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SOLID PHASE SYNTHESIS OF SOMATOSTATIN-28

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SUMMARY

The synthesis of ovine hypothalamic somatostatin-28 (Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH) has been accomplished by solid phase methodology. The structure of the synthetic material was verified by: (1) direct sequence analysis with a Beckman 890C sequencer, (2) correlation of the amino acid analyses of the isolated tryptic peptide fragments with their theoretical compositions, and (3) comparison, using high performance liquid chromatography, of the synthetic methionine-sulfoxide and methionine-sulfone modified NH_2-terminal peptides (residues 1-11) with the corresponding tryptic fragment from somatostatin-28.

The tetradecapeptide somatostatin has been isolated and characterized from both ovine (1) and porcine (2) hypothalami as well as pigeon (3) and anglerfish (4) pancreases. However, many laboratories have reported the observation of somatostatin-like substances having molecular weights greater than the tetradecapeptide in hypothalamic and pancreatic extracts (2,3). Very recently, Pradayrol et al. (5) reported the isolation and primary structure of an NH_2-terminally extended somatostatin containing 28 amino acids from porcine intestinal extracts which was designated as SS-28[1]. We have recently isolated from ovine hypothalamic extracts a bioactive somatostatin containing 28 amino acid residues as well as a shorter 25 amino acid residue peptide (manuscript in preparation). The primary structure of

[1]Symbols for amino acids are according to IUPAC-IUB recommendations published in Arch. Biochem. Biophys. (1966) 115, 1-12. Other abbreviations are as follows: SS = somatostatin; Boc = t-butyloxycarbonyl; TFA = trifluoroacetic acid; DMF = dimethylsulfoxide; HOBt = 1-hydroxybenzotriazole; PTH = phenylthiohydantoin; TLC = thin layer chromatography; DCC = dicyclohexylcarbodiimide; HPLC = high performance liquid chromatography; Met(O) = methionine-sulfoxide; Met(O_2) = methionine-sulfone.

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the larger ovine hypothalamic somatostatin is Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH, which is identical to the sequence of the intestinal SS-28 reported by Pradayrol et al. (5). The shorter 25 residue hypothalamic somatostatin has the same primary structure as SS-28 except the first three NH_2-terminal residues are deleted. We have synthesized SS-28 by solid phase technique (6) to provide enough material for extended biological testing. In this paper, we present the synthesis of SS-28 and the verification of its chemical structure.

MATERIALS AND METHODS

Derivatized amino acids used in the synthesis were of the L-configuration and purchased from Bachem, Inc. The N^{α}-amino function was protected exclusively with the Boc group. Side-chain functional groups were protected as follows: 2-chlorobenzyloxycarbonyl for lysine; p-methoxybenzyl for cysteine; benzyl for threonine, serine and glutamic acid; tosyl for arginine. TFA and DMF were distilled before use. HOBt was recrystallized from MeOH. All other solvents were reagent grade and used without further purification. Distilled water was degassed by purging with helium. PTH-amino acid standards were purchased from Pierce Chem. Co. while the gamma-methyl ester of PTH-Glu was synthesized according to Edman (7).

Peptides were hydrolyzed in sealed evacuated tubes containing 6 N HCl and 2% thioglycollic acid for 24 hours at 110° C. To quantitate cysteine peptide samples were oxidized with performic acid and then hydrolyzed in 6 N HCl only. Amino acid analyses of peptide hydrolysates were determined with either a Beckman/Spinco 119 or a Kontron Liquimat III amino acid analyzer. Optical rotations of peptide solutions (1% w/v in 1% HOAc) were measured in a Perkin-Elmer 141 polarimeter. Ascending TLC was performed on 0.25 mm thick precoated silica gel 60 plates (EM-Laboratories) using the solvent system, N-BuOH:pyridine:HOAc:H_2O (6:6:1.2:4.8).

Synthesis of SS-28. Coupling of Boc-(p-methoxybenzyl)cysteine to the chloromethyl resin (0.9 meq Cl/g, Lab. Systems, Inc.) was performed by the Monahan and Gilon procedure (8) and resulted in a substitution of 0.40 mmol cysteine per gram resin according to the Gisin analysis (9). The successive Boc-amino acids were coupled manually to 1.75 g of the protected cysteine-resin according to a previously described procedure (10) using 1 mmol of Boc-amino acid per gram resin in CH_2Cl_2 (or 10% DMF/CH_2Cl_2 for tryptophan and arginine) plus one equivalent of 2 M DCC in CH_2Cl_2 for two hours. Boc-asparagine was coupled overnight as its p-nitrophenyl ester using one equivalent of HOBt (11) in 50% DMF/CH_2Cl_2. The resulting protected peptide-resin (3.98 g) was treated with 1.5 ml anisole, 0.25 ml methylethyl sulfide and 10 ml hydrogen fluoride per gram peptide-resin at -20° for 0.5 hr and 0° for 0.5 hr. The work-up of the hydrogen fluoride cleavage reaction was as before (10) and 1.27 g crude peptide was recovered. This material was dissolved in 270 ml 1% HOAc and added dropwise to a potassium ferricyanide solution (319 mg in 128 ml 0.01 M NH_4OAc, pH 6.8) to form the disulfide bond as described by Rivier et al (12). After cyclization the peptide was chromatographed on both anion- and cation-

exchange resins as described (12) and lyophilized, yielding 0.52 g. The peptide was purified by CM-32 carboxymethyl cellulose (Whatman) cation-exchange chromatography (1.8 X 18 cm, V_{bed} = 50 ml) using a concave gradient generated by dropping 1 L 0.5 M NH_4OAc, pH 6.5 into a mixing flask containing 400 ml 0.01 M NH_4OAc, pH 4.5. Final purification was carried out using partition chromatography on Sephadex G-50 fine (Pharmacia) with a n-BuOH:pyridine:0.1% HOAc (5:3:11) solvent system. The chromatographic fractions were monitored by TLC and pooled to favor purity rather than quantity. The yield of product from the final purification step was 61 mg.

Synthesis of [Met(O)^8]SS-28-(1-11) and [Met(O_2)^8]SS-28-(1-11). The methionine-sulfoxide and methionine-sulfone substituted 8th position analogs corresponding to the NH_2-terminal tryptic fragment of SS-28 were prepared using the Beckman 990 synthesizer and purified as described (13).

Sequence Determination of SS-28. The dipeptide Phe-Leu-OH (100 nmol) and polybrene (5 mg, Aldrich) were subjected to three degradative cycles in the Beckman 890C sequencer using a Beckman 0.1 M Quadrol program (No. 121078). Synthetic SS-28 (500 nmol) was then added to the sequencer cup, dried and subjected to 28 degradative cycles. The recovered anilinothiazolinone derivatives of the cleaved amino acids were converted to their respective PTH products by incubation with 1 M HCl/MeOH at 50° for 10 min. The PTH-amino acids were dried under a stream of nitrogen, dissolved in 20 µl MeOH and analyzed on 0.20 mm thick high performance silica gel 60 F-254 plates (EM Laboratories) as described (14).

Trypsin Digestion and HPLC Separation of the Digest. Synthetic SS-28 (600 nmol) was digested with trypsin (38 µg, Calbiochem B grade) in 200 µl 1% NH_4HCO_3 for 4 hr at 37°. The digestion was terminated by lyophilization. The tryptic fragments were separated using an Altex Model 332 HPLC system with a 4.6 x 250 mm, 10 µ, RP-18 HPLC column (Brownlee). Gradient elution at a flow-rate of 0.6 ml/min was accomplished with a pyridine formate/n-propanol buffer system (15) at room temperature as described in the legend to Figure 1. The column effluent was collected in 1.8 ml fractions and monitored with an automatic fluorescamine detection system as previously described (16).

RESULTS AND DISCUSSION

SS-28 was synthesized using solid phase methodology with a final yield of 2.7%. The physical properties of SS-28 as well as those of the

TABLE 1. PHYSICAL CONSTANTS AND YIELDS OF SS-PEPTIDES

Peptides	$[\alpha]_D$	R_t-HPLC* (purity)	R_f-TLC	Yield+
SS-28	-81.7°	11.95 min (97.5%)	0.43	2.7%
[Met(O)^8]SS-28-(1-11)	-127.7°	10.88 min (97.5%)	0.21	34.6%
[Met(O_2)^8]SS-28-(1-11)	-122.3°	13.65 min (97.5%)	0.27	34.9%

* HPLC was performed on a Waters Associate Model 204 liquid chromatography system with solvent A composed of 0.25 N triethylammonium phosphate at pH 3.00 and solvent B containing 80% CH_3CN in solvent A (13). The column was eluted isocratically with 19% B for SS-28 and 6% B for the two methionine modified peptides.

Yields are based on the amine content in the protected Boc-amino acid resin as determined by the method of Gisin (9).

TABLE 2. AMINO ACID COMPOSITION OF SYNTHETIC SS-PEPTIDES

	SS-28	[Met(O)^8]SS-28-(1-11)	[Met(O_2)^8]SS-28-(1-11)
Lys	2.95	-	-
Arg	2.06	1.05	1.12
Trp	0.99	-	-
Asp	2.91	1.78	1.84
Thr	1.93	-	-
Ser	2.90	1.68	1.82
Glu	1.03	-	-
Pro	1.94	2.12	2.09
Gly	1.00	-	-
Ala	4.03	2.99	3.18
Cys	1.93	-	-
Met	0.93	1.06	-
Met(O_2)	-	-	0.93
Phe	2.95	-	-
NH_3	3.16	2.15	2.23

methionine-sulfoxide and methionine-sulfone substituted SS-28-(1-11) tryptic fragments are presented in Table 1. Each synthetic peptide was judged to be at least 97% pure by HPLC analysis based on the area under the major peak relative to the total integrated areas. Amino acid analyses of the synthetic SS-28 and its two methionine modified NH_2-terminal tryptic fragments gave the expected composition as shown in Table 2.

Lacking enough native material for thorough comparative studies with our synthetic SS-28, we resorted to rigorous analytical techniques to verify the structure of the synthetic substance. The synthetic peptide was sequenced directly in the Beckman 890C sequencer; PTH-amino acid analyses of the released residues were consistent with the primary structure of the SS-28 (data not shown).

HPLC analysis of the tryptic peptides from the synthetic SS-28 showed five major peaks (a to e) as shown in Figure 1. Amino acid analyses of the peptides in these peaks (Table 3) are consistent with the theoretical compositions of the tryptic peptides from SS-28 as shown in Table 4.

To verify that the methionine residue of SS-28 was not oxidized during the synthesis or purification, two NH_2-terminal tryptic fragments corresponding to SS-28-(1-11), one substituting methionine-sulfoxide and the

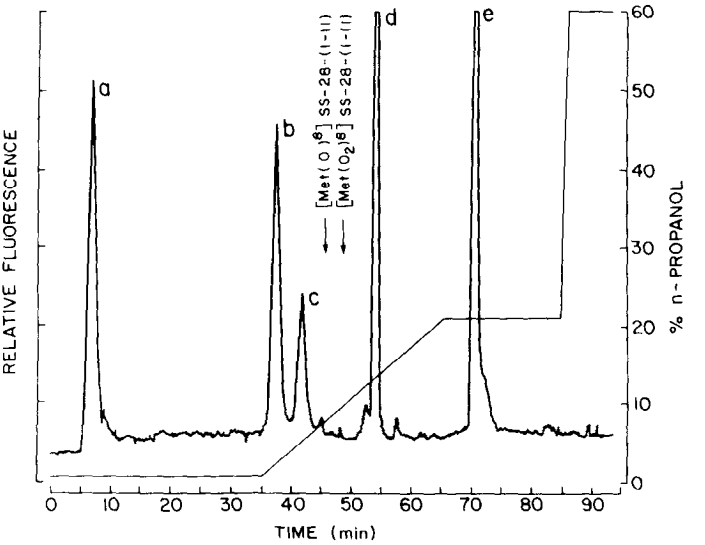

Figure 1: HPLC chromatography of the trypsin digestion product from synthetic SS-28. The system employed has been described in Materials and Methods. The column was eluted isocratically for 35 min with a solvent composed of 60 ml formic acid, 29 ml pyridine and 1% n-propanol made up to 1 L with H_2O, followed by a 30 min linear gradient from 1% to 22% n-propanol and then isocratically at 22% n-propanol for another 22 min, followed by washing the column with 60% n-propanol while maintaining the same concentration of formic acid and pyridine throughout the analysis. Peaks a,b,c,d, and e were collected and after hydrolyses analyzed on the Kontron Liquimat III amino acid analyzer.

other methionine-sulfone for the methione at the 8th residue were prepared and compared with the NH_2-terminal tryptic fragment of SS-28 by HPLC. As shown in Figure 1, the methionine-sulfoxide and methionine-sulfone

TABLE 3. AMINO ACID COMPOSITIONS OF THE HPLC PURIFIED TRYPTIC PEPTIDES FROM SS-28

	a	b	c	d	e
Lys	0.18	1.31	0.74	-	1.01
Arg	0.99	-	-	1.13	-
Trp	-	-	-	-	0.93
Asp	-	-	-	1.76	0.96
Thr	-	1.87	1.86	-	-
Ser	-	0.87	1.07	1.99	-
Glu	1.00	-	-	-	-
Pro	-	-	-	2.02	-
Gly	-	1.12	1.28	-	-
Ala	-	0.96	1.30	3.07	-
Cys	-	1.92	1.65	-	-
Met	-	-	-	0.47	-
Phe	-	0.92	1.06	-	2.09

TABLE 4. CORRELATION OF THE HPLC ISOLATED PEAKS WITH THEORETICAL PEPTIDES GENERATED FROM TRYPSIN DIGESTION OF SYNTHETIC SS-28

Peaks	Theoretical Peptides
a	Glu-Arg + Glu-Arg-Lys
b	Lys-Ala-Gly-Cys-Lys Thr-Phe-Thr-Ser-Cys
c	Ala-Gly-Cys-Lys Thr-Phe-Thr-Ser-Cys
d	Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg
e	Asn-Phe-Phe-Trp-Lys

containing peptides have completely different retention times than the NH_2-terminal tryptic fragment of SS-28 under identical chromatographic conditions.

Thus, it is concluded that we have accomplished the synthesis of the recently isolated SS-28 by solid phase methodology and the structure of the synthetic material was ascertained by rigorous chemical analyses. Preliminary bioassays using dissociated rat anterior pituitary cell culture showed that the synthetic SS-28 is more active than SS-14 on the inhibition of growth hormone release.

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