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# Synthesis of β-$^3$H-Mitotane for Use in a Rapid Assay for Mitotane Metabolism

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## SUMMARY

A $^3H^+$-release method has been developed for the assay of β-hydroxylation of the adrenolytic drug mitotane. β-$^3$H-mitotane was synthesized by the reduction of 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethane by an aluminium-$Hg_2Cl_2$ couple in the presence of $^3H_2O$. For β-hydroxylation of mitotane, the $^3H^+$-release assay is more efficient and sensitive than a method utilizing $^{14}$C-mitotane and chromatographic separation of metabolites by HPLC. The $^3H^+$-release assay has been used to evaluate the ability of adrenal tumors to metabolize mitotane via the β-hydroxylation route.

Key words: Mitotane, tritiated, deuteriated; tritium release assay; adrenocortical cancer.

## INTRODUCTION

Mitotane (*o,p'*-DDD) is used for the treatment of adrenal carcinoma and Cushing's disease (1,2). In regard to the biochemical mechanism of action by which mitotane causes its adrenolytic effect or interferes with steroid synthesis, our studies to date (3) and the

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literature indicate that a mitochondrial cytochrome P-450 is involved in mitotane metabolic transformations (4). We have previously identified *o,p'*-DDA as a major metabolite of mitotane (5) and proposed (3) its formation by a P450-catalyzed hydroxylation of mitotane at the β-carbon to give an unstable *gem* dichloro-hydroxymethyl metabolite. Subsequent dehydrochlorination of the hydroxylated product will form the corresponding acyl chloride that, in the presence of water, will form the acidic metabolite, *o,p'*-DDA, or could bind to tissue nucleophiles (Fig. 1).

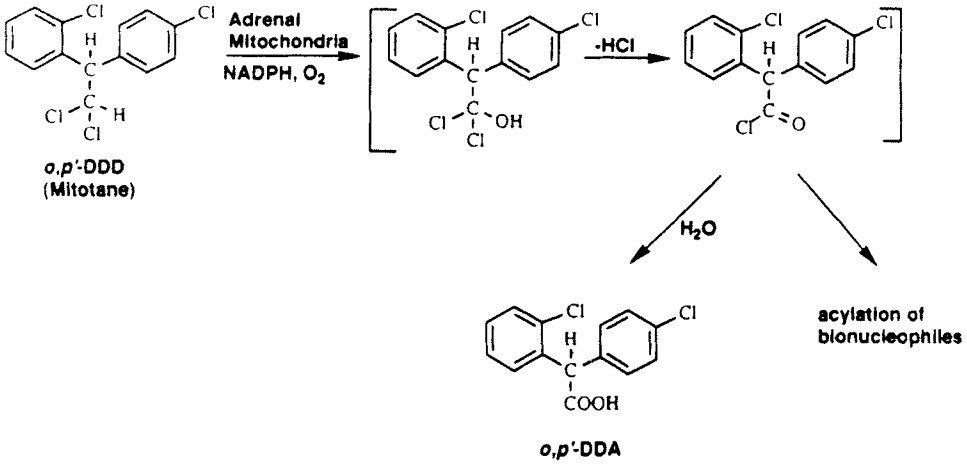


Fig. 1 Proposed metabolic pathway for mitotane with β-hydroxylation.

To further study the role of β-hydroxylation in the activation of mitotane, the enzyme(s) involved in this metabolic route and to evaluate the ability of adrenal tumors to activate mitotane *via* β-hydroxylation, a rapid and specific assay for the β-hydroxylation of mitotane has been developed. The method is comparable to the $^{3}H$-release method developed for the assay of aromatase (6,7) and lanosterol 14α-demethylase (8) in which a P450-catalyzed oxidative reaction results in the oxidation of a specific C-H bond releasing the hydrogen atom to the solvent medium as a proton. After organic solvent extraction of the unreacted substrate the amount of $^{3}H^{+}$ released to the aqueous media can be readily determined.

## RESULTS AND DISCUSSION

The reaction conditions required to label mitotane at the desired position were developed using deuterated reagents and evaluation of the reaction products by $^{1}H$-NMR.

Attempts to deuterate the β- position by the addition of $^2H_2O$ to a reaction of mitotane and n-butyllithium (9) or by selective reduction of the iodo group from β-I-*o,p'*-DDD were unsuccessful. In the former reaction, only low incorporation of deuterium was achieved in a process where labelled mitotane could not be separated from starting mitotane. In the second approach, reaction of β-I-*o,p'*-DDD with $NaCNBH_3$ resulted primarily in dehydroiodination to yield 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethylene.

The successful synthesis of β-$^3$H-*o,p'*-DDD was based upon the approach used by Gold and Brunk (10) to synthesize 1,1-bis(4-chlorophenyl)-2-$^2$H-2,2-dichloroethane from 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethane (*p,p'*-DDT). Our reaction conditions with *o,p'*-DDT were developed with $^2H_2O$ in anticipation of the use of high specific activity $^3H_2O$ instead of their use of $^2H_4$-methanol. Accordingly the solvent system chosen was dry THF which maintained the *o,p'*-DDT, $Hg_2Cl_2$ and $^2H_2O$ in solution. It was determined that an excess of $^2H_2O$ in the order of 65 mole to 1 mole of *o,p'*-DDT was required to obtain the maximal yield of deuterated mitotane. NMR analysis of the deuterated reaction product showed a singlet at δ 5.19 for the aliphatic proton in place of a singlet at 5.68 for *o,p'*-DDT and doublets at 6.34 and 5.15 for *o,p'*-DDD. Chemical yields of 50-60% and the NMR integration indicated 94-97% incorporation of deuterium. Mass spectral analysis of the deuterated mitotane indicated 97% deuterium incorporation based upon the signal intensities at *m/z* 318 to 319 peaks which were 0.2 and 5.9 respectively. When M was set to 100%, mass spectral analysis also yielded values of M + 2 (123%), M + 4 (61%), and M + 6 (14%), peaks characteristic of a tetrachlorinated compound. After loss of the $CDCl_2$ side chain to yield an ion of *m/z* 235, the residual fragmentation pattern was identical to that observed for mitotane.

When the reaction was run using $^3H_2O$ (18 mCi/mmol) as the proton source, only a trace of the starting *o,p'*-DDT could be detected at the completion of the reaction. After purification by TLC, the calculated specific activity for the product was 8.36 mCi/mmol, with a chemical yield of 53%. The radioisotopic purity of the final product was measured by C-18 reversed phase HPLC with a 65:35 acetonitrile:water solvent system and 97% of the radioactivity co-chromatographed with reference mitotane. Also, 99% of the activity co-chromatographed with mitotane on silica TLC with hexane ($R_f$ 0.38) or with pyridine ($R_f$ 0.86) as the solvents. On a reverse phase TLC system with acetonitrile:water:acetic

acid (90:10:0.2, pH=3.5), 99% of the activity co-chromatographed ($R_f$ 0.55). The high percent of radioactivity that co-chromatographed in the basic and acidic solvent systems is also an indication of the stability of the $^3$H-C bond.

The validity of using $^3H^+$-release from β-$^3$H-mitotane in assays for mitotane metabolism was examined by comparing the results of this method with that of the literature for the subcellular distribution of mitotane metabolism in dog and bovine adrenal cortex preparations (11,12 ). The results, after incubation with 1 mg of protein, were as follows: mitochondria, 133.71 ± 2.51; cytosol, 0.85 ± 0.22; microsomes, 5.42 ± 0.48 pmol/min/mg protein. The predominant activity in the mitochondria is in agreement with the literature. In other experiments, as summarized in Table 1, the results for mitotane metabolism using the $^3$H-release assay were also compared to incubations with 1-(2-chlorophenyl)-1-(4-chlorophenyl-$^{14}$C)-2,2-dichloroethane ($^{14}$C-mitotane, 83.2 μCi/mmol) (13) using a $^{14}$C-HPLC assay (14). The activities determined with the $^3$H-release assay correlated well with the values obtained with the $^{14}$C-HPLC assay when both free and bound metabolites are considered for the latter method.

Table 1. The comparison of β-$^3$H-mitotane and $^{14}$C-mitotane-HPLC results

| Enzyme Source | Percent Transformation | | |
|---|---|---|---|
| | $^3H^+$-released[a] | $^{14}$C-HPLC | |
| | | metabolites[b] | bound[a] |
| Acetone Powder[c] | 8.34 ± 0.18[d] | 6.34 ± 0.66 | ____[e] |
| Human Adrenal Cortex | 0.51 ± 0.03[f] | 0.25 ± 0.04 | 0.21 ± 0.04 |
| Adrenal Tumor I | 0.30 ± 0.02[f] | 0.18 ± 0.02 | 0.14 ± 0.02 |
| Adrenal Tumor II | 0.01 ± 0.002[f] | ____[e] | ____[e] |

[a] Percent of total radioactivity used for the incubation ± S.D. (N=3).
[b] Percentage of total radioactivity injected to HPLC that co-chromatographed with *o,p'*-DDA (N=3).
[c] Acetone powder of bovine adrenal mitochondria (15).
[d] Assay conditions: [β-$^3$H-mitotane] = 80 nmol, time = 30 min, protein = 2.65 mg, $volume_{total}$ = 1 mL.
[e] Not determined.
[f] Assay conditions: [β-$^3$H-mitotane] = 80 nmol, time = 20 min, protein = 6 mg $volume_{total}$ = 1 mL.

Based upon our hypothesis that mitotane requires metabolic activation to act as an adrenolytic agent, it is clinically important to establish the ability of human adrenal tumors to metabolize mitotane. The $^3$H-release assay provides a more efficient and sensitive method than the $^{14}$C-HPLC assay to monitor the capacity of adrenal tumors to promote β-oxidation of mitotane. That is, a major advantage of the $^3$H-release method to measure mitotane metabolism is the rapidity of the assay. For example, a typical assay utilizing the $^3$H-release method took between 2-3 hrs for completion and between 20-40 reactions can be processed in a single experiment. In contrast the $^{14}$C-HPLC assay previously used required two days to process four samples. In addition, there is a significant increase in the sensitivity of the $^3$H-release assay due to the higher specific activity readily obtainable for the $^3$H-reagent versus a $^{14}$C-mitotane reagent.

## EXPERIMENTAL

### *Chemicals*

1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD, mitotane) and 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2,2-tichloroethane (*o,p'*-DDT) were purchased from Aldrich Co. (Milwaukee, WI). The cofactors glucose-6-phosphate, glucose-6-phosphate dehydrogenase and $NADP^+$ were purchased from Sigma Chemical Co. (St. Louis, Mo).

**1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloro-2-iodoethane (β-I-*o,p'*-DDD):** Diisopropylamine (0.5 mL, 3.4 mmol) was added to a reaction flask containing dry THF (3.3 mL) and anhydrous ether (6.7 mL) under a nitrogen atmosphere at -5°C. n-Butyllithium (1.72 mL, 3.4 mmol) was added and after 10 min the temperature was lowered to -78°C (dry ice/acetone). After 30 min, mitotane (1.0 g, 3.13 mmol) dissolved in ether (6.7 mL) was added dropwise to the cooled reaction mixture. The reaction was stirred for an additional 50 min followed by addition of n-iodosuccinimide (1.5 g, 6.7 mmol) in THF (5 mL). After 15 min, the reaction was quenched by the slow addition of distilled water (2 mL). The reaction mixture was allowed to warm up to room temperature and hydrochloric acid (1.0 N, 2 mL) was added. The layers were separated and the remaining aqueous phase was extracted with ether (3 x 10 mL). The crude product was purified by column chromatography (silica with hexane). This product

showed the following properties: 1H NMR ($CDCl_3$) 5.82δ (s, 1H, -C**H**-$CCl_2I$) 7.1-7.6 (m, 8H, aryl **H**). Mass spectrum (70 eV) *m/z* (rel intensity) 446 (0.3), 444 (0.2), 410 (0.6), 408 (0.7), 323 (17.8), 321 (54), 319 (100), 317 (81.2), 283 (66.7), 281 (66.3), 248 (40.4), 246 (55.1), 237 (40.3), 235 (54.8), 199 (22.8), 176 (41), 165 (45.9). *Anal* Calcd for $C_{14}H_9Cl_4I$: C, 37.67; H, 2.02. Found: C, 38.06; H, 1.83

**β-$^3$H-mitotane:** Under anhydrous conditions, *o,p'*-DDT (15 mg; 0.042 mmol), mercuric chloride (1.5 mg; 0.0055 mmol) and aluminum foil (10 mg; 0.371 mmol) were added to a reaction vial. The vial was sealed and dry THF (400 µL) was added to dissolve *o,p*-DDT and mercuric chloride, followed by $^3H_2O$ (50 mCi in 50 µL; 2.78 mmol). The reaction was maintained overnight with stirring at 50-55°C in an oil bath. At the end of the reaction time, the reaction vial was centrifuged and the solution transferred via a Hamilton gas-tight syringe to a septum-capped glass tube. The residue was washed successively with hexane (3 x 200 µL). The combined hexane extracts and solution were back-extracted with water (3 x 300 µL) and the organic layer dried over magnesium sulfate. The organic layer was evaporated under nitrogen and the residue purified by TLC (silica, hexane) resulting in a 53% yield (8.36 mCi/mmol). NMR analysis and mass spectrum of the deuterated compound synthesized in preliminary experiments by the same procedure but with $^2H_2O$ showed 94-97% incorporation of deuterium at the β-carbon with yields of 50-60%. NMR (300 Mz, $CDCL_3$) δ 5.19 (s, 1H, -C**H**-$CDCl_2$), 7.1-7.45 (m, 8H, aryl **H**), 94-97% deuterium incorporation. Mass spectrum (70 eV) *m/z* (rel intensity) 326(0.1), 325(0.8), 324(0.6), 323(3.6), 322(1.3), 321(7.2), 320(1.0), 319(5.9), 318(0.2), 235(100), 199(20), 165(41.4), 89(8.9).

**Enzymatic assays with β-$^3$H-Mitotane:** A 15-mL centrifuge tube contained β-$^3$H-mitotane (80 nmol; 1.14 mCi/mmol) solubilized with the aid of Tween 80, 11 µmol glucose-6-phosphate, 0.7 units glucose-6-phosphate dehydrogenase, 5.5 µmol $MgCl_2$, 50 µmol HEPES buffer, 0.92 µmol $NADP^+$ and the appropriate amount of protein as indicated in the text and Table 1, in a final volume of 1 mL. The reaction was started by the addition of $NADP^+$ and samples were incubated for 20 min. The reaction was stopped by addition of 5 mL of chloroform to each tube and tubes were vigorously mixed for 1 min and then centrifuged for 10 min at 3000xg. An aliquot of the aqueous phase (800 µL) was transferred to another tube and Norit A in 200 µL of HEPES buffer (pH 7.4) was added. The suspension was shaken for 20 sec, the samples allowed to stand on

ice for 25 min and then centrifuged for 10 min. An 800-μL aliquot was removed and added to 6.5 mL of Ecolite scintillation fluid and counted with an LS 5000 scintillation counter. Results were expressed as pmol/min/mg protein or percent transformation.

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