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Novel sst_2-selective somatostatin agonists. Three-dimensional consensus structure by NMR

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Abstract

The three-dimensional NMR structures of six octapeptide agonist analogues of somatostatin (SRIF) in the free form are described. These analogues, with the basic sequence H-DPhe/Phe^2-c[Cys^3-Xxx^7-DTrp^8-Lys^9-Thr^{10}-Cys^{14}]-Thr-NH_2 (the numbering refers to the position in native SRIF), with Xxx^7 being Ala/Aph, exhibit potent and highly selective binding to human SRIF type 2 (sst_2) receptors. The backbone of these sst_2-selective analogues have the usual type-II' β-turn reported in the literature for $sst_{2/3/5}$-subtype-selective analogues. Correlating biological results and NMR studies led to the identification of the side chains of DPhe^2, DTrp^8 and Lys^9 as the necessary components of the sst_2 pharmacophore. This is the first study to show that the aromatic ring at position 7 (Phe^7) is not critical for sst_2 binding and that it plays an important role in sst_3 and sst_5 binding. This pharmacophore is therefore different from that proposed by others for $sst_{2/3/5}$ analogues.

Introduction

Somatostatin (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), a cyclic tetradecapeptide, isolated from the hypothalamus as a growth hormone inhibitor, is now known to be a multifunctional peptide located in most of the brain regions and in peripheral organs.[1,2] Cells containing SRIF are typically neurons or endocrine-like cells which are found in high density throughout the central and peripheral nervous systems, in the endocrine pancreas, and in the gut and in small numbers in the thyroid, adrenals, submandibular glands, kidneys, prostrate and placenta.[1,2] The activities of SRIF are mediated through a family of five different high affinity membrane receptors, sst_1-sst_5 (sst_s). Due to its broad spectrum of physiological activities and its short duration of action due to rapid proteolytic degradation *in vivo*,[3] SRIF continues to be a target for the development of subtype-specific analogues.[4-7] (and references therein)

Indeed, over the last three decades, hundreds of SRIF analogues have been reported and tested in different biological systems including affinity and selectivity towards the five receptor-subtypes.[8-10] Correspondingly, early structure-activity relationship (SAR) studies ruled out

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Abbreviations: The abbreviations for the common amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (Eur. J. Biochem. 1984, 138:9−37). The symbols represent the L-isomer except when indicated otherwise.

the specific involvement of the side chains of all residues but Phe^7-DTrp^8-Lys^9 for biological recognition.[11-13] (**Note**: the numbering of residues follows that of native SRIF.) Extensive structural studies including NMR and x-ray diffraction[14] were carried out to elucidate the pharmacophore and the consensus structural motif of analogues binding predominantly to $sst_{2/5}$ (and sst_3) receptors. On the basis of these 3D structures of the free peptides, a pharmacophore model was proposed for SRIF analogues binding to $sst_{2/5}$ (and sst_3).[15-18] In this model, the side chains and the relative spatial arrangement of DPhe^2, Phe^7, DTrp^8 and Lys^9 constitute the most essential elements necessary for binding (Figure 3C). The side chain of DTrp^8 is in close proximity to the side chain of Lys^9 (~5 Å), whereas the side chain of Phe^7 is about 7 − 9 Å away from the side chain of DTrp^8 and 9 − 11 Å from the side chain of Lys^9. The aromatic side chain at position 2 outside the disulfide bond was not conserved in its position in most of these analogues. The distance between DPhe^2 and DTrp^8, Lys^9, Phe^7 were 11−15 Å, 12−15 Å, 5−11 Å respectively.[19] In this model of the pharmacophore, the rotamer of the side chains of DTrp^8 and Phe^7 are in the *trans* configuration and that of Lys^9 is in the *gauche* configuration.

Recently, we have proposed models of the sst_4 and the sst_1 pharmacophores derived from the NMR consensus structures of several receptor selective analogues.[20,21] These models of the pharmacophores are different from that of the reported $sst_{2/3/5}$ selective pharmacophore in the following manner. The sst_4 pharmacophore has one aromatic side chain, either Phe^6 or Phe^{11} involved in binding in addition to the DTrp^8 and Lys^9 pair. The distance between DTrp^8 and Lys^9 is 4.5−6.5 Å, between DTrp^8 and Phe$^{6/11}$ is 5.5−9.5 Å and between Lys^9 and Phe$^{6/11}$ is 4.5−6.5 Å. The aromatic side chain of Phe$^{6/11}$ is closer to the side chain of Lys^9 than that of DTrp^8 (Figure 3E). On the other hand, the sst_1 pharmacophore has two aromatic side chains, at position 6 or 7 and 11, involved in binding in addition to the DTrp^8 and IAmp^9 pair. The positioning of the two aromatic side chains at the back of the peptide plane has to be noted, which is opposite to that of the sst_4 and $sst_{2/3/5}$ pharmacophores. The sst_1 receptor selectivity is mainly achieved by the amino acid IAmp, which replaces Lys at position 9. The distance between DTrp^8 and IAmp^9 is 7−8 Å, between DTrp^8 and Phe$^{6/7}$ is 6−7.5 Å and between IAmp^9 and Phe^{11} is 8−10 Å (Figure 3D).

In a similar manner, we present a model of the sst_2 pharmacophore based on the 3D NMR structures of the free peptides. The three-dimensional structures of six analogues in DMSO-d_6 are presented including the structure of octreotide amide. These analogues have the typical octreotide scaffold with a type-II' β-turn backbone conformation similar to that of the reported $sst_{2/3/5}$ selective analogues, yet their pharmacophore has one aromatic side chain (Phe^7) less than that of the reported $sst_{2/3/5}$ selective analogues.

Results

Peptide synthesis and characterization are described in Table 1 and experimental section. The proton resonance assignment and the structure determination by NMR for each of the sst_2-selective analogues **1** − **6** (Table 1) are presented in Tables 2 and 3.

Assignment of proton resonances, collection of structural restraints, and structure determination

The nearly complete chemical shift assignment of proton resonances (Table 2) for analogues **1** − **6** (Table 1) has been carried out using two-dimensional (2D) NMR experiments applying the standard procedure described in the Experimental Section. The N-terminal amino protons for analogues **5** and **6** were not observed due to fast exchange with the solvent. The amide proton of DPhe^2/Phe for analogues **2** and **3** were observed at high field due to the carbamoylation of the N-terminus. Chemical shifts can provide insight into the structure of the peptides, which is particularly true for SRIF analogues. The ring current of the indole ring leads

to a distinct shift of the C^{γ} protons of the sequential Lys side chain to lower frequencies when these groups are closer in space, as in a β-turn. The downfield shift of C^{γ} protons has been observed in all of these sst_2-selective analogues, which is similar to the observations found in the non-selective analogues binding to $sst_{2/3/5}$.[17,22-30] This indicates the presence of a β-turn around these residues in these analogues.

A large number of experimental NOEs is observed for all of the six analogues in the NOESY spectrum measured with a mixing time of 100 ms, leading to over 100 to 120 meaningful distance restraints per analogue and concomitantly ~15 restraints per residue, which is a typical number for folded proteins (Table 3). These structural restraints are used as input for the structure calculation with the program CYANA[31] followed by restrained energy minimization using the program DISCOVER.[32] The resulting bundle of 20 conformers per analogue represents the 3D structure of each analogue in solution. For each analogue, the small residual constraint violations in the distances for the 20 refined conformers (Table 3) and the coincidence of the experimental NOEs and short interatomic distances (data not shown) indicate that the input data represent a self-consistent set, and that the restraints are well satisfied in the calculated conformers (Table 3). The deviations from ideal geometry are minimal, and similar energy values are obtained for all of the 20 conformers for each analogue. The quality of the structures determined is reflected by the small backbone RMSD values relative to the mean coordinates of ~0.5 Å (see Table 3 and Figure 2).

Three-dimensional structure of H-DPhe^2- c[Cys^3-Ala^7-DTrp^8-Lys^9-Thr^{10}-Cys^{14}]-Thr-NH$_2$(1)

Analogue **1** binds to receptor 2 with high affinity (IC_{50} < 10 nM) and has an Ala at position 7. The quality of the structure is reflected by the small RMSD (Table 3), which can also be visually depicted from Figure 2 showing the bundle of 20 conformers representing the 3D structure. From the backbone torsion angles (Table 4), it can be seen that the backbone contains a β-turn of type-II' around DTrp^8-Lys^9. The β-turn is supported by the strong sequential $d_{\alpha N}(i,i+1)$ NOEs and medium-range $d_{\alpha N}(i,i+2)$ NOE (Figure 1) between DTrp^8 and Thr^{10}, as well as the hydrogen bond between Thr^{10}NH-O'Ala^7 in all the 20 structures. The low temperature coefficient observed for the amide proton of Thr^{10} (−1.1 ppb/K) confirms the presence of the hydrogen bond.[33] From the torsion angles listed in Table 4, it can be seen that the side chain of DTrp^8 is in the *trans* rotamer, Lys^9 is in the *gauche*$^+$ rotamer and that of DPhe^2 is in the *gauche*$^-$ rotamer.

Three-dimensional structure of H$_2$N-CO-DPhe^2-c[Cys^3-Aph(CONH$_2$)^7-DTrp^8-Lys^9-Thr^{10}-Cys^{14}]-Thr-NH$_2$ (2)

Analogue **2** differs from analogue **1** at position 7 by the Aph($CONH_2$) group as well as the N-terminal carbamoylation (Table 1). The backbone torsion angles (Table 4) indicate a β-turn of type-II' conformation around DTrp^8-Lys^9, which is supported by the medium range $d_{\alpha N}(i,i+2)$ NOE between DTrp^8-Thr^{10} (Figure 1). The turn structure is further stabilized by the observed hydrogen bond between Thr^{10}NH-O'Aph^7 in most of the structures. The presence of the hydrogen bond is further confirmed by the unshifted amide proton resonance of Thr^{10} when the temperature was raised from 298K to 313K. The long range medium NOE observed between DPhe^2(NH) and Thr^{10}(HN) as well as the strong αH NOE observed between the cysteins stabilize the structure. The side chain of DPhe^2 is in the *gauche*$^+$ rotamer, Aph^7 and DTrp^8 are in the *trans* rotamer and that of Lys^9 is in the *gauche*$^-$ rotamer (Table 4).

Three-dimensional structure of H$_2$N-CO-Phe^2-c[Cys^3-Aph(CONH$_2$)^7-DTrp^8-Lys^9-Thr^{10}-Cys^{14}]-Thr-NH$_2$(3)

Analogue **3** binds selectively with high affinity to the sst_2 receptor and is different from analogue **2**, with a Phe at position 2. From the backbone torsion angles (Table 4), it can be seen that the backbone contains a β-turn of type-II' around DTrp^8-Lys^9. The β-turn is supported by

the medium-range $d_{\alpha N}(i,i+2)$ NOE and the weak $d_{NN}(i,i+2)$ NOE (Figure 1) between DTrp[8] and Thr[10], as well as the hydrogen bond Thr[10]NH-O'Aph[7] observed in most of the 20 structures. The low temperature coefficient observed for the amide proton of Thr[10] (−0.4 ppb/K) confirms the presence of the hydrogen bond.[33] In addition long range medium NOEs are observed between the amide protons of Phe[2] and Thr[10] and the cysteins, which stabilizes the structure. The side chains of Phe[2], Aph[7] and Lys[9] are in the *gauche*$^+$ rotamer and that of DTrp[8] is in the *trans* rotamer (Table 4).

Three-dimensional structure of H_2N-CO-DPhe[2]-c[Cys[3]-Aph(CONHOCH$_3$)[7]-DTrp[8]-Lys[9]-Thr[10]-Cys[14]]-Thr-NH_2(4)

Analogue **4** binds selectively with nanomolar affinity to the sst_2 receptor. It differs from analogues **2** and **3** by the longer chain of Aph($CONHOCH_3$) at position 7, which inhibits the binding to receptors 3 and 5 completely (Table 1). The backbone torsion angles indicate a β-turn of type-II' conformation around DTrp[8]-Lys[9] (Table 4), which is supported by the medium-range $d_{\alpha N}(i,i+2)$ NOE and the weak $d_{NN}(i,i+2)$ NOE observed between DTrp[8] and Thr[10] (Figure 1). The unshifted amide proton resonance of Thr[10] at 7.63 ppm (from 298K to 313K) suggests the presence of a hydrogen bond involving this amide proton, which has been observed in all the 20 structures. The side chain of DPhe[2] is in the *gauche*$^+$ rotamer, Aph[7] and DTrp[8] are in the *trans* rotamer and that of Lys[9] is in the *gauche*$^-$ rotamer (Table 4). The other long range NOEs, which stabilize the structure, are observed between the amide proton of DPhe[2] and αH proton of Cys[14] and the αH protons of the cysteins.

Three-dimensional structure of H-c[Cys[3]-Phe[7]-DTrp[8]-Lys[9]-Thr[10]-Cys[14]]-OH (5)

Analogue **5** is the shortest analogue, a cyclic hexapeptide containing only the core residues. Analogue **5** was synthesized to identify the role of $_D$Phe[2] in sst_2 binding. Indeed, it did not bind to all the five receptors (Table 1). From the backbone torsion angles it can be seen that it has a type-II' β-turn around DTrp[8] and Lys[9](Table 4) and the turn is supported by the presence of the medium range $d_{\alpha N}(i,i+2)$ NOE observed between DTrp[8] and Thr[10] (Figure 1) as well as the hydrogen bond observed between Thr[10]NH-O'Phe[7] in all of the 20 structures. The low temperature coefficient observed for the amide proton of Thr[10] (−1.4 ppb/K) confirms the presence of the hydrogen bond.[33] The side chain of Phe[7] and DTrp[8] are in the *trans* rotamer and that of Lys[9] is in the *gauche*$^+$ rotamer (Table 4).

Three-dimensional structure of H-DPhe[2]-c[Cys[3]-Phe[7]-DTrp[8]-Lys[9]-Thr[10]-Cys[14]]-Thr-NH_2 (6)

Analogue **6**, which is very similar to octreotide (Thr-ol is substituted by Thr-NH_2), binds to the $sst_{2/3/5}$ receptors with moderately high affinity (Table 1). The 3D structure of this analogue was obtained in order to compare the structure of octreotide with the other analogues under identical conditions. Melacini et al. reported that the backbone of Sandostatin (octreotide) could be in a conformational equilibrium between β-turn and helical structures in solvents different from DMSO.[19,28] From the torsion angles, we observe a type-II' β-turn conformation for the backbone, which is supported by the presence of the medium range $d_{\alpha N}(i,i+2)$ NOE observed between DTrp[8] and Thr[10] (Figure 1) as well as the hydrogen bond observed between Thr[10]NH-O'Phe[7] in all of the 20 structures. The unshifted amide proton resonance of Thr[10] at 7.58 ppm (from 298K to 313K) confirms that this amide proton is involved in a hydrogen bond. The side chain of Phe[7] and DTrp[8] are in the *trans* rotamer and that of Lys[9] is in the *gauche*$^+$ rotamer (Table 4).

Discussion

We have recently proposed models of the sst_1 and sst_4 pharmacophores based on the 3D NMR structures of several receptor selective analogues. The sst_1 and sst_4 receptors have maximum sequence similarity and hence were assumed to be part of one close family, different from the

family of $sst_{2/3/5}$ receptors. Yet, the proposed sst_1 and sst_4 pharmacophores are completely distinct from each other. For example, the position and the number of aromatic rings involved in binding are different for the two ligands, suggesting that these hydrophobic residues interact with different regions of the sst_1 and sst_4 receptors, respectively (Figure 3D and 3E). On the other hand, most of the previously published analogues binding with high affinity to sst_2 receptor were also binding to sst_5 receptors with nM affinity and sometimes to sst_3 receptors as well. Melacini et al., proposed a pharmacophore model for these analogues binding non-selectively to all of the three receptors (Figure 3C).[19] Here, we have identified the crucial residues involved in selective sst_2 binding, revealing subtle differences in the pharmacophores between the only sst_2-selective analogues and the less selective $sst_{2/3/5}$-selective analogues. In the $sst_{2/3/5}$-selective pharmacophore found in octreotide and homologues, we identified two aromatic side chains (at position 2 and 7) in addition to the $DTrp^8$ and Lys^9 residues. Removal of the aromatic amino acid at position 2 (analogue **5**) resulted in loss of binding to all of the three receptors (compare with analogue **6**). This clearly confirms that residue 2 is important for binding to $sst_{2/3/5}$ receptors (Table 1). On the other hand, replacement of Phe at position 7 by Ala (analogue **1**) resulted in selective binding (nM) to receptor 2. Hence, it can be concluded that this aromatic ring is not crucial for sst_2 binding, and is crucial for sst_3 and sst_5 binding, as the binding to receptors 3 and 5 decreased (Table 1). Replacement of Phe at position 7 by an amino acid having a longer side chain, such as Aph has also decreased the binding to sst_3 and sst_5 receptors, retaining high binding affinity to sst_2 receptors.

Three-dimensional structures of the sst_2-selective analogues and the pharmacophore model

Most of the bioactive analogues of SRIF reported so far have a β-turn type either of type II' or type VI for the backbone conformation.[13,15,17,18,24,34-39] The structures of all of the presented four sst_2-selective analogues have also a β-turn of type-II' (Figure 2, Table 4). In these analogues, the side chain of $DTrp^8$ is in the *trans* conformer and the side chain of Lys^9 is either in the *gauche*$^+$ or *gauche*$^-$ conformer, bringing the two side chains adjacent to each other in a close proximity. The side chain of Phe or DPhe at position 2 is either in the *gauche*$^+$ or *gauche*$^-$conformer, far from the DTrp-Lys pair and their position is conserved in all of the four analogues studied here (Figure 3A). Hence, we propose a pharmacaphore model for sst_2-selective analogues involving the three side chains, namely, the indole (DTrp) at position 8, the amino-alkyl (Lys) at position 9 and an aromatic ring at position 2 (Figure 3B). In addition, the right spatial arrangement of the indole ring, the lysine side chain and the aromatic ring of a phenylalanine is also important for sst_2-selective binding. The proposed distances between the Cγ of residue 8 and Cγ of Phe^2 is 12−13.5 Å; Cγ of residue 8 and Cγ of Lys^9 is 4−5 Å and Cγ of Phe^2 and Cγ of Lys^9 is 12.5−15 Å. Conservative replacements of these residues do not change the binding affinities and receptor selectivity evidently.

Comparison of the sst_2-selective versus $sst_{2/3/5}$-selective pharmacophore

With the consensus structural motif for the sst_2-selective SRIF analogues (Figures 3A and B), comparison between the sst_2 and the pharmacophore for $sst_{2/3/5}$-selective analogues (Figure 3C) is possible. Interestingly, Goodman and coworkers illustrated that in SRIF analogues, which bind selectively to $sst_{2\ 2/3/5}$, the side chains of $DTrp^8$, Lys^9, Phe^7 and Phe^2 constitute the most essential elements necessary for binding.[15,16] In their pharmacophore model, $DTrp^8$ and Lys^9 were at a close proximity of ~5 Å, which is similar to the sst_2 pharmacophore. The aromatic ring at position 7, Phe^7 is farther away from $DTrp^8$ (7 to 9 Å) and Lys^9 (9 to 11 Å), and is not present in the sst_2 pharmacophore. Hence Phe^7 is not crucial for sst_2 receptor binding. The position of $DPhe^2$ in the sst_2 pharmacophore is highly conserved whereas it varies in the $sst_{2/3/5}$ pharmacophore. This difference can easily be seen from the superposition of the sst_2-selective analogue **1** and the $sst_{2/3/5}$-selective octreotide (Figure 4).[16] They differ mostly in the position of the phenylalanine at position 2. Based on our studies reported here and the observation of Melacini et al.,[19] octreotide undergoes a conformational exchange between β-

turn and helical backbone conformation, it can be proposed that octreotide undergoes conformational change to fit into both the sst_2 and sst_5 pharmacophores. It should prefer the β-turn conformation, where the $DPhe^2$ is close to the sst_2 pharmacophore (Figure 4A) and must prefer the helical conformation to fit the sst_5 pharmacophore (Figure 4B), where $DPhe^2$ is further away from the sst_2 pharmacophore (this hypothesis is further supported by unpublished results of sst_5 selective analogues). This conformational flexibility is therefore necessary to explain the non-selective binding of the octreotide type of analogues to $sst_{2/3/5}$ receptors simultaneously.

Comparison of the sst_2-selective versus sst_1- and sst_4-selective pharmacophores

Since three different pharmacophores are available for the subtype-selective analogues, namely, 1, 2 and 4, the differences among these subtype-selective pharmacophores can be discussed with respect to sst_2 pharmacophore (Figure 3B). The sst_1 pharmacophore has two aromatic side chains important for binding and they are closer to DTrp-IAmp pair and are present on the back side of the peptide, compared to the sst_2 pharmacophore, where one aromatic ring important for binding is far from DTrp-Lys pair. Also IAmp at position 9 crucial for sst_1 selectivity is further away from $DTrp^8$, compared to the other pharmacophores, where Lys at position 9 is closer to $DTrp^8$. The sst_4 pharmacophore is somewhat similar to sst_2 pharmacophore in the number of interacting residues with the receptors, namely $DTrp^8$, Lys^9 and one aromatic side chain either at position 6 or 11. But the position of the aromatic side chain is closer to the DTrp-Lys pair in the sst_4 pharmacophore, whereas it is farther away in the sst_2 pharmacophore. Hence, these subtype-selective pharmacophores explain why the sst_2-selective analogues did not bind to other receptors of SRIF, specifically to 1 and 4.

Conclusions

The 3D conformations of four cyclic SRIF octapeptide analogues with a hexapeptide core having high binding affinity and selectivity to the sst_2 receptor have been presented. These studies indicate that these analogues have a β-turn of type-II' for the backbone conformation which orients the side chains of the essentially important residues, namely indole at position 8, amino alkyl group at position 9 and an aromatic ring outside the cycle, in their respective positions for effective receptor-ligand binding. Based on this, we have proposed the SRIF binding motif for the sst_2 receptor consisting of these three side chains, namely $DTrp^8$, Lys^9 and $DPhe^2$. This binding motif differs from the binding motif for sst_2/sst_3/sst_5-selective receptors by one less aromatic side chain at position 7 (Figure 3). Furthermore, the model proposed also explains the selective binding of the non-peptoid analogues of SRIF agonists.[40-43] The pharmacophore models proposed so far enable us to understand the binding of several other SRIF analogues to somatostatin receptors and will play an important role in designing highly selective peptides as well as non-peptide ligands of SRIF.

Experimental Section

Peptide synthesis, purification and characterization

Starting Materials—4-methylbenzhydrylamine resin (MBHA) with the capacity of 0.4 mmol/g and Boc-Cys(Mob)-CM resin with a capacity of 0.3−0.4 mmol/g were used. All Boc-Nα–protected amino acids were commercially available (Chem Impex, Wood Dale, IL, Reanal Finechemical Co., Budapest, Hungary), except Boc-Aph(Fmoc) which was synthesized in our laboratory. [44]

Synthesis and Purification—Peptides were synthesized by SPPS methodology following the Boc strategy and purified as we published earlier.[45] The -CO-NH_2 (Cbm) ureido group at the 4-amino function of Aph and at the N-terminus of the peptides was introduced

simultaneously on the resin. The 4-amino function of Aph was freed with 20% piperidine in NMP and the N-terminus Boc was deprotected with 50% TFA in DCM after completion of the synthesis, then the carbamoylation was carried out with NaOCN (200 mg, 1.3 mmol) in NMP (8 mL) and glacial acetic acid (6 mL) per gram of initial resin. The ureido group -CO-$NHOCH_3$ (Cbm-OMe) at the 4-amino function of Aph (analogue 4) was also introduced on the resin. The Nα-Boc protected resin-bound hexapeptide Boc-Aph(Fmoc)-DTrp-Lys[Z(2Cl)]-Thr(Bzl)-Cys(Mob)-Thr(Bzl)-MBHA was treated with 20% piperidine in NMP to free the 4-amino function of Aph and CH_3O-NH-$COOC_6H_4$-NO_2 (10 fold excess) in the presence of DIPEA in NMP was added to the resin which was shaken at RT for 4 h as we described earlier.[46] After completion of the synthesis, N-terminus Boc was deprotected with 50% TFA in DCM and the carbamoylation was carried out as described above. The completed peptides were cleaved from the resin by HF containing the scavengers anisole (10% v/v) and methyl sulfide (5% v/v) for 60 min at 0 °C. The diethyl ether precipitated crude peptides were cyclized in 75% acetic acid (200 mL) by addition of iodine (10% solution in methanol) until the appearance of a stable orange color. Forty minutes later, ascorbic acid was added to quench the excess of iodine. The crude, lyophilized peptides were purified by preparative RP-HPLC.[47]

Characterization—The purity of the final peptides was determined by analytical RP-HPLC, CZE analysis.[48] (See legend of Table 1 for details.) Each peptide showed a purity of >95% by these methods. The observed monoisotopic $(M + H)^+$ values of each peptide corresponded with the calculated $(M + H)^+$ values.

Sample preparation and NMR Experiments

Peptides were synthesized on solid-phase with Boc chemistry on a CS-Bio Peptide Synthesizer (Model CS536).[4-6] Anhydrous HF cleaved the products from the resin support with simultaneous side chain deprotection. The diethyl ether precipitated crude peptides were cyclized in 75% acetic acid by the addition of iodine (10% solution in methanol). All peptides were purified by preparative RP-HPLC. The purity of the final products was determined by analytical RP-HPLC and CZE. They were >95% pure by both methods. The products were also characterized by mass spectroscopy; the observed $(M+H)^+$ values coincided with the calculated $(M+H)^+$ values.

NMR samples were prepared by dissolving 2 mg of the analogue in 0.5 mL of DMSO-d_6. The ^{1}H NMR spectra were recorded on a Bruker 700 MHz spectrometer operating at proton frequency of 700 MHz. Chemical shifts were measured using DMSO (δ = 2.49 ppm) as an internal standard. The 1D spectra were also acquired at temperatures between 298 and 318 K and were utilized to measure the temperature coefficients of the amide resonances. All the 2D spectra were acquired at 298 K. Resonance assignments of the various proton resonances have been carried out using total correlation spectroscopy (TOCSY);[49,50] double-quantum filtered spectroscopy (DQF-COSY)[51] and nuclear Overhauser enhancement spectroscopy (NOESY).[52-54] The TOCSY experiments employed the MLEV-17 spin-locking sequence suggested by Davis and Bax,[49] applied for a mixing time of 50 ms. The NOESY experiments were carried out with a mixing time of 100 ms. The TOCSY and NOESY spectra were acquired using 800 complex data points in the ω_1 dimension and 1024 complex data points in the ω_2 dimension with t_{1max} = 47 ms and a t_{2max} = 120 ms and were subsequently zero-filled to 1024 × 2048 before Fourier transformation. The DQF-COSY spectra were acquired with 1024 × 4096 data points and were zero-filled to 2048 × 4096 before Fourier transformation. The TOCSY, DQF-COSY and NOESY spectra were acquired with 8, 8 and 16 scans, respectively, with a relaxation delay of 1 s. The signal from the residual water of the solvent was suppressed using pre-saturation during the relaxation delay and during the mixing time. The TOCSY and NOESY data were multiplied by 75° shifted sine-function in both dimensions. All the spectra were

processed using the software PROSA.[55] The spectra were analyzed using the software X-EASY.[56]

Structure Determination

The chemical shift assignment of the major conformer (the population of the minor conformer was < 10%) was obtained by the standard procedure using DQF-COSY and TOCSY spectra for intra-residual assignment and the NOESY spectrum was used for the sequential assignment.[57] The collection of structural restraints was based on the NOEs and vicinal $^{3}J_{NH\alpha}$ couplings. Dihedral angle constraints were obtained from the $^{3}J_{NH\alpha}$ couplings, which were measured from the 1D ^{1}H NMR spectra and from the intra-residual and sequential NOEs along with the macro GRIDSEARCH in the program CYANA.[31] The calibration of NOE intensities versus ^{1}H-^{1}H distance restraints and appropriate pseudo-atom corrections to the non-stereo specifically assigned methylene, methyl and ring protons were performed using the program CYANA. On an average, approximately 100 NOE constraints and 20 angle constraints were utilized while calculating the conformers (Table 3). A total of 100 conformers were initially generated by CYANA and a bundle containing 20 CYANA conformers with the lowest target function values were utilized for further restrained energy minimization, using the CFF91 force field[58] with the energy criteria fit 0.1 kCal/mol/Å[59] in the program DISCOVER with steepest decent and conjugate gradient algorithms.[60] The resulting energy minimized bundle of 20 conformers was used as a basis for discussing the solution conformation of the different SRIF analogues. The structures were analyzed using the program MOLMOL.[61]

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Additional abbreviations

Amp, 4-aminomethylphenylalanine
Aph, 4-amino-phenylalanine
CYANA, Combined assignment and dynamics algorithm for NMR applications
DMSO, dimethylsulfoxide
DQF-COSY, double quantum filtered correlation spectroscopy
IAmp, 4-(N-Isopropyl)-aminomethyl-phenylalanine
NMR, nuclear magnetic resonance
NOESY, nuclear Overhauser enhancement spectroscopy
3D, three-dimensional
PROSA, Processing algorithms
RMSD, root mean square deviation
SAR, structure activity relationships
SRIF, somatostatin
TOCSY, total correlation spectroscopy.

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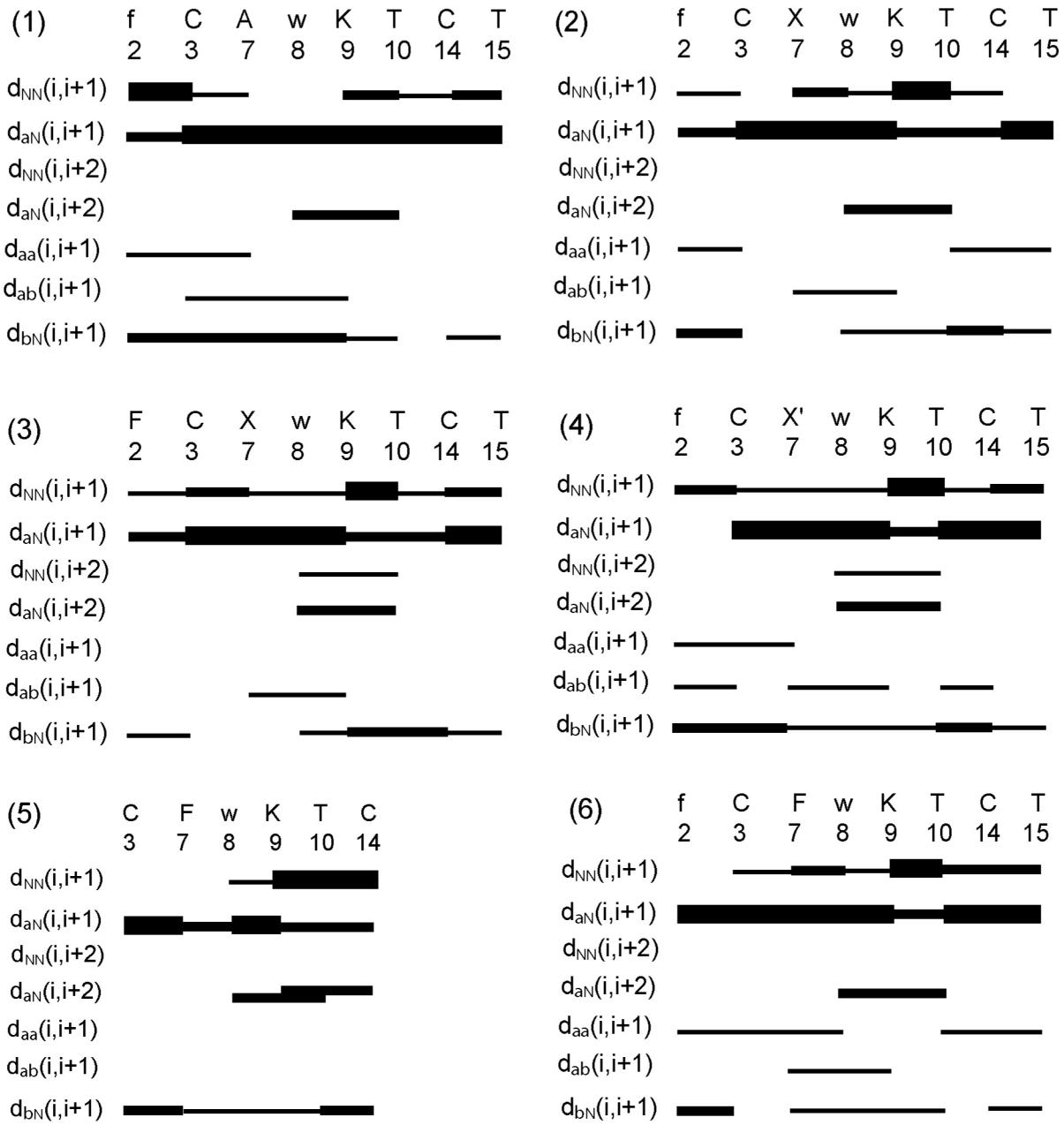

Figure 1.

Survey of characteristic NOEs used in CYANA for structure calculation for analogues **1**-**6**. Thin, medium and thick bars represent weak (4.5 to 6 Å), medium (3 to 4.5 Å) and strong (< 3 Å) NOEs observed in the NOESY spectrum. The medium-range connectivities $d_{NN}(i,i+2)$, $d_{\alpha N}(i,i+2)$, and $d_{\beta N}(i,i+2)$ are shown by lines starting and ending at the positions of the residues related by the NOE. Residues designated with X and X' correspond to the amino acid Aph ($CONH_2$) and Aph($CONHOCH_3$), respectively.

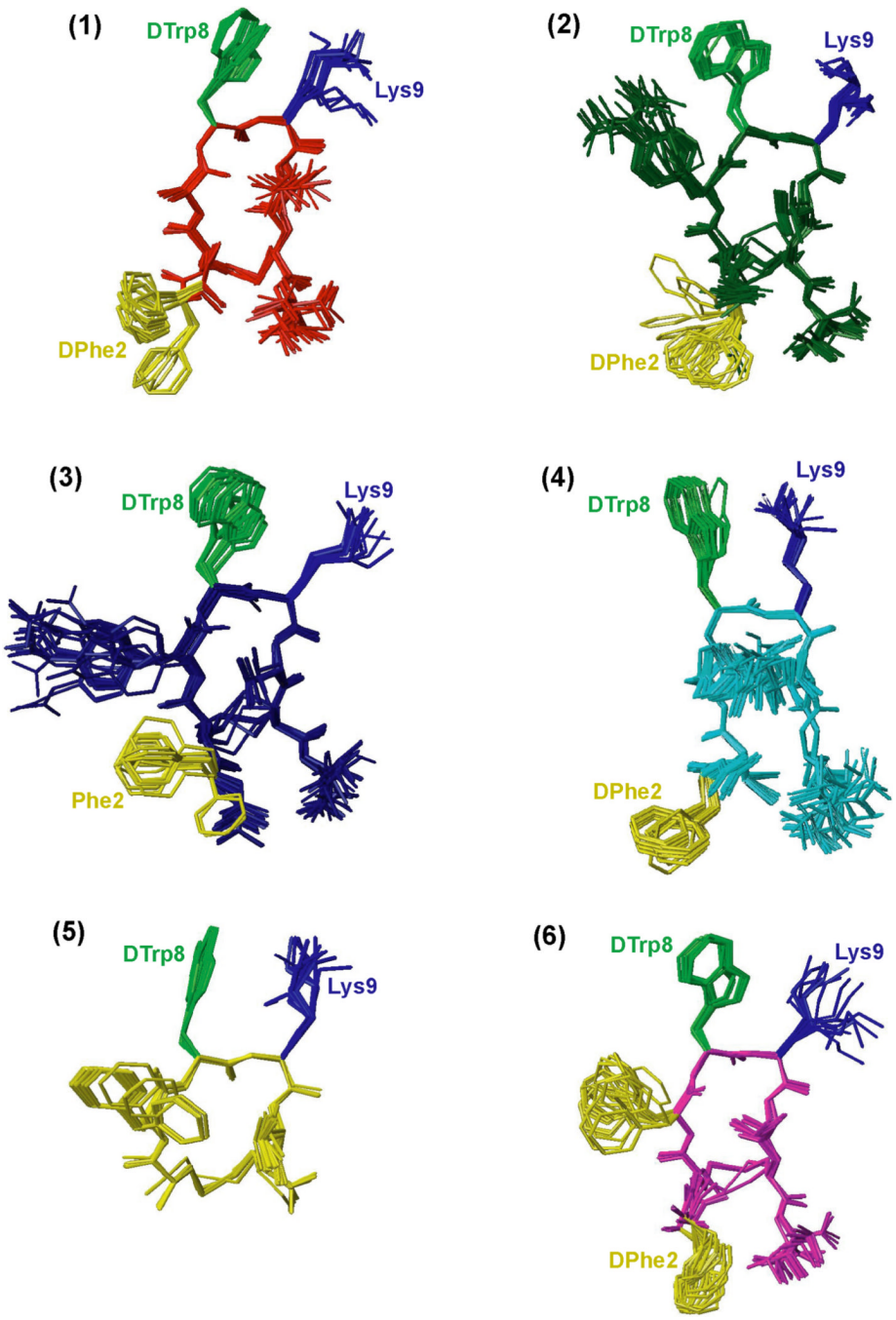

Figure 2.

NMR structures of analogues **1**–**6**. For each analogue, twenty energy-minimized conformers with the lowest target function are used to represent the 3D NMR structure. The bundle is obtained by overlapping the Cα atoms of the residues 2–9. The backbone and the side chains are displayed including the disulfide bridge. The following color code is used: red (**1**) H-DPhe-c[Cys-Ala-DTrp-Lys-Thr-Cys]-Thr-NH_2, dark green (**2**) H_2NCO-DPhe-c[Cys-Aph($CONH_2$)-DTrp-Lys-Thr-Cys]-Thr-NH_2, navy blue (**3**) H_2NCO-Phe-c[Cys-Aph($CONH_2$)-DTrp-Lys-Thr-Cys]-Thr-NH_2, cyan (**4**) H_2NCO-DPhe-c[Cys-Aph($CONHOCH_3$)-DTrp-Lys-Thr-Cys]-Thr-NH_2, yellow (**5**) H-c[Cys-Phe-DTrp-Lys-Thr-Cys]-OH, magenta (**6**) H-DPhe-c[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2. The side chains that are involved in sst_2-binding are

highlighted; these are DTrp at position 8 in light green, Lys at position 9 in blue and DPhe or Phe at position 2 in yellow.

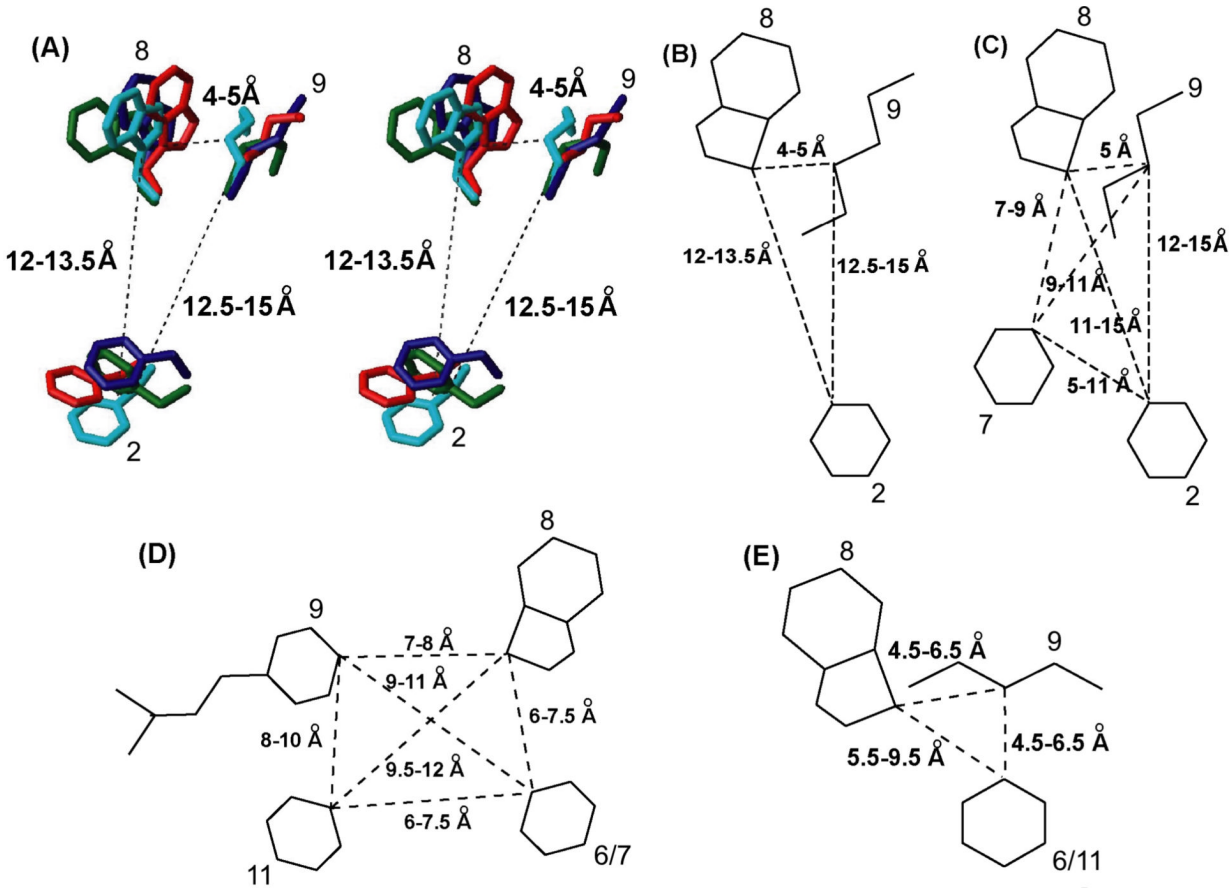

Figure 3.

Consensus structural motif of sst_2-selective SRIF analogues. (A) Stereo view of the consensus structural motif for the sst_2-selective analogues **1** (red), **2** (green), **3** (navy blue) and **4** (cyan). Only the side chains of the residues DPhe[2], DTrp[8] and Lys[9] for **1**, **2**, **3** and **4** are shown. The distances between the Cγ atoms of DTrp[8], Lys[9] and DPhe[2] are displayed. For each analogue, the conformer with the lowest target function is displayed. (B) Schematic drawing of the pharmacophore for the sst_2-selective SRIF analogues. (C) Schematic drawing of the pharmacophore for the $sst_{2/3/5}$-selective SRIF analogues.[16] (D) Schematic drawing of the pharmacophore for the sst_1-selective SRIF analogues.[62] (E) Schematic drawing of the pharmacophore for the sst_4-selective SRIF analogues.[20] In all of the pharmacophores, the distance between the Cγ atoms of the respective residues is displayed.

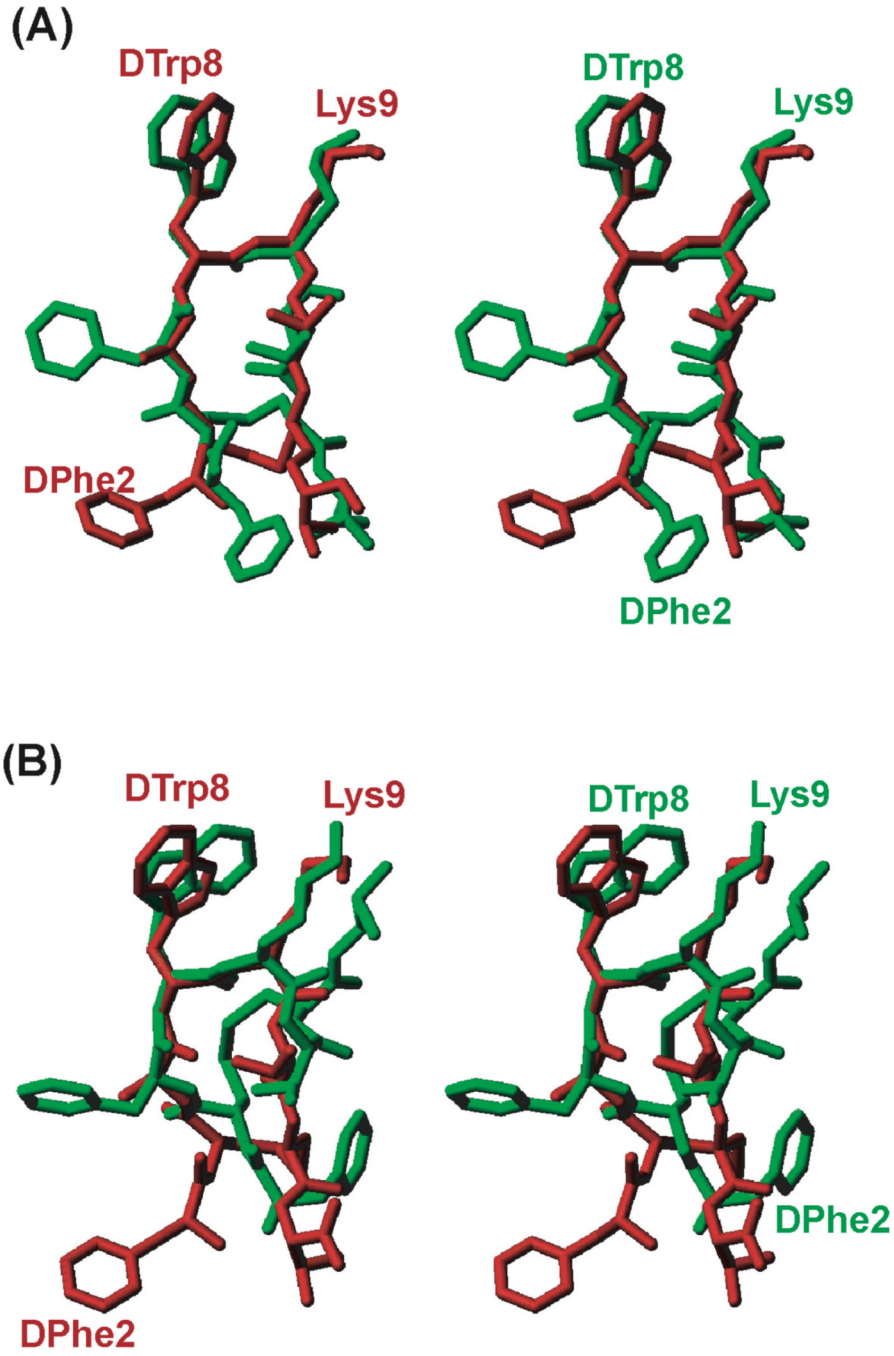

Figure 4.

Comparison of the 3D structure of sst_2-selective (analogue **1**) and $sst_{2/3/5}$-selective (octreotide and analogue **6**) SRIF analogues. Stereo view of the superposition of the 3D structure of the sst_2-selective analogue **1** (red) with the 3D structure of the $sst_{2/3/5}$-selective octreotide (green) [16] (A) in β-turn conformation and (B) in helical conformation. It must be noted that $DPhe^2$ labeled, in both of the analogues is important for selective binding, but differs in its spatial orientation relative to the $DTrp^8$-Lys^9 pair.

Table I

Physico-chemical properties and sst_{1-5} binding affinities (IC_{50}s, nM) of analogues studied by NMR.

ID #	Compound	Purity (%)		MS[c]		IC_{50} nM[d]				
		HPLC[a]	CZE[b]	M (mono) calc.	MH^+(mono) obs.	sst_1	sst_2	sst_3	sst_4	sst_5
1	H-DPhe-c[Cys-Ala-DTrp-Lys-Thr-Cys]-Thr-NH_2	99	99	955.40	956.38	>1K (3)	8.6;4.5;3.6	772;~1472;543	>1K (3)	158;807;226
2	H_2N-CO-DPhe-c[Cys-Aph($CONH_2$)-DTrp-Lys-Thr-Cys]-Thr-NH_2	98	97	1132.45	1133.17	>1K (3)	6.4;9;6.2	210;725;394	>1K; 905;494	400;77;100
3	H_2N-CO-Phe-c[Cys-Aph($CONH_2$)-DTrp-Lys-Thr-Cys]-Thr-NH_2	98	99	1132.46	1133.44	>1K (2)	7.5;20	942;1094	957;872	109;260
4	H_2N-CO-DPhe-c[Cys-Aph($CONHOCH_3$)-DTrp-Lys-Thr-Cys]-Thr-NH_2	99	99	1119.46	1120.42	>1K (2)	3.1;4.4	>1K;958	>1K (2)	808;539
5	H-c[Cys-Phe-DTrp-Lys-Thr-Cys]-OH	97	98	78430	785.27	>1K	378	>1K	>1K	>1K
6	H-DPhe-c[Cys-Phe-DTrp-Lys-Thr-Cys]-Thr-NH_2	95	99	1031.43	1032.00	>1K	2.7;2.2	80;38	>10K	2.7;3.9

[a] Percent purity determined by HPLC (Hewlett-Packard Series II 1090 Liquid Chromatograph) using buffer system: A = TEAP (pH 2.5) and B = 60% CH_3CN/40% A with a gradient slope of 1% B/min, at flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5-μm particle size, 300 Å pore size). Detection at 214 nm.

[b] Capillary zone electrophoresis (CZE) was done using a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 Model 50Z and using a ChromJet integrator. Field strength of 15 kV at 30 °C, mobile phase: 100 mM sodium phosphate (85:15, H_2O:CH_3CN) pH 2.50, on a Supelco P175 capillary (363 μm OD x 75 μm ID X 50 cm length). Detection at 214 nm.

[c] Mass spectra (MALDI-MS) were measured on an ABI-Perseptive DE-STR instrument. The instrument employs a nitrogen laser (337 nm) at a repetition rate of 20 Hz. The applied accelerating voltage was 20 kV. Spectra were recorded in delayed extraction mode (300 ns delay). All spectra were recorded in the positive reflector mode. Spectra were sums of 100 laser shots. Matrix α-cyano-4-hydroxycinnamic acid was prepared as saturated solutions in 0.3% trifluoroacetic acid and 50% acetonitrile. The observed monoisotopic $(M + H)^+$ values of each peptide corresponded with the calculated $(M + H)^+$ values.

[d] The IC_{50} values (nM) were derived from competitive radioligand displacement assays reflect the affinities of the analogues for the cloned somatostatin receptors using the non-selective ^{125}I-[Leu^8,$DTrp^{22}$,Tyr^{25}]SRIF-28, as the radioligand.

Table 2

Proton chemical shifts of analogues 1−6*

Residue	^{1}H	Analogues					
		1	2	3	4	5	6
H_2N-CO			5.42	5.66	7.59		
DPhe/Phe[2]	NH	7.99	6.33	6.42	8.01		
	αH	4.20	4.55	4.53	4.19		4.15
	βH	3.22,2.96	3.08,2.72	3.00,2.80	3.25,2.95		3.24,2.95
	H2,6	7.38	7.28	7.14	7.38		7.38
	H3,5	7.29	7.23	7.21	7.30		7.29
Cys[3]	NH	9.22	8.72	8.59	9.23		9.22
	αH	5.27	5.22	5.16	5.27	4.14	5.28
	βH	2.76,2.82	2.77,2.77	2.81,2.81	2.82,2.82	3.07,2.98	2.81,2.81
		Ala	Aph($CONH_2$)	Aph($CONH_2$)	Aph($CONHOCH_3$)	Phe	Phe
Aph/Ala[7]	NH	8.44	8.49	8.45	8.52	8.36	8.55
	αH	4.47	4.58	4.60	4.65	4.67	4.66
	βCH_3	1.17					
	βH		2.77,2.77	2.81,2.81	2.79,2.79	2.81,2.81	2.84,2.84
	H2,6		6.96	6.99	7.03	7.03	7.08
	H3,5		7.22	7.28	7.44	7.16	7.17
	HT		8.46	8.54	8.84		
	NH_2		5.86	5.92	9.55		
	CH_3				3.62		
DTrp[8]	NH	8.92	8.82	8.80	8.81	8.74	8.77
	αH	4.30	4.20	4.19	4.19	4.58	4.19
	βH	3.07,2.99	2.98,2.73	2.98,2.72	2.98,2.73	3.14,2.86	2.96,2.71
	HD1	7.19	6.93	6.91	6.97	6.99	6.95
	HE1	10.87	10.76	10.77	10.74	10.80	10.81
	HZ2	7.32	7.29	7.30	7.29	7.32	7.32
	HH2	7.06	7.04	7.06	7.04	7.08	7.06
	HZ3	6.99	6.98	7.00	6.99	7.00	6.99
	HE3	7.54	7.42	7.41	7.41	7.47	7.43
Lys[9]	NH	8.43	8.36	8.39	8.40	8.50	8.46
	αH	3.99	4.01	4.00	3.97	3.89	3.99
	βH	1.70,1.25	1.72,1.27	1.73,1.29	1.70,1.26	1.69,1.37	1.72,1.30
	γH	0.72,0.72	0.77,0.74	0.80,0.76	0.76,0.72	0.86,0.86	0.79,0.79
	δH	1.29,1.29	1.30,1.30	1.32,1.32	1.32,1.32	1.35,1.35	1.35,1.35
	εH	2.55,2.55	2.56,2.56	2.58,2.58	2.55,2.55	2.60,2.60	2.57,2.57
	εNH	7.60	7.56	7.62	7.58	7.63	7.62

Residue	^{1}H	Analogues					
		1	2	3	4	5	6
Thr[10]	NH	7.68	7.58	7.58	7.63	7.72	7.62
	αH	4.48	4.49	4.51	4.48	4.29	4.50
	βH	3.99	3.97	3.97	4.02	4.18	4.00
	γH	1.05	1.06	1.06	1.08	1.09	1.07
	OH	4.88	4.80	4.83	4.87		
Cys[14]	NH	8.42	8.46	8.47	8.49	8.08	8.49
	αH	5.15	5.30	5.21	5.15	4.58	5.17

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Residue	^{1}H	Analogues					
		1	**2**	**3**	**4**	**5**	**6**
	βH	2.86,2.86	2.85,2.85	2.84,2.84	2.86,2.86	3.14,2.86	2.86,2.86
Thr^{15}	NH	8.06	8.17	8.13	8.05		8.07
	αH	4.22	4.21	4.23	4.23		4.21
	βH	4.02	4.06	3.93	4.03		4.03
	γH	1.05	1.07	0.99	1.07		1.06
	OH	5.15	5.13	5.07	5.15		
NH_2		7.53,7.36	7.42,7.26	7.43,7.35	7.53,7.36		7.35,7.52

* The chemical shifts were measured at 298K in DMSO in ppm with the internal reference of the DMSO signal at 2.49 ppm.

Table 3

Characterization of the NMR structures of analogues 1−6*

ID#	NOE distance restraints	Angle restraints***	CYANA Target function**	Backbone RMSD (Å)	Overall RMSD (Å)	CFF91 energies (Kcal/mol)			Residual restraint violations on			
						Total energy	Van der Waals	Electrostatic	Distances		Dihedral Angles	
									No. ≥ 0.1 Å	Max (Å)	No. ≥ 1.5 deg	Max (deg)
1	131	24	0.005	0.52 ± 0.09	1.03 ± 0.20	178 ± 7	89 ± 4	89 ± 7	0.7 ± 0.1	0.11 ± 0.01	0 ± 0	0 ± 0
2	135	17	0.001	0.44 ± 0.24	0.96 ± 0.30	211 ± 10	118 ± 5	93 ± 8	0.2 ± 0.1	0.05 ± 0.01	0 ± 0	0 ± 0
3	130	25	0.003	0.47 ± 0.28	1.07 ± 0.45	204 ± 14	124 ± 11	81 ± 7	0.3 ± 0.1	0.07 ± 0.03	0 ± 0	0 ± 0
4	130	19	0.008	0.73 ± 0.25	1.21 ± 0.31	188 ± 11	116 ± 7	72 ± 6	0.5 ± 0.1	0.10 ± 0.02	0 ± 0	0 ± 0
5	80	14	0.0002	0.08 ± 0.09	0.62 ± 0.19	158 ± 10	86 ± 7	73 ± 13	0.1 ± 0.0	0.03 ± 0.00	0 ± 0	0 ± 0
6	115	24	0.002	0.56 ± 0.14	1.06 ± 0.28	210 ± 14	121 ± 10	89 ± 8	0.4 ± 0.1	0.11 ± 0.02	0 ± 0	0 ± 0

* The bundle of 20 conformers with the lowest residual target function was used to represent the NMR structures of each analogue.

** The target function is zero only if all the experimental distance and torsion angle constraints are fulfilled and all non-bonded atom pairs satisfy a check for the absence of steric overlap. The target function is proportional to the sum of the square of the difference between calculated distance and isolated constraint or van der Waals restraints and similarly isolated angular restraints are included in the target function. For the exact definition see reference.[32]

*** Meaningful NOE distance restraints may include intra-residual and sequential NOEs.[32]

Table 4

Torsion angles ϕ, ψ and χ_1 (in degrees) of the bundle of 20 energy minimized conformers.

Analogue	Angle	DPhe[2]	Cys[3]	Ala[7]	DTrp[8]	Lys[9]	Thr[10]	Cys[14]	Thr[15]
1	ϕ	-	−33 ± 60	−156 ± 13	72 ± 3	−91 ± 0	−140 ± 4	−156 ± 16	−11 ± 46
	ψ	67 ± 17	87 ± 6	132 ± 3	−146 ± 2	26 ± 1	−149 ± 23	87 ± 8	121 ± 56
	χ_1	108 ± 6	132 ± 3	170 ± 95	−151 ± 3	−128 ± 2	−22 ± 86	176 ± 3	173 ± 54
2				**Aph(CONH$_2$)[7]**					
	ϕ	121 ± 45	−84 ± 35	−178 ± 19	97 ± 14	−103 ± 7	−74 ± 5	87 ± 2	−19 ± 37
	ψ	172 ± 9	117 ± 16	88 ± 9	−113 ± 14	2 ± 6	−137 ± 8	81 ± 8	110 ± 76
	χ_1	115 ± 32	−121 ± 40	−175 ± 14	150 ± 6	−83 ± 7	−43 ± 7	−143 ± 24	−163 ± 58
3		**Phe[2]**		**Aph(CONH$_2$)[7]**					
	ϕ	−149 ± 9	174 ± 38	179 ± 22	95 ± 22	−103 ± 12	−63 ± 5	83 ± 28	−109 ± 59
	ψ	−145 ± 51	110 ± 11	98 ± 20	−108 ± 7	−8 ± 1	−140 ± 5	78 ± 8	48 ± 87
	χ_1	−132 ± 30	−144 ± 24	−126 ± 18	−171 ± 6	−105 ± 1	−39 ± 2	−110 ± 34	−129 ± 76
4		**DPhe[2]**		**Aph(CONHOCH$_3$)[7]**					
	ϕ	175 ± 6	43 ± 2	−111 ± 8	44 ± 5	−141 ± 4	−143 ± 10	167 ± 71	−83 ± 83
	ψ	41 ± 4	53 ± 5	59 ± 5	−104 ± 3	45 ± 8	165 ± 52	78 ± 14	105 ± 78
	χ_1	98 ± 6	−76 ± 34	−177 ± 5	160 ± 5	−45 ± 8	−52 ± 7	−155 ± 13	118 ± 49
5				**Phe[7]**					
	ϕ		–	−154 ± 8	156 ± 3	−93 ± 3	−93 ± 3	−54 ± 5	
	ψ		168 ± 2	23 ± 6	−128 ± 3	12 ± 0	2 ± 4	–	
	χ_1		−28 ± 3	160 ± 38	166 ± 1	−116 ± 0	−62 ± 8	−156 ± 4	
6				**Phe[7]**					
	ϕ	–	−71 ± 28	−173 ± 11	102 ± 2	−93 ± 3	−104 ± 5	−168 ± 5	−89 ± 64
	ψ	−160 ± 0	−153 ± 66	98 ± 6	−121 ± 0	1 ± 3	45 ± 5	81 ± 3	98 ± 70
	χ_1	32 ± 0	−117 ± 5	−150 ± 29	−168 ± 1	−126 ± 4	140 ± 8	−148 ± 12	175 ± 4