SYNTHESIS OF [^{3}H]TERT-BUTYL 8-CHLORO-5,6-DIHYDRO-5-METHYL-6-OXO-4H-IMIDAZO[1,5-a][1,4]BENZODIAZEPINE 3-CARBOXYLATE, A SELECTIVE, HIGH AFFINITY LIGAND FOR THE DIAZEPAM INSENSITIVE (DI) SUBTYPE OF THE BENZODIAZEPINE RECEPTOR

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SUMMARY

The preparation of [^{3}H]-labelled tert-butyl 8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine 3-carboxylate (TCIB, **6**), a high affinity ligand for the diazepam insensitive (DI) subtype of the benzodiazepine receptor (BZR) is described. Synthesis of [^{3}H]TCIB was accomplished in 4 steps starting from 5-chloroisatoic anhydride. Tritium-label introduction was achieved in the final step by selective catalytic tritiolysis in 62% radiochemical yield with quantitative isotopic incorporation.

Key words: Imidazobenzodiazepine, Diazepam Insensitive, Benzodiazepine Receptor, Ro 15-4513, Tritium Labelling, $GABA_A$ Receptor

INTRODUCTION

Ligands binding to the benzodiazepine receptor (BZR) elicit a wide variety of pharmacological actions through an allosteric interaction with γ-aminobutyric acid (GABA) receptors and its associated chloride ion channel [reviewed in 1]. The diazepam insensitive (DI) subtype of benzodiazepine receptor (BZR) represents a novel isoform with a markedly different ligand binding profile than other diazepam sensitive (DS) isoforms of this receptor [2,3]. Thus, DI are characterized by low affinities (>1μM) for prototypical 1,4-benzodiazepines (e.g. diazepam, flunitrazepam), triazolobenzodiazepines (e.g. triazolam), and triazolopyridazines (e.g. CL 218,872) which are high affinity (nM) ligands at DS, and high affinities for imidazobenzodiazepines, such as Ro 15-4513 (K_i 3.1 nM) [2,4,5]. Compounds from several other

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chemical classes, such as pyrazoloquinolines, quinolines and β-carbolines also bind to DI with high affinities [6,7].

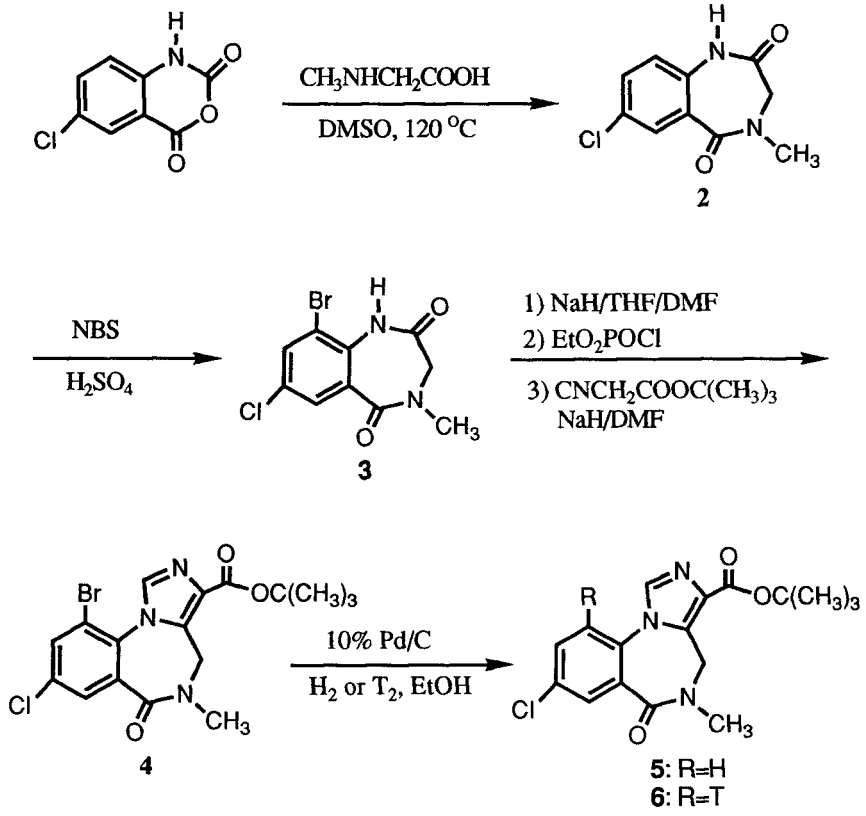
Recent studies have implicated DI in the alcohol antagonist action of several BZR ligands for: a) the ability of Ro 15-4513 and 19-4603 to antagonize some of the biochemical and behavioral effects of ethanol [8-11], b) the high affinity of these compounds for DI [5], c) the lack of measurable DI in alcohol non-tolerant rats [3] and d) the predominant cerebellar localization of DI sites in humans, rodents and pigeons [12,13]. Molecular biological studies have provided insight into the subunit heterogeneity of the $GABA_A$/benzodiazepine receptor complex [reviewed in 14,15]. A pharmacological profile similar to that of native cerebellar DI sites can be reconstituted in cell lines transfected with cDNA's encoding α6, β2, γ2 subunits [16] but not other α subunits. Nonetheless, the involvement of DI sites in the alcohol antagonist actions of BZR ligands remains controversial, in part due to the paucity of selective DI ligands. To date, [^{3}H]Ro15-4513 has been the only tritiated ligand utilized to discriminate DI receptors, since other radiolabelled ligands commonly used for the study of DS (e.g. [^{3}H]Ro15-1788, [^{3}H]β-CCM, [^{3}H]flunitrazepam) exhibit low affinities at DI [2,4]. Some problems encountered with [^{3}H]Ro15-4513 include high nonspecific binding as well as a photoreactive azide substituent [17]. Recent efforts in the elucidation of DI structure-affinity relationships led to the design and synthesis of tert-butyl 8-chloro-5,6-dihydro-5-methyl-6-oxo-imidazo[1,5-a][1,4]benzodiazepine 3-carboxylate (TCIB), the most potent (K_i 1.7 nM) and selective ligand (DI/DS ratio 0.4 vs 0.6 for [^{3}H]Ro15-4513 [5,18]) described to date. The tritiated TCIB should be a valuable tool to study the structure, function, and pharmacological role of DI receptors.

SYNTHESIS

The synthetic route for preparing tritium labelled TCIB (**6**) is outlined in Scheme 1. Commercially available 5-chloroisatoic anhydride was heated with sarcosine in dimethyl sulfoxide to afford 1,4-benzodiazepine skeleton **2** [19] in high yield. Bromination of **2** at the 9-position was achieved by treatment with N-bromosuccinimide in concentrated sulfuric acid to afford **3**. Compound **3** was deprotonated with sodium hydride in THF/DMF, followed by reaction with diethyl chlorophosphate to form the corresponding enol phosphate [19,20]. This was subsequently reacted with a solution of tert-butyl isocyanoacetate and sodium hydride in DMF to afford the desired tritiation precursor **4**. Selective catalytic hydrogenation of **4** over 10% Pd-C for 1 hour afforded the unlabelled **5** in high yield with no significant hydrogenolysis of the chlorine atom. Longer reaction times (>2 h) however resulted in significant amounts of side products corresponding to loss of both halogen atoms. Thus, catalytic tritiation of **4** over 10% Pd-C with 25 Ci of carrier free tritium gas [21] during 1 hour followed by TLC purification afforded [^{3}H]tert-butyl 8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (**6**) (62% radiochemical yield, 29 Ci/mmol) in greater than 99%

radiochemical purity. The labelled compound **6** co-migrated on TLC with the corresponding unlabelled reference compound **5**. The purified material was stored at -70 °C in absolute ethanol at a concentration of 1.47 mCi/mL since higher dilutions generally promote higher radiochemical stability.

Scheme 1



DISCUSSION

The paucity of ligands which bind to DI with high affinity and selectivity has hindered an understanding of the pharmacologic and physiologic function(s) of this BZR isoform. Several recent reports described novel high affinity ligands to the DI receptor; nonetheless all of the ligands except Ro15-4513 lacked selectivity [5,6,7,12]. We thus wished to synthesize a more selective radioligand to address this problem. Preliminary radioligand binding experiments demonstrated that [^{3}H]TCIB (**6**) is potent (K_d 2.6 nM) and selective (DI/DS ratio 0.2). While the increase in affinity and selectivity of **6** over [^{3}H]Ro15-4513 is modest, [^{3}H]TCIB has a GABA shift of 1.16 *at*

DS [18] and lacks the photoreactive azido group of Ro15-4513 [22]. These factors may make [^{3}H]TCIB a desirable alternative to [^{3}H]Ro15-4513.

In order to introduce bromine in structure **2** for introduction of tritium in the final step, attempts were made to brominate the aromatic ring of **2** using mild conditions. However typical conditions such as bromine in acetic acid proved unsuccessful. This is most likely due to deactivation of the phenyl ring by the chloro substituent [23]. However, bromination of **2** was achieved in 70% yield under strongly acidic conditions using N-bromosuccinimide in concentrated sulfuric acid. The position of the bromine atom was as expected from substituent and electronic directing effects on the ring. The acylamino group, a moderate activating group, has a major directing effect (para>ortho) in this case [23,24], so that bromine incorporation on the 9-position can be rationalized on this basis since the 7-position (para) is occupied by a chloro group. The proton NMR spectrum of **4** showed signals corresponding to two aromatic protons with chemical shifts at 7.89 and 7.75 ppm, respectively, downfield of TMS. These two protons have the same coupling constant of 2.4 Hz, indicating that they are meta (6- and 8-positions) to each other. Comparison with the NMR spectrum of precursor **2** supports the 9-bromo-substitution of structure **3**.

Selective tritiation of **4** was achieved by controlling the reaction time and temperature of the reaction. This condition was established by catalytic hydrogenation of **4** at room temperature for different times and monitoring by TLC. The optimal condition was found to be 1 hour at room temperature where starting material had been completely consumed and only very small amounts of the deschloro side-product had formed. This was easily separated by TLC. Tritium labelled compound **6** was obtained in 62% chemical yield with quantitative tritium incorporation. This high degree of isotopic incorporation was obtained despite the potential ease of palladium catalyzed isotopic dilution of tritium with hydrogen from the ethanol solvent.

EXPERIMENTAL

Melting points were determined on a Mel-Temp II capillary apparatus and are uncorrected. Elemental analyses were performed at Atlantic Microlab, Norcross, Georgia. Chemical Ionization Mass Spectra (CIMS) were obtained using a Finnigan 1015 mass Spectrometer. Electron ionization mass spectra (EIMS) were obtained using a V. G. Micro Mass 7070F mass spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded using Varian XL-300 Fourier transform spectrometers in $CDCl_3$ or DMSO-d_6. Chemical shifts are expressed in parts per million (ppm) on the δ scale relative to a TMS internal standard. Ultraviolet (UV) spectra were recorded using a Hewlett-Packard 8450A UV/VIS spectrophotometer. Thin layer chromatography (TLC) was performed on 500 micron Analtech GHLF silica gel plates eluting with methylene chloride-ethyl acetate-methanol (6 : 3 : 0.5). For radiolabelled compound **6**, the TLC plate was analysed using a Bertold model LB 2760 TLC scanner. Radioactivity

determinations were carried out with a Packard model 2200 CA "Tri-Carb" liquid scintillation analyzer using hydrofluor scintillation cocktail.

7-Chloro-3,4-dihydro-4-methyl-2H-1,4-benzodiazepine-2,5(1H)-dione (2) A mixture of 5-chloroisatoic anhydride (10 g, 50 mmol) and sarcosine (5.4 g, 60 mmol) in dimethyl sulfoxide (35 ml) was stirred at 140 °C for 2 hours. The clear brown solution was poured into ice-water (200 ml) and the off-white precipitate formed while stirring. The product was collected by filtration and washed thoroughly with water, yielding **2** (9 g, 90%): mp 250-252 °C (lit mp 259-262°C [25]); ^{1}H-NMR 10.57 (br s,1H), 7.71 (d,1H, *J*=2.3 Hz), 7.58 (dd,1H, *J*=2.4, 8.5 Hz), 7.13 (d,1H, *J*=8.5 Hz), 3.89 (s, 3H), 3.12 (s, 9H); MS (EIMS) m/z 226, 224 (M), 197, 195, 155, 153, 125, 98, 90, 63.

9-Bromo-7-chloro-3,4-dihydro-4-methyl-2H-1,4-benzodiazepine-2,5(1H)-dione (3) N-Bromosuccinimide (7.1 g, 40 mmol) was dissolved in concentrated sulfuric acid (25 ml) by stirring and gently heating. To this solution **2** was added portionwise. The reaction mixture was allowed to stir at room temperature for 4 hours and the red-brown solution was poured onto ice (200 g) and neutralized by addition of excess aqueous ammonium hydroxide. The precipitate was collected by filtration and washed with water (400 ml) followed by ether to afford **3** (2.85 g, 70%) as a light brown powder. An analytically pure sample was obtained by recrystallization from ethyl acetate: mp 175-176 °C; ^{1}H-NMR 7.89 (d,1H, *J*=2.4 Hz), 7.75 (d, 1H, *J*=2.4 Hz), 7.67 (br s, 1H), 3.89 (s, 2H), 3.28 (s, 3H); ^{13}C-NMR 167.9, 164.8, 135.1, 132.1, 130.9, 130.8, 129.0, 115.3, 52.2, 36.6; MS (CIMS) m/z 322, 320, 305 (M+H), 303 (M+H), 275, 242, 227, 225. Anal. (Found) C, 39.63; H, 2.71; N, 9.22% Anal. (Calculated for $C_{10}H_8BrClN_2O_2$) C, 39.57; H, 2.66; N, 9.23%.

tert-Butyl 10-bromo-8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine 3-carboxylate (4) A solution of **3** (1.5 g, 5 mmol) in a mixture of DMF (10 ml) and THF (25 ml) was cooled in ice-water and then treated with sodium hydride (200 mg, 8 mmol). After 20 min, diethyl chlorophosphate (1.7 ml, 10 mmol) was added dropwise and the solution was continuously stirred for 30 min with cooling from an ice-bath. A solution of tert-butyl isocyanoacetate (1.12 ml, 8 mmol) and sodium hydride (250 mg, 10 mmol) in DMF (10 ml), which had been stirred for 15 min with ice-bath cooling, was added slowly. After stirring for another 30 min with cooling, the reaction mixture was allowed to stir at room temperature overnight. Acetic acid (5 ml) was added to quench the reaction and it was then poured into ice water (200 ml) and extracted with ethyl acetate. The combined extracts were washed with water, brine and dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure and the residue was crystallized from ethyl acetate to afford **4** (715 mg, 34%) as light yellow crystals: mp 208-210 °C;^{1}H-NMR 8.12 (s, 1H), 7.92 (d, 1H *J*=2.2 Hz), 7.88 (d, 1H *J*=2.3 Hz), 5.15

(d, 1H *J*=15.6 Hz), 4.27 (d, 1H *J*=15.6 Hz), 3.20 (s, 3H), 1.66 (s, 9H); ^{13}C-NMR 164.4, 161 (1C), 137.3 (1C), 136.3 (1C), 135.0 (1C), 134.8 (1C), 133.8 (1C), 131.0 (1C), 129.5, 129.0, 117.4, 82.2, 42.3, 35.5, 28.5, 28.4, 28.3; MS (CIMS) m/z 428 (M+H), 426 (M+H)), 372, 370, 348, 292, 243, 196, 136. Anal. (Found) C, 47.62; H, 4.04; N, 9.72%. Anal. (Calculated for $C_{17}H_{17}BrClN_3O_3$) C, 47.85; H, 4.02; N, 9.85%.

tert-Butyl 8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]-[1,4]benzodiazepine 3-carboxylate (5) To a stirred solution of **4** (35 mg, 8.2 μmol) in ethyl alcohol (4 ml) was added 10% palladium on activated carbon (35 mg). The reaction mixture was stirred for 1 hour at room temperature under an atmosphere of hydrogen gas. It was then filtered through a pad of celite and washed with methylene chloride (20 ml). The filtrate was evaporated under reduced pressure and the residue was crystallized from ethyl acetate to afford **5**•HBr (22 mg, 63%) as an off-white crystalline solid: mp 254-256 °C; ^{1}H-NMR (free base) 8.05 (d, 1H, *J*=2.4 Hz), 7.89 (s, 1H), 7.59 (dd, 1H, *J*=2.4 Hz, 8.5 Hz), 7.37 (d, 1H, *J*=8.5 Hz), 5.18 (br s, 1H), 4.35 (br s, 1H), 3.26 (s, 3H), 1.67 (s, 9H); ^{13}C-NMR 164.9, 161.6, 134.4, 134.2, 132.4, 132.3, 132.2, 130.4, 130.2, 129.9, 81.8, 42.3, 35.7, 28.4, 28.3, 28.2; MS (CIMS) m/z 367, 350 (M+H), 348 (M+H), 314, 309, 292. Anal. (Found) C, 58.71; H, 5.22; N, 12.08%. Anal. (Calculated for $C_{17}H_{18}ClN_3O_3$) C, 58.74; H, 5.25; N, 12.14%.

[^{3}H]tert-Butyl 8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a][1,4]benzodiazepine 3-carboxylate (6). A solution of **4**, (10 mg, 0.0234 mmol) in ethanol (1 mL) containing 10% Pd-C (10 mg) was stirred for 1 hour at room temperature under an atmosphere of carrier free tritium gas (25 Ci, 0.431 mmol) at New England Nuclear, Boston, MA. The solution was filtered, evaporated under a stream of argon (to remove labiles) and then diluted with absolute ethanol for storage (720 mCi). A 250 mCi (crude product) portion of this in 25 mL of absolute ethanol was used for purification in our laboratory. Excess aqueous ammonia was added to the crude product (to liberate **6** free base) prior to solvent evaporation. The pure product **6** was obtained by TLC purification on one 20 cm x 20 cm x 0.5 mm plate eluting with the TLC solvent system followed by extraction of the band co-migrating with unlabelled **5** with 30 mL of the solvent system. After evaporating the solvent, the residue was reconstituted with 100 mL of absolute ethanol. Liquid sintillation counting indicated a yield of 147 mCi of **6** from 250 mCi crude product which corresponds to a total yield of 423 mCi (62%) in greater than 99% radiochemical purity as determined by TLC analysis and UV analysis (EtOH, λ = 287 nM (sh), ε_{289} =1960 cm^{-1}. M^{-1}). The specific activity determined by UV analysis at 287 nM was 29 Ci/mmol corresponding to quantitative isotopic incorporation.

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