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Synthesis and sst_2 binding profiles of new (Tyr^3)octreotate analogs

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Abstract: One of the main objectives of our current work is the development of new somatostatin analogs that would retain the general characteristics of [Tyr^3]octreotate (Tate) while showing potential for clinical application. In this respect, study of their interaction with the sst_2 is crucial in providing preliminary structure-activity relationships data. In the present work we report on the synthesis and the preliminary biological evaluation of a total of 15 new structurally modified [Tyr^3]octreotate analogs. The binding affinities were determined during competition binding assays in sst_2-positive rat acinar pancreatic AR4-2J cell membranes using [^{125}I-Tyr^3]octreotide as the radioligand. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: somatostatin analogs; [Tyr^3]octreotate; sst_2; binding affinity; AR4-2J cells

INTRODUCTION

Somatostatin [Somatotropin Release Inhibiting Factor: SRIF] [H-Ala^1-Gly^2-c(Cys^3-Lys^4-Asn^5-Phe^6-Phe^7-Trp^8-Lys^9-Thr^{10}-Phe^{11}-Thr^{12}-Ser^{13}-Cys^{14})-OH] is a regulatory peptide hormone first isolated in 1973 from bovine hypothalami [1]. The first described action of SRIF was the inhibition of growth hormone (GH) release from the anterior pituitary. SRIF suppresses the secretion of many hormones, such as thyroid stimulating hormone (TSH), prolactin, insulin and glucagon [2–5]. Furthermore, SRIF acts as a neurotransmitter in the central nervous system [6], e.g. by modulating locomotor activity and cognitive function [7]. As a gut peptide, SRIF inhibits intestinal motility and regulates the secretion of several gut hormones [8]. Another outstanding reported action of SRIF is the inhibition of cell proliferation [9].

Two native isoforms of somatosatin have been identified so far: the tetradecapeptide SRIF-14 and the octacosapeptide SRIF-28 [10]. Both isoforms are biologically active and are found in the periphery and in the central nervous system, but SRIF-14 is the predominant isoform. The physiological actions of SRIF are mediated by high affinity receptors located on the cell membrane of target cells. Until now five receptor subtypes (sst_{1-5}) have been identified and characterized [11–15], all coupled via nucleotide binding proteins (G-proteins) to multiple cellular effector systems [13]. Both SRIF isoforms bind to all five sst subtype receptors with a high affinity.

Of particular clinical relevance is the high density expression of sst, and especially of sst_2, in a large variety of primary tumors and their metastases, such as gastroenteropancreatic neuroendocrine tumors (GEP-NETs), gliomas, meningiomas and breast tumors [16–19]. The antiproliferative action of SRIF and its analogs has been documented in several cell lines, but most importantly, *in vivo* in animal tumor models and in human. However, for *in vivo* application, the development of highly specific and metabolically stabilized SRIF analogs is required. Veber *et al.* [20] at Merck synthesized a cyclic hexapeptide analog of somatostatin and demonstrated that the central tetrapeptide Phe^7-(D)-Trp^8-Lys^9-Thr^{10} contains the somatostatin pharmacophore. Since then, hundreds of hexa- and octapeptide analogs of somatostatin have been synthesized and characterized. Among these, octreotide, (D)-Phe-c[Cys-Phe-(D)-Trp-Lys-Thr-Cys]-Thr-ol (Sandostatin®, Novartis) [21], is a potent inhibitor of growth hormone secretion and is clinically used in the treatment of acromegaly and neuroendocrine tumors. Octreotide binds predominantly to sst_2 and to a lesser extent to sst_5 while displaying low affinity to sst_3. Octreotide has been the subject of extensive structural studies including NMR and X-ray diffraction [22–25]. Taken together these studies have demonstrated that octreotide in solution exists in two conformational families, differing mainly in the conformation of the *C*-terminal tail. The molecule adopts an overall antiparallel β-sheet conformation, with a type II′ β-turn centered at the D-Trp^4-Lys^5 region. In one conformational family, the residues following this β-turn continue the β-sheet structure. On the basis

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of elaborate structure-activity relationship (SAR) studies of various somatostatin analogs, Goodman *et al.* [22] concluded that this family contains the active conformation(s). In the second conformational family, the residues following the β-turn adopt a 3_{10}-helical conformation.

Substitution of Phe^3 by Tyr^3 as well as *C*-terminal $Thr(ol)^8$ by Thr^8 in the parent octreotide sequence leads to [Tyr^3]octreotate (H-D-Phe-c(Cys-Tyr-D-Trp-Lys-Thr-Cys)-Thr-OH) showing enhanced affinity and selectivity for the sst_2 [26]. Synthetic somatostatin analogs, as those based on octreotide and octreotate, radiolabeled with a variety of metallic radionuclides have been used in the diagnosis, staging and radionuclide therapy of sst_2-positive tumors [27–29]. The first radioactive SRIF analog used for imaging was ^{111}In-diethylentriamine pentaacetic acid (DTPA)-octreotide [30]. This radiopharmaceutical, commercially available under the trade name OctreoScan (Mallinckrodt Inc., MO/USA), is clinically used in the sst_2-targeted diagnostic imaging of neuroendocrine tumors [31]. Although hundreds of sst-specific somatostatin analogs have been synthesized, there is a need for more potent ligands to better explore the physiological role of somatostatin and its cognate receptors as well as to make available more effective somatostatin-based drugs.

As part of an ongoing research on the development of new SRIF analogs with potential clinical application, we report herein on the synthesis and the sst_2-binding affinity of a total of 15 structurally modified [Tyr^3]octreotate analogs. Structural modifications on the original [Tyr^3]octreotate sequence were introduced to provoke changes in the polarity or/and net charge or charge distribution on the molecule. More specifically, we replaced position 1 and 3 aromatic amino acids by more or less polar residues, and the *C*-terminal Thr^8 with charged residues. Furthermore, elongations of the peptidic chain with polar amino acids, like Asn and Ser, were attempted. We also present affinities of two previously reported analogs [$BzThi^3$]octreotate and [$Nal(1)^3$]octreotate (with benzothienylalanine and 1-naphtylalanine in position 3, respectively) [32], which were resynthesized for comparison purposes. Study of the interaction of new SRIF analogs with the sst_2 is crucial in providing preliminary structure-activity relationships data.

MATERIALS AND METHODS

Materials

The 9-fluorenylmethoxycarbonyl-protected amino acids and peptide reagents were obtained from CBL (Patras, Greece), Bachem (Bubendorf, Switzerland), and Novabiochem (Läufelfingen, Switzerland). All solvents and reagents used for solid-phase synthesis were of analytical quality and used without further purification.

Iodine-125 was obtained from MDS Nordion, SA (Fleurus, Belgium). All culture media and supplements were supplied by Gibco BRL, Life Technologies (Grand Island, NY) and by Biochrom KG Seromed (Berlin, Germany). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide) was obtained from Sigma–Aldrich (St. Louis, USA). For protein measurements, the protein microdetermination kit (Procedure No. P 5656) by Sigma Diagnostics (St. Louis, USA) was utilized. The Brandel M-48 Cell Harvester (Adi Hassel Ingenieur Büro, Munich, Germany) was used for filtrations during binding assays. Radioactivity measurements were conducted in an automated well-type gamma counter [NaI(Tl) crystal, Canberra Packard Auto-Gamma 5000 series model] calibrated for ^{125}I.

Methods

Peptide synthesis and purification. The analogs were synthesized by Fmoc solid phase methodology [33] utilizing a 2-chlorotrityl-chloride resin [34] as solid support. Fmoc-protected amino acids were used with the *t*-Butyl group (Bu^t) as side-chain protection group for Tyr, Thr and Asp, *t*-butoxycarbonyl group (Boc) for Trp and Lys, trityl (Trt) group for Asn and 4-methoxytrityl (Mmt) group for Cys.

Stepwise synthesis of the peptide analogs was achieved with DIC/HOBt in DMF as coupling agents [35]. Couplings were performed with Fmoc-amino acid, DIC and HOBt in DMF in a 3, 3.3 and 4.5 molar excess, respectively, for 2.5 h at room temperature. Completeness of the reaction was monitored by the Kaiser test [36], the Chloranil test [37] and TLC. The Fmoc groups were removed by treatment with 20% piperidine in DMF for 40 min. After synthesis and removal of the Fmoc group of the peptide's final amino group, the resin was treated with a TFA solution (15 ml/g peptide resin) in the presence of scavengers (TFA/1,2-ethanedithiol/triethylsilane/water/anisole, 88/4/3/2/3 v/v/v/v/v) to liberate the fully deprotected crude peptides. The released peptides were precipitated upon concentration of solvent and addition of cold ether. The precipitates were collected by filtration, washed twice with cold ether and dried in vacuum over KOH. The formation of the disulfide bridge (cyclization) was mediated either by the DMSO/water method in a solution of 25% DMSO/H_2O for 24–36 h at room temperature [38] or via the CCl_4/TBAF method [39] in a solution of 20% CCl_4 in acetonitrile in the presence of 150 µl of 1 M TBAF in dry tetrahydrofuran. In both methods of cyclization 1.5 ml of the oxidative solution per mg of crude linear peptide was used. Completeness of the formation of the disulfide bond was monitored either by the Ellman's test or by analytical HPLC. All the products were purified by gel filtration chromatography on Sephadex G-15 using 15% acetic acid as the eluent. Final purification was achieved by preparative high performance liquid chromatography (HPLC, Mod.10 ÄKTA, Amersham Biosciences, Piscataway, USA) equipped with a Lichrosorb RP18 column (C_{18} column 7 µm particle size; 250 mm × 8 mm) with a linear gradient from 10 to 70% acetonitrile (0.1% TFA) for 35 min and 70 to 100% acetonitrile (0.1% TFA) for 5 min at a flow rate of 1.5 ml/min and UV detection at 220 and 254 nm. The appropriate fractions were pooled and lyophilized. All products gave single spots on TLC (Merck precoated silica gel plates, type G_{60}-F_{254}) in the solvent systems: (i) 1-butanol–acetic acid–water–pyridine (4 : 1 : 1 : 2) (ii) 1-butanol–acetic acid–water (4 : 1 : 5 upper phase). Analytical HPLC (Äcta Purifier) equipped with a Nucleosil 100 C_{18}

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column (5 μm particle size; 250 mm × 4.6 mm, Agilent Technologies, Waldbronn, Germany) produced single peaks with at least 97% of the total peptide peak integrals. ESI-MS (Micromass–Platform LC instrument) results were consistent with the expected peptide formulae. The physicochemical properties of the new analogs are summarized in Table 1.

Cell culture. Rat pancreatic carcinoma AR4-2J cells were grown confluent in F-12 K nutrient mixture supplemented by 10% (v/v) fetal bovine serum, 1 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin in humidified air containing 5% CO_2 at 37 °C. Subculturing was performed employing a trypsin/EDTA (0.05%/0.02% w/v) solution.

Determination of Binding Affinity

Membrane preparation from AR4-2J cells. Cell membranes from AR4-2J cells were prepared as mentioned in detail previously [40]. AR4-2J cells were grown to confluence, mechanically disaggregated, washed twice with cold PBS buffer pH 7.0 and resuspended in homogenization buffer (1 ml/flask) containing 10 mM Tris pH 7.4 and 0.1 mM EDTA. Cells were homogenized using a Bioblock Scientific homogenizer (50 strokes/5 ml) and the homogenized suspension was centrifuged at 2600 rpm for 10 min at 4 °C in a J2-MC Beckman centrifuge. The supernatant was removed and recentrifuged at 26 000 rpm for 15 min at 4 °C in a CS120GX micro ultracentrifuge (Hitachi). The pellet was resuspended in homogenization buffer (100 μl/flask) and stored at −80 °C in aliquots of 100 μl.

Protein measurement. The protein concentration of the samples was determined according to the method of Lowry [41] using bovine serum albumin as the standard.

Radioiodination. Radioiodination was performed using the chloramine-T method. To an Eppendorf tube containing 30 μl 0.2 M phosphate buffer, pH 7.4, 15 μg [Tyr^3] octreotide dissolved in 7.5 μl 0.1% acetic acid/ethanol (8/2) and 15 μl radioiodide solution in 0.05 M NaOH/saline (2/40) ([^{125}I]NaI (nca), 7–10 MBq) were added followed by 3 μl of a freshly prepared solution of chloramine-T in water (1 μg/μl). The labeling reaction was allowed to proceed for 60s at room temperature. After dilution with 50 μl MeOH/saline (1/1) the mixture was immediately subjected to HPLC purification. [^{125}I–Tyr^3]octreotide (74 TBq/mmol) was isolated by analytical RP-HPLC (Waters, Symmetry Shield RP-18, 5 μm, 150 mm × 3.9 mm) applying a linear gradient system at a 1 ml/min flow rate from 10 to 40% B in 60min, where solvent A = 0.1% TFA in water and solvent B = pure acetonitrile. The fraction containing the radioligand (≈2 ml, t_R [^{125}I–Tyr^3]octreotide = 33.0 min; t_R [Tyr^3]octreotide = 26.9 min) was diluted with 5 ml saline and loaded onto an OASIS^{TM} cartridge (Waters, Milford, MA) preactivated by ethanol followed by water and saline. After washing the cartridge with saline, the radioligand was eluted with 50% ethanolic saline. The ethanol was removed under reduced pressure followed by dilution with 50 mM phosphate buffered saline, pH 7.4 containing 1% BSA to an activity concentration of approximately 500 000 cpm/10 μl. The purified tracer was stored at −20 °C.

Competition binding assays. Receptor binding assays were performed in AR4-2J cell membrane homogenates using [^{125}I –

Table 1 Physicochemical properties of synthesized SRIF analogs

	Analog	MW	[a][M + 1]$^+$	[b]Yield (%)	Melting point (°C)	[c]TLC		[d]HPLC t_R (min)
						Rf_1[A]	Rf_2[B]	
i	[D-Tyr^1]Tate	1065.24	1066.61	46	189–191	0.57	0.29	14.35
ii	[D-Tyr(Et)^1]Tate	1093.3	1094.85	53	184–185	0.59	0.36	16.17
iii	[D-Phg^1]Tate	1035.22	1036.91	43	179–181	0.52	0.64	14.78
iv	[Asp^1]Tate	1017.15	1017.94	53	205–207	0.30	0.16	16.65
v	[Phg^3]Tate	1019.22	1019.64	51	201–203	0.61	0.21	16.21
vi	[Tic^3]Tate	1045.26	1045.92	43	194–196	0.34	0.21	19.06
vii	[D-Phg^1,Phg^3]Tate	1005.19	1005.59	44	170–172	0.49	0.31	15.86
vii	[Lys^8]Tate	1076.31	1077.27	45	172–174	0.33	0.20	16.19
ix	[Asp^0, Lys^8]Tate	1191.41	1192.15	40	182–184	0.26	0.17	16.26
x	[Asp^8]Tate	1063.22	1063.95	48	230–232	0.56	0.30	15.12
xi	[Asn^0]Tate	1163.41	1164.25	40	196–198	0.53	0.46	15.32
xii	[Ser^9]Tate	1136.20	1137.31	51	211–213	0.51	0.49	14.94
xiii	[Asp^0,Asp^8]Tate	1178.22	1178.94	55	205–207	0.52	0.25	14.71
xiv	[Asn^0,D-Tyr(Et)^1]Tate	1207.30	1208.17	41	190–192	0.56	0.31	15.41
xv	[Asn^0,D-Phg^1,Phg^3]Tate	1119.19	1120.12	40	187–189	0.47	0.22	15.21
xvi	[BzThi^3]Tate[e]	1089.31	1090.16	48	196–198	0.65	0.41	16.93
xvii	[Nal(1)^3]Tate[e]	1083.22	1084.12	47	215–217	0.64	0.40	16.73

[a] Data obtained by ESI-MS.
[b] Yields were calculated on the basis of the amino acid content of the resin. All peptides were at least 97% pure.
[c] Solvent systems: (A) 1-butanol-acetic acid-water-pyridine (4:1:1:2), (B) 1-butanol-acetic acid-water (4:1:5 upper phase).
[d] For elution conditions see Experimental Section.
[e] Resynthesized analog.

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Tyr^3]octreotide as the radioligand. Similar experiments were performed for [Tyr^3]octreotate, [BzThi^3]Tate and [1 – Nal^3]Tate for comparison. Typically, 40 000 cpm of radioligand was incubated in triplicates for 40 min at 37 °C with 15 μg protein in the presence of increasing concentrations of tested analog ranging from 10^{-13} to 10^{-6} M in a total volume of 300 μl of 50 mM HEPES (pH 7.6, 0.3% BSA, 5 mM $MgCl_2$, 10 μM bacitracin). Incubation was terminated by addition of ice-cold washing buffer (1 ml, 10 mM HEPES, 150 mM NaCl, pH 7.6) and rapid filtration over Whatman GF/B filters (Adi Hassel Ingenieur Büro, Munich, Germany, presoaked in binding buffer) on a Brandel cell harvester. The filters were rinsed thoroughly with washing buffer (4 × 2 ml) and filter activity was measured in an automatic NaI(Tl) gamma counter. Binding data were analyzed by nonlinear regression according to a one-site model using the PRISM 2 program (GraphPad software, San Diego, CA).

RESULTS

Peptide Synthesis and Purification

All analogs shown in Table 1 were synthesized either manually or automatically on the 2-chlorotrityl chloride resin using the standard Fmoc/Bu^t strategy. Except for the cases of the Fmoc-Cys(Mmt)–OH coupling to Thr(Bu^t) whereby the coupling reaction lasted 1 h longer, couplings were complete within 2.5 h. Solid-phase synthesis yields ranged within 40–53%, when calculated on the amount of Thr(Bu^t) initially coupled to the resin. Peptides with *N*-terminal Asn were obtained in lower yields. As 40 min treatment with piperidine was not sufficient for the deprotection of the end amino group of Asn, the resin was treated for 3 min with a solution of 2% DBU in DMF. Using a lower concentration of TFA (85% instead of 88%) and dimethylsulfide in addition to the other scavengers in the cleavage mixture higher purity products were obtained.

Formation of the disulfide bond was quantitative by both the applied methods. By the CCl_4/TBAF method disulfide-bridge formation was completed within 10 min. Therefore, this was the method of choice despite its disadvantage of residual TBAF left in the crude peptide after solvent evaporation.

Binding Affinity for the sst$_2$

The IC_{50} values of [Tyr^3]octreotate analogs, as determined during competition-binding assays on rat sst$_2$-positive AR4-2J cell membranes, are summarized in Table 2. Replacements of D-Phe^1 were undertaken to affect the polarity and/or charge at the *N*-terminus. Insertion of D-Tyr^1 instead of D-Phe^1 in analog **i** reduced affinity ($IC_{50} = 2 \pm 0.20$ nM) with respect to parent [Tyr^3]octreotate ($IC_{50} = 0.30 \pm 0.03$ nM), implying that, the presence of a polar free phenolic group in this position is unfavorable for sst$_2$ binding. This finding was further supported by the fact that analog **ii** with D-Tyr(Et)^1, wherein the phenolic group of Tyr was transformed to an ether of diminished polarity, exhibited similar binding affinity ($IC_{50} = 0.22 \pm 0.01$ nM) with Tate. Likewise, substitution of D-Phe^1 by nonpolar D-Phg^1 (D-Phenylglycine) in analog **iii** slightly affected the affinity ($IC_{50} = 0.47 \pm 0.03$ nM). On the other hand, replacement of D-Phe^1 by the negatively charged L-Asp^1 in analog **iv** provoked an almost ten-fold reduction of binding affinity ($IC_{50} = 2.70 \pm 0.09$ nM).

In another set of compounds, we investigated the role of Tyr^3 in sst$_2$ binding capacity. Thus, in analogs **v** and **vi** Tyr^3 was replaced by the nonpolar L-Phg^3 (Phenylglycine) and L-Tic^3 (1,2,3,4,-tetrahydro-isoquinoline-3-carboxylic acid), respectively. For comparison purposes, we also synthesized *de novo* analogs **xvi** and **xvii** containing the lipophilic BzThi (Benzothienylalanine) and Nal(1) [1-Naphthylalanine] at position 3, respectively. In the case of the Phg^3 (analog **v)** binding affinity was only moderately reduced ($IC_{50} = 1 \pm 0.10$ nM), while analogs **xvi** and **xvii** displayed a high affinity for the rat sst$_2$ ($IC_{50} = 0.27 \pm 0.03$ nM and $IC_{50} = 0.28 \pm 0.04$ nM, respectively). Conversely, the binding affinity for the sst$_2$ deteriorated dramatically in the case of Tic^3 (analog **vi)**, ($IC_{50} = 200 \pm 58$ nM). Double replacement of aromatic residues in positions 1 and 3 by D- and L-Phg, respectively, afforded analog **vii** with a slightly higher affinity ($IC_{50} = 0.50 \pm 0.03$ nM) than the singly substituted Phg^3 (analog **v)** and a similar affinity to the other singly substituted D-Phg^1 (analog **iii)**.

Table 2 Binding affinities of [Tyr^3]octreotate analogs

Peptide analog	Formula	Binding affinity[a] IC_{50} (nM)
Tate	[Tyr^3]octreotate	0.30 ± 0.03
i	[D-Tyr^1]Tate	2 ± 0.2
ii	[D-Tyr(Et)^1]Tate	0.22 ± 0.01
iii	[D-Phg^1]Tate	0.47 ± 0.03
iv	[Asp^1]Tate	2.70 ± 0.09
v	[Phg^3]Tate	1 ± 0.1
vi	[Tic^3]Tate	200 ± 58
vii	[D-Phg^1,Phg^3]Tate	0.50 ± 0.03
viii	[Lys^8]Tate	4.40 ± 0.76
ix	[Asp^0, Lys^8]Tate	15 ± 1.29
x	[Asp^8]Tate	0.51 ± 0.09
xi	[Asn^0]Tate	0.28 ± 0.05
xii	[Ser^9]Tate	1.16 ± 0.05
xiii	[Asp^0, Asp^8]Tate	6.46 ± 0.73
xiv	[Asn^0, D-Tyr(Et)^1]Tate	0.55 ± 0.04
xv	[Asn^0, D-Phg^1, Phg^3]Tate	1.36 ± 0.16
xvi	[BzThi^3]Tate	0.27 ± 0.03
xvii	[Nal(1)^3]Tate	0.28 ± 0.04

[a] IC_{50} values of [Tyr^3]octreotate analogs, as determined by competition binding assays in rat sst$_2$-positive AR4-2J cell membranes using [^{125}I – Tyr^3]octreotide as the radioligand; values are the mean ±SEM and are expressed in nM.

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Modifications at the *C*-terminal involved substitution of Thr^8 by either Asp^8, introducing an extra negative charge, or by Lys^8, introducing an alkyl chain with a positive charge at the *C*-terminus While the Asp^8 (analog **x)** displayed conserved affinity for the sst_2 ($IC_{50} = 0.51 \pm 0.09$ nM), the Lys^8 (analog **viii)** showed significantly reduced sst_2-binding capacity with an approximately 15-fold higher IC_{50} value. Concomitant *N*-terminal elongation by a negatively charged Asp^0-residue afforded the corresponding analogs **xiii** and **ix**, both exhibiting significantly lower affinity for the sst_2 ($IC_{50} = 6.46 \pm 0.73$ nM and $IC_{50} = 15 \pm 1.29$ nM, respectively). On the other hand, *C*-terminal elongation of the Tate sequence by a polar but noncharged residue (Ser) in analog **xii** slightly deteriorated the affinity ($IC_{50} = 1.16 \pm 0.05$ nM).

N-terminal elongation of Tate by the polar and not charged Asn^0 in analog **xi** had no impact on binding affinity ($IC_{50} = 0.28 \pm 0.05$ nM). Conversely, the same Asn^0-elongation carried out in analogs **ii** and **vii** provided analogs **xiv** and **xv**, respectively, with reduced affinities in comparison with their respective parent peptides ($IC_{50} = 0.55 \pm 0.04$ nM and $IC_{50} = 1.36 \pm 0.16$ nM, respectively).

DISCUSSION

We have synthesized 15 new structurally modified [Tyr^3]octreotate analogs and evaluated their binding affinity to sst_2-positive rat acinar pancreatic AR4-2J cell membranes.

Our findings showed that the existence of a free phenolic hydroxylic group in position 1 seems to be a negative factor for the peptide's ability to bind sst_2. When the phenolic hydroxylic group was protected with an alkyl chain as ether the binding affinity was improved (compared to values for **i** and **ii,** respectively). Substitution of the D-Phe^1 by a residue with a shorter and compact side-chain (D-Phg) decreased the affinity slightly (analog **iii**). Additional modification of analog **iii** by the introduction of Phg in position 3 (analog **vii**) led to almost no change in the binding profile of the resultant molecule. On the other hand, replacement of the aromatic D-Phe^1 by an aliphatic and polar negatively charged residue, such as L-Asp, deteriorated the binding affinity of the corresponding analog (**iv).** Our findings led to the suggestion that the D-configuration and hydrophobicity of the amino acid in position 1 are of primary importance for the sst_2 binding affinity of the analogs.

From NMR and X-Ray experiments [28–31] it is known that a hydrogen bond between the C=O of the residue in position 3 and the NH of the residue in position 3 stabilizes the β-turn. Replacement of Tyr^3 by Tic^3 (an interesting conformational restricted and bulky residue known to enhance the β-turn character in the peptide sequence where is being inserted) caused a sharp decrease in the affinity (analog **vi**), while analog **v,** with Phg in position 3, exhibited slightly lower affinity. It is notable that the analogs **xvi** and **xvii**, with BzThi and Nal(1) respectively, in the same position exhibited comparable affinity as Tate. These results further confirm the fact that the orientation of the β-turn at position 3 of the analogs is crucial for receptor binding and reveal the significance of one methylene group (β-carbon) at the side chain not participating in a ring (phenyl in the case of Phg or isoquinolinic in the case of Tic). Whether the omission of this critical methylene group leads to restriction of conformational freedom or just imposes steric hindrance due to the vicinity of the bulky residue is not clear at this stage and warrants further investigation employing NMR methodology.

Substitution of Thr^8 (*C*-terminal) by a negatively charged residue (Asp) did not affect the ability of the analog to bind to the sst_2 with a high affinity. On the other hand, the introduction of a positively charged residue in combination with a long aliphatic chain in the same position seems to be the restraining factors for receptor binding to the receptor. Furthermore, addition of Ser^9 beyond Thr^8 at the *C*-terminal part provoked a slight decrease in the binding affinity of the resultant analog **xii**. Therefore, structural modifications of the side-chain moieties in the *C*-terminal part of the parent [Tyr^3]octreotate influence interactions of the analogs with the receptor.

Enlargement of the peptidic chain to the amino terminal with Asp (negatively charged residue) in combination with a positive or a negative charge in position 8 (analogs **ix** and **xiii**) caused a significant decrease of binding affinity. On the other hand, insertion of the neutral Asn at the *N*-terminal part of the [Tyr^3]octreotate improved the binding affinity profile. In contrast, multiple structural modifications on [Tyr^3]octreotate's chain in combination with insertion of Asn at the *N*-terminal part slightly reduced the binding affinity. These results seem to support the idea that the proper topological arrangement of the binding elements at the *N*-terminal part of the analogs make the molecules more or less 'visible' to the receptor and demonstrate the importance of neutral and lipophilic character at the *N*-terminal part of the analogs.

In conclusion, our present study provides new information on structure-activity relationships and new evidence for the design of [Tyr^3]octreotate analogs regarding sst_2 binding profile. As noted above, of all 15 new analogs, analogs **ii** and **xi** are the most promising lead structure(s) as they show a high affinity binding for the sst_2.

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