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PENTACYCLIC TRITERPENES FROM THE FUNGUS, *LEPTOSPHAERIA MACULANS*

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Key Word Index—*Leptosphaeria maculans*; fungus; pentacyclic triterpenoids; maculaniol; 3α-hydroxy-urs-18,20-dien-28-oic acid.

Abstract—Fermented broth of the fungus, *Leptosphaeria maculans* gave two pentacyclic triterpenoid compounds. Based on spectral analysis these compounds were identified as maculaniol and 3α-hydroxy-urs-18,20-dien-28-oic acid.

INTRODUCTION

Leptosphaeria maculans (Desm.) Ces. et de Not. causes blackleg in rapeseed (*Brassica napus* and *B. campestris*) and other crucifers [1]. It produces a major phytotoxin, sirodesmin PL, and the corresponding deacetyl derivative [2]. In addition, *L. maculans* produces the phytotoxins sirodesmin H and phomalirazine in liquid culture [3, 4]. In the present study, the isolation and characterization of triterpenoid type metabolites from the avirulent-isolate of the fungus is reported.

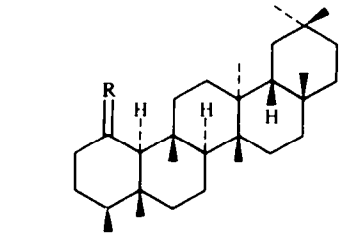
RESULTS AND DISCUSSION

Compound **1**, analysed for $C_{30}H_{52}O$ (HRMS:$[M]^+$ at *m/z* 428.4012, calc. 428.4016). It gave a positive Liebermann–Burchard test and a violet coloration with ceric sulphate, indicative of a triterpenoid. The IR spectrum shows absorption for a hydroxyl (3560 cm^{-1}) group, and the ^1H NMR spectrum shows signals for seven methyl groups as singlets at δ0.76, 0.88, 0.96, 0.98, 1.02, 1.04, 1.18 and a doublet of a secondary methyl group at δ0.89. A one-proton multiplet at δ3.49 was assigned to a carbinylic proton. The ^{13}C NMR spectrum showed the presence of 30 carbon atoms. The multiplicity assignment of each carbon atom was determined by using DEPT experiments [5] with the last polarization pulse $\theta = 45°$, 90° and 135°. It indicated the presence of eight methyl, 11 methylene and five methine carbons.

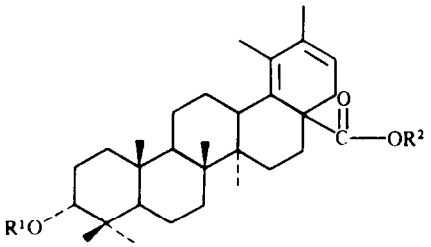
The secondary nature of the alcoholic group in compound **1** was indicated by the formation of an acetate (**1a**) and oxidation to a ketone (**1b**). The latter can be reduced back to the parent alcohol with sodium and isoamyl alcohol, showing the equatorial configuration of the hydroxyl group in **1**. This was also confirmed by the multiplicity of the carbinylic proton in the 2D-*J*-resolved spectrum. It appeared as a sextet owing to two axial–axial ($J_{ax,ax} = 9.4$ Hz) and one equatorial–axial ($J_{eq,ax} = 4.5$ Hz) coupling. All these spectral data indicated characteristic features of pentacyclic triterpenes with the frieds skeleton carrying a hydroxy group in ring A (Fig. 1). The spectral data of compound **1** closely resemble those of 3α-hydroxyfriedelane [6]. The two compounds, however, differ widely in melting points and optical rotation. The ketone (**1b**) is likewise different from friedeline [7]. It does not give the Zimmermann colour reaction, showing the absence of a 3-oxo group. In the ^{13}C NMR spectrum of compound **1**, the chemical shifts of the carbon atoms of rings B–E showed close agreement with those reported earlier [8, 9] but differed slightly in ring A. The ^{13}C chemical shift assignments were confirmed by the hetero-COSY spectrum (Table 1).

The position of the hydroxyl group in ring A was ascertained by selective decoupling experiments. Irradiation at δ0.88 (23-Me) simplified the multiplet at δ1.42 to a double doublet. On the other hand, irradiation at δ1.42 collapsed the doublet of 23-methyl to a singlet and also simplified the multiplet at δ1.38. The multiplets at δ1.42 and 1.38 can, therefore, be assigned to protons joined to C-4 and C-3, respectively. Consequently, the hydroxyl group must be at C-1 or C-2. The formation of a sextet for the carbinylic proton at δ3.48 indicated that the hydroxyl group should be at an α and equatorial position at C-1 because, at C-2, it would be expected to give an octet. This is supported by homonuclear ^1H–^1H chemical shift correlation measurement (COSY 45°) which showed coupling of the carbinylic proton to three protons rather than four. Moreover, in the ^1H NMR spectrum of ketone **1b**, three protons were exchangeable with D_2O which provided conclusive evidence for the presence of the carbonyl group in **1b** and, hence the hydroxyl group in compound **1**, at C-1. The heteronuclear ^1H–^{13}C chemical shift correlation spectrum (hetero COSY) is in agreement with the structure assigned to compound **1**, which I have named maculaniol.

Compound **2** was assigned the molecular formula $C_{32}H_{48}O_4$ (EIMS: $[M]^+$ at *m/z* 496.3581 calc 496.3552). It gave rise to IR bands at 3300–2520 (CO_2H), 2900–2840 (C–H), 1720 (ester-carbonyl), 1700 (carboxyl) and 1640 (C=C) cm^{-1}. The molecular formula suggested nine double bond equivalents, one of which has been accounted for by the carbonyl of the carboxyl group, one by the carbonyl of the ester function, and five for the five rings of the pentacyclic triterpene skeleton, which was indicated by the presence of seven methyl signals in the ^1H NMR



1 R = α-OH, β-H
1a R = β-OAc, β-H
1b R = O



	R^1	R^2
2	Ac	H
2a	Ac	Me

Fig. 1.

spectrum at δ1.62, 1.67, 0.92, 0.89, 0.86, 0.84, 0.72 (3H, *s*, each).

The presence of two vinylinic methyls, only one olefinic proton (δ5.12, 1H, *dd*, $J_{21,22\alpha}$ = 5.08, Hz and $J_{21,22\beta}$ = 6.66 Hz, H-21), and the conjugated nature of the diene as indicated by the UV spectrum (λ_{max} 272 nm) showed that both the double bonds are located in ring E. The relatively lower absorption maximum in the UV spectrum has been attributed to the crowding of the substituents in the cisoid system [10].

The ^{1}H NMR spectrum further showed an acetoxy function (δ2.02) which has been placed at C-3 on biogenetic grounds and which was supported by a one-proton triplet at δ4.86 (*J* = 3.0 Hz) for H-3. The coupling constant showed that H-3 is equatorial and the acetoxy function has the α-disposition. The presence of the carboxyl group, as indicated by the IR spectrum, was supported by the methylation of compound **2** with diazomethane to give the methylester **2a**. Fragments at *m/z* 150.25, 164.08 and 178.09 in the high resolution mass spectrum of compound **2** corresponding to $C_9H_{10}O_2$, $C_{10}H_{12}O_2$ and $C_{11}H_{14}O_2$, respectively, confirmed the position of the carboxyl at C-17. In the light of these observations and the ^{13}C NMR data (Table 2) the structure of compound **2** has been assigned as 3α-acetoxy-4rs-18,20-dien-28-oic acid (maculanoic acid).

This seems to be the first report of the natural occurrence of pentacyclic triterpenoids in a fungus such as *Leptosphaeria maculans*. The role of these compounds in virulence or pathogenicity of *L. maculans* on canola or other crucifer plants remains to be elucidated.

EXPERIMENTAL

Fungal isolate. The fungus, *Leptosphaeria maculans*, used in the present study was isolated from the canola stubbles collected from a field near Leduc, Alberta, Canada.

Table 1. ^{13}C NMR chemical shifts of compounds **1**, **1a** and **1b**

C	1	1a	1b
1	74.6 (41.3)*	74.6	74.5
2	32.1 (41.5)	32.2	32.1
3	16.4 (213.0)	16.3	16.2
4	48.0 (59.5)	48.0	48.0
5	37.8 (42.1)	37.8	37.8
6	41.8 (35.6)	41.6	41.6
7	17.6 (30.5)	17.6	17.6
8	53.2 (53.1)	53.2	53.2
9	37.0 (37.4)	37.0	37.0
10	60.9 (58.2)	60.9	60.9
11	35.4 (32.4)	35.3	35.4
12	30.6 (36.0)	30.6	30.6
13	38.2 (38.3)	38.3	38.2
14	39.6 (39.7)	39.6	39.6
15	32.3 (31.8)	32.3	32.4
16	36.0 (32.8)	36.0	36.0
17	29.8 (30.0)	29.8	29.8
18	42.8 (42.8)	42.8	42.8
19	35.6 (39.2)	35.6	35.6
20	28.2 (28.1)	28.2	28.3
21	32.8 (35.0)	32.8	32.8
22	39.2 (35.3)	39.2	39.3
23	11.2 (6.8)	11.2	11.2
24	15.8 (22.3)	15.7	15.8
25	18.2 (14.6)	18.2	18.2
26	18.6 (17.9)	18.6	18.7
27	20.0 (18.2)	20.0	20.0
28	31.7 (20.3)	31.7	31.8
29	34.9* (32.1)	34.9	34.9
30	32.0* (18.6)	32.0	32.0
CO_2Me		51.5	

*Values are interchangeable.
*Values in parentheses are friedline [8].

Growth conditions. The fungus was cultivated on Pablum (baby food); 40 g l^{-1} supplemented with 1 g l^{-1} of yeast extract and 1 g l^{-1} tomato paste (pH 6.5). The fungus was grown in conical flasks (1 l) containing 100 ml media broth. Flasks were incubated in a New Brunswick Rotary shaker at 200 rpm, temperature 25 ± 2° for 30 days.

Extraction and purification. Fermented broth was (5 l) blended with EtOAc in a Waring blender, filtered, and the EtOAc frs, pooled. The EtOAc extract was concd *in vacuo* yielding 942 mg of a yellow oily residue, which was subjected to CC on silica gel (200 g) using as solvent system $CHCl_3$–EtOAc (19:1). Frs which contained **1** and **2** were combined and evapd to dryness, yielding 216 mg of solids. The solids were subjected to HPLC analysis using a silica ODS column (25 × 1.00 cm i.d. Terochem Scientific, Edmonton, Alberta, Canada) and a solvent mixt. of MeCN–H_2O–H_3PO_4 (1600:400:1) to give 162 mg of a powder. This powder was further purified on the silica ODS column developed with a mixt. of MeCN–H_2O–H_3PO_4 (1700:300:1). H_3PO_4 in the eluate was removed by gel filtration to yield 23 mg **1** and 27 mg **2**.

Spectral analysis. UV: EtOH; IR: $CHCl_3$; MS: VG-analytical spectrometer using direct injection probe; ^{1}H NMR and ^{13}C NMR: 300 and 000 MHz, respectively, with TMS as an int. standard.

Table 2. ^{13}C NMR spectral data for compound **2** (25.2 MHz, $CDCl_3$, TMS as an int. standard)

C	[9]	
1	38.6	38.6
2	23.6	23.6
3	80.8	79.9
4	37.7	37.7
5	55.5	55.5
6	18.0	18.2
7	33.3	33.2
8	40.7	40.6
9	51.0	51.0
10	37.0	36.9
11	21.1	21.1
12	26.0	26.0
13	38.3	38.2
14	43.2	43.2
15	27.4	27.3
16	37.3	37.2
17	34.2	34.2
18	142.5	142.6
19	129.6	129.4
20	32.2	32.4
21	34.5	34.6
22	37.6	37.8
23	27.8	28.0
24	16.6	16.6
25	16.0	16.0
26	16.4	16.6
27	14.4	14.4
28	25.1	25.2
29	31.2	31.2
30	29.1	29.1
OAc	170.7	

Compound **1**. Mp 188–189°, $[\alpha]_D$ +25°; EIMS *m/z* (rel. int.): 428.4012 $[M]^+$, 413 $[M-Me]^+$ (7), 410 $[M-H_2O]^+$ (12), 395 $[M-H_2O-Me]^+$ (18), 341 (15), 304 (20), 275 (32) 207 (65) and 205 (100); ^1H NMR: text; ^{13}C NMR: Table 1.

Acetylation of compound **1**. Compound **1** (20 mg) was refluxed with Ac_2O (10 ml) and pyridine (5 ml) for 3 hr. Usual work-up provided the acetate **1a** (13.6 mg) which was crystallized from aq. Me_2CO, mp 218–220° $[\alpha]_D$: +19° ($CHCl_3$; *c* 0.125); IR ν_{max} cm^{-1}: 1730, 1200 (CO_2Me); EIMS *m/z* (rel. int.): 470 $[M]^+$ (12), 455 $[M-Me]^+$ (6), 410 $[M-HOAc]^+$ (16), 395 $[M-Me-HOAc]^+$ (10), 341 (20), 346 (15), 317 (23), 249 (85), 205 (55), 125 (40); ^1H NMR ($CDCl_3$); δ0.76 (3H, *s*, Me-24), 0.86 (3H, *s*, Me-25), 0.88 (3H, *s*, *J* = 6.7 Hz, Me-23), 0.96 (3H, *s*, Me-29), 0.98 (3H, *s*, Me-26), 1.02 (3H, *s*, Me-30), 1.04 (3H, *s*, Me-27), 1.18 (3H, *s*, Me-28), 2.01 (3H, *s*, OAc), 4.62 (1H, *sex*, $J_{ax,eq}$ = 5.6 Hz, H-1).

Oxidation of compound **1**. A soln of compound **1** (20 mg) and pyridine (3 ml) cooled to 15° was added to CrO_3–pyridine complex [11] prepared from CrO_3 (20 mg) and the mixt. left room temp. for 24 hr, Excess CrO_3 was decomposed by addition of MeOH. The mixt. was then digested with EtOAc and filtered. The filtrate yielded a solid residue which crystallized with Me_2CO (16.2 mg), mp 280°; $[\alpha]_D$ +14° ($CHCl_3$; *c* 0.20). IR ν_{max} cm^{-1}; 1710 (C=O); EIMS *m/z* (rel. int.): 426 $[M]^+$ (6), 411 $[M-Me]^+$ (12), 341 (18), 302 (26), 273 (12), 205 (100), 125 (3); ^1H NMR ($CDCl_3$); δ0.76 (3H, *s*, Me-24), 0.86 (3H, *s*, Me-25), 0.86 (3H, *d*, *J* = 6.2 Hz, Me-23), 0.99 (3H, *s*, Me-29), 0.98 (3H, *s*, Me-26), 1.04 (3H, *s*, Me-27), 1.2 (3H, *s*, Me-30), 1.18 (3H, *s*, Me-28).

Reduction of compound **1b**. Na (80 mg) was slowly added to a refluxing soln of compound **1b** (16 mg) in isoamylalcohol (2 ml) and refluxing continued until tracted with Et_2O. The organic layer was washed with H_2O, dried (Na_2SO_4), and evapd to a gummy solid which crystallized from Me_2O to yield (12 mg) compound **1**, mp 188–190° $[\alpha]_D$ +25° ($CHCl_3$; *c* 0.18).

Compound **2** was obtained as needles (aq. MeOH), mp 184–185°; $[\alpha]_D^{24}$ + 52.6° ($CHCl_3$); EIMS *m/z* (rel. int.): 496.3581 $[M]^+$, 436.3344 $[M-HOAc]^+$ (3), 318 (8), 285 (12), 257 (3), 239 (2), 201 (6), 190 (3), 189 (10), 187 (4), 178 (7), 173 (5), 164 (4), 150 (6), 133 (2), 119 (20), 105 (26), 97 (18), 83 (100) and 69 (38); ^1H NMR: text; ^{13}C NMR: Table 2.

Methylation of compound **2**. To an ethereal soln of **2**, freshly prepared CH_2N_2 was added in excess and the mixt. kept at room temp. overnight. The reaction mixt. afforded **2a** which formed needles on keeping its concd methanolic soln in the cold. Mp 168–170°; $[\alpha]_D^{24}$ − 20.40 ($CHCl_3$); IR ν_{max} $CHCl_3$ cm^{-1}, 2900, 2840, 1720 (*br*), 1645. EIMS *m/z* (rel. int.): 510.3710 $[M]^+$ (2) (required for $C_{33}H_{50}O_4$; 510.3708), 495.3462 $[C_{32}H_{47}O_4]^+$ (30), 480 (16), 450.3481 $[(M-MeCO_2H)^+, 7]$, 285 (38), 201 (28), 175 (16), 161 (14), 133 (26), 122 (42), 119 (16), 105 (46), 81 (50); ^1H NMR (100 MHz; $CDCl_3$); 0.72 (3H, *s*), 0.86 (3H, *s*), 0.86 (3H, *s*), 0.89 (3H, *s*), 0.92 (3H, *s*), 1.60 and 1.67 (2 × Me, *s*, H-29 and H-30), 2.18 (3H, *s*, Ac), 3.72 (3H, *s*, OMe), 5.12 (1H, *dd*, $J_{21,22\alpha}$ = 5.08 Hz and $J_{21,22\beta}$ = 6.66 Hz, H-21), 5.84 (1H, +, *J* = 3.0 Hz, H-3).

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