Microbiological Hydroxylation. Part XIX.[1] The Action of an Ant Fungus ('Acromyrmex Fungus') on Oxygenated Androstanes, Pregnanes, and Cholestanes

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The title fungus readily metabolises oxygenated derivatives of androstane and pregnane, but not cholestane. With the androstanes and pregnanes three basic processes occur: hydroxylation at positions determined by the directing influences of the substrates' oxygenated groups, Baeyer–Villiger type oxidation of 3- and 20-ketones (the latter corresponding to side-chain degradation of the pregnanes), and dehydrogenation(s) in ring A. With the exception, of substrates which direct hydroxylation to the 17- (or 16-) position, the processes all occur at comparable rates; and in general complex mixtures are formed from most substrates.

WHILE investigating various fungi which had not previously been tested as potential steroid hydroxylators we had occasion to screen an unusual fungus † associated with the ant species *Acromyrmex octospinosus* Reich (Hymenoptera: Myrmicinae). The marked activity of this fungus revealed by the initial tests prompted a detailed study, reported here, with oxygenated substrates of the androstane, pregnane, and cholestane types.

Each species of attine (fungus-growing) ant cultivates in its nest a fungus which is symbiotic with the ant (and possibly peculiar to it).[2] The fungi are deficient in some proteolytic enzymes, and are consequently unable to utilise protein nitrogen; growth requires repeated applications of faecal material (containing free amino-acids) by the ants. Under natural conditions the ants use the fungi as their primary food source thereby preventing the mycelia from producing the sporophores which are essential for complete identification of the fungi.[3] Thus, since the fungus used here cannot be formally identified, it is given the loose description 'Acromyrmex fungus'.

Table 1 summarises the microbiological results. The n.m.r. spectra of the steroids, substrates, and products, involved here for which spectroscopic data have not appeared in the earlier publications are listed in Table 2: the arabic serial number sequence of steroids discussed earlier is used in this Table which contains steroids nos. 850—873. The structures of new compounds were elucidated, as usual, from a combination of chemical and spectrometric methods. [Identification of the seven-membered ring A lactones may be illustrated as follows. In addition to the spectral features associated with the 17β-hydroxy- and 11-oxo-groups, 17β-hydroxy-4-oxa-A-homo-5α-androstane-3,11-dione (no. 862) showed $\nu_{max.}$ ($CHCl_3$) 1 725 cm^{-1}, consistent with a ring-A lactone function, and τ ($CDCl_3$) 5.70 (4 lines, J 13 and 8 Hz) and 6.32 (d, J 13 Hz) indicating an O·CH_2·CH< unit; these results, together with the origin of the product, lead to a >CO·O·C(4a)H_2·C(5)H< structure.] For new compounds the n.m.r. signals appear in Table 2, and the other information required for their characterisation is given in Table 3. Since the microbiological and chemical operations of the present work are routine applications of techniques fully described in earlier parts, and with the new compounds being adequately reported in the Tables, the whole of the Experimental section has been deposited as Supplementary Publication No. SUP 21370.‡

The complexity of the mixture obtained in many of the incubations does not stem from promiscuous attack on the steroid nucleus by the fungus. Three different basic processes may occur, *viz.* (i) hydroxylation at sites determined by the directing influences[4,5] of the substrates' oxygenated groups, (ii) Baeyer–Villiger type oxidation of 3- or 20-ketones, and (iii) dehydration(s) in ring A of a 3-ketone. In general processes (ii) and (iii) occur only when position 11 has an oxygen substituent [which may be present in the substrate, or introduced by process (i)]. With 20-ketones process (ii) may be followed by hydrolysis to give an alcohol; such an alcohol, or one formed by direct hydroxylation [process (i)], may be further oxidised to a ketone. These possibilities, and the ensuing combination of them, account for the diversity of products. The relative rates of the various reactions clearly vary according to the substrates' structures, but this variation is not so marked as to allow one process, or sequence of processes, to dominate the outcome with a particular substrate.

The hydroxylations [process (i)] can be rationalised in terms of a triangular arrangement of three active sites on the enzyme surface.[4,5] Binding and hydroxylation in the normal mode is visualised as involving sites 3, 11, and 17 (or 16); in the reverse mode the corresponding positions are 17, 7 (or 6), and 3. Microbiological removal

† A sample of the fungus, obtained from an ant nest in Trinidad in 1971, was provided by Dr. J. M. Cherrett (University College of North Wales, Bangor), who also identified the ant species.

‡ For details of Supplementary Publications see Notice to Authors No. 7 in *J.C.S. Perkin I*, 1974, Index issue.

[1] Part XVIII, A. M. Bell, A. D. Boul, Sir Ewart R. H. Jones, G. D. Meakins, J. O. Miners, and A. L. Wilkins, *J.C.S. Perkin I*, 1975, 1364.

[2] N. A. Weber, *Science*, 1966, **153**, 587; A. Moeller, *Botan. Mitt. Tropen.*, 1893, **6**, 127; S. E. Craven, M. W. Dix, and G. E. Michaels, *Science*, 1970, **169**, 184.

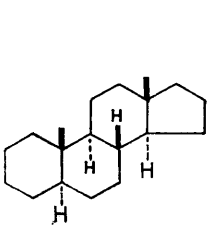
[3] For details see R. Singer, 'The Agaricoles in Modern Taxonomy,' J. Cramer, Weinheim, 1962.

[4] A. M. Bell, P. C. Cherry, I. M. Clark, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins, and P. D. Woodgate, *J.C.S. Perkin I*, 1972, 2081.

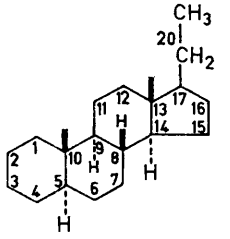
[5] V. E. M. Chambers, W. A. Denny, J. M. Evans, Sir Ewart R. H. Jones, A. Kasal, G. D. Meakins, and J. Pragnell, *J.C.S. Perkin I*, 1973, 1500.

TABLE 1

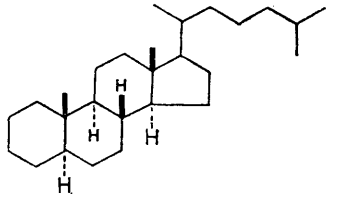
Hydroxylation of 5α-androstane (I), 5α-pregnane (II), and 5α-cholestane (III) derivatives with 'Acromyrmex fungus'



(I)



(II)



(III)

Substrates are indicated by abbreviated names or symbols, *e.g.* 3β-OH–20-CO–Δ^5 represents 3β-hydroxypregn-5-en-20-one. In the 'product' column those oxygen functions introduced during the incubation are in bold type, and n.i. indicates no products isolated. The entries under conditions refer to the use of ethanol (E) as solvent for the substrate, and to the time of incubation (in days). The yields are calculated after making allowance for recovered starting material

5α-Androstane substrates

	Conditions	Recovered substrate	Main product(s)			Other product(s)		
3-CO–Δ^4	E4	27%	**11**α-OH–**17**- CO–Δ^1	19%		**11**α,	**16**β-$(OH)_2$	6%
			11α-OH–**17**- CO	11		**11**α,	**16**β-$(OH)_2$–Δ^1	4
			16β- OH–Δ^1	10				
3,11-$(CO)_2$	E4	0	**17**β- OH–$\Delta^{1,4}$	13	**4**-oxa-A-homo–		**17**β- OH	5
			16β- OH–$\Delta^{1,4}$	7	**4**-oxa-A-homo–		**16**- CO	3
		4-oxa-A-homo–	**17**- CO	7			**16**- CO–$\Delta^{1,4}$	3
3,17-$(CO)_2$	E1.5	0	**11**α- OH–$\Delta^{1,4}$	30		**11**α-	OH–Δ^1	9
		4-oxa-A-homo–**11**α-	OH	22	3β,	**11**α-	$(OH)_2$	7
		11α-	OH–Δ^4	14		**11**α-	OH	3
	E4	0	**4**-oxa-A-homo–**11**α-	OH	25	**4**-oxa-A-homo–**11**α,	**17**β-$(OH)_2$	8
			11α-	OH–Δ^1	18		**11**α, **17**β-$(OH)_2$–Δ^1	8
			11α-	OH–$\Delta^{1,4}$	12	3β,	**11**α $(OH)_2$	5
							11α, **17**β-$(OH)_2$–$\Delta^{1,4}$	4
7,17-$(CO)_2$	E4	31	**3**β,	**17**β-$(OH)_2$	23		**11**α- OH	4
			3β-	OH	11	3β,	**11**α- $(OH)_2$	4
3β-OH–6-CO	E4	8		**17**β- OH	40		**17**- CO	3
				16β- OH	27		**16**- CO	3
3β-OH–7-CO	E4	0	**11**α-	OH	21			
				17β- OH	19			
				16β- OH	17			
3β-OH–17-CO	E3	0	3-CO–	**11**α- OH–$\Delta^{1,4}$	13	3-CO–	**11**α, **17**β- OH–$\Delta^{1,4}$	11
				11α, **17**β-$(OH)_2$	13	3-CO–	**11**α- OH–Δ^1	10
			3-CO–	**11**α, **17**β-$(OH)_2$–Δ^1	12	3-CO–	**11**α- OH	5
				11α- OH	12	**4**-oxa-A-homo–**11**α OH		4
17β-OH–11-CO	E4	31	**3**β-	OH	23	**3**-	CO–Δ^1	5
			3-	CO–$\Delta^{1,4}$	22	**3**,	**17**- $(CO)_2$–$\Delta^{1,4}$	4
3,6$(CO)_2$–4,4-$(CH_3)_2$	E4	15	**4**-oxa-A-homo–	**17**β- OH	18			

5α-Pregnane substrates

3,20-$(CO)_2$–Δ^4	E4	0	**11**α-OH–**17**- CO	26		**11**α,	**17**β-$(OH)_2$–Δ^1	8
			11α-OH–**17**- CO–Δ^1	19		**11**α-OH–**17**- CO–Δ^1		3
			11α, **17**β-$(OH)_2$	18				
3β-OH–20-CO–Δ^5			**11**α-OH–3,**17**-$(CO)_2$–$\Delta^{1,4}$	22	3-CO–	**11**α,	**17**β-$(OH)_2$–Δ^4	12
			11α-OH–3,**17**-$(CO)_2$–Δ^4	21	3-CO–	**11**α,	**17**β-$(OH)_2$–$\Delta^{1,4}$	9

5α-Cholestane substrates

3,6-$(CO)_2$	E4	>90	n.i.	
3β-OH–Δ^5	E4	>90	n.i.	

TABLE 2

N.m.r. signals

The results, presented in the form used earlier,[a] were obtained by examining solutions in $CDCl_3$ at 100MHz

No.	Compound		τ_2	τ_2 (calc) [b]	—CH·OH *etc.*		
850	Androsta-1,4-diene-3,11,6-trione	19	8.55	8.52			
		18	9.10	9.08			
851	4-Oxa-A-homo-5α-androstane-3,11,16-trione	19	8.80	8.80	H-4a	6.32	d (13)
		18	9.17	9.17	H-4a	5.68	*4* (13, 8)
852	Androsta-1,4-diene-3,11,17-trione	19	8.56	8.53			
		18	9.12	9.09			
853	4-Oxa-A-homo-5α-androstane-3,11,17-trione	19	8.82	8.82	H-4a	6.32	d (13)
		18	9.17	9.17	H-4a	5.69	*4* (13, 8)
854	16β-Hydroxyandrosta-1,4-dien-3-one	19	8.75	7.89	H-16	5.60	m (15)
					H-4	3.94	d (2)
					H-2	3.85	*4* (10,2)
		18	8.96	8.98	H-1	2.95	d (10)
855	11α-Hydroxy-5α-androst-1-ene-3,17-dione	19	8.84	8.85	H-11	5.85	*6* (10, 10, 5)
					H-2	4.21	d (10.5)
		18	9.08	9.07	H-1	1.70	d (10.5)
856	11α-Hydroxyandrost-4-ene-3,17-dione	19	8.66	8.66	H-11	5.95	*6* (10, 10, 5)
		18	9.06	9.03	H-4	4.27	s
857	11α-Hydroxyandrosta-1,4-diene-3,17-dione	19	8.66	8.67	H-11	5.90	*6* (10, 10, 5)
					H-4	3.93	d (2)
					H-2	3.87	*4* (10, 2)
		18	9.05	9.03	H-1	2.21	d (10)
858	11α-Hydroxy-4-oxa-A-homo-5α-androstane-3,17-dione	19	8.93	8.92	H-11	6.06	*6* (10, 10, 5)
					H-4a	6.32	d (13)
		18	9.13	9.11	H-4a	5.70	*4* (13, 8)
859	16β-Hydroxyandrosta-1,4-diene-3,11-dione	19	8.55	8.57	H-16	5.47	m (18)
					H-4	3.93	d (2)
					H-2	3.82	*4* (10, 2)
		18	9.00	9.01	H-1	2.32	d (10)
860	17β-Hydroxy-5α-androst-1-ene-3,11-dione	19	8.74	8.77	H-17	6.10	t (8)
					H-2	4.21	d (10)
		18	9.26	9.27	H-1	2.52	d (10)
861	17β-Hydroxyandrosta-1,4-diene-3,11-dione	19	8.54	8.59	H-17	6.12	t (8)
					H-4	3.94	m (2)
					H-2	3.83	*4* (10, 2)
		18	9.21	9.23	H-1	2.34	d (10)
862	17β-Hydroxy-4-oxa-A-homo-5α-androstane-3,11-dione	19	8.84	8.84	H-17	6.13	t (8)
					H-4a	6.35	d (13)
		18	9.31	9.31	H-4a	5.70	*4* (13, 8)
863	3β,17β-Dihydroxy-5α-androstan-11-one	19	8.97	8.97	H-3	6.45	*7* (10,10 5, 5)
		18	9.31	9.30	H-17	6.15	t (8)
864	11α,16β-Dihydroxyandrosta-1,4-dien-3-one	19	8.68	8.67	H-11	5.93	*6* (10, 10, 5)
					H-16	5.58	m (20)
		18	8.94	8.95	H-4	3.91	d (2)
					H-2	3.85	*4* (10, 2)
					H-1	2.22	d (10)
865	11α,17β-Dihydroxy-5α-androst-1-en-3-one	19	8.87	8.87	H-11	5.90	*6* (10, 10, 5)
					H-17	6.34	t (8)
		18	9.21	9.21	H-2	4.22	d (10.5)
					H-1	1.70	d (10.5)
866	11α,17β-Dihydroxyandrost-4-en-3-one	19	8.68	8.68	H-11	5.98	*6* (10, 10, 5)
					H-17	6.34	t (8)
		18	9.18	9.17	H-4	4.27	s
867	11α,17β-Dihydroxyandrosta-1,4-dien-3-one	19	8.68	8.69	H-11	5.98	*6* (10, 10, 5)
					H-17	6.34	t (8)
		18	9.18	9.17	H-4	3.93	d (2)
					H-2	3.87	*4* (10, 2)
					H-1	2.20	d (10)
868	11α,17β-Dihydroxy-4-oxa-A-homo-5α-androstan-3-one	19	8.94	8.94	H-11	6.05	*6* (10, 10, 5
					H-17	6.14	t (8)
		18	9.27	9.25	H-4a	6.32	d (13)
					H-4a	5.70	*4* (13, 8)
869	3β,11α-Dihydroxy-5α-androstane-7,17-dione	19	8.79	8.7	H-3	6.42	m (21)
		18	9.13	9.09	H-11	5.92	m (18)
870	4,4-Dimethylandrost-5-en-3-one	19	9.14		H-6	4.43	*4* (5, 2)
		18	9.28				
		4-Me	8.76, 8.76				
871	4,4-Dimethyl-5α-androstane-3,6-dione	19	8.88				
		18	9.28				
		4-Me	8.87, 8.52				
872	4,4-Dimethyl-5α-androstane-3β,6α-diol	19	9.12		H-3	6.80	*4* (9, 6)
		18	9.32		H-6	6.04	*6* (10, 10,5
		4-Me	9.00, 8.70				
873	17β-Hydroxy-4α,4α-dimethyl-4-oxa-A-homo-5α-androstane-3,6-dione	19	9.01		H-17	6.34	t (8)
		18	9.26				
		4-Me	8.60, 8.21				

[a] Ref. 4. [b] Substituent effect calc. for 4-oxa-A-homo-3-CO; 19-H, −0.15; 18-H, 0.00.

of the side-chain from progesterone and pregnenolone has been observed with many bacteria and with some fungi.[6] The very marked activity of the Acromyrmex fungus in degrading 20-ketones may be a consequence of 17-hydroxylation rather than Baeyer–Villiger type oxidation; however, the inability to degrade the cholesterol side-chain suggests, but does not establish, the occurrence of the latter route with the 20-oxo-pregnanes.

An unexpected feature of the present results is that lactone formation occurs readily with 3-ketones, but not with 17-ketones. In previous microbiological work on steroids the cyclic ketone ⟶ lactone transformation, which has been shown to involve incorporation of atmospheric oxygen,[7] has been encountered frequently with 17-ketones, but only once with a 3-ketone.[8] As would be predicted, the presence of a 4,5-double bond in a 3-ketone substrate inhibits Baeyer–Villiger type oxidation, and dehydrogenation becomes the preferred process.

TABLE 3

Characterisation of new compounds

Compound	M.p. (°C) *	$[\alpha]_D$ (°) †	Analytical figures (%)		
4-Oxa-A-homo-5α-androstane-3,11,16-trione	224—226	−160	(Found M^+	318.1827)	
		(0.05)	($C_{19}H_{26}O_4$ req. M	318.1831)	
4-Oxa-A-homo-5α-androstane-3,11,17-trione	256—257	+100	(Found M^+	318.1831)	
		(0.1)	($C_{19}H_{26}O_4$ req. M	318.1831)	
Androsta-1,4-diene-3,11,16-trione	(Oil)	−57	Found	76.3	7.6
		(0.1)	$C_{19}H_{24}O_3$ req.	76.5	7.4
11α-Hydroxy-4-oxa-A-homo-5α-androstane-3,17-dione	218—220	+22	Found	71.4	8.7
		(1.0)	$C_{19}H_{28}O_4$ req.	71.2	8.8
16β-Hydroxyandrosta-1,4-diene-3,11-dione	218—219	+134	Found	76.0	8.2
		(1.0)	$C_{19}H_{24}O_3$ req.	76.0	8.05
17β-Hydroxy-5α-androst-1-ene-3,11-dione	152—154	+70	Found	75.4	8.5
		(0.1)	$C_{19}H_{28}O_3$ req.	75.5	8.7
17β-Hydroxy-4-oxa-A-homo-5α-androstane-3,11-dione	251—252	+28	Found	71.1	8.7
		(0.1)	$C_{18}H_{26}O_4$ req.	71.2	8.8
11α,16β-Dihydroxyandrosta-1,4-dien-3-one	216—218	−42	Found	75.35	8.7
		(0.35)	$C_{19}H_{26}O_3$ req.	75.5	8.7
11α,17β-Dihydroxy-5α-androst-1-en-3-one	165—168	+11	Found	75.1	9.5
		(0.6)	$C_{19}H_{28}O_3$ req.	75.0	9.3
11α,17β-Dihydroxy-4-oxa-A-homo-5α-androstan-3-one	239—240	−49	Found	71.1	9.4
		(0.3)	$C_{18}H_{30}O_4$ req.	70.8	9.4
4,4-Dimethyl-5α-androstane-3β,6α-diol	170—175	+7	Found	78.9	11.2
		(0.4)	$C_{21}H_{36}O_2$ req.	78.7	11.3
4,4-Dimethyl-5α-androstane-3,6-dione	169—171	−63	Found	79.8	10.2
		(1.0)	$C_{21}H_{32}O_2$ req.	79.7	10.2
17β-Hydroxy-4α-4α-dimethyl-4-oxa-A-homo-5α-androstane-3,6-dione	191—194	−57	Found	72.0	9.2
		(0.15)	$C_{21}H_{32}O_4$ req.	72.4	9.3

* From Me_2CO–petrol. † $CHCl_3$ as solvent.

In this context it is noteworthy that a 3β-hydroxy-group, present initially in the substrate, or introduced microbiologically, is surprisingly stable towards oxidation.

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[6] G. Wix, K. Buki, E. Tomorkeny, and G. Ambrus, *Steroids*, 1968, **11**, 401; D. R. Brannon, J. Martin, A. C. Oehlschlager, N. N. Durham, and L. H. Zalkow, *J. Org. Chem.*, 1965, **30**, 760; K. Singh, S. N. Sehlgal, and C. Vezina, *Steroids*, 1963, **2**, 513; E. Vischer and A. Wettstein, *Experientia*, 1953, **9**, 371; J. Fried, R. W. Thomas, and A. Klingsberg, *J. Amer. Chem. Soc.*, 1953, **75**, 5764.

[7] R. L. Prairie and P. Talalay, *Biochemistry*, 1963, **2**, 203.

[8] A. S. Clegg, W. A. Denny, Sir Ewart R. H. Jones, G. D. Meakins, and J. T. Pinhey, *J.C.S. Perkin I*, 1973, 2137.