Bioconversion of Triterpenes by Mycobacteria. Structure and Conformation of the Products of Degradation of 7,11-Dioxodihydrolanosterol by *Mycobacterium phlei*

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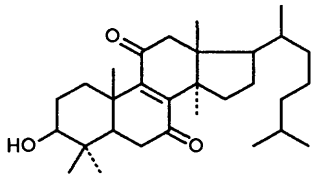
While mycobacteria are unable to degrade lanosterol and dihydrolanosterol, principal components of wool fat, we observed the transformation of some of their autoxidation products by *Mycobacterium phlei*. By analogy with the mechanism of degradation of cholesterol, this difference was assumed to be due to the requirement for the presence of an enone group before the side-chain can be degraded. This paper reports the spectroscopic determination of the structure of the major metabolites of 7,11-dioxodihydrolanosterol. The side-chain is degraded from eight carbon atoms to three, the terminal carbon atom being oxidized to a primary alcohol or a methyl ester. The tetracyclic skeleton can undergo regioselective oxidation–reduction modifications at the 3- and 7-position. Their conformational analysis, carried out by 2D-NMR methods, indicates a chair form for ring A of 3β-hydroxy derivatives, while it is highly deformed for 3-keto compounds as predicted formerly by Mislow for this lanostane series.

In the course of a study of the bioconversion of wool fat by mycobacteria, we investigated the behaviour of polycyclic compounds other than cholesterol[1] whose metabolism is well described. Wool fat is rich in tetracyclic triterpenes of the lanosterol family,[2] especially lanosterol and dihydrolanosterol. There are no reports in the literature of the degradation of these compounds by mycobacteria. Our own attempts using strains of mycobacteria which degrade sterols were unsuccessful, although we did observe transformation of some of their autoxidation products which are natural constituents of wool fat.



I

We describe herein the bioconversion of 7,11-dioxodihydrolanosterol I by *Mycobacterium phlei* strain ATCC 11758. This strain degrades oxygenated triterpenes and transforms compound I into a large number of metabolites. We describe herein the structures of the major products.

Results and Discussion

Synthesis of the Substrate and Bioconversion.—Since it is not easy to separate the major oxidized triterpene, 3β-hydroxylanost-7,11-dione-8-ene I, from the highly complex mixture which constitutes wool fat, we synthesized it from lanosterol by classical methods. Commercial lanosterol consists of a mixture of lanosterol and dihydrolanosterol, and so was hydrogenated catalytically[3] to give a quantitative yield of dihydrolanosterol. Dihydrolanosterol was acetylated,[3] and the product was then oxidized by chromic acid to give a mixture of products oxidized in the 7- and 11-position. After deacetylation under alkaline conditions,[4] compound I was separated by LC [silica gel 60 M, Merck; hexane–ethyl acetate (8:2)].

It is known that mycobacteria are able to completely degrade tetracyclic compounds such as sterols.[5] After a rapid screening of the action of several non-pathogenic mycobacterial strains from our collection towards substrate I, we selected a *Mycobacterium phlei* strain exhibiting good degradative activity. This fast growing strain, ATCC 11758, was cultured on a synthetic medium, and substrate I was added halfway through the exponential growth phase. After 3 days of contact under aerobic conditions the mixture was extracted, and the products were separated by HPLC on silica. With a UV detector at 260 nm we observed ten new products. The structures of six of these compounds were determined.

Identification of the Products.—The products could be divided into two main groups on the basis of their UV and ^{13}C NMR spectra (Table 1). The compounds in group A contained the same ene-dione chromophore as the starting compound, λ_{max}(EtOH) 270 nm (ε 7000 dm^3 mol^{-1} cm^{-1}). Resonance peaks of the two conjugated carbonyl groups ($200 < \delta_C < 203$) and the ethylenic carbon atoms ($149 < \delta_C < 152$) were observed in the ^{13}C NMR spectra. The small difference in resonance between these two latter peaks was assigned to crossed conjugation in this ene-dione system.

For the compounds in group B, there was a hypsochromic (λ_{max} 256 nm) and a hyperchromic shift (ε 9000 dm^3 mol^{-1} cm^{-1}) of the UV absorption band, corresponding to the reduction of one of the carbonyl groups of the starting ene-dione chromophore. The position of the peak was in agreement with the value of 252 nm calculated from the Woodward–Fieser parameters. This partial reduction of the conjugated system was confirmed by ^{13}C NMR spectroscopy, which showed a

Table 1 Spectroscopic properties of the substrate and the bioconversion products

	UV spectrum $\lambda_{max}(\varepsilon_{max})$	HRMS ($M^{\cdot+}$) Calc.; Found (mol. form.)	^{13}C NMR[a] C=O	C=C	C(OH)	OMe
I (substrate)	270 (7400)	456.715; 456.714 ($C_{30}H_{48}O_3$)	202.2 202.5	150.7 151.8	77.6 (C-3)	
Group A						
1	269 (7500)	428.574; 428.572 ($C_{26}H_{36}O_5$)	201.1 201.7 214.5 (C-3) 176.6 (ester)	150.5 151.6		51.6
2	270 (7200)	430.272; 430.273 ($C_{26}H_{38}O_5$)	201.6 201.9 176.7 (ester)	150.4 151.8	77.6 (C-3)	51.6
4	268 (6900)	400.261; 400.259 ($C_{25}H_{36}O_4$)	201.3 201.9 215.6 (C-3)	149.7 151.5	67.6 (C-22)	
5	267 (7000)	402.276; 402.278 ($C_{25}H_{38}O_4$)	202.1 202.2	150.5 151.8	77.6 (C-3) 67.7 (C-22)	
Group B						
3	254 (9000)	430.272; 430.274 ($C_{26}H_{38}O_5$)	199.8 218.0 (C-3) 176.8 (ester)	138.8 160.1	67.0	51.6
6	258 (9100)	402.276; 402.275 ($C_{25}H_{38}O_4$)	200.3 218.1 (C-3)	139.9 160.2	67.0 67.7 (C-22)	

[a] δ_C; internal $SiMe_4$ for $CDCl_3$ solution.

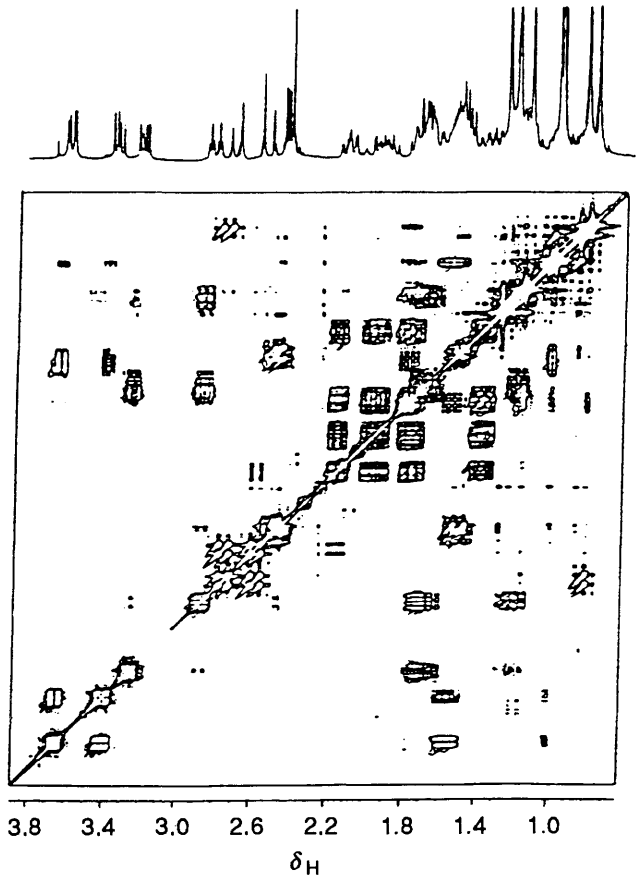
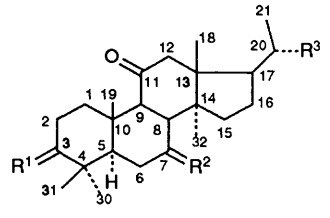


Fig. 1 300 MHz ^1H COSY spectrum of compound **5**

single carbonyl peak between δ_C 199 and 201, and two distinct ethylene carbon resonances, δ_C 139 and 160, characteristic of an α,β unsaturated ketone. All compounds of this group also exhibited a peak at δ_C 67.0, which was not present in the spectrum of the starting compound **I**. This peak corresponds to a carbon bearing an alcoholic group, indicating that a carbonyl group at position 7 or 11 had been reduced.

^{13}C NMR analysis enabled further division of the products into subgroups based on the degree of oxidation of the carbon at position 3. Some of the products exhibited a δ_C 77.6 signal characteristic of a hydroxy group at position 3, also present in the starting compound **I**. This peak was absent from the spectrum of other products which presented a new peak between δ_C 215 and 218, characteristic of an unconjugated ketone carbonyl group formed by oxidation of the alcohol group at position 3. Inspection of the ^{13}C NMR spectra could therefore readily localize the sites on the tetracyclic skeleton that had undergone oxidation–reduction.



	R	R′	R^3
1	=O	=O	CO_2Me
2	H, OH	=O	CO_2Me
3	=O	OH, H	CO_2Me
4	=O	=O	CH_2OH
5	H, OH	=O	CH_2OH
6	=O	OH, H	CH_2OH

^{13}C NMR analysis was also employed to determine the nature of the side-chain. With respect to the substrate **I**, six

Table 2 Chemical shifts (δ_H; $SiMe_4$) for the protons of rings A, B and C

Compound	1α	1β	2α	2β	3α	5α	6α	6β	7	12α	12β
1	1.74	2.95	2.61	2.49		2.26	2.38	2.53		2.82	2.56
2	~1.3[a]	2.88	1.73	1.68	3.27	1.51	2.47	2.49		2.83	2.53
4	1.75	2.96	2.60	2.49		2.25	2.38	2.53		2.75	2.63
5	~1.2[a]	2.89	1.73	1.74	3.27	1.51	2.47	2.49		2.76	2.60
3	1.70	2.98	2.61	2.44		2.09	~1.7[a]	~1.7[a]	4.45	2.76	2.43
6	1.70	2.98	2.61	2.44		2.09	~1.7[a]	~1.7[a]	4.46	2.69	2.47

[a] Overlapping lines.

Table 3 Chemical shifts (δ_H; $SiMe_4$) for the protons of ring D and the side-chain

Compound	15α	15β	16α	16β	17α	20	22-H^{A}[a]	22-H^{B}	OMe
1	2.17	1.68	1.91	1.38	2.17	2.41			3.67
2	2.16	1.81	1.88	1.37	2.17	2.44			3.66
4	2.17	1.68	2.00	1.41	1.83	1.57	3.42	3.66	
5	2.17	1.77	1.97	1.39	1.83	1.57	3.41	3.66	
3	~2.0[b]	~1.7[b]	~1.9[b]	~1.45[b]	2.28	2.46			3.67
6	*c*	*c*	*c*	*c*	1.89	1.58	3.42	3.67	

[a] The most deshielded diastereotopic 22-H proton is labelled B. [b] Overlapping lines. [c] Ambiguous assignment in the range 1.4–2.0.

peaks in the paraffin carbon region ($\delta_C < 60$) disappeared, and new peaks appeared, corresponding to an alcoholic carbon (δ_C 67–68) or to methyl ester carbons [δ(C=O) 175–178; δ(OMe) 51.6]. The side-chain is therefore degraded from eight carbon atoms to three, and the terminal carbon is oxidized to either a primary alcohol or a methyl ester. Mass spectrometry confirmed the molecular masses of these structures.

2D NMR spectroscopy was used to obtain stereochemical and conformational information on the products identified. The various protons were assigned from ^1H–^1H shift-correlated spectra from the unambiguously identified spin systems of deshielded protons in the neighbourhood of polar groups. An example, for compound **5**, is shown in Fig. 1.

(a) *Rings* A *and* B (*Table* 2). For the compounds with a hydroxy group at position 3, the deshielded proton on the carbon at this position is readily identified (X part of an ABX system). All the protons of rings A and B could be assigned from the ^2J, ^3J couplings, and ^4Js with the angular methyl (position 19). More distant correlations with protons in ring C were not observable owing to the presence of an ethylenic double bond at positions 8, 9. The values of the chemical shifts of the 1-H$^{\alpha}$ and 1-H$^{\beta}$ protons were somewhat unexpected. The 1-H$^{\beta}$ proton is strongly deshielded, whereas the 1-H$^{\alpha}$ proton is highly shielded and its multiplet is masked by the methyl peaks. Examinations of molecular models indicated that this behaviour could be accounted for by the fact that the 1-H$^{\beta}$ proton is in the plane of the carbonyl group at position 11 close to the oxygen atom. It is, therefore, strongly deshielded by the diamagnetic anisotropy of this group. On the other hand, the 1-H$^{\alpha}$ proton lies within the shielding cone and is therefore strongly shielded.

Similar behaviour is observed for the 1-H protons in the 3-keto compounds in which the alcohol group of the starting compound had been oxidized to a 3-keto group. This was exploited to assign the protons in rings A and B. The 3-H proton multiplet is no longer present, but it is replaced as starting reference for the connectivities by the directly identifiable 1-H$^{\beta}$ proton resonance.

From the UV and ^{13}C spectra, we concluded that some of the bioconversion products were reduced at the C-7 or C-11 carbonyl position. ^1H NMR spectroscopy could identify the site of reduction since all the products analysed had a highly deshielded 1-H$^{\beta}$ proton whose resonance could only be accounted for by the influence of the carbonyl at position 11. Therefore only the carbonyl group at position 7 was attacked during the bioconversion.

(b) *Ring* C (*Table* 2). An AB system between δ_H 2.4 and 2.8 (^2J 16–18 Hz) was observed for all products studied, assigned to the 12-H$^{\alpha}$ and 12-H$^{\beta}$ protons, providing additional evidence for an intact carbonyl group at position 11. The two protons could be distinguished by the fact that the 12-H$^{\alpha}$ proton is correlated with the 18β angular methyl group by ^4J coupling.

(c) *Ring* D *and side-chain* (*Table* 3). The peaks in this region of the molecule were assigned by successive correlations from either the 22-H protons α to the alcohol group (AB part of ABX system) or the 20-H proton α to the ester group (doublet of quadruplets), which was identified unambiguously. The 15-H$^{\alpha}$ and 15-H$^{\beta}$ protons were discriminated by ^5J coupling of the 15-H$^{\alpha}$ proton with proton 12-H$^{\beta}$ and ^4J coupling of the 15-H$^{\beta}$ proton with the 32α angular methyl group.

These analyses were verified in a selective decoupling study. Some spin systems exhibited partially overlapping resonances, and in some cases close to first-order, 2D/J (J-resolved)[6,7] experiments allowed the analysis to be undertaken. In other cases, when larger samples were available, inspection of the multiplets in the cross-sections of ^{13}C–1 H shift-correlated spectra[8] should allow us to resolve the problem. When the spin systems were analysed, the results were verified by simulation of spectra with the LAOCOON program.

Conformational Analysis.—(a) *Ring A.* A number of studies have been carried out on the conformation of ring A in 4,4-dimethyl-3-keto steroids or 3-keto triterpenoids.[9] Burkert and Allinger[10] determined the $J_{1\alpha(a)-2\beta(a)}$ coupling constants for 4,4-dimethylandrostanone (compound **A**). The value of J (13.7 Hz) indicated an antiperiplanar arrangement between 1-H^{a} and 2-H^{a}, and these authors concluded that ring A was in the chair conformation. A similar order of magnitude for J (14.8 Hz) has been reported for a 3-keto-triterpenoid, 3-oxours-12-en-24,28-dioic acid dimethyl ester (compound **B**[1]) using J-resolved and J-correlated spectroscopy.[7] The authors assumed that the methoxycarbonyl group at position 4β of this compound conferred less hindrance than did a methyl group. A half-chair conformation was proposed[11] for the analogue with a methyl group in the 4β position (compound **B**[2]) due to interaction with the 8β-Me.

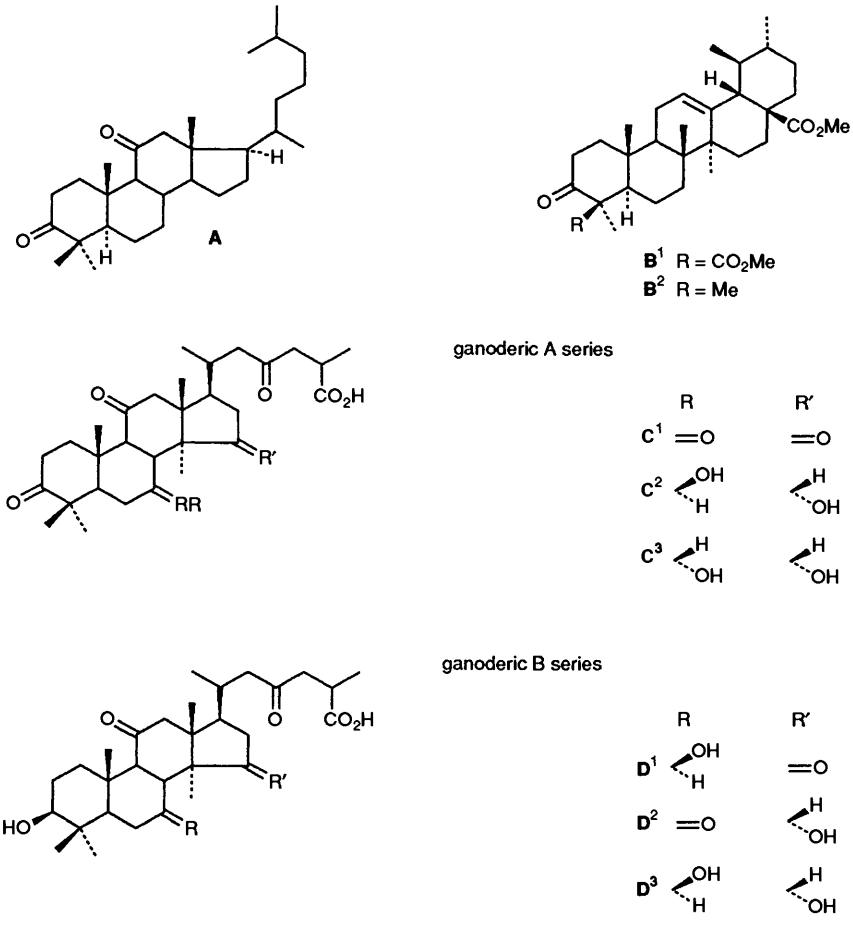


Table 4 ^1H–^1H Coupling constants (J/Hz) in ring A

Compound	1α–1β	1α–2α	1α–2β	1β–2α	1β–2β	2α–2β	2α–3α	2β–3α	5α–6α	5α–6β	6α–6β
1	14.5	9.0	6.0	6.0	8.0	15.0			3.0	14.7	14.7
2	13.5	3.5	13.0	3.5	3.5	13.0	3.5	13.0	3.0	14.0	16.0
4	14.5	9.0	6.5	5.5	8.0	15.5			2.5	15.0	14.4
5	13.7	3.6	14.5	3.6	3.6	14.0	3.0	13.3	2.0	15.2	15.4
3	14.0	10.0	6.5	5.5	8.5	15.5					
6	14.5	10.0	6.0	6.0	8.5	15.5					

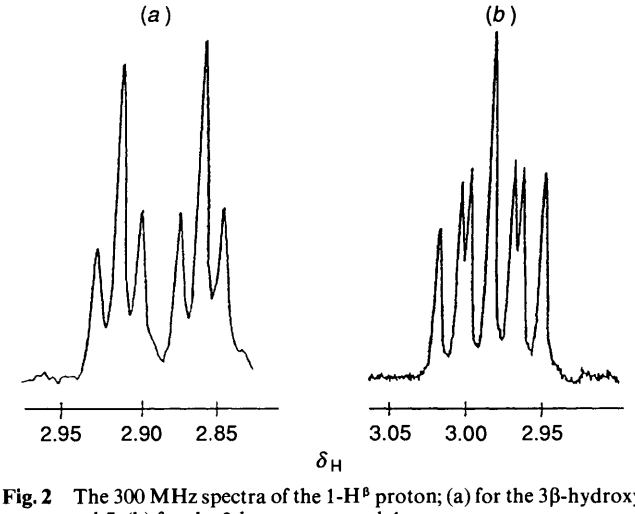


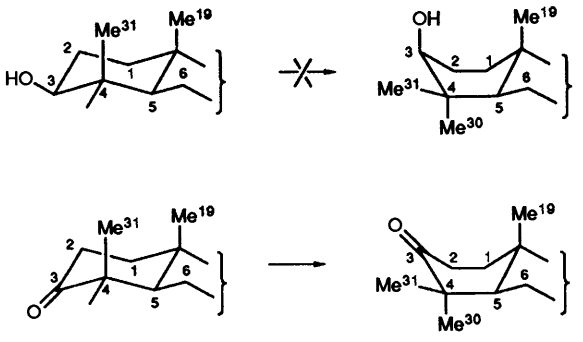
Fig. 2 The 300 MHz spectra of the 1-H$^\beta$ proton; (a) for the 3β-hydroxy compound **5**; (b) for the 3-keto compound **4**

In other 3-keto terpenoids derived from ganoderic acid A (compounds **C**), two Japanese groups[12,13] reported ^3J couplings between the 1-H and 2-H protons ranging from 5.5 to 9.5 Hz. It was suggested that ring A of ganoderic acid A was in the boat conformation,[12] which was confirmed by X-ray analysis. However, the coupling constants of the derivatives of ganoderic acid B (compounds **D**) with an alcohol group at the 3β position, indicated a chair conformation for ring A.[12,13]

In the present study, the highly deshielded 1-H$^\beta$ proton could be exploited as a conformational probe, the spin system being strongly influenced by the nature of the group at the 3 position (hydroxy or keto; see Fig. 2).

We observed coupling constants between 6 and 10 Hz for the four protons in positions 1 and 2 of the 3-keto compounds. $J_{1\alpha(a)-2\beta(a)}$ was close to 6 Hz (compounds **1**, **3**, **4**, **6**). On the other hand, we found coupling constants of 13.0 and 14.5 Hz for compounds **2** and **5**, respectively, indicating a chair conformation for ring A of the 3-hydroxy derivatives. These observations indicated that ring A in the 3-keto derivatives is highly deformed, although not sufficiently to turn it into the boat conformation. A sofa conformation in which carbons C-1, -2, -3, -4 and -5 are all in the same plane was suggested by the values of the coupling constants (Table 4).

The large chemical shift of the 1-H$^\beta$ proton, whose orientation

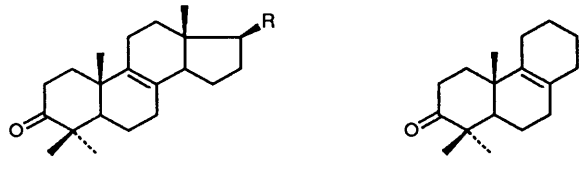


Scheme 1

is unchanged towards the deshielding carbonyl at position 11, was further evidence for this conformational modification. NOESY correlations analysis of compounds **4** and **5** in which ring A is in chair and sofa conformations, respectively, demonstrated a nuclear Overhauser effect (NOE) for methyls C-19 and -31 of compound **5** whose chair conformation maintains a diaxial orientation of these two groups. No correlation between these two methyl groups was observed in compound **4**, lending further support for the modified conformation indicated by the values of the coupling constants.

In this lanostane series, assignment of these conformations was tentatively made by analysis of interactions between methyls C-30 and -31 on the carbon at position 4 and the angular methyl C-19. In the proposed chair conformation for the 3-hydroxy series, there would be a strong 1,3-diaxial interaction between methyl C-19 and methyl C-31 (Scheme 1). Although this interaction would be reduced in the boat conformation, it is not energetically favourable, as apart from the eclipsing interactions of the boat form there is also a strong interaction between methyl C-19 and the hydroxy group on position 3.

On the other hand, in the 3-keto derivatives, the 1,3 diaxial interaction MeC-31/MeC-19 is suppressed, since the 3-carbon is hybrided sp^2 and the ring adopts a sofa conformation. This interpretation based on the minimization of interactions on ring A in isolation is not, however, in line with the empirical force-field calculations of Mislow and co-workers[9] for the 3-keto-4,4-dimethyl steroids and lanostane. Among the compounds studied, calculation indicated a marked distortion of ring A for lanost-8-en-3-one. For the tricyclic compound



Lanost-8-en-3-one

without ring D, ring A adopts the chair conformation. This distortion of ring A was interpreted as an extreme case of conformational transmission of ring D to ring A *via* the double bond at C-8–C-9, and our results provide experimental conformation of these theoretical predictions.

(b) *Ring B.* In the metabolites retaining the 7-keto group of the starting compound (group A), the coupling constants for the 5α angular proton and the 6α and 6β protons indicated a 5α(a)–6α(pseudo-e)/6β(pseudo-a) linkage characteristic of half-chair or twist structures.[14] In compounds **3** and **6** of group B, the 7-keto group is reduced to an alcohol, posing the problem of the configuration of this group. The spin pattern of ring B is of the ABXY type involving protons 5-H^{α}, 6-H^{α}, 6-H^{β} and

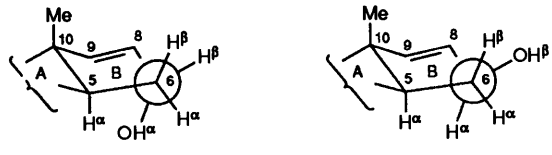
Table 5 ^1H–^1H Coupling constants (J/Hz) in ring B

Compound	5α–6α	5α–6β	6α–6β	7β–6α	7β–6β
1	3.0	14.7	14.7		
2	3.0	14.0	16.0		
3		Σ 15.5[a]	*b*	Σ 6.0[c]	
4	2.5	15.0	14.4		
5	2.0	15.2	15.4		
6		Σ 15.0[a]	*b*	Σ 5.0[c]	

[a] Measured on the 5-H^{α} system. [b] Overlapping lines. [c] Measured on the 7-H system.

proton 7-H (α or β). Unfortunately the 6-H^{α} and 6-H^{β} proton resonances were masked by other peaks, and so we could only analyse the multiplets of protons 5-H^{α} and 7-H, which nevertheless provided sufficient information to enable us to identify the configuration of carbon 7 (see Table 5).

The *trans* junction of ring B with ring A restricts its (ring B's) conformational mobility between an half chair and a boat form. For the least strained half-chair form, a geometry between protons 5-H^{α}, 6-H^{α} and 6-H^{β} similar to that in the 7-keto series was found, as indicated by the sum of the coupling constants for the 5-H^{α} proton (ΣJ 15 Hz).



(a) 7-OH^{α} (b) 7-OH^{β}

Fig. 3

The coupling constants for proton 7-H will depend on the configuration at this position. For the 7-OH^{α} configuration, the 7-H^{β} proton is pseudo-equatorial, and the sum of the coupling constants will be low. On the other hand, in the other configuration, the 7-H^{α} proton will be in a pseudo-axial orientation and the sum of the coupling constants will be much larger. The observed value in the two cases studied (ΣJ 5–6 Hz) indicated a 7α configuration for the hydroxy group. We also measured the values of these coupling constants for the pair of stereoisomers, 3β,7ξ-dihydroxylanost-8-en-11-one, obtained by reduction of substrate **I** with sodium borohydride.[15] We found values for ΣJ of 14.4 and 5.2 Hz for the 7β- and 7α-hydroxy derivatives, respectively. Similar values have been reported in the ganoderic acid A series[13] for the 7-OH^{α} derivative (compound **C**[3], ΣJ 6.8 Hz), and in the ganoderic acid B series for the two 7-OH^{β} derivatives (compound **D**[1], ΣJ 17.5 Hz; and compound **D**[3], ΣJ 17.8 Hz).

(c) *Ring D.* The coupling constants for the protons on ring D were comparable to those reported by Sanders[16,17] and Schneider[8] for steroids, and are given in Table 6. This indicated that the 17β configuration of the side-chain is conserved in the bioconversion products.

Conclusion.—The presence of an ene-dione system on the lanosterol skeleton enabled degradation of these derivatives by *Mycobacterium phlei.* Lanosterol itself is not attacked by this bacterium. We observed similar behaviour with 4,4-dimethylcholesterol which is not attacked by these mycobacteria, although the 7-keto analogue is degraded.[18] This difference was assumed to be due to the requirement for the presence of an enone group before the side-chain can be degraded. Indeed, for cholesterol, degradation only starts after formation of an enone group in ring A following action of cholesterol dehydrogenase and 3-keto Δ^5-cholestenone isomerase.[1]

The major products of degradation of the side-chain of 8,9-

Table 6 ^1H–^1H Coupling constants (J/Hz) in ring D

Compound	15α–15β	15α–16α	15α–16β	15β–16α	15β–16β	16α–16β	16α–17α	16β–17α
1	12.5	9.5	2.5	7	13.0	13.5	9.5	8.5
2	13.0	9.5	2.5	7	12.5	13.5	9.0	9.5
4	12.5	9.5	2.2	6.5	12.5	13	9.5	9.5
5	13.2	9.4	2.2	7	12.5	13.5	9.5	9.5
a	11.4	9.9		5.6	11.4	13.3	9.4	9.4
b	11.8		3.0		8.5			
c	11.8	9.0	1.0	8.5	11.2	18.3		8.8

[a] Values reported by Sanders and co-workers[16,17] for 11β-hydroxyprogesterone. [b] Values reported by Schneider *et al.*[8] for a progesterone analogue. [c] Values reported by Schneider *et al.*[8] for 5αH-androstan-17-one and the parent Δ^5-compound.

dioxolanosterol all had the side-chain cleaved at C-3. We did not detect any metabolite in which this chain had undergone complete degradation. This was accounted for by hindrance from ring D especially from the axial methyl group in the 14α-position towards the 17α hydrogen. The tetracyclic skeleton undergoes regioselective oxidation–reduction modifications at the 3- and 7-position. While the ring A of 3β-hydroxy derivatives remains in the chair form as in the initial substrate, proton coupling constants indicate an important conformational change for 3-keto derivatives as predicted formerly by Mislow[9] in this lanostane series. When the 7-keto group is reduced to an alcohol function this transformation is stereoselective, leading to the 7-OH$^{\alpha}$ compounds.

Experimental

Bioconversion Experiments.—Mycobacterium phlei strain ATCC 11758 exists as four varieties following their pigmentation properties. The variety cb, used in our experiments, was cultured at 37 °C for 72 h in an Erlenmeyer flask containing stirred Sauton[19] medium (100 cm^3). A solution of 3β-hydroxylanost-8-ene-7,11-dione substrate (10 mg) in dimethylformamide (2 cm^3) was added and the mixture was stirred 72 h. After acidification of the medium by 10% HCl (10 cm^3), the products were extracted with CH_2Cl_2. The organic phase was washed with water to neutrality, dried (Na_2SO_4) and evaporated. The separation of the various components was performed with a Waters 501 HPLC apparatus (Si-60-5 column; hexane–ethyl acetate gradient 8:2 to 0:10 in 30 min).

NMR Methods.—NMR spectra were obtained at 300.13 MHz with a Bruker AM 300 WB spectrometer with samples in $CDCl_3$ solution at 24 °C in 5 mm tubes. For 2D-COSY (two-dimensional chemical-shift-correlated spectroscopy) the applied pulse sequence was (90°, t_1, 90°, acquisition). The data size of the time domain was 256 (f_1) × 1024 (f_2) with a spectral width of 1600 Hz. The time-domain matrix was extended by zero-filling to 2048 points. Sine bell enhancement was used in both directions and the number of transients for each FID was 16.

For 2D-NOESY (two-dimensional nuclear overhauser and exchange spectroscopy) the applied pulse sequence was (90°, t_1, 90°, τ_m, 90°, acquisition). The solutions were degassed. The data size of the time domain was 128 (f_1) × 512 (f_2). The time-domain matrix was extended by zero-filling to 1024. Sine bell enhancement was used in both directions and the number of transients for each FID was 8 with a mixing time set to 0.6 s.

Homonuclear proton 2D-J experiments were carried out with the pulse sequence (90°, t_1, 180°, t_1, acquisition). The data size of the time domain was 64 (f_1) × 1024 (f_2). The time-domain matrix was extended by zero-filling to 2048.

References

1 C. J. Sih, J. C. Wancz and H. H. Tai, *J. Am. Chem. Soc.*, 1967, **89**, 1956.
2 D. Daniel, E. Lederer and L. Velluz, *Bull. Soc. Chim. Biol.*, 1945, **27**, 218.
3 R. E. Marker, E. L. Wittle and L. W. Mixon, *J. Am. Chem. Soc.*, 1937, **59**, 1308.
4 L. Ruzicka, E. Rey and A. C. Muhr, *Helv. Chim. Acta*, 1944, **27**, 472.
5 H. Iizuka and A. Naito, *Microbial Conversion of Steroids and Alkaloids*, Springer-Verlag, Berlin, Heidelberg and New York, 1981.
6 K. Nagayama, P. Bachmann, K. Wuthrich and R. R. Ernst, *J. Magn. Reson.*, 1978, **31**, 133.
7 D. Loganathan, G. K. Triveri and K. V. R. Chari, *J. Org. Chem.*, 1986, **51**, 3366.
8 H. J. Schneider, U. Buchheit, N. Becker, G. Schmidt and U. Siehl, *J. Am. Chem. Soc.*, 1985, **107**, 7027.
9 As a leading reference see: D. A. Dougherty, K. Mislow, J. W. Huffman and J. Jacobus, *J. Org. Chem.*, 1979, **44**, 1585.
10 U. Burkert and N. L. Allinger, *Tetrahedron*, 1978, **34**, 807.
11 D. Loganathan, K. V. R. Chari and G. K. Triveri, in the press (see ref. 7).
12 T. Kubota, Y. Asaka, I. Miura and H. Mori, *Helv. Chim. Acta*, 1982, **65**, 611.
13 T. Kikuchi, S. Kanomi, S. Kadota, Y. Murai, K. Tsubono and Z. Ogita, *Chem. Pharm. Bull.*, 1986, **34**, 3695.
14 P. Tisnes, P. Maroni and L. Cazaux, *Org. Magn. Reson.*, 1979, **12**, 481.
15 J. R. Dias, *J. Org. Chem.*, 1974, **39**, 1767.
16 L. D. Hall and J. K. M. Sanders, *J. Am. Chem. Soc.*, 1980, **102**, 5703.
17 L. D. Hall and J. K. M. Sanders, *J. Org. Chem.*, 1981, **46**, 1132.
18 J. C. Promé, unpublished results.
19 B. Sauton, *C. R. Acad. Sci. (Paris)*, 1912, **155**, 860.

Paper 1/00395J
Received 28*th January* 1991
Accepted 15*th April* 1991