LC–MS- and (1)H NMR Spectroscopy–Guided Identification of Antifungal Diterpenoids from Sagittaria latifolia

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · SEPTEMBER 2015

Impact Factor: 3.8 · DOI: 10.1021/acs.jnatprod.5b00470

JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

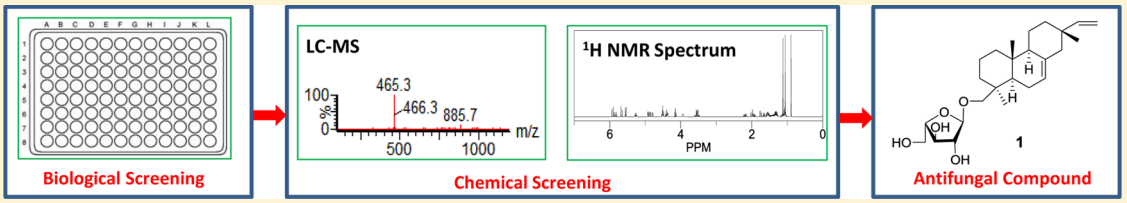
LC-MS- and ^{1}H NMR Spectroscopy-Guided Identification of Antifungal Diterpenoids from *Sagittaria latifolia*

Ranga Rao Ravu,[†] Melissa R. Jacob,[†] Cynthia Jeffries,[§] Ying Tu,[§] Shabana I. Khan,[†,‡] Ameeta K. Agarwal,[†,‡] R. Kiplin Guy,[§] Larry A. Walker,[†,‡] Alice M. Clark,[†,‡] and Xing-Cong Li*[,†,‡]

[†]National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences, and [‡]Department of BioMolecular Sciences, School of Pharmacy, The University of Mississippi, University, Mississippi 38677, United States

[§]Department of Chemical Biology and Therapeutics, St Jude Children's Research Hospital, Memphis, Tennessee 38105, United States

Supporting Information



ABSTRACT: Antifungal screening of small-molecule natural product libraries showed that a column fraction (CF) derived from the plant extract of *Sagittaria latifolia* was active against the fungal pathogen *Cryptococcus neoformans*. Dereplication analysis by liquid chromatography−mass spectrometry (LC-MS) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR) indicated the presence of new compounds in this CF. Subsequent fractionation of the plant extract resulted in the identification of two new isopimaradiene-type diterpenoids, **1** and **2**. The structures of **1** and **2** were determined by chemical methods and spectroscopic analysis as isopimara-7,15-dien-19-ol 19-*O*-α-L-arabinofuranoside and isopimara-7,15-dien-19-ol 19-*O*-α-L-(5′-acetoxy)-arabinofuranoside, respectively. Compound **1** exhibited IC_{50} values of 3.7 and 1.8 μg/mL, respectively, against *C. neoformans and C. gattii*. Its aglycone, isopimara-7,15-dien-19-ol (**3**), resulting from acid hydrolysis of **1**, was also active against the two fungal pathogens, with IC_{50} values of 9.2 and 6.8 μg/mL, respectively. This study demonstrates that utilization of the combined LC-MS and ^{1}H NMR analytical tools is an improved chemical screening approach for hit prioritization in natural product drug discovery.

Our previous work has shown that small-molecule natural product libraries consisting of chromatographically tractable column fractions (CFs) are a valuable resource for natural product drug discovery and that UPLC-MS-ELSD-PDA (abbreviated as LC-MS below) is a powerful dereplication tool to facilitate compound identification.[1] The present study further demonstrates the utility of this resource for antifungal natural products discovery. The in vitro antifungal screening of greater than 14 000 CFs was conducted against the opportunistic fungal pathogen *Cryptococcus neoformans*, which causes life-threatening systemic cryptococcosis in immunocompromised patients.[2] Using LC-MS and ^{1}H NMR spectroscopic analytical tools for hit dereplication, an active CF, coded 80680-c4, from the ethanol extract of *Sagittaria latifolia* Willd. (Alismataceae), was predicted to possess new compounds.

S. latifolia, an herbaceous aquatic plant, has long been an important food source to indigenous peoples of the Americas. This genus consists of nearly 25 species distributed through the tropical regions of North and South America.[3] Previous phytochemical investigations of other *Sagittaria* species revealed the presence of various chemotypes of diterpenoids.[3−12] Several *ent*-rosane and *ent*-kaurane diterpenoids showed potent antimicrobial activities against oral pathogens.[10,11] To identify the antifungal compound(s), a scale-up extraction of the flowers and leaves of *S. latifolia*, followed by isolation and structure elucidation of bioactive components, was conducted, leading to the identification of two new isopimaradienes (**1** and **2**). Using this as an example, the study reported here further illustrates the advantage of the combined LC-MS and ^{1}H NMR spectroscopic analytical tools for hit prioritization in natural products drug discovery.

RESULTS AND DISCUSSION

The present dereplication analysis is based on comparison of the molecular weight (MW), mass spectrometry (MS) fragmentation patterns, UV absorptions, and characteristic ^{1}H NMR signals with previously reported data in databases such as SciFinder Scholar (Chemical Abstracts Service, Columbus, OH, USA) and the *Dictionary of Natural Products* (CRC Press, www.dnp.chemnetbase.com), as well as chemotaxonomic considerations that structurally similar compounds are present in the same species, genus, or even family of the plant.

CF80680-c4 showed promising antifungal activity against *C. neoformans* ATCC 90113 with an IC_{50} of 12.7 μg/mL and was

Received: May 26, 2015
Published: September 15, 2015

DOI: 10.1021/acs.jnatprod.5b00470
J. Nat. Prod. 2015, 78, 2255−2259

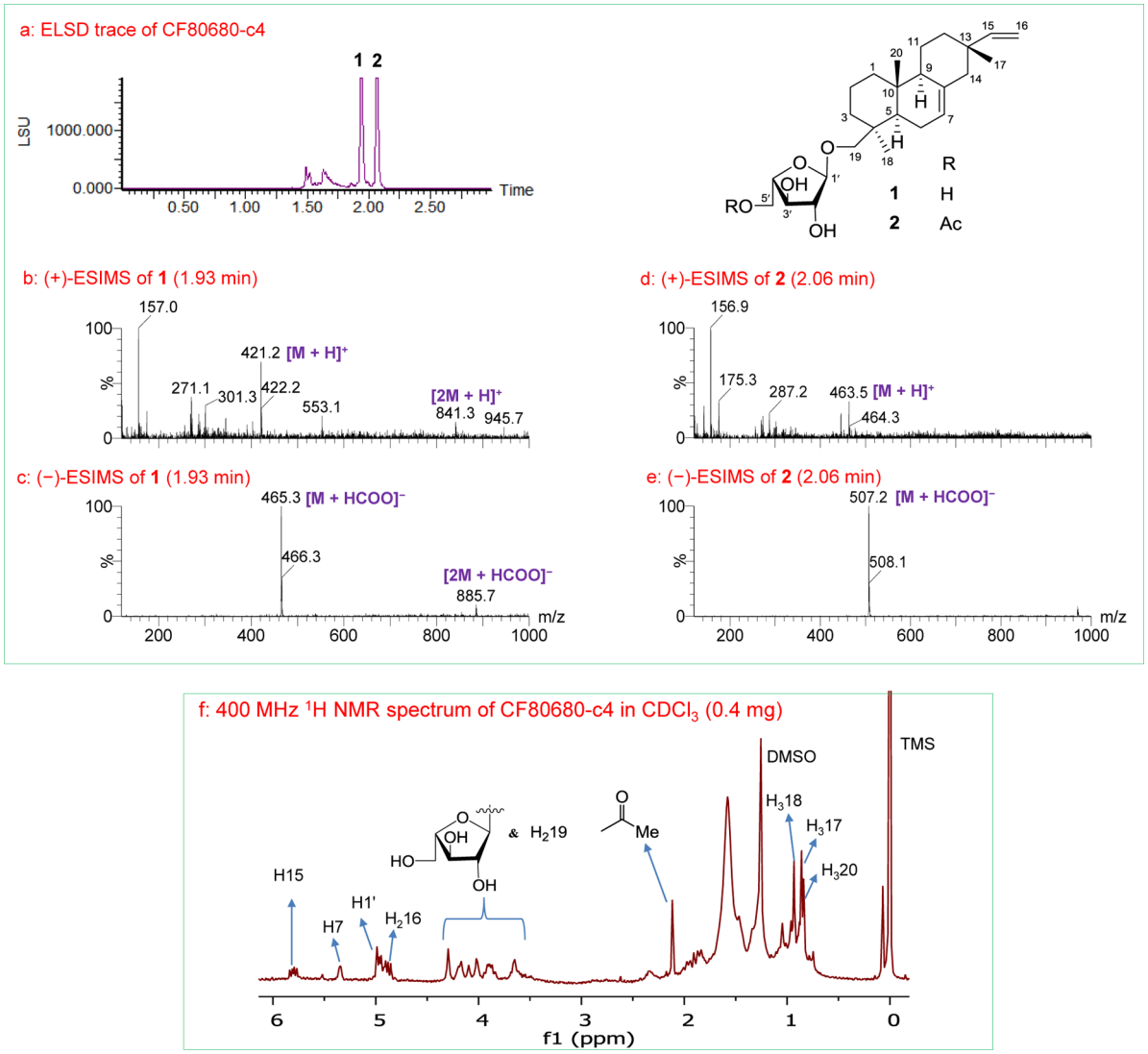


Figure 1. LC-MS and ^{1}H NMR analysis of the antifungal chromatographic fraction CF80680-c4 from *Sagittaria latifolia*. LC-MS: Acquity UPLC BEH C_{18} column (2.1 × 50 mm, 1.7 μm); gradient elution starting at 15%, ramping to 20% in 0.2 min, then to 95% CH_3CN in water with 0.1% HCOOH in 2.65 min at a flow rate of 1.0 mL/min. Ionization and detection of natural products were carried out on a Waters SQ mass spectrometer using both the positive and negative ESI modes. ^{1}H NMR: Recorded on a Bruker DRX NMR spectrometer operating at 400 (^{1}H) using TMS as an internal standard. (a) ELSD chromatogram showing compounds **1** and **2** with retention times of 1.93 and 2.06 min, respectively; (b and c) positive and negative ESIMS total-ion chromatograms (TIC) of compound **1**, respectively; (d and e) positive and negative ESIMS TIC of compound **2**, respectively; and (f) 400 MHz ^{1}H NMR spectrum of CF80680-c4 indicating the structural information on compounds **1** and **2**.

the most active of all CFs generated from the plant extract of *S. latifolia* by an automated high-throughput fractionation system.[13] It is worth noting that the parent ethanol extract of *S. latifolia* had an IC_{50} of >50 μg/mL against this fungal pathogen and was not considered as a hit, highlighting the importance of activity enrichment by this fractionation approach. The LC-MS data of CF80680-c4 are shown in Figure 1. The evaporative light scanning detected (ELSD) chromatogram indicated that it contained two major compounds (**1** and **2**) in a ratio of approximately 1:1 at retention times (t_R) of 1.93 and 2.06 min, both of which lacked UV absorptions (data not shown). Compound **1** gave a protonated molecular ion at m/z 421.2 $[M + H]^+$ in the positive ESIMS and a $[M + HCOO]^-$ ion at m/z 465.3 in the negative ESIMS, indicating a MW of 420. For compound **2**, the ionized molecular ions at m/z 463.5 $[M + H]^+$ and 507.2 $[M + HCOO]^-$ in the positive and negative ESIMS, respectively, indicated a MW of 462. The difference of 42 mass units between **1** and **2** suggested that an acetyl group was likely present in **2**, explaining the slightly longer retention time of **2**. Since several diterpenoids have been isolated from other *Sagittaria* species, the MW ranges and the non-UV absorption nature of compounds **1** and **2** suggested they could be diterpenoid derivatives. Using the MW and taxonomic information to search the aforementioned databases, it was determined that one diterpenoid named sagittine B from *S. sagittifolia* had a MW of 420, and two diterpenoids named sagittine C and sagittine D from *S. sagittifolia* had the same MW of 462.[10] Since the same MW (or molecular formula) generally corresponds to multiple chemical structures, especially for small MWs less than 500, it was undetermined as to whether **1** and **2** were the known compounds mentioned above, and the LC-MS data were unable to address this without available authentic

DOI: 10.1021/acs.jnatprod.5b00470
J. Nat. Prod. 2015, 78, 2255−2259

Table 1. NMR Spectroscopic Data for Compounds 1−3 in $CDCl_3$ (δ, ppm; J, Hz)[a]

	1		2		3	
H/C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.03 α/1.84 β	39.8	0.96 α/1.78 β	39.7	1.04 α/1.88 β	39.9
2	1.44	18.7	1.38	18.5	1.48/1.36	18.6
3	1.82 α/0.96 β	36.0	1.76 α/0.91 β	36.0	1.91 α/0.99 β	35.5
4		37.0		36.8		38.0
5	1.26 dd (12.3, 4.2)	51.7	1.20 dd (12.1, 4.1)	51.5	1.27 dd (12.2, 4.3)	51.6
6	1.83 α/1.98 β	23.3	1.79 α/1.92 β	23.2	1.89 α/2.00 β	23.1
7	5.33 br s	121.5	5.28 br s	121.5	5.34 br s	121.7
8		135.8		135.4		135.7
9	1.66	52.3	1.60	52.1	1.66	52.3
10		35.5		35.3		35.4
11	1.55 α/1.33 β	20.5	1.48 α/1.25 β	20.3	1.55 α/1.34 β	20.5
12	1.32 α/1.47 β	36.3	1.27 α/1.41 β	36.1	1.32 α/1.46 β	36.3
13		37.0		36.8		37.0
14	1.96 α/1.88 β	46.2	1.90 α/1.83 β	46.0	1.98 α/1.83 β	46.2
15	5.79 dd (17.5, 10.7)	150.4	5.72 dd (17.4, 10.8)	150.2	5.79 dd (17.5, 10.8)	150.5
16	4.91 dd (17.5, 1.4)		4.85 dd (17.2, 1.4)		4.91 d (17.2)	
	4.85 dd (10.8, 1.4)	109.4	4.79 dd (10.6, 1.4)	109.2	4.85 d (10.7)	109.3
17	0.84 s	21.6	0.79 s	21.5	0.85 s	21.6
18	0.92 s	28.1	0.88 s	27.9	0.96 s	27.1
19	3.65 d (9.3)	70.8	3.60 d (9.4)	70.8	3.89 d (10.7)	65.3
	3.62 d (9.3)		3.53 d (9.0)		3.47 d (10.9)	
20	0.83 s	16.3	0.78 s	16.1	0.83 s	16.3
C=O				171.4		
$CO\underline{C}H_3$			2.05 s	20.9		
1′	4.96 br s	108.9	4.86 br s	108.1		
2′	4.01	78.8	4.02	80.7		
3′	3.98	78.1	3.79	77.9		
4′	4.13	87.2	4.06	82.3		
5′	3.88 br d (11.7)	62.0	4.20	64.3		
	3.80 br d (11.5)					

[a]Data obtained at 500 (^{1}H) and 125 (^{13}C) MHz for compound **1** and at 400 (^{1}H) and 100 (^{13}C) MHz for compounds **2** and **3**. Assignments were based on DEPT and 2D NMR including DQF-COSY, HMQC, HMBC, and ROESY. Well-resolved ^{1}H NMR couplings are expressed with coupling patterns and constants in Hz in parentheses. Some geminal protons were assigned as α- or β-oriented on the ring system.

compounds for direct comparison of retention times and MS data.

The advantage of using ^{1}H NMR spectroscopy for hit dereplication lies in its fingerprinting nature providing excellent structural information that can significantly narrow down possible hits when combined with LC-MS for this purpose. In this study, a 400 MHz ^{1}H NMR spectrum of CF80680-c4 was acquired in deuterated chloroform with a quantity of 0.4 mg (dried from 200 μL of the sample at 2 mg/mL in DMSO). This ^{1}H NMR spectrum (Figure 1f) showed methyl groups of the diterpenoid skeleton in the range δ_H 0.6−1.0, an acetyl group at δ_H 2.05, sugar moiety signals in the range δ_H 3.5−4.5, and vinylic protons of the aglycone moiety and anomeric protons of the sugar moiety in the range δ_H 4.7−6.0. Comparison of the characteristic methyl resonances with those of sagittines B−D indicated distinct differences,[10] suggesting that compounds **1** and **2** had a different diterpenoid skeleton and thus might be new natural products.

To isolate compounds **1** and **2**, a 95% ethanol extract of the dried leaves and flowers of *S. latifolia* was fractionated into CFs by normal-phase silica gel chromatography using a solvent system of chloroform with an increasing percentage of methanol. The CFs containing the two compounds were located readily by direct comparison of TLC behavior and ^{1}H NMR spectra with those of CF80680-c4. Subsequent isolation of these CFs by reversed-phase C_{18} column chromatography resulted in the purified compounds **1** and **2**.

The ^{1}H and ^{13}C NMR data of compound **1** (Table 1) due to the aglycone moiety differed from those of any diterpenoid previously isolated from *Sagittaria* spp., while its sugar moiety appeared to be a single β-arabinofuranose that was present in several diterpenoid glycosides that have been isolated from *Sagittaria* spp.[10,11] Acid hydrolysis of **1** afforded an aglycone (**3**) and L-arabinose. The aglycone was identified as isopimara-7,15-dien-19-ol by comparison of its ^{1}H NMR spectrum and optical rotation data with those reported in the literature.[14,15] Its ^{13}C NMR data were assigned by 2D NMR experiments and are reported for the first time in Table 1. The ^{1}H and ^{13}C NMR assignments of compound **1** were facilitated by 2D NMR experiments. The linkage of the arabinose to the C-19 hydroxy group in **1** was confirmed by the HMBC correlations between H_2-19 at δ_H 3.65 and 3.62 (1H, each, d, J = 9.3 Hz) and C-1′ at δ_C 108.9 and between H-1′ at δ_H 4.96 (1H, br s) and C-19 at δ_C 70.8, as well as the NOE correlation between H-19 and H-1′. In addition, the key NOE correlations, H_3-20 and H_2-19, H-5 and H-9, and H_3-17 and H-11β, supported the isopimara-7,15-dien-19-ol skeleton exhibiting a conformation of chair/chair-like/chair for the tricyclic A/B/C ring system, as shown in the optimized geometry by ChemBio3D Ultra 12.0 (Figure 2).

DOI: 10.1021/acs.jnatprod.5b00470
J. Nat. Prod. 2015, 78, 2255−2259

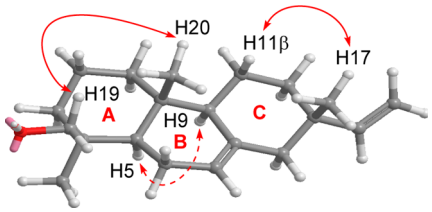


Figure 2. Key NOE correlations of aglycone **3**.

The ^{1}H and ^{13}C NMR data of compound **2** (Table 1) indicated that it had the same aglycone as **1**, and an additional acetyl group was confirmed to be attached to the C-5 hydroxy group of the α-L-arabinofuranose by the HMBC correlation between H-5′ and the carbonyl carbon of the acetyl group. This acetylation resulted in a downfield shift for both C-5′ and H-5′ compared to those for compound **1**. The chemical correlation between the two compounds was achieved by converting **2** into **1** through a mild alkaline hydrolysis procedure. The NOE correlations of **2** obtained from a 2D ROESY experiment showed exactly the same patterns as those for compound **1**, supporting the configurational and conformational assignment for its aglycone (**3**).

In vitro antifungal testing showed that compound **1** had an IC_{50} value of 3.7 μg/mL against *C. neoformans* ATCC 90113, while compound **2** was inactive at the highest test concentration of 20.0 μg/mL. The IC_{50} value of **1** was approximately one-third of that observed for CF80680-c4. It was thus concluded that compound **1** is responsible for the antifungal activity of CF80680-c4. Compound **3**, the aglycone of compound **1**, was also active against *C. neoformans* ATCC 90113, with an IC_{50} of 9.2 μg/mL. Since the recent cryptococcosis outbreak throughout the Pacific Northwest was caused by *Cryptococcus gattii*, a highly virulent fungal pathogen that generally affects immunocompetent hosts,[16,17] compounds **1** and **3** were tested further against *C. gattii* ATCC 32609, showing IC_{50}/MIC values of 1.8/20.0 and 6.8/20.0 μg/mL, respectively. For comparison, the positive control amphotericin B exhibited IC_{50}/MIC values of 0.2/0.6 and 0.1/0.3 μg/mL against *C. neoformans* ATCC 90113 and *C. gattii* ATCC 32609, respectively. It was noted that acetylation of **1** resulted in the loss of activity for compound **2**, while deglycosylation of **1** retained the activity for compound **3**. The retention of activity for aglycone **3** implies that more chemically tractable, structurally similar natural and synthetic diterpenoids can be explored for anticryptococcal activity.

The cytotoxicity of compounds **1** and **3** was evaluated against two mammalian cell lines to determine their selectivity of antifungal activity. The IC_{50} values of **1** were 30.0 and 21.5 μg/mL against Vero (African green monkey kidney fibroblast) and LLC-PK_1 (pig kidney epithelial) cells, respectively, while for compound **3** the values were 37.5 and 42.5 μg/mL, respectively. Both compounds were not cytotoxic at the concentrations that were responsible for antifungal activity and are significantly less cytotoxic than the positive control doxorubicin (IC_{50} values of 5.4 and 0.4 μg/mL against the same two cell lines, respectively).

In conclusion, the LC-MS and ^{1}H NMR spectroscopic analysis of the active CF80680-c4 from our small-molecule natural product libraries has led to the identification of antifungal diterpenoids. The combination of LC-MS and ^{1}H NMR spectroscopy is an improved chemical dereplication tool for structural analysis of compounds in semipurified CFs. The efficiency of rapid isolation of active compounds was proven in this study, whereas the conventional bioassay-guided fractionation often requires multiple steps of fractionation and bioassay before isolation of the desired active compounds. The inclusion of ^{1}H NMR spectroscopic analysis for dereplication is particularly powerful for identifying known compounds without time-consuming isolation from CFs that contain structurally similar compounds. Two such examples that illustrate rapid dereplication of known antifungal compounds are further shown in the Supporting Information. Thus, the application of this improved dereplication tool will enhance discovery potential in a natural product discovery program.

EXPERIMENTAL SECTION

General Experimental Procedures. Specific rotations were measured on an Autopol IV polarimeter. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Bruker DRX NMR spectrometer operating at 400 (^{1}H) and 100 (^{13}C) MHz or a 500 MHz Bruker DRX NMR spectrometer operating at 500 (^{1}H) and 125 (^{13}C) MHz. Chemical shifts are expressed in ppm relative to the residual solvent signals. High-resolution ESIMS data were obtained on an Agilent Series 1100 SL mass spectrometer. Column chromatography was performed on silica gel (40 μm, J. T. Baker) and reversed-phase silica gel (C_{18}, 40 μm, J. T. Baker). Silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany) and reversed-phase TLC plates (C_{18}, Merck, Darmstadt, Germany) were used for analytical TLC. The plates were visualized by spraying 10% H_2SO_4 followed by heating. UPLC-MS-ELSD-PDA data of CFs were obtained with a Waters Acquity UPLCMS system (Waters Corp., Milford, MA, USA). The general conditions are shown in Figure 1, while the detailed instrumentation protocol has been described previously.[1]

Plant Material. The leaves and flowers of *Sagittaria latifolia* Willd. were collected in Marinette, WI, USA, with coordinates of 45°05′12″ N 087°35′15″ W by Andrew Townesmith and Greg Gust on August 19, 2005, and identified by A. Townesmith. A voucher specimen (collection no. 272) is deposited in the Herbarium of the Missouri Botanical Garden, St Louis, MO, USA.

Extraction and Isolation. The air-dried and powdered leaves and flowers of *S. latifolia* (40 g) were extracted three times with 95% EtOH (360 mL) at 37 °C for 10 min using an accelerated solvent extractor to yield a crude EtOH extract (9.8 g). The extract (9.8 g) was chromatographed on silica gel using $CHCl_3$ first and then a gradient elution of $CHCl_3$–MeOH (0–30% MeOH) to yield 29 fractions, which were subjected to TLC analysis by comparison with CF80680-c4. Compound **2** was present in fractions 11 and 12, which were combined (540 mg) and chromatographed on reversed-phase C_{18} silica gel using 80–100% MeOH in H_2O to yield compound **2** (159.1 mg). The combined fractions 16–18 (280 mg) contained compound **1**, which was obtained in a quantity of 172.5 mg by reversed-phase C_{18} silica gel chromatography using 85–100% MeOH in H_2O. While conducting the isolation work, antifungal testing of the 29 CFs against *C. neoformans* ATCC 90113 was performed, indicating that compound **1**-containing fractions were most active, with IC_{50} values of ~5 μg/mL, thereby excluding the possibility of missing any antifungal compounds from other CFs.

*Isopimara-7,15-dien-19-ol 19-O-α-L-arabinofuranoside (**1**):* white powder; $[\alpha]^{25}_{D}$ −89.9 (*c* 0.1, $CHCl_3$); NMR data, Table 1; HRESIMS *m*/*z* 465.2860 (calcd for $[C_{25}H_{40}O_5 + HCOO]^-$, 465.2858; Δ = −0.41 ppm).

*Isopimara-7,15-dien-19-ol 19-O-α-L-(5′-acetoxy)arabinofuranoside (**2**):* colorless liquid; $[\alpha]^{25}_{D}$ −65.9 (*c* 0.1, $CHCl_3$); NMR data, Table 1; HRESIMS *m*/*z* 507.2967 (calcd for $[C_{27}H_{42}O_6 + HCOO]^-$, 507.2963; Δ = −0.68 ppm).

Acid Hydrolysis of Compound 1. A solution of **1** (25 mg) in 2 M HCl–1,4-dioxane (1:1, 2 mL) was stirred at 80 °C for 45 min. After cooling, the reaction mixture was diluted with H_2O (10 mL) and extracted with $CHCl_3$ (10 mL × 3). The combined organic layers were washed with H_2O (5 mL × 3), dried over Na_2SO_4, and evaporated to

DOI: 10.1021/acs.jnatprod.5b00470
J. Nat. Prod. 2015, 78, 2255–2259

Table 2. In Vitro Antifungal Activity and Cytotoxicity of Compounds 1 and 3[a]

compound	antifungal activity (IC_{50}/MIC, μg/mL)[b]		cytotoxicity (IC_{50}, μg/mL)[c]	
	C. neoformans ATCC 90113	*C. gattii* ATCC 32609	Vero	LLC-PK_1
1	3.7 ± 0.3/>20.0	1.8 ± 0.6/20.0 ± 0	30.0 ± 0	21.5 ± 4.9
3	9.2 ± 1.1/>20.0	6.8 ± 0.6/20.0 ± 0	37.5 ± 3.5	42.5 ± 3.5
amphotericin B	0.2 ± 0.02/0.7 ± 0	0.1 ± 0.06/0.3 ± 0.1		
doxorubicin			5.4 ± 0.2	0.4 ± 0.2

[a]Both antifungal activity and cytotoxicity are expressed as mean values of three experimental values with standard deviations. [b]IC_{50}: concentration responsible for 50% growth inhibition of fungal cells; MIC: minimum inhibitory concentration (lowest concentration that allows no detectable growth). The highest test concentration used for both compounds **1** and **3** was 20 μg/mL. [c]IC_{50}: concentration responsible for 50% growth inhibition of mammalian cells. The highest test concentration used for both compounds **1** and **3** was 50 μg/mL.

dryness to yield a residue (21 mg). The residue was subjected to C_{18} reversed-phase silica chromatography using MeOH−H_2O (98:2) to give compound **3** (7.0 mg): colorless liquid: $[\alpha]^{25}_D$ −13.9 (*c* 0.1, $CHCl_3$) {ref.:[14] $[\alpha]^{20}_D$ −15 ($CHCl_3$)}.

The H_2O layer was passed through an Amberlite MB-150 resin column (8 g) eluting with H_2O (50 mL) to give L-arabinose (4.5 mg): $[\alpha]^{25}_D$ +93.9 (*c* 0.1, H_2O), which was comparable to $[\alpha]^{25}_D$ +119.9 (*c* 0.1, H_2O) for standard L-(+)-arabinose from Sigma-Aldrich. L-Arabinose gave an R_f value of 0.7 on normal-phase silica gel TLC developed with $CHCl_3$−MeOH−H_2O (6:4:1).

Alkaline Hydrolysis of Compound 2. A solution of **2** (1.0 mg) in MeOH−1% KOH (1:1, 200 μL) was kept at room temperature for 15 min. The reaction mixture was directly subjected to TLC analysis using $CHCl_3$−MeOH (95:5) as a developing system. Compound **1** was detected with an R_f value of 0.3.

In Vitro Antifungal Assay. A modified version of the CLSI (formerly NCCLS) method was used for susceptibility testing. Organisms (*C. neoformans* ATCC 90113 and *C. gattii* ATCC 32609) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Amphotericin B (ICN Biomedicals, Aurora, OH, USA) was used as a positive control. The detailed procedure has been described previously.[18,19]

In Vitro Cytotoxicity Assay. The two mammalian cell lines Vero (African green monkey kidney fibroblast) and LLC-PK_1 (pig kidney epithelial) used in this study were obtained from ATCC. Cytotoxicity was determined by the neutral red method.[20] Doxorubicin (ICN Biomedicals) was used as a positive control. The detailed procedure has been described in a previous paper.[18]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00470.

Two additional examples for dereplication of known antifungal compounds using LC-MS and ^{1}H NMR; NMR and HRESIMS spectra of compounds **1**−**3** (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 662-915-6742. Fax: 662-915-7989. E-mail: xcli7@olemiss.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Ms. M. Bennett for extraction of the plant material, Ms. M. Wright for in vitro antifungal testing, Mr. J. Trott for cytotoxicity testing, Dr. B. Avula for high-resolution mass spectrometry analyses, and Mr. F. T. Wiggers for acquiring the 2D NMR spectra. This work was supported by the NIH, NIAID, Grant No. AI27094, and the USDA Agricultural Research Service Specific Cooperative Agreement No. 58-6408-2-0009.

■ REFERENCES

(1) Yang, J.; Liang, Q.; Wang, M.; Jeffries, C.; Smithson, D.; Tu, Y.; Boulos, N.; Jacob, M. R.; Shelat, A. A.; Wu, Y.; Ravu, R. R.; Gilbertson, R.; Avery, M. A.; Khan, I. A.; Walker, L. A.; Guy, R. K.; Li, X.-C. *J. Nat. Prod.* **2014**, *77*, 902−909.

(2) Perfect, J. R. In *Antifungal Therapy*; Ghannoum, M. A., Perfect, J. R., Eds.; Informa Healthcare: New York, 2010; Chapter 18, pp 308−319.

(3) Tanaka, C. M. A.; Radke, V. S. C. O.; da Silva, C. C.; Nakamura, C. V.; de Oliveira, P. L.; Kato, L.; de Oliveira, C. M. A. *Quim. Nova* **2010**, *33*, 30−32.

(4) Sharma, S. C.; Tandon, J. S.; Dhar, M. M. *Phytochemistry* **1975**, *14*, 1055−1057.

(5) Juneja, R. K.; Sharma, S. C.; Tandon, J. S. *Indian J. Chem. Sect B: Org. Chem.* **1986**, *25B*, 748−749.

(6) Yoshikawa, M.; Yamaguchi, S.; Murakami, T.; Matsuda, H.; Yamahara, J.; Murakami, N. *Chem. Pharm. Bull.* **1993**, *41*, 1677−1679.

(7) Yoshikawa, M.; Yoshizumi, S.; Murakami, T.; Matsuda, H.; Yamahara, J.; Murakami, N. *Chem. Pharm. Bull.* **1996**, *44*, 492−499.

(8) Radke, V. S. C. O.; Tanaka, C. M. A. *Biochem. Syst. Ecol.* **2004**, *32*, 529−531.

(9) Radke, V. S. C. O.; Amaral, M. C. E.; Schuquel, I. T. A.; da Silva, C. C.; de Oliveira, C. M. A.; Tanaka, C. M. A. *J. Braz. Chem. Soc.* **2007**, *18*, 444−447.

(10) Liu, X.-T.; Pan, Q.; Shi, Y.; Williams, I. D.; Sung, H. H.-Y.; Zhang, Q.; Liang, J.-Y.; Ip, N. Y.; Min, Z. D. *J. Nat. Prod.* **2006**, *69*, 255−260.

(11) Liu, X.-T.; Shi, Y.; Yu, B.; Williams, I. D.; Sung, H. H.-Y.; Zhang, Q.; Liang, J.-Y.; Ip, N. Y.; Min, Z. D. *Planta Med.* **2007**, *73*, 84−90.

(12) Liu, X.-T.; Shi, Y.; Liang, J.-Y.; Min, Z.-D. *Zhongguo Tianran Yaowu* **2009**, *7*, 341−345.

(13) Tu, Y.; Jeffries, C.; Ruan, H.; Nelson, C.; Smithson, D.; Shelat, A. A.; Brown, M. K.; Li, X.-C.; Hester, P. J.; Smillie, T.; Khan, I. A.; Walker, L. A.; Guy, R. K.; Yan, B. *J. Nat. Prod.* **2010**, *73*, 751−754.

(14) Cagnoli, N.; Ceccherelli, P.; Curini, M. *J. Chem. Res. (S)* **1982**, 254.

(15) Passannanti, S.; Paternostro, M.; Piozzi, F. *J. Nat. Prod.* **1984**, *47*, 885−889.

(16) Kidd, S. E.; Hagen, F.; Tscharke, R. L.; Huynh, M.; Bartlett, K. H.; Fyfe, M.; MacDougall, L.; Boekhout, T.; Kwon-Chung, K. J.; Meyer, W. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 17258−17263.

(17) Byrnes, E. J., 3rd; Li, W.; Lewit, Y.; Ma, H.; Voelz, K.; Ren, P.; Carter, D. A.; Chaturvedi, V.; Bildfell, R. J.; May, R. C.; Heitman, J. *PLoS Pathog.* **2010**, *6*, e1000850.

(18) Yang, C.-R.; Zhang, Y.; Jacob, M. R.; Khan, S. I.; Zhang, Y.-J.; Li, X.-C. *Antimicrob. Agents Chemother.* **2006**, *50*, 1710−1714.

(19) Li, X.-C.; Jacob, M. R.; Khan, S. I.; Ashfaq, M. K.; Babu, K. S.; Agarwal, A. K.; Elsohly, H. N.; Manly, S. P.; Clark, A. M. *Antimicrob. Agents Chemother.* **2008**, *52*, 2442−2448.

(20) Borenfreund, E.; Puerner, J. *Toxicol. Lett.* **1985**, *24*, 119−124.

DOI: 10.1021/acs.jnatprod.5b00470
J. Nat. Prod. 2015, 78, 2255−2259