Broomeanamides: Cyclic Octapeptides from an Isolate of the Fungicolous Ascomycete *Sphaerostilbella broomeana* from India

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**ABSTRACT:** The genus *Sphaerostilbella* comprises fungi that colonize basidiomata of wood-inhabiting fungi, including important forest pathogens. Studies of fermentation cultures of an isolate (TFC201724) collected on the foothills of Himalayas, and closely related to *S. broomeana* isolates from Europe, led to the identification of a new cyclic octapeptide along with two closely related analogues (1–3) and four dioxopiperazines (4–7). The structure of the lead compound, broomeanamide A (1), was assigned mainly by analysis of 2D NMR and HRESIMS data. The structure consisted of one unit each of *N*-MeVal, Ala, *N*-MePhe, Pro, Val, and Ile and two *N*-MeLeu units. The amino acid sequence was determined on the basis of 2D NMR and HRESIMSMS data. NMR and HRMS data revealed that the other two new peptides have the same amino acid composition except that the Ile unit was replaced with Val in one instance (2) and the *N*-MeVal unit was replaced with Val in the other (3). The absolute configuration of 1 was assigned by analysis of the acid hydrolysate by application of Marfey’s method using both C18 and C3 bonded-phase columns. Broomeanamide A (1) showed antifungal activity against *Cryptococcus neoformans* and *Candida albicans*, with MIC values of 8.0 and 64 μg/mL, respectively.

The high rate of morbidity and mortality associated with microbial infections and the development of multidrug resistance by infectious agents continue to intensify the need for new antimicrobial agents. The proven track record of fungi makes them an attractive resource for the discovery of novel compounds with antimicrobial activity. Fungicolous fungi, which colonize other fungi, are well known for their ability to produce antimicrobial secondary metabolites. Among ascomycetes, the family Hypocreaceae (Hypocreales) includes the highest diversity of fungicolous fungi, the majority of which occur on wood-decaying basidiomycetes. The genus *Sphaerostilbella* is exceptional in the family because of the tendency to specialize on wood-inhabiting members of the Russulales, an order well known for its agaric members from the large genera of *Russula* and *Lactarius*. One of these species, *S. broomeana*, grows exclusively on basidiomata of *Heterobasidion* species, some of which are major forest pathogens. It was known only from Europe until a morphologically very similar, but genetically distinct anamorph was recently collected on the foothills of the Himalayas in India. Fermentation of this *Sphaerostilbella* isolate (TFC201724) afforded an antifungal extract, which was found to contain three new cyclic octapeptides (1–3) along with four dioxopiperazines (4–7). Among the latter, 4 and 6 do not appear to have been previously encountered from a natural source, although 4 has been reported as a synthetic product. The most abundant cyclic octapeptide metabolite (1) showed significant antifungal activity. Relatively few cyclic octapeptides have been reported from fungi, but such compounds show a wide range of bioactivities.

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For example, the cyclic octapeptide fungosporin, produced by various *Penicillium* and *Aspergillus* spp., is considered to be a mycotoxin.\(^3\) Epichilin, isolated from the endophytic fungus *Epichloë typhina*, exhibited potent inhibitory activity toward spore germination of *Cladosporium pheii*, a fungal pathogen of the timothy plant, with an IC\(_{50}\) value of 22 nM.\(^5\) Shearamide A was isolated from the stromata of *Eupenicillium shearii* (now *Talaromyces pinophilus*) and displayed insecticidal effects against *Helicoverpa zea* larvae.\(^6\) Two other new cyclic octapeptides, mariannamides A and B, have been reported from *Mariannaea elegans* and showed antimicrobial activity against *Escherichia coli* and *Cryptococcus neoformans*.\(^8\)

Recently, we reported a pair of peptaibol-type metabolites called sphaerotibilins A and B from an isolate of *S. toxica*,\(^7\) a species closely related to the isolate used in this study.\(^3\) To our knowledge, there are no other reports of secondary metabolites from any member of *Sphaerotilus*. Herein, we describe the structure elucidation and characterization of 1−7, as well as the results of antibacterial and antifungal assays for a subset of these compounds.

**RESULTS AND DISCUSSION**

A solid-substrate rice fermentation of *S. broomeana* TFC201724 was extracted with EtOAc, and the resulting extract was partitioned between acetonitrile and hexanes. The acetonitrile-soluble fraction was subjected to silica gel column chromatography and reversed-phase HPLC to afford broomeanamides A (1), B (2), and C (3) and dioxopiperazines 4−7.

Broomeanamide A (1) was obtained as a white powder with a molecular formula of C\(_{49}\)H\(_{80}\)N\(_{8}\)O\(_{8}\) (14 degrees of unsaturation), as determined by HRESIMS. Characteristic signals observed in the \(^{1}H\) NMR spectrum (CDCl\(_{3}\); Table 1) indicated that 1 is a peptide. Analysis of its \(^{1}H\) and \(^{13}C\) NMR and HSQC data revealed the presence of three amid N-H protons, 15 methyl groups (including four N-methyls), six methylenes, 14 methines (eight of which are heteroatom-bonded), one phenyl group, and eight carboxylic carbons (\(\delta_{c}\) 168.3−174.8). Interpretation of \(^{1}H−^{1}H\) TOCSY (Figure 1) and HSQC data of 1 established the presence of individual valine (Val), alanine (Ala), proline (Pro), isoleucine (Ile), N-methylvaline (N-MeVal), and N-methylphenylalanine (N-MePhe) units, along with two N-methylleucine (N-MeLeu\(^1\), N-MeLeu\(^2\)) residues. These data accounted for all the NMR resonances of 1 and 13 of the 14 unsaturations, indicating that 1 is a cyclic octapeptide.

The complete amino acid sequence was determined by extensive analysis of HMBC and ROESY correlations (Figure 1), supported by HRESIMSMS data (Figure S10). Interpretation of the NMR data collected in CDCl\(_{3}\) was complicated somewhat by overlap of three \(\alpha\)-proton signals at \(\delta_{H}\) 4.96, which were identified as those of Ala, Ile, and N-MeLeu\(^2\) on the basis of the TOCSY data, but other resonances were generally well-resolved, and collection of the full 2D NMR data set overcame this issue. The carbonyl resonance of Ala (\(\delta_{C}\) 174.8) and its \(\alpha\)-carbon (\(\delta_{C}\) 45.3) were identified based on HMBC correlations of the \(\beta\)-CH\(_{3}\) of Ala to \(\delta_{C}\) 174.8 and \(\delta_{C}\) 45.3. The N-MePhe unit was acylated by Pro based on an HMBC correlation from the N-CH\(_{3}\), of N-MePhe to the Pro carbonyl, which was assigned by HMBC correlations with the Pro \(\beta\)-proton signals. The latter were somewhat upfield shifted relative to typical Pro signals, possibly due to shielding associated with spatial proximity to the aryl group. This connection was supported by ROESY correlations between the \(\alpha\)-protons of the N-MePhe and Pro units. An HMBC correlation from the Ala amide NH to the carbonyl of the N-MePhe unit and a ROESY correlation between the \(\alpha\)-proton of N-MePhe and the amide NH of Ala indicated that N-MePhe acylated Ala in the sequence. HMBC correlations of the \(\alpha\)-proton and the N-CH\(_{3}\) of N-MeVal to the carbonyl of Ala extended the sequence by indicating acylation of the N-MeVal unit by the Ala unit. A ROESY correlation from the overlapped signal for the Ala unit \(\alpha\)-proton to the \(\alpha\)-proton of the N-MeVal was consistent with this connection, completing the partial sequence Pro → N-MePhe → Ala → N-MeVal. This was further supported by observation of an m/z 443.2647 ion in the HRESIMSMS data corresponding to the expected C\(_{24}\)H\(_{23}\)N\(_{5}\)O\(_{4}\) fragment. The N-MeLeu\(^2\) unit was acylated by N-MeVal based on an HMBC correlation from the N-MeLeu\(^2\)-N-CH\(_{3}\) signal to the carbonyl of N-MeVal. HMBC correlations from the Ile NH to carbonyls at \(\delta_{C}\) 168.3 and 172.8 and of the N-CH\(_{3}\) signal of N-MeLeu\(^1\) to the carbonyl at \(\delta\) 172.8 indicated that Ile acylates the N-MeLeu\(^1\) unit and identified the Ile carbonyl shift as \(\delta\) 168.3. HMBC correlations from the \(\alpha\)-proton of N-MeLeu\(^2\) to the carbonyl of Ile further extended the partial sequence to Pro → N-MePhe → Ala → N-MeVal → N-MeLeu\(^2\) → Ile → N-MeLeu\(^3\). This extended connectivity was supported by HRESIMSMS ions at m/z 683.4479 and 810.5466, consistent with the formulas for the sequence through Ile and through N-MeLeu\(^4\), respectively. Additional ROESY correlations were consistent with this connectivity. This effectively completed the structure, as the only remaining unit to insert was the Val residue, and its location between the N-MeLeu\(^4\) and Pro units was supported by an HMBC correlation from the Val amide NH to the carbonyl carbon of the N-MeLeu\(^1\) unit and a ROESY correlation between the \(\alpha\)-proton of N-MeLeu\(^3\) and the amide NH of Val, as well as a ROESY correlation between the \(\alpha\)-proton of Val and the \(\beta\)-protons of Pro, indicating that Val acylated Pro in the sequence. Thus, the gross structure of broomeanamide A was assigned as shown in structure 1.

Marfey’s method\(^9,10\) was applied to assign the absolute configurations of the amino acid residues resulting from acid hydrolysis of broomeanamide A. The 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide derivatives of the amino acids present in the acid hydrolysate of 1 were analyzed by LC-MS along with those of authentic L- and D-amino acids. Initial efforts employed a C\(_{18}\) column and did not resolve all of the derivatives, a problem that is known to arise, especially for
certain N-methyl amino acids. However, complementary chromatography on a C3 column led to separation of those that were unresolved on C18, ultimately enabling assignment of the L-configuration for all of the amino acid residues (Figure S1).

Broomeamides B (2) and C (3) were obtained in considerably lesser amounts. The molecular formula of broomeanamide B (2) was determined to be C48H78N8O8 (14 degrees of unsaturation) by HRESIMS, indicating the presence of one CH2 unit less than that of 1. Analysis of the 1H NMR spectroscopic data (Table S1) confirmed that 2 was a close analogue of 1 with the same amino acid composition except that the Ile unit was replaced with Val. Specifically, the data showed the appearance of two methyl doublets at δH 0.76 (δC 17.9) and δH 0.88 (δC 14.1) in place of the methyl triplet at δH 0.72 (δC 11.0) and the methyl doublet at δH 0.72 (δC 16.5) for the Ile residue in 1. The α-proton signals were somewhat better resolved in this case, but the sequence was otherwise identical, as supported by nearly identical NMR shifts for the remainder of the molecule. The TOCSY, HSQC, and HMBC results were also consistent with the structure proposed for 2.

The small sample of broomeanamide C (3) obtained was not completely freed of minor homologues, but could be identified based on NMR and MS data, as it was clearly closely related to 1 and 2. HRESIMS data indicated that it is an isomer of 2. The 1H NMR spectrum of 3 (Table S2) was similar to that of 1 except for the appearance of a new amide

Table 1. NMR Spectroscopic Data for Broomeanamide A (1) in CDCl3

| Amino acid | position | 13Cδ | 1H (mult., J in Hz)b | HMBC | ROESY |
|------------|----------|------|----------------------|------|-------|
| Pro        |          |      |                      |      |       |
| o-CH       | 173.5    | 55.7 | 4.05 (t, 7.0)        | 28.9 |       |
| β-CH2      | 2.9      | 1.03 (m)                  | 173.5, 48.2, 25.5 |
| γ-CH2      | 173.5    | 28.9 | 0.55 (m)              | 173.5, 48.2, 25.5 |
| δ-CH3      | 25.5     | 1.97 (m)                  | 55.7, 28.9 |
| e-CH3      | 25.5     | 1.52 (m)                  | 55.7 |
| s-CH3      | 48.2     | 3.53 (t, 6.5)            | 55.7, 25.5, 28.9 |
| N-MePhe    |          |      |                      |      |       |
| CO         | 169.2    | 29.1 | 2.83 (s)              | 173.5 |
| α-CH       | 62.6     | 34.1 | 5.10 (dd, 10.7, 4.0)  | 169.2 |
| β-CH3      | 138.3    | 34.1 | 3.02 (m)              | 138.3, 129.8, 62.6 |
| γ-CH3 (ar.)| 138.3    | 179.8 | 7.15 (d, 7.7)        | 129.8, 127.0, 34.1 |
| δ-CH3 (ar.)| 128.9    | 7.29 (t, 8.0)            | 138.3, 129.8 |
| e-CH3 (ar.)| 127.0    | 7.26 (t, 7.4)            | 129.8 |
| Ala        |          |      |                      |      |       |
| CO         | 174.8    | 175.4 | 8.88 (d, 8.1)        | 169.2 |
| NH         | -        | 45.3 | 4.96 (m)              | 174.8, 17.5 |
| β-CH3      | 17.5     | 1.72 (d, 6.3)            | 174.8, 45.3 |
| N-MeVal    |          |      |                      |      |       |
| CO         | 171.5    | 31.0 | 3.40 (s)              | 174.8, 57.1 |
| α-CH       | 57.1     | 31.0 | 5.28 (d, 10.7)        | 174.8, 171.5, 31.0, 28.5, 20.1 |
| β-CH3      | 28.5     | 179.8 | 2.36 (m)              | 20.1, 57.1 |
| γ-CH3      | 20.1     | 179.8 | 0.99 (d, 6.4)        | 57.1, 28.5, 20.1 |
| δ-CH3      | 20.1     | 179.8 | 0.95 (d, 6.4)        | 57.1, 28.5, 20.1 |
| N-Me.eu2   |          |      |                      |      |       |
| CO         | 168.3    | 29.1 | 2.86 (s)              | 171.5, 59.2 |
| NH         | -        | 59.2 | 4.93 (m)              | 168.3 |
| β-CH3      | 39.1     | 39.1 | 2.40 (m)              | 23.8 |
| β-CH3      | 39.1     | 39.1 | 1.07 (m)              | 22.3 |
| γ-CH3      | 25.4     | 39.1 | 1.45 (m)              | 39.1, 25.4 |
| δ-CH3      | 22.3     | 39.1 | 0.89 (d, 6.4)        | 39.1, 25.4 |
| e-CH3      | 23.3     | 39.1 | 0.94 (d, 6.4)        | 39.1, 25.4 |
| N-Me.eu1   |          |      |                      |      |       |
| CO         | 172.8    | 11.0 | 0.72 (t, 7.3)        | 168.3 |
| NH         | -        | 54.7 | 4.96 (m)              | 172.8, 38.4 |
| α-CH       | 38.4     | 16.5 | 1.77 (m)              | 22.6 |
| β-CH3      | 16.5     | 25.3 | 0.92 (d, 6.3)        | 38.4, 23.8 |
| γ-CH3      | 25.3     | 11.0 | 1.36 (m)              | 1.77, 1.36, 1.27 |
| δ-CH3      | 11.0     | 11.0 | 0.72 (t, 7.3)        | 1.77, 0.92, 0.72 |
| N-Me.eu1   |          |      |                      |      |       |
| CO         | 171.0    | 30.9 | 2.92 (s)              | 172.8, 55.3 |
| NH         | -        | 55.3 | 5.07 (t, 7.3)        | 172.8, 171.0, 35.1, 29.6, 25.9 |
| α-CH       | 55.3     | 35.1 | 1.77 (m)              | 172.8, 171.0, 35.1, 29.6, 25.9 |
| β-CH3      | 55.3     | 35.1 | 1.60 (m)              | 171.0, 55.3, 25.8, 22.3 |
| γ-CH3      | 25.8     | 35.1 | 1.60 (m)              | 22.3 |
| δ-CH3      | 22.3     | 35.1 | 0.86 (d, 6.4)        | 34.9, 24.4, 22.3 |
| e-CH3      | 22.3     | 35.1 | 0.93 (d, 6.4)        | 34.9, 24.4, 22.3 |
| Val        |          |      |                      |      |       |
| CO         | 170.4    | 18.8 | 8.20 (d, 8.7)        | 171.0 |
| NH         | -        | 56.7 | 4.30 (t, 8.7)        | 170.4, 33.7, 18.8 |
| α-CH       | 33.7     | 33.7 | 1.73 (m)              | 170.4, 56.7, 18.8 |
| β-CH3      | 19.1     | 8.85 (d, 6.3)           | 56.8, 33.7, 18.8 |

a150 MHz; CDCl3 signal reference set at δ 77.2. b600 MHz; residual CHCl3 signal reference set at δ 7.26.
N-H doublet at $\delta_{\text{H}}$ 6.65 and the absence of the N-methyl signal corresponding to the N-MeVal unit in 1, revealing that the N-MeVal residue in 1 was replaced by a Val unit in 3.

Several dioxopiperazines were also isolated from these cultures. Dioxopiperazines are frequently encountered from fungi, bacteria, and plants and display a variety of biological activities. Analysis of NMR and MS data and comparison with literature values enabled identification of the gross structures of dioxopiperazines 4, 5, and 6, all of which contain a dehydrophenylalanine unit. Among these, only 5 had been previously reported from a natural source, although compound 4 had been described as a synthetic product. The olefin geometry in such compounds is sometimes not discussed in literature reports, although it has been shown that the chemical shift of the olefinic signal is somewhat diagnostic for such assignments, with the olefinic $^1$H NMR signal for the Z-isomer typically appearing ca. 0.5 ppm downfield relative to that of the E-isomer in cases where both isomers are available for comparison. The double bond in 4 between C-9 and C-10 was confirmed to have the Z-geometry on the basis of a NOESY correlation between NH-8 and the H-2′/6′ resonance of the aromatic ring, as well as the olefinic $^1$H NMR shift ($\delta_{\text{H}}$ 6.99).

The absolute configuration at C-6 in 4 was not initially assigned, as there was no $[\alpha]_D$ literature value for direct comparison. Crystals of 4 were later obtained, enabling analysis by X-ray crystallography with an eye toward unambiguously assigning the configuration. Interestingly, in addition to confirming the gross structure and the olefin geometry, these data revealed that the sample of 4 was obtained as a racemate. Notably, closely related compound 5 was also obtained from this extract and identified as the N-8 methyl analogue of 4, which had been previously reported from *Penicillium pinophilum* and assigned the 6S configuration. Prior to that, 5 had also been reported as a synthetic product. $^1$H NMR and MS data matched well with literature values for 5, and the $[\alpha]_D$ had the same sign as that reported for the 6S isomer, though it was significantly lower in magnitude. Upon analysis of these literature descriptions, however, some confusing issues were noted. First of all, both prior references show structures clearly depicting the 6R configuration even though the text indicates that the assignment was 6S in each case. Presumably, this was a graphical typo that was carried over in the second report, which referenced the first. Moreover, the earlier report described synthetic 5, for which a $[\alpha]_D$ value of +535 was reported using CHCl$_3$ as solvent. The later report (of naturally occurring 5 from *P. pinophilum* as noted above) gave a value of +90 using MeOH as solvent. Measurements of our sample gave values of +15 and +20 in CHCl$_3$ and MeOH, respectively. The rather large discrepancies among these numbers could be explained by varying levels of epimerization at C-6 in the two nonsynthetic samples. Other literature reports describe some tendency for Pro-containing dioxopiperazines to epimerize under certain conditions. Given the finding that 4 crystallized as a racemate, it seems that the sample of 5 obtained was likely scalenic. It may be that the sample of 4 initially obtained was also scalemic, but that there is some preference for crystallization in the racemic form.

Related compound 6 was also obtained, differing from 4 by hydroxylation at C-6. Although 6 does not appear to have been reported previously, close analogues have been described from a plant source (*Claoxylon polot*) that bear a hydroxy group or methoxy group at C-6 of the proline residue and methyl groups at the C-4 and N-8 positions. Compound 6 crystallized from MeOH and was also subjected to X-ray crystallographic analysis, leading to confirmation of the structure and to recognition that it was also obtained as a racemate. This was perhaps less unexpected given that the $\alpha$-position of the Pro unit is modified. ORTEP representations of 4 and 6 are shown in Figure 2. Compound 7 was identified as the well-known cyclo(1-Pro-1-Phe) by comparing the $^1$H NMR, MS, and specific rotation data with literature values.

In standard disk assays against *Candida albicans* (ATCC 10231) and *Staphylococcus aureus* (ATCC 29213), broomeanamides A and B (1 and 2) both showed inhibition at the 50 µg/disk level against *S. aureus*, while broomeanamide A displayed inhibition against *C. albicans* at the 50 µg/disk level. Compounds 4–7 showed no activity in these screens. Broomeanamide A (1) was separately tested for antifungal activity against *Cryptococcus neoformans* (H99), *C. albicans*, and *S. aureus* in MIC assays (Table 2). Broomeanamide A displayed a significant inhibitory effect against *C. neoformans*,...
with an MIC value of 8 μg/mL at 37 °C, while showing lesser inhibitory effects against C. albicans and S. aureus with MIC values of 64 and 128 μg/mL, respectively (Figure S20).

### EXPERIMENTAL SECTION

#### General Experimental Procedures.

Specific rotations were measured on an AUTOPOL III automatic polarimeter. ¹H and ¹³C NMR spectra were recorded using Bruker AVANCE-600 or 1220 Infinity-C₁₈ column; S μm; 9.4 × 250 mm; gradient elution 40–100% MeCN in H₂O over 60 min; 2 mL/min) to afford 6 (1.5 mg, t₅₀ 9.1 min), and fraction 5 (43 mg), eluted with 100% EtOAc in hexanes, was further separated by semipreparative RP-HPLC (Agilent 1260 Infinity-C₁₈ column; S μm; 9.4 × 250 mm; gradient elution 40–100% MeCN in H₂O over 60 min; 2 mL/min) to afford 3 (0.5 mg, t₅₀ 39.0 min). Known compounds 5 and 7 were identified by comparison of their NMR and specific rotation data with literature values.¹⁵,¹⁷

**Broomeanamide A (I):** white solid; [α] D₂₀ = −6 (c 0.05, MeOH); ¹H and ¹³C NMR and HMBC data, see Table 1; positive ion HRESIMS m/z 909.6197 [M + H]+ (calcd for C₄₉H₇₈N₈O₈ + H, 909.6177).

**Broomeanamide B (II):** white solid; [α] D₂₀ = −18 (c 0.03, MeOH); ¹H and ¹³C NMR and HMBC data, see Table S1; positive ion HRESIMS m/z 895.6032 [M + H]+ (calcd for C₄₉H₇₄N₈O₈ + H, 895.6021).

**Broomeanamide C (III):** white solid; [α] D₂₀ = −42 (c 0.03, MeOH); ¹H and ¹³C NMR and HMBC data, see Table S2; negative ion HRESIMS m/z 893.5887 [M − H]− (calcd for C₄₉H₇₄N₈O₈ − H, 893.5864).

**Compound 4:** white solid; [α] D₂₀ = 7.74 (1H, br s, NH-8), 7.43 (2H, br t, J = 7.3 Hz, H-3′, 5′), 7.57 (2H, br d, J = 7.6 Hz, H-2′, 6′), 7.34 (1H, br t, J = 7.3 Hz, H-4′), 6.99 (1H, s, H-10), 4.31 (1H, dd, J = 10.2, 6.5 Hz, H-6), 3.82 (1H, m, H-3a), 3.65 (1H, m, H-3b), 2.47 (1H, m, H-5a), 2.10 (1H, m, H-5b), 2.03 (1H, m, H-4a), 1.96 (1H, m, H-4b), ¹¹C NMR (CDCl₃, 150 MHz) 166.0 (C, C-7), 158.0 (C, C-1), 133.3 (C, C-9), 129.5 (CH, C-2′, 6′), 128.8 (C, C-1′), 128.6 (CH, C-3′, 5′), 127.5 (CH, C-4′), 116.0 (CH, C-10), 59.3 (CH, C-6), 45.8 (CH₂, C-3), 29.1 (CH₂, C-5), 22.0 (CH₂, C-4), negative ion HRESIMS m/z 241.0977 [M − H]− (calcd for C₁₇H₂₄N₂O₂ − H, 241.0978).

**Compound 6:** white solid; [α] D₂₀ = 7.74 (1H, br s, NH-8), 7.43 (2H, br t, J = 7.6 Hz, H-3′, 5′), 7.39 (2H, br d, J = 7.3 Hz, H-2′, 6′), 7.35 (1H, br t, J = 7.3 Hz, H-4′), 7.06 (1H, s, H-10), 3.89 (1H, m, H-3a), 3.77 (1H, m, H-3b), 3.19 (1H, s, OH-6), 2.38 (1H, m, H-5a), 2.29–2.21 (2H, m, H-5b, H-4a), 2.08 (1H, m, H-4b), ¹¹C NMR (CDCl₃, 150 MHz) 165.1 (C, C-7), 158.3 (C, C-1), 133.0 (C, C-9), 129.6 (CH, C-2′, 6′), 128.7 (CH, C-3′, 5′), 126.6 (CH, C-4′), 117.7 (CH, C-10), 97.2 (C-2), 45.8 (CH₂, C-3), 36.8 (CH₂, C-5), 19.5 (CH₂, C-4), negative ion HRESIMS m/z 257.0921 [M − H]− (calcd for C₂₁H₁₈N₂O₄ − H, 257.0926).

**Marfrey’s Analysis of Broomeanamide A.** Determination of the absolute configuration of the amino acid units in I was accomplished using Marfrey’s method in conjunction with both C₃ and C₁₈ chromatographic separation.⁹−¹¹ For the derivatization reaction, 0.2 mg of I was transferred to a 2 mL glass tube, to which 500 μL of 6 M HCl was added and kept at 110 °C for 16 h. After hydrolysis, the liquid was evaporated under a stream of air, and 50 μL of H₂O, 20 μL of 1 M NaH₂O₃, and 100 μL of 1% Marfrey’s reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, L-FDAA) in acetone were added. The tube was sealed and kept at 40 °C with occasional agitation. The reaction was quenched with addition of 40 μL of 2 M HCl and dried under a stream of air. The reaction product was dissolved in 200 μL of MeOH, filtered with a 0.22 μm hydrophilic PTFE filter, and submitted to C₃ and C₁₈ analysis using an Agilent 1260 HPLC coupled to an Agilent 6120 single quadrupole MS, collecting positive and negative ESIMS at m/z 160–1500. Mobile phases consisted of 0.1% formic acid in MeCN (A), 0.1% aqueous formic acid (B), and

| organism                  | compound (MIC, μg/mL) | 1 | control* |
|---------------------------|-----------------------|---|----------|
| Staphylococcus aureus ATCC 43300 | 128 | 0.16 |          |
| Candida albicans ATCC 10231   | 64  | 1.70 |          |
| Cryptococcus neoformans H99 (23 °C) | 128 | 0.85 |          |
| Cryptococcus neoformans H99 (37 °C) | 8  | 0.85 |          |

Table 2. Minimum Inhibitory Concentration Assay Results for 1

“Chlortetracycline + streptomycin was the control for S. aureus. Ampicillin B was the control for fungal strains.
0.1% formic acid in MeOH (C). C1 chromatography employed a Zorbax SB-C3 column (150 × 4.6 cm, 5 μm), with a 55 min gradient elution from 5:80:15 to 5:35:60 A:B:C, with a column temperature of 50 °C. For separation of t-FDAA-N-MePhe isomers, an Ace Equivance C4u column (150 × 4.6 mm, 5 μm) maintained at 40 °C was used, employing a gradient of 20:80 to 50:40 A:B over 45 min. Authentic standards of both d- and t-isomers of alanine (Ala), valine (Val), proline (Pro), isoleucine (Ile), α-isoalloleucine (allo-Ile), N-methyl-valine (N-MeVal), N-methyl-leucine (N-MeLeu), and N-methyl-phenylalanine (N-MePhe) were subjected to Marfey’s reaction conditions and analyzed using the same LC-M5 protocol. The t-FDAA-N-MeVal and t-FDAA-L-Ile isomers coeluted and showed the same major m/z fragment. However, all t-FDAA-L-leucine derivatives produce another fragment at m/z 338, which was used to confirm the absence of t-allo-Ile. As such, the peak at 33.6 min with m/z 384 could be characterized as t-FDAA-L-N-MeVal. Thus, all seven amino acids (Ala, Val, Pro, Ile, N-MeLeu, N-MeVal, N-MeLeu, and N-MePhe), each having the L-configuration, were detected in the hydrolysate of S.

X-ray Crystallographic Analysis of 4. Upon crystallization from CH3OH using the vapor diffusion method, colorless crystals were obtained. A crystal (0.020 mm × 0.030 mm × 0.365 mm) was separated from the sample and mounted on a glass fiber, and data were collected using Incoatec microsource 3.0 Cu Kα radiation. Data were collected using Incoatec microsource 3.0 Cu Kα radiation. A = 1.541 Å × 1.541 Å × 1.541 Å at 150(2) K. Crystal data: C14H14N3O7, M = 242.27 g/mol, space group tetragonal, P42hc; unit cell dimensions a = 14.8338(3) Å, b = 14.8338(3) Å, c = 11.0209(4) Å, V = 2425.06(13) Å3, Z = 8, Dc (calcld) = 1.327 g/cm3, μ = 0.733 mm−1, F(000) = 1024. A total of 3036 frames were collected. The total exposure time was 42.6 h. The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using a tetragonal unit cell yielded a total of 30 899 reflections to a maximum θ angle of 66.62° (0.84 Å resolution), of which 20 987 were independent (average redundancy 21.12 h. The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using a tetragonal unit cell yielded a total of 30 899 reflections to a maximum θ angle of 66.62° (0.84 Å resolution), of which 20 987 were independent (average redundancy 8.83% [I > 2σ(I)] for all data. Crystallographic data for 4 have been deposited with the Cambridge Crystallographic Data Centre (CCDC deposition number 2060794).

Bioassays. Petri plate antifungal assays against Candida albicans (ATCC 10231) and Cryptococcus neoformans (H99) and antibacterial assays against Staphylococcus aureus (ATCC 29213) were conducted using procedures reported previously. Briefly, 100 mL of yeast malt agar and tryptic soy agar (Difco) were prepared, sterilized by autoclaving, and cooled to 45 °C. One to three milliliters of the inoculum suspension was transferred to the warm fluid and mixed thoroughly by gentle swirling to avoid bubbles. Then the agar was poured into Petri plates (100 × 15 mm; 5 mL each). In conducting the disk diffusion assay, each filter paper disk (6.25 mm in diameter) was impregnated with the sample to be tested (50 μg/disk). After evaporation of the solvent, the disk was placed on the agar surface and incubated at room temperature for 24–72 h. The antifungal agent amphotericin B and the antibiotic gentamicin (Sigma Chemical Co.) at 25 μg/disk were used as positive controls.

Minimum Inhibitory Concentration (MIC). To quantify the inhibitory concentrations of compound 1 against bacterial and fungal pathogens, MICs were measured using species-specific modifications to standard CLSI testing methods. Briefly, overnight cultures of Staphylococcus aureus ATCC 43500, C. albicans ATCC 10231, and C. neoformans H99 were sequentially diluted to OD 0.8 in PBS and 100 μL of each dilution was added in triplicate in RPMI-1640 buffered with MOPS. The final cell suspension was incubated with serial dilutions of 1 ranging from 0.25 to 128 μM. Growth was assessed by adding 10% alamarBlue (Bio-Rad) followed by incubation for 24 h (S. aureus ATCC 43300 at 37 °C) or 48 h (C. albicans ATCC 10231 at 23 °C and C. neoformans H99 at both 23 and 37 °C). Chlortetracycline + streptomycin was the control for S. aureus, while amphotericin B was the control for fungal strains.

ASSOCIATED CONTENT
Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.1c00414.

Additional figures (PDF)

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Notes
The authors declare no competing financial interest.

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