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Application of structure-based thermodynamic calculations to the rationalization of the enantioselectivity of subtilisin in organic solvents

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Abstract

The effect of organic solvents on the selectivity of lyophilized or CLEC (cross-linked enzyme crystals)-subtilisin in the resolution of *sec*-phenethyl alcohol and *trans*-sobrerol was studied. A theoretical model, that tries to predict solvent effects on enantioselectivity only as a function of the activity coefficients of the desolvated part of the substrate in the relevant transition state of the reaction (Ke, T.; Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.*, **1996**, *118*, 3366) was examined and shown to agree poorly with the experimental data. The tetrahedral intermediate was studied with MonteCarlo molecular mechanics, and the activity coefficients were calculated with UNIFAC.

1. Introduction

The study of enzyme selectivity in nearly anhydrous organic solvents is a particularly interesting and intriguing field of research.[1] In these media, the absence of a continuous aqueous phase around the enzymes allows their direct interaction with the solvents, and confers to the biocatalysts new properties with respect to stability, activity and specificity-selectivity.[2] Furthermore, in organic media, enzymes such as hydrolases can catalyze esterifications and transesterifications readily, with high product yields.[2]

A deep understanding of enzyme–substrate–solvent interactions is necessary to improve further enzyme performance. Thus several theories have been proposed to explain the mechanisms by which solvents influence enzymatic properties. For instance, the selectivity could be altered if the solvent molecules could bind within the active site and alter either the interaction between enzyme and substrate

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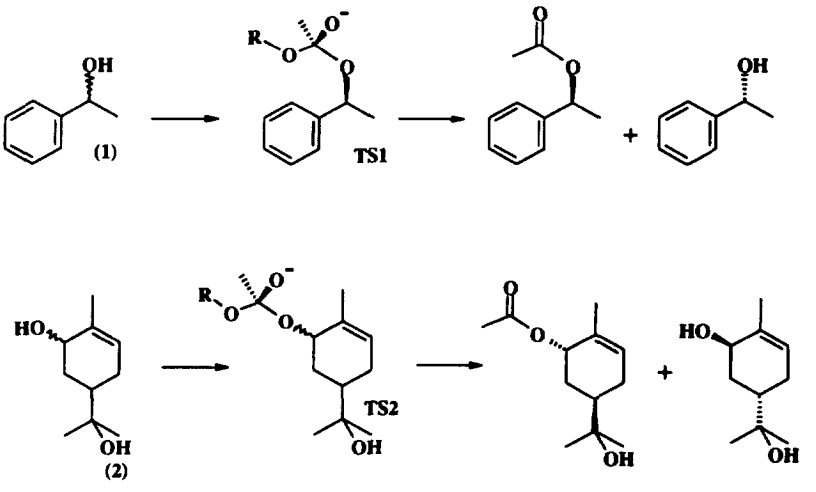

Fig. 1. Subtilisin-catalyzed transesterification reactions of *sec*-phenethyl alcohol **1** and *trans*-sobrerol **2** with vinyl-acetate

or the structural characteristics of the complex.[3] According to another theory, the solvent could modify the enzyme conformation and thus influence the selectivity by altering the molecular recognition process between substrate and enzyme.[4] A third model introduces a dependence of selectivity on the energetics of substrate solvation, regardless of the presence of other mechanisms.[5] Based on this last rationale, Klibanov and coworkers have proposed a quantitative model to rationalize the solvent dependence of enzymatic selectivity solely on the basis of the thermodynamics of substrate solvation.[6] The validity of this model is examined in the present paper in the case of two different substrates and two different forms (crystalline and lyophilized) of subtilisin Carlsberg.

2. Results and discussion

The quantitative model that tries to rationalize the solvent dependence of enzyme selectivity solely on the basis of the thermodynamics of substrate solvation was elaborated by Klibanov and coworkers,[6] and is represented by this simple equation:

$$\log E = \log(\gamma'_S/\gamma'_R) + \text{constant} \quad (1)$$

where E is the enantiomeric ratio, and γ'_S and γ'_R are defined as the activity coefficients of the desolvated substrate moiety in the transition state for the *S*- and *R*-enantiomer. This model predicts that the logarithm of enzyme selectivity (E) should be proportional to the logarithm of the ratio of the thermodynamic activity coefficients of the desolvated portions of the substrates in the transition state of the reaction, plus a constant. We have applied this model to the subtilisin-catalyzed transesterification reactions of *sec*-phenethyl alcohol **1** and *trans*-sobrerol **2** with vinyl-acetate or butyrate, carried out in several organic solvents (Fig. 1).

Table 1 shows the enantiomeric ratio (E) and the relative velocity (V_{rel}) of our model reactions with the organic solvents most commonly used for enzymatic transesterifications. Regarding the acylating agent, the enantioselectivity was higher with vinyl butyrate than with vinyl-acetate in all the solvents tested. It can also be seen that the enzyme was markedly more selective with *trans*-sobrerol than with *sec*-phenethyl alcohol, and that the enantioselectivities of lyophilized and CLEC-subtilisin were comparable. The transesterification rates in the various solvents don't show a clear trend: the selectivity was found to

Table 1
Enantiomeric ratio (E) and relative velocity (V_{rel}) of subtilisin catalyzed transesterification in various organic solvents

solvent	*sec*-phenetyl alcohol						*trans*-sobrerol	
	lyophilized subtilisin				CLEC subtilisin		lyophilized subtilisin	
	VA		VB		VB		VA	
	E	V_{rel}[a]	E	V_{rel}[b]	E	V_{rel}[c]	E	V_{rel}[d]
triethylamine			18	100				
benzene	5.2	32.0	16	26.9	14	100.0		
CCl_4	3.8	5.7	24	29.1	18	28.0		
CH_3CN	1.5	25.1	4	41.1	4	10.7		
dioxane	4.9	21.2	21	96.9	18	17.3		
DMF			12	12.4	6	0.03		
MTBE	6.4	100	21	29.6				
CH_2Cl_2	7	24.4	15.4	9.2				
THF	7.4	41.0	17	1.7			133	100.0
$CHCl_3$			17	9.2				
acetone							88	81.8
nitrobenzene							62	63.3
3-pentanone							101	93.2
t-amylalcohol							75	45.5

[a] 3.6×10^{-5} mmol/min mg enzyme is taken as 100. [b] 5.2×10^{-5} mmol/min mg enzyme is taken as 100. [c] 1.7×10^{-2} mmol/min mg enzyme is taken as 100. [d] 1.0×10^{-5} mmol/min mg enzyme is taken as 100. VA, vinyl acetate; VB, vinyl butyrate.

vary erraticly and to depend on the acylating agent and enzyme form. It should be emphasized that all the experiments were carried out at a constant water activity (<0.1) by working in the presence of molecular sieves or by using reactants pre-equilibrated with molecular sieves.[7,14]

In order to generate a model of the solvated and desolvated portions of the substrates in the transition state, a conformational analysis of the tetrahedral intermediates TS1 and TS2 was carried out. The intermediates were generated starting from the X-ray structure of the subtilisin Carlsberg–Eglin complex, as described in the computational section (*vide infra*). After an initial minimization of the entire structure, the conformational space of the *R*- and *S*-acetyl intermediates was investigated by MC/EM technique[8] on a substructure which included the substrate and all atoms within 15 Å of the tetrahedral carbon. All the minima found within 50 kJ/mol of the global minimum were fully reminimized to allow a more accurate determination of the relative energies.

In qualitative agreement with the experimental data, the complexes formed by the *S*-substrates were found to be more stable than those formed by the *R*-enantiomers. The calculated ΔE_{S-R} were 29 kJ/mol for **1** and 180 kJ/mol for **2**. In both cases, the predicted selectivity was one order of magnitutide larger

Fig. 2. Schematic representation of binding of *S*- and *R*-enantiomers of *sec*-phenethyl alcohol (*a*), and *trans*-sobrerol (*b*) in the active site of subtilisin. The aminoacids reported are those of the catalytic triad and of the oxyanion hole

than the experimentally observed one in all the solvents examined. However, transesterification of **2** was correctly predicted to be more selective than the transesterification of **1**.

The lowest minimum conformation of the *sec*-phenethyl alcohol complex S_0 showed that the hydrogen of Nε of His64 was directed towards the alcoholic oxygen of the substrate and the Oγ of Ser221. This arrangement seemed to favor a stacking interaction between the benzene ring of the substrate and the imidazole ring of His64. In the first structure above the global minimum (S_1) the methyl group present in the alcoholic moiety was directed outside the catalytic pocket.

The stabilizing hydrogen bond network and the stacking interaction were not found in the *R*-complex model, and the lack of these favorable interactions can be invoked as a reason for the preference of subtilisin for the *S*-enantiomer (Fig. 2a) (ΔE_{S-R}=29 kJ/mol).

In the case of *trans*-sobrerol, the S_0-enantiomer was much more stable than the R_0-enantiomer because it was less sterically congested. In the S_0-complex, the substrate was directed outside the protein and was stabilized by a network of H-bonds, which were absent in the case of the R_0-complex (Fig. 2b).

Once the structures of the intermediates were determined, UNIFAC was applied to calculate the activity coefficients.[9] The solvent accessible areas of the tetrahedral intermediate were evaluated both in the enzyme-bound and free form and, in the latter case, the enzyme was replaced by a hydrogen atom. The surface areas for each UNIFAC group were calculated by the Connolly algorithm[12] and the results are reported in Table 2 and Table 3. The upper part of the table shows the desolvation areas of the alcoholic moiety of the intermediate and the lower part, the data for the acetyl moiety. The linear regression of Eq. 1 between the experimentally determined E in the various solvents and the calculated γ'_S/γ'_R in the same solvents gave the slopes reported in Table 4.

According to the literature,[6] two different strategies were employed for the calculation of the γ' values. The groups with areas greater than 50% desolvated were considered completely desolvated and the groups with areas less than 50% desolvated were considered completely solvated (Table 4, *all or nothing* column). Alternatively, the precise percentages of desolvation were used for the calculation of

Table 2
Solvent accessible areas of *R*- and *S-sec*-phenethyl alcohol transition states

group	free	enzyme bound									
		(R_0)-complex		(R_1) -complex		(R_2) -complex		(S_0) -complex		(S_1) -complex	
	Å^2	Å^2	des%	Å^2	des%	Å^2	des%	Å^2	des%	Å^2	des%
Ph	176.5	39.5	77.6	49.4	72.0	128.7	27.1	61.4	65.2	43.9	75.1
CH_3	72.8	21.7	70.2	43.5	40.2	12.9	82.3	16.5	77.3	45.7	37.2
[a]OH	33.3	2.0	94.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
CH	21.6	0.0	100.0	0.4	98.1	0.0	100.0	3.6	83.3	1.4	93.5
C	0.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0
[b]OR	28.8	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100
CH_3	75.7	16.0	78.9	13.4	82.3	14.8	80.4	21.0	72.3	13.6	82.0
O^-	35.5	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0

[a]OH group is part of the alcohol moiety. [b] R = H in "free" tetrahedral intermediate; R = Enz when the tetrahedral intermediate is complexed with subtilisin

Table 3
Solvent accessible areas of *R*- and *S-trans*-sobrerol transition states

group	free	enzyme bound			
		(R_0)-complex		(S_0) -complex	
	Å^2	Å^2	desolvation %	Å^2	desolvation %
C^7H_3	75.1	58.7	21.8	40.7	45.8
[a]OH	36.9	0.0	100.0	0.0	100.0
iPr-OH	152	1.9	98.8	82.5	45.7
C^2H	18.6	5.4	71.0	0.0	100.0
C^1=C^6H	31.7	10.6	66.6	19.3	39.1
C^5H_2	28.7	2.2	92.3	28.2	1.7
C^4H	5.6	0.0	100.0	0.4	92.9
C^3H_2	24.9	7.2	71.1	0.9	96.4
C	0.0	0.0	100.0	0.0	100.0
[b]OH	43.9	0.0	100.0	0.0	100.0
CH_3	50.1	3.8	92.4	10.3	79.4
O^-	18.7	1.8	90.4	0.0	100.0

[a]OH group is part of the alcohol moiety. [b] R = H in "free" tetrahedral intermediate; R = Enz when the tetrahedral intermediate is complexed with subtilisin

Table 4
Slopes and correlation coefficients obtained from the linear regression of Eq. 1 applied to the experimentally determined E values, for subtilisin catalyzed transesterification in different organic solvents, and calculated γ'_S/γ'_R

	substrate	intermediate	acylating agent	lyophilized				CLEC			
				all or nothing		*fractional*		*all or nothing*		*fractional*	
Entry				slope	R^2	slope	R^2	slope	R^2	slope	R^2
1	*sec*-phenetyl alcohol	S_0-R_0	VA	nc	nc	0.82	0.01				
2		S_0-R_1	VA	-2.51	0.70						
3		S_0-R_2	VA	-0.12	0.03						
4		S_1-R_0	VA	2.42	0.66	4.60	0.56				
5		S_1-R_1	VA	0.39	0.07						
6		S_1-R_2	VA	0.06	0.01						
7		alcohol (S_1-R_1)	VA	0.98	0.43	2.64	0.36				
8		S_0-R_0	VB	nc	nc	-1.59	0.04	nc	nc	-1.62	0.04
9		S_0-R_1	VB	-0.07	0.01						
10		S_0-R_2	VB	-0.08	0.01						
11		S_1-R_0	VB	2.06	0.60	1.97	0.18	2.67	0.79	3.43	0.29
12		S_1-R_1	VB	0.28	0.16						
13		S_1-R_2	VB	0.17	0.09						
14		alcohol (S_1-R_0)	VB	0.75	0.22	1.90	0.33	2.81	0.71	2.91	0.64
15	*trans*-sobrerol	S_0-R_0	VA	-0.28	0.62	0.49	0.33				
16		alcohol (S_0-R_0)	VA	0.17	0.25	0.23	0.23				

nc: not calculated, the two structures were identical in terms of desolvation of different groups

γ' (Table 4, *fractional* column). In the original Klibanov procedure the γ'_S/γ'_R ratios were calculated on the alcohol moiety only. We followed this procedure (entries 7 and 14) and extended the calculation to the entire intermediate (Table 4, entries 1–6 and 8–13). For *sec*-phenethyl alcohol the two global minima R_0 and S_0 were indistinguishable in terms of desolvation surface area (Table 2) and thus by UNIFAC calculations (Table 4, entry 1). The calculation was therefore extended to all the minima found for the tetrahedral intermediate in an energy range of 25 kJ/mol from the global minimum, so six structures for the *R*-complex (R_0–R_5) and two structures for the *S*-complex (S_0–S_1) were taken into account (Table 2). In terms of desolvation of different groups, the first two structures for the *S*-complex differed in the position of the methyl group in the alcoholic moiety, which in S_1 is more exposed to the solvent. For the *R*-complex the six structures (R_0–R_5) fell into three clusters: one containing structures R_0, R_3 and R_5 (Table 2, R_0-complex), one containing R_2 and R_4 (Table 2, R_2-complex), and one containing R_1 alone (Table 2, R_1-complex).

Each *R*- and *S*-structure were compared to one another for vinyl acetate (entries 1–7) and vinyl butyrate (entries 8–14) transesterifications and linear regressions are reported in Table 4. In all cases the slopes obtained differed greatly from unity, which is the theoretical value, and spanned from −2.51 to 3.43. This basically means that the model in some cases tends to underestimate the influence of the solvent (slope>1, i.e., the variations of E values were higher than those of γ'_S/γ'_R), and in others to overestimate

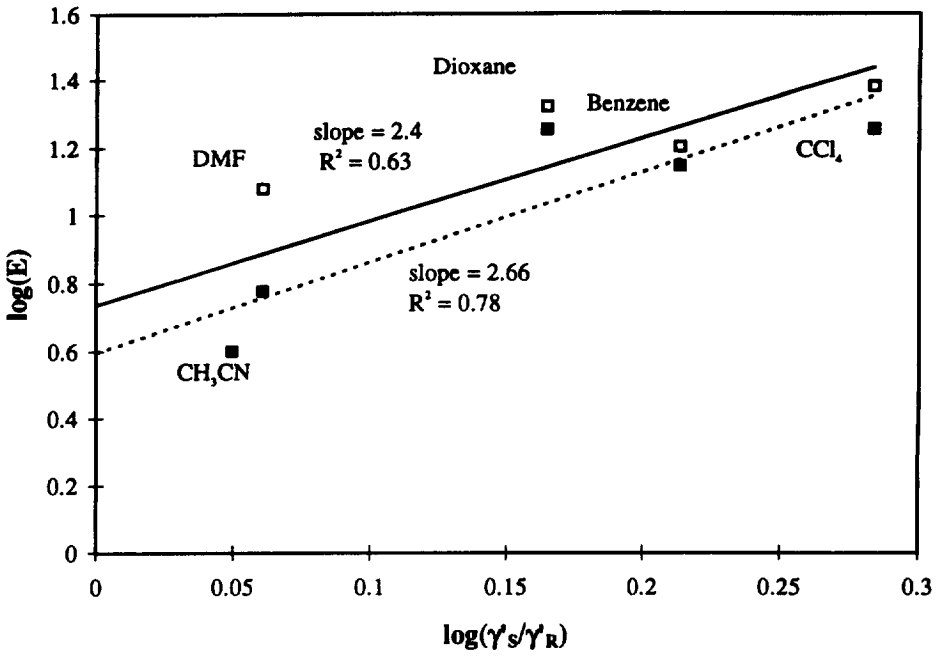

Fig. 3. Dependence of the enantiomeric ratio (E) of lyophilized (open squares, —) and CLEC-subtilisin (filled squares, ·····) on the ratio of the activity coefficients, calculated with the *all or nothing* approach, of the desolvated parts of *sec*-phenethyl alcohol in various organic solvents in the transesterification with vinyl butyrate

it (slope<1, i.e., the variations of E values were lower than those of γ'_S/γ'_R). The case of negative slope values is even worse because the predicted solvent effects on enantioselectivity were opposite to the experimental ones. The use of the *fractional* instead of the *all or nothing* model did not change the essence of the results, because in both cases the slopes differed greatly from unity. These data also show that this model was unsuitable for both lyophilized[10] and CLEC-subtilisin, since both enzyme forms experimentally displayed identical trends with the same solvents. For instance, Fig. 3 depicts the dependence of the enantiomeric ratio of both lyophilized and CLEC-subtilisin on the ratio of activity coefficients, using the *all or nothing* approach in the transesterification of *sec*-phenethyl alcohol with vinyl butyrate.

The same considerations apply to the transesterification of *trans*-sobrerol with vinyl acetate (Table 4, entries 15 and 16). In this case, clear differences in terms of desolvation were observed in the S_0- and R_0-complexes (Table 3 and Fig. 2b). However no correlation was found between the UNIFAC calculation and experimental selectivities either by considering the alcohol moiety or by extending the calculation.

It could be argued that these results were a consequence of an inaccurate determination of E values. However, these values were obtained through simple and reliable chiral chromatographic tecniques[14] and, indeed, the standard deviations were less than 20%. One point to be taken into account is that all calculations were run *in vacuo*, which could have led to some artifacts in the determination of the structure of the global minimum, that could have been different for the different solvents if their presence had been considered explicitly.[11] Such an approach, however, would be extremely demanding if a large number of organic solvents were to be taken into consideration. One more point is that UNIFAC is unable to distinguish between slightly different structures in terms of desolvation (*e.g.* in the case of R_0- and S_0-complexes of *sec*-phenthyl alcohol) and it may be suitable only for markedly different complexes.

Finally, it must be considered that the solvent-dependence of enzyme-selectivity could not be entirely due to substrate desolvation phenomena, but also to the presence of solvent molecules at the active site, that could change the molecular recognition process by steric, electrostatic and conformational factors.[3,4] Thus, even though the method developed by Klibanov and coworkers[6] to rationalize and predict enzyme selectivity is very appealing for its simplicity, and gives excellent results for some systems, it does not seem to be generally applicable to enzymatic resolutions in organic solvents.[6]

3. Materials and methods

(±)-*sec*-Phenethyl alcohol and (±)-*trans*-*p*-menth-6-ene-2,8-diol (*trans*-sobrerol), vinyl acetate and vinyl butyrate were purchased from Aldrich. All organic solvents were distilled and equilibrated on 4 Å molecular sieves before use. Subtilisin Carlsberg (protease Type VIII) was purchased from Sigma and, before use, dissolved in 20 mM potassium buffer (pH 7.8) and lyophilized.[13] Cross-linked enzyme crystals (CLEC) of subtilisin (dry powder, ChiroCLEC©-BL) was a generous gift from Altus Biologics, Cambridge, MA, USA.

3.1. Enzyme catalyzed transesterifications

sec-Phenethyl alcohol and lyophilized subtilisin: To a 3 mL screw-capped vial were added 1 mL of organic solvent, 0.01 mmol of *sec*-phenethyl alcohol, 0.2 mmol of acylating agent (vinyl acetate or butyrate), 50 mg of molecular sieves and 10 mg of subtilisin; the reaction mixture was shaken at 180 rpm, 45°C and, at different times, aliquots were withdrawn and the degree of conversion and the enantiomeric excess of the product determined by chiral GC as already described.[14a]

sec-Phenethyl alcohol and CLEC-subtilisin: The reaction was carried out as described above using 0.083 mmol of *sec*-phenethyl alcohol, 0.35 mmol of vinyl butyrate and 1–5 mg of CLEC-subtilisin without adding molecular sieves to the solution. Prior to mixing, all components were brought to $a_w < 0.1$ by equilibration (2 days) with molecular sieves in sealed containers.[14]

trans-Sobrerol and lyophilized subtilisin: The reaction was carried out as above using 0.05 mmol of sobrerol, 0.07 mmol of vinyl acetate and 30 mg of subtilisin. The degree of conversion and the enantiomeric excess of the products were determined by chiral GC as already described.[14a] The enantiomeric ratio was calculated according to Chen et al.[15]

3.2. Molecular modeling

Calculations and graphic simulations were performed on a Silicon Graphics Indigo2 R10000. Starting geometries of the studied compounds were generated and subjected to simulation using the program Macro Model-BatchMin (version 5.5). The initial coordinates of subtilisin Carlsberg complexed with the polypeptidic inhibitor Eglin C (2sec.pdb) were obtained the from Brookhaven Data Bank. The inhibitor, except for the residues Leu59 and Asp60, and all water molecules except one, which is placed close to Leu217, His67 and Pro225 and is highly conserved and present in a large number of subtilisins from different sources, were removed from the enzyme structure. The incorrect bond lengths in His39 and His67 were corrected and the acidic hydrogens added. Only polar hydrogens and substrate hydrogens were considered explicitly, while for the rest of the protein a united atom model was used. The enzyme was completely minimized under vacuum using the AMBER* force field,[16] Polak–Ribiere conjugated gradient (PRCG) as minimization algorithm, and 0.05 kJ/Å mol as gradient convergence criterion.

The residual part of the inhibitor (Leu-Asp dimer) was used to create the structures of the tetrahedral intermediates of our substrates inside the active site and then deleted.

In the case of *sec*-phenethyl alcohol, both *R*- and *S*-acyl-intermediates were subjected to conformational analysis using the Montecarlo/Energy Minimization technique, PRCG, AMBER*.[17] The study was limited to the atoms included in a 15 Å range from the carbonyl carbon in the tetrahedral intermediate.[18] During the energy minimization step the atoms in this range are kept fixed in their crystallographic positions with a distance-dependent harmonic force constant that varies as a function of the distance from the tetrahedral intermediate. To better reproduce the H-bond network in the active site, no restraint was imposed on the coordinates of polar hydrogens. The rest of the protein was completely neglected in this phase of the calculation. From the conformational analysis, 184 structures for the *S*-enantiomer and 224 structures for the *R*-enantiomer were obtained in 50 kJ/mol. These were fully minimized using PRCG, AMBER* until a gradient of 0.05 kJ/mol was reached, giving 3 structures for the *S*-enantiomer complex and 10 structures for the *R*-enantiomer complex in 41 kJ/mol. *trans*-Sobrerol was first studied as a free uncomplexed molecule to find the most stable conformation, performing three systematic-pseudo-MonteCarlo/Energy Minimization conformational searches (2000 steps each)[19] to obtain the global minimum. This structure was then used to build the transition state intermediate that was placed inside the active site as already described for *sec*-phenethyl alcohol, and the conformational search on this intermediate was run using the same restraints as described before.[17] From the conformational analysis, 90 structures were obtained for the *S*-enantiomer and 28 structures for the *R*-enantiomer in 50 kJ/mol. These were fully minimized using PRCG, AMBER* until a gradient of 0.05 kJ/mol was reached, giving in 41 kJ/mol two structures for the *S*-enantiomer complex and two structures for the *R*-enantiomer complex. Solvent accessible surface areas for our substrates were calculated using the Connolly algorithm[12] with a probe radius of 1.4 Å and the 'free' tetrahedral intermediates were obtained by replacing the subtilisin with an atom of hydrogen. The percentages of desolvation were calculated according to Eq. 2.

$$\% = 100 * (\text{Å}_{\text{free}} - \text{Å}_{\text{enzyme bound}}) / \text{Å}_{\text{free}} \quad (2)$$

3.3. UNIFAC application

All the activity coefficients (γ) were calculated using the UNIFAC computer algorithm.[9] The desolvated fraction of the substrate was determined measuring the Connolly surface area[12] for each group. For *sec*-phenethyl alcohol the desolvated portions were calculated on the global minimum for the *R*-enantiomer, while for the *S*-enantiomer they were calculated on the two lowest minima. For *trans*-sobrerol we compared the two global minima structures. For the calculation of γ values the concentrations of acylating agents and substrates reported above (*enzyme catalyzed transesterifications*) were used.

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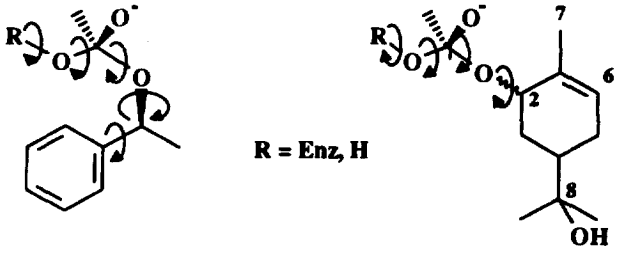
17. The torsion angles considered for the analysis are represented:



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