The isolation, identification, and bioassay of the antifungal metabolites produced by *Monocillium nordinii*

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The metabolites produced when *Monocillium nordinii* (Bourchier) W. Gams, a destructive mycoparasite of pine stem rusts, is grown in liquid culture have been separated and identified.

The metabolites include the known compound monorden (1) and five new substances, monocillin I (2), monocillin II (4), monocillin III (3), monocillin IV (5), and monocillin V (6). Structural assignments and chemical correlations of the five new compounds are reported and the absolute configuration of monorden is assigned. The antifungal spectra of the three major metabolites are reported. Monorden and monocillin I show pronounced activity against a wide variety of fungi, including *Ceratocystis ulmi*, the cause of Dutch elm disease. Extraction of the mycelium yielded averufin (13), along with a pigment C_{18}H_{22}O_{6}, as yet unidentified.


*Monocillium nordinii* (Bourchier) W. Gams est un mycoparasite des rouilles de la tige du pin, qui, lorsque mis en croissance sur milieux liquides, produit des métabolites que les auteurs ont pu séparer et identifier. Ces métabolites incluent le composé connu monorden (1) et cinq nouvelles substances: monocilrine I (2), monocilrine II (4), monocilrine III (3), monocilrine IV (5) et monocilrine V (6). Les spécificités structurales et les corrélations chimiques des cinq nouveaux composés sont rapportées et la configuration absolue de la monorden est établie. Les spectres antifongiques des trois métabolites majeurs sont également rapportés. La monorden et la monocilrine I présentent une activité prononcée contre une grande variété de champignons, incluant le *Ceratocystis ulmi* qui cause la maladie hollandaise de l'orme. Des extraits de mycélium donnent de l'averufine (13), ainsi qu'un pigment C_{18}H_{22}O_{6} non encore identifié.

[Traduit par le journal]

**Introduction**

Recently Tsuneda and Hiratsuka (in preparation) have reported that *Monocillium nordinii* (Bourchier) W. Gams is a destructive mycoparasite of pine stem rusts, *Cronartium coleosporioides* Arth. and *Endocronartium harknessii* (J. P. Moore) Y. Hiratsuka. These workers isolated the crude ether soluble metabolites produced when *M. nordinii* is grown on potato dextrose agar and showed that the metabolites were active against the pine stem rusts as well as against a variety of other fungi, including *Ceratocystis ulmi*, the cause of Dutch elm disease.

We report herein: (1) the isolation and identification of the antifungal compounds; (2) the antifungal spectra of the three major components and their microscopic effects on the morphology of spores and germtubes of several fungi.

**Materials and methods**

High resolution mass spectra were recorded on an AEI MS-50 mass spectrometer, coupled to a DS 50 computer. Infrared spectra were recorded on a Unicam SP 1000 or a Nicolet 7199 FT spectrometer and uv spectra on a Cary model 14 M spectrometer. ^1H nmr spectra were determined on a Varian HA-100, or Bruker WH 200, or Bruker WH 400 spectrometer with TMS as internal standard. Melting points were recorded on a Fisher- Johns melting point apparatus and are uncorrected. Specific rotations at the D line were determined using a Perkin-Elmer 141 automatic polarimeter.

Silica gel tlc plates were 0.5-mm silica gel G (E. Merck, Darmstadt) containing 1% electronic phosphor (General Electric, Cleveland). Compounds were detected by viewing under uv light or by spraying with 30% sulfuric acid and charing. All solvents were reagent grade and were distilled before use. Unless otherwise specified, anhydrous magnesium sulfate was used as a drying agent for organic solutions and silica gel 60 (BDH Chemicals, 70–230 mesh) as adsorbant for column chromatography.

**Preparation of metabolites**

The strain of *M. nordinii* (C-678) used in this study was isolated from a spores of *C. coleosporioides* collected in Alberta and cultures were maintained at 4°C in slant tubes containing Difco potato dextrose agar (PDA). The liquid culture medium was Difco potato dextrose broth with 0.2% yeast extract added, 200
mL in each of 10 500-mL Erlenmeyer flasks fitted with foam plugs. Each flask was inoculated with ca. 2 mL of an aqueous suspension of mycelium from a 2-week-old culture. After incubation the flasks were kept at room temperature for 14 days. The culture broth was then decanted from the mycelium and continuously extracted with ether for 24 h. The ether solution was dried and evaporated to give a viscous gum (0.77 g) which was separated as described below.

**Separation of metabolites**

The crude metabolites (0.77 g) were subjected to chromatography over silica gel (40 g) using chloroform as eluant with a flow rate of 16 mL/h. The known compound monorden (1, 250 mg) was eluted first; followed by a compound C₆H₄NO (52 mg), which we have named monocillin I (2); then mixed fractions; then a compound C₁₃H₂₀O₃ (61 mg), hereafter called monocillin I (3); then further mixed fractions; and finally a compound C₁₂H₈O₄, named monocillin V (6). The mixed fractions were purified by preparative thin-layer chromatography (ptlc) over silica gel to give additional monocillin I (22 mg); a compound C₁₄H₉O₅ (5 mg), named monocillin II (4); and a compound C₁₂H₆O₄ (6 mg), named monocillin IV (5). The numbers in the names reflect the order of appearance on tlc plates (i.e., monocillin I, least polar; monocillin V, most polar).

**Characterization of metabolites**

**Monorden (I)** was crystallized from chloroform-ether, mp 195°C (reported 195°C (McCrapa et al. 1964; Mirington et al. 1966)). The H nmr spectrum, the ir spectrum, and the uv spectrum were identical with those previously reported (McCrapa et al. 1964; Mirington et al. 1966).

Monocillin I (2) was obtained as a colorless oil which could not be induced to crystallize; ir (film): 3300, 1651 cm⁻¹; uv (ethanol, 0°H) λ max (c 20 000): 273 (c 24 200), 319 nm (c 13 000); 1H nmr (CDCl₃) δ: 1.60 (d, J = 7 Hz, 3H), 1.95 (d, J = 15, 10, 4 Hz, 1H), 2.40 (dd, J = 15, 4, 2.5 Hz, 1H), 3.09 (dd, J = 10, 2.5, 2.5 Hz, 1H), 3.26 (broad s, 1H), 3.60 (d, J = 15 Hz, 1H), 5.22 (d, J = 15 Hz, 1H), 5.55 (m, 1H), 5.92 (dd, J = 11, 2.5 Hz, 1H), 6.0 (d, J = 16 Hz, 1H), 6.24 (dd, J = 11, 10 Hz, 1H), 6.42 (d, J = 3 Hz, 1H), 6.46 (d, J = 3 Hz, 1H), 6.94 (dd, J = 16, 10 Hz, 8.74 (broad s, 1H), 11.40 (s, 1H). Exact Mass calcd. for C₁₅H₁₈O₄: 330.1104; found (ms): 330.1120.

Monocillin II (4) was obtained as a colorless, crystalline solid from ether, mp 198–200°C; ir (film): 3400, 1645, 1615 cm⁻¹; 1H nmr (CDCl₃) δ: 1.56 (d, J = 7 Hz, 3H), 3.82 (d, J = 16 Hz, 1H), 4.22 (d, J = 16 Hz, 1H), 5.30 (m, 2H), 5.86 (d, J = 16 Hz, 1H), 6.28 (d, J = 2 Hz, 1H), 6.42 (d, J = 2 Hz, 1H), 6.75 (dd, J = 16, 9, 5 Hz, 1H), 10.28 (s, 1H). Exact Mass calcd. for C₁₆H₁₈O₅: 316.1311; found (ms): 316.1313.

Monocillin III (3) was obtained as white crystals from ether, mp 204–205°C; ir (film): 3400, 1645, 1620 cm⁻¹; 1H nmr (CDCl₃) δ: 1.42 (d, J = 7 Hz, 3H), 3.56 (d, J = 18 Hz, 1H), 4.47 (d, J = 18 Hz, 1H), 5.24 (m, 1H), 6.40 (d, J = 16 Hz, 1H), 6.19 (d, J = 2 Hz, 1H), 6.37 (d, J = 2 Hz, 1H), 6.82 (dd, J = 16, 9, 5 Hz, 1H), 11.10 (s, 1H). Exact Mass calcd. for C₁₅H₁₄O₅: 323.1260; found (ms): 323.1265.

Monocillin IV (5) was obtained as white crystals and recrystallized from ether, mp 139–140°C; ir (CHCl₃): 3600, 3400, 1705, 1645 cm⁻¹; 1H nmr (CDCl₃) δ: 1.42 (d, J = 7 Hz, 3H), 2.46 (m, J = 6 Hz, 1H), 2.64 (dd, J = 9, 3 Hz, 1H), 2.80 (dd, J = 5, 3 Hz, 1H), 3.62 (d, J = 18 Hz, 1H), 4.37 (d, J = 18 Hz, 1H), 5.26 (m, 1H), 6.20 (d, J = 2 Hz, 1H), 6.38 (d, J = 2 Hz, 1H), 11.2 (s, 1H). Exact Mass calcd. for C₁₄H₁₂O₄: 334.1416, found (ms): 334.1420.

**Chemical transformations of monorden and monocillins**

**Hydrogenation of monocillin I**

A solution of monocillin I (14 mg) in 0.01 N KOH in 95% ethanol (5 mL) containing 5% Pd/C (5 mg) was hydrogenated for 1 h. After removal of the catalyst, the solution was acidified with 0.1 N HCl and extracted with ether (3 × 20 mL). The ether extract was washed with 5% NaHCO₃ solution, dried, concentrated, and purified by column chromatography on silica gel (eluting with 10% ether–benzene as eluant) to furnish a crystalline solid (670 mg).

**Epoxidation of monocillin II**

A mixture of monocillin II (5.1 mg), 85% m-chloroperbenzoic acid (3 mg) and methylene chloride (2 mL) was stirred at room temperature for 4 h. The reaction mixture was diluted with water (3 mL) and extracted with ether (3 × 20 mL). The ether extract was then washed with 5% NaHCO₃ solution, dried, and concentrated to give a crude oil (5 mg). Purification by column chromatography on silica gel using 10% ether–benzene as eluant furnished an oil (3.1 mg) identical (tlc, mp, 1H nmr) with monocillin III (3).

**Hydrogenation of monocillin II**

A mixture of monocillin II (10 mg), W-2 Raney nickel (0.2 mL), and 5% CHCl₃ in ethanol (2 mL) was stirred at room temperature for 2 h. The reaction mixture was then filtered and concentrated to give a crude oil (0.5 mg). Recrystallization from ether gave monocillin IV (8), identical (tcl, mp, 1H nmr) with an authentic sample.

**Hydrogenation of monocillin III**

A mixture of monocillin III (5 mg), W-2 Raney nickel (0.2 mL), and 5% CHCl₃ in ethanol (2 mL) was stirred at room temperature for 45 min. The reaction mixture was then filtered and concentrated to give a crude oil (0.5 mg). Purification by column chromatography on silica gel using 5% ether–benzene as eluant furnished an oil (3.1 mg) identical (tcl, 1H nmr) with monocillin V (6).

**Preparation of monorden dimethyl ether**

A mixture of monorden (50 mg), potassium carbonate (100 mg), methyl iodide (0.1 mL), and acetone (5 mL) was heated under reflux for 2 h. Filtration and concentration of the solid which was recrystallized from ether to give monorden dimethyl ether (45 mg), mp 186–188°C (reported 187°C (Mirington et al. 1966)), identical in properties with those reported (Mirington et al. 1966).

**Deoxygenation of monorden dimethyl ether**

Triphenylphosphine selenide (50 mg) and trifluoroacetic acid (20 μL) were added to a solution of monorden dimethyl ether (10 mg) in CH₂Cl₂ (2 mL). After being stirred for 1 h the reaction mixture was diluted with water and extracted with ether (2 × 30 mL). The extract was washed with 10% NaHCO₃ solution, dried, and evaporated to give an oil. Excess triphenylphosphine selenide was removed by chromatography over silica gel (eluant benzene) and the crude product (eluted with ether) was purified by preparative thin-layer chromatography (ptlc) over silica gel to give triene 8 as an oil (3 mg) which decolorized very rapidly in air: 1H nmr (CDCl₃) δ: 1.48 (d, J = 7 Hz, 1H), 2.58 (m, 2H), 3.15 (s, 3H), 3.91 (d, J = 16 Hz, 1H), 3.92 (s, 3H), 4.36 (d, J = 6 Hz, 1H), 5.24 (m, 1H), 5.70 (dd, J = 15, 7.7 Hz, 1H), 6.04 (m, 4H), 6.48 (s, 1H), and 7.20 (dd, J = 16, 10 Hz, 1H). Because of the instability of this compound, no further analytical data were obtained.
nickel (1 mL), and 95% ethanol (10 mL) was stirred at room temperature for 2.5 h. The reaction mixture was then filtered and concentrated to give an oil (50 mg) which was chromatographed over silica gel (5 g) using benzene–ether (17:3) as eluant. The first eluted compound was tetrahydroxymonordenin dimethyl ether (10, 15 mg), whose spectral properties (IR, H nmr, mass spectrum) were identical with those reported (Mirington et al. 1966). The more polar component was the alcohol II (20 mg) obtained as a colorless glass; [α]$_D^{25}$ = 27.3$^\circ$ (c 6.2 × 10$^{-3}$, CHCl$_3$); ir (film): 3500 1725 cm$^{-1}$; H nmr (CDCl$_3$): δ 1.38 (d, J = 7 Hz, 3H), 3.68 (m, 1H), 3.78 (s, 3H), 3.90 (s, 3H), 3.92 (d, J = 18 Hz, 1H), 4.24 (d, J = 18 Hz), 5.20 (m, 1H), 6.48 (s, 1H).

Mol. Wt. calcd. for C$_{18}$H$_{20}$O$_{6}$Cl: 398; found (ms): 398.

Preparation of benzoxaze 12
The alcohol II (6 mg) in CHCl$_3$ (1 mL) was treated with pyridine (0.1 mL) and benzoyl chloride (50 µL) for 1 h. After work-up, the crude benzoxaze was purified by chromatography over silica gel using 2.5% ether in benzene as eluant to give the benzoxaze (4.2 mg) as a colorless oil: [α]$_D^{25}$ = 30.7$^\circ$ (c 2.5 × 10$^{-3}$, CHCl$_3$); ir (film): 1740, 1720 cm$^{-1}$; H nmr (CDCl$_3$): δ 1.38 (d, J = 7 Hz, 3H), 3.80 (s, 3H), 3.90 (s, 3H), 3.92 (d, J = 18 Hz, 1H), 4.24 (d, J = 18 Hz, 1H), 5.15 (m, 1H), 5.25 (m, 1H), 6.46 (s, 1H), 7.42 (m, 3H), 8.00 (m, 2H). Mol. Wt. calcd. for C$_{20}$H$_{20}$O$_{6}$Cl: 502; found (ms): 502.

Isolation and identification of pigments from mycelium
Sokhlet extraction of the dried mycelium from 10 culture flasks with chloroform for 24 h yielded a dark orange viscous oil (ca. 0.5 g). Examination of the crude extract by tlc revealed the presence of two pigments in addition to monorden and the monocilins.Column chromatography over silica gel using ether–benzene (1:9) as eluant led to the separation of a yellow pigment and an orange pigment. The orange pigment, mp 228–227°C (dec.) from acetone, had the molecular formula C$_{20}$H$_{20}$O$_{6}$, and was shown to be identical (mp, ir, H nmr, ms (Donkersloot et al. 1972) with averufin (13). The yellow pigment, mp 245–247°C (dec.) from chloroform–ether, shows the following properties: ir (CHCl$_3$): 3200(weak, broad), 1645, 1630, 1601, 1580 cm$^{-1}$; H nmr (CDCl$_3$): δ 3.98 (s, 3H), 4.78 (dt, J = 8, 2Hz, 1H), 5.41 (dd, J = 2, 1.5 Hz, 1H), 6.40 (s, 1H), 6.47 (dd, J = 2, 1.5 Hz, 1H), 6.70 (dd, J = 8, 1 Hz, 1H), 6.77 (dd, J = 8, 1 Hz, 1H), 6.79 (d, J = 8 Hz, 1H), 7.49 (dd, J = 8, 1 Hz, 1H), 13.25 (s, 1H); uv (CH$_3$CN) $\lambda_{max}$: 240 (c 9100). Exact Mass calcd. for C$_{18}$H$_{19}$O$_{6}$: 324.0620; found (ms): 324.0623. Anal. calcd. for C$_{18}$H$_{19}$O$_{6}$: C 66.66, H 3.70; found: C 66.28, H 3.71.

Antifungal spectra and microscopic effects on fungal morphology
Antifungal activities of the three major compounds, monorden (I) and monocillin I (2) and III (3), were examined against the 16 fungi listed in Table 1. The effect of each compound was tested on the germination of asexual spores for the first nine fungi and on mycelial growth for others. For C. ulmi, effects on both phases were examined. Cultures of all the test fungi except E. harknessii were maintained on Difco PDA at 20°C in the dark.

For the germination tests, spore suspensions of the first eight fungi were prepared by adding sterile distilled water to a 10- to 14-day-old culture of the fungus and scraping the culture surface with a needle. The spores were washed by centrifugation at 2000 rpm for 5 min and suspended in sterile distilled water. Spore density was adjusted to about 4 × 10$^4$ spores/mL except for A. brassicae (10$^4$ spores/mL), and the spore suspension of each fungus was mixed with an equal volume of sterile 4% Difco malt extract broth. Heat shock was applied to the sporangiospores of Phycomyces blakesleeanus by keeping the spore suspension at 48°C for 1 h. Aliquots of a 4% ethanol solution of each antibiotic (0, 20, 50, 100, 200, and 400 µg/mL and also 1000 µg/mL for monocillin III) were then mixed with an equal volume of the spore suspension of each fungus. About 0.05 mL of each mixture was pipetted on to sterile clean cover slips that were placed in petri dish moist chambers.

Rust spores (peridermoid teliospores) did not germinate well in any liquid media tested; therefore, an agar medium was used for the germination tests. Equal volumes of melted 0.6% water agar and various dilutions (same as described above) of the antibiotic in 4% ethanol were thoroughly mixed on a Vortex mixer and a thin agar layer was made on sterile clean cover slips with the mixture, about 0.3 mL for each slip. Mature rust spores

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**Table 1. Effect of antibiotics produced by Monocillium nordinii on spore germination or mycelial growth of fungi**

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Monorden</th>
<th>Monocillin I</th>
<th>Monocillin III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L.C.</td>
<td>M.I.C.</td>
<td>L.C.</td>
</tr>
<tr>
<td><strong>Phycomyces blakesleeanus</strong></td>
<td>Burgeff</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Alternaria brassicae</em> (Berk.)</td>
<td>Sacc.</td>
<td>25</td>
<td>NL</td>
</tr>
<tr>
<td><em>Colletotrichum graminicola</em></td>
<td>(Ces.) G. W. Wils.</td>
<td>200</td>
<td>NL</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em> (Cda ex Fr.)</td>
<td>Sacc.</td>
<td>200</td>
<td>NL</td>
</tr>
<tr>
<td><em>Ceratoystis minor</em> (Hedge.)</td>
<td>Hunt</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Ceratoystis morata</em> (Rumb.)</td>
<td>Hunt</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Ceratoystis ulmi</em> (Buism.) C. Moreau</td>
<td>10</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td><em>Leposphaeria maculans</em> (Desm.) Ces. &amp; de Not.</td>
<td>10</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td><em>Endocronartium harknessii</em> J. P. Moore Y. Hiratsuka</td>
<td>10</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td><em>Pythium debaryanum</em> Hesse</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Rhizoctonia solani</em> Kuhn</td>
<td>10</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Sclerotinia sclerotiorum</em> (Lib.) de Bary</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td><em>Contophora puteana</em> (Shum. ex Fr.) Karst.</td>
<td>10</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td><em>Fomes pini</em> (Brot. ex Fr.) Karst.</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><em>Merulius ambiguous</em> Berk.</td>
<td>10</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td><em>Schizophyllum commune</em> Fr.</td>
<td>10</td>
<td>200</td>
<td>10</td>
</tr>
</tbody>
</table>

*Antibiotics were tested in the effect on spore germination of the first nine fungi listed and on mycelial growth of others at 0, 10, 25, 50, 100, 200 µg/mL, and also at 500 µg/mL only for monocillin III.  
*L.C. least concentration (micrograms per milliliter) among those tested which was required to reduce germination or growth rate of the test fungus.  
*NL indicates that 100% inhibition of spore germination or mycelial growth did not occur at the highest concentration.  
*N' indicates that significant reduction of germination did not occur at the highest concentration.
collected from a fresh rust sorus were then dusted evenly on the solidified agar surface and kept in petri dish moist chambers. Five cover slips per test fungus were removed after incubating for 24 h at 20°C in the dark and germination rates were determined by counting 200 spores per slip. Morphological alterations of spores and germ tubes caused by the antibiotics were observed periodically up to 48 h of incubation. Inhibitory effect on the mycelial growth of test fungi was tested on 6-cm petri plates containing PDA supplemented with various dilutions (same as above) of each antibiotic. The PDA plates were inoculated with the test fungi (three replicates for each fungus) and incubated at 20°C in the dark. Colony diameters were measured at daily intervals.

Results and discussion

Chemistry of the metabolites

In the earlier work (A. Tsuneda and Y. Hiratsuka, in preparation) the crude metabolites of *M. noridini* were obtained by ether extraction of the PDA on which the fungus was grown. The same mixture of metabolites, as determined by tlc, were produced in good yield (0.335 g/mL) when the fungus was grown on potato dextrose liquid medium. Separation of the crude metabolites by column and thin layer chromatography led to the isolation of six compounds: monorden (1), and five new compounds, named, in order of increasing polarity, monocillin I, II, III, IV, and V.

Monorden (1) was identified by comparison of its properties (mp, ir, uv, 1H nmr, and ms spectra) with those published (McCrapa et al. 1964; Mirrington et al. 1964, 1966) for the antibiotic first isolated by Delmotte and Delmotte-Plaquée (1953) as a metabolite of *Monosporium bonorden*. This same compound 1 has also been isolated from *Nectria radicicola* cultures (Miryington et al. 1964) where it was given the name radicicol. Since the compound was first isolated from a *Monosporium* species, and is here isolated from a *Monocillinum* species, we use the name monorden for 1.

The structure of monocillin I (2), which differs from monorden (1) only in that the chlorine on the aromatic ring is replaced by hydrogen, was readily apparent from the 400-MHz 1H nmr spectrum which shows two aromatic protons (δ 6.42 and 6.46) with meta coupling (3 Hz) in place of the one-proton singlet at δ 6.63 in the spectrum of monorden. In other respects the 1H nmr (there are some differences in some chemical shifts), ir, and uv spectra of 1 and 2 are very similar.

Monocillin III differs from monocillin I in that it has one less unsaturation. The 1H nmr spectrum shows two less olefinic protons but the ir spectrum still shows the conjugated ketone carbonyl group (1645 cm⁻¹, coincident with the intramolecularly hydrogen-bonded ester). The spectroscopic evidence thus suggested structure 3 for monocillin III and, indeed, when monocillin I was allowed to ab-

\[
\begin{align*}
1 & \quad R = \text{Cl}; R' = \text{H} \\
2 & \quad R = \text{H}; R' = \text{H} \\
7 & \quad R = \text{Cl}; R' = \text{CH}_3
\end{align*}
\]

\[
\begin{align*}
3 & \quad R = \text{H} \\
4 & \quad R = \text{CH}_3
\end{align*}
\]

\[
\begin{align*}
5 & \quad R = \text{H} \\
6 & \quad R = \text{H} \\
7 & \quad R = \text{Cl}
\end{align*}
\]

\[
\begin{align*}
8 & \quad R = \text{Cl} \\
9 & \quad R = \text{H} \\
10 & \quad R = \text{H}
\end{align*}
\]

\[
\begin{align*}
11 & \quad R = \text{H} \\
12 & \quad R = \text{COPh}
\end{align*}
\]

\[
\begin{align*}
13 & \quad R = \text{H} \\
14 & \quad R = \text{H}
\end{align*}
\]
when it was found that treatment of monocillin II (4) with one equivalent of \( m \)-chloroperbenzoic acid gave monocillin III (3) in high yield, confirming the structure as 4.

Monocillin IV (5) and monocillin V (6) both show saturated ketone absorption (1705 cm\(^{-1}\)) in the infrared. The \(^1\)H nmr spectrum of monocillin IV reveals the presence of two olefinic hydrogens. Assuming that this unsaturation might occupy the same position as the unconjugated double bond in monocillin II (4), monocillin II was treated with hydrogen loaded Raney nickel in chloroform–ethanol (1:19), conditions which favor hydrogenation of the conjugated double bond of an \( \alpha,\beta \)-unsaturated ketone (Cornubert et al. 1952). The product, formed in good yield, was identical with monocillin IV, confirming structure 5 for the latter. Similar hydrogenation of monocillin III (3) gave monocillin V, showing that it possesses structure 6.

Attempts to directly correlate monocillin I (2) with monorden (1) by chlorination of the former were unsuccessful, as were attempts to substitute hydrogen for chlorine in monorden (1). The source of the chlorine found in the metabolite I was not immediately obvious to us, but was eventually traced to the Difco potato dextrose, which was shown by analysis to contain about 0.3% chloride.

An independent proof of the trans nature of the epoxide in monorden, first suggested earlier (Mingrington et al. 1966), was obtained in the following way. The epoxide ring in monorden dimethyl ether (7) was deoxygenated to the corresponding olefin 8 by treatment with triphenylphosphine selenide in the presence of a catalytic amount of trifluoroacetic acid (Clive and Denyer 1973). This reaction has been shown by Clive and Denyer (1973) to be stereospecific with retention of configuration. The proton at C-8' (zearalenone numbering, see 9) in the \(^1\)H nmr spectrum of 8 appears at \( \delta \) 5.70 as an eight-line signal \( (d, d, d, 15, 7, 1 \text{ Hz}) \). When the methylene group at C-9' (multiplet at \( \delta \) 2.68) was simultaneously irradiated (concurrent spin-decoupling of the C-10' hydrogen (\( \delta \) 5.24) to a quartet shows that the C-9' protons are being irradiated), the signal for the C-8' proton appears as a doublet, coupled to the proton on C-7' by 15 Hz, indicating (Jackman and Sternhell 1969) the trans stereochemistry of the C-7', C-8' double bond, and thus of the original epoxide.

The absolute stereochemistry depicted for the epoxide ring of monorden (1), monocillin I (3), and monocillin V (6), was determined by application of Brewster's benzoate rule (Brewster 1961). Treatment of monorden dimethyl ether (7) with hydrogen-loaded Raney nickel in 95% ethanol gave a mixture of tetrahydromonorden dimethyl ether (10), identical with that previously reported (Mingrington et al. 1966), and the alcohol 11, formed by hydrogenolysis and hydrogenation. The alcohol 11 was benzoylated to give 12. Application of the benzoate rule \( ([M]_D^{12} - [M]_D^{11} = -46) \) indicates that C-8' in 11 has the R-configuration, and thus the epoxides in 1, 3, and 6 have the absolute configuration shown. Experiments directed toward determination of the configuration at C-10' are underway.

Extraction of the mycelium of \( M. \) nordii obtained from the liquid cultures provided small amounts of monorden and the monocillins, along with an orange pigment identified as averufin (13) (Donkersloot et al. 1972), and a yellow pigment \( C_{18}H_{12}O_6, \text{mp} 246^\circ \text{C (dec.),} \) which has not been identified.

**Antifungal activity of the three major metabolites**

The antifungal spectra of monorden (1), monocillin I (2), and monocillin III (3) are shown in Table 1. Fungi susceptible to both monorden and monocillin I include a wide variety of fungi such as \( P. \) blakesleeanus (Zygomycotina), \( P. \) debaryanum (Mastigomycotina), \( C. \) ulmi (Ascomycotina), and \( F. \) pini (Basidimycotina), indicating that their antifungal activities are nonspecific. The inhibitory effect on monorden is only slightly stronger than that of monocillin I toward most of the test fungi. This suggests that the chloride on the aromatic ring of monorden does not play an important role in the bioactivity. The only exception is \( E. \) harknessii, the host fungus of \( M. \) nordii (A. Tsuena and Y. Hiratsuka, in preparation); concentrations of monorden and monocillin I which cause complete inhibition of the rust spore germination are 10 and 200 \( \mu \)g/mL, respectively. This suggests that monorden may play a key role in the mycoparasitic action of \( M. \) nordii on the rust sori in nature. The question arises, however, as to whether the rust galls provide a sufficient source of chlorine for the production of an effective amount of monorden. This aspect is worthy of further investigation.

The antifungal activity of monocillin III is negligible. Preliminary experiments showed that monocillin II, IV, and V also have very weak activity. It seems that alterations in the macrocyclic ring of monorden (1) and monocillin I (2) result in a significant loss of activity.

Both monorden and monocillin I cause morphological alterations and degeneration in spores and germ tubes of the test fungi, except in \( C. \) graminicola, \( F. \) avenaceum, and \( L. \) maculans, which show only re-
tarded growth of germ tubes. The effects on the morphology of Ceratocystis spp. are especially prominent, and these as they appear in C. ulmi are illustrated in Figs. 1-4. Other Ceratocystis spp. respond similarly. As illustrated in Fig. 1, asexual reproduction of C. ulmi appears to be divided into three stages: (1) swelling of ungerminated spores, (2) emergence of germ tubes, and (3) conidiation. A nearly complete suppression of the first stage is caused by both compounds at 200 μg/mL, the second stage at 25 μg/mL, and the third stage at 10 μg/mL. At the lowest concentration, large parts of the germ tubes show signs of degeneration and eventually the growing tips are degenerated (Fig. 2). Bursting of abnormally swollen spores is frequent at concentrations between 25-100 μg/mL (Fig. 4). The abnormal swelling also occurs in the hyphal cells formed on PDA containing 10 μg/mL of monorden or monocillin I. On the PDA plates, C. ulmi ceases to grow when the colony size becomes about 2 mm and the colonies consist of densely packed swollen hyphal cells (Fig. 3). Some of these cells separate readily to become arthrosporelike structures (Fig. 3).

Germination of A. brassicace conidia (Fig. 5) is not completely suppressed by any of the compounds (Table 1), however, both monorden and monocillin I induce abnormal bulb formation in the germ tubes at and above 25 μg/mL; the higher the concentration, the more extensive the formation of the bulbs (Figs. 6-8). The terminal bulbs eventually lose their cell contents or sometimes proliferate into filamentous germ tubes (Fig. 7, arrow), but only at lower concentration. At 100 and 200 μg/mL, germ tubes often become inflated right after their emergence to form thin-walled saclike structures.
Figs. 5–8. Effects of monorden and monocillin I on *Alternaria brassicae*. Fig. 5. Germination of a conidium in the absence of the antibiotics (control). Fig. 6. Terminal swelling of a germtube caused by monocillin I at 25 µg/mL. Fig. 7. Terminal swellings of germtubes caused by monorden at 50 µg/mL. Reemergence of a filamentous germtube (arrow) sometimes occurs from the swollen bodies at this concentration. Fig. 8. Saclike swellings (arrows) emerged from conidial cells under the influence of monorden at 100 µg/mL. These structures appear to be devoid of most of the cell contents.

Figs. 9–12. Effects of monocillin I (100 µg/mL) on spores of *Endocononartium harknessii*. Fig. 9. Germination in the absence of the compound (control). Fig. 10. Terminal swelling of a germtube. Fig. 11. Swelling of an entire germtube and subsequent degeneration of the cell. Fig. 12. Reemergence of filamentous germtubes from a swollen body. Figs. 13–15. Effects of monorden and monocillin III on *Phycomyces blakesleeanus*. Fig. 13. Germination of sporangiospores in the absence of the antibiotics (control). Fig. 14. Bursting of a swollen spore caused by monorden at 10 µg/mL. Fig. 15. Abnormal branching (arrows) and degeneration of a germtube induced by monocillin III at 300 µg/mL. Figs. 5–15. x 570.
(Fig. 8) that eventually collapse. A similar phenomenon occurs in *E. harknessii* in response to monocillin I. At 10 and 25 μg/mL, spores germinate as well as in the control (Fig. 9) and produce morphologically normal germ tubes. However, at 50 and 100 μg/mL the tips, or the entire length of the germ tubes become swollen (Figs. 10, 11), forming bulbs or saclike structures that eventually degenerate. In some cases, filamentous germ tubes reemerge from the swollen structures (Fig. 12), but they soon cease to grow.

The heat-treated sporangiospores of *P. blakesleeanus* germinate quickly in the absence of the antibiotics (Fig. 13). However, both monorden and monocillin I suppress germination completely at 10 μg/mL and often cause an abnormal swelling and bursting of the spores (Fig. 14). Monocillin III induces abnormal branching and subsequent degeneration of the germ tubes at 50 μg/mL (Fig. 15).

Evans and White (1966) reported that radicicol (= monorden), a metabolite of *Nectria radicicola*, caused terminal and intercalary swellings in hyphae of *Aspergillus niger* and *Septoria lycopersici*. Our results show that both monorden and monocillin I cause not only the abnormal swelling of spores and germ tubes but also an eventual degeneration or bursting of the swollen cells in some fungi. The exact mechanism which induces this phenomenon has not been determined, but we speculate that the antibiotics either impair the permeability control of the plasma membrane or inhibit cell wall synthesis of susceptible fungi, or perhaps upset both of these functions. Tewari and Skoropad (1979) reported that hyphae of *A. brassicaceae* formed terminal or intercalary swellings and one or more cells of the conidia developed saclike swellings in the presence of polynoxins, antibiotics produced by *Streptomyces cacaoi* var. *asoenis*. These morphological abnormalities are strikingly similar to those induced by monorden and monocillin I. Polynoxins cause an imbalance between the growth of the cell wall and other cellular elements by competitively inhibiting chitin-UDP acetylglucosaminyltransferase (Endo et al. 1970; Hori et al. 1974; Benitez et al. 1976). The structures of monorden and monocillin I, however, are quite different from those of the polynoxins, and their mode of action on cell wall synthesis, if any, is probably different from that of the polynoxins. Similar morphological abnormalities were also reported to be caused in *Alternaria alternata* by bulbiformin, a metabolite of *Bacillus subtilis* (Vasudeva et al. 1958).

Our preliminary experiments indicate that neither monorden nor monocillin I are toxic to higher plants and animals. Greenhouse experiments and field trials to test the feasibility of using the two compounds to control plant diseases, especially Dutch elm disease, are planned.

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