at the α-position following the same procedure already described in Chapter 3 (Scheme 3.15), this is, by treatment with different alkyl halides in basic medium using acetone as solvent (Scheme 4.14.A). After purification by column chromatography α-alkylated β-keto amides **3a-h** were obtained in low to moderate yields (38-60%).

Scheme 4.14. (A) Synthesis of racemic α-substituted β-keto amides **3a-h**. **(B)** Chemical reduction of β-keto amides **2a-d** and α-alkyl-β-keto amides **3a-h** into the corresponding racemic β-hydroxy amides **4a-d** and **5a-h**.



Afterwards, the racemic β-hydroxy amides **4a-d** and α-alkyl-β-hydroxy amides **5a-h** that would serve as GC and HPLC standards were synthesised by addition of 1 equiv of sodium borohydride to a solution of the corresponding keto amides **2a-d** and **3a-h** in dry dichloromethane (Scheme 4.14.B). Reactions were stirred at room temperature for two hours until no starting material was detected and the products were obtained after liquid-liquid extraction in high yields (89-98%) and excellent purity.

4.2.2. Testing the suitability of β-keto amides 2a-d as substrates for overexpressed ketoreductases

Before attempting the DKR of the α-alkylated substrates, different ADHs were tested towards the bioreduction of β-keto amides **2a-d**. Therefore, (*S*)-selective

ADHs from *Rhodococcus ruber* (ADH-A),[203] *Thermoanaerobacter ethanolicus* (TeSADH),[204] *Thermoanaerobacter* sp. (ADH-T),[205] *Sphingobium yanoikuyae* (SyADH),[206] *Ralstonia* sp. (RasADH),[207] and the (*R*)-selective ADHs from *Lactobacillus brevis* (LbADH)[208] and the commercially available evo-1.1.200 were screened. Thus, substrate **2a-d** (25 mM) was added into a reaction mixture containing Tris·HCl 50 mM pH 7.5 and NAD(P)H (1 mM). DMSO was used as cosolvent in all cases using a 2.5% v/v ratio and either large amounts of isopropanol (^{i}PrOH) or the glucose/GDH system were employed to regenerate the cofactor. All reactions were incubated at 30 ºC for 24 hours (Table 4.1). After this time, ADH-A and ADH-T revealed good results with all substrates, leading to the synthesis of the (*S*)-hydroxy amides with high conversions and *ee* values (entries 1, 3, 8, 10, 15, 17, 22 and 24, 96->99% conv. and 96->99% *ee*). (*S*)-Selective TeSADH, that achieved encouraging results with substrate **2a** (entry 2, 89% conv. and >99% *ee*), mostly lost its activity with the other substrates (entries 9, 16 and 23). Additionally, the commercial evo-1.1.200 only led to the formation of (*R*)-**4d** with a moderate conversion value (entry 28, 40% conv. and >99% *ee*) but it fully converted **2a**, **2b** and **2c** into the enantiopure (*R*)-hydroxy amides **4a-c** (entries 7, 14 and 21, >99% conv. and >99% *ee*).

From these results, it was rather clear that even though these substrates were suitable for some ADHs (ADH-A and ADH-T), the amide moiety and the substitution pattern in R^1 had an important influence in some ADHs behaviour. For this reason, the next step in our research project was the study of the influence that the substitution pattern at the α-position had in the bioreduction. Then, ADH-catalysed DKR transformations were attempted.

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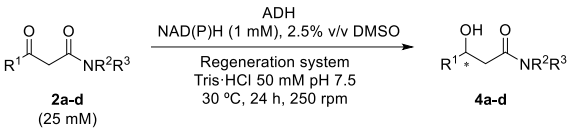
[203] W. Stampfer, B. Kosjek, C. Moitzi, W. Kroutil, K. Faber, *Angew. Chem. Int. Ed.* **2002**, *41*, 1014-1017.
[204] C. Heiss, M. Laivenieks, J. G. Zeikus, R. S. Phillips, *Bioorg. Med. Chem.* **2001**, *7*, 1659-1666.
[205] Z. Findrik, D. Vasić-Racki, S. Lütz, T. Daussmann, C. Wandrey, *Biotechnol. Lett.* **2005**, *27*, 1087-1095.
[206] (a) I. Lavandera, G. Oberdofer, J. Gross, S. de Wildeman, W. Kroutil, *Eur. J. Org. Chem.* **2008**, 2539-2543; (b) I. Lavandera, A. Kern, V. Resch, B. Ferreira-Silva, A. Glieder, W. M. F. Fabian, S. de Wildeman, W. Kroutil, *Org. Lett.* **2008**, *11*, 2155-2158.
[207] (a) I. Lavandera, A. Kern, B. Ferreira-Silva, A. Glieder, S. de Wildeman, W. Kroutil, *J. Org. Chem.* **2008**, *15*, 6003-6005; (b) H. Man, K. Kędziora, J. Kulig, A. Frank, I. Lavandera, V. Gotor-Fernández, D. Röther, S. Hart, J. P. Turkerburg, G. Grogan, *Top. Catal.* **2014**, *5*, 356-365.
[208] (a) M. Wolberg, W. Hummel, C. Wandrey, M. Müller, *Angew. Chem. Int. Ed.* **2000**, *39*, 4306-4308; (b) S. Leuchs, L. Greiner, *Chem. Biochem. Eng. Q.* **2011**, *2*, 267-281.

Table 4.1. ADH-catalysed bioreduction of β-keto amides **2a-d**.[a]



Entry	Substrate	R^1	R^2	R^3	ADH	*c* (%)[b]	*ee* (%)[c]
1	**2a**	Me	H	Bn	ADH-A	>99	96 (*S*)
2	**2a**	Me	H	Bn	TeSADH	89	>99 (*S*)
3	**2a**	Me	H	Bn	ADH-T	>99	>99 (*S*)
4	**2a**	Me	H	Bn	SyADH	<1	n.d.
5	**2a**	Me	H	Bn	RasADH	73	10 (*S*)
6	**2a**	Me	H	Bn	LbADH	18	84 (*R*)
7	**2a**	Me	H	Bn	evo-1.1.200	>99	>99 (*R*)
8	**2b**	Et	H	Bn	ADH-A	96	96 (*S*)
9	**2b**	Et	H	Bn	TeSADH	2	n.d.
10	**2b**	Et	H	Bn	ADH-T	96	>99 (*S*)
11	**2b**	Et	H	Bn	SyADH	<1	n.d.
12	**2b**	Et	H	Bn	RasADH	51	5 (*S*)
13	**2b**	Et	H	Bn	LbADH	<1	n.d.
14	**2b**	Et	H	Bn	evo-1.1.200	>99	>99 (*R*)
15	**2c**	Me	H	Allyl	ADH-A	>99	>99 (*S*)
16	**2c**	Me	H	Allyl	TeSADH	7	n.d.
17	**2c**	Me	H	Allyl	ADH-T	>99	>99 (*S*)
18	**2c**	Me	H	Allyl	SyADH	<1	n.d.
19	**2c**	Me	H	Allyl	RasADH	33	40 (*S*)
20	**2c**	Me	H	Allyl	LbADH	8	n.d.
21	**2c**	Me	H	Allyl	evo-1.1.200	>99	>99 (*R*)
22	**2d**	Me	-[$(CH_2)_5$]-		ADH-A	>99	>99 (*S*)
23	**2d**	Me	-[$(CH_2)_5$]-		TeSADH	<1	n.d.
24	**2d**	Me	-[$(CH_2)_5$]-		ADH-T	>99	>99 (*S*)
25	**2d**	Me	-[$(CH_2)_5$]-		SyADH	<1	n.d.
26	**2d**	Me	-[$(CH_2)_5$]-		RasADH	15	60 (*S*)
27	**2d**	Me	-[$(CH_2)_5$]-		LbADH	42	95 (*R*)
28	**2d**	Me	-[$(CH_2)_5$]-		evo-1.1.200	40	>99 (*R*)

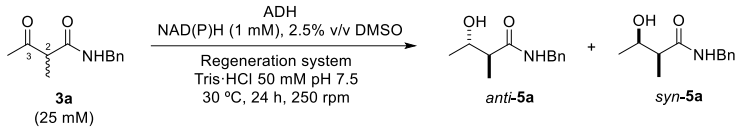
[a] Reaction conditions: substrate **2a-d** (25 mM), ADH (15 mg made-in-house ADH or 50 µL commercial stock of evo-1.1.200), DMSO (2.5% v/v), NAD(P)H (1 mM), $MgSO_4 \cdot 6H_2O$ (1 mM, LbADH and evo-1.1.200), isopropanol (5% v/v, ADH-A, TeSADH, ADH-T, SyADH, LbADH, evo-1.1.200), glucose (50 mM, RasADH), glucose dehydrogenase (5 U, RasADH), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24 h, 250 rpm. [b] Measured by GC. [c] Measured by HPLC.

4.2.3. ADH-catalysed DKR of model substrate 3a. Influence of the α-substitution (substrates 3a-e)

The α-methylated β-keto amide **3a** was chosen as model substrate for our research, starting with the bioreduction of **3a** (25 mM) using DMSO as cosolvent.

The reaction mixture (Tris·HCl 50 mM pH 7.5) was implemented with NAD(P)H and the corresponding regeneration system. Finally, made-in-house ADH or the commercial evo-1.1-200 were added and the reactions were incubated at 30 ºC for 24 hours (Table 4.2).

Table 4.2. Bioreduction of model substrate **3a** to produce the corresponding diastereo- and enantioenriched alcohol **5a**.[a]



Entry	ADH	*c* (%)[b]	*de* (%)[c,d]	*ee anti* (%)[c]	*ee syn* (%)[c]
1	ADH-A	>99	90	n.d.	99 (2*R*,3*S*)
2	TeSADH	<1	n.d.	n.d.	n.d.
3	ADH-T	30	n.d.	n.d.	n.d.
4	SyADH	<1	n.d.	n.d.	n.d.
5	RasADH	>99	30	28 (2*S*,3*S*)	30 (2*R*,3*S*)
6	LbADH	6	n.d.	n.d.	n.d.
7	evo-1.1.200	>99	94	n.d	>99 (2*S*,3*R*)

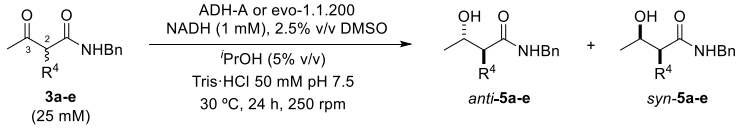
[a] Reaction conditions: substrate **3a** (25 mM), ADH (15 mg made-in-house ADH or 50 µL commercial stock of evo-1.1.200), DMSO (2.5% v/v), NAD(P)H (1 mM), $MgSO_4 \cdot 6H_2O$ (1 mM, LbADH and evo-1.1.200), isopropanol (5% v/v, ADH-A, TeSADH, ADH-T, SyADH, LbADH, evo-1.1.200), glucose (50 mM, RasADH), glucose dehydrogenase (5 U, RasADH), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24 h, 250 rpm. [b] Measured by GC. [c] Measured by HPLC. [d] ADH-A and evo-1.1.200 produced preferentially the *syn*-diastereoisomer, while RasADH (entry 5) led preferentially to the formation of the *anti*-diastereoisomer.

On the one hand, it was observed that full conversion was achieved when using ADH-A (entry 1), RasADH (entry 5) or evo-1.1.200 (entry 7). Remarkably, ADH-A and evo-1.1.200 led to very high diastereo- and enantioselectivity (entries 1 and 7), obtaining in both cases the *syn* diastereoisomer as the major one. Overall, (2*R*,3*S*)-**5a** was obtained with 90% *de* and 99% *ee* using ADH-A as biocatalyst (entry 1) and evo-1.1.200 led to the formation of (2*S*,3*R*)-**5a** with 94% *de* and >99% *ee* (entry 7). On the other hand, even though RasADH displayed good results in terms of conversion, it could only achieve the synthesis of the *anti*-diastereoisomer (2*S*,3*S*)-**5a** with 30% *de* and 28% *ee* (entry 5).

After this preliminary screening with keto amide **3a**, the influence of different alkyl chains in the α-position was studied. For this purpose, the bioreduction of substrates **3b-e** into the corresponding hydroxy amides **5a-e** was attempted, finding

remarkable results only with ADH-A and evo-1.1.200 (Table 4.3). As observed with the model substrate **3a**, both enzymes favoured the formation of the *syn*-product. Therefore, (2*R*,3*S*)-hydroxy amides were obtained with ADH-A in high conversions (78->99% conv, entries 1-5) and moderate to high diastereoselectivity (72-92% *de*) and very high enantioselectivity (99% *ee*). Additionally, (2*S*,3*R*)-**5b-d** were synthesised using evo-1.1.200 in moderate to high conversion values (60->99% conv, entries 6-9) and high diastereo- and enantioselectivity (90-94% *de* and 99->99% *ee*). However, this enzyme lost its activity towards the synthesis of the α-benzylated β-hydroxy amide **5e** (entry 10).

Table 4.3. Dynamic kinetic resolution of α-alkylated β-keto amides **3a-e** bearing different pattern substitution at the α-position.[a]



Entry	Substrate	R^4	ADH	*c* (%)[b]	*de* (%)[c,d]	*ee syn* (%)[c]
1	**3a**	Me	ADH-A	>99	90	99 (2*R*,3*S*)
2	**3b**	Et	ADH-A	>99	88	99 (2*R*,3*S*)
3	**3c**	*n*-Pr	ADH-A	99	72	99 (2*R*,3*S*)[e]
4	**3d**	Allyl	ADH-A	99	92	99 (2*R*,3*S*)
5	**3e**	Bn	ADH-A	78	78	99 (2*R*,3*S*)[e]
6	**3a**	Me	evo-1.1.200	>99	94	>99 (2*S*,3*R*)
7	**3b**	Et	evo-1.1.200	96	92	>99 (2*S*,3*R*)
8	**3c**	*n*-Pr	evo-1.1.200	60	90	99 (2*S*,3*R*)
9	**3d**	Allyl	evo-1.1.200	98	92	>99 (2*S*,3*R*)
10	**3e**	Bn	evo-1.1.200	<1	n.d.	n.d.

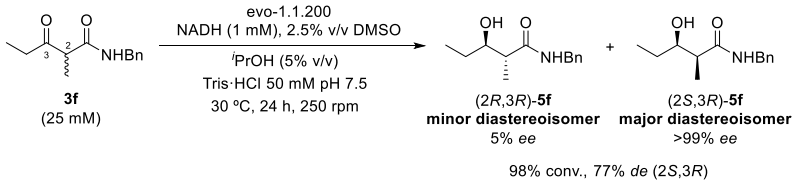
[a] Reaction conditions: substrate **3a-e** (25 mM), ADH-A (15 mg) or evo-1.1.200 (50 µL commercial stock), DMSO (2.5% v/v), NADH (1 mM), $MgSO_4 \cdot 6H_2O$ (1 mM, evo-1.1.200), isopropanol (5% v/v), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24 h, 250 rpm. [b] Measured by GC.
[c] Measured by HPLC. [d] Both ADH-A and evo-1.1.200 led preferentially to the formation of the *syn*-diastereoisomer. [e] Enantiomeric excess of the *anti*-diastereoisomer was also determined, finding 99% *ee* for (2*S*,3*S*)-**5c** and **5e**.

At this point, both ADH-A and evo-1.1.200 had led to satisfactory results in terms of conversion and selectivity in the DKR of α-alkyl-β-keto amides bearing different pattern substitution at the α-position, with the exception of evo-1.1.200 when **3e** was employed as substrate.

4.2.4. Influence of the R^1 length and the amide moiety. DKR of substrates 3f-h

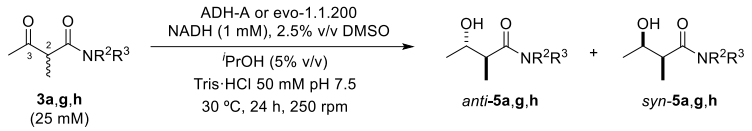
Encouraged by these results and looking for a further exploitation of the synthetic approach, we decided to undertake the ADH-catalysed DKR of substrate **3f**, bearing an ethyl group at R^1 position. Using the same reaction conditions than before, all ADHs were screened finding that only evo-1.1.200 was capable of effectively reduced **3f** (Scheme 4.15). This enzyme led to the preferential formation of β-hydroxy amide (2*S*,3*R*)-**5f** in high conversion (98%), moderate *de* (77%) and excellent *ee* values (>99%).

Scheme 4.15. DKR of α-methylated β-keto amide **3f** catalysed by evo-1.1.200.



At this stage, only the study of the influence that different amide moieties had in the process was left. For this reason, the bioreduction protocol was set up using the allyl and piperidinyl substrates **3g** and **3h** (Table 4.4). On the one hand, as previously observed, benzyl substrate **3a** was a suitable substrate for ADH-A and evo-1.1.200, being the *syn*-hydroxy amide the major product in both cases (entries 1 and 4). (2*R*,3*S*)-**5g** and (2*S*,3*R*)-**5g** were obtained with high conversion and selectivity (99% conv., 82% *de* and 99% *ee* and 98% conv., 86% *de* and >99% *ee*, entries 2 and 5). On the other hand, **3h** was transformed in high conversion, low *de* and high enantioselectivity towards the formation of (2*R*,3*S*)-**5h** (96% conv, 12% *de* and 99% *ee*, entry 3) when using ADH-A as biocatalyst, observing a dramatic loss of activity with evo-1.1.200 (entry 6).

Table 4.4. Influence of the amide moiety in the ADH-catalysed DKR of α-methyl-β-keto amides **3a**, **3g** and **3h**.[a]



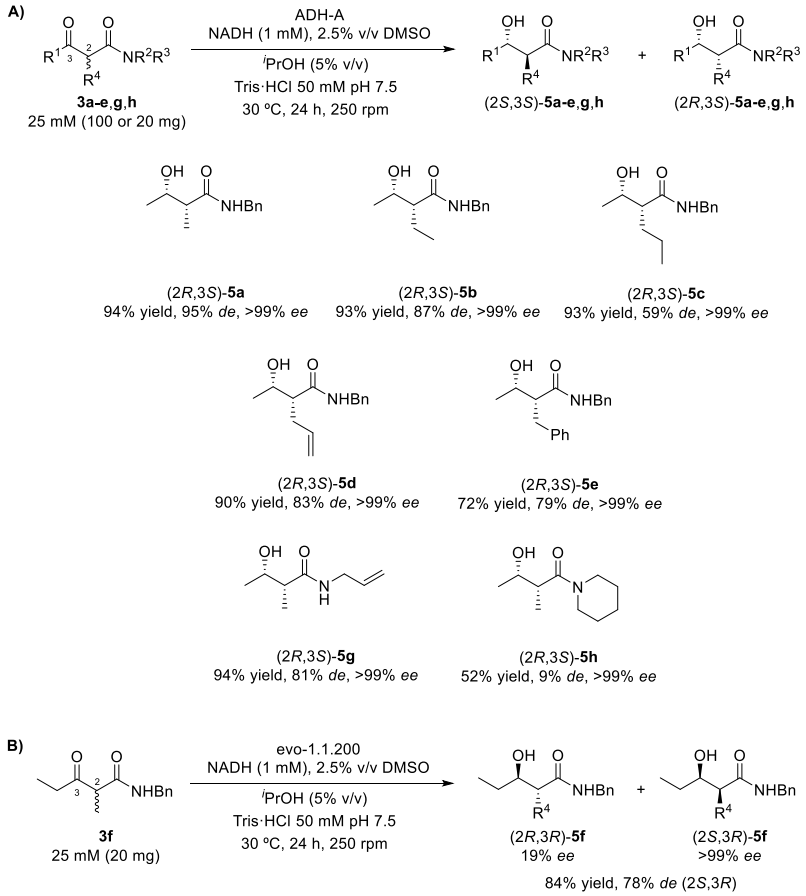
Entry	Substrate	R^2	R^3	ADH	*c* (%)[b]	*de* (%)[c,d]	*ee syn* (%)[c]
1	**3a**	H	Bn	ADH-A	>99	90	97 (2*R*,3*S*)
2	**3g**	H	Allyl	ADH-A	99	82	99 (2*R*,3*S*)
3	**3h**	-[$(CH_2)_5$]-		ADH-A	96	12	99 (2*R*,3*S*)[e]
4	**3a**	H	Bn	evo-1.1.200	>99	94	>99 (2*S*,3*R*)
5	**3g**	H	Allyl	evo-1.1.200	98	86	>99 (2*S*,3*R*)
6	**3h**	-[$(CH_2)_5$]-		evo-1.1.200	8	n.d.	n.d.

[a] Reaction conditions: substrate **3a**, **3h** or **3g** (25 mM), ADH-A (15 mg) or evo-1.1.200 (50 µL commercial stock), DMSO (2.5% v/v), NADH (1 mM), $MgSO_4 \cdot 6H_2O$ (1 mM, evo-1.1.200), isopropanol (5% v/v), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24 h, 250 rpm. [b] Measured by GC. [c] Measured by HPLC. [d] Both ADH-A and evo-1.1.200 led preferentially to the formation of the *syn*-diastereoisomer. [e] Enantiomeric excess of the *anti*-diastereoisomer was also determined, finding 99% *ee* for (2*S*,3*S*)-**5g**.

4.2.5. Semi-preparative bioreduction of α-alkyl-β-keto amides 3a-h

In order to demonstrate the applicability of the method, we decided to scale-up the ADH-catalysed DKR transformations. For this purpose, ADH-A was the enzyme of choice as it had demonstrated to be the most efficient ketoreductase, providing good or excellent results in the bioreduction of benzylic substrates **3a-e**, allylic **3g** and piperidinyl **3h**. Thus, 100 mg of model substrate **3a** and 20 mg of the other substrates (**3b-e**, **3g** and **3h**) were transformed into the corresponding enantiopure hydroxy amides. After 24 hours, similar results to those obtained at analytical scale were found (Scheme 4.16.A). Therefore, the *syn*-(2*R*,3*S*)-diastereoisomer of each alcohol was obtained as the major one in moderate to high yields (52-94%) and excellent enantioselectivity (>99% *ee*). The diastereoselectivity of ADH-A remained high with the exception of substrates **3c** (59% *de*) and, especially, **3h** (9% *de*).

Scheme 4.16. A) Semi-preparative DKR of α-alkyl-β-keto amides **3a-e**, **3g** and **3h** catalysed by ADH-A. **B)** Semi-preparative bioreduction of β-keto amide **3f** catalysed by evo-1.1.200.

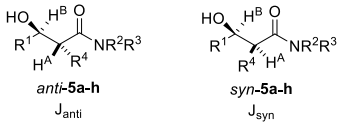


Additionally, in order to fully characterised the *N*-benzyl-3-hydroxy-2-methylpentanamide (**5f**), 20 mg of the corresponding ketone **3f** were reduced using evo-1.1.200 in Tris·HCl 50 mM buffer pH 7.5 (Scheme 4.16.B). 2.5% v/v of DMSO was used as co-solvent and isopropanol (5% v/v) was added to regenerate the cofactor (NADH, 1 mM). Again, after 24 hours, we found similar results to those obtained at analytical scale.

All the so-obtained hydroxy amides were fully characterised by ^{1}H, ^{13}C and DEPT-NMR experiments, IR and HR-MS. Furthermore, homodecoupling NMR experiments were performed and the coupling constants between H^{A} and H^{B} were

calculated in order to determine which diastereoisomer was the major one (Table 4.5).

Table 4.5. Comparison of coupling constants between H^A and H^B obtained for racemic hydroxy amides.



	5a	**5b**	**5c**	**5d**	**5e**	**5f**	**5g**	**5h**
J_{anti} (Hz)	5.8	5.7	6.8	4.4	6.0	5.4	5.9	6.0
J_{syn} (Hz)	3.1	3.4	3.2	3.6	2.7	2.4	3.0	2.0

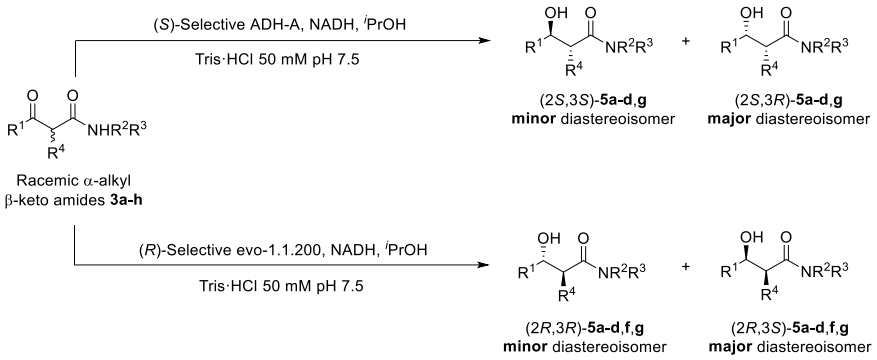
Comparison between ^{1}H-NMR experiments of **5a-h** obtained from bioreduction and chemical reduction using sodium borohydride, confirmed that the *syn*-diastereoisomer was obtained as the major one in the DYRKR protocol. This result was confirmed with the method showed by Kalaitzakis and Smonou with α-alkyl-β-hydroxy carbonyl compounds,[209] together with the measured $^3J_{HAHB}$ for similar derivatives[195] and the known diastereopreference with the same enzymes with α-alkyl-β-keto esters.[173] The absolute configuration was determined due to the known stereospecificity of these ADHs.[203-208]

4.2.6. Final remarks

Different α-alkyl-β-keto amides **3a-h** have been transformed into the corresponding diastereo- and enantioenriched hydroxy amides through a DKR protocol catalysed by ketoreductases. The (*S*)-selective ADH-A from *R. ruber* and the commercial (*R*)-selective evo-1.1.200 have been found as robust enzymes, which were capable of producing the *syn*-products in a highly selective manner (Scheme 4.17). Especially ADH-A led to good results, being applied in semi-preparative DKR transformations. This way, it was possible to synthesise and characterise a wide panel of enantiopure and diastereoenriched α-alkyl-β-hydroxy amides in moderate to high yields. These results have proven the reproducibility and applicability of this method.

[209] D. Kalaitzakis, I. Smonou, *J. Org. Chem.* **2008**, *73*, 3919-3921.

Scheme 4.17. Overview of the ADH-catalysed DKR of α-alkyl-β-keto amides.



4.3. Experimental section

4.3.1. General information

Chemical reagents were purchased from different commercial sources and used without further purification. The solvents employed, dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), diethyl ether (Et_2O), hexane (Hex), dimethylsulfoxide (DMSO), acetonitrile (MeCN) and isopropanol (^{i}PrOH) were employed without previous drying. 1,4-Dioxane, methanol (MeOH) and acetone were previously distilled and dried over sodium, calcium hydride and calcium sulphate, respectively. β-Keto amides **2a-b**,**d** and **3a-f**,**h** have been synthesised as described in Chapter 3 (Sections 3.3.2 and 3.3.3). Likewise, β-keto amide **2c** was obtained in 91% yield using the enzymatic protocol described in Section 3.3.2.2.[158] Afterwards, the alkylation protocol described in Section 3.3.3 was employed to obtain the α-methylated β-keto amide **3g** in moderate yield (51%).

Alcohol dehydrogenases from *Rhodococus ruber* (ADH-A),[203] *Thermoanaerobacter ethanolicus* (TeSADH),[204] *Thermoanaerobacter* sp. (ADH-T),[205] *Sphingobium yanoikuyae* (SyADH),[206] *Ralstonia* sp. (RasADH)[207] and *Lactobacillus brevis* (LbADH)[208] overexpressed in *E. coli* cells were obtained as previously described in the literature and used as lyophilised preparations. Evo-1.1.200 was acquired from evoxx technologies GmbH.

4.3.2. Synthesis of racemic β-hydroxy amides 4a-d and α-alkyl β-hydroxy amides 5a-h

Sodium borohydride (38 mg, 1 mmol) was carefully added to a solution of racemic keto amides **2a-d** and **3a-h** (1 mmol, 0.1 M) in dry CH_2Cl_2 (10 mL) under N_2 atmosphere. The mixture was stirred for 2 hours until no starting material was detected by TLC analysis. Then, the reaction was quenched with H_2O (10 mL) and extracted with CH_2Cl_2 (3 x 10 mL). Afterwards, the organic layers were collected, dried over Na_2SO_4, filtered and the solvent evaporated under reduced pressure. This procedure afforded the corresponding racemic β-hydroxy amides **4a-d** and **5a-h** in excellent yields (89-98% yields).

4.3.3. General procedure for the bioreduction of β-keto amides 2a-d and α-alkyl-β-keto amides 3a-h using ADH-A overexpressed in *E. coli*

Lyophilised *E. coli*/ADH-A cells (15 mg), 2.5% v/v DMSO (15 µL), 1 mM NADH (60 µL of a 10 mM stock solution) and ^{i}PrOH (30 µL) were successively added into an Eppendorf tube containing the β-keto amide (**2a-d**, 25 mM) or α-substituted β-keto amide (**3a-h**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5 (540 µL). The reaction was shaken at 30 ºC and 250 rpm for 24 h. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into β-hydroxy amides **4a-d** and α-alkyl-β-keto amides **5a-h** were determined by GC, and *de* and *ee* values were measured by HPLC.

4.3.4. General procedure for the bioreduction of β-keto amides 2a-d and α-alkyl-β-keto amides 3a-h using RasADH overexpressed in *E. coli*

Lyophilised *E. coli*/RasADH cells (15 mg), 2.5% v/v DMSO (15 µL), 1 mM NADPH (60 µL of a 10 mM stock solution), 50 mM glucose (60 µL of a 500 mM stock solution) and glucose dehydrogenase (60 µL, 3 U) were added into an Eppendorf tube containing the corresponding β-keto amide (**2a-d** or **3a-h**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5 (420 µL). The reaction was shaken at 30 ºC and 250 rpm for 24 h. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into β-hydroxy amides **4a-d** and α-alkyl-β-hydroxy amides **5a-h** were determined by GC, and *de* and *ee* values were measured by HPLC.

4.3.5. General procedure for the bioreduction of β-keto amides 2a-d and α-alkyl-β-keto amides 3a-h using LbADH overexpressed in *E. coli*

Lyophilised *E. coli*/LbADH cells (15 mg), 2.5% v/v DMSO (15 µL), 1 mM NADPH (60 µL of a 10 mM stock solution), 1 mM $MgCl_2$ (60 µL of a 10 mM stock solution) and ^{i}PrOH (30 µL) were added into an Eppendorf tube containing the β-keto amide (**2a-d**, 25 mM) or α-substituted β-keto amide (**3a-h**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5 (480 µL). The reaction was shaken at 30 ºC and 250 rpm for 24 h. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into β-hydroxy amides **4a-d** and α-

alkyl-β-keto amides **5a-h** were determined by GC, and *de* and *ee* values were measured by HPLC.

4.3.6. General procedure for the bioreduction of β-keto amides 2a-d and α-alkyl-β-keto amides 3a-h using SyADH, TeSADH or ADH-T overexpressed in *E. coli*

Lyophilised *E. coli*/SyADH, *E. coli*/TeSADH or *E. coli*/ADH-T cells (15 mg), 2.5% v/v DMSO (15 µL), 1 mM NADPH (60 µL of a 10 mM stock solution), and ^{i}PrOH (30 µL) were added into an Eppendorf tube containing the corresponding β-keto amide (**2a-d** or **3a-h**, 25 mM) in Tris·HCl 50 mM pH 7.5 (540 µL). The reaction was shaken at 30 ºC and 250 rpm for 24 h. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into β-hydroxy amides **4a-d** and **5a-h** were determined by GC, and *de* and *ee* values were measured by HPLC.

4.3.7. General procedure for the bioreduction of β-keto amides 2a-d and α-alkyl-β-keto amides 3a-h using evo-1.1.200

50 µL of commercial evo-1.1.200 stock were added in an Eppendorf tube containing the corresponding β-keto amide (**2a-d** or **3a-h**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5 (312 µL). The reaction media was implemented with 2.5% v/v DMSO (12.5 µL), 1 mM NADH (50 µL of 10 mM stock solution), 1 mM $MgCl_2$ (50 µL of a 10 mM stock solution) and isopropanol (25 µL). The reaction was shaken at 30 ºC and 250 rpm for 24 h. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversions into β-hydroxy amides **4a-d** and **5a-h** were determined by GC, and *de* and *ee* values were measured by HPLC.

4.3.8. Semi-preparative bioreduction of α-alkyl-β-keto amides 3a-e, 3g and 3h using the alcohol dehydrogenase from *Rhodococcus ruber* (ADH-A)

Lyophilised *E. coli*/ADH-A cells (100 mg for β-keto amide **3a** and 50 mg for β-keto amides **3b-e**, **3g** and **3h**), DMSO (2.5% v/v), NADH (1 mM) and ^{i}PrOH (5% v/v) were successively added into an Erlenmeyer flask containing the β-keto amide (100 mg for **3a** and 20 mg for **3b-e**, **3g** and **3h**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5. The reaction was shaken at 30 ºC and 250 rpm for 24 h and then

extracted with EtOAc (3 x 15 mL). The organic layers were separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The solvent was concentrated under vacuum furnishing the α-alkyl-β-hydroxy amides **5a-e**, **5g** and **5h** in moderate to excellent isolated yields (52-94%).

4.3.9. Preparative bioreduction of α-methyl-β-keto amide 3f using the commercial alcohol dehydrogenase evo-1.1.200

β-Keto amide **3f** (20 mg, 25 mM) was added to an Erlenmeyer flask containing DMSO (2.5% v/v), $MgCl_2 \cdot 6H_2O$ (1 mM), NADH (1 mM) and ^{i}PrOH (5% v/v) in Tris·HCl buffer 50 mM pH 7.5 (final volume: 3.6 mL). Next, of lyophilised evo-1.1.200 (75 mg) was added and the reaction shaken at 30 ºC and 250 rpm for 24 h. After this time, the reaction was extracted with EtOAc (3 x 5 mL). The organic layers were separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The solvent was concentrated under vacuum, achieving the α-methyl-β-hydroxy amide **5f** in high isolated yield (17 mg, 84%).

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Advanced Synthesis & Catalysis

Synthesis of α-Alkyl-β-Hydroxy Amides through Biocatalytic Dynamic Kinetic Resolution Employing Alcohol Dehydrogenases

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Abstract: Chiral (α-substituted) β-hydroxy amides are interesting derivatives as they are useful building blocks of many biologically active compounds. Herein, the biocatalytic stereocontrolled synthesis of various acyclic *syn*-α-alkyl-β-hydroxy amides through a dynamic kinetic resolution is shown. Hence, a series of overexpressed alcohol dehydrogenases (ADHs) in *Escherichia coli* was used to reduce the corresponding racemic β-keto amides. Among them, ADH-A from *Rhodococcus ruber* and commercial evo-1.1.200 afforded the best activities and selectivities, giving access to the opposite enantiomers with high diastereomeric excess and excellent enantiomeric excess. Some of these compounds were obtained at semipreparative scale.

Keywords: Alcohol dehydrogenases; Biocatalysis; Chiral synthesis; Dynamic kinetic resolutions; β-Hydroxy amides

Chiral β-hydroxy amides are highly interesting derivatives as they are versatile and useful building blocks of different biologically active compounds. Among them, oxazolines,[1] oxazoles,[2] pyrrolidines,[3] azetidines,[4] and 1,3-amino alcohols such as fluoxetine,[5] can be mentioned. They are also present in the core structure of anticancer families like bengamides,[6] and can be utilised as ligands to induce chirality in organic transformations.[7]

Different synthetic approaches have been designed in order to synthesise these derivatives (Figure 1A). Hence, the aldol condensation between a (chiral) amide and an aldehyde[4,8] or an acylsilane[9] has been demonstrated as a valuable methodology to obtain these compounds with high enantiomeric excess (*ee*). Likewise, the hydrogenation of β-keto amide precursors employing ruthenium[10] or iridium[11] complexes, the hydroboration of unsaturated amides mediated by rhodium[12] or copper[13] complexes, the regioselective ring-opening of α,β-epoxy amides with sodium bis(2-methoxyethoxy)aluminium hydride (Red-Al),[5] the palladium-catalysed β-acyloxylation of amides,[14] and the ruthenium-mediated reduction of β-keto nitriles[15] have been shown as attractive strategies.

However, the synthesis of α-substituted β-hydroxy amides in diastereo- and enantioselective manner is more challenging due to the formation of four possible diastereoisomers. Again, various synthetic protocols have been developed to successfully get access to these compounds (Figure 1A). Aldol additions have provided good results although at the expense of using very low reaction temperatures.[16] The opening of chiral epoxides with different nucleophiles has also been demonstrated as another powerful tool. However, enantiopure synthons must be previously synthesised.[6,17] A very simple and direct method is the stereoselective reduction of the racemic α-substituted β-keto amides under dynamic conditions.[18] Since these substrates can easily racemise due to the high acidity of the α-hydrogen, a dynamic kinetic resolution (DKR)[19,20] can be achieved, thus providing in the ideal case one out of four diastereoisomer products. Various chemical agents such boranes[21] and hydrides[22] have afforded the corresponding racemic β-hydroxy amides with high diastereomeric excess (*de*), while the Ru-[3,23] and Ir-mediated[24] hydrogenation of α-substituted β-keto

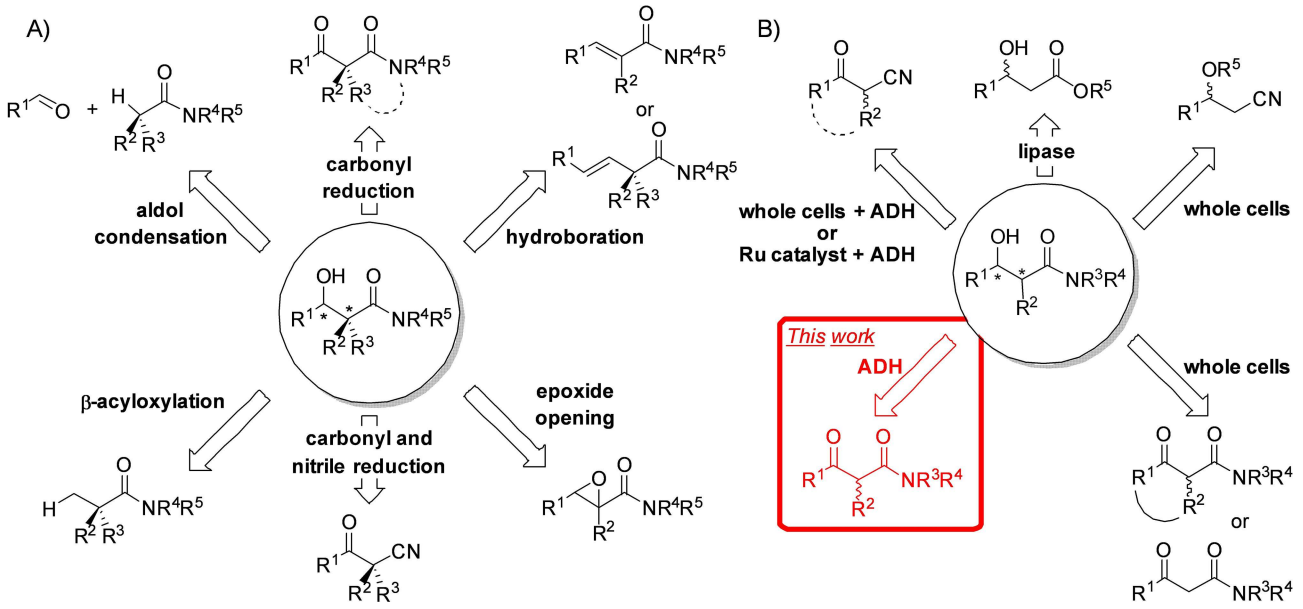


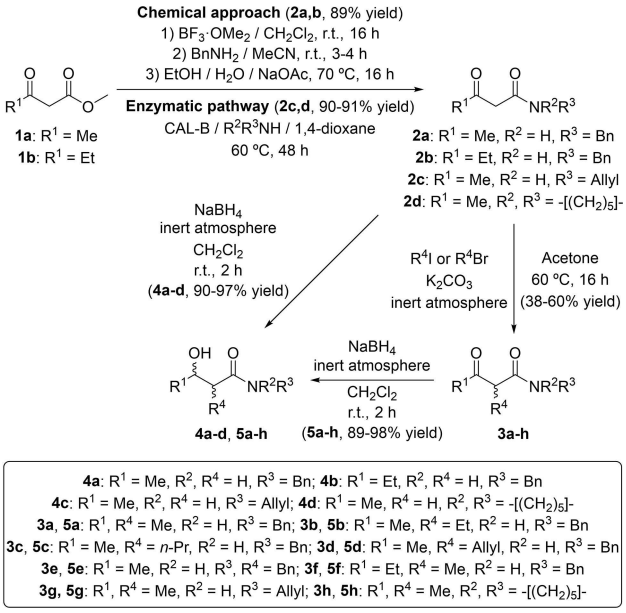
Figure 1. A) Chemical approaches; and B) (chemo)enzymatic methodologies to synthesise chiral (α-substituted) β-hydroxy amides.

lactams gave the alcohols with excellent *de* and *ee*. There is just one report demonstrating the asymmetric hydrogen transfer of acyclic substrates with a ruthenium complex, obtaining the final compounds with very high *de* and *ee* values after recrystallisation of the reaction crude.[25]

From an enzymatic point of view (Figure 1B), the kinetic resolution of racemic β-hydroxy esters through lipase-catalysed aminolysis,[26] or β-*O*-protected nitriles with *Rhodococcus erythropolis* whole cells[27] and the reduction of β-keto amides using yeasts or fungi,[28] have provided the chiral alcohols but with low yields and/or selectivities. Very recently, the combination of *Rhodococcus rhodochrous* whole cells[29] or a ruthenium catalyst[30] with alcohol dehydrogenases (ADHs)[31] delivered different cyclic β-hydroxy amides starting from the corresponding racemic β-keto nitriles through a DKR process with excellent *de* and *ee*.

While the bioreduction under dynamic conditions of α-substituted β-keto esters using whole cells or isolated ADHs has been recurrently studied,[20,32] this has not been the case for the amide analogues. Herein (Figure 1B, red), a set of α-alkyl-β-keto amides has been successfully reduced with different lyophilised preparations containing overexpressed ADHs in *E. coli* affording the corresponding β-hydroxy amides in many cases with outstanding enantio- and diastereoselectivities.

For this purpose, the chemical synthesis of a wide panel of α-substituted β-keto amides was developed (Scheme 1). They differed in the amide protecting group and the substitution pattern at α-position. We used as starting materials commercially available β-keto esters **1a** and **1b** following two independent



Scheme 1. Synthesis of (α-substituted) β-keto amides and racemic β-hydroxy amides.

synthetic methodologies in order to obtain the corresponding β-keto amides **2a–d**. Better results were found for the synthesis of N-benzylated keto amides **2a** and **2b** (89% yield) through the formation of 1,3,2-dioxaborinane intermediates,[33] while to get access to compounds **2c** (91% yield) and **2d** (90% yield) the lipase-mediated approach was preferred,[34] finding less than 20% yield when benzylamine was used as nucleophile in the biotransformations for the synthesis of **2a** and **2b**. Later on, the β-keto amides were

alkylated at α-position by treatment with different alkyl halides in basic medium using acetone as solvent.[32] After purification by column chromatography, α-alkylated β-keto amides **3a–h** were obtained in moderate yields (38–60%). Finally, the racemic β-hydroxy amides **4a–d** and α-substituted β-hydroxy amides **5a–h** were synthesised by addition of sodium borohydride to a solution of the corresponding keto amide in dry dichloromethane, obtaining the products after extraction in high yields (89–98%) and excellent purities.

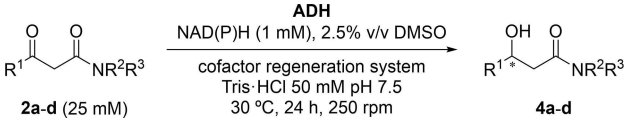
In order to study the suitability of the DKR processes with the α-substituted substrates, different ADHs were tested first towards the bioreduction of β-keto amides **2a–d**. Thus, the effect of the amide protecting group could be considered. This way, lyophilised *E. coli* preparations containing overexpressed (*S*)-selective ADHs from *Rhodococcus ruber* (ADH-A),[35] *Thermoanaerobacter ethanolicus* (TeSADH),[36] *Thermoanaerobium sp.* (ADH-T),[37] *Sphingobium yanoikuyae* (SyADH),[38] *Ralstonia sp.* (RasADH),[39] and the (*R*)-selective ADH from *Lactobacillus brevis* (LbADH)[40] were screened. Also, commercially available evo-1.1.200[41] was studied.

The bioreductions were performed at 25 mM concentration of the substrate in a reaction mixture containing Tris·HCl 50 mM pH 7.5 and 1 mM of the nicotinamide cofactor NAD(P)H. DMSO was used as cosolvent in all cases in a 2.5% v/v ratio, and either large amounts of 2-propanol (^{i}PrOH) or the glucose/GDH system were employed to regenerate the cofactor. All reactions were incubated at 30 °C for 24 hours (Table 1). After this time, ADH-A and ADH-T revealed good results with all substrates, leading to the synthesis of the (*S*)-alcohols with high conversions and *ee* values (entries 1, 2, 4, 5, 7, 8, 10 and 11). Furthermore, the commercial evo-1.1.200 fully converted **2a–c** into the enantiopure (*R*)-alcohols **4a–c** (entries 3, 6 and 9). However, it led to the formation of enantiopure (*R*)-**4d** with a moderate conversion value (entry 12). The other ADHs afforded worse results (see SI, Table S1).

From these results, it was clear that even though these substrates were suitable for some ADHs, the amide moiety (R^2 and R^3) and the substitution pattern in R^1 had an important influence in some ADHs behaviour. For this reason, the next step was to study the influence that the substitution pattern at the α-position had in the bioreduction, and also to look if DKR transformations were possible.

The α-methylated β-keto amide **3a** was chosen as model substrate (Table 2 and Table S2 in the Supporting Information). Under the same reaction conditions previously described, it was observed that full conversion into **5a** was achieved when using ADH-A (entry 1), RasADH (entry 2) or evo-1.1.200 (entry 3). On the one hand, ADH-A and evo-1.1.200 led to very high diastereo- and enantioselectivity, obtaining in both cases the *syn* diastereoisomer as the major one. This way, (2*R*,3*S*)-**5a** was obtained with 90% *de* and 99% *ee* using ADH-A as biocatalyst and evo-1.1.200 led to the formation of (2*S*,3*R*)-**5a** with 94% *de* and >99% *ee*. On the other hand, even though RasADH showed good results in terms of conversion, it achieved the synthesis of the *anti*-diastereoisomer (2*S*,3*S*)-**5a** but with only 30% *de* and 28% *ee*.

Table 1. ADH-catalysed bioreduction of β-keto amides **2a–d**.[a]



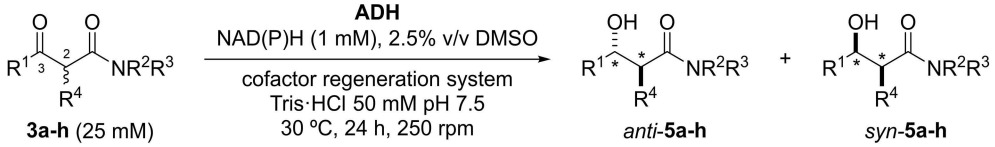
Entry	Substrate	ADH	*c* [%][b]	*ee* [%][c]
1	**2a**	ADH-A	>99	96 (*S*)
2	**2a**	ADH-T	>99	>99 (*S*)
3	**2a**	evo-1.1.200	>99	>99 (*R*)
4	**2b**	ADH-A	96	96 (*S*)
5	**2b**	ADH-T	96	>99 (*S*)
6	**2b**	evo-1.1.200	>99	>99 (*R*)
7	**2c**	ADH-A	>99	>99 (*S*)
8	**2c**	ADH-T	>99	>99 (*S*)
9	**2c**	evo-1.1.200	>99	>99 (*R*)
10	**2d**	ADH-A	>99	>99 (*S*)
11	**2d**	ADH-T	>99	>99 (*S*)
12	**2d**	evo-1.1.200	40	>99 (*R*)

[a] For reaction conditions and the complete set of data, see the Supporting Information.
[b] Conversion values were measured by GC analyses.
[c] Enantiomeric excess values were measured by HPLC analyses. Major enantiomer in parentheses.

After this screening, the influence of different alkyl chains at α-position was studied. For this purpose, the bioreduction of substrates **3b–e** into the corresponding alcohols **5b–e** was attempted, finding remarkable results only with ADH-A and evo-1.1.200 (Tables S3 and S4 in the Supporting Information; Table 2, entries 4–11). As observed with the model substrate **3a**, both enzymes favoured the formation of the *syn*-alcohols. In this manner, (2*R*,3 *S*)-enantiomers were obtained with high conversions (78–>99% conv) and moderate to high diastereoselectivity (72–92% *de*) and very high enantioselectivity (99% *ee*) with ADH-A. Additionally, (2*S*,3*R*)-**5b–d** were synthesised in moderate to high conversion values (60–98% conv) and high diastereo- and enantioselectivities (90–92% *de* and 99–>99% *ee*) with evo-1.1.200. However, this biocatalyst lost its activity towards the synthesis of the α-benzylated β-hydroxy amide **5e** (entry 11).

Encouraged by these results and looking for a further exploitation of the synthetic approach, we decided to undertake the ADH-catalysed DKR of substrate **3f**, bearing an ethyl group at R^1 position.

Table 2. Dynamic kinetic resolution of α-substituted β-keto amides **3a–h** bearing different pattern substitution at α-position.[a]



Entry	Substrate	ADH	*c* [%][b]	*de* [%][c][d]	*ee anti* [%][c]	*ee syn* [%][c]
1	**3a**	ADH-A	>99	90	n.d.	99 (2*R*,3*S*)
2	**3a**	RasADH	>99	30	28 (2*S*,3*S*)	30 (2*R*,3*S*)
3	**3a**	evo-1.1.200	>99	94	n.d.	>99 (2*S*,3*R*)
4	**3b**	ADH-A	>99	88	n.d.	99 (2*R*,3*S*)
5	**3b**	evo-1.1.200	96	92	n.d.	>99 (2*S*,3*R*)
6	**3c**	ADH-A	99	72	99 (2*S*,3*S*)	99 (2*R*,3*S*)
7	**3c**	evo-1.1.200	60	90	n.d.	99 (2*S*,3*R*)
8	**3d**	ADH-A	99	92	n.d.	99 (2*R*,3*S*)
9	**3d**	evo-1.1.200	98	92	n.d.	>99 (2*S*,3*R*)
10	**3e**	ADH-A	78	78	99 (2*S*,3*S*)	99 (2*R*,3*S*)
11	**3e**	evo-1.1.200	<1	n.d.	n.d.	n.d.
12	**3f**	ADH-A	<1	n.d.	n.d.	n.d.
13	**3f**	evo-1.1.200	98	77	5 (2*R*,3*R*)	>99 (2*S*,3*R*)
14	**3g**	ADH-A	99	82	n.d.	99 (2*R*,3*S*)
15	**3g**	evo-1.1.200	98	86	n.d.	>99 (2*S*,3*R*)
16	**3h**	ADH-A	96	12	99 (2*S*,3*S*)	99 (2*R*,3*S*)
17	**3h**	evo-1.1.200	8	n.d.	n.d.	n.d.

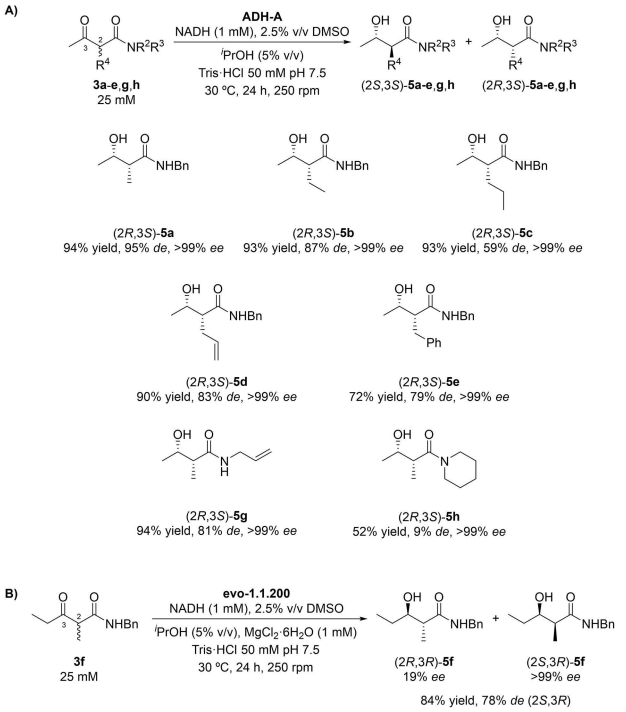
[a] For reaction conditions and the complete set of data, see the Supporting Information.
[b] Conversion values were measured by GC analyses.
[c] Diastereomeric and enantiomeric excess values were measured by HPLC analyses. Major diastereoisomer shown in parentheses.
[d] ADH-A and evo-1.1.200 produced preferentially the *syn*-diastereoisomer, while RasADH (entry 2) led preferentially to the formation of the *anti*-diastereoisomer. n.d. not determined.

Using the same reaction conditions, all enzymes were screened (Table S5 in the Supporting Information and Table 2, entries 12 and 13). Unfortunately, only evo-1.1.200 led to the preferential formation of alcohol (2*S*,3*R*)-**5f** with high conversion (98%), moderate *de* (77%) and excellent *ee* (>99%) values.

At this point, the influence that different amide moieties had in the process was studied. For this reason, the bioreduction protocol was set up using α-methylated substrates **3g** and **3h** (Table S5 in the Supporting Information; Table 2, entries 14–17). On the one hand, as previously observed with compound **3a**, the keto amide **3g** was a suitable substrate for ADH-A and evo-1.1.200, being the *syn*-alcohol the major product in both cases (entries 14 and 15). Hence, (2*R*,3*S*)- and (2*S*,3*R*)-**5g** were obtained with high conversion and selectivity (99% conv., 82% *de* and 99% *ee* and 98% conv., 86% *de* and >99% *ee*, respectively). On the other hand, **3h** led to high conversion and low *de* values, while high enantioselectivity towards the formation of (2*R*,3*S*)-**5h** (96% conv, 12% *de* and 99% *ee*, entry 16) when using ADH-A as biocatalyst and a complete loss of activity when utilising evo-1.1.200. From these results it became clear that the N-protecting had a large effect in the enzyme recognition, being the benzyl and the allyl moieties the most appropriate ones to perform these DKR transformations.

The relative *syn* configuration of the final products was assigned based on the use of NMR homonuclear decoupling experiments (see the Supporting Information). This result was confirmed with the method showed by Kalaitzakis and Smonou with α-alkyl-β-hydroxy carbonyl compounds,[42] together with the measured $^{3}J_{H2H3}$ for similar derivatives[25] and the known diastereopreference with the same enzymes with α-alkyl-β-keto esters.[32] The absolute configuration was determined due to the known stereospecificity of these ADHs.[32,35–41]

In order to demonstrate the applicability of the method, ADH-catalysed DKR transformations were performed at semipreparative scale. For this purpose, ADH-A was the enzyme of choice as it was the most efficient ketoreductase, providing good or excellent results in the bioreduction of substrates **3a–e**, **g**, **h**. This way, 100 mg of model β-keto amide **3a** and 20 mg of the other compounds were transformed into the corresponding enantioenriched alcohols. 2.5% v/v of DMSO was employed as co-solvent and the reaction media (Tris·HCl 50 mM pH 7.5) was implemented with NADH (1 mM). 2-Propanol (5% v/v) was employed to regenerate the nicotinamide cofactor.



Scheme 2. Semipreparative DKR of α-substituted β-keto amides: A) **3a–e**, **g**, **h** catalysed by overexpressed ADH-A; and B) **3f** catalysed by evo-1.1.200.

After 24 hours, similar results to those obtained at analytical scale were found (Scheme 2). Thus, the *syn*-(2*R*,3*S*)-diastereoisomers of the alcohols were obtained as the major one in moderate to high yields (52–94%) and excellent enantioselectivities (>99% *ee*). The diastereoselectivity of ADH-A remained high with the exception of β-hydroxy amide **5c** (59% *de*) and, especially, alcohol **5h** (9% *de*). Finally, the transformation of **3f** (20 mg) with evo-1.1.200 was performed, obtaining the enantiopure (2*S*,3*R*)-**5f** diastereoisomer with 78% *de*.

Overall, herein the reduction of various acyclic α-alkyl-β-keto amides has been described, affording the corresponding *syn*-α-alkyl-β-hydroxy amides with high diastereo- and enantioselectivities through DKR processes, employing lyophilised *E. coli* cells containing overexpressed ADHs. The high acidity of the α-proton ensured a fast substrate racemisation yielding the enantioenriched products at conversions close to 100% even at almost neutral pH. Enantiocomplementary ADH-A from *Rhodococcus ruber* and commercially available evo-1.1.200 afforded the best results. An important effect of the alkyl chain at α-position and also of the amide protecting group was observed in these bioreductions. Thus, higher *de* values were obtained for short alkyl moieties and N-benzylated amides. This methodology allows to get access to a new family of compounds with selectivities comparable to the ones obtained with metal catalysts,[25] thus demonstrating the great potential of enzymes to obtain valuable derivatives under straightforward, simple, and environmentally-friendly conditions.

Experimental Section

Alcohol dehydrogenases from *Ralstonia sp.* (RasADH), *Lactobacillus brevis* (LbADH), *Sphingobium yanoikuyae* (SyADH), *Thermoanaerobacter ethanolicus* (TeSADH), *Thermoanaerobacter sp.* (ADH-T), *Rhodococcus ruber* (ADH-A) overexpressed on lyophilised *E. coli* cells were obtained as previously described in the literature.[32] evo-1.1.200 was acquired from evoxx technologies GmbH. Chemical reagents were purchased from different commercial sources and used without further purification.

Semipreparative Scale Bioreduction of α-Substituted β-Keto Amides 3a–e, g, h Using the Alcohol Dehydrogenase from *Rhodococcus ruber* (ADH-A)

Lyophilised *E. coli*/ADH-A cells (100 mg for β-keto amide **3a** and 50 mg for β-keto amides **3b–e**, **g**, **h**), DMSO (2.5% v/v), NADH (1 mM) and ^{i}PrOH (5% v/v) were successively added into an Erlenmeyer flask containing β-keto amide (100 mg for **3a** and 20 mg for **3b–e**, **g**, **h**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5. The reaction was shaken at 30 °C and 250 rpm for 24 h and then extracted with EtOAc (3×15 mL). The organic layers were separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The solvent was concentrated under vacuum, furnishing the α-substituted β-hydroxy amides **5a–e**, **g**, **h** in moderate to excellent isolated yields (52–94%).

Semipreparative Scale Bioreduction of α-Substituted β-Keto Amide 3f Using the Commercial Alcohol Dehydrogenase evo-1.1.200

20 mg of β-keto amide **3f** (25 mM) was added in an Erlenmeyer flask containing DMSO (2.5% v/v), $MgCl_2 \cdot 6H_2O$ (1 mM), NADH (1 mM) and ^{i}PrOH (5% v/v) in Tris·HCl buffer 50 mM pH 7.5 (final volume: 3.6 mL). Finally, 75 mg of lyophilised evo-1.1.200 were added and the reaction was shaken at 30 °C and 250 rpm for 24 h. After this time, the reaction was extracted with EtOAc (3×5 mL). The organic layers were separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. The solvent was concentrated under vacuum, achieving the α-substituted β-hydroxy amide **5f** in high isolated yield (17 mg, 84%).

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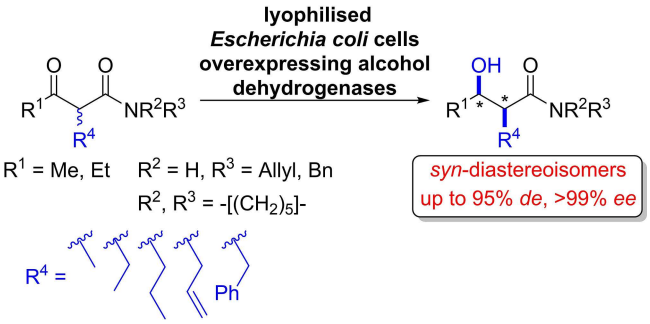
UPDATES

Synthesis of α-Alkyl-β-Hydroxy Amides through Biocatalytic Dynamic Kinetic Resolution Employing Alcohol Dehydrogenases

Adv. Synth. Catal. **2019**, *361*, 1–8

D. Méndez-Sánchez, Á. Mourelle-Insua, V. Gotor-Fernández*, I. Lavandera*



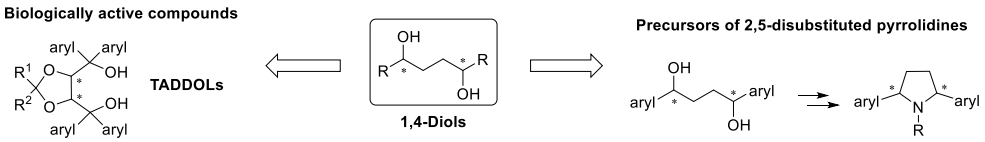
Chapter 5

Stereoselective synthesis of 1,4-diaryl-1,4-diols mediated by alcohol dehydrogenases

5.1. Bibliographic background

Optically active 1,4-diols can be found in biologically active compounds such as pharmaceuticals, flavours and fragrances as well as they can be used as chiral ligands and auxiliaries for asymmetric synthesis (Scheme 5.1).[210] Furthermore, they are precursors of important ligands employed in asymmetric hydrogenation as 2,5-disubstituted pyrrolidines and phosphine derivatives.[211] Due to all these reasons, the asymmetric synthesis of 1,4-diol scaffolds is a field of interest in organic chemistry, so different non-enzymatic and enzymatic synthetic approaches have been disclosed in the last two decades as it will be discussed in the next sections.

Scheme 5.1. Importance of chiral 1,4-diol scaffold in organic chemistry.



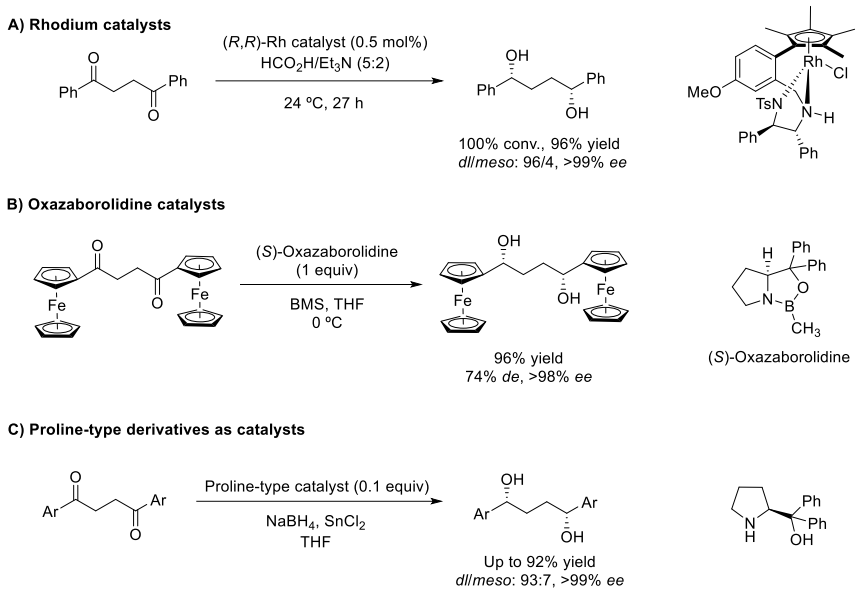
5.1.1. Synthesis of optically active 1,4-diols

Catalytic asymmetric transfer hydrogenation (CATH) has been commonly used to synthesise chiral 1,4-diols from the corresponding prochiral 1,4-dicarbonyl compounds in quantitative yields.[212] However, the hydroxy ketone intermediate as well as the four possible diol diastereoisomers can be accomplished. Thus, several methodologies towards the stereoselective asymmetric reduction of 1,4-dicarbonyl compounds have been developed using chiral catalysts and reducing agents (Scheme 5.2). For instance, Zhen *et al.* tested several Rh(III)/Cp* complexes bearing the ligand *N*-(*p*-tolylsulfonyl)-1,2-diphenylethylene-1,2-diamine.[213] The system formic acid/triethylamine was employed as hydrogen source and the reactions took place under mild conditions (24-30 ºC). Several prochiral ketones were tested as substrates as well as the 1,4-diphenylbutane-1,4-dione. In particular,

[210] (a) D. Seeback, A. K. Beck, A. Heckel, *Angew. Chem. Int. Ed.* **2001**, *40*, 92-138; (b) A. Robinson, V. K. Aggarwal, *Angew. Chem. Int. Ed.* **2010**, *49*, 6673-6675.
[211] (a) P. Melchiorre, K. A. Jørgensen, *J. Org. Chem.* **2003**, *68*, 4151-4157; (b) H. Chen, J. A. Sweet, K.-C. Lam, A. L. Rheingold, D. V. McGrath, *Tetrahedron: Asymmetry* **2009**, *20*, 1672-1682; (c) S. E. Denmark, W.-T. T. Chang, K. N. Houk, P. Liu, *J. Org. Chem.* **2015**, *80*, 313-366.
[212] F. Foubelo, C. Nájera, M. Yus, *Tetrahedron: Asymmetry* **2015**, *26*, 769-790.
[213] L.-S. Zheng, Q. Llopis, P.-G. Echevarria, C. Férard, G. Guillamot, P. Phansavath, V. Ratovelomana-Vidal, *J. Org. Chem.* **2017**, *82*, 5607-5615.

this dicarbonyl compound was successfully reduced into the corresponding (1*R*,4*R*)-diol with excellent conversion and enantioselectivity, attaining a high *dl*/*meso* ratio (96:4) using the Rh catalyst shown in Scheme 5.2.A.

Scheme 5.2. Metal- and organocatalysed asymmetric reduction of 1,4-dicarbonyl compounds.



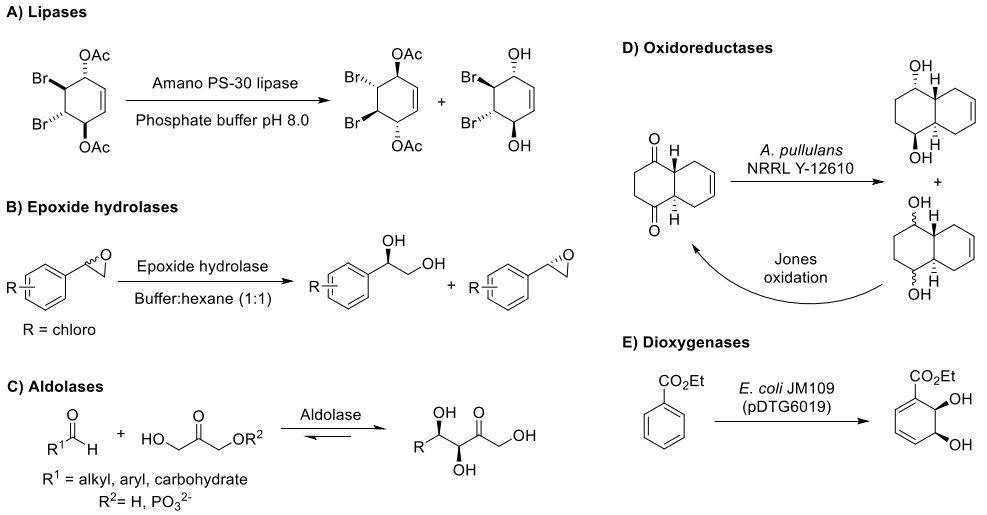
Some borane complexes such as oxazaborolidines have also served as catalysts for the synthesis of chiral 1,4-diols.[214] For example, the system formed by an optically active methyl-substituted oxazaborolidine and the dimethylsulfide borane complex ($BH_3 \cdot Me_2S$, BMS) was capable of catalysing the asymmetric reduction of 1,2-bis(ferrocenoyl)ethane into the corresponding diol in high yield, enantio- and diastereoselectivity (Scheme 5.2.B). Finally, borane reagents generated *in situ* from proline-type derivatives in combination with a reducing agent (*e.g.* $NaBH_4$) have also been employed in these reductive transformations.[215] For instance, Li *et al.* reported the asymmetric reduction of aryl 1,4-diketones using $NaBH_4$ and $SnCl_2$ in combination with (*S*)-(–)-α,α-diphenyl-2-pyrrolidinemethanol (Scheme 5.2.C).[215b] Thus, five 1,4-diarylbutane-1,4-diones

[214] (a) L. Schwink, P. Knochel, *Tetrahedron Lett.* **1997**, *38*, 3711-3714; (b) E. J. Corey, C. J. Helal, *Angew. Chem. Int. Ed.* **1998**, *37*, 1986-2012.
[215] (a) D. J. Aldous, W. M. Dutton, P. G. Steel, *Tetrahedron: Asymmetry* **2000**, *11*, 2455-2462; (b) X. Li, G. Zhao, W.-G. Cao, *Chin. J. Chem.* **2006**, *24*, 1402-1405.

differing in the ring substitution were successfully converted into the corresponding diols in high yields, enantio- and diastereoselectivities.

Biocatalysis has emerged in the last decades as a good alternative to produce optically active (poly)alcohols by using different types of enzymes (Scheme 5.3). Among others, lipases (A), epoxide hydrolases (B), aldolases (C), reductases (D) and dioxygenases (E) can be the enzymes of choice to synthesise diols in a diastereo- and enantioselective manner.[216]

Scheme 5.3. Biocatalysis towards the synthesis of optically active diols.



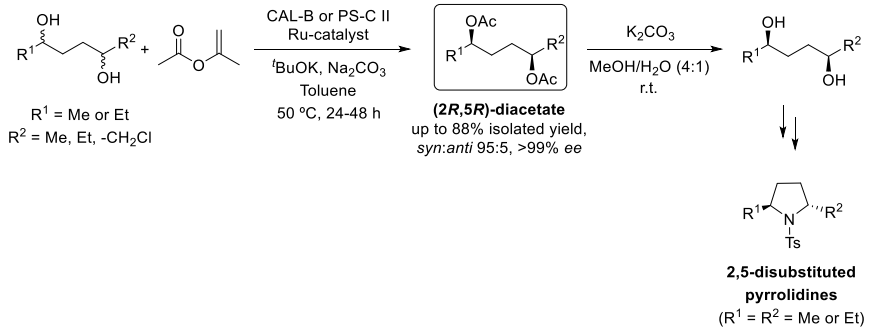
Even though several biocatalytic methodologies have been developed towards the synthesis of chiral diols, the production of optically active 1,4-dialkyl-1,4-diols have been mainly described by lipase-mediated resolution protocols. The first example dates back to 1993 when Norin and co-workers reported the transesterification of hexane-2,5-diol catalysed by CAL-B using ethyl thiooctanoate as acyl donor.[217] The process led to the synthesis of the (2*S*,5*S*)-hexane-2,5-diol in high isolated yield and with good stereoselectivity (>99% *ee*). Later, similar procedures were described using other lipases such as the one from *Pseudomonas cepacia* (PSL), from *Candida rugosa* (CRL), from *Alcaligenes* sp.

[216] (a) T. Hudlicky, J. W. Reed, *Chem. Soc. Rev.* **2009**, *38*, 3117-3132; (b) C. M. Clouthier, J. N. Pelletier, *Chem. Soc. Rev.* **2012**, *41*, 1585-1605; (c) G. Torrelo, U. Hanefeld, F. Hollmann, *Catal. Lett.* **2015**, *145*, 309-345.
[217] A. Mattson, N. Öhrner, K. Hult, T. Norin, *Tetrahedron: Asymmetry* **1993**, *5*, 925-930.

(lipase AH) or the porcine pancreatic lipase (PPL) using vinyl acetate as acyl donor.[218]

Interestingly, Bäckvall and co-workers have been very active in the development of dynamic kinetic resolutions for this class of secondary diols by coupling a ruthenium catalyst with CAL-B. Thus, the treatment of hexane-2,5-diol with 4 mol% of a ruthenium catalyst in combination with CAL-B and 4-chlorophenyl acetate as acyl donor led to the formation of the diacetate as a 86:14 mixture of (2*R*,5*R*)-diacetate and *meso*-diacetate in moderate yield and excellent enantioselectivity.[219] The same research group proposed a mechanism explaining the formation of the *meso*-diacetate which is the *anti*-Kazlauskas product,[220] and they also extended this DKR approach to different 1,4-dialkyl-1,4-diols.[221] In this case, CAL-B and an immobilised PSL form (PS-C II) were used as biocatalysts using isopropenyl acetate as acyl donor. After 24 or 48 hours at 50 ºC in toluene, the corresponding diacetates were obtained in high isolated with good diastereoselectivity and excellent enantioselectivity. After basic hydrolysis to form the optically active 1,4-diols, it was possible to synthesise chiral 2,5-disubstituted pyrrolidines with interesting applications as chiral ligands (Scheme 5.4).

Scheme 5.4. Dynamic kinetic resolution of 1,4-alkyl-1,4-diols for their application in the synthesis of optically active 2,5-disubstituted pyrrolidines.

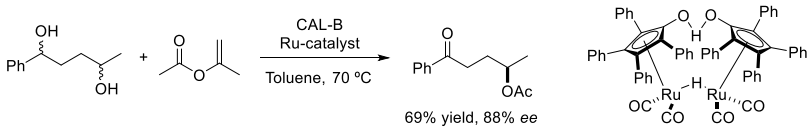


In terms of bulkier substitutions, only two examples describing the resolution of the 1-phenylpentane-1,4-diol or its acetate have been found, also described by

[218] (a) H. Nagai, T. Morimoto, K. Achiwa, *Synlett* **1994**, 289-290; (b) G. Caron, R. J. Kazlauskas, *Tetrahedron: Asymmetry* **1994**, *4*, 657-664.
[219] (a) B. A. Persson, A. L. E. Larsson, M. Le Ray, J.-E. Bäckvall, *J. Am. Chem. Soc.* **1999**, *121*, 1645-1650; (b) B. A. Persson, F. F. Huerta, J.-E. Bäckvall, *J. Org. Chem.* **1999**, *64*, 5237-5240.
[220] M. Edin, J.-E. Bäckvall, *J. Org. Chem.* **2003**, *68*, 2216-2222.
[221] L. Borén, K. Leijondahl, J.-E. Bäckvall, *Tetrahedron Lett.* **2009**, *50*, 3237-3240.

Bäckvall and co-workers. A dynamic kinetic asymmetric transformation (DYKAT) of unsymmetrical 1,4-diols, including 1-phenylpentane-1,4-diol, was performed leading to the acetylation of the least hindered alcohol.[222] Later on, the oxidation of the remaining hydroxyl group took place under the reaction conditions, obtaining the enantiopure γ-acetoxy ketones (Scheme 5.5). The second example dates back to 2014 when the same research group studied the behaviour of CAL-B in the kinetic resolution through hydrolytic reactions in phosphate buffer of different compounds, including the diacetylated 1-phenylpentane-1,4-diol.[223]

Scheme 5.5. 1-Phenylpentane-1,4-diol DYKAT and oxidation to achieve the corresponding γ-acetoxy ketone.



Aside from lipases, alcohol dehydrogenases have been employed in the synthesis of optically active 1,4-dialkyl-1,4-diols. In the next section, we will present some interesting results involving the utilisation of these enzymes.

5.1.2. Alcohol dehydrogenases towards the synthesis of chiral diols

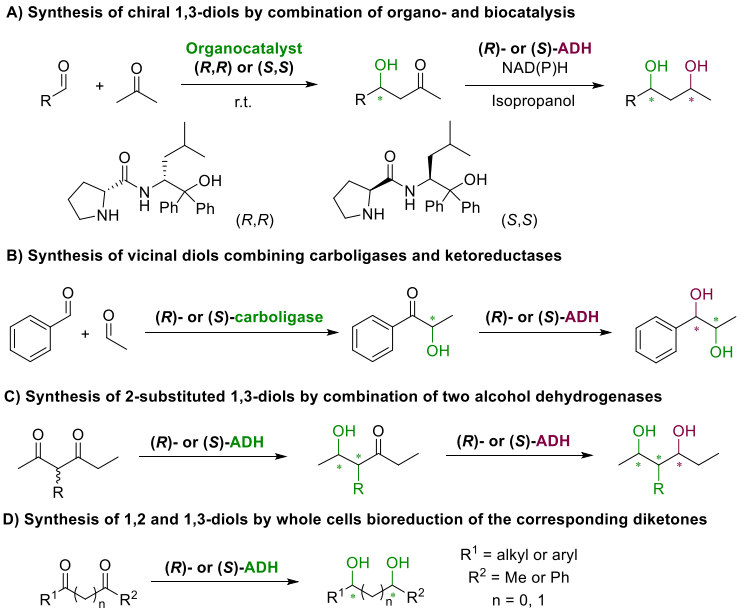
As previously introduced in Chapter 4, ADHs have been widely used for the synthesis of chiral alcohols.[166,168] Herein, we will focus in their applicability towards the production of optically active diols. In this way, several methodologies have been developed employing oxidoreductases as biocatalysts.

In this section, we will focus on examples regarding the stereoselective synthesis of optically active 1,4-diols *via* bioreduction reactions. Ketoreductases are versatile catalysts for the production of several diol scaffolds, fact that has been illustrated in Scheme 5.6 displaying the synthesis of 1,2 and 1,3-diols from the corresponding dicarbonyl compounds. Thus, ADHs can act in combination with organocatalysts (Scheme 5.6.A),[224] other biocatalysts such as lyases (Scheme

[222] B. Martín-Matute, J.-E. Bäckvall, *J. Org. Chem.* **2004**, *69*, 9191-9195.
[223] B. Yang, R. Lihammar, J.-E. Bäckvall, *Chem. Eur. J.* **2014**, *20*, 13517-13521.
[224] K. Baer, M. Kraußer, E. Burda, W. Hummel, A. Berkessel, H. Gröger, *Angew. Chem. Int. Ed.* **2009**, *48*, 9355-9358.

5.6.B)[225] or other ketoreductases (Scheme 5.6.C),[226] but also by using whole cells (Scheme 5.6.D).[227]

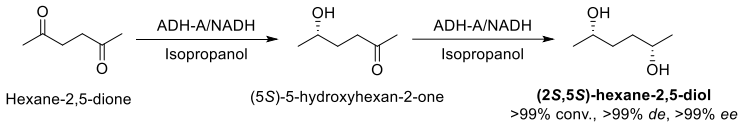
Scheme 5.6. Some examples of ketoreductases applied to the synthesis of optically active diols.



In terms of production of 1,4-dialkyl-1,4-diols, some examples have been described using ADHs, leading to the synthesis of optically active hexane-2,5-diol (Scheme 5.7). Liese, Kroutil and co-workers made use of the ADH from *Rhodococcus ruber* (ADH-A) to selectively reduce hexane-2,5-dione using isopropanol (^{i}PrOH) as coupled-substrate.[228] An *in situ* product removal technique (ISPR) was reported to eliminate the acetone (co-product) in order to shift the thermodynamic equilibrium towards the alcohol formation in order to achieve full conversion.

[225] J. Wachtmeister, A. Jakoblinnert, D. Rother, *Org. Process Res. Dev.* **2016**, *20*, 1744-1753.
[226] D. Kalaitzakis, I. Smonou, *J. Org. Chem.* **2010**, *75*, 8658-8661.
[227] (a) P. Mahajabeen, A. Chadha, *Tetrahedron: Asymmetry* **2015**, *26*, 1167-1173; (b) M. Kurina-Sanz, F. R. Bisogno, I. Lavandera, A. A. Orden, V. Gotor, *Adv. Synth. Catal.* **2009**, *351*, 1842-1848.
[228] (a) K. Goldberg, K. Edegger, W. Kroutil, A. Liese, *Biotechnol. Bioeng.* **2006**, *95*, 192-198; (b) K. Edegger, W. Stampfer, B. Seisser, K. Faber, S. F. Mayer, R. Oehrlein, A. Hafner, W. Kroutil, *Eur. J. Org. Chem.* **2006**, 1904-1909.

Scheme 5.7. Bioreduction of hexane-2,5-dione to produce enantiopure (2*S*,5*S*)-hexane-2,5-diol.



ADH-A from *Rhodococcus ruber* has also been employed by the same group for the production of this enantiopure diol employing a micro-aqueous system as reaction media (99% hexane and buffer).[42] After 24 hours at 30 ºC and using ^{i}PrOH as cosubstrate, the enantiopure diol was obtained with high conversion values (76% conv). Furthermore, van der Oost and co-workers successfully created thermostable and evolved variants of the alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* (AdhA).[229] After screening around 1500 mutants, a ten-fold improvement in the specific activity of the enzyme at 30 ºC retaining its stability was achieved, leading to an efficient process to produce enantiopure (2*S*,5*S*)-hexane-2,5-diol. Finally, Lütz and co-workers made use of a bimembrane reactor system which enabled a continuous biocatalytic ketone reduction process with concurrent *in situ* removal of acetone.[230] For this purpose, the (*R*)-selective ADH from *Lactobacillus brevis* was employed, allowing the formation of (2*R*,5*R*)-hexane-2,5-diol in high space-time yield (>170 $g \cdot L^{-1} \cdot d^{-1}$).

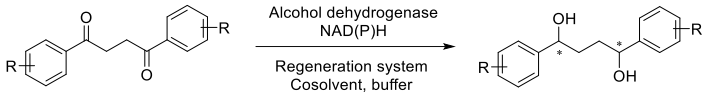
Despite their already discussed importance, no examples about the bioreduction of 1,4-diaryl-1,4-diketones using ADHs have been found in the bibliography, probably due to steric reasons. For this reason, the use of ADHs for the stereoselective reduction of different 1,4-diaryl-1,4-diketones under mild reaction conditions will be described next.

[229] R. Machielsen, N. G. H. Leferink, A. Hendriks, S. J. J. Brouns, H.-G. Hennemann, T. Daußmann, J. van der Oost, *Extremophiles* **2008**, *12*, 587-594.
[230] K. Schroer, S. Lütz, *Org. Process Res. Dev.* **2009**, *13*, 1202-1205.

5.2. Discussion

Herein, we present the asymmetric synthesis of optically pure 1,4-diaryl-1,4-diols bearing different pattern substitutions in the aromatic ring (Scheme 5.8). This approach consists in the bioreduction of the corresponding prochiral ketones, which were previously chemically synthesised through already reported methods. Special attention was paid to the use of several made-in-house ADHs, along with the optimisation of several reaction parameters including the use of organic cosolvents, the regeneration system or the reaction time.

Scheme 5.8. ADH-mediated bioreduction of prochiral 1,4-diaryl-1,4-diketones.



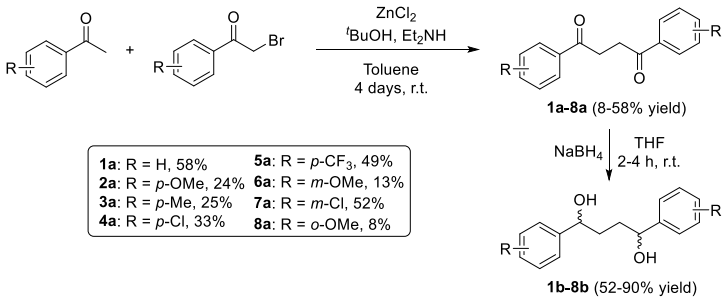
5.2.1. Chemical synthesis of 1,4-diaryl-1,4-diones 1a-8a

In order to synthesise a representative panel of 1,4-diaryl-1,4-diones that could serve as substrates in the bioreduction process, we made use of an already described protocol which involved the cross aldol condensation of 4'-substituted acetophenones with α-bromo-4'-substituted acetophenones.[231] The cross aldol products underwent first a 1,3-dehydrobromination and then the cleavage of the corresponding activated cyclopropyl intermediates in the presence of zinc chloride and diethylamine and *tert*-butanol (Scheme 5.9). These reactions were carried out for 4 days at room temperature in dry toluene, achieving the desired 1,4-diaryl-1,4-diones **1a-8a** in low to moderate yields (8-58%).

Once the diketones **1a-8a** were obtained, we synthesised the racemic 1,4-diols **1b-8b** that would serve as standards for the development of HPLC methods. For this purpose, $NaBH_4$ was added to a solution of the corresponding diketone in dry THF, obtaining the desired products after 2-4 h in moderate to high yields (52-90%).

[231] N. M. Nevar, A. V. Kel'in, O. G. Kulinkovich, *Synthesis* **2000**, 1259-1262.

Scheme 5.9. Synthesis of 1,4-diaryl-1,4-diones (**1a**-**8a**) and 1,4-diaryl-1,4-diols (**1b**-**8b**) bearing different pattern substitutions in the aromatic ring.



At this point, a representative panel of substrates was obtained. Therefore, they were tested as substrates in the bioreduction process catalysed by different ADHs.

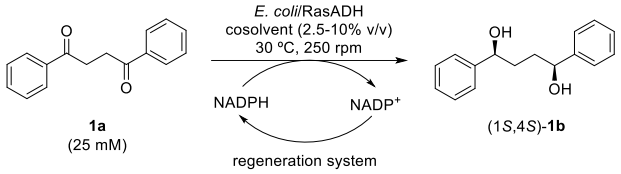
5.2.2. Preliminary studies using 1,4-diphenylbutane-1,4-dione (1a) as model substrate

The less hindered substrate, this is 1,4-diphenylbutane-1,4-dione (**1a**), was chosen as model substrate for the study. First of all, lyophilised cells of *E. coli* overexpressing different ketoreductases were tested as biocatalysts in the bioreduction of this diketone. Thus, ADHs from *Ralstonia* sp. (RasADH),[207] *Lactobacillus brevis* (LbADH),[208] *Sphingobium yanoikuyae* (SyADH),[206] *Thermoanaerobacter ethanolicus* (TeSADH)[204] and *Rhodococcus ruber* (ADH-A)[203] were used. With that purpose, 25 mM substrate concentration was initially considered and 2.5% v/v of DMSO was used as cosolvent to solubilise the lipophilic diketone in a Tris·HCl 50 mM pH 7.5 medium, containing the cofactor (1 mM of NAD(P)H). ^{i}PrOH or a glucose/glucose dehydrogenase (GDH) system were employed to regenerate the cofactor. After 24 hours at 30 ºC, only RasADH led to the formation of 1,4-diphenylbutane-1,4-diol (**1b**). Even though a moderate conversion value was obtained (Table 5.1, entry 1, 56% conv), the selectivity of the process revealed to be excellent towards the formation of (1*S*,4*S*)-**1b** (98% *de* and >99% *ee*), while a longer reaction time (48 h) allowed to recover (1*S*,4*S*)-**1b** in 82% conv maintaining the excellent selectivity (entry 2).

Since RasADH was the only made-in-house ketoreductase which allowed the formation of the desired diol, we decided to optimise the bioreduction using this enzymatic preparation. For this purpose, we screened several parameters such as

the recycling system, type and amount of cosolvent and the reaction time. First, we set up the bioreduction in the absence of cosolvent using the glucose/GDH system but also ^{i}PrOH as recycling system (entries 3 and 4, respectively). After 48 hours, lower conversion values were found (72% conv). Additionally, using IPA as coupled-substrate led to a significantly loss of diastereoselectivity (from 98% to 78% *de*).

Table 5.1. RasADH-catalysed bioreduction of model substrate 1,4-diphenylbutane-1,4-diol (**1a**).[a]



Entry	Regeneration system	Cosolvent	Cosolvent (% v/v)	t (h)	*c* (%)[b]	*dr*[b,c]	*ee* (%)[b,d]
1	Glucose/GDH	DMSO	2.5	24	56	99:1	>99
2	Glucose/GDH	DMSO	2.5	48	82	99:1	>99
3	Glucose/GDH	-	-	48	72	99:1	>99
4	^{i}PrOH	-	-	48	72	89:11	>99
5	Glucose/GDH	EtOH	2.5	24	57	99:1	>99
6	Glucose/GDH	1,4-dioxane	2.5	24	72	99:1	>99
7	Glucose/GDH	1,4-dioxane	5	24	78	>99	>99
8	Glucose/GDH	1,4-dioxane	10	24	79	99:1	>99
9	Glucose/GDH	1,4-dioxane	10	48	80	99:1	>99
10	Glucose/GDH	MTBE	2.5	24	70	>99	>99
11	Glucose/GDH	MTBE	5	24	83	>99	>99
12	Glucose/GDH	MTBE	10	24	77	>99	>99
13	Glucose/GDH	MTBE	10	48	77	>99	>99
14	Glucose/GDH	THF	2.5	24	72	99:1	>99
15	Glucose/GDH	THF	5	24	88	>99	>99
16	Glucose/GDH	THF	10	24	80	99:1	>99
17	Glucose/GDH	THF	10	48	82	99:1	>99

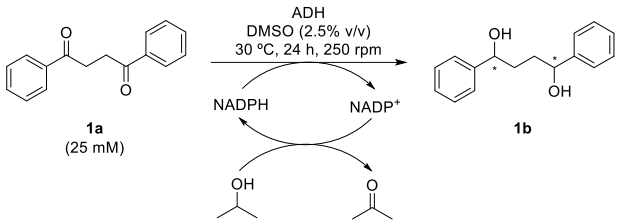
[a] Reaction conditions: substrate **1a** (25 mM), RasADH (15 mg), cosolvent (2.5-10% v/v), NADPH (1 mM), glucose (50 mM, entries 1-3 and 5-17), glucose dehydrogenase (5 U, entries 1-3 and 5-17), ^{i}PrOH (5% v/v, entry 4), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24-48 h, 250 rpm. [b] Conversions measured by HPLC. [c] Ratio of (1*S*,4*S*)-**1b** to *meso*-(1*R*,4*S*)-**1b**. [d] Enantiomeric excess of (1*S*,4*S*)-**1b**.

These experiments demonstrated the importance of using a cosolvent due to the low solubility of the diketone in the reaction media, while glucose/GDH system revealed to be the best option to regenerate the cofactor. Taking this data into

account, we performed an exhaustive cosolvent screening (entries 5-17). Different organic solvents such as ethanol (EtOH), 1,4-dioxane, *tert*-butyl methyl ether (MTBE) and tetrahydrofuran (THF) were tested as cosolvents for the synthesis of diol **1b**. They were employed in different amounts between 2.5% v/v to 10% v/v. When using 2.5% v/v of cosolvent (entries 1, 5, 6, 10 and 14), 1,4-dioxane, MTBE and THF revealed the best results in terms of conversion, affording very high enantio- and diastereoselectivities. When the amount of these cosolvents was increased up to 5% v/v (entries 7, 11 and 15), an increase in the conversion value was observed in all cases and perfect diastereoselectivity was found (>99% *de*).

Encouraged by these results, we decided to increase the amount of cosolvent up to 10% v/v (entries 8, 12 and 16). However, these experiments did not lead to better results, even when the bioreductions were incubated for 48 hours (9, 13 and 17). At this point, 5% v/v THF was chosen as the best conditions (entry 15), leading to the synthesis of the diastereo- and enantiopure (1*S*,4*S*)-**1b** in 88% conv. It is important to point out that, during this optimisation process, the hydroxy ketone intermediate was not detected in the reaction medium.

Table 5.2. Bioreduction of model substrate **1a** using commercial ADHs.



Entry	ADH	*c* (%)[b]	*dr*[b,c]	*ee* (%)[b,d]
1	P1-B02	85	>99	>99
2	P1-B10	79	>99	>99
3	P1-B12	75	99:1	>99
4	P2-D03	89	87:13	>99[e]
5	P2-D11	69	98:2	>99

[a] Reaction conditions: substrate **1a** (25 mM), KRED (2 mg), DMSO (2.5% v/v), NADPH (1 mM), $MgSO_4$ (1.25 mM), ^{i}PrOH (10% v/v), phosphate 125 mM pH 7 (1 mL final volume), 30 ºC, 24 h, 250 rpm. [b] Conversions measured by HPLC. [c] Ratio of (1*S*,4*S*) and (1*R*,4*R*)-**1b** to *meso*-(1*R*,4*S*)-**1b**. [d] Enantiomeric excess of (1*S*,4*S*)-**1b**. [e] Enantiomeric excess of (1*R*,4*R*)-**1b**.

After trying several made-in-house enzymes, commercial ketoreductases purchased from Codexis Inc. were also tested as biocatalysts for this process. Again, 25 mM of the model substrate was initially considered and 2.5% v/v DMSO

was added in order to solubilise the diketone **1a**. In this case, ^{i}PrOH was used as cosubstrate and phosphate buffer 125 mM pH 7.0 containing 1 mM NADPH was employed as reaction medium. After 24 hours at 30 ºC, we found that diketone **1a** was a suitable substrate for five of these enzymes (Table 5.2). Again, the hydroxy ketone was not detected in any case. Interestingly, the (*R*)-selective KRED-P2-D03 allowed the synthesis of (1*R*,4*R*)-**1b** in 89% conv, 74% *de* and excellent enantioselectivity (entry 4), the opposite enantiomer of that obtained with RasADH and the other commercial ADHs. Thus, it could be demonstrated that by using complementary ADHs, both enantiomers of 1,4-diphenylbutane-1,4-diol could be produced.

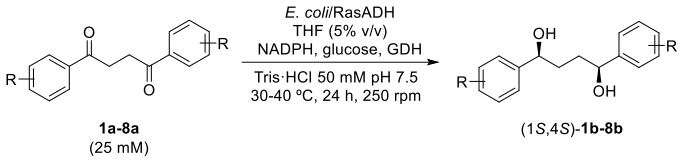
5.2.3. Enlarging the substrate scope. *E. coli*/RasADH towards the synthesis of optically active 1,4-diols 2b-8b

Once the best reaction conditions were set for the model substrate (Table 5.3, entry 1), we decided to study the bioreduction of similar substrates differing in the ring substitution pattern. First of all, diketones that differed at the *para* substitution of the aromatic ring were considered, so biotransformations at 25 mM substrate concentration were set up for **2a**-**5a** using RasADH as biocatalyst. 5% THF was employed as cosolvent and the bioreductions were incubated at 30 ºC for 24 hours (Table 5.3, entries 2-5). In all cases, the synthesis of the (1*S*,4*S*)-diol was achieved with excellent diastereo- and enantioselectivity (>99% *de* and >99% *ee*). In terms of conversions, the best result was obtained with the *p*-chlorinated substrate **4a** (entry 4, 82% conv.), while the worst was achieved with bulkier trifluoromethylated diketone **5a** (entry 5, 50% conv). Ketones bearing the *p*-methoxy (**2a**) and *p*-methyl (**3a**) substitutions led to 72% and 77% conv, respectively (entries 2 and 3).

Later on, we decided to widen the study including *meta* and *ortho*-substituted diketones. Therefore, bioreduction of diketones **6a**-**8a** were set up under the same reaction conditions that bioreduction of *para*-substituted substrates (entries 6, 7 and 10). Unfortunately, low conversion values were achieved using these diketones as starting material, especially with the *m*-methoxylated substrate (**6a**) that led to only 8% conv of the corresponding (1*S*,4*S*)-diol **6b** (entry 6). The *o*-methoxylated substrate (**8a**) also led to the synthesis of the corresponding diol **8b** in 8% conv (entry 10). However, in this particular case, the production of the hydroxy ketone intermediate was observed in much higher conversion than the diol (36% conv).

The bioreduction of the *m*-chlorinated substrate (**7a**) reached 22% conv (entry 7) and two additional experiments were carried out in order to increase this value. So, the amount of enzyme was doubled (entry 8) and in addition the temperature was increased up to 40 ºC (entry 9), leading to the synthesis of the diastereo- and enantiopure (1*S*,4*S*)-**7b** in 34% and 43% conversion, respectively.

Table 5.3. Selective bioreduction of diketones **1a-8a** using RasADH as biocatalyst.[a]



Entry	Substrate	RasADH (mg)	T (ºC)	*c* (%)[b]	*de* (%)[b,c]	*ee* (%)[b,d]
1	H (**1a**)	15	30	88	>99	>99
2	*p*-OMe (**2a**)	15	30	72	>99	>99
3	*p*-Me (**3a**)	15	30	77	>99	>99
4	*p*-Cl (**4a**)	15	30	82	>99	>99
5	*p*-CF_3 (**5a**)	15	30	50	>99	>99
6	*m*-OMe (**6a**)	15	30	8	>99	>99
7	*m*-Cl (**7a**)	15	30	22	>99	>99
8	*m*-Cl (**7a**)	30	30	34	>99	>99
9	*m*-Cl (**7a**)	30	40	43	>99	>99
10	*o*-OMe (**8a**)	15	30	8[e]	>99	>99

[a] Reaction conditions: substrate **1a-8a** (25 mM), RasADH (15-30 mg), THF (5% v/v), NADPH (1 mM), glucose (50 mM), glucose dehydrogenase (5 U), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30-40 ºC, 24 h, 250 rpm. [b] Measured by HPLC. [c] Ratio of (1*S*,4*S*)-**1b-8b** to *meso*-diol. [d] Enantiomeric excess of (1*S*,4*S*)-**1b-8b**. [e] Hydroxy ketone was produced in 36% conv.

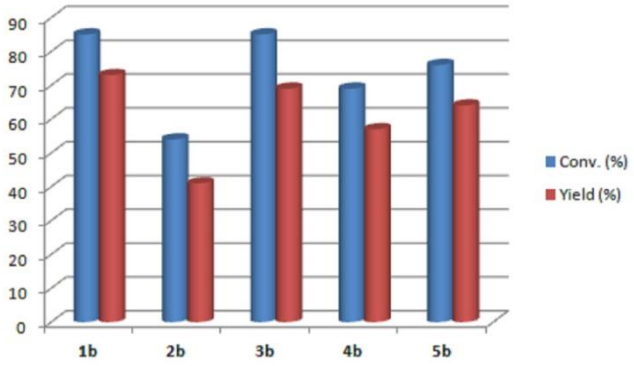
Finally, the bioreductions of these compounds were also attempted using the commercial ketoreductases, although no conversion was observed in any case. These results showed that the substitution pattern had a remarkable effect on the enzymatic activity, achieving the best results with the *para*-substituted diketones. Furthermore, the difficulty of these biotransformations has been demonstrated as, among several ADHs, they could only be achieved using RasADH as biocatalyst.

5.2.4. Semi-preparative bioreduction of 1,4-diaryl-1,4-diketones 1a-5a,7a

Finally, in order to demonstrate the applicability of this methodology, semi-preparative biotransformations were set up using *E. coli*/RasADH and the optimised reaction conditions. This is, 25 mM substrate concentration, THF as cosolvent (5% conv), 30 ºC and glucose/GDH system as NADPH-regeneration

system in a Tris·HCl 50 mM pH 7.5 medium (Figure 5.1). After 24 hours and purification by column chromatography, diols (1*S*,4*S*)-**1b**-**5b** and **7b** were obtained with excellent diastereo- and enantioselectivity (>99% *de* and >99% *ee*). The unsubstituted diol (**1b**) along with *p*-methylated (**3b**) and *p*-trifluoromethylated (**5b**) diols were obtained in high yields (73%, 69% and 64%) while the *p*-methoxylated (**2b**) and the *p*-chlorinated (**4b**) diols were achieved in moderate yields (41% and 57%). Predictably, the worst results were obtained for the *m*-chlorinated substrate (**7a**) that allowed the synthesis of the corresponding diol in 10% yield. All the diols obtained were fully characterised by NMR experiments and HRMS. Moreover, in order to confirm the absolute configuration, the optical rotation was measured and compared with those described in the literature.[232]

Figure 5.1. Semi-preparative bioreductions of diketones **1b**-**5b** using RasADH as biocatalyst.



Additionally, the bioreduction of the *o*-methoxylated substrate (**8a**) was set up at 20 mg-scale, isolating the (4*S*)-4-hydroxy-1,4-bis(2-methoxyphenyl)butan-1-one

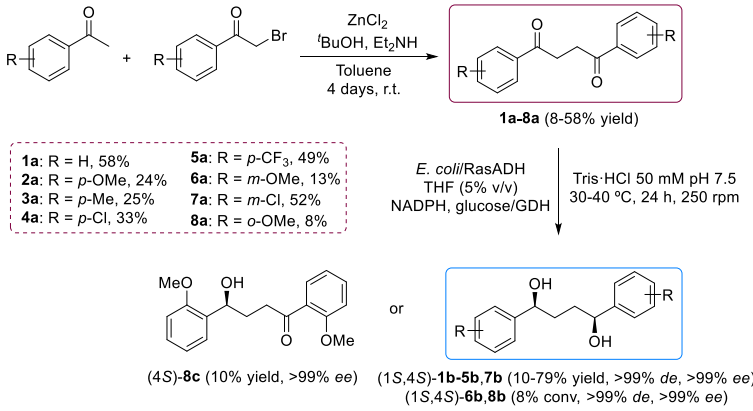
[232] (a) (–)-(1*S*,4*S*)-1,4-Diphenylbutane-1,4-diol (**1b**): $[\alpha]_D^{21} = -57.2$ (c = 0.8, $CHCl_3$). Described in the literature: $[\alpha]_D^{25} = -59.0$ (c = 1.0, $CHCl_3$) for the *syn*-(1*S*,4*S*)-diol. D. Domin, D. Benito-Garagorri, K. Mereiter, C. Hametner, J. Fröhlich, K. Kirchner, *J. Organomet. Chem.* **2007**, *692*, 1048-1057; (b) (–)-(1*S*,4*S*)-1,4-Bis(4-methoxyphenyl)butane-1,4-diol (**2b**): $[\alpha]_D^{21} = -43.0$ (c = 0.1, $CHCl_3$). Described in the literature [215b]: $[\alpha]_D^{25} = +41.6$ (c = 1.0, $CHCl_3$) for the *syn*-(1*R*,4*R*)-diol; (c) (–)-(1*S*,4*S*)-Bis(4-methylphenyl)butane-1,4-diol (**3b**): $[\alpha]_D^{21} = -45.2$ (c = 0.2, $CHCl_3$). Described in the literature [211b]: $[\alpha]_D^{25} = -47.0$ (c = 1.0, $CHCl_3$) for the *syn*-(1*S*,4*S*)-diol; (d) (–)-(1*S*,4*S*)-1,4-Bis(4-chlorophenyl)butane-1,4-diol (**4b**): $[\alpha]_D^{21} = -22.1$ (c = 0.2, $CHCl_3$). Described in the literature [215b]: $[\alpha]_D^{21} = +24.4$ (c = 1.05, $CHCl_3$) for the *syn*-(1*R*,4*R*)-diol; (e) (–)-(1*S*,4*S*)-1,4-Bis[4-(trifluoromethyl)phenyl]butane-1,4-diol (**5b**): $[\alpha]_D^{21} = -20.1$ (c = 0.4, $CHCl_3$). Described in the literature [211c]: $[\alpha]_D^{24} = +19.0$ (c = 0.1, $CHCl_3$) for the *syn*-(1*R*,4*R*)-diol; (f) (–)-(1*S*,4*S*)-1,4-Bis(3-chlorophenyl)butane-1,4-diol (**7b**): $[\alpha]_D^{21} = -41.0$ (c = 0.1, $CHCl_3$).

in low yield (10% conv). This hydroxy ketone **8c** was characterised by ^{1}H, ^{13}C and DEPT-NMR experiments and HRMS.

5.2.5. Final remarks

The chemical synthesis and bioreduction of a series of 1,4-diaryl-1,4-diones has been developed using the (*S*)-selective alcohol dehydrogenase from *Ralstonia* sp. (Scheme 5.10).

Scheme 5.10. Overview of the asymmetric synthesis of diols (1*S*,4*S*)-**1b-8b** and hydroxy ketone (4*S*)-**8c**.



First of all, a wide panel of diketones differing in the substitution of the aromatic ring was synthesised from commercial 4'-substituted and α-bromo-4'-substituted acetophenones in low to moderate yield (8-58%) following a reported protocol. Next, RasADH was found to be the best biocatalyst to achieve their bioreductions towards optically active (1*S*,4*S*)-diols. After optimisation of the reaction conditions (regeneration system, cosolvent, temperature and reaction time), diols **1b-8b** were synthesised in low to moderate conversions (8-79%) and excellent selectivity (>99% *ee* and >99% *de*) under mild reaction conditions. Only for the *o*-methoxylated diketone **8a**, the formation of the hydroxy ketone (4*S*)-**8c** was observed, which in fact was obtained in slightly higher conversion than the desired diol.

5.3. Experimental section

5.3.1. General information

The 4'-substituted acetophenones and α-bromo-4´-substituted acetophenones were purchased from TCI Europe (Zwijndrecht, Belgium). NADPH as enzyme cofactor and all the chemical reagents were obtained with the highest quality available from Sigma-Aldrich-Fluka (Steinheim, Germany). Alcohol dehydrogenases and GDH were obtained from Codexis Inc. (Redwood City, CA, USA). The production of *E. coli* overexpressed ADHs has been previously reported.[203-208]

5.3.2. General procedure for the synthesis of 1,4-diaryl-1,4-diketones 1a-8a

For the preparation of diketones **1a-8a** we followed a similar methodology to the one described in the literature.[231] Commercial anhydrous $ZnCl_2$ (2.72 g, 20 mmol) was placed into a one-neck 250 mL round-bottom flask and dried by melting under vacuum (1 torr) at 250-350 ºC for 15-20 min. After cooling under vacuum to room temperature, toluene (10 mL), diethylamine (1.03 mL, 10 mmol) and ^{t}BuOH (0.95 mL, 10 mmol) were successively added. The mixture was stirred until zinc chloride was fully dissolved (approx. 2 h), and then the corresponding acetophenone (8.0 mmol) and α-bromoacetophenone (5.0 mmol) were successively added. The mixture was stirred at room temperature for 4 days. After this, CH_2Cl_2 (300 mL) was added and the resulting organic phase was successively washed with an aqueous H_2SO_4 2.0 M solution (2 x 120 mL), water (1 x 150 mL) and brine (1 x 150 mL). The organic phase was dried over Na_2SO_4, filtered, and the solvents were evaporated under reduced pressure. Crude solids, except compounds **6a** and **8a** that were purified by column chromatography on silica gel (EtOAc/hexane 20%), were purified by crystallisation in absolute EtOH in order to yield the corresponding 1,4-diaryl-1,4-diketones **1a-8a** (8-58% yield).

5.3.3. Synthesis of racemic 1,4-diaryl-1,4-diols 1a-8b

Diketones **1a-8a** (0.3 mmol) were dissolved in THF (2.0 mL) and $NaBH_4$ (90.8 mg, 2.4 mmol) was slowly added at room temperature. Reactions were stirred for 2-4 h until completion. After this, an aqueous HCl 1.0 M solution (2.0 mL) was added and the mixtures extracted with CH_2Cl_2 (2 x 5 mL). The organic phases were

dried over Na_2SO_4, filtered and the solvents evaporated under reduced pressure. The reaction crudes were purified by column chromatography on silica gel (EtOAc/hexane mixtures), isolating the racemic 1,4-diaryl-1,4-diols **1a-8b** (62-90% yield).

5.3.4. Synthesis of racemic 4-hydroxy-1,4-bis(2-methoxyphenyl)butan-1-one 8c

Diketone **8a** (0.13 mmol) was dissolved in THF (0.9 mL) and $NaBH_4$ (21 mg, 0.54 mmol) was added at room temperature. The reaction was stirred for 30 min until the formation of the hydroxy ketone was observed by TLC analysis. After this, an aqueous HCl 1.0 M solution (2.0 mL) was added and the mixture extracted with CH_2Cl_2 (2 x 5 mL). The organic layers were dried over Na_2SO_4, filtered and the solvents evaporated under reduced pressure. The reaction crude was purified by column chromatography on silica gel (EtOAc/hexane mixtures), isolating the racemic hydroxy ketone **8c** in 5% yield.

5.3.5. Enzymatic conversion of 1,4-diaryl-1,4-diols 1b-8b using overexpressed *E. coli*/RasADH

Lyophilised *E. coli*/RasADH cells (15 or 30 mg), the cosolvent (2.5-10% v/v), 1 mM NADPH (60 µL of a 10 mM stock solution), 50 mM glucose (60 µL of a 500 mM stock solution) and glucose dehydrogenase (5 U) were added into an Eppendorf tube containing 1,4-diaryl-1,4-diketones (**1a-8a**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5 (420 µL). The reaction was shaken at 30-40 ºC and 250 rpm for 24-48 h. After this time, the mixture was extracted with ethyl acetate (2 x 500 µL), the organic layers separated by centrifugation (2 min, 5700 x *g*), combined and finally dried over Na_2SO_4. Conversion, diastereomeric excess and enantiomeric excess values of 1,4-diaryl-1,4-diols **1b-8b** were determined by HPLC.

5.3.6. Bioreduction of 1,4-diphenylbutane-1,4-dione 1a using commercial alcohol dehydrogenases

In a 2.0 mL Eppendorf tube, KRED (2 mg) was added to 900 µL phosphate buffer 125 mM pH 7.0 (1.25 mM $MgSO_4$, 1 mM $NADP^+$) containing 1,4-diphenylbutane-1,4-dione (**1a**, 25 mM), DMSO (25 µL) and ^{i}PrOH (100 µL). The reaction was incubated at 250 rpm and 30 ºC for 24 h. Then, the mixture was extracted with ethyl acetate (2 x 500 µL), the organic layers separated by centrifugation (2 min, 5700 x *g*), combined and finally dried over Na_2SO_4.

Conversion, diastereomeric excess and enantiomeric excess values of 1,4-diphenylbutane-1,4-diol **1b** were determined by HPLC.

5.3.7. Semi-preparative bioreductions of 1,4-diaryl-1,4-diones 1a-5a, 7a and 8a using overexpressed *E. coli*/RasADH

Lyophilised *E. coli*/RasADH cells (50 mg for 1,4-diketon **1a**, 20 mg for 1,4-diketones **2a**-**4a**, 100 mg for 1,4-diketone **5a** and 150 mg for 1,4-diketone **7a**), THF (5% v/v), NADPH (1 mM), glucose (50 mM) and GDH (50-100U) were added into an Erlenmeyer flask containing a suspension of the corresponding 1,4-diketone (25 mM) in Tris·HCl buffer 50 mM pH 7.5. The reaction was incubated at 30 ºC and 250 rpm for 24 h (**1a**-**5a**) or at 40 ºC and 250 rpm for 48 h (**7a**). Then, the mixture ethyl acetate (3 x 15 mL). The organic layers were separated by centrifugation (5 min, 400 x *g*), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography on silica gel (EtOAc/hexane mixtures), isolating the enantiopure (1*S*,4*S*)-diols **1b**-**5b**, **7b** and **8b** in moderate to high yields (10-73%).

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Stereoselective Enzymatic Reduction of 1,4-Diaryl-1,4-Diones to the Corresponding Diols Employing Alcohol Dehydrogenases

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Abstract: Due to the steric hindrance of the starting prochiral ketones, the preparation of chiral 1,4-diaryl-1,4-diols through the asymmetric hydrogen transfer reaction has been mainly restricted to the use of metal-based catalysts, oxazaborolidines, or organocatalysts. Herein, we demonstrated the versatility of oxidoreductases, finding overexpressed alcohol dehydrogenase from *Ralstonia* sp. (*E. coli*/RasADH) as the most active and stereoselective biocatalyst. Thus, the preparation of a set of 1,4-diaryl-1,4-diols bearing different pattern substitutions in the aromatic ring was achieved with complete diastereo- and enantioselectivity under mild reaction conditions.

Keywords: alcohol dehydrogenases; asymmetric synthesis; bioreduction; 1,4-diols; diketones

1. Introduction

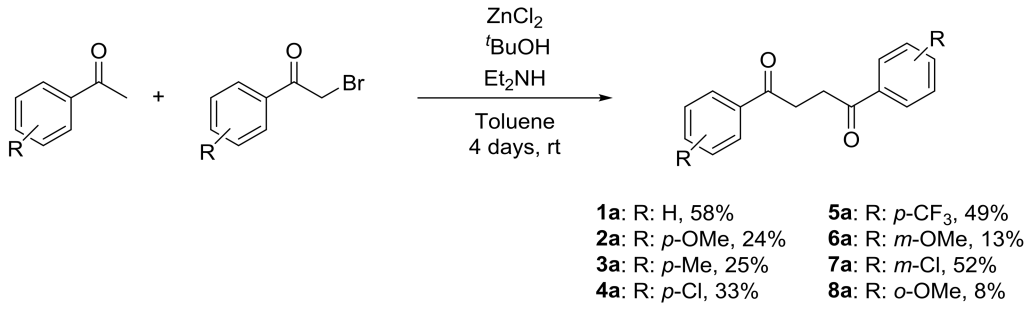
Optically active 1,4-diols can be found as structural motifs in a huge number of biologically active compounds as pharmaceuticals, flavors, and fragrances, but also in chiral ligands and auxiliaries for asymmetric synthesis purposes [1,2]. Their synthetic versatility has also been utilized as precursors of valuable compounds such as 2,5-disubstituted pyrrolidines and phosphine derivatives used as ligands in asymmetric hydrogenations [3–5]. In this context, 1,4-butanediol plays a key role due its applications in the food and cosmetic industry, but also as precursor of plastics, pharmaceuticals, fibers, solvents, and biologically active lactones, among others. For these reasons, the preparation of enantioenriched 1,4-diols using efficient and selective methodologies is a field of interest in organic chemistry. Different asymmetric strategies have been reported for their synthesis, the catalytic asymmetric transfer hydrogenation (CATH) of prochiral 1,4-dicarbonylic compounds being one of the most recurrent methods [6,7]. CATH is the most straightforward approach for obtaining the optically pure diols in quantitative yield, but presents difficulty in that the hydroxy ketone intermediate can also be attained and that (up to) four possible diastereoisomers can be formed. Therefore, several selective chiral catalysts and reducing reagents have been developed for the stereoselective asymmetric reduction of these prochiral diketones, including rhodium or iridium complexes [8], different borane complexes such as oxazaborolidines [9,10], or proline-type derivatives as catalysts in combination with the appropriate reducing agents [11,12].

Biocatalysis has emerged in the last decades as a mature technology for the production of optically active (poly)alcohols by means of the stereoselection displayed by different enzymes [13–17].

Among the wide set of (poly)alcohol structures that can be obtained using biocatalysts, the production of optically active 1,4-dialkyl-1,4-diols and their corresponding acetates has been described mainly by the action of lipases through resolution procedures involving acylation and transesterification processes [18–25], and only the resolution of the bulkier 1-phenylpentane-1,4-diol or its acetate has been successfully achieved [26,27]. Alcohol dehydrogenases (ADHs) are valuable biocatalysts that have been widely employed for the synthesis of chiral alcohols due to their usually excellent selectivity [28–31], including dicarbonyl bioreduction processes [32,33]. Among the wide set of alcohol structures that can be obtained with these enzymes, optically active 1,4-dialkyl-1,4-diols have been described. Efforts have been especially focused on the preparation of chiral 2,5-hexanediol, a key starting material for the preparation of several catalysts and drugs [34–37]. However, until now there are no examples about the enzymatic reduction of 1,4-diaryl-1,4-diketones to obtain the corresponding chiral 1,4-diaryl-1,4-diols, as these substrates are probably too bulky for ADH recognition. In the present paper, we describe for the first time the use of ADHs for the stereoselective preparation under mild reaction conditions (aqueous buffer and 30 °C) of a representative panel of 1,4-diaryl-1,4-diols bearing different pattern substitutions in the aromatic ring.

2. Results and Discussion

As a first step, the chemical synthesis of prochiral 1,4-diketones **1**–**8a** was developed to later study the biocatalyzed stereoselective synthesis of the optically active 1,4-diols **1**–**8b**. Different methods have been described for the preparation of these substrates [38,39]. Among them, we have carried out the synthesis of these compounds through the cross aldol condensation of 4′-substituted acetophenones with α-bromo-4′-substituted acetophenones, followed by 1,3-dehydrobromination of these products, and cleavage of the corresponding activated cyclopropyl intermediates in the presence of $ZnCl_2$,·^{t}BuOH,·and Et_2NH (Scheme 1) [40]. Thus, 1,4-diketones **1**–**8a** were obtained in yields varying from 8% for the *o*-methoxy derivative (**8a**) to 58% for 1,4-diphenylbutane-1,4-dione (**1a**).



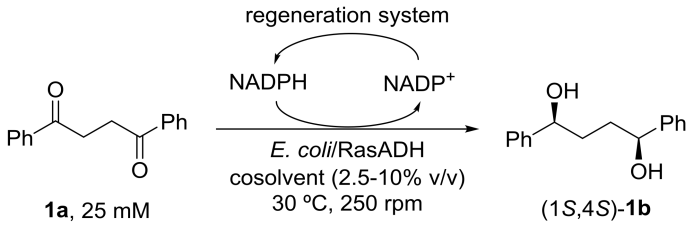
Scheme 1. Synthesis of diketones **1**–**8a** from the corresponding 4′-substituted acetophenones and the α-bromo-4′-substituted acetophenones.

Once synthesized, the obtained diketones were tested in the bioreduction processes catalyzed by a set of commercially available and "made in house" alcohol dehydrogenases. As model substrate 1,4-diphenylbutane-1,4-dione (**1a**) was chosen. Thus, lyophilized cells of *E. coli* overexpressing alcohol dehydrogenases from *Ralstonia* sp. (*E. coli*/RasADH) [41], *Lactobacillus brevis* (*E. coli*/LBADH) [42], *Sphingobium yanoikuyae* (*E. coli*/SyADH) [43], *Thermoanaerobacter ethanolicus* (*E. coli*/TeSADH) [44] and *Rhodococcus ruber* (*E. coli*/ADH-A) [45] were tested. From previous studies, 25 mM substrate concentration was initially considered and 2.5% *v*/*v* of dimethylsulfoxide (DMSO) was used as cosolvent due to the low solubility of the ketones in the aqueous media, using Tris·HCl buffer 50 mM pH 7.5, and isopropanol (IPA) or glucose dehydrogenase (GDH) with glucose as reducing agents to recycle the nicotinamide cofactor [46]. The reactions were incubated for 24 h at 30 °C, observing only activity when *E. coli*/RasADH was utilized (Table 1). This result is not entirely surprising, as this NADP-dependent enzyme has been previously described as a valuable biocatalyst for the reduction

of sterically hindered substrates [47]. Hence, a conversion of 56% was reached by using this enzyme, leading to the formation of diol (1*S*,4*S*)-**1b** in a highly selective manner (98% *de*, >99% *ee*), not observing the hydroxy ketone intermediate (Table 1, entry 1). Remarkably, 82% conversion was obtained after 48 h maintaining the excellent selectivity (Table 1, entry 2).

In order to optimize the bioreduction using this enzymatic preparation, different conditions were studied (Table 1, entries 3–17). Firstly, in similar conditions the absence of cosolvent led to a decrease in the conversion (72%–82% conversion, entries 2–3). When the usual regeneration system (glucose/GDH) was changed to IPA (entry 4), the diastereoselectivity of the process dropped from 98% *de* to 78% *de*.

Table 1. Bioreduction of 1,4-diphenylbutane-1,4-dione (**1a**) using *E. coli*/RasADH [a].



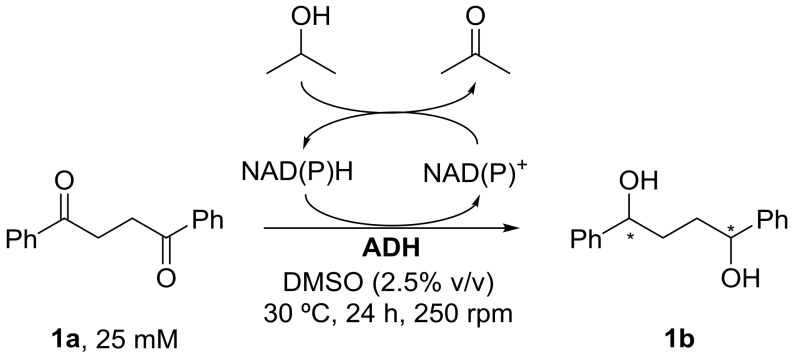
Entry	Regeneration System	Cosolvent	% (*v*/*v*)	t (h)	Conversion (%) [b]	*de* [b,c]	*ee* (%) [b,d]
1	Glucose/GDH	DMSO	2.5	24	56	99:1	>99
2	Glucose/GDH	DMSO	2.5	48	82	99:1	>99
3	Glucose/GDH	-	-	48	72	99:1	>99
4	IPA	-	-	48	72	89:11	>99
5	Glucose/GDH	EtOH	2.5	24	57	99:1	>99
6	Glucose/GDH	1,4-dioxane	2.5	24	72	99:1	>99
7	Glucose/GDH	1,4-dioxane	5	24	78	>99	>99
8	Glucose/GDH	1,4-dioxane	10	24	79	99:1	>99
9	Glucose/GDH	1,4-dioxane	10	48	80	99:1	>99
10	Glucose/GDH	MTBE	2.5	24	70	>99	>99
11	Glucose/GDH	MTBE	5	24	83	>99	>99
12	Glucose/GDH	MTBE	10	24	77	>99	>99
13	Glucose/GDH	MTBE	10	48	77	>99	>99
14	Glucose/GDH	THF	2.5	24	72	99:1	>99
15	Glucose/GDH	THF	5	24	88	>99	>99
16	Glucose/GDH	THF	10	24	80	99:1	>99
17	Glucose/GDH	THF	10	48	82	99:1	>99

[a] For reaction details, see Materials and methods section; [b] Measured by High Performance Liquid Chromatography (HPLC); [c] Ratio of (1*S*,4*S*) and (1*R*,4*R*) to *meso*-diol; [d] Enantiomeric excess of (1*S*,4*S*)-**1b**.

Taking all these data into account, an exhaustive cosolvent screening was performed (entries 5–17). Ethanol (EtOH), 1,4-dioxane, methyl *tert*-butyl ether (MTBE), and tetrahydrofuran (THF) were chosen as suitable cosolvents for the biotransformation and employed in different quantities. When using 2.5% *v*/*v* of the organic solvent, for instance 1,4-dioxane, MTBE, and THF (entries 6, 10, and 14), better conversions than DMSO (entry 1) and EtOH (entry 5) were achieved, also affording excellent selectivities. An increase in the amount of these cosolvents from 2.5% to 5% *v*/*v* revealed even better conversions (entries 7, 11 and 15) and perfect diastereoselectivities (>99% *de*). Finally, the amount of cosolvent was increased up to 10% *v*/*v* (entries 8, 12, and 16), but unfortunately this did not lead to any improvement. Moreover, higher reaction times (from 24 h to 48 h, entries 9, 13, and 17) did not show better results. It must be pointed out that the hydroxy ketone intermediate was not observed in any case.

Commercial ADHs purchased from Codexis were also tested, as these enzymes have recently demonstrated interesting applications [48]. In this case, IPA was utilized as hydrogen source and phosphate buffer 125 mM pH 7.0 as suitable reaction medium. DMSO (2.5% *v*/*v*) was added again to solubilize the ketone. Thus, diketone **1a** was a suitable substrate for five of them (69–89% conversion, 74–99% *de*, Table 2). ADH-P2-D03 led to the formation of the opposite enantiomer (1*R*,4*R*)-**1b** (entry 4) with excellent enantioselectivity and 74% *de*. Thus, by modifying the biocatalyst, both enantiomers of diol **1b** could be achieved. Again, the hydroxy ketone intermediate was not detected.

Table 2. Bioreduction of 1,4-diphenylbutane-1,4-dione (**1a**) using commercially available alcohol dehydrogenases (ADHs) [a].



Entry	ADH	Conversion (%) [b]	*de* [b,c]	*ee* (%) [b,d]
1	P1-B02	85	>99	>99
2	P1-B10	79	>99	>99
3	P1-B12	75	99:1	>99
4	P2-D03	89	87:13	>99 [e]
5	P2-D11	69	98:2	>99

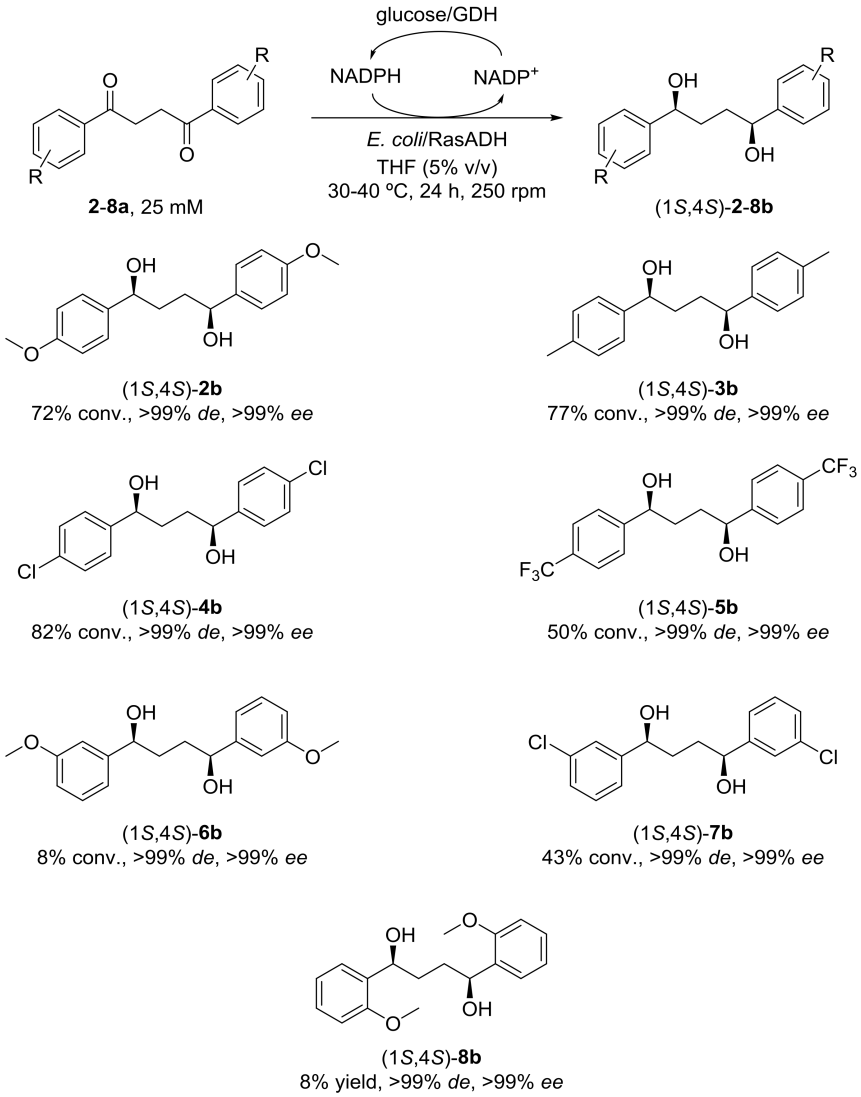
[a] For reaction conditions, see Materials and methods section; [b] Measured by HPLC; [c] Ratio of (1*S*,4*S*) and (1*R*,4*R*) to *meso*-(1*R*,4*S*); [d] Enantiomeric excess of (1*S*,4*S*)-**1b**; [e] Enantiomeric excess of (1*R*,4*R*)-**1b**.

Seeking further exploitation of the synthetic approach, first we considered the study of similar substrates that differed at the *para* substitution of the aromatic ring. For this purpose, biotransformations with *E. coli*/RasADH and substrates **2**–**5a** were set up using the best conditions found in the reduction of diketone **1a** (Table 1, entry 15, 5% *v*/*v* THF, 30 °C, 24 h). The results are summarized in Scheme 2. In all cases, sole formation of (1*S*,4*S*)-diols **2**–**5b** was observed (>99% *de* and >99% *ee*), with no detection of the corresponding hydroxy ketone intermediates in any case. For chlorinated substrate **4a** (82% conversion), the results were comparable to those obtained with the model substrate (88% conversion). A small drop in the conversion was observed for methoxylated (**2a**) and methylated (**3a**) compounds (72% and 77%, respectively), while bulkier trifluoromethylated diketone **5a** showed a significant loss of activity (50% conversion).

Then, the study was also performed on substrates bearing *meta* or *ortho* substitution at the phenyl ring. Hence, diketones **6**–**8a** were used as substrates for RasADH. However, very low yields (8%) were observed for the corresponding enantiopure methoxylated derivatives (1*S*,4*S*)-**6b** and **8b**. A special mention is deserved for *ortho*-substituted diketone **8a**, that provided hydroxy ketone **8c** at higher extent (36%) than the corresponding diol **8b**. Regarding the *m*-chloro derivative **7a**, that showed higher enzymatic conversion (22%), two different experiments were also carried out. On the one hand, the amount of enzyme was doubled leading to the formation of (1*S*,4*S*)-**7b** in 34% conversion after 24 h. On the other hand, the temperature was raised up to 40 °C, obtaining (1*S*,4*S*)-**7b** in 43% conversion after 24 h. These results showed that the substitution pattern had an effect on the enzymatic activity, achieving the most valuable results for the *para*-substituted diketones.

Next, the bioreductions of diketones **2**–**5a** were also attempted with the commercially available ADHs reported in Table 2. Unfortunately, no conversion was observed in any case, demonstrating the difficulty of these bioconversions, only achieved with RasADH.

Finally, preparative biotransformations up to 100-mg scale were carried out using *E. coli*/RasADH under the optimized reaction conditions (Figure 1), using THF (5% *v*/*v*) as cosolvent at 30 °C and glucose/GDH as NADPH regeneration system. After 24 h, the conversions obtained were between 40% and 90%, isolating the corresponding diastereo- and enantiopure diols (1*S*,4*S*)-**1**–**5**,**7b** in moderate to high yields. The highest yield was obtained for the unsubstituted diol (1*S*,4*S*)-**1b**, which was recovered in 73% yield, while the *m*-chloro compound (**7b**) led to the lowest yield (10%).



Scheme 2. Selective bioreduction of diketones **2**–**8a** using *E. coli*/RasADH and tetrahydrofuran (THF) as cosolvent.

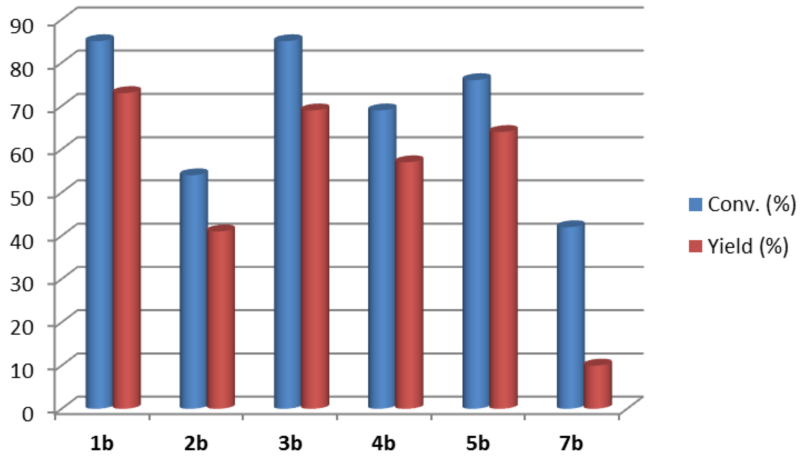


Figure 1. Conversions and isolated yields for the RasADH-catalyzed preparative biotransformations to obtain the optically active diols (1*S*,4*S*)-**1**–**5**,**7b**.

3. Conclusions

A series of 1,4-diaryl-1,4-diols were synthesized via ADH-catalyzed bioreduction from the corresponding bulky 1,4-diaryl-1,4-diketones. Among the different enzymatic preparations used, ADH from *Ralstonia* sp. (RasADH) overexpressed in *E. coli* afforded the best results in terms of conversions and diastereo- and enantiomeric excess, usually obtaining the (1*S*,4*S*)-diols, and only observing the formation of the hydroxy ketone intermediate in the case of the *o*-methoxylated derivative. The cofactor regeneration system and the addition of an organic cosolvent were studied, showing that glucose/GDH and THF were the best options. Herein we have shown that the bioreduction can be a useful alternative under mild conditions to obtain these appealing chiral compounds, which can be further employed as synthons for the preparation of other valuable derivatives.

4. Materials and Methods

As for the substrates and products studied in this contribution, HPLC separations, HPLC chromatograms of optically active 1,4-diaryl-1,4-diols and NMR spectra, please see the Supplementary Materials.

4.1. General Materials and Methods

The 4′-Substituted acetophenones and α-bromo-4′-substituted acetophenones were purchased from TCI Europe (Zwijndrecht, Belgium). NADPH as enzyme cofactor and all the other chemical reagents were obtained with the highest quality available from Sigma-Aldrich-Fluka (Steinheim, Germany). Alcohol dehydrogenases and GDH were obtained from Codexis Inc., (Redwood City, CA, USA). The production of *E. coli* overexpressed ADHs has been previously reported and these enzymatic preparations have been provided by Prof. Wolfgang Kroutil (University of Graz) [35,41,43].

NMR spectra were recorded on a Bruker AV300 MHz spectrometer (Bruker Co., Faellanden, Switzerland). All chemical shifts (δ) are given in parts per million (ppm) and referenced to the residual solvent signal as internal standard. Measurement of the optical rotation values was carried out at 590 nm on a Autopol IV Automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). High performance liquid chromatography (HPLC) analyses were performed for conversion, enantiomeric excess, and diastereomeric excess value measurements using a Hewlett Packard 1100 chromatograph UV detector at 210 nm (Agilent Technologies, Inc., Wilmington, DE, USA). As chiral columns, Chiralpak AD-H (25 cm × 4.6 mM) and Chiralpak IA (25 cm × 4.6 mM) were used (Chiral Technologies, Mainz, Germany). Thin-layer chromatography (TLC) analyses were conducted with Merck Silica Gel 60 F254 precoated plates (Merck KGaA, Darmstadt, Germany) and visualized with UV and potassium permanganate stain. Column chromatography purifications were performed using Merck Silica Gel 60 (230–400 mesh, Merck KGaA, Darmstadt, Germany).

4.2. General Procedure for the Synthesis of 1,4-Diaryl-1,4-Diketones **1–8a**

For the preparation of diketones **1–8a** we followed a similar methodology to the one described in the literature [40]. Commercial anhydrous $ZnCl_2$ (2.72 g, 20 mMol) was placed into a one-neck 250-mL round-bottom flask and dried by melting under vacuum (1 torr) at 250–350 °C for 15–20 min. After cooling under vacuum to room temperature, toluene (10 mL), diethylamine (1.03 mL, 10 mmol) and ^{t}BuOH (0.95 mL, 10 mmol) were successively added. The mixture was stirred until zinc chloride was fully dissolved (approx. 2 h), and then the corresponding acetophenone (8.0 mMol) and α-bromoacetophenone (5.0 mMol) were successively added. The mixture was stirred at room temperature for 4 days. After this, CH_2Cl_2 (300 mL) was added and the resulting organic phase was successively washed with an aqueous H_2SO_4 2.0 M solution (2 × 120 mL), water (1 × 150 mL) and brine (1 × 150 mL). The organic phase was dried over Na_2SO_4, filtered, and the solvents were evaporated under reduced pressure. Crude solids, except compounds **6a** and **8a** that were purified by column chromatography (Hexane/EtOAc 4:1), were purified by crystallization in absolute EtOH in

order to yield the corresponding 1,4-diaryl-1,4-diketones (see Supplementary Materials) **1a** (0.69 g, 58% yield), **2a** (0.36 g, 24%), **3a** (0.33 g, 25%), **4a** (0.51 g, 33%), **5a** (0.91 g, 49%), **6a** (0.19 g, 13%), **7a** (0.79 g, 52%) and **8a** (0.11 g, 8%) [38–40,49–51].

*4.3. General Procedure for the Synthesis of Racemic 1,4-Diaryl-1,4-Diols **1–8b***

Diketones **1–8a** (0.3 mMol) were dissolved in THF (2.0 mL) and $NaBH_4$ (90.8 mg, 2.4 mMol, 8.0 equiv.) was slowly added at room temperature. Reactions were stirred for 2–4 h until completion. After this, an aqueous HCl 1.0 M solution (2.0 mL) was added and the mixtures were extracted with CH_2Cl_2 (2 × 5 mL). The organic phases were dried over Na_2SO_4, filtered and the solvents were evaporated under reduced pressure. The reaction crudes were purified by column chromatography (EtOAc/hexane mixtures), isolating the racemic 1,4-diaryl-1,4-diols (±)-**1b** (61.7 mg, 85% yield), (±)-**2b** (65.2 mg, 72%), (±)-**3b** (65.6 mg, 81%), (±)-**4b** (71.9 mg, 77%), (±)-**5b** (102.1 mg, 90%), (±)-**6b** (30.0 mg, 52%), (±)-**7b** (58.3 mg, 62%) and (±)-**8b** (33.0 mg, 66%).

4.4. General Procedure for the Synthesis of Racemic 4-Hydroxy-1,4-Bis(2-methoxyphenyl)butan-1-one **8c**

Diketone **8a** (0.13 mMol) was dissolved in THF (0.9 mL) and $NaBH_4$ (21 mg, 0.54 mMol, 4.0 equiv.) was added at room temperature. The reaction was stirred for 30 min until the formation of the hydroxy ketone was observed by TLC analysis. After this, an aqueous HCl 1.0 M solution (2.0 mL) was added and the mixture was extracted with CH_2Cl_2 (2 × 5 mL). The organic layers were dried over Na_2SO_4, filtered and the solvents were evaporated under reduced pressure. The reaction crude was purified by column chromatography (EtOAc/hexane mixtures), isolating the racemic hydroxy ketone (±)-**8c** (2 mg, 5% yield).

4.5. General Procedure for the Enzymatic Conversion of 1,4-Diaryl-1,4-Diols **1–8b** *Using Overexpressed E. coli/RasADH*

Lyophilized *E. coli*/RasADH cells (15–30 mg), the cosolvent (2.5%–10% *v*/*v*), 1 mM $NADP^+$ (60 µL of a 10 mM stock solution), 50 mM glucose (60 µL of a 500 mM stock solution) and glucose dehydrogenase (5 U) were added into an Eppendorf tube containing 1,4-diaryl-1,4-diketones (**1–8a**, 25 mM) in Tris·HCl buffer 50 mM pH 7.5 (420 µL). The reaction was shaken at 30–40 °C and 250 rpm for 24–48 h. After this time, the mixture was extracted with ethyl acetate (2 × 500 µL), the organic layers separated by centrifugation (2 min, 5700× *g*), combined and finally dried over Na_2SO_4. Conversion, diastereomeric excess and enantiomeric excess values of 1,4-diaryl-1,4-diols **1–8b** were determined by HPLC (see Supplementary Materials).

4.6. General Procedure for the Bioreduction of 1,4-Diphenylbutane-1,4-Dione **1a** *Using Commercial Alcohol Dehydrogenases*

In a 2.0 mL Eppendorf tube, KRED (2 mg) was added to 900 µL phosphate buffer 125 mM pH 7.0 (1.25 mM $mgSO_4$, 1 mM $NADP^+$) containing 1,4-diphenylbutane-1,4-dione (**1a**, 25 mM), DMSO (25 µL) and ^{i}PrOH (100 µL). The reaction was incubated at 250 rpm and 30 °C for 24 h. Then, the mixture was extracted with ethyl acetate (2 × 500 µL), the organic layers separated by centrifugation (2 min, 5700× *g*), combined and finally dried over Na_2SO_4. Conversion, diastereomeric excess and enantiomeric excess values of 1,4-diphenylbutane-1,4-diol (**1b**) were determined by HPLC.

4.7. Preparative Bioreductions of 1,4-Diarylbutane-1,4-Diones **1–5a**, **7a**, *and* **8a** *Using Overexpressed E. coli/RasADH*

Lyophilized *E. coli*/RasADH cells (50 mg for 1,4-diketone **1a**, 20 mg for 1,4-diketones **2–4a**, 100 mg for 1,4-diketone **5a**, and 150 mg for 1,4-diketone **7a**). THF (5% *v*/*v*), $NADP^+$ (1 mM), glucose (50 mM), and GDH (50–100 U), were added into an Erlenmeyer flask containing a suspension of the corresponding 1,4-diketone (25 mM) in Tris·HCl buffer 50 mM pH 7.5. The reaction was incubated at 30 °C and 250 rpm for 24 h (**1a–5a**) or at 40 °C and 250 rpm for 48 h (**7a**). Then, the mixture was

extracted with ethyl acetate (3 × 15 mL). The organic layers were separated by centrifugation (5 min, 4000× *g*), combined, and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography (EtOAc/hexane mixtures), isolating the enantiopure (1*S*,4*S*)-diols in moderate to high yields (10%–73%).

(–)-(1*S*,4*S*)-1,4-Diphenylbutane-1,4-diol [(1*S*,4*S*)-**1b**]. Yield: 37 mg (73%). R_f = 0.36 (40% EtOAc/hexane). Mp: 74–75 °C. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.76–1.96 (m, 2H), 1.88–2.02 (m, 2H), 2.59 (s, 2OH), 4.72 (dd, J_{HH} = 6.5, 3.9 Hz, 2H), 7.08–7.50 (m, 10H) ppm. ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 35.9 ($2CH_2$), 74.6 (2CH), 125.8 (4CH), 127.5 (2CH), 128.4 (4CH), 144.6 (2C) ppm. HRMS (ESI$^+$, *m/z*): calcd for $(C_{16}H_{18}NaO_2)^+$ (M + Na)$^+$ 265.1204; found 265.1199. $[\alpha]_D^{21}$ = −57.2 (*c* = 0.5, $CHCl_3$), described in the literature [52]: $[\alpha]_D^{25}$ = −59.0 (*c* = 1.0, $CHCl_3$) for *syn*-(*S*,*S*)-diol.

(–)-(1*S*,4*S*)-1,4-Bis(4-methoxyphenyl)butane-1,4-diol [(1*S*,4*S*)-**2b**]. Yield: 9 mg (41%). R_f = 0.21 (40% EtOAc/hexane). ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.71–1.85 (m, 2H), 1.85–2.00 (m, 2H), 3.82 (s, 6H), 4.68 (m, 2H), 6.88 (d, J_{HH} = 8.5, 4H), 7.27 (d, J_{HH} = 8.1 Hz, 4H) ppm. ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 34.4 ($2CH_2$), 53.6 ($2CH_3$), 81.0 (2CH), 113.9 (4CH), 127.5 (2CH), 135.2 (2C), 159.1 (2C) ppm. HRMS (ESI$^+$, *m/z*): calcd for $(C_{18}H_{22}NaO_4)^+$ (M + Na)$^+$ 325.1415; found 325.1410. $[\alpha]_D^{21}$ = −43.0 (*c* = 0.1, $CHCl_3$), described in the literature [11]: $[\alpha]_D^{22}$ = +41.6 (*c* = 1.0, $CHCl_3$) for *syn*-(*R*,*R*)-diol.

(–)-(1*S*,4*S*)-1,4-Bis(4-methylphenyl)butane-1,4-diol [(1*S*,4*S*)-**3b**]. Yield: 14 mg (69%). R_f = 0.27 (40% EtOAc/hexane). Mp: 114–115 °C. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.72–1.80 (m, 2H), 1.88–2.04 (m, 2H), 2.28 (s, 2OH), 2.36 (s, 6H), 4.70 (dd, J_{HH} = 6.8, 4.4 Hz, 2H), 7.16 (d, J_{HH} = 8.0 Hz, 4H), 7.24 (d, J_{HH} = 8.0 Hz, 4H) ppm. ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 21.1 ($2CH_3$), 35.8 ($2CH_2$), 74.5 (2CH), 125.8 (4CH), 129.1 (4CH), 137.2 (2C), 141.7 (2C) ppm. HRMS (ESI$^+$, *m/z*): calcd for $(C_{18}H_{22}NaO_2)^+$ (M + Na)$^+$ 293.1517; found 293.1512. $[\alpha]_D^{21}$ = −45.2 (*c* = 0.2, CH_2Cl_2), described in the literature [4]: $[\alpha]_D^{25}$ = −47.0 (*c* = 1.0, $CHCl_3$) for *syn*-(*S*,*S*)-diol.

(–)-(1*S*,4*S*)-1,4-Bis(4-chlorophenyl)butane-1,4-diol [(1*S*,4*S*)-**4b**]. Yield: 11 mg (57%). R_f = 0.45 (40% EtOAc/hexane). Mp: 112–113 °C. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.69–2.01 (m, 4H), 2.31 (s, 2OH), 4.71 (d, J_{HH} = 5.9 Hz, 2H), 7.09–7.46 (m, 8H) ppm. ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 35.8 ($2CH_2$), 73.8 (2CH), 127.1 (4CH), 128.5 (4CH), 133.2 (2C), 142.9 (2C) ppm. HRMS (ESI$^+$, *m/z*): calcd for $(C_{16}H_{16}Cl_2NaO_2)^+$ (M + Na)$^+$ 333.0425; found 333.0419. $[\alpha]_D^{21}$ = −22.1 (*c* = 0.2, $CHCl_3$), described in the literature [11]: $[\alpha]_D^{21}$ = +24.4 (*c* = 1.05, $CHCl_3$) for *syn*-(*R*,*R*)-diol.

(–)-(1*S*,4*S*)-1,4-Bis[4-(trifluoromethyl)phenyl]butane-1,4-diol [(1*S*,4*S*)-**5b**]. Yield: 63 mg (64%). R_f = 0.51 (40% EtOAc/hexane). Mp: 159–160 °C. ^{1}H NMR (300.13 MHz, MeOD): δ 1.67–1.77 (m, 2H), 1.78–1.92 (m, 2H), 3.30 (s, 2OH), 4.73 (apparent t, J_{HH} = 5.6 Hz, 2H), 7.49 (d, J_{HH} = 8.2 Hz, 4H), 7.60 (d, J_{HH} = 8.2 Hz, 4H) ppm. ^{13}C NMR (300.13 MHz, MeOD): δ 34.8 ($2CH_2$), 72.5 (2CH), 124.4 (q, J_{CF} = 271.2 Hz, $2CF_3$), 124.7 (q, J_{CF} = 3.6 Hz, 4CH), 126.1 (4CH), 128.9 (q, J_{CF} = 32.0 Hz, 2C), 149.6 (2C) ppm. HRMS (ESI$^+$, *m/z*): calcd for $(C_{18}H_{16}F_6NaO_2)^+$ (M + Na)$^+$ 401.0952; found 401.0951. $[\alpha]_D^{25}$ = −20.1 (*c* = 0.4, $CHCl_3$), described in the literature [3]: $[\alpha]_D^{24}$ = +19.0 (*c* = 0.1, $CHCl_3$) for *syn*-(*R*,*R*)-diol.

(–)-(1*S*,4*S*)-1,4-Bis(3-chlorophenyl)butane-1,4-diol [(1*S*,4*S*)-**7b**]. Yield: 8 mg (10%). R_f = 0.35 (40% EtOAc/hexane). ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 1.80–1.93 (m, 4H), 2.54 (s, 2OH), 4.71 (m, 2H), 7.02–7.51 (m, 8H) ppm. ^{13}C NMR (300.13 MHz, $CDCl_3$): δ 35.8 ($2CH_2$), 74.0 (2CH), 124.0 (2CH), 126.1 (2CH), 127.8 (2CH), 129.9 (2CH), 134.5 (2C), 146.7 (2C) ppm. HRMS (ESI$^+$, *m/z*): calcd for $(C_{16}H_{16}Cl_2NaO_2)^+$ (M + Na)$^+$ 333.0425; found 333.0426. $[\alpha]_D^{19}$ = −41.0 (*c* = 0.1, $CHCl_3$).

To obtain the hydroxy ketone **8c**, lyophilized *E. coli*/RasADH cells (80 mg), THF (5% *v*/*v*), $NADP^+$ (1 mM), glucose (50 mM), and GDH (50 U), were added into an Erlenmeyer flask containing a suspension of the 1,4-diketone **8a** (20 mg, 25 mM) in Tris·HCl buffer 50 mM pH 7.5. The reaction was

incubated at 30 °C and 250 rpm for 24 h. Then, the mixture was extracted with ethyl acetate (3 × 15 mL) and the organic layers were separated by centrifugation (5 min, 4000× *g*), combined and finally dried over Na_2SO_4. The reaction crude was purified by column chromatography (EtOAc/hexane mixtures), isolating **8c** (2 mg, 10% yield).

4-Hydroxy-1,4-bis(2-methoxyphenyl)butan-1-one (**8c**). R_f = 0.41 (40% EtOAc/hexane). ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 2.13–2.36 (m, 2H), 3.00 (s, OH), 3.05–3.29 (m, 2H), 3.85 (s, OCH_3), 3.88 (s, OCH_3), 6.79–7.07 (m, 4H), 7.25 (m, 1H), 7.38 (d, J_{HH} = 7.5 Hz, 1H), 7.40–7.51 (t, J_{HH} = 7.8 Hz, 1H), 7.69 (dd, J_{HH} = 7.7, 1.8 Hz, 1H) ppm. HRMS (ESI$^+$, *m/z*): calcd for $(C_{18}H_{20}NaO_4)^+$ $(M + Na)^+$ 323.1259; found 323.1260.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/8/4/150/s1. Substrates and products studied in this contribution, 2. HPLC separations, 3. HPLC chromatograms of optically active 1,4-diaryl-1,4-diols, 4. NMR spectra.

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Chapter 6

A designer natural deep eutectic solvent to recycle the cofactor in alcohol dehydrogenase-catalysed processes

6.1. Bibliographic background

In the Preface of this Doctoral Thesis the interest that neoteric solvents have awaken in the last years as environmentally friendly solvents for (bio)catalytic processes has already been introduced. In particular, deep eutectic solvents (DES) have risen up as environmentally benign alternatives to traditional volatile organic solvents.[52] They are prepared by simply mixing the individual components (generally a HBA and a HBD) at moderate temperature, which results in a waste-free process. Natural deep eutectic solvents (NADES) have attracted special attention since they are composed by plant based primary metabolites (organic acids, sugars, alcohols, amines and amino acids).[53]

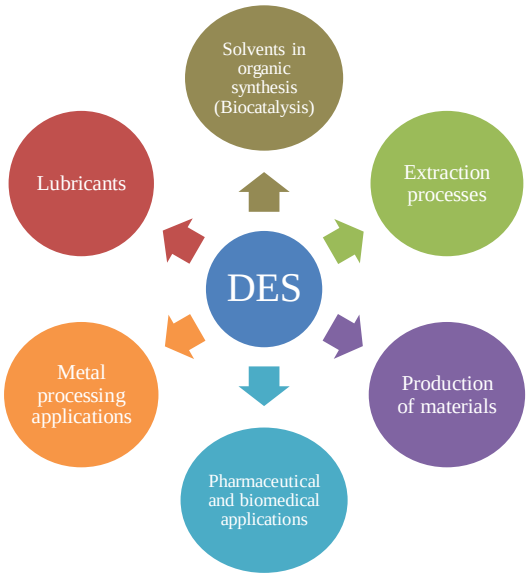

Figure 6.1. DES applications.

Due to their simple preparation, low cost and biodegradable properties, DES have been applied in several research fields (Figure 6.1) including their role in synthetic applications as reaction media[52b,233] in organocatalysis,[234] in organometallic chemistry[235] and also in Biocatalysis.[236] Furthermore, the use of

[233] (a) Q. H. Zhang, K. D. Vigier, S. Royer, F. Jerome, *Chem. Soc. Rev.* **2012**, *41*, 7108-7146; (b) P. Liu, J.-W. Hao, L.-P. Mo, Z.-H. Zhang, *RSC Adv.* **2015**, *5*, 48675-48704.
[234] N. Guajardo, C. R. Müller, R. Schrebler, C. Carlesi, P. Domínguez de María, *ChemCatChem* **2016**, *8*, 1020-1027.
[235] J. García-Álvarez, E. Hevia, V. Capriati, *Eur. J. Org. Chem.* **2015**, *31*, 6779-6799.

DES have been successfully achieved in extraction processes,[237] materials production,[238] pharmaceutical and biomedical uses,[239] metal processing applications[52a] and as potential lubricants.[240]

6.1.1. DES and Biocatalysis

The first publication reporting enzyme-catalysed biotransformations in this media dates back to 2008 when Kazlauskas and co-workers investigated the performance of hydrolytic enzymes in DES.[55] For this purpose, lipases such as CAL-B were tested in the transesterification and aminolysis of ethyl valerate using different mixtures of DES as solvent. Comparable or even better results than those obtained in toluene were found, demonstrating the applicability of DES in lipase-catalysed transformations. Additionally, the hydrolysis of esters and epoxides were attempted using esterases and epoxide hydrolases, respectively. Interestingly, the addition of 10% to 25% v/v of DES resulted in a significant enzyme activity improvement.

Since this pioneer research, the combination of lipases and DES has acquired maturity through the development of biocatalysed processes in pure DES, mixtures of DES and water or mixtures of DES and organic solvents.[241] For example, our research group developed the first example of a lipase-catalysed aldol reaction using a DES consisting of a mixture of choline chloride/glycerol (1:2 mol/mol) and water as solvent and porcine pancreas lipase (PPL) as biocatalyst (Scheme 6.1).[58b] After optimising the reaction between 4-nitrobenzaldehyde and acetone, different benzaldehydes and ketones were tested to synthesise a series of β-hydroxy ketones in high conversions with a low percentage of the dehydrated product formation.

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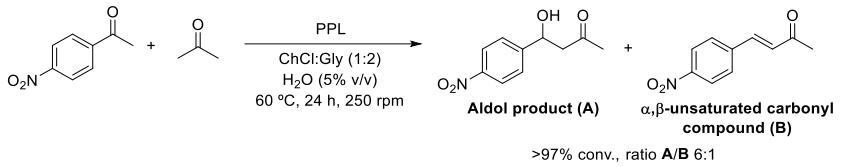
[236] P. Domínguez de María, Z. Maugeri, *Curr. Opin. Chem. Biol.* **2011**, *15*, 220-225.
[237] (a) Z. Maugeri, W. Leitner, P. Domínguez de María, *Tetrahedron Lett.* **2012**, *51*, 6968-6971; (b) M. Krystof, M. Pérez-Sánchez, P. Domínguez de María, *ChemSusChem* **2013**, *4*, 630-634; (c) F. Pena-Pereira, J. Namieśnik, *ChemSusChem* **2014**, *7*, 1784-1800.
[238] D. V. Wagle, H. Zhao, G. A. Baker, *Acc. Chem. Res.* **2014**, *47*, 2299-2308.
[239] (a) Y. P. Mbous, M. Hayyan, A. Hayyan, W. F. Wong, M. A. Hashim, C. Y. Looi, *Biotechnol. Adv.* **2017**, *35*, 105-134; (b) S. Cherukuvada, A. Nangia, *Chem. Commun.* **2014**, *50*, 906-923.
[240] A. P. Abbott, E. I. Ahmed, R. C. Harris, K. S. Ryder, *Green Chem.* **2014**, *16*, 4156-4161.
[241] (a) E. Durand, J. Lecomte, P. Villeneuve, *Eur. J. Lipid. Sci. Technol.* **2013**, *115*, 379-385; (b) V. Gotor-Fernández, C. E. Paul, *Sustainable Catalysis in Ionic Liquids*, Ed. P. Lozano, CRC Press, Boca Raton, USA, **2018**, Chapter 7, pp. 137-171.

Scheme 6.1. Lipase-catalysed aldol reaction in DES/water mixtures.



Apart from hydrolases, the combination of DES with other classes of enzymes such as lyases[60] and oxidoreductases[242] has gained recent attention for synthetic purposes. In the next section, the most representative examples using whole-cells or isolated ADHs in combination with DES will be introduced.

6.1.2. Ketoreductases and deep eutectic solvents

The first example of an ADH-catalysed process in DES-water mixtures was reported by Maugeri and Domínguez de María in 2014,[243] describing the Baker's yeast-catalysed bioreduction of ethyl acetoacetate using up to 30% v/v of ChCl:Gly (1:2 mol/mol) (Scheme 6.2.A). Interestingly, two main achievements were reported:

(a) The yeast seems to be active at long reaction times (up to 12 days) and at high DES contents (up to 90% v/v).

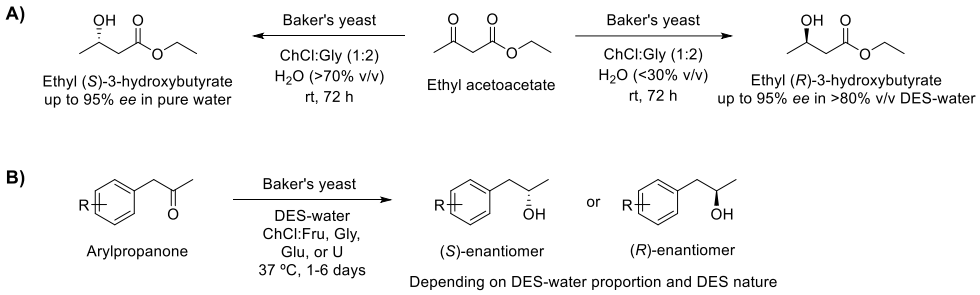
(b) A different stereoselectivity was observed depending on the DES-water proportion. Thus in the absence of DES, the (*S*)-alcohol was obtained with excellent selectivity (95% *ee*). Increasing amounts of DES (up to 20% v/v DES) still favoured the formation of the (*S*)-alcohol. When using around 30% DES volume content, the racemic alcohol was virtually obtained, observing the formation of the (*R*)-enantiomer as the major enantiomer when higher DES contents were employed (around a 95% *ee* at 90% v/v DES content). The authors considered that the addition of the eutectic mixture presumably led to the inhibition of the (*S*)-ketoreductases present in the BY, reinforcing in this way the (*R*)-stereoselectivity of the enzymatic preparation.

Other remarkable publications involving BY-catalysed bioreductions in DES-water mixtures are those published by Bubalo *et al.*[244] and by Capriati and co-

[242] V. Gotor-Fernández, C. E. Paul, *J. Biotechnol.* **2019**, *293*, 24-35.
[243] Z. Maugeri, P. Domínguez de María, *ChemCatChem* **2014**, *6*, 1535-1537.
[244] M. C. Bubalo, M. Mazur, K. Radošević, I. R. Redovniković, *Process Biochem.* **2015**, *50*, 1788-1792.

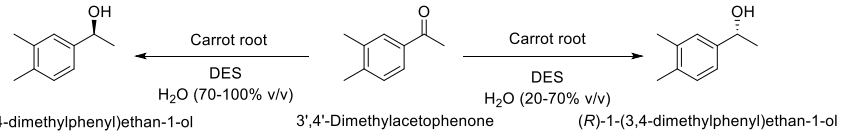
workers.[245] In the first one, ethyl acetoacetate was employed as substrate while in the second one the bioreductions of arylpropanones were attempted with different ChCl-based DES as solvents, differing in the HBD component, and also at different DES-water ratios (Scheme 6.2.B). In both cases, the stereoinversion of these biotransformations was demonstrated depending on the eutectic solvent and its concentration in the reaction medium.

Scheme 6.2. Baker's yeast-catalysed bioreduction of prochiral ketones in DES-water reaction media. A) Bioreduction of ethyl acetoacetate. B) Bioreduction of arylpropanones.



Another example of inversion of the stereoselectivity by adding high amounts of DES was reported by Panić *et al.* very recently.[246] Herein, the bioreduction of 3',4'-dimethylacetophenone was attempted using carrot root as biocatalyst in the presence of variable DES ratios (Scheme 6.3). On the one hand, high conversion and enantioselectivity towards the formation of the (*S*)-alcohol was found in pure water. On the other hand, even though the conversion value decreased when using DES-water mixtures, the major formation of the (*R*)-alcohol was observed when using more than 30% v/v of DES.

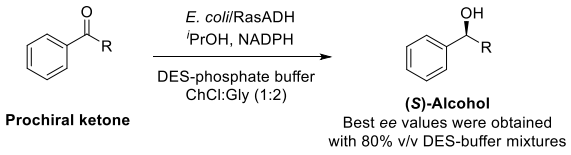
Scheme 6.3. Bioreduction of 3',4'-dimethylacetophenone using carrot root in DES-water mixtures.



[245] P. Vitale, V. M. Abbinante, F. M. Perna, A. Salomone, C. Cardellicchio, V. Capriati, *Adv. Synth. Catal.* **2017**, *359*, 1049-1057.
[246] M. Panić, M. M. Elenkov, M. Roje, M. C. Bubalo, I. R. Redovniković, *Process Biochem.* **2018**, *66*, 133-139.

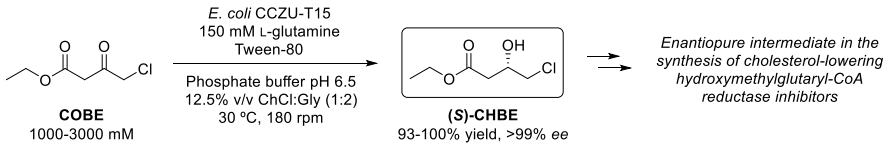
Müller *et al.* reported the bioreduction of a series of prochiral ketones using lyophilised preparations of different ADHs overexpressed in *E. coli* cells.[61b] ADH from *Thermoanaerobacter ethanolicus* (TeSADH), horse liver ADH (HLADH) and ADH from *Ralstonia* sp. (RasADH) were found to be active in DES-buffer mixtures. RasADH revealed to be the most robust one as it remained fully active up to 60-70% v/v of DES. For that reason, a wide panel of aromatic ketones was reduced using this enzymatic preparation and a reaction media composed by different ratios of an eutectic mixture formed by ChCl:Gly (1:2 mol/mol) and a phosphate buffer (Scheme 6.4). Interestingly, an increasing amount of DES resulted in higher *ee* values, finding the best results using 80% v/v of DES. Additionally, other HBD components such as U and EG were tested, finding slightly lower conversions but the same trend in the stereoselectivity of the process.

Scheme 6.4. RasADH-catalysed bioreduction of aromatic ketones in DES-phosphate buffer mixtures.



Interestingly, Xu *et al.* reported the asymmetric reduction of octan-2-one in a biphasic system composed by a DES and a water-immiscible IL.[247] *Acetobacter pasteurianus* GIM1.158 cells were employed as biocatalyst. After testing different choline chloride-based DES, ChCl:EG displayed the best results. Additionally, the biphasic system formed by the ChCl:EG DES and an imidazole-based IL (1-butyl-3-methylimidazolium hexafluorophosphate) allowed a highly efficient synthesis of (*R*)-octan-2-ol with an enhanced substrate concentration (1.5 M).

Scheme 6.5. Asymmetric synthesis of ethyl (*S*)-4-chloro-3-hydroxybutyrate (CHBE) from ethyl 4-chloro-3-oxobutyrate (COBE) catalysed by *E. coli* CCZU-T15 cells in DES-buffer mixtures.

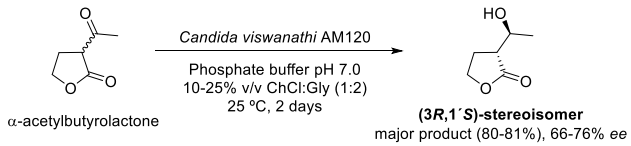


[247] P. Xu, P.-X. Du, M.-H. Zong, N. Li, W.-Y. Lou, *Sci. Rep.* **2016**, *6*, 26158.

Later on, in 2017, He and co-workers developed the asymmetric synthesis of ethyl (*S*)-4-chloro-3-hydroxybutyrate (CHBE), which is a key chiral precursor of enantiopure intermediates in the synthesis of cholesterol-lowering hydroxymethylglutaryl-CoA reductase inhibitors.[248] As starting material, ethyl 4-chloro-3-oxobutanoate (COBE) was employed, which was reduced by the ketoreductase activity of *E. coli* CCZU-T15 cells in mixtures of a choline chloride-based DES [ChCl:Gly (1:2 mol/mol)] and a buffer solution at pH 6.5 (Scheme 6.5). The reaction conditions were optimised finding the best reductase activity when a 12.5% v/v of DES was employed. Additionally, the addition of several additives as precursors in the cofactor synthesis was studied. In this way, by adding 150 mM of L-glutamine it was not necessary the external addition of cofactor. Finally, the performance of several surfactants facilitating the mass transfer of COBE was studied, finding the best results using Tween-80.

Very recently, Żarowska and co-workers reported the asymmetric synthesis of the *anti*-diastereoisomers of α'-1'-hydroxyethyl-γ-butyrolactone through the yeast-catalysed bioreduction of α-acetylbutyrolactone under dynamic conditions.[249] Seven yeast strains were tested in combination with different organic solvents and the eutectic mixture formed by ChCl:Gly (1:2 mol/mol) as cosolvents. Interestingly, the utilisation of the DES in 10 and 25% v/v led to shorter reaction times and better enantio- and diastereoselectivity when using *Candida viswanathi* AM120 as biocatalyst (Scheme 6.6).

Scheme 6.6. Yeast-catalysed bioreduction of α-acetylbutyrolactone in DES-buffer mixtures.

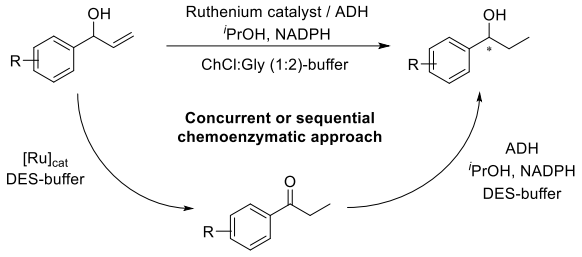


Until now, we have just introduced reduction processes catalysed by whole cell or overexpressed ADHs. However, there are some examples involving immobilised and isolated ADHs as well as some oxidation protocols. For instance, Xu *et al.* made use of *Acetobacter sp.* CCTCC M209061 immobilised cells to

[248] Y. Dani, B. Huan, H.-S. Zhang, Y.-C. He, *Appl. Biochem. Biotechnol.* **2017**, *181*, 1347-1359.
[249] W. Mączka, K. Wińska, M. Grabarczyk, B. Żarowska, *Appl. Sci.* **2018**, *8*, 1334.

reduce 3-chloropropiophenone.[250] Different choline chloride-based DES were tested finding that mixtures of 5% v/v ChCl:U (1:2 mol/mol) and TEA-HCl buffer pH 5 led to the best results in terms of biocompatibility and flow cytometry studies. By using these conditions, it was possible to synthesise the desired (*S*)-3-chloro-1-phenylpropan-1-ol up to 500 mL scale. In another contribution, González-Sabín and co-workers reported the first example using purified alcohol dehydrogenases in the presence of DES.[251] Additionally, DES-buffer mixtures revealed to be a suitable reaction media for running sequential and concurrent processes such as the ruthenium-catalysed isomerisation of racemic allylic alcohols coupled with the ADH-catalysed bioreduction of the resulting propiophenones (Scheme 6.7). The best results were accomplished with ChCl:Gly (1:2 mol/mol) and ChCl:sorbitol (1:1 mol/mol) eutectic mixtures and it was observed that increasing amounts of DES, led to better results in terms of *ee*.

Scheme 6.7. Ruthenium-catalysed isomerisation of allylic alcohols coupled with ADH-catalysed reduction of the corresponding propiophenones in DES-buffer mixtures.



Furthermore, some ADH-catalysed oxidation reactions have been described in DES-buffer mixtures.[52c,252] For instance, Xu *et al*. reported the kinetic resolution of 1-(4-methoxyphenyl)ethan-1-ol (MOPE) using *Acetobacter sp.* CCTCC M209061 cells and DES as co-solvents.[252a] ChCl:Gly (1:2 mol/mol) mixture added in 20% v/v led to the best results (49.4% conv), isolating the enantioenriched (*S*)-MOPE in 98.7% *ee*. The use of DES-buffer mixtures as reaction media allowed increasing the substrate concentration from 30 mM up to 55 mM without affecting the stereoselectivity of the process.

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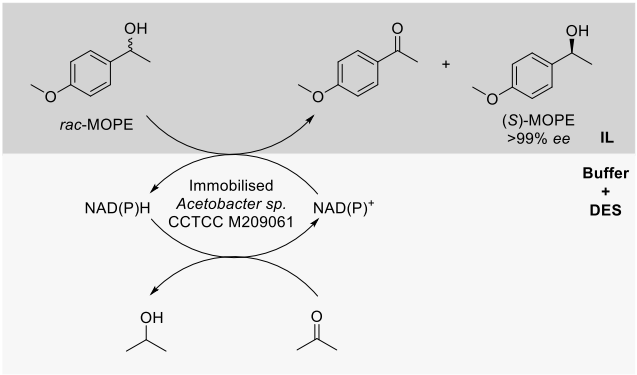
[250] P. Xu, Y. Xu, X.-F. Li, B.-Y. Zao, M.-H. Zong, W.-Y. Lou, *ACS Sustainable Chem. Eng.* **2015**, *3*, 718-724.
[251] L. Cicco, N. Ríos-Lombardía, M. J. Rodríguez-Álvarez, F. Morís, F. M. Perna, V. Capriati, J. García-Álvarez, J. González-Sabín, *Green Chem.* **2018**, *20*, 3468-3475.
[252] (a) P. Xu, J. Cheng, W.-Y. Lou, M.-H. Zong, *RSC Adv.* **2015**, *5*, 6357-6364; (b) S. H. Mao, L. Yu, S. X. Si, X. G. Liu, F. P. Lu, *J. Chem. Technol. Biotechnol.* **2016**, *91*, 1099-1104.

Later on, the same research group improved this process by using a biphasic system composed by an IL and a DES (Scheme 6.8).[52c] In this case, the combination of 1-butyl-3-methylimidazolium hexafluorophosphate and a 10% v/v of ChCl:Gly (1:2 mol/mol) led to the production of the enantiopure (*S*)-MOPE after 7 hours using higher substrate concentrations (from 50 mM to 80 mM). Additionally, immobilised cells were used demonstrating that they were able to retain the 72% of their initial activity after 9 cycles. Furthermore, by using the immobilised cells, it was possible to perform the kinetic resolution in a total volume of 500 mL.

Scheme 6.8. Synthesis of enantiopure (*S*)-1-(4-methoxyphenyl)ethan-1-ol (MOPE) through the ADH-catalysed kinetic resolution of the racemic MOPE in a biphasic IL/DES-buffer system.

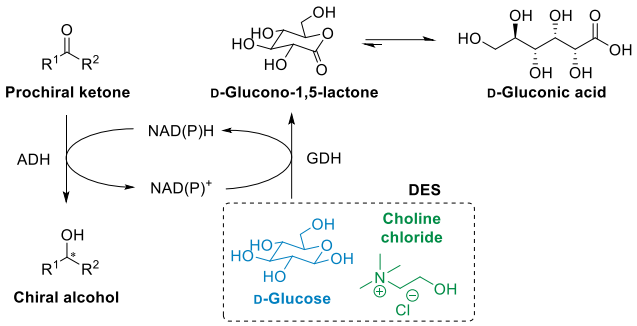


At this point, the capability of deep eutectic solvents to be used as cosolvents in redox processes has been proven. However, in the examples described in the literature, choline chloride/glycerol (ChCl:Gly) mixtures have been mainly employed, being the choice of glucose as HBDs scarcely reported. As already discussed in the Preface of this thesis (Scheme 0.1.B), the use of a glucose dehydrogenase (GDH) as the coupled-enzyme is one of the most popular methods to regenerate the nicotinamide cofactor. For this reason, we decided to explore the possibility to use a designer glucose-based NADES, which could be beneficial in order to simplify the cofactor regeneration system while helping at the same time to solubilise the organic compounds to provide a more productive biotransformation. Thus, we will introduce a new protocol in which a ChCl:Glu DES will be used as both cosolvent and source of glucose for the cofactor regeneration system of different ADH-catalysed bioreduction processes.

6.2. Discussion

The bioreduction of prochiral ketones with different overexpressed alcohol dehydrogenases has been studied using a NADES-buffer reaction media. The DES of choice was composed by choline chloride (ChCl) and D-glucose (Glu) in a 1.5:1 molar ratio. The presence of D-glucose in the eutectic mixture provided a designer DES performing two important roles: (i) behaving as co-solvent to improve the solubility of lipophilic substrates, allowing a higher substrate concentration; and (ii) providing the necessary co-substrate (glucose) for the cofactor regeneration system, as GDH will be employed (Scheme 6.9).

Scheme 6.9. ADH-catalysed bioreduction of prochiral ketones using a DES composed by D-glucose and choline chloride as both co-solvent and co-substrate for the nicotinamide cofactor recycling.

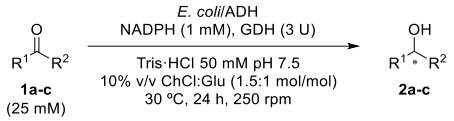


6.2.1. Made-in-house alcohol dehydrogenases in NADES-buffer mixtures

As starting point in this research project, the (*R*)-selective ADH from *Lactobacillus brevis* (LbADH)[208] and the (*S*)-selective ADHs from *Thermoanaerobacter ethanolicus* (TeSADH),[204] *Thermoanaerobacter* sp. (ADH-T),[205] *Sphingobium yanoikuyae* (SyADH)[206] and *Ralstonia sp.* (RasADH)[207] were initially tested in the bioreduction of their model substrate. Each substrate was added in 25 mM concentration in a reaction media composed by Tris·HCl 50 mM pH 7.5, including NADPH as nicotinamide cofactor in all cases. As coupled-enzyme for the cofactor-recycling system, the commercially available GDH-105 (3 U) from Codexis Inc. was employed. Initially, a proportion of 10% v/v of ChCl:Glu mixture (1.5:1 mol/mol) was added. This glucose-composed NADES was chosen in order to have the higher amount of glucose for cofactor recycling purposes without the detriment of using a highly dense and viscous NADES, which

would make it difficult to handle.[253] As shown in Table 6.1, all ADHs led to high conversions and enantioselectivities. However, LbADH, ADH-T and RasADH led to better results than TeSADH and SyADH. Thus, they were capable of transforming acetophenone, octan-2-one and propiophenone, respectively (entries 1, 3 and 5), into the corresponding enantiopure alcohols with full conversion and excellent enantioselectivity (>99% *ee*).

Table 6.1. ADH-catalysed bioreduction of prochiral ketones using a NADES composed by ChCl and Glu as cosolvent and as source of glucose for the cofactor-recycling system.[a]



Entry	ADH	Substrate	Conv. (%)[b]	*ee* (%)[b]
1	LbADH	Acetophenone (**1a**)	>99	>99 (*R*)
2	TeSADH	Octan-2-one (**1b**)	86	92 (*S*)
3	ADH-T	Octan-2-one (**1b**)	99	>99 (*S*)
4	SyADH	Propiophenone (**1c**)	81	>99 (*S*)
5	RasADH	Propiophenone (**1c**)	>99	>99 (*S*)

[a] Reaction conditions: substrate **1a-c** (25 mM), *E. coli*/ADH (10 mg), 10% v/v NADES ChCl:Glu (1.5:1 mol/mol), $MgCl_2 \cdot 6H_2O$ (1 mM, entry 1), NADPH (1 mM), GDH-105 (3 U), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24 h, 250 rpm. [b] Conversion and *ee* values were measured by GC.

Due to their excellent performances, we decided to study the influence of different parameters such as the NADES-buffer ratio or the substrate concentration in LbADH, ADH-T and RasADH-catalysed bioreductions. However, before that, the integrity of the NADES of choice in the reaction media was evaluated.

6.2.2. Integrity of NADES in the aqueous medium

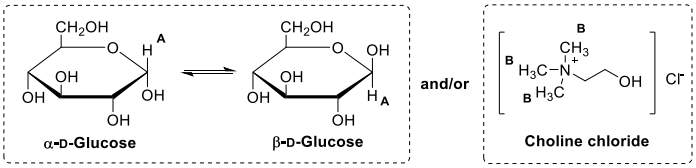
It was already known from previous studies that using NADES in water at >50% w/w ratios, the nanostructure of the eutectic solvent is perfectly retained.[254] As the density of ChCl:Glu (1.5:1 mol/mol) had been estimated to be 1.27 g/mL at 30 ºC,[253] 50% v/v was more than 50% w/w. For this reason, we decided to verify the integrity of the NADES nanostructure at lower concentrations. For this

[253] A. Hayyan, F. S. Mjalli, I. M. Alnashef, Y. M. Al-Wahaibi, T. Al-Wahaibi, M. A. Hashim, *J. Mol. Liq.* **2013**, *178*, 137-141.
[254] O. S. Hammond, D. T. Bowron, K. J. Edler, *Angew. Chem. Int. Ed.* **2017**, *56*, 9782-9785.

purpose, a series of ^{1}H-NMR experiments were performed in D_2O trying to mimic the reaction conditions for the biotransformation:

(a) Choline chloride and glucose as standards.
(b) Choline chloride and glucose directly mixed in the NMR tube (quantities corresponding to 10% v/v).
(c) NADES (ChCl:Glu 1.5:1 mol/mol) 10%, 20%, 30% and 50% v/v in the deuterated solvent.

Table 6.2. ^{1}H-NMR signals of glucose and choline chloride components and different NADES ratios in D_2O solution.



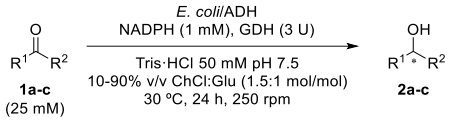
Entry	NMR sample	δ_A (ppm)	δ_B (ppm)
1	Glucose	4.65 and 4.67 (β-D-glucose) 5.25 and 5.26 (α-D-glucose)	-
2	Choline chloride	-	3.25
3	Choline chloride and glucose (directly mixed in the NMR tube, 10% v/v)	4.67 and 4.69 (β-D-glucose) 5.26 and 5.27 (α-D-glucose)	3.24
4	10% v/v NADES	4.67 and 4.69 (β-D-glucose) 5.26 and 5.27 (α-D-glucose)	3.24
5	20% v/v NADES	4.67 and 4.69 (β-D-glucose) 5.27 and 5.27 (α-D-glucose)	3.25
6	30% v/v NADES	4.69 and 4.71 (β-D-glucose) 5.28 and 5.29 (α-D-glucose)	3.27
7	50% v/v NADES	4.73 and 4.75 (β-D-glucose) 5.33 and 5.32 (α-D-glucose)	3.33

As show in Table 6.2, when using low concentrations of NADES (10% and 20% v/v, entries 4 and 5), the components seemed to do not interact, so basically ChCl and Glu were in solution and the NADES nanostructure appeared to be destroyed. However, at higher proportions of NADES (30% and 50% v/v, entries 6 and 7), the NMR signals exhibited a downfield shift in comparison with the individual compounds. This effect reveals an interaction implying that at least part of the NADES is behaving as a pair rather than such as individual components.

6.2.3. Influence of NADES-buffer ratio and substrate concentration

As the NADES nanostructure seemed to be destroyed at low concentrations of the eutectic mixture, we decided to increase the NADES-buffer ratio.

Table 6.3. Influence of DES-buffer ratio in bioreductions catalysed by LbADH, ADH-T and RasADH.[a]



Entry	ADH	Substrate	NADES (% v/v)	*c* (%)[b]	*ee* (%)[b]
1	LbADH	Acetophenone (**1a**)	10	>99	>99 (*R*)
2	LbADH	Acetophenone (**1a**)	20	>99	>99 (*R*)
3	LbADH	Acetophenone (**1a**)	30	>99	>99 (*R*)
4	LbADH	Acetophenone (**1a**)	40	>99	>99 (*R*)
5	LbADH	Acetophenone (**1a**)	50	>99	>99 (*R*)
6	LbADH	Acetophenone (**1a**)	60	>99	>99 (*R*)
7	LbADH	Acetophenone (**1a**)	70	92	>99 (*R*)
8	LbADH	Acetophenone (**1a**)	80	15	>99 (*R*)
9	LbADH	Acetophenone (**1a**)	90	<1	n.d.
10	ADH-T	Octan-2-one (**1b**)	10	99	>99 (*S*)
11	ADH-T	Octan-2-one (**1b**)	20	99	>99 (*S*)
12	ADH-T	Octan-2-one (**1b**)	30	98	>99 (*S*)
13	ADH-T	Octan-2-one (**1b**)	40	98	>99 (*S*)
14	ADH-T	Octan-2-one (**1b**)	50	97	>99 (*S*)
15	ADH-T	Octan-2-one (**1b**)	60	46	>99 (*S*)
16	ADH-T	Octan-2-one (**1b**)	70	39	>99 (*S*)
17	ADH-T	Octan-2-one (**1b**)	80	5	n.d.
18	ADH-T	Octan-2-one (**1b**)	90	2	n.d.
19	RasADH	Propiophenone (**1c**)	10	>99	>99 (*S*)
20	RasADH	Propiophenone (**1c**)	20	>99	>99 (*S*)
21	RasADH	Propiophenone (**1c**)	30	>99	>99 (*S*)
22	RasADH	Propiophenone (**1c**)	40	>99	93 (*S*)
23	RasADH	Propiophenone (**1c**)	50	>99	90 (*S*)
24	RasADH	Propiophenone (**1c**)	60	>99	90 (*S*)
25	RasADH	Propiophenone (**1c**)	70	>99	89 (*S*)
26	RasADH	Propiophenone (**1c**)	80	52	80 (*S*)
27	RasADH	Propiophenone (**1c**)	90	2	n.d.

[a] Reaction conditions: substrate **1a-c** (25 mM), *E. coli*/ADH (10 mg), 10-90% v/v NADES ChCl:Glu (1.5:1 mol/mol), $MgCl_2 \cdot 6H_2O$ (1 mM, entries 1-5), NADPH (1 mM), GDH-105 (3 U), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24 h, 250 rpm. [b] Conversion and *ee* values were measured by GC. n.d. not determined.

Thus, we studied the bioreduction of acetophenone, octan-2-one and propiophenone using LbADH, ADH-T and RasADH, respectively, at NADES proportions higher than 10% v/v (Table 6.3). On the one hand, ADH-T led to worse results than the others at high contents of NADES, losing half of its activity when using 60% v/v NADES (entry 15). Gratifyingly, LbADH and RasADH were capable to perform the bioreduction of acetophenone and propiophenone, respectively, in high conversions by using up to 70% v/v NADES (entries 7 and 25, respectively). On the other hand, the selectivity of LbADH and ADH-T revealed a remarkable performance in all cases but when using RasADH as biocatalyst and proportion equal or higher than 40% v/v of NADES (entries 22-26), a drop in the selectivity was observed (80-93% *ee*). As already commented in the bibliographic background, this surprising change in the stereoselectivity has been reported for several authors when using different ADHs such as RasADH,[61b] being typically associated with the inhibition of other biocatalytic activities present in the enzyme preparation or due to protein conformational changes.[242]

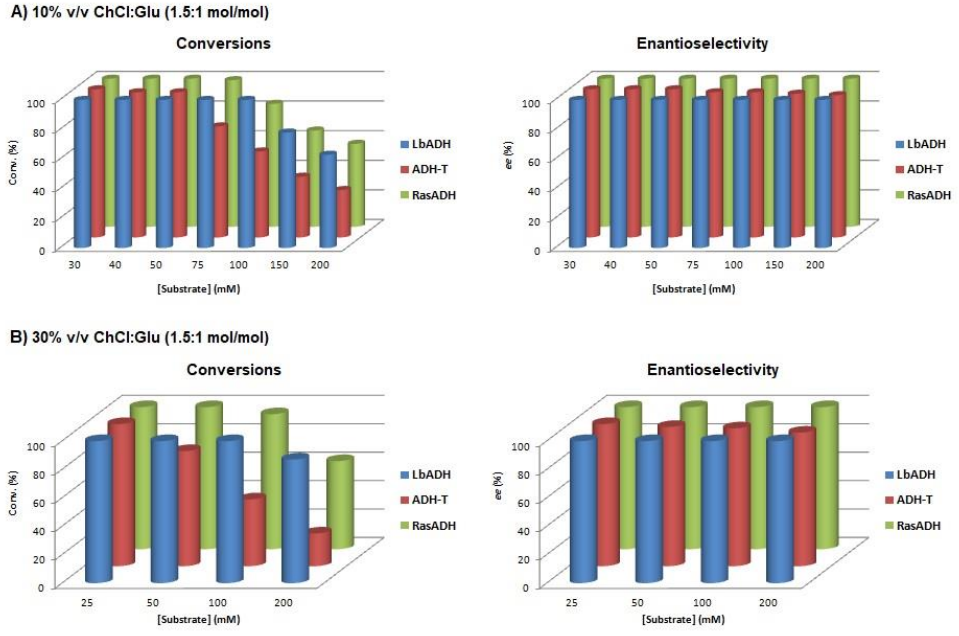


Figure 6.2. Influence of the substrate concentration in the conversion and selectivity of ADH-catalysed bioreductions of acetophenone (LbADH), octan-2-one (ADH-T) and propiophenone (RasADH) using ChCl:Glu (1.5:1 mol/mol) as cosolvent and as source of glucose at: **A)** 10% v/v; and **B)** 30% v/v.

Next, in order to demonstrate the applicability of this methodology, selected biotransformations were attempted at higher ketone concentrations. For this study, a 10% v/v of the eutectic mixture was chosen as starting point and the substrate concentration varied from 25 mM up to 200 mM (Figure 6.2.A), studying the effect of increasing the substrate conversion for both the conversion (Figure 6.2.A left) and the stereoselectivity (Figure 6.2.A right).

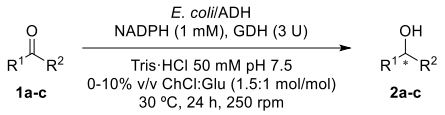
After 24 hours, the reaction mixture was analysed by GC analysis, observing that LbADH was the most robust enzyme as it was able to catalyse the synthesis of enantiopure (*R*)-1-phenylethanol in full conversions up to 100 mM of acetophenone. A decrease in the conversion was observed at higher substrate concentrations (78% and 63% conv. at 150 mM and 200 mM acetophenone, respectively) but always achieving the alcohol in its enantiopure form. Furthermore, ADH-T was capable of catalysing the reduction of octan-2-one up to 50 mM with full conversion and yielding enantiopure (*S*)-octan-2-ol. However, from 75 mM to 200 mM substrate concentration, lower conversion values were gradually observed (from 75% to 32% conv.) with a slight loss of selectivity (96-98% *ee*). Finally, RasADH led to full conversions up to 75 mM of propiophenone while higher substrate concentrations led to a significantly decrease in the conversions (56-83% conv. at 100-200 mM) but maintaining an excellent selectivity towards the formation of (*S*)-1-phenylpropan-1-ol in all cases.

It is important to point out that the concentration of glucose decreased along the reaction as this constituent is employed as co-substrate for the cofactor recycling system. For this reason, we considered employing higher amounts of NADES, this is 30% v/v, at different ketone concentrations (Figure 6.2.B). Under these conditions, higher conversion values were observed for both LbADH and RasADH without affecting the selectivity. However, worse results in terms of conversions were observed for ADH-T for reactions with >25 mM concentration, noticing a slight but gradually drop in the selectivity at higher substrate concentrations.

At this point, in order to demonstrate the improvements of our system regarding the typically used glucose/GDH system, we decided to set up the bioreductions without the addition of NADES. For this purpose, the best conditions in terms of substrate concentration and conversion with 10% v/v of NADES were selected for each enzyme. Later on, the same transformations were set up with

external addition of glucose (Table 6.4). Satisfactorily, using the same amount of glucose, the biotransformations with the NADES/GDH system (even entries) worked out better that the reductions performed with the traditional glucose/GDH system (odd entries). While these results can be surprising since the NMR experiments revealed that the nanostructure of the NADES is destroyed at low concentrations, there are reports that have described that choline chloride can exert a stabilising effect in the structure of biomolecules such as proteins[255] or DNA.[256]

Table 6.4. Bioreduction of prochiral ketones using glucose as co-substrate in pure buffer or using 10% v/v of NADES.[a]



Entry	NADES (% v/v)	ADH	Substrate	*c* (%)[b]	*ee* (%)[b]
1	0	LbADH	**1a** (100 mM)	59	>99 (*R*)
2	10	LbADH	**1a** (100 mM)	>99	>99 (*R*)
3	0	ADH-T	**1b** (50 mM)	89	>99 (*S*)
4	10	ADH-T	**1b** (50 mM)	98	>99 (*S*)
5	0	RasADH	**1c** (100 mM)	61	>99 (*S*)
6	10	RasADH	**1c** (100 mM)	83	>99 (*S*)

[a] Reaction conditions: substrate (**1a-c**), *E. coli*/ADH (10 mg), $MgCl_2 \cdot 6H_2O$ (1 mM, entries 1 and 2), NADPH (1 mM), GDH-105 (3 U), 10% v/v NADES (even entries) or 240 mM glucose (odd entries), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 24 h, 250 rpm. [b] Conversion and *ee* values were measured by GC.

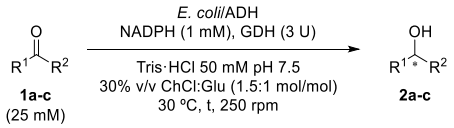
6.2.4. Recycling studies

Once the benefits of using the NADES/GDH system were proven, the recyclability of the enzyme and the NADES was investigated using a substrate concentration of 25 mM and 30% v/v NADES-buffer. However, before trying to recycle the catalytic system, the bioreduction of each substrate was monitored to determine the time needed for each enzyme to complete the bioreduction (Table 6.5). Under these conditions, LbADH was capable of synthesising the enantiopure (*R*)-1-phenylethanol in full conversion after 1 hour (entry 2) while ADH-T needed 90 minutes to fully convert octan-2-one into (*S*)-octan-2-ol (entry 5). Finally,

[255] A. R. Harifi-Mood, R. Ghobadi, A. Divsalar, *Int. J. Biol. Macromol.* **2017**, *95*, 115-120.
[256] I. Mamajanov, A. E. Engelhart, H. D. Bean, N. V. Hud, *Angew. Chem. Int. Ed.* **2010**, *49*, 6310-6314.

RasADH only required 20 minutes to achieve the formation of (*S*)-1-phenylpropan-1-ol in full conversion (entry 8).

Table 6.5. Monitoring the reaction time in the ADH-catalysed bioreductions using 30% v/v of NADES as cosolvent and source of glucose.[a]



Entry	ADH	Substrate	t (min)	*c* (%)[b]	*ee* (%)[b]
1	LbADH	**1a**	30	70	>99 (*R*)
2	LbADH	**1a**	60	>99	>99 (*R*)
3	ADH-T	**1b**	30	82	>99 (*S*)
4	ADH-T	**1b**	60	95	>99 (*S*)
5	ADH-T	**1b**	90	>99	>99 (*S*)
6	RasADH	**1c**	10	97	>99 (*S*)
7	RasADH	**1c**	15	98	>99 (*S*)
8	RasADH	**1c**	20	>99	>99 (*S*)

[a] Reaction conditions: substrate (**1a-c**, 25 mM), *E. coli*/ADH (10 mg), 30% v/v NADES ChCl:Glu (1.5:1 mol/mol), $MgCl_2 \cdot 6H_2O$ (1 mM, entries 1 and 2), NADPH (1 mM), GDH-105 (3 U), Tris·HCl 50 mM pH 7.5 (600 µL final volume), 30 ºC, 10-90 min, 250 rpm. [b] Conversion and *ee* values were measured by GC.

Considering these reaction times and reaction conditions, the recyclability of the enzyme and the NADES was studied (Figure 6.3). Once each bioreduction was complete (1 hour for LbADH, 90 minutes for ADH-T and 20 minutes for RasADH), the reaction mixture was extracted with ethyl acetate (upper phase). As expected, all ADHs led to full conversions and excellent stereoselectivity. The aqueous phase (bottom phase), containing the NADES and both enzymes (ADH and GDH), was employed for a second biotransformation. For this purpose, the corresponding substrate was added and the reactions were incubated at 30 ºC for 1 h (LbADH), 90 min (ADH-T) or 20 min (RasADH). Sadly, a significant loss of activity was observed, especially for the LbADH. This inactivation effect could be related to the use of the large amount of EtOAc as extracting solvent. Furthermore, we observed a decrease in the pH of our buffer at the end of the process, which can be explained due to the transformation of glucose into D-glucono-1,5-lactone that spontaneously hydrolyses to D-gluconic acid as by-product varying the pH of the medium (Scheme 6.9).

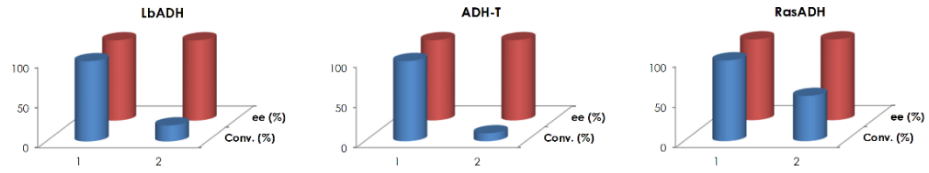


Figure 6.3. Recycling of the enzyme and the buffer-NADES (30% v/v) mixture after extracting with EtOAc the bioreduction of acetophenone (LbADH), octan-2-one (ADH-T) or propiophenone (RasADH).

In order to test the influence of these two parameters, the use of pentane instead of EtOAc as extracting solvent (Figure 6.4, blue bars), and the development of biotransformations in a more concentrated buffer (Tris·HCl 200 mM pH 7.5) (Figure 6.4, red bars) was studied. Remarkably, an improvement in the conversion values was attained when using pentane, leading to a successful first recycling with LbADH and RasADH. In addition, the use of the Tris·HCl buffer 200 mM pH 7.5 made possible to recycle RasADH system three times without significant loss of its activity. Additionally, in order to reduce the interaction between the ADH and the organic solvent, we perform new recycling experiments filtering the enzyme before the extraction (Figure 6.4, green bars), considering only pentane as extracting solvent. Afterwards, the enzyme was resuspended in the reaction medium again, more substrate was added and bioreductions were incubated at 30 ºC. Slightly better results were found with LbADH and RasADH following this procedure.

Finally, we decided to measure the pH value of the reaction medium after each biotransformation. Hence, a pH value of 3.5 was found when the bioreductions were carried out in the Tris·HCl buffer 50 mM while a value around 5.0 was found by using the buffer 200 mM. Thus, the important effect that the production of D-gluconic acid had in the pH was confirmed. In order to overcome this limitation, the pH was readjusted up to 7.5 before each recycling experiment and after enzyme filtration and extraction with pentane (Figure 6.4, purple bars). A great improvement was found using this protocol, especially when using LbADH (98% conversion and 98% *ee* of (*R*)-**2a** after 5 cycles) and RasADH (98% conversion and >99% *ee* of (*S*)-**2c** after 8 cycles).

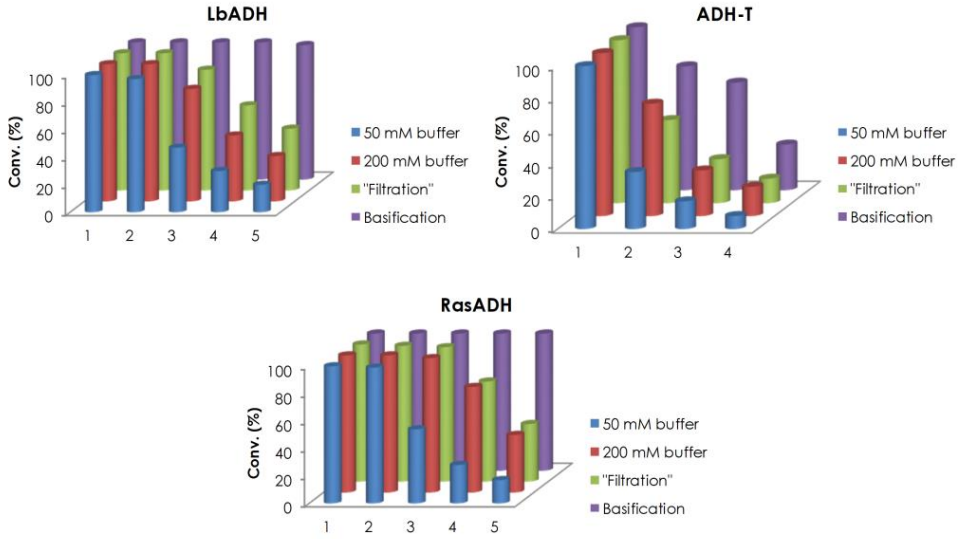
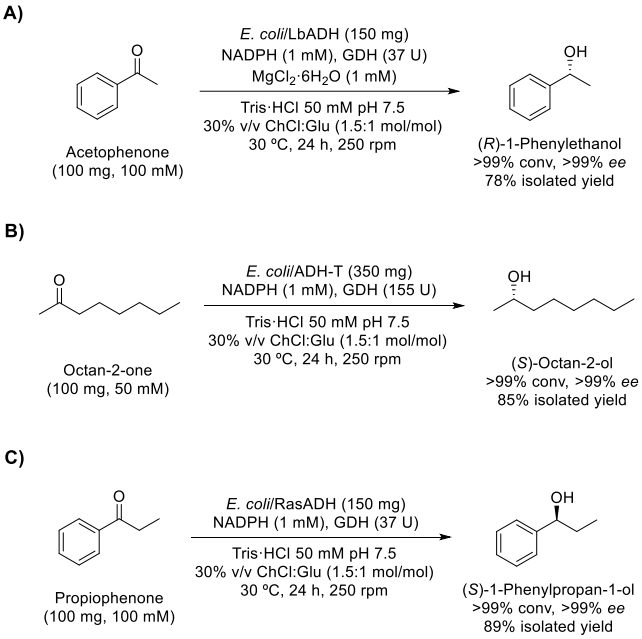


Figure 6.4. Recycling of the enzyme and the buffer-NADES (30% v/v) mixture after extraction in the bioreductions of acetophenone with LbADH, octan-2-one with ADH-T and propiophenone with RasADH. In all cases the reaction was extracted with pentane. **A)** Blue bars correspond to reactions set up in a buffer 50 mM. **B)** Red bars are reactions carried out in a buffer 200 mM. **C)** Green bars correspond to reactions set up in a buffer 200 mM and in which the enzyme was filtered before extraction. **D)** Purple bars are reactions carried out in a buffer 200 mM, the ADH filtered before extraction and the pH of the NADES-buffer mixture readjusted up to 7.5 before reusing.

6.2.5. Semi-preparative bioreductions in NADES-buffer mixtures

Finally, we set up some semi-preparative biotransformations in order to demonstrate the applicability of this methodology (Scheme 6.10). Thus, 100 mg of each ketone **1a-c** were successfully converted into the enantiopure alcohols **2a-c** (>99% *ee*), finding conversion values in accordance to those obtained at analytical scale, this is full conversions in all cases. After extraction, alcohols were recovered in high isolated yields: (*R*)-1-phenylethanol in 78% yield using LbADH, (*S*)-octan-2-ol in 85% yield with ADH-T and (*S*)-1-phenylpropan-1-ol in 89% yield utilising RasADH.

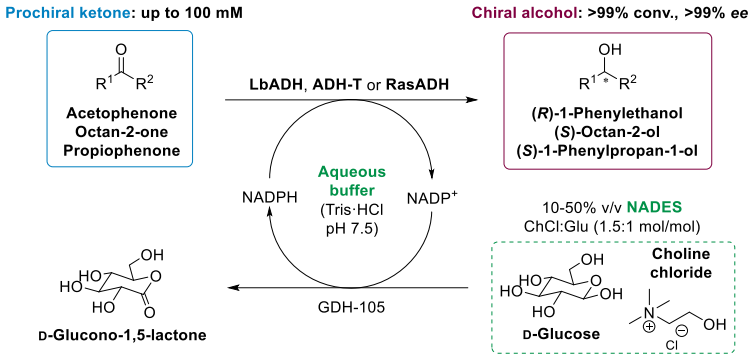
Scheme 6.10. Semi-preparative bioreductions of prochiral ketones in NADES-buffer mixtures. **A)** LbADH-catalysed reduction of acetophenone into (*R*)-1-phenylethanol. **B)** Synthesis of (*S*)-octan-2-ol catalysed by ADH-T. **C)** RasADH-catalysed transformation of propiophenone into the enantiopure (*S*)-1-phenylpropan-1-ol.



6.2.6. Final remarks

The bioreduction of different prochiral ketones (acetophenone, octan-2-one and propiophenone) has been developed using different ketoreductases (LbADH, ADH-T and RasADH) as biocatalyst in a designer NADES-buffer system. The DES of choice was composed by ChCl:Glu (1.5:1 mol/mol) with a clear intention. Thus, the glucose used as HBD served as co-substrate for the GDH-catalysed reaction crucial in the NADPH recycling. Additionally, the combination of the NADES with an aqueous buffer phase made possible to employ higher substrate concentrations (up to 100 mM) in comparison with the buffer system. In this manner, it was possible to scale-up some bioreductions achieving the enantiopure alcohols in high isolated yields and full conversion values (Scheme 6.11).

Scheme 6.11. ADH-catalysed bioreduction of prochiral ketones in NADES-buffer mixtures.



Furthermore, the recyclability of the enzyme and the NADES was explored finding that the use of pentane as extracting solvent preserved better from enzyme inactivation than using EtOAc. Besides, D-gluconic acid was formed as co-product in the cofactor recycling reaction, leading to a dramatic change in the pH especially when using more diluted buffers (Tris·HCl 50 mM). This effect was countered by using a more concentrated buffer (Tris·HCl 200 mM) and readjusting the NADES-buffer mixture pH after extraction with pentane and before reusing, especially LbADH and RasADH leading to greater improvements.

6.3. Experimental section

6.3.1. General information

Acetophenone, 1-phenylethanol, propiophenone, 1-phenylpropan-1-ol, octan-2-one, octan-2-ol, NADPH as enzyme cofactor and all the other chemical reagents were obtained with the highest quality available from Sigma-Aldrich-Fluka (Steinheim, Germany).

Glucose dehydrogenase (GDH-105, 48 U/mg) was obtained from Codexis Inc. (Redwood City, CA, US). Overexpressed ADHs from *Lactobacillus brevis* (LbADH), from *Thermoanaerobacter ethanolicus* (TeSADH), from *Thermoanaerobacter* sp. (ADH-T), from *Sphingobium yanoikuyae* (SyADH) and from *Ralstonia* sp. (RasADH) have been produced following the methodology previously described.[204-208]

6.3.2. Bioreduction of acetophenone using LbADH

Lyophilised *E. coli*/LbADH cells (10 mg), 1 mM NADPH (60 µL of a 10 mM stock solution), 1 mM $MgCl_2 \cdot 6H_2O$ (60 µL of a 10 mM stock solution) and glucose dehydrogenase (GDH-105, 3 U) were added into an Eppendorf tube containing acetophenone (**1a**, 25-200 mM) in a mixture (total volume: 600 µL) of Tris·HCl buffer 50 mM pH 7.5 and NADES ChCl:Glu (1.5:1 mol/mol, 10-50% v/v). The reaction was incubated at 30 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversion and enantiomeric excess of 1-phenylethanol (**2a**) were determined by GC analysis.

6.3.3. Bioreduction of octan-2-one or propiophenone using TeSADH, ADH-T, SyADH or RasADH

Lyophilised *E. coli*/TeSADH, ADH-T, SyADH or RasADH cells (10 mg), 1 mM NADPH (60 µL of a 10 mM stock solution) and glucose dehydrogenase (GDH-105, 3 U) were added into an Eppendorf tube containing octan-2-one (**1b**, for TeSADH and ADH-T, 25-200 mM) or propiophenone (**1c**, for SyADH and RasADH, 25-200 mM) in a mixture (total volume: 600 µL) of Tris·HCl buffer 50

mM pH 7.5 and NADES ChCl:Glu (1.5:1 mol/mol, 10-50% v/v). The reaction was incubated at 30 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversion and enantiomeric excess of octan-2-ol (**2b**) and 1-phenylpropan-1-ol (**2c**) were determined by GC analysis.

6.3.4. Bioreduction of acetophenone using LbADH and the glucose/GDH system (in the absence of NADES)

Lyophilised *E. coli*/LbADH cells (10 mg), 1 mM NADPH (60 µL of a 10 mM stock solution), 1 mM $MgCl_2 \cdot 6H_2O$ (60 µL of a 10 mM stock solution), 240 mM glucose (60 µL of a 2.4 M stock solution) and glucose dehydrogenase (GDH-105, 3 U) were added into an Eppendorf tube containing acetophenone (**1a**, 100 mM) in Tris·HCl buffer 50 mM pH 7.5 (total volume: 600 µL). The reaction was incubated at 30 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversion and enantiomeric excess of 1-phenylethanol (**2a**) were determined by GC analysis.

6.3.5. Bioreduction of octan-2-one or propiophenone using ADH-T or RasADH and the glucose/GDH system (in the absence of NADES)

Lyophilised *E. coli*/ADH-T or RasADH cells (10 mg), 1 mM NADPH (60 µL of a 10 mM stock solution), 240 mM glucose (60 µL of a 2.4 M stock solution) and glucose dehydrogenase (GDH-105, 3 U) were added into an Eppendorf tube containing **1b** (for ADH-T, 50 mM) or **1c** (for RasADH, 100 mM) in a mixture of Tris·HCl buffer 50 mM pH 7.5 (total volume: 600 µL). The reaction was incubated at 30 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with EtOAc (2 x 500 µL), the organic layers separated by centrifugation (2 min, 13000 rpm), combined and finally dried over Na_2SO_4. Conversion and enantiomeric excess of octan-2-ol (**2b**) and 1-phenylpropan-1-ol (**2c**) were determined by GC analysis.

6.3.6. Semi-preparative conversion of acetophenone using LbADH

Acetophenone (100 mg, 100 mM) was added to a mixture of 30% v/v NADES ChCl:Glu (1.5:1 mol/mol, 2.5 mL) and Tris·HCl buffer 50 mM pH 7.5 (5.8 mL). The reaction media was implemented with 1 mM NADPH and 1 mM $MgCl_2 \cdot 6H_2O$. Later on, glucose dehydrogenase (GDH-105, 37 U) and *E. coli*/LbADH (150 mg) were added. The reaction was incubated at 30 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with EtOAc (2 x 10 mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. Enantiopure (*R*)-1-phenylethanol (**2a**) was obtained with full conversion and high isolated yield (>99% *ee*, >99% conv., 78% yield).

6.3.7. Semi-preparative conversion of octan-2-one using ADH-T

Octan-2-one (100 mg, 50 mM) was added to a mixture of 30% v/v NADES ChCl:Glu (1.5:1 mol/mol, 4.68 mL) and Tris·HCl buffer 50 mM pH 7.5 (7.32 mL) implemented with 1 mM NADPH. Afterwards, glucose dehydrogenase (GDH-105, 155 U) and *E. coli*/ADH-T (350 mg) were added. The reaction was incubated at 30 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with EtOAc (2 x 10 mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. Enantiopure (*S*)-octan-2-ol (**2b**) was obtained with full conversion and high isolated yield (>99% *ee*, >99% conv., 85% yield).

6.3.8. General procedure for the conversion of propiophenone using RasADH

Propiophenone (100 mg, 100 mM) was added to a mixture of 30% v/v NADES ChCl:Glu (1.5:1 mol/mol, 2.24 mL) and Tris·HCl buffer 50 mM pH 7.5 (5.2 mL). The reaction media was implemented with 1 mM NADPH and glucose dehydrogenase (GDH-105, 37 U) and *E. coli*/RasADH (150 mg) were finally added. The reaction was incubated at 30 °C and 250 rpm for 24 hours. After this time, the mixture was extracted with EtOAc (2 x 10 mL), the organic layers separated by centrifugation (5 min, 4900 rpm), combined and finally dried over Na_2SO_4. Enantiopure (*S*)-1-phenylpropan-1-ol (**2c**) was obtained with full conversion and high isolated yield (>99% *ee*, >99% conv., 89% yield).

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A designer natural deep eutectic solvent to recycle the cofactor in alcohol dehydrogenase-catalysed processes†

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Deep eutectic solvents (DESs) nowadays represent a sustainable alternative to traditional organic solvents in (bio)transformations. Herein, the use of a solvent composed of an aqueous buffer and choline chloride : glucose (1.5 : 1 mol/mol) is proposed, a natural DES (NADES) serving as both a cosolvent and efficient system to recycle the nicotinamide cofactor. Thus, glucose from the NADES served as a co-substrate required for several alcohol dehydrogenases to reduce different prochiral ketones, and also helped to solubilise the organic compounds to develop effective biotransformations at higher substrate concentrations.

The application of oxidoreductases (EC.1) in organic synthesis has gained maturity in the last decades due to their high levels of activity and selectivity displayed in redox processes.[1] Particularly, alcohol dehydrogenases (ADHs, EC.1.1.1.x) represent a sustainable alternative to non-enzymatic methods for the production of optically active alcohols from racemic and prochiral carbonylic compounds.[2] This class of redox catalyst requires the use of a cofactor, β-nicotinamide adenine dinucleotide, which exists in a phosphorylated ($NADP^+$) and non-phosphorylated (NAD^+) oxidised form, or in their reduced versions [NAD(P)H]. These cofactors can mediate as electron acceptors/donors in either oxidation or reduction processes. Since the use of cofactors in stoichiometric amounts is hampered due to inhibition effects and economic hurdles, the employment of cofactor recycling systems is compulsory for the development of efficient and economic feasible redox transformations.[3] For the case of reductive reactions, this normally implies that the coupling of a second enzymatic reaction is typically mediated by glucose dehydrogenase (GDH)[4] or formate dehydrogenase (FDH) at the expense of a sacrificial cosubstrate, glucose or formate, respectively, in the so-called coupled-enzyme approach. Other methodologies involve the use of chemical, electrochemical or photochemical transformations,[5] or the use of cheaper synthesised nicotinamide cofactor mimetics in stoichiometric amounts.[6]

Deep eutectic solvents (DESs) have recently emerged as a new generation of ionic liquids (ILs).[7] A DES is typically composed of a quaternary ammonium salt acting as a hydrogen bond acceptor (HBA, *i.e.* choline chloride), and a hydrogen bond donor (HBD, such as glycerol, urea or a sugar), so the intermolecular interactions between its components provide specific properties for these neoteric solvents. Their straightforward preparation by simply mixing a HBA and a HBD to form a liquid represents an excellent waste-free process providing a significant advantage in comparison with the preparation of traditional ILs. Moreover, the possibility of utilising natural and degradable compounds is also highly valuable from an environmental point of view.

In this context, natural deep eutectic solvents (NADESs) are defined as a mixture of two or more compounds that are generally plant based primary metabolites (organic acids, sugars, alcohols, amines and amino acids), that interact *via* intermolecular forces and liquefy if combined in specific molar ratios.[8] The NADES concept dates from 2011 and it created great expectations in the context of green chemistry as they are less toxic and more environmentally friendly than ILs and traditional metal-based DESs.

Interestingly, the use of (NA)DESs as green (co)solvents in enzymatic transformations has rapidly gained attention,[9] including their application in redox processes.[10] In this context, the development of bioreductions using ADHs appears as a robust technique for the asymmetric synthesis of alcohols using aqueous-(NA)DES mixtures, where mainly choline chloride : glycerol (ChCl : Gly) has been employed as a cosolvent.[11] In some cases, other HBDs have also been used, with the choice of glucose being scarcely reported and in all cases for whole cell-mediated biotransformations.[12] Herein, we have focused on the development of efficient global

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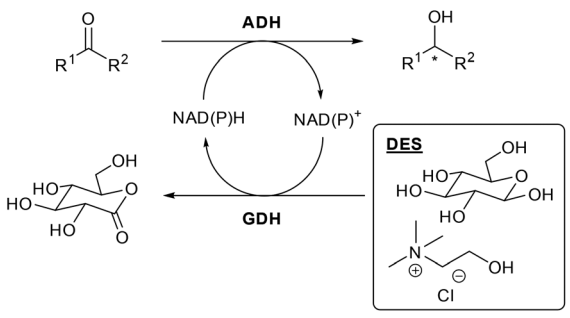
†Electronic supplementary information (ESI) available: Bioreduction experiments, analytics, copies of HPLC chiral analyses, and ^1H, ^{13}C and DEPT NMR spectra for the described organic compounds. See DOI: 10.1039/c9gc00318e

reduction processes by combining an overexpressed ADH for the reduction of prochiral ketones accomplished with an appropriate cofactor recycling system, considering in this case GDH, which allows the cofactor reduction by transforming D-glucose into D-glucono-1,5-lactone that later spontaneously hydrolyses in the reaction medium towards the formation of D-gluconic acid. For that reason, we decided to investigate the use of D-glucose as a HBD in a designer NADES with an efficient solution for: (a) better solubility of the lipophilic substrates and (b) a suitable cofactor regeneration system (Scheme 1).

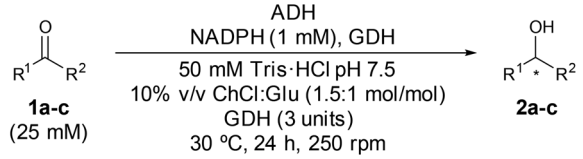
For this study, commercially available GDH-105 from Codexis Inc. was selected for cofactor recycling, and the behaviour of different made in house overexpressed NADPH-dependent ADHs was studied. We used the (*R*)-selective one from *Lactobacillus brevis* (LbADH)[13] and the (*S*)-selective ADHs from *Thermoanaerobacter ethanolicus* (TeSADH),[14] *Thermoanaerobacter* sp. (ADH-T),[15] *Sphingobium yanoikuyae* (SyADH)[16] and *Ralstonia* sp. (RasADH).[17] To check the enzyme activity in the presence of NADES, the model substrate for each ADH was added in 25 mM concentration under ideal conditions for this class of redox enzymes, and commercial GDH-105 was used as the coupled-enzyme (3 units). From ChCl : Glu mixtures, 1.5 : 1 mol/mol was selected in order to have a significant amount of glucose for cofactor recycling purposes without the detriment of using a highly dense and viscous NADES at considerable glucose concentrations, which would make it difficult to handle.[18] 10% v/v of the NADES formed by ChCl and glucose (1.5 : 1 mol/mol) was added into the reaction medium (50 mM Tris·HCl pH 7.5, 1 mM NADPH). Finally, 10 mg of the lyophilised ADH was resuspended and the reactions were incubated at 30 °C for 24 h. The results from the bioreduction experiments are shown in Table 1.

Gratifyingly, complete conversions and selectivities were attained for LbADH, ADH-T and RasADH in the bioreduction of acetophenone, 2-octanone and propiophenone, respectively (entries 1, 3 and 5), while TeSADH (entry 2) and SyADH (entry 4) also led to high conversions and good to excellent enantiomeric excess. As a result of their remarkable performances, we decided to delve into bioreductions catalysed by LbADH, ADH-T and RasADH using this designer NADES as both a co-solvent and co-substrate.



Scheme 1 Use of a designer deep eutectic solvent formed by D-glucose and ChCl to recycle the nicotinamide cofactor [NAD(P)H] in alcohol dehydrogenase-catalysed processes.

Table 1 Bioreduction of ketones using ADHs in an aqueous medium with 10% v/v of NADES ChCl : Glu (1.5 : 1 mol/mol) as a cosolvent[a]



Entry	ADH	Substrate	Conv.[b] (%)	ee[b] (%)
1	Lb	Acetophenone (**1a**)	>99	>99(*R*)
2	TeS	2-Octanone (**1b**)	86	92(*S*)
3	T	2-Octanone (**1b**)	99	>99(*S*)
4	Sy	Propiophenone (**1c**)	81	>99(*S*)
5	Ras	Propiophenone (**1c**)	>99	>99(*S*)

[a] See the ESI for the detailed reaction conditions and protocols. [b] Conversion and enantiomeric excess values were determined by GC analysis. The absolute configuration of the alcohol appears in brackets.

As one could argue regarding the integrity of the NADES in an aqueous medium at low concentrations, we decided to increase its concentration in order to study this effect. Edler and co-workers recently showed that by using DES in water at >50% (w/w) concentrations,[19] the nanostructure of the eutectic solvent could be perfectly retained (see also NMR experiments in the ESI, Fig. S2–S8†). Therefore, we increased the proportion of NADES employed in the biotransformation up to 90% v/v [20] (see Fig. 1).

Gratifyingly, LbADH and ADH-T revealed perfect results in terms of conversion and enantioselectivity at high NADES contents. On the one hand, LbADH quantitatively catalyzed the bioreduction of acetophenone even at 60% v/v NADES concentration, maintaining complete selectivity even at 80% v/v of NADES (>99% ee). On the other hand, RasADH led to perfect conversion until 70% v/v of NADES in the bioreduction of propiophenone, but a drop in the selectivity was observed when using a proportion equal or higher than 40% v/v of NADES (93% ee). Interestingly, this surprising change in the stereoselectivity has been reported for several authors when using different alcohol dehydrogenases (*e.g.*, RasADH),[11b] and it is usually associated with the inhibition of other biocatalytic activities present in the enzyme preparation or due to protein conformational changes.[10]

To show the applicability of this methodology, the use of higher substrate concentrations was explored, ranging from 30 to 200 mM (Fig. 2) under similar reaction conditions to those previously reported at 25 mM. First of all, 10% v/v of NADES was employed (Fig. 2A) and different trends were observed. On the one hand, ADH-T and RasADH catalysed the complete reductions of 2-octanone and propiophenone up to 50 mM, yielding both alcohols in the enantiopure form. ADH-T displayed lower activities at a 75 mM substrate concentration (75% conversion) with a slight decrease in the selectivity

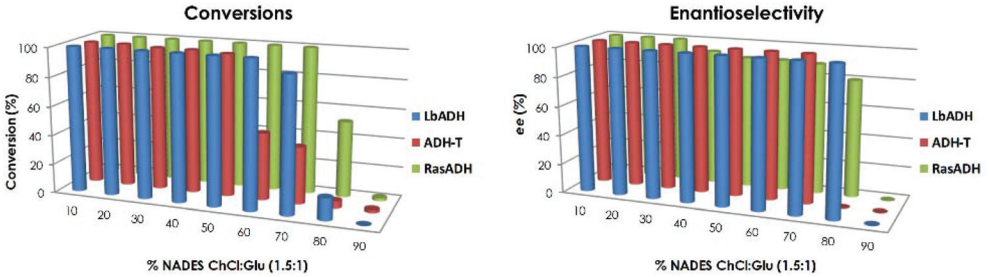


Fig. 1 Effect of activity and selectivity using different amounts of NADES ChCl : Glu (1.5 : 1 mol/mol) in the bioreduction of acetophenone (LbADH), 2-octanone (ADH-T) and propiophenone (RasADH). Numerical values are given in the ESI.†

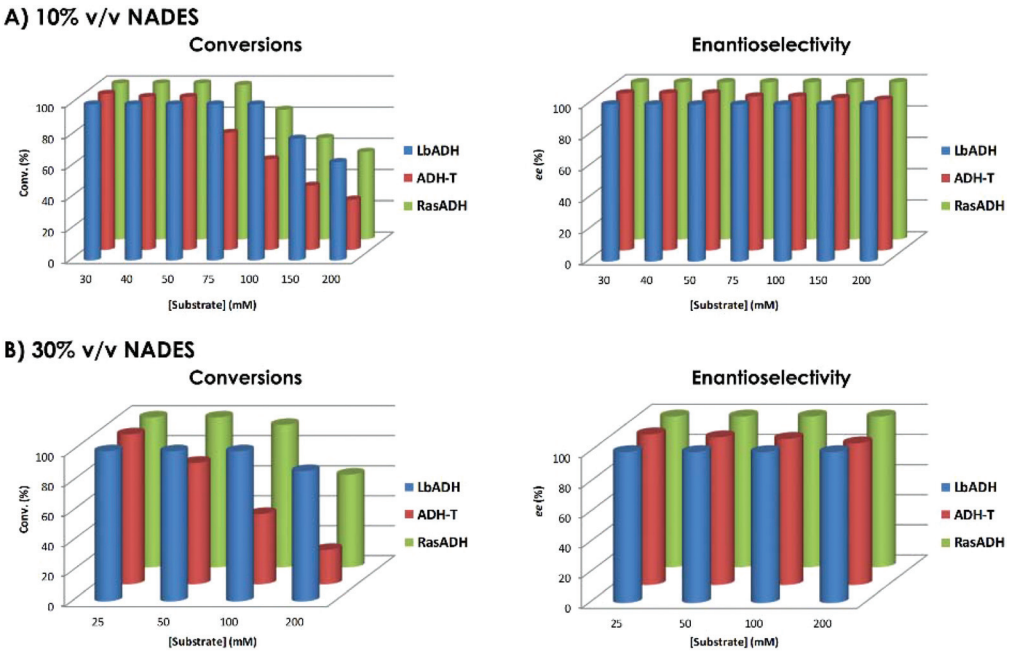


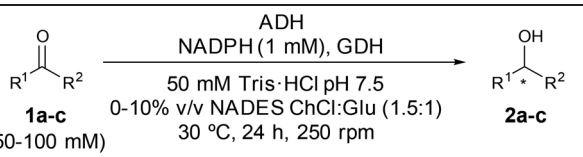
Fig. 2 Effect of activity and selectivity using different amounts of NADES ChCl : Glu (1.5 : 1 mol/mol) at variable substrate concentrations in the bioreduction of acetophenone (LbADH), 2-octanone (ADH-T) and propiophenone (RasADH). Numerical values are given in the ESI.†

(96–98% ee at 75–200 mM), while RasADH led to 83% conversion at 100 mM, to later significantly decrease the activity although the selectivity was optimum in all cases. On the other hand, LbADH was capable of transforming up to 100 mM of acetophenone into enantiopure (*R*)-1-phenylethanol with complete conversion.

Having in mind that glucose is employed as a co-substrate, so that its concentration decreased with the advance of the reaction, higher amounts of NADES were employed (30% v/v of NADES) at different ketone concentrations (25–200 mM, Fig. 2B). Remarkably, the three ADHs worked with excellent levels of selectivity, and although the activity of ADH-T was worse in comparison when using 10% v/v of NADES, LbADH and RasADH acted in a similar manner to that in the reaction with 10% NADES.

The best substrate concentration *vs.* conversion ratio for each enzyme was selected in order to compare the effect of our system regarding the standard glucose/GDH system typically employed (Table 2). Using the same amount of glucose for all the bioreductions, the NADES/GDH system was superior (even

Table 2 Bioreduction of ketones using ADHs with glucose as a co-substrate in pure buffer or using a 10% v/v ChCl : Glu mixture as a cosolvent at 30 °C and 250 rpm for 24 h[a]



Entry	NADES (%)	ADH-substrate[b]	Conv.[c] (%)	ee[c] (%)
1	0	Lb and **1a** (100 mM)	59	>99(*R*)
2	10	Lb and **1a** (100 mM)	>99	>99(*R*)
3	0	T and **1b** (50 mM)	89	>99(*S*)
4	10	T and **1b** (50 mM)	98	>99(*S*)
5	0	Ras and **1c** (100 mM)	61	>99(*S*)
6	10	Ras and **1c** (100 mM)	83	>99(*S*)

[a] See the ESI for the detailed reaction conditions and protocols. [b] The ketone concentration is shown in brackets, while the glucose concentration is 240 mM. [c] Conversion and enantiomeric excess values were determined by GC analysis. The absolute configuration of the alcohol appears in brackets.

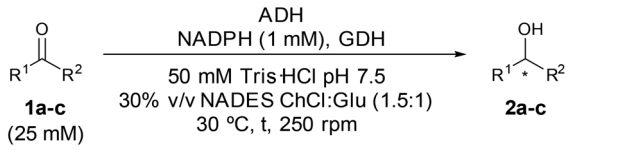
entries) to the biotransformations performed by just adding glucose/GDH (odd entries), especially for the bioreduction of acetophenone with LbADH (entries 1 and 2). While this effect remains unclear, there are reports that have described that choline chloride can exert a stabilizing effect on the structure of biomolecules such as proteins[21] or DNA.[22]

Selected bioreduction processes were monitored within the time, trying to find optimal conditions for the selective bioreduction of ketones (Table 3). 30% v/v NADES, 25 mM ketone concentrations and 30 °C were fixed, achieving full conversions for each substrate at 1 h (acetophenone, LbADH, entry 2), 1.5 h (2-octanone, ADH-T, entry 5) or 20 min (propiophenone, RasADH, entry 8).

Also, the recyclability of the enzyme and NADES (30% v/v) was subjected to study (Fig. 3). After the first transformation was completed, the reaction mixture was extracted in high yields with ethyl acetate (EtOAc, upper phase), showing full conversions and excellent selectivities, and the aqueous medium formed by the NADES and the enzymes (ADH and GDH, bottom phase) was employed for running a second biotransformation by simply adding the corresponding ketone **1a–c**. Unfortunately, a significant activity loss was observed (10–56% conversion), which was dramatic for LbADH (Fig. 3, left) and ADH-T (Fig. 3, middle).

There are two possible reasons to explain this inactivation effect. On the one hand, the use of a great amount of an organic solvent such as EtOAc could lead to a loss of enzyme activity. On the other hand, D-glucono-1,5-lactone is produced through glucose oxidation that later spontaneously hydrolyses to D-gluconic acid as a by-product, decreasing the pH of our aqueous system (Tris·HCl 50 mM pH 7.5). In order to test the influence of these two parameters in the enzyme activity, firstly, pentane was used instead of EtOAc to extract the product (Fig. 4, blue bars), and later the Tris·HCl buffer 200 mM pH 7.5 was also employed instead of the previous 50 mM buffer (Fig. 4, red bars) in order to see if a higher buffer concentration could balance out the production of D-gluconic acid without altering the pH of the buffer. The results are shown in Fig. 4, observing significant benefits when using pentane and 200 mM Tris HCl buffer.

Table 3 Conversion *vs.* time of alcohol dehydrogenase-catalysed bioreductions using 30% v/v NADES ChCl : Glu (1.5 : 1)[a]



Entry	ADH	Substrate	t (min)	Conv.[b] (%)	ee[b] (%)
1	Lb	**1a**	30	70	>99(*R*)
2	Lb	**1a**	60	>99	>99(*R*)
3	T	**1b**	30	82	>99(*S*)
4	T	**1b**	60	95	>99(*S*)
5	T	**1b**	90	>99	>99(*S*)
6	Ras	**1c**	10	97	>99(*S*)
7	Ras	**1c**	15	98	>99(*S*)
8	Ras	**1c**	20	>99	>99(*S*)

[a] See the ESI for the detailed reaction conditions and protocols. [b] Conversion and enantiomeric excess values were determined by GC analysis. The absolute configuration of the alcohol appears in brackets.

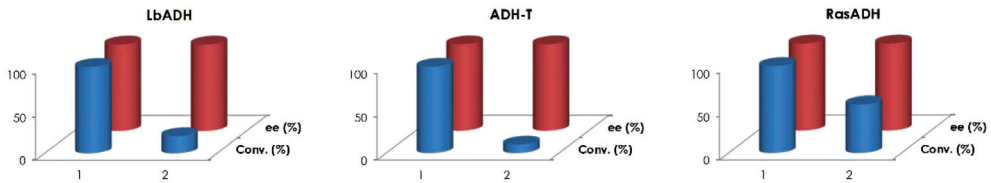


Fig. 3 Recycling of the enzyme and 50 mM buffer-NADES (30% v/v) mixture after extraction with EtOAc in the bioreductions of acetophenone with LbADH (left), 2-octanone with ADH-T (middle) and propiophenone with RasADH (right). Numerical values are given in the ESI.†

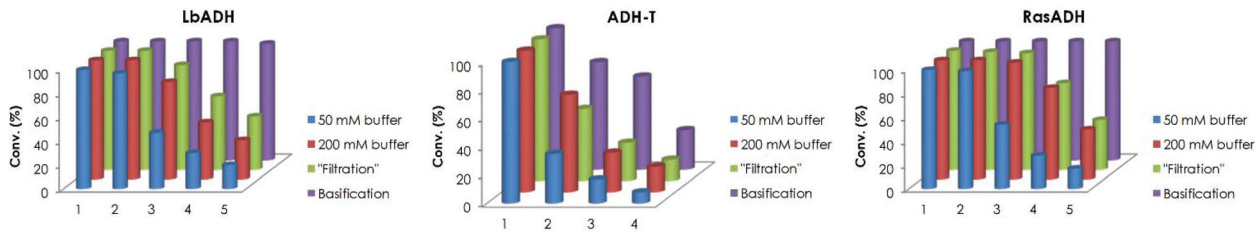


Fig. 4 Recycling of the enzyme and NADES (30% v/v) after extraction with pentane in the bioreductions of acetophenone with LbADH (left), 2-octanone with ADH-T (middle) and propiophenone with RasADH (right): (A) blue bars are reactions carried out in 50 mM buffer, extracting with pentane and reusing the NADES-buffer plus the enzyme; (B) red bars are reactions carried out in 200 mM buffer, extracting with pentane and reusing the NADES-buffer plus the enzyme; (C) green bars are reactions carried out in 200 mM buffer, filtering the enzyme, extracting with pentane and reusing the NADES-buffer plus the enzyme; and (D) purple bars are reactions carried out in 200 mM buffer, filtering the enzyme, extracting with pentane, readjusting the pH of the NADES-buffer mixture up to 7.5 and reusing the NADES-buffer plus the enzyme. Numerical values are given in the ESI.†

Interestingly, the use of pentane already led to better results allowing a successful first recycling with LbADH (Fig. 4 left, 97% conversion) and RasADH (Fig. 4 right, 99% conversion). In order to explore the possible enzyme deactivation due to the extracting solvent, additional experiments (Fig. 4, green bars) were carried out by filtering the ADH after the bioreduction, to later extract the reaction medium and perform the next reuse. In this case, only slight improvements were found for LbADH (Fig. 4, left), while no significant changes were found for the recycling of ADH-T (Fig. 4, middle) and RasADH (Fig. 4, right). When the pH value of the reaction medium after a biotransformation was measured, it was found to be 3.5 for those performed in the Tris·HCl buffer 50 mM, while a value around 4.9–5.0 was attained in the ones carried out at a 200 mM buffer concentration due to the release of D-gluconic acid. For that reason, the pH was readjusted up to 7.5 before each recycling experiment after enzyme filtration and extraction with pentane (Fig. 4, purple bars), finding in this manner a great improvement, especially when using LbADH (98% conversion and 98% ee after 5 cycles, Fig. 4 left) and RasADH (98% conversion and >99 ee after 8 cycles, Fig. 4 right and the ESI†).

Finally, in order to demonstrate the applicability of the method, we set up some semi-preparative biotransformations (100 mg of ketone) finding results in accordance with those obtained at the analytical scale. Full conversions into the enantiopure alcohols were reached, producing (*R*)-1-phenylethanol in 78% isolated yield with LbADH (150 mg of the lyophilised preparation and 100 mM ketone concentration), (*S*)-2-octanol in 85% yield using ADH-T (350 mg of the lyophilised preparation, 50 mM) and (*S*)-1-phenyl-1-propanol in 89% yield with RasADH (150 mg of the lyophilised preparation, 100 mM), after extractions with EtOAc.

For these semi-preparative transformations, we performed a simple quantification of the *E*-factor[23] to obtain an overview of the environmental impact of this methodology. Hence, the EATOS tool[24] was used focusing on the relevance of the reaction conditions regarding the reagents, catalysts and solvents employed (excluding water), and taking into account the waste generated. As can be seen in the ESI (Fig. S1†), the values obtained were between 236 and 286. While these numbers are still high, it can be seen that the highest percentage of these numbers comes from the solvents employed, in particular from EtOAc used to extract the final products. Since on the large scale the recycling of organic solvents is a commonly applied technique, we are sure that these numbers could be further optimised.

Overall, a ChCl : Glu mixture has been used as a designer natural DES applied to ketone bioreduction transformations using five different ADHs. The combination of an aqueous buffer system with the NADES up to a 50% v/v ratio has provided two main advantages. On one hand, the presence of glucose provides the co-substrate for the GDH-catalysed reaction for the nicotinamide cofactor recycling. On the other hand, the bioreductions were run at higher substrate concentrations in comparison with the buffer system employing glucose/GDH, and the development of practical protocols in terms of excellent conversions were possible in up to 100 mM concentration with excellent selectivities. After optimisation of the reaction conditions, the bioreductions were carried out on the semi-preparative scale finding that the use of pentane as the extracting organic solvent presented great advantages for enzyme recycling in comparison with EtOAc, the traditional solvent used in these work-up procedures, as pentane is more resistant to enzyme inactivation. It is also worth mentioning that the use of concentrated buffers (*i.e.* 200 mM Tris HCl buffer) highly improved the enzyme activity of ADHs in these reactions, since D-gluconic acid is formed as a co-product in the cofactor recycling reaction, dramatically changing the pH in the presence of a more diluted buffer with the advancement of the reaction course.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Conclusions

To summarise the main achievements of this Doctoral Thesis, it must be mentioned that several (chemo)enzymatic methodologies have been developed in order to synthesise optically pure compounds in an efficient, selective and straightforward way under mild reaction conditions. For this purpose, enzymes as lipases and transaminases have been employed in the Part I of this Doctoral Thesis as biocatalysts in acylation and transamination processes. Furthermore, in Part II, the bioreduction of several prochiral ketones using alcohol dehydrogenases and the performance of these enzymes in NADES-buffer mixtures has been studied.

In Chapter 1, the synthesis of a wide panel of racemic 1-[2-bromo(het)aryloxy]propan-2-amines has been developed from the corresponding 2-bromophenols. After optimisation of the reaction conditions (acyl donor, amount of enzyme, solvent, temperature and reaction time), the kinetic resolution of these compounds was successfully performed using CAL-B as biocatalyst and ethyl methoxyacetate as acyl donor. In this manner, conversion values closed to the ideal 50% and high to excellent enantioselectivities for the preparation of the corresponding (*S*)-amines and (*R*)-amides were achieved in short reaction times.

In Chapter 2, a general cascade transaminase-catalysed protocol was disclosed for the synthesis of optically active γ- and δ-lactams starting from the corresponding γ- and δ-keto esters. In this way, making use of the appropriate TA in each case, the process happened through the biotransamination of these substrates to form the corresponding chiral amino esters, intermediates that were never observed as they underwent a spontaneous cyclisation in the reaction medium giving rise to the optically active lactams. The process has been carefully studied taking into account the amount of amine donor, temperature, co-solvent and substrate concentration, in order to finally demonstrate its scalability and reproducibility.

In Chapters 3 and 4, a chemical process for synthesising a representative group of α-alkyl-β-keto amides from the corresponding β-keto esters was initially developed. Hence, in Chapter 3, the role displayed by TAs has been deeply examined in the transformation of these compounds into the corresponding optically active β-amino amides through a DKR process. After optimisation of several reaction parameters (amount and source of TA, temperature and pH), it has been possible to obtain the corresponding diastereoenriched and enantiopure α-alkyl-β-amino amides. In a similar approach, ADHs have been employed in

Conclusions

Chapter 4 as biocatalysts in the bioreduction of the β-keto amides. Thus, the corresponding diastereoenriched and enantiopure α-alkyl-β-hydroxy amides were successfully synthesised. Both amino and hydroxy amides have been fully characterised and their relative configuration proved using homodecoupling ^{1}H-NMR experiments.

In Chapter 5, the chemoenzymatic synthesis of chiral 1,4-diaryl-1,4-diones has been reported. First of all, 4'-substituted and α-bromo-4'-substituted acetophenones differing in the substitution pattern of the aromatic ring have been employed to synthesise the corresponding 1,4-diketones. Afterwards, the bioreductions of those derivatives catalysed by (*S*)-selective RasADH was studied and, after optimisation of the reaction conditions (regeneration system, cosolvent, temperature and reaction time), the synthesis of the corresponding optically active (1*S*,4*S*)-diols was successfully achieved.

Finally, in Chapter 6, a designer NADES formed by choline chloride and glucose (ChCl:Glu, 1.5:1 mol/mol) has been used as both co-solvent and co-substrate in the ADH-catalysed bioreduction of different prochiral ketones. Thus, it has been possible to employ higher substrate concentrations (up to 100 mM) especially if compared with those reactions carried out in the absence of this co-solvent. The glucose used as DES component serving as HBD, was crucial in order to regenerate the NADPH since GDH was employed as coupled enzyme. Additionally, the recyclability of this system has been extensively studied, focusing on the optimisation of the utilised organic solvent for the liquid-liquid extraction separation and the buffer concentration.

Conclusiones

A modo de conclusiones de esta Tesis Doctoral y de manera general se puede decir que se han desarrollado varias metodologías (quimio)enzimáticas para sintetizar compuestos ópticamente activos de manera directa, eficiente y selectiva empleando condiciones de reacción suaves. Para ello, en la Parte I de esta Tesis Doctoral se han empleado lipasas y transaminasas como biocatalizadores en procesos de acilación y transaminación. Además, en la Parte II, se ha estudiado la biorreducción de diferentes cetonas empleando alcohol deshidrogenasas, así como el comportamiento de estos enzimas en mezclas de disolventes eutécticos naturales y disoluciones tampón.

En el Capítulo 1, se ha sintetizado un extenso panel de 1-[2-bromo(hetero)ariloxi]propan-2-aminas racémicas a partir de los correspondientes 2-bromofenoles. Tras optimizar las condiciones de reacción (donador de acilo, cantidad de enzima, disolvente, temperatura y tiempo de reacción), la resolución cinética de estos compuestos se llevó a cabo empleando la CAL-B como biocatalizador y el metoxiacetato de etilo como donador de acilo. De esta forma, en tiempos de reacción cortos, se alcanzaron conversiones próximas al 50% y elevadas o excelentes enantioselectividades para la obtención tanto de las correspondientes (*S*)-aminas como de las (*R*)-amidas.

En el Capítulo 2, se describe un proceso en cascada catalizado por transaminasas para la síntesis de γ- y δ-lactamas a partir de los correspondientes γ- y δ-cetoésteres. De esta forma, haciendo uso de la TA adecuada en cada caso, el proceso transcurre a través de la biotransaminación de dichos sustratos para dar lugar a los correspondientes aminoésteres quirales, intermedios que nunca llegaron a ser observados ya que ciclaban espontáneamente en el medio de reacción dando lugar a las lactamas ópticamente activas. El proceso ha sido estudiado minuciosamente atendiendo a la cantidad del donador de amino, temperatura, cosolvente utilizado y concentración del sustrato, con el fin de demostrar finalmente la escalabilidad y reproducibilidad del proceso.

En los Capítulos 3 y 4, se desarrolló inicialmente un proceso químico general para sintetizar un grupo representativo de β-cetoamidas alquiladas en α a partir de los correspondientes β-cetoésteres. De esta forma, en el Capítulo 3, se ha explorado el papel que juegan una serie de transaminasas en la transformación de estos compuestos en β-aminoamidas ópticamente activas mediante procesos de DKR. Tras optimizar diversos parámetros de reacción (tipo y cantidad de TA,

temperatura y pH), ha sido posible obtener las correspondientes β-aminoamidas alquiladas en α diastereoenriquecidas y enantiopuras. De forma similar, en el Capítulo 4 se han empleado ADHs como biocatalizadores para la biorreducción de las β-cetoamidas. Así, se han sintetizado las correspondientes β-hidroxiamidas alquiladas en α de manera estereoselectiva. Todas las aminoamidas e hidroxiamidas fueron caracterizadas y su configuración relativa se ha determinado mediante experimentos de desacoplamiento homonuclear de ^{1}H-RMN.

En el Capítulo 5, se ha presentado la síntesis quimioenzimática de 1,4-diaril-1,4-dioles quirales. Primeramente, se utilizaron acetofenonas 4'-sustituidas y α-bromo-4'-sustituidas que diferían en la sustitución del anillo aromático para sintetizar las correspondientes 1,4-dicetonas. Posteriormente, se estudió la biorreducción de dichos derivados catalizada por la RasADH y, tras optimizar las condiciones de reacción (sistema de regeneración, cosolvente, temperatura y tiempo de reacción), la síntesis de los correspondientes (1*S*,4*S*)-dioles ópticamente activos se llevó a cabo satisfactoriamente.

Finalmente en el Capítulo 6, un NADES formado por cloruro de colina y glucosa (ChCl:Glu, 1.5:1 mol/mol), se ha utilizado como cosolvente y, al mismo tiempo, cosustrato en la biorreducción de diferentes cetonas proquirales catalizada por ADHs. Así, ha sido posible utilizar altas concentraciones de sustrato (hasta 100 mM) que son mucho mayores en comparación con los procesos que transcurren en ausencia de este cosolvente. La glucosa empleada como parte del DES ha sido además crucial a la hora de regenerar el NADPH ya que se utilizó una GDH como enzima acoplado. Además, la reciclabilidad de este sistema fue estudiada en detalle, siendo necesaria la optimización del disolvente orgánico empleado para la extracción, así como la concentración de la disolución tampón empleada en el proceso.

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