Design and Synthesis of Conformationally Constrained Somatostatin Analogues with High Potency and Specificity for μ Opioid Receptors

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A series of cyclic, conformationally constrained peptides related to somatostatin were designed and synthesized in an effort to develop highly selective and potent peptides for the μ opioid receptor. The following new peptides were prepared and tested for their μ opioid receptor potency and selectivity in rat brain binding assays: D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (**2**, CTOP); D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (**3**, CTAP); D-Phe-Cys-Tyr-D-Trp-Nle-Thr-Pen-Thr-NH_2 (**4**); D-Phe-Cys-Tyr-D-Trp-Lys-Val-Pen-Thr-NH_2 (**5**); D-Phe-Cys-Tyr-D-Trp-Lys-Gly-Pen-Thr-NH_2 (**6**); D-Phe-Cys-Tyr-Trp-Lys-Thr-Pen-Thr-NH_2 (**7**); D-Tyr-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH (**8**); D-PhGly-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (**9**); and D-PhGly-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr-OH (**10**). The most selective peptide, **2** (CTOP), displayed both high affinity (IC_{50} = 3.5 nM) and exceptional selectivity (IC_{50} δ/IC_{50} μ = 4000) for μ opioid receptors. Furthermore, **2** exhibited very low affinity for somatostatin receptors in the rat brain (IC_{50} > 24 000 nM), with an IC_{50} somatostatin/IC_{50} μ receptor selectivity of 8750. These conformationally constrained cyclic peptides should provide new insight into the structural and conformational requirements for the μ opioid receptor and the physiological role of this receptor.

The cyclic tetradecapeptide somatostatin, H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys--OH is an important regulatory hormone that is distributed throughout the central and peripheral nervous system and exhibits a broad range of biological activities.[2-4] These physiological activities of the hormone have been shown to be related to somatostatin's structural and conformational properties.[5,6] These studies have provided the basis for the development of new analogues that are more potent, and in some cases more selective, than somatostatin in their biological activities. For example, a cyclic hexapeptide analogue of somatostatin *cyclo*[Pro-Phe-D-Trp-Lys-Thr-Phe] has been prepared and was found to be 50–100 times more potent than somatostatin for the inhibition of insulin, glucagon, and growth hormone release,[7] biological activities believed to be of physiological significance for endogenous somatostatin. As yet another example, two cyclic analogues of somatostatin, D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr(ol)[8] (SMS201-995) and the bicyclic analogue *cyclo*[Ala-Cys-Phe-D-Trp-Lys-Thr-Cys], were used to identify two different types of somatostatin receptors in the rat brain.[9] These and other studies[10] have demonstrated the value of conformational structure considerations in somatostatin structure–biological activity analysis.

While the physiological role of somatostatin in regulating growth hormone, insulin, and glucagon release seems well-established, its role in other systems, especially in the CNS, is less well defined. One such interaction is that with the opioid receptor in the brain where it has been demonstrated that somatostatin has a very weak interaction with the opioid receptor.[11,12] These interesting activities have led us to examine the possibility of developing somatostatin-like analogues with potent and receptor-specific opioid activity and little or no somatostatin-like activity.

For this purpose, we have utilized various structural considerations in conjunction with conformational constraints[13,14] to modify the somatostatin structure in a manner which enhances opioid-like activities and greatly decreases its binding activity at somatostatin receptors. In our previous investigations toward these goals[14,15] we prepared a series of cyclic somatostatin analogues containing penicillamine (Pen; β,β-dimethylcysteine) to further restrict the conformational properties of the cyclic peptide analogues. These structure–activity studies resulted in the development of analogues that displayed increasingly good affinity and selectivity for μ opioid receptors in the rat brain. These analogues have also provided important new information concerning the steric and electronic requirements of the μ receptor. To date, our most potent and

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977). All optically active amino acids are of the L variety unless otherwise stated. Other abbreviations include the following: DCC, dicyclohexylcarbodiimide; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; 2-ClZ, 2-chlorobenzyloxycarbonyl; 2-BrZ, 2-bromobenzyloxycarbonyl; HOBt, *N*-hydroxybenzotriazole; For, formyl; Tos, tosyl; N^{α}-Boc, N^{α}-*tert*-butyloxycarbonyl; Pen, penicillamine; PhGly, phenylglycine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; FABMS, fast atom bombardment mass spectrometry; CGP23,996, des-Ala^1,Gly^2-[desamino-Cys^3,Tyr^{11}]-3,14-dicarbasomatostatin.
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Table I. Binding Potencies and Selectivities of CTP Analogues in Competition With [^{3}H]Naloxone and [^{3}H]DPDPE Receptor Binding to Rat Brain Membranes[a,b]

peptide	IC_{50}, nM, binding vs. [^{3}H]naloxone	IC_{50}, nM binding vs. [^{3}H]DPDPE	ratio[c]
1, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	3.7 ± 0.6	8400 ± 2000	2250
2, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2	2.8 ± 0.5	13500 ± 2750	4800
3, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2	3.5 ± 0.5	4500 ± 772	1300
4, D-Phe-Cys-Tyr-D-Trp-Nle-Thr-Pen-Thr-NH_2	9.6 ± 2.4	5600 ± 600	580
5, D-Phe-Cys-Tyr-D-Thr-Lys-Val-Pen-Thr-NH_2	5.5 ± 2.0	2000 ± 380	360
6, D-Phe-Cys-Tyr-D-Trp-Lys-Gly-Pen-Thr-NH_2	539 ± 315	58000 ± 3000	110
7, D-Phe-Cys-Tyr-Trp-Lys-Thr-Pen-Thr-NH_2	1350 ± 106	>70000	>52
8, D-Tyr-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH	9100 ± 2000	27500 ± 7000	3
9, D-PhGly-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	350 ± 97	2800 ± 570	8
10, D-PhGly-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr-OH	2100 ± 370	32000 ± 8800	15

[a] All values are the arithmetic mean ± SD from at least three separate experiments done in duplicate. [b] Related data from ref 17. [c] Ratio = IC_{50} (vs. [^{3}H]DPDPE)/IC_{50} (vs. [^{3}H]naloxone).

selective analogue has been D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (**1**, CTP).[16] This compound was found to be about 270-fold selective for the μ vs. δ opioid receptors as measured by its binding to rat brain receptors utilizing [^{3}H]naloxone and [^{3}H][D-Ala^2,D-Leu^5]enkephalin ([^{3}H]DADLE), and to bind about 200-fold less potently to somatostatin binding sites in the brain as compared to somatostatin. Interestingly, the radioreceptor binding studies with [^{3}H]naloxone gave Hill numbers near unity[15] suggesting that these analogues were interacting at μ opioid receptor binding sites in a manner similar to that of the labeled antagonist. The Hill coefficients obtained from binding with [^{3}H]DADLE were less than 1 suggesting interactions with multiple receptor sites or conformational states.

Our previous studies have demonstrated that potent and selective μ opioid activity could be obtained, and the structure–biological activity analysis indicated that the Tyr^3 and Pen^7 residues and a C-terminal carboxamide group in **1** are particularly important for developing potent and selective μ opioid peptides. Utilizing these results as a starting point, we have further explored those structural features that would show increased μ receptor selectivity and at the same time lead to decreased binding potency to somatostatin receptor. In this paper we report the design, synthesis, and purification of analogues of CTP, **1**, with exceptional μ vs. δ receptor selectivity, and which is addition, display little affinity for somatostatin receptors in the rat brain.

Results and Discussion

To enhance the selectivity and potency of somatostatin-like analogues for the opioid receptor, we have examined the effect of various structural modifications of our conformational-constrained lead compounds with μ selectivity, D-Phe-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-NH_2 (Cys^2,Pen^{7}A, CP) and D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (Cys^2,Tyr^3,Pen^{7}A, **1**, CTP). In our previous studies we found that the use of Tyr in position 3, penicillamine (Pen) in position 7, and a carboxamide terminal[15] was particularly important for increasing μ receptor potency and selectivity. Though somatostatin-like activity was substantially decreased, we felt that a greater selectivity for μ opioid vs. somatostatin receptors would be desirable.

Peptides were prepared by the solid-phase method of peptide synthesis utilizing either a *p*-methylbenzhydrylamine resin for carboxamide terminal peptides or a standard chloromethylated Merrifield resin for carboxylate terminal peptides. Preformed symmetrical anhydrides of the N-protected amino acids were employed in all the coupling reactions. Peptides were cyclized to their disulfide form under high dilution using $K_3Fe(CN)_6$ at pH 8.4 as the oxidizing agent. Purification was made by a combination of gel filtration, SP-Sephadex cation exchange chromatography, and either partition chromatography on Sephadex G-25 or preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a Vydac C_{18} reverse-phase column. Purity was assessed by thin-layer chromatography, paper electrophoresis, analytical RP-HPLC, amino acid analysis, and fast atom bombardment mass spectrometry (FABMS). See the Experimental Section for details.

Nine new analogues (**2–9**) related to CTP (**1**) were prepared which primarily examined the effect of modifications at positions 1, 4, 5, and 6 of CTP (**1**) on the relative potency of the new compounds at the μ vs. the δ opioid receptor and at the μ opioid vs. the somatostatin receptor. The receptor binding affinities and specificities were assessed by examining the ability of the analogue to inhibit binding of receptor-specific μ ([^{3}H]naloxone), δ ([^{3}H]DPDPE), and somatostatin ([^{125}I]CGP23, 996) peptides (see the Experimental Section for the methods used in the bioassay studies). The results of the binding studies that assess the μ and δ opioid receptor binding potencies and μ receptor selectivities of the new analogues are shown in Table I, and the studies that assess the μ opioid receptor vs. somatostatin receptor binding potencies and selectivities of the new analogues are summarized in Table II. Inspection of the results shown in these tables demonstrate that (a) we have designed peptides which are highly potent for binding to μ opioid receptors; (b) at the same we have developed analogues that show very weak binding affinity for somatostatin receptors; and (c) in view of the wide variation in selectivity of the new peptide analogues for μ opioid vs. δ opioid receptors and for μ opioid vs. somatostatin receptors, it is apparent that these three brain receptors have quite different conformational structure requirements for high potency.

In every case examined in this study, the new analogues (Table I) have a higher affinity in displacing the μ opioid

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Table II. Inhibitory Potency and Selectivity of CTP Analogues on [^{125}I]CGP23,996 and [^{3}H]Naloxone Receptor Binding to Rat Brain Membranes[a,b]

peptide	[^{125}I]CGP23,996 IC_{50}, nM	[^{3}H]naloxone IC_{50}, nm	ratio[c]
1, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	690 ± 220	3.7 ± 0.2	187
2, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2	24330 ± 5600	2.8 ± 0.5	8700
3, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2	14330 ± 4330	3.5 ± 0.5	4090
6, D-Phe-Cys-Tyr-D-Trp-Lys-Gly-Pen-Thr-NH_2	13300 ± 2200	539 ± 315	25
7, D-Phe-Cys-Tyr-Trp-Lys-Thr-Pen-Thr-NH_2	740 ± 200	1350 ± 106	0.55
8, D-Tyr-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH	18500 ± 3500	9100 ± 2000	2.0
9, D-PhGly-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2	2300 ± 100	350 ± 97	6.6
10, D-PhGly-Pen-Phe-D-Trp-Lys-Thr-Cys-Thr-OH	947 ± 165	2100 ± 420	0.45
somatostatin[c]	6.0 ± 1.7	2000 ± 1800	0.0003

[a] All values are the arithmetic mean ± SD from at least three separate experiments done in duplicate. [b] Data from ref 16; related data see ref 17. [c] Ratio = IC_{50} (vs. [^{125}I]CGP/IC_{50} (vs. [^{3}H]naloxone).

receptor specific [^{3}H]naloxone than in displacing the δ opioid receptor specific [^{3}H]DPDPE. This is in keeping with our previous findings that the Cys^2, Tyr^3, Pen^7, and C-terminal carboxamide substitutions are particularly suitabile for the μ vs. δ opioid receptor specificity. Thus, D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (Cys^2,Tyr^3,Pen^{7}A, **1**, CTP, Table I), the parent compound of our new cyclic analogues, has an IC_{50} value of 3.7 nM in inhibiting [^{3}H]naloxone binding, and a very high IC_{50} value of 8400 nM in inhibiting [^{3}H]DPDPE binding. This compound therefore shows high μ opioid receptor vs. δ receptor specificity with a ratio of 2250. However, **1** still retains fairly high binding potency at somatostatin receptors with an IC_{50} value of 690 nM in inhibiting [^{125}I]CGP23,996 binding. Though this peptide has considerable specificity for μ opioid vs. somatostatin receptor in the rat brain with a selectivity ratio of 197 (Table II), we sought to find analogues with much lower potencies at somatostatin vs. opiate receptors in the brain.

The importance of the 1-position to potency and selectivity for these receptors was examined by replacing the D-Phe residue with the more conformationally constrained amino acid D-phenylglycine (D-PhGly). The resulting analogue, D-PhGly-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2, **9** (Table I), was about 100-fold less potent in binding to the μ receptor than **1** (Table I), but interestingly was about 2.5 times more potent binding to the δ receptor (Table I), and had only a 3.3 decreased binding to the somatostatin receptor relative to **1** (Table II). Interestingly, when in addition to the D-PhGly substitution the Tyr^3 residue is replaced by Phe^3 and the carboxamide terminal by a carboxylate terminal, D-PhGly-Cys-Phe-D-Trp-Lys-Thr-Pen-Thr-OH, **10** (Table I), which was weakly potent at all receptors, was obtained, but unexpectedly **10** was more selective than **9** in the opioid binding assay (Table I), and in addition **10** showed increased potency relative to **9** in the somatostatin binding assay (Table II). Thus, analogue **10** actually is a weak but nonetheless somatostatin receptor selective analogue. Finally, when the D-Phe^1 is replaced by D-Tyr^1, Pen^7 by Cys^7, and the C-terminal is a carboxylate, D-Tyr-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH, **8** (Table I), is obtained, with little selectivity and very weak potency at all three receptors (Tables I and II).

The importance of the Thr^6 residue in CTP (**1**) to its high receptor potency and selectivity was next examined by either replacing Thr^6 with the more lipophilic pseudoisosteric amino acid Val (D-Phe-Cys-Tyr-D-Trp-Lys-Val-Pen-Thr-NH_2, **5**, Table I) or by completely removing the side chain in position 6 by replacing Thr^6 with Gly^6 (D-Phe-Cys-Tyr-D-Trp-Lys-Gly-Pen-Thr-NH_2, **6**, Table I). In the former analogue only a slight decrease in binding at the μ receptor was noted, but a 4-fold increase in binding at the δ receptor was observed (Table I) giving a less receptor-specific analogue. The Gly^6 analogue **6** (Table I) showed a further 100-fold decrease in binding to the μ opioid receptor and a further 29-fold decrease in binding at the δ opioid receptor making it a weak and less selective opioid receptor binding peptide. This analogue also showed greatly reduced binding at the somatostatin receptor relative to **1** (Table II) and hence showed only a 23-fold binding ratio for the μ opioid receptor vs. the somatostatin receptor.

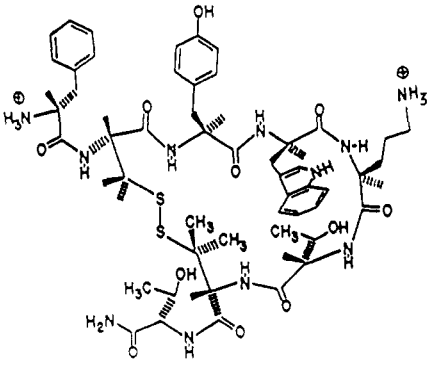

Figure 1. Structure of H-D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP, **2**).

The most interesting results came from examining the steric and electronic requirements for the 5-position of CTP (**1**). Previous structure–activity studies of somatostatin have shown the importance of Lys^9 in the native hormone for somatostatin biological activity.[17] Thus, replacing the Lys^5 residue with Arg gave analogue **3**, which was equipotent in binding at the μ receptor and about 2-fold more potent in binding at the δ receptor relative to CTP (**1**, Table I). The most interesting finding, however, was the nearly 21-fold *decrease* in binding potency at the somatostatin receptor of **3** vs. **1** (Table II). Hence, the

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analogue D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP, **3**) is about 1200-fold selective for the μ vs. δ receptor binding sites and over 4000-fold selective for μ opioid receptor binding vs. the somatostatin receptor binding in the rat brain. This result prompted us to replace the Lys^5 residue with Orn^5. This substitution results in the shortening of the side chain group by one methylene group while retaining a basic functional group. The analogue thus obtained, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, (CTOP, **2**, Figure 1), again retains almost the same binding potency at the μ receptor, but is over 1.6 times less potent in binding to the δ receptor than CTP (**1**, Table I). Hence, this highly potent peptide for binding at the μ receptor is about 4000-fold selective for the μ vs. δ receptor in the rat brain. Furthermore, as shown in Table II, CTOP (**2**) has very weak binding to the somatostatin receptor in the rat brain with an IC_{50} of 24 330 nM (about 4000 times that of somatostatin). This peptide has 8700-fold selectivity for μ opioid receptor binding sites vs. somatostatin sites. Essentially, we have designed a peptide, CTOP (**2**), initially based on the somatostatin structure, that has virtually no somatostatin-like binding properties (1/4000 the binding potency of somatostatin—Table II), but instead is a highly opioid receptor specific and potent peptide (7100-fold more potent than somatostatin—Table II). Interestingly, replacement of Orn^5 with the pseudoisosteric but now neutral and lipophilic residue norleucine (Nle) provides analogue **4**, D-Phe-Cys-Tyr-D-Trp-Nle-Thr-Pen-Thr-NH_2 (Table I), with only a 2.7-fold loss in potency in binding to the μ opioid receptor. However, analogue **4** binds to the δ opioid receptor about 2.3 times better than CTOP (**2**) and hence is not as opioid receptor selective.

The studies demonstrate that the substitution of Lys^5 for an Orn^5 residue in the Pen^7-containing cyclic peptides such as CTP (**1**) resulted in a ligand with high potency and selectivity for μ opioid receptor binding sites and very weak affinity for somatostatin receptor binding sites. Therefore, it should be a useful ligand for furthermore characterization at the μ opioid receptor due to its high affinity and selectivity toward this class of opioid receptors and its very weak interactions with somatostatin receptors. The availability of these conformationally constrained cyclic peptides should provide new insights into the structural and conformational requirements for the μ opioid receptor and the physiological roles of these receptors.

Experimental Section

General Methods. Amino acid analysis was performed on a Beckman Model 120C amino acid analyzer after acid hydrolysis in sealed tubes with either 4 M methanesulfonic acid or 6 M HCl containing 0.1% phenol, at 110 °C for 22 h in vacuo. Fast atom bombardment mass spectra (FABMS) were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas. Ascending TLC was performed on Baker 250-μm analytical silica gel glass plates in the following solvent systems: (I) butanol/acetic acid/water, 4:1:5 (v/v), upper phase; (II) butanol/acetic acid/water/pyridine, 15:3:10:12 (v/v); (III) butanol/acetic acid/water/pyridine, 6:1.2:4.8:6 (v/v); and (IV) isopropyl alcohol/ammonia/water, 3:1:1 (v/v). Paper electrophoresis was at 450 V for 90 min at 4 °C; peptides moved toward the cathode (reported as centimeters from the origin). Buffer systems are as follows: pH 2.2, 88% formic acid/acetic acid/water, 12:10:75 (v/v), and pH 5.6, pyridine/acetic acid/water, 23:6:970 (v/v). Peptides were visualized by UV fluorescence quenching, ninhydrin, Pauly reagent, and iodine. All peptides gave a positive Ehrlich test. HPLC was performed with the following solvent systems: (V) Vydac 218TP15-16 C_{18}RP column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 78:22 (v/v), at a flow rate of 2.5 mL/min; (VI) Vydac 218TP5 C_4RP column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 77:23 (v/v), at a flow rate of 1.5 mL/min; and (vII) Vydac 218TP5 C_4RP column (25 cm × 4.5 mm) with 6.1% trifluoroacetic acid/CH_3CN, 77:23 (v/v), at a flow rate of 1.5 mL/min. Optical rotations were determined with a Perkin-Elmer 241 MC polarimeter at the sodium D line.

Solid-Phase Peptide Synthesis of Cyclic Peptides. Analogues were prepared by standard solid-phase synthetic techniques previously used in our laboratory.[19] Briefly, *N-tert*-butyloxycarbonyl (Boc) protected amino acids were used throughout the synthesis and were purchased from Vega Biotechnology (Tucson, AZ), Bachem (Torrance, CA), or were prepared by published methods. Peptides with a COOH-terminal carboxylic acid groups were prepared by first attaching N^{α}-Boc-*O*-Bzl-L-threonine (Bzl = benzyl) to chloromethylated copoly-(styrene–1% divinylbenzene) beads (Lab Systems, 0.71 mequiv of Cl/g of resin).[20] Carboxamide peptides were prepared by using a *p*-methylbenzhydrylamine resin (pMBHA resin, Peptides International, 0.35 mmol/g of resin). Reactive side chains were protected as follows: Thr, benzyl ether; Lys, 2-chlorobenzyloxycarbonyl; Orn, benzyloxycarbonyl; Arg, tosyl; Tyr, 2-bromobenzyloxycarbonyl; Cys and Pen, *p*-methylbenzyl; Trp was used without protection of the indole nitrogen. Peptides were synthesized on a Vega Model 250 peptide synthesizer. A 1.5 M excess of preformed symmetrical anhydrides[21] was used for all coupling reactions, which were monitored by ninhydrin[22] and/or chloranil[23] tests and repeated as necessary. Boc protection was removed at each step by two treatments with 40% TFA in methylene chloride for 5 and 20 min each, except that after tryptophan was incorporated into the growing peptide, the TFA solution was modified to also contain 10% ethane dithiol and 5% carbon disulfide. Peptides were deprotected and removed from the resin with anhydrous liquid HF (10 mL/g of resin) containing 10% anisole at 4 °C for 45 min. After removal of the HF in vacuo, the free peptides were extracted with 30% acetic acid and the aqueous solution washed with ethyl ether and lyophilized. After cyclization with 0.01 M $K_3Fe(CN)_6$ at pH 8.4, the analogues were purified by gel filtration with 5% acetic acid on Sephadex G-15, SP-Sephadex cation-exchange chromatography, and either partition chromatography (butanol/benzene/pyridine/0.1% acetic acid, 6:2:1:9, v/v) on Sephadex G-25 (block polymerizate)[18,24] or preparative HPLC (Vydac C_{18}RP column, 1 in. × 25 cm, 0.1% TFA/CH_3CN buffer). Purity was assessed by thin-layer chromatography in a minimum of four solvent systems, by paper electrophoresis at two different pH values, and by analytical reversed-phase HPLC (Table III). Integration of the HPLC chromatograms (at 214 nm) indicated purities were in excess of 97%. Amino acid analysis after acid hydrolysis gave the proper molar ratios (±7.0%) of the constituent amino acids. Cysteine was determined as cysteic acid; penicillamine and tryptophan were not determined.

D-Phe-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH_2 (CTP, 1). N^{α}-Boc-Thr(*O*-Bzl) was coupled to *p*-methylbenzhydrylamine resin using a 2-fold excess of the preformed symmetrical anhydride in methylene chloride. No reactive amino groups on the resin were detected by the ninhydrin test after 30 min. The substitution of protected threonine on the resin was 0.33 mmol/g of resin. The protected peptide resin corresponding to the title compound was obtained after stepwise coupling of the following N^{α}-Boc-protected amino acids (in order of addition): N^{α}-Boc-Pen(*S*-4-MeBzl); N^{α}-Boc-Thr(*O*-Bzl); N^{α}-Boc-Lys(N^{ϵ}-2-ClZ); N^{α}-Boc-D-Trp; N^{α}-Boc-Tyr(*O*-2-BrZ); N^{α}-Boc-Cys(*S*-4-MeBzl); N^{α}-Boc-D-Phe. After coupling the last amino acid, the N^{α}-Boc protecting group was

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Table III. Chemical Characteristics of Synthetic Peptides

peptide[a]	thin-layer chromatography[b] R_f values				paper electrophoresis[c]		HPLC (k')[d]		FABMS[e]	
	I	II	III	IV	pH 2.2	pH 5.6	V	VI	$[M + H]_{obsd}$	$[M + H]_{calcd}$
2, Cys^2,Tyr^3,Orn^5,Pen^{7}A	0.24	0.71	0.64	0.87	11.2	8.7	2.6	2.5	1062.6	1062.4
3, Cys^2,Tyr^{3}Arg^5,Pen^{7}A	0.25	0.74	0.68	0.85	11.3	8.8	3.6	3.2	1104.9	1104.5
4, Cys^2,Tyr^3,Nle^5,Pen^{7}A	0.46	0.83	0.79	0.96	8.4	6.4	>50.0	>30.0	1061.5	1061.4
5, Cys^2,Tyr^3,Val^6,Pen^{7}A	0.31	0.74	0.67	0.86	11.1	8.7	N.D.	5.4	1073.4	1073.4
6, Cys^2,Tyr^3,Gly^6,Pen^{7}A	0.22	0.70	0.65	0.88	11.2	8.6	3.9	2.4	1032.4	1032.4
7, Cys^2,Tyr^3,Trp^4,Pen^{7}A	0.26	0.70	0.64	0.85	11.2	8.7	2.8	3.9	1076.5	1075.4
8, D-Tyr^1,Cys^2,Tyr^3,Cys^7	0.26	0.75	0.64	0.84	11.2	6.7	2.1	3.6	1065.4	1065.4
9, D-PhGly^1,Cys^2,Tyr^3,Pen^{7}A	0.22	0.69	0.65	0.83	11.2	8.7	2.7	4.1	1062.5	1062.4
10, D-PhGly^1,Pen^2,Cys^7	0.15	0.65	0.61	0.81	11.2	6.8	5.5	4.7	1047.5	1047.4

[a] Cys^2,Tyr^3,Trp^4,Pen^{7}A = D-Phe-Cys-Tyr-Trp-Lys-Thr-Pen-Thr-NH_2. [b] Baker 250-mm analytical silica gel glass plates were used. Solvent systems are as follows: (I) butanol/acetic acid/water, 4:1:5 (v/v) (upper phase); (II) butanol/acetic acid/water/pyridine, 15:3:10:12 (v/v); (III) butanol/acetic acid/water/pyridine, 6:1:2:4.8:6 (v/v); (IV) isopropyl alcohol/ammonia/water, 3:1:1 (v/v). [c] Electrophoresis was at 450 V for 90 min at 4 °C; peptides moved toward the cathode (reported as centimeters from the origin). [d] Capacity factor for the following systems: (V) Vydac 218TP15-16 C_{18} reversed-phase (RP) column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 78:22 (v/v), at a flow rate of 2.5 mL/min; (VI) Vydac 218TP5 C_4RP column (25 cm × 4.6 mm) with 0.1% trifluoroacetic acid/CH_3CN, 77:23 (v/v), at a flow rate of 1.0 mL/min. All peptides were monitored at λ = 214 nm. ND, not determined. [e] FABMS were obtained on a Varian 311A spectrometer equipped with an Ion Tech Ltd. source with xenon as the bombarding gas. Molecular weights are in grams per mole.

removed, the amino group neutralized, and the resulting protected peptide resin H-D-Phe-Cys(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Lys(N^ϵ-2-ClZ)-Thr(*O*-Bzl)-Pen(*S*-4-MeBzl)-Thr(O-Bzl)-pMBHA resin, dried in vacuo. A portion of the protected peptide resin (1.0 g) was cleaved from the resin by liquid HF (vide supra). After evaporation of the HF and anisole in vacuo at 0 °C, the dried product was washed with ethyl ether (3 × 30 mL), extracted with 30% aqueous HOAc (3 × 30 mL), and lyophilized. The peptide powder was dissolved in 1.5 L of 0.1% aqueous acetic acid under a blanket of nitrogen, and the pH was adjusted to 8.4 with aqueous ammonia. Then 0.01 M $K_3Fe(CN)_6$ was added until a yellow color persisted using an excess of $K_3Fe(CN)_6$. After 60 min at room temperature, the reaction was terminated by adjusting the pH to 4.5 with acetic acid. Excess ferro- and ferricyanide were removed by the addition of 15 mL of Bio Rad 3 × 4A (Cl^- form) anion exchange resin. After stirring for 30 min, the resin was filtered off, the filtrate lyophilized, and the peptide powder purified by gel filtration on a 100 × 2.5 cm Sephadex G-15 column using 30% acetic acid as eluent solvent. The major peptide peak was isolated and lyophilized to give a white powder. The desalted peptide was dissolved in a 10% acetic acid/20 mM sodium acetate buffer and applied to a SP-Sephadex cation-exchange column (40 × 2.4 cm). The peptide was eluted with a linear salt (NaCl) gradient from 0.3 to 1.4 M in the same buffer. The major peak, eluting at approximately 0.8 M NaCl, was isolated and lyophilized. After desalting on Sephadex G-10 with 30% acetic acid the compound was finally purified by partition chromatography (butanol/benzene/pyridine/0.1% acetic acid, 6:2:1:9, v/v) on Sephadex G-25 (block polymerizate), R_f = 0.16. The purified peptide was isolated by lyophilization to give a white powder: yield 16% based on starting substitution on the resin; $[\alpha]^{25}_D$ −25.6° (c 1.0, 0.2 M HOAc). Amino acid anal.: Phe 1.02 (1.0); Cys, 0.97 (1.0); Tyr 1.00 (1.0); Lys 0.98 (1.0); Thr, 1.91 (2.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (2, CTOP). The protected peptide resin to the title compound was prepared as reported for 1, except that N^α-Boc-Orn(N^ϵ-Z) was used instead of N^α-Boc-Lys(N^ϵ-2-ClZ) in the coupling scheme to give D-Phe-Cys(*S*-4MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Orn(N^ϵ-Z)-Thr(*O*-Bzl)-Pen(S-4-MeBzl)-Thr(*O*-Bzl)-pMBHA resin. The peptide was removed from the resin, the protecting groups cleaved, the compound cyclized, and the title compound purified as reported for 1, except that final purification was by preparative HPLC (see above for conditions) to give the product **2** as a white powder: yield 14%; $[\alpha]^{25}_D$ −26.6° (c 1.0, 0.2 M HOAc). Amino acid anal.: Phe, 1.06 (1.0); Tyr, 1.00 (1.0); Orn, 0.96 (1.0); Thr 1.88 (2.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (3, CTAP). The protected peptide resin was prepared as reported for 1 (vide supra), except that N^α-Boc-Arg(N^G-Tos) was used instead of N^α-Boc-Lys(N^ϵ-2-ClZ) in the coupling scheme to give D-Phe-Cys(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Arg(N^G-Tos)-Thr(*O*-Bzl)-Pen(*S*-4-MeBzl)-Thr(*O*-Bzl)-pMBHA resin. The peptide was cleaved from the resin, the protecting groups removed, the compound cyclized, and the title compound purified as reported for **2** to give the title compound **3** as a white powder: yield 11%. Amino acid anal.: Phe, 1.00 (1.0); Tyr, 1.03 (1.0); Arg, 0.97 (1.0); Thr, 1.91 (2.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-Phe-Cys-Tyr-D-Trp-Nle-Thr-Pen-Thr-NH_2 (4). The protected peptide resin to the title compound was prepared as reported for 1, except that N^α-Boc-Nle was used instead of N^α-Boc-Lys(N^ϵ-2-ClZ) in the coupling scheme to give D-Phe-Cys-(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Nle-Thr(*O*-Bzl)-Pen(*S*-4-MeBzl)-Thr(*O*-Bzl)-pMBHA resin. The cyclic peptide **4** was obtained in a purified form as outlined for compound **2** to give a white powder of **4**: yield 8%. Amino acid anal.: Phe, 0.94 (1.0); Tyr 1.00 (1.0); Nle, 1.06 (1.0); Thr, 1.84 (2.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-Phe-Cys-Tyr-D-Trp-Lys-Val-Pen-Thr-NH_2 (5). The protected peptide resin to the title compound was prepared as reported for 1, except that N^α-Boc-Val was used instead of the N^α-Boc-Thr(*O*-Bzl) in the 6-position of the peptide in the coupling scheme to give D-Phe-Cys(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Lys(N^ϵ-2-ClZ)-Val-Pen(*S*-4-MeBzl)-Thr(*O*-Bzl)-pMBHA resin. The cyclic peptide **5** was obtained in a purified form as outlined for compound **2** to give a white powder of **5**: yield 16%. Amino acid anal.: Phe, 0.94 (1.0); Tyr, 0.95 (1.0); Lys, 1.04 (1.0); Val, 1.00 (1.0); Thr 0.96 (1.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-Phe-Cys-Tyr-D-Trp-Lys-Gly-Pen-Thr-NH_2 (6). The protected peptide resin to the title compound was prepared as reported for 1, except that N^α-Boc-Gly was used instead of the N^α-Boc-Thr(*O*-Bzl) in the 6-position of the peptide in the coupling scheme to give D-Phe-Cys(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Lys(N^ϵ-2-ClZ)-Gly-Pen(*S*-4-MeBzl)-Thr(*O*-Bzl)-pMBHA resin. The cyclic peptide **6** was obtained in a purified form as outlined for compound 1 to give a white powder of **6**: yield 13%. Amino acid anal.: Phe, 0.96 (1.0); Tyr, 0.94 (1.0); Lys, 1.10 (1.0); Gly, 1.00 (1.0); Thr, 0.93 (1.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-Phe-Cys-Tyr-Trp-Lys-Thr-Pen-Thr-NH_2 (7). The protected peptide resin to the title compound was prepared as in 1, except that N^α-Boc-L-Trp was used instead of N^α-Boc-D-Trp in the 4-position of the peptide in the coupling scheme to give D-Phe-Cys(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-Trp-Lys(N^ϵ-2-ClZ)-Thr(*O*-Bzl)-Pen(*S*-4-MeBzl)-Thr(*O*-Bzl)-pMBHA resin. The cyclic peptide **7** was obtained in a purified form as outlined for compound **2** to give a white powder of **7**: yield 13%. Amino acid anal.: Phe, 0.96 (1.0); Tyr, 0.96 (1.0); Cys 0.99 (1.0); Lys, 1.04 (1.0); Thr,

1.89 (2.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-Tyr-Cys-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH (8). The protected peptide resin to the title compound was prepared as reported for 1, except that a standard chloromethylated copoly(styrene–1% divinylbenzene) resin (vida supra, Merrifield resin) was used. The initial C-terminal amino acid N^{α}-Boc-Thr(*O*-Bzl) was attached to the resin by the method of Gisin[20] to give the resin substituted with Thr to the extent of about 0.40 mmol/g of resin. This resin was then used directly in the synthesis. The protected peptide resin corresponding to the title compound was obtained by stepwise coupling of the following N^{α}-Boc-protected amino acids (in order of addition): N^{α}-Boc-Cys(*S*-4-MeBzl); N^{α}-Boc-Thr(*O*-Bzl); N^{α}-Boc-Lys(N^{ϵ}-2-ClZ); N^{α}-Boc-D-Trp; N^{α}-Boc-Tyr(*O*-2-*BrZ*); N^{α}-Boc-Cys(*S*-4-MeBzl); N^{α}-Boc-D-Tyr(*O*-2-BrZ). After removal of the N^{α}-Boc protecting group and drying in vacuo there was obtained D-Tyr(*O*-2-BrZ)-Cys(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Lys(N^{ϵ}-2-ClZ)-Thr(*O*-Bzl)-Cys(*S*-4-MeBzl)-Thr(*O*-Bzl)-*O*-resin. The peptide was cleaved from the resin and the protected groups removed in the usual manner using HF; the compound was cyclized and the title compound purified as reported in 1 to give 8 as a white powder: yield 9%. Amino acid anal.: Tyr, 1.94 (2.0); Lys, 0.93 (1.0); Thr, 1.84 (2.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-PhGly-Cys-Tyr-D-Trp-Lys-Thr-Pen-Thr-NH$_2$ (9). The protected peptide resin to the title compound was prepared as reported for 1, except that N^{α}-Boc-D-PhGly was used instead of N^{α}-Boc-D-Phe in the 1-position of the peptide in the coupling scheme to give D-PhGly-Cys(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Lys(N^{ϵ}-2-ClZ)-Thr(*O*-Bzl)-Pen(*S*-4-MeBzl)-Thr(*O*-Bzl)-pMBHA resin. The cyclic peptide was obtained in a purified form by methods outlined for compound 1 to give 9 as a white powder: yield 10%. Amino acid anal.: Cys, 1.02 (1.0); Tyr, 1.06 (1.0); Thr, 1.86 (2.0); Lys, 0.94 (1.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

D-PhGly-Pen-Tyr-D-Trp-Lys-Thr-Cys-Thr-OH (10). The protected peptide resin to the title compound was prepared as above except that N^{α}-Boc-Pen(*S*-4-MeBzl) was used instead of N^{α}-Boc-Cys(*S*-4-MeBzl) in the 2-position and N^{α}-Boc-D-PhGly instead of N^{α}-Boc-D-Tyr(*O*-2-BrZ) in the 1-position to give D-PhGly-Pen(*S*-4-MeBzl)-Tyr(*O*-2-BrZ)-D-Trp-Lys(N^{ϵ}-2-ClZ)-Thr(*O*-Bzl)-Cys(*S*-4-MeBzl)-Thr(O-Bzl)-*O*-resin. The peptide was cleaved from the resin, the protecting groups removed, the peptide cyclized, and the title compound obtained in a highly purified form as outlined for 8 to give the product 10 as a white powder: yield 16%. Amino acid anal.: Tyr, 0.94 (1.0); Thr, 1.88 (2.0); Lys, 1.00 (1.0). The TLC, paper electrophoresis, analytical HPLC, and FABMS data are given in Table III.

Radioreceptor Assays. Adult Sprague-Dawley rats (150–200 g) were killed and the brains were rapidly removed and homogenized (10% w/v) in 0.32 M sucrose in a glass homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged at 1000*g* for 10 min, and the resulting pellet was resuspended in 50 mM Tris·HCl buffer (pH 7.4 at 25 °C) containing 5 mM $MgCl_2$, bovine serum albumin at 2 mg/mL, and bacitracin at 20 μg/mL by using a Polytron homogenizer (15 s, setting 5). The centrifugation and resuspension step was repeated once.

Rat brain plasma membranes (100 μL) were incubated at 25 °C for 120 min (180 min using [^{3}H]DPDPE) in a total volume of 1 mL of 50 mM Tris·HCl buffer (pH 7.4, as above) containing 0.2 nM [^{125}I]CGP23,996, 1 nM [^{3}H]naloxone, or [^{3}H]DPDPE and at least 10 freshly prepared concentrations of our synthetic somatostatin analogues. All incubations were done in duplicate using polypropylene test tubes, and every compound was tested at least three times. The concentration of test compounds was determined by quantitative amino acid analysis or from published molar extinction coefficients. Incubation was terminated by rapid filtration (Brendel M-24R cell harvester) of the incubation mixture through a GF/B glass fiber filter that had been pretreated with 0.1% polyethylenimine in order to reduce filter binding. Specific binding to somatotstatin or μ or δ opioid receptors was defined as the difference in the amounts of radioligands bound in the absence or presence of 1 μM somatostatin, 1 μM naltrexone, or 1 μM DPDPE, respectively. The data were analyzed by using nonlinear least-squares regression analysis on the Apple II+ computer. Programs were generously provided by SHM Research Corp., Tucson, AZ.

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5-HT$_1$ and 5-HT$_2$ Binding Characteristics of Some Quipazine Analogues

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Arylpiperazines, such as 1-(3-trifluoromethylphenyl)piperazine (TFMPP) and its chloro analogue mCPP, are 5-HT$_1$ agonists, whereas quipazine, i.e., 2-(1-piperazino)quinoline, appears to be a 5-HT$_2$ agonist. Radioligand binding studies using rat cortical membrane homogenates and drug discrimination studies using rats trained to discriminate a 5-HT$_1$ agonist (i.e., TFMPP) or a 5-HT$_2$ agonist (i.e., 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (DOM)) from saline reveal that quipazine and its 1-deaza analogue 2-naphthylpiperazine (2-NP) bind at 5-HT$_1$ and 5-HT$_2$ sites but produce stimulus effects similar to those of DOM. A structurally related compound, 1-naphthylpiperazine (1-NP), possesses a high affinity for 5-HT$_1$ (K_i = 5 nM) and 5-HT$_2$ (K_i = 18 nM) sites. 1-NP produces stimulus effects similar to those of TFMPP and is able to antagonize the stimulus effects produced by DOM. The present results suggest that the unsubstituted benzene ring of quipazine, and of its 1-deaza analogue 2-naphthylpiperazine, makes a significant contribution to the binding of these agents to 5-HT$_2$ sites and, more importantly, may account for their 5-HT$_2$ agonist properties.

Central serotonin (5-HT) binding sites have been divided into two major populations: 5-HT$_1$ sites, which display a high affinity for tritiated 5-HT, and 5-HT$_2$ sites, which display a high affinity for tritiated spiperone and ketanserin.[1,2] Arylpiperazines constitute a group of fairly se-

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