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Lipases in β-Dipeptide Synthesis in Organic Solvents

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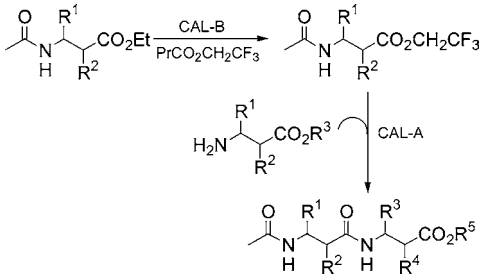
ABSTRACT



A number of β-dipeptides were prepared by two-step lipase-catalyzed reactions where *N*-acetylated β-amino esters were first activated as 2,2,2-trifluoroethyl esters with *Candida antarctica* lipase B (CAL-B). The activated esters were then used to acylate a β-amino ester in the presence of *Candida antarctica* lipase A (CAL-A) in dry Et_2O or *i*-Pr_2O.

Proteases (also called peptidases) are widely used as catalysts for enzymatic peptide synthesis, although many synthesis limiting facts (enzymatic hydrolysis of the formed peptide chain, enzymatic instability under nonaqueous conditions where hydrolysis could be prevented, and often limited substrate specificity to certain coded amino acids) exist.[1] On the other hand, lipases are relatively stable and show wide substrate specificity, and although they catalyze peptide bond formation, they do not possess amidase activity which is essential for splitting peptide bonds. Thus far, the use of lipases in peptide synthesis has been limited to PPL (porcine pancreatic lipase)- and CRL (lipase from *Candida rugosa*)-catalyzed reactions of *N*-protected amino esters as electrophiles and free amino esters as nucleophiles.[2] These lipase-catalyzed methods are typically based on the use of coded or noncoded α-amino acids. In the case of PPL, some doubts have been expressed due to possible contamination by proteases.[2b]

During the past few years, interest in the enantiomers of β-amino acids and further in β-peptides has increased tremendously from both pharmaceutical and chemical aspects.[3] Contrary to α-peptides, in vivo tests with proteolytic enzymes have shown β-peptides to be completely stable toward proteolysis owing to very high metabolic stability.[3a] Peptidomimetic activities of some β-peptides are well recognized. A β-tetrapeptide (*N*-Ac-β^{3}hThr-β^{2}hLys-β^{3}hTrp-β^{3}hPhe-NH_2) is an example.[3b] It forms a β-turn similar to that in a peptide hormone somatostatin where α-L-Trp and α-L-Lys residues situate in the turn. Accordingly, the above-mentioned β-tetrapeptide nicely imitates the α-peptidic ligand, acting as a potent agonist at the human somatostatin $hsst_4$ receptor.

Research interest in our laboratory has focused on using lipases as chiral catalysts in the enantioselective *N*-acylation

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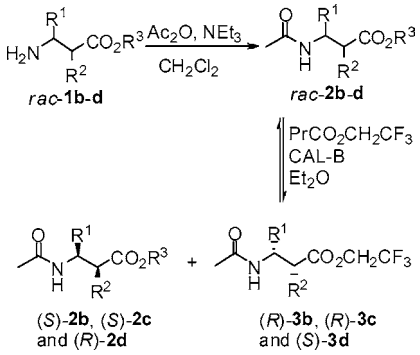
of β-amino esters with achiral esters in organic media.[4] In these studies, the two *Candida antarctica* lipases, lipases A (CAL-A) and B (CAL-B), revealed an interesting behavior, CAL-A being a highly chemo- and enantioselective *N*-acylation catalyst.[4c,e] On the other hand, CAL-B gave reactions both at the amino (*N*-acylation) and ester (interesterification) functions of an amino ester, chemoselectivity varying with the structure of an amino ester and an achiral acyl donor.[4b,d,e]

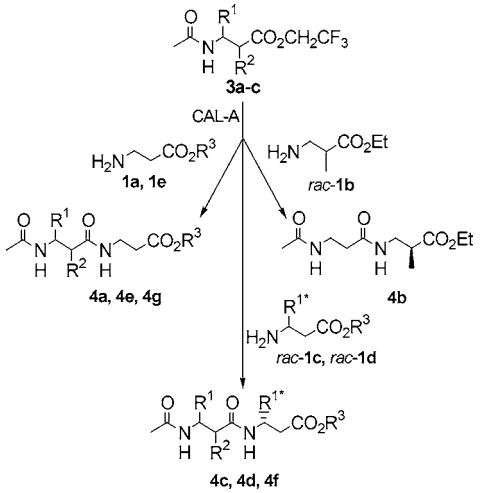
Thus far, enzymatic methods have not been used for the preparation of β-peptides. Inspired by the above results, we have now initiated studies of lipase-catalyzed β-peptide synthesis, where CAL-B allows the activation of a *N*-protected amino ester **2** as the 2,2,2-trifluoroethyl ester **3** (Scheme 1). This product is then used without purification

Scheme 1. CAL-B-Catalyzed Interesterification Leading to Activated *N*-Acylated Amino Esters



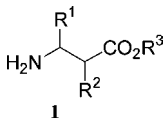
to acylate the amino group of **1** in the presence of CAL-A (Scheme 2). For this purpose, the amino esters **1a**−**e** (Table 1) were prepared in both racemic and enantiomeric forms

Scheme 2. CAL-A-Catalyzed Dipeptide Synthesis[a]



[a] Due to various combinations of **1** and **3** in the obtained dipeptides, R^1 (= R^{1*}), R^2, and R^3 (= R^{3*}) have the definition originally given for **1a**−**e** in Table 1.

Table 1. Used Amino Esters



1

compd	R^1	R^2	R^3	ee$^{(R)}$ (%)	ee$^{(S)}$ (%)
1a	H	H	Et		
1b	H	Me	Et	88	68
1b	H	Me	Et	95	99
1c	Me	H	Et	99	99
1d	Ph	H	Me	99	99
1e	H	H	Me		

(enantiomeric excess values, ee, are given in Table 1), as previously described.[4b−e] From these, **1a**, *rac*-**1b**−**d**, (*R*)- and (*S*)-**1b**, and (*S*)-**1c** were *N*-protected with the aid of acetic anhydride, leading to the corresponding substrates **2** for interesterification. Accordingly, one of the substrates, **2** (0.1 M), was incubated with CAL-B (20−50 mg mL^{-1}, Novozym 435) in the presence of 2,2,2-trifluoroethyl butanoate (0.2 M) in dry diethyl ether (Table 2). *tert*-Butyl methyl ether

Table 2. CAL-B-Catalyzed Transformation of **2a**−**d** into **3a**−**d** with 2,2,2-Trifluoroethyl Butanoate[a]

entry	substrate (ee)	time (h)	ee$^{(S)-\mathbf{2}}$ (%)	ee$^{(R)-\mathbf{3}}$ (%)	C (%)
1	**2a**	24			60
2	*rac*-**2b**	48	41	62	40[b]
3	(*S*)-**2b** (99%)	48	99	99 (*S*)	40[b]
4	(*R*)-**2b** (95%)	48	90 (*R*)	99	40[b]
5	*rac*-**2c**	3	50	90	36
6	*rac*-**2c**	24	64	57	53
7	(*S*)-**2c** (99%)	24	99	99 (*S*)	40
8	*rac*-**2d**	106	2 (*R*)	73 (*S*)	3[c]

[a] **2** (0.1 M) + 2,2,2-trifluoroethyl butanoate (0.2 M) in diethyl ether. Enzyme: CAL-B (Novozym 435, 20 mg mL^{-1}). [b] CAL-B (40 mg mL^{-1}). [c] CAL-B (50 mg mL^{-1}).

(TBME) was also a possible solvent. After 24−48 h, the transformation into the corresponding **3** tended to stop at an equilibrium at 40−60% conversion. Because the interesterification rate of *rac*-**2d** was extremely slow (Table 2, entry 8), **3d** was not used for further preparative purposes. The preferred enantioselection of CAL-B for *rac*-**2** is shown in Scheme 1.

For the CAL-B-catalyzed interesterification of *rac*-**2b**−**d** with 2,2,2-trifluoroethyl butanoate, enantioselectivities are relatively low (Scheme 1, Table 2). Due to the equilibrium nature of the interesterification, clear drops in ee values are

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Table 3. CAL-A-Catalyzed Dipeptide Synthesis[a]

entry	acyl donor (ee)	nucleophile (ee)	time (h)	dipeptide	yield[b] (%)	$[\alpha]^{23}_{D}$[c] (10^{-1}deg cm^2 g^{-1})	ee (%)
1	**2a**	*rac*-**1d**	24	no reaction			
2	**3a**	1e	7	**4a**	92		
3	**3a**	*rac*-**1b**	20	(*S*)-**4b**	87	+6.0	37
4	**3a**	(*R*)-**1b** (88%)	26	(*R*)-**4b**	75	−21.1	77
5	**3a**	(*S*)-**1b** (68%)	23	(*S*)-**4b**	82	+17.8	74
6	**3a**	*rac*-**1c**	24	(*R*)-**4c**	82	+12.9	68
7	**3a**	(*R*)-**1c** (99%)	44	(*R*)-**4c**	77	+18.9	99
8	**3a**	(*S*)-**1c** (99%)	44	(*S*)-**4c**	76	−18.9	99
9	**3a**	*rac*-**1d**	20	(*S*)-**4d**	83	−40.4	92
10	**3a**	(*R*)-**1d** (99%)	21	(*R*)-**4d**	61	+43.4	99
11	**3a**	(*S*)-**1d** (99%)	22	(*S*)-**4d**	85	−43.4	99
12	(*R*)-**3b** (62%)	1a	10	(*R*)-**4e**	90	−33.5	62
13[d]	(*R*)-**3b** (99%)	(*R*)-**1d** (99%)	81	(*R,R*)-**4f**	70	−22.5	99
14[d]	(*R*)-**3b** (99%)	(*S*)-**1d** (99%)	75	(*R,S*)-**4f**	85	−103.2	99
15[d]	(*S*)-**3b** (99%)	(*R*)-**1d** (99%)	80	(*S,R*)-**4f**	61	+103.2	99
16[d]	(*S*)-**3b** (99%)	(*S*)-**1d** (99%)	72	(*S,S*)-**4f**	82	+22.5	99
17	(*R*)-**3c** (57%)	1a	8	(*R*)-**4g**	89	+13	57
18	(*R*)-**3c** (57%)	*rac*-**1c**	168	no reaction			
19	(*S*)-**3c** (57%)	*rac*-**1c**	168	no reaction			

[a] **3a**−**c** (0.1 M) + **1a**−**e** (0.2 M) in diethyl ether. Enzyme: CAL-A preparation (Chirazyme L5, lyo., Boehringer-Mannheim, 30 mg mL^{-1} of the preparation on Celite containing 20% (w/w) of the original enzyme powder).[5] [b] Isolated yield calculated from an acyl donor. [c] (*c* 1.0, $CHCl_3$). [d] DIPE as solvent.

evident when high conversion rates are reached. This is clearly shown for *rac*-**2c**, where ee$^{(R)-\mathbf{3c}}$ drops from a value of 90% to 57% while the respective ee$^{(S)-\mathbf{2}}$ increases only from 50% to 64% when the reaction proceeds from 36% to 53% conversion (Table 2, entries 5 and 6). It has not been possible to affect the equilibrium position by changing the 2,2,2-trifluoroethyl butanoate concentration from 0.2 to 0.3 M. In the activation step, low enantioselectivity can be seen as a benefit, allowing both enantiomers of **2** to be turned into the 2,2,2-trifluoroethyl ester in a reasonable amount of time when the enantiomers are available. However, the use of racemic **2** in interesterification is less beneficial because the reaction then produces enantiomerically enriched products **3** (Table 2).

Interesterification as shown in Scheme 1 was stopped by filtering off CAL-B at conversions shown in Table 2. After evaporation of the excess 2,2,2-trifluoroethyl butanoate (and the solvent), the resulting mixture of one of the unreacted ethyl esters **2** and the formed 2,2,2-trifluoroethyl ester **3** (0.1 M) was dissolved in diethyl ether or in diisopropyl ether (DIPE) without further purification. One of the amino esters **1** (0.2 M) and the CAL-A preparation (30 mg mL^{-1}, containing 20% (w/w) of the lipase and 12% (w/w) of sucrose on Celite)[5] were added to start the peptide synthesis (Scheme 2, Table 3). Reactions were stopped when **3** was totally consumed. The obtained dipeptide **4** and the unreacted **1** and **2** were purified by column chromatography on silica eluting with dichloromethane containing methanol (3% (v/v)). As a crucial point, **2** was shown to be unable to acylate the amino group of **1** in the presence of CAL-A (Table 3, entry 1), and the separation of **1** and **2** from the dipeptide made their reuse possible. The secondary enzymatic hydrolysis of the formed peptide **4** was not detected. On the other hand, enzymatic hydrolysis of **3** by the water in the seemingly dry CAL-A preparation and the competitive chemical peptide formation were observed. However, the proportion of the hydrolyzed **3** was only approximately 5%. For the formation of **4f**, dry DIPE was used as a solvent since the reaction in diethyl ether was very slow.

Alkyl activation in **3** exposes the carbonyl group to a chemical peptide bond formation in addition to the enzymatic one. The nature of a nucleophile affects both chemical and enzymatic reactivities. Accordingly, a chemical reaction alone was responsible for the dipeptide formation when **1a** or **1e** was a nucleophile (Table 3, entries 2, 12, and 17). In the formation of dipeptide (*S*)-**4d** (ee = 92%, entry 9), the maximal proportion of a chemical transformation was approximately 20% in 20 h. This was detected by GC (a Chrompack CP-Chirasil-L-Val column, 25 m) following the disappearance of **3a** and *rac*-**1d** with time without and in the presence of the CAL-A preparation. For a chemical transformation, 20% is an overestimation because the competitive situation between chemical and enzymatic reactions lacks in the absence of the enzyme. It is clear that CAL-A recognizes the stereostructures of the added nucleophiles (entries 3, 6, and 9). In the case of CAL-A, the *S*-enantiomer of β^2-substituted *rac*-**1b** was slightly more reactive than the *R*-counterpart, while the *R*-enantiomer was faster for the CAL-B-catalyzed interesterification. In β^3-substituted **1c** and **1d**, the same stereostructures were recognized by CAL-A (acylation) and CAL-B (interesterification). This is in accordance with our earlier observations.[4b−e]

Thus far, CAL-A has been used only in cases where activated achiral esters have reacted with a racemic nucleo-

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phile, recognizing the stereostructure of the nucleophile (the β-amino group of the β-amino ester).[4] In the present work, the acyl donors, in addition to nucleophiles, can be chiral (Table 3, entries 12−19). When enantiopure substrates **1d** and **3b** are used in the CAL-A-catalyzed dipeptide formation the resulting product is a pure stereoisomer as shown in Table 3 (entries 13−16). According to these results, the formation of *R*,*S*- and *S*,*S*-stereoisomers reaches higher conversions in a shorter period of time than their antipodes do. The present results indicate that β^2-substituted **3b** is accepted as an acyl donor by CAL-A, whereas the corresponding β^3-substituted acyl donor is unreactive (entries 13−16 vs 18 and 19). Evidently, the reactions of (*R*)-**3b** (entry 12) and (*R*)-**3c** (entry 17) with **1a** were not enzyme catalyzed.

Lipase-catalyzed reactions ($RCONu^1 + Nu^2H = Nu^1H + RCONu^2$) are generally accepted to proceed through a so-called ping-pong bi−bi mechanism where the first product (Nu^1H) leaves the active site in the formation of an acyl-enzyme intermediate (RCO_2Enz). This intermediate then reacts with an added nucleophile (Nu^2H), leading to the liberation of another product $RCONu$,[2] and the free enzyme is ready for another catalytic cycle.[4] On the basis of this mechanism, the terms interesterification (a reaction between two esters) and transesterification (a reaction between an ester and an alcohol) are often used as synonyms because in both cases an alcohol serves as Nu^2H. We found it necessary to differentiate the terms and to point out that the nucleophile (Nu^1H) does not necessarily leave the active site (as expected on the basis of the mechanism) but rather may stay there and react as Nu^2H when such an enzyme forms an acyl-enzyme intermediate with **2**, leading to the formation of 2,2,2-trifluoroethyl ester **3**. As an experimental basis to this conclusion, 2,2,2-trifluoroethanol which is formed from the butanoate ester through the CAL-B-catalyzed formation of an acyl enzyme intermediate is able to form products **3**, whereas a separately added 2,2,2-trifluoroethanol is not. We have observed similar behavior previously.[4]

In summary, we have demonstrated a two-step lipase-catalyzed kinetic approach for the preparation of a number of β-dipeptides under mild reaction conditions (at room temperature (23 °C) and without the use of coupling reagents). The method is based on an activation step (formation of a *N*-protected amino ester as a 2,2,2-trifluoroethyl ester) through CAL-B-catalyzed interesterification followed by the CAL-A-catalyzed *N*-acylation of an amino ester without purifying the products while replacing the lipase with another. In peptide synthesis, the amino ester was used in excess, and the unreacted counterpart was easily separated and thus recyclable.

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Supporting Information Available: Experimental procedures and spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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