Indole Alkaloids Produced by a Marine Fungus Isolate of *Penicillium janthinellum* Biourge

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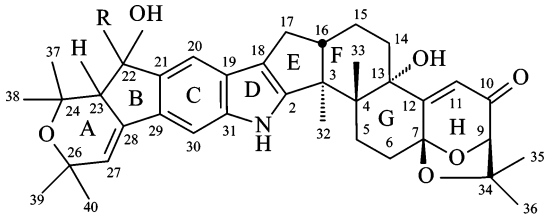
Three new indole alkaloids, shearinines D, E, and F (**1**−**3**), together with the known shearinine A (**4**) were isolated from the marine-derived strain of the fungus *Penicillium janthinellum* Biourge. The chemical structures of **1**−**4** were established by 2D NMR and HREIMS data. Shearinines A, D, and E induce apoptosis in human leukemia HL-60 cells, and shearinine E also inhibits EGF-induced malignant transformation of JB6 P^+ Cl 41 cells in a soft agar.

Marine-derived fungi are a rich source of structurally new natural products with a wide range of biological activities.[1−3] In our search for new compounds from marine-derived fungi, three new indole-diterpenoid alkaloids, shearinines D, E, and F (**1**−**3**), together with the known shearinine A (**4**)[4] were obtained from the fungus *Penicillium janthinellum* Biourge, which was isolated from marine sediments. These metabolites are analogues of paspalinine (**5**)[5,6] and janthitrems B−G (**6a**, **6b**, **7a**, **7b**, **7c**)[7,8] previously discovered from terrestrial fungi. Here we describe the isolation and structural elucidation of new alkaloids produced by the marine fungus *P. janthinellum.*

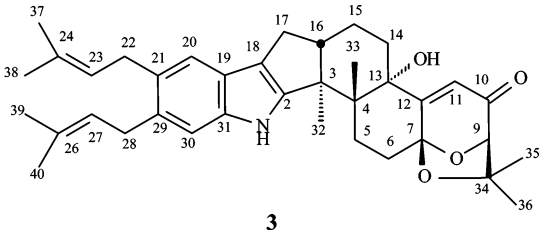
Results and Discussion

The EtOAc extract of the culture of *P. janthinellum* was fractionated by Si gel column chromatography using a step gradient−elution technique employing mixtures of hexane−EtOAc as solvent to yield individual compounds **1**−**4**. The structures of compounds **1**−**4** were established by the interpretation of physical data (^{1}H NMR, ^{13}C NMR, HSQC, HMBC, COSY, NOESY, and HREIMS). The physical data of the compounds were compared with literature data of the related compounds.[4−6]



1 R=H (H_{22}, H_{23}-*trans*)

2 R=H (H_{22}, H_{23}-*cis*)



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The molecular formula of compound **1** was determined as $C_{37}H_{45}O_6N$ on the basis of HREIMS and ^{13}C NMR analysis. Inspection of the ^{1}H and^{13} C NMR data (Table 1) of **1** revealed the presence of eight quaternary methyls (δ 16.2, C-32; 23.6, C-33; 23.1, C-35; 28.8, C-36; 30.0, C-37; 23.0, C-38; 31.9, C-39; 30.0, C-40), five methylenes (δ 27.0, C-5; 28.2, C-6; 33.9, C-14; 21.1, C-15; 27.5, C-17), six methines (δ 88.0, C-9; 117.7, C-11; 48.5, C-16; 76.4, C-22; 60.1, C-23; 120.9, C-27) including two olefinic and two oxygen-bearing ones, two aromatic proton singlets (δ_H 7.44, H-20; 7.34, H-30), and an N-H singlet (δ_H 7.71). Absorptions at 233 nm (log ϵ 4.1), 256 (sh) (4.4), 263 (4.3), and 330 (4.1) in the UV spectrum of the shearinines, e.g., shearinine D (**1**), suggested the presence of a 2,3-disubstituted indole nucleus with a double bond in conjugation with the aromatic ring system as in janthitrems E, F, and G[8] and shearinine A.[4] The general features of the ^{1}H and ^{13}C NMR spectra (Table 1) of **1** closely resembled those of shearinine A (**4**),[4] with the exception of proton and carbon resonances corresponding to one additional secondary hydroxy group (δ_H 4.96, δ_C 76.4). HMBC correlations between H-22 and C-20, C-28, and C-29 placed this hydroxy group at C-22 of ring B. The NOESY spectrum exhibited cross-peaks H-22/H_3-38, indicating that these protons are cofacial (Figure 1). The cross-peak between H_3-38 and H-23 in the COSY-45 spectrum demonstrated that these protons were oriented on opposite faces of the molecule. The coupling constants[9] of the resonances at δ 4.96 (H-22) and 2.67 (H-23) indicated that the gross structure of shearinine D (**1**) was H_{22},H_{23}-*trans*-22-hydroxyshearinine A.

The relative configuration of **1** was clarified by analysis of the NOESY spectrum. Thus, from the presence of NOESY correlations from H-16 to H_3-33 and from H_3-32 to H-17α and the absence of the NOE between H-16 and H_3-32 the *trans*-3,16-ring junction was deducted. The steric relationship between the indole portion of **1** and the diterpene part is determined by NOESY correlations between H-20 and H-17.

Shearinine E (**2**) has the same molecular formula as shearinine D (**1**). The NMR data of **2** were very similar to those obtained for shearinine D (Tables 1, 2) with the exception of the C-22 and C-23 resonances (Tables 1, 2). The NOESY spectrum revealed correlations between H-22 and H-23, H-20, and H_3-37. The coupling constants[10] of the resonances at δ 4.96 (H-22) and 2.67 (H-23) (Figure 2) suggested that **2** is an isomer of **1**. Therefore, the structure of shearinine E (**2**) was determined as H_{22},H_{23}-*cis*-22-hydroxy-shearinine A.

The molecular formula of compound **3** was determined as $C_{37}H_{47}O_4N$ on the basis of HREIMS and NMR spectra. The general features of the ^{1}H and ^{13}C NMR spectra of **3** (Table 3) closely resembled those of shearinines D (**1**) and E (**2**) (Tables 1, 2) and also of 21-isopentenylpaxilline[4] and paspalinine (**5**),[5] with the

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Table 1. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for Shearinine D (**1**)

position	δ_C	DEPT	δ_H (*J* in Hz)	NOESY (350 ms) and 1D NOE	HMBC[a]
1(NH)			7.71 brs	5, 33, 30	2, 18, 19, 31
2	153.8	C			
3	51.7	C			
4	39.9	C			
5	27.0	CH_2	2.69 m	32	3, 4, 6, 7
			1.82 m	1	3, 4, 6
6	28.2	CH_2	2.81 m		7, 5
			2.05 m		7, 12, 4
7	104.3	C			
9	88.0	CH	4.32 d (1.3)	35, 36	7, 10, 11, 36
10	196.9	C			
11	117.7	CH	5.84 brs	14	7, 9, 10, 13
12	169.5	C			
13	77.6	C			
14	33.9	CH_2	2.00 m	11	
15	21.1	CH_2	2.06 m	32	
			1.80 m		
16	48.5	CH	2.82 m	33	
17	27.5	CH_2	2.75 dd (13.2, 6.2)		2, 3, 16, 18
			2.44 dd (13.1, 10.5)	32	2, 15, 16, 18
18	117.5	C			
19	126.9	C			
20	113.9	CH	7.44 s	22, 17	18, 19, 29, 30, 31
21	138.7[b]	C			
22	76.4	CH	4.96 brd (5.3)	20, 37, 38	20, 24, 28, 29
23	60.1	CH	2.67 dd (5.7, 2.9)	37	22, 24, 27, 28, 37
24	73.8	C			
26	72.4	C			
27	120.9	CH	5.96 d (2.9)	30, 39, 40	23, 26, 29, 39, 40
28	135.1[b]	C			
29	131.9	C			
30	102.6	CH	7.34 s	27, 1	19, 20, 21, 28
31	141.1	C			
32	16.2	CH_3	1.38 s	5, 17a, 15	2, 3, 4, 16
33	23.6	CH_3	1.23 s	1, 16, 14	3, 4, 5, 13
34	78.8	C			
35	23.1	CH_3	1.18 s	9	9, 34, 36
36	28.8	CH_3	1.44 s	9	9, 34, 35
37	30.0	CH_3	1.49 s	38, 22, 23	23, 24, 38
38	23.0	CH_3	1.12 s	40, 37, 22	23, 24, 37
39	31.9	CH_3	1.34 s	27	26, 27, 40
40	30.0	CH_3	1.35 s	38, 27	26, 27, 39

[a] HMBC correlations, optimized for 8 Hz, are from proton(s) to the indicated carbon. [b] Assigments may be reversed.

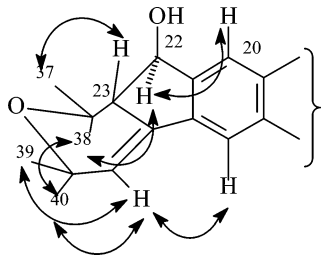

Figure 1. Selected NOESY correlations in **1**.

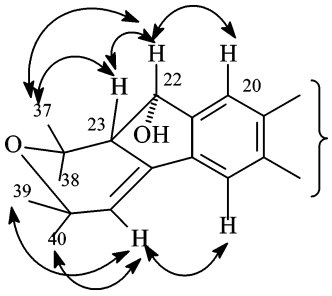

Figure 2. Selected NOESY correlations in **2**.

exception of proton and carbon resonances belonging to rings A and B. The resonances of four methyl groups at double bonds (δ_C 17.9−δ_H 1.74; δ_C 17.8−δ_H 1.72; δ_C 25.8−δ_H 1.75; δ_C 25.8−δ_H 1.74 ppm) and two methylene groups (δ_C 32.0−δ_H 3.39; δ_C 31.9−

Table 2. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for Shearinine E (**2**)

position	δ_C	DEPT	δ_H (*J* in Hz)	NOESY (350 ms)	HMBC[a]
1(NH)			7.72 brs	33, 5, 30	2, 18, 19, 31
2	153.9	C			
3	51.7	C			
4	39.8	CH_2			
5	27.0	CH_2	2.70 brt (10.3)	32	3, 4, 6, 7, 33
			1.83 m	1, 33	3, 6, 13, 33
6	28.2	CH_2	2.81 m		
			2.05 m		
7	104.3	C			
9	87.9	CH	4.32 d (1.3)	35, 36	7, 10, 11, 36
10	197.1	C			
11	117.7	CH	5.85 d (1.2)	14	7, 9, 13
12	169.5	C			
13	77.6	C			
14	33.9	CH_2	2.00 td (12.4, 4.3)		13, 15, 16
			1.90 m	11, 33	
15	21.1	CH_2	2.08 m	32	
			1.80 m		
16	48.5	CH	2.82 m	33	
17	27.5	CH_2	2.73 dd (6.3, 13.2)	20	2, 3, 1, 18
			2.44 dd (10.7, 13.2)	32	2, 15, 16, 18
18	117.6	C			
19	126.8	C			
20	114.9	CH	7.51 s	22, 17	18, 22, 29, 30, 31, 19
21	139.9[b]	C			
22	74.3	CH	5.35 brd (7.0)	20, 37, 23	20, 21, 28, 29
23	52.5	CH	2.90 dd (2.9, 7.3)	22, 37	21, 24, 27, 37, 38
24	74.8	C			
26	72.8	C			
27	121.1	CH	6.04 d (2.9)	39, 40, 30	23, 26, 29, 39, 40
28	136.7[b]	C			
29	132.5	C			
30	102.9	CH	7.38 s	1, 27	19, 20, 21, 28
31	141.4	C			
32	16.0	CH_3	1.39 s	5, 17, 15	2, 3, 4, 16
33	23.6	CH_3	1.23 s	1, 16	3, 4, 5, 13
34	78.7	C			
35	23.1	CH_3	1.19 s	9	9, 34, 36
36	28.9	CH_3	1.45 s	9	9, 34, 35
37	29.8	CH_3	1.53 s	22, 23	23, 24, 38
38	24.9	CH_3	1.38 s		23, 24, 37
39	32.2	CH_3	1.32 s	27	26, 27, 40
40	29.3	CH_3	1.41 s	27	26, 27, 39

[a] HMBC correlations, optimized for 8 Hz, are from proton(s) to the indicated carbon. [b] Assigments may be reversed.

δ_H 3.39 ppm) in the ^1H and ^{13}C NMR spectra and HMBC correlations of H-23 to C-37 and C-38 and H-27 to C-39 and C-40 proposed isopentenyl fragments connected at C-21 and C-29 atoms in **3**. This supposition was confirmed by HMBC correlations of H-20 to C-18, C-22, C-29, and C-31 and H-30 to C-19, C-21, and C-28. Therefore, the structure of shearinine F (**3**) was determined as 21,22-diisopentenylpaspalinine. The relative configuration of ring fusions in **3** was the same as in **1** and **2** (based on the NOESY correlations: H-16/H-33, H-17/H-32, H-11/H-14, H-5α/H-32).

The molecular formula of compound **4** was determined as $C_{37}H_{45}NO_5$ on the basis of HREIMS and NMR spectra. The remaining ^1H and ^{13}C NMR assignments were similar to those of shearinine A.[4] The connectivity of **4** and all ^1H and ^{13}C NMR assignments were verified by analysis of HMBC, HSQC, and COSY experiments. The relative configuration of **4** was proposed on the basis of coupling constants, NOESY data, and structural identity to shearinine A.[4] The good agreement of NMR spectra of **1** with those of **2**, **3**, and **4**, together with NOESY data and proton coupling constants, supported that these compounds have identical relative configurations of ring fusions. The α-orientation of the hydroxy group at C-13 was supported by NOESY correlations, which were observed between H-17α and H_3-32, and also between H_3-33 and H-16, and, additionally, NOE correlation between H-11 and H-14α.

Shearinines A, D, E, and F were tested for cytotoxic properties against mouse epidermal JB6 P^+ Cl 41 cells using the MTS method.[11] The compounds did not show any cytotoxicity up to 200 μM concentration. Some of these compounds showed cancer preventive and antileukemic properties. Shearinine E inhibited EGF-

Table 3. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for Shearinine F (**3**)

position	δ_C	DEPT	δ_H (J in Hz)	NOESY (350 ms)	HMBC[a]
1(NH)			7.52 brs	33, 5, 30	2, 18, 19, 31
2	151.4	C			
3	51.4	C			
4	39.9	C			
5	26.9	CH_2	2.65 brt (10.5)	32	6, 7, 33
			1.79 m	1	
6	28.2	CH_2	2.78 m		5, 7
			2.02 m	33	4, 7, 12
7	104.4	C			
9	88.0	CH	4.30 d (1.2)	35, 36	7, 11, 36
10	197.1	C			
11	116.8	CH	5.83 brs	14	7, 9, 13
12	169.8	C			
13	77.7	C			
14	33.9	CH_2	1.99 m	11	
			1.89 m	11	
15	21.1	CH_2	2.05 m	32	
			1.77 m		
16	48.5	CH	2.79 m	33	
17	27.6	CH_2	2.70 dd (13.1, 6.3) (β)		2, 3, 16, 18
			2.40 dd (13.1, 10.6) (α)	32	2, 16, 18
18	116.8	C			
19	123.6	C			
20	118.4	CH	7.20 s	22, 17	18, 22, 29, 31
21	131.3	C			
22	32.0[b]	CH_2	3.39 brd (7.1)	20, 37	23, 24
23	124.3[b]	CH	5.31 m[b]	38	37, 38
24	131.9[b]	C			
26	131.5[b]	C			
27	123.8[b]	CH	5.30 m[b]	39	39, 40
28	31.9[b]	CH_2	3.39 brd (7.1)	30, 40	26, 27
29	132.6	C			
30	111.4	CH	7.09 s	1, 28	19, 21, 28
31	139.0	C			
32	16.2	CH_3	1.34 s	5, 17, 15	2, 3, 4, 16
33	23.5	CH_3	1.21 s	1, 16, 6	3, 4, 5, 13
34	78.7	C			
35	23.1	CH_3	1.18 s	9	9, 34, 36
36	28.8	CH_3	1.44 s	9	9, 34, 35
37	17.9[b]	CH_3	1.74 brs[c]	22	23, 24, 38
38	25.8	CH_3	1.75 brs[b]	23	23, 24, 37
39	25.8	CH_3	1.74 brs[b]		26, 27, 40
40	17.8[b]	CH_3	1.72 brs[c]	28	26, 27, 39

[a] HMBC correlations, optimized for 8 Hz, are from proton(s) to the indicated carbon. [b, c] Assigments may be reversed.

induced malignant transformation of JB6 P^+ Cl 41 cells in a soft agar with $INCC_{50}$ (inhibition of number of the colonies$_{50}$) equal to 13 μM concentration. The anchorage-independent transformation assay in a soft agar is a well-accepted tool to determine whether a compound can be a potentially effective cancer preventive agent in either humans or animals.[12,13] Shearinines A, D, and E induced apoptosis in human leukemia HL-60 cells at 100 μM concentration by 10%, 39%, and 34% of the apoptotic cells compared to control cells, respectively.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 141 polarimeter, and melting points were taken with a Leica Galen III instrument. UV spectra were recorded on a Specord UV−vis spectrometer in MeOH. IR spectra were determined on a Specord M 82 Carl Zeiss Jena in $CHCl_3$. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker DRX-500 spectrometer operating at 500 and 125.6 MHz, respectively, using TMS as an internal standard. HREIMS were obtained with a JEOL MS Route mass spectrometer. MALDI MS analyses were carried out with a Bruker Biflex TOF mass spectrometer equipped with a UV-nitrogen laser (337 nm). Column chromatography was carried out using silica gel L (40/100 μm, Chemapol, Czechoslovakia). Sephadex LH-20 was purchased from Pharmacia Biotech, Sweden. Plates (4.5 × 6.0 cm) precoated with Si gel (5−17 μm, Sorbfil, Russia) were used for thin-layer chromatography.

Cultivation of *P. janthinellum*. The fungus *P. janthinellum* was isolated from marine bottom sediments collected at a depth of 11 m (Sea of Japan, Amursky Bay, near Vladivostok). The fungus was cultivated for 2 weeks at 30 °C in 20 0.5 L flasks, each containing 60 g of the nutrient medium RM[14] of the following composition (g): sodium tartrate, 0.01; yeast extract, 0.02; rice, 20; KH_2PO_4, 0.01; seawater, 40.

Extraction and Isolation. The fungus mycelia with the medium were extracted for 24 h with 3 L of EtOAc. Evaporation of the solvent under reduced pressure gave a brown oil (3 g), to which 500 mL of H_2O−EtOH (4:1) was added, and the combination was thoroughly mixed to yield a suspension. It was extracted successively with hexane (500 mL × 2), $CHCl_3$ (500 mL × 2), and *n*-BuOH (500 mL × 2). The $CHCl_3$ fraction was concentrated *in vacuo* to give a residue (700 mg), which was separated on a silica gel column (30 × 3 cm) eluted with a hexane−EtOAc acetate gradient (hexane−EtOAc = 100:0; 95:5; 90:10; 87:13; 85:15; 80:20, each 1.5 L) to afford 10 fractions. Gel filtration of fractions 2, 4, 5, and 7 over a Sephadex LH-20 column with $CHCl_3$ gave **1** (10 mg), **2** (16 mg), **3** (8 mg), and **4** (60 mg).

Shearinine D (1): white solid (hexane−EtOAc), mp 180−185 °C (dec); $[\alpha]^{20}_D$ +21.8 (*c* 0.13, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 233 (4.13), 256 (sh) (4.36), 263 (4.34), 330 nm (4.08); IR ($CHCl_3$) ν_{max} 3585, 3471, 3013, 2979, 2933, 2865, 1688, 1602, 1456, 1377, 1365, 1354 cm^{-1}; HREIMS *m*/*z* 599.3273 (calcd for $C_{37}H_{45}O_6N$, 599.3247).

Shearinine E (2): white solid (hexane−EtOAc), mp >300 °C (dec); $[\alpha]^{20}_D$ +3.5 (*c* 0.17, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 231 (4.20), 256 (sh) (4.36), 264 (4.38), 286 (4.03), 330 nm (4.04); IR ($CHCl_3$) ν_{max} 3585, 3471, 3014, 2980, 2933, 2870, 1688, 1602, 1456, 1376, 1356 cm^{-1}; HREIMS *m*/*z* 599.3221 (calcd for $C_{37}H_{45}O_6N$, 599.3247).

Shearinine F (3): white solid (hexane−EtOAc), mp 128−130 °C; $[\alpha]^{20}_D$ +76 (*c* 0.25, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 234 (3.90), 300 nm (3.28); IR ($CHCl_3$) ν_{max} 3475, 3052, 3042, 3036, 3006, 2930, 2856, 1687, 1602, 1456, 1376, 1336 cm^{-1}; HREIMS *m*/*z* 569.3500 (calcd for $C_{37}H_{47}O_4N$, 569.3505).

Shearinine A (4): white solid (hexane−EtOAc), mp >300 °C (dec); IR ($CHCl_3$) ν_{max} 3582, 3474, 2979, 2937, 2871, 1688, 1455, 1377, 1365 cm^{-1}; MALDI-MS *m*/*z* 584 $[M + H]^+$; other properties of **4** have been previously reported.[4]

Anchorage-Independent Transformation Assay. The mouse epidermal JB6 P^+ Cl 41 cells were kindly donated by Prof. Zigang Dong (The Hormel Institute, University of Minnesota). The cancer preventive effect of shearinine E was evaluated using the JB6 P^+ Cl 41 cell line and an anchorage-independent neoplastic transformation assay in a soft agar. EGF (10 ng/mL) was used for stimulating neoplastic transformation of JB6 P^+ Cl 41 cells. The assay was carried out in six-well tissue culture plates. Mouse JB6 P^+ Cl 41 cells (8 × 10^3/mL) were treated with various concentrations of shearinine E in 1 mL of 0.33% BME (basal medium Eagle) agar containing 10% FBS over 3 mL of 0.5% BME agar containing 10% FBS and various concentrations of shearinine B. The cultures were maintained in a 37 °C, 5% CO_2 incubator for 1 week. Cell colonies were then scored using a LEICA DM IRB inverted research microscope (Leica Mikroskopie und Systeme GmbH, Germany) and Image-Pro Plus software, version 3.0 for Windows (Media Cybernetics, Silver Spring, MD).

Apoptosis Assay Assessed by Flow Cytometry. The induction of early and late apoptosis by shearinines A, D, or E was analyzed by flow cytometry using a Becton Dickinson FACs calibur flow cytometer (BD Biosciences, San Jose, CA) after annexin V−fluorescein-isothiocyanate (FITC) and propidium iodide staining according to the manufacturer's protocol (BD Pharmingen, Flanklin Lakes, NJ). In brief, HL-60 cells (2 × 10^5 cells/well) were grown in six-well plates for 12 h in 10% FBS/RPMI 1640 medium and then treated with 100 μM concentration of shearinine A, D, or E for 24 h. After treatment with shearinines, the cells were washed and collected by centrifugation at 1000 rpm (170 rcf) for 5 min and resuspended in 500 μL of 1× binding buffer. Then, 5 μL of annexin V−FITC and 500 ng/mL of propidium iodide were added, and the cells were incubated at room temperature for 15 min in the dark and analyzed by flow cytometry.

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Supporting Information Available: Structural formulas of compounds **4**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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