Isolation and Structural Elucidation of a Novel Sterol Metabolite of *Fusarium sporotrichioides* 921

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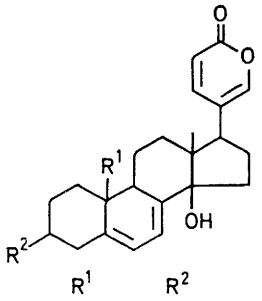
The isolation and identification of 12β-acetoxy-4,4-dimethyl-24-methylene-5α-cholesta-8,14-diene-3β,11α-diol (3) from *Fusarium sporotrichioides* 921, previously associated with a fatal outbreak of alimentary toxic aleukia, is described.

ALIMENTARY toxic aleukia (ATA) is a disease that has been recorded in Russia since the beginning of the 19th century.[1,2] This disease has been associated with the consumption of food made from grain which remained unharvested under snow and became mouldy from contamination with a variety of micro-organisms. The most prevalent of the organisms are toxic species of *Fusarium sporotrichioides* and *F. poae*.[2] Olifson has previously reported the isolation of two steroidal glycosides, sporofusarin (1) and poaefusarin (2) and their



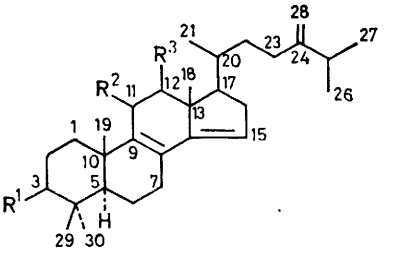
	R^1	R^2
(1)	Me	glucose — glucose — ribose
(2)	CHO	xylose

aglycones from *F. sporotrichioides* and *F. poae*, respectively, and suggested these steroids to be the toxins responsible for ATA.[3,4] However, subsequent work on an ' authentic sample of poaefusarin '[5] and on *Fusarium* species from eastern Europe[6] has failed to detect any steroidal compounds. Only the presence of trichothecenes and zearalenone were confirmed. We have previously shown that ATA is caused by the trichothecene 3α-hydroxy-4β,15-diacetoxy-8α-(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene (T-2 toxin).[7,8,9] During the course of our investigations with an extract derived from strain 921, one of the most toxic strains of *F. sporotrichioides* previously associated with a fatal outbreak of ATA, we were successful in isolating six non-toxic steroids in low yield.[7] Four of these were the known compounds β-sitosterol, camphesterol, stigmasterol, and ergosterol. The remaining two steroids appeared to be unknown compounds. We report here the characterization of the most abundant of these two steroids by spectroscopic and chemical methods.

RESULTS AND DISCUSSION

The steroids isolated represent all the steroids detectable by spectroscopic and chromatographic techniques with polarity (determined by chromatography) less than that of T-2 toxin. The steroids were separated on a Florisil column into three fractions. The first fraction contained the known steroids β-sitosterol, camphesterol, stigmasterol, and ergosterol. The second fraction contained an as yet unidentified oil and the third fraction contained a steroid (3) whose identification is the subject of this report.

The compound (3) (white crystals, m.p. 181 °C, $[\alpha]$ +32.7°) was obtained in 1.4% yield from the extract. The mass spectrum of (3) had M^+ at *m/e* 498 and showed a strong peak at *m/e* 438 indicative of the loss of $MeCO_2H$.



	R^1	R^2	R^3
(3)	OH	OH	OAc
(4)	OH	OH	OH
(5)	OAc	OAc	OAc
(6)	=O	=O	OAc

Further loss of Me resulted in the base peak at *m/e* 423. The ^1H n.m.r. spectrum showed a singlet at δ 2.07 (3 H, acetate Me) and two singlets at δ 4.73 (1 H) and 4.78 (1 H) indicative of a 1,1-disubstituted ethylene group. The high-resolution mass spectrum established the formula $C_{32}H_{50}O_4$ for (3). The u.v. spectrum showed an absorption at 248 nm (ε 18 200) suggestive of a heteroannular diene. The i.r. spectrum contained a band attributable to a 1,1-disubstituted ethylene (880 cm^{-1}), an ester band (1 712 cm^{-1}), and a hydroxyl-band (3 460 cm^{-1}).

The ^{13}C n.m.r. spectrum (Table) of (3) exhibits a total of 32 peaks, in agreement with the molecular formula. Hydrolysis of (3) results in a compound (4) containing

30 carbon peaks, whereas acetylation of (3) results in a compound (5) containing two additional acetate groups. Mild oxidation of (3) produces a diketone (6). Thus, from these observations, it is clear that compound (3) contains an acetate group and two secondary hydroxy-groups.

In addition to the peaks for the acetate group, examination of the ^{13}C n.m.r. spectrum of (3) (Table) reveals 6 olefinic carbons and 24 aliphatic carbons. The six olefinic carbons were classified further on the basis of the SFORD spectrum as one methylene, one methine, and

^{13}C N.m.r. chemical shifts [a]

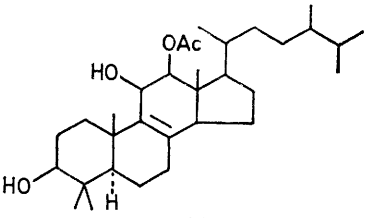
	(3)	(4)	(5)	(6)	(7)
C-1	34.36	34.47	34.16	34.16	34.40
C-2	27.38	27.56	23.64	34.16	27.62
C-3	79.16 [b]	78.60	79.83	216.41	78.07
C-4	39.04	39.08	37.78	47.05	39.04
C-5	50.15	50.19	50.34	50.98	50.43
C-6	18.07	18.06	17.68	18.59	18.22
C-7	26.94	26.99	27.03	27.85	27.90
C-8	125.85	125.25	128.91	142.12	131.00
C-9	138.39	139.00	134.24	137.09	134.79
C-10	37.17	37.19	36.71	36.58	37.01
C-11	68.55	71.70	68.84	194.87	69.68
C-12	79.02 [b]	76.18	73.58	80.18	80.73
C-13	46.45	48.87	46.51	50.08	42.33
C-14	146.69	146.24	145.82	145.08	45.88
C-15	128.82	122.81	122.37	127.39	22.92
C-16	35.17	35.43	35.17	35.54	28.54
C-17	48.97	48.27	48.79	46.75	47.23
C-18	16.68	16.43	16.33	19.74	10.92
C-19	22.05	22.37	21.47	20.83	20.32
C-20	33.25	33.71	32.99	33.72	35.00
C-21	18.07	18.06	18.17	18.04	19.69
C-22	33.25	33.71	32.99	33.18	33.43
C-23	30.89	30.91	30.67	30.83	29.72
C-24	156.38	156.40	155.95	155.93	38.20
C-25	33.81	33.71	33.53	34.15	32.82
C-26	21.85	21.83	21.57	21.86	16.87
C-27	21.99	21.92	21.66	21.86	19.39
C-28	105.99	105.90	105.86	106.08	14.66
C-29	15.51	15.59	16.14	15.32	14.83
C-30	28.19	28.27	27.85	26.88	27.44
Me*C*O	170.73		168.59, 168.68, 170.32	169.07	170.94
*Me*CO	21.26		21.32, 21.17, 20.98	21.07	19.69

[a] In p.p.m. downfield from $SiMe_4$. [b] Assignments may be reversed.

four quaternary carbons. Chemical-shift considerations [10] and the SFORD results confirm that the carbons attached to the two hydroxy-groups and the acetate group are methine carbons. Classification of the remaining aliphatic carbons on the basis of the SFORD spectrum shows (3) to contain three quaternary carbons, four methine carbons, seven methylene carbons, and seven methyl carbons.

The presence of additional methyl carbons in (3) compared to the number in cholesterol suggests the possibility of a lanosterol-type structure for (3). Comparison of the spectrum of (3) with that of (5) shows several peaks that undergo shifts identical to those reported for lanosterol and its acetate [11] [*e.g.* CH_2 at δ 27.38 in (3) to δ 23.64 in (5) and C at δ 39.04 in (3) to δ 37.78 in (5)]. Similar comparisons between (3) and (6) are observed for lanosterol and its ketone derivative.[11] These data suggest that the structure of ring A in (3) is identical to that of lanosterol.

Further comparison of the ^{13}C n.m.r. spectrum of (3) with those reported for 5α-cholestane [12] and 5α-androstane [13,14] derivatives suggests the following concerning the spectrum of (3): (*a*) the quaternary carbon peak due to C-14 (δ 56.7 in cholestane) [12] is absent or shifted in (3); (*b*) the methine carbon peaks due to C-17, C-9, and C-8 (δ 56.4, 54.9, and 33.6, respectively in cholestane) [12] are absent or shifted in (3); (*c*) the methylene carbon peak due to C-12 (δ 40.2 in cholestane) [12] is absent or shifted in (3); and (*d*) the methyl carbon peaks due to C-18 and C-19 (δ 12.1 and 12.3, respectively, in cholestane) [12] are shifted considerably downfield in (3).



(7)

The above observations suggest that two of the double bonds involve C-8, C-9, and C-14. The SFORD results suggest the possibility of either $\Delta^{8,9;14,15}$ or $\Delta^{8,14;9,11}$ for the location of two of the double bonds in (3). The former possibility is the only one consistent with the ^{13}C data on (4), (5), (6), and the partially reduced derivative, (7) (see later discussion), and the u.v. data on the derivative (6). The remaining double bond is located at C-24 and C-28 as indicated by the ^{13}C chemical shifts of the derivatives, the mass-spectral fragmentation, and the results of the spectra of (3) with added lanthanide shift reagent.

The locations of the acetate group and the hydroxy-groups were confirmed by comparison of the ^{13}C spectra of (3), (4), and (5). Removal of the acetate group in (3) to form (4) results in downfield shifts of the peaks in (3) at δ 120.82 (CH, to δ 122.81 p.p.m.) and 46.45 (C, to δ 48.87). Smaller changes are observed for other peaks (Table). Assuming the peak at δ 46.45 to be due to C-13, the shifts suggest that the acetate group is located at C-12. Furthermore, comparison with the shifts reported for other hydroxy-steroids and their acetates suggests that the configuration is β at C-12.[15,16]

In addition to the changes observed in the chemical shifts of ring A (lanosterol *vs.* lanosterol acetate), formation of the acetate (5) from (3) results in an upfield shift for C-12 (5.5 p.p.m.) and C-9 (4.2 p.p.m.) and a downfield shift for C-8 (3.0 p.p.m.). These shifts suggest that the hydroxy-group is located on C-11.[15,16] The upfield shift for C-9 and downfield shift for C-8 are similar to the shifts observed for the allyl alcohol moiety of other sterols.[17,18] The C-11 hydroxy-group was confirmed by formation of the diketone (6) from (3). In addition to the shifts expected for the change from an allyl alcohol to an α,β-unsaturated ketone (shifts of

C-8 and C-9), significant shifts were also observed for C-12, C-13, C-18, and C-19. The bathochromic shift of $\lambda_{max.}$ in the u.v. spectrum from 248 nm in (3) to 299 nm in (6) also indicates that one of the free hydroxy-groups is *in an* allylic position to the diene system. The appearance of a band at 1 675 cm^{-1} in the i.r. spectrum of (6) is also consistent with a ketone in conjugation with a double bond.

Based on the ^{13}C shifts observed for 11α- and 11β-androstanol and their acetates,[15] the magnitude of the shift change for C-12 on formation of (5) from (3) would suggest that the configuration at C-11 is α. Thus, the structure of (3) has been established by chemical and spectroscopic methods as 12β-acetoxy-4,4-dimethyl-24-methylene-5α-cholesta-8,14-diene-3β,11α-diol. A large number of hydroxylated tetracyclic triterpenes have been isolated previously from fungal sources.[19] In the biosynthetic route for the conversion of lanosterol into cholesterol, dealkylation of lanosterol to 4,4-dimethylcholesta-8,14-diene-3β-ol is established.[20] *C*-Alkylation at the C-30 (lanosterol) level is well documented.[21] The 24-methylenedihydrolanosterol and possibly 14-demethyl-24-methylenelanosterol were isolated from *Phycomyces blakesleeanus* and *Aspergillus fumigatus*.[22] Most likely, lanosterol was methylated by *Fusarium sporotrichioides* 921, then demethylated at C-14 and this intermediate was hydroxylated at the C-11α and C-12β positions. Hydroxylation of the steroid ring by *Fusarium* is well established. Hydroxylation of digitoxigenin at the 11α position [23] and at the 12β position [24] by *Fusarium* species has been reported. Partial acetylation performed by *F. sporotrichioides* has been shown in the production of trichothecenes.[25] It is difficult to comment as to why the sterically hindered hydroxy-group at C-12 was acetylated, leaving the less-hindered hydroxys at C-3 and C-11 unacetylated. In general, the composition of sterol esters of the fungi has received little attention in the literature. The structure of the additional steroid in the ethanol extract of *F. sporotrichioides* 921 is as yet unidentified. This compound may be an artifact since it was not always detected. The ^1H n.m.r. spectrum of this steroid does not have any signals lower than δ 6.6.[26] On the basis of the available data, we feel that this steroid could not have the structure of sporofusarin (1) proposed by Olifson.[3] Therefore, the occurrence of (1) in the extract [3,27] must be considered doubtful. All the steroids isolated from this strain were non-toxic in a rabbit skin test.[2] T-2 toxin isolated from the same extract was primarily responsible for the rabbit skin irritation [28] and accordingly for ATA in man.

In summary, a very careful analysis of an extract from a toxic, authentic strain of *F. sporotrichioides* 921 involved in ATA revealed no steroid corresponding to the structure of sporofusarin (1). Instead a new steroid, which could be formed by hydroxylation of a biogenetic intermediate from lanosterol to ergosterol, was isolated and its structure proved by physical methods.

EXPERIMENTAL

All reactions were conducted under nitrogen. Light petroleum refers to the fractions having b.p. 60—80 °C. T.l.c. was performed on Kieselgel GF_{254}. M.p.s were determined with a Kofler hot-stage apparatus. I.r. spectra of crystalline compounds were recorded with a Perkin-Elmer 621 spectrophotometer in KBr discs; u.v. spectra were measured in ethanol with a Cary 17 spectrophotometer. Optical rotations were measured in ethanol on a Perkin-Elmer 141 polarimeter. Mass spectra were determined on a VG Micromass ZAB-2F mass spectrometer. ^1H N.m.r. spectra were recorded on a Varian T-60 or on a Varian XL-100-12 spectrometer. Natural abundance, proton-decoupled ^{13}C n.m.r. spectra were obtained at 25.2 MHz on a Varian Associates XL-100-12 spectrometer equipped with the Varian 620-L disc data system. Typical operating conditions were as follows: pulse angle 30°; data points 8 000; pulse delay 1.0 s; acquisition time 0.8 s, corresponding to a sweeep width of 5 000 Hz; square-wave, modulated proton-decoupling 100 Hz; and exponential broadening of −0.5. Samples were prepared to a concentration of 25 mg per 0.5 ml in [^2H]chloroform in 5-mm sample tubes with a small amount of tetramethylsilane added as an internal reference. The deuterium signal from the solvent served as the internal lock. Single-frequency off-resonance, proton-decoupled (SFORD) spectra were obtained by off-setting the decoupler frequency 500 Hz from $SiMe_4$ to aid in the assignment of the chemical shifts. A total of four spectra of (3) with successive addition of the lanthanide shift reagent $Eu(fod)_3$ were obtained to aid in the assignment of the chemical shifts.

Assignment of the Chemical Shifts.—The ^{13}C chemical shifts for (3)—(7) (Table) are based on comparisons with the spectra of structurally related compounds, SFORD spectra, known hydroxy and acetate substituent effects on ^{13}C n.m.r. chemical shifts of steroids, and from the results of lanthanide-induced shift (LIS) experiments. Carbons 1—7, 10, 29, and 30 of (3)—(7) were assigned by analogy with lanosterol, its acetate and ketone, and dihydrolanosterol.[11] The side-chain carbons (C-20—28) were assigned from comparison of the shifts for (3)—(7), from the results of LIS experiments on (3) (the side-chain carbons shift very little with added lanthanide shift reagent), from the SFORD results, and from comparison with the shifts for the side-chain carbons of ergostanol.[14] The assignments for C-26 and C-27 are based on those of cholesterol, and must be regarded as tentative. The chemical shifts of C-8, -9, -14—16 are based on analogy with those of 5α-cholesta-8,14-dien-3β-ol [17,18] and from shifts induced by the various substituents at C-11. For the tetrahydro-derivative (7), comparison with the chemical shifts of 5α-cholest-8-en-3β-ol was used to assign the shifts of C-8,9,14—16. The chemical shift of C-17 was assigned as the only methine carbon (other than C-5) in the region characteristic of this chemical shift. Finally, the shifts of C-11 and C-12 were assigned on the basis of comparison with C-11 and C-12 of 11α- and 12β-androstanol [15] and from shifts induced in the derivatives (4)—(7).

Isolation of Constituents.—The toxic *F. sporotrichioides* 921 was inoculated and cultured on 1 kg of wheat divided among 10 1-l flasks at 12 °C for 21 days. The extraction of the metabolites from the grain was done by blender maceration of the grains in ethyl alcohol. The ethanolic extracts were concentrated by evaporation under reduced pressure. The residue was diluted with water and extracted with light

petroleum, diethyl ether, ethyl acetate, chloroform, and methylene chloride. The light petroleum extracts were combined with the diethyl ether extracts and treated with diazomethane, in order to convert the fatty acids into their methyl esters. After 2 h the mixture was concentrated *in vacuo* (9.8 g) and the residue diluted with light petroleum and kept at 4 °C for 16 h, to give white needles, m.p. 151 °C (2.3 g), which were identified as T-2 toxin.[7] The mother-liquors were subjected to chromatography on Florisil 100—200 (1.5 kg). Graded elution was effected with light petroleum followed by light petroleum–ethyl acetate mixtures. A total of 110 fractions of 250 ml each were collected and mixed on the basis of t.l.c. Fractions 26—38 (30% ethyl acetate in light petroleum) were combined (0.140 g) and crystallized from acetone–hexane to afford 12β-acetoxy-4,4-dimethyl-24-methylene-5α-cholesta-8,14-diene-3β,11α-diol (3) as needles, m.p. 180—181 °C, (Found: M^+ — AcOH, 438.347 2. $C_{30}H_{46}O_2$ requires M — AcOH, 438.349 7); $[\alpha]^{25}$ +32.7° (*c* 0.44); $\lambda_{max.}$ 248 (ε 18 200); $\nu_{max.}$ 3 450, 1 712, 1 260, 1 060, 880 cm^{-1}; δ 2.07 (s, 12-OAc), 3.33 (m, H-3), 4.3 (s, H-11), 4.75 (2 H, d, *J* 5 Hz, H-28), 5.03 (s, H-12), and 5.67 (s, H-15); *m/e* 498 (M^+), 438 (M — 60), and 423 (M — 60 —15) (base peak).

4,4-*Dimethyl*-24-*methylene*-5α-*cholesta*-8,14-*diene*-3β,11α,-12β-*triol* (4).—The monoacetate (3) (30 mg) in methanol (9 ml) was added to a stirred solution (3 ml) of 2N NaOH, and the mixture stirred for 16 h. The solvent was then removed giving the free alcohol (4) as an oil (22 mg), m.p. 158—159 °C (from acetone–light petroleum) (Found: M^+, 456.359 6. $C_{30}H_{48}O_3$ requires M, 456.360 3); $[\alpha]_D^{25}$ −10.4 (*c* 0.29); $\lambda_{max.}$ 249 (ε 22 200); $\nu_{max.}$ 3 400, 1 005, and 875 cm^{-1}; δ 3.23 (m, H-3), 3.75 (s, H-12), 4.37 (s, H-11), 4.67 (2 H, s, H-28), and 5.63 (s, H-15).

3β,11α,12β-*Triacetoxy*-4,4-*dimethyl*-24-*methylene*-5α-*cholesta*-8,14-*diene* (5).—Acetylation of (3) (20 mg) with pyridine–acetic anhydride and 4-dimethylaminopyridine for 12 h gave 3β,11α,12β-triacetoxy-4,4-dimethyl-24-methylene-5α-cholesta-8,14-diene (5) (24 mg) as an oil. Attempts to recrystallize this compound failed. Precipitation by the addition of water to a methanol solution of (5) resulted in a white powder, m.p. 63—68 °C (Found: M^+ — AcOH, 522.371 9. $C_{34}H_{50}O_4$ requires M — AcOH, 522.370 9); $\nu_{max.}$ 1 730, 1 230, 1 015, and 880 cm^{-1}; δ 2.05 (9 H, s, OAc), 4.67 (2 H, d, *J* 7 Hz, H-28), 5.06 (s, H-12), 5.44 (s, H-11), and 5.68 (s, H-15).

12β-*Acetoxy*-4,4,24-*trimethyl*-5α-*cholest*-8-*ene*-3β,11α-*diol* (7).—A solution of the monoacetate (3) (25 mg) was hydrogenated in ethyl alcohol (25 ml) with 10% palladium–charcoal (10 mg) under pressure of 60 lb in^{-2} for 16 h at 22 °C; isolation in the usual way gave the product (7) (20 mg) as an oil. Two recrystallizations from ethyl acetate–light petroleum yielded crystals, m.p. 199 °C (Found: M^+ — AcOH, 442.383 7, $C_{30}H_{50}O_2$ requires M — AcOH, 442.381 0); $\nu_{max.}$ (neat) 3 450, 1 710, 1 240, and 1 020 cm^{-1}; δ 2.07 (s, OAc), 3.28 (m, H-3), 4.17 (s, H-11), and 4.92 (s, H-12).

12β-*Acetoxy*-4,4-*dimethyl*-24-*methylene*-5α-*cholesta*-8,14-*diene*-3,11-*dione* (6).—To a stirred solution of pyridinium dichromate (123 mg) and sodium acetate (3 mg) in dry methylene chloride (5 ml) was added (3) (40 mg) in methylene chloride (5 ml) under nitrogen. After 3 h, ether (200 ml) was added, the resulting mixture was filtered, and the residue washed with ether (100 ml). The combined organic layers were passed through a pad of Florisil and evaporated to furnish (6) (28 mg) as an oil (Found: M^+, 494.336 9. $C_{32}H_{46}O_4$ requires M, 494.339 5). $\lambda_{max.}$ 292 (ε 12 700); $\nu_{max.}$ (neat) 1 755, 1 705, 1 675, 1 220, 1 030, and 885 cm^{-1}; δ 2.05 (s, 12-OAc), 4.68 (2 H, s, H-28), 4.95 (s, 12-H), and 5.95 (s, 15-H).

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