New Bioactive Metabolites from a Freshwater Isolate of the Fungus Kirschsteiniothelia sp.

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NEW BIOACTIVE METABOLITES FROM A FRESHWATER ISOLATE OF THE FUNGUS *KIRSCHSTEINIOTHELIA* SP.

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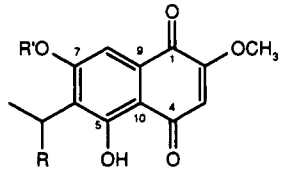
ABSTRACT.—The new cytotoxic naphthoquinone dimer kirschsteinin [**5**], two new chlorinated diphenyl ethers **8** and **9**, three known naphthoquinone derivatives **1**, **2**, and **7**, a monoacetyl derivative of **2**, and the (−)-enantiomer of *O*-methylasparvenone [**4**], have been isolated from a previously undescribed species of *Kirschsteiniothelia*. The structures of these compounds were assigned primarily by nmr studies and by spectral comparisons.

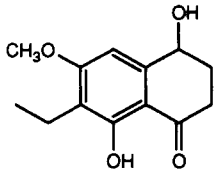
During our investigations of marine and aquatic fungi as sources of novel biologically active secondary metabolites (1–3), we examined an isolate of a previously undescribed species of *Kirschsteiniothelia* (Pleosporales, Loculoascomycetes). Organic extracts of both the culture filtrate and the mycelium from submerged cultures of this isolate showed antimicrobial activity. Chemical investigations of these extracts have led to the isolation of a new cytotoxic, unsymmetrically-substituted naphthoquinone dimer, along with two new chlorinated diphenyl ethers, four naphthoquinone derivatives, and a partially reduced naphthoquinone derivative. These studies represent the first reported chemical examination of a *Kirschsteiniothelia* species.

An isolate of *Kirschsteiniothelia* sp., C-76-1, originally obtained from submerged wood in a freshwater stream, was grown in liquid shake culture with a peptone/yeast extract/glucose medium. After 12 days, the EtOAc-Me_2CO (9:1) extract of the culture filtrate was subjected to cc on Si gel to afford five major components. Four of these metabolites were identified as 6-ethyl-2,7-dimethoxyjuglone [**1**], 6-(1-hydroxyethyl)-2,7-dimethoxyjuglone [**2**], a monoacetyl derivative of **2** [**3**], and (−)-*O*-methylasparvenone [**4**]. The structures of these compounds were established by comparison of their spectral properties (nmr, ms, ir, uv) to literature values for compounds **1** and **2** and the (+)-enantiomer of **4**, all of which had been previously reported (4–6).

Analysis of the fifth metabolite (**5**) by hreims led to establishment of the molecular formula $C_{26}H_{20}O_{11}$ ($[M]^+$ 508.0986; Δ 1.9 mmu). The uv and ir spectra suggested the presence of a naphthoquinone subunit in the molecule. The ^{13}C-nmr data (Table 1)



1 R=H, R′=Me
2 R=OH, R′=Me
3 R=OAc, R′=Me
7 R=R′=H



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[1]Alfred P. Sloan Fellow (1990–92) and NIH Research Career Development Awardee (1990–95; K04 CA 01571).

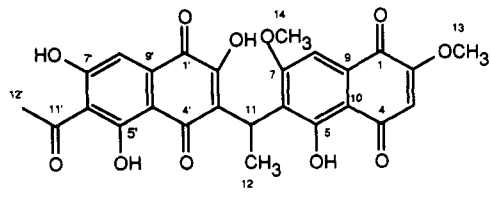
TABLE 1. Nmr Data for Kirschsteinin [**5**].[a]

Position	^{1}H	^{13}C	Selective INEPT Correlations
1	—	179.4 (s)	
2	—	160.5 (s)	
3	6.01 (s)	110.0 (d)	1, 2, 4, 5[b], 10
4	—	189.9 (s)	
5	—	160.8 (s)	
6	—	126.8 (s)	
7	—	162.7 (s)	
8	7.24 (s)	103.4 (d)	1, 6, 7, 9, 10
9	—	130.4 (s)	
10	—	109.0 (s)	
11	4.94 (q, 7 Hz)	28.2 (d)	5, 6, 7, 12, 2′, 3′, 4′
12	1.69 (d, 7)	17.1 (q)	6, 11, 3′
13	3.87 (s)	56.5 (q)[c]	2
14	3.97 (s)	56.4 (q)[c]	7
1′	—	180.7 (s)	
2′	—	153.9 (s)	
3′	—	125.6 (s)	
4′	—	189.7 (s)	
5′	—	166.4 (s)	
6′	—	113.5 (s)	
7′	—	169.4 (s)	
8′	7.15 (s)	109.4 (d)	1′, 6′, 7′, 10′
9′	—	128.0 (s)	
10′	—	106.5 (d)	
11′	—	205.4 (s)	
12′	2.76 (s)	33.5 (q)	6′, 11′
5-OH	12.81 (s)	—	5, 6
2′-OH	7.57 (br s)	—	
5′-OH	14.58 (s)	—	5′, 10′
7′-OH	14.12 (s)	—	7′

[a]Data recorded in $CDCl_3$ at 360 and 90.7 MHz, respectively.
[b]A four-bond correlation. All other signals represent two- or three-bond correlations.
[c]These carbon assignments are interchangeable.

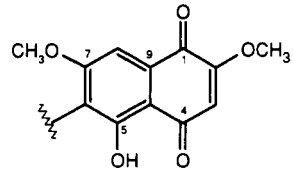
confirmed the presence of twenty-six carbons, and included signals for one ketone carbon, four quinone carbonyl groups, six oxygenated aromatic or ester carbons, ten other sp^2 carbons, two methoxy groups, and three aliphatic carbons at 33.5 (q), 28.2 (d), and 17.1 ppm (q). The ^{1}H-nmr spectrum contained resonances corresponding to three aromatic or vinylic protons, two methoxy groups, an acyl or aryl methyl group, and an isolated CH-Me unit.

These data were consistent with the presence of two naphthoquinone subunits in **5**.

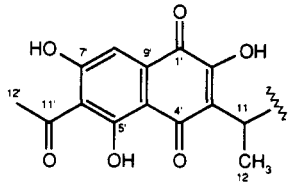


5

The close similarity of the ^{1}H- and ^{13}C-nmr data for compound **1** to selected signals for compound **5** provided strong evidence that the latter compound contained a similar hydroxydimethoxynaphthoquinone subunit (**a**). One-bond carbon-proton correlations for **5** were based on the results of single-frequency heteronuclear decoupling experiments. A series of selective INEPT (7) experiments confirmed the connectivity of the hydroxydimethoxynaphthoquinone subunit as shown in **a**. This assignment was further supported by ^{1}H-^{1}H decoupling experiments which clearly showed sharpening of each individual MeO singlet upon irradiation of the neighboring (ortho) aromatic proton signal. ^{13}C-nmr chemical shift assignments for **1** (6) and subunit **a** are in very close agreement, with each carbon shift in **a** differing by no more than 0.8 ppm from the corresponding value for **1**.



a



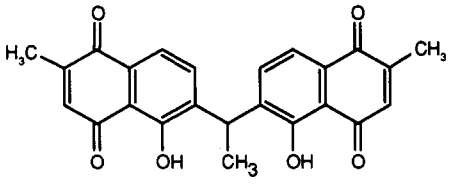
b

A major fragment ion at *m/z* 274.0476 in the hreims of **5** could not be attributed to subunit **a**, and must correspond to the remaining portion of **5**, which has the formula $C_{14}H_{10}O_6$. Ten of the carbons and two oxygens could be accounted for by a second naphthoquinone skeleton. This left the isolated CH-Me unit, the acyl unit, three exchangeable (phenolic) protons, and one isolated aromatic or olefinic proton to be assigned. Further selective INEPT experiments (Table 1) were performed to determine the substitution pattern of the second subunit, **b**, which was ultimately assigned as a substituted trihydroxynaphthoquinone moiety. Irradiation of the aliphatic methine proton (H-11) produced correlations with one of the quinone carbonyl carbons (C-4′), and two other carbons of **b**, locating the CH-Me group on the quinone ring at C-3′, identifying carbons 2′, 3′, and 4′, and implying hydroxylation at C-2′ (153.9 ppm). Irradiation at the isolated proton singlet (7.15 ppm) afforded correlations with three of the aromatic carbons, verifying its location on the aromatic ring of subunit **b**. A key correlation was also observed to the second quinone carbon signal of **b** (180.7 ppm, C-1′) in an experiment optimized for 14 Hz, indicating placement of the isolated proton at C-8′. The upfield carbon shift of C-8′ (109.4 ppm) indicates that H-8′ is ortho and/or para to at least one oxygenated carbon. The substituents remaining to be placed on C-5′, C-6′, and C-7′ included two OH groups and one acetyl moiety. Irradiation of the acetyl group methyl proton signal (H_3-12′) resulted in a correlation with the signal at 113.5 ppm (C-6′), securing the position of this group. The extreme downfield shifts of the two hydroxylated carbon signals (166.4 and 169.4 ppm) are only consistent with a meta relationship, requiring the substitution pattern shown for **b**. Observation of correlations between H-8′ and all of the aromatic carbon signals for **b** except 166.4 ppm permitted tentative assignment of this carbon as C-5′.

Linkage of the two subunits as shown in **5** was clearly indicated by the selective INEPT results for H-11, which showed polarization transfer to carbons 5, 6, 7, 2′, 3′, and 4′. Irradiation at 1.69 ppm (H_3-12) resulted in confirmatory correlations to carbons 3′, 6, and 11. The absolute stereochemistry at C-11 remains to be assigned.

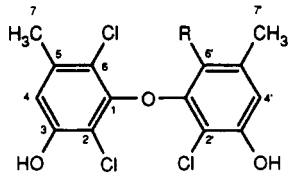
Compound **5**, which we have named kirschsteinin, is a rare example of a naphthoquinone dimer in which the monomer units are connected by an ethylidene link-

age. Most of the known naphthoquinone dimers occur in a direct head-to-head or tail-to-tail linkage. There are a few examples of symmetrical dimers linked by an ethylidene unit [e.g., ethylidene-6,6′-biplumbagin [**6**] from the fruit of *Diospyros maritima* (8)]. Kirschsteinin is to our knowledge the first unsymmetrically substituted example of this type, and the first to exhibit a head-to-tail linkage. Kirschsteinin showed antimicrobial activity versus *Bacillus subtilis* and *Staphylococcus aureus* in disk assays at 1.0 and 10 μg/disk, respectively. In screens for cytotoxicity toward human solid tumor cell lines (9), kirschsteinin afforded ED_{50} values of 3.1, 5.2, and 2.7 μg/ml against human non-small lung carcinoma A-549, breast adenocarcinoma MCF-7, and colon adenocarcinoma HT-29 cells, respectively.



6

The EtOAc-Me_2CO (9:1) extract of the mycelium also showed antimicrobial activity and was subjected to cc resulting in the isolation of two major components and three minor metabolites. The major components were found to be **1** and **5**, while one of the minor constituents was found to be the known compound 6-ethyl-7-hydroxy-2-methoxyjuglone [**7**] by comparison of its spectral data (nmr, ms, ir, uv) to those of its methylated analogue **1** (4,5), and by comparison of its mp to that previously reported for **7** (10). Analysis of a second minor component **8** by hrgc-eims suggested the molecular formula $C_{14}H_{11}O_3Cl_3$ ($[M]^+$ 331.9781; Δ 0.7 mmu). The ^{13}C-nmr data revealed the presence of four oxygenated sp^2 carbons, eight other sp^2 carbons, and two sp^3 carbons. The ^1H-nmr spectrum contained signals for three aromatic protons and two aryl methyl groups. Although most of the nmr data for this compound was measured in CD_3OD due to better solubility, a spectrum taken in $CDCl_3$ revealed two phenolic OH proton resonances. The eims data for the remaining minor component **9** indicated that it has one additional chlorine and one less hydrogen atom than **8** ($C_{14}H_{10}O_3Cl_4$). Analysis of the ^1H- and ^{13}C-nmr data clearly indicated a symmetrical structure for **9**, with two proton signals and seven carbon signals virtually identical to signals present in the spectra for **8**. The only signals observed in the ^1H-nmr spectrum of component **9** were indicative of an aromatic proton (6.31 ppm) and an aryl methyl group (2.28 ppm). The remaining protons must be exchangeable protons of two identical phenolic OH groups. Based on these data and the elemental composition, compound **9** must be a symmetrical diphenyl ether with both rings substituted by a proton, a methyl group, an hydroxyl group, and two chlorine atoms. Proton decoupling and selective INEPT experiments revealed that the aromatic proton and the methyl group are located ortho to each other. In addition, these data showed that the methyl group is not ortho to either oxygen substituent, and must by default be ortho to a chloro group. No further conclusions could safely be drawn from selective INEPT correlations to the aromatic proton signal. The downfield chemical shifts of the oxygenated carbons indicated that they must be meta to one another, and this was consistent with the upfield shifts of the other non-oxygenated carbons (112.1, 112.2, and 118.4). The only structures that satisfy these conditions are **9** and an alternate structure, in which the 4-H and the 6-Cl are interchanged on each ring.



8 R=H
9 R=Cl

Analogous proton decoupling and selective INEPT experiments indicated that **8** is also a polysubstituted diphenyl ether, and revealed the possible substitution patterns for each ring. One phenyl ring of **8** showed ^{1}H- and ^{13}C-nmr shifts virtually identical to those of **9**, suggesting an identical substitution pattern. The second ring of **8** differed only in that one chloro group was replaced by a proton. The ^{1}H-^{1}H coupling (1.8 Hz) is consistent with a meta relationship between the two protons. Selective INEPT and proton decoupling experiments located the methyl group between the two aromatic protons. Both protons are shifted upfield (112.4 ppm and 113.9 ppm), suggesting that both are ortho and/or para to oxygenated aromatic carbons. However, as in **9**, the substitution patterns could not be conclusively determined from these data.

The structures of **8** and **9** were verified by ^{1}H-nmr experiments conducted on the dimethylated derivative of **8** formed by reaction with CH_2N_2. The new methoxy proton signals showed NOESY correlations to H-4 and H-4′, and irradiation of each aromatic proton signal resulted in sharpening of a different OMe proton signal. These data indicated that the aromatic protons at C-4 and C-4′ were ortho to phenolic groups

TABLE 2. Nmr Data for **8** and **9**.

Position	^{1}H	^{13}C	Selective INEPT Correlations
Compound **8**			
1	—	152.0 (s)	
2	—	112.1 (s)	
3	—	152.9 (s)	
4	6.31 (q, 0.7)	112.5 (d)	1, 2, 3, 6, 7
5	—	137.0 (s)	
6	—	118.4 (s)	
7	2.28 (d, 0.7)	20.6 (q)	4, 5, 6
1′	—	154.1 (s)	
2′	—	110.6 (s)	
3′	—	156.0 (s)	
4′	6.57 (dd, 0.6, 1.8)	113.9 (d)	2′, 3′, 6′, 7′
5′	—	139.5 (s)	
6′	6.21 (dd, 0.6, 1.8)	112.4 (d)	1′, 2′, 4′, 7′
7′	2.19 (br s)	21.5 (q)	4′, 5′, 6′
Compound **9**			
1	—	152.1 (s)	
2	—	112.1 (s)	
3	—	152.2 (s)	
4	6.31 (br s)	112.5 (d)	2, 3, 6
5	—	137.0 (s)	
6	—	118.3 (s)	
7	2.25 (br s)	20.4 (q)	4, 5, 6

[a]Data recorded in CD_3OD at 360 and 90.7 MHz, respectively.

in the natural product. This result permitted assignment of the structure of **8** as shown, and the structure of **9** was proposed by analogy based on the chemical shift comparison described above. Compounds **8** and **9** each showed antibacterial activity versus *B. subtilis* and *S. aureus* in disk assays at 5 and 1 μg/disk, respectively. Although halogenated aromatics are not uncommon among the fungi, neither **8** nor **9** has been previously reported to our knowledge.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—General procedures and instrumentation employed in this work have been described previously (1).

ISOLATION AND DESCRIPTION OF *KIRSCHSTEINIOTHELIA* SP.—*Kirschsteiniothelia* sp. C-76-1 (ATCC 76730) was originally isolated from submerged wood collected in December, 1984 from a natural thermal stream in Puyehue, Chile. The name *Kirschsteiniothelia funicularia* will be proposed for this new species in a manuscript that is in preparation. A subculture has been deposited in the American Type Culture Collection in Rockville, MD (accession number ATCC 76730), and specimens will also be deposited at the Herbarium of the New York Botanical Garden. This new species is most closely related to *Kirschsteiniothelia aethiops* (Berk. and Curtis) D. Hawksw. (11). It differs from *K. aethiops* by having ascomata with longer beaks, larger asci, and an endoascus with an elongated and coiled base. The asci of *K. aethiops* have a more complex ascus apical apparatus, and the warts on the ascospores are more prominent and irregular in shape.

CULTIVATION AND CHEMICAL ISOLATION PROCEDURES.—This culture was used to inoculate ten 2-liter Erlenmeyer flasks, each containing 400 ml of a medium containing peptone (1.3%), yeast extract (1.3%), and glucose (3.0%). Flask cultures were inoculated at 25–28° and aerated by agitation on an orbital shaker at 180 rpm for 12 days. The cultures were filtered, and the mycelium and the filtrate were independently extracted with EtOAc-Me_2CO (9:1). In each case, the organic phase was dried ($MgSO_4$) and evaporated to afford an orange oil (ca. 700 mg each). Due to differences in composition indicated by tlc, the oils were chromatographed separately on Si gel ($CHCl_3$/MeOH gradient), followed by partition of active fractions with Sephadex LH-20 using CH_2Cl_2/Me_2CO/hexane mixtures (12).

METABOLITES ISOLATED FROM THE FILTRATE.—The major component **1** (32.8 mg total) eluted from a Sephadex LH-20 column as an orange-yellow crystalline solid from CH_2Cl_2-hexane (4:1) and was identified as 6-ethyl-2,7-dimethoxyjuglone. Physical and spectral data for **1** have been reported previously (4,5).

6-(1-Hydroxyethyl)-2,7 dimethoxyjuglone [**2**] (4.3 mg) and its monoacteyl derivative **3** (25.4 mg) both eluted as yellow crystalline solids from CH_2Cl_2-Me_2CO (3:2). Compound **2** has been reported (5), and only data not included in the prior report are given here: $[\alpha]_D$ −7.6° (c = 0.5 g/dl, CH_2Cl_2); eims (70 eV) *m/z* $[M]^+$ 278 (rel. int. 15%), 260 (100), 245 (31), 231 (86), 217 (37), 201 (17), 189 (21), 175 (18), 161 (38), 145 (13), 131 (11), 118 (12), 115 (18), 105 (23), 93 (10), 77 (40), 69 (55); ^{13}C nmr ($CDCl_3$) 190.0 (s), 179.2 (s), 162.0 (s), 160.7 (s), 160.1 (s), 130.8 (s), 126.8 (s), 109.3 (s), 109.2 (d), 103.2 (d), 63.7 (d), 56.6 (q), 56.4 (q), 22.8 (q). The monoacetyl derivative **3** has not been reported previously. Compound **3** gave the following data: $[\alpha]_D$ −41.6° (c = 1.8, CH_2Cl_2); uv max (MeOH) 381 (ε 1310), 303 (980), 268 (6090), 262 (8160), 232 (14800); eims (70 eV) *m/z* $[M]^+$ 320 (rel. int. 1.1%), 277 (10), 260 (68), 245 (23), 231 (54), 217 (27), 201 (18), 189 (14), 177 (20), 175 (20), 161 (24), 135 (20), 115 (15), 105 (31); ^1H nmr ($CDCl_3$) 7.21 (1H, s), 6.34 (1H, q, J = 6.8), 6.00 (1H, s), 3.98 (3H, s), 3.88 (3H, s), 2.03 (3H, s), 1.61 (3H, d, J = 6.8); ^{13}C nmr ($CDCl_3$) 190.3 (s), 179.8 (s), 171.0 (s), 163.0 (s), 161.6 (s), 160.9 (s), 132.0 (s), 123.1 (s), 110.0 (d), 109.5 (s), 103.5 (d), 65.2 (d), 57.0 (q), 56.9 (q), 21.5 (q), 18.7 (q).

Component **4** (12.1 mg) was eluted as a white crystalline solid from CH_2Cl_2-Me_2CO (3:1) and was found to be *O*-methylasparvenone, although it had an optical rotation opposite in sign to that of the *O*-methylasparvenone isomer reported in the literature. The $[\alpha]_D$ value was determined to be −25.1° (c = 0.6, MeOH), as compared to the reported value of +22° (c = 2.6, MeOH) for the literature compound (6). However, the absolute stereochemistry has not been determined.

Kirschsteinin [**5**] (8.8 mg) eluted from CH_2Cl_2-Me_2CO (3:2) as an orange crystalline solid, and gave the following data: mp 185–186°, $[\alpha]_D$ +59° (c = 1.3, CH_2Cl_2); uv max (MeOH) 419 (ε 2950), 306 (6040), 264 (7260), 218 (21900); ir 1674, 1627 cm^{-1}; eims (70 eV) *m/z* $[M]^+$ 508 (rel. int. 25%), 274 (30), 260 (18), 248 (20), 234 (100), 219 (13), 203 (29), 175 (10), 163 (25), 135 (34), 129 (10), 115 (10); ^1H nmr and ^{13}C nmr see Table 1; hreims found *m/z* $[M]^+$ 508.0986, calcd for $C_{26}H_{20}O_{11}$ 508.0967; major fragment ion observed at *m/z* 274.0476, calcd for $C_{14}H_{10}O_6$ 274.0477.

METABOLITES ISOLATED FROM THE MYCELIUM.—Components **1** (114 mg) and **5** (60.1 mg) were eluted from Sephadex LH-20 with hexane-CH_2Cl_2 (1:4) and CH_2Cl_2-Me_2CO (3:2), respectively. Component **7** (2.7 mg) eluted at CH_2Cl_2-Me_2CO (2:3) as a yellow crystalline solid and was found to be 6-ethyl-7-hydroxy-2-methoxyjuglone (10). ^{1}H-nmr data for **7** have not been reported previously: ($CDCl_3$) 12.65 (1H, s), 7.09 (1H, s), 6.00 (1H, s), 3.87 (3H, s), 2.72 (2H, q, $J=6$), 1.16 (3H, t, $J=6$).

Two minor compounds eluting with CH_2Cl_2-Me_2CO (4:1) were isolated as white crystalline solids **8** (4.3 mg) and **9** (5.0 mg). Compound **8** gave the following data: mp 171–172°; uv max (MeOH) 282 (2580), 242 (5740); ir (neat) 3366 (br), 2917, 1559, 1190, 1082 cm^{-1}; eims (70 eV) *m/z* $[M]^+$ 332 (rel. int. 15.4%), 334 (16), 336 (5.4), 297 (11), 262 (100), 227 (3.8), 198 (2.9), 131 (1.4); ^{1}H nmr and ^{13}C nmr see Table 2; hreims observed *m/z* $[M]^+$ 331.9781, calcd for $C_{14}H_{11}O_3Cl_3$ 331.9789. Compound **9** gave the following data: mp 176–177°; uv max (MeOH) 298 (ε 3920), 258 (5600); ir 3366, 2917, 1159, 1437, 1190, 1082 cm^{-1}, eims (70 eV) *m/z* $[M]^+$ 366 (rel. int. 13.7%), 368 (19), 370 (9.5), 331 (6.3), 296 (100), 261 (6.8), 232 (3.5), 148 (6.8), 111 (16); ^{1}H and ^{13}C nmr see Table 2.

METHYLATION OF COMPOUND **8**.—A sample of **8** (1.4 mg) was treated with an ethereal solution of CH_2N_2. The solution was allowed to stand for 30 min, and the solvent was then evaporated to afford the dimethylated derivative (1.4 mg) as a colorless oil. Compound **8**: eims (70 eV) *m/z* $[M]^+$ 360 (10), 362 (9.6), 364 (2.7), 325 (9.1), 290 (100), 275 (62), 260 (19), 212 (10), 144 (57); ^{1}H nmr (CD_3OD); 6.76 (1H, br s), 6.54 (1H, br s), 6.37 (1H, br s), 3.89 (3H, s), 3.88 (3H, s), 2.40 (3H, s), 2.37 (3H, s).

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