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Enzymatic Fragment Condensation of Side Chain-Protected Peptides using Subtilisin A in Anhydrous Organic Solvents: A General Strategy for Industrial Peptide Synthesis

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Abstract: Herein, the enzymatic condensation of side chain-protected peptide fragments using subtilisin A in anhydrous organic solvents is described. A screening with dipeptide Cbz-Val-Xxx carboxamidomethyl esters with H-Xxx-Val-NH_2 nucleophiles was performed, wherein Xxx stands for every (side chain-protected) amino acid residue. Finally, it was demonstrated that it is feasible to enzymatically condense larger peptide fragments (up to the 10-mer level) bearing multiple side chain-protecting groups with very high conversion.

Keywords: anhydrous conditions; fragment condensation; organic solvents; peptide synthesis; subtilisin A

Although a few pharmaceutically relevant peptides can be produced relatively cost-efficiently on a large scale by fermentation, whereas most pharmaceutical peptides on the large scale for short peptide sequences are synthesized by chemical means.[1] Solution phase stepwise chemical peptide synthesis is only feasible for medium-sized and long peptides (containing 10–50 amino acid residues), solid phase peptide synthesis (SPPS) is most commonly applied.[2] The main disadvantage of SPPS is that extremely high yields should be realized for each coupling and deprotection cycle.[3] For instance, when per step (coupling, Fmoc-deprotection and final cleavage) a yield of 95% is realized in the synthesis of a decamer, an overall yield of only 34% of the crude peptide is obtained, which should be purified by a laborious and cost-inefficient HPLC method. Therefore, on the industrial scale, peptides longer than 10–15 amino acids are not cost-efficiently synthesized in one run on the solid phase. Furthermore, during synthesis, long side chain-protected peptides tend to form tertiary structures (a process which is called "hydrophobic collapse")[4] making peptide elongation very troublesome so that a large excess of reagents and amino acid building blocks is needed. Additionally the purification of the final product is often very difficult due to the presence of significant amounts of truncated peptides and peptides containing deletions.[5]

Taking into account the drawbacks of both SPPS and solution phase peptide synthesis, it is generally impossible to synthesize, for instance, a 30-mer peptide solely by only one of these two strategies. To remedy these limitations, a hybrid approach can be used wherein protected peptide fragments are synthesized by means of SPPS and subsequently chemically coupled in solution. On paper, the "ideal" approach for the synthesis of a 30-mer peptide would be a fully symmetric (convergent) fragment condensation strategy wherein the length of the protected peptides synthesized by SPPS does not exceed 10 amino acids, e.g., a 10+10+10 strategy (Figure 1, A). However, if the desired peptide does not contain Gly or Pro residues at the *C*-terminal coupling position of the fragments, racemization is inevitable. Although with carefully selected coupling positions and reagents or condensation techniques such as the azide method,[6] racemization can be minimized, it can never be fully eliminated. Especially in the case of pharmaceutical peptides, even a few tenths of percent of diastereoisomers in the final product is unacceptable. Therefore, a convergent approach is most often not feasible and one has to adapt the peptide fragment length to the positions of the Gly and Pro residues, if they are present at all. This leads to the SPPS synthesis of undesirably short (<5 amino acids) or long (>10 amino

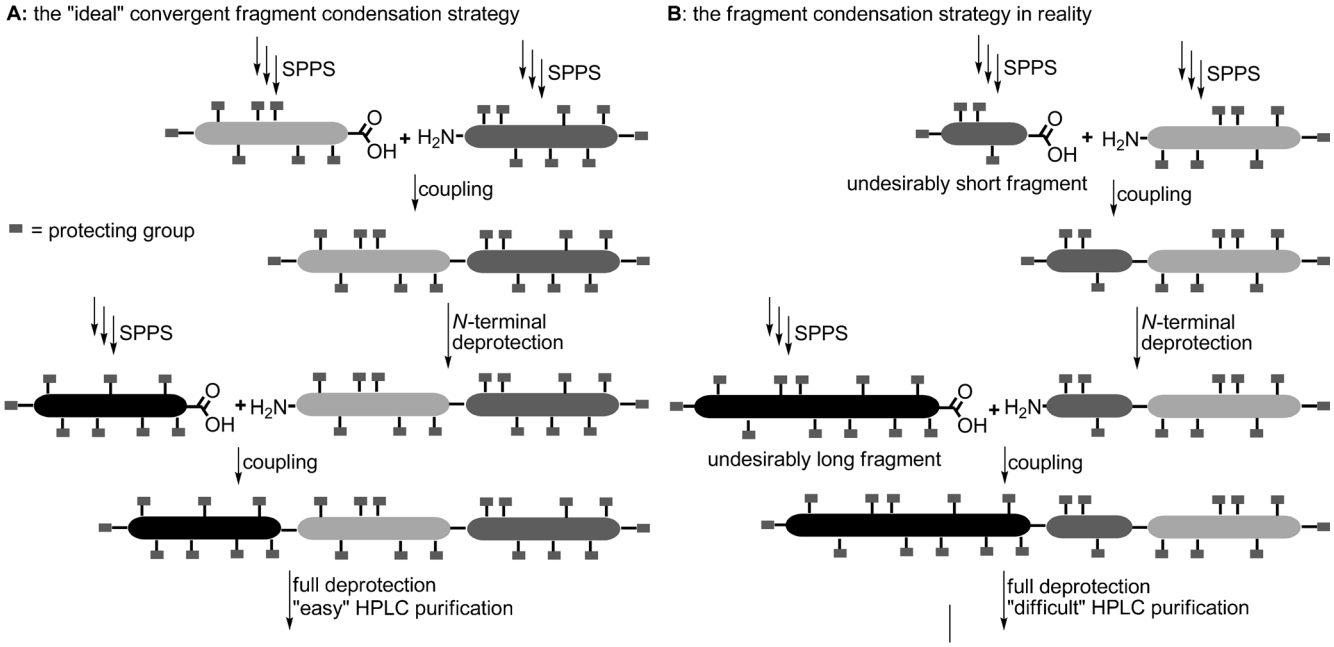


Figure 1. The hybrid peptide synthesis approach using solid phase synthesis of protected peptide fragments and condensation of these fragments in solution.

acids) protected peptide fragments, which are often very poorly soluble in organic solvents (Figure 1, B). Alternatively, other coupling positions are chosen and racemization is taken for granted. As a consequence, a difficult preparative HPLC purification is required due to the similar retention times of the two diastereoisomers leading to (partial) overlap of peaks. Mixed fractions have to undergo an additional round of preparative HPLC purification leading to much larger expenses and a lower product yield.

In contrast to chemical fragment condensation, the enzymatic coupling of peptide fragments is totally free of racemization.[7] Therefore, the 10+10+10 fragment condensation strategy for the synthesis of a 30-mer peptide is at least in theory feasible when using enzymes. Although many enzymes, i.e., proteases and lipases, have been applied to the synthesis of short peptide sequences in aqueous buffered solution, only very few examples of enzymatic peptide fragment condensations have been reported.[8] The major drawback is that the presence of water, which is essential for enzyme activity and stability, leads to undesired hydrolysis of the peptide backbone.[9] For instance, when Wong et al. compared several commercially available proteases for peptide synthesis, partial hydrolysis proved inevitable for all enzymes, even when a 2.5 molar excess of nucleophile was used.[10] Especially when longer fragments are used, with cost prices up to €100,000 per kg for a 10-mer peptide, partial hydrolysis is unacceptable. To ensure a fast coupling reaction and thereby reducing proteolysis, a peptide *C*-terminal activated ester is required as the acyl donor. Specific esters have been designed, so-called substrate mimetics, which have such a high affinity for the enzyme so that side reactions could be virtually eliminated.[11] However, peptides containing such a *C*-terminal ester moiety are notoriously difficult to synthesize chemically in high stereoisomeric purity. In fact, the coupling reagents required to synthesize these substrate mimetics are often identical to those used for chemical peptide bond formation.[12] A few solid-phase methodologies have been reported for the synthesis of peptide substrate mimetics, but these strategies are complicated, low yielding and require the use of resins which are not suitable on an industrial scale.[13] Recently, we reported the SPPS of peptide carboxamidomethyl (Cam) esters, which are highly active in subtilisin A-catalyzed peptide synthesis, using industrially applicable resins.[14] There are several examples from the literature wherein subtilisin is used for peptide fragment condensation with esters as the acyl donor.[15] However, there is always 1–5% of water present leading to partial hydrolysis of the acyl donor ester, a relatively large excess of amine is used and peptide fragments never exceed three amino acids. Recently, we described the use of subtilisin A for dipeptide synthesis in anhydrous organic solvents. Molecular sieves were used to fully eliminate the hydrolytic side reactions and only 1.1 equivalents of nucleophile were needed for full conversion of the acyl donor ester.[16] Herein, we report the fragment condensation of oligopeptide Cam esters with oligopeptide amines under anhydrous reaction conditions with subtilisin A. To our surprise, peptide fragments

Table 1. Peptide fragment condensations using Subtilisin A in anhydrous organic solvent.

Entry[a]	Acyl donor Cam ester	Nucleophile	Conversion to product [%][b]
1	Cbz-Phe-Leu-Ala-OCam	H-Ala-Leu-Phe-NH_2	hexapeptide, quant.
2	Cbz-Ala-Leu-Phe-OCam	H-Leu-Phe-NH_2	pentapeptide, 95
3	Cbz-Gly-Ile-Ala-OCam	H-Gly-Phe-NH_2	pentapeptide, quant.
4	Cbz-Ala-Pro-Leu-OCam	H-Gly-Phe-NH_2	pentapeptide, 87
5	Cbz-Ala-Pro-Leu-OCam	H-Gly-Leu-Met-NH_2	hexapeptide, quant.
6	Cbz-Gly-Ile-Ala-OCam	H-Ser(*t*-Bu)-Leu-Leu-NH_2	hexapeptide, 95
7	Cbz-Ala-Pro-Leu-OCam	H-Trp(Boc)-Met-Asp(O-*t*-Bu)-Phe-NH_2	heptapeptide, 97
8	Ac-Leu-Ser(*t*-Bu)-Lys(Boc)-Gln(Trt)-Met-OCam	H-Leu-Phe-NH_2	heptapeptide, 96

[a] *Conditions:* Alcalase-CLEA-OM, DMF/THF (1/9, v/v), 1.5 equiv. nucleophile, 3 Å molecular sieves, 37 °C, 16 h (Procedure A).

[b] Conversions estimated using HPLC-MS by integration of the acyl donor starting material and the product peaks, assuming identical response factors.

carrying the bulky protective groups on the side chain functionalities are also well accepted by the enzyme. To the best of our knowledge, enzymatic fragment condensation of fully protected peptides has never been described before.

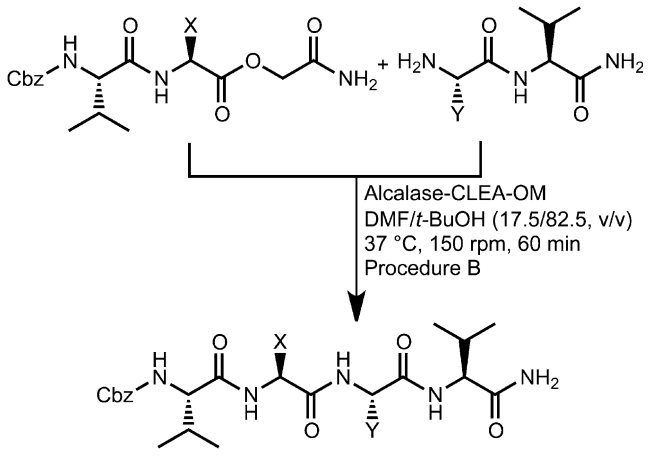
Initially, it was investigated whether Cam esters could also be used to condense peptide fragments longer than dipeptides. As shown in Table 1, a number of peptide fragments without any functionalized side chain could be successfully coupled by subtilisin A (Alcalase) in anhydrous organic solvent (entries 1–5). Excellent conversions were observed by analytical HPLC with only 1.5 equiv. of nucleophile. After work-up and purification, a yield of 73% could be obtained for Cbz-Phe-Leu-Ala-Ala-Leu-Phe-NH_2. Unfortunately, peptide fragments containing amino acids with unprotected functional groups in the side chain, are very poorly soluble in organic solvents. Therefore, it was investigated whether peptides containing amino acids with protected side chain functionalities, which are better soluble in organic solvents, were also accepted by subtilisin A. As shown in Table 1 (entries 6–8), side chain-protected peptide fragments, up to the pentamer level, were well accepted by subtilisin A and high conversions to the desired products were obtained, according to HPLC-MS analysis. It is rather surprising that large, sterically demanding protecting groups still fit in the substrate binding pockets of the enzyme.

To explore the scope and limitations of this peptide fragment condensation strategy, two libraries of dipeptides were synthesized, i.e., a Cbz-Val-Xxx-OCam and an H-Yyy-Val-NH_2 library, in which Xxx and Yyy represent all proteinogenic amino acids with protected side chain functionalities. It was decided to take a Val residue at both penultimate positions because this usually results in lower coupling rates, so that the differences between the Xxx and Yyy residues become more clearly visible. The efficiencies of all 400 possible coupling reactions were investigated as shown in Scheme 1 and Figure 2.

As is clear from Figure 2, A, the coupling reactions of a quite large number of dipeptide coupling combinations proceed smoothly and high conversions were observed despite the fact the sterically demanding Val residues are located at the penultimate positions.

With respect to the Cbz-Val-Xxx-OCam library with protected side chain functionalities, there is a clear preference for hydrophobic amino acids. However, also amino acids containing sterically demanding protecting groups, such as Gln(Trt) and Glu(O-*t*-Bu), are very well accepted by the enzyme. It must be noted that, when reaction times were extended and/or more enzyme was applied, almost all reactions could be brought to complete conversion.

Some acyl donors containing amino acids with sterically demanding protecting groups proved difficult to incorporate, such as Cbz-Val-Ser(*t*-Bu)-OCam and Cbz-Val-Thr(*t*-Bu)-OCam. Therefore, a second Cbz-



Scheme 1. Coupling reactions between Cbz-Val-Xxx-OCam and H-Yyy-Val-NH_2.

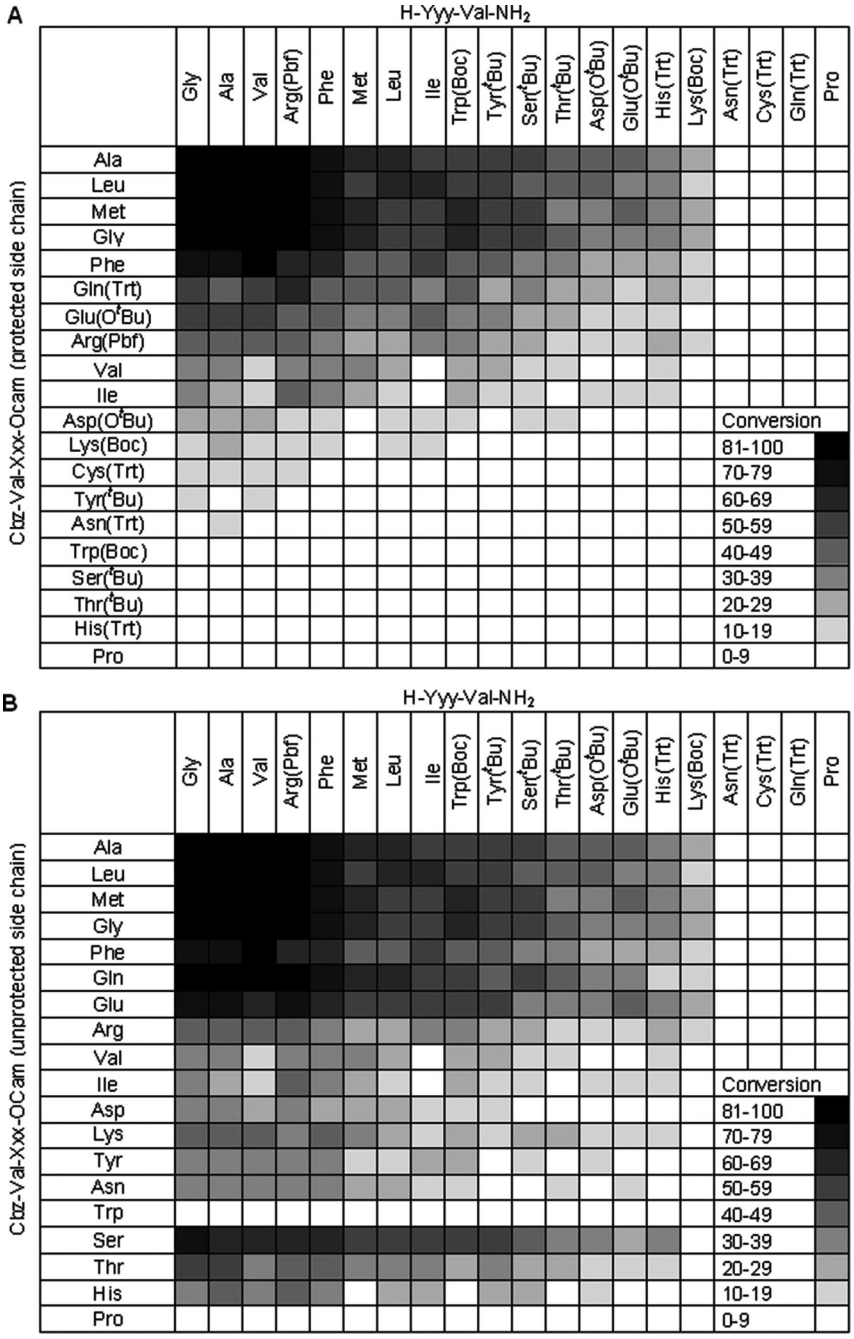


Figure 2. Comparison of coupling efficiencies (HPLC conversion to the desired tetramers) between Cbz-Val-Xxx-OCam and H-Yyy-Val-NH_2 dipeptides in which A: all functional side chains were protected and B: all functional side chains of the Cbz-Val-Xxx-OCam were unprotected, and all functional side chains of the H-Yyy-Val-NH_2 were protected.

Val-Xxx-OCam library was synthesized containing no protecting groups on the side chain functionalities. As evident from Figure 2, B, the efficiency of several coupling reactions in the case of acyl donors without protected side chain functionalities was increased compared to the fully protected acyl donors. For instance, *t*-Bu-protected Ser and Thr residues gave poor coupling efficiencies, while the unprotected analogues were readily accepted by subtilisin A, thereby broadening the scope of the enzymatic peptide fragment condensation strategy. Another large difference was observed for an unprotected Gln, which was found to be one of the most efficient acyl donors.

To show the versatility of this fragment condensation strategy, wherein only the *C*-terminal amino acid residue of the Cam ester was unprotected, two antimicrobial peptides containing ten amino acid residues were synthesized, as shown in Table 2.[17,18] These pep-

Table 2. Synthesis of two antimicrobial peptides using an acyl donor with an unprotected *C*-terminal residue.

Entry[a]	Acyl donor Cam ester	Nucleophile	Conversion to product [%]	Decapeptide product after full deprotection[b]	Isolated yield [%][c]
1	Boc-Leu-Asp(O*t*-Bu)-Gln(Trt)-Ser(*t*-Bu)-**Gln**-OCam	H-Phe-Val-Gly-Ser(*t*-Bu)-Arg(Pbf)-NH_2	92	H-Leu-Asp-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2	63
2	Boc-His(Trt)-Lys(Boc)-Thr(*t*-Bu)-Asp(O-*t*-Bu)-**Ser**-OCam	H-Phe-Val-Gly-Leu-Met-NH_2	96	H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2	55

[a] *Conditions:* Alcalase-CLEA-OM, DMF/MTBE (1/9, v/v), 3 Å molecular sieves, 37 °C, 16 h.
[b] *Conditions:* TFA/TIS/H_2O (95/2.5/2.5, v/v/v).
[c] Isolated yield based on acyl donor starting material.

tides show activity against a number of Gram-positive and Gram-negative bacteria from the skin, the oral, the respiratory and the gastrointestinal tracts.

Clearly, an almost complete conversion was obtained for the peptide fragment condensation reactions. The semi-protected acyl donor with a *C*-terminal unprotected Ser residue could be prepared by using a very acid labile protecting group, i.e., Trt, which was simultaneously deprotected during the acidic cleavage of the peptide from the solid support. During this mild acidic cleavage, only partial deprotection of His(Trt) was observed, the other side chain protecting groups were left intact. After enzymatic fragment condensation, full deprotection of the decapeptides was performed to obtain the natural antimicrobial peptides in good yield (63, 55% respectively).

After these successful 5+5 amino acid fragment condensations, it was investigated whether even longer peptide fragments could be condensed. As demonstrated in Table 3, the solvent mixture suitable for the 5+5-mer (entries 1 and 2) fragment condensation, i.e., DMF/MTBE (1/9, v/v), was not applicable to longer fragment condensations (entries 3 and 4) due to the limited solubility of the peptides. However, CH_2Cl_2 was found to be a very good alternative, since protected peptides containing more than 5–7 amino acid residues tend to be highly soluble in this solvent. In fact, when CH_2Cl_2 was used as the solvent, nearly quantitative conversion was obtained for a number of different peptide fragment condensations. When longer reaction times were applied, even an enzymatic 9+10-mer side chain-protected peptide fragment condensation proved well feasible and an excellent conversion to the 19-mer product was observed (Table 3, entry 4). It was surprising that such large peptides, bearing bulky side chain protecting groups, were still accepted by the enzyme and that the coupling proceeded smoothly, despite the anhydrous reaction conditions. This racemization-free enzymatic side chain protected peptide fragment condensation strategy opens new doors for the (industrial) synthesis of long peptides.

In conclusion, we have identified a novel enzymatic fragment condensation strategy of side chain protected peptides up to the coupling of 10+9-mer frag-

Table 3. Fragment condensation of side chain protected peptides of different lengths in DMF/MTBE (1/9, v/v) and CH_2Cl_2.

Entry[a]	Acyl donor Cam ester	Nucleophile	Conversion in DMF/MTBE [%][b]	Conversion in CH_2Cl_2 [%][b]
1	Ac-Asp(O-*t*-Bu)-Leu-Ser(*t*-Bu)-Lys(Boc)-**Gln**-OCam (5-mer)	H-Met-Glu(O-*t*-Bu)-Glu(O-*t*-Bu)-Glu-(O-*t*-Bu)-Ala-NH_2 (5-mer)	quant.	quant.
2	Ac-Thr(*t*-Bu)-Ser(*t*-Bu)-Asp(O-*t*-Bu)-Leu-Ser(*t*-Bu)-Lys(Boc)-**Gln**-OCam (7-mer)	H-Met-Glu(O-*t*-Bu)-Glu(O-*t*-Bu)-Glu-(O-*t*-Bu)-Ala-NH_2 (5-mer)	62	98
3	Ac-Thr(*t*-Bu)-Phe-Thr(*t*-Bu)-Ser(*t*-Bu)-Asp(O-*t*-Bu)-Leu-Ser(*t*-Bu)-Lys(Boc)-**Gln**-OCam (9-mer)	H-Met-Glu(O-*t*-Bu)-Glu(O-*t*-Bu)-Glu-(O-*t*-Bu)-Ala-NH_2 (5-mer)	not soluble	78
4	Ac-Thr(*t*-Bu)-Phe-Thr(*t*-Bu)-Ser(*t*-Bu)-Asp(O-*t*-Bu)-Leu-Ser(*t*-Bu)-Lys(Boc)-**Gln**-OCam (9-mer)	H-Ala-Met-Val-Ser(*t*-Bu)-Tyr(*t*-Bu)-Pro-Arg(Pbf)-Glu(O-*t*-Bu)-Asn(Trt)-His(Trt)-NH_2 (10-mer)	not soluble	95[c]

[a] *Conditions:* Alcalase-CLEA-OM, DMF/MTBE (1/9, v/v) or CH_2Cl_2, 3 Å molecular sieves, 37 °C, 16 h (Procedures A and C, respectively).
[b] Conversions estimated using HPLC by integration of the acyl donor starting material and the product peaks, assuming identical response factors.
[c] After 1 week.

ments in neat organic solvent using subtilisin A. It was shown that the strategy is broadly applicable, especially when the side chain functionality of the *C*-terminal amino acid Cam ester is left unprotected.

Experimental Section

General Condtions

Before use, Alcalase-CLEA-OM was dried as follows: 3 g Alcalase-CLEA-OM (CLEA technologies, 850 AGEU/g), 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 and the solid was dried for 1 min at ambient temperature. Protected peptide nucleophiles were synthesized on a Sieber resin using standard Fmoc SPPS protocols.[18] Protected peptide Cam esters were synthesized using literature procedures.[12] The side chain-protected Cbz-Val-Xxx-OCam library was deprotected using 5 μmol peptide in TFA/H_2O (1 mL, 95/5, v/v) and stirred for 1 h at ambient temperature. Afterwards, the volatiles were removed by a nitrogen flow and the residue was lyophilized from CH_3CN/H_2O (3/1, v/v). The resulting lyophilized powders were dissolved in DMF (90 μL) containing piperidine (10 μmol, 1 μL) and used as such for the coupling efficiency assays described in general procedure B.

General Procedure A

Enzymatic peptide coupling reactions in DMF/THF or DMF/MTBE (1/9, v/v): to a suspension of Alcalase-CLEA-OM (10 mg) and 3 Å molecular sieves (10 mg) in THF or MTBE (900 μL), a solution of the acyl donor Cam ester (30 μmol) and protected peptide nucleophile (45 μmol, 1.5 equiv.) in DMF (100 μL) was added. The obtained reaction mixture was shaken at 37 °C with 200 rpm for 16 h and subjected to LC-MS analysis.

General Procedure B

Coupling efficiencies between Cbz-Val-Xxx-OCam and H-Yyy-Val-NH_2 with protected side chain functionalities: fragment condensations were performed in a 96-well format with glass vial inserts. To a glass insert was added a solution of Cbz-Val-Xxx-OCam (2.5 μmol) in DMF (45 μL) and a solution of H-Yyy-Val-NH_2 (5 μmol, 2 equiv.) in DMF (45 μL). Subsequently, a suspension of crushed molecular sieves (5 mg) and Alcalase-CLEA-OM (4.5 mg) in *t*-BuOH (410 μL) were added. The plate was covered with an alumina lid and shaken at 37 °C with 150 rpm for 1 h. Afterwards, samples of the supernatant (300 μL) were taken and added to DMSO (700 μL) and analyzed by HPLC. The identity of the tetrapeptide products was confirmed by LC-MS analysis.

General Procedure C

Enzymatic peptide coupling reactions in CH_2Cl_2: to a suspension of Alcalase-CLEA-OM (10 mg) and 3 Å molecular sieves (10 mg) in CH_2Cl_2 (500 μL), was added a solution of the peptide Cam ester (3.0 μmol) and the peptide nucleophile (4.5 μmol) in CH_2Cl_2 (500 μL). The obtained reaction mixture was shaken at 37 °C with 200 rpm for 16 h, and the progress of the condensation reaction was monitored with LC-MS.

Cbz-Phe-Leu-Ala-Ala-Leu-Phe-NH_2

To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) in THF (9 mL) was added a solution of Cbz-Phe-Leu-Ala-OCam (300 μmol, 162 mg) and H-Ala-Leu-Phe-NH_2 (450 μmol, 157 mg, 1.5 equiv.) in DMF (1 mL). The obtained reaction mixture was shaken at 37 °C with 200 rpm for 16 h. Afterwards, the reaction mixture was filtered and the solid enzyme particles washed with CH_2Cl_2 (10 mL × 2) and DMF (5 mL × 2). The combined organic phases were concentrated under vacuum to a volume of 10 mL and purified by preparative HPLC. The pure fractions were pooled and lyophilized yielding Cbz-Phe-Leu-Ala-Ala-Leu-Phe-NH_2 as a white solid; yield: 176 mg (73%). ^{1}H NMR (DMSO-d_6, 300 MHz): δ = 0.77–0.95 (m, 12H), 1.20 (dd, J = 7.2 and 9.9 Hz, 6H), 1.30–1.67 (m, 6H), 2.68–2.85 (m, 2H), 2.98–3.04 (m, 2H), 4.13–4.44 (m, 6H), 4.94 (s, 2H), 7.09–7.32 (m, 17H), 7.48 (d, J = 8.4 Hz, 1H), 7.72 (d, J = 7.8 Hz, 1H), 7.83 (d, J = 7.8 Hz, 1H), 7.93 (d, J = 7.2 Hz, 1H), 8.05–8.09 (m, 2H); ^{13}C NMR (DMSO-d_6, 75 MHz): δ = 17.7, 21.5, 22.8, 22.9, 23.9, 24.0, 37.4, 48.1, 51.0, 51.3, 53.3, 55.9, 65.1, 126.1, 127.3, 127.5, 127.9, 128.1, 129.0, 136.9, 137.6, 137.9, 155.7, 171.3, 171.4, 171.7, 171.9, 172.5; LC-MS: R_t = 22.07 min and m/z = 814, $[M + H]^+$, calcd. for $C_{44}H_{60}N_7O_8^+$: 814.

H-Leu-Asp-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2

To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) in THF or MTBE (9 mL), was added a solution of Boc-Leu-Asp(O*t*Bu)-Gln(Trt)-Ser(*t*-Bu)-Gln-OCam (0.3 mmol, 330 mg) and H-Phe-Val-Gly-Ser-(*t*-Bu)-Arg(Pbf)-NH_2 (0.45 mmol, 1.5 equiv., 393 mg) in DMF (1 mL). The obtained reaction mixture was shaken at 37 °C with 200 rpm for 16 h. Afterwards, the reaction mixture was filtered and the solid enzyme particles and molecular sieves washed with CH_2Cl_2 (100 mL × 5) and DMF (5 mL × 2). The combined organic phases were concentrated under vacuum and the residue dissolved in TFA/TIS/H_2O (10 mL, 95/2.5/2.5, v/v/v) and stirred at ambient temperature for 60 min. The reaction mixture was added to MTBE/*n*-heptanes (100 mL, 1/1, v/v) and the precipitates collected by centrifugation (4000 rpm, 10 min). The residue was purified by preparative HPLC, the pure fractions were pooled and lyophilized affording H-Leu-Asp-Gln-Ser-Gln-Phe-Val-Gly-Ser-Arg-NH_2 as a white solid; yield: 214 mg (63%). LC-MS: R_t = 13.67 min and m/z = 568 $[M+2H]^{2+}$, calcd. for $C_{48}H_{80}N_{16}O_{16}^{2+}$: 568.

H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2

To a suspension of Alcalase-CLEA-OM (100 mg) and 3 Å molecular sieves (100 mg) in THF or MTBE (9 mL), was added a solution of Boc-His(Trt)-Lys(Boc)-Thr(*t*-Bu)-Asp-(O-*t*-Bu)-Ser-OCam (0.3 mmol, 313 mg) and H-Phe-Val-Gly-Leu-Met-NH_2 (0.45 mmol, 1.5 equiv., 251 mg) in DMF (1 mL). The obtained reaction mixture was shaken at 37 °C with 200 rpm for 16 h. Afterwards, the reaction mixture was filtered and the solid enzyme particles and molecular sieves

washed with CH_2Cl_2 (100 mL × 5) and DMF (5 mL × 2). The combined organic phases were concentrated under vacuum and the residue dissolved in TFA/TIS/H_2O (10 mL, 95/2.5/2.5, v/v/v) and stirred at ambient temperature for 60 min. The reaction mixture was added to MTBE/*n*-heptanes (100 mL, 1/1, v/v) and the precipitates collected by centrifugation (4000 rpm, 10 min). The residue was purified by preparative HPLC, the pure fractions were pooled and lyophilized affording H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2 as a white solid; yield: 187 mg (55%). LC-MS: $R_t = 10.23$ min and $m/z = 379$ $[M+3H]^{3+}$, calcd. for $C_{50}H_{83}N_{14}O_{14}S^{3+}$: 379.

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