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# A nonpeptide oxytocin receptor antagonist radioligand highly selective for human receptors

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**Abstract**

A novel, potent nonpeptide oxytocin receptor antagonist (1-(1-(2-(2,2,2-trifluoroethoxy)-4-(1-methylsulfonyl-4-piperidinyloxy) phenylacetyl)-4-piperidinyl)-3,4-dihydro-2(1*H*)-quinolinone) has been identified that can be labeled to high specific activity with [$^{35}$S]. In binding studies, this compound exhibits sub-nanomolar affinity and a high degree of selectivity (900–1800-fold) for human oxytocin receptors compared to human vasopressin receptors. This compound appears suitable for studying the pharmacology of oxytocin receptors in human and nonhuman primate tissues, for which there is currently a paucity of highly selective tools. It may also be useful as a nonlabeled competitor or as a radioligand in autoradiographic studies of oxytocin receptor localization in these tissues.


*Keywords:* Oxytocin receptor antagonist; Oxytocin; Radioligand; Oxytocin receptor; Vasopressin

## 1. Introduction

Oxytocin and arginine vasopressin are closely related nonapeptides that are synthesized and released by hypothalamic neurons, and that exert a variety of hormonal and neurotransmitter-like effects. Oxytocin has well-established actions in parturition and lactation, and vasopressin plays a key role in renal water resorption and vasoconstriction. Aside from their well-known peripheral roles, both hormones have also been reported to act centrally, with oxytocin playing possible roles in analgesia, drug dependence, and social, sexual and maternal behaviors, and vasopressin affecting learning, memory and social and reproductive behaviors (selected reviews: Argiolas and Gessa, 1991; Barberis and Tribollet, 1996; Verbalis, 1999; Gimpl and Fahrenholz, 2001).

The effects of these peptides are mediated by a family of four G-protein-coupled receptors—the oxytocin receptor and the vasopressin receptors $V_{1a}$, $V_{1b}$, and $V_2$. These four receptors have been classified on the basis of the signal transduction systems to which they are coupled as well as by differences in their affinities for a variety of peptide analogues (e.g., Peter et al., 1995; Barberis and Tribollet, 1996). All four members of this receptor family have been cloned and expressed in cell lines, facilitating the study of the biochemical pharmacology of each receptor (Kimura et al., 1992; Morel et al., 1992; Thibonnier et al., 1994; Sugimoto et al., 1994; De Keyzer et al., 1994; Birnbaumer et al., 1992; Lolait et al., 1992).

Centrally administered oxytocin and vasopressin elicit behavioral effects in rodents and primates, and in some cases, oxytocin has the opposite effect of vasopressin. For example, in studies of avoidance behaviors in rodents, oxytocin has been reported to decrease learning and memory functions while vasopressin has been reported to enhance these functions, which may suggest differential localization of these receptor subtypes in the central nervous system (see Argiolas and Gessa, 1991; De Wied et al., 1993). Localization of these receptors in rat brain has been studied using a number of different radioligands for autoradiography, with antibodies for immunohistochemical studies and with mRNA probes for in situ hybridization

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(recently reviewed by Barberis and Tribollet, 1996). The results of these studies suggest that the oxytocin and vasopressin $V_{1a}$ receptors are the predominant receptor subtypes of this family in rat brain and spinal cord, and that they are differentially distributed. The pharmacological tools used to distinguish between the rat oxytocin and vasopressin $V_{1a}$ receptors have shown good selectivity for one of these two receptor subtypes. However, the selectivity of these tools (e.g., [d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin (Elands et al., 1987) can be much lower for human (and other primate) receptors, making the results of autoradiographic studies in these species difficult to interpret at present (see Barberis and Tribollet, 1996; Pettibone et al., 1992).

We have identified a novel, potent nonpeptide oxytocin receptor antagonist, (1-(1-(2-(2,2,2-trifluoroethoxy)-4-(1-methylsulfonyl-4-piperidinyloxy) phenylacetyl)-4-piperidinyl)-3,4-dihydro-2(1*H*)-quinolinone) (Compound A), which can be labeled to high specific activity with [$^{35}$S]. This compound exhibits a high degree of selectivity for human oxytocin receptors compared to human vasopressin receptors, and appears to be suitable for studying the pharmacology of oxytocin receptors in human tissues, as well as serving as a nonlabeled competitor or as a radioligand in autoradiographic studies of oxytocin receptor localization in these tissues. In this paper, we report the in vitro pharmacological characterization of the unlabeled antagonist, and the radioligand binding properties of the [$^{35}$S]-labeled compound.

## 2. Materials and methods

### 2.1. Synthesis of compound A

Compound A was prepared as shown in Fig. 1. Details of the synthesis are given as Supplementary information.

### 2.2. Materials

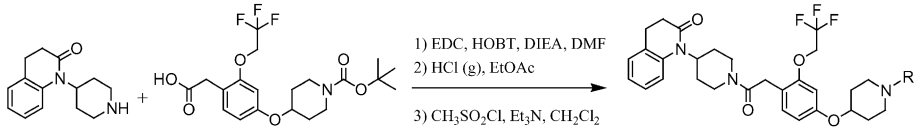
Oxytocin, arginine vasopressin, desamino [D-arg$^8$]vasopressin, Thr$^4$Gly$^7$oxytocin, Phe$^2$Orn$^8$oxytocin/vasotocin, [1-(β-mercapto-β, β-cyclo-pentamethylene propionic acid), 2-(*O*-methyl)tyrosine]Arg$^8$-vasopressin, were from Peninsula Laboratories, (Belmont, CA, USA) or Peptides International (Louisville, KY, USA). [d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin was from Bachem (King of Prussia, PA, USA), [$^3$H]oxytocin, [$^3$H]arginine vasopressin, and [$^{125}$I][d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin were from New England Nuclear (Boston, MA, USA) and [$^3$H]*myo*-inositol was from Amersham-Pharmacia (Piscataway, NJ, USA). Fluo-4 acetoxymethyl ester (Fluo-4 AM) was obtained from Molecular Probes (Eugene, OR, USA). Other chemicals were from Sigma (St. Louis, MO, USA).

### 2.3. Cell culture

Cloned human oxytocin receptors were expressed in Chinese hamster ovary (CHO) cells (Gordon Ng, Merck Research Laboratories, Montreal). Cells were grown in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum; 2 mM L-glutamine (GIBCO, Rockville, MD, USA); penicillin/streptomycin (0.2 mM hypoxanthine, 0.032 mM thymidine GIBCO); 10 μg/ml puromycin (Clontech, Palo Alto, CA, USA). Human embryonic kidney (HEK/293) cells expressing cloned human oxytocin receptors or cloned human vasopressin receptors $V_{1b}$ or $V_2$ (M. Jacobson/C. Salvatore, Merck Research Laboratories, West Point) were grown in Dulbecco's modified Eagle medium with 10% fetal bovine serum, 250 μg/ml geneticin, 100 μg/ml gentamycin, and 250 μg/ml hygromycin-B. Cultured cells in Costar (Cambridge, MA, USA) (CHO cells) or NUNC (Roskilde, Denmark) (HEK/293 cells) flasks were incubated at 37 °C in a humidified incubator with 95% $O_2$ and 5% $CO_2$.

### 2.4. Preparation of membranes from cultured cells expressing cloned human receptors

The preparation of membranes from cultured cells was essentially the same as described in Pettibone et al. (1991). Briefly, cells expressing the desired receptors at about 90–100% confluence were collected in phosphate-buffered saline/1 mM EDTA (pH = 7.4) and centrifuged at 500 × *g* at 4 °C for 5 min. The resulting pellets were homogenized with a Polytron in 50 mM Tris buffer, 5 mM $MgCl_2$ (pH = 7.4), and centrifuged at 50,000 × *g* at 4 °C for 20 min. The membrane pellets were re-homogenized and resuspended in appropriate




**1** **2**

**3** R = $CO_2C(CH_3)_3$
**4** R = H
**Compound A** R = $SO_2CH_3$

Fig. 1. Synthesis of Compound A. Standard peptide coupling conditions using amine **1** (Ogawa et al., 1993) and carboxylic acid **2** (Williams et al., 1999) readily afforded amide derivative **3**. The *tert*-butyl carbamate protecting group in **3** was cleanly removed with HCl gas in ethyl acetate to give the hydrochloride salt of compound **4**. Mesylation of compound **4** using standard conditions provided Compound A in high yield. Use of [$^{35}$S]methanesulfonyl chloride in the mesylation step provided [$^{35}$S]Compound A (Dean et al., 1995).

volumes of 50 mM Tris, 5 mM $MgCl_2$ (pH = 7.4). Aliquots of the membrane suspension were centrifuged at 17,000 × *g* and stored in 10% glycerol in 50 mM Tris buffer, 5 mM $MgCl_2$ (pH = 7.4) at − 70 °C. Membrane concentration was determined using the method of Lowry et al. (1951).

### 2.5. Preparation of human platelet membranes

The preparation of vasopressin $V_{1a}$ receptor-rich membranes from human platelets is adapted from Swart et al. (1985). Briefly, human platelet-rich supernatant was obtained from the American Red Cross. The units were pooled and centrifuged at 150 × *g* for 15 min at 4 °C to remove erythrocytes. The supernatants were then centrifuged at 16,000 × *g* at 4 °C. The pellets were resuspended and homogenized in 80 ml of hypotonic buffer (10 mM Tris/HCl, pH 7.4, 5 mM EDTA). The resuspended pellets were centrifuged at 27,500 × *g* for 25 min at 4 °C. The pellets were washed two more times by resuspension and high-speed centrifugation as above. Then the final pellets were resuspended in binding buffer 10 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$, divided into aliquots and stored at − 70 °C.

### 2.6. Radioligand binding to cloned receptors

Radioligand (1 nM [$^3$H]oxytocin, 0.1 nM [$^{125}$I][d($CH_2$)$_5$, Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin, 0.5 nM [$^3$H]arginine vasopressin, or 30 pM [$^{35}$S]Compound A) in 50 mM Tris buffer, 5 mM $MgCl_2$ (pH = 7.4) was mixed with test compounds in 1% dimethyl sulfoxide or with 1 μM unlabeled ligand (oxytocin, arginine vasopressin, or Compound A) to estimate nonspecific binding (final [dimethyl sulfoxide] = 0.17%). Unlabeled oxytocin was used to estimate nonspecific binding in the [$^{125}$I][d($CH_2$)$_5$,Tyr(Me)$^2$, Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin assays. Membranes were then added and mixed, and the reaction was incubated for 60 min at 30 °C. The assay mixture was filtered onto 96-well glass fiber filter plates (Packard, Meriden, CT, USA) presoaked with 0.2% polyethylenimine. The plates were dried, scintillation cocktail was added, and the plates were counted with a Packard TopCount.

### 2.7. [$^3$H]arginine vasopressin binding to human platelet membranes (vasopressin $V_{1a}$ receptors)

To measure specific [$^3$H]arginine vasopressin binding, 100 μl of human platelet membranes at 1:3 dilution was added to triplicate tubes containing either buffer (10 mM Tris/HCl, pH 7.4, 5 mM $MgCl_2$) for total binding, test compounds or 1 μM arginine vasopressin for determination of nonspecific binding and 0.5 nM of [$^3$H]arginine vasopressin (final volume = 300 μl). After incubation at 30 °C for 60 min, the incubation mixtures were filtered through a Wallac (Gaithersburg, MD, USA) glass fiber filtermat (presoaked in 0.1% bovine serum albumin) using a Tomtec cell harvester (Gaithersburg, MD, USA), and rapidly washed with 8.0 ml/well of ice-cold buffer Tris/HCl (50 mM, pH = 7.4) containing 5 mM $MgCl_2$. Filtermats were dried and sealed in a plastic sample bag with 24 ml Wallac BetaScint scintillation cocktail. The radioactivity on the filter plates was counted by Wallac Beta Plate reader.

### 2.8. [$^3$H]oxytocin and [$^3$H]vasopressin binding to rat tissues

All harvesting of animal tissues was carried out using protocols approved by the West Point Institutional Animal Care and Use Committee in accordance with the provisions of the ILAR Guide for the Care and Use of Laboratory Animals. The preparation of membranes from rat uterus, liver, and kidney medulla is adapted from Fuchs et al. (1985). Briefly, female Sprague–Dawley rats were pre-treated for 24 h with 0.3 mg/kg diethylstilbestrol dipropionate administered i.p. The uterus was excised and fatty tissue was removed. The liver and kidneys were excised from male Sprague–Dawley rats and the medulla was dissected out of the kidney. All tissues were homogenized in hypotonic buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.5 mM dithiothreitol). The liver homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The liver supernatant, as well as the uterus and kidney medulla homogenates were centrifuged at 48,000 × *g* for 30 min. The resulting pellets were washed two more times by resuspension and high-speed centrifugation as above. The final pellets were resuspended in binding buffer and [$^3$H]radioligand binding was performed as described above except that filtration was performed on a Skatron cell harvester using GF/B filters (presoaked with 0.2% polyethylenimine) purchased from Skatron (Lier, Norway). The filters were counted with a Packard TriCarb 1900.

### 2.9. Measurement of $Ca^{2+}$ flux in CHO and HEK/293 cells expressing cloned human oxytocin receptors

CHO and HEK/293 cells expressing cloned human oxytocin receptors were grown as described above. The cells were plated in poly-D-lysine-coated 384-well plates from Becton-Dickinson (40K cells/well for CHO and 20K cells/well for HEK/293) using a Labsystems Multidrop (Helsinki, Finland). The plated cells were grown overnight at 6% $CO_2$ and 37 °C. The cells were washed with 3 × 100 μl assay buffer (Hanks Balanced Salt Solution (Gibco 14025-076) containing extra $MgCl_2$ to a final concentration of 5 mM, 20 mM HEPES, 2.5 mM probenicid, and 0.1% bovine serum albumin) using a Skatron Embla384 cell washer. The cells were loaded with 1 μM Fluo-4 AM in the presence of 0.02% pluronic acid and 1% bovine serum albumin for 1 h at 6% $CO_2$ and 37 °C. The cells were washed as above. $Ca^{2+}$ flux was measured using Molecular Devices $FLIPR_{384}$ fluorometric imaging plate reader (Sunnyvale, CA, USA). For antagonist potency determinations, the cells were pre-incubated for 5 min with various concentrations of Compound A and then stimulated with oxytocin for 3 min (5 nM for HEK/

293 cells; 15 pM for CHO cells). For $K_i$ determinations, the cells were pre-incubated with various concentrations of Compound A and stimulated with various concentrations of oxytocin for 3 min.

### 2.10. Phosphatidylinositol hydrolysis assay

CHO cells expressing cloned human oxytocin receptors were grown in 96-well plates and loaded overnight with 1 μCi [$^3$H]*myo*-inositol/well. The following day, the medium was removed and plates were washed twice with 200 ml warmed, oxygenated buffer A (127 mM NaCl, 25 mM $NaHCO_3$, 10 mM glucose, 2.5 mM KCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, and 1.8 mM $CaCl_2$) using a Skatron SkanWasher. Then 100 μl/well of buffer B (buffer A plus 10 mM LiCl and 0.01% human serum albumin) or an appropriate concentration of antagonist in buffer B was added and allowed to incubate for 10 min at room temperature. The buffer B was then removed, and an appropriate concentration of oxytocin in buffer B was added to each well. The concentration of oxytocin used was 10 nM, near its $EC_{50}$ value in this assay. The plate was placed in a 37 °C incubator for 30 min. The reaction was terminated by aspirating off the contents of the wells and washing the plate once with 200 ml ice-cold phosphate-buffered saline using the SkanWasher. Then 100 μl ice-cold 5% trichloroacetic acid was added to each well, and the plate was kept on ice for 15 min. The trichloroacetic acid was neutralized by the addition of 100-μl 0.5 M Tris to each well. The plate was mixed by aspiration and dispense cycles in a Tomtek Quadra 96, and 150 μl of the contents of each well were transferred from the assay plate to tubes containing 400 μl water. These tubes were again mixed and 400 μl of the contents was transferred to 96-well large volume filter plates filled with washed Dowex 1 × 8 (formate counter-ion). The sample-loaded column plates were washed twice with 1 ml water, followed by a 1.2-ml wash of 60 mM ammonium formate, 5 mM sodium tetraborate solution. Washing was performed by Quadra and filtered using a Whatman Polyfiltronics manifold system (Clifton, NJ, USA). A 2-ml 96-well collection plate was then placed in the filter chamber, and the [$^3$H]inositol phosphates were eluted from the column plate into the empty collection plate with 1.2 ml of 1 M ammonium formate, 0.1 M formic acid solution. A 1-ml aliquot of the eluate was transferred into 20-ml scintillation vials, and 10-ml Packard Aquassure scintillation cocktail was added to the vials. Radioactivity in each sample vial was determined with a Packard CA 1900 liquid scintillation counter.

### 2.11. Binding kinetics studies

On-rate studies were performed at 30 °C by incubating [$^{35}$S]Compound A (15, 30, or 60 pM) in the presence or absence of 15 μM unlabeled Compound A with membranes prepared from CHO cells expressing the cloned human oxytocin receptor. The binding reactions were stopped at various times by filtration, and the filters counted as described above. The apparent on-rate, $K_{app}$, for each ligand concentration was determined by nonlinear curve fitting of specific binding vs. time (SigmaPlot), and these $K_{app}$ values were plotted against ligand concentration. The off-rate, $k_2$, was determined indirectly from the ordinate intercept of the line fit to these data, and the on-rate, $k_1$, was determined from the slope. Off-rates were also determined directly by allowing [$^{35}$S]Compound A to come to equilibrium with the membranes above (60 min), and adding unlabeled Compound A to a final concentration of 15 μM. The dissociation reaction was stopped at various time points by filtration and counted as described above. The specific counts bound were plotted as a function of time after addition of excess unlabeled compound, and $k_2$ was determined directly from nonlinear curve fitting of these data (SigmaPlot, SPSS, Chicago, IL, USA).

## 3. Results

### 3.1. Binding studies

The binding affinity and selectivity of Compound A were tested with both human and rat receptors. In competition binding studies using [$^3$H]oxytocin or [$^3$H]arginine vasopressin, the compound was found to be highly selective for human oxytocin receptors compared to human vasopressin $V_{1a}$, $V_{1b}$, or $V_2$ receptors. $K_i$ values for the binding of Compound A to these receptors were determined from competition binding curves (examples are shown in Fig. 2) by the method of Cheng and Prussoff (1973), and are presented in Table 1. However, in the case of the rat receptors, the selectivity of the compound for the oxytocin receptors compared to vasopressin $V_{1a}$ receptors decreases from 1800-fold to 3-fold, largely due to a higher affinity for the rat, compared to the human, vasopressin $V_{1a}$ receptor (Table 1; Fig. 2).

The binding affinities and selectivities of Compound A were compared to those of unlabeled oxytocin and [d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin. Both of these peptide ligands bind to the oxytocin receptor, and are available commercially as radioligands. The data for this comparison are summarized in Table 1. Oxytocin is essentially nonselective for the oxytocin receptor compared to the vasopressin $V_{1a}$ receptor in human, but is about 100-fold selective in rat. [d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin is also nonselective in human but is about 1300-fold selective for the oxytocin receptor compared to the $V_{1a}$ receptor in rat. Thus, the selectivity of these ligands for the oxytocin receptor and vasopressin $V_{1a}$ receptors is dependent on species: [d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin shows high selectivity for oxytocin receptors in the rat system, while Compound A shows high selectivity for human oxytocin receptors. The testing of Compound A against a panel of 175 receptors, transporters, ion channels, and enzymes (MDS Panlabs, Bothell, WA,

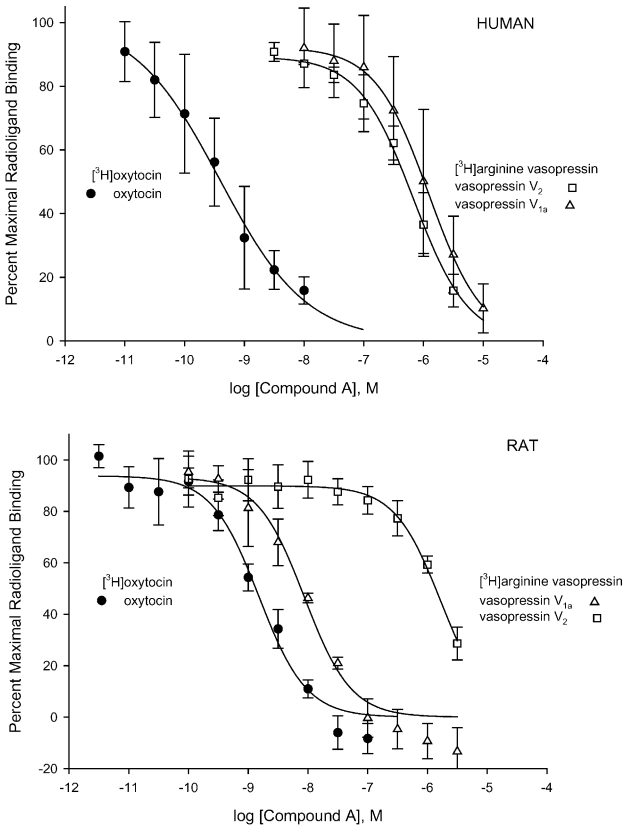



Fig. 2. Top panel: Comparison of Compound A inhibition of [$^3$H]oxytocin binding to cloned human oxytocin receptors expressed in CHO cells (●), and [$^3$H]arginine vasopressin binding to human vasopressin $V_{1a}$ receptors (△) expressed in platelets and to human $V_2$ (□) receptors expressed in HEK/293 cells. Error bars are S.E.M. ($n=3$). Bottom panel: Comparison of Compound A inhibition of [$^3$H]oxytocin binding to rat uterus (●), and [$^3$H]arginine vasopressin binding to rat liver (vasopressin $V_{1a}$ receptors) (△) and to rat kidney (vasopressin $V_2$ receptors) (□). Error bars are S.E.M. ($n=3$).

USA) indicated sub-micromolar activity in only two cases: the $\alpha_1$ adrenergic receptor ($K_i=437$ nM) and at the monoamine transporter ($K_i=547$ nM), suggesting Compound A is relatively free of potentially confounding activities.

To confirm the $K_i$ of Compound A for the human oxytocin receptor, [$^3$H]oxytocin saturation studies were performed with membranes prepared from CHO cells expressing the cloned human oxytocin receptor in the presence and absence of fixed concentrations of Compound A. In the absence of Compound A, [$^3$H]oxytocin bound to a single class of high affinity sites with a $K_d$ value of $0.33 \pm 0.12$ nM ($n=4$). When the $K_d$ for [$^3$H]oxytocin binding was plotted as a function of the fixed concentrations of Compound A (Fig. 3), curve fitting of the points indicated a $K_i$ value of $0.14 \pm 0.08$ nM ($n=4$) for this compound binding to oxytocin receptor, similar to the values obtained from competition binding studies (Table 2). Co-incubation with Compound A did not alter the apparent $B_{max}$ value for [$^3$H]oxytocin (data not shown). In a parallel study, a 60-min pre-incubation of the membranes with 0.3 nM Compound A *before* addition of

Table 1
Species dependence of ligand selectivity: inhibition of [$^3$H]oxytocin binding or [$^3$H]arginine vasopressin binding

| | $K_i$ (nM) | | |
|---|---|---|---|
| | Oxytocin | OVTA[a] | Compound A |
| *Oxytocin receptor* | | | |
| Human | 0.21 ± 0.08 (7) | 0.28 ± 0.30 (3) | 0.29 ± 0.17 (5) |
| Rat | 0.44 ± 0.23 (3) | 0.02 | 1.1 ± 0.73 (3) |
| *Vasopressin $V_{1a}$ receptor* | | | |
| Human | 1.18 ± 0.38 (3) | 1.39 ± 2.36 (17) | 556 ± 216 (14) |
| Rat | 49 ± 4 (3) | 27 ± 9 (2) | 3.5 ± 1.7 (4) |
| *Vasopressin $V_{1b}$ receptor* | | | |
| Human | 241 ± 56 (3) | >1000 (3) | >1000 (2) |
| Rat | n/d | n/d | n/d |
| *Vasopressin $V_2$ receptor* | | | |
| Human | 303 ± 142 (4) | 657 ± 269 (4) | 275 ± 113 (3) |
| Rat | 37 ± 9 (3) | 44 ± 21 (2) | >1000 (4) |

$K_i$ determined from binding competition $IC_{50}$: $K_i = IC_{50}/(1+[L]/K_d)$ (Cheng and Prussoff, 1973). Values are group mean ± standard deviation ($n$).
Source of receptors—Human: oxytocin receptor, CHO cells expressing human oxytocin receptors; vasopressin $V_{1a}$ receptors, human platelets; vasopressin $V_{1b}$ receptors, HEK/293 cells expressing human $V_{1b}$ receptors; vasopressin $V_2$ receptors, HEK/293 cells expressing human $V_2$ receptors. Rat: oxytocin receptor, diethylstilbestrol dipropionate-primed rat uterus; vasopressin $V_{1a}$ receptors, rat liver; vasopressin $V_2$ receptors, rat kidney medulla.
[a] [d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin.

[$^3$H]oxytocin produced $B_{max}$ values for [$^3$H]oxytocin binding similar to those obtained in the co-incubation studies (data not shown), indicating that this compound binds reversibly to the oxytocin receptor.

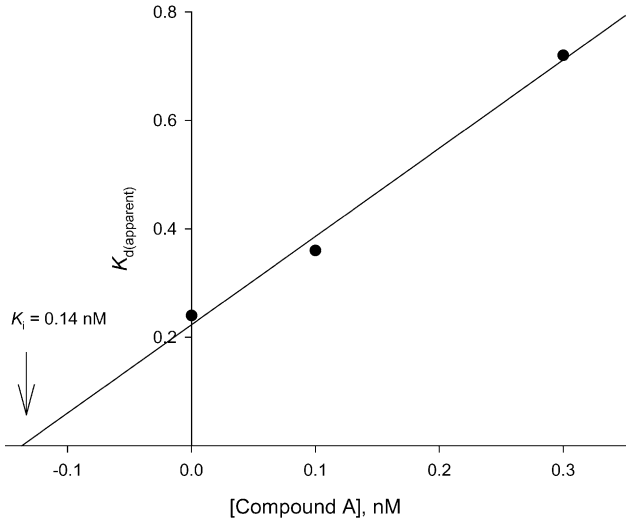



Fig. 3. Determination of the $K_i$ value for Compound A from saturation experiments. Apparent $K_d$ values were determined for [$^3$H]oxytocin binding to membranes prepared from CHO cells expressing human cloned oxytocin receptors in the presence and absence of 0.1 and 0.3 nM Compound A. The $K_i$ for Compound A was determined from the equation $K_{d(apparent)}=(K_d/K_i)[antagonist]+K_d$. A plot of $K_{d(apparent)}$ vs. [antagonist] has intercepts at $K_d$ on the ordinate and $K_i$ on the abscissa.

Table 2
Comparison of Compound A affinity determinations for human oxytocin receptors

| | Compound A |
|---|---|
| | $K_i$ (nM) ± S.D. (*n*) for oxytocin receptors |
| [$^3$H]oxytocin binding competition | 0.29 ± 0.17 (5) |
| [$^3$H]oxytocin binding saturation | 0.14 ± 0.08 (4) |
| [$^{125}$I]OVTA[a] binding competition | 0.23 ± 0.16 (2) |
| Schild plot analysis FLIPR | [b]0.50 ± 0.12 (2) |

$K_i$ (nM) ± S.D. (*n*) for CHO cells expressing the human oxytocin receptor.
[a] [$^{125}$I][d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin.
[b] $K_{Db}$ (nM) estimated from p$A_2$ value obtained from FLIPR experiments performed with HEK cells expressing the human oxytocin receptor.

### 3.2. Functional studies

The oxytocin receptor is coupled to phospholipase C by $G_{\alpha q}$. To determine if Compound A acts as an agonist or antagonist at the oxytocin receptor, it was evaluated in functional assays using CHO cells expressing the cloned human oxytocin receptor. In phosphatidylinositol hydrolysis assays, Compound A alone did not stimulate accumulation of inositol phosphates over basal accumulation, indicating Compound A was not an agonist of the oxytocin receptor. However, a 10-min preincubation with Compound A inhibited oxytocin (10 nM) induced stimulation of phosphatidylinositol hydrolysis with an $IC_{50}$ value of 3.6 ± 0.9 nM, indicating Compound A is a potent oxytocin receptor antagonist (data not shown). In an assay of agonist-induced increases in intracellular calcium in cultured cells expressing the cloned human oxytocin receptor, Compound A alone had no effect, while it inhibited oxytocin-stimulated increases in intracellular calcium with an $IC_{50}$ value of 0.54 ± 0.066 nM ($n$ = 3) for the oxytocin receptor-expressing HEK/293 cells and 1.1 ± 0.17 nM ($n$ = 3) for oxytocin receptor-expressing CHO cells (data not shown), consistent with potent antagonist activity observed in the phosphatidylinositol hydrolysis assay.

To further characterize the oxytocin receptor antagonism by Compound A, Schild analysis was performed using $Ca^{2+}$ flux data collected from HEK oxytocin receptor-expressing cells. The $EC_{50}$ of the oxytocin concentration–response was shifted to the right by increasing concentrations of Compound A (Fig. 4). When these data were plotted according to the method of Schild (inset, Fig. 4), the slope of the plot was 1.13 and the $K_{Db}$ determined was 0.50 ± 0.12 nM, consistent with values determined with other methods (Table 3). The maximum response to oxytocin was decreased at high concentrations of Compound A, a phenomenon commonly observed with very potent antagonists in functional assays.

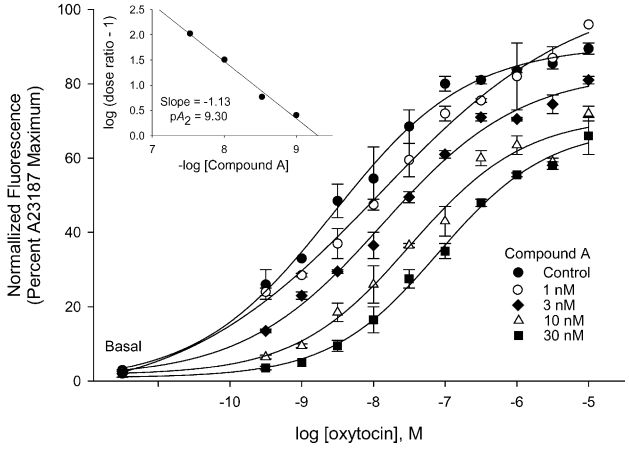



Fig. 4. Schild plot determination of Compound A $K_{Db}$ value using $Ca^{2+}$ flux data determined by fluorometry from HEK/293 cells expressing cloned human oxytocin receptors.

Table 3
Comparison of binding saturation results with three different radioligands for human oxytocin receptors

| | $B_{max}$ (fmol/mg) | $K_d$ (nM) |
|---|---|---|
| [$^3$H]oxytocin | 1060 ± 223 (3) | 1.24 ± 0.40 (3) |
| [$^{125}$I]OVTA[a] | 1380 ± 69 (3) | 0.24 ± 0.01 (3) |
| [$^{35}$S]Compound A | 418 ± 38 (4) | 0.042 ± 0.012 (4) |

[a] [$^{125}$I][d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin. Values are group mean ± standard deviation (*n*).

### 3.3. Characterization of [$^{35}$S]Compound A binding

Compound A was synthesized with the methyl sulfonyl group radiolabeled with [$^{35}$S], and its properties as a radioligand were characterized. Initial binding studies indicated that [$^{35}$S]Compound A binding to oxytocin receptors comes to equilibrium in 20 min at 30 °C (data not shown). At the membrane concentration used in subsequent assays (30 μg protein/ml), percent nonspecific binding was 27%. At 600 μg/ml, nonspecific binding was 10% (data not shown).

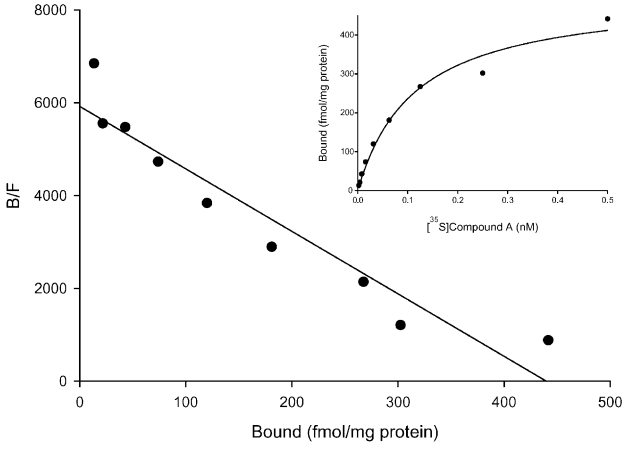



Fig. 5. Representative Scatchard analysis of binding of [$^{35}$S]Compound A to membranes prepared from CHO cells expressing the cloned human oxytocin receptor. Actual saturation curve is shown in the inset. $K_d$ values of four determinations was 0.042 nM ± 0.012. $B_{max}$ was 418 ± 38 fmol/mg protein.

Specific [$^{35}$S]Compound A binding to membranes prepared from CHO cells expressing the cloned human oxytocin receptor was saturable (Fig. 5, inset). Nonlinear curve fitting indicated a single class of binding site with a mean $K_d$ value of 0.042 ± 0.012 nM ($n$ = 4), about 3.8-fold less than the $K_i$ value determined in saturation studies with the unlabeled compound, and about 7-fold less than the $K_i$ value determined in competition studies. The $B_{max}$ value derived from these studies was 418 ± 38 fmol/mg protein, 2–3-fold lower than that obtained for the other two radioligands studied. These data are presented as a Scatchard plot (Fig. 5) and are included in the summary in Table 3. Kinetics of [$^{35}$S]Compound A binding to cloned human oxytocin receptors were determined at 30 °C. The on-rate for Compound A binding to these receptors was $7.4 \pm 5.8 \times 10^9$ M$^{-1}$ min$^{-1}$ determined at three different ligand concentrations ($n$ = 2 at each concentration). The off-rate, determined indirectly from the ordinate intercept of a plot of the $K_{app}$ values obtained in the on-rate studies vs. [ligand], as well as directly from studies using addition of a large excess of competing ligand, was 0.075 ± 0.036 min$^{-1}$ ($n$ = 4) (data not shown). The $K_d$ calculated from these rates was 10.1 pM, comparable with the $K_d$ determined in the saturation binding studies.

### 3.4. Comparison of [$^{35}$S]Compound A, [$^{3}$H]oxytocin and [$^{125}$I][d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin in binding competition studies

A number of reference compounds of various structural classes (peptides and nonpeptides) and functional activities (agonists and antagonists) were compared in competition binding assays measuring specific [$^{35}$S]Compound A, [$^{3}$H]oxytocin, and [$^{125}$I][d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin binding to membranes prepared from CHO oxytocin receptor-expressing cells. The $K_i$ values from these studies are shown in Table 4. The absolute potencies of these reference compounds are in good agreement with these three radioligands. $K_i$ values for these reference compounds determined with each radioligand are within a factor of approximately 3 with the exception of oxytocin, Phe$^2$Orn$^8$oxytocin and [d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin (within a factor of 4–5). It should be noted that in these assays a small component of [$^{35}$S]Compound A binding (< 10%) was not competed by any of the peptide or nonpeptide ligands, but was competed by Compound A.

Table 4
Comparison of reference compound affinities for human oxytocin receptors from binding competition studies using three different radioligands

| | $K_i$ (nM) | | |
|---|---|---|---|
| | [$^{35}$S]Compound A | [$^{3}$H]oxytocin | [$^{125}$I]OVTA[b] |
| Oxytocin | 1.12 ± 0.44 (7) | 0.21 ± 0.08 (6) | 0.69 ± 0.74 (3) |
| Arginine vasopressin | 3.60 ± 1.51 (3) | 1.90 ± 0.90 (4) | 3.84 ± 2.82 (3) |
| Thr$^4$Gly$^7$oxytocin | 6.57 ± 3.07 (3) | 6.86 ± 2.57 (6) | 18.79 ± 6.85 (3) |
| Desamino [D-arg$^8$] vasopressin | 129 ± 10 (3) | 47.7 ± 18.9 (6) | 156 ± 43 (3) |
| Phe$^2$Orn$^8$oxytocin/ vasotocin | 13.7 ± 0.5 (3) | 4.27 ± 2.11 (6) | 7.00 ± 1.24 (3) |
| Kruszynski compound[a] | 8.59 ± 3.13 (3) | 6.11 ± 4.16 (5) | n/d |
| OVTA[b] | 1.39 ± 0.01 (2) | 0.28 ± 0.30 (3) | n/d |
| Compound A | 0.10 ± 0.02 (8) | 0.29 ± 0.17 (5) | 0.23 ± 0.16 (3) |
| L-368,899[c] | 6.94 ± 0.32 (4) | 7.20 ± 4.15 (4) | 7.09 ± 3.58 (3) |

$K_i$ values determined from $IC_{50}$ values: $K_i = IC_{50}/(1+[L]/K_d)$ (Cheng and Prussoff, 1973), and represent group means ± standard deviation ($n$).

[a] [1-(β-mercapto-β, β-cyclo-pentamethylene propionic acid), 2-(*O*-methyl)tyrosine]-Arg$^8$-Vasopressin (Kruszynski et al., 1980).

[b] [d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin (Elands et al., 1987).

[c] 1-((7,7-dimethyl-2(*S*)-(2(*S*)-amino-4-(methylsulfonyl)butyramido)bicyclo [2.2.1]-heptan-1(*S*)-yl)methyl)sulfonyl)-4-(2-methylphenyl)piperazine (Williams et al., 1994).

## 4. Discussion

Potent, selective ligands are important in the identification, characterization, and localization of G-protein-coupled receptors, and if the ligand can be radiolabeled to high specific activity, it holds additional interest for the investigator. A large number of peptide and peptide-related ligands have been developed by the laboratories of Manning and Sawyer, which have proven very useful in understanding the pharmacology of oxytocin and vasopressin receptors (e.g., Manning and Sawyer, 1993). For the most part, these peptide analogues have been characterized and optimized using three biological assays in the rat: in vitro uterine contraction, and in vivo vasopressor and antidiuretic activity (e.g., Chan et al., 1996, 2000). In several cases, peptide analogue oxytocin agonists or antagonists have been radiolabeled, further increasing their utility (e.g., Elands et al., 1987, 1988). In binding studies using membrane preparations from rat tissues, these compounds have behaved largely as expected. However, significant species differences have been observed in the ligand selectivity of oxytocin and vasopressin receptors (e.g., Pettibone et al., 1992), which have seriously complicated interpretation of results when using these ligands in species other than rat. An example is shown in Table 1 where [d(CH$_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-NH$_2^9$]ornithine vasotocin is seen to be highly selective for oxytocin receptors vs. vasopressin $V_{1a}$ receptors in rat, but not in human. Recently, radiolabeled and photoactivable or fluorescent peptide analogues have been reported that have been characterized using human oxytocin and vasopressin receptors (Durroux et al., 1999; Carnazzi et al., 2001; Breton et al., 2001). However, where the comparison between oxytocin receptors and vasopressin $V_{1a}$ receptors was made, at best only modest (17-fold) selectivity was observed (for vasopressin $V_{1a}$ receptors (Durroux et al., 1999)).

Compound A was selected from a collection of nonpeptide oxytocin receptor antagonists, and was shown to be well behaved in competition binding assays using both

[$^3$H]oxytocin and [$^3$H]arginine vasopressin as radioligands as well as in [$^3$H]oxytocin saturation binding assays as an unlabeled competitor. $K_i$ values for Compound A were similar between these two types of assays, and preincubation of membranes with Compound A in [$^3$H]oxytocin saturation binding assays did not alter the $B_{max}$ values in the saturation studies, indicating that Compound A binds reversibly to the oxytocin receptor. Interestingly, while Compound A was found to be highly selective (900–1800-fold) for the oxytocin receptors vs. the vasopressin receptors in the human system, it is not selective in the rat, and may not be the ligand of choice in this species. Functional studies indicated that Compound A was an oxytocin receptor antagonist with no agonist properties.

Radiolabeling of Compound A with [$^{35}$S] in the final synthetic step afforded a radioligand of high specific activity. Saturation binding studies of human oxytocin receptors with [$^{35}$S]Compound A indicated that the ligand bound to a single high affinity site with $K_d$ values comparable to the $K_i$ values determined for the unlabeled compound. The $B_{max}$ values obtained from these studies with [$^{35}$S]Compound A were somewhat lower (2–3 ×) than those observed in similar studies with [$^3$H]oxytocin or [$^{125}$I][d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin. The reason for this difference is not clear, but it does indicate that [$^{35}$S]Compound A is not binding to membrane sites in addition to the oxytocin receptors. In support of this, the $K_i$ values of oxytocin receptor agonists and antagonists from a variety of structural classes, determined in competition binding studies using [$^{35}$S]Compound A, were very similar to those obtained with two other well-behaved and widely used radioligands, [$^3$H]oxytocin and [$^{125}$I][d($CH_2$)$_5$,Tyr(Me)$^2$,Thr$^4$,Tyr-$NH_2^9$]ornithine vasotocin.

The data presented above indicate that Compound A is a potent oxytocin receptor antagonist with a high degree of selectivity for human oxytocin receptor compared to the human vasopressin receptors. In addition, the data also indicate that [$^{35}$S]Compound A is a well-behaved, highly selective radioligand for human oxytocin receptors. The use of a methane [$^{35}$S]sulfonamide as the radiolabeled functionality (Dean et al., 1996) has the benefit of affording a very high specific radioactivity without the drawbacks due to structural perturbations (lower affinity, higher lipophilicity) often observed with [$^{125}$I] radiolabeling of small molecule ligands. In preliminary studies, competition binding data indicate Compound A binding to membranes prepared from rhesus monkey tissues (uterus, liver, and kidney are highly enriched in oxytocin, vasopressin $V_{1a}$ and $V_2$ receptors, respectively) are similar to those observed for human membranes (data not shown). This is not surprising, considering the high degree of identity (97%) between rhesus and human oxytocin receptors (Salvatore et al., 1998). The high selectivity for human oxytocin receptors suggests that Compound A may be useful as a novel, highly specific, tool in studying the pharmacology of oxytocin receptors in human (and nonhuman primate) tissues. In addition, Compound A or [$^{35}$S]Compound A may serve as a novel, highly specific, nonlabeled competitor or radioligand, respectively, in autoradiographic studies of oxytocin receptor localization in tissues from these species.

## Appendix A. Supplementary information

### *A.1. Details of synthesis of Compound A*

#### *A.1.1. General*

Abbreviations: EtOAc = ethyl acetate, DMF = dimethylformamide, HOBT = 1-hydroxybenzotriazole, DIEA = diisopropylethylamine, EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride.

Thin layer chromatographic analysis was accomplished using Analtech Uniplate silica gel GF (250 μm) TLC plates. For preparative silica gel column chromatography, EM Science Silica Gel 60 (230–400 mesh) was utilized.

A Thermo Separations Spectra System P4000 HPLC instrument was utilized. For the stationary phase, a Hewlett-Packard Zorbax SB-C8 column (75 × 4.6 mm, 3.5 μm) was employed. The mobile phase consisted of a binary gradient, 95:5 A/B to 0:100 A/B over 4.5 min, where A is water containing 0.1% by volume trifluoroacetic acid and B is acetonitrile containing 0.1% by volume trifluoroacetic acid, using a flow rate of 3.0 ml/min. Peaks were detected by UV absorption at 215 nm.

A Waters 2690 HPLC and a ZMD quadrupole mass spectrometer were used to record LC-MS information. For the HPLC stationary phase, a YMC PRO column was employed (3 mm × 5 cm, C18 stationary phase, 5 μM particle size, 120 Å pore size). The mobile phase consisted of a binary gradient, 92:8 A/B to 0:100 A/B over 3.4 min, then 0:100 A/B for 0.3 min, where A is water containing 0.05% by volume trifluoroacetic acid and B is acetonitrile containing 0.05% by volume trifluoroacetic acid, using a flow rate of 2.0 ml/min. The ionization method for the mass spectrometer was electrospray, with positive ion detection.

Proton NMR spectra were recorded at 400 MHz using a Varian spectrometer. Tetramethylsilane was employed an internal standard (0.00 ppm).

#### *A.1.2. Procedure*

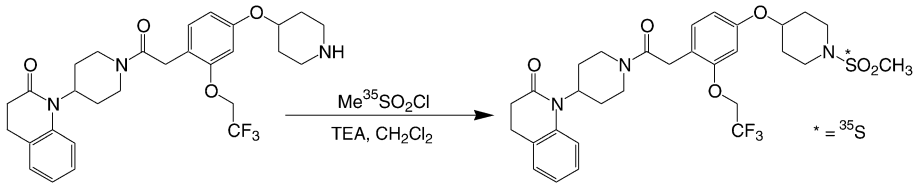
Refer to Fig. 1. To a stirred solution of compound **1** (0.20 g, 0.87 mmol), compound **2** (0.37 g, 0.85 mmol), EDC (0.21 g, 1.1 mmol), HOBT hydrate (0.14 g, 0.92 mmol) in DMF (10 ml) was added enough DIEA (approximately 0.2 ml) to obtain a reading of pH 7 on wetted E. Merck pH indicator strips. The mixture was stirred at ambient temperature for 16 h. The solvent was removed under reduced pressure and the residue was partitioned between EtOAc (50 ml) and saturated aqueous $NaHCO_3$ (50 ml). The organic phase was dried over anhydrous $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The residue was purified using silica gel column chromatography with 3:1 EtOAc/

hexanes as eluant. Compound **3** was obtained as a gum (0.53 g, 94%). TLC $R_f$ = 0.7 (3:1 EtOAc/hexanes); HPLC RT = 3.91 min; LC-MS *m/z* = 646; NMR ($CDCl_3$) 7.24 ppm (d, *J* = 8.4 Hz, 1H), 7.15–7.21 ppm (m, 2H), 6.98–7.02 ppm (m, 2H), 6.61 ppm (dd, *J* = 2.2, 8.4 Hz, 1H), 6.42 ppm (d, *J* = 2.2 Hz, 1H), 4.81 ppm (br d, *J* ~ 13 Hz, 1H), 4.45 ppm (m, 2H), 4.34 ppm (q, *J* = 8.2 Hz, 2H), 4.08 ppm (br d, *J* ~ 14 Hz, 1H), 3.64–3.52 (m, 4H), 3.33 ppm (m, 2H), 3.09 ppm (br t, *J* ~ 14 Hz, 1H), 2.81 ppm (br t, *J* ~ 7 Hz, 2H), 2.64 ppm (br t, *J* ~ 12 Hz, 1H), 2.56 ppm (dd, *J* = 5.1, 7.8 Hz, 2H), 2.35–2.52 ppm (m, 2H), 1.90 ppm (m, 2H), 1.74 ppm (m, 4H), 1.47 ppm (s, 9H).

A stirred solution of compound **3** (0.50 g, 0.78 mmol) in EtOAc (25 ml) was cooled to 0 °C. HCl gas was bubbled through the cold solution for 10 min. The mixture was stirred at 0 °C for 10 min and then at ambient temperature for 45 min. The solvent was removed under reduced pressure and the resulting solid was triturated in a small volume of EtOAc and collected by filtration to give the hydrochloride salt of compound **4** as an amorphous white powder (0.43 g, 93%). TLC $R_f$ = 0.3 (90:10:0.5 $CH_2Cl_2$/$CH_3OH$/$NH_4OH$); HPLC RT = 2.93 min; LC-MS *m/z* = 546; NMR (DMSO-$d_6$) 8.9 ppm (br s, 2H), 7.18-7.25 (m, 3H), 7.12 ppm (d, *J* = 8.2 Hz, 1H), 7.01 ppm (t, *J* = 7.0 Hz, 1H), 6.76 ppm (d, *J* = 2.2 Hz, 1H), 6.68 ppm (dd, *J* = 2.2, 8.4 Hz, 1H), 4.74 ppm (q, *J* = 8.8 Hz, 2H), 4.66 ppm (m, 1H), 4.50 ppm (br d, *J* ~ 12 Hz, 1H), 4.28 ppm (m, 1H), 4.00 ppm (br d, *J* ~ 13 Hz, 1H), 3.58 ppm (AB q, *J* ~ 12 Hz, 2H), 3.22 ppm (m, 2H), 3.0–3.18 ppm (m, 3H), 2.77 ppm (br t, *J* ~ 7 Hz, 2H), 2.64 ppm (br t, *J* ~ 11 Hz, 1H), 2.43 ppm (br t, *J* ~ 8 Hz, 2H), 2.33 ppm (dq, *J*d = 4.0 Hz, *J*q = 12.3 Hz, 2H), 2.30 ppm (m, 2H), 1.84 ppm (m, 2H), 1.66 ppm (m, 2H).

A stirred solution of compound **4** (0.20 g, 0.34 mmol) and triethylamine (0.51 mmol) in $CH_2Cl_2$ (15 ml) was cooled to 0 °C. To the solution was added methanesulfonyl chloride (0.36 mmol). The cooling bath was removed and the mixture was stirred at ambient temperature for 1 h. The reaction was diluted with $CH_2Cl_2$ (20 ml) and extracted with saturated aqueous $NaHCO_3$ (20 ml). The organic phase was dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure. The residue was purified using silica gel column chromatography with 97:3 $CH_2Cl_2$/$CH_3OH$ as eluant. Compound A was obtained as an amorphous solid (0.20 g, 92% yield). TLC $R_f$ = 0.4 (97:3 $CH_2Cl_2$/$CH_3OH$); HPLC retention time = 3.49 min; LC-MS *m/z* = 624; NMR ($CDCl_3$), 7.25 ppm (d, *J* = 8 Hz, 1H), 7.15–7.22 ppm (m, 2H), 7.01 ppm (t, *J* = 8 Hz, 2H), 6.60 ppm (dd, *J* = 2.4, 8.3 Hz, 1H), 6.42 ppm (d, *J* = 2.4 Hz, 1H), 4.81 ppm (br d, *J* ~ 10 Hz, 1H), 4.51 ppm (m, 1H), 4.42 ppm (m, 1H), 4.35 ppm (q, *J* = 8.0 Hz, 2H), 4.08 ppm (br d, *J* ~ 10 Hz, 1H), 3.69 ppm (AB quartet, *J* ~ 10 Hz, 2H), 3.3–3.4 ppm (m, 4H), 3.10 ppm (br t, *J* ~ 12 Hz, 1H), 2.82 ppm (s and overlapping m, 5H), 2.64 ppm (br t, *J* ~ 12 Hz, 1H), 2.

*Detailed Synthesis of [$^{35}$S] Compound A*




[$^{35}$S]Methanesulfonyl chloride (Dean et al. (1995)) in anhydrous dichloromethane (5 mCi, SA = ~ 1100 Ci/mmol) was transferred in to a 15-ml pear-shaped flask. The solution was concentrated by distillation at atmospheric pressure (oil bath temperature 60–64 °C) to a volume of 50–100 μl. The flask was removed from the oil bath and briefly allowed to cool. This solution was then transferred via blunt-end syringe to a solution containing compound **4** (free base, ~ 5 mg) in 50 μl of anhydrous dichloromethane. The distillation flask was rinsed with anhydrous dichloromethane (2 × 50 μl) and transferred to the reaction mixture. To this solution was added 10 μl of anhydrous triethylamine (stored over molecular sieves 4 Å). The reaction mixture was stirred at room temperature for 2 h, then diluted with 1 ml dichloromethane and 200 μl of water. The dichloromethane layer was separated, washed with saturated sodium bicarbonate (2 × 2 ml). The dichloromethane solution was dried over anhydrous sodium sulfate twice and then evaporated under a nitrogen stream at 30 °C. The resulting residue was dissolved in 75 μl of acetonitrile/water (1:1 v/v) and purified by using preparative HPLC (Zorbax-SB-C8, 9.4 × 250 mm semi-prep column, 60% water containing 0.1% TFA–40% acetonitrile, UV = 230 nm, flow rate = 4 ml/min). The pure product-containing fractions were combined and neutralized with dilute sodium bicarbonate solution. The acetonitrile was removed by evaporation and the aqueous solution passed through a Sep-Pak (Waters, C-18) cartridge. The cartridge was washed with water, followed by elution of [$^{35}$S]Compound A with ethanol. The total activity of the ethanol fraction (10 ml) collected was 2.9 mCi (58% radiochemical yield). The radiochemical purity was determined to be >99% by HPLC analysis (Zorbax-SB-C8, 4.6 × 250 mm column, 55% water containing 0.1% TFA–45% acetonitrile, UV = 230 nm, flow rate = 1 ml/min) and the specific activity obtained was 1116 Ci/mmol as determined by LC/MS.

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