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Total enzymatic synthesis of cholecystokinin CCK-5

H. Xiang[1], **G. Y. Xiang**[2], **Z. M. Lu**[2], **L. Guo**[3], and **H. Eckstein**[4]

[1] School of Pharmacy, China Pharmaceutical University, Nanjing, P.R. China
[2] School of Pharmacy, Huazhong University of Science and Technology, Wuhan, P.R. China
[3] West China School of Pharmacy, Sichuan University, Chengdu, P.R. China
[4] Institute of Organic Chemistry, University of Tübingen, Tübingen, Germany

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Summary. This paper describes the enzymatic synthesis of the *C-terminal* fragment H-Gly-Trp-Met-Asp-Phe-NH_2 of cholecystokinin. Immobilized enzymes were used for the formation of all peptide bonds except thermolysin. Beginning the synthesis with phenylacetyl (PhAc) glycine carboxamidomethyl ester (OCam) and H-Trp-OMe by using immobilized papain as biocatalyst in buffered ethyl acetate, the dipeptide methyl ester was then coupled directly with Met-OEt · HCl by α-chymotrypsin/Celite 545 in a solvent free system. For the $3+2$ coupling PhAc-Gly-Trp-Met-OEt had to be converted into its OCam ester.

The other fragment H-Asp(OMe)-Phe-NH_2 resulted from the coupling of Cbo-Asp(OMe)-OH with H-Phe-NH_2 · HCl and thermolysin as catalyst, followed by catalytic hydrogenation.

Finally PhAc-Gly-Trp-Met-Asp-Phe-NH_2 was obtained in a smooth reaction from PhAc-Gly-Trp-Met-OCam and H-Asp(OMe)-Phe-NH_2 with α-chymotrypsin/Celite 545 in acetonitrile, followed by basic hydrolysis of the β-methyl ester. The PhAc-group is removed with penicillin G amidase and CCK-5 is obtained in an overall isolated yield of 19.6%.

Keywords: Enzymatic peptide synthesis – Immobilized enzymes – Organic solvent system – Solvent free system – CCK-5 – Pentagastrin

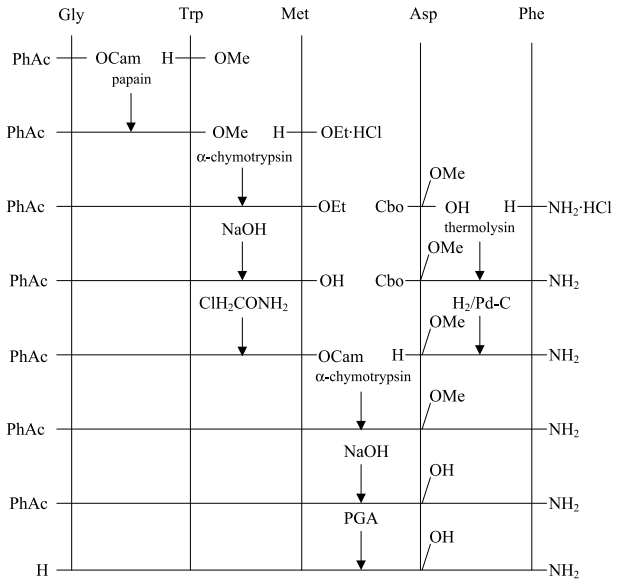
Abbreviations: ACN, acetonitrile; Cam, carboxamidomethyl; CCK-5, cholecystokinin *C-terminal* pentapeptide; DCM, dichloromethane; DMF, dimethylformamide; Et, ethyl; FABMS, fast atom bombardment mass spectrum; HPLC, high pressure liquid chromatography; Me, methyl; PGA, penicillin G amidase; TEA, triethylamine; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Cbo, benzyloxycarbonyl; R_t, retention time in min

Introduction

The synthesis of the cholecystokinin *C-terminal* octapeptide CCK-8 has been widely investigated for its gastrointestinal bioactivity (e.g. Plata-Salaman et al., 1988; Baile et al., 1986; Smith, 1984). The total enzymatic synthesis of peptides containing more than three residues is less studied (Fite et al., 2002).

In this work we investigated the total enzymatic synthesis of the pentapeptide CCK-5, which is a key intermediate of the synthesis of CCK-8. Beyond that, it is used as the commercially available therapeutical drug pentagastrin, which has the same sequence, because CCK and gastrin have the same five amino acids in the *C-terminal* sequence. The synthetic strategy, which is outlined in Scheme 1, involved five steps by enzymatic catalysis. As *N-terminal* protecting group the phenylacetyl group (PhAc) was chosen as an alternative to the conventional benzyloxycarbonyl group (Cbo) or tert-butyloxycarbonyl group (Boc) (Kullmann, 1987), because PhAc can be removed enzymatically by penicillin G amidase (PGA) (Fite et al., 1998). The convergent strategy was started from PhAc-Gly-OCam. Trp-OMe, Met-OEt · HCl, and H-Asp(OMe)-Phe-NH_2 were employed as nucleophiles successively.

The three key steps involved in the strategy are: (1) The enzymatic condensation of PhAc-Gly-Trp-OMe and Met-OEt · HCl, (2) the synthesis of H-Asp(OMe)-Phe-NH_2 from Cbo-Asp(OMe)-OH and H-Phe-NH_2 · HCl, and (3) the coupling of PhAc-Gly-Trp-Met-OCam and H-Asp(OMe)-Phe-NH_2. Capellas et al. (1997) reported the enzymatic coupling between Cbo-Gly-Trp-OCam and Met-OEt catalyzed by α-chymotrypsin in organic solvent media and tested the reactivity of three *C-terminal* ester derivatives. Benzyl- (OBzl) and carboxamidomethyl- (OCam) are more active than the methyl ester. In this work we were successful in coupling the dipeptide methyl ester directly with Met-OEt hydrochloride in a solvent free system in analogy to the method published by Cerovsky, 1992. The pure tripeptide was obtained efficiently in 71.4% isolated yield.



Scheme 1. Enzymatic synthesis of the pentapeptide H-Gly-Trp-Met-Asp-Phe-NH_2

In the preparation of H-Asp(OMe)-Phe-NH_2, the Cbo-group was removed from Cbo-Asp(OMe)-Phe-NH_2 by catalytic hydrogenation. The deprotection was complete in one hour and the isolated yield was satisfactory (97.6%). The free pentapeptide H-Gly-Trp-Met-Asp(OH)-Phe-NH_2 was obtained by two deprotection steps: (1) The removal of the β-methyl ester from the aspartic acid residue followed by (2), the enzymatic removal of the PhAc-group from PhAc-Gly-Trp-Met-Asp(OH)-Phe-NH_2 with immobilized PGA.

Materials and methods

Materials

Silica gel plates 60 F_{254}, 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 (Darmstadt, Germany). Nucleosil C18, 5 μm and 7 μm were from Macherey-Nagel (Düren, Germany). 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 (St. Louis, MO, U.S.A.). Penicillin G Amidase (PGA) (EC 3.5.1.11), immobilized on Eupergit (powder, 109 U/g) and Celite 545 (particle size 20–45 μm) were obtained from Fluka (Buchs, Switzerland). Papain immobilized on VA Biosynth is reported elsewhere (Eckstein, 1990). α-Chymotrypsin was adsorbed on Celite 545 according to Basso et al. (2000). PhAc-Gly-OCam was synthesized according to Martinez et al. (1983, 1985). The amino acid derivatives were prepared by standard procedures in our laboratory. The boric buffer was obtained by adjusting 0.1 M borax to a pH of 8.2 by addition of 1 M HCl. All other chemicals and solvents used were of analytical grade.

HPLC analysis

Analytical HPLC system: Gilson pumps 305/307, Merck LaChrom-7400 detector, set to 260 nm.

Column: Nucleosil C18, 5 μm, 100 × 2 mm.

Solvent system: (A): 0.1% aqueous TFA, (B): 80% ACN/0.1% TFA; flow rate: 0.3 ml/min.

HPLC system I: Gradient elution 30% B to 70% B over 12 min: 0–3 min., 30% B; 3–5 min., 30% B to 70% B; 5–9 min., 70% B; 9–9.5 min., 70% to 30% B; 9.5–12 min., 30% B.

HPLC system II: Gradient elution 35% B to 80% B over 12 min: 0–2 min., 35% B; 2–9 min., 35% B to 80%; 9–10 min., 80% to 35% B; 10–12 min., 35% B.

Preparative HPLC system: Gilson pumps 305/307, Detector: ISCO UA-6, 260 nm.

Column: Nucleosil, C18, 7 μm, 250 × 8 mm.

Solvent system: (A): 0.1% aqueous TFA, (B): 80% ACN/0.1% TFA, 23% B; flow rate: 5 ml/min.

Peptide syntheses

PhAc-Gly-Trp-OMe

125 mg (0.5 mmol) PhAc-Gly-OCam and 164 mg (0.75 mmol) H-Trp-OMe were dissolved in 50 ml ethyl acetate containing 270 μl 0.2 M borax buffer (pH 8.5), 30 μl β-mercaptoethanol, and 1 mg EDTA. To this solution 300 mg immobilized papain were added. After 3 h the reaction was complete and the HPLC yield was 97%. The mixture was filtered to remove the immobilized enzyme which was washed with a mixture of 40 ml ethyl acetate and 10 ml water. The combined filtrates were extracted successively with 1 M citric acid (3 × 100 ml), 5% $NaHCO_3$ (3 × 100 ml), and saturated sodium chloride (1 × 100 ml). The organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was lyophilized yielding a white solid (174 mg, 91%, mp. 126–128°C).

Fast atom bombardment mass spectrum (FABMS) m/z 394.2 $[M + H^+]$, m/z 416.2 $[M + Na^+]$, $C_{22}H_{23}N_3O_4$ requires 393.5.

HPLC data (system I): $R_t = 6.95$ (PhAc-Gly-Trp-OMe), $R_t = 1.66$ (PhAc-Gly-OCam).

PhAc-Gly-Trp-Met-OEt

The synthesis of this tripeptide was carried out under solvent free conditions (Cerovsky et al., 1989). 1.97 g (5.0 mmol) PhAc-Gly-Trp-OMe, 4.28 g (20.0 mmol) Met-OEt · HCl, 10 g (100 mmol) $KHCO_3$ and 5.72 g (20.0 mmol) $Na_2CO_3 \cdot 10H_2O$ were mixed. 2 g immobilized α-chymotrypsin (10 mg α-chymotrypsin/g Celite 545) were added and stirred manually every 20 min. The reaction was monitored by HPLC (system I). After 2 h the reaction was complete and the HPLC yield was 96%. The mixture was washed with water until a pH of 7.0 was reached, diluted with 100 ml 80% EtOH and then sonicated to extract the tripeptide. After removal of the immobilized enzyme by filtration, the solvent was evaporated to dryness under vacuum. The tripeptide ester was obtained by recrystallization with EtOH as a white solid (1.92 g, 71.4%, mp. 174–176°C).

FABMS m/z 539.1 $[M + H^+]$, m/z 561.2 $[M + Na^+]$, $C_{28}H_{34}N_4O_5S_1$ requires 538.6.

HPLC data (system I): $R_t = 6.96$ (PhAc-Gly-Trp-OMe), $R_t = 7.89$ (PhAc-Gly-Trp-Met-OEt), $R_t = 9.38$ (PhAc-Gly-Trp-Met-Met-OEt).

PhAc-Gly-Trp-Met-OH

To obtain Phac-Gly-Trp-Met-OH, 3.8 g (0.7 mmol) PhAc-Gly-Trp-Met-OEt were dissolved in 150 ml H_2O/acetone (1:1) and 5 ml 4 N NaOH were added. After stirring for 1 h, no starting material was detected by TLC. The acetone was removed under vacuum. The product was precipitated by acidification with 1 N HCl to pH 3 and filtered. The residue was

washed with 100 ml water and dried. The crystallization was performed in a mixture of ethyl acetate/petroleum ether yielding a white solid (3.45 g, 98.4%, mp. 180–182°C).

FABMS m/z 511.1 $[M+H^+]$, m/z 533.2 $[M+Na^+]$, $C_{26}H_{30}N_4O_5S_1$ requires 510.6.

TLC data (DCM/MeOH = 9/1): Rf = 0.15 (Phac-Gly-Trp-Met-OH), Rf = 0.8 (Phac-Gly-Trp-Met-OEt).

PhAc-Gly-Trp-Met-OCam

PhAc-Gly-Trp-Met-OCam was synthesized according to Capellas et al. (1997). 2.0 g (4.0 mmol) PhAc-Gly-Trp-Met-OH were dissolved in 75 ml MeOH/H_2O (2:1) and 3.4 ml 20% Cs_2CO_3 were added. After 1 h the reaction was complete and no starting material was detected by TLC. The solvent was removed under vacuum. The residue was evaporated to dryness with 3×50 ml toluene and then dried overnight under high vacuum obtaining a white glassy Cs-salt (2.9 g).

2.9 g PhAc-Gly-Trp-Met-OCs were dissolved in 60 ml anhydrous DMF and 0.56 g (6.0 mmol) 2-chloroacetamide were added and stirred at 35°C. After 72 h the reaction was complete and no starting material was detected by TLC. The mixture was evaporated to dryness. The residue was dissolved in 50 ml ethyl acetate, extracted successively with 1 M citric acid (3×50 ml), 5% $NaHCO_3$ (3×50 ml), and saturated sodium chloride (1×50 ml). The organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum. The crystallization was performed in a mixture of ethyl acetate/petroleum ether yielding a white solid (2.15 g, 84.1%, mp. 189–191°C).

FABMS m/z 568.2 $[M+H^+]$, m/z 590.1 $[M+Na^+]$, $C_{28}H_{33}N_5O_6S_1$ requires 567.6.

TLC data (DCM/MeOH = 7/3): Rf = 0.55 (Phac-Gly-Trp-Met-OH), Rf = 0.65 (Phac-Gly-Trp-Met-OCs).

TLC data (DCM/MeOH = 9/1): Rf = 0.15 (Phac-Gly-Trp-Met-OCs), Rf = 0.5 (Phac-Gly-Trp-Met-OCam).

Cbo-Asp(OMe)-Phe-NH_2

5.0 g (17.8 mmol) Cbo-Asp(OMe)-OH and 4.6 g (23.0 mmol) H-Phe-$NH_2 \cdot$ HCl were suspended in 80 ml H_2O and the pH was adjusted to 7.0 with 1 N NaOH. After addition of 25 mg thermolysin, the mixture was stirred at 40°C. After 7 h the reaction was complete and no starting material was detected by TLC. The precipitated product was isolated by filtration, dissolved in 100 ml ethyl acetate, and extracted successively with 1 M citric acid (3×100 ml), 5% $NaHCO_3$ (3×100 ml) and saturated sodium chloride (1×50 ml). The organic phase was dried over anhydrous Na_2SO_4. The solvent was removed under vacuum yielding a white solid (6.88 g, 90.6%, mp. 185–186°C).

FABMS m/z 428.1 $[M+H^+]$, m/z 450.1 $[M+Na^+]$, $C_{22}H_{25}N_3O_6$ requires 427.5.

TLC data (DCM/MeOH = 9/1): Rf = 0.5 (Cbo-Asp(OMe)-Phe-NH_2), Rf = 0.2 (Cbo-Asp(OMe)-OH), Rf = 0.1 (Phe-NH_2).

H-Asp(OMe)-Phe-NH_2

1.0 g (2.34 mmol) Cbo-Asp(OMe)-Phe-NH_2 were dissolved in 200 ml MeOH and 50 mg 10% Pd-C added. The mixture was poured into a 500 ml shaked-bottle and then gaseous hydrogen was led in. After 45 min the reaction was complete and no starting material was detected by TLC (n-butanol/AcOH/H_2O = 3:1:1). The catalyst was removed by filtration and washed with 50 ml MeOH. The combined filtrates were concentrated to dryness yielding a white solid (0.67 g, 97.6%, mp. 120–122°C).

FABMS m/z 294.1 $[M+H^+]$, m/z 316.1 $[M+Na^+]$, $C_{14}H_{19}N_3O_4$ requires 293.3.

TLC data (DCM n-butanol/AcOH/H_2O = 3:1:1): Rf = 0.5 (H-Asp(OMe)-Phe-NH_2), Rf = 0.7 (Cbo-Asp(OMe)-Phe-NH_2).

PhAc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2

0.56 g (1.0 mmol) PhAc-Gly-Trp-Met-OCam and 0.74 g (2.50 mmol) H-Asp(OMe)-Phe-NH_2 were dissolved in 10 ml acetonitrile containing 50 μl 0.05 M Tris-HCl buffer (pH 9.0) and 50 μl triethylamine. 1 g α-chymotrypsin/Celite 545 (10 mg α-chymotrypsin/g Celite 545) was added. After 2 h the reaction was complete and the mixture was worked up by the same procedure as used for PhAc-Gly-Trp-Met-OEt. The residue was lyophilized yielding a white solid (0.56 g, 73.0%, decomposition at 220°C).

FABMS m/z 786.3 $[M+H^+]$, m/z 808.3 $[M+Na^+]$, $C_{40}H_{47}N_7O_8S_1$ requires 785.9.

HPLC data (system II): R_t = 9.06 (PhAc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2), R_t = 6.99 (PhAc-Gly-Trp-Met-OCam).

Phac-Gly-Trp-Met-Asp(OH)-Phe-NH_2

0.4 g (0.51 mmol) PhAc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 were dissolved in 100 ml H_2O/acetone (1:1) and 1 ml 4 N NaOH were added. After 1 h the acetone was removed under vacuum. The product was precipitated by acidification with 1 N HCl to pH 3 and filtered. The residue was washed with 50 ml water and dried yielding a white solid (0.34 g, 86.8%, 214–217°C).

FABMS m/z 772.9 $[M+H^+]$, m/z 794.2 $[M+Na^+]$, $C_{39}H_{45}N_7O_8S_1$ requires 771.9.

HPLC data (system II): R_t = 7.75 (PhAc-Gly-Trp-Met-Asp(OH)-Phe-NH_2), R_t = 9.06 (PhAc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2).

H-Gly-Trp-Met-Asp(OH)-Phe-NH_2

0.2 g (0.26 mmol) PhAc-Gly-Trp-Met-Asp(OH)-Phe-NH_2 were suspended in 10 ml H_2O and the pH was adjusted with 1 N NaOH to 7.6. After addition of 0.4 g immobilized PGA, the mixture was stirred at 35°C for 24 h. To extract the product, the mixture was diluted with 20 ml 80% EtOH, sonicated, and filtered. The filtrate was evaporated to dryness under vacuum. The residue was separated by preparative HPLC with an eluent of 23% B. The residue was dissolved in 5 ml 23% B and the desired product was eluted as the second peak. The pooled fractions were lyophilized twice yielding a white powder (97 mg, 57.4%, decomposition at 230°C).

FABMS m/z 654.3 $[M+H^+]$, m/z 676.0 $[M+Na^+]$, $C_{31}H_{39}N_7O_7S_1$ requires 653.7.

HPLC data (system II): R_t = 1.81 (H-Gly-Trp-Met-Asp(OH)-Phe-NH_2), R_t = 7.75 (PhAc-Gly-Trp-Met-Asp(OH)-Phe-NH_2).

Results and discussion

Opposite to the strategy which is used in chemical peptide synthesis, we prefer to begin the synthesis at the amino terminal of the peptide. To shift the equilibrium of the enzyme catalyzed peptide bond formation, both is necessary, an excess of one fragment and the highest possible concentration. This inherits the danger of a possible ''product inhibition'' of the enzyme by the carboxy group containing fragment. Therefore it is better to apply the easily available nucleophile (amino acid ester or amide) in excess. Beyond that it more economic as well. In contrast to the chemical peptide bond formation, in enzyme catalyzed peptide bond formation this strategy is possible, because there is no danger of racemization during the activation of the carboxyl group of peptides.

Unfortunately in enzymatic peptide synthesis there exists no general method for peptide bond formation. Therefore each coupling step was optimized. A series of reaction parameters were investigated to find the best conditions for each enzymatic coupling step, such as reaction media, optimal acyl donor and nucleophile.

Influence of the reaction media

The coupling reaction between PhAc-Gly-OCam (10 mmolar) and Trp-OMe (15 mmolar), catalyzed by papain-VA, was investigated in both solvents, acetonitrile and ethyl acetate. In this coupling step, ethyl acetate was superior (HPLC yield 97%) to acetonitrile (HPLC yield 67%).

The condensation reaction between PhAc-Gly-Trp-OMe and Met-OEt · HCl was studied in organic solvent systems and α-chymotrypsin adsorbed on Celite 545. In this case no desired tripeptide was found in either acetonitrile or ethyl acetate. However, under solvent free conditions, peptide bond formation was observed. Different inorganic salt systems with crystalline water were investigated to optimize the pH of the reaction. Three systems of inorganic salts were studied (experimental details not published). The lowest conversion rate, 23.9%, was observed with the mixture of sodium sulfate and sodium carbonate decahydrate. A better yield, 40.2%, could be obtained with sodium carbonate and its decahydrate. With 96% HPLC yield the system potassium hydrogen carbonate/sodium carbonate decahydrate was clearly superior. Applying the last salt system with a 4 fold excess of the nucleophile Met-OEt the tripeptide OEt ester was obtained in an isolated yield of 71.4%. At a higher excess of the nucleophile the Met–Met formation increased considerably.

In contrast to the first coupling step the ethyl acetate system was not successful to obtain the pentapeptide Phac-Gly-Trp-Met-Asp(OMe)-Phe-NH_2 in the $3+2$ coupling of PhAc-Gly-Trp-Met-OCam at a 0.1 molar concentration and H-Asp(OMe)-Phe-NH_2 at a 0.25 molar concentration. However, in acetonitrile containing 0.5% Tris-HCl buffer and 0.5% TEA the desired protected pentapeptide was formed and isolated in a yield of 73%.

Influence of the ester group of the acyl-donor

Cbo-Gly-Trp-OAl was reported as a suitable acyl-donor substrate to prepare the CCK-5 peptide (Capellas et al., 1996). In their paper the reactivity difference among carboxamidomethyl (OCam), benzyl (OBzl), allyl (OAl) and methyl (OMe) esters was assayed. 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 obtained. We also observed, that Phac-Gly-OMe could not be coupled with Trp-OMe with immobilized papain as catalyst. As expected, the OCam ester was necessary and the dipeptide was isolated in a good yield (91.1%) in an organic solvent system.

In the next coupling step the methyl ester of the dipeptide was active enough to be coupled with Met-OEt · HCl. The coupling in the solvent free system was successful and the tripeptide could be isolated in 71.4% yield. With this strategy three reaction steps could be avoided in comparison to the published method by Capellas et al. (1996).

An alternative strategy would be the use of Met-OCam as nucleophile. The resulting tripeptide would then, as OCam ester, be ready for direct coupling with the dipeptide Asp(OMe)-Phe-NH2. We did not follow this route, because the more active tripeptide OCam ester would probably react more easily with another Met-OCam and more of the byproduct Phac-Gly-Trp-Met-Met-OCam would be obtained. Beyond this, Met-OEt is synthesized in one step in contrast to the multi step synthesis of Met-OCam. An the other hand, there is a risk of racemization by converting the tripeptide ethyl ester to the tripeptide OCam ester. If some racemization occurred in our synthesis, the D-methionin containing tripeptide would not be coupled in the following chymotrypsin catalyzed $3+2$ coupling. The isolated yield of 73% of the pentapeptide is within the usual range. Therefore racemization did not occur in larger amounts, if at all.

For formation of the peptide bond Asp-Phe we choose to protect the β-carboxyl group of Cbo-Asp according to the results of an earlier investigation (Eckstein et al., 2001). Additionally to the already reported methyl ester we also tried OBzl and OEt for β-protection. We expected better conversion rates in case of OBzl followed by OEt and the lowest with OMe. But the conversion rates were 80%, 67% and the highest, 90.6%, in the case of OMe, respectively, with a 0.22 molar concentration of Cbo-Asp(OMe)-OH and a 0.29 molar concentration of Phe-NH_2 · HCl.

In the synthesis of PhAc-Gly-Trp-Met-Asp(OMe)-Phe-NH_2, PhAc-Gly-Trp-Met-OEt was not reactive enough as an acyl-donor, and no coupling reaction was observed. The best result was achieved by using PhAc-Gly-Trp-Met-OCam as an acyl-donor ester and α-chymotrypsin adsorbed on Celite 545.

Influence of the C-α carboxyl group of the nucleophiles

Generally, the selection of the C-α terminal protecting group was made taking into account its reactivity as well as the fact that the product of one reaction will be the acyl-donor for the next one. In the present work, Trp-OMe was a good nucleophile for the synthesis of PhAc-Gly-Trp-OMe with a very good isolated yield of 91.1%, catalyzed by papain-VA.

The dipeptide methyl ester was a tolerable acyl-donor for the following reaction with Met-OEt · HCl as a nucleophile and reacted smoothly with the dipeptide ester. PhAc-Gly-Trp-Met-OEt was obtained in a good isolated yield of 71.4% within 2 hours. The by-product PhAc-Gly-Trp-$(Met)_n$-OEt, $n = 2$, could be kept below 5%.

Removal of the PhAc group by penicillin G amidase

The cleavage of the PhAc group is a typical equilibrium reaction. To achieve a high percentage of cleavage we choose the low molar concentration of 0.026 for the PhAc-pentapeptide. After 24 h at 35°C more than 95% of the PhAc group have been removed according to the HPLC control. To avoid a possible increase of byproducts, the reaction was stopped and the CCK-5 peptide was isolated.

Conclusion

The enzymatic synthesis of H-Gly-Trp-Met-Asp-Phe-NH_2 was carried out avoiding chemical reaction steps, whenever it was possible. Unfortunately we needed two OCam esters, PhAc-Gly-OCam and we had to transfer the tripeptide PhAc-Gly-Trp-Met-OEt to PhAc-Gly-Trp-Met-OCam. However, applying the solvent-free system, we could use the OMe ester directly for the coupling of PhAc-Gly-Trp-OMe as acyl-donor and Met-OEt · HCl as nucleophile. Thus three more reaction steps of preparing an OCam ester, as reported (Capellas et al., 1996), could be avoided. Despite of the Asp-Phe coupling using thermolysin all peptide couplings could be performed with immobilized enzymes exclusively. This is important for the large scale synthesis of peptides.

At the end of the synthesis the amino protecting group PhAc could also be removed enzymatically from PhAc-Gly-Trp-Met-Asp(OH)-Phe-NH_2 with immobilized PGA. The overall isolated yield was 19.6 %.

In this paper we could again demonstrate, that ''green chemistry'', the application of the enzymatic technology, is indeed a good and versatile alternative to the ''chemical'' peptide synthesis. We are now trying to optimize the strategy further by avoiding the OCam ester at the tripeptide fragment and the catalytic hydrogenation of the Cbo-Asp(OMe)-Phe-NH_2.

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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: 07071-29-4193