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Candida antarctica lipase B catalysed amidation of pyroglutamic acid derivatives. A reaction survey

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Abstract

Pyroglutamic acid esters, both (*S*)- and (*R*)-enantiomers, have been studied as substrates of the *Candida antarctica* lipase B catalyzed amidation in anhydrous organic solvents. They behaved as very good substrates when primary amines or ammonia were used as nucleophiles, affording the corresponding secondary and primary amides, respectively, but did not react with secondary amines. The reaction was enantioselective for the (*R*)-enantiomer of chiral amines although little kinetic difference was observed between (*S*)- and (*R*)-pyroglutamates as acyl donors. As an example of an infrequent reaction, free (*S*)-pyroglutamic acid may also act as a substrate of the reaction, but is much less reactive than its esters.

Keywords: Pyroglutamic acid derivatives; Enzyme catalysis; *Candida antarctica* lipase B; Aminolysis; Ammoniolysis

1. Introduction

Lipase-catalysed *N*-acylation in anhydrous organic solvents is a known but still infrequent reaction. Some functionalized ester groups such as α-halo [1], propiolic and acrylic [2], β-keto [3], β-hydroxy and epoxy [4], β-furyl and phenyl [5], have been successfully used as substrates but reports on α-amino acid esters as acyl donors of this reaction, besides the original Margolin and Klibanov [6] and Matos et al.'s [7] papers and those from our own group, are nearly unknown. A line of our research is aimed to study the influence of some structural features of the molecule on the activity and regioselectivity of the lipase catalysed aminolysis of dicarboxylic N-blocked α-amino acids substrates, centered on glutamic acid derivatives as a representative, cheap and easily available model [8]. We found that the N-protecting group of the L-glutamic acid diethylester displays an important effect on the reaction rate while α-regioselectivity remains unchanged [9]. As an extension of our study, we attempted the amidation of diethyl N-free glutamate under the same experimental conditions. The reaction was expected to be different because this simpler substrate lacks the steric and electronic interactions of the neutral N-blocking groups while it can form stronger acid–base interactions and hydrogen bonds. Our initial results [10] showed that it was a two-step

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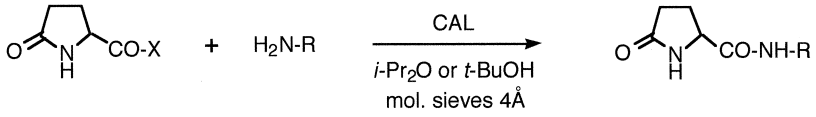
reaction: initially, a fast chemical cyclization to ethyl pyroglutamate occurred, followed by the enzyme-catalysed aminolysis of the newly formed cyclic derivative. This spontaneous cyclization is an already known reaction [11] although unexpected in experimental conditions as mild as a mere solution in anhydrous diisopropylether at 60°C. Then, we applied this reaction to obtain the benzyl (*S*)- and (*R*)-pyroglutamates from the dibenzylesters of N-free L- and D-glutamic acids (see Section 2). The enzymatic reaction displayed a poor enantioselectivity to the pyroglutamic acyl donor, L = (*S*)- or D = (*R*)-enantiomer [in all, the natural chiral amino acids except cysteine, the L-series corresponds to (*S*)-configuration and D- to (*R*). (*S*) and (*R*) notation is used in this paper for pyroglutamic derivatives], but a high reactivity when compared to N-protected glutamic derivatives [9].

Both (*S*)- and (*R*)-pyroglutamic acids [(*S*)- and (*R*)-2-pyrrolidine-5-carboxylic acids] and their ethyl esters are commercially available compounds [(*S*)-pyroglutamic acid is a very cheap product] in optically pure form. A few examples of their usefulness as chiral building blocks have been reported in the syntheses of pharmaceuticals [12] and natural products such as alkaloids [13], neurotoxins [14] and others [15]. Potentially, (*S*)-pyroglutamic derivatives could also be applied to resolve racemic mixtures by forming diastereomeric species or as the substrate of nucleophile-enantioselective enzymatic reactions. In this paper, we report a general survey on the enzymatic amidation of pyroglutamic derivatives. Several acyl donors and nucleophiles have been studied, both to investigate the limits of the reaction and their synthetic potential as well as a part of our research line of lipase catalysed amidation of dicarboxylic amino acid derivatives. *Candida antarctica* lipase B (CAL from here on) was the unique enzyme used in all reactions and, except ammoniolysis, all of them were performed at the same experimental conditions although the temperature and amount of enzyme were increased when the substrate was the less reactive free acid (Scheme 1). *N*-Methylacetanilide or *N*-methylbutyranilide were used as internal standards [9] in the kinetic studies.

2. Experimental

2.1. Analytical methods and materials

The structural assignment of all compounds was made according to their ^{1}H and $^{13}C-NMR$ spectra recorded in $CDCl_3$ solution [CD_3OD for (*S*)-pyroglutamic acid amide] using a Varian-Gemini-200 or a Varian XL-300 spectrometers. Elemental analyses were performed on a Perkin-Elmer 240C instrument. Melting points were determined with a Reichert-Jung apparatus equipped with a microscope and are uncorrected. Optical rotations were determined on a



(*S*) or (*R*)-**1** **4** (*S*) or (*R*)-**5a-d**

1 X = OEt
2 X = OBn
3 X = OH

a R = *n*-Pn
b R = *c*-Hx
c R = CH(Me)-Ph
d R = H

Scheme 1. Amidation of pyroglutamic acid derivatives with aliphatic primary amines or ammonia. No reaction was detected with a secondary amine.

Perkin-Elmer 241-C polarimeter. Analytical HPLC was performed on a Beckman Chromatograph (flow rate 1 ml/min) using a UV detector at $\lambda = 200$ nm. A Deltapak C_{18} 5 μ (3.9 × 15 mm) was employed with acetonitrile:H_2O (containing trifluoroacetic acid 0.5 ml/l) 80:20 as eluent. A Spherisorb S5CHI2 (250 × 4.6 mm) column was used for analysis involving chiral amines, *i*-PrOH:hexane 30:70 as eluent and $\lambda = 215$ nm. Analytical TLC was performed on aluminium sheets coated with a 0.2-mm layer of silicagel 60 F_{254} (Merck). Chromatographic separations were performed on columns using the flash chromatographic technique on silicagel 230–240 mesh (Merck). Diisopropylether was refluxed on sodium wire, distilled and stored on molecular sieves 4 Å. Amines were obtained from commercial sources, distilled and stored on KOH pellets. The rest of the commercial chemicals were used without further purification. Benzyl esters (*S*)- and (*R*)-**2** and minor amide (*S,S*)-**5c** were chemically synthesized by us. Novozym 435, a Novo Nordisk commercial immobilized preparation of CAL was used as received.

2.2. Chemical reactions

2.2.1. Benzyl (S)- and (R)-pyroglutamate *2*

Molecular sieves 4 Å (1 g) were added to solutions of L- or D-glutamic acid dibenzylester (130 mg) in diisopropylether (20 ml) and the resulting suspensions shaken in an orbital shaker at 250 r.p.m. at 60°C for 1 h. The solvent was evaporated and the residues chromatographed (EtAcO:MeOH 20:1), yielding benzyl (*S*)- or (*R*)-pyroglutamate. Benzyl (*S*)-pyroglutamate (*S*)-**2**: (76%) Colorless syrup $[\alpha]_D$: +3.8° (c = 1, $CHCl_3$). $^1H-$ NMR: 7.33 (m, 5H, aromatic); 6.75 (d, 1H, NH); 5.15 (s, 2H, CH_2–Bn); 4.25 (m, 1H, CH-α); 2.49–2.16 (m, 4H, CH_2-γ, CH_2-β). $^{13}C-$ NMR: 178.0 (CO-γ); 171.8 (CO-α);135.0, 128.6, 128.5, 128.3 (Ph); 67.2 (CH_2–Bz); 55.4 (C-α); 29.1 (C-γ); 24.7 (C-β). Benzyl (*R*)-pyroglutamate (*R*)-**2**: (81%) Colorless syrup $[\alpha]_D$: −6.1° ($c = 1$, $CHCl_3$). It showed the same ^1H and $^{13}C-$ NMR spectral data reported above for the (*S*)-enantiomer.

2.2.2. (S,S)-Methylbenzyl-pyroglutamic amide (S,S)-*5c*

$SOCl_2$ (2.8 ml) was added to a suspension of (*S*)-pyroglutamic acid (500 mg) in dry toluene (15 ml). After 2 h, the solvent was evaporated under vacuum and the resulting residue was dissolved again in dry CH_2Cl_2 (20 ml). Triethylamine (2.7 ml) was added to the solution and then (*S*)-methylbenzylamine (1 ml) dropwise. After 5 h, the reaction was stopped and the solvent evaporated. The residue was chromatographed (EtAcO:MeOH 20:1) to yield 40% of a colorless solid, m.p. 149°C, $[\alpha]_D$: −66.0° (c = 1, $CHCl_3$). $^1H-$ NMR: 7.29 (m, 5H, aromatic); 7.23–6.99 (m, 2H, α-NH, NH-amide); 5.07 (q, 1H, CH-amide); 4.05 (m, 1H, CH-α); 2.23 (m, 2H, CH_2-γ); 2.46–2.02 (m, 2H, CH_2-β); 1.44 (d, 3H, CH_3). $^{13}C-$ NMR: 179.6 (CO-γ); 171.1 (CO-α); 142.9, 128.6, 127.4, 126.3 (Ph); 57.1 (C-α); 29.3 (C-γ); 25.6 (C-β); 48.9 (CH-amide); 21.6 (CH_3).

2.3. Enzymatic reactions

2.3.1. Aminolysis of ethyl and benzyl pyroglutamates *1* and *2*

2.3.1.1. General procedure. The reactions were initially carried out at an analytical scale in screw-cap 2 ml vials: CAL (10 mg/ml) and 4 Å molecular sieves (50 mg/ml) were added to a diisopropylether solution of the pyroglutamic ester **1** or **2** (20 mM), *N*-methylacetanilide or butyranilide (5 mM) as an internal standard, and the corresponding amine (50 mM). The reactions were incubated at 45°C in an orbital shaker at 250 r.p.m. Aliquots (20 μl) were periodically withdrawn, evaporated to dryness, dissolved in acetonitrile, filtered off and analyzed by HPLC. The analysis of reactions with chiral amines were made with both a reverse phase and a chiral column. Control reactions without enzyme were also checked.

Preparative reactions were performed at a 0.64-mmol scale, using the above described experimental conditions except the absence of the internal standard. After conclusion, the enzyme and molecular sieves were filtered off and washed with acetonitrile. The combined organic extracts were evaporated to dryness and the residue recrystallized (*i*-Pr_2O:MeOH).

*2.3.1.2. N-Pentyl-(S)-pyroglutamic amide (S)-**5a***. (74%) Colorless solid, m.p. 88–89°C $[\alpha]_D$: −58.8° ($c = 1$, CH_2Cl_2). ^1H-NMR: 7.48 (s, 1H, α-NH); 6.80 (t, 1H, NH–Pn); 4.12 (m, 1H, CH-α); 3.21 (m, 2H, CH_2–NH); 2.30 (m, 2H, CH_2-γ); 2.52–2.09 (m, 2H, CH_2-β); 1.48 (m, 2H, NH–CH_2–CH_2; 1.34–1.22 (m, 4H, CH_2–CH_2–CH_3); 0.86 (t, 3H, CH_3). $^{13}C-NMR$: 179.5 (CO-γ); 172.2 (CO-α); 57.1 (C-α); 29.4 (C-γ); 25.7 (C-β); 28.95, 29.91, 22.20 (CH_2-amine); 39.5 (NH–CH_2); 13.8 (CH_3). Calc. (%) for $C_{10}H_{18}N_2O_2$: C: 60.58; H: 9.15; N: 14.13. Found (%): C: 60.48; H: 9.15; N: 13.95.

*2.3.1.3. N-Pentyl-(R)-pyroglutamic amide (R)-**5a***. (65%) Colorless solid, m.p. 90°C $[\alpha]_D$: +54.6° ($c = 1$, CH_2Cl_2). This compound showed the same ^1H and $^{13}C-NMR$ spectral data reported above for the (*S*)-enantiomer. Calc. (%) for $C_{10}H_{18}N_2O_2$: C: 60.58; H: 9.15; N: 14.13. Found (%): C: 60.72; H: 9.08; N: 14.03.

*2.3.1.4. N-Cyclohexyl-(S)-pyroglutamic amide (S)-**5b***. (75%) Colorless solid, m.p. 155°C $[\alpha]_D$: +8.0° ($c = 1$, CH_3OH). ^1H-NMR: 6.99 (d, 1H, α-NH); 6.19 (d, 1H, NH-cyclohexyl); 4.05 (m, 1H, CH-α); 3.69 (m, 1H, CH-cyclohexyl); 2.27 (m, 2H, CH_2-γ); 2.49-2.04 (m, 2H, CH_2-β); 1.88 (m, 2H, $(CH_2)_2$CH-cyclohexyl, eq); 1.69–1.51 (m, 3H, CH_2–CH_2–CH_2-cyclohexyl, eq); 1.29 (m, 2H, $(CH_2)_2$CH-cyclohexyl, ax); 1.21–1.03 (m, 3H, CH_2–CH_2–CH_2-cyclohexyl, ax). $^{13}C-NMR$: 179.3 (CO-γ); 171.0 (CO-α); 57.2 (C-α); 29.4 (C-γ); 25.9 (C-β); 48.5 (CH-cyclohexyl); 32.9, 32.8, 25.4, 24.8 (CH_2, cyclohexyl).

*2.3.1.5. N-Cyclohexyl-(R)-pyroglutamic amide (R)-**5b***. (64%) Colorless solid, m.p. 156°C $[\alpha]_D$: −9.1° ($c = 1$, CH_3OH). This compound showed the same ^1H and $^{13}C-NMR$ spectral data reported above for the (*S*)-enantiomer. Calc. (%) for $C_{11}H_{18}N_2O_2$: C: 62.82; H: 8.63; N: 13.32. Found (%): C: 62.71; H: 8.56; N: 13.05.

*2.3.1.6. N-(S,R)-Methylbenzyl-pyroglutamic amide (S,R)-**5c***. (36%) Colorless solid, m.p. 145°C $[\alpha]_D$: +9.2° ($c = 1$, $CHCl_3$). ^1H-NMR: 7.29 (m, 6H, aromatic, α-NH); 7.04 (d, 1H, J = 7.4, NH-amide); 5.11 (q, 1H, CH-amide); 4.15 (m, 1H, CH-α); 2.25 (m, 2H, CH_2-γ); 2.50–2.05 (m, 2H, CH_2-β); 1.49 (d, 3H, CH_3). $^{13}C-NMR$: 179.6 (CO-γ); 171.1 (CO-α); 142.9, 128.5, 127.3, 126.0 (Ph); 57.1 (C-α); 29.3 (C-γ); 25.6 (C-β); 48.8 (CH-amide); 21.6 (CH_3). Calc. (%) for $C_{13}H_{16}N_2O_2$: C: 67.22; H: 6.94; N: 12.06. Found (%): C: 67.03; H: 6.96; N: 11.98.

*2.3.1.7. N-(R,R)-Methylbenzyl-pyroglutamic amide (R,R)-**5c***. (52%) Colorless solid, m.p. 148–149°C $[\alpha]_D$: +71.3° ($c = 1$, $CHCl_3$). This compound showed the same ^1H and $^{13}C-NMR$ spectral data reported above for the (*S*)-enantiomer.

*2.3.2. Amidation of (S)-pyroglutamic acid (S)-**3***

Analytical reactions were carried out in screw-cap 2 ml vials, each containing CAL (50 mg), molecular sieves 4 Å (50 mg), acid (*S*)-**3** (4 mg, 0.03 mmol) and a solution of **4a** (50 mM) and *N*-methylacetanilide (5 mM) in anhydrous diisopropylether (1 ml). These vials were incubated at 60°C in an orbital shaker (250 r.p.m.). Instead of withdrawing aliquots, a whole vial was processed for each analysis: the enzyme and molecular sieves were filtered off, washed with boiling MeOH, the solvent of the combined organic solutions evaporated and the residue redissolved in MeOH:acetonitrile for HPLC analysis.

Preparative reactions were also performed at 60°C. CAL (300 mg) and molecular sieves 4 Å

(300 mg) were added to a suspension of (*S*)-**3** (24 mg) in a 50-mM solution of each amine in diisopropylether (6 ml). After 24 h, the pentylamine reaction was stopped while the rest were allowed to react for a total of 96 h. Following the same procedure as that for the analytical reactions, the final residue was chromatographed, first hexane:EtAcO 1:1 and then EtAcO:MeOH 20:1. The corresponding pentyl, cyclohexyl and methylbenzyl amides were obtained: (*S*)-**5a** (43%), (*S*)-**5b** (43%), and (*S,R*)-**5c** (37%). These compounds showed the same spectroscopic and physical characteristics as those obtained via aminolysis of the esters.

2.3.3. Ammoniolysis of ethyl (S)-pyroglutamate (S)-*1*

*2.3.3.1. (S)-Pyroglutamic acid amide (S)-**5d**.* NH_3-saturated *t*-BuOH (1 ml) was added to CAL (25 mg), 4 Å molecular sieves (50 mg), *N*-methylacetanilide (1.5 mg) and (*S*)-**1** (3 mg). A control reaction without enzyme was also carried out. Both reactions were incubated at 45°C at 250 r.p.m. and aliquots (20 μl) were periodically withdrawn, evaporated, dissolved in acetonitrile:MeOH, filtered off and analysed by HPLC and TLC. The blank reaction remained unchanged while the enzymatic one was completed in about 30 min. The preparative reaction was carried out under the same experimental conditions except for the absence of internal standard and with an initial amount of 90 mg of (*S*)-**1** and 30 ml of NH_3-saturated *t*-BuOH. After filtering the enzyme and sieves, washing with boiling MeOH and evaporating the solvent, the residue was chromatographed (acetone: MeOH 10:1). The amide (*S*)-**5d** was obtained in 60% yield (44 mg). Colorless solid, m.p. 156–158°C $[\alpha]_D$: $-15.4°$ ($c = 1$, CH_3OH). $^1H-$NMR: 5.07 (s, 3H, NH, NH_2); 4.39 (m, 1H, CH-α); 2.78–2.38 (m, 3H, CH_2-γ, CH-β_1); 2.27 (m, 1H, CH-β_2). $^{13}C-$NMR: 181.8 (CO-γ); 178.0 (CO-α); 58.2 (C-α); 30.8 (C-γ); 27.2 (C-β).

3. Results and discussion

3.1. Aminolysis of esters of (S)- and (R)-pyroglutamic acid

3.1.1. Achiral amines

Ethyl and benzyl (*S*)- and (*R*)-pyroglutamic acid esters **1** and **2** as acyl donors and two achiral aliphatic amines, **4a** and **4b**, as nucleophiles were examined. In spite of significant differences in structure, all six reactions proceeded at a high rate (Table 1). A secondary amine, dipropylamine, was also examined but no changes were detected after a 5-h period at 60°C, and using 50 mg/ml of enzyme while the other experimental conditions were unchanged.

The reaction exhibits some preference for the (*S*)-substrate but, in contrast with the N-protected glutamic acid esters, the enantioselectivity is rather poor. As expected from our experience, amino groups located on a secondary carbon atom are poorer as nucleophiles, and benzyl esters are poorer as acyl donors than the corresponding to amines located on a primary carbon atom and ethyl esters.

3.1.2. Chiral amines

In the first paper of this research line [8], we reported that the CAL-catalysed amidation of diethyl *N*-Cbz-L-glutamate exhibited an excel-

Table 1
Amidation[a] of esters (*S*)- and (*R*)-**1** and -**2** with achiral amines **4a,b**. Conversion[b] (%) into amides **5a,b**

Reaction	At 5 min (%)	> 98% (min)
(*S*)-**1** + **4a**	84	20
(*R*)-**1** + **4a**	24	45
(*S*)-**1** + **4b**	42	45
(*R*)-**1** + **4b**	25	> 60
(*S*)-**2** + **4a**	40	30[c]
(*R*)-**2** + **4a**	20	30[c]

[a] Ester (20 mM), amine (50 mM), CAL (10 mg/ml), mol. sieves 4 Å (50 mg/ml) in anhydrous diisopropylether at 45°C.
[b] Analytical scale, HPLC data using an internal standard.
[c] Conversion after 5, 10, 20 and 30 min for (*S*)-**2** + **4a**: 40, 68, 86 and > 98%; for (*R*)-**2** + **4a**: 20, 42, 72 and > 98%, respectively.

Table 2
Calculated molecular volumes and areas

Acyl group-H	Volume (Å^3)	Area (Å^2)
(*S*)-Pyroglutamyl	109.5	131.2
(*R*)-Pyroglutamyl	109.1	130.2
Cbz-L-Glu(OEt)	294.1	318.6
Cbz	109.8	143.1

lent enantioselectivity towards the nucleophile when it was a chiral amine such as α-methylbenzylamine. On the other hand, it is known [16] that the acyl moiety may influence the activity and nucleophile enantioselectivity of the reaction (stereochemical controller), as the final step of reactions catalysed by lipases and, in general, serine hydrolases, depends on the accessibility by the nucleophile (amine in this case) for the attack and cleavage of the acyl–serine bond inside the active site cavity. A big acyl moiety makes the cavity more crowded and enantiomerically restricted than a small one. Unlike the parent glutamic derivatives, the structure of pyroglutamic acyl group consist of a regular-shape small rigid ring without any attached polar side chain. Its calculated molecular volume (Table 2) [2] is smaller than that of the Cbz-L-Glu(OEt) acyl group, and similar to the Cbz group when compared with that of the N-blocking groups reported in Ref. [9] although the pyroglutamyl molecular area is smaller than the corresponding to the Cbz group. The whole pyroglutamyl moiety could be set in a hypothetical box measuring $3.94 \times 5.45 \times 3.05$ Å. So, its steric hindrance of the catalytic serine bond is probably very small and a lower nucleophile enantioselectivity than that of the acyclic glutamic derivatives could be expected.

In order to clear up this point, the usual experimental conditions were applied to the aminolysis of ethyl (*S*)-pyroglutamate **1** with (*R,S*)-α-methylbenzylamine **4c**. The reaction displayed an excellent enantioselectivity (Table 3) ($E \approx 100$) [17] at a higher rate than all the previously tested acyclic glutamic derivatives. From these results, we can conclude that the nucleophile enantioselectivity of the CAL-catalysed reactions is due to the enzyme narrow and well-defined channel to the active site [18], probably enhanced by a sterically precise conformation of the nucleophile needed to fit in the cavity in order to attack the acyl–serine bond [19]. The higher reaction rate shown by (*S*)-**1** suggests an easy passage across the acyl channel to reach the acyl site of the active pocket, described [18] as more spacious than the nucleophile one, and a lack of hindrances to reach the catalytic serine.

On the other hand, the sequential transition states are stabilised by polar amino acid residues located in precise points of the active site (oxyanion hole). In the case of CAL, the region surrounding the catalytic Ser105 is electronically defined by three residues (Thr40, Asp134 and Gln157) that, in addition to His224 of the catalytic triad, have their polar side chains within a 5-Å range from the O_γ of the active serine [20] and form the hydrogen bond network largely responsible for the acyl selectivity. The small volume and rigid shape of **1** allow it to reach the catalytic serine in a fast and efficient manner (high activity as a substrate) but, on the other hand, it is too small to fit precisely in the

Table 3
Amidation[a] of enantiopure and racemic esters **1** with chiral amines **4c**. Conversion[b] (%) into amides **5c**

React./Prod.	5 min	30 min	45 min	60 min
(S)-1* + *(R,S)-4c				
(*S,R*)-**5c**	12.7	48.0	53.0	54.9
(*S,S*)-**5c**	n.d.	< 1	1.3	1.7
(S,R)-1* + *(R)-4c				
(*S,R*)-**5c**	11.5	33.6	41.9	48.7
(*R,R*)-**5c**	3.7	12.4	13.5	14.1

[a] Ester (20 mM), amine (50 mM), CAL (10 mg/ml), mol. sieves 4 Å (50 mg/ml) in anhydrous diisopropylether at 45°C.
[b] Analytical scale, HPLC data with internal standard.

[2] Calculated using SYBIL 6.3 Molecular Modelling Software (Tripos Associates, 1699 South Hanley Road, St. Louis, MO 63144, USA) after a complete geometric optimization following the AM1 semiempirical method.

active site and both (*S*)- and (*R*)-enantiomers can reach and bond the catalytic serine with little differences of energy and the consequent lack of acyl enantioselectivity. The amidation of racemic **1** with (*R*)-**4c** under the usual experimental conditions afforded the amide **5c** with a modest enantioselectivity (Table 3) ($E = 11$).

3.2. Amidation of free (S)-pyroglutamic acid

Amidation of carboxylic acids catalysed by lipases in organic solvents is a very infrequently reported reaction [21,22]. Free acids are not convenient substrates of lipase-catalysed amidations because they are less reactive and less soluble in organic solvents than their esters. In addition, they form salts with amines, the nucleophile of this reaction, and it may be expected that the carboxylate anion will interact with the polar residues of the tunnel and active site in an undesired way, inhibiting the reaction.

Free (*S*)-pyroglutamic acid **3** was checked as a substrate for amidation by **4a** in anhydrous diisopropylether catalysed by CAL [3] (temperature and amount of enzyme used were higher than with esters) and, although in a slower manner than its ethyl ester (time was measured in hours instead of minutes), it did proceed and the corresponding amide (*S*)-**5a** was formed (TLC, EtAcO:MeOH 20:1, and HPLC). Kinetic studies of this reaction were difficult to carry out because the free acid and the probably formed salt are not soluble in the organic media used. So, each aliquot had to be obtained from a whole reaction vial, evaporated, the residue extracted with boiling methanol and then analysed (Table 4).

When the reactions were performed at a preparative scale with the amines **4a–c**, the corresponding amides (*S*)-**5a** (1 day, 43%), (*S*)-**5b** (4 days, 43%) and (*S,R*)-**5c** (4 days, 37%), identical to the samples afforded by the ester reactions, were obtained. No amide formation was detected in blank reactions without enzyme. Although not interesting as a synthetic method, at least when compared with its esters, it is remarkable from the enzyme catalysis point of view that free pyroglutamic acid is able to work as a substrate of lipase-catalysed amidations.

Table 4
Amidation[a] of (*S*)-pyroglutamic acid (*S*)-**3** with *n*-PnNH$_2$ **4a**. Conversion[b] (%) into **5a**

Time (h)	0.5	1.5	5.5	9.5	24
Conversion (%)	< 1	2.3	10.5	15.6	32.6

[a]Acid (20 mM), amine (50 mM), CAL (50 mg/ml), mol. sieves 4 Å (50 mg/ml) in anhydrous diisopropylether at 60°C.
[b]Analytical scale, HPLC data with internal standard.

3.3. Ammoniolysis of ethyl (S)-pyroglutamate

Another still unusual reaction catalysed by lipases is the ammoniolysis of esters, that is, the use of ammonia as nucleophile. Little more [23] has been added to the initial reports beyond recent work by García et al. [3] and de Zoete et al. [24]. The reaction was initially performed by us at an analytical scale with (*S*)-**1** and NH_3-saturated *t*-BuOH catalysed by CAL at 35°C and controlled by TLC (acetone:MeOH 10:1). After 30 min, the ester had disappeared while a new spot, not corresponding to the free acid, arose as the only product. After a preparative scale reaction, it was identified as the primary amide (*S*)-**5d**. A blank reaction without enzyme remained unchanged after 1 h.

4. Conclusions

Pyroglutamic esters are highly reactive substrates in CAL-catalysed aminolysis reactions with primary amines and displaying a good enantioselectivity for the (*R*)-enantiomer of the chiral ones. As (*S*)-pyroglutamic acid and its

[3] Lipases PS and AP-6 (Amano), Lipozyme IM20 (Novo Nordisk) and *C. rugosa* and porcine pancreatic lipases (Sigma) were also checked but no reaction was detected after a 3-day period at 45°C and 50 mg/ml of lipase.

ethyl ester are inexpensive and commercially available in optically pure form, the work reported here is a convenient method to synthesize optically pure amides as chiral building blocks or to resolve racemic amines via the formation of the diastereomerically pure (S,R)-amide. Esters may also afford the primary amide when ammonia instead of amines is used as the nucleophile and even the free acid is reactive enough to undergo amidation with amines.

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