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# Studies on Peptides. CXLVIII.[1,2] Application of a New Deprotecting Procedure with Trimethylsilyl Trifluoromethanesulfonate for the Syntheses of Two Porcine Spinal Cord Peptides, Neuromedin U-8 and Neuromedin U-25

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The usefulness of a new deprotecting procedure was demonstrated in the solution syntheses of two porcine spinal cord peptides, designated neuromedin U-8 and neuromedin U-25. Protected neuromedin U-8 (8-residue peptide), prepared by condensation of two fragments, served as a C-terminal amino component for the synthesis of neuromedin U-25 (25-residue peptide). Onto this fragment, five peptide fragments were successively condensed by the azide procedure to construct the entire amino acid sequence of neuromedin U-25, a possible biosynthetic precursor of neuromedin U-8. All protecting groups were cleaved from protected neuromedin U-8 and neuromedin U-25 by 1 M trimethylsilyl trifluoromethanesulfonate–thioanisole in trifluoroacetic acid. The results were compared with those obtained by trifluoromethanesulfonic acid deprotection. In terms of contractile activity in rat uterus, neuromedin U-25 was twice as active as neuromedin U-8.

**Keywords**——porcine spinal cord peptide; neuromedin U-8; neuromedin U-25; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; deprotecting reagent; trimethylsilyl trifluoromethanesulfonate; uterus contractile activity

Recently, we[3] found that trimethylsilyl trifluoromethanesulfonate (trimethylsilyl triflate, TMSOTf) cleaved various protecting groups currently employed in peptide synthesis more readily than TFMSA.[4] By using this newly found deprotecting reagent, we synthesized two peptides isolated by Minamino *et al.*[5] from porcine spinal cord; an 8-residue peptide and a 25-residue peptide, designated neuromedin U-8 and neuromedin U-25, respectively. Their sequence analysis revealed that U-25 contained the U-8 sequence, preceded by paired Arg residues, a typical biosynthetic processing signal.[6] Thus, U-8 is thought very likely to be processed biologically from U-25. Their C-terminal amide structures were confirmed by the solid phase synthesis.[5] Following our syntheses of neuromedins B, C,[7] K and L,[8] we synthesized U-8 and U-25 in order to examine the relationship between structure and function of these neurocandidate peptides. In combination with the TFA-labile Z(OMe) group,[9] amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole/TFA[4] were employed, *i.e.*, Arg (Mts),[10] Asp(OBzl), Glu (OBzl), and Lys (Z). Later it was found that these side chain protecting groups could be cleaved more smoothly when TFMSA was replaced by TMSOTf.[3] Thus, in the final steps of the syntheses, deprotections were performed in two ways and the results were compared. Six peptide fragments served to construct the entire amino acid sequence of U-25, as shown in Fig. 1. U-8 was ob-

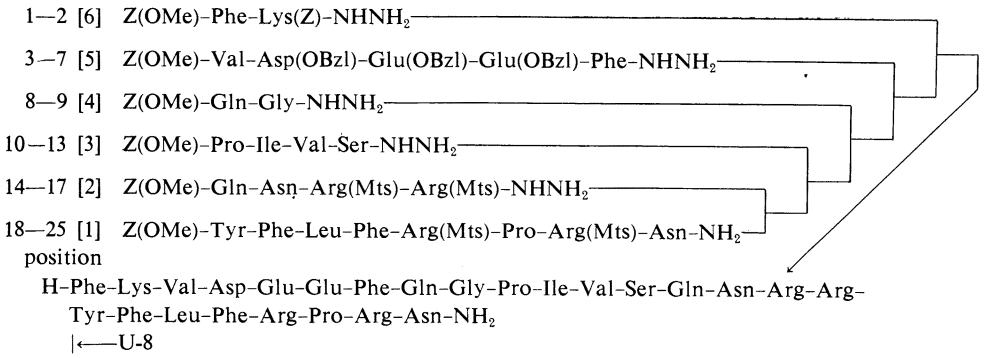



Fig. 1. Synthetic Route to Neuromedin U-25

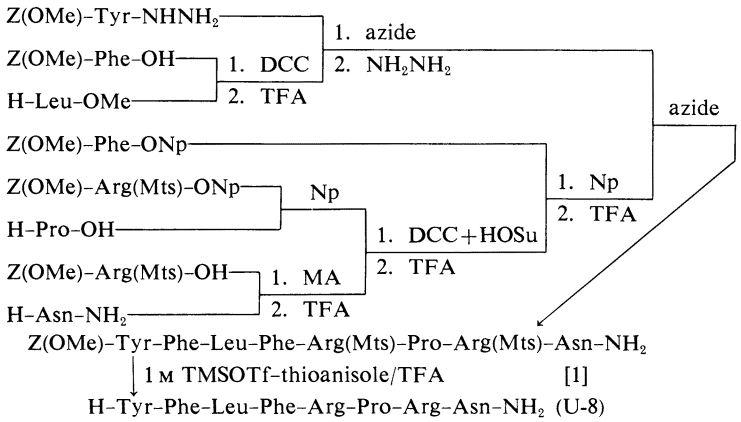



Fig. 2. Synthetic Scheme for the Protected Octapeptide Amide [1] and U-8

tained by deprotection of the C-terminal octapeptide fragment [1].

Fragment [1], Z(OMe)–Tyr–Phe–Leu–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (positions 18—25 of U-25, protected U-8), was prepared, according to the scheme shown in Fig. 2, by azide condensation[11] of two components, Z(OMe)–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (component 1) and Z(OMe)–Tyr–Phe–Leu–$NHNH_2$ (component 2). First, Z(OMe)–Arg(Mts)–Pro–OH, prepared by the Np method,[12] was condensed by DCC in the presence of HOSu[13] with a TFA-treated sample of Z(OMe)–Arg(Mts)–Asn–$NH_2$ prepared by the mixed anhydride (MA) method.[14] The resulting protected tetrapeptide amide was treated with TFA, then condensed with Z(OMe)–Phe–OH by the Np method to give component 1. Component 2 was prepared by the azide condensation of Z(OMe)–Tyr–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Phe–Leu–OMe,[15] followed by the usual hydrazine treatment of the resulting protected tripeptide ester. The purity of fragment [1], prepared by condensation of these two components, was ascertained by thin layer chromatography (TLC), elemental analysis and amino acid analysis after 6 N HCl hydrolysis, as was done with other fragments. This protected octapeptide [1] served as a key intermediate for the synthesis of U-25, and its deprotection with 1 M TMSOTf–thioanisole/TFA afforded U-8. In this deprotecting step, the time required for complete removal of the two Mts groups from protected U-8 was less than 60 min in an ice-bath, while removal by 1 M TFMSA–thioanisole/TFA required 120 min.

Fragment [2], Z(OMe)–Gln–Asn–Arg(Mts)–Arg(Mts)–$NHNH_2$ (positions 14—17), was prepared by stepwise Np additions of two residues, Asn and Gln, to a TFA-treated sample of

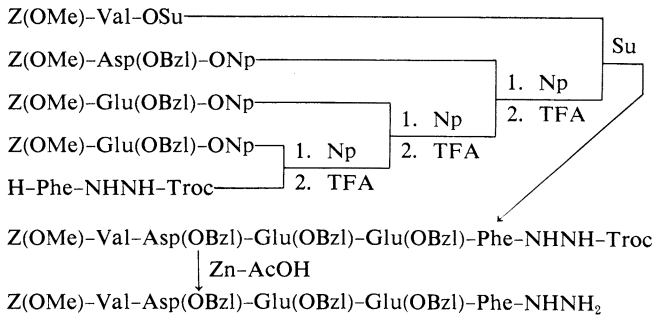



Fig. 3. Synthetic Scheme for the Protected Pentapeptide Hydrazide [5] (Positions 3—7)

Z(OMe)–Arg(Mts)–Arg(Mts)–OMe,[16] followed by the usual hydrazine treatment of the resulting protected tetrapeptide ester. Fragment [3], Z(OMe)–Pro–Ile–Val–Ser–$NHNH_2$ (position 10—13), was prepared in a stepwise manner also starting from a TFA-treated sample of Z(OMe)–Val–Ser–OMe.[17] The active Np and the MA methods were employed to introduce two residues, Ile and Pro, respectively and the resulting tetrapeptide ester was converted to [3] by hydrazine treatment as stated above. Fragment [4], Z(OMe)–Gln–Gly–$NHNH_2$ (positions 8—9), was easily obtained by hydrazine treatment of the known dipeptide ester, Z(OMe)–Gln–Gly–OMe.[18]

Fragment [5], Z(OMe)–Val–Asp(OBzl)–Glu(OBzl)–Glu(OBzl)–Phe–$NHNH_2$ (positions 3—7) was prepared in a stepwise manner starting from H–Phe–NHNH–Troc as shown in Fig. 3. The Np method was employed for condensations of two Glu(OBzl) and one Asp(OBzl) residues and the Su method[19] for Z(OMe)–Val–OH. The Troc group was removed from the resulting protected pentapeptide derivative by treatment with Zn powder[20] to give [5]. The N-terminal fragment, Z(OMe)–Phe–Lys(Z)–$NHNH_2$ (positions 1 and 2), was prepared by the usual hydrazine treatment of the corresponding dipeptide ester, which was easily prepared by the Np method.

Six peptide fragments thus obtained were assembled successively by the azide procedure to minimize racemization. DMF was employed as a solvent, and the amount of the acyl component was increased from 1.2 to 2 equivalents as chain elongation progressed. The products were purified either by precipitation from DMF with an appropriate solvent, such as AcOEt or MeOH or by gel-filtration on Sephadex LH-20 using DMF as an eluant. Throughout the synthesis, Leu was used as a diagnostic amino acid in acid hydrolysis in order to ascertain the homogeneity of each product. Recovery of Leu was compared with those of newly added amino acids after each condensation. Thus, satisfactory incorporation of each fragment was ascertained (Table I).

In the final step, protected U-25 was treated with 1 M TMSOTf–thioanisole/TFA in the presence of *m*-cresol and EDT in an ice-bath for 60 min to remove all protecting groups employed. The deprotected peptide was briefly treated with dil. ammonia to reverse any possible N→O shift[21] at the Ser residue. In this step, ammonium fluoride was added to ensure the complete hydrolysis of the O-trimethylsilyl groups possibly attached at the Ser and Tyr residues. The treated peptide was purified by gel-filtration on Sephadex G-25, followed by reversed-phase high performance liquid chromatography (HPLC) on a TSK-GEL LS-410KG column using isocratic elution of 26% acetonitrile in 0.1% TFA. Synthetic U-25 thus purified exhibited a sharp single spot on TLC and a single peak in analytical HPLC and behaved as a single component on disc isoelectrophoresis. Its 6 N HCl hydrolysate (Table I) and its leucine-aminopeptidase (LAP) digest contained amino acids in the ratios predicted by theory. The

TABLE I. Amino Acid Ratios in 6N HCl Hydrolysates of Synthetic Neuromedin U-25 and Its Intermediates

| | Protected peptides | | | | | | Syn. | Residue |
|---|---|---|---|---|---|---|---|---|
| | 18—25 | 14—25 | 10—25 | 8—25 | 3—25 | 1—25 | U-25 | |
| Asp | 1.03 | 2.20 | 2.16 | 1.95 | 3.30 | 3.29 | 3.02 | (3) |
| Ser | | | 0.94 | 0.79 | 0.92 | 0.82 | 0.78 | (1) |
| Glu | | 1.09 | 1.10 | 2.01 | 4.46 | 4.47 | 4.02 | (4) |
| Gly | | | | 1.02 | 1.14 | 1.11 | 1.14 | (1) |
| Val | | | 0.72 | 0.73 | 1.64 | 1.94 | 1.87 | (2) |
| Ile | | | 0.69 | 0.75 | 0.80 | 0.93 | 0.94 | (1) |
| Leu | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | (1) |
| Tyr | 0.94 | 0.97 | 0.97 | 0.98 | 0.96 | 1.02 | 1.01 | (1) |
| Phe | 1.99 | 2.11 | 2.07 | 1.97 | 3.19 | 4.10 | 3.71 | (4) |
| Lys | | | | | | 0.93 | 1.04 | (1) |
| Arg | 2.07 | 4.38 | 4.28 | 3.92 | 4.24 | 4.03 | 3.84 | (4) |
| Pro | 0.97 | 1.07 | 2.09 | 1.99 | 2.15 | 2.35 | 1.93 | (2) |
| Recov. (%) | 92 | 83 | 80 | 90 | 80 | 85 | 78 | |

overall yield from protected U-25 in the present new deprotecting procedure was 52%. For comparison, deprotection by using 1 M TFMSA–thioanisole/TFA was carried out. Protected U-25 was treated with the above reagents for 150 min, then purified as stated above, except for ammonium fluoride treatment. The overall yield in this experiment was 47%. To determine optimal deprotecting conditions, we preliminarily treated a small amount of each sample with either one of the reagents and examined the purity of the product by HPLC. The rate of deprotection by TMSOTf was clearly faster than that by TFMSA. In preparative experiments, U-8 and U-25 were obtained in better yields by the new procedure than by the TFMSA deprotecting procedure as stated above. On the basis of these experimental results, we consider that our new deprotecting procedure with TMSOTf is applicable to practical peptide synthesis, and is superior to the previous TFMSA deprotecting procedure.

The biological activity of neuromedin U-25 on rat uterus contraction was compared with that of U-8. Isolated uterus from female rats of the Wistar strain (about 200 g body weight) was suspended in a bath containing modified Locke-Ringer solution (35 °C) bubbled with 95% $O_2$–5% $CO_2$ and the contraction was recorded with a recticorder (KN 260, Natsume Seisakusho Co., Ltd.) *via* an isotonic transducer (KN 259). With regard to uterus stimulating activity (molar basis), neuromedin U-25 was about twice as potent as neuromedin U-8.

## Experimental

General experimental procedures described herein are essentially the same as described in part CXXXIX[22] of this series. Prior to condensation, the $N^{\alpha}$-Z(OMe) group was removed by treatment with TFA in the presence of anisole. The active ester reaction was performed at room temperature. An azide was prepared with isoamyl nitrite and the reaction was performed at 4 °C. A mixed anhydride (MA) was prepared with isobutyl chloroformate, then the reaction was performed in an ice-bath. Unless otherwise stated, products were purified by one of the following procedures. A (extraction procedure): The product was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$–NaCl, dried over $Na_2SO_4$ and concentrated. The residue was recrystallized or precipitated from appropriate solvents. B (washing procedure): After evaporation of the solvent, the residue was treated with 5% citric acid and ether, then the resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and $H_2O$, and recrystallized or precipitated from appropriate solvents. C (gel-filtration procedure): The product partially purified by procedure B was dissolved in a small amount of DMF and a solution was applied to a column of Sephadex LH-20, which was eluted with DMF. Fractions (6.5 ml each) corresponding to the front main peak (monitored by ultraviolet absorption (UV) measurement at 275 nm) were combined and the solvent was removed by

evaporation *in vacuo*. The residue was precipitated from DMF with ether.

*Rf* values in TLC performed on silica gel (Kieselgel G, Merck) refer to the following solvent systems: $Rf_1$ $CHCl_3$–MeOH (10:0.5), $Rf_2$ $CHCl_3$–MeOH–$H_2O$ (8:3:1), $Rf_3$ *n*-BuOH–AcOH–pyridine–$H_2O$ (4:1:1:2), $Rf_4$ *n*-BuOH–AcOH–pyridine–$H_2O$ (30:6:20:24). HPLC was conducted with a Waters 204 compact model. LAP was purchased from Sigma (Lot. No. L-6007).

**Z(OMe)–Arg(Mts)–Asn–$NH_2$**——The title compound was prepared by the MA method and purified by procedure B, followed by precipitation from DMF with AcOEt; yield 86%, mp 191—195 °C, $[\alpha]_D^{25}$ −5.3° ($c$=0.9, DMF), $Rf_2$ 0.61. *Anal.* Calcd for $C_{28}H_{39}N_7O_8S$: C, 53.07; H, 6.20; N, 15.47. Found: C, 53.40; H, 6.34; N, 15.16.

**Z(OMe)–Arg(Mts)–Pro–OH**——A mixture of DCC (5.67 g, 27.5 mmol), Z(OMe)–Arg(Mts)–OH (13.01 g, 25.0 mmol) and Np–OH (5.06 g, 27.5 mmol) in THF (150 ml) was stirred for 5 h. The filtered solution was added to a solution of H–Pro–OH (8.64 g, 75.0 mmol) in $H_2O$ (30 ml) containing $Et_3N$ (13.9 ml, 0.1 mol) and the mixture was stirred for 24 h. The solvent was evaporated off and the residue was dissolved in 5% $NH_4OH$. The aqueous phase, after being washed with AcOEt, was acidified with 5% citric acid and the resulting precipitate was extracted with AcOEt. The extract was washed with $H_2O$–NaCl, dried over $Na_2SO_4$ and concentrated. The residue was recrystallized from AcOEt and ether; yield 12.30 g (80%), mp 101—104 °C, $[\alpha]_D^{25}$ −23.3° ($c$=0.9, MeOH), $Rf_2$ 0.54. *Anal.* Calcd for $C_{29}H_{39}N_5O_8S$: C, 56.38; H, 6.36; N, 11.34. Found: C, 56.38; H, 6.41; N, 11.41.

**Z(OMe)–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$**——A mixture of Z(OMe)–Arg(Mts)–Pro–OH (10.19 g, 16.5 mmol), SuOH (2.09 g), 18.2 mmol) and DCC (3.75 g, 18.2 mmol) in THF (100 ml) was stirred for 5 h and filtered. The filtrate was added to a solution of a TFA-treated sample of Z(OMe)–Arg(Mts)–Asn–$NH_2$ (9.51 g, 15.0 mmol) and $Et_3N$ (4.59 ml, 32.9 mmol) in DMF (100 ml) and the mixture was stirred overnight. The solvent was removed by evaporation and the residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 12.58 g (78%), mp 137—139 °C, $[\alpha]_D^{23}$ −18.7° ($c$=1.0, DMF), $Rf_2$ 0.58. *Anal.* Calcd for $C_{48}H_{68}N_{12}O_{12}S_2 \cdot H_2O$: C, 53.02; H, 6.49; N, 15.46. Found: C, 53.10; H, 6.65; N, 15.28.

**Z(OMe)–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (Positions 21—25)**——A mixture of Z(OMe)–Phe–ONp (4.32 g 9.60 mmol), $Et_3N$ (2.45 ml, 17.6 mmol) and a TFA-treated sample of the above protected tetrapeptide amide (8.55 g, 8.0 mmol) in DMF (100 ml) was stirred overnight and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 7.59 g (78%), mp 144—147 °C, $[\alpha]_D^{23}$ −15.7° ($c$=1.0, DMF), $Rf_2$ 0.68. Amino acid ratios in a 6 N HCl hydrolysate: Phe 1.00, Arg 2.05, Pro 0.95, Asp 1.00 (recovery of Asp, 83%). *Anal.* Calcd for $C_{57}H_{77}N_{13}O_{13}S_2 \cdot 1/2H_2O$: C, 55.86; H, 6.42; N, 14.86. Found: C, 55.74; H, 6.39; N, 14.75.

**Z(OMe)–Tyr–Phe–Leu–OMe**——The azide [prepared from 6.47 g (18.0 mmol) of Z(OMe)–Tyr–$NHNH_2$] in DMF (60 ml) and $Et_3N$ (2.51 ml, 18.0 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Phe–Leu–OMe[20] (6.85 g, 15.0 mmol) in DMF (60 ml) containing $Et_3N$ (2.09 ml, 15 mmol), then the solution was stirred overnight and the solvent was removed by evaporation. The residue was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 7.90 g (85%), mp 183—185 °C, $[\alpha]_D^{23}$ −28.8° ($c$=1.8, MeOH), $Rf_1$ 0.37. *Anal.* Calcd for $C_{34}H_{41}N_3O_8 \cdot 1/2H_2O$: C, 64.95; H, 6.73; N, 6.68. Found: C, 65.07; H, 6.74; N, 6.92.

**Z(OMe)–Tyr–Phe–Leu–$NHNH_2$ (Positions 18—20)**——The above tripeptide ester (6.20 g, 10.0 mmol) in MeOH (60 ml) was treated with 80% hydrazine hydrate (1.90 ml, 3 eq) at room temperature overnight. The solution was concentrated and the residue was precipitated from DMF with MeOH, yield 5.32 g (86%), mp 224—226 °C, $[\alpha]_D^{23}$ −37.0° ($c$=1.0, DMF), $Rf_2$ 0.59. Amino acid ratios in a 6 N HCl hydrolysate: Tyr 0.97, Phe 1.01, Leu 1.00 (recovery of Leu, 93%). *Anal.* Calcd for $C_{33}H_{41}N_5O_7$: C, 63.96; H, 6.67; N, 11.30. Found: C, 63.88; H, 6.59; N, 11.27.

**Z(OMe)–Tyr–Phe–Leu–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ [1] (Positions 18—25)**——The azide [prepared from 3.72 g (6 mmol) of Z(OMe)–Tyr–Phe–Leu–$NHNH_2$] in DMF (40 ml) and $Et_3N$ (0.84 ml, 6 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (4.87 g, 4.0 mmol) in DMF (50 ml) containing $Et_3N$ (0.56 ml, 4.0 mmol). The solution was stirred overnight and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.88 g (74%), $[\alpha]_D^{23}$ −49.5° ($c$=1.0, DMF), $Rf_2$ 0.57. Amino acid ratios in a 6 N HCl hydrolysate: Tyr 0.91, Phe 1.94, Leu 0.97, Arg 2.01, Pro 0.95, Asp 1.00 (recovery of Asp, 95%). *Anal.* Calcd for $C_{81}H_{106}N_{16}O_{17}S_2 \cdot 3/2H_2O$: C, 58.36; H, 6.59; N, 13.45. Found: C, 58.16; H, 6.59; N, 13.68.

**Z(OMe)–Asn–Arg(Mts)–Arg(Mts)–OMe**——A mixture of Z(OMe)–Asn–ONp (3.51 g, 8.40 mmol), $Et_3N$ (2.15 ml, 15.4 mmol) and a TFA-treated sample of Z(OMe)–Arg(Mts)–Arg(Mts)–OMe[16] (6.11 g, 7.0 mmol) in DMF (100 ml) was stirred overnight and concentrated. The residue was purified by procedure A, followed by recrystallization from MeOH and ether; yield 5.64 g (82%), mp 127—130 °C, $[\alpha]_D^{23}$ −13.9° ($c$=1.4, MeOH), $Rf_2$ 0.64. *Anal.* Calcd for $C_{44}H_{62}N_{10}O_{12}S_2 \cdot 3/2H_2O$; C, 52.11; H, 6.46; N, 13.81. Found; C, 52.26; H, 6.39; N, 13.31.

**Z(OMe)–Gln–Asn–Arg(Mts)–Arg(Mts)–OMe**——A mixture of Z(OMe)–Gln–ONp (4.66 g, 10.8 mmol), $Et_3N$ (2.75 ml, 19.8 mmol) and a TFA-treated sample of the above protected tripeptide (8.88 g, 9.0 mmol) in DMF (120 ml) was stirred overnight and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 7.22 g (72%), mp 125—128 °C, $[\alpha]_D^{23}$ −13.8° ($c$=1.3, DMF), $Rf_2$ 0.56. *Anal.* Calcd for $C_{49}H_{70}N_{12}O_{14}S_2 \cdot 1/2H_2O$: C, 52.34; H, 6.37; N, 14.95. Found: C, 52.45; H, 6.71; N, 14.85.

**Z(OMe)–Gln–Asn–Arg(Mts)–Arg(Mts)–$NHNH_2$[2] (Positions 14—17)**——The above protected tetrapeptide

ester (3.92 g, 3.51 mmol) in DMF (40 ml) was treated with 80% hydrazine hydrate (1.1 ml, 5 eq) at room temperature overnight. The solvent was removed by evaporation and the residue was precipitated from DMF with MeOH; yield 3.15 g (80%), mp 146—148 °C, $[\alpha]_D^{23}$ −6.0° ($c$=0.7, DMF), $Rf_2$ 0.55. Amino acid ratios in a 6 N HCl hydrolysate: Glu 1.00, Asp 1.03, Arg 1.99 (recovery of Glu, 83%). *Anal.* Calcd for $C_{48}H_{70}N_{14}O_{13}S_2 \cdot H_2O$: C, 50.87; H, 6.40; N, 17.30. Found: C, 50.74; H, 6.23; N, 17.29.

**Z(OMe)–Ile–Val–Ser–OMe**——A mixture of Z(OMe)–Ile–ONp (7.50 g, 18.0 mmol), $Et_3N$ (4.60 ml, 33.0 mmol) and a TFA-treated sample of Z(OMe)–Val–Ser–OMe[17] (5.74 g, 15.0 mmol) in DMF (100 ml) was stirred overnight and concentrated. The residue was purified by procedure B, followed by precipitation from DMF with ether; yield 6.53 g (88%), mp 225—228 °C, $[\alpha]_D^{23}$ −2.2° ($c$=0.9, DMF), $Rf_2$ 0.88. *Anal.* Calcd for $C_{24}H_{37}N_3O_8$: C, 58.17; H, 7.53; N, 8.48. Found: C, 58.35; H, 7.75; N, 8.74.

**Z(OMe)–Pro–Ile–Val–Ser–OMe**——A MA [prepared from 4.97 g (10.8 mmol) of Z(OMe)–Pro–OH · DCHA] in THF (50 ml) was added to an ice-chilled solution of a TFA-treated sample of the above protected tripeptide ester (4.46 g, 9.0 mmol) in DMF (50 ml) containing $Et_3N$ (1.25 ml, 9.0 mmol) and the solution was stirred for 5 h. The solution was concentrated and the residue was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.03 g (76%), mp 217—219 °C, $[\alpha]_D^{23}$ −34.1° ($c$=0.9, DMF), $Rf_2$ 0.82. *Anal.* Calcd for $C_{29}H_{44}N_4O_9$: C, 58.77; H, 7.48; N, 9.45. Found: C, 58.93; H, 7.63; N, 9.62.

**Z(OMe)–Pro–Ile–Val–Ser–NHNH$_2$ [3] (Positions 10—13)**——The above protected tetrapeptide ester (5.93 g, 10.0 mmol) in DMF (100 ml) was treated with 80% hydrazine hydrate (3.13 ml, 5 eq) overnight. The solvent was evaporated off and the residue was precipitated from DMF with MeOH; yield 5.22 g (88%), mp 244—247 °C, $[\alpha]_D^{23}$ −26.1° ($c$=1.3, DMF), $Rf_2$ 0.58. Amino acid ratios in a 6 N HCl hydrolysate: Pro 0.98, Ile 1.02, Val 0.97, Ser 1.00 (recovery of Ser, 86%). *Anal.* Calcd for $C_{28}H_{44}N_6O_8$: C, 56.74; H, 7.48; N, 14.18. Found: C, 56.48; H, 7.47; N, 14.00.

**Z(OMe)–Gln–Gly–NHNH$_2$ [4] (Positions 8—9)**——Z(OMe)–Gln–Gly–OMe[18] (3.81 g, 10.0 mmol) in DMF (30 ml) was treated with 80% hydrazine hydrate (3.13 ml, 5 eq) as stated above and purified by precipitation from DMF with MeOH; yield 3.13 g (82%), mp 195—197 °C, $[\alpha]_D^{23}$ −2.1° ($c$=0.9, DMF), $Rf_2$ 0.38. Amino acid ratios in a 6 N HCl hydrolysate: Glu 0.98, Gly 1.00 (recovery of Gly, 90%). *Anal.* Calcd for $C_{16}H_{23}N_5O_6$: C, 50.39; H, 6.08; N, 18.36. Found: C, 50.29; H, 5.97; N, 18.54.

**Z(OMe)–Glu(OBzl)–Phe–NHNH–Troc**——A mixture of Z(OMe)–Glu(OBzl)–ONp (11.29 g, 21.6 mmol), $Et_3N$ (5.52 ml, 39.6 mmol) and a TFA-treated sample of Z(OMe)–Phe–NHNH–Troc (9.34 g, 18.0 mmol) in DMF (150 ml) was stirred overnight and concentrated. The product was purified by procedure A, followed by recrystallization from MeOH; yield 9.75 g (73%), mp 151—154 °C, $[\alpha]_D^{23}$ −16.4° ($c$=0.8, DMF), $Rf_2$ 0.78. *Anal.* Calcd for $C_{33}H_{45}Cl_3N_4O_9$: C, 53.70; H, 4.78; N, 7.59. Found: C, 53.89; H, 4.76; N, 7.63.

**Z(OMe)–Glu(OBzl)–Glu(OBzl)–Phe–NHNH–Troc**——A mixture of Z(OMe)–Glu(OBzl)–ONp (5.96 g, 11.4 mmol), $Et_3N$ (2.91 ml, 20.9 mmol) and a TFA-treated sample of the above dipeptide derivative (7.01 g, 9.50 mmol) in DMF (100 ml) was stirred overnight and concentrated. The product was purified by procedure A, followed by recrystallization from MeOH; yield 7.64 g (84%), mp 120—121 °C, $[\alpha]_D^{23}$ −12.5° ($c$=1.0, DMF), $Rf_2$ 0.75. *Anal.* Calcd for $C_{45}H_{48}Cl_3N_5O_{12}$: C, 56.46; H, 5.05; N, 7.32. Found: C, 56.37; H, 5.22; N, 7.70.

**Z(OMe)–Asp(OBzl)–Glu(OBzl)–Glu(OBzl)–Phe–NHNH–Troc**——A mixture of Z(OMe)–Asp(OBzl)–ONp (4.27 g, 8.40 mmol), $Et_3N$ (2.15 ml, 15.4 mmol) and a TFA-treated sample of the above protected tripeptide derivative (6.70 g, 7.0 mmol) in DMF (100 ml) was stirred overnight and concentrated. The product was purified by procedure A, followed by precipitation from DMF with ether; yield 6.53 g (80%), mp 130—133 °C, $[\alpha]_D^{23}$ −10.6° ($c$=1.0, DMF), $Rf_1$ 0.50. *Anal.* Calcd for $C_{56}H_{59}Cl_3N_6O_{15}$: C, 57.86; H, 5.12; N, 7.23. Found: C, 58.00; H, 5.09; N, 7.28.

**Z(OMe)–Val–Asp(OBzl)–Glu(OBzl)–Glu(OBzl)–Phe–NHNH–Troc**——A mixture of Z(OMe)–Val–OSu (2.27 g, 6.0 mmol), $Et_3N$ (1.53 ml, 11.0 mmol) and a TFA-treated sample of the above protected tetrapeptide derivative (5.81 g, 5.0 mmol) in DMF (100 ml) was stirred overnight and concentrated. The product was purified by procedure B, followed by precipitation from DMF with AcOEt; yield 4.93 g (78%), mp 208—211 °C, $[\alpha]_D^{23}$ −26.7° ($c$=0.8, DMF), $Rf_2$ 0.75. *Anal.* Calcd for $C_{61}H_{68}Cl_3N_7O_{16}$: C, 58.07; H, 5.43; N, 7.77. Found: C, 57.90; H, 5.48; N, 7.98.

**Z(OMe)–Val–Asp(OBzl)–Glu(OBzl)–Glu(OBzl)–Phe–NHNH$_2$ [5] (Positions 3—7)**——The above Troc-derivative (3.78 g, 3.0 mmol) in DMF–AcOH (40 ml–10 ml) was treated with Zn powder (1.96 g, 10 eq) at room temperature for 8 h. The solution was filtered, the filtrate was concentrated, and the residue was treated with 3% EDTA. The resulting powder was washed with 5% $NaHCO_3$ and $H_2O$ and precipitated from DMF with MeOH; yield 2.67 g (82%), mp 215—217 °C, $[\alpha]_D^{23}$ −21.4° ($c$=1.0, DMF), $Rf_2$ 0.59. Amino acid ratios in a 6 N HCl hydrolysate: Val 0.89, Asp 1.04, Glu 2.11, Phe 1.00 (recovery of Phe, 82%). *Anal.* Calcd for $C_{58}H_{67}N_7O_{14}$: C, 64.13; H, 6.22; N, 9.03. Found: C, 63.92; H, 6.11; N, 8.97.

**Z(OMe)–Phe–Lys(Z)–OMe**——The title compound was prepared by the Np method and purified by procedure B, followed by precipitation from DMF with ether; yield 85%, mp 137—138 °C, $[\alpha]_D^{23}$ −31.0° ($c$=0.8, DMF), $Rf_2$ 0.79. *Anal.* Calcd for $C_{33}H_{39}N_3O_8$: C, 65.44; H, 6.49; N, 6.94. Found: C, 65.37; H, 6.41; N, 6.81.

**Z(OMe)–Phe–Lys(Z)–NHNH$_2$ [6] (Position 1—2)**——The above protected dipeptide ester was treated with 80% hydrazine hydrate (5 eq) as stated above and the product was purified by precipitation from DMF with MeOH; yield 87%, mp 180—183 °C, $[\alpha]_D^{23}$ −24.5° ($c$=0.9, DMF), $Rf_2$ 0.63. Amino acid ratios in a 6 N HCl hydrolysate: Phe 1.07, Lys 1.00 (recovery of Lys, 80%). *Anal.* Calcd for $C_{32}H_{39}N_5O_7$: C, 63.46; H, 6.49; N, 11.56. Found: C, 63.44; H,

6.47; N, 11.50.

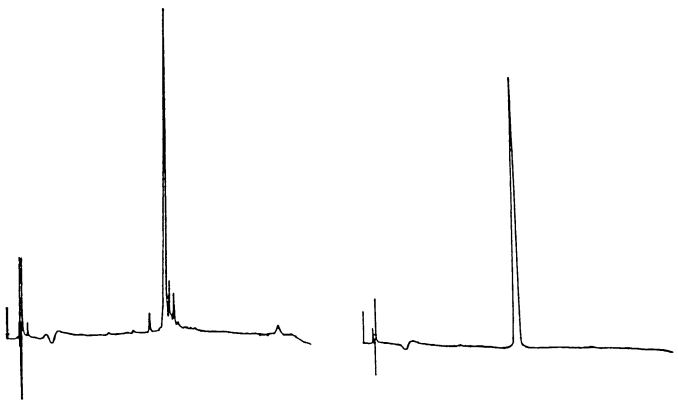
**Z(OMe)–Gln–Asn–Arg(Mts)–Arg(Mts)–Tyr–Phe–Leu–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (Positions 14—25)**——The azide [prepared from 2.68 g (2.40 mmol) of fragment [2]] in DMF (40 ml) and $Et_3N$ (0.40 ml, 2.88 mmol) were added to an ice-chilled solution of a TFA-treated sample of fragment [1] (3.28 g, 2.0 mmol) in DMF (30 ml) containing $Et_3N$ (0.28 ml, 2.0 mmol) and the mixture, after being stirred for 24 h, was concentrated. The product was purified by procedure B, followed by precipitation from DMF with EtOH; yield 3.55 g (69%), mp 176—180 °C, $[\alpha]_D^{23}$ −17.9° ($c$=1.1, DMF), $Rf_2$ 0.56. *Anal.* Calcd for $C_{120}H_{164}N_{28}O_{27}S_4 \cdot 3H_2O$: C, 55.15; H, 6.56; N, 15.01. Found: C, 55.32; H, 6.73; N, 14.70.

**Z(OMe)–Pro–Ile–Val–Ser–Gln–Asn–Arg(Mts)–Arg(Mts)–Tyr–Phe–Leu–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (Positions 10—25)**——The azide [prepared from 1.20 g (2.03 mmol) of fragment [3]]in DMF (10 ml) and $Et_3N$ (0.34 ml, 2.44 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected dodecapeptide amide (4.00 g, 1.56 mmol) in DMF (40 ml) containing $Et_3N$ (0.22 ml, 1.56 mmol) and the mixture, after being stirred for 24 h, was concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 3.21 g (69%), mp 203—206 °C, $[\alpha]_D^{23}$ −47.1° ($c$=0.4, DMF), $Rf_2$ 0.47. *Anal.* Calcd for $C_{139}H_{196}N_{32}O_{32}S_4 \cdot 4H_2O$: C, 55.14; H, 6.79; N, 14.81. Found: C, 54.87; H, 6.86; N, 14.63.

**Z(OMe)–Gln–Gly–Pro–Ile–Val–Ser–Gln–Asn–Arg(Mts)–Arg(Mts)–Tyr–Phe–Leu–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (Positions 8—25)**——The azide [prepared from 0.76 g (2.0 mmol) of fragment [4]] in DMF (10 ml) and $Et_3N$ (0.33 ml, 2.4 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above hexadecapeptide amide (2.96 g, 1.0 mmol) in DMF (30 ml) containing $Et_3N$ (0.14 ml, 1.0 mmol) and the mixture, after being stirred for 24 h, was concentrated. The product was purified by procedure B, followed by precipitation from DMF with MeOH; yield 2.70 g (86%), mp 188—192 °C, $[\alpha]_D^{23}$ −11.0° ($c$=0.8, DMF), $Rf_2$ 0.46. *Anal.* Calcd for $C_{146}H_{207}N_{35}O_{35}S_4 \cdot 5H_2O$: C, 54.27; H, 6.77; N, 15.18. Found; C, 54.11; H, 6.58; N, 14.99.

**Z(OMe)–Val–Asp(OBzl)–Glu(OBzl)–Glu(OBzl)–Phe–Gln–Gly–Pro–Ile–Val–Ser–Gln–Asn–Arg(Mts)–Arg(Mts)–Tyr–Phe–Leu–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$ (Positions 3—25)**——The azide [prepared from 276 mg (0.26 mmol) of fragment [5]] in DMF (10 ml) and $Et_3N$ (43 μl, 0.31 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above protected octadecapeptide (400 mg, 0.13 mmol) in DMF (15 ml) containing $Et_3N$ (18 μl, 0.13 mmol) and the mixture, after being stirred for 24 h, was concentrated. The product was purified by procedure C, followed by precipitation from DMF with MeOH; yield 430 mg (84%), mp 246—248 °C, $[\alpha]_D^{23}$ −46.7° ($c$=0.4, DMF), $Rf_2$ 0.60. *Anal.* Calcd for $C_{195}H_{262}N_{40}O_{46}S_4 \cdot 9H_2O$: C, 55.86; H, 6.73; N, 13.36. Found: C, 55.60; H, 6.54; N, 13.16.

**Z(OMe)–Phe–Lys(Z)–Val–Asp(OBzl)–Glu(OBzl)–Glu(OBzl)–Phe–Gln–Gly–Pro–Ile–Val–Ser–Gln–Asn–Arg(Mts)–Arg(Mts)–Tyr–Phe–Leu–Phe–Arg(Mts)–Pro–Arg(Mts)–Asn–$NH_2$, Protected U-25**——The azide [prepared



(a) (b)

Fig. 4. HPLC of Synthetic Neuromedin U-25

a), crude sample; b), purified sample.

(+) (−)

Fig. 5. Disc Isoelectrofocusing of Synthetic Neuromedin U-25

from 135 mg (0.20 mmol) of fragment [6]] in DMF (10 ml) and $Et_3N$ (28 $\mu$l, 0.20 mmol) were added to an ice-chilled solution of a TFA-treated sample of the above tricosapeptide (403 mg, 0.10 mmol) in DMF (15 ml) containing $Et_3N$ (14 $\mu$l, 0.10 mmol) and the solution, after being stirred for 24 h, was concentrated. The product was purified by procedure C; yield 350 mg (79%), mp 238—240 °C, $[\alpha]_D^{23}$ −9.1° ($c$=0.8, DMF), $Rf_2$ 0.54. *Anal.* Calcd for $C_{218}H_{289}N_{43}O_{50}S_4 \cdot 4H_2O$: C, 58.02; H, 6.63; N, 13.35. Found: C, 58.13; H, 6.58; N, 13.11.

**Synthetic U-25**——(a) Protected U-25 (50 mg) was treated with 1 M TMSOTf–thioanisole/TFA (2.25 ml, 200 eq) in the presence of *m*-cresol (118 $\mu$l, 100 eq) and EDT (18.9 $\mu$l, 20 eq) in an ice-bath for 60 min, then dry ether was added. The resulting powder was collected by centrifugation, washed with dry ether and dissolved in $H_2O$ (10 ml). Under cooling with ice, the pH of the filtered solution was adjusted to 8.0 with 5% $NH_4OH$, and 1 M $NH_4F/H_2O$ (282 $\mu$l, 25 eq) was added. After 30 min, the solution was adjusted to pH 6.0 with 1 N AcOH. The solution was applied to a column of Sephadex G-25 (3.3 × 103 cm), which was eluted with the same solvent. The fractions (9.2 ml each) corresponding to the front main peak (tube Nos. 39—49, monitored by UV absorption measurement at 275 nm) were collected and the solvent was removed by lyophilization to give a powder; yield 34.6 mg (98%). A part of the crude product (17 mg) thus obtained was purified by HPLC on a TSK-GEL LS-410KG column (21.5 × 300 mm), by isocratic elution with 26% acetonitrile in 0.1% TFA aq. at a flow rate of 8.0 ml per min. The eluate corresponding to the main peak (retention time 33 min, Fig. 4-a) was collected. The rest of the sample was similarly purified by HPLC. The eluates were combined and the solvent was removed by evaporation. The residue was applied to a column of Sephadex G-25 (1.8 × 55 cm), which was eluted with 0.1 N AcOH. The desired fractions (monitored as described above) were collected and the solvent was removed by lyophilization to give a fluffy white powder, yield 18.5 mg (53%). The overall yield from protected U-25 was 52%; $[\alpha]_D^{28}$ −78.6° ($c$=0.4, $H_2O$), a single spot on TLC: $Rf_3$ 0.39, $Rf_4$ 0.46. The retention time was 25 min in HPLC on an analytical Nucleosil 5C18 column (4.0 × 150 mm), eluted with a gradient of acetonitrile (15% to 40%, 40 min) in 0.1% TFA aq. at a flow rate of 0.8 ml per min (Fig. 4-b). The mobility in disc isoelectrofocusing on 7.5% polyacrylamide gel containing Pharmalyte (pH 3—10) was 6.3 cm from the origin toward the cathodic end of the gel, after running at 200 V for 4 h (Fig. 5). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in a LAP digest (numbers in parentheses are theoretical): Asp 0.97 (1), Ser 0.96 (1), Glu 1.83 (2), Pro 1.70 (2), Gly 0.94 (1), Val 1.71 (2), Ile 0.90 (1). Leu 1.00 (1), Tyr 0.92 (1), Phe 3.42 (4), Lys 0.99 (1), Arg 3.37 (4), Asn and Gln were not determined (recovery of Leu, 70%).

(b) Protected U-25 (100 mg) was treated with 1 M TFMSA–thioanisole/TFA (4.5 ml) in the presence of *m*-cresol (0.24 ml, 100 eq) and EDT (37.7 $\mu$l, 20 eq) in an ice-bath for 150 min, then dry ether was added. The resulting powder was treated with 5% $NH_4OH$ at pH 8.0 for 30 min and purified by gel-filtration on Sephadex G-25, followed by HPLC as stated above; yield 33.2 mg (47% from the protected peptide). A mixture of the samples obtained in (a) and (b) emerged as a single peak (retention time 25 min) from a Nucleosil 5C18 HPLC column (4.0 × 150 mm), which was eluted with a gradient of acetonitrile (15% to 40%, 40 min) in 0.1% TFA aq. at a flow rate of 0.8 ml per min. Amino acid ratios in a 6 N HCl hydrolysate: Asp 2.87, Ser 0.80, Glu 3.92, Gly 1.01, Val 1.68, Ile 0.89, Leu 1.00, Tyr 1.04, Phe 3.78, Lys 0.92, Arg 3.81, Pro 1.96 (recovery of Leu, 69%).

**Synthetic U-8**——(a) Protected U-8 (50 mg) was treated with 1 M TMSOTf–thioanisole in TFA (0.9 ml, 30 eq) in the presence of *m*-cresol (48 $\mu$l, 15 eq) in an ice-bath for 60 min, then dry ether was added. The deprotected peptide was treated with 5% $NH_4OH$ containing $NH_4F$ (10 eq) at pH 8.0, then submitted to gel-filtration on Sephadex G-10 (2.8 × 87 cm) as stated above. Yield 28.3 mg (84%). The final purification was achieved similarly by HPLC on a TSK-GEL LS-410KG column (21.5 × 300 mm) using isocratic elution with 22% acetonitrile in 0.1% TFA aq. The purified peptide (retention time 50 min) was passed through a column of Sephadex G-10 using 0.1 N AcOH and finally lyophilized to give a fluffy powder; yield 14.4 mg (51%), overall yield from the protected peptide was 42%. $[\alpha]_D^{28}$ −44.7° ($c$=0.2, $H_2O$), a single spot on TLC: $Rf_3$ 0.55, $Rf_4$ 0.75. The retention time was 20 min in HPLC on a Cosmosil 5C18 column (4 × 150 mm) on gradient elution with acetonitrile (15—40%, 40 min) in 0.1% TFA aq. at a flow rate of 0.8 ml/min; Amino acid ratios in a 6 N HCl hydrolysate and a LAP digest (numbers in parentheses): Asp 1.01, Leu 1.00 (1.00), Tyr 0.99 (0.93), Phe 1.97 (1.89), Arg 1.91 (1.73), Pro 0.99 (0.86), Asn was not determined, recovery of Leu 81% (79%).

(b) Protected U-8 (200 mg) was treated with 1 M TFMSA–thioanisole/TFA (3.7 ml, 30 eq) in the presence of *m*-cresol (0.4 ml, 30 eq) in an ice-bath for 120 min, then dry ether was added. The deprotected peptide was treated with 5% $NH_4$ OH at pH 8.0 and then purified by gel-filtration on Sephadex G-10, followed by HPLC as described above; yield 43.3 mg (32%). A mixture of the samples obtained in (a) and (b) emerged as a single peak (retention time, 20 min) from a Cosmosil 5C18 HPLC column on gradient elution with acetonitrile (15% to 40%, 40 min) in 0.1% TFA aq. at a flow rate of 0.8 ml per min. Amino acid ratios in a 6 N HCl hydrolysate: Asp 1.03, Leu 1.00, Pro 0.97, Tyr 0.94, Phe 1.99, Arg 2.07 (recovery of Leu, 72%).

## References and Notes

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used:

Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Mts = mesitylenesulfonyl, Troc = 2,2,2-trichloroethyloxycarbonyl, Np = *p*-nitrophenyl, Su = *N*-hydroxysuccinimidyl, DCC = dicyclohexylcarbodiimide, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid, THF = tetrahydrofuran, DMF = dimethylformamide, EDT = ethanedithiol, EDTA = ethylenediaminetetraacetic acid disodium salts.


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