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CYTOCHALASINS AND PHYTOTOXINS FROM THE FUNGUS *XYLARIA OBOVATA*

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Key Word Index—*Xylaria obovata*; Ascomycotina; cytochalasins; xylobovatin; clonostachydiol; xylobovide; phaseolinon; phytotoxin.

Abstract—The new metabolites xylobovatin, 19,20-epoxycytochalasin C, deacetyl-19,20-epoxy-cytochalasin C, xylobovide and 4,6,10,12-tridecatetraene-2,8-diol, and the known clonostachydiol and phaseolinon were isolated from the culture broth of the Ethiopian fungus *Xylaria obovata*. While the cytochalasins are cytotoxins, clonostachydiol is an anthelmintic, and phaseolinon and xylobovide show phytotoxic effects. The formation of these metabolites was studied and the more oxidized cytochalasins were found to be formed late in the fermentation process on moist sterile rice. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Species of the genus *Xylaria* are widespread from the temperate to the tropical zones of the Earth. Stromata of *Xylaria* spp. are common on stumps and fallen branches of decidous trees. Most species are active wood-rotting fungi inducing decay of the white-rot type. *X. obovata* is a wood-inhabiting tropical fungus. Specimens were collected from the Munesa forest in southern Ethiopia and slants are maintained as strain ADA 228. Extracts of this fungus were tested in a screen for biologically active compounds. Dagne *et al.* [1] reported recently on the isolation of the new deacetyl 19,20-epoxycytochalasin Q (**1**) and 19,20-epoxy-cytochalasin Q (**2**) from *X. obovata* ADA-228, and identified them as the main cytotoxic compounds of the extract. A reinvestigation of this strain led to the isolation and identification of some further compounds, three of them being hitherto unknown cytochalasins. These compounds and some other metabolites are discussed here in detail.

RESULTS AND DISCUSSION

In addition to cytochalasins **1** and **2**, their probably precursor, cytochalasin Q (**4**), was isolated from the culture broth of *X. obovata* and identified by NMR. A fourth cytochalasin (**3**) was found, displaying resonances in the NMR which are very similar to those of cytochalasin **2**. The main differences between these two compounds was in an additional 3-proton doublet of a secondary methyl group in **3**, while one of the methyl singlets of **2** was missing. Two-dimensional (2D) $^1H\{^1H\}$ NMR studies revealed that this additional methyl doublet was isolated at C-18, while the substitutions at the five- and six-membered rings were unchanged. The ^{13}C NMR spectrum corroborated the finding that the hydroxyl group at C-18 was absent (Table 1). The H-18, H-19 coupling constant of 9 Hz requires a diaxial conformation of these two hydrogen atoms (Table 2). Model studies with computer software minimizing the interaction within the molecule [2] showed that such a conformation was only possible with an 18-methyl group in the β-position. Taking all of this information together, compound **3** was identified as 18-desoxy-19,20-epoxycytochalasin Q, and named xylobovatin, which is a new compound not previously mentioned in the literature. The lack of an oxygen atom at C-18 is rather unusual in cytochalasins, and until now had been only observed in zygosporin E [3].

Two additional cytochalasins were isolated in much lower concentrations. They differed from **1**–**4** by the presence of resonances in the ^1H NMR spectra of two allylic methyl groups. Because the resonances of the 11- and 12-methyl groups at δ_H 0.8 and 1.2 were missing, a double bond in the cyclohexyl ring was assumed.

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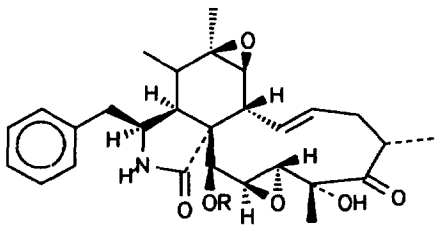

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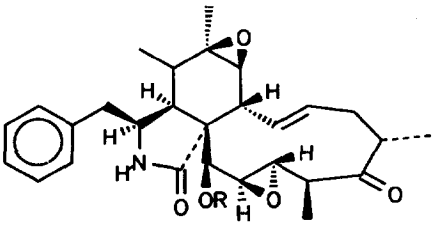

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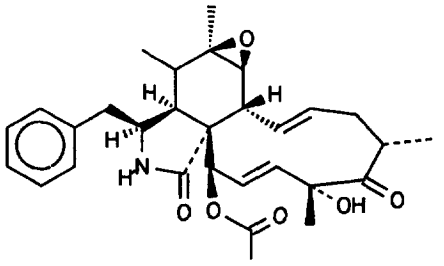

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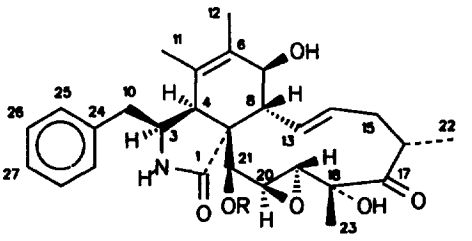

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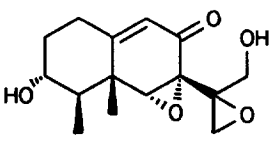

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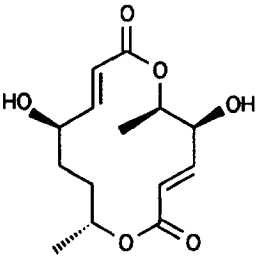
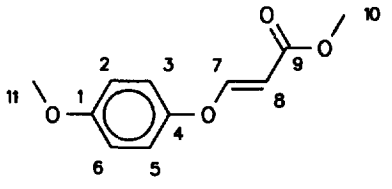


8 Clonostachydiol



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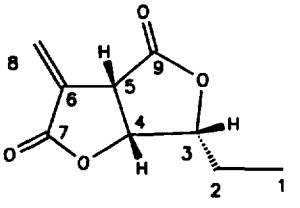
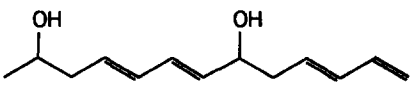


10 Xylobovide



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Analysis of the 2D NMR spectra of these metabolites identified these two compounds as deacetyl 19,20-epoxy-cytochalasin C (**5**) and 19,20-epoxy-cytochalasin C (**6**). They were always formed in low concentrations and at the end of the fermentation period. Both compounds are reported here for the first time.

Table 1. ^{13}C NMR of components **3**, **9** and **10** (100 MHz, $CDCl_3$)

C	3	9	10
1	170.0 0	156.9 *s*	9.7 *q*
2	–	115.0 *d*	22.1 *t*
3	54.5 +	119.4 *d*	77.1 *d*
4	50.9 +	149.6 *s*	84.3 *d*
5	36.7 +	119.4 *d*	46.3 *d*
6	57.2 0	115.0 *d*	129.9 *s*
7	62.5 +	160.5 *d*	167.7 *s*
8	44.5 +	110.9 *d*	127.3 *t*
9	54.1 0	167.8 *s*	172.4 *s*
10	45.8 –	51.3 *q*	
11	12.7 +	55.7 *q*	
12	19.7 +		
13	131.4 +		
14	130.8 +		
15	37.5 –		
16	43.6 +		
17	215.9 0		
18	50.6 +		
19	58.6 +		
20	57.4 +		
21	72.8 0		
22	18.6 +		
23	14.8 +		
24	136.9 0		
25	129.2 +		
26	129.0 +		
27	127.2 +		
COMe	174.7 0		
COMe	20.6 +		

A UV-active compound was isolated in small amounts. The IR spectrum revealed absorptions of an unsaturated ester group. In the ^1H NMR spectrum this metabolite displayed two sets of *trans* configurated double bonds adjacent to ester carbonyl groups. The ^1H NMR 2D spectra helped to identify the compound as the dilactone **8**. This compound was described only very recently as an anthelmintic compound from *Clonostachys cylindrospora*, a fungus belonging to the order Moniliales, and named clonostachydiol [4]. The absolute stereochemistry of clonostachydiol shown in our article was clarified by synthesis [5]. It is worth remembering that *X. obovata* belongs to the Ascomycotina, section Xylariales, so clonostachydiol is now also known from a rather unrelated species.

The least polar metabolite produced by *X. obovata* displayed a strong absorption in the UV and only a few ^1H NMR resonances. Two methoxy group resonances were observed at δ_H 3.72 and 3.81 and two doublets at δ_H 6.99 and 7.74 with a coupling constant of 12 Hz, as well as an AA'XX' spin system of a 1,4-disubstituted aromatic ring. The ^1H NMR data, together with the molecular formula $C_{11}H_{12}O_4$, determined by high resolution (HR)-MS led to the identification of this metabolite from the literature as the (*E*)-methyl 3-(4-methoxyphenoxy)propenoate (**9**). It was first described from the culture broth of *Poronia punctata* [6], which belongs to a genus that is closely related to the genus *Xylaria*.

In minor amounts, another lactone, named xylobovide (**10**), was isolated, as revealed by its IR absorption at 1770 and 1768 cm^{-1}. The ^1H NMR spectrum showed resonances of an exomethylene group in conjugation with a carbonyl and two protons at low fields at δ_H 4.55 and 5.14. $2D^1H\{^1H\}$ NMR served to assign all protons leading, together with the IR data, to a bis-γ-lactone. All of the NMR data were very similar to those for sporothriolide [7] (isolated from a *Sporothrix* sp.) which possesses a hexyl side-chain instead of the ethyl side-chain of xylobovide. The reversal of the substituents at C-4 and C-5 in xylobovide, leading to ethiosolide [8], is excluded because the resonance of H-5 would shift from δ_H 5.14 to $\approx$3.5. Xylobovide is opened by methanol under mildly acidic conditions to the methyl ester of the saturated acid. Its fragmentation pattern is shown in Fig. 1. Xylobovide is a phytotoxin inhibiting the germination of *Eragrostis tef* seeds at 50–100 $\mu g\ ml^{-1}$.

Two sterols belonging to the ergosterane group were also detected. One of these was ergosterol, while the other proved to be more polar. Extensive ^1H and ^{13}C NMR studies including various 2D techniques led to the identification of this sterol as cerevisterol [9]. The assignment of almost all protons in the ^1H NMR spectrum results in a reassignment of the ^{13}C NMR data using long-range $^{13}C\{^1H\}$ NMR spectra [10] (Table 3). The HMBC [11] spectra were optimized for $^2J_{(C,H)}$ and $^3J_{(C,H)}$ couplings [12]. Interestingly, we also observed two $^4J_{(C,H)}$ couplings between C-23 and H-26 and C-23 and H-27 which require dihedral angles near 180° (W-coupling) according to common theories. Cerevisterol has been reported from various marine invertebrates and higher fungi, e.g. *Lactarius hysginus* [13], *Cryptoporus volvatus* [14] and *Fusarium oxysporum* [15].

Finally, a bisdienediol was found in very low yield. Extensive ^1H NMR studies in $CDCl_3$ and C_6D_6 were performed to assign the overlapping olefinic protons. The resulting assignments led to the structure of (*all-E*)-trideca-4,6,10,12-tetraene-2,8-diol (**11**). Due to the scarcity of the material, the absolute configuration could not be assigned. This novel metabolite, which displayed no antibiotic or phytotoxic activity in our assays, could be a precursor of clonostachydiol (**8**). *Xylaria obovata* ADA-228 proved to be a rich source of various metabolites of different biosynthetic origins. Besides cytotoxic and antibiotic compounds, such as the cytochalasins, phytotoxins were also found. The ecological role of the phytotoxins remains an open question, because species of the genus *Xylaria* grow on dead wood with no obvious requirement for phytotoxins. Of interest is the observation of the anthelmintic clonostachydiol. Its role might be in the protection of the fruit bodies against invasive insects

Table 2. ^{1}H NMR compounds of **3**, **5**, **6**, **10** and **11** (400 MHz; **3**–**10** in $CDCl_3$, **11** in C_6H_6)*

H	3	5	6	10	11
1	—	—	—	1.05 *t* (7.5)	1.11 *d* (6)
2	—	—	—	1.85 *qtd* (7.5, 7.5, 4.5)	3.66 *tq* (6, 6)
3	3.60 *ddd* (7, 7, 2.5)	3.35 *m*	3.35 *ddt* (8, 6, 1)	4.55 *td* (7.5, 4.5)	2.14 dd (7.5, 6)
4	2.12 *dd* (5.7, 2.5)	2.84 *s*	2.54 *s*	5.14 *dd* (7, 4.5)	5.65 *dt* (15, 7.5)
5	1.61 *qd* (7.2, 5.7)	—	4.01 *dt* (7, 2)	—	6.10 *dd* (15, 10.5)
6	—	—	—	—	6.27 *dd* (10, 10.5)
7	2.74 (5.5)	3.75 *d* (10)	3.81 *d* (10)	—	5.61 *dd* (15, 6)
8	2.38 (10, 5.5)	2.40 *t* (10)	2.26 *t* (10)	6.40 *d* (2)	4.08 *q* (6)
8′-	—	—	—	6.10 *d* (2)	—
9	—	—	—	—	2.40 *dd* (7.5, 6)
10	2.83 *m*	2.96 *dd* (13, 6)	3.08 *dd* (13, 6)	—	5.75 *dt* (5.2, 7.5)
	—	2.88 *dd* (1, 8)	3.01 *dd* (13, 8)	—	—
11	0.79 *d* (7.2)	1.42 *s*, 1.43 *s*	—	—	6.16 *dd* (15.2, 10.2)
12	1.20 *s*	1.66 *s*, 1.67 *s*	—	—	6.38 *dt* (16.8, 10.2)
13	6.27 *dd* (15, 10, 1)	5.98 *dd* (15.5, 10)	6.16 *dd* (16, 10)	—	5.17 *d* (16.8)
13′-	—	—	—	—	5.04 *d* (10.2)
14	5.57 *ddd* (15, 10.5, 5)	5.65 *ddd* (15.5, 10, 6)	5.72 *ddd* (16, 10, 6)	—	—
15	2.50 *td* (13, 10.5)	2.09 *ddd* (12, 6, 2, 1)	2.12 *dddd* (12, 6, 2, 1)	—	—
15′-	2.17 *dddd* (13, 5, 3.5, 1)	2.60 *td* (12, 10)	2.68 *td* (12,10)	—	—
16	2.97 *dqd* (13, 6.7, 3.5)	3.21 *dqd* (12, 7, 2)	3.24 *dqd* (12, 7, 2)	—	—
17	—	—	—	—	—
18	2.26 *dq* (9, 7)	—	—	—	—
19	2.94 *dd* (9, 2)	3.47 *d* (2)	3.21 *d* (2)	—	—
20	3.55 *dd* (2, 1)	3.31 *m*	3.47 *dd* (2, 1)	—	—
21-	5.55 *s*	4.13 *s*	—	5.76 *s*	—
22-	1.14 *d* (6.7)	1.19 *d* (7)	1.22 *d* (7)	—	—
23	1.30 *d* (7)	1.56 *s*	—	1.55 *s*	—
24	—	—	—	—	—
25-	7.20 *m*	7.20 *m*	—	7.20 *m*	—
26	7.33 *m*	7.32 *m*	—	7.33 *m*	—
27	7.25 *m*	7.26 *m*	—	7.25 *m*	—
Ac	2.12 *s*	—	—	2.16 *s*	—

* *J* (MHz) in parentheses.

but, since the specificity of this toxin against the predators of this fungus was not tested, no precise conclusions could be drawn. The finding of (*E*)-methyl-3-(4-methoxyphenoxy)propenoate (**9**), first described from *Poronia punctata*, corroborates the taxonomic proximity between these two genera of Ascomycotina.

EXPERIMENTAL

General. ^{1}H and ^{13}C NMR: 400 and 75.5 MHz, respectively, $CDCl_3$, with TMS as int. standard. MS: 70 eV. IR: KBr. UV; MeOH. Optical rotations: $CHCl_3$, if not stated otherwise. EI^+-, $FAB^\pm$- and MS-MS: four-sector tandem mass spectrometer at 10 kV accelerating voltage. Resolution of both MS was set to 1:1000, except for HR measurements, where the first MS was set to a resolution of 1:10000. In EI^+ mode (70 eV), the sample was introduced via a heated direct inlet probe (25–200°, 4° min^{-1}). For $FAB^\pm$ measurements, thioglycerol and 3-NBA served as matrices. The FAB gun was operated at 6 kV with xenon as the FAB gas. Collision-induced dissociation took place in the third field-free region (collision gas He, precursor intensity 30%). MS-MS spectra (linked scans of MS2 at constant B/E ratio) were recorded at 300 Hz filtering.

Xylaria obovata ADA-228 was grown in 3 1-l Erlenmeyer flasks filled with a solid medium consisting of rice (250 g) and 300 ml water, and autoclaved for 20 min at 121°. After 20 or 40 days the mouldy rice was ground in a mortar and pestle and extracted with 1.5 l $CHCl_3$. After drying with Na_2SO_4 the solvent was evaporated and the crude extract separated on a Si-60 column with an *n*-hexane–EtOAc gradient (19:1 to 0:1). When necessary, the collected frs were purified further by prep. TLC.

After 20 days the fermentation of *X. obovata* on 250 g rice yielded (mg): **1**, 11; **2**, 201; **4**, 37; **8**, 5; **9**, 1; **11**, 1.5; cerevisterol, 2; ergosterol, 5. After 40 days the yields were (mg): **1**, 35; **2**, 365; **3**, 6; **5**, 1; **6**, 2; **7**, 2; **8**, 10; **9**, 5; **10**, 32; **11**, 3; cerevisterol, 5; ergosterol, 42; indolyl-3-carboxylic acid methyl ester, 2.

Xylobovatin (**3**). R_f 0.71 (*n*-hexane–EtOAc 1:2). IR λ_{max}: 3430, 1745, 1710, 1685 cm^{-1}. MS *m/z* (rel. int.): 507.2586 (507.2621 calc. f. $C_{30}H_{37}NO_6$) (17), 489.2557 (489.2515 calc. f. $C_{30}H_{35}NO_5$) $[M-H_2O]^+$ (7), 448.2484 (448.2488 calc. f. $C_{28}H_{34}NO_4)^+$ $[M-Ac]^+$ $(6)^+$, 416.2032 (416.2073 calc. f. $C_{23}H_{30}NO_6$) [M – ben-

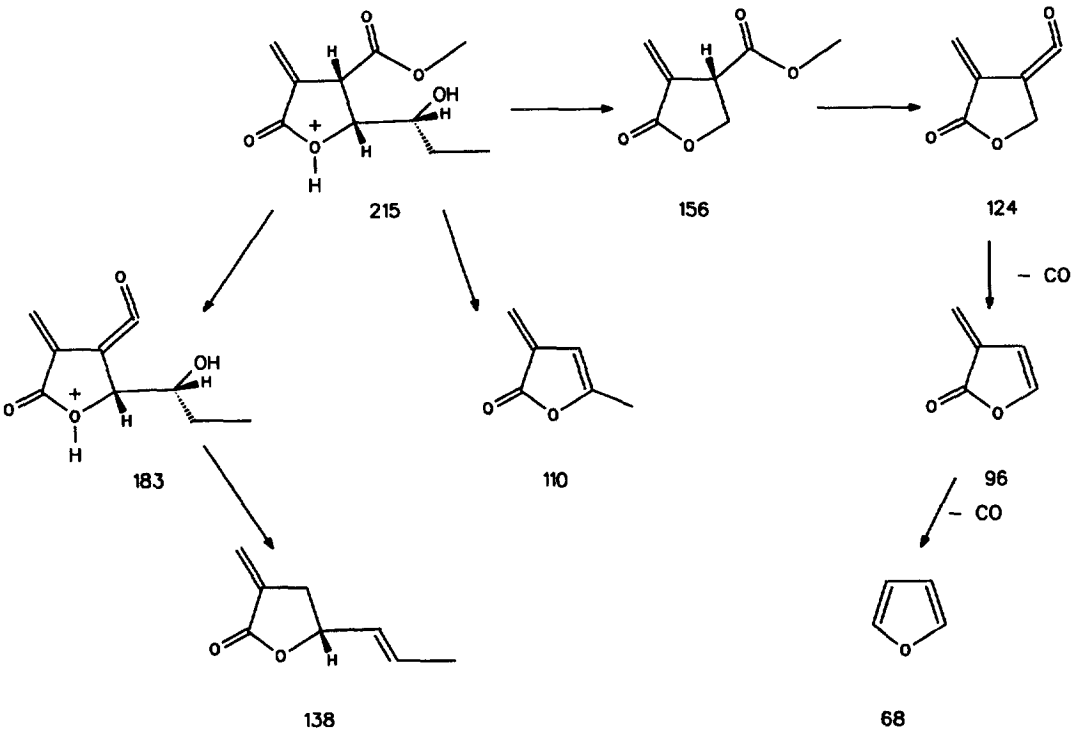

Fig. 1. MS fragmentation of xylobovide methyl ester.

Table 3. ^{13}C and ^{1}H NMR of cerevisterol (600 MHz, $CDCl_3$)

C	^{13}C	^{1}H		Couplings in HMBC
1	33.0	1.64 (α-H)	1.55 (β-H)	1.17
2	30.4	1.79 (α-H)	1.46 (β-H)	1.80, 1.55
3	57.3	4.10 (α-H)	—	2.16, 1.80, 1.46
4	39.2	1.80 (α-H)	2.16 (β-H)	—
5	75.9	—	—	5.38, 2.16, 1.80
6	73.1	3.66 (α-H)	—	—
7	117.4	5.38	—	1.97, 1.93
8	143.5	—	—	1.97, 1.93, 1.59
9	43.2	1.97 (α-H)	—	5.38, 2.08, 1.17
10	37.0	—	—	1.97, 1.80, 1.79, 1.64, 1.17
11	21.9	1.88	1.60	1.97
12	38.9	1.36 (α-H)	2.08 (β-H)	0.59
13	43.6	—	—	1.93, 1.59, 1.36, 0.59
14	54.7	1.93 (α-H)	—	5.38, 2.08
15	22.8	1.59 (α-H)	1.59 (β-H)	—
16	27.9	ND	ND	1.31
17	55.9	1.31 (α-H)	—	5.20, 2.05, 1.06, 0.59
18	12.2	0.59	—	—
19	18.4	1.17	—	—
20	40.6	—	2.05 (β-H)	5.27, 5.20, 1.06
21	21.0	1.06	—	—
22	135.4	5.20	—	2.05, 1.87, 1.06
23	132.0	5.27	—	2.05, 1.87, 1.49, 0.97, 0.875, 0.865
24	42.7	—	1.87 (β-H)	5.27, 5.20, 1.49, 0.97, 0.875, 0.865
25	32.8	1.49	—	5.27, 0.97, 0.875, 0.865
26	19.8	0.875	—	—
27	19.5	0.865	—	—
28	17.5	0.97	—	5.27

ND: not determined.

zyl]$^+$ (100), 356.1854 (356.1862 calc. f. $C_{21}H_{26}NO_4$) [416−HOAc]$^+$ (59), 338.1779 (338.1756 calc. f. $C_{21}H_{24}NO_3$) [356−H_2O]$^+$ (60), 328.1923 (328.1913 calc. f. $C_{20}H_{26}NO_3$) [356−CO]$^+$ (13), 320.1672 (320.1651 calc. f. $C_{21}H_{22}NO_2$) [338−H_2O]$^+$ (9), 310.1770 (310.1807 calc. f. $C_{20}H_{24}NO_2$) [338−CO]$^+$ (18), 1 296.1651 calc. f. $C_{19}H_{22}NO_2$) [310−CH_2]$^+$(9), 91 (21), 43 (12). $[\alpha]^{27}$: 296.1626 (589 nm, +44.5°; 578 nm, +45.4°; 546 nm, +49.6°; 436 nm, +79.6°; 365 nm, +139.6° (c = 1.3, MeOH).

*Deacetyl-*19,20-*epoxy cytochalasin C* (**5**). R_f 0.46 (*n*-hexane–i-PrOH 7:3). IR (λ_{max}): 3423, 3347, 1708, 1684 cm^{-1}. MS *m/z* (rel. int.): 481.2450 (481.2464 calc. f. $C_{28}H_{35}NO_6$) (10), 463 (6), 453 (9), 435 (28), 321 (48), 91 (100). $[\alpha]^{27}$ 589 nm, +11.6°; 578 nm, +14.8° (c = 0.25).

19,20-*Epoxy cytochalasin C* (**6**). R_f 0.52 (*n*-hexane–i-PrOH 7:3). IR λ_{max}: 3438, 1745, 1708, 1685 cm^{-1}. EI$^+$-MS *m/z* (rel. int.): 523.2560 (523.2570 calc. f. $C_{30}H_{37}O_7N$) (15), 480 (10), 477 (13), 463 (10), 435 (14), 432 (13), 420 (22), 405 (20), 372 (34), 362 (31), 334 (13), 316 (9), 312 (15), 252 (40), 238 (22), 187 (16), 185 (17), 174 (24), 169 (16), 163 (9), 162 (16), 161 (19), 160 (20), 133 (18), 120 (43), 98 (9), 91 (61), 43 (100). FAB$^+$-MS *m/z*: 524.2, [M+H]$^+$ 546.2, [M+Na]$^+$. $[\alpha]_D$ −6.8° (c = 0.25).

Xylobovide (**10**). R_f 0.78 (*n*-hexane–EtOAc 1:2). Crystals, mp 106°. IR λ_{max}: 1770, 1768, 1666, 1568 cm^{-1}. Xylobovide is rather sensitive to acid catalysed solvolysis to the saturated ester. MS of the 9-methyl-ester *m/z* (rel. int.): 215.096 (215.0919 calc. f. $C_{10}H_{15}O_5$) [M+H]$^+$] (6), 183.0654 (183.0657 calc. f. $C_9H_{11}O_4$) [M−MeOH]$^+$ (19), 156 [M$^-$−C_3H_7O]$^+$ (100), 138 [M−CO_2CH_3−H_2O]$^+$ (21), 124.0207 [M$^+$−C_3H_7O−MeOH]$^+$ (124.0160 calc. f. $C_6H_4O_3$) (42), 110.0377 (110.0368 calc. f. $C_6H_6O_4$) (14), 96.0244 [124−CO]$^+$ 96.0211 calc. f. $C_5H_4O_2$) (32), 81.0327 [110−CO]$^+$ (81.0340 calc. f. C_5H_5O) (6), 68.0261 (68.0262 calc. f. C_4H_4O) (20), 67.0814 (67.0814 calc. f. C_4H_3O (32). $[\alpha]^{27}$: 589 nm, −87.2°; 578 nm, −90.5°; 5 746 nm, −102.1°; 436 nm, −181.0° (c = 1.00, CH_3OH).

(4,6,10,12)-*Tridecatetraene*-2,8-*diol* (**11**). R_f 0.56 (*n*-hexane–i-PrOH 8:2). IR λ_{max}: 3380 cm^{-1}. $[\alpha]_D$ −1.5° (c = 0.66).

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