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Etoxadrol-*meta*-isothiocyanate: a potent, enantioselective, electrophilic affinity ligand for the phencyclidine-binding site

Andrew Thurkauf, Mariena V. Mattson, Philip N. Huguenin*, Kenner C. Rice and Arthur E. Jacobson

Section on Drug Design and Synthesis, Laboratory of Neuroscience, National Institute of Diabetes, Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

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Etoxadrol-*meta*-isothiocyanate (2*S*,4*S*,6*S*-2-ethyl-2-(3-isothiocyanatophenyl)-2-piperidyl)1,3-dioxolane, **4a**) has been synthesized and characterized as an irreversible ligand for the phencyclidine (PCP)-binding site. It is the first chiral electrophilic affinity ligand for this site to have been described. This affinity ligand is based upon etoxadrol, a 1,3-dioxolane known to have PCP-like effects in vivo and in vitro. Etoxadrol-*meta*-isothiocyanate was found to be four–five times more potent in vitro than metaphit (1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine), the only previously known electrophilic affinity ligand for the PCP-binding site. The binding was shown to be highly enantioselective for etoxadrol-*meta*-isothiocyanate (**4a**). The 2*R*,4*R*,6*R*-enantiomer of **4a** was essentially inactive. The ability of the 2*S*,4*S*,6*S*-enantiomer (**4a**) to interact with the benzodiazepine, muscarinic, and mu opioid receptor systems was also examined, and it was found not to interact with these receptor systems. It seems likely that **4a** will prove to be a valuable tool in the study of structure and function of the PCP-binding site.

Phencyclidine-binding site; Etoxadrol-*meta*-isothiocyanate; Etoxadrol; 1,3-Dioxolane; Electrophilic affinity ligand; Enantioselective irreversible ligand

1. INTRODUCTION

The acylating agent metaphit (1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine, **1**, fig.1) [1] has been utilized with great success as a tool for the characterization of the PCP-binding site. The encouraging results obtained from the many studies [2–11] involving this agent have initiated efforts in our laboratory toward the synthesis and biochemical characterization of more potent affinity ligands related to structurally diverse PCP agonists. The dissociative anesthetics dexoxadrol (4*S*,6*S*-2,2-diphenyl-4-(2-piperidyl)-1,3-dioxolane, **2**) and etoxadrol (2*S*,4*S*,6*S*-2-ethyl-2-phenyl-4-(2-piperidyl)-1,3-dioxolane, **3**) have been shown to competitively inhibit the binding of [^{3}H]TCP to stereoselective, saturable-binding sites in rat brain [12]. The availability of an electrophilic affinity ligand structurally related to these dioxolanes could serve to complement metaphit in further studies of this binding site. This goal has been made more pertinent by recent studies which indicate that some heterogeneity may exist between the binding sites of these two classes of compounds [13]. We now report the synthesis and characterization of an electrophilic affinity ligand, 2*S*,4*S*,6*S*-2-ethyl-2-(3-isothiocyanatophenyl)-4-(2-piperidyl)-1,3-dioxolane (etoxadrol-*meta*-isothiocyanate, **4a**), which is stereochemically and struc-

Correspondence address: A.E. Jacobson, Building 8, Room B1-22, Section on Drug Design and Synthesis, LN, NIDDK, NIH, Bethesda, MD 20892, USA

* *Present address:* F. Hoffmann-La Roche, ZFE, Isotopenlabor, Grenzacherstr., 4002 Basel, Switzerland

Abbreviations: etoxadrol-*meta*-isothiocyanate, 2*S*,4*S*,6*S*-2-ethyl-2-(3-isothiocyanatophenyl)-2-piperidyl)-1,3-dioxolane; metaphit, 1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine; PCP, phencyclidine

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turally related to etoxadrol, shows good affinity for the [^{3}H]TCP-binding site in rat brain homogenates, and which is enantioselective. The enantiomeric 2*R*,4*R*,6*R*-etoxadrol-*meta*-isothiocyanate does not acylate nor have affinity for the PCP-binding site, except at very high concentrations.

2. MATERIALS AND METHODS

2.1. *Chemical synthesis*

The synthesis of 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate (**4a**) is shown in fig.1. The 2*S*,3*S*-2-piperidyl-1,2-ethanediol was obtained by the hydrolysis of dexoxadrol as previously described [14]. Synthesis of the (+)-2*R*,4*R*,6*R*-enantiomer was carried out in a similar fashion using 2*R*,3*R*-2-piperidyl-1,2-ethanediol [14] as the starting material. Spectra from nuclear magnetic resonance (Varian XL300 spectrometer), infrared (Beckmann 3001 instrument), and chemical ionization (CI) mass spectra (Finnegan Mat-311 spectrometer) were in accord with the assigned structures. Gas chromatographic (GC) analysis utilized a Hewlett-Packard 5880A instrument with a carbowax capillary column and flame ionization detector. Melting points were obtained using a Thomas-Hoover Unimelt apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on Analtech silica gel GF plates eluted with a mixture of chloroform/methanol/conc. ammonium hydroxide (90:9:1) and visualized by iodine staining. All new compounds gave combustion analyses for carbon, hydrogen and nitrogen within ± 0.4% of the calculated value and were performed by Atlantic Microlabs, Atlanta, GA.

2.2. *2-(3-Nitrophenyl)-2-ethyl-4-(2-piperidyl)-1,3-dioxolanes (**5a,b**)*

A solution of 2*S*,3*S*-2-piperidyl-1,2-ethanediol (1.19 g, 8.21 mmol) and *p*-toluenesulfonic acid (1.71 g, 9.03 mmol) in 5 ml of acetonitrile was evaporated to dryness. The residue was dissolved in nitromethane and extracted (Soxhlet extractor containing neutral active alumina (2 g) in the stock; the mixture was refluxed; and *m*-nitropropiophenone dimethyl acetal (2.03 g, 9.0 mmol) [15] was then added in one portion). After 1 h reaction time, the cooled (25°C) reaction mixture was separated by extraction with a mixture of ether (40 ml) and 1.0 N sodium hydroxide solution (20 ml). The crude material from the organic layer, after drying ($MgSO_4$) and concentration, was purified by silica gel chromatography (90% $CHCl_3$:9% MeOH:1% NH_4OH) to give 2.05 g (81%) of a mixture of the diastereometric ketals **5a,b**. TLC R_f 0.54 and 0.50, m.p. 176–178°C (HCl salt).

2.3. *2-(3-Nitrophenyl)-2-ethyl-4-(1-(t-butoxy carbonyl)-2-piperidyl)-1,3-dioxolanes (**6a,b**)*

To a solution of **5a,b** (1.1 g, 3.6 mmol) in pentene-stabilized chloroform (15 ml) was added di-*t*-butyl dicarbonate (0.942 g, 1.2 equivalents). A second layer of 2.90 N sodium bicarbonate was added and the solution stirred overnight. The chloroform layer was removed, dried ($MgSO_4$), and concentrated to give 1.42 g (97%) of a mixture of isomers. GC of the mixture indicated a 65:35 ratio of 2*S*:2*R* isomers, **6a** and **6b**, respectively. A portion of the diastereometric mixture was separated by flash chromatography (88% $CHCl_3$:11% MeOH:1% NH_4OH). The diastereometric configuration at the C-2 carbon of **6a** and **6b** was assigned by comparison of their NMR spectra with the NMR spectra of etoxadrol and epietoxadrol [16]. Exact mass calculated for $C_{21}H_{30}N_2O_6$ was 406.2104 (found, 406.2111).

2.4. *2-(3-Aminophenyl)-2-ethyl-4-(1-(t-butoxycarbonyl)-2-piperidyl)-1,3-dioxolanes (**7a,b**)*

A solution of **6a** and **6b** (980 mg) in methanol (20 ml) was hydrogenated over 60 mg of 10% Pd on carbon at 3 atmospheres pressure for 5 h. After filtration of the catalyst, the amine products (**7a,b**) were obtained (99%) by concentration of the reaction mixture. Analysis by GC indicated no change in the ratio of isomers during the hydrogenation. Exact mass calculated for $C_{21}H_{32}N_2O_4$ was 376.2362 (found, 376.2384).

2.5. *2-(3-Isothiocyanatophenyl)-2-ethyl-4-(1-t-butoxycarbonyl)-2-piperidyl)-1,3-dioxolanes (**8a,b**)*

Thiophosgene in pentene-stabilized chloroform (0.71 ml of a 2.78 N solution) was added to a two-phase mixture of a solution of **7a,b** (739 mg, 1.96 mmol) in 4 ml of pentene-stabilized chloroform and a saturated sodium bicarbonate solution (4 ml). After 20 min the organic layer was removed by pipette and dried by passage through a short packed column of anhydrous $MgSO_4$. Concentration of the resulting filtrate gave a mixture of isomeric isothiocyanates (**8a,b**), m.p. 63–66°C, which could be separated by column chromatography. However, attempts to deprotect the individual isothiocyanate diastereomers **8a** and **8b** resulted in the scrambling of the stereochemistry at C-2. Thus, the isomeric mixture **8a,b** was subjected to the deprotection conditions and the desired product **4a** was separated from its epimer **4b** by recrystallization (see below).

2.6. *2S,4S,6S-2-(3-Isothiocyanatophenyl)-2-ethyl-4-(2-piperidyl)-1,3-dioxolane (**4a**)*

The *t*-BOC protected isothiocyanate **8a,b** (625 mg, 1.5 mmol) was dissolved in dry diethyl ether (10 ml) in a small pressure bottle. A dry solution of diethyl ether saturated with HCl gas (3 ml) was added and the bottle sealed and brought to 40°C using a warm sand bath. The deprotected product **4a** separated as its hydrochloride salt in about 20 min. The solvent was decanted and the residual semicrystalline product was rinsed twice with dry ether. The salt (a 65:35 mixture of **4a** and its C-2 epimer **4b**) was dissolved in hot ethyl acetate and crystallized by careful trituration with isooctane to give a 92:8 mixture of **4a** and **4b**. The mixture was recrystallized twice from ethyl acetate and triturated with isooctane to give a 98:2 mixture of **4a** and **4b**. Further recrystallization (2 × from ethyl acetate) gave pure **4a**. HCl, m.p. 184–185°C; $[\alpha]_{23}^{D}$ −5.2° (*c* 0.34, $CHCl_3$). Attempts to prepare alternate salts through the free base resulted in varying degrees of polymerization with resultant oiling out of the material. Although improved overall yields of the product could be obtained by initial deprotection as the hydrobromide, the resultant salt proved to be less amenable to isomeric purification through recrystallization. In addition, the hydrobromide salt was considerably less water soluble.

2.7. *Assignment of configuration of* **4a** *and* **4b**

The absolute configuration at C-2 was assigned spec-

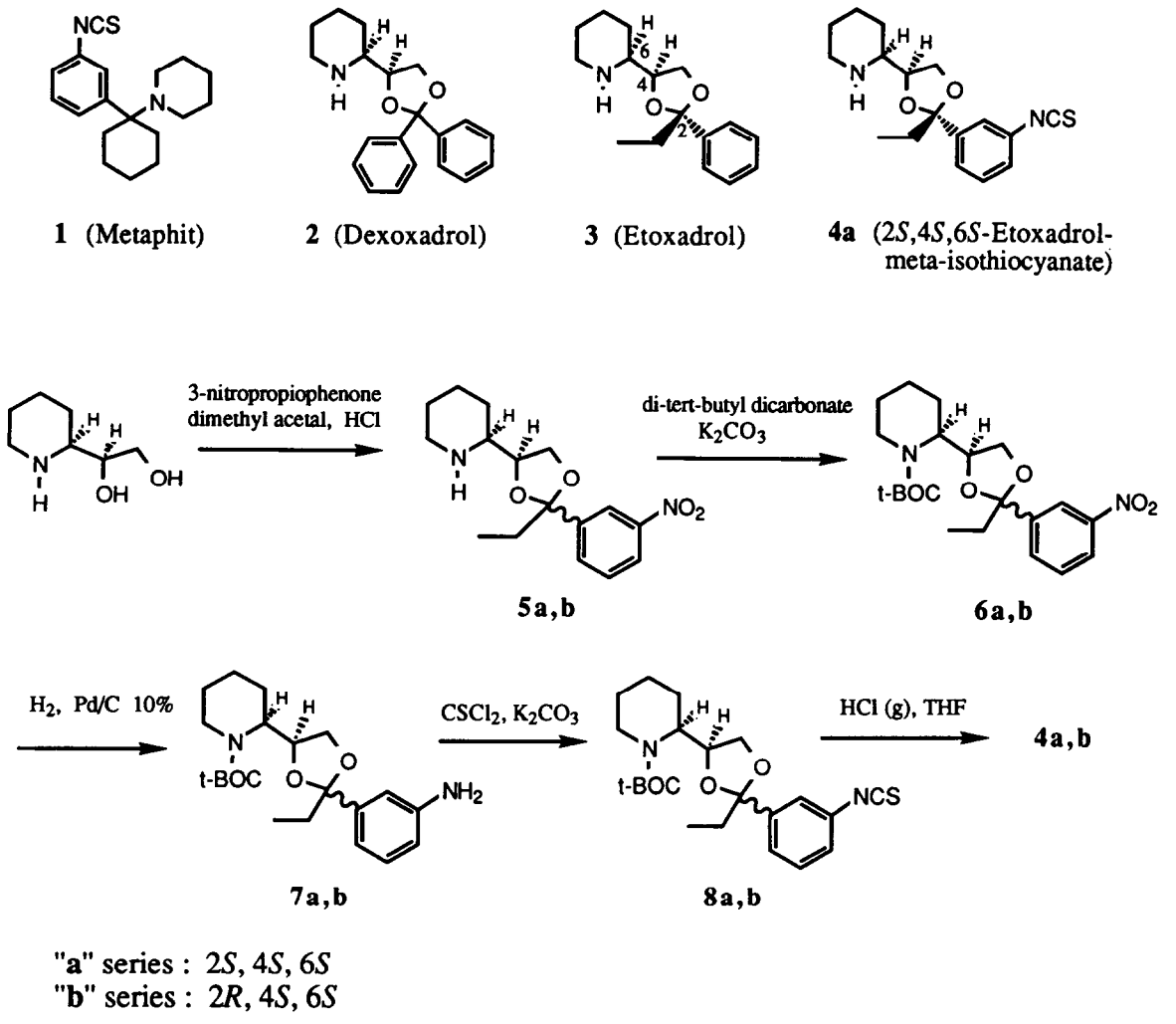

Fig.1. Structures of compounds and reaction sequence.

troscopically by analogy to etoxadrol (**3**). Examination of nuclear magnetic resonance spectra show the C-4 proton of etoxadrol to be deshielded relative to the corresponding C-4 resonance for its C-2 epimer epietoxadrol (3.79 vs 4.03 ppm). A similar effect was seen for **4a** in relation to its C-2 epimer **4b** (4.30 vs 4.42 ppm). In addition, similar isomeric ratios and relative GC retention times for **4a**/**4b** and etoxadrol/epietoxadrol provide further indication that **4a** and etoxadrol possess identical C-2 stereochemistry.

2.8. *2R,4R,6R-2-(3-Isothiocyanatophenyl)-2-ethyl-4-(2-piperidyl)-1,3-dioxolane)*

The dextrorotatory 2*R*,4*R*,6*R*-enantiomer of **4a** was prepared from 2*R*,3*R*-2-piperidyl-1,2-ethanediol by the same series of reactions which gave **4a**. Spectroscopic data (infrared and nuclear magnetic resonance) for the intermediate products leading to the dextrorotatory 2*R*,4*R*,6*R*-enantiomer of **4a** were analogous to those obtained in the epimeric series. The hydrochloride salt of the dextrorotatory 2*R*,4*R*,6*R*-enantiomer of **4a** was recrystallized from tetrahydrofuran, m.p. 184–185°C, $[\alpha]_{23}^{D}$ +4.2° (*c* 0.64, $CHCl_3$).

2.9. *Binding assays*

Competitive binding experiments were performed as previously described [17] except that a tissue homogenate preparation of fresh whole rat brain minus cerebellum was used. Incubation was carried out at 5°C with [^{3}H]TCP as the radioligand. Rapid filtration was carried out through filters presoaked in 0.03% polylysine. Experiments were performed in triplicate, and 10 μM TCP was used for the determination of nonspecific binding.

Irreversible binding experiments were performed using fresh tissue homogenates. The homogenates were incubated with **4a** or the dextrorotatory 2*R*,4*R*,6*R*-enantiomer of **4a** for 30 min at 5°C and extensively washed to remove unreacted material. This wash procedure was found to be sufficient to remove a 10 μM

concentration of etoxadrol and a 50 μM concentration of the dextrorotatory 2*R*,4*R*,6*R*-enantiomer of **4a**, without loss of binding. Data from these experiments were analyzed using GraphPAD software [18]. Binding methodologies for benzodiazepine, muscarinic, and mu opioid receptors were taken from the literature [19–21].

3. RESULTS

Examination of the results of the displacement of [^{3}H]TCP from untreated control tissue and tissue pretreated with 1 μM 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate (**4a**) revealed a loss of about 50% of the total binding capacity in the treated tissue. Complete acylation could be obtained at a 20 μM concentration. A Scatchard plot of the data (fig.2) shows that the loss of binding sites occurs without changing the relative affinity of the remaining sites (K_d for control, 19.2×10^{-9} M; K_d for treated, 21.7×10^{-9} M; B_{max} for control, 1.36 pmol/mg protein; B_{max} for treated, 0.66 pmol/mg protein). The ligand displays an apparent IC_{50} of 590 nM. In contrast, the dextrorotatory 2*R*,4*R*,6*R*-enantiomer of **4a** showed an apparent IC_{50} of 51 μM and, up to a 50 μM concentration, did not irreversibly interact with the PCP site under conditions known to be effective for acylation by the active enantiomer **4a**. An examination of the time requirements for acylation by **4a** revealed that less than 5 min was required to acylate all of the available sites, using a 1 μM concentration. Longer exposure time (to 60 min) showed no appreciable change in total irreversible binding. The stability of 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate (**4a**) in aqueous solution was examined by gas chromatographic analysis and ultraviolet spectroscopy. No detectable decomposition of etoxadrol-*meta*-isothiocyanate hydrochloride could be observed after 24 h which was typical of aromatic isothiocyanates, which are known to react slowly with hydroxyl groups and much more rapidly with amines or thiol moieties.

Table 1

Ability of 1 μM 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate (**4a**) to reversibly and irreversibly antagonize binding of various receptor ligands

Ligand	Maximum % acylation by 1 μM of **4a**	IC_{50} (μM) for **4a**	IC_{50} (μM) for TCP
[^{3}H]TCP[a]	50	0.77	0.02
[^{3}H]QNB[b]	0	3.6	40.3
[^{3}H]Flunitrazepam	0	>100	>100
[^{3}H]FOXY[c]	0	18	2.0

[a] ^{3}H-1-(1-[2-Thienyl]cyclohexyl)piperidine
[b] ^{3}H-3-Quinuclidinyl benzilate
[c] ^{3}H-3,14-Dihydroxy-4,5-epoxy-6β-fluoro-17-methylmorphinan

The ligand concentrations used were: 1.8 nM [^{3}H]TCP, 0.65 nM [^{3}H]QNB, 0.87 nM [^{3}H]flunitrazepam and 1.6 nM [^{3}H]FOXY. The binding methodology for TCP was modified from [17], using a tissue homogenate preparation of fresh whole rat brain minus cerebellum, and the others were from the literature [19–21]. Acylation by 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate refers to the % acylation in washed, treated tissue compared with similarly washed controls. IC_{50} values for 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate were estimated from competitive displacement curves

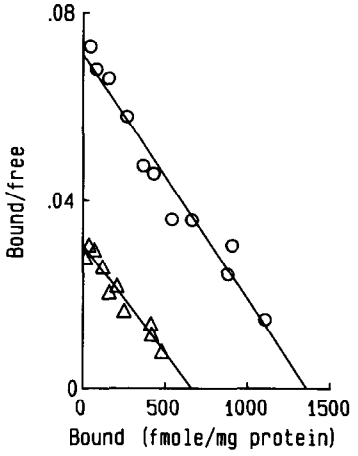

Fig.2. Scatchard plots of [^{3}H]TCP binding in the presence (△) or absence (○) of 1 μM 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate.

2*S*,4*S*,6*S*-Etoxadrol-*meta*-isothiocyanate (**4a**) showed no affinity for the benzodiazepine or muscarinic receptor systems in competitive binding experiments using [^{3}H]flunitrazepam and [^{3}H]-QNB, respectively, as the competitive ligands. Etoxadrol-*meta*-isothiocyanate was found to weakly displace [^{3}H]FOXY from mu opioid receptors (IC_{50} = 25 μM) but did not irreversibly inactivate these receptors.

4. DISCUSSION

2*S*,4*S*,6*S*-Etoxadrol-*meta*-isothiocyanate shows

an in vitro pharmacological profile similar to metaphit in that it will irreversibly bind to [^{3}H]TCP sites in brain tissues and is specific for these sites. However, it is more potent than metaphit in its affinity to the PCP-binding site (apparent K_i of 590 nM and 2800 nM, respectively). Both the reversible and irreversible binding of etoxadrol-*meta*-isothiocyanate to the PCP-binding site are enantioselective; the 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate is the first example of an enantioselective electrophilic affinity ligand for the PCP site.

Recently, a report by Domino et al. [22] casts doubt on the use of metaphit as a valid affinity ligand for the PCP-binding site. They noted that the in vivo (intracerebroventricular administration in mice) actions of phenylisothiocyanate, an organic isothiocyanate which should be indiscriminate in its acylation of amines or thiol-containing amino acids, were similar to those found with metaphit (i.e., there were similar acute and delayed effects). While the enantioselective acylating properties of 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate do not conclusively contradict the in vivo work with metaphit by Domino et al., the enantioselectivity which we have found lends credence to the idea that the PCP site is not available for irreversible binding, at least in vitro, by reasonable concentrations of a potentially competing ligand possessing a similar acylating functionality. It is, of course, conceivable that introduction of a sufficiently large concentration of any electrophilic acylation agent will nonspecifically interact with all proteins containing thiols or amino functions, in vivo or in vitro.

The fact that a wide variety of structural classes has been shown to interact with the PCP-binding site has both facilitated and complicated the study of the binding site. In particular, the diversity of structures has made the identification of structural determinants for binding more complicated. The availability of new affinity ligands, like 2*S*,4*S*,6*S*-etoxadrol-*meta*-isothiocyanate, structurally based on different PCP agonist structural classes should aid in this area and thus, indirectly, in the elucidation of the nature of the binding site surface.

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