Improvement in Potency of an Oxytocin Antagonist after Systematic Substitutions with L-Tryptophan†

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We report twelve analogues (1–12) of [Pmp¹,D-Trp²,Arg⁸]oxytocin, ANTAG (Pmp = β,β-pentamethylene-β-mercaptopropionic acid), which is a potent antagonist (pA_2 = 7.77) of the uterotonic effect of oxytocin (OT) in rats, as measured in a uterotonic assay. Nine of the following analogues were designed by replacement of each of the nine residues in ANTAG with an L-tryptophan residue: [Ac-Trp¹,D-Trp²,Val⁶,Arg⁸]OT (1), [Pmp¹,Trp²,Arg⁸]OT (2), [Pmp¹, D-Trp²,Trp³,Arg⁸]OT (3), [Pmp¹,D-Trp²,Trp⁴,Arg⁸]OT (4), [Pmp¹,D-Trp²,Trp⁵,Arg⁸]OT (5), [Aaa¹,D-Trp²,Trp⁶,Arg⁸]OT (6), [Aaa¹,D-Trp²,Val⁶,Arg⁸]OT (7), [Pmp¹,D-Trp²,Ica⁷,Arg⁸]OT (8), [Pmp¹,D-Trp²,Trp⁷,Arg⁸]OT (9), [Pmp¹,D-Trp²,Trp⁸]OT (10), [Pmp¹,D-Trp²,Arg⁸,Trp⁹]OT (11), [Pmp¹,D-Trp²,Arg⁸,Trp(For)⁹]OT (12). In these analogues Aaa = 1-adamantaneacetic acid, and Ica = indoline-2-carboxylic acid. All linear analogues and analogues featuring Trp substitutions in the ring sequence of ANTAG were OT antagonists of lower potency than the parent peptide. All the analogues featuring Trp subtitutions in the tail sequence of ANTAG were OT antagonists of equal or better potency than the parent peptide. Replacement with Ica⁷ gave analogue 8, equipotent with ANTAG, but replacement with Trp⁷ gave analogue 9, which shows almost a two-fold increase in potency (pA_2 = 8.06). Replacement with Trp⁹ gave analogue 11 (pA_2 = 8.03) which is about 1.8 times more potent than the parent antagonist, although Trp(For)⁹ had lower potency. Of great interest is that substitution with Trp⁸ leads to a more potent analogue, 10 (pA_2 = 8.22), which, unlike most antidiuretic hormone anatagonists, lacks any cationic charge in the molecule. The antidiuretic assay shows antagonists 9–11 to be weak antagonists of [Arg⁸]vasopressin, the antidiuretic hormone, with $pA_2 \leq 6.0$; hence, they may be interesting leads for future design of more potent and specific OT antagonists.

Introduction

There is great interest in designing highly potent and reversible antagonists of oxytocin (OT) as pharmacological tools to evaluate the role of this hormone in labor and to inhibit preterm labor.[1] A problem in their design is that OT may have developed in mammalians as an interesting example of molecular evolution.[2] In this view, the ancestral hormone arginine vasotocin gave rise to OT, for milk let down in the nursing female and to enhance the contractions of labor during delivery, and to its sister hormone arginine vasopressin (AVP), which is the antidiuretic hormone, for water conservation. Since the sequence of OT differs from that of AVP in only two amino acids, both hormones bind to OT and to AVP receptors and show similar biological properties, although with different affinities and different potencies in each target organ. Thus, it is not surprising that reversible antagonists to these hormones designed to date act as reversible antagonists for both AVP and OT.[3]

We have recently designed an interesting OT antagonist [Pmp¹,D-Trp²,Phe³,Ile⁴,Arg⁸]oxytocin.[4] In designing this antagonist we used D-tryptophan at position 2, which was of interest since [D-Trp²]OT had been reported to have antagonistic properties.[5,6] We selected β,β-pentamethylene-β-mercaptopropionic acid (Pmp)[7] because this substituent has led to stronger antagonists of OT;[7,8] and we chose the rest of the sequence because [Pmp¹,D-Phe²,Phe³,Ile⁴,Arg⁸]OT was among the most potent antagonists of OT in a rat uterotonic assay.[9] We found that our D-Trp² analogue was a potent antagonist, similar in potency to its D-Phe² counterpart. Further substitutions with isoleucine at position 3 and glutamine at position 4 made the sequence of the antagonist closer to that of the hormone itself and led to a more potent and selective antagonist [Pmp¹,D-Trp²,Arg⁸]OT (ANTAG) with higher specificity of action on the uterus than the kidney.[10]

Because [Trp⁸]OT was reported to be both an agonist and an antagonist of OT in the oxytocic bioassay using the rat uterus in vivo and in vitro,[11] we thought it desirable to replace position 8 of ANTAG with tryptophan. Once we found this analogue to be a very potent antagonist, we

† All symbols and abbreviations comply with the recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* 1984, *138*, 9). Unless otherwise indicated, amino acids are of the L configuration. Other abbreviations used are as follows: OT, oxytocin; AVP, [Arg⁸]vasopressin; Pmp,β,β-pentamethylene-β-mercaptopropionic acid; ANTAG, [Pmp¹,D-Trp², Arg⁸]oxytocin; Aaa, 1-adamantaneacetic acid; Ica, indoline, 2-carboxylic acid; MBHA, 4-methylbenzhydrylamine; DCM, dichloromethane; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; CHL, chloroform; TEA, triethylamine; Boc, *tert*-butyloxycarbonyl; ONp, 4-nitrophenyl ester; Tos, p-toluenesulfonyl; Meb, 4-methylbenzyl; For, formyl; Ac, acetyl; Bzl, benzyl; Pyr, pyridine; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamyl; NMR, nuclear magnetic resonance.

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Table I. Physicochemical Characteristics of OT Antagonists (OTAs)[a]

OTA no.	MW	yield,[b] mg (%)	OR,[c] deg	TLC[d] R_f A	B	C	D	HPLC t_R,[e] min
1	1196	112 (60)	−60	0.22	0.36	0.16	0.56	4.0
2	1126	44 (19)	−52	0.21	0.37	0.27	0.60	3.2
3	1199	70 (25)	−101	0.23	0.35	0.23	0.59	4.0
4	1184	40 (13)	−64	0.34	0.48	0.17	0.50	14.4
5	1198	32 (15)	−57	0.50	0.59	0.54	0.73	7.6
6	1231	68 (28)	−31	0.43	0.48	0.34	0.69	22.6
7	1144	92 (48)	−68	0.34	0.44	0.23	0.67	12.4
8	1174	40 (12)	−95	0.48	0.44	0.35	0.65	6.0
9	1215	42 (18)	−101	0.55	0.49	0.42	0.67	7.3
10	1156	37 (11)	−73	0.62	0.59	0.42	0.73	13.8
11	1256	41 (16)	−93	0.39	0.48	0.13	0.41	7.4
12	1284	36 (20)	−63	0.43	0.44	0.39	0.63	8.8

[a] The structures for the free peptides (1–12) is given in the Experimental Section. [b] These yields are based on the milliequivalents of Boc-amino acid-resin, or 4-methylbenzhydrylamine-resin used as starting material. [c] OR = optical rotation. OR was determined as $[\alpha]^{27}_D$, in degrees (c 1, 1 N AcOH). Peptide 10 was dissolved in 30% AcOH. [d] The composition of solvents A–D is given in the Experimental Section. [e] Solvent composition = 58% B, flow rate 1.8 mL/min. From the HPLC pattern, it is estimated that all the antagonists were at least 95–98% pure.

Table II. Amino Acid Analyses of OT Antagonists

amino acid[a]	amino acid ratios 1	2	3	4	5	6	7	8	9	10	11	12
Asp	1.01	1.04	1.03	1.00		0.97	0.99	0.94	1.00	1.00	1.00	0.94
Glu	1.00	1.04	1.06		1.06	0.98	0.97	0.95	0.96	1.05	0.95	0.94
Gly	0.99	0.96	1.02	0.97	0.94	1.00	1.02	0.98	1.04	0.98		
Arg	1.01	1.02	1.00	1.04	1.00	1.03	1.02	1.00	1.07		1.02	1.06
Pro	0.99	0.90	0.90	0.92	0.90	1.07	0.99			0.94	0.98	1.00
Val	1.00						1.00					
Cys		0.46	0.43	0.50	0.44			0.32	0.50	0.50	0.46	0.56
Pmp-SS-Cys + Ile	0.98	1.55	0.57	1.42	1.57	0.95	0.98	1.63	1.40	1.58	1.49	1.50
Trp[b]	1.70	0.87	1.52	1.50	1.48	1.46	0.79	0.82	1.48	1.84	1.66	1.46
Ica[c]								1.02				
Trp(For)[d]												0.79
Aaa[e]						0.82	0.95					

[a] PTC-Pmp-SS-Cys has UV absorption similar to and coelutes with PTC-Ile.[10] We report the combined values for Pmp-SS-Cys and Ile. [b] Tryptophan in peptides was determined by its UV absorption at 280 nm as we have reported.[38] The values found for tryptophan are still somewhat low and suggest that the peptide has several moles of AcOH, TFA, and/or H_2O, as has been our experience with tryptophyl peptides.[38] [c] A standard of indoline-2-carboxylic acid (Ica) was prepared for this determination. [d] Trp(For) in peptides was estimated by its UV absorption at 300 nm. [e] 1-Adamantaneacetic acid was determined by HPLC of hydrolysates at 220 nm.

decided to systematically replace each position of the sequence of ANTAG with tryptophan and to compare the biological effects of the resulting antagonists to those of the parent molecule. The analogues prepared were tested as antagonists of OT in the rat oxytocic bioassay[12] in the presence of magnesium salts, in vitro, by measuring the area under the peak recorded for uterine contractions.[13] We report here (Tables I and II) twelve new OT antagonists (1–12) and biological data for them and for [Pmp^1,D-Trp^2,Arg^8]OT (Table III) for comparison.

Results and Discussion

Peptide Synthesis. All peptides were assembled either manually by the solid phase (SP) method,[14,15] with modifications previously described,[16] or by an automated method employing an Applied Biosystems (ABS) 430A automated peptide synthesizer. The starting resin for the manual synthesis was Boc-Gly-resin, 1% cross-linked with divinylbenzene prepared by esterification of the chloromethylated resin with the cesium salt of Boc-Gly.[17] For the automated SP syntheses we used either a Boc-Gly-Pam-resin[18] or a Boc-Trp(For)-Pam-resin, and for the antagonist with the Trp(For)-NH_2[9] substituent, we coupled Boc-Trp(For) to a 4-methylbenzhydrylamine-resin.[19] For

Table III. pA_2 of Competitive Antagonists of OT Contractile Action in the Rat Uterus and pA_2 of Competitive Antagonism to AVP Antidiuretic Action in the Rat[a]

OT antagonist	no.	pA_2 OT[b]	pA_2 AVP[c]
[Pmp^1,D-Trp^2,Arg^8] OT, ANTAG[d]		7.77 ± 0.03	<5.9
[Ac-Trp^1,D-Trp^2,Val^6,Arg^8] OT	1	5.43 ± 0.24	
[Pmp^1,Trp^2,Arg^8] OT	2	6.50 ± 0.11	
[Pmp^1,D-Trp^2,Trp^3,Arg^8] OT	3	6.95 ± 0.12	
[Pmp^1,D-Trp^2,Trp^4,Arg^8] OT	4	6.83 ± 0.03	
[Pmp^1,D-Trp^2,Trp^5,Arg^8] OT	5	5.21 ± 0.27	
[Aaa^1,D-Trp^2,Trp^6,Arg^8] OT	6	6.52 ± 0.07	
[Aaa^1,D-Trp^2,Val^6,Arg^8] OT	7	5.37 ± 0.03	
[Pmp^1,D-Trp^2,Ica^7,Arg^8] OT	8	7.92 ± 0.18	
[Pmp^1,D-Trp^2,Trp^7,Arg^8] OT	9	8.06 ± 0.1	<5.8
[Pmp^1,D-Trp^2,Trp^8] OT	10	8.22 ± 0.14	<6.0
[Pmp^1,D-Trp^2,Arg^8,Trp^9] OT	11	8.03 ± 0.10	<6.0
[Pmp^1,D-Trp^2,Arg^8,Trp(For)^9] OT	12	7.67 ± 0.09	

[a] Preliminary results presented in part at the European Peptide Symposium, in Platja d'Aro, Spain, September 1990 and in the Proceedings of the Symposium.[43] [b] Rat oxytocic assay in vitro in the presence of Mg^{2+}. [c] Rat antidiuretic assay. These antagonists show also very weak agonism in this bioassay. [d] This analogue was previously reported.[10]

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the automated synthesis, the standard ABS protocol was used, using Boc-amino acids,[20] scission of the Boc-group with TFA to avoid side reactions with glutaminyl peptides,[21] neutralization with DIEA, and coupling of either symmetric anhydrides[22] or HOBt esters.[23] For the manual syntheses, the Boc group was removed at each cycle with 30% trifluoroacetic acid in DCM. However, Boc-D-Trp-peptide-resins were freed from Boc-groups by treatment with 30% TFA in DCM containing 1% mercaptoethanol to inhibit destruction of Trp[24,25] and 5% anisole to inhibit the alkylation of the indole ring by *tert*-butyl cations.[24] After neutralization of the resin with 10% DIEA in DCM, coupling was performed with the appropriate Boc-amino acid and DCC,[26] except for Boc-Asn and Boc-Gln, which were coupled as the 4-nitrophenyl esters to avoid dehydration of carboxamide functionalities to nitriles,[27] and Pmp(S-Bzl)[7] or Pmp(S-Meb),[28] which were incorporated by activation with DCC in the presence of HOBt. In the case of linear analogues lacking a disulfide ring, 1-adamantaneacetic acid was introduced in place of the Pmp residue. Introduction of Boc-Trp at position 1, acidolysis, neutralization, and acetylation with acetic anhydride gave the desired Ac-Trp substituent. Completion of coupling was monitored by means of the ninhydrin test.[29]

Except for peptide **12**, the final protected peptides were removed from the resin by ammonolysis,[21] which removed the *N*-formyl group from tryptophan.[30] Most of the protected peptides were freed from blocking groups by reduction with Na in liquid ammonia[31] and the disulfhydryl peptides were cyclized in very dilute solution[32] to the corresponding cyclic disulfide by oxidation with potassium ferricyanide.[33] In the case of analogues **1**, **6** and **7**, sodium and liquid ammonia gave several products, probably due to peptide bond scission after Val[6] and Trp[6] as suggested by amino acid analyses. Therefore, peptide precursors of analogues **1**, **6**, and **7** were more suitably freed from blocking groups by treatment with liquid HF/anisole.[34] Cleavage of the protected peptide-MBHA-resin for analogue **12** by direct HF/anisole treatment, followed by oxidative cyclization, gave the desired Trp(For)-amide analogue. The free peptides were desalted and freed from scavengers by gel filtration[35] on Sephadex G-15 with 50% AcOH,[36] followed by a final purification by preparative high-performance liquid chromatography (HPLC).

Amino acid analyses were performed for each analogue by using the HPLC–Picotag method,[37] using standards of indoline-2-carboxylic acid and Pmp-SS-Cys.[10] Tryptophan in peptides, was estimated by UV spectrophotometry[10,36] and 1-adamantane acetic acid in hydrolyzates was quantified by HPLC using a standard of the acid and monitoring at 220 nm.

Bioassays. All analogues were tested as antagonists of OT uterotonic action as previously described.[13] Isolated uteri from rats in natural estrus were incubated in Van Dyke-Hastings solution containing 0.5 mM Mg^{2+}. Isometric contractions in response to doses of OT were recorded with a force-displacement transducer and a Grass polygraph Model 79 and the response was quantified by integrating the area under the peaks obtained. The standard was a pure synthetic OT (450 units/mg) from Calbiochem. The pA_2 for each antagonist was determined by the method of Schild[12] in at least four separate assays. The most potent antagonists of the series were also tested in a rat antidiuretic assay performed in water-loaded rats under ethanol anesthesia, as previously described.[13] The AVP standard used was from Bachem Inc. An in vivo pA_2 was estimated by dividing the effective dose by the estimated volume of distribution of 67 mL/kg. The effective dose is referred to as the molar concentration of an antagonist that reduces the response to an agonist by one-half. Antagonists of OT having agonistic and antagonistic activity have the antagonistic potencies approximated.

Structure–Activity Relationships. The recent finding that linear OT analogues without disulfide bridges are moderately potent OT antagonists[39] led us to also substitute positions 1 and 6 of ANTAG with tryptophan. When introducing tryptophan at position 1, we chose to protect N-terminal tryptophan with an acetyl group, because past experience suggested that a free amino group might lead to agonistic activity,[6] and we chose valine-6 instead of cysteine. When introducing tryptophan at position 6 we introduced 1-adamantaneacetic acid at position 1, as chosen by Manning, to maintain similarity to his substituents. Finally, we prepared analogue **7**, [Aaa[1],D-Trp[2],Val[6],Arg[8]]OT in order to have both substituents in the same molecule as a reference to evaluate the tryptophyl substitution. Whereas analogue **7** and analogue **1**, [Ac-Trp[1],D-Trp[2],Val[6],Arg[8]]OT, were weak antagonists with $pA_2 = 5.37$ and 5.43, respectively, substitution of Val

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with Trp gave the more potent antagonist 6, [Aaa^1,D-Trp^2,Trp^6,Arg^8]OT, with pA_2 = 6.52. Nevertheless, all three analogues have sharply lower potency than the parent antagonist, ANTAG. Hruby et al. studied the conformation of OT and its competitive inhibitor [1-penicillamine]OT and analyzed the results in terms of conformational and dynamic properties.[45] In his view oxytocin has a flexible conformation whereas [1-penicillamine]OT has a more restricted conformation. This and subsequent results have suggested that cyclic sulfide analogues of OT featuring β,β-dialkyl substituents, or the similar Pmp^1 substitution, may support a more defined conformation which would lead to suitable requirements for antagonist potency.[46] Our linear analogues are probably too flexible and also suffer from important structural differences from the parent molecule. Alternatively, they may be susceptible to enzymatic cleavages not operative for the cyclic structures,[47] and they may bind to other receptors or participate in other unknown biological effects which may decrease potency.

Replacement of D-Trp^2 with L-Trp^2 gave [Pmp^1,Trp^2,Arg^8]OT, with pA_2 = 6.50, showing a substantial drop in biological potency, confirming the need for D-Trp^2 to maintain higher potency. We have previously hypothesized that Tyr^2 and Asn^5 have a critical role in occupation and activation of receptors.[40] It has also been suggested on conformational grounds that certain amino acids, for example aromatic amino acids of the D-configuration with para substituents, may possess high inhibitory potency.[46] The peptide sequence of the analogue may promote high binding affinity; however, the side chain of the aromatic D-amino acid instead of being located on top of the plane of the peptide ring, in proximity to the asparagine side chain, would be located away from the plane of the 20-membered ring, unable to participate in binding leading to transduction of the hormonal message.

Replacement of Ile^3 and Gln^4 with tryptophan gave analogues 3, [Pmp^1,D-Trp^2,Trp^3,Arg^8]OT, and 4, [Pmp^1,D-Trp^2,Trp^4,Arg^8]OT, with pA_2 = 6.95 and 6.93, respectively, which had lowered biological potency. The results might be caused by the substantial structural difference between tryptophan and Ile^3 and Gln^4.

Replacement of Asn^5 with Trp gave analogue 5, [Pmp^1,D-Trp^2,Trp^5,Arg^8]OT, pA_2 = 5.21, with the expected low biological activity, since L-Asn seems to be essential for binding to the neurohypophyseal hormone receptors and initiation of the hormonal message[40] and its substitution usually leads to substantial loss of potency.[40,41,6] Hence, all of the Trp substitutions in the ANTAG ring led to poorer antagonists.

The interesting replacement of Pro^7 with indoline-2-carboxylic acid, which has the same ring structure as in proline with an added aromatic ring, resulted in antagonist 8, [Pmp^1,D-Trp^2,Ica^7,Arg^8]OT, pA_2 = 7.92, which is equipotent with ANTAG. This amino acid would probably maintain conformational effects similar to those of proline.

In general, the replacement with tryptophan anywhere in the tail portion of ANTAG led to more potent analogues. Thus, analogue 9, [Pmp^1,D-Trp^2,Trp^7,Arg^8]OT, pA_2 = 8.06 is almost twice as potent as ANTAG. Tryptophan would introduce more conformational flexibility to the tail portion than would be possible with the more rigid prolyl residue.

Of great interest is analogue 10, [Pmp^1,D-Trp^2,Trp^8]OT, pA_2 = 8.22, featuring replacement of Arg^8 with tryptophan, which is 2.8 times more potent than ANTAG and, unlike the usual AVP antagonists, lacks a cationic charge anywhere in the molecule. Analogue 10 is an interesting new lead since it is uniquely different in structure from AVP and its antagonists, and may hopefully have higher specificity for OT receptors. The design of OT antagonists faces a possible stumbling block in the solubility of very lipophilic analogues lacking a cationic charge. Hence, it is possible that the use of arginine at position 8 confers solubility to the antagonists, but it is not essential to generate antagonism. Analogue 11, [Pmp^1,D-Trp^2,Arg^8,Trp^9]OT, pA_2 = 8.03, bearing Trp^9, was about 1.8 times more potent than ANTAG, but its Trp(For) derivative, analogue 12, pA_2 = 7.67, was weaker than the parent ANTAG. Since it has been suggested that the tail portion of OT and analogues may be important in increasing affinity for the receptor, it is probable that certain, as yet undefined, lipophilic substituents at position 7, 8, or 9 might enhance affinity, hence may increase potency. However, since the introduction of an aromatic amino acid in the tail portion of this peptide will alter the enzymatic susceptibility of the peptide, it is not apparent whether conformational or enzymatic effects, or even altered movement across tissues, binding to other receptors, or other biological effects, are responsible for the patterns of potencies of these antagonists.

Antidiuretic studies showed analogues 9–11 to be weaker than ANTAG as antagonists of AVP in the antidiuretic assay, with pA_2 = 6.0 or less.

Conclusions. We have shown that the potency of [Pmp^1,D-Trp^2,Arg^8]OT, ANTAG, a reversible antagonist of OT in the uterotonic assay with a pA_2 value of 7.77 is enhanced by the replacement of amino acids in the tail sequence by either Trp^7, Trp^8, or Trp^9, leading to analogues 9, 10, and 11 with values for pA_2 of 8.07, 8.22, and 8.03, respectively. Additionally, these compounds, 9–11, were weak antagonists of AVP in the antidiuretic assay; hence they may be useful leads for future design of more highly potent and specific OT antagonists.

We are undertaking further uterotonic studies in our baboon models[44] to ascertain the efficacy of analogue 10. These studies are essential to verify that this antagonist is potent and specific not only in the rat but also in primates.

Experimental Section

All peptides were synthesized by the SP method, either manually with a special vessel and a mechanical shaker or with an Applied Biosystems (ABS) 430A automated peptide synthesizer. The synthetic reagents for the automated synthesizer were obtained from ABS. For the manual synthesis of peptides, Boc-amino acid derivatives were purchased from Bachem Inc. The 4-methylbenzhydrylamine-resin, the Boc-Gly-Pam-resin, and the Boc-Trp(For)-Pam-resin were purchased from ABS, and chloromethylated resin and ion-exchange resins were supplied by Bio-Rad. We prepared β,β-pentamethylene-β-mercaptopropionic acid (Pmp) with the thiol group protected as the Pmp(S-Bzl) or Pmp(S-Meb) derivative as described elsewhere.[28] For all synthetic steps we used HPLC solvents supplied by Fisher Scientific, including dichloromethane (DCM), dimethylformamide (DMF). Other reagents were of analytical grade and were purchased from Aldrich Chemical Co., Pierce Chemical Co., or Chemical Dynamics. For the removal of blocking groups, some of the peptides were treated with liquid HF,[34] using an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan). Peptides were desalted and freed from scavengers by gel-filtration chromatography[35] with Sephadex G-15 (Pharmacia) using 50% AcOH.[36] Elution profiles were determined at 254 nm in a Varian DMS 70 UV spectrophotometer. The peptides were finally purified by preparative

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HPLC as described below. For preparative HPLC we used a Gilson autopreparative HPLC System 71, with three Model 303 pumps, a Model 811 dynamic mixer, a Model 803C manometric module, a Holochrome variable-wavelength detector, an Apple IIe computer as a controller, and a Linear Instruments Corp. recorder. The preparative column module used (2.14 × 25 cm) had a guard module (5 cm) both packed with Dynamax-60A (8 μm, C_{18}, Rainin). The purity of peptides was checked by analytical HPLC with a Millipore apparatus consisting of a U6K injector, two Model 6000A solvent pumps, a 660 programmer, a Model 450 variable-wavelength detector, a Houston Instruments dual-pen recorder, and an analytical μBondapak C_{18} column (30 × 0.39 cm). Peptides were detected at 220 nm. The solvents used were HPLC grade and were supplied by Fisher Scientific. The water used was purified by passing institutionally deionized and distilled water through a D5041 mixed ion-exchange cartridge (Barnstead Sybron Corp., Boston, MA) and distilling in an all-glass still. The solvent systems used both for analytical or preparative HPLC were (A) 0.05% TFA, (B) 60% MeCN–40% solvent A. Thin-layer chromatography (TLC) was performed on silica gel G precoated TLC plates (Analtech Uniplates, 0.25 mm). The following solvent systems were used (ratios given by volume): (A) *n*-BuOH–AcOH–H_2O (4:1:1), (B) *n*-BuOH–AcOH–H_2O (4:1:5, upper phase), (C) *n*-BuOH–AcOH–H_2O (5:1:1), (D) *n*-BuOH–AcOH–H_2O–Pyr (5:1:1:1). For analysis, the peptides (40–80 μg) were applied in solution to the thin-layer plate and, after development of at least 10 cm with a solvent system, they were visualized with Ehrlich reagent or chlorine–tolidine.[15] For amino acid analysis the peptides were hydrolyzed with 6 N HCl for 24 h at 110 °C and the amino acids in the hydrolysate were derivatized with phenyl isothiocyanate. The resulting PTC-amino acids were analyzed by the Waters Associates Picotag method,[37] using a Pierce Chemical Co. amino acid standard. The analytical set up consists of a Waters Picotag column (3.9 × 150 mm), two Waters 501 pumps, a Waters automated gradient controller, a Waters Model 441 absorbance detector, a Waters temperature-controlling Module, and a Waters 745B data module for data analysis. The optical rotations of peptides were measured with a Rudolph polarimeter (precision ± 0.01°). Microanalyses were performed by Galbraith Laboratories Inc. (Knoxville, TN). The ^1H NMR spectrum of Boc-Ica was obtained in a Varian XLA-400 spectrophotometer.

Solid-Phase Synthesis of Protected Peptides. Boc-amino acids were used for the synthesis, for protection of side-chain functionalities, Boc-Arg(Tos), Cys(Meb), Pmp(S-Meb) or Pmp(S-Bzl) was used, and where appropriate, Boc-Trp(For), Boc-D-Trp, or Boc-D-Trp(For) was used. For manual syntheses, the starting Boc-Gly-resin (0.7 mmol of Gly/g) was prepared on a 200–400 mesh chloromethylated resin (Bio-Rad), 1% cross-linked with divinylbenzene, by esterification with the cesium salt of Boc-Gly.[17] For syntheses in the peptide synthesizer either a Boc-Gly-Pam-resin (0.7 mmol/g, ABS) or Boc-Trp(For)-Pam-resin (0.69 mmol/g, ABS) was used. For the synthesis of the Trp(For)[9] antagonist we coupled Boc-Trp(For) to a 4-methylbenzhydrylamine-resin.[19] The Boc-Gly-resin (0.5–0.7 mmol/g) was taken manually through the required number of coupling cycles by the SP method as previously modified.[16] In each cycle the Boc group was removed with 30% trifluoroacetic acid in DCM and, after neutralization of the resin with 10% DIEA in DCM, coupling was performed with a 3-fold excess of Boc-amino acid and DCC. Six molar excess of Boc-Asn-ONp or Boc-Gln-ONp in DMF was used at the appropriate steps. Completion of the coupling step was monitored by means of the ninhydrin test which usually gave a negative response.[29] If the test was positive the coupling step was repeated, but if only faintly positive, the unreacted peptide was acetylated by treatment with Ac_2O–DIEA–DCM (1:1:8). The dicyclohexylurea byproduct was removed by washing the resin three times at the end of the coupling step with MeOH–CHL (1:2). When Boc-D-Trp was introduced at position 2 in a manual synthesis, the Boc-group was removed from the Boc-D-Trp-peptide-resin by treatment with 30% TFA in DCM containing 1% mercaptoethanol and 5% anisole to inhibit the alkylation of the indole ring. Pmp(S-Meb) or Pmp(S-Bzl) were incorporated in 3 mol excess in DMF solution by activation with DCC in the presence of HOBt.

Alternatively, peptides were assembled on a Boc-Gly-Pam-resin (0.7 mmol/g) using the automated ABS 430A peptide synthesizer, in which case, Boc-Cys(Meb) and Boc-Trp(For) or Boc-D-Trp(For) were the derivatives of choice. The Pmp(S-Meb) residue was introduced by manual coupling. The final peptides were removed from the resin by ammonolysis with MeOH (25 ml) saturated with ammonia, which removed the formyl group from tryptophyl residues at the same time. After 3 days, the resin was removed by filtration and extracted three times with hot DMF. The peptide amide was precipitated from the pooled extracts by treatment with water or Et_2O–petroleum ether, usually yielding 400–600 mg of protected peptide. Since sodium/liquid ammonia cleavage of protected peptides containing valine or tryptophan at position 6 gave mixtures with several components, protected precursors of analogues 1, 6, and 7 were freed from blocking groups by treatment with liquid HF/anisole. Peptide 12 was obtained by coupling Boc-Trp(For) to a 4-methylbenzhydrylamine-resin,[20] using the automated synthesis with the standard ABS protocol using Boc-amino acids and Pmp(S-Meb),[28] which was incorporated into the peptide resin in the last step by manual SP synthesis by treating a DMF solution of this acid with DCC in the presence of HOBt. Analogue 12 was obtained as the Trp(For)-amide by direct HF/anisole cleavage of the peptide from the MBHA resin, followed by the usual oxidative cyclization.

TLC analysis of protected peptides usually showed one major component with minor impurities. These peptides were very insoluble, making chromatographic purification difficult, and reprecipitation improved their homogeneity only slightly. Hence, they were used directly for preparation of the free analogues.

Boc-indoline-2-carboxylic Acid (Boc-Ica). Indoline-2-carboxylic acid (3 g, 18.4 mmol) was dissolved in a solution of sodium hydroxide (0.804 g, 20.1 mmol) in water (2 mL). To this solution, *tert*-butyl alcohol (4 mL) was added all at once and then di-*tert*-butyl dicarbonate (4.83 g, 22.1 mmol) over a period of 1 h.[42] A pH of 10 was maintained by the addition of additional sodium hydroxide solution. Additional *tert*-butyl alcohol was added (2 mL) and the solution was stirred overnight. The reaction mixture was diluted with water (10 mL) and was extracted with three 15-mL portions of petroleum ether. The aqueous solution was acidified with 10% citric acid and was extracted three times with EtOAc (20 mL). The pooled EtOAc extracts were washed with water and saturated salt, dried ($MgSO_4$), and evaporated to dryness. The residual oil was dissolved in EtOAc (5 mL) and the solution was treated with petroleum ether (200 mL), which resulted in crystallization of product (3.48 g, 72% yield) with mp 122–124 °C; recrystallization from cyclohexane gave the analytical sample: mp 128–129 °C; optical rotation, $[\alpha]^{27}_D$ –78° (c 2, EtOH). A ^1H NMR spectrum was obtained with a $CDCl_3$ solution of Boc-Ica and a standard program (Varian Associates) using a pulse width of 14.2 μs, an acquisition time of 2.5 s with 32 scans, and tetramethylsilane as internal reference, and the number of protons was ascertained by integration. The nine methyl protons were detected at 1.599, 1.499, and 1.422 ppm; the α-carbon proton was detected as a multiplet at 4.87–4.9 ppm; the two β-methylene group protons were detected as a multiplet at δ 3.20–3.53 ppm; the four aromatic protons were detected as a multiplet at 6.945–7.207 ppm. Anal. ($C_{14}H_{17}N_1O_4$): C, H, N.

Pmp-D-Trp-Ile-Gln-Asn-Cys-Pro-Arg-Trp-NH_2, [Pmp^1,D-Trp^2,Arg^8,Trp^9]OT (11, Table I). Pmp(S-Meb)-D-Trp-Ile-Gln-Asn-Cys(Meb)-Pro-Arg(Tos)-Trp-NH_2 assembled by the SP method as described above (215 mg) was dissolved in liquid ammonia (200 mL) freshly distilled from sodium and treated with sodium under anhydrous conditions until a pale blue color lasted for about 15–30 s.[31] After evaporation of ammonia in a vacuum, the solid residue was dissolved in 30 mL of 50% AcOH and the solution was added to water (2 L).[32] The pH of the resulting solution was adjusted to 7.0 with ammonium hydroxide and oxidative cyclization of sulfhydryl peptide was accomplished by titration with 0.01 N potassium ferricyanide until uptake ceased[33] and then adding 20% excess of ferricyanide solution. After 20 min, the ferrocyanide and ferricyanide salts were removed by stirring for 10 min with AG1 X-2 (Cl^-) ion-exchange resin (15 g) and then by passing the suspension through a column containing ion-exchange resin (15 g), using additional 0.2 N AcOH (100 mL) for washings. The combined filtrate and washings were lyophilized. For analysis, a solution of the peptide obtained was injected into an analytical μBondapak C_{18} column (30 × 0.39 cm) and was eluted isocratically with 60% solvent B, at a rate of 1.8

mL/min, with monitoring at 220 nm. Isocratic elution with 50% solvent B delayed the peptide substantially and resulted in resolution of impurities. For purification, the lyophilized peptide-containing salts were dissolved in 0.2 N AcOH (50 mL) and applied to a preparative Dynamax-60A (8 μm, C_{18}, Rainin) column (2.14 × 25 cm), with a 5-cm guard module. A gradient was run from 0 to 50% B over 30 min, eluting at a rate of 4 mL/min, with monitoring of the eluent at 280 nm. Center portions of the main component eluted after ca. 1.5 h. The purer fractions, determined by analytical HPLC, were pooled and lyophilized, yielding antagonist 11 (41 mg). Analogue purity was established by thin-layer chromatography (TLC) in four separate solvent systems (Table I), by analytical HPLC and by amino acid analysis (Table II).

For amino acid analyses (Table II) a standard of Pmp-SS-Cys was prepared[10] and was derivatized with PITC at the same time as the hydrolysis product of the analogue and a standard amino acid mixture (Pierce Chemical Co.), in order to form the PTC-amino acids for analysis by the Picotag method.[37] An aliquot of the PTC-derivative of Pmp-SS-Cys was injected into the Picotag column and eluted with the normal protocol, in order to determine its UV absorption and elution time, and in a separate run, it was coinjected with the PTC-amino acid standard into the Picotag column, in order to verify its position of elution. PTC-Pmp-SS-Cys has UV absorption similar to and coelutes with PTC-Ile; hence, for peptide hydrolyzates we report the combined values for Pmp-SS-Cys and Ile. The value of Pmp-SS-Cys is somewhat low for the Pmp component, because symmetric disulfide, Pmp-SS-Pmp, can also form. The value of Cys added to the value estimated for Pmp-SS-Cys gives an estimate for Cys. Except for tryptophan, all analogues gave the expected amino acid analysis ratios ± 10%. Tryptophan, was estimated in peptides by UV spectrophotometry at 280 nm, and Trp(For) was estimated at 300 nm. The lower values found for tryptophan suggest that the peptide may have several moles of AcOH, TFA, and H_2O, as we have observed with other peptides.[10,38] 1-Adamantaneacetic acid in hydrolysates was quantified by HPLC using a standard of this acid and monitoring at 220 nm.

This procedure, with only minor variations, was used to prepare all the antagonists (Table I) except for analogues 10 and 12, shown below.

Pmp-D-Trp-Ile-Gln-Asn-Cys-Pro-Arg-Trp(For)-NH_2, [Pmp^1,D-Trp^2,Arg^8,Trp(For)^9]OT (12, Table I). Pmp(S-Meb)-D-Trp-Ile-Gln-Asn-Cys(Meb)-Pro-Arg(Tos)-Trp(For)-MBHA-resin assembled by the SP method as described above (0.25 mmol) was treated with anisole (1 mL) and liquid HF[34] (9 mL) for 60 min at 0 °C. After removal of HF under vacuum, the residue was extracted four times with petroleum ether and then three times with 50% AcOH (10 mL). Following the usual oxidative cyclization as described above, HPLC purification yielded purified analogue 12 (36 mg).

Pmp-D-Trp-Ile-Gln-Asn-Cys-Pro-Trp-Gly-NH_2, [Pmp^1,D-Trp^2,Trp^3]OT (10, Table I). Pmp(*S*-Meb-D-Trp-Ile-Gln-Asn-Cys(Meb)-Pro-Trp-Gly-NH_2 (200 mg), obtained by the SP method was treated with sodium in liquid ammonia, as described above, the residue was dissolved in 50% AcOH (20 mL) and the solution was added to distilled water (600 mL). Since a cloudiness appeared, acetone (200 mL) was added until a clear solution resulted and then distilled water (1200 mL) was added. This solution was subjected to oxidative cyclization as described for antagonist 11 except that after concentrating in a vacuum to remove acetone, the solution was lyophilized. The residue obtained was dissolved in the smallest possible volume of 50% acetic acid and was applied to a Sephadex G-15 column (115 × 2.7 cm) and eluted with the same solvent at a rate of about 50–60 mL/h.[36] The eluate was monitored in a UV spectrophotometer at 254 nm. The fractions corresponding to the major peak were monitored by analytical HPLC, eluting isocratically with 75% solvent B, and the purer ones were pooled and lyophilized, yielding desalted peptide (80 mg). During analytical HPLC, isocratic elution with 55% solvent B delayed the peptide about 25 min and resulted in resolution of impurities. The peptide was dissolved in 50% AcOH (3 mL) and was diluted with water (9–15 mL) until faintly cloudy, the cloudiness being cleared with an additional drop of glacial AcOH. The solution was applied to a preparative Dynamax-60A (8 μm, C_{18}, Rainin) column (2.14 × 25 cm), with a 5-cm guard module. A gradient was run from 0 to 50% B over 30 min, eluting at a rate of 5 mL/min, with monitoring of the eluent at 280 nm. Center portions of the main component eluted after ca. 3 h. The purer fractions, determined by analytical HPLC, were pooled and lyophilized, yielding purified antagonist 10 (37 mg). The structures of the antagonists prepared are as follows: [Ac-Trp^1,D-Trp^2,Val^6,Arg^8]OT (1); [Pmp^1,Trp^2,Arg^8]OT (2), [Pmp^1,D-Trp^2,Trp^3,Arg^8]OT (3); [Pmp^1,D-Trp^2,Trp^4,Arg^8]OT (4), [Pmp^1,D-Trp^2,Trp^5,Arg^8]OT (5), [Aaa^1,D-Trp^2,Trp^6,Arg^8]OT (6), [Aaa^1,D-Trp^2,Val^6,Arg^8]OT (7), [Pmp^1,D-Trp^2,Ica^7,Arg^8]OT (8), [Pmp^1,D-Trp^2,Trp^7,Arg^8]OT (9), [Pmp^1,D-Trp^2,Trp^8]OT (10), [Pmp^1,D-Trp^2,Arg^8,Trp^9]OT (11), [Pmp^1,D-Trp^2,Arg^8,Trp(For)^9]OT (12). The physicochemical properties, amino acid analyses, and biological properties of these analogues are shown on Tables I–III, respectively.

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Stereospecific Synthesis of (*R*)- and (*S*)-*S*-Adenosyl-1,8-diamino-3-thiooctane, a Potent Inhibitor of Polyamine Biosynthesis. Comparison of Asymmetric Induction vs Enantiomeric Synthesis

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Two diastereomers of the potent spermidine synthase inhibitor *S*-adenosyl-1,8-diamino-3-thiooctane have been prepared in high (>96% de) stereochemical purity. Two synthetic routes were investigated, one based on asymmetric induction and the other involving an enantiomeric synthesis. The latter route gave the desired products in >96% de, whereas the synthesis based on asymmetric induction resulted in only 80% de in the final product. Evaluation of the two diastereomers as inhibitors of spermidine synthase showed that the *R* diastereomer is a more potent inhibitor than the *S* diastereomer.

The polyamines spermidine and spermine are synthesized in vivo by a pair of closely related aminopropyltransferases (APT), spermidine synthase (putrescine aminopropyltransferase, PAPT, EC 2.5.1.16) and spermine

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