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Fully Enzymatic Peptide Synthesis using *C*-Terminal *tert*-Butyl Ester Interconversion

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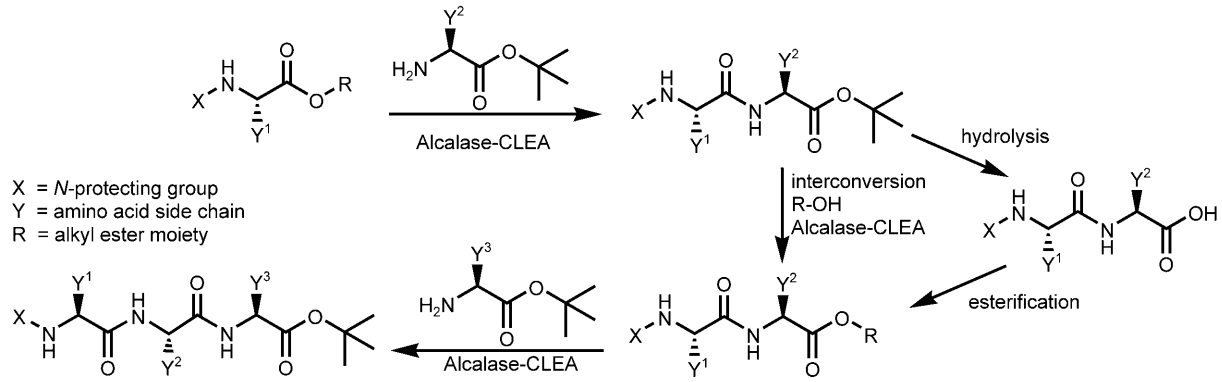
Abstract: Chemoenzymatic peptide synthesis is potentially the most cost-efficient technology for the synthesis of short and medium-sized peptides with some important advantages. For instance, stoichiometric amounts of expensive coupling reagents are not required and racemisation does not occur, thus rendering purification easier compared to chemical peptide synthesis. The economically most attractive synthesis runs in the *N*→*C* terminal direction wherein a cheap *C*-terminally protected amino acid is employed as the building block for elongation. However, *C*-terminal deprotection and activation after an elongation step – without cleavage of the side-chain protective groups or the peptide bonds – was hitherto still a challenge. In this paper we describe a novel *C*-terminal ester interconversion catalysed by the serine endopeptidase Alcalase. *C*-Terminally protected peptide *tert*-butyl esters were enzymatically converted into primary alkyl esters in quantitative yield and used directly in the next enzymatic elongation step with another amino acid *tert*-butyl ester. This fully enzymatic *N*→*C* elongation strategy by *C*-terminal ester interconversion was applied toward the synthesis of biologically active peptides up to the pentamer level.

Keywords: cross-linked enzyme aggregates (CLEA); enzyme catalysis; peptides; serine protease alcalase; transesterification

Peptides, from simple dipeptides to complex oligopeptides, are increasingly abundant on the market as (pro)drugs or in clinical development.[1] Additionally, peptides have also gained importance as nutritional and cosmetic ingredients.[2] During the past decades, an increased interest for peptide synthesis has arisen but this is still an expensive and time-consuming procedure up to date.[3]

There are four main approaches available for peptide synthesis, i.e., fermentation, solid-phase or solution-phase chemical peptide synthesis, and chemoenzymatic peptide synthesis.[4] Currently, fermentation is only well feasible for long peptides (>50 amino acids). Solid-phase and solution-phase chemical peptide syntheses are the most commonly used methods, but they require full protection of the side-chain functionalities, except in the case of fragment ligation techniques where a *C*-terminal thioester is involved, which is difficult to introduce. Moreover, to suppress racemisation, expensive and environmentally unfriendly coupling reagents are required in stoichiometric amounts. Chemoenzymatic peptide synthesis, wherein peptides are stepwise elongated enzymatically has been studied in academia during the past decades and proved to be suitable for certain short peptide sequences (up to 5 amino acids).[5] The amino acid side chains do not require protection and, most importantly, racemisation is completely absent so that purification is potentially much easier. However, (*de*)-protection of the *N*- and *C*-termini still requires harsh reaction conditions.[6] When the peptide is elongated in the *C*→*N* direction, expensive *N*-protected and *C*-activated amino acid building blocks are employed and after each coupling step the *N*-protective group should be removed. Elongation in the *N*→*C* direction (Scheme 1) would require the less expensive *C*-protected amino acid building blocks, such as carboxy amides or *tert*-butyl esters, but a selective *C*-deprotection should then be at hand.

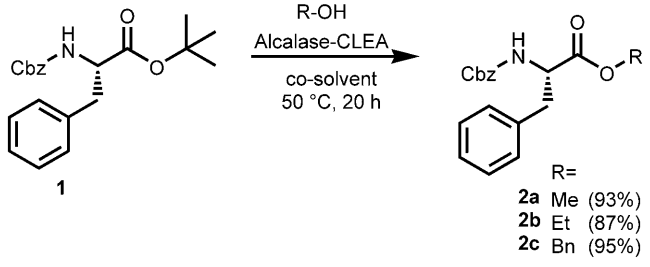


Scheme 1. Chemoenzymatic peptide synthesis in the *N*→*C* direction using *C*-terminal *t*-Bu ester interconversion.

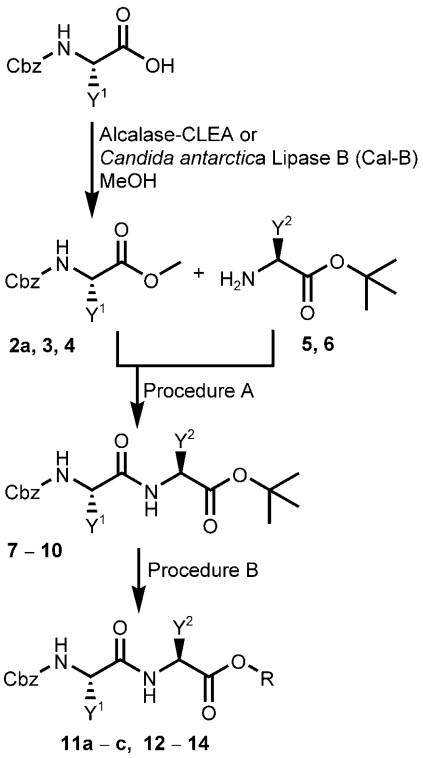
Enzymatic deprotection of *tert*-butyl esters has been described in the literature for a few enzymes under very mild reaction conditions.[7] More recently, it was discovered that the serine protease Alcalase[8] can hydrolyse *C*-terminal peptide *tert*-butyl esters.[9] However, simultaneous hydrolysis of the peptide bonds was mostly inevitable. It was earlier reported by Liu et al.[10] that the amidolytic activity of Alcalase is significantly reduced in alcoholic solvents. We now envisioned that it is possible to combine *C*-terminal deprotection and activation in one single step by interconverting *tert*-butyl esters into primary alkyl esters with Alcalase in organic solvents with an alcohol as the nucleophilic species (see Scheme 1), thus avoiding peptide bond hydrolysis since water is absent.

As a proof-of-principle for the interconversion reaction Alcalase-CLEA (cross-linked enzyme aggregates of Alcalase)[11] was used with Cbz-Phe-O-*t*-Bu **1** as the model substrate, as shown in Scheme 2.

Initial experiments showed that the interconversion of *tert*-butyl ester **1** into primary alkyl esters **2a–c** indeed proceeded smoothly. When Alcalase-CLEA was used in pure methanol or ethanol, rapid deactivation of the enzyme occurred. However, when >90% of a cosolvent (e.g., MTBE or THF) was used along with the alcohol, quantitative conversion of the *tert*-butyl ester was observed on HPLC without detectable Alcalase-CLEA deactivation. We observed some *tert*-butyl ester hydrolysis, which we could prevent by adding 3 Å molecular sieves, resulting in almost quantitative conversion of **1** into the primary alkyl esters **2a–c** (Scheme 2). We then applied this interconversion (Procedure B) on the dipeptide *C*-terminal *tert*-butyl esters **7–10** (see Scheme 3 and Table 1) which were obtained in satisfactory yield by Alcalase-CLEA mediated coupling of Cbz-protected methyl esters[12] **2a**, **3** and **4** in the presence of H-Phe-O-*t*-Bu (**5**) or H-Leu-O-*t*-Bu (**6**) (Procedure A). Since peptide bond formation and *tert*-butyl ester interconversion are catalysed by the same enzyme, one would expect *tert*-butyl to methyl ester interconversion during peptide coupling where methanol (up to 1 equiv.) is liberated



Scheme 2. Interconversion of Cbz-Phe-O-*t*-Bu into primary alkyl esters (Procedure B).



Scheme 3. Enzymatic dipeptide synthesis (Procedure A) and *C*-terminal *t*-Bu ester interconversion (Procedure B).

Table 1. Yields for the methyl esterification, coupling (Procedure A) and interconversion (Procedure B) reactions[a] as shown in Scheme 3.

Compound	Enzyme	Yield [%]
Cbz-Phe-OMe (**2a**)	Alcalase	93
Cbz-Phe-OEt (**2b**)	Alcalase	87
Cbz-Phe-OBn (**2c**)	Alcalase	95
Cbz-Ala-OMe (**3**)	Alcalase	91
Cbz-Gly-OMe (**4**)	Cal-B	93
Cbz-Phe-Leu-O-*t*-Bu (**7**)	Alcalase	77
Cbz-Ala-Leu-O-*t*-Bu (**8**)	Alcalase	85
Cbz-Ala-Phe-O-*t*-Bu (**9**)	Alcalase	86
Cbz-Gly-Phe-O-*t*-Bu (**10**)	Alcalase	81
Cbz-Phe-Leu-OMe (**11a**)	Alcalase	93
Cbz-Phe-Leu-OEt (**11b**)	Alcalase	94
Cbz-Phe-Leu-OBn (**11c**)	Alcalase	89
Cbz-Ala-Leu-OMe (**12**)	Alcalase	96
Cbz-Ala-Phe-OMe (**13**)	Alcalase	91
Cbz-Gly-Phe-OMe (**14**)	Alcalase	92

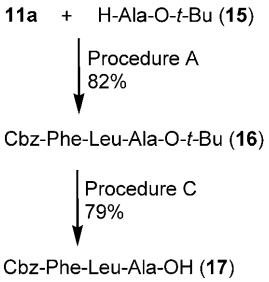
[a] Compounds **3** and **4**: methyl esterification,[12] compounds **7**–**10**: coupling (Procedure A), compounds **11**–**14**: interconversion (Procedure B).

leading to subsequent coupling of a second *tert*-butyl ester building block. Indeed this "double coupling" side reaction was observed in some cases, but only to a small extent since peptide bond formation is preferred over the much slower interconversion. Furthermore, this could always be minimised (<2%) when 4 Å molecular sieves were used to absorb the methanol.

Gratifyingly, the dipeptide *tert*-butyl esters **7**–**10** were smoothly converted into the corresponding primary alkyl esters using Alcalase-CLEA in high yields (Procedure B, Table 1, **11a–c**, **12**–**14**). Noteworthy, even the use of prolonged reaction times (48 h) did not result in any detectable peptide bond cleavage.

The applicability and versatility of this efficient and fully enzymatic peptide elongation strategy was further illustrated by the synthesis of three bioactive peptides, as shown in Scheme 4, Scheme 5 and Scheme 6.

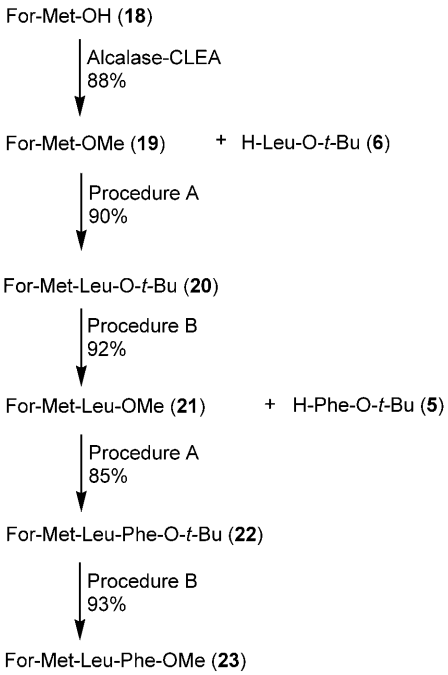
The synthesis of the thermolysine tripeptide assay substrate Cbz-Phe-Leu-Ala-OH[13] (**17**) (Scheme 4) was performed by condensing Cbz-Phe-Leu-OMe (**11a**) with H-Ala-O-*t*-Bu (**15**) using Alcalase-CLEA (82% yield) and subsequent hydrolysis of the *tert*-butyl ester **16** (Procedure C) giving the desired tripeptide **17** with a yield of 79% and with 98% purity (according to HPLC). Along with the *tert*-butyl ester deprotection, 6% peptide bond hydrolysis was observed by HPLC.



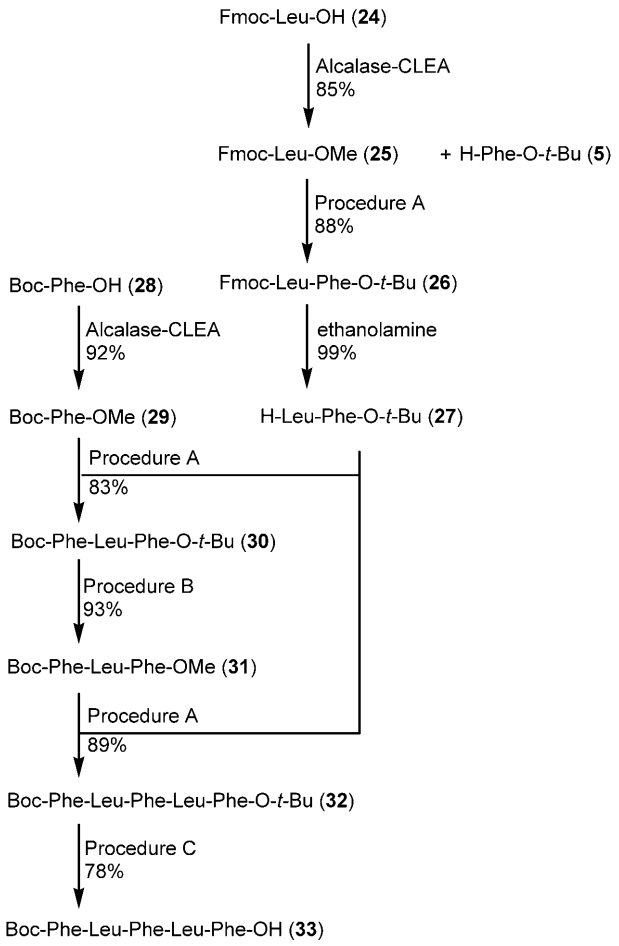
Scheme 4. Fully enzymatic synthesis of the thermolysine assay tripeptide substrate Cbz-Phe-Leu-Ala-OH.[13]

The anti-inflammatory peptide For-Met-Leu-Phe-OMe[14] (**23**) (Scheme 5) was prepared analogously by stepwise enzymatic elongation and interconversion using Alcalase-CLEA for all individual steps. The methyl esterification[12] (to furnish methyl ester **19**), coupling (to give **20** and **22**) and interconversion (yielding product **21**) reactions all proceeded smoothly and high yields were obtained (85–92%). The final transesterification of tripeptide *t*-Bu ester **22** into the corresponding Me ester yielded the anti-inflammatory tripeptide **23** with 93% yield and 97% purity (according to HPLC).

Finally, the anti-inflammatory pentapeptide Boc-Phe-Leu-Phe-Leu-Phe-OH[14a–c] (**33**) (Scheme 6), a competitive antagonist of tripeptide **23**, was synthesised by means of Alcalase-CLEA mediated interconversions and fragment condensations. Methyl esterification of acids **24** and **28** proceeded smoothly (with 85% and 92% yields, respectively) indicating that the nature of the *N*-protective group seems irrelevant for substrate recognition. The fragment condensations (products **30** and **32**) proceeded even faster than the



Scheme 5. Fully enzymatic synthesis of the anti-inflammatory tripeptide For-Met-Leu-Phe-OMe[14]; For = formyl *N*-protecting group.



Scheme 6. Chemoenzymatic synthesis of the anti-inflammatory pentapeptide Boc-Phe-Leu-Phe-Leu-Phe-OH.[14a–c]

coupling of single amino acids (giving **26**), which is in accordance with the fact that Alcalase is an *endo*protease and thus prefers peptides over single amino acids. Transesterification as well as final hydrolysis of the *tert*-butyl ester functionality (in **30** and **32**, respectively), which is chemically usually not orthogonal to *N*-Boc deprotection, proved to be well feasible using Alcalase-CLEA. The anti-inflammatory pentapeptide **33** was obtained with a yield of 78% and with 95% purity (according to HPLC).

In this paper, the fully enzymatic peptide synthesis strategy has only been demonstrated for a relatively small number of amino acids without any functional groups, but since Alcalase can accept a wide variety of (functionalised) amino acids and peptides,[8,11] we are confident that the scope of this strategy is wide. Nevertheless, we are currently investigating the robustness of this enzymatic peptide elongation strategy by using more challenging amino acid building blocks.

In conclusion, we have shown for the first time the feasibility of fully enzymatic peptide synthesis in the $N \rightarrow C$ terminal direction with simultaneous *C*-deprotection and activation of the growing oligopeptide. Cheap amino acid *tert*-butyl ester building blocks, as well as peptide fragments, could be used for the elongation. To synthesise the bioactive oligopeptides shown in this paper the use of only one enzyme, Alcalase-CLEA, was sufficient.

Experimental Section

General Methods

Before use, 3 g Alcalase-CLEA (Type OM, CLEA-Technologies, 580 U/g) was suspended in 100 mL of *t*-BuOH and crushed with a spatula. After filtration, the enzyme was re-suspended in 50 mL of MTBE or THF followed by filtration. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 MHz NMR spectrometer and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm), DMSO-d_6 (2.50 ppm for ^{1}H or 39.9 ppm for ^{13}C) or $CDCl_3$ (77.0 ppm for ^{13}C). Column chromatography was carried out using silica gel, Merck grade 9385 60 Å. Analytical HPLC chromatograms were recorded on an HP1090 liquid chromatograph, using a reversed-phase column (Inertsil ODS-3, C18, 5 μm particle size, 150×4.6 mm) at 40 °C. The flow-injection analysis (FIA) experiments to determine the exact mass were performed on an Agilent 1100 LC-MS system (Agilent, Waldbronn, Germany). Preparative HPLC was performed on a Varian PrepStar system using a stationary-phase column (Pursuit XRs, C18, 10 μm particle size, 500× 41.4 mm). Pure fractions were pooled, concentrated under vacuum and the volatiles removed by coevaporated with toluene (25 mL, 2×) and $CHCl_3$ (25 mL, 2×). The 3 Å and 4 Å molecular sieves (Acros, 8 to 12 mesh) were activated (200 °C under vacuum overnight) and used as such (uncrushed).

Typical Procedure for Enzymatic Peptide Coupling Reactions (Procedure A)

Alcalase-CLEA (1.0 g) and 4 Å molecular sieves (1.0 g) were added to a solution of Cbz-Phe-OMe (**2a**, 0.4 g, 1.3 mmol), H-Leu-O-*t*-Bu (0.48 g, 2.6 mmol, 2 equiv.) in 30 mL of MTBE (or THF). The mixture was shaken at 50 °C with 150 rpm for 20 h. After filtration, the solids were re-suspended in MeOH (50 mL) followed by filtration (3×). The combined filtrates were concentrated and the resulting residue was redissolved in 100 mL of EtOAc and washed with saturated aqueous $NaHCO_3$ solution (75 mL, 2×), aqueous HCl solution (pH 1, 75 mL, 2×), brine (75 mL) and dried (Na_2SO_4). The solution was concentrated under vacuum and the resulting crude dipeptide was purified by preparative HPLC giving Cbz-Phe-Leu-O-*t*-Bu **7** as a white solid; yield: 0.46 g (77%).

Typical Procedure for Transesterification of *t*-Bu Esters (Procedure B)

Alcalase-CLEA (1.0 g) and 3 Å molecular sieves (2 g) were added to a solution of Cbz-Phe-O-*t*-Bu (**1**, 0.50 g, 1.67 mmol) in MTBE or THF (28 mL) and MeOH (2 mL). The mixture was shaken at 50 °C with 150 rpm for 48 h. After filtration, the solids were re-suspended in MeOH

(50 mL) followed by filtration (3×). The combined filtrates were concentrated under vacuum and the remaining solid was redissolved in 100 mL of EtOAc. This solution was washed with saturated aqueous $NaHCO_3$ solution (75 mL, 2×), aqueous HCl solution (pH 1, 75 mL, 2×), brine (75 mL) and dried (Na_2SO_4). The organic phase was concentrated under vacuum and the resulting crude material was purified by preparative HPLC giving Cbz-Phe-OMe **2a** as a colourless oil; yield: 0.41 g (93%).

Typical Procedure for the Hydrolysis of Peptide *t*-Bu Esters (Procedure C)

Alcalase-CLEA (0.5 g) was added to a solution of Cbz-Phe-Leu-Ala-O-*t*-Bu (**6**, 540 mg, 1 mmol) in 18 mL of dioxane and 2 mL of water. The mixture was shaken at 37 °C with 150 rpm for 36 h. After filtration, the solids were re-suspended in MeOH (50 mL) followed by filtration (3×). The combined filtrate were concentrated under vacuum and the resulting crude acid was purified by preparative HPLC giving Cbz-Phe-Leu-Ala-OH **23** as a white solid; in 79% yield: 0.38 g (79%).

Cbz-Phe-Leu-Ala-O-*t*-Bu (16): yield: 0.56 g (82%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.24–7.05 (10H, m), 6.89 (1H, d, J = 6.9 Hz), 6.82 (1H, d, J = 7.2 Hz), 5.64 (1H, d, J = 8.4 Hz), 5.00–4.90 (2H, m), 4.42–4.29 (3H, m), 3.02–2.90 (2H, m), 1.55–1.33 (12H, m), 1.24 (3H, d, J = 7.2 Hz), 0.79 (6H, d, J = 5.7 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 18.2, 22.1, 22.7, 24.5, 27.9, 38.4, 41.3, 48.6, 51.7, 56.0, 81.8, 126.9, 127.9, 128.0, 128.4, 128.5, 129.3, 136.3, 156.0, 171.0, 171.1, 171.7; FIA-ESI(+)-TOF-MS: m/z = 540.3040, calcd. for $C_{30}H_{42}N_3O_6$ $[M+H]^+$: 540.3068.

For-Met-Leu-O-*t*-Bu (20): yield: 0.40 g (90%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.13 (1H, s), 6.51–6.46 (2H, m), 4.68 (1H, q, J = 6.9 Hz), 4.23–4.35 (1H, m), 2.59–2.53 (2H, dd), 2.07–1.87 (5H, m), 1.66–1.34 (12H, m), 0.87 (6H, dd, J = 3.9 and 6.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.0, 22.0, 22.7, 24.9, 28.0, 31.7, 41.5, 50.4, 51.7, 82.1, 160.6, 170.1, 171.4; FIA-ESI(+)-TOF-MS: m/z = 347.2003, calcd. for $C_{16}H_{32}N_2O_4S$ $[M+H]^+$: 347.1999.

For-Met-Leu-OMe (21): yield: 0.28 g (92%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.10 (1H, s), 7.24–7.18 (2H, m), 4.78 (1H, q, J = 7.8 Hz), 4.50–4.43 (1H, m), 3.04 (3H, s), 2.54 (2H, dd, J = 7.2 Hz), 2.07–1.61 (5H, m), 1.61–1.48 (3H, m), 0.85 (6H, dd, J = 3.3 and 5.7 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 15.0, 21.6, 22.6, 24.7, 29.6, 31.9, 40.7, 50.3, 50.9, 52.2, 161.0, 170.9, 172.8; FIA-ESI(+)-TOF-MS: m/z = 305.1539, calcd. for $C_{13}H_{25}N_2O_4S$ $[M+H]^+$: 305.1530.

Fmoc-Leu-OMe (25): yield: 0.44 g (85%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.69 (2H, d, J = 7.5 Hz), 7.54–7.51 (2H, m), 7.35–7.18 (5H, m), 5.07 (1H, d, J = 8.1 Hz), 4.36–4.30 (3H, m), 4.16 (1H, t, J = 6.9 Hz), 3,67 (2H, s), 1.63–1.47 (3H, m), 0.89–0.87 (6H, dd); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.8, 22.8, 24.7, 41.8, 47.2, 52.3, 67.0, 120.0, 125.1, 127.0, 127.7, 141.3, 143.8, 155.9, 173.9; FIA-ESI(+)-TOF-MS: m/z = 368.1855, calcd. for $C_{22}H_{26}NO_4$ $[M+H]^+$: 368.1856.

Fmoc-Leu-Phe-O-*t*-Bu (26): yield: 0.63 g (88%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.67 (2H, d, J = 7.5 Hz), 7.50 (2H, d, J = 6.9 Hz), 7.33–7.04 (9H, m), 6.38 (1H, d, J = 7.2 Hz), 5.16 (1H, d, J = 8.4 Hz), 4.64 (1H, q, J = 7.5 Hz), 4.38–4.22 (2H, m), 4.14–4.10 (2H, m), 2.99 (2H, dd, J = 6.0 Hz), 1.50–1.25 (12H, m), 0.84 (6H, dd, J = 3.3 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.9, 22.9, 24.6, 27.9, 38.0, 41.5, 47.1, 53.6, 67.0, 82.3, 119.9, 125.0, 125.1, 126.9, 127.0, 127.7, 128.3, 129.5, 136.0, 141.3, 143.8 (2C), 156.0, 170.2, 171.5; FIA-ESI(+)-TOF-MS: m/z = 557.2982, calcd. for $C_{34}H_{41}N_2O_5$ $[M+H]^+$.

H-Leu-Phe-O-*t*-Bu (27): yield: 0.37 g (99%); ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 8.65 (1H, d, J = 7.5 Hz), 7.24–7.20 (5H, m), 4.41 (1H, q, J = 7.2 Hz), 3.57–3.52 (1H, m), 2.97 (2H, dd, J = 2.4 and 7.8 Hz), 1.73–1.30 (1H, m), 1.54–1.32 (11H, m), 0.88 (6H, dd, J = 6.9 Hz); ^{13}C NMR ([D$_6$]DMSO, 75 MHz): δ = 21.7, 22.8, 23.5, 27.4, 36.7, 41.7, 51.3, 53.9, 80.7, 126.4, 128.1, 129.1, 136.8, 163.3, 170.0, 171.4; FIA-ESI(+)-TOF-MS: m/z = 335.2351, calcd. for $C_{19}H_{31}N_2O_3$ $[M+H]^+$: 335.2329.

Boc-Phe-Leu-Phe-O-*t*-Bu (30): yield: 0.62 g (83%); ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.23–7.07 (10H, m), 6.49 (1H, d, J = 7.8 Hz), 6.36 (1H, d, J = 7.8 Hz), 4.98 (1H, d, J = 7.8 Hz), 4.61 (1H, q, J = 7.8 Hz), 4.64–4.57 (2H, m), 2.97 (2H, dd, J = 6.3 Hz), 1.56–1.31 (21H, m) 0.79 (6H, dd, J = 6.0 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 22.0, 22.8, 24.5, 27.9, 28.2, 38.1, 41.1, 51.7, 53.7, 55.6, 80.2, 82.2, 126.9, 128.3, 128.6, 129.3, 129.5, 136.1, 136.6, 155.4, 170.2, 170.9, 171.1; FIA-ESI(+)-TOF-MS: m/z = 582.3572, calcd. for $C_{33}H_{48}N_3O_6$ $[M+H]^+$: 582.3538.

Boc-Phe-Leu-Phe-Leu-Phe-O-*t*-Bu (32): yield: 0.49 g (starting from 0.65 mmol; 89%); ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 8.22 (1H, d, J = 7.2 Hz), 7.99–7.86 (3H, m), 7.29–7.11 (15H, m), 6.90 (1H, d, J = 8.4 Hz), 4.57–4.48 (1H, m), 4.29–4.22 (3H, m), 4.06–4.05 (1H, m), 3.02–2.64 (6H, m), 1.20–1.06 (24H, m), 0.87–0.77 (1H, m); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 21.5, 22.9, 23.9, 27.4, 36.6, 37.0, 41.1, 41.2, 50.5, 50.8, 53.2, 54.0, 55.5, 77.9, 80.4, 125.9, 126.0, 126.3, 127.8, 128.0, 129.0 (2C), 137.0, 137.5, 138.1, 155.0, 170.1, 170.2, 170.4, 171.2, 171.6; FIA-ESI(+)-TOF-MS: m/z = 842.5035, calcd. for $C_{48}H_{68}N_5O_8$ $[M+H]^+$: 842.5062.

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