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X-RAY STRUCTURES OF TWO STEPHACIDINS, HEPTACYCLIC ALKALOIDS FROM THE MARINE-DERIVED FUNGUS *ASPERGILLUS OSTIANUS*

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Abstract – Two stephacidins, 21-hydroxystepahcidin and notoamide F, were isolated from the cultivation medium of the marine-derived fungus *Aspergillus ostianus*, and their structures were confirmed by X-ray crystallography.

Stephacidins are a unique class of alkaloids possessing a complex heptacyclic skeleton isolated from terrestrial and marine fungi.[1] Stephacidins A [**(+)-1**] and B [**(-)-5**] were isolated by Qian-Cutrone and co-workers from a mitosporic marine-derived fungus *Aspergillus ochraceus* WC76466.[2] Another stephacidin analogue, avrainvillamide [(+)-**4**], was obtained by Fenical and co-workers from a marine fungus *Aspergillus* sp. as a cytotoxic natural product.[3] The same alkaloid was isolated by Sugie and co-workers from a terrestrial fungus, *Aspergillus ochraceus*, under the name of CJ-17665.[4] Of these stephacidins, stephacidin B (**5**), whose structure was determined by X-ray analysis,[2] is attracting perhaps the most attention among chemists, since it "is one of the most complex indole alkaloids isolated from fungi" according to von Nussbaum,[1] and the Meyers',[5] Baran's,[6] and Williams'[7] groups have recently succeeded in total syntheses of this remarkable alkaloid together with avrainvillamide (CJ-17665) (**4**) and stephacidin A (**1**). It was found that avrainvillamide reversibly dimerized to stephacidin B.[5,6] The absolute configurations of these stephacidins, **(+)-1**, **(+)-4**, and **(-)-5**, have been firmly established by Baran and co-workers.[6]

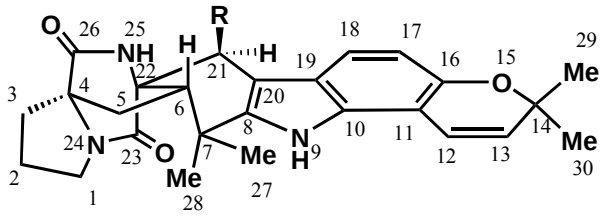
Here we report the isolation and structural establishment of two stephacidins, **(+)-2** (designated 21-hydroxystephacidin A) and **(+)-3** (notoamide F). This paper is the first to report the X-ray structures of 'monomeric' stephacidins.

Compound **(+)-2**, $[\alpha]^{23}_{D}$ +80.2 (*c* 0.15, MeOH), showed the pseudo-molecular formula ($[M+Na]^+$ *m*/*z* 470.2054; calc for $C_{26}H_{29}N_3O_4Na$: 470.2056) (HRTOFMS), indicating 14 degrees of unsaturation. Indeed, the ^{1}H-NMR spectrum (400 MHz, CD_3OD) was rather simple for a compound composed of 29 carbons; it showed two pairs of doublets due to *Z*-olefin and ortho-aromatic protons [δ 7.44 (1H, *J* = 8.4 Hz)/6.60 (1H, *J* = 8.4 Hz) and 6.91 (1H, *J* = 10.0 Hz)/5.71 (1H, *J* = 10.0 Hz)], together with two methine [δ 5.34 (1H, s), 2.84 (1H, dd, *J* = 8.6, 6.3 Hz)], eight methylene [δ 3.51 (m)/3.40 (m), 2.76 (m)/2.00 (m), 2.19 (2H, deformed t), 2.12 (m)/2.02 (m)], and four methyl signals [δ 1.45 (9H, s), 1.15 (3H, s)]. The ^{13}C NMR spectrum suggested the presence of two amide (or ester) carbons (δ 175.3, 170.6), ten sp^2 carbons (δ 149.7, 142.4, 134.9, 130.2, 122.9, 119.4, 119.0, 110.8, 109.4, 106.6), two sp^3-methine carbons (δ 61.2, 48.4), four sp^3-methylene carbon (δ 45.1, 31.4, 30.0, 25.4), and four methyl (δ 28.5, 27.7, 27.5, 22.5) carbons. Although we were able to construct several structural fragments by analyzing the 2D NMR spectra (COSY, HSQC, HMBC), it was impossible for us to establish the definitive structure for this complex compound. Fortunately, compound **(+)-2** was crystallized in MeOH, and its structure was finally determined by X-ray analysis (**X-ray-2** in Figure 1: relative configuration).

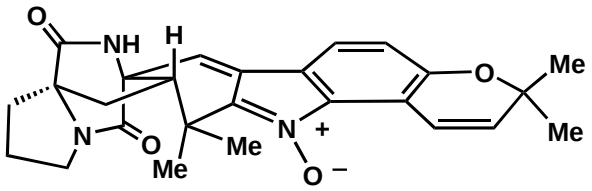
It turned out that compound **(+)-2** is an analogue of stephacidin A possessing a hydroxy group at C-21. The CD spectra of both enantiomers (natural and synthetic) of stephacidin A are reported.[6] The CD spectrum (Figure 2) of **(+)-2** was superimposable on that of natural stephacidin A [**(+)-1**], establishing the absolute configuration of **(+)-2** as shown in Figure 1.

The molecular formula of compound **(+)-3**,[8] $C_{27}H_{31}N_3O_5$, $[\alpha]^{23}_{D}$ +52.8 (*c* 0.24, MeOH), suggested that this compound was a methylated derivative of **(+)-2**. The ^{1}H- and ^{13}C-NMR spectra of **(+)-3** were quite similar to those of **(+)-2** except for the presence of the methoxy signals [δ_H 3.51 (3H, s), δ_C 57.6 (q)] in the former. The position of the methoxy moiety at C-21 was deduced by the HMBC correlations from the OMe protons to C-21. Keeping a chloroform solution of **(+)-3** in a refrigerator afforded a crystal suitable for X-ray analysis. The result is shown as **3-X-ray** in Figure 1. The absolute configuration of **(+)-3** was determined by the CD spectrum in the same manner as described above. The same compound was very recently reported by Tsukamoto and co-workers under the name of notoamide F.[10]

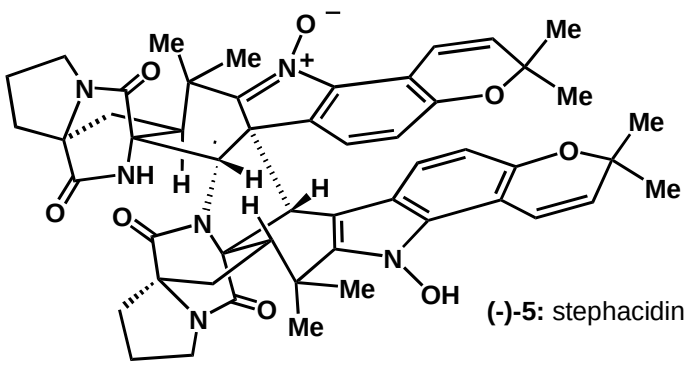
Biogenetically, 21-hydroxystephacidin A [**(+)-2**] is supposed to be produced by the enzymatic oxidation of C-21 (a chemically active benzylic site) followed by O-methylation giving **(+)-3**. Another possibility



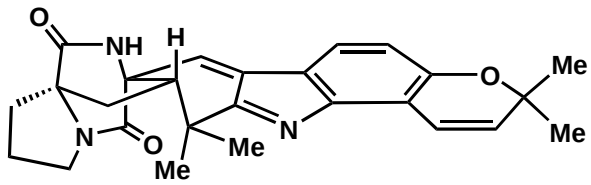
(+)-1: stephacidin A
(+)-2: R = OH: 21-hydroxystephacidin A
(+)-3: R = OMe: notoamide F



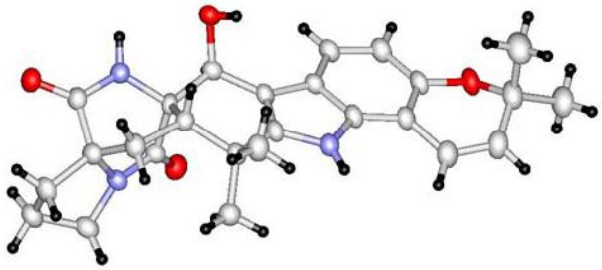
(+)-4: avrainvillamide



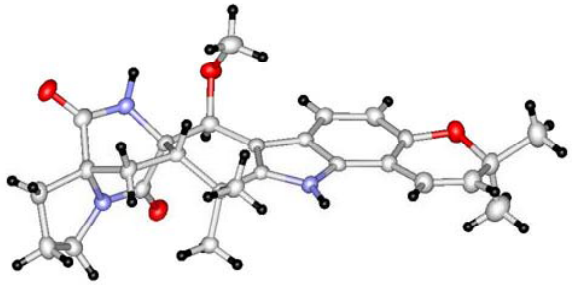
(-)-5: stephacidin B



6: aspergamide



2-X-ray



3-X-ray

Figure 1. Structures of a new stephacidin, 21-hydroxystephacidin A [**(+)-2**], and notoamide F [**(+)-3**] and other stephacidins [**(+)-1**, **(+)-4**, **(-)-5**, and **6**] and X-ray structures (relative stereochemistry), **2-X-ray** and **3-X-ray**, determined for **(+)-2** and **(+)-3**.

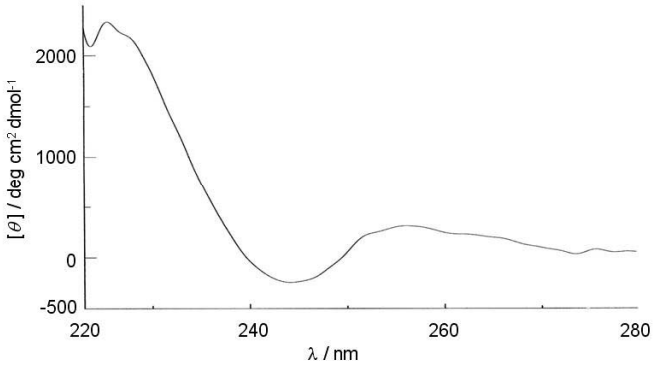


Figure 2. CD Spectrum of 21-hydroxystephacidin A [**(+)-2**] measured in methanol.

is that both compounds are artificially formed by a Michael-type addition of water and methanol to aspergamide (**6**)[9] during the separation experiments. However, this possibility seems rather unlikely because we were unable to detect (^{1}H-NMR) aspergamide in any of the HPLC fractions during the purification processes. Myers observed that deutero-methanol attacked the β-position (C-21) of the vinylnitrone of [**(-)-4**] to give rise to a diastereomeric mixture of the adducts in a 15:1 ratio (stereochemistry not assigned).[5] In the present experiments no trace of the diastereomers of **(+)-2** and **(+)-3** was obtained, implying that **(+)-2** and **(+)-3** are genuine natural products, and, if so, **(+)-2** could be a biogenetic precursor of aspergamide (**6**).

EXPERIMENTAL

General Experimental Procedures: Optical rotation values were determined on a JASCO P-1010 polarimeter. High-resolution mass spectra were obtained with a Waters LCT-Premier 2695 mass spectrometer. 1D and 2D NMR were recorded on Bruker ARX-400, JEOL-JNM-AL400, and JEOL-GSX-400 spectrometers. IR spectra were recorded on a JASCO FT/IR-420 spectrometer. UV spectra were determined on a Beckman DU-650 spectrometer. Chemical shifts (δ) are expressed in parts per million (ppm) with reference to the solvent signals [^{1}H NMR: CD_3OD (δ 3.30). ^{13}C NMR: CD_3OD (δ 49.0)]. Multiplicities of ^{13}C signals were determined by DEPT and HSQC spectra. Flash column chromatography (FCC) was carried out on silica gel (40-63 μm, Merck Co.). Thin layer chromatography was carried out on silica gel 60 F_{254} plates (Merck Co.). Cultivation of the titled fungus was performed in a Sanyo Grows Cabinet NLR-350H. HPLC was done using a Tosoh CCPS. Recycle-HPLC was done using a Japan Analytical Industry Co., Ltd LC-908.

Fungal Isolation and Identification: The fungus, *Aspergillus ostianus* designated as strain 01F313, was isolated from an unidentified marine sponge collected at Pohnpei in 2001. The fungus is maintained on 1/10 YSA medium at the Marine Natural Products Laboratory, Faculty of Pharmaceutical Sciences, The University of Tokushima, Japan.

Cultivation of Fungus: Using bromine-modified artificial seawater: The fungus was cultured for five weeks at 20 °C two times. First cultivation was done in sixty seven 500 mL Erlenmeyer flasks each containing 150 mL of a 1/2 PD medium [potato (100 g) and D-glucose (10 g) were boiled with 1 L of an aqueous (distilled water) solution of NaBr (47.50 g), KBr (1.14 g), $CaBr_2$ (2.48 g), and $MgSO_4$ (5.94 g) (bromine-modified artificial seawater) for 10 min.] Acidity of the medium was adjusted to pH 8.3 with 1 M HBr and 1 M NaOH.]. Second cultivation was done in one hundred and sixty seven 500 mL Erlenmeyer flasks under the same conditions.

Extraction and Isolation of Metabolites: After the cultivation, the broth was filtered, and the filtrate was passed through a column packed with HP-20SS (335 g). After washing with 2 L of distilled water, MeOH (2.5 L) was passed through the column. The MeOH solution was concentrated. The brown extract (7.1 g) was separated by NP-FCC (350 g of silica gel) eluted with $CHCl_3$-acetone gradient (1:0 to 0:1) to give 13 fractions. Fraction 9 (270.5 mg) was separated by ODS-FCC (ϕ 2.5 cm x 15 cm) eluted with H_2O-MeOH gradient (9:1 to 0:1) to give 8 fractions. Fraction 9-4 (18.0 mg) was separated by NP-HPLC using a silica gel column [Mightysil Si60 250-20 (5 μm); flow rate 6.0 mL/min; *n*-hexane/2-propanol (80:20)] into 7 fractions. Fraction 9-4-7 (3.4 mg) was 21-hydroxystephacidin A. Fraction 9-6 (34.0 mg) was separated by NP-HPLC using a silica gel column [Mightysil Si60 250-20 (5 μm); flow rate 6.0 mL/min; *n*-hexane/2-propanol (80:20)] into 3 fractions. Fraction 9-6-3 (3.3 mg) was notoamide F.

21-hydroxystephacidin A (2): colorless needles. ^{1}H NMR (400 MHz, CD_3OD) δ 7.44 (d, 1H, J = 8.4 Hz, H-18), 6.91 (d, 1H, J = 10.0 Hz, H-12), 6.60 (d, 1H, J = 8.4 Hz, H-17), 5.71 (d, 1H, J = 10.0 Hz, H-13), 5.34 (s, 1H, H-21), 3.51 (m, 1H, H-1), 3.40 (m, 1H, H-1), 2.84 (dd, 1H, J = 8.6, 6.3 Hz, H-6), 2.76 (m, 1H, H-3), 2.191 (d, 1H, J = 8.6 Hz, H-5), 2.188 (d, 1H, J = 6.3 Hz, H-5), 2.12 (m, 1H, H-2), 2.02 (m, 1H, H-2), 2.00 (m, 1H, H-3), 1.45 (s, 3H, H-27), 1.45 (s, 3H, H-29), 1.45 (s, 3H, H-30), 1.15 (s, 3H, H-28); ^{13}C NMR (75 MHz, CD_3OD) δ 175.3 (C-26), 170.6 (C-23), 149.7 (C-16), 142.4 (C-8), 134.9 (C-10), 130.2 (C-13), 122.9 (C-19), 119.4 (C-18), 119.0 (C-12), 110.8 (C-17), 109.4 (C-20), 106.6 (C-11), 76.5 (C-14), 68.7 (C-4), 64.3 (C-22), 61.2 (C-21), 48.4 (C-6), 45.1 (C-1), 36.2 (C-7), 31.4 (C-5), 30.0 (C-3), 28.5 (C-27), 27.7 (C-29), 27.5 (C-30), 25.4 (C-2), 22.5 (C-28).

Crystallographic Data: CCDC 712059 for **2**, and CCDC 712060 for **3** contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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