Studies of Unusual Amino Acids and Their Peptides. XVII.[1,2] The Synthesis of Peptides Containing *N*-Carboxymethyl Amino Acids. II[3,4]

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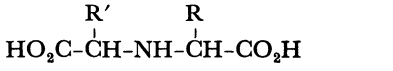
The synthetic routes were investigated to four kinds of tetrapeptides made up of three usual amino acid residues and one *N*-carboxymethyl (Cm-) amino acid residue. The application of vacuum distillation made the isolation of a Cm-amino acid diester more convenient and efficient compared with the chromatographic methods which had been used previously. The efficiency of peptide bond formation at the imino group of a Cm-amino acid by the acid chloride method was remarkably improved under suitable reaction conditions. In the elongation of the peptide chain from a peptide containing a Cm-amino acid at the C-terminal position, the coupling efficiency was usually poorer than that in the case of the corresponding peptide composed only of the usual amino acid residues, and it depended greatly on the coupling methods, the 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide–1-hydroxybenzotriazole method being generally the most desirable.

In our previous publications,[3,5,6] we have reported on the preparation of *N*-carboxymethyl (Cm-) amino acids (**I**, R′=H) and their derivatives using the corresponding usual amino acids as the starting materials. Cm-amino acids are simple-structured members of a group of unusual amino acids represented by the general structural formula of **I**.



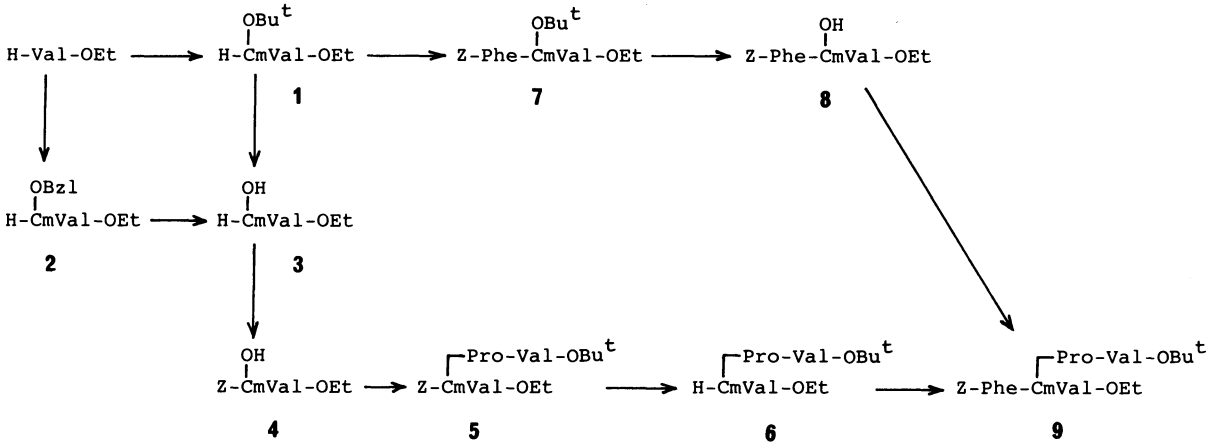
I

These amino acids (**I**) have been found in increasing numbers in nature, *e.g.*, in many marine animals and in crown gall tumors, and have recently been receiving more attention.[7,8] Furthermore, a series of synthetic peptides containing substituted Cm-amino acids have recently been demonstrated to be active as inhibitors of angiotensin-converting enzyme,[9] thermolysin,[10] enkephalinase,[11] and malate dehydrogenase.[12]

In Part I[3] of this series, we dealt with some basic problems in the synthesis of peptides containing Cm-amino acids, *e.g.*, the protection of the carboxyl and the imino groups of a Cm-amino acid, and the peptide bond formation at its carboxyl groups and imino group. As an extension of this work, the present paper will deal with the synthetic routes to four kinds of tetrapeptides (**9**, **15**, **19**, and **20**) made up of three usual amino acid residues (Pro, Val, and Phe) and one Cm-amino acid residue (CmVal),[13] the latter occupying four different positions in the peptide chains. These tetrapeptides were intended as the starting materials for a cyclic peptide model containing a Cm-amino acid.[14] The peptide chains were lengthened mainly from the dipeptide (**7**), which contained the Cm-amino acid at the C-terminal position and was protected by three kinds of selectively removable groups. Such a way of peptide chain elongation has not yet been attempted, and it has often been demonstrated that the efficiency of peptide bond elongation from a peptide containing a Cm-amino acid residue as either the amino or the carboxyl component is much poorer than that in the case of the corresponding usual peptide.

Preparation of Tetrapeptide **9**. According to the route adopted in the previous study,[3] Z-CmVal-OEt (**4**) was coupled with H-Pro-Val-OBu^t by the DCC, DCC–HOBt, or EDC method, to afford the tripeptide **5** (Scheme 1). For this purpose, **4** was prepared first *via* the H-Val-OEt→**2**→**3**→**4** route,[3] and later *via* a new route, H-Val-OEt→**1**→**3**→**4**. The debenzyloxycarbonylation of **5** was accomplished by hydrogenation, using Pd on carbon as the catalyst in the absence of any acid. It is noteworthy that the resulting product, **6**, though having a free imino group, was stable at room temperature for a long period of time, like the diesters of Cm-amino acids.[5] Then, **6** was coupled with Z-Phe-OH by the acid chloride method. Though the coupling efficiency by this method had generally been far from satisfactory,[3] the improvement of the procedure described below gave the tetrapeptide **9** in a fairly good yield (60%).

As an alternative to the desired tetrapeptide (**9**), the route *via* the dipeptide **7**, which contains CmVal as the C-terminal residue and is useful as a key compound for the preparation of two other tetrapeptides (**15** and **19**) as well, was examined next. For



Scheme 1.

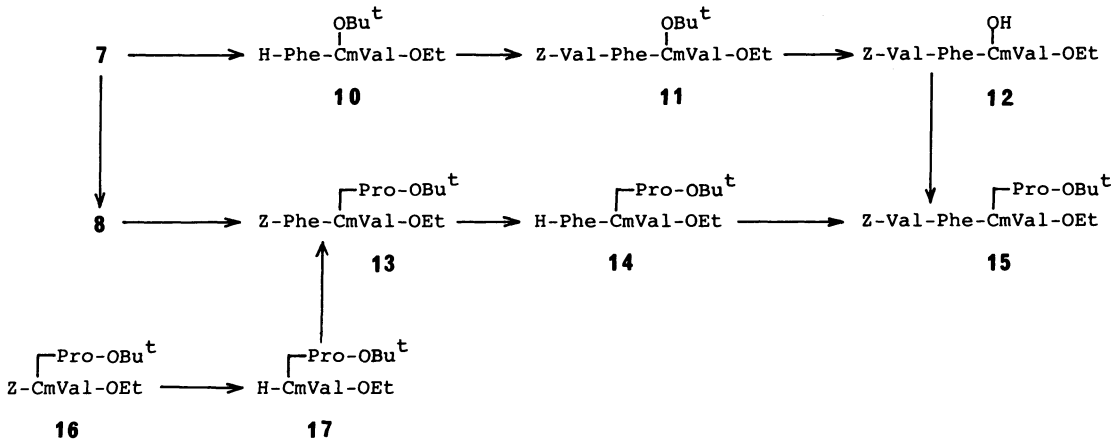
that purpose, the Cm-valine ethyl *t*-butyl ester **1** was prepared according to the general procedure described previously,[5] using ethyl valinate and *t*-butyl bromoacetate in the presence of triethylamine (TEA). Though the purification of Cm-amino acid diesters had hitherto been carried out by silica-gel column chromatography or TLC,[5] **1** was found to be isolable by means of vacuum distillation, thus making it possible to prepare such Cm-amino acid diesters as **1** more conveniently and in quantity.

As stated in the previous papers[3,5] the imino group of a Cm-amino acid has a remarkably poor reactivity, showing that the attempted couplings of Z-Phe-OH with a diester of Cm-valine by several conventional condensation methods all ended in failure, except by the acid chloride method. The coupling efficiency by the last method, however, was rather poor (*ca.* 40%). Recently we have attempted to overcome this difficulty by the use of the high pressure technique in the couplings of Z-amino acids with Cm-amino acid diesters by means of the *N*-hydroxysuccinimide ester method.[16] The application of high pressure (10 kbar) enabled some of the coupling reactions to be carried out so as to give much higher yields than at atmospheric pressure, while the coupling yields remained low in some reactions between relatively bulky amino acid residues. Moreover, a large-scale preparation at high pressure must be attended with some difficulties under the present conditions. Accordingly, the optimum reaction conditions for the coupling by the acid chloride method was investigated in the reaction of Z-Phe-Cl with the Cm-valine diester **1**; it was revealed that the best yield of **7** (84%) was obtained by the use of 2 to 3 equivalents of Z-Phe-Cl and by conducting the reaction at 0 °C from beginning to end instead of at 0 °C for several hours and thereafter at room temperature.[3]

The removal of the *t*-butyl group from **7** was carried out by treating **7** with hydrogen chloride in ethyl acetate for a few hours, without causing any damage to the coexistent Z group. Then, as an attempt to elongate the peptide chain from a C-terminal Cm-amino acid residue, the de-*t*-butylated product **8** was coupled with H-Pro-Val-OBu^t by means of the EDC or EDC–HOBt method. The yield of the reaction, **8**→**9**, (45%) as conducted according to the EDC method, was poorer than that of the corresponding reaction, **4**→**5**, (66%) under comparable conditions. This illustrates the large steric hindrance of a peptide containing a Cm-amino acid compared with that of a Cm-amino acid itself. On the other hand, the application of the EDC–HOBt method to the present coupling gave **9** in a good yield (80%). The superiority of this coupling method seems to be a general trend in the preparation of peptides containing Cm-amino acids, as will be indicated below by several examples.

The optical rotation of the product (**9**) obtained from **8** and H-Pro-Val-OBu^t by the EDC or EDC–HOBt method was identical with that of the product obtained *via* the first-mentioned route (**4**→**5**→**6**→**9**). No racemization occurs during the segment coupling at the carboxyl group in the glycine moiety of a Cm-amino acid residue, as might be expected. On the other hand, when we compare the overall yields of the tetrapeptide **9** starting from ethyl valinate *via* the two routes mentioned above, it is apparent that the route *via* the dipeptide **7** is more desirable, so much the more because the route *via* Z-CmVal-OEt (**4**) contains more steps (H-Val-OEt→**2**→**3**→**4** or H-Val-OEt→**1**→**3**→**4**).

Preparation of Tetrapeptide 15. For the preparation of the tetrapeptide **15**, the following two routes were examined starting from the dipeptide **7** mentioned above: (i) **7**→**10**→**11**→**12**→**15** and (ii)



Scheme 2.

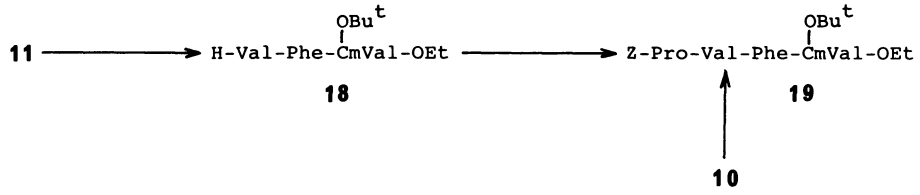
7→8→13→14→15 (Scheme 2). The elongation of the peptide chain at the N-terminal residue of a peptide containing a C-terminal Cm-amino acid residue has not previously been attempted. In the (i) route, the Z group of **7** was first removed, and the resulting product (**10**) was coupled with Z-Val-OH by several methods. Generally, the coupling efficiency was less satisfactory than that of the corresponding coupling, Z-Val-OH+H-Phe-Val-OBu^t, under comparable conditions (see Experimental). This presumably indicates the extention of the influence of the bulkiness of the Cm-amino acid to the amino group of the adjacent amino acid residue. Thus, the coupling method and conditions had a profound influence on the coupling efficiency. The *N*-hydroxysuccinimide ester method in dioxane gave only a 25% yield after 3 d, while it gave a 46% yield in dichloromethane. The mixed anhydride method and the EDC method gave better yields (45 and 54% respectively). The best yield (70%) was obtained by the EDC-HOBt method. The comparison of the yields of the couplings of Z-Val-OSu and Z-Ala-OSu with **10** in dichloromethane under comparable reaction conditions (46 and 65% respectively) also reveals the influence of the steric hindrance. Then the removal of the *t*-butyl group from **11** was attained as in the **7→8** step to give **12**. The coupling of **12** with H-Pro-OBu^t was carried out by the EDC-HOBt method, thus obtaining the desired tetrapeptide **15** in a 61% yield.

On the other hand, in the (ii) route, **8** was first coupled with H-Pro-OBu^t by means of the EDC-HOBt method to give the tripeptide **13** in a 65% yield. This yield was somewhat better than that of the corresponding coupling of the tripeptide **12** with H-Pro-OBu^t described above. The tripeptide **13** was also prepared *via* another route. The dipeptide **16**[3] was debenzyloxycarbonylated by hydrogenation as usual to give **17**, and this was then coupled with Z-Phe-Cl by the improved procedure described above (77% yield). The product thus obtained was identical with that obtained by the (ii) route. Then the tripeptide **13** was debenzyloxycarbonylated by hydrogenation, and the resulting **14** was coupled with Z-Val-OH by several methods. The yields were 34% for the *N*-hydroxysuccinimide ester method, 41% for the mixed anhydride method, and 51% for the EDC-HOBt method. These yields were rather poor compared with those of the corresponding coupling of Z-Val-OH with **10**. This presumably reflects the difference in the bulkiness of the two amino components containing the Cm-amino acid (**14** *vs.* **10**).

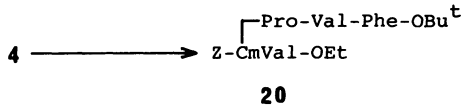
The final products (**15**) obtained *via* the two routes, (i) and (ii), were identical. On the other hand, a comparison of the overall yields (from the dipeptide **7**) of the tetrapeptide **15** obtained by the EDC-HOBt method *via* Routes (i) and (ii) revealed that the (i) route (43%) was somewhat better than the (ii) route (33%).

Preparation of Tetrapeptides 19 and 20. As is shown in Scheme 3, the tripeptide **11** was debenzyloxycarbonylated by hydrogenation to give **18**, which was then coupled with Z-Pro-OH by several different methods. The yields were 26% for the *N*-hydroxysuccinimide ester method, 44% for the mixed anhydride method, and 77% for the EDC-HOBt method. In this case also, the last method was the most desirable one. The yields were generally comparable with those of the preceeding step, **7→10→11**. Moreover, there was a close resemblance in the relationship between the coupling method and the coupling efficiency in both cases.

The tetrapeptide **19** was also prepared in a 59%



Scheme 3.



Scheme 4.

yield by the coupling of Z-Pro-Val-OH with **10** by the EDC-HOBt method. This yield was poorer than that of the corresponding coupling of Z-Val-OH with **10**, but the overall yield *via* the **7**→**10**→**19** route (segment coupling) was somewhat better than that *via* the **7**→**10**→**11**→**18**→**19** route (stepwise elongation). The optical rotation of the product obtained by the segment coupling was identical with that of the product obtained by the stepwise elongation, indicating that no racemization occurred.

The EDC coupling of Z-CmVal-OEt (**4**) with H-Pro-Val-Phe-OBu^t, which had been prepared by the stepwise elongation starting from the C-terminal phenylalanine, gave the tetrapeptide **20** in a 55% yield (Scheme 4). This yield was better than that reported previously[3] of the corresponding coupling of **4** with H-Pro-Val-Val-OBzl by the DCC method (37%).

In conclusion, the results obtained in the present work indicate that, in the elongation of the peptide chain from a peptide containing a Cm-amino acid residue, the coupling efficiency is generally poor compared with that in the case of the corresponding peptide composed only of usual amino acid residues, and depends largely on the coupling methods. The EDC-HOBt method is the method of choice.

Experimental

All the melting points are uncorrected. The optical rotations were measured with a JASCO DIP-4 polarimeter. The TLC and preparative TLC were performed on Merck Kieselgel 60 F_{254} and Kieselgel GF_{254} (Type 60) respectively. Merck Kieselgel 60 was used for the column chromatography. The ^{1}H NMR spectra were recorded on a Hitachi R-24B spectrometer.

H-CmVal-OEt (1). To a stirred mixture of H-Val-OEt·TosOH (59.10 g) and TEA (37.96 g) in THF (180 ml) we added, drop by drop, a solution of *t*-butyl bromoacetate[17] (43.58 g) in THF (10 ml) over a period of 2 h. The reaction mixture was then stirred at room temperature for 10 d. The precipitates were subsequently filtered off, and the filtrate was evaporated under reduced pressure. The residue was dissolved in EtOAc, washed with water, and dried over Na_2SO_4. After the solvent had been removed, the residue was distilled under reduced pressure; yield, 29.22 g (61%); oil, bp 85 °C/0.7 Torr (1 Torr=133.322 Pa); $[\alpha]_D^{25}$ −18.0° (*c* 1.0, MeOH); n_D^{22} 1.4345. ^{1}H NMR ($CDCl_3$) δ= 0.96 (6H, d, *J*=6 Hz, $CH(C\underline{H}_3)_2$), 1.26 (3H, t, *J*=7 Hz, $CH_2C\underline{H}_3$), 1.44 (9H, s, $C(C\underline{H}_3)_3$), 1.7—2.2 (1H, m, $C\underline{H}(CH_3)_2$), 2.27 (1H, s, NH), 3.00 (1H, d, *J*=5.5 Hz, $NHC\underline{H}(C_3H_7)CO$), 3.22 (2H, s, $NHC\underline{H}_2CO$), and 4.14 (2H, q, *J*=7 Hz, $C\underline{H}_2CH_3$).

Picrolonate: mp 136.5—138 °C (EtOH); $[\alpha]_D^{25}$ +1.0° (*c* 1.0, MeOH). Found: C, 52.79; H, 6.43; N, 13.35. Calcd for $C_{23}H_{33}N_5O_9$: C, 52.77; H, 6.35; N, 13.38.

DCHA Salt of Z-CmVal-OEt (4). The diester **1** (1.56 g) was treated with 4 M HCl (1 M=1 mol dm^{-3}) in EtOAc (30 ml) at room temperature for 3 h. After the solvent had been removed under reduced pressure, the residue (**3**·HCl) was dissolved in water (11 ml) with $NaHCO_3$ (1.76 g). To the resulting solution, we added a solution of Z-Cl (1.13 g) in ether (2 ml) in 8 portions over a period of 3.5 h. Then the mixture was stirred below 5 °C overnight. After the usual work-up, the raw product was mixed with DCHA (1.09 g) to afford the corresponding salt, which was then recrystallized from hexane; yield, 2.14 g (69%); mp 109.5—110 °C; $[\alpha]_D^{25}$ −40.1° (*c* 1.0, $CHCl_3$). [lit,[3] mp 109.5—110 °C, $[\alpha]_D^{25}$ −39.8° (*c* 1.0, MeOH).]

Z-Pro-Val-OBu^t. This was prepared from Z-Pro-OH and H-Val-OBu^t by the mixed anhydride method using isobutoxycarbonyl chloride in THF; yield, 79%; mp 111—113 °C (EtOAc-petroleum ether); $[\alpha]_D^{23}$ −32.8° (*c* 1.0, DMF). This compound had previously been prepared by the DCC-HOBt method;[18] mp 114—115 °C, $[\alpha]_D^{23}$ −34.3° (*c* 1.0, DMF).

Tripeptide 5. This was prepared from Z-CmVal-OEt (**4**) (recovered from the DCHA salt by treatment in EtOAc with 1 M HCl as usual) and H-Pro-Val-OBu^t (prepared through the debenzyloxycarbonylation of Z-Pro-Val-OBu^t by catalytic hydrogenation in MeOH) by the three different methods described below, according to a previous report.[3] The final purification was performed by preparative TLC on silica gel with benzene-EtOAc (3:1), while recrystallization was done from cyclohexane;

mp 96—97 °C; $[\alpha]_D^{25}$ −107.4° (*c* 1.0, MeOH). Found: C, 63.22; H, 8.23; N, 7.16. Calcd for $C_{31}H_{47}N_3O_8$: C, 63.14; H, 8.03; N, 7.13.

The yields were 54% by the DCC method, 59% by the DCC-HOBt method, and 66% by the EDC method.

Dipeptide **7**. To a stirred solution of **1** (3.65 g) and TEA (1.57 g) in THF (10 ml), we added a solution of Z-Phe-Cl (freshly prepared by the reaction of Z-Phe-OH (10.48 g) with PCl_5 (7.29 g) in ether according to the literature[19]) in THF (20 ml) at 0 °C over a period of 20 min. The mixture was stirred overnight in a thermostatted bath at 0 °C. The precipitates were then filtered off, and the filtrate was evaporated under reduced pressure. The residue was distributed between EtOAc and water, and the separated organic layer was washed successively with 10% citric acid, water, 4% $NaHCO_3$, and water, and dried over Na_2SO_4. After the solvent had been removed under reduced pressure, the residue was purified on a silica-gel column, with hexane-EtOAc (3:1) as the eluent; yield, 6.42 g (84%); syrup, $[\alpha]_D^{25}$ −52.1° (*c* 1.0, MeOH). Found: C, 66.77; H, 7.72; N, 5.05. Calcd for $C_{30}H_{40}N_2O_7$: C, 66.65; H, 7.46; N, 5.18.

De-t-butylation of **7**. **7** (3.81 g) was treated with 4.5 M HCl (9.5 ml) in EtOAc at room temperature for 3.5 h. The oily residue (**8**) obtained after the removal of the solvent under reduced pressure was used for the next reaction without any further purification. On the other hand, a part of this product was converted to the DCHA salt in the usual manner; mp 125—127 °C (EtOAc-hexane); $[\alpha]_D^{25}$ −47.2° (*c* 1.0, MeOH). Found: C, 68.03; H, 8.34; N, 6.30. Calcd for $C_{38}H_{55}N_3O_7$: C, 68.54; H, 8.33; N, 6.31.

Tetrapeptide **9**. a) Into a solution of **8** (prepared from **7** (3.35 g) as described above) and HOBt (0.98 g) in THF (10 ml), we stirred EDC·HCl (1.44 g) at 0 °C. After 15 min, a solution of H-Pro-Val-OBu^t (prepared from Z-Pro-Val-OBu^t (3.00 g) by catalytic hydrogenation) in THF (10 ml) was added, and the reaction mixture was stirred at 0 °C for several hours and then at room temperature overnight. After the mixture had been evaporated under reduced pressure, the residue was dissolved in EtOAc, washed successively with water, 10% citric acid, water, 4% $NaHCO_3$, and water, and dried over Na_2SO_4. The subsequent removal of the solvent afforded a syrup, which was purified on a silica-gel column, with hexane-EtOAc (1:1) as the eluent; yield, 3.63 g (80%); syrup, $[\alpha]_D^{25}$ −76.8° (*c* 1.0, MeOH). Found: C, 64.91; H, 7.91; N, 7.50. Calcd for $C_{40}H_{56}N_4O_9$: C, 65.20; H, 7.66; N, 7.60.

The EDC method in acetonitrile gave the desired compound (**9**) in a 45% yield.

b) The tripeptide **5** (617 mg) was hydrogenated in THF in the presence of 5% Pd-C (150 mg). After the filtration of the catalyst and the removal of the solvent, the residue (**6**) was treated with Z-Phe-Cl (freshly prepared from Z-Phe-OH (877 mg) and PCl_5 (677 mg)) in the presence of TEA (110 mg) in a manner similar to that described for the preparation of the dipeptide **7**. The final purification was performed by preparative TLC on silica gel with benzene-EtOAc (3:2); yield, 466 mg (60%); $[\alpha]_D^{25}$ −76.9° (*c* 1.0, MeOH).

Tripeptide **11**. To a solution of Z-Val-OH (2.26 g) in THF (15 ml), we added EDC·HCl (1.73 g) and HOBt (1.22 g) at 0 °C. After 30 min, a solution of **10** (prepared through the debenzyloxycarbonylation of **7** (4.05 g) by catalytic hydrogenation) in THF (5 ml) was added, after which the reaction mixture was stirred at 0 °C for several hours and then at room temperature overnight. After a work-up similar to that described for the preparation of **9** (Method (a)), the residual syrup was purified on a silica-gel column, with hexane-EtOAc (1:1) as the eluent; yield, 3.37 g (70%); syrup, $[\alpha]_D^{25}$ −55.0° (*c* 1.0, MeOH). Found: C, 65.76; H, 7.85; N, 6.62. Calcd for $C_{35}H_{49}N_3O_8$: C, 65.71; H, 7.72; N, 6.57.

The *N*-hydroxysuccinimide ester method gave a 25% yield in dioxane and a 46% yield in CH_2Cl_2 after 3 d at room temperature. The mixed anhydride method using isobutoxycarbonyl chloride in THF gave a 45% yield, and the EDC method in acetonitrile, a 54% yield.

Z-Val-Phe-Val-OBu^t. Z-Val-OH (1.26 g) and H-Phe-Val-OBu^t (prepared through the debenzyloxycarbonylation of Z-Phe-Val-OBu^t[20] (2.27 g) by catalytic hydrogenation) were reacted in THF (11 ml) by the EDC-HOBt method in the same manner as has been described for the preparation of **11**. The raw product was recrystallized from EtOAc-petroleum ether; yield, 2.52 g (91%); mp 178–179 °C; $[\alpha]_D^{25}$ −46.2° (*c* 1.0, MeOH). [lit,[21] mp 178 °C, $[\alpha]_D$ −45.5° (*c* 1, MeOH).]

The *N*-hydroxysuccinimide ester method in dioxane gave a 72% yield, the mixed anhydride method using isobutoxycarbonyl chloride in THF gave a 78% yield, and the EDC method in acetonitrile gave a 84% yield, under the same reaction conditions as were used for the preparation of **11**.

Z-Ala-Phe-$^{OBu^t}_{|}$CmVal-OEt. A sloution of Z-Ala-OSu (222 mg) and **10** (prepared from **7** (308 mg) by catalytic hydrogenation) in CH_2Cl_2 (2 ml) was stirred at room temperature for 3 d. 1-(2-Aminoethyl)piperazine (30 mg) was then added, and the mixture was stirred for an additional hour. After the solvent had been removed under reduced pressure, the residue was dissolved in EtOAc and washed as usual. The final purification was performed by preparative TLC on silica gel with benzene-EtOAc (3:1); yield, 228 mg (65%); syrup, $[\alpha]_D^{25}$ −65.4° (*c* 0.51, MeOH). Found: C, 65.04; H, 7.52; N, 6.80. Calcd for $C_{33}H_{45}N_3O_8$: C, 64.79; H, 7.41; N, 6.87.

Tripeptide **13**. a) **8** (prepared through the de-*t*-butylation of **7** (1.62 g) with HCl in EtOAc as described above) and H-Pro-OBu^t (prepared from Z-Pro-OBu^t (1.01 g) by catalytic hydrogenation) were coupled by the EDC-HOBt method in a manner similar to that described for the preparation of **9** (Method (a)). The final purification was performed on a silica-gel column, with hexane-EtOAc (1:1) as the eluent; yield, 1.24 g (65%); syrup, $[\alpha]_D^{25}$ −64.9° (*c* 1.0, MeOH). Found: C, 65.86; H, 7.56; N, 6.45. Calcd for $C_{35}H_{47}N_3O_8$: C, 65.91; H, 7.43; N, 6.59.

b) The dipeptide **16**[3] (250 mg) was hydrogenated in THF in the presence of 5% Pd-C (55 mg). After the filtration of the catalyst and the removal of the solvent, the residue was treated with Z-Phe-Cl (freshly prepared from Z-Phe-OH (460 mg) and PCl_5 (320 mg)) in the presence of TEA (57 mg) in a manner similar to that described for the preparation of the dipeptide **7**. The final purification was performed by preparative TLC on silica gel with

benzene–EtOAc (3:1); yield, 251 mg (77%); syrup, $[\alpha]_D^{25}$ −64.6° (*c* 1.0, MeOH).

Tetrapeptide 15. a) The tripeptide **11** (3.19 g) was treated with 4 M HCl in EtOAc (15 ml) at room temperature for 1.5 h. After the solvent had been removed under reduced pressure, the oily residue (**12**) was coupled with H-Pro-OBu^t (prepared from Z-Pro-OBu^t (1.67 g)) in THF by the EDC–HOBt method in a manner similar to that described for the preparation of **9** (Method (a)). The final purification was performed on a silica-gel column, with hexane–EtOAc (1:1) as the eluent; yield, 2.23 g (61%); syrup, $[\alpha]_D^{25}$ −78.8° (*c* 1.0, MeOH). Found: C, 65.00; H, 7.91; N, 7.38. Calcd for $C_{40}H_{56}N_4O_9$: C, 65.20; H, 7.66; N, 7.60.

b) The tripeptide **13** (322 mg) was hydrogenated in MeOH in the presence of 5% Pd–C (200 mg). After the filtration of the catalyst and the removal of the solvent, the oily residue (**14**) was coupled with Z-Val-OH (152 mg) in THF (2 ml) by the EDC–HOBt method in a manner similar to that described for the preparation of **9** (Method (a)). The final purification was performed by preparative TLC on silica gel with benzene–EtOAc (3:1); yield, 196 mg (51%); $[\alpha]_D^{25}$ −78.1° (*c* 1.0, MeOH).

The *N*-hydroxysuccinimide ester method in CH_2Cl_2 gave a 34% yield after 3 d at 30 °C, while the mixed anhydride method using isobutoxycarbonyl chloride in THF gave a 41% yield.

Tetrapeptide 19. a) The tripeptide **11** (570 mg) was hydrogenated in MeOH in the presence of 5% Pd–C (400 mg). After the filtration of the catalyst and the removal of the solvent, the oily residue (**18**) was coupled with Z-Pro-OH (266 mg) in THF (3 ml) by the EDC–HOBt method in a manner similar to that described for the preparation of **9** (Method (a)). The raw product was purified by recrystallization from EtOAc–petroleum ether; yield, 506 mg (77%); mp 108—110 °C; $[\alpha]_D^{25}$ −84.1° (*c* 1.0, MeOH). Found: C, 64.98; H, 7.71; N, 7.54. Calcd for $C_{40}H_{56}N_4O_9$: C, 65.20; H, 7.66; N, 7.60.

The *N*-hydroxysuccinimide ester method in CH_2Cl_2 (reactant concentration, 0.34 M) gave a 26% yield after 3 d at room temperature. The mixed anhydride method, using isobutoxycarbonyl chloride in THF, gave a 44% yield (duration and temperature, 3 h at 0 °C and then overnight at 25 °C).

b) Z-Pro-Val-OBu^t (140 mg) was treated with 4 M HCl in EtOAc (2 ml) at room temperature for 1.5 h. After the solvent had been removed under reduced pressure, the residue was coupled with **10** (prepared from **7** (185 mg) by catalytic hydrogenation) in THF (2 ml) by the EDC–HOBt method in a manner similar to that described for the preparation of **9** (Method (a)). Subsequent recrystallization from EtOAc–petroleum ether afforded a pure product; yield, 149 mg (59%); mp 108.5—110 °C; $[\alpha]_D^{25}$ −84.4° (*c* 1.0, MeOH).

Z-Val-Phe-OBu^t. This was prepared from Z-Val-OH and H-Phe-OBu^t by the mixed anhydride method, using isobutoxycarbonyl chloride in THF; yield, 72%; mp 102—103 °C (ether–petroleum ether); $[\alpha]_D^{25}$ −32.5° (*c* 1.0, MeOH). Found: C, 68.79; H, 7.60; N, 6.17. Calcd for $C_{26}H_{34}N_2O_5$: C, 68.70; H, 7.54; N, 6.16.

Z-Pro-Val-Phe-OBu^t. This was prepared from Z-Pro-OH and H-Val-Phe-OBu^t (prepared through the debenzyloxycarbonylation of Z-Val-Phe-OBu^t by catalytic hydrogenation) by means of the EDC–HOBt method in THF; yield, 73%; mp 164—166 °C (EtOAc–petroleum ether); $[\alpha]_D^{25}$ −78.5° (*c* 1.0, MeOH). Found: C, 67.34; H, 7.39; N, 7.74. Calcd for $C_{31}H_{41}N_3O_6$: C, 67.49; H, 7.49; N, 7.62.

Tetrapeptide 20. To a solution of **4** (0.69 g) in THF (5 ml), we added EDC·HCl (0.41 g) at 0 °C. After 20 min, a solution of H-Pro-Val-Phe-OBu^t (prepared through the debenzyloxycarbonylation of Z-Pro-Val-Phe-OBu^t (1.01 g) by catalytic hydrogenation with 5% Pd–C (0.28 g) in THF) in THF (5 ml) was added. After the resulting mixture had been stirred at 0 °C overnight, it was evaporated under reduced pressure. The residue was dissolved in EtOAc, washed successively with water, 10% citric acid, water, 4% $NaHCO_3$ and water, and dried over Na_2SO_4. The subsequent removal of the solvent afforded a syrup (1.19 g). A part of this raw product was chromatographed on preparative layers of silica gel with benzene–EtOAc (1:1); yield, 55% (based on Z-Pro-Val-Phe-OBu^t used); syrup, $[\alpha]_D^{25}$ −101.3° (*c* 0.94, MeOH). Found: C, 65.11; H, 7.77; N, 7.60. Calcd for $C_{40}H_{56}N_4O_9$: C, 65.20; H, 7.66; N, 7.60.

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13) a) The amino acids and their derivatives used here are all of the L-configuration. b) The following abbreviations are used for Cm-amino acids and their derivatives: CmAA for a Cm-amino acid derived from an amino acid (AA), and X-CmAA-Z for its derivative (X-NC(R)HCO-Z). In these abbreviations, H of the imino group and/or OH of the carboxyl groups are sometimes omitted. c) Abbreviations given by the IUPAC-IUB Commission (*J. Biol. Chem.*, **247**, 977 (1972)) are used throughout. Additional abbreviations: Z, benzyloxycarbonyl; Bu^t, *t*-butyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; TosOH, *p*-toluenesulfonic acid; THF, tetrahydrofuran,; Z-Cl, benzyloxycarbonyl chloride; DCHA, dicyclohexylamine; DMF, *N,N*-dimethylformamide; HOSu, *N*-hydroxysuccinimide.

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