in position two, may perturb the geometry of the ethylamine fragment and realign the orientation of the Leu[5] side chain differently from **1-c** once the phenolic and phenyl rings bind to their respective sites affording a conformer with enhanced μ-receptor preference. In accordance with this hypothesis is the observation that the D-Leu[5] diastereomer of **1-d** has considerably reduced selectivity (by a factor of 4 based on the IC_{50} ratio between the MVD and GPI assays).[16]

Conclusion

In the series of 7α-(1-hydroxy-1-methylphenalkyl)oripavines maximal analgesic activity has been reported to be associated with a 19-phenylpropyl chain as in PEO. The fact that analgesic potency is drastically reduced by shortening or lengthening the phenylalkyl chain implicates an additional well-defined binding site. We found that the conformation reported for the cyclic opioid peptide H-Tyr-*cyclo*[-D-N^{δ}-Orn-Gly-Phe-Leu-] provides the possibility of topographical congruency between the C_{21} aromatic ring in PEO and that of the phenylalanyl side chain of the peptide. Accordingly, the two may interact with a common lipophilic receptor site. Low-energy peptide conformers were obtained by a bimolecular energy minimization procedure with PEO, and it was found that the ethylamine fragment of the tyramine moiety in the former assumes a different conformation than its counterpart in the latter such that the basic nitrogens occupy distinct spatial loci approximately 1.1 Å apart. The variation in receptor selectivity among the members of the homologous series shown in Figure 1 may arise from subtle differences in backbone conformation which governs the direction of the leucine side chain and the geometrical orientation of the ethylamine fragment once the phenolic and phenylalanyl rings are bound to the receptor.

Previous conformational comparisons of flexible opioid peptides with rigid opiates resulted in intermediate- to high-energy conformers for those peptides having maximum resemblence to rigid alkaloids.[36] Since the rigidity of the cyclic opioid derivative **1-c** precludes any major backbone rearrangement, we found that an alternate approach is to anchor the minimal common element (the phenolic group) and attach the basic nitrogens and the secondary aromatic rings of the respective molecules by "spring constants" followed by the relaxation. Since the procedure balances mutual spatial orientations of pharmacophoric elements, it may be regarded analogous to an induced fit.

Acknowledgment. We are indebted to the financial assistance of the Natural Sciences and Engineering Research Council of Canada (operating Grant A-1529) to J.D. while at the University of Sherbrooke and to A.M. (operating Grant A-0329). We also acknowledge Isabelle Lepage for typing the manuscript.

Registry No. 1c, 83159-95-9; 2, 14521-98-3.

(36) Loew, G. H.; Burt, S. K. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 7.
(37) Hudson, D.; Sharpe, R.; Szelke, M. *Int. J. Peptide Protein Res.* **1980**, *15*, 122.

In Vitro Labeling of Serotonin-S_2 Receptors: Synthesis and Binding Characteristics of [^{3}H]-7-Aminoketanserin

Walter Wouters,*† Cornelus G. M. Janssen,‡ Jacky Van Dun,† Jos B. A. Thijssen,‡ and Pierre M. Laduron†

Department of Biochemical Pharmacology and Department of Drug Metabolism & Pharmacokinetics, Janssen Pharmaceutica, B-2340 Beerse, Belgium. *Received November 25, 1985*

[^{3}H]-7-Aminoketanserin (7-amino-3-[2-[4-(2-tritio-4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4-(1*H*,3*H*)-quinazolinedione), an amino derivative of the selective serotonin-S_2 antagonist ketanserin, was synthesized and tested for in vitro labeling of serotonin-S_2 receptors. The compound showed a very high affinity for both membrane-bound and detergent-solubilized serotonin-S_2 receptors with K_D values of 0.35 and 2.03 nM, respectively. At nanomolar concentrations, binding to serotonin-S_1 sites was totally absent. Serotonin-S_2 receptor binding was characterized by a slow dissociation and a very low nonspecific binding. In rat frontal cortex preparations, binding could be displaced by nanomolar concentrations of different serotonin antagonists and micromolar concentrations of serotonin agonists. Compounds with other pharmacological profiles were poorly or not active. Introduction of an amino function in this new radioligand led to a decreased lipophilicity. Therefore, besides being a valuable radioligand for routine binding studies, [^{3}H]-7-aminoketanserin will probably be a good ligand for labeling serotonin-S_2 receptors on intact cells.

Serotonin-S_2 receptors have been identified in the central nervous system as well as in the periphery. These receptor sites were shown to mediate the antagonism of various serotonergic effects, measured both in vivo and in vitro, such as behavioral excitation in rodents induced by serotonin mimetic agents ("the serotonin syndrome"), serotonin-induced contractions in isolated arteries, and serotonin-induced shape changes and aggregation of blood platelets.[1]

Several radioligands have been used for the in vitro biochemical characterization of serotonin-S_2 receptors, including [^{3}H]spiperone,[2] [^{3}H]LSD,[3] [^{125}I]LSD,[4] [^{3}H]mianserin,[5] and [^{3}H]metergoline.[6] However, most of these ligands were either not selective for serotonin-S_2 sites or showed a too high nonspecific binding. Recently [^{3}H]-ketanserin was introduced as a high-affinity, selective

† Department of Biochemical Pharmacology.
‡ Department of Drug Metabolism & Pharmacokinetics.

(1) Leysen, J. E.; de Chaffoy de Courcelles, D.; De Clerck, F.; Niemegeers, C. J. E.; Van Nueten, J. M. *Neuropharmacology* **1984**, *23*, 1493.
(2) Leysen, J. E.; Niemegeers, C. J. E.; Tollenaere, J. P.; Laduron, P. M. *Nature (London)* **1978**, *272*, 168.
(3) Peroutka, S. J.; Snyder, S. H. *Mol. Pharmacol.* **1979**, *16*, 687.
(4) Hartig, P. R.; Kadan, M. J.; Evans, M. J.; Krohn, A. M. *Eur. J. Pharmacol.* **1983**, *89*, 321.
(5) Peroutka, S. J.; Snyder, S. H. *J. Pharmacol. Exp. Ther.* **1981**, *216*, 142.
(6) Hamon, M.; Mallat, M.; Herbet, A.; Nelson, D. L.; Audinot, M.; Pichat, L.; Glowinski, J. *J. Neurochem.* **1981**, *36*, 613.

0022-2623/86/1829-1663$01.50/0

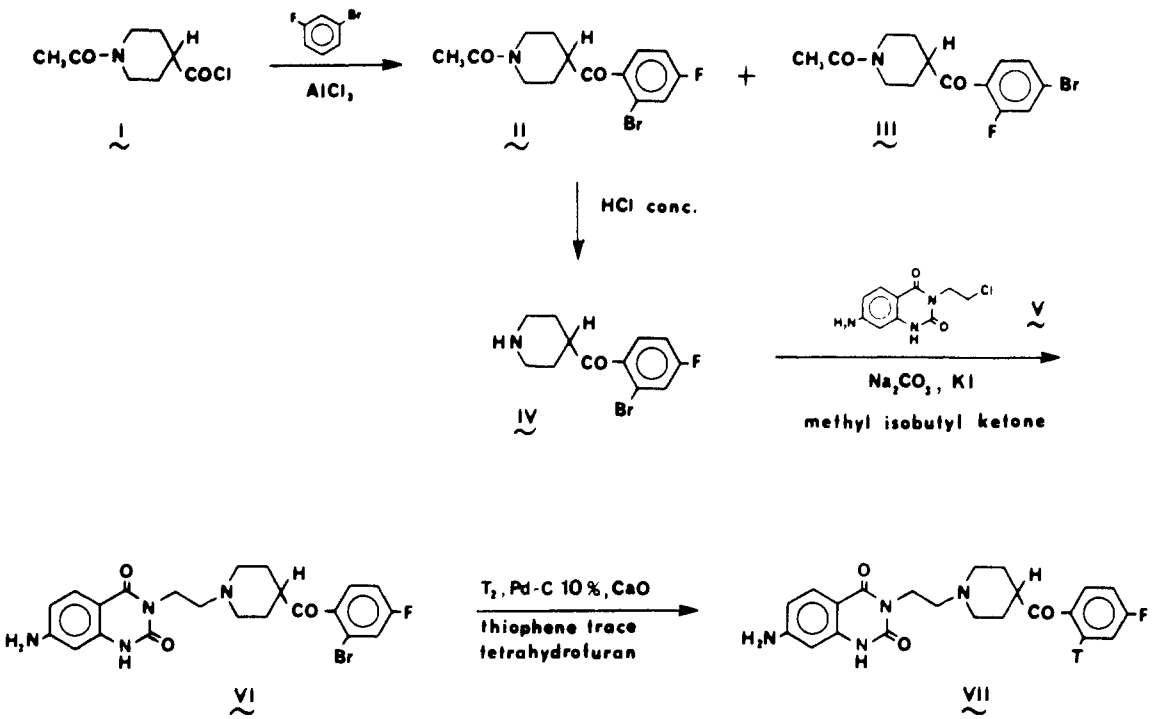


Figure 1. Synthesis of [^{3}H]-7-aminoketanserin.

serotonin-S_2 receptor ligand that did not bind to serotonin-S_1 sites (a serotonin binding site labeled by nanomolar concentrations of [^{3}H]serotonin).[7] By use of [^{3}H]ketanserin, membrane-bound as well as detergent-solubilized serotonin-S_2 receptors could be specifically labeled.[8,9] However, in both cases there was a relatively high amount of nonspecific binding.

In the present paper we describe the synthesis and binding characteristics of a derivative of ketanserin namely [^{3}H]-7-aminoketanserin. This new radioligand combines the selectivity of ketanserin with a very low nonspecific binding.

Results

Synthesis of [^{3}H]-7-Aminoketanserin (VII). In order to obtain specifically ortho-labeled VII (Figure 1), to avoid unnecessary loss of radioactivity and to acquire a high specific activity, the synthetic strategy toward tritiated aminoketanserin required the introduction of the tritium label via dehalogenation in the latest possible stage. Friedel–Craft acylation of 3-fluorobromobenzene with I[10] afforded an isomeric mixture of II and III that was separated by HPLC. Deacetylation of II and coupling of the deprotected piperidine derivative IV with V[11] furnished bromoaminoketanserin VI, which was reductively dehalogenated (tritium; Pd 10% on charcoal) to title compound VII. Some physicochemical properties of 7-aminoketanserin are listed in Table II.

Table I. Inhibition by 7-Aminoketanserin of Radioligand Binding in Nine Binding Models[a]

binding model	[^{3}H]ligand	−log IC_{50}, M	K_i,[b] nM	K_i/K_i (serotonin-S_2)
serotonin-S_2	ketanserin	9.25	0.17	1
serotonin-S_1	serotonin	5.25	4416	25 976
histamine-H_1	pyrilamine	8.65	1.22	7.2
α_1-adrenergic	WB-4101	7.82	5.49	32.3
dopamine-D_2	haloperidol	6.38	164	965
α_2-adrenergic	clonidine	<6		
β-adrenergic	dihydroalprenolol	<6		
opiate	sufentanil	<6		
muscarinic-acetylcholine	dexetimide	<6		

[a] Binding was performed as described in the Experimental Section. [b] For calculation of K_i values, dissociation constants (K_D) of the various radioligands were taken from the literature.[7,8,13]

Receptor Profile of 7-Aminoketanserin. 7-Aminoketanserin was tested in nine different binding models from rat and guinea pig brain. The −log IC_{50} values and calculated K_i values are shown in Table I. The compound selectively bound to serotonin-S_2 receptors; binding to serotonin-S_1 sites showed an affinity that was 25 000 times lower. 7-Aminoketanserin was 7 times less potent at the histamine-H_1 receptor than at the serotonin-S_2 receptor and 32 times less active at the α_1-adrenergic receptor. Finally, the compound was only very weakly active at the dopamine-D_2 receptor and completely inactive in the other binding models at concentrations up to 1 μM.

Binding Characteristics of [^{3}H]-7-Aminoketanserin. Saturation curves for [^{3}H]-7-aminoketanserin binding to an (M + L + P) fraction (combined heavy and light mitochondrial and microsomal fractions) from rat frontal cortex and to a solubilized preparation are shown in Figure 2. Results were obtained from three (membrane preparation) or two (solubilized preparation) independent experiments, each performed in triplicate. Nonspecific binding was very low; in routine binding experiments the nonspecific binding level reached about 7% of total binding in a membrane preparation (using 1 nM [^{3}H]-7-aminoketanserin) and about 16% of total binding to a solubilized preparation (using 2 nM [^{3}H]-7-aminoketan-

(7) Leysen, J. E.; Awouters, F.; Kennis, L.; Laduron, P. M.; Vandenberk, J.; Janssen, P. A. J. *Life Sci.* **1981**, *28*, 1015.
(8) Leysen, J. E.; Niemegeers, C. J. E.; Van Nueten, J. M.; Laduron, P. M. *Mol. Pharmacol.* **1982**, *21*, 301.
(9) Wouters, W.; Van Dun, J.; Leysen, J. E.; Laduron, P. M. *Eur. J. Pharmacol.* **1985**, *115*, 1.
(10) Duncan, R. L., Jr.; Helsley, G. C.; Welstead, W. J., Jr.; Da Vanzo, J. P.; Funderburk, W. H.; Lunsford, C. D. *J. Med. Chem.* **1970**, *13*, 1.
(11) Unlabeled VII was obtained in a 35% yield by coupling V (this reference) with 4-(4-fluorobenzoyl)piperidine (this reference). Satisfactory elemental combustion analyses were obtained (C, H ±0.3%). The compound was TLC and HPLC identical to tritiated VII: U.S. Patent Application 678 422, 1985.

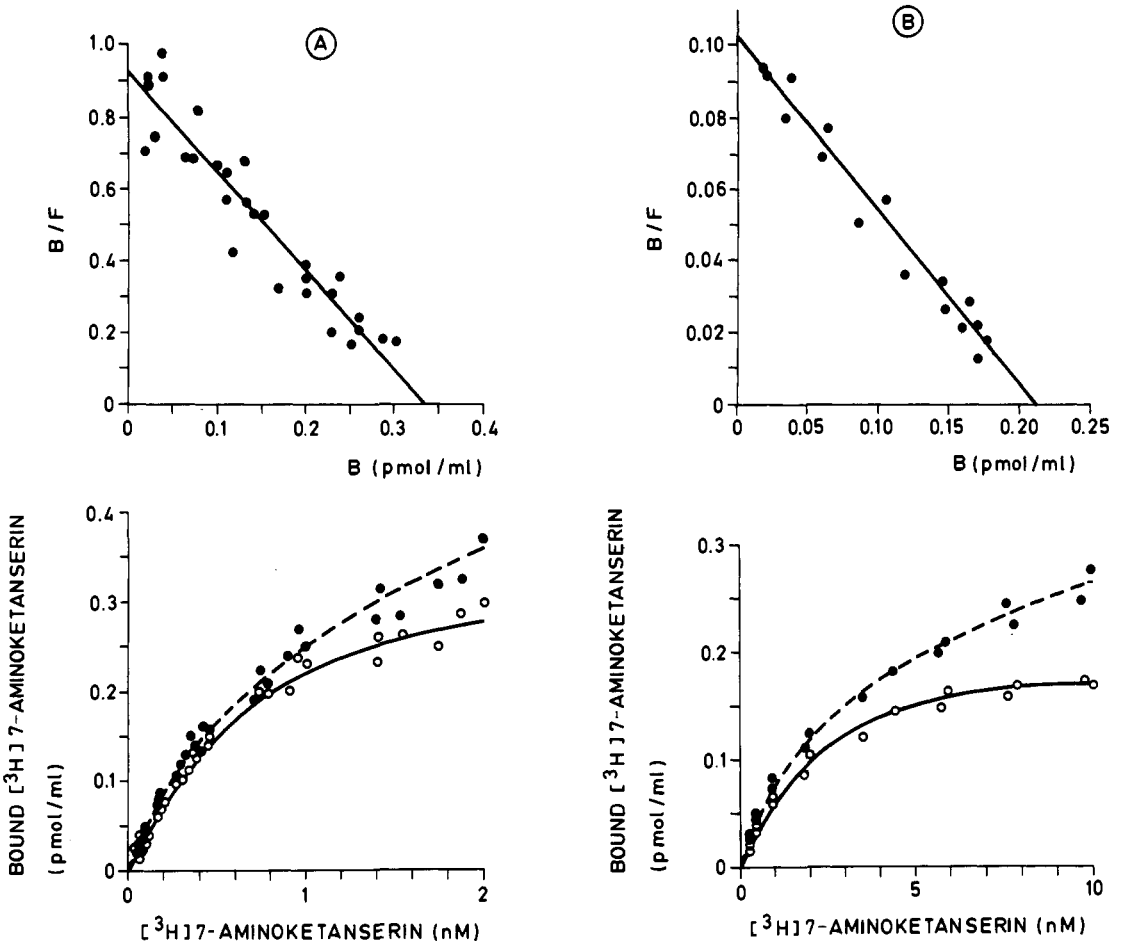


Figure 2. Saturation curves of total (-●-) and specific (-○-) [^{3}H]-7-aminoketanserin binding with Scatchard plots of specific binding to an (M + L + P) fraction (A) and a CHAPS/salt-solubilized preparation (B) from rat frontal cortex. Binding was performed as described in the Experimental Section. Specific binding was calculated as the difference between total binding and binding in the presence of a 1000-fold excess of methysergide. The figure represents points obtained from three (M + L + P) or two (solubilized) independent experiments, each performed in triplicate.

Table II. Physical Constants and Kinetic Binding Parameters for [^{3}H]-7-Aminoketanserin[a]

parameter	membrane-bound receptors	solubilized receptors
association k_1, nM^{-1} min^{-1}	0.385	0.183
dissociation[b] k_{-1}, min^{-1}	0.088	0.056
$t_{1/2}$, min	7.8	12.4
K_D,[c] nM	0.35 ± 0.04 (3)	2.03 ± 0.01 (2)
B_{max},[c] fmol/mg of tissue	36.97 ± 2.65 (3)	4.18 ± 0.24 (2)

[a] log P = 2.44; pK_{a_1} = 8.20, pK_{a_2} (base) = 1.25. P is the octanol/water partition coefficient at pH 9.8. [b] Association was performed during 20 min; dissociation was started by the addition of a 1000-fold excess of pipamperone. [c] Values are means ± SD. Numbers in parentheses are numbers of independent experiments performed in triplicate.

serin). Scatchard analysis of the data yielded straight lines, indicating binding to only one class of binding sites. K_D and B_{max} values were calculated for each separate experiment. Their mean values are given in Table II.

Both association and dissociation of [^{3}H]-7-aminoketanserin binding were relatively slow (see Table II). Association constants were calculated as described in the Experimental Section and gave values of 0.385 and 0.183 nM^{-1} min^{-1} for membrane-bound and solubilized receptors, respectively. Dissociation showed first-order reaction kinetics with dissociation constants of 0.088 min^{-1} ($t_{1/2}$ = 7.8 min) and 0.056 min^{-1} ($t_{1/2}$ = 12.4 min), respectively.

Pharmacological Characterization of [^{3}H]-7-Aminoketanserin Binding. Serotonin agonists and antagonists from several different chemical classes were tested for their potency to inhibit [^{3}H]-7-aminoketanserin binding to an (M + L + P) fraction and a solubilized preparation from rat frontal cortex; the inhibition curves are shown in Figure 3. For all the compounds, monophasic inhibition curves were obtained. There was no displacement under the threshold of nonspecific binding.

The -log IC_{50} and K_i values derived from competition experiments with antagonists and agonists of different pharmacological classes are summarized in Table III. Serotonin antagonists such as pirenperone, ketanserin, ritanserin, methysergide, and pipamperone showed nanomolar affinity; serotonin agonists (serotonin and bufotenine) showed micromolar affinity. There was a pronounced stereospecificity with a 1000-fold difference between the affinities of (+) and (-)-butaclamol. Compounds with other pharmacological profiles such as pyrilamine (histamine-H_1 antagonist), prazosin and WB-4101 (α_1-adrenergic antagonist), and domperidone (dopamine-D_2 antagonist) were only active at micromolar concentrations.

For solubilized receptors the different compounds showed a same rank order of potencies to displace [^{3}H]-7-aminoketanserin binding, but the affinities were generally lower than those obtained for membrane-bound receptors.

Discussion and Conclusion

Chemical modification of ketanserin by amino substitution resulted in an improved serotonin-S_2 receptor ligand. [^{3}H]-7-Aminoketanserin showed an affinity of 0.35

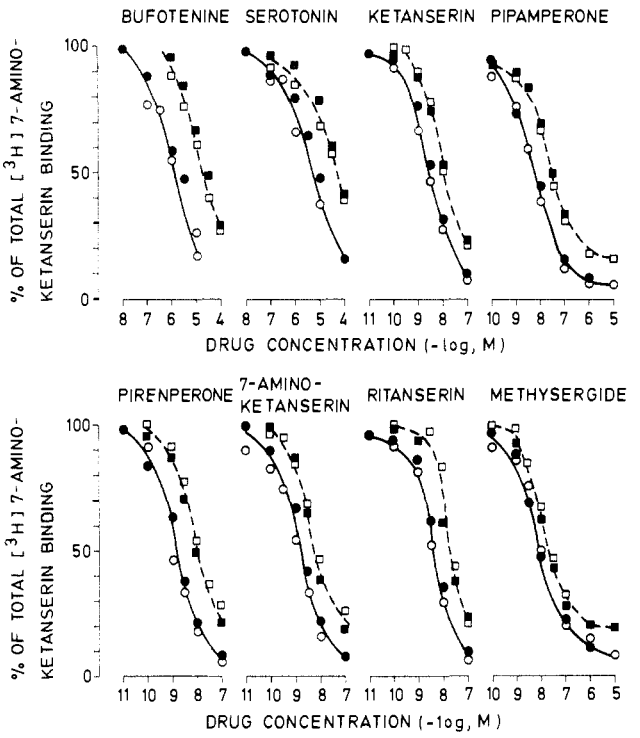


Figure 3. Displacement of [^{3}H]-7-aminoketanserin binding to an (M + L + P) fraction (—) and a CHAPS/salt-solubilized (---) preparation from rat frontal cortex by different serotonin antagonists and agonists. Binding experiments were performed as described in the Experimental Section. [^{3}H]-7-Aminoketanserin was used as 1 nM (M + L + P) or 2 nM (solubilized) final concentration. Filled and open symbols represent the results from two independent experiments, each performed in triplicate.

Table III. Competition of Various Compounds in [^{3}H]-7-Aminoketanserin Binding to an (M + L + P) Fraction and to a CHAPS/Salt-Solubilized Preparation from Rat Frontal Cortex[a]

	membrane-bound receptors		solubilized receptors	
compds	-log IC_{50}, M	K_i, nM	-log IC_{50}, M	K_i, nM
7-aminoketanserin	8.86 ± 0.20	0.36	8.35 ± 0.07	2.23
pirenperone	8.74 ± 0.09	0.47	8.15 ± 0.07	3.54
spiperone	8.62 ± 0.03	0.62	8.11 ± 0.09	3.88
ketanserin	8.58 ± 0.09	0.68	8.10 ± 0.05	3.97
ritanserin	8.46 ± 0.09	0.90	7.85 ± 0.04	7.06
pipamperone	8.27 ± 0.10	1.39	7.72 ± 0.03	9.53
methysergide	8.13 ± 0.12	1.92	7.87 ± 0.03	6.74
mianserin	7.93 ± 0.06	3.04	7.57 ± 0.07	13.46
(+)-butaclamol	7.88 ± 0.00	3.41	7.45 ± 0.01	17.74
domperidone	6.23 ± 0.02	152	5.70 ± 0.00	998
WB-4101	5.57 ± 0.04	697	5.11 ± 0.013	3881
pyrilamine	5.40 ± 0.03	1032	4.70 ± 0.01	9976
(-)-butaclamol	4.90 ± 0.14	3261	4.56 ± 0.16	13771
prazosin	4.35 ± 0.07	11572	4.40 ± 0.06	19905
bufotenine	5.88 ± 0.11	341	4.90 ± 0.14	6295
serotonin	5.45 ± 0.35	919	4.55 ± 0.14	14092

[a] IC_{50} values are means ± standard deviation of two experiments, performed in triplicate. K_i values were calculated according to the Cheng–Prusoff equation. Final concentrations of [^{3}H]-7-aminoketanserin were 1 nM for membrane-bound and 2 nM for solubilized receptors; equilibrium dissociation constants (K_D) were as in Table II.

nM for binding to membrane-bound serotonin-S_2 receptors, which makes it (together with [^{3}H]ketanserin; K_D = 0.42 nM)[8] the radioligand with the highest specific serotonin-S_2 receptor affinity, presently available. Affinity for binding to solubilized serotonin-S_2 receptors was about 6 times lower. This difference in affinity is a common feature of detergent-solubilized receptors; it was also encountered when other radioligands were used to label solubilized serotonin-S_2 receptors.

The binding of [^{3}H]-7-aminoketanserin was characterized by very low levels of nonspecific binding. In routine binding assays nonspecific binding was typically lower than 10% for membrane-bound and 20% for solubilized receptors. Another interesting feature of [^{3}H]-7-aminoketanserin binding was its dissociation rate, which was more than 2 times smaller than that reported for [^{3}H]-ketanserin.[8]

Addition of an amine function to ketanserin drastically reduced the lipophilicity of the compound. 7-Aminoketanserin showed a log P of 2.44 with a pK_a of 8.20. This means that under physiological conditions (pH 7.4), the octanol/water partition coefficient (P_{app}) is only 38. Preliminary experiments showed that [^{3}H]-7-aminoketanserin cannot label brain serotonin-S_2 receptors in in vivo conditions (results not shown). Presumably the ligand cannot cross the blood–brain barrier, a finding which is compatible with the hydrophilic properties of the ligand.

Taken together, [^{3}H]-7-aminoketanserin was shown to be a valuable radioligand for serotonin-S_2 receptor studies. Due to its physicochemical properties one may expect it to be a good ligand for the labeling of serotonin-S_2 receptors on intact cells.

Experimental Section

General Methods. Melting points were taken on a Mettler melting point apparatus and are uncorrected. Elemental combustion analyses were obtained with a Carlo Erba elemental analyzer 1106. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AM 360 spectrometer. Chemical shifts are reported as values in parts per million relative to tetramethylsilane (δ 0.00) as an internal standard. Liquid scintillation counting was performed on a Packard Tri-Carb 460 CD; the radioactivity of the samples was measured in Instagel II (Packard) as a scintillation cocktail. Large-scale preparative HPLC (compound II) was conducted on a Jobin-Yvon Module prep, using a column with 80 mm i.d., charged with 500 g of silica gel (15–25 μm Lichroprep Si-60, Merck and Co., Darmstadt, Germany), which was eluted with n-hexane–ethyl acetate (25% by volume). For small-scale preparative and analytical HPLC the apparatus consisted of two Waters Model 660 solvent programmers for gradient elution. The samples were injected by a Waters Model U6K universal injector. On-line detection of the HPLC eluate was carried out with a Varian UV 50 variable-wavelength detector. For the labeled material only, analysis was carried out by on-line radioactivity detection of the HPLC eluate with a Berthold radioactivity monitor BF 5025 HP system, using a flow-through cell of 0.400 mL. In this case, the eluate was mixed with Pico-fluor TM30 (Packard, used as scintillation cocktail) in a LKB Ultrograd mixing unit. The normalized areas of the peaks were computed by a SP 4270 system (Spectra Physics). The stainless-steel columns used (30-cm narrow bore, 4.6-mm i.d.) were packed by the balance density slurry method with a Haskel air driven liquid pump, applying Hypersil ODS (5 μm) as stationary phase. The specific activity of the labeled compound was measured as follows: a calibration curve was formed by HPLC–UV analysis of known injected quantities of unlabeled reference material under conditions as described above. On this basis the unknown weight of a labeled sample was determined, and the eluted compound was collected and counted. Calculation from both quantities gave the desired specific activity.

All solvents used were of p.a. quality, unless stated otherwise. The reactants were of "reagent grade" and were used as such. Diisopropyl ether, methanol, 2-propanol, sodium hydroxide, sodium carbonate, and Pd-10% on charcoal were supplied by Merck and Co, (Darmstadt, Germany). Chloroform, acetone, methyl isobutyl ketone, tetrahydrofuran, hydrochloric acid, and calcium oxide were purchased from J. T. Baker Chemicals B. V. (Deventer, Holland). Acetonitrile was obtained from U.C.B. (Leuven,

Belgium). Aluminum chloride, 3-fluorobromobenzene, charcoal, thiophene, and potassium iodide were supplied by Janssen Chimica (Beerse, Belgium). Silica gel columns for gravity chromatography utilized E. Merck silica gel 60 (70–230-mesh ASTM).

CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) and bufotenin were supplied by Serva (Heidelberg, Germany), and serotonin was obtained from Janssen Chimica (Beerse, Belgium). All other drugs were kindly provided by their respective companies of origin.

[^{3}H]Pyrilamine (24.1 Ci/mmol), [^{3}H]dihydroalprenolol (42 Ci/mmol), [^{3}H]clonidine (41.5 Ci/mmol), and [^{3}H]WB-4101 (19.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA), [^{3}H]serotonin (14.4 Ci/mmol) from Amersham (Amersham, UK), and [^{3}H]dexetimide (16 Ci/mmol) and [^{3}H]haloperidol (22 Ci/mmol) from Janssen Life Sciences Products (Beerse, Belgium).

1-Acetyl-4-(2-bromo-4-fluorobenzoyl)piperidine (II). A mixture of 70 g of 1-bromo-3-fluorobenzene (0.4 mol) and 80 g of ground aluminum chloride (0.6 mol) was stirred at 60 °C. Then 61 g of 1-acetyl-4-piperidinecarbonyl chloride I[10] (0.32 mol) was added in small portions over a period of 1 h. The reaction mixture was stirred for another 2 h at 80 °C and cooled to 60 °C whereupon 300 mL of chloroform was introduced. The formed slurry was poured onto well-stirred ice–water. The layers were separated, and the water layer was extracted with chloroform (3×). The organic phases were combined and washed, respectively, with water (3×), a 10% NaOH solution (3×), and water (3×). Drying and evaporation at aspirator pressure gave 70 g of a mixture consisting mainly of isomers II and III; HPLC separation yielded 23 g of TLC and ^{1}H NMR pure II (22%) together with 43 g of TLC and ^{1}H NMR pure isomer III (41%). Compound II: mp 83 °C; ^{1}H NMR ($CDCl_3$) δ 1.59 and 1.73 (qd, 2, $N(CCH_2)_2$; ax), 1.90 and 1.94 (m, 2, $N(CCH_2)_2$; eq), 2.10 (s, 3, $COCH_3$), 2.75 and 3.16 (ddd, 2, $N(CH_2C)_2$; ax), 3.30 (tt, 1, $N(CC)_2CHCO$), 3.87 and 4.52 (m, 2, $N(CH_2C)_2$; eq), 7.11 (ddd, 1, ArH; *o*-F-*p*-Br), 7.34 (dd, 1, ArH; *m*-F-*m*-Br), 7.37 (dd, 1, ArH; *o*-F-*o*-Br). Anal. ($C_{14}H_{15}BrFNO_2$) C, H, N.

Compound III: mp 112 °C; ^{1}H NMR ($CDCl_3$) δ 1.56 and 1.71 (qd, 2, $N(CCH_2)_2$; ax), 1.94 (m, 2, $N(CCH_2)_2$; eq), 2.10 (s, 3, $COCH_3$), 2.80 and 3.19 (ddd, 2, $N(CH_2C)_2$; ax), 3.31 (tt, 1, $N(CC)_2CHCO$), 3.88 and 4.55 (m, 2, $N(CH_2C)_2$; eq), 7.36 (ddd, 1, ArH; *o*-F-*p*-Br), 7.41 (dd, 1, ArH; *p*-F-*o*-Br), 7.68 (t, 1, ArH; *m*-F-*m*-Br). Anal. ($C_{14}H_{15}BrFNO_2$) C, H, N.

4-(2-Bromo-4-fluorobenzoyl)piperidine (IV). A mixture of 7 g of II (0.021 mol) and 30 mL of concentrated hydrochloric acid was stirred and refluxed for 3 h. The solution was cooled, and the formed precipitate was filtered and washed twice with 20 mL of acetone. The residue was recrystallized from 160 mL of 2-propanol–methanol (1:1) to furnish 5.3 g (78%) of pure IV: mp 242 °C; ^{1}H NMR ($CDCl_3$) δ 1.62 (qd, 2, $N(CCH_2)_2$; ax), 1.86 (m, 2, $N(CCH_2)_2$; eq), 2.10 (br s, 1, NH), 2.67 (td, 2, $N(CH_2C)_2$; ax), 3.15 and 3.17 (m, 3, $N(CH_2C)_2$; eq and $N(CC)_2CHCO$), 7.08 (ddd, 1, ArH; *o*-F-*p*-Br), 7.32 (dd, 1, ArH; *m*-F-*m*-Br), 7.35 (dd, 1, ArH; *o*-F-*o*-Br). Anal. ($C_{12}H_{13}BrFNO$·HCl) C, H, N.

7-Amino-3-[2-[4-(2-bromo-4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1*H*,3*H*)-quinazolinedione (VI). A mixture of 370 mg of 7-amino-3-(2-chloroethyl)-2,4(1*H*,3*H*)-quinazolinedione V[11] (1.55 mmol), 484 mg of IV (1.50 mmol), 320 mg of sodium carbonate (3 mmol), and a catalytic amount of potassium iodide in 20 mL of technical methyl isobutyl ketone (MIBK) was stirred and refluxed for 20 h. Hot MIBK was introduced, and the reaction was filtered hot over a sintered glass funnel, washed twice with 15 mL of hot MIBK, and evaporated to leave 890 mg of residue. This was stirred with 5 mL of methanol and filtered in order to remove solid impurities. Evaporation of the filtrate and stirring with 10 mL of MIBK–diisopropyl ether (1:4) for 30 min gave 400 mg of crude solid, which was chromatographed over silica with chloroform–methanol (95:5) as eluent, to yield 180 mg of 83% HPLC pure product. The material was further purified by HPLC (stainless-steel column, 4.6 mm × 30 cm, Hypersil ODS 5 μm), using acetonitrile–water–diisopropylamine (30:70:0.2) as eluate in an isocratic run with a flow rate of 2 mL/min, to furnish 110 mg (15%) of 99.9% HPLC pure VI: mp 229 °C; ^{1}H NMR ($CDCl_3$) δ 1.75 and 1.83 (m, 4, $N(CCH_2)_2$; ax and eq), 2.15 (td, 2, $N(CH_2C)_2$; ax), 2.65 (t, 2, CH_2N–piper.), 3.02 (tt, 1, $N(CC_2)CHCO$), 3.07 (m, 2, $N(CH_2C)_2$; eq), 4.15 (t, 2, CH_2CN–piper.), 5.40 (br s, 2, NH_2), 6.14 (d, 1, ArH; *o*-NH_2-*o*-NH–quin.), 6.47 (dd, 1, ArH; *o*-NH_2-*p*-NH–quin.), 7.07 (td, 1, ArH; *o*-F-*p*-Br), 7.28 (dd, 1, ArH; *m*-F-*m*-Br), 7.35 (dd, 1, ArH; *o*-F-*o*-Br), 7.87 (d, 1, ArH; *m*-NH_2-*m*-NH–quin.), 8.70 (br s, 1, NHCO). Anal. ($C_{22}H_{22}BrFN_4O_3$) C, H, N.

7-Amino-3-[2-[4-(2-tritio-4-fluorobenzoyl)-1-piperidinyl]ethyl]-2,4(1*H*,3*H*)-quinazolinedione (VII). To a solution of 50 mg of VI (0.10 mmol) in $2^1/_2$ mL of tetrahydrofuran were successively added 50 mg of Pd-10% on charcoal, 100 mg of powdered calcium oxide, and 0.01 mL of a 3% thiophene solution in tetrahydrofuran. Tritiation (I.R.E., Fleurus, Belgium) of VI was performed at room temperature for 18 h, using approximately 30 Ci of tritium gas. The excess of tritium was adsorbed on active charcoal. The catalyst was removed by millipore filtration, and the filtrate was lyophilized twice with methanol to remove labile tritium. The residue was purified by HPLC (stainless-steel column, 4.6 mm × 30 cm, Hypersil ODS 5 μm; isocratic run with acetonitrile–water–diisopropylamine (28:72:0.2) and a flow rate of 3 mL/min) to afford 7.38 mg of VII[11] (18%) with a total radioactivity of 223 mCi, a specific activity of 12.54 Ci/mmol, and a 99.9% HPLC purity.

Tissue Preparation. Wistar rats and Pirbright guinea pigs were killed by decapitation, and their brains were removed from their skulls. Different brain regions were rapidly dissected and immediately homogenized either in buffer to prepare a total particulate membrane preparation or in 0.25 M sucrose for the preparation of a (M + L + P) (total heavy (M) and light (L) mitochondrial and microsomal (P)) or (P) fraction following procedures previously described.[8,12]

Solubilization Procedure. Serotonin-S_2 receptor from rat frontal cortex were solubilized with a mixture of CHAPS and sodium chloride as previously described.[9] In short, an aliquot of a microsomal (P) fraction (dilution 1:2 w/v) was incubated with an equal volume of solubilization medium (20 mM sodium phosphate pH 7.2, 0.5 M sucrose, 2 mM EDTA, 13.6 mM CHAPS, and 2.8 M sodium chloride) during 12 min at 4 °C. Then, the solubilization mixture was diluted 2-fold with buffer (10 mM sodium phosphate pH 7.2, 0.25 M sucrose, and 1 mM EDTA) and centrifuged at 180000*g* (r_{av}) for 60 min in a Beckman SW65 Ti rotor. The supernatant was diluted 2-fold with water (corresponding to a final dilution of 1:16 w/v) and used as such in the binding assay.

Binding Assays. (a) Membrane Preparations. Binding of [^{3}H]-7-aminoketanserin was assayed in 50 mM Tris-HCl, pH 7.7. Two milliliter aliquots of an (M + L + P) fraction (dilution 1:100 w/v) of rat frontal cortex were incubated for 15 min at 37 °C with [^{3}H]-7-aminoketanserin and unlabeled drugs or their solvent in a final volume of 2.2 mL. Incubation was stopped by rapid filtration through Whatman GF/B glass fiber filters using a Multividor 40-well filtration manifold (Janssen Scientific Instruments Division, Beerse, Belgium). Filters were rinsed twice with ice-cold buffer and transferred to counting vials. Eight milliliters of Insta-Gel II was added, and the vials were counted for radioactivity.

Binding of [^{3}H]ketanserin to serotonin-S_2 receptors and radioligand binding to serotonin-S_1 sites, α_1-adrenergic, α_2-adrenergic, β-adrenergic, dopamine-D_2, histamine-H_1, opiate, and muscarinic-acetylcholine receptors were performed according to the same technique. Brain tissues, buffers, ligands, and incubation conditions for these assays were as previously described.[7,8,13]

(b) Soluble Preparations. Aliquots (0.4 mL) of the soluble preparation were incubated for 15 min at 30 °C with [^{3}H]-7-aminoketanserin in the presence of various unlabeled drugs or their solvent in a final volume of 0.5 mL. Bound and free radioactivity were separated by the addition of 50 μL of an activated charcoal suspension (10% charcoal and 2% bovine serum albumin in water) followed by centrifugation at 14000 rpm for 3 min. Aliquots (200 μL) of the supernatant were transferred to counting vials, and radioactivity was measured as described above.

Mathematical Analysis. Fitting of Scatchard plots and calculation of equilibrium dissociation constants (K_D) and maximal number of binding sites (B_{max}) were done by using a computerized

(12) Laduron, P. M. *Int. Rev. Neurobiol.* **1977**, *30*, 251.
(13) Leysen, J. E.; Gommeren, W.; Niemegeers, C. J. E. *Eur. J. Pharmacol.* **1983**, *87*, 209.

least-squares fitting program. K_i values were calculated from IC_{50} values according to the equation of Cheng and Prusoff:[14] $K_i = IC_{50}/(1 + (C/K_D))$, where C is the concentration and K_D the equilibrium dissociation constant of the radioligand.

Association velocity constants were derived from the slope of a plot according to $\ln [B_{max}(L_0 - B_t)/L_0(B_{max} - B_t)] = k_1[1/(L_0 - B_{max})]t$, where L_0 is the radioligand concentration, B_{max} the maximal stereospecific binding, and B_t the stereospecific binding at time t.[15] Dissociation velocity constants were derived according to the equation $\ln B_t = \ln (B_{max} - k_{-1}t)$.

P_{app}, the octanol/water partition coefficient at any given pH, was calculated from $P_{app} = P/(1 + 10^{pK_a - pH})$, where P is the partition coefficient of the uncharged molecule and pK_a the acid dissociation constant.

Acknowledgment. We gratefully acknowledge W. Verluyten and L. Roevens for HPLC separations and analyses, Dr. J. Bracke for NMR spectroscopy, and Dr. R. Crauwels for physicochemical determinations. We also thank D. Verkuringen for typing the manuscript. Part of this work was supported by a grant from IWONL.

Registry No. I, 59084-16-1; II, 102745-97-1; III, 98295-50-2; IV, 102779-93-1; V, 101389-50-8; VI, 102745-98-2; VII, 102745-99-3; 1-bromo-3-fluorobenzene, 1073-06-9.

(14) Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.
(15) Frost, P. A.; Pearson, R. G. *Kinetics and Mechanism. A Study of Homogeneous Chemical Reactions*, 2nd ed.; Wiley: New York, 1961.

Influence of Alkyl Chain Ramification on Estradiol Receptor Binding Affinity and Intrinsic Activity of 1,2-Dialkylated 1,2-Bis(4- or 3-hydroxyphenyl)ethane Estrogens and Antiestrogens

Rolf W. Hartmann

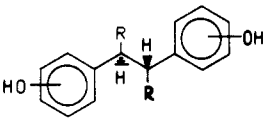
Sonderforschungsbereich 234, Institute of Pharmacy, Lehrstuhl Pharmazeutische Chemie II, University of Regensburg, 8400 Regensburg, Federal Republic of Germany. *Received December 3, 1985*

The influence of a symmetrical introduction of CH_3 substitutents in the α or β positions of the 1,2-dialkyl-1,2-bis(4-hydroxyphenyl)ethane estrogens hexestrol (ethyl, HES) and octestrol (*n*-propyl, OCES) [isopropyl (**1**), *tert*-butyl (**2**), *sec*-butyl (**3**), isobutyl (**4**)] and the 1,2-dialkyl-1,2-bis(3-hydroxyphenyl)ethane antiestrogens metahexestrol (ethyl, MetaHES) and metaoctestrol (*n*-propyl, MetaOCES) [isopropyl (**5**), *tert*-butyl (**6**), *sec*-butyl (**7**), isobutyl (**8**)] on estradiol receptor (E_2R) binding affinity and intrinsic activity is described. The synthesis of compounds 1–8 was accomplished by reductive coupling of (a) the corresponding α-alkylbenzyl alcohols with $TiCl_3/LiAlH_4$ and separation of the meso diastereoisomers (compounds **2–4**, **7**, and **8**), (b) α-*tert*-butyl-3-methoxybenzyl chloride with $CoCl_2$/EtMgCl and isolation of the meso configurated isomer (**6**), and (c) the isopropyl phenyl ketones with $TiCl_4$/Zn and subsequent hydrogenation of the corresponding *cis*-hex-3-enes (compounds 1 and **5**). The binding affinity of 1–8 to the calf uterine E_2R was measured relative to that of [^{3}H]E_2 by a competitive binding assay. Compounds 1 and **5** showed relative binding affinity (RBA) values exceeding those of HES and MetaHES, respectively. All other derivatives showed RBA values smaller than the corresponding parent compounds. The intrinsic activity was monitored in terms of uterotrophic and antiuterotrophic activity. It is striking that the introduction of a CH_3 group in the α positions of MetaHES and MetaOCES led to compounds with full intrinsic activity (**5–7**), i.e., estrogens without antiestrogenic properties. No correlation between E_2R binding affinity and intrinsic activity was found.

We have recently shown that displacement of the phenolic hydroxy groups of the synthetic estrogen hexestrol (HES, Chart I) to the 3,3′ positions only led to a moderate reduction of the binding affinity for the estradiol receptor (E_2R).[1,2] Interestingly metahexestrol [*meso*-3,4-bis(3-hydroxyphenyl)hexane, MetaHES] turned out to be a partial antiestrogen, i.e., it exhibited strongly decreased uterotrophic properties compared to HES and showed a strong inhibition of the estrone-stimulated uterine growth of the immature mouse.[1-3] This compound is of great interest for the treatment of hormone-dependent human breast cancer, since it shows strong inhibitory activity on several rodent mammary tumors.[1-3]

Structural modifications of MetaHES have been performed in order to increase the binding affinity for the E_2R and thus enhance tumor inhibiting properties,[1,4,5] since antitumor activity appears to be correlated with the E_2R binding affinity.[1] Symmetrical substitution in the 4,4′, 5,5′

Chart I



4 OH compd	R	3 OH compd
HES	CH_2CH_3	Meta HES
1	$CHCH_3$	5
2	CCH_3	6
OCES	$CH_2CH_2CH_3$	Meta OCES
3	$CHCH_2CH_3$	7
4	CH_2CHCH_3	8

(1) Hartmann, R. W.; Buchborn, H.; Kranzfelder, G.; Schönenberger, H. *J. Med. Chem.* **1981**, *24*, 1192.
(2) Kranzfelder, G.; Hartmann, R. W.; von Angerer, E.; Schönenberger, H.; Bogden, A. E. *J. Cancer Res. Clin. Oncol.* **1982**, *103*, 165.
(3) For recent reviews of the pharmacology of metahexestrol, see: (a) Engel, J.; Hartmann, R. W.; Schönenberger, H. *Drugs Future* **1983**, *8*, 413. (b) Hartmann, R. W. *Cancer Treat. Rev. (Suppl. A)* **1984**, *11*, 155.
(4) Hartmann, R. W.; Heindl, A.; Schönenberger, H. *J. Med. Chem.* **1984**, *27*, 577.

0022-2623/86/1829-1668$01.50/0