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Synthesis of Deuterium Labeled 2,4-Dipyrrolidinylpyrimidine as a Chemical Probe for P450 Mediated Oxidation of Tirilazad Mesylate.

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SUMMARY

Selective deuterium labelled pyrrolidinylpyrimidine analogs were synthesized to provide a probe for the metabolite identification of Tirilazad. Synthesis of 2-[D_8]-pyrrolidinyl-4-pyrrolidinylpyrimidine (**1**) and 4-[D_8]-pyrrolidinyl-2-pyrrolidinylpyrimidine (**2**) was accomplished by reaction of pyrrolidine (**3**) with 2,4-dichloropyrimidine (**4**), separation of the two reaction products (**5** and **6**) and reaction of these products with [D_8]pyrrolidine (**7**). [D_{16}]-2,4-dipyrrolidinylpyrimidine (**8**) was prepared by reaction of [D_8]pyrrolidine (**7**) with 2,4-dichloropyrimidine (**4**).

Keywords: 2,4-dipyrrolidinylpyrimidine, deuterium, probe, Tirilazad

INTRODUCTION

Alicyclic tertiary amines (aziridine, pyrrolidine, piperidine, piperazine, hexamethylenimine, heptamethylenimine) and substituted derivatives thereof, are common moieties of drugs. Thus, there have been widespread investigations as to

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the metabolic fate of these functional groups and the role that metabolism may play in the elimination and toxicity of drugs containing alicyclic tertiary amines. The oxidative metabolic transformations of tertiary amines in mammalian systems can be divided into two general routes; α-carbon oxidation and N-oxidation. The α-carbon oxidation of N-alkylamines, a common reaction for many drugs, leads to the formation of carbinolamines which can either be oxidized to an α-carbonyl (amides; lactam) metabolite or rearranged to a secondary amine accompanied by ring opening or *N*-dealkylation.

Tirilazad (**T**[1], Figure 1; Freedox®, Tirilazad mesylate, 21-[4-(2,6-di-1-pyrrolidinyl)-4-pyrimidinyl-1-piperazinyl]-16α-methyl-pregna-1,4,9(11)-triene-3,20-dione mesylate), is currently under clinical development for the treatment for subarachnoid hemorrhage and head injury. **T** has been shown to be an inhibitor of lipid peroxidation both *in vitro* and *in vivo* (1,2). The identification of the oxidized metabolites of **T** is of considerable interest as this provides insights into the mechanisms of action and metabolic clearance of **T**. In preliminary animal studies, biological transformation of **T** *in vivo* and *in vitro* involved complex metabolism on both the steroidal and heterocyclic portion of the molecule. The structures of the metabolites of the heterocyclic amine portion of **T** have been partially characterized by mass spectrometry as oxidized and desaturated metabolites of the pyrrolidine rings (3,4). However, due to the chemical similarity of the 2-pyrrolidine and the 4-pyrrolidine moieties, the chemical instability of metabolites, full structural characterization of the metabolites was not possible (3,4). In a recent investigation[5], we have employed 2,4-dipyrrolidinylpyrimidine (2,4-DPP) as a chemical probe to further characterize P450 mediated oxidation of the heterocyclic domain of **T**. In pursuit of this objective, deuterium labelled analogs of 2,4-DPP have been prepared and employed as metabolic probes in conjunction with LC-MS techniques to characterize *in vitro* biological transformations of 2,4-DPP in rat liver microsomes.

RESULTS AND DISCUSSION

Structures of metabolic probes and synthetic intermediates are shown in Figure 1. Structures were characterized by MS, proton NMR and ^{13}C NMR. Synthesis of 2-[D_8]-pyrrolidinyl-4-pyrrolidinylpyrimidine (**1**) and 4-[D_8]-pyrrolidinyl-2-pyrrolidinylpyrimidine (**2**) was accomplished by reaction of pyrrolidine (**3**) with 2,4-dichloropyrimidine (**4**), separation of the two reaction

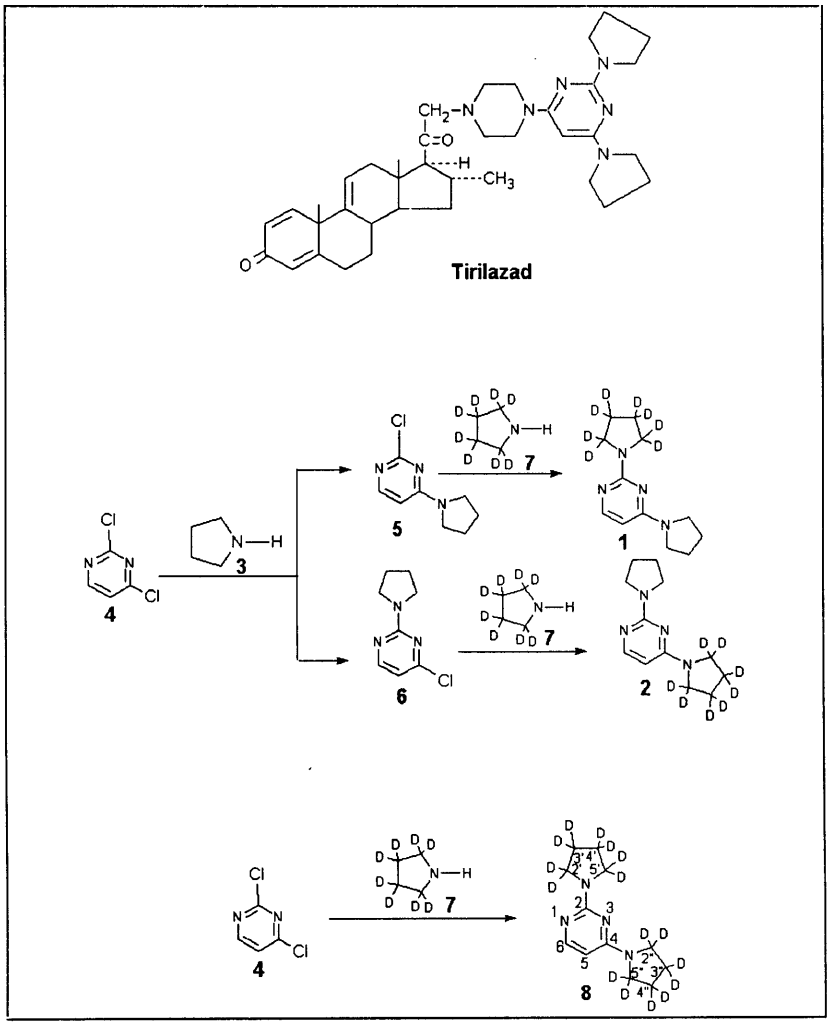

Figure 1. Structure of Tirilazad and synthetic pathways for the deuterated pyrrolidinylpyrimidine analogs.

products (**5** and **6**) and reaction of these products with [D_8]pyrrolidine (**7**). Specific additions to either the 2- (**1**) or 4-positions (**2**) was accomplished by full structural characterization of 2-chloro-4-pyrrolidinylpyrimidine (**5**), 4-chloro-2-pyrrolidinylpyrimidine (**6**) intermediates (**5** and **6**, respectively). [D_{16}]-2,4-dipyrrolidinylpyrimidine (**8**) was prepared by reaction of [D_8]pyrrolidine (**7**) with 2,4-dichloropyrimidine (**4**). Correct assignment of the structures of the synthetic intermediates **5** and **6** were made using NMR data. The carbon chemical shifts of C-5 and C-6 (Table 1) were assigned based on known values. C-2 and C-4 were differentiated from heteronuclear multiple bond correlation (HMBC) experiments where cross peaks are seen from H-5 to C-4 & C-6, and, H-6 to C-2 & C-5. Replacement of the chlorine was evidenced on the upfield shift of C-5 in **5** due to C-5 being β to three nitrogens. This assignment was confirmed in **6** from an HMBC cross peak seen from H-2′ to C-2. ^{1}H NMR data of synthetic intermediates indicate that the pyrrolidine in the 2 position is freely rotating on the NMR time scale at room temperature while the pyrrolidine in the 4 position is hindered as indicated by several broad signals.

EXPERIMENTAL

NMR Analyses. ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer and chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS; Table 1). ^{1}H and HMBC spectra were recorded on a Bruker ARX-400 spectrometer (Bruker Instr. Inc., Billerica, MA). Data were processed on a Bruker Aspect X-32 computer using Bruker UXNMR software. Spectra were recorded at 300 K using a 5 mm broadband inverse probe. One dimensional proton spectra were recorded as free induction decays of 32K complex points with sweep widths of 6,024 Hz. Four dummy scans were used. The receiver gain and the number of scans recorded were optimized for each sample.

The accumulated free induction decays were Fourier transformed with no zero-filling after application of a resolution enhancing Gaussian window function (LB = -1, GB = 0.2). A polynomial baseline correction function was applied after phase correction and before integration. Samples were dissolved in 500 µl of $CDCl_3$ (Cambridge Isotope Labs. Inc., Andover, MA) and placed in 5 mm NMR tubes (Wilmad, Buena NJ). All data are referenced to TMS and reported in ppm (δ).

TABLE 1. 13C NMR Chemical Shift Assignments for 1,2,5,6 and 8

Assignments	Compound Number - Chemical Shifts[a],d				
	1	2	5	6	8
2	159.51	159.30	160.14	159.90	159.84
4	160.44	160.38	160.57	160.72	160.23
5	93.00	92.84	101.91	108.03	92.61
6	153.84	154.08	155.70	158.45	154.86
2',5'	b	46.30	46.38	----	46.07
3',4'	b	25.41	24.50 & 25.19	----	25.30
2", 5"	46.05	b	----	46.64	45.66
3",4"	25.20	b	----	25.24	24.99

[a]In $CDCl_3$ relative to internal TMS
[b]Signals cannot be seen.

Gas Chromatography and Liquid Chromatography Mass Spectrometry. Gas chromatography mass spectrometry was performed on a Nermag R10-10L (Delsi Instruments, Rueil-Malmaison, France) mass spectrometer with a Hewlett Packard 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with a split/splitless injector. The instrument was operated in electron ionization mode with the electron energy set at 70 eV. The instrument was scanned from m/z 450-50 every 1 second. Instrument control and data acquisition were carried out using Teknivent Vector II software (Teknivent,

Maryland Heights, MO). The injector and transfer lines were both operated at 300 °C. A 30 m x .25 mm ID DB-1 column (0.25 μm film thickness, J&W Scientific, Folsom, CA) was used for all analyses. Helium (99.995% pure, AGA, Maumee, OH) at a head pressure of 5 psi was used as the carrier gas. The oven temperature was held at 100 °C for the first two minutes then increased at 25 °C/min to 290 °C, held at 290 °C for five minutes before returning to the initial conditions. The HPLC system consisted of a PE410 (Perkin Elmer, Norwalk, CT) pump, a Waters 490 MS ultraviolet detector (Waters, Milford, MA) set at λ = 305 nm, and an ISS 100 autosampler (Perkin Elmer). Chromatographic separations were performed using a linear gradient (90% aqueous to 100% organic in 40 minutes) with a binary mobile phase consisting of 0.1 M ammonium acetate in water against acetonitrile with a flow rate of 0.5 ml/min on a 4.6 X 250 mm Zorbax SB-CN column (MAC-MOD Analytical, Chadds Ford, PA). Column effluent was then passed directly into the particle beam nebulizer of the mass spectrometer.

Yield and Purity. Percent yields were not determined by weighing reactants and product(s) since our objectives were purity of the synthetic standard for in vitro studies and the ability to ascertain positions of deuterium unambiguously. However, analysis by LC-MS/GC-MS gave a highly reliable estimate of actual yields which we refer to as purity by LC-MS/GC-MS.

2-Chloro-4-pyrrolidinylpyrimidine (5), 4-Chloro-2-pyrrolidinylpyrimidine (6). **5** and **6** were prepared by reacting 0.1 mol 2,4-dichloropyrimidine (Aldrich Chemical Co,, Milwaukee WI) with 0.1 mol of pyrrolidine in 100 mL of diethyl ether (Mallincrodt, Paris, KY) at 0 °C for 2 h. The ether was stripped and the resulting white solid was dissolved in 2 mL of 90/10 $CHCl_3/CH_3OH$ (Burdick & Jackson, McGraw Park, IL). GC-MS analysis of the product showed the presence of two chromatographically resolved peaks both having molecular weights corresponding to **5** and **6** ($M^{+\cdot}$ = m/z 183). The products

were separated on a 150 mL silica gel (230 - 400 mesh; EM Science, Gibbstown NJ) column packed and equilibrated with 90/10 $CHCl_3/CH_3OH$. The products were flash chromatographed using argon, and 20 mL fractions were collected. GC-MS analysis of the fractions showed that two distinct peaks were collected each exhibiting $M^{+\cdot}$ = m/z 183. Proton NMR analysis of the isolated materials determined that the first peak to elute was **5**: m.p. = 91 - 92 °C; purity by GC-MS 100 %; ^{1}H NMR (400 MHz), δ 6.19 (d, J_6=6.04 Hz, 1H, H-5), 7.98 (d, J_5=6.04 Hz, 1H, H-6), 3.63 - 3.35 (broad s, 4H, H-2" and H-5"), 2.06 and 2.00 (broad s, 4H, H-3" and H-4"). Rotomers are seen for the pyrrolidine ring at 300 K. At 317 K the signals at 2.00 & 2.06 have collapsed to a singlet at 2.03 d and the signals at 3.35 - 3.63 have started to coalesce. The second peak was characterized as **6**: m.p. = 90.5 - 92 °C; purity by GC-MS = 100%; ^{1}H NMR (400 MHz), δ 6.50 (d, J_6=5.18 Hz, 1H, H-5), 8.18 (d, J_5=5.18 Hz, 1H, H-6), 3.60 (t, J=6.76 Hz, 4H, H-2′ and H-5′), 2.01 (t, J=6.76 Hz, 4H, H-3′ and H-4′). The percent purity by LC-MS of **5** and **6** was 25% and 75%, respectively.

2-[D_8]-Pyrrolidinyl-4-pyrrolidinylpyrimidine (1). Excess [D_8]-pyrrolidine (**7**) (99.2 atom % D enriched, C/D/N Isotopes, Vaudreuil, Quebec, Canada) was then added to neat **5** (0.01 moles) and the mixtures were allowed to react at room temperature under argon overnight. **1** was then purified and analyzed in the same manner as the intermediates **5**. **1**: m.p. = 90.5 - 92 °C; purity by GC-MS and LC-MS ≈ 100%; GC-MS and LC-MS characterization, co-eluted with authentic 2,4-dipyrrolidinylpyrimidine, $M^{+\cdot}$ = 226; ^{1}H NMR (400 MHz), d 7.89 (d, J_5=6.06 Hz, 1H, H-6), 5.66 (d, J_6=6.06, 1H, H-5), 3.46 (broad s, 2H, H-2" & 5"), 1.97 (broad s, 2H, H-3" & 4").

4-([D_8]-Pyrrolidinyl-2-pyrrolidinylpyrimidine **(2).** 2 was prepared in the same manner as **1** by reacting **6** with **7**. 2: m.p. = 90.5 - 92 °C, purity by GC-MS and LC-MS ≈ 100%; GC-MS and LC-MS characterization, co-eluted with authentic

2,4-dipyrrolidinylpyrimidine, M^+ = 226; ^1H NMR (400 MHz), d 7.89 (d, J_5=6.08 Hz, 1H, H-6), 5.65 (d, J_6=6.08 Hz, 1H, H-5), 3.57 (t, $J_{3',4'}$=6.72 Hz, 4H, H-2' & 5'), 1.96 (t, $J_{2',5'}$=6.72, 4H, H-3' &4').

[D_{16}]-2,4-dipyrrolidinylpyrimidine (8). **8** was prepared by addition of two equivalents (0.2 mol) of [D_8]-pyrrolidine to neat 2,4-dichloropyrimidine. The resulting white solid was taken up in 90/10 chloroform/methanol and purified as described above. **8**: m.p. = 91 - 92.5 °C, purity by GC-MS and LC-MS ≈ 100%; GC-MS and LC-MS characterization, co-eluted with authentic 2,4-dipyrrolidinylpyrimidine, M^+ = 234.

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FOOTNOTES

1) Abbreviations: **T**, tirilazad; 2,4-DPP, 2,4-dipyrrolidinylpyrimidine