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TRITERPENOIDS OF THE FUNGUS *PISOLITHUS TINCTORIUS**

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Key Word Index—*Pisolithus tinctorius*, Sclerodermataceae, Basidiomycete, polyhydroxylated triterpenes, X-ray, differential nuclear Overhauser effect

Abstract—Several new triterpenoids were isolated from *Pisolithus tinctorius*. Their molecular structures were established from spectroscopic studies, X-ray analysis and chemical correlation.

INTRODUCTION

Pisolithus tinctorius (Mich. ex Pers.) is a fungus of great economic interest, since its basidiospore inoculum forms specific ectomycorrhizae important for the development of pine seedlings, thus enabling the creation of artificial forestation zones [1] This fungus occurs widely in Portugal, in two varieties, *crassipes* and *tuberosus* [2]

Previous work established the structure of three biosynthetically interesting triterpenes, pisolactone [3] (**1**), 24-methyllanosta-8,24(28)-diene-3β,22ξ,23ξ-triol 22-acetate (**2**) and 24-ethyllanosta-8,24(28)*E*-diene-3β,22ξ,23ξ-triol 22-acetate [4] (**3**) We report here details concerning other new triterpenes, as well as on the absolute stereochemistry of **2** and **3**

RESULTS AND DISCUSSION

Powdered fruiting bodies of the *tuberosus* variety of *P. tinctorius* were exhaustively extracted with diethyl ether and the resulting brown oil was purified by chromatography on a column of silica gel, to afford three major triterpenoid fractions.

From the first fraction, a new crystalline compound, mp 248–250°, $C_{31}H_{48}O_3$ (M^+ 468), was isolated and found to be identical (mmp, TLC, IR) with the product obtained by Jones' oxidation of pisolactone (**1**) and thus assigned structure **4**

The second fraction was found to contain ergosterol and pisolactone, plus a complex mixture which upon acetylation yielded the acetates of 5,6-dihydroergosterol and 5,6,22,23-tetrahydroergosterol in a ratio of 19·6 (by GLC analysis), as well as a minor triterpene (**5**), M^+ at *m/z* 540, consistent with the molecular formula $C_{35}H_{56}O_4$, mp 165–170°, having the lanosterol nucleus, two secondary acetate groups (by ^{1}H NMR) and an exocyclic methylene [ν_{max} 900 cm^{-1}, δ 4.78 (*s*, 1H), 4.85 (*s*, 1H)]. Mild basic hydrolysis of **5** afforded diol **6** (no carbonyl by IR), the mass spectrum of which showed a diagnostic peak at *m/z* 314, corresponding to the loss of the side chain to give the basic lanosterol nucleus [5] This suggests that, apart from the ubiquitous 3-hydroxy function, the second hydroxyl group was part of an alkylated side chain

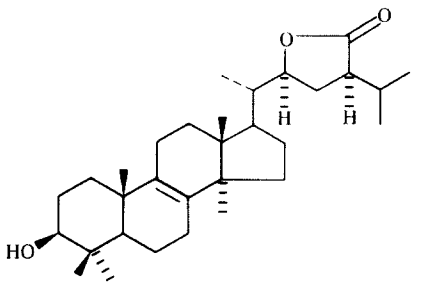
The ^{13}C NMR spectrum of **6** exhibited four sp^2 carbon resonances at δ 128 6, 153 9, 134.8 and 135.0, the first pair corresponding to the carbon atoms of an exocyclic methylene, and the other to the lanosterol Δ^8-double bond [6]. Also two peaks at δ 78.3 and 71 0 confirmed the presence of two oxygenated carbons, the first one corresponding to C-3 and the other to a carbon of the side chain [7].

Silylation of **6** with bis-trimethylsilylacetamide [8] gave a bis-silylated derivative **7** Its mass spectrum contained the ion *m/z* 185 (50%) resulting from fragmentation between C-20 and C-22, which by analogy with the fragmentation obtained for the silylated derivative of 22-hydroxyergosterol [9], is characteristic of an oxygenated function at C-22. The values for the optical rotation of **6** and those of lanosterol, eburicol, inotodiol and its 22-epimer are listed in Table 1.

While introduction of a (22*R*)-chiral centre in the side chain of lanosterol, as in inotodiol, does not affect the rotation, introduction of a (22*S*)-centre lowers the value, in agreement with empirical findings [11, 14] Since the value of the rotation of eburicol is very close to that of lanosterol, it is conceivable that introduction of *R* or *S*-chirality at C-22 in the former has a similar effect. Thus the rotation of **6** and the value Δ[M] (−158) observed in relation to eburicol as well as the value of Δ[M] of (−53) relating 22-*epi*-inotodiol to lanosterol, both suggest the (22*S*)-configuration as the more probable one for **6** However, this conclusion is to be taken with caution given the fact that considerable discrepancies are found in the literature for the optical rotations recorded [15, 16]

Based on the previous discussion we suggest that **6** is (22*S*)-lanost-8,24(28)-diene-3β,22-diol. The scarcity of the material precluded any further characterization.

* Part 3 in the series 'Terpenoids from Basidiomycetes', Ref. [4] constitutes Part 2 in this series.



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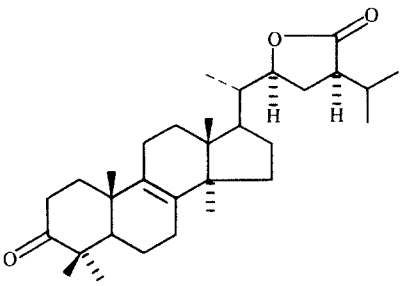

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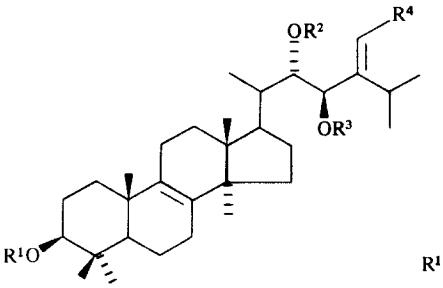
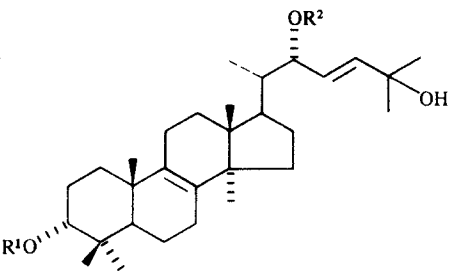
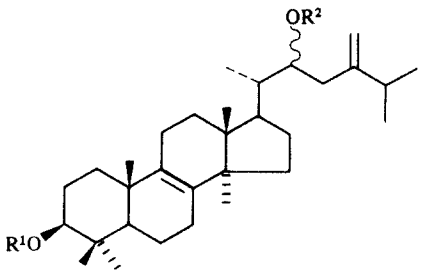


	R¹	R²	R³	R⁴
2	H	Ac	H	H
3	H	Ac	H	Me
10	Ac	H	Ac	H
11	Ac	H	H	H
12	H	H	H	Me
13	H	acetonide		Me
14	H	H	H	H
15	H	acetonide		H
16	Ac	acetonide		H



	R¹	R²
8	Ac	Ac
9	H	Ac

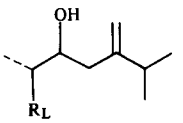
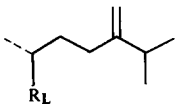
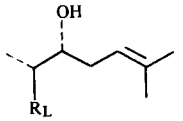
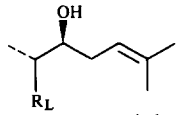
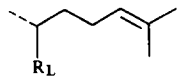


	R¹	R²
5	Ac	Ac
6	H	H
7	TMS	TMS

The third fraction yielded, upon acetylation, the acetates of ergosterol endoperoxide and $\Delta^{9(11)}$-ergosterol endoperoxide and a new diacetate **8** with the gross lanosterol skeleton (by ^{1}H NMR) The mass spectrum was consistent with the molecular formula $C_{34}H_{54}O_5$, mp, 190–192°, $[\alpha]_D$ + 6°, δ 2 05 (*s*, 3H) and 2 06 (*s*, 3H) which was identical (TLC, mmp) with the product obtained on acetylation of the natural monoacetate **9**, $C_{32}H_{52}O_4$, 187–190°, δ2.05 (*s*, 3H, MeCO$_2$R) isolated directly from the methanolic extract of the fungus The presence of signals at δ 5 65 (1H, *dd*, J_1 = 14 7 Hz, J_2 = 7.5 Hz) and 5 86 (1H, *d*, J = 14 7 Hz) attributed to the *trans*-olefinic protons [17], as well as a resonance at 5 30 (1H, *dd*, J_1 = 7 5 Hz, J_2 = 3 7 Hz) assigned to the allylic proton, strongly suggested the structural fragment R_2CH-CH(OAc)-CH=CH-CR_3 in **9** The tertiary alcoholic function (ν_{max} *ca* 3400 cm^{-1}) in **8** and **9** was placed on C-25 since the characteristic doublet assigned to the two methyl groups attached to this carbon was absent, and a singlet at δ 1.32 (6H) was present Placement of one of the secondary alcoholic functions (3α) at C-3 (3.42, *d*, $W_{1/2}$ = 7 Hz, on **9**, shifting to 4.57 on **8**), would be consistent with the structures proposed which were confirmed unambiguously by an X-ray structure analysis of **8** (Fig 1). The ether fraction of the extract of the *crassipes* variety of *P. tinctorius* yielded besides **2** and **3**, ergosterol, ergosterol endoperoxide and its $\Delta^{9(11)}$-derivative

Application of Horeau's method [18, 19] to the diacetate **10** obtained by partial acetylation of **11** led to the assignment of the absolute configuration at C-22 as *S* The identical configuration was assumed to be present at the corresponding position in the ethylidene triterpenoid **12** In order to establish the configuration at C-23, this carbon was locked in the five-membered ring of the

Table 1

Compound *	$[\alpha]_D$	[M]	Ref
	+29°	132	—
 eburicol	+66°	290	[10]
 inotodiol	+59°	261	[11]
 22-*epi* inotodiol	+44°	194	[12]
	+58°	247	[13]

* R_L basic skeleton of lanosterol.

acetonide **13** of triol **12**, obtained by saponification of **3**. Since a strong N.O.E., implying a *cis*-relationship, was observed (cf. Fig 2) for the C-22, C-23 protons of **13**, the absolute configuration at C-23 was assigned as *R**. From the methanolic extracts of *P. tinctorius* (var *tuberosus*), mannitol was also isolated and identified.

EXPERIMENTAL

^{1}H NMR, data are for solns in $CDCl_3$ with TMS as int. standard, and were recorded at 300 and 250 MHz ^{13}C NMR data were recorded at 25 15 MHz for $CDCl_3$ solns with tetramethylsilane as int. standard The X-ray analysis used a Nicolet R 3m diffractometer

Crystal data $C_{34}H_{54}O_5$, $M_r = 542.75$, monoclinic, space group $C2$, $a = 26\,690(20)$, $b = 7\,122(4)$, $c = 23\,244(27)$ Å, $\beta = 1^3$, $z = 4$, $D_c = 1\,14$ g/cm^3, $\mu(Cu-K_\alpha) = 5.48$ cm^{-1} 1922 Independent observed reflections [$|F_0| > 3\,\sigma(|F_0|)$, $\Theta \leq 58°$] were measured on a Nicolet R3m Diffractometer with Cu–K_α radiation (graphite monochromator) using ω-scans. The structure was solved by direct methods and the non-hydrogen atoms refined anisotropically to $R = 0.065$, $R_\omega = 0\,064$. Computations were carried at on an Eclipse S140 computer using the SHELXTL program system [20] The atomic coordinates for this work are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U K Any request should be accompanied by a full literature citation for this communication

Extraction of P tinctorius (*var* tuberosus) *fruiting bodies.* Powdered, dried fruiting bodies (31 kg) were extracted by percolation with Et_2O followed by MeOH. Removal of the Et_2O and of the MeOH gave brown oils (42 and 164 g, respectively) which were chromatographed separately on a column of silica gel.

The Et_2O extract was purified on a chromatographic column eluted with hexane–EtOAc (6 4) Three fractions were collected (F-1, F-2 and F-3 by order of the compounds eluted), and analysed by TLC (silica gel GF_{254}, hexane–EtOAc, 7 3).

Fraction F-1 Purification by flash column chromatography (silica gel, hexane–EtOAc, 9 1) gave a white crystalline solid of 3-*oxopisolactone* (**4**) (21 mg), mp 248–250° (from MeOH) Found: M^+, m/z 468 3598, $C_{31}H_{48}O_3$ requires 468 3603). $[\alpha]_D + 79°$ ($CHCl_3$, c 1 4), IR ν_{max}^{KBr} cm^{-1} 1705 and 1740 (C=O); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0 71 (3H, *s*), 0 87 (3H, *s*), 0.89 (3H, *d*, $J = 7$ Hz), 0 91 (3H, *d*, $J = 7$ Hz), 0 98 (3H, *d*, $J = 7$ Hz), 1 02 (3H, *s*), 1 07 (3H, *s*), 1 08 (3H, *s*), 2 47 (1H, *m*), 2.50 (2H, *m*), 4 35 (1H, *m*); MS m/z (rel int) 468 [M, $C_{31}H_{48}O_3$]$^+$ (20), 453 [M – Me]$^+$ (35), 435 [M – Me – H_2O]$^+$ (10), 313 [M – $C_9H_{15}O_2$]$^+$ (5), 298 [M–$C_9H_{15}O_2$ – Me]$^+$ (15)

Fraction F-2 Fractional crystallization from EtOAc gave pisolactone (**1**), (238 mg), ergosterol (135 mg), and a mixture of pisolactone and ergosterol (1 136 g) The remainder of F-2 was acetylated using Ac_2O (2 ml) in pyridine (2 ml) and purified by CC (silica gel, hexane–EtOAc, 9 1), yielding by order of elution a mixture of the acetates of 5,6-dihydroergosterol and 5,6,22,23-tetrahydroergosterol (ratio 19/6), (90 mg), mp 163–172° (from MeOH), analysed by GC-MS on a column SE-54 (25 m × 0 18 mm), column temperature 270°, injection temperature 300°, ionic source temperature 300°, and compared with authentic samples

Further elution yielded triterpene **5** (5 mg), mp 165–170° (MeOH), IR ν_{max}^{KBr} cm^{-1} 1730, 1640, 900, ^{1}H NMR (300 MHz, $CDCl_3$) δ 0 66 (3H, *s*), 0 87 (9H, *br s*), 0 97 (12H, *br s*), 2 02 (3H, *s*), 2.07 (3H, *s*), 4 50 (1H, *m*), 4 78 (1H, *s*), 4 85 (1H, *s*), 5 20 (1H, *m*); MS m/z (rel int.) 540 [M, $C_{35}H_{56}O_4$]$^+$ (10), 525 [M – Me]$^+$ (2), 480 [M – AcOH]$^+$ (3), 465 [M – AcOH – Me]$^+$ (20), 420 [M – 2AcOH]$^+$ (0 5), 405 [M – 2AcOH – Me]$^+$ (9), 356 [M – $C_{11}H_{20}O_2$]$^+$ (4), 281 [M – $C_{11}H_{20}O_2$ – AcOH – Me]$^+$ (5) Hydrolysis of **5** (KOH, 10%, reflux under nitrogen for 1 hr yielded **6**, mp 161–165° (MeOH), $[\alpha]_D + 29°$ ($CDCl_3$, c 0.16), IR ν_{max}^{KBr} cm^{-1} 3450 (OH), 890 (CH_2=CR_2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0 70 (3H, *s*), 0 81 (3H, *s*), 0 91 (3H, *s*), 0 92 (3H, *d*, $J = 6$ Hz), 0.98 (3H, *s*), 1 01 (3H, *d*, $J = 6$ Hz), 1.04 (3H, *s*), 1.06 (3H, *d*, $J = 6$ Hz), 3 23 (1H, *m*), 3.80 (1H, *m*), 4 78 (1H, *s*), 4 88 (1H, *s*); ^{13}C NMR (25.15 MHz, $CDCl_3$). δ 153 9, 135 0, 134 8, 128 6, 78 3, 71 0; MS m/z (rel. int) 456 [M, $C_{31}H_{52}O_2$]$^+$ (26), 441 [M – Me]$^+$ (7), 438 [M – H_2O]$^+$ (1), 423 [M – H_2O – Me]$^+$ (9), 372 [M – C_6H_{12}]$^+$ (16), 357 [M – C_6H_{12} – Me]$^+$(100), 339 [M – C_6H_{12} – H_2O – Me]$^+$ (45), 314 [M – $C_9H_{18}O$]$^+$ (10), 281 [M – $C_9H_{18}O$ – H_2O]$^+$ (18)

Fraction F-3 Acetylation of this fraction using the same conditions as for F-2, followed by CC purification (silica gel, hexane–EtOAc, 7 3), yielded by order of elution a mixture of the acetates of ergosterol endoperoxide and $\Delta^{9(11)}$-ergosterol endoperoxide, (4 1, by NMR) (779 mg), mp 199–201° (from MeOH) Separation by $AgNO_3$–silica gel TLC yielded pure ergosterol

*The absence of any detectable equivalent N.O E in the parent diol (cf ref [4]) is an indication of very different side-chain conformations in the diol and in the corresponding acetonide The previously suggested assignment of a (23*S*)-chirality should therefore be reversed

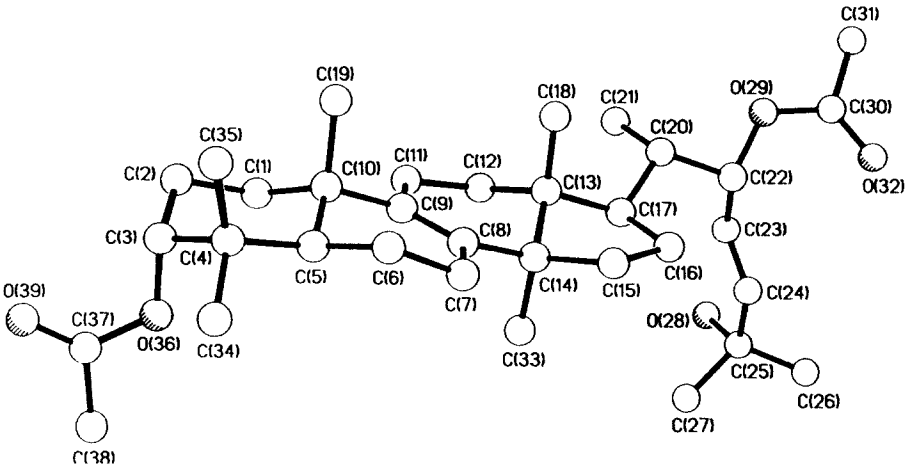


Fig 1 3-D structural drawing with atomic labelling of compound **8** Hydrogen atoms are omitted for clarity

endoperoxide acetate, mp 200–202° (lit [21] mp 202–205°) Basic hydrolysis of this compound as described before, afforded ergosterol endoperoxide [mp 177–178° from MeOH (lit [22] mp 176–178°)] identical (TLC, mmp) with an authentic sample

Further elution of the column yielded (22*R*)-25-hydroxylanosta-8,23-diene-3α,22-diacetate (**8**), (328 mg), mp 190–192 (from MeOH) (Found M^+, *m/z* 542 3964, $C_{34}H_{54}O_5$ requires 542 3971) $[\alpha]_D + 6°$ ($CHCl_3$, *c* 1 5), IR ν_{max}^{KBr} cm^{-1} 3450 (OH), 1730 and 1720 (C=O), 970 (RCH=CHR), ^1H NMR (300 MHz, $CDCl_3$) δ0 72 (3H, *s*), 0 87 (6H, *s*), 0 92 (3H, *s*), 0 94 (3H, *d*, *J* = 6 Hz), 1 00 (3H, *s*), 1 32 (6H, *s*), 2 05 (3H, *s*), 2 06 (3H, *s*), 4 57 (1H, $W_{1/2}$ = 7 Hz), 5 25 (1H, *dd*, J_1 = 7 5 Hz, J_2 = 3 7 Hz), 5 56 (1H, *dd*, J_1 = 15 Hz, J_2 = 7 5 Hz), 5 79 (1H, *d*, *J* = 15 Hz), MS *m/z* 542 [M, $C_{34}H_{54}O_5$]$^+$ (44), 482 [M–AcOH]$^+$ (5), 467 [M–AcOH–Me]$^+$ (32), 449 [M–AcOH–Me–H_2O]$^+$ (42), 407 [M–2AcOH–Me]$^+$ (26), 389 [M–2AcOH–Me–H_2O]$^+$ (26), 356 [M–$C_{10}H_{18}O_3$]$^+$ (11), 341 [M–$C_{10}H_{18}O_3$]$^+$ (11), 325 [M–$C_8H_{13}O_3$–AcOH]$^+$ (14)

From the methanolic extract (164 g) after 'flash chromatography' (silica gel, hexane–EtOAc, 7 3) **9** was obtained (3 mg), mp 187–190 (from MeOH), IR ν_{max}^{KBr} cm^{-1} 3370 (OH), 1720 (C=O), ^1H NMR (300 MHz, $CHCl_3$) δ0 70 (3H, *s*), 0 82 (3H, *s*), 0 87 (3H, *s*), 0 94 (6H, *d*, *J* = 6 Hz), 0 97 (3H, *s*), 0 98 (3H, *s*), 1 32 (6H, *s*), 2 05 (3H, *s*), 3 42 (1H, $W_{1/2}$ = 7 Hz), 5 30 (1H, *dd*, J_1 = 7 5 Hz, J_2 = 3 7 Hz), 5 65 (1H, *dd*, J_1 = 14 7 Hz, J_2 = 7 5 Hz), 5 86 (1H, *d*, *J* = 14 7 Hz), MS *m/z* 500 (rel int) [M, $C_{32}H_{52}O_4$]$^+$ (*10*), 485 [M–Me]$^+$ (1), 482 [M–H_2O]$^+$ (1), 467 [M–H_2O–Me]$^+$ (2), 440 [M–AcOH]$^+$ (26), 425 [M–AcOH–Me]$^+$ (30), 407 [M–AcOH–Me–H_2O]$^+$ (40), 389 [M–AcOH–2H_2O–Me]$^+$ (4), 325 [M–$C_8H_{13}O_3$–H_2O]$^+$ (14), 314 [M–$C_{10}H_{18}O_3$]$^+$ (8) Further elution of the column yielded mannitol (2 g), identical in all respects with an authentic sample

Extraction of Pisolithus tinctorius (*var* crassipes) *fruiting bodies* The fungus (2 6 kg) was extracted as described before CC (silica gel, hexane–EtOAc, 7 3) of the ethereal extract (5 6 g) yielded ergosterol, ergosterol endoperoxide and $\Delta^{9(11)}$-ergosterol endoperoxide identical with authentic samples, as well as a mixture of **2** and **3** (ratio 4 1, by ^1H NMR) (1 9 g)

Oxidation of pisolactone To pisolactone (100 mg) in Me_2CO (30 ml) was added Jones' reagent (0 2 ml), and after 10 m at room temp, the reaction mixture was diluted with H_2O (30 ml), the organic solvent removed under red pres, and the aq phase extracted with CH_2Cl_2 (3 × 10 ml), washed (aq $NaHCO_3$), and the organic phase, dried ($MgSO_4$) Evapn of the solvent under red pres gave 3-oxopisolactone (**4**), (95 mg), identical in all respects (TLC, mmp, and spectroscopic data) with the natural compound

(22*S*,23*R*)-22,23-*Dihydroxylanosta*-8,24(28)-*diene*-3β-*yl-acetate* A soln of **14** (obtained by alkaline hydrolysis of **2**) (456 mg) in Me_2CO (10 ml) and *p*-toluenesulphonic acid (8 mg) was heated under reflux during 2 hr Sodium bicarbonate was added, and the reaction mixture worked-up as in the oxidation of pisolactone After CC purification the acetonide **15** (410 mg) was obtained, mp 197–200° (from Me_2CO), (Found M^+, *m/z* 512 4224, $C_{34}O_{56}O_3$ requires 512 4229) $[\alpha]_D$ + 57 ($CHCl_3$, *c* 3 1), ^1H NMR (300 MHz, $CDCl_3$) 0 66–1 16 (30 H, Me groups), 1 40 (3H, *s*), 1 54 (3H, *s*), 2 38 (1H, *h*, *J* = 9 Hz), 3 23 (1H, *m*), 4 29 (1H, *d*, *J* = 7 2 Hz), 4 74 (1H, *d*, *J* = 7 2 Hz), 5 04 (1H, *s*), 5 25 (1H, *s*) The acetonide **13** derived from **3** was similarly prepared and had mp 225–229°, ^1H NMR (300 MHz, $CDCl_3$) 0 65–1 16 (24H, Me groups), 1 32 (3H, *s*), 1 46 (3H, *s*), 2 50 (1H, *m*), 3 14 (1H, *m*), 4 10 (1H, *d*, *J* = 7 2 Hz), 4 54 (1H, *d*, *J* = 7 2 Hz), 5 67 (1H, *m*) The acetonide **15** was acetylated (Ac_2O, pyridine, room temp, 8 hr) to give the 3-acetate acetonide **16**, (432 mg), mp 200–204° (from Me_2CO), which was dissolved in THF (10 ml), containing MeOH (5 ml) and aq AcOH (75%) (5 ml), and heated to reflux for 2 hr The solvents were removed under vacuum and the residue after CC purification yielded (22*S*,23*R*)-22,23-dihydroxylanosta-8,24(28)-diene-3β-yl-acetate (**11**), (231 mg), mp 181–185° (from MeOH), $[\alpha]_D$ + 39° ($CHCl_3$, *c* 1 4), IR ν_{max}^{KBr} cm^{-1} 3450 (OH) and 1700 (C=O), ^1H NMR (300 MHz, $CDCl_3$) δ0 73–1 10 (24H), 2 04 (3H, *s*), 2 38 (1H, H, *J* = 9 Hz), 3 62 (1H, *d*, *J* = 9 Hz), 4 07 (1H, *d*, *J* = 9 Hz), 4 50 (1H, *dd*, J_1 = 9 Hz, J_2 = 4 5 Hz), 5 09 (1H, *s*), 5 19 (1H, *s*)

Diacetate **10** The monoacetate **11** was acetylated (Ac_2O, pyridine) at room temp for 1 hr After the usual work-up the residue was purified by flash chromatography to yield pure 3,23-diacetate **10** (19 mg), mp 188–192° (from MeOH), ^1H NMR (300 MHz, $CDCl_3$) δ0 70 (3H, *s*), 0 88 (6H, *s*), 0 90 (3H, *s*), 0 91 (3H, *d*, *J* = 7 2 Hz), 1 01 (3H, *s*), 1 05 (3H, *d*, *J* = 7 Hz), 1 11 (3H, *d*, *J* = 7 Hz), 2 05 (6H, *s*), 2 36 (1H, *h*, *J* = 6 Hz), 3 74 (1H, *d*, *J* = 9 Hz), 4 50 (1H, *m*), 5 13 (1H, *s*), 5 15 (1H, *d*, *J* = 9 Hz)

Determination of absolute configuration by Horeau's method The diacetate **10** (9 6 mg) was dissolved in dry pyridine (0 5 ml) containing (±)-α-phenylbutyric anhydride (31 5 mg) and left at room temp for 24 hr H_2O (0.1 ml) was added and the reaction mixture was heated for 30 m in a water bath C_6H_6 (3 ml) was added and the free phenylbutyric acid was titrated with 0 05 M

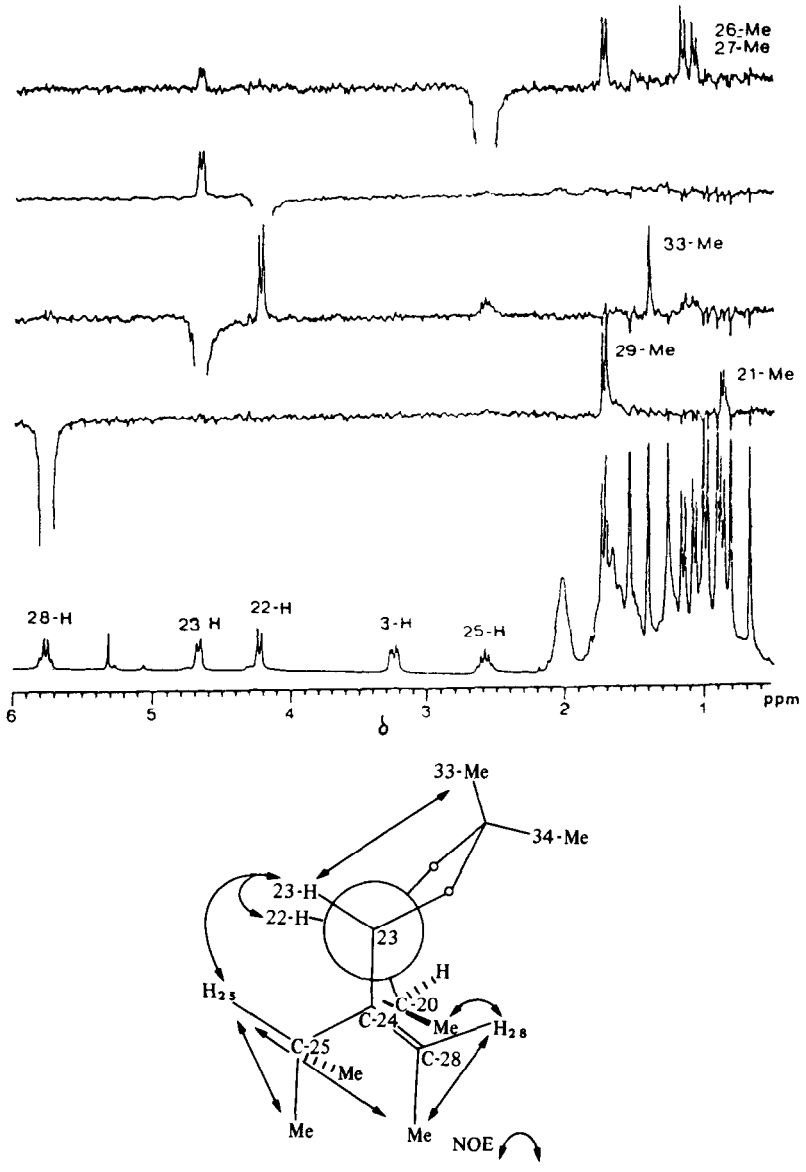


Fig 2 NOE obtained for the side chain of compound **13**

NaOH (2.01 ml) in the presence of phenolphthalein. The aq phase was extracted with C_6H_6 (2 × 2 ml), acidified with 0.05 HCl (1 ml) and extracted again with C_6H_6 The organic phase was dried ($MgSO_4$) and the solvent evapd to yield the pure phenylbutyric acid (32 8 mg, esterification yield 13%), $[\alpha]_D^{25°}$ − 0 175° (C_6H_6, *c* 0 34) (optical yield 17%)

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