Peptide drugs

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Fine-tuning the π–π Aromatic Interactions in Peptides: New Somatostatin Analogs Containing Mesityl Alanine.

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Understanding non-covalent interactions between aromatic moieties is essential in medicinal chemistry and lead optimization for drug design. These interactions are fundamental in controlling diverse phenomena: for example, vertical stacking interactions provide stability to duplex DNA.[1] Other important examples include the spike-nucleocapsid interaction in viruses;[2a] molecular self-assembly in supramolecular systems;[2b] and host-guest molecular recognition events.[2c] Aromatic amino acids strongly contribute to protein architecture and stability[3,4] as it has been observed in SH3 and WW domains,[5a] and of peptides, including the antimicrobial Tachiplesin I[5b] or the pharmacologically important hormone somatostatin.[6]

Somatostatin, also known as *somatotropin release-inhibiting factor* (SRIF), is a 14 amino-acid natural peptide whose sequence is shown in Figure 1 (left). In clinical practice, somatostatin is currently used as a gastric anti-secretory drug, to treat growth hormone secretion disorders, and to treat endocrine tumors.[7] It is involved in multiple biological functions mediated by direct interactions between it and at least five characterized G-protein-coupled receptors, named SSTR1-5.[8] These receptors differ in their tissue distribution and pharmacological properties.

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The 3D structure of somatostatin has been a matter of debate during the last three decades. Initially, Hirschmann and co-workers[9] postulated the existence of an interaction between aromatic residues Phe6-Phe11. This interaction could enable in the stabilization of some of the biologically active conformations of the hormone. Few years later, the same authors detected the Phe6-Phe11 interaction by NMR,[10a] and hypothesized that it should be perpendicular (edge-to-face) rather than parallel (face-to-face).[10a,b] However, other authors subsequently reported that they were unable to observe the Phe6-Phe 11 interaction by NMR (either in aqueous solution or in methanol) and that the only interaction that they could observe was that between Phe6 and Phe7.[11a,b] Attempts to further characterize its structure have been unsuccessful. Presently, there is a consensus that the native structure of somatostatin in solution is as an ensemble of several conformations in equilibrium, a few of which partially structured.[12] This scenario probably explains researchers' failure to obtain detailed NMR or X-ray data on the 3D structure of the peptide.

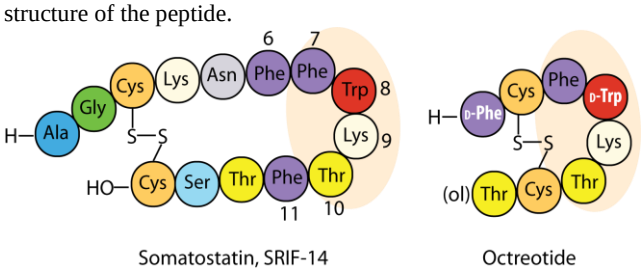


Figure 1. Amino acid sequences of somatostatin and of octreotide, showing their respective proposed pharmacophores.

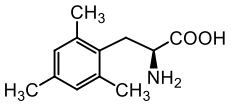
To date, most of the work done on somatostatin analogs has focused on the synthesis of molecules with smaller, more rigid rings; some of these compounds have shown enhanced selectivity and stability.[13] The best examples are octreotide (shown in Figure 1, right) and lanreotide, the only two somatostatin-analog drugs on the market.[14] Both of these compounds have strong affinity for SSTR2 but only moderate to low affinity for the other receptors. Like most of the somatostatin analogs currently under research, octreotide and lanreotide are octapeptides that include part of the somatostatin pharmacophore[15] and feature a covalent disulfide bridge as a surrogate of the proposed non-covalent interaction between Phe6 and Phe11.

Given recent advances in peptide chemistry which have greatly facilitated synthesis of large cyclic peptides, we reasoned that we could introduce point modifications into the 14-residue scaffold to fine tune rigidity, specificity and stability to produce new analogs structurally much closer to the natural hormone than the octapeptides. In previous studies,[16] we explored substitution of Trp8 with 3-(3'-quinolyl) alanine (Qla, both enantiomers), finding that the corresponding analogs exhibit more conformational variability than does somatostatin itself. Remarkably, these analogs were selective for SSTR1 and SSTR3 receptors. Thus, we deduced

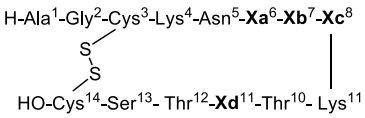
that structural flexibility is advantageous for activity in certain receptors and that relative to the parent compound, these Qla-analogs have a greater proportion of highly flexible conformers in solution.

Seeking new somatostatin analogs that would be conformationally stabilized (by π–π interactions) relative to the parent compound, we substituted key amino acids in somatostatin with non-natural residues. We prepared various analogs by replacing the aromatic ring of the phenylalanine with a mesityl group (2,4,6-trimethylphenyl), by substituting one Phe with 3-mesityl alanine (Msa, **1**). We chose Msa based on the higher electronic density that the methyl groups confer to the aromatic moiety, and on the lesser conformational mobility of the mesityl ring, relative to Phe.[17] Hence, we expected that the π–π interactions between the Msa and the remaining Phe residues would be stronger than those among the Phe of the parent compound, and envisaged that the intrinsic rigidity of the Msa amino acid could shift the conformational equilibrium towards more rigid conformations (relative to those of the natural compound).

We initially prepared two different peptides containing Msa instead of Phe at position 6 or 11, respectively. Additionally, to increase the physiological stability of the resulting peptides in blood plasma, we used D-Trp (instead of L-Trp) at position 8 (peptides **2** and **3**, Figure 2), a modification known to enhance stability while maintaining the biological activity of the peptide[7a,9,18]. Since the aromatic interaction had been postulated to occur either between residues 6 and 11 (Veber et al.)[9] or between residues 6 and 7 (Jans et al.),[11a] we were also interested in the effects of substituting Phe7 with Msa (peptide **4**). The resulting analog exhibited outstanding receptor affinity, which prompted us to study the effects of D-Trp substitution in this compound; thus, we then prepared the same sequence with L-Trp8 (peptide **5**).



L-3-Mesityl alanine (Msa), **1**

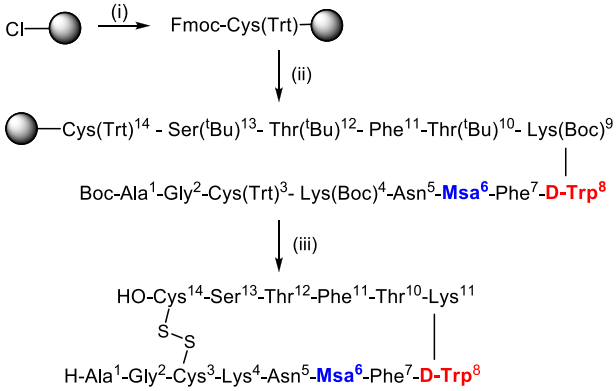


Xa = Msa; Xb = Xd = Phe; Xc = D-Trp **[L-Msa6,D-Trp8]-SRIF**, **2**
Xa = Xb = Phe; Xd = Msa; Xc = D-Trp **[L-Msa11,D-Trp8]-SRIF**, **3**
Xa = Xd = Phe; Xb = Msa; Xc = D-Trp **[L-Msa6,D-Trp8]-SRIF**, **4**
Xa = Xd = Phe; Xb = Msa; Xc = L-Trp **[L-Msa6,D-Trp8]-SRIF**, **5**

Figure 2. New somatostatin analogs (**2-5**) with L-3-mesityl alanine (Msa)

Here we present how the structural studies confirmed that the aromatic interactions do exist, and significantly contribute to both the greater stability and structural rigidity of our peptide analogs relative to somatostatin. Moreover, we have also evaluated the interaction of these derivatives with the five receptors in cellular cultures. We have found that each of these peptides exhibits a unique profile of strong affinity and selectivity for one or more of SSTR1-5. Furthermore, we have correlated this selectivity to the presence of aromatic clusters on the basis of the NMR data, thus paving the way for a rational design of new efficacious somatostatin-based analogs. We have also characterized the relative orientation of the aromatic rings in the clusters, and found that each peptide displays a particular π-π interaction fingerprint, including parallel, offset-stacked and perpendicular orientations as has been described in proteins.[3]

We obtained Fmoc-L-3-mesityl alanine by following a procedure previously developed by our group.[19] The four peptides containing Msa, at either position 6 [L-Msa6,D-Trp8]-SRIF (**2**), position 11 [L-Msa11,D-Trp8]-SRIF (**3**) or position 7 [L-Msa7,D-Trp8]SRIF (**4**) and [L-Msa7]-SRIF (**5**), were prepared by solid-phase peptide synthesis on 2-chlorotrityl chloride resin, using the Fmoc/^{t}Bu strategy. Scheme 1 shows the preparation of [L-Msa6,D-Trp8]SRIF (**2**). Peptides **3-5** were prepared using the same strategy. When the non-natural amino acid was coupled, only 1.5 eq were used.



[L-Msa6,D-Trp8]-SRIF, **2**

Scheme 1.(i) (a) Fmoc-L-Cys(Trt)-OH (3 eq), DIEA (3 eq) (b) MeOH; (ii) (a) Piperidine 20% DMF, (b) Fmoc-AA-OH (1.5-3 eq), DIPCDI (3 eq), HOBt (3 eq), DMF, (c) Boc-Ala-OH, DIPCI, HOBT, DMF (iii) (a) DCM/TFE/AcOH, (b) I_2, (c) TFA/DCM/anisole/H_2O

With the purified peptides **2-5** in hand, we first measured the selectivity of each one for each of the five receptors (SSTR1-5) in binding assays using stable CHO (*Chinese hamster ovary*) cell lines. The efficacy of the interaction against each receptor was assessed in competitive assays, using the membranes of the cultured cells and ^{125}I-labeled somatostatin. Somatostatin, [D-Trp8]-SRIF and octreotide were used as controls (Table 1).

Table 1. Affinity of somatostatin, [D-Trp8]-SRIF, octreotide and peptides **2-5** to receptors SSTR1-5. Values represent mean ± SEM

	SSTR1 Ki (nM)	**SSTR2** Ki (nM)	**SSTR3** Ki (nM)	**SSTR4** Ki (nM)	**SSTR5** Ki (nM)	**$t_{1/2}$ (h)**
Somatostatin (SRIF)	0.43 ± 0.08	0.0016 ± 0.0005	0.53 ± 0.21	0.74 ± 0.07	0.23 ± 0.04	2.75
[D-Trp8]-SRIF	**0.32 ± 0.11**	**0.001 ± 0.0007**	**0.61 ± 0.02**	5.83 ± 0.44	**0.46 ± 0.24**	19.7
Octreotide	300 ± 85	**0.053 ± 0.011**	15.2 ± 5.9	>10^3	11.53 ± 1.91	200
[L-Msa6,D-Trp8]-SRIF (2)	3.08 ± 0.9	4.55 ± 0.66	**0.78 ± 0.1**	4.70 ± 0.92	**0.36 ± 0.003**	**26**
[L-Msa11,D-Trp8]-SRIF (3)	3.35 ± 1.32	0.14 ± 0.06	1.31 ± 0.2	>10^3	**0.73 ± 0.19**	**41**
[L-Msa7,D-Trp8]-SRIF (4)	**0.33 ± 0.09**	**0.0024 ± 0.001**	7.49 ±0.63	>10^3	>10^3	**25**
[L-Msa7]-SRIF (5)	4.17 ± 1.45	**0.019 ± 0.009**	>10^3	28.72 ± 6.9	>10^3	**5.2**

Bold colored numbers represent data in close proximity or below the SRIF values (blue and red respectively).

[L-Msa6,D-Trp8]-SRIF **(2)** showed high affinity towards receptors 3 and 5; in fact, its Ki for these receptors is similar to those of somatostatin, which are 20 to 30 times lower than those of

octreotide (Table 1). [L-Msa11,D-Trp8]-SRIF (**3**) exhibited high affinity for SSTR5, and significant affinity (although lower than that of somatostatin) towards SSTR1, SSTR2 and SSTR3 (Table 1).

[L-Msa7,D-Trp8]-SRIF **(4)** showed striking affinity for SSTR2 — even higher than that of octreotide. Its inhibition constant for SSTR2 (1.5 times that of natural SRIF) compares very favorably to that of octreotide (33 times lower than that of SRIF). Moreover, **4** showed impressive affinity towards SSTR1, in remarkable difference to octreotide. Finally [L-Msa7]-SRIF (**5**) which lacks D-Trp8, shows a similar profile than **4** albeit with higher dissociation constants (Ki) (Table 1).

To evaluate the structural effects of these site-directed modifications and subsequently correlate them with the biological activity observed, we used NMR spectroscopy to analyze the conformations of these four peptides in aqueous solution and then compared each one to that of somatostatin. In all cases proton resonance assignments were identified using 2D TOCSY and NOESY homonuclear experiments.[20a] Somatostatin, as deduced from the pattern of NOEs, populates several conformations in solution. All the analogs (**2-5**) showed more intense NOEs than did somatostatin, although the majority of the peaks present in the NMR of these analogs are also detected in the parent compound. This indicates that each analog is structurally similar to one of the characteristic conformations of the hormone in solution. As shown in the structures below, the side-chain orientation of peptide **2** is very different from that of the remaining peptides, explaining why the restraints observed in somatostatin cannot be fitted to a unique conformation.[12]

Each of the well-defined bidimensional spectra of compounds **2-5**, enabled us to characterize their main conformation in solution using the software Crystallography & NMR System (CNS).[20b] To generate the list of experimental restraints for calculation, the volume of all assigned peaks was integrated, and then transformed into distances. Three sets of calculations (120 structures each) were run until the best match between assignments and final structures was obtained. After calculations all the obtained structural results supported the NMR data.

We first analyzed the structural effects of replacing Phe6 with Msa. Analysis of the 2D-NOESY spectrum of [L-Msa6,D-Trp8]-SRIF **(2)** suggested the presence of a well-defined structure in solution (Figure 3). The dominant conformer of **2** contains a cluster of three aromatic rings (residues 6, 7 and 11) defined by a large set of NOEs among them. As expected, the two aromatic rings of Msa6 and Phe11 are in close proximity, due to a strong face-to-face π–π interaction.[21] Numerous contacts between residues Lys9 and D-Trp8 are also observed in this peptide.

Peptide [L-Msa11,D-Trp8]-SRIF **(3)** is also highly structured**.** A superimposition of 30 selected low-energy conformers of this peptide reveals that the region containing residues Phe6-Phe7-D-Trp8-Lys9-Thr10-Msa11 is well ordered (RMSD value of 0.384 for the backbone, Figure 4). Peptide **3** also exhibited a strong π–π interaction between the Phe6 and Msa11 rings, whereby the Phe7 is located on the opposite face of the molecule. The orientation is defined by contacts between the Phe6 ring and the Msa11 ring plus its methyl groups. This aromatic interaction most likely fixes the backbone conformation (a detailed geometric analysis of the π–π aromatic interaction is given in Supporting Information). Interestingly, the π–π interaction occurs at the opposite molecular face relative to that observed in analog **2**. However, the relative orientation of Lys9 and Trp8 side-chains is the same in both compounds.

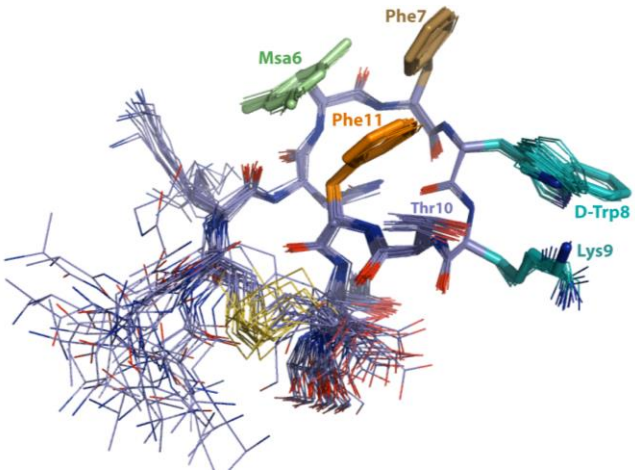


Figure 3. Superimposition of 28 minimum energy conformers of [L-Msa6,D-Trp8]-SRIF (**2**) as calculated based on NMR data using the backbone and the side-chains of residues 6 to 11 for the fitting. [21]

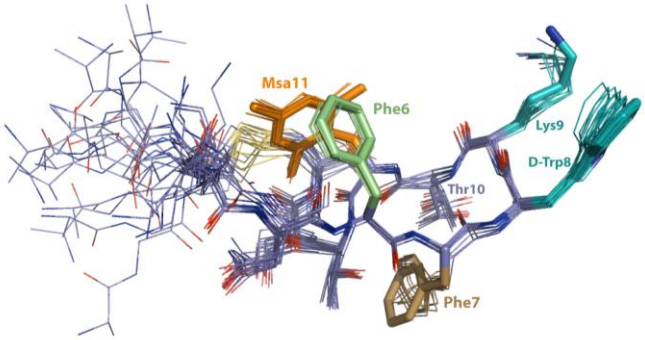


Figure 4. The most stable conformers of [L-Msa11,D-Trp8]-SRIF (**3**) as deduced based on NMR data.[22]

As predicted, substituting Phe with Msa either in position 6 or 11, afforded highly structured peptides, that feature an internal π–π aromatic interaction between the two aromatic residues.[21] However, consequences of introducing Msa into the vicinal position 7 were difficult to predict. To this end, we investigated structures (in solution) of [L-Msa7,D-Trp8]-SRIF **(4)** and [L-Msa7]-SRIF **(5)**. As deduced from bidimensional NMR data combined with CNS calculations, peptide **4** showed a well-ordered family of structures showing a hairpin in the region encompasing residues 6 to 11. This conformation is defined by contacts between the aromatic rings of Phe6 and of Phe11 and by a set of contacts between side-chains of D-Trp8 and of Lys9 which restrict the hairpin register (Figure 5). Interestingly, peptide **4** is structurally very similar to peptide **3** although in the former the aromatic interaction is distinctly *edge-to-face*, defined by contacts between the Phe6 aromatic ring with both Phe11 and the side-chain of Lys4. Peptide **5** with Trp8 in its natural configuration was also sufficiently structured to have its 3D structure determined by NMR. The calculated 24 minimum energy conformers that fit the experimental data are shown in Figure 6. As it seen in the Figure, this structure also contains the hairpin, indicating that D-Trp8 further stabilizes a conformation that already exists in the natural sequence, and that has been suggested to be essential for the biological activity of somatostatin.[18] The Trp8-

Lys9 interactions are reflected in the upfield shifted Lys γ-protons which are shielded by the aromatic indole ring.

Based on the NMR data that we obtained for each structure, we deduced that the Msa7 residue is not involved in forming π–π-interactions with either Phe6 or Phe11 since it is located on the opposite face of the molecule. However the position occupied by the aromatic ring in position 7 (below the hairpin, peptides **3**, **4** and **5**; as shown in Figures 4, 5 and 6, respectively) helps to stabilize the π–π interaction between the aromatic rings in positions 6 and 11. To the best of our knowledge, peptides **3-5** are the best structurally defined 14-residue somatostatin analogs described to date. Peptide **4** is the least flexible among these exhibiting the most highly structured conformation due to the combined effects of reinforced aromatic interaction between Phe6 and Phe11 and the presence of D-Trp8 in the structure. (RMSD = 0.3)

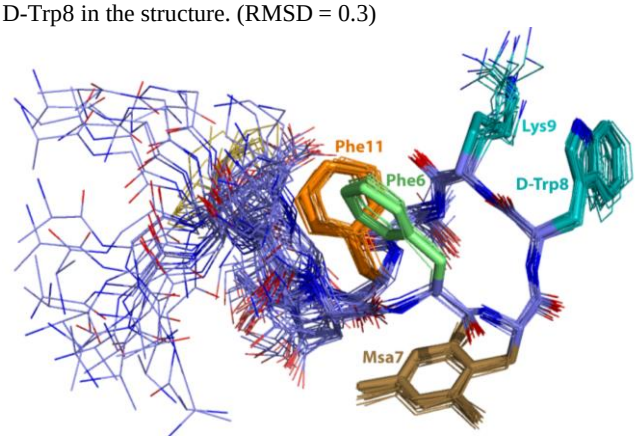


Figure 5. Superimposition of the NMR calculated 35 minimum energy conformers of [L-Msa7,D-Trp8]-SRIF (**4**) that were calculated based on NMR data.[21]

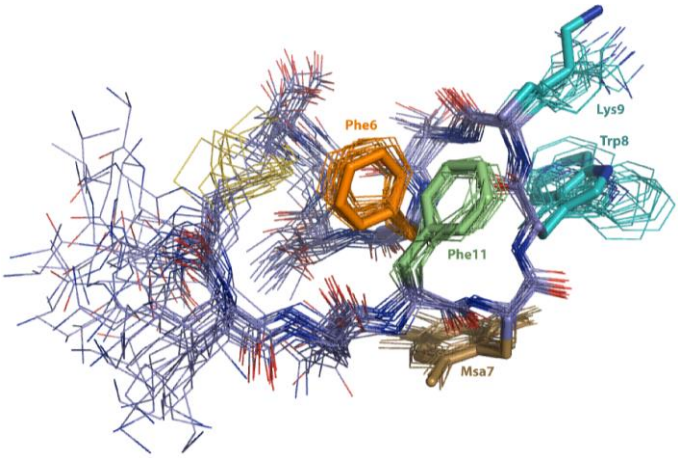


Figure 6. Superimposition of the NMR calculated 24 minimum energy conformers of [L-Msa7]-SRIF (**5**) that were calculated based on NMR data. [21] (RMSD value of 0.7 for the backbone).

During the development of short peptide analogs in the 1990's, the structural rigidity of compounds containing the somatostatin pharmacophore was correlated to their affinity for SSTR2.[23] The outstanding affinity of peptide **4** for SSTR2 is in good agreement with its high rigidity. The reasoned that the well-defined conformation that we found for **4** is probably very close to the native conformation of somatostatin when it binds to SSTR2. Moreover, we could also hypothesized that the significant activity of peptide **4** against SSTR1 derives from the π–π interaction between Msa7 and the Phe^{195} present in SSTR1, according to the pharmacophore proposed by Kaupmann et al.[24] In peptide **4**, Msa7 could interact with Phe^{195} through a reinforced π–π interaction, this scenario would explain the fact that this analog showed more affinity for SSTR1 than the parent compound. Thus, in line with the suggested induced-fit mechanism for SSTR1,[25] the enhanced aromatic-aromatic interactions between Msa7 and Phe^{195} would be essential for the affinity of **4** (which is much more rigid than peptide **5**) to receptor SSTR1.

Peptide **2** (Msa in position 6) was found to have a completely different selectivity profile than **4**, in good agreement with its distinct 3D structure: it binds SSTR3 and SSTR5 with affinities of the same order of magnitude as that of somatostatin.

In summary, we obtained two conformationally rigid somatostatin analogs with complementary selectivity, that mimic two of the different conformations that coexist in the native hormone.

Finally, we measured the serum stability of our peptides as well as that of octreotide, somatostatin and [D-Trp8]-SRIF, for the sake of comparison. Although the half-life of peptides **2-4** in serum is not as high as that of octreotide, these three analogs were on average 10 to 20 times more stable than somatostatin. Their greater stability probably stems from the presence of unnatural amino acids and, very likely, from the stronger interaction between the residues at positions 6 and 11 relative to that in the parent compound. As expected, the half-life of peptide **5** with L-Trp8 is only twice as high as that of somatostatin. The surprisingly long half-life of analog **3** (Msa in position 11) relative to peptides **2** and **4**, may corroborate the fact that the unnatural aromatic residue in position 11 shields the residue at position 6, as previously suggested.[10b]

To date, the most conformationally restricted somatostatin analogs have been developed via deletion of amino acids, reduction of ring size, or formation of a covalent bridge. These modifications usually improve the intrinsic pharmacological properties associated with peptide drugs. However, we have followed a different approach to obtain analogs with greater rigidity obtaining four new somatostatin analogs with a unique activity and selectivity profiles for SSTR1-5, by fine-tuning the electronic and steric properties of specific aromatic residues. We have exploited the non-covalent interactions between aromatic residues to modulate the conformational flexibility and provide major advantages in receptor selectivity and serum stability. By enhancing aromatic-aromatic interactions in somatostatin analogs, we have obtained four peptides with high receptor selectivities and with restricted conformations that enabled us to determine their 3D structures by NMR. Furthermore, we have elucidated the key aspects of the selectivity against the five somatostatin receptors by simply introducing an unnatural Msa amino acid in the original sequence. Our results prove that the modification of non-covalent interactions is a promising strategy in drug discovery and opens new possibilities for designing unprecedented peptide analogs of natural compounds.

Experimental Section

NMR assignment and structure calculation: NMR data were acquired at 285 K, using trifluoroacetate as a counter-ion at a pH 4.5 on a Bruker Avance III 600-MHz spectrometer equipped with a z-pulse field gradient unit. All spectra were processed with NMRPipe/NMRDraw software[26a] and were analyzed with CARA.[26b] The volume of all manually assigned peaks in the NOESY spectrum was integrated to generate the list of experimental restraints. The structures were water refined and ranked based on minimum values of energy-terms and violations of the experimental restraints. Molecular images were generated using PyMOL.

Receptor ligand-binding assay. All receptor-binding assays were performed with membranes isolated from CHO-K1 cells expressing human SRIF-14 receptor.[27a] IC_{50} values were calculated using a curve-fitting program (GraphPad Prism). The Ki values for the compounds were determined as previously described.[27b] Data represent the mean ± S.E.M. of values from at least three separate experiments, each of which was performed in triplicate.

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[21] The topology of the mesityl precludes that the mesityl alanine play the "edge" role in an edge-to-face interaction.

[22] The side chains of Trp 8 and of Lys 9 are depicted in cyan, and the disulfide bridge, in gold. The side chains of residues 6, 7 and 11 are shown in light green, brown, and orange, respectively and are labeled for clarity. More detailed structural data for each peptide, including a text file with the 35 low-energy conformers in PDB format, are provided in Supporting Information.

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Peptide drugs

Pablo Martín-Gago, Marc Gomez-Caminals, Rosario Ramón, Xavier Verdaguer, Pau Martin-Malpartida, Eric Aragón, Jimena Fernández-Carneado, Berta Ponsati, Pilar López-Ruiz, Alicia Cortes, Begoña Colas, Maria J. Macias* and Antoni Riera*

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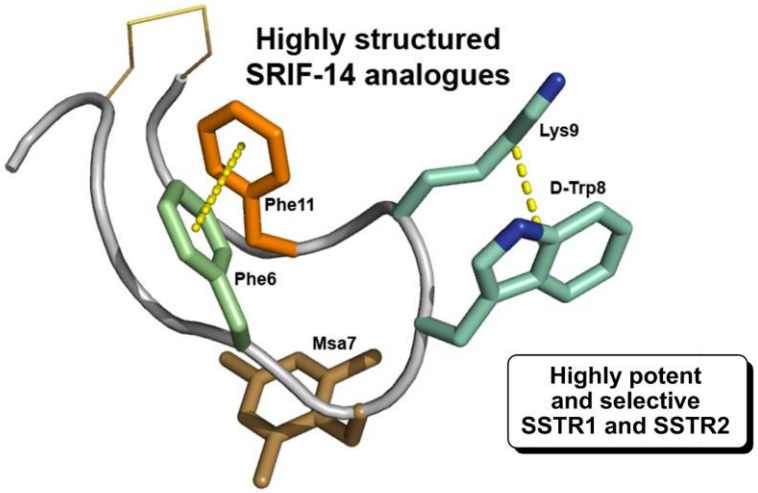
Fine-tuning the π–π Aromatic Interactions in Peptides: New Somatostatin Analogs Containing Mesityl Alanine.



Four new somatostatin analogs with greater conformational rigidity than the parent compound and interesting biological profiles have been prepared by substituting residues Phe6, Phe7 or Phe11 in the native sequence with mesityl alanine (Msa) and by substituting L-Trp8 with D-Trp8. Detailed structures have been established based on NMR data. [L-Msa6,D-Trp8]-SRIF exhibits high affinity for receptors SSTR3 and 5, whereas [L-Msa7,D-Trp8]-SRIF shows high affinity for SSTR1 and SSTR2. Our results demonstrate that fine-tuning of non-covalent interactions between the side-chains of aromatic amino acids can be exploited for modulating affinity and selectivity in peptide-drug design.