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# Studies on Peptides. LII.[1,2] Application of the Trifluoromethanesulphonic Acid Procedure to the Synthesis of a Peptide with Somatostatin Activity

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Trifluoromethanesulphonic acid was applied to remove the protecting groups, Z and MBzl, from Z–Ala–Gly–Cys(MBzl)–Lys(Z)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH to produce the peptide with somatostatin activity.

The acidolysis of various protecting groups currently employed in peptide synthesis by trifluoromethanesulphonic acid was recently examined[4] and the usefulness of this reagent was demonstrated preliminarily in the synthesis of a model peptide, H–Thr–Lys–Pro–Arg–OH, named tuftsin.[5,6] We wish to add an another example of application of this deblocking procedure to the synthesis of a more complex peptide. For our present purpose, synthesis of somatostatin (I), the tetradecapeptide known as a growth hormone release inhibitory factor (SRIF), was selected.

The structure of somatostatin from ovine hypothalamic tissue was elucidated by Guillemin, *et al.*[7,8] at the Salk Institute in 1973 and its solid phase synthesis was reported by the same group of investigators.[7,9] Conventional way of synthesis of this hypothalamic principle,[10] as well as its solid phase synthesis,[11] were subsequently reported, because of its great biological interests.

Our synthetic scheme of somatostatin (I) is illustrated in Fig. 1. This was carried out by condensation of two peptide fragments, Z–Ala–Gly–Cys(MBzl)–Lys(Z)–$NHNH_2$ (II) and Z(OMe)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH (III), followed by deblocking of all protecting groups with trifluoromethanesulphonic acid and subsequent air oxidation to form the disulfide bridge.

1) Part LI. H. Yajima, M. Kurobe, I. Yo, N. Fujii, and Y. Baba, *Chem. Pharm. Bull.* (Tokyo), **23**, 1622 (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=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, MBzl=*p*-methoxybenzyl, ONP=*p*-nitrophenyl ester.
3) Location: *Sakyo-ku, Kyoto.*
4) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *Chem. Commun.*, **1974**, 107.
5) K. Nishioka, A. Constantopoulos, P.S. Satoh, W.M. Mitchell, and V.A. Najjar, *Biochim. Biophys. Acta*, **310**, 217 (1973), A. Constantopoulos and V.A. Najjar, *J. Biol. Chem.*, **248**, 3819 (1973), K. Nishioka, A. Constantopoulos, P.S. Satoh, and V.A. Najjar, *Biochem. Biophys. Res. Commun.*, **47**, 172 (1972); K. Nishioka, P.S. Satoh, A. Constantopoulos, and V.A. Najjar, *Biochim. Biophys. Acta*, **310**, 230 (1973).
6) H. Yajima, H. Ogawa, H. Watanabe, N. Fujii, M. Kurobe, and S. Miyamoto, *Chem. Pharm. Bull.* (Tokyo), **23**, 371 (1675).
7) P. Brazeau, W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier, and R. Guillemin, *Science*, **179**, 77(1973).
8) 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).
9) J.E.F. Rivier, *J. Am. Chem. Soc.*, **96**, 2986 (1974).
10) *a*) D. Sarantakis and W.A. McKinley, *Biochem. Biophys. Res. Commun.*, **54**, 234 (1973); *b*) H.U. Immer, K. Sestanj, V.R. Nelson, and M. Götz, *Helv. Chim. Acta*, **57**, 730 (1974).
11) *a*) D. Yamashiro and C.H.Li, *Biochem. Biophys. Res. Commun.*, **54**, 882 (1973); *b*) D.H. Coy, E.J. Coy, A. Arimura, and A.V. Schally, *Biochem. Biophys. Res. Commun.*, **54**, 1267 (1973).

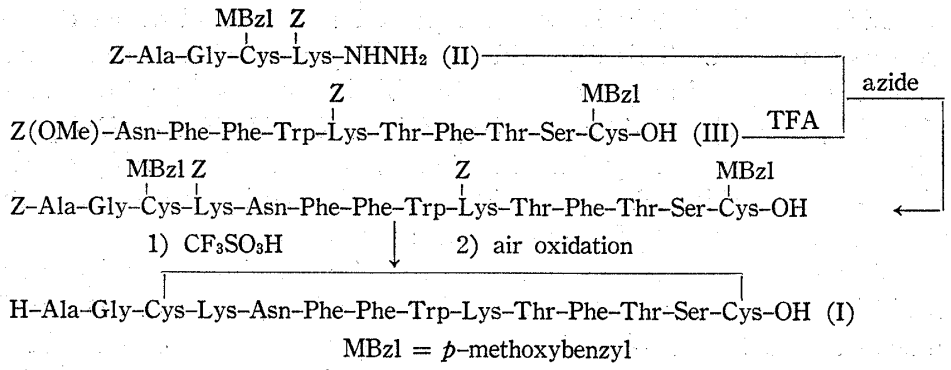


Fig. 1. Synthetic Route to Somatostatin

The Z(OMe) group removable by trifluoroacetic acid (TFA)[12] served as a temporal protecting group for the $\alpha$-amino function of necessary intermediates containing Cys. The *p*-methoxybenzyl (MBzl) and the Z group removable by trifluoromethanesulphonic acid or hydrogen fluoride[13] were adopted for protection of the side chain functional groups of Cys and Lys respectively. To construct the entire tetradecapeptide sequence of somatostatin, three protected dipeptide hydrazides served as building blocks. Therefore the azide procedure of Honzl and Rudinger[14] was the sole tool to elongate the peptide chain except for incorporation of Trp and Asn. These two amino acid residues were introduced by the *p*-nitrophenyl ester procedure.[15]

Synthetic scheme of the protected decapeptide (III) is illustrated in Fig. 2. Z(OMe)–Ser–azide derived from the corresponding hydrazide by the Honzl and Rudinger procedure was

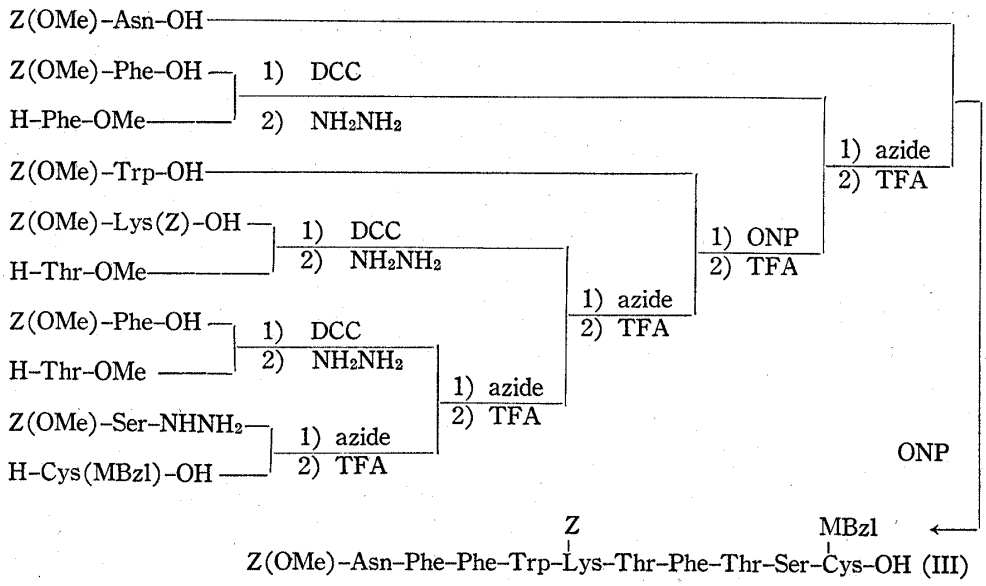


Fig. 2. Synthetic Scheme of the Protected Decapeptide (III) (Positions 5 ot 14)

allowed to react with the triethylammonium salt of H–Cys(MBzl)–OH to give Z(OMe)–Ser–Cys(MBzl)–OH. This protected dipeptide is a compound not easily soluble in ethyl acetate.

12) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962); H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **17**, 1962 (1969); H. Yajima, F. Tamura, and Y. Kiso, *ibid.*, **18**, 2574 (1970); H. Yajima, F. Tamura, Y. Kiso, and M. Kurobe, *ibid.*, **21**, 1380 (1973).
13) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).
14) J. Honzl and J. Rudinger, *Coll. Czech. Chem. Commun.*, **26**, 2333 (1961).
15) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

Therefore, for purification the batchwise washing procedure with acid rather than the extraction procedure was employed. This dipeptide was finally purified by recrystallization from dimethylformamide (DMF) and ethyl acetate. In addition of this dipeptide, three protected dipeptide hydrazides, Z(OMe)–Phe–Thr–$NHNH_2$, Z(OMe)–Lys(Z)–Thr–$NHNH_2$ and Z(OMe)–Phe–Phe–$NHNH_2$ were prepared by the dicyclohexylcarbodiimide (DCC) condensation of respective Z(OMe)–amino acids and amino acid esters, followed by treatment of the resulting protected dipeptide esters with hydrazine in the usual manner.

Next, the Z(OMe) group was removed from the above Z(OMe)–Ser–Cys(MBzl)–OH by treatment with TFA in the presence of anisole and the resulting dipeptide, H–Ser–Cys(MBzl)–OH, was condensed with Z(OMe)–Phe–Thr–$NHNH_2$ by the above stated azide procedure. Again, the batchwise washing and recrystallization procedures were found effective to purify the resulting protected tetrapeptide, Z(OMe)–Phe–Thr–Ser–Cys(MBzl)–OH. Such purification procedures were extended to later steps of this synthesis. The yield of this coupling reaction of two dipeptide units was somewhat low.

The above protected tetrapeptide, after treatment with TFA, was similarly condensed with Z(OMe)–Lys(Z)–Thr–$NHNH_2$ by the above stated azide procedure. In order to remove the unreacted tetrapeptide from the product, the batchwise washing with dilute ammonia, in addition to the washing with a citric acid solution, was necessary, though it was unexpected. After such washing, followed by recrystallization, the protected hexapeptide, Z(OMe)–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH, was obtained in chromatographically and analytically pure form.

Incorporation of Z(OMe)–Trp–OH to the TFA treated product of the above hexapeptide was perfomed by the *p*-nitrophenyl ester procedure. Following to the suggestion of König and Geiger,[16] N-hydroxybenzotriazole (HOBT) was added as a catalyst in this active ester reaction. This coupling reaction proceeded smoothly and the product, Z(OMe)–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH, was isolated in nearly quantitative yield.

The α-amino deprotection of the above heptapeptide by TFA gave some technical problem, since it contains the acid sensitive Trp residue. Before addition of TFA, the sample was immersed well with anisole containing a small amount of mercaptoethanol[17] and the air of the flask was replaced by nitrogen gas. The deblocking reaction was performed under ice-cooling for 30 minutes. The product, which was somewhat brownish, was precipitated by addition of dry ether and then submitted to the next coupling reaction with Z(OMe)–Phe–Phe–$NHNH_2$ by the azide procedure. The usual batchwise washing and recrystallization from DMF and ethyl acetate gave the protected nonapeptide, Z(OMe)–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH without particular difficulty. From this protected nonapeptide, the Z(OMe) group was removed by the similar careful treatment with TFA. To the resulting deblocked peptide, Z(OMe)–Asn–OH was incorporated by the *p*-nitrophenyl ester procedure in the presence of HOBT as did in the introduction of Z(OMe)–Trp–OH. The product, Z(OMe)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH (III), was isolated after similar purification, in chromatographycally and analytically pure form. Its homogeneity was further assessed by amino acid analysis.

Next, the N-terminal tetrapeptide unit (II) Z–Ala–Gly–Cys(MBzl)–Lys(Z)–$NHNH_2$, was prepared as illustrated in Fig. 3. This unit was also adopted by Sarantakis and McKinley[10a] for this somatostatin synthesis, but with different protecting groups from ours. Z(OMe)–Cys(MBzl)–Lys(Z)–OMe, prepared by the DCC condensation of Z(OMe)–Cys(MBzl)–OH with H–Lys(Z)–OMe was treated with TFA and the resulting product, after neutralization with sodium bicarbonate, was extracted with ethyl acetate. This solution containing H–Cys-

16) W. König and R. Geiger, *Chem. Ber.*, **106**, 3626 (1973).
17) G.R. Marshall, *Advan. Exp. Med.*, **2**, 48 (1969); H. Yajima, H. Kawatani, and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **18**, 1333 (1970).

(MBzl)–Lys(Z)–OMe was submitted to the DCC coupling reaction with Z–Ala–Gly–OH.[18] HOBT was employed in this fragment condensation reaction to suppress the acylurea formation.[19] and the product, Z–Ala–Gly–Cys(MBzl)–Lys(Z)–OMe, was isolated without difficulty in excellent yield. This was then converted to the corresponding hydrazide, Z–Ala–Gly–Cys(MBzl)–Lys(Z)–$NHNH_2$ (II), in the usual manner. Its homogeneity was confirmed by thin-layer chromatography with ceric sulphate[12c] and hydrazine tests[20] and elemental analysis.

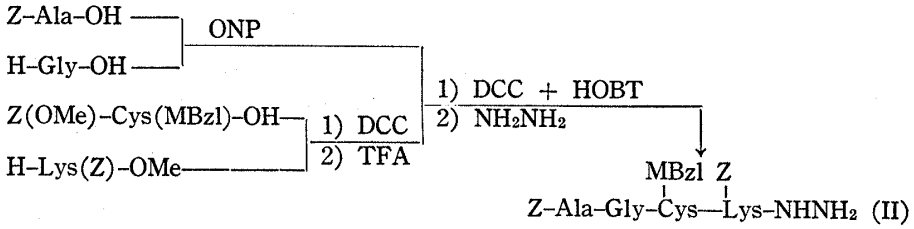


Fig. 3. Synthetic Scheme of the Protected Tetrapeptide Hydrazide (II) (Position 1 to 4)

The final coupling reaction between the N-terminal tetrapeptide unit (II) and the decapeptide unit (III) prepared above was then conducted. Removal of the Z(OMe) group from Z(OMe)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH was conducted as stated above. The resulting TFA salt, after neutralization with triethylamine, was submitted as an amino component to the azide coupling reaction of Z–Ala–Gly–Cys(BMzl)–Lys(Z)–$NHNH_2$ to give the protected tetradecapeptide, Z(OMe)–Ala–Gly–Cys(MBzl)–Lys(Z)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH. This, after purification by usual washing and recrystallization, was isolated in pure form with satisfactory elemental and amino acid analysis.

According to our preliminary observations,[4] the protected tetradecapeptide obtained above was treated with trifluoromethanesulphonic acid in the presence of anisole in an ice-bath for 30 minutes to remove the Z and MBzl groups. The deblocked peptide precipitated by addition of dry ether as fine powder exhibited a single spot positive to ninhydrin and Ehrich tests on thin-layer chromatography. In order to establish the intramolecular disulfide bridge, the product was then air oxidized in high diluted conditions at pH 6.5 at room temperature for 72 hr. As stated by Yamashiro and Li,[11a] the solution became turbid during this oxidation step, indicating the formation of polymeric substances in some extent. Decrease in the colour by the Ellman reagent[21] was the guide of this oxidation reaction. For concentration of the desired peptide from this diluted solution, a column chromatography on IRC-50 worked out nicely as mentioned by Yamashiro and Li.[11a] However the yield of the product at this stage was 41%. Rivier[9] referred also that air oxidation brought about cyclization with relatively poor yield (*ca.* 25%). The peptide thus concentrated was purified by partition column chromatography on Sephadex G-25F.[22] As reported,[9,10a,11b] the solvent system of *n*-butanol, acetic acid and water (4: 1: 5) was quite effective to isolate, as the third fraction (Fig. 1), a homogeneous peptide with the *Rf* value (hold-up volume/elution volume) which was very close to that of the natural source reported by Rivier.[9] The rotation figure and *Rf* value on thin-layer chromatography of the peptide thus isolated were very close to those of literatures.[9,11a,b] From these results, this peptide can be judged as a monomeric form of

18) *a*) E. Schröder, *Ann. Chem.*, **679**, 207 (1964); *b*) F.H.C. Stewart, *Aust. J. Chem.*, **21**, 2543 (1968); F. Weygand and W. Steglich, *Chem. Ber.*, **93**, 2983 (1960).
19) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
20) H. Ertel and L. Horner, *J. Chromatg.*, **7**, 268 (1962); K. Hofmann, R. Schmiechen, R.D. Wells, Y. Wolman, and N. Yanaihara, *J. Am. Chem. Soc.*, **87**, 611 (1965).
21) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959); A.F. S.A. Habeeb, in "Methods in Enzymology," ed. C.H.W. Hirs and Timasheff, Academic Press, New York, 1972, p. 457.
22) D. Yamashiro, *Nature*, **201**, 76 (1964).

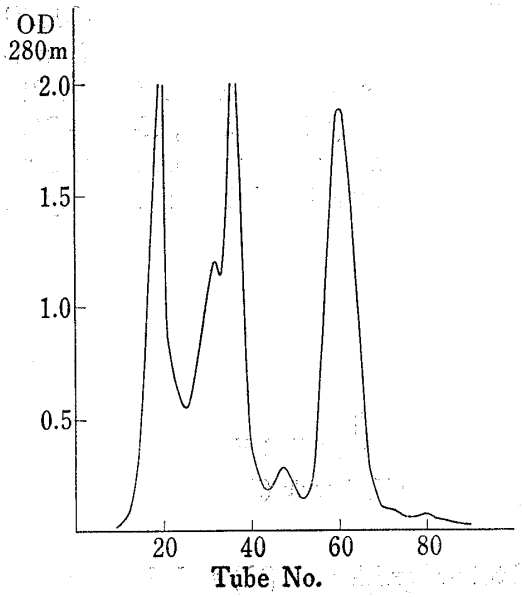


Fig. 4. Purification of Synthetic Somatostatin by Column Chromatography on Sephadex G-25

solvent system *n*-BuOH–AcOH–$H_2O$ (4: 1: 5)

somatostatin. The hydrolysate of this peptide by 3N *p*-toluenesulphonic acid[23] contained the constituent amino acids in ratios predicted by theory. The contents of Trp and cystine were determined by the enzymatic hydrolysis also. Two fractions separated prior to the above third fraction (Fig. 4) are presumably a mixture of polymeric substances and both are less soluble in water. The yield of the monomer of somatostatin was only 7%. Coy, *et al.*[11b] reported the isolation of this substance in 6% overall yield in the purification step. These low yields seem due to difficulty in settlement of optimum conditions to establish such a large intramolecular disulfide bridge.

Our synthetic peptide was assayed by Professor H. Imura of the Internal Medicine, Kobe University and found that all of three fractions separated above had an ability to inhibit the secretion of growth hormone in rats. These results will be published elsewhere.

From these results, it can be justify the conclusion that as far as protecting groups are concerned, trifluoromethanesulphonic acid removed the Z and MBzl groups from the peptide containing Cys and Lys under controlled conditions to produce the biologically active peptide. We wish to emphasize the important role of controlled removal conditions as we adopted above, since H–Cys(MBzl)–OH was treated with this reagent under more drastic conditions, such as without any solvent at room temperature for 90 minutes, the recovery of cysteine+cystine became considerably low. It seems noteworthy that the Trp residue survived also fully intact under conditions settled in this experiment.

## Experimental

Thin-layer chromatography was performed on silica gel (Kieselgel, 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: 20: 6: 24), $Rf_4$ *n*-BuOH–AcOH–$H_2O$–AcOEt (1: 1: 1: 1).

**Z(OMe)-Ser-Cys(MBzl)-OH**——To a solution of Z(OMe)–Ser–$NHNH_2$ (28.30 g) in DMF (150 ml), 5.4N HCl–DMF (37 ml) and isoamylnitrite (16 ml) were added at −5° and the mixture was stirred for 5 min. When the hydrazine test[20] became negative, the solution, after neutralization with $Et_3N$ (27.6 ml) was combined with a solution of H–Cys(MBzl)–OH (24.13 g) in $H_2O$ (150 ml) containing $Et_3N$ (27.6 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the resulting solid residue was washed batchwisely with 10% citric acid and $H_2O$ and ether. It was recrystallized from DMF and AcOEt; yield 34.12 g (69%), mp 161—162°, $[\alpha]_D^{28}$ −5.3° ($c$=0.8, DMF), $Rf_1$ 0.50. *Anal.* Calcd. for $C_{23}H_{28}O_8N_2S$: C, 56.08; H, 5.73; N, 5.69. Found: C, 56.27; H, 5.86; N, 5.56.

**Z(OMe)-Phe-Thr-OMe**——DCC (22.60 g) was added to a solution of Z(OMe)–Phe–OH (32.90 g) and H–Thr–OMe (prepared from 17.0 g of the hydrochloride with 19.3 ml of $Et_3N$) in DMF (300 ml) and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt, which was washed with 10% citric acid, 5% $Na_2CO_3$ and $H_2O$, dried over $Na_2SO_4$ and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 31.0 g (73%), mp 134—136°, $[\alpha]_D^{25}$ −11.5° ($c$=1.0, DMF), $Rf_1$ 0.86. *Anal.* Calcd. for $C_{23}H_{28}O_7N_2$: C, 62.14; H, 6.35; N, 6.30. Found: C, 62.43; H, 6.58; N, 6.51.

**Z(OMe)-Phe-Thr-$NHNH_2$**——To a solution of Z(OMe)–Phe–Thr–OMe (31.0 g) in MeOH (300 ml), aqueous 80% hydrazine hydrate (35 ml) was added. The gelatinous mass formed on standing overnight, was collected by filtration and recrystallized from DMF and MeOH; yield 30.07 g (97%), mp 199—200°, $Rf_1$ 0.52. *Anal.* Calcd. for $C_{22}H_{28}O_6N_4$: C, 59.44; H, 6.35; N, 12.61. Found: C, 59.18; H, 6.34; N, 12.58.

23) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).

**Z(OMe)-Lys(Z)-Thr-OMe**——DCC (23.0 g) was added to a solution of Z(OMe)–Lys(Z)–OH (44.50 g) and H–Thr–OMe (prepared from 17.0 g of the hydrochloride with 17.6 ml of $Et_3N$) in DMF (400 ml) and the mixture was stirred at room temperature for 48 hr. The solution, after filtration, was condensed *in vacuo* and the residue was dissolved in AcOEt, which was washed with 10% citric acid, 5% $Na_2CO_3$ and $H_2O$, dried over $Na_2SO_4$ and then evaporated. The residue was triturated with ether and recrystallized from AcOEt and ether; yield 42.02 g (75%), mp 128—130°, $[\alpha]_D^{26}$ −0.9° ($c$=1.2, DMF). $Rf_1$ 0.80. *Anal.* Calcd. for $C_{28}H_{37}O_9N_3$: C, 60.09; H, 6.66; N, 7.51. Found: C, 60.21; H, 6.63; N, 7.57.

**Z(OMe)-Lys(Z)-Thr-NHNH$_2$**——In the usual manner, aqueous hydrazine hydrate (80%, 37.5 ml) was added to a solution of Z(OMe)–Lys(Z)–Thr–OMe (42.0 g). The gelatinous mass formed on standing overnight was collected by filtration and recrystallized from dioxane and MeOH; yield 36.10 g (86%), mp 158—161°, $Rf_1$ 0.67. *Anal.* Calcd. for $C_{27}H_{37}O_8N_5$: C, 57.94; H, 6.66; N, 12.52. Found: C, 57.73; H, 6.68; N, 12.66.

**Z(OMe)-Trp-ONP**——DCC (24.0 g) was added to a solution of Z(OMe)–Trp–OH (37.0 g) and *p*-nitrophenol (16.70 g) in AcOEt (300 ml) and the mixture was stirred at room temperature for 24 hr. The solution, after filtration was washed with 5% $Na_2CO_3$ and $H_2O$, dried over $Na_2SO_4$ and then evaporated. The residue was triturated with petroleum ether and recrystallized from AcOEt and ether; yield 26.66 g (55%), mp 118—119°; *Anal.* Calcd. for $C_{26}H_{23}O_7N_3$: C, 63.80; H, 4.78; N, 8.57. Found: C, 63.75, H, 4.74, N, 8.59.

**Z(OMe)-Phe-Phe-OMe**——DCC (22.60 g) was added to a solution of Z(OMe)–Phe–OH (32.93 g) and H–Phe–OMe (prepared from 21.57 g of the hydrochloride with 16.6 ml of $Et_3N$) in DMF (300 ml) and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting solid was washed batchwisely with 10% citric acid and ether; yield 34.32 g (70%), mp 135—137°, $[\alpha]_D^{25}$ −19.4° ($c$=1.0, DMF), $Rf_1$ 0.99. *Anal.* Calcd. for $C_{28}H_{30}O_6N_2$: C, 68.55; H, 6.16; N, 5.71. Found: C, 68.41; H, 5.97; N, 5.58.

**Z(OMe)-Phe-Phe-NHNH$_2$**——To a solution of Z(OMe)–Phe–Phe–OMe (21.0 g) in DMF (150 ml), hydrazine hydrate (80%, 25 ml) was added and the solution was kept on standing overnight. The solvent was condensed to one third of the original volume and MeOH (100 ml) was added. The forming gelatinous mass was collected by filtration and washed with hot MeOH; yield 20.10 g (96%), mp 216—220°, $Rf_1$ 0.69. *Anal.* Calcd. for $C_{27}H_{30}O_5N_4$: C, 66.10; H, 6.16; N, 11.42. Found: C, 66.24; H, 6.01; N, 11.46.

**H-Ser-Cys(MBzl)-OH. • TFA Salt**——Z(OMe)–Ser–Cys(MBzl)–OH (24.98 g) was treated with TFA (60 ml) in the presence of anisole (16 ml) under cooling with ice for 30 min. Dry ether was added to form the fine powder, which was collected by filtration and dried over KOH pellets *in vacuo*; yield 22.26 g (99%), mp 135—140°, $Rf_1$ 0.21. *Anal.* Calcd. for $C_{14}H_{20}O_5N_2S \cdot CF_3COOH$: C, 43.43; H, 4.78; N, 6.33. Found: C, 43.23; H, 4.58; N, 6.34.

**Z(OMe)-Phe-Thr-Ser-Cys(MBzl)-OH**——To a solution of Z(OMe)–Phe–Thr–NHNH$_2$ (27.50 g) in DMF (200 ml), 3.75N HCl–DMF (30.4 ml) and isoamylnitrite (9.9 ml) were added and the mixture was stirred at −5° for 10 min. When the hydrazine test became negative, the solution, after neutralization with $Et_3N$ (18.8 ml), was added to a solution of H–Ser–Cys–(MBzl)–OH (prepared from 22.0 g of the TFA salt with 25.1 ml of $Et_3N$) in $H_2O$ (250 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was triturated with 10% citric acid and ether. The resulting powder was washed batchwisely with 10% citric acid and $H_2O$ and then recrystallized from DMF and MeOH; yield 16.51 g (44%); mp 161—165°, $[\alpha]_D^{25}$ −11.5° ($c$=1.2, DMF). $Rf_1$ 0.46. *Anal.* Calcd. for $C_{36}H_{44}O_{11}N_4S \cdot H_2O$: C, 56.97; H, 6.11; N, 7.38. Found: C, 56.93; H, 5.87; N, 7.13.

**Z(OMe)-Lys(Z)-Thr-Phe-Thr-Ser-Cys(MBzl)-OH**——Z(OMe)–Phe–Thr–Ser–Cys(MBzl)–OH (7.59 g) was treated with TFA (20 ml) in the presence of anisole (4 ml) under cooling with ice for 30 min. The excess TFA was removed by evaporation *in vacuo* and the residue was treated with ether to form the fine powder, which was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (100 ml). To this solution, $Et_3N$ (5.3 ml) and the azide (prepared from 8.39 g of Z(OMe)–Lys(Z)–Thr–NHNH$_2$ with 8.8 ml of 3.75N HCl–DMF, 2.4 ml of isoamylnitrite and 4.6 ml of $Et_3N$) in DMF (100 ml) were added. The mixture, after stirring at 4° for 48 hr, was condensed *in vacuo*. The residue was treated with $H_2O$ and ether. The resulting powder was washed with 5% $NH_4OH$, 5% citric acid and $H_2O$ and recrystallized from DMF and MeOH; yield 7.96 g (71%); mp 181—186°, $[\alpha]_D^{25}$ −20.6° ($c$=1.0, DMF). $Rf_1$ 0.37. *Anal.* Calcd. for $C_{54}H_{69}O_{16}N_7S \cdot H_2O$: C, 57.79; H, 6.38; N, 8.74. Found: C, 57.78; H, 6.35; N, 8.79.

**Z(OMe)-Trp-Lys(Z)-Thr-Phe-Thr-Ser-Cys(MBzl)-OH**——In the usual manner, the above protected hexapeptide (6.10 g) was treated with TFA (20 ml) in the presence of anisole (5 ml) at 0° for 30 min, when dry ether was added. The resulting fine powder was collected by filtration, dried over KOH pellets *in vacuo*; yield 5.60 g. This was then dissolved in DMF (100 ml), to which $Et_3N$ (1.6 ml), Z(OMe)–Trp–ONP (3.96 g) and HOBT (1.12 g) were combined and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was treated with 10% citric acid and ether. The resulting powder was washed batchwisely with 10% citric acid and $H_2O$ and then recrystallized from DMF and MeOH; yield 6.42 g (90%), mp 178—180°, $[\alpha]_D^{25}$ −20.3° ($c$=1.0, DMF), $Rf_1$ 0.45. *Anal.* Calcd. for $C_{65}H_{79}O_{17}N_9S \cdot H_2O$: C, 59.66; H, 6.24; N, 9.64. Found: C, 59.89; H, 6.18; N, 9.48.

**Z(OMe)-Phe-Phe-Trp-Lys(Z)-Thr-Phe-Thr-Ser-Cys(MBzl)-OH**——Z(OMe)–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH (3.73 g) immersed well in anisole (5 ml) containing mercaptoethanol (0.01 ml) was treated

with TFA (12 ml) in an ice-bath for 30 min. The air of the flask was replaced by nitrogen gas before this treatment. Dry ether was added and the resulting powder was collected by filtration. It was dried over KOH pellets *in vacuo* and then dissolved in 90% aqueous DMF (10 ml). To which, $Et_3N$ (1.4 ml) and the azide (prepared from 1.68 g of Z(OMe)–Phe–Phe–$NHNH_2$, 2.1 ml of 3.58N HCl–DMF, 0.6 ml of isoamylnitrite and 1.1 ml of $Et_3N$) in DMF (5 ml) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with 10% citric acid and ether. The resulting powder was washed batchwisely with 10% citric acid and $H_2O$ and then recrystallized from DMF and MeOH; yield 3.46 g (88%), mp 194—196°, $[\alpha]_D^{25}$ −20.1° ($c$=1.1, DMF), $Rf_2$ 0.82. *Anal.* Calcd. for $C_{83}H_{97}O_{19}N_{11}S \cdot H_2O$: C, 62.19; H, 6.23; N, 9.61. Found: C, 61.97; H, 6.02; N, 9.54.

**Z(OMe)-Asn-Phe-Phe-Trp-Lys(Z)-Thr-Phe-Thr-Ser-Cys(MBzl)-OH (III)**——The above protected nonapeptide (3.36 g) immersed well in anisole (5 ml) containing mercaptoethanol (0.01 ml) was treated, under nitrogen gas, with TFA (10 ml) at 0° for 30 min. Dry ether was added and the resulting powder was collected by filtration, dried over KOH pellets *in vacuo*. This was then dissolved in DMF (40 ml), to which $Et_3N$ (0.7 ml), Z(OMe)–Asn–ONP (0.96 g), HOBT (0.44 g) were combined and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated and the residue was treated with 10% citric acid and ether as stated above. The resulting powder was washed with 10% citric acid and $H_2O$ and then recrystallized from DMF and MeOH; yield 2.72 g (76%); mp 216—219°, $[\alpha]_D^{25}$ −13.1° ($c$=1.2, DMF), $Rf_1$ 0.38, $Rf_2$ 0.88. *Anal.* Calcd. for $C_{87}H_{103}O_{21}N_{13}S \cdot H_2O$: C, 60.86; H, 6.16; N, 10.61. Found: C, 60.77; H, 6.42; N, 10.82.

**Z-Ala-Gly-OH**——The title compound was prepared by the *p*-nitrophenyl ester procedure.[15] yield 58%, mp 129—130°, $[\alpha]_D^{26}$ −16.7° ($c$=1, 3, EtOH). (lit[18a] mp 125—128°, $[\alpha]_D$ −16° in EtOH, lit.[18b] mp 134—135°, $[\alpha]_D$ −17° in EtOH, lit.[18c] mp 127—128°, $[\alpha]_D$ −17° in EtOH). *Anal.* Calcd. for $C_{13}H_{16}O_5N_2$: C, 55.71; H, 5.75; N, 10.00. Found: C, 55.96; H, 5.86; N, 10.26.

**Z(OMe)-Cys(MBzl)-Lys(Z)-OMe**——In the usual manner, DCC (13.0 g) was added to a solution of Z(OMe)–Cys(MBzl)–OH (20.0 g) and H–Lys(Z)–OMe (prepared from 17.0 g of the hydrochloride with 9 ml of $Et_3N$) in DMF (400 ml) and the mixture was stirred at room temperature for 48 hr. The solution was filtered, the filtrate was condensed *in vacuo* and the residue was treated with ether. The resulting powder was washed batchwisely with 10% citric acid, 5% $Na_2CO_3$ and $H_2O$ and then recrystallized from tetrahydrofuran and MeOH; yield 20.73 g (59%), mp 112—115°, $[\alpha]_D^{25}$ −25.8° ($c$=1.2, DMF), $Rf_1$ 0.98. *Anal.* Calcd. for $C_{35}H_{43}O_9N_3S$: C, 61.65; H, 6.38; N, 6.16. Found: C, 61.41; H, 6.27; N, 6.41.

**Z-Ala-Gly-Cys(MBzl)-Lys(Z)-OMe**——Z(OMe)–Cys(MBzl)–Lys(Z)–OMe (2.07 g) was treated with TFA (2 ml) in the presence of anisole (1 ml) at 0° for 30 min. The excess TFA was evaporated *in vacuo* and the residue was dissolved in AcOEt, which was washed with 5% $Na_2CO_3$ and $H_2O$–NaCl, dried over $Na_2SO_4$ and then filtered. The filtrate was condensed *in vacuo* at 20° and the residue was dissolved in tetrahydrofuran (20 ml). To this solution, Z–Ala–Gly–OH (0.94 g), HOBT (0.7 g) and DCC (0.88 g) were combined. 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 powder was washed batchwisely with 10% citric acid, 5% $Na_2CO_3$ and $H_2O$ and then recrystallized twice from tetrahydrofuran and AcOEt; yield 1.90 g (80%), mp 113—117°, $[\alpha]_D^{25}$ −27.4° ($c$=1.1, DMF). $Rf_1$ 0.77. *Anal.* Calcd. for $C_{39}H_{49}O_{10}N_5S$: C, 60.06; H, 6.33; N, 8.98. Found: C, 60.24; H, 6.23; N, 9.08.

**Z-Ala-Gly-Cys(MBzl)-Lys(Z)-$NHNH_2$ (II)**——In the usual manner, hydrazine hydrate (80%, 1 ml) was added to a solution of Z–Ala–Gly–Cys(MBzl)–Lys(Z)–OMe (1.50 g) in MeOH (20 ml) and the solution was kept on standing overnight. The resulting gelatinous mass was collected by filtration and recrystallized from DMF and MeOH; yield 1.40 g (93%), mp 167—169°, $Rf_1$ 0.73. *Anal.* Calcd. for $C_{38}H_{49}O_9N_7S$: C, 58.52; H, 6.33; N, 12.57. Found: C, 58.36; H, 6.35; N, 12.72.

**Z-Ala-Gly-Cys(MBzl)-Lys(Z)-Asn-Phe-Phe-Trp-Lys(Z)-Thr-Phe-Thr-Ser-Cys(MBzl)-OH**——As stated above, Z(OMe)–Asn–Phe–Phe–Trp–Lys(Z)–Thr–Phe–Thr–Ser–Cys(MBzl)–OH (2.44 g) immersed well in anisole (2 ml) containing mercaptoethanol (0.01 ml) was treated with TFA (5 ml) at 0° for 60 min. The air of the flask was replaced by nitrogen gas before this treatment. Dry ether was added and the resulting powder was collected by filtration, dried over KOH pellets *in vacuo* and then dissolved in DMF (20 ml). To this solution, $Et_3N$ (0.7 ml) and the azide (derived from 1.35 g of Z–Ala–Gly–Cys(MBzl)–Lys(Z)–$NHNH_2$ with 1.06 ml of 3.58N HCl–DMF, 0.28 ml of isoamylnitrite and 0.53 ml of $Et_3N$) in DMF (10 ml) were combined and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was treated with 10% citric acid and AcOEt. The resulting powder was washed batchwisely with 10% citric acid and $H_2O$ and recrystallized twice from DMF and MeOH; yield 2.36 g (88%), mp 226—231°, $[\alpha]_D^{25}$ −14.9° ($c$=1.1, DMF), $Rf_1$ 0.36. Amino acid ratios in a 3N Tos–OH hydrolysate: Ala 1.01, Gly 1.00, Lys 1.90, Asp 1.07, Phe 3.09, Thr 2.08, Ser 0.85, Trp 0.74, 1/2Cys 1.43 (average recovery 93%). *Anal.* Calcd. for $C_{116}H_{140}O_{27}N_{18}S_2 \cdot H_2O$: C, 60.56; H, 6.22; N, 10.96. Found: C, 60.56; H, 6.42; N, 11.25.

**Isolation of the Tetradecapeptide with Somatostatin Activity**——The above protected tetradecapeptide (231 mg) in TFA (3 ml) was treated with trifluoromethanesulphonic acid (2 ml) in the presence of anisole (0.2 ml) in an ice-bath for 30 min. Dry ether was added and the resulting fine powder was collected by filtration ($Rf_2$ 0.72, single spot positive to ninhydrin and Ehrlich tests). This was then dissolved in $H_2O$ (10 ml) and the solution, after treatment with Amberlite IR-4B (acetate form, approximately 2 g) for 30 min

under $N_2$ gas, was filtered. The filtrate was then diluted with $H_2O$ to 1000 ml. The pH of the solution was adjusted to 6.5 with 10% $NH_4OH$ and the solution was kept on standing at room temperature for 72 hr. During this period, the solution became turbid and the absorbancy at 412 mμ by the Ellman's reagent[21] decreased from 0.520 to the constant value, 0.075. The solution, after filtration, was then applied to a column of Amberlite IRC-50 (3×6.5 cm), with a flow rate of 30 ml per 60 min. The column was then eluted with pyridine–AcOH–$H_2O$ (30: 4: 66 v/v).[11a] Individual fractions (10 ml each) were collected and the content of these tubes was examined by thin-layer chromatography. Fractions (tube No. 6—11), which were positive to ninhydrin and Ehrlich tests, were combined. The solvent was evaporated *in vacuo* and the residue was lyophilized to give fluffy powder; yield 82 mg (41%). $Rf_2$ 0.50 (broad main spot). Amino acid ratios in a HCl hydrolysate: Ala 1.00, Gly 1.02; 1/2 Cys 1.77, Lys 2.17, Asp 0.87, Phe 3.26, Thr 2.14, Ser 0.83 (recovery 89%). This was then applied to a column of Sephadex G-25F (1.75×120 cm), previously equilibrated with the lower phase of *n*-BuOH–AcOH–$H_2O$ (4: 1: 5 v/v). The column was then developed by the upper solution of the above solvent system with a flow rate of 12 ml per 60 min. Individual fractions (5 ml each) were collected and the absorbancy at 280 mμ was determined. Three main peaks were detected (Fig. 1). Fractions of these peaks were combined respectively and the solvents were evaporated *in vacuo*. The respective residues were lyophilized. Peak 1 (tube No. 17—21), yield 26.6 mg (13%), amino acid ratios in a 3N Tos-OH hydrolysate: Ala 1.00, Gly 1.05, 1/2 Cys 1.39, Lys 2.26, Asp 1.12, Phe 3.30, Try 0.91, Thr 2.18, Ser 1.00 (recovery 91%). Peak II (tube No. 34—40), yield 11.3 mg (5.7%), amino acid ratios in a 3N Tos-OH hydrolysate: Ala 1.00, Gly 1.03; 1/2Cys 1.79, Lys 1.90, Asp 1.00, Phe 2.87, Trp 0.54, Thr 1.87, Ser 0.75 (recovery 94%). Peak III (tube No. 56—66), yield 15.0 mg (7.5%), *Rf* (hold-up volume/elution volume) 0.31 (lit.[9] 0.36). $Rf_2$ 0.68, $Rf_3$ 0.65 (lit.[11a] 0.47, lit.[11b] 0.78), $Rf_4$ 0.58 (lit.[11b] 0.50). $[\alpha]_D^{25}$ −34.8° (*c*=0.5, 1% AcOH). (lit.[9] −32.3°, natural, lit.[10a] −36°, lit.[10b] −34.5°, lit.[11b] −39.5°). Amino acid ratios in a 3N Tos-OH hydrolysate: Ala 1.00, Gly 1.02, 1/2Cys 1.85, Lys 1.76, Asp 0.90, Phe 3.10, Trp 0.92, Thr 2.03, Ser 1.00 (average recovery 94%). Amino acid ratios in an APM digest: Ala 1.00, Gly 0.99, 1/2Cys 1.92, Lys 1.59, Phe 3.03, Trp 0.90, Thr 2.13, Asn+Ser (not determined) (average recovery 95%). *Anal.* Calcd. for $C_{76}H_{104}O_{19}N_{18}S_2 \cdot 2CH_3COOH \cdot 7H_2O$: C, 51.00; H, 6.74; N, 13.38. Found: C, 50.76; H, 6.35; N, 12.82.

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