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Studies on Peptides. LVI.[1,2] Synthesis of N^1-Tyr-Somatostatin

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In order to obtain an useful derivative for radioimmunoassay, The Tyr residue was attached at the N-terminus of somatostatin. N^1-Tyr-somatostatin prepared by the conventional method was found active as synthetic somatostatin. It was confirmed that des-[Ala^1-Gly^2]-somatostatin is an active compound.

In the preceding paper,[4] we described an alternate synthesis of ovine somatostatin, the structure of which was determined by Guillemin, *et al.*[5] as a growth hormone release inhibiting factor (GIF). We added the Tyr residue to the N-terminus of somatostatin to obtain an useful derivative for a specific radioimmunoassay of this hypothalamic principle. Arimura, *et al.*[6] replaced the Ala residue at position 1 by Tyr by the solid method for this purpose. Conventional synthetic way of N^1-Tyr-somatostatin is illustrated in Fig. 1.

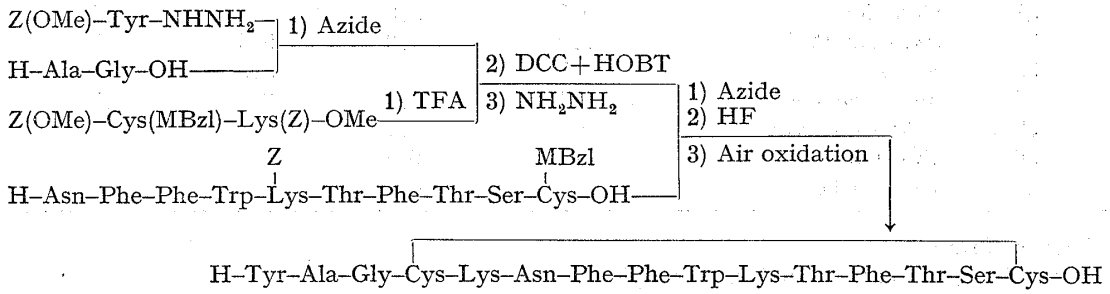

Fig. 1. Synthetic Route to N_1–Tyr–Somatostatin

According to the procedure of Honzl and Rudinger,[7] Z(OMe)–Tyr–$NHNH_2$ was condensed with H–Ala–Gly–OH to give Z(OMe)–Tyr–Ala–Gly–OH, which was further condensed, by means of dicyclohexylcarbodiimide (DCC) plus N-hydroxybenzotriazole (HOBT) procedure,[8] with H–Cys(MBzl)–Lys(Z)–OMe derived from the corresponding Z(OMe)-derivative by the usual trifluoroacetic acid (TFA) treatment.[9] The resulting protected pentapeptide ester, Z(OMe)–Tyr–Ala–Gly–Cys(MBzl)–Lys(Z)–OMe, was smoothly converted to the corresponding

1) Part LV: Y. Kai, H. Kawatani, H. Yajima, and Z. Itoh, *Chem. Pharm. Bull.* (Tokyo), **23**, 2346 (1975).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC-IUB Commission of Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z(OMe)=*p*-methoxybenzyloxycarbonyl, Z=benzyloxycarbonyl, MBzl=*p*-methoxybenzyl.
3) Location: *Sakyo-ku, Kyoto.*
4) N. Fujii and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **23**, 1596 (1975).
5) P. Brazeau, W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier, and R. Guillemin, *Science*, **179**, 77 (1973); N. Ling, R. Burgus, J. Rivier, W. Vale, and P. Brazeau, *Biochem. Biophys. Res. Commun.*, **50**, 127 (1973); R. Burgus, N. Ling, M. Butcher, and R. Guillemin, *Proc. Natl. Acad. Sci. U.S.* **70**, 684 (1973).
6) A. Arimura, H. Satoh, and D.H. Coy, Program of the 56th Annual Meeting, The Endocrine Society, U.S. p. A-153, 1974.
7) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
8) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
9) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).

hydrazide in the usual manner. Its acid hydrolysis gave constituent amino acids in ratios predicted by theory, except for Tyr, the recovery of which is generally low as mentioned by Iselin.[10]

Again, according to the above stated modified azide procedure, this protected pentapeptide hydrazide, Z(OMe)–Tyr–Ala–Gly–Cys(MBzl)–Lys(Z)–$NHNH_2$, was condensed with the partially protected decapeptide, H–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH, an intermediate of our previous synthesis of somatostain.[4] The product, Z(OMe)–Tyr–Ala–Gly–Cys(MBzl)–Lys(Z)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH, was purified by batchwise washing with ether and 10% citric acid and finally by precipitation from dimethylformamide with methanol. Its homogeneity was confirmed by thin-layer chromatography, elemental analysis and further by hydrolysis with 3N *p*-toluenesulfonic acid.[11] In the latter experiment, the contents of Tyr and Trp were confirmed.

In our previous experiments,[4] trifluoromethanesulphonic acid was employed to remove all protecting groups. Careful controlled conditions were necessary to prevent certain destruction of the Cys(MBzl) residue and after air oxidation, at least three components, a monomer and two other polymeric substances, were isolated. The yield of a monomer was only 7%. However, all of three components were found equally to possess a significant growth hormone release inhibiting activity. Considering these experimental results, in the present synthesis, hydrogen fluoride[12] were employed to remove all protecting groups from the protected pentadecapeptide obtained above and after air oxidation, the product was concentrated by column chromatography on Amberlite IRC-50 according to Yamashiro and Li.[13] Further purification was abandoned. However, it was found that the product, presumably a mixture of a monomer and polymeric substances, was active as a monomer of synthetic somatostatin.[4]

In connection with these experiments, we wish to mention that a shorter chain peptide, des-[Ala[1]–Gly[2]]-somatostatin[14] prepared by the conventional method, is also an active compound. Biological data of these synthetic peptides will be reported in a separated paper.

Experimental

Thin-layer chromatography was performed on silicagel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems. Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–pyridine–AcOH–H_2O (4:1:1:2).

Z(OMe)-Tyr-OMe——Z(OMe)-azide (6.0 g) was added to solution of H–Tyr–OMe (prepared from 4.64 g of the hydrochloride with 6.0 ml of Et_3N) in DMF (20 ml) and the solution was stirred at room temperature for 24 hr. After evaporation of the solvent, the residue was dissolved in AcOEt, which was washed with 5% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. Treatment of the residue with petroleum ether afforded the solid, which was recrystallized from AcOEt and petroleum ether; yield 6.28 g (87%), mp 99—101°, $[\alpha]_D^{26}$ −16.5° (c=0.9, MeOH). Rf_1 0.90. *Anal.* Calcd. for $C_{16}H_{21}O_6N$: C, 63.50; H, 5.89, N, 3.90. Found: C, 63.38; H, 5.88; N, 3.67.

Z(OMe)-Tyr-$NHNH_2$——To a solution of Z(OMe)–Tyr–OMe (2.86 g) in MeOH (30 ml), 80% hydrazine hydrate (4 ml) was added. The crystalline solid formed on standing at room temperature overnight was collected by filtration and recrystallized from MeOH; yield 2.61 g (91%). mp 223—225°, $[\alpha]_D^{26}$ −12.8° (c=1.0, DMF). Rf_1 0.60. *Anal.* Calcd. for $C_{18}H_{21}O_5N_3$: C, 60.15; H, 5.89; N, 11.69. Found: C, 60.37; H, 5.70; N, 11.68.

Z(OMe)-Tyr-Ala-Gly-OH——Z(OMe)–Tyr–$NHNH_2$ (2.70 g) in DMF (20 ml) was converted to the corresponding azide, according to Honzl and Rudinger,[7] with 3.78N HCl-DMF (4.4 ml), isoamylnitrite (1.1 ml) and Et_3N (2.3 ml). This solution was combined with a solution of H–Ala–Gly–OH (1.1 g, derived from the Z-derivative by catalytic hydrogenation)[4] in H_2O (10 ml) containing Et_3N (3.5 ml). The solution was stirred at 4° for 48 hr and then the solvent was evaporated. The residue was dissolved in 5% NH_4OH, which after washing with AcOEt, was acidified with citric acid. The resulting oily precipitate was extracted with AcOEt,

10) B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962).
11) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
12) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
13) D. Yamashiro and C.H. Li, *Biochem. Biophys. Res. Commun.*, **54**, 882 (1973).
14) P. Brazeau, W. Vale, J. Rivier, and R. Guillemin, *Biochem. Biophys. Res. Commun.*, **60**, 1202 (1974).

which was washed with H_2O, dried over Na_2SO_4 and then evaporated. Treatment of the residue with ether afforded the solid, which was recrystallized from AcOEt and ether; yield 1.38 g (55%), mp 149—153°, $[\alpha]_D^{25}$ −16.2° (c=1.1, MeOH). Rf_1 0.22. *Anal.* Calcd. for $C_{23}H_{27}O_8N_3 \cdot H_2O$: C, 56.20; H, 5.95; N, 8.55. Found: C, 56.28; H, 5.83; N, 8.43.

Z(OMe)-Tyr-Ala-Gly-Cys(MBzl)-Lys(Z)-OMe——Z(OMe)–Cys(MBzl)–Lys(Z)–OMe[4] (1.02 g) was treated with TFA (1 ml) in the presence of anisole (0.5 ml) at 0° for 30 min. The excess TFA was removed by evaporation and the residue was dissolved in ice-cold AcOEt, which was washed with 10% K_2CO_3 and H_2O–NaCl, dried over Na_2SO_4 and then filtered. To this filtrate, Z(OMe)–Tyr–Ala–Gly–OH (0.71 g), HOBT (0.26 g) and DCC (0.31 g) were successively combined and the mixture was stirred at room temperature for 24 hr. The solution was filtered, the filtrate was condensed *in vacuo*. Treatment of the residue with ether gave the solid, which was washed batchwisely with 10% citric acid, 5% Na_2CO_3 and H_2O and then recrystallized from THF and MeOH; yield 1.38 g (95%), mp 151—154°, $[\alpha]_D^{26}$ −26.2° (c=1.0, DMF). Rf_1 0.70. *Anal.* Calcd. for $C_{49}H_{60}O_{13}N_6S$: C, 60.48; H, 6.22; N, 8.64. Found: C, 60.35; H, 6.15; N, 8.36.

Z(OMe)-Tyr-Ala-Gly-Cys(MBzl)-Lys(Z)-NHNH$_2$——To a solution of Z(OMe)–Tyr–Ala–Gly–Cys(MBzl)–Lys(Z)–OMe (0.85 g) in MeOH (20 ml), 80% hydrazine hydrate (0.5 ml) was added. The gelatinous mass formed on standing at room temperature overnight, was collected by filtration and then recrystallized from 90% MeOH; yield 0.67 g (71%), mp 179—184°, $[\alpha]_D^{26}$ −19.2° (c=1.0, DMF). Rf_1 0.50. Amino acid ratios in an acid hydrolysate: Tyr 0.45, Ala 1.02, Gly 1.00, Lys 0.87 (average recovery 99%).[10] *Anal.* Calcd. for $C_{48}H_{60}O_{12}N_8S$: C, 59.24; H, 6.22; N, 11.52; S, 3.30. Found: C, 59.04; H, 6.20; N, 11.43; S, 3.40.

Z(OMe)-Tyr-Ala-Gly-Cys(MBzl)-Lys(Z)-Asn-Phe-Phe-Trp-Lys(Z)-Thr-Phe-Thr-Ser-Cys(MBzl)-OH——Z(OMe)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH[4] (0.50 g) was treated as usual with TFA (4 ml) in the presence of anisole (2 ml) containing ethanedithiol (0.1 ml) in an ice-bath for 45 min. Dry ether was added and the resulting fine powder (Rf_1 0.50) was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr; yield 0.44 g (91%). The TFA salt thus obtained (0.25 g) was dissolved in DMF (1 ml) containing Et_3N (0.07 ml). To this ice-cold solution, the azide (prepared according to Honzl and Rudinger[7] from 0.17 g of Z(OMe)–Tyr–Ala–Gly–Cys(MBzl)–Lys(Z)–$NHNH_2$ with 0.13 ml of 3.13N HCl-DMF, 0.03 ml of isoamylnitrite and 0.06 ml of Et_3N) in DMF (1 ml) was combined and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with ether and 10% citric acid. The resulting fine powder was washed batchwisely with 10% citric acid and ether and then precipitated from DMF with MeOH; yield 0.30 g (81%), mp 231—239°, $[\alpha]_D^{26}$ −14.1° (c=1.1, DMF). Rf_1 0.44. Amino acid ratios in a hydrolysate by 3N Tos-OH: Tyr 0.84, Ala 0.86, Gly 1.00, Lys 2.01, Asp 0.97, Phe 3.09, Trp 0.62, Thr 2.42, Ser 1.00 (average recovery 97%). *Anal.* Calcd. for $C_{126}H_{151}O_{30}N_{19}S_2 \cdot H_2O$: C, 60.68; H, 6.18; N, 10.67; S. 2.57. Found: C, 60.94; H, 6.06; N, 10.66; S, 2.85.

N^1-Tyr-Somatostatin——According to Sakakibara, *et al.*[12] the above protected pentadecapeptide (0.25 g) was treated with HF (approximately 10 ml) in the presence of anisole (0.5 ml) at 0° for 60 min. The excess HF was evaporated under reduced pressure at 0°. Dry ether was added and the resulting powder was collected by filtration, washed with ether and then dissolved in H_2O (5 ml), which was treated with Amberlite IR-4B (acetate form, approximately 1.5 g) for 30 min. The resin was removed by filtration, the filtrate was diluted with H_2O to 1000 ml. The solution, after adjusting the pH to 6.5 with 10% NH_4OH, was kept on standing at 20° for 72 hr. The Ellman test of the solution decreased from OD 0.660 to 0.160 at 412 mμ. The solution was applied to a column of Amberlite IRC-50 (3×5.2 cm) for concentration. The product retained in the column was eluted with the solvent system of pyridine–AcOH–H_2O (30: 4: 66, v/v).[13] Individual fractions (10 ml each) were examined by thin-layer chromatography. Fractions (tube No. 6—16) positive to ninhydrin and Ehrich tests (Rf_2 0.51) were combined and the solvent was evaporated and the residue was lyophilized to give fluffy powder; yield 0.13 g (62%). Amino acid ratios in a Tos-OH hydrolysate: Tyr 0.90, Ala 1.00, Gly 0.98, 1/2 Cys 1.35, Lys 1.75, Asp 1.37, Phe 3.39, Trp 0.85, Thr 2.35, Ser 1.14 (average recovery 85%).

Z(OMe)-Cys(MBzl)-Lys(Z)-NHNH$_2$——To a solution of Z(OMe)–Cys(MBzl)–Lys(Z)–OMe (1.01 g) in MeOH (10 ml), 80% hydrazine hydrate (1 ml) was added. The crystalline mass formed on standing at room temperature overnight, was collected by filtration and recrystallized from THF and MeOH; yield 0.96 g (95%), mp 172—176°, $[\alpha]_D^{26}$ −17.9° (c=0.9, DMF). *Anal.* Calcd. for $C_{34}H_{43}O_8N_5S$: C, 59.89; H, 6.36; N, 10.27. Found: C, 59.96; H, 6.30; N, 10.13.

Z(OMe)-Cys(MBzl)-Lys(Z)-Asn-Phe-Phe-Trp-Lys(Z)-Thr-Phe-Thr-Ser-Cys(MBzl)-OH——The TFA salt of H–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH prepared as stated above (0.19 g) was dissolved in DMF (4 ml). To this solution, Et_3N (0.06 ml) and the azide (prepared from 0.09 g of Z(OMe)–Cys(MBzl)–Lys(Z)–$NHNH_2$, 0.1 ml of 3.13N HCl–DMF, 0.02 ml of isoamylnitrite and 0.04 ml of Et_3N) in DMF (4 ml) were combined and the solution was stirred at 4° for 20 hr. The solvent was evaporated and the residue was treated with ether and 10% citric acid. The resulting powder was washed with 10% citric acid and H_2O and then precipitated from DMF with MeOH; yield 0.19 g (75%), mp 219—222°, $[\alpha]_D^{25}$ −15.9° (c=1.2, DMF). Rf_1 0.50. Amino acid ratios in a 3N Tos-OH hydrolysate: Lys 1.75, Asp 1.00, Phe 2.79, Trp 0.64, Thr 2.36, Ser 0.94 (average recovery 94%). *Anal.* Calcd. for $C_{112}H_{134}O_{26}N_{16}S_2 \cdot H_2O$: C, 61.07; H, 6.22; N, 10.18. Found: C, 61.32; H, 6.19; N, 10.03.

Des-[Ala^1-Gly^2]-Somatostatin——According to Sakakibara, *et al.*,[12] the above protected dodecapeptide (0.15 g) was treated with HF (approximately 5 ml) in the presence of anisole (0.5 ml) at 0° for 45 min. The excess HF was removed by evaporation and the residue was treated with dry ether. The resulting fine powder was then dissolved in H_2O (1200 ml) and the solution, after adjusting the pH to 6.5 with 10% NH_4OH, was kept on standing at room temperature for 72 hr. The solution was applied to a column of Amberlite IRC-50 (3×5.6 cm), which was washed with H_2O (100 ml). The product retained in the column was eluted with the solvent system of pyridine–AcOH–H_2O (30: 4: 66) as stated above. Fractions (tube No. 7—17) positive to ninhydrin and Ehrich tests (main spot Rf_4 0.44) were combined and the solvent was evaporated and the residue was lyophilized; yield 52 mg (43%). Amino acid ratios in a 3N Tos–OH hydrolysate: 1/2 Cys 1.34, Lys 1.90, Asp 0.93, Phe 3.37, Trp 0.79, Thr 2.27; Ser 1.00 (average recovery 89%).

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Isolation of a New Isoflavone from Chinese *Pueraria* Flowers

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A new isoflavone, 6,4′-dihydroxy-7-methoxy-isoflavone, was isolated from Chinese *Pueraria* flowers.

Pueraria flowers have been used for the treatment of crapulence as a folk medicine in China, Korea, Formosa and Japan. In our previous paper,[2] it has been reported that irisolidone-7-O-glucoside from Japanese *Pueraria* flowers (*Pueraria lobata* (Will.)Ohwi)and tectoridin from Formosan *Pueraria* flowers (*P. montana*(Lour.)Merril)were isolated. Recently, isolation of irisolidone, genistein, daizein and biochanin A as the isoflavonoids and of quercetin as the flavonoid in addition to the essential oily components from the fresh flowers of *Pueraria thunbergiana* Benth.(=*P. lobata* (Will.) Ohwi) was reported by Kurihara and Kikuchi.[3] The present paper is concerned with the isolation and the structure of 6,4′-dihydroxy-7-methoxy-isoflavone from Chinese *Pueraria* flowers[4] (Chinese crude drug, "Gehua, 葛花").

Thin-layer chromatogram (TLC) of 70% methanol extract of the flowers on silica gel plate revealed the presence of several components. Isolation of the components was carried out as shown in Chart 1. The mixture of the isoflavones was subjected to silica gel column chromatography to give an isoflavone named kakkatin (I), mp over 290°, showing one spot on TLC. Final purification was effected by recrystallization from methanol. I was analyzed for $C_{16}H_{12}O_5$. The ultraviolet (UV) spectrum of I exhibited the charactristics of the isoflavone.[5] Additionally, color tests also indicated the isoflavone character of I; a yellow color appeared when the compound was added to aqueous sodium hydroxide, to concentrated

1) Location: *a) 3-4, Kowakae, Higashiosaka, Osaka; b) 33-94, Enoki-cho, Suita, Osaka.*
2) M. Kubo, K. Fujita, H. Nishimura, S. Naruto, and K. Namba, *Phytochemistry*, **12**, 2547 (1973).
3) T. Kurihara and M. Kikuchi, *Yakugaku Zasshi*, **93**, 1201 (1973).
4) Original plant is unidentified.
5) W.D. Ollis, "The Chemistry of Flavonoid Compounds," ed. by T.A. Geissman, Pergamon Press, New York, 1962, p. 353.