ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 6 (1999) 83–88

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Microbial hydroxylation of 2-oxatestosterone

Herbert L. Holland *, Gingipalli Lakshmaiah

Department of Chemistry, Brock University, St. Catharines, Ontario, Canada L2S 3A1

Received 30 March 1998; accepted 13 July 1998

Abstract

An investigation has been undertaken of the microbial biotransformation of 2-oxatestosterone by microorganisms known to hydroxylate conventional steroids, using *Aspergillus ochraceus*, *Bacillus megaterium*, *Curvularia lunata* and *Rhizopus arrhizus*. *A. ochraceus* and *B. megaterium* gave products of 11α- and 15β-hydroxylation, respectively. Biotransformation by *C. lunata* gave C-11β- and C-14α-hydroxylated products, whereas *R. arrhizus* produced only the 6β-hydroxy derivative.

Keywords: *Aspergillus ochraceus*; *Bacillus megaterium*; Biocatalysis; Biotransformation; *Curvularia lunata*; Hydroxylation; Oxasteroid; *Rhizopus arrhizus*; Steroid

1. Introduction

The hydroxylation of steroids by fungal or bacterial biocatalysts has been known for many years [1,2]. This procedure remains one of the most useful preparative methods for the introduction of hydroxyl groups at sites of the steroid nucleus remote from other functionality, and the value of microbial steroid hydroxylation in the preparation of pharmacologically active steroids is well-established. However, although the introduction of a heteroatom (O, N, or S) in place of a carbon in the steroid skeleton results in notable modifications of biological activity [3], the potential pharmacological properties of hydroxylated derivatives of heterosteroids remains largely unexplored as microbial transformations of ring heteroatom-substituted steroids have not been systematically studied. Although the hydroxylation of some 17-aza-D-homosteroids in low yields by *Cunninghamella elegans* [4] and the 11α-hydroxylation of 4-aza-5α-pregnane-3,20-dione by *Aspergillus ochraceus* [5] have been reported, no microbial hydroxylations or other biotransformations of oxasteroids appear in the literature, and the effect on the outcome of microbial hydroxylation reactions of the presence of an oxygen heteroatom in the substrate has not been systematically examined.

In view of this, the valuable pharmacological properties of 2-oxasteroids [6,7], and the fact that such compounds can be readily converted to 2-azasteroids (themselves possessing unique physiological properties [8]) by simple treatment with aqueous ammonia [6,9], we have undertaken a study of the biotransformations of 2-oxatestosterone (**1**) by the fungi *Aspergillus*

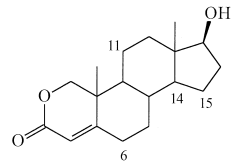
* Corresponding author. Tel.: +1-905-6885550; Fax: +1-905-6829020; E-mail: holland@chemiris.labs.brocku.ca

1381-1177/99/$ - see front matter
PII: S1381-1177(98)00134-9

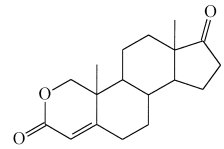
ochraceus, Bacillus megaterium, Curvularia lunata and *Rhizopus arrhizus*, microorganisms known to be efficient hydroxylators of conventional steroids at the pharmacologically significant C-11α, C-15β, C-11β, and C-6β positions, respectively [10,11].

2. Results and discussion

2-Oxatestosterone (**1**) was prepared by the method of Frimer et al. [7]. The ^{13}C NMR data for this compound and for its biotransformation products are summarised in Table 1. Biotransformation of **1** by *A. ochraceus*, summarised in Table 2, afforded a single product in 82% isolated yield, identified as the 11α-hydroxy derivative **1a** by spectral analysis. This and other products described below were identified by spectral analysis, in particular by the characteristic ^{1}H NMR shifts of their C-18 and C-19 methyl groups produced by hydroxylations at C-6β, C-11α, C-11β, C-15β [12], or C-14α [13], and by characteristic shifts of α, β, γ, and δ carbons in their ^{13}C NMR spectra, reported in Table 1 [13,14]. *A. ochraceus* is known to convert a variety of androstanes and pregnanes to their 11α-hydroxylated derivatives [15–17], and the formation of **1a** therefore serves to extend the range of substrates usefully hydroxylated by this microorganism.



1
1a 11αOH
1b 15βOH
1c 11βOH
1d 14αOH
1e 6βOH



2
2a 15βOH
2b 6βOH

Biotransformation of **1** by *B. megaterium* (Table 2) gave a mixture of products, including some in which the C-17β alcohol group had been oxidised to carbonyl. Two products of 15β hydroxylation, diol **1b** and ketoalcohol **2a**, were

Table 1
^{13}C NMR shifts of substrate 1 and products 1a–2b

C	Compound								
	1 [7]	**1a**	**1b**	**1c**	**1d**	**1e**	**2**	**2a**	**2b**
1	77.4	78.4	76.9	77.6	77.4	77.4	77.2	77.2	78.2
3	165.2	165.7	164.6	164.9	164.5	164.8	164.9	164.9	164.6
4	113.9	114.5	113.0	112.4	114.0	115.8	114.3	114.3	115.2
5	168.3	168.8	168.4	169.8	167.5	166.5	167.3	167.2	166.0
6	30.9[a]	30.8[a]	30.3[a]	31.3	30.7	69.1	31.0[a]	31.2	70.2
7	30.6[a]	30.4[a]	30.2[a]	30.5	25.6	37.2	30.9[a]	30.6	36.9
8	35.3	34.9	29.9	30.3	38.7	29.9	35.3	30.0	31.0
9	49.7	55.9	49.5	52.0	42.8	49.0	49.6	50.0	49.4
10	38.3	39.6	37.9	39.1	38.4	37.7	38.3	38.5	38.4
11	21.6	68.3	21.0	68.4	20.7	21.2	21.3	21.2	21.2
12	36.5	47.8	37.5	46.6	33.4	36.1	30.7[a]	32.8	30.6
13	43.0	44.0	42.9	42.2	47.3	42.6	47.7	47.4	47.4
14	50.6	50.1	54.8	53.0	83.4	50.1	51.0	55.1	51.2
15	23.8	23.6	67.9	23.9	29.8	23.2	22.1	67.4	21.8
16	31.0[a]	31.8	41.9	32.3	28.7	28.9	35.9	46.9	35.8
17	81.8	81.1	80.4	82.4	78.9	80.8	220.2	219.0	220.1
18	11.4	12.6	13.3	13.8	15.1	11.0	14.0	17.9	13.8
19	16.9	17.2	16.2	19.8	16.7	18.5	16.8	16.7	18.8

[a] Similar shifts may be interchanged in vertical columns.

Table 2
Summary of biotransformations of 2-oxatestosterone (**1**)

Microorganism	Product (hydroxylation site) (percentage yield)
A. ochraceus	**1a** (11α)(82)
B. megaterium	**1b** (15β)(30) **2** (−)(15) **2a** (15β)(35) **2b** (6β)(2)
C. lunata	**1c** (11β)(35) **1d** (14α)(40)
R. arrhizus	**1e** (6β)(59)

thus isolated, together with a small amount of the 6β alcohol **2b**. Although 15β hydroxylation is the predominant reaction reported for *B. megaterium* with steroidal substrates, hydroxylation at C-6β of Δ^4-3-ketosteroid substrates has also been previously observed [18].

C. lunata is an efficient 11β-hydroxylator of steroidal substrates [19], and is also known to carry out the 14α-hydroxylation reaction [20]. Both activities were observed in the present study, with the 11β-alcohol **1c** and the 14α-alcohol **1d** being formed in approximately equal amounts, as summarised in Table 2.

The regio-and stereoselectivities of steroid hydroxylations by *R. arrhizus* are known to depend on the structure of the substrate; saturated 5α-androgens are hydroxylated at C-6α, C-7α, C-7β, and C-11α (*inter alia*) [21], whereas, for mechanistic reasons [22], Δ^4-3-keto substrates are hydroxylated predominantly at C-6β [23]. The latter mode of reaction was exclusively observed in the present case, where the 6β-hydroxylated derivative **1e** was obtained as the sole product in 59% isolated yield.

None of the biotransformations discussed above has been optimised, but the processes are readily amenable to the routine scale-up and optimisation procedures already developed for microbial steroid hydroxylations [24,25], and the organisms used are well-characterised commercial strains that have previously been extensively examined for use in the hydroxylation of conventional steroids by the investigation of such techniques as cell immobilisation [24,26–28] and the application of organic solvent methodology [28]. The hydroxylations of 2-oxatestosterone reported herein are therefore of potential preparative utility. They are also of value in that they extend the range of substrates known to be efficiently hydroxylated by the biocatalysts used, in addition to providing a possible entry into the preparation of analogous hydroxyazasteroids by substitution of the heteroatom [6,9].

The observation that microbial hydroxylations of 2-oxatestoterone proceed at sites identical to those observed for hydroxylations of analogous carbocyclic steroids by the same microorganisms is also significant as it implies that, in contrast to some other microbial hydroxylations (notably those of aromatic hydrocarbons by *Mortierella isabellina*) [29], the presence of an oxygen heteroatom in the substrate has no directing influence on the outcome of the hydroxylation of a steroidal substrate. The generality of this statement remains to be established by investigation of the microbial hydroxylation of other heterosteroids by a wider range of microbial biocatalysts.

3. Experimental

3.1. Apparatus, materials, and methods

Melting points were determined on a Kofler heating stage. Infrared spectra were recorded with an ATI Matson Research Series FT spectrometer. The NMR spectra were recorded at 300 MHz (^{1}H) or 75 MHz (^{13}C) with a Bruker Avance series 300 spectrometer using $CDCl_3$ as solvent and $CHCl_3$ as internal standard. EI mass spectra were obtained with a Kratos 1S instrument. Thin layer chromatography was performed on Merck silica gel 60F-254 and column chromatography used silica gel, 230–400 mesh.

3.2. Maintenance and growth of microorganisms

A. ochraceus ATCC 18500 (NRRL 405) was maintained on Czapek agar slopes, grown at 24°C, stored at 4°C and grown in submerged culture at 24°C in a medium composed of glucose (5 g), malt extract (1 g), beef extract (1 g), yeast (1 g) and cornsteep liquor (1 ml) per liter of distilled water, adjusted to pH 5.5 prior to the addition of sucrose (2 g/l). *B. megaterium* ATCC 13368 was maintained on nutrient agar slopes at 30°C and grown in submerged culture at 30°C in a medium composed of soytone (15.5 g) and yeast extract (40 g) per liter of distilled water, adjusted to pH 6.2. *C. lunata* ATCC 12017 was maintained on rabbit food agar slopes (ATCC medium no. 340), grown at 27°C and stored at 4°C; this fungus was grown in submerged culture at 27°C in a medium composed of sucrose (10 g), tryptone (10 g), sodium nitrate (2 g), magnesium sulfate (0.5 g), potassium chloride (0.5 g), ferrous sulfate (0.1 g), dibasic potassium phosphate (1 g) and calcium carbonate (0.25 g) per liter of distilled water. *R. arrhizus* ATCC 11145 was maintained on malt agar slopes, grown at 27°C, stored at 4°C, and grown in submerged culture at 27°C in a medium composed of glucose (40 g), peptone (20 g) and corn steep liquor (5 ml) per liter of distilled water.

3.3. Biotransformation procedures

3.3.1. A. ochraceus, R. arrhizus

Fungi were grown in the media specified above (200 ml in each of the five 1-l Erlenmeyer flasks), placed on a rotary shaker at 180 rpm for 72 h. The fungal mass was then collected by filtration, washed with water and resuspended in five 1-l Erlenmeyer flasks, each containing 200 ml of distilled water. A solution of substrate (0.3 g) in 95% ethanol (10 ml) was added to the flasks, which were then replaced on the rotary shaker at 180 rpm, 27°C, for 48 h. After this time, the biotransformation medium was collected by filtration and extracted with dichloromethane by continuous extraction for 72 h. The extract was evaporated, and subjected to chromatography using dichloromethane/methanol gradient elution.

3.3.2. B. megaterium

B. megaterium was grown in the medium specified above (200 ml in each of the five 1-l Erlenmeyer flasks), placed on a rotary shaker at 180 rpm for 72 h. The cells were then collected by centrifugation and then resuspended in five 1-l Erlenmeyer flasks, each containing 200 ml of 0.1 M phosphate buffer, pH 7.24. A solution of substrate (0.3 g) in 95% ethanol (10 ml) was added to the flasks, which were then replaced on the rotary shaker at 180 rpm, 27°C, for 48 h. After this time, the cells were again removed by centrifugation, and the medium was extracted with dichloromethane by continuous extraction for 72 h. The extract was evaporated, and subjected to chromatography as outlined above.

3.3.3. C. lunata

Biotransformations with *C. lunata* were performed as described above for *A. ochraceus* and *R. arrhizus*, with the biotransformation period reduced to 12 h.

3.4. Biotransformation products

Spectral data for isolated products are listed below. All products gave infrared spectra consistent with the proposed structures (C-3 C=O at 1700–1715 cm^{-1}, C-17 C=O at 1740–1745 cm^{-1}, OH at 3400–3500 cm^{-1} [7]. Yields are reported in Table 2.

11α,17β-Dihydroxy-2-oxa-androst-4-en-3-one ***(1a)***; m.p. 200–202°C; ^{1}H NMR included signals at δ 0.84 (3H, s, C-18H), 1.34 (3H, s, C-19H), 3.75 (1H, t, C-17αH), 4.04 (1H, m, C-11βH), 4.08 and 4.97 (2H, ABq, C-1H) and 5.71 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 306(14), 288(6), 276(8), 164(30) relative to 84(100).

15β,17β-Dihydroxy-2-oxa-androst-4-en-3-one ***(1b)***; m.p. 238–240°C; ^{1}H NMR included signals at δ 1.07 (3H, s, C-18H), 1.34 (3H, s,

C-19H), 3.60 (1H, t, C-17α H), 4.03 and 4.26 (2H, ABq, C-1H), 4.28 (1H, t, C-15α H), and 5.71 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 306(8), 288(6), 262(4), 234(11) relative to 66(100).

11β,17β-Dihydroxy-2-oxa-androst-4-en-3-one ***(1c)***; m.p. 213–215°C; ^{1}H NMR included signals at δ 1.04 (3H, s, C-18H), 1.48 (3H, s, C-19H), 3.63 (1H, t, C-17α H), 4.18 and 4.40 (2H, ABq, C-1H), 4.22 (1H, m, C-11α H), and 5.65 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 306(38), 288(69), 273(22), 259(42), 243(54), 214(60) relative to 55(100).

14α,17β-Dihydroxy-2-oxa-androst-4-en-3-one ***(1d)***; m.p. 196–198°C; ^{1}H NMR included signals at δ 0.92 (3H, s, C-18H), 1.26 (3H, s, C-19H), 4.07 and 4.25 (2H, ABq, C-1H), 4.33 (1H, t, C-17α H), and 5.70 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 306(5), 288(30), 273(100), 243(23).

6β,17β-Dihydroxy-2-oxa-androst-4-en-3-one ***(1e)***; m.p. 255–259°C; ^{1}H NMR included signals at δ 0.82 (3H, s, C-18H), 1.42 (3H, s, C-19H), 3.67 (1H, t, C-17α H), 4.02 and 4.24 (2H, ABq, C-1H), 4.40 (1H, t, C-6α H), and 5.84 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 306(100), 288(14), 277(8), 229(15).

2-Oxa-androst-4-ene-3,17-dione ***(2)***; m.p. 177–180°C (Ref. [30], m.p. 178–183°C); ^{1}H NMR included signals at δ 0.92 (3H, s, C-18H), 1.25 (3H, s, C-19H), 4.02 and 4.25 (2H, ABq, C-1H), and 5.71 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 288(13), 273(3), 258(10), 243(8) relative to 66(100).

15β-Hydroxy-2-oxa-androst-4-ene-3,17-dione ***(2a)***; m.p. 209–212°C; ^{1}H NMR included signals at δ 1.24 (3H, s, C-18H), 1.30 (3H, s, C-19H), 4.03 and 4.26 (2H, ABq, C-1H), 4.62 (1H, t, C-15α H), and 5.73 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 304(100), 286(16), 271(14), 260(23), 191(85).

6β-Hydroxy-2-oxa-androst-4-ene-3,17-dione ***(2b)***; m.p. 215–217°C; ^{1}H NMR included signals at δ 0.96 (3H, s, C-18H), 1.45 (3H, s, C-19H), 4.02 and 4.26 (2H, ABq, C-1H), 4.48 (1H, t, C-6α H), and 5.88 (1H, s, C-4H) ppm; ^{13}C NMR (Table 1); MS m/z(%) 304(100), 286(8), 259(5), 230(9).

Acknowledgements

We are grateful to Mr. T. Jones (Brock University) for assistance in obtaining NMR and mass spectral data. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada.

References

[1] D.H. Peterson, H.C. Murray, S.H. Eppstein, L.M. Reineke, A. Weintraub, P.D. Meister, H.M. Leigh, J. Am. Chem. Soc. 74 (1952) 5933.
[2] H.L. Holland, Chem. Soc. Rev. 11 (1982) 371.
[3] H. Suginome, J.B. Wang, Steroids 55 (1990) 353.
[4] T.A. Crabb, P.J. Dawson, R.O. Williams, J. Chem. Soc., Perkin Trans. 1 (1982) 571.
[5] T.A. Crabb, N.M. Ratcliffe, J. Chem. Res. Synop. (1989) 280.
[6] R. Pappo, R.J. Chorvat, Tetrahedron Lett. (1972) 3237.
[7] A.A. Frimer, J. Hamieri-Buch, S. Ripshtos, P. Gilinsky-Sharon, Tetrahedron 42 (1986) 5693.
[8] P. Lupon, L. Plana, F. Serra, P. Dalmases, J.J. Bonet, A. Giner-Sorollo, F. Schmid, Eur. J. Med. Chem. 18 (1983) 41.
[9] M. Gut, M. Uskokovic, J. Org. Chem. 26 (1961) 1943.
[10] W. Charney, H.L. Herzog, Microbial Transformations of Steroids, Academic Press, New York, 1967.
[11] S.B. Mahato, S. Garai, Steroids 62 (1997) 332.
[12] J.E. Bridgeman, P.C. Cherry, A.S. Clegg, J.M. Evans, E.R.H. Jones, A. Kasal, V. Kumar, G.D. Meakins, Y. Morisawa, E.E. Richards, P.D. Woodgate, J. Chem. Soc. C (1970) 250.
[13] H.L. Holland, E. Riemland, Can. J. Chem. 63 (1985) 1121.
[14] J.W. Blunt, J.B. Stothers, Org. Mag. Res. 9 (1977) 439.
[15] A.M. Bell, J.W. Browne, W.A. Denny, E.R.H. Jones, A. Kasal, G.D. Meakins, J. Chem. Soc., Perkin Trans. 1 (1972) 2930.
[16] A.S. Clegg, W.A. Denny, E.R.H. Jones, G.D. Meakins, J.T. Pinhey, J. Chem. Soc., Perkin Trans. 1 (1973) 2137.
[17] P. Somal, C.L. Chopra, Appl. Microbiol. Biotechnol. 21 (1985) 267.
[18] A. Berg, J.-A. Gustafsson, M. Ingelman-Sundberg, K. Carlstrom, J. Biol. Chem. 251 (1976) 2831.
[19] G.M. Shull, D.A. Kita, J. Am. Chem. Soc. 77 (1955) 763.
[20] Y.Y. Lin, L.L. Smith, Biochim. Biophys. Acta 218 (1970) 515.
[21] A.M. Bell, J.M. Clark, W.A. Denny, E.R.H. Jones, G.D. Meakins, W.E. Muller, E.E. Richards, J. Chem. Soc., Perkin Trans 1 (1973) 2131.
[22] H.L. Holland, P.R.P. Diakow, Can. J. Chem. 56 (1978) 694.
[23] H.L. Holland, Acc. Chem. Res. 17 (1984) 398.

[24] V. Bihari, P.P. Goswami, S.H.M. Riva, A.W. Khan, S.K. Basu, V.C. Vora, Biotechnol. Bioeng. 26 (1984) 1403.
[25] E.L. Dulaney, U.S. Pat. 2,902,593 (September 22 1959), Chem Abs. 54 (1960) 1659h.
[26] P.O. Larssen, K. Mosbach, Methods Enzymol. 44 (1976) 183.
[27] T.K. Mazumder, K. Sonomoto, A. Tanaka, S. Fukui, Appl. Microbiol. Biotechnol. 21 (1985) 154.
[28] K. Sonomoto, K. Nomura, A. Tanaka, S. Fukui, Eur. J. Appl. Microbiol. Biotechnol. 16 (1982) 57.
[29] H.L. Holland, L.J. Allen, M.J. Chernishenko, M. Diez, A. Kohl, J. Ozog, J.-X. Gu, J. Mol. Cat. B: Enzymatic 3 (1997) 311.
[30] R. Pappo, U.S. Pat. 3,128,283 (April 7 1964), Chem Abs. 61 (1964) 3171b.