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Acylation of β-Amino Esters and Hydrolysis of β-Amido Esters: *Candida antarctica* Lipase A as a Chemoselective Deprotection Catalyst

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N-Acylation by lipase A from *Candida antarctica* (CAL-A) in ethyl butanoate was applied to the kinetic resolution of *tert*-butyl esters of 3-amino-3-phenylpropanoic acid ($E > 100$), 3-amino-4-methylpentanoic acid ($E > 100$) and 3-aminobutanoic acid ($E = 60$) on 1.0–2.0 M scale. With the N-acylated resolution products, the exceptional ability of CAL-A to hydrolyse amides and bulky *tert*-butyl esters was then studied. In all N-acylated *tert*-butyl esters, chemoselectivity favoured the amide bond cleavage. The *tert*-butyl ester bond was left intact with 3-amino-3-phenylpropanoate, whereas with 3-amino-4-methylpentanoate and 3-aminobutanoate the CAL-A-catalysed hydrolysis of *tert*-butyl ester followed the amide hydrolysis.

Introduction

The serine hydrolase superfamily includes enzymes, such as lipases (EC 3.1.1.3) and endopeptidases (EC 3.4.21.–), which are pivotal for life. The natural function of lipases is in digestion where they mainly hydrolyse ester bonds of triacylglycerols. Endopeptidases, in turn, typically hydrolyse amide bonds in various physiological processes like in cell signalling and destruction, virulence and immune response, in addition to their function in digestion.[1] Apart from the different functions of serine hydrolase enzymes, their catalytic triad, consisting of Ser, His and Asp (or Glu) residues, provides them with the potential to catalyse reactions by the formation and cleavage of both ester and amide bonds through similar reaction mechanisms. However, only restricted number of lipases (lipases from *Candida rugosa*,[2a] *Aspergillus niger*,[2b] *Rhizomucor miehei* and *Pseudomonas stutzeri*,[2c] *Pseudomonas aerigunosa*,[2d] and lipases A (CAL-A)[2e,f] and B (CAL-B)[2f–n] from *Candida antarctica*) have been reported to cleave amide bonds, although usually very slowly. The mechanistic difference between the two enzyme types was previously proposed to reside in the transition-state-stabilising hydrogen bond at the amide hydrogen of endopeptidases, which is lacking in lipases.[3]

Enantiomers of β-amino acids are important structural units of peptides, peptidomimetics and many biologically active natural products.[4] Among biocatalytic methods used for their preparation, lipase-catalysed kinetic resolution of racemic β-amino esters by N-acylation in organic solvents has been thoroughly studied and reviewed.[5] If successful, the method produces one enantiomer as an N-acyl ester **2** (protocol I, Scheme 1) and the other enantiomer as the unreacted β-amino ester enantiomer **1**. In addition to N-acylations, the kinetic resolution of racemic β-lactams by enantioselective ring hydrolysis and the hydrolysis of β-amino esters are well reported in the presence of lipases.[6] As a mechanistic difference, the β-amino acid derivative binds at different pockets of the enzyme in N-acylation of a β-amino ester and hydrolysis of a β-lactam or a β-amino ester. Thus, for lipase-catalysed N-acylation, an achiral acyl donor (for instance ethyl butanoate) is bound at the acyl binding site forming an acyl–enzyme intermediate with the serine hydroxyl of the enzyme, and the β-amino ester as an amine nucleophile reacts with the intermediate in the second mechanistic step. For the hydrolysis of a β-lactam or a β-amino ester, these molecules themselves are bound at the acyl binding site as acyl donors, and water acts as a nucleophile in the second step.

We have long been interested in the exceptional properties of CAL-A among lipases. CAL-A is a chemoselective N-acylation catalyst, for instance, for the acylation of ethyl 3-aminobutanoate with carboxylic acid esters, whereas many other lipases tend to form multiple products through both N-acylation and interesterification.[7] As CAL-A has already shown interesting amidase activity,[2e,f] the present paper focuses on attaining a deeper insight into the utility of CAL-A on this activity. CAL-A was previously shown to contain two active forms,[2e] the native enzyme and the one possibly lacking the active site flap[8] that usually restricts access to the active site. Both CAL-A forms were shown to catalyse the amide hydrolysis of ethyl 3-butanamidobutanoate.[2e] In addition, CAL-A-CLEA preparation was previously used for deprotection of a number of racemic arylethanamides where amide hydrolyses were completed in 4–6 days.[2f]

In the present work, we focus on the utility of CAL-A as a commercial CAT#NZL-101-IMB preparation (herein CAL-A) in the kinetic resolution of *rac*-**1a–c** ($R^1 = CH_2CF_3$ or Et; $R^2 = t$Bu) through N-acylation to learn about concentration effects on re-

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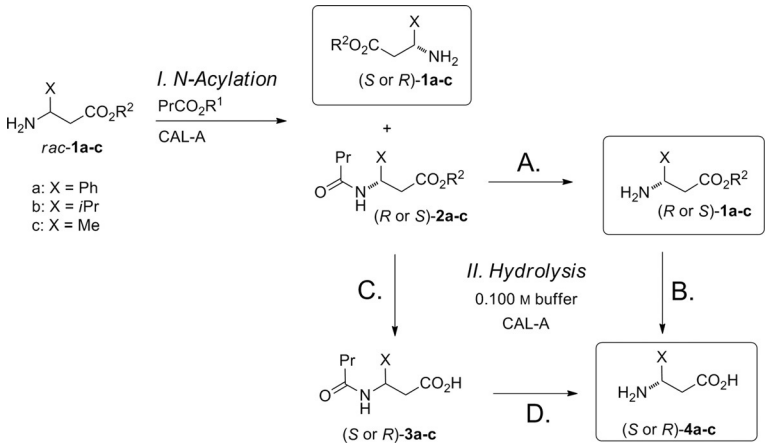


Scheme 1. N-Acylation of β-amino esters **1a–c** and hydrolysis of the amido esters **2a–c** using CAL-A.

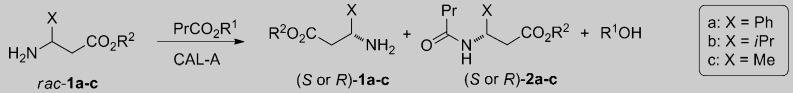
activity and enantioselectivity (Scheme 1, protocol I). As the kinetic resolution produces the reactive enantiomer as an amide product **2**, another focus has been on the study of the chemo- and enantioselectivity of the CAL-A-catalysed hydrolysis of the amido esters *rac*-**2a–c** ($R^2 = t$Bu) and the possibility to transform the enzymatic N-acylation products (*R* or *S*)-**2a–c** ($R^2 = t$Bu) into the corresponding enantiomers of **1a–c** ($R^2 = t$Bu; protocol II). Therefore, three structurally different *tert*-butyl esters of β^3-amino esters *rac*-**1a–c** were prepared by using known methods.[9a–c] Alkyl butanoates ($PrCO_2R^1$) were used as acyl donors in N-acylations because the acyl-binding site of CAL-A was previously shown to be a shallow binding pocket favouring accommodation of long unbranched carbon chains in the acyl part of the acyl donor.[10] Thereafter the β^3-butanamido esters **2a–c** ($R^2 = t$Bu) were prepared as racemates or using CAL-A catalysis, followed by N-deacylation through CAL-A-catalysed hydrolysis. As our focus has been on the lipase-catalysed chemo- and enantioselective formation and cleavage of the amide bond rather than the cleavage of the ester bond by hydrolysis, *tert*-butyl esters were used as substrates *rac*-**1a–c**.

Results and Discussion

N-Acylation of amino esters *rac*-1a–c ($R^2 = t$Bu)

CAL-A is well-known for its ability to catalyse the chemoselective N-acylation of various β-amino esters in organic solvents.[5,7b,d,11,12] Thus, we previously reported CAL-A adsorbed on celite as an N-acylation catalyst in the kinetic resolution of *rac*-**1a–c** (Scheme 1, $R^2 = $ Et) with 2,2,2-trifluoroethyl butanoate in diisopropyl ether (DIPE; Table 1, entries 1–3).[7b] These reactions were shown to proceed with relatively moderate enantio-

Table 1. CAL-A-catalysed N-acylation of *rac*-**1a–c** at 23 °C.



Entry	Substrate	c [mol L^{-1}]	Enzyme [mg mL^{-1}]	Acyl donor	t [h]	ee_s(1) [%]	ee_p(2) [%]	c [%]	E
1	**1a** (R^2 = Et)[a]	0.10	40[b]	$PrCO_2CH_2CF_3$[c]	1	98	90	52	75
2	**1b** (R^2 = Et)[a]	0.10	40[b]	$PrCO_2CH_2CF_3$[c]	2.5	99	87	53	106
3	**1c** (R^2 = Et)[a]	0.10	40[b]	$PrCO_2CH_2CF_3$[c]	25	–	–	50	6
4	**1a** ($R^2 = t$Bu)	0.050	20	$PrCO_2CH_2CF_3$[c]	0.75	>99 (*R*)	96 (*S*)	51	>100
5	**1a** ($R^2 = t$Bu)	0.10	20	$PrCO_2CH_2CF_3$[c]	2	>99 (*R*)	93 (*S*)	52	>100
6	**1a** ($R^2 = t$Bu)	0.50	20	$PrCO_2CH_2CF_3$[c]	2	98 (*R*)	98 (*S*)	50	>100
7	**1a** ($R^2 = t$Bu)	1.0	20	$PrCO_2CH_2CF_3$[c]	6	>99 (*R*)	94 (*S*)	51	>100
8	**1a** ($R^2 = t$Bu)	2.0	20	$PrCO_2CH_2CF_3$[c]	24	>99 (*R*)	79 (*S*)	56	43±3
9	**1a** ($R^2 = t$Bu)	0.25	20	$PrCO_2Et$[d]	24	>99 (*R*)	98 (*S*)	51	>100
10	**1a** ($R^2 = t$Bu)	0.50	20	$PrCO_2Et$[d]	24	92 (*R*)	>99 (*S*)	48	>100
11	**1a** ($R^2 = t$Bu)	0.50	40	$PrCO_2Et$[d]	24	>99 (*R*)	97 (*S*)	51	>100
12	**1a** ($R^2 = t$Bu)	1.0	80	$PrCO_2Et$[d]	24	>99 (*R*)	96 (*S*)	51	>100
13	**1a** ($R^2 = t$Bu)	2.0	80	$PrCO_2Et$[d]	24	>99 (*R*)	99 (*S*)	50	>100
14	**1b** ($R^2 = t$Bu)	1.0	80	$PrCO_2Et$[d]	24	>99 (*R*)	>99 (*S*)	50	>100
15	**1b** ($R^2 = t$Bu)	2.0	80	$PrCO_2Et$[d]	24	88 (*R*)	>99 (*S*)	47	>100
16	**1c** ($R^2 = t$Bu)	1.0	80	$PrCO_2Et$[d]	24	99 (*S*)	84 (*R*)	54	53
17	**1c** ($R^2 = t$Bu)	2.0	80	$PrCO_2Et$[d]	24	56 (*S*)	93 (*R*)	38	60±1

[a] Ref. [7b]. [b] CAL-A on celite. [c] 2 equiv. 2,2,2-trifluoroethyl butanoate in DIPE. [d] A solvent and an acyl donor.

selectivity, strongly depending on the substrate structure, as measured by the values of enantiomeric ratio (*E*).

Herein, *rac*-**1a** [0.050–2.0 M, (R^2 = *t*Bu)] was subjected to CAL-A-catalysed N-acylation with 2,2,2-trifluoroethyl butanoate (2 equiv.) in DIPE (Table 1, entries 4–8). Elevation of the substrate concentration took place at the expense of the time needed to reach the theoretical 50% conversion of kinetic resolution. On the other hand, the reactions at 0.050–1.0 M substrate concentrations in the presence of 20 mg mL^{-1} of CAL-A proceeded with excellent enantioselectivity ($E > 100$), whereas the enantioselectivity dropped at 2.0 M concentration (entry 8), indicating an unfavourable solvent effect caused by high proportions of 2,2,2-trifluoroethyl butanoate or 2,2,2-trifluoroethanol produced by the acylation. Therefore, the medium 2,2,2-trifluoroethyl butanoate in DIPE was replaced by ethyl butanoate as an acyl donor and a solvent (entries 9–13). N-Acylation turned slow compared to the acylation with alkyl-activated 2,2,2-trifluoroethyl butanoate, and elevation on enzyme content was necessary with increasing content of *rac*-**1a** to reach 50% conversion in 24 h. With the enzyme content of 80 mg mL^{-1}, even 2.0 M substrate concentration gave excellent enantioselectivity after 24 h ($E > 100$, entry 13, compared to $E = 43$, entry 8). The change of *rac*-**1a** to aliphatic *rac*-**1b** and *rac*-**1c** in ethyl butanoate resulted in reduction in reactivity (entries 14–17), as shown in lower conversions after 24 h at 2.0 M substrate concentrations even with enzyme content 80 mg mL^{-1} (entries 15 and 17). Moreover, *rac*-**1c** was not as good a substrate for CAL-A, as deduced from the moderate *E* values 53 and 60 for the kinetic resolution of *rac*-**1c** (R^2 = *t*Bu; entries 16 and 17) in ethyl butanoate. However, even then the enantioselectivities were clearly higher than with *rac*-**1c** (R^2 = Et) where $E = 6$ was observed with 2,2,2-trifluoroethyl butanoate in DIPE (entry 3).

For the separation of the enantiomers, one gram of *rac*-**1a** (2.00 M), *rac*-**1b** (1.00 M) and *rac*-**1c** (2.00 M) as *tert*-butyl esters were resolved with CAL-A (80 mg mL^{-1}) in ethyl butanoate, yielding the unreacted **1** as the respective (*R*)-, (*R*)- and (*S*)-enantiomers and the butanamide **2** as the respective (*S*)-, (*S*)- and (*R*)-enantiomers as shown in Experimental Section. Notably, the spatial structures of the asymmetric centres are equivalent, and the observed variations in *R* and *S* configurations in the series of resolution products **1a–c** and **2a–c** stem from the CIP rules. The enantiomeric products were all obtained at *ee* > 99%, except (*R*)-**2c** for which *ee* = 71% was detected in accordance with the moderate enantioselectivity (Table 1, entries 16 and 17).

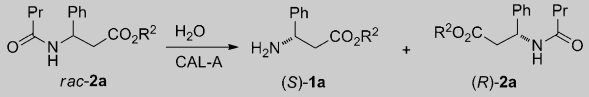
The absolute configurations are in accordance with the previous studies for the N-acylation of *rac*-**1a–c** (R^2 = Et) with 2,2,2-trifluoroethyl butanoate where (*S*)-**1a** (R^2 = Et), (*S*)-**1b** (R^2 = Et) and (*R*)-**1c** (R^2 = Et) are the more reactive enantiomers in the presence of CAL-A on celite.[7b] The configurations are further in accordance with the specific rotations given in literature as described in the Experimental Section.[13]

Hydrolysis of amido esters *rac*-2a–c (R^2 = *t*Bu)

As discussed in the Introduction, lipases can, in principle, hydrolyse both ester and amide bonds, although natural chemoselectivity should be in favour of ester hydrolysis. In accordance with this, the hydrolysis of ethyl 3-butanamidobutanoate *rac*-**2c** (R^2 = Et, X = Me, Scheme 1) was previously shown to take place at the ester and amide functional groups with CAL-A in phosphate buffer (0.100 M, pH 7.5), yielding amido acid (*S*)-**3c** through ester hydrolysis (step C), amino ester (*R*)-**1c** through amide hydrolysis (step A) and amino acid (*R*)-**4c** through sequential hydrolysis (steps A + B).[2e] In the present work, *rac*-**2a–c** as sterically hindered *tert*-butyl esters rather than as *n*-alkyl esters were used as substrates for CAL-A. Although CAL-A is able to hydrolyse *tert*-butyl esters,[14] hydrolysis can be expected to be considerably reduced compared to the hydrolysis of the corresponding ethyl esters.

The effect of pH (pH range 1.0–9.0) on enantioselectivity and conversion for the hydrolysis of *rac*-**2a** (R^2 = *t*Bu) by CAL-A (100 mg mL^{-1}) was studied in suitable buffer solutions (0.100 M; Table 2). At pH 1.0 the reaction gave *rac*-**3a** (R^2 = *t*Bu) through acid-catalysed ester hydrolysis (Table 2, row 1). Increase in pH resulted in conversions between 22 and 33 (rows 2–8), the samples containing traces of amino acid (*S*)-**4a** (R^2 = *t*Bu) after one day. As for the sequential reactions, the *E* values cannot be considered as a measure of enantioselectivity. On the other hand, $ee^{(R)\text{-}2a}$ is lowest at pH 5.0 (row 5) and enantioselectivity is highest at pH 9.0 (row 8). Accordingly, ammonium acetate buffer (pH 9.0; 0.100 M) was chosen for further studies. The easy removal of ammonium acetate by evaporation was another reason to use this buffer.

Table 2. CAL-A-catalysed (100 mg mL^{-1}) hydrolysis of *rac*-**2a** (0.050 M; R^2 = *t*Bu) and formation of (*S*)-**1a** (R^2 = *t*Bu) in various buffers (0.100 M; *t* = 24 h) at 47 °C.



rac-**2a** (*S*)-**1a** (*R*)-**2a**

Entry	pH	ee_s(2) [%]	ee_p(1) [%]	*c* [%]	*E*
1[a]	1.0	*rac*	*rac*	40	–
2	2.0	27 (*R*)	95 (*S*)	22	51
3	3.0	34 (*R*)	83 (*S*)	29	15
4	4.0	41 (*R*)	87 (*S*)	32	21
5	5.0	27 (*R*)	80 (*S*)	25	12
6	6.6	31 (*R*)	88 (*S*)	26	21
7	7.5	45 (*R*)	93 (*S*)	33	43
8	9.0	33 (*R*)	96 (*S*)	26	68

[a] Non-enzymatic hydrolysis into **3a**.

The hydrolysis of *rac*-**2a** (R^2 = *t*Bu; 0.050 M) with CAL-A (100 mg mL^{-1}) in ammonium acetate buffer (pH 9.0; 0.100 M) chemoselectively produced (*S*)-**1a**, even though traces of **4a** were detected through steps A + B (Figure 1). **3a** was not formed at all. This result is a strong indication about high chemoselectivity toward the amide hydrolysis of *rac*-**2a**. Notably,

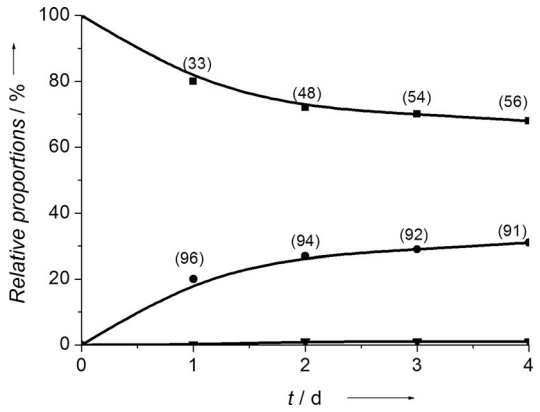


Figure 1. CAL-A-catalysed (CAT#NZL-101-IMB; 100 mg mL^{-1}) hydrolysis of *rac*-**2a** (■, 0.050 M; R^2 = *t*Bu) and formation of the products (*S*)-**1a** (●) and (*S*)-**4a** (▼) in ammonium acetate buffer (0.100 M, pH 9.0) at 47 °C; *ee* values in parentheses.

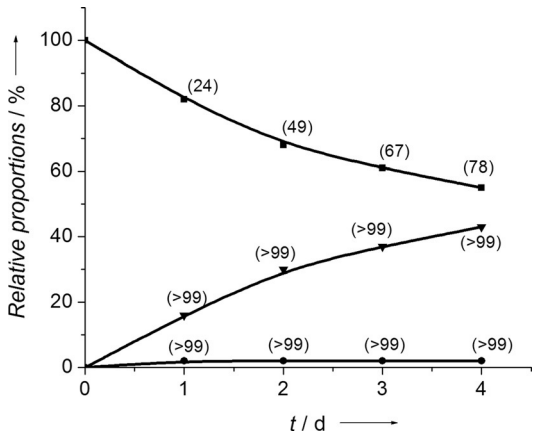


Figure 2. CAL-A-catalysed (CAT#NZL-101-IMB; 100 mg mL^{-1}) hydrolysis of *rac*-**2b** (■, 0.050 M; R^2 = *t*Bu) and formation of the products (*S*)-**4b** (▼) and (*S*)-**1b** (●) in ammonium acetate buffer (0.100 M, pH 9.0); *ee* values in parentheses.

the unbranched butanoyl part of **2a** is bound into the acyl binding pocket of CAL-A for amide hydrolysis, whereas for ester hydrolysis the acyl part of **2a** might be large and too polar for accommodating in the pocket. In accordance with this, the fact that amino ester (*S*)-**1a** hardly hydrolysed into amino acid (*S*)-**4a** indicates that the phenyl group of the substrate is not accepted into the acyl binding pocket. As an unexpected drawback, the reaction tended to stop at approximately 30% conversion after 4 days. If the substrate concentration was lowered to 0.020 M under the otherwise same conditions, the reaction proceeded slightly further (≈35% conversion). If the initial reaction mixture contained both *rac*-**1a** (R^2 = *t*Bu; 0.050 M) and *rac*-**2a** (R^2 = *t*Bu; 0.050 M), the hydrolysis of *rac*-**2a** (R^2 = *t*Bu) ceased (c = 13% instead of 20% after 24 h in Figure 1), proposing product inhibition behind the phenomenon. Accordingly, (*S*)-**1a** is expected to have high affinity to the nucleophile binding site in aqueous medium, preventing binding of (*S*)-**2a**.

Applicability of the reaction was studied by subjecting (*S*)-**2a** (0.55 g, 1.89 mmol, *ee* > 99%; 0.050 M), obtained from the N-acylation reaction described above, to hydrolysis in ammonium acetate buffer (0.100 M, pH 9.0; 38 mL) at 47 °C. In accordance with analytical-scale reactions, amino ester (*S*)-**1a** (0.16 g; 0.72 mmol; 38%; *ee* > 99%) and the unreacted (*S*)-**2a** (0.28 g; 0.96 mmol; *ee* = 98%) were isolated after 3 days.

The hydrolysis of *rac*-**2b** (R^2 = *t*Bu; 0.050 M) by CAL-A (100 mg mL^{-1}) in ammonium acetate buffer (pH 9.0; 0.100 M) chemoselectively yielded the amino ester (*S*)-**1b**, which immediately reacted into the amino acid (*S*)-**4b** with *ee* > 99% (Figure 2; Scheme 1, steps A + B). The concentration of (*S*)-**1b** stayed minimal all the time. Accordingly, CAL-A enabled sequential deprotection by hydrolysis of both amide and *tert*-butyl groups in the formation of (*S*)-**4b** (R^2 = *t*Bu).

Hydrolysis of *rac*-**2c** (R^2 = *t*Bu; 0.050 M) with CAL-A (100 mg mL^{-1}) in ammonium acetate buffer (pH 9.0; 0.100 M) was faster than with the other substrates, evidently leading to the total hydrolysis of the substrate in the formation of **4c** at reaction times long enough (Figure 3). All possible hydrolysis

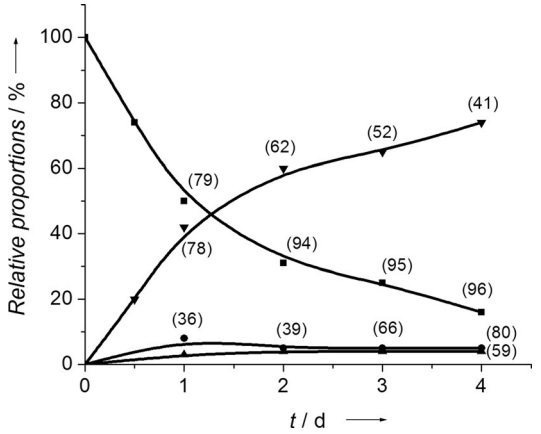


Figure 3. CAL-A-catalysed (CAT#NZL-101-IMB; 100 mg mL^{-1}) hydrolysis of *rac*-**2c** (■, 0.050 M; R^2 = *t*Bu) and formation of the products (*R*)-**4c** (▼), **1c** (●) and (*R*)-**3c** (▲) in ammonium acetate buffer (0.100 M, pH 9.0); *ee* values in parentheses.

products were detected, albeit amido ester (*R*)-**3c** only in low amount (< 5%). (*R*)-**3c** did not react further as tested separately with *rac*-**3c**. Enantioselectivity for the hydrolysis of *rac*-**2c** was low as concluded from $ee^{(S)\text{-}\mathbf{2c}}$ = 96% at 80% conversion. If (*R*)-**2c** (0.64 g, 2.80 mmol, *ee* 71%; 0.050 M), obtained from the gram-scale N-acylation described above, was hydrolysed by CAL-A (100 mg mL^{-1}) in ammonium acetate buffer (0.100 M, pH 9.0; 55.8 mL) at 47 °C, (*R*)-**4c** (0.18 g; 1.8 mmol; 64%; *ee* 96%) and the unreacted (*S*)-**2c** (0.14 g; 0.61 mmol; *ee* 4%) were isolated after two days. Formations of **1c** and **3** were so low that they were not able to isolate.

Overall, the obtained results suggest that CAL-A cleaves, although slowly, the amide over the *tert*-butyl ester of β-amido esters in highly chemo- and enantioselective fashion under mild conditions.

Conclusions

We have studied kinetic resolutions based on the N-acylation of *tert*-butyl esters of 3-amino-3-phenylpropanoic acid (*rac*-**1a**), 3-amino-4-methylpentanoic acid (*rac*-**1b**) and 3-aminobutanoic acid (*rac*-**1c**) over the concentration range 0.10–2.0 M with 2,2,2-trifluoroethyl butanoate in diisopropyl ether and with ethyl butanoate as an acyl donor and a solvent. We have shown that even 2.0 M substrate concentrations are possible for the enantioselective N-acylation of the substrates, producing butanamido esters **2a–c** with (*S*)-, (*S*)- and (*R*)-configurations, respectively.

Kinetic resolutions based on the hydrolysis of *tert*-butyl esters of *rac*-**2a–c** (0.050 M) by CAL-A in ammonium acetate buffer (0.100 M, pH 9.0) were shown to depend strongly on the substrate structure. The formation of the amido acid **3c** was detected only with **2c**, and even then chemoselectivity strongly favoured amide hydrolysis over that of *tert*-butyl ester. On the other hand, merely the hydrolysis of *rac*-**2a** allowed the preparation of the amino ester (*S*)-**1a** in which the *tert*-butyl ester remained intact. Accordingly, hydrolysis of *rac*-**2b** gave the corresponding amino acid (*S*)-**4b** (*ee* > 99%) in a highly enantioselective manner, and hydrolysis of *rac*-**2c** enantiomerically enriched (*R*)-**4c** (*ee* 41%) with 75% yield after 4 days.

Thus, the present results show that CAL-A is able to catalyse not only the hydrolysis of amide bonds but also the hydrolysis of *tert*-butyl esters of β-amino esters. These abilities make CAL-A unique not only among lipases, but among enzymes in general. These unusual reaction types can be further utilised in the removal of protective groups of this type of substrates. Slow reaction time may be overcome by enzyme engineering in the future studies.

Experimental Section

Materials and Methods

Reagents were purchased from Acros, Aldrich, Fluka, J. T. Baker, Lancaster or Merck and used as received. Lipase A from *C. antarctica* (NZL-101-IMB) immobilised on a resin was a product of Biocatalytics (now Codexis). Lipase B from *C. antarctica* (CAL-B, Novozym 435) was obtained from Novozymes.

The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer operating at 500 MHz with tetramethylsilane as an internal standard. High-resolution mass spectrometry was performed in ESI$^+$ mode with Bruker Avance microOTOF-Q quadrupole-TOF spectrometer. Melting points were recorded with a Gallenkamp apparatus. The optical rotations were determined with PerkinElmer 341 polarimeter, and $[\alpha]_D$ values were given in units of 10^{-1} deg cm^{-2} g^{-1}. The determination of *E* for the N-acylation reactions was based on the equation $E = \ln[(1-c)(1-ee_S)]/\ln[(1-c)(1+ee_S)]$ with $c = ee_S/(ee_S+ee_P)$ using linear regression (*E* as the slope of the line $\ln[(1-c)(1-ee_S)]$ versus $\ln[(1-c)(1+ee_S)]$).[15] Here ee_S and ee_P denote enantiomeric excesses of the substrate and the product, respectively.

Analytical-scale hydrolyses of *rac*-**2a–c** into **1a–c**, **3a–c** and **4a–c** took place in New Brunswick Scientific G24-incubator (47 °C). The reaction samples were analysed by gas chromatography with flame ionisation detection (Agilent 6850) using VARIAN CP Chirasil-Dex CB FAST GC (CP99927; 15 m × 0.15 mm × 0.15 μm) and Varian WCOT CP Chirasil-Dex CB columns (CP7050215; 25 m × 0.25 mm × 0.25 μm). The whole reaction volume (1.00 mL) was used for analysis at each time interval. The enzyme used was carefully washed with dichloromethane. Water and dichloromethane were evaporated from the samples, and the products were derivatised into amido esters by adding acetic anhydride (80.0 μL) and after 10 min MeOH (320 μL). The derivatisation reaction was let to proceed for at least 2 h before injecting a sample into GC for analysis. The relative proportions of the four compounds were then determined from the peak areas by taking into account the peak responses, which were determined by making a solution containing all the four compounds in equal amounts.

In analytical-scale N-acylations, *rac*-**1a–c** (0.050–2.0 M) was dissolved into the solvent followed by addition of acyl donor and enzyme. Reactions proceeded under shaking (170 rpm) at 23 °C. Samples (0.1 mL) were taken to follow the reaction by filtering off the enzyme and derivatising the free amine with acetic anhydride (10 μL). The samples were diluted with dichloromethane and analysed by GC.

In the synthesis of the racemic starting materials, thin-layer chromatography (TLC) was performed by using Merck Kieselgel 60F^{254} sheets. Spots were visualised by treatment with 5% ethanolic phosphomolybdic acid solution and heating.

Determination of absolute configurations

(*R*)-**1a** (R^2 = *t*Bu): CAL-A-catalysed N-acylation of *rac*-**1a** yielded (*R*)-**1a** (*ee* > 99%) with $[\alpha]_D^{25}$ +19.6 (*c* = 1.00, $CHCl_3$). Literature value for (*S*)-**1a** is −21.0 (*c* = 1.00, 20 °C, $CHCl_3$)[13a] and +20.0 (*c* = 0.71, 20 °C, $CHCl_3$)[13b] for (*R*)-**1a** (*ee* 99%).

(*R*)-**1b** (R^2 = *t*Bu): CAL-A-catalysed N-acylation of *rac*-**1b** yielded (*R*)-**1b** (*ee* > 99%) with $[\alpha]_D^{22}$ +23.4 (*c* = 0.46, $CHCl_3$). Literature value for (*S*)-**1b** is −26.4 (*c* = 0.67, 19 °C, $CHCl_3$)[13c] and +19.1 (*c* = 0.68, 25 °C, $CHCl_3$)[13b] for (*R*)-**1b**.

(*S*)-**1c** (R^2 = *t*Bu): Hydrolysis of the N-acylation product (*R*)-**2c** yielded amino acid (*R*)-**4c** (*ee* 96%) with $[\alpha]_D^{22}$ −35.9 (*c* = 0.50, H_2O). Literature value for (*S*)-**4c** is +32.0 (*c* = 0.6, 25 °C, H_2O).[13d] Thus, the N-acylation products were (*S*)-**1c** and (*R*)-**2c**.

Synthesis of the racemic starting materials

β-Amino acid *rac*-**4c** was commercially available. *rac*-**4a** and -**4b** were prepared according to the literature, that is, ammonium acetate, malonic acid and an aldehyde (benzaldehyde or isobutyraldehyde, respectively) were used as starting materials in the formation of β-amino acids.[9a] *rac*-**1a** (R^2 = Et) was further prepared from the corresponding β-amino acid using $SOCl_2$/EtOH, and *rac*-**1a–c** (R^2 = *t*Bu) using *t*BuOAc/$HClO_4$.[9b,c] The N-acyl group was attached to amino esters *rac*-**1a–c** from the corresponding acyl chloride or anhydride to yield *rac*-**2a–c**.

rac-**4a** (3-amino-3-phenylpropanoic acid): Benzaldehyde (90.0 mmol, 9.55 g, 9.15 mL), malonic acid (90.9 mmol, 9.43 g) and ammonium acetate (121 mmol, 9.30 g) were dissolved in ethanol (150 mL), and the mixture was kept at for 5 h. *rac*-**4a** was filtered, washed with ethanol and separated as white crystals (38.7 mmol, 6.40 g, 43%; R_f = 0.7 (80% EtOH, 20% H_2O); m.p. 222–223 °C). ^{1}H NMR (500 MHz, D_2O): δ = 2.71 (dd, 1H, *J* = 16 Hz, 6.5 Hz, ArCHCH_2), 2.81 (dd, 1H, *J* = 16 Hz, 8.5 Hz, ArCHCH_2), 4.54 (m, 1H,

ArC*H*), 4.70 (s, 2H, ArCHNH_2), 4.70 (s, 1H, COO*H*), 7.34–7.40 ppm (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, D_2O) δ = 40.41, 52.69, 126.87, 129.21, 129.24, 135.96, 177.19 ppm; MS–ESI$^+$: *m/z* 165.0791, $C_9H_{11}NO_2$ requires 165.0789.

rac-**4b** (3-amino-4-methylpentanoic acid): *rac*-**4b** was prepared as *rac*-**4a**, except that after evaporation of ethanol, the crude product was re-dissolved into ethanol (50.0 mL) followed by refluxing. Addition of acetone (50.0 mL) precipitated the product. The mixture was let cool to room temperature, and the product was filtered and washed with acetone. *rac*-**4b** was obtained as white crystals (48.8 mmol, 6.40 mg, 54%; R_f = 0.5 (90% EtOH, 10% H_2O), m.p. 202–203 °C). ^{1}H NMR (500 MHz, D_2O): δ = 0.89 (d, 3H, *J* = 7 Hz, $(CH_3)_2$CH), 0.91 (d, 3H, *J* = 7 Hz, $(CH_3)_2$CH), 1.85 (m, 1H, $(CH_3)_2CH$), 2.31 (dd, 1H, *J* = 16 Hz, 9.5 Hz, CHNH_2CH_2), 2.48 (dd, 1H, *J* = 16 Hz, 4 Hz, (CHNH_2CH_2), 3.24 (m, 1H, $(CH_3)_2$CHCH), 4.70 (s, 1H, COO*H*), 4,70 ppm (s, 2H, NH_2); ^{13}C NMR (125 MHz, D_2O) δ = 17.21, 17.38, 29.98, 35.92, 54.81, 178.51 ppm; MS–ESI$^+$: *m/z* 131.0945, $C_6H_{13}NO_2$ requires 131.0946.

rac-**1a** (R^2 = *t*Bu; 3-amino-3-phenylpropanoic acid *tert*-butyl ester): *tert*-Butyl acetate (80.0 mL) was added on *rac*-**4a** (18.2 mmol, 3.00 g). After mixing for 5 min, perchloric acid (27.3 mmol, 2.74 g, 2.35 mL) was added, and the reaction mixture was stirred for 15 h at RT. The product was extracted by water (4×50 mL) and 0.5 M HCl (50.0 mL). The aqueous phases were combined, and the pH was adjusted to 8.00 with 2 M NaOH. The product was extracted with dichloromethane (4×50.0 mL). The combined phases were washed with water (50 mL) and saturated NaCl (50 mL). The organic phase was dried with sodium sulfate. The product (10.7 mmol, 2.36 mg, 59%) was obtained as slightly yellow oily liquid after filtration and evaporation [R_f = 0,5 (90% DCM, 10% MeOH)]. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.42 (s, 9H, COOC(CH_3)$_3$), 2.70 (m, 2H, ArCHCH_2), 3.26 (s, 2H, NH_2), 4.42 (m, 1H, ArC*H*), 7.08–7.26 ppm (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 28.05, 44.27, 52.72, 80.98, 126.57, 127.66, 128.64, 143.04, 170.89 ppm; MS–ESI$^+$: *m/z* 199.1567, $C_{13}H_{20}NO_2$ requires 199.1596.

rac-**1b** (R^2 = *t*Bu; 3-amino-4-methylpentanoic acid *tert*-butyl ester) was prepared similarly to *rac*-**1a** (R^2 = *t*Bu). The obtained product (11.2 mmol, 2.09 g, 49%) was a bright oily liquid [R_f = 0.5 (90% DCM, 10% MeOH]. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.94 (q, 6H, *J* = 3,5 Hz, $(CH_3)_2$CH), 1.47 (s, 9H, COOC(CH_3)$_3$), 1.65 (m, 1H, $(CH_3)_2CH$), 1.79 (s, 2H, NH_2), 2,18 (dd, 1H, *J* = 15.5 Hz, 10 Hz, CH_3CHNH_2CH_2), 2.40 (dd, 1H, *J* = 15.5 Hz, 4 Hz, CH_3CHNH_2CH_2), 3.01 ppm (m, 1H, $(CH_3)_2$CHCHNH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 17.83, 18.83, 28.14, 33.21, 40.76, 53.66, 80.51, 172.41 ppm; MS–ESI$^+$: *m/z* 187.1573, $C_{10}H_{21}NO_2$ requires 187.1572.

rac-**1c** (R^2 = *t*Bu; 3-aminobutanoic acid *tert*-butyl ester) was prepared similarly to *rac*-**1a** (R^2 = *t*Bu). The obtained product (14.8 mmol, 2.36 mg, 51%) was a yellowish oily liquid [R_f = 0.4 (90% dichloromethane, 10% MeOH]. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.12 (d, 3H, *J* = 6.5 Hz, CH_3CHNH_2), 1.46 (s, 9H, COOC(CH_3)$_3$), 1.83 (s, 2H, CH_3CHNH_2), 2.23 (dd, 1H, *J* = 15.5 Hz, 8.0 Hz, CHNH_2CH_2), 2.33 (dd, 1H, *J* = 15.5 Hz, 4.5 Hz, (CHNH_2CH_2), 3.34 ppm (m, 1H, CH_3CHNH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 23.30, 28.13, 44.20, 45.39, 80.51, 171.77 ppm; MS–ESI$^+$: *m/z* 159.1253, $C_8H_{17}NO_2$ requires 159.1259.

rac-**2a** (R^2 = *t*Bu; 3-butanamido-3-phenylpropanoic acid *tert*-butyl ester): The reaction mixture of *rac*-**1a** (R^2 = *t*Bu) (2.3 mmol, 0.50 g), triethylamine (6.78 mmol, 0.45 g, 0.95 mL) and butanoic anhydride (2.94 mmol, 0.45 g, 0.47 mL) was stirred for 60 min. The reaction was stopped by adding methanol (30.0 mL) and mixing for 60 min. The crude product was purified by column chromatography on silica gel (90% petroleum ether, 10% acetone).The product *rac*-**2a** (R^1 = *t*Bu; 2.2 mmol, 0.65 g, 99%) was a yellow, crystalline substance [R_f = 0.1 (90% petroleum ether, 10% acetone); m.p. 69–70 °C]. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.98 (t, 3H, *J* = 7.5 Hz, NHCOCH$_2$CH$_2$CH_3), 1.33 (s, 9H, COOC(CH_3)$_3$), 1.70 (dt, 2H, *J* = 22.5 Hz, 7.5 Hz, NHCOCH$_2$CH_2), 2.23 (t, 2H, *J* = 8 Hz, NHCOCH_2), 2.75 (dd, 2H, *J* = 16 Hz, 6 Hz, ArCHCH_2), 2.84 (dd, 2H, *J* = 16 Hz, 6 Hz, ArCHCH_2), 5.43 (m, 1H, ArC*H*), 6.72 (d, 1H, *J* = 7.5, N*H*), 7.26–7.35 ppm (m, 5H, C_6H_5); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 13.78, 19.17, 27.90, 38.74, 41.17, 49.54, 81.40, 126.31, 127.44, 128.55, 140.76, 170.72, 172.27 ppm; MS-ESI$^+$: *m/z* 291.1829, $C_{17}H_{25}NO_3$ requires 291.1834.

rac-**2b** (R^2 = *t*Bu; 3-butanamido-4-methylpentanoic acid *tert*-butyl ester): *rac*-**1b** (R^2 = *t*Bu; 2.67 mmol, 0.50 g) was used as the starting material, and the synthesis took place as with *rac*-**2a** (R^2 = *t*Bu), except that the eluent for column chromatography was 90% dichloromethane, 10% methanol. The product *rac*-**2b** (R^2 = *t*Bu; 2.64 mmol, 0.68 g, 99%) was a white crystalline substance [R_f = 0.9 (90% dichloromethane, 10% methanol), m.p. 46–47 °C]. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.93–1.01 (m, 3H, NHCOCH$_2$CH$_2$CH_3), 0.93–1.01 (m, 6H, $(CH_3)_2$CH), 1.46 (s, 9H, COOC(CH_3)$_3$), 1.69 (m, 2H, NHCOCH$_2$CH_2), 1.84 (m, 1H, $(CH_3)_2CH$), 2.20 (m, 2H, NHCOCH_2), 2.45 (m, 2H, CHNHCH_2), 4.06 (m, 1H, $(CH_3)_2$CHC*H*NH), 6.21 ppm (s, 1H, N*H*). ^{13}C NMR (125 MHz, $CDCl_3$) δ = 13.78, 19.07, 19.28, 19.30, 28.03, 31.60, 37.73, 38.94, 51.51, 171.65, 172.44 ppm; MS-ESI$^+$: *m/z* 257.1986, $C_{14}H_{27}NO_3$ requires 257.1991.

rac-**2c** (R^2 = *t*Bu; 3-butanamidobutanoic acid *tert*-butyl ester): *rac*-**1c** (R^2 = *t*Bu; 3.1 mmol, 0.50 g) was used as the starting material, and the synthesis took place as with *rac*-**2a** (R^2 = *t*Bu). The product *rac*-**2c** (R^2 = *t*Bu; 3.1 mmol, 0.72 g, 99%) was a greyish liquid [R_f = 0.2 (90% petroleum ether, 10% acetone)]. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.95 (t, 3H, *J* = 7.5 Hz, NHCOCH$_2$CH$_2$CH_3), 1.22 (d, 3H, *J* = 7.0 Hz, CH_3CHNH_2), 1.47 (s, 9H, COOC(CH_3)$_3$), 1.66 (dt, 2H, *J* = 22.5, 7.5 Hz, NHCOCH$_2$CH_2), 2.15 (t, 2H, *J* = 7.5 Hz, NHCOCH_2), 2.43 (m, 2H, CH_3CHNH_2CH_2), 4.34 (m, 1H, CH_3C*H*NH_2), 6.29 ppm (s, 1H, N*H*); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 13.69, 19.17, 20.00, 28.08, 38.78, 41.15, 42.07, 81.17, 171.24, 172.26 ppm; MS-ESI$^+$: *m/z* 229.1673, $C_{12}H_{23}NO_3$ requires 229.1678.

Preparative-scale N-acylation and hydrolysis

rac-**1a** (R^2 = *t*Bu; 1.00 g, 4.52 mmol) was dissolved into ethyl butanoate (2.26 mL) to obtain a 2.0 M solution. Addition of CAL-A-preparation (CAT#NZL-101-IMB; 80.0 mg mL^{-1}) started the reaction. The reaction mixture was shaken at RT and stopped at 50% conversion after 24 h. (*S*)-**2a** (0.62 g, 94%, *ee* > 99%) and (*R*)-**1a** [0.47 g, 94%, *ee* > 99%, $[\alpha]_D^{25}$ +19,6 (*c* = 1.00, $CHCl_3$)] were separated by column chromatography using dichloromethane/methanol (9:1) as an eluent.

(*S*)-**2a** (R^2 = *t*Bu; 0.55 g, 1.89 mmol, *ee* > 99%) was further hydrolysed by CAL-A (CAT#NZL-101-IMB; 100 mg mL^{-1}) in 0.050 M ammonium acetate buffer (0.100 M, pH 9.0, 38.0 mL) at 47 °C. The reaction was stopped after three days. Purification by column chromatography using dichloromethane/methanol (95:5) as an eluent yielded (*S*)-**2a** (0.28 g, ee = 98%, $[\alpha]_D^{22}$ −48.2 (*c* = 0.55, $CHCl_3$)) and (*S*)-**1a** [0.16 g, 38%, *ee* > 99%, $[\alpha]_D^{25}$ −20.0 (*c* = 1.00, $CHCl_3$)].

rac-**1b** (R^2 = *t*Bu; 1.00 g, 5.34 mmol) was dissolved into ethyl butanoate (5.34 mL) to obtain a 1.0 M solution. Addition of CAL-A-preparation (CAT#NZL-101-IMB; 80.0 mg mL^{-1}) started the reaction. The reaction mixture was shaken at RT and stopped at 50% conversion after 24 h. (*S*)-**2b** (0.64 g, 93%, *ee* > 99%) and (*R*)-**1b** [0.45 g, 90%,

ee > 99%, $[\alpha]_D^{22}$ +23.4 (*c* = 0.46, $CHCl_3$)] were separated by column chromatography using dichloromethane/methanol (9:1) as an eluent.

rac-**1c** (R^2 = *t*Bu; 1.00 g, 6.28 mmol) was dissolved into ethyl butanoate (6.28 mL) to obtain a 2.0 M solution. Addition of CAL-A-preparation (CAT#NZL-101-IMB; 80.0 mg mL^{-1}) started the reaction. The reaction mixture was shaken in an incubator (*t* = 47 °C) and stopped at 58% conversion after 29 h. (*R*)-**2c** (0.74 g, 88%, *ee* 71%) and (*S*)-**1c** as butanoate salt (0.20 g, 48%, *ee* > 99%, $[\alpha]_D^{22}$ +15.3 (*c* = 0.50, $CHCl_3$)) were separated by column chromatography using dichloromethane/methanol (9:1) as an eluent.

(*R*)-**2c** (R^2 = *t*Bu; 0.64 g, 2.80 mmol, *ee* 71%) was further hydrolysed by CAL-A (CAT#NZL-101-IMB; 100 mg mL^{-1}) in 0.050 M ammonium acetate buffer (0.100 M, pH 9.0, 55.8 mL) at 47 °C. The reaction was stopped after two days. Purification twice by column chromatography using dichloromethane:methanol (9:1) and ethanol:water (9:1) as eluents yielded (*R*)-**4c** [0.18 g, 1.8 mmol, 64%, *ee* 96%, $[\alpha]_D^{22}$ −35.9 (*c* = 0.50, H_2O)] and (*S*)-**2c** [0.14 g, 0.61 mmol, *ee* = 4%, $[\alpha]_D^{22}$ −1.45 (*c* = 0.50, $CHCl_3$)].

The spectral data of the isolated products in the enzymatic reactions were identical to those of the racemic starting materials.

Keywords: acylation · chemoselectivity · enzyme catalysis · kinetic resolution · hydrolysis

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