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Synthesis of α-Aminophosphonic Octapeptide, Phe–Gly–Ser–Leu–Ala^P–Phl–Leu–Pro, an Analog with Partial Sequence of *erb* B-2 Gene Product[1)]

Kaoru Inami, Tadashi Teshima, Hideaki Miyashita,† and Tetsuo Shiba*

Peptide Institute, Protein Research Foundation, 4-1-2 Ina, Minoh, Osaka 562

†Protein Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565

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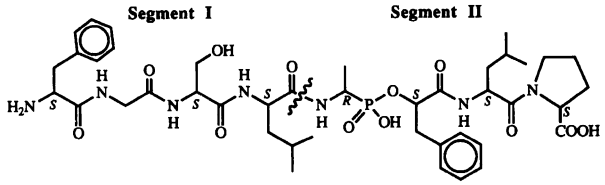
A chemical synthesis was performed of an octapeptide with a phosphonic ester linkage, which corresponds to a variation of the partial sequence of a gene product of *erb* B-2. The phosphonic ester linkage was successfully prepared through a coupling reaction of magnesium salt of the hydroxypeptide with phosphonochloridate, without damage of the peptide bond. The free aminophosphonic peptide as the final product gave a single peak on the ODS column, and a reasonable ratio of the component amino acids. The reaction series exploited here might be useful as a general procedure for preparation of the phosphonic peptide.

α-Aminoethylphosphonic acid occurs naturally as a constituent of the phospholipids of various organisms, e.g., a starfish, *Asterias forbesi*, a seaflower, *Tealia felina*, etc.[2)] Inhibitory activities of the phosphonic peptides for zinc proteases, such as thermolysin and carboxypeptidase, were reported by Bartlett and his coworkers as tetrahedral transition-state analogs of the parent peptides and proteins.[3)] Furthermore, the phosphonic analogs attracted many researchers as haptens for the catalytic antibodies, especially as the proteolytic abzyme, since those models fit to the hydrated form of the carboxyl function in the transition state. In view of such particular properties of the phosphonic analogs, we tried to synthesize an octapeptide Phe–Gly–Ser–Leu–Ala^P–Phl–Leu–Pro (**1**), which is a substitution analog with the partial sequence of an oncogene *erb* B-2 product. The octapeptide seemed to be worthwhile as a candidate of the hapten of the catalytic antibody. We can expect formation of an antibody with activity of a sequence-specific protease like the restriction enzyme for nucleic acid. (Fig. 1)

This peptide contains α-aminoethylphosphonic acid (Ala^P), and 3-phenyllactic acid (Phl)[4)] as substitutes for an alanine and a phenylalanine residue, respectively, in the original oncogene. Both residues are linked by the ester linkage, giving a more stable bond than the labile phosphonamide one. For the synthesis, the octapeptide was divided into two segments: i.e., Phe–Gly–Ser–Leu (segment I) and Ala^P–Phl–Leu–Pro (segment II), which were condensed by the fragment condensation in the final stage. This strategy is due to our intention to shorten the synthetic pathway after introduction of the phosphonic acid residue. A final deprotection reaction



Phe-Gly-Ser-Leu-Ala-Phe-Leu-Pro

(Partial sequence of gene product of oncogen *erb* B-2)

Fig. 1. Structure of candidate of hapten of a proteolytic abzyme.

was carried out in a neutral medium, since the phosphonic ester is either cleaved in an acidic media or decomposed under basic conditions. For this reason, benzyloxycarbonyl (Z) and benzyl (Bzl) groups were chosen as the protecting groups for α-amino group, as well as hydroxyl and carboxyl groups.

The *N*-terminal protected tetrapeptide, Z–Phe–Gly–Ser(Bzl)–Leu–OH (Segment I) (**7**), was prepared starting from Boc–L-Leu–OH (**2**) according to the usual stepwise elongation, by use of WSCD–HOBt method, as shown in Fig. 2.

Another tetrapeptide with phosphonic ester linkage, Ala^P–Phl–Leu–Pro (Segment II), was prepared starting from Boc–L-Pro–OH (**9**) as shown in Fig. 3. The condensation of Boc–L-Leu–OH with HCl·H–L-Pro–OPac was carried out by a mixed-anhydride method to avoid the racemization due to the formation of a charged Schiff base of H–Pro–OPac,[5)] followed by the deprotection of Boc group with HCl/dioxane. A coupling of the dipeptide with *O*-acetyl-3-phenyllactic acid (**12**)

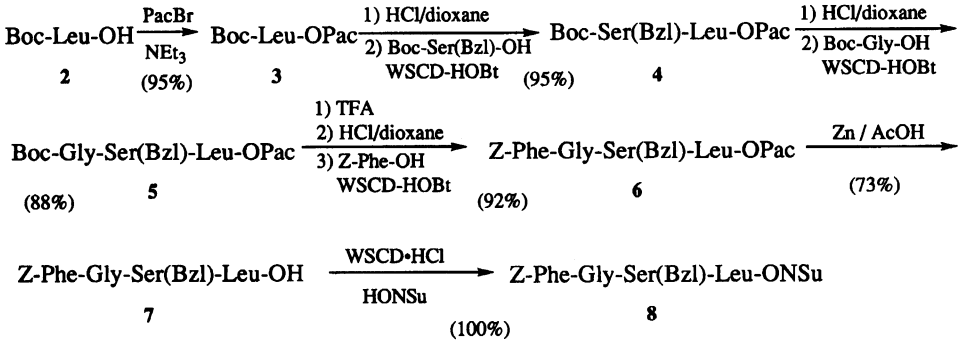


Fig. 2. Preparation of segment I.

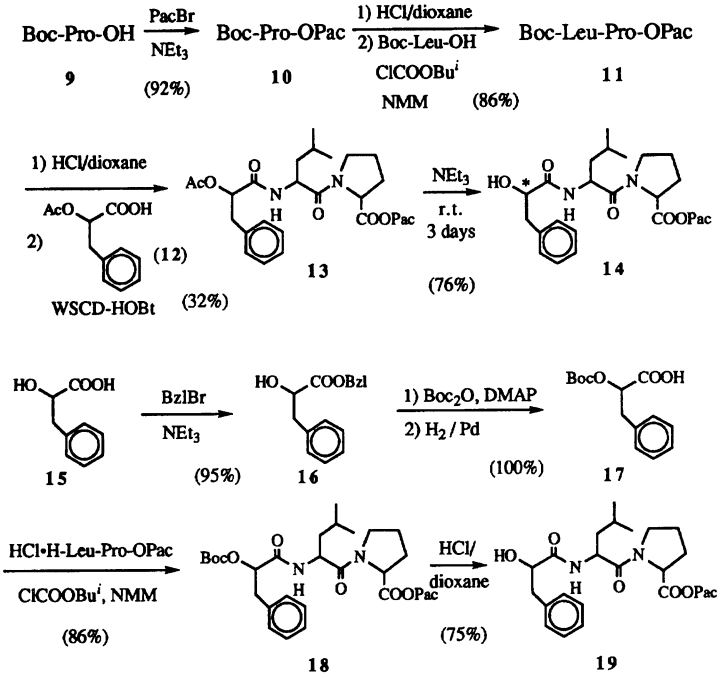


Fig. 3. Preparation of segment II.

was carried out by WSCD–HOBt method to give the desired tripeptide (**13**) in 32% yield; dioxopiperazine of Leu–Pro also formed. An acetyl group of this obtained tripeptide was removed with triethylamine to afford a partially racemized **14**.[6] Thus, the Boc group was then chosen as the protecting group of the hydroxyl group of Phl, whose α-proton seemed to be labile in basic media. Boc–Phl–OH (**17**) was prepared from 3-phenyllactic acid by successive procedures: an esterification with benzyl bromide and triethylamine, an introduction of Boc group with Boc_2O and DMAP, and then removal of the benzyl group by catalytic hydrogenation. Boc–Phl–OH was coupled with a dipeptide, HCl·H–L-Leu–L-Pro–OPac, by the mixed anhydride method using isobutyl chloroformate to give the Boc–tripeptide–OPac in 86% yield. No formation of dioxopiperazine of Leu–Pro, which was formed in the condensation by WSCD–HOBt method, was observed in this reaction. The Boc group was removed under ordinary conditions to afford the hydroxypeptide **19** without racemization.

Bartlett and his coworkers previously prepared the *N*-protected aminophosphonic diester **21** by heating the mixture of the *N*-protected aminophosphonic acid and trimethyl orthoformate at 120 °C for 48 h, and then converted **21** to its mono ester **22** by the half-saponification with sodium hydroxide.[3] (Fig. 4) In our reaction, by heating a mixture of the same reagents at 120 °C for 38 h,[7] we recognized a formation of the mono ester **22** in 28% yield, beside the dimethyl ester **21** in 68% yield. This result showed us that the esterification reaction might proceed slowly enough to be able to be stopped at a stage of the formation of the mono ester **22**. Therefore, we tried to optimize the reaction

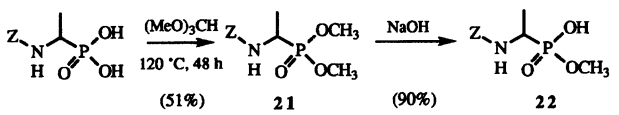
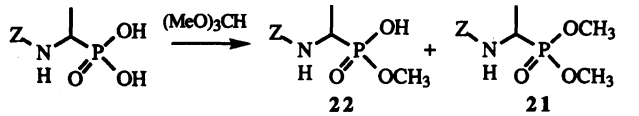


Fig. 4. Preparation of phosphonic ester by Bartlett et al.

conditions for predominant formation of the mono ester **22**. The result of the reactions is shown in Table 1. While no esterification reaction took place below 100 °C, the reaction at 120 °C for 45 min gave the desired mono ester **22** predominantly. After transformation of the mono ester with thionyl chloride to the phosphonyl chloride, a coupling of the chloride obtained with the hydroxytripeptide **19** afforded no desired phosphonic peptide **23** under ordinary coupling conditions. In order to activate a hydroxyl function of the tripeptide **19**, we then tried a reaction of the phosphonyl chloride with the magnesium alkoxide, which was derived from the tripeptide and *t*-butylmagnesium chloride. A similar procedure was reported by Hayakawa et al. in case of the phosphorylation of the sugar moiety in a nucleotide in the presence of the amide functions.[8] (Fig. 5) First, the reaction conditions reported by Hayakawa et al. were applied to our reaction. Thus, *t*-butylmagnesium chloride (1 equiv) was reacted with the hydroxypeptide **19** (1 equiv) in THF, and then the *N*-protected aminophosphonyl chloride (1 equiv) was added to the reaction mixture, which was allowed to stand for 1 h to afford the desired phosphonic peptide (**23**) in 15%. In the investigation for an optimal reaction condition, as mentioned in Table 2, the condition (Entry 5) was found to be best to avoid the side reaction (transesterification and cleavage) due to the base-labile phenacyl ester.

The Z–tetrapeptide–OPac (**23**) was hydrogenated in the presence of Pd-black to afford a peptide with the free amino and carboxyl groups, since the Pac ester was removed at the same time under these conditions, but the phenethyl ester which was produced by the reduction of the phenacyl group was also formed. This reaction mixture without purification was allowed to condense with the *N*-terminal tetrapeptide by the active ester method to give the desired peptide **25** in 37% yield (Fig. 6). The low yield seemed to arise from the incomplete hydrogenolysis of the phenacyl group. Thus, we now changed the carboxyl protection in segment I to benzyl group. Thus, the deprotection was completed before the condensation step. The Z–tetrapeptide–OBzl **28** was prepared as shown in Fig. 7. The simultaneous removal of the benzyloxycarbonyl and benzyl groups in compound **28** was carried out by catalytic hydrogenation using Pd as the catalyst under the same conditions as above. This free phosphonic tetrapeptide was coupled with the *N*-terminal tetrapeptide by the active ester method to give the protected octapeptide **29** in 89% yield. The methyl group of the phosphonic acid residue in the octapeptide was removed with sodium iodide (15 equiv) in 2-butanone at 80 °C for 1.5 h in yield of 78% and then the deprotection of three benzyl-type protecting groups was carried out by catalytic hydrogenation to afford the desired free octapeptide **1** quantitatively (Fig. 7). The final product was isolated and purified by the reverse-phase HPLC (Nacalai Cosmosil $5C_{18}$). An amino acid analysis of the octapeptide obtained after acid hydrolysis gave reasonable values for composite amino acids. The optical purity of the product was checked by Marfey's method,[9] as shown in Fig. 8.

Table 1. Yields of Phosphonic Esters



22 **21**

	Monoester (%)	Diester (%)
120 °C, 38 h	27.7	67.4
120 °C, 1.5 h	49.9	32.3
120 °C, 45 min	**82.0**	**9.9**

The synthetic strategy for the aminophosphonic peptide mentioned above, including the phosphonylation using the magnesium salt of the hydroxypeptide in the presence of amide bonds and deprotection of a methyl group of the phosphonate with NaI, may be widely applied as a general synthetic method of phosphopeptides as well as phosphonic peptides. Moreover the peptide obtained here would be a useful candidate as a hapten of the proteolytic abzyme, though such an experiment has not yet been done.

Experimental

All melting points are uncorrected. The ^{1}H NMR spectra were obtained with a JEOL JNM-EX-270 spectrometer. The chemical shifts are given in δ, with TMS as the internal standard. The specific rotations were obtained with a JASCO DIP-360 Digital Polarimeter. The high performance liquid chromatography was carried out with a Shimadzu LC-9A liquid chromatograph. Amino acid analysis was performed with a JEOL JLC-300 Fully Automated Liquid Chromatograph. TLC was done by the ascending method on a Merck Silica gel 60 F_{254}. The silica gel column chromatography was carried out on a Merck Silica gel 60 (0.040—0.063 mm).

Boc–L-Leu–OPac (3). To a solution of Boc–L-Leu–OH·H_2O (7.48 g, 30.0 mmol) and triethylamine (4.58 ml, 33.0 mmol) in acetone (60 ml), phenacyl bromide (6.57 g, 33.0 mmol) was added. The reaction mixture was stirred at room temperature for 3 h. After removal of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate and washed with brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure to the residue, which was washed with hexane; yield 10.0 g (95.4 %).

Boc–L-Ser(Bzl)–L-Leu–OPac (4). To Boc–L-Leu–OPac (9.00 g, 25.8 mmol), 5.7 M hydrogen chloride (1 M=1 mol dm^{-3}) in 1,4-dioxane solution (90 ml) was added. The solution was allowed to stand at room temperature for 30 min. Hexane was added to the solution, and the precipitate was collected by suction filtration. The solid was dissolved in a solution of Boc–L-Ser(Bzl)–OH (7.62 g, 25.8 mmol) and HOBt (3.66 g, 27.1 mmol) in DMF (50 ml) under ice-cooling. WSCD (4.80 ml, 27.1 mmol) was added to the solution, which was then stirred for 30 min, and then the additionally at room temperature for 18 h. After re-

Table 2. Optimization of Ester Formation Reaction

Entry	Starting material	*t*-BuMgCl equiv	Temp	Reaction time of activation	Reaction time of coupling	Yield of **23** or **28** (%)
1	**19**	1.0	R.T.	10 min	1 h	15.1
2	**19**	1.5	R.T.	5 min	20 h	ca. 0
3	**19**	1.5	R.T.	5 min	5 min	44.1
4	**19**	1.5	−78 °C	2 min	−78 °C,2 h→ R.T.,10 min	37.0
5	**19**	1.5	4 °C	1 min	5 min	69.6
6	**27**	1.5	4 °C	1 min	5 min	82.0

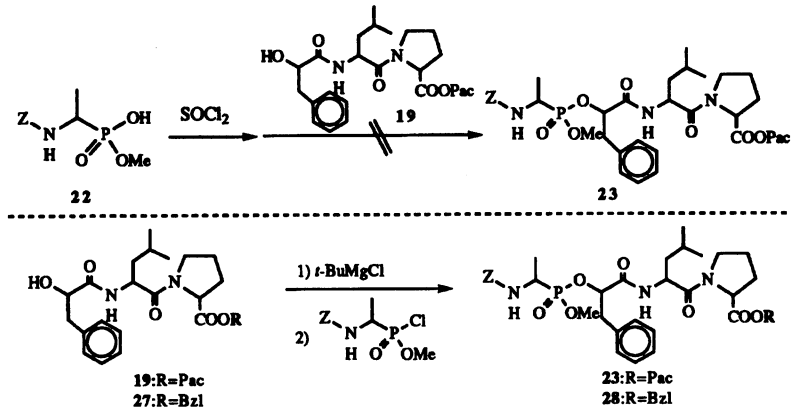


Fig. 5. Formation of phosphonic ester linkage.

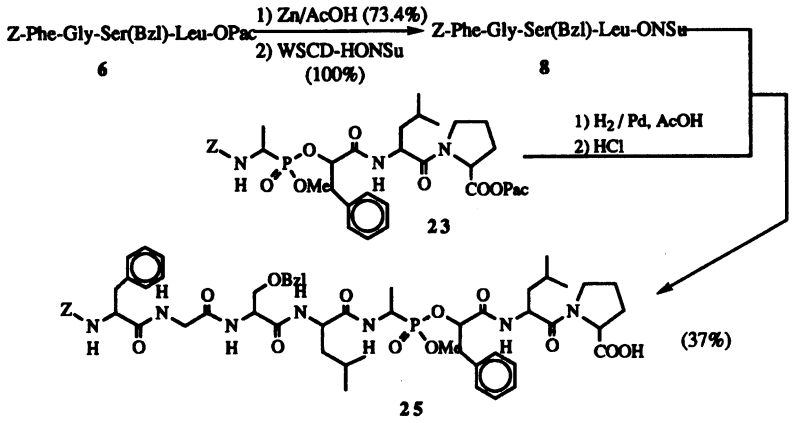


Fig. 6. Octapeptide prepared from tetrapeptide phenacyl ester.

moval of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate; this was washed successively with 10% aqueous citric acid, brine, saturated aqueous sodium hydrogencarbonate, and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. Purification by silica gel column chromatography using developing solvent of chloroform gave a colorless solid; yield 12.9 g (94.9%). Mp 87—89 °C. Found: C, 66.11; H, 7.45; N, 5.30%. Calcd for $C_{29}H_{38}N_2O_7$: C, 66.14; H, 7.27; N, 5.32%. $[\alpha]_D^{28}$ −18.8° (*c* 0.642, DMF). ^{1}H NMR ($CDCl_3$) δ=4.74 (1H, m, Ser α), 4.30 (1H, m, Leu α), 3.89 (1H, dd, J=4.0 and 9.2 Hz), and 3.57 (1H, dd, J=7.0 and 9.1 Hz).

Boc–Gly–L-Ser(Bzl)–L-Leu–OPac (5). To Boc–L-Ser(Bzl)–L-Leu–OPac (10.7 g, 20.3 mmol), 5.7 M hydrogen chloride in 1,4-dioxane solution (110 ml) was added; the mixture was allowed to stand at room temperature for 30 min. Hexane was added to the solution, and the precipitate was collected by suction filtration. The solid was dissolved into the solution of Boc–Gly–OH (3.56 g, 20.3 mmol) and HOBt (2.89 g, 21.3 mmol) in DMF (50 ml). WSCD (3.77 ml, 21.3 mmol) was added to the solution under ice-salt cooling. The mixture was stirred for 30 min, and then additionally at room temperature for 18 h. After removal of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate, and washed successively with 10% aqueous citric acid, brine, saturated aqueous sodium hydrogencarbonate, and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure to a solid, which was washed with hexane;

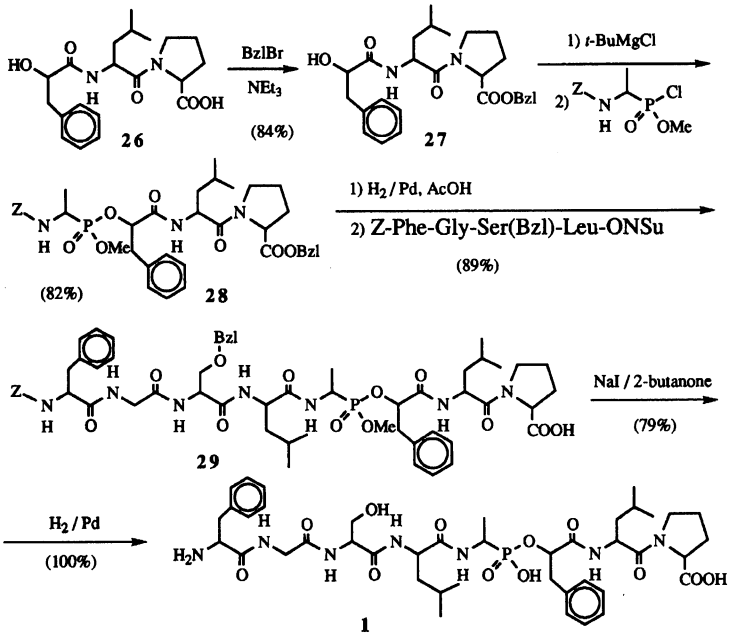
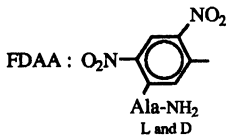


Fig. 7. Synthesis of the octapeptide.

	AAA	D-AA Content (%) in Acid Hydrolyzate*
Ala^P	0.96(1)	0.28
Ser	0.87(1)	0.32
Pro	1.00(1)	2.5
Gly	1.01(1)	
Leu	2.00(2)	2.0
Phe	0.96(1)	1.8

*FDAA Derivatives
Hydrolysis (6M HCl, 110 °C, 22 h)



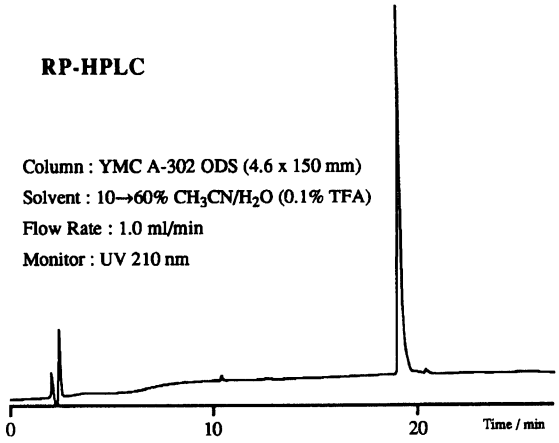


Fig. 8. Characterization of phosphonic octapeptide analog **1**.

yield 10.3 g (87.5%). Found: C, 62.98; H, 7.07; N, 7.36%. Calcd for $C_{31}H_{41}N_3O_8 \cdot 0.5H_2O$: C, 62.82; H, 7.14; N, 7.09%. ^{1}H NMR ($CDCl_3$) δ=3.82 (2H, d, J=5.6 Hz, Gly α).

Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–OPac (6). To Boc–Gly–L-Ser(Bzl)–L-Leu–OPac (10.0 g, 17.1 mmol), trifluoroacetic acid (52.7 ml, 0.684 mol) was added. The solution was allowed to stand at room temperature for 30 min. After concentration of the solution under reduced pressure, 5.7 M hydrogen chloride in 1,4-dioxane solution (9.32 ml) and ether (50 ml) were added, and the precipitate which formed was collected by suction filtration. The solid was added to a solution of Z–L-Phe–OH (3.56 g, 20.3 mmol) and HOBt (2.89 g, 21.3 mmol) in DMF (50 ml). WSCD (3.77 ml, 21.3 mmol) was then added to the solution under ice-salt cooling. This mixture was stirred for 30 min, and then at room temperature for 18 h. After removal of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate, and washed successively with 10% aqueous citric acid, brine, saturated aqueous sodium hydrogencarbonate, and brine. The gel which precipitated from the ethyl acetate solution was collected by suction filtration; yield 12.0 g (91.8%). Mp 45—46 °C. Found: C, 65.46; H, 6.35; N, 7.17%. Calcd for $C_{43}H_{48}N_4O_9 \cdot 1.5H_2O$: C, 65.22; H, 6.49; N, 7.08%. $[\alpha]_D^{26}$ −24.8° (c 1.08, DMF). ^{1}H NMR (DMSO-d_6) δ=3.08 (1H, dd, J=3.7 and 13.7 Hz) and 2.77 (1H, dd, J=10.8 and 13.7 Hz).

Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–OH (7). To a solution of Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–OPac (3.82 g, 4.99 mmol) in 90% aqueous acetic acid, zinc dust (16.3 g, 0.250 mol) was added under ice-cooling. The reaction mixture was vigorously stirred at 0 °C for 10 min, and then at room temperature for 30 min. Insoluble material was filtered off, and the filtrate was concentrated under reduced pressure. The residue was collected by suction filtration and washed with hexane; yield 2.37 g (73.4%). Found: C, 61.15; H, 6.28; N, 8.12%. Calcd for $C_{35}H_{42}N_4O_8 \cdot 2.25H_2O$: C, 61.17; H, 6.82;

N, 8.15%.

Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–ONSu (8). To a solution of Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–OH (647 mg, 1.00 mmol) in DMF (ml), *N*-hydroxysuccinimide (121 mg, 1.05 mmol) and WSCD·HCl (201 mg, 1.05 mmol) were added under ice-cooling. The reaction mixture was stirred at 0 °C for 30 min, and then at room temperature for 17 h. The reaction mixture was concentrated under reduced pressure, extracted with ethyl acetate, and then washed with brine. The organic layer was dried over anhydrous magnesium sulfate, and concentrated under reduced pressure. The oily residue was crystallized in hexane and collected by suction filtration; yield 757 mg (100%). ^{1}H NMR (DMSO-d_6) δ=2.79 (4H, s, ONSu).

Boc–L-Pro–OPac (10). To a solution of Boc–L-Pro–OH (6.46 g, 30.0 mmol) and triethylamine (4.58 ml, 33.0 mmol) in acetone (60 ml), phenacyl bromide (6.57 g, 33.0 mmol) was added. The reaction mixture was stirred at room temperature for 2 h. After removal of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate and washed with brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure to a residue, which was washed with hexane; yield 9.23 g (92.3%).

Boc–L-Leu–L-Pro–OPac (11). Boc–L-Pro–OPac (8.50 g, 25.5 mmol) was dissolved in 5.7 M hydrogen chloride in 1,4-dioxane solution (90 ml) which was allowed to stand at room temperature for 30 min. Hexane was added to this solution, and the precipitate which formed was collected by suction filtration. Isobutyl chloroformate (3.64 ml, 28.1 mmol) was added to the solution of Boc–L-Leu–OH·H_2O (6.36 g, 25.5 mmol) and *N*-methylmorpholine (6.17 ml, 56.1 mmol) in DMF (50 ml) under ice-salt cooling. The solution was stirred for 10 min. Then H–L-Pro–OPac hydrochloride prepared above was added to it and this mixture was stirred under ice-salt cooling for 30 min, and then at room temperature for 18 h. After removal of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate, and washed successively with 10% aqueous citric acid, brine, saturated aqueous sodium hydrogencarbonate, and brine, The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. Purification by silica gel column chromatography using chloroform as developing solvent gave a colorless solid; yield 9.84 g (86.3%). Mp 41—43 °C. Found: C, 64.07; H, 7.79; N, 6.09%. Calcd for $C_{24}H_{34}N_2O_6$·0.2H_2O: C, 64.04; H, 7.70; N, 6.22%. $[\alpha]_D^{26}$ −96.0° (*c* 3.73, DMF). ^{1}H NMR ($CDCl_3$) δ=4.70 (1H, dd, J=4.4 and 8.0 Hz, Leu α), 4.49 (1H, m, Pro α), 0.98 (3H, d, J=6.5 Hz, Leu δ), and 0.92 (3H, d, J=6.5 Hz, Leu δ).

Ac–L-Phl–L-Pro–OPac (13). Boc–L-Leu–L-Pro–OPac (447 mg, 1.00 mmol) was dissolved in 5.5 M hydrogen chloride in 1,4-dioxane solution (5.45 ml) which was allowed to stand at room temperature for 30 min. Ether was added to this solution, and the precipitate which formed was collected by suction filtration. To the solution of the deprotected dipeptide thus obtained in DMF (2 ml), Ac–L-Phl–OH (208 mg, 1.00 mmol), WSCD (186 μl, 1.05 mmol) and HOBt (142 mg, 1.05 mmol) were added under ice-salt cooling. The solution was stirred at room temperature for 4.5 h. After removal of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate, and washed successively with 10% aqueous citric acid, brine, saturated aqueous sodium hydrogencarbonate, and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. Purification by silica gel column chromatography using a developing solvent of benzene–ethyl acetate (3 : 1) gave a colorless oil; yield 171 mg (31.9%).

Deacetylation of Compound 13 with Triethylamine. A mixed solution of Ac–L-Phl–L-Leu–L-Pro–OPac (46.5 mg, 86.7 μmol) and triethylamine (13.2 μl, 95.4 μmol) in methanol (300 μl) was stirred at room temperature for 3 d. After removal of the solvent under reduced pressure, the residue was purified by preparative silica gel thin-layer chromatography (developing solvent: benzene–ethyl acetate 3 : 1) to give a partially racemized compound **14**; yield 31.8 mg (75.9%). ^{1}H NMR ($CDCl_3$) δ=4.46 and 4.32 (dd, α proton of Phl, the ratio of the signals is 1 : 4.)

H–L-Phl–OBzl (16). To a solution of (*S*)-3-phenyllactic acid (4.83 g, 29.1 mmol) in acetone (60 ml), triethylamine (4.44 ml, 32.0 mmol), and benzyl bromide (3.78 ml, 32.0 mmol) were added. The reaction mixture was stirred at room temperature for 6 h. After concentration under reduced pressure, the residue was dissolved in ethyl acetate and washed with water. The organic layer was dried over anhydrous magnesium sulfate. Concentration under reduced pressure gave oily product; yield 6.71 g (90.1%).

Boc–L-Phl–OH (17). A mixed solution of H–L-Phl–OBzl (1.24 g, 4.84 mmol), di-*t*-butyl dicarbonate (1.11 ml, 4.84 mmol), and 4-dimethylaminopyridine (177 mg, 1.45 mmol) in ethyl acetate (20 ml) was stirred at room temperature for 30 min. The reaction mixture was washed with 10% aqueous citric acid and brine. An organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. The oily product was dissolved in methanol (40 ml). Hydrogen was bubbled through the solution at room temperature for 2.5 h in the presence of Pd-black (100 mg). Concentration of the solution under reduced pressure after filtration of the catalyst gave an oily product; yield 1.22 g (94.6%). ^{1}H NMR ($CDCl_3$) δ=7.5 (5H, m, aromatic), 5.13 (2H, dd, J=4.4 and 8.0 Hz, benzyl), 4.95 (1H, dd, J=5.4 and 8.0 Hz, Phl α), 3.2 (2H, m, Phl β), 1.44 (9H, s).

Boc–L-Phl–L-Leu–L-Pro–OPac (18). A mixture of Boc–L-Leu–L-Pro–OPac (3.80 g, 8.25 mmol), and 5.5 M hydrogen chloride in 1,4-dioxane solution (46.5 ml) was allowed to stand at room temperature for 40 min. Ether was added to the solution, and the precipitate was collected by suction filtration. Dipeptide Pac ester hydrochloride prepared above was added to a cold solution of Boc–L-Phl–OH (2.27 g, 8.52 mmol), *N*-methylmorpholine (1.87 ml, 17.0 mmol), and isobutyl chloroformate (1.11 ml, 8.52 mmol) in DMF (50 ml) and stirred under ice-salt cooling for 10 min, and then at room temperature for 18 h. After removal of the solvent by concentration under reduced pressure, the residue obtained was dissolved in ethyl acetate, and washed successively with 10% aqueous citric acid, brine, saturated aqueous sodium hydrogencarbonate, and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. Purification by silica gel column chromatography using toluene–ethyl acetate 10 : 1 as developing solvent gave a colorless powder; yield 4.26 g (97.0%). Mp 50—53 °C. Found: C, 66.22; H, 7.30; N, 5.07%. Calcd for $C_{33}H_{42}N_2O_8$: C, 66.65; H, 7.12; N, 4.71%. $[\alpha]_D^{28}$ −77.2°

(*c* 0.624, DMF). ^{1}H NMR ($CDCl_3$) δ=5.23 (1H, m, Phl α), 4.83 (1H, m, Pro α), and 4.72 (1H, m, Leu α).

H–L-Phl–L-Leu–L-Pro–OPac (19). A solution of Boc–L-Phl–L-Leu–L-Pro–OPac (3.80 g, 8.25 mmol) and 5.5 M hydrogen chloride in 1,4-dioxane solution (151 ml) was allowed to stand at room temperature at 40 °C for 1 h. Purification by silica gel column chromatography using toluene–ethyl acetate (9:1) as the developing solvent gave a colorless powder; yield 3.09 g (75.2%). Mp 130—131 °C. Found: C,66.78; H, 7.20; N, 5.57%. Calcd for $C_{28}H_{34}N_2O_6 \cdot 0.5H_2O$: C, 66.78; H, 7.01; N, 5.56%. $[\alpha]_D^{28}$ −108.6° (*c* 1.63, DMF). ^{1}H NMR ($CDCl_3$) δ=4.32 (1H, m, Phl α).

Z–L-Ala^P(OMe)–OH (22). To a suspension of aminophosphonic acid (5.00 g, 39.2 mmol) in a mixture of dioxane–water (1:1) (40 ml), 2-(benzyloxycarbonyloxy)iminophenylacetonitrile (15.0 g, 53.5 mmol) was added. The reaction mixture was stirred at room temperature for 2 h. After concentration under reduced pressure, the residual oil obtained was suspended in saturated aqueous sodium hydrogencarbonate and washed three times with ethyl acetate. The aqueous layer was acidified to pH 1 with concentrated hydrochloric acid, extracted three times with ethyl acetate, then washed with brine. An organic layer was dried over anhydrous magnesium sulfate. The residual oil after concentration under reduced pressure was dissolved in trimethyl orthoformate (200 ml), and heated at 110 °C for 30 min. Again the residual oil obtained after concentration under reduced pressure was suspended in a saturated aqueous sodium hydrogencarbonate solution and washed three times with ethyl acetate. An aqueous layer was acidified to pH 1 with concentrated hydrochloric acid, extracted three times with ethyl acetate, and then washed with brine. An organic extract was dried over anhydrous magnesium sulfate. Concentration under reduced pressure gave a colorless solid; yield 8.60 g (78.9%). Found: C, 48.25; H, 5.90; N, 5.07%. Calcd for $C_{11}H_{16}NO_5P$: C, 48.35; H, 5.90; N, 5.13%. ^{1}H NMR ($CDCl_3$) δ=7.33 (s, 1H), 5.30 (br., 1H), 5.11 (s, 2H), 4.20 (br., 1H), 3.71 (d, J=9.6 Hz, 3H), and 1.35 (dd, 3H).

Z–L-Ala^P(OMe)–L-Phl–L-Leu–L-Pro–OPac (23). To a suspension of Z–L-Ala^P(OMe)–OH (1.24 g, 4.55 mmol) in carbon tetrachloride (10 ml), thionyl chloride (664 μl, 9.10 mmol) was added. The reaction mixture was stirred at room temperature for 1.5 h and finally concentrated to oil. On the other hand, a mixed solution of H–L-Phl–L-Leu–L-Pro–OPac (1.50 g, 3.03 mol) in tetrahydrofuran (THF) (5 ml) and 1.12 M *t*-butylmagnesium chloride in THF solution (4.06 ml, 4.55 mmol) was prepared and stirred at 0 °C for 5 min. To the solution, the above residual oil dissolved in THF (2 ml) was added under ice-cooling and the mixture was stirred for 1 min. Immediately, the mixture was transferred to 10% aqueous citric acid and extracted twice with ethyl acetate. An organic layer was washed successively with brine, saturated aqueous sodium hydrogencarbonate, and brine. The solution was dried over anhydrous magnesium sulfate and concentrated under reduced pressure. Purification by silica gel column chromatography using chloroform–methanol with ratio of 19:1 as developing solvent gave a colorless solid; yield 1.58 g (69.6%). Mp 60—62 °C. Found, C, 62.04; H, 6.72; N, 5.39%. Calcd for $C_{39}H_{48}N_3O_{10}P \cdot 0.3H_2O$: C, 62.03; H, 6.49; N, 5.56%. $[\alpha]_D^{26}$ −66.0° (*c* 1.22, DMF). ^{1}H NMR ($CDCl_3$) δ=3.42 (3H, d, J=10.8 Hz, Ala^P OCH_3), and 1.45 (3H, dd, J=7.5 and 17.5 Hz, Ala^P β).

H–L-Phl–L-Leu–L-Pro–OH (26). To a solution of H–L-Phl–L-Leu–L-Pro–OPac (90.2 mg, 0.182 mmol) in 90% aqueous acetic acid, zinc dust (595 mg, 9.10 mmol) was added under ice-cooling. The mixture was vigorously stirred at 0 °C for 10 min, and then at room temperature for 30 min. Insoluble material was filtered off, and the filtrate was concentrated under reduced pressure. The residue was collected by suction filtration and washed with hexane; yield 57.6 mg (84.0%). Mp 222—225 °C. Found: C, 63.54; H, 7.66; N, 7.28%. Calcd for $C_{20}H_{28}N_2O_5$: C, 63.81; H, 7.50; N, 7.44%. $[\alpha]_D^{26}$ −87.5° (*c* 0.04, DMF).

H–L-Phl–L-Leu–L-Pro–OBzl (27). A mixed solution of H–L-Phl–L-Leu–L-Pro–OH (51.8 mg, 0.138 mmol), triethylamine (19.2 μl, 0.138 mmol), and benzyl bromide (16.3 μl, 0.138 mmol) in DMF (500 μl) was stirred at room temperature for 1 h. After removal of the solvent by concentration under reduced pressure, the residue was dissolved in ethyl acetate, and washed successively with water, saturated aqueous sodium hydrogencarbonate, and brine. An organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced pressure to give an oily product; yield 53.9 mg (83.7%). ^{1}H NMR ($CDCl_3$) δ=5.15 (2H, d, J=12.2 Hz, Benzyl) and 5.04 (2H, d, J=12.2 Hz, Benzyl).

Z–L-Ala^P(OMe)–L-Phl–L-Leu–L-Pro–OBzl (28). To a suspension of Z–L-Ala^P(OMe)–OH (410 mg, 1.50 mmol) in carbon tetrachloride (3 ml), thionyl chloride (219 μl, 3.00 mmol) was added. The reaction mixture was stirred at room temperature for 1.5 h, and then concentrated to residual oil. The residue obtained was dissolved in THF (2 ml), to which a mixed solution of H–L-Phl–L-Leu–L-Pro–OBzl (467 mg, 1.00 mmol) in tetrahydrofuran (THF) (5 ml), and 1.12 M *t*-butylmagnesium chloride in THF solution (1.34 ml, 1.50 mmol) was added under ice cooling; this mixture was stirred for 1 min. Immediately, the mixture was transferred to 10% aqueous citric acid and extracted twice with ethyl acetate. An organic layer was washed successively with brine, saturated aqueous sodium hydrogencarbonate, and brine again. The extract was dried over anhydrous magnesium sulfate and then concentrated under reduced pressure. Purification by silica gel column chromatography using chloroform–methanol (100:1) as developing solvent gave a colorless solid; yield 589 mg (81.6%). Mp 37—40 °C. Found: C, 63.23; H, 6.89; N, 5.76%. Calcd for $C_{38}H_{48}N_3O_9P$: C, 63.24; H, 6.70; N, 5.82%. $[\alpha]_D^{28}$ −57.1° (*c* 2.97, DMF). ^{1}H NMR ($CDCl_3$) δ=3.71 and 3.39 (3H, d, J=10.8 Hz, OCH_3).

Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–L-Ala^P(OMe)–L-Phl–L-Leu–L-Pro–OH (29). Hydrogen was bubbled through a solution of Z–L-Ala^P(OMe)–L-Phl–L-Leu–L-Pro–OBzl (151 mg, 0.209 mmol) and acetic acid (2 ml) in methanol (50 ml) in the presence of Pd-black (100 mg) for 40 min. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure. The residual oil was dissolved in DMF (500 μl). To the solution, Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–ONSu (155 mg, 0.209 mmol) was added, and then this mixture was stirred at room temperature for 1 d. After removal of the solvent by concentration under reduced pressure, the residue obtained was dissolved in ethyl acetate, and washed with 10% aqueous citric acid and brine successively. An organic layer was dried over anhydrous magnesium sulfate and concentrated under reduced

pressure. Purification by silica gel column chromatography using developing solvent of chloroform–methanol–acetic acid (95 : 5 : 3) gave a colorless solid; yield 210 mg (89.3%). Mp 124—126 °C. Found: C, 60.35; H, 6.89; N, 8.50%. Calcd for $C_{58}H_{76}N_7O_{14}P \cdot 1.5H_2O$: C, 60.41; H, 6.90; N, 8.50%. $[\alpha]_D^{28}$ −44.3° (*c* 0.377, DMF). ^{1}H NMR ($CDCl_3$) δ=3.22 (3H, d, J=10.8 Hz, OCH_3), and 0.82 (12H, m, Leu δ).

Z–L- Phe– Gly–L- Ser(Bzl)–L- Leu–L- Ala^P(ONa)–L-Phl–L-Leu–L-Pro–OH. A solution of Z–L-Phe–Gly–L- Ser(Bzl)–L- Leu–L- Ala^P(OMe)–L- Phl–L- Leu–L- Pro– OH (39.8 mg, 35.3 μmol) and sodium iodide (79.8 mg, 0.530 mmol) in 2-butanone (4 ml) was heated at 80 °C for 1.5 h. After removal of the solvent by concentration under reduced pressure, purification of demethylated phosphonic acid product by high-performance liquid chromatography (Nacalai Cosmosil 5C_{18} (10×250 mm), 50→80→80% (0→20→30 min) CH_3CN–H_2O (0.1% TFA), 3.0 ml min^{-1}) gave a colorless powder; yield 31.7 mg (78.9%).

H–L- Phe–Gly–L- Ser–L- Leu–L- Ala^P(ONa)–L- Phl–L-Leu–L-Pro–OH (1). Hydrogen was bubbled through a solution of Z–L-Phe–Gly–L-Ser(Bzl)–L-Leu–L-Ala^P(ONa)–L-Phl–L-Leu–L-Pro–OH (27.0 mg, 23.8 μmol) obtained above in acetic acid (0.5 ml) and methanol (20 ml) in the presence of Pd-black (50 mg) for 3 h. The catalyst was filtered off, and the filtrate was concentrated under reduced pressure, followed by lyophilization. Purification of the final product by high-performance liquid chromatography (Nacalai Cosmosil 5C_{18} (10×250 mm), 30→80→80% (0→25→30 min) CH_3CH–H_2O (0.1% TFA), 3.0 ml min^{-1}) gave a colorless powder; yield 22.0 mg (100%). Found: C, 51.89, H, 6.78, N, 9.74%. Calcd for $C_{42}H_{61}N_7O_{12}PNa \cdot 0.5TFA \cdot 3H_2O$: C, 51.75; H, 6.81, N, 9.82%. Amino Acid Analysis: Ala^P 0.96 (1), Ser 0.87 (1), Pro 1.00 (1), Gly 1.01 (1), Leu 2.00 (2), Phe 0.96 (1). ^{1}H NMR (DMSO-d_6) δ=7.3 (10H, m, aromatic ×2), 1.10 (3H, dd, J=7.3 and 15.3 Hz, Ala^P β), and 0.80 (12H, dd, J=6.6 and 10.3 Hz, Leu δ×2).

References

1) K. Inami, T. Teshima, and T. Shiba, "63rd National Meeting of the Chemical Society of Japan," Higashi-Osaka, March 1992, Abstr., No. 3E7 42.

2) K. Yamauchi, *Yuki Gosei Kagaku Kyokaishi*, **46**, 654 (1988); K. Yamauchi, *Nippon Nogeikagaku Kaishi*, **65**, 1493 (1991).

3) J. E. Hanson, A. P. Kaplan, and P. A. Bartlett, *Biochemistry*, **29**, 6294 (1989); P. A. Bartlett, J. E. Hansen, and P. P. Giannousis, *J. Org. Chem.*, **55**, 6268 (1990); M. A. Phillips, A. P. Kaplan, W. J. Rutter, and P. A. Bartlett, *Biochemistry*, **31**, 959 (1992).

4) Abbreviations: Ala^P: α-aminoethylphosphonic acid; Phl: 3-phenyllactic acid; Z: benzyloxycarbonyl; Bzl: benzyl; Boc: *t*-butoxycarbonyl; WSCD : water-soluble carbodiimide (*N*-ethyl-*N'*-[3-(dimethylamino)propyl]carbodiimide); HOBt: 1-hydroxybenzotriazole; Pac: phenacyl (benzoylmethyl); DMAP: 4-(dimethylamino)pyridine; HONSu: 1-hydroxysuccinimide; NMM: *N*-methylmorpholine.

5) H. Kuroda, S. Kubo, N. Chino, T. Kimura, and S. Sakakibara, *Int. J. Pept. Protein Res.*, **40**, 114 (1992).

6) Two double doublets appeared at 4.32 and 4.46 (4 : 1) which should be assigned as the α-protons of L- and D-3-phenyllactic acid residues, respectively.

7) The starting material disappeared after 38 h.

8) Y. Hayakawa, S. Watanabe, T. Nobori, and R. Noyori, *Tetrahedron Lett.*, **28**, 2259 (1987); M. Uchiyama, Y. Aso, R. Noyori, and Y. Hayakawa, *J. Org. Chem.*, **58**, 373 (1993).

9) P. Marfey, *Carlsberg Res. Commun.*, **49**, 591 (1984).