JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

The Halicylindramides, Farnesoid X Receptor Antagonizing Depsipeptides from a *Petrosia* sp. Marine Sponge Collected in Korea

Dongyup Hahn,[†] Hiyoung Kim,[‡] Inho Yang,[‡] Jungwook Chin,[†] Hoosang Hwang,[‡] Dong Hwan Won,[‡] Byoungchan Lee,[‡] Sang-Jip Nam,[§] Merrick Ekins,[⊥] Hyukjae Choi,*[,∥] and Heonjoong Kang*[,‡]

[†]New Drug Development Center, Daegu-Gyeongbuk Medical Innovation Foundation, Daegu, 41061, Republic of Korea

[‡]School of Earth and Environmental Sciences, Seoul National University, NS-80, Seoul, 08826, Republic of Korea

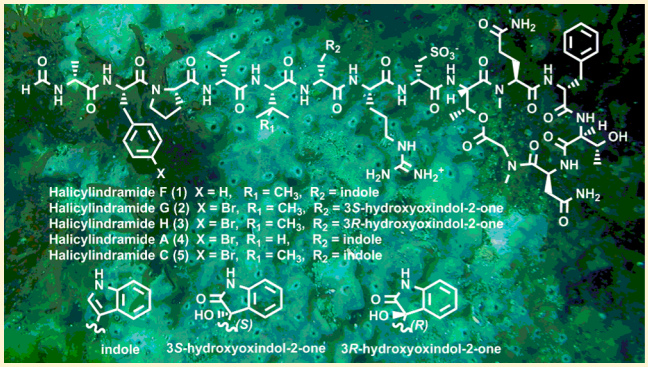
[§]Department of Chemistry and Nano Science, Global Top 5 Program, Ewha Womans University, Seoul, 03760, Republic of Korea

[⊥]Queensland Museum, P.O. Box 3300, South Brisbane, Queensland 4101, Australia

[∥]College of Pharmacy, Yeungnam University, Gyeongsan, 38541, Republic of Korea

Supporting Information

ABSTRACT: Three new structurally related depsipeptides, halicylindramides F−H (**1**−**3**), and two known halicylindramides were isolated from a *Petrosia* sp. marine sponge collected off the shore of Youngdeok-Gun, East Sea, Republic of Korea. Their planar structures were elucidated by extensive spectroscopic data analyses including 1D and 2D NMR data as well as MS data. The absolute configurations of halicylindramides F−H (**1**−**3**) were determined by Marfey's method in combination with Edman degradation. The absolute configurations at C-4 of the dioxyindolyl alanine (Dioia) residues of halicylindramides G (**2**) and H (**3**) were determined as 4*S* and 4*R*, respectively, based on ECD spectroscopy. The C-2 configurations of Dioia in **2** and **3** were speculated to both be 2*R* based on the shared biogenesis of the halicylindramides. Halicylindramides F (**1**), A (**4**), and C (**5**) showed human farnesoid X receptor (hFXR) antagonistic activities, but did not bind directly to hFXR.



Marine sponges are one of the richest sources of bioactive natural products, including peptides with complex structures and molecular weight exceeding 1000 Da. These peptides, isolated from marine sponges, have intriguing structural features, such as halogen atoms, macrocyclic rings, and uncommon amino acids with high levels of modification, such as *N*-methylation, epimerization (D-amino acids), and unusual branches.[1] Peptides found in marine sponges have also been reported to be potent cytotoxins (e.g., arenastatin A, IC_{50} value of 8.3 pM against KB cells;[2] hemiasterlin, ED_{50} value of 0.5 nM for antiproliferative activity against MCF-7 cells[3]), anti-inflammatory agents (halipeptin A, 60% inhibition of paw edema in mice at 0.3 mg/kg ip),[4] antibacterial agents (discodermins),[5] antifungal agents (theonellamide F, MIC of 1.8−7.3 μM against pathogenic fungi;[6] the discobahamins against *Candida albicans*;[7] the halicylindramides against *Mortierella ramanniana*;[8] jaspamide[9] and the theopapuamides[10] against amphotericin B-resistant *C. albicans*), and antiviral agents (papuamide A, EC_{50} of 2.6 nM for inhibition of HIV-1_{RF} infection;[11] homophymine A, cytoprotective activity against HIV-1 infection with an IC_{50} of 75 nM).[12]

The farnesoid X receptor (FXR) is expressed in the liver and in the intestine and is a nuclear hormone receptor of bile acids (BAs; e.g., chenodeoxycholic acid). In addition, its ligand-dependent transcription regulates cholesterol metabolism at multiple stages, such as synthesis, transport, elimination, and reabsorption of BAs.[13,14] Therefore, FXR has attracted attention as a therapeutic target for the treatment of dyslipidemia and cholestasis. In an animal model of non-obstructive cholestasis, FXR activation improved BA clearance from hepatocytes and protected against cholestatic liver damage.[15] However, in an animal model of obstructive cholestasis, FXR activation aggravated hepatocyte injury and parenchymal necrosis.[16] Although potential risks of FXR activation have been raised by studies based on preclinical models of obstructive cholestasis, FXR antagonists are considered to offer a potential means of treating obstructive and/or severe cholestasis.[17]

During our search for nonsteroidal human FXR (hFXR) antagonists in marine organisms, we isolated three new and two known halicylindramides, which are a type of depsipeptide,[8] from a *Petrosia* sp. collected in Korean waters. Herein, the

Special Issue: Special Issue in Honor of John Blunt and Murray Munro

Received: October 1, 2015

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX−XXX

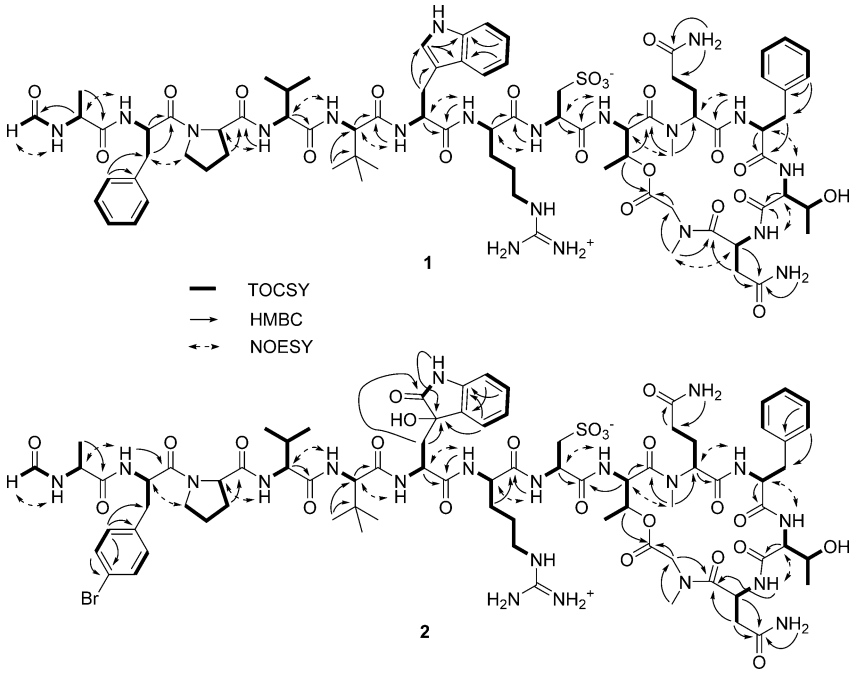


Figure 1. Key TOCSY, HMBC, and NOESY correlations in halicylidramides F (**1**) and G (**2**).

isolation and structure elucidation of the three new compounds and their hFXR antagonistic activities are described.

RESULTS AND DISCUSSION

Halicylindramide F (**1**) was isolated as a pale yellow oil, and its molecular formula was determined as $C_{79}H_{113}N_{20}O_{22}S$ by HRESIMS. The IR spectrum of **1** displayed absorption bands at 1655 and 1745 cm^{-1}, indicating the presence of amide and ester carbonyls, respectively. Amide carbonyl signals (δ_C 167.6–173.6), amide NH signals (δ_H 6.78–8.45), and α proton signals (δ_H 3.46–4.98) suggested that compound **1** was a peptide. A formyl group signal (δ_H 7.89, δ_C 160.5) in the 1D NMR spectra and a negative ninhydrin reaction suggested that the N-terminus of **1** was blocked with a formyl group.

Spectroscopic analyses of 1D and 2D NMR data, including COSY, HMQC, HMBC, and TOCSY data, allowed us to assign the constitutive amino acids of **1** (Figure 1 and Table S1, Supporting Information). The ^1H and ^{13}C NMR peaks of *N*-formylated Ala, Pro, Val, Arg, Cys(SO_3H), Thr, and Asn were assigned using COSY, TOCSY, and HMBC spectra (Figure 1). The assignment of *tert*-Leu (*t*-Leu) was established by the COSY correlation between amide NH and the α proton and the HMBC correlation from singlet methyl protons (9H, δ_H 0.70) to the α and β carbons (δ_C 60.4 and 33.7, respectively, Table 1).

*N*MeGln and *N*MeGly (Sar) were also assigned by COSY, TOCSY, and HMBC spectroscopic analysis (Figure 1). The ^1H and ^{13}C NMR chemical shifts of two singlet methyl signals (δ_H 2.93/δ_C 30.6 and δ_H 2.73/δ_C 35.6) and the HMBC correlations from these two methyl protons to α carbons (δ_C 54.9 and 48.6, respectively) indicated the presence of *N*MeGln and Sar (Figure 1 and Table 1).

The COSY correlation between the most deshielded exchangeable proton signal (δ_H 10.6) and one singlet aromatic proton signal (δ_H 7.15) and the COSY correlations between four aromatic protons (δ_H 7.60, 6.96, 7.03, and 7.31) suggested the presence of an indole of Trp. Two sets of Phe were also assigned on the basis of COSY, TOCSY, and HMBC spectra analyses (Figure 1 and Table 1).

Connections between neighboring amino acids were determined by HMBC and NOESY data analyses (Figure 1). The deshielded ^1H NMR signal of Hβ in Thr-1 at 5.14 ppm and the strong HMBC correlation from the Hβ of Thr-1 to the carbonyl carbon of Sar supported the presence of an ester linkage between Thr-1 and Sar to form a macrocyclic lactone ring.

The conformation of Pro of **1** was deduced to be *trans* because the value of $\Delta\delta_{\beta\gamma}$ (differential value between ^{13}C chemical shift carbon β and carbon γ of proline) is smaller than 9 ppm.[18] The NOESY correlation between Hδ of Pro and Hβ of Phe-1 also supported the *trans* conformation (Figure 1).

The absolute configurations of the constituent amino acids of halicylindramide F (**1**) were determined using Marfey's method and the advanced Marfey's method (Table S2).[19,20] As a result, D-Ala, L-Pro, D-Val, L-*t*-Leu, D-Trp, L-Arg, D-Cys(SO_3H), L-Thr, L-*N*MeGln, D-Asn, D-Phe, and L-Phe were found to be the amino acids constituting **1**. The absolute configurations of both Thr-1 and Thr-2 residues were identified as L-Thr. The exact positions of D- and L-Phe were assigned by coupled Edman degradation[21] followed by advanced Marfey's analysis. Compound **1** was deformylated and subjected to two Edman reaction cycles to remove two amino acids (Ala and Phe-1) from the N-terminus of halicylindramide F. The resulting dodecapeptide was analyzed by the advanced Marfey's method and found to contain D-Phe without L-Phe, and thus the configuration of Phe-1 was assigned as L.

The molecular formula of **2** was determined as $C_{79}H_{112}{}^{79}BrN_{20}O_{24}S$ based on the protonated molecule peak at m/z 1835.7062 $[M + H]^+$ as determined by HRESIMS. The presence of a bromine atom in compound **2** was confirmed by the pattern of the ion cluster of the protonated molecule at m/z 1835.7062:1836.6591:1837.6467:1838.6130 with an intensity ratio of 6:7:9:10, and this isotope pattern is similar to that calculated for a compound with the assigned molecular formula

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. NMR Data of Halicylindramides F−H (1−3) in DMSO-d_6 at 600 MHz (^{1}H) and 150 MHz (^{13}C)

	1			2		3	
position	δ_C, mult.[a]	δ_H, (J in Hz)	position	δ_C, mult.[a]	δ_H, (J in Hz)	δ_C, mult.[a]	δ_H, (J in Hz)
NCHO	160.5, C	7.89, s	NCHO	160.5, C	7.88, s	161.4, C	7.90, s
Ala α	46.4, CH	4.33, q (7.3)	Ala α	46.4, CH	4.31, q (7.3)	47.6, CH	4.31, m[b]
β	19.4, CH_3	0.89, d (7.2)	β	18.6, CH_3	0.93, d (7.0)	19.5, CH_3	0.92, d (7.0)
NH		8.14, brd (7.8)	NH		8.18, brs		8.18, brs
CO	171.4, C		CO	171.4, C		172.3, C	
Phe-1 α	51.7, CH	4.72, m	BrPhe α	51.9, CH	4.71, m[b]	52.6, CH	4.72, m[b]
β	34.7, CH_2	2.74, d (13.8)	β	36.2, CH_2	2.69−2.72 m	37.1, CH_2	2.71, m
		3.01, m			3.02, m[b]		3.01, m[b]
C1	137.7, C		C1	137.1, C		138.2, C	
C2/C6	129.3, CH	7.25, m	C2/C6	131.8, CH	7.26, m[b]	132.6, CH	7.26, m[b]
C3/C5	128.0, CH	7.22, m	C3/C5	130.8, CH	7.42, m	131.7, CH	7.43, d (8.1)
C4	126.7, CH	7.16, m	C4	119.5, C		120.3, C	
NH		8.45, brd (8.4)	NH		8.44, brd (8.3)		8.42, m[b]
CO	169.4, C		CO	171.0, C		171.4, C	
Pro α	59.8, CH	4.46, m	Pro α	59.5, CH	4.46, m	59.8, C	4.40, m[b]
β	29.8, CH_2	1.88, m	β	29.5, CH_2	1.89, m	30.5, CH_2	1.80, m[b]
		2.07 m			2.10, m		2.02, m[b]
γ	24.4, CH_2	1.89, m	γ	24.6, CH_2	1.90, m	25.2, CH_2	1.83, m[b]
δ	47.0, CH_2	3.67, m	δ	46.9, CH_2	3.70−3.62 m	47.8, CH_2	3.60, m
							3.71, m
CO	171.1, C		CO	169.8, C		168.7, C	
Val α	57.2, CH	4.38, m[b]	Val α	57.2, CH	4.38, m[b]	58.1, CH	4.37, m[b]
β	31.2, CH	2.07, m	β	30.7, CH	2.08, m	31.8, CH	2.09, m[b]
γ	17.3, CH_3	0.78, d (6.7)	γ	17.4, CH_3	0.76, d (6.7)	18.2, CH_3	0.77, d (6.7)
γ'	18.7, CH_3	0.83, d (7.2)	γ'	19.4, CH_3	0.81, d (6.7)	20.3, CH_3	0.82, d (6.5)
NH		7.81, m	NH		7.80, m[b]		7.78, m[b]
CO	171.0, C		CO	171.9, C		171.4, C	
t-Leu α	60.4, CH	4.18, d (8.7)	t-Leu α	60.2, CH	4.13, m[b]	61.3, CH	4.13, m[b]
β	33.7, C		β	34.1, C		34.9, C	
γ	26.5, CH_3	0.70, s	γ	26.6, CH_3	0.86, s	27.6, CH_3	0.89, s
NH		7.76, m	NH		7.82, m[b]		7.91, m[b]
CO	169.9, C		CO	170.0, C		170.9, C	
Trp α	54.0, CH	4.57, m	Dioia α	48.5, CH	4.46, m[b]	50.8, CH	4.42, m[b]
β	26.8, CH_2	3.16, m	β	39.0, CH_2	2.30, m	39.9, CH_2	2.18, m
		2.96, m			1.89, m		1.96, m
C2	124.1, CH	7.15, s	C2	178.2, C		179.8, C	
C3	109.8, C		C3	74.0, C		75.2, C	
C3a	136.6, C		C3a	131.7, C		132.0, C	
C7a	119.5, C		C7a	141.7, C		142.1, C	
C4	118.1, CH	7.60, d (7.9)	C4	124.0, CH	7.35, d (7.6)	125.0, CH	7.23, m[b]
C5	120.8, CH	6.96, dd (7.5, 7.1)	C5	122.0, CH	6.90, dd (7.6, 7.3)	122.6, CH	6.94, brt (7.3)
C6	111.2, CH	7.03, dd (7.4, 7.6)	C6	128.0, CH	7.18, m[b]	129.3, CH	7.19, m[b]
C7	127.2, CH	7.31, d (8.0)	C7	109.8, CH	6.78, d (8.0)	110.8, CH	6.80, brd (7.5)
1-NH		10.6, brs	1-NH		10.24, brs		10.32, brs
			3-OH		6.16, brs		6.18, brs
NH		8.29, brd (6.5)	NH		8.21, brd (7.9)		8.33, brs
CO	170.0, C		CO	169.8, C		169.1, C	
Arg α	51.5, CH	4.39, m	Arg α	52.0, CH	4.22, m[b]	53.0, CH	4.27, m[b]
β	29.5, CH_2	1.61, m	β	29.1, CH_2	1.65, m	29.5, CH_2	1.68, m[b]
γ	23.6, CH_2	1.28, m	γ	23.2, CH_2	1.40, m	24.8, CH_2	1.45, m
δ	39.7, CH_2	2.94, m	δ	40.2, CH_2	3.01, m[b]	41.2, CH_2	3.01, m[b]
guanidine	156.7, C		guanidine	156.8, C		157.7, C	
NH		7.99, brs	NH		7.81, brs[b]		7.82, brs[b]
CO	171.4, C		CO	171.0, C		172.3, C	
Cys(SO_3H) α	50.9, CH	4.60, m	Cys(SO_3H) α	50.9, CH	4.59, m	51.8, CH	4.59, m
β	52.4, CH_2	2.93, m	β	52.5, CH_2	2.90, m	53.4, CH_2	2.96, m[b]
NH		8.35, brd (6.5)	NH		8.30, brd (8.3)		8.30, brd (7.2)
CO	170.8, C		CO	169.4, C		169.0, C	
Thr-1 α	51.8, CH	4.83, d (8.5)	Thr-1 α	51.9, CH	4.84, d (8.5)	52.8, CH	4.83, d (8.6)

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 1. continued

	1			2		3	
position	δ_C, mult.[a]	δ_H, (J in Hz)	position	δ_C, mult.[a]	δ_H, (J in Hz)	δ_C, mult.[a]	δ_H, (J in Hz)
β	69.5, CH	5.14, d (6.7)	β	69.2, CH	5.14, d (6.7)	69.8, CH	5.13, brq (6.6)
γ	17.3, CH_3	1.16, d (6.5)	γ	17.4, CH_3	1.17, d (6.5)	18.2, CH_3	1.16, d (6.5)
NH		7.98, m	NH		7.96, m		8.01, brs
CO	167.9, C		CO	169.2, C		170.5, C	
*N*MeGln α	54.9, CH	4.97, m	*N*MeGln α	54.5, CH	4.98, m[b]	55.2, CH	4.96, m[b]
β	24.7, CH_2	1.70, m	β	24.7, CH_2	1.70, m	26.7, CH_2	1.70, m[b]
		1.89, m					1.89, m[b]
γ	31.6, CH_2	1.87, m	γ	31.5, CH_2	1.89, m	32.4, CH_2	1.87, m[b]
							1.92, m[b]
*N*Me	30.6, CH_3	2.93, s	*N*Me	30.4, CH_3	2.94, s	31.3, CH_3	2.94, s
$CONH_2$	173.6, C	6.68, brs	$CONH_2$	173.5, C	6.64, brs	174.4, C	6.67, brs
		7.17, brs			7.17, brs[b]		7.16, brs[b]
CO	168.8, C		CO	167.5, C		168.6, C	
Phe-2 α	54.2, CH	4.67, d (7.2)	Phe α	54.0, CH	4.69, d (7.2)	55.0, CH	4.69, m[b]
β	38.0, CH_2	3.00, m	β	37.9, CH_2	2.98, m	39.2, CH_2	2.96, m[b]
							3.02, m[b]
C1	137.3, C		C1	137.1, C		138.0, C	
C2/C6	129.4, CH	7.22, m	C2/C6	129.3, CH	7.18, m[b]	130.2, CH	7.18, m[b]
C3/C5	128.0, CH	7.14, m	C3/C5	129.0, CH	7.23, m[b]	128.9, CH	7.23, m[b]
C4	126.3, CH	7.12, m	C4	126.2, CH	7.15, m[b]	127.2, CH	7.17, m[b]
NH		7.55, brd (6.6)	NH		7.56, brd (6.6)		7.56, brs
CO	171.1, C		CO	170.7, C		171.9, C	
Thr-2 α	60.9, CH	3.85, m	Thr-2 α	60.7, CH	3.85, m	61.9, CH	3.86, m
β	65.6, CH	3.96, m	β	65.5, CH	3.96, m	66.3, CH	3.96, brs
γ	20.2, CH_3	0.89, d (4.8)	γ	20.1, CH_3	0.89, d (6.3)	21.0, CH_3	0.85, m[b]
OH		4.87, d (5.1)	OH		4.88, d (4.9)		4.90, brs
NH		7.92, brd (7.9)	NH		7.95, brs		7.96, brd (6.8)
CO	169.8, C		CO	169.6, C		170.1, C	
Asn α	45.9, CH	4.98, m	Asn α	46.0, CH	4.98, m[b]	46.8, CH	4.99, m[b]
β	36.9, CH_2	2.08, m	β	36.9, CH_2	2.09, m	38.1, CH_2	2.08, m[b]
		2.64, m			2.30, m		2.66, m[b]
NH		7.50, brd (9.6)	NH		7.51, brd (7.6)		7.50, brd (9.1)
$CONH_2$	171.2, C	6.78, brs	$CONH_2$	172.1, C	6.72, brs	173.0, C	6.77, brs
		7.32, brs			7.33, brs		7.32, brs
CO	172.1, C		CO	169.4, C		170.2, C	
Sar α	48.6, CH_2	3.46, d (15.7)	Sar α	49.5, CH_2	3.49, d (17.3)	50.6, CH_2	3.48, m[b]
		4.40, m			4.42, m[b]		4.42, m[b]
*N*Me	35.6, CH_3	2.73, s	*N*Me	35.4, CH_3	2.74, s	36.4, CH_3	2.73, s
CO	167.6, C		CO	167.5, C		168.5, C	

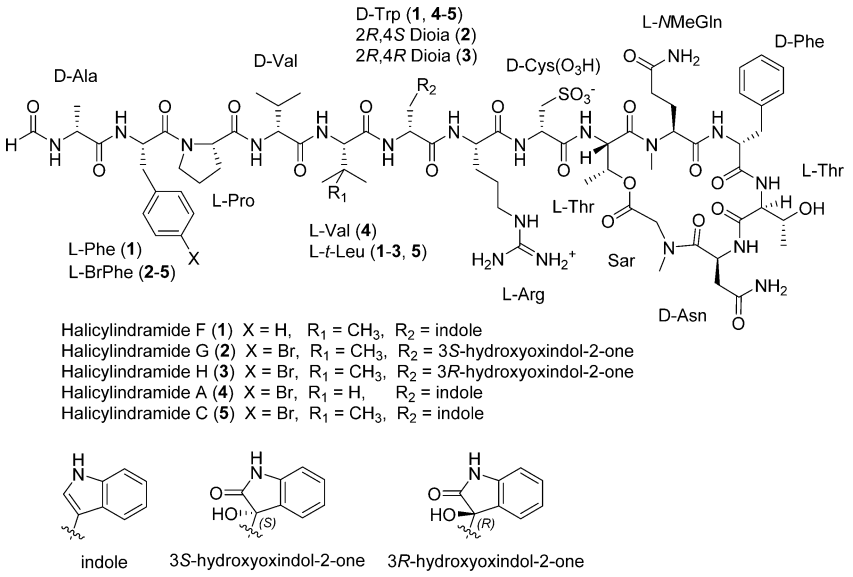
[a]Derived from the combination of ^{13}C NMR, HSQC, and HMBC data. [b]Signals overlapped.

(S28, Supporting Information). Like compound **1**, the NMR data of **2** also showed characteristic features of a peptide, such as amide carbonyl signals (δ_C 167.5−173.5), amide NH signals (δ_H 6.64−8.44), and α proton signals (δ_H 3.49−4.98). The 1D and 2D NMR spectra of **2** were closely related to those of compound **1**, indicating that compound **2** was also a halicylindramide (Tables 1 and S3). However, aromatic proton and carbon signals (δ_H 7.15, δ_C 124.1, C-2 of Trp) and the nonprotonated carbon signal (δ_C 109.8, C-3 of Trp) of **1** were not found in the ^1H and ^{13}C NMR spectra of **2**, and a deshielded carbonyl carbon at 178.2 ppm and an oxygenated carbon at 74.0 ppm were additionally observed in the ^{13}C NMR spectrum of **2**, which suggested the Trp residue of **1** was modified in **2**. Furthermore, the methylene protons at 2.30 and 1.89 ppm of **2** showed HMBC correlations to the carbons at 178.2 and 74.0 ppm. In addition, a deshielded exchangeable proton (δ_H 10.24) exhibited an HMBC correlation with the carbon at 74.0 ppm. These spectroscopic features indicated the presence of a dioxindolyalanine (Dioia) group in compound **2** rather than the indole of Trp. In addition, the ^1H and ^{13}C NMR spectra of compound **2** showed a 1,4-disubstituted benzene ring and a monosubstituted benzene ring rather than the two monosubstituted benzene rings in the spectra of **1**. The ^{13}C NMR chemical shift of C-4 of the 1,4-disubstituted benzene of **2** was 119.5 ppm, which corresponded to bromination at the C-4 position. Furthermore, spectroscopic data analyses enabled us to assign N-formylated Ala, Pro, Val, *t*-Leu, Arg, Cys(SO_3H), Thr, *N*MeGln, Asn, and Sar. Their assignments were secured based on HMBC and NOESY correlations, and the structure of halicylindramide G (**2**) was determined (Figure 1).

The molecular formula of **3** was also determined to be $C_{79}H_{112}{}^{79}BrN_{20}O_{24}S$ based on its protonated molecule peak at m/z 1835.7119 in $[M + H]^+$ and its ion cluster by HRESIMS. The ^1H NMR and ^{13}C NMR spectra of compound **3** were similar to those of compound **2** (Table 1). Spectroscopic data analysis including 1D and 2D NMR data analysis indicated that

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX−XXX

Chart 1



the planar structure of compound **3** was identical to the planar structure of compound **2** (Table S4).

The conformation of Pro in compounds **2** and **3** was assigned to be *trans* based on the small $\Delta\delta_{\beta\gamma}$ values and on the NOESY correlations between Hδ of Pro and Hα of BrPhe (Figure 1).[18]

The absolute configurations of the constitutive amino acids, except for the Dioia unit of halicylindramides G (**2**) and H (**3**), were determined by Marfey's method and the advanced Marfey's method. They were found to possess identical absolute configurations of the amino acids except for the Dioia unit (Table S5). The Dioia unit decomposed during the acid hydrolysis, and attempts to prepare Marfey's derivatives from the Dioia standards also failed.[22,23] The absolute configurations of its stereogenic centers were investigated by comparing the ECD absorption of the parent compounds with four authentic Dioia standards (Figure 2).[24,25] The ECD spectra of **2** at 240 and 265 nm showed positive and negative Cotton effects, respectively, indicating a Dioia unit with the 4*S* configuration. On the other hand, the ECD absorption spectra of **3** showed negative and positive Cotton effects at 240 and 265 nm, respectively, and thus, the absolute configuration of C-4 of Dioia in **3** was assigned as *R*. The Trp residue of previously reported halicylindramides A–E and halicylindramide F (**1**) was found to be a D-type amino acid, and thus, the absolute configurations of the α position of Dioia in **2** and **3** were speculated to be *R* based on a shared biogenesis of the halicylindramides.

Along with compounds **1**–**3**, compounds **4** and **5** were also obtained, and their spectroscopic data including LRESIMS data and 1D/2D NMR data were identical to those of previously reported data of halicylindramides A and C.[8]

Due to the structural similarity, compounds **2** and **3** were assumed to be oxidation artifacts derived from compound **5**. A portion of compound **5** in a dried vial and a portion of **5** dissolved in MeOH (1 mg/mL) were stored at room temperature and −20 °C under dark conditions for 8 weeks, and no changes were obversed in this examination. Therefore, it could be speculated that the oxidation from Trp to Dioia occurs under the highly oxidative conditions in living organisms such as cyanobacteria or marine sponges or the Dioia unit is prepared before the halicylindramides' biosynthesis.

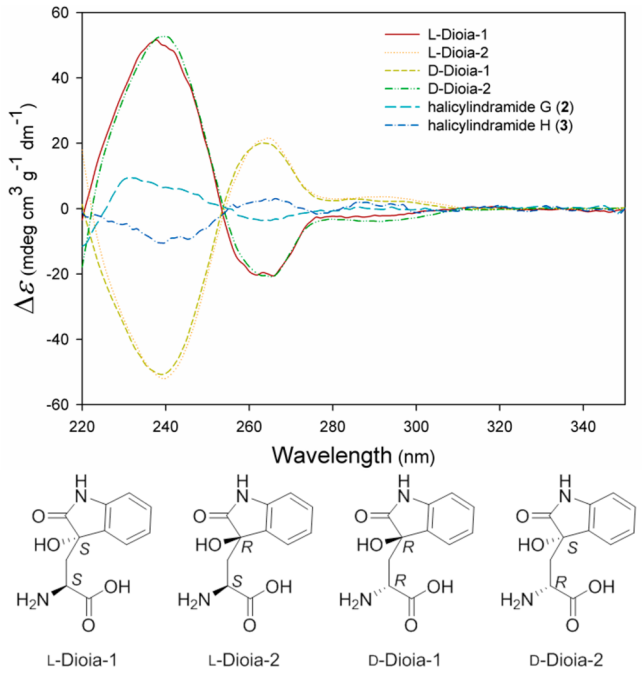


Figure 2. ECD spectra of halicylindramides G (**2**) and H (**3**) and four authentic standards of Dioia.

The antagonistic activities of compounds **1**–**5** against hFXR were evaluated using a co-transfection assay. Compounds **1**, **4**, and **5** showed potent inhibitory effects of hFXR transactivation with IC_{50} values of 6.0, 0.5, and 5.0 μM, respectively, while **2** and **3** were inactive up to 100 μM. Compounds **1**–**5** are not cytotoxic against HepG2 cells (IC_{50} values of 100, 100, 100, 20, and 100 μM, respectively; IC_{50} value of doxorubicin = 2.1 μM, cytotoxicity = IC_{50} values less than 10 μM). In particular, halicylindramide A (**4**) most potently inhibited hFXR transactivation with an IC_{50} value of 0.5 μM, which is much lower than that of the well-known FXR antagonist *E*-guggulsterone (IC_{50} 41.0 μM,).[26,27] Interestingly, halicylindramides G (**2**) and

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX−XXX

H (**3**) did not antagonize ligand-dependent hFXR transactivation, and the presence of Trp in the halicylindramides is speculated to be crucial for their hFXR antagonistic activity. In direct binding experiments using the BIAcore system, **1**, **4**, and **5** did not show any significant inhibition of the recruitment of cofactor SRC-1 peptide to hFXR at concentrations higher than their IC_{50} values determined in a cell-based co-transfection assay. Therefore, it seems that the hFXR antagonism of the halicylindramides F, A, and C is achieved not by their direct binding to LBD of the receptor but rather by an indirect manner such as through a membrane-bound receptor or a kinase/phosphatase pathway. Halicylindramides A–E have been previously reported as antifungal agents against *Mortierella ramanniana* and cytotoxic agents against P388 murine leukemia cells.[8]

All of the previously reported halicylindramides A–E were discovered from *Halichondria cylindrata*, while the two known halicylindramides A and C along with three new analogues (**1**–**3**) in this study were isolated from *Petrosia* sp. 4921. The structures of the halicylindramides show high degrees of modification such as N-methylation, epimerization to D-amino acids, and halogenation, which is reminiscent of cyanobacterial peptides.[28] Due to the structural features and the occurrence of the halicylindramides in two different genera of marine sponges, the producers of the halicylindramides may be sponge-associated cyanobacteria.

In conclusion, three new and two previously reported halicylindramides were isolated from the extract of a Korean *Petrosia* sp. marine sponge. The structures of compounds **1**–**3** are closely related to halicylindramide C (**5**). The L-BrPhe of **5** is substituted with L-Phe in **1**. The Dioia unit in compounds **2** and **3**, oxidation products of Trp, have not been reported in the previously reported halicylindramides. Halicylindramides F (**1**), A (**4**), and C (**5**) were found to be potent antagonists of hFXR, with IC_{50} values as low as 0.5 μM. Currently, target identification of the halicylindramides is under way.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Autopol III using a 5 cm cell. UV spectra were acquired on a Scinco UVS-2100 using a path length of 1 cm. ECD spectra were collected in the wavelength range 200 to 400 nm using a JASCO J-715 (path length: 0.01 dm; cell volume: 400 μL; scan speed: 50 nm/min; resolution: 0.5 nm). IR spectra were obtained using a Nicolet ThermoElectron Nicolet 5700 FT-IR spectrometer using KBr plates. NMR spectra were recorded in DMSO-d_6 (δ_C 39.5, δ_H 2.50) using solvent peaks as internal standards on a Bruker Avance DPX-600 spectrometer (600 and 150 MHz for ^1H and ^{13}C NMR, respectively). LR- and HRFABMS were obtained using a JEOL JMS-AX505WA mass spectrometer. HPLC was carried out using a Younglin SDV 30 Plus HPLC system equipped with a Younglin M 720 UV detector.

Animal Material. The *Petrosia* sp. 4921 specimen (HK11) was collected by scuba at a depth of 10–20 m off the shore of Youngdeok-Gun, East Sea, Republic of Korea. The identification of the specimen was done by an Australian sponge taxonomist, Merrick Ekins (S84, Supporting Information). A voucher specimen (G331811) is deposited at the Queensland Museum, Australia, and at the Center for Marine Natural Products and Drug Discovery, Seoul National University, Korea (registered as HK11).

Isolation of Halicylindramides F–H, A, and C (1–5). Collected sponges (18 kg, wet weight) were lyophilized, and the dried materials (2.1 kg) were powdered and extracted three times with 50% MeOH in CH_2Cl_2 at room temperature (rt) for 3 days. The extract (570 g) was partitioned between hexanes and MeOH, and MeOH solubles were further partitioned between EtOAc and H_2O. Finally, the H_2O-soluble fraction was extracted with 1-BuOH. Fractions were assayed for their hFXR antagonizing activities. The 1-BuOH fraction exhibited the most potent bioactivity on both activity screening platforms. 1-BuOH solubles were fractionated by size exclusion chromatography through Sephadex LH-20 (1000 × 50 mm) using 70% MeOH in CH_2Cl_2. The bioactive compounds were finally purified by RP-HPLC [Phenomenex HydroRP, 250 × 10 mm, flow rate 2.0 mL/min, UV detection at 210 nm] with 30% CH_3CN in H_2O. Finally, three new depsipeptides, halicylindramides F (**1**, 10 mg), G (**2**, 8 mg), and H (**3**, 7 mg) and two known analogue, halicylindramides A (**4**, 30 mg)[8] and C (**5**, 260 mg),[8] were obtained.

*Halicylindramide F (**1**):* pale yellow oil; $[\alpha]^{25}_D$ −8.0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 207 nm (log ε 4.32), 287 nm (log ε 3.06); IR (KBr) ν_{max} 1745, 1655 cm^{-1}; ^1H, ^{13}C, and 2D NMR data, Table 1; HRESIMS *m*/*z* 1725.7976 $[M + H]^+$ (calcd for $C_{79}H_{113}N_{20}O_{22}S$ 1725.8059).

*Halicylindramide G (**2**):* pale yellow oil; $[\alpha]^{25}_D$ −10 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 207 nm (log ε 4.40), 261 nm (log ε 3.32); ECD (1.0 mg/mL, CH_3CN), λ_{max} ($\Delta\varepsilon$) 322 (0.59), 265 (−3.65), and 231 (9.39) nm; IR (KBr) ν_{max} 1735, 1640 cm^{-1}; ^1H, ^{13}C, and 2D NMR data, Table 1; HRESIMS *m*/*z* 1835.7062 $[M + H]^+$ (calcd for $C_{79}H_{112}{}^{79}BrN_{20}O_{24}S$ 1835.7063).

*Halicylindramide H (**3**):* pale yellow oil; $[\alpha]^{25}_D$ −10 (*c* 0.1, MeOH); UV (MeOH) λ_{max} 207 nm (log ε 4.41), 260 nm (log ε 3.31); ECD (1.0 mg/mL, CH_3CN), λ_{max} ($\Delta\varepsilon$) 319 (0.88), 266 (2.99), and 239 (−10.54) nm; IR (KBr) ν_{max} 1735, 1640 cm^{-1}; ^1H, ^{13}C, and 2D NMR data, Table 1; HRESIMS *m*/*z* 1835.7119 $[M + H]^+$ (calcd for $C_{79}H_{112}{}^{79}BrN_{20}O_{24}S$ 1835.7063).

*Halicylindramide A (**4**):* $[\alpha]^{25}_D$ −1.5 (*c* 0.1, MeOH) (reported $[\alpha]^{23}_D$ −1.4).[8]

*Halicylindramide C (**5**):* $[\alpha]^{25}_D$ −6.0 (*c* 0.1, MeOH); (reported $[\alpha]^{23}_D$ −6.1).[8]

Advanced Marfey's Method.[19,20] A peptide (200 μg) was treated with 100 μL of 6 N HCl at 110 °C for 30 min, and the resulting acid hydrolysates were lyophilized. Dried products were dissolved in 100 μL of 1 M $NaHCO_3$, and samples were halved and transferred to two separate vials. A 25 μL amount of 1% L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucine amide) in acetone was added to one, and 25 μL of a 1% D- and L-FDLA mixture in acetone was added to the other. Solutions were vortexed and incubated at 40 °C for 60 min, reactions were quenched by adding 25 μL of 2 N HCl, reaction mixtures were diluted with 100 μL of MeOH, and 10 μL of each solution was injected into a HPLC-ESIMS.

The reaction products from advanced Marfey's method were separated on an RP-HPLC column (Capcell Pak C-18 MGII column, 20 × 50 mm, 5.0 μm, Shiseido, Japan) by step gradient elution [0.1% TFA in H_2O (A) and 100% CH_3CN (B); 0–10 min, B, 30%; 10–50 min, B, 30–70%; 50–60 min, B, 70%; 60–70 min, B, 100%; flow rate, 100 μL/min; column temperature, 30 °C] and detected by ESIMS (sheath gas flow rate, 20 arbitrary units; auxiliary gas flow rate, 1.40 arbitrary units; sweep gas flow rate, 1.5 arbitrary units; capillary voltage, 36 V; capillary temperature, 277 °C; tube lens, 120 V). Retention times of individual Marfey's derivatives are included in the Supporting Information (Table S2).

Preparation of Dodecapeptide from 1 by Modified Edman Degradation.[21] Halicylindramide F (**1**, 300 μg) was deformylated by treating it with 100 μL of 1.5 N HCl at rt for 24 h, and reaction products were lyophilized. To couple peptide N-termini with the Edman reagent, the products were dissolved in 60 μL of Edman coupling solution (phenylisothiocyanate/H_2O/EtOH/triethylamine = 1:1:7:1), vortexed, and heated at 50 °C for 8 min. Mixtures were then desolvated in a centrifugal evaporator at 50 °C for 5 min, and the residue were resuspended in 100 μL of deionized H_2O and washed three times with 100 μL of heptane/EtOAc (7:1, v/v). The resulting aqueous layers were dried in a centrifugal evaporator at 50 °C for 15 min. To perform cyclization and cleavage, 50 μL of 8 mM BF_3/Et_2O in CH_3CN was added to the residue, and the reaction mixture was vortexed and heated at 50 °C for 5 min. The product mixture was dried under a N_2 stream, resuspended in 100 μL of H_2O, and washed three times with 100 μL of heptane/EtOAc (1:5, v/v). The aqueous

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX–XXX

layers were concentrated in a centrifugal evaporator to obtain the dealanyl peptide, which was subjected to a second Edman reaction to prepare the dodecapeptide that lacks 2-Phe.

Preparation of Authentic Dioia Standards from D- and L-Trp.[23] DMSO (450 μL) was slowly added to a vial of 2.5 mL of 12 N HCl at rt. Phenol (50 mg) was then added followed by D-Trp (500 mg) in 15 mL of acetic acid. The reaction mixture was stirred at rt for 5 h, and the acetic acid, HCl, and DMSO were then removed *in vacuo*. The resulting syrup was resuspended in 750 μL of acetic acid, stored at 4 °C for 12 h, and dried under vacuum, and the oxyindolyl-D-alanine C-4 epimers (D-Oia, 243 mg, 45%) produced were obtained by RP-HPLC (Phenomenex Polar RP, 10 × 250 mm, 4 μm, 100% H_2O, 3.0 mL/min, 250 nm, retention time 24 min).

D-Oia diastereomers: $C_{11}H_{12}N_2O_3$, ESIMS m/z $[M + H]^+$ 221; UV (H_2O) 252 nm; ^1H NMR (DMSO-d_6) 11.01 (1H, brs), 10.65 (1H, brs), 9.18 (2H, brs), 8.50 (2H, brs), 7.64−6.83 (8H, m), 4.68 (1H, m), 4.45 (1H, m), 3.10−2.90 (2H, m), 2.35 (2H, m), 2.15 (2H, m).

D-Oia diastereomers (100 mg) were dissolved in 10 mL of 0.1 N NaOH, and the solution was aerated with O_2 for 12 h at rt. Conversion was confirmed by the formation of two products, which were both more polar than D-Oia on NP-TLC (1-BuOH/EtOAc/H_2O/AcOH = 1:1:1:1) and produced spots of almost equal intensity (ninhydrin). The reaction mixture was adjusted to pH 4 with acetic acid and refrigerated overnight, and the residual syrup was dried *in vacuo*. The two diastereomers were separated by RP-HPLC (YMC AQ S-5, 10 × 250 mm, 5 μm, 280 nm) by step gradient elution [100% H_2O (eluent A) and 100% CH_3CN (eluent B); 0−18 min, A, 100%, 2.5 mL/min; 18−28 min, B: 25%, 3.5 mL/min]. D-Dioia-1 and -2 were eluted with retention times of 22 and 26 min, respectively. In addition, L-Dioia-1 and -2 were also prepared using the same procedure from L-Trp via L-Oia.

D-Dioia-1: $[\alpha]^{25}_D$ +20 (*c* 0.1, H_2O); ECD (0.5 mg/mL, CH_3CN), λ_{max} ($\Delta\varepsilon$) 318 (−0.24), 265 (21.49), and 239 (−50.77) nm; LRESIMS m/z $[M + H]^+$ 237.

D-Dioia-2: $[\alpha]^{25}_D$ −39 (*c* 0.1, H_2O); ECD (0.5 mg/mL, CH_3CN), λ_{max} ($\Delta\varepsilon$) 315 (0.42), 263 (−20.07), and 240 (52.64) nm; LRESIMS m/z $[M + H]^+$ 237.

L-Dioia-1: $[\alpha]^{25}_D$ −20 (*c* 0.1, H_2O); ECD (0.5 mg/mL, CH_3CN), λ_{max} ($\Delta\varepsilon$) 318 (0.57), 266 (−20.53), and 238 (51.54) nm; LRESIMS m/z $[M + H]^+$ 237.

L-Dioia-2: $[\alpha]^{25}_D$ +39 (*c* 0.1, H_2O); ECD (0.5 mg/mL, CH_3CN), λ_{max} ($\Delta\varepsilon$) 315 (−0.42), 263 (20.07), and 240 (−52.64) nm; LRESIMS m/z $[M + H]^+$ 237.

Cell-Based Co-transfection Assay. CV-1 cells were seeded in 96-well plates in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% resin-charcoal-stripped fetal bovine serum in humidified air containing 5% CO_2 at 37 °C for 24 h. Transient co-transfections with pCMX-hFXR, CMX-β-GAL, and Tk-(EcRE)$_6$-LUC were carried out using SuperFect (Qiagen), according to the manufacturer's instructions. After incubation for 24 h, co-transfected cells were treated with a control vehicle (DMSO) or indicated compounds for the hFXR agonist test. Ligands were treated with 50 μM chenodeoxycholic acid (CDCA) to test for antagonism on hFXR. Cells were harvested at 24 h, and luciferase activities were assayed as described previously.[13,14] Luciferase activities were normalized versus β-galactosidase activity expressed by the control plasmid CMX-β-GAL. Cotransfections were performed in triplicate.

Surface Plasmon Resonance (SPR) Spectroscopy. Direct bindings of **1**, **4**, and **5** to the ligand binding domain (LBD) of hFXR were monitored by SPR spectroscopy using a BIAcore system.[29] hFXR LBD (4 μM), preincubated for 1 h with *E*-guggulsterone or **1**−**5** in the presence of 50 μM CDCA, was injected over a sensor chip surface coated with immobilized coactivator peptide (SRC-1). Changes in resonance units (RUs) were used to monitor the ligand-induced association of the hFXR LBD and SRC-1 peptide.

Cytotoxicity Assay. HepG2 human liver carcinoma cells (5.0 × 10^3 cells/mL) were plated to 96-well plates containing 0.1 mL of Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% resin-charcoal-stripped fetal bovine serum. Cells were incubated overnight (37 °C, 5% CO_2) to allow recovery before being treated with test compounds. Serially diluted samples were added to wells, and doxorubicin was used as a positive control. Plates were incubated for approximately 24 h at 37 °C in humidified air containing 5% CO_2 before staining with MTT. MTT assessment of cytotoxicity was performed as a modification of Mosmann's method,[30] replacing the overlying medium with 60 μL of a 1:6 dilution of MTT stock (5 mg/mL PBS, pH = 7.4) in complete culture medium. Mitochondrial dehydrogenases in live cells converted the MTT to an insoluble purple formazan crystal. Following the incubation (30 min at 37 °C) with the MTT media mixture, the remaining solution was removed from the wells and the crystals were solubilized by the addition of 50 μL of DMSO. After incubating the plate at rt in the dark for 15 min, absorbance was read at 550 nm with a VICTOR2 (PerkinElmer).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00871.

One-dimensional (^1H and ^{13}C) and two-dimensional (HMQC, COSY, TOCSY, NOESY, and HMBC) NMR spectra of **1**−**3** and surface plasmon resonance spectroscopy data of **1**, **4**, and **5** (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel (H. Choi): (82) 53-810-2824. Fax: (82) 53-810-4654. E-mail: h5choi@yu.ac.kr.

*Tel (H. Kang): (82) 2-880-5730. Fax: (82) 2-883-9289. E-mail: hjkang@snu.ac.kr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the 2013 Yeungnam University research grant.

■ REFERENCES

(1) Ebada, S. S.; Proksch, P. In *Handbook of Marine Natural Products*; Fattorusso, E.; Gerwick, W. H.; Taglialatela-Scafati, O., Eds.; Springer: New York, 2012; Vol. *1*, Chapter 4, pp 191−293.

(2) Kobayashi, M.; Aoki, S.; Ohyabu, N.; Kurosu, M.; Wang, W.; Kitagawa, I. *Tetrahedron Lett.* **1994**, *35*, 7969−7972.

(3) Anderson, H. J.; Coleman, J. E.; Andersen, R. J.; Roberge, M. *Cancer Chemother. Pharmacol.* **1997**, *39*, 223−226.

(4) Randazzo, A.; Bifulco, G.; Giannini, C.; Bucci, M.; Debitus, C.; Cirino, G.; Gomez-Paloma, L. *J. Am. Chem. Soc.* **2001**, *123*, 10870−10876.

(5) Ryu, G.; Matsunaga, S.; Fusetani, N. *Tetrahedron Lett.* **1994**, *35*, 8251−8254.

(6) Matsunaga, S.; Fusetani, N.; Hashimoto, K.; Waelchli, M. *J. Am. Chem. Soc.* **1989**, *111*, 2582−2588.

(7) Gunasekera, S. P.; Pomponi, S. A.; McCarthy, P. J. *J. Nat. Prod.* **1994**, *57*, 79−83.

(8) Li, H.; Matsunaga, S.; Fusetani, N. *J. Med. Chem.* **1995**, *38*, 338−343.

(9) Scott, V. R.; Boehme, R.; Matthews, T. R. *Antimicrob. Agents Chemother.* **1998**, *32*, 1154−1157.

(10) Plaza, A.; Bifulco, G.; Keffer, J. L.; Lloyd, J. R.; Baker, H. L.; Bewley, C. A. *J. Org. Chem.* **2009**, *74*, 504−512.

(11) Ford, P. W.; Gustafson, K. R.; McKee, M. C.; Shigematsu, N.; Maurizi, L. K.; Pannell, L. K.; Williams, D. E.; de Silva, D.; Lassota, P.; Allen, T. M.; van Soest, R.; Andersen, R. J.; Boyd, M. R. *J. Am. Chem. Soc.* **1999**, *121*, 5899−5909.

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX−XXX

(12) Zampella, A.; Sepe, V.; Luciano, P.; Bellotta, F.; Monti, M. C.; D'Auria, M. V.; Jepsen, T.; Petek, S.; Adeline, M.; Laprévote, O.; Aubertin, A.; Debitus, C.; Poupat, C.; Ahond, A. *J. Org. Chem.* **2008**, *73*, 5319−5327.
(13) Makishima, M.; Okamoto, A. Y.; Repa, J. J.; Tu, H.; Learned, R. M.; Luk, A.; Hull, M. V.; Lustig, K. D.; Mangelsdorf, D. J.; Shan, B. *Science* **1999**, *284*, 1362−1365.
(14) Parks, D. J.; Blanchard, S. G.; Bledsoe, R. K.; Chandra, G.; Consler, T. G.; Kliewer, S. A.; Stimmel, J. B.; Willson, T. M.; Zavacki, A. M.; Moore, D. D.; Lehmann, J. M. *Science* **1999**, *284*, 1365−1368.
(15) Liu, Y.; Binz, J.; Numerick, M. J.; Dennis, S.; Luo, G.; Desai, B.; MacKenzie, K. I.; Mansfield, T. A.; Kliewer, S. A.; Goodwin, B.; Jones, S. A. *J. Clin. Invest.* **2003**, *112*, 1678−1687.
(16) Fiorucci, S.; Antonelli, E.; Rizzo, G.; Renga, B.; Mencarelli, A.; Riccardi, L.; Orlandi, S.; Pellicciari, R.; Morelli, A. *Gastroenterology* **2004**, *127*, 1497−1512.
(17) Fiorucci, S.; Distrutti, E.; Ricci, P.; Giuliano, V.; Donini, A.; Baldelli, F. *Expert Opin. Ther. Targets* **2014**, *18*, 1449−1459.
(18) Siemion, I. Z.; Wieland, T.; Pook, K. *Angew. Chem., Int. Ed. Engl.* **1975**, *14*, 702−703.
(19) Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591−596.
(20) Fuji, K.; Ikai, Y.; Oka, H.; Suzuki, M.; Harada, K. *Anal. Chem.* **1997**, *69*, 5146−5151.
(21) Edman, P. *Acta Chem. Scand.* **1950**, *4*, 283−293.
(22) Simat, T. J.; Steinhart, H. *J. Agric. Food Chem.* **1998**, *46*, 490−498.
(23) Labroo, R. B.; Cohen, L. A. *J. Org. Chem.* **1990**, *55*, 4901−4904.
(24) Takayama, H.; Shimizu, T.; Sada, H.; Harada, Y.; Kitajima, M.; Aimi, N. *Tetrahedron* **1999**, *55*, 6841−6846.
(25) Robinson, S. J.; Morinaka, B. I.; Amagata, T.; Tenney, K.; Bray, W. M.; Gassner, N. C.; Lokey, R. S.; Crews, P. *J. Med. Chem.* **2010**, *53*, 1651−1661.
(26) Urizar, N. L.; Liverman, A. B.; Dodds, D. T.; Silva, F. V.; Ordentlich, P.; Yan, Y.; Gonzalez, F. J.; Heyman, R. A.; Mangelsdorf, D. J.; Moore, D. D. *Science* **2002**, *296*, 1703−1706.
(27) Burris, T. P.; Montrose, C.; Houck, K. A.; Osborne, H. E.; Bocchinfuso, W. P.; Yaden, B. C.; Cheng, C. C.; Zink, R. W.; Barr, R. J.; Hepler, C. D.; Krishnan, V.; Bullock, H. A.; Burris, L. L.; Galvin, R. J.; Bramlett, K.; Stayrook, K. R. *Mol. Pharmacol.* **2005**, *67*, 948−954.
(28) Choi, H.; Pereira, A. R.; Gerwick, W. H. In *Handbook of Marine Natural Products*; Fattorusso, E.; Gerwick, W. H.; Taglialatela-Scafati, O., Eds.; Springer: New York, 2012; Vol. *1*, Chapter 2, pp 55−152.
(29) Fujino, T.; Sato, Y.; Une, M.; Kanayasu-Toyoda, T.; Yamaguchi, T.; Shudo, K.; Inoue, K.; Nishimaki-Mogami, T. *J. Steroid Biochem. Mol. Biol.* **2003**, *87*, 247−252.
(30) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.

DOI: 10.1021/acs.jnatprod.5b00871
J. Nat. Prod. XXXX, XXX, XXX−XXX