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MICROBIAL TRANSFORMATIONS OF STEROIDS—VI. TRANSFORMATION OF TESTOSTERONE AND ANDROSTENEDIONE BY *BOTRYOSPHAERICA OBTUSA*

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Summary—The 7β progesterone-hydroxylating microorganism *Botryosphaerica obtusa* [1] was tested for its ability to hydroxylate at this site the C-19 androstene-based compounds, androstenedione (androst-4-ene-3,17-dione) and testosterone (17β-hydroxyandrost-4-en-3-one). Only very limited 7β hydroxylation of both substrates was observed. The products included traces of 7β-monohydroxytestosterone and 6β,7β-dihydroxyandrostenedione from testosterone, and of 6β,7β-dihydroxyandrostenedione from androstenedione. 6β,7β-Dihydroxyandrostenedione does not appear to have been reported previously as a microbial transformation product. Both substrates were monohydroxylated in significant amounts at the isomeric 7α site and at the 6β site. Testosterone was also significantly monohydroxylated at the 15α site and in minor amounts at the 11α and 12β sites. Some monohydroxytestosterones had also been oxidised at their 17-OH group, converting them into the corresponding monohydroxy androstenediones. The 7α-hydroxy metabolites and 15α-hydroxytestosterone being chemically demanding to synthesis are valuable microbial transformation products.

INTRODUCTION

We have found *Botryosphaerica obtusa* to be an efficient 7-hydroxylator of progesterone [1]. Short incubations produced 7β-monohydroxyprogesterone as the predominant metabolite and small amounts of 7α-monohydroxyprogesterone. Trace amounts of 6β-, 9α-, 14α-, and 15β-monohydroxyprogesterone, together with their corresponding 7β-hydroxylated diol derivatives, were also produced. Longer transformation times considerably increased the conversion to these 7β-dihydroxylated progesterones. A third set of products was also present in 3-day aqueous transformation media. These were C(6)-C(7) unsaturated monohydroxyprogesterones (monohydroxy-6-dehydroprogesterones), produced by dehydration of precursor diols derived from 7β-hydroxyprogesterone [1, 2].

7β-Hydroxylated steroidal 4-en-3-ones are not commercially available. No chemical synthesis seems to have been described, and they do not appear to have been made in large quantities by microbiological means probably because microbial producers of these compounds are uncommon, mostly being filamentous fungi [3–6]. 7β-Hydroxytestosterone is known mainly as a product of metabolism in the liver [7]. Therefore, it was of considerable importance for us to know if our strain of *B. obtusa* would 7β-hydroxylate steroids other than progesterone. In particular, a microbial synthesis of 7β-hydroxytestosterone and 7β-hydroxyandrostenedione would enable us to supply these materials through the Steroid Reference Collection, to aid studies of liver hydroxylation of the steroid hormones.

As in previous investigations in this series [1–2, 8–10], high-field ^{1}H-nuclear magnetic resonance (NMR) spectroscopy has been used for rapid and efficient structure determination of sub-milligram quantities of products isolated by high-pressure liquid chromatography (HPLC), to avoid the need for tedious chemical purification on a larger scale.

EXPERIMENTAL

Cultivation of B. obtusa strain 38560 and steroid transformation

B. obtusa was cultivated and steroid transformations were performed as previously described [1].

Steroid metabolite purification and analysis by nuclear magnetic resonance spectroscopy

At the end of 3 days of androstenedione or testosterone transformation, steroids were extracted from the aqueous media by use of chloroform, then purified by semi-preparative reverse-phase HPLC using 5 micron ODS 2 columns eluted with 60% aqueous methanol. Structures were determined by high-field ^{1}H-NMR spectroscopy (250–500 MHz) as previously described [1–2, 8–10]. Compounds for which authentic spectra were already available were

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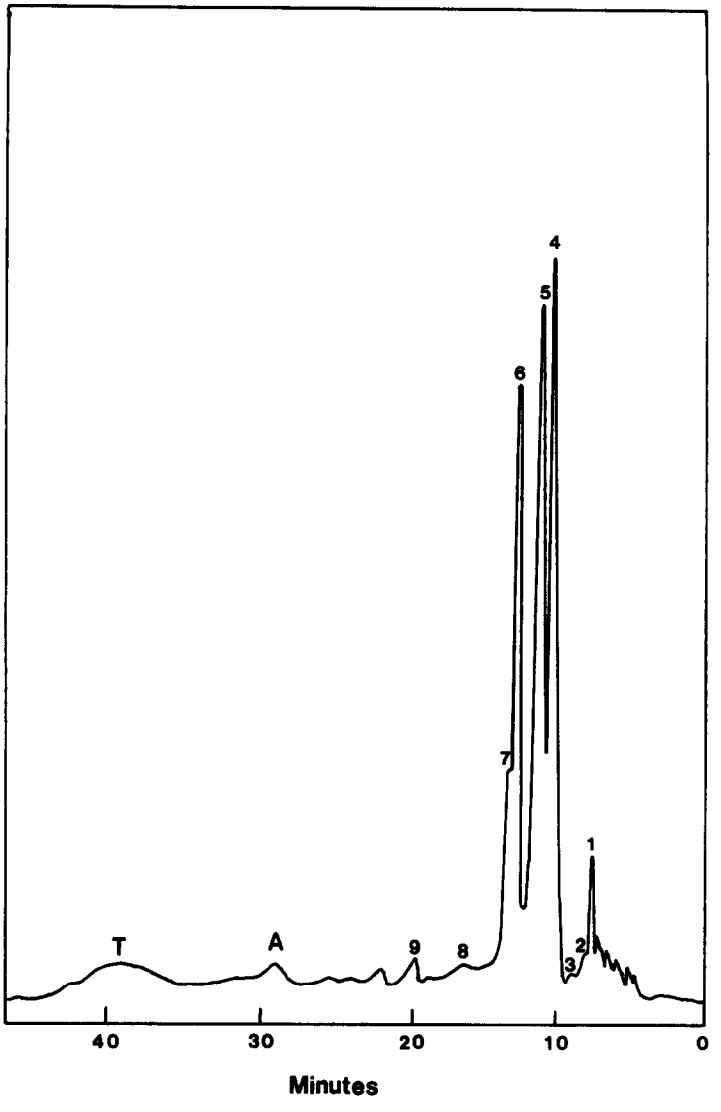

Fig. 1. Analytical 5 μ ODS 2 reverse-phase HPLC trace of *B. obtusa* testosterone metabolites eluted with 60% aqueous methanol. The peak labelled A is androstenedione and that labelled T is testosterone.

identified by "fingerprint" spectral comparisons, especially of signals for the geminal H in C*H*OH systems and for the 4-H, 17-H (in testosterones), 18-H_3 and 19-H_3 protons. Where no reference spectrum was available, a 2D ^{1}H homonuclear shift-correlated (COSY) spectrum was obtained and the structure was assigned by determining signal connectivities of the skeletal protons.

RESULTS

The testosterone-transforming ability of *B. obtusa* is unusual because, unlike most filamentous fungi we have tested, it is capable of producing after 3 days of incubation a large number of metabolites. We were able to isolate nine metabolites in sufficient quantities for structural analyses by high-field 1D and 2D COSY ^{1}H-NMR spectroscopy. The peaks for these compounds are shown in the analytic 5 μ ODS 2 reverse-phase HPLC trace in Fig. 1, and are labelled 1–9 in order of increasing retention time on the column. By contrast, *B. obtusa*'s androstenedione-transforming ability was more in line with expectation, being limited compared with that for testosterone. The analytical reverse-phase HPLC trace of the androstenedione transformation metabolites is shown in Fig. 2. The compounds labelled 10–13 in the trace were those for which we were able to determine structures.

The presence in the NMR spectra of signals for 4-H (s* or narrow d, δ *ca* 5.7–6.0), 18-H_3 *methyl* (s, δ *ca* 0.7–1.0) and 19-H_3 *methyl* (s, δ *ca* 1.2–1.5) showed that the androstenedione and testosterone metabolites had retained their fundamental androst-4-en-3-one skeleton. Six of the testosterone products (labelled 3, 4, 5, 7, 8 and 9 in Fig. 1) had mid-field

*s, singlet; d, doublet; t, triplet; m, multiplet.

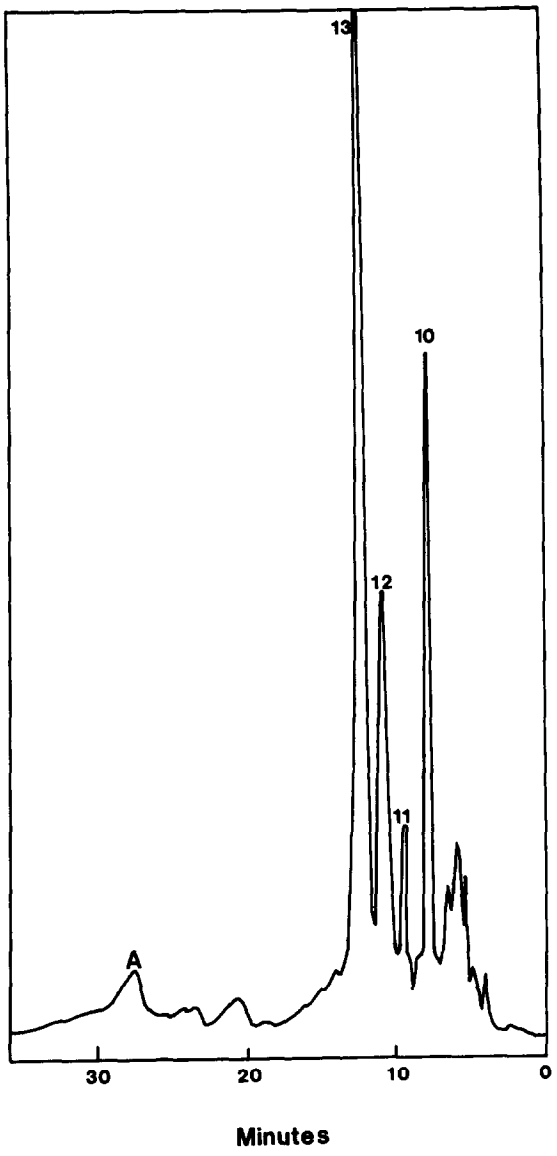

Fig. 2. Analytical 5 μ ODS 2 reverse-phase HPLC trace of *B. obtusa* androstenedione metabolites (see legend to Fig. 1). The peak labelled A is androstenedione.

17α-H signals (t, δ *ca* 3.6–4.0) showing that the 17β-OH group had not been oxidised. Three compounds (labelled 1, 2 and 6 in Fig. 1) lacked this mid-field signal and their 18-H_3 methyl signal was shifted down-field (δ *ca* 0.9–1.0) showing that these metabolites were androstenedione derivatives. Spectra of all the compounds, except compound 1, had new mid-field multiplets (δ 3.5–4.5) characteristic of geminal methine protons of C*H*OH systems, indicating that they had been monohydroxylated. Compound 1 had two of these mid-field multiplets showing it to be dihydroxylated. The HPLC polarities (reverse-phase) of all products were consistent with the degree of hydroxylation indicated: none showed the additional chemical shifts which, in our earlier work, had indicated hydroxylation at tertiary sites (C-9 or C-14) [8].

Androstenedione gave only hydroxylated androstenediones.

NMR data for individual testosterone and androstenedione transformation metabolites

NMR features of structural significance for those testosterone and androstenedione metabolites which have not previously been reported by us are summarised in the paragraphs below, with fuller details in Table 1. Calculated chemical shifts, in parentheses in Table 1, are derived by applying hydroxylation increments obtained from the corresponding monohydroxyprogesterones. The very good agreement between observed and calculated δ values demonstrates once again the constancy and additivity of substituent increments.

Compounds 1 and 10. 6β,7β-Dihydroxyandrostenedione. This compound was present in tiny amounts from testosterone transformations but was a major product of androstenedione transformation. It does not appear to have been reported previously as a microbial transformation product, but has been synthesised chemically as a product of hydroxylation of androst-4,6-diene-3,17-dione with osmium tetroxide [11]. The two methine proton signals (δ 4.28, d, $J_{6\alpha,7\alpha}$ 3.5 Hz, 6α-H, and δ 3.51, td, collapsing to dd on adding D_2O, $J_{6\alpha,7\alpha}$ 3.5 Hz, $J_{7\alpha,8}$ 10 Hz, 7αH) were seen from the COSY spectrum to be mutually spin-coupled. The presence of a 6β-hydroxy group was indicted by the characteristic shift and singlet profile of the 4-H signal and the strong deshielding of 19-H_3 (δ 1.39) and 8β-H (δ 2.00) [9]. The 18-H_3 singlet (δ 0.96) was in the normal range for an androstan-17-one with no substituent in rings C or D.

Compounds 2 and 11. 15α-Hydroxyandrostenedione. This metabolite is a transformation product of both testosterone and androstenedione. Its NMR features and polarity matched those reported previously [8].

Compound 3. 7β-Hydroxytestosterone. The 7β location for the additional hydroxy group was indicated by close similarities of the NMR spectrum to that of 7β-hydroxyprogesterone [1], in particular the 7α-H signal (δ 3.47, m, $W_{\frac{1}{2}}$ *ca* 36 Hz). The spin-coupled (COSY) 6α-H (δ 2.53, dd, $J_{6\alpha,6\beta}$ 14 Hz, and $J_{6\alpha,7\beta}$ 5 Hz) and 6β-H (δ 2.43, dd, $J_{6\alpha,6\beta}$ 14 Hz, $J_{6\beta,7\beta}$ 2 Hz) also had the chemical shifts and unusual multiplicities indicative of 7β-hydroxylation. Other signals in the NMR spectrum were normal for a testosterone derivative.

Compound 4. 15α-Hydroxytestosterone. The methine proton signal (15β-H) was a characteristic broad doublet, coupled (COSY) to 16α-H and 16β-H which were also coupled to 17α-H. We have previously reported a similar set of couplings on the corresponding signals for 15α-hydroxyprogesterone, to establish the location of the hydroxy group. Its configuration is clearly α, from the absence of any major down-field shift of the 18-methyl proton signal, together with

Table 1. ^{1}H-NMR data[a] for testosterone, androstenedione and hydroxylated derivatives

	18-H_3	19-H_3	17α-H	Other C*H*OH	4-H	Other significant proton signals				
Testosterone	0.80	1.19	3.65	—	5.73	1.70 (1α-H) 2.03 (1β-H)	2.29 (6α-H) 2.42 (6β-H)	1.57 (8β-H) 0.93 (9α-H)	1.60 (11α-H) 1.43 (11β-H)	2.09 (16α-H) 1.48 (16β-H)
7β-Hydroxytestosterone	0.82 (0.84)	1.22 (1.22)	3.64 (3.59)	3.47 (7α-H) (3.41)	5.77 (5.78)	—	2.53 (6α-H) (2.55) 2.43 (6β-H) (2.46)	—	—	2.10 (16α-H) (2.15) 1.51 (16β-H) (1.50)
11α-Hydroxytestosterone	0.82 (0.84)	1.32 (1.31)	3.70 (3.67)	4.05 (11β-H) (4.01)	5.72 (5.74)	2.01 (1α-H) (2.00) 2.66 (1β-H) (2.66)	—	—	—	—
12β-Hydroxytestosterone	0.87	1.21	3.90	3.53 (12α-H)	5.73	—	—	—	1.76 (11α-H) 1.48 (11β-H)	2.10 (16α-H) 1.51 (16β-H)
15α-Hydroxytestosterone	0.82 (0.83)	1.22 (1.19)	3.92 (3.91)	4.13 (15β-H) (4.16)	5.72 (5.75)	—	—	1.80 (8β-H) (1.77)	—	1.97 (16β-H) (2.09)
Androstenedione	0.93	1.22	—	—	5.76	—	2.35 (6α-H) 2.40 (6β-H)	1.75 (8β-H) 1.00 (9α-H)	—	2.12 (16α-H) 2.48 (16β-H)
6β-Hydroxyandrostenedione	0.96 (0.96)	1.42 (1.42)	—	4.41 (6α-H) (4.42)	5.84 (5.83)	—	—	2.18 (8β-H) (2.17) 0.98 (9α-H) (0.98)	— —	2.15 (16α-H) (2.16) 2.49 (16β-H) (2.48)
7α-Hydroxyandrostenedione	0.93 (0.94)	1.22 (1.22)	—	4.11 (7β-H) (4.12)	5.81 (5.85)	—	2.47 (6α-H) (2.49) 2.68 (6β-H) (2.65)	1.29 (9α-H) (1.48)		2.14 (16α-H) (2.18) 2.48 (16β-H) (2.51)
6β,7β-Dihydroxyandrostenedione[b]	0.96 (1.00)	1.39 (1.45)	—	4.28 (6α-H) 3.51 (7α-H)	5.90 (5.91)	—	—	2.00 (8β-H) 0.89 (9α-H)	—	1.61 (16α-H) 2.48 (16β-H)

[a] δ, ppm from internal Me_4Si: solvent, $CDCl_3$. Calculated δ values, in parentheses, were derived by use of hydroxylation increments from the corresponding monohydroxyprogesterones. [b] Literature values, in DMSO-d_6/D_2O instead of $CDCl_3$, 18-H_3, 0.87; 19-H_3, 1.32; 7α-H, 3.30; 6α-H, 4.10; 4-H, 5.75 [11].

Table 2. Relative percentage yields of testosterone and androstenedione metabolites

Substrate	Metabolite		Relative yield (%)	Amount recovered per 10 mg incubation (mg)
Testosterone	6β,7β-Dihydroxyandrostenedione	(Compound 1)	4	0.3
	6β-Hydroxyandrostenedione 6β-Hydroxytestosterone	(Compound 6) (Compound 7)	35	2.4
	7α-Hydroxytestosterone	(Compound 5)	28	1.9
	7β-Hydroxytestosterone	(Compound 3)	<2	<0.1
	11β-Hydroxytestosterone	(Compound 8)	<2	<0.1
	12β-Hydroxytestosterone	(Compound 9)	2	0.1
	15α-Hydroxyandrostenedione	(Compound 2)	<2	<0.1
	15α-Hydroxytestosterone	(Compound 4)	29	2.0
Androstenedione	6β,7β-Dihydroxyandrostenedione	(Compound 10)	22	1.6
	6β-Hydroxyandrostenedione	(Compound 13)	47	3.4
	7α-Hydroxyandrostenedione	(Compound 12)	18	1.3
	15α-Hydroxyandrostenedione	(Compound 11)	2	0.2

significant deshielding of 17α-H as observed for 15α-hydroxyprogesterone [9].

Compound 5. 7α-Hydroxytestosterone. We have already described the NMR features of this compound [9].

Compounds 6 and 13. 6β-Hydroxyandrostenedione. This is a minor metabolite in testosterone transformation mixtures but a major one in androstenedione counterparts. It has the typical features of a 6β-hydroxy-4-en-3-one, already described for 6β-hydroxyprogesterone [9] and 6β-hydroxytestosterone [9].

Compound 7. 6β-Hydroxytestosterone. The NMR features were identical with those already described [9].

Compound 8. 11α-Hydroxytestosterone. Like 11α-hydroxyprogesterone [2], this compound was recognised from the broad 11β-H signal, and large down-field shifts of the 19-H_3 and 1α- and 1β-H signals, by virtue of their spatial proximity to 11α-OH. The 1β-OH signal, a distinctive pair of triplets at the low-field extreme of the methylene envelope (δ 2.66), is particularly easy to recognise. The metabolite was identical with an authentic sample.

Compound 9. 12β-Hydroxytestosterone. The detailed ^{1}H-NMR features of a 12β-hydroxy steroid do not appear to have been described previously. 12β-Hydroxytestosterone showed a distinctive 12α-H signal as a double doublet (δ 3.53), being coupled only to the vicinal 11α- and 11β-protons. The equatorial position of the hydroxy substituent was indicated by the magnitudes of the two coupling constants affecting the geminal 12α-proton ($J_{11\alpha,12\alpha}$ 4.5 Hz; $J_{11\beta,12\alpha}$ 11 Hz). The new hydroxy substituent shifted the 11α-H, 17α-H and 18-H_3 signals down-field compared with testosterone, but had little effect on any others. These shifts can be attributed to spatial proximity.

Compound 12. 7α-Hydroxyandrostenedione. The characteristic features of the ^{1}H-NMR spectrum of this compound corresponded very closely to those already described for 7α-hydroxyprogesterone [9] and 7α-hydroxytestosterone [9]. Its chromatographic mobility was identical with that of an authentic synthetic sample.

Yields of testosterone and androstenedione metabolites

The relative proportions and amounts of androstenedione and testosterone metabolites chloroform-extractable from aqueous transformation media were determined by computing integration of HPLC metabolite traces as previously described [8,10] and the results are presented in Table 2. Testosterone gave 6β-hydroxyandrostenedione and 6β-hydroxytestosterone in rougly equal proportions and their combined amounts approximated those of 7α-hydroxytestosterone and 15α-hydroxytestosterone, each being in the region of 30% of the total products and about 2 mg of product formed per 10 mg of added substrate. The three other metabolites, 6β,7β-dihydroxyandrostenedione, and 11α- and 12β-hydroxytestosterone were formed in trace amounts and together accounted for less than 5% of the total products. With androstenedione as substrate, 6β-hydroxyandrostenedione was the predominant metabolite accounting for just under 50% of products and equivalent to a yield of 3.5 mg per 10 mg of added substrate. 6β,7β-Dihydroxyandrostenedione and 7α-hydroxyandrostenedione were also formed in reasonable amounts. They comprised about 20% each of the total metabolites (about 1.5 mg per 10 mg incubation). Unlike its testosterone counterpart, 15α-hydroxyandrostenedione was a very minor product of androstenedione transformation.

DISCUSSION

We have found *B. obtusa* to be a very efficient 7β-hydroxylator of progesterone [1]. Minor monohydroxylated products, and dihydroxyprogesterone formed by longer incubations, show it to be capable also of hydroxylating the 6β-, 7α-, 9α-, 11α-, 12α-, 14α-, 15α- and 15β-sites, as well as dehydrating 7β-hydroxylated progesterones to 6-dehydroprogesterones.

Compared with many other microorganisms, *B. obtusa* metabolises testosterone efficiently. However, fewer products are produced with this substrate than with progesterone and hydroxylation site usage is different. The 6β-, 7α-, 11α- and 15α-positions, which are minor progesterone hydroxylation sites, are the major ones hydroxylated on testosterone (Fig. 1). Only trace amounts of 7β-monohydroxytestosterone, and 6β,7β-dihydroxyandrostenedione, are detectable in transformation mixtures showing that the 7β site in testosterone, unlike the progesterone counterpart, is rarely attacked by *B. obtusa*.

Site usage with androstenedione is even more restricted (Fig. 2). Only the 6β-, 7α- and 15α-positions are significantly hydroxylated. Neither 7β-hydroxyandrostenedione nor 7β-monohydroxytestosterone was observed, but some 6β,7β-dihydroxyandrostenedione (compound 10) was produced, showing some capacity to 7β-hydroxylate androstene-based steroids. No other dihydroxylated metabolites were found, in contrast to progesterone transformation mixtures from this organism [1].

It is unclear why modification of the C-17 side-chain functionality should change *B. obtusa* from a major 7β-hydroxylator of progesterone to a major 6β-, 7α- and 15α-hydroxylator of androstene-based steroids. Presumably, the explanation must be in the way the different steroids are bound to the active site of the hydroxylase enzyme and presented to the catalytic centre.

Although we were disappointed to find that *B. obtusa* would not be a suitable organism for producing gramme-scale quantities of 7β-hydroxylated androstene-based compounds, we are gratified that it produces in reasonable yields three valuable steroidal transformation products, 7α-hydroxyandrostenedione, 7α-hydroxytestosterone and 15α-hydroxytestosterone, each of which is demanding to make chemically. This organism could be developed for producing these three compounds.

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