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Enantioselective enzyme catalysed ammoniolysis of amino acid derivatives. Effect of temperature

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Abstract—The lipases from *Candida antarctica* (B type), *Thermomyces lanuginosus* and *Pseudomonas alcaligenes* catalysed the enantioselective ammoniolysis of free amino acid esters. In the ammoniolysis of phenylalanine methyl ester catalysed by *T. lanuginosus* lipase a decrease in temperature to –20°C significantly enhanced the enantioselectivity up to an enantiomeric ratio (E) of 84. Several proteases efficiently catalysed the ammoniolysis of *N*-BOC protected amino acid esters with nearly absolute enantioselectivity.

1. Introduction

Since the first reports by us[1,2] and Gotor et al.,[3] the number of publications on the ammoniolysis of carboxylic esters catalysed by lipases has substantially increased.[4] We recently showed[5] that the enantioselectivity of the *Candida antarctica* lipase B (CaLB) catalysed ammoniolysis of phenylglycine methyl ester **1** increased substantially on lowering the temperature of the reaction. The enantiomeric ratio (E) increased from 17 at 40°C to 52 at –20°C. We now present a more detailed study of the effect of temperature on the ammoniolysis of three structurally related amino acid esters catalysed by three lipases from widely different sources. We also report our further investigation of the enantioselective ammoniolysis of two *N*-*tert*-butoxycarbonyl (*N*-BOC) protected amino acid esters.

Variation of the temperature to enhance the enantioselectivity has been known for years,[6] but the use of temperatures below 0°C in enzymatic reactions is not common because most enzymes are not active at these temperatures. Lipases, in particular *Pseudomonas cepacea* lipase, are an exception, and have been reported to maintain their activity even at temperatures as low as –60°C.[7]

The enantiomeric ratio E[8] in enzymatic kinetic resolutions is often temperature dependent.[9,10] This can be explained by Eqs. (1)–(3) where $\Delta H^{\ddagger}$ is the enthalpy of activation, $\Delta S^{\ddagger}$ the free entropy of activation and $\Delta G^{\ddagger}$ the free energy of activation.

$$\Delta\Delta G^{\ddagger} = -RT \ln E \tag{1}$$

and

$$\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger} \tag{2}$$

Hence,

$$\ln E = \Delta\Delta S^{\ddagger}/R - \Delta\Delta H^{\ddagger}/(RT) \tag{3}$$

Another relevant parameter is the racemic temperature (T_r in Eq. (4)), at which there is no discrimination of the enantiomers.

$$T_r = \Delta\Delta H^{\ddagger}/\Delta\Delta S^{\ddagger} \tag{4}$$

Studies on the effect of the temperature on the enantioselectivity of lipases are few[7,11] and, with a single exception,[5] concern the enantiodiscrimination of alcohols.

2. Results and discussion

2.1. Ammoniolysis of α-amino esters with lipases

We have investigated the ammoniolysis of the methyl esters of phenylglycine **1**, phenylalanine **2** and homophenylalanine **3** (see Fig. 1). They form a series of three amino acid esters in which the phenyl group is separated from the stereogenic centre by zero, one and two methylene groups, respectively.

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The reactions were carried out in sealed 2 mL vials. Enzyme and zeolite NaA were added to a solution of the free amino acid ester in *tert*-butanol saturated with ammonia. Reactions at temperatures below 20°C were performed with addition of ammonia saturated *tert*-butyl methyl ether (TBME) to avoid solidification of the reaction medium.

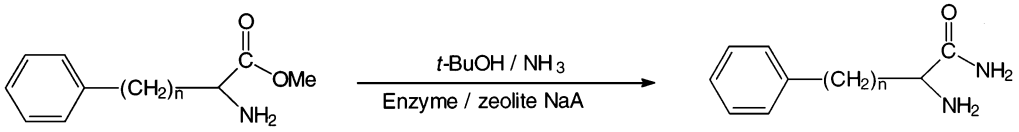
Besides *C. antarctica* lipase B (CaLB, Novo SP611), we have used the lipases from *Thermomyces lanuginosus* (TlL, Novo SP398) and *Pseudomonas alcaligenes* (PaL). All were immobilised on Accurel EP100.[12] Taking the ammoniolysis of **2** as a test reaction, a number of other lipases, as well as related hydrolases[†] proved to be inactive. The effect of the temperature on the ammoniolysis of **1**–**3** in the presence of the three lipases mentioned above was investigated (Tables 1–3).

CaLB showed a preference for the (*R*)-enantiomer in all reactions (Table 1). The *E* value of the reaction of **1** and **3** increased at low temperature, the reaction of **1** being the most selective one. The ammoniolysis of **2** in the presence of this enzyme proceeded with very low enantioselectivity ($E \sim 1$) and no significant improvement at −10°C was detected.

This order of enantioselectivity could be explained based on the notion that the more inefficient the interaction of the (*S*)-enantiomer with the active site the higher the (*R*)-enantioselectivity. The high reactivity and low enantioselectivity of **2** at all temperatures suggests that (*S*)-**2** (as well as (*R*)-**2**) is a good substrate with a favourable orientation. In the case of (*S*)-**1**, the phenyl group cannot adopt this orientation (Fig. 2), resulting in a less effective fit in the active site. Consequently, the enantioselectivity towards (*R*)-**1** would be enhanced. In the case of (*S*)-**3**, the molecule is more flexible, and the phenyl group could adopt a closer orientation to that of (*S*)-**2**, but not the same. This species would then interact less efficiently than (*S*)-**2** (higher enantioselectivity towards the (*R*)-enantiomer than in the case of **2**), but more than (*S*)-**1** (lower (*R*)-enantioselectivity than in the case of **1**).

TlL and PaL, which did not accept **1** as substrate, preferentially converted the (*S*)-enantiomers of **2** and **3** (Tables 2 and 3); *E* was higher in the ammoniolysis of **2** than in that of **3**. Enhanced *E* factors resulted when the reactions were performed at −20°C, even reaching a value of 84 in the ammoniolysis of **2** catalysed by TlL. This lipase acted more enantioselectively than PaL in the ammoniolysis of both **2** and **3**. The ammoniolysis of **2** catalysed by PaL did not show an increase in selectiv-

[†] *C. rugosa* lipase, *R. arrhizous* lipase, cholesterol esterase from *C. cylindracea*, porcine pancreas lipase, amino acylase I from *A. melleus*, penicillin acylase from *E. coli* and the proteases SP458, SP539 (serine type endoprotease), Maxatase (alkaline protease, subtilisin, from *Bacillus licheniformis*), Maxacal (high alkaline protease, subtilisin, from *Bacillus alkalophilus*), chymotrypsine and trypsine.



n = 0 **1**
n = 1 **2**
n = 2 **3**

n = 0 **4**
n = 1 **5**
n = 2 **6**

Figure 1. Enzyme catalysed ammoniolysis of **1**–**3**.

Table 1. CaLB catalysed ammoniolysis of **1**–**3** at different temperatures[a]

Substrate	Selectivity	Temp. (°C)	Time (h)	Conv. (%)	Ee_P (%)	E[b]
1[c]	*R*	40		46	78	16
1[c]	*R*	−20		33	94	52
2	*R*	40	0.5	46	7	~1
2	*R*	20	0.5	37	13	~1
2	*R*	4	3	24	11	~1
2	*R*	−10	3	15	13	~1
3	*R*	40	0.5	29	56	4
3	*R*	20	0.5	18	62	5
3	*R*	4	5	41	65	7
3	*R*	−10	5	33	74	9
3	*R*	−20	5	16	80	10
3	*R*	−30	24	18	86	15

[a] Reaction conditions: substrate 50 mM solution in ammonia saturated *tert*-butyl alcohol, 50 mg mL^{-1} zeolite NaA, 50 mg mL^{-1} enzyme. TBME (30%) was added in reactions below 20°C.
[b] Calculated as $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$.
[c] Data from Ref. 5.

Table 2. TlL catalysed ammoniolysis of **2** and **3** at different temperatures[a]

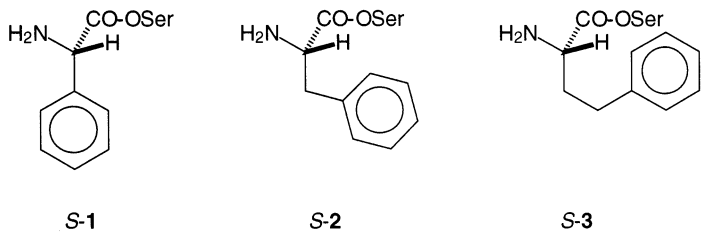
Substrate	Selectivity	Temp. (°C)	Time (h)	Conv. (%)	Ee_P (%)	E[b]
2	*S*	40	1	8	84	13
2	*S*	20	1	11	89	20
2	*S*	4	5	29	91	30
2	*S*	−10	24	40	93	52
2	*S*	−20	5	8	97	84
3	*S*	40	5	29	68	6
3	*S*	20	5	18	72	7
3	*S*	4	5	41	81	11
3	*S*	−10	5	33	83	13
3	*S*	−20	5	16	87	15

[a] Reaction conditions: substrate 50 mM solution in ammonia saturated *tert*-butyl alcohol, 50 mg mL^{-1} zeolite NaA, 50 mg mL^{-1} enzyme. TBME (30%) was added in reactions below 20°C.
[b] Calculated as $E=\ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$.

Table 3. PaL catalysed ammoniolysis of **2** and **3** at different temperatures[a]

Substrate	Selectivity	Temp. (°C)	Time (h)	Conv. (%)	Ee_P (%)	E[b]
2	*S*	40	1	23	65	6
2	*S*	20	1	21	67	6
2	*S*	4	5	45	56	6
2	*S*	−20	24	26	76	9
3	*S*	40	1	7	26	~1
3	*S*	20	1	7	32	2
3	*S*	4	5	14	50	3
3	*S*	−20	5	4	69	6

[a] Reaction conditions: substrate 50 mM solution in ammonia saturated *tert*-butyl alcohol, 50 mg mL^{-1} zeolite NaA, 50 mg mL^{-1} enzyme. TBME (30%) was added in reactions below 20°C.
[b] Calculated as $E=\ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$.



S-1 *S*-2 *S*-3

Figure 2. Model of possible conformations of the (*S*)-enantiomers of **1**–**3**.

ity at moderately low temperatures. However, at −20°C, the reactivity of the enzyme decreased considerably and an increase in enantioselectivity was then detected. In the case of **3**, the decrease in reactivity and increase in enantioselectivity at lower temperatures were smoother.

Finally, $\ln E$ was plotted against $1/T$ (Eq. (1)). In general, a good linear correlation was obtained (Figs. 3–5). The ammoniolysis of **2** in the presence of PaL was an exception, and the data points could not be fitted to a straight line; E remained constant from 40 to 4°C and improved only at −20°C (Fig. 5).

The differential activation parameters $\Delta\Delta H^{\ddagger}$ and $\Delta\Delta S^{\ddagger}$ as well as the racemic temperature were calculated, using Eq. (1), from the data in Figs. 3–5; they are summarised in Table 4. The differential activation enthalpy ($\Delta\Delta H^{\ddagger}$) is the major contributor to $\Delta\Delta G^{\ddagger}$ in all cases; hence, the enantioselectivity is enhanced at low temperatures. This parameter is related to the steric interactions of the enantiomers with the active site. The values for $\Delta\Delta S^{\ddagger}$ give an indication of losses in rotational and translational freedom caused by interaction with the system.

2.2. Ammoniolysis of α-amino acid esters with proteases

The free amino acid esters **1**–**3** were not converted by any of the proteases tested, as noted above. Presumably, an acylation of the amino group is required to

ensure proper binding of the reactant in the S_2 subsite.[13] We previously showed that proteases can catalyse the ammoniolysis of *N*-protected α-amino acid esters (in particular *Z*-amino acid esters) with high enantioselectivity.[14] In a continuation of this study, we have now performed the ammoniolysis of the *N*-*tert*-butoxycarbonyl (*N*-BOC) methyl esters of phenylalanine **7** and homophenylalanine **8** (see Fig. 6). We surmised that the BOC group could enhance the enantioselectivity due to its steric bulk. Moreover, the BOC group is commonly used in organic chemistry as it can be very easily removed by treatment with mild acid.

The reactions were carried out in sealed 2 mL vials at 40°C. The protease (immobilised on Accurel EP100) and zeolite NaA were added to a solution of the *N*-BOC amino acid ester in *tert*-butyl alcohol containing ammonia (see Section 4). The results are summarised in Table 5.

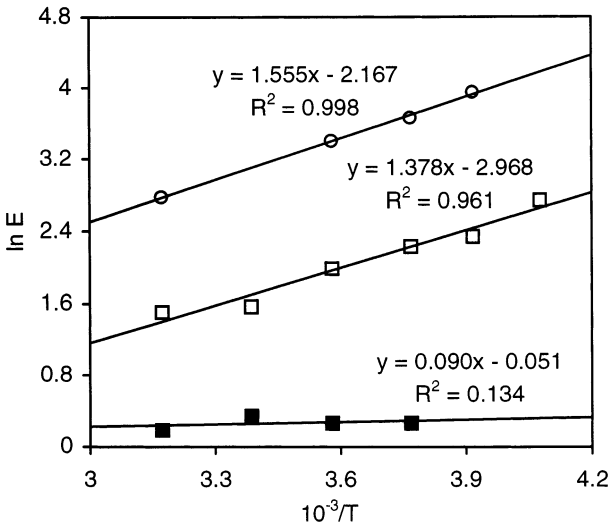


Figure 3. Plot of ln *E* versus $1/T$ for CaLB catalysed ammoniolysis of **1** (○), **2** (■) and **3** (□).

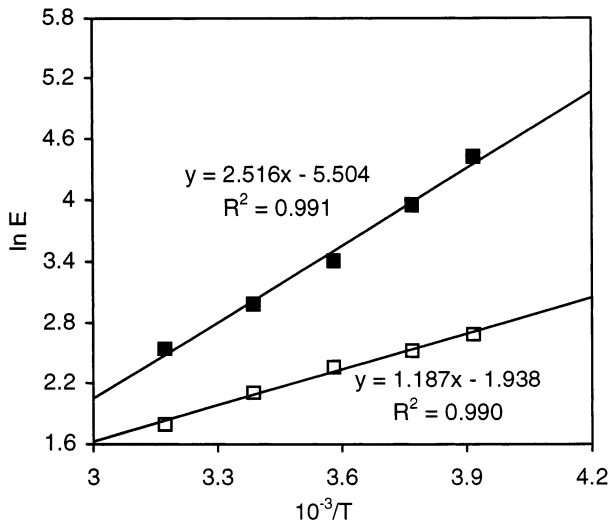


Figure 4. Plot of ln *E* versus $1/T$ for TlL catalysed ammoniolysis of **2** (■) and **3** (□).

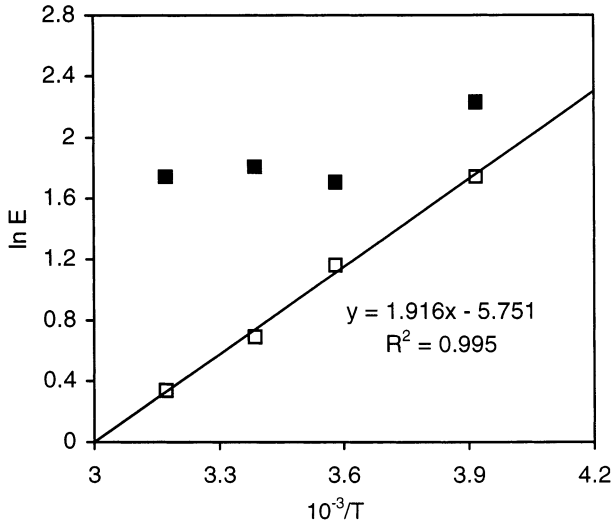


Figure 5. Plot of ln *E* versus $1/T$ for PaL catalysed ammoniolysis of **2** (■) and **3** (□).

Table 4. Calculated differential thermodynamic parameters for the ammoniolysis of **1**–**3**[a]

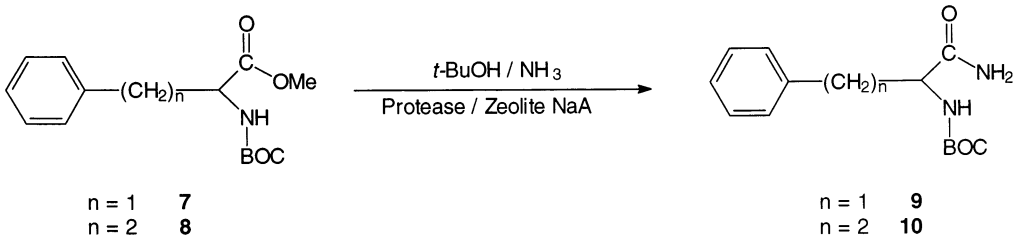
Lipase	Substrate	$\Delta\Delta S^{\ddagger}$ (cal K^{-1} mol^{-1})	$\Delta\Delta H^{\ddagger}$ (kcal mol^{-1})	T_r (°C)
CaLB	**1**	−4.30	−3.09	443
CaLB	**2**	−0.10	−0.18	1525
CaLB	**3**	−5.89	−2.73	188
TlL	**2**	−10.93	−5.00	182
TlL	**3**	−3.85	−2.36	337
PaL	**3**	−11.42	−3.80	57

[a] Difference in $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ of the fast reacting enantiomer minus the slow reacting enantiomer.

All reactions showed an extraordinarily high (*S*)-selectivity. No traces of the (*R*)-amide were detected except for the reaction of **7** with SP539. The presence of the blocking BOC group gave, in general, higher enantioselectivities than the Z group.[14] In all cases **7** was a better substrate than **8**, the reactions of **8** being very slow, with a conversion of only 10% in 24 hours in the best case (Maxatase). Since these reactions were all highly enantioselective at room temperature, we did not examine the effect of decreasing the temperature on enantioselectivity.

3. Conclusions

Lipases of *C. antarctica* (Type B), *T. lanuginosus* and *P. alcaligenes* catalyse the enantioselective ammoniolysis of free α-amino esters. The enantiorecognition is generally improved at low temperatures, reaching an *E* of 84 at –20°C in the ammoniolysis of phenylalanine catalysed by TlL. CaLB was *R* selective whereas TlL and PaL were *S* selective. Proteases catalysed the (*S*)-enantioselective ammoniolysis of *N*-BOC protected α-amino acid esters with practically total enantioselectivity. Free amino esters were not substrates of proteases.



n = 1 **7**
n = 2 **8**

n = 1 **9**
n = 2 **10**

Figure 6. Protease catalysed ammoniolysis of **7** and **8**.

Table 5. Protease catalysed ammoniolysis of **7** and **8**[a]

Substrate	Protease	Selectivity	Time (h)	Conv. (%)	E^b	E^c
7	Maxatase	*S*	2	15	≫100	54
7	Maxacal	*S*	2	5	≫100	8
7	SP539	*S*	2	7	65	89
7	SP458	*S*	2	1		
8	Maxatase	*S*	24	10	≫100	
8	Maxacal	*S*	24	<1		
8	SP539	*S*	24	3	≫100	
8	SP458	*S*	24	<1		

[a] Reaction conditions: substrate 50 mM solution in *tert*-butyl alcohol–ammonia saturated *tert*-butanol (9:1, v/v), 50 mg mL^{-1} zeolite NaA, 30 mg mL^{-1} enzyme, 40°C.
[b] Calculated as $E=\ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$.
[c] E values for the ammoniolysis of *N*-*Z*-PheOMe (Ref. 14).

4. Experimental

4.1. Materials and methods

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ solution with TMS as internal standard using a 400 MHz Varian-VXR 400S spectrometer. The reactions were monitored by chiral HPLC on a Daicel Chemical Industries Ltd 4.6×150 mm 5 μ Crownpak CR (+) column using a Waters 625 pump and a Waters 486 UV detector operating at 215 nm. The eluent was aqueous $HClO_4$ at a flow rate of 0.6 mL min^{-1}. Details about pH and temperature are specified for each case. Solvents were dried over zeolite CaA (Uetikon, activated at 400°C for 24 hours). All substrates and final products used as standards were obtained from common commercial sources except homophenylalanine methyl ester and homophenylalanine amide, which were synthesised (see below). All enzymes were immobilised on Accurel EP100 according to a published procedure.[12] SP539, SP458 and SP398 were kindly donated by Novo Nordisk; *C. antarctica* lipase B on Accurel EP100 was received from Uniqema as a gift. *P. alcaligenes* lipase, penicillin acylase from *E. coli*, Maxacal and Maxatase were obtained from Gist-brocades (now DSM-Life Sciences), as gifts. *R. arrhizus* lipase, chymotrypsine, trypsine and cholesterol esterase were gifts from Roche Diagnostics. *C. rugosa* lipase and porcine pancreas lipase were purchased from Sigma; amino acylase I from *A. melleus* was from Fluka.

4.2. Synthesis of substrates and standards

4.2.1. Homophenylalanine methyl ester 3. Hydrogen chloride was bubbled through a dispersion of homophenylalanine (710 mg) in methanol (60 mL) for 30 minutes and stirred at room temperature for 16 hours. The solvent was evaporated in vacuo. Then $NaHCO_3$ saturated aqueous solution (50 mL) was added and the mixture extracted with dichloromethane (3×30 mL). The organic layer was washed with water (50 mL), dried over anhydrous Na_2SO_4 and evaporated. Homophenylalanine (61%) was obtained as a colourless liquid. ^{1}H NMR: 7.23 (m, 5H, Ar); 3.70 (s, 3H, CH_3); 3.48 (m, 1H, CH-α); 2.78 (m, 2H, CH_2-γ); 2.01 (d, 2H, NH_2); 2.08 (m, 1H, CH_2-β_1); 1.87 (m, 1H, CH_2-β_2). ^{13}C NMR: 176.18 (CO); 141.16, 128.47, 128.45, 126.05 (Ar); 53.85 (CH-α); 52.02 (CH_3); 36.25 (CH_2-γ); 31.90 (CH_2-β).

4.2.2. Homophenylalanine amide 6. Homophenylalanine methyl ester (300 mg) was added to saturated aqueous ammonium hydroxide solution (20 mL) and stirred at room temperature for 24 hours. Water (30 mL) was added and the mixture was extracted with dichloromethane (3×30 mL). The organic solvent was evaporated in vacuo to afford homophenylalanine amide (95%) as a white solid. ^{1}H NMR: 7.26 (m, 5H, Ar); 7.04 (s, 1H, $CONH_2$), 5.86 (s, 1H, $CONH_2$); 3.37 (m, 1H, CH-α); 2.74 (m, 2H, CH_2-γ); 2.18 (m, 1H, CH_2-β_1); 1.84 (m, 1H, CH_2-β_2); 1.57 (s, 2H, CH-NH_2).

^{13}C NMR: 178.07 (CO); 141.12, 128.52, 128.43, 126.11 (Ar); 54.70 (CH-α); 36.62 (CH_2-γ); 32.09 (CH_2-β). Mp 90–91°C.

4.2.3. *N*-BOC-phenylalanine methyl ester 7. Phenylalanine methyl ester (666 mg, 3.72 mmol) was dissolved in anhydrous dichloromethane (15 mL). Triethylamine (1.03 mL, 7.44 mmol) was added, the mixture was cooled in an ice-water bath and then a solution of di-*tert*-butyldicarbonate (487 mg, 2.23 mmol) in dichloromethane (10 mL) was added dropwise. The reaction mixture was left stirring at room temperature for 17 hours and then washed with 0.1N HCl (2×20 mL), $NaHCO_3$ saturated solution (20 mL) and finally water (30 mL). The product **7** was obtained as a white solid (520 mg, 50%). ^{1}H NMR: 7.32–7.11 (m, 5H, Ar); 4.98 (d, 1H, NH); 4.58 (m, 1H, CH-α); 3.71 (s, 3H, OCH_3); 3.08 (m, 2H, CH_2-β); 1.41 (s, 9H, CH_3-BOC). ^{13}C NMR: 172.36 (*CO*OMe); 155.08 (CO-BOC); 136.03, 129.30, 128.55, 127.03 (Ar); 79.91 ($C(CH_3)_3$); 54.43 (CH-α); 52.19 (CH_3O); 38.37 (CH_2-β); 28.29 (CH_3-BOC). Mp 46°C.

4.2.4. *N*-BOC-homophenylalanine methyl ester 8. Following the same procedure described for **7**, from homophenylalanine (719 mg), the methyl ester **8** was obtained as a white solid (625 mg, 57%). ^{1}H NMR: 7.32–7.14 (m, 5H, Ar); 5.10 (d, 1H, NH); 4.35 (m, 1H, CH-α); 3.71 (s, 3H, OCH_3); 2.67 (m, 2H, CH_2-γ); 2.14 (m, 1H, CH_2-β_1); 1.95 (m, 1H, CH_2-β_2); 1.45 (s, 9H, CH_3-BOC). ^{13}C NMR: 173.17 (*CO*OMe); 155.36 (CO-BOC); 140.77, 128.49, 128.41, 126.16 (Ar); 79.94 ($C(CH_3)_3$); 53.25 (CH-α); 52.02 (CH_3O); 34.37 (CH_2-γ); 31.64 (CH_2-β); 28.32 (CH_3-BOC). Mp 102°C.

4.3. Enzymatic reactions

4.3.1. General procedure for lipase ammoniolysis. *tert*-Butyl alcohol and TBME were saturated with ammonia by bubbling the gas through the dried solvent for 1 hour. The reactions were carried out in 2 mL vials at 1 mL scale. To a solution of the substrate 50 mM in *tert*-butanol saturated with ammonia, 50 mg enzyme and 50 mg zeolite NaA powder were added. For reactions at 4°C and lower, 30% TBME saturated with ammonia was added to avoid solidification of the reaction medium. At the specified time for each analysis the vial was damped in 9 mL perchloric acid aqueous solution (pH 1) and shaken for 2 minutes. An aliquot (200 μL) was withdrawn and added to 200 μL internal standard solution in the same solvent and analysed by chiral HPLC (conditions specified for each case).

4.3.2. General procedure for protease ammoniolysis. The reactions were carried out in 2 mL vials at 1 mL scale. To a solution of the substrate (50 mM) in *tert*-butanol–*tert*-butanol saturated with ammonia (9:1, v/v), protease immobilised on Accurel EP100 (30 mg) and zeolite NaA (50 mg) were added. Reactions with free amino esters were performed following the same procedure described for lipases. For reactions with *N*-BOC protected amino acid esters, at the specified time for each analysis the vial was damped in 1N HCl solution (9 mL) and shaken at 40°C for 1 hour, when deprotection of the amino group was complete. An aliquot was then withdrawn and the same procedure as in the case of lipases was followed.

4.3.3. Ammoniolysis of 1. Following the general procedure for lipases, (*R*)-phenylalanine amide in the same concentration as the substrate was used as standard. HPLC conditions: perchloric acid solution pH 1, 35°C.

4.3.4. Ammoniolysis of 2 and 7. Following the general procedure for lipases and proteases, (*R*)-phenylglycine amide in the same concentration as the substrate was used as standard. HPLC conditions: perchloric acid solution pH 1, 35°C.

4.3.5. Ammoniolysis of 3 and 8. Following the general procedure for lipases and proteases, (*R*)-phenylglycine amide in the same concentration as the substrate was used as internal standard. HPLC conditions: perchloric acid solution pH 2, 49°C.

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