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Cutting Short the Asymmetric Synthesis of the Ramatroban Precursor by Employing ω-Transaminases

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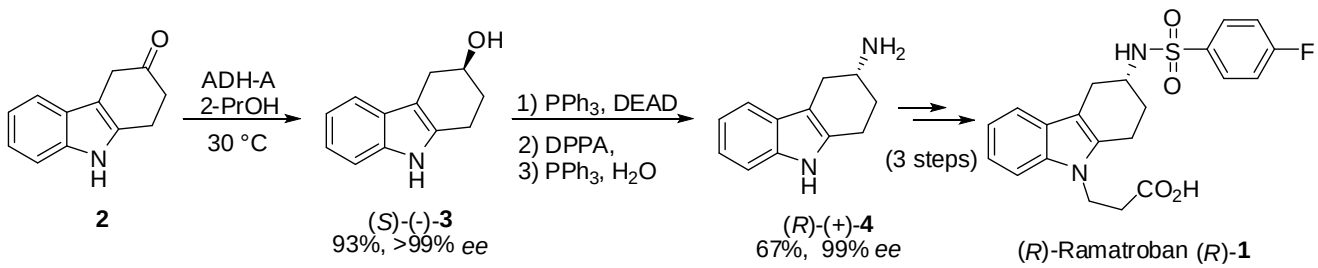
Abstract. Starting from an adequate ketone precursor previous reports required three step for the preparation of (*R*)-2,3,4,9-tetrahydro-1*H*-carbazol-3-amine, a key intermediate for the synthesis of the anti allergic drug Ramatroban. A single biocatalytic step was sufficient to prepare the target amine with >97% *ee* (HPLC) via reductive amination of the corresponding ketone using a ω-transaminase as biocatalyst. Since the ketone was barely soluble under the reaction conditions employed, it was provided as a solid and still the reaction went to completion within 4 hours at 50 mM substrate concentration. Although 2-propylamine is regarded as an ideal amine donor, it turned out to be detrimental for the specific ketone precursor leading to the formation of various side products. These could be avoided by using (*R*)-1-phenylethylamine as the best suited amine donor. An alternative work up was developed via freeze drying of the reaction mixture, enabling the isolation of the desired (*R*)-amine in excellent yield (96%) and enantiopure form on a preparative scale (500 mg). No purifications step (e.g. column chromatography, crystallization) was required.

Keywords: amines; asymmetric synthesis; biotransformations; nitrogen heterocycles; transaminases;

(*R*)-Ramatroban, Baynas® (*R*)-**1** is employed for the treatment of allergic rhinitis and asthma but also shows promising applications for the treatment of coronary artery diseases.[1] Since exclusively the (*R*)-enantiomer is linked to the pharmacological activity,[2] efficient asymmetric routes for its preparation are required.

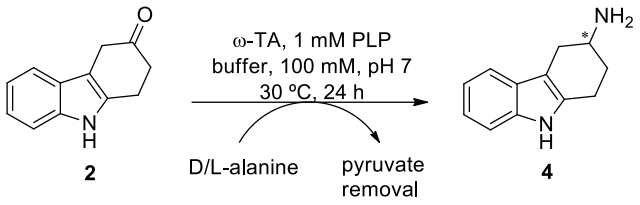
To date, the preparation of the enantiomerically pure drug has been described by the formation of diastereomeric salts[3] or asymmetric Fischer indolization using chiral phosphoric acids.[4] Since biocatalysis offers various methods to prepare pharmaceutically active ingredients or natural products[5] e.g. employing hydrolases,[6] the chemoenzymatic synthesis of Ramatroban has been reported using lipases or oxidoreductases for the asymmetric key step, giving the enantioenriched alcohol (*S*)-**3** (Scheme 1).[7] The main drawback of this route results from the Mitsunobu inversion of the alcohol to afford the amine (*R*)-**4** which is prone to racemization. Moreover, large excesses of various reagents such as diethyl azodicarboxylate (DEAD), diphenylphosphoryl azide (DPPA) or triphenyl phosphine (PPh_3) are required, whereby some require special handling. Consequently, this step is the main limitation to establish an industrial chemoenzymatic process where the production of intermediates on a multi-kg scale is required.



Scheme 1. Key steps of the published chemoenzymatic synthesis of (*R*)-Ramatroban involving the asymmetric bio reduction of the ketone **2** and the Mitsunobu inversion of the alcohol (*S*)-**3** to afford the enantiopure amine (*R*)-**4**.[7]

As an alternative the direct asymmetric amination of the prochiral ketone **2** is described here employing ω-transaminases (ω-TAs, EC 2.6.1.x).[8,9] ω-TAs have been used already successfully for the preparation of pharmaceutical compounds[10] or natural products[11] even on a multi-kg scale.[12]

The amination of the ketone **2** was initially studied at a 25 mM substrate concentration using a set of ω-TAs overexpressed in *E. coli*: the (*R*)-selective ω-TAs from (*R*)-*Artrhobacter*,[13] *Hyphomonas neptunium*[14] and *Aspergillus terreus* and the (*S*)-selective ω-TAs from *Bacillus megaterium*,[15] *Vibrio fluvialis*,[16] *Arthrobacter citreus*,[17] and *Chromobacterium violaceum*[18] (Scheme 2). D- and L-alanine, respectively were initially used as amine source. The equilibrium was shifted towards product formation by removing/recycling the pyruvate to L-alanine with an alanine dehydrogenase (AlaDH) at the expense of ammonia and formate.[19]



Scheme 2. Amination of ketone **2** employing ω-transaminases (ω-TA). Pyruvate was removed/recycled to L-alanine employing an alanine dehydrogenase; formate dehydrogenase was employed for NADH recycling; PLP: pyridoxal-5'-phosphate.

From the (*S*)-selective ω-TAs tested only the ω-TA from *A. citreus* and *C. violaceum* were active leading to complete consumption of the ketone after 24 h (Table 1). While *A. citreus* displayed a moderate enantiopreference (66% *ee*, entry 1), a perfect *ee* >97% (HPLC) was obtained with *C. violaceum* (entry 2). The amination was performed on preparative scale isolating (*S*)-**4** in enantiopure form and good isolated yield (62%).

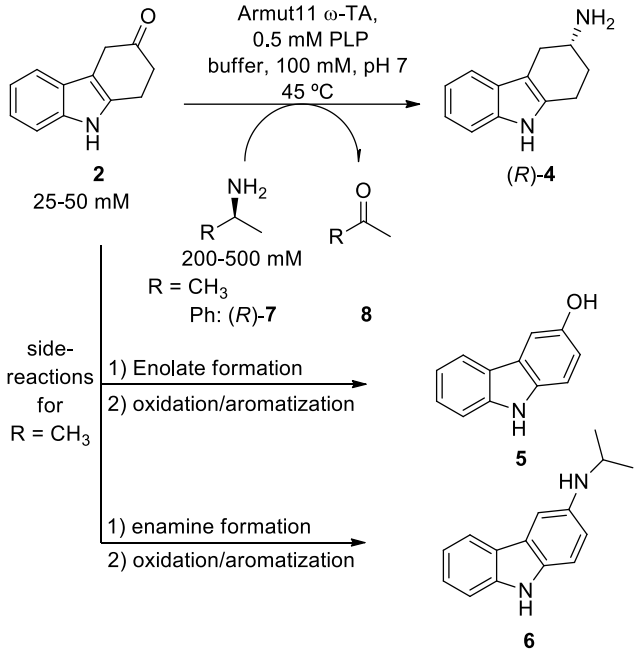
Table 1. Biocatalytic reductive amination of **2** employing ω-TAs and L-alanine as amine donor.

Entry	ω-TA	Conv. [%][a]	**4** [%][b]	*ee* **4** [%][d]
1	*A. citreus*	>97	90	66 (*S*)
2	*C. violaceum*	>97	93 (62)[c]	>97 (*S*)

[a] Measured by GC-FID. Reaction conditions: phosphate buffer (100 mM, pH 7), ketone **2** (25 mM), lyophilised cells E. coli/ω-TA (30 mg), PLP (1 mM), NAD^+ (1 mM), D- or L-alanine (10 equiv.), Ala-DH (11 U), FDH (11 U), ammonium formate (150 mM), 24 h at 30 ºC and 750 rpm. [b] An unidentified side product was formed, therefore the percentage of **4** is lower than the conversion. [c] Isolated yield in brackets. [d] Determined for the corresponding acetamide by HPLC on a chiral phase.

It is worth mentioning that the substrate **2** was barely soluble in buffer (1.6 mM), thus at 25 mM calculated substrate concentration most of the ketone **2** was present as a solid, consequently it is remarkably that the transformation worked so well for both (*S*)-enzymes.

Unfortunately, none of the (*R*)-selective ω-TAs of the first set of enzymes transformed ketone **2** providing access to the target amine (*R*)-**4**. Consequently, a variant of (*R*)-*Arthrobacter* (ArRmut11-ω-TA) was tested, which was developed for the amination of sterically hindered ketones.[12b] This enzyme was evolved to show an improved thermal stability and is described to preferentially accept 2-propylamine as amine donor. Consequently 2-propylamine was employed in excess (500 mM) to shift the equilibrium towards the formation of the amine **4** at 45 °C (Scheme 3).



Scheme 3. Single enzyme asymmetric amination of ketone **2** using ArRmut11-ω-TA.

After 24 h the ketone **2** was completely consumed, obtaining the amine (*R*)-**4** in enantiopure form although with low isolated yield (26%, Table 2, entry 1), due to the formation of side products, which were isolated and characterized by NMR and mass spectroscopy. These products were identified as a mixture of the phenol **5** and isopropylamino carbazol **6** (see SI for details). The formation of **5** can be rationalized by base promoted formation of the enolate and the subsequent spontaneous aromatization. The second side-product, the isopropylamino carbazol **6**, can be explained by the formation of the corresponding enamine followed by spontaneous aromatization. Trying to improve the yield for the target (*R*)-**4**, we considered the use of different organic co-solvents, which might help also with respect to the low solubility of the substrate.

However, the addition of a miscible organic co-solvent such as DMSO favoured the formation of the enamine leading to even lower isolated yields (entry 2). On the other hand, better results were obtained with a non-miscible solvent such as EtOAc where the organic phase acts as a reservoir for the ketone reducing the formation of side products but slowing down the overall reaction (entry 3).

Table 2. Reductive amination of **2** employing ArRmut11-ω-TA and 2-propylamine (500 mM) as amine donor.

Entry	Co-solvent	**4** [%][a]	*ee* **4** [%][b]
1	--	26	>97 (*R*)
2	10% DMSO	9	>97 (*R*)
3	10% EtOAc	43	>97 (*R*)

[a] Isolated yields after flash chromatography. Reaction conditions: ketone (4.6 mg, 25 mM), lyophilised cells containing ArRmut11-ω-TA (30 mg), PLP (0.5 mM), 2-propylamine (500 mM), after 24 h at 30 ºC and 750 rpm. [b] Determined for the corresponding acetamide by HPLC on a chiral phase.

To minimise or even avoid the formation of the side-products **5** and **6**, (*R*)-1-phenylethylamine (*R*)-**7** as a sterically more demanding amine donor was investigated. Due to its bulkiness and lower basicity, less base catalysed enol formation as well as also less enamine formation was expected. Additionally, the deamination of (*R*)-**7** is thermodynamically favoured meaning that a lower excess of the amine donor should be required.[20]

Optimization of the biocatalytic amination of **2** was performed at 45 °C in phosphate buffer at pH 7 at varied concentrations of the starting ketone and the amine donor (Table 3). The reaction was first performed at a 25 mM substrate concentration employing 4 equivalents of (*R*)-**7** as amine source. Employing a semi purified enzyme preparation (heat treatment) of ArRmut11-ω-TA all the starting material was exclusively transformed into the desired amine (*R*)-**4** already after 4 h (entry 1). The amine was isolated after flash chromatography in good yield and enantiopure form. However, a significant mass loss (24%) was noticed during the isolation/purification steps. For that reason, we designed an alternative work-up procedure. Instead of extraction after basification, the crude reaction was alkalinized and freeze-dried to remove the water. The so obtained powder was suspended in a CH_2Cl_2/EtOH mixture and filtered through Celite®, then the solvent was removed under reduced pressure. Using this protocol (*R*)-**4** was obtained in enantiopure form with 95% isolated yield and excellent chemical purity (see supporting information for NMR data), without any additional purification step (entry 2).

When reducing the equivalents of amine donor to make the process more attractive, 2 equivalents of (*R*)-**7** were found to be the optimum (entries 3 and 4). Below this level, longer reaction times were noticed leading also to the formation of trace amounts of side products. In a next step, the substrate concentration was increased. Finally at 50 mM substrate concentration the amine was still isolated in excellent yield, enantiopure form and without the formation of side products (entry 5).

Table 3. Reductive amination of **2** employing ArRmut11-ω-TA and varied concentrations of (*R*)-1-phenylethylamine **7** as amine donor at 45 °C and pH 7 after 4 h.

Entry	Ketone (mM)	Eqs. **7**	**4** [%]	*ee* **4** [%][c]
1	25	4	76[a]	>97 (*R*)
2	25	4	95[b]	>97 (*R*)
3	25	3	90[b]	>97 (*R*)
4	25	2	94[b]	>97 (*R*)
5	50	2	95[b]	>97 (*R*)

[a] Isolated yields after flash chromatography. [b] Isolated yields after alternative work-up. [b] Determined by HPLC on a chiral phase of the acetamide derivative.

The synthetic applicability of the approach was demonstrated on a 500 mg scale (2.70 mmol, 50 mM) with just 0.5 mM PLP. The enantiopure amine (*R*)-**4** was obtained in 96% isolated yield and enantiopure form (>97% *ee*).

Finally, the reductive amination was compared with the "old chemoenzymatic route" that involves the bioreduction of the ketone **2** and the Mitsunobu inversion (Figure 1).[7]

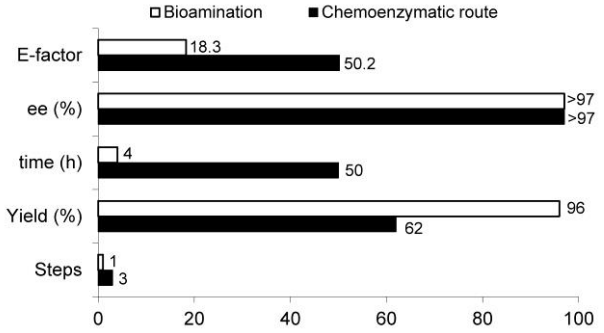


Figure 1. Comparison between the biocatalytic amination and the chemoenzymatic route (ADH reduction plus Mitsunobu) in terms of optical purity, time, yield, E-factor and number of steps.

The biocatalytic amination provides access to the enantiopure amine within only 4 h, while a longer reaction time (50 h) and three steps were required in the previous approach. Additionally, higher yields were obtained with the present route (96% versus 62%) not requiring a chromatographic purification of the amine. In terms of waste production the E-factor[21] defined as total waste (kg)/product (kg) diminished since over-stoichiometric reagents were minimized (see SI for details). Moreover, the direct

amination is much more attractive from an environmental point of view since the amination is performed in aqueous buffer avoiding the use of hazardous reagents such as DPPA or DEAD.

In summary, an efficient one-step protocol was developed for the amination of ketone **2** to the enantiopure amine (*R*)-**4**, which is a key intermediate for the synthesis of the anti allergic drug Ramatroban. The practical applicability of the approach has been demonstrated performing the amination on a 500 mg scale affording the enantiopure amine with excellent chemical purity and very high isolated yield (96%). Last but not least, the total yield of the formal total synthesis of the drug has been significantly improved from 30% in the previous route to 50% and the number of steps was reduced from 6 to 4.

Experimental Section

All starting materials were obtained from commercial suppliers and were used as received. TLC analyses were carried out with precoated aluminium sheets (TLC silica gel 60 F254, Merck) with detection by UV or staining with potassium permanganate. Preparative chromatographic separations were performed by flash chromatography on Merck silica gel (0.063-0.200 μm). Optical rotation was measured at 20 °C with a Perkin-Elmer polarimeter at the sodium D-line. GC-MS spectra were recorded with an Agilent 7890A GC system mass selective detector and HP-5 MS column [30 m × 0.25 mm × 0.25 μm; helium as carrier gas (flow = 0.55 mL/min)]. ^{1}H and ^{13}C NMR spectra were recorded at 20 °C with a Bruker 300 MHz unit; chemical shifts are given in ppm relative to the resonance of the solvent. Formate dehydrogenase from *Candida boidinii* catalogue no. FDH 002 [lyophilised powder 2.2 U/mg] and NAD^+ free acid were purchased from Codexis. Lyophilised *E. coli* cells containing overexpressed ω-transaminases were prepared as previously reported.[13a,22] Activity for alanine dependent ω-TAs and ArRmut11 was determined for the deamination of (*R*)- or (*S*)-1-phenylethanamine with pyruvate. Purified L-alanine dehydrogenase was prepared as described recently.[12a] Small scale reactions were shaken in 2 mL thermo shaker Eppendorf® Comfort. Preparative scale reactions were performed in an Infors Unitron shaker.

Biotransformations (AlaDH; analytical scale): The ketone **2** (4.6 mg, 25 μmol) was suspended in K-phosphate buffer (pH 7, 100 mM, 550 μL) and the following reagents dissolved in K-phosphate buffer (pH 7, 100 mM) were added: L-alanine (250 μL, 1 M), NH_4HCOO (150 μL, 1.5 M), PLP (20 μL, 50 mM), NAD^+ (20 μL, 50 mM) Ala-DH (10 μL, 11 U) FDH (5 mg, 11 U) and the lyophilised cells containing ω-TA (30 mg). The reductive amination was carried out at 30 °C in an orbital shaker (750 rpm, Eppendorf thermo shaker) for 24 h. After that time the reaction was stopped by adding a saturated solution of Na_2CO_3 (300 μL). The crude was extracted with CH_2Cl_2 (2 × 700 μL), dried over Na_2SO_4 and injected in the GC for conversion measurement.

Biotransformations (2-propylamine; analytical scale): The ketone **2** (4.6 mg, 25 μmol) was suspended in a K-phosphate buffer (pH 7, 100 mM, 750 μL) containing PLP (0.5 mM) and 2-propylamine (0.75 M). Then, semi purified ArRmut11-ωTA (250 μL) was added and the mixture was shaken at 45 °C in an Eppendorff thermo shaker (750 rpm,) for 24 h. The reaction was quenched and worked-up as described above.

Biotransformations [(*R*)-1-phenylethylamine; analytical scale]: The ketone **2** (4.6 mg, 25 μmol) was suspended in K-phosphate buffer (pH 7, 100 mM, 750 μL) containing PLP (0.5 mM) and different concentrations of (*R*)-1-phenylethylamine. Then, semi purified ArRmut11-ωTA (250 μL) was added to the suspension and the mixture was shaken at 45 °C in an Eppendorff thermo shaker (750 rpm) for 4 h. The reaction was quenched and worked-up as described above.

Biotransformations on preparative scale using ω-TA from *C. violaceum*: The ketone **2** (27.6 mg, 149 μmol) was suspended in K-phosphate buffer (pH 7, 100 mM, 3.3 mL) and the following aqueous solutions were added to the suspension: L-alanine (1.5 mL, 1 M), NH_4HCOO (900 μL, 1.5 M), PLP (120 μL, 50 mM), NAD^+ (120 μL, 50 mM) Ala-DH (100 μL 110 U) FDH (50 mg, 110 U) and the lyophilised cells containing ω-TA (180 mg). The reductive amination was carried out at 30 °C in a 15 mL Falcon tube and shaken in an Infors orbital shaker (120 rpm) for 24 h. After that time the reaction was stopped by adding a saturated solution of Na_2CO_3 (1.8 mL) and H_2O (10 mL) and the crude was centrifuged to remove the protein. The aqueous phase was extracted with a CH_2Cl_2/EtOH (95:5) (3 × 20 mL). The organic phases were combined, dried over Na_2SO_4, and the solvent evaporated under vacuum. The crude was purified by flash chromatography (0-2% NH_3/MeOH) affording the amine as a white solid (17.3 mg, 0.093 mmol, 62%). R_f (1% NH_3/MeOH): 0.20. ^{1}H NMR (CD_3OD, 300.13 MHz): δ 1.68-1.81 (m, 1H), 2.07-2.11 (m, 1H), 2.37-2.45 (m, 1H), 2.77-2.83 (m, 2H), 2.95-3.05 (m, 1H), 3.12-3.20 (m, 1H), 6.94-7.05 (m, 2H), 7.24 (d, $^{3}J_{HH}$= 7.9 Hz, 1H), 7.35 (d, $^{3}J_{HH}$= 7.9 Hz, 1H). ^{13}C NMR (CD_3OD, 75.5 MHz): δ 22.6 (CH_2), 31.3 (CH_2), 33.1 (CH_2), 49.3 (CH), 108.2 (C), 111.5 (CH), 118.1 (CH), 119.3 (CH), 121.5 (CH), 129.0 (C), 134.4 (C), 138.1 (C). $[\alpha]_D^{20}$= -52.5 (*c* 0.8, MeOH) for >97% *ee*. Lit.[7] for (*R*)-amine in 99% *ee* $[\alpha]_D^{20}$= +62.8 (*c* 2.0, MeOH) for 99% *ee*.

Biotransformations on preparative scale using ArRmut11-ωTA: The ketone (500 mg, 2.70 mmol) was equally distributed in two Falcon tubes (50 mL) containing K-phosphate buffer (pH 7, 100 mM, 13.5 mL), PLP (0.5 mM) and (*R*)-1-phenylethylamine (327 mg, 348 μL, 100 mM). Then, semi purified ArRmut11-ωTA (13.5 mL, 360 mg crude protein, 216 U) was added to the suspension and the reductive amination was carried out at 45 °C in an Infors orbital shaker (200 rpm) for 4 h. After that time the reactions were combined and basified with a saturated solution of Na_2CO_3 (10 mL) and the mixture was freeze-dried. The so obtained white powder was resuspended with a CH_2Cl_2/EtOH solution (95:5, 100 mL) and filtered trough

a Celite® plug (5 g). The solvent was evaporated under reduced pressure affording a solid containing (*R*)-1-phenylethylamine. The final high vacuum drying (0.5 mbar, 40 °C, 4 h) afforded the enantiopure amine (*R*)-**4** as a light-green solid (485 mg, 2.60 mmol, 96%) containing less than 2% of (*R*)-1-phenylethylamine.

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