# Total Enzymatic Synthesis of the Cholecystokinin Octapeptide (CCK-8)

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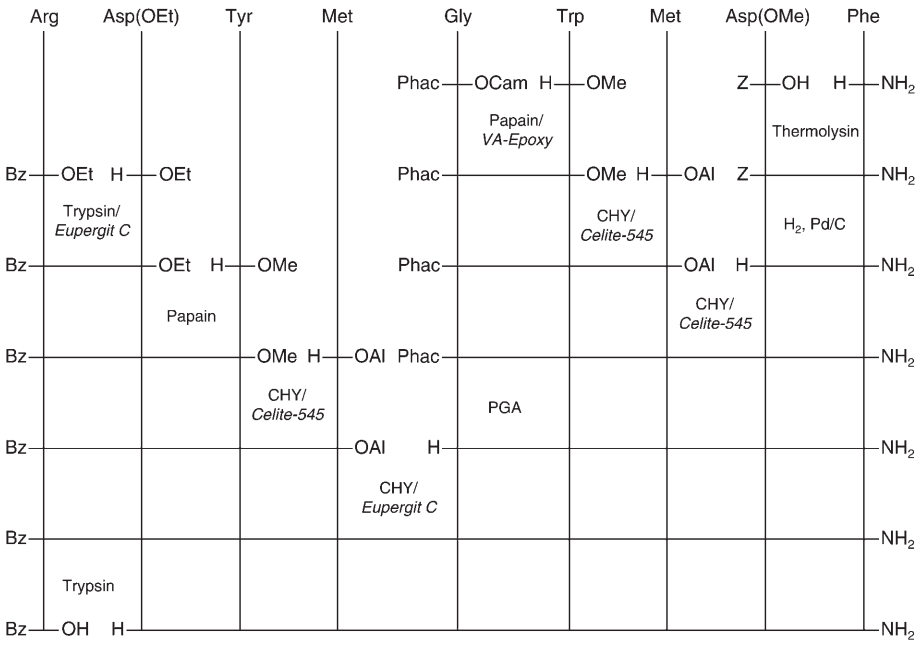
The enzymatic synthesis of the cholecystokinin octapeptide (CCK-8) is reported. The target octapeptide CCK-8 is the minimum active sequence with the same biological activity as naturally occurring cholecystokinin and is a potential therapeutic agent in the control of gastrointestinal function as well as a drug candidate for the treatment of epilepsy and obesity. The protected CCK-8 was obtained by incubation of Bz-Arg-Asp(OEt)-Tyr-Met-OAl and Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ with immobilized $\alpha$-chymotrypsin. The Bz-Arg group was used as an N-terminal protecting group in the synthesis of the tripeptide fragment. The protected CCK-8 was treated with trypsin to remove the Bz-Arg group successfully. Free or immobilized enzymes were used as catalysts. The effect of the acyl donor ester structure, the C($\alpha$) protecting group of the nucleophile, reaction media, enzyme, and the carrier of the enzymes on the outcome of the coupling reaction was studied.

**Introduction.** – Cholecystokinin (CCK) is a polypeptide hormone of 33 amino acid residues and is involved in the food absorption and digestion. It is also widely distributed in the vertebrate central nervous system. The cholecystokinin C-terminal octapeptide ($CCK_{26–33}$, CCK-8) is the minimum active sequence with the same biological activity as the naturally occurring cholecystokinin. It is a potential therapeutic agent in the control of gastrointestinal function [1] and is a drug candidate for the treatment of epilepsy [2], obesity [3], and inflammatory diseases [4]. In contrast to the chemical synthesis, in enzymatic peptide synthesis, there occurs no racemization, and all the coupling steps can be conducted under mild conditions with a minimum of side-chain protection. The enzyme-catalyzed peptide synthesis is widely reported in the literature [5–7]. Many efforts were devoted in the past to the enzymatic synthesis of CCK-8 [8–10]. Unfortunately, there is no general common protocol for the enzymatic peptide synthesis. To suppress the competing hydrolytic reaction, the undesired oligomerization and product cleavage, each coupling step needs to be optimized. A series of reaction parameters were investigated to find the best condition for each peptide-bond formation such as acyl donor, nucleophile, reaction media, and enzyme.

In our previous work [11–13], we reported the enzymatic synthesis of fragments of CCK-8. We hereby report the enzymatic synthesis of the protected (Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$) and N-terminal free octapeptide (Asp-(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$). As it is difficult to estimate the optimal fragments leading to a successful condensation, different fragments of CCK-8 were synthesized. In this communication, we report only the successful coupling steps.

Bz-Arg-Asp(OEt)-Tyr-Met-OAl was synthesized by stepwise chain elongation from the N- to the C-terminus. The protected pentapeptide obtained by fragment condensation between Phac-Gly-Trp-Met-OAl and Asp(OMe)-Phe-$NH_2$ was subjected to the enzymatic cleavage of the Phac group by penicillin G amidase (PGA) to obtain the N-terminal free pentapeptide Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$. The final fragment coupling in the [3 + (3 + 2)] approach was successful by coupling Bz-Arg-Asp(OEt)-Tyr-Met-OAl with Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (*Scheme*).

Scheme. *Selected Strategy for the Synthesis of the N-Terminal Free Octapeptide CCK-8* (CHY: $\alpha$-chymotrypsin)




One of the objectives was to use the N-terminal protecting groups, which are cleaved by enzymes under mild conditions. In the present work, phenylacetyl (Phac) and benzoyl-arginine (Bz-Arg) were used as enzymatically labile N-terminal protecting groups. We have found earlier that the cleavability of the Phac group depends on the peptide sequence, and, in special cases, it cannot be cleaved at all. Therefore, Bz-Arg was used as an alternative N-terminal protecting group as suggested by *Glass* [14]. The Bz-Arg group was introduced and removed easily with trypsin. Trypsin is highly specific towards basic amino acids in the $P_1$ position, and, because there is no basic amino acid in the target sequence, the use of Bz-Arg as a protecting group is possible. The enzymatic removal of the N-terminal protecting group from protected CCK-8 is not yet reported. *Fite et al.* [15] described the synthesis of the Phac-protected CCK-8. However, the cleavage of the Phac group is not reported. Other authors [8–10] used chemically cleavable N-terminal protecting groups.

In this work, the reactions were catalyzed by immobilized enzymes as far as possible. For each reaction, the enzymatic preparation leading to the maximum product yield was selected. Substrate concentrations and acyl donor/nucleophile ratios were chosen to obtain a good product yield and to minimize the oligomerization.

The selection of the nucleophile ester was made taking into account its reactivity as well as the possibility that the product of the reaction would be used as the acyl donor for the next step. For instance, the methionine allyl ester was chosen as a nucleophile to prepare the allyl ester at the C-terminal end of the peptide. The peptide allyl ester was then used directly as an acyl donor without any chemical modification in the following fragment condensation step. The use of allyl esters in fragment condensation is reported in [15].

**Results and Discussion.** – *Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-OAl.* The protecting group Bz-Arg was introduced easily to Asp(OEt)-OEt according to the method described elsewhere [16]. The reaction was catalyzed by trypsin/*Eupergit C*. Enzymatic introduction and cleavage of protecting groups have advantages over chemical methods due to their selectivity, specificity, and mild operation conditions. To shift the equilibrium of the enzymatically catalyzed synthesis to the product side, an excess of one reactant and the highest possible concentration is needed. In the stepwise elongation from Bz-Arg-OEt · HCl to Bz-Arg-Asp(OEt)-Tyr-Met-OAl, easily available nucleophiles (amino acid esters) were used in excess. The reaction between Bz-Arg-OEt · HCl and Asp(OEt)-OEt · HCl under solvent-free condition similar to the method described by *Cerovsky* [17] to yield Bz-Arg-Asp(OEt)-OEt was kinetically controlled. After a reaction time of 4 h, the yield of isolated Bz-Arg-Asp(OEt)-OEt was 71.5%. If the reaction was not terminated at its optimal point, side reactions were observed. Two by-products were due to the esterase activity of trypsin, which hydrolyzed the esters of both starting material (Bz-Arg-OEt · HCl) and product (Bz-Arg-Asp(OEt)-OEt) giving rise to Bz-Arg-OH and Bz-Arg-Asp(OEt)-OH, respectively. Because of the proteolytic action of trypsin/*Eupergit C*, Bz-Arg-OH has also resulted from cleavage of the product Bz-Arg-Asp(OEt)-OEt. Both by-products could be kept below 10% by monitoring the kinetic of the reaction with HPLC. The immobilized trypsin could be used at least three times. When the reaction was carried out in *Tris* · HCl buffer (0.1M, pH 8.0), the only products were Bz-Arg-OH and Bz-Arg-Asp(OEt)-OH. For the extension of this sequence to Bz-Arg-Asp(OEt)-Tyr-OH, two different acyl donors were chosen in solvent-free conditions in the presence of papain. The first acyl donor, Bz-Arg-Asp(OEt)-OH did not react with Tyr-OMe · HCl, but the diester Bz-Arg-Asp(OEt)-OEt reacted with Tyr-OMe · HCl to give the product in 30.5% yield. This is generally the case in peptide synthesis where peptide esters react better than peptides with a free carboxylic acid function. From the kinetics of papain-catalyzed peptide synthesis, esterified acyl donors are preferable than acyl donors with a free carboxy group [18]. When the molar ratio of Bz-Arg-Asp(OEt)-OEt and Tyr-OMe · HCl was 1:4 and 1:1.5, the yield of Bz-Arg-Asp(OEt)-$(Tyr)_n$-OH ($n = 2$, 3) determined by HPLC was 10 and 5%, respectively. The sequence was further extended to Bz-Arg-Asp(OEt)-Tyr-Met-OAl by reacting Bz-Arg-Asp(OEt)-Tyr-OH and Met-OAl · Tos in MeCN containing 3.75% *Tris* · HCl buffer (0.1M, pH 8.1). Kinetically controlled peptide synthesis with a free carboxylic acid is not reported often but similar

cases were observed many times in our previous work. A hydrolyzed product (Bz-Arg-Asp(OEt)-Tyr-Met-OH) was also formed, but its yield was kept below 5% by optimizing the conditions. In this reaction, the amount of buffer played a crucial role to direct the reaction either towards the target product or towards the hydrolyzed product. When the reaction was conducted in *Tris* · HCl buffer (0.1M, pH 8.1) containing 10% MeCN, only Bz-Arg-Asp(OEt)-Tyr-Met-OH was formed. However, when the concentration of MeCN was increased to 96.2%, Bz-Arg-Asp(OEt)-Tyr-Met-OAl was isolated in a good yield (63.9%). For the final fragment coupling, Bz-Arg-Asp(OEt)-Tyr-Met-OAl was used.

*Synthesis of CCK-5* (Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$). *Capellas et al.* [19] reported the reactivity difference among carboxamidomethyl (OCam), benzyl (OBzl) and methyl (OMe) esters of Z-Gly-Trp during the coupling reaction with Met-OEt in organic media. They reported that the methyl ester was least reactive and carboxamidomethyl ester was the most reactive. We also observed that Phac-Gly-OMe could not be coupled with Trp-OMe catalyzed by immobilized papain. As expected, the OCam ester could be used and the dipeptide was isolated in 90% yield. For the extension of this sequence from Phac-Gly-Trp-OMe to Phac-Gly-Trp-Met-OAl, two conditions were studied. When the reaction between Phac-Gly-Trp-OMe and Met-OAl · Tos was carried out in AcOEt containing 0.2% Tris · HCl buffer (0.1M, pH 8.2) in presence of $\alpha$-chymotrypsin, only 3% of the product (Phac-Gly-Trp-Met-OAl) but 20% of the by-product (Phac-Gly-Trp-OH) was obtained. However under solvent-free condition, the condensation reaction was successful. Basic, neutral, and acidic solvent-free systems were created by using the salts $KHCO_3/Na_2CO_3 \cdot 10\ H_2O$, $Na_2SO_4 \cdot 10\ H_2O$, and $KHSO_4/Na_2SO_4 \cdot 10\ H_2O$, respectively. The highest yield was 60.1% after 4 h by using the basic salt system.

The synthesis of another fragment, Asp(OMe)-Phe-$NH_2$, was carried out as reported elsewhere [11]. In an earlier investigation of our group [11], a tripeptide OCam ester (Phac-Gly-Trp-Met-OCam) was condensed with the dipeptide amide (Asp(OMe)-Phe-$NH_2$). For the synthesis of the tripeptide fragment, Phac-Gly-Trp-OMe was coupled with Met-OEt · HCl. The conversion of the ethyl ester to the OCam ester needed three steps. Here, we report the successful application of the allyl ester. The tripeptide allyl ester could be obtained by using Met-OAl instead of Met-OEt. The pentapeptide Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was obtained by direct fragment condensation of the tripeptide allyl ester Phac-Gly-Trp-Met-OAl and the dipeptide amide Asp(OMe)-Phe-$NH_2$. The condensation of Phac-Gly-Trp-Met-OAl with Asp-(OMe)-Phe-$NH_2$ · HCl was investigated in different reaction systems (*Table*). No reaction took place under the solvent-free conditions and in MeCN. However, the coupling was observed in AcOEt containing a small amount of buffer and $Et_3N$. The yield of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was 24.3% (HPLC) when the reaction was carried out in AcOEt containing 0.35% *Tris* · HCl buffer. When the *Tris* · HCl buffer was replaced by borax buffer containing $Et_3N$, the yield increased to 34.2%. The best result (66.7%) was obtained when a buffer of *Tris*, borax, and $Et_3N$ was added.

The selective deprotection of Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was conducted with immobilized PGA. Unfortunately, the yield of the reaction was low.

*Synthesis of Bz-Arg-Protected CCK-8 [3 + (3 + 2)].* The crucial fragment condensation between Bz-Arg-Asp(OEt)-Tyr-Met-OAl and Gly-Trp-Met-Asp(OMe)-

Table. *Isolated Amount* (HPLC) *of Product* (Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$), *By-product* (Phac-Gly-Trp-Met-OH), *and Acyl Donor* (Phac-Gly-Trp-Met-OAl). Acyl donor and nucleophile were mixed in different reaction systems. $\alpha$-Chymotrypsin/*Celite-545* was used to catalyze the reaction.

| Reaction System (Conditions) | Reactants | | Time [h] | Conversion [%][a]) | | |
|---|---|---|---|---|---|---|
| | Acyl donor [mmol] | Nucleophile [mmol] | | Product | By-product | Acyl donor |
| Solvent free (0.2 mmol $KHCO_3$, 0.04 mmol $Na_2CO_3 \cdot 10\ H_2O$) | 0.1 | 0.2 | 72 | – | 15.3 | 42.9 |
| MeCN System[b]) (2 ml MeCN, 10 µl 0.1M *Tris*·HCl, pH 8.1) | 0.1 | 0.2 | 72 | – | – | 82.9 |
| AcOEt System (2 ml AcOEt, 7 µl 0.1M *Tris*·HCl, pH 8.1) | 0.05 | 0.1 | 96 | 24.3 | 12.0 | 36.8 |
| AcOEt System[b]) (2 ml AcOEt, 8 µl 0.1M Borax, pH 8.2) | 0.1 | 0.2 | 72 | 34.2 | 9.5 | 3.9 |
| AcOEt System[c]) (6 ml AcOEt, 240 µl 0.1M Borax, pH 8.2, 400 µl 0.1M *Tris*·HCl, pH 8.1) | 0.2 | 0.5 | 95 | 66.7 | 2.0 | – |

[a]) Determined by HPLC. [b]) 10 µl of $Et_3N$ were added. [c]) 20 µl of $Et_3N$ were added.

Phe-$NH_2$ with $\alpha$-chymotrypsin/*Eupergit C* gave a protected CCK-8. Unexpectedly, free $\alpha$-chymotrypsin did not catalyze the reaction. However, when the enzyme was immobilized on *Eupergit C* or *Celite-545*, 17 and 5% yield was obtained, respectively. The main cause of the low yield was due to the hydrolysis of the allyl ester of Bz-Arg-Asp(OEt)-Tyr-Met-OAl to Bz-Arg-Asp(OEt)-Tyr-Met-OH. It was tried to control the hydrolytic action of the enzyme by lowering the $H_2O$ content in the system; however, the yield could not be improved. Unlike in other coupling steps reported here, the acyl donor in this reaction was added in double molar ratio to the nucleophile because of much easier preparation of Bz-Arg-Asp(OEt)-Tyr-Met-OAl than Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.

*Cleavage of Bz-Arg from Protected CCK-8.* The action of trypsin is restricted to the linkage of the carboxy group of arginine or other basic amino acids. As expected, trypsin cleaved the Arg-Asp bond with the formation of Bz-Arg-OH and the deprotected peptide. But several other peptide bonds were also attacked, especially after longer reaction times. However, applying 'tosyl-amido-2-phenylethyl chloromethyl ketone' (TPCK)-treated trypsin resulted in highly selective cleavage and relatively pure Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ was obtained as evidenced by ESI-MS (*Fig. 1*). *Fig. 2* shows the ESI-MS of the target octapeptide after purification. At pH 7, there was no cleavage at all but at a pH of 8.5, the cleavage underwent smoothly. In contrast to the Phac group, the Bz-Arg protecting group proved to be useful in longer peptide sequences provided they are free of basic amino acids.

**Conclusions.** – A complete enzymatic synthetic route for the N-terminal protected and deprotected CCK-8 has been developed. The cleavability of the enzymatically

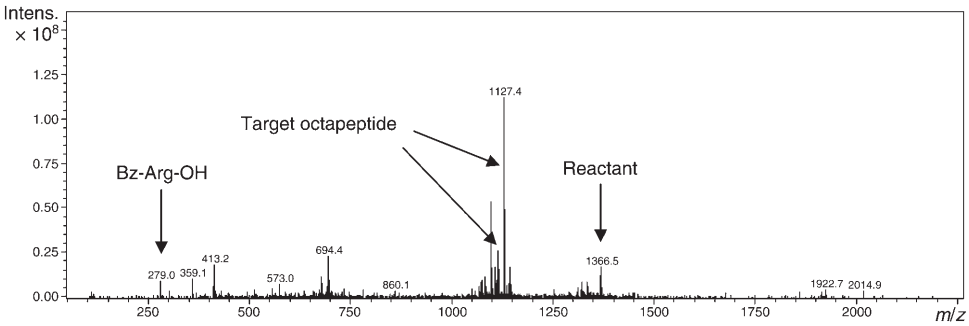



Fig. 1. *ESI-MS of the reaction mixture of the aqueous suspension of protected CCK-8 and TPCK-treated trypsin at pH 8.5*

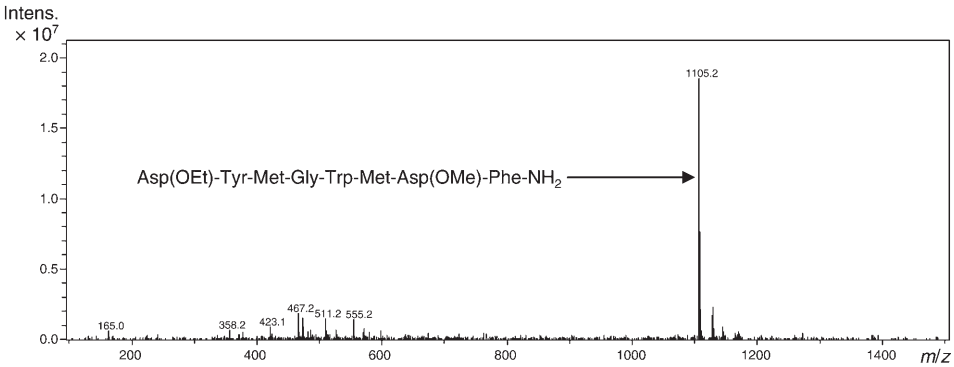



Fig. 2. *ESI-MS of Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$* (CCK-8)

labile Phac group depends on the peptide sequence, and sometimes it cannot be cleaved at all. It could be demonstrated that the Bz-Arg group is superior to other reported N-terminal protecting groups in CCK-8 syntheses, as it is cleaved easily with trypsin. Additionally, it could be shown that the fully enzymatic peptide synthesis even of longer peptides is possible if all available condensation systems are applied. Especially in some cases, the solvent-free conditions proved to be very useful. The enzymatic technology, 'Green Chemistry', is a versatile alternative to the chemical peptide synthesis.

## Experimental Part

*Materials.* Papain (EC 3.4.22.2) from *Carica papaya* ($H_2O$-soluble, 30000 USP-U/mg using casein as substrate) and $\alpha$-chymotrypsin (EC 3.4.21.1) from bovine pancreas (Type II, 3 × crystallized, lyophilized powder, 350 U/mg using *N*-acetyl-L-tyrosine ethyl ester (ATEE) as substrate) were obtained from *Merck*. Thermolysin (EC 3.4.24.2) from *Bacillus thermoproteolyticus rokko* (Protease X, lyophilized powder containing calcium and sodium buffer salts, 50 U/mg protein, casein assay) was from *Sigma*. Trypsin (EC 3.4.21.4) from porcine pancreas (crystallized, lyophilized, 40 U/mg using *N*-benzoyl-L-arginine ethyl ester (BAEE) as substrate) was obtained from *Merck*. 'Tosyl-amido-2-phenylethyl chloromethyl ketone' (TPCK)-treated trypsin (EC 3.4.21.4) from bovine pancreas (10138 U/mg), *Celite-*

*545* (particle size 20–45 μm) and penicillin G amidase (PGA) immobilized on *Eupergit C* (109 U/g) were obtained from *Fluka*. *Nucleosil C18* (5 μm) and *Polygosil C18* (50–60 μm) were from *Macherey-Nagel*. α-Chymotrypsin was adsorbed on *Celite-545* according to *Basso et al.* [20]. α-Chymotrypsin was immobilized on *Eupergit C* as mentioned below. *Eupergit C* (10 g) washed with $KH_2PO_4$ buffer (1M, pH 8.0) was added to the soln. of α-chymotrypsin (600 mg) in $KH_2PO_4$ buffer (1M, 100 ml, pH 8.0). After rotating for 3 d, NaCl (1M, 100 ml) was added to the suspension. The solvent was sucked off and the α-chymotrypsin/*Eupergit C* was washed with $KH_2PO_4$ buffer (0.05M, pH 8.0) and stored at −20°. Trypsin was immobilized on *Eupergit C* as reported elsewhere [21]. The vinyl acetate resin was epoxidized according to *Burg et al.* [22]. Papain was immobilized on *VA-Epoxy* as reported elsewhere [23]. Methionine allyl ester tosylate (Met-OAl·Tos) was purchased from *Fluka*. All L-amino acids were gifts from *Degussa*. Phac-Gly-OCam was synthesized according to *Martinez et al.* [24][25]. Bz-Arg-OH was synthesized according to the standard procedure [26][27]. The amino acid derivatives were prepared by standard procedures in our laboratory. All other chemicals and solvents used were of anal. grade.

*Anal. HPLC.* The HPLC system consisted of *Gilson* pumps *305* and *307*, the *Gilson* control software *712* and a *Merck LaChrom-7400* detector set to 260 nm. The anal. columns (100 × 2 mm) were filled with *Nucleosil C18* (5 μm) and the flow rate was adjusted to 0.3 ml/min.

The MeOH solvent system consisted of 0.05M $AcONH_4$ (pH 6.5) (*A*) and 80% MeOH/$H_2O$ containing 0.05M $AcONH_4$ (*B*). The gradient elution systems are listed below.

*HPLC System i:* Gradient elution from 40 to 55% *B* over 24 min: 0–3.60 min, 40% *B*; 3.60–7.50 min, 40–55% *B*; 7.50–16.50 min, 55% *B*; 16.50–17.20 min, 55–40% *B*; 17.20–24.00 min, 40% *B*.

*HPLC System ii:* Gradient elution from 40 to 85% *B* over 19.50 min: 0–5.00 min, 40% *B*; 5.00–8.00 min, 40–85% *B*; 8.00–12.00 min, 85% *B*; 12.00–13.00 min, 85–35% *B*; 13.00–16.00 min, 35% *B*; 16.00–17.00 min, 35–40% *B*; 17.00–19.50 min, 40% *B*.

*HPLC System iii:* Gradient elution from 55 to 85% *B* over 22 min: 0–4.00 min, 55% *B*; 4.00–7.00 min, 55–85% *B*; 7.00–11.50 min, 85% *B*; 11.50–12.50 min, 85–50% *B*; 12.50–13.51 min, 50% *B*; 13.51–14.50 min, 50–55% *B*; 14.50–22.00 min, 55% *B*.

The MeCN solvent system consisted of 0.1% aq. TFA (*A*) and 80% MeCN/$H_2O$ containing 0.1% TFA (*B*). The used gradient systems are listed below.

*HPLC System iv:* Gradient elution from 35 to 60% *B* over 19.50 min: 0–4.30 min, 35% *B*; 4.30–5.50 min, 35–60% *B*; 5.50–13.00 min, 60% *B*; 13.00–14.20 min, 60–35% *B*; 14.20–19.50 min, 35% *B*.

*HPLC System v:* Gradient elution from 40 to 85% *B* over 19.50 min: 0–5.00 min, 40% *B*; 5.00–8.00 min, 40–85% *B*; 8.00–12.00 min, 85% *B*; 12.00–13.00 min, 85–35% *B*; 13.00–16.00 min, 35% *B*; 16.00–17.00 min, 35–40% *B*; 17.00–19.50 min, 40% *B*.

*HPLC System vi:* Gradient elution from 50 to 85% *B* over 19.50 min: 0–5.00 min, 50% *B*; 5.00–8.00 min, 50–85% *B*; 8.00–12.00 min, 85% *B*; 12.00–13.00 min, 85–45% *B*; 13.00–16.00 min, 45% *B*; 16.00–17.00 min, 45–50% *B*; 17.00–19.50 min, 50% *B*.

*Prep. HPLC.* The HPLC system was the same as in the anal. HPLC. The columns (250 × 8 mm) were filled with *Nucleosil C18* (7 μm), and the flow rate was set to 4 ml/min. The solvent system consisted of 0.1% aq. TFA (*A*) and 80% MeCN/$H_2O$ containing 0.1% TFA (*B*).

*Prep. Chromatography* (MPLC). The samples were loaded to the column with a peristaltic pump *P-1* (*Pharmacia Fine Chemicals*), and the elution was achieved with a *ProMinent electronic E-0803* pump. The UV absorption of the eluent was monitored with a *ISCO Model UA-5* detector set to 254 nm, and fractions of *ca.* 20 ml were collected with a *ISCO-328* fraction collector. The column (30 × 4 cm) was filled with *Polygosil C18* (50–60 μm), and the flow rate was set to 5 ml/min.

The solvent system consisted of aq. 0.05M $AcONH_4$ (pH 6.5) (*A*) and MeOH containing 25 ml 2M $AcONH_4$ per 1000 ml (*B*). The peptides were eluted with a step gradient, beginning with a MeOH concentration of the sample up to 80% *B* according to the elution of the products.

*Peptide Syntheses. Bz-Arg-Asp(OEt)-OEt.* The synthesis of Bz-Arg-Asp(OEt)-OEt was carried out under solvent-free conditions. Bz-Arg-OEt·HCl (2.1 g, 6 mmol), Asp(OEt)-OEt·HCl (2.7 g, 12 mmol), $Na_2CO_3 \cdot 10\ H_2O$ (0.8 g, 2.8 mmol), $Na_2CO_3$ (0.8 g, 7.6 mmol), and grinded NaOH (0.34 g, 8.4 mmol) were mixed thoroughly with a glass rod. Trypsin/*Eupergit C* (4 g) was added, and the mixture was stirred manually every 20 min. The reaction was monitored with HPLC system *ii*. When the reaction was complete, the mixture was diluted with 15 ml of 80% EtOH containing 2 ml of AcOH, and the pH was

adjusted to 7. This suspension was then transferred to a sintered glass frit and washed with 80% EtOH until the product was extracted completely from the solid residue. The filtrate was evaporated to dryness *in vacuo*. The remaining residue was dissolved in 40% aq. MeOH and separated by MPLC. The pooled fractions of Bz-Arg-Asp(OEt)-OEt were lyophilized twice yielding a white powder (1.925 g, 71.5%, $t_R$ 11.83 min in HPLC system *ii*). The product was verified by comparing with the reference sample of our laboratory.

*Bz-Arg-Asp(OEt)-Tyr-OH.* Bz-Arg-Asp(OEt)-OEt (450 mg, 1 mmol), Tyr-OMe · HCl (350 mg, 1.5 mmol), $KHCO_3$ (1.0 g, 10 mmol), $Na_2SO_4 \cdot 10\ H_2O$ (75 mg, 0.25 mmol), EDTA (0.1M, 50 µl), and 2-sulfanylethanol (60 µl) were mixed thoroughly. Free papain (80 mg) was added, and the mixture was stirred manually every 20 min. The reaction was monitored with HPLC system *i*. When the reaction was complete, the mixture was diluted with 20 ml of $H_2O$, transferred into a sintered glass frit, and washed with $H_2O$ until the filtrate was neutral. The remaining filter cake was washed with pure MeOH, and the filtrate was collected. The filtrate was evaporated to dryness *in vacuo*. The obtained residue was dissolved in 35% aq. MeOH and separated by MPLC. The pooled fractions of Bz-Arg-Asp(OEt)-Tyr-OH were lyophilized twice yielding a white powder (179 mg, 30.5%). $t_R$ 7.0 min (HPLC system *i*). FAB-MS ($C_{28}H_{36}N_6O_8$; calc. 584.3): 585.2 ($[M+H]^+$), 607.2 ($[M+Na]^+$).

The by-products were isolated and characterized by FAB-MS as Bz-Arg-Asp(OEt)-Tyr-Tyr-OH (13 mg, 1.7%, $t_R$ 9.0 min in HPLC system *i*; FAB-MS ($C_{37}H_{45}N_7O_{10}$; calc. 747.3): 748.2 ($[M+H]^+$), 770.3 ($[M+Na]^+$)) and Bz-Arg-Asp(OEt)-Tyr-Tyr-Tyr-OH (7 mg, 0.7%). $t_R$ 11.0 min (HPLC system *i*). FAB-MS ($C_{46}H_{54}N_8O_{12}$; calc. 910.4): 911.5 ($[M+H]^+$), 933.7 ($[M+Na]^+$).

*Bz-Arg-Asp(OEt)-Tyr-Met-OAl.* Bz-Arg-Asp(OEt)-Tyr-OH (180 mg, 0.3 mmol) and Met-OAl · Tos (272 mg, 0.75 mmol) were dissolved in MeCN (8 ml) containing *Tris* · HCl buffer (0.1M, 300 µl, pH 8.1). To this soln., α-chymotrypsin/*Celite-545* (500 mg) was added, and the mixture was stirred. The reaction was monitored with HPLC system *iv*. After 47 h, the reaction was stopped by adding a few drops of AcOH, and the mixture was filtered to remove α-chymotrypsin/*Celite-545*. The filter cake was washed with pure MeOH, and the filtrate was collected and evaporated to dryness *in vacuo*. The residue was dissolved in 45% aq. MeOH and separated by MPLC. The pooled fractions were lyophilized yielding a white powder of Bz-Arg-Asp(OEt)-Tyr-Met-OAl (290 mg, 63.9%). $t_R$ 10.09 min (HPLC system *iv*). FAB-MS ($C_{36}H_{49}N_7O_9S$; calc. 755.3): 756.2 ($[M+H]^+$), 778.2 ($[M+Na]^+$).

*Phac-Gly-Trp-OMe.* This dipeptide was synthesized in a yield of 90% according to the method described elsewhere [11].

*Phac-Gly-Trp-Met-OAl.* Phac-Gly-Trp-OMe (158 mg, 0.4 mmol), Met-OAl · Tos (200 mg, 0.55 mmol), $KHCO_3$ (40 mg, 0.4 mmol), and $Na_2CO_3 \cdot 10\ H_2O$ (35 mg, 0.12 mmol) were mixed thoroughly. α-Chymotrypsin/*Celite-545* (150 mg) was added and stirred manually every 20 min. The reaction was monitored with HPLC system *v*. The mixture was then washed with $H_2O$ until neutrality to remove the salts. The filter cake was suspended in 100 ml of warm 80% EtOH, sonicated to extract the tripeptide, and filtered. The solvent was evaporated to dryness *in vacuo*. The tripeptide ester Phac-Gly-Trp-Met-OAl was obtained by recrystallization with EtOH as a white solid (134 mg, 60.1%). M.p. 164–167°. $t_R$ 11.63 min (HPLC system *v*). FAB-MS ($C_{29}H_{34}N_4O_5S$; calc. 550.2): 551.2 ($[M+H]^+$), 573.1 ($[M+Na]^+$).

*Z-Asp(OMe)-Phe-NH$_2$.* The synthesis of this dipeptide was performed according to the procedure reported elsewhere [11]. However, the workup was different. The precipitated white product Z-Asp(OMe)-Phe-$NH_2$ was isolated by filtration, and washed with 5% cold citric acid (3 × 100 ml) and cold $H_2O$ (3 × 100 ml) successively. Finally, the product was air-dried: 5.87 g (96.8%). M.p. 180–183°. $t_R$ 11.57 min (HPLC system *iii*).

*Asp(OMe)-Phe-NH$_2$.* Z-Asp(OMe)-Phe-$NH_2$ (4 g, 9.3 mmol) was completely dissolved in warm MeOH (700 ml) followed by addition of HCl (6M, 1.7 ml) and then of 10% Pd/C (200 mg) catalyst. The mixture was hydrogenated in two portions in a 500-ml shaked-bottle with $H_2$. The reaction was monitored with HPLC system *iii*. The reaction was complete within 20 min. The hydrogenated portions were combined, filtered, and the filtrate was concentrated *in vacuo* to yield Asp(OMe)-Phe-$NH_2$ · HCl as a white solid (2.97 g, 96.4%). M.p. 155–158°. $t_R$ 2.10 min (HPLC system *iii*).

*Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH$_2$.* Phac-Gly-Trp-Met-OAl (110 mg, 0.2 mmol) and Asp-(OMe)Phe-$NH_2$ · HCl (165 mg, 0.5 mmol) were dissolved in AcOEt (6 ml) containing borax buffer

(0.1M, 240 µl, pH 8.2), *Tris*·HCl buffer (0.1M, 400 µl, pH 8.1), and $Et_3N$ (20 µl). To this soln., α-chymotrypsin/*Celite-545* (600 mg) was added, and the mixture was stirred. The reaction was monitored with HPLC system *vi*. After 95 h, the reactant Phac-Gly-Trp-Met-OAl had completely vanished, and the HPLC conversion of the target product was 66.7%, and, thereafter, the concentration of the product started to decrease. The reaction was stopped by adding a few drops of AcOH. The mixture was filtered, and the filter cake was washed with 80% MeOH followed by addition of AcOEt/$H_2O$ 4:1 until the product was extracted completely. The combined filtrates were dried *in vacuo*, and the crude product was dissolved in MeOH and separated on a *Sephadex LH-20* (25–100 µm, 90 × 4 cm) column, equilibrated with MeOH, followed by elution with MeOH. Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ eluted first, and the pooled fractions were collected and dried *in vacuo* yielding a white solid (85 mg, 54.0%). $t_R$ 6.11 min (HPLC system *vi*). FAB-MS ($C_{40}H_{47}N_7O_8S$; calc. 785.3): 786.2 ($[M+H]^+$).

*Cleavage of Phac from Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.* Phac-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (112 mg, 0.14 mmol) was suspended in $H_2O$ (10 ml), and the pH was adjusted to 7.6 with 0.1M NaOH. After addition of PGA/*Eupergit C* (350 mg), the mixture was stirred at 35° for 30 h. The reaction was monitored with HPLC system *v*. To extract the product, the mixture was diluted with 80% EtOH (20 ml), sonicated, and filtered. The filtrate was evaporated to dryness. The residue was dissolved in 40% aq. MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder (15 mg, 16.1%). $t_R$ 3.85 min (HPLC system *iv*). FAB-MS ($C_{32}H_{41}N_7O_7S$; calc. 667.3): 668.2 ($[M+H]^+$).

*Synthesis of Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.* Bz-Arg-Asp(OEt)-Tyr-Met-OAl (46 mg, 0.06 mmol) and Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$ (21 mg, 0.03 mmol) were dissolved in MeCN (4 ml) containing *Tris*·HCl buffer (0.05M, 18 µl, pH 8.0) and $Et_3N$ (18 µl). To this mixture, α-chymotrypsin/*Eupergit C* (70 mg) was added, and the mixture was stirred at r.t. The reaction was monitored with HPLC system *v*. After 118 h, the reaction was stopped, and the mixture was filtered to remove the immobilized enzyme. The filter cake was washed with 80% MeCN in which the solubility of reactants and product was higher in comparison to other solvents like MeOH, EtOH, or AcOEt. The combined filtrates were dried *in vacuo*, and the residue was dissolved in 50% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white solid (7 mg, 17.1%). $t_R$ 11.02 min (HPLC system *v*). FAB-MS ($C_{65}H_{84}N_{14}O_{15}S_2$; calc. 1364.6): 1365.4 ($[M+H]^+$).

*Cleavage of Bz-Arg from Bz-Arg-Asp(OEt)-Tyr-Met-Gly-Trp-Met-Asp(OMe)-Phe-$NH_2$.* The Bz-Arg-protected octapeptide (15 mg, 0.01 mmol) was suspended in $H_2O$ (3.5 ml), and pH was adjusted to 8.5 with 0.1M NaOH. TPCK-Treated trypsin (2 mg) was added followed by stirring at r.t. for 4 h. The reaction was monitored with HPLC system *iv*. The reaction was stopped by adjusting the pH to 6.0 with a few drops of AcOH. The mixture was evaporated to dryness *in vacuo*, and the residue was dissolved in 50% MeCN and separated isocratically by prep. HPLC with 52% *B* of the MeCN system. The pooled fractions were lyophilized yielding a white powder (2 mg, 16.5%). $t_R$ 10.09 min (HPLC system *iv*). ESI-MS ($C_{52}H_{68}N_{10}O_{13}S_2$; calc. 1104.4): 1105.2 ($[M+H]^+$), 1127.4 ($[M+Na]^+$).

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