[Chem. Pharm. Bull. 27(9)2238—2242(1979)]

UDC 547.466.1.04 : 547.269.3.04

Studies on Peptides. LXXXII.[1,2] Synthesis of [4-Gln]-Neurotensin by the Methanesulfonic Acid Deprotecting Procedure

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(Received March 12, 1979)

In order to examine the side reaction at the tyrosine residue, *i.e.*, O-sulfonation, that occurs during the methanesulfonic acid deprotection of Arg(*p*-methoxybenzenesulfonyl), [4-Gln]-neurotensin was synthesized as a model peptide. A mixture of cation scavengers, anisole-thioanisole-*o*-cresol (1 : 1 : 1), suppressed this side reaction sufficiently for practical peptide synthesis.

Keywords——neurotensin; [4-Gln]-neurotensin; Arg(*p*-methoxybenzenesulfonyl); Arg(mesitylene-2-sulfonyl); methanesulfonic acid deprotection; anisole-thioanisole-*o*-cresol as cation scavengers; O-sulfonation of tyrosine as a side reaction; contractile activity on isolated rabbit duodenum

The occurrence of neurotensin, H–Pyr–Leu–Tyr–Glu–Asn–Lys–Pro–Arg–Arg–Pro–Tyr–Ile–Leu–OH (I), in bovine hypothalami, as well as in bovine intestine, was first reported by Carraway and Leeman[4,5] and its solid phase synthesis was simultaneously reported in 1975.[6] More recently, Folkers *et al.*,[7] in collaboration with the research group of Leeman, reported solid phase syntheses of three analogs; neurotensin amide, [4-Gln]-neurotensin, and its amide. The possible natural occurrence of the [4-Gln]-analog was postulated.

In this paper, we report a conventional synthesis of [4-Gln]-neurotensin (II) as an example of synthesis of a peptide containing arginine and tyrosine by the MSA deprotecting procedure.[8] We are particularly interested in a combination of these two amino acids because of our recent observation of a side reaction which took place at the tyrosine residue[9] during the MSA deprotection of Arg (MBS)[10] or Arg (Mts)[11] present in the same sequence. These two protecting groups for the guanidino group of arginine are known to be cleaved smoothly by MSA, but the corresponding cations liberated by MSA partially attacked the phenolic group of tyrosin to form Tyr(MBS) or Tyr (Mts), even in the presence of a cation scavenger, anisole. In a model experiment, we were able to suppress this side reaction effectively by

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2) Amino acids, peptides and their derivatives used are of the L-configuration. The following abbreviations are used: Z(OMe)=*p*-methoxybenzyloxycarbonyl, Z=benzyloxycarbonyl, Bzl=benzyl, MBS=*p*-methoxybenzenesulfonyl, Mts=mesitylene-2-sulfonyl, DCC=dicyclohexylcarbodiimide, HOBT=N-hydroxybenzotriazole, TFA=trifluoroacetic acid, DMF=dimethylformamide, MSA=methanesulfonic acid.
3) Location: *a, b*) *Sakyo-ku, Kyoto, 606, Japan*; *c*) *Fukakusa, Fushimi, Kyoto, 612, Japan.*
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using an alternative scavenger system, namely, a mixture of anisole-thioanisole-*o*-cresol (1:1:1, v/v).[11] The effectiveness of this scavenger system in practical peptide synthesis was first demonstrated in our recent synthesis of neurotensin,[1] in which the side reaction resulting from Arg (Mts) was examined. In the present synthesis, a combination of the above scavenger system and the MSA deprotection of Arg (MBS) was examined.

The synthetic scheme for [4-Gln]-neurotensin (II) is illustrated in Fig. 1. This scheme differs from that employed for our recent synthesis of neurotensin[1] mainly in two respects: 1. Z(OMe)–Arg(MBS)–Arg(MBS)–Pro–OH, an available fragment used for the synthesis of granuliberin-R (a frog skin peptide isolated from *Rana rugosa*),[12] was employed. 2. The glutamine-containing fragment (position 4) was newly prepared starting with Z(OMe)–Asn–Lys(Z)–Pro–OH.[1] A combination of N^{α}-deprotection by TFA[13] and subsequent condensation by either the azide[14] or the DCC–HOBT procedure[15] permitted us to elongate the peptide chain without particular difficulty. The final incorporation of Z–Pyr–OH[16] by the *p*-nitrophenyl ester procedure[17] was performed using N-methylmorpholine as a base, instead of triethylamine, to minimize O-acylation at the tyrosine residue.[18]

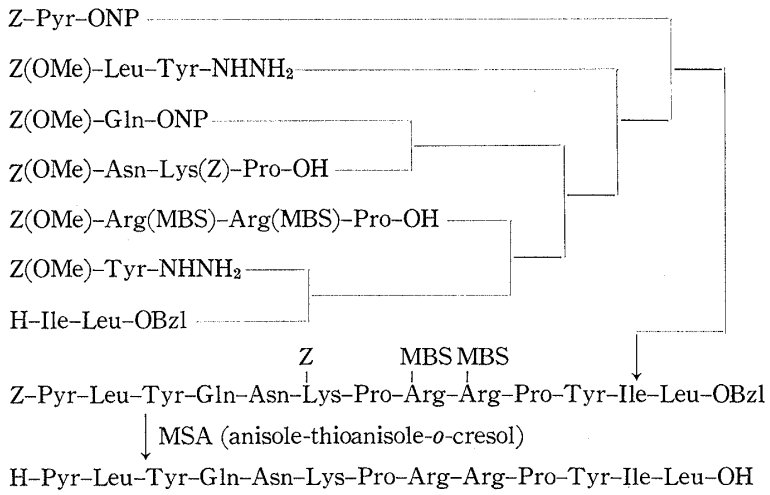

Fig. 1. Synthetic Route to [4-Gln]-neurotensin

For deprotection, the protected tridecapeptide ester, Z–Pyr–Leu–Tyr–Gln–Asn–Lys(Z)–Pro–Arg(MBS)–Arg(MBS)–Pro–Tyr–Ile–Leu–OBzl, was treated with MSA in the presence of a mixture of anisole, thioanisole ane *o*-cresol as cation scavengers, as mentioned earlier, in an ice-bath for 10 minutes and at room temperature for 60 minutes. This treatment was repeated once more to ensure complete removal of the MBS group from the two arginine residues. The deprotected peptide was converted to the corresponding acetate on Amberlite CG-4B and subsequently purified by partition chromatography[19] on Sephadex G-25 with the solvent system *n*-BuOH–AcOH–H_2O (4:1:5). The chromatographic pattern showed 5 peaks (Fig. 2). After the front peak (F-1), due to contaminating scavengers, three minor peaks (F-2, 3 and 4) were separated. The product, obtained from the main peak (F-5) in 57% yield, gave

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a sharp single spot on TLC, and its homogeneity was further assessed by hydrolysis with 4 N MSA[20] and elemental analysis. When this product was treated with aminopeptidase M (Merck), the hydrolysate contained no proline or lysine and lacked one mole each of leucine and arginine, as well as pyroglutamic acid. Enzymatic assessment of the purity of the synthetic peptide was thus abandoned, because of the absence of prolidase and pyroglutaminase-like activities in this commercial preparation. We were also unable to obtain complete recovery of tyrosine, because of this incomplete hydrolysis. However, we emphasize that tyrosine recovery in the 4 N MSA hydrolysate was satisfactory, indicating that the tyrosine residue in the desired product had not suffered side reaction, *i.e.*, Tyr (MBS) formation.[9] This product was found to be stable under these hydrolytic conditions and gave no tyrosine recovery.

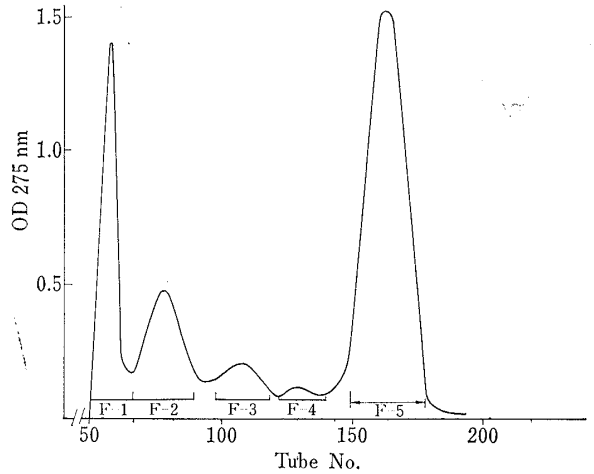

Fig. 2. Purification of Synthetic [4-Gln]-neurotensin by Partition Chromatography on Sephadex G-25

Sample: derived from the protected peptide (400 mg).
Column: 2.9×136 cm
Solvent: *n*-BuOH–AcOH–H_2O (4: 1: 5).
Fraction: 7.5 ml each.

The chromatographic pattern in the present synthesis was quite similar to that observed, when Arg (Mts) was employed for the synthesis of neurotensin, giving 53% yield in the purification step.[1] Among the side products mentioned above, fractions F-3 and F-4 were very minor components and were not examined further. The fraction F-2, isolated in 13% yield (though it was not a single component), was examined by hydrolysis with 4 N MSA. Recovery of tyrosine was somewhat low, but was still 83%. This result implied that this fraction contained very little of Tyr (MBS) derivative, but rather consisted mostly of the Tyr (3-benzyl) derivative[21] or derivatives due to incomplete deprotection. In these parallel experiments, we demonstrated that Arg(MBS) and Arg(Mts) could be applied to the synthesis of peptides containing tyrosin, when MSA was employed as a deprotecting reagent in the presence of a mixture of cation scavengers, anisole–thioanisole–*o*-cresol, though more efficient scavengers might still be desirable.

When the contractile response on isolated rabbit duodenum was compared with that of synthetic neurotensin[1] (taken as 1), the relative potency of synthetic [4-Gln]-neurotensin was 0.7. Roughly equivalent potency of these two compounds on guinea pig ileum was reported by Folkers *et al.*[7]

Experimental

General experimental procedures used were the same as those described in Part LXII[22] of this series. An azide was prepared according to Honzl and Rudinger.[14] Under cooling with ice-NaCl, isoamylnitrite (1 equiv.) was added to a stirred solution of a hydrazide (1 equiv.) in DMF and HCl–DMF (2 equiv.). After 15 min, when the hydrazine test[23] became negative, the solution was neutralized with Et_3N (2 equiv.) and then mixed with an ice-chilled solution of an amino component. Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems: Rf^1 $CHCl_3$–MeOH (9: 1), Rf^2 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf^3 *n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2).

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Z(OMe)-Tyr-Ile-Leu-OBzl——The azide (prepared from 9.34 g, 26 mmol, of Z(OMe)–Tyr–$NHNH_2$) in DMF (40 ml) was added to a solution of H–Ile–Leu–OBzl (prepared from 10.0 g, 20 mmol, of the Z(OMe) derivative by treatment with 30 ml of TFA in the presence of 10 ml of anisole as reported previously) in AcOEt (50 ml). After further addition of Et_3N (3.6 ml, 26 mmol), the mixture was stirred at 4° for 48 hr and then condensed. The residue was extracted with AcOEt. The extract was washed with 5% citric acid and H_2O–NaCl, dried over Na_2SO_4 and condensed. The residue was treated with ether and the resulting powder was recrystallized from AcOEt and ether; yield 10.60 g (80%), mp 143—145°, $[\alpha]_D^{20}$ −19.1° (c=0.9, DMF), Rf^1 0.59. *Anal.* Calcd. for $C_{37}H_{47}N_3O_8$: C, 67.15; H, 7.16; N, 6.35. Found: C, 66.93; H, 6.99; N, 6.12.

Z(OMe)-Arg(MBS)-Arg(MBS)-Pro-Tyr-Ile-Leu-OBzl——Z(OMe)–Tyr–Ile–Leu–OBzl (5.50 g, 8.3 mmol) was treated with TFA (18 ml) in the presence of anisole (4.5 ml, 5 equiv.) in an ice-bath for 60 min, then excess TFA was removed by evaporation. The residue was treated with H_2O saturated with $NaHCO_3$ and the basic mixture was extracted with AcOEt (60 ml). The extract was washed with H_2O–NaCl, dried over Na_2SO_4 and filtered. The filtrate was then combined with a solution of Z(OMe)–Arg(MBS)–Arg(MBS)–Pro–OH (6.37 g, 7 mmol) and HOBT (1.18 g, 7.7 mmol) in DMF (40 ml). After addition of DCC (1.73 g, 8.4 mmol), the mixture was stirred at room temperature for 48 hr, filtered and then condensed. Treatment of the residue with 5% citric acid and ether afforded a powder, which was purified by batchwise washing with 5% citric acid, 5% $NaHCO_3$ and H_2O followed by recrystallization three times from MeOH and ether; yield 7.55 g (76%), mp 130—132°, $[\alpha]_D^{20}$ −46.4° (c=1.4, MeOH), Rf^2 0.79. Amino acid analysis (6 N HCl hydrolysis with phenol): Pro 1.15, Tyr 1.00, Ile 0.92, Leu 1.00, Arg 2.17 (recovery 86%). *Anal.* Calcd. for $C_{68}H_{90}N_{12}O_{17}S_2$: C, 57.85; H, 6.43; N, 11.91. Found: C, 57.90; H, 6.39; N, 11.73.

Z(OMe)-Gln-Asn-Lys(Z)-Pro-OH——Z(OMe)–Asn–Lys(Z)–Pro–OH (5.41 g, 8.3 mmol) was treated with TFA (10 ml) and anisole (2.7 ml, 3 equiv.) as mentioned above and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (50 ml), together with Et_3N (2.31 ml, 17 mmol), HOBT (0.5 g, 3.3 mmol) and Z(OMe)–Gln–ONP (3.56 g, 8.3 mmol). The mixture was stirred at room temperature for 48 hr and then condensed. Treatment of the residue with 5% citric acid and ether afforded a powder, which was purified by batchwise washing with 5% citric acid and H_2O, followed by precipitation from DMF with MeOH; yield 5.48 g (85%), mp 157—158°, $[\alpha]_D^{20}$ −25.9° (c=0.7, DMF), Rf^2 0.27. Amino acid analysis: Asp 0.95, Glu 1.06, Pro 1.00, Lys 0.90 (recovery 95%). *Anal.* Calcd. for $C_{37}H_{49}N_7O_{12}$: C, 56.69; H, 6.30; N, 12.50. Found: C, 56.52; H, 6.41; N, 12.70.

Z(OMe)-Gln-Asn-Lys(Z)-Pro-Arg(MBS)-Arg(MBS)-Pro-Tyr-Ile-Leu-OBzl——The protected hexapeptide, Z(OMe)–Arg(MBS)–Arg(MBS)–Pro–Tyr–Ile–Leu–OBzl, (6.25 g, 4.4 mmol) was treated with TFA (11.5 ml) and anisole (2.9 ml, 6 equiv.) as usual, and excess TFA was removed by evaporation. The residue was treated with ether and H_2O saturated with $NaHCO_3$. The resulting gummy precipitate was dried over P_2O_5 *in vacuo* for 3 hr then dissolved in DMF (20 ml) together with Z(OMe)–Gln–Asn–Lys(Z)–Pro–OH (3.47 g, 4.4 mmol) and HOBT (0.68 g, 4.4 mmol). After further addition of DCC (0.92 g, 4.8 mmol), the mixture was stirred at room temperature for 48 hr, filtered and then condensed. The residue was treated with 5% citric acid and the resulting powder was purified by batchwise washing with 5% citric acid, 5% $NaHCO_3$ and H_2O, followed by precipitation from MeOH with AcOEt; yield 7.01 g (79%), mp 151—153°, $[\alpha]_D^{20}$ −28.8° (c=0.9, DMF), Rf^2 0.56. Amino acid analysis (hydrolysis with phenol): Asp 1.08, Glu 1.04, Pro 2.23, Ile 0.86, Leu 1.00, Tyr 0.86, Lys 1.17, Arg 2.15 (recovery 88%). *Anal.* Calcd. for $C_{96}H_{129}N_{19}O_{25}S_2 \cdot 3.5H_2O$: C, 55.53; H, 6.60; N, 12.82. Found: C, 55.45; H, 6.36; N, 13.23.

Z(OMe)-Leu-Tyr-Gln-Asn-Lys(Z)-Pro-Arg(MBS)-Arg(MBS)-Pro-Tyr-Ile-Leu-OBzl——The above protected decapeptide ester (6.02 g, 3 mmol) was treated with TFA (13 ml) and anisole (3.2 ml, 10 equiv.) as usual and dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 hr and then dissolved in DMF (20 ml) containing Et_3N (0.45 ml, 3.3 mmol). The azide (prepared from 1.55 g, 3.3 mmol of Z(OMe)–Leu–Tyr–$NHNH_2$) and Et_3N (0.45 ml, 3.3 mmol) in DMF (7 ml) were added to this ice-chilled solution. After stirring at 4° for 48 hr, the mixture was condensed and the residue was treated with 5% citric acid and ether. The resulting powder was purified by batchwise washing as mentioned above, followed by precipitation from MeOH with AcOEt; yield 4.26 g (62%), mp 157—158°, $[\alpha]_D^{20}$ −34.5° (c=0.8, DMF), Rf^2 0.49. Amino acid analysis (hydrolysis with phenol): Asp 1.00, Glu 1.00, Pro 2.00, Ile 0.85, Leu 1.77, Tyr 1.75, Lys 1.06, Arg 1.94 (recovery 77%). *Anal.* Calcd. for $C_{111}H_{149}N_{21}O_{28}S_2 \cdot 2H_2O$: C, 57.32; H, 6.63; N, 12.65. Found: C, 57.19; H, 6.61; N, 12.66.

Z-Pyr-Leu-Tyr-Gln-Asn-Lys(Z)-Pro-Arg(MBS)-Arg(MBS)-Pro-Tyr-Ile-Leu-OBzl——The above protected dodecapeptide ester (4.26 g, 1.9 mmol) was treated with TFA (10 ml) and anisole (2.41 ml, 12 equiv.) as usual and the deprotected peptide isolated as mentioned above was dissolved in DMF (20 ml) together with N-methylmorpholine (0.47 ml, 4.1 mmol) and Z–Pyr–ONP (0.79 g, 2.1 mmol). After stirring at room temperature for 48 hr, the solution was condensed and the residue was treated with 5% citric acid and ether. The resulting powder was purified by batchwise washing as mentioned above, followed by precipitation three times from DMF with MeOH; yield 3.61 g (82%), mp 190—192°, $[\alpha]_D^{20}$ −33.5° (c=1.0, DMF). Rf^2 0.45. Amino acid analysis (hydrolysis with phenol): Asp 1.00, Glu 2.14, Pro 2.06, Ile 0.84, Leu 1.93, Tyr 1.84, Lys 1.05, Arg 1.87 (recovery 83%). *Anal.* Calcd. for $C_{115}H_{152}N_{22}O_{29}S_2 \cdot H_2O$: C, 57.82; H, 6.50; N, 12.90. Found: C, 57.59; H, 6.62; N, 13.18.

H-Pyr-Leu-Tyr-Gln-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH——The above protected tridecapeptide ester (400 mg, 0.17 mmol) was treated with MSA (3 ml) in the presence of anisole–thioanisole–*o*-cresol (1 : 1 : 1, v/v, 0.5 ml) in an ice-bath for 10 min and at room temperature for 60 min, then dry ether was added. The residue was again treated with MSA and precipitated with ether as described above. The deprotected product was dissolved in a small amount of H_2O and the solution was treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min. The pH of the filtrate was adjusted to 9 with 10% NH_4OH/in an ice-bath and then to 6 with AcOH. After lyophilization, the resulting powder was dissolved in a small amount of the upper phase of a solvent system consisting of *n*-BuOH–AcOH–H_2O (4 : 1 : 5), and the solution was applied to a column of Sephadex G-25 (fine) equilibrated with the lower phase of the above solvent system. The column was then developed with the upper phase and individual fractions (7.5 ml each) were collected. Determination of the UV absorbancy at 275 nm indicated the presence of five peaks (Fig. 2). The front peak (F-1, tube No. 50—67) was found to be due to contaminating scavengers and two other peaks (F-3, tube No. 99—118 and F-4, tube No. 122—140) were minor components. Fractions corresponding to the main peak (F-5, tube No. 149—178) were collected and the solvent was evaporated off. Lyophilization of the residue afforded a white fluffy powder; yield 178 mg (57%), $[\alpha]_D^{20}$ −94.1° (c=0.4, H_2O), Rf[3] 0.57. Amino acid ratios in 4 N MSA hydrolysate: Asp 1.00, Glu 2.29, Pro 2.02, Ile 0.90, Leu 1.96, Tyr 1.95, Lys 1.05, Arg 2.00 (average recovery 92%). Amino acid ratios in aminopeptidase M (Merck) digest: Asn 1.40, Gln 0.72, Pro=0, Ile 1.00, Leu 1.00, Tyr 1.48, Lys=0, Arg 1.09 (Ile recovery was taken as the basis of calculation). *Anal.* Calcd. for $C_{78}H_{122}N_{22}O_{19}\cdot 3CH_3COOH\cdot 8H_2O$: C, 50.54; H, 7.57; N, 15.44. Found: C, 50.67; H, 7.45; N, 15.60.

The material obtained from F-4 (Tube No. 68—89); 41 mg (13%), Rf[3] 0.63 (tailing spot). Amino acid ratios in 4 N MSA hydrolysate: Asp 1.00, Glu 2.43, Pro 2.18, Ile 0.91, Leu 1.97, Tyr 1.67, Lys 0.97, Arg 1.84 (average recovery 72%).

Acknowledgement This investigation was supported in part by a grant from the Ministry of Education, Science and Culture (No. 211208). The authors are also grateful for the support of the Mitsubishi Foundation.

[Chem. Pharm. Bull. 27(9)2242—2245(1979)]

UDC 547.963.32.04 : 542.8.04

Selective Removal of Protecting Groups for Phosphomonoesters of Nucleotides by Anodic Oxidation

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(Received March 14, 1979)

Cyclic volutammetry of phenyl derivatives of various nucleotides was carried out, using the parent nucleosides as a control. The *p*-*N*-benzylaminophenyl ester of 3′-O-acetylthymidine 5′-phosphate showed a peak at low anodic potential, and the *N*-benzylaminophenyl group could be removed by controlled potential electrolysis at this potential. *p*-*N*-Tritylaminophenyl-, *p*-*N*-acetylaminophenyl and *p*-methoxyphenyl esters of nucleotides were also subjected to controlled potential electrolysis; the oxidative removal of these phenyl groups was less effective than that of the *N*-benzylaminophenyl ester.

Keywords——*p*-*N*-benzylaminophenyl phosphate; *p*-*N*-tritylaminophenyl phosphate; *p*-*N*-acetylaminophenyl phosphate; *p*-methoxyphenyl phosphate; cyclic voltammetry; controlled potential electrolysis

We have previously reported that the *N*-trityl-*p*-aminophenyl group served as a useful protecting group for terminal phosphate residues in the synthesis of oligonucleotides.[2] This protecting group could be removed by oxidative hydrolysis with iodine from protected oligo-

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