TRITIUM LABELED DIFLORASONE DIACETATE

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SUMMARY

Diflorasone diacetate* (III) is catalytically reduced with tritium gas
to give the A-ring saturated 1,2,4,5-tetratritiated compound. The
double bonds in the 1,2- and 4,5-positions are restored by dehydrogen-
ation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The
tritium labeled diflorasone diacetate thus prepared has a specific
activity of 7.69 Ci/mM after chromatographic purification. The low
tritium retention indicates that the introduction of tritium during
reduction and its removal during dehydrogenation occurred predominantly
from the same side of the steroid molecule. The $\Delta^{1,4}$ product and the
Δ^1 and Δ^4 by-products are readily separated by thin-layer chroma-
tography (tlc) on silica gel plates impregnated with ferric chloride.

Key Words: Tritium, Diflorasone Diacetate, Purification

INTRODUCTION

Diflorasone diacetate is a potent anti-inflammatory agent in topical treat-

ment of acute eczematous dermatoses. This communication describes the prepara-

tion of tritium labeled diflorasone diacetate for conducting in vitro membrane

diffusion studies and in vivo percutaneous absorption, metabolism, and excretion

studies in test animals and man.

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*Generic name for FLORONE® (The Upjohn Co.), $6\alpha,9\alpha$-difluoro-$11\beta,17\alpha,21$-trihyd-
roxy-16β-methylpregna-1,4-diene-3,20-dione 17,21-diacetate.

The reduction of A-ring double bonds in Δ^1 and $\Delta^{1,4}$-3-keto steroids with tritium gas, followed by the regeneration of double bonds with an oxidizing agent, such as selenium dioxide or DDQ, represents a convenient two-step procedure for introducing non-exchangeable tritium into A-ring unsaturated 3-keto steroids. The degree of success of this method depends on the stereochemical course of the reduction step, since dehydrogenation occurs predominantly from the α-face of the molecule (1). Thus, selective reduction of the 1,2-double bond in 17β-hydroxyandrosta-1,4-diene-3-one with tritium gas introduces tritium predominantly from the β-face. Oxidation at C-17 followed by removal of the labile label at C-2 by exchange and dehydrogenation with DDQ from the α-face leads to androsta-1,4-diene-3,17-dione labeled at C-1 with good retention of tritium. In contrast, the same product, obtained from tritium reduction of androst-1-ene-3,17-dione followed by exchange at C-2, introduction of 4,5-double bond, and DDQ treatment, has a much lower specific activity, because in this case entry of tritium occurs predominantly from the α-face (2).

Tritium has also been incorporated into the 1,2 and 4-positions of $\Delta^{1,4}$-3-keto steroids using this reduction-oxidation procedure, in which both the 1,2 and 4,5-double bonds are reduced before the dehydrogenation. Examples are 1,2,4-tritiated 9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione 21-acetate (3) (Ia) and 9α-fluoro-11β,17α-21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione 21-acetate (4) (Ib). Evidently, entry of tritium from the α-face predominates in both instances, since retention of tritium is low. Introduction of tritium from the β-face is enhanced by carrying out selective A-ring reduction on the $\Delta^{1,4,9\,(11)}$-3-keto system. Thus, Wren, *et al* (5) obtained tritiated 17α,21-dihydroxy-6α-fluoro-16α-methylpregna-1,4,9(11)-triene-3,20-dione 21-acetate (IIa) with a specific activity two to three times greater than those of Ia and Ib.
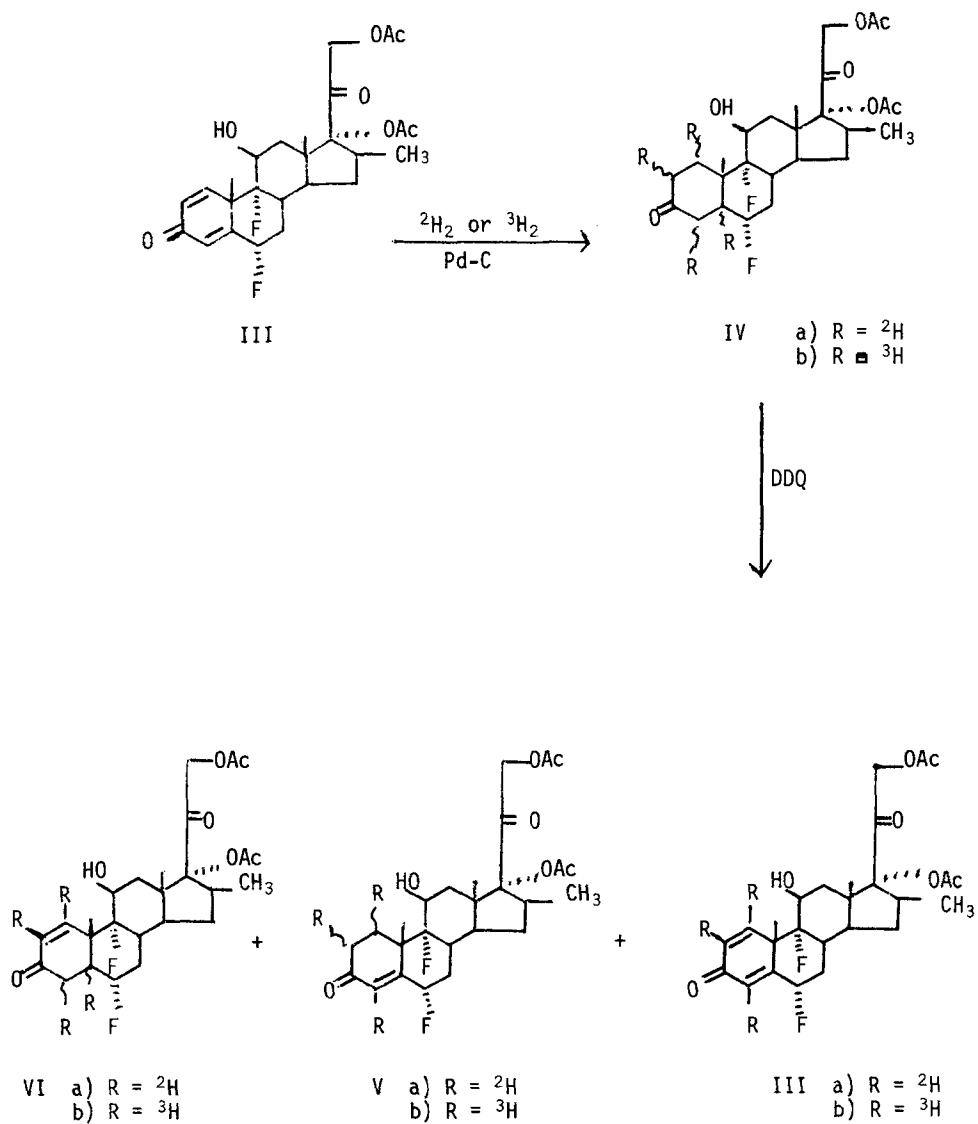
I a) R = α-CH₃
   b) R = β-CH₃
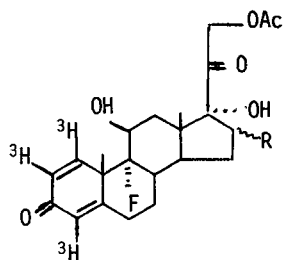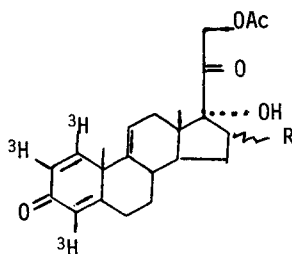
II a) R = α-CH₃
   b) R = β-CH₃

Compound IIb or the corresponding 17,21-diacetate would serve as a suitable intermediate for preparing tritium labeled III, since the 9,11-double bond would provide an entry to the desired 9α-fluoro and 11β-hydroxy functions. However, direct reduction of III with tritium gas followed by dehydrogenation with DDQ would be a shorter approach, albeit the resulting product would be expected to have a lower specific activity. Because of the large amount of tritium which can be incorporated during reduction, even relatively low retention during dehydrogenation can still provide a product with high enough specific activity suitable for the anticipated membrane diffusion and ADME studies. We therefore chose to label III by the direct approach.

In several trial experiments carried out to determine the stability and behavior of III and its A-ring saturated analog under the reaction conditions, the deuterium label was used to estimate the extent of isotope incorporation.

III

IV   a) R = ^{2}H
      b) R = ^{3}H

$\xrightarrow{\text{^{2}H$_2$ or ^{3}H$_2$}}$ over Pd-C

DDQ

VI   a) R = ^{2}H
     b) R = ^{3}H

V   a) R = ^{2}H
     b) R = ^{3}H

III a) R = ^{2}H
     b) R = ^{3}H

Reduction of III in dioxane at atmospheric pressure and room temperature with deuterium gas over 10% palladium on charcoal catalyst afforded the tetra-deuterated IVa in 95% yield. IVa was treated with an excess (3 to 5 fold) of DDQ in dioxane at 100-105° to afford IIIa and the Δ^1 compound VIa in 14.3% and 13.1% yields, respectively, after chromatographic purification (silica gel column eluted with 10% v/v Me$_2$CO-benzene). Trace amounts of the corresponding Δ^4 compound (Va) were also detected in the dehydrogenation mixture. The proton magnetic resonance (pmr) spectrum of IIIa indicated that it was approximately 10% deuterated at C-1, C-2, and C-4.

During the dehydrogenation, disappearance of IVa was complete in about 20 hours. Examination of the reaction mixture by tlc at this point showed presence of primarily the $\Delta^{1,4}$ and Δ^1 compounds in addition to DDQ-related materials. Further prolonged heating and addition of fresh DDQ decreased the yield of the Δ^1 compound but did not significantly increase the yield of the $\Delta^{1,4}$ product. In a separate experiment, treatment of VIa with DDQ at 100°C led only to tarry unidentifiable materials and no significant amounts of IIIa. The Δ^4 compound, on the other hand, underwent dehydrogenation with DDQ to give IIIa. These results would suggest that IVa is converted to VIa, and, either directly to IIIa or first to Va, which is further dehydrogenated to IIIa. The sequence of IVa → VIa → IIIa, if it is operative at all, cannot be a major reaction path. Also, the yields of IIIa (14.3%) and VIa (13.1%) from IVa would suggest a 1:1 ratio of the 5α and 5β isomers, since it has been reported (6) that dehydrogenation of 5α-3-keto-steroids with DDQ gives Δ^1-3-keto compounds while 5β-3-keto-steroids are converted to Δ^4-3-keto compounds.

Two tlc systems were used to monitor reaction mixtures and analyze products: silica gel GF coated plates and silica gel GF coated plates impregnated with FeCl$_3$ (7), both developed with 20% v/v Me$_2$CO in benzene. On the plain silica gel plate, the motilities of the A-ring saturated (R$_f$ 0.59) and the Δ^1 (R$_f$ 0.58) compounds are virtually identical. Both compounds are well resolved from the Δ^4

(R_f 0.48) and $\Delta^{1,4}$ (R_f 0.44) compounds, which are only partially resolved from one another. However, ultraviolet (uv) illumination readily distinguishes the uv absorbing Δ^1 compound from the A-ring saturated compound. The latter is detected by exposure to I_2 vapor. The $FeCl_3$ impregnated plate provides excellent resolution among the Δ^1 (R_f 0.56), Δ^4 (R_f 0.50), and $\Delta^{1,4}$ (R_f 0.42) compounds with detection by charring with phosphomolybdic acid. An attempt to effect separation of these compounds preparatively with a silica gel-$FeCl_3$ column was unsuccessful because of massive elution of $FeCl_3$.

The preparation of tritiated III was based on the procedures developed for deuterium labeled III. Compound III was reduced with carrier-free tritium gas in dioxane over 10% palladium on charcoal catalyst at room temperature and dehydrogenated with DDQ. The resulting crude mixture was subjected to repeated chromatographic purification (silica gel columns eluted with 10% v/v Me_2CO in benzene) to give IIIb which was 98.5% radiochemically pure by tlc (silica gel-$FeCl_3$, 20% v/v Me_2CO in benzene) and contained <1% each radiochemically of the Δ^1 (VIb) and Δ^4 (Vb) materials. The specific activity of IIIb, 7.69 Ci/mM, compared well with those of compounds reported in the literature prepared in a similar manner, *e.g.*, 6.55 Ci/mM for Ia(3) and 9.07 Ci/mM for Ib(4). Successive dilutions of IIIb with non-radioactive material gave crystalline products with specific activities of 2.14 Ci/mM and 0.183 Ci/mM.

EXPERIMENTAL

Radioactivity determinations were carried out in Diotol cocktail (Burdick-Jackson) on a Packard Tri-Carb Model 2425 liquid scintillation spectrometer by the external standard method. Melting points were obtained using a Thomas-Hoover Unimelt and were uncorrected. Uv spectra were obtained with a Cary Model 15 spectrometer and pmr spectra with a Varian A-60A spectrometer using tetramethyl-

silane as internal standard. Thin-layer chromatography plates were analyzed with a Vanguard Model 880 Autoscanner equipped with a Model 885 Glass Plate Scanner. The ratios of the radioactive components on the plates were determined by scraping the zones into Diotol cocktail containing 3% H_2O for counting.

The tlc plates used were 2.5 x 10 cm. or 5 x 20 cm. glass plates coated with a 250 μm thick layer of silica gel GF (Analtech, Inc.) or silica gel GF impregnated with $FeCl_3$*. All plates were developed with 20% v/v Me_2CO in benzene. With the smaller plates, best results were obtained after a development period of 20-30 min, during which the solvents were allowed to evaporate over the top end of the plate. The spots on silica gel GF plates were visualized by either illumination with uv light (254 nm) or exposure to I_2 vapor. The $FeCl_3$ impregnated plates were sprayed with a 10% solution of phosphomolybdic acid in ethanol and heated either in an oven at 100°C for 15 min or on a hot plate for about 1 min to give deep blue spots. Chromatographic columns were packed with silica gel, 70-230 mesh (Brinkman Cat. No. 7734).

$6\alpha,9\alpha$-Difluoro-11β,17α,21-trihydroxy-16β-methylpregnane-3,20-dione-1,2,4,5-^{2}H$_4$ 17,20-Diacetate (IVa)

A mixture of 25 ml of dioxane and 371 mg of 10% Pd-C was stirred at room temperature and atmospheric pressure under deuterium gas. The deuterium uptake in 1 hr was 9.8 ml. To the prereduced mixture was added a solution of 371 mg (0.75 mmole) of III in 5 ml of dioxane. Reduction with deuterium gas was continued, and the uptake of 37.8 ml (Th. 33.6 ml) was complete in 2 hrs. The stirring was stopped at 2.5 hrs and the mixture was kept under deuterium gas

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*$FeCl_3$ impregnated plates were prepared by thrice dipping the silica gel plates in a solution of 100 g of $FeCl_3 \cdot 6H_2O$ in 1 ℓ of Me_2CO and air drying for 15 min, followed by 20 min of drying at 100°C. The dark brown plates were stored over $CaSO_4$ (Drierite).

overnight. The catalyst was filtered and washed with 15 ml of benzene. The
combined filtrate and washings were concentrated at 50°C and 75 mm Hg pressure
to give 900 mg of syrup which crystallized at room temperature on standing. The
supernatant liquid was removed and the crystals were rinsed with 2 ml of dioxane,
2 x 2 ml of Et_2O and finally several portions of Skellysolve B (SSB). The crys-
tals were filtered, washed with SSB, and dried, 356 mg, 95.2% yield; m.p. 226-
227°C (dec); tlc showed presence of a single component; pmr spectrum compatible
with structure IVa with 75% deuteration among C-1, C-2, C-4, and C-5; m/e 502
(0.5)*, 501 (1.0), 500 (0.9), 499 (0.4).

6α,9α-Difluoro-11β,17α,21-trihydroxy-16β-methylpregn-1-ene-3,20-dione-1,2,4,5-$^2H_4$ 17,20-Diacetate (VIa)

A slurry of 251 mg (0.5 mmole) of IVa and 454 mg (2.0 mmoles) of DDQ in
7.5 ml of anhydrous dioxane (freshly distilled over lithium aluminum hydride)
was stirred under a nitrogen atmosphere in an oil bath at 105°C. After 21 hrs
another portion of DDQ (182 mg, 0.8 mmole) was added to the dark red-brown
solution, and heating with stirring was continued for another 30 hrs. The
reaction mixture was examined by tlc at 21, 41 and 51 hrs. In all cases the
mixture contained, in addition to DDQ-related materials, VIa and IIIa. The
addition of fresh DDQ appeared to increase the ratio of IIIa to VIa somewhat,
but the last 10 hrs of heating seemed to be of no consequence. The mixture was
cooled to room temperature and filtered to remove solids which were washed with
10 ml of dioxane. The combined filtrate and washings were concentrated at 50°C
and 75 mm Hg pressure. The residue was chromatographed on a 3 x 30 cm column of
90g of silica gel eluted with 1.5 *l* of 10% v/v Me_2CO in benzene. After a fore-
run of one column volume (∿135 ml), the eluate was collected in 10 ml fractions
at 1.5 min/fraction. Fractions 41 through 55 were pooled and concentrated. The

*Numbers in parentheses are relative intensities of mass peaks.

residue, 43 mg, was crystallized from a mixture of 0.1 ml of EtOAc and 0.15 ml of SSB with cooling overnight to give 33 mg of VIa, 13.1% yield, mp 205-208°C (dec); λ_{max}^{EtOH} 223 nm (ε 10,850); single component by tlc.

6α,9α-Difluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione-1,2,4-^{2}H$_3$ 17,21-Diacetate (IIIa)

The pooled eluate collected in fractions 76 through 137 from the above column was concentrated to give 60 mg residue. The latter was crystallized from a mixture of 0.1 ml of EtOAc and 0.15 ml of SSB to give 36 mg of IIIa, 14.3% yield, mp 228-230°C (dec); λ_{max}^{EtOH} 238 nm (ε 16,300); single component by tlc identical to authentic III*; pmr spectrum indicated ~10% deuteration among C-1, C-2, and C-4.

6α,9α-Difluoro-11β,17α,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione-1,2,4-^{3}H$_3$ 17,21-Diacetate (IIIb)

A solution of 250 mg (0.5 mmole) of III in 12 ml of dioxane was stirred with 250 mg of 10% Pd-C under 100 Ci of carrier-free tritium gas at room temperature and atmospheric pressure with an uptake of 25 ml**. Labile tritium was removed by evaporation with a dioxane-methanol mixture twice. The entire mixture was brought to dryness and residue dissolved in 7.5 ml of dioxane. DDQ (454 mg, 2.0 mmoles) was added and mixture was stirred at 100°C for 24 hrs. Fresh DDQ (227 mg, 1.0 mmole) was added and mixture was further heated with stirring for another 24 hrs. Labile tritium was again removed by evaporation with dioxane-methanol. The crude, ~11 Ci, was chromatographed on a 3 x 30 cm column of 90 g of silica gel eluted with 10% v/v Me$_2$CO in benzene. Two fractions were collected: 500 ml containing 670 mCi and 1200 ml with 999 mCi. The latter was shown by tlc to contain the Δ^1 and Δ^{1,4} products.

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*Preparation of compounds III and V has been described; Great Britain Patent No. 1,403,962 (August 25, 1975).

**Reduction with tritium gas and dehydrogenation were carried out at New England Nuclear Corp., Boston, Mass.

The residue from the 1200 ml fraction was again chromatographed on a 3 x 30 cm column of 90 g of silica gel eluted with 2.25 l of 10% v/v Me_2CO in benzene. The eluate was collected, after a 100 ml forerun, in 10 ml fractions at 1.5 min per fraction. Radioactivity in the eluate was monitored by counting a 1 µl aliquot from each of the chosen fractions in 10 ml of Diotol cocktail. The residue from the pooled fractions 86 through 115, 339 mCi, was shown by tlc to be composed radiochemically of 91.5% $\Delta^{1,4}$, 5.0% Δ^4 and 3.5% Δ^1 compounds. The mixture was, therefore, again chromatographed on a 2.2 x 43 cm column of 90 g of silica gel the same way as described above. The residual oil from pooled fractions 71 through 120, 300 mCi, was found by tlc to contain radiochemically 97.6% $\Delta^{1,4}$, 1.2% Δ^4 and 0.23% Δ^1 compounds. This material was diluted with 50 mg of non-radioactive III and crystallized from a mixture of 1.2 ml of THF and 9 ml of H_2O to give 63 mg of IIIb, sp. act. 4.33 mCi/mg or 2.14 Ci/mM; λ_{max}^{ETOH} 238 nm (ϵ 16,100); radiochemical purity 98.5% by tlc with 1.0% Δ^4 and 0.5% Δ^1 compounds. From the specific activity of the diluted material, the weight of the added III, and total activity of the undiluted material, the weight (19.3 mg) and specific activity (7.69 Ci/mM) of the undiluted IIIb were calculated.

The 2.14 Ci/mM material (30 mg) was further diluted with 318 mg of non-radioactive III and recrystallized from a mixture of 3.4 ml of EtOAc and 6.8 ml of SSB to give 342 mg of IIIb, sp. act. 0.371 mCi/mg or 0.183 Ci/mM; λ_{max}^{ETOH} 238 nm (ϵ 16,150); *anal.* calc'd for $C_{26}H_{32}F_2O_7$(494.52): C, 63.14; H, 6.52; found: C, 62.73; H, 6.70.

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