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A Large-Scale Synthesis of Somatostatin for Clinical Use by a Novel Alternating Solution/Solid-Phase Procedure[1,2]

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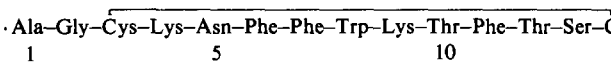
A large-scale synthesis of somatostatin was developed. A stepwise $C \rightarrow N$ approach in solution was used, employing $N(\alpha)$-t-butoxycarbonyl amino acid active esters. The scheme of semi-permanent protection utilized 2-(methylsulfonyl)-ethoxycarbonyl for the ε-amino group of lysine; acetamidomethyl for the β-thiol groups of cysteine; the orange-colored 2-[4-(phenylazo)-phenylsulfonyl]-ethoxy group for the C-terminal carboxy group of cysteine. All condensations and $N(\alpha)$-deprotections were carried out in homogeneous solution, while isolation and purification of peptides carrying the colored group was achieved by precipitation and washing of the solid products. Thus, the "alternating solution/solid-phase peptide synthesis" combines advantages of both the classical solution synthesis and the Merrifield solid-phase technique. The overall yield was 5%, or 16 g of somatostatin from 100 g of the novel amino acid derivative, $N(\alpha)$-t-butoxycarbonyl-*S*-acetamidomethyl-L-cysteine 2-[4-(phenylazo)-phenylsulfonyl]-ethyl ester. An improved method for the preparation of *S*-acetamidomethyl-L-cysteine, free of thiazolidine carboxylic acid, is described.

INTRODUCTION

The hypothalamic growth hormone release-inhibiting hormone, somatostatin, was isolated and its structure elucidated by Guillemin and co-workers (*1*). It is a heterodetic cyclic tetradecapeptide with a disulfide bond joining the cysteine residues in positions 3 and 14:



[1] This paper is dedicated to the memory of the late Professor George Kenner, Liverpool.

[2] All amino acids except glycine are of the L-configuration. Standard abbreviations for amino acids and peptides as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.*, **247**, 977 (1972): three-letter symbolism) and in the "Atlas of Protein Sequence and Structure" (Vol. 5, M. O. Dayhoff, National Biomedical Research Foundation, Silver Spring, Md., 1972: one-letter symbolism). For our purposes, the correlation is as follows: Ala = A; Asn = N; Cys = C; Gly = G; Lys = K; Phe = F; Ser = S; Thr = T; Trp = W. Abbreviations for protecting groups are (to avoid confusion with amino acids, uppercase symbols are used in connection with the three-letter, lowercase symbols with the one-letter abbreviations for amino acids): ACM, a = acetamidomethyl; BOC, boc = t-butoxycarbonyl; MSC, m = 2-(methylsulfonyl)-ethoxycarbonyl; OPSE, opse = 2-[4-(phenylazo)-phenylsulfonyl]-ethoxy. Components of active esters are: ONP = 4-nitrophenoxy; ONSU = *N*-succinimidoxy; OTCP = 2,4,5-trichlorophenoxy.

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Somatostatin exhibits a wide variety of biological effects, mainly in the central nervous system and the digestive tract, that make it look very promising as an experimental and therapeutic agent (*2*). It would therefore be desirable to produce a sufficient supply of very pure material for clinical investigation. As in the case of angiotensin, corticotropin, and the melanotropins (*3*), this goal was achieved by a classical peptide synthesis that allows the purification of every intermediate and of the final product. However, strategy and tactics were different from those used earlier (*3*).

The choice of the novel *S*-acetamidomethyl-L-cysteine 2-[4-(phenylazo)-phenylsulfonyl]-ethyl ester as the C-terminal starting point of the synthesis proved to be especially advantageous, because it allowed the use of a special procedure that can be described as "alternating solutions/solid-phase peptide synthesis" without having to revert to polymeric carriers (*4*).

EXPERIMENTAL

General remarks. Evaporation (30°C) means removal of solvent by distillation from a rotatory distillation apparatus at a bath temperature of 30°C. Thin-layer chromatography (tlc) is performed on silica gel plates; spots of compounds were revealed by their inherent color and by standard spray techniques (ninhydrin, Pauli, Reindel–Hoppe, iodine, etc.). Solvent systems for tlc development were (all ratios expressed as v/v): CMA = $CHCl_3$/MeOH/AcOH, 95:5:3; BAW1 = 2-butanol/AcOH/H_2O, 72:7:21; BPAW1 = *n*-butanol/AcOH/pyridine/H_2O, 50:12:12:25; EBPAW1 = EtOAc/*n*-butanol/pyridine/AcOH/H_2O, 42:24:21:6:10; EPAW1 = EtOAc/pyridine/HCO_2H/H_2O, 63:21:10:6; EPAW2 = same solvents, 70:16:8:6. References for the preparation of protected amino acid intermediates can be found (if they are not given in the text) in Ref. (*5*).

N-Hydroxymethyl acetamide. Acetamide (250 g), polyoxymethylene (paraformaldehyde, 150 g), and potassium carbonate (2.5 g) were combined and pulverized, and the mixture was melted at 100°C. After 1 hr, the melt was cooled to 50–60°C, treated with aqueous 12 *N* potassium carbonate (0.5 ml) and kept overnight in the refrigerator. The solid mass was dissolved at room temperature in the minimal amount of acetone (about 200 ml), the solution was cooled to −30°C for a few hours, and the crystals (170 g, mp 45–50°C) were gathered by rapid filtration and dried. A second crop of 50 g was obtained by evaporating some of the solvent and cooling. Total yield was about 55–60%. This product is superior to that produced with aqueous formaldehyde, giving better reproducible results upon condensation with cysteine.

S-Acetamidomethyl-L-cysteine hydrochloride. Crystalline *N*-hydroxymethyl acetamide (75 g, 0.84 mol) was dissolved in dioxane (300 ml) and this solution added dropwise at room temperature to a well-stirred mixture of L-cysteine hydrochloride monohydrate (88 g, 0.5 mol), dioxane (1250 ml), concentrated HCl (22 ml), and water (40 ml). Stirring was continued for 30 min after complete addition; the oily product that was formed was separated and freed of solvent by evaporation (30°C). Water was removed by dissolving the residue in ethanol and evaporating the solvent (30°C). The residue then crystallized. The crystals were gathered by vacuum filtration, washing successively with 2-propanol and diethyl ether, and drying: 90 g (80%), mp 162–165°C, $[\alpha]_D^{25}$ =

$-27.5°$ ($c = 1$, H_2O). A sample was recrystallized from 2-propanol/water (8:2): mp 166–169°C, $[\alpha]_D^{25} = -27°$ ($c = 1$, H_2O). Veber *et al.* (*6*) reported mp 159–163°C, $[\alpha]_D^{25} = -30.7°$ ($c = 1$, H_2O).

S-Acetamidomethyl-L-cysteine monohydrate, Cys(ACM). S-Acetamidomethyl-L-cysteine hydrochloride (2.1 g) was dissolved in water (20 ml) and treated with silver oxide (2.3 g). After 15 min, the mixture was filtered and the filtrate treated with H_2S. After filtration through alumina, the filtrate was evaporated to dryness, the residue triturated with ethanol, and the solid separated and dried: 1 g, $[\alpha]_D^{25} = -39.5°$ ($c = 1$, H_2O). tlc: only one spot, R_f 0.23 (EPAW1). The presence of 1,3-thiazolidine-4-carboxylic acid, R_f 0.35 (EPAW1), could not be demonstrated with Reindel–Hoppe reagent. Cys(ACM) had mp 205–206°C (decomp.), $[\alpha]_D^{25} = -40.3°$ ($c = 1$, H_2O). The literature reports mp 193–195°C (decomp.), $[\alpha]_D^{22} = -43.0°$ ($c = 1$, H_2O) (*6*), and mp 187°C (decomp.), $[\alpha]_D^{25} = -42.5°$ ($c = 1$, H_2O) (*7*).

N-t-Butoxycarbonyl-S-acetamidomethyl-L-cysteine 2-4-(phenylazo)-phenylsulfonyl]-ethyl ester, BOC·Cys(ACM)·OPSE, 1. BOC·Cys(ACM)·OH (32 g, 0.11 mol, prepared from Cys(ACM) according to Hermann and Schreier (*7*) in 83% yield with mp 112–114°C and $[\alpha]_D^{25} = -33.6°$ ($c = 1$, H_2O) and 2-[4-(phenylazo)-phenylsulfonyl]-ethyl alcohol (*8*) (PSE·OH; 30.4 g, 0.10 mol) were dissolved in pure pyridine (350 ml). The homogeneous solution was then cooled to 0°C and treated in small portions with a solution of dicyclohexylcarbodiimide (21.6 g, 0.105 mol) in pure pyridine (40 ml). The mixture was stirred overnight in a refrigerator; the reaction is complete only after 6–8 hr (it was followed by tlc in ethyl acetate/methanol, 96:4, wherein PSE·OH has R_f 0.50–0.55 and BOC·Cys(ACM)·OPSE has R_f 0.42–0.50). In case of incompleteness, a small amount of BOC·Cys(ACM)·OH was added, because unreacted PSE·OH is difficult to remove from **1**.

The isolation of **1** was accomplished through the following steps: filtration from dicyclohexyl urea; evaporation of pyridine (30°C); dissolution of the residue in ethyl acetate and filtration from dicyclohexyl urea; washing of the filtrate successively with aqueous $K_2SO_4/KHSO_4$ (pH 2.0), water, 5% aqueous $NaHCO_3$, and water; drying of the organic phase with MgSO; filtration and evaporation; trituration of the residue with dry ether (400 ml); filtration of the orange-colored solid; washing with ether and drying. Yield 53.3 g (92%, variations from 92 to 97%).

S-Acetamidomethyl-L-cysteine 2-[4-(phenylazo)-phenylsulfonyl]-ethyl ester hydrochloride (and trifluoroacetate), H·Cys(ACM)·OPSE, h·C(a)·opse, 1a. Hydrochloride: BOC·Cys(ACM)·OPSE (5.78 g, 10 mmol) was added within 1–2 min to a solution of concentrated HCl (1 ml) in 98% formic acid (100 ml and kept for 10 min at room temperature. The solvent was evaporated as rapidly as possible (temperature $\leq$ 30°C), the oily residue dissolved in methanol (15 ml), and this solution added dropwise at 0°C with stirring to anhydrous ether (150 ml). The product was filtered, rinsed with ether, and dried: 5.0 g (97%), mp 160–162°C (decomp.). The oily residue obtained after evaporation can also be triturated with 2-propanol (instead of the methanol/ether precipitation): yield 90–95%, mp 162–164°C.

Trifluoroacetate: **1** (2.0 g) was dissolved in trifluoroacetic acid (16 ml) at room temperature, and the solvent removed by evaporation after 5 min. Treatment of the residue with anhydrous ether provided a solid sample of **1a** trifluoroacetate, 2.01 g (98%).

BOC · Ser–Cys(ACM) · OPSE, 2. A solution of HCl, H · Cys(ACM) · OPSE, **1a**, (5.15 g, 0.01 mol) and *N*-ethylmorpholine (1.26 ml, 0.01 mol) in pure, anhydrous dimethylformamide was cooled to −10°C and treated rapidly with BOC · Ser · OH (2.05 g, 0.01 mol) and then dropwise with a solution of dicyclohexylcarbodiimide (2.47 g, 0.012 mol) and 1-hydroxybenzotriazole (2.70 g, 0.02 mol) in dimethylformamide (20 ml). After 1 hr at −10°C, the solution was slowly brought to room temperature, avoiding any spontaneous heating above 20°C (especially with larger quantities). The reaction was complete after 6 hr (tlc), and the mixture was subjected to the following procedure: evaporation of the solvent (30–35°C); trituration of the residue with ethyl acetate (200 ml); filtration from dicyclohexylurea; washing of the filtrate successively with aqueous $K_2SO_4/KHSO_4$ (pH 2), water, 5% aqueous $NaHCO_3$, and water; drying over $MgSO_4$; evaporation of the solvent to a residual volume of about 50 ml; cooling to 0°C for 16 hr. The product precipitated as a gelatinous solid; it was filtered off and washed with dry ethyl acetate and diethyl ether: 5.3 g (80%).

The use of the 2,4-dinitrophenyl and the 4-chlorophenylthio esters of t-butoxycarbonylserine afforded **2** with practically identical properties, but only in 66 and 75% yields, respectively.

Acidolysis of the BOC group. Procedure A. H · Ser-Cys(ACM) · OPSE trifluoroacetate, 2a. BOC · Ser–Cys(ACM) · OPSE (38.5 g, 0.058 mol) was rapidly added to cold (0°C) trifluoroacetic acid (300 ml). The reaction was accompanied by a temperature rise to about 18°C and was complete in 3–5 min. After this time, the solvent was immediately evaporated (20°C), and the distillate trapped at −70°C; it can be reused without additional purification. The residue was triturated with diethyl ether, and the pure solid product gathered by filtration: 38.5 g (97%). In case of the presence of residual dicyclohexylurea (from the previous step), this can conveniently be removed by washing twice at room temperature with 2-propanol (50 ml each).

The trifluoroacetic acid procedure in this form was also used for the preparation of **3a**, **4a**, and **5a**, i.e., for peptides without tryptophan. Procedure A was also carried out in a modified form with a 1 : 1 mixture of trifluoroacetic acid/methylene chloride, using 8 ml of the mixture for 1 g of BOC peptide. In this case, the reaction time was 15–20 min.

The hydrochloride of **2a** was prepared in 94% yield by the procedure exemplified in the preparation of **1a**.

Procedure B. H · Trp–Lys(MSC)–Thr–Phe–Thr–Ser–Cys(ACM) · OPSE trifluoroacetate, 7a. BOC · Trp–Lys(MSC)–Thr–Phe–Thr–Ser–Cys(ACM) · OPSE (13.3 g, 9 mmol) was rapidly added to a vigorously stirred, ice-cold mixture of trifluoroacetic acid (130 ml), ethane dithiol (13 ml), and anisole (6 ml) surrounded by an ice/water bath. After removal of the bath, the mixture was stirred at room temperature for about 20 min. The solvent was then evaporated; the residue was treated with 2-propanol (50 ml), and this solvent was also evaporated. The residue was again triturated with 2-propanol (50 ml) and then gathered by filtration, washed with 2-propanol/diethyl ether, diethyl ether, and dried: 13.4 g (100%).

The reagent mixture can also be composed as follows (giving equally good results after 45 min reaction time): trifluoroacetic acid (70 ml), methylene chloride (40 ml), ethane dithiol (14 ml), and anisole (14 ml) for the same amount of **7**, as above.

Procedure B was applied in the preparation of all other tryptophan-containing peptides, i.e., **8a**, **10a**, **11a**, **12a**, and **14a**.

Condensation of active esters. BOC·Phe–Trp–Lys(MSC)–Thr–Phe–Thr–Ser–Cys(ACM)·OPSE, 8. A cold (0°C) solution of H·Trp–Lys(MSC)–Thr–Phe–Thr–Ser–Cys(ACM)·OPSE trifluoroacetate, **7a** (13.4 g, 9 mmol), in pure dimethylformamide (130 ml) was treated successively with *N*-ethylmorpholine (2.3 ml, 18 mmol), 1-hydroxybenzotriazole (2.43 g, 18 mmol), and BOC·Phe·ONP (4.25 g, 11 mmol), the ice bath removed, and the reaction allowed to proceed for 1 hr at room temperature. Control of completeness was accomplished with tlc. The solvent was then evaporated in a high vacuum (30°C). The residue solidified upon cooling to 0°C; it was gathered by vacuum filtration, washed with water, dried, and then washed with warm solvents (40°C), twice with 2-propanol and three times with acetone. Yield: 13.6 g (93%) of chromatographically pure **8**.

All active ester couplings in the preparation of **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10**, **11**, **12**, and **14** were carried out in a practically identical manner.

S(3),S(14)-bis-Acetamidomethyl dihydrosomatostatin diacetate, H·Ala–Gly–Cys–(ACM)–Lys–Asn–Phe–Phe–Trp–Lys–Thr–Phe–Thr–Ser–Cys(ACM)·OH, 14b. A solution of H·Ala–Gly–Cys(ACM)–Lys(MSC)–Asn–Phe–Phe–Trp–Lys(MSC)–Thr–Phe–Thr–Ser–Cys(ACM)·OPSE trifluoroacetate, **14a** (57.5 g, 23 mmol), in dimethylformamide (1150 ml) and methanol (115 ml) was stirred and cooled to $\leq 5°C$ in an ice bath and slowly treated with ice-cold 0.2 *N* $Ba(OH_2)$ solution (1150 ml, 0.23 mol). The addition was complete after 40 min; care was taken to keep the temperature from rising above 15°C. The ice bath was removed and, after the temperature had risen to about 18°C, a current of CO_2 was bubbled through the solution until it was slightly acid (pH 5–6). The $BaCO_3$ was removed in a centrifuge (Sorvall, 6000 rpm), and the supernatant evaporated (35–40°C) to a residual volume of about 600 ml wherein the product was still completely dissolved. This solution was poured (with vigorous stirring!) into ethyl acetate (4000 ml). After 1 hr, the fine, solid precipitate was gathered on a glass porous filter (No. 2), washed with ethyl acetate, and dried: 42 g (100%) of a crude reaction product, **14b**. Purification was achieved either with partition chromatography on Sephadex G25F or, better with countercurrent distribution using Partridge systems (n-butanol/water/acetic acid). Purity according to hplc was better than 98% (Fig. 3). Yield: 20.2 g (48%).

Somatostatin diacetate, 14e. *S*(3), *S*(14)-bis-Acetamidomethyl dihydrosomatostatin diacetate, **14b** (7 g, 3.6 mmol), was dissolved in a degassed mixture of glacial acetic acid (350 ml) and water 315 ml), covered with oxygen-free nitrogen, and stirred at room temperature. Then, a 1 *N* aqueous solution of $AgNO_3$ (35.6 ml) was added and stirring continued for 16 hr after which the rather slow reaction is complete. The reaction mixture was evaporated to dryness and the insoluble silver salt (**14c**) washed with degassed water. It was then suspended in a mixture of dimethylformamide/water (60:40) and treated with H_2S. Silver sulfide was removed by centrifugation, and the supernatant evaporated to dryness. The residue was dissolved in degassed water (50 ml) and lyophilized: 5.65 g **14d**, purity 70% according to hplc and assay of sulfhydryl groups.

This compound was dissolved in a small amount of (degassed) dimethylformamide and diluted with 5% aqueous acetic acid (4.6 litres). The dilute solution was then slowly introduced over 18 hr into a solution of $K_3\{Fe(CN)_6\}$ (12 g) and ammonium acetate (24 g); the pH was kept at 6.9 using 8% ammonia (automatic pH-stat). During the whole reaction, the mixture was well stirred and blanketed with N_2. At the end of the

reaction, the mixture was acidified to pH 5 with acetic acid. Complex ions were removed with Bio-Rad AG-3X4, and then the somatostatin was adsorbed on a weakly acidic ion exchange resin (for example, Bio-Rex 70). The product was washed with 5% acetic acid and eluted with 50% acetic acid. Evaporation at room temperature and lyophilization from water yielded 5.2 g of crude somatostatin.

Final purification was achieved by chromatography in 1 *N* acetic acid over a column (2000 × 50 cm) of Sephadex G25. Lyophilization yielded 2.9 g of somatostatin, **14e**, as a white powder with a purity of 98.4% (analytical hplc, Fig. 3).

RESULTS

The General Approach

In order to minimize racemization and to allow for the purification and characterization of intermediates (Tables 1–3), a stepwise synthesis beginning at the C-terminus and proceeding towards the N-terminus was adopted. Amino acid active esters were chosen as building units (Figs. 1 and 2). Such a strategy had proved to be useful in earlier instances (*9*). We departed only twice from this scheme: For the preparation of the C-terminal dipeptide, **2**, condensation with dicyclohexyl carbodiimide (*10*) and 1-hydroxybenzotriazole (*11*) afforded somewhat better yields than either the 2,4-dinitrophenyl or the 4-chlorophenylthio esters of serine. At the N-terminus, the last elongation step consisted in the attachment of the dipeptide unit, Ala-Gly (no danger of racemization), instead of the individual amino acids.

TABLE 1

THIN-LAYER CHROMATOGRAPHY OF SOMATOSTATIN PEPTIDES ON SILICA GEL (R_f)[a]

Solvent	1	1a	2	2a	3	3a	4	4a	5	5a	6	6a	7	7a
CMA	0.50[b]								0.05					
BAW1	0.69[b]	0.36	0.63		0.69		0.75	0.36[c]	0.66	0.35[c]	0.57		0.70[d]	0.36
BPAW1	0.77[b]	0.62	0.70	0.60		0.56[b]	0.75	0.65						
EPAW2			0.74	0.30	0.70[e]	0.30	0.67	0.39	0.63	0.33	0.50	0.25	0.50	0.25[f]
EPAW1						0.52	0.84	0.47	0.79	0.50	0.75	0.50	0.76[g]	0.36
EBPAW1		0.72		0.50	0.86			0.65		0.69			0.77[g]	0.65[g]

	8	8a	9	9a	10	10a	11	11a	12	12a	14	14a	14b	14c
CMA														
BAW1	0.67	0.42					0.50							
BPAW1	0.69		0.70						0.65		0.60		0.40	0.40
EPAW2	0.58	0.32	0.58[d]	0.40	0.70	0.60	0.70		0.75		0.70	0.50		
EPAW1	0.77	0.38	0.82[d]					0.50		0.40				
EBPAW1	0.91	0.75	0.94											

[a] Detected by the inherent orange color, by ninhydrin, Reindel–Hoppe, I_2, or other suitable means. Unless otherwise indicated, the *N*(*α*)-BOC or *N*(*α*)-unprotected trifluoroacetate salts (a series) were applied to the plates and were found to give one spot only after development.

[b] Hydrochloride.

[c] "Unstable" in this solvent: anion exchange?

[d] The *N*(*α*)-BPOC derivative (instead of BOC).

[e] Very slight impurity, $R_f = 0.75$.

[f] Elongated spot, $R_f = 0.20$–0.30.

[g] Elongated spot, $R_f = 0.55$–0.75.

TABLE 2

ASPECT (A), MELTING POINT (M), SPECIFIC ROTATION (R), AND SPECTROSCOPIC DATA OF SOMATOSTATIN PEPTIDES

	1	2	3	4	5	6	7[a]	8[a]	9[a]	10	11	12	14	14e
A[b]	s	s	c	s	s	s	s	s	c	c	s	s	s	s
M[c]	75–77	111–113	138–140	153–155	169–172	172–174	158–160	171–173	222–224	—	—	—	—	—
R[d]	−24.0°[e]	−25.5°[e]	−29.0°[e]	−16.0°[f]	−20.0°[f]	−19.5°[f]	−16.5°[f]	−13.0°[g]	−9.1°[g]	−16.0°[g]	−14.5°[g]	—	−15.0°[g]	−39.0°[h]
S[i]	320	320	320	320	320	320	320	320	—	445[j]	445[j]	445[j]	—	—
	22 500	22 400	23 300	20 800	20 700	20 800	17 500	18 400	—	520	520	480	—	—

[a] The corresponding $N(\alpha)$-BPOC derivative (the BOC compound, **7**, had $[\alpha]_D^{20} = -17.5°$, $c = 1$ in glacial acetic acid).
[b] s = solid, c = cryst.
[c] °C.
[d] $[\alpha]_D^{20}$.
[e] $c = 1$ in methanol.
[f] $c = 1$ in glacial acetic acid.
[g] $c =$ in dimethylformamide.
[h] $c = 1$ in water.
[i] $\lambda_{max}/\varepsilon$, in methanol.
[j] In dimethylformamide.

TABLE 3

ANALYTICAL DATA OF SOMATOSTATIN PEPTIDES

	1	2	3	4	5	6	7[a]
A[b]	$C_{26}H_{34}O_7N_4S_2$	$C_{29}H_{39}O_9N_5S_2$	$C_{33}H_{46}O_{11}N_6S_2$	$C_{42}H_{55}O_{12}N_7S_2$	$C_{46}H_{62}O_{14}N_8S_2$	$C_{56}H_{80}O_{19}N_{10}S_3$	$C_{78}H_{96}O_{20}N_{12}S_3$
W[c]	578.7	665.8	766.9	914.1	1015.2	1293.5	1617.9
Carbon	53.97/54.12[d]	52.31/52.44	51.68/51.86	55.20/54.78	54.50[e]/53.74	52.00[e]/50.91	56.42/56.35
Hydrogen	5.92/6.08	5.90/6.14	6.95/6.21	6.07/6.14	6.15/6.17	6.24/6.05	6.03/5.97
Nitrogen	9.66/9.65	10.52/9.96	10.96/10.67	10.73/10.52	11.03/10.65	10.80/10.44	9.82/9.94
Sulfur	—	—	—	7.00/7.35	6.31/6.43	7.45/7.45	6.20/6.45

	8[a]	9[a]	10	11	12	14	14c
A	$C_{87}H_{105}O_{21}N_{13}S_3$	$C_{96}H_{114}O_{22}N_{14}S_3$	$C_{89}H_{114}O_{24}N_{16}S_3$	$C_{99}H_{132}O_{29}N_{18}S_4$	$C_{105}H_{142}O_{31}N_{20}S_5$	$C_{110}H_{150}O_{33}N_{22}S_5$	$C_{76}H_{104}O_{19}N_{18}S_2$
W	1765.1	1912.2	1888.0	2166.5	2341	2470	1638
Carbon	59.20[e]/57.75	59.32[f]/59.28	—	—	—	—	—[g]
Hydrogen	5.97/6.00	5.97/6.05	—	—	—	—	—
Nitrogen	10.30/9.96	10.09/9.83	—	—	—	—	—
Sulfur	5.44/5.79	4.95/5.20	—	—	—	—	—

[a] The corresponding *N*(*α*)-BPOC derivative.
[b] Calcd molecular composition.
[c] Calcd molecular weight.
[d] % calculated/% found.
[e] The calculated values are not corrected for found water (**5**, 2.5%; **6**, 3.5%; **8**, 2.3%).
[f] Calculated for **9** (the corresponding *N*(*α*)-BPOC derivative) with 1.6% water.
[g] Correct amino acid analysis.

		YIELD% step	YIELD% overall	Expt. No.
BOC-Ser-OH + DCCI	h[C^{a}]opse	97	97	1a
	boc[SC^{a}]opse	80	77.6	2
BOC-Thr-ONSU	h[SC^{a}]opse	97	75.3	2a
	boc[TSC^{a}]opse	98	73.8	3
BOC-Phe-ONP	h[TSC^{a}]opse	94	69.3	3a
	boc[FTSC^{a}]opse	90	62.4	4
BOC-Thr-ONSU	h[FTSC^{a}]opse	97	60.5	4a
	boc[TFTSC^{a}]opse	90	54.5	5
BOC-Lys(MSOC)-ONP	h[TFTSC^{a}]opse	97	52.8	5a
	boc[K^{m}TFTSC^{a}]opse	88	46.5	6
BOC-Trp-ONP	h[K^{m}TFTSC^{a}]opse	98	45.6	6a
	boc[WK^{m}TFTSC^{a}]opse	85	38.7	7
BOC-Phe-ONP	h[WK^{m}TFTSC^{a}]opse	100	38.7	7a
	boc[EWK^{m}TFTSC^{a}]opse	93	36.0	8
BOC-Phe-ONP	h[FWK^{m}TFTSC^{a}]opse	97	34.9	8a
	boc[FFWK^{m}TFTSC^{a}]opse	95	33.2	9
BOC-Asn-ONP	h[FFWK^{m}TFTSC^{a}]opse	100	33.2	9a
	boc[NFFWK^{m}TFTSC^{a}]opse	87	28.9	10
BOC-Lys(MSOC)-ONP	h[NFFWK^{m}TFTSC^{a}]opse	100	28.9	10a

FIG. 1. Stepwise synthesis of [Lys(MSC)^{9}, Cys(ACM)^{14}]-dihydrosomatostatin-(5-14)-decapeptide 2-[4-(phenylazo)-phenylsulfonyl]-ethyl ester, **10a**. The reagents used for lengthening the chains are indicated on the left-hand side opposite the $N(\alpha)$-deprotected peptides (or amino acid) with which they react to produce the next $N(\alpha)$-BOC peptide.

$N(\alpha)$-Protection

The choice of the t-butoxycarbonyl (BOC, boc) group allowed us to adopt a technically easy, uniform method for its removal after every elongation step (Figs. 1 and 2). Acidolysis with trifluoroacetic acid (with or without added methylene chloride) was adopted as the standard procedure, as it proved to be easier to perform and to give better results than the HCl/formic acid method (*5*). The trifluoroacetic acid was almost quantitatively recovered after each step. At first, we had envisaged the use of the BOC group only up to the hexapeptide stage, **6**, and then—after the introduction of the very sensitive tryptophan residue—to proceed with the 2-biphenylyl-isopropyloxycarbonyl (BPOC) group, because it can be cleaved by milder procedures, e.g., in acetic or formic acid (*12*). In our case, however, mild cleavage proceeded poorly, and we had to revert to trifluoroacetic acid. We therefore decided to use BOC throughout the synthesis,

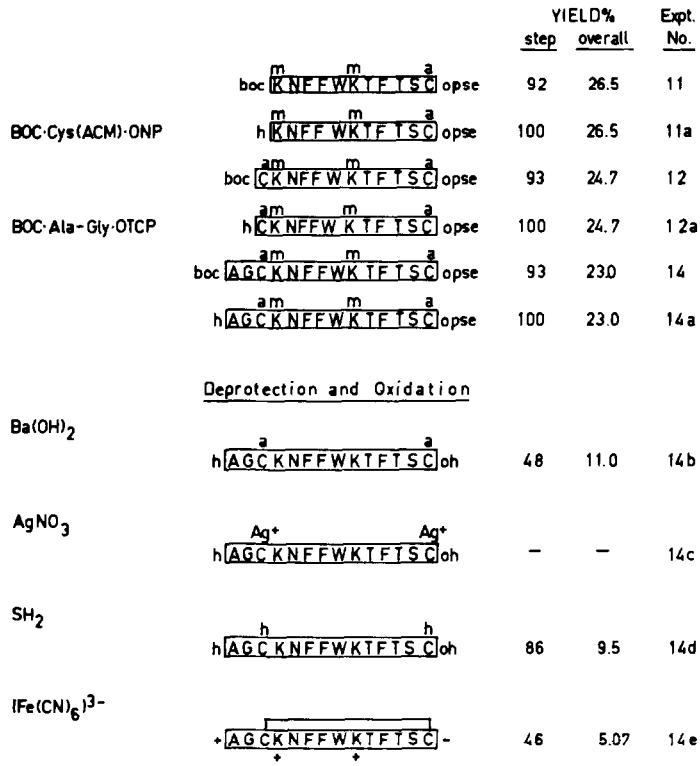


FIG. 2. Synthesis of somatostatin acetate salt, **14e**, from BOC-[Lys(MSC)[4,9], Cys(ACM)[14]]-dihydro-somatostatin-(4-14)-undecapeptide 2[4-(phenylazo)-phenylsulfonyl]-ethyl ester, **11**. Reagents indicated on the left-hand side.

although the usual procedure with or without anisole and mercaptoethanol as carbonium ion scavengers was also unsatisfactory. Only after having developed a new formula utilizing trifluoroacetic acid with about 6% anisole and 10% *ethane dithiol* (with or without methylene chloride) were the excellent results (high yields and practically no side products) indicated in Figs. 1 and 2 achieved.

Side-Chain Protection

The hydroxyl functions of threonine and serine were left unprotected. We decided to use differential protection for lysine and cysteine in order to facilitate the introduction of modifications on either the amino or the mercapto groups for later structure–activity studies or other purposes, if desired. The groups also had to be resistant to the acidolytic conditions required for the removal of the $N(\alpha)$-protection. Thus, the acetamidomethyl group (ACM, a) (*6*) was chosen for Cys, and the 2-(methane-sulfonyl)-ethoxycarbonyl group (MSC, m) (*13*) for lysine.

The preparation of large amounts of pure *S*-acetamidomethyl-L-cysteine according to the usual procedure (*6*, *7*) proved to be difficult, mostly due to the formation of thiazolidine carboxylic acid. The difficulty was surmounted by using new methods for the preparation of *N*-hydroxymethyl acetamide (obtained in crystalline form from

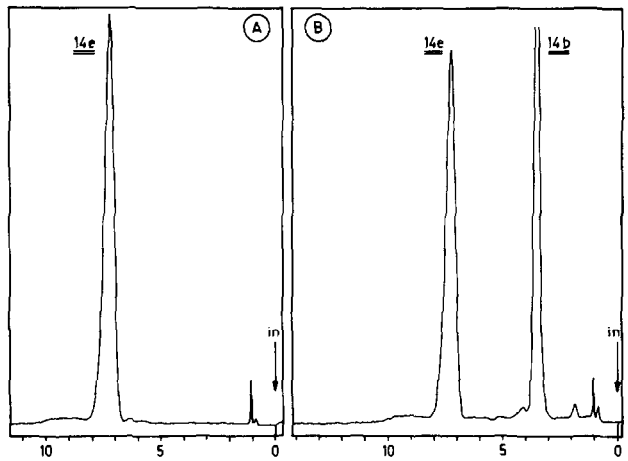
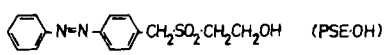


FIG. 3. High-pressure liquid chromatography of 0.861 mg of somatostatin acetate salt, **14e**, alone (panel A), and in a mixture containing added 0.674 mg of [Cys(ACM)^{3,14}]-dihydrosomatostatin acetate salt, **14b**, (panel B). Ordinates, arbitrary absorption units at $\lambda = 210$ *nm*; abscissas elution time in *minutes*, injection (in) at 0. Apparatus: Waters Associates liquid chromatograph with modified UGK injector, modified 6000A pump, modified 450 multiwavelength detector, and Sefram (Paris) "Servotrace" chart recorder. Chromatographic conditions: 300 x 3.9-mm column filled with μBondapak C_{18} absorbent (10-μm particles); a mixture of 700 ml of 10^{-2} *M* ammonium acetate, pH = 4 (adjusted with acetic acid, and 250 ml of acetonitrile (spectroscopic grade) as eluant; flow rate 4 ml min^{-1} at 300 psi; detector set at $\lambda = 210$ nm and range = 2 AUFS; chart recorder set at 10 mV FS and 1 cm min^{-1}. The experiments were carried out by Drs. Briot and Guimbard of the analytical department of Clin-Midy.

acetamide and paraformaldehyde) and for its condensation with cysteine hydrochloride (carried out in dioxane containing less than 5% water). These procedures probably eliminate the formation of free formaldehyde during the reaction, which is supposedly responsible for the cyclization of cysteine to thiazolidine carboxylic acid.

C-Terminal Carboxy Group Protection

At the C-terminal position, we wanted a protective group that would allow all peptide intermediates to be directly visible on chromatograms or in countercurrent distributions, and that could easily be removed under the same alkaline conditions that are required to eliminate the MSC groups from the lysine side chains. We therefore chose the orange-colored 2-[4-(phenylazo)-phenylsulfonyl]-ethoxy (OPSE, opse) group (*8*).



This group shows facilitated hydrolysis of its carboxylic acid esters; whether this is due partly to β-elimination is questionable. At any rate, it was readily cleaved at the end of the synthesis together with the MSC groups. The choice of OPSE not only allowed a rapid assessment by tlc of the reaction progress (condensation, removal of the BOC group) but also had an additional, unexpected advantage: Whereas all peptides were easily soluble in dimethylformamide or trifluoroacetic acid during reactions, they were *insoluble* or only very slightly soluble in the usual organic solvents and aqueous systems used in the isolation steps. It therefore sufficed to evaporate the solvent after the

reaction and to wash the residual, orange-colored solid product in order to obtain it in excellent yield (Figs. 1 and 2) and high purity (Tables 1–3).

Peptide Bond Formation

The condensations were routinely carried out in dimethylformamide solution, and their progress followed by tlc. At practically every step, a number of active esters were investigated in order to choose the one that would give the best results. For example, in the preparation of **2**, the 2,4-dinitrophenyl ester of BOC·Ser·OH gave a 66% yield, its 4-chlorophenylthio ester a 75% yield, and the dicyclohexylcarbodiimide/1-hydroxybenzotriazole procedure an 80% yield of products of equal quality. In the preparation of **3**, carbodiimide condensation gave an excellent yield but a product of poorer quality than was obtained with the *N*-hydroxysuccinimide ester of BOC·Thr·OH. In the preparation of **4**, the *p*-nitrophenylester procedure gave an absolutely pure product, whereas the products obtained both with the *N*-hydroxysuccinimide ester and with carbodiimide were somewhat impure. In the preparation of **5**, BOC·Thr·ONSU gave excellent results, and **6** was best prepared via the *p*-nitrophenyl ester. The slight difference observed in the preparation of **4** between the nitrophenyl and the *N*-hydroxysuccinimide esters of BOC·Phe·OH was greatly magnified in the preparation of **7**; BOC·Trp·ONP reacted very well, whereas BOC·Trp·ONSU was almost unreactive. For the rest of the synthesis, *p*-nitrophenyl esters remained the reagents of choice, except in the last stage, where the 2,4,5-trichlorophenyl ester of BOC·Ala–Gly·OH gave the best results. Characteristics of the peptides and analytical results are displayed in Tables 1–3 and Fig. 3.

Deprotection and Oxidation

The last steps of the synthesis (Fig. 2) consisted of the removal of all protecting groups and oxidative cyclization of dihydrosomatostatin, **14d**, to somatostatin, **14e**. The MSC and PSE groups were removed with the barium hydroxide procedure (*13*) that proved to be superior to that using sodium or potassium hydroxide, mainly because Ba^{2+} can conveniently be eliminated with CO_2. *S*(3),*S*(14)-bis-Acetamidomethyl somatostatin was obtained in 48% yield and over 98% purity after either partition chromatography or (preferably) countercurrent distribution. The elimination of the ACM groups was accomplished with silver ion in dilute acetic acid, a rather slow reaction. After removal of Ag^{+} with dihydrogen sulfide, the product was oxidized as usual in the syntheses of somatostatin with ferricyanide at high dilution. After chromatography on Sephadex G25, the product was obtained in 98.4% purity. The amino acid analysis was correct, and the sample was identical with a reference kindly provided by Dr. Jean Rivier. All of the many clinical and biological tests performed so far prove qualitatively and quantitatively that the compound is, indeed, somatostatin (reports in preparation).

DISCUSSION

The "alternating solution/solid-phase peptide synthesis" used here was made possible by the special combination of protecting groups (and unprotected hydroxyl functions)

chosen. It allows a rapid synthesis of somatostatin with a minimal expenditure of time and effort for the condensation and purification steps. It can easily be handled on a large scale. A somatostatin of high purity is produced in a yield of 16 g per 100 g of the novel first intermediate, BOC · Cys(ACM) · OPSE, **1**.

A number of syntheses with the Merrifield solid-phase technique and with fragment condensation in solution have been reported. In the first category (for example, (*14*)), with one exception, benzyl-type side-chain protection was used that had to be removed by liquid hydrogen fluoride. Since the drawbacks of this treatment are well known, Chang and Meienhofer (*15*) used t-butyl-type side chain protection, an acid-labile linkage to the resin, and the alkali-labile 9-fluorenylmethyloxycarbonyl group for $N(\alpha)$-protection. Somatostatin was obtained in high yield and purity. The second category (*16*) comprises both types of side-chain protection. An interesting approach by Fujii and Yajima (*17*) used the azide procedure in connection with deblocking of benzyl-type protecting groups by trifluoromethanesulfonic acid. They also exploited the insolubility of intermediates for isolation and purification (Thr and Ser were unprotected).

The procedure described here combines a number of advantages: (i) It has been applied on the 10- to 100-g scale, and there are no obvious reasons preventing scale-up. (ii) $N(\alpha)$-t-Butoxycarbonyl amino acid active esters are easily prepared on a large scale and in high purity. (iii) Their condensations are carried out in solution without macromolecules bound to the growing peptide as in Ref. (*4*), thus ensuring a condensation reaction with a minimum of steric restraints. (iv) The uniform cleavage of the *t*-butoxycarbonyl group with trifluoroacetic acid allows for a standardization and automatization of this recurring step. (v) The presence of $HS \cdot CH_2–CH_2 \cdot SH$ during acidolysis with trifluoroacetic acid completely prevented damage to tryptophan; in this respect it proved to be superior to $HS \cdot CH_2–CH_2 \cdot OH$. (vi) The side-chain protecting groups are absolutely resistant to acidolysis and are completely removed under relatively mild conditions. (vii) The C-terminal colored protecting group shows facilitated removal with alkali; it confers an orange color to the peptides (an analytical advantage), and it helps to make all peptides sufficiently insoluble to enable them to be completely purified by simple washing procedures that require much less solvent than the Merrifield technique. (viii) Finally, the synthesis of *S*-acetamidomethyl-L-cysteine was improved.

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