A New 1-Hydroxy-2,6-pyrazinedione Associated with Hypovirulent Isolates of *Sclerotinia minor*[⊥]

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A new 1-hydroxy-2,6-pyrazinedione, sclerominol (**1**), was isolated from cultures of hypovirulent isolates of *Sclerotinia minor*, a fungal plant pathogen associated with lettuce drop and other plant diseases. This compound was characterized by NMR, mass spectrometry, and X-ray crystallography. One other 1-hydroxy-2,6-pyrazinedione, flutimide, has been reported. Flutimide has activity as an inhibitor of influenza virus endonuclease, and therefore, sclerominol was evaluated for related biological activity. Sclerominol (**1**) displayed some activity against cancer cell lines but little activity against three influenza virus strains. The role of **1** in the physiology of hypovirulent isolates of *S. minor* has not been determined, but **1** has also been recovered from debilitated isolates of *S. sclerotiorum*.

Hypovirulent isolates of fungal plant pathogens, which have a reduced ability to cause disease, are being examined for potential application in plant disease management. Hypovirulence has been most extensively studied in *Cryphonectria parasitica*, the causal agent of chestnut blight,[1−4] where it has been associated with unencapsidated double-stranded RNA (dsRNA) *Hypovirus* species,[5,6] dsRNA viruses,[7] and mitochondrial mutations.[8] Considerable variability in the hypovirulent phenotype exists among different dsRNA-containing isolates of *C. parasitica* and, in addition to reduced virulence, often includes altered colony morphology; reduced conidiation, oxalate accumulation, laccase and cutinase production, and pigmentation; and modulation of specific mRNAs and polypeptides.[3,6,9] *Hypovirus*-mediated hypovirulence has been associated with alterations in several fungal processes, including virulence, by selectively affecting the expression of specific fungal genes and regulatory pathways.[6,10]

Hypovirulent isolates also have been reported from *Sclerotinia minor*, the causal agent of lettuce drop and other diseases, and may be associated with the presence of specific dsRNAs.[11] Three isolates of *S. minor* from a sample of 30 were significantly less virulent, slow growing, had atypical colony morphologies and, therefore, were considered hypovirulent. DsRNA was detected in all hypovirulent isolates but also was detected in several virulent isolates. The hypovirulent phenotype was transmissible to selected recipient isolates, and when hypovirulent isolates were applied as cell suspensions to established lesions on lettuce, lesion expansion and production of sclerotia (e.g., survival structures of fungus) were reduced by up to 100%.[11] These studies on the use of hypovirulence as a disease management strategy have been encouraging, but an increased understanding of hypovirulence in *S. minor* is needed, particularly on the specific virulence-associated pathways affected in hypovirulent isolates.

During studies of the hypovirulent isolates of *S. minor*, a zone of yellow precipitate was observed around the margins of colonies of the hypovirulent isolates and, occasionally, directly on the colonies of hypovirulent isolates. No precipitate was observed on, or around, the colonies of typical, virulent isolates.[12] This putative correlation between hypovirulence and precipitate may reflect a virulence-associated pathway being affected in hypovirulent isolates of *S. minor*. Therefore, the objectives of this study were to characterize and identify the precipitate associated with hypovirulent isolates of *S. minor*.

Isolation of the yellow precipitate from agar was achieved essentially by filtration of the melted agar and then washing with H_2O. This simple procedure produced a complete recovery of crude material. Analysis by GC/MS showed the presence of three compounds, one major and two minor, in an approximate ratio of 90:5:5. Mass spectra showed that they were related, with the major compound, sclerominol (**1**), having a molecular weight of 306, and the minor compounds having molecular weights of 290 and 306. Initial attempts to separate the three compounds by chromatography failed, as they decomposed on a silica gel column and were not separated on a reversed-phase column. The ready loss of 17 mass units in its mass spectrum suggested that the main compound contained a hydroxyl group. Assuming that acetates would be more stable, the mixture was acetylated. Both compounds with molecular weights of 306 produced monoacetates, but the other minor component remained unchanged, indicating that this compound lacked an OH group. This compound was, therefore, called deoxysclerominol. In the mass spectrometric analysis of the mixture, the first fragmentation of the minor component of molecular weight 306, isosclerominol, was the loss of 91 Da, even when acetylated. This apparent loss of a benzyl group may produce a more stable rearrangement product by aromatization or other means. The small amount of material available precluded any further characterization of deoxysclerominol or isosclerominol.

Sclerominol (**1**) was purified by recrystallization from MeOH, and more material was eventually obtained from the mother liquor by HPLC using a Synergi Polar RP column and a gradient from 10% acetonitrile in H_2O to 100% acetonitrile. Infrared analysis showed an OH absorption at 3338 cm^{-1}, aromatic absorptions at 3032 and 724−785 cm^{-1} (multiple sharp bands), carbonyl absorptions at

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Table 1. ^{1}H NMR Data for **1** and Its Acetate (**2**) and *p*-Bromobenzoate (**3**) Derivatives

position	scleorminol (**1**)	acetate (**2**)	*p*-bromobenzoate (**3**)
9, 13	7.93, d(7.8), 2H[a]	7.87	7.90
7	7.83, s, 1H	7.77	7.80
11	7.43, t(7.4), 1H	7.41	7.43
17, 19	7.36, t(7.3), 2H	7.36	7.36
16, 20	7.33, d(5), 2H	7.33	7.32
10, 12	7.315, t(7.8), 2H	7.31	7.31
18	7.31, t(7.8), 1H	7.31	7.30
14	4.21, s, 2H	4.20, d(13), 1H	4.23, d(13), 1H
		4.18, d(13), 1H	4.21, d(13), 1H
acetate		2.38, s, 3H, Ac	
p-bromobenzoate			8.03, dt(8.6, 2.0), 2H
p-bromobenzoate			7.67, dt(8.6, 2.0), 2H

[a] Chemical shift (δ) with reference to TMS, multiplicity (coupling constant in hertz), number of hydrogens.

Table 2. ^{13}C NMR Data (δ) for **1** and Its Acetate (**2**) and *p*-Bromobenzoate (**3**) Derivatives

position	scleorminol (**1**)	acetate (**2**)	*p*-bromobenzoate (**3**)
acyl C=O		165.8	161.6
C-2	159.1	158.5	158.5
C-6	156.5	156.8	156.7
C-5	151.7	152.8	153.0
C-7	144.8	144.2	144.4
C-15	135.3	135.3	135.3
C-9, C-13	134.7	134.5	134.5
C-8	133.2	133.3	133.3
C-11	132.6	132.3	132.4
p-bromobenzoate			132.3
p-bromobenzoate			131.9
p-bromobenzoate			130.3
C-3	131.4	132.3	132.4
C-16, C-20	130.0	130.1	130.1
C-10, C-12	128.8	128.8	128.8
C-17, C-19	128.7	128.7	128.7
C-18	127.0	127.0	127.0
p-bromobenzoate			124.5
C-14	39.8	39.8	39.9
acetate CH_3		17.6	

1716 cm^{-1}, and a C=N absorption at 1660 cm^{-1}. From its NMR data (Tables 1 and 2), **1** consisted of three ring systems, one phenyl, one benzyl, and a central unit, which contained at least three quaternary carbons and one CH. The phenyl and benzyl rings were easily identified and assigned with the use of homo- and heteronuclear two-dimensional NMR spectra. HRMS and elemental analysis of the acetate (**2**) provided the molecular formula $C_{20}H_{16}N_2O_4$, corresponding to $C_{18}H_{14}N_2O_3$ for the parent compound. Considering the NMR information already obtained and the implied presence of benzyl and phenyl rings as well as an OH, the central part of the molecule had the formula $C_5HN_2O_2$. The structure of this unit could not be determined by NMR because of the presence of four heteroatoms and only one hydrogen. The compound's molecular formula implied the presence of 13 rings or double bonds in the molecule. The two aromatic rings accounted for eight of these; therefore, the middle portion of the compound had to contain five rings or double bonds. The carbon NMR spectrum did show three carbonyl-like signals between 150 and 160 ppm, which implied the presence of at least one ring and another double bond.

The NMR signals of five protons for each aromatic ring were assigned from the ^{1}H/^{1}H (COSY) spectrum, indicating that each ring was monosubstituted and, therefore, attached to the middle ring system. A ^{1}H/^{13}C (HETCOR) correlation spectrum allowed the assignment of the carbons and protons of both aromatic rings and correlation of the other protons with their respective carbons. Long-range heteronuclear coupling, through an HMBC experiment

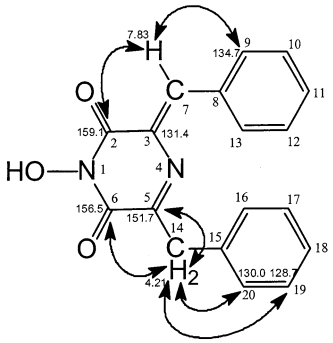
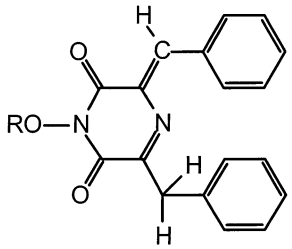


Figure 1. Structure of scleorminol, **1**, showing the numbering of the different carbons along with the connectivities determined by HMBC, with the relevant chemical shifts.

(Figure 1), was visible not only within the aromatic rings but also between the benzylic protons (δ 4.21) and two of the quaternary carbons of the middle section (δ 151.7 and 156.5), as well as all carbons of the benzylic ring except the para carbon. By analogy to a similar compound, flutimide,[13] and with the help of the long-range heteronuclear coupling NMR experiment, the signal at δ 156.5 was assigned to C-6, and the signal at 151.7 was assigned to C-5. Similarly, the proton at δ 7.83 showed long-range coupling to the ortho carbons (C-9 and C-13) of the phenyl ring (δ 134.7) and a quaternary carbon (δ 159.1). For this to be possible, this CH group had to be adjacent to the phenyl ring and two carbons away from the carbon at δ 159.1 (C-2). No coupling was observed for C-3 except possibly for a very faint long-range heteronuclear coupling to H-14.



To determine the position of the OH group, **1** was acetylated (**2**). However, no NOEs were observed for the acetate group. A bulkier *p*-bromobenzoate (**3**) was then prepared, to look for NOEs and to obtain a crystal. However, **3** produced no useful NOEs either, suggesting that there were no hydrogens close to the OH group. Both

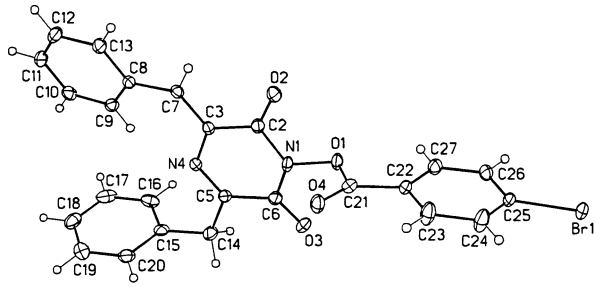


Figure 2. ORTEP II drawing of the X-ray structure of scleromino*l* *p*-bromobenzoate (**3**).

2 and **3** showed a split signal for the benzylic protons but without a significant change in chemical shift. This suggested that the hydroxyl group was not close to either of the aromatic rings of **1**, but that acylation was sufficient to put the two hydrogens of the benzylic group in different environments. In both cases, very small changes were observed in the NMR spectra of the derivatives (Tables 1 and 2), except that a distinct signal for C-3 was not visible. It is assumed that C-3 is under the C-11 signal as it would undergo a shift of about 1 ppm, similar to that of C-5. The C-11 peak in both derivatives was larger than in **1** when compared to that of C-18, its equivalent in the other aromatic ring.

A crystal suitable for X-ray analysis was obtained from the *p*-bromobenzoate (**3**) by slow evaporative crystallization in CH_2Cl_2/hexane. The ORTEP II structure obtained is depicted in Figure 2.[14] All hydrogens being accounted for, the nitrogen atoms had to be at positions 1 and 4, showing **1** to be a 1-hydroxy-2,6-pyrazinedione, more specifically, (3*Z*)-5-benzyl-3-benzylidene-1-hydroxypyrazine-2,6(1*H*,3*H*)-dione. This structure fits well with the NMR data and explains why no NOEs were observed in the two derivatives since there are no hydrogens close to the derivatized hydroxyl group.

2,6-Pyrazinediones are rarely found in nature. To our knowledge, only one other report exists of a 1-hydroxy-2,6-pyrazinedione. This compound, flutimide, was the isobutyl/isobutylidene analogue of our benzyl/benzylidene compound and was isolated from *Delitschia confertaspora*, a fungus found in dassie dung in Namibia.[13] Flutimide was isolated through its activity as an inhibitor of influenza virus endonuclease. On the basis of this report, scleromino*l* was tested for biological activity. Testing against a number of cancer cell lines by the U.S. National Cancer Institute originally yielded promising results, but did not warrant testing beyond a hollow-fiber in vivo assay. Following the example of flutimide, scleromino*l* was then tested against different influenza virus cell lines by the U.S. National Institute of Allergy and Infectious Diseases, but was found to be inactive.

Little information is available to suggest a physiological role for flutimide in *D. confertaspora* or for scleromino*l* (**1**) in hypovirulent isolates of *S. minor*. Initial speculation suggested that **1** may possess antiviral activity against the viral-like double-stranded RNA (dsRNA) associated with hypovirulence in selected isolates of *S. minor*, or possibly be a response to infection by this agent. The potential role of flutimide in *D. confertaspora* is even more speculative because no information was presented on the possible presence of dsRNA in this fungus.[13] Scleromino*l* was also identified from debilitated isolates of *S. sclerotiorum* in the present study, but these isolates were not characterized for the possible presence of dsRNA. In combination, however, it appears that overproduction of **1** in *Sclerotinia* spp. is associated with debilitated growth in these isolates.

Experimental Section

General Experimental Procedures. GC/MS analysis was performed on a Finnigan/MAT 4500 MS system operating in the EI mode, with a 15 m DB5MS column (120−280 °C, 15 deg/min). IR spectra were recorded with a Perkin-Elmer 16F PC FT-IR spectrophotometer. UV spectra were obtained on a Varian DMS 200 UV−vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AM500 spectrometer in $CDCl_3$ at 500 and 125 MHz, respectively. Chemical shifts are referenced to residual $CHCl_3$ at 7.24 ppm for ^{1}H spectra and $CDCl_3$ at 77.0 ppm for ^{13}C spectra and reported (δ) relative to TMS. Homonuclear correlation spectra were obtained using the 90−t_1−45 FID pulse sequence (COSY-45). Direct and indirect heteronuclear correlation spectra (HETCOR, HMBC) were obtained using standard Bruker sequences.

Isolation of Metabolites. Three hypovirulent isolates (isolates 6, 13, and 23)[11] of *Sclerotinia minor* were grown on potato-dextrose-agar (PDA) medium at 21 °C. Colonies producing precipitate were selected for harvesting and chemical characterization.

PDA-containing yellow precipitate was removed from areas adjacent to colonies and placed in 1.5 mL Eppendorf microfuge tubes with 2 to 3 volumes of H_2O. The mixture was heated to 95−100 °C in a steam bath to melt and dilute the PDA and centrifuged at 8000*g* for 3 min, and the supernatant was removed. The precipitate was dried and stored at −20 °C. GC/MS analysis of precipitate: a. Deoxyscleromino*l*: (Retention time: 10 min, 9 s) *m/z* 290 (100), 261 (26), 218 (12), 212 (50), 117 (39), 102 (27), 91 (90), 89 (12), 77 (19). b. Isoscleromino*l*: (Retention time: 10 min, 21 s) *m/z* 306 (68), 215 (85), 199 (20), 185 (25), 115 (10), 101 (5), 91 (100). c. Scleromino*l*: (Retention time: 11 min, 12 s) *m/z* 306 [M^+] (40), 289 (21) (M − OH), 261 (35), 246 (5), 233 (8), 218 (30), 116 (50), 102 (25), 91 (100) (C_6H_5-$CH_2^{+\bullet}$), 89 (24), 77 (25) ($C_6H_5^{+\bullet}$).

Scleromino*l* (1): IR (KBr) ν_{max} 3338, 3032, 1716, 1660, 1610, 1580, 1564, 1470, 785−724 cm^{-1} (multiple sharp bands); UV ($CHCl_3$) λ_{max} 246 (sh), 251, 260 (sh), 372; NMR, see Tables 1 and 2; HREIMS *m/z* 306.1016 (calcd for $C_{18}H_{14}N_2O_3$, 306.1004).

Scleromino*l* Acetate (2). An aliquot of the crude scleromino*l* (2 mg) was stirred overnight in 500 μL of CH_2Cl_2 and 50 μL of acetyl chloride. The next morning, the solvent was removed under nitrogen and the residue was chromatographed on silica gel with $CHCl_3$, followed by 1% MeOH in $CHCl_3$: EIMS *m/z* 348 [M^+] (5), 306 (95), 289 (63), 261 (60), 259 (20), 246 (12), 233 (10), 218 (58), 116 (50), 102 (45), 91 (93), 89 (21), 77 (21), 43 (100); NMR, see Tables 1 and 2; HREIMS *m/z* 348.1117 (calcd for $C_{20}H_{16}N_2O_4$, 348.1110); *anal.* C 68.49%, H 4.29%, N 7.82%, calcd for $C_{20}H_{16}N_2O_4$, C 68.97%, H 4.60%, N 8.05%.

Scleromino*l* *p*-Bromobenzoate (3). An aliquot of the crude **1** (5 mg) was dissolved in THF (1 mL) and stirred in an ice bath. An excess of *p*-bromobenzoyl chloride (7 mg) was added, followed by a 1% solution of Et_3N in THF (340 μL). Following this addition, the solution went from yellow to orange and back to yellow within 2 s. After 5 min, the solution was concentrated under nitrogen and the residue was chromatographed on silica gel with 1:1 $CHCl_3$/hexane followed by 2:1 $CHCl_3$/hexane for a 4.5 mg yield. (See NMR data in Tables 1 and 2.) Recrystallization of the *p*-bromobenzoate from a variety of solvent systems only yielded small needles (0.2 mm) arranged around a central nucleus. However, slow evaporative recrystallization from CH_2Cl_2/hexane yielded crystals suitable for X-ray analysis.

Crystallography. a. Data Collection. A crystal of the *p*-bromobenzoate (**3**) ($C_{25}H_{17}BrN_2O_4$) having approximate dimensions of 0.2 × 0.2 × 0.2 mm was mounted on a glass capillary. All measurements were made on a Siemens Smart CCD diffractometer with Mo Kα radiation. Cell constants and an orientation matrix for data collection were obtained from

least-squares refinement using the setting angles of 3436 reflections corresponding to a triclinic cell with dimensions $a = 8.6720(1)$ Å, $b = 10.5631(1)$, $c = 12.6419(3)$ Å, $\alpha = 84.565(1)°$, $\beta = 73.862(1)°$, $\gamma = 69.871(1)°$. For $Z = 2$ and fw = 489.32, the calculated density is 1.98 g/cm^3. Based on the systematic absences, the space group was determined to be $P\bar{1}$. The data were collected at a temperature of −150 °C using the $\omega-2\theta$ scan technique to a maximum 2θ value of 46.4°.

b. Data Reduction. A total of 4191 reflections were collected. The unique set contains only 2954 reflections. The data were corrected for Lorentz and polarization effects.[15] No absorption corrections were made.

c. Solution and Refinement. The structure was solved by direct methods. All atoms were refined anisotropically except the hydrogens. The hydrogen positions were found by difference Fourier mapping. The final cycle of full matrix least-squares refinement was based on 2775 observed reflections ($I > 2.5\sigma(I)$) and 357 variable parameters. Weights based on counting statistics were used. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.430 and −0.560 e/Å^3, respectively.

All calculations were performed using the NRCVAX crystallographic software package.[16]

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(14) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre under CCDC # 196204. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Rd., Cambridge CB2 1Ez, UK [fax: +44 (0)1223−336033 or e-mail: deposit@ccdc.cam.ac.uk].
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