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An unusual ring—A opening and other reactions in steroid transformation by the thermophilic fungus *Myceliophthora thermophila*

A. Christy Hunter*, Kieran R. Watts, Cinzia Dedi, Howard T. Dodd

Molecular Targeting and Polymer Toxicology Group, School of Pharmacy, University of Brighton, East Sussex BN2 4GJ, UK

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ABSTRACT

A series of steroids (progesterone, testosterone acetate, 17β-acetoxy-5α-androstan-3-one, testosterone and androst-4-en-3,17-dione) have been incubated with the thermophilic ascomycete *Myceliophthora thermophila* CBS 117.65. A wide range of biocatalytic activity was observed with modification at all four rings of the steroid nucleus and the C-17β side-chain.

This is the first thermophilic fungus to demonstrate the side-chain cleavage of progesterone. A unique fungal transformation was observed following incubation of the saturated steroid 17β-acetoxy-5α-androstan-3-one resulting in 4-hydroxy-3,4-seco-pregn-20-one-3-oic acid which was the product generated following the opening of an A-homo steroid, presumably by lactonohydrolase activity. Hydroxylation predominated at axial protons of the steroids containing 3-one-4-ene ring-functionality. This organism also demonstrated reversible acetylation and oxidation of the 17β-alcohol of testosterone.

All steroidal metabolites were isolated by column chromatography and were identified by ^{1}H, ^{13}C NMR, DEPT analysis and other spectroscopic data. The range of steroidal modification achieved with this fungus indicates that these organisms may be a rich source of novel steroid biocatalysis which deserve greater investigation in the future.

1. Introduction

Thermophilic fungi are a potentially diverse biocatalytic resource for the generation of new steroidal compounds and discovery of novel metabolic behaviour [1,2]. These fungi have a remarkable range of secretary enzymes (e.g. amylases, cellulases, lipases, proteases, xylanases) and cell associated enzymes (e.g. trehalase, invertase, β-glycosidase) [3–5] but as of yet, very little is known about their ability to transform steroids. The only study [6] in this area to date determined that *Rhizomucor tauricus* IMI 23312 had patterns of metabolism similar to mesophilic fungi, with hydroxylation reactions predominating.

To gain further insight into the handling of steroids by thermophilic fungi we have employed the thermophilic ascomycete *Myceliophthora thermophila* CBS 117.65. This organism is known to generate exogenous enzymes such as laccase [7], xylanases [8,9] and cellulases [10] which, have found practical application in the food industry [11], as biocatalysts [12] in spectroscopic science [13] and biosensor technologies [14]. In order to determine the pattern of metabolic handling of steroids by this fungus, a range of structurally diverse steroidal compounds namely progesterone (**1**), testosterone acetate (**2**), 17β-acetoxy-5α-androstan-3-one (**3**), testosterone (**4**) and androst-4-en-3,17-dione (**5**) have been used as biochemical probes.

2. Materials and methods

2.1. Chemicals and reagents

All steroids were purchased from Steraloids Ltd. (UK) and were used as supplied. Solvents were of analytical grade; petroleum ether refers to the fraction with a boiling point of 60–80 °C. Silica for column chromatography was Merck 9385 and TLC was performed with Macherey–Nagel Alugram® SIL G/UV$_{254}$

2.2. Microorganism

M. thermophila CBS 117.65 was obtained from the collection at the Centraalbureau voor Schimmelcultures (Ned). Stock cultures were grown on potato dextrose agar (Oxoid, UK) slopes (3 days) and maintained at 4 °C until use. Steroid transformation studies were carried out in 3% malt extract medium (Oxoid, UK).

2.3. Conditions of cultivation and transformation

Spores were transferred aseptically in a category 2 biological safety cabinet into 500 mL Erlenmeyer flasks containing 300 mL

* Corresponding author. Tel.: +44 01273 642088; fax: +44 01273 642674.
E-mail address: c.hunter@bton.ac.uk (A. Christy Hunter).

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Table 1
^{1}H NMR data for steroidal starting material and transformation products determined in $CDCl_3$.

Reference data	4-H	18-H_3	19-H_3	21-H_3	Other significant signals
Progesterone (**1**)	5.73	0.67	1.19	2.13	
Testosterone acetate (**2**)	5.72	0.84	1.19	2.01	4.59 (1H, t, J = 7.9 Hz, 17α-H)
17β-Acetoxy-5α-androstan-3-one (**3**)		0.80	1.02	2.04	4.59 (1H, t, J = 9 Hz, 17α-H)
Testosterone (**4**)	5.71	0.79	1.19		3.64 (1H, t, J = 8.8 Hz, 17α-H)
Androst-4-en-3,17-dione (**5**)	5.74	0.92	1.22		
Transformation products					
5α-Androstan-3α,17β-diol (**6**)		0.73	0.79		3.63 (1H, t, J = 8.6 Hz, 17α-H),
4.04 (1H, t, J = 2.6 Hz, 3β-H)					
11β-Hydroxy-progesterone (**7**)	5.82	0.80	1.38	2.29	4.37 (1H, t, J = 2.5 Hz, 11α-H)
11α-Hydroxy-progesterone (**8**)	5.73	0.70	1.32	2.13	4.03 (1H, td, J = 5 Hz, J = 10 Hz, 11β-H)
14α-Hydroxy-androst-4-en-3,17-dione (**9**)	5.75	1.05	1.22		
17β-Hydroxy-5α-androstan-3-one (**10**)		0.76	1.02		3.64 (1H, t, J = 8.6 Hz, 17α-H)
4-Hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**)		0.81	0.77	2.03	3.84 (2H, dd, J = 4 Hz, J = 10 Hz, 4-H)
					4.57 (1H, t, J = 8 Hz, 17α-H)
11α-Hydroxytestosterone (**12**)	5.73	0.82	1.32		3.65 (1H, t, J = 8.3 Hz, 17α-H) 4.03 (1H, td, J = 4.5 Hz, J = 8 Hz, 11α-H)
6β-Hydroxy-androst-4-en-3,17-dione (**13**)	5.84	0.95	1.41		4.35 (1H, t, J = 3 Hz, 6α-H)
7α-Hydroxy-androst-4-en-3,17-dione (**14**)	5.82	0.92	1.22		4.11 (1H, br s, 7β-H)

of sterile media and were incubated for 72 h at 40 °C. The cultures were shaken at 180 rpm on an orbital shaker. Aliquots (5 mL) from the seed flask were transferred aseptically to 10 flasks and grown for a further 72 h as above, at the end of which the fungus is in log phase growth. After this time period steroid dissolved in dimethylformamide (DMF) was evenly distributed between the flasks (1 mg/mL) under sterile conditions and incubated for a further 5 days after which the metabolites were extracted from the broth.

2.4. Extraction and identification of metabolites

The fungal mycelium was separated from the broth by filtration under vacuum. Following completion the mycelium was rinsed with ethyl acetate (0.5 L) to ensure the entire available steroid was removed. The mycelial broth was then extracted thrice with ethyl acetate (1.5 L). The organic extract was dried over sodium sulfate and the solvent evaporated *in vacuo* to give a gum. This gum was adsorbed onto silica and chromatographed on a column of silica; the steroidal metabolites were eluted with increasing concentrations of ethyl acetate in petroleum ether. The solvent was collected in aliquots (10 mL) and analysed by thin layer chromatography (TLC) to identify the separated metabolite fractions. The solvent systems used for running the TLC plates were 50:50 petroleum ether in ethyl acetate or pure ethyl acetate. A 50:50 sulfuric acid in methanol spray was used to develop the TLC plates.

2.5. Analysis and identification of metabolites

Characteristic splitting patterns [15] and shift values [16] in the ^{1}H and ^{13}C NMR spectra in comparison to the starting compounds were used to determine metabolite structure in combination with DEPT analysis to identify the nature of the carbon. Spectra were recorded on a Bruker WM 360 Spectrometer, all samples were analysed in deuteriochloroform using tetramethylsilane as the internal standard. High resolution mass measurement (HRMS) was determined in electrospray ionization (ESI) mode using a Bruker Daltonics Microtof spectrometer. Infra-red absorption spectra were recorded directly on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate® (Tables 1–4).

Table 2
^{13}C NMR data for steroidal starting material and transformation products determined in $CDCl_3$.

	Compounds													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Carbon atom														
1	35.68	35.70	38.48	35.09	35.29	32.23	37.10	37.53	35.73	38.61	37.33	35.28	37.14	35.41
2	33.91	33.92	38.10	33.28	33.46	29.04	34.21	34.17	33.90	38.20	36.84	32.01	34.18	33.89
3	199.37	199.32	211.93	198.95	198.82	66.57	200.25	200.16	199.34	211.01	162.00	198.25	200.15	198.75
4	123.89	123.93	44.64	123.49	123.71	36.77	126.47	124.61	124.19	44.73	71.00	122.32	126.57	127.16
5	170.90	171.06	46.60	170.53	169.80	39.22	167.95	170.80	169.78	46.82	46.84	169.15	167.66	167.00
6	32.73	32.72	28.75	32.08	32.11	28.44	33.51	33.57	32.31	28.87	27.59	31.43	72.85	41.00
7	31.85	31.48	31.21	31.85	30.84	31.59	29.92	31.60	25.59	31.39	28.46	28.95	38.06	67.12
8	35.50	35.39	35.16	38.02	34.72	35.57	30.94	34.95	37.96	35.58	37.41	33.15	29.42	38.36
9	53.60	53.70	53.71	53.27	53.38	54.49	60.40	59.01	46.87	54.00	45.13	57.05	53.66	45.39
10	38.53	38.59	35.70	37.92	38.19	36.21	37.96	39.92	38.66	35.95	34.64	37.84	39.37	38.56
11	20.98	20.51	20.90	18.97	19.87	23.38	73.06	68.90	19.12	21.17	18.82	66.71	20.28	20.16
12	38.61	36.60	36.81	35.79	30.31	35.90	49.89	50.47	24.51	36.72	31.42	46.28	31.28	30.92
13	43.87	42.44	42.60	42.18	47.05	43.00	48.44	44.12	52.51	43.06	45.83	41.40	47.63	47.31
14	55.97	50.23	50.55	49.84	50.41	51.10	53.10	55.35	80.75	50.97	45.59	47.65	50.92	45.66
15	24.32	23.45	23.51	22.69	21.30	20.38	23.95	24.23	30.29	23.44	24.31	21.09	21.72	21.26
16	22.79	27.46	27.51	29.04	35.26	30.55	20.42	22.96	33.04	30.55	27.27	28.33	35.76	35.69
17	63.45	82.43	82.70	82.15	219.87	82.00	63.69	63.12	218.07	81.91	77.60	78.83	220.45	220.26
18	13.28	12.00	12.12	10.40	13.25	11.16	15.56	14.50	17.32	11.10	13.69	10.10	13.78	13.50
19	17.33	17.39	11.46	16.61	16.93	11.22	19.57	18.31	17.88	11.52	10.06	16.23	19.57	17.01
20	209.24	170.84	171.15	–	–	–	211.76	208.78	–		170.20	–	–	–
21	31.45	21.13	21.17	–	–	–	27.98	31.35	–		22.24	–	–	–

Table 3
Significant metabolite infra-red absorption signals cm^{-1}.

Compound	>C=O	>C=CH—	—OH
5α-Androstan-3α,17β-diol (**6**)			3322 br
11β-Hydroxy-progesterone (**7**)	1701, 1663	1615	3428
11α-Hydroxy-progesterone (**8**)	1654	1610	3379
14α-Hydroxy-androst-4-en-3,17-dione (**9**)	1741, 1653	1609	3419
17β-Hydroxy-5α-androstan-3-one (**10**)	1657		3444
4-Hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**)	1732, 1708		3507
11α-Hydroxytestosterone (**12**)	1656	1609	3391
6β-Hydroxy-androst-4-en-3,17-dione (**13**)	1730, 1664	1620	3446
7α-Hydroxy-androst-4-en-3,17-dione (**14**)	1728, 1654	1620	3400

Table 4
Product yields following chromatography.

Starting material	Metabolites	Yield (%)
Progesterone (**1**)		18.00
	Testosterone acetate (**2**)	6
	Testosterone (**4**)	2
	5α-Androstan-3α,17β-diol (**6**)	10
	11β-Hydroxy-progesterone (**7**)	4
	11α-Hydroxy-progesterone (**8**)	6
Testosterone acetate (**2**)		20.31
	Testosterone (**4**)	10
	Androst-4-en-3,17-dione (**5**)	10
	14α-Hydroxy-androst-4-en-3,17-dione (**9**)	6
17β-Acetoxy-5α-androstan-3-one (**3**)		10
	17β-Hydroxy-5α-androstan-3-one (**10**)	4
	4-Hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**)	22
Testosterone (**4**)		23
	Testosterone acetate (**2**)	10
	Androst-4-en-3,17-dione (**5**)	8
	11α-Hydroxytestosterone (**12**)	6
Androst-4-en-3,17-dione (**5**)		24.8
	Testosterone acetate (**2**)	16
	Testosterone (**4**)	4
	6β-Hydroxy-androst-4-en-3,17-dione (**13**)	4
	7α-Hydroxy-androst-4-en-3,17-dione (**14**)	8

2.6. Time course experiment

Conditions were identical to those in Section 2.3 except the steroid (500 mg) dissolved in DMF was evenly distributed between five 250 mL Erlenmeyer flasks (each containing 100 mL of media) for each of the substrates (**1–5**). One flask was harvested after 8 h then one every 24 h and extracted as in Section 2.4. Following 6 h under high vacuum, the product ^{1}H NMR spectra were determined in $CDCl_3$ to confirm the presence of and steroidal nature of the extracts (Table 5).

3. Results

3.1. Products of metabolism and structural identification

Transformation of progesterone (**1**) generated five products of metabolism (Fig. 1). The structure of testosterone acetate (**2**) was determined by comparison of its' NMR spectra to that of (**1**). A new signal was observed in the ^{1}H NMR spectrum of (**2**) at 4.59 ppm (1H, t) coupled with a downfield shift of the 18-H_3 signal (0.17 ppm), but no loss of the 21-H_3 signal suggested insertion of an oxygen via Baeyer–Villiger oxidation of the C-17β side-chain. This was supported by accurate mass determination HRMS ESI M+Na^+ obsd. 353.209 $C_{21}H_{30}NaO_3$ req. 353.209. The ^{13}C NMR spectrum of (**2**) demonstrated downfield shift for both C-16 (4.67 ppm) and C-17 (18.98 ppm) with concomitant upfield shift for C-20 (38.40 ppm) and C-21 (10.32 ppm) which was consistent with literature values [16].

Testosterone (**4**) was identified by comparison of its ^{1}H and ^{13}C NMR spectra to that of (**2**) where loss of the 21-H_3 (2.01 ppm) signal and an upfield shift of the 17α-H from 4.59 ppm (1H, t) in (**2**) to 3.64 ppm (1H, t) in (**4**) suggested hydrolysis of the side-chain, generating a 17β-alcohol. The ^{13}C NMR spectrum of (**4**) was devoid of the C-20 (170.84 ppm) and C-21 (21.13 ppm) resonance signals. These and all other ^{13}C NMR signals were consistent with the stated molecular structure [16] as was HRMS ESI M+Na^+obsd. 311.197 $C_{19}H_{28}NaO_2$ req. 311.198.

5α-Androstan-3α,17β-diol (**6**) was initially identified by comparison of its ^{1}H and ^{13}C NMR spectra to that of testosterone (**4**). The ^{1}H NMR spectrum of (**6**) was completely devoid of the double bond 4-H (5.71 ppm) resonance signal indicating hydrogenation had taken place, as did absence of a double bond absorption signal in the infra-red spectrum. Further support was afforded by a significant upfield shift for the 19-H_3 signal (0.4 ppm) due to loss of π-electrons and by upfield shifts for C-4 (86.72 ppm), C-5 (131.31 ppm), C-6 (3.64 ppm) and C-19 (5.39 ppm). A new C*H*OH signal was present in the ^{1}H NMR spectrum of (**6**) at 4.04 ppm (1H, t) consistent with a reduction of the C-3 ketone to a 3α-alcohol. Also, loss of the C-3 ketone ^{13}C NMR signal at 198.95 ppm and replaced by 3α-alcohol C*H*OH signal at 66.57 ppm was consistent with literature values [16], HRMS ESI M+Na^+ obsd. 315.225 $C_{19}H_{32}NaO_2$ req. 315.229 agreed with the proposed structure.

The accurate mass measurement of (**7**) (HRMS ESI M+Na^+obsd. 353.207 $C_{21}H_{30}NaO_3$ req. 353.208) indicated that this metabolite was a monohydroxylation product of progesterone (**1**). Comparison of the ^{1}H NMR spectrum of (**7**) with progesterone (**1**) revealed that both the 18-H_3 and 19-H_3 had undergone downfield shifts (0.13 ppm and 0.19 ppm respectively). This coupled with the new C*H*OH signal at 4.37 ppm confirmed hydroxylation at C-11 with β-stereochemistry giving this metabolite the assignment of 11β-hydroxyprogesterone (**7**). This was fully supported by the ^{13}C NMR spectra, in comparison to that of (**1**), where C-11 underwent a downfield shift (52.08 ppm) as did β-carbons C-9 (6.80 ppm) and C-12 (11.28 ppm) and a γ-carbon upfield shift was observed for C-8 (4.56 ppm) which is consistent with literature shift values [16].

Table 5
Time course experiment.

Time points	8 h	24 h	48 h	72 h
Metabolites identified following metabolism of progesterone (**1**)	**7**,**8**			**2**,**4**,**6**
Metabolites identified following metabolism of testosterone acetate (**2**)	**4**,**5**	**9**		
Metabolites identified following metabolism of 17β-acetoxy-5α-androstan-3-one (**3**)	**10**,**11**			
Metabolites identified following metabolism of testosterone (**4**)		**2**,**5**,**12**		
Metabolites identified following metabolism of androst-4-en-3,17-dione (**5**)		**2**,**4**,**13**,**14**		

Metabolites isolated following incubation of starting materials are: testosterone acetate (**2**); testosterone (**4**); androst-4-en-3,17-dione (**5**); 5α-androstan-3α,17β-diol (**6**); 11β-hydroxy-progesterone (**7**); 11α-hydroxy-progesterone (**8**); 14α-hydroxy-androst-4-en-3,17-dione (**9**); 17β-hydroxy-5α-androstan-3-one (**10**); 4-hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**); 11α-hydroxytestosterone (**12**); 6β-hydroxy-androst-4-en-3,17-dione (**13**); 7α-hydroxy-androst-4-en-3,17-dione (**14**).

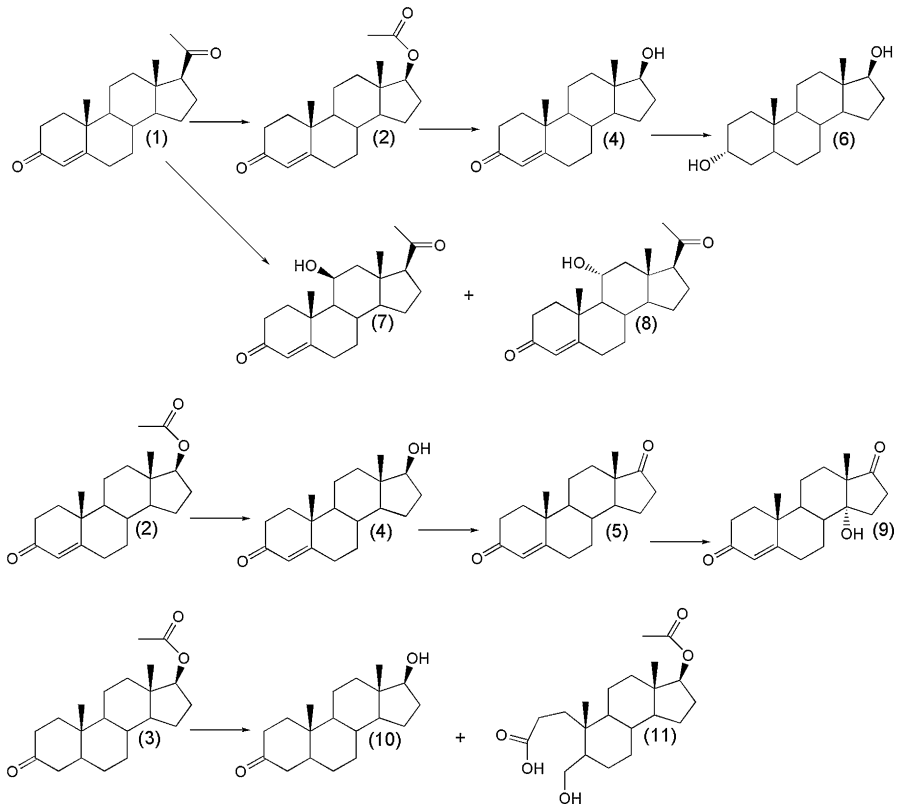

Fig. 1. Metabolites isolated following transformation of progesterone (**1**), testosterone acetate (**2**) and 3β-acetoxy-5α-androstan-3-one (**3**) by *Myceliophthora thermophila*.

11α-Hydroxyprogesterone (**8**) was identified by comparison of its ^{1}H and ^{13}CNMR spectra with that of the starting material progesterone (**1**). The ^{1}H NMR spectrum of (**8**) demonstrated a significant downfield shift for the 19-methyl group, this combined with a new resonance signal at 4.03 ppm (td) suggested that monohydroxylation had taken place at an equatorial proton, possibly 11α-H. Accurate mass measurement fully supported a product of monohydroxylation (HRMS ESI M+Na^{+} obsd. 353.213 $C_{21}H_{30}NaO_3$ req. 353.208). The product ^{13}C NMR spectrum demonstrated β-carbon downfield shifts for C-9 (5.41 ppm) and C-12 (11.86 ppm) and other downfield shifts for C-1 (1.85 ppm) and C-10 (1.39 ppm) all of which are consistent with substitution at C-11α.

Incubation of testosterone acetate (**2**) resulted in the isolation of three steroidal metabolites. The structure of testosterone (**4**) was readily confirmed as above. Androst-4-en-3,17-dione (**5**) was identified following comparison of its' ^{1}H and ^{13}C NMR spectrum with that of (**4**). The ^{1}H NMR spectrum of (**5**) was devoid of the 17α-H signal at 3.64 ppm (1H, t), present in testosterone (**4**), indicating oxidation of the alcohol had taken place. This was supported by a downfield shift (0.13 ppm) for the 18-H_3 in (**5**). The ^{13}C NMR spectrum of (**5**) contained a resonance at 219.87 ppm consistent with a relatively sterically strained 5-ring ketone. Its position at C-17 was supported by β-carbon increases for C-13 (4.87 ppm) and C-16 (6.22 ppm) and overall structure was confirmed by comparison with an authentic sample [17] and accurate mass determination of the isolated steroid HRMS ESI M+Na^{+} obsd. 309.183 $C_{19}H_{26}NaO_2$ req. 309.182

Comparison of the ^{1}H and ^{13}C NMR spectra of androst-4-en-3,17-dione (**5**) to 14α-hydroxy-androst-4-en-3,17-dione (**9**) revealed a significant downfield shift for the 18-methyl signal (0.13 ppm) with the absence of any new signals in the spectrum suggesting that a tertiary hydroxyl was present at C-14. Accurate mass measurement (HRMS ESI M+Na^{+}obsd. 325.177 $C_{19}H_{26}NaO_3$ req. 325.177) supported the monohydroxylation at this tertiary carbon centre, as did the ^{13}C NMR spectra that demonstrated β-carbon downfield shifts for C-8 (3.24 ppm), C-13 (5.46 ppm), C-15 (8.99 ppm) and γ-carbon upfield shift for C-9 (6.51 ppm) all of which are consistent with the proposed structure [16]

Following incubation of 17β-acetoxy-5α-androstan-3-one (**3**) two metabolites were isolated. 17β-Hydroxy-5α-androstan-3-one (**10**) was readily identified by comparison of the ^{1}H and ^{13}C NMR of compound (**10**) to that of compound (**3**). The ^{1}H NMR spectrum of (**10**) did not contain the 21-H_3 (2.04 ppm) signal but showed an upfield shift for the 17α-H signal (0.95 ppm) indicating hydrolysis had taken place. This was confirmed by the ^{13}C NMR spectrum of (**10**) which was devoid of the resonance signals for C-20 (171.15 ppm) and C-21 (21.17 ppm). The structure was fully supported by HRMS ESI M+Na^{+}obsd. 313.213 $C_{19}H_{30}NaO_2$ req. 313.213.

Comparison of the ^{1}H and ^{13}C NMR data of 4-hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**) to the starting material (**3**) demonstrated that the C-17β-acetate was intact [21-H_3 (2.03 ppm); C-20 (170.20 ppm); C-21 (22.24 ppm)]. The ^{1}H NMR spectrum of (**11**) contained a new resonance signal at 3.84 ppm (2H, dd) for the protons at C-4 and a signal at 4.57 ppm (1H, t) consistent with the 17α-H. The ^{13}C NMR spectrum showed an upfield shift in the C-3 quaternary carbon ketone resonance signal (49.93 ppm) which indicated the possibility of ring-A opening. The presence of two new oxygen's was revealed by accurate mass measurement HRMS ESI M+Na^{+} obsd. 389.230 $C_{21}H_{34}NaO_5$ req. 389.229. This was further supported by elemental analysis that was performed following crystallization of the compound from ethyl acetate as needles m.p. 228–229 °C (found C, 68.86; H, 9.60 $C_{21}H_{34}O_5$ calc. for C, 68.82; H, 9.35%). Ultimately the molecular structure was unequivocally defined by X-ray crystallography (Fig. 2).

Three products of metabolism were isolated following incubation of testosterone (**4**) (Fig. 3). The first of which was readily identified as testosterone acetate (**2**) by a new methyl signal in the ^{1}H NMR spectrum at 2.01 ppm consistent with an acetate group.

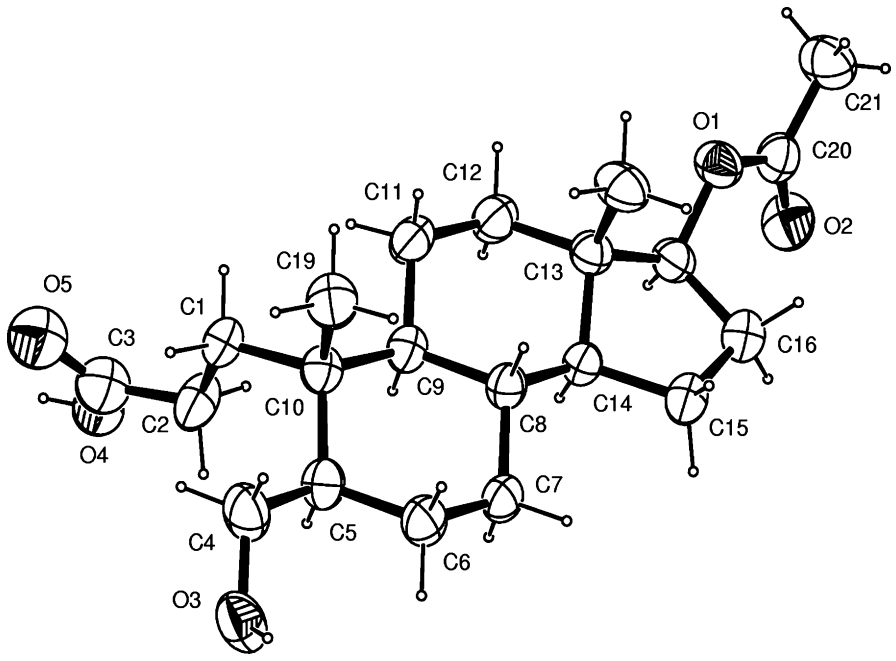

Fig. 2. Single X-ray crystal structure of 4-hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**).

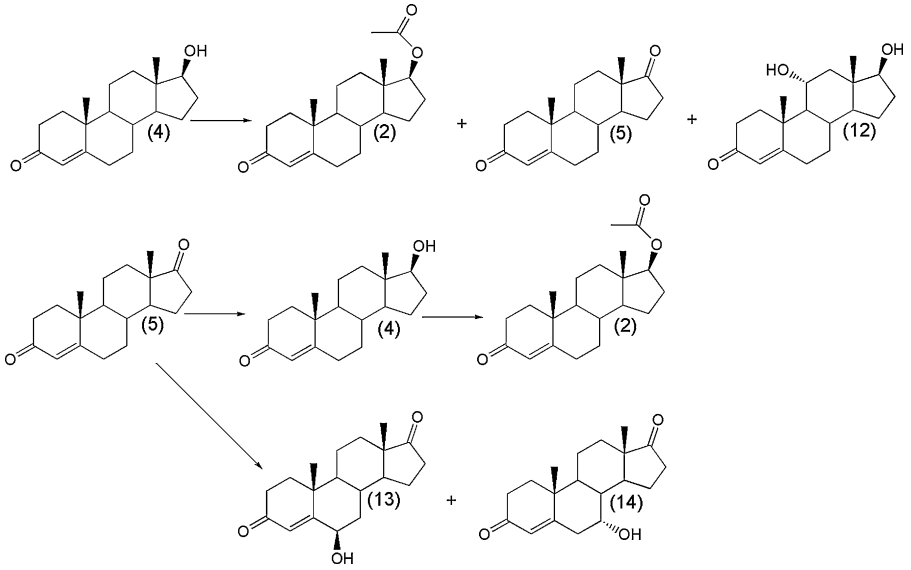

Fig. 3. Metabolites isolated following transformation of testosterone (**4**) and androst-4-en-3,17-dione (**5**) by *Myceliophthora thermophila*.

A downfield shift in the 17α-H of 0.95 ppm demonstrated that the acetylation occurred on the 17β-alcohol. New signals in the ^{13}C NMR spectrum of (**2**) at 170.84 ppm (C-20) and 21.13 ppm (C-21) further supported the presence of the acetate as did HRMS ESI M+Na$^+$obsd.311.200 $C_{19}H_{28}NaO_2$ req. 311.198 of the isolated steroid. The structure of androst-4-en-3,17-dione (**5**) was identified as above.

The final metabolite, 11α-hydroxytestosterone (**12**) was found to have an accurate mass measurement of HRMS ESI M+Na$^+$obsd. 327.192 $C_{19}H_{28}NaO_3$ req. 327.193, consistent with monohydroxylation of (**4**). In comparison to the NMR spectra of (**4**) the ^{1}H NMR spectrum of (**12**) had a new resonance signal at 4.03 ppm (1H, td) consistent with substitution at an equatorial proton. Downfield β-carbon shifts in the ^{13}C NMR spectra for C-9 (3.78 ppm) and C-12 (10.49 ppm) and upfield γ-carbon shifts for C-8 (4.87 ppm), and C-13 (0.78 ppm) confirmed hydroxylation at C-11.

Androst-4-en-3,17-dione (**5**) facilitated the isolation of 4 metabolites, compounds (**2**) and (**4**) were identified as described above. 6β-Hydroxy-androstan-4-en-3,17-dione (**13**) was identified by comparison of its NMR spectra with that of (**4**). A new signal was observed at 4.35 ppm (1H, t) in the ^{1}H NMR spectrum suggesting monohydroxylation, and that was confirmed by mass spectrometry (HRMS ESI M+Na$^+$obsd. 325.177 $C_{19}H_{28}NaO_3$ req. 325.177). A significant downfield shift was observed for the 19-methyl (0.19 ppm) in the ^{1}H NMR spectrum indicating a hydroxyl group in close proximity to this group. The ^{13}C NMR spectrum of (**13**) revealed downfield β-carbon shifts for C-4 (2.86 ppm), C-7 (7.22 ppm) and C-19 (2.64 ppm) and an upfield shift for C-8 (5.30 ppm) confirmed hydroxylation at C-6 as did comparison to an authentic sample [6].

The spectra of 7α-hydroxy-androst-4-en-3,17-dione (**14**) were compared to (**5**) and revealed a new resonance signal in the ^{1}H NMR spectrum at 4.11 ppm (br s) indicating an axial hydroxylation.

Monohydroxylation was confirmed by accurate mass measurement (HRMS ESI M+Na$^+$ obsd. 325.177 $C_{19}H_{28}NaO_3$ req. 325.177). There were no significant differences observed in the methyl resonance signals indicating that this substitution was at distance from these centres. The ^{13}C NMR spectrum demonstrated β-carbon downfield shifts for C-6 (8.89 ppm) and C-8 (3.64 ppm) and γ-carbon upfield shifts for C-9 (7.99) and C-14 (4.75 ppm) confirming hydroxylation at C-7, as did comparison with an authentic sample [6].

3.2. Crystallographic structural determination of 4-hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**)

$C_{21}H_{34}O_5$, Mr: 366.48, orthorhombic space group $P2_12_12_1$ (no. 19), $a = 9.2910(4)$, $b = 9.3913(3)$, $c = 22.5610(9)$ Å, $\alpha = \beta = \gamma = 90^\circ$, $V = 1968.55(13)$ Å^3, $Z = 4$, $D'_{calc} = 1.24$ Mg/m^3, $u = 0.09$ mm^{-1}, $F(000)$ 800. Data were collected using a crystal of size 0.3 mm × 0.2 mm × 0.2 mm on a KappaCCD diffractometer. A total of 8711 independent reflections were collected for $3.47 < \theta < 26.01^\circ$ and $-11 \le h \le 11$, $-10 \le k \le 11$, $-23 \le l \le 27$. There were 3677 independent reflections and 3104 reflections with $I > \sigma(I)$ were used in the refinement. No absorption correction was applied. The structure was solved by direct methods and refined using SHELXL-97. The diagrams used ORTEP-3 for windows. The final R indices were $[I > 2\sigma(I)]$ $R_1 = 0.065$, $wR_2 = 0.161$ and R indices (all data) $R_1 = 0.079$, $wR_2 = 0.173$. The goodness-of-fit on F^2 was 1.046 and the largest difference peak and hole was 0.57 and −0.30 e Å^{-3}.

3.3. Time course experiment

^1H NMR was used to determine time of metabolite formation and structural nature. For clarity only a key metabolite ^1H NMR signal is noted here, with full metabolite assignments available in Table 1. Metabolism of progesterone (**1**) occurred in two distinct phases initiating at the 8 h time point with hydroxylation at C-11 in both stereochemical positions. This was supported by key resonance signals in the ^1H NMR at 4.37 ppm (11α-H) and 4.03 ppm (11β-H) for metabolites (**7**) and (**8**) respectively. The second phase of metabolism was observed at the 72 h time point with the presence of selective resonance signals in the ^1H NMR spectrum for metabolites (**2**) at 4.59 ppm (17α-H), (**4**) at 3.64 ppm (17α-H) and (**6**) 4.04 ppm (3α-H).

Hydrolysis of testosterone acetate (**2**) forming the metabolite (**4**) and its oxidation generating (**5**) occurred within the first 8 h, with key resonance signals present in the ^1H NMR spectrum at 3.64 ppm (17α-H) and 4.04 ppm (3β-H). The occurrence of 14α-hydroxylation of (**5**) was evident at the 24 h time point with a new 18-H_3 resonance signal 1.05 ppm present.

The fully saturated steroid 17β-acetoxy-5α-androstan-3-one (**3**) underwent hydrolysis to form metabolite (**10**) with the presence of 17α-H signal at 3.64 ppm and ring-A Baeyer–Villiger oxidation forming (**11**) with the signatory resonance signal at 3.84 ppm (4-H) present at the 8 h time point.

All of the metabolites of testosterone (**4**) were present in the media at the 24 h time point with key ^1H NMR resonance signals for metabolite (**2**) at 4.59 ppm (17α-H), (**5**) at 0.92 ppm (18-H_3) and (**12**) at 4.03 ppm (11α-H) observed.

Androst-4-en-3,17-dione (**5**) underwent two distinct routes of metabolism with ring-D modification and ring-B hydroxylation in generation of the four observed metabolites by the 24 h time point. Testosterone (**4**) was readily identified by the 17α-H resonance signal at 3.64 ppm and its acetylated analogue metabolite (**2**) with the 17α-H resonance signal at 4.59 ppm. The hydroxylated metabolites were identified by resonance signals for (**13**) at 4.35 ppm (6α-H) and (**14**) at 4.11 ppm (7β-H)

4. Discussion

Baeyer–Villiger oxidation of steroids has been reported in a wide range of mesophilic fungi and predominantly occurs on ring-D, frequently resulting in the generation of testololactone [17–20]. *M. thermophila* CBS 117.65 is the first thermophilic fungus reported to perform Baeyer–Villiger oxidation. A novel Baeyer–Villiger oxidation of the ring-A ketone [21] was evident following transformation of the saturated steroid 17β-acetoxy-5α-androstan-3-one (**3**) with subsequent lactone opening, presumably brought about by the action of lactonohydrolase resulting in the generation of 4-hydroxy-3,4-seco-pregn-20-one-3-oic acid (**11**) (Fig. 1). As none of the intact ring-A lactone was isolated it would appear that the mechanism of ring opening is rapid. With respect to the generation of a 7 membered lactone ring-A other *Aspergillus species* (*A. terreus*, *A. foetidus*) have been shown to lactonize comparatively smaller and less rigid 6-carbon ring monocyclic ketones to 7 membered ring systems. Some monocyclic ketones transformed by *Aspergillus strains* have also demonstrated lactonohydrolase activity [22].

Baeyer–Villiger oxidation of the 17β-methylketone side-chain of progesterone (**1**) (Fig. 1) was confirmed by isolation of testosterone acetate (**2**). The timed experiment revealed that this transformation occurred a significant time after the hydroxylation products (**7**,**8**) being detected at the 72 h time point. This would suggest that the enzyme responsible is induced possibly by progesterone (**1**) or its C-11 hydroxylated analogues (**7**,**8**). The formed testosterone acetate (**2**) then underwent subsequent hydrolysis forming testosterone (**4**), a frequently observed fungal catalytic side-chain cleavage pathway [17]. However, although oxidation of 17β-alcohol to the ketone occurred, subsequent Baeyer–Villiger oxidation generating a D-homo lactone was not observed. Instead, in this ultimately reductive part of the putative pathway (Fig. 1), progesterone (**1**) undergoes a dual enzymatic attack on ring-A resulting in an unusual reduction of the 3 ketone, to a 3α-alcohol and hydrogenation of the C-4-C-5 alkene. It could be argued that as none of the other 4-ene-3-one functionalized steroids underwent hydrogenation or keto-reduction that the enzymes responsible are also induced. The hydrogenation of the C-4-C-5 alkene has previously been observed in mesophilic fungi [23].

Incubation of testosterone acetate (**2**) resulted in a significantly different pattern of metabolism in comparison to progesterone (**1**), notably being devoid of hydroxylation at C-11. The transformation initiated with hydrolysis of testosterone acetate (**2**), generating testosterone (**4**) which was then oxidised to androst-4-en-3,17-dione (**5**) and then hydroxylated at C-14. This result further supports the notion that the progestogens (**1**,**7**,**8**) have a role in switching on the enzymatic attack observed on ring-A resulting in (**6**). In support of this, single functional groups on steroids transformed by fungi, have already been shown to control specific enzyme induction and therefore metabolic fate [2,20].

Incubation of testosterone (**4**) demonstrated this organism's ability for reverse metabolism with the occurrence of acetylation generating testosterone acetate (**2**) and oxidation resulting in androst-4-en-3,17-dione (**5**) (Fig. 3). Incubation of androst-4-en-3,17-dione (**5**) following reduction of the C-17 ketone to the β-alcohol also underwent acetylation generating testosterone acetate (**2**). Hence pathway reversibility was clearly demonstrated by this fungus (Fig. 4) with these biocatalytic reactions. Interestingly following incubation of androst-4-en-3,17-dione (**5**) the only attack on ring-B of the steroid nucleus was observed with hydroxylation occurring at C-6β and C-7α. The organism also appears to have a preference for stereochemistry of hydroxylation, with attack predominantly at axial protons (6β, 7α, 11β, 14α), the exception being the equatorial 11α-H.

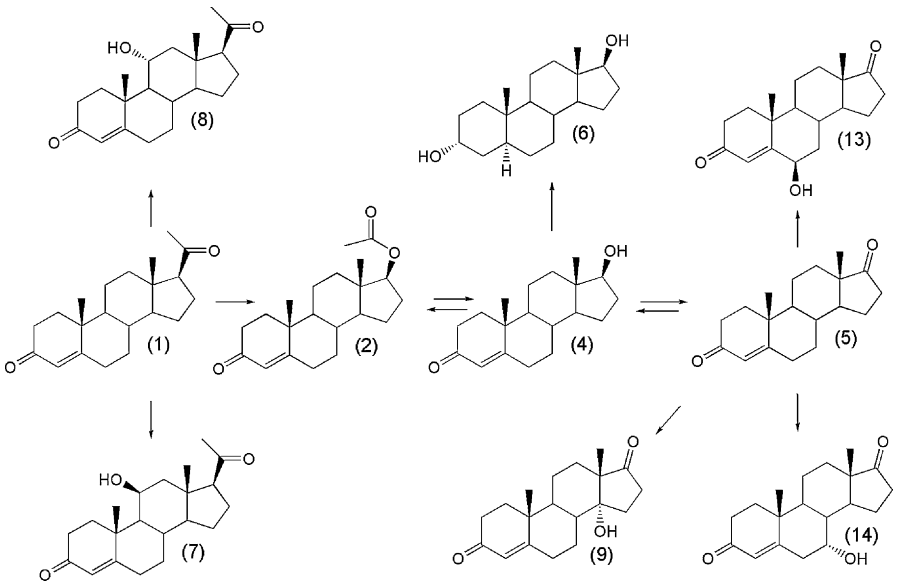

Fig. 4. Putative metabolic pathways present in *Myceliophthora thermophila*.

The experimental results demonstrate the remarkable range of biocatalytic activity achieved by this organism. This included a unique ring-A transformation that generated a highly novel steroid that may be of interest in the future within academia and clinical medicine. Unusually, *M. thermophila* has demonstrated the flexibility of enzyme systems to chemically interact with all four of the steroidal rings and the C-17β-side chain utilizing putative hydroxylation and reductive pathways (Fig. 4). The range of reactions encountered with this fungus indicates that these organisms may be a rich source of novel steroid biocatalysis which deserve greater investigation in the future.

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