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Synthesis and evaluation of optically pure [^{3}H]-(+)-pentazocine, a highly potent and selective radioligand for σ receptors

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Tritium-labeled (+)-pentazocine ([^{3}H]-**1b**) of specific activity 26.6 Ci/mmol was synthesized in 3 steps starting with (+)-normetazocine (**2**) of defined optical purity. [^{3}H]-**1b** has been characterized as a highly selective ligand for labeling of σ receptors. Competition data revealed that [^{3}H]-**1b** could be displaced from guinea pig brain membrane preparations with a number of commonly used σ receptor ligands. [^{3}H]-**1b** exhibited saturable, enantioselective binding with a K_d of 5.13 ± 0.97 nM and a B_{max} of 1146 ± 122 fmol/mg protein. Phencyclidine (PCP) displaced [^{3}H]-**1b** with low affinity while MK-801 was inactive, thus indicating insignificant activity at the PCP-binding site; apomorphine failed to displace [^{3}H]-**1b** indicating lack of dopamine receptor cross-reactivity. Since the affinity of [^{3}H]-**1b** is about 6 times that of the two commonly employed σ ligands ((+)-3-[^{3}H]PPP and [^{3}H]DTG) and since it is more selective for σ receptors than the benzomorphan [^{3}H]SKF-10,047, it represents the first example of a highly selective benzomorphan based σ receptor ligand. [^{3}H]-**1b** should prove useful for further study of the structure and function of σ receptors.

Pentazocine, (+)-[^{3}H]-; Receptor, σ; Scatchard analysis; Radiolabeling

1. INTRODUCTION

Numerous attempts have been made to identify a potent analgesic without the addiction liability and side effects associated with the use of morphine and other classical narcotics. Highly significant advances have been made in this area with the introduction of the agonist-antagonist drugs buprenorphine (Buprenex), nalbuphine (Nubaine) and butorphanol (Stadol). Early work in this area following the introduction of the 6,7-benzomorphans as analgesics by May and his associates [1] resulted in evaluation of a series of racemic *N*-alkyl substituted 2-hydroxy-5,9-dimethyl-6,7-benzomorphans of which the 3,3-dimethylallyl derivative [(±)-pentazocine, Talwin] [2] emerged as the first clinically useful narcotic agonist-antagonist. Initial studies showed the analgesic activity to reside predominantly in the (−)-enantiomer, **1a** (fig.1) [3], as the (+)-isomer, **1b**, was found to be 1/20 as potent as **1a** [3].

Later investigations revealed that (+)-pentazocine (**1b**) is a highly potent and selective ligand for σ receptors [4,5]. σ receptors bind certain antipsychotic drugs, psychotomimetic (+)-opiates, and phencyclidine (PCP) [6]. However, σ sites are pharmacologically distinct from either dopamine, opiate, or PCP receptors. Studies both in vivo and in vitro have demonstrated that σ sites are functional. They have been implicated in control of motor behavior [7], regulation of smooth muscle contraction [8,9], regulation of neurotransmitter release [9], and modulation of the phosphoinositide second messenger system [10]. σ sites have also been implicated in the motor disorder, dysto-

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nia [7,11]. It is also possible that these sites mediate at least some of the psychotomimetic actions of benzomorphans such as (+)-*N*-allylnormetazocine, and PCP related compounds, as well as the antipsychotic effects of drugs such as haloperidol.

In order to study the binding characteristics of σ sites in vivo and in vitro, it is necessary to have radiolabeled probes with high affinity and selectivity for the receptor. At present, there are only four available radiolabeled probes for σ receptors. These are [^{3}H]haloperidol [12], (+)-3-[^{3}H]-(3-hydroxyphenyl)-*N*-(1-propyl)piperidine ((+)-3-[^{3}H]-PPP) [12], 1,3-[^{3}H]di-(*o*-tolyl)guanidine ([^{3}H]-DTG) [13], and (+)-*N*-[^{3}H]allylnormetazocine ((+)-[^{3}H]SKF-10,047 [4]). Two of these, [^{3}H]haloperidol and (+)-[^{3}H]SKF-10,047, are limited by their substantial cross-reactivity with other receptors. We thus wished to synthesize optically pure (+)-[^{3}H]pentazocine ([^{3}H]-**1b**) (fig.1) for evaluation as a σ ligand and now report this compound is a highly potent and selective drug which promises to be an extremely valuable σ receptor probe. The requirement of optically pure ligands for most meaningful drug receptor studies has been discussed earlier and is well known [14].

2. MATERIALS AND METHODS

2.1. *Chemical synthesis*

Spectra from nuclear magnetic resonance (Varian XL300 spectrometer), infrared (Beckman 3001 instrument), and chemical ionization (CI) mass spectra (Finnegan Mat-311 spectrometer) were in accord with the assigned structures. Gas chromatographic analysis (GC) was performed on a Hewlett-Packard 5880A instrument with a carbowax capillary column and a flame ionization detector. Melting points were obtained using a Thomas-Hoover Unimelt apparatus, and are uncorrected. Specific rotation values were recorded at the sodium-D line using a Perkin-Elmer 241 MC polarimeter. Thin layer chromatographic analysis (TLC) was performed on Analtech silica gel (GHF) plates. Solvent system A refers to concentrated aqueous NH_3-MeOH-$CHCl_3$ (1:9:90); solvent system B refers to concentrated aqueous NH_3-MeOH-$CHCl_3$ (2:18:80). All new compounds gave combustion analyses for carbon, hydrogen and nitrogen within ± 0.4% of the calculated value and were performed at Atlanta Microlabs, Atlanta, GA, USA. All tritium-labeled compounds co-migrated with their unlabeled counterparts on TLC as above; their radiochemical purity was assessed using a Berthold model LB2760 TLC scanner. Specific activity measurements for radiolabeled compounds were determined by ultraviolet (UV) analysis with a Hewlett-Packard HP 8450A UV/VIS spectrophotometer.

2.2. *Optically pure (+)-cis-2'-hydroxy-5,9-dimethyl-6,7-benzomorphan [(+)-2], [(+)-normetazocine)]*

In a modification of the procedure of Tullar et al. [3], racemic **2** [1] (20.0 g, 0.092 mol) was dissolved in 240 ml of distilled water containing 6.92 g (0.046 mol) of (−)-tartaric acid. After 1 h, a white precipitate had formed which was filtered and washed with a small amount of cold water. The material was recrystallized twice from water and dried in vacuo at 60°C overnight to give 11.42 g of (+)-**2**·(−)-tartrate, m.p. 306–307°C. Reported [3] m.p. 305–308°C.

The base was regenerated and the optical purity determined as follows: a small portion of the salt (1.0 g) was placed in a 50 ml flask with $CHCl_3$ (15 ml), 3 ml water and 3 ml conc. NH_4OH solution and stirred for 20 min. The $CHCl_3$ layer was filtered through Na_2SO_4 and concentrated to provide (+)-**2** free base, m.p. 260–262°C, $[\alpha]^{25}D = +70.0°$ (c = 0.5, methanol). Reported [3] m.p. 260–262°C, $[\alpha]^{25}D = +70.1°$ (c = 1, ethanol). The (+)-**2** (50 mg) was dissolved in 2 ml of dry DMF and optically pure (*S*)-(−)-α-methylbenzylisocyanate (27 mg, 0.8 equiv.) [14] was added in one portion via syringe and the resulting solution was stirred overnight. The solution was then partitioned between 5 ml water and 5 ml diethyl ether. The aqueous layer was removed, and the ethereal layer was extracted with 5 ml of 1 N HCl solution. The ethereal layer was then dried (Na_2SO_4). A small amount of impurity [*N,N'*-bis-(α-methylbenzyl)urea] which could be detected by GC was removed by filtration through a short pad of silica gel. The filtrate was concentrated to give urea **5**. Similarly, the previously described procedure was carried out using a sample of (±)-**2** to provide a mixture of diastereomeric ureas. The 300 MHz ^{1}H-NMR spectra of the urea prepared from the (±)-base showed methyl doublets at δ 1.51 and 1.46 ppm for the diastereomeric α-methyl groups. No doublet at δ 1.46 was detectable in the spectrum of the urea prepared from the (+)-base indicating 99+% optical purity [15].

2.3. *(+)-cis-2'-Hydroxy-1',3'-dibromo-2,6-dimethyl-6,7-benzomorphan (3)*

To a stirred solution of (+)-normetazocine base (**2**) (0.5 g, 2.3 mmol) and triethylamine (0.70 g, 6.9 mmol) in acetic acid (5.0 ml) at room temperature was added dropwise, for 10 min, a solution of bromine (freshly redistilled) (0.74 g, 4.6 mmol) in acetic acid (5.0 ml). After the addition was complete, the reaction mixture was stirred for a further 10 min (complete by TLC; solvent system A), and then poured into a mixture of 50 g ice and excess concentrated aqueous ammonia. The copious white precipitate was filtered (slow) and the solid was washed with distilled water (100 ml) and air dried overnight to afford 0.87 g (quantitative) of crude **3**. Two recrystallizations of **3**·HCl from 5.0 ml of ethyl acetate afforded 0.75 g (79%) of **3**·HCl: m.p. 248–249°C; $[\alpha]^{25}D = +67.4°$ (c = 0.29, MeOH); anal. calc. ($C_{14}H_{18}ClBr_2NO$): C 40.86, H 4.41, N 3.40%; found: C 40.99, H 4.45, N 3.46%.

2.4. *(+)-cis-2'-Hydroxy-1'-bromo-2,6-dimethyl-6,7-benzomorphan (4)*

A solution of **3**·HCl 0.30 g, 0.729 mmol) in MeOH (30 ml) containing 10% Pd/C (0.30 g) was stirred at room temperature and 1 atm. pressure under an atmosphere of hydrogen gas (excess). Analysis of the reaction mixture after 2 h byTLC (solvent system A) indicated the presence of one major (higher R_f) pro-

duct which did not comigrate with the desired (+)-normetazocine. Continued hydrogenation failed to effect any further change. The reaction mixture was filtered through celite and the filtrate was evaporated in vacuo to afford a crystalline residue (quantitative as **4**·HBr salt). The residue was dissolved in MeOH (5 ml) and treated with an excess of HBr gas in MeOH. Distillation of the MeOH and addition of 2-propanol, keeping the volume constant at 10 ml, afforded **4**·HBr (0.21 g, 76%): m.p. 245–246°C; $[\alpha]^{25}D = +60.5°$ (c = 0.33, MeOH); anal. calc. ($C_{14}H_{19}Br_2NO$): C 44.59, H 5.08, N 3.71%; found: C 44.66, H 5.11, N 3.68%.

2.5. *(+)-[^{3}H]Normetazocine ([^{3}H]-2)*

A solution of **3**·HCl (20.0 mg, 0.049 mmol) in 2.0 ml of methanolic KOH (2.0%, w/v) containing 20 mg of 10% Pd/C was stirred for 2 h at room temperature under an atmosphere of carrier-free tritium gas (20.0 Ci, 0.338 mmol). The reaction mixture was filtered, evaporated under a stream of argon to remove labiles and acidified by dissolving in 25 ml of 2.0% acetic acid in MeOH (for storage): activity = 1.80 Ci. The solution was evaporated under a stream of nitrogen and the residue was treated with water (3.0 ml) followed by concentrated aqueous ammonia (3.0 ml). The basified solution was extracted with 3 × 3 ml of CH_2Cl_2 and the organic extract was evaporated under a stream of nitrogen and applied to one 20 cm × 20 cm × 0.5 mm silica gel preparative TLC plate (Analtech GF). The plate was eluted with solvent system A and the band comigrating with unlabeled (+)-normetazocine (**2**) was removed, and extracted with 40 ml of solvent system B. The extract was filtered through a plug of glass wool and evaporated under a stream of nitrogen. Reconstitution of the residue with methanol (50 ml) afforded [^{3}H]-**2** (0.68 Ci, 24% radiochemical yield) in greater than 99% radiochemical purity: specific activity = 26.6 Ci/mmol (by UV analysis of the solution at λ = 282 nm; $\epsilon_{282} = 2175\ l \cdot mol^{-1} \cdot cm^{-1}$ in MeOH). This material was found to be unstable on storage at 13.6 mCi/ml in MeOH and was therefore diluted to a final dilution of 1 mCi/ml of MeOH.

2.6. *(+)-[^{3}H]Pentazocine ([^{3}H]-**1b**)*

50 ml (50 mCi, 0.00188 mmol) of diluted MeOH stock solution of [^{3}H]-**2** was evaporated under a stream of nitrogen gas at 60°C and the residue was transferred to a small test tube with 3 × 1 ml of CH_2Cl_2. The CH_2Cl_2 was evaporated under a stream of nitrogen gas and to the residue was added 0.00226 mmol of 4-bromo-2-methyl-2-butene in 110 μl of *N,N*-dimethylformamide (DMF) and 0.5 mg of anhydrous $NaHCO_3$. The reaction mixture was heated at 60°C under a nitrogen atmosphere and after 12 h the reaction was diluted with 1 μl distilled water. The aqueous mixture was extracted with 2 × 1 ml of $CHCl_3$ and the organic extract was evaporated under a stream of nitrogen. The residue was chromatographed on one 20 cm × 20 cm × 0.5 mm preparative TLC plate, eluting with solvent system A. The band comigrating with unlabeled **1b** was removed and extracted with 20 ml of solvent system B and the extract filtered through a plug of glass wool. Evaporation of the solvent under a stream of nitrogen and reconstitution of the residue with 3.0 ml of ethanol afforded [^{3}H]-**1b**, 4.67 mCi (9.3% chemical yield), specific activity 26.6 Ci/mmol. The material was adjusted to a final dilution of 1 mCi/ml of 95% EtOH for storage.

2.7. *Receptor-binding studies*

Binding studies were carried out using the crude P_2 fraction of guinea pig brain. Frozen guinea pig brains (Pel-Freeze, Rogers, AK) were allowed to thaw slowly on ice after removal of the cerebella. Crude P_2 membranes were prepared as previously described for rat brain [16]. Membranes were incubated with the designated concentration of (+)-[^{3}H]pentazocine (26.6 Ci/mmol) and 500 mg of membrane protein in a total volume of 500 μl of 50 mM Tris-HCl, pH 8.0. Incubations were carried out for 120 min at 25°C. Non-specific binding was determined in the presence of 10 mM unlabeled (+)-pentazocine. Assays were terminated by dilution with 5 ml of ice-cold 10 mM Tris-HCl, pH 8.0, and vacuum filtration through glass fiber filters (Schleicher and Schuell, Keene, NH) using a Brandel Cell Harvester (Gaithersburg, MD). Filters were then washed twice with 5 ml of ice-cold 10 mM Tris-HCl, pH 8.0. Filters were soaked in 0.5% polyethyleneimine for at least 30 min at 25°C prior to use. Filters were counted on a Packard model 4450 scintillation spectrometer using Ecoscint cocktail (National Diagnostics, Manville, NJ) after overnight extraction of counts.

For Scatchard analysis, 12 concentrations of (+)-[^{3}H]pentazocine ranging from 0.05 nM to 25 nM were incubated as described above. Data were analyzed using the iterative curve fitting program BDATA (EMF Software Inc., Baltimore, MD). Competition binding studies were carried out as described below.

3. RESULTS

We have developed an efficient route (fig.1) to high specific activity [^{3}H]-**1b** starting from (+)-normetazocine (**2**) [3] which renders [^{3}H]-**1b** available for the first time. Thus, dibromination of **2** (base) in glacial acetic acid afforded (+)-*cis*-2′-hydroxy-1′,3′-dibromo-2,6-dimethyl-6,7-benzomorphan (**3**) in 79% yield. Attempts to tribrominate **2** with *N,N′*-dibromoisocyanuric acid in concentrated sulfuric acid [17] were unsuccessful. Hydrogenation of **3**·HCl over 10% Pd/C in MeOH afforded the monobromide **4** as the only product. The more hindered 1′-bromine of **4** appeared to be resistant to hydrogenolysis under these conditions. However, hydrogenation of **3** in 2% methanolic KOH in the presence of 10% Pd/C proceeded smoothly to give **2**, identical by ^{1}H-NMR, TLC and GC to an authentic sample [3]. It was therefore necessary to convert **3** to its phenoxide anion before complete hydrogenolysis to **2** was possible. Thus, tritiolysis of **3** in 2% methanolic KOH in the presence of 10% Pd/C afforded [^{3}H]-**2** in 24% radiochemical yield. This procedure afforded [^{3}H]-**2** which was converted to the desired [^{3}H]-**1b** of specific activity 26.6 Ci/mmol in 9.3% chemical yield by *N*-alkylation with 4-bromo-2-

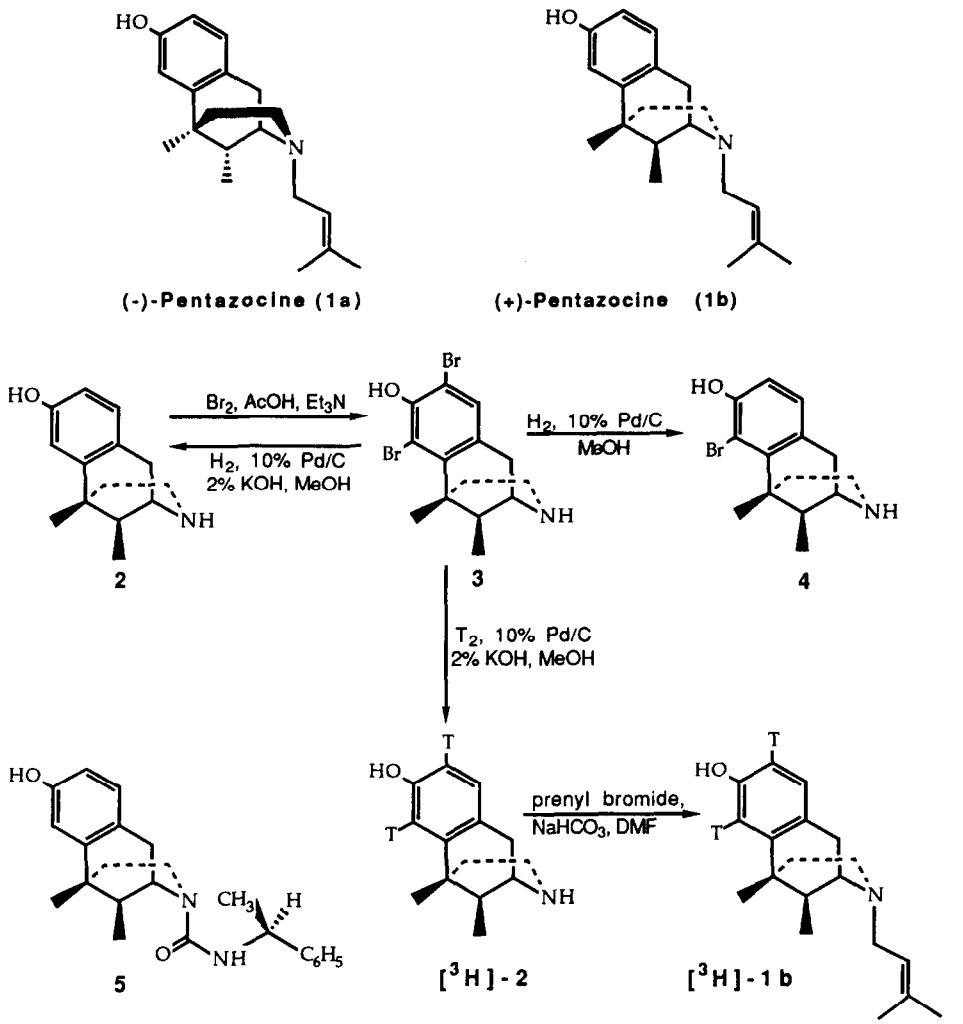

Fig.1. Structures of compounds and reaction sequence.

methyl-2-butene in DMF containing $NaHCO_3$ as described previously [2]. Racemic [^{3}H]pentazocine of low specific activity (0.053 Ci/mmol) has been previously synthesized [18] by a route employing $NaBT_4$ reduction which is not applicable to synthesis of high specific activity ligand required for receptor-binding studies. Scatchard analysis of [^{3}H]-**1b** binding to guinea pig brain membranes was carried out as described in section 2. [^{3}H]-**1b** exhibited saturable binding with K_d = 5.13 ± 0.97 nM and B_{max} = 1146 ± 122 fmol/mg of protein. Results are based on the average ± SE of two experiments, each performed in duplicate. All plots are linear suggesting labeling of a single class of sites. Specific binding was greater than 90% at all concentrations examined (up to 25 nM).

In order to determine whether [^{3}H]-**1b** labels σ receptors, the ability of several unlabeled compounds to compete for sites labeled by [^{3}H]-**1b** was determined. Results are shown in table 1. The typical σ receptor ligands haloperidol, DTG, and (+)-3-PPP competed with high affinity. (+)-Pentazocine bound with higher affinity than (−)-pentazocine, demonstrating proper enantioselectivity for σ sites. PCP bound with low affinity while the selective PCP receptor ligand, MK-801, was inactive. Finally, the dopamine receptor agonist apomorphine, was inactive. This ligand

Table 1

Ligand selectivity of site labeled by (+)-[^{3}H]pentazocine

Ligand	IC_{50} (nM)
(+)-Pentazocine	5.0 ± 0.7
Haloperidol	5.3 ± 0.9
DTG	45 ± 10
(+)-3-PPP	48 ± 2
(+)-SKF-10,047	97 ± 6
(−)-Pentazocine	136 ± 14
PCP	2871 ± 735
Apomorphine	>10000
(+)-MK801	>10000

Assays were carried out under the conditions described in section 2, using 3 nM [^{3}H]-(+)-pentazocine and 12 concentrations of unlabeled test ligand ranging from 0.05–10000 nM. Competition data were analysed using the iterature curve fitting program CDATA (EMF Software Inc., Baltimore, MD). IC_{50} values are averages of two experiments ± SE. Each experiment was carried out in duplicate. Greater than 10000 nM denotes less than 20% inhibition at 10000 nM concentration of test drug

selectivity pattern is identical to that reported for sites labeled by (+)-3-[^{3}H]PPP and [^{3}H]DTG, two commonly used σ receptor probes [12,13].

4. DISCUSSION

The results of this study conclude that (+)-pentazocine is amenable to high specific activity tritiation and that [^{3}H]-**1b** labels σ sites. The prototypical σ ligands DTG and (+)-3-PPP exhibit high affinity for the site labeled by [^{3}H]-**1b**. The inactivity of apomorphine and high potency of haloperidol demonstrates failure to label dopamine receptors, whereas the weak affinity of PCP and the absence of potency of MK-801 demonstrates failure to label PCP receptors. Thus, [^{3}H]-**1b** binds with high selectivity to a site with identical ligand selectivity as the σ receptor.

The high binding affinity of [^{3}H]-**1b** (K_d = 5.1 nM) is also noteworthy. The only other benzomorphan-based radiolabeled σ ligand, (+)-[^{3}H]SKF-10,047, has been reported to exhibit a K_d of 40 nM in guinea pig brain membranes [4]. Thus, [^{3}H]-**1b** binds to σ sites with about 8-fold higher affinity than (+)-[^{3}H]SKF-10,047. This is qualitatively consistent with the results of competition against [^{3}H]-**1b**, where unlabeled (+)-pentazocine exhibited 20-fold higher affinity than unlabeled (+)-SKF-10,047. Compared to (+)-SKF-10,047, the higher affinity of (+)-pentazocine for σ receptors and its much lower affinity for PCP receptors ([5,19] and unpublished observations) suggest that [^{3}H]-**1b** will be a more useful σ ligand than (+)-[^{3}H]SKF-10,047. The other typical radiolabeled σ ligands, (+)-3-[^{3}H]PPP and [^{3}H]DTG exhibit high selectivity for σ receptors [12,13]. However, these ligands bind with 5–6-fold lower affinity than [^{3}H]-**1b**. The only other radioligand with comparable affinity to [^{3}H]-**1b** is [^{3}H]haloperidol (K_d = 2.0 nM) [12]. However, use of this compound is severely limited by its nearly equal affinity for dopamine receptors and the necessity to mask these receptors in σ binding protocols [12].

In summary, the high affinity, high selectivity and low level of non-specific binding render [^{3}H]-**1b** a highly suitable tool for study of the structure and function of σ receptors. This ligand offers several advantages over the currently available σ receptor probes. In addition, since recent evidence suggests that (+)-benzomorphan and nonbenzomorphan ligands interact differently with σ receptors [20], a better radiolabeled benzomorphan based ligand probe is needed in order to further investigate this phenomenon. A more detailed assessment of the binding characteristics of (+)-[^{3}H]pentazocine and a comparison to those of other radiolabeled σ ligands is therefore warranted.

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