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Enzymatic synthesis of activated esters and their subsequent use in enzyme-based peptide synthesis

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ABSTRACT

Chemoenzymatic peptide synthesis is potentially the most cost-efficient technology for the synthesis of short and medium-sized peptides. However, there are still some limitations when challenging peptides, e.g. containing sterically demanding acyl donors, non-proteinogenic amino acids or proline residues, are to be synthesized. To remedy these limitations, special ester moieties have been used that are specifically recognized by the enzyme, e.g. guanidinophenyl, carboxamidomethyl (Cam) or trifluoroethyl (Tfe) esters, which, unfortunately, are notoriously difficult to synthesize chemically. Herein, we demonstrate that Cam and Tfe esters are very useful for Alcalase-CLEA mediated peptide synthesis using sterically demanding and non-proteinogenic acyl donors as well as poor nucleophiles, and combinations thereof. Furthermore, these esters can be efficiently synthesized by using the lipase Cal-B or Alcalase-CLEA. Finally, it is shown that the ester synthesis by Cal-B and subsequent peptide synthesis by Alcalase-CLEA can be performed simultaneously using a two-enzyme-one-pot approach with glycolamide or 2,2,2-trifluoroethanol as additive.

1. Introduction

In the past few decades, a large number of peptides have been introduced onto the market since they can be used either as a therapeutic or as prodrug [1], and an even increasing number is in clinical trails. Additionally, peptides have found applications as nutritional additive or as a cosmetic ingredient [2]. Despite this demand for peptides, their production on large scale remains expensive and time consuming [3]. Common peptide synthesis approaches are fermentation, solid-phase or solution-phase chemical peptide synthesis, and chemo-enzymatic peptide synthesis [4]. Currently, the fermentative approach is well feasible for large peptides (>50 amino acid residues) and proteins containing natural fragments and requires a large development effort for each individual peptide/protein. Solid phase peptide synthesis (SPPS) is the most commonly used method for medium-sized and long peptides (10–50 amino acid residues). However, SPPS requires besides full protection of the functionalized side chains, also expensive and environmentally unfriendly coupling reagents in at least stoichiometric amounts. Furthermore, the requirement of functionalized resins and the use of reagent excess makes SPPS an expensive method. Solution phase chemical peptide synthesis is most commonly used for the synthesis of small peptides containing two to ten amino acid residues. Also this approach requires expensive coupling reagents and of major concern is the uncontrolled *C*-terminal racemization during fragment assembly. Finally, chemo-enzymatic peptide synthesis, wherein peptide fragments are elongated enzymatically, has been studied in academia during the past decades and proved to be suitable for certain short peptide sequences up to five amino acid residues [4]. The application of enzymes as coupling reagent is a promising alternative since functionalized amino acid side chain do not require full protection and most importantly, *C*-terminal racemization is completely absent during fragment assembly, which is beneficial for the characterization and purification of the final peptide.

There are two approaches toward enzymatic peptide synthesis, either the thermodynamically, or the kinetically controlled approach [5]. In thermodynamically controlled peptide synthesis, an *N*-terminally protected acyl donor reacts with a *C*-terminally protected amino acid acceptor as the nucleophile, resulting in the formation of the peptide bond, and one water molecule is expelled. Thermodynamically controlled peptide synthesis is, however, rather slow and the thermodynamic equilibrium between product and starting materials needs to be shifted into the synthetic direction, for example by product precipitation, by water withdrawal, or by using organic solvents, to obtain a high product yield. This is in contrast to the kinetically controlled peptide synthesis, in which an *N*-terminally protected and *C*-terminally activated

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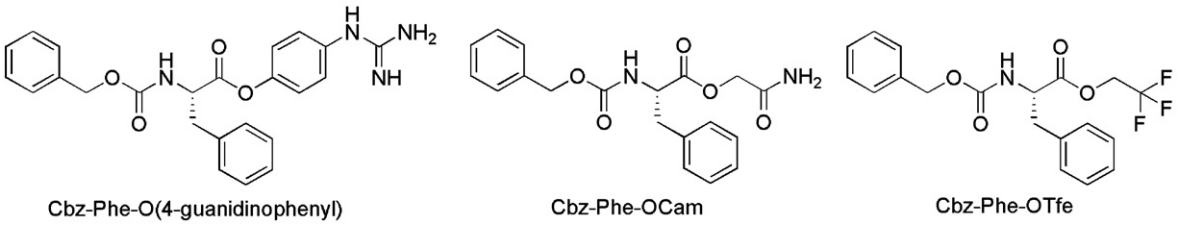


Fig. 1. Representative examples of acyl donors that are specifically recognized by certain proteases.

amino acid ester reacts preferentially with a *C*-terminally protected acyl acceptor, to give a high product yield in a generally shorter reaction time.

The high selectivity of enzymes restricts the number of amino acids that will be recognized. Therefore, coupling of sterically demanding amino acid as acyl donors (valine, isoleucine, threonine), notoriously weak nucleophiles (proline), or D- and other non-proteinogenic amino acid residues, remains rather challenging. To remedy the restricted access toward the primary specificity pocket, designed acyl donors with an ester moiety that is specifically recognized by the enzyme, among others, guanidinophenyl (Gp) [6], carboxamidomethyl (Cam) [7] or trifluoroethyl (Tfe) [8] esters have been used (Fig. 1). Thus, successful couplings of sterically demanding acyl donors decorated with these activated ester moieties have been reported. Also, in the presence of these activated esters, enzyme mediated couplings of weak nucleophiles and non-proteinogenic amino acid residues, became feasible [9]. Although these active esters broaden the scope of enzymatic peptide synthesis, their chemical synthesis is however not straightforward [10] since highly reactive coupling reagents are required to couple the rather poor nucleophilic alcohol derivatives which increases the risk of racemization [11].

Herein, we report a highly promising enzymatic approach toward the synthesis of Cam and Tfe active esters by means of cross-linked enzyme aggregates of Alcalase (Alcalase-CLEA) [12] and immobilized *Candida antartica* lipase B (Cal-B). Additionally, their subsequent application is demonstrated in an enzymatic peptide synthesis approach using a number of challenging acyl donors and nucleophiles to obtain not easily accessible dipeptides. Furthermore, we explored a 'two-enzyme-one-pot' approach, in which the activated ester is synthesized by Cal-B and simultaneously used as a substrate by Alcalase-CLEA to elongate the peptide sequence.

2. Materials and methods

2.1. Chemicals and enzyme preparation

Unless stated otherwise, chemicals were obtained from commercial sources and used without further purification. Prior to use, Alcalase-CLEA (3 g, Type OM, CLEA-technologies, 580 U/g) was suspended in ^{t}BuOH (100 mL) and crushed with a spatula. After being collected by filtration, the enzyme was resuspended in MTBE or THF (50 mL) and isolated by filtration. A stock solution of 2-hydroxyacetamide (CAS 598-42-5 from Sigma–Aldrich) was prepared by dissolving 2-hydroxyacetamide (1.25 g) in HPLC grade acetonitrile (200 mL) and $MgHPO_4$ (5.0 g) was added. The obtained suspension was stirred for 30 min at room temperature followed by filtration. Cal-B was purchased from Novozymes (immobilized Novozym®-435, LC 200204). Molsieves (3 Å, 8–12 Mesh, Acros) were activated under reduced pressure at 200 °C. ^{t}BuOH was dried on activated molsieves prior to use. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a Bruker Avance NMR spectrometer. ^{1}H chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm) or DMSO-d_6 (2.50 ppm) and ^{13}C NMR chemical shifts relative to DMSO-d_6 (39.50 ppm) or $CDCl_3$ (77.00). Column chromatography was carried out using silica gel, Merck grade 9385 60 Å. Analytical HPLC diagrams were recorded on an HP1090 Liquid Chromatograph, using a reversed-phase column (Inertsil ODS-3, C18, 5 μm particle size, 150 mm × 4.6 mm internal diameter) at 40 °C. UV detection was performed at 220 nm using a UV–VIS 204 Linear spectrometer (Varian). The gradient program was: 0–25 min linear gradient ramp from 5% to 98% eluent B and from 25.1 to 30 min at 5% eluent B (eluent A: 0.5 mL/L methane sulfonic acid (MSA) in H_2O; eluent B: 0.5 mL/L MSA in acetonitrile). Preparative HPLC was performed on a Varian PrepStar system using a stationary-phase column (Pursuit XRs, C18, 10 μm particle size, 500 mm × 41.4 mm) at room temperature. UV detection was performed at 220 nm and 254 nm using a UV–VIS Varian ProStar spectrometer. The gradient program was 20% eluent B and 80% eluent A to 95% eluent B and 5% eluent A in 30 min (eluent A: 0.1 mL/L formic acid in H_2O; eluent B: 0.1 mL/L formic acid in acetonitrile) with a flow rate of 80 mL/min and an injection volume of 10 mL. Pure fractions were pooled and lyophilized. The flow-injection analysis (FIA) experiments to determine the exact mass were performed on an Agilent 1100 LC-MS system (Agilent, Waldbronn, Germany). The ESI-MS was run in positive mode, with the following conditions: *m*/*z* 50–3200, 175 V fragmentor, 0.94 cycl/s, 350 °C drying gas temperature, 10 L N_2/min drying gas, 45 psi g nebuliser pressure and 4 kV capillary voltage. The exact mass was determined using an internal referent to recalibrate the *m*/*z* axis for each measurement. Reference dipeptides [13] as well as *N*-Cbz-protected amino acid methyl [14], Cam [15] and Tfe [16] esters were synthesized according to the literature procedures. Analytical data of all known compounds was compared to those reported in the literature which is cited in the supplementary information.

2.1.1. Cbz-D-Phe-OTfe

This compound was synthesized from Cbz-D-Phe-OH according to the literature procedure [16] and obtained as a white solid; R_t(HPLC) 22.37 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 2.99–3.10 (m, 2H), 4.37–4.46 (m, 2H), 4.65–4.71 (m, 1H), 5.02–5.09 (m, 3H), 7.02–7.30 (m, 10H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 37.8, 54.5, 60.6, 61.1, 67.1, 127.3, 128.0, 128.2, 128.4, 128.7, 129.0, 134.9, 170.1; FIA-ESI(+)-TOF-MS: *m*/*z* $[M+H]^+$ calcd for $C_{19}H_{19}F_3NO_4$: 382.1261; found: 382.1249.

2.2. Enzymatic peptide synthesis

Alcalase-CLEA (100 mg) was added to a mixture of MTBE or THF (3 mL) containing activated 3 Å molsieves (200 mg). Subsequently, the *N*-Cbz-protected amino acid or dipeptide ester (100 mg) followed by a *C*-terminally protected amino acid or dipeptide (1.5 equiv) were added to the enzyme suspension. The reaction mixture was shaken at 150 rpm at 50 °C for 16 h. After filtration, the remaining solids were subsequently washed with EtOAc (3 × 10 mL), CH_2CL_2 (3 × 10 mL) and MeOH (3 × 10 mL). The combined filtrate was concentrated *in vacuo* and the resulting residue was purified by one of the following three different methods.

In first method, depending on the solubility of the product, the peptides were purified by column chromatography using EtOAc/*n*-heptane or CH_2Cl_2/MeOH as eluent. In the second method, the residue was redissolved in EtOAc or CH_2Cl_2 (50 mL) and the solution was washed with sat. aq. $NaHCO_3$ (25 mL), 1 mM aq. HCl (25 mL), and brine (25 mL). The organic layer was dried (Na_2SO_4), concentrated *in vacuo*, and the volatiles were co-evaporated with toluene (2 × 20 mL) and $CHCl_3$ (2 × 20 mL). The third method was purification of the peptides by preparative HPLC.

2.2.1. Cbz-L-Phe-L-Pro-NH_2

White solid; R_t(HPLC) 16.53 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.65–1.69 (m, 3H), 2.00–2.25 (m, 1H), 2.85–3.01 (m, 2H), 3.37–3.57 (m, 1H), 4.33–4.48 (m, 1H), 4.68 (q, 1H, J = 15.0 and 7.5 Hz), 5.00 (q, 2H, J = 18.9 and 11.4 Hz) 5.45 (s, 1H), 5.72 (d, 1H, J = 8.7 Hz), 6.32 (s, 1H), 7.15–7.26 (m, 11H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 24.4, 28.2, 38.4, 47.8, 54.6, 60.0, 67.1, 127.3, 127.9, 128.2, 128.5, 128.6, 129.3, 135.7, 136.1, 156.4, 171.4, 173.5; FIA-ESI(+)-TOF-MS: m/z $[M+H]^+$ calcd for $C_{22}H_{26}N_3O_4$: 396.1918; found: 396.1925.

2.2.2. Cbz-D-Phe-L-Pro-NH_2

White solid; R_t(HPLC) 16.54 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.49–2.12 (m, 5H), 2.50 (q, 1H, J = 7.5 Hz), 2.93 (d, 2H, J = 6.6 Hz), 3.49–3.54 (m, 1H), 4.33–4.50 (m, 2H), 4.98 (d, 2H, J = 3.3 Hz), 5.41 (s, 1H), 5.85 (d, 1H, J = 6.6 Hz), 6.71 (s, 1H), 7.14–7.27 (m, 11H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 24.4, 28.2, 38.4, 47.8, 54.6, 60.0, 67.1, 127.3, 127.9, 128.2, 128.5, 128.6, 129.3, 135.7, 136.1, 156.4, 171.4, 173.5; FIA-ESI(+)-TOF-MS: m/z $[M+H]^+$ calcd for $C_{22}H_{26}N_3O_4$: 396.1918; found: 396.1920.

2.2.3. Cbz-D-Phe-L-Leu-NH_2

White solid; R_t(HPLC) 18.37 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.77 (dd, 6H, J = 10.2 and 6.3 Hz), 1.24–1.43 (m, 3H), 2.74–2.95 (m, 2H), 4.13–4.32 (m, 2H), 4.95 (s, 1H), 7.01 (s, 1H), 7.25–7.32 (m, 11H), 7.57 (d, 1H, J = 7.8 Hz), 8.14 (d, 1H, J = 8.1 Hz); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 21.2, 23.0, 23.9, 37.4, 50.6, 56.2, 65.2, 126.1, 127.4, 127.6, 127.9, 128.2, 129.1, 136.8, 137.5, 155.8, 171.0, 173.9; FIA-ESI(+)-TOF-MS: m/z $[M+H]^+$ calcd for $C_{23}H_{30}N_3O_4$: 412.2231; found: 412.2215.

2.2.4. Cbz–L-Val–L-Pro-NH_2

White solid; R_t(HPLC) 14.52 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 0.90 (dd, 6H, J = 11.1 and 6.9 Hz), 1.87–2.31 (m, 6H), 3.50–3.58 (m, 1H), 3.64–3.70 (m, 1H), 4.24–4.29 (m, 1H), 4.50–4.54 (m, 1H), 5.02 (d, 2H, J = 5.1 Hz), 5.45–5.51 (m, 2H), 6.74 (s, 1H), 7.19–7.29 (m, 6H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.3, 18.9, 24.4, 29.1, 29.7, 46.9, 57.8, 59.1, 65.3, 79.1, 127.5, 127.6, 128.2, 137.0, 156.1, 170.0, 173.3; FIA-ESI(+)-TOF-MS: m/z $[M+H]^+$ calcd for $C_{18}H_{26}N_3O_4$: 348.1918; found: 348.1908.

2.2.5. Cbz-L-Ala-L-Ala-Gly-L-Phe-NH_2

White solid; R_t(HPLC) 14.28 min; ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 1.16–1.29 (m, 6H), 2.74–3.06 (m, 2H), 3.56–3.77 (m, 2H), 4.05–4.12 (m, 1H), 4.22–4.46 (m, 1H), 5.02 (d, 2H, J = 6.9 Hz), 7.10–7.48 (m, 14H), 7.93–8.21 (m, 3H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.9, 48.2, 53.7, 65.3, 65.4, 126.1, 127.6, 127.9, 128.2, 129.0, 136.9, 137.9, 138.0, 155.5, 168.2, 168.3, 172.3, 172.6, 172.7; FIA-ESI(+)-TOF-MS: m/z $[M+H]^+$ calcd for $C_{25}H_{32}N_5O_6$: 498.2347; found: 498.2346.

2.3. Cam or Tfe ester synthesis using Alcalase-CLEA

Alcalase-CLEA (100 mg) was added to a mixture of MTBE or THF (3 mL), 3 Å molsieves (200 mg), glycolamide or 2,2,2-trifluoroethanol (200 mg), and *N*-Cbz-protected amino acid (50 mg). The reaction mixture was shaken at 150 rpm at 50 °C for 72 h. Purification of the ester was performed as described in Section 2.2.

2.4. Cam or Tfe ester synthesis using Cal-B

Cal-B (100 mg) was added to a mixture of MTBE or acetonitrile (3 mL), 3 Å molsieves (100 mg), carbamoylmethanol or 2,2,2-trifluoroethanol (200 mg), and *N*-Cbz-protected amino acid or dipeptide (50 mg). The reaction mixture was shaken at 150 rpm at 50 °C for 16 h. Purification of the ester was performed as described in Section 2.2.

2.4.1. Cbz-L-Ala-L-Ala-OTfe

White solid; R_t(HPLC) 18.06 min; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.30-1.36 (m, 6H), 4.21–4.59 (m, 4H), 5.03 (s, 2H), 5.37 (d, 1H, J = 6.6 Hz), 6.67 (s, 1H), 7.19–7.26 (m, 5H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 17.6, 18.4, 47.9, 50.3, 60.1, 60.6, 61.1, 61.6, 67.1, 67.2, 120.8, 124.5, 128.0, 128.2, 128.5, 136.1, 156.0, 171.2, 172.1; FIA-ESI(+)-TOF-MS: m/z $[M+H]^+$ calcd for $C_{16}H_{20}F_3N_2O_5$: 377.1319; found: 377.1341.

2.5. Dipeptide synthesis with simultaneous use of Alcalase-CLEA and Cal-B

To a mixture of Alcalase-CLEA (25 mg) and Cal-B (100 mg) in acetonitrile (3 mL) was added 3 Å molsieves (200 mg) and the appropriate alcohol (200 mg). Then, the *N*-Cbz-protected amino acid (50 mg) and subsequently the amino acid amide (1.0 equiv) were added. The obtained reaction mixture was shaken at 150 rpm at 50 °C for 16 h.

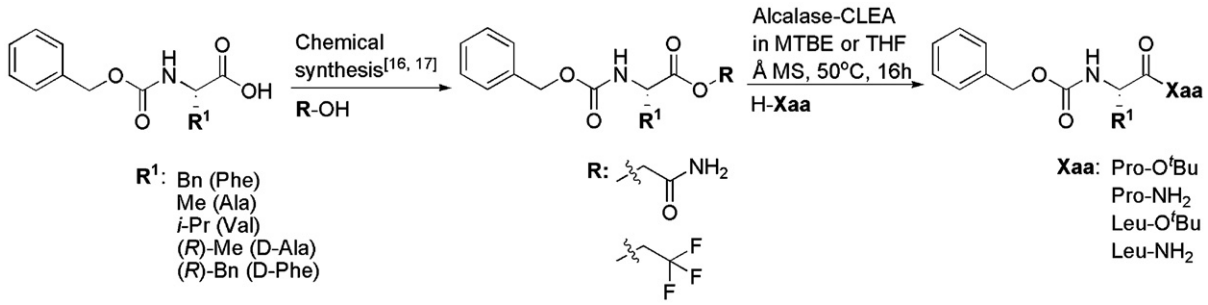
2.6. ^{t}Bu-ester hydrolysis using Alcalase-CLEA

Alcalase-CLEA (20 mg) was suspended in dioxane/water (2 mL, 9/1, v/v) or DMF/water (2 mL, 1/1, v/v) and then the dipeptide ^{t}Bu-ester (50 mg) was added. The reaction mixture was shaken at 150 rpm at 37 °C for 16 h. Purification of the dipeptide carboxylic acid was performed as described in Section 2.2.

3. Results and discussion

3.1. Alcalase-CLEA catalyzed peptide synthesis using Cam and Tfe esters

As reported previously by us, Alcalase-CLEA mediated peptide synthesis was highly efficient in anhydrous organic solvents for the coupling of *N*-terminally protected amino acid *C*-terminal methyl esters with *C*-terminally protected amino acid nucleophiles [17]. Encouraged by these results, we decided to explore the scope of coupling reactions in which the poor nucleophile proline was used. Although the subtilisin catalyzed coupling of proline was described in the literature [18], others observed that proline as nucleophile gave no conversion at all [19]. In our hands, coupling of Cbz-L-Phe-OMe with H-L-Pro-O^{t}Bu in the presence of Alcalase-CLEA in anhydrous THF gave an almost quantitative conversion (98%) after 24 h to the dipeptide) as judged by HPLC analysis, and the dipeptide Cbz-L-Phe-L-Pro-O^{t}Bu was isolated in 92% yield. Noteworthy, only 50 mol% excess of H-L-Pro-O^{t}Bu was used, which is a rather small amount for enzymatic coupling reactions. Despite these promising data, in case of more challenging acyl donors like Cbz-D-Phe-OMe or Cbz-L-Val-OMe, the conversion toward dipeptides Cbz-D-Phe-Pro-O^{t}Bu and Cbz-L-Val-Pro-O^{t}Bu dropped significantly to 24% and 32%, respectively. In order to improve the coupling yield, we investigated the versatility of Cam and Tfe active esters in Alcalase-CLEA mediated peptide synthesis using a number of challenging acyl



Scheme 1. Alcalase mediated synthesis of dipeptides using Tfe and Cam esters.

Table 1
Alcalase-CLEA mediated dipeptide synthesis using Cam and Tfe esters[a].

Entry	Acyl donor	Nucleophile	Dipeptide	Yield (%)[b]
1	Cbz-L-Phe-OCam	H-L-Leu-O^{t}Bu	Cbz-L-Phe-L-Leu-O^{t}Bu	87
2	Cbz-L-Phe-OCam	H-L-Pro-O^{t}Bu	Cbz-L-Phe-L-Pro-O^{t}Bu	92
3	Cbz-L-Phe-OCam	H-L-Leu-NH_2	Cbz-L-Phe-L-Leu-NH_2	91
4	Cbz-L-Phe-OCam	H-L-Pro-NH_2	Cbz-L-Phe-L-Pro-NH_2	91
5	Cbz-L-Phe-OTfe	H-L-Pro-NH_2	Cbz-L-Phe-L-Pro-NH_2	90
6	Cbz-L-Phe-OTfe	H-L-Pro-NH_2	Cbz-L-Phe-L-Pro-NH_2	93
7	Cbz-L-Ala-OTfe	H-L-Leu-NH_2	Cbz-L-Ala-L-Leu-NH_2	94
8	Cbz-L-Ala-OTfe	H-L-Pro-NH_2	Cbz-L-Ala-L-Pro-NH_2	90
9	Cbz-D-Ala-OTfe	H-L-Leu-NH_2	Cbz-D-Ala-L-Leu-NH_2	45
10	Cbz-D-Ala-OCam	H-L-Leu-NH_2	Cbz-D-Ala-L-Leu-NH_2	92
11	Cbz-D-Ala-OCam	H-L-Pro-NH_2	Cbz-D-Ala-L-Pro-NH_2	69
12	Cbz-D-Phe-OTfe	H-L-Leu-NH_2	Cbz-D-Phe-L-Leu-NH_2	36
13	Cbz-D-Phe-OCam	H-L-Leu-NH_2	Cbz-D-Phe-L-Leu-NH_2	93
14	Cbz-D-Phe-OCam	H-L-Pro-NH_2	Cbz-D-Phe-L-Pro-NH_2	50
15	Cbz-L-Val-OCam	H-L-Leu-NH_2	Cbz-L-Val-L-Leu-NH_2	93
16	Cbz-L-Val-OCam	H-L-Pro-NH_2	Cbz-L-Val-L-Pro-NH_2	76

[a] Reaction conditions: see Section 2.2.
[b] Isolated yield.

donors and nucleophiles, as is shown in Table 1 and Scheme 1. Gp esters were not included in this study because they have poor solubility in anhydrous organic solvents and are recognized by arginine specific proteases, such as chymotrypsin and not by Alcalase.

Evidently, the Cam as well as Tfe active esters were found to be very good acyl donors in Alcalase-CLEA mediated dipeptide syntheses. Good yields were obtained in entries 1–8 and in entries 9–16 using more challenging acyl donors. Not only D-amino acids but also valine could be used in combination with the poor nucleophilic proline. There was almost no difference in efficiency in case of H-L-Leu-NH_2 or H-L-Pro-NH_2 as the nucleophilic species when the acyl donors Cbz-L-Phe and Cbz-L-Ala were used irrespective of the active ester. However, in case of more challenging acyl donors as Cbz-D-Phe, Cbz-D-Ala and Cbz-D-Val, the Cam esters turned out to be superior, while the coupling efficiency of proline was lower compared to leucine. These results triggered us to investigate the type of active ester in more detail in order to optimize such difficult coupling reactions.

3.2. Differences between the amino acid methyl, Cam and Tfe ester species and their respective enzymatic synthesis

In order to optimize the efficiency of the enzyme mediated dipeptide synthesis, the coupling of several amino acid methyl, Cam, and Tfe esters to H-L-Pro-NH_2 was investigated in the presence of Alcalase-CLEA, as shown in Table 2.

Gratifyingly, these results showed that the Cam esters, and to a lesser extent the Tfe esters, were in all cases more efficient acyl donors than the commonly used methyl ester.

During the course of our research we found that much more amino acid esters like methyl, ethyl, benzyl, as well as *tert*-butyl were accessible by Alcalase-CLEA mediated ester synthesis [20].

Table 2
Alcalase-CLEA mediated dipeptide synthesis with H-L-Pro-NH_2 as nucleophile[a].

Entry	Acyl donor	Initial rate[b]	Conversion[e] (%)
1	Cbz-L-Ala-OMe	10.6	77[c]
2	Cbz-L-Ala-OTfe	11.9	90[c]
3	Cbz-L-Ala-OCam	12.8	95[c]
4	Cbz-L-Phe-OMe	11.0	81[c]
5	Cbz-L-Phe-OTfe	12.8	93[c]
6	Cbz-L-Phe-OCam	13.1	98[c]
7	Cbz-D-Ala-OMe	1.7	35[d]
8	Cbz-D-Ala-OTfe	2.8	52[d]
9	Cbz-D-Ala-OCam	5.1	97[d]
10	Cbz-D-Phe-OMe	0.8	12[d]
11	Cbz-D-Phe-OTfe	1.9	29[d]
12	Cbz-D-Phe-OCam	3.5	65[d]
13	Cbz-L-Val-OMe	0.9	11[d]
14	Cbz-L-Val-OTfe	1.4	21[d]
15	Cbz-L-Val-OCam	2.9	50[d]

[a] Reaction conditions: see Section 2.2, only 25 mg of Alcalase-CLEA was used and the products were not isolated
[b] Initial rates were determined by HPLC and are expressed as %conversion per hour per 25 mg of Alcalase-CLEA.
[c] HPLC conversion after: 10 h.
[d] HPLC conversion after: 24 h.
[e] Conversions to dipeptide product compared to acyl donor starting material.

Therefore, we tried to synthesize Cbz-L-Phe-OCam as well as Cbz-L-Phe-OTfe in the presence of the respective alcohols and Alcalase-CLEA (reaction conditions see Section 2.3). Both esters could be obtained, however, their formation was rather slow and as a result of this the yield was relatively low (24% and 57%, respectively). Since the lipase Cal-B is known to catalyze ester synthesis in anhydrous organic solvents, we used this enzyme for the synthesis of the desired Cam and Tfe active esters. We found that Cal-B was highly efficient, and several amino acids as well as dipeptides were accepted as substrate, as shown in Table 3.

3.3. Fully enzymatic peptide synthesis

Since the active esters were synthesized enzymatically, and on their turn can be used as versatile acyl donors in the next

Table 3
Cal-B mediated synthesis of Tfe and Cam esters[a].

Entry	Ester product	Yield (%)[b]
1	Cbz-Gly-OTfe	93
2	Cbz-L-Pro-OTfe	91
3	Cbz-L-Ala-OTfe	96
4	Cbz-L-Ala-OCam	80
5	Cbz-D-Ala-OTfe	93
6	Cbz-D-Ala-OCam	65
7	Cbz-L-Ala-L-Ala-OTfe	77

[a] Reaction conditions: see Section 2.4.
[b] Isolated yield.

Table 4
Fully enzymatic peptide synthesis using Cal-B and Alcalase-CLEA.

Entry	Ester product[a]	Peptide product[b]	Yield (%)[c]	Deprotected product[d]	Yield (%)[e]
1	Cbz-Gly-OTfe	Cbz-Gly-L-Phe-NH_2 [22]	89		
2	Cbz-Gly-OTfe	Cbz-Gly-L-Leu-NH_2 [22]	88		
3	Cbz-Gly-OTfe	Cbz-Gly-L-Phe-O^{t}Bu	90	Cbz-Gly-L-Phe-OH [22]	88
4	Cbz-L-Ala-OTfe	Cbz-L-Ala-L-Leu-O^{t}Bu	91	Cbz-L-Ala-L-Leu-OH [22]	86
5	Cbz-L-Ala-OTfe	Cbz-L-Ala-L-Phe-O^{t}Bu	92	Cbz-L-Ala-L-Phe-OH [22]	90
6	Cbz-L-Phe-OTfe	Cbz-L-Phe-L-Leu-O^{t}Bu	87	Cbz-L-Phe-L-Leu-OH [22]	84
7	Cbz-L-Ala-L-Ala-OTfe	Cbz-L-Ala-L-Ala-Gly-L-Phe-NH_2	85		
8	Cbz-D-Ala-OCam	Cbz-D-Ala-D-Ala-O^{t}Bu	75		

[a] Reaction conditions: Sections 2.4 and 2.3 for entry 6.
[b] Reaction conditions Section 2.2.
[c] Purified yield compared to acyl donor.
[d] Reaction conditions: Section 2.6.
[e] Purified yields compared to acyl donor.

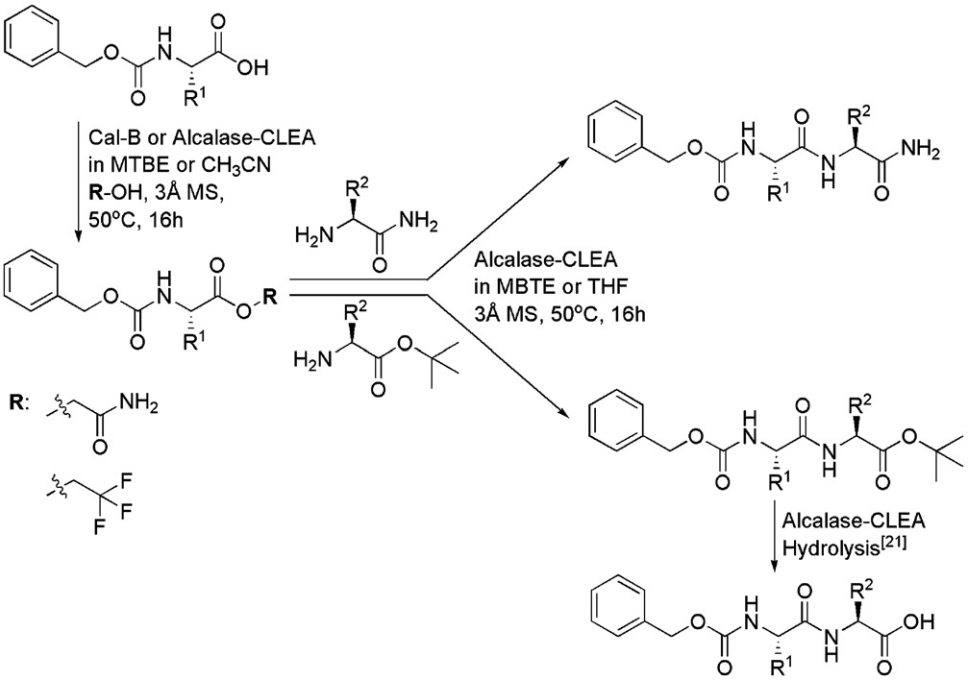
coupling step, we explored the fully enzymatic synthesis of several biologically interesting peptides, as shown in Table 4 and Scheme 2.

Dipeptide amides were easily accessible (entries 1–2), as well as dipeptide *tert*-butyl esters (entries 3–6). With respect to the latter, enzymatic hydrolysis [21] by Alcalase-CLEA gave the dipeptide acid that could be used as substrate for Cal-B to give the corresponding Tfe active ester as the dipeptide acyl donor for the next coupling step. Interestingly, also a dipeptide Tfe ester was recognized by the enzyme and reacted with a dipeptide amide to give a tetrapeptide in high yield (entry 7). Furthermore, not only proteinogenic amino acids, also H-D-alanine *tert*-butyl ester was recognized by the enzyme as a nucleophile to give the all D-dipeptide, Cbz-D-Ala-D-Ala-O^{t}Bu, (entry 8).

3.4. Simultaneous esterification and peptide coupling: two-enzymes-one-pot approach

Finally, we investigated the possibility of performing the esterification and subsequent dipeptide formation in the presence of Cal-B and Alcalase-CLEA in a one pot approach. Indeed, starting from an *N*-terminally protected amino acid as the acyl donor and an amino acid amide as the nucleophile, in the presence of both enzymes with either 2,2,2-trifluoroethanol or glycolamide as additive, the corresponding dipeptide was obtained, as shown in Table 5 and Scheme 3.

Although Alcalase-CLEA and Cal-B were separately capable of catalyzing dipeptide formation, the obtained yields were significantly higher if both enzymes were combined, probably because the esterification equilibrium as mediated by Cal-B is shifted to the ester side since the ester is consumed in the Alcalase-CLEA mediated coupling reaction. Without addition of an alcohol the dipeptide yields remained low. No deactivation of the enzymes was observed, probably due to the fact both enzymes were immobilized and water was absent. For optimal results, only one equivalent of nucleophile was necessary. Thus, by applying this two-enzyme-one-pot approach, dipeptides were obtained in high yields starting from readily accessible amino acids with a free carboxylic acid functionality acyl donor and an amino acid with a free N^{α}-amino moiety nucleophile.

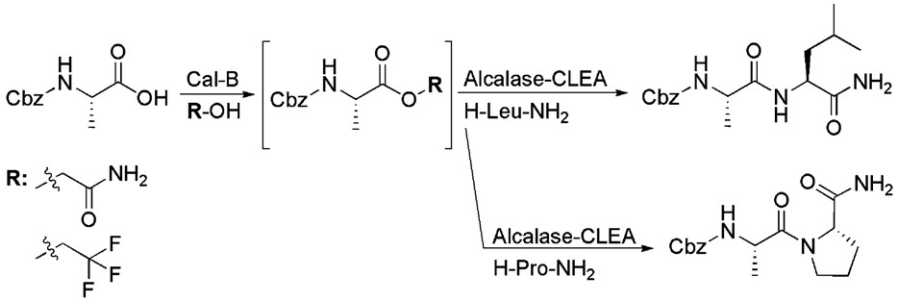


Scheme 2. Cal-B or Alcalase-CLEA mediated Tfe or Cam ester synthesis followed by Alcalase-CLEA catalyzed peptide synthesis and subsequent *tert*-butyl ester hydrolysis according to literature reference [21] (for entry 3–6 Table 4).

Table 5
Dipeptide synthesis using a Cal-B and Alcalase-CLEA two-enzyme-one-pot approach with 2,2,2-trifluoroethanol or glycolamide as additive[a].

Product	Enzyme(s)	Alcohol	Ester (%)	Dipeptide (%)
Cbz-L-Ala-L-Leu-NH_2	Alcalase-CLEA	Tfe-OH	1	13
Cbz-L-Ala-L-Leu-NH_2	Cal-B	Tfe-OH	30	31
Cbz-L-Ala-L-Leu-NH_2	Cal-B and Alcalase-CLEA	Tfe-OH	2	87
Cbz-L-Ala-L-Pro-NH_2	Alcalase-CLEA	Tfe-OH	0	9
Cbz-L-Ala-L-Pro-NH_2	Cal-B	Tfe-OH	32	14
Cbz-L-Ala-L-Pro-NH_2	Cal-B and Alcalase-CLEA	Tfe-OH	1	27
Cbz-L-Ala-L-Leu-NH_2	Alcalase-CLEA	Cam-OH	0	15
Cbz-L-Ala-L-Leu-NH_2	Cal-B and Alcalase-CLEA	Cam-OH	1	88
Cbz-L-Ala-L-Pro-NH_2	Alcalase-CLEA	Cam-OH	0	11
Cbz-L-Ala-L-Pro-NH_2	Cal-B and Alcalase-CLEA	Cam-OH	3	31

[a] Reaction conditions: Section 2.5, conversions were calculated by HPLC assuming that absorption coefficients of starting material and products were identical.



Scheme 3. Simultaneous esterification and peptide synthesis using a two-enzyme-one-pot approach.

4. Conclusions

Herein, we have shown that Cam and Tfe active esters are very useful to achieve highly efficient Alcalase-CLEA mediated peptide synthesis. These active esters allow the use of sterically demanding and non-proteinogenic acyl donors as well as poor nucleophiles, and combinations thereof. Furthermore, the Cam and Tfe active esters based on amino acids can be enzymatically synthesized by the lipase Cal-B. Finally, a fully enzymatic peptide synthesis approach was developed by combination of two enzymes in which the esterification is performed by Cal-B, while Alcalase-CLEA is responsible for peptide synthesis, also in a two-enzyme-one-pot approach. We are currently exploring whether this technology can be used for the synthesis of longer peptides using also the more hydrophilic amino acid residues.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.03.012.

References

[1] (a) B. Steffansen, C.U. Nielsen, S. Frokjaer, Eur. J. Pharm. Biopharm. 60 (2005) 241–245;
(b) K. Adermann, Curr. Prot. Pept. Sci. 6 (2005) 205–206.
[2] (a) H. Korhonen, A. Pihlanto, Curr. Pharm. Des. 9 (2003) 1297–1308;
(b) J. Torres-Llanez, B. Vallejo-Cordoba, A.F. González-Córdova, Arch. Latinoam. Nutr. 55 (2005) 111–117;
(c) H. Korhonen, A. Pihlanto, Curr. Pharm. Des. 13 (2007) 829–843;
(d) M.B. Roberfroid, J. Nutr. 137 (2007) 2493–2502.
[3] D. Hans, P.R. Young, D.D.P. Fairlie, Med. Chem. 2 (2006) 627–646.
[4] F. Guzmán, S. Barberis, A. Illanes, J. Biotechnol. 10 (2007) 279–314.
[5] F. Bordusa, Chem. Rev. 102 (2002) 4817–4867.
[6] V. Čeřovsky, F. Bordusa, J. Pept. Res. 55 (2000) 325–329.
[7] T. Miyazawa, E. Ensatsu, N. Yabuuchi, R. Yanagihara, T. Yamada, J. Chem. Soc. Perkin Trans. 1 (2002) 390–395.
[8] T. Miyazawa, S. Nakajo, M. Nishikawa, K. Hamahara, K. Imagawa, E. Ensatsu, R. Yanagihara, T. Yamada, J. Chem. Soc. Perkin Trans. 1 (2001) 82–86.
[9] (a) S.M.A. Salam, K.-I. Kagawa, K. Kawashiro, Biotechnol. Lett. 27 (2005) 1199–1203;
(b) F. Bordusa, Braz. J. Med. Biol. Res. 33 (2000) 469–485.
[10] M. Capellas, M.D. Benaiges, G. Caminal, G. Gonzalez, J. Lopez-Santín, P. Clapés, Biotechnol. Bioeng. 50 (2000) 700–708.
[11] M.K. Dhaon, R.K. Olsen, K. Ramasamy, J. Org. Chem. 47 (1982) 1962–1965.
[12] R.A. Sheldon, Biochem. Soc. Trans. 35 (2007) 1583.
[13] W. König, R. Geiger, Chem. Ber. 103 (1970) 788–798.
[14] W. H. J. Boesten, P.J. L. M. Quaedflieg, WO Patent No. 6222013, 1999.
[15] T. Miyazawa, E. Ensatsu, M. Hiramatsu, R. Yanagihara, T. Yamada, J. Chem. Soc. Perkin Trans. 1 (2002) 396–401.
[16] O.J. Park, H.G. Park, J.W. Yang, Biotechnol. Lett. (1996) 473–478.
[17] T. Nuijens, C. Cusan, T.J.G.M. van Dooren, H.M. Moody, R. Merkx, J.A.W. Kruijtzer, D.T.S. Rijkers, R.M.J. Liskamp, P.J.L.M. Quaedflieg, Adv. Synth. Catal. 352 (2010) 2399–2404.
[18] S.T. Chen, S.Y. Chen, C.L. Kao, K.T. Wang, Bioorg. Med. Chem. Lett. 4 (1994) 443–448.
[19] J.U. Klein, A. Prykhodzka, V. Čeřovsky, J. Pept. Sci 6 (2000) 541–549.
[20] (a) T. Nuijens, C. Cusan, J.A.W. Kruijtzer, D.T.S. Rijkers, R.M.J. Liskamp, P.J.L.M. Quaedflieg, Synthesis 5 (2009) 809–814;
(b) T. Nuijens, J.A.W. Kruijtzer, C. Cusan, D.T.S. Rijkers, R.M.J. Liskamp, P.J.L.M. Quaedflieg, Tetrahedron Lett. 50 (2009) 2719–2721.
[21] F. Gini, I.F. Eggen, D.J. van Zoelen, C. Boeriu, Chimica Oggi 27 (2009) 24–26.
[22] (a) D.I. Mundy, W.J. Strittmatter, Cell 40 (1985) 645–656;
(b) A. Koide, M. Yoshizawa, K. Kurachi, Eur. J. Biochem. 117 (1981) 383–388;
(c) K. Olesen, M.C. Kielland-Brandt, Protein Eng. 6 (1993) 409–415;
(d) Y. Bai, R. Hayashi, J. Biol. Chem. 254 (1979) 8473–8479.