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Stephacidin A and B: Two Structurally Novel, Selective Inhibitors of the Testosterone-Dependent Prostate LNCaP Cells

Jingfang Qian-Cutrone,*,† Stella Huang,† Yue-Zhong Shu,† Dolatrai Vyas,† Craig Fairchild,‡ Ana Menendez,‡ Kimberly Krampitz,† Richard Dalterio,† Steven E Klohr,† and Qi Gao†

Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492, and Princeton, New Jersey 08543

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Fungi have proven to be a very rewarding source of bioactive and structurally diverse natural products.[1] In the course of our ongoing screening program aimed at the discovery of novel and selective antitumor agents potentially acting at molecular targets, we have discovered two new antitumor alkaloids, stephacidin A (**1**) and B (**2**).[2] Compounds **1** and **2**, produced by *Aspergillus ochraceus*WC76466, are structurally related to the cytotoxic marine natural product, avrainvillamide (**3**), isolated first from a marine fungal strain of *Aspergillus* sp. by Fenical and co-workers,[3] and later from the fermentation broth of *Aspergillus ochraceus* by Sugie and co-workers under the name of CJ-17,665.[4] Both compounds **1** and **2** demonstrated in vitro cytotoxic activity against various human tumor cell lines, but **2** exhibited more potent and selective antitumor activities, especially against testosterone-dependent prostate LNCaP cell line with an IC_{50} value of 0.06 μM. The structures of **1** and **2** (Figure 1) were established on the basis of NMR data and X-ray crystallography. In this paper, we report the isolation, characterization and preliminary in vitro antitumor screening of these two novel fungal alkaloids.

Aspergillus ochraceus WC76466, is a mitosporic fungus that was isolated from light brown clay collected from Sirsaganj, Uttar Pradesh, India. A biologically pure culture of *Aspergillus ochraceus* strain WC76466 has been deposited with the American Type Culture Collection under the accession number ATCC-74432. The fungus was grown on 24.5 cm × 24.5 cm Nunc fermentation plates. The Nunc plates were extracted with methanol, and the aqueous methanol extracts were combined and concentrated. The remaining aqueous solution was then partitioned against ethyl acetate. The ethyl acetate fraction was subjected to Sephadex LH-20 gel filtration chromatography. Final purification of **1** and **2** was achieved by preparative reverse-phase HPLC on a C18 column.

Compound **1** was obtained as a white amorphous solid. The UV spectrum with λ_{max} in methanol at 242, 309, and 335 (sh) nm was indicative of aromatic functionality with an extended conjugation. High-resolution FAB-MS analysis of **1** suggested a molecular formula of $C_{26}H_{29}O_3N_3$, indicating 14 degrees of unsaturation and the presence of an odd number of nitrogen atoms. This molecular formula was also in agreement with the carbon and proton counts from the ^{13}C- and ^1H NMR spectra ($CDCl_3$/methanol-d_4). The IR spectrum showed absorption bands at 1640−1690 and 3440 cm^{-1}, which are characteristic of amides/lactams. The presence of amides/lactams was also supported by the carbonyl signals at δ 174.0 and 169.3 in the ^{13}C NMR spectrum. The 10 ^{13}C NMR signals between δ 148.1 and 104.0 suggested the presence of five double bonds.

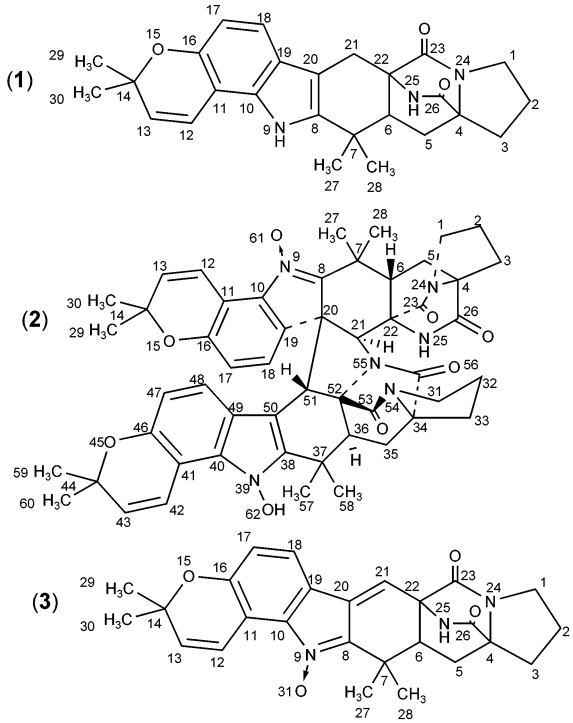

Figure 1. Chemical structures of compounds **1**, **2**, and **3**.

Furthermore, signals due to the following groups were observed in the ^{13}C- and ^1H NMR spectra: four methyls, five methylenes, one methine, and four-quarternary carbons. The above-mentioned groups and structural fragments represent only seven degrees of unsaturation. Thus, there must be seven rings in the structure. Detailed 1D- and 2D-NMR spectra studies (DEPT, COSY, HETCOR, HMBC, HMQC, and NOE) led to the establishment of the connectivity of functional groups and, in turn, the molecular structure (**1**) (Figure 1).

Compound **2** was isolated as an off-white amorphous solid. UV absorptions with λ_{max} in methanol at 240, 268 (sh), 301, and 346 (sh) nm were indicative of aromatic functionality with extended conjugation. High-resolution FAB-MS analysis revealed that the compound has a molecular formula of $C_{52}H_{54}O_8N_6$, which indicated 29 degrees of unsaturation. The IR spectrum showed absorption bands at 1640−1690 and 3440 cm^{-1}, which are characteristic of amides/lactams. It was challenging to find a suitable NMR solvent for **2**. The ^{13}C- and ^1H NMR spectra showed severe signal broadening and overlapping in single solvent of methanol-d_4 or $CDCl_3$. In addition, compound **2** appeared to be unstable in several common NMR solvents such as acetone-d_6 and DMSO-d_6, resulting in a

* To whom correspondence should be addressed. E-mail: jingfang.cutrone@bms.com.
† Wallingford, Connecticut.
‡ Princeton, New Jersey.

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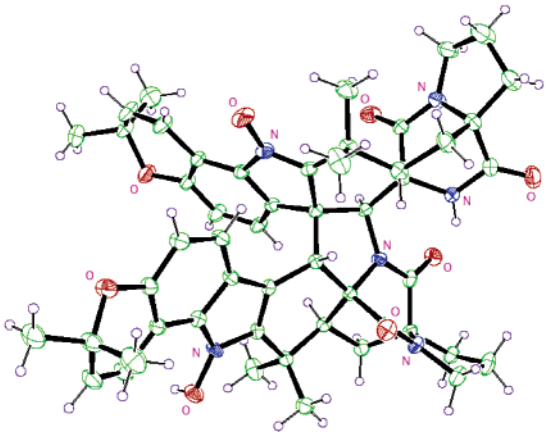

Figure 2. ORTEP drawing of **2** with non-H atoms drawn at 30% probability level and H-atoms shown as small spheres of arbitrary radii.

decomposed mixture after spectral acquisition. A solvent mixture of DMSO-d_6 and acetonitrile-d_3 (1:1) that would give well-resolved ^{13}C- and ^{1}H NMR spectra was finally found. Initial analyses of ^{13}C NMR and ^{1}H NMR spectra suggested that compound **2** should be structurally related to **1**. On the basis of its molecule weight as well as the numbers of the carbon and proton signals, **2** approximately represented a dimer of **1**. The connection of the monomers, however, was not easily recognized. The ^{13}C NMR signals at δ 175.1, 173.8, 167.5, and 167.0 implied the presence of four carbonyl functional groups. The 19 signals between δ 153.0 and 98.0 indicated the structure should have 9−10 double bonds. Extensive 2D-NMR studies including COSY, HMBC, NOE, and NOESY led to the establishment of all structural fragments; however, the nature of the dimer linkage point remained unestablished.

The final structure and the relative stereochemistry of **2** were unambiguously determined by single-crystal X-ray analysis (Figure 2).[5] Colorless platelike crystals grown from acetonitrile were found to be a 1:1 acetonitrile solvate of the compound. A dimeric structure of **1** is revealed. Two molecules of **1** are connected simply by forming two bonds between them (C20−C51 and C21−N55), in such a fashion that a butterfly-like molecule results. A fused five-membered ring, which has an envelope conformation, is introduced. The length of the N9→O61 bond is 1.301 Å compared to 1.375 Å of the N39−OH62 bond. The two N−C bonds (N39−C38, N39−C40) in one pyrrole ring have a similar length, 1.390 versus 1.374 Å. However, in the other pyrrole ring, the length of N9−C8 (1.312 Å) is significantly shorter than that of N9−C10 (1.443 Å), which is in agreement with its double bond nature.

Compounds **1** and **2** demonstrated in vitro cytotoxicity against a panel of tumor cell lines (Table 1), while **2** is more potent than **1**. Good selectivity was also observed in the testosterone-dependent LNCaP cells, especially with **2**. It is noteworthy that the effects of these compounds are not mediated by p53, mdr, or bcl2, and they are also not tubulin- or topoisomerase II-mediated, indicating a novel mechanism of action.

Table 1: In Vitro Cytotoxicity of **1** and **2** (IC_{50} in μM)

cell line	histotype	characteristic	**1** (IC_{50})	**2** (IC_{50})
PC3	prostate	testosterone-independent	2.10	0.37
LNCaP	prostate	testosterone-sensitive	1.00	0.06
A2780	ovarian	parental	4.00	0.33
A2780/DDP	ovarian	mutp53/bcl2+	6.80	0.43
A2780/Tax	ovarian	taxol-resistant	3.60	0.26
HCT116	colon	parental	2.10	0.46
HCT116/mdr+	colon	overexpress mdr+	6.70	0.46
HCT116/topo	colon	resistant to etoposide	13.10	0.42
MCF-7	breast	estradiol-sensitive	4.20	0.27
SKBR3	breast	estradiol-independent	2.15	0.32
LX-1	lung	sensitive	4.22	0.38

Compounds **1** and **2** are isolated from the same fungal species that can produce **3**. All three compounds are structurally related. Compound **1** could be oxidized to **3**, an imine oxide with C8−N9 and C20−C21 double bonds. Compound **2** is a dimer that could be formed from **1** via **3**. The dimerization could be initiated by the protonation of the imine oxide on O62, which would generate a carbocation at C51 that could attack the double bond at C20−C21 of other monomeric unit. The resulting cation at C21 could in turn attack the amide N55 to finish the dimerization. The double cross-links of two monomers produce a five-membered ring and three additional chiral centers in the structure. Thus, **2** possesses a total of 15 rings and 9 chiral centers in its structure, which represents one of the most structurally complex and novel alkaloids occurring in nature.

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Supporting Information Available: Atomic coordinates of the crystal structure have been deposited with the Cambridge Crystal Structure Database (CCDC 191152). Tables of physical-chemical data including UV, IR, MS, ^{1}H NMR, and ^{13}C NMR of stephacidin A and B (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Dreyfuss, M. M.; Chapela, I. H. *Biotechnol. Ser.* **1994**, *26*, 49−80.
(2) Qian-Cutrone, J.; Krampitz, K. D.; Shu, Y. Z.; Chang, L. P. Stephacidin Antitumor Antibiotics. U.S. Patent 6,291,461, 2001.
(3) Fenical, W.; Jensen, P.; Cheng, X. C. Avrainvillamide, a Cytotoxic Marine Natural Product, and the Derivatives thereof. U.S. Patent 6,066,635, 2000.
(4) Sugie, Y.; Hirai, H.; Inagaki, T.; Ishiguro, M.; Kim, Y.-J.; Kojima, Y.; Sakakibara, T.; Sakemi, S.; Sugiura, A.; Suzuki, Y.; Brennan, L.; Duignan, J.; Huang, L.; Sutcliffe, J.; Kojima, N. *J. Antibiot.* **2001**, *54*, 911−916.
(5) Single-crystal X-ray diffraction data were collected at room temperature using a Nonius CAD-4 diffractometer equipped with Cu Kα radiation. Colorless platelike crystal (0.10 mm × 0.20 mm × 0.70 mm) grown from acetonitrile, $C_{52}H_{54}N_6O_8 \cdot CH_3CN$, space group $P2_1$, a = 12.689(1) Å, b = 11.091(1) Å, c = 16.796(5) Å, α = 90°, β = 97.61(2)°, γ = 90°, Z = 2, d_x = 1.263 g cm^{-3}, V = 2343.0(8) Å^3. 2571 reflections measured, 2443 observed ($I \geq 2\sigma$); 622 parameters refined; $R(F)$ = 0.037; $wR(F^2)$ = 0.092; S = 1.103.

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