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Tyrosylated Analogues of Somatostatin

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The total synthesis of [Tyr[1]]-, [Tyr[6]]-, [Tyr[7]], [D-Tyr[8]]-, [Tyr[11]]-, and [Tyr[1],D-Trp[8]]somatostatin is described. The biological potencies of these analogues in vitro (inhibition of spontaneous secretion of radioimmunoassayable growth hormone by cultured rat anterior pituitary cells) as compared with that of somatostatin (100) were found to be 116, 29, 108, 10, 65, and 400, respectively; in vivo, in the rat, these peptides were assayed for their ability to inhibit the spontaneous release of insulin and glucagon. No statistically significant discrepancies were observed in the potency values obtained in vitro and in vivo further indicating similarities of specificities and sensitivities of the pituitary and pancreatotropic receptors.

The observation that des-(Ala[1],Gly[2])somatostatin or even des-(Ala[1],Gly[2])-desamino-Cys[3]-somatostatin were very potent analogues of somatostatin[1] (65 and 60%, respectively) led us to synthesize several acylated analogues of Cys[3]-somatostatin in an effort to obtain molecules which (a) could exhibit higher affinity for the receptor, which (b) might be more resistant to degradation by exopeptidases or that (c) could, because of their more hydrophobic character, be dissolved in oil and administered as a depot so that a protracted effect could be obtained. The biological evaluation in vitro and in vivo of such analogues has been reported by Brazeau et al.[2] and reevaluated by Brown et al.[3] Our conclusion was that the 38-membered ring of somatostatin contains all the information necessary for recognition and binding by the pancreatotropic and pituitary receptors and thus the side chain could be manipulated to yield tailored molecules. We predicted, for example, that [Tyr[1]]somatostatin would have full biological potency. This analogue, as well as other tyrosylated analogues of somatostatin, can easily be iodinated with radioactive I_2 or be specifically coupled by the bis-diazotized benzidine procedure[4] as hapten to larger proteins in order to raise specific antibodies against somatostatin (Vale et al., in preparation).

In view of the multiple possible use of tyrosylated analogues and as part of our studies on structure–activity relationships, we systematically substituted tyrosine for the phenylalanine residues at positions 6, 7, and 11.

The observation that [D-Trp[8]]somatostatin was eight times more potent than somatostatin in all systems tested[5] led us to synthesize analogues having other D- and L-amino acids at that position. The low biological potency of [Gly[8]]-, [L-Ala[8]]-, and [D-Ala[8]]somatostatin (<1%)[6–9] (and Rivier et al., in preparation) led us to conclude that, in contradistinction to luteinizing hormone releasing factor (LRF) for which substitution at the 6 position by any D-amino acids[10] yielded analogues as potent if not many times more potent than LRF, the indole ring at the eighth position of the somatostatin molecule was an absolute requisite for high biological potency. The synthesis of [D-Tyr[8]]somatostatin was to confirm this hypothesis.

[Tyr[1],D-Trp[8]]somatostatin, combining two modifications, the first yielding a compound as active as somatostatin and the other making it superactive, was predicted to be more potent than somatostatin and could be used in binding studies and characterization of the somatostatin receptor (Lazarus et al., in preparation).

This paper describes the total synthesis, physical constants, and biological evaluation in vitro and in vivo of materials that, through their ability of being easily radiolabeled, could be used in immunological, biological, and biochemical studies.

Synthesis, Purification, and Characterization. The tyrosylated analogues of somatostatin reported in Table I were synthesized by the solid-phase method[11] previously described for somatostatin[12] and analogues.[1] Boc-Cys-(SpOMe-Bzl) was esterified to the chloromethylated resin by a modification of Monahan and Gilon's procedure.[13] The stepwise buildup of the peptide on the resin was done using a standard procedure previously reported[12] with the exception that all couplings but those of Boc-Asn-PNP and Boc-Trp, for which dimethylformamide was used, were done in CH_2Cl_2. α-*tert*-Butyloxycarbonyllysine (ϵ-2ClZ)[14] was coupled at the ninth position while, for reasons of economy, α-*tert*-butyloxycarbonyllysine (ϵ-Z) was used at the fourth position. For threonine and serine, *O*-Bzl protection was chosen. For tyrosine the 2,6-Cl_2Bzl protecting group turned out to live up to its promises.[15] When present in the peptide, alanine and glycine were introduced as the Z-protected dipeptide.

After cleavage and cyclization (potassium ferricyanide[16]), the peptides were purified by gel filtration[17] and countercurrent distribution.[18] R_f values on TLC in five different solvent systems or after paper electrophoresis, optical rotation, yields, and partition coefficient in a 1-butanol–acetic acid–water two-phase system are reported in Table II. Amino acid analyses[19] of peptide hydrolysates are reported in Table III.

Biological Activity. In vitro, the peptides were assayed for their ability to inhibit the spontaneous secretion of radioimmunoassayable growth hormone by rat anterior pituitary cells in monolayered culture according to Vale et al.[20] In vivo the peptides were assayed for their ability to inhibit the spontaneous release of insulin and glucagon in the rat. Test samples were injected in the jugular vein and blood samples collected from the hepatic portal vein of animals under ether anesthesia.[21] Hormone levels (growth hormone, insulin, and glucagon) were measured by specific radioimmunoassays.[6] Potency values were calculated by multiple dose bioassays with somatostatin

Table I. Specific Biological Activity of Somatostatin Analogues

Compd	% specific act. of somatostatin (95% confidence limits)		
	In vitro,[a] growth hormone	In vivo[b] Glucagon	In vivo[b] Insulin
Somatostatin	100	100	100
[Tyr[1]]somatostatin (1)	116 (68-206)	78 (27-247)	70 (23-209)
[Tyr[6]]somatostatin (2)	29 (18-45)	14 (4.3-47)	12 (3.8-36)
[Tyr[7]]somatostatin (3)	108 (66-187)	109 (33-364)	75 (25-228)
[Tyr[11]]somatostatin (4)	65 (18-267)	65 (23-202)	252 (87-822)
[D-Tyr[8]]somatostatin (5)	10 (5-19)	<10	28 (11-72)
[Tyr[1]-D-Trp[8]]somatostatin (6)	400 (200-1200)		659 (238-1600)

[a] Three culture dishes per point; potencies calculated from results of multiple dose bioassays. [b] Six rats per point; potencies calculated from results of four-point bioassays.

Table II. Physical Constants and Yield of Somatostatin Analogues

Compd	TLC system[a] 2-BA	IaPyA	IpA	BPyA	BAW	Electrophoresis[b]	$[\alpha]^{22}$D,[c] deg	Yield,[d] %	K value[e]
1	0.32	0.63	0.81	0.54	0.45	0.33	−27.2	5	0.41
2	0.24	0.46	0.74	0.43	0.35	0.38	−32.4	11	0.23
3	0.24	0.47	0.73	0.41	0.33	0.44	−30.4	15	0.23
4	0.24	0.48	0.74	0.44	0.39	0.45	−32.1	11	0.24
5	0.22	0.43	0.74	0.41	0.34	0.41	−48.0	25	0.24
6	0.30	0.65	0.82	0.55	0.45	0.38	−38.3	5	0.34

[a] 2-BA, 2-butanol-0.1 N acetic acid (1:1, upper phase); IaPyA, isoamyl alcohol-pyridine-0.1% acetic acid (5:3:11, upper phase); IpA, 2-propanol-1 N acetic acid (2:1); BPyA, 1-butanol-pyridine-0.1 N acetic acid (5:3:11, upper phase); BAW, 1-butanol-acetic acid-water (4:1:5, upper phase). I_2, ninhydrin spray, and Pauly reagent were successively used. Loads varied from 20 to 40 μg per spot. Under those conditions, very closely related impurities in the amount of 1-2% would not be detected. [b] R_f values relative to lysine and alanine, 200-μg loads on Whatman 3MM (pH 4.7 buffer containing 2.5% acetic acid, 2.5% pyridine, 5% 1-butanol, and 90% water) at 3500 V, 2.0-h run. The six analogues and somatostatin (R_f 0.43) were run on the same chromatogram. Ninhydrin spray and Pauly reagent were successively used. [c] Concentration in 1% AcOH = 1. [d] Peptide yields are calculated on the basis of millimoles of peptides isolated after final purification relative to the total millimoles of starting *tert*-butylcarbonylamino acid, viz., as resin ester. Yields were not optimized. [e] Partition coefficient K in the system BAW (4:1:5) was obtained from the CCD purification step where $K = N/(n - N)$: N = abcissa for peak tube; n = number of transfers.

Table III. Amino Acid Analyses of Peptide Hydrolysates[a]

Compd	Ala	Gly	Cys	Lys	Asp	Phe	Tyr	Trp	Thr	Ser	NH_3
1		1.00	1.78	1.99	1.04	3.13	1.00	0.78	1.96	0.84	1.18
2	1.00	1.00	1.83	1.96	1.01	2.03	1.00	0.82	1.92	0.79	1.07
3	1.00	1.00	1.81	1.97	1.03	2.04	0.98	0.84	1.88	0.75	1.08
4	1.00	1.00	1.83	1.95	1.01	2.02	0.98	0.83	1.95	0.86	1.17
5	1.01	1.00	1.92	1.94	1.03	3.10	0.98		1.85	0.79	1.47
6		1.00	1.77	1.97	1.02	3.14	1.00	0.80	1.94	0.81	1.10

[a] Hydrolyses were carried out in 6 N HCl containing 2.5% thioglycolic acid at 110° in evacuated sealed tubed for 20 h. No correction was made for decomposition of Trp. Cys and Ser values were obtained after performic oxidation as reported in ref 1.

as a reference standard. All the analogues reported in Table I have statistically parallel log dose-response functions when compared with somatostatin in vitro and in vivo. While the specific activities of the various analogues vary considerably, the intrinsic activity of each of them is maximal indicating that none of them acts as a partial agonist.

Subsequent to our original report on the synthesis, biological properties, and potential of [Tyr[1]]somatostatin,[22] Arimura et al.[23] and Patel and Reichlin[24] used this analogue as a trace in somatostatin radioimmunoassays. The higher potency of [Tyr[1],D-Trp[8]]somatostatin indicates that, as it is the case for des-Asn[5]-somatostatin and des-Asn[5]-[D-Trp[8]]somatostatin,[25] some structural modifications, at least, are additive in their biological effects.

The lower potency of [Tyr[6]]somatostatin (15%) as compared to that of [Tyr[7]]somatostatin (100%) would indicate that each phenylalanine plays a different role. The Phe[6] residue might be functionally involved in the receptor activation process whereas the Phe[7] could be involved in the internal stabilization of somatostatin tertiary structure through the stacking of aromatic rings. Holladay and Puett[26] have indeed proposed a model for the tertiary structure of somatostatin. One of the most striking features of that model is the stacking of the three aromatic side chains in the order Trp[8], Phe[11], and Phe[7]. The high potency of [Tyr[11]]somatostatin could indicate that the phenyl side chain of Phe[11] is more involved in building up a hydrophobic aromatic nucleus at one end of the somatostatin molecule rather than be independently required for recognition by the receptors on either the anterior pituitary or endocrine pancreas. Physicochemical studies are being carried out with those analogues to prove or refute this working hypothesis.

The low potency of [D-Tyr[8]]somatostatin (approximately 100 times less than that of [D-Trp[8]]somatostatin) is best explained by the unique properties of the indole ring, which will not only be involved in the buildup of the aromatic hydrophobic nucleus previously mentioned but also would be specifically recognized.

Experimental Section

Syntheses. Chloromethylated polystyrene resin cross-linked with 1% divinylbenzene was obtained from Lab. Systems, Inc.

(LS 601-0.75 mM/g as Cl^-). Esterification of α-Boc-Cys-(SpOMe-Bzl) to the resin was performed as follows.[5,13] To α-Boc-Cys(SpOMe-Bzl) (7.5 mequiv) dissolved in dry, redistilled Me_2SO (25 ml) is added the chloromethylated resin (10 g, 7.5 mequiv of Cl). A thick slurry is obtained to which KO-*t*-Bu (6.8 mequiv) is added as a dry fluffy powder. After ensuring that the base is properly dissolved by vigorous shaking, the slurry is kept at 80 °C for 2 h. After extensive washing [Me_2SO, Me_2SO–water (9:1), MeOH, and CH_2Cl_2], the resin is dried and can be kept refrigerated for a long period of time. A substitution of 0.20–0.22 mmol of protected cysteine per gram of resin was deduced either from the weight gain of the resin or by quantitative amino acid analysis of the peptides made on such resin.

Deblocking was achieved in 20 min by TFA–CH_2Cl_2 (50:50) containing 5% ethanedithiol, followed by neutralization with 12.5% Et_3N in CH_2Cl_2. Successive coupling of each amino acid (5 M excess) was mediated by dicyclohexylcarbodiimide (5 M excess) for an average of 2 h in CH_2Cl_2 with the exception of Boc-Trp which was coupled in CH_2Cl_2–DMF (1:1) and Boc-Asn which was introduced as its *p*-nitrophenyl ester in DMF overnight. The reaction was controlled by the ninhydrin test of Kaiser et al.[27] Washes included DMF, CH_2Cl_2, and MeOH.

The protected amino acids [α-Boc-Cys(*p*-OMe-Bzl), α-Boc-Ser(OBzl), α-Boc-Thr(OBzl), α-Boc-Trp, α-Boc-Phe, α-Boc-Lys(ε-2ClZ), α-Boc-Lys(ε-Z), α-Boc-Asn-PNP, Z-Ala-Gly, α-Boc-Gly, α-Boc-Tyr(2,6-Cl_2-Bzl)] were bought from BACHEM and were found to be homogeneous and optically pure by determination of their optical rotation.

Cleavage, Deprotection, Oxidation, and Purification. The protected peptide resin (6 g) was treated with HF[28] (~100 ml) for 40 min at −20 °C and for 20 min at 0 °C in the presence of anisole (10 ml). After rapid removal of HF and drying under vacuum (the temperature is kept around 0 °C), the light-brown-colored resin was washed with ether (4 × 50 ml). The dried resin was immediately extracted with 25% AcOH (150 ml) and diluted to 3500 ml with degassed H_2O (N_2).

Dropwise titration of this solution (pH adjusted to 6.7 with NH_4OH) with potassium ferricyanide[16] (1 g/500 ml of H_2O) was terminated upon apparition of a yellow color or complete disappearance of free sulfhydryls as monitored by Habeeb's method[29] using Ellman's reagent.[30]

The solution sat for 10 min and the pH was adjusted to 5.0 with glacial AcOH; Bio Rad AG3-X4A (100–200 mesh, chloride form, 10–15 g) was added to the turbid solution and stirred for 15 min. The solution was filtered over Celite and applied successively onto two columns: (a) Bio Rad AG3-X4A chloride form (10 ml); (b) Bio Rex-70 (50 ml) cation form. The Celite + resin cake was thoroughly washed with water (500 ml) which was applied onto columns a and b as a wash.

The Bio Rex-70 column (3.0 × 7 cm) was further washed with 5% AcOH (200 ml). The peptide material was displaced with 50% AcOH. The peptide-containing fractions were diluted with water and lyophylized (yield 0.80–1.20 g). This crude, off-white colored material was applied onto a Sephadex G-25F gel column (3 × 200 cm) equilibrated and eluted with 10% AcOH. The elution pattern, as observed at 280 nm (when Trp is present) or at 272 mm for [D-Tyr[8]]somatostatin, showed one major symmetrical peak centered at 2 V_0. This material (400–700 mg) could be evaluated by TLC to be from 40 to 80% pure. Final purification was achieved by countercurrent distribution[18] on a 100-tube Post apparatus (10 ml lower and upper phases) using the two-phase system (1-BuOH–AcOH–H_2O, 4:1:5). The analogue (400–700 mg) was applied in tube 1 and 200–250 transfers were performed. The *K* values obtained for each analogue are reported in Table II. Air oxidation of the crude deprotected peptides, under mild stirring at pH 6.8 (1 week), was also found to be applicable and even advantageous in the case of 1.

Characterization. Homogeneity of peptides 1–6 was demonstrated by thin-layer chromatography on Eastman chromatogram sheets (6061 silica gel with fluorescent indicator) in five solvent systems and by paper electrophoresis; see Table II. Amino acid analyses (see Table III) were performed on peptide hydrolysates using a Beckman/Spinco Model 119 amino acid analyzer. Peak areas were determined by an Infotronics Model CRS-100A electronic integrator. Optical rotations were measured on a Perkin-Elmer Model 141 polarimeter.

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somatostatin on inhibition of insulin release in vivo. It is, however, less potent than somatostatin on inhibition of glucagon release. Des-Asn[5]-[D-Trp[8]]somatostatin is thus the first analogue of somatostatin with high potency and dissociated biological activity.
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Nucleic Acids. 16. Orally Active Derivatives of *ara*-Cytidine[1,2]

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Water-soluble derivatives of *ara*-cytidine (cytarabine, Cytosar) were prepared and tested for antitumor, immunosuppressive, and antiarthritic activities in animals after oral administration. The compounds tested included the 5′-palmitate, 5′-benzoate, and 5′-adamantoate esters of *ara*-cytidine, made water soluble by use of their hydrochloride salts or peptidyl derivatives, and two basic 5′ esters (5′-nicotinoate and 5′-quinuclidinate) as their hydrochloride salts. Five of the compounds had antitumor activity superior to that found with *ara*-cytidine itself after oral administration in the L1210 leukemic mouse assay. One of these, 5′-adamantoyl-*ara*-cytidine hydrochloride, had antitumor activity after oral administration approaching that achieved with parenterally administered *ara*-cytidine.

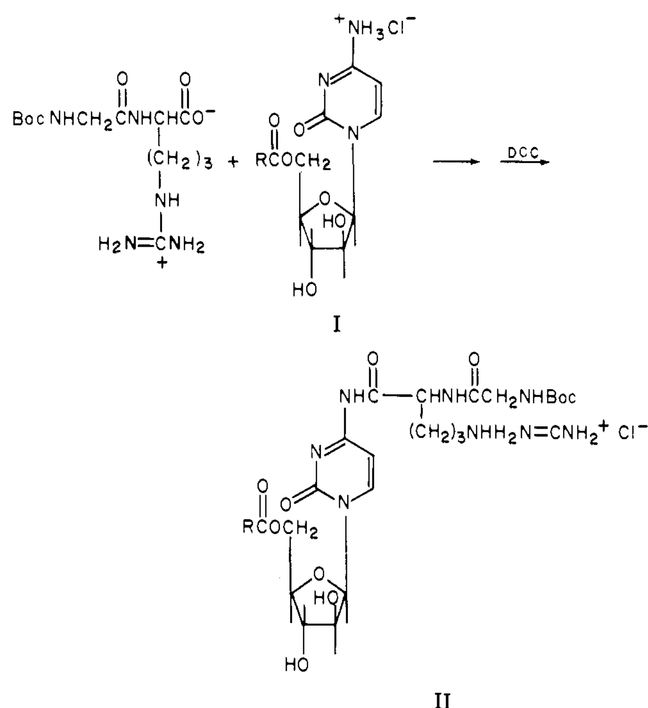
In previous papers[1] we described the systematic modification of relatively insoluble 5′-acylates (esters) of the antitumor, antiviral, immunosuppressive nucleoside, *ara*-cytidine (cytarabine, Cytosar), in order to adjust predictably the nucleoside's pharmacologic properties. By these structural modifications we were able to affect the solubility, the enzymatic hydrolysis of the ester to the active nucleoside, and the catabolic conversion to *ara*-uridine and thus increase the depot activity. This paper describes results of our modifications of the most biologically interesting of these 5′-esters in an attempt to prepare a derivative that will produce as much activity in man when administered orally as *ara*-cytidine administered parenterally.

Orally administered *ara*-cytidine has not been shown to be active in man, although in mice it is about one-fifth as active when administered orally as when given parenterally.[3] Pharmacokinetic analyses suggest low rate and extent of absorption and rapid elimination (deamination and excretion) so that plasma levels are never sufficient to produce therapeutic activity.[4]

Desirable Properties. Ideally, an orally active derivative of *ara*-cytidine should have adequate dissolution and absorption rates in the gastrointestinal tract and resistance to deamination by pyrimidine aminohydrolase found in the gut and other tissues. If the drug, once absorbed, is rapidly localized in lipid depots, it should be protected from deamination. Diffusion from the depot sites should maintain therapeutic blood levels. Finally, the substituent groups used to confer these properties upon the compound should be subject to rapid removal in vivo[1] to release *ara*-cytidine for subsequent conversion to *ara*-CTP, the active form of the drug.[5]

The presence of an appropriate ester group at the 5′ position achieves localization of the drug at the site of parenteral administration and/or in lipid depots. It also prevents deamination. However, such esters are only sparingly soluble in water and, apparently because of slow dissolution, produce only minimal activity when administered orally in aqueous suspensions. It occurred to us that they might be made adequately soluble by forming acid addition salts of the esters or by attaching a suitable peptidyl substituent at N[4]. The covalently bonded substituents could then be removed enzymatically in vivo. Our earlier results with N^4-amide derivatives of *ara*-cy-

Scheme I



tidine suggested that such derivatives were inactive,[6,7] but these results were from in vitro experiments, which have not quantitatively predicted in vivo activity[1,8]—presumably because the systems lack the requisite amidase activity. We reasoned that if a substituent placed on the N^4-amino group were an amino acid or peptide, hydrolytic enzymes ubiquitous in vivo would convert it to the free amine at N[4]. This should be especially likely if the amino acid linked to N[4] were lysine or arginine, because trypsin or trypsin-like enzymes capable of splitting lysyl and arginylamido bonds are found in the gut, blood, and tissues.

For our studies we chose the *tert*-butoxycarbonylglycyl-L-arginyl derivatives (II in Scheme I) in which the carboxyl group of arginine is attached to the N^4-amino group of cytosine. We thought that combining a glycine residue with a protonated arginine residue would confer water solubility on the derivative, while the arginylamido