Synthesis and Characterization of Fluorescent Antagonists and Agonists for Human Oxytocin and Vasopressin V_{1a} Receptors

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The fluoresceinyl (Flu) group has been linked by an amide bond to the side chain amino group at position 8 of (a) two oxytocin (OT) antagonists, to give d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Orn^8(5/6C-Flu),Tyr-NH_2^{9}]VT (Orn^8(5/6C-Flu)OTA) (**1**) and desGly-NH_2,d(CH_2)$_5$[D- Tyr^2,Thr^4,Orn^8(5/6C-Flu)]VT (**2**), and (b) eight Lys^8 and Orn^8 analogues of potent OT agonists, to give d[Lys^8(5/6C-Flu)]VT (**3**), d[Thr^4,Lys^8(5/6C-Flu)]VT (**4**), [HO^1][Lys^8(5/6C-Flu)]VT (**5**), [HO^1][Thr^4,Lys^8(5/6C-Flu)]VT (**6**), d[Orn^8(5/6C-Flu)]VT (**7**), d[Thr^4,Orn^8(5/6C-Flu)]VT (**8**), [HO^1][Orn^8(5/6C-Flu)]VT (**9**), and [HO^1][Thr^4,Orn^8(5/6C-Flu)]VT (**10**). The tetramethylrhodamyl (Rhm) group was attached to the precursor peptide of **9** to give [HO^1][Orn^8(5/6C-Rhm)]VT (**11**). All 11 fluorescent peptides were evaluated in human OT and vasopressin V_{1a} (vasoconstrictor), V_{1b} (pituitary), and V_2 (antidiuretic) receptor binding and functional assays. With K_d = 6.24, 217, >10000, and >10000 nM for the OT, V_{1a}, V_{1b}, and V_2 receptors, peptide **1** is a potent and selective fluorescent OT antagonist and may be useful for specifically labeling OT receptors while peptide **2** exhibits low affinities for all the receptors. The fluorescent peptides **3**−**10** are all very potent agonists for the human OT receptor. They exhibit the following K_d values (nM) for the human OT, V_{1a}, V_{1b}, and V_2 receptors, respectively: (**3**) 0.29, 57, 124, >10000; (**4**) 1.8, 25.5, 150, >10000; (**5**) 0.34, 13.7, 66, nd (not determined); (**6**) 0.32, 17.3, 53, >10000; (**7**) 0.25, 107, 393, >10000; (**8**) 0.40, 30, 282, >10000; (**9**) 0.18, 12.2, 126, nd; (**10**) 0.17, 11.8, 87, >1000; (**11**) 0.092, 7.36, nd, nd. Peptide **7** exhibits both a high affinity and a high selectivity for human OT receptors. Peptides **7** and **11** were utilized to study the internalization of the OT receptor−ligand complex. Preliminary studies indicate that this process is similar to that observed for the vasopressin V_{1a} receptor and differs from that observed for vasopressin V_2 receptors. Some or all of the fluorescent OT antagonists and agonists reported here are very promising new fluorescent ligands for labeling cells which express the human OT receptor and are also useful tools to follow endocytosis of the receptor−ligand complex.

Introduction

G protein coupled receptors (GPCRs) are involved in the recognition and the transduction of many extracellular signals which are as diverse as light, ions, odorants, small molecules such as peptides, and proteins. The control of the activities of GPCRs is thus very important in regulating physiological functions. Like activation, desensitization and internalization play a crucial role in the control of GPCR activities. Internalization processes have been extensively studied for several receptors and clearly indicate that although high sequence homologies exist between related receptors, processes of internalization may be different.[1] For example, it has been shown that AT_{1a} receptors expressed in CHO (see the Abbreviations following the Experimental Section) cells undergo an internalization upon agonist stimulation while AT_2 receptors remain at the cell surface in the same conditions of stimulation.[2]

Vasopressin and oxytocin are two neurohypophysial peptides involved in the regulation of various physiological functions. Among them, vasopressin participates in the control of blood pressure and of water reabsorption while oxytocin induces contractions of uterine myocytes during parturition and mammary gland myocytes during lactation. Although these two hormones have different roles, large homologies have been observed in their structures and in the structures of the three vasopressin (V_{1a}, V_{1b}, and V_2) receptors and the OT receptor.[3] Except for the V_2 receptor, which is coupled to adenylyl cyclase, the other three receptors are coupled to phospholipase C.

A few studies have been performed to investigate the internalization of vasopressin V_{1a} and V_2 receptors. To carry out these studies, some fluorescent ligands[4] were developed and used to label vasopressin receptors expressed in cell lines. It has been shown that vasopressin fluorescent agonists were internalized and remained dispersed throughout the cytoplasm in vascular smooth muscle A10 cells which expressed V_{1a} receptors, while they were addressed to a perinuclear region in LLC-PK1 cells which expressed V_2 receptors.[5]

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Table 1. Physicochemical Properties of Free Peptides **3a**−**10a**

compd no.	peptide	yield, %	$[\alpha]_D^{25}$, deg (c = 1, 1 N AcOH)	TLC, R_f[b] a	b	c	HPLC,[c] T_r, min	formula	MW(calcd)	MS(found) (ESMS)
3a	d[Lys8]VT	56.7	112.0	−0.23	0.02	0.12	17.9	$C_{43}H_{66}O_{12}N_{12}S_2$	1007.2	1007.2
4a	d[Thr^4,Lys^8]VT	68.2	98.0	−0.24	0.02	0.19	19.0	$C_{42}H_{65}O_{12}N_{11}S_2$	980.2	980.2
5a	HO^1[Lys^8]VT	47.1	84.0	−0.29	0.38	0.11	17.0	$C_{43}H_{66}O_{13}N_{12}S_2$	1023.2	1023.3
6a	HO^1[Thr^4,Lys^8]VT	69.7	76.0	−0.25	0.10	0.17	19.2	$C_{42}H_{65}O_{13}N_{11}S_2$	996.2	996.2
7a	d[Orn^8]VT	55.1	44.0	−0.16	0.10	0.15	17.4	$C_{42}H_{64}O_{12}N_{12}S_2$	993.2	993.2
8a	d[Thr^4,Orn^8]VT	65.5	99.0	−0.21	0.20	0.23	18.8	$C_{41}H_{63}O_{12}N_{11}S_2$	966.2	966.2
9a	HO^1[Orn^8]VT	22.6	56.0	−0.14	0.07	0.14	17.0	$C_{42}H_{64}O_{13}N_{12}S_2$	1009.2	1009.2
10a	HO^1[Thr^4,Orn^8]VT	32.6	109.0	−0.17	0.12	0.18	19.0	$C_{41}H_{63}O_{13}N_{11}S_2$	982.2	982.1

[a] Yields are based on the amount of the protected peptide in the reduction−reoxidation step in each case and are uncorrected for acetic acid and water content. [b] Solvent systems and conditions are given in the Experimental Section. [c] All peptides were at least 95% pure. For elution a linear gradient of 90:10 to 30:70 (0.05% aqueous TFA/0.05% TFA in MeCN) over 60 min with a flow rate of 1.0 mL/min was utilized.

More recently, the same pattern of internalization has been observed when using transiently transfected HEK 293 cells expressing hemaglutinin-tagged vasopressin V_{1a} and V_2 receptors.[6] These data clearly indicate that the processes of internalization are different despite the high homologies between these two receptors.

OT receptor activation is very important for the parturition process and is thus a promising therapeutic target for the control of preterm labor. Analyses have been performed to characterize hormone binding sites on OT receptors and the coupling to Gq and Gi. Desensitization of OT receptors has also been reported in the human myometrium,[7,8] but their internalization after the activation by oxytocin is still questionable. Yet, no direct analysis using fluorescent ligands has been performed. Since vasopressin V_{1a} and OT receptors are expressed in the uterus, the analysis of OT receptor endocytosis should be carried out with fluorescent ligands which are very selective for OT receptors. Some fluorescent derivatives of OT have been designed by Buku and collaborators,[9,10] but none have been used for human OT receptor labeling. Moreover, no pharmacological characterization of the vasopressin and oxytocin receptors has been performed with these fluorescent ligands.

Here we report the design and synthesis of fluorescent oxytocin antagonists and agonists and their pharmacological properties for all the human vasopressin and oxytocin receptor subtypes. Among them, some are very selective for the OT receptor and are very potent tools for labeling of OT receptors. Furthermore, we also report preliminary findings with some of these fluorescent ligands from studies on the internalization of the OT receptors in CHO cell lines and in transiently transfected HEK 293 cell lines.

Design and Synthesis of Fluorescent Analogues

For the design of fluorescent OT antagonists, we selected two of our previously reported OT antagonists: (**1a**) [1-β-mercapto-β,β-cyclopentamethylenepropionic acid,2-O-methyltyrosine,4-threonine,8-ornithine,9-tyrosylamide]vasotocin (d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH$_2^9$]VT) (OTA)[11,12] and (**2a**) desglycinamide [1-β-mercapto-β,β-cyclopentamethylenepropionic acid,2-D-tyrosine,4-threonine,8-ornithine]vasotocin (desGly-NH$_2$,d(CH$_2$)$_5$[D-Tyr^2,Thr^4,Orn^8]VT).[13] Neither peptide contains an α-NH$_2$ group at position 1. Both peptides have an ornithine residue, suitable for attachment of a fluoresceinyl group, at position 8. Peptides **1a**[11,12] and **2a**[13] were converted to their respective Orn^8 (5/6-Flu) derivatives **1** and **2** by coupling with the 5(and 6)-carboxyfluorescein succinimide ester (5/6C-Flu-OSu). For the design of fluorescent OT agonists, our strategy, essentially an expansion of that reported by Buku for the synthesis of d[Thr^4,DH-Pro,Lys^8(5/6C-Flu)]VT[9] and that which we utilized for the design of linear fluorescent V_{1a} antagonists,[14] was to select a series of potent OT agonists which lacked an α-NH$_2$ group at position 1 and replace the Leu residue at position 8 in each one with both Orn and Lys residues for attachment of a fluoresceinyl group. We selected the following four OT agonists: (**A**) [1-deamino]oxytocin (dOT),[15] (**B**) [1-deamino,4-threonine]oxytocin (d[Thr^4]OT),[16] (**C**) [1-L-(−)-2-hydroxy-3-mercaptopropanoic acid]oxytocin ([HO^1]OT),[17] and (**D**) [1-L-(−)-2-hydroxy-3-mercaptopropanoic acid,4-threonine]oxytocin ([HO^1,Thr^4]OT.[18] With the exception of d[Thr^4]OT,[16] the others are 2−10 times more potent than OT as oxytocic agonists in rat bioassays.[15−17,18] Four Lys^8 analogues and four Orn^8 analogues of **A**−**D** were synthesized to give the following eight peptides: (**3a**) d[Lys^8]VT, (**4a**) d[Thr^4,Lys^8]VT, (**5a**) HO^1[Lys^8]VT, (**6a**) HO^1[Thr^4,Lys^8]VT, (**7a**) d[Orn^8]VT, (**8a**) d[Thr^4,-Orn^8]VT, (**9a**) HO^1[Orn^8]VT, (**10a**) HO^1[Thr^4,Orn^8]VT (Table 1). These were subsequently converted to the desired Lys^8(5/6-Flu) and Orn^8(5/6- Flu) vasotocin agonists by coupling with 5/6C-Flu-OSu. Subsequently, for internalization studies, the tetramethylrhodamine (Rhm) group was attached to the Orn^8 residue of peptide **9a** by coupling with 5(and 6)-carboxytetramethylrhodamine succinimide ester (5/6C-Rhm-OSu).

Ten Flu^8 peptides and one Rhm^8 peptide (see Table 2) were designed according to the above rationale.

The Flu^8 OT antagonists **1** and **2** (Table 2) have the following general structure:

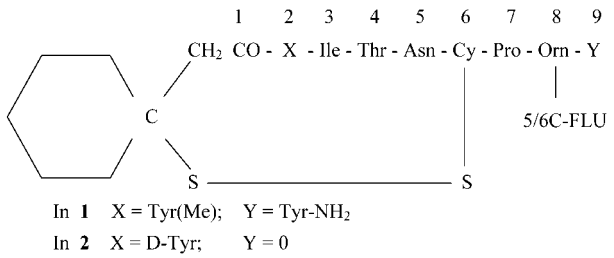


Table 2. Physicochemical Properties of Fluorescent Peptides **1**−**11**

compd no.	peptide	TLC,[a] R_f a	b	c	d	HPLC,[b] T_r, min	formula	MW (calcd)	MS(found)[c] (ESMS)
1	d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Orn^8(5/6C-Flu),Tyr- $NH_2{}^9$]VT[d]	0.68	0.59	0.72	0.71	37.7	$C_{75}H_{89}O_{19}N_{12}S_2$	1512.7	1512.5
2	desGly-NH_2,d$(CH_2)_5$[D-Tyr^2,Thr^4,Orn^8(5/6C-Flu)]VT[d]	0.54	0.44	0.45	0.63	34.1	$C_{65}H_{77}O_{18}N_9S_2$	1336.5	1336.4
3	d[Lys^8(5/6-Flu)]VT[d]	0.46	0.31	0.51	0.56	27.2	$C_{64}H_{76}O_{18}N_{12}S_2$	1365.5	1365.3
4	d[Thr^4,Lys^8(5/6C-Flu)]VT[d]	0.47	0.36	0.57	0.56	29.0	$C_{63}H_{75}O_{18}N_{11}S_2$	1338.5	1338.4
5	[HO^1][Lys^8(5/6C-Flu)]VT[d]	0.47	0.37	0.51	0.62	26.6	$C_{64}H_{76}O_{19}N_{12}S_2$	1381.5	1381.4
6	[HO^1][Thr^4,Lys^8(5/6C-Flu)]VT[d]	0.52	0.36	0.56	0.55	28.7	$C_{63}H_{75}O_{18}N_{11}S_2$	1354.5	1354.4
7	d[Orn^8(5/6C-Flu)]VT[d]	0.39	0.37	0.61	0.56	26.4	$C_{63}H_{74}O_{18}N_{12}S_2$	1351.5	1351.4
8	d[Thr^4,Orn^8(5/6C-Flu)]VT[d]	0.45	0.42	0.65	0.60	28.6	$C_{62}H_{73}O_{18}N_{11}S_2$	1324.5	1324.4
9	[HO^1][Orn^8(5/6C-Flu)]VT[d]	0.38	0.50	0.56	0.56	29.2	$C_{63}H_{74}O_{19}N_{12}S_2$	1367.5	1367.4
10	[HO^1][Thr^4,Orn^8(5/6C-Flu)]VT[d]	0.45	0.36	0.52	0.56	28.3	$C_{62}H_{73}O_{19}N_{11}S_2$	1340.5	1340.4
11	[HO^1][Orn^8(5/6C-Rhm)]VT[d]	0.19	0.07	0.09	0.43	29.8	$C_{67}H_{84}O_{17}N_{14}S_2$	1421.6	1421.3

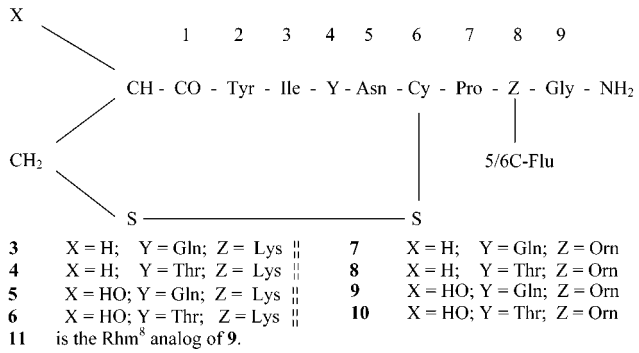
[a−c] TLC solvent systems and HPLC and ESMS conditions are described in the Experimental Section. [d] This paper.

Table 3. Receptor Binding Affinities (Ki) of the Fluorescent and Related Ligands for the Various Human Vasopressin and Oxytocin Receptors[a]

compd no.	ligand	K_i (nM) R OT	R V_{1a}	R V_{1b}	R V_2*
OTA	d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Orn^8,Tyr-$NH_2{}^9$]VT	0.21	3.9	>10000	930
1	d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Orn^8(5/6C-Flu),Tyr- $NH_2{}^9$]VT	6.24 ± 0.46	217 ± 32	>10000*	>10000
2	desGly-NH_2,d$(CH_2)_5$[D-Tyr^2,Thr^4,Orn^8(5/6C-Flu)]VT	126 ± 20	656 ± 7.5*	>1000*	>10000
OT	OT	0.8 ± 0.2	120 ± 40	nd	3500 ± 300
3	d[Lys^8(5/6-Flu)]VT	0.29 ± 0.04	57 ± 10	124 ± 33	>10000
4	d[Thr^4,Lys^8(5/6C-Flu)]VT	1.8 ± 0.1	25.5 ± 2	150 ± 64	>10000
5	[HO^1][Lys^8(5/6C-Flu)]VT	0.34 ± 0.05	13.7 ± 6.8	66 ± 14	nd
6	[HO^1][Thr^4,Lys^8(5/6C-Flu)]VT	0.32 ± 0.03	17.3 ± 4.3	53 ± 14.3	>10000
7	d[Orn^8(5/6C-Flu)]VT	0.25 ± 0.03	107 ± 12	393 ± 203	>10000
8	d[Thr^4,Orn^8(5/6C-Flu)]VT	0.40 ± 0.16	30 ± 7	282 ± 138	>10000
9	[HO^1][Orn^8(5/6C-Flu)]VT	0.18 ± 0.07	12.2 ± 2.8	126 ± 30	nd
10	[HO^1][Thr^4,Orn^8(5/6C-Flu)]VT	0.17 ± 0.05	11.8 ± 1.4	87 ± 23	>1000
11	[HO^1][Orn^8(5/6C-Rhm)]VT	0.09 ± 0.02	7.6 ± 0.8	nd	nd

[a] All values are expressed as the mean ± SE, calculated from three independent determinations except for the V_2 receptor subtype or when an asterisk is present: in these cases two independent experiments were performed. nd = not determined.

The eight Flu^8 OT agonists **3**−**10** (Table 2) and one Rhm^8 OT agonist **11** have the following general structure:



3 X = H; Y = Gln; Z = Lys ‖ **7** X = H; Y = Gln; Z = Orn
4 X = H; Y = Thr; Z = Lys ‖ **8** X = H; Y = Thr; Z = Orn
5 X = HO; Y = Gln; Z = Lys ‖ **9** X = HO; Y = Gln; Z = Orn
6 X = HO; Y = Thr; Z = Lys ‖ **10** X = HO; Y = Thr; Z = Orn
11 is the Rhm^8 analog of **9**.

Preliminary communications on the properties of peptides **1**−**10** have been reported.[19,20]

Results

Affinities for Oxytocin and Vasopressin Receptors. The affinities of all the ligands (peptides **1**−**11**; Table 2) for the human OT receptor have been evaluated by competition experiments using [^{125}I]OTA[12] or [^{125}I]-HO-LVA[21] as radiolabeled ligands. The inhibition constants are reported in Table 3. In comparing the data of compounds **1** (Orn^8(5/6C-Flu)OTA) and **2**, both derivatives of the previously reported oxytocin antagonists **1a** and **2a**, only **1** exhibits a good affinity for the OT receptor (K_i = 6.24 nM). By contrast, analogue **2**, which differs from **1** by a D-Tyr^2/Tyr(Me)^2 interchange and by deletion of the tyrosyl-NH_2 residue, has a 20-fold lower affinity for the oxytocin receptor. Concerning ligands **3**−**11**, which are derived from the Orn^8 and Lys^8 OT agonists **3a**−**10a**, they exhibit very good affinities for the OT receptor with K_i values in the 0.5 nM range, except that for the ligand **4**, which is 1.8 nM.

To investigate their selectivities, we determined their affinities for the human vasopressin V_{1a}, V_{1b}, and V_2 receptors by competition experiments using [^{125}I]HO-LVA or [^{3}H]AVP as labeled ligands (Table 3). All of them exhibit very low affinities for the vasopressin V_2 receptor, since none of them were able to bind efficiently to the V_2 receptor at a concentration lower than 1 μM. Moreover, only the ligands **5**, **6**, **10**, and **11** exhibit medium affinities for the V_{1b} receptor, while the others have low affinities for this receptor (Table 3). The selectivities of the ligands for the V_{1a} receptor vs OT receptor are illustrated in Figure 1A and reported in Table 3. Compounds **1** and **2** show low affinities for the vasopressin V_{1a} receptor, while most of the other compounds exhibit good affinities for this receptor subtype. Indeed, the inhibition constants are between 7 and 60 nM except that for compound **7**, which for the V_{1a} receptor is greater than 100 nM. Compound **7** is thus more selective for the OT receptor versus V_{1a} receptor. It is of interest to note that, as illustrated in Figure 1B, it can be used to bind selectively to OT receptors, while compounds **10** and **11** can be used to label both OT and V_{1a} receptors (Figure 1C).

Antagonist and Agonist Properties of the Fluorescent Ligands. We evaluated inositol phosphate

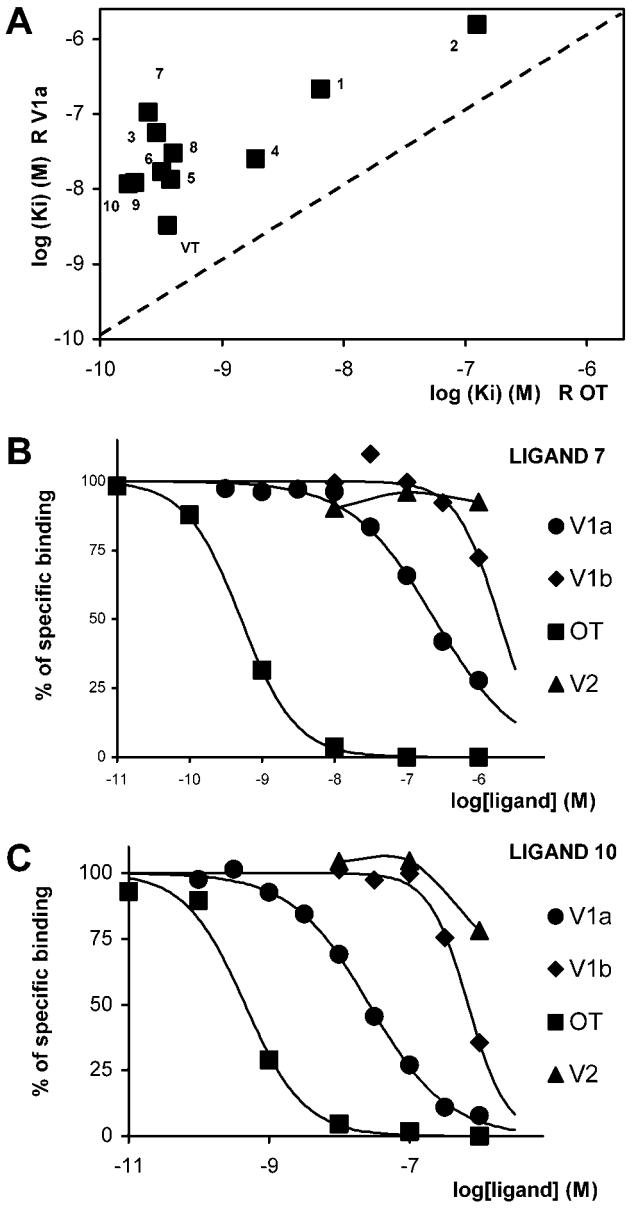


Figure 1. Graphical representation of the selectivities of the fluorescent peptides for OT and V_{1a} vasopressin receptor subtypes (panel A): abscissa, log(K_i) of the ligand for the OT receptor; ordinate, log(K_i) of the ligand for the V_{1a} vasopressin receptor. K_i values were calculated with the program Ligand and are indicated in Table 2. The dashed line indicates equipotency in affinity for the OT and V_{1a} vasopressin receptor subtypes. The indicated number refers to the number of the component as mentioned in Table 2. VT = vasotocin. Determination of the affinities of fluorescent oxytocin analogues **7** (panel B) and **10** (panel C) for the various human oxytocin and vasopressin receptor subtypes by concentration-displacement binding experiments. Membranes were prepared from CHO cells expressing one human vasopressin or oxytocin receptor subtype. They were incubated for 1 h at 30 °C in the presence of increasing concentrations of fluorescent analogue and 140 pM [^{125}I]HO-LVA for the OT receptor subtype, 60 pM [^{125}I]HO-LVA for the V_{1a} receptor subtype, or 2 nM [^{3}H]AVP (for the V_{1a}, V_{1b}, and V_2 receptor subtypes). Membranes were used at 0.5−1 μg of protein/assay for radioiodinated ligands and 5−10 μg of protein/assay for [^{3}H]AVP. Values of specific binding measured in the presence of fluorescent analogues are expressed as a percentage of the specific total binding measured in the absence of competitor. Concentration-displacement curves are representative of three independent experiments, each performed in triplicate.

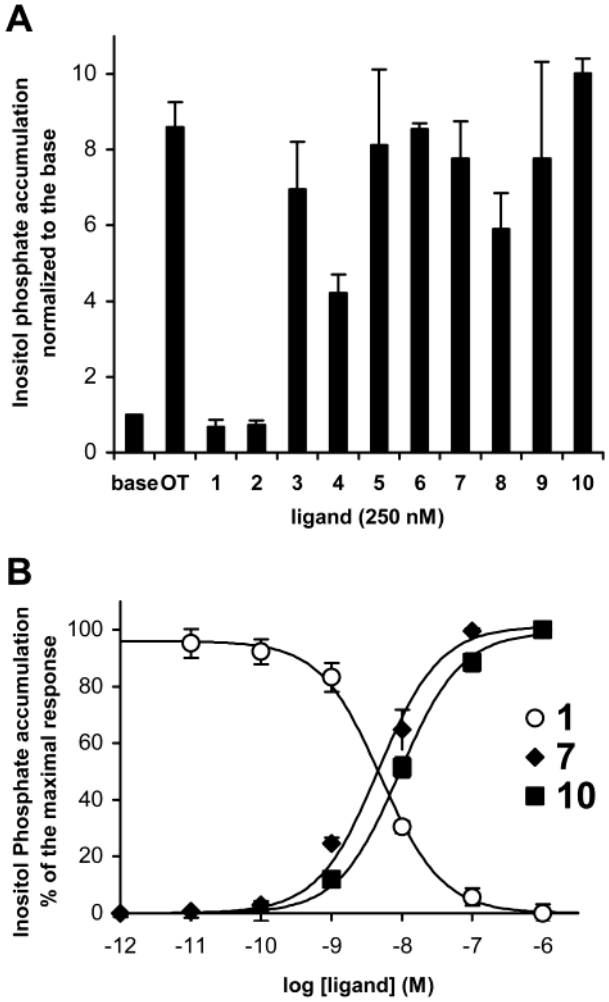


Figure 2. (A) Determination of the agonist properties of the fluorescent ligands. *myo*-[^{3}H]Inositol-prelabeled CHO-OT cells were incubated for 20 min at 37 °C with OT or with a fluorescent ligand (250 nM). Total inositol phosphates were determined. The values are normalized to the cells' response in control conditions. All values are expressed as the mean ± SE, calculated from two independent determinations. (B) Determination of the inhibition constants of the fluorescent analogue **1** (open circles) and of the activation constants of fluorescent agonists **7** (closed tilted squares) and **10** (closed squares). Fluorescent ligand **1** was added at various concentrations, and 20 min later, cells were stimulated with 10 nM OT for 20 min. Total inositol phosphates were determined, and the values were normalized to the response of OT-stimulated cells in the absence of fluorescent ligand. Fluorescent agonists **7** and **10** were added at various concentrations, and the values were normalized to maximal responses. All values are expressed as the mean ± SE calculated from three (for **1**) or four (for **7** and **10**) independent determinations. The apparent inactivation constants were calculated as $K_{inact} = IC_{50}/(1 + [OT]/K_{act})$, where IC_{50} is the concentration of fluorescent ligand leading to 50% inhibition of specific binding, [OT] = 10 nM, and K_{act} is the concentration of OT inducing half-maximal stimulation of total inositol phosphate accumulation (K_{act} = 10 nM).

accumulation induced by the various ligands at 250 nM on a CHO cell line stably expressing human OT receptors (CHO-OT). As illustrated in Figure 2A, ligands **1** and **2** are devoid of any agonistic properties since no accumulation of second messengers was observed. The antagonistic properties of compound **1** have been investigated. As shown in Figure 2B (open circles), it is a full antagonist. It completely inhibited the OT (10 nM)

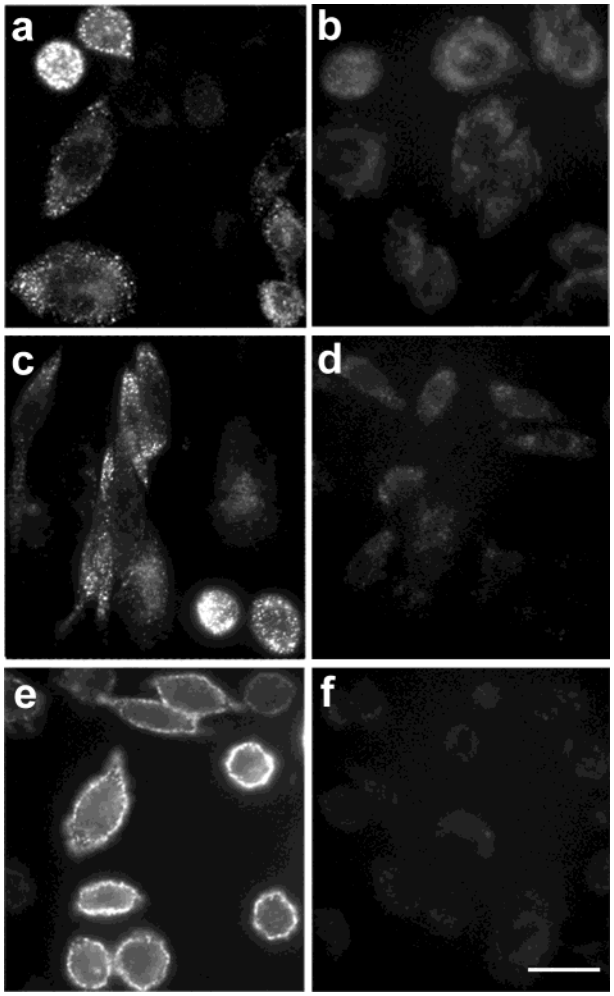


Figure 3. Labeling of CHO cells expressing OT receptors. CHO-OT cells were incubated with fluorescent ligand (2.5 nM) in the absence (panels a, c, and e) or in the presence (panels b, d, and f) of unlabeled ligand and observed by fluorescence microscopy. (a, b) Cells were incubated with 2.5 nM d[Orn^8-(5/6C-Flu)]VT (**7**) for 1 h at 37 °C. (c, d) Cells were incubated with 2.5 nM [HO^1][Orn^8(5/6C- tetramethylrhodamyl)]VT (**11**) for 1 h at 37 °C. (e, f) Cells were incubated with 2.5 nM **11** for 5 h at 4 °C. (Bar = 10 μM.)

induced inositol phosphate accumulation at a concentration higher than 100 nM. The value of the inhibition constant (K_{inact}) is 2.74 ± 0.35 nM.

By contrast, compounds **3**−**10** all have agonistic properties since, as shown in Figure 2A, at a concentration of 250 nM, they induced an increase in inositol phosphate accumulation. Most of them appear to be full agonists. We investigated more precisely the agonist properties of compounds **7** (Figure 2B, closed tilted squares) and **10** (Figure 2B, closed squares). The inositol phosphate accumulation is dependent on ligand concentration. The activation constants (K_{act}) of compounds **7** and **10** were, respectively, 5.3 ± 1.5 and 6.6 ± 2.2 nM.

Fluorescent Labeling of CHO Cells Expressing Human OT Receptors. We used the fluorescent agonist **7** to label human OT receptors stably expressed in the CHO cell line (Figure 3) or in the transiently transfected HEK 293 cell line. CHO-OT cells (panels a and b) were incubated for 1 h at 37 °C in the presence of 2.5 nM agonist **7**. CHO-OT cells observed by fluorescent microscopy presented a punctate labeling (panel a). This labeling was not observed when CHO-OT cells were incubated with an excess of nonfluorescent peptide (1 μM OTA) (panel b). The same pattern was also observed in HEK 293 cells transfected with the cDNA encoding for the OT receptor and was not observed when HEK 293 cells were transfected only with a carrier plasmid (data not shown). Because tetramethylrhodamine is less sensitive to photobleaching than fluorescein, we synthesized a derivative of ligand **9** with a tetramethylrhodamine on the ornithyl^8 to give [HO^1]-[Orn^8(5/6C-Rhm)]VT (peptide **11**; Table 2). The same punctate labeling was observed when CHO-OT cells were incubated for 1 h at 37 °C in the presence of 2.5 nM [HO^1][Orn^8(5/ 6C-Rhm)]VT (panel c). No labeling was observable when cells were incubated with an excess of nonfluorescent peptide (1 μM OTA) (panel d). By contrast, incubation of cells in the presence of 2.5 nM [HO^1][Orn^8(5/6C-Rhm)]VT for 5 h at 0 °C exhibited a pericellular labeling (panel e), which was no more observable when cells were incubated with an excess of unlabeled ligand (panel f).

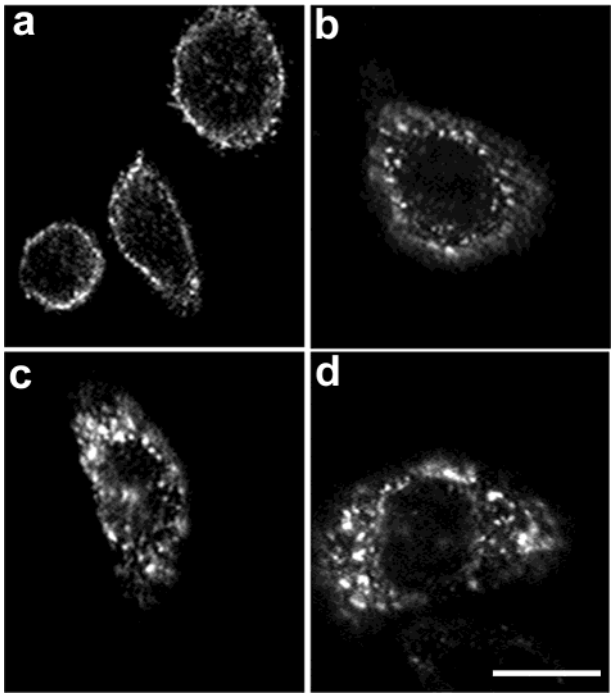


Figure 4. Confocal microscopic imaging of CHO-OT cells incubated with 2.5 nM **11** for 5 h at 4 °C (panel a) or for 15 min (panel b), 1 h (panel c), and 3 h (panel d) at 37 °C. Images were acquired as midcellular 200 nM thick optical sections. (Bar = 10 μM.)

Because the punctate labeling we observed at 37 °C and not at 0 °C suggests an endocytosis of the ligand−receptor complex, we undertook a confocal analysis of cells labeled with [HO^1][Orn^8(5/6C-Rhm)]VT for various periods of time. As illustrated in Figure 4, images acquired as midcellular optical sections show small fluorescent endosome-like particles in the cytoplasmic area when cells were incubated at 37 °C (Figure 4b−d) and not when cells were incubated at 4 °C (Figure 4a). Internalization of receptors was observed within 15 min (Figure 4b), but endosome-like particles were distributed in the periphery of the cells. After a 1 h incubation (Figure 4c) endosome-like particles were widespread in the cytoplasmic area but were not concentrated in the perinuclear region, even after a 3 h incubation (Figure 4d).

Discussion

We previously developed fluorescent analogues of linear vasopressin antagonists[14] and demonstrated that the addition of a fluorophore on a Lys^8 residue allows the ligand to maintain a high affinity for the human vasopressin V_{1a} receptor. To develop a fluorescent antagonist selective for human OT receptors, we have designed a fluorescent analogue of OTA[12] (peptide **a**, Table 1) which was previously characterized as an OT antagonist with a high affinity for rat and human OT receptors.[12,22] As in the case of [Lys^8(5C-Flu/Rhm)]-PVA,[14] we decided to link a fluoresceinyl group on residue 8, which has an ornithyl residue in the OTA structure. The resulting compound, Orn^8(5/6C-Flu)OTA (**1**) (Tables 2 and 3), exhibits a good affinity for OT receptors with a K_i of 6.24 nM. Moreover, because its affinity for the V_{1a} receptor is 34 times lower than that for the OT receptor, **1** is much more selective for OT receptors than [Lys^8(5C-Flu/Rhm)]PVA[14] and iodinated OTA[12] itself. Indeed, the selectivity indices (K_i(OT)/K_i-(V_{1a})) are 0.029 for **1**, 9.4 for [Lys^8(5C-Flu/Rhm)]PVA, and 0.5 for IOTA. Thus, Orn^8(5/6C-Flu)OTA may be a very useful tool to specifically label OT receptors. However, compound **1** has quite high binding affinity for the OT receptor, but its affinity is 30-fold lower than that of OTA (Table 3). The reason for such a decrease remains unclear since it was not seen for other compounds,[14] and it may be due to the global rigidity of this antagonist derivated with a bulky group such as the fluorescein.

In contrast to **1**, compound **2** did not show a good affinity for any of the four receptors. The D-Tyr/Tyr(Me) interchange at position 2 combined with the deletion of the Tyr-NH_2[9] residue in its structure induces a 20-fold decrease in its affinity for the oxytocin receptor. This decrease in the affinity of compound **2** is more probably due to the presence of an acidic function at the C-terminus than to the position 2 interchange or to the deletion of the tyrosyl residue. Indeed, such an effect has already been observed for other ligands such as d(CH_2)$_5$AVP,des-Gly[9]-d(CH_2)$_5$-AVP and des-Gly(NH_2)[9]-d(CH_2)$_5$-AVP for which the inhibition constants for the V_{1a} receptor are 0.3, 0.23, and 7.2 nM, respectively.[23]

As noted earlier, fluorescent agonists of oxytocin have been previously reported. Buku and collaborators[9] reported three fluorescent analogues of oxytocin which differ by the position of the fluoresceinyl group on the peptide. The authors noted that the addition of fluorescein at position 1 or 4 in a series of analogues of d[DH-Pro^7]OT leads to weak and moderate fluorescent agonists, respectively, while the addition of fluorescein at residue 8 of [1-desamino,Thr^4,Lys^8]OT confers to the agonist a good oxytocic activity in the rat.[9] They also developed a fluorescent vasotocin agonist, d[Phe^9(Flu)]-AVT, which yielded half-maximal hydrosmotic responses in the toad urinary bladder at 2 nM.[10] However, none of these ligands have been tested on human OT receptors, and no comparative pharmacology on the different human vasopressin and OT receptor subtypes has been performed.

Nevertheless, all these results corroborate computational modeling data[24] and photoaffinity labeling studies:[25] indeed, the side chain of the vasopressin Arg^8 residue is close to the first extracellular loop and is also close to the extracellular medium, while residue 9 should be embedded deeper in the binding cleft of the receptor. Many analogues are probably capable of supporting bulky groups such as biotin or photoactivatable or fluorescent groups on residue 8 without losing their affinities for the vasopressin receptor. Therefore, we considered position 8 as the best position to add the fluoresceinyl group. Because [Lys^8]vasotocin (also known as [Lys^8]oxytocin) exhibits a good affinity for the human OT receptor (1.87 + 0.4 nM),[26] we chose to develop fluorescent agonists by incorporating Lys^8 and Orn^8 residues in OT agonists to give peptides **3a**−**10a**, Table 1, for derivatization to Lys^8- and Orn^8(5/6C-Flu)OT agonists. The resulting fluorescent analogues **3**−**11** (Tables 2 and 3) exhibit very high affinities for the OT receptors since their inhibition constants are in the 0.5 nM range. Whatever the modification introduced in the ligand, be it the substitution of an ornithyl residue for a lysyl residue, substitution of a threonyl residue for a glutamyl residue, or hydroxylation of the N-terminus of the ligand, no significant change in the inhibition was observed, except for compound **4** for which a slight increase of K_i was observed.

As concerns their affinities for the vasopressin receptors, it is worth noting that the addition of a fluorophore induces a 4−30-fold decrease of the inhibition constant for the V_{1a} receptor. The decrease is even more drastic for the V_2 receptor since we were not able to determine any inhibition constant of the ligands for this receptor subtype. Moreover and contrary to the OT receptor, the V_{1a} receptor is more sensitive to subsequent modifications of the fluorescent ligands, especially for the Orn^8 series. Indeed substitution of threonyl for glutamyl in position 4 or hydroxylation of the N-terminus induces an increase in the affinity of the ligand for the vasopressin V_{1a} receptor. However, these modifications are not additive since compounds **9** and **10** have the same inhibition constant (12 nM). Both of these peptides and peptide **11** are potent ligands to label human vasopressin V_{1a} and oxytocin receptors. Compound **7**, which has a higher dissociation constant for vasopressin V_{1a} receptor (107 nM), is much more selective for labeling OT receptors.

To our knowledge, and in contrast to the vasopressin receptor (for a review see ref 27), very few studies on desensitization and internalization of the OT receptor have been undertaken, and contradictory results on a possible internalization have been reported. Indeed, a time-dependent decrease in the number of oxytocin binding sites by exposing myometrial cells to oxytocin was observed by Phaneuf and his collaborators[7] and by Adachi and Oku.[8] However, according to Phaneuf and his collaborators,[7] flow cytometry experiments demonstrated that OT receptors were not internalized during this treatment, while Adachi and Oku reported the internalization of OT receptors which could be blocked by concanavalin A and by incubation at 4 °C.[8] The development of fluorescent OT agonists may thus offer new possibilities to study the internalization process of OT receptors. Indeed, as we have found, the fluorescent agonists we have developed are efficient ligands to label OT receptors. The punctate labeling observed is very similar to what has already been observed for other receptors such as vasopressin V_{1a} receptors[5] and soma-

tostatin sst2a receptors.[28] It results from an internalization of the complex ligand−OT receptor when a CHO cell line stably expressing OT receptor as well as transiently transfected HEK 293 cells were incubated in the presence of fluorescent agonists. Because vasopressin V_{1a} and V_2 receptors, which share large structural homologies with the OT receptor, used different intracellular routes when internalized,[5] the analysis of OT receptor internalization may be a relevant question. The data presented here suggest that the internalization process of the OT receptor is similar to what has been observed for the vasopressin V_{1a} receptor and different from the vasopressin V_2 receptor internalization.[27] Indeed, the OT receptor internalization can be clearly seen within 15 min, but endosome-like particles remained widespread in the cytoplasmic area after a longer time of incubation with the fluorescent peptide and did not concentrate in the perinuclear region, suggesting a recycling of OT receptors. Such results obtained on cell lines have to be confirmed on human myometrial cells to better understand mechanisms underlying myometrium contraction during parturition.

In conclusion, the fluorescent antagonist **1** and the fluorescent agonists **7**, **9**, **10**, and **11** we have developed will be helpful to investigate more precisely the behavior of OT receptors at the cellular level and especially to study the process of internalization of OT receptors. A few strategies based on the expression of chimeric receptors such as green fluorescent protein (GFP) receptors or tagged receptor have been used to follow receptors during the internalization and to better understand such processes. Nevertheless, these strategies were performed on cell lines in which receptors were transiently or stably overexpressed, and their application on a primary culture cell model is far from being simple. By contrast, fluorescent ligands are relevant tools to study this dynamic process in freshly dissociated cells or in primary culture cells. Thus, the fluorescent ligands we have developed, especially agonists **7**, **10**, and **11**, may be useful ligands to study desensitization and internalization of the ligand−OT receptor complex in cells such as myocytes of human myometrium during parturition. Finally these fluorescent ligands can also be used to perform fluorescence recovery after photobleaching to study the diffusion of molecules in a tissue or in one cell[29] or to obtain structural information for ligand−receptor interactions by fluorescence quenching,[30] polarization,[31] or resonance energy-transfer experiments.[32]

Experimental Section

Materials and Methods. All reagents used were analytical grade. L-(−)-2-Hydroxy-3-benzylthiopropanoic acid (HO-Mpr(Bzl))[33] and 3-tritylthiopropanoic acid (Mpr(Trt))[34] were synthesized as previously described.[33,34] The Mpr(Meb),N^{α}-Boc-protected amino acids and Boc-Gly resin were purchased from Bachem (Torrance, CA) and Chem-Impex International (Wood Dale, IL). The 5(and 6)-carboxyfluorescein succinimide ester (5/6-Flu-OSu) was from Pierce Chemical Co. (Rockford, IL), and the 5(and 6)-carboxytetramethylrhodamine succinimide ester (5/6-Rhm-OSu) was from Molecular Probes, Inc. (Eugene, OR). Peptides d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH_2^{9}]VT (**1a**) (OTA)[12] and desGly-NH_2,d$(CH_2)_5$[D-Tyr^2,Thr^4,Orn^8]VT (**2a**)[13] were synthesized as previously described.[12,13] Peptides d[Lys^8]-VT (**3a**)[35,36] and d[Orn^8]VT (**7a**)[37] (Table 1) were resynthesized as described below. Peptides d[Thr^4,Lys^8]VT (**4a**), [HO^1][Lys^8]-VT (**5a**), [HO^1][Thr^4,Lys^8]VT (**6a**), d[Thr^4,Orn^8]VT (**8a**), [HO^1]-[Orn^8]VT (**9a**), and [HO^1][Thr^4,Orn^8]VT (**10a**) (Table 1) were synthesized specifically for this study. All peptides were synthesized by the Merrifield solid-phase method[38,39] as previously described[11−13] or with modifications previously described.[18] TLC was run on precoated silica gel plates (60F-254, E. Merck) with the following solvent systems: (a) 1-butanol/AcOH/H_2O (4:1:5, upper phase); (b) 1-butanol/AcOH/H_2O (4:1:1); (c) 1-butanol/AcOH/H_2O/pyridine (15:3:3:10); (d) 1-butanol/AcOH/H_2O (2:1:1); (e) 1-butanol/AcOH/H_2O (4:1:2). Loads of 10−15 μg each were applied, and chromatograms were developed at a minimal length of 10 cm. For detection, a combination of monitoring on a UV lamp (model UVGL-58, UVP Inc., San Gabriel, CA) and the chlorine gas procedure for the KI-starch reagent[39] was used. Analytical HPLC was performed on a Waters 810 instrument under the following conditions: 90:10 to 30:70 0.05% aqueous TFA/0.05% TFA in MeCN, linear gradient over 60 min at 1.0 mL/min (λ = 254 or 214 nm), on a Microsorb C_{18} column (Rainin Instruments Co., Inc.). All peptides were at least 95% pure. Electrospray mass spectrometry (ESMS) was done by the University of Oklahoma Health Sciences Center Molecular Biology Resource Facility on a PE Sciex Q-STAR Quadropole TOF mass spectrometer using 50:50 MeCN/H_2O with 0.5% AcOH as a solvent.

General Procedure for Peptide Synthesis. Peptides **3a**[35,36] and **7a**[37] (Table 1), precursors of the fluorescent peptides **3** and **7**, respectively (Tables 2 and 3), were resynthesized for the purpose of this investigation. We report here the synthesis of peptides **4a**−**6a** and **8a**−**10a** (Table 1), precursors of the fluorescent peptides **4**−**6** and **8**−**10**, respectively (Tables 2 and 3). All peptides were synthesized by the Merrifield solid-phase method[38,39] with the modifications previously described[11−13,18] as follows. Boc-Gly resin (0.25 mmol) was converted to the corresponding protected acyl octapeptidyl resin in eight cycles of deprotection, neutralization, and coupling with the following amino acids: position 8, Boc-Orn(2ClZ) or Boc-Lys(2ClZ); position 7, Boc-Pro; position 6, Boc-Cys(Bzl) or Boc-Cys (Mob); position 5, Boc-Asn-ONp; position 4, Boc-Gln-ONp or Boc-Thr-(Bzl); position 3, Boc-Ile; position 2, Boc-Tyr(Bzl); position 1, Mpr(Meb), HO-Mpr(Bzl), or Mpr(Trt). A 1 M HCl/AcOH mixture was used in all the deprotection steps,[38] except those involving Boc-Gln in which TFA was employed.[40] Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. All coupling reactions (except when Boc-Asn and Boc-Gln were involved) were performed by the DCC/HOBt procedure[41] in CH_2Cl_2/DMF (9:1, v/v). Boc-asparagine and Boc-glutamine were coupled as their nitrophenyl esters[42] in DMF. The acylpeptide resins were cleaved by ammonolysis in methanol.[43] All of the protected precursors were purified by the same general method: extraction or dissolving with warm DMF followed by reprecipitations with H_2O and EtOH/Et_2O until judged pure by TLC as previously described[11−13,18] to give the required protected peptides **III**−**X** (Table 4). The physicochemical properties of all protected peptides **III**−**X** are given in Table 4. All protected peptides **III**−**X** were deprotected by the sodium in liquid ammonia procedure[44] as previously described.[11−13,18] Prior to the reduction of peptides **III** and **IV**, the *S*-Trt protecting group was removed by treatment with 1 M HCl/AcOH and precipitation with ether. All of the resulting disulfydryl compounds were oxidatively cyclized with $K_3[Fe(CN)_6]$ using the modified reverse procedure.[45] The free peptides were desalted and purified by a two-step gel filtration procedure[46] on Sephadex G-15 (eluent 50% AcOH) and Sephadex LH-20 or G-15 (eluent 2 M AcOH). For some peptides an additional purification on Sephadex G-15 and/or on Sephadex LH-20 with 0.2 M AcOH as eluent was carried out. The purity of the free peptides **3a**−**10a** (Table 1) was checked by TLC and by HPLC. Their masses were confirmed by ESMS. The TLC, HPLC, and ESMS data and some other physicochemical properties of the free peptides **3a**−**10a** are presented in Table 1.

General Procedure for Coupling of a Fluorophore. The preparation of the fluorescent peptides **1**−**11** (Tables 2 and 3) was performed as described by Tota and co- workers[31] with some modifications. The purification of the fluorescent peptides

Table 4. Physicochemical Properties of Protected Peptides **IIIa**−**Xa**

compd no.	peptide	yield, %	mp, °C	$[\alpha]_D^{25}$, deg (c = 1, DMF)	TLC, R_f a	b	c
III	Mpr(Trt)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro- Lys(2ClZ)-Gly-NH_2	61.8	232−33	−22.4	0.63	0.55	0.74
IV	Mpr(Trt)-Tyr(Bzl)-Ile- Thr(Bzl)-Asn-Cys(Bzl)- Pro-Lys(2ClZ)-GlyNH_2	60.4	235−36	−29.0	0.81	0.67	0.78
V	HO-Mpr(Bzl)-Tyr(Bzl)- Ile-Gln-Asn-Cys(Bzl)-Pro-Lys(2ClZ)-GlyNH_2	59.8	218−19	−29.6	0.65	0.59	0.80
VI	HO-Mpr(Bzl)-Tyr(Bzl)- Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Lys(2ClZ)-Gly-NH_2	70.2	222−23	−18.2	0.79	0.65	0.71
VII	Mpr(Meb)-Tyr(Bzl)-Ile-Gln-Asn-Cys(Bzl)-Pro- Orn(2ClZ)-Gly-NH_2	68.6	236−38	−34.2	0.80	0.67	0.77
VIII	Mpr(Meb)-Tyr(Bzl)-Ile- Thr(Bzl)-Asn-Cys(Bzl)- Pro-Orn(2ClZ)-GlyNH_2	74.0	236−38	−24.0	0.83	0.67	0.73
IX	HO-Mpr(Bzl)-Tyr(Bzl)- Ile-Gln-Asn-Cys(Bzl)-Pro-Orn(2ClZ)-GlyNH_2	82.9	226−28	−18.8	0.88	0.69	0.79
X	HO-Mpr(Bzl)-Tyr(Bzl)- Ile-Thr(Bzl)-Asn-Cys(Bzl)-Pro-Orn(2ClZ)-Gly-NH_2	72.1	227−28	−30.0	0.61	0.59	0.70

[a] The protected peptides **III**−**X** are the immediate protected precursors for the peptides **3a**−**11a** given in Table 1. [b] Yields are based on the amino acid content of the resin. [c] Solvent systems are described in the Experimental Section.

was by one-step gel filtration on Sephadex LH-20 as described below. Both the fluorescent labeling and purification were performed in the dark as follows. The precursor free peptide (10 μM) was dissolved in 1 mL of 0.1 M $NaHCO_3$, the solution was cooled in an ice bath, and 12 μM 5/6-Flu-OSu (or 5/6-Rhm-OSu) in 0.5 mL of DMF was added with stirring in two portions for 30 min. The reaction was incubated for an additional 1 h at room temperature with continuous stirring, TLC, and pH (∼8.5) monitoring. The coupling mixture was then subjected to gel filtration on Sephadex LH-20 (100 × 1.5 cm) eluting with 25% AcOH with a flow rate of 4 mL/min. The desired fluorescently labeled peptide was eluted in a single peak (absorbance at 254 nm). Lyophilization of the pertinent fractions gave the desired [Orn^8(5/6-Flu)]VT, [Lys^8(5/6-Flu)]-VT, and [Orn^8(5/6-Rhm)]VT analogues **1**−**11** (Tables 2 and 3) with yields in the range of 20−40%.

Cell Culture. CHO cell lines expressing the vasopressin receptor subtypes or the oxytocin receptor were maintained in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented fetal calf serum, 4 mM glutamine, and 500 units/mL penicillin and streptomycin, in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Membrane Preparation. Cells were treated overnight with 5 mM sodium butyrate to increase receptor expression.[47] This treatment does not modify the pharmacological properties of the receptor. Culture dishes of CHO cells expressing one receptor subtype were washed twice with PBS without Ca^{2+} and Mg^{2+}. Lysis buffer (15 mM Tris/HCl, 2 mM $MgCl_2$, 0.3 mM EDTA, pH 7.4) was added, and the cells were scraped, polytron homogenized, and centrifuged at 100*g* for 5 min. The supernatant was then centrifuged at 44000*g* for 20 min at 4 °C. The pellets were resuspended in buffer A (50 mM Tris/HCl, 5 mM $MgCl_2$, pH 7.4) and centrifuged a second time at 44000*g* for 20 min at 4 °C. The pellets were then resuspended in the appropriate volume of buffer A. The protein concentration was estimated. The membranes were immediately used or aliquotted and stored in liquid nitrogen.

Binding Experiments. The affinities of the various ligands for the vasopressin and oxytocin receptor subtypes were determined by competition experiments using 200−250 pM [^{125}I]OTA or 120−180 pM [^{125}I]HO-LVA antagonist for the OT receptor, 40−60 pM [^{125}I]HO-LVA antagonist for the V_{1a} vasopressin receptor, and 1−3 nM [^{3}H]AVP for the V_{1b} and V_2 vasopressin receptors as radioligands. Nonspecific binding was determined in the presence of 1 μM unlabeled ligand. The concentrations of fluorescent ligand varied from 1 pM to 1 μM. The membranes were used at 0.5−1.5 μg of protein/assay for competition with iodinated ligands and 5−10 μg of protein/assay with [^{3}H]AVP. Binding assays were performed at 30 °C for 1 h. Ligand binding data were analyzed with the computer program Ligand, and the inhibition constants, K_i, were determined with a nonlinear least-squares regression from at least three independent competition experiments (two independent experiments as concern the V_2 receptor) performed each in triplicate.

Inositol Phosphate Assays. Inositol phosphate accumulation was determined as previously described.[48] Briefly, CHO-OT cells were labeled overnight with *myo*-[2-^{3}H]inositol (10−20 Ci/mmol; DuPont New England Nuclear) at a final concentration of 1 μCi/mL in a serum- and inositol-free medium. Then the cells were washed twice in PBS, incubated for 1 h in PBS at 37 °C, and incubated with 10 mM LiCl for 15 min and with or without antagonists at various concentrations. The cells were then stimulated with 10 nM OT for 20 min. The reaction was stopped with perchloric acid, inositol phosphates were extracted on an anion-exchange column (Dowex AG1-8, formate form, 200−400 mesh; Bio-Rad) and collected, and the radioactivity was determined by scintillation counting. Results are expressed as the amount of IP produced over the radioactivity contained in the membrane fraction. K_{inact} values were determined from three independent concentration−response curves (ligand **1**) and from four independent concentration−response curves (ligands **7** and **10**), performed in triplicate. K_{inact} was calculated as $IC_{50}/(1 + [OT]/K_{act})$, where IC_{50} is equal to the concentration of antagonist leading to half-inhibition of the OT-stimulated cells, [OT] = 10 nM, and K_{act} is the concentration of OT inducing half-maximal stimulation of total inositol phosphate accumulation (K_{act} = 13 nM) in CHO cells.

Fluorescent Labeling of CHO Cells Expressing the Human OT Receptor. CHO cells expressing OT receptors were washed twice with culture medium without fetal calf serum and incubated at 37 or 4 °C with fluorescent ligand at a concentration of 10 K_i and with or without an excess of unlabeled ligand. Then the cells were washed three times, fixed with 4% paraformaldehyde for 30 min, washed three times for 10 min with 0.1 M glycine solution (pH 7.4), and quickly rinsed with PBS. The cells were observed on a Zeiss axiovert 2 microscope equipped for epifluorescence (excitation range, 450−495 nm; barrier filter, 515 nm) using a 63-oil immersion objective. Confocal images were acquired as mid-cellular optical sections on a Noran Odyssee XL confocal microscope with a slit of 10 μm and a 63-oil immersion objective.

Abbreviations

Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (*Eur. J. Biochem*. **1989**, *180*, A9−A11) and IUPHAR (*Trends Pharmacol. Sci.* **2001**). All amino acids are in the L-configuration unless otherwise noted. Other abbreviations: [^{125}I]HO-LVA, 4-HO,3-[^{125}I]PhCH$_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; HO-LVA, linear vasopressin antagonist, 4-HOPhCH$_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; [Lys^8(5C-Flu/Rhm)]PVA, [Lys^8(5C-Flu/Rhm)] phenylpropionic linear vasopressin antagonist, 4-HOPh-(CH$_2$)$_2$CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Lys(5C-Flu/Rhm)-NH_2; [HO^1][Orn^8]VT, [1-L-(−)-2-hydroxy-3-thiopropanoic acid,8-ornithine]vasotocin; [HO^1][Thr^4,Orn^8]VT, [1-L-(−)-2-hydroxy-3-thiopropanoic acid,4-threonine,8-ornithine]vasotocin; [HO^1][Thr^4,Lys^8]VT, [1-L-(−)-2-hydroxy-3-thiopropanoic acid,4-threonine,8-lysine]vasotocin; [HO^1][Lys^8]VT, [1-L-(−)-2-hydroxy-3-thiopropanoic

acid,8-lysine]vasotocin; [Lys^8]VT, [8-lysine]vasotocin; 5/6C-Flu, 5(or 6)-carboxyfluoresceinyl; 5/6C-Rhm, 5(or 6)-carboxytetramethylrhodamyl; AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; Meb, *p*-methylbenzyl; CHO cells, Chinese hamster ovary cells; [DH-Pro^7]OT, [dehydroproline^7]oxytocin; d[Lys^8]VT, [1-deamino,8-lysine]vasotocin; d[Orn^8]VT, [1-deamino,8-ornithine]vasotocin; d[Thr^4,Orn^8]VT, [1-deamino,4-threonine,8-ornithine]vasotocin; d[Thr^4,Lys^8]VT, [1-deamino,4-threonine,8-lysine]vasotocin; DCC, dicyclohexylcarbodiimide; desGly-NH$_2$,d(CH$_2$)$_5$[D-Tyr^2,Thr^4,Orn^8]VT, desglycinamide [1-β-mercapto-β,β- cyclopentamethylenepropionic acid,2-D-tyrosine,4-threonine,8- ornithine]vasotocin; DMF, dimethylformamide; ESMS, electrospray mass spectrometry; Et$_3$N, triethylamine; Et$_2$O, ethyl ether; HEK 293 cells, human embryonic kidney 293 cells; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MeCN, acetonitrile; Mob, *p*-methoxybenzyl; 2ClZ, 2-chlorobenzyloxycarbonyl; Mpr, thiopropanoic acid; HO-Mpr, L-(−)-2-hydroxy-3-thiopropanoic acid; ONp, *p*-nitrophenyl ester; OSu, *N*-hydroxysuccinimide ester; OT, oxytocin; OTA, oxytocin antagonist, (CH$_2$)$_5$C(S)-CH$_2$CO-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Tyr-NH$_2$; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Trt, trityl; Tyr(Me), *O*-methyl-L-tyrosine; d(CH$_2$)$_5$[Tyr(Me)^2,Thr^4,Orn^8,Tyr-NH$_2$^{9}]VT (OTA), [1-β-mercapto-β,β-cyclopentamethylenepropionic acid,2-*O*-methyl-L-tyrosine,4-threonine,8-ornithine, 9-tyrosylamide]vasotocin; AT, angiotensin; VP, vasopressin; VT, vasotocin.

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References

(1) Tsao, P.; Cao, T.; von Zastrow, M. Role of endocytosis in mediating downregulation of G-protein-coupled receptors. *Trends Pharmacol. Sci.* **2001**, *22*, 91−96.

(2) Heim, L.; Meinel, L.; Pratt, R. E.; Dzau, V. J.; Kobilka, B. K. Intracellular trafficking of angiotensin II and its AT1 and AT2 receptors: evidence for selective sorting of receptor and ligand. *Mol. Endocrinol.* **1997**, *11*, 1266−1277.

(3) Barberis, C.; Morin, D.; Durroux, T.; Mouillac, B.; Guillon, G.; Seyer, R.; Hibert, M.; Tribollet, E.; Manning, M. Molecular pharmacology of AVP and OT receptors and therapeutic potential. *Drug News Perspect.* **1999**, *12*, 279−292.

(4) Lutz, W. H.; Londowski, J. M.; Kumar, R. The synthesis and biological activity of four novel fluorescent vasopressin analogues. *J. Biol. Chem.* **1990**, *265*, 4657−4663.

(5) Lutz, W.; Salisbury J. L.; Kumar, R. Vasopressin receptor mediated endocytosis: current view. *Am. J. Physiol.* **1991**, *261*, F1−F13.

(6) Innamorati, G.; Le Gouill, C.; Balamotis, M.; Birnbaumer, M. The long and the short cycle. Alternative intracellular routes for trafficking of G-protein-coupled receptors. *J. Biol. Chem.* **2001**, *276*, 13096−13103.

(7) Phaneuf, S.; Asboth, G.; Carrasco, M. P.; Linares, B. R.; Kimura, T.; Harris, A.; Lopez Bernal, A. L. Desensitization of oxytocin receptors in human myometrium. *Hum. Reprod. Update* **1998**, *4*, 625−633.

(8) Adachi, S.; Oku, M. The regulation of oxytocin receptor expression in human myometrial monolayer culture. *J. Smooth Muscle Res.* **1995**, *31*, 175−187.

(9) Buku, A.; Yamin, N.; Gazis, D. Fluorescent, photoaffinity, and biotinyl analogs of oxytocin. *Peptides* **1988**, *9*, 783−786.

(10) Eggena, P.; Lu, M.; Buku, A. Internalization of fluorescent vasotocin- receptor agonist and antagonist in the toad bladder. *Am. J. Physiol.* **1990**, *259*, C462−C470.

(11) Manning, M.; Kruszynski, M.; Bankowski, K.; Olma, A.; Lammek, B.; Cheng, L. L.; Klis, W. A.; Seto, J.; Haldar, J.; Sawyer, W. H. Solid-Phase Synthesis of 16 Potent (Selective and Non-selective) *in vivo* Antagonists of Oxytocin. *J. Med. Chem.* **1989**, *32*, 383−391.

(12) Elands, J.; Barberis, C.; Jard, S.; Tribollet, E.; Dreifuss, J.-J.; Bankowski, K.; Manning, M.; Sawyer, W. H. ^{125}I-labelled d(CH$_2$)$_5$-[Tyr(Me)^2, Thr^4, Tyr-NH$_2$^{9}]OVT: a selective oxytocin receptor ligand. *Eur. J. Pharmacol.* **1988**, *147*, 197−207.

(13) Manning, M.; Miteva, K.; Pancheva, S.; Stoev, S.; Wo, N. C.; Chan, W. Y. Design and Synthesis of Highly Selective *In Vitro* and *In Vivo* Uterine Receptor Antagonists of Oxytocin: Comparison with Atosiban. *Int. J. Pept. Protein Res.* **1995**, *46*, 244−252.

(14) Durroux, T.; Peter, M.; Turcatti, G.; Chollet, A.; Balestre, M.-N.; Barberis, C.; Seyer, R. Fluorescent pseudo-peptide linear vasopressin antagonists: design, synthesis, and application. *J. Med. Chem.* **1999**, *42*, 1312−1319.

(15) Hope, D. B.; Murti, V. V. S.; du Vigneaud, V. A highly potent analogue of oxytocin, deamino-oxytocin. *J. Biol. Chem.* **1962**, *237*, 1563−1566.

(16) Manning, M.; Coy, C.; Sawyer, W. H. The Deamino Derivatives of [4- Threonine]Oxytocin and [4-Threonine]Mesotocin; Analogs Possessing a Surprising Spectrum of Diminished Pharmacological Activities. *Experientia* **1971**, *27*, 1372−1374.

(17) Hope, D. V.; Walti, M. [1-(L-2-hydroxy-3-mercaptopropanoic acid]-oxytocin, a highly potent analogue of oxytocin not bound to neurophysin. *Biochem. J.* **1971**, *125*, 909−911.

(18) Manning, M.; Lowbridge, J.; Haldar, J.; Sawyer, W. H. Synthesis and Some Pharmacological Properties of [1-(L-2-Hydroxy-3-mercaptopropanoic acid), 4-threonine]oxytocin (Hydroxy[4-Thr]-oxytocin), a Peptide with Strikingly High Oxytocic Potency, and of [1-(L-2-Hydroxy-3- mercaptopropanoic acid)]oxytocin (Hydroxy-oxytocin). *J. Med. Chem.* **1976**, *19*, 376−380.

(19) Manning, M.; Cheng, L. L.; Stoev, S.; Durroux, T.; Mouillac, B.; Guillon, G.; Morin, D.; Derick, S.; Barberis, C. Design and Discovery of Vasopressin and Oxytocin Agonists, Antagonists, and Fluorescent Ligands with High Affinities for Human Receptors. In *Peptides 2000. Proceedings of the 26th European Peptide Symposium*; Martinez, J., Fehrentz, J. A., Eds.; EDK, Editions Medicales et Scientifiques: Paris, France, 2001; pp 105−106.

(20) Durroux, T.; Mouillac, B.; Seyer, R.; Balestre, M.-N.; Terrillon, S.; Taulan, M.; Widmer, H.; Barberis, C.; Cheng, L. L.; Stoev, S.; Manning, M. Fluorescent Oxytocin Agonists and Antagonists: Design, Synthesis and Activities. In *Peptides 2000. Proceedings of the 26th European Peptide Symposium*; Martinez, J., Fehrentz, J. A., Eds.; EDK, Editions Medicales et Scientifiques: Paris, France, 2001; pp 627−628.

(21) Barberis, C.; Balestre, M.-N.; Jard, S.; Tribollet, E.; Arsenijevic, Y.; Dreifuss, J.-J.; Bankowski, K.; Manning, M.; Chan, W. Y.; Schlosser, S. S.; Holsboer, F.; Elands, J. Characterization of a novel linear radioiodinated vasopressin antagonist: an excellent radioligand for vasopressin V1a receptors. *Neuroendocrinology* **1995**, *62*, 135−146.

(22) Chini, B.; Mouillac, B.; Ala, Y.; Balestre, M.-N.; Trumpp-Kallmeyer, S.; Hoflack, J.; Elands, J.; Hibert, M.; Manning, M.; Jard, S.; Barberis, C. Tyr115 is the key residue for determining agonist selectivity in the V1a vasopressin receptor. *EMBO J.* **1995**, *14*, 2176−2182.

(23) Jard, S.; Gaillard, R. C.; Guillon, G.; Marie, J.; Schoeneberg, P.; Muller, A. F.; Manning, M.; Sawyer, W. H. Vasopressin antagonists allow demonstration of a novel type of vasopressin receptor in the rat adenohypophysis. *Mol. Pharm.* **1986**, *30*, 171−177.

(24) Mouillac, B.; Chini, B.; Balestre, M.-N.; Elands, J.; Trump-Kallmeyer, S.; Hoflack, J.; Hibert, M.; Jard, S.; Barberis, C. The binding site of neuropeptide vasopressin V1a receptor: evidence for a major localization within transmembrane regions. *J. Biol. Chem.* **1995**, *270*, 25771−25777.

(25) Phalipou, S.; Seyer, R.; Cotte, N.; Breton, C.; Barberis, C.; Hibert, M.; Mouillac, B. Docking of Linear Peptide Antagonists into the Human V1a Vasopressin Receptor. Identification of binding domains by photoaffinity labeling. *J. Biol. Chem.* **1999**, *274*, 23316−23327.

(26) Bussolati, G.; Chinol, M.; Chini, B.; Nacca, A.; Cassoni, P.; Paganelli, G. ^{111}In-labeled 1,4,7,10-Tetraazacyclododecane-*N*, N′, N″, N‴-tetraacetic Acid-Lys 8-Vasotocin: A New Powerful Radioligand for Oxytocin Receptor-expressing Tumors. *Cancer Res.* **2001**, *61*, 4393−4397.

(27) Carmichael, M. C.; Lutz, W.; Kumar, R. Endocytosis of the vasopressin receptor. In *Vasopressin 1993*; Gross, P., Richter, D., Robertson, G. L., Eds.; John Libbey Eurotext: Montrouge, France, 1993; pp 79−92.

(28) Nouel, D.; Gaudriault, G.; Houle, M.; Reisine, T.; Vincent, J.-P.; Mazella, J.; Beaudet, A. Differential internalization of somatostatin in Cos-7 cells transfected with SST$_1$ and SST$_2$ receptor subtypes: a confocal microscopic study using novel fluorescent somatostatin derivatives. *Endocrinology* **1997**, *138*, 296−306.

(29) Zagyansky, Y. A.; Jard, S. Does lectin-receptor complex formation produce zones of restricted mobility within the membrane? *Nature* **1979**, *280*, 591−593.

(30) Turcatti, G.; Vogel, H.; Chollet, A. Probing the binding domain of the NK2 receptor with fluorescent ligands: evidence that heptapeptide agonists and antagonists bind differently. *Biochemistry* **1995**, *34*, 3972−3980.
(31) Tota, M. R.; Daniel, S.; Sirotina, A.; Mazina, K. E.; Frong, T. M.; Longmore, J.; Strader, C. D. Characterization of a fluorescent substance P analogue. *Biochemistry* **1994**, *33*, 13079−13086.
(32) Turcatti, G.; Nemeth, K.; Edgerton, M.; Knowles, J.; Vogel, H.; Chollet, A. Fluorescent labeling of NK2 receptor at specific sites in vivo and fluorescence energy transfer analysis of NK2 ligand-receptor complexes. *Recept. Channels* **1997**, *5*, 201−207.
(33) Hope, D. B.; Walti, M. Synthesis of α-Hydroxy-analogues of S-Benzylcysteine and Cysteine. *J. Chem. Soc. (C)* **1970**, 2475−2478.
(34) Photaki, I.; Tsougraki, C.; Kotsira-Engonopoulos, C. Synthesis and some pharmacological properties of [Glu(OMe)^4]oxytocin and [Mpr^1, Glu(OMe)^4]oxytocin. *Int. J. Pept. Protein Res.* **1979**, *13*, 426−433.
(35) Rimpler, M. [1-Desamino]Lysinvasotocin, ein Disulfidpolypeptid mit Oxytocin-Wirkung. *Justus Liebigs Ann. Chem.* **1971**, *745*, 8−19.
(36) Fahrenholz, F.; Eggena, P.; Kojro, E.; Gazis, D.; Schwartz, I. L. Synthesis and biological activities of a photoaffinity probe for vasotocin and oxytocin receptors. *Int. J. Pept. Protein Res.* **1987**, *30*, 577−582.
(37) Huguenin, R. L. Syntheses de la desamino^1-Orn^8-vasopressine, de la desamino^1-Phe^2-Orn^8-vasopressine, de la desamino^1-Ile^3-Orn^8- vasopressine (=desamino-Orn^8-oxytocine) et de la desamino^1-Phe^2-Ile^3- Orn^8-vasopressine (=desamino^1-Phe^2-Orn^8-oxytocine). *Helv. Chim. Acta* **1966**, *49*, 711−725.
(38) Merrifield, R. B. Solid-phase peptide synthesis. III. An improved synthesis of bradykinin. *Biochemistry* **1964**, *3*, 1385−1390.
(39) Stewart, J. M.; Young, J. D. *Solid Phase Peptide Synthesis*; Pierce Chemical Co.: Rockford, IL, 1984.
(40) Manning, M.; Coy, E.; Sawyer, W. H. Solid-phase synthesis of [4-threonine]-oxytocin. A more potent and specific oxytocic agent than oxytocin. *Biochemistry* **1970**, *9*, 3925−3929.
(41) Koenig, W.; Geiger, R. A new method for the synthesis of peptides: Activation of the carboxyl group with dicyclohexyl-carbodiimide by using 1-hydroxy-benzotriazole as additives. *Chem. Ber.* **1970**, *103*, 788−798.
(42) Bodansky, M.; du Vigneaud, V. A method of synthesis of long peptide chains using a synthesis of oxytocin as an example. *J. Am. Chem. Soc.* **1959**, *81*, 5688−5691.
(43) Manning, M. Synthesis by the Merrifield method of a protected nonapeptide amide with the amino acid sequence of oxytocin. *J. Med. Chem.* **1968**, *11*, 1348−1349.
(44) du Vigneaud, V.; Gish, D. T.; Katsoyannis, P. G. A synthetic preparation possessing biological properties associated with arginine-vasopressin. *J. Am. Chem. Soc.* **1954**, *76*, 4751−4752.
(45) Rivier, J.; Kaiser, R.; Galyean, R. Solid-phase synthesis of somatostatin and glucagon-selective analogs in gram quantities. *Biopolymers* **1978**, *17*, 1927−1938.
(46) Manning, M.; Wuu, T. C.; Baxter, J. W. M. The purification of synthetic oxytocin and analogues by gel filtration on Sephadex G-15. *J. Chromatogr.* **1968**, *38*, 396−398.
(47) Kassis, S.; Henneberry, R. C.; Fishman, P. H. Induction of catecholamine- responsive adenylate cyclase in HeLa cells by sodium butyrate. *J. Biol. Chem.* **1984**, *259*, 4910−4916.
(48) Kirk, C. J.; Guillon, G.; Balestre, M.-N.; Jard, S. Stimulation, by vasopressin and other agonists, of inositol-lipid breakdown and inositol phosphate accumulation in WRK1 cells. *Biochem. J.* **1986**, *240*, 197−20.

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