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# Fully Enzymatic *N*→*C*-Directed Peptide Synthesis Using *C*-Terminal Peptide α-Carboxamide to 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 rendering purification easier compared to chemical peptide synthesis. In this paper, a novel interconversion reaction of peptide *C*-terminal α-carboxamides into primary alkyl esters with alcalase was used to develop a fully enzymatic peptide synthesis strategy. For each elongation step a cost-efficient amino acid carboxamide building block was used followed by the interconversion of the elongated peptide carboxamide to the corresponding primary alkyl ester. These peptide esters are the starting materials for the next enzymatic peptide elongation step.

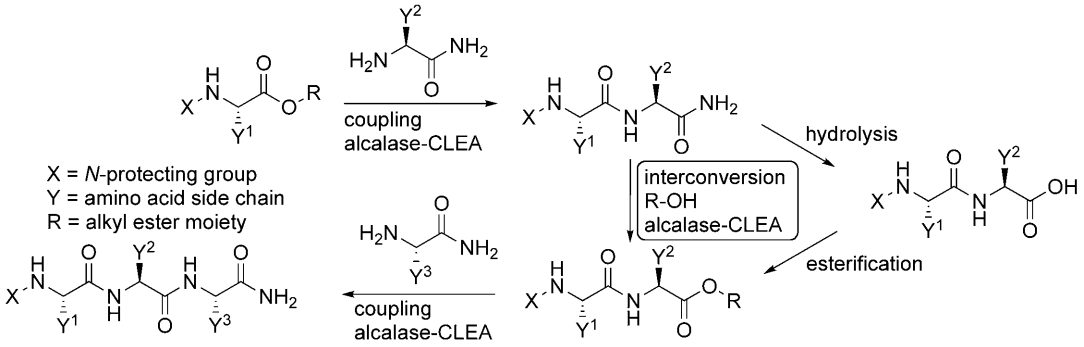
**Keywords:** cross-linked enzyme aggregates (CLEAs); enzyme catalysis; interconversion; peptides; serine protease alcalase

Although an increasing amount of peptides is used as therapeutic, (pro)drug, nutritional or cosmetic ingredient,[1] their large-scale and cost-effective production remains a huge challenge.[2] Commonly used production methods are very time-consuming and expensive.[3] There are four main approaches toward the synthesis of peptides, i.e., solid-phase or solution-phase chemical peptide synthesis, chemoenzymatic peptide synthesis and fermentation. Mostly applied are solid-phase and solution-phase chemical peptide synthesis. However, both methods require full protection of the functionalized amino acid side chains. Furthermore, expensive coupling reagents have to be used in stoichiometric amounts and in the case of fragment assembly uncontrolled racemisation of the *C*-terminally activated amino acid residue occurs during the coupling reaction.

During the past two decades, chemoenzymatic peptide synthesis has proved to be useful for the cost-effective synthesis of certain peptide sequences (up to 5 amino acids) on a large scale.[2] Herein, the amino acids are coupled enzymatically in a step-wise manner wherein the functionalised amino acid side chains require no protection and, more imporantly, racemisation is completely absent during the fragment assembly. Nevertheless, the *N*- and *C*-termini still require protection and, after each elongation step, a selective deprotection of one of the termini needs to be performed. When the peptide sequence is synthesised in the *C*→*N* terminal direction, expensive *N*-terminally protected and *C*-terminally activated amino acid building blocks are required and, after each coupling step, the *N*-terminal protecting group must be removed. Furthermore, with the growing peptide being the acyl acceptor which is generally applied in an excess over the acyl donor ,this strategy is highly cost-inefficient. Elongation of the peptide sequence in the *N*→*C* terminal direction requires an excess of the much cheaper *C*-terminally protected amino acids as building blocks, such as carboxamides (Scheme 1) or *tert*-butyl (*t*-Bu) esters. After peptide elongation, the *C*-terminus must be selectively deprotected and subsequently activated for the next enzymatic coupling step. However, the acid-catalyzed chemical hydrolysis of the *C*-terminal *t*-Bu ester or carboxamide function requires rather harsh reaction conditions and is unselective.[4]

The enzymatic hydrolysis of *t*-Bu esters under mild reaction conditions has been reported for a number




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

of lipases and proteases.[5] Unfortunately, lipases most often do not recognise peptides as their substrates, while proteases are fraught with undesired peptide bond cleavage. Recently, we described a fully enzymatic peptide synthesis strategy in which *C*-terminal interconversion of peptide *t*-Bu esters into primary alkyl esters by the industrial protease alcalase is the key step.[6] In any case, the preferred *C*-terminal protective group is the α-carboxyamide, since the amino acid α-amide building blocks are easily accessible from amino acids in a one-pot process using mineral acid-catalysed methyl esterification followed by quenching with an excess of ammonia.

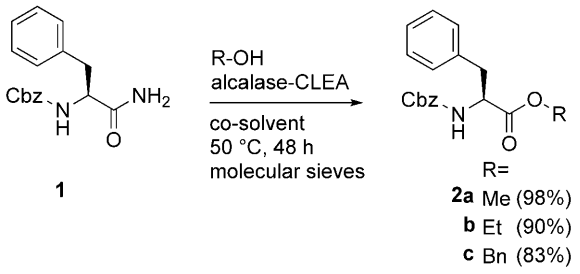
The enzymatic hydrolysis of peptide α-carboxamides is a known reaction. Selective peptide deamidases with a wide substrate tolerance have been identified, among others the peptide amidase from the *flavedo* of oranges (PAF).[7] In a single example, PAF has been used for the interconversion of peptide α-carboxamides into methyl esters,[8] albeit in a maximum yield of 40%. Moreover, PAF is not well characterised which makes fermentation by a micro-organism impossible, while extraction from orange peel requires 1000 kg to give 1 g of enzyme.

Recently, a versatile amidation of *C*-terminal peptide alkyl esters using ammonium salts as amine donor in the presence of the protease alcalase was described.[9] We envisioned that this reaction could be reversed for developing an interconversion reaction of *C*-terminal peptide α-carboxamides into alkyl esters. Subsequently, these esters can serve as intermediate for further enzymatic peptide elongation, as shown in Scheme 1.

Therefore, as a proof-of-principle for the interconversion reaction, alcalase was applied to Cbz-Phe-$NH_2$ **1** as model substrate in the presence of alcohols, as depicted in Scheme 2. The interconversion of carboxamides into primary alkyl esters (**2a–c**) proceeded smoothly and in high yield. Immobilised alcalase cross-linked enzyme aggregates (alcalase-CLEAs)[10] were used for optimal enzyme stability in anhydrous organic solvent but also for a convenient work-up (filtration) and recycling. Performing this interconversion reaction in the presence of 3 Å molecular sieves nullified the hydrolysis of the α-carboxyamide. The reverse reaction, i.e., carboxamide synthesis from the alkyl ester, by the ammonia released during interconversion, could be minimised by using a large excess of the corresponding alcohol and 5 Å molecular sieves to absorb the ammonia. To avoid deactivation of the enzyme in anhydrous methanol or ethanol, a co-solvent, i.e., tetrahydrofuran (THF) or methyl *tert*-butyl ether (MTBE), was used. When these optimal reaction conditions were used, full conversion of the carboxamide into the corresponding esters was observed (HPLC analysis), and **2a–c** were isolated in good yields (83–98%).

To investigate the scope of this novel carboxamide interconversion reaction, several dipeptide *C*-terminal carboxamides (**3a–i**) were converted into their corresponding methyl esters (**4a–h**), as is shown in Table 1.

Gratifyingly, several dipeptide α-carboxyamides were smoothly converted into their corresponding methyl esters in a good yield. It should be noted that also in cases of moderate conversion after 72 h, satisfactory yields could be obtained after prolonged reaction times. For instance, Cbz-Val-Asp-$NH_2$ (entry 8) was isolated in 78% yield after 15 days of stirring. Interestingly, the hydroxy functionality of serine




**Scheme 2.** Interconversion of Cbz-Phe-$NH_2$ into primary alkyl esters using alcalase-CLEA.

**Table 1.** Interconversion of Cbz-Val-Xaa-$NH_2$ into their corresponding methyl esters using alcalase-CLEA.

| Entry | Cbz-Val-Xaa-$NH_2$ | Cbz-Val-Xaa-OMe yield[a] |
|---|---|---|
| 1 | Cbz-Val-Ala-$NH_2$ (**3a**) | 97% (**4a**) |
| 2 | Cbz-Val-Ser-$NH_2$ (**3b**) | 89% (**4b**) |
| 3 | Cbz-Val-Leu-$NH_2$ (**3c**) | 88% (**4c**) |
| 4 | Cbz-Val-Gln-$NH_2$ (**3d**) | 73% (**4d**) |
| 5 | Cbz-Val-Phe-$NH_2$ (**3e**) | 71% (**4e**) |
| 6 | Cbz-Val-Glu-$NH_2$ (**3f**) | 67% (**4f**) |
| 7 | Cbz-Val-Asn-$NH_2$ (**3g**) | 36% (**4g**) |
| 8 | Cbz-Val-Asp-$NH_2$ (**3h**) | 16% (**4h**) |
| 9 | Cbz-Val-Gly-$NH_2$ (**3i**) | trace |

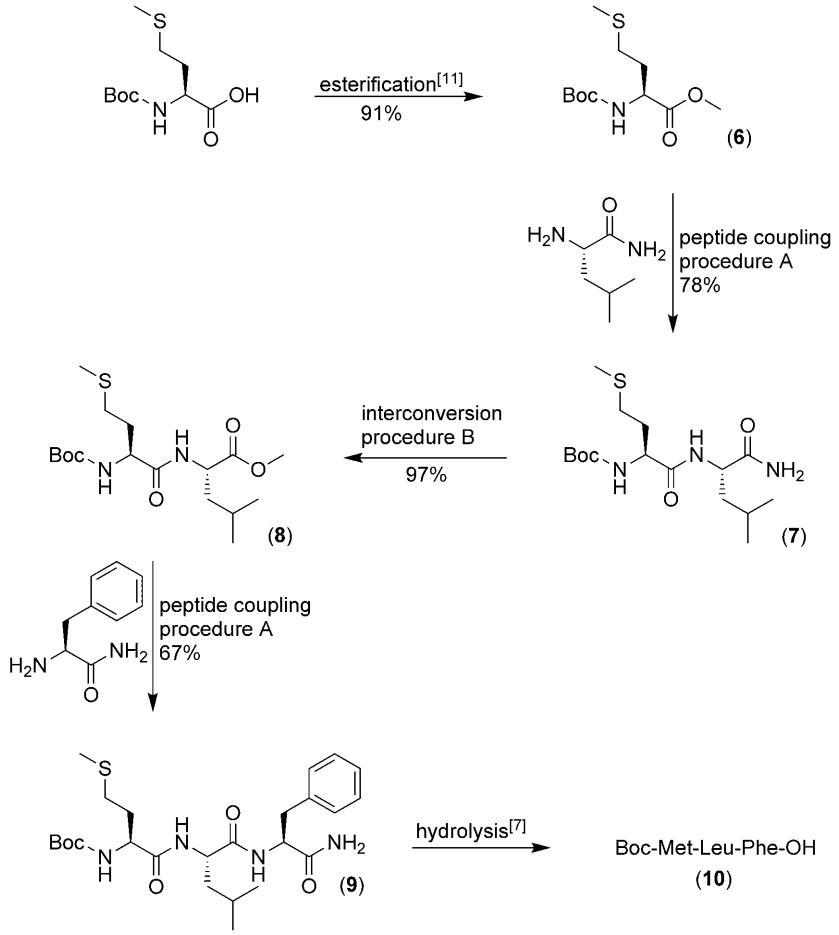
[a] Isolated yield after 72 h reaction time.

(entry 2), and even the β- and γ-carboxamides of asparagine and glutamine (entries 7 and 4, respectively), were not affected during the α-carboxamide interconversion reaction. The dipeptide product Cbz-Val-Gln-OMe (**4d**) was compared with reference compounds including Cbz-Val-Glu(OMe)-$NH_2$ (**5**), to verify that the amide interconversion was fully selective.[11]

The interconversion was also feasible with *Candida antarctica* lipase B (Cal-B) under identical reaction conditions with Cbz-Ala-$NH_2$ as substrate (93% yield). However, the interconversion of (di)peptide amides catalysed by Cal-B gave no or low yields.

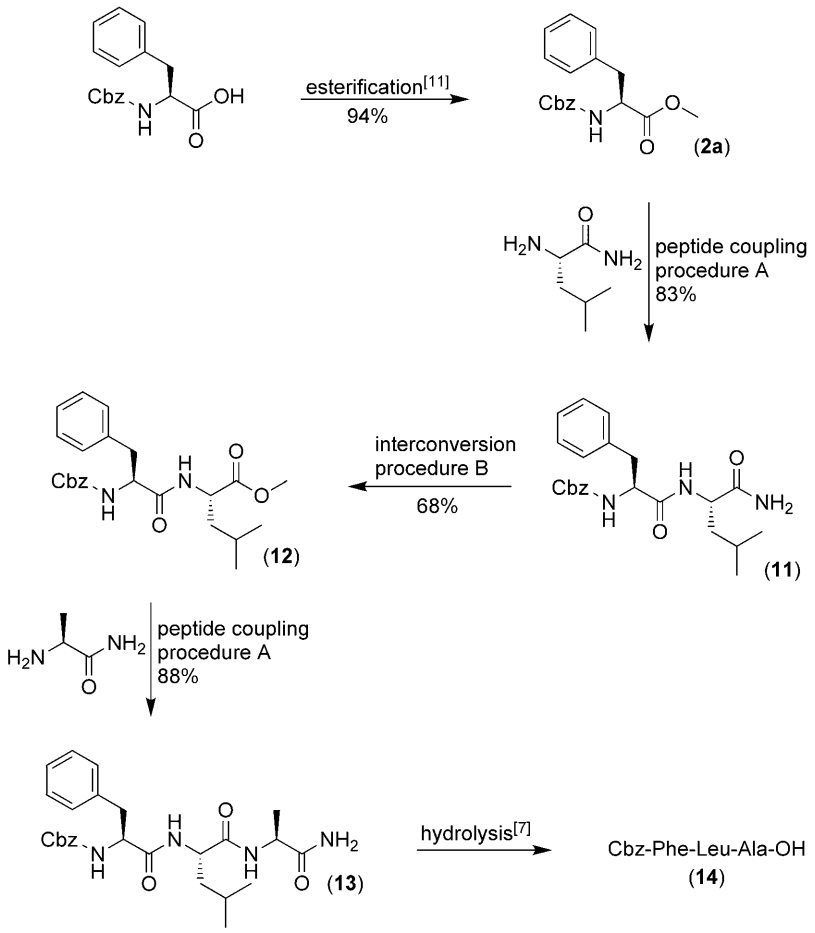
To show the versatility of the fully enzymatic peptide elongation strategy by *C*-terminal carboxyamide interconversion, two biologically interesting tripeptides were synthesised, as shown in Scheme 3 and Scheme 4.

As a first example, the synthesis of the chemotactic peptide antagonist[12] Boc-Met-Leu-Phe-OH (**10**) (Scheme 3) was performed using alcalase-CLEA for all individual reaction steps, except for the final hydrolysis of Boc-Met-Leu-Phe-$NH_2$ (**9**), which was performed with PAF (quantitative conversion according to HPLC analysis).[7] High yields were obtained for the esterification (**6**, 91%), peptide coupling (**7**, 78% and **9**, 67%) and the interconversion (**8**, 97%). The tripeptide Boc-Met-Leu-Phe-$NH_2$ (**9**) was isolated in a somewhat lower yield of 67%, since it appeared that the tetrapeptide Boc-Met-Leu-Phe-Phe-$NH_2$ (11%, verified by LC-MS analysis) had also been formed.




**Scheme 3.** Fully enzymatic $N \rightarrow C$ directed synthesis of the chemotactic peptide antagonist Boc-Met-Leu-Phe-OH[12] (**10**).




**Scheme 4.** Fully enzymatic *N*→*C* directed synthesis of the thermolysine assay substrate Cbz-Phe-Leu-Ala-OH[13] (**14**).

Since peptide coupling and interconversion are catalysed by the same enzyme, one could think of performing both reactions in one pot without isolation of the coupling product. However, for full conversion of the acyl donor generally an excess (approximately 1.5 equiv.) of the nucleophile amide is required, which would immediately be coupled with the methyl ester product of the interconversion and interconverted itself to the methyl ester which would also be prone to further coupling. Hence, this would lead to a lower yield of the interconversion product and to a laborious work-up and purification due to the presence of several by-products.

In the second example, the thermolysine substrate[13] Cbz-Phe-Leu-Ala-OH (**14**) (Scheme 4) was synthesised analogously with high yields for all individual steps. The interconversion of Cbz-Phe-Leu-$NH_2$ (**11**) into Cbz-Phe-Leu-OMe (**12**) proved to be difficult due to alcoholysis of the dipeptide backbone into Cbz-Phe-OMe (**2a**, 10%, as shown by HPLC). This side reaction, very much depending on the peptide sequence, appeared to be more pronounced when longer peptides were used. For instance, when the interconversion was performed on the pentapeptide Boc-Gly-Trp-Met-Asp(OBn)-Phe-$NH_2$, a reasonable conversion into the desired product was observed [61% of Boc-Gly-Trp-Met-Asp(OBn)-Phe-OMe as verified by LC-MS analysis]. However, also significant peptide backbone alcoholysis (up to 38%) resulting in the formation of Boc-Gly-Trp-Met-Asp(OBn)-OMe, Boc-Gly-Trp-Met-OMe and Boc-Gly-Trp-OMe as identified by LC-MS analysis, was observed. To avoid premature cleavage of the peptide backbone, the reaction should be stopped before full conversion of the starting material is obtained.

Since alcalase has a very broad substrate tolerance,[14] the fully enzymatic peptide synthesis strategy *via* amide to ester interconversion will be applicable to a large variety of industrially interesting peptides. However, for a limited number of peptide sequences the application is hampered due to undesired backbone alcoholysis which may become more prominent for longer peptides Therefore we think that the stepwise *N*→*C*-directed peptide synthesis strategy will only be applicable on a large scale for peptides up to five amino acids length.

In conclusion, we have disclosed a versatile and fully enzymatic peptide synthesis strategy based on si-

multaneous *C*-terminal deprotection and activation of the growing oligopeptide, using the easily accessible amino acid α-carboxyamides as the building blocks, and alcalase as the biocatalyst. It was shown that in case of longer peptides side-chain groups were not affected. However, a certain amount of alcoholysis was observed.

## Experimental Section

Before use, 3 g alcalase-CLEA (Type OM, CLEA-Technologies, 580 U/g) was suspended in 100 mL *t*-BuOH and crushed with a spatula. After filtration, the enzyme was re-suspended in 50 mL 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). 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 the positive mode, with the following conditions: $m/z = 50$–3200, 175 V fragmentor, 0.94 cycl/sec, 350 °C drying gas temperature, 10 L $N_2$/min drying gas, 45 psig nebuliser pressure and 4 kV capillary voltage. The exact mass was determined using an internal referent to re-calibrate the $m/z$ axis for each measurement. 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. Massa. 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 coevaporation with toluene (25 mL, 2×) and $CHCl_3$ (25 mL, 2×). The 3 Å and 5 Å molecular sieves (Acros, 8 to 12 mesh) were activated (200 °C under vacuum overnight) and used as such (uncrushed). Dipeptide amide starting materials **3a–i** and reference compound **5** were synthesised by solution-phase peptide synthesis using the EDC/HOAt strategy or by standard SPPS on a Rink-amide resin. Analytical data of compounds **2b**,[15] **4a**,[16] **4b**,[17] **4c**,[18] **4e**,[19] **6**,[20] **11**,[21] **12**[22] and **13**[23] were found identical to those reported in the literature. The identity of compounds **2a**, **2c, 10** and **15** was ascertained by comparison with commercially available samples (Bachem, Switzerland).

### Typical Procedure for Enzymatic Peptide Coupling Reactions (Procedure A)

Alcalase-CLEA (500 mg) and 4 Å molecular sieves (500 mg) were added to a solution of Boc-Met-OMe (**6**, 500 mg, 1.9 mmol), H-Leu-$NH_2$ (371 mg, 2.9 mmol, 1.5 equiv.) in 30 mL MBTE (or THF). The mixture was shaken at 50 °C with 150 rpm for 20 h. After filtration, the solids were re-suspended in DMF (50 mL) and shaken at 50 °C for 30 min, followed by filtration. This procedure was repeated three times. The combined filtrates were concentrated under vacuum and the resulting oil was purified by preparative HPLC and lyophilised from $CH_3CN/H_2O$ (3/1, v/v) giving Boc-Met-Leu-$NH_2$ **7** as a white solid; yield: 534 mg (78%).

### Typical Procedure for Enzymatic α-Carboxyamide to Ester Interconversion (Procedure B)

Alcalase-CLEA (250 mg), 3 Å molecular sieves (400 mg) and 5 Å molecular sieves (200 mg) were added to a solution of Cbz-Phe-$NH_2$ (**1**, 250 mg, 0.84 mmol) in MTBE (or THF, 14 mL) and MeOH (1 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) and shaken at 50 °C for 30 min followed by filtration. This procedure was repeated three times. The combined filtrates were concentrated under vacuum and the volatiles co-evaporated with toluene (100 mL, 2×) and $CHCl_3$ (100 mL, 2×) giving Cbz-Phe-OMe as a colorless oil; yield: 258 mg (98%). The crude compounds were purified by preparative HPLC followed by lyophilisation from $CH_3CN/H_2O$ (3/1, v/v).

**Cbz-Val-Gln-OMe (4d):** The synthesis of this compound started from Cbz-Val-Gln-$NH_2$ and **4d** was obtained as a white solid; $R_t$ (HPLC): 14.77 min; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta = 0.86$ (dd, $J = 12.3$ and 6.6 Hz, 6H, $CH_3$ 2×), 1.73–2.16 (m, 5H, CH, $CH_2$ 2×), 3.60 (s, 3H, $COOCH_3$), 3.88–3.93 (m, 1H, CH), 4.17–4.24 (m, 1H, CH), 5.02 (s, 2H, $CH_2$), 6.78 (s, 1H, NH), 7.24–7.40 (m, 7H, Ph and NH 2×), 8.33 (d, $J = 7.8$ Hz, 1H, NH); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta = 18.0$, 18.9, 26.4, 30.3, 30.9, 51.6, 59.6, 65.3, 127.5, 127.6, 128.2, 137.0, 156.0, 171.3, 172.1, 173.1; FIA-ESI(+)-TOF-MS: $m/z = 394.1986$, calcd. for $C_{19}H_{28}N_3O_6$ $[M+H]^+$: 394.1973.

**Cbz-Val-Glu-OMe (4f):** White solid; $R_t$ (HPLC): 15.69 min; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta = 0.86$ (dd, $J =$ 11.1 and 6.6 Hz, 6H, $CH_3$ 2×), 1.74–2.02 (m, 3H, CH, $CH_2$), 2.28–2.33 (m, 2H, $CH_2$), 3.60 (s, 3H, $COOCH_3$), 3.86–3.91 (m, 1H, CH), 4.25–4.28 (m, 1H, CH), 5.03 (s, 2H, $CH_2$), 7.27–7.39 (m, 6H, Ph and NH), 8.30 (d, $J = 7.2$ Hz, 1H, NH), 12.18 (s, 1H, COOH); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta = 18.1$, 19.0, 25.9, 29.7, 30.2, 51.1, 51.7, 59.8, 65.3, 127.6, 127.7, 128.2, 137.0, 156.0, 170.4, 172.0, 173.6; FIA-ESI(+)-TOF-MS: $m/z = 395.1798$, calcd. for $C_{19}H_{27}N_2O_7$ $[M+H]^+$: 395.1813.

**Cbz-Val-Asn-OMe (4g):** White solid; $R_t$ (HPLC): 14.24 min; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta = 0.84$ (dd, $J =$ 11.1 and 6.6 Hz, 6H, $CH_3$ 2×), 1.92–1.99 (m, 1H, CH), 3.00 (s, 3H, $COOCH_3$) 3.88–3.93 (m, 1H, CH), 4.57–4.61 (m, 1H, CH), 5.03 (s, 2H, $CH_2$), 6.94 (s, 1H, NH), 7.27–7.40 (m, 7H, Ph and NH 2×), 8.26 (d, $J = 7.5$ Hz, 1H, NH); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta = 18.8$, 18.9, 30.4, 36.4, 48.6, 51.7, 59.7, 65.3, 127.5, 127.7, 128.2, 137.0, 156.0, 170.7, 171.0, 171.7; FIA-ESI(+)-TOF-MS: $m/z = 380.1813$, calcd. for $C_{18}H_{26}N_3O_6$ $[M+H]^+$: 380.1816.

**Cbz-Val-Asp-OMe (4h):** White solid; $R_t$ (HPLC): 15.47 min; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta = 0.86$ (dd, $J =$ 11.1 and 6.6 Hz, 6H, $CH_3$ 2×), 1.92–2.02 (m, 1H, CH), 2.59–2.78 (m, 2H, $CH_2$), 3.16 (s, 3H, $COOCH_3$) 3.90–3.95 (m, 1H, CH), 4.61–4.63 (m, 1H, CH), 5.04 (s, 2H, $CH_2$), 7.27–7.38 (m, 6H, Ph and NH), 8.37 (d, $J = 7.5$ Hz, 1H, NH), 12.66 (s, 1H, COOH); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta = 17.8$, 18.9, 30.4, 36.6, 48.4, 51.9, 59.7, 65.3, 127.5, 127.7, 128.2, 137.0, 156.0, 171.0, 171.2, 171.3; FIA-ESI(+)-TOF-

MS: $m/z = 381.1666$, calcd. for $C_{18}H_{25}N_2O_7$ $[M+H]^+$: 381.1656.

**Cbz-Val-Glu(OMe)-$NH_2$ (5):** White solid; $R_t$ (HPLC): 14.52 min; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta = 0.86$ (dd, $J =$ 7.8 Hz, 6H, $CH_3$ 2×), 1.76–2.33 (m, 5H, CH, $CH_2$ 2×), 3.57 (s, 3H, $COOCH_3$) 3.84–3.89 (m, 1H, CH), 4.19–4.27 (m, 1H, CH), 5.03 (s, 2H, $CH_2$), 7.08 (s, 1H, NH), 7.30–7.49 (m, 7H, Ph and NH 2×), 8.01 (d, $J = 7.8$ Hz, 1H, NH); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta = 18.0$, 19.1, 27.2, 30.0, 51.2, 60.2, 65.3, 127.5, 127.6, 136.9, 156.1, 170.9, 172.7; FIA-ESI(+)-TOF-MS: $m/z = 394.1998$, calcd. for $C_{19}H_{28}N_3O_6$ $[M+H]^+$: 394.1973.

**Boc-Met-Leu-$NH_2$ (7):** White solid; $R_t$ (HPLC): 16.07 min; $^1$H NMR ($CDCl_3$, 300 MHz): $\delta = 0.86$ (dd, $J =$ 6.3 Hz, 6H, $CH_3$ 2×), 1.38 (s, 9H, $CH_3$ 3×), 1.38–1.47 (m, 3H, CH and $CH_2$), 1.73–1.83 (m, 2H, $CH_2$), 1.96–2.04 (m, 3H, $SCH_3$), 2.51 (dd, $J = 7.2$ Hz, 2H, $CH_2$), 4.11–4.17 (m, 1H, CH), 4.38–4.45 (m, 1H, CH), 5.23 (d, $J = 3.9$ Hz, 1H, NH), 5.53 (s, 1H, NH), 6.39 (s, 1H, NH), 6.65 (d, $J = 6.9$ Hz, 1H, NH); $^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta = 15.4$, 21.7, 23.0, 24.8, 28.3, 30.3, 40.5, 51.3, 54.1, 80.7, 171.7, 174.2; FIA-ESI(+)-TOF-MS: $m/z =$ calcd. for $C_{16}H_{32}N_3O_4S$ $[M+H]^+$: 362.2108; found: 362.2112.

**Boc-Met-Leu-OMe (8):** White solid; $R_t$ (HPLC): 18.63 min; $^1$H NMR ($CDCl_3$, 300 MHz): $\delta = 0.86$ (d, $J =$ 5.7 Hz, 6H, $CH_3$ 2×), 1.38 (s, 9H, $CH_3$ 3×), 1.40–1.64 (m, 3H, CH and $CH_2$), 1.81–2.06 (m, 5H, $CH_2$ and $SCH_3$), 2.54 (dd, $J = 6.9$ Hz, 2H, $CH_2$), 3.66 (s, 3H, $COOCH_3$), 4.15–4.30 (m, 1H, CH), 4.50–4.57 (m, 1H, CH), 5.08 (d, $J = 7.5$ Hz, 1H, NH), 6.65 (d, $J = 8.4$ Hz, 1H, NH); $^{13}$C NMR ($CDCl_3$, 75 MHz): $\delta = 15.5$, 22.2, 23.2, 25.1, 28.7, 30.5, 31.7, 41.8, 51.1, 52.7, 80.7, 171.6, 173.4; FIA-ESI(+)-TOF-MS: $m/z =$ 377.2099, calcd. for $C_{17}H_{33}N_3O_5S$ $[M+H]^+$: 377.2105.

**Boc-Met-Leu-Phe-$NH_2$ (9):** White solid; $R_t$ (HPLC): 20.14 min; $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta = 0.82$ (dd, $J =$ 14.1 and 5.4 Hz, 6H, $CH_3$ 2×), 1.38–1.79 (m, 15H, CH, $CH_2$ 2× and $CH_3$ 3×), 2.02 (s, 3H, $SCH_3$), 2.41–2.50 (m, 2H, $CH_2$), 2.79–3.02 (m, 2H, $CH_2$), 3.80–4.15 (m, 1H, CH), 4.21–4.30 (m, 1H, CH), 4.38–4.54 (m, 1H, CH), 7.00–7.23 (m, 2H, NH 2×), 7.26–7.34 (m, 6H, NH and Ph), 7.80–7.88 (m, 2H, NH 2×); $^{13}$C NMR (DMSO-$d_6$, 75 MHz): $\delta = 14.5$, 21.5, 22.9, 23.8, 28.0, 29.6, 31.3, 51.0, 53.3, 53.4, 78.0, 126.1, 127.9, 129.0, 137.6, 155.2, 171.4 (2×), 172.4; FIA-ESI(+)-TOF-MS: $m/z = 509.2820$, calcd. for $C_{25}H_{41}N_4O_5S$ $[M+H]^+$: 509.2792.

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