Chem. Pharm. Bull.
30(9)3147—3153(1982)

An Extensive Survey by the Use of High Performance Liquid Chromatography on Racemization during the Coupling of Benzyloxycarbonyl-L-phenylalanyl-L-valine with L-Proline *tert*-Butyl Ester[1,2]

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(Received February 26, 1982)

The extent of racemization and the coupling efficiency during the coupling of benzyloxycarbonyl-L-phenylalanyl-L-valine with L-proline *tert*-butyl ester were conveniently and rapidly determined by the use of high performance liquid chromatography with high accuracy. An extensive examination of various known coupling methods revealed that the coupling methods using diphenyl phosphorazidate (DPPA) and, especially, diethyl phosphorocyanidate (DEPC) afforded the best results. Reported results using DPPA and DEPC as peptide coupling reagents are also summarized (Table I).

Keywords——amino acid; peptide; racemization; high performance liquid chromatography; peptide bond formation; fragment condensation; organophosphorus reagent

Two organophosphorus reagents, diphenyl phosphorazidate (DPPA, $(C_6H_5O)_2P(O)N_3$)[3,4] and diethyl phosphorocyanidate (DEPC, $(C_2H_5O)_2P(O)CN$),[3,5] in combination with triethylamine, have been proved to be excellent coupling reagents in peptide synthesis by both

TABLE I. Racemization Tests using DPPA and DEPC in Dimethylformamdie

Run	Coupling reaction	Extent of racemization (%) DPPA method	Extent of racemization (%) DEPC method	Method of detection[a]	Ref.
1	Boc–Leu–Ile–OH + H–Asn–Leu–OBu^t	14.1±2.1	11.9	AA	10
2	Boc–Ala–Met–Leu–OH + H–Ile–OBu^t	1.8	0.4	AA	10
3	Boc–Ala–Met–Leu–OH + H–Leu–OBu^t	3.9	—	AA	10
4	Boc–Ala–Met–Leu–OH + H–Asp(OBu^t)–OBu^t	6.7	—	AA	10
5	Z–Pro–Val–OH + H–Pro–Val–OMe	5.0±0.0	0.2±0.1	GLC	11
6	Z–Pro–Val–OH + H–Pro–Val–OMe[b]	1.6±0.0	2.8±0.5	GLC	11
7	Z–Pro–Val–OH + H–Pro–Val–OMe[c]	1.7±1.2	0.6±0.0	GLC	11
8	Boc–Pro–Val–OH + H–Pro–Val–OMe	6.0±1.0	0.1±0.1	GLC	11
9	Tfa–Pro–Val–OH + H–Pro–Val–OMe	1.7±0.2	0.0±0.0	GLC	11
10	Tfa–Pro–Val–OH + H–Pro–Val–OMe[b]	3.0±1.0	0.8±0.1	GLC	11
11	Tfa–Pro–Val–OH + H–Pro–Val–OMe[c]	3.0±1.0	2.0±1.0	GLC	11
12	Z–Pro–Val–OH + H–Val–Pro–OMe	4.5±0.1	2.7±0.5	GLC	11
13	Z–Pro–Ala–OH + H–Val–Pro–OMe	2.1	0.2	GLC	11
14	Z–Pro–Leu–OH + H–Pro–Leu–OMe	1.2	0.8	GLC	11
15	Z–Ala–Val–OH + H–Val–OMe	0.8	—	HPLC	12
16	Z–Ala–Val–OH + H–Val–OMe[d]	2.5	—	HPLC	12
17	Z(OMe)–Gly–Ala–OH + H–Phe–OBzl	3.4	—	HPLC	13
18	Z(OMe)–Gly–Ala–OH + H–Phe–OBzl[c]	2.4	—	HPLC	13
19	Cyclization of H–(Sar–Ala)$_3$–OH	<1	—	TLC	14
20	Cyclization of H–(D-Pro–Phe)$_3$–OH	<1	—	TLC	14

a) AA, by amino acid analyzer; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography.
b) Carried out in tetrahydrofuran.
c) Carried out in ethyl acetate.
d) After the reaction, DMF was removed *in vacuo*. See the text.

solution and solid-phase methods.3–5) Little racemization during fragment coupling has been observed by the Young test6,7) in the solution method or by the Izumiya test8) on the solid-phase method. The utility of DPPA and DEPC has been well demonstrated by the successful synthesis of porcine motilin, a gastrointestinal hormone, by a combination of the solution and the solid-phase methods.8a,9)

Since then, several reports10–14) have appeared on racemization during fragment coupling using either DPPA or DEPC; the results are summarized in Table I.15) In general, the DEPC method appears to be superior to the DPPA method.

$$\text{Z–Phe–Val–OH} + \text{H–Pro–OBu}^t \xrightarrow{\text{in DMF}} \underset{\text{I}}{\text{Z–Phe–L/D–Val–Pro–OBu}^t}$$

$$\xrightarrow{\text{TFA}} \underset{\text{II}}{\text{Z–Phe–L/D–Val–Pro–OH}} \xrightarrow{\qquad} \text{HPLC analysis}$$

Chart 1

We have now extensively examined, by the use of high performance liquid chromatography (HPLC), the extent of racemization during fragment coupling using the DPPA and DEPC methods together with other representative coupling methods. We chose a model reaction from the synthesis of the *N*-terminal tripeptide portion of porcine motilin, which involved the coupling of Z–Phe–Val–OH with H–Pro–OBu^t, as shown in Chart 1. A standard sample of Z–Phe–Val–Pro–OBu^t (L-I) was prepared by the coupling of Z–Phe–Val–OH with H–Pro–OBu^t using DPPA or DEPC.16) A sample of Z–Phe–D-Val–Pro–OBu^t (D-I) was analogously prepared from the D-Val derivative.16) Separation of L- and D-I by HPLC was not successful under various conditions. Thus, the corresponding *C*-terminal free peptides, Z–Phe–Val–Pro–OH (L-II) and its D-Val isomer (D-II), were prepared from L- and D-I, respectively, by treatment with TFA. After several trials, effective conditions were found to separate L- and D-II by HPLC, as shown in Fig. 1. The retention volume of L-II was 30.41 ml while that of D-II was 25.63 ml. The separation factor (α) and the value of peak resolution (Rs) were 1.27 and 1.59, respectively. This system gave quantitative results, and the detection limit of D-isomer was approximately 0.01%.

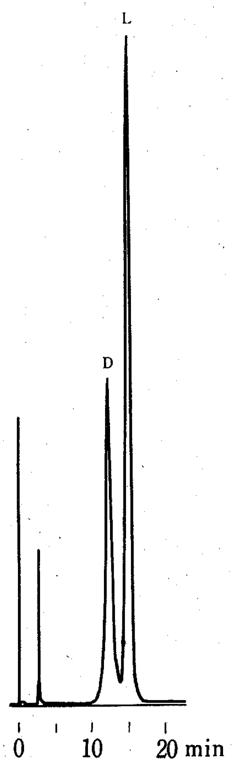


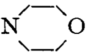
Fig. 1. Separation of Diastereoisomeric Tripeptides (L- and D-II)

Column: SN-01 (ϕ 4.6×250 mm).
Temperature: room temperature.
Eluent: 0.02 M ammonium formate in methanol–water (95: 5).
Flow rate: 2 ml/min.
Range: 0.08 absorbance unit.
Detection: 254 nm.

Using this analytical HPLC procedure, we examined the extent of racemization by various coupling methods. The chemical yield of the coupling was determined by HPLC of L- and D-I. The results are summarized in Table II. Each coupling reaction was conducted in DMF except for run 20, in which a mixture of ethyl acetate and chloroform was used. The standard reaction procedures and work-ups for the DPPA and DEPC methods are as follows: DPPA or DEPC is added to a mixture of Z–Phe–Val–OH and H–Pro–OBu^t in DMF, followed by the addition of TEA at or below 0 °C. After the reaction, the reaction mixture is diluted with a mixture of benzene and ethyl acetate and successively washed with aqueous acid and alkali. This procedure was employed throughout runs 1—12 except for run 5. Both the DPPA and DEPC methods, in general, gave satisfactory results in terms of the chemical yield as well as the degree of racemization, but the DEPC method was slightly better. The use of a slight excess (1.1 equivalents) of the amine component (H–Pro–OBu^t), DPPA or DEPC, and TEA furnished the best result, as in run 1 or run 7, In run 5, DMF was removed at below 50 °C (bath temperature) immediately after the coupling reaction, which afforded a more racemized product than the other runs using the standard work-ups.[17] The use of an excess of TEA (run 6) should be avoided because it causes a greater extent of racemization. Bis(*p*-nitrophenyl)phosphorazidate(*p*-NO$_2$-DPPA)[18] afforded the coupling product (I) in good yield, but racemization occurred to a greater extent even when the reaction was conducted at −20 °C. This coincides with the result of the Young test,[18] and may be due to the fact that *p*-NO$_2$-DPPA will activate the carboxyl group of Z-Phe–Val–OH more strongly than DPPA, so formation of the oxazolone derivative of Z–Phe–Val–OH, with racemization,[19] will occur more easily.

The DCCD method gave rise to greater racemiazation, as expected. Furthermore, *N*,*N'*-dicyclohexyl-*N*-(benzyloxycarbonylphenylalanylvalyl)urea, a well-known by-product of the

Table II. Extent of Racemization during the Formation of Tripeptide (I)

Run	Coupling method	Coupling			D-Isomer(%)[b]
		Temp. (°C)	Time (h)	Yield (%)[a]	
1	DPPA–TEA (1.1 eq/1.1 eq)[c]	0 r.t.	4 20	86.8	2.2
2	DPPA–TEA (1 eq/1 eq)	0	24	75.4	3.2
3	DPPA–TEA (1 eq/1 eq)	−20	24	71.6	2.5
4	DPPA–TEA (1.1 eq/1.1 eq)	0	24	77.2[d]	2.2
5	DPPA–TEA (1.1 eq/1.1 eq)	0	24	59.8[e]	9.6
6	DPPA–TEA (1.1 eq/3.3 eq)	0	24	71.6	7.1
7	DEPC–TEA (1.1 eq/1.1 eq)[c]	0 r.t.	4 20	87.6	1.4
8	DEPC–TEA (1 eq/1 eq)	0	24	76.7	3.0
9	DEPC–TEA (1 eq/1 eq)	−20	24	71.4	2.0
10	DEPC–TEA (1.1 eq/1.1 eq)	0	24	74.9[d]	2.0
11	*p*-NO$_2$–DPPA–TEA (1 eq/1 eq)	0	24	82.1	6.2
12	*p*-NO$_2$–DPPA–TEA (1 eq/1 eq)	−20	24	80.7	5.0
13	DCCD (1.5 eq)	0	24	49.3[d]	19.3
14	DCCD–HOSu (1.5 eq/2 eq)	0	24	16.9[d]	2.4
15	DCCD–HOBt (1.5 eq/2 eq)	0	24	46.9[d]	4.7
16	DCCD–HONB (1.5 eq/2 eq)	0	24	20.5[d]	4.5
17	Ph$_3$P–(PyS)$_2$[f] (1 eq/1 eq)	0	24	80.6	40.4
18	Ph$_3$P–(Py(O)S)$_2$[g]–Py(O)SH[h] (1 eq/1 eq/2 eq)	0 r.t.	0.5 5	55.7	2.6
19	ClCO$_2$Bu^i–MeNO (1 eq/1 eq)	−20 0	4 20	29.9	6.8
20	Azide (Curtius)	0	24	38.7	2.6
21	Azide (Rudinger)	0	24	61.9	2.3
22	Azide (*in situ*)	0	24	52.9	3.2
23	Stepwise (DPPA)	0	24	82.3[i]	0.3[j]
24	Stepwise (DEPC)	0	24	79.6[i]	0.1[j]

a) HPLC yield. *b*) (L-D-L)/(L-L-L+L-D-L). *c*) H-Pro-OBu^t (1.1 eq). *d*) Purified over silica gel. *e*) After the reaction, DMF was removed *in vacuo*. *f*) 2,2'-dipyridyl disulfide. *g*) 2,2'-dithiobis(pyridine-*N*-oxide). *h*) 2-mercaptopyridine *N*-oxide. *i*) Yield of Z–Phe–OH+H–Val–Pro–OBu^t. *j*) Range, 0.04 absorbance unit.

DCCD method,[20] was also isolated in 26.8% yield. Addition of *N*-hydroxysuccinimide (HOSu), *N*-hydroxybenzotriazole (HOBt), or *N*-hydroxy-5-norbornene-2,3-dicaboximide (HONB) with DCCD greatly decreased the racemization. However, the coupling efficiency also decreased and the formation of the urea derivative was inevitable. Although substantial racemization was observed in the oxidation-reduction method (run 17),[21] the use of *N*-oxide type reagents[22] suppressed racemization strongly (run 18). The carbonic mixed anhydride method caused more racemization with less chemical yield. Slight racemization was detected in various azide methods (run 20—22).

As far as examined, the DPPA and DEPC methods caused less racemization and gave better chemical efficiency than the other methods. In particular, the DEPC method gave the best results in terms of the extent of racemization as well as the coupling efficiency. Since the present procedure using HPLC is convenient and rapid with high accuracy, it represents a new peptide racemization test which should be useful to evaluate new coupling methods developed in the future.

Experimental

All melting points are uncorrected. ^{1}H-NMR spectra were recorded on a JEOL PMX-60 or MH-100 spectrometer with tetramethylsilane as an internal standard. Optical rotations were measured with a JASCO DIP-140 automatic polarimeter. HPLC was carried out with a JASCO Tri Rotar-II high pressure liquid chromatograph. Silica gel (70—230 mesh ASTM, Merck Art. 7734) was used for column chromatography.

Z-Phe-Val-OBu^t——DPPA (6.054 g, 22 mmol) was added to a stirred solution of Z–Phe–OH (5.986 g, 20 mmol) and H–Val–OBu^t[23] (3.812 g, 22 mmol) in DMF (80 ml) at 0°C, followed by the addition of TEA (2.226 g, 22 mmol) in DMF (10 ml) at 0°C. The mixture was stirred at 0°C for 4 h, then at room temperature overnight. After dilution with ethyl acetate–benzene (4: 1, 500 ml), the mixture was successively washed with 10% aq. citric acid ($\times 2$), water ($\times 2$), sat. aq. sodium chloride ($\times 1$), sat. aq. sodium bicarbonate ($\times 2$), water ($\times 2$), and sat. aq. sodium chloride ($\times 2$), then dried over sodium sulfate (this washing and drying process being described as "treated as usual"). Removal of the solvent by concentration *in vacuo* gave an oil, which was crystallized from a small volume of hexane. Recrystallization from ethyl acetate–hexane afforded Z–Phe–Val–OBu^t (8.574 g, 94%) as colorless crystals, mp 102—103°C, $[\alpha]_D^{20}$ $-19.60°$ (c=1, MeOH) (lit.[24] mp 104—106°C, $[\alpha]_D^{20}$ $-19.85\pm0.5°$ (c=1.5, MeOH)). The DEPC method was also applied to the preparation of Z–Phe–Val–OBu^t in 71% yield.

Z-Phe-Val-OMe——Prepared as described above in 91% yield using H–Val–OMe. Colorless crystals, mp 111—113°C, $[\alpha]_D^{20}$ $-16.3°$ (c=1, MeOH) (lit.[25] mp 113°C, $[\alpha]_D^{20}$ $-16.7°$ (MeOH)).

Z-Phe-Val-NHNH$_2$——Hydrazine hydrate (100%, 13.2 ml) was added to a solution of Z–Phe–Val–OMe (6.80 g, 16.5 mmol) in DMF (40 ml). The mixture was stirred at room temperature overnight. After dilution with water (100 ml), the precipitates were collected, washed with water, and purified by recrystallization from methanol to give Z–Phe–Val–NHNH$_2$ (6.107 g, 90%) as colorless crystals, mp 214—216°C, $[\alpha]_D^{20}$ $-7.8°$ (c=1, DMF) (lit.[26] mp 209—214°C, $[\alpha]_D^{26}$ $-7.7°$ (c=0.7, DMF)).

Z-Phe-Val-OH——Z–Phe–Val–OBu^t (5.000 g, 11 mmol) was dissolved in TFA (15 ml) and the mixture was stirred at room temperature for 2 h. The solution was concentrated *in vacuo* to give a residue, which was dissolved in benzene (10 ml) and concentrated. This work-up was carried out three times. The final residue was crystallized from hexane. Recrystallization from ethyl acetate–hexane gave Z–Phe–Val–OH (3.480 g, 79%) as colorless crystals, mp 143—145°C, $[\alpha]_D^{20}$ $-3.43°$ (c=1, THF) (lit.[27] mp 141—143°C, $[\alpha]_D^{20}$ $-3.4°$ (c=1, THF)).

Z-Phe-Val-Pro-OBu^t (L-I)——DPPA (605 mg, 2.2 mmol) was added to a stirred solution of Z–Phe–Val–OH (797 mg, 2 mmol) and H–Pro–OBu^t[23] (377 mg, 2.2 mmol) in DMF (11 ml) at 0°C, followed by the addition of TEA (223 mg, 2.2 mmol) in DMF (3 ml) at 0°C. The mixture was stirred at 0°C for 4 h, then at room temperature overnight. After dilution of the mixture with ethyl acetate–benzene (4: 1, 150 ml), the mixture was treated as usual. The solution was concentrated *in vacuo* to give a residue, which was purified by column chromatography over silica gel with ethyl acetate–hexane (1: 1) and recrystallized from ethyl acetate–hexane to give L-I[16] (759 mg, 69%) as colorless crystals, mp 115—116°C, $[\alpha]_D^{20}$ $-76.11°$ (c=1, MeOH). NMR δ ppm (CDCl$_3$): 0.94 (6H, dd, $\mathrm{CH(C\underline{H}_3)_2}$, J=6 Hz), 1.24 (1H, m, $\mathrm{C\underline{H}(CH_3)_2}$), 1.43 (9H, s, $\mathrm{C(CH_3)_3}$), 2.02 (4H, m, $\mathrm{CH_2CH_2}$), 3.06 (2H, d, $\mathrm{C\underline{H}_2C_6H_5}$, J=7 Hz), 3.68 (2H, m, $\mathrm{CH_2}$), 4.14 (1H, s, NH), 4.3—4.7 (3H, m, CH$\times$3), 5.06 (2H, s, $\mathrm{OC\underline{H}_2C_6H_5}$), 5.70 (1H, s, NH), 7.18 (5H, s, $\mathrm{C_6H_5}$). *Anal.* Calcd for $\mathrm{C_{31}H_{41}N_3O_6}$: C, 67.49; H, 7.49; N, 7.62. Found: C, 67.25; H, 7.38; N, 7.61.

Z–Phe–Val–Pro–OBu^t (L-I) was prepared analogously by the DEPC method in 67% yield.

Z-Phe-Val-Pro-OH (L-II)——TFA (3 ml) was added to L-I (1.103 g, 2 mmol) and the mixture was stirred at room temperature for 2 h. The mixture was treated as described for the preparation of Z–Phe–Val–

OH. Recrystallization from ethyl acetate–diethyl ether afforded L-II[16] (843 mg, 87%) as colorless crystals, mp 92—94°C, $[\alpha]_D^{20}$ −61.90° (c=1, MeOH) (lit.[26] mp 93—95°C, $[\alpha]_D^{20}$ −62.0° (c=1, MeOH)).

Z-Phe-D-Val-Pro-OH (D-II)——Prepared from Z–Phe–D-Val–OH[28] (mp 144—147°C, $[\alpha]_D^{20}$ −15.07° (c=1, DMF), synthesized from Z–Phe–OH and H-D-Val–OBu^t) and H–Pro–OBu^t using DPPA or DEPC by the procedure described for the preparation of L-II. Recrystallization of the crude product from ethyl acetate–diethyl ether afforded pure D-II[16] as colorless crystals, mp 80—82°C, $[\alpha]_D^{20}$ −20.19° (c=1, MeOH). NMR δ ppm ($CDCl_3$): 0.84 (6H, dd, $CH(C\underline{H}_3)_2$, J=6 Hz), 2.06 (5H, m, $C\underline{H}(CH_3)_2$, CH_2CH_2), 3.60 (2H, m, CH_2), 4.3—4.7 (3H, m, CH×3), 5.00 (2H, s, $OC\underline{H}_2C_6H_5$), 6.06 (1H, s, NH), 7.12 (5H, s, C_6H_5), 7.14 (5H, s, C_6H_5), 7.78 (1H, s, NH). *Anal.* Calcd for $C_{27}H_{33}N_3O_6$: C, 65.44; H, 6.71; N, 8.48. Found: C, 65.28; H, 6.64; N, 8.42.

Determination of Coupling Efficiency during the Coupling of Z-Phe-Val-OH with H-Pro-OBu^t——A crude sample (*ca.* 100 mg) of L-I containing D-I obtained by each coupling reaction was weighed accurately, dissolved in ethyl acetate, and made up to 5.0 ml. The solution (4 μl) was injected with a microsyringe into the HPLC apparatus under the following conditions: column, SS-10-ODS-B (ϕ 4.6×250 mm); temperature, room temperature; eluent, ethyl acetate–hexane (1: 1); flow rate, 1 ml/min; range, 0.16 absorbance unit; detection, 254 nm.

Determination of Extent of Racemization during the Coupling of Z-Phe-Val-OH with H-Pro-OBu^t——A crude sample (100 mg) of L-II containing D-II was dissolved in methanol, and made up to 3.0 ml. The solution (5 μl) was injected with a microsyringe into the HPLC apparatus under the conditions shown in Fig. 1.

The detection limit of D-II was determined to be 0.0085% by using an internal standard (Z–Phe–Pro–OH) in two steps as follows. The peak of L-II was maximized in a chart at 0.08 absorbance unit using *ca.* 1 μl of a mixture of L/D-II and the internal standard, and the quantity of L-II was estimated to be 100.17 μg from the standard calibration curve. The least detectable area of the internal standard was 1.4 and no peak of D-II could be detected in this case. Next, using *ca.* 100 μl of the mixture of L/D-II and the internal standard, the peak of the internal standard was maximized in a chart and its peak area was estimated to be 153.8. The least detectable quantity of D-II was 0.94 μg. The peak of L-II was too high to be within the chart in this case, but its quantity was calculated to be 11004.39 μg. The detection limit of D-II was calculated by means of the equation:

$$\frac{\text{the quantity of D-II}}{\text{the quantities of L-II and D-II}} \times 100 = 0.0085\%.$$

Racemization Test in Table II——Run 1: DPPA (303 mg, 1.1 mmol) in DMF (3 ml) was added to a stirred solution of Z–Phe–Val–OH (398 mg, 1 mmol) and H–Pro–OBu^t (188 mg, 1.1 mmol) in DMF (4 ml) at 0°C, followed by the addition of TEA (111 mg, 1.1 mmol) in DMF (3 ml) at 0°C. The mixture was stirred at 0°C for 4 h, then at room temperature for 20 h. The mixture was diluted with ethyl acetate–benzene (4: 1, 100 ml) and treated as usual. The solvent was removed *in vacuo* to give a crude I, a part of which was subjected to HPLC to determine the coupling yield (87%). Without purification, the crude I was dissolved in TFA (1.63 ml) and the mixture was stirred at room temperature for 2 h. TFA was removed *in vacuo* to give a residue, which was dissolved in benzene and concentrated. This work-up was repeated three times. The final residue was dissolved in 1 N aq. sodium hydroxide (1.5 ml) and diluted with water (50 ml). The aqueous layer was washed with diethyl ether (10 ml×3), adjusted to pH 3 by the addition of 10% aq. citric acid, and extracted with methylene chloride (30 ml×3). The extracts were dried over sodium sulfate and concentrated *in vacuo* (this TFA treatment and work-up process being described as "treated with TFA"). The residue of II was subjected to HPLC.

Runs 2—12: Carried out as described for run 1. The experimental conditions and results are summarized in Table II.

Run 13: DCCD (309 mg, 1.5 mmol) in DMF (3 ml) was added to a stirred solution of Z–Phe–Val–OH (398 mg, 1 mmol) and H–Pro–OBu^t (171 mg, 1 mmol) in DMF (7 ml) at 0°C. The mixture was stirred at 0°C for 24 h, and filtered to remove *N,N'*-dicyclohexylurea. The filtrate was diluted with ethyl acetate–benzene, and treated as usual. The crude residue was purified by silica gel column chromatography with ethyl acetate–hexane (1: 1). The first fraction to be eluted (*Rf* 0.69) contained *N,N'*-dicyclohexyl-*N*-(benzyloxycarbonylphenylalanylvalyl)urea (162 mg, 26.8%) as an amorphous solid. IR ν_{max}^{KBr} cm^{-1}: 3300, 1700, 1650, 1530, 1390, 1160, 740, 700. NMR δ ppm ($CDCl_3$): 0.83 (3H, d, J=6 Hz, $CH(C\underline{H}_3)_2$), 0.93 (3H, d, J=6 Hz, $CH(C\underline{H}_3)_2$), 1.1—2.3 (23H, $C\underline{H}(CH_3)_2$, C_6H_{11}×2), 3.10 (2H, d, J=6.6 Hz, $CC\underline{H}_2C_6H_5$), 4.0—4.8 (2H, m, -CH-×2), 5.10 (2H, s, $OC\underline{H}_2C_6H_5$), 5.37 (1H, d, J=7.6 Hz, NH), 6.54 (1H, d, J=7 Hz, NH), 7.20 (5H, s, C_6H_5), 7.30 (5H, s, C_6H_5), 7.50 (1H, s, NH). *Anal.* Calcd for $C_{35}H_{48}N_4O_5$: C, 69.51; H, 8.00; N, 9.26. Found: C, 69.43; H, 8.13; N, 9.00.

The second fraction to be eluted (*Rf* 0.36) contained I (49.3% yield), which was treated with TFA to give II.

Run 14: DCCD (309 mg, 1.5 mmol) in DMF (3 ml) was added to a stirred solution of Z–Phe–Val–OH (398 mg, 1 mmol), H–Pro–OBu^t (171 mg, 1 mmol), and HOSu (230 mg, 2 mmol) in DMF (7 ml) at 0°C. The mixture was treated as described for run 13.

Run 15—16: Carried out as described for run 14.

Run 17: Triphenylphosphine (262 mg, 1 mmol) in DMF (5 ml) was added to a stirred solution of Z–Phe–Val–OH (398 mg, 1 mmol), H–Pro–OBu^t (171 mg, 1 mmol), and 2,2′-dipyridyl disulfide (220 mg, 1 mmol) in DMF (5 ml). The mixture was stirred at 0°C for 24 h, and treated as described for run 1.

Run 18: Carried out as described for run 17 using 2,2′-dithiobis(pyridine-*N*-oxide) and 2-mercaptopyridine-*N*-oxide.

Run 19: Isobutyl chlorocarbonate (137 mg, 1 mmol) in DMF (2 ml) was added to a stirred solution of Z–Phe–Val–OH (398 mg, 1 mmol) and *N*-methylmorpholine (101 mg, 1 mmol) in DMF (5 ml) at −20°C. The mixture was stirred at −20°C for 20 min, then H–Pro–OBu^t (171 mg, 1 mmol) in DMF (3 ml) was added. The mixture was stirred at −20°C for 4 h, then at 0°C for 20 h, and worked up as usual.

Run 20: Hydrochloric acid (1 N, 1.1 ml) was added to a stirred solution of Z–Phe–Val–$\mathrm{NHNH_2}$ (412 mg, 1 mmol) in acetic acid (5 ml) and DMF (1 ml) at −5°C, followed by the addition of sodium nitrite (69 mg), then further hydrochloric acid (1 N, 1.1 ml). The whole was stirred for 10 min at −5°C, then cold water (20 ml) was added and the mixture was extracted with cold ethyl acetate (5 ml × 3). The organic extracts were washed with cold sat. aq. sodium bicarbonate (5 ml × 2), and cold water (5 ml × 2), then dried over sodium sulfate for 20 min. The azide solution thus prepared was added to a solution of H–Pro–OBu^t (171 mg, 1 mmol) and TEA (0.14 ml) in chloroform (5 ml) at 0°C. The mixture was stirred at 0°C for 24 h, diluted with ethyl acetate–benzene, and treated as described for run 1.

Run 21: Hydrogen chloride in ethyl acetate (10%, 0.73 ml) was added to a stirred solution of Z–Phe–Val–$\mathrm{NHNH_2}$ (412 mg, 1 mmol) in DMF (4 ml) at −20°C, followed by the addition of isoamylnitrite (0.16 ml). The mixture was stirred for 10 min at −20°C, then neutralized with TEA (0.28 ml) and combined with a solution of H–Pro–OBu^t (171 mg, 1 mmol) in DMF (2 ml) containing TEA (0.17 ml). The mixture was stirred at 0°C for 24 h, diluted with ethyl acetate–benzene, and treated as described for run 1.

Run 22: Hydrochloric acid (1 N, 1.1 ml) was added to a stirred solution of Z–Phe–Val–$\mathrm{NHNH_2}$ (412 mg, 1 mmol) in DMF (4 ml) at −20°C, followed by the addition of sodium nitrite (69 mg), then further hydrochloric acid (1 N, 1.1 ml). The mixture was stirred for 10 min at −20°C, then neutralized with TEA (0.28 ml) and combined with a solution of H–Pro–OBu^t (171 mg, 1 mmol) in DMF (2 ml) containing TEA (0.17 ml). The mixture was stirred at 0°C for 24 h, diluted with ethyl acetate–benzene, and treated as described for run 1.

Run 23: DPPA (4.541 g, 16.5 mmol) was added to a stirred solution of Z–Val–OH (3.769 g, 15 mmol) and H–Pro–OBu^t (2.825 g, 16.5 mmol) in DMF (50 ml) at 0°C, followed by the addition of TEA (1.670 g, 16.5 mmol) in DMF (10 ml) at 0°C. The mixture was stirred at 0°C for 24 h, diluted with ethyl acetate–benzene (4 : 1, 500 ml), and treated as usual. Removal of the solvent by concentration *in vacuo* gave an oil, which was purified by silica gel column chromatography with ethyl acetate–hexane (1 : 1) to give Z–Val–Pro–OBu^t (3.678 g, 61%) as a colorless oil. NMR δ ppm ($\mathrm{CDCl_3}$): 0.96 (3H, d, $\mathrm{CH(C\underline{H}_3)_2}$, J=6.7 Hz), 1.06 (3H, d, $\mathrm{CH(C\underline{H}_3)_2}$, J=6.7 Hz), 1.44 (9H, s, $\mathrm{C(CH_3)_3}$), 1.99 (5H, m, $\mathrm{C\underline{H}(CH_3)_2}$, $\mathrm{CH_2CH_2}$), 3.71 (2H, d, $\mathrm{CH_2}$, J=6.7 Hz), 4.2—4.6 (2H, m, CH × 2), 5.11 (2H, $\mathrm{OC\underline{H}_2C_6H_5}$), 5.59 (1H, d, NH, J=8 Hz), 7.36 (5H, s, $\mathrm{C_6H_5}$).

A stirred solution of Z–Val–Pro–OBu^t (3.330 g, 8.2 mmol) in THF (40 ml) was hydrogenated over 5% palladium-carbon (1 g) for 6 h. After removal of the catalyst, the solvent was evaporated off *in vacuo* to give H–Val–Pro–OBu^t (1.816 g, 82%).

DPPA (303 mg, 1.1 mmol) was added to a stirred mixture of Z–Phe–OH (299 mg, 1 mmol) and H–Val–Pro–OBu^t (270 mg, 1 mmol) in DMF (7 ml) at 0°C, followed by the addition of TEA (111 mg, 1.1 mmol) in DMF (3 ml) at 0°C. The mixture was stirred at 0°C for 24 h, diluted with ethyl acetate–benzene, and treated as described for run 1.

Run 24: Carried out as described for run 23 but using DEPC.

Acknowledgement We are grateful to Prof. S. Yamada (Jōsai University) for his interest in this research. We thank Dr. R. Matsueda (Sankyo Co., Ltd.) for the gift of *N*-oxide-type reagents and for valuable information about the reaction conditions for the oxidation–reduction method, and Prof. S. Hara (Tokyo College of Pharmacy) for his kind suggestions on HPLC. This work was supported in part by grants from the Foundation for the Promotion of Research on Medicinal Resources, the Research Foundation for Oriental Medicine, and the Japan Research Foundation for Optically Active Compounds.

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