

RADIOCHEMICAL SYNTHESSES.

PART III ⁽¹⁾ - THE SYNTHESIS OF TRITIATED α -ECDYSONE AND 25-DEOXY- α -ECDYSONE OF HIGH SPECIFIC ACTIVITY⁽²⁾

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SUMMARY

23,24-³H- α -ecdysone and 23,24-25-³H-25-deoxy- α -ecdysone were prepared by catalytic hydrogenation of 2 β ,3 β ,14 α ,22R,25-pentahydroxy-5 β -cholest-7-en-23-yn-6-one with tritium gas and palladium on charcoal in ethanol. Radioactivity and purity measurements of the stored highly labelled compounds are described.

INTRODUCTION

α -Ecdysone, a hormone controlling the moulting of insects, was synthesized⁽³⁾ shortly after elucidation of its structure⁽⁴⁾. The synthetic methods developed in our laboratories opened access to high specific activity tritiated ecdysone which was essential

for the study of hormone metabolism⁽⁵⁾, transport⁽⁶⁾, binding, and particularly localization.⁽⁷⁾ Ecdysone had previously been labelled⁽⁸⁾ by Wilzbach-exchange⁽⁹⁾, but the specific activity after purification (1.3 mC/mM) was far below the value desirable for localization studies by autoradiography.

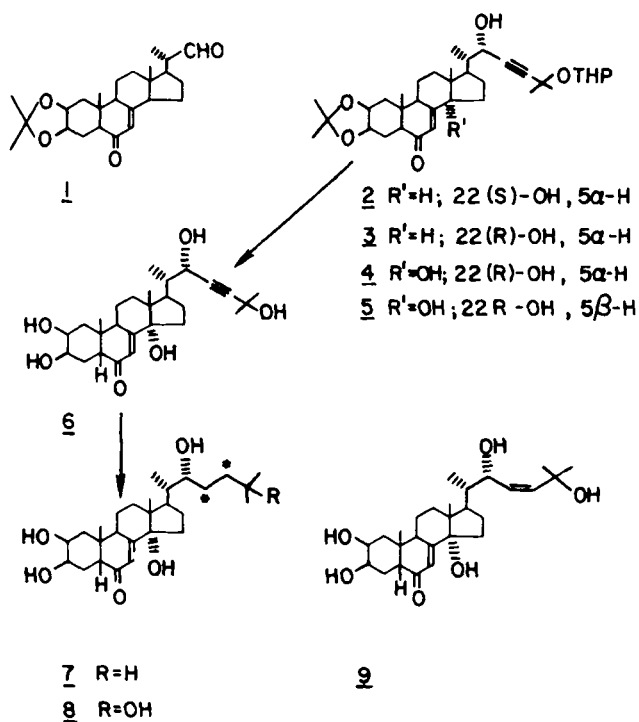
We wish to report the synthesis of sidechain-tritiated α -ecdysone of specific activity 50C/mMole, and of tritiated 25-deoxy- α -ecdysone of specific activity 33C/mMole.

Results and Discussion

The catalytic tritiation of unsaturated precursors, the least destructive way of labelling, is frequently used⁽¹⁰⁾ for preparing highly tritiated organic compounds. In general, aprotic solvents are used to obtain high tritium incorporation and minimal radioactive waste. The following tritiation was performed in ethanol and gave, as in several other cases with this solvent, highly tritiated products. The position of the label was expected to be preferentially at the position of prior unsaturation, but additional scrambling along the carbon chain⁽¹¹⁾ was expected. A suitably unsaturated derivative 6 of α -ecdysone was synthesized in four steps from an aldehyde 1 which was in hand from an earlier synthesis⁽¹²⁾ of β -ecdysone.

Selective alkylation of the aldehyde 1 with the bromomagnesium derivative of 3-methyl-3-(tetrahydropyran-2'-yloxy)-butyne gave predominantly a mixture of 22S and 22R isomers 2 and 3 in a ratio of 5:4, with minor amounts of reduction of the formyl group in 1 and apparently no attack on the unsaturated 6-ketone. Separation of the required 22R isomer 3 was simplified by partial removal of 2 by fractional crystallization and was completed by

extensive preparative thin-layer chromatography (t.l.c.). Assignment of configurations at C_{22} to the isomers 2 and 3 was based entirely on conversion of 3 to α -ecdysone identical with authentic material (3). Nuclear magnetic resonance (n.m.r.) spectra of the 22R isomer 3 showed the 21-H doublet at lower field than in 2.



The epimerization of 3 to its 5 β -isomer is attended by excessive deconjugation to an 8(14) olefinic isomer and by unwanted epimerization at C-14. These side-reactions were prevented by prior hydroxylation of 3 at C-14 with selenium dioxide in warm dioxane to give 4, which was cleanly equilibrated with the

required 5 β -isomer 5 in methanolic potassium carbonate. The 2:3 mixture of isomers 4 and 5 was separated by t.l.c. and protecting groups were carefully removed from 5 using 0.05N hydrochloric acid in tetrahydrofuran-water (7:3) to give 6, purified by t.l.c. until homogeneous but still non-crystalline. Saturation of 6 with hydrogen and 5% palladium on charcoal (Pd/C) produced crystalline α -ecdysone identified by infra-red, ultraviolet and n.m.r. spectroscopy and mixed m.p. with an authentic sample⁽³⁾.

Under platinum catalyzed hydrogenation of 6, a hydrolytic loss of the 25-hydroxyl group occurred with production of 25-deoxy- α -ecdysone (7). This structure was assigned on the basis of 100 MHz n.m.r. spectra in deuterio-pyridine which showed a 21-H doublet at 1.24 p.p.m. ($J=6.5\text{Hz}$) very similar in chemical shift to the 21-H of α -ecdysone, indicating a 22R-hydroxyl group. A high field doublet for six hydrogens at 0.83 ppm ($J=6.0\text{Hz}$) established that the hydroxyl at C₂₅ had been lost by hydrogenolysis, since the mass spectral molecular ion appeared at m/e 448.

This interesting compound 8 has been examined as a possible biosynthetic precursor of β -ecdysone.⁽¹³⁾

Treatment of (22R)-2 β ,3 β ,14 α ,22,25-pentahydroxy-5 β -cholest-7-en-23-yn-6-one (6) with 5% Pd/C and tritium gas in ethanol yielded a mixture of (22R)-[23,24-³H]-2 β ,3 β ,14 α -22,25-pentahydroxy-5 β -cholest-7-en-6-one (8, α -ecdysone), (22R)-[23,24,25-³H]-2 β ,3 β ,14 α ,22,25-pentahydroxy-5 β -cholesta-7,23-dien-6-one (9) and (22R)-[23,24,25-³H]-2 β ,3 β ,14 α -22-tetrahydroxy-5 β -cholest-7-en-6-one (7, 25-deoxy- α -ecdysone) from which 7 and 8 were obtained after purification by t.l.c. The compounds were stored in benzene-alcohol mixtures. After approximately one year, radiodecomposition was found to agree with storage data of other tritiated steroids of similar specific activity⁽¹⁴⁾.

Radioactivity Measurements: The usually applied procedure of diluting a labelled steroid of medium specific activity with benzene and counting aliquots with 10,000-100,000 cpm gave highly erratic results with ^3H -ecdysone and (to a lesser extent) with ^3H -25-deoxyecdysone. Owing to the lack of sufficient unlabelled material, the addition of adequate cold carrier (10,15,16) to the counting-dilutions was not possible.

It was found, however, by diluting high specific activity ecdysone with benzene-methanol for counting, that adsorption and precipitation effects were avoided. Similar specific activity values obtained in both high and low polarity scintillation fluids indicated that correct radioactivity measurements with 4π geometry (no adsorption at the vial surface) had been attained. Combustion in an oxygen flask (17) was used to verify this result.

Dilution analysis is frequently used to verify radio-activity chemical purity. Its precision depends on the availability of a purified carrier, an accurate radioactivity assay, and a dry sample. If only small amounts of carrier are available, microweighing techniques are required. The last two difficulties can be avoided if ultraviolet extinction measurements are used for mass determination. To increase the precision of radioactivity measurements of tritiated samples in mixed solvents, a series of 5 different aliquots was prepared and counted. Mean values and standard deviations of specific activity were calculated.

EXPERIMENTAL (18)

Alkylation of (20S)-2 β ,3 β -isopropylidenedioxy-20-formyl-5 α -pregn-7-en-6-one (1).

A suspension of 5.95 gm of the aldehyde 1 (12) in 90 ml of tetrahydrofuran was stirred at 20° during addition of 165 ml

of a 0.1 M solution of the bromomagnesium derivative of 3-methyl-3-(tetrahydropyran-2'-yloxy)-butyne in tetrahydrofuran, over 2 hr. The mixture was then hydrolyzed with saturated aqueous Rochelle salt and the products isolated with ethyl acetate. Evaporation of the solvent gave a solid residue which was triturated with 200 ml of hot petrol ether (60/80). Filtration gave a solid (5.0 gm) which was recrystallized from methylene dichloride-hexane to give 2.55 gm and 2.22 gm crops of almost pure 22S isomer 2. An analytical sample of from t.l.c. purification of the 2.55 gm crop showed m.p. 230-232 (ethyl acetate); $[\alpha]_D^{25} \text{CHCl}_3 -5^\circ$; u.v. (max) 245 nm, ($\epsilon = 14,260$); n.m.r. (100 MHz, CDCl_3) 0.65 (s, 18-H), 1.04 (s, 19-H), 1.09 (d, $J=6.5$ Hz, 21-H), 1.33, 1.49 (2s, acetonide), 1.53 (broad s, 26-H and 27-H), 4.47 (d, $J=3.5$ Hz, 22-H), 5.05 (m, OCHO of THP), 5.73 (m, 7-H). Anal. Calcd. for $\text{C}_{35}\text{H}_{52}\text{O}_6$: C, 73.91; H, 9.22. Found: C, 73.71; H, 9.22.

By combining the original petrol ether filtrate with mother liquors from the above crystallizations, a residue rich in the required 22R isomer 3 was available for t.l.c. purification (plates, 100 x 20 x 0.13 cm of Merck silica gel HF). Elution of the faster moving zone and crystallization from methanol afforded 1.51 gm (20%) of 3 having m.p. 195-97°C; $[\alpha]_D^{25} \text{CHCl}_3 + 12.6$, u.v. max 245 nm ($\epsilon = 14,050$); n.m.r. (100 MHz, CDCl_3) 0.62 (s, 18-H), 1.03 (s, 19-H), 1.13 (d, $J=6$ Hz, 21-H); 1.32, 1.47 (2s, acetonide), 1.52 (s, 26-H and 27-H), 4.48 (d, $J=1.0$ Hz, 22-H), 5.04 (m, OCHO), 5.72 (m, 7-H). Anal. Calcd. for $\text{C}_{35}\text{H}_{52}\text{O}_6$: C, 73.91; H, 9.22. Found: C, 74.09; H, 9.07.

(22R)-14 α ,22-dihydroxy-2 β ,3 β -isopropylidenedioxy-25-(tetrahydropyran-2'-yloxy)-5 α -cholest-7-en-23-yn-6-one (4).

A mixture containing 0.5 gm of the 22 R isomer 3 in 50 ml of dry dioxane and 0.15 gm of selenium dioxide was stirred in an oil bath at about 60°C for 2 hours. The reaction mixture was cooled to room temperature, filtered through Celite directly into saturated aqueous potassium bicarbonate solution, and the products isolated with methylene chloride. The crude product (0.6 gm) was recrystallized from methylene chloride-hexane to afford 4 having m.p. 232-35°C; $[\alpha]_D^{25}$ CHCl₃ + 6°, u.v. max 242 nm ($\epsilon = 12,360$), Anal. Calcd. for C₃₅H₅₂O₇: C, 71.88; H, 8.96. Found: C, 71.08; H, 8.72.

Epimerization of (4) \rightarrow (5).

To 0.4 gm of the preceding 5 α -H compound 4, a solution of 40 ml, prepared from 0.33 gm of potassium carbonate in 100 ml of 1:9 water-methanol, was added and the mixture stirred under nitrogen for 18 hr. at 25°C. Water was added and most of the methanol was removed under reduced pressure, before extraction with methylene chloride. The product (0.4 gm), comprising a mixture of the 5 α and 5 β isomers (ratio 2:3), was purified by t.l.c. on silica gel plates. The less polar compound was isolated (0.23 gm) and recrystallized from methylene chloride-ether-hexane to give the 5 β -isomer 5 having m.p. 225-227°C; $[\alpha]_D^{25}$ CHCl₃ -50°, u.v. max 242 nm ($\epsilon = 13,500$). Anal. Calcd. for C₃₅H₅₂O₇·H₂O, C, 69.74, H, 9.03. Found: C, 71.39; H, 8.28; H, 8.75.

(22R)-2 β ,3 β ,14 α ,22,25-pentahydroxy-5 β -cholest-7-en-23-yn-6-one (6)

A solution of 0.25 gm of 5 in 50 ml of 0.05N hydrochloric acid in tetrahydrofuran-water (7:3) was allowed to stand at room temperature for 6 hr. The reaction mixture was then diluted with saturated aqueous potassium bicarbonate solution and extracted with tetrahydrofuran. The combined tetrahydrofuran extracts were "dried" over sodium sulfate, filtered, and the solvent evaporated under reduced pressure. The residue consisted of a mixture of two compounds which were separated by silica gel t.l.c. using 8% methanol in chloroform as developing solvent. The less polar by-product was found by n.m.r. spectroscopy to be the acetone derivative of 6. Elution of the more polar major product with tetrahydrofuran gave an amorphous solid 6 showing n.m.r. (100 MHz, deuteriopyridine) 0.76 (s, 18-H), 1.03 (s, 19-H), 1.44 (d, J=6.5Hz, 21-H), 1.71 (s, 26-H and 27-H), and 6.13 (m, 7-H).

25-Deoxy- α -ecdysone (7)

Hydrogenation of 5 mg of the acetylene 6 over platinum in 2 ml ethanol solution gave predominantly 25-deoxy- α -ecdysone (7) purified by t.l.c. and isolated as a non-crystalline solid showing u.v. max 241 nm ($\epsilon = 13,100$); nmr (100 MHz deuteriopyridine) 0.72 (s, 18-H), 0.83 (d, J=6.0Hz, 26 and 27-H), 1.05 (s, 19-H), 1.24 (d, J=6.5Hz, 21-H), 6.16 (bs, 7-H); MS m/e 448 (M^+), 430 (M-18).

Tritiation: A solution of 20 mg of 6 in 2 ml ethanol and 20 mg of 5% palladium on charcoal was connected with a suitable manifold, cooled to -196°C , evacuated, degassed, and excess tritium gas was then introduced by Toepler pumping. The reaction mixture

was stirred magnetically for 4 hr. at room temperature with a tritium partial pressure of approximately 300 mm Hg. Unreacted tritium gas was removed, the reaction mixture was filtered over Celite, and labile tritium was removed by distillation with methanol. After titulation with benzene the methanol residue was applied to a 20 x 20 cm silica-plate (1.5 mm layer) and developed with chloroform-methanol (10:1) until three separate bands were detectable by u.v. extinction (254 nm) and Bremsstrahlung (Geiger-Muller counter). The zones were collected, eluted with acetone, evaporated quickly to dryness in vacuo, weighed, and dissolved in 100 ml methanol-benzene (1:10) and stored at room temperature. The slowest moving band (R_f ca 0.15) was found to be ^3H - α -ecdysone (ca 5.3 mg, 600 mC, 27% yield); the next zone (R_f ca 0.25) was chromatographically identified as (9) (ca 6.3 mg, 31%); the highest band (R_f ca 0.7) was similarly identified as ^3H -25-deoxyecdysone 7 (ca 5.8 mg, 440 mC, 30%). An aliquot of the ^3H -ecdysone stock solution, containing 0.21 mg was mixed with 24.41 mg inactive carrier, dissolved in methanol and recrystallized four times in a small centrifuge tube by adding water to the boiling methanol solution. After removal of samples for dilution analysis, 9.8 mg purified ^3H -ecdysone, total activity 7.4 mC, was obtained and stored as described above.

Chromatographic Identification, Radiochemical Purity and Storage

^3H -Ecdysone: Mobility identical with reference material was seen on silica t.l.c. (chloroform or ether containing 10% methanol). and paper chromatography (Bush C system). Identity of the ^3H - α -ecdysone was established by dilution analysis.

The radiochemical purity of undiluted material was high soon after the preparation, judged by radioscanning of thin-layer chromatoplates.

After 6 months of storage in methanol:benzene (1:9) a distinct impurity, less polar than α -ecdysone was seen. At 18 months, this became more intense and overrun paper chromatography (Bush C system/ 300 hr development) showed a radiochemical purity of 70% with six distinct by-products.

Less radiodecomposition was seen when ^3H - α -ecdysone was diluted with cold carrier, crystallized, and then stored in the above solvents. In this way, a sample stored for 8 months showed radiochemical purity of ca 95% by t.l.c.

25-Deoxy- ^3H - α -ecdysone

This compound was more stable than α -ecdysone in storage and showed initial purity of 95%, which decreased to 90% after 18 months storage in pure benzene.

Determination of non-exchangeable tritium in ^3H - α -ecdysone (8)

Extended steam distillation from a ca 13 μg sample of (8) under neutral conditions showed a loss of only 2.4% of the label. Continued distillation from alkaline (NaOH) solution led to a further small loss of 1.1% of the label, determined by counting aliquots of the distillate in dioxane-toluene-methanol scintillation solution, showing a total of 3.5% exchangeable tritium.

Specific Activity Determinations.

^3H -Ecdysone: Reproducible values (50.4 ± 0.7 C/mM) in toluene scintillation solution and ($52.5 \pm 0.4^*$ C/mM) in dioxane-

toluene-methanol scintillation were obtained when benzene-methanol (2:1) was used for dilution and channel ratio was used to correct for quenching. Similar, but less precise values ($55.6 \pm 2.4^* \text{ C/mM}$), were obtained by internal standardization with tritiated toluene. In addition, the specific activity values before dilution carry possible weighing errors. The specific activity of the diluted crystallized material was determined to be $409 \pm 24 \text{ mC/mM}$.

25-Deoxy- ^3H -ecdysone:

Reproducible specific activities (a) $32.7 \pm 0.3 \text{ C/mM}$, (b) $35.6 \pm 0.3^* \text{ C/mM}$ were obtained when the benzene stock solution was (a) diluted with methanol-water (4:1) and counted in a dioxane-toluene-methanol scintillation solution, and (b) when a benzene-methanol (9:1) dilution was counted in toluene scintillation solution. This is in agreement with 33.5 C/mM , obtained when an aliquot of the stock solution was allowed to evaporate on the combustion paper of a Schoniger-flask, and counted after combustion and dilution with water.

Dilution Analysis of ^3H -Ecdysone:

(a) Gravimetric: The specific activity of a one year stored, freshly diluted sample was $502 \pm 22 \text{ mC/mM}$, and after two, three and four crystallizations it was found to be 417 ± 32 , 437 ± 37 , and $409 \pm 24 \text{ mC/mM}$, indicating a radiochemical purity of $82 \pm 4\%^{**}$. After

*Standard deviation of 5 different samples from the same dilution.

**Standard deviation allowing for propagation of the fractional error

high vacuum drying, 0.5 - 1.5 mg samples were weighed on a micro-analytical balance for the determination.

(b) Spectroscopic: A few milligrams authentic ecdysone were mixed with a few lambda of 18 month stored ^3H -ecdysone stock solution and dissolved in (ca 0.1 ml) methanol. The u.v. extinction of 5 different concentrations of suitable methanol dilutions was measured at 242 nm. The radioactivity of an aliquot diluted with methanol was determined in toluene scintillation solution and corrected for channel ratio quenching. The material was crystallized from aq. methanol, re-dissolved after centrifugation, and its extinction and radioactivity were measured. Based on a molar extinction coefficient of 12,400 the following specific activities were calculated. A radiochemical purity of 76±3%** is indicated.

Total crude:	7.06 <u>±</u> 0.3**	mC/mM			
1 x recryst., crop:	5.07 <u>±</u> 0.06	"	} mother liquors:	12.6 <u>±</u> 0.1mC/mM	
2 x " "	5.28 <u>±</u> 0.07	"		" "	5.34 <u>±</u> 0.05mC/mM
3 x " "	5.40 <u>±</u> 0.05	"		" "	5.47 <u>±</u> 0.14mC/mM

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[#]Standard deviation allowing for propagation of the fractional error.

^{**}Standard deviation of five independently measured and counted samples.

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18 Melting points are uncorrected. N.m.r. spectra were recorded using a Varian HA-100 spectrometer and are quoted in p.p.m. downfield from tetramethylsilane as interval reference. Substantial amounts of tritiated material were manipulated in glove boxes. Liquid scintillation counting was performed with a Packard "Tri-carb" 314 A spectrometer. The term "toluene scintillation solution" is used for a solution of 0.4% 2,5-diphenyloxazole (PPO) and 0.05% p-bis 2-(5-phenyloxazolyl)-benzene (POPOP) in scint. grade toluene. "Dioxane-toluene-methanol scintillation solution" is used for 1 ℓ spect. grade dioxane, 1 ℓ toluene and 0.6 ℓ methanol containing 208 gm naphthalene, 13 g PPO and 0.26 g POPOP, which can hold ca 5% water. A Vanguard "Autoscanner 880" was used for measuring radioactivity on paper chromatograms; a model 885 glass plate scanner was used for t.l.c. plates.

Mass spectra were recorded on an Hitachi Perkin-Elmer RMU-6D spectrometer by direct inlet probe at 250-350°C.

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