Microbiological Degradation of Bile Acids

THE PREPARATION OF HEXAHYDROINDANE DERIVATIVES AS SUBSTRATES FOR STUDYING CHOLIC ACID DEGRADATION

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Relatively large amounts of 3-(3 α -hexahydro-7 $\alpha\beta$ -methyl-1,5-dioxoindan-4 α -yl)propionic acid (IIb), which is believed to be one of the intermediates involved in the degradation of cholic acid (I), were needed to identify its further degradation products. A simple method for the preparation of this compound was then investigated. *Arthrobacter simplex* could degrade 3-oxoandrost-4-ene-17 β -carboxylic acid (IIIa) to 3-(1 β -carboxy-3 α -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVa) in good yield, the structure of which was established by partial synthesis. It was therefore expected that, if a similar degradation by this organism occurred with 17 α -hydroxy-3-oxoandrost-4-ene-17 β -carboxylic acid (IIIb), which is easily obtained by chemical oxidation of commercially available 17 α -hydroxydeoxycorticosterone, the resulting product, 3-(1 β -carboxy-3 α -hexahydro-1 α -hydroxy-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVb), could be readily converted chemically into the required dioxocarboxylic acid (IIb). Exposure of compound (IIIb) to *A. simplex* produced, as expected, compound (IVb) which was then oxidized with NaBiO₃ to give a reasonable yield of compound (IIb).

In our previous study on the degradation of cholic acid (3 α ,7 α ,12 α -trihydroxy-5 β -cholan-24-oic acid) (I) by *Streptomyces rubescens*, we reported that, since 3-(3 α -hexahydro-7 $\alpha\beta$ -methyl-1,5-dioxoindan-4 α -yl)propionic acid (IIb) might be a common intermediate involved in the microbiological degradation of some kinds of steroids including cholic acid, studies on the degradation of this compound would give an insight into the mechanism of the degradation of not only cholic acid but also other steroids (Hayakawa *et al.*, 1976b) (Scheme 1). To continue such studies we needed relatively large amounts of compound (IIb). However, our synthesis of this compound previously reported was unfavourable for the present purpose, since it comprised multiple steps starting from oestrane-3,17 β -diol and the total yield of this compound was low (Hayakawa *et al.*, 1976b).

Since we found the biochemically interesting degradation of cholic acid (I) by *Arthrobacter simplex* to (4*R*)-4-[4 α -(2-carboxyethyl)-3 α -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-1 β -yl]valeric acid (IIa) (Hayakawa *et al.*, 1967) and also the inability of this organism to degrade this metabolite further (Hayakawa *et al.*, 1968), we have extended this biotransformation to other bile acids and related substances for the following two purposes: (1) the preparation

of hypothetical metabolites involved in cholic acid degradation; (2) the preparation of compounds containing the hexahydroindane nucleus, which are not directly related to cholic acid metabolites but which probably serve as substrates. These degradation products possibly offer information on the further degradation of compounds (IIa) and (IIb). We have already reported some of the former results (Hayakawa *et al.*, 1976a). During the course of the latter study, we found that 3-oxoandrost-4-ene-17 β -carboxylic acid (IIIa) could be degraded by *A. simplex* to 3-(1 β -carboxy-3 α -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVa). We expected therefore that, if the 17 α -hydroxy derivative of compound (IIIa), 17 α -hydroxy-3-oxoandrost-4-ene-17 β -carboxylic acid (IIIb), is similarly degraded by this organism to the corresponding hexahydroindane derivative, 3-(1 β -carboxy-3 α -hexahydro-1 α -hydroxy-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVb), this α -hydroxy acid could be readily converted chemically into the required dioxocarboxylic acid (IIb).

The present paper describes the microbiological degradation of both compounds (IIIa) and (IIIb) to form the corresponding hexahydroindane derivatives (IVa) and (IVb) and the chemical conversion of compound (IVb) into compound (IIb). The metabolism of compound (IIb) by *S. rubescens* is reported in the following paper (Hashimoto & Hayakawa, 1977).

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Scheme 1. Microbiological degradation of cholic acid (I), 3-oxoandroster-4-ene-17 β -carboxylic acid (IIIa) and 17 α -hydroxy-3-oxoandroster-4-ene-17 β -carboxylic acid (IIIb) to form the respective hexahydroindane derivatives (IIa) and (IIb), (IVa) and (IVb)

Results and Discussion

Microbiological degradation of 3-oxoandroster-4-ene-17 β -carboxylic acid (IIIa) and its 17 α -hydroxy derivative (IIIb)

As described by Hayakawa *et al.* (1969), compound (IIIa) was exposed to *A. simplex* in a flask and a new metabolite was isolated as crystals. The structure was assigned as 3-(1 β -carboxy-3 $\alpha\alpha$ -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVa) by a

comparison of the physical data such as i.r. and n.m.r. (nuclear magnetic resonance) of this metabolite and its dimethyl ester with those of authentic samples of compound (IIa) and its nor and dinor derivatives and their corresponding dimethyl esters previously prepared by Hayakawa *et al.* (1969, 1976a). The exact proof of the structure was given by its partial synthesis as described below. A large-scale incubation in jar fermentors produced crude crystals of this metabolite, in approx. 89% yield.

Similarly compound (IIIb) was exposed to *A. simplex* and converted into a new metabolite, which was isolated as crystals, with a yield of approx. 86%. 3-(1 β -Carboxy-3 $\alpha\alpha$ -hexahydro-1 α -hydroxy-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVb) was assigned to the structure of this metabolite as described above for compound (IVa). A more definitive proof of the structure was established by the chemical conversion of this metabolite into compound (IIb) as described below.

Chemical proof of structures of the degradation products (IVa) and (IVb)

Partial synthesis of compound (IVa). This was carried out by a route that had been used for the partial syntheses of compounds (IIa) and (IIb) (Hayakawa *et al.*, 1969, 1976b) (Scheme 2). The key intermediate in this sequence was 17 β -hydroxymethyl-oestra-4,9-dien-3-one (VIIa). This compound was prepared from methyl 3-methoxyoestra-1,3,5(10)-triene-17 β -carboxylate (V) through the modified Birch reduction (Sandoval *et al.*, 1955) into (3-methoxyoestra-2,5(10)-dien-17 β -yl)methanol (VI), hydrolysis with oxalic acid into the $\Delta^{5(10)}$ -3-one, bromination with trimethylphenylammonium perbromide to the 5,10-dibromo-3-one and finally dehydrobromination with pyridine. The purification of the resulting $\alpha\beta,\gamma\delta$ -unsaturated ketone (VIIa) presented certain difficulties, but the corresponding acetate (VIIb) was obtained in a pure form, and the structure was confirmed by the typical u.v.-absorption maximum at 305 nm and i.r.-absorption maxima at 1660, 1613 and 1588 cm⁻¹ characteristic of a $\Delta^{4,9}$ -3-one structure. The ozonolysis of this acetate gave 3-(1 β -acetoxymethyl-3 $\alpha\alpha$ -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (VIIIa). This compound was treated with aqueous alkali to yield the hydroxy-oxo acid (VIIIb), which with diazomethane gave the corresponding methyl ester (VIIIc). Oxidation of this ester with chromic anhydride in acetic acid and subsequent hydrolysis resulted in the formation of 3-(1 β -carboxy-3 $\alpha\alpha$ -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVa). Identity with a sample obtained from growing cultures was established by mixed m.p. and by comparison of the $[\alpha]_D$ values and the i.r. spectrum. The dimethyl ester of this synthetic sample could not be induced to

Scheme 2. Partial synthesis of 3-(1 β -carboxy-3 $\alpha\alpha$ -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVa)

crystallize, but the i.r. and n.m.r. spectra were identical with those of the oily dimethyl ester of the isolated sample. This sequence of reactions conclusively established the structure of the degradation product (IVa), including its stereochemistry. The results show that in the degradative sequence the original stereochemistry of compound (IIIa) remains undisturbed.

Chemical conversion of compound (IVb) into the dioxocarboxylic acid (IIb). Oxidation of compound (IVb) in aqueous acetic acid with sodium bismuthate, which oxidizes disubstituted glycollic acids to the corresponding ketones (cf. Rigby, 1950), gave compound (IIb), which was identical in all respects (m.p., mixed m.p., $[\alpha]_D$ and i.r.) with an authentic sample prepared by Hayakawa *et al.* (1976b), with a yield of approx. 67%. The overall yield of this compound, based on compound (IIIb) and 17 α -hydroxydeoxycorticosterone (17 α ,21-dihydroxy-pregn-4-ene-3,20-dione), was approx. 57 and 46% respectively. The results established the structure of compound (IVb), except for the configuration of both the carboxyl and hydroxyl groups at C-1, and afforded a simple method for the preparation of compound (IIb). With regard to this configuration

at C-1, we confirmed that the degradation of bile acids and their analogues by *A. simplex* or *S. rubescens* to the corresponding C₁₈, C₁₇ and C₁₆ hexahydroindane-oxodicarboxylic acids occurred with retention of configuration (Hayakawa *et al.*, 1969, 1976a,b). This was also true in the degradation of compound (IIIa) to compound (IVa) as described above. Therefore the configuration at C-1 in compound (IVb) is probably the same as that at C-17 in compound (IIIb), but the definitive conclusion must await further investigations.

Experimental

General

The methods of incubation, physical measurements and chromatography and the micro-organism, *A. simplex*, were as described by Hayakawa & Fujiwara (1969) and Hayakawa *et al.* (1969). Except where otherwise stated, crystallization or recrystallization from a mixture of solvent A/solvent B such as methanol/water, acetone/diethyl ether or acetone/light petroleum (b.p. 40–60°C) was carried out by the addition of the solvent B to a solution of certain products in the solvent A. An ethereal solution of

toxic and explosive diazomethane was prepared from *N*-nitrosomethylurea, a potent carcinogen reported by Graffi & Hoffmann (1966), and was used for methylations. These operations were conducted with the utmost care as described by Fieser & Fieser (1967). Unless otherwise stated, optical rotations and i.r.-absorption spectra were determined in chloroform solution and u.v.-absorption spectra were measured in ethanol.

Other materials

As substrates for incubation, 3-oxoandrost-4-ene-17 β -carboxylic acid (IIIa) and its 17 α -hydroxy derivative (IIIb) were prepared as follows. Oxidation of deoxycorticosterone (21-hydroxypregn-4-ene-3,20-dione) with NaBiO₃ by the method of Brooks & Norymberski (1953) gave compound (IIIa), m.p. 243–246°C, $[\alpha]_D^{25} + 159.8 \pm 2^\circ$ (*c* 1.071), i.r. max. (in Nujol) at approx. 2600–3100 (OH of CO₂H), 1725 (C=O of CO₂H), 1635 ($\alpha\beta$ -unsaturated C=O) and 1606 (C=C) cm⁻¹ and $\lambda_{\max}$. 242nm (ϵ = 16600) (Found: C, 76.0; H, 8.9. Calc. for C₂₀H₂₈O₃: C, 75.9; H, 8.9%). Brooks & Norymberski (1953) give m.p. 237–241°C and $[\alpha]_D + 161^\circ$ (in chloroform) for this compound. A solution of 17 α -hydroxydeoxycorticosterone (92.5g) in dioxan (1.9 litres) was treated with an aqueous solution of HIO₄·2H₂O (61g) in water (280ml) at room temperature (approx. 20°C), with stirring. The mixture was stirred for 1 h at the same temperature, diluted with water (4 litres) and concentrated *in vacuo* to approx. 2.5 litres below 30°C. The resultant crystals were collected by filtration, washed with water and then dried under reduced pressure. Recrystallization from methanol/water gave a crude pale-yellowish compound (IIIb) [70.8g, m.p. 225–228°C (decomp.)]. This product was used for incubation without further purification. Further recrystallization from chloroform/methanol (1:1, v/v) did not raise the melting point but gave colourless crystals, which exhibited $[\alpha]_D^{25} + 105.9 \pm 1.5^\circ$ (*c* 1.003), i.r. max. at 3506 (OH), approx. 2400–3200 (OH of CO₂H), 1759 (shoulder), 1726 (shoulder) and 1703 (C=O of CO₂H), 1662 ($\alpha\beta$ -unsaturated C=O) and 1619 (C=C) cm⁻¹ and $\lambda_{\max}$. 242.5nm (ϵ 15000) (Found: C, 72.4; H, 8.5. Calc. for C₂₀H₂₈O₄: C, 72.3; H, 8.5%). Reichstein *et al.* (1939) give m.p. 232–240°C (decomp.) for this compound.

As a starting material for the partial synthesis of compound (IVa), [3-methoxyoestra-2,5(10)-dien-17 β -yl]methanol (VI) was prepared as follows. The modified Birch reduction of methyl 3-methoxyoestra-1,3,5(10)-triene-17 β -carboxylate (V) (m.p. 160.5–162°C) was carried out essentially as reported by Sandoval *et al.* (1955), except that a mixture of 2-methylpropan-2-ol/tetrahydrofuran (1:1, v/v) was used as a solvent instead of diethyl ether (cf. Hayakawa *et al.*, 1969). The enol ether (VI) (m.p. 117–120°C, no appreciable u.v. absorption at near

280 nm) thus obtained as a crude product was used for the next step without further purification. Sandoval *et al.* (1955) give m.p. 114–117°C for this crude compound. Other materials were obtained from commercial sources and where necessary recrystallized or distilled before use. Light petroleum refers to the fraction of b.p. 40–60°C.

Degradation of compounds (IIIa) and (IIIb) by *A. simplex*

As previously described for the degradation of cholic acid (Hayakawa *et al.*, 1969), compound (IIIa) (200mg) was exposed to *A. simplex* in a flask and converted into a mixture of degradation products (180mg). On recrystallization from acetone, the mixture gave 3-(1 β -carboxy-3 α -hexahydro-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVa) in prisms, m.p. 226.5–230.5°C, $[\alpha]_D^{25} + 34.5 \pm 2^\circ$ (*c* 1.064 in ethanol) and i.r. max. (in Nujol) at approx. 2600–3100 (OH of CO₂H) and 1694 (broad) (C=O and C=O of CO₂H) cm⁻¹ (Found: C, 62.5; H, 7.5; C₁₄H₂₀O₅ requires C, 62.7; H, 7.5%). The large-scale incubation of compound (IIIa) (16.5g) was carried out with the use of jar fermentors in a manner similar to that described by Hayakawa & Fujiwara (1969) and yielded the crude crystals (12.4g) of compound (IVa). Repeated recrystallization from methanol gave analytically pure product (9.06g).

The dimethyl ester, prepared with ethereal diazomethane, gave an oil and showed i.r. max. at 1733 (ester) and 1709 (shoulder) (C=O) cm⁻¹ and n.m.r. absorptions at 1.01 (3H; singlet; 7 $\alpha\beta$ -Me), 3.67 (3H; singlet; CO₂Me) and 3.71 (3H; singlet; CO₂Me) p.p.m.

As described above, exposure of compound (IIIb) (900mg) to *A. simplex* yielded a mixture of products (665mg), which, on crystallization from acetone/diethyl ether, gave 3-(1 β -carboxy-3 α -hexahydro-1 α -hydroxy-7 $\alpha\beta$ -methyl-5-oxoindan-4 α -yl)propionic acid (IVb) in prisms, m.p. 153–154°C, $[\alpha]_D^{25} - 2.6 \pm 0.4^\circ$ (*c* 0.962 in ethanol) and i.r. max. (in Nujol) at 3494 and 3450 (OH), approx. 2600 (OH of CO₂H) and 1698 (C=O and C=O of CO₂H) cm⁻¹ (Found: C, 59.4; H, 7.2; C₁₄H₂₀O₆ requires C, 59.1; H, 7.1%). A mixture of products (56.11g), resulting from the large-scale incubation of compound (IIIb) (70.8g) in jar fermentors, was treated in a similar manner as above and gave crystals of compound (IVb): the first crop (38g, m.p. 151.5–154°C) and second one (14.12g, m.p. 147–153°C).

The dimethyl ester, prepared with ethereal diazomethane, crystallized from acetone/diethyl ether in prisms, m.p. 93–94.5°C, $[\alpha]_D^{24} - 2.1 \pm 0.2^\circ$ (*c* 2.020), i.r. max. at 3530 (OH) and 1726 (shoulder) and 1710 (ester and C=O) cm⁻¹ and n.m.r. absorptions at 1.02 (3H; singlet; 7 $\alpha\beta$ -Me), 3.06 (1H; doublet; J 1.7Hz; OH), 3.66 (3H; singlet; CO₂Me) and 3.80

(3H; singlet; CO₂Me) p.p.m. (Found: C, 61.8; H, 7.8; C₁₆H₂₄O₆ requires C, 61.5; H, 7.8%).

Partial synthesis of compound (IVa)

Hydrolysis and bromination-dehydrobromination of the enol ether (VI). A solution of the crude enol ether (3.02g) in methanol (300ml) was chilled to approx. 0°C and a solution of oxalic acid dihydrate (4.63g) in water (36ml) was added with cooling to approx. 5°C, and with stirring. The mixture was stirred for 1 h at room temperature, neutralized with 5% (w/v) NaHCO₃, concentrated *in vacuo* to approx. 30ml below 40°C and then extracted with diethyl ether. Evaporation of the diethyl ether *in vacuo* left an oily mass (2.77g), which was dissolved in pyridine (90ml) and chilled to approx. -10°C. Trimethylphenylammonium perbromide (3.28g) was added in small portions to the chilled solution over a period of 15 min, with stirring. The mixture was left overnight at room temperature (approx. 20°C) and then heated on a boiling-water bath for 1 h; the orange-coloured mixture was concentrated *in vacuo*, diluted with 2% (w/v) Na₂S₂O₃ (200ml) and then extracted with diethyl ether. The extract was successively washed with dilute HCl, water, 5% (w/v) NaHCO₃ and water and dried over anhydrous Na₂SO₄. Evaporation of the diethyl ether *in vacuo* left an oily residue (2.70g), which is believed to contain mainly 17β-hydroxymethylcholestra-4,9-dien-3-one (VIIa), but attempts to obtain this compound in a pure state were unsuccessful. It was then treated with acetic anhydride/pyridine (1:1, v/v; 27ml) at room temperature for 16 h, and the acetylation mixture was chromatographed on alumina (neutral, grade III; 86g). Elution with light petroleum/benzene (from 4:1, v/v, to 3:7, v/v) gave 17β-acetoxymethylcholestra-4,9-dien-3-one (VIIb), which crystallized from acetone/light petroleum in rods (1.2g), m.p. 134–137°C, $[\alpha]_D^{25} -247.6 \pm 2^\circ$ (c 0.847), $\lambda_{\max}$ 305 nm (ϵ 21000) and i.r. max. (in Nujol) at 1730 and 1246 (acetate), 1660 ($\alpha\beta,\gamma\delta$ -unsaturated C=O) and 1613 and 1588 (C=C) cm⁻¹ (Found: C, 77.0; H, 8.7; C₂₁H₂₈O₃ requires C, 76.8; H, 8.6%).

The free alcohol (VIIa), obtained by the alkaline hydrolysis of the above acetate with methanolic 0.1 M-KOH at room temperature under a stream of N₂, crystallized from acetone in prisms, m.p. 135–136.5°C, $[\alpha]_D^{25} +315.1 \pm 2^\circ$ (c 1.014), $\lambda_{\max}$ 307 nm (ϵ 21300) and i.r. max. (in Nujol) at 3370 (OH), 1643 ($\alpha\beta,\gamma\delta$ -unsaturated C=O) and 1589 (C=C) cm⁻¹ (Found: C, 79.4; H, 9.2; C₁₉H₂₆O₂ requires C, 79.7; H, 9.2%).

Ozonolysis of the dienone acetate (VIIb). The acetate (1.04g) was dissolved in a mixture of ethanol-free ethyl acetate (85ml) and acetic acid (8.5ml), and a stream of 4% (w/w) ozonized O₂ was passed into the solution at approx. -12°C at the rate of 7 litres/h for 75 min. Water (25ml) was added and the mixture was stirred for 1 h with ice cooling. Then

30% (w/w) H₂O₂ (5ml) was added and the mixture was kept overnight in a refrigerator. The mixture was diluted with water, saturated with NaCl and then extracted with diethyl ether. The ethereal solution was extracted several times with 5% (w/v) NaHCO₃, and the combined alkaline extracts were acidified with dilute HCl, saturated with NaCl and then extracted with diethyl ether to recover an acidic fraction. Evaporation of the diethyl ether *in vacuo* left an oily mass (750mg), which was believed to be 3-(1β-acetoxymethyl-3α-hexahydro-7αβ-methyl-5-oxoindan-4α-yl)propionic acid (VIIIa). It could not be induced to crystallize but showed i.r.-absorption maxima at approx. 2600–3200 (OH of CO₂H), 1729 (shoulder) (acetate) and 1718 and 1700 (C=O and C=O of CO₂H) cm⁻¹.

The acetate (830mg), obtained by repeating the above ozonolysis, was dissolved in methanol (13.5ml) and treated with 1 M-KOH (6ml) at room temperature (approx. 20°C) for 1 h. The mixture was neutralized with dilute HCl, evaporated *in vacuo* to remove the methanol, acidified with dilute HCl, saturated with NaCl and then extracted with diethyl ether. The ethereal solution was evaporated *in vacuo* to yield a residue, which was chromatographed on silicic acid (26g). Elution with dichloromethane/acetone (from 4:1, v/v, to 7:3, v/v) gave 3-(3α-hexahydro-1β-hydroxymethyl-7αβ-methyl-5-oxoindan-4α-yl)propionic acid (VIIIb), which crystallized from acetone in rods (550mg), m.p. 145–147°C, $[\alpha]_D^{25} +4.2 \pm 2^\circ$ (c 1.013 in methanol) and i.r. max. (in Nujol) at 3404 (OH), approx. 2600–3100 (OH of CO₂H), 1721 and 1694 (C=O and C=O of CO₂H) cm⁻¹ (Found: C, 66.4; H, 8.8; C₁₄H₂₂O₄ requires C, 66.1; H, 8.7%).

Methylation, oxidation and hydrolysis of the hydroxy-oxo acid (VIIIb). The methyl ester (VIIIc), prepared with ethereal diazomethane from the acid, was an oily mass and showed i.r. max. at 3439 (OH), 1726 (ester) and 1704 (C=O) cm⁻¹. A solution of the ester (200mg) in acetic acid (4ml) was treated with a solution of CrO₃ (111 mg) in acetic acid/water (4:1, v/v; 0.5 ml) under cooling at approx. 5°C for 1 h. The mixture was kept overnight at room temperature (approx. 20°C), methanol was added to destroy the excess of CrO₃, and the solution was concentrated *in vacuo*, diluted with water, saturated with NaCl and then extracted with diethyl ether. The extract was partitioned into a neutral fraction (38mg) and an acidic fraction (160mg) by the usual NaHCO₃ extraction procedure, which was described above in the ozonolysis of compound (VIIb). The acidic fraction was chromatographed on silicic acid (6.4g). Elution with dichloromethane and dichloromethane/methanol (199:1, v/v) gave methyl 3-(1β-carboxy-3α-hexahydro-7αβ-methyl-5-oxoindan-4α-yl)propionate (VIIIId), which crystallized from diethyl ether/light petroleum in needles (90mg), m.p. 35–40°C, $[\alpha]_D^{25} +28.4 \pm 2^\circ$ (c 1.083 in ethanol) and i.r. max. (in Nujol)

at approx. 2600–3180 (OH of CO₂H), 1730 (ester), 1712 (C=O of CO₂H) and 1692 (C=O) cm⁻¹ (Found: C, 63.9; H, 8.1; C₁₅H₂₂O₅ requires C, 63.8; H, 7.9%).

The above half-ester (70 mg) was dissolved in a mixture of methanol (0.5 ml) and 1 M-KOH (0.55 ml) and left at room temperature (approx. 20°C) for 1.5 h. The mixture was diluted with water (approx. 5 ml), acidified with dilute HCl, saturated with NaCl and then extracted with diethyl ether. The extract was washed with saturated NaCl, dried over anhydrous Na₂SO₄ and then evaporated *in vacuo* to yield a crystalline solid (67 mg), which was recrystallized from acetone to give 3-(1β-carboxy-3α-hexahydro-7αβ-methyl-5-oxoindan-4α-yl)propionic acid (IVa) as prisms (45 mg), m.p. 223–226°C and $[\alpha]_D^{22} +31.9 \pm 3^\circ$ (c 0.712 in ethanol) (Found: C, 63.0; H, 7.7; C₁₄H₂₀O₅ requires C, 62.7; H, 7.5%). Identity with the product obtained from growing cultures was established by mixed m.p. and by comparison of the $[\alpha]_D$ values and the i.r. spectrum.

Oxidation of compound (IVb) with NaBiO₃

Exactly as described above for deoxycorticosterone, this compound was oxidized with NaBiO₃ and gave 3-(3α-hexahydro-7αβ-methyl-1,5-dioxoindan-4α-yl)propionic acid (IIb). On recrystallization from acetone/light petroleum, it showed m.p. 107.5–108°C and $[\alpha]_D^{24.5} +100 \pm 1.4^\circ$ (c 1.023) (Found: C, 65.4; H, 7.7. Calc. for C₁₃H₁₈O₄: C, 65.5; H, 7.6%). Identity with an authentic sample previously synthesized by Hayakawa *et al.* (1976b) was established by mixed m.p. and by comparison of the $[\alpha]_D$ values and the i.r. spectrum.

A large-scale oxidation was carried out as follows: NaBiO₃ (82.5 g) was added to a solution of compound (IVb) (52.12 g) in 50% (v/v) acetic acid (1.5 litres) and the suspension was vigorously stirred on a mechanical shaker at approx. 28°C for 43 h. The mixture was filtered through a Hyflo-Super-Cel (Johns-Manville Corp., New York, NY, U.S.A.) pad to remove fine precipitates and the precipitates were washed well with 50% (v/v) acetic acid (approx. 100 ml). The washings were combined with the filtrate, concentrated *in vacuo*, diluted with water and then thoroughly extracted with chloroform (2.5

litres). The extract was washed with water, dried over anhydrous Na₂SO₄ and evaporated *in vacuo* to yield a brown-coloured syrup (36.95 g). This was dissolved in water (approx. 100 ml) with the use of NaHCO₃ powder and ice-cooled. Acidification with dilute HCl precipitated the fine colourless crystals of compound (IIb), which were collected by filtration, washed with water and then dried under reduced pressure to give the first crop (27.24 g, m.p. 109–110.5°C) of crystals. The filtrate was combined with the washings, saturated with NaCl and then extracted with chloroform. Evaporation of the chloroform left a residue (6.54 g), which crystallized from acetone/light petroleum to yield the second crop (2.17 g, m.p. 108–110°C) of crystals of compound (IIb).

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