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Influence of Organic Solvents on Enzyme Chemoselectivity and Their Role in Enzyme-Substrate Interaction

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Abstract: Chemoselectivity of lipoprotein lipase from *Pseudomonas specie* is strongly influenced by solvation phenomena and can be modulated by changing the reaction medium. Experimental results obtained from enzymatic acylation of L-Ser-β-naphthylamide indicated that chemoselectivity depends on the nature of hydrogen bonds taking place among the molecular species present in the reaction medium. Inverted selectivity was observed upon moving from hydrogen bond donor solvents to hydrogen bond acceptor solvents. Solvent hydrophobicity can also influence chemoselectivity inducing intramolecular electrostatic interactions which alter the reactivity of the nucleophilic group(s). Solvation phenomena were studied by means of ^{1}H NMR. An explanation of interactions between the solvated substrate and the enzyme is also proposed.

INTRODUCTION

It is largely recognized that enzyme selectivity in organic solvents is strongly influenced by the nature of the medium[1]. Additional effects on selectivity may be due to steric features of both substrate and solvent[2]. Solid state NMR studies of proteases[3] have shown how their catalytic triad remains unaltered when the biocatalyst is suspended in anhydrous acetone or octane. Moreover, X-ray data of Subtilisin Carlsberg crystals grown in acetonitrile[4] and γ-chymotrypsin soaked in hexane[5] indicated that their structures are identical to those grown in water. These observations lead to the belief that factors other than conformational modifications have a prominent role for explaining variation of selectivity, for instance, solvation phenomena[6].

Lipase should be viewed as a system on the boundary of two phases, namely the surrounding solvent and the active site. The energy of moving the reacting system from the solvent to the active site is included in the effective rate constant, k_{cat}/K_m. During binding of the substrate to the enzyme a fraction of solvent present on the substrate molecule is removed and returns to the bulk solvent. As a consequence, each organic solvent can affect the partitioning behaviour of substrates into enzyme's active site according to their physico-chemical nature. This behaviour leads to profound changes in substrate specificity and catalytic efficiency, since the ability of the enzyme to utilize the free energy of binding with the substrate reflects the difference between binding energies of substrate-enzyme and substrate-solvent interactions[7].

Several examples of enzymatic chemoselective acylations in organic solvents have been reported, often with chemoselectivities opposite to those of chemical reactions[2,8]. Klibanov suggested an explanation for the solvent dependence of chemoselectivity considering that when a nucleophilic group is participating in a hydrogen bond with the solvent it cannot attack the acyl-enzyme complex. As a hydroxyl group is better hydrogen bond donor than amino group, its ability to act as nucleophile is hindered to a greater extent by hydrogen-bond-forming solvents than that of amino group[1].

Halling[6] reported that solvation phenomena should be discounted before seeking explanations for the residual effects in terms of direct interactions with the enzyme molecules and substrate solvation in some cases completely explains the observed effects of changing solvents.

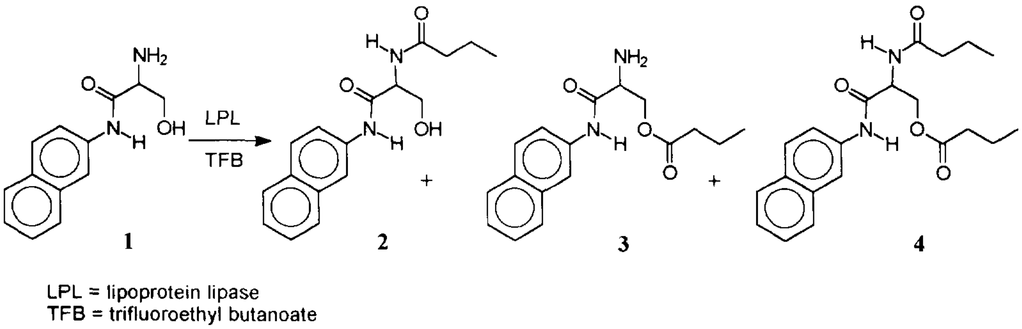
Further insights into the effect of the reaction medium on catalytic activity and selectivity have come from the studies of Dordick on peroxidase's active site[9]. By using electron paramagnetic resonance it was shown that active site is exposed to organic solvent. Penetration of organic solvent into the active site of an enzyme would be expected to alter the balance of forces which determine the ground-state of enzyme and substrate as well as transition state energies.

In the present work particular attention has been paid to solvation phenomena in different reaction media using ^1H NMR to investigate the solvent-substrate interactions. This data has been used to explain chemoselectivity of lipoprotein lipase from *Pseudomonas specie* (LPL).

RESULTS

Kinetic study of solvent effect on enzymatic acylation

The chemoselective acylation of L-Ser-β-naphthylamide (**1**) catalyzed by purified LPL was studied employing trifluoroethyl butanoate as the acyl donor (Scheme 1). No acylation was observed in the absence of the enzyme in any of the reaction media tested. In the enzymatic reaction no diacylated product (**4**) was detected below 40% of total conversion.



Scheme 1

The effect of reaction medium on enzyme chemoselectivity, expressed as the ratio of the specificity constants k_{cat}/K_m of acylation of -OH and -NH_2 groups has been evaluated. Experiments were performed in different solvents with varying concentrations of water and poly(ethylene glycol) (PEG), in order to assess the effect of hydration and of an amphiphilic hygroscopic polymer[10] on the chemoselectivity. We selected three solvents according to their disposition to engage different types of interactions with solutes: benzene, an apolar aprotic solvent, *tert*-amyl alcohol, a dipolar protic solvent and pyridine, a dipolar aprotic solvent. The differences between the three solvents tested concern their polarity and ability to accept hydrogen bonds and can be described quantitatively by log P (partition coefficient), ε (dielectric constant) and AN (acceptor number)[11].

The most significant result obtained was the inversion of enzyme selectivity upon changing the solvent from benzene or *tert*-amyl alcohol to pyridine (Table 1). The strong effect of reaction medium on chemoselectivity can be quantified by the ratio between the specificity constants ($(k_{cat}/K_m)_{OH}/(k_{cat}/K_m)_{NH_2}$) which changes from a minimum value of 0.05 when working in *tert*-amyl alcohol/water/PEG to a maximum value of 10.5 obtained in anhydrous pyridine as solvent, that corresponds to a 210 fold increase.

Table 1. Kinetic Data of Enzymatic Acylation of L-Serine-β-naphthylamide at 40°C.

Solvent	$[H_2O]$ (% v/v)	[PEG] (mM)	v_0 (OH)•10^4 (mM/mg•s)	v_0 (NH_2)•10^4 (mM/mg•s)	$\frac{(k_{cat}/K_m)_{OH}}{(k_{cat}/K_m)_{NH_2}}$
Pyridine	0	0	14.5	1.4	10.5
Pyridine	0.4	0	1.8	0.70	2.6
Pyridine	0	0.5	0.15	0.05	3.0
Pyridine	0.4	0.5	2.7	0.45	5.9
Benzene	0	0	3.1	7.1	0.44
Benzene	0	0.5	4.0	12.4	0.32
Benzene, pyridine (2% v/v)	0	0	0.41	0.64	0.64
tert-amyl alcohol	0	0	0.32	3.0	0.11
tert-amyl alcohol	0.4	0	0.22	2.2	0.10
tert-amyl alcohol	0	0.5	0.65	3.7	0.18
tert-amyl alcohol	0.4	0.5	0.35	6.5	0.05

In *tert*-amyl alcohol the acylation rate of the amino group is about one order of magnitude higher than that for the hydroxyl group, under any combination of experimental conditions used (Table 1). Pyridine was the only solvent tested which led to preferential acylation of the -OH group which was attacked with an initial rate 10 times higher than that for the $-NH_2$ group.

It should be noted that the enzymatic reaction in pyridine was strongly influenced by the presence of water in the reaction medium (Table 1). The value of $(k_{cat}/K_m)_{OH}/(k_{cat}/K_m)_{NH_2}$ dropped from 10.5 to 2.6 when 0.4% of water was added to pyridine. Moreover, the overall reaction rate was reduced, and the rate of acylation of the -OH group decreased by one order of magnitude.

To verify whether the effect of pyridine on the substrate could be ascribed to a simple base catalysis process, an enzymatic reaction was conducted in benzene in the presence of 2% (v/v) of pyridine (about 10 fold excess based on substrate concentration). No preference for attack at the -OH group was noticed, and the reaction followed a similar behaviour to the case of pure benzene as solvent (Table 1). Therefore, pyridine affects reaction chemoselectivity only when it represents the bulk solvent.

When L-Ser-β-naphthylamide was acylated chemically using an equimolar amount of butanoyl chloride as acylating agent[12], the N-acylated derivative **2** was the main product (>65%) in both benzene and *tert*-amyl alcohol. In pyridine the N-acylated derivative was also formed in high yield (70%) and other products including the diacylated product **4** (12%); the O-acylated product **3** (6%) and unreacted substrate (12%) were formed. It is remarkable that in pyridine the behaviour of the chemical reaction is opposite to that of the enzymatic reaction carried out in the same solvent. However, in pyridine the two reactions, enzymatic and chemical, are not comparable on the basis of their mechanism. The acylating species are different (trifluoroethyl butanoate and N-butanoylpyridinium chloride respectively) as well as the transition states and the rate determining steps (nucleophilic attack on acyl-enzyme and nucleophilic attack on N-butanoylpyridinium chloride). Nevertheless, it is noteworthy that the solvent effect is considerable and is observed for the enzymatic reaction.

In a previous communication[10] we reported that chemoselectivity can be induced in LPL by chemical modification of the enzyme and we observed that the lipase covalently modified with PEG can catalyze the chemospecific acylation of $-NH_2$ group of L-Ser-β-naphthylamide in benzene, whereas the native enzyme did not give the same selectivity. In the present investigation a mixture of 0.5mM of PEG (MW=20,000) and native LPL was employed. In benzene acylation was not specific, although the N-acylated derivative **2** was predominantly formed. This indicated that the chemoselectivity previously observed[10] was a consequence of the covalent modification of the lipase and not simply an effect due to the presence of PEG molecules in the reaction medium. PEG exerts a negative effect on enzyme activity when working in pyridine. Although PEG slightly favours the acylation of the $-NH_2$ group in the reactions performed in benzene or in a *tert*-amyl alcohol/water mixture, the variations were negligible and no further investigation was conducted.

^1H NMR investigation of solvent-substrate interactions

Trying to give a rational explanation of experimental data, attention was focused on solvation phenomena taking place in the different reaction media. NMR spectroscopy is one of the main techniques used for studying solvent effects on dissolved compounds[11].

Significant variations of the signals relative to the methylene protons (CH_2-β) of L-Ser-β-naphthylamide were observed as a function of the nature of the solvent and water content. The variations of chemical shifts of L-Ser-β-naphthylamide protons were correlated to physico-chemical characteristics of solvents and to alterations in the reactivity of the -OH and $-NH_2$ groups of the substrate. Each solvent presents peculiar values of chemical shifts and coupling constants (J), indicating that the prevailing conformation changes significatively (Table 2).

Table 2. ^1H NMR Data of L-Serine-β-naphthylamide in Different Solvents[a].

Solvent	T (°K)	D_2O (% v/v)	Δδ (ppm ± 0.001)	$^3J_{Ha,X}$ (Hz ± 0.1)	$^3J_{Hb,X}$ (Hz ± 0.1)
H_2O	313	0	<0.04	5.6_5	4.8_5
benzene	297	0	0.267	5.0	5.5
benzene - pyridine (2% v/v)	313	0	0.263	5.2_5	5.1_5
benzene - pyridine (2% v/v)	333	0	0.217	5.3_5	5.1_5
pyridine	297	0	0.119	5.7_5	4.4
pyridine	297	2.9	0.072	5.7_5	4.6_5
pyridine	297	6.5	<0.04	5.6	4.9
pyridine	317	0	0.104	5.7_5	4.6_5
pyridine	367	0	0.070	5.7_5	4.6_5
tert-amyl alcohol	313	0	0.104	5.1_5	6.1
tert-amyl alcohol	313	3.8	0.122	4.9	6.3_5
tert-amyl alcohol	313	7.4	0.127	4.8	6.5
tert-amyl alcohol	333	0	0.083	5.2	6.1
tert-amyl alcohol	353	0	0.067	5.3_5	5.6

[a] $^2J_{Ha,Hb}$ = -10.6Hz and the variations are smaller than experimental error.

Figure 1 shows the signal, a doublet, relative to methylene protons in pure D_2O. These protons are magnetically equivalent, thus indicating that the $HC-CH_2OH$ bond is free to rotate and the structure is mobile.

In anhydrous benzene the methylene protons are not magnetically equivalent ($\Delta\delta = \delta_{Ha} - \delta_{Hb} =$ 0.267ppm at 297°K), and the molecule appears rigid (Figure 2a). Rotation of the $HC-CH_2OH$ bond is hindered because of the formation of an intramolecular hydrogen bond, most likely between the oxygen of the -OH group and the hydrogen of the amide group, as such an electrostatic interaction is greatly favoured by the hydrophobic nature of the solvent.

In anhydrous pyridine the signal of methylene protons give two complex multiplets, as the two protons are not magnetically equivalent (Figure 3a), although the difference in chemical shift between the two signals is smaller ($\Delta\delta$ = 0.119ppm at 297°K) than in benzene. The molecule appears to be less rigid than in benzene, because pyridine is able to solvate both the -OH and amide -NH groups, acting as hydrogen bond acceptor and therefore preventing the formation of the intramolecular hydrogen bond.

Upon addition of small quantities of water (1-6.5%, v/v) to the pyridine the signals of the CH_2-β protons change (Figure 3b,c) and the multiplets coalesce and become progressively similar to that signal observed in D_2O, as can be deduced also from coupling constants values in Table 2. This can be explained considering that water replaces pyridine solvating the substrate's polar groups. Water molecules remove those interactions which cause rigidity and allow $HC-CH_2OH$ bond to rotate freely and hence the methylene protons tend to become magnetically equivalent. It is interesting to notice that the effect of augmented mobility can not be mimicked by raising the temperature. At 367°K (Figure 3e) the signals of the two protons are not equivalent, even though $CH-CH_2OH$ bond appears to be more mobile compared to the situation at room temperature. This indicated that the interactions taking place between the substrate and pyridine were particularly strong.

Upon addition of 2-6% (v/v) of pyridine to benzene the NMR signals of CH_2-β protons did not change appreciably ($\Delta\delta$ = 0.263ppm at 313°K) compared to pure benzene, and it seems that pyridine is unable to perturb the intramolecular hydrogen bond that is induced by benzene (Figure 2b). On increasing the temperature to 333°K no appreciable variation of chemical shift values were detectable and this confirmed the presence of a substantial conformational constraint in the molecule.

In anhydrous the signals of the methylene protons were a multiplet and the $HC-CH_2OH$ bond looked partially rigid (Figure 4). On increasing the temperature to 353°K the signals of the two protons tend to collapse. Upon addition of water the difference in chemical shift of the two protons increased, indicating that water causes a further differentiation between the CH_2-β protons and induces an enhancement of rigidity. This observation is also supported by the coupling constants values. This is in accordance with the fact that in dilute alcohol-water mixtures molecules have restricted motion because of hydrogen bonding interactions between alcohol hydroxyl groups and water molecules[13]. In *tert*-amyl alcohol the coupling constants values differ form those observed in the other solvents, indicating that different conformational constraints exist.

DISCUSSION

The results obtained from ^1H NMR studies indicated that the observed change in chemoselectivity

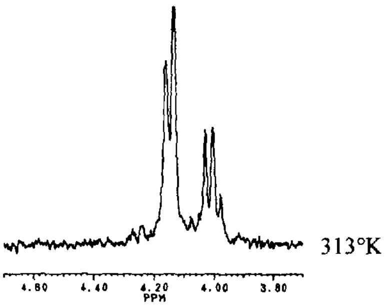


Figure 1. ^{1}H NMR of L-Ser-β-naphthylamide in D$_2$O

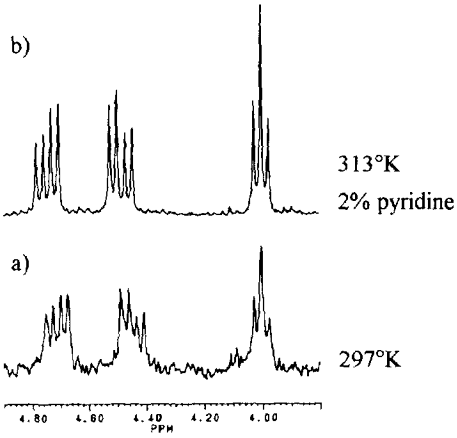


Figure 2. ^{1}H NMR of L-Ser-β-naphthylamide in benzene

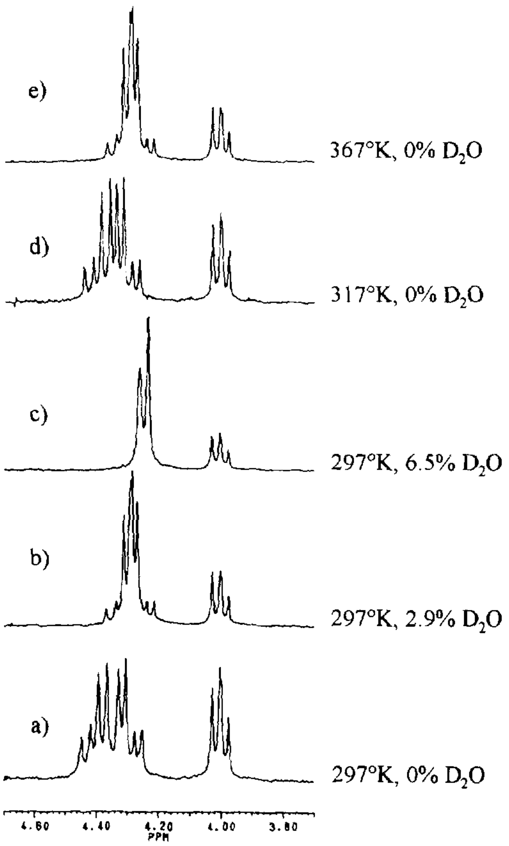


Figure 3. ^{1}H NMR of L-Ser-β-naphthylamide in pyridine

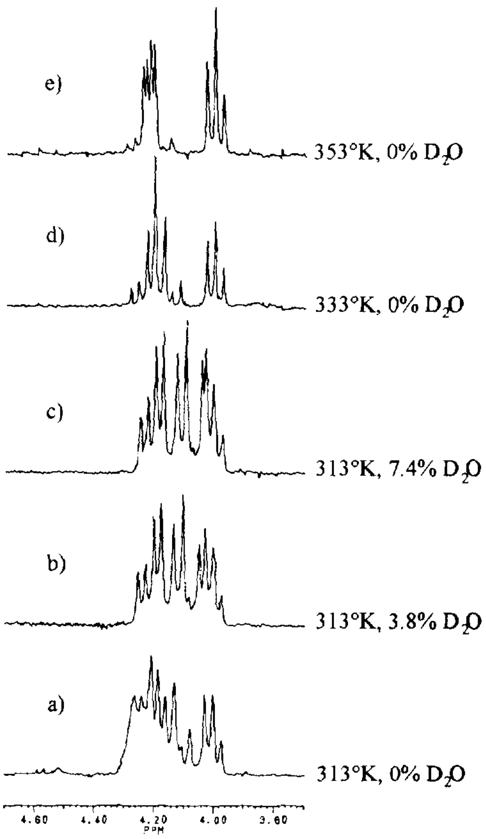


Figure 4. ^{1}H NMR of L-Ser-β-naphthylamide in *t-amyl*-alcohol

could be explained, at least partially, by variations in the solvation of the substrate, L-Ser-β-naphthylamide.

Benzene, an apolar aprotic solvent, is unable to influence the reactivity of the nucleophilic groups by engaging hydrogen bonds. However, in the case of L-Ser-β-naphthylamide, benzene induces an intramolecular hydrogen bond because of its hydrophobicity. On accepting a hydrogen bond from the amido group the hydroxyl group is reduced in nucleophilicity and in its ability to attack the acyl-enzyme.

Tert-amyl alcohol, a dipolar protic solvent, can act as a hydrogen bond donor. This solvent is very efficient in solvating anions or groups having a negative charge density, expressed as the ratio between charge and volume of the group, and this tendency becomes stronger as the charge density increases. Therefore, -OH group is more solvated and undergoes a larger reduction in nucleophilicity compared to amino group.

Pyridine, a dipolar aprotic solvent, can solvate anions or groups having a negative charge density by means of ion-dipole or ion-induced dipole forces. In the specific case of L-Ser-β-naphthylamide, pyridine engages a hydrogen bond with the -OH group inducing the enhancement of the negative charge density on the oxygen. Polarization of O-H bond translates into an increase of the nucleophilicity of the -OH group which can attack the acyl-enzyme intermediate more effectively. However, this would be possible only in the case the pyridine-substrate interaction persists during substrate binding in the active site. The polarization due to pyridine causes rigidity of the molecule, but this rigidity decreases upon addition of water, which solvates the -OH group of L-Ser-β-naphthylamide side chain acting as a hydrogen bond donor solvent. As a consequence, the negative charge density of -OH group is dispersed and its nucleophilicity reduced, as shown by the kinetic data.

Although no direct information is available about the way the substrate approaches the enzyme's active site and whether the solvent can affect the energies involved in the transition state, on the basis of the experimental data obtained it is possible to offer different interpretations of the interactions occurring between organic solvent and enzyme.

It is generally accepted that the substrate binding energy can be divided into two components, the first is due to removal of solvent molecules from the portion of the substrate excluded from the solvent in the enzyme-bound state. The second component is the energy of interaction between the enzyme and the substrate molecule. It has been suggested that the energy of desolvation of the area of substrate interacting with the enzyme is the only portion of substrate binding energy which changes from solvent to solvent[1b,14]. The case described in the present work does not follow this rule. In fact, acylation of -OH group would be unfavourable in pyridine because hydroxyl group is more solvated than amino group and removing of the solvent molecules from the hydroxyl group would require more energy than desolvation of the -NH_2 group.

A further interpretation of the solvent effect on the chemoselectivity of the reaction could be that pyridine ($pK_a = 5.25$) acts as a base deprotonating the hydroxy group of L-Ser-β-naphthylamide ($pK_a \approx 15$) so creating a strongly nucleophilic species which, after desolvation, enters the active site and reacts readily with acyl-enzyme complex. However, the comparison of pK_a values together with the results obtained performing the experiment in benzene in the presence of 2% (v/v) of pyridine lead us to exclude this idea.

Our hypothesis is that pyridine establishes a strong electrostatic interaction with the -OH group of the substrate. In its activated form LPL lifts its *lid* leaving a large hydrophobic surface accessible to the substrate[15] and guiding it to the active site. Approaching the active site, the substrate drags the solvent molecules and these become involved in the microenvironment that accommodates and stabilizes the transition state. The presence of molecules of organic solvent inside enzymes' active sites have been already reported by Klibanov in a study of the X-ray structure of subtilisin's crystals grown in acetonitrile[4] which described that few molecules of organic solvent can be accommodated inside the active site of the enzyme, probably by displacing weakly bound disordered water molecules[16]. Work by Halling[6] has also suggested that organic solvents may participate in solvating part of the substrate molecules in the Michaelis complex and/or the transition state, and that solvent molecules may also compete with substrate for binding at the active site of the enzyme. Our hypothesis is also supported by the experimental observation that the enzymatic reaction performed in benzene with 2% (v/v) of pyridine shows no selectivity of acylation towards the -OH group. ^{1}H NMR spectra indicate that when L-Ser-β-naphthylamide is dissolved in a benzene-pyridine mixture, interactions between the substrate and pyridine are negligible, and that the hydrophobic interactions between benzene and the substrate are predominant in determining solvation, conformation and reactivity of the nucleophilic groups of the substrate.

CONCLUSIONS

Our data have indicated that the reaction medium exerts a strong influence on enzymatic selectivity through different mechanisms. The formation of intermolecular hydrogen bonds between the organic solvent and the substrate can cause the inversion of the chemoselectivity upon moving from hydrogen bond donor to hydrogen bond acceptor solvents. Solvation can also induce the stabilization of intramolecular hydrogen bonds that alter the reactivity of the nucleophilic groups. Penetration of solvent molecules into the active site and control of rate determining step by solvent changes cannot be excluded[17]. Finally, direct interactions of the solvent with enzyme have to be considered, especially in the case of polar solvents.

However, a considerable part of solvent effect on selectivity can be ascribed to solvation of the substrate and ^{1}H NMR has proved to be a valuable tool for the investigation and description of solvation phenomena.

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EXPERIMENTAL SECTION

Materials: All chemicals were of analytical or HPLC grade, bottled and dried over 4Å molecular sieves (100g/L) and used without further purification. 2,2,2-Trifluoroethyl butanoate was synthesized using a standard procedure[18]. Crude LPL was purified according to the procedure described in ref. 10 and stored at 4°C over P_2O_5.

Kinetic measurements: 2,2,2-Trifluoroethyl butanoate (50mM) and L-Serine-β-naphthylamide (15mM) were added to a solution of 5mg of LPL in 1mL of solvent. Water and PEG were added according to the concentrations reported in Table 2. The mixture was thermostatted at 40°C and magnetically stirred. Samples were withdrawn and analyzed by HPLC. Initial rates were determined following the reaction up to 10% of total conversion.

Chemical acylation of L-Serine-β-naphthylamide: 0.22mmoles (50 mg) of L-Serine-β-naphthylamide were dissolved in the organic solvent (14.5mL) and an equimolar amount (23μL) of butanoyl chloride was added under vigorous stirring. The reaction was stopped after 1h and the mixture analyzed by HPLC. The products were isolated and purified by preparative TLC.

N-butanoyl-(L)-Serine-β-naphthylamide (**2**). $^1\underline{\mathrm{H}}\ \underline{\mathrm{NMR}}$ ($CDCl_3$) δ 0.95 (t, 3H, $C\underline{H}_3(CH_2)_2CONH$-), 1.70 (m, 2H, $CH_3C\underline{H}_2CH_2CONH$-), 2.30 (t, 2H, $CH_3CH_2C\underline{H}_2CONH$-), 3.70 (dd, J = 11.2, 4.1, 1H, β-$C\underline{H}H$), 4.34 (dd, J = 11.2, 2.9, 1H, β-$CH\underline{H}$), 4.61 (m, 1H, α–CH), 6.78 (d, J = 6.7, 1H, -CONH-), 7.35 (m, 3H, naphthyl), 7.45-7.50 (m, 3H, naphthyl), 7.79 (s, 1H, C_1H- (naphthyl)), 9.23 (s, 1H, CONH-naphthyl)

O-butanoyl-(L)-Serine-β-naphthylamide (**3**). $^1\underline{\mathrm{H}}\ \underline{\mathrm{NMR}}$ ($CDCl_3$) δ: 1.00 (t, 3H, $C\underline{H}_3(CH_2)_2COO$-), 1.75 (m, 2H, $CH_3C\underline{H}_2CH_2COO$-), 2.38 (t, 2H, $CH_3CH_2C\underline{H}_2COO$-), 4.56 (d, J = 9.2, 2H, β-CH_2), 4.80 (t, J = 9.2, 1H, α–CH), 7.38 (m, 3H, naphthyl), 7.78 (m, 3H, naphthyl), 8.23 (s, 1H, C_1H-(naphthyl)), 8.55 (s, 1H, CONH-naphthyl)

N,O-dibutanoyl-(L)-Serine-β-naphthylamide (**4**). $^1\underline{\mathrm{H}}\ \underline{\mathrm{NMR}}$ ($CDCl_3$) δ: 0.92 (t, 3H, $C\underline{H}_3(CH_2)_2CONH$-), 0.97 (t, 3H, $C\underline{H}_3(CH_2)_2COO$-), 1.68 (m, 2H, $CH_3C\underline{H}_2CH_2CONH$-), 1.72 (m, 2H, $CH_3C\underline{H}_2CH_2COO$-), 2.30 (t, 2H, $CH_3CH_2C\underline{H}_2CONH$-), 2.33 (t, 2H, $CH_3CH_2C\underline{H}_2COO$-), 4.44 (dd, J = 12.2, 5.0, 1H, β-$C\underline{H}H$), 4.60 (dd, J = 12.2, 7.1, 1H, β-$CH\underline{H}$), 5.06 (dd, J = 7.1, 5.0, 1H, α–CH), 6.71 (d, 1H, CONH), 7.75 (m, 3H, naphthyl), 7.35-7.50 (m, 3H, naphthyl), 8.18 (s, 1H, C_1H-(naphthyl)), 9.17 (s, 1H, CONH-naphthyl).

$^{13}\underline{\mathrm{C}}\ \underline{\mathrm{NMR}}$ ($CDCl_3$) δ: 13.63 ($\underline{C}H_3(CH_2)_2CONH$-), 13.69 ($\underline{C}H_3(CH_2)_2COO$-), 18.34 ($CH_3\underline{C}H_2CH_2CONH$-), 19.12 ($CH_3\underline{C}H_2CH_2COO$-), 35.99 ($CH_3CH_2\underline{C}H_2CONH$-), 38.44 ($CH_3CH_2\underline{C}H_2COO$-), 53.11 ($C_\alpha H$), 63.51($C_\beta H_2$), 117.07, 119.98, 125.13, 127.54, 127.66, 128.70, 130.80, 133.773, 135.09, 167.55 (COO-), 173.68 (CONH-naphthyl), 174.12 (CONH-).

Product(s) analysis: The analyses of the products were performed by HPLC. A Perkin Elmer Series 10 Liquid Chromatograph, equipped with a LC-235 diode array UV-visible detector set at 284nm and a C-18 reverse phase NOVAPAK (Waters), l=15cm, i.d.=3.9cm column, was employed to measure the concentration of product(s) and substrate. A multistep gradient analysis was performed using two mobile phases: A (acetonitrile:water - 70:30) and B (water). The gradient elution was programmed in order to run from 100% A to 100% B. Flow rate was 1.2mL/min. The retention times were determined by comparison with pure compounds. Indomethacin was used as internal standard.

^{1}H NMR measurements: ^{1}H NMR spectra were measured on a Bruker AC 200 spectrometer using a multinuclear 5mm probe. 500μL of solution were used for all the measurements with a typical sample concentration of 4mg/mL. All the samples were prepared using non deuterated solvents except for the D_2O solution. The benzene signal was presaturated using a standard continuous wave irradiation. The *tert*-amyl alcohol spectra presented a broad signal relative to the solvent hydroxyl proton whose chemical shift was dependent on the temperature and the content of water: at low temperature this signal is overlapped with the -CH_2 resonance from the L-Serine-β-naphthylamide so that presaturation could not be applied. These two signals had different longitudinal relaxation times and the suppression was performed using an inversion recovery pulse sequence. A detailed baseline correction was necessary to clean the distorted residual component of the solvent hydroxyl signal. In the other solutions, no solvent suppression was used.

In all the spectra reported the -CH signal has been set arbitrary at 4.00ppm.

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