[Chem. Pharm. Bull. 26(9)2899—2903(1978)]

UDC 547.466.1.057 : 546.161.04

# Synthesis of Two Substance P Analogs, [8-Tyr] and [5-Asn] Substance P[1]

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(Received March 13, 1978)

In a conventional manner, two substance P analogs, [8-Tyr] and [5-Asn]-substance P, were synthesized. When contractility on isolated guinea-pig ileum was examined, [8-Tyr] and [5-Asn]-analogs exhibited the activities of 1.73 and 2.08 times higher than that of synthetic substance P respectively.

**Keywords**——hypothalamic peptide; synthetic substance P analog; [8-Tyr]-substance P; [5-Asn]-substance P; hydrogen fluoride deprotection; *in vitro* contractility on guinea-pig ileum

Complete amino acid sequence of substance P from bovine hypothalami and horse intestine was determined by Leeman *et al.*[3] and Studer *et al.*[4] respectively. Following to its solid phase synthesis by Tregear *et al.*,[5] this hypothalamic principle was synthesized by the conventional method,[6,7] and further by the solid phase method.[8–10] As far as the conventional method is concerned, we reported the first synthesis of substance P in 1973[6] and later $Tyr^1$-substance P was prepared for its radioimmunoassay.[11]

We wish to record further the synthesis of two analogs, named [8-Tyr] and [5-Asn]-substance P. The former is also a useful compound for its radioimmunoassay and its solid phase synthesis was reported by Dowell *et al.*[12] and Folkers *et al.*[13]

For the synthesis of [8-Tyr]-substance P in a conventional manner, Z(OMe)-Gln-Gln-Phe-Tyr-Gly-OH was newly synthesized starting with the known tripeptide, Z-Phe-Tyr-Gly-OH.[14] This tripeptide was prepared alternatively by the azide condensation[15] of Z-Phe-Tyr-$NHNH_2$ and the triethylammonium salt of Gly. After removal of the Z group by hydro-

1) Amino acids, peptides and their derivatives are of the L-configuration. Following abbreviations were used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, TFA=trifluoroacetic acid, NP=*p*-nitrophenyl, HOBT=N-hydroxybenzotriazole, DMSO=dimethylsulfoxide.

2) Location: *a) Shomachi, Tokushima, 770, Japan; b) Kasumi, Hiroshima, 734, Japan; c) Sakyo-ku, Kyoto, 606, Japan.*

3) M.M. Chang, S.E. Leeman and H.D. Niall, *Nature New Biol.*, **232**, 86 (1971).
4) R.O. Studer, A. Trzeciak and W. Lergier, *Helv. Chim. Acta*, **56**, 860 (1973).
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7) J. Bergmann, M. Bienert, H. Niedrich, B. Mehlis and P. Oehme, *Exp.*, **30**, 401 (1974); J. Bergmann, P. Oehme, M. Bienert and H. Niedrich, *ibid.*, **30**, 1315 (1974).
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9) G.H. Fisher, J. Humphries and K. Folkers, *J. Med. Chem.*, **17**, 843 (1974).
10) R.W. Bury and M.L. Mashford, *J. Med. Chem.*, **19**, 854 (1976).
11) H. Yajima, K. Kitagawa, T. Segawa and Y. Nakata, *Chem. Pharm. Bull.* (Tokyo), **24**, 544 (1976).
12) D. Powell, S. Leeman, G.W. Tregear, H.D. Niall and J.T. Potts, Jr., *Nature New Biol.*, **241**, 252 (1973).
13) G.H. Fisher and K. Folkers, *J. Med. Chem.*, **19**, 325 (1976).
14) H. Yajima, K. Kawasaki, Y. Kinomura, T. Oshima, S. Kimoto and M. Okamoto, *Chem. Pharm. Bull.* (Tokyo), **16**, 1342 (1968).
15) J. Honzl and J. Rudinger, *Coll. Czech. Chen. Commum.*, **26**, 2333 (1961).

genolysis, the tripeptide chain was elongated stepwisely by two successive additions of Z-(OMe)-Gln-OH by the *p*-nitrophenyl ester procedure.[16]

Next, according to the scheme shown in Fig. 1, the protected pentapeptide obtained above was brought into the undecapeptide chain by successive assembling with the two known peptide fragments,[6] H-Leu-Met-$NH_2$ and Z-Arg($NO_2$)-Pro-Lys(Z)-Pro-OH. The DCC plus HOBT procedure[17] served to unit these fragment without risk of racemization. The resulting undecapeptide amide, Z-Arg($NO_2$)-Pro-Lys(Z)-Pro-Gln-Gln-Phe-Tyr-Gly-Leu-Met-$NH_2$, was then treated with hydrogen fluoride[18] to remove all protecting groups. In addition to anisole, Met was added as a scavenger to minimize the possible alkylation at the Met residue during this treatment. The deprotected peptide, after conversion to the corresponding acetate with Amberlite IR-4B, was purified by column chromatography on Sephadex G-10.

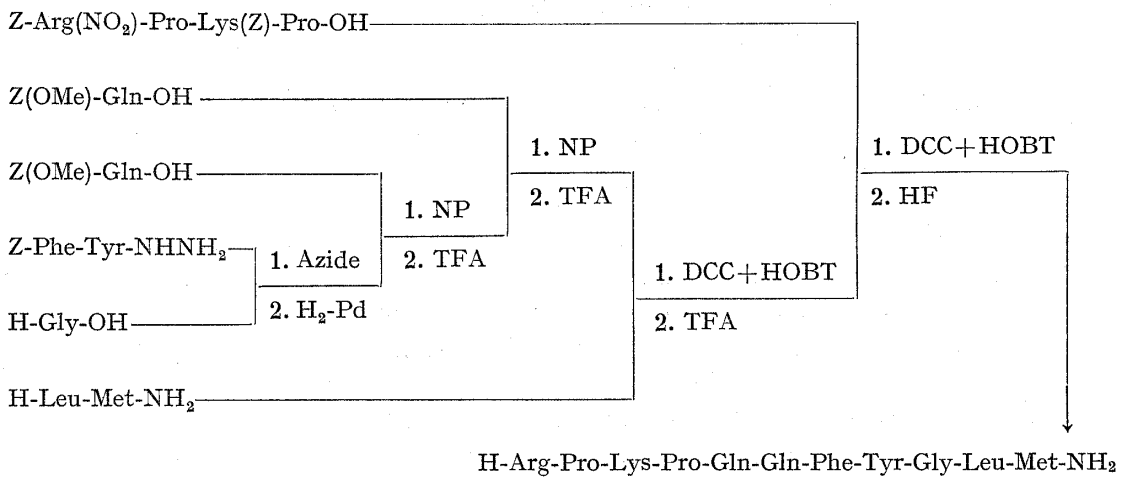


Fig. 1. Synthetic Route to [8-Tyr]-Substance P

In the above synthesis, one of the Phe residues was replaced by an analogous amino acid, Tyr. Synthesis of [5-Asn]-substance P was next performed to examine the change in biological spectra caused by sequence alternation from Gln to Asn, since this position is easy to manipulate synthetically.

Z(OMe)-Asn-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$ was prepared by two alternative routes; the one by addition of Z(OMe)-Asn-OH to the known hexapeptide amide, H-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$[6] and the other by the DCC plus HOBT condensation of H-Leu-Met-$NH_2$ with Z(OMe)-Asn-Gln-Phe-Phe-Gly-OH, which was obtained, after addition of Z(OMe)-Asn-OH to

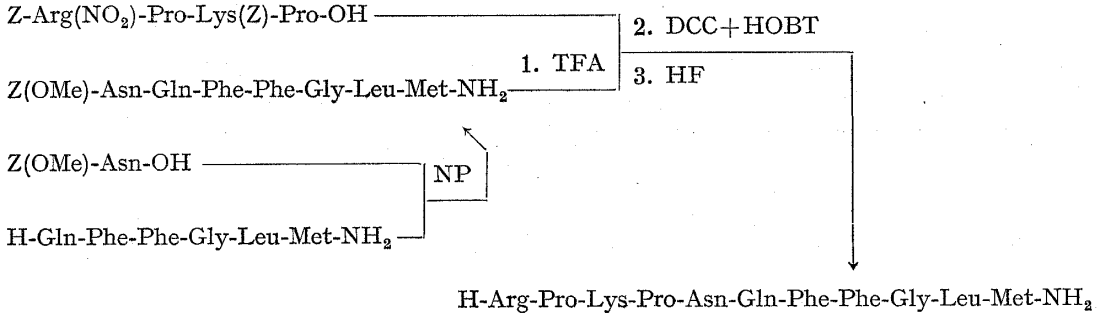


Fig. 2. Synthetic Route to [5-Asn]-Substance P

16) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
17) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
18) S. Sakakibara, Y. Shimonishi, Y. Kishida, H. Okada and H. Sugihara, *Bull. Chem. Soc. Japan.*, **40**, 2164 (1967).

the known tetrapeptide, H-Gln-Phe-Phe-Gly-OH.[19] The chain elongation of the above heptapeptide amide to Z-Arg($NO_2$)-Pro-Lys(Z)-Pro-Asn-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$ and subsequent removal of all protecting groups were performed in essentially the same manner as described in the preparation of [8-Tyr]-analog as shown in Fig. 2.

When contractility on isolated guinea-pig ileum was examined, synthetic (8-Tyr] and [5-Asn]-substance P exhibited the activities about two times higher than that of synthetic substance P respectively. Relative potency of [8-Tyr]-analog was 1.73±0.06 and [5-Asn]-analog was 2.08±0.12 respectively. Folkers *et al.*[13] mentioned that [8-Tyr]-substance P showed the same contractile activity response as solid phase synthetic substance P.

## Experimental

Thin-layer chromatography was performed on silicagel (Kiesel gel 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).

### [I] Synthesis of [8-Tyr]-Substance P

**Z-Phe-Tyr-$NHNH_2$**——To a solution of Z-Phe-Tyr-OMe[20] (35.0 g) in MeOH (250 ml), 80% hydrazine hydrate (35 ml) was added. The crystalline mass formed on standing at room temperature was collected by filtration and washed with MeOH; yield 32.5 g (92%), mp 225—227°, $[\alpha]_D^{26}$ −21.5° ($c$=0.4, DMF), $Rf_1$ 0.58. *Anal.* Calcd. for $C_{26}H_{28}N_4O_5 \cdot 1/2H_2O$: C, 64.31; H, 6.02; H, 11.54. Found: C, 64.50; H, 5.74; N, 11.63.

**Z-Phe-Tyr-Gly-OH**——Isoamylnitrite (4.0 ml) was added to an ice-chilled mixture of Z-Phe-Tyr-$NHNH_2$ (14.31 g) in DMF (50 ml) and 1 N HCl–DMF (60 ml). The solution was stirred for 15 min, neutralized with $Et_3N$ (8.4 ml) and then combined with a solution of Gly (4.50 g) in $H_2O$ (20 ml) containing $Et_3N$ (11.2 ml). The mixture was stirred at 4° for 48 hr, the solvent was evaporated and the residue was dissolved in $H_2O$, which was washed with AcOEt. The aqueous phase was acidified with citric acid and the resulting precipitate was extracted with AcOEt, which was washed with $H_2O$–NaCl, dried over $Na_2SO_4$ and then evaporated. The residue was recrystallized from AcOEt; yield 14.75 g (95%), mp 151—153°, $[\alpha]_D^{26}$ −29.4° ($c$=0.6, DMF). (lit.[14] mp 159—165°, $[\alpha]_D$ −25.5° in MeOH). $Rf_1$ 0.29. *Anal.* Calcd. for $C_{28}H_{29}N_3O_7$: C, 64.72; H, 5.62; N, 8.08. Found: C, 64.11; H, 5.83; N, 8.00.

**Z(OMe)-Gln-Phe-Tyr-Gly-OH**——Z-Phe-Tyr-Gly-OH (4.32 g) in 80% aqueous MeOH (100 ml) was hydrogenated for 8 hr over a Pd catalyst in the presence of a few drops of AcOH. The solution was filtered, the filtrated was condensed and the residue was treated with EtOH. The resulting powder was dissolved in DMF (50 ml), to which $Et_3N$ (3.5 ml) and Z(OMe)-Gln-ONP (3.63 g) were added. The mixture was stirred at room temperature for 48 hr and then condensed *in vacuo*. The residue was treated with ether and the resulting powder, after washing with 10% citric acid and $H_2O$, was precipitated from DMF with AcOEt; yield 5.53 g (97%), mp 186—191°, $[\alpha]_D^{26}$ −32.5° ($c$=1.6, DMF). $Rf_1$ 0.06. *Anal.* Calcd. for $C_{34}H_{39}N_5O_{10} \cdot H_2O$: C, 58.69; H, 5.94; N, 10.07. Found: C, 58.84; H, 5.90; N, 9.88.

**Z(OMe)-Gln-Gln-Phe-Tyr-Gly-OH**——Z(OMe)-Gln-Phe-Tyr-Gly-OH (4.75 g) was treated with TFA (10 ml)-anisole (2.0 ml) in an ice-bath for 60 min and dry ether was added. The resulting powder was dissolved in DMF (40 ml), to which $Et_3N$ (3.5 ml) and Z(OMe)-Gln-ONP (3.02 g) were added. The product was isolated as stated above; yield 5.22 g (93%), mp 218—221°, $[\alpha]_D^{26}$ −25.2° ($c$=1.0, DMF). $Rf_2$ 0.44. *Anal.* Calcd. for $C_{39}H_{47}N_7O_{12} \cdot H_2O$: C, 56.85; H, 6.00; N, 11.90. Found: C, 56.69; H, 6.01; N, 11.99.

**Z(OMe)-Gln-Gln-Phe-Tyr-Gly-Leu-Met-$NH_2$**——DCC (1.03 g) was added to a stirred mixture of H-Leu-Met-$NH_2$ (prepared from 2.98 g of Z(OMe)-derivative as reported[6]), HOBT (0.68 g) and Z(OMe)-Gln-Gln-Phe-Tyr-Gly-OH (4.0 g) in DMF–DMSO (30—15 ml). After 48 hr, the solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwise as stated above and then precipitated twice from DMF with AcOEt; yield 3.52 g (67%), mp 217—222°, $[\alpha]_D^{26}$ −37.7° ($c$=1.8, DMF), $Rf_1$ 0.68. Amino acid ratios in an acid hydrolysate: Glu 2.19, Phe 1.30, Tyr 0.66, Gly 1.00, Leu 0.84, Met 0.88 (average recovery 87%). *Anal.* Calcd. for $C_{50}H_{68}N_{10}O_{13}S \cdot 3H_2O$: C, 54.43; H, 6.76; N, 12.70. Found: C, 54.36; H, 6.65; N, 12.21.

**Z-Arg($NO_2$)-Pro-Lys(Z)-Pro-Gln-Gln-Phe-Tyr-Gly-Leu-Met-$NH_2$**——The above protected heptapeptide amide (1.05 g) was treated with TFA (2.0 ml)–anisole (0.5 ml) as usual and dry ether was added. The resulting powder was dissolved in a small amount of DMF and 1.33 N HCl–DMF (1 ml) was added. Dry ether was added and the resulting powder was then dissolved in DMF (50 ml), to which $Et_3N$ (0.14 ml), HOBT (0.17 g), Z-Arg($NO_2$)-Pro-Lys(Z)-Pro-OH[6] (1.0 g) and DCC (0.25 g) were successively added. The mixture,

19) K. Kitagawa, Y. Ban, T. Akita, T. Segawa, Y. Nakata and H. Yajima, *Chem. Pharm. Bull.* (Tokyo), **26**, 1604 (1978).

20) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970); P.G. Katsoyannis, J. Ginos, A. Cosmatos and G. Schwartz, *J. Am. Chem. Soc.*, **95**, 6427 (1973).

after stirring at room temperature for 48 hr, was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwise as stated above and then dissolved in a small amount of the solvent consisting of $CHCl_3$–MeOH–$H_2O$ (8: 3: 1). The solution was applied to a column of silica (2.5×40 cm), which was eluted with the same solvent system. Eluates which contained the substance of $Rf_1$ 0.39 were combined and the solvent was evaporated. The residue was treated with $H_2O$ and the resulting powder was recrystallized from MeOH; yield 0.87 g (52%), mp 140—145°, $[\alpha]_D^{25}$ −51.6° ($c$=0.5, DMF). $Rf_1$ 0.38. Amino acid ratios in an acid hydrolysate: Arg 0.81, Pro 1.56, Orn+Lys 1.11, (calcd. as Lys) Glu 1.90, Phe 0.96, Tyr 0.34, Gly 1.00, Leu 0.94, Met 0.87 (average recovery 87%).[21] *Anal.* Calcd. for $C_{79}H_{109}N_{19}O_{20}S \cdot 5H_2O$: C, 53.70; H, 6.79; N, 15.06. Found: C, 54.16; H, 6.51; N, 14.54.

**H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Tyr-Gly-Leu-Met-$NH_2$**——The above protected undecapeptide amide (319 mg) was treated with HF (approximately 10 ml) in the presence of anisole (1.0 ml) and Met (30 mg) in an ice-bath for 45 min and the excess HF was removed by evaporation *in vacuo*. The residue was dissolved in a small amount of $H_2O$ (30 ml), which was treated with Amberlite IR-4B (approximately 5 g) for 30 min. The resin was removed by filtration, the filtrate was washed with AcOEt and then lyophilized. The residue (330 mg) was dissolved in 30% AcOH (30 ml) and the solution was incubated with a few drops of 2-mercaptoethanol at 37° overnight and then applied to a column of Sephadex G-10 (2.8×86 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and the absorbancy at 275 mμ was determined. The desired fractions (tube No. 30—45) were collected and the solvent was evaporated. The residue was lyophilized to give a fluffy white powder; yield 216 mg (73%), $[\alpha]_D^{20}$ −54.1° ($c$=0.3, 3% AcOH). (lit.[13] −53.8° in MeOH). Amino acid ratios in an acid hydrolysate: Arg 1.11, Pro 1.88, Lys 0.95, Glu 2.27, Phe 1.00, Tyr 0.99, Gly 1.00, Leu 0.96, Met 1.02 (average recovery 90%). $Rf_2$ 0.28. *Anal.* Calcd. for $C_{63}H_{98}N_{18}O_{14}S \cdot 3CH_3COOH \cdot 9H_2O$: C, 48.58; H, 7.56; N, 14.78. Found: C, 48.57; H, 7.01; N, 14.34.

**[II] Synthesis of [5-Asn]-Substance P**

**Z(OMe)-Asn-Gln-Phe-Phe-Gly-OH**——Z(OMe)-Gln-Phe-Phe-Gly-OH (6.62 g)[19] was treated with TFA (13 ml) in the presence of anisole (3.3 ml) as usual and dry ether was added. The resulting powder was collected by filtration and then dissolved in DMF (100 ml), to which $Et_3N$ (4.2 ml) and Z(OMe)-Asn-ONP (4.17 g) were added. The mixture was stirred at room temperature for 48 hr and condensed *in vacuo*. The residue was treated with ether and the resulting powder, after washing batchwise with 10% citric acid and $H_2O$, was precipitated from DMF with ether; yield 4,30 g (55%), mp 207—211°, $[\alpha]_D^{25}$ −38.3° ($c$=1.0, DMF). $Rf_1$ 0.29. *Anal.* Calcd. for $C_{38}H_{45}N_7O_{11}$: C, 58.83; H, 5.85; N, 12.64. Found: C, 59.06; H, 5.84; N, 12.33.

**Z(OMe)-Asn-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$**——(a) DCC (1.03 g) was added to a mixture of Z(OMe)-Asn-Gln-Phe-Phe-Gly-OH (3.10 g), HOBT (0.68 g) and H-Leu-Met-$NH_2$ (prepared from 3.40 g of Z(OMe)-derivative as reported)[6] in DMF (50 ml). The mixture was stirred at room temperature for 48 hr and then filtered. The filtrate was condensed and the residue was treated with AcOEt. The resulting mass was washed batchwise with 10% citric acid, 5% $NaHCO_3$ and $H_2O$ and precipitated twice from DMF with AcOEt yield 3.55 g (87%), mp 249—250°, $[\alpha]_D^{24}$ −27.5° ($c$=0.5, DMSO), $Rf_1$ 0.47. *Anal.* Calcd. for $C_{49}H_{66}N_{10}O_{12}S \cdot 1/2H_2O$: C, 57.24; H, 6.57; N, 13.62. Found: C, 57.28; H, 6.35; N, 13.27. (b) Z(OMe)-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$ (1.81 g) was treated with TFA (4.0 ml)-anisole (1.0 ml) as usual and dry ether was added. The resulting powder was then dissolved in DMF (20 ml), to which $Et_3N$ (0.84 ml) and Z(OMe)-Asn-ONP (0.85 g) were added. After the reaction as mentioned above, the product was similarly isolated; yield 0.86 g (43%), mp 249—250°, $Rf_1$ 0.47.

**Z-Arg($NO_2$)-Pro-Lys(Z)-Pro-Asn-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$**——The above protected heptapeptide amide (1.02 g) was treated with TFA (2.0 ml)-anisole (0.5 ml) as usual and dry ether was added. The resulting powder was then dissolved in DMF (20 ml), to which $Et_3N$ (0.14 ml), Z-Arg($NO_2$)-Pro-Lys(Z)-Pro-OH[6] (1.62 g), HOBT (0.27 g) and DCC (0.42 g) were successively added. The mixture was stirred at room temperature for 48 hr, the solvent was evaporated and the residue was treated with AcOEt and the resulting powder, after washing batchwise as stated above, was precipitated twice from DMF with AcOEt; yield 0.75 g (46%), mp 190—195°, $[\alpha]_D^{25}$ −41.9° ($c$=0.8, DMF). $Rf_1$ 0.57. Amino acid ratios in an acid hydrolysate: Arg 0.93, Pro 2.11, Lys+Orn 1.13 (Calcd. as Lys), Asp 0.92, Glu 0.96, Phe 2.08, Gly 1.02, Leu 1.00, Met 0.80 (average recovery 87%). *Anal.* Calcd. for $C_{78}H_{107}N_{19}O_{19}S \cdot H_2O$: C, 56.27; H, 6.60; N, 15.99. Found: C, 56.24; H, 6.41; N, 15.55.

**H-Arg-Pro-Lys-Pro-Asn-Gln-Phe-Phe-Gly-Leu-Met-$NH_2$**——The above protected undecapeptide amide (348 mg) was treated with HF (approximately 15 ml) in the presence of anisole (1.0 ml) and Met (50 mg) in an ice-bath for 45 min. The excess HF was evaporated and the residue was dissolved in $H_2O$ (30 ml). The solution was treated with Amberlite IR-4B (acetate form, approximately 3 g) for 30 min and then filtered. The filtrate was lyophilized and the residue was purified by column chromatography on Sephadex G-10 as stated above; yield 187 mg (59%), $[\alpha]_D^{24}$ −61.5° ($c$=0.2, 3%AcOH). $Rf_2$ 0.38. Amino acid ratios in

21) Low recovery of tyrosine in an acid hydrolysate of protected peptides was mentioned by B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962).

an acid hydrolysate: Arg 0.97, Pro 2.06, Lys 0.96, Asp 1.08, Glu 1.16, Phe 2.21, Gly 1.02, Leu 1.00, Met 0.95 (average recovery 80%). *Anal.* Calcd. for $C_{62}H_{96}N_{18}O_{13}S \cdot 3CH_3COOH \cdot 9H_2O$: C, 48.73; H, 7.58; N, 15.05. Found: C, 48.62; H, 7.05; N, 15.09.

**Acknowledgement** This investigation has been supported in part by the grant of Ministry of Education, Science and Culture (No. 211208). The authers express their sincere appreciations to the support of the Mitsubishi Foundation.

[Chem. Pharm. Bull. 26(9)2903—2905(1978)]

*UDC 615.375.011.5.074 : 547.586.2.04*

# Preparation of Specific Antibodies to Catecholamines and L-3,4-Dihydroxyphenylalanine. II.[1] The Site of Attachment on Catechol Moiety in the Conjugates

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(Received March 22, 1978)

In order to estimate the site of attachment on hapten in the conjugates of catecholamines to protein prepared by the Mannich reaction, a model compound was prepared from 4-methylcatechol and ethylamine. The structure of the compound was confirmed as 5-(ethylamino)methyl-4-methylcatechol from the infrared, $^1H$- and $^{13}C$-nuclear magnetic resonance spectra.

**Keywords**——catechol; Mannich reaction; catecholamine; formaldehyde; C-13-NMR;

In the previous papers[1,3] we reported a method for preparation of the antigens of catecholamines and L-3,4-dihydroxyphenylalanine, in which the haptens were conjugated to bovine serum albumin by the Mannich reaction with formaldehyde perhaps through ε-amino groups of lysine. The site of attachment on the hapten molecule in a conjugate is closely related to the specificity of the antibody to the hapten. Burckhalter and Leib suggested that *ortho*-substitution was predominant in aminomethylation by the Mannich reaction on phenols.[4] On catechols, *ortho*-substitution was also proposed, but no strict demonstration on the structures of the products was performed.[5] In order to estimate the site of attachment on catecholamines in the conjugates, a model compound (HC–EA) was prepared from 4-methylcatechol and ethylamine, and investigated for its structure.

## Experimental

**Materials**——4-Methylcatechol, ethylamine hydrochloride, ethylamine hydrobromide, N-methylbenzylamine and Silica gel plates (2.5 × 10 cm) were obtained from Tokyo Chemical Industries Co., Ltd. N-Methylbenzylamine was converted to its hydrochloride crystal. Formaldehyde was obtained from Wako Pure

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2) Location: *Hongo, 7-3-1, Bunkyo-ku, Tokyo.* (Address to M. Yoshioka)
3) A. Miwa, M. Yoshioka, A. Shirahata, Y. Nakagawa and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **24**, 1422 (1976).
4) J. H. Burckhalter and R.I. Leib, *J. Org. Chem.*, **26**, 4078 (1961).
5) J.H. Burckhalter, F.H. Tendick, E.M. Jones, W.F. Holcomb and A.L. Rawlins, *J. Am. Chem. Soc.*, **68**, 1894 (1946); D.S. Bariana, J. Krupey, L.M. Scarpati, S.O. Freedman and A.H. Sehon, *Nature*, **207**, 1155 (1965).