PERKIN 1

α-Chymotrypsin-catalysed segment condensations *via* the kinetically controlled approach using carbamoylmethyl esters as acyl donors in organic media †

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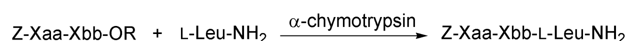
The superiority of the carbamoylmethyl ester as an acyl donor for the α-chymotrypsin-catalysed segment condensations *via* the kinetically controlled approach is demonstrated in several model systems carried out in organic media with low water content. Furthermore, this approach is successfully applied to the construction of the Leu-enkephalin sequence *via* a 4 + 1 segment coupling.

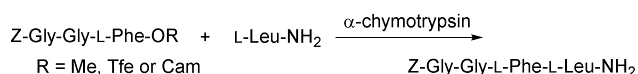
Introduction

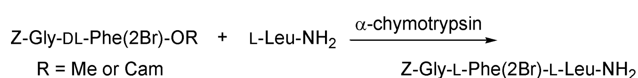
In modern peptide synthesis, a high degree of chiral integrity of products is required.[1] Stepwise elongation of a peptide chain using urethane-protected amino acids is a method generally free from racemisation. Much greater difficulties are encountered during the coupling of peptide segments, because activated *C*-terminal amino acid residues can be racemised easily. Nevertheless, segment condensation is indispensable for building long peptide chains. Accordingly, a great deal of effort has been exerted to prevent racemisation during segment couplings.[2] In this regard enzymatic methodologies have attracted the attention of researchers. In particular, enzymatic peptide synthesis using proteases[3] is becoming recognised as an alternative or complement to chemical synthesis of biologically active peptides. Besides freedom from racemisation, enzymatic methodologies have also several other advantages: among others, high regio- and stereoselectivity and minimal side-chain protection. On the other hand, the following are counted as major disadvantages: narrow substrate specificity often shown by enzymes, and secondary hydrolysis of a growing peptide. In previous papers,[4] we have reported on the broadening of substrate tolerance of a serine protease, α-chymotrypsin (EC 3.4.21.1), by using such activated esters as the 2,2,2-trifluoroethyl (Tfe) or carbamoylmethyl (Cam) ester as acyl donors in the kinetically controlled approach of peptide-bond formation. Among the activated esters examined, the Cam ester proved to have a particularly noteworthy ameliorating effect. Furthermore, the method employing this ester as the acyl donor was applied to the amide-bond formation between an amino acid residue and a chiral amine.[5] In order to clarify the scope and limitations we intended to apply this approach to several model segment condensations and also to the construction of the Leu-enkephalin sequence.[6] In the present paper, we describe the details of our relevant work.

Results and discussion

Initially, in order to ascertain the usefulness of the Cam ester in α-chymotrypsin-catalysed segment condensations *via* the kinetically controlled approach, several model coupling systems of the 2 + 1 or 3 + 1 type were investigated (Scheme 1). In these segment condensations the possibility of racemisation of the *C*-terminal residue of the carboxylic component and the fission of peptide bond(s) within the carboxylic component must be taken into consideration. Fortunately, we could resort to HPLC analysis for the close examination of these undesirable side reactions on the basis of our recent researches.[4,5] Table 1 shows the results of the α-chymotrypsin-catalysed couplings of several kinds of fragments with L-Leu-NH_2 as a typical amino component (molar ratio, 1 : 4).[7] This Table includes also the results obtained using the Me or Tfe ester for the purpose of comparison. The reaction conditions were chosen to be similar to the dipeptide syntheses in acetonitrile containing 4% Tris buffer (pH 7.8).[4b] The amounts of the donor ester, the desired peptide and its epimer, and other possible by-products were determined by HPLC analysis on an ODS column using aqueous acetonitrile or MeOH containing H_3PO_4 as a mobile phase. As a typical example, HPLC separation of compounds relevant to the coupling of Z-L-Phe-L-Phe-OR (R = Me, Tfe or Cam) with L-Leu-NH_2 is shown in Table 2.







Scheme 1

The couplings of fragments bearing a *C*-terminal Ala residue as carboxylic component were examined first (Table 1, entries 1–4). The reaction profile in the coupling of Z-L-Phe-L-Ala-OCam with L-Leu-NH_2 is shown as an example in Fig. 1. When the Me esters were used as acyl donors, the yields of the desired peptides were low. The use of the Cam esters once again resulted in a marked increase in the peptide yields. In addition, HPLC analysis revealed that no racemisation of the L-Ala

† Electronic supplementary information (ESI) available: elemental analyses and HPLC separation data. See http://www.rsc.org/suppdata/p1/b1/b108738j/

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Table 1 α-Chymotrypsin-catalysed fragment couplings with L-Leu-NH_2 as an amino component[a]

Entry	Carboxylic component	Time (min)	Yield (%) Peptide[b]	Yield (%) Hydrolysis product[c]	Other by-products[d]
1	Z-Gly-L-Ala-OMe	30	8.3	1.1	
2	Z-Gly-L-Ala-OCam	30	86.0	12.6	
3	Z-L-Phe-L-Ala-OMe	60	1.7	0	
		300	8.7	2.1	A
4	Z-L-Phe-L-Ala-OCam	60	70.8	9.8	
		120	79.4	10.7	B
5	Z-Gly-L-Phe-OMe	5	14.3	1.1	
6	Z-Gly-L-Phe-OTfe	5	56.2	1.2	
7	Z-Gly-L-Phe-OCam	5	95.3	4.7	
8	Z-Gly-Gly-L-Phe-OMe	5	14.9	1.0	
9	Z-Gly-Gly-L-Phe-OTfe	5	62.3	2.4	
10	Z-Gly-Gly-L-Phe-OCam	5	88.3	4.5	
11	Z-L-Phe-L-Phe-OMe	60	12.8	0.9	C
12	Z-L-Phe-L-Phe-OTfe	60	50.6	4.2	
13	Z-L-Phe-L-Phe-OCam	60	83.4	8.1	
14	Z-Gly-DL-Phe(2Br)-OMe[e]	30	4.8[f]	1.6	
15	Z-Gly-DL-Phe(2Br)-OCam[e]	30	39.6[f]	10.6	

[a] A mixture of 0.05 mmol of a carboxylic component, 0.2 mmol of L-Leu-NH_2·HCl, 0.2 mmol of TEA, and 150 mg of the immobilised α-chymotrypsin was incubated with shaking in a solvent composed of 2 ml of acetonitrile and 83 µl of Tris buffer (pH 7.8) at 30 °C. [b] Desired peptide. [c] Hydrolysis product of the donor ester. [d] A, 1.4% of Z-L-Phe-L-Leu-NH_2; B, 4.1% of Z-L-Phe-L-Leu-NH_2; C, 0.5% of Z-L-Phe-L-Leu-NH_2. [e] Using 0.1 mmol of the carboxylic component. [f] L-L Peptide.

Table 2 HPLC separation of compounds relevant to the α-chymotrypsin-catalysed coupling of Z-L-Phe-L-Phe-OR with L-Leu-NH_2[a]

Compound	k'[b] Mobile phase A	k'[b] Mobile phase B
Z-L-Phe	5.1	2.8
Z-L-Phe-L-Leu-NH_2	5.4	2.8
Z-L-Phe-L-Phe-OCam	7.3	3.5
Z-L-Phe-L-Phe	9.7	4.5
Z-L-Phe-L-Phe-L-Leu-NH_2	12.5	5.1
Z-L-Phe-D-Phe-L-Leu-NH_2	13.7	
Z-L-Phe-L-Phe-OMe	22.7	9.4
Z-L-Phe-L-Phe-OTfe		19.2

[a] HPLC conditions: see the Experimental section. Column temp., 40 °C. Mobile phase A, 46% aq. acetonitrile containing H_3PO_4 (0.01 M); mobile phase B, 54% aq. acetonitrile containing H_3PO_4 (0.01 M). [b] Capacity factor.

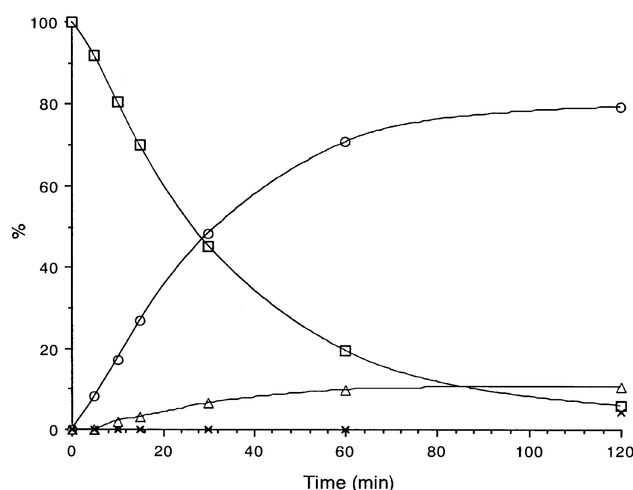


Fig. 1 α-Chymotrypsin-catalysed coupling of Z-L-Phe-L-Ala-OCam with L-Leu-NH_2. Symbols: □, Z-L-Phe-L-Ala-OCam; ○, Z-L-Phe-L-Ala-L-Leu-NH_2; △, Z-L-Phe-L-Ala; ×, Z-L-Phe-L-Leu-NH_2.

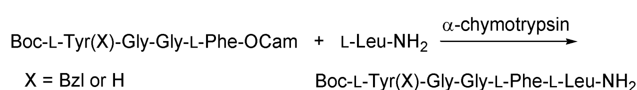
residue accompanied the couplings. In entries 3 and 4, the Phe-Ala bond was expected to be susceptible to cleavage by α-chymotrypsin on account of its substrate specificity.[8] In fact, when the Me ester was used as the acyl donor, the production of 1.4% of Z-L-Phe-L-Leu-NH_2 was observed besides the desired peptide (8.7%) after 5 h of incubation. In this case no production of Z-L-Phe was detected, which indicates that Leu-NH_2 served as a much better nucleophile than water under the present reaction conditions. In the case of the Cam ester as the acyl donor, the production of the defective peptide was not observed after 1 h, but it became detectable (4.1%) after 2 h together with the maximum yield of the desired peptide (79%). The couplings of fragments bearing a *C*-terminal Phe residue were next examined (Table 1, entries 5–10). As expected from the fact that the Phe residue is one of α-chymotrypsin's good amino acid substrates, these couplings were rather fast even when the Me ester was used as the acyl donor. The Tfe ester moderately increased the coupling yield, while it was significantly enhanced by the use of the Cam ester. No racemisation of the L-Phe residue accompanied these couplings. The coupling of a fragment bearing the L-Phe-L-Phe sequence was further tried where the bond between the two Phe residues was susceptible to enzymatic cleavage (Table 1, entries 11–13). In fact, when the Me ester was used as the acyl donor, the production of a small amount (0.5%) of Z-L-Phe-L-Leu-NH_2 (and no formation of Z-L-Phe) was observed after 1 h of incubation. The coupling yield was greatly improved by the use of the Cam ester without either the formation of the defective peptide or the racemisation of the *C*-terminal L-Phe residue.

Fragment couplings of a carboxylic component bearing a sterically demanding non-protein amino acid residue at the *C*-terminal position were also examined (Table 1, entries 14 and 15). In the synthesis of dipeptides containing halogenophenylalanines, the *o*-Br-Phe residue was found to exert an especially deleterious effect.[4a,5] The low coupling efficiency with the Me ester as an acyl donor was significantly improved by the use of the Cam ester. With the latter ester the peptide yield reached a maximum (40%) within 30 min, indicating that the Cam ester of the dipeptide carboxylic component reacted more rapidly than that of the amino acid carboxylic component.[5] Moreover, HPLC analysis revealed that starting from the racemic substrate, the L-ester reacted specifically and the D-counterpart remained unchanged, affording only the L-L peptide with L-Leu-NH_2 as the amino component, even when the Cam ester was employed. Thus, this approach using the Cam ester must surely be very useful for the incorporation of non-protein amino acids into peptides, because racemic amino

acids can be directly used for coupling without resolving them before use.

The Cam esters of *N*-protected peptides used in the above experiments were prepared mainly through the EDC–HOBT-mediated {EDC = 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide} coupling of an *N*-protected peptide with an amino acid Cam ester. They were also prepared *via* the reaction of the Cs salt of an *N*-protected peptide with 2-chloroacetamide in DMF. The samples prepared by both routes were identical with each other in terms of their physical properties including the specific rotation (see the Experimental section). This means that during the substitution reaction of 2-chloroacetamide with the Cs salt no epimerisation of the peptide fragment occurs, and thus the Cam ester necessary for the fragment condensation can be prepared directly from the corresponding *C*-free compound *via* its Cs salt.[9]

In order to demonstrate further the potential usefulness of the Cam ester as the acyl donor in the kinetically controlled peptide synthesis we further tried to prepare the Leu-enkephalin sequence[2b] *via* a 4 + 1 segment condensation (Scheme 2). In this case, the Tyr-Gly bond in the carboxylic



Scheme 2

component and the peptide formed might be cleaved and the phenolic hydroxy group of the Tyr residue might be attacked. First, the carboxylic component carrying the *O*-benzyl-Tyr residue was allowed to couple with L-Leu-NH_2 (molar ratio, 1 : 4) under similar reaction conditions to those above. Reversed-phase HPLC analysis permitted not only the quantification of the desired protected pentapeptide but also the detection and quantification of its epimer [Boc-L-Tyr(Bzl)-Gly-Gly-D-Phe-Leu-NH_2] and other possible by-products [*i.e.*, Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe, Boc-L-Tyr(Bzl) and Boc-L-Tyr(Bzl)-L-Leu-NH_2]. These compounds were well separated one from another by choosing appropriate analytical conditions, especially by adjusting the eluent composition. The Cam ester of the carboxylic component necessary for the study was prepared by the following two routes: (i) the EDC–HOBT-mediated coupling of Boc-L-Tyr(Bzl)-Gly-Gly with L-Phe-OCam; (ii) the substitution of 2-chloroacetamide with the Cs salt of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe in DMF. In this case, the sample prepared by the latter route was completely identical with that prepared by the former route. The profile of the coupling reaction is shown in Fig. 2. After only 5 min of incubation, the desired peptide was produced in 67% yield. The acyl donor disappeared completely within 30 min and the peptide yield reached to 96%. No epimeric peptide was formed and no products attributable to the fission of the Tyr-Gly bond were detected. However, the concomitant formation of a small amount (4.0%) of the hydrolysis product of the donor ester was inevitable. Next, the carboxylic component carrying the Tyr residue with a free hydroxy group was used for the coupling with the same amino component. In this case, the acyl donor disappeared almost completely within 10 min and the peptide yield reached to 95%; the yield of the hydrolysis product of the donor ester was 5.0%. Neither racemisation of the L-Phe residue nor cleavage of the Tyr-Gly bond occurred during the coupling. Moreover, the chromatogram of the reaction mixture on HPLC showed no indication of the occurrence of side reactions at the hydroxy group of the Tyr residue.

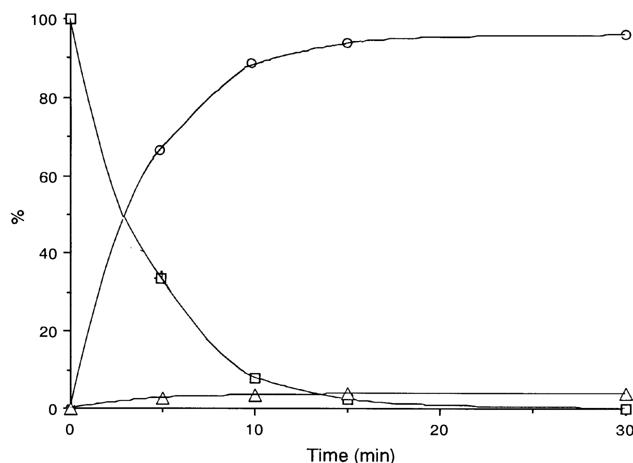


Fig. 2 α-Chymotrypsin-catalysed synthesis of the Leu-enkephalin sequence *via* a 4 + 1 segment condensation. Symbols: □, Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OCam; ○, Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-Leu-NH_2; △, Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe.

As illustrated in the above examples, when the Cam ester is used as a donor ester which exceptionally facilitates the production of the desired peptide, the enzymatic cleavage of peptide bond(s) within the carboxylic component is suppressed completely or to a very low level. In addition, no racemisation of the *C*-terminal residue accompanies the coupling. Thus, these results indicate the usefulness of the Cam ester as an acyl donor in the α-chymotrypsin-catalysed kinetically controlled segment condensations.

Experimental

General

^1H NMR spectra were obtained at 300 MHz on a Varian Unity 300 spectrometer using DMSO-d_6 as a solvent with TMS as an internal standard unless otherwise noted. Mps were determined on a Yamato MP-21 apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-4 digital polarimeter. $[a]_D$-Values are given in units of 10^{-1} deg cm^2 g^{-1}. MALDI-TOF mass spectra were recorded on a PerSeptive Biosystems Voyager DE PRO Biospectrometry Workstation, where α-cyano-4-hydroxycinnnamic acid was used as a matrix reagent. All organic solvents were distilled following standard protocols and dried over molecular sieves prior to use. Petroleum spirit refers to the fraction with distillation range 30–70 °C. L-Leu-NH_2·HCl was purchased from Kokusan Chemical Works. α-Chymotrypsin (type II, *ex* bovine pancreas) was purchased from Sigma and had a specific activity of 48 units per mg solid with Bz-Tyr ethyl ester. It was immoblised on Celite as described before.[4a] Elemental analysis data for all new compounds are available as Supplementary material. †

Preparation of the Me and Tfe esters of *N*-protected peptides

The Me and Tfe esters of *N*-protected peptides were prepared through the coupling of an *N*-protected amino acid or Z-Gly-Gly with an amino acid ester hydrochloride or hydrobromide by the EDC–HOBT method in 79–96% yield, as illustrated below for the preparation of Z-L-Phe-L-Phe-OTfe. The Me esters were prepared also through the treatment of *N*-protected peptides with an ethereal solution of diazomethane in nearly quantitative yield. The mps and $[a]_D$-values of the following *N*-protected peptide Me esters have been reported: Z-Gly-L-Ala-OMe,[10] Z-L-Phe-L-Ala-OMe[11,12] and Z-L-Phe-L-Phe-OMe.[12,13]

To a stirred solution of Z-L-Phe (898 mg, 3.0 mmol), L-Phe-OTfe·HBr (prepared through the debenzyloxycarbonylation of Z-L-Phe-OTfe with 25% HBr in AcOH) (985 mg, 3.0 mmol), triethylamine (TEA) (304 mg, 3.0 mmol) and HOBT (406 mg, 3.0 mmol) in DMF (12 ml) was added EDC·HCl (579 mg, 3.0 mmol) under ice-cooling. After stirring at this temperature for 2 h and then at ambient temperature overnight, the reaction mixture was diluted with EtOAc, washed successively with 1 M HCl, water, 1 M aq. $NaHCO_3$ and brine, and dried over Na_2SO_4. Evaporation of the solvent *in vacuo* afforded white

crystals, which were recrystallised from EtOAc–petroleum spirit; yield, 1.38 g (87%); mp 165–165.5 °C; $[\alpha]_D^{25}$ +13.9 (*c* 1.0, $CHCl_3$); δ_H ($CDCl_3$) 2.94–3.13 (4H, m, 2 × Phe β-CH_2), 4.35–4.51 (3H, m, Phe α-CH + CH_2CF_3), 4.83 (1H, q, *J* 6.6, Phe α-CH), 5.07 (2H, s, PhC*H*$_2$O), 5.22 (1H, br, NH), 6.22 (1H, br d, *J* ≈7, NH), 6.95–7.39 (15H, m, 3 × Ph).

Z-Gly-L-Phe-OMe. Oil, $[\alpha]_D^{25}$ +2.7 (*c* 1.0, DMF); δ_H 2.86–3.03 (2H, m, Phe β-CH_2), 3.58 (3H, s, OMe), 3.56–3.62 (2H, m, Gly CH_2), 4.47 (1H, q-like, *J* ≈6, Phe α-CH), 5.01 (2H, apparent s, PhC*H*$_2$O), 7.17–7.35 (10H, m, 2 × Ph), 7.39 (1H, br t, *J* ≈6, Gly NH), 8.30 (1H, d, *J* 7.5, Phe NH).

Z-Gly-L-Phe-OTfe. Mp 85.5–86.5 °C (from EtOAc–petroleum spirit), $[\alpha]_D^{25}$ +28.7 (*c* 1.0, $CHCl_3$); δ_H 2.93–3.08 (2H, m, Phe β-CH_2), 3.58–3.61 (2H, m, Gly CH_2), 4.54 (1H, q-like, *J* ≈6, Phe α-CH), 4.71 (2H, q, *J* 8.7, CH_2CF_3), 5.00 (2H, apparent s, PhC*H*$_2$O), 7.19–7.33 (10H, m, 2 × Ph), 7.41 (1H, br t, *J* ≈7, Gly NH), 8.46 (1H, d, *J* 7.5, Phe NH).

Z-Gly-Gly-L-Phe-OMe. Mp 98–101 °C (from MeOH), $[\alpha]_D^{25}$ +8.0 (*c* 1.0, MeOH) (lit.,[14] mp 98–101 °C); δ_H 2.84–3.06 (2H, m, Phe β-CH_2), 3.57 (3H, s, OMe), 3.69–3.87 (4H, m, 2 × Gly CH_2), 4.46 (1H, q-like, *J* ≈7, Phe α-CH), 5.01 (2H, s, PhC*H*$_2$O), 7.18–7.30 (10H, m, 2 × Ph), 7.49 (1H, br t, *J* ≈6, Gly NH), 8.04 (1H, br t, *J* ≈6, Gly NH), 8.31 (1H, br d, *J* ≈8, Phe NH).

Z-Gly-Gly-L-Phe-OTfe. Mp 108–110 °C (from $CHCl_3$–petroleum spirit), $[\alpha]_D^{25}$ +21.6 (*c* 1.0, $CHCl_3$); δ_H 2.89–3.16 (2H, m, Phe β-CH_2), 3.61–3.77 (4H, m, 2 × Gly CH_2), 4.42 (2H, AB q, *J* 18, CH_2CF_3), 4.54–4.60 (1H, m, Phe α-CH), 5.00 (2H, s, PhC*H*$_2$O), 7.19–7.33 (10H, m, 2 × Ph), 7.48 (1H, br t, *J* ≈6, Gly NH), 8.10 (1H, br t, *J* ≈6, Gly NH), 8.38 (1H, br d, *J* ≈7, Phe NH).

Z-L-Phe-L-Phe-OTfe. Mp 165–165.5 °C (from EtOAc–petroleum spirit), $[\alpha]_D^{25}$ +13.9 (*c* 1.0, $CHCl_3$); δ_H ($CDCl_3$) 2.94–3.13 (4H, m, 2 × Phe β-CH_2), 4.35–4.51 (3H, m, Phe α-CH + CH_2CF_3), 4.83 (1H, q, *J* 6.6, Phe α-CH), 5.07 (2H, s, PhC*H*$_2$O), 5.22 (1H, br, NH), 6.22 (1H, br d, *J* ≈7, NH), 6.95–7.39 (15H, m, 3 × Ph).

Z-Gly-DL-Phe(2Br)-OMe. Mp 110–110.5 °C (from EtOAc–petroleum spirit); δ_H 2.94–3.25 [2H, m, Phe(2Br) β-CH_2], 3.58 (3H, s, OMe), 3.49–3.66 (2H, m, Gly CH_2), 4.52–4.60 [1H, m, Phe(2Br) α-CH], 5.01 (2H, apparent s, PhC*H*$_2$O), 7.14–7.59 (11H, m, ArH), 7.38 (1H, t, *J* 6.9, Gly NH), 8.43 [1H, d, *J* 8.1, Phe(2Br) NH].

Preparation of *N*-protected peptide carbamoylmethyl esters

The Cam esters of *N*-protected peptides were prepared mainly through the coupling of an *N*-protected peptide with an amino acid Cam ester hydrobromide (prepared through the debenzyloxycarbonylation of the *N*-Z-amino acid Cam ester; used in the presence of an equimolar amount of TEA) by the EDC–HOBT method in DMF in a similar manner to that described above for the preparation of other esters (85–94% yield). They were also prepared *via* the reaction of the Cs salt of an *N*-protected peptide with 2-chloroacetamide. The samples prepared by both routes were identical in terms of their physical properties including the $[\alpha]_D$-value. The preparation of Z-L-Phe-L-Ala-OCam by the latter route is shown below as a typical example. To a solution of Z-L-Phe-L-Ala (3.72 g, 10 mmol) in MeOH (60 ml) was added aq. Cs_2CO_3 (1.63 g, 5 mmol in 7.5 ml), and the mixture was evaporated under reduced pressure. After repeated evaporation to dryness with toluene, the residue was stored over P_4O_{10} in a vacuum desiccator. Z-L-Phe-L-Ala-OCs thus obtained was mixed with 2-chloroacetamide (0.94 g, 10 mmol) in DMF (40 ml) and the mixture was stirred at 60 °C overnight. The reaction mixture was partitioned between EtOAc and water, and the aqueous phase was extracted further with EtOAc, and the combined organic extracts were washed successively with 1 M aq. $NaHCO_3$ and water and dried over Na_2SO_4. Evaporation of the solvent afforded white crystals, which were recrystallised from EtOAc–petroleum spirit; yield 2.66 g (64%); mp 153–154 °C, $[\alpha]_D^{25}$ −15.0 (*c* 1.0, DMF); δ_H 1.34 (3H, d, *J* 7.2, Ala Me), 2.68–3.02 (2H, m, Phe β-CH_2), 4.24–4.32 (1H, m, Phe α-CH), 4.37 (1H, quintet, *J* 7.2, Ala α-CH), 4.43 (2H, s, OCH_2CO), 4.92 (2H, apparent s, PhC*H*$_2$O), 7.10–7.34 (12H, m, 2 × Ph + NH_2), 7.52 (1H, d, *J* 8.1, Phe NH), 8.60 (1H, d, *J* 7.2, Ala NH). On the other hand, the sample prepared through the coupling of Z-L-Phe with L-Ala-OCam showed mp 153.5–154 °C and $[\alpha]_D^{25}$ −15.4 (*c* 1.0, DMF).

Z-Gly-L-Ala-OCam. Mp 135–137 °C (from EtOAc–petroleum spirit), $[\alpha]_D^{25}$ −18.6 (*c* 1.0, DMF); δ_H 1.30 (3H, d, *J* 7.2, Ala Me), 3.56–3.70 (2H, m, Gly CH_2), 4.36 (1H, quintet, *J* 7.2, Ala α-CH), 4.42 (2H, s, OCH_2CO), 5.01 (2H, apparent s, PhC*H*$_2$O), 7.29–7.35 (7H, m, Ph + NH_2), 7.43 (1H, t, *J* 6.0, Gly NH), 8.36 (1H, d, *J* 7.2, Ala NH).

Z-Gly-L-Phe-OCam. Mp 141–142.5 °C (from EtOAc–petroleum spirit), $[\alpha]_D^{25}$ −9.6 (*c* 1.0, DMF); δ_H 2.88–3.17 (2H, m, Phe β-CH_2), 3.52–3.67 (2H, m, Gly CH_2), 4.43 (2H, AB q, *J* 13, OCH_2CO), 4.55–4.62 (1H, m, Phe α-CH), 5.00 (2H, s, PhC*H*$_2$O), 7.19–7.33 (12H, m, 2 × Ph + NH_2), 7.41 (1H, t, *J* 6.9, Gly NH), 8.35 (1H, d, *J* 7.8, Phe NH).

Z-Gly-Gly-L-Phe-OCam. Mp 170–172 °C (from propan-2-ol–petroleum spirit), $[\alpha]_D^{25}$ −6.8 (*c* 1.0, DMF); δ_H 2.87–3.16 (2H, m, Phe β-CH_2), 3.61–3.77 (4H, m, 2 × Gly CH_2), 4.42 (2H, AB q, *J* 17, OCH_2CO), 4.54–4.61 (1H, m, Phe α-CH), 5.00 (2H, s, PhC*H*$_2$O), 7.19–7.34 (12H, m, 2 × Ph + NH_2), 7.47 (1H, br t, *J* ≈6, Gly NH), 8.08 (1H, br t, *J* ≈6, Gly NH), 8.37 (1H, d, *J* 8.1, Phe NH).

Z-L-Phe-L-Phe-OCam. Mp 156–158 °C (from EtOAc–petroleum spirit), $[\alpha]_D^{25}$ −32.3 (*c* 1.0, MeOH); δ_H 2.63–3.19 (4H, m, 2 × Phe β-CH_2), 4.23–4.30 (1H, m, Phe α-CH), 4.42 (2H, AB q, *J* 15, OCH_2CO), 4.57–4.63 (1H, m, Phe α-CH), 4.91 (2H, apparent s, PhC*H*$_2$O), 7.17–7.35 (17H, m, 3 × Ph + NH_2), 7.49 (1H, d, *J* 9.0, NH), 8.35 (1H, d, *J* 7.5, NH).

Z-Gly-DL-Phe(2Br)-OCam. Mp 153–154.5 °C (from acetone–petroleum spirit); δ_H 2.98–3.34 [2H, m, Phe(2Br) β-CH_2], 3.52–3.66 (2H, m, Gly CH_2), 4.45 (2H, AB q, *J* 15, OCH_2CO), 4.62–4.71 [1H, m, Phe(2Br) α-CH], 5.01 (2H, apparent s, PhC*H*$_2$O), 7.15–7.59 (12H, m, Ar + Gly NH + NH_2), 8.47 [1H, d, *J* 7.5, Phe(2Br) NH].

Preparation of authentic *N*-protected peptide amides

The authentic samples of *N*-protected peptide amides were prepared through the coupling of an *N*-protected amino acid or Z-Gly-Gly with a dipeptide amide hydrochloride or hydrobromide (in the presence of an equimolar amount of TEA) by the EDC–HOBT method in DMF (65–88% yield) as described previously for the preparation of *N*-protected dipeptide amides.[4] On the other hand, the mixtures of epimers (L-L + D-L or L-L-L + L-D-L) of tripeptides Z-Xaa-Xbb-Leu-NH_2 (Xaa = Gly or Phe; Xbb = Ala or Phe) were prepared through the coupling of Z-Xaa-L-Xbb with L-Leu-NH_2 (in the form of its hydrochloride with an equimolar amount of TEA) using EDC·HCl in DMF (L-Xbb is prone to racemisation under these reaction conditions). A mixture of epimers (L-L + D-L) of Z-Gly-Gly-Phe-Leu-NH_2 was prepared in the same manner. Each sample thus prepared showed only two main peaks, corresponding to both the epimers on reversed-phase HPLC. The faster

eluting epimer proved to be L-L or L-L-L by comparison with an authentic sample.

Z-Gly-L-Ala-L-Leu-NH$_2$. Mp 164–165 °C (from MeOH–petroleum spirit), $[\alpha]_D^{25}$ −22.5 (*c* 1.0, DMF); δ_H 0.82 and 0.86 (6H, 2 d, *J* 6.0, Leu Me$_2$), 1.20 (3H, d, *J* 6.0, Ala Me), 1.46 (2H, t-like, *J* 6.6, Leu β-CH$_2$), 1.49–1.61 (1H, m, Leu γ-CH), 3.56–3.69 (2H, m, Gly CH$_2$), 4.12–4.29 (2H, m, Ala α-CH + Leu α-CH), 5.01 (2H, s, PhC*H$_2$*O), 6.97 and 7.13 (2H, 2 s, NH$_2$), 7.28–7.38 (5H, m, Ph), 7.50 (1H, t, *J* 6.0, Gly NH), 7.79 (1H, d, *J* 8.7, Leu NH), 8.09 (1H, d, *J* 6.0, Ala NH).

Z-L-Phe-L-Ala-L-Leu-NH$_2$. Mp 202 °C (decomp.) (from MeOH–petroleum spirit), $[\alpha]_D^{25}$ −15.8 (*c* 1.0, DMF); δ_H 0.83 and 0.86 (6H, 2 d, *J* 6.6, Leu Me$_2$), 1.22 (3H, d, *J* 7.2, Ala Me), 1.44 (2H, t-like, *J* 7.2, Leu β-CH$_2$), 1.51–1.63 (1H, m, Leu γ-CH), 2.66–3.02 (2H, m, Phe β-CH$_2$), 4.17–4.30 (3H, m, Ala α-CH + Leu α-CH + Phe α-CH), 4.92 (2H, apparent s, PhC*H$_2$*O), 6.97 (1H, s, NH$_2$), 7.16–7.34 (11H, m, 2 × Ph + NH$_2$), 7.51 (1H, d, *J* 8.4, Phe NH), 7.76 (1H, d, *J* 8.4, Leu NH), 8.19 (1H, d, *J* 7.5, Ala NH).

Z-Gly-L-Phe-L-Leu-NH$_2$. Mp 205–206.5 °C (from MeOH–petroleum spirit), $[\alpha]_D^{25}$ −18.8 (*c* 1.0, DMF); δ_H 0.81 and 0.86 (6H, 2 d, *J* 6.3, Leu Me$_2$), 1.43–1.61 (3H, m, Leu β-CH$_2$ + Leu γ-CH), 2.73–3.03 (2H, m, Phe β-CH$_2$), 3.46–3.67 (2H, m, Gly CH$_2$), 4.19 (1H, q-like, Leu α-CH), 4.46–4.54 (1H, m, Phe α-CH), 4.99 (2H, apparent s, PhC*H$_2$*O), 6.98 and 7.09 (2H, 2 s, NH$_2$), 7.15–7.34 (10H, m, 2 × Ph), 7.43 (1H, t, *J* 4.5, Gly NH), 7.97 (1H, d, *J* 8.4, Leu NH), 8.07 (1H, d, *J* 7.8, Phe NH).

Z-L-Phe-L-Phe-L-Leu-NH$_2$. Mp 235–235.5 °C (from MeOH), $[\alpha]_D^{25}$ −29.3 (*c* 1.0, DMF); δ_H 0.82 and 0.87 (6H, 2 d, *J* 6.3, Leu Me$_2$), 1.42–1.62 (3H, m, Leu β-CH$_2$ + Leu γ-CH), 2.61–3.08 (4H, m, 2 × Phe β-CH$_2$), 4.19–4.24 (2H, m, Leu α-CH + Phe α-CH), 4.51–4.58 (1H, m, Phe α-CH), 4.91 (2H, apparent s, PhC*H$_2$*O), 6.99 and 7.11 (2H, 2 s, NH$_2$), 7.19–7.32 (15H, m, 3 × Ph), 7.46 (1H, d, *J* 8.7, Leu NH), 7.97 (1H, d, *J* 8.4, Phe NH), 8.16 (1H, d, *J* 8.1, Phe NH).

Z-Gly-(L/D)-Phe(2Br)-L-Leu-NH$_2$. (mixture of L-L and D-L isomers). Mp 196.5–198.5 °C (from MeOH–diethyl ether–petroleum spirit), $[\alpha]_D^{25}$ −29.2 (*c* 1.0, DMF).

Z-Gly-Gly-L-Phe-L-Leu-NH$_2$. Mp 195–196 °C (from MeOH–petroleum spirit), $[\alpha]_D^{25}$ −18.1 (*c* 1.0, DMF); δ_H 0.81 and 0.86 (6H, 2 d, *J* 6.5, Leu Me$_2$), 1.42–1.60 (3H, m, Leu β-CH$_2$ + Leu γ-CH), 2.71–3.06 (2H, m, Phe β-CH$_2$), 3.53–3.76 (4H, m, 2 × Gly CH$_2$), 4.14–4.23 (1H, m, Leu α-CH), 4.44–4.51 (1H, m, Phe α-CH), 5.01 (2H, s, PhC*H$_2$*O), 6.96 and 7.07 (2H, 2 s, NH$_2$), 7.13–7.34 (10H, m, 2 × Ph), 7.48 (1H, t, *J* 4.5, Gly NH), 7.93 (1H, d, *J* ≈8, Leu NH), 8.07 (1H, d, *J* ≈8, Phe NH), 8.08 (1H, t-like, Gly NH).

α-Chymotrypsin-catalysed segment condensations to tri- or tetrapeptides

The preparation of Z-L-Phe-L-Phe-L-Leu-NH$_2$ is described as a typical example. A mixture of Z-L-Phe-L-Phe-OCam (25 mg, 0.05 mmol), L-Leu-NH$_2$·HCl (33 mg, 0.2 mmol), TEA (28 µl, 0.2 mmol) and the immobilised enzyme on Celite (150 mg, corresponding to 4.7 mg of α-chymotrypsin) was incubated with shaking (180 strokes min^{-1}) in a solvent composed of acetonitrile (2 ml) and 0.05 M Tris buffer (pH 7.8) (83 µl) at 30 °C. An aliquot (10 µl) of the reaction mixture was withdrawn periodically, diluted with AcOH (100 µl), and analysed by HPLC. The targeted peptide and its epimer (Z-L-Phe-D-Phe-L-Leu-NH$_2$), the remaining donor ester and possible by-products (Z-L-Phe-L-Phe, Z-L-Phe and Z-L-Phe-L-Leu-NH$_2$) were quantified by HPLC analysis on an ODS column (see below).

Preparation of samples related to the synthesis of the Leu-enkephalin sequence

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OCam. This was prepared by the following two routes. (i) Boc-L-Tyr(Bzl)-Gly-Gly[2b] was coupled with L-Phe-OCam·HBr (in the presence of an equimolar amount of TEA) by the EDC–HOBT method in DMF; 79% yield; mp 190–191 °C (from acetone), $[\alpha]_D^{25}$ −10.9 (*c* 1.0, MeOH). Or (ii) Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe[2b] was converted to the Cs salt and treated with 2-chloroacetamide in DMF at 60 °C overnight as described above, and the crude product was purified by recrystallisation from acetone; 65% yield; mp 190–191 °C, $[\alpha]_D^{25}$ −11.2 (*c* 1.0, MeOH); δ_H 1.18 and 1.27 (9H, 2 s, Me$_3$CO), 2.61–3.17 (4H, m, Tyr β-CH$_2$ + Phe β-CH$_2$), 3.69–3.73 (4H, m, 2 × Gly CH$_2$), 4.06–4.14 (1H, m, Tyr α-CH), 4.43 (2H, AB q, *J* 16, OCH$_2$CO), 4.53–4.62 (1H, m, Phe α-CH), 5.04 (2H, AB q, *J* 18, PhC*H$_2$*O), 6.89 and 7.16 (4H, 2 d, *J* 8.0, Tyr ArH), 6.95 (1H, d, *J* 7.2, Tyr NH), 7.20–7.44 (12H, m, 2 × Ph + NH$_2$), 8.05 (1H, br t, *J* ≈6, Gly NH), 8.21 (1H, br t, *J* ≈6, Gly NH), 8.39 (1H, d, *J* 7.8, Phe NH).

Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-L-Leu-NH$_2$. This authentic sample was prepared by the following two routes. (i) Boc-L-Tyr(Bzl)-Gly-Gly was coupled with L-Phe-L-Leu-NH$_2$·HCl (in the presence of an equimolar amount of TEA) by the EDC–HOBT method in DMF; 51% yield. Or (ii) Boc-L-Tyr(Bzl) was coupled with Gly-Gly-L-Phe-L-Leu-NH$_2$ (prepared by hydrogenolysis of Z-Gly-Gly-L-Phe-L-Leu-NH$_2$ with 5% Pd/C in MeOH) by the EDC–HOBT method in DMF; 89% yield; mp 165.5–167 °C (from aq. EtOH), $[\alpha]_D^{25}$ − 15.7 (*c* 1.0, DMF); δ_H 0.82 and 0.87 (6H, 2 d, *J* 6.5, Leu Me$_2$), 1.18 and 1.27 (9H, 2 s, Me$_3$CO), 1.42–1.63 (3H, m, Leu β-CH$_2$ + Leu γ-CH), 2.60–2.95 (2H, m, Tyr β-CH$_2$), 2.72–3.04 (2H, m, Phe β-CH$_2$), 3.57–3.71 (4H, m, 2 × Gly CH$_2$), 4.06–4.14 (1H, m, Tyr α-CH), 4.18 (1H, apparent q, *J* ≈7, Leu α-CH), 4.46–4.53 (1H, m, Phe α-CH), 5.03 (2H, s, PhC*H$_2$*O), 6.89 and 7.16 (4H, 2 d, *J* 8.4, Tyr ArH), 6.92 (1H, d, *J* ≈9, Tyr NH), 6.99 and 7.10 (2H, 2 s, NH$_2$), 7.20–7.41 (10H, m, 2 × Ph), 7.97 (1H, d, *J* 8.1, Leu NH), 8.02 (1H, br t, *J* ≈5.5, Gly NH), 8.09 (1H, d, *J* 7.8, Phe NH), 8.19 (1H, br t, *J* ≈5.5, Gly NH); MALDI-TOF MS [Found: *m*/*z*, 767.34. $(C_{40}H_{52}N_6O_8 + Na)^+$ requires *m*/*z*, 767.37].

Boc-L-Tyr(Bzl)-Gly-Gly-D-Phe-L-Leu-NH$_2$. This authentic sample was prepared through the coupling of Boc-L-Tyr(Bzl)-Gly-Gly with D-Phe-L-Leu-NH$_2$·HBr (prepared through the treatment of Boc-D-Phe-L-Leu-NH$_2$ with 25% HBr in AcOH), in the presence of an equimolar amount of TEA, by the EDC–HOBT method in DMF; 45% yield; mp 196.5–198 °C (from aq. EtOH), $[\alpha]_D^{25}$ −6.0 (*c* 1.0, DMF; MALDI-TOF MS [Found: *m*/*z*, 767.35. $(C_{40}H_{52}N_6O_8 + Na)^+$ requires *m*/*z*, 767.37].

Boc-L-Tyr(Bzl)-L-Leu-NH$_2$. This authentic sample was prepared through the coupling of Boc-L-Tyr(Bzl) with L-Leu-NH$_2$·HCl (in the presence of an equimolar amount of TEA) by the EDC–HOBT method in DMF; 58% yield; mp 183–183.5 °C (from CHCl$_3$), $[\alpha]_D^{25}$ −11.0 (*c* 1.0, DMF); δ_H 0.82 and 0.86 (6H, 2 d, *J* 6.6, Leu Me$_2$), 1.23 and 1.30 (9H, 2 s, Me$_3$CO), 1.42–1.63 (3H, m, Leu β-CH$_2$ + Leu γ-CH), 2.62–2.91 (2H, m, Tyr β-CH$_2$), 4.02–4.11 (1H, m, Tyr α-CH), 4.20–4.27 (1H, m, Leu α-CH), 5.04 (2H, s, PhC*H$_2$*O), 6.89 and 7.15 (4H, 2 d, *J* 8.4, Tyr ArH), 6.92 (1H, d, *J* ≈9, Tyr NH), 6.99 and 7.20 (2H, 2 s, NH$_2$), 7.28–7.43 (5H, m, Ph), 7.81 (1H, d, *J* 8.1, Leu NH).

Boc-L-Tyr-Gly-Gly-L-Phe. This was prepared by hydrogenolysis of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe with 5% Pd/C in MeOH; quantitative yield; mp 153–155 °C, $[\alpha]_D^{25}$ +23.6 (*c* 1.0, MeOH); δ_H 1.20 and 1.28 [9H, 2 s (15 : 85), Me$_3$CO], 2.57–3.07 (4H, m, Tyr β-CH$_2$ + Phe β-CH$_2$), 3.67–3.71 (4H, m, 2 × Gly CH$_2$), 4.03–4.10 (1H, m, Tyr α-CH), 4.32–4.39 (1H, m, Phe α-CH), 6.62 and 7.01 (4H, 2 d, *J* 8.4, Tyr ArH), 6.88 (1H, d,

J 8.1, Tyr NH), 7.17–7.27 (5H, m, Ph), 7.94 (1H, br t, J ≈6, Gly NH), 8.03 (1H, d, J 7.8, Phe NH), 8.14 (1H, t, J 5.4, Gly NH), 9.14 (1H, s, ArOH), 12.6 (1H, br, COOH).

Boc-L-Tyr-Gly-Gly-L-Phe-OCam. This was prepared by hydrogenolysis of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OCam with 5% Pd/C in DMF; 30% yield; mp 177–179 °C (from $CHCl_3$–EtOAc–petroleum spirit), $[\alpha]_D^{25}$ −8.8 (c 1.0, DMF); δ_H 1.18 and 1.27 (9H, 2 s, Me_3CO), 2.56–3.17 (4H, m, Tyr β-CH_2 + Phe β-CH_2), 3.67–3.73 (4H, m, 2 × Gly CH_2), 4.06–4.14 (1H, m, Tyr α-CH), 4.42 (2H, AB q, J 15.5, OCH_2CO), 4.53–4.60 (1H, m, Phe α-CH), 6.61 and 7.01 (4H, 2 d, J 7.0, Tyr ArH), 6.89 (1H, d, J 7.5, Tyr NH), 7.18–7.33 (7H, m, Ph, NH_2), 8.01 (1H, br t, J ≈6, Gly NH), 8.15 (1H, br t, J ≈6, Gly NH), 8.35 (1H, d, J ≈8, Phe NH), 9.13 (1H, s, OH). When the deprotection by hydrogenolysis of the Bzl group from the protected Cam ester was conducted in MeOH, Boc-L-Tyr-Gly-Gly-L-Phe-OMe was obtained instead.

Boc-L-Tyr-Gly-Gly-L-Phe-L-Leu-NH_2. This was prepared by hydrogenolysis of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-L-Leu-NH_2 with 5% Pd/C in MeOH; 81% yield; mp 162–164.5 °C (from aq. EtOH), $[\alpha]_D^{25}$ −17.7 (c 1.0, DMF); δ_H 0.82 and 0.87 (6H, 2 d, J 6.2, Leu Me_2), 1.20 and 1.28 [9H, 2 s (2 : 98), Me_3CO], 1.43–1.63 (3H, m, Leu β-CH_2 + Leu γ-CH), 2.57–3.05 (4H, m, Tyr β-CH_2 + Phe β-CH_2), 3.58–3.7l (4H, m, 2 × Gly CH_2), 4.03–4.10 (1H, m, Tyr α-CH), 4.19 (1H, apparent q, J ≈7, Leu α-CH), 4.47–4.54 (1H, m, Phe α-CH), 6.62 and 7.01 (4H, 2 d, J 7.8, Tyr ArH), 6.87 (1H, d, J 8.1, Tyr NH), 6.96 and 7.08 (2H, 2 s, NH_2), 7.16–7.24 (5H, m, Ph), 7.97 (1H, d, J ≈8, Leu NH), 7.98 (1H, apparent t, Gly NH), 8.08 (1H, d, J 7.5, Phe NH), 8.15 (1H, br t, J ≈6, Gly NH), 9.14 (1H, br, OH).

Synthesis of the Leu-enkephalin sequence *via* the α-chymotrypsin-catalysed segment condensation

The α-chymotrypsin-catalysed coupling of Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe-OCam (34 mg, 0.05 mmol) with L-Leu-NH_2·HCl (33 mg, 0.2 mmol) in the presence of an equimolar amount of TEA was done in the same manner as described above. The targeted peptide and its epimer, the remaining donor ester and possible by-products [Boc-L-Tyr(Bzl)-Gly-Gly-L-Phe, Boc-L-Tyr(Bzl) and Boc-L-Tyr(Bzl)-L-Leu-NH_2] were quantified by HPLC analysis (see below). The coupling of Boc-L-Tyr-Gly-Gly-L-Phe-OCam with L-Leu-NH_2 was likewise undertaken.

HPLC analyses

The liquid chromatograph employed was a GL Sciences PU-610 instrument equipped with a Rheodyne 8125 sample injector, a GL Sciences UV-620 variable-wavelength UV monitor and a Shimadzu C-R6A data processor. HPLC analyses were undertaken under the following conditions: column, Inertsil ODS 3 (5 µm; 1.5 mm i.d. × 150 mm, GL Sciences); mobile phase, 42–54% aq. acetonitrile containing H_3PO_4 (0.01 M) or 50–65% aq. MeOH containing H_3PO_4 (0.01 M); flow rate, 0.1 ml min^{-1}; column temperature, 30, 35 or 40 °C; detection, UV at 254 nm. The epimers (L-L and D-L, or L-L-L and L-D-L) of the resulting peptides were also separated well on the same column by decreasing the amount of acetonitrile or MeOH in the mobile phase. The HPLC separation of compounds relevant to the α-chymotrypsin-catalysed coupling of Z-L-Phe-L-Phe-OR with L-Leu-NH_2 is shown in Table 2. The HPLC separation of compounds relevant to the synthesis of the Leu-enkephalin sequence is also available as Supplementary material. †

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