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Production of Unusual Dispiro Metabolites in *Pestalotiopsis virgatula* Endophyte Cultures: HPLC-SPE-NMR, Electronic Circular Dichroism, and Time-Dependent Density-Functional Computation Study

Julie R. Kesting,[†] Lars Olsen,[†] Dan Staerk,[†,‡] Mysore V. Tejesvi,[§,⊥] Kukkundoor R. Kini,[⊥] Harishchandra S. Prakash,[⊥] and Jerzy W. Jaroszewski*[,†]

[†]Department of Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, DK-2100 Copenhagen, Denmark

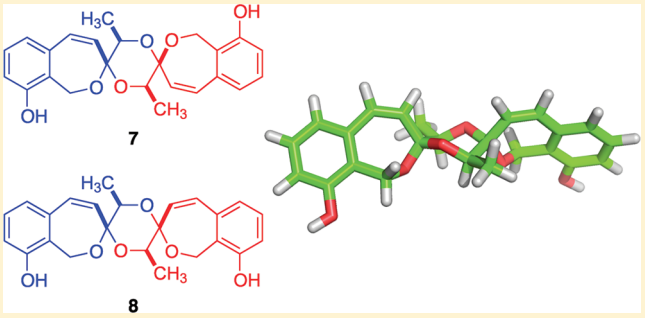
[‡]Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg, Denmark

[§]Department of Biology, Faculty of Science, University of Oulu, P.O. Box 3000, FI-90014 Oulu, Finland

[⊥]Department of Studies in Biotechnology, University of Mysore, Mysore-Karnataka, India

Supporting Information

ABSTRACT: The endophytic fungus *Pestalotiopsis virgatula*, derived from the plant *Terminalia chebula* and previously found to produce a large excess of a single metabolite when grown in the minimal M1D medium, was induced to produce a variety of unusual metabolites by growing in potato dextrose broth medium. Analysis of the fermentation medium extract was performed using an HPLC-PDA-MS-SPE-NMR hyphenated system, which led to the identification of a total of eight metabolites (**1**−**8**), six of which are new. Most of the metabolites are structurally related and are derivatives of benzo[*c*]oxepin, rare among natural products. This includes dispiro derivatives **7** and **8** (pestalospiranes A and B), having a novel 1,9,11,18-tetraoxadispiro[6.2.6.2]octadecane skeleton. Relative and absolute configurations of the latter were determined by a combination of NOESY spectroscopy and electronic circular dichroism spectroscopy supported by time-dependent density-functional theory calculations (B3LYP/TZVP level). This work demonstrates that a largely complete structure elucidation of numerous metabolites present in a raw fermentation medium extract can be performed by the HPLC-SPE-NMR technique using only a small amount of the extract, even with unstable metabolites that are difficult to isolate by traditional methods.



Endophytic fungi are microorganisms living in the intercellular space of plant tissues and are able to produce a large number of natural products belonging to various structural classes.[1−3] Endophytes have been shown to produce important anticancer agents originally isolated from plants, and it has been shown that many of these compounds are in fact produced both by the host plants and by the fungi.[4,5] *Pestalotiopsis* species (Amphisphaeriaceae) are distributed all over the world, with most species associated with living plants, and some being saprobes in soil or plant debris.[6−11] Various species of this genus have been reported to yield structurally diverse secondary metabolites.[12−21]

Previous reports on *Pestalotiopsis* species from several medicinally important plants indicated that these fungal extracts would also contain interesting natural products.[22,23] Recently, we reported that a *Pestalotiopsis virgatula* culture derived from the plant *Terminalia chebula* produces a simple metabolite with a benzo[*c*]oxepin skeleton,[24] which is novel in natural products. Detection and structure determination of this metabolite was accomplished using a small amount of crude extract of the fermentation broth and employing a coupling (hyphenation) of solid-phase extraction (SPE) with HPLC and NMR.[24] This hyphenated technique, HPLC-SPE-NMR, has proven to be an invaluable tool for fast dereplication of natural products extracts of both plant and fungal origin.[25−32] It enables complete structure elucidation of new compounds in complex mixtures following separation by analytical-scale HPLC. HPLC-SPE-NMR can serve as a preparatory step for targeted larger-scale isolation of selected metabolites initially identified by this technique.

In this report, we describe identification by HPLC-SPE-NMR of several new metabolites, including interesting structures with an unusual dispiro skeleton from the same isolate of *P. virgatula* as investigated previously,[24] but grown in a different culture medium. Thus, this report provides a striking example of

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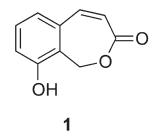
induction of production of an array of novel metabolites by alteration of fermentation conditions. Combination of HPLC-SPE-NMR with electronic circular dichroism (ECD) spectroscopy supported by time-dependent density-functional theory calculations[33] (TDDFT) of chiral electronic transitions provided a largely complete picture of structures and stereochemistry of these metabolites.



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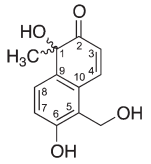

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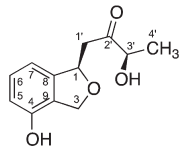

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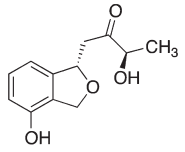

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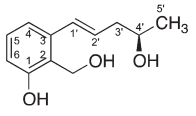

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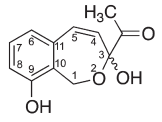

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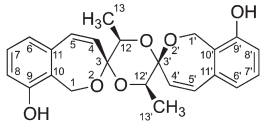

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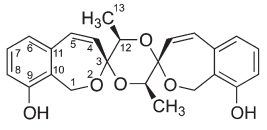

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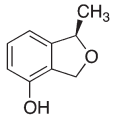
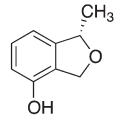


9a



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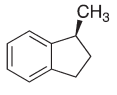

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RESULTS AND DISCUSSION

In the original study, *P. virgatula* was cultured in the minimal M1D medium.[24] Under these conditions, the culture produced practically exclusively a single metabolite, **1**.[24] Subsequently, we carried out cultivation of the same fungal isolate in potato dextrose broth (PDB) medium, a general-purpose medium for yeasts and molds. HPLC analysis revealed a striking difference in the metabolite pattern as compared to the M1D grown fungus. Thus, rather than being dominated by a previously observed single peak,[24] the chromatogram displayed a number of well-separated major constituents (Figure 1). Parallel HPLC-MS and HPLC-SPE-NMR analyses provided MS (both positive- and negative-ion mode) and NMR data for all major peaks (*a*−*h*) in the chromatogram. For the HPLC-SPE-NMR analysis, eight consecutive HPLC separations were performed, and the analytes adsorbed cumulatively on a general-purpose (GP) resin SPE stationary phase using UV traces at 235, 254, and 275 nm for peak trapping based on absorbance thresholds. After removal of chromatographic solvents from the trapping columns, the analytes were eluted into an NMR flow probe with CD_3CN. Alternatively, the SPE cartridges were eluted with CH_3CN for ECD measurements. 1D ^1H NMR spectra as well as 2D homo- and heteronuclear correlations, supported by MS data, led to the identification of the previously isolated compound **1**, eluted as peak *d*, with compounds **2**−**8** (all of which, except **2**, are new) eluting as the remaining peaks. HPLC-SPE-NMR analyses were performed using less than 40 mg of the crude extract.

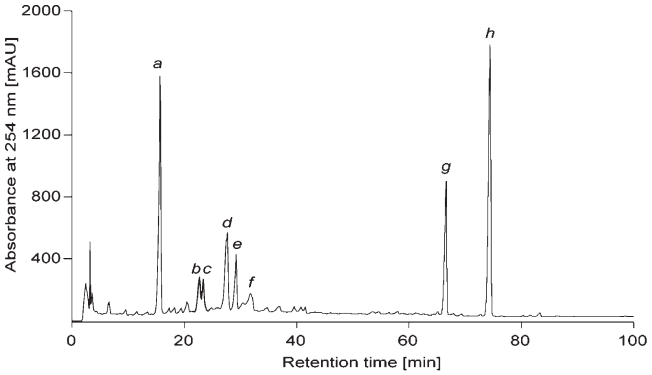

Figure 1. HPLC trace (254 nm) of the crude extract of *Pestalotiopsis virgatula* endophyte (isolate code TC-320[24]) grown in potato dextrose broth medium; 150 × 4.6 mm i.d. Phenomenex C18(2) Luna column, 3 μm, 40 °C, elution rate 0.8 mL/min, elution gradient as described in the Experimental Section.

Using data recorded in the hyphenated mode, the constituent eluted as peak *a* (t_R = 15.7 min; *m*/*z* 243.2, $[M + Na]^+$; *m*/*z* 219.1, $[M - H]^-$) was identified as the known fungal metabolite cyclosordariolone (**2**).[34,35] Following isolation of a sample of this material by preparative HPLC, it was found to be devoid of optical activity and ECD absorption. Thus, the material is evidently racemate or a scalemic mixture with a low enantiomeric excess of one enantiomer. Only two previous reports on this compound exist, one describing a (−)-form isolated from *Sordaria macrospora*[34] ($[\alpha]^{20}_D$ −78, CH_3OH), and the other reporting a (+)-form isolated from an unidentified hyphomycete ($[\alpha]^{20}_D$ +168, CH_3OH).[35] Since the reported specific rotations of these isolates differ strongly in spite of being determined in the same solvent, also the *S. macrospora* material[34] appears to be partly racemic.

The constituents eluted in the closely spaced and equally intense peaks *b* and *c* (t_R = 22.5 and 23.1 min) had the same molecular mass (*m*/*z* 245.2, $[M + Na]^+$; *m*/*z* 220.9, $[M - H]^-$). Their gross structures could be determined in the NMR hyphenated mode as the diasteromers **3** and **4** or their stereoisomers.

Table 1. NMR Data for Compounds 3 and 4[a]

	3		4		2D correlations[c]		
position	δ_H[b]	δ_C	δ_H[b]	δ_C	COSY	NOESY	HMBC[d]
1	5.60, m	81.1	5.59, m	81.2	1′a, 1′b	3a, 7, 1′a, 1′b	6, 7, 3a, 3b, 1′a, 1′b
3	a: 5.03, dd (11.9, 2.5)	71.3	a: 5.02, dd (12.1, 2.8)	71.2	3b	1, 3b	7
	b: 4.94, br d (11.9)		b: 4.93, dd (12.1, 0.8)		3a	3a	
4		152.1		152.1			3a, 3b, 5, 6
5	6.71, d (7.7)	115.1	6.71, d (7.7)	115.1	6	6	3a, 3b, 6, 7
6	7.13, t (7.7)	130.3	7.14, t (7.7)	130.3	5, 7	5, 7	5, 7
7	6.71, d (7.7)	113.7	6.73, d (7.7)	113.6	6	1, 6, 1′	5, 6
8		144.8		144.9			3a, 3b, 6, 7
9		126.4		126.4			3a, 3b, 5, 7
1′	a: 2.96, dd (16.6, 7.9)	45.3	a: 2.94, dd (16.5, 7.4)	45.2	1, 1′b	7, 1′b, 3′, 4′	3a, 3b
	b: 2.90, dd (16.6, 4.6)		b: 2.90, dd (16.5, 5.0)		1, 1′a	7, 1′a, 3′, 4′	
2′		211.9		211.9			1′a, 1′b, 3′, 4′
3′	4.19, m	74.0	4.18, m	73.2	4′, 3′-OH	1′, 4′	4′
4′	1.28, d (7.0)	19.8	1.26, d (7.0)	19.6	3′	1′, 3′	3′
4-OH	7.07, br s		7.10, br s				
3′-OH	3.54, br d (4.6)		3.55, br d (4.5)				

[a] ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra in CD_3CN (δ relative to internal TMS) of compounds isolated by preparative HPLC (see Supporting Information for spectra acquired in the hyphenated mode). [b] Coupling constants in Hz are given in parentheses; d, doublet; t, triplet; m, multiplet, br, broad signal. [c] The correlations observed for both compounds. [d] From the indicated proton to carbon in the same row.

Thus, the ^{1}H NMR spectra were similar and revealed the presence of a 1,2,3-trisubstitued benzene ring, two spin systems corresponding to CH_3-CH and CH_2-CH fragments, both with oxygenated methine carbons, and an additional oxygenated methylene group. Both methylene groups displayed geminally coupled diastereotopic proton resonances. The assembly of fragments was based on COSY, NOESY, HSQC, and HMBC data recorded in the HPLC-SPE-NMR mode.

In order to gain more information about the stereochemistry of the isomeric metabolites, they were isolated in a preparative-scale procedure to give a pure compound eluted as peak *b*, whereas the compound eluted as peak *c* was obtained admixed with 25% of the other isomer (^{1}H NMR). This enabled unambiguous individual NMR characterization (Table 1). ECD spectra of these compounds together with the UV spectrum of the fast-eluting stereoisomer are shown in Figure 2. ECD spectra of aromatic compounds with a directly attached stereogenic center display Cotton effects around 250−280 nm, corresponding to the ^1L_b transition of the benzene chromophore.[36] The sign of the ^1L_b Cotton effect depends on the configuration of the contiguous stereogenic center, but not those further apart.[36] Moreover, the sign of the ^1L_b Cotton effect depends on the type and position of additional, achiral substituents of the benzene ring;[36] this is due to the fact that ring substitution introduces bond transition moments that modulate coupling of the ^1L_b transition with the chiral group. On the basis of the reported UV absorption of the 1-alkyl-substituted 1,3-dihydroisobenzofuran-4-ol chromophore[37,38] and indeed the observed UV spectrum of **3** (see Figure 2; the UV spectrum of **4** was identical), the chiral ^1L_b transition in the ECD spectra of **3** and **4** is expected to be at 260−275 nm. From the large difference between molecular ellipticity of **3** and **4** in this region, it follows that the two compounds differ by the configuration of C-1; since they are diastereomers, the configuration of C-3′ must be the same. This conclusion is in agreement with previous reports on related fungal metabolites, i.e., (1,3-dihydro-4-hydroxy-1-isobenzofuran)acetic acid,[34] 1-(1,3-dihydro-4-hydroxy-1-isobenzofuranyl)butan-2,3-diol,[35] and 1-(1,3-dihydro-4-hydroxy-1-isobenzofuranyl)hex-4-en-2,3-diol,[37] which were also found to occur as mixtures differing in the configuration of C-1 in the 1,3-dihydroisobenzofuran moiety.

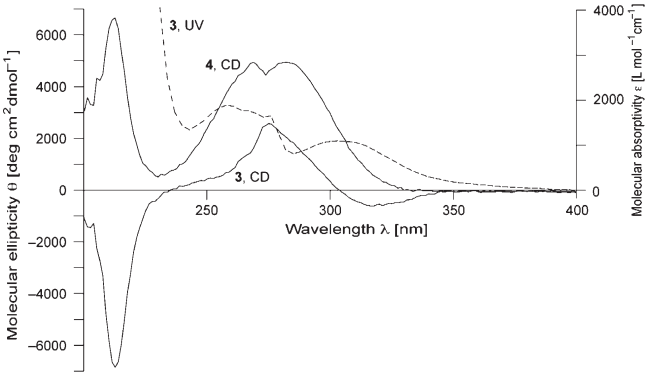

Figure 2. UV spectrum of compound **3** (stippled line) and ECD spectra of compounds **3** and **4**, eluted as peaks *b* and *c* (Figure 1), respectively, in CH_3CN; the ECD spectrum of **4** was corrected for the content of **3** (25% as determined by ^{1}H NMR spectroscopy).

Further indication for the lability of the C-1 stereocenter was obtained from hydrogen-to-deuterium exchange experiments. When **4** (1 mg) in CD_3OD (500 μL) was treated with diethylamine (30 μL) and the solution immediately examined by ^{1}H NMR spectroscopy (400 MHz), the signals of H-1′ disappeared due to deuterium exchange, while the H-3′ signal was unaffected. No such exchange was observed in the presence of pyridine, even after prolonged standing at room temperature (24 h) or heating to 50 °C. The base-catalyzed deprotonation of C-1′ gives the possibility of a reversible opening of the dihydrofuran ring by 1,2-elimination and hence epimerization at C-1, while the absence of

deuterium exchange at C-3′ confirms the configurational stability of this center under weakly basic conditions. The preferential deuteration at C-1′ as compared to C-3′ is explainable by destabilization of the alternative enolate by the electron-withdrawing effect of the hydroxy substituent.

In order to correlate the opposite contributions of the chiral aromatic chromophore to the ECD absorption of **3** and **4** (Figure 2) with the absolute configuration of C-1, TDDFT calculations[33] of ECD spectra of model compounds **9a** and **9b** were performed. Such calculations have previously proved useful for prediction of ECD spectra,[39−41] including ECD spectra of substituted aromatics.[42,43] Initially, the calculations were performed for the indane **10**, for which a negative Cotton effect at 274 nm was reported.[44] Compound **10** was geometry-optimized using the hybrid functional B3LYP method with the DZP or the TZVP basis set, and the UV and ECD transitions were determined using the TZVP or TZVPP basis sets (see Supporting Information). Since B3LYP calculations systematically overestimate electronic transition energies resulting in a blue shift of the calculated spectra, the predicted ECD spectra were corrected by parallel shift toward longer wavelengths by a value corresponding to the difference between experimental and calculated (from the same molecular geometry) UV absorption maxima (see Supporting Information).[45] Both basis sets yielded for **10** a negative Cotton effect around 275 nm, in agreement with experimental results.[44,46] For the model compounds **9a** and **9b**, for which no experimental data have been reported, two practically isoenergetic conformations were identified, with the hydroxy group periplanar to the aromatic ring and the hydroxy group proton either *anti* or *syn* to the methylene group. However, the hydroxy group conformation had no influence on the sign of the calculated Cotton effects associated with the long-wavelength transitions, which was negative for the *R*-enantiomer **9a** (as for **10**) and positive for the *S*-enantiomer **9b** (see Supporting Information).

On the basis of these results, we conclude that compounds **3** and **4**, apparently with negative and positive contributions from the ^1L_b Cotton effect to the total ECD absorption (Figure 2), have the *R*- and the *S*-configuration at C-1, respectively. That **3** and **4** as well as the related fungal metabolites with the 1,3-dihydroisobenzofuran moiety[34,35,37] are epimers at C-1 is presumably the result of their formation by nonstereospecific addition of a hydroxymethyl group to a double bond in open-chain biosynthetic precursors, this stereocenter being also configurationally labile via reversible opening of the dihydrofuran ring. Direct TDDFT calculations of ECD curves of the full structures **3** and **4** were not performed because of the flexibility of the molecules with four rotatable single bonds, resulting in difficulties with reliable Boltzmann weighting of ECD spectra of numerous conformers present, the relative energies of which are dependent on the computational method used. Thus, no information about absolute configuration of C-3′ was derived from the ECD spectra of **3** and **4**. However, information about the configuration of the corresponding stereogenic center in the conformationally more rigid metabolite eluted as peak *h* was obtained from TDDFT calculations (see below), and the same *R*-configuration at this stereocenter is proposed to be preserved on biosynthetic grounds in all metabolites identified in this work, including **3** and **4**.

The constituent eluted as peak *d* (t_R = 27.7 min; *m*/*z* 199.2, $[M + Na]^+$; *m*/*z* 175.1, $[M - H]^-$) was identical with the reported compound **1**.[24] This compound was also isolated from fruiting bodies of a basidomycete, *Xylaria polymorpha*.[47]

The constituent eluted as peak *e* (t_R = 29.2 min; *m*/*z* 231.1, $[M + Na]^+$; *m*/*z* 207.1, $[M - H]^-$) was identified as **5** on the basis of spectra recorded in the hyphenated mode. Thus, the compound displayed the characteristic ^{1}H NMR coupling pattern of a 1,2,3-trisubstituted benzene ring and two double-bond protons appearing as a broadened doublet (J = 15.8 Hz) and a double triplet (J = 15.8 and 7.1 Hz), compatible with a *trans*-disubstituted double bond next to a methylene group. A COSY spectrum recorded in the HPLC-SPE-NMR mode revealed the presence of an extended CH═CH−CH_2−CH−CH_3 spin system. On the basis of these data, the compound has structure **5**. Owing to loss of material during preparative HPLC, the absolute configuration of **5** was not established, but is tentatively proposed to be the same as that of the corresponding center in **3** and **4** on biosynthetic grounds. This new metabolite closely resembles 5-[3-hydroxy-2-(hydroxymethyl)phenyl]pent-4-ene-2,3-diol (sordariol) isolated from the fungus *S. macrospora*,[38] differing from **5** by the presence of another hydroxy group at C-3′.

The material eluted as the small peak *f* (t_R = 31.8 min; *m*/*z* 243.2, $[M + Na]^+$; *m*/*z* 219.2, $[M - H]^-$) also showed the presence of a trisubstituted benzene ring and a disubstituted double bond, the latter having a *cis* configuration (J = 12.3 Hz). The oxygenated methylene group was characterized by a large chemical shift difference between the diastereotopic protons ($\Delta\delta$ = 0.65 ppm, J = 13.8 Hz). In addition, a sharp resonance was observed at δ 4.90, which did not show any correlation in the HSQC spectrum. This resonance exhibited an exchange cross-peak to a residual water resonance in a NOESY spectrum, strongly suggesting the presence of a tertiary hydroxy group. On first glance, no other resonances were present. However, a clear signal was observed in the HSQC spectrum at δ_H 1.98 and δ_C 23.3, indicative of a methyl group that coincides with the solvent (CD_3CN) resonance that was suppressed in the 1D ^{1}H NMR experiment. In addition, the methyl group resonance at δ_H 1.98 displayed an HMBC correlation to a ^{13}C signal at δ_C 204.6, and the diastereotopic methylene protons showed correlations to a ^{13}C signal at δ_C 101.3. These data are in excellent agreement with structure **6**. In spite of the small amount of material present, heteronuclear correlations enabled assignment of all ^{13}C NMR chemical shifts (see Experimental Section). However, attempts to isolate this compound by preparative HPLC proved unsuccessful, which is presumably a result of instability and the minute amount of **6** present in the extract. Because of configurational lability of the hemiketal function, we believe that **6** is a racemate. A related compound in which the carbonyl group is reduced to an alcohol (relative and absolute configuration unknown) has previously been reported from *S. macrospora*.[34]

The compounds eluted as peaks *g* and *h* (t_R = 66.7 and 74.5 min, respectively) were isomers (*m*/*z* 431.1, $[M + Na]^+$, *m*/*z* 407.1, $[M - H]^-$) with a molecular mass that is considerably higher than those of the metabolites described above. These compounds were assigned the unusual dispiro structures **7** and **8**, respectively, with the absolute configuration as shown, based on combined use of NMR hyphenation, ECD spectroscopy, and TDDFT calculations as detailed below.

The ^{1}H NMR spectrum of the compound eluted as peak *h* showed the presence of fragments familiar from the spectra of the above-mentioned compounds: a 1,2,3-trisubstituted benzene ring, a *cis*-disubstituted double bond without further vicinal couplings, an oxygenated methylene group with diastereotopic protons, and a CH−CH_3 spin system with the methine group oxygenated. A comprehensive 2D NMR data set was acquired in

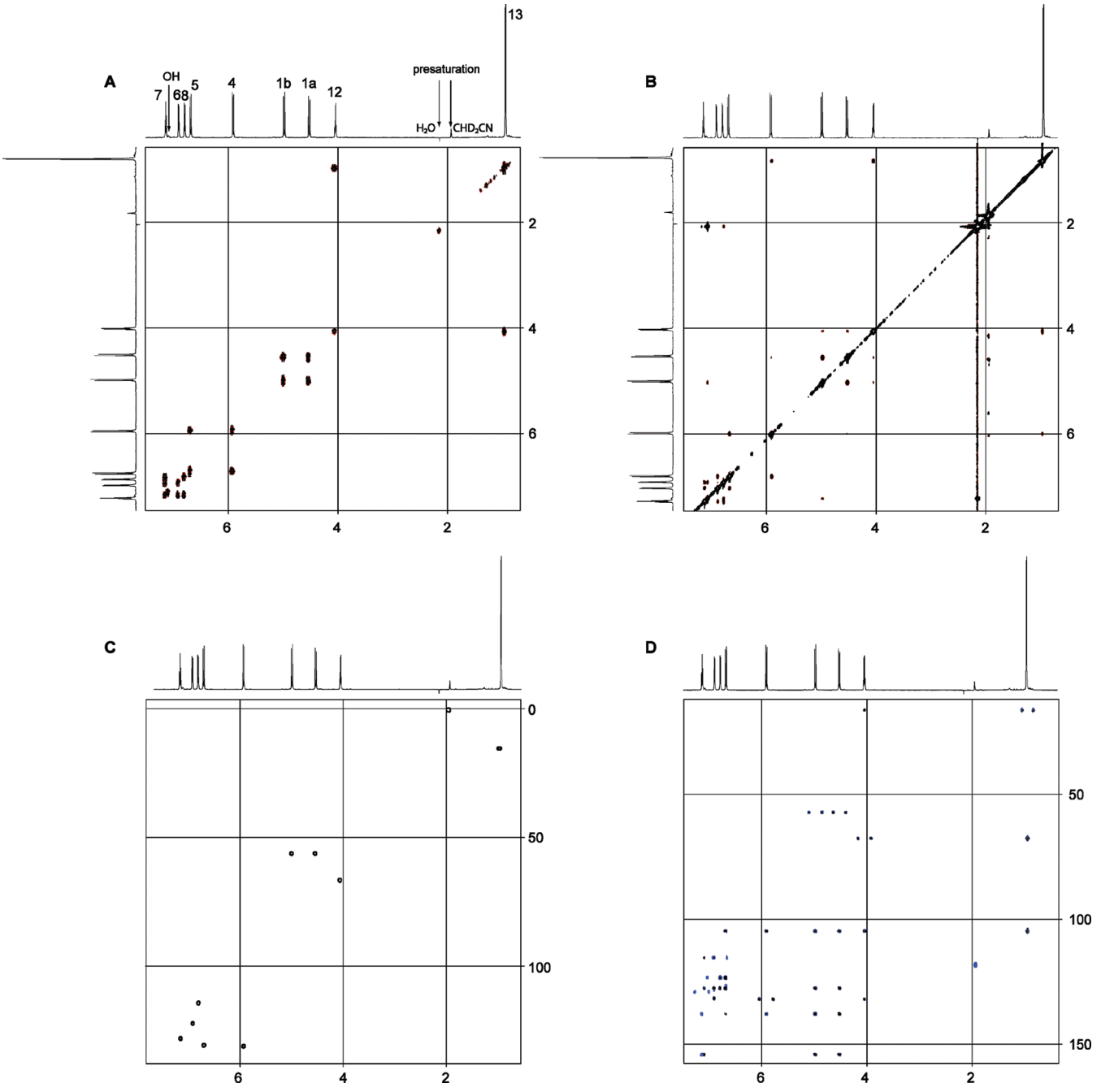

Figure 3. COSY (A), NOESY (B), HSQC (C), and HMBC (D) spectra of compound **8** (peak *h*) recorded in the HPLC-SPE-NMR mode (600 MHz, CD_3CN).

the HPLC-SPE-NMR mode (Figure 3), and analysis of HMBC connectivities showed this compound to have a 1,9,11,18-tetraoxadispiro[6.2.6.2]octadecane framework assembled from fragments corresponding to carbon skeletons present in compounds **3**−**6**. For a molecule with four stereogenic centers, up to 16 stereoisomers are generally possible. However, because of the presence of symmetry elements, the total number of stereoisomers in the present case is reduced to 12, corresponding to seven diastereomeric structures, A−G shown in Figure 4. Of these, molecules A, F, and G have no symmetry elements and their NMR spectra will show anisochronous signals for the corresponding atoms of two halves of the molecule. Therefore, they cannot represent the compound eluted with peak *h*, which exhibits isochronous chemical shifts (Figure 3). One is thus left with the centrosymmetric structures B and C, and structures D and E, which have a 2-fold (C_2) symmetry axis. Because the compound eluted as peak *h* exhibited an ECD spectrum (generated from material trapped in an HPLC-SPE run using the hyphenated setup[27]), the centrosymmetric and thus achiral structures B and C can be ruled out, the remaining task being to differentiate between the possible structures D and E and their enantiomers. The differentiation between D and E can be achieved considering a NOESY spectrum. Thus, in a chair conformation of the central 1,4-dioxolane ring of both structures

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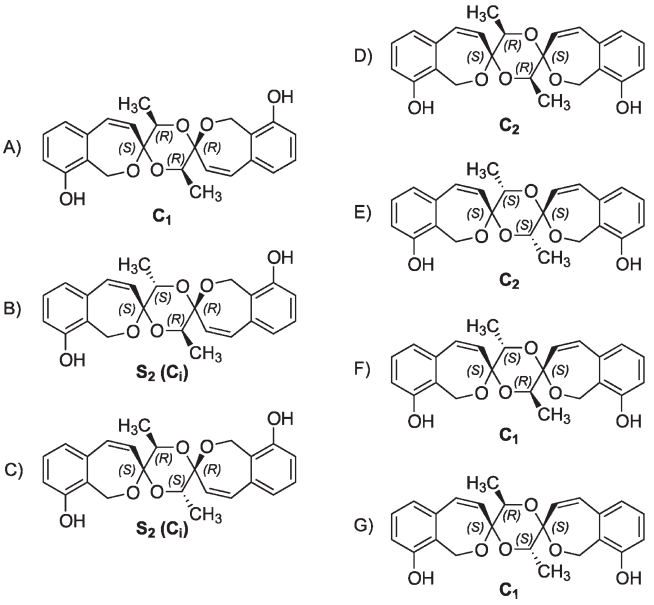

Figure 4. Theoretically possible stereoisomers of the dispiro compounds eluted as peaks *g* and *h* with average molecular symmetries as shown (C_1, no symmetry elements; C_2, 2-fold symmetry axis; S_2, 2-fold alternating symmetry axis corresponding to an inversion center, C_i). Structures A and D−G are chiral, whereas the remaining are achiral, giving a total of 12 stereoisomers.

D and E, the methyl groups are *cis* relative to each other, with one axial and one equatorial methyl group and one of the exocyclic C−O ketal bonds axial and the other equatorial, the axial ketal bond providing stabilization due to the anomeric effect.[48] A flip of the dioxolane ring yields a superimposable conformation, and the two average to the C_2-symmetric structure. Notably, in both chair conformations of D the double bond and the methyl group are *cis* (with either axial−equatorial or equatorial−axial orientation), whereas in those of E the double bond and the methyl group are *trans* (either equatorial−equatorial or axial−axial arrangement). The reverse is true for the relationship between the double bond and the methine proton of the dioxolane ring. In the NOESY spectrum of the compound eluted as peak *h*, the methyl group showed strong correlation to the neighboring olefinic proton signal (Figure 3B), while no NOE correlation was observed between the olefinic and the methine proton. On the other hand, the methine proton showed a weaker correlation to the OCH_2 group, in agreement with the *cis* (axial−equatorial or equatorial−axial) arrangement of the two substituents expected for structure D. In addition to the above-mentioned degenerate conformations with the dioxolane ring in the chair conformation, a Monte Carlo conformational search identified another low-energy conformation of structure D, with the dioxolane ring in a skew-boat conformation and both methyl groups quasiequatorial (Figure 5). Also this conformation is in excellent agreement with the observed NOE effects. The structure D (in terms of relative configuration) is therefore concluded to be the correct representation of the compound eluted as peak *h*.

In order to determine its absolute configuration, TDDFT calculations (B3LYP method, energy optimization with the DZP basis set, calculation of ECD transitions with the TZVP basis set) were performed for both enantiomers corresponding to D. The calculations for the chair and skew-boat conformations (Figure 5) showed a negative long-wavelength and a positive

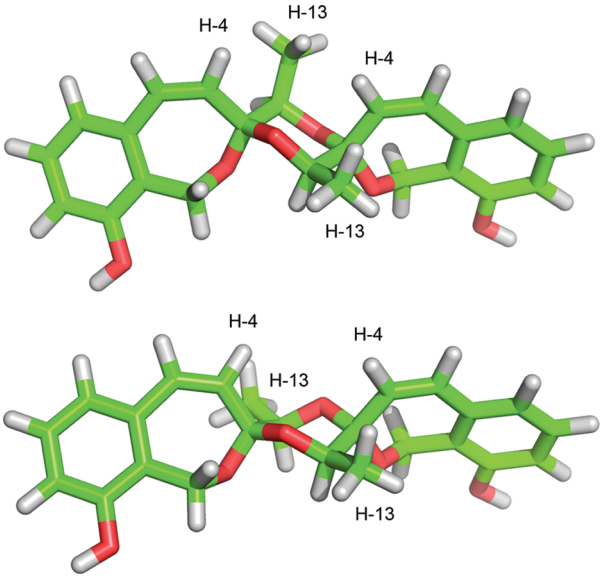

Figure 5. Low-energy conformations of compound **8** with chair (top) and skew-boat (bottom) conformations of the 1,4-dioxolane ring, showing proximity of methyl groups (H-13) and the olefinic protons (H-4).

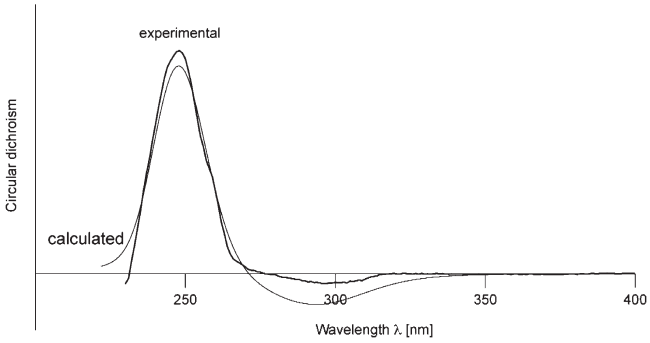

Figure 6. Observed ECD spectrum (thicker line) of the compound eluted in peak *h* (CH_3CN) and B3LYP/TZVP-calculated ECD spectrum of **8** (thinner line), obtained by averaging the calculated spectra of the conformers with the 1,4-dioxolane ring in a chair and in a skew-boat conformation (Figure 5). The ECD axis is arbitrary.

short-wavelength Cotton effect for the enantiomer shown in D, in agreement with the experimental result for the compound eluted with peak *h* (Figure 6). The calculated spectrum shown in Figure 6 corresponds to a 1:1 average of calculated ECD spectra of the chair and the skew-boat conformation, red-shifted by 8 nm to account for the difference between calculated (λ_{max} = 282−284 nm) and experimental long-wavelength UV absorption (λ_{max} = 292 nm, in CH_3CN). Thus, this compound has structure **8**. As already mentioned, the *R*-configuration of the oxygenated methine group in **8** is tentatively extended to **3**−**5** on the basis of biosynthetic grounds.

The ^{1}H NMR spectrum of the compound eluted as peak *g* contained the same kind of resonances as the compound eluted with peak *h* (compound **8**) except that all resonances were doubled (Figure 7). The isomers with anisochronous NMR signals of the two parts of the molecule could be A, F, or G (Figure 4) or any of their enantiomers. However, since the

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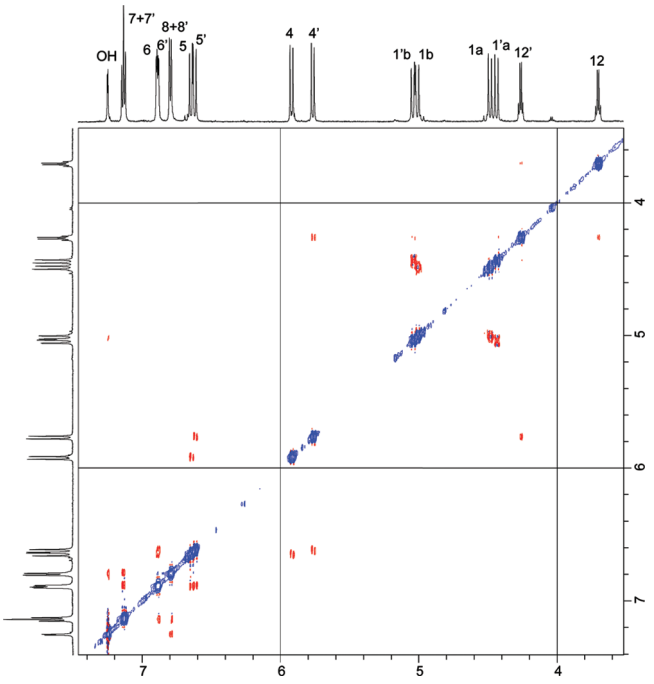

Figure 7. 1D ^{1}H NMR and NOESY spectrum of compound **7** (peak *g*) recorded in the HPLC-SPE-NMR mode (600 MHz, CD_3CN).

configuration of the stereocenters bearing the methyl groups may be assumed to be the same in both dispiro compounds on biosynthetic grounds, the likely candidate for the stereoisomer eluted as peak *g* is A. In fact, in a NOESY spectrum of this compound (Figure 7), one of the methyl groups exhibited a correlation to one of the anisochronous olefinic protons similarly to that in **8**, whereas no such correlation was observed for the other methyl group. On the other hand, the methine group next to the latter methyl group exhibited correlation to the other anisochronous olefinic proton signal, unlike the other methine group and unlike the situation observed in compound **8**. It can thus be concluded that while both methyl groups in the dioxolane ring of compound **8** are *cis* to the double bonds, the compound eluted with peak *g* has one methyl group *cis* and the other *trans* to the double bond, in agreement with A. Thus, the compound eluted with peak *g* is assigned the structure **7**. Compounds **7** and **8** can be viewed as dimers of a compound corresponding to the seco form of **3** and **4** with a *cis* double bond;[34] **7** and **8** are thus formed by dimerization of the seco form upon formation of either the opposite or the same configurations at the two newly formed ketal centers, respectively. A summary of NMR data for the two isomers is given in Table 2. We propose to assign the names pestalospiranes A and B to compounds **7** and **8**, respectively. These interesting metabolites are representatives of a small but growing group of natural benzo[*c*]oxepin derivatives of fungal origin.[24,34,35,38,49]

This work further demonstrates the power of HPLC-SPE-NMR in structure elucidation of natural products on a small scale (only less than 40 mg of crude culture broth extract was used for all hyphenated experiments described here, including preparatory steps). Thus, the technique is valuable as an exploratory tool for identification of extract constituents at a raw extract stage and assessment of their value based on exact chemical structures obtained. This can be followed by preparative-scale isolation in quantity if, for whatever reason, the identified constituents are deemed valuable. It should be emphasized, however, that although some 900 mg of the extract was available in total and a preparative-scale fractionation was performed, most of the crude extract constituted an insoluble tar and the preparative fractionation resulted in progressive loss of material, so that only compounds **1**−**4** could be isolated in this work by traditional means. This illustrates the advantage of postcolumn solid-phase extraction in HPLC-SPE-NMR, which in addition to NMR spectra was used to obtain ECD spectra after a relatively rapid analytical-scale HPLC separation of the crude extract. This approach is more advantageous than traditional preparative procedures applied with the purpose of identification of extract constituents. Although traditional fractionation procedures involve repeated and lengthy chromatographic steps and evaporation of large amounts of solvents, which can contribute to loss of tautomeric and otherwise labile compounds such as the cyclizable compound **5** and the hemiketal and ketals **6**−**8**, they might in general be considered indispensable in order to obtain sufficient amounts of compounds for pharmacological evaluation. However, the use of postcolumn SPE trapping with the HPLC-SPE-NMR system to obtain samples for bioassays alongside spectroscopic data is currently being evaluated in our laboratories. Thus, an automated HPLC-SPE-NMR hyphenated system supported by MS and ECD is potentially useful for both rapid chemical dereplication and pharmacological screening without classical preparative-scale fractionation of extracts originating from natural sources.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were recorded using a Perkin-Elmer 241 polarimeter. UV spectra were obtained using a Thermo Scientific NanoDrop 100 spectrophotometer. ECD spectra were measured using an Olis DSM10 spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer. 1D and 2D NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer equipped with an inverse ^{1}H{^{13}C} flow-probe with a 30 μL active volume and 60 μL total volume (hyphenated mode spectra) or on a Bruker Avance 400 MHz spectrometer equipped with a 5 mm ^{13}C{^{1}H} probe (remaining spectra). Standard Bruker library pulse sequences were used for NMR data acquisition; NOESY spectra were recorded with mixing time of 600 ms, and HMBC spectra were optimized for $^nJ_{CH}$ = 8 Hz. Hyphenated experiments were performed using a system composed of an Agilent 1100 liquid chromatograph, consisting of a degasser, a quaternary pump, an autosampler, a column oven, and a photodiode array (PDA) detector, a Bruker Esquire LC ion-trap mass spectrometer operating in the ESI mode via a 1:20 flow splitter, a Knauer K100 Wellchrom pump for postcolumn eluent dilution, a Spark Holland Prospekt 2 solid-phase extraction device, and the above-mentioned 600 MHz NMR spectrometer. Analytical chromatographic separations were performed with a Shimadzu LC-10 system consisting of a degasser, a quaternary pump, an autosampler, a column oven, and a PDA detector. Preparative separations were performed with an Agilent model 1100 preparative chromatograph consisting of two preparative pumps, an autosampler, a multiple-wavelength detector, and a fraction collector. Water was purified by 0.22 μm membrane filtration and deionization (Millipore system); other solvents used were HPLC grade.

Fungal Material. The fungal material used (code TC-320) was *Pestalotiopsis virgatula* (Kleb.) Steyaert (Amphisphaeriaceae), which was isolated and identified as described elsewhere.[22,23] The host plant, *Terminalia chebula* Retz., Combretaceae, was identified by Mr. S. Kumara, PhytoMyco Research Ptv. Ltd., Nanjangud, India; the voucher

Table 2. NMR Data for Compounds 7 and 8[a]

	pestalospirane A (7)					pestalospirane B (8)				
			2D correlations					2D correlations		
position	δ_H[b]	δ_C[c]	COSY	NOESY	HMBC[d]	δ_H[b]	δ_C[c]	COSY	NOESY	HMBC[d]
1	a: 4.49 d (13.8)	57.47	1b	1b, 12		a: 4.52, d (13.8)	57.30	1b	1b, 4, 12	
	b: 5.02, d (13.8)		1a	1a, 12		b: 4.97, d (13.8)		1a	1a, 9-OH, 12	
3		102.07			1a, 1b, 4, 5, 13		104.97			1a, 1b, 4, 5, 12, 13
4	5.93, d (12.5)	132.85	5	5, 13	6	5.92, d (12.5)	132.04	5	5, 13	5, 6, 12
5	6.65, d (12.5)	132.10	4	4, 6	6	6.67, d (12.5)	131.42	4	4, 6	6
6	6.90, d (7.6)	123.48	7	5, 7	5, 8	6.89, d (7.6)	123.45	7	5, 7	5, 8
7	7.13, dd (7.6, 8.1)	129.24	6, 8	6, 8	6	7.13, dd (7.6, 8.1)	129.25	6, 8	6, 8	6
8	6.78, d (8.1)	115.65	7	7, 9-OH	6	6.78, d (8.1)	115.50	7	7, 9-OH	6
9		153.80			1a, 1b, 7		154.17			1a, 1b, 7
10		127.93			1b, 5, 6, 8, 9-OH		127.72			1a, 1b, 5, 6, 8, 9-OH
11		137.60			1b, 7		137.84			1a, 1b, 4, 7
12	3.71, q (6.7)	72.91	13	1a, 1b, 13, 12′	13	4.03, q (6.5)	67.81	13	1a, 1b, 13	13
13	1.37, d (6.6)	19.99	12	4, 12		0.94, d (6.5)	16.47	12	4, 12	12
9-OH	7.25, br			8		7.08, br			8	
1′	a: 4.45 d (13.8)	57.02	1′b	1′b, 12′						
	b: 5.05, d (13.8)		1′a	1′a, 12′						
3′		100.24			1′a, 1′b, 4′, 5′, 12, 13′					
4′	5.78, d (12.5)	133.33	5′	5′	6′					
5′	6.63, d (12.5)	132.41	4′	4′, 6′	6′					
6′	6.89, d (7.6)	123.48	7′	5′, 7′	5′, 8′					
7′	7.13, dd (7.6, 8.1)	129.24	6′, 8′	6′, 8′	6′					
8′	6.78, d (8.1)	115.65	7′	7′, 9′-OH	6′					
9′		153.80			1′a, 1′b, 7′					
10′		127.93			1′b, 5′, 6′, 8′, 9′-OH					
11′		137.60			1′b, 7′					
12′	4.27, q (6.5)	67.74	13′	12, 4′ 13′	13′					
13′	0.92, d (6.5)	15.02	12′	12′						
9′-OH	7.25, br			8′						

[a] Spectra obtained in hyphenated mode in CD_3CN at 600 MHz. [b] Coupling constants in Hz in parentheses; d, doublet; q, quartet; br, broad signal. [c] From HSQC and HMBC spectra. [d] From the indicated proton to carbon in the same row.

specimen (accession number BSI/SC/5/23/08-08/tech-2) was deposited in herbarium MH (Botanical Survey of India, Southern Circle, Tamil Nadu Agricultural University). The isolate was fermented under static conditions in 10 1-L Erlenmeyer flasks containing 500 mL of potato dextrose broth medium at 23 °C for 21 days. Fungal mycelia were separated from the culture filtrate by passing through four layers of cheese cloth. Each filtrate was extracted with EtOAc (2 × 1 L), and the combined extracts were dried by flash evaporation to yield 0.9 g of a dark, resinous residue.

HPLC-PDA-MS-SPE-NMR Experiments. Separations were performed on a 150 × 4.6 mm i.d. Phenomenex C18(2) Luna column (3 μm) operated at 40 °C and eluted at a flow rate of 0.8 mL/min. The linear gradient elution profile was made of H_2O-CH_3CN (95:5) + 0.1% formic acid (eluent A) and CH_3CN-H_2O (95:5) + 0.1% formic acid (eluent B), with 0% B at 0 min, 35% B at 50 min, 100% B at 115−123 min, and 0% B at 133 min. The chromatography was monitored at four wavelengths (235, 254, 275, and 315 nm), and trapping of analytes was controlled by absorbance threshold levels after dilution of the column eluate with H_2O (2 mL/min). Following eight repeated chromatographic separations (injection volume 25 μL, extract concentration 2.5 mg/mL), the analytes were trapped on GP-phase SPE cartridges (10 × 2 mm i.d.). The cartridges were dried with pressurized N_2, and the analytes were eluted with CD_3CN to the NMR probe using optimized elution and push volumes of the solvent. The spectra were calibrated to the residual ^{1}H NMR signal of CD_2HCN set to δ_H 1.94 and to the ^{13}C NMR signal of the methyl group of CD_3CN set to δ_C 1.32.

Preparative-Scale Isolation. Fungal extract (0.8 g) was dissolved in 20 mL of CH_3CN-H_2O (1:1), diluted with 180 mL of H_2O, and applied to three 10 g (60 mL) LC-18 Supelco SPE columns, rinsed with CH_3CN and preconditioned with 100 mL of CH_3CN-H_2O (5:95). The columns were successively eluted with 100 mL portions of CH_3CN-H_2O (1:3, 1:1, 3:1, and 1:0). Corresponding fractions from the three identical columns were pooled, concentrated in vacuo, freeze-dried, and subjected to ^{1}H NMR analysis. The fraction eluted with CH_3CN-H_2O (1:3) (240 mg) was redissolved in 10 mL of CH_3CN-H_2O (15:85) and subjected to repeated separation on a 250 × 21.2 mm i.d. Supelco Discovery C18 column (5 μm), eluted with 14 mL/min of an isocratic flow of 15% CH_3CN in H_2O (injection volume 1−1.5 mL). This yielded compounds **1** (4 mg), **2** (0.8 mg), **3** (5.7 mg), and **4** (7.8 mg, containing 25% of **3**). The fraction eluted with CH_3CN-H_2O (1:1) (24.5 mg) contained compounds **7** and **8**, but failed, after repeated preparative HPLC, to yield any pure material.

TDDFT Calculations. A Monte Carlo conformational search was performed using Macromodel ver. 9.7 in the Schrödinger software package (Schrödinger, LLC, New York, NY, USA). After that, Turbomole ver. 6.1 (Turbomole GmbH, Karlsruhe, Germany) was used to

optimize ground-state conformations of **8**, **9a**, **9b**, and **10**.[50−52] For that purpose, B3LYP[53−55] with the DZP or TZVP basis set[56,57] was used. ECD and UV spectra were calculated using TDDFT[33] with B3LYP and the TZVP basis set and also the TZVPP basis set for compound **10**. The number of transitions included in the TDDFT calculations was 15. Dipole-velocity rotational strengths were employed to calculate ECD spectra using the equation $\Delta\varepsilon(\sigma) = [\sigma_0/(2.296 \times 10^{-39} \times \pi^{0.5} \times \Delta\sigma)] \times R_{vel} \times \exp\{-[(\sigma - \sigma_0)/\Delta\sigma]^2\}$,[58] where σ is wavenumber, σ_0 is the center of the band, $\Delta\sigma$ is the bandwidth (all in cm^{-1}), and R_{vel} is dipole-velocity rotational strength (in 10^{-40} erg). Frequency correction of σ_0 was applied by comparing observed and calculated UV spectra.[45]

Cyclosordariolone[34,35] **[1,6-Dihydroxy-5-(hydroxymethyl)-1-methylnaphthalen-2(1*H*)-one] (2):** yellowish solid; $[\alpha]^{25}_D$ 0 (*c* 0.1, CH_3OH); ^1H NMR (CD_3OD, 400 MHz) δ 7.98 (1H, dd, *J* = 10.3, 0.6 Hz, H-4), 7.49 (1H, dd, *J* = 8.5, 0.6 Hz, H-8), 6.92 (1H, d, *J* = 8.5 Hz, H-7), 6.15 (1H, d, *J* = 10.3 Hz, H-3), 4.89 and 4.85 (each 1H, d, *J* = 11.8 Hz, CH_2), 1.46 (3H, s, CH_3); ^{13}C NMR (CD_3OD, 100 MHz) δ 207.1 (C, CO) 156.8 (C, C-6), 143.6 (CH, C-4), 139.3 (C, C-9), 130.2 (C, C-10), 127.6 (CH, C-8), 126.6 (C, C-5), 124.6 (CH, C-3), 118.6 (CH, C-7), 77.8 (C, C-1), 55.4 (CH_2), 32.6 (CH_3).

(*R*)-3-Hydroxy-1-[(*R*)-4-hydroxy-1,3-dihydroisobenzofuran-1-yl]butan-2-one (3): colorless film; $[\alpha]^{25}_D$ −19.4 (*c* 0.19, CH_3OH); UV (CH_3CN) λ_{max} (log ε) 227 (2.72), 259 (2.30), 304 (2.08) nm; IR (KBr) ν_{max} 3416, 2912, 2887, 2862, 1716, 1627, 1599, 1469, 1373, 1291, 1005, 784 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1.

(*R*)-3-Hydroxy-1-[(*S*)-4-hydroxy-1,3-dihydroisobenzofuran-1-yl]butan-2-one (4): colorless film; $[\alpha]^{25}_D$ +43.9 (*c* 0.25, CH_3OH); UV (CH_3CN) λ_{max} (log ε) 226 (2.71), 268 (2.21), 298 (1.84) nm; IR (KBr) ν_{max} 3407, 2940, 2887, 2859, 2578, 1685, 1466, 1386, 1362, 1270, 1236, 1208, 1187, 1045, 1032, 996 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1.

(*E*)-2-(Hydroxymethyl)-3-(4-hydroxypent-1-enyl)phenol (5): ^1H NMR (CD_3CN, 600 MHz, hyphenated mode) δ 7.08 (1H, t, *J* = 7.8 Hz, H-5), 6.97 (1H, d, *J* = 7.8 Hz, H-4), 6.70 (1H, d, *J* = 7.8 Hz, H-6), 6.69 (1H, br d, *J* = 15.8 Hz, H-1′), 6.10 (1H, dt, *J* = 15.8, 7.1 Hz, H-2′), 4.78 (2H, s, CH_2O), 3.81 (1H, m, H-4′), 2.30 (intensity diminished due to solvent suppression of the residual water peak at δ 1.97, br t, *J* = 7.1 Hz, H-3′), 1.15 (3H, d, *J* = 6.3 Hz, CH_3).

1-(3,9-Dihydroxy-1,3-dihydrobenzo[*c*]oxepin-3-yl)ethanone (6): ^1H NMR (CD_3CN, 600 MHz, hyphenated mode) δ 7.22 (1H, t, *J* = 7.8 Hz, H-7), 7.00 (1H, d, *J* = 7.8 Hz, H-6), 6.87 (1H, d, *J* = 7.7 Hz, H-8), 6.83 (1H, d, *J* = 12.3 Hz, H-5), 5.75 (1H, d, *J* = 12.3 Hz, H-4), 5.17 and 4.51 (each 1H, d, *J* = 13.8, H-1), 4.90 (1H, s, 3-OH), 1.98 (CH_3, observed only in HSQC and HMBC spectra due to solvent presaturation applied at this frequency in ^1H NMR spectra); ^{13}C NMR (CD_3CN, 600 MHz HSQC and HMBC spectra acquired in hyphenated mode) δ 204.6 (C, CO), 154.0 (C, C-9), 137.4 (C, C-11), 134.1 (CH, C-5), 131.1 (CH, C-4), 129.3 (CH, C-7), 128.3 (C, C-10), 123.5 (CH, C-6), 115.7 (CH, C-8), 101.3 (C, C-3), 58.6 (CH_2, C-1), 23.3 (CH_3).

Pestalospirane A (7): ^1H and ^{13}C NMR data, see Table 2.

Pestalospirane B (8): ^1H and ^{13}C NMR data, see Table 2.

■ ASSOCIATED CONTENT

Supporting Information. Selected NMR spectra (Figures S1−S9) and B3LYP-calculated energies, atomic coordinates, and ECD and UV transitions. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: (45) 35336372. Fax: (45) 35336041. E-mail: jj@farma.ku.dk.

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