 © 1988 The Chemical Society of Japan Bull. Chem. Soc. Jpn., 61, 4315—4321 (1988)

Synthesis of Fluorescent Amino Acids and Peptides with 2-Aminopyridine at the Carboxyl or Amino Terminus

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(Received April 16, 1988)

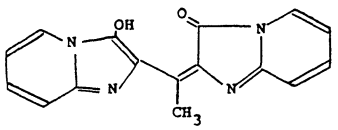
N-(2-Pyridyl) derivatives of glycine (**1**), DL-alanine (**2**), DL-valine (**3**), and DL-norleucine (**4**) were synthesized as fluorescence labeled amino acids. These amino acids were used for the synthesis of N-terminal fluorescence-labeled peptides. *N*-(2-Pyridyl)-1,2-ethanediamine (**7**) was synthesized and used for the C-terminal labeling of amino acids and peptides. With these amino acid derivatives, some peptides labeled with 2-aminopyridine were synthesized and their spectroscopic characteristics were examined.

Fluorescent labeling of the reducing ends of sugars with 2-aminopyridine has become a useful method in the structure analyses of the sugar moiety of glycoconjugates.[1] This is because of the stability of the 2-pyridylamino group against acid, alkaline, and other reagents, and also the facility both for the detection and purification of 2-pyridylaminosugars during electrophoresis or in several chromatographies including HPLC, owing to its positive charge in acidic conditions or the hydrophobic nature in alkaline conditions. Fluorescence-labeled sugars has been used as substrates for some carbohydrases[2] and glycosyltransferases.[3] This labeling can be employed in the preparation of fluorescent amino acids and peptides that can be used as substrates for enzymes participating in the post-translational modification of the proteins or peptides. Labelling of the N-terminus, C-terminus, or both termini of a peptide may protect the molecule against exopeptidase digestion and serve as the fluorescence marker for the compound. No peptide with 2-aminopyridine has been synthesized. This prompts us to synthesize the *N*-2-pyridylamino peptides. *N*-(2-Pyridyl)glycine (**1**), the sole amino acid reported as a 2-pyridylamino derivative, seemed to be a suitable compound for use in the synthesis of N-terminal and C-terminal fluorescence-labeled peptides, but it was found not to be suitable for the labeling of the C-terminus of peptides because of the low reactivity of its imino group to acylation and because acylation causes the loss of its fluorescence. We then selected *N*-(2-pyridyl)-1,2-ethane diamine (**7**) as a reagent for C-terminal fluorescence-labeling of amino acids and peptides, and found **1** is synthesized by the reduction of *N*-(2-pyridylamino)acetaldehyde oxime with $LiAlH_4$. Here, we develop a new easy route for the preparation of **7**, synthesize some fluorescent peptides with **7** at the C-terminal together with peptides with **1** or **2** at their N-terminal, and examine their fluorescent and UV spectra at different pH's.

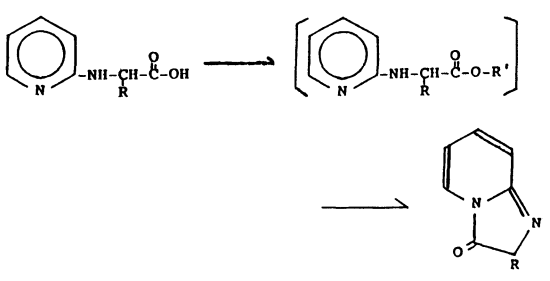
Results and Discussion

Four methods have been reported for the synthesis of **1**.[4] We synthesized **1** from formaldehyde via its nitrile by the method of Ohta and Masaki.[4b] This method could be applied to the synthesis of other *N*-(2-pyridyl) derivatives of DL-alanine (**2**), DL-valine (**3**), and DL-norleucine (**4**) using acetaldehyde, isobutyraldehyde, and valeraldehyde as the starting material, respectively. These four *N*-(2-pyridyl) α-amino acids can be used similarly for the syntheses of N-terminal fluorescence-labeled peptides. Here, the synthesis of some peptides starting from **1** and **2** and the characteristics of the peptides were examined.

Blocking the imino group of **1** or **2** by a benzyloxycarbonyl or *t*-butoxycarbonyl group was unsuccessful. *N*-Acetylation of **1** by acetic anhydride in pyridine gave a green by-product, which was not identified, but seemed to be mesomethyloxonol, because **1** is reported to give a green dye with the structure shown below when treated with hot acetic anhydride.[5]



Accordingly, the coupling of **1** to amino acids or peptides was done without blocking the imino group in chloroform with dicyclohexylcarbodiimide (DCC) or in water with water soluble carbodiimide (WSC). When peptide synthesis was carried out in chloroform or methylene dichloride, powdered **1** was used because of the low solubility of **1** in the solvents. The reaction solution of **1** became greenish in a few minutes, and the color increased to dark green or dark purple depending upon the pH of the solution. The yield of the desired peptide was about 50—60% on several conditions and did not exceed 70%. The synthesis of active esters of **1** was unsuccessful, because the active ester may be unstable and convert to 1,3a-diazaindan-3-one.[5]



The coupling reaction with **2** proceeded like that with **1**, but the reaction solution turned yellow, because of the formation of unknown by-products.

N-(2-Pyridyl)-1,2-ethanediamine (**7**) has been synthesized from *N*-(2-pyridylamino)acetaldehyde oxime by reduction with $LiAlH_4$.[4a] We found that **7** could be prepared by heating a mixture of 2-chloropyridine and ethylenediamine in a good yield.

Coupling of **7** to carboxyl components proceeded with a good yield in the presence of DCC or WSC without formation of the colored by-products. The 2-pyridylamino derivatives were usually soluble in water at acidic pH and in organic solvents at alkaline pH. These characteristics were sometimes useful and sometimes disadvantageous in the purification of the product. As a whole, the synthesis of 2-pyridylamino peptides was more troublesome than the normal peptide synthesis.

Since the spectroscopic characteristics of *N*-2-pyridyl amino acids or peptides have not been reported, the effect of pH on the UV and fluorescence spectra of these compounds was examined first. Figures 2-A and 2-B present the emission and excitation spectra of **1** at different pH's. The figures show that the fluorescence intensity of **1** is influenced strongly by pH, but the maximum wavelengths for excitation and emission are not influenced. Figure 2-C shows the emission spectra of the four compounds **1**, **2**, **5**, and **7** under the same conditions. The fluorescence intensity decreased in the order of **5**>**7**>**1**>**2**. Compound **2** seems not to be very useful for the synthesis of fluorescent peptides because of its low fluorescence intensity. But the peptide produced from **2** had the normal fluorescence intensity (as shown in Fig. 3-B).

Figure 3 shows the pH dependence of the fluorescence intensities of nine compounds. The fluorescence intensities of these compounds were lowest at around pH 5, and high at around pH 10—11, becoming very low above pH 12. These characteristics were common to all of the 2-pyridylamino derivatives examined. The fluorescence intensities of the *N*-2-pyridylamino acids **2** (Fig. 3-B), **3** (Fig. 3-C), and **4** (data not shown) were weaker than those of the other 2-pyridylamino compounds, but gradually increased when **2** and **3**

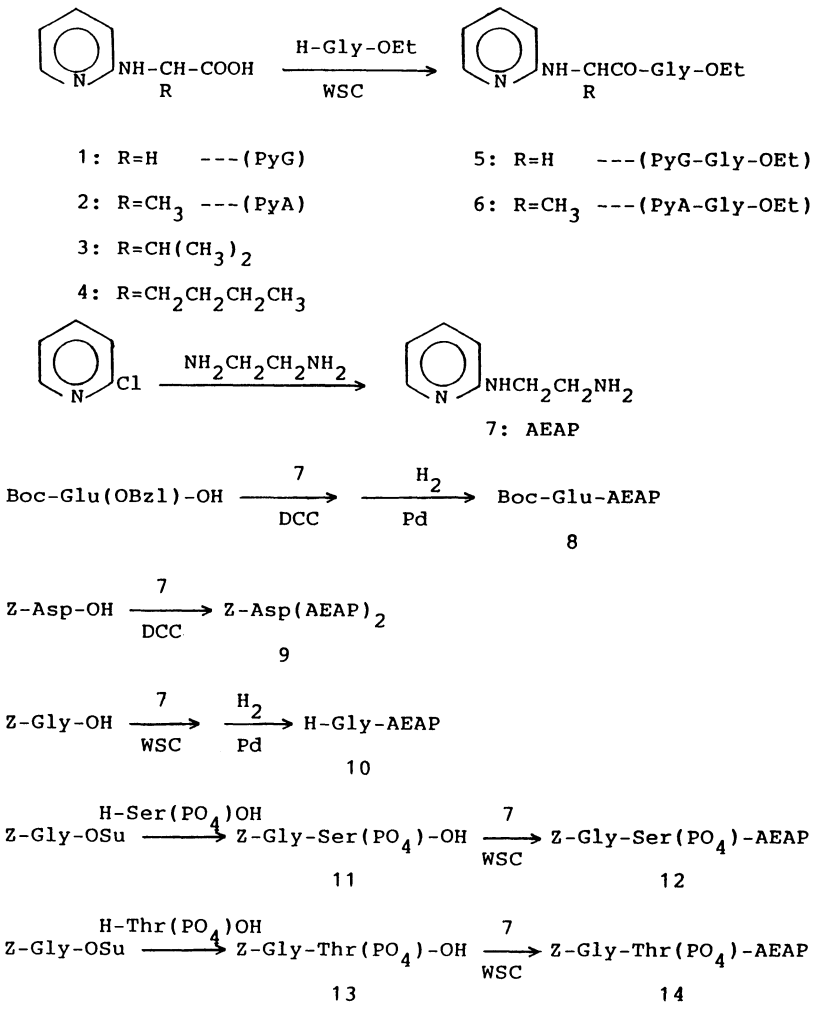


Fig. 1. Structures and syntheses of several fluorescent amino acids and peptides labeled with 2-aminopyridine.

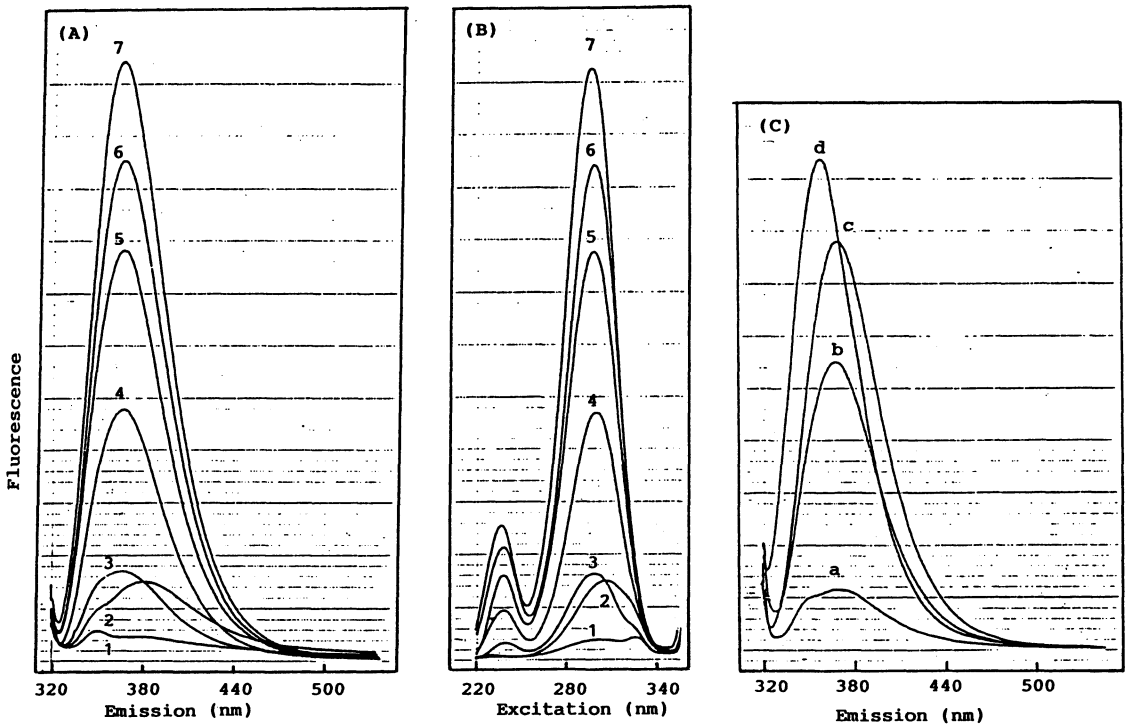


Fig. 2. (A) Emission spectra of **1** in different buffers.
Fluorescence was measured at the excitation wavelength of 304 nm and at the concentration of 2.4 μM. Solvents used were: 1, pH 5 (acetate); 2, 0.1 M HCl; 3, pH 6 (acetate); 4, 0.1 M NaOH; 5, pH 7 (acetate); 6, pH 8 (borate); 7, pH 10 (borate).
(B) Excitation spectra of **1** at the emission wavelength of 375 nm in different solvents. Solvents used were the same as those in A.
(C) Emission spectra of some 2-pyridylamino derivatives measured in borate buffer, pH 10, at the excitaton wavelength of 304 nm and at the concentration of 1.2 μM. Compounds were as follows: a, *N*-(2-pyridyl)-DL-alanine; b, *N*-(2-pyridyl)glycine; c, *N*-(2-pyridyl)-1,2-ethanediamine, and d, PyG-Gly-OEt.

were kept acidic.

The fluorescent intensities of N-terminal-labeled peptides (**5** and **6**) were higher than those of the component amino acids **1** or **2** (Fig. 3-A or 3-B). Fluorescence intensities of the C-terminal-labeled amino acids **8** (Fig. 3-E) and **10** (Fig. 3-D) were higher than those of N-terminal-labeled derivatives.

Figure 4 shows the absorption spectra of **1** in 0.1 M HCl (1 M=1 mol dm^{-3}), 0.1 M NaOH, and acetate buffer, pH 5. Compound **1** had two maxima around 240 and 306 nm. The absorbance at 306 nm in acidic pH was higher than in alkaline pH. The absorption maxima and molar extinction coefficients of compounds **2** and **3** in either 0.1 M HCl or 0.1 M NaOH were almost the same as those of **1** (Table 1). Compounds **6**, **7**, and **8** also had similar UV absorption spectra.

These spectroscopic characteristics were convenient for the detection of the 2-pyridylamino derivatives by TLC and HPLC.

Compound PyG-Gly-OEt (**5**), DL-PyA-Gly-OEt (**6**), Boc-Glu-AEAP (**8**), and Z-Asp(AEAP)$_2$ (**9**) were synthesized to test the coupling reaction and to use as starting materials for the synthesis of many fluorescent peptides. We also synthesized fluorescent phosphopeptides Z-Gly-Ser(PO_4)-AEAP and Z-Gly-Thr(PO_4)-AEAP to study the β-elimination reaction of phosphoserine and phosphothreonine peptides and as substrates for phosphatases. In the coupling of Z-Gly-Ser(PO_4)-OH (**11**) and **7** with WSC, one main product (peak c) appeared (Fig. 5), it was found to be **12**, Z-Gly-Ser(PO_4)-AEAP. Peaks a and b were **7** and **11**, respectively. This shows that *N*-blocked phosphoserine or phosphothreonine could be used as an acid component without blocking the phosphate. The reaction was completed in 2 h and the product (peak c) was isolated using HPLC.

In a study of hen riboflavin-binding protein (RBP), we have found that RBP in hen blood, the precursor of RBP in egg yolk, was 11 or 13 amino acid residues longer than the protein in egg yolk.[6] It is likely that the C-terminal part is removed by protease(s) in the oocyte membrane during the incorporation of RBP from the blood to the oocyte (egg yolk). To search for a specific protease that modifies the C-terminal of the protein, a fluorescent peptide (**15**), PyG-Gln-Lys-Lys-Leu-Leu-Lys-Phe-Glu-βAla having a similar amino acid sequence to that of the C-terminal part was

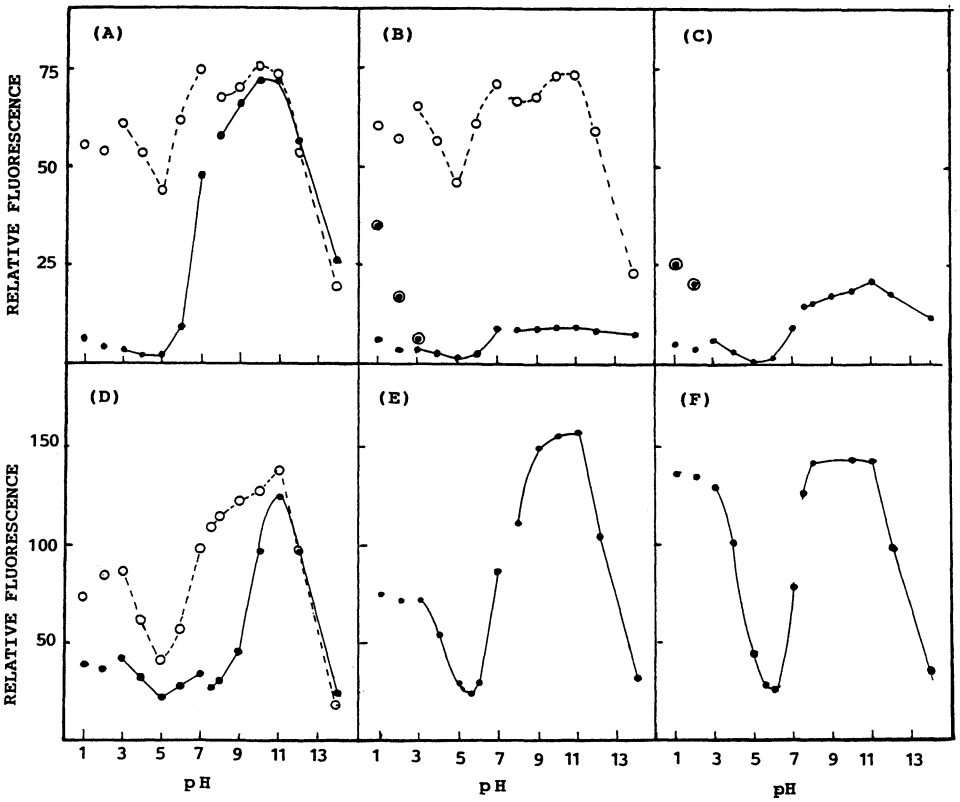


Fig. 3. Relationship of fluorescence and pH for some 2-pyridylamino derivatives at the concentration of 1.2 μM.
(A), **1** (●) and **5** (○); (B), **2** (●) and **6** (○); (C), **4**; (D),**7** (●) and **10** (○); (E), **8** and (F), **9**. The marks (○) in (B) and (C) were the values after 24 h. The excitation wavelength was 304 nm and the emission wavelength was 375 nm. Solvents were: pH 1, 0.1 M HCl; pH 2, 0.02% TFA; pH 3—7, 0.1 M acetate buffer; pH 7.5—12, 0.1 M borate buffer; pH 13, 0.1 M NaOH.

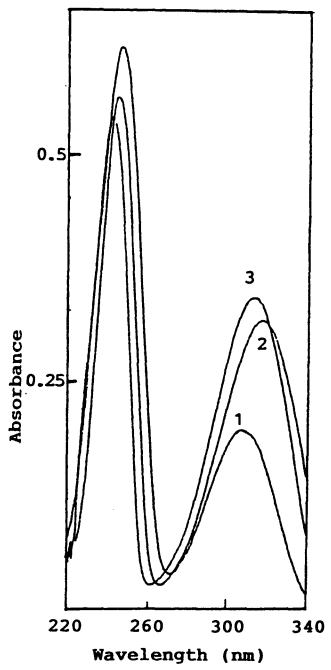


Fig. 4. Absorption spectra of **1** in 0.1 M NaOH (1), 0.1 M acetate buffer (pH 5) (2), and 0.1 M HCl (3) at the concentration of 50 μM.

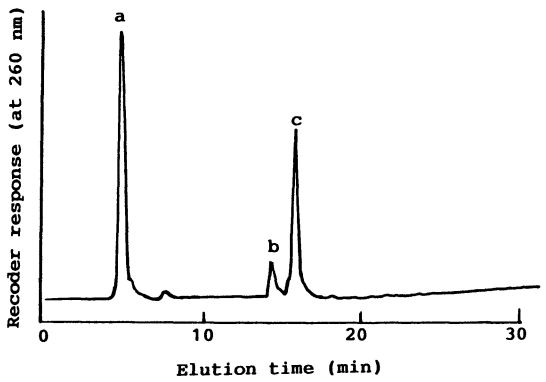


Fig. 5. HPLC of the reaction mixture of **11** and **7** with WSC. Peaks a, b, and c were **7**, **11**, and **12**. Elution was with an exponential gradient of CH_3CN from 0% to 50% in 0.05% TFA, and the starting volume was 50 ml. Column, ODS-120 T (7×250 mm); flow rate, 3 ml min^{-1}.

synthesized. The synthesis of **15** was performed by the solid phase peptide synthesis. In the coupling of **1** with peptides on the resin, the reaction was performed in the CH_2Cl_2 with the suspension of **1** because of its low solubility in the solvent. The reaction solution

Table 1. UV-Spectra of 2-Pyridylamino Derivatives

Compound	Solvent (0.1 M)	λ_{max}	ε_{max}[a)]	λ_{max}	ε_{max}[a)]
		nm	$l\,mol^{-1}cm^{-1}$	nm	$l\,mol^{-1}cm^{-1}$
1	HCl	308	7200	237	11400
	NaOH	302	4300	242	13200
	pH 5[b)]	316	6500	244	11500
2	HCl	308	7200	237	11700
	NaOH	302	4100	242	12700
3	HCl	307	7300	235	12000
	NaOH	302	3800	240	13400
5	HCl	308	7100	238	11100
	NaOH	304	4200	245	12300
7	HCl	308	7200	237	11800
	NaOH	302	4100	243	13200
8	HCl	310	6400	238	11300
	NaOH	307	3800	248	12300
9	HCl	312	12600	242	22100
	NaOH	308	7500	248	24100
10	HCl	308	6400	236	11600
	NaOH	301	3600	239	12200

a) The value is at its maximum wavelength. b) Sodium acetate.

colored dark purple and the resin was stained with the same color. In spite of the strong staining of the resin and using the suspension of **1**, the yield of the coupling was fairly good.

Since fluorescence labeled peptides with 2-aminopyridine can be synthesized by the usual method or with reagents as described above, the labeled peptides are expected to work as useful compounds for the analyses of some enzymes or some reactions owing to its unique spectroscopic characteristics.

Experimental

General. Melting points were measured on a Yanagimoto melting point apparatus and were uncorrected. ^1H NMR spectra were recorded on a Varian XL-100 (100 MHz) or a JEOL FX-90 (90 MHz) spectrometer with Me_4Si as an internal standard, unless otherwise stated.

TLC was done on silica-gel plates (Merck Kieselgel 60 F254). The solvent system was *n*-BuOH-AcOH-H_2O (4 : 1 : 1) unless otherwise stated. HPLC conditions were as follows: column, TSK ODS-120T (7×250 mm); flow rate, 3 ml min^{-1}; detection of pyridylamino compounds at 310 nm or of benzyloxycarbonyl compounds at 260 nm; elution systems; (1), exponential or linear gradient of 0.05% TFA and 50% CH_3CN-0.05% TFA; (2) several isocratic conditions. Fluorescence was measured by a Hitachi spectrophotometer 650-10S.

N-(2-Pyridyl)glycine (**1**) and 2-(2-pyridylamino)propionitrile were synthesized by the method of Ohta and Masaki.[4b)]

***N*-(2-Pyridyl)-DL-alanine (2):** A solution of 2-(2-pyridylamino) propionitrile (7 g) in 6 M HCl (100 ml) was refluxed for 5 h and concentrated. Crystals (NH_4Cl) that appeared were filtered off and washed with ethanol. The washings and filtrate were combined and concentrated. The crystals that appeared were filtered off. The filtrate was applied to a Dowex 50 H^+ column (2×12 cm). Compound **2** was eluted with 0.2 M ammonium acetate as a yellow fraction. The solution was concentrated and **2** was crystallized with ethanol; yield 4.7 g (59%); mp 153 °C; pale yellow needles; ^1H NMR (D_2O) δ=1.48 (3H, d, CH_3), 4.0—4.2 (1H, q, CH), 6.8—7.0 (2H, m, pyridine), 7.7—8.0 (2H, m, pyridine). Found: C, 57.82; H, 6.07; N, 16.86%. Calcd for $C_8H_{10}O_2N_2$: C, 57.73; H, 6.10; N, 16.65%.

***N*-(2-Pyridyl)-DL-alanine (2):** A solution of 2-(2-pyridylamino) propionitrile (7 g) in 6 M HCl (100 ml) was refluxed for 5 h and concentrated. Crystals (NH_4Cl) that appeared were filtered off and washed with ethanol. The washings and filtrate were combined and concentrated. The crystals that appeared were filtered off. The filtrate was applied to a Dowex 50 H^+ column (2×12 cm). Compound **2** was eluted with 0.2 M ammonium acetate as a yellow fraction. The solution was concentrated and **2** was crystallized with ethanol; yield 4.7 g (59%); mp 153 °C; pale yellow needles; ^1H NMR (D_2O) δ=1.48 (3H, d, CH_3), 4.0—4.2 (1H, q, CH), 6.8—7.0 (2H, m, pyridine), 7.7—8.0 (2H, m, pyridine). Found: C, 57.82; H, 6.07; N, 16.86%. Calcd for $C_8H_{10}O_2N_2$: C, 57.73; H, 6.10; N, 16.65%.

***N*-(2-Pyridyl)-DL-alaline Hydrochloride:** Mp 160—161 °C. Found: C, 47.42; H, 5.47; N, 13.83; Cl, 17.50%. Calcd for $C_8H_{10}O_2N_2HCl$: C, 47.27; H, 5.46; N, 13.69; Cl, 17.48%.

***N*-(2-Pyridyl)-DL-valine Hydrochloride (3):** Isobutyraldehyde (7.2 g) was mixed with an aqueous solution of $NaHSO_3$ (10 g in 10 ml H_2O) and warmed (60—70 °C) for 0.5 h. 2-Aminopyridine (9.4 g) was added and warmed 0.5 h more. Finally NaCN (7.6 g in 16 ml of water) was added and the mixture was heated under continuous stirring in a boiling water bath for 6 h. The dark brown product of the nitrile (α-(2-pyridylamino)isovaleronitrile) was extracted with 40 ml of chloroform five times, and the organic layer dried over $MgSO_4$ and concentrated. The nitrile was directly hydrolyzed in 6 M HCl (400 ml) under reflux for 6 h. The hydrolysate was concentrated to remove NH_4Cl as crystals and filtered. The filtrate was neutralized with 4 M NaOH, washed with chloroform, and evaporated. The residue was dissolved in water, acidified with 6 M HCl, and concentrated. The crystals that appeared (NaCl) were removed by filtration and washed with acetone. The filtrate and washings were combined and concentrated, giving the desired product in crystalline form. The product was recrystallized from water: Yield, 7.2 g (62%); mp 151—152 °C; ^1H NMR (D_2O) δ=1.7 (6H, d, 2×CH_3), 2.28—2.62 (1H, m, CH), 4.39 (1H, d, CH), 6.9—7.2 (2H, m, pyridine), 7.8—8.1 (2H, m, pyridine). Found: C, 51.94; H, 6.53; N, 12.14%. Calcd for $C_{10}H_{14}N_2O_2HCl$: C, 52.06; H, 6.55; N, 12.14%.

***N*-(2-Pyridyl)-DL-norleucine Hydrochloride (4):** Compound **4** was synthesized similarly as **3** from valeraldehyde. Mp 132—134 °C; ^1H NMR (D_2O) δ=0.9 (3H, t, CH_3), 4.4—5.6 (1H, m, CH), 6.9—7.2 (2H, m, pyridine), 7.8—8.2 (2H, m, pyridine). Found: C, 53.91; H, 6.96; N, 11.42%. Calcd for $C_{11}H_{16}N_2O_2HCl$: C, 53.99; H, 7.00; N, 11.45%.

PyG-Gly-OEt (5): To a solution of **1** (1.9 g), glycine ethyl ester hydrochloride (2.8 g), triethylamine (2 ml) in $CHCl_3$ (80 ml) and DMF (20 ml), and WSC (2.1 g) were added. The mixture was stirred for 2 days at room temperature. The dark green reaction mixture was diluted with 0.1 M HCl, washed with $CHCl_3$ twice and neutralized with triethylamine to pH 7.5. The product was extracted 4 time with $CHCl_3$. The extracts were combined, dried over $MgSO_4$, and concentrated, giving crystalline **5**. The crystals were washed with ethyl acetate. The yield was 1.4 g (61%). Recrystallization was done with ethanol: mp 121.5—122 °C.

^{1}H NMR ($CDCl_3$) δ=1.2—1.34 (3H, t, CH_3), 5.47 (1H, br, NH), 6.4—6.8 (2H, m, pyridine), 7.4—7.6 (1H, m, pyridine), 8.08—8.16 (1H, m, pyridine). Found: C, 55.65; H, 6.38; N, 17.76%. Calcd for $C_{11}H_{15}N_3O_3$: C, 55.69; H, 6.37; N, 17.71%.

DL-PyA-Gly-OEt: WSC (21 mg) was added to a cooled mixture of **2** (17 mg), glycine ethyl ester hydrochloride (28 mg), triethylamine (15 μl), DMF (0.1 ml), and $CHCl_3$ (0.9 ml). The mixture was stirred for 3 h at room temperature. Product **6** was isolated by HPLC with a C_{18}-column. The peak around 7 min on isocratic elution with 25% acetonitrile- 0.01 M NH_4OAc buffer (pH 5.6) was collected, concentrated, and crystallized with ethanol: Yield, 12 mg (48%), mp 109—110 °C. ^{1}H NMR ($CDCl_3$) δ=1.24 (3H, t, $CH_2C\underline{H}_3$), 1.51 (3H, d, $CHC\underline{H}_3$), 5.8 (1H, br, NH), 6.4—6.72 (2H, m, pyridine), 7.3—7.6 (1H, m, pyridine), and 8.08—8.16 (1H, m, pyridine). Found: C, 57.47; H, 6.82; N, 16.78%. Calcd for $C_{12}H_{17}N_3O_3$: C, 57.36; H, 6.82; N, 16.72%.

***N*-(2-Pyridyl)-1,2-ethanediamine Dihydrochloride (7):** A mixture of 1,2-ethanediamine (100 ml) and 2-chloropyridine (10 g) was refluxed for 8 h and concentrated to remove as much of the unreacted 1,2-ethanediamine as possible under reduced pressure. Then 2 M NaOH (40 ml) was added to the residue and the product extracted 8 times with 50 ml of $CHCl_3$ each time. The extracts were evaporated under reduced pressure. Hydrochloric acid was added to the residue and concentrated to crystallize **7**. Recrystallization from a mixture of water and ethanol after charcoal treatment gave **7** as faintly yellow needles: yield 11 g (62%); mp 215—216 °C; ^{1}H NMR (D_2O) δ=3.35 (2H, t, CH_2), 3.79 (2H, t, CH_2), 6.9—7.2 (2H, m, pyridine), and 7.8—8.1 (2H, m, pyridine). Found: C, 39.04; H, 6.22; N, 19.95%. Calcd for $C_7H_{11}N_3$, 2HCl : C, 40.02; H, 6.24; N, 20.00%.

Boc-Glu(OBzl)-AEAP: A solution of Boc-Glu(OBzl)-OH (1.6 g, 5 mmol) in chloroform (20 ml) was mixed with a solution of **7** (1.0 g) in 20 ml of chloroform and 0.7 ml of triethylamine. DCC (1.0 g) was added to the cooled upper solution. After 3 h, the reaction mixture was filtered and evaporated. Ethyl acetate (40 ml) was added to the residue and insoluble materials were removed by filtration. The filtrate was washed twice with 2.5% Na_2CO_3 and then with water, dried over Na_2SO_4, and concentrated to a syrup. The yield was about 80% from the absorbance at 310 nm, the purity was confirmed by TLC (*n*-BuOH-AcOH-H_2O, 4 : 1 : 1) and the compound was used for the next step without further purification.

Boc-Glu-AEAP Acetate (8): The *O*-benzyl group of Boc-Glu(OBzl)-AEAP was removed by hydrogenation over Pd-black in methanol-acetic acid (10 : 1). After filtration and evaporation of the reaction solution, the product was crystallized as acetate from methanol-ethyl acetate: mp 136—137 °C. Found: C, 53.15; H, 7.04; N, 13.14%. Calcd for $C_{19}H_{30}N_4O_7$: C, 53.51; H, 7.09; N, 13.14%.

Z-Asp(AEAP)$_2$ (9): Z-Asp (0.62 g, 2.3 mmol), **7** (1.0 g, 5 mmol), and triethylamine (0.8 ml) were dissolved in a mixture of 5 ml of H_2O and 10 ml of dioxane. WSC (1.1 g, 5.5 mmol) was added to the mixture at 0 °C. After 2 h, the reaction mixture was evaporated; the residue was dissolved in 0.1 M HCl and washed three times with chloroform. After the addition of a sat. $NaHCO_3$ solution to the aqueous layer, the product was extracted twice with $CHCl_3$. The chloroform layer was dried over $MgSO_4$ and concentrated. Compound **9** was crystallized by the addition of ethyl acetate; yield, 0.7 g (60%); mp 103—104 °C; ^{1}H NMR ($CDCl_3$) δ= 2.57—2.72 (2H, m, CH_2), 3.36 (8H, s, 2×CH_2-CH_2), 4.4—4.6 (1H, m, CH), 5.02 (2H, s, $C\underline{H}_2$Ph), 5.5 (2H, br, NH), 6.3—6.7 (4H, m, pyridine), 7.3 (5H, s, Ph), 7.3—7.32 (2H, m, pyridine), 7.5 (1H, s, NH), 7.7 (1H, s, NH), and 7.9—7.96 (2H, m, pyridine). Found: C, 61.22; H, 6.11; N, 19.30%. Calcd for $C_{26}H_{31}N_7O_4$: C, 61.77; H, 6.18; N, 19.39%.

Gly-AEAP (10): WSC (192 mg) was added to a cooled solution of Z-Gly (209 mg), **7** (210 mg), triethylamine (150 μl), chloroform (18 ml), and DMF (2 ml) and stirred for 3 h at room temperature. The product was extracted with 0.1 M HCl, and washed with chloroform. Saturated $NaHCO_3$ was added, and the mixture was extracted with chloroform. The organic layer was concentrated and hydrogenated over Pd-black in 0.1 M HCl to give **10**. Compound **10** was crystallized with ethanol: yield 163 mg (61%); mp 190—192 °C; ^{1}H NMR (D_2O) δ=3.62 (4H, s, CH_2-CH_2), 3.89 (2H, s, CH_2), 6.9—7.2 (2H, m, pyridine), and 7.8—8.1 (2H, m, pyridine). Found: C, 37.78; H, 6.45; N, 19.67%. Calcd for $C_9H_{14}N_4O$, 2HCl, H_2O: C, 37.91; H, 6.36; N, 19.65%.

Z-Gly-Ser(P)OH (11): A solution of Z-Gly-OSu (400 mg) in 2 ml of dioxane was added with stirring to the phosphoserine solution (200 mg) in 4 ml of water and 2 ml of dioxane, the pH of which had been adjusted to 8.5. The mixture was stirred at room temperature for 8 h and concentrated under reduced pressure. The residue was dissolved in water, washed with ethyl acetate three times, and applied to HPLC to purify **11**. Compound **11** was eluted around 13 min, concentrated, and crystallized with chloroform: 246 mg (58%); mp 164—165 °C. ^{1}H NMR (D_2O) δ=3.80 (2H, s, NCH_2), 4.1—4.3 (2H, m, CH_2OP), 5.0 (2H, s, CH_2-O), and 7.26 (5H, s, Ph). Found: C, 41.38; H, 4.43; N, 7.39%. Calcd for $C_{13}H_{17}N_2O_9P$: C, 41.49; H, 4.56; N, 7.44%.

Z-Gly-Thr(P)OH (13): Compound **13** was synthesized in a similar way to **11** with 90 mg (0.45 mmol) of phosphothreonine and 300 mg (0.98 mmol) of Z-Gly-OSu. Compound **13** could not be obtained as a crystal even after purification by HPLC: Yield, 143 mg (78%); ^{1}H NMR (D_2O) δ=1.06 (3H, d, CH_3), 3.74 (2H, s, $NHC\underline{H}_2CO$), 4.42—4.56 (1H, m, CHOP), 4.86 (2H, s, $C\underline{H}_2$Ph), 7.12 (5H, m, Ph).

Z-Gly-Ser(P)-AEAP (12): To a solution of **11** (19.6 mg) and **7** (20.6 mg) in water (0.5 ml), the pH of which had been adjusted to 6, WSC (9.5 mg) was added. The reaction was monitored by the use of HPLC (Fig. 5) to confirm the production of **12** (elution time, 15.5 min). The reaction was completed in 2 h and **12** was isolated by HPLC: Yield, 18 mg (62%); ^{1}H NMR (D_2O) δ=3.48 (4H, s, 2×CH_2), 3.91 (2H, s, NCHCO), 4.4—4.2 (1H, m, CHOP), 5.12 (2H, s, CH_2Ph), 6.28—7.06 (2H, m, pyridine), 7.37 (5H, s, Ph), and 7.76—8.0 (2H, m, pyridine).

Z-Gly-Thr(P)-AEAP (14): Compound **14** was synthesized in a similar way to **12**. The elution time of **14** on HPLC was 15.6 min: Yield, 66%; ^{1}H NMR (D_2O) δ=1.26 (3H, d, CH_3), 3.45 (4H, s, 2×CH_2), 3.92 (2H, s, NCH_2CO), 5.21 (2H, s, $C\underline{H}_2$Ph), 6.8—7.06 (2H, m, pyridine), 7.38 (5H, s, Ph), and 7.7—8.0 (2H, m, pyridine).

PyG-Gln-Lys-Lys-Leu-Leu-Lys-Phe-Glu-βAla (15): Compound **15** was synthesized by the solid phase peptide synthesis.[7] Boc-β-alanine (5 mmol) was coupled to 10 g of chloromethylated polystyrene resin (Peptide Institute, Inc.) according to the method of Gisin[8] and obtained a β-alanyl resin (0.25 mmol g^{-1}). Three fold of Boc-amino acid (26 mmol) was coupled to the resin (3.5 g) stepwise with DCC. The coupling reaction was repeated in the introduction of

Boc-Phe-DCAA and Boc-Gln. In the introduction of *N*-(2-pyridyl)glycine, **1** was powdered in a mortar because of its low solubility in methylene dichloride.

Peptide **15** was purified by HPLC after HF treatment (0 °C, 1 h). The yield was about 55% from the β-alanyl resine. The structure of **15** was confirmed by FAB-mass,[9] (Found: *m/z* 1238.8. Calcd for the structure: M, 1238) and also by amino acid analysis of the peptide. The amino acid composition of **15** was $Glu_{2.1}$ $Leu_{2.0}$ $Phe_{1.0}$ $Lys_{3.0}$ β-$Ala_{1.2}$. PyG could not be analyzed under ordinary analysis conditions.

The authors are grateful to Prof. Yasutsugu Shimonishi, Dr. Toshifumi Takao, and Mr. Hiroyuki Watanabe, Institute for Protein Research, Osaka University, for technical help in the solid phase peptide synthesis and for the FAB-MS measurements.

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