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Lowdenic Acid: A New Antifungal Polyketide-Derived Metabolite from a New Fungicolous *Verticillium* sp.

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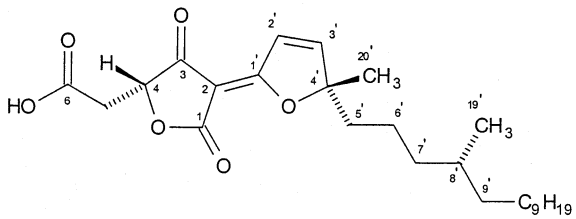
Lowdenic acid (**1**), a new antifungal metabolite with an unusual structure and a mixed biogenetic origin, has been obtained from nonsporulating cultures of a previously undescribed *Verticillium* sp. (MYC-406 = NRRL 29280) that was isolated as a colonist of polypore basidiomata. The gross structure of **1** was proposed by analysis of NMR data and confirmed by X-ray diffraction analysis, which enabled assignment of relative stereochemistry. Compound **1** occurs as an equilibrium *E*/*Z* mixture. The known antifungal metabolite canescin A (**2**) was also isolated.

Several species of the fungal genus *Verticillium* are known to be mycoparasitic, and some of these species have been used or proposed as biocontrol agents.[1−3] In a few cases, chemical studies have revealed that the biocontrol effects of these mycoparasites are due at least in part to the production of antifungal metabolites.[4] During our ongoing chemical investigations of mycoparasitic and fungicolous fungi as potential sources of new antifungal agents,[5,6] an isolate of a new *Verticillium* sp. (mitosporic fungi; MYC-406 = NRRL 29280 = CBS 102427) was obtained from basidiomata of a polypore found growing on the underside of a dead hardwood log at Lowden State Park near Oregon, IL. The EtOAc extract of solid-substrate fermentation cultures of this isolate displayed activity against *Aspergillus flavus*. Bioassay-guided fractionation of the extract by Sephadex LH-20 column chromatography, followed by semipreparative reversed-phase HPLC, afforded a new antifungal compound that we named lowdenic acid (**1**) as an equilibrium mixture of *E*- and *Z*-isomers, along with the known metabolite canescin A (**2**).[7]



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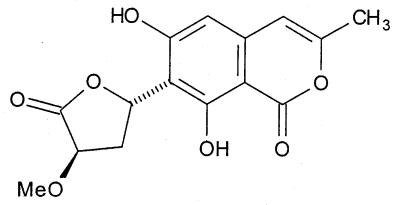

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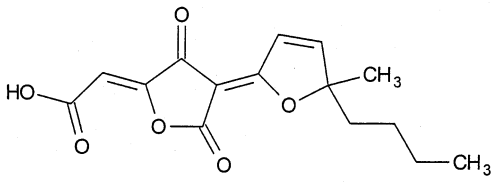

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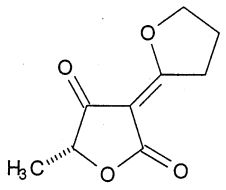

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Lowdenic acid (**1**) was assigned the molecular formula $C_{26}H_{40}O_6$ (seven unsaturations) on the basis of HRFABMS data ($[M + H]^+$ at *m*/*z* 449.2902; $\Delta = 0.1$ mmu). The ^1H and ^{13}C NMR spectra (Table 1) revealed that most of the signals appeared in duplicate in a ratio of approximately 1:1, suggesting the presence of an epimeric/isomeric mixture. HPLC conditions were found that afforded a baseline separation of the two isomers. However, collection of each component, followed by reanalysis, resulted in the appearance of both peaks in each case, indicating that equilibration occurs spontaneously. Therefore, structural analysis was carried out on the equilibrium mixture. The NMR data for the two components were nearly identical, so only the data for one isomer (**1**) are described in detail here. The ^{13}C NMR spectrum showed signals for an α,β-unsaturated ketone carbonyl, a 1,2-disubstituted olefin unit, and three carbons in the carboxy carbonyl region (δ_C 167.6, 173.7, 180.1). Signals were also observed for two nonprotonated carbons in the upfield sp^2 and/or the downfield oxygenated sp^3 carbon regions (δ_C 93.3, 102.0). In addition, the NMR spectra contained signals for three methyl groups, an oxygenated methine, an aliphatic methine, and multiple methylene units, indicative of a lengthy aliphatic side chain. When the ^1H NMR spectrum was recorded using DMSO-d_6 as the solvent, a broad signal corresponding to an exchangeable proton was also observed at δ_H 12.5.

COSY data defined two isolated proton spin systems corresponding to the C-2′, C-3′ and C-4, C-5 units in **1**. Analysis of HMBC data for H-4 and H-5 showed numerous correlations that enabled assembly of partial structure **1a**

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Table 1. NMR Data for Lowdenic Acid (**1**) in $CDCl_3$

	δ_H (mult., *J* in Hz)[a]		δ_C[b]	
no.	*E*	*Z*	*E*	*Z*
1			170.1	167.6
2			93.6	93.3
3			194.2	196.3
4	4.87 (dd; 7.2, 4.2)	4.91 (dd; 7.2, 4.2)	78.2	78.5
5a	3.05 (dd; 17, 4.2)	3.02 (dd; 17, 4.2)	35.8	35.8
5b	2.81 (dd; 17, 7.2)	2.83 (dd; 17, 7.2)		
6			173.7	173.7
1′			179.3	180.1
2′	7.50 (d; 6.0)	7.55 (d; 6.0)	123.3	123.1
3′	7.44 (d; 6.0)[c]	7.45 (d; 6.0)[c]	160.3[d]	160.4[d]
4′			102.5	102.0
5′	1.92 (m)	1.95 (m)	38.3	38.2
6′	1.26 (m)	1.26 (m)	21.6[e]	21.7[e]
7′	1.07 (m)	1.07 (m)	36.8	36.8
8′	1.31 (m)	1.31 (m)	32.5	32.5
9′	1.27 (m)	1.27 (m)	37.0	37.0
10′−15′	1.22−1.27		27.0−29.9	
16′	1.25 (m)	1.25 (m)	31.9	31.9
17′	1.28 (m)	1.28 (m)	22.7[f]	22.6[f]
18′	0.88 (t; 7.2)	0.88 (t; 7.2)	14.1	14.1
19′	0.80 (d; 6.6)[g]	0.82 (d; 6.6)[g]	19.4	19.4
20′	1.62 (s)	1.63 (s)	23.2	23.3

[a] Recorded at 600 MHz. [b] Recorded at 90 MHz. [c−g] These signals occur at slightly different positions, but the assignments are interchangeable. The positions of some of the aliphatic side-chain signals could be assigned on the basis of analysis of 2D NMR data, even though they were not resolved from the methylene envelope.

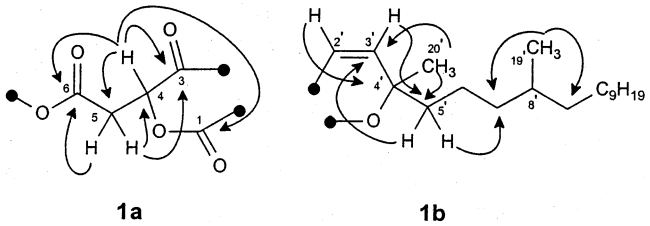

Figure 1. Partial structures **1a** and **1b** with selected HMBC correlations.

(Figure 1). Chemical shift considerations were consistent with assignment of this structural unit. However, no further correlations to any of the three carbonyl carbons (C-1, C-3, and C-6) were observed.

A second partial structure (**1b**; Figure 1) was also assembled by analysis of HMBC data. Both olefinic protons (H-2′ and H-3′) showed HMBC correlations to the nonprotonated carbon C-4′, and the downfield shift of C-4′ (δ_C 102.0) indicated its attachment to at least one oxygen atom. HMBC correlations of H_3-20′ (δ_H 1.63) to C-3′, C-4′, and C-5′ indicated connection of C-3′, C-5′, and C-20′ to C-4′ and revealed that C-4′ must be a singly oxygenated sp^3 carbon, despite its downfield shift. The 6 Hz coupling constant between H-2′ and H-3′ suggested that the corresponding olefin is located in a ring, most likely a five-membered ring.[8] Further HMBC correlations permitted extension of this structural unit to include a long aliphatic side chain. These data enabled location and spectral assignment of the C-5′, C-6′, C-7′, C-8′(C-19′), C-9′ fragment, as well as the terminal C-16′, C-17′, C-18′ unit. Overlapping of the remaining methylene NMR signals precluded further specific assignments, but because the number of methylene units was unambiguously established by the molecular formula, and because these methylene signals could not be located elsewhere, the side chain could be extended to that shown in **1b**.

Two carbon signals (δ_C 180.1 and 93.3) remained unassigned at this point. Both protons of the C-2′, C-3′ olefin unit showed correlations to the signal at δ_C 180.1, suggesting its direct attachment to C-2′. Unfortunately, no other HMBC correlations were observed for this signal, and none were observed for the signal at δ_C 93.3, so these carbons could not be unambiguously located. Moreover, there were no correlations relating the two substructures **1a** and **1b**. Assignment of these two carbon signals to a tetrasubstituted, oxygenated olefin unit joining **1a** and **1b** would lead to the proposal of gross structure **1**. However, it was felt that the NMR data did not provide sufficient positive evidence for a conclusive assignment.

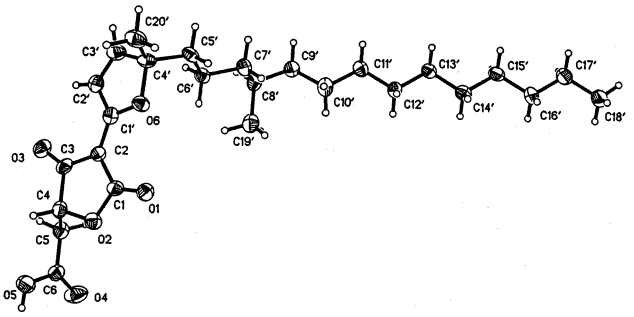

Figure 2. X-ray crystallographic model of lowdenic acid (**1**).

Fortunately, crystals of **1** that proved suitable for crystallographic analysis were obtained from a CH_3CN/H_2O (1:1) solution of freshly collected material corresponding to the second peak in the HPLC trace. The X-ray structure (Figure 2) allowed assignment of the structure and relative stereochemistry and revealed that the crystal examined corresponded to the *Z*-isomer (as depicted in **1**). The data verified the structural features assigned by NMR analysis and confirmed that the compound possesses a free carboxylic acid unit. The two carbons not unambiguously assigned by 2D NMR analysis of **1** (δ_C 180.1 and 93.3) must indeed correspond to the central C1′−C2 oxygenated olefin unit. Compound **1** possesses an unusual bicyclic structure containing a furylidene ring linked via a carbon−carbon double bond to a tetrahydrofurandione ring. As expected, the two five-membered rings are nearly coplanar, and the three stereogenic centers (C-4, C-4′, and C-8′) are well separated from each other. This is consistent with the observed absence of any NOESY correlations that would have been relevant to determining the relative stereochemistry of **1**.

Although only the *Z*-isomer of **1** is shown, as noted earlier, the two sets of signals in the ^1H and ^{13}C NMR spectra of **1** are due to the occurrence of an approximate 1:1 ratio of the *E*- and *Z*-isomers. Italicic acid (**3**),[9] reported as a product of *Penicillium italicum*, contains a furylidene ring similarly linked via a carbon−carbon double bond to a tetrahydrofurandione ring and appears to be the closest known analogue of **1**.[9] As is the case with **1**, compound **3** was described as an equilibrium mixture of the *E*- and *Z*-isomers at the furylidene double bond. However, spectral assignments for the individual isomers of **3** were not reported.

Isomerization at a furylidene double bond has been previously studied in the case of carolic acid (**4**), another fungal metabolite, originally isolated from *Penicillium charlesii*.[10−13] Compound **4** reportedly exists in $CDCl_3$ solution as a 4:5 mixture of the *E*- and *Z*-isomers.[11] However, when crystals of **4** obtained from EtOH were redissolved in the dark in CD_3CN, NMR signals for only one isomer were observed. Comparison of the chemical shifts of this isomer with those of *E*- and *Z*-methyl 3-methoxyacrylate indicated that it is the *E*-isomer,[11] and this was confirmed by X-ray crystallography.[12] The NMR

assignments for the two isomers of **1** are consistent with those reported for the carolic acid isomers.[11] However, in this case, the crystal obtained for **1** represented the *Z*-isomer (Figure 2). A freshly prepared solution of the crystalline sample of *Z*-lowdenic acid in CD_3CN showed only one set of NMR signals, but signals for the *E*-isomer were soon in evidence, and after 18 h at ambient light, a 6:4 mixture of the *Z*- and *E*-isomers was present. As was proposed for carolic acid,[11] this isomerization could be envisioned to involve a light-initiated (radical) process or could be catalyzed by traces of acid.

A second compound was isolated from the *Verticillium* sp. culture, and analysis of EIMS, ^{1}H NMR, ^{13}C NMR, and DEPT data for this metabolite enabled its identification as canescin A (**2**), an antifungal agent originally isolated from *Penicillium canescens*.[7]

The biosynthesis of lowdenic acid (**1**) appears to involve condensation of a modified 20-carbon polyketide unit (which forms the side chain, the furylidene ring, and C-1 and C-2 of the tetrahydrofurandione ring) with a four-carbon dicarboxylic acid unit. Such a process would be analogous to that experimentally determined for italicic acid[9] and carolic acid.[11−13] Interestingly, a similar biogenetic pathway has also been proposed for canescin A (**2**) involving aldol condensation of a polyketide with a tricarboxylic acid unit through a multistep process.[14]

Lowdenic acid (**1**) represents a relatively rare structural class. Italicic acid (**3**) and carolic acid (**4**) appear to be the most closely related metabolites reported to date, and no bioactivity has been reported for either of these compounds. Lowdenic acid (**1**) exhibited activity in disk assays against *Aspergillus flavus* (NRRL 6541), causing a zone of inhibition of 14 mm at 250 μg/disk, and showed an MIC value[15] against *A. flavus* of approximately 6 μg/mL. It also caused a 9 mm zone of inhibition in standard disk assays at 200 μg/disk when tested against *C. albicans* (ATCC 90029). Inhibitory zones of 12 and 17 mm were observed for **1** in standard disk assays against *S. aureus* (ATCC 29213) and *B. subtilis* (ATCC 6051), respectively, at 200 μg/disk.

Experimental Section

General Experimental Procedures. ^{13}C NMR data were recorded at 90 MHz (Bruker WM-360). UV absorptions were recorded using a Beckman DU 640 spectrophotometer. Low-resolution electron impact (EI) mass spectra were obtained with a VG Trio-1 quadrupole mass spectrometer operating at 70 eV with direct probe. Low- and high-resolution ESI mass spectra were obtained using a Micromass Autospec instrument. Details of other instrumentation and general experimental procedures have been described elsewhere.[16]

Fungal Material. An unidentified isolate of *Verticillium* sp. (MYC-406 = NRRL 29280 = CBS 102427) was obtained from basidiomata of a polypore, *Poria* sp. (Coriolaceae), collected by Dr. H. D. Thiers on September 28, 1996, from the underside of a dead hardwood log at Lowden State Park, near the town of Oregon, Ogle County, IL. Procedures employed for plating and isolation of cultures from basidiocarp surfaces have been described previously.[6] The isolate was recognized as *Verticillium* by Dr. Walter Gams of the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. However, the characteristic features of this species did not match those of any known *Verticillium*, leading to its classification as a new, previously undescribed species of the *Albo-erecta* taxonomic group.

The fungus was grown on several slants of PDA for 14 days at 25 °C. A hyphal fragment-spore suspension prepared from the PDA slants served as inoculum for solid-substrate fermentation, which was carried out in six 500 mL flasks each containing 50 g of rice. Distilled water (50 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 lb/in.^{2} for 30 min. After the flasks were cooled to room temperature, they were inoculated with 1.0 mL of the hyphal fragment-spore suspension and incubated at 25 °C for 40 days.

Extraction and Isolation. The fermented rice substrate was first fragmented with a spatula and then extracted with EtOAc (50 mL). The combined EtOAc extracts were filtered and evaporated to yield 690 mg of crude extract. Solvent partitioning of the crude extract between hexane (3 × 40 mL) and CH_3CN (30 mL) provided 580 mg of a CH_3CN-soluble fraction. This fraction was subjected to Sephadex LH-20 column chromatography (45 × 1.5 cm), eluting with 350 mL of hexane/CH_2Cl_2 (1:4), then CH_2Cl_2−acetone (200 mL of 3:2; 150 mL of 1:4). Finally, the column was washed with 100 mL of acetone. Fractions were collected and pooled on the basis of TLC and ^{1}H NMR analysis. The seventh fraction (88 mg), eluted with 1:4 hexane/CH_2Cl_2, was subjected to Sephadex LH-20 column chromatography (23 × 1.2 cm) using a less polar solvent system (100 mL of 3:7 hexane/CH_2Cl_2). The major fraction from this column (63 mg) was a mixture of the *E*- and *Z*-isomers of **1**. A portion of this fraction (38 mg) was further purified by RP HPLC (Alltech HS Hyperprep 100 BDS C_{18}; 10 × 250 mm; flow rate, 2 mL/min; 100% CH_3CN over 25 min) to afford samples of the *E*- (9 mg; t_R 12.9 min) and *Z*-isomers (10 mg; t_R 14.1 min), each of which slowly equilibrated back to the *E*:*Z* mixture. The twelfth fraction (68 mg) from the first column, eluted with 3:2 CH_2Cl_2/acetone, was further fractionated by Sephadex LH-20 column chromatography (20 × 1.5 cm) using the same solvent gradient to afford six fractions. One fraction that was eluted with 3:2 CH_2Cl_2/acetone (35 mg) was purified by RP HPLC (Alltech HS Hyperprep 100 BDS C_{18}; 10 × 250 mm; flow rate, 2 mL/min; 20−100% CH_3CN in H_2O over 40 min) to afford canescin A (**2**; 19 mg; t_R 23.5 min). Compound **2** was identified by comparison of its spectral data (^{1}H NMR, ^{13}C NMR, $[\alpha]_D$, and MS) with literature values.[7]

Lowdenic acid (1): (*E*:*Z* mixture) white powder; mp 68−70 °C; $[\alpha]_D$ −39° (*c* 0.02 g/100 mL, $CHCl_3$, 27 °C); UV λ_{max} (EtOH) 214 (ϵ 7900), 318 (ϵ 16000); IR ν_{max} ($CHCl_3$) 3300−2500 (br), 2954, 2924, 2853, 1758, 1738, 1704, 1567, 1462, 1167, 1048, 1023, 991, 822 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 1; HMBC (600 MHz; H → C#): H-4 → C-1, C-3, C-5, C-6; H_2-5 → C-3, C-4, C-6; H-2′ → C-1′, C-3′, C-4′; H-3′ → C-1′, C-2′, C-4′, C-5′, C-20′; H_2-5′ → C-3′, C-4′, C-6′, C-7′, C-20′; H_2-7′ → C-5′, C-8′, C-9′, C-19′; H_3-18′ → C-16′, C-17′; H_3-19′ → C-7′, C-8′, C-9′; H_3-20′ → C-3′, C-4′, C-5′; FABMS (thioglycerol/TFA) obsd *m*/*z* 449 $[M + H]^+$; HRFABMS obsd *m*/*z* 449.2902 $[M + H]^+$, calcd for $C_{26}H_{41}O_6$, 449.2903.

Single-Crystal X-ray Diffraction Analysis of Lowdenic Acid (1).[17] Crystals of **1** were recovered from CH_3CN/H_2O (1:1), and a single crystal (0.22 × 0.14 × 0.04 mm) was selected for analysis. Data were collected on a Nonius Kappa CCD diffractometer (Mo Kα radiation, λ = 0.71073 Å, graphite monochromator) at 190 K (N_2 cold gas stream). Standard CCD techniques were employed, yielding 10 562 data. Cell dimensions were determined from analysis of 7970 reflections to be a = 7.4840(15) Å, b = 5.1270(10) Å, c = 66.518(13) Å, α = 90°, β = 90.65(3)°, and γ = 90°, and the calculated density is 1.188 mg/m^3. The systematic absence corresponded to space group *I*2 (the nonstandard space group was chosen to facilitate refinement as a pseudo-merohedral twin). Equivalent data were averaged, yielding 1708 unique data [R_{int} = 0.056, 1320F > 4$\sigma(F)$]. Data were corrected for absorption (multiscan technique), as well as for Lorentz and polarization effects. The computer programs (Denzo, Scalepack) from the HKLint package were used for data reduction. The structure was solved using XS, a direct methods program, and refined by full-matrix least-squares performed with the XL computer program. Illustrations were made with the XP program, and tables were prepared with the XCIF program, all of which are in the SHELXTL v5.1 package. Thermal ellipsoids shown are at the 35% level. All non-hydrogen atoms were refined with anisotropic thermal parameters, and the final refinement, based on 1320 reflections, gave R_1 = 0.0566, R_2 = 0.1115 standard deviation. A water molecule (located an a 2-fold axis) is included in the crystal structure of **1** and serves as a

hydrogen bond donor and acceptor to **1** (O5−H5 = 0.84 Å, H5···O10$^{(a)}$ = 1.83 Å, O5···O10$^{(a)}$ = 2.654(8) Å, O5−H5···O10$^{(a)}$ = 165.4°, (a) = 1+X, −1+Y, Z; O10−H10 = 0.84 Å, H10···O3 = 2.14 Å, O10···O3 = 2.724(6) Å, O10−H10···O3 = 126.7°). The two five-membered rings are planar [RMS deviation from planarity for ring (C1,C2,C3,C4,O2) = 0.020 Å and for ring (C1′,C2′,C3′,C4′,O6) = 0.011 Å] with dihedral angle = 8.2(6)°. The crystal is a pseudo-merohedral twin; the twinning law is a 2-fold rotation about the reciprocal *c*-axis. The twin fraction refined to 0.085(2).

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra and stereoviews of ORTEP and unit cell representations for lowdenic acid (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Crystallographic data for compound **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition number CCDC 213830). Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223-336033; or e-mail: deposit@ccdc.cam.ac.uk).

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