Design, Synthesis and Pharmacological Characterization of a Potent Radioiodinated and Photoactivatable Peptidic Oxytocin Antagonist[∞]

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Received March 19, 2001

Using a segment strategy, we have synthesized four iodinated photoactivatable cyclic peptidic ligands of oxytocin, bearing a β-mercapto-$\beta\beta$-cyclopentamethylene propionic group (Pmp) on their *N*-terminus. All the syntheses were RP-HPLC monitored, and the compounds were HPLC purified. They were characterized by ^{1}H NMR, MALDI-TOF, or FAB mass spectrometries. The affinities of Pmp-**Tyr(Me)**-Ile-Thr-Asn-Cys-**Gly**-Orn-Phe(3I,4N_3)-NH_2 (**20**), Pmp-**Tyr**-Ile-Thr-Asn-Cys-**Gly**-Orn-Phe(3I,4N_3)-NH_2 (**21**), Pmp-**Tyr(Me)**-Ile-Thr-Asn-Cys-**Pro**-Orn-Phe(3I,4N_3)-NH_2 (**22**), and Pmp-**Tyr**-Ile-Thr-Asn-Cys-**Pro**-Orn-Phe(3I,4N_3)-NH_2 (**23**) were evaluated as inhibition constants (K_i, in nM) for the human oxytocin receptor expressed in Chinese hamster ovary cells by displacement of a radioiodinated disulfide-cyclized antagonist (Elands et al. *Eur. J. Pharmacol.* **1987**, *147*, 197−207). The most potent of them, compound **22**, was synthesized by another method in order to allow its radiolabeling by ^{125}I. Its dissociation constant (K_d) for the human oxytocin receptor, directly measured in saturation studies, was 0.25 ± 0.04 nM, and its antagonist properties were determined by inactivation of phospholipase C, thus obtaining an inactivation constant (K_{inact}) of 0.18 ± 0.02 nM, evaluated by inositol phosphate accumulation. This compound is a very good tool for the mapping of peptidic antagonist binding sites in the human oxytocin receptor.

Introduction

Oxytocin (OT) is a neurohypophyseal nonapeptide that regulates several physiological functions. It stimulates the contraction of uterine and mammary gland muscles during parturition and lactation, respectively.[1] OT acts through receptors that belong to the G-protein-coupled receptor, characterized by seven putative transmembrane domains.[2] In the previous decade, several OT receptors have been cloned from various mammalian species.[3−5] Functionally coupled to phospholipase C, oxytocin receptors[6] share a high degree of peptide sequence homology with the different vasopressin receptor subtypes. As well, the peptide sequence of OT is structurally very close to that of vasopressin, and numerous analogues have been described to date for both neuropeptides.[7] On careful and meticulous survey of the literature,[8] we considered that the V_{1a} vasopressin receptor subtype could serve as a good model for the study of structure−activity relationships. Furthermore, because OT and vasopressin receptors belong to the same family, we thought that the structural information obtained for the V_{1a} vasopressin receptor subtype could be very useful for the study and understanding of ligand−receptor interactions of the OT receptor itself. Therefore, we were interested in designing and synthesizing a series of OT antagonists because of their potential therapeutic interest, for instance, in the treatment of premature labor.[9−11]

Site-directed mutagenesis studies[12] and construction of chimeric OT receptor have been performed;[13] however, limited information was available regarding the OT receptor binding domains. A complementary approach is the use of photoactivatable analogues. On the basis of the formation of a covalent bond between the ligand and its receptor upon UV irradiation, this method allows the mapping of membrane proteins, particularly under denaturing conditions.[14] To date, several ligands for the photolabeling of OT receptor have been re-

[∞] Abbreviations: Abbreviations used are in accordance to the IUPAC−IUB Commission (*Eur. J. Biochem.* **1984**, *138*, 9−37). The following abbreviations were also used: BHA resin, benzhydrylamine resin; Boc, *tert*-butyloxycarbonyl; BOP, (1*H*-1,2,3-benzotriazol-1-yl-oxy)-tris(dimethylamino)phosphonium hexafluorophosphate; BSA, bovine serum albumine; CHO, Chinese hamster ovary cells; COSY, correlated spectroscopy; Dcb, dichlorobenzyl; DFQ-COSY, double-quantum-filtered correlated spectroscopy; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediamine tetraacetate; FAB, fast atom bombardment; HPLC, high-performance liquid chromatography; (i-Pr)$_2$EtN, diisopropylethylamine; [^{125}I]-OTA, Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Tyr(3^{125}I)-NH_2; K_{act}, activation constant; K_{inact}, inactivation constant; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MBHA resin, *p*-methylbenzhydrylamine resin; mol equiv, molar equivalent; MS, mass spectroscopy; NMR, nuclear magnetic resonance spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; Orn, ornithine; OT, oxytocin, Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2; OTA, oxytocin antagonist, Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Tyr-NH_2; PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; Pmp, β-mercapto-$\beta\beta$-cyclopentamethylene propionic acid; PyBOP, (benzotriazolyloxy)tripyrrolidinophosphonium hexafluorophosphate; SDS, sodium dodecyl sulfate; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane; Xan, xanthyl; Z, benzyloxycarbonyl; ZOTA, azido oxytocin antagonist (**18**).

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10.1021/jm010125u CCC: $20.00
Published on Web 08/02/2001

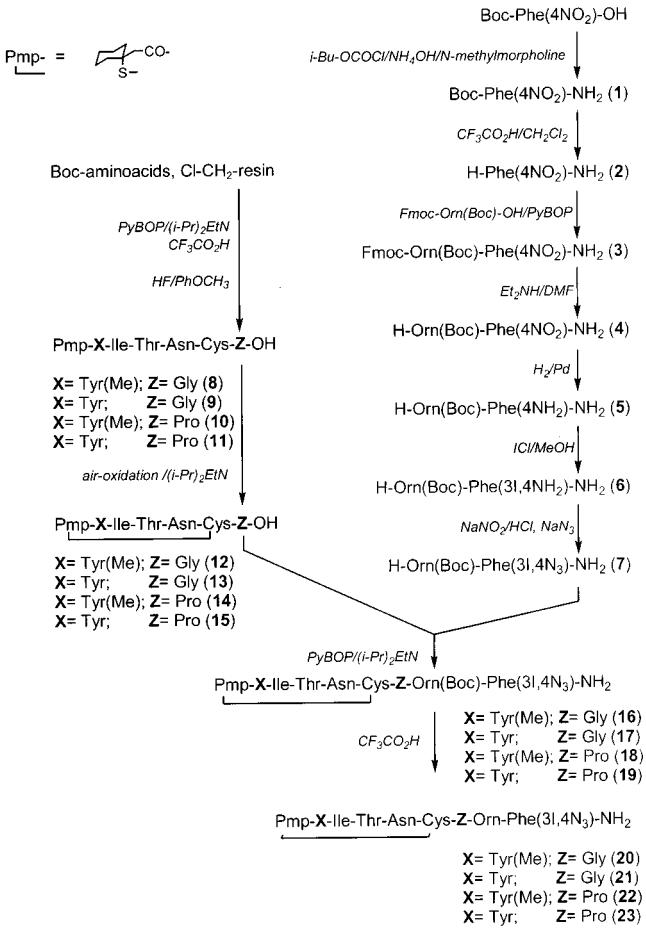
ported.[15−19] However, they were mainly synthesized by direct coupling of bulky aromatic groups on functional amino acid side chains, and no direct identification of receptor residues in close interaction with the probe has been reported. Thus, we exploited OT analogues specifically designed according to a different approach, consisting of the minimal structural modification of the ligand peptide sequence.[20] We synthesized them using a combination of solid phase and solution synthesis and characterized them pharmacologically by measuring their affinities on CHO cells, stably expressing the human OT receptor. We selected the analogue from the series displaying the highest affinity and subsequently developed a synthesis strategy for radiolabeling. The structure of the nonradioactive iodinated analogue was ascertained by ^{1}H NMR spectrometry, and its pharmacological properties were determined using saturation binding experiments and also measure of inositol phosphate accumulation.

Results

Probe Design. It was generally observed that structural modifications of the cyclic part of the OT analogue family often resulted in a loss of both affinity and activity of the ligands while chemical modifications of the C-terminal section were better tolerated.[21] Accordingly, we designed photoactivatable OT analogues on the basis of the following: (i) the replacement of the N-terminal Cys residue by the Pmp group[22] increases both the affinity and the antagonist potency of the analogues, (ii) the substitution of the Gln^4 and Pro^7 residues for Thr^4 and Gly^7 residues, respectively, results in an increased selectivity of the ligand for the OT receptor,[23] and (iii) O-alkylation of the Tyr^2 residue also increases the antagonist potency of the analogues.[24,25] Thus, we selected, as lead compounds, antagonist Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Tyr(3^{125}I)-NH_2 ([^{125}I]-OTA), on the basis of its maximum affinity, and analogue Cys-Tyr-Ile-Thr-Asn-Cys-Gly-Leu-Gly-NH_2, on the basis of its maximum selectivity for the OT receptor.[23,26,27] To maximally conserve the structure of [I]-OTA, we selected the azido group to replace the *p*-hydroxyl group of the C-terminal Tyr^9 residue,[28] resulting in residue Phe(4N_3)^9. We also explored two positions by introducing either a Tyr or a Tyr(Me) residue in position 2 and a Gly or a Pro residue in position 7 (Scheme 1). We finally chose to introduce the iodine atom on the same aromatic ring as the azido group (Phe(3I,4N_3)^9, keeping in view that the close proximity of the ^{125}I tracer to the covalent bond established on photoactivation would lower the risk of losing signal detection owing to enzymatic cleavage of the ligand. Though it has been suggested that partial deiodination might occur during the irradiation step due to intramolecular rearrangements of the highly reactive nitrene intermediate,[29,30] many results were reported with such a configuration,[31−33] and several reagents offering such structural characteristic are currently available commercially (see Pierce catalog).

To synthesize analogues **20**−**23**, the general structure of which is given in Scheme 1, we had to (i) conciliate the catalytic hydrogenation of a Phe(4NO_2) precursor residue and the presence of a disulfide bridge, (ii) control the reactivity of the diazonium intermediate produced during the synthesis of the azido group and enable the reaction with the Tyr^2 residue, and (iii) perform the selective iodination of the Phe^9 residue at the Phe(4NH_2)^9 intermediate stage without any unexpected reaction at position 2 (Tyr^2-containing analogues). Accordingly, we selected a segment-coupling strategy taking advantage of the presence of the nonracemizable Gly or Pro residues at position 7. We combined solid-phase and solution peptide synthesis to obtain, respectively, the *N*-terminal heptapeptide segments (residues 1−7) and the common C-terminal dipeptide **7** (residues 8−9) for the final coupling.

Scheme 1. Synthesis of Analogues 20−23 for Preliminary Affinity Determination[a]



[a] Right part of the scheme: solution synthesis of dipeptide **7**. Left part of the scheme: solid-phase synthesis of heptapeptides **8**−**11** and disulfide-cyclization giving heptapeptides **12**−**15**. Lower part of the scheme: coupling of heptapeptides **12**−**15** to dipeptide **7** giving compounds **16**−**19**, followed by their Boc deprotection giving analogues **20**−**23**.

Probes Synthesis. All heptapeptide fragments (compounds **12**−**15**) were synthesized on a chloromethylated resin, using Boc as protecting group and PyBOP as coupling reagent;[34] HF deprotected; cyclized by air-oxidation (Scheme 1); and subsequently purified by RP-HPLC. Dipeptide **7** was synthesized in solution according to the synthetic route described in Scheme 1. The reactions of cyclization, coupling, hydrogenation, iodination, and azidation were monitored by HPLC, using a dual wavelength detection (214 and 254 nm).[35] The second UV detector was set at 254 nm to take advantage of the high absorbance properties of the aryldiazonium and arylazido groups at that wavelength. Indeed, the

presence of these chromophores in the different peptide intermediates always resulted in an important decrease of the corresponding $\epsilon_{214}/\epsilon_{254}$ ratio, which was particularly helpful for peptide identification during HPLC monitoring. In a similar way, the disulfide cyclization of compounds **12**−**15** to compounds **16**−**19** resulted in a 2-fold decrease of the $\epsilon_{214}/\epsilon_{254}$ ratio in each case. This observation, along with the increased polarity of the cyclized peptides (1−3% in CH_3CN elution difference), facilitated the identification of the synthesized compounds, subsequenly confirmed by Ellman's reagent and MALDI-TOF MS. Compounds **20**−**23** were obtained by coupling each heptapeptide **12**−**15** to the dipeptide **7**, followed by deprotection of the Orn(Boc)[8] residue and RP-HPLC purification. The removal of the Boc group resulted in an important shift in the HPLC elution, approximately 7% lower CH_3CN as compared to the Boc precursor, but no change of the $\epsilon_{214}/\epsilon_{254}$ ratios was observed. The purities of compounds **20**−**23** were assessed by HPLC and their structures were confirmed by FAB-MS. It is also noticeable that O-methylation of the phenol group of Tyr^2 resulted in a 6% CH_3CN increase of elution and that all Pro^7-containing peptides and intermediates elute 1−2% higher than the corresponding Gly^7-containing peptides. According to this synthetic route, compound **18** was always obtained as the major product of a mixture of three compounds and was particularly difficult to purify because of very close HPLC elution percent at 55, 55.7, and 56% CH_3CN, respectively. This observation, together with the need for a convenient radiolabeling of the compounds, prompted us to design another synthesis strategy.

NMR Study. The chemical structure of peptide **22**, the analogue observed to be the most promising candidate for photolabeling experiments, was confirmed by two-dimensional ^1H NMR. All expected spin systems were identified in the TOCSY experiment, and their assignment was obtained by using the sequential procedure described by Wüthrich.[36] The combination of the COSY, TOCSY, and NOESY spectra allowed us to unambiguously assign all expected spins systems as well as aromatic signals of substituted rings of $Tyr(Me)^2$ and $Phe(3I,4N_3)^9$. Indeed, in the case of $Phe(3I,4N_3)^9$, due to the 3-iodo and 4-azido substitutions, the three-signal system composed of two doublets (6-H and 5-H) and a singlet (2-H) was clearly observed with the TOCSY. Of the two doublets, the 6-H assignment was performed on the basis of NOE with $C_\beta H_2$ protons. In a similar way, the 2,6-H and 3,5-H aromatic signals of the $Tyr(Me)^2$ were assigned on the basis of their NOE with the $C_\beta H_2$ and the methoxy group, respectively. The observation of a strong NOE between the H_α-proton of Cys^6 with the H_δ–H_δ protons of Pro^7 is indicative of the trans conformation for the Cys^6/Pro^7 amide bond.

Affinities of the Nonradioactive Iodinated Ligands 20−**23.** The affinities of ligands **20**−**23** were determined on membrane preparations from CHO cells stably expressing the human OT receptor. We performed concentration-dependent binding inhibitions using the specific radiolabeled antagonist [^{125}I]-OTA as radioligand[23] (Figure 1). The data suggest that (i) analogues containing a Pro residue in position 7 (**22** and **23**, $K_i \approx 0.3$ and 0.6 nM respectively) display a better affinity than those containing a Gly residue (**20** and **21**

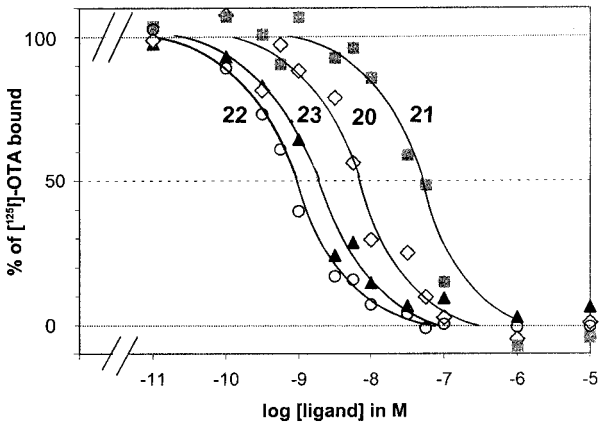


Figure 1. Concentration-dependent binding experiments of analogues **20**−**23** to human OT receptor stably expressed in CHO cells. Membrane preparations (2−3 μg) were incubated for 1 h at 30 °C in darkness with increasing amounts of each peptide (10 pM to 10 μM) and 200−220 pM of [^{125}I]-OTA. Values of specific binding, measured in the presence of unlabeled ligands, are expressed as the percentage of the total binding measured in the absence of competitor. The apparent dissociation constants (K_i) of the nonradioactive ligands were calculated from the relation $K_i = IC_{50}/(1 + [L]/K_d)$, where IC_{50} is the concentration of analogue resulting in 50% inhibition of total binding and [L] and K_d are the concentration and the dissociation constant of [^{125}I]-OTA, respectively, with $K_d = 0.18$ nM. Results are the mean of triplicate values and are representative of, at least, three independent experiments.

$K_i \approx 2.3$ and 18 nM respectively) and (ii) for each Pro^7- or Gly^7-containing analogue, O-alkylation of the Tyr^2 residue with a methyl group resulted in a slight increase in affinity (K_i shifting from 18 to 2.3 nM for analogues **21** and **20** and from 0.6 to 0.3 nM for analogues **23** and **22**). Ligand **22**, whose structure differs from antagonist [I]-OTA only by the presence of an azido group in place on the hydroxyl group, showed the best affinity for the human OT receptor expressed in CHO cells. On the basis of these findings, we selected compound **22** for radioiodination (becoming ligand **29**), to determine its ability to interact directly with specific receptors, employing saturation experiments.

Synthesis of the Radioactive Ligand 29 ([^{125}I]-ZOTA). To synthesize ligand **29**, we developed a different schedule in order to reduce the handling of radioactive intermediate compounds that would have been generated at several steps of the segment coupling strategy. Indeed, since the iodine atom may only be introduced at the $Phe(4NH_2)$ stage, because of the structural delocalization of the π-electrons on the meta position of the aromatic ring (Figure 2), the following reactions of azidation, segment coupling, and Boc deprotection would have to be performed on radioactive compounds. Therefore, we designed a strategy consisting of the solid-phase synthesis of nonapeptide **25**, with the direct incorporation of a Phe(4NHZ) residue and its subsequent iodination and complete azidation.[37] Peptide **25** has been synthesized on a *p*-methylbenzhydrylamine resin, using Boc as temporary protecting group, PyBOP as coupling reagent, and a *p*-aminophenylalanine residue with a benzyloxycarbonyle group as side chain protection. The product was cyclized by air-oxidation and RP-HPLC purified and its structure checked by MALDI-TOF MS (Figure 2a).

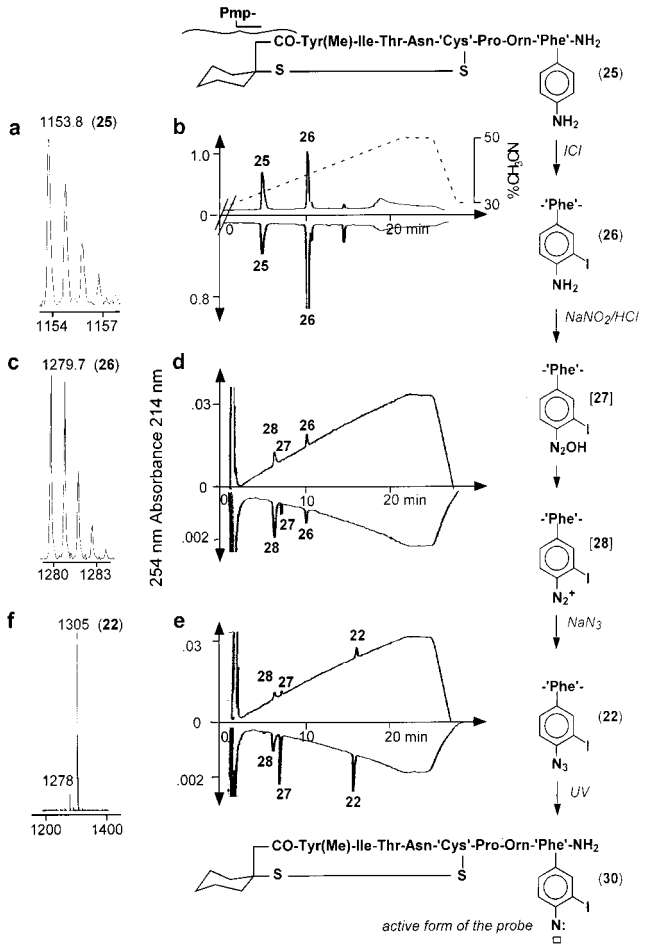


Figure 2. MS and HPLC monitoring of analogue **22** synthesis according to the strategy designed for obtaining radioactive analogue **29**. Upper part of the figure: detailed sequence of precursor **25**. Right part of the figure: description of the "Phe^8" moiety at different steps of the synthesis, respectively, as intermediate "Phe($4NH_2$)" (**25**), "Phe($3I,4NH_2$)" (**26**), "Phe($3I,4N_2OH$)" (hypothetic intermediate **27**), "Phe($3I,4N_2^+$)" (**28**), and "Phe($3I,4N_3$)" (**22**). Lower part of the figure: photoactivated form of analogue **22** represented as its highly reactive nitrene (short-life intermediate **30**). Panels a and c: MALDI-TOF mass spectra of compound **25** and **26**. Panel f: FAB mass spectra of azidopeptide **22**. The M + 1 molecular main peak (1305) is always accompanied by an unidentified degradation product resulting from bombardment. Panels b, d, and e: chromatograms illustrating the main steps for the synthesis of analogue **22**. RP-HPLC monitoring was performed on a C_{18}-column at a flow rate of 2 mL/min in the linear CH_3CN gradient mode (dotted line as shown on panel b) using 0.1% CF_3CO_2H acidified eluents. The detection was performed at two distinct wavelengths (214 nm upward and 254 nm downward). Panel b: The iodination of compound **25** by ICl in MeOH gives the expected monoiodinated derivative **26** and traces of the more hydrophobic diiodinated compound (third peak). Panel d: The diazotization of compound **26** was performed at 0 °C in darkness using an excess of $NaNO_2$/HCl and results in the formation of two products (**27** and **28**) having very different absorbance properties ($\epsilon_{214}/\epsilon_{254}$). Panel e: Once compound **26** reacted completely, an excess of NaN_3 was added and the reaction mixture was allowed to reach room temperature to give the hydrophobic azidopeptide **22**.

The radiolabeling procedure of compound **25** was first optimized using nonradioactive iodine (^{127}I) at the usual radioiodination concentration scale.[38] Owing to 50% of iodine incorporation, for a reaction with 1 mCi $Na^{125}I$ (specific activity of 2200 Ci/mmol), we, therefore, developed procedures using either ICl or NaI/Iodo-Gen for nonradioactive iodinations at a nanomolar scale (Figure 2b). Monoiodinated peptide **26** was obtained by an equimolar reaction of ICl in MeOH with freshly dissolved peptide **25** (≈1 nmol), purified by RP-HPLC and characterized by MALDI-TOF MS (Figure 2c). Compound **26** (approximately 0.5 nmol) was then progressively diazotized with an excess of $NaNO_2$ in HCl at 0 °C, in darkness, to obtain a mixture of two compounds that could not be identified by MALDI-TOF MS, probably because of their photosensitivity (Figure 2d, peaks **27**, **28**). Peak **28** might be the corresponding diazonium intermediate since it disappeared progressively upon addition of NaN_3 in excess (2 mol equiv toward $NaNO_2$) at room temperature to give ligand **22** ([I]-ZOTA, Figure 2e), which was characterized by FAB-MS (Figure 2f). Identical strategy was employed for synthesizing the radioactive ligand **29** ([^{125}I]-ZOTA), except that compound **25** was iodinated with $Na^{125}I$ (1 mCi) in the presence of Iodo-Gen and HPLC purified for diazotization and azidation. Ligand **29** was purified by HPLC and stored in liquid nitrogen. The identity of radioactive ligand **29** was determined by HPLC coinjection of compounds **22** and **29** and measuring the radioactivity associated with the 0.5-mL HPLC fractions.

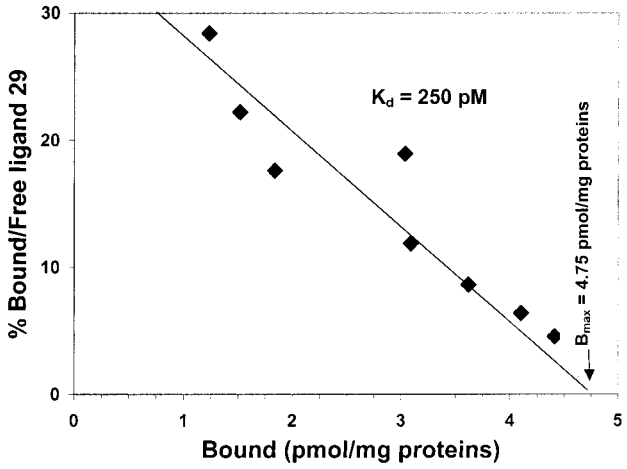


Figure 3. Scatchard representation of a saturation binding experiment performed with radioiodinated analogue **29**. Membrane preparations of CHO cells stably expressing the human OT receptor (2−3 μg) were incubated in the presence of increasing concentrations of peptide **29** (10−900 pM) for 1 h at 30 °C in darkness. Specific binding was expressed as the difference between total and nonspecific binding determined in the presence of 800 nM of the corresponding nonradioactive iodinated analogue (**22**). The results are the mean of triplicate values and are representative of three independent experiments, giving a K_d value of 0.25 ± 0.04 nM.

Pharmacological Studies. The Scatchard representation of the saturation binding experiments of ligand **29** is linear (Figure 3), indicating that this compound interacts with a single class of receptors (Hill coefficient = 0.97). The determination of its affinity gave a K_d value of 0.25 ± 0.04 nM, suggesting that the replacement of the initial hydroxyl group by an azido had nearly no influence on the affinity of the parent ligand (K_d of [^{125}I]-OTA = 0.18 ± 0.04 nM).

To determine its biological activity, we measured the ability of ligand **22** (the nonradioactive iodinated form of **29**) to inhibit OT-induced inositol phosphate accumulation in CHO cells, in a concentration-dependent

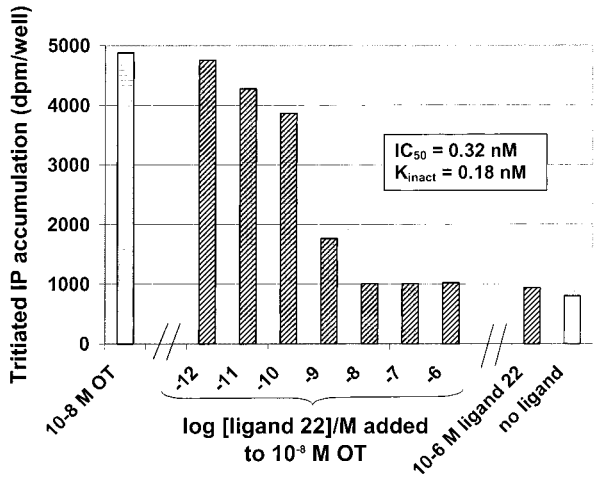


Figure 4. Determination of the antagonist properties of analogue **22**. The ability of analogue **22** to inhibit OT-induced inositol phosphate accumulation was determined in CHO cells stably expressing the human OT receptor. *myo*-[2-^{3}H]Inositol-prelabeled- and PBS-equilibrated CHO cells were incubated for 10 min in PBS supplemented with 10 mM LiCl, in the absence or presence of increasing concentrations of ligand **22** (1 pM to 1 μM, all hatched open and gray-colored bars). Cells were then incubated in the presence of 10 nM OT (all gray-colored open and hatched bars) for an additional 15 min period, and the reaction was stopped by addition of ice-cold perchloric acid followed by neutralization of the samples. The radioactivity associated to extracted inositol phosphates was measured, and the results were expressed as disintegrations per min/well, with no correction for the basal activity (open bar). Results are from a single experiment representative of three independent experiments each performed in triplicate. The apparent inactivation constant $K_{inact} = 0.18 \pm 0.02$ nM was calculated from $K_{inact} = IC_{50}/(1 + [OT]/K_{act})$, where IC_{50} is the concentration of analogue **22** yielding 50% inhibition of specific total inositol phosphate accumulation, [OT] = 10 nM, and K_{act} is the concentration of OT inducing half-maximum accumulation of inositol phosphates (K_{act} = 12.9 nM for CHO cells expressing the wild-type human OT receptor).

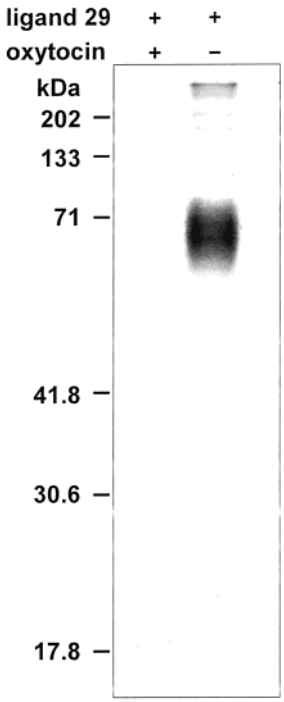


Figure 5. Photolabeling of the human oxytocin receptor. Membrane preparations of CHO cells stably expressing the OT receptor (500 μg, 4 mL) were incubated with freshly radioiodinated analogue **29** (≈2 nM) in a Tris:HCl buffer (Tris 50 mM, $MgCl_2$ 5 mM, pH 7.4, 0.5 mg/mL BSA) with (left lane) or without (right lane) an excess of 10^{-5} M oxytocin, for 1 h at 30 °C. Membranes were then washed twice using cold Tris:HCl buffer (without BSA, 10 mL) to remove unbound ligand **29** and were resuspended in the same buffer for UV irradiation (254 nm, 1 min) in a Petri dish, on ice. The membranes were washed twice again and solubilized in Laemmli buffer, and the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (gel with 12% bisacrylamide) as were molecular mass standards. Equivalent amounts of proteins (25 μg) were loaded into each well. The gel was further fixed, dried, and autoradiographed on Kodak film for approximately 10 h at −70 °C. Positions of protein standard masses are indicated on the left part of the figure. The autoradiogram of the dried gel is representative of at least three distinct experiments.

manner.[12,39] As shown in Figure 4, the ligand **22** was able to inhibit the action of 10 nM OT, at a concentration displaying half-maximal stimulation of inositol phosphate production, with a calculated K_{inact} of 0.18 ± 0.02 nM. In addition, the incubation of high concentrations of ligand **22** on CHO cells (up to 1 μM) resulted in the production of inositol phosphate levels comparable to those of the basal activity, suggesting that this ligand is a potent antagonist for the human OT receptor.

To determine whether analogue **22** ([I]-ZOTA) was able to covalently and specifically bind to the human OT receptor, membrane preparations of transfected CHO cells were incubated with analogue **29** ([^{125}I]-ZOTA), the radioactive form of ligand **22**. Then, membranes were washed, irradiated at 254 nm, and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) for autoradiography. As shown in Figure 5, the labeling (right lane) was specific since it could be completely displaced when the incubation was performed with an excess of oxytocin (left lane). Irradiation of the membranes resulted in the strong labeling of a major protein band migrating at an apparent molecular mass of 70−75 kDa, which is in agreement with the expected molecular mass for the native glycosylated human OT receptor. An additional faint band at around 42 kDa was also specifically labeled and could correspond to the molecular mass of the proteic core of the human OT receptor, as deduced from its cDNA sequence. This labeling was 254 nm-dependent and easily observable, even under denaturing conditions such as for SDS−PAGE, and no labeling at all could be observed in the absence of UV irradiation (data not shown).[44]

Discussion

A survey of the literature reveals that the photoactivatable OT derivatives previously described bear the photoreactive group and the ^{125}I-tracer on separate residues and were obtained by introduction of bulky arylazido groups to functional amino acids side chains.[17−19,40] Though the affinity criterion is not considered to be crucial for photolabeling experiments, we always obtained the best results using high-affinity ligands, particularly because of the absence of non-specific labeling.[41] Therefore we selected, as lead compounds, analogues having high affinity and high selectivity for the OT receptors, and we designed new photoreactive analogues in a manner so as to maintain

as much as possible their close interactions with the targeted receptor. Thus, we modified an aromatic residue already present in the peptide sequence. We replaced the Tyr[9] *p*-hydroxyl group of antagonist [I]-OTA with an azido group, because it is activated in a nitrene intermediate (Figure 2, active form **30**)[42] that, among the known reactive groups, possesses structural similarity to the hydroxyl function.[28] This position of the azido group should allow a more precise and representative mapping of the receptor-binding site, as compared to ligands previously reported,[17−19,40] for which the photoreactive groups were more distant because of the additional aromatic structures necessary for their introduction. It has also been reported that the *C*-terminal part of OT analogues was barely sensitive to chemical modifications, suggesting that the last two residues were probably not important for affinity. Accordingly, we showed that the replacement of the hydroxyl group with a hydrophobic azido group did not modify significantly the affinity of the molecule (ligand **22**, $K_d = 0.25 \pm 0.02$ nM) compared to [I]-OTA (0.18 ± 0.04 nM).

The introduction of the photoreactive group on the Phe[9] residue offers the opportunity to further functionalize these ligands. Indeed, the remaining free amino group of the Orn[8] side chain could serve as an anchor point for the introduction of fluorophores, biotin, or other groups, allowing the study of ligand−receptor interactions by different biochemical approaches. These derivatizations can be easily performed using the segment coupling strategy, particularly adapted for the preparation, in high amounts, of various chemically modified C-terminal dipeptides (8−9) in solution. We also developed in this work an experimental procedure allowing the complete azidation of radioiodinated compounds, suggesting that such syntheses can be performed routinely with efficiency and reproducibility, though subnanomolar amounts of compounds are usually involved in these reactions. Among the series of photoactivatable OT ligands we designed and synthesized, analogue **22** displayed the highest affinity toward the human OT receptor (stably expressed in CHO cells). It was able to inhibit the OT-induced inositol phosphate accumulation, indicating that the introduction of the photoactivatable azido group did not modify the antagonist properties of the parent compound. Furthermore, the trans conformation, mainly observed for the Cys[6]-Pro[7] amide bond by NMR studies, seemed not to alter these antagonistic properties, unlike what had been reported recently.[43]

Thus we can conclude that ligand **22** is a promising candidate for the study of antagonist−receptor interactions, as observed from the results obtained on the mapping of the human OT receptor-binding site.[44] Furthermore, the synthesis procedure can serve for the design of novel photoactivatable ligands. As an example, it would be possible to isolate the aryldiazonium intermediate (compound **28**), using appropriate stabilizing counterions ($CF_3CO_2^-$, BF_4^-).[45] Aryldiazonium groups are photoreactive groups generating arylcations upon UV irradiation.[46] These are positively charged and are characterized by a different reactivity compared to arylnitrenes. Therefore, such compounds, used in combination with arylazido derivatives, could possibly complete the mapping of the OT receptor antagonist-binding site.

Experimental Section

General. Protected L-amino acids, Boc-L-Tyr(Me)-OH, Pmp(Mob), Boc-Phe(*p*-NHZ)-OH, PyBOP, BHA, and MBHA resins were from either Propeptide, Bachem, or Novabiochem. Solid-phase peptide synthesis was performed using a manual device as previously described,[34] and solvents used were of analytical grade. The pH of the organic solutions during solid-phase and solution synthesis was determined using moistened pH indicator paper. Peptides were deprotected using HF (from Matheson, provided by Interchim) on a Kel-F apparatus (from the Protein Research Foundation). Couplings of the heptapeptidic segments to H−Orn(Boc)-Phe(3I,4N_3)-NH_2 segment were performed in UV spectroscopy grade quality Me_2SO and DMF. The ligand Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Tyr-NH_2 (OTA) was a generous gift from Dr Manning and was radioiodinated as previously described.[26] All diazo and azido peptides were handled in darkness.

HPLC. Analytical HPLC monitorings were performed using three types of conditions. For system I, a Merck Lichrosorb C_{18} column (5-μm particle size, 4 × 250 mm, 100 Å porosity) was fitted to a Shimadzu LC 9A pump equipped with two Waters 440 and 441 photometers, operating at 214 and 254 nm, respectively. Elution was performed with linear gradients of mobile phase B (CF_3CO_2H:CH_3CN, 0.1/100, v/v) in mobile phase A (CF_3CO_2H:H_2O, 0.1/100, v/v) at a flow rate of 2 mL/min and gradients of 1% B/min (void volume, $v_0 \approx 1.7$ mL). The values reported as percent elution correspond to the percent of CH_3CN of the eluent passing through the detector cell at the time of UV detection and were corrected for the void volume (v_0). For system II, a Merck Lichrospher C_{18} end-capped column (5-μm particle size, 100 Å porosity, 4 × 250 mm) was fitted, instead of the Lichrosorb column described in system I, and run at a 1 mL/min flow rate. For system III, the radioiodinated compounds were handled on a specially devoted installation, including a Lichrocart Purospher C_{18} column (250 × 4 mm; 5-μm particle size, end-capped) connected to a high-pressure Beckman gradient system with two 114 M pumps and a 421 A controller, a 440 Waters detector set at 254 nm, and a LKB fraction collector. Elution was performed with linear gradients of mobile phase B (CF_3CO_2H:CH_3CN:H_2O, 0.1/60/40, v/v/v) in mobile phase A (CF_3CO_2H:H_2O, 0.1/100, v/v) at a flow rate of 1 mL/min and gradients of 1% B/min. Highly radioactive 0.5-mL fractions were measured on a Mini-Assay type 6−20 γ-counter (Numelec) equipped with an attenuator.

Semipreparative HPLC was performed at each step of the synthesis utilizing the high-pressure gradient mode employing a dual pump system (model 400, Applied Biosystem), driven by a 738A controller (Applied Biosystem) and fitted with either a Whatman Partisil ODS 3 Magnum 20 column (10-μm particle size, 22 × 500 mm, $v_0 \approx 110$ mL) or a Vydac 218TP1022 column (10-μm particle size, 100 Å porosity, 22 × 250 mm, $v_0 \approx 50$ mL). UV detection was performed using a Waters 440 absorbance detector (254 nm) and an ABI 738A spectrophotometric detector (set on 214 nm). Peptide purifications were done using linear gradients of 0.2%, 0.5%, or 1% CH_3CN/min at a flow rate of 10 mL/min, as previously reported.[35] Briefly, the peak corresponding to the desired peptide was collected in several fractions using a Gilson model 202 fraction collector, coupled to the 214-nm detector and operating in the peak detection mode. Each fraction was further checked by analytical HPLC and finally selected on the basis of the purity rather than synthetic yield. The fractions were then flash frozen and lyophilized. The stock solutions of 10^{-3} M ligands were periodically checked by analytical HPLC before use, and their purity was assessed by integration of the peaks at 214 and 254 nm.

NMR Spectroscopy. The lyophilized sample (2 mg) was solubilized in [$^2H_6$]Me_2SO, and all NMR experiments were recorded at 27 °C on a Bruker AMX spectrometer, operating at 600 MHz for ^1H nucleus. Chemical shifts are quoted relative to the [$^2H_5$]Me_2SO resonance fixed at 2.5 ppm. Typically, DQF−COSY spectra[47] were collected into an 800 × 1024 data matrix with 32 scans per t_1 value and TOCSY spectra[48] with

a mixing time of 80 ms into a 512 × 1024 data matrix with 16 scans per t_1 value. NOESY spectra[49] were acquired in the phase-sensitive mode using time-proportional phase incrementation with a mixing time of 200 ms. All the data were processed with the UXNMR software. One zero-filling and a $\pi/4$ phase-shifted sine bell window function were applied in both dimensions before Fourier transformation. Routine characterization of heptapeptide intermediates and of dipeptide **6** were performed on a Bruker AMX 360 spectrometer operating at 360 Hz.

Mass Spectrometry. MALDI-TOF. The samples were prepared by mixing an equal volume of a 10^{-5} M peptide solution and of a saturated solution of α-cyano-4-hydroxycinnamic acid in CF_3CO_2H:CH_3CN:H_2O, 0.1/50/50 (v/v/v). The mixture was then applied (0.5 μL) on the target of a Bruker Biflex III mass spectrometer and dried at room temperature.[50] Desorption of the samples was performed using a 337-nm laser, and the positive ion mass spectra were obtained in a linear and reflector mode with an acceleration of 19 kV. External mass calibration was performed with low-mass peptide standards (angiotensine I and II). FAB-MS was performed at the Université de Montpellier 2, using thioglycerol as matrix.

Synthesis of the H-Orn(Boc)-Phe(3I,4N3)-NH_2 Segment (7). The following reactions were monitored using the analytical HPLC system I. Boc-Phe(4NO_2)-OH (10 mmol; HPLC elution %: 38; $\epsilon_{214}/\epsilon_{254}$: 1.3) was amidated in dimethylformamide using *N*-methylmorpholine (10 mmol), isobutylchlorocarbonate (10 mmol), and an excess of 14 M ammonia (2 mL) to give Boc-Phe(4NO_2)-NH_2 (**1**) as a precipitate after a 10 min reaction (HPLC elution %: 32; $\epsilon_{214}/\epsilon_{254}$: 1.2). The Boc protecting group of compound **1** was then removed by a mixture of CF_3CO_2H:CH_2Cl_2 (1/1, v/v) to give H-Phe(4NO_2)-NH_2 (**2**), which was isolated by precipitation in diethyl ether (HPLC elution %, 13; $\epsilon_{214}/\epsilon_{254}$, 1.0). Compound **2** (4.5 mmol) was then coupled to Fmoc-Orn(Boc)-OH (4.5 mmol; HPLC elution %, 50; $\epsilon_{214}/\epsilon_{254}$, 1.9) in DMF using PyBOP (4.5 mmol) and (i-Pr)$_2$EtN (4 mol equiv). The dipeptide Fmoc-Orn(Boc)-Phe(4NO_2)-NH_2 (**3**) (HPLC elution %, 53; $\epsilon_{214}/\epsilon_{254}$, 1.9) was obtained, in 89% yield, by precipitation in water and was then alternately rinsed with diethyl ether and hexane. The Fmoc protecting group of compound **3** was then removed using diethylamine in dimethylformamide (10%, v/v) to give H-Orn(Boc)-Phe(4NO_2)-NH_2 (**4**) (HPLC elution %, 27; $\epsilon_{214}/\epsilon_{254}$, 1.4), which was further precipitated in diethyl ether.

Compound **4** was then reduced by hydrogen bubbling over catalyst (10% Pd on charcoal) in a solution of MeOH:dimethylformamide:12 M HCl (10/90/1, v/v/v). The resulting peptide H-Orn(Boc)-Phe(4NH_2)-NH_2 (**5**) (HPLC elution %, 15; $\epsilon_{214}/\epsilon_{254}$, 37.5) was obtained in 90% yield following removal of the catalyst by filtration of the reaction mixture on Celite. Peptide **5** (0.45 mmol) was then iodinated using 0.01 M ICl (1 mol equiv) in MeOH to give a mixture of **5** and mono- and diiodinated compounds. The reaction mixture was applied on the HPLC column to isolate the desired monoiodinated peptide H-Orn(Boc)-Phe(3I,4NH_2)-NH_2 (**6**) (HPLC elution %, 25; $\epsilon_{214}/\epsilon_{254}$, 7.5). ^{1}H NMR data (ppm): Orn residue, H_α, 3.74; $H_{\beta\beta}$, 1.67; $H_{\gamma\gamma}$, 1.43; $H_{\delta\delta}$, 2.92; side chain NH_2, 6.83; Boc protecting group, 1.39; Phe residue, NH, 8.5; H_α, 4.4; $H_{\beta\beta}$, 2.83−2.65; H_6, H_5, and H_2 (aromatic), 6.99, 6.79, and 7.49, respectively; NH_2 (aromatic amine), 8.02; NH_2-C-terminus amide, 7.51−7.07. Dipeptide **6** was finally diazotized and azidated in darkness in 2 M HCl using solutions of 1 M $NaNO_2$ (1.1 mol equiv, 10 min at 0 °C) and of 1 M NaN_3 (1.05 mol equiv), with HPLC-monitoring at room temperature. The azidopeptide H-Orn(Boc)-Phe(3I,4N_3)-NH_2 (**7**) (HPLC elution %, 37; $\epsilon_{214}/\epsilon_{254}$, 1.6) was then purified by semipreparative HPLC.

Syntheses of Segments 12−15. The following reactions were monitored using the analytical HPLC system II. Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Gly-OH (**8**), Pmp-Tyr-Ile-Thr-Asn-Cys-Gly-OH (**9**), Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-OH (**10**), and Pmp-Tyr-Ile-Thr-Asn-Cys-Pro-OH (**11**) were synthesized according to a general protocol previously described.[34] Briefly, the peptides were synthesized on a chloromethylated polystyrene (1% cross-linked, 200−400 mesh), using a Boc strategy with PyBOP as coupling reagent, (i-Pr)$_2$EtN as tertiary amine, and CH_2Cl_2 or dimethylformamide as solvents. The following side-chain-protected amino acids were used: Boc-Pmp(Mob)-OH, Boc-Cys(Mob)-OH, Boc-Tyr(Dcb)-OH, and Boc-Asn(Xan)-OH. All couplings were monitored by the qualitative ninhydrin test. Recouplings were necessary for the incorporation of Boc-Asn(Xan)-OH and Boc-Tyr(Dcb)-OH residues. The peptides were then deprotected (including peptide−resin bond) by HF treatment, purified by semipreparative HPLC, and lyophilized.

Peptides **8** (HPLC elution %, 43; $\epsilon_{214}/\epsilon_{254}$, 24), **9** (HPLC elution %, 38; $\epsilon_{214}/\epsilon_{254}$, 24), **10** (HPLC elution %, 44; $\epsilon_{214}/\epsilon_{254}$, 33), and **11** (HPLC elution %, 39; $\epsilon_{214}/\epsilon_{254}$, 22) were then cyclized by air-oxidation in an aqueous solution at low concentration (1 mg/mL) maintained at pH 8, using (i-Pr)$_2$EtN, to give the corresponding disulfide cyclic peptides Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Gly-OH (**12**; HPLC elution %, 41; $\epsilon_{214}/\epsilon_{254}$, 13), Pmp-Tyr-Ile-Thr-Asn-Cys-Gly-OH (**13**; HPLC elution %, 35; $\epsilon_{214}/\epsilon_{254}$, 12), Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-OH (**14**; HPLC elution %, 43; $\epsilon_{214}/\epsilon_{254}$, 16), and Pmp-Tyr-Ile-Thr-Asn-Cys-Pro-OH (**15**; HPLC elution %; 36; $\epsilon_{214}/\epsilon_{254}$, 14; MALDI-TOF $(M + H)^+ = 864.7$; $(M + Na)^+ = 886.7$; $(M + K)^+ = 902.7$; calculated exact mass = 863.4).

Syntheses of Compounds 16−19. The following reactions were monitored using the analytical HPLC system II. Peptide segments **12**, **13**, **14**, and **15** were coupled to dipeptide **7**, the reaction mixtures were then acidified using CF_3CO_2H and applied on a HPLC column to give Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Gly-Orn(Boc)-Phe(3I,4N_3)-NH_2 (**16**), Pmp-Tyr-Ile-Thr-Asn-Cys-Gly-Orn(Boc)-Phe(3I,4N_3)-NH_2 (**17**), Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn(Boc)-Phe(3I,4N_3)-NH_2 (**18**), and Pmp-Tyr-Ile-Thr-Asn-Cys-Pro-Orn(Boc)-Phe(3I,4N_3)-NH_2 (**19**). Compounds **16−19** were then deprotected on their Orn side chain. For 10-mg scale preparations, the Boc group was removed using CF_3CO_2H:CH_2Cl_2 (1/1, v/v) for 1 h, and the trifluoroacetate salt was precipitated using cold diethyl ether and recovered by centrifugation (10 mL conical glass tubes). For small scale preparations (<1 mg), the Boc compounds were deprotected in CH_3CN:CF_3CO_2H, (1/1, v/v, 200 μL) for 1−2 h, under HPLC monitoring, and were directly injected on the preparative HPLC column to give, respectively, Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Gly-Orn-Phe(3I,4N_3)-NH_2 (**20**; HPLC elution %, 44; $\epsilon_{214}/\epsilon_{254}$, 2.6), Pmp-Tyr-Ile-Thr-Asn-Cys-Gly-Orn-Phe(3I,4N_3)-NH_2 (**21**; HPLC elution %, 38; $\epsilon_{214}/\epsilon_{254}$, 2.1), Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Phe(3I,4N_3)-NH_2 (**22**; HPLC elution %, 48; $\epsilon_{214}/\epsilon_{254}$, 2.3), and Pmp-Tyr-Ile-Thr-Asn-Cys-Pro-Orn-Phe(3I,4N_3)-NH_2 (**23**; HPLC elution %, 39; $\epsilon_{214}/\epsilon_{254}$, 2.6).

Synthesis of the Radioiodinated Peptide 29 ([^{125}I]-ZOTA). The synthesis was first performed using nonradioactive iodine (^{127}I) according to a modified synthesis protocol, and all reactions were monitored under the conditions of HPLC system I (Figure 2). Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Phe(4NH_2)-NH_2 (**24**) was synthesized on a MBHA resin using a Boc/PyBOP/HF strategy, followed by air-oxidation cyclization, to give Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Phe(4NH_2)-NH_2 (**25**), purified by HPLC and characterized by MALDI-TOF mass spectrometry (found M + 1 = 1153.8, calculated exact mass = 1152.6) (Figure 2a). Iodination was then performed by reacting with an equimolar amount of freshly dissolved **25** in MeOH with ICl in MeOH, to give a mixture of compound **25**, Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Phe(3I,4NH_2)-NH_2 (**26**), and diiodinated compound (Figure 2b). Compound **26** was purified using HPLC and characterized by MALDI-TOF MS (found M + 1 = 1279.7, calculated exact mass = 1278.4) (Figure 2c). Diazotization and azidation of **26** were then done using the same amounts as for the further radiolabeling procedure. Briefly, compound **26** (0.1 nmol) was diazotized with an excess of 10^{-3} M $NaNO_2$ (10 μL approximately) at 0 °C in darkness, in a solution of 12 M HCl (10 μL) for 1 h before addition of NaN_3 (2 mol equiv over $NaNO_2$, allowed to reach room temperature), yielding compound **22** (Figure 2e), which was characterized by FAB MS (M + 1 = 1305, calculated exact mass = 1304.4) (Figure 2f). The synthesis of Pmp-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-Phe(3^{125}I,4N_3)-NH_2 (**29**, [^{125}I]-

ZOTA) was performed according to the same procedure, except that the iodination of peptide **25** (10^{-3} M, 40 μL) was done with $Na^{125}I$ (Amersham, 1 mCi) in a phosphate buffer (pH 6.8, 55 μL) using an Iodo-Gen-coated Eppendorf vessel (300 μL) for 5 min. Radiolabeled peptide **29** ([^{125}I]-ZOTA) was purified under the conditions of HPLC system III, concentrated under vacuum (Speed-Vac apparatus), and stored in liquid nitrogen. The identity of **29** was determined by measure of the radioactivity of 0.5-mL HPLC-eluted fractions of coinjected compounds **22** and **29**.

Membrane Preparations. The human OT receptor cDNA was a generous gift from Dr T. Kimura.[3] OT receptor was stably expressed in CHO cells as previously described.[12] Cells were washed twice, in PBS without Ca^{2+} and Mg^{2+}, followed by the addition of a lysis buffer (15 mM Tris:HCl, 2 mM $MgCl_2$, 0.3 mM EDTA, pH 7.4). The cells were then scrapped, homogenized (Polytron), and centrifuged at 100*g* for 5 min at 4 °C. Supernatants were collected and centrifuged at 44000*g* for 20 min at 4 °C. Pellets were suspended in a Tris:HCl buffer (15 mM Tris:HCl, 5 mM $MgCl_2$, pH 7.4) and centrifuged again at 44000*g* for 20 min at 4 °C. The pellets were resuspended in a convenient volume of the same buffer and proteins were determined. Membranes were stored in liquid nitrogen or used immediately.

Binding Experiments. Affinities of analogues **20**−**23** were determined from competition experiments using [^{125}I]-OTA (200−220 pM) as radioligand with 2−3 μg of membrane proteins.[8,26] The ligands were tested at concentrations ranging from 10 pM to 10 μM, and the experiments were performed in triplicate. The K_d of compound **29** ([^{125}I]-ZOTA) was determined in saturation experiments with 3 μg of membrane proteins using concentrations ranging from 10 to 900 pM. Incubations were performed in triplicate at 30 °C for 1 h in a Tris:HCl buffer (15 mM Tris:HCl, 5 mM $MgCl_2$, pH 7.4) containing 1 mg/mL BSA.[8] Bound and free ligand were separated by filtration (Brandel apparatus) over Whatman GF/C filters previously soaked with a 10 mg/mL BSA solution for 3−4 h. The binding data were analyzed by nonlinear least-squares regression using the computer program Ligand.[51]

Inositol Phosphate Assays. The antagonist potency of compound **22** ([I]-ZOTA) was determined by measuring the accumulation of inositol phosphates as previously described.[12] Briefly, CHO cells expressing the OT receptor were cultured in 6-well dishes for 48 h in Dulbecco's modified Eagle's medium-supplemented medium and labeled for 24 h with *myo*-[2-^{3}H]inositol (Dupont New England Nuclear) at 1 μCi/mL, in a serum- and inositol-free medium. Cells were then washed twice with PBS and incubated in PBS for 1 h. They were then incubated in PBS containing 10 mM LiCl with or without analogue **22**, at various concentrations for 10 min. The cells were then stimulated using 10 nM OT for an additional period of 15 min. Incubation was stopped with perchloric acid. Inositol phosphate extraction was performed using anion-exchange chromatography (Dowex AG-1 × 8, formate form, 200−400 mesh, Bio-Rad) and the radioactivity was measured. A K_{inact} value was calculated as $K_{inact} = IC_{50}/(1 + [OT]/K_{act})$, where IC_{50} corresponds to the concentration of analogue leading to the half-inhibition of the stimulation induced by OT, [OT] = 10 nM and K_{act} = 12.9 nM.[12]

Photolabeling of the Human Oxytocin Receptor. CHO cells membranes expressing the human OT receptor (500 μg, 4 mL) were suspended in buffer A (50 mM Tris:HCl, 5 mM Mg^{2+}, pH 7.4) containing BSA (0.5 mg/mL) in low-adsorption, borosilicated tubes (Corex). Incubations were performed using ≈2 nM of freshly radioiodinated ligand **29** for 1 h at 30 °C, in darkness, in the presence or absence of 10^{-5} M OT for specific labeling determination. To remove unbound ligand, membranes were washed with cold buffer A without BSA (10 mL) and centrifuged (44000*g*, 20 min) twice. The remaining pellet was resuspended in cold buffer A (1 mL) and UV-irradiated for 1 min at 254 nm, in ice-cold siliconized Petri dish. Membranes were then washed twice again using buffer A (1 mL) and finally solubilized in Laemmli buffer for SDS−PAGE (12% acrylamide gel, 1 mm thickness) with prestained standards (Kaleidoscope, Bio-Rad). The gel was fixed in $MeCO_2H$/MeOH/Me_2SO/H_2O, 16:40:2:42 (v/v), dried under vacuum, and autoradiographed using Kodak XAR-5 films at −70 °C.

Acknowledgment. This work was supported by INSERM, CNRS, and MRT. Thanks are due to Dr N. Galéotti for help in MALDI-TOF experiments, Dr P. Jouin for constant interest and advice, M. Passama and L. Charvet for illustrations, and Dr Tuhinadri Sen for reading the manuscript.

Supporting Information Available: A table of the chemical shifts of 22, a table of the structures and properties of 20−23, and parts of the TOCSY and NOESY spectra of 22. This material is available free of charge via the Internet at http/::pubs.acs.org.

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JM010125U