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Wortmannin and Wortmannine Analogues from an Undescribed *Niesslia* sp.

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Abstract

In the course of our studies of coprophilous fungi as sources of antifungal agents, a strain of an undescribed species in the genus *Niesslia* (TTI-0426) was isolated from horse dung collected in Texas. An extract from fermentation cultures of this strain afforded two new antifungal wortmannin derivatives, wortmannins C and D (**1** and **2**), as well as four additional new related compounds, wortmannines B1–B4 (**3–6**), containing an unusual ring system. The structures of these metabolites were established mainly by analysis of HRESIMS and 2D NMR data. Relative configurations were assigned using NOESY data, and the structure assignments were supported by NMR comparison with similar compounds. Wortmannins C and D showed activity against *Cryptococcus neoformans* and *Candida albicans* in disk assays, but low MIC potency observed for **1** was suggested to be due in part to efflux processes on the basis of assay results for a *Schizosaccharomyces pombe* efflux mutant in comparison to wild-type.

Cryptococcus species are among the most common causes of invasive fungal infections worldwide, and are especially problematic for immunocompromised individuals such as those with HIV/AIDS.^{1–2} Current treatments still rely on a small number of antifungal agents discovered many years ago which remain unsatisfactory because of toxicity and drug resistance issues. Historically, antifungal screening programs have centered mainly on *Candida* and *Aspergillus* spp. There is still a need for treatments effective against these pathogens, but *Cryptococcus* spp. have been relatively neglected in such programs.³ Given that natural products from fungi have been important in the development of antifungal agents, we have initiated a *Cryptococcus*-centric exploration of fungi for antifungal natural products, with an emphasis on fungal sources that remain relatively unexplored to date.

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Notes

The authors declare no competing financial interest.

DEDICATION

Dedicated to Dr. Rachel Mata, National Autonomous University of Mexico, Mexico City, Mexico and Dr. Barbara N. Timmermann, University of Kansas, for their pioneering work on bioactive natural products.

Coprophilous fungi are those that colonize animal dung, and comprise an especially conspicuous component of the microbiota of herbivore dung.^{4–6} Past studies of coprophilous fungi have afforded a variety of new metabolites with bioactivities that often included antifungal effects.^{7–8} Even so, this ecological niche group remains relatively underexplored. During our continuing studies of coprophilous fungi, a strain, TTI-0426, was isolated from horse dung collected at Lake Houston in Montgomery County, Texas. Morphological characteristics and rDNA sequence analysis identified TTI-0426 as an undescribed *Niesslia* sp. The rDNA sequences were most similar to metagenome sequences amplified from cotton field soil in northern Texas and soil-borne strains of *Monocillium indicum*. An extract of a solid-substrate fermentation of TTI-0426 exhibited activity against *Cryptococcus neoformans* and *Candida albicans*, and was therefore selected for chemical investigation. Fractionation of this extract led to the isolation of six new wortmannin and wortmannine derivatives (**1–6**), which we named wortmannins C and D and wortmannines B1–B4.

RESULTS AND DISCUSSION

Strain TTI-0426 displayed morphological features reminiscent of several fungi in the Niessliaceae, especially species of *Monocillium* and *Niesslia*. Similarity searches of public databases (NCBI, Mycobank) with the large subunit (LSU) rDNA sequences retrieved *Niesslia exilis* (AY489720, 96% identity) as the most similar sequence, and searches with the internal transcribed spacer region (ITS) of the rDNA retrieved Canadian soil-borne strains identified as *Monocillium indicum* (e.g., CBS 182.65, 97% identity) and a series of metagenomic ITS partial sequences from cotton fields near Lubbock, Texas (e.g., JX319019, JX366671, JX321580, 99% identity). Maximum likelihood analysis of the LSU rDNA placed TTI-0426 close to strains of *Monocillium indicum* (85% branch support). Maximum likelihood analysis of the ITS rDNA also indicated that TTI-0426 was closely related to *M. indicum* or possibly even a sister species. Furthermore, TTI-0426 grouped close with partial ITS metagenomic sequences amplified from soils of northern Texas cotton fields, indicating that it might be the same species. Therefore, we concluded that TTI-0426 represents a yet unnamed species of *Niesslia*.

Methyl ethyl ketone (MEK) extracts of TTI-0426 grown on a solid vermiculite matrix⁹ infused with sucrose-yeast extract medium (YES)¹⁰ or on an agitated liquid sucrose-tomato paste medium (STP)¹¹ caused growth inhibition zones against *C. albicans* and *C. neoformans*, but did not affect *Staphylococcus aureus* (Fig. S26). The fermentation was scaled up on YES medium infused into vermiculite. The solid mass was extracted with MEK and concentrated under vacuum. Solvent partitioning, followed by silica gel chromatography and reversed phase HPLC afforded compounds **1–6**.

Wortmannin C (**1**) was obtained as a yellow oil. The molecular formula was determined to be C₂₆H₃₀O₈ (12 degrees of unsaturation) based on HRESIMS data. The ¹H and ¹³C NMR data of **1** (Table 1) revealed the presence of three methyl singlets (one methoxy), one methyl doublet and one methyl triplet. Resonances for five methylene units, five methines (two oxygenated and one aromatic/olefinic), and 11 nonprotonated carbons were also observed. These data closely resembled those of the known compound wortmannin (**7**),¹² with the only major differences being the absence of a resonance for an acetyl methyl group and the

presence of additional signals for two methyl groups (a doublet and a triplet), a methylene unit, and a methine. These differences suggested that the acetyl group in wortmannin is replaced with a 2-methylbutyryl group in **1**. This hypothesis was supported by analysis of 2D NMR data. HMBC correlations shown in Figure 1 indicated the planar connectivity of carbons C-1 through C-21 to be the same as that of wortmannin. No correlations to cross-conjugated carbonyl carbon C-7 were observed, but a signal at δ_C 172.7 for the corresponding carbon was present in the ^{13}C NMR spectrum, matching well with literature data.¹² Correlations from both H₃-26 and H₃-25 to a methylene carbon at δ_C 27.2 and a methine carbon at δ_C 40.8, together with additional correlations from H₃-24 and H-23 to ester carbon C-22 completed the 2-methylbutyrate unit. Finally, a correlation from oxymethine H-11 to C-22 confirmed the placement of the 2-methylbutyrate unit at C-11, leading to the assignment of structure **1**.

NOESY correlations (Figure 2) from H-1 to H₃-19 and H-11, as well as from H-11 to H₃-18 and H_β-12, indicated that these are all on the same face of the ring system, while a correlation from H_α-12 to H-14 placed these protons on the opposite face. These correlations were fully consistent with a relative configuration analogous to that of **7**. The absolute configuration of **1** is assumed to match that of **7** at the relevant stereocenters. Though not conclusive, the specific rotation of **1** is similar to that of **7** (**1**: $[\alpha]_D^{20} = +72$; **7**: $[\alpha]_D^{20} = +86.5$). Unfortunately, small-scale hydrolysis of a sample of **1** failed to afford a 2-methylbutyric acid degradation product for comparison to standards, so the absolute configuration at the corresponding stereocenter was not assigned.

The HRESIMS data for wortmannin D (**2**) indicated a molecular formula with one less CH₂ unit than **1**. The 1H NMR spectrum again resembled that of wortmannin. In comparison to **1**, **2** lacked signals for the 2-methylbutyryl unit, instead showing signals for an isopropyl group. These data suggested that the 2-methylbutyryl unit in **1** is replaced by an isobutyryl unit in **2**, and all NMR data for **2** (Table 1) were fully consistent with this assignment, as all other spectroscopic data were nearly identical to those of **1**. It is assumed that the stereochemical configuration of **2** is analogous to that of wortmannin and **1**. The 2-methylbutyryl and isobutyryl units are somewhat rare as constituents of fungal metabolites

Wortmannin (**7**) is a well known potent phosphoinositide-3-kinase inhibitor originally described from *Penicillium wortmanni* as a selective antifungal agent,¹³ and subsequently reported from several other fungal sources. Multiple analogues have been prepared synthetically in efforts to optimize its kinase inhibitory effects, but only a few analogues have been isolated from other fungal sources, including wortmannin B, which lacks the C-2 methoxy methyl group, while replacing the acetyl group of **7** with a methyl group.¹⁴ Given the prior nomenclature, the names wortmannin C and D are proposed for **1** and **2**.

The NMR data for wortmannine B1 (**3**) resembled those of **1** and **2**, but **3** was found to incorporate a nitrogen atom, as it was assigned the molecular formula C₂₆H₃₃NO₉ (11 degrees of unsaturation) on the basis of HRESIMS data. The 1H and ^{13}C NMR data (Table 2) again showed resonances for a 2-methylbutyryl unit, along with three methyl singlets (including one methoxy), one oxygenated methylene, three other aliphatic methylene units, five other methines (including three oxygenated), and nine other nonprotonated carbons (two

olefinic, two other ester/amide carbons, two ketones, and three quaternary sp^3 carbons). These data closely matched those of the recently reported wortmannine B,¹⁵ which contains a previously undescribed ring system, and whose structure was confirmed by X-ray crystallographic analysis. The NMR data suggested that the structure of **3** matches that of wortmannine B with acylation of the C-11 oxygen by a 2-methylbutyryl unit. Wortmannine B lacks an acyl unit altogether at that position. COSY and HMBC (Figure 1) correlations for **3**, including HMBC correlations from H-23 and H-11 to C-22, independently confirmed this hypothesis. NOESY correlations (Figure 2) from H-11 to H_β-12, H₃-18, and H₃-19, as well as from H₃-19 to H-1, placed all of these hydrogens on the same face of the molecule, while correlations from H_α-12 to H-14 and from H₃-21 to H-4 placed these units on the opposite face. The coupling constant observed between H-4 and H-20 matched well with that reported for wortmannine B, indicating the same relative configuration at C-5, and establishing an overall relative configuration, and presumably absolute configuration, matching that of wortmannine B and wortmannin at analogous stereocenters.

Wortmannine B2 (**4**) was determined to be an isomer of **3**. The ¹H NMR spectrum of **4** was very similar to that of **3** with the main differences being the shift of H-4 from δ 4.03 in **3** to 3.80 in **4** and a difference in J_{H4-H20} from 6.5 Hz in **3** to ~0 in **4** suggesting that **4** is an epimer of **3**, likely differing only in the configuration at C-20. COSY and HMBC data for **4** confirmed the same planar structure as **3** and NOESY data indicated the same relative configuration at all stereocenters except for C-20. Energy-minimized models of each epimer, generated using Spartan 16, were utilized to measure the dihedral angle between H-4 and H-20. The 6.5-Hz coupling constant in **3** is consistent with a predicted dihedral angle of 41°, closely matching the data for wortmannine B and indicating that H-20 is α-oriented. Conversely, the value in **4** is near zero, consistent with a predicted dihedral angle closer to 90°, indicating that H-20 is β-oriented in **4**. These data establish **4** as an epimer of **3** at C-5, leading to assignment of the absolute configuration shown.

Compound **5** was assigned a molecular formula containing one CH₂ unit less than **3** and **4** on the basis of HRESIMS data. The ¹H NMR spectrum of **5** was very similar to that of **3**, with the signals for the 2-methylpropyl unit replaced by those of an isopropyl group, indicating that the 2-methylbutyryl unit in **3** was replaced by an isobutyryl unit in **5**. HRESIMS data for **6** showed it to be an isomer of **5**. NMR data revealed that the only significant difference between **5** and **6** was analogous to the difference between **3** and **4**, indicating that **6** is an epimer of **5** at position C-20. The names wortmannines B3 and B4 are proposed for **5** and **6**, respectively.

Compounds **1-6** were evaluated for activity against *C. neoformans*, *C. albicans*, and *S. aureus* in disk diffusion assays at levels of 50 μg/disk. Compounds **1** and **2** showed 16- and 13-mm inhibitory zones, respectively, against *C. neoformans* (amphotericin B gave a 13-mm zone at 25 μg/disk). Both compounds also showed 25-mm zones at 50 μg/disk against *C. albicans* (amphotericin B gave a 10-mm zone at 25 μg/disk), but did not show activity against *S. aureus*. Compounds **3-6** were inactive in all of these assays. To better define the antifungal effect of representative compound **1**, minimal inhibitory concentration (MIC) values were determined for **1** by the broth microdilution method according to NCCLS

protocols, testing against *Aspergillus fumigatus*, as well as *C. albicans* and *C. neoformans*. Disappointingly, at 48 hours, the MIC values for *C. albicans* and *A. fumigatus* were each 200 μ M, while the MIC for *C. neoformans* (as measured at 72 hours), was 100 μ M (ca. 47 μ g/mL). Interestingly, visual inspection of growth at 24 hours indicated a significant growth delay of the *C. albicans* cultures at drug concentrations as low as 25 μ M. However, inhibition at lower concentrations was temporary, with growth similar to untreated controls at the 48 hour end-point at concentrations below the formal MIC. This observation suggests an initial fungistatic effect that is eventually overcome with longer incubation times.

To further explore the potential mechanism of this temporal growth inhibition, we performed MIC testing for **1** in the *Schizosaccharomyces pombe* isolate MJ1682, a yeast strain with mutations in several efflux pump genes, as well as a mutation in the gene encoding a transcription factor regulating drug efflux. This strain has been used to assess the role of MDR-type efflux pumps in antifungal drug resistance and fungal cell physiology.^{16,17} Compared to the isogenic wild-type control strain SAK1, the MJ1982 efflux mutant was 10-times more susceptible to compound **1**. Taken together, these observations suggest that efflux from the fungal cell might limit the potency and durability of the antifungal activity of this compound.

Wortmannin (**7**) and related compounds such as viridin and its analogues, are known to be derived from the steroid pathway via distinctive steps that involve oxidative removal of carbons from sterol precursors.¹⁸ The recent publication of the viridin biosynthetic gene cluster¹⁸ helped guide the identification a putative gene cluster in a draft genome sequence of strain TTI-0426 that could potentially encode the biosynthesis of wormannin and wortmannine analogues (Supplemental information, Figures S31–S33 and Tables S1–S3). Members of this class that contain the furan unit characteristic of **1–2** and **7** are referred to as furanosteroids, and compounds retaining this unit are well known to display a variety of bioactivities. The mechanism leading to the skeletal change in **3–6** or the stage of the biosynthetic process at which it occurs is not clear, but the absence of this unit in **3–6** is consistent with loss of activity among these analogues.

EXPERIMENTAL SECTION

General Experimental Procedures.

Optical rotations were measured on a Rudolph Research Autopol III automatic polarimeter. UV data were obtained using a Varian Cary III UV/vis spectrophotometer. ¹H and ¹³C NMR spectra were recorded using an AVANCE-500 spectrometer with a 5-mm probe or an AVANCE-600 spectrometer with a 1.3-mm probe. Chemical shift values were referenced to residual solvent signals for acetone-*d*₆ ($\delta_{\text{H}}/\delta_{\text{C}}$, 2.05/29.8), CDCl₃ ($\delta_{\text{H}}/\delta_{\text{C}}$, 7.24/77.2), or methanol-*d*₄ ($\delta_{\text{H}}/\delta_{\text{C}}$, 3.31/49.0). HSQC, HMBC, COSY, and NOESY data were recorded using an AVANCE-600 spectrometer with a 1.3-mm probe or a Bruker AVANCE-500 MHz spectrometer with a 5-mm triple-resonance cryoprobe. HRESIMS data were recorded using a Waters Q-TOF Premier mass spectrometer. Reversed phase HPLC separations were carried out using a semipreparative Apollo C₁₈ column (Alltech; 1.0 $\times$ 25 cm, 5- μ m particles; 2 mL/min) interfaced with an Agilent 1220 Infinity instrument equipped with a variable-

wavelength UV detector or a Gilson model 322 instrument equipped with a model 155 variable-wavelength detector.

Fungal Material.

Horse dung was collected at Lake Houston Wilderness Park, Montgomery Co., Texas, USA. The producing strain was isolated by plating washed dung particles onto Miru's agar medium.¹⁹ Mycelia emerging from these dung particles were transferred to YMA medium (1% maltose extract, 0.2% yeast extract, and 2% agar) to establish axenic cultures. The isolate was assigned the accession number TTI-0426 in Texas Therapeutics Institute's culture collection at the Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center at Houston, Houston, TX, USA. Strain TTI-0426 grew on agar media to form slow-growing white mycelium bearing pink, slimy, sporodochial conidiomata. Thick-walled lanceolate and capitate setae formed among conidiogenous cells. The phialidic conidiogenous cells produce slimy masses of curved elliptical to allantoid conidia. This combination of features was reminiscent of several fungi in the Niessliaceae, especially species of *Monocillium* and *Niesslia* with thick-walled conidiogenous cells and capitate setae. Key morphological features that identify the fungus as a species of *Niesslia* are illustrated in Fig. S27 and S28.

To corroborate the morphological identification, we estimated its approximate phylogenetic relationships based on the internal transcribed spacer (ITS) and large subunit (LSU) rDNA sequences. DNA extraction from cultured mycelium was conducted as previously described. The ITS and LSU DNA sequences were amplified by primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and LR7 (5'-TACTACCACCAAGATCT-3').⁹ DNA was amplified in 50- μ L reaction volumes by the following procedures: initial denaturation at 94 °C for 2 min, followed by 32 cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 55 °C, and extension for 2 min at 72 °C, with final extension at 72 °C for 10 min. Amplicons were sequenced using the primers ITS1, ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), LROR (5'-ACCCGCTGAACTTAAGC-3'), LR5 (5'-TCCTGAGGGAACTTCG-3') and LR7.^{20–21} The ITS+LSU rDNA sequence (1921 bp) was accessioned in the NCBI GenBank ([MH997627](#)).

The sequence of the LSU region of TTI-0426 was aligned with sequences from other species of *Monocillium*, *Niesslia*, and *Hyaloseta* retrieved from GenBank. These three genera may eventually all be merged into the genus *Niesslia* (W. Gams, personal communication). A second alignment was made using the ITS region and similar sequences from Genbank. Maximum likelihood analyses of the two sets of aligned sequences were executed in Mega 7.0²² produce phylogenetic trees (Figures S29–30). Based on the phylogenies of its LSU and ITS sequences, TTI-0426 appears to be a possible sister species of *M. indicum*, the type species of the genus *Monocillium*.^{23,24} The close relationship to *M. indicum* was also evident in the ITS phylogeny. However, unlike *M. indicum*, the conidiogenous cells of TTI-0426 are aggregated in sporodochial masses and the conidia are allantoid in shape. TTI-0426 was even more closely related to, and possibly conspecific with, a group of metagenomic sequences amplified from northern Texas cotton fields. Therefore, we refer to

TTI-0426 as *Niesslia* sp. based on its morphological features and its phylogenetic proximity to other species of *Niesslia* and *Monocillium*.

Fermentation and Extraction.

The strategy to generate small-scale extracts from fungi for antifungal screening against *C. neoformans* and *C. albicans* has been detailed previously.^{25–26} Briefly, the strain was grown at a 12-mL scale of on five media selected for proven ability to stimulate production of secondary metabolites. After 14 d, each fermentation was extracted with an equal volume of methyl ethyl ketone (MEK). After thorough mixing of the solvent with the culture for at least 2 h, 5 mL of the organic phase was dried. The dried residue was re-suspended in 500 μ L of DMSO. Aliquots (20 μ L) of the crude DMSO extracts were applied to the wells of the assay plates as described above along with the corresponding controls, e.g., amphotericin B or a mixture of streptomycin sulfate and chlortetracycline (Fig. S26).

To scale up the growth of TTI-0426, agar plugs from YMA cultures were inoculated into 250-mL Erlenmeyer flasks each containing 50 mL of SMYA seed medium (1% Bacto neopeptone, 4% maltose, 1% yeast extract and 0.4% agar). Seed media were incubated for 5 days with agitation at 220 rpm at 23 °C. A scaled-up fermentation was then carried out in eight 1-L Pyrex bottles each containing 340 mL of coarse sterilized vermiculite. Liquid YES medium¹⁰ consisting of 150 g sucrose; 20 g yeast extract; 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$; and 0.5 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1 L of deionized H_2O , was prepared in 125-mL aliquots in separate flasks. Five mL of seed culture was mixed into each flask and the mixture from each flask was poured onto a bottle of the sterilized vermiculite. The bottles were incubated and rolled on a tissue culture roller machine at 23 °C until the fungal growth penetrated and immobilized the vermiculite (4 d). After 14 d, the growth was extracted with an equal volume of MEK by rolling the bottles with the solid matrix for 2 hr.

Isolation and Purification.

The crude extract (980 mg) was partitioned between hexane (3×15 mL) and MeCN (20 mL). The MeCN-soluble portion (858 mg) was subjected to silica gel column chromatography, eluting with a stepwise gradient of hexane-EtOAc (200 mL each of 1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:1) and EtOAc-MeOH (100 mL of 99:1, 97:3, 95:5, 85:15, 75:25, 50:50, 25:75, 0:100, 0:100) to provide 20 fractions. Fraction 7 (25 of 27 mg) was purified by reversed-phase HPLC with 65% MeCN- H_2O for 15 min, followed by a linear gradient to 100% MeCN- H_2O over 15 min to afford **2** (1.3 mg, t_R = 14.6 min), and **1** (5.4 mg, t_R = 17.5 min). Fraction 9 (37 of 48 mg) was separated using reversed-phase HPLC with 40% MeCN- H_2O for 20 min, followed by a linear gradient to 100% MeCN- H_2O over 10 min to give **6** (1.6 mg, t_R = 21.5 min) and **4** (4.6 mg, t_R = 28.1 min). Fraction 10 (48 of 51 mg) was purified using reversed-phase HPLC with 20% MeCN- H_2O for 20 min, followed by a linear gradient to 100% MeCN- H_2O over 10 min to afford **5** (3.2 mg, t_R = 18.3 min), **6** (1.9 mg, t_R = 21.6 min), **3** (5.4 mg, t_R = 25.9 min), and **4** (3.0 mg, t_R = 28.2 min). Fraction 11 (28 of 32 mg) was separated using reversed-phase HPLC with 40% MeCN- H_2O for 20 min, followed by a linear gradient to 100% MeCN- H_2O over 10 min to give **5** (2.4 mg, t_R = 18.1 min) and **3** (3.3 mg, t_R = 25.7 min).

Wortmannin C (1).

yellow oil; $[\alpha]_D^{25} +72$ (c 0.003, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 205 (4.0), 256 (3.8), 289 (3.7) nm; ^1H and ^{13}C NMR data, see Table 1; HMBC data, H-1 $\rightarrow$ C-2, 3, 5, 10, 19; H_a-2 $\rightarrow$ C-1, 21; H_b-2 $\rightarrow$ C-10; H-11 $\rightarrow$ C-8, 9, 10, 12, 22; H_a-12 $\rightarrow$ C-11, 13, 14, 17, 18; H_β-12 $\rightarrow$ C-9, 11, 13, 14, 17, 18; H-14 $\rightarrow$ C-8, 9, 13, 15, 16, 18; H_a-15 $\rightarrow$ C-13, 14, 17; H_β-15 $\rightarrow$ C-8, 14, 16; H_a-16 $\rightarrow$ C-15, 17; H_b-16 $\rightarrow$ C-14, 17; H₃-18 $\rightarrow$ C-12, 13, 14, 17; H₃-19 $\rightarrow$ C-1, 5, 9, 10; H-20 $\rightarrow$ C-4, 5, 6; H₃-21 $\rightarrow$ C-2; H-23 $\rightarrow$ C-22, 24, 25, 26; H_a-24 $\rightarrow$ C-22, 23, 25, 26; H_b-24 $\rightarrow$ C-22, 23, 25, 26; H₃-25 $\rightarrow$ C-22, 23, 24; H₃-26 $\rightarrow$ C-23, 24; key NOESY data, H-1 $\leftrightarrow$ H₃-19; H-1 $\leftrightarrow$ H-11; H-11 $\leftrightarrow$ H₃-18; H_β-12; H_a-12 $\leftrightarrow$ H-14; H-14 $\leftrightarrow$ H_a-15; HRESITOFMS m/z 493.1833 $[\text{M}+\text{Na}]^+$ (calcd for C₂₆H₃₀O₈Na, 493.1838).

Wortmannin D (2).

yellow oil; $[\alpha]_D^{25} +30$ (c 0.00087, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 204 (3.6), 256 (3.3), 284 (2.7) nm; ^1H NMR data, see Table 1; HRESITOFMS m/z 479.1681 $[\text{M}+\text{Na}]^+$ (calcd for C₂₅H₂₈O₈Na, 479.1682).

Wortmannine B1 (3).

white powder; $[\alpha]_D^{25} +40$ (c 0.0033, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 203 (3.6), 249 (3.6) nm; ^1H and ^{13}C NMR data, see Table 2; HMBC data, NH $\rightarrow$ C-4, 5, 6, 20; H-1 $\rightarrow$ C-3, 5, 10, 19; H_a-2 $\rightarrow$ C-1, 10, 21; H_b-2 $\rightarrow$ C-1, 10, 21; H-4 $\rightarrow$ C-3, 5, 7, 10, 20; H-11 $\rightarrow$ C-8, 9, 12, 22; H_a-12 $\rightarrow$ C-11, 13, 14, 18; H_β-12 $\rightarrow$ C-9, 11, 13, 14, 18; H-14 $\rightarrow$ overlap; H_a-15 $\rightarrow$ C-8, 14, 16; H_β-15 $\rightarrow$ overlap; H_a-16 $\rightarrow$ C-14, 15, 17; H_β-16 $\rightarrow$ overlap; H₃-18 $\rightarrow$ C-12, 13, 14, 17; H₃-19 $\rightarrow$ C-1, 5, 9, 10; H-20 $\rightarrow$ C-5, 6; H₃-21 $\rightarrow$ C-2; H-23 $\rightarrow$ C-22, 24, 25, 26; H_a-24 $\rightarrow$ C-22, 23, 25, 26; H_b-24 $\rightarrow$ C-22, 23, 25, 26; H₃-25 $\rightarrow$ C-22, 23, 24; H₃-26 $\rightarrow$ C-23, 24; key NOESY data, H-1 $\leftrightarrow$ H₃-19; H₃-19 $\leftrightarrow$ H-11; H-11 $\leftrightarrow$ H₃-18; H-11 $\leftrightarrow$ H_β-12; H_a-12 $\leftrightarrow$ H-14; H₃-18 $\leftrightarrow$ H_β-15; H-4 $\leftrightarrow$ H₃-21; HRESITOFMS m/z 526.2054 $[\text{M}+\text{Na}]^+$ (calcd for C₂₆H₃₃NO₉, 526.2053).

Wortmannine B2 (4).

white powder; $[\alpha]_D^{25} +51$ (c 0.0032, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 204 (3.8), 246 (3.8) nm; ^1H and ^{13}C NMR data, see Table 2; HMBC data, NH $\rightarrow$ C-4, 5; H-1 $\rightarrow$ C-3, 5, 10, 19; H_a-2 $\rightarrow$ C-1, 10, 21; H_b-2 $\rightarrow$ C-1, 10, 21; H-4 $\rightarrow$ C-3, 5, 6, 7, 20; H-11 $\rightarrow$ C-8, 9, 12, 22; H_a-12 $\rightarrow$ C-11, 13, 18; H_β-12 $\rightarrow$ C-9, 11, 13, 14, 17, 18; H-14 $\rightarrow$ C-8, 9, 12, 13, 15, 18; H_a-15 $\rightarrow$ C-13, 14, 16, 17; H_β-15 $\rightarrow$ C-8, 14, 16; H_a-16 $\rightarrow$ C-15, 17; H_β-16 $\rightarrow$ C-14, 15, 17; H₃-18 $\rightarrow$ C-12, 13, 14, 17; H₃-19 $\rightarrow$ C-1, 5, 9, 10; H-20 $\rightarrow$ C-3, 5, 6; H₃-21 $\rightarrow$ C-2; H-23 $\rightarrow$ C-22, 24, 25, 26; H_a-24 $\rightarrow$ C-22, 23, 25, 26; H_b-24 $\rightarrow$ C-22, 23, 25, 26; H₃-25 $\rightarrow$ C-22, 23, 24; H₃-26 $\rightarrow$ C-23, 24; key NOESY data, H-1 $\leftrightarrow$ H₃-19; H₃-19 $\leftrightarrow$ H-11; H-11 $\leftrightarrow$ H₃-18; H₃-18 $\leftrightarrow$ H_β-15; H_a-15 $\leftrightarrow$ H-14; H-14 $\leftrightarrow$ H_a-12; H-14 $\leftrightarrow$ H_a-16; H-4 $\leftrightarrow$ H₃-21; H₃-21 $\leftrightarrow$ H-14; HRESITOFMS m/z 526.2060 $[\text{M}+\text{Na}]^+$ (calcd for C₂₆H₃₃NO₉Na, 526.2053).

Wortmannine B3 (5).

white powder; $[\alpha]_D^{25} +42$ (c 0.0013, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 203 (3.6), 248 (3.5) nm; ^1H NMR data, see Table 2; HRESITOFMS m/z 512.1898 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{31}\text{NO}_9\text{Na}$, 512.1897).

Wortmannine B4 (6).

white powder; $[\alpha]_D^{25} +20$ (c 0.0011, MeOH); UV (MeOH) $\lambda_{\max}$ (log ϵ) 204 (3.4), 243 (3.2) nm; ^1H NMR data, see Table 1; HRESITOFMS m/z 512.1898 $[\text{M}+\text{Na}]^+$ (calcd for $\text{C}_{25}\text{H}_{31}\text{NO}_9\text{Na}$, 512.1897).

Antifungal Assays.

In vitro antifungal activity was measured according to methods recommended by the National Committee for Clinical Laboratory Standards (NCCLS).²⁷ The strains and methods for agar zone of inhibition assays with *C. albicans* ATCC 10231, *C. neoformans* H99, and *S. aureus* ATCC 43330 have been detailed previously.²⁴ MIC values were also measured according to NCCLS recommendations, using the serial broth microdilution method in 96-well plates. The test medium was RPMI-1640 (Sigma) supplemented with 0.165 M MOPS, pH 7.0. Compounds were dissolved in DMSO and serially diluted in the medium. The spectrophotometric MIC value was defined as the lowest concentration of a test compound that resulted in a culture with a density consistent with 50% growth inhibition (MIC-50) when compared to the growth of the untreated control. The strains were incubated at 37 °C, and the MICs were determined at 48 h for *C. albicans* and *A. fumigatus*, and 72 hours for *C. neoformans* due to its slower growth rate. Due to nutritional auxotrophies, the *S. pombe* strains were incubated in YES medium for 72 hours.²⁸ Viability was determined with the aid of a plate reader using Alamar Blue (BioRad) as the viability indicator. The strains employed were efflux mutant MJ1682 (h90 ade6 leu1 ura4-D18 caf5::bsdR pap1-del pmd1-del mfs1-del bfr1-del) and wild-type control SAK1(h+ ade6-M210 leu1 ura4-D18). Itraconazole was used as a positive control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.COSY and key HMBC correlations for **1** (CDCl₃) and **4** (acetone-*d*₆)

Figure 2.
Key NOESY correlations for **1** (CDCl₃) and **4** (acetone-*d*₆)

Table 1.¹H and ¹³C NMR Spectroscopic Data for Wortmannins C (**1**) and D (**2**)

position	1^a		2^b
	δ_{H}	δ_{C}^c	δ_{H}
1	4.76, dd (2.1, 6.9)	88.4	4.76, dd (2.1, 6.8)
2a	3.00, dd (6.9, 11.1)	72.8	3.00, dd (6.8, 11.1)
2b	3.43, dd (2.1, 11.1)		3.43, dd (2.1, 11.1)
3		157.7	
4		114.3	
5		143.0	
6		144.8	
7		172.7	
8		140.3	
9		149.7	
10		40.8	
11	6.17, ddd (2.8, 7.5, 9.0)	70.2	6.16, ddd (2.7, 7.5, 8.8)
12 α	1.55, m		1.58, dd (8.8, 12.7)
12 β	2.54, m	36.1	2.70, m
13		49.3	
14	2.88, ddd (2.8, 5.9, 12.8)	44.2	2.88, ddd (2.7, 6.0, 12.8)
15 α	3.13, m	23.2	3.18, m
15 β	2.04, tt (9.1, 12.8)		2.05, tt (9.2, 12.8)
16a	2.23, td (9.1, 18.6)	35.8	2.23, td (9.2, 18.8)
16b	2.56, m		2.70, m
17		216.4	
18	0.95, s	14.7	0.95, s
19	1.74, s	26.5	1.71, s
20	8.22, s	150.2	8.22, s
21	3.14, s	59.5	3.13, s
22		175.6	
23	2.43, m	40.8	2.70, m
24a	1.55, m	27.2	1.19, d (7.1)
24b	1.68, m		
25	1.18, d (6.8)	15.8	1.20, d (6.8)
26	0.89, t (7.4)	11.5	--

^aRecorded in CDCl₃, at 600, 150 MHz.^bRecorded in CDCl₃ at 500 MHz.^cAll ¹³C NMR multiplicities are consistent with the assignments.

Table 2.

Selected NMR Spectroscopic Data for Wortmannines B1–B4 (3–6)

position	δ_H^a	$\delta_C^{b,f}$	δ_H^c	$\delta_C^{d,f}$	δ_H^e	δ_H^c	$\delta_C^{d,f}$	5	6
1	4.43, d (2.7)	85.4	4.49, d (2.4)	85.8	4.51, d (2.5)	4.54, t (2.7)	85.4	4.46, d (2.6)	4.42, t (2.6)
2a	3.27, d (10.5)	72.1	3.42, d (10.6)	73.4	3.37, d (10.7)	3.52, dd (2.7, 11.4)	73.2	3.30, d (10.5)	3.44, dd (2.6, 11.4)
2b	3.61, dd (2.7, 10.5)		3.60, dd (2.4, 10.6)		3.62, dd (2.5, 10.7)	3.74, dd (2.7, 11.4)		3.63, dd (2.6, 10.5)	3.76, dd (2.6, 11.4)
3		169.1		168.0			169.2		
4	4.00, d (6.4)	46.3	3.77, d (6.2)	47.3	3.83, d (6.6)	3.64, d (1.2)	50.7	4.03, d (6.4)	3.80, s
5		64.3		65.5			66.5		
6		172.1		173.5			170.7		
7		199.0		200.7			202.3		
8		141.8		142.7			142.9		
9		164.4		164.6			167.2		
10		45.7		46.2			47.3		
11	6.00, ddd (3.4, 7.2, 9.7)	68.7	5.96, ddd (3.5, 7.2, 9.9)	69.7	5.99, ddd (3.5, 7.0, 10.0)	5.99, ddd (3.4, 7.0, 10.0)	69.2	6.02, ddd (3.2, 7.2, 10.0)	6.04, ddd (3.4, 7.0, 10.0)
12a	1.47, m	36.1	1.64, m	36.6	1.59, m	1.64, m	36.4	1.42, m	1.48, dd (10.0, 12.7)
12b	2.75, dd (7.2, 12.7)		2.54, m		2.56, m	2.54, m		2.75, dd (7.2, 12.6)	2.74, dd (7.0, 12.7)
13		50.5		51.0			50.9		
14	2.58, m	42.3	2.94, ddd (3.5, 5.7, 12.9)	42.4	2.88, dd (3.5, 6.0, 12.9)	2.99, ddd (3.4, 5.9, 12.9)	42.2	2.55, m	2.59, m
15a	2.58, m	20.0	2.51, m	20.8	2.49, m	2.51, m	20.9	2.55, m	2.59, m
15b	1.89, tt (8.9, 12.5)		1.83, tt (9.0, 12.9)		1.80, tt (9.0, 12.9)	1.85, tt (9.0, 12.9)		1.89, tt (8.9, 12.2)	1.92, tt (9.1, 13.1)
16a	2.18, dt (8.9, 19.5)	35.5	2.16, m	35.4	2.24, dt (9.0, 18.8)	2.18, dt (9.0, 19.1)	36.0	2.19, dt (8.9, 18.4)	2.24, dt (9.1, 19.4)
16b	2.58, m		2.59, m		2.56, m	2.59, m		2.55, m	2.59, m
17		215.4		215.3			215.0		
18	1.13, s	13.9	1.09, s	14.2	1.10, s	1.06, s	14.1	1.11, s	1.08, s
19	1.51, s	17.0	1.52, s	17.4	1.53, s	1.39, s	17.7	1.48, s	1.44, s
20	5.59, d (6.4)	77.0	5.57, d (6.2)	77.8	5.48, d (6.6)	5.52, s	80.3	5.62, d (6.4)	5.65, s
21	3.08, s	58.2	3.05, s	58.0	3.08, s	3.16, s	58.2	3.10, s	3.18, s
22		174.4		175.6			175.5		

position	δ_{H}^a	$\delta_{\text{C}}^{b,f}$	δ_{H}^c	$\delta_{\text{C}}^{d,f}$	δ_{H}^e	δ_{H}^c	$\delta_{\text{C}}^{d,f}$	δ_{H}^a	δ_{H}^a
23	2.33, m	41.2	2.57, m	41.5	2.56, m	2.57 (m)	41.4	2.55, m	2.59, m
24a	1.51, m	26.9	1.53, m	27.6	1.59, m	1.55 (m)	27.6	1.163, d (7.0)	1.19, d (7.0)
24b	1.64, m		1.67, m		1.66, m	1.67 (m)			
25	1.14, d (6.9)	16.5	1.16, d (6.8)	16.5	1.18, d (6.9)	1.15 (d, 6.9)	16.4	1.158, d (7.0)	1.19, d (7.0)
26	0.90, t (7.5)	11.6	0.92, t (7.4)	11.6	0.95, t (7.5)	0.92 (t, 7.4)	11.6		
NH	6.31, s		8.04, s			8.17 (s)		6.22, s	6.25, s
OH	3.42, s								

Data for **3** are given in three different solvents to foster comparison with literature analogues as well as **4-6**.

^a Measured in CDCl₃ at 500 MHz.

^b Measured in CDCl₃ at 150 MHz.

^c Measured in acetone-*d*₆ at 600 MHz.

^d Measured in acetone-*d*₆ at 125 MHz.

^e Measured in methanol-*d*₄ at 500 MHz.

^f All ¹³C NMR multiplicities are consistent with the assignments.