20.4 mg/kg of compound XIV in 5 mL/mg sterile water intragastrically through the nasal route with an infant feeding tube.

Registry No. **1**, 103336-05-6; **2**, 130985-34-1; **3**, 130985-35-2; **4**, 128657-34-1; **5**, 52709-42-9; **6**, 130985-36-3; **7**, 130985-37-4; **8**, 86170-45-8; **9**, 91970-62-6; **10**, 130985-38-5; **11**, 130985-39-6; **12**, 130985-40-9; **13**, 130985-41-0; **14**, 638-10-8; **15**, 85532-42-9; **16**, 130985-42-1; **17**, 129765-95-3; **18**, 130985-43-2; **19**, 130985-44-3; **20**, 130985-45-4; **21**, 130985-46-5; **22**, 130985-47-6; **23**, 130985-48-7; **24**, 130985-49-8; **25**, 130985-50-1; **26**, 130985-51-2; I, 131064-22-7; II, 131064-23-8; III, 131100-22-6; III·Na, 129272-65-7; IV, 131064-24-9; IV·Na, 129272-73-7; V, 130985-52-3; VI, 130985-53-4; VII, 130985-54-5; VIII, 130985-55-6; IX, 130985-56-7; X, 130985-57-8; XI, 130985-58-9; XII, 130985-59-0; XIII, 131064-25-0; XIII·K, 130985-60-3; XIV, 130985-61-4; suleptanic acid, 121807-13-4; di-*tert*-butyl-*N*,*N*-diethylphosphoramidite, 117924-33-1; N^{α}-(*tert*-butyloxycarbonyl)-L-glutamic acid α-*tert*-butyl ester, 24277-39-2.

Design of Potent Oxytocin Antagonists Featuring D-Tryptophan at Position 2

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We prepared nine analogues (1–9) of MCPA-D-Phe-Phe-Ile-Asn-Cys-Pro-Arg-Gly-NH_2, [MCPA^1, D-Phe^2, Phe^3, Ile^4, Arg^8]oxytocin (MCPA = β-mercapto-β,β-pentamethylenepropionic acid), a potent antagonist of the rat uterotonic action of oxytocin (OT). We replaced D-Phe with D-Trp and made [MCPA^1,D-Trp^2,Phe^3,Ile^4,Arg^8]OT (**1**), which had OT pA_2 of 7.51, somewhat higher than that of the D-Phe^2 antagonist which has OT pA_2 = 7.35 in our rat uterotonic assay. Both compounds are equipotent as antagonists of [Arg^8]vasopressin in the rat antidiuretic assay, with pA_2 = 8.1. Other substitutions gave [MCPA^1,D-Trp^2,4-Cl-Phe^3,Ile^4,Arg^8]OT (**2**), OT pA_2 7.44; [MCPA^1,D-Trp^2,Phe^3,Ile^4,3,4-dehydro-Pro^7,Arg^8]OT (**3**), OT pA_2 = 7.42; [MCPA^1, D-Trp^2,Phe^3,Arg^8]OT (**4**), OT pA_2 = 7.58; [MCPA^1,D-Trp^2,Phe^3,Arg^8,Gly^9-NHEt]OT (**5**), OT pA_2 = 7.49; [MCPA^1,D-Trp^2,Ile^4,Arg^8]OT (**6**), OT pA_2 = 7.46; [MCPA^1,D-Trp^2,Val^4,Arg^8]OT (**7**), OT pA_2 = 7.58; [MCPA^1,D-Trp^2,Thr^4,Arg^8]OT (**8**), OT pA_2 = 7.48; and finally, [MCPA^1,D-Trp^2,Arg^8]OT (**9**), which was a more potent and more selective OT antagonist, with OT pA_2 = 7.77 in the uterotonic assay and ADH pA_2 < 5.9 in the antidiuretic assay and hence is an important lead for the design of OT antagonists.

Introduction

In recent years, reversible antagonists of the uterotonic action of OT (oxytocin) have been sought as inhibitors of preterm labor[1] and as pharmacological tools in assessing the possible role of OT in labor. However, the main thrust in the synthesis of neurohypophyseal analogues has been in the development of potent antagonists of arginine vasopressin (AVP), the antidiuretic hormone, in the hope of developing new therapeutic agents selective either as as diuretics to counteract edema or as antivasopressors to treat hypertension. Unfortunately, these antagonists, in general, are not specific and act as reversible antagonists of AVP or OT, showing diuretic, antivasopressor, and antioxytocic actions.[2]

The use of D-tryptophan to substitute OT amino acids has been infrequent. The analogue [D-Trp^2]OT has been prepared[3] and reported to be an inhibitor of OT uterotonic action, although Hruby and Smith report that no potency was specified.[4] Analogues of OT with lipophilic amino acids at position 2, among them D-tryptophan, combined with substitutions of a basic amino acid at position 8 and a β-mercaptopropionic acid at position 1 resulted in strong OT antagonists in the rat uterotonic assay.[5]

We decided to prepare OT antagonists featuring substitutions with D-tryptophan and to study the feasibility of enhancing their potency and specificity as antagonists to OT uterotonic action. In designing the desired antagonists we took advantage of the knowledge that (a) replacement at position 1 with β-mercapto-β,β-pentamethylenepropionic acid (MCPA)[6,36] instead of β-mercaptopropionic acid has led to stronger antagonists of OT[6,7] and (b) the analogues [MCPA^1,D-Phe^2,Phe^3,Ile^4,Arg^8]OT and [MCPA^1,D-Phe^2,Phe^3,Gln^4,Arg^8]OT were among the most potent antagonists of OT in the rat uterotonic assay.[8] We prepared [MCPA^1,D-Trp^2,Phe^3,Ile^4,Arg^8]OT (ANTAG) as our initial antagonist. We tested this compound as antagonist of OT in the rat oxytocic bioassay[9] in the presence of magnesium salts, in an assay measuring the area under the peak resulting from a uterine contraction.[10] We found that ANTAG was a potent antagonist, as good or somewhat better than its D-Phe^2 counterpart, which we also prepared as a reference compound. Hence, we prepared additional analogues attempting to bring the sequence of the antagonists closer to that of the hormone itself, in the hope of attaining higher affinity for OT receptors, and hence higher potency, as well as higher specificity of action on the uterus rather than the kidney.

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

OTA no.	MW	% yield[b]	OR,[c] deg	TLC[d] R_f				HPLC[e] rentention time, min
				A	B	C	D	
1	1145	34	−112	0.45	0.51	0.27	0.77	14.2
2	1180	20	−135	0.47	0.53	0.27	0.82	19.3
3	1143	34	−141	0.50	0.53	0.29	0.80	14.2
4	1160	33	−100	0.38	0.43	0.43	0.66	5.4
5	1188	34	−83	0.31	0.46	0.14	0.73	7.2
6	1111	32	−79	0.47	0.51	0.25	0.83	14.6
7	1097	27	−100	0.40	0.50	0.23	0.75	10.0
8	1099	45	−89	0.39	0.47	0.17	0.73	5.8
9	1126	48	−76	0.27	0.43	0.11	0.66	4.2

[a] The structures for the free peptides (1–9) is given in the Experimental Section. [b] These yields are based on the amount of Boc-Gly-resin used as starting material. [c] OR = optical rotation. OR was determined as $[\alpha]^{25}_{D}$, in degrees (c 1, 1 N AcOH). [d] The composition of solvents A–D is given in the Experimental Section. [e] Solvent composition = 58% solvent B, flow rate 1.8 mL/min. All compounds were at least 95–98% pure as shown by the HPLC pattern.

Table II. Amino Acid Analyses of OT Antagonists

amino acid[a]	amino acid ratios								
	1	2	3	4	5	6	7	8	9
Asp	1.05	1.00	0.96	1.00	0.94	0.98	0.97	0.94	1.00
Glu				1.03	1.04				1.00
Gly	1.00	0.99	1.08	0.99	0.92	1.05	1.07	0.98	1.06
Arg	0.90	1.04	0.90	0.98	1.00	1.10	1.10	1.00	1.08
Thr								1.01	
Pro	0.96	0.90		1.10	1.04	1.04	0.93	1.07	0.94
3,4-dehydro-Pro			0.91						
Val							0.90		
Cys	0.30	0.33	0.39	0.22	0.47	0.50	0.54	0.31	0.48
MCPA-SS-Cys + Ile	1.53	1.73	1.47	0.58	0.51	2.10	1.25	1.52	1.59
Phe	1.02		0.99	0.93	1.07				
4-Cl-Phe		1.10							
Trp[b]	0.92	0.80	0.89	0.86	0.77	0.79	0.81	0.79	0.78

[a] Standards of PTC derivatives of 3,4-dehydro-Pro, MCPA-SS-Cys, and 4-Cl-Phe were used to determine the UV absorption and were coinjected with PTC derivatives of an amino acid standard mixture to identify their position. PTC-MCPA-SS-Cys has UV absorption similar to and coelutes with PTC-Ile. Hence we report the combined values for MCPA-SS-Cys and Ile. Values for MCPA-SS-Cys may be somewhat low for the MCPA component, because MCPA-SS-MCPA is also a byproduct. The value of Cys added to that for MCPA-SS-Cys gives an estimate for Cys. [b] Tryptophan in peptides was estimated by its UV absorption as we have reported.[33] The values found for tryptophan are still somewhat low and suggest that the peptide has several moles of H_2O, AcOH, and/or TFA, as has we have observed with other peptides.[33]

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

OT antagonist	OT bioassay		ADH bioassay	
	OT pA_2[b]	ED_{50}	ADH pA_2[c]	ED_{50}
[MCPA[1],D-Phe[2],Phe[3],Ile[4],Arg[8]]OT[d]	7.35 ± 0.04	$4.66 \pm 0.37 \times 10^{-8}$	8.09 ± 0.10	$0.87 \pm 0.17 \times 10^{-8}$
[MCPA[1],D-Trp[2],Phe[3],Ile[4],Arg[8]]OT (1)	7.51 ± 0.03	$3.09 \pm 0.11 \times 10^{-8}$	8.10 ± 0.09	$0.83 \pm 0.17 \times 10^{-8}$
[MCPA[1],D-Trp[2],4-Cl-Phe[3],Ile[4],Arg[8]]OT (2)	7.44 ± 0.06	$4.02 \pm 0.63 \times 10^{-8}$		
[MCPA[1],D-Trp[2],Phe[3],Ile[4],3,4-dehydro-Pro[7],Arg[8]]OT (3)	7.42 ± 0.03	$3.86 \pm 0.25 \times 10^{-8}$		
[MCPA[1],D-Trp[2],Phe[3],Arg[8]]OT (4)	7.58 ± 0.04	$2.70 \pm 0.19 \times 10^{-8}$		
[MCPA[1],D-Trp[2],Phe[3],Arg[8],Gly[9]*N*-ethylamide]OT (5)	7.49 ± 0.03	$3.29 \pm 0.19 \times 10^{-8}$		
[MCPA[1],D-Trp[2],Ile[4],Arg[8]]OT (6)	7.46 ± 0.02	$3.53 \pm 0.17 \times 10^{-8}$		
[MCPA[1],D-Trp[2],Val[4],Arg[8]]OT (7)	7.58 ± 0.03	$2.69 \pm 0.17 \times 10^{-8}$		
[MCPA[1],D-Trp[2],Thr[4],Arg[8]]OT (8)	7.48 ± 0.06	$4.68 \pm 0.94 \times 10^{-8}$		
[MCPA[1],D-Trp[2],Arg[8]]OT (9)	7.77 ± 0.03	$1.75 \pm 0.11 \times 10^{-8}$	<5.86 ± 0.19	$<1.68 \pm 0.78 \times 10^{-6}$

[a] Preliminary results presented in part at the European Peptide Symposium, in Tubingen, FRG, September 1988 and in the proceedings of the symposium.[34] [b] Rat oxytocic assay in vitro in the presence of Mg^{2+}, mean ± SE. [c] Rat antidiuretic assay, mean ± SE. [d] This analogue was prepared synthetically and was homogeneous on HPLC and on TLC; its amino acid analysis showed the expected amino acids, and its optical rotation was similar to that reported.[8]

We report here (Tables I and II) nine new antagonists of OT (1–9) and biological data (Table III) on [MCPA[1],D-Phe[2],Phe[3],Ile[4],Arg[8]]oxytocin and the analogues 1–9.

Results and Discussion

Peptide Synthesis. All peptides were prepared either manually by the solid-phase (SP) method,[11,12] introducing modifications previously described,[13] and/or by an automated method employing an Applied Biosystems (ABS) 430A automated peptide synthesizer, to compare both methods. The starting resin for the manual syntheses was a Boc-Gly-resin, 1% cross-linked with divinylbenzene prepared by esterification of the chloromethylated resin with the cesium salt of Boc-Gly.[14] A Boc-Gly-Pam resin[15]

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(ABS) was used also, either for manual SP or for automated SP synthesis. For the automated syntheses, the standard ABS protocol was used, using Boc-amino acids,[16] and Boc-D-Trp(For) to protect the indole group. Treatment with TFA to remove the Boc group was chosen to avoid side reactions with glutaminyl peptides,[17] followed by neutralization with DIEA and coupling of either symmetric anhydrides[18] or HOBt esters.[19] For the manual syntheses, the Boc group was removed at each cycle with 30% trifluoroacetic acid in DCM. Because Boc-D-Trp was used, without protection of the indole group, Boc-D-Trp-peptide-resins were treated with 30% TFA in DCM containing 1% mercaptoethanol to inhibit destruction of D-Trp during removal of the Boc group.[20] After neutralization of the resin with 10% DIEA in DCM, coupling was performed with Boc-amino acid and DCC,[21] except for Boc-Asn and Boc-Gln, which were coupled as the 4-nitrophenyl esters to avoid dehydration of carboxamide functionalities to nitriles,[22] and MCPA(*S*-Bzl) or MCPA(*S*-Meb),[23] which were incorporated by activation with DCC in the presence of HOBt. Completion of coupling was monitored by means of the ninhydrin test.[24] The final protected peptides were removed from the resin by ammonolysis[17] or, for one of the analogues, by aminolysis with ethylamine.[12] We found that both ammonolysis[25] or aminolysis removed the *N*-formyl group from trytophan. Most of the protected peptides were freed from blocking groups by reduction with Na in liquid ammonia[26] and the disulfhydryl peptides were cyclized in very dilute solution[27] to the corresponding cyclic disulfide by oxidation with potassium ferricyanide.[28] In the case of analogue 2, which contain 4-chlorophenylalanine, MCPA(S-Meb) and Boc-Cys(Meb) were used for synthesis and removal of protecting groups from the isolated protected peptide obtained after ammonolysis was accomplished with liquid HF/anisole[29] followed by the usual oxidative cyclization. This procedure avoids the dehalogenation which would occur with Na in liquid ammonia. The free peptides were desalted and purified by a two-step procedure of gel filtration[30] on Sephadex G-15 and elution with 50% AcOH for desalting in one step and 0.2 N AcOH in a second step.[31] Alternatively, peptides were more conveniently purified by preparative high-performance liquid chromatography (HPLC).

The synthesis of peptides either by manual or by automated methods led to protected and free peptides in similar yields and with similar degrees of purity. The physicochemical characteristics of the antagonists are listed in Table I. The amino acid analyses (Table II) were determined by the HPLC–Picotag method.[32] The mixed disulfide of β-mercapto-β,β-pentamethylenepropionic acid was prepared to facilitate its identification in the amino acid analysis.

Bioassays. All peptides were tested as antagonists of OT action in the uterus as previously described.[10] Isolated uteri from rats in natural estrus were incubated in Van Dyke–Hastings solution with 0.5 mM Mg^{2+}. The isometric contractions in response to OT were recorded with a force transducer and a Grass polygraph Model 79 and the response was quantified by integrating the area under the peaks obtained. The standard used was pure synthetic OT from Calbiochem. The pA_2 for each antagonist was determined by the method of Schild.[9] The effective dose is referred to as the molar concentration of an antagonist that reduces the response to an agonist by one-half. All OT antagonists were tested in at least four separate assays. The D-Phe[2] antagonist of Manning, our antagonist 1, and the most potent antagonist of the series were also tested in the antidiuretic assay as previously described.[10] This assay was performed in water-loaded rats under ethanol anesthesia. The ADH 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. In the ADH bioassay, analogues of OT having both agonistic and antagonistic activity have the antagonistic potencies approximated.

Structure–Activity Relationships. It may be seen that analogue 2, featuring 4-Cl-Phe at position 3, did not show an improvement in potency when compared to our starting analogue 1. However, 2 was converted to 1, albeit in low yields, by catalytic hydrogenation. Hence, 2 is a useful potential precursor for the radioactive labeling of 1 by catalytic tritiation, which is desirable to study its metabolism.

Analogue 3, featuring 3,4-dehydro-Pro at position 7, was prepared with the same aim. However, it was found that hydrogenation of 7 could not be followed by HPLC because both 1 and 7 have the same retention times, hence the hydrogenation could not be optimized since the starting peptide 7 could not be separated from 1.

Analogues featuring substitution of Ile[4] with Gln, antagonists 4 and 5, had approximately similar potencies to that of 1, hence offered no special advantage. Analogues featuring substitution of Phe[3] with Ile and of Ile[4] with Val or Thr were approximately equipotent with 1. Only compound **9**, featuring simultaneous replacement with Ile at position 3 and glutamine at position 4, was substantially more potent. This pattern of substitution gives the analogue with the most oxytocin-like sequence.

For the purpose of comparison, we studied the antidiuretic effect of the D-Phe[2] antagonist of Manning, our antagonist 1, and our most potent antagonist in the oxytocic assay, antagonist 9. It may be seen from Table III that 1 is more potent than the D-Phe[2] antagonist, and that both compounds have identical potency in the ADH

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bioassay. The finding that antagonist **9** has a higher potency as an OT antagonist and a lower potency than **1** as an antagonist in the antidiuretic assay suggests that this analogue may be a rather significant lead in the design of more potent and selective OT antagonists.

We have previously reported the testing of antagonist 1 in vivo in the rat.[10] We found that antagonist 1 can (1) inhibit uterine contraction of an estrus rat in response to exogenous OT, (2) inhibit milk letdown in lactating rats, (3) delay labor in rats. In addition, we showed that antagonist 1 can inhibit in vitro uterine contractions in response to OT in tissue obtained from women at cesarian section.[10]

Recently we showed that antagonist 1 can inhibit spontaneous nocturnal uterine contractions and labor in the baboon.[33] These data suggest that (1) OT is a primary regulator of uterine activity during pregnancy and (2) an OT antagonist might be effective in preventing preterm labor in humans. The development of more potent and specific OT antagonists such as compound **9** should lead to effective and specific inhibitors of labor.

Conclusions

We have shown the development of potent antagonists of OT uterotonic action featuring D-Trp in position 2, with values for pA_2 in the range of 7.42–7.77. When Ile[3] and Gln[4] were introduced simultaneously as substituents in the same analogue, antagonist **9** was obtained, showing substantially increased antioxytocic potency in vitro (pA_2 = 7.77) while its antagonistic effect in the ADH antidiuretic assay was markedly diminished (pA_2 < 5.9).

These studies indicate that compound **9** would appear to be a significant lead for the design of more potent and selective OT antagonists.

Experimental Section

All peptides were prepared by the SP method.[11,12] either manually with a special vessel and a mechanical shaker and/or by an automated method employing an Applied Biosystems (ABS) 430A automated peptide synthesizer. All synthetic reagents for the automated synthesizer were obtained from ABS. For manual synthesis of the same peptides, Boc-amino acid derivatives were purchased from Chemical Dynamics Corp. or Bachem Inc. Chloromethylated resins and ion-exchange resins were supplied by Bio-Rad. We prepared β-mercapto-β,β-pentamethylenepropionic acid (MCPA) with the thiol group protected as the MCPA(*S*-Bzl)[6] or MCPA(*S*-Meb)[22] derivative and were able to compare each of these products to genuine samples generously provided by W. Huffman, SmithKline and French, and R. Machineni, Bachem, respectively. For all synthetic and chromatographic steps we used HPLC solvents purchased from Fisher Scientific, including dichloromethane (DCM) and dimethylformamide (DMF). All other reagents were of analytical grade and were supplied either by Chemical Dynamics, Pierce Chemical Co., or Aldrich Chemical Co. Where suitable, some peptides were treated with liquid HF,[29] in order to remove blocking groups, using an all-Teflon apparatus (Protein Research Foundation, Osaka, Japan) as described below. Peptides were desalted by gel permeation chromatography[30] with Sephadex G-15 (Pharmacia) using 50% AcOH.[31] Elution profiles were determined by UV spectrophotometry at 254 nm in a Varian DMS 70 spectrophotometer. For preparative HPLC we used a Gilson autopreparative HPLC System 71, with three Model 303 pumps, a Model 803C manometric module, a Model 811 dynamic mixer, 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) and was packed with Dynamax-60A (8 μm, C_{18}, Rainin). 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. Water 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 following solvent systems were used both for analytical or preparative HPLC: (A) 0.05% TFA, (B) 60% MeCN and 40% solvent A.

Thin-layer chromatography (TLC) was performed on Uniplates, which are silica gel G precoated TLC plates (0.25 mm, Analtech). Solvent systems used were (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 (4:1:1:1). Peptides (40–80 μg) were applied, and after development of at least 10 cm with a solvent system, the peptides on the plate were visualized with Ehrlich reagent or chlorine–tolidine.[12]

For amino acid analysis peptides were hydrolyzed with 6 N HCl for 24 h at 110 °C and the hydrolysates were analyzed by the Picotag method, using an amino acid standard mixture purchased from Pierce Chemical Co. The analytical set up uses 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. Optical rotations were measured with a Rudolph polarimeter (precision ±0.01°).

Mixed Disulfide of β-Mercapto-β,β-pentamethylenepropionic Acid and Cysteine [1(L)-Amino-5,5-pentamethylene-3,4-dithiahexane-1,6-dicarboxylic Acid, MCPA-SS-Cys]. PMP(S-Bzl) (1.06 g, 4 mmol) dissolved in liquid ammonia was freed from the *S*-benzyl protecting group by treating the solution with sodium until the blue color lasted for about 15-30 s. Cysteine (0.97 g, 8 mmol) was added and the ammonia was removed in a vacuum to a solid residue. The products were dissolved in water (50 mL), the pH of the solution was decreased to 7.1 with 2 N HCl, and the resulting solution was treated with 1 N potassium ferricyanide (17 mL). The precipitate of cystine was collected. The filtrate was treated with AG1-X2 (Cl^-) (30 g) and was added to a column with additional resin (30 g), and the resin was washed with additional water (50 mL). The pooled eluent was evaporated to dryness in a vacuum, the residue was dissolved in water (30 mL), and 2 N HCl was added to pH 3. The solid formed was collected, washed with small volumes of cold water, then petroleum ether, and air-dried. Collected material (667 mg) had mp 187 °C dec. Recrystallization from water gave the analytical sample. Anal. ($C_{11}H_{19}N_1O_4S_2$): C, H, N.

Solid-Phase Synthesis of Protected Peptides. Boc-amino acids were used for the synthesis, and for protection of side chain functionalities, Boc-Arg(Tos), Cys(Bzl) or Cys(Meb), and Boc-Thr(Bzl) were used. 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.[14] Sometimes a Boc-Gly-Pam resin (0.69 mmol/g, ABS) was used. The Boc-Gly-resin (0.5–0.7 mmol/g) was taken manually through the required number of coupling cycles by the SP method of synthesis as previously modified.[13] In each cycle the Boc group was removed with 30%

(33) White, W. F.; Hedlund, M. T.; Rippel, R. H.; Arnold, W.; Flouret, G. *Endocrinology* **1973**, *93*, 96.
(34) Flouret, G.; Brieher, W.; Mahan, K.; Wilson, L. In *Peptides*; Jüng, G., Bayer, E., Eds.; Walter de Gruyter: New York, 1989; p 549.
(35) All symbols and abbreviations comply with recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9). Amino acids are of the L configuration unless otherwise indicated. Other abbreviations used are as follows: OT, oxytocin; AVP, [Arg[8]]vasopressin; MCPA,β-mercapto-β,β-pentamethylenepropionic acid; TFA, trifluoroacetic acid; DCM, dichloromethane; DMF, dimethylformamide; DIEA, diisopropylethylamine; TEA, triethylamine; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; Boc, *tert*-butyloxycarbonyl; ONp, 4-nitrophenyl ester; Tos, *p*-tolylsulfonyl; Meb, 4-methylbenzyl; For, formyl; Bzl, benzyl; Pyr, pyridine; CHL, chloroform; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet; PITC, phenyl isothiocyanate; PTC, phenylthiocarbamyl.
(36) MCPA is often referred to in the literature as β-mercapto-β,-β-cyclopentamethylenepropionic acid.

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. A 6 molar excess of Boc-Asn-ONp or Boc-Gln-ONp in DMF was used at the appropriate step. Completion of the coupling step was monitored by means of the ninhydrin test and was usually negative.[23] If the test was strongly positive, the coupling step was repeated, but if only faintly positive, the peptide was capped 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). The Boc group was removed from Boc-D-Trp-peptide-resins by treatment with 30% TFA in DCM containing 1% mercaptoethanol. MCPA(*S*-Bzl) or MCPA(*S*-Meb) were incorporated in 3 molar excess in DMF solution by activation with DCC in the presence of HOBt.

Alternatively, the same peptides were assembled on a Boc-Gly-Pam resin (0.69 mmol/g) with the automated ABS 430A peptide synthesizer, in which case Boc-Cys(Meb) and Boc-D-Trp(For) were the derivatives of choice. In either case, the final assembled peptide was removed from the resin by ammonolysis with MeOH (25 mL) saturated with ammonia. One of the peptides was removed by treatment with MeOH (15 mL) and ethylamine (10 mL). 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 precipitation with water and/or $EtOH-Et_2O$. Usually 400–600 mg of protected peptide was obtained. TLC analysis of peptides thus obtained usually showed one major component with minor impurities moving faster or slower. The protected peptides prepared either by the manual or the automated method usually had similar patterns of impurities. These peptides were very insoluble in most common solvents, making chromatographic purification difficult, and reprecipitation improved their homogeneity only slightly, resulting in substantial waste of the main peptide. Hence, they were not purified at this stage and were used directly for preparation of the free analogues.

MCPA-D-Trp-Phe-Ile-Asn-Cys-Pro-Arg-Gly-NH_2, [$MCPA^1$,D-Trp^2,Phe^3,Ile^4,Arg^8]oxytocin (1, Table I). MCPA(*S*-Bzl)-D-Trp-Phe-Ile-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 assembled by the solid-phase method as described above (153 mg) was dissolved in liquid ammonia (200 mL) freshly distilled from sodium and treated under anhydrous conditions with sodium until a pale blue color lasted for about 15–30 s.[26] After evaporation of ammonia in a vacuum, the solid residue was dissolved in 20 mL of 50% AcOH. The dissolved peptide was added to deaerated water (1.5 L),[27] the pH was adjusted to 7.0 by the addition of concentrated ammonium hydroxide, and cyclization to the peptide disulfide was brought about by titrating the disulfhydryl peptide solution with 0.01 N potassium ferricyanide until uptake ceased,[28] and then adding 20% excess of the potassium 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 solution 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. The residue 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.[31] The eluate was monitored in a UV spectrophotometer at 254 nm. The fractions corresponding to the major peak were monitored by analytical HPLC, with an analytical μBondapak C_{18} column (30 × 0.39 cm), eluting with 70% solvent B, and detecting peptides at 220 nm. The pure fractions by HPLC criteria were pooled and lyophilized. The residue (85 mg) was dissolved in 2 mL of 0.2 N AcOH and was subjected to gel filtration on a Sephadex G-15 column (115 × 2.7 cm), eluting with the same solvent at a rate of 50–60 mL/h. The peptide eluted in one main peak, which was monitored by UV absorbance at 254 nm. Lyophilization of the suitable portions of the peak as determined by HPLC gave the desired analogue 1 (55 mg). Alternatively, this antagonist could be more conveniently purified by preparative HPLC on a preparative column (2.14 × 25 cm) with a guard module (5 cm), both packed with Dynamax-60A (8 μm, C_{18}, Rainin), by running a gradient with 0–55% solvent B over a period of 30 min, at a rate of 5 mL/min, monitoring at 280 nm. The main peak eluted after about 3 h and the purity of fractions was checked by HPLC, and the suitable ones were pooled and lyophilized. Analogue purity was established by thin-layer chromatography (TLC) in four separate solvent systems and by analytical HPLC (Table I), as well as by amino acid analysis (Table II). This procedure, with only minor variations, was used to prepare all of the antagonists (1–9) except when 4-Cl-Phe (see below) was part of the sequence (antagonist 2). The physicochemical characteristics of the antagonists are shown on Table I.

For amino acid analysis by the Picotag method (Table II), standards of 3,4-dehydro-Pro, MCPA-SS-Cys, and 4-Cl-Phe were prepared and were derivatized with PITC at the same time as a standard amino acid mixture and peptide hydrolysate, in order to form the PTC-amino acids. The PTC-amino acids obtained were used to determine their UV absorption and were coinjected with the PTC-amino acid standard to identify their position of elution. PTC-MCPA-SS-Cys has UV absorption similar to and coelutes with PTC-Ile. Hence we report the combined values for MCPA-SS-Cys and Ile. The value for MCPA-SS-Cys is somewhat low for the MCPA component, because symmetric disulfide, MCPA-SS-MCPA, can also form. The value of Cys added to the value estimated for MCPA-SS-Cys gives an estimate for Cys. All analogues gave expected amino acid ratios ±10%, except for tryptophan, which gave lower yields due to destruction during acid hydrolysis. However tryptophan in peptides was estimated in 0.1 N HCl solution by UV spectrophotometry at 280 nm, as we have previously reported.[33]

MCPA-D-Trp-4-Cl-Phe-Ile-Asn-Cys-Pro-Arg-Gly-NH_2, [PMP^1,D-Trp^2,4-Cl-Phe^3,Ile^4,Arg^8]oxytocin (2, Table I). MCPA(*S*-Meb)-D-Trp-4-Cl-Phe-Ile-Asn-Cys(Meb)-Pro-Arg-(Tos)-Gly-NH_2 assembled by the solid-phase method as described above (159 mg) was treated with anisole (1 mL) and liquid HF[29] (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). The pooled extracts were added to deaerated water (1.6 L), the pH was adjusted to 7.0, then the ferricyanide titration was accomplished as above.

The structures of all the other free antagonists prepared are as follows: MCPA-D-Trp-Phe-Ile-Asn-Cys-3,4-dehydro-Pro-Arg-Gly-NH_2 or [$MCPA^1$,D-Trp^2,Phe^3,Ile^4,3,4-dehydro-Pro^7,Arg^8]OT (3), MCPA-D-Trp-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 or [$MCPA^1$,D-Trp^2,Phe^3,Arg^8]OT (4), MCPA-D-Trp-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NHEt or [$MCPA^1$,D-Trp^2,Phe^3,Arg^8,Gly^9-NHEt]OT (5), MCPA-D-Trp-Ile-Ile-Asn-Cys-Pro-Arg-Gly-NH_2 or [$MCPA^1$,D-Trp^2,Ile^4,Arg^8]OT (6), MCPA-D-Trp-Ile-Val-Asn-Cys-Pro-Arg-Gly-NH_2 or [$MCPA^1$,D-Trp^2,Val^4,Arg^8]OT (7), MCPA-D-Trp-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH_2 or [$MCPA^1$,D-Trp^2,Thr^4,Arg^8]OT (8), MCPA-D-Trp-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2 or [$MCPA^1$,D-Trp^2,Arg^8]OT (9).

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