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Alternative Synthesis of Substance P[1]

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A bovine hypothalamic peptide, substance P was synthesized alternatively by successive condensation of three subunits, N-terminal di-, middle hepta- and C-terminal dipeptides. Two newly synthesized peptides, PCA-Phe-Phe-Gly-Leu-Met-NH_2 and PCA-Gln-Phe-Phe-Gly-Leu-Met-NH_2 were both found to be approximately two times active than synthetic substance P, when the contractile activity was determined in isolated guinea-pig ileum.

Keywords——substance P; hypothalamic peptide; [6-PCA]-substance P-(6—11) hexapeptide amide; [5-PCA]-substance P-(5—11) heptapeptide amide; smooth muscle contractile activity

Following to the first solid phase synthesis of substance P by Tregear *et al.*,[3] this bovine hypothalamic principle was synthesized alternatively in a conventional manner[4,5] as well as modified solid phase methods.[6–8]

As far as a conventional method is concerned, we reported in 1973 the first synthesis of the undecapeptide amide corresponding to the entire amino acid sequence of substance P.[4] In this synthesis and others,[5,9] the peptide chain was elongated stepwise starting with H-Phe-Gly-Leu-Met-NH_2, though different α-amino protecting groups were employed in each instance.

As a part of our accumulated synthetic data related to substance P since 1973, we wish to record its alternative synthesis in this paper. The TFA-labile Z(OMe) group was adopted and the Gly-terminal peptide chain was elongated stepwise by the *p*-nitrophenyl ester procedure[10] starting with the known dipeptide, Z(OMe)-Phe-Gly-OH,[4] as illustrated in Fig. 1. The protected pentapeptide, Z(OMe)-Gln-Gln-Phe-Phe-Gly-OH thus newly synthesized, after deprotection, was coupled with Z(OMe)-Lys(Z)-Pro-OH by the pentachlorophenyl ester procedure[11] to afford the protected heptapeptide, Z(OMe)-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-OH, which was then condensed with H-Leu-Met-NH_2 by means of DCC in the presence of HOBT.[12]

1) Amino acids, peptides and their derivatives are of the L-configuration. The following abbreviations were used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, DCC=dicyclohexylcarbodiimide, HOBT=1-hydroxybenzotriazole, PCP=pentachlorophenyl, NP=*p*-nitrophenyl, TFA=trifluoroacetic acid.
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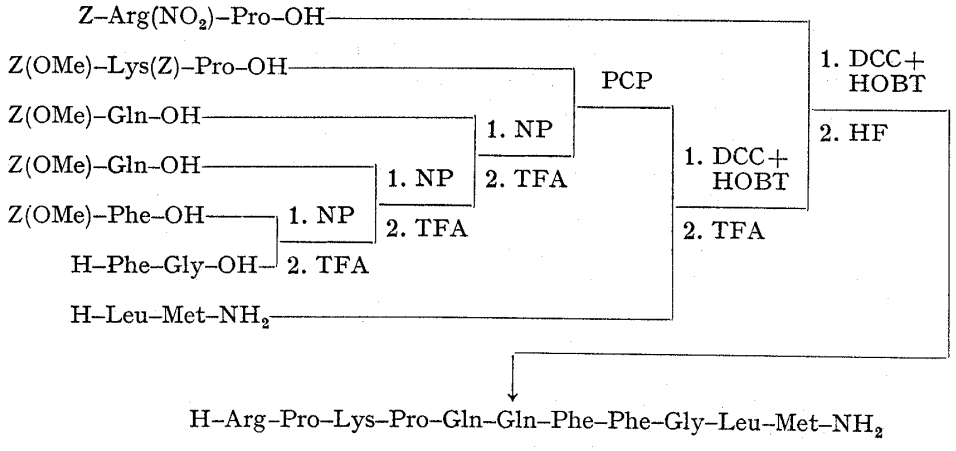

Fig. 1. Alternative Synthetic Route to Substance P

The known protected nonapeptide amide, Z(OMe)-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2[4] was thus obtained. Subsequent condensation of Z-Arg(NO_2)-Pro-OH[13] with this deprotected nonapeptide amide afforded alternatively the known protected substance P. After deprotection by hydrogen fluoride,[14] synthetic substance P was purified by Sephadex G-10 using 30% acetic acid, instead of CM-cellulose previously employed, since synthetic substance P was less soluble in water and therefore a large amount of the material was retained in CM-cellulose.

Using available intermediates mentioned above, pyroglutamyl(PCA)-hexa and heptapeptide amides were newly synthesized and assayed for comparison with the corresponding Gln-peptides, which exhibited much higher activity than the parent compound.[4] When contractile activity of synthetic peptides was assayed in isolated guinea-pig ileum, alternatively synthesized substance P was confirmed as active as our previous sample.[4] The newly synthesized PCA-peptides exhibited much higher activity than that of synthetic substance P; relative potency of PCA-Phe-Phe-Gly-Leu-Met-NH_2 to synthetic substance P was 1.97±0.16 and PCA-Gln-Phe-Phe-Gly-Leu-Met-NH_2 was 2.04±0.07 respectively. This tendency was thus found quite similar to that of the corresponding Gln-peptides.[4]

Experimental

Thin-layer chromatography was performed on silica gel (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–AcOH–pyridine–H_2O (4:1:1:2), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24).

[I] Alternative Synthesis of Substance P

Z(OMe)-Phe-Phe-Gly-OH——Z(OMe)-Phe-Gly-OH[4] (19.30 g) was treated with TFA (35 ml) in the presence of anisole (10 ml) in an ice-bath for 45 min and dry ether was added. The resulting fine powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (150 ml). To this solution Et_3N (21 ml) and Z(OMe)-Phe-ONP (22.50 g) were added. After stirring at room temperature for 48 hr, the solvent was evaporated and the residue was dissolved in H_2O. The aqueous phase, after washing with ether, was acidified with 10% citric acid. The resulting solid precipitate was collected by filtration, washed betchwise with 10% citric acid and H_2O and then recrystallized from tetrahydrofuran and MeOH; yield 21.47 g (81%), mp 167—168°, $[\alpha]_D^{26}$ −28.5° (c=1.0, DMF). Rf_1 0.48. *Anal.* Calcd. for $C_{29}H_{31}N_3O_7 \cdot 1/2\ H_2O$: C, 64.19; H, 5.94; N, 7.74. Found: C, 64.19; H, 5.82; N, 7.83.

Z(OMe)-Gln-Phe-Phe-Gly-OH——Z(OMe)-Phe-Phe-Gly-OH (10.66 g) was treated with TFA (15 ml)–anisole (3.5 ml) and the deprotected peptide isolated as stated above was dissolved in DMF (150 ml). To this solution, Et_3N (8.4 ml) and Z(OMe)-Gln-ONP (8.62 g) were added. After stirring at room temperature for 48 hr, the solvent was evaporated and the residue was treated with ether and 5% citric acid. The resulting powder was washed betchwise with 5% citric acid and H_2O and then precipitated from DMF with ether;

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yield 12.29 g (93%), mp 201—204°, $[\alpha]_D^{26}$ −32.0° (c=0.5, DMF). Rf_1 0.20, Rf_2 0.73. *Anal.* Calcd. for $C_{34}H_{39}N_5O_9 \cdot 1/2\ H_2O$: C, 60.88; H, 6.01; N, 10.44. Found: C, 61.05; H, 6.02; N, 10.62.

Z(OMe)-Gln-Gln-Phe-Phe-Gly-OH——The above tetrapeptide (6.62 g) was treated with TFA (13 ml)–anisole (3.3 ml) and the deprotected peptide isolated as stated above was dissolved in DMF (100 ml). To this solution, Et_3N (4.2 ml) and Z(OMe)-Gln-ONP (4.31 g) were added. After stirring at room temperature for 48 hr, the solution was condensed. Treatment of the residue with AcOEt gave the gelatinous mass, which was washed batchwise as stated above and then precipitated from DMF with AcOEt; yield 6.45 g (82%), mp 234—237°, $[\alpha]_D^{26}$ −22.0° (c=0.5, DMF). Rf_1 0.17. *Anal.* Calcd. for $C_{39}H_{47}N_7O_{11} \cdot H_2O$: C, 57.98; H, 6.11; N, 12.13. Found: C, 57.65; H, 5.96; N, 12.19.

Z(OMe)-Lys(Z)-Pro-OPCP——To a solution of Z(OMe)-Lys(Z)-Pro-OH[4] (10.80 g) and PCP-OH (5.32 g) in AcOEt (100 ml), DCC (4.12 g) was added and the mixture was stirred at room temperature for 24 hr. The solution was filtered, the filtrate was condensed and the residue was treated with a small amount of EtOH. The resulting solid was recrystallized from AcOEt and petroleum ether; yield 7.36 g (47%), mp 68—73°, $[\alpha]_D^{25}$ −49.4° (c=1.5, DMF). Rf_1 0.81. *Anal.* Calcd. for $C_{34}H_{34}Cl_5N_3O_8$: C, 51.69; H, 4.33; N, 5.32. Found: C, 52.28; H, 4.43; N, 5.28.

Z(OMe)-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-OH——The above protected pentapeptide, Z(OMe)-Gln-Gln-Phe-Phe-Gly-OH (3.16 g) was treated with TFA (6 ml)–anisole (1.4 ml) as stated above. The TFA salt, formed as a fine powder by addition of dry ether, was dissolved in DMF (70 ml). To this solution, Et_3N (1.7 ml) and Z(OMe)-Lys(Z)-Pro-OPCP (3.20 g) were added. The mixture, after stirring at room temperature for 48 hr, was condensed *in vacuo*. Treatment of the residue with ether afforded a solid, which was washed batchwise with 10% citric acid and H_2O and then precipitated from DMF with AcOEt; yield 4.09 g (89%), mp 210—215°, $[\alpha]_D^{25}$ −42.0° (c=0.5, DMF). Rf_1 0.18. *Anal.* Calcd. for $C_{58}H_{72}N_{10}O_{15}$: C, 60.61; H, 6.31; N, 12.18. Found: C, 60.35; H, 6.27; N, 12.18.

Z(OMe)-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (by 7+2 Procedure)——DCC (0.20 g) was added to a stirred solution of the above protected heptapeptide (0.87 g), H-Leu-Met-NH_2 (prepared from 0.43 g of the Z(OMe)-derivative[4]) and HOBT (0.14 g) in DMF (25 ml). After 48 hr, the solution was filtered and the filtrate was condensed. The product was isolated as described previously in the preparation by the 2+7 procedure;[4] yield 0.88 g (85%), mp 252—255°, $[\alpha]_D^{25}$ −36.2° (c=0.6, DMF). (lit.[4] mp 248°, $[\alpha]_D$ −37.0° in DMF). Rf_1 0.59. Amino acid ratios in an acid hydrolysate: Lys 1.09, Pro 1.06, Glu 2.15, Phe 2.02, Gly 1.07, Leu 1.00, Met 0.90 (average recovery 90%). *Anal.* Calcd. for $C_{69}H_{93}N_{13}O_{16}S$: C, 59.50; H, 6.73; N, 13.07. Found: C, 59.23; H, 6.77; N, 13.14.

Z(OMe)-Arg(NO_2)-Pro-Lys(Z)-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (by 2+9 Procedure)——The above protected nonapeptide amide (420 mg) was treated with TFA (0.9 ml)–anisole (0.2 ml) as usual and the TFA salt, precipitated by ether as a powder, was converted to the corresponding HCl salt by 1 N HCl–DMF (0.3 ml). To a solution of this hydrochloride in DMF (20 ml), Et_3N (0.04 ml), Z-Arg(NO_2)-Pro-OH (272 mg), HOBT (81 mg) and DCC (124 mg) were successively added. After stirring at room temperature for 48 hr, the solution was filtered and the filtrate was condensed. Treatment of the residue with AcOEt afforded the powder, which was washed batchwise as stated above and then purified by column chromatography on silica (2.0×60 cm) using $CHCl_3$–MeOH–H_2O (8:3:1) and finally precipitated from DMF with AcOEt to give the protected undecapeptide amide identical with that prepared by the 4+7 procedure; yield 230 mg (46%), mp 215—220°, $[\alpha]_D^{26}$ −43.3° (c=0.9, DMF). (lit.[4] mp 224°, $[\alpha]_D$ −39.8° in DMF). Rf_1 0.50. Amino acid ratios in an acid hydrolysate: Arg 0.81, Pro 1.61, (Calcd. Lys+Orn as Lys) 1.15, Glu 2.10, Phe 1.96, Gly 1.00, Leu 0.95, Met 0.81 (average recovery 91%). *Anal.* Calcd. for $C_{79}H_{109}N_{19}O_{19}S$: C, 57.12; H, 6.61; N, 16.02. Found: C, 57.06; H, 6.61; N, 15.78.

H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2——The above protected undecapeptide amide (224 mg) was treated with HF (approximately 10 ml) in the presence of anisole (0.5 ml) and Met (45 mg) as stated previously.[4] The excess HF was removed by evaporation, the residue was dissolved in H_2O (60 ml), which was treated with Amberlite IR-4B (acetate form, approximately 3 g). The resin was removed by filtration, the filtrate was washed with AcOEt and then condensed *in vacuo* to the one-third of the original volume and a few drops of mercaptoethanol was added. The solution was incubated at 40° for 8 hr and then applied to a column of Sephadex G-10 (2.8×96 cm), which was eluted with 30% AcOH. Individual fractions (4 ml each) were collected and absorbancy at 260 mμ was determined. Fractions corresponding to the front main peak (tube No. 47—60) were collected and the solvent was removed by lyophilization to give a fluffy white powder; yield 175 mg (81%). $[\alpha]_D^{26}$ −69.0° (c=0.1, 5% AcOH), Rf_3 0.55. (lit.[4] $[\alpha]_D^{27}$ −76.0° in 5% AcOH, Rf_3 0.55). Amino acid ratios in an acid hydrolysate: Arg 0.92, Pro 2.05, Lys 0.97, Glu 2.35, Phe 2.15, Gly 1.22, Leu 1.00, Met 0.98 (average recovery 89%).

[II] Synthesis of Pyroglutamyl (PCA)-peptides

Z-PCA-Phe-Phe-Gly-Leu-Met-NH_2——To a solution of the TFA salt of H-Phe-Phe-Gly-Leu-Met-NH_2 (derived from 1.56 g of Z(OMe)-derivative)[4] in DMF (20 ml), Et_3N (0.4 ml) and Z-PCA-ONP[15] (0.80 g) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was treated with ether. The resulting powder was washed batchwise with 10% citric acid, 5%

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Na_2CO_3, and H_2O and then precipitated from DMF with ether; yield 0.82 g (49%), mp 214—216°, $[\alpha]_D^{25}$ −33.5° (c=0.9, DMF). Rf_1 0.63. Amino acid ratios in an acid hydrolysate: Glu 0.98, Phe 1.93, Gly 1.00, Leu 0.98, Met 0.80 (average recovery 81%). *Anal.* Calcd. for $C_{44}H_{55}N_7O_9S \cdot H_2O$: C, 60.32; H, 6.55; N, 11.19. Found: C, 60.32; H, 6.56; N, 11.18.

H-PCA-Phe-Phe-Gly-Leu-Met-NH_2——Z-PCA-Phe-Phe-Gly-Leu-Met-NH_2 (0.40 g) was treated with HF (approximately 5 ml) in the presence of anisole (0.5 ml) in an ice-bath for 30 min. The excess HF was removed by evaporation and the residue was dissolved in H_2O (5 ml), which was treated with Amberlite IR-4B (acetate form, approximately 3 g) for 30 min and then filtered. The filtrate was condensed *in vacuo* and the residue was treated with 5% NH_4OH. The resulting powder was washed with H_2O and recrystallized from MeOH; yield 0.21 g (62%), mp 225—230°, $[\alpha]_D^{26}$ −40.4° (c=0.3, DMF). Rf_1 0.67. Amino acid ratios in an acid hydrolysate: Glu 0.84, Phe 1.95, Gly 0.92, Leu 1.00, Met 0.86 (average recovery 83%). *Anal.* Calcd. for $C_{36}H_{49}N_7O_7S \cdot 1/2\ H_2O$: C, 58.99; H, 6.88; N, 13.37. Found: C, 58.98; H, 6.78; N, 12.77.

Z-PCA-Gln-Phe-Phe-Gly-Leu-Met-NH_2——To a solution of the TFA salt of H-Gln-Phe-Phe-Gly-Leu-Met-NH_2 (prepared from 1.36 g of the Z(OMe)-derivative[4]) in DMF (20 ml), Et_3N (0.4 ml) and Z-PCA-ONP (0.60 g) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was treated with AcOEt. The resulting powder was washed batchwise as stated above and then precipitated from DMF with AcOEt; yield 1.17 g (79%), mp 230—232°, $[\alpha]_D^{26}$ −39.6° (c=0.8, DMF). Rf_1 0.52. Amino acid ratios in an acid hydrolysate: Glu 2.01, Phe 2.04, Gly 1,00. Leu 0.99, Met 0.75 (average recovery 84%). *Anal.* Calcd. for $C_{49}H_{63}N_9O_{11}S$: C, 59.67; H, 6.43; N, 12.78. Found: C, 59.40; H, 6.54; N, 12.58.

H-PCA-Gln-Phe-Phe-Gly-Leu-Met-NH_2——The above protected heptapeptide amide (0.40 g) was treated with HF (approximately 5 ml) in the presence of anisole (0.5 ml) and the product was isolated as stated above; yield 0.19 g (56%), mp 268—270°, $[\alpha]_D^{26}$ −51.7° (c=0.3, DMF). Rf_1 0.32. Amino acid ratios in an acid hydrolysate: Glu 1.87, Phe 1.96, Gly 1.00, Leu 1.00, Met 0.78 (average recovery 80%). *Anal.* Calcd. for $C_{41}H_{57}N_9O_9S \cdot H_2O$: C, 56.60; H, 6.84; N, 14.49. Found: C, 56.59; H, 6.78; N, 13.81.

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Studies on the Constituents of Bezoar. Characterization of Fatty Acids and Their Cholesteryl Esters

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The chemical investigation of Argentine bezoar showed the existence of cholesteryl esters of fatty acids (**1**), lithocholic acid, methyl cholate, methyl deoxycholate, methyl chenodeoxycholate, oleanolic acid, and ursolic acid besides the constituents already reported to be. The fatty acid compositions of **1**, as well as of free fatty acids (**2**) were scrutinized using gas chromatography and mass chromatography by converting them into the corresponding mixtures of methyl esters (**1e**) and (**2e**). These analyses showed that the mixtures consisted of C_{14} to C_{18} fatty acid esters with varing degree of relative abundance, palmitate and stearate being the major components in each of the mixtures.

Keywords——bezoar; fatty acid cholesteryl esters; fatty acids; bile acid methyl esters; oleanolic acid; ursolic acid; lithocholic acid; mass chromatography

The oriental drug "Bezoar", which is the dried gallstone of the ox, *Bos taurus* Linné var. *domesticus* Gmelin, has been used from ancient times as cardiotonics, antipyretics, analge-

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