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Three-dimensional consensus structure of sst₂-selective somatostatin (SRIF) antagonists by NMR

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Abstract

The three-dimensional NMR structures of seven octapeptide analogs of somatostatin (SRIF), based on octreotide, with the basic sequence H-Cpa/Phe²-c[DCys³-Xxx⁷-DTrp/Daph(Cbm)⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Yyy-NH₂ (the numbering refers to the position in native SRIF), with Xxx⁷ being Aph(Cbm)/Tyr/Arg(NMe,benzoyl) and Yyy being Nal/DTrp/Thr, are presented here. Most of these analogs exhibit potent and highly selective binding to sst₂ receptors and all of the analogs are antagonists inhibiting receptor signaling. Based on their consensus 3D structure, the pharmacophore of the sst₂-selective antagonist has been defined. The pharmacophore involves the side chains of Cpa², DTrp/Daph(Cbm)⁸ and Lys⁹ with the backbone, for most of sst₂-selective antagonists are comprised of a type-II' β -turn. Hence, the sst₂-selective antagonist pharmacophore is very similar to the sst₂-selective agonist pharmacophore previously described.

Introduction

Somatostatin, a cyclic tetradecapeptide, (SRIF, H-Ala¹-Gly²-c[Cys³-Lys⁴-Asn⁵-Phe⁶-Phe⁷-Trp⁸-Lys⁹-Thr¹⁰-Phe¹¹-Thr¹²-Ser¹³-Cys¹⁴]-OH), inhibits the release of several hormones including growth hormone, glucagon, insulin, secretin and gastrin.^{1,2} SRIF initiates its functions by binding to a family of five different receptors, sst₁-sst₅ (sst_s) with nanomolar (nM) affinity. The important role of SRIF and its short duration of action due to rapid proteolytic degradation *in vivo*³ led to the design of various SRIF analogs which are small and receptor subtype-specific.⁴⁻⁹ Though the mechanism behind the receptor signaling (agonism) is not completely understood, extensive structural studies of these peptide analogs have been reported that identified the role of the residues involved in binding and selectivity towards the five receptors. In particular, structure activity relationship (SAR) studies led to the identification of consensus structural motifs for agonist analogs binding selectively to sst₁, sst₂ and sst₄ receptors.¹⁰⁻¹² Also, a non-selective pharmacophore for agonists binding to sst₂/sst₅ (and partially to sst₃) receptors has been proposed of which octreotide is a member.¹³⁻²² Each of these agonist pharmacophores required a certain number of aromatic side chains, in addition to the DTrp⁸-Lys⁹ pair, at a certain fixed distance, with respect to DTrp⁸ and Lys⁹. For example, in the non-selective sst_{2/5} pharmacophore, there were two aromatic side chains at position 2 and 7, which were suggested to be crucial for receptor

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binding.²³ Our group showed that the aromatic side chain at position 7 is crucial only for sst₃ and sst₅ receptor binding and therefore, we proposed the sst₂-selective pharmacophore is a subset of the non-selective octreotide pharmacophore.¹² Although there is no report of sst₃ or sst₅-selective peptide agonists, sst₃-selective antagonists have been reported.²⁴ The sst₃-selective antagonist analogs have amino-glycine (Agl) at position 8, instead of the usual Trp/DTrp and have been shown to be crucial for sst₃ selectivity as well as inhibiting the signaling. The structure of one of these peptides in water and DMSO shows that two conformations are observed, in the ratio 60:40 for most of the backbone resonances, due to the *cis/trans* conformational change in Agl.^{8,25}; and Grace et al., unpublished results. Non-peptide antagonists binding selectively to each the five somatostatin receptor have also been reported.^{26,27}

It is the aim of the present work to elucidate the pharmacophore for sst₂-selective antagonists. Such antagonists are based on octreotide with an L-aromatic amino acid at position 2 and DCys at position 3.^{28,29} Numerous examples³⁰ suggest that such substitutions (an L-aromatic residue at position 2 and DCys at position 3) are crucial for inhibiting the signaling at the receptors. However, since these antagonists were based on octreotide, they bind to sst_{2/3/5} receptors and hence were non-selective. Based on our knowledge of sst₂-selective agonists that the aromatic side chain at position 7 is critical only for sst_{3/5} receptors, we designed sst₂-selective antagonist analogs.³¹ These antagonists have a longer aromatic side chain at position 7, in addition to DCys at position 3. Here we present the 3D NMR structures of seven potent sst₂-selective antagonists and propose the first sst₂-selective antagonist pharmacophore model. Analogs **1-7** were tested for antagonism using an *in vitro* calcium release assay and an immunohistochemical sst₂ internalization assay as described in detail in Cescato et al.³¹

Results

In this section, we present general details about the chemical shift assignment and the description of the 3D NMR structure for each SRIF antagonists **1 - 7** given in Table 1.

Chemical shift assignment, collection of structural restraints, and structure determination

The three-dimensional structures of the seven antagonists, with Dcys³ have been studied in the solvent DMSO-d₆. Almost nearly complete chemical shift assignments of the proton resonances (Table 2) for analogs **1 - 7** (Table 1) have been carried out using two-dimensional (2D) NMR experiments and the standard procedure described in the Experimental Section. The N-terminal amino protons for analogs **1, 3**, and **4** were not observed in the NMR spectra attributed to fast exchange with the solvent. The amide proton of Cpa/Phe² for analogs **2, 5, 6** and **7** were observed in the NMR spectra since the N-terminal protection by an acetyl group in **2**, DOTA in **5** and by a carbamoyl group in **6** and **7** (Table 2) slows down the exchange of NH protons. The bulky DOTA group at the N-terminus as well as the amide proton resonance of Cpa² in **5** showed very broad resonances. The line broadening could be due to the *cis-trans* isomerism about the amide bond connecting the DOTA group and Cpa².³² The carbamoylation of the N-terminus resulted in high field shifted resonances for the amide protons of Cpa² for **6** and **7**. In octreotide types of analogs, with a β -turn around DTrp⁸ and Lys⁹, the ring current of the indole group leads to a distinct up-field shift for the C γ protons of the sequential Lys side chain because of their close proximity. Up-field shift has been observed for the C γ protons for all of the seven sst₂-selective antagonists (Table 2), similar to the sst₂-selective agonists¹² as well as non-selective agonists binding to sst₂, sst₃ and sst₅ receptors.¹³⁻²² Similar shifts were also observed for the C γ protons of Lys⁹ in **4** and **5**, having DAph(Cbm) at position 8, instead of DTrp⁸ (Table 2).

A large number of experimental NOEs is observed for six of the analogs (except analog **3**) in the NOESY spectrum measured with a mixing time of 100 ms, leading to over 100 meaningful distance restraints per analog and concomitantly ~15 restraints per residue, which is a typical number for folded proteins (Table 3). For analog **3**, two sets of resonances were observed with a ratio of 60:40 indicative of the presence of two conformations. The accompanied overlap of resonances resulted in a reduced number of assigned NOEs, compared to the other analogs with a single set of resonances (Table 3). For all analogs, structural restraints from NOEs were used as input for the structure calculation with the program CYANA33 followed by restrained energy minimization using the program DISCOVER.³⁴ The resulting bundle of 20 conformers per analog represents the 3D solution structure of each analog. For each analog, the small residual violations in the distance constraints only for the 20 refined conformers (Table 3) and the coincidence of 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 were obtained for all 20 conformers of each analog. 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-Cpa²-c[Dcys³-Aph(Cbm)⁷-DTrp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Nal¹⁵-NH₂ (**1**)

Analog **1** binds to receptor 2 with high affinity (IC₅₀ <10 nM) and has moderate affinity for receptors 3 and 5. It differs from octreotide by Cpa², Aph(Cbm)⁷, Nal¹⁵ in addition to DCys at position 3. The 3D NMR structure shows that the backbone has a β-turn of type II' around DTrp⁸ and Lys⁹, similar to octreotide types of analogs (Figure 2, Table S1). The β-turn is supported by the weak d_{NN}(i, i+2) NOE observed between the amide protons of DTrp⁸ and Thr¹⁰. The side chains of Cpa², Lys⁹ are in the *gauche*⁺ configuration, the side chains of Aph(Cbm)⁷, DTrp⁸ are in the *trans* configuration and the side chain of Nal¹⁵ is in the *gauche*⁻ configuration (Table S1).

Three-dimensional structure of Ac-4NO₂Phe²-c[Dcys³-Tyr⁷-DTrp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Dtyr¹⁵-NH₂ (**2**)

Analog **2** differs from analog **1** by 4NO₂Phe², Tyr⁷ and Dtyr¹⁵ as well as the N-terminal acetylation (Table 1) and binds selectively to sst₂ with high affinity. The 3D NMR structure shows that the backbone has a β-turn of type II' around DTrp⁸ and Lys⁹ (Figure 2, Table S1). The turn is supported by the weak d_{NN}(i, i+2) NOE observed between the amide protons of DTrp⁸ and Thr¹⁰ (Figure 1). Furthermore, all of the calculated twenty conformers were stabilized by a hydrogen bond between the amide proton of Tyr⁷ and the carbonyl of Thr¹⁰ and also between the carbonyl of Tyr⁷ and the amide proton of Thr¹⁰. The side chain of 4NO₂Phe² is in the *gauche*⁻ configuration, the side chains of Tyr⁷, Lys⁹ are in the *gauche*⁺ configuration, and the side chains of DTrp⁸, Dtyr¹⁵ are in the *trans* configuration (Table S1).

Three-dimensional structure of H-Cpa²-c[Dcys³-Agl(NMe,Benzoyl)⁷-DTrp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Nal¹⁵-NH₂ (**3**)

Analog **3** binds selectively to the sst₂ receptor with high affinity and is different from analog **1** by Agl(NMe,Benzoyl) at position 7 (Table 1). Two sets of peaks in the ratio of 60:40 were observed in the NMR spectrum of this analog because of the *cis/trans* isomerization of the side chain of Agl(NMe,Benzoyl)⁷. The 3D structure of the major conformer is determined here and is assumed to be the active conformation. Since the number of assigned NOEs is much less compared to the NOEs observed for the other analogs with a single conformation, the 3D structure of analog **3** is poorly defined compared to the structures of the other

analogs (Figure 2, Table 3). Though two conformations were observed, the number of intramolecular NOE's observed for the minor conformer were few and hence were not sufficient to perform the structure calculation. Hence the major conformation was presumed to be bioactive, based on its conformational similarity with that of the other analogs. The 3D structure shows that the backbone has a β -turn of type-II' around DTrp⁸ and Lys⁹ (Table S1), which is supported by the weak sequential $d_{\alpha N}(i, i+2)$ NOE observed between DTrp⁸ and Thr¹⁰ (Figure 1). The side chains of Cpa², Agl(NMe,Benzoyl)⁷ are in the *gauche*⁻ configuration, the side chain of DTrp⁸ is in the *trans* configuration and the side chains of Lys⁹, Nal¹⁵ are in the *gauche*⁺ configuration (Table S1).

Three-dimensional structure of H-Cpa²-c[Dcys³-Tyr⁷-DAph(Cbm)⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Nal¹⁵-NH₂ (4)

Analog **4** binds selectively with nanomolar affinity to the sst₂ receptor and differs from analog **1** by Tyr at position 7, and DAph(Cbm) at position 8 (Table 1). The 3D NMR structure shows that the backbone has a type-II' β -turn around residues DAph(Cbm)⁸ and Lys⁹ (Figure 2, Table S1), which is supported by the weak $d_{\alpha N}(i, i+2)$ NOE observed between DAph(Cbm)⁸ and Thr¹⁰ (Figure 1). The side chains of Cpa², Tyr⁷ and Lys⁹ are in the *gauche*⁺ configuration, the side chain of DAph(Cbm)⁸ is in the *trans* configuration and the side chain of Nal¹⁵ is in the *gauche*⁻ configuration (Table S1).

Three-dimensional structure of DOTA-Cpa²-c[Dcys³-Tyr⁷-DAph(Cbm)⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Nal¹⁵-NH₂ (5)

Analog **5** is identical to analog **4** except for the N-terminal DOTA group, which enhances its selectivity for receptor 2 (Table 1). The 3D NMR structure shows that the backbone has a type-II' β -turn around DAph(Cbm)⁸ and Lys⁹ (Figure 2, Table S1), which is supported by the weak sequential $d_{\alpha N}(i, i+2)$ NOE observed between DAph(Cbm)⁸ and Thr¹⁰ (Figure 1). The turn conformation is stabilized by the hydrogen bonds observed between the amide proton of Tyr⁷ and the carbonyl of Thr¹⁰ in all of the 20 conformers calculated. Experimentally measured small temperature coefficient of -0.03 ppm/K for the amide proton of Tyr⁷ suggests that it could be involved in a hydrogen bond. The side chains of Cpa², Tyr⁷ and DAph(Cbm)⁸ are in the *trans* configuration, the side chain of Lys⁹ is in the *gauche*⁺ configuration and the side chain of Nal¹⁵ is in the *gauche*⁻ configuration (Table S1).

Three-dimensional structure of Cbm-Phe²-c[Dcys³-Aph(Cbm)⁷-DTrp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Nal¹⁵-NH₂ (6)

Analog **6** differs from analog **1** by the N-terminal carbamoylation and the Phe² substitution. Analog **6** has low binding affinity to sst₂ and has moderate binding affinities for sst₃, sst₄ and sst₅ and does not bind to sst₁ (Table 1). The 3D structure shows that the backbone has a type-I β -turn around DTrp⁸ and Lys⁹ (Figure 2, Table S1), which is supported by the weak sequential $d_{NN}(i, i+2)$, $d_{\alpha N}(i, i+2)$ NOEs observed between DTrp⁸ and Thr¹⁰ (Figure 1). In addition, long range $d_{\alpha N}(i, i+3)$ NOE observed between Aph(Cbm)⁷ and Thr¹⁰ (Figure 1) stabilizes the β -turn. Hydrogen bonds observed between the amide proton of Aph(Cbm)⁷ and the carbonyl of Thr¹⁰ in most of the calculated 20 structures suggest the β -turn conformation. Experimentally measured small temperature coefficient of -0.04 ppm/K for the amide proton of Aph(Cbm) suggests that it could be involved in a hydrogen bond. The side chains of Phe², Lys⁹ and Nal¹⁵ are in the *gauche*⁺ configuration and the side chains of Aph(Cbm)⁷, DTrp⁸ are in the *trans* configuration (Table S1).

Three-dimensional structure of Cbm-Phe²-c[Dcys³-Aph(Cbm)⁷-DTrp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-Thr¹⁵-NH₂ (7)

Analog **7** is similar to analog **6** except for the C-terminal amino acid Thr and it binds with low affinity to sst₂ but does not bind to the other receptors (Table 1), however inhibits the signaling. The 3D structure shows that the backbone has type-I' turn around residues DTrp⁸ and Lys⁹ (Figure 2, Table S1), which is supported by the weak sequential d_{NN}(*i*, *i*+2), d_{αN}(*i*, *i*+2) NOEs observed between DTrp⁸ and Thr¹⁰ (Figure 1). Hydrogen bonds observed in most of the 20 calculated conformers between the amide proton of Thr¹⁰ and the carbonyl of Aph(Cbm)⁷ also indicates the presence of the β-turn conformation. Experimentally measured small temperature coefficient of −0.02 ppm/K for the amide proton of Thr¹⁰ indicates that it could be involved in a hydrogen bond. The side chains of Phe², Aph(Cbm)⁷ and Lys⁹ are in the *trans* configuration and the side chain of DTrp⁸ is in the *gauche*⁺ configuration (Table S1).

Discussion

Our group has proposed agonist pharmacophore models for the sst₁, sst₂ and sst₄ subtype-selective cyclic analogs of SRIF, based on their 3D NMR structures.¹⁰ Each one of the pharmacophore models requires Trp/DTrp⁸, Lys⁹, in addition to a certain number of aromatic side chains necessary for efficient interaction with their corresponding receptors. The agonist pharmacophore models are revisited before defining the antagonist pharmacophore for sst₂-selective analogs of SRIF.

Existing agonist pharmacophore models for sst₁, sst₂, sst_{2/5} and sst₄ selective analogs

The sst₁ selective agonist pharmacophore proposed has two aromatic side chains, at position 6 or 7 and 11, in addition to the DTrp⁸ and IAMP⁹ pair.¹¹ It is noteworthy to mention that, in this pharmacophore, the aromatic side chains are present at the back of the peptide plane, different from the other sst pharmacophores. The sst₁ selectivity is achieved through the amino acid IAMP at position 9, which replaces Lys. The distances reported for efficient receptor-ligand interactions are as follows: between DTrp⁸ and IAMP⁹ is 7-8 Å, between DTrp⁸ and Phe^{6/7} is 6-7.5 Å, between DTrp⁸ and Phe¹¹ is 9.5-12 Å, between IAMP⁹ and Phe^{6/7} is 9-11 Å and between IAMP⁹ and Phe¹¹ is 8-10 Å. The sst₂ and sst₄ selective agonist pharmacophores require one aromatic side chain, in addition to the DTrp⁸ and Lys⁹ side chains. But the aromatic side chains are placed differently with respect to DTrp⁸-Lys⁹ pair in sst₂ and sst₄ pharmacophores. In the sst₄ agonist pharmacophore, the aromatic side chain is closer to the side chain of Lys⁹ than to the side chain of DTrp⁸, with the following distances: between DTrp⁸ and Lys⁹ is 4.5-6.5 Å; DTrp⁸ and Phe^{6/11} is 5.5-9.5 Å and between Lys⁹ and Phe^{6/11} is 4.5-6.5 Å.¹⁰ In contrast, the sst₂ pharmacophore, the distances between DTrp⁸ and Phe² is 12-13.5 Å; DTrp⁸ and Lys⁹ is 4-5 Å and Phe² and Lys⁹ is 12.5-15 Å and therefore, this aromatic side chain is far from the DTrp⁸-Lys⁹ pair.¹² In addition, this pharmacophore happens to be a subset of the sst_{2/5}-partially selective agonist pharmacophore proposed by Melacini et al. for analogs binding to both sst₂ and sst₅ receptors non-selectively.²³ Sometimes, these analogs also exhibited a low binding affinity for sst₃ receptor. In this pharmacophore, the side chains of two aromatic residues Dphe², Phe⁷ along with DTrp⁸ and Lys⁹ are proposed to be crucial for binding. The side chain of DTrp⁸ is in close proximity to the side chain of Lys⁹ (~5 Å), whereas the side chain of Phe⁷ is about 7-9 Å away from the side chain of DTrp⁸ and 9-11 Å from the side chain of Lys⁹. The position of the aromatic side chain at the N-terminus was not conserved for most of the analogs. The distances between Dphe² and DTrp⁸, Lys⁹, Phe⁷ were 11-15 Å, 12-15 Å, 5-11 Å, respectively.²³ Also, it has been shown that octreotide types of analogs undergo conformational change between α-helix and a β-sheet resulting in two different positions for the aromatic residue at position 2.³⁵ All of these observations lead to the fact that the

flexibility in the position of Dphe² is necessary for the analogs to bind to two different receptors simultaneously.

Three-dimensional structures of the sst₂-selective antagonists and the antagonist pharmacophore model

In octreotide types of agonists, either the removal of the aromatic side chain at position 7 by an Ala or replacement by an amino acid having a longer side chain, such as Aph(Cbm) resulted in analogs with decreased binding affinity to sst₃ and sst₅ receptors, but still having high affinity binding to the sst₂ receptor.¹² The 3D NMR structures of such analogs led to the identification of the sst₂-selective agonist pharmacophore.¹² Here, the same idea has been extended to the antagonist analogs based on octreotide having DCys at position 3 and L-Phe at position 2.²⁸⁻²⁹ The antagonists have a longer side chain at position 7, instead of Phe and hence do not bind to sst_{3/5} receptors, but do have high binding affinity to the sst₂ receptor (Table 1). Comparison of the 3D NMR structures of these analogs with high binding affinity (**1** to **5**) shows that all of the analogs are comprised of a backbone conformation of β -turn of type II' (Figure 2, Table S1) similar to that reported for the sst₂-selective agonists.¹² In the antagonists, the side chain of DTrp/DAph(Cbm)⁸ is in the *trans* conformer and the side chain of Lys⁹ is in the *gauche*⁺ conformer, bringing the two side chains adjacent to each other. Such a conformational preference has also been reported for the agonists binding to sst₂.¹² Superposition of the 3D NMR structures of the sst₂ antagonists **1** to **5** shows that in addition to the side chains of DTrp/DAph(Cbm)⁸ and Lys⁹ that are conserved in their position, the side chain of Cpa/Phe at position 2 is also highly conserved (Figure 3A). The distances between the C γ atoms of the side chains are as follows: Cpa² and DTrp⁸ is 11.5-14 Å, Cpa² and Lys⁹ is 13-15 Å and DTrp⁸ and Lys⁹ is 4.5-5.5 Å (Figure 3A). Comparing the sst₂ antagonist pharmacophore, with the 3D structures of analogs **6** and **7**, having low binding affinity for sst₂ shows that the loss in the binding is mainly due to the position of the Phe at position 2, which does not fit the sst₂ antagonist pharmacophore (Figure 3D). The carbomoyl group at the N-terminus in combination with DCys³ constrain possibly the side chain of Phe² not to fit into the sst₂ pharmacophore.

Comparison of the sst₂-selective agonist versus antagonist pharmacophores

With the introduction of the pharmacophore model for the sst₂-selective antagonists of SRIF (Figures 3A and B), this motif can be compared with the existing pharmacophore for sst₂-selective agonists (Figure 3C). We suggested earlier that in SRIF agonists, which bind selectively to sst₂, the side chains of DTrp⁸, Lys⁹ and Phe² constitute the most essential elements necessary for binding.¹² In the sst₂-selective agonist pharmacophore model, the distances between DTrp⁸ and Lys⁹ is 4 to 5 Å, but Phe² is farther away from DTrp⁸ (12 to 13.5 Å) and Lys⁹ (12.5 to 15 Å). The sst₂-selective antagonist pharmacophore also requires DTrp⁸, Lys⁹ and Phe² with the following distances: DTrp⁸ and Lys⁹ is 4.5-5.5 Å, and Phe² is farther away from DTrp⁸ (11.5 to 14 Å) and Lys⁹ (13 to 15 Å). Comparing the two pharmacophores as well as the distances between the corresponding residues suggests that the sst₂ selective antagonist pharmacophore is very similar to the agonist pharmacophore.

Conclusions

The 3D conformations of seven cyclic SRIF antagonists having high binding affinity and selectivity to the sst₂ receptor have been studied in DMSO. These studies suggest that the backbone conformations of these analogs have a β -turn similar to the agonists binding selectively to the sst₂ receptor. Based on the conserved positions of the side chains, we propose that the sst₂-antagonist pharmacophore, consisting of DTrp/DAph(Cbm)⁸, Lys⁹ and Cpa² is very similar to the sst₂-agonist pharmacophore. Since the consensus structural motifs of sst₂-selective agonists and antagonists are structurally indistinguishable, the nature

of antagonism *versus* agonism in somatostatin analogs is not understood. However, the presented structural studies, in combination with activity measurements, indicates that the action of antagonism *versus* agonism of somatostatin analogs is not based on significant structural differences. The studies rather suggest that either small structural differences including changes in the chemical structure of the analogs, or different dynamics may be responsible for antagonist *versus* agonist action. Studies are in progress to elucidate the mechanism of action of antagonism *versus* agonism in somatostatin analogs.

Materials and Methods

Sample preparation and NMR Experiments

Analogues were synthesized by the solid-phase approach either manually or on a CS-Bio analog synthesizer model CS536.31 In vitro Ca^{++} release assay and immunohistochemical sst_2 internalization assay were carried out as described in detail earlier.³¹

NMR samples were prepared by dissolving 2.5 mg of the analog in 0.5 mL of DMSO-d_6 . The ^1H NMR spectra were recorded on a Bruker 700 MHz spectrometer operating at proton frequency of 700 MHz. Chemical shifts were measured using DMSO ($\delta = 2.49$ ppm) as an internal standard. The 1D spectra were also acquired at temperatures between 293 and 323 K and were used to measure the temperature coefficients of the amide resonances. The 2D spectra were acquired at 298 K. Resonance assignments of the various proton resonances have been carried out using total correlation spectroscopy (TOCSY);^{36,37} double-quantum filtered spectroscopy (DQF-COSY)³⁸ and nuclear Overhauser enhancement spectroscopy (NOESY).³⁹⁻⁴¹ The TOCSY experiments employed the MLEV-17 spin-locking sequence suggested by Davis and Bax,³⁶ applied for a mixing time of 50 or 70 ms. The NOESY experiments were carried out with a mixing time of 100 ms or 150 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 $t_{1\text{max}} = 47$ ms and a $t_{2\text{max}} = 120$ 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 16, 16 and 64 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 spectra were processed using the software PROSA.⁴² The spectra were analyzed using the software X-EASY.⁴³

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.⁴⁴ The collection of structural restraints is based on the NOEs and vicinal $^3J_{\text{NH}\alpha}$ couplings. Dihedral angle constraints were obtained from the $^3J_{\text{NH}\alpha}$ couplings, which were measured from the 1D ^1H NMR spectra and from the intra-residual and sequential NOEs along with the macro GRIDSEARCH in the program CYANA.³³ The calibration of NOE intensities *versus* ^1H - ^1H 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 field⁴⁵ with the energy criteria fit 0.1 kcal/mol/ $\text{\AA}^4$ ⁴⁶ in

the program DISCOVER with steepest decent algorithm⁴⁷ as described by Koerber et al.⁴⁶ The resulting energy minimized bundle of 20 conformers was used as a basis for discussing the solution conformation of the different SRIF analogs. The structures were analyzed using the program MOLMOL.⁴⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Agl aminoglycine

Aph	aminophenylalanine
Cbm	Carbomoyl
Cpa	Chlorophenylalanine
CYANA	Combined assignment and dynamics algorithm for NMR applications
DQF-COSY	double quantum filtered correlation spectroscopy
Nal	naphthylalanine
NOESY	nuclear Overhauser enhancement spectroscopy
PROSA	Processing algorithms
SRIF	somatotropin release inhibiting factor, also somatostatin
TOCSY	total correlation spectroscopy

Figure 1.

Survey of characteristic NOEs used in CYANA for structure calculation for analogs **1-7**. 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 NOE's $d_{NN}(i, i+2)$, $d_{\alpha N}(i, i+2)$, and $d_{\beta N}(i, i+2)$ are shown by lines starting and ending at the positions of the residues related by the NOE. The symbols F^{Cl} , F^{NO_2} , F^{NH_2} , G^{AB} , A^N , w, c and y refer to Cpa, 4NO₂Phe, Aph(Cbm), Agl(NMe, Benzoyl), Nal, DTrp, DCys and DTyr, respectively.

Figure 2.

NMR structures of sst_2 -selective antagonists **1-7**. For each analog, twenty energy-minimized conformers with the lowest target function are used to represent the 3D NMR structure. The bundle is obtained by superposition of C^α atoms of all the residues. The backbone and all the side chains are displayed including the disulfide bridge. The following color code is used: royal blue (**1**) H-Cpa-c[DCys-Aph(Cbm)-DTrp-Lys-Thr-Cys]-2Nal-NH₂, red (**2**) Ac-4NO₂Phe-c[DCys-Tyr-DTrp-Lys-Thr-Cys]-DTyr-NH₂, cyan (**3**) H-Cpa-c[DCys-Agl(NMe,benzoyl)-DTrp-Lys-Thr-Cys]-2Nal-NH₂, dark green (**4**) H-Cpa-c[DCys-Tyr-DAph(Cbm)-Lys-Thr-Cys]-Nal-NH₂, hot pink (**5**) DOTA-Cpa-c[DCys-Tyr-DAph(Cbm)-Lys-Thr-Cys]-Nal-NH₂, grey (**6**) Cbm-Phe-c[DCys-Aph(Cbm)-DTrp-Lys-Thr-Cys]-Nal-NH₂, purple (**7**) Cbm-Phe-c[DCys-Aph(Cbm)-DTrp-Lys-Thr-Cys]-Thr-NH₂. For **3**, the structure of the major conformer is shown. The side chains which are involved in sst_2 -binding are highlighted, which are DTrp/DAph(Cbm) at position 8 in light green, Lys at position 9 in blue and Cpa at position 2 in yellow. N-terminal DOTA group in (**5**) is shown in light pink for clarity.

Figure 3.

Consensus structural motif of sst₂-selective antagonists of SRIF. (A) Stereo view of the consensus structural motif for the sst₂-selective antagonists **1** (royal blue), **2** (red), **3** (cyan), **4** (dark green) and **5** (hot pink). Only the side chains of residues Cpa²/Nal¹⁵, DTrp⁸ and Lys⁹ for **1**, **2**, **3**, **4** and **5** are shown. The distances between the Cγ atoms of DTrp⁸, Lys⁹ and Cpa²/Nal¹⁵ are shown. For each analog, the conformer with the lowest target function is used in defining the pharmacophore. (B) Schematic drawing of the pharmacophore for the sst₂-selective antagonists of SRIF. (C) Schematic drawing of the pharmacophore for the sst₂-selective agonists of SRIF.¹² (D) Superposition of the sst₂-antagonist **4** (dark green) on the structures of analogs **6** (in grey) and **7** (in dark violet).

Table 1

Sst1-sst5 binding affinities (IC₅₀, nM) of analogs studied by NMR

No	Peptide	sst ₁	sst ₂	sst ₃	sst ₄	sst ₅
1	H-Cpa-c[DCys-Aph(Cbm)-DTrp-Lys-Thr-Cys]-2Nal-NH ₂	>1K (2)	6.9 ± 0.7	155 ± 29	479 ± 8	149 ± 37
2	Ac-4NO ₂ -Phe-c[DCys-Tyr-DTrp-Lys-Thr-Cys]-DTyr-NH ₂ ²⁸	>1K	3.6 ± 0.4	ca. 1K	349 ± 30	276 ± 119
3	H-Cpa-c[DCys-LAgl(NMe,benzoyl)-DTrp-Lys-Thr-Cys]-2Nal-NH ₂	>1K	17 ± 5	827 ± 244	>1K	442 ± 254
4	H-Cpa-c[DCys-Tyr-Daph(Cbm)-Lys-Thr-Cys]-Nal-NH ₂	>1K (2)	3.7 ± 1.3	346 ± 81	>1K (2)	>1K (2)
5	DOTA-Cpa-c[DCys-Tyr-Daph(Cbm)-Lys-Thr-Cys]-Nal-NH ₂	>1K (2)	1.4 ± 0.5	>1K (2)	>1K (2)	>1K (2)
6	Cbm-Phe-c[DCys-Aph(Cbm)-DTrp-Lys-Thr-Cys]-Nal-NH ₂	>1K (2)	89; 89	293; 111	108; 83	502; 170
7	Cbm-Phe-c[DCys-Aph(Cbm)-DTrp-Lys-Thr-Cys]-Thr-NH ₂	>1K (2)	405; 957	>1K (2)	>1K (2)	>1K (2)

The IC₅₀ values (nM) were derived from competitive radioligand displacement assays reflecting the affinities of the analogs for the cloned receptors using the non-selective [¹²⁵I]-Leu⁸,DTrp²²,Tyr²⁵]SRIF-28, as the radioligand. Mean values ± SEM when N ≥ 3 (shown in parentheses). In the other cases (N = 2), individual values are shown.

Table 2

Assignment of the various proton resonances in analogs 1-7*

Residue	¹ H	Analog						
		1	2	3	4	5	6	7
Cbm/DOA						3.49, 2.84	5.59	5.60
		Cpa	pNO ₂ -Phe	Cpa	Minor	Cpa		
Phe ²	NH	-	8.16	-	-	8.50	6.20	6.21
	αH	3.86	4.58	4.03	3.97	4.69	4.34	4.34
	βCH ₂	3.09, 2.76	3.09, 2.82	3.09, 2.89	3.14, 2.81	3.08, 2.64	2.94, 2.69	2.94, 2.71
	H2,6	7.30	7.49	7.27	7.32	7.25	7.21	7.22
	H3,5	7.36		7.46	7.37	7.00	7.00	
Dcy ³	NH	8.70	8.55	8.91	8.96	8.45	8.35	8.40
	αH	4.53	4.34	4.66	4.71	4.42	4.33	4.36
	βCH ₂	3.28, 2.47	3.14, 2.51	3.07, 2.84	3.27, 2.39	3.20, 2.45	3.17, 2.66	3.20, 2.71
		Aph(Cbm)		LAgl(NMe-Benzoyl)			Aph(Cbm)	Aph(Cbm)
Tyr ⁷	NH	8.29	8.10	9.23	9.15	8.24	8.06	8.10
	αH	4.64	4.50	5.68	6.42	4.61	4.54	4.47
	βCH ₂	2.97, 2.74	2.90, 2.64, 6.87	-	-	2.96, 2.70	2.93, 2.67	2.82, 2.75
	H2,6	7.04	6.58	7.29	7.37	6.97	6.92	6.97
	H3,5	7.29	9.13	7.46	-	6.67	6.63	7.22
	HH	-		-	-	9.17	-	-
	HT	8.46		-	-		8.45	8.40
	NH ₂	5.85		-	-		5.85	5.83
		γCH ₃		2.79	3.07			
						DAph(Cbm)	DAph(Cbm)	
DTp ⁸	NH	8.66	8.55	8.26	8.12	8.57	8.46	8.57

Residue	¹ H	Analog						
		1	2	3	4	5	6	7
	αH	4.43	4.38	4.55	4.45	4.35	4.36	4.33
	βH	3.05, 2.84	3.06, 2.80	3	2.81, 2.68	2.82, 2.67	3.08, 2.86	3.08, 2.84
	HD1	7.01	7.04	7.25	7.18	-	7.03	7.00
	HE1	10.82	10.75	10.92	10.87	-	10.77	10.75
	HZ2	7.31	7.29	7.35	-	-	7.30	7.30
	HH2	7.05	7.03	7.07	-	-	7.05	7.05
	HZ3	6.98	6.95	6.97	6.98	-	6.98	6.97
	HE3	7.50	7.51	7.61	7.55	-	7.51	7.49
	H2,6				7.01	6.99		
	H3,5				7.26	7.22		
	HT				8.51	8.45		
	NH ₂				5.85	5.79		
Lys ⁹	NH	8.40	8.34	8.38	8.34	8.34	8.30	8.25
	αH	4.10	4.11	4.07	3.98	4.11	4.16	4.20
	βCH ₂	1.72, 1.30	1.70, 1.28	1.55, 1.31	1.48, 1.25	1.74, 1.37	1.72, 1.36	1.73, 1.36
	γCH ₂	0.85, 0.80	0.85, 0.85	0.93, 0.93	0.82, 0.82	0.97, 0.92	0.93, 0.93	0.97, 0.97
	δCH ₂	1.31, 1.31	1.33, 1.33	1.35, 1.35		1.38, 1.38	1.38, 1.38	1.38, 1.38
	εCH ₂	2.56, 2.56	2.56, 2.56	2.60, 2.60		2.64, 2.64	2.60, 2.60	2.61, 2.61
	εNH	7.31	7.54	7.63	7.56	7.60	7.62	7.57
Thr ¹⁰	NH	7.53	7.40	7.44	7.50	7.43	7.52	7.56
	2H	4.53	4.51	4.14		4.53	4.40	4.38
	βH	3.84	3.92	3.91	3.96	3.86	3.94	4.00
	γH	0.88	1.03	0.95		0.93	0.96	1.11
	OH	5.39	5.49	5.11	5.26	5.38	5.34	4.89
Cys ¹⁴	NH	8.51	8.58	8.10	8.16	8.49	8.40	8.48
	αH	4.68	4.59	4.44	4.54	4.63	4.62	4.72
	βCH ₂	3.05, 2.83	3.02, 2.71	3.08, 2.86		3.04, 2.79	3.02, 2.79	3.21, 2.94

Residue	¹ H	Analog						
		1	2	3	4	5	6	7
			DTyr					Thr
Nal ¹⁵	NH	8.51	8.30	8.16	8.48	8.37	8.33	7.83
	αH	4.55	4.37	4.49	4.57	4.54	4.52	4.13
	βCH ₂	3.19, 2.98	2.89, 2.63	3.16, 2.94	3.19, 2.99	3.17, 2.97	3.18, 2.97	4.00
	H1	7.71	-	7.68	7.72	7.69	7.70	-
	H3	7.43	-	7.37	7.43	7.40	7.43	-
	H4	7.77	-	7.77	7.76	7.70	7.79	-
	H5	7.77	-	7.79	7.80	7.77	7.82	-
	H6	7.42	-	7.44	7.43	7.42	7.45	-
	H7	7.44	-	7.44	7.42	7.43	7.42	-
	H8	7.79	-	7.82	7.79	7.76	7.77	-
	2,6H	-	6.95					-
	3,5H	-	6.53					-
	γCH ₃	-	-					1.02
	OH	-	9.09					5.22
NH ₂		7.50, 7.15	7.42, 7.11	7.38, .14	7.57, 7.16	7.44, 7.08	7.43, 7.16	7.20, 7.14

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

Table 3

Characterization of the NMR structures of analogs 1-7*

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. $\geq$ 0.1 Å	Max (Å)	No. $\geq$ 1.5 deg	Max (deg)
1	136	11	0.031	0.34 $\pm$ 0.12	1.05 $\pm$ 0.25	219 $\pm$ 3	176 $\pm$ 3	44 $\pm$ 1	0.4 $\pm$ 0.1	0.5 $\pm$ 0.09	0 $\pm$ 0	0 $\pm$ 0
2	148	10	0.057	0.39 $\pm$ 0.03	1.12 $\pm$ 0.11	187 $\pm$ 5	139 $\pm$ 4	48 $\pm$ 2	0.6 $\pm$ 0.1	0.8 $\pm$ 0.11	0 $\pm$ 0	0 $\pm$ 0
3	87	15	0.200	1.02 $\pm$ 0.29	2.22 $\pm$ 0.56	225 $\pm$ 7	178 $\pm$ 10	48 $\pm$ 6	0.1 $\pm$ 0.1	0.2 $\pm$ 0.08	0 $\pm$ 0	0 $\pm$ 0
4	102	19	0.005	0.33 $\pm$ 0.18	1.15 $\pm$ 0.34	217 $\pm$ 7	166 $\pm$ 5	52 $\pm$ 3	0.1 $\pm$ 0.1	0.3 $\pm$ 0.11	0 $\pm$ 0	0 $\pm$ 0
5	151	14	0.030	0.09 $\pm$ 0.06	0.61 $\pm$ 0.15	232 $\pm$ 8	185 $\pm$ 8	47 $\pm$ 2	0.5 $\pm$ 0.2	0.9 $\pm$ 0.16	0 $\pm$ 0.1	0.2 $\pm$ 0.24
6	138	20	0.025	0.50 $\pm$ 0.22	1.20 $\pm$ 0.47	232 $\pm$ 10	172 $\pm$ 9	59 $\pm$ 4	0.3 $\pm$ 0.2	0.6 $\pm$ 0.18	0 $\pm$ 0	0 $\pm$ 0
7	155	22	0.097	0.40 $\pm$ 0.16	0.82 $\pm$ 0.17	157 $\pm$ 4	108 $\pm$ 4	50 $\pm$ 4	0.9 $\pm$ 0.1	1.1 $\pm$ 0.19	0 $\pm$ 0	0 $\pm$ 0

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

** 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. For the exact definition see reference.³²

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