Pergamon

Bioorganic & Medicinal Chemistry, Vol. 3, No. 3, pp. 245–255, 1995

0968-0896/95 $9.50 + .00

0968-0896(95)00019-4

Enzymatic Peptide Synthesis in Low Water Content Systems: Preparative Enzymatic Synthesis of [Leu]- and [Met]-Enkephalin Derivatives

Pere Clapés,[a*] Josep-Lluis Torres[a] and Patrick Adlercreutz[b]

[a]*Unit for Protein Chemistry and Biochemistry, Centre d'Investigació i Desenvolupament (C.I.D.–C.S.I.C.), Jordi Girona 18–26, 08034 Barcelona, Spain*

[b]*Department of Biotechnology, Chemical Center, University of Lund. P.O. Box 124, S-22100 Lund, Sweden*

Abstract—A novel total enzymatic synthesis of [Leu]- and [Met]-enkephalin derivatives was accomplished in low-water content systems at a preparative scale. α-Chymotrypsin, papain, thermolysin and bromelain adsorbed on Celite were used as catalysts. Organic solvents such as acetonitrile and ethyl acetate with small amounts of buffer added or at specific water activity were used as reaction media. Simple readily available amino acid ester derivatives were used as starting building blocks. This feature allowed the possibility of using the products in one step directly as acyl-donor ester, without any chemical or enzymatic modification, in the next enzymatic coupling. The optimal strategy for the synthesis of the enkephalin derivatives was different depending on the carboxy terminal group. The preparation of the carboxy-terminal amide derivatives (R-Tyr-Gly-Gly-Phe-Leu[Met]-NH_2) was achieved via 4 + 1 fragment condensation catalyzed by α-chymotrypsin. The carboxy-terminal ethyl ester derivatives (R-Tyr-Gly-Gly-Phe-Leu[Met]-OEt) were obtained via 2 + 3 condensation catalyzed by bromelain, a quite unusual protease for peptide synthesis but more effective than papain in this coupling. Both syntheses were carried out in four enzymatic steps and one or two chemical deprotection steps routinely used in peptide synthesis. The overall yields of pentapeptide derivatives were between 40–54% of pure product.

Introduction

The parallel advances of medicine, biochemistry and lately biotechnology have demonstrated that peptides are involved in the regulation and maintenance of many biological processes.[1] As a result, peptides represent new opportunities as drugs, diagnostic reagents, agricultural chemicals and even as additives in food industries to preserve and modify the taste of food.[2,3] Thus, the strong interest in the peptide area is not surprising, and, as a consequence, the methodology for obtaining peptides in high yield, good purity and in large scale production is a field of growing interest.

In this concern, enzymatic methodology[4] is a promising area in peptide synthesis which was improved a few years ago with the use of proteases in organic solvents.[5,6] Apart from their regio- and stereo-selectivity and the possibility of carrying out the reaction under mild conditions, the organic media offer good solubility for hydrophobic substrates (i.e. amino acid derivatives), and undesirable hydrolytic side reactions can be suppressed in condensation reactions.[7] In this case the driving force of the synthesis is the low water activity rather than the precipitation of product, a common practice in peptide synthesis in aqueous media.[8] A biocatalyst configuration that has been shown to be quite useful in organic media is enzyme adsorbed on a solid support material. Small amounts of buffer or water are added to activate the enzyme. This kind of biocatalyst has been used for peptide synthesis reactions catalyzed by proteases.[9] The application of these systems to protease catalyzed enzymatic coupling has opened new tactics in both fragment condensation and stepwise peptide synthesis strategies.[10] Several advantages have already been demonstrated in systematic studies using dipeptide syntheses as model reactions. However, the general suitability of these systems has to be proved in the synthesis of larger peptides. In this sense, a good opportunity is the preparation of biologically active peptides, i.e. the [Leu]- and [Met]-enkephalin opiate pentapeptides. Although both syntheses have already been accomplished either by convergent[11] or stepwise enzymatic total synthesis strategies,[12] some problems are still unsolved making this technology tedious and uncompetitive over conventional chemical methods.

The work presented in this paper is a new approach to the total enzymatic synthesis of Leu- and Met-enkephalin derivatives using predominantly organic media to perform the enzymatic reactions. Z-Tyr-Gly-

Abbreviations—Al: Allyl; Boc: *tert*-butyloxycarbonyl; Bzl: benzyl; Cam: carboxamidomethyl; ESMS: electrospray mass spectrometry; Et: ethyl; FABMS: fast atom bombardment mass spectrometry; Me: methyl; MOPS: 3-[*N*-morpholino]propane sulfonic acid (biological buffer); ^{t}Bu: *tert*-butyl; TEAP: triethylamine-phosphoric acid buffer; TFA: trifluoroacetic acid; Z: benzyloxycarbonyl.

Gly-Phe-Leu-NH_2, Z-Tyr-Gly-Gly-Phe-Leu-OEt, Boc-Tyr-Gly-Gly-Phe-Met-NH_2 and Boc-Tyr-Gly-Gly-Phe-Met-OEt were prepared by means of α-chymotrypsin, papain, thermolysin and bromelain adsorbed on Celite. α-Chymotrypsin has a primary specificity for bulky and hydrophobic amino acids (Phe, Tyr, Trp, Leu, Met) and it was selected for the Tyr–Gly, Phe–Leu and Phe–Met peptide bonds. Papain has a broad substrate specificity and according to the literature data it was selected for Gly–Gly and Gly–Phe peptide bonds. Bromelain is very similar to papain as far as the specificity, but it is not a well-known protease. All of these proteases are serine- or cysteine-type and the peptide bond formation can be done under kinetically controlled conditions. Thermolysin is an aspartic protease and it is specific towards hydrophobic residues at P'_1 position (Schecter and Becter nomenclature). Since this enzyme has no esterase activity, it is useful in the synthesis of some fragments bearing C-terminal esters. It was selected mainly for Phe–Leu and Gly–Phe bonds. Acetonitrile, ethyl acetate and methyl caproate were used as solvents with a controlled amount of buffer or at fixed water activity.

Following a convergent strategy, the nucleophiles used were methyl, ethyl or benzyl esters of amino acids or peptide fragments. Hence, the product obtained could be used directly, without any chemical or enzymatic modification, as acyl donor ester in the following enzymatic step. As N-α protecting groups, benzyloxycarbonyl (Z) was used preferentially for the leucine containing derivatives and *tert*-butyloxycarbonyl (Boc) was used for methionine derivatives.

Results

Enzymatic synthesis of Z-Tyr-Gly-Gly-Phe-Leu-NH_2 and Boc-Tyr-Gly-Gly-Phe-Met-NH_2

Two different strategies were investigated for the enzymatic synthesis of the pentapeptide amides (Figs 1 and 2). Both strategies have the same number of steps and start with the synthesis of I and III (Z protection) or II and IV (Boc protection).

The synthesis of I and II was achieved via α-chymotrypsin catalysis from Z-Tyr-OR and Boc-Tyr-OR (where R: Me, Cam) and H-Gly-OBzl. It was observed that carboxamidomethyl acyl-donor esters rendered higher yields (91%) than the methyl ester (71%) derivatives. In addition, during the chymotryptic coupling between Z-Tyr-OMe and H-Gly-OBzl the formation of Z-Tyr-Gly-OH and Z-Tyr-Gly-Gly-OBzl, in both cases less than 6% were observed. These undesired side reactions are a consequence of the esterase activity of chymotrypsin towards glycine observed previously in other condensation reactions using H-Gly-OMe, H-Gly-OEt or H-Ala-OEt as nucleophiles in predominantly aqueous systems.[13] When acyl-donor carboxamidomethyl esters were used, the by-product formation, on the time scale of the ester aminolysis reaction, was minimized to less than 0.1%. This could be explained, among other things, by the difference between the reaction rates of product and by-product formation. The higher reactivity of carboxamidomethyl esters with α-chymotrypsin is related to the orientational effects of hydrogen bonding interaction between the enzyme and the ester.[14] Thus, the use of Cam derivatives was preferred for preparative purposes.

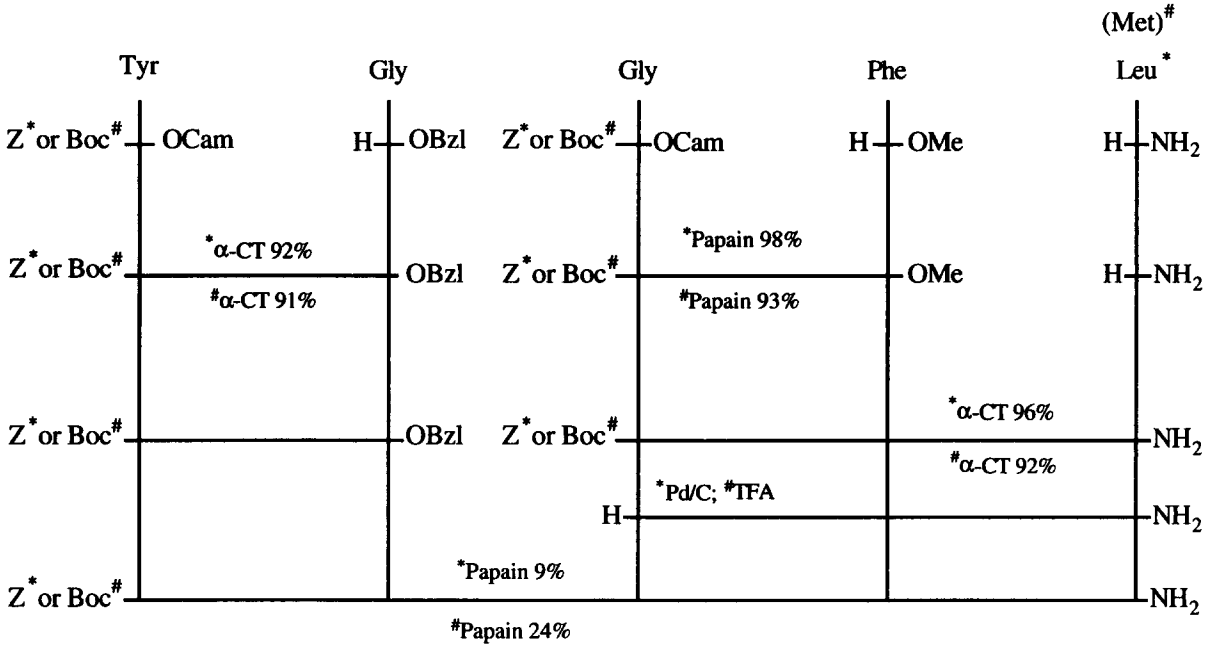

Figure 1. Enzymatic synthesis of Z-Leu- and Boc-Met-enkephalin amides. Synthetic scheme based on 2 + 3 enzymatic fragment condensation. Z-Tyr-Gly-OBzl (I), Boc-Tyr-Gly-OBzl (II), Z-Gly-Phe-OMe (III), Boc-Gly-Phe-OMe (IV), Z-Gly-Phe-Leu-NH_2 (V), Boc-Gly-Phe-Met-NH_2 (VI), Z-Tyr-Gly-Gly-Phe-Leu-NH_2 (XIII) and Boc-Tyr-Gly-Gly-Phe-Met-NH_2 (XIV).

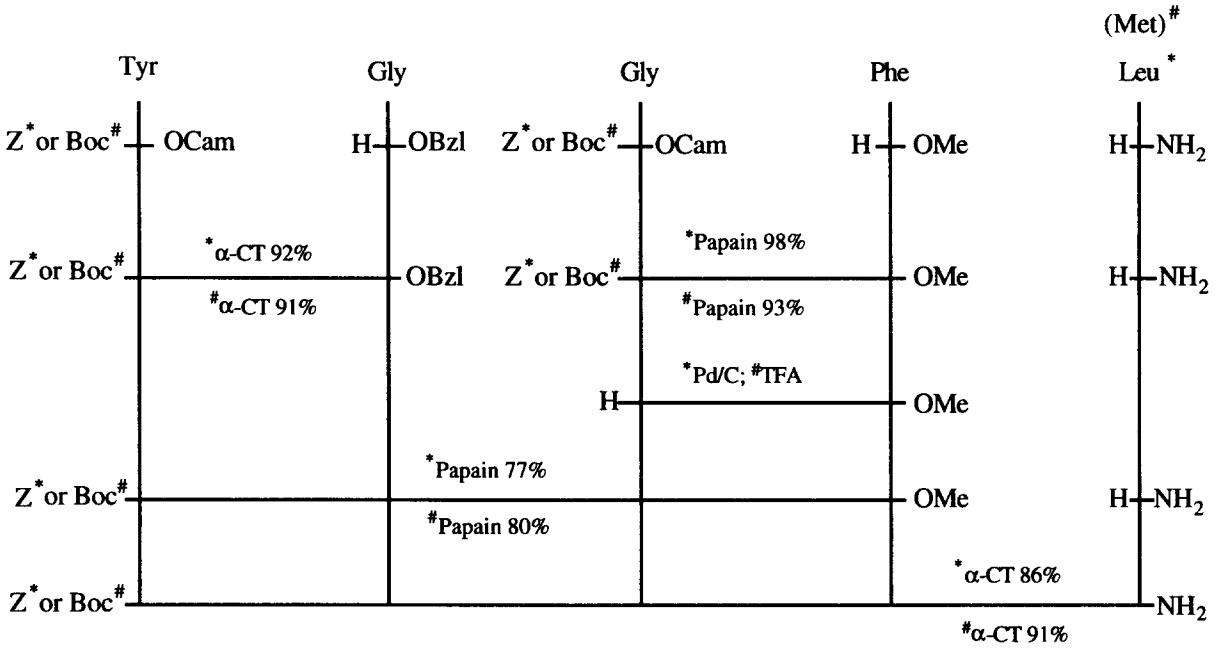

Figure 2. Enzymatic synthesis of Z-Leu- and Boc-Met-enkephalin amides. Synthetic scheme based on 4 + 1 enzymatic fragment condensation. Z-Tyr-Gly-OBzl (I), Boc-Tyr-Gly-OBzl (II), Z-Gly-Phe-OMe (III), Boc-Gly-Phe-OMe (IV), Z-Tyr-Gly-Gly-Phe-OMe (VII), Boc-Tyr-Gly-Gly-Phe-OMe (VIII), Z-Tyr-Gly-Gly-Phe-Leu-NH_2 (XIII) and Boc-Tyr-Gly-Gly-Phe-Met-NH_2 (XIV).

The preparation of III and IV was accomplished by papain in quantitative yields using either Z-Gly-OCam or Boc-Gly-OCam as acyl donors and H-Phe-OMe as nucleophile. The reaction rate was higher with Z-Gly-OCam than with Boc-Gly-OCam. With respect to solvents, ethyl acetate at 0.936 of water activity gave faster reaction rates than acetonitrile containing 4% of buffer.

The difference between the two strategies for the pentapeptide synthesis was in the next steps. A first attempt to prepare the target peptides was via 2 + 3 fragment condensation as shown in Figure 1. Prior to that, the tripeptide precursors, V and VI were obtained in quantitative yields from the pairs Z-Gly-Phe-OMe, H-Leu-NH_2 and Boc-Gly-Phe-OMe, H-Met-NH_2 respectively, by means of α-chymotrypsin catalysis. The performance of these reactions was very similar to the one described for the synthesis of Z-Phe-Leu-NH_2.[15] After deprotection of the Z or Boc groups, the fragment condensation reaction between dipeptides I, II and H-Gly-Phe-Leu-NH_2, H-Gly-Phe-Met-NH_2 failed. Neither papain nor bromelain catalyzed the desired reaction in a reasonable yield. Bromelain did not catalyze the synthesis and only hydrolysis of acyl donor ester was observed. With papain 9% yield of Z-Tyr-Gly-Gly-Phe-Leu-NH_2 was obtained in 48 h and 24% yield of Boc-Tyr-Gly-Gly-Phe-Met-NH_2 was achieved in 24 h.[16] In addition, the formation of undesired peptides Z-Tyr-Gly-Phe-Leu-NH_2 and Boc-Tyr-Gly-Phe-Met-NH_2 was observed. The target pentapeptide appears to further react, probably via transpeptidation, with the nucleophiles H-Gly-Phe-AA-NH_2, to yield the tetrapeptides as the final products after long incubation times. This side reaction was observed mainly with papain during the Z-Tyr-Gly-Gly-Phe-Leu-NH_2 synthesis.

The second strategy tested is depicted in Figure 2. In this case I and II were coupled with H-Gly-Phe-OMe via papain catalysis in high yields (80%) and without detectable by-products. The last step was the 4 + 1 fragment condensation via chymotryptic catalysis. The reactions between VII, VIII and H-Leu-NH_2, H-Met-NH_2, proceeded in high yields, 85% and 91% respectively, and no Tyr–Gly bond split was observed. The reactions were carried out in both acetonitrile with 4% of buffer and ethyl acetate at 0.936 of water activity, and no significant differences were observed between these two solvent systems.

Enzymatic synthesis of Z-Tyr-Gly-Gly-Phe-Leu-OEt and Boc-Tyr-Gly-Gly-Phe-Met-OEt

A second type of [Leu]- and [Met]-enkephalin derivatives was also prepared enzymatically. The strategies used for the synthesis are shown in Figure 3. In this case an ethyl ester carboxy terminal group was chosen because it can be removed easily to obtain the Leu- and Met-enkephalins. Alternatively, the ethyl ester derivatives can be used directly as acyl-donors to incorporate new amino acids or peptide fragments. An attempt to synthesize these derivatives via 4 + 1 fragment condensation failed. No product or just trace amounts were obtained in the reaction between VII, VIII and H-Met-OEt, H-Leu-OEt using either papain or α-chymotrypsin as a catalyst. Other leucine or methionine derivatives such as H-Leu-OR_2 and H-Met-OR_2 (R_2: ^{t}Bu, Me or Al) showed the same behavior. These results are clearly different from those obtained for the previously described terminal amides. It appears that the C-terminal group exerts a significant influence on the enzymatic reaction. Since the 4 + 1 fragment condensation did not work for terminal ethyl esters the

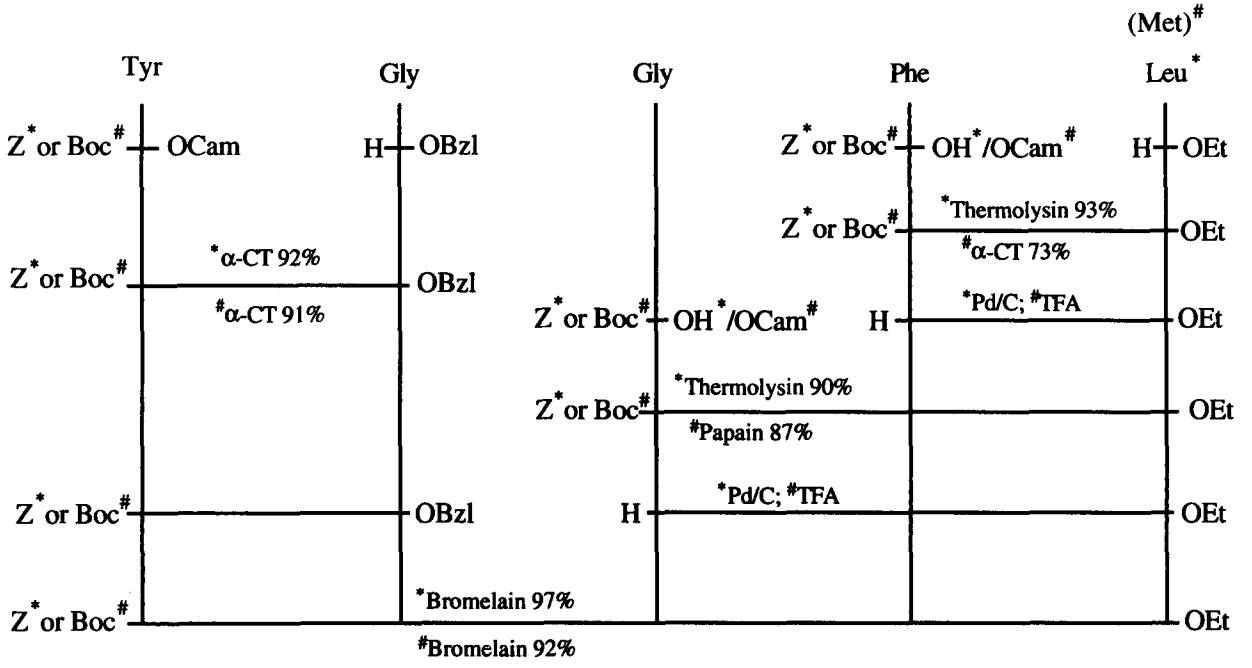

Figure 3. Protease-catalyzed synthesis of Z-Leu- and Boc-Met-enkephalin ethyl ester derivatives. Synthetic scheme based on 2 + 3 enzymatic fragment condensation. Z-Tyr-Gly-OBzl (I), Boc-Tyr-Gly-OBzl (II), Z-Phe-Leu-OEt (IX), Z-Gly-Phe-Leu-OEt (X), Boc-Phe-Met-OEt (XI), Boc-Gly-Phe-Leu-OEt (XII), Z-Tyr-Gly-Gly-Phe-Leu-OEt (XV) and Boc-Tyr-Gly-Gly-Phe-Met-OEt (XVI).

2 + 3 strategy was considered again. For this, the tripeptide C-terminal segments H-Gly-Phe-Leu-OEt and H-Gly-Phe-Met-OEt were prepared.

The precursor X was synthesized in 90% yield via thermolysin catalysis from Z-Gly-OH and H-Phe-Leu-OEt. Another possibility tested was the reaction between Z-Gly-OCam and H-Phe-Leu-OEt catalyzed by papain. The reaction worked but rendered low tripeptide production (< 37%). Z-Phe-Leu-OEt (IX) was prepared from Z-Phe-OH and H-Leu-OEt in 93% yield using thermolysin. This coupling was faster than in the synthesis of tripeptide, and none of the reactions produced detectable amounts of by-products. Compound IX was also obtained via kinetically controlled papain catalysis in methyl caproate at 0.936 of water activity from Z-Phe-OCam and H-Leu-OEt, but the yield (76%) was in this case lower.

Another possibility assayed for the tripeptide synthesis was the reaction between III and H-Leu-OEt via chymotrypsin catalysis. This reaction failed even with different leucine esters (^{t}Bu, Me) and different solvents (acetonitrile, methyl caproate) giving in all cases Z-Gly-Phe-OH as the major product.

The tripeptide precursor XII was obtained via papain catalysis between Boc-Gly-OCam and H-Phe-Met-OEt. Since the reaction rate was low, the amount of papain-Celite used (8 g) was higher than in other syntheses with this protease. The reaction was clean without detectable by-products and the yield (87%) remarkably high. Boc-Phe-Met-OEt (XI) was prepared in 73% yield by α-chymotrypsin controlled coupling between Boc-Phe-OCam and H-Met-OEt. For this particular reaction, the presence of triethylamine in the reaction media was necessary even in the case when the free base of H-Met-OEt was used. In absence of this organic base, the enzyme lost the activity and neither dipeptide nor hydrolysis of the acyl-donor ester was observed. Probably, the role played by the triethylamine is not only to neutralize the amino acid or peptide hydrochlorides, but also to act as water-mimicking cosolvent.[17] Here again, the importance of acyl-donor ester structure was clearly demonstrated since, Boc-Phe-OMe gave lower yields (20% after 24 h) and reaction rates. Thermolysin was also tested as catalyst for the synthesis of the dipeptide derivative XI. This protease did not catalyze the coupling, even using other methionine ester derivatives such as H-Met-OMe and H-Met-OAl.

Alternatively, the synthesis of the tripeptide precursor XII via α-chymotrypsin catalyzed coupling between Boc-Gly-Phe-OMe and H-Met-OEt was attempted. In this case, the yield was very low (< 10%) and Boc-Gly-Phe-OH was obtained as the main product.

The final 2 + 3 fragment condensation between I, II and H-Gly-Phe-AA-OEt (AA: Leu, Met) was successfully accomplished via bromelain catalysis. The results are listed in Table 2. Bromelain turned out to be a more efficient catalyst than papain. It is noteworthy that the change of the protecting group in P_4' (Schecter and Berger nomenclature) amino acid position dramatically increased the nucleophilicity of the tripeptide.

Discussion

Many efforts have been devoted to the enzymatic synthesis of Leu- and Met-enkephalin in predominantly

Table 1. Conditions for protease adsorption onto Celite

Protease	mg protease/g Celite	Buffer solution, pH
α-Chymotrypsin	15 or 30	Tris–HCl 50 mM, pH 7.8–9
Thermolysin	30	MOPS (Na^+) 50 mM, pH 7
Papain	15	sodium borate 0.1 M, pH 8.2
Bromelain	80	sodium borate 0.1 M, pH 9

Table 2. Protease-catalyzed synthesis of Leu- and Met-enkephalin derivatives. Results corresponding to the final 2 + 3 fragment condensation reaction between Z-Tyr-Gly-OBzl (I), Boc-Tyr-Gly-OBzl (II) and different nucleophiles. Reactions were carried out in acetonitrile (30 mL, reactions 1, 2; 50 mL reactions 3,4) containing sodium borate 0.1 M buffer (4% v/v) (pH 8.2 reactions 1, 3; pH 9 reactions 2, 4) and incubated with a preparation of the protease (papain or bromelain) adsorbed on Celite (3 g reactions 1, 2; 4 g reactions 3, 4)

# Reaction	Protease	Donor ester	Nucleophile	Time (h)	Yield %
1	Papain	I	H-Gly-Phe-Leu-OEt	48	50
2	Bromelain	I	H-Gly-Phe-Leu-OEt	24	97
3	Papain	II	H-Gly-Phe-Met-OEt	24	52
4	Bromelain	II	H-Gly-Phe-Met-OEt	8	92

aqueous media.[18] Under aqueous conditions, the peptide bond-forming steps are favored by the precipitation of the product. For instance, phenylhydrazine moiety has been used as α-carboxy protecting group of the amino component. Hence, the poor solubility of the resulting protected peptides cause a favorable shift of the chemical equilibrium and simplify the purification procedures.[11] However, the phenylhydrazine group has to be chemically transformed into an ester or acid to be used in the next enzymatic step. In addition most of the reactive side chain groups of the amino acids have to be protected with the purpose of lowering the solubility of the products, therefore making this approach as tedious as any conventional chemical synthesis. In a similar way, the stepwise Met-enkephalin synthesis has been performed by means of the exoprotease carboxypeptidase Y, using amino acid amides as nucleophiles.[12] Although the yields were remarkably high (60–90%), this strategy is darkened by transpeptidation phenomena which may occur during each elongation step. As in the case of phenylhydrazine, the terminal amides also have to be transformed enzymatically or chemically into an ester or free acid in order to use them in the next enzymatic coupling.

Using low water systems some important improvements over the strategies in aqueous solutions have already been reported. Vulfson *et al.*[10] used similar systems as in this work to perform the enzymochemical synthesis of Leu- and Met-enkephalin amide derivatives in three steps. However, the already performed H-Gly-Gly-OEt dipeptide derivative was used and only the amide terminal derivatives were reported.

The synthetic routes presented here are an improvement in terms of yields and number of chemical and enzymatic steps with respect to other total synthetic schemes. Our results represent another confirmation that organic-low water content media are useful systems for the preparative scale synthesis of peptides catalyzed by proteases adsorbed on Celite.

The strategy followed permitted the preparation of [Leu]- and [Met]-enkephalin derivatives in four enzymatic steps and one or two chemical deprotections routinely used in chemical peptide synthesis. Simple and readily available amino acid derivatives were used as starting materials. The purification of the intermediates was achieved easily by simple crystallization or by flash chromatography on silica. In most cases a simple solvent extraction was enough. The intermediates can also be used directly without purification. This was possible because one of the advantages of enzymatic methods is the fact that the main impurities of the intermediates, which are normally the hydrolysis of the acyl-donor ester and the nucleophile, do not interfere in the next coupling.[19] The overall yields obtained (60–71%) are comparable or even higher than the ones obtained by conventional solution-phase chemical synthesis.[20,21] Recently, racemization free chemical methodology using KOBt (potassium salt of 1-hydroxybenzotriazole) has been developed to improve the chemical solution method.[22] However, in that case the Fmoc amino acid chlorides have to be prepared prior to the synthesis, increasing considerably the number of steps.

An important feature of the present approach, is the use of esters of amino acid or peptide fragment nucleophiles. Thus, the resulting condensation products can be used directly, without any chemical modification as acyl-donors in the next enzymatic step. In predominantly aqueous media and in some low-water content systems, amino acid esters are usually poor nucleophiles[23,24] and a high molar excess is needed to obtain reasonable yields.[25,26] In this work, we have found that modification of the acyl-donor ester structure (i.e. using carboxamidomethyl esters instead of methyl esters) allowed the incorporation of amino acid esters in high yields. Furthermore, it is known that in aqueous media, high concentrations of amine component are used to inhibit the aminolytic activity on the newly formed peptide bond.[27] In our case, the low water activity minimized the hydrolytic reactions. This

allowed us to optimize the yields by using a slight excess of one of the fragments only to direct the reaction towards the final product with the simplest substrate, independently of whether it was the nucleophile or the acyl-donor.

Most of the amino acid derivatives are soluble in polar solvents such as acetonitrile and dimethylformamide. Some others are soluble in non-polar solvents like ethyl acetate and dichloromethane. However, solvents that offer good solubility are not the best for enzymatic activity. Acetonitrile shows good solubility for these compounds and, among the water-miscible solvents, is the most compatible with enzymatic activity and stability.[9] This makes it possible to choose within a wide range of protecting groups and esters usually used in enzymatic peptide synthesis. These groups can influence the yield and the activity of the enzyme in a given reaction.[14,28] For instance, carboxamidomethyl and benzyl esters of amino acids or peptides were better acyl-donors than the methyl esters.

The introduction of organic low water systems in enzymatic peptide synthesis is an important improvement that can make this methodology more competitive over chemical methods, in particular in large scale synthesis. The time consuming optimization processes pay off when high yields are obtained and the number of steps reduced. The optimization include not only reaction medium engineering but also the search for good acyl-donors and nucleophiles, as well as new proteases to cover all possibilities of peptide bond formation. In this sense, we want also to make a contribution to the use of the protease bromelain, a well know enzyme but with little application in the enzymatic synthesis of peptide bonds.

Experimental

Materials

α-Chymotrypsin (EC 3.4.21.1) from bovine pancreas (type II), papain (EC 3.4.22.2) from papaya latex (2 × crystallized, lyophilized powder), thermolysin (EC 3.4.24.2) from *Bacillus thermoproteolyticus* Rokko (type X) and bromelain (EC 3.4.22.4) (crude powder containing approx. 50% of protein) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Celite (30–80 mesh) was from BDH (Poole, U.K.). Amino acid derivatives, Z-Tyr-OMe, Z-Phe-OMe, H-Gly-OBzl•HCl, H-Leu-OEt•HCl, H-Leu-OMe•HCl, H-Leu-O^{t}Bu•HCl, Z-Gly-OH, Z-Phe-OH, H-Met-OEt•HCl, H-Met-OMe•HCl, H-Met-O^{t}Bu•HCl, H-Leu-NH_2, H-Met-NH_2•HCl, Boc-Gly-OH, Boc-Tyr-OMe, Boc-Tyr-OH and H-Phe-OMe•HCl were purchased from Bachem Feinchemikalien AG (Switzerland). H-Met-OAl•*p*-tosylate was from Novabiochem AG (Switzerland). Z-Gly-OCam, Boc-Gly-OCam, Z-Tyr-OCam, Boc-Tyr-OCam, Z-Phe-OCam and Boc-Phe-OCam were synthesized in our laboratory by standard procedures.[29] All other chemicals and solvents used were of analytical grade and obtained from Merck (Merck–Igoda S.A., Barcelona, Spain).

Methods

Enzyme immobilization. The enzymes were adsorbed being dried onto Celite. Enzyme–Celite preparations were obtained by mixing a solution (2 mL) of the protease in the adequate buffer with Celite (2 g). After mixing thoroughly, the preparation was dried under vacuum overnight. The amount of enzyme loaded on the Celite and the buffer and pH used in each immobilized preparation are summarized in Table 1.

Enzymatic peptide synthesis. Enzymatic reactions were started by adding a preparation of the protease on Celite to the organic media containing the substrates and a controlled amount of buffer. In some cases reactions were performed at a fixed water activity of 0.936, using a similar procedure as described in previous papers.[30,31] When the amine component was used in hydrochloride form an equimolar amount of triethylamine was always added. The reactions were carried out in 250 mL closed flasks, placed on a reciprocal shaker (120 rpm) at 25 °C. Samples were withdrawn from the reaction medium at different times from initial to 48 h, depending on the synthesis, and analyzed by HPLC. When the reaction was completed, the mixture was filtered off to remove the enzyme–Celite preparation and worked-up as follows. The solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate and washed successively with citric acid 5% (3 × 100 mL), $NaHCO_3$ 10% (3 × 100 mL) and saturated NaCl (1 × 100 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. Depending on the reaction the product was then further purified or crystallized. Crystallization was performed using hexane/ethyl acetate mixtures unless noted otherwise.

De-acylation of Z-peptides was achieved by catalytic hydrogenation using Pd over charcoal under hydrogen atmosphere. Boc-peptides were deprotected by treatment with TFA during 40 min. The solvents were evaporated under reduced pressure and the residue triturated with ether or petroleum ether.

HPLC analysis and purification. All the reactions were followed by HPLC analysis (Merck–Hitachi D-6000) using a LichroCART 250-4 HPLC cartridge column (Lichrosphere 100, RP-18, 5 μm, 250 × 4 mm). The solvent system used was the following: solvent (A) aqueous 0.1% TFA/(B) 0.1% TFA in CH_3CN; flow rate 1 mL min^{-1}; detection 254 nm. Elution conditions for each product are described below. The yields were calculated from the peak areas of the carboxyl and amino components, acyl-donor ester and peptide product.

Purity of the peptides was assessed by HPLC under the following conditions. Column VYDAC C-18, 5 μm 0.46 × 25 cm; solvent system (A) aqueous 0.1% TFA/ (B)

0.085% TFA in water:CH_3CN 1:4 (v/v); gradient elution from 10 to 70% B over 45 min, flow rate 1.5 mL min^{-1}, detection 215 nm.

Purification of final products by HPLC was carried out as follows. The crude peptides were loaded onto a preparative VYDAC C-18 15–20 μm (5 × 30 cm) cartridge and eluted using CH_3CN gradients in TEAP buffer systems at different pH values, at a flow rate of 100 mL min^{-1}, with detection at 230 nm. Analysis of the fractions was accomplished under isocratic conditions using VYDAC C-18, 5 μm 0.46 × 25 cm column eluted with (A) 0.1% aqueous TFA/(B) 0.085% TFA in water:CH_3CN 1:4, flow rate 1.5 mL min^{-1} and detection at 215 nm (0.016 AUF). The pure fractions were pooled and desalted on the same preparative cartridge by a CH_3CN gradient in 0.1% (v/v) TFA. The eluates were then lyophilized. Elution conditions for products XIII and XIV are described below.

Enzymatic synthesis of Z-Tyr-Gly-OBzl (I). Z-Tyr-OMe (1.94 g, 5.9 mmol) and H-Gly-OBzl•HCl (2.42 g, 12 mmol) were dissolved in acetonitrile (100 mL) containing Tris–HCl 50 mM, pH 7.8 buffer (1% v/v) and triethylamine (1.66 mL, 12 mmol). A completely clear solution was obtained. Then, a preparation of α-chymotrypsin on Celite (3 g, 15 mg of α-chymotrypsin g^{-1} of Celite) was added. After 24 h the yield by HPLC was 80%. The product was worked up as described above and the residue crystallized, yielding 1.7 g (62%) of dipeptide as a white solid.

The same reaction was carried out using Z-Tyr-OCam as donor ester. In this case Z-Tyr-OCam (2.9 g, 8.0 mmol) and H-Gly-OBzl•HCl (2.7 g, 13.6 mmol) were dissolved in the same solvent system containing triethylamine (2.5 mL, 17.7 mmol). Then a preparation of α-chymotrypsin on Celite (6 g, 20 mg of α-chymotrypsin g^{-1} of Celite) was added. After 12 h the HPLC dipeptide yield was 92%. The product was worked-up as described and crystallized obtaining 2.9 g (79%) of the dipeptide derivative.

HPLC reaction monitoring: 40% B for 5 min and gradient 40 to 80% B over 20 min, *K'*(Z-Tyr-Gly-OBzl) = 6.1, mp = 156–158 °C; $[\alpha]_D^{23}$ = –19.4; $[\alpha]_{578}^{23}$ = –21.3 (*c* 1, DMF). FABMS m/z 463.2 $[(M + 1)^+]$ $C_{26}H_{27}N_2O_6$ requires 462.4; ^1H NMR (d_6-DMSO, 300 MHz): 9.19 *s* (1H), 8.51 *br t* (1H), 7.48 *d* (*J* = 8.7 Hz, 1H), 7.36 *m* (10 H), 7.05 *d* (*J* = 8.4 Hz, 2H), 6.63 (*J* = 8.4 Hz, 2H), 5.12 *s* (2H), 4.93 *d* (2H), 4.19 *br m* (1H), 3.93 *m* (2H), 2.87 *m* (1H), 2.60 *m* (1H).

Enzymatic synthesis of Boc-Tyr-Gly-OBzl (II). The procedure was similar to the one described for compound I. Boc-Tyr-OMe (1.5 g, 5.1 mmol) and H-Gly-OBzl•HCl (1.51 g, 7.5 mmol) were dissolved in acetonitrile (100 mL) containing Tris–HCl 50 mM, pH 9 buffer (2% v/v) and triethylamine (1.3 mL, 9.0 mmol). To this solution, a preparation of α-chymotrypsin–Celite (2 g, 30 mg of α-chymotrypsin g^{-1} of Celite) were added. After 48 h the yield by HPLC was 71%. The reaction was worked up as described. The product was further purified by flash chromatography on silica (eluent; hexane:ethyl acetate 9:11 (v/v)) yielding 1.2 g (57%) of the pure product.

Boc-Tyr-OCam was also used as acyl-donor ester. In this case, Boc-Tyr-OCam (1.69 g, 5.0 mmol) and H-Gly-OBzl•HCl (1.61 g, 8.0 mmol) were dissolved in acetonitrile (100 mL) containing Tris–HCl 50 mM, pH 7.8 buffer (1% v/v) and triethylamine (2 mL, 14.4 mmol). To this solution, a preparation of α-chymotrypsin–Celite (2 g, 30 mg g^{-1} of Celite) was added. After 24 h, the yield by HPLC was 91% and no Boc-Tyr-OCam was detected by HPLC. The product was worked-up as above, except that in this case no flash chromatography was required since the impurities were removed by simple extraction. The pure peptide was obtained as a white solid (1.8 g, 86%).

HPLC reaction monitoring: 30% B for 3 min and gradient 30 to 70% B over 15 min, *K'* (Boc-Tyr-Gly-OBzl) = 7.4, mp = 140–142 °C; $[\alpha]_D^{23}$ = –6.1; $[\alpha]_{578}^{23}$ = –7.2 (*c* 1, DMF). ESMS m/z 451.1 $[(M + Na)^+]$, $C_{23}H_{29}N_2O_6$ requires 428.4; ^1H NMR (d_6-DMSO, 300 MHz) : 9.15 *s* (1H), 8.32 *br t* (1H), 7.36 *m* (5H), 7.03 *d* (*J* = 8.4 Hz, 2H), 6.86 *d* (*J* = 8.7 Hz, 1H), 6.62 *d* (*J* = 8.4 Hz, 2H), 5.12 *s* (2H), 4.00 *m* (3H), 2.83 *br m* (1H), 2.59 *br m* (1H), 1.28 *s* (9H).

Enzymatic synthesis of Z-Gly-Phe-OMe (III). Z-Gly-OCam (2.13 g, 8 mmol) and H-Phe-OMe (2.87 g, 16 mmol) were dissolved in separate flasks with water saturated ethyl acetate (50 mL) containing mercaptoethanol (0.15 mL). These solutions were equilibrated with a saturated salt solution of KNO_3 for two days in order to obtain a water activity of 0.936.[30,31] After the equilibration process, the solutions containing the substrates were mixed. Then, a preparation of papain on Celite (1 g, 15 mg of papain g^{-1} of Celite) previously equilibrated at the same water activity was added. After 2 h of reaction time the yield by HPLC was 98%. The reaction was worked-up as described. The pure dipeptide was obtained as a pale yellow oil (2.7 g, 91%).

HPLC reaction monitoring: 30% B for 5 min and gradient 30 to 70% B over 15 min; *K'* (Z-Gly-Phe-OMe) = 8.4; $[\alpha]_D^{23}$ = 13.0; $[\alpha]_{578}^{23}$ = 14.1 (*c* 1, EtOH). FABMS m/z 371 $[(M + 1)^+]$, $C_{20}H_{23}N_2O_5$ requires 370.4. ^1H NMR (300 MHz) ($CDCl_3$) 7.35 *m* (5H), 7.25 *m* (3H), 7.05 *m* (2H), 6.52 *br d* (1H), 5.45 *br t* (H), 5.11 *s* (2H), 4.87 *m* (1H), 3.85 *m* (2H), 3.70 *s* (3H), 3.10 *m* (2H).

Enzymatic synthesis of Boc-Gly-Phe-OMe (IV). The procedure was similar to the one described for compound III. In this case Boc-Gly-OCam (1.53 g, 6.6 mmol), H-Phe-OMe (1.77 g, 9.9 mmol) and a preparation of papain on Celite (1.5 g, 15 mg papain g^{-1} of Celite) were used. After 4 h the yield by HPLC was 93%. The product was worked up as described, yielding the pure dipeptide product as a pale yellow oil (1.9 g, 86%).

HPLC reaction monitoring: isocratic elution, 40% B, K' (Boc-Gly-Phe-OMe) = 4.1; $[\alpha]_D^{23}$ = 14.2; $[\alpha]_{578}^{23}$ = 14.7 (*c* 2, EtOH). ESMS m/z 359.0 [(M + Na)$^+$], $C_{17}H_{25}N_2O_6$ requires 336.4. ^{1}H NMR (200 MHz) ($CDCl_3$) 7.27 *m* (3H), 7.10 *m* (2H), 6.66 *br d* (*J* = 8 Hz, 1H), 5.21 *br t* (1H), 4.87 *dt* (*J* = 8, *J* = 6 Hz, 1H), 3.77 *br t* (2H), 3.7 *s* (3H), 3.11 *dd* (*J* = 6, *J* = 2 Hz, 2H), 1.44 *s* (9H).

Enzymatic synthesis of Z-Gly-Phe-Leu-NH$_2$ (V). Z-Gly-Phe-OMe (2.36 g, 6.4 mmol) and H-Leu-NH$_2$ (1.66 g, 13 mmol) were dissolved in water-saturated methyl hexanoate (100 mL). To this solution, a preparation of α-chymotrypsin on Celite (1 g, 15 mg g^{-1} Celite) was added. The reaction was performed at 0.936 of water activity, following the same procedure described for compound III. The product precipitated in the reaction medium. After 48 h no Z-Gly-Phe-OMe was detected by HPLC. Dimethylformamide (100 mL) was then added to dissolve the precipitate. The reaction mixture was then worked up as described, yielding the pure tripeptide (2.9 g, 94%).

This reaction was also carried out in acetonitrile containing Tris–HCl 50 mM, pH 7.8 buffer (4% v/v). In this case Z-Gly-Phe-OMe (2.8 g, 7.6 mmol) and H-Leu-NH$_2$ (1.67 g, 12.8 mmol) were used. No precipitation was observed. After 24 h the product yield by HPLC was 96% and no Z-Gly-Phe-OMe was detected. The product was worked up as above yielding 2.6 g (92%) of tripeptide.

HPLC reaction monitoring: isocratic elution 45% B, K' (Z-Gly-Phe-Leu-NH$_2$) = 4.1; mp 210–212 °C; $[\alpha]_D^{23}$ = −18.43; $[\alpha]_{578}^{23}$ = −19.6. FABMS (de-acylated form) m/z 335.2 [M + H]$^+$ $C_{17}H_{27}N_4O_3$ requires 334.4. FABMS m/z 469.1 [M + H]$^+$ $C_{25}H_{33}N_4O_5$ requires 468.6. ^{1}H NMR (300 MHz, d_6-DMSO) 8.05 *d* (*J* = 8.0 Hz, 1H), 7.99 *d* (*J* = 8 Hz, 1H), 7.45 *br t* (*J* = 6.0 Hz, 1H), 7.33 *s* (5H), 7.21 *s* (5H), 7.11 *br s* (1H), 7.00 *br s* (1H), 4.99 *s* (2H), 4.50 *br m* (1H), 4.19 *dt* (*J* = 7.8, *J* = 7.5 Hz, 1H), 3.55 *m* (2H), 2.87 *m* (2H), 1.6–1.4 *m* (3H), 0.86 *d* (*J* = 6 Hz, 3H), 0.81 *d* (*J* = 6 Hz, 3H).

Enzymatic synthesis of Boc-Gly-Phe-Met-NH$_2$ (VI). Boc-Gly-Phe-OMe (1.9 g, 5.7 mmol) and H-Met-NH$_2$ (1.69 g, 11.4 mmol) were dissolved in acetonitrile (100 mL) containing Tris–HCl 50 mM, pH 7.8 buffer (4% v/v). To this solution, a preparation of α-chymotrypsin on Celite (2 g, 15 mg g^{-1} Celite) was added. After 24 h the yield of tripeptide by HPLC was 92%. The product was then worked-up as described, yielding (2.2 g, 87%) of pure tripeptide.

HPLC reaction monitoring: gradient elution 30 to 80% B over 50 min, K' (Boc-Gly-Phe-Met-NH$_2$) = 6.5; mp 135–137 °C; $[\alpha]_D^{23}$ = −14.6; $[\alpha]_{578}^{23}$ = −15.3 (*c* 1, DMF). ESMS (de-acylated form) m/z 353.3 [M + H]$^+$, 375.3 [M + Na]$^+$, $C_{26}H_{25}N_4O_3S_1$ requires 352.5. FABMS m/z 453.1 $C_{21}H_{33}N_4O_5S_1$ requires 452.2. ^{1}H NMR (300 MHz, d_6-DMSO) 8.06 *d* (*J* = 7.4 Hz, 1H), 7.96 *d* (*J* = 7.4 Hz, 1H), 7.22 *m* (6H), 7.09 *d* (*J* = 4.5 Hz, 1H), 6.94 *t* (*J* = 6 Hz, 1H), 4.49 *m* (1H), 4.23 *m* (1H), 3.47 *m* (2H), 3.01 *m* (1H), 2.79 *m* (1H), 2.42 *m* (2H), 2.02 *s* (3H), 1.91 *m* (1H), 1.78 *m* (1H), 1.35 *s* (9H).

Enzymatic synthesis of Z-Tyr-Gly-Gly-Phe-OMe (VII). H-Gly-Phe-OMe•TFA (1.7 g, 4.9 mmol) and Z-Tyr-Gly-OBzl (1.5 g, 3.2 mmol) were dissolved in acetonitrile (100 mL) containing sodium borate 0.1 M, pH 8.2 buffer (4% v/v) and triethylamine (0.75 mL). To this solution, a preparation of papain on Celite (1.5 g, 15 mg papain g^{-1} Celite) was added. After 6 h of reaction time, the yield by HPLC was 80%. The reaction mixture was worked-up as described. The pure tetrapeptide was obtained by crystallization, yielding a white solid (1.4 g, 74%).

HPLC reaction monitoring: elution as described for compound VI, K' (Z-Tyr-Gly-Gly-Phe-OMe) = 7.3; mp 113–115 °C; $[\alpha]_D^{23}$ = −18.2; $[\alpha]_{578}^{23}$ = −19.0 (*c* 1, DMF). FABMS m/z 591.1 [M + H]$^+$ $C_{31}H_{35}N_4O_8$ requires 590.6. ^{1}H NMR (300 MHz, d_6-DMSO) 9.12 *s* (1H), 8.34 *d* (*J* = 4.5 Hz, 1H), 8.27 *br t* (*J* = 5.4 Hz, 1H), 8.02 *br t* (*J* = 5.4 Hz, 1H), 7.47 *d* (*J* = 8.4 Hz, 1H), 7.10–7.40 m (10H), 7.04 *d* (*J* = 8.4 Hz, 2H), 6.62 *d* (*J* = 8.4 Hz, 2H), 4.92 *d* (*J* = 6.6 Hz, 2H), 4.47 *m* (1H), 4.18 *m* (1H), 3.72 *m* (4H), 3.56 *s* (3H), 3.10–2.50 *c m* (4H).

Enzymatic synthesis of Boc-Tyr-Gly-Gly-Phe-OMe (VIII). Boc-Tyr-Gly-OBzl (1 g, 2.3 mmol) and H-Gly-Phe-OMe•TFA (1.41 g, 4.2 mmol) were dissolved in acetonitrile (100 mL) containing boric acid–Borax 50 mM, pH 9 buffer (4% v/v) and triethylamine (0.70 mL). To this solution a preparation of papain on Celite (1.5 g, 15 mg of papain g^{-1} of Celite) was added. After 9 h the product yield by HPLC was 80%. The reaction was worked-up following the procedure described above. Crystallization yielded the pure tetrapeptide (0.99 g, 77%).

HPLC reaction monitoring: same as compound II, K' (Boc-Tyr-Gly-Gly-Phe-OMe) = 5.7; mp 90–92 °C; $[\alpha]_D^{23}$ = −7.3; $[\alpha]_{578}^{23}$ = −7.8 (*c* 1, DMF). ESMS m/z 579.4 [M + Na]$^+$, $C_{28}H_{37}N_4O_8$ requires 556.6. ^{1}H NMR (300 MHz, d_6-DMSO) 9.15 *s* (1H), 8.32 *d* (*J* = 7.8 Hz, 1H), 8.17 *t* (*J* = 5.7 Hz, 1H), 8.00 *t* (*J* = 5.7 Hz, 1H), 7.22 *m* (5H), 7.02 *d* (*J* = 8.4 Hz, 2H), 6.90 *d* (*J* = 6.1 Hz, 1H), 6.62 *d* (*J* = 8.4 Hz, 2H), 4.45 *m* (1H), 4.07 *m* (1H), 3.70 *br t* (4H), 3.57 *s* (3H), 2.80–3.05 *m* (3H), 2.50–2.65 *m* (1H), 1.28 *s* (9H).

Enzymatic synthesis of Z-Phe-Leu-OEt (IX). Z-Phe-OH (2.1 g, 7.1 mmol) and H-Leu-OEt (3.4 g, 21.3 mmol) were dissolved in acetonitrile (100 mL) containing MOPS (Na^+) 50 mM, pH 7 buffer (4% v/v), and incubated in the presence of a preparation of thermolysin on Celite (2 g, 30 mg thermolysin g^{-1} of Celite). After 6 h, the dipeptide yield by HPLC was 93%. The reaction was worked-up as described above. Crystallization yielded a white solid chromatographically homogeneous product (2.6 g, 83%).

HPLC reaction monitoring: 50% B for 5 min and gradient 50 to 85% B over 5 min, *K'* (Z-Phe-Leu-OEt) = 6.1; mp 117–120 °C; $[\alpha]_D^{23}$ = –23.0; $[\alpha]_{578}^{23}$ = –24.4 (*c* 2, EtOH). ESMS m/z 463.4 $[M + Na]^+$, $C_{25}H_{33}N_2O_5$ requires 440.5. ^1H NMR (300 MHz, $CDCl_3$) 7.27 *m* (10H), 6.29 *br* (1H), 5.37 *br* (1H), 5.08 *s* (2H), 4.50 *m* (2H), 4.15 *q* (*J* = 7.2 Hz, 2H), 3.07 *m* (2H), 1.50 *m* (3H), 1.26 *t* (*J* = 7.2 Hz, 3H), 0.89 *t* (*J* = 5.1, 6H).

Enzymatic synthesis of Z-Gly-Phe-Leu-OEt (X). Z-Gly-OH (2.5 g, 12.0 mmol) and H-Phe-Leu-OEt (2.5 g, 8.0 mmol) were dissolved in acetonitrile (100 mL) containing MOPS (Na^+) 50 mM, pH 7 buffer (4% v/v). To this solution, a preparation of thermolysin on Celite (4 g, 30 mg thermolysin g^{-1} of Celite) was added. After 24 h tripeptide yield by HPLC was 90%. The product was worked-up as described previously. Crystallization rendered a white solid (3 g, 76%).

HPLC reaction monitoring: 45% B for 5 min and gradient 45 to 80% B over 10 min, *K'* (Z-Gly-Phe-Leu-OEt) = 6.7; mp 99–100 °C; $[\alpha]_D^{23}$ = –19.8; $[\alpha]_{578}^{23}$ = –20.7 (*c* 2, EtOH). FABMS (de-acylated form) m/z 364.0 $[M + H]^+$, $C_{19}H_{30}N_3O_4$ requires 363.4. FABMS m/z 498.1 $[M + H]^+$ $C_{27}H_{36}N_3O_6$ requires 497.3. ^1H NMR (300 MHz, $CDCl_3$) 7.25 *m* (10H), 6.70 *d* (*J* = 7.8 Hz, 1H), 6.31 *d* (*J* = 8.4 Hz, 1H), 5.43 *br t* (1H), 5.10 *s* (2H), 4.70 *dt* (*J* = 7.8, *J* = 6 Hz, 1H), 4.49 *m* (1H), 4.15 *q* (*J* = 7.2 Hz, 2H), 3.83 *d* (*J* = 6.0 Hz, 2H), 3.09 *m* (2H), 1.52 *m* (3H), 1.26 *t* (*J* = 7.2 Hz, 3H), 0.88 *d* (*J* = 6.0 Hz, 6H).

Enzymatic synthesis of Boc-Phe-Met-OEt (XI). Boc-Phe-OCam (2.26 g, 7.0 mmol) and H-Met-OEt (1.99 g, 11.2 mmol) were dissolved in acetonitrile (100 mL) containing Tris–HCl 50 mM, pH 7.8 buffer (1% v/v) and triethylamine (1.6 mL, 11.2 mmol). To this solution, a preparation of α-chymotrypsin on Celite (2 g, 30 mg α-chymotrypsin g^{-1} of Celite) was added. After 24 h the tripeptide yield by HPLC was 73%. The product was worked-up as described previously and crystallized yielding the pure dipeptide (2 g, 70%).

HPLC reaction monitoring: same as compound X, *K'* (Boc-Phe-Met-OEt) = 5.2; mp 118–119 °C; $[\alpha]_D^{23}$ = –15.5; $[\alpha]_{578}^{23}$ = –17.0 (*c* 1, EtOH). ESMS m/z 447.0 $[M + Na]^+$, $C_{21}H_{33}N_2O_5S_1$ requires 424.5. ^1H NMR (300 MHz, $CDCl_3$) 7.25 *m* (5H), 6.57 *d* (*J* = 5 Hz, 1H), 5.0 *br d* (1H), 4.61 *m* (1H), 4.36 *m* (1H), 4.16 *m* (2H), 3.08 *m* (2H), 2.40 *br t* (2H), 2.06 *s* (3H), 2.02–2.18 *m* (1H), 2.02–1.90 *m* (1H), 1.42 *s* (9H), 1.27 *t* (*J* = 7.2 Hz, 3H).

Enzymatic synthesis of Boc-Gly-Phe-Met-OEt (XII). Boc-Gly-OCam (1.59 g, 6.8 mmol) and H-Phe-Met-OEt.TFA (1.5 g , 3.4 mmol) were dissolved in acetonitrile (100 mL) containing sodium borate 0.1 M, pH 8.2 buffer (4% v/v), triethylamine (0.57 mL, 4.1 mmol) and mercaptoethanol (0.53 mL). To this solution, a preparation of papain on Celite (8 g, 15 mg papain g^{-1} of Celite) was added. After 24 h the tripeptide yield by HPLC was 87%. The product was worked-up as described previously and crystallized yielding the pure tripeptide (1.3 g, 82%).

HPLC reaction monitoring: 50% B for 5 min and gradient 50 to 74% B over 11 min, *K'* (Boc-Gly-Phe-Met-OEt) = 3.2; mp 99–101 °C; $[\alpha]_D^{23}$ = –18.5; $[\alpha]_{578}^{23}$ = –19.9 (*c* 2, EtOH). ESMS (de-acylated form) m/z 382.3 $[M + H]^+$, $C_{18}H_{28}N_3O_4S_1$ requires 381.5. FABMS m/z 482.2 $[M+H]^+$ $C_{23}H_{36}N_3O_6S_1$ requires 481.2. ^1H NMR (300 MHz, $CDCl_3$) 7.26 *m* (5H), 6.91 *d* (*J* = 8.1 Hz, 1H), 6.85 *d* (*J* = 6.2 Hz, 1H), 5.30 *m* (1H), 4.66 *m* (2H), 4.17 *q* (*J* = 7.2 Hz, 2H), 3.77 *br m* (2H), 3.06 *m* (2H), 2.41 *br t* (2H), 2.05 *s* (3H), 1.86–2.16 m (2H), 1.43 *s* (9H), 1.27 *t* (*J* = 7.2 Hz, 3H).

Enzymatic synthesis of Z-Tyr-Gly-Gly-Phe-Leu-NH_2 (XIII). Z-Tyr-Gly-Gly-Phe-OMe (0.56 g, 0.95 mmol) and H-Leu-NH_2 (0.25 g, 1.91 mmol) were dissolved in acetonitrile (50 mL) containing Tris–HCl 50 mM, pH 7.8 buffer (4% v/v). To this solution, a preparation of α-chymotrypsin on Celite (2 g, 15 mg α-chymotrypsin g^{-1} of Celite) was added. After 1.5 h the product yield by HPLC was 85% and no Z-Tyr-Gly-Gly-Phe-OMe was detected.

The same reaction was carried out in ethyl acetate. Z-Tyr-Gly-Gly-Phe-OMe (0.48 g, 0.81 mmol) and H-Leu-NH_2 (0.21 g, 1.6 mmol) were dissolved in water-saturated ethyl acetate (50 mL). This solution was pre-equilibrated to obtain a water activity of 0.936 following the same procedure as with compound III. A preparation of α-chymotrypsin on Celite (0.8 g, 15 mg α-chymotrypsin g^{-1} of Celite), at the same water activity, was added. After 2 h the product yield by HPLC was 86%.

The crude peptide was purified by preparative HPLC as indicated above. First the peptide was chromatographed using a TEAP system at pH 6.7 and gradient elution from 21 to 45% CH_3CN in 60 min. Analysis of the fractions were performed as described in methods using 45% B for elution. Pure fractions were pooled, loaded onto the cartridge and eluted using 0.1% TFA system under gradient conditions 27 to 43% CH_3CN in 30 min. Impure fractions from the previous TEAP run were re-chromatographed on the TFA system using a gradient from 31 to 39% CH_3CN in 25 min. The best fractions from both 0.1% TFA runs were pooled and lyophilized (0.37g, 66%). Purity 99.7% by HPLC.

HPLC reaction monitoring: 40% B for 5 min and gradient 40 to 80% B over 20 min, *K'* (Z-Tyr-Gly-Gly-Phe-Leu-NH_2) = 2.3; mp (lyophilized) 170–173 °C; $[\alpha]_D^{23}$ = –24.1; $[\alpha]_{578}^{23}$ = –25.4 (*c* 1, DMF). FABMS m/z 711.2 $[M + Na]^+$ $C_{36}H_{45}N_6O_8$ requires 688.8. ^1H NMR (500 MHz, d_6-DMSO) 9.12 *s* (1H), 8.19 *t* (*J* = 5.6 Hz, 1H), 8.03 *d* (*J* = 8.1 Hz, 1H), 7.97 *t* (*J* = 5.6 Hz, 1H), 7.90 *d* (*J* = 8.3 Hz, 1H), 7.39 *d* (*J* = 8.2 Hz, 1H), 7.32 *m* (3H), 7.27–7.14 *m* (8H), 7.08–6.88 *cs* (4H), 6.63 *d* (*J* = 8.4 Hz, 2H), 7.04 *d* (*J* = 8.3 Hz, 2H), 4.97 *d* (*J* = 12.6 Hz, 1H), 4.91 *d* (*J* = 12.6 Hz, 1H) (AB system), 4.51 *m* (1H), 4.20 *m* (2H), 3.73 *m* (3H), 3.63 *m* (1H), 3.04 *m* (1H), 2.93 *m* (1H), 2.80 *m* (1H), 2.64 *m* (1H), 1.56 *m* (1H), 1.47 *m* (*J* = 7.5 Hz, 2H), 0.87 *d* (*J* = 6.6 Hz, 3H), 0.82 *d* (*J* = 6.6 Hz, 3H).

Enzymatic synthesis of Boc-Tyr-Gly-Gly-Phe-Met-NH_2 (XIV). Boc-Tyr-Gly-Gly-Phe-OMe (0.56 g, 1.0 mmol) and H-Met-NH_2 (0.30 g, 2 mmol) were dissolved in acetonitrile (50 mL) containing Tris–HCl 50 mM, pH 9 buffer (4% v/v). To this solution, a preparation of α-chymotrypsin on Celite (2 g, 15 mg α-chymotrypsin g^{-1} of Celite) was added. After 2 h the pentapeptide yield by HPLC was 91%. The crude product was purified by preparative HPLC as indicated in the Experimental section. First the peptide was chromatographed using a TEAP system at pH 2.5 and gradient elution from 19 to 35% CH_3CN over 60 min. Analysis of the fractions was performed as described in methods using 39% B for elution. Two fraction pools were obtained. The impure pool was re-chromatographed using TEAP pH 6.5 with the same CH_3CN gradient. The best fractions from both TEAP runs were pooled and desalted using the 0.1% TFA system and a steep CH_3CN gradient and lyophilized (0.47 g, 70%). Purity 99.7% by HPLC.

HPLC reaction monitoring: same as compound II, *K'* (Boc-Tyr-Gly-Gly-Phe-Met-NH_2) = 4.7; mp (lyophilized) 110–113 °C; $[\alpha]_D^{23} = -18.1$; $[\alpha]_{578}^{23} = -19.2$ (*c* 1, DMF). FABMS m/z 673.3 [M + H]$^+$ $C_{32}H_{45}N_6O_8S_1$ requires 672.8. ^{1}H NMR (500 MHz, d_6-DMSO) 9.1 *br s* (1H), 8.1 *t* (*J* = 5.6 Hz, 1H), 8.0 *d* (*J* = 7.8 Hz, 1H), 7.9 *d* (*J* = 8.2 Hz, 1H), 7.35–7.15 complex *m* (6H), 7.1–6.95 complex system (4H), 6.81 *d* (*J* = 8.5 Hz, 1H), 6.63 *d* (*J* = 8.4 Hz, 2H), 4.51 *m* (1H), 4.25 *m* (1H), 4.1 *br m* (1H), 3.8–3.6 *m* (4H), 3.2–2.6 complex system (4H), 2.48–2.30 complex system (2H), 2.03 *s* (3H), 2.0–1.9 m (1H), 1.85–1.75 m (1H), 1.3 *s* (9H).

Enzymatic synthesis of Z-Tyr-Gly-Gly-Phe-Leu-OEt (XV). Z-Tyr-Gly-OBzl (0.40 g, 0.9 mmol) and H-Gly-Phe-Leu-OEt (0.25 g, 0.7 mmol) were dissolved in acetonitrile (30 mL) containing sodium borate 0.1 M, pH 9 buffer (4% v/v), and mercaptoethanol (0.25 mL). To this solution, a preparation of Bromelain on Celite [3 g, 80 mg Bromelain (crude powder) g^{-1} of Celite] was added. After 24 h the pentapeptide yield by HPLC was 97%. The product was worked-up as described above and further purified by flash chromatography on silica. First, the crude was eluted with ethyl acetate to remove the excess of Z-Tyr-Gly-OBzl. Then, the pure pentapeptide was eluted with ethyl acetate:methanol 19:1 (v/v) yielding 0.35 g (70%) of pentapeptide. Purity 99.9% by HPLC.

HPLC reaction monitoring: same as compound XII, *K'* (Z-Tyr-Gly-Gly-Phe-Leu-OEt) = 7.1; mp (lyophilized) 93–95 °C; $[\alpha]_D^{23} = -25.8$; $[\alpha]_{578}^{23} = -27.2$ (*c* 1, DMF). FABMS m/z 718.2 [M + H]$^+$ $C_{38}H_{48}N_5O_9$ requires 717.8. ^{1}H NMR (500 MHz, d_6-DMSO) 9.11 *s* (1H), 8.31 *d* (*J* = 7.7 Hz, 1H), 8.18 *t* (*J* = 5.5 Hz, 1H), 8.00 *d* (*J* = 8.5 Hz, 1H), 7.92 *t* (*J* = 5.6 Hz, 1H), 7.37 *d* (*J* = 8.5 Hz, 1H), 7.35–7.12 *m* (10H), 7.04 *d* (*J* = 8.4 Hz, 2H), 6.63 *d* (*J* = 8.5 Hz, 2H), 5.01–4.87 *m* (2H), 4.57 *m* (1H), 4.24 *m* (2H), 4.07 *m* (2H), 3.71 *m* (3H), 3.62 *m* (1H), 3.06–2.60 complex system (4H), 1.7–1.4 *m* (3H), 1.17 *t* (*J* = 7.1 Hz, 3H), 0.90 *d* (*J* = 7.5 Hz, 3H), 0.84 *d* (*J* = 7.5 Hz, 3H).

Enzymatic synthesis of Boc-Tyr-Gly-Gly-Phe-Met-OEt (XVI). Boc-Tyr-Gly-OBzl (0.87 g, 2 mmol) and H-Gly-Phe-Met-OEt (0.60 g, 1.6 mmol) were dissolved in acetonitrile (50 mL) containing sodium borate 0.1 M, pH 9 buffer (4% v/v), and mercaptoethanol (0.30 mL). To this solution, a preparation of Bromelain on Celite (4 g, 80 mg Bromelain (crude powder) g^{-1} of Celite) was added. After 8 h the pentapeptide yield by HPLC was 92%. The product was worked-up and purified as above (compound XV) yielding 0.80 g (71%) of pentapeptide. Purity 99.9% by HPLC.

HPLC reaction monitoring: same as compound II, *K'* (Boc-Tyr-Gly-Gly-Phe-Met-OEt) = 4.9; mp (lyophilized) 92–94 °C; $[\alpha]_D^{23} = -8.7$; $[\alpha]_{578}^{23} = -9.7$ (*c* 1, EtOH). FABMS m/z 702.3 [M + H]$^+$ $C_{34}H_{48}N_5O_9S_1$ requires 701.3. ^{1}H NMR (500 MHz, d_6-DMSO) 9.1 *br s* (1H), 8.35 *d* (*J* = 7.6 Hz, 1H), 8.1 *t* (*J* = 5.6 Hz, 1H), 8.0 *d* (*J* = 8.4 Hz, 1H), 7.9 *t* (*J* = 5.4 Hz, 1H), 7.25 *m* (4H), 7.2 *m* (1H), 7.0 *d* (*J* = 8.3 Hz, 2H), 6.8 *d* (*J* = 8.2 Hz, 1H), 6.6 *d* (*J* = 8.3 Hz, 2H), 4.55 *m* (1H), 4.35 *m* (1H), 4.1 *m* (3H), 3.7–3.6 *m* (4H), 3.1–2.6 complex system (4H), 2.5 *m* (2H), 2.05 *s* (3H), 2.0–1.8 complex *m* (2H), 1.3 *s* (9H), 1.2 *t* (3H).

Acknowledgments

This project was financially supported by the Comisión Interministerial para la Ciencia y la Tecnología SAF92-0261-C02-01. Furthermore, the project was supported in both countries by the SCIENCE program of the Commission of the European Countries.

References

1. Andersen, A. J.; Fomsgaard, J.; Thorbek, P.; Aasmul-Olsen, S. *Chimicaoggi (Chemistry Today)* **1991**, March, 16.

2. Delente, J. J. *TIBTECH* **1986**, April, 80.

3. Vulfson, E. N. *Trends Food Sci. Technol.* **1993**, *4*, 209.

4. Jakubke, H.-D.; Kuhl, P.; Könnecke, A. *Angew. Chem. Int. Ed. Engl.* **1985**, *24*, 85.

5. Gupta, M. N. *Eur. J. Biochem.* **1992**, *203*, 25.

6. Mattiasson, B.; Adlercreutz, P. *TIBTECH* **1991**, *9*, 25.

7. Klibanov, A. M. *Chemtech.* **1986**, *16*, 354.

8. Kullman, W. *Enzymatic Peptide Synthesis*, CRC Press; Boca Raton, 1987.

9. Reslow, M.; Adlercreutz, P.; Mattiasson, B. *Eur. J. Biochem.* **1988**, *177*, 313; Clapés, P.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. Appl. Biochem.* **1989**, *12*, 376.

10. Gill, I.; Vulfson, E. N. *J. Chem Soc. Perkin Trans. 1* **1992**, *6*, 667; Richards, A. O.; Gill, I. S.; Vulfson, E. N. *Enzyme Microb. Technol.* **1993**, *15*, 928.

11. Kullman, W. *Biochim. Biophys. Res. Commun.* **1979**, *2*, 693.

12. Widmer, F.; Breddam, K.; Johansen, J. T. In: *Peptides 1980, Proc. 16th Europ. Peptide Symp.*, p. 46, Brunfeldt, K., Ed.; Scriptor; Kopenhagen, 1981.

13. Luisi, P. L.; Saltman, R.; Valch, D.; Guarnaccia, R. *J. Mol. Catalysis* **1977**, *2*, 133.

14. Cohen, S.G.; Torem, B.; Vaidya, V.; Ehret, A. *J. Biol. Chem.* **1976**, *215*, 4722.

15. Clapés, P.; Adlercreutz, P.; Mattiasson, B. *J. Biotechnol.* **1990**, *15*, 323.

16. Both reactions were carried out in acetonitrile (2 mL) containing sodium borate 0.1 M, pH 8.2 buffer (4% v/v). The acyl-donor ester (20 mM) and the nucleophile (30 mM) were incubated with a preparation of papain on Celite (0.150 g).

17. Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272.

18. See references 10–12; Kimura, Y.; Nakanishi, K.; Matsuno, R. *Enzyme Microb. Technol.* **1990**, *12*, 272.

19. Andersen, A. J.; Widmer, F.; Johansen, J. T. In: *Peptides 1986, Proc. 19th Eur. Peptide Symp.*, pp. 183–188, Walter de Gruyter & Co.; Berlin, 1987.

20. Wang, S. S.; Guisin, B. F.; Winter, D. P.; Makofske, R.; Kulesha, I. D.; Tzougraki, C.; Meienhofer, J. *J. Org. Chem.* **1977**, *42*, 1286.

21. Hoeg-Jensen, T.; Jakobsen, M. H.; Holm, A. *Tetrahedron Lett.* **1991**, *32*, 6387.

22. Sivanandaiah, K. M.; Suresh Babu, V. V.; Shankaramma, S. C. *Int. J. Peptide Protein Res.* **1994**, *44*, 24.

23. Morihara, K.; Oka, T. *Biochem. J.* **1977**, *163*, 531.

24. Nagashima, T.; Watanabe, A.; Kise, H. *Enzyme Microb. Technol.* **1992**, *14*, 842.

25. Widmer, F.; Ohno, M.; Smith, M.; Nelson, N.; Afinsen, C.B. In: *Peptides 1982, Proc. 17th Eur. Peptide Symp.*, pp. 375–379, Walter de Gruyter & Co; Berlin, 1983.

26. West, J. B.; Wong, C.-H. *J. Org. Chem.* **1986**, *51*, 2728.

27. Fastrez, J.; Fersht, A. R. *Biochemistry* **1973**, *12*, 2025.

28. Calvet, S.; Clapés, P.; Torres, J. L.; Valencia, G.; Feixas, J.; Adlercreutz, P. *Biochim. Biophys. Acta* **1993**, *1164*, 189.

29. Martinez, J.; Laur, J.; Castro, B. *Tetrahedron Lett.* **1983**, *24*, 5219.; Martinez, J.; Laur, J.; Castro, B. *Tetrahedron* **1985**, *41*, 739.

30. Adlercreutz, P. *Eur. J. Biochem.* **1991**, *199*, 604.

31. Clapés, P.; Valencia, G.; Adlercreutz, P. *Enzyme Microb. Technol.* **1992**, *14*, 575.

(*Received in U.S.A.* 14 *September* 1994; *accepted* 16 *December* 1994)