Synthesis of ^3H Labeled Dihydrorotenone

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SUMMARY

The catalytic tritiation of rotenone results in the preparation of two products, the expected tritiated 6',7'-dihydrorotenone ([^{3}H]DHR) and tritiated 6',7'-dihydrorotenol ([^{3}H]DHR-ol). The ratio of [^{3}H]DHR to [^{3}H]DHR-ol is 9 to 1. Reversed-phase HPLC provided the purified [^{3}H]DHR and [^{3}H]DHR-ol with estimated specific activities of 45 and 60 Ci/mmol, respectively.

KEYWORDS: Tritium, Dihydrorotenone, Dihydrorotenol, ^{3}H-NMR

INTRODUCTION

Rotenone 1 is a natural insecticide and fish poison found in the roots of tropical plants from the *Leguminosae* family. Rotenone toxicity is imparted by irreversible inhibition of Complex I (NADH-CoQ reductase, EC 1.6.99.3) of the mitochondrial electron transport chain (ETC). We have been interested in developing a radiotracer for Complex I density as the loss of Complex I has been linked to several neurologic disorders and implicated in Parkinson's and Huntington's diseases (1, 2) as well as the normal aging process.(3)

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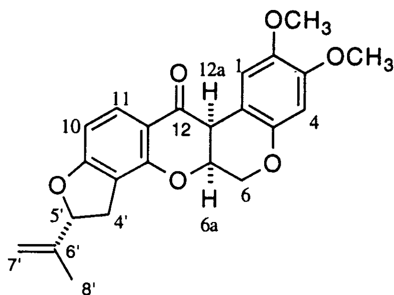
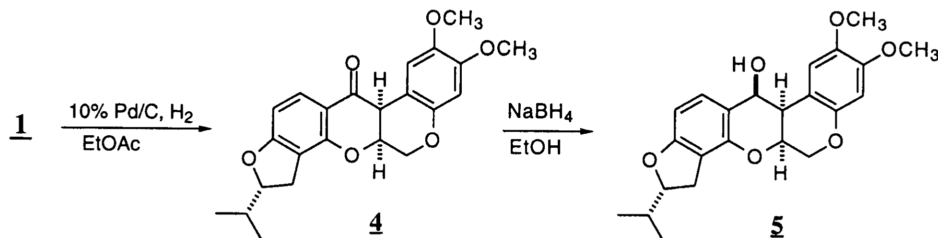


Rotenone 1

Tritiated 6',7'-dihydrorotenone ([^{3}H]DHR) 2 has been used to assay the density of Complex I in rat brain tissue (4), in intact human platelets (5), and in post-mortem Alzheimer's and normal human brain tissue.(6) It has also been used to identify the rotenone binding site on Complex I.(7) As part of our ongoing effort to evaluate radiolabeled derivatives of rotenone as potential Complex I imaging agents (8, 9) we synthesized [^{3}H]DHR as a standard for in vitro binding assays. While the use of commercially prepared [^{3}H]DHR from the catalytic tritiation of rotenone 1 has been reported (4, 7), full synthetic details have never been published. We report here the synthesis of [^{3}H]DHR, identification of a tritiated byproduct, proton NMR data and analyses of their respective ^{3}H-NMR spectra.

Scheme 1

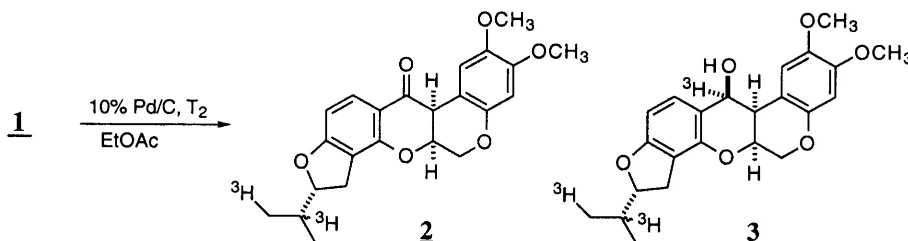


RESULTS AND DISCUSSION

A review of the literature revealed that several catalysts (Pd/C, platinum oxide, palladium-barium sulfate, Raney nickel) and a variety of solvents (acetone, benzene, butyl acetate, dichloromethane, ethanol, ethyl acetate) have been used in the catalytic hydrogenation of rotenone. (10-15) Most of the reactions gave a significant yield of the desired DHR. Some authors reported the exclusive production of DHR (11, 12) while others showed significant production of byproducts including rotenonic acid, dihydrorotenonic acid (13-15) and dihydrorotenol. (13, 14) Before performing the tritiation for this effort, we established the conditions for the production of the desired DHR as

shown in scheme 1. The catalytic hydrogenation of rotenone over 10% palladium on carbon in ethyl acetate at low hydrogen pressure yielded a single reduction product, 6',7'-dihydrorotenone **4** (DHR), in 94% isolated yield. The reaction was found to be >95% complete in under 20 minutes at room temperature.

Scheme 2



Unlike the catalytic hydrogenation, the reductive tritiation with 10% palladium on carbon in ethyl acetate gave two tritiated products, tritiated 6',7'-dihydrorotenone **2** ([^{3}H]DHR) and a second product in a 9:1 ratio (Scheme 2). The reduction byproduct was thought to be 6',7'-dihydrorotenen-12-ol, the result of reduction of the 12-ketone functionality. In order to positively identify this second product, DHR was reduced at room temperature with sodium borohydride in ethanol and purified to provide 6',7'-dihydroroten-12-ol **5** (DHR-ol) (Scheme 1) in 75% isolated yield. Coinjection of DHR-ol **5** and the crude tritiation mixture on reversed-phase HPLC confirmed that the byproduct was indeed [^{3}H]DHR-ol. Comparison of the DHR-ol **5** proton NMR with the proton NMR of the crude tritiation mixture corroborated the identification of [^{3}H]DHR-ol. This over-reduction product had not been previously identified in the catalytic hydrogenation of rotenone over palladium on carbon in acetone, dichloromethane or ethyl acetate (11, 12) including our own reaction where the reagents and solvents were proportional to those used in the tritiation reaction. It is also interesting to note that the only production of DHR-ol during hydrogenation was in butyl acetate with Ni turnings as the catalyst. (13, 14)

Tritium NMR analysis of the labeled product showed three multiplets, centered at 1.51 (13%), 0.75 (44%) and 0.59 ppm (43%). These signals are attributed to tritium incorporation at the 6' (exocyclic methine), 7' and 8' (methyl) positions. There are several features of the tritium spectra to which we would like to draw attention. First, the bulk of the tritium incorporation is in the combined methyl positions, relative to the methine (87:13). Although not rigorously explained, this uneven

incorporation of tritium across unsymmetrical double bonds is often observed, dating from the first high resolution tritium NMR spectrum, analyzing the catalytic tritiation of phenylacetylene.(16) Second, the tritium is incorporated equally into both methyl groups, which have distinct chemical shifts, despite starting from a single vinylic precursor. Third, each multiplet represents a series of isotopomers having different numbers of tritium atoms in the methyl groups and methine positions. Such complex tritium spectra from a seemingly simple hydrogenation are also well-known, and a thorough analysis of an analogous tritiation product has been published.(17)

Preparative separation of the two tritiation products was accomplished by reversed-phase HPLC. The specific activities of the [^{3}H]DHR and [^{3}H]DHR-ol were measured by HPLC and calculated to be 45 Ci/mmol and 60 Ci/mmol, respectively.

EXPERIMENTAL

All solvents and reagents were used as received from the various manufacturers. Tritium gas (97.9%) was purchased from EG&G Mound Applied Technologies, Miamisburg, OH. HPLC of non-radioactive products was performed on a Waters 590 liquid chromatograph equipped with an LDC UV absorbance detector set at 295 nm. HPLC of tritiated products was performed on a Waters Model 510 liquid chromatograph with a Supelco LC-18 column (.25 x 25 cm) and a mobile phase of 50:50 acetonitrile/water (v/v). Mass detection was performed using a Hewlett Packard 1040A diode array spectrophotometer set at 295 nm and radioactivity was monitored by an in line INUS β RAM HPLC flow detector using a lithium glass solid scintillator with an efficiency of about 0.1%. Proton and tritium NMR spectra were obtained on a Bruker AC-300 NMR spectrometer. ^{1}H (at 300 MHz) and ^{3}H (at 320 MHz) spectra were recorded in C_6D_6 using a 5 mm ^{3}H/^{1}H dual probe. Samples were made to a volume of about 250 μL in Teflon tubes (Wilmad #6005), which were then placed inside 5 mm glass NMR tubes having a screw-cap (Wilmad 507-TR-8"). Spectra were acquired at 298 °K. Liquid scintillation counting was performed with a Packard 1500 liquid scintillation counter using Optifluor cocktail. Specific activities were determined by HPLC, comparing UV absorbance with that of a standard sample followed by liquid scintillation counting of the isolated HPLC peak effluent.

6',7'-Dihydrorotenone (4) To a magnetically stirred, room temperature solution of rotenone (0.25 mmol, 100 mg) in 2.5 mL ethyl acetate was added 10% palladium on carbon (10 mg). The reaction vial was then capped with a septa and twice vacuum degassed and backfilled with

hydrogen gas. The reaction was allowed to proceed under balloon pressure of hydrogen and was monitored by TLC with no apparent change in R_f over a one hour period. The ethyl acetate solution was filtered through a short plug of celite/silica and the solvent removed in vacuo to provide a glassy solid (95 mg, 94% based on the desired product) which was analyzed by reversed-phase analytical HPLC (Waters μbondaPak-C_{18}, 3.9 x 300 mm, isocratically eluted at 1 mL/min. with 60/40 acetonitrile/water; t_R=18.3 min. for rotenone and 22.0 min. for dihydrorotenone) and found to contain only the dihydrorotenone product. A second reaction was run on a 10 mg scale and was shown to be >95% complete as determined by HPLC analysis of an aliquot withdrawn at 20 min. 300 MHz ^1H NMR (C_6D_6) δ ppm 8.15 (d, J = 8.5, 1 H, C_{11}-H), 7.21 (s, 1 H, C_1-H), 6.54 (s, 1 H, C_4-H), 6.38 (d, J = 8.5, 1 H, C_{10}-H), 4.29 (dd, J = 12.0, 2.8, 1 H, C_6-αH), 4.05 (m, 2 H, C_{6a}-H, $C_{5'}$-H), 3.59 (d, J = 3.9, C_{12a}-H), 3.50 (d, J = 12.0, C_6-βH), 3.38 (s, 3 H, OCH_3), 3.24 (s, 3 H, OCH_3), 2.23 (m, 2 H, $C_{4'}$-CH_2), 1.19 (dqq, J = 6.7, 6.7, 6.6, 1 H, $C_{6'}$-H) , 0.80 (d, J = 6.6, 3 H, $C_{7'\text{ or }8'}$-CH_3), 0.64 (d, J = 6.7, 3 H, $C_{8'\text{ or }7'}$-CH_3). 75 MHz ^{13}C NMR (C_6D_6) δ ppm 189.0-C_{12}, 167.7-C_9, 157.9-C_{7a}, 149.5-C_3, 147.4-C_{4a}, 143.9-C_2, 129.8-C_{11}, 113.4-C_{11a}, 113.0-C_8, 110.4-C_1, 104.9-C_{12b}, 104.8-C_{10}, 100.9-C_4, 90.6-$C_{5'}$, 72.2-C_{6a}, 66.3-C_6, 56.3-OCH_3, 55.9-OCH_3, 44.6-C_{12a}, 33.2-$C_{6'}$, 29.4-$C_{4'}$, 17.9-$C_{7'}$, 17.6-$C_{8'}$.

6',7'-Dihydroroten-12β-ol (5) A room temperature, magnetically stirred solution of dihydrorotenone (0.038 mmol, 15 mg) in 2.5 mL of absolute ethanol was treated with an excess of solid sodium borohydride (0.38 mmol, 14 mg). The reduction was monitored by TLC and found to be complete within 5 min. The reaction was quenched by adding the solution to aqueous acid (15 mL, 0.1 N HCl). After the addition of 10 mL of brine, the aqueous solution was extracted with diethyl ether (3 x 20 mL) and the combined extracts were dried over magnesium sulfate. The ether solution was passed through a short column of silica gel (15 mm x 20 mm) and the solvent removed in vacuo to provide the desired alcohol as a slightly yellow oil (17 mg, 113 %). 6',7'-Dihydroroten-12β-ol was purified by preparative reversed-phase HPLC (Whatman ODS-3, 9mm x 50 cm column eluted isocratically at 5 mL/min. with 60/40 acetonitrile/water). A 12 mg sample was purified and found to elute at 10.3 min. under the above conditions to provide 8 mg of pure 6',7'-Dihydroroten-12β-ol after extraction from the aqueous solvent with diethyl ether. 300 MHz ^1H NMR (C_6D_6) δ ppm 6.96 (d, J = 8.1, 1 H, C_{11}-H), 6.59 (d, J = 8.1, 1 H, C_{10}-H), 6.53 (s, 1 H, C_1-H), 6.51 (s, 1 H, C_4-H), 4.87 (dd, J = 10.1, 10.1, 1 H, $C_{6\alpha}$ or β –H), 4.66 (m, 1 H, C_{6a}-H), 4.56 (d, J = 3.7, 1 H, C_{12}-H), 4.29 (m, 1 H, $C_{5'}$-H), 4.24 (dd, J = 11.3, 10.1, 1 H, $C_{6\beta}$ or α–H), 3.52 (s, 3 H,

OCH_3), 3.30 (s, 3 H, OCH_3), 2.94 (d, J = 9.4, 1 H, C_{12a}-H), 2.89 (m, 2 H, $C_{4'}$-CH_2), 1.71 (dqq, J = 6.7, 6.7, 6.7, 1 H, $C_{6'}$-H) , 0.94 (d, J = 6.7, 3 H, $C_{7' \text{ or } 8'}$-CH_3), 0.77 (d, J = 6.7, 3 H, $C_{8' \text{ or } 7'}$-CH_3).

6'-[^{3}H],7'-[^{3}H]-Dihydrorotenone (2) A solution was prepared consisting of rotenone (0.025 mmol, 10 mg), 10% Pd/C (10 mg) and ethyl acetate (1 mL). The solution was frozen with liquid nitrogen and thawed under an atmosphere of dry nitrogen for three cycles. The solution was then twice cycled through a freeze-thaw degas process followed by a repeat of the initial freeze and dry nitrogen atmospheric thaw process three additional cycles. The reaction vessel was cooled to liquid nitrogen temperature and tritium gas was transferred from a UT_3 source to the reaction vessel to a final pressure of 550 mm Hg. The reaction was warmed to room temperature providing a vessel pressure of ca. 1 atmosphere and the incorporation reaction allowed to proceed for 1 h. The tritium was then transferred back to the source and the solvent removed in vacuo. The substrate was then treated with 1 mL of methanol followed by in vacuo drying and the process repeated to remove any exchangeable tritium. The catalyst was filtered off through a porosity C glass fiber filter and the catalyst washed with 5 mL ethyl acetate. The crude tritiation product was isolated by lyophilization to provide 1.52 Ci of product which was found to contain a 9:1 ratio of the desired dihydrorotenone 2 as well as the dihydrorotenol 3 over reduction byproduct. 6'-[^{3}H],7'-[^{3}H]-Dihydrorotenone was isolated with a retention time of 11.5 min. from the crude product by reversed phase HPLC on a Supelco LC-18 25 x 250 mm column eluting isocratically at 1 mL/min. with 50/50 acetonitrile/water while monitoring radioactivity and UV. Specific activity was calculated to be 45 Ci/mmol by comparison to an absorbance standard of non-radioactive dihydrorotenone. 320 MHz ^{3}H NMR (C_6D_6) δ ppm 1.53 (mult., 13.3% incorporated activity), 0.75 (mult, 44.2%), 0.59 (mult, 42.5%).

6'-[^{3}H],7'-[^{3}H]-Dihydroroten-12β-ol (3) The alcohol was prepared as an over reduction byproduct from the above tritium incorporation. Isolation by HPLC from the crude reaction mixture was as described above. The alcohol had a retention time of 9.5 min. and a calculated specific activity of 60 Ci/mmol. 320 MHz ^{3}H NMR (C_6D_6) δ ppm 4.51 (s, 29.1% incorporated activity), 1.68 (mult, 9.3%), 0.89 (mult, 31.4%), 0.72 (mult, 30.2%).

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