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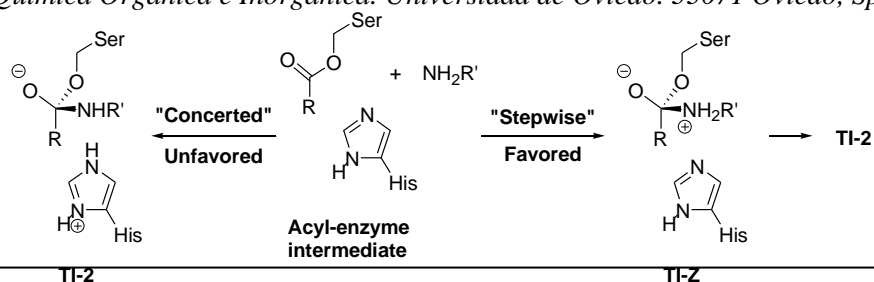
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Redesigning the mechanism of the lipase-catalysed aminolysis of esters

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TETRAHEDRON:
ASYMMETRY

Redesigning the mechanism of the lipase-catalysed aminolysis of esters

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Abstract—In previous works, the lipase B from *Candida antarctica* (CAL-B) catalysed the resolution of several 2-phenylcycloalkanamines by aminolysis of ethyl acetate. In these processes, the size of the cycle and the stereochemistry of the chiral centres of the amines had a strong influence on both the enantiomeric ratio and the reaction rate of these reactions. In this paper, molecular modelling approach has been used to revise the lipase-catalysed aminolysis mechanism. Thus, complexes of CAL-B with the phosphoramidate analogs related to substrates in the kinetic resolution of several 2-phenylcycloalkanamines by this enzyme were built and minimised. This computational study suggests the formation of zwitterionic species (named TI-Z), resulting from the direct His-unassisted attack of the amine to the carbonyl group of the acyl-enzyme, as the most plausible intermediate for the CAL-B-catalysed aminolysis. This proposal slightly differs from the commonly accepted serine-mediated mechanism, where the removal of the proton from the amine occurs simultaneously to the nucleophile attack to the acyl-enzyme complex (TI-2). Subsequently, His-assisted deprotonation of the resulting ammonium group takes place, and a molecule of water could be necessary in some cases to facilitate the transfer of the proton to the catalytic histidine. © 2016 Elsevier Science. All rights reserved

1. Introduction

The aminolysis of esters is a model reaction between an electrophile and a nucleophile that has been subjected to a great number of experimental and theoretical studies.¹ Base-catalysed models of this reaction were early reported by Bruice,² Jencks,³ and Bunnett.⁴ The mechanism has been widely revised and, even today, there is not a definitive outcome. Thus, in general, three different pathways have been considered when this reaction has been studied: (a) the concerted mechanism, where the transfer of the proton from the amine to the leaving group occurs simultaneously to the cleavage and formation of the bonds involved in the process; (b) the stepwise mechanism where no charged-intermediates are present (the typical “addition/elimination” pathway); and (c) the stepwise mechanism through zwitterionic species.

The lipase-catalysed acylation of amines is an example of aminolysis process assisted by a weak base.⁵ Although this reaction has been widely used to obtain optically active amines,⁶ in some cases on a multi-ton scale,⁷ its mechanism has been scarcely studied, and it is assumed, and therefore accepted, that the pathway for acylation of amines is completely analogous to that for acylation of alcohols (serine-mediated mechanism).⁸ In spite of the higher nucleophilicity of amines compared to alcohols, most hydrolase-catalysed acylations involve alcohols not amines.

Further, reactions with amines are often slower, requiring larger amounts of catalyst or longer reaction times.

In the last years, we have investigated the utility of some lipases, especially the lipase B from *Candida antarctica* (CAL-B) to catalyse the enantio-,⁹ and regioselective¹⁰ aminolysis of esters. Thus, we have recently described the CAL-B-catalysed kinetic resolutions of racemic *trans*- and *cis*-2-phenylcycloalkanamines (*rac*-1–*rac*-5, Figure 1).¹¹ These type of substrates are very interesting due to their pharmacological properties¹² and also to their potential applicability as chiral auxiliaries, bases and ligands.

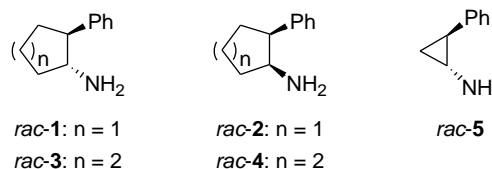
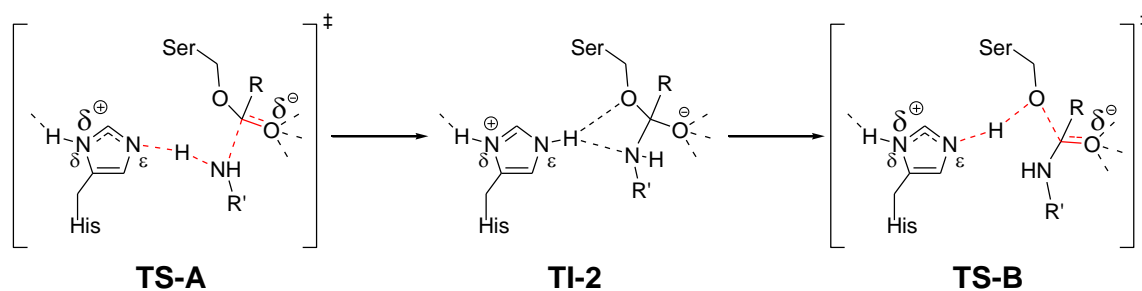


Figure 1. 2-Phenylcycloalkanamines.

The efficacy of the CAL-B-catalysed resolution of 2-phenylcycloalkanamines was dramatically influenced by the stereochemistry and the size of the cycle of the amine (Table 1).¹¹ When the resolution of *rac*-1–*rac*-5 was carried out by CAL-B-catalysed acylation under the simplest reaction conditions, that is, using ethyl acetate as the acyl donor and the solvent, (1*R*,2*S*) enantiomers of *trans*

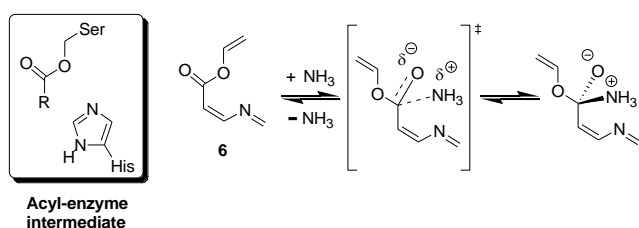


Scheme 1. Mechanism for the lipase-catalysed aminolysis based on the analog for the transesterification reaction.

For better understanding, this section has been divided in three subheadings. Firstly, the molecular modelling study over 2-phenylcyclopentanamines (*rac-1* and *rac-2*), then over *trans*-2-phenylcyclopropanamine (*rac-5*), and lastly, over 2-phenylcyclohexanamines (*rac-3* and *rac-4*) will be shown.

2.1. 2-Phenylcyclopentanamines *rac-1* and *rac-2*

We started this study with the *trans* diastereoisomer *rac-1* (Figure 1), that had been perfectly resolved by CAL-B,^{11a} and we tried to find a catalytically productive conformation for the fast-reacting enantiomer (1*R*,2*S*)-**1** (Table 1, entry 1). Thus, the phosphoramidate analog of TI-2 for (1*R*,2*S*)-**1** was modelled. However, although several arrangements of the substrate in the active site were examined, non-productive conformations were found (Figure S1 in Supplementary Material). The minimal distance found for the bond *c* between the N_ε-H of His224 and the N of the nucleophile was still too large (3.49 Å, Table 2, entry 1) for an H-bond interaction to exist.²¹ This result indicates that the concerted attack of the amine on the acyl-enzyme carbonyl with the proton removal by the catalytic histidine is not favoured, and as a consequence, a different mechanism to that operating in the transesterification of alcohols might be plausible.



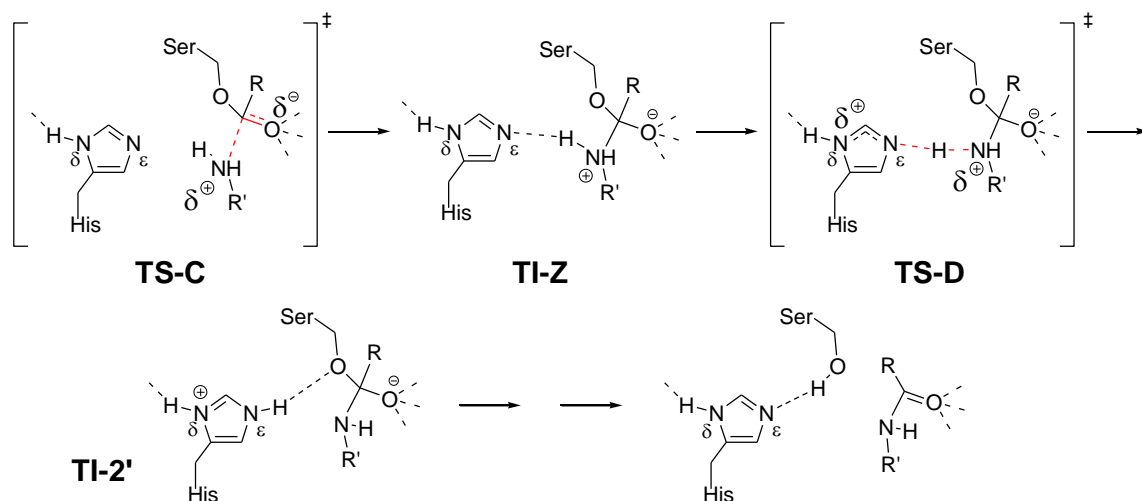
Scheme 2. An example of the stepwise mechanism through zwitterionic intermediates for the aminolysis reaction of **6**.²² The acyl-enzyme intermediate in the serine-mediated mechanism could be visualized as an analog to compound **6**.

Looking at the literature, computational studies related to the aminolysis process have shown that stepwise mechanisms are usually more favoured energetically than the concerted one, and in some cases, it takes place through

zwitterionic species. Thus, Adalsteinnsson and Bruice,²² who investigated the intramolecularly catalysed aminolysis of substituted phenyl esters of quinoline derivatives. In these reactions assisted by a weak base, computational approaches demonstrated that the mechanism going *via* zwitterionic intermediates was the most favourable. They modelled the ammonolysis of compound **6**, which was used as a model of a quinoline carboxylic ester, obtaining as the lowest energy pathway for the ammonia attack that is shown in Scheme 2.

In the case of the lipase-catalysed aminolysis, zwitterionic species²³ could be favoured if we consider the following facts: (1) the proton removal catalysed by a base such as imidazole from alcohols is much easier than from amines; (2) amines are stronger nucleophiles than alcohols; and (3) the acyl-enzyme complex is a particularly activated ester due to the side chains of the amino acids that shape the oxyanion hole. Thus, we study the direct attack of the amine to the acyl-enzyme carbonyl without the initial participation of the catalytic His (Scheme 3). The result would be a tetrahedral intermediate that we have named as “zwitterionic tetrahedral intermediate” (TI-Z),²⁴ which can go on to TI-2²⁵ by the subsequent His-mediated removal of the proton. In the last step, the transfer of the proton between [His-H]⁺ and Ser would take place affording the amide.

Assuming this pathway, enantiodiscrimination could be determined by TS-C, TS-D, or both. Thus, we decided to model the protonated phosphoramidate analog that would mimic TI-Z (Figure 2B). This intermediate presents five key-hydrogen bonds, *c'* being the essential for the subsequent His-mediated removal of the proton. So, in this case a catalytically productive conformation was defined as one that maintains all five key-hydrogen bonds presented in Figure 2B (a,c',d,e,f). Starting from the best phosphoramidate obtained for TI-2 for the fast-reacting enantiomer, we built and modelled the protonated intermediate for (1*R*,2*S*)-**1** (see details in Experimental Section), obtaining the structure that is shown in Figure 3A.



Scheme 3. Proposed mechanism through zwitterionic intermediates for the lipase-catalysed aminolysis.

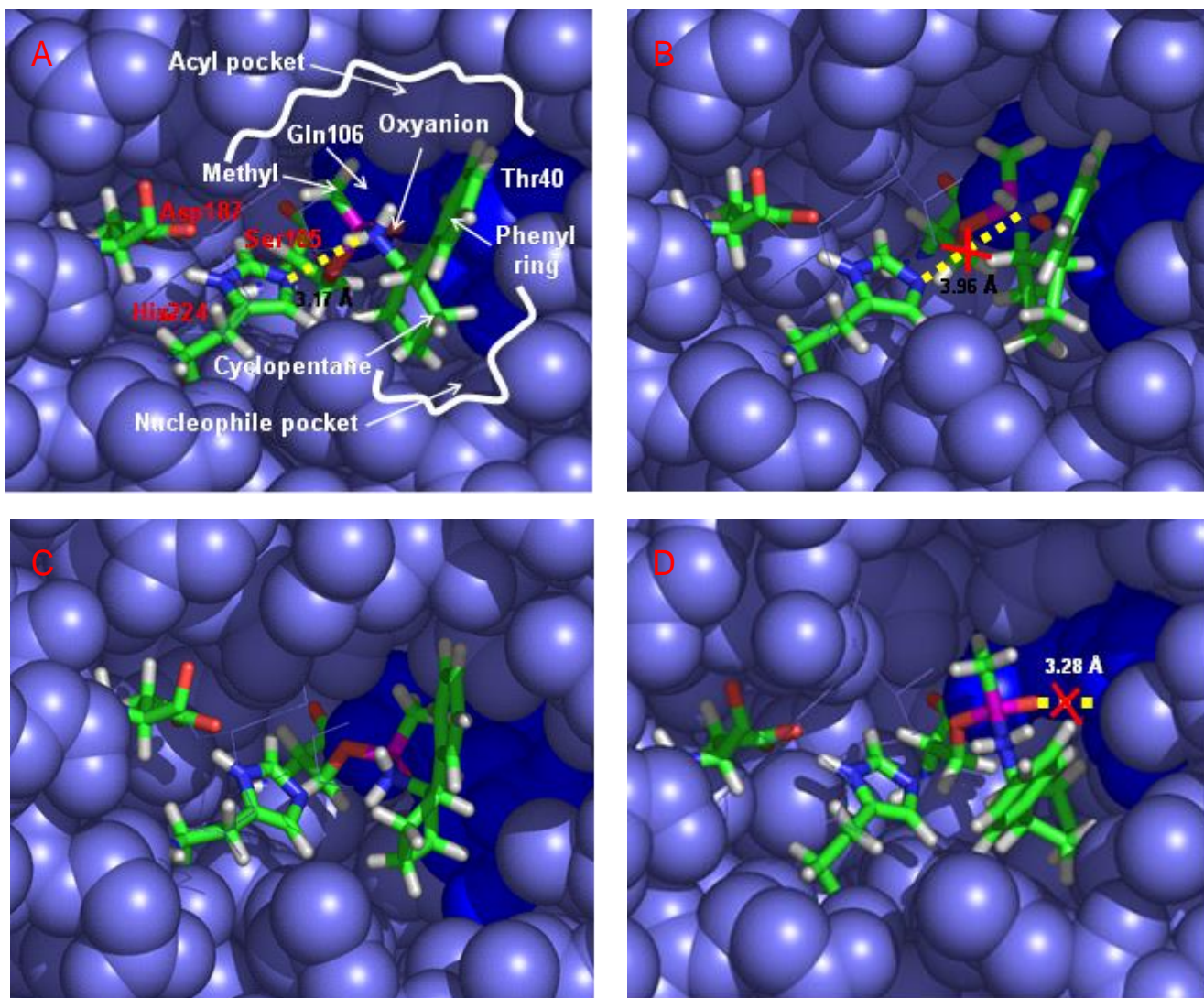


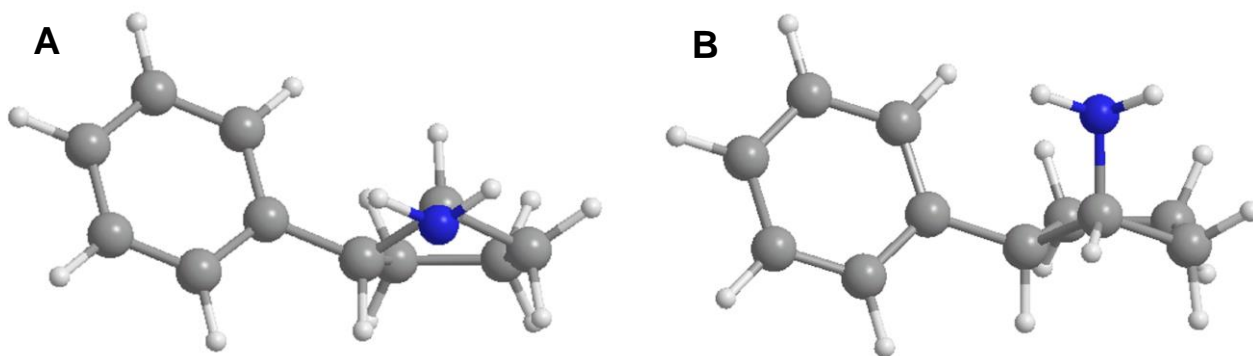
Fig. 3. Best conformations of the analogs for: A) TI-Z of (1*R*,2*S*)-1; B) TI-Z of (1*S*,2*R*)-1; C) TI-Z of (1*R*,2*R*)-2; and D) TI-Z of (1*S*,2*S*)-2. Lost key-hydrogen bonds appear with a red cross. The above images display Glu188 and Ile189 in a line representation to allow a better view of the large pocket of the lipase.

Table 2. Distances and angles of the key hydrogen bonds in the most productive conformations obtained for the intermediates in the CAL-B-catalysed acetylation of *rac-1*, *rac-2*, and *rac-5*.

Entry	TI	Compound	H-bond distance, Å (angle, °) ^a					
			a	b	c or c'	d	e	f
1	TI-2	(1 <i>R</i> ,2 <i>S</i>)- 1	2.97 (132)	2.84 (145)	3.49 (148)	2.87 (151)	2.78 (175)	2.95 (173)
2	TI-Z	(1 <i>R</i> ,2 <i>S</i>)- 1	2.80 (149)	-- ^b	3.17 (138)	2.92 (154)	2.78 (176)	3.00 (170)
3	TI-Z	(1 <i>S</i> ,2 <i>R</i>)- 1	2.83 (165)	-- ^b	3.96 (90)	2.87 (151)	2.80 (167)	2.78 (170)
4	TI-Z	(1 <i>R</i> ,2 <i>R</i>)- 2	2.89 (166)	-- ^b	3.12 (125)	3.08 (161)	2.83 (172)	2.82 (173)
5	TI-Z	(1 <i>S</i> ,2 <i>S</i>)- 2	2.88 (164)	-- ^b	3.18 (125)	3.13 (160)	2.74 (165)	3.28 (128)
6	TI-Z	(1 <i>R</i> ,2 <i>S</i>)- 5	2.87 (169)	-- ^b	2.90 (131)	3.27 (141)	2.77 (155)	2.97 (163)
7	TI-Z	(1 <i>S</i> ,2 <i>R</i>)- 5	2.87 (166)	-- ^b	2.94 (125)	3.17 (159)	2.77 (171)	2.78 (173)

^a Figure 2 defines the hydrogen bonds. Distances are between heteroatoms involved in the H- bonds (N-N, N-O, O-O), and angles refer to the N-H-O or similar. Lost hydrogen bonds appear in bold.

^b Not relevant for TI-Z (see Figure 2B).

**Figure 4.** Main conformation using DFT calculations for: A) *rac-1*; and B) *rac-2*.

The methyl of the acetate lay in the large subsite, while the carbocycle was perfectly bound into the medium-sized pocket, with the phenyl ring in a *pseudo*-equatorial position at the fold of the envelope and the amino group at the flap in a nearly equatorial disposition. This conformation was also the most stable found using DFT calculations (Becke3LYP/6-31+G* level of theory; see Figure 4A).²⁶ The phenyl moiety was placed near to the right side of the large hydrophobic subsite allowing the existence of favourable van der Waals interactions with Ile189 and Ile285. As shown in Table 2 (entry 2) the distance between the N_ε of His224 and the amine diminished and was appropriate for the existence of the H-bond (bond c'). Furthermore, all five essential hydrogen bonds were present and no steric impediments between the substrate and the lipase were found.

To verify the validity of this novel intermediate, next we built and modelled the TI-Z analog for the slow-reacting enantiomer (1*S*,2*R*)-**1**, and the obtained results were in agreement with the experimental facts. The best conformation obtained is shown in Figure 3B. In it, the cyclopentane presented a more unstable distorted envelope conformation and it shared out between the medium and the large pockets, with the phenyl hydrophobic group pointing toward the solvent. Moreover, the distance between the N_ε of His224 and the N of the protonated

amine was too large (bond c', Table 2, entry 3). Thus, the best conformation of the cycle for (1*R*,2*S*)-**1** and its improved disposition into the active site of the lipase (better TS-C), as well as the loss of one of the key hydrogen bonds in the intermediate of (1*S*,2*R*)-**1** (and therefore, worse TS-D) can explain the excellent enantioselectivity obtained in the resolution of *rac-1*.

The CAL-B-catalysed resolution of (±)-*cis*-2-phenylcyclopentanamine (*rac-2*) was not as efficient as in the case of the *trans* isomer, although the (1*R*,2*R*) enantiomer reacted 16 times faster than *S* one (Table 1, entry 2).^{11a} To explain these differences, we built and modelled the zwitterionic intermediates for both enantiomers of the *cis* isomer *rac-2*. Thus, in the best conformation obtained for the fast-reacting enantiomer (1*R*,2*R*)-**2** (Figure 3C), the carbocycle was bound into the medium-sized pocket in a conformation slightly different from the most stable disposition found by DFT calculations (Figure 4B).²⁶ The amino group was placed at the flap of the envelope in a *pseudo*-axial position and the phenyl ring at the fold of the envelope fitted in a nearly equatorial disposition pointing out to the solvent. The interaction between the catalytic histidine and the ammonium group was not very strong since the angle was scarcely higher than 120° (bond c', Table 2, entry 4). We think that these

facts could be an explanation for the 7-fold lower reactivity of this enantiomer compared to the *trans* isomer (1*R*,2*S*)-1.

Next, we searched for a productive conformation of the TI-Z analog for the enantiomer (1*S*,2*S*)-2, but we did not find it (Figure 3D). For the best one, the cyclopentane presented a distorted envelope conformation with both substituents in *pseudo*-equatorial positions. Furthermore, the phenyl ring was closer to His224 forcing the substrate to move next to Thr40, losing thus an H-bond interaction (bond f, Table 2, entry 5). This loss of one of the key hydrogen bonds for the catalysis and the less adequate disposition of the substrate can explain the worse reactivity of (1*S*,2*S*)-2, and therefore, the enantioselectivity exhibited by the enzyme.

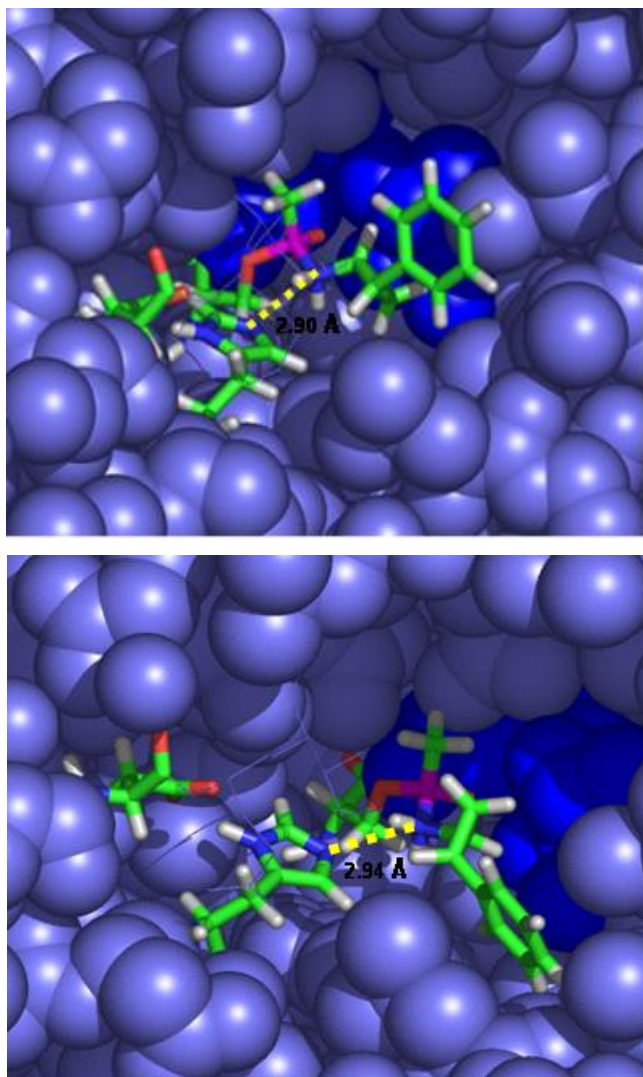


Figure 5. Best conformations of the analogs for: A) TI-Z of (1*R*,2*S*)-5; and B) TI-Z of (1*S*,2*R*)-5. The above images display Glu188 and Ile189 in a line representation to allow a better view of the large pocket of the lipase.

2.2. *trans*-2-Phenylcyclopropanamine *rac*-5

As shown previously (Table 1, entry 5), CAL-B catalysed the acetylation of *rac*-5 with high efficiency (90% of

conversion was obtained after 2h of reaction) but with very low enantioselectivity ($E = 4$), that is, both enantiomers were good substrates for the lipase.^{11b} When we built and minimised the TI-Z analogs for both enantiomers of *rac*-5, results obtained were in good agreement with those experimental (Table 2, entries 6 and 7). Both structures presented all key-hydrogen bonds with no intra- or intermolecular steric clashes. The smaller size of the carbocycle allowed a minor distance between the catalytic histidine and the ammonium group (bond c', around 2.90 Å), and thus, a very efficient H-bond interaction.

The protonated phosphoramidate of the enantiomer (1*R*,2*S*)-5 (Figure 5A) showed a disposition in the active site similar to that for (1*R*,2*S*)-1 (Figure 3A), with the phenyl group on the right side of the large hydrophobic pocket pointing out to the solvent, but interestingly, the intermediate for (1*S*,2*R*)-5 (Figure 5B) presented a more favoured unexpected binding of the phenyl moiety with regards to the five-membered analog (1*S*,2*R*)-1 (compare with Figure 3B). The smaller size of the cycle of (1*S*,2*R*)-5 allowed the phenyl ring to be deeply fitted into the medium subsite, thus making favourable van der Waals interactions with the hydrophobic side chains of Trp104, Leu278, Ala282, and Ile285. These results indicate that modelling of the TI-Z analogs can also predict the poor resolution of *rac*-5 because both enantiomers can perfectly bind into the active site of CAL-B.

2.3. 2-Phenylcyclohexanamines *rac*-3 and *rac*-4

Next, we studied the CAL-B-catalysed acylation of (\pm)-*trans*-2-phenylcyclohexanamine (*rac*-3). This compound was efficiently resolved with CAL-B^{11b} and ethyl acetate but, significantly, this process was much slower than that with (\pm)-*trans*-2-phenylcyclopentanamine (*rac*-1) (Table 1, entries 1 and 3).²⁷ Cyclohexane derivatives are present in a two chair-equilibrium in solution, which lies on the side of the thermodynamically more stable one. *trans*-1,2-Disubstituted derivatives virtually exist only in that conformation placing both substituents in equatorial positions. Thus, only those structures with both the amino and the phenyl groups placed in equatorial positions were taken into account during the computer modelling study of *rac*-3.²⁸

Since good results had been obtained using the zwitterionic intermediates, we built and minimised the corresponding one for the fast-reacting enantiomer (1*R*,2*S*)-3. However, although we systematically searched for several dispositions of the cycle, the H-bond between the N_e of the catalytic histidine and the ammonium group (bond c', see Figure 2B) was always lost, being 3.32 Å the shortest distance found (Figure S2 in Supplementary Material and Table 3, entry 1), indicating that the direct proton transfer between both groups would not be favoured.

Table 3. Distances and angles of the key hydrogen bonds in the most productive conformations obtained for the intermediates in the CAL-B-catalysed acetylation of *rac*-3 and *rac*-4.

Entry	Compound	H-bond distance, Å (angle, °) ^a						
		a	c'	d	e	f	g	h
1	(1 <i>R</i> ,2 <i>S</i>)-3	2.81 (155)	3.32 (131)	2.92 (156)	2.80 (170)	2.92 (172)	-- ^b	-- ^b
2	(1 <i>R</i> ,2 <i>S</i>)-3	2.82 (158)	-- ^c	2.93 (148)	2.77 (178)	3.28 (167)	4.14 (161)	3.17 (152)
3	(1 <i>R</i> ,2 <i>S</i>)-3	2.82 (158)	-- ^c	2.87 (146)	2.75 (179)	3.07 (171)	3.34 (166)	2.96 (160)
4	(1 <i>S</i> ,2 <i>R</i>)-3	2.83 (156)	-- ^c	2.91 (146)	2.77 (167)	2.80 (173)	3.29 (126)	3.06 (139)
5	(1 <i>R</i> ,2 <i>R</i>)-4	2.87 (172)	-- ^c	3.12 (163)	2.83 (168)	2.80 (172)	3.51 (136)	3.18 (140)
6	(1 <i>S</i> ,2 <i>S</i>)-4	2.84 (169)	-- ^c	3.02 (160)	3.03 (160)	2.79 (164)	3.41 (134)	3.25 (133)

^a Figures 2B and 6 define the hydrogen bonds. Distances are between heteroatoms involved in the H-bonds (N-N, N-O, O-O), and angles refer to the N-H-O or similar. Lost hydrogen bonds appear in bold.

^b This H-bond is not present in TI-Z (see Figure 2B).

^c This distance is not present in “assisted TI-Z” (see Figure 6).

To be plausible as catalytically productive intermediates, this mechanism should provide an alternative path for this proton transfer. Previous reports based on modelling studies have suggested that a proton can be carried between the catalytic His and the nucleophile/leaving group by means of another molecule which would act as a proton transfer (PT, Figure 6). Thus, proton transfer-aided pathways have been previously proposed for the CAL-B-catalysed enantioselective ring opening of β -lactams,²⁹ and for the CAL-B-catalysed regioselective aminolysis of 3',5'-diaminonucleosides.¹⁵

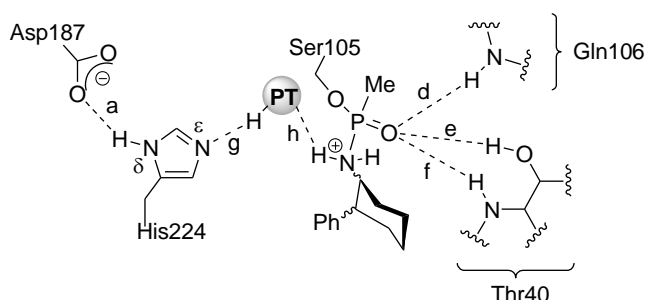


Figure 6. Analogs of “assisted TI-Z” used in the molecular modelling study for the acetylation of 2-phenylcyclohexanamines catalysed by CAL-B. Essential hydrogen bonds for the catalysis are shown as dashed lines (a-h). Novel interactions are now named as g and h. PT= proton transfer.

Although reactions were performed with anhydrous solvents and amines were stored under nitrogen atmosphere, in all cases the acyl donor was partially hydrolysed such as indicated the analysis of the ¹H-NMR spectra of the reaction crudes.³⁰ Thus, either ethanol coming from ethyl acetate or water present in the medium reaction could play the role of a proton transfer.³¹ Starting from the TI-Z analog obtained for (1*R*,2*S*)-3, we first modelled an ethanol molecule in different positions near the catalytic triad, obtaining as the most stable structure that one, in which this molecule was midway between the protonated amine and the catalytic histidine (Figure S3 in Supplementary Material). Although the substrate fitted correctly into the active site, the calculated N_ε-O distance was too large (bond g, 4.14 Å) for the existence of an H-bond interaction (Table 3, entry 2).

Better results were obtained when a molecule of water was added as PT and modelled in several positions close to the catalytic triad. We found a stable intermediate with a similar binding of the substrate in the active site of the lipase (Figure 7A) to that obtained in the ethanol-assisted TI-Z, where the smaller size of the water molecule allowed that all key-hydrogen bonds were present in the intermediate (Table 3, entry 3).³²

Next, we modelled the analog of “assisted TI-Z” with a molecule of water for the slow-reacting enantiomer (1*S*,2*R*)-3, but no adequate conformations were found (Figure 7B). In all cases, the interaction between His224 and the molecule of water was very weak or was not present (bond g, Table 3, entry 4). Furthermore, the comparison of the modelled intermediates for both enantiomers (Figure 8) shows for (1*S*,2*R*)-3 a worse binding of the phenyl moiety in the acyl subsite. Thus, in analogy, the phenyl group was placed close to both the catalytic histidine and the molecule of water (average phenyl-water distance: 5.25 Å), meanwhile in the (1*R*,2*S*)-3 intermediate this group was fitted on the right side of the large pocket, moving from the proton transfer (average phenyl-water distance: 5.70 Å) and leaving free the space over His224. So, in this case, modelling predicts that a PT is necessary to transfer the proton from the nucleophile to the catalytic histidine. Better bindings of the substrate and the molecule of water in the (1*R*,2*S*)-3 intermediate can justify the enantioselectivity exhibited by CAL-B towards this substrate.

Finally, we studied the enzymatic resolution of *rac*-4. This amine proved to be a very poor substrate for CAL-B, since after 2 days of reaction only 5% of amine was converted in acetamide of very low *ee* (13%, Table 1, entry 4).^{11b} To carry out the modelling of the corresponding intermediate derived from *rac*-4, we have only taken into account the conformation observed in the ¹H-NMR spectrum of amine *rac*-4. The analysis of the coupling constants values measured for the signals of H-1 and H-2 indicated an equatorial position for the phenyl group and an axial position for the amino group.³⁵

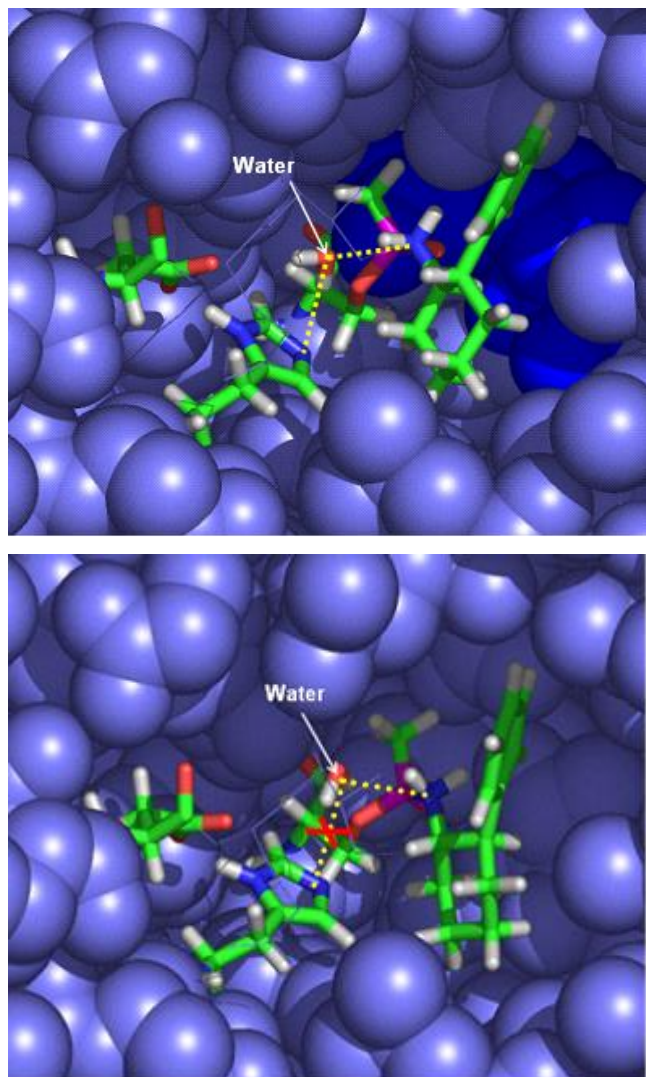


Figure 7. Best conformations of the analogs for: A) “assisted TI-Z” with a molecule of water of (1*R*,2*S*)-3; and B) “assisted TI-Z” with a molecule of water of (1*S*,2*R*)-3. Lost key-hydrogen bond appears with a red cross. The above images display Glu188 and Ile189 in a line representation to allow a better view of the large pocket of the lipase.

By analogy with the *trans* isomer *rac*-3, the water proton transfer-assisted model was used for the *cis* isomer *rac*-4. We searched for productive conformations of (1*R*,2*R*)-4 analog, but in the best structure found (see Figure S4 in Supplementary Material), the carbocycle rotated pointing the axial hydrogens H-3 and H-5 towards the catalytic histidine and placing the phenyl group in the acyl pocket exposed to the solvent. Due to the *cis* arrangement of phenyl and amino groups, the aromatic ring was placed very close to the molecule of water (average phenyl-water distance: 4.95 Å). As a result, the binding of the water molecule was destabilised, thus losing an H-bond interaction (bond g, Table 3, entry 5).

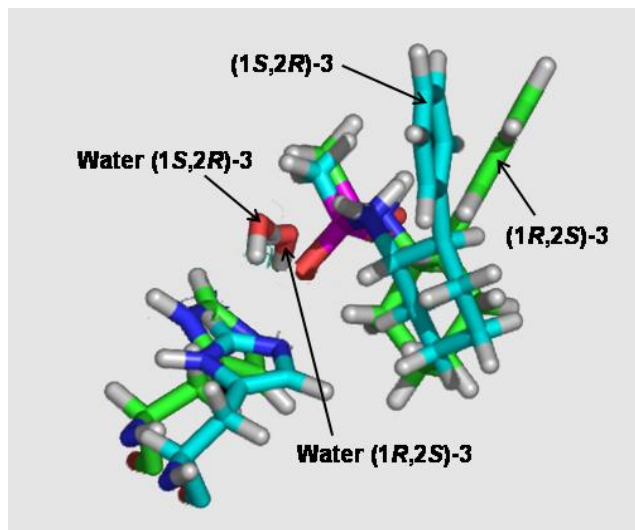


Figure 8. Structural comparison of “assisted TI-Z” analogs of (1*R*,2*S*)-3 (green) and (1*S*,2*R*)-3 (blue) with a molecule of water acting as a proton transfer. The phenyl moiety in the (1*S*,2*R*)-3 structure was closer to the molecule of water, hindering a good fitting of the proton transfer.

When the “assisted TI-Z” model with a molecule of water for (1*S*,2*S*)-4 was built and minimised, the best structure obtained (Figure S5 in Supplementary Material) showed that the cyclohexane was not bound into the medium pocket and the phenyl moiety was in the acyl subsite extending some parts into the solvent. In this case, although the phenyl ring was not as close to the molecule of water (average phenyl-water distance: 5.60 Å), the H-bond interaction with His224 was lost (bond g, Table 3, entry 6).

For *rac*-4, the proton transfer-aided model can also explain its poor reactivity. For both enantiomers, the substrate is not well fitted into the lipase active-site and the transport of the proton from the nucleophile to the catalytic histidine seems to be hindered.

3. Conclusions

Results obtained in the enzymatic resolution of some 2-phenylcycloalkanamines catalysed by CAL-B show that the size of the cycle and the relative configuration of the chiral centres play an important role in determining both the reaction rate and the enantioselectivity. Molecular modelling studies on the aminolysis of these 2-phenylcycloalkanamines (1-5) suggest the favoured formation of zwitterionic intermediates resulting from the direct attack of the amine to the carbonyl group of the acyl-enzyme without the activation of the catalytic histidine, which is in contrast to the concerted pathway predicted in the serine-mediated mechanism. In a second step, deprotonation of the formed ammonium group by the catalytic histidine would take place. Alternatively, a molecule of water to assist the transfer of the proton could be necessary.

Furthermore, this computer-aided modelling study can also explain the different reaction rates observed in these enzymatic processes. Thus, the requirement of a molecule of water to transport a proton from the nucleophile to the catalytic histidine could be responsible for the lower reaction rate of the *trans*-cyclohexanamine *rac*-**3** with regards to its cyclopentyl and cyclopropyl analogs. Finally, the poor reactivity of *rac*-**4** has been also explained, because in its major ring conformation, the binding of both enantiomers does not allow an adequate transfer of the proton to the histidine.

4. Experimental Section

The program Insight II, version 2000.1, was used for viewing the structures. The geometric optimisations were performed using Discover, version 2.9.7 (*Accelrys*, San Diego CA, USA), using the AMBER force field.¹⁸ The distance dependent dielectric constant was set to 4.0 to mimic the electrostatic shielding of the solvent and the 1-4 van der Waals interactions were scaled to 50%. The crystal structure for the CAL-B (1lbs)¹⁷ was obtained from the Protein Data Bank (www.rcsb.org/pdb/), and includes a phosphonate. Protein structures shown in this paper and in the Electronic Supplementary Information were created using PyMol v0.97.

The crystal structure of CAL-B was relaxed before addition of the substrate to the experimental structure. The PDB file 1lbs was opened with all atoms of the crystal structure, including water molecules. Hydrogen atoms were added to the structure using Biopolymer module, tested for partial charge balance and corrected for atom types within the AMBER force field. Geometry optimizations were carried out in two steps. First, the steepest descent algorithm corrected major high energy structural problems and got the molecule to a local minimum with a RMS deviation of about 0.02 Å mol⁻¹. Second, conjugate gradient algorithm gave a faster and more precise minimization near the local minima, obtaining a RMS value of 0.005 Å mol⁻¹ or lower. The entire structure was relaxed by a systematic manner that avoided large structural changes. First only the water molecules were optimized. After the side chains of amino acids were released and optimized as well. The entire complex of enzyme and solvent molecules was then released for the final optimization. For all further geometry optimizations in this study the solvent molecules were never fixed. This was to ensure that water molecules were mobile and that their position did not hinder the substrate.

Geometry Optimization of Phosphoramidate Core. A phosphoramidate core of the tetrahedral intermediate mimicking acetylation of ethylamine was assembled manually in the crystal structure. The partial charges on the intermediate atoms were set to those of the tetrahedral intermediate (carbon, not phosphorus), the values of which were obtained by semi-empirical calculation performed using the AM1 parameters.

At this stage, proton linked to the nucleophile amine was minimized in the two possible positions where it can be placed since it can interconvert its position with the electron lone-pair in order to choose the best intermediate.

For the optimization of the phosphoramidate core a systematic approach of releasing different motifs was applied. Initially, the intermediate was allowed to adjust to the enzyme active site by keeping the entire enzyme fixed during the geometry optimization. The side chains of the lipase were then released and allowed to adjust and finally the entire complex was allowed to adjust. This approach avoided drastic changes in the lipase structure caused by non-optimal conformations of the intermediate. A hydrogen bond calculation was performed after each minimization in order to assure the presence of the critical hydrogen bonds around the catalytic site.

Phosphoramidate analogues of TI-2 including 2-phenylcycloalkanamine group. After obtaining a catalytically productive intermediate core, the remainder of the cyclic amine was added, firstly the cycle and then the phenyl group, and different conformations were produced by manual adjustment of dihedral angles identified in Figure 9. Geometry optimizations were again performed using the same approach used to optimize the phosphoramidate core with systematic releasing of the substrate, amino acid side chains and the entire enzyme complex. Again, minimizations were carried out with both the steepest descent and conjugate gradients until an RMS value of at least 0.005 Å mol⁻¹ was obtained.

Adjustments of approximately 10-20° were performed for dihedral angles involving *a* and *b* bonds when a local minima structure was found. Structures with obvious steric problems were ignored. Additional manual adjustments were done to orient substrate moieties into visible pockets.

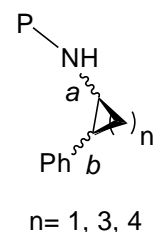


Figure 9. Manipulated dihedral angles for 2-phenylcycloalkanamine structures. Dihedral angles corresponding to all the bonds labelled above were adjusted during manual conformational searching. Adjustments of 120° were made of each dihedral angle to produce possible conformations. In addition, different conformations of the cycle were explored.

Zwitterionic Intermediates (TI-Z). To obtain these structures, the amino group was replaced by an ammonium fragment, and the catalytic histidine was deprotonated, that is, we started minimizing the acetylation of ethylammonium, and then we built the rest of the molecule, first, the cycle and then the phenyl moiety. For the optimization of the zwitterionic core, the same systematic approach of releasing was applied. First, only the intermediate was allowed to adjust to the enzyme active

site. Second, the side chains of the amino acids were released too, and finally the entire complex was allowed to adjust, allowing the molecules of water to adjust their position in all steps.

Adjustments of approximately 10-20° were performed for dihedral angles when a local minima structure was found. Structures with obvious steric problems were ignored. Additional manual adjustments were done to orient substrate moieties into visible pockets.

“Assisted” Zwitterionic Intermediates. Finally, in the TI-Z analogues obtained for 2-phenylcyclohexanamines, a molecule of ethanol or water was added in several positions near from the catalytic histidine. For the optimization of this structure, a similar systematic approach of releasing was applied. First, only the proton transfer was allowed to adjust to the enzyme structure. Second, the substrate and the side chains of the amino acids were released too, and finally the entire complex was allowed to adjust, with all the molecules of water releasing in each step.

Acknowledgments

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