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OXIDATION OF 3β-HYDROXYANDROSTENES BY THE 3β-HYDROXY-STEROID OXIDASE (CHOLESTEROL OXIDASE) FROM *BREVIBACTERIUM STEROLICUM* PRIOR TO THEIR ANALYSIS BY GAS–LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

The 3β-hydroxysteroid oxidase from *Brevibacterium sterolicum* has been applied to the oxidation of a number of 3β-hydroxyandrostenes, including polar steroids containing up to three other hydroxylic groups. The substrates, products, and derivatives thereof have been examined by gas–liquid chromatography. Retention index increments for these conversions, and for parallel transformations of other steroids, show considerable regularities, and together with mass spectrometric data afford characteristic structural information.

INTRODUCTION

The use of cholesterol oxidase (E.C. 1.1.36) from *Nocardia erythropolis* for the selective oxidation of 26-hydroxycholesterol[1] and of a wide variety of other 3β-hydroxysteroids has been described. The method was applied in particular to pairs of Δ^5- and 5α, 3β-hydroxysteroids which were difficult to separate by gas–liquid chromatography (GLC). The respective products, Δ^4- and 5α,3-ketosteroids, are widely separated[2,3]. Further investigations illustrated the usefulness of the enzyme in the characterisation of natural sterols[4] and in the production of 3-ketosteroids, especially for sterols containing additional hydroxyl groups[5–8]. Unfortunately, although most sterols

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and other 3β-hydroxysteroids were oxidised at satisfactory rates, C_{19} 3β-hydroxysteroids (with no side-chain at C-17) were oxidised extremely slowly[9–11]. This difficulty was partially overcome for 17-keto-5-androstenes by the formation of the 17-O-isopentyloximes[11,12] (to mimic the sterol side-chain) prior to incubation with the enzyme.

In contrast with the enzyme from *N. erythropolis*, the 3β-hydroxysteroid oxidase from *Brevibacterium sterolicum*[13] oxidises dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) at a satisfactory rate[14]. We have therefore explored the utility of this enzyme in the oxidation of other C_{19} 3β-hydroxysteroids, in connection with its application to the characterisation of steroids of this type in biological samples such as amniotic fluid and new-born infants' urine.

MATERIALS AND METHODS

3β-Hydroxysteroid oxidase was obtained from *Brevibacterium sterolicum* as described previously[13]. The preparation used contained 0.4 mg protein/mg solid (20.3 Units). 5-Androsten-3β-ol, 3β-hydroxy-D-homo-5-androsten-17a-one, 3β-hydroxy-5-androstene-11,17-dione, 3β,7β-dihydroxy-5-androsten-17-one, 3β,19-dihydroxy-5-androsten-17-one, 5α-androstane-3β,17α-diol and 5α-androstane-3α,17β-diol were obtained from the M.R.C. Steroid Reference Collection (Professors W. Klyne and D. N. Kirk). 3β,15α-Dihydroxy-5-androsten-17-one, 5-androstene-3β,15α,16α,17β-tetrol and 5-androstene-3β,15β,16β,16β-tetrol were kindly donated by Dr. E. M. Chambaz (University of Grenoble, Grenoble, France). Other steroids were purchased from BDH (Poole, Great Britain), Ikapharm (Ramat-Gan, Israel), and Koch-Light (Colnbrook, Great Britain). Methoxyamine hydrochloride was supplied by Kodak (Kirby, Great Britain) and N,O-bis-(trimethylsilyl)trifluoroacetamide was obtained from Pierce and Warriner (Chester, Great Britain).

Steroids (50 μg) were mainly oxidised at 30° by addition in propan-2-ol (50 μl) to 50 m*M* NaH_2PO_4–Na_2HPO_4 buffer (3 ml, pH 7.0) containing 100 μg of enzyme preparation. Some incubations were performed on a smaller scale. The progress of oxidation was usually monitored for Δ^5-3β-hydroxysteroids by the increase in extinction at 240 nm due to the formation of the Δ^4-3-ketone chromophore. Products were extracted with ethyl acetate (2.5 ml) and the extracts were washed with water (2 ml).

O-Methyloximes were prepared by heating the steroids in pyridine at 80° with 3 mole-equiv. of methoxyamine hydrochloride for 1 h. Trimethylsilyl (TMS) ethers were formed by heating the steroids with N,O-bis(trimethylsilyl)trifluoroacetamide (20 μl) at 80° for 30 min.

GLC was performed on a Pye 104 gas chromatograph fitted with columns (2.5 m × 4 mm I.D.) of 1% OV-1 or 1% OV-17 on Gas-Chrom Q (100–120 mesh) at 235° with nitrogen as the carrier gas (50 ml/min). The data in Table III(a) were obtained on a Carlo Erba Fractovap GB gas chromatograph with a column (3.6 m × 3 mm I.D.) packed with 1% SE-30 on Gas-Chrom Q and temperature programmed from 190° at 1°/min. Mass spectra were obtained by GLC–mass spectrometry (MS) using LKB 9000 or DuPont 21-490 F instruments each with GLC columns (2.5 m × 3 mm I.D.) of 1% OV-1. Helium was used as the carrier gas (30 ml/min), the source temperatures were 260–280° (LKB) and 240–250° (DuPont) and spectra were obtained at 70 eV. The mass spectrometers were linked to a VG 2035 data system.

RESULTS AND DISCUSSION

The 3β-hydroxysteroid oxidase from *Brevibacterium sterolicum* has been used by Yamaguchi *et al.*[15] in the estimation of urinary dehydroepiandrosterone. In further work the unreactivity of 3α-hydroxysteroids with the enzyme was exploited in the determination of the relative contributions of 3α- and 3β-hydroxysteroids to the total 17-ketosteroids of human urine[16]. We were interested in separating the pair of epimers 5α-androstane-3α,17β-diol and 5α-androstane-3β,17α-diol by thin-layer chromatography (TLC). In a protocol for the separation and identification by TLC of testosterone metabolites of human hyperplastic prostate, Beastall was unable to distinguish these compounds[17]. Separation was achieved less conveniently by preparative GLC of the trimethylsilyl ethers. Selective enzymic oxidation of the 3β-hydroxysteroid to 17α-hydroxy-5α-androstan-3-one followed by TLC readily afforded a simple procedure of indirect analysis (Table I).

TABLE I

SEPARATION OF 5α-ANDROSTANE-3β,17α-DIOL (VIA SELECTIVE TRANSFORMATION) AND 5α-ANDROSTANE-3α,17β-DIOL FOLLOWING INCUBATION WITH 3β-HYDROXYSTEROID OXIDASE

A mixture of 5α-androstane-3β,17α-diol (100 μg) and 5α-androstane-3α,17β-diol (200 μg) was incubated with enzyme (100 μg) overnight as described in Materials and methods. After extraction, the products were analysed by thin-layer chromatography on silica gel with chloroform–ethyl acetate (3:1, v/v) as the mobile phase.

Steroids	R_F
5α-Androstane-3β,17α-diol	0.35
5α-Androstane-3α,17β-diol	0.36
17α-Hydroxy-5α-androstan-3-one	0.52
Mixture of diols	0.35
Products after incubation	0.35 0.53

In previous work, cholesterol oxidase from *Nocardia erythropolis* has been used to oxidise numerous Δ^5-3β-hydroxysteroids in good yield to the Δ^4-3-ketones, which were analysed by GLC and GLC–MS.

Unfortunately, the low reactivity of C_{19} steroids limited the investigations of androstenes. However, using the enzyme from *B. sterolicum* we were able to oxidise a number of Δ^5-3β-hydroxyandrostenes. These and the oxidation products were analysed by GLC as their trimethylsilyl ethers (Table II). The increases observed in the Kováts retention indices on oxidising a Δ^5-3β-hydroxysteroid to the corresponding Δ^4-3-ketone were similar to those previously reported for Δ^5-3β-hydroxyandrosten-17-one isopentyloximes[11] and for Δ^5-stenols[4]. An application of the method to the analysis of the 5α- and Δ^5-sterols of certain sponges has been reported[4]. As can be seen in Table II, 11-ketodehydroepiandrosterone (3β-hydroxy-5-androstene-11,17-dione) was not oxidised by the enzyme under the conditions employed. Other derivatives of dehydroepiandrosterone which failed to react were the 6-methyl, 7-keto, and 11β,16α-dihydroxy analogues. Examination of the mass spectra of the ketones formed from reactive substrates, after treatment with N,O-bis(trimethylsilyl)trifluoroacetamide,

TABLE II

RETENTION INDICES, *I*, OF SOME 3β-HYDROXY-Δ⁵-ANDROSTENES BEFORE AND AFTER INCUBATION WITH 3β-HYDROXYSTEROID OXIDASE

For experimental details, see Materials and methods. The steroids were derivatised as the TMS ethers prior to GLC.

Substrate	$I^{235°}_{OV\text{-}1}$		
	Before oxidation	*After oxidation*	*ΔI*
5-Androsten-3β-ol	2360	2425	+65
3β-Hydroxy-5-androsten-17-one	2590	2650	+60
3β-Hydroxy-D-homo-5-androsten-17α-one	2705	2810	+105
3β,7β-Dihydroxy-5-androsten-17-one	2705	2750	+45
3β,19-Dihydroxy-5-androsten-17-one	2685	2835	+150
3β-Hydroxy-5-androstene-11,17-dione	2730	2730*	—
5-Androstene-3β,17α-diol	2600	2630	+30
5-Androstene-3β,17β-diol	2620	2675	+55
5-Androstene-3β,16β,17β-triol	2890	2945	+55
3β,17β-Dihydroxy-5-androsten-16-one	2795	2845	+50

* Unchanged substrate.

showed that partial formation of enol trimethylsilyl ethers occurred. In a further series of oxidations, products and substrates were converted to the methyloximes before trimethylsilylation. On OV-1 phase, relatively small increases in retention time were observed: *e.g.* dehydroepiandrosterone methyloxime trimethylsilyl ether, $I^{245°}$ =

TABLE III

EFFECT OF ENZYMIC OXIDATION ON THE GLC RETENTION INDICES OF SOME 3β-HYDROXYANDROSTENES AND 3β-HYDROXYANDROSTANES CHROMATOGRAPHED AS THE METHYLOXIMES

For experimental details, see Materials and methods. Hydroxylic groups were derivatised as the TMS ethers.

Substrate	$I^{190 \times 1°/min}_{SE\text{-}30}$			
	Before oxidation (TMS or methyloxime–TMS)	*After oxidation (methyloxime–TMS)*		*ΔI To mid point*
(a) Data for temperature programmed GLC on 1% SE-30 column				
3β,7α-Dihydroxy-5-androsten-17-one	2635	2675	2695	50
3β,15α-Dihydroxy-5-androsten-17-one	2780	2790	2815	20
3β,18-Dihydroxy-5-androsten-17-one	2765	2790	2805	30
3β,19-Dihydroxy-5-androsten-17-one	2705	2765	2775	65
5-Androstene-3β,15α,16α,17β-tetrol	3035	3095		60
5-Androstene-3β,15β,16β,17β-tetrol	3095	3155	3165	65
(b) Data for isothermal GC on 1% OV-17 column		$I^{235°}_{OV\text{-}17}$		
5-Androsten-3β-ol	2475	2665	2690	200
3β-Hydroxy-5-androsten-17-one	2910	3025	3040	125
3β-Hydroxy-5α-androstan-17-one	2905	2985	3000	90
3β-Hydroxy-D-homo-5-androsten-17α-one	3035	3170	3195	145
3β-Hydroxy-5-androsten-16-one	2930	3065	3095	150
5-Androstene-3β,17β-diol	2725	2915	2935	200

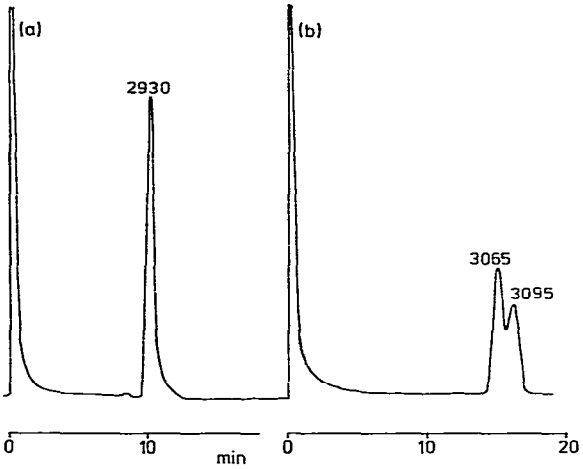

Fig. 1. GLC chromatograms of (a) 3β-hydroxy-5-androsten-16-one as the methyloxime trimethylsilyl ether and (b) 4-androstene-3,16-dione formed after oxidation by the 3β-hydroxysteroid oxidase from *B. sterolicum* and derivatised as the 3,16-dimethyloxime. GLC column 1% OV-17 (2.5 m × 4 mm I.D.) at 235° with 50 ml/min of nitrogen.

2640; 4-androstene-3,17-dione dimethyloxime, $I^{245°} = 2660$. In addition there was only a small difference in retention indices between the Δ^4-3-ketone and 5α-3-ketone methyloximes (5α-androstane-3,17-dione dimethyloxime $I^{245°} = 2655$), in contrast with the characteristic difference ($\Delta I_{OV\text{-}1} \approx 80$) observed with the free ketones[3]. When SE-30 phase was used with temperature programming, retention increments accompanying oxidation were somewhat larger (Table III (a)). Analysis of the methyloxime trimethylsilyl ethers on OV-17 liquid phase however, gave more substantial increases in retention indices after oxidation and a greater distinction between 5α- and Δ^4-3-ketones (Table III(b)). Two peaks were observed for the methyloximes of 3-keto-

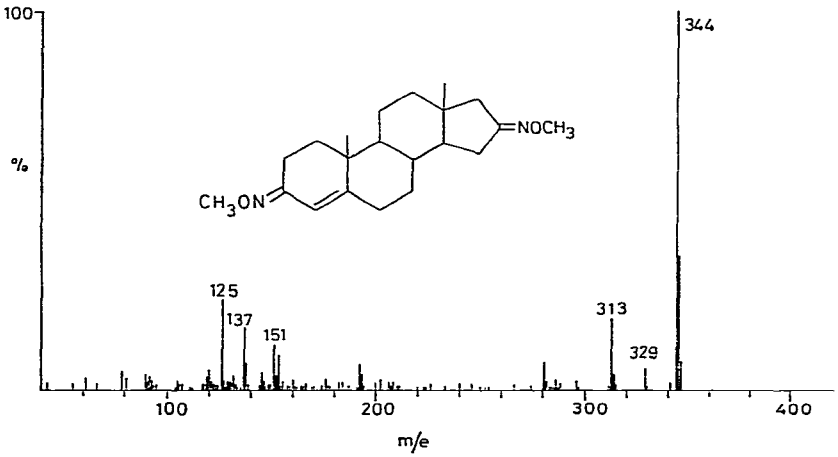

Fig. 2. Mass spectrum (70 eV) of the 4-androstene-3,16-dione (derivatised as the 3,16-dimethyloxime) formed during oxidation of 3β-hydroxy-5-androsten-16-one by 3β-hydroxysteroid oxidase from *B. sterolicum*.

TABLE IV

REPRESENTATIVE RETENTION INDEX INCREMENTS ACCOMPANYING CHOLESTEROL OXIDASE OXIDATIONS

Substrate type	*Derivatives of*		*ΔM*	*Column*	*Temp.*	*ΔI*	*No.*	*Range*
	Substrate 3-ol	*Product 3-one*						
5-en-3β-ols								
Sterols[3]	—	—	−2	OV-1	275	+115	8	105–125
Sterols[4]	TMS	—	−74	OV-1	275	+50	4	45–50
Side-chain hydroxy sterols[5]	TMS	—	−74	OV-1	265	+55	7	50–85
Side-chain hydroxy sterols[5]	TMS	—	−74	OV-17	265	+225	7	205–235
Pregnenols[3,*]	TMS	—	−74	OV-1	250	+50	3	45–55
Androstenols[18]	—	—	−2	OV-1	210	+75	3	70–85
Androstenols[**]	TMS	—	−74	OV-1	235	+50	7	30–65
19-Hydroxyandrostenols[**]	TMS	—	−74	OV-1	235	+150	1	—
Androstenols[19]	TMS	Methyloxime	−45	SE-30	190 × 1°/min	+55	4	40–65
Androstenols[18]	—	—	−2	OV-17	230	+160	3	155–165
Androstenols[**]	TMS	Methyloxime	−45	OV-17	235	+160	5	125–200
5α-3β-ols								
Sterols[3]	—	—	−2	OV-1	275	+25	7	15–35
Sterols[4]	TMS	—	−74	OV-1	275	−30	9	−25–−45
Pregnanols[3,*]	TMS	—	−74	OV-1	250	−30	3	−20–−35
Androstanols[**]	TMS	Methyloxime	−45	OV-17	235	+90	1	—

* 17α-Hydroxy-20-ones (1 example of each) gave different increments and are excluded pending further investigations.

** This paper.

steroids, corresponding to the *syn* and *anti* isomers. During GLC–MS on the 2.5 m OV-1 column only one unresolved peak was usually observed. Gas chromatograms (OV-17) illustrating the enzymic oxidation of 3β-hydroxy-5-androsten-16-one are shown in Fig. 1, while Fig. 2 shows the mass spectrum of the dimethyloxime of the 4-androstene-3,16-dione formed in the reaction.

From a survey of data collected in the course of several investigations it is possible to list typical retention index increments for the oxidation of various substrate types and for various derivatives used for GLC. Examples are summarised in Table IV: some indication of the regularity to be expected is given by the ranges of values observed. Substituents in the vicinity of the site of oxidation affect the increments, as exemplified by the value of +150 found for the conversion of 19-hydroxydehydroepiandrosterone (as diTMS ether) to 19-hydroxyandrostenedione (as TMS ether). Similarly the conversion[8] of 19-hydroxycholesterol (as diTMS ether) into 19-hydroxycholestenone (as TMS ether) yielded a ΔI value of +275, compared with +225 for side-chain hydroxycholesterol oxidations (Table IV).

The results indicate that the *Brevibacterium* cholesterol oxidase is effective in catalysing the oxidation of 5-androsten-3β-ols containing as many as three additional hydroxyl groups. The enzyme has accordingly been found applicable to the characterisation of dihydroxy and trihydroxy-5-androsten-17-ones in human amniotic fluid[19]. The different substrate specificities of the cholesterol oxidases from *Brevibacterium* and *Nocardia* enhance the analytical power of selective enzymic oxidation in the study of natural 3-hydroxysteroids. A comparative study of the characteristics of these cholesterol oxidases, and of two similar enzymes from *Schizophyllum* and *Streptomyces* spp., has recently been reported[20].

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