

Protease-catalyzed synthesis of the tripeptide CCK_{26–28}, a fragment of CCK-8

L. P. Meng^{1,2}, R. Joshi², and H. Eckstein²

¹ Pharmacy School of Tongji Medical College, HUST, Wuhan, China

² Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

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Summary. Two enzymatically synthetic strategies of the tripeptide derivative PhAc-Asp(OMe)-Tyr-Met-OAl are reported. The second strategy gains the advantage of more economical starting materials, less reaction steps and a higher overall isolated yield of this tripeptide fragment over the first strategy. The effect of the acyl-donor ester concentration and structure, the C- α protecting group of the nucleophile, reaction media, enzyme and the carrier on the tripeptide derivative synthesis were studied. This tripeptide selected is a fragment of the cholecystokinin C-terminal octapeptide (CCK-8), a potential therapeutic agent in the control of gastrointestinal function and also a drug candidate for the treatment of epilepsy.

Keywords: Enzymatic peptide synthesis – Immobilized enzymes – Organic solvent system – Solvent free system – CCK fragment

Abbreviations: ACN, acetonitrile; AcONH₄, ammonium acetate; Al, allyl; Cam, carboxamidomethyl; CCK, cholecystokinin; CCK-8, cholecystokinin C-terminal octapeptide; CHY, α -chymotrypsin; DCM, dichloromethane; DMF, dimethylformamide; Et, ethyl; EtOAc, ethyl acetate; FABMS, fast atom bombardment mass spectrometry; HPLC, high pressure liquid chromatography; Me, methyl; MPLC, middle pressure liquid chromatography; TEA, triethylamine; TFA, trifluoroacetic acid; Tos, *p*-tosylate; Tris, tris-(hydroxymethyl)-aminomethane; R_t, retention time in min; Z, benzyloxycarbonyl

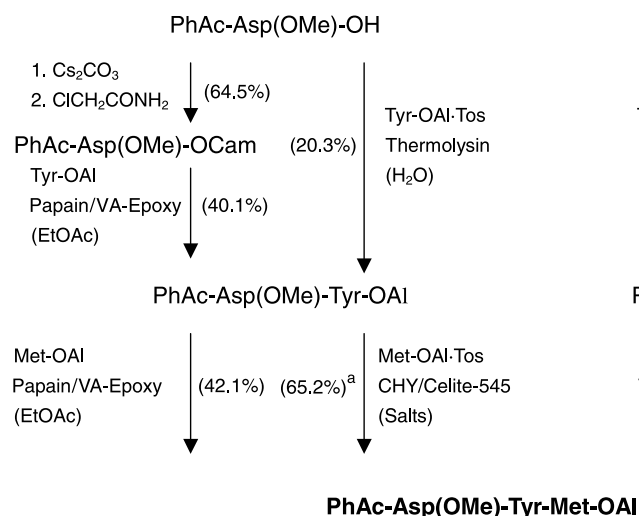
Introduction

In 1898, van't Hoff pointed out the possibility of enzymatic peptide bond formation by reversal of the hydrolytic action of proteases into synthetic action (van't Hoff, 1898). The protease-catalyzed peptide synthesis has been widely investigated (Clapes et al., 2001; De Martin et al., 2001; Nagayasu et al., 1994). Many efforts have also been devoted to the enzymatic synthesis of the cholecystokinin C-terminal octapeptide CCK-8 (CCK_{26–33}) (Cerovsky et al., 1988; Kullman, 1982; Sakina et al., 1988), which is a potential therapeutic agent in the control of gastro-

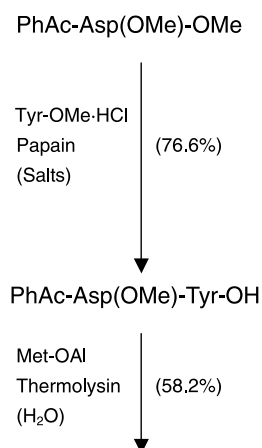
intestinal function (Baile et al., 1986) and also a drug candidate for the treatment of epilepsy (Tirassa et al., 2005).

However, for the synthesis of the N-terminal tripeptide (CCK_{26–28}), there was little knowledge about the synthetic strategy and reaction conditions (Fite et al., 2002). Hence, in this paper, we describe the total enzymatic synthesis of the tripeptide PhAc-Asp(OMe)-Tyr-Met-OAl, which is a key intermediate for the synthesis of CCK-8. Two different strategies to synthesize the tripeptide with protease catalysts are presented in Scheme 1. In the first strategy, one way was the coupling of PhAc-Asp(OMe)-OCam and Tyr-OAl to obtain PhAc-Asp(OMe)-Tyr-OAl. The alternative approach to obtain PhAc-Asp(OMe)-Tyr-OAl is that PhAc-Asp(OMe)-OH as acyl-donor reacted with Tyr-OAl · Tos under thermolysin-controlled peptide synthesis. And then PhAc-Asp(OMe)-Tyr-OAl incubated with Met-OAl, Met-OAl · Tos in organic media and under solvent free conditions, respectively. In the second strategy, the first enzymatic coupling between PhAc-Asp(OMe)-OMe and Tyr-OMe · HCl was performed in a solvent free system in analogy to the method published by Cerovsky (1992). The pure dipeptide fragment (PhAc-Asp(OMe)-Tyr-OH) was obtained efficiently in 76.6% isolated yield. Met-OAl was utilized as the following nucleophile to condense with the dipeptide fragment in water under thermolysin catalysis. The methionine derivative, Met-OAl, was chosen as the nucleophile, because the tripeptide obtained can be used directly as acyl-donor in the next enzymatic coupling. Therefore, the tripeptide can be easily integrated into a final assembly of CCK-8 via the fragment

a) the first strategy:



b) the second strategy:



Scheme 1. Enzymatic synthesis of the N-terminal tripeptide fragment PhAc-Asp(OMe)-Tyr-Met-OAl. The isolated yields of products are shown in parentheses. ^aThe isolated yield was calculated after the unreacted educts were recycled for a repeated conversion

condensation from the tripeptide and a CCK-5. In addition, we also investigated the influence of the acyl-donor ester concentration and structure, the C- α protecting group of the nucleophile and reaction medium on the enzymatic synthesis of tripeptide.

Materials and methods

Materials

Papain (EC 3.4.22.2) from *Carica papaya* (water-soluble, 30000 USP-U/mg using casein as substrate) and α -chymotrypsin (EC 3.4.21.1) from bovine pancreas (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. Celite 545 (particle size 20–45 μ m) were obtained from Fluka. Nucleosil C18 (5 μ m) and Polygosil C18 (60–80 μ m) were from Macherey-Nagel. Papain immobilized on VA Biosynth is reported elsewhere (Eckstein, 1990). α -Chymotrypsin was adsorbed on Celite 545 according to Basso et al. (2000). Tyrosine allyl ester *p*-tosylate (Tyr-OAl·Tos) was purchased from Senn Chemicals AG and methionine allyl ester *p*-tosylate (Met-OAl·Tos) was from Fluka. The amino acid derivatives PhAc-Asp(OMe)-OH, PhAc-Asp(OMe)-OMe, Tyr-OMe·HCl, and Met-OEt·HCl were prepared by standard procedures in our laboratory. The boric buffer was obtained by adjusting 0.1 M borax to a desired pH value by addition of 1 M HCl. All other chemicals and solvents used were of analytical grade.

Methods

Analytical HPLC

Gilson pumps 305 and M307; Merck LaChrom-7400 detector, set to 260 nm; column: Nucleosil C18 (5 μ m), 100 \times 2 mm; flow rate: 0.3 ml/min.

Solvent system: (A): 0.05 M AcONH₄ (pH 6.5), (B): 80% MeOH/H₂O containing 0.05 M AcONH₄.

Elution: Adapted gradient 40 to 85% B: 0–4.5 min, 40% B: 4.5–7 min, 40 to 85% B: 7–11.5 min, 85% B: 11.5–12.5 min, 85 to 35% B: 12.5–13.5 min, 35% B: 13.5–14.5 min, 35 to 40% B: 14.5–17.5 min, 40% B.

Preparative chromatography (MPLC)

Peristaltic pump P-1 (Pharmacia fine chemicals) and ProMinent electronic E-0803 pump; detector: ISCO Model UA-5; fraction collector: ISCO-328. Column: Polygosil C18 (50–60 μ m), 30 \times 4 cm; flow rate: 5 ml/min.

Solvent system: (A): 0.05 M AcONH₄ (pH 6.5), (B): 100% MeOH/H₂O containing 0.05 M AcONH₄.

Elution: Step gradient, beginning with 30 to 80% B according to the elution of the products.

The first strategy of peptide syntheses

PhAc-Asp(OMe)-OCam

PhAc-Asp(OMe)-OCam was synthesized according to the procedure of Martinez et al. (1983). The crystallization of the product was performed in a mixture of ethyl acetate/petroleum ether yielding a white solid (3.30 g, 64.5%, mp 100–101 °C).

FABMS m/z 323.0 [M+H⁺], m/z 345.1 [M+Na⁺], C₁₅H₁₈N₂O₆ requires 322.2.

HPLC data: R_t = 1.13 (PhAc-Asp(OMe)-OH), R_t = 3.86 (PhAc-Asp(OMe)-OCam).

PhAc-Asp(OMe)-Tyr-OAl

a) PhAc-Asp(OMe)-OCam and Tyr-OAl: 110 mg (0.5 mmol) Tyr-OAl and 256 mg (0.8 mmol) PhAc-Asp(OMe)-OCam were dissolved in 12 ml EtOAc containing 100 μ l ACN, 24 μ l 0.1 M borax buffer (pH 6.9), 16 μ l β -mercaptoethanol, and 1.6 mg EDTA. To this solution 1 g papain/VA-Epoxy were added and thoroughly mixed by rotating at room temperature. The reaction was monitored by HPLC. After 6 h, the mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 ml ACN and 10 ml water. The combined filtrates were evaporated to dryness in vacuo and the residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder PhAc-Asp(OMe)-Tyr-OAl (94 mg, 40.1%, mp 91–93 °C).

FABMS m/z 469.1 [M+H⁺], m/z 491.0 [M+Na⁺], C₂₅H₂₈N₂O₇ requires 468.3.

HPLC data: R_t = 3.86 (PhAc-Asp(OMe)-OCam), R_t = 12.88 (PhAc-Asp(OMe)-Tyr-OAl).

A by-product (HPLC yield around 9%) was isolated and characterized as PhAc-Asp(OMe)-Tyr-Tyr-OAl by FABMS m/z 632.2 [M+H⁺], m/z 654.2 [M+Na⁺], C₃₄H₃₇N₃O₉ requires 631.3.

HPLC data: $R_t = 13.78$ (PhAc-Asp(OMe)-Tyr-Tyr-OAl).

b) PhAc-Asp(OMe)-OH and Tyr-OAl·Tos: 265 mg (1 mmol) PhAc-Asp(OMe)-OH and 786 mg (2 mmol) Tyr-OAl·Tos were suspended in 1.5 ml H₂O and the pH was adjusted to 7.0 with 1 M NaOH. After addition of 100 mg thermolysin, the mixture was stirred at 40 °C. After 5 days, the mixture was diluted with 50 ml ACN and filtered. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 30% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder PhAc-Asp(OMe)-Tyr-OAl (95 mg, 20.3%, mp 91–93 °C).

PhAc-Asp(OMe)-Tyr-Met-OAl

a) In organic media: 142 mg (0.75 mmol) Met-OAl and 113 mg (0.25 mmol) PhAc-Asp(OMe)-Tyr-OAl were dissolved in 7.5 ml EtOAc containing 15 μ l 0.1 M borax buffer (pH 6.9), 10 μ l β -mercaptoethanol, and 1 mg EDTA. To this solution 1 g papain/VA-Epoxy were added and thoroughly mixed by rotating at room temperature. After 3 h, the maximum HPLC yield was 64.4%. The mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 ml ACN and 10 ml water. The combined filtrates were evaporated to dryness in vacuo and the residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder PhAc-Asp(OMe)-Tyr-Met-OAl (63 mg, 42.1%, mp 161–163 °C).

FABMS m/z 600.3 [$M + H^+$], m/z 622.2 [$M + Na^+$], C₃₀H₃₇N₃O₈S₁ requires 599.2.

HPLC data: $R_t = 12.88$ (PhAc-Asp(OMe)-Tyr-OAl), $R_t = 14.36$ (PhAc-Asp(OMe)-Tyr-Met-OAl).

A by-product (HPLC yield around 10%) was isolated and characterized as PhAc-Asp(OMe)-Tyr-Met-Met-OAl by FABMS m/z 731.2 [$M + H^+$], m/z 753.2 [$M + Na^+$], C₃₅H₄₆N₄O₉S₂ requires 730.2.

HPLC data: $R_t = 16.37$ (PhAc-Asp(OMe)-Tyr-Met-Met-OAl).

b) In the solvent free system: 113 mg (0.25 mmol) PhAc-Asp(OMe)-Tyr-OAl, 361 mg (1 mmol) Met-OAl·Tos, 50 mg (0.5 mmol) KHCO₃ and 120 mg (0.4 mmol) Na₂CO₃·10H₂O were mixed. 750 mg immobilized α -chymotrypsin (10 mg α -chymotrypsin/g Celite 545) were added and stirred manually every 20 min. The reaction was monitored by HPLC. After 5 h the reaction was stopped as there was no substantial improvement in the conversion. The mixture was washed with EtOAc and filtered. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 40% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder PhAc-Asp(OMe)-Tyr-Met-OAl (63 mg, 42.1%, mp 161–163 °C). To increase the yield, unreacted substrates were recycled for a second conversion and an overall yield of 65.2% could be obtained.

The second strategy of peptide syntheses

PhAc-Asp(OMe)-Tyr-OH

556 mg (2.4 mmol) Tyr-OMe·HCl, 3.2 g (32 mmol) KHCO₃ and 686 mg (2.4 mmol) Na₂CO₃·10H₂O were mixed. 446 mg (1.6 mmol) PhAc-Asp(OMe)-OMe and 240 mg papain were added and stirred manually from time to time. After 1 d, the mixture was washed with ACN and filtered. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 30% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder PhAc-Asp(OMe)-Tyr-OH (328 mg, 76.6%, mp 158–159 °C).

FABMS m/z 429.1 [$M + H^+$], m/z 451.1 [$M + Na^+$], C₂₂H₂₄N₂O₇ requires 428.2.

HPLC data: $R_t = 1.95$ (Tyr-OMe), $R_t = 7.29$ (PhAc-Asp(OMe)-OMe), $R_t = 3.38$ (PhAc-Asp(OMe)-Tyr-OH).

PhAc-Asp(OMe)-Tyr-Met-OAl

220 mg (0.5 mmol) PhAc-Asp(OMe)-Tyr-OH and 189 mg (1 mmol) Met-OAl were dissolved in 750 μ l H₂O with 20 μ l ACN and the pH was adjusted to 7.0 with 1 M NaOH. After addition of 30 mg thermolysin, the mixture was stirred at 40 °C. After 30 min, the reaction was complete and the HPLC yield was 81.9%. The mixture was washed with ACN and filtered. The filtrate was evaporated to dryness under vacuum. The residue was dissolved in 30% MeOH and separated by MPLC. The pooled fractions were lyophilized twice yielding a white powder PhAc-Asp(OMe)-Tyr-Met-OAl (174 mg, 58.2%, mp 161–163 °C).

Results and discussion

In contrast to the chemical synthesis, the application of enzymes to form the peptide bond can avoid the racemization and all the couplings can be conducted under mild conditions with minimum side-chain protection. Unfortunately there is no general protocol available for enzymatic peptide synthesis. In order to suppress the competing hydrolytic reactions, the undesired oligomerization and product cleavage, each coupling step has to be optimized. A series of reaction parameters were investigated to find the best conditions for each peptide bond formation, such as optimal acyl donor, nucleophile, reaction media and enzyme.

Influence of the structure of the acyl-donor

Capellas et al. (1996) reported the reactivity difference among carboxamidomethyl (OCam), benzyl (OBzl) and methyl (OMe) esters of Z-Gly-Trp in the coupling with Met-OEt in organic media. It was found that the methyl ester was the least reactive acyl-donor with the lowest yield of product but the highest yield of by-products. We also observed that PhAc-Asp(OMe)-OMe could not be coupled with Tyr-OAl with immobilized papain in EtOAc. As expected, the OCam ester was necessary to obtain PhAc-Asp(OMe)-Tyr-OAl in organic media. However, in the solvent free system, PhAc-Asp(OMe)-OMe, the methyl ester was active enough to be coupled with Tyr-OMe·HCl and PhAc-Asp(OMe)-Tyr-OH was isolated in a good yield (76.6%). With this strategy not only two reaction steps for synthesizing the Cam ester, but also a possible racemization during this activation could be avoided.

In the synthesis of the dipeptide PhAc-Asp(OMe)-Tyr-OAl, the Cam ester might be avoided, in case PhAc-Asp(OMe)-OH or PhAc-Asp(OH)-OH could be coupled with Tyr-OAl directly under the catalysis of thermolysin. The condensation between PhAc-Asp(OH)-OH with the free β -carboxyl group and Tyr-OAl·Tos with thermolysin was tried, but no coupling could be observed neither

in aqueous nor in organic media at pH 7.0. By way of contrast, as mentioned earlier, the condensation between PhAc-Asp(OMe)-OH, where the β -carboxyl group is protected with a methyl group, and Tyr-OAl·Tos or Tyr-OAl could be achieved in water at pH 7.0 with thermolysin. The isolated yield of PhAc-Asp(OMe)-Tyr-OAl was 20.3%. The result of the low yield is in accordance with the report (Isowa and Ichikawa, 1979) that the tyrosine residue is a poor acyl group acceptor in thermolysin-catalyzed synthesis due to the “aversion” of thermolysin for tyrosine residues in the P₁' site.

Both dipeptides PhAc-Asp(OMe)-Tyr-OAl and PhAc-Asp(OMe)-Tyr-OH were good acyl-donors for the tripeptide synthesis of PhAc-Asp(OMe)-Tyr-Met-OAl and the isolated yields of both reactions in different reaction systems (solvent free system and water system) with different enzymes (α -chymotrypsin on celite-545 and thermolysin) were above 52%.

Influence of the C- α carboxyl group of the nucleophiles

In organic media, for PhAc-Asp(OMe)-OCam, both Tyr-OMe and Tyr-OAl were good nucleophiles for the corresponding synthesis of PhAc-Asp(OMe)-Tyr-OMe and PhAc-Asp(OMe)-Tyr-OAl in EtOAc containing low-water content with papain/VA-Epoxy. The HPLC yields of the reactions were above 25%. However, generally, the selection of the C- α terminal protecting group was made taking into account its reactivity as well as the possibility that the product of one reaction will be the acyl-donor for the next one. As mentioned above, it has been already demonstrated by Capellas et al. (1996) that the methyl ester was the least reactive acyl-donor for the next coupling step. Consequently, for the strategy 1, Tyr-OAl was chosen as the nucleophile to afford PhAc-Asp(OMe)-Tyr-OAl (see Scheme 1).

In the second strategy, however, the much cheaper Tyr-OMe could be used as nucleophile for the synthesis of PhAc-Asp(OMe)-Tyr-OH in the solvent free system. Another advantage is that the end product of the coupling reaction is directly the free carboxyl group, which is necessary for the next coupling step with thermolysin (see Scheme 1).

For the synthesis of the tripeptide the coupling reaction between PhAc-Asp(OMe)-Tyr-OAl and Met-OEt or Met-OAl in the low water content system (EtOAc containing small amounts of buffer) with papain/VA-Epoxy was investigated. It was found that the HPLC yields 55.2 and 64.4% were obtained, respectively. In the solvent free system, using Met-OEt as the nucleophile, the HPLC yield

was 11.0%, while using Met-OAl, the yield increased to 46.3%. In both systems the highest active nucleophile was Met-OAl. However, in the low water system the starting material also was hydrolyzed to PhAc-Asp(OMe)-Tyr-OH and could not be recycled as is the case in the solvent free system. In the solvent free system, the isolated yield increased to 65.2% with a second conversion of the recycled unreacted educts.

Influence of the concentration of substrate

To shift the equilibrium of the enzyme catalyzed peptide bond formation, both are necessary, an excess of one reactant and the highest possible concentration. In the cases of the synthesis of PhAc-Asp(OMe)-Tyr-OH and PhAc-Asp(OMe)-Tyr-Met-OAl, it is better to apply the easily available nucleophile (amino acid ester) in excess, which is more economic as well, because the carboxyl component inherits the danger of a possible “product inhibition” of the enzyme. But in the synthesis of PhAc-Asp(OMe)-Tyr-OAl, an excess of PhAc-Asp(OMe)-OCam with 1.6-fold molar ratio to the nucleophile Tyr-OAl was applied to minimize the formation of the oligomer PhAc-Asp(OMe)-Tyr-Tyr-OAl.

Influence of the reaction media

Solvent plays an important role in enzymatic peptide synthesis in organic media (Dordick, 1989; Cassells and Halling, 1989). Previous fundamental studies by Kise et al. (1990, 1988) showed that both acetonitrile and ethyl acetate were the best solvents providing high enzymatic activities and product yields in some enzymatic dipeptide syntheses.

There was no coupling reaction detected between PhAc-Asp(OMe)-OH and Tyr-OAl·Tos with thermolysin/Celite 545 in acetonitrile with low buffer content. However, with free thermolysin in water PhAc-Asp(OMe)-Tyr-OAl could be obtained in an isolated yield of 20.3%. The condensation between PhAc-Asp(OMe)-OCam and Tyr-OAl with papain/VA-Epoxy in acetonitrile with low buffer content, the HPLC yield of PhAc-Asp(OMe)-Tyr-OAl was only 14.9%, while in ethyl acetate with low buffer content, the HPLC yield reached 57.3% with an isolated yield of 40.1%.

The coupling between PhAc-Asp(OMe)-OCam and Tyr-OAl in EtOAc was investigated in 0.1 M borax buffers with three different pH values, 5.9, 6.9 and 8.2. As shown in Table 1, at the highest pH (pH 8.2) the reaction rate was the fastest and the HPLC yield of over-reacted by-product

Table 1. pH effect on the synthesis of PhAc-Asp(OMe)-Tyr-OAl from PhAc-Asp(OMe)-OCam and Tyr-OAl^a

| pH | Time (h) | Components (%) ^b | |
|-----|----------|-----------------------------|------------|
| | | Product | By-product |
| 5.9 | 168 | 24.3 | 7.3 |
| 6.9 | 6 | 57.3 | 9.4 |
| 8.2 | 3 | 30.1 | 27.1 |

^a Reactions conditions: EtOAc, containing 0.2% (v/v) 0.1 M borax buffer, 0.1% (v/v) β -mercaptoethanol and EDTA, 41.1 mM Tyr-OAl, 65.8 mM PhAc-Asp(OMe)-OCam, 1 g papain/VA-Epoxy

^b The concentrations at the "kinetic" optimum were determined by HPLC; product = PhAc-Asp(OMe)-Tyr-OAl, by-product = PhAc-Asp(OMe)-Tyr-Tyr-OAl

was the highest, which was opposite to the case with the lowest pH (pH 5.9).

The highest yield of the dipeptide allyl ester (57.3%) was obtained at pH 6.9. Interestingly, when this coupling was carried out at the optimal pH of 6.9 in H₂O/ACN, the isolated yield of the dipeptide allyl ester also changed with the content of ACN in the reaction media: 33.8% (without ACN), 40.1% (0.8% ACN) and 26.1% (7.7% ACN). The explanation therefore is that a small amount of ACN is needed to increase the solubility of reactants but a higher concentration of ACN denatures papain.

In the tripeptide synthesis, the HPLC yields of PhAc-Asp(OMe)-Tyr-Met-OAl from PhAc-Asp(OMe)-Tyr-OAl and Met-OAl in buffered ethyl acetate with papain/VA-Epoxy were only slightly different with the pH value of the 0.1 M borax buffer: 67.5% at pH 5.9, 64.4% at pH 6.9 and 62.9% at pH 8.0.

Influence of the enzyme and the carrier

Free papain and papain/VA-Epoxy, respectively, were applied for the coupling of PhAc-Asp(OMe)-OMe and Tyr-OMe·HCl in the solvent free system. With papain/VA-Epoxy, only PhAc-Asp(OMe)-Tyr-OMe and over-reacted esters were formed. The resulting ester has to be hydrolyzed to PhAc-Asp(OMe)-Tyr-OH in a second step, because the free carboxyl group was needed for the following coupling to the tripeptide. With free papain, however, the necessary carboxyl component PhAc-Asp(OMe)-Tyr-OH was the main product.

Conclusion

After having investigated many parameters in the different pathways for synthesizing the N-terminal tripeptide frag-

ment, the best option is the second strategy starting from PhAc-Asp(OMe)-OMe and Tyr-OMe·HCl with free papain in the solvent free system followed by the coupling of obtained PhAc-Asp(OMe)-Tyr-OH and Met-OAl in water with thermolysin. In respect to the easily available Tyr-OMe·HCl, this strategy is more economic and the overall yield is 45%. This is much higher than the overall yield of strategy 1 of ca. 17%.

The second strategy also has the advantage that two reaction steps to synthesize an OCam ester could be avoided and the overall yield (45%) is higher than the synthesis of the tripeptide PhAc-Asp(OBu^t)-Tyr-Met-OAl in an overall yield of 24% as reported by Fite et al. (2002).

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- Authors' address:** Prof. Dr. Heiner Eckstein, Institute of Organic Chemistry, University of Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany,
Fax: +49 7071 294193, E-mail: heiner.eckstein@uni-tuebingen.de