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PAPER

Improving the carboxyamidomethyl ester for subtilisin A-catalysed peptide synthesis†

Roseri J. A. C. de Beer,‡[a] Timo Nuijens,‡[b] Lotte Wiermans,[b] Peter J. L. M. Quaedflieg[b] and Floris P. J. T. Rutjes*[a]

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A series of novel glycine esters was evaluated for efficiency in subtilisin A-CLEA-catalysed peptide synthesis. The reactivity of the easily accessible carboxyamidomethyl (Cam) ester was further enhanced by elongating it with an amino acid residue, thereby creating more recognition space for subtilisin A.

Introduction

A large number of peptides have been introduced into the market as therapeutics or prodrugs,[1] and an even increasing number is in clinical trials. Additionally, peptides have found applications as nutritional additives or as cosmetic ingredients.[2] Despite this demand for peptides, their production on a large scale remains expensive and time consuming.[3] Common peptide synthesis approaches include fermentation, solid-phase or solution-phase chemical peptide synthesis, and chemoenzymatic peptide synthesis.[4] Currently, the fermentative approach is only well feasible for very large peptides (>50 amino acid residues) and proteins containing natural fragments and requires a large development effort for each individual target product. Solution-phase chemical peptide synthesis is most commonly used for the synthesis of small peptides containing two to ten amino acid residues. Solid-phase peptide synthesis (SPPS)[5] is the most commonly applied method for medium-sized and long peptides (10–50 amino acid residues). However, peptides longer than 10–15 amino acids are not cost-efficiently synthesised on the solid phase because they tend to form tertiary structures (by so-called "hydrophobic collapse") making peptide elongation troublesome so that a large excess of reagents and amino acid building blocks is needed.[6] Additionally the purification of the final product is often cost-inefficient due to the presence of significant amounts of peptides of similar length. Therefore, peptides longer than 10 amino acids are often produced using a combination of solid-phase synthesis of protected oligopeptide fragments which are subsequently chemically condensed in solution, *e.g.* by a 10 + 10 condensation to make a peptide of 20 amino acids. The major drawback of chemical fragment condensation is that upon activation of the C-terminal amino acid residue racemisation occurs, except when C-terminal Gly or Pro residues are used. Therefore, the chemical fragment condensation strategy is usually limited to using C-terminally activated Gly and Pro residues, or one has to deal with a difficult purification due to the formation of undesired diastereoisomers.

Chemoenzymatic peptide synthesis has been examined by several groups during the past decades and proved to be suitable for certain short peptide sequences up to five amino acid residues.[4] The application of proteases as a coupling reagent is a promising alternative since functionalised amino acid side chains do not require protection and, most importantly, C-terminal racemisation is completely absent during fragment assembly, which is beneficial for the characterisation and purification of the final peptide.

There are two approaches to performing enzymatic peptide synthesis, the thermodynamically and the kinetically controlled approach.[7] In thermodynamically controlled peptide synthesis, an N-terminal protected acyl donor having a free C-terminal carboxylic acid function reacts with a C-terminal protected amino acid nucleophile, resulting in the formation of the peptide bond and expulsion of one water molecule. Thermodynamically controlled peptide coupling is, however, rather slow and the thermodynamic equilibrium between the product and starting materials needs to be shifted into the synthetic direction, for example by product precipitation, water withdrawal, or by using organic solvents, to obtain a high product yield. This is in contrast to kinetically controlled peptide synthesis, in which an N-terminal protected and C-terminal activated amino acid or peptide ester reacts preferentially with a C-terminal protected acyl acceptor, usually giving a higher product yield in a shorter reaction time.

The high selectivity of proteases restricts the number of amino acids that will be recognised. Therefore, coupling of different peptide sequences using one single enzyme remains challenging. To remedy these limitations, designed acyl donors with an ester

[a]*Institute for Molecules and Materials, Radboud University Nijmegen, Heyendaalseweg 135, NL-6525 AJ Nijmegen, The Netherlands. E-mail: f.rutjes@science.ru.nl*
[b]*DSM Innovative Synthesis B.V., P.O. Box 18, NL-6160 MD Geleen, The Netherlands*

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‡Both authors contributed equally.

moiety that is specifically recognised by the enzyme, among others, guanidinophenyl (Gp),[8] carboxamidomethyl (Cam)[9] or 2,2,2-trifluoroethyl (Tfe)[10] esters, have been used. In the presence of these activated esters, protease-mediated couplings of weak nucleophiles and non-proteinogenic amino acid residues have become feasible.[11] Although these active esters broaden the scope of enzymatic peptide synthesis, their preparation is not straightforward[12] since highly reactive reagents are required to couple the poorly nucleophilic alcohol derivatives, which increases the risk of racemisation.[13] In fact, the coupling reagents used are often identical to those used for chemical peptide bond formation.[14] A few solid-phase methodologies have been reported for the synthesis of peptide Gp esters, but these strategies are complicated and require the use of resins which are not available on an industrial scale.[15]

Recently, we reported the use of amino acid and peptide Cam-esters in subtilisin A-catalysed peptide synthesis in anhydrous organic solvents.[16] Subtilisin A, a serine endoprotease, is industrially available in the form of Alcalase. Although the enzyme has a slight preference for large uncharged hydrophobic residues in both the P_1 and the P_1' positions,[17] a broad range of substrates is accepted and it can therefore be applied to the synthesis of numerous peptides.[18] It was shown that the use of Cam esters broadened the scope of Alcalase-catalysed peptide synthesis even further and that the coupling of very challenging substrates became feasible in high yields without any hydrolytic side reactions. However, coupling reactions sometimes remained rather slow and a relatively large amount of enzyme was required, especially when longer peptide fragments were to be coupled. Clearly, there is room for improvement of the acyl donor ester for Alcalase-catalysed peptide synthesis.

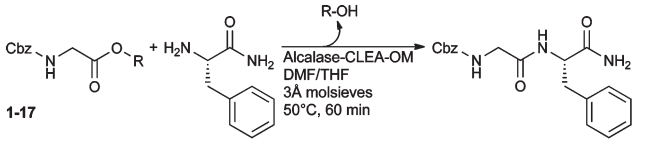
In this paper we compare various new active esters for Alcalase-catalysed peptide synthesis in organic solvents, including Cam esters that are elongated with any of the proteinogenic amino acid residues. These elongated Cam esters could be conveniently synthesised in high yield and purity using specifically designed solid-phase peptide synthesis techniques with readily available starting materials and resins.

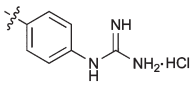
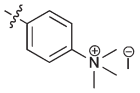
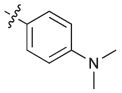
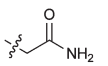
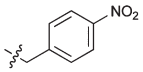
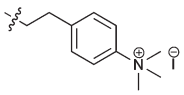
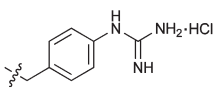
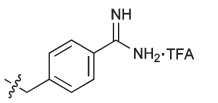
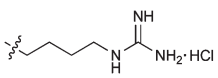
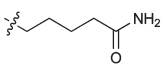
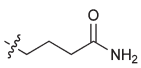
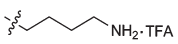
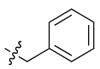
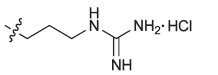
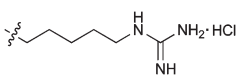
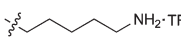
Results and discussion

To test the coupling efficiencies in Alcalase-catalysed dipeptide synthesis, with H-Phe-NH_2 functioning as the nucleophile, a library of Cbz-Gly-OR esters was synthesised. The library was synthesised using previously described chemical techniques and was originally designed to identify improved activating esters for papain.[19] Alcalase cross-linked enzyme aggregates (CLEAs)[20] were used for convenient handling and workup. A solvent mixture of dimethylformamide–tetrahydrofuran (DMF–THF 1 : 9, v/v) ensured good solubility of all starting materials. Molecular sieves were added to remove water from the reaction mixture, to prevent any hydrolytic side reactions. The results of the screening are given in Table 1.

Clearly, the substituted phenyl esters (entries 1–3) are the most active species for the Alcalase-CLEA-catalysed peptide coupling, followed by the Tfe and Cam ester derivatives (entries 4 and 5). This might be explained by their strong electron-withdrawing properties, which render the carbonyl more susceptible to nucleophilic attack by the active site serine. In addition, it also enhances the leaving group ability of the corresponding alcohols. The drawbacks of these phenyl esters are that, due to their high activation level, they are relatively difficult to synthesise, show

Table 1 Relative activity of various Cbz-Gly-OR esters in Alcalase-CLEA-catalysed peptide coupling with H-Phe-NH_2



Entry	R =		Relative activity
1		**1**	100
2		**2**	95
3		**3**	70
4		**4**	68
5		**5**	60
6		**6**	34
7		**7**	30
8		**8**	31
9		**9**	28
10		**10**	23
11		**11**	11
12		**12**	9
13		**13**	8
14		**14**	8
15		**15**	7
16		**16**	4
17		**17**	4

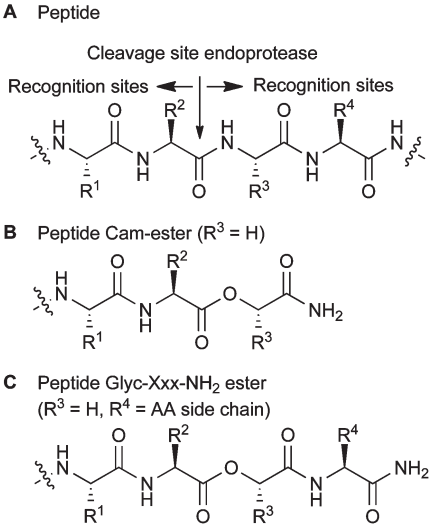


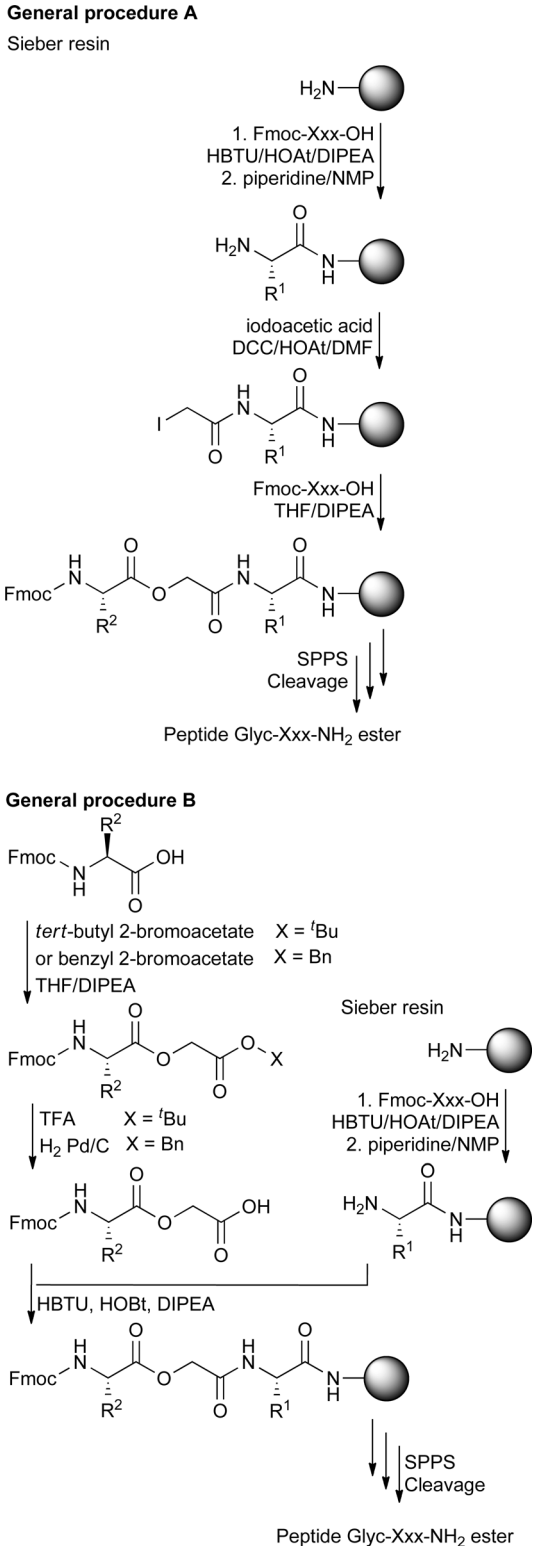
Fig. 1 Similarity of the natural endopeptidase peptide cleavage site with the Cam- and Cam-Xxx-NH_2 ester.

spontaneous peptide coupling and other side reactions. The Cam-ester (entry 5), which is chemically[21] and enzymatically[16] much easier to synthesise and does not give any spontaneous peptide coupling or racemisation, also displays very good activity. This activity cannot only be explained by its electron-withdrawing properties. It is believed that the amide group of the Cam ester moiety binds to the enzyme *via* a hydrogen bond in the same fashion as an amide of a peptide backbone binds when it is recognised and cleaved by an endoprotease (Fig. 1A). Wells *et al.* already showed for subtiligase, a mutant of subtilisin BPN wherein the active site serine is replaced by a cysteine, that the Cam ester activation (Fig. 1B) could be improved by elongating it with an amino acid amide, thereby creating additional binding interactions with the enzyme (Fig. 1C).[22]

To investigate if the Cam ester could be further improved for Alcalase, a library of Fmoc-Val-Ala-OGlyc-Xxx-NH_2 esters, wherein Xxx stands for all 20 proteinogenic amino acids, with an either protected or unprotected side chain functionality, was synthesised using specifically designed SPPS techniques. Two methods were developed, one approach is based on the attachment of iodoacetic acid to a Sieber resin (Scheme 1A) while the other method is based on the attachment of Fmoc-Xxx-OCH_2CO_2H building blocks to the same resin (Scheme 1B).

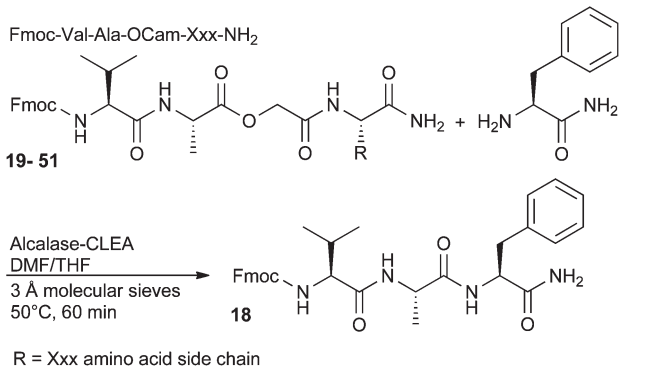
Both methods have their appealing characteristics. The advantage of method A is that no special amino acid building blocks have to be used, however, the esterification has to be performed at 50 °C for 24 h, which complicates automated peptide synthesis. Although method B requires the use of special Fmoc amino acid building blocks, the advantage is that all reactions on the resin are performed using standard SPPS protocols at ambient temperature. Furthermore, the Fmoc-Xxx-OCH_2CO_2H building blocks are easily accessible. The dipeptide Glyc-Xxx-NH_2 esters were obtained in very high yield and purity and no side reactions were observed upon cleavage from the resin with trifluoroacetic acid.

The relative activity of the Fmoc-Val-Ala-OGlyc-Xxx-NH_2 esters was tested in an Alcalase-CLEA-catalysed peptide coupling using H-Phe-NH_2 as the nucleophile, as shown in Table 2. The product peak was analysed using HPLC and the yields were calculated using a calibration curve of Fmoc-Val-Ala-Phe-NH_2 (**18**).There are large differences between the



Scheme 1 Two different solid phase methods developed for the synthesis of peptide Glyc-Xxx-NH_2 esters.

Table 2 Relative activity of various Fmoc-Val-Ala-OCam-Xxx-NH_2 esters in the Alcalase-CLEA-catalysed peptide coupling with H-Phe-NH_2

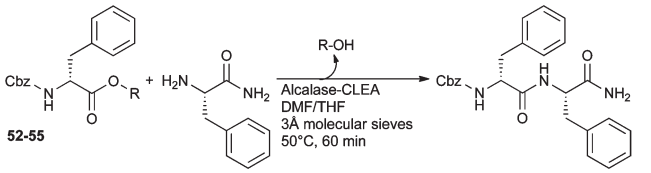


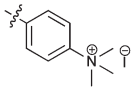
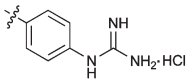
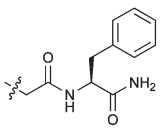
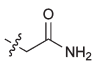
Entry	Xxx =		Relative activity
1	Phe	**19**	204
2	Tyr	**20**	198
3	Ser	**21**	169
4	Leu	**22**	165
5	Arg	**23**	163
6	Asp(O^{t}Bu)	**24**	143
7	Ser(^{t}Bu)	**25**	143
8	Gln(Trt)	**26**	133
9	Ala	**27**	131
10	Gly	**28**	124
11	Cys	**29**	114
12	Thr	**30**	108
13	Glu(O^{t}Bu)	**31**	108
14	Arg(Pbf)	**32**	106
15	Pro	**33**	104
16	**--- (= Cam)**	**34**	**100**
17	Cys(Trt)	**35**	92
18	Trp(Boc)	**36**	90
19	Tyr(^{t}Bu)	**37**	88
20	Ile	**38**	88
21	Gln	**39**	84
22	Thr(^{t}Bu)	**40**	84
23	Lys	**41**	82
24	Trp	**42**	63
25	Met	**43**	53
26	Lys(Boc)	**44**	45
27	Asn(Trt)	**45**	41
28	Glu	**46**	37
29	Val	**47**	33
30	Asn	**48**	31
31	His	**49**	16
32	His(Trt)	**50**	16
33	Asp	**51**	12

Glyc-Xxx-NH_2 esters and almost an equal part of them is better (entries 1–15) or worse (entries 17–33) than the non-elongated Cam ester (entry 16). The best results were obtained using Phe and Tyr (**19** and **20**, entries 1 and 2), resulting in a two-fold enhancement.

The optimal Glyc-Xxx-NH_2 ester (Table 2, entry 1, Glyc-Phe-NH_2, **19**) was compared to the most active substituted phenol esters from the first Cbz-Gly-OR screening (Table 1, entries 1 and 2). To clearly discern the intrinsic reactivities, the challenging substrate Cbz-D-Phe was chosen as the acyl donor with H-Phe-NH_2 as the nucleophile (Table 3). D-Amino acids are notoriously difficult substrates for Alcalase, in fact, Chen *et al.* reported that no peptide product was obtained at all using Alcalase and Cbz-D-Phe as the acyl donor.[18]

Table 3 Relative activity of various Cbz-D-Phe-OR esters in Alcalase-CLEA-catalysed peptide coupling with H-Phe-NH_2



Entry	R =		Relative activity
1		**52**	100
2		**53**	98
3		**54**	90
4		**55**	54

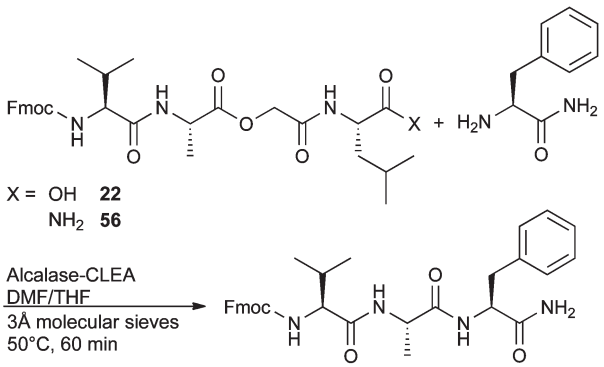
As is evident from Table 3, the elongated Cam ester (entry 3) shows comparable reactivity to the substituted phenol esters (entries 1 and 2). An equally active, but more conveniently accessible ester was thus developed for Alcalase-CLEA-catalysed peptide synthesis. Another advantage is that no racemisation occurred on the activated amino acid ester, *i.e.* D-Phe, using the Cbz-D-Phe-OGlyc-Phe-NH_2 ester (**54**, entry 3, ee of D-Phe >99.5), this is in contrast to the Cbz-D-Phe-OTMAP (**52**, entry 1, ee of D-Phe 85.8%) ester.

Although the Glyc-Xxx-NH_2 esters can be conveniently synthesised on the solid phase, the Sieber or Rink resins required to obtain the C-terminal amide functionality are relatively expensive. The resins which are commonly used to obtain a C-terminal carboxylic acid functionality (Wang or 2-chlorotritylchloride resin) are generally much cheaper and thus better suited for large-scale applications. Therefore, the relative activity of a C-terminal carboxamide functionality (Fmoc-Val-Ala-OGlyc-Leu-NH_2, **22**) was compared to a C-terminal carboxylic acid functionality (Fmoc-Val-Ala-OGlyc-Leu-OH, **56**) in the Alcalase-CLEA-catalysed peptide coupling with H-Phe-NH_2 as the nucleophile. Gratifyingly, as demonstrated in Table 4, comparable reaction rates were obtained.

Conclusions

In conclusion, the performance of various novel and known C-terminal esters in subtilisin A-catalysed peptide synthesis in anhydrous organic solvents was compared. It appeared that C-terminal phenolic esters were most active but they are difficult

Table 4 Relative activity of Cam-Leu-NH_2 and Cam-Leu-OH esters in the Alcalase-CLEA-catalysed peptide coupling with H-Phe-NH_2



Entry	X =		Relative activity
1	NH_2	**56**	91
2	OH	**22**	100

to synthesise in a chemically and stereochemically pure form. The slightly less reactive C-terminal carboxamidomethyl (Cam) esters, however, are easily accessible and are not prone to racemisation. It was demonstrated that the activity of Cam esters can be increased to the level of the most active phenolic esters by elongating them with (apolar) amino acids and amino acid amides. Additionally, the convenient accessibility of these substituted Cam-esters *via* two different solid-phase strategies was demonstrated.

Experimental

General information

All resins and reagents used for SPPS were purchased from GL Biochem (Changhai, China) and all peptide grade solvents from Biosolve (Valkenswaard, The Netherlands). Before use, 3 g Alcalase-CLEA (type OM, CLEA-Technologies, 580 U g^{-1}) was suspended in 100 mL ^{t}BuOH and crushed with a spatula. After filtration, the enzyme was resuspended in 50 mL MTBE followed by filtration. Large enzyme particles were removed by a sieve (0.5 mm pore size). ^{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). Analytical HPLC chromatograms were recorded on an HP1090 Liquid Chromatograph, using a reversed-phase column (Phenomenex, C18, 5 µm particle size, 150 × 4.6 mm) at 40 °C. The gradient program was: a 0–25 min linear gradient ramp from 5% to 98% eluent B and from 25.1–30 min with 5% eluent B (eluent A 0.5 mL L^{-1} methane sulfonic acid (MSA) in H_2O, eluent B 0.5 mL L^{-1} MSA in acetonitrile). The flow was 1 mL min^{-1} from 0–25.1 min and 2 mL min^{-1} from 25.2–29.8 min, then back to 1 mL min^{-1} until stopping at 30 min. Injection volumes were 20 µL. 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, followed by lyophilisation. The 3 Å molecular sieves (Acros, 8–12 mesh) were activated (200 °C under vacuum overnight), crushed and sieved (0.5 mm pore size) to remove large particles. To determine the ee of Phe the samples were concentrated *in vacuo* and the residue suspended in excess 6 N HCl and refluxed overnight. Chiral HPLC was performed on a crown ether (+) column (150 mm length, 4.0 mm internal diameter, 5 µm particle size) at 25 °C with 30 mM aqueous $HClO_4$ (pH = 2.0) as the eluent. UV detection was performed at 210 nm using a UV-VIS linear spectrometer. The flow was 1 mL min^{-1}. Injection volumes were 5 µL. R_t (D-Phe) = 6.90 min, R_t (L-Phe) = 8.82 min, synthesis details and characterisation data of the Cbz-Gly-OR library have been published previously.[19] Synthesis[16] and analytical data[23] of Cbz-D-Phe-OCam were identical to those reported in the literature.

General procedure A: synthesis of peptide Glyc-Xxx-NH_2 esters

The Fmoc protecting group was removed from a Sieber resin (1 g, loading = 0.5 mmol g^{-1}) using piperidine in *N*-methyl-2-pyrrolidone (NMP, 10 mL, 1 : 4, v/v) followed by washing steps with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×). Fmoc-Xxx-OH was coupled to the amine using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluorophosphate (HBTU, 2 mmol, 758 mg), 1-hydroxy-7-azabenzotriazole (HOAt, 2 mmol, 272 mg) and diisopropylethylamine (DIPEA, 4 mmol, 697 µL) in NMP (10 mL, 60 min). The resin was washed with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×) followed by removal of the Fmoc protecting group using piperidine in NMP (10 mL, 1 : 4, v/v). After washing with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and *N*,*N*-dimethylformamide (DMF, 10 mL, 2 min, 3×) the free amine was reacted with iodoacetic acid (1 mmol, 186 mg), diisopropylcarbodiimide (DIC, 1 mmol, 155 µL) and HOAt (1 mmol, 136 mg) in DMF (10 mL, 30 min) followed by washing steps with DMF (10 mL, 2 min, 2×), CH_2Cl_2 (10 mL, 2 min, 2×), and DMF (10 mL, 2 min, 2×). Subsequently, Fmoc-Xxx-OH (2 mmol) and DIPEA (2.5 mmol, 436 µL) in DMF–tetrahydrofuran (DMF–THF, 1 : 1, v/v, 10 mL) were added and the mixture was shaken at 50 °C with 200 rpm for 20 h. Afterwards, the resin was washed with 10 vol% H_2O in DMF (10 mL, 2 min, 2×) and DMF (10 mL, 2 min, 3×). Further Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[5] The peptide Cam ester was cleaved from the resin using 2.5 vol % trifluoroacetic acid (TFA) in CH_2Cl_2 (20 mL) for 30 min. After filtration the volatiles were partly concentrated *in vacuo* to a volume of 10 mL. Subsequently, ^{i}PrOH in H_2O (1 : 3, v/v, 50 mL) was added followed by partial evaporation of the volatiles to a volume of 30 mL. The precipitates were removed by filtration and washed with H_2O (5 mL, 2×), followed by lyophilisation from MeCN–H_2O (3 : 1, v/v).

General procedure B: synthesis of peptide Glyc-Xxx-NH_2 esters

Coupling and Fmoc deprotection of the first amino acid was identical to general procedure A. Fmoc-Xxx-OCH_2COOH (1 mmol) (Fmoc-Val-Ala-OCH_2COOH for the library synthesis)

was coupled to the free amine using HBTU (1 mmol, 379 mg), 1-hydroxybenzotriazole (HOBt, 1 mmol, 136 mg) and DIPEA (2 mmol, 349 μL) in NMP (10 mL, 90 min). Further Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[5] After synthesis of the desired sequence, the peptide was cleaved from the resin and precipitated as described in general procedure A.

General procedure C: side chain deprotection of Fmoc-Val-Ala-OGlyc-Xxx-NH_2 peptides

Fmoc-Val-Ala-OGlyc-Xxx-NH_2 (0.02 mmol) was dissolved in TFA–H_2O (1 mL, 95 : 5, v/v) and stirred for 1 h. Afterwards, the volatiles were concentrated by nitrogen flow and the residue lyophilised from CH_3CN–H_2O (3 : 1, v/v). The lyophilised powders were dissolved in DMF (100 μL) and used as such for the relative activity determination assays described below. Additional piperidine (0.02 mmol, 2 μL) was added to the stock solutions of Fmoc-Val-Ala-OGlyc-Asp-NH_2 and Fmoc-Val-Ala-OGlyc-Glu-NH_2.

General procedure D: synthesis of peptide Glyc-Xxx-OH esters

2-Chlorotritylchloride resin (1 g, loading = 1.2 mmol g^{-1}) was reacted with Fmoc-Xxx-OH (2 mmol) and DIPEA (5 mmol) in CH_2Cl_2 (10 mL, 30 min) followed by washing with DMF (10 mL, 2×). Afterwards, the unreacted tritylchloride moieties were capped with MeOH–CH_2Cl_2–DIPEA (10 mL, 15 : 85 : 5, v/v/v) followed by washing steps with NMP (10 mL, 2 min, 3×), CH_2Cl_2 (10 mL, 2 min, 3×) and NMP (10 mL, 2 min, 3×). Fmoc-Xxx-OCH_2COOH (1 mmol) was coupled to the free amine using HBTU (1 mmol, 379 mg), HOBt (1 mmol, 136 mg) and DIPEA (2 mmol, 349 μL) in NMP (10 mL, 90 min). Further Fmoc deprotection and Fmoc amino acid coupling cycles were performed using standard SPPS protocols.[5] After synthesis of the desired sequence, the peptide was cleaved from the resin and precipitated as described in general procedure A.

General procedure E: relative activity determination for Alcalase-CLEA catalysed peptide coupling

To a suspension of Alcalase-CLEA (4.5 mg), H-Phe-NH_2 (0.54 mg) and crushed 3 Å molecular sieves (4.5 mg) in THF (900 μL), amino acid or peptide ester stock solution (20 mM) in DMF (100 μL) was added. The reaction mixture was shaken at 50 °C with 200 rpm for 60 min. Afterwards, the reaction mixture was filtrated and analysed by analytical HPLC by integrating the peptide coupling product peak. Integration areas of different reactions were compared to each other to determine the relative activity (ester which gave the highest peptide product integration area = 100%).

HCl·H-Ala-$OCH_2C(=O)$O-Bn

To a solution of Boc-Ala-OH (1 mmol, 189 mg) in THF (50 mL) were added DIPEA (2.5 mmol, 436 μL) and benzyl iodoacetate (2.0 mmol, 552 mg). This mixture was shaken for 20 h at 50 °C with 200 rpm and after this period of stirring, the reaction mixture was concentrated *in vacuo*. The residue was resuspended in EtOAc (100 mL) and this solution was washed with sat. aq. $NaHCO_3$ (100 mL, 2×), 0.1 N HCl (100 mL, 2×), brine (100 mL) and dried (Na_2SO_4). The solution was concentrated *in vacuo* and the residue was resuspended in 2 N HCl in dioxane (25 mL). After stirring for 1 h, cold diethyl ether (Et_2O, 100 mL) was added and the precipitate was filtered off and washed with cold Et_2O (20 mL, 2×). HCl·H-Ala-$OCH_2C(=O)$O-Bn was dried at 40 °C *in vacuo* for 24 h and obtained in an overall yield of 87%. R_t(HPLC) 5.19 min; purity = 98%; ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 1.62 (d, J = 6.9 Hz, 3H), 4.19–4.28 (m, 1H), 4.51–4.77 (m, 2H), 5.03 (s, 2H), 7.21 (s, 5H), 8.64 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 16.0, 49.2, 61.8, 67.4, 128.5, 128.6, 128.7, 134.8, 166.9, 169.7.

Fmoc-Val-Ala-OCH_2COOH

To a solution of Fmoc-Val-OH (1 mmol, 339 mg) in CH_2Cl_2 (50 mL) which was cooled on ice to 0 °C, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCl, 1.1 mmol, 211 mg) and HOBt (1.1 mmol, 150 mg) were added. After stirring for 30 min, HCl·H-Ala-$OCH_2C(=O)$O-Bn (1.1 mmol, 300 mg) and DIPEA (1.1 mmol, 192 μL) were added. The obtained reaction mixture was stirred at ambient temperature for 6 h, followed by washing with sat. aq. $NaHCO_3$ (100 mL, 2×), 0.1 N HCl (100 mL, 2×), and brine (100 mL). The CH_2Cl_2 solution was dried (Na_2SO_4) and subsequently concentrated *in vacuo* and the residue was resuspended in MeOH–toluene (1 : 1, v/v, 250 mL). Then, the mixture was hydrogenated using Pd/C (10 mol%) with 5 bar H_2 at 25 °C for 24 h. Afterwards, the reaction mixture was filtrated over Celite followed by concentration of the solvents *in vacuo* and purification of the residue by preparative HPLC. Fmoc-Val-Ala-OCH_2COOH was obtained in an overall yield of 73%. R_t(HPLC) 15.76 min; purity = 99%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.88 (2 × d, J = 6.6 and 6.9 Hz, 6H), 1.34 (d, J = 7.2 Hz, 3H), 2.92–2.03 (m, 1H), 3.88–3.94 (m, 1H), 4.18–4.39 (m, 4H), 4.51–4.65 (m, 2H), 7.29–7.43 (m, 5H), 7.75 (q, J = 3.6 and 3.3 Hz, 2H), 7.89 (d, J = 7.5 Hz, 2H), 8.42 (d, J = 6.6 Hz, 1H), 13.05 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.3, 46.6, 47.2, 59.6, 60.7, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 168.7, 171.1, 172.0.

Fmoc-Val-Ala-Phe-NH_2 (18)

R_t(HPLC) 22.48 min; purity = 99%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.82 (2 × d, J = 2.7 and 3.6 Hz, 6H), 1.15 (d, 3H, J = 7.2 Hz), 1.92–1.99 (m, 1H), 2.79–3.02 (m, 2H), 3.86 (q, 1H, J = 7.2 Hz and 1.2 Hz), 4.19–4.43 (m, 5H), 7.05 (s, 1H), 7.16–7.44 (m, 11H), 7.73 (t, 2H, J = 6.6 Hz), 7.84–8.00 (m, 4H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 19.0, 21.2, 22.7, 24.1, 30.3, 46.6, 47.5, 49.9, 59.6, 62.0, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.2, 171.3, 171.8, 173.5.

Fmoc-Val-Ala-OGlyc-Phe-NH_2 (19)

R_t(HPLC) 16.85 min; purity = 98%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.87 (2 × d, J = 7.2 and 7.8 Hz, 6H), 1.32

(d, J = 7.2 Hz, 3H), 1.87–2.15 (m, 1H), 2.77–3.05 (m, 2H), 3.86–3.92 (m, 1H), 4.20–4.46 (m, 7H), 7.11–7.46 (m, 11H), 7.72–7.76 (m, 2H), 7.89 (d, J = 7.5 Hz, 2H), 8.09 (d, J = 8.4 Hz, 1H), 8.44 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.3, 37.5, 46.6, 47.5, 53.5, 59.6, 62.1, 65.6, 120.0, 125.3, 126.1, 126.9, 127.5, 129.0, 137.7, 140.6, 143.7, 143.8, 166.0, 171.1, 171.7, 172.4.

Fmoc-Val-Ala-OGlyc-Leu-NH$_2$ (22)

R_t(HPLC) 16.60 min; purity = 98%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.82–0.90 (m, 12H), 1.33–1.61 (m, 6H), 1.94–2.00 (m, 1H), 3.86–3.92 (m, 1H), 4.18–4.38 (m, 5H), 4.53 (m, 2H), 7.01 (s, 1H), 7.29–7.44 (m, 6H), 7.74 (q, J = 4.5 Hz and 2.4 Hz, 2H), 7.88–7.98 (m, 3H), 8.46 (d, J = 6.0 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 21.4, 22.9, 24.1, 30.3, 40.8, 46.6, 47.8, 50.5, 59.6, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.0, 171.3, 171.9, 173.6.

Fmoc-Val-Ala-OGlyc-Asp(O^{t}Bu)-NH$_2$ (24)

R_t(HPLC) 17.00 min; purity = 98%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.87 (2 × d, J = 10.2 and 7.1 Hz, 6H), 1.32–1.37 (m, 12H), 1.93–2.00 (m, 1H), 2.41–2.70 (m, 2H), 3.87–3.93 (m, 1H), 4.17–4.58 (m, 7H), 7.15 (s, 1H), 7.31–7.44 (m, 6H), 7.73 (m, 2H), 7.89 (d, J = 7.5 Hz, 2H), 8.18 (d, J = 8.1 Hz, 1H), 8.45 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 27.5, 30.3, 31.2, 37.4, 46.6, 47.5, 49.0, 59.6, 62.2, 65.6, 80.0, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 166.1, 169.3, 171.3, 171.8.

Fmoc-Val-Ala-OGlyc-Ser(^{t}Bu)-NH$_2$ (25)

R$_t$(HPLC) 16.85 min; purity = 97%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.87 (2 × d, J = 7.2 and 8.4 Hz, 6H), 1.10 (s, 9H), 1.35 (d, J = 7.2 Hz, 3H), 1.94–2.00 (m, 1H), 3.30–3.53 (m, 2H), 3.87–3.92 (m, 1H), 4.20–4.36 (m, 5H), 4.50–4.63 (m, 2H), 7.11 (s, 1H), 7.29–7.44 (m, 6H), 7.73–7.75 (m, 2H), 7.87–7.90 (m, 3H), 8.42 (d, J = 6.0 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 27.1, 30.3, 31.2, 46.6, 47.4, 52.9, 59.6, 61.7, 62.2, 65.6, 72.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 156.0, 166.1, 171.2, 171.2, 171.2.

Fmoc-Val-Ala-OGlyc-Gln(Trt)-NH$_2$ (26)

R_t(HPLC) 19.63 min; purity = 97%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.80 (2 × d, J = 6.6 Hz, 6H), 1.26 (d, J = 7.2 Hz, 3H), 1.55–1.68 (m, 1H), 1.74–1.93 (m, 2H), 2.20–2.25 (m, 2H), 3.80–3.85 (m, 1H), 4.06–4.28 (m, 5H), 4.40–4.53 (m, 2H), 7.00–7.37 (m, 22H), 7.67 (q, J = 3.6 Hz, 2H), 7.82 (d, J = 7.5 Hz, 2H), 7.96 (d, J = 8.1 Hz, 1H), 8.37 (d, J = 6.6 Hz, 1H), 8.54 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.6, 18.1, 19.0, 27.2, 27.6, 30.3, 31.1, 46.6, 47.5, 51.3, 59.6, 62.2, 65.6, 79.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.2, 171.3, 171.5, 171.9, 172.6.

Fmoc-Val-Ala-OGlyc-Ala-NH$_2$ (27)

R_t(HPLC) 14.69 min; purity = 98%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.87 (2 × d, J = 7.2 and 7.8 Hz, 6H), 1.32 (d, J = 7.2 Hz, 3H), 1.87–2.15 (m, 4H), 2.77–2.85 (m, 1H), 2.99–3.05 (m, 1H), 3.86–3.92 (m, 1H), 4.20–4.61 (m, 6H), 7.12–7.46 (m, 11H), 7.72–7.76 (m, 2H), 7.90 (d, J = 7.5 Hz, 2H), 8.09 (d, J = 8.4 Hz, 1H), 8.44 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.6, 18.2, 19.0, 30.3, 46.6, 47.5, 47.7, 59.6, 62.3, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 165.8, 171.3, 171.9, 173.7.

Fmoc-Val-Ala-OGlyc-Gly-NH$_2$ (28)

R_t(HPLC) 13.65 min; purity = 92%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.88 (2 × d, J = 7.2 and 7.5 Hz, 6H), 1.34 (d, J = 6.9 Hz, 3H), 1.93–2.00 (m, 1H), 3.66 (d, J = 5.4 Hz, 2H), 3.87–3.93 (m, 1H), 4.22–4.59 (m, 6H), 7.08 (s, 1H), 7.30–7.44 (m, 6H), 7.74–7.90 (m, 4H), 8.12–8.16 (m, 1H), 8.47 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.3, 41.5, 46.6, 47.5, 59.6, 62.4, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 166.6, 170.4, 171.3, 171.9.

Fmoc-Val-Ala-OGlyc-Glu(O^{t}Bu)-NH$_2$ (31)

R_t(HPLC) 17.30 min; purity = 98%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.88 (2 × d, J = 6.9 and 7.2 Hz, 6H), 1.33–1.39 (m, 12H), 1.66–1.79 (m, 1H), 1.87–2.02 (m, 2H), 2.17–2.27 (m, 2H), 3.87–3.93 (m, 1H), 4.17–4.37 (m, 5H), 4.48–4.60 (m, 2H), 7.11 (s, 1H), 7.29–7.44 (m, 6H), 7.74 (dd, J = 4.5 Hz and 2.7 Hz, 2H), 7.89 (d, J = 7.5 Hz, 2H), 8.00 (d, J = 8.1 Hz, 1H), 8.46 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 28.0, 30.3, 31.2, 32.5, 46.6, 47.5, 51.9, 59.6, 62.2, 65.6, 69.1, 120.0, 125.3, 126.2, 126.9, 127.3, 127.4, 127.5, 128.4, 140.6, 143.7, 143.8, 144.8, 166.1, 171.2, 171.3, 171.9, 172.0.

Fmoc-Val-Ala-OGlyc-Arg(Pbf)-NH$_2$ (32)

R_t(HPLC) 17.94 min; purity = 94%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.86 (2 × d, J = 6.9 and 7.1 Hz, 6H), 1.10 (s, 3H), 1.32–1.40 (m, 9H), 1.96–2.02 (m, 4H), 2.94–3.03 (m, 3H), 3.80–3.92 (m, 1H), 4.17–4.35 (m, 5H), 4.53 (m, 2H), 7.07 (s, 1H), 7.28–7.43 (m, 5H), 7.75 (q, J = 4.8 Hz and 2.4 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H), 7.99 (d, J = 8.1 Hz, 1H), 8.45 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 12.1, 16.7, 17.5, 18.1, 18.8, 19.0, 28.2, 30.3, 31.2, 42.4, 46.6, 47.4, 51.6, 59.6, 62.2, 65.6, 86.2, 116.1, 120.0, 124.2, 125.3, 126.9, 127.5, 131.3, 137.2, 140.6, 143.7, 143.8, 156.0, 157.3, 166.1, 171.3, 171.9, 173.0.

Fmoc-Val-Ala-OGlyc-Pro-NH$_2$ (33)

R_t(HPLC) 14.42 min; purity = 97%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.87 (2 × d, J = 6.9 and 9.9 Hz, 6H), 1.36 (d, J = 7.2 Hz, 3H), 1.78–2.03 (m, 5H), 3.30–3.47 (m, 2H), 3.87–3.93 (m, 1H), 4.15–4.45 (m, 5H), 4.61–4.89 (m, 2H), 6.94 (s, 1H), 7.22–7.44 (m, 6H), 7.73–7.77 (m, 2H), 7.89 (d, J = 7.5 Hz, 2H),

8.39 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 16.8, 18.2, 19.0, 24.0, 29.0, 30.3, 45.1, 46.6, 47.3, 59.6, 61.6, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 164.6, 171.1, 171.9, 173.3.

Fmoc-Val-Ala-OCam (34)

R_t(HPLC) 14.67 min; purity = 98%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.88 (2 × d, J = 6.6 and 6.9 Hz, 6H), 1.33 (d, J = 7.2 Hz, 3H), 1.93–2.00 (m, 1H), 3.86–3.91 (m, 1H), 4.18–4.48 (m, 6H), 7.32–7.46 (m, 7H), 7.75 (q, J = 3.9 Hz and 2.7 Hz, 2H), 7.89 (d, J = 7.2 Hz, 2H), 8.49 (d, J = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 16.6, 18.2, 18.9, 30.3, 46.6, 47.6, 59.7, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 156.0, 168.4, 171.4, 171.8.

Fmoc-Val-Ala-OGlyc-Cys(Trt)-NH$_2$ (35)

R_t(HPLC) 20.91 min; purity = 91%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.87 (2 × d, J = 6.0 and 6.9 Hz, 6H), 1.34 (d, J = 7.2 Hz, 2H), 1.93–2.00 (m, 1H), 2.19–2.50 (m, 2H), 3.87–3.93 (m, 1H), 4.13–4.38 (m, 4H), 4.48–4.64 (m, 2H), 6.43–7.10 (m, 20H), 7.46–7.76 (m, 2H), 7.89 (d, J = 6.0 Hz, 2H), 8.20 (d, J = 8.4 Hz, 1H), 8.44 (d, J = 6.9 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 14.0, 14.3, 16.7, 18.1, 18.2, 19.0, 22.1, 30.3, 33.8, 46.6, 47.4, 55.7, 120.0, 125.3, 126.1, 126.6, 126.9, 127.4, 127.5, 127.7, 127.9, 128.2, 128.9, 129.0, 140.6, 143.7, 143.8, 144.1, 166.0, 171.1, 171.8.

Fmoc-Val-Ala-OGlyc-Trp(Boc)-NH$_2$ (36)

R_t(HPLC) 19.42 min; purity = 92%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.84 (2 × d, J = 6.9 and 7.2 Hz, 6H), 1.31 (d, J = 7.2 Hz, 3H), 1.61 (s, 9H), 1.89–2.08 (m, 1H), 2.90–3.16 (m, 1H), 3.85–3.91 (m, 1H), 4.19–4.58 (m, 7H), 7.18–7.81 (m, 13H), 7.88 (d, J = 7.5 Hz, 2H), 8.02 (d, J = 8.1 Hz, 1H), 8.18 (d, J = 8.1 Hz, 1H), 8.44 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 16.6, 18.1, 18.9, 27.2, 27.6, 30.3, 46.6, 47.5, 52.0, 59.6, 62.1, 65.6, 83.3, 114.5, 116.4, 119.2, 120.0, 122.3, 123.7, 124.1, 125.6, 126.9, 127.5, 130.2, 134.5, 140.6, 143.7, 143.8, 148.9, 155.9, 166.1, 171.2, 171.7, 172.4.

Fmoc-Val-Ala-OGlyc-Tyr(^{t}Bu)-NH$_2$ (37)

R_t(HPLC) 17.86 min; purity = 94%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.87 (2 × d, J = 6.9 and 8.4 Hz, 6H), 1.25 (s, 9H), 1.32 (d, J = 7.5 Hz, 3H), 1.93–2.00 (m, 1H), 2.72–3.01 (m, 2H), 3.86–3.92 (m, 1H), 4.17–4.45 (m, 7H), 6.84 (d, J = 7.5 Hz, 2H), 6.98 (d, J = 7.5 Hz, 2H), 7.29–7.44 (m, 6H), 7.72–7.76 (m, 2H), 7.89 (d, J = 7.5 Hz, 2H), 8.05 (d, J = 8.4 Hz, 1H), 8.45 (d, J = 6.3, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 16.1, 17.6, 18.4, 27.9, 29.7, 46.0, 46.9, 52.9, 59.1, 61.5, 65.0, 76.9, 114.2, 119.3, 122.6, 124.7, 126.4, 127.0, 129.0, 129.4, 131.7, 140.0, 143.1, 143.2, 152.7, 155.4, 165.4, 170.7, 171.1, 171.9.

Fmoc-Val-Ala-OGlyc-Ile-NH$_2$ (38)

R_t(HPLC) 16.46 min; purity = 96%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.79–0.93 (m, 12H), 1.01–1.44 (m, 4H), 1.68–1.75 (m, 2H), 1.93–2.00 (m, 1H), 3.86–3.92 (m, 1H), 4.13–4.35 (m, 5H), 4.48–4.71 (m, 2H), 7.05 (s, 1H), 7.29–7.44 (m, 6H), 7.72–7.90 (m, 5H), 8.45 (d, J = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 11.0, 15.3, 16.7, 18.1, 190.0, 24.0, 30.3, 36.5, 46.6, 47.4, 56.3, 59.6, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.0, 171.2, 171.9, 172.5.

Fmoc-Val-Ala-OGlyc-Thr(^{t}Bu)-NH$_2$ (40)

R_t(HPLC) 17.32 min; purity = 98%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.88 (2 × d, J = 6.9 Hz, 6H), 1.00 (d, J = 6.3 Hz, 3H), 1.13 (s, 9H), 1.35 (d, J = 7.2 Hz, 3H), 1.94–2.00 (m, 1H), 3.84–3.98 (m, 2H), 4.13–4.36 (m, 5H), 4.52–4.69 (m, 2H), 7.16–7.44 (m, 7H), 7.62–7.76 (m, 3H), 7.89 (d, J = 7.5 Hz, 2H), 8.43 (d, J = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 16.8, 171.1, 19.0, 19.4, 27.9, 30.3, 46.6, 47.4, 57.3, 59.6, 62.3, 65.6, 66.7, 73.4, 120.0, 120.0, 121.3, 125.3, 126.9, 127.2, 127.5, 128.8, 140.6, 143.7, 143.8, 155.9, 166.2, 171.1, 171.2, 171.4, 171.7.

Fmoc-Val-Ala-OGlyc-Met-NH$_2$ (43)

R_t(HPLC) 15.93 min; purity = 91%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.87 (2 × d, J = 7.2 and 7.8 Hz, 6H), 1.33 (d, J = 6.9 Hz, 3H), 1.72–2.02 (m, 6H), 2.41–2.50 (m, 2H), 3.82–3.92 (m, 1H), 4.20–4.35 (m, 5H), 4.54 (m, 2H), 7.10 (s, 1H), 7.29–7.43 (m, 5H), 7.74 (dd, J = 3.6 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H), 8.04 (d, J = 8.1 Hz, 1H), 8.47 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 14.5, 16.6, 18.1, 19.0, 29.5, 30.3, 31.7, 46.5, 47.5, 51.3, 59.6, 62.3, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.3, 171.3, 172.0, 172.7.

Fmoc-Val-Ala-OGlyc-Lys(Boc)-NH$_2$ (44)

R_t(HPLC) 17.06 min; purity = 95%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.86 (2 × d, J = 6.9 and 7.1 Hz, 6H), 1.21–1.63 (m, 15H), 1.92–2.00 (m, 1H), 2.83–2.89 (m, 2H), 3.80–3.91 (m, 1H), 4.12–4.34 (m, 5H), 4.50–4.60 (m, 2H), 6.70–6.75 (m, 1H), 7.02 (s, 1H), 7.28–7.43 (m, 5H), 7.71–7.95 (m, 5H), 8.45 (d, J = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d$_6$, 75 MHz) δ = 12.1, 16.7, 17.5, 18.1, 18.8, 190.0, 22.5, 28.2, 29.1, 30.3, 31.1, 46.6, 47.5, 51.6, 59.6, 62.2, 65.6, 86.2, 120.0, 124.2, 125.3, 126.9, 127.5, 128.8, 131.3, 140.6, 143.7, 143.8, 156.0, 166.1, 171.3, 171.9, 173.0.

Fmoc-Val-Ala-OGlyc-Asn(Trt)-NH$_2$ (45)

R_t(HPLC) 19.68 min; purity = 96%; ^{1}H NMR (DMSO-d$_6$, 300 MHz) δ = 0.88 (2 × d, J = 6.6 Hz, 6H), 1.32 (d, J = 7.2 Hz, 2H), 1.97–2.02 (m, 1H), 2.63–2.65 (m, 2H), 3.79–3.94 (m, 1H), 4.17–4.33 (m, 4H), 4.44–4.61 (m, 3H), 7.11–7.43 (m, 21H), 7.74 (q, J = 4.5 Hz and 2.1 Hz, 2H), 7.89 (d, J = 7.5 Hz, 2H),

8.23 (d, J = 8.1 Hz, 1H), 8.46 (d, J = 6.3 Hz, 1H), 8.59 (s, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 18.1, 19.0, 30.4, 46.6, 47.5, 49.6, 62.1, 65.6, 69.3, 120.0, 125.3, 126.2, 126.9, 127.3, 127.5, 128.4, 140.6, 144.6, 166.0, 168.9, 171.3, 171.9, 172.0.

Fmoc-Val-Ala-OGlyc-Val-NH$_2$ (47)

R_t(HPLC) 15.80 min; purity = 98%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.81–0.90 (m, 12H), 1.34 (d, J = 7.2 Hz, 3H), 1.91–2.04 (m, 2H), 3.86–3.92 (m, 1H), 4.11–4.38 (m, 5H), 4.56 (m, 2H), 7.06 (s, 1H), 7.29–7.44 (m, 6H), 7.72–7.90 (m, 5H), 8.45 (d, J = 6.6 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.7, 17.7, 18.1, 19.0, 19.1, 30.3, 46.6, 47.4, 57.1, 59.6, 62.2, 65.6, 120.0, 125.3, 126.9, 127.5, 140.6, 143.7, 143.8, 155.9, 166.1, 171.2, 171.8, 172.5.

Fmoc-Val-Ala-OGlyc-His(Trt)-NH$_2$ (50)

R_t(HPLC) 14.08 min; purity = 90%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.85 (2 × d, J = 6.0 and 6.3 Hz, 6H), 1.30 (d, J = 7.2 Hz, 3H), 1.90–1.98 (m, 1H), 2.73–3.10 (m, 2H), 3.87–3.92 (m, 1H), 4.17–4.32 (m, 4H), 4.57–4.58 (m, 3H), 6.97 (s, 1H), 7.09–7.44 (m, 24H), 7.73 (dd, J = 4.5 and 2.4 Hz, 2H), 7.89 (d, J = 7.5 Hz, 2H), 8.14 (d, J = 8.1 Hz, 1H), 8.46 (d, J = 6.3 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 16.6, 18.1, 18.9, 30.3, 46.6, 47.5, 51.5, 55.7, 59.6, 62.1, 65.6, 120.0, 120.2, 125.3, 126.1, 126.5, 126.9, 127.4, 127.5, 127.6, 128.2, 128.4, 128.4, 128.9, 129.1, 136.7, 140.6, 140.8, 143.7, 143.8, 166.1, 171.3, 171.8, 171.8.

Cbz-D-Phe-OGlyc-Phe-NH$_2$ (54)

ee D-Phe > 99.5; R_t(HPLC) 17.43 min; purity = 97%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 2.79–2.89 (m, 2H), 3.01–3.16 (m, 2H), 4.37–4.57 (m, 5H), 4.98 (s, 2H), 7.14–7.49 (m, 17H), 7.86 (d, J = 8.1 Hz, 1H), 8.19 (d, J = 8.4 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 36.2, 37.3, 55.6, 55.2, 63.9, 65.3, 126.1, 126.4, 127.4, 127.6, 127.9, 128.1, 128.2, 129.0, 136.7, 137.4, 137.8, 156.0, 165.9, 171.1, 172.5.

Fmoc-Val-Ala-OGlyc-Leu-OH (56)

R_t(HPLC) 21.45 min; purity = 92%; ^{1}H NMR (DMSO-d_6, 300 MHz) δ = 0.85–0.87 (m, 12H), 1.34 (d, J = 3.5 Hz, 3H), 1.54–1.60 (m, 3H), 1.98–2.02 (m, 1H), 3.87–3.92 (m, 1H), 4.22–4.36 (m, 5H), 4.46–4.60 (m, 2H), 7.32–7.44 (m, 5H), 7.74 (q, J = 4.5 and 2.7 Hz, 2H), 7.89 (d, J = 3.6 Hz, 2H), 8.15 (d, J = 3.6 Hz, 1H), 8.46 (d, J = 2.7 Hz, 1H); ^{13}C NMR (DMSO-d_6, 75 MHz) δ = 18.2, 19.0, 21.4, 22.9, 24.1, 30.3, 46.5, 50.5, 51.5, 59.7, 62.3, 65.6, 69.1, 120.0, 125.2, 126.2, 126.9, 127.3, 127.5, 128.2, 128.4, 140.6, 143.6, 143.8, 144.7, 156.0, 166.1, 170.9, 171.3, 171.6, 173.6.

Notes and references

1 B. Steffansen, C. U. Nielsen and S. Frokjaer, *Eur. J. Pharm. Biopharm.*, 2005, **60**, 241; K. Adermann, *Curr. Protein Pep. Sci.*, 2005, **6**, 205.
2 H. Korhonen and A. Pihlanto, *Curr. Pharm. Des.*, 2003, **9**, 1297; J. Torres-Llanez, B. Vallejo-Cordoba and A. F. González-Córdova, *Arch. Latinoam. Nutr.*, 2005, **55**, 111; H. Korhonen and A. Pihlanto, *Curr. Pharm. Des.*, 2007, **13**, 829; M. B. Roberfroid, *J. Nutr.*, 2007, **137**, 2493.
3 D. Hans, P. R. Young and D. D. P. Fairlie, *Med. Chem.*, 2006, **2**, 627.
4 F. Guzmán, S. Barberis and A. Illanes, *J. Biotech.*, 2007, **10**, 279.
5 W. C. Chan, *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, Oxford University Press, 2000.
6 J. Bedford, C. Hyde, T. Johnson, W. Jun, D. Owen, M. Quibell and R. C. Sheppard, *Int. J. Pept. Protein Res.*, 1992, **40**, 300.
7 F. Bordusa, *Chem. Rev.*, 2002, **102**, 4817.
8 V. Čeřovský and F. Bordusa, *J. Pept. Res.*, 2000, **55**, 325.
9 T. Miyazawa, E. Ensatsu, N. Yabuuchi, R. Yanagihara and T. Yamada, *J. Chem. Soc., Perkin Trans. 1*, 2002, 390.
10 T. Miyazawa, S. Nakajo, M. Nishikawa, K. Hamahara, K. Imagawa, E. Ensatsu, R. Yanagihara and T. Yamada, *J. Chem. Soc., Perkin Trans. 1*, 2001, 82.
11 S. M. A. Salam, K.-I. Kagawa and K. Kawashiro, *Biotechnol. Lett.*, 2005, **27**, 1199; F. Bordusa, *Braz. J. Med. Biol. Res.*, 2000, **33**, 469.
12 M. Capellas, M. D. Benaiges, G. Caminal, G. Gonzalez, J. Lopez-Santín and P. Clapés, *Biotechnol. Bioeng.*, 2000, **50**, 700.
13 M. K. Dhaon, R. K. Olsen and K. Ramasamy, *J. Org. Chem.*, 1982, **47**, 1962.
14 F. Bordusa, C. Dahl, H.-D. Jakubke, K. Burger and B. Koksch, *Tetrahedron: Asymmetry*, 1999, **10**, 307.
15 N. Wehofsky, N. Koglin, S. Thust and F. Bordusa, *J. Am. Chem. Soc.*, 2003, **125**, 6126.
16 T. Nuijens, C. Cusan, C. H. M. Schepers, J. A. W. Kruijtzer, D. T. S. Rijkers, R. M. J. Liskamp and P. J. L. M. Quaedflieg, *J. Mol. Catal. B: Enzym.*, 2011, **71**, 79–84.
17 D. F. Tai, *Curr. Org. Chem.*, 2003, **7**, 515–554.
18 S.-T. Chen, S.-Y. Chen and K.-T. Wang, *J. Org. Chem.*, 1992, **57**, 6960.
19 R. J. A. C. de Beer, B. Zarzycka, M. Mariman, H. I. V. Amatdjais-Groenen, M. J. Mulders, P. J. L. M. Quaedflieg, F. L. van Delft, S. B. Nabuurs and F. P. J. T. Rutjes, *ChemBioChem*, 2012, **13**, 1319–1326.
20 R. A. Sheldon, *Biochem. Soc. Trans.*, 2007, **35**, 1583.
21 T. Miyazawa, E. Ensatsu, M. Hiramatsu, R. Yanagihara and T. Yamada, *J. Chem. Soc., Perkin Trans. 1*, 2002, 396.
22 L. Abrahmsen, J. Tom, J. Burnier, K. Butcher, A. A. Kossiakoff and J. A. Wells, *Biochemistry*, 1991, **30**, 4151.
23 S. M. A. Salam, K. Kagawa and K. Kawashiro, *Tetrahedron: Asymmetry*, 2006, **17**, 22.