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Article

Biosurfactants from Marine Cyanobacteria Collected in Sabah, Malaysia

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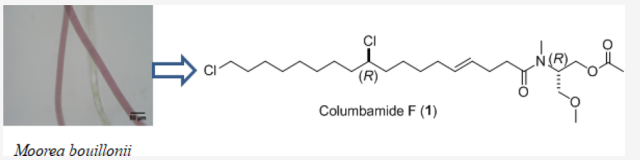
ABSTRACT: Chemical investigation of the organic extract from *Moorea bouillonii*, collected in Sabah, Malaysia, led to the isolation of three new chlorinated fatty acid amides, columbamides F (**1**), G (**2**), and H (**3**). The planar structures of **1**−**3** were established by a combination of mass spectrometric and NMR spectroscopic analyses. The absolute configuration of **1** was determined by Marfey's analysis of its hydrolysate and chiral-phase HPLC analysis after conversion and esterification with Ohrui's acid, (1*S*,2*S*)-2-(anthracene-2,3-dicarboximido)cyclohexanecarboxylic acid. Compound **1** showed biosurfactant activity by an oil displacement assay. Related known fatty acid amides columbamide D and serinolamide C exhibited biosurfactant activity with critical micelle concentrations of about 0.34 and 0.78 mM, respectively.



Biosurfactants are promising amphipathic compounds derived from microorganisms and can exhibit a diversity of bioactivities such as cytotoxic, antimicrobial, antiadhesive, and antibiofilm activities against human pathogens.[1] Due to their chemical diversity and surface activities, biosurfactants are widely used in different fields including food, agriculture, cosmetics, and petrochemical industries.[2−6] According to a 2017 market research report, the global market for biosurfactants was valued at USD 3.99 billion in 2016 and is expected to reach USD 5.52 billion by 2022.[7] A number of studies have reported biosurfactant properties of known natural product structures including the cytotoxic somocystinamide A and apratoxins,[1] 2-acyloxyethylphosphonate,[8] and exopolysaccharides[9] from cyanobacteria. The high structural diversity and bioactivities of cyanobacterial secondary metabolites present them as potential candidates for novel biosurfactants. As part of our continuing study of bioactive compounds from marine cyanobacteria collected in Sabah, Malaysia,[10] here we report the biosurfactant-assay-guided isolation and structure elucidations of the three new compounds columbamides F (**1**), G (**2**), and, H (**3**) from *Moorea bouillonii.* Marfey's analysis of the *N*,*O*-dimethylserinol moiety and Ohrui's method followed by chiral-phase HPLC were executed to determine the absolute configuration. The biosurfactant activities were measured by the oil displacement assay for **1** and by the ring method for the previously isolated compounds from cyanobacteria. Cytotoxicities of these compounds were evaluated using MCF-7 breast cancer cells.

RESULTS AND DISCUSSION

The mat-forming *M. bouillonii* sample was collected from Mantanani Island, Sabah, Malaysia, and the identification was done on the basis of its morphology[11] and phylogenetic studies based on 16S rRNA gene sequencing analysis (Supporting Information S1, S2, and S3). The cyanobacterial sample was extracted with MeOH, and solvent partitioning was done by using EtOAc, H_2O, and BuOH. The chemical profiling of the EtOAc fraction by LC-MS exhibited the presence of a number of known compounds including apratoxins A and C, wewakazole, lyngbyabellin A, laingolides, and additional possibly new chlorinated compounds. The EtOAc fraction was subjected to normal-phase silica gel column chromatography and divided into 12 fractions. The silica gel fractions of hexane/EtOAc (50:50 v/v), 100% EtOAc, and EtOAc/MeOH (90:10 v/v) exhibited a higher value for the diameter of the clear zone (∼140 mm) compared to other fractions in an oil displacement assay.[12] On the basis of their bioactivity and chemical profile these fractions were chosen for the isolation of new halogenated compounds. The compounds that eluted with a 50:50 (v/v) mixture of hexane/EtOAc were determined as columbamides F (**1**) and G (**2**) with di- and trichlorinated patterns, respectively (Figure S4). Another new compound, columbamide H (**3**), was purified from the 100% EtOAc fraction along with known compounds apratoxin A[13] and columbamide D,[10] confirmed by comparing their ^{1}H and ^{13}C NMR chemical shift data.

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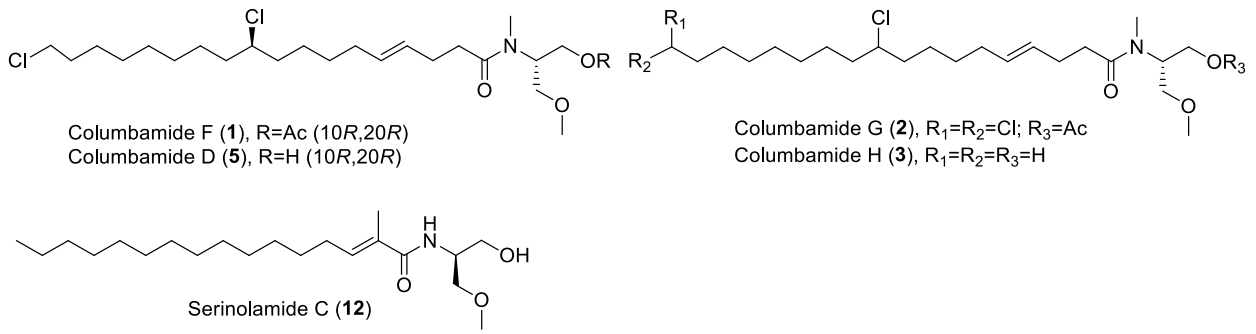
Chart 1



The planar structure of **1** was established by analyzing NMR and MS data (Figure 1 and Table 1). The molecular formula of

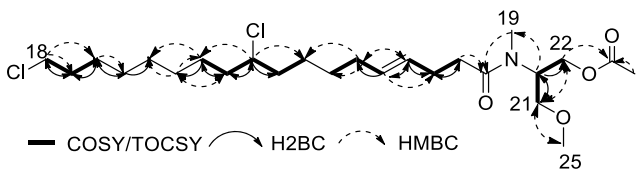


Figure 1. 2D NMR correlations for **1**.

1 was determined to be $C_{25}H_{45}Cl_2NO_4$ by ESITOFMS, suggesting three degrees of unsaturation. The presence of carbonyls (δ_C 173.3, 170.7), two olefinic methines (δ_C 129.1, 130.9) with identical protons (δ_H 5.46), a chlorinated methine (δ_C 64.2, δ_H 3.88), an *N*-methyl (δ_C 31.7, δ_H 2.95), an *N*-methine (δ_C 51.9, δ_H 4.90), a chlorinated methylene (δ_C 45.1, δ_H 3.53), a methoxy (δ_C 58.9, δ_H 3.34), two oxo-methylenes (δ_C 70.9, δ_H 3.57 and δ_C 62.0, δ_H 4.26), and other methylenes (δ_C 26.4−38.4, δ_H 1.31−2.42) revealed the similarities of this compound to the columbamides.[10,14] The mass difference between columbamide D and **1** is 42, indicating the possibility of the acetylation of columbamide D for **1**. Columbamide A has an acetyl function as well. An acetylated *N*,*O*-dimethylserinol moiety was elucidated by HMBC correlations from both the methyl singlet H_3-24 and the deshielded methylene protons H_2-22 to an acetoxy carbonyl at δ_C 170.7, H-20 to H-21 and H_2-22 by COSY, H-20 to C-19 and H_3-25 to C-21 by HMBC, and H-20 to C-21 and C-22 by H2BC. The connection between C-1 and H-19 was also made by HMBC correlations. COSY and H2BC correlations allowed the assignment from H-3 to H-4. The double bond between C-4 and C-5 was established by H2BC signals from H-4/5 to C-3 and C-6 and corresponding HMBC signal from H-5 to C-4. COSY signals from H-5 to H-6, H-6 to H-7, and H-8 to H-9 allowed the connections of these protons to the adjacent methylenes. The position of the chlorine atom at position C-10 was established by the following signals: COSY correlations from H-10 to H-9 and H-11, H2BC correlations from H-10 to C-9 and C-11, and HMBC correlations from H-10 to C-8, C-9, C-11, and C-12. The assignment of methylenes from C-11 to C-17 was confirmed by COSY, HMBC, and H2BC correlations. The position of the second chlorine at the terminal C-18 was supported by COSY signals from H-17 to H-18 and vice versa. The corresponding H2BC and HMBC signals completed the planar structure of columbamide F (**1**).

ESITOFMS analysis of **2** showed the $[M + H]^+$ ion at m/z 528.2451, corresponding with the molecular formula $C_{25}H_{44}Cl_3NO_4$, indicating three degrees of unsaturation. The mass difference between columbamide G (**2**) and columbamide F (**1**) was 34 Da, suggesting the presence of an additional chlorine. The position of the additional chlorine was confirmed by the triplet proton signal at δ_H 5.75 and associated carbon signal at δ_C 73.6 ppm, which indicated a terminal dichloromethine at C-18, similar to columbamide E.[10]

The molecular formula of columbamide H (**3**) was determined as $C_{23}H_{44}ClNO_3$ by ESITOFMS, indicating two degrees of unsaturation. The NMR signals for columbamide H (**3**) are similar to columbamide F (**1**) except for the replacement of the acetoxy group at C-22 by a hydroxy group (δ_H 3.02), resulting in the shielded shift of the adjacent methylene group to H-22 (δ_H 3.79) and the lack of a terminal chlorine at position C-18, confirmed by the triplet signal for the methyl group at H-18 (δ_H 0.88 and δ_C 14) (Table 1).

An *E* configuration of the double bond for compounds **1**−**3** was obtained by measuring the coupling constant ($^3J_{H,H}$ = 16−17 Hz) from the ^{13}C satellites observed by nondecoupled HSQC analysis[15] (Figure S28). The absolute configuration of the stereogenic center of the dimethylated and acetylated serinol moiety was determined by Marfey's analysis.[16] The two standards, (*R*)- and (*S*)-*N*,*O*-dimethylserinol (**4**), were synthesized[17] (Figure S29) and derivatized with Marfey's reagent. In a similar way derivatization was done for the hydrolysates of compounds **1**−**3**. The *R*-configurations for *N*,*O*-dimethylserinol residues were established (Figure S30) by comparing the retention times.

In our previous study, the absolute configuration of the chloromethine of columbamide D was determined by total synthesis of all four isomers followed by chiral-phase HPLC analysis. For this study we examined the potential of a chiral labeling reagent for determining the configuration of the chloromethine on the long alkyl chain of columbamide F (**1**). The absolute configuration of the chloromethine at C-10 was determined by chemical conversion of columbamide D (**5**) standards and natural compound **1** followed by esterification with Ohrui's acid (**6**).[18] The use of the highly potent fluorescent chiral labeling reagent (*R*,*R*)- and (*S*,*S*)-2-(anthracene-2,3-dicaroximido)cyclohexanecarboxylic acid (Figure 2) has been well reported for the discrimination of compounds having remote stereocenters.[19] Because our team previously synthesized columbamide D stereoisomers,[10] (10*S*,20*R*)- and (10*R*,20*R*)-(**5**) were used as standards in the Ohrui reaction for columbamide F (**1**). Synthesized diastereomers of columbamide D and natural **1** were converted to diol **7** by dihydroxylation and then cleaved oxidatively with sodium periodate ($NaIO_4$) to form two aldehydes (**8**, **9**). After separation, **8** was reduced with $NaBH_4$ to furnish a linear

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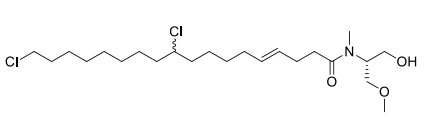
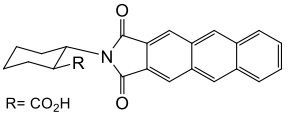
Table 1. NMR Spectroscopic Data for Columbamides F (1), G (2), and H (3) in $CDCl_3$

	columbamide F (1)				columbamide G (2)		columbamide H (3)	
position	δ_C,[a] type	δ_H (J in Hz)[b]	COSY/ TOCSY[b]	HMBC/ H2BC[b]	δ_C,[c] type	δ_H (J in Hz)[b]	δ_C,[c] type	δ_H (J in Hz)[b]
1	173.3, C				173.2, C		174.3, C	
2	34.02, CH_2	2.38, m	3	1, 3′	34.02, CH_2	2.38, m	33.9, CH_2	2.40, m
3	28.0, CH_2	2.33, m	4	2′, 4′	28.0, CH_2	2.33, m	28.0, CH_2	2.34, m
4	129.1, CH	5.46, m	3, 3′	3′, 5′, 6	129.1, CH	5.45, m	128.9, CH	5.46, m
5	130.9, CH	5.46, m	6	3, 4, 4′, 6′	130.9, CH	5.45, m	131.1, CH	5.46, m
6	32.6, CH_2	2.00, m	5, 7	4, 5, 7, 8	32.3, CH_2	2.00, m	32.3, CH_2	2.0, m
7	28.8, CH_2	1.31, m[d]	6		28.9, CH_2	1.33, m	28.04, CH_2	1.32, m
8	25.9, CH_2	1.51, 1.39, m	9		25.8, CH_2	1.39, m	25.9, CH_2	1.38, m
9	38.5, CH_2	1.69, m	8, 10′		38.5, CH_2	1.70, m	38.3, CH_2	1.70, m
10	64.2, CH	3.88, m	9, 9′, 11	8, 9, 9′, 11′, 11, 12	64.2, CH	3.88, m	64.3, CH	3.88, m
11	38.4, CH_2	1.69, m	10, 12	10, 10′, 12′, 12, 13	38.5, CH_2	1.70, m	38.3, CH_2	1.70, m
12	26.4, CH_2	1.51, 1.39, m	11	11, 11′, 13′	26.4, CH_2	1.54, m 1.38, m	26.5, CH_2	1.51, m
								1.38, m
13	28.8, CH_2	1.31, m[d]		11, 12, 14	28.4, CH_2	1.32, m	28.0, CH_2	1.28, m
14	29.0, CH_2	1.31, m[d]			29.2, CH_2	1.29, m	29.2, CH_2	1.29, m
15	28.7, CH_2	1.31, m[d]		14′, 16′	28.7, CH_2	1.32, m	28.0, CH_2	1.29, m
16	26.8, CH_2	1.40, m	17	15, 17, 15′, 17′	26.8, CH_2	1.42, m	26.5, CH_2	1.38, m
17	32.5, CH_2	1.76, m	16, 18, 18′	16, 16′, 18, 18′	43.6, CH_2	2.2, m	22.6, CH_2	1.27, m
18	45.1, CH_2	3.53, t (6.8)	17	16, 17, 17′	73.6, CH	5.75, t (6.1)	14.0, CH_3	0.88, t (6.8)
19	31.7 (27.3), CH_3	2.95, s (2.82, s)		1	31.5 (27.2), CH_3	2.95, s (2.82, s)	31.8, CH_3	3.02, s (2.84, s)
20	51.9, CH	4.90, m	21, 22	19, 21′, 22′	51.9, CH	4.90, m	57.9, CH	4.39, m
21	70.9, CH_2	3.57, dd (10.3, 6.8) 3.48, dd (10.3, 5.5)	20	20′, 20, 22, 25	70.9, CH_2	3.59, dd (10.3, 6.8) 3.48, dd (9.6, 4.8)	71.0, CH_2	3.57, dd (10.3, 4.8)
22	62.0, CH_2	4.26, dd (11.0, 7.5) 4.20, dd (12.3, 4.8)	20	20, 21, 23	62.0, CH_2	4.26, dd (11.6, 7.5) 4.20, dd	62.3, CH_2	3.79, m
22-OH								3.02, bs
23	170.7, C				170.7, C		58.9, CH_3	3.34, s
24	20.8, CH_3	2.04, s (2.05, s)		23	20.8 CH_3	2.04, s (2.05, s)		
25	58.9, CH_3	3.32, s 3.34, s		21	59.0, CH_3	3.32, s		
						3.34, s		

[a]Measured at 150 MHz. [b]Measured at 600 MHz. Values in parentheses are from TOCSY and H2BC experiments. [c]Derived from HSQC, HMBC, and H2BC data. [d]Overlapping signals.



Synthetic Columbamide D standard **-5** (10*S*,20*R*) and (10*R*,20*R*) diastereomers



(1*S*,2*S*)-2-(anthracene-2,3-dicarboximido) cyclohexanecarboxylic acid (Ohrui's acid)-(**6**)

Figure 2. Synthetic standards and Ohrui's acid for determining the configuration at a remote stereocenter.

alcohol (**10**), which was esterified with (*S*,*S*)-2-(anthracene-2,3-dicarboximido)cyclohexanecarboxylic acid (**6**) in the presence of EDCI and DMAP to afford **11**. Natural columbamide F (**1**) was converted and esterified with **6** in a similar way (Figure 3). Chiral-phase HPLC analysis of synthetic 10*S* and 10*R* standards revealed that a co-injection experiment can separate both standards. Then by a coelution experiment, natural **1** gave a single peak with the synthetic 10*R* isomer, while two peaks were observed with the synthetic 10*S* isomer, confirming the *R*-configuration for the chloromethine of **1** (Figure S32). Although in Ohrui's original method, the HPLC separation of the diastereomers was done by using a C18 or C30 column and fluorescence detection using different temperatures ranging from 40 to −50 °C,[19−21] here we report the significance of chiral-phase HPLC for the separation of diastereomers using a UV detector at room temperature. Ohrui's acid was used to determine the absolute chloromethine using a small amount of sample, and this can be a powerful approach for determining the absolute configuration of natural products having a remote stereogenic center.

Fatty acid amides columbamides A and B[14] isolated from cultured *M. bouillonii* showed cannabinomimetic activity

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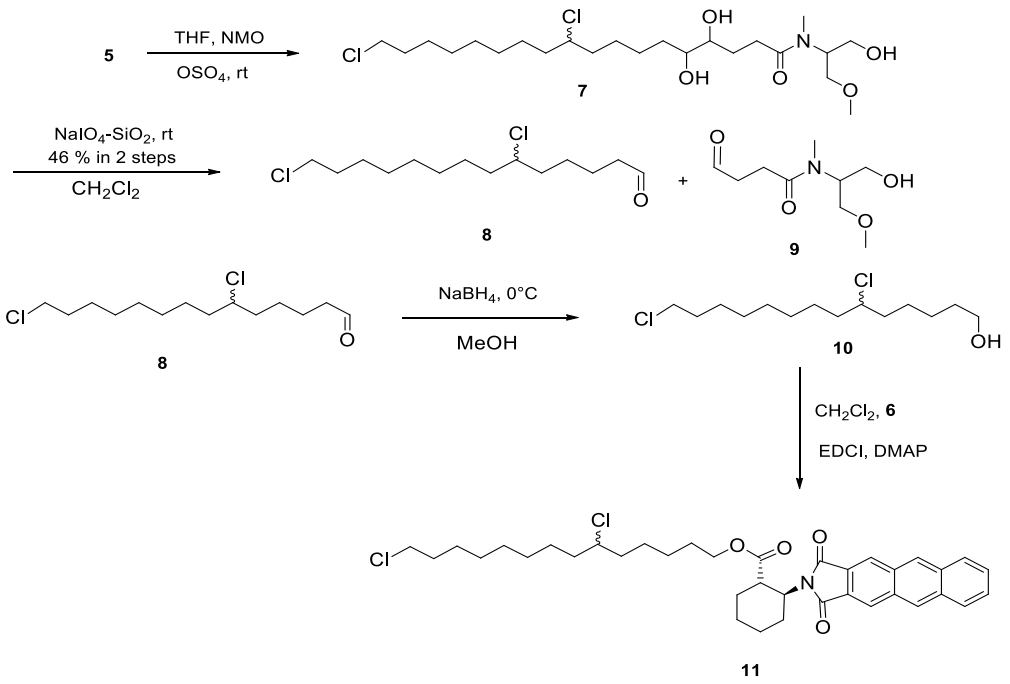

Figure 3. Chemical conversion to apply Ohrui's method for determining the configuration of the chloromethine of columbamide F (**1**).

similar to other fatty acid amides.[22,23] Here we report the surface tension lowering activity of fatty acid amides columbamide D (**5**) and serinolamide C (**12**)[24] (isolated by our group). Because of the amphiphilic properties, these compounds reduced the surface tension of aqueous solutions at concentrations of 10−1200 μg/mL, as determined by the ring method. The critical micelle concentrations (CMCs) of **5** and **12** were 0.34 and 0.78 mM, respectively, which are lower than the CMC value of the synthetic surfactant SDS (8.2 mM)[25] and comparable to pluronic F-68 (0.07−0.35 mM)[26] and triton X-100 (0.33 mM).[27] Serinolamide C (**12**) isolated from *Okeania* sp. showed potent antifouling activity (0.88 μg/mL) against *Amphibalanus amphitrite* larvae. The biosurfactant properties of serinolamide C could reduce the surface tension of seawater in the microplate well, and therefore the larvae cannot adhere easily. Because of the small amount of sample, the biosurfactant activity of columbamide F (**1**) was checked by an oil displacement assay, which is an indirect measurement of surface activity. The diameter of the clear zone was measured as ∼90 mm for a concentration of 10 mg/mL, which is a slightly higher displacement area compared to SDS (∼84 mm) and pluronic F-68 (∼54 mm) for the same concentration. In addition columbamides F−H (**1**−**3**) were tested for the cytotoxicity against the MCF-7 breast cancer cell line but were inactive (IC_{50} > 10 μg/mL).

In summary, the new columbamides F (**1**), G (**2**), and H (**3**) were isolated from *M. bouillonii* collected in Sabah, Malaysia. In combination with NMR and MS data the planar structures of these three new columbamides were established. The absolute configuration of columbamide F (**1**) has been determined to be (10*R*,20*R*) by Marfey's analysis and chiral-phase HPLC analysis of synthetic standards and columbamide F (**1**) after derivatization with the Ohrui's acid. Ohrui's method combined with chiral-phase HPLC analysis at room temperature may increase its application to determine the absolute configuration of natural compounds having a remote stereogenic center. Because of having surface-tension-lowering properties with a low CMC value and weak cytotoxicity, the fatty acid amides columbamide D (**5**) and serinolamide C (**12**) may have potential for applications in various fields such as healthcare, food, and cosmetics. The current work reports the discovery of new potential biosurfactants and has uncovered greater diversity in the columbamide family of compounds.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation data were measured on a Horiba SEPA-300 polarimeter using chloroform as a solvent. IR spectra were measured on a JASCO FT/IR-4100 type A. The ^{1}H NMR, ^{13}C NMR, and 2D NMR spectra for compounds **1**, **2**, and **3** were acquired using a JEOL 600 MHz spectrometer. For all NMR experiments, $CDCl_3$ (Cambridge Isotope Laboratories, Inc.) was used as solvent as an internal standard (δ_H 7.26 and δ_C 77.0). LC-MS data were acquired on an Agilent 1100 series HPLC system coupled with a Bruker Daltonics microTOF-HS mass spectrometer. The HPLC system was equipped with a Cadenza CD-C18 column (2 × 150 mm, 3 μm, flow rate 0.2 mL/min at 25 °C) and operated under the following conditions: 0−15 min 50−80% MeCN with 0.1% (v/v) formic acid (FA) in Milli-Q H_2O; 15−30 min, isocratic 80% MeCN with 0.1% (v/v) FA. Preparative HPLC systems used a Cosmosil Cholester column (10 × 250 mm, 5 μm) at a flow rate of 3 mL/min, with UV detection at 210 nm.

Biological Material and Gene Sequencing. The cyanobacterial sample (M1705) was collected from Rocky Point, Mantanani Island, Sabah, Malaysia (06°42′16″ N; 116°19′23″ E) in November 2017 by using scuba diving at a depth of 5−10 m. The sample was cleaned by removing foreign particles, and the seawater was squeezed out by hand before storing in MeOH for transportation. Small pieces of the collected cyanobacterial sample were preserved in 8 mL of RNA*later* solution for genetic analysis. The sample was identified by 16S rRNA gene sequence analysis (detailed procedure reported in Supporting Information S2) as *Moorea bouillonii* (accession no. MN759382).

Extraction and Isolation. The *M. bouillonii* cyanobacterium sample was homogenized at 10 000 rpm for 10 min with MeOH and extracted three times. The extract was filtered and concentrated *in vacuo*. The residue of the MeOH extract was partitioned between EtOAc and H_2O and then BuOH. These fractions were dried and subjected to an oil displacement assay for biosurfactant activity and a

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cytotoxicity assay against the MCF7 breast cancer cell line. The chemical profiling of EtOAc and BuOH fractions revealed the presence of known cytotoxic compounds including apratoxins, wewakazole, and possible new halogenated compounds according to the MarinLit database. The EtOAc extract (600 mg) was then applied onto a silica gel (Merck) column with the size 0.063−0.200 mm and eluted with a stepwise gradient starting with 100% *n*-hexane to 100% EtOAc (98:2, 95:5, 90:10, 80:20, 50:50 v/v) followed by 100% EtOAc to 100% MeOH (95:5, 90:10, 80:20, 50:50) to yield 12 major fractions (F1 to F12). The solvents were removed *in vacuo* using a rotary evaporator, and the sample was moved to a clean small vial to be weighed. The 50:50 (v/v) hexane/EtOAc fraction was then subjected to semipreparative reversed-phase HPLC (gradient 0−30 min 50−80% MeCN; 30−40 min 80−100% MeCN, Cosmosil cholester, 10 × 250 mm, 3 mL/min and UV detection at 210 nm) using HPLC-grade MeCN (Wako) and Milli-Q H_2O to obtain columbamides F (**1**) (5 mg, t_R = 43.3 min) and G (**2**) (2 mg, t_R = 44.7 min). The silica gel fraction that eluted with 100% EtOAc was subjected to RP-HPLC with similar conditions to obtain columbamide H (**3**) (3.5 mg, t_R = 39.1 min). Then compounds **1** and **3** were further purified using RP-HPLC (gradient 0−30 min, 80−100% MeCN, Cosmosil cholester, 4.6 × 250 mm, 1 mL/min and UV detection at 210 nm) to yield **1** (3.2 mg, t_R = 13.9 min) and **3** (2 mg, t_R= 12 min).

Columbamide F (1): colorless oil; $[\alpha]^{22}_D$ +6.2 (*c* 0.29, $CHCl_3$); IR (neat) λ_{max} 2929, 2856, 1743, 1648, 1456, 1109, 1042 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m*/*z* 494.2816 $[M + H]^+$ (calcd for $C_{25}H_{46}Cl_2NO_4$, 494.2804).

Columbamide G (2): colorless oil; $[\alpha]^{23}_D$ +9 (*c* 0.07, $CHCl_3$); IR (neat) λ_{max} 2923, 2853, 1741, 1651, 1456, 1106 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m*/*z* 528.2451 $[M + H]^+$ (calcd for $C_{25}H_{45}Cl_3NO_4$, 528.2414).

Columbamide H (3): colorless oil; $[\alpha]^{23}_D$ +23.1 (*c* 0.06, $CHCl_3$); IR (neat) λ_{max} 3394, 2925, 2855, 1624, 1456, 1121 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HRESIMS *m*/*z* 418.3042 $[M + H]^+$ (calcd for $C_{23}H_{45}ClNO_3$, 418.3087).

Acid Hydrolysis and Marfey's Analysis. The absolute configurations of the *N*,*O*-dimethylserinol moiety were determined by comparisons with synthetic *N*,*O*-dimethylserinol standards prepared according to the method of Gao et al.[17] Columbamides F (**1**) and G (**2**) (0.2 mg) were hydrolyzed using 12 M HCl at 120 °C for 24 h to cleave both the amide and acetate groups. Columbamide H (**3**) (0.2 mg) was hydrolyzed using 6 M HCl at 120 °C for 16 h. The reaction was quenched with $NaHCO_3$ and evaporated to dryness. The hydrolysates from the columbamides and the synthesized *N*,*O*-dimethyserinol were subjected to Marfey's analysis. The hydrolysates and standards were first dissolved in 100 μL of H_2O, and then 200 μL of FDAA (5-fluoro-2,4-dinitrophenyl-L-alaninamide) solution (in 1% acetone) was added, followed by addition of 40 μL of 1 M $NaHCO_3$. The solutions were heated in an oven at 40 °C for 1 h, and then the reaction was quenched with 20 μL of 2 M HCl. A 200 μL amount of MeCN was added, and this mixture was directly analyzed by LC-MS (gradient 0−80 min 10−50% MeCN with 0.1% FA in Milli-Q H_2O, Cosmosil 5C18-AR II column, 4.6 × 250 mm, flow rate 0.2 mL/min at 25 °C, UV detection at 340 nm). The retention times for synthetic (*R*)- and (*S*)-*N*,*O*-dimethylserinol (**4**) derivatives were 82.4 and 85.1 min, respectively, whereas the retention times for derivatized hydrolysates from columbamide F−H (**1**−**3**) were 82.4, 83.0, and 83.1 min, respectively.

Ohrui's Reaction and Chiral-Phase HPLC Analysis. To a solution of synthetic **5**-(10*R*,20*R*) (4.9 mg, 0.0108 mmol) in THF (0.30 mL) were added NMO (50.0% in H_2O, 3.3 μL, 0.0162 mmol) and OsO_4 (0.020 M in H_2O, 108 μL, 0.00216 mmol) at room temperature. The mixture was stirred for 20 h, quenched with saturated aqueous Na_2SO_3, and extracted with EtOAc. The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and concentrated under reduced pressure. To a solution of crude diol **7** in CH_2Cl_2 (0.50 mL) was added $NaIO_4/SiO_2$ (50.0 mg), at rt.[28] The mixture was stirred for 30 min, then filtered through a pad of Celite (elution with EtOAc), and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc/hexane = 2.5:97.5) to give aldehyde **8** (1.40 mg, 0.00499 mmol, 46% in two steps) as a colorless oil. To a solution of aldehyde **8** (1.40 mg, 0.00499 mmol) in MeOH (0.10 mL) was added $NaBH_4$ (0.50 mg, 0.0132 mmol) at 0 °C. The mixture was stirred for 30 min, quenched with saturated $NaHCO_3$, extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude alcohol **10** was used for the next step. To a solution of the crude alcohol **10** in CH_2Cl_2 (0.30 mL) were added (*S*,*S*)-Orui's acid (3.7 mg, 0.0114 mmol), EDCI (2.80 mg, 0.0146 mmol), and catalytic DMAP at rt under an Ar atmosphere. The mixture was stirred for 18 h, quenched with saturated NH_4Cl, extracted with EtOAc, washed with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc/hexane = 20:80) to give **11** (4.4 mg), which was used for HPLC analysis (HPLC conditions: 90:10 hexane/isopropanol, Chiralcel OJ-H, 4.6 × 250 mm, 1 mL/min, UV 300 nm). The synthetic **5**-(10*S*,20*R*) (4.8 mg, 0.0106 mmol) and natural columbamide F (**1**) (3 mg, 0.006 mmol) were degraded and derivatized in a similar method to that described previously to obtain **6**-ester of *S*-**10** (4.0 mg) and **6**-ester of columbamide F (**1**)−**10** (2.0 mg), respectively. Using a chiral-phase HPLC system, the co-injection experiments of the synthetic standards (**11**) were separated into two peaks at 22.2 and 24.9 min. Again in a coelution experiment of natural columbamide F (**1**) with synthetic standards, 10*R* gave a single peak at 25.2 min while 10*S* was separated from natural **1** and gave two peaks at 22.5 and 25.1 min.

Oil Displacement Assay. The oil displacement test was performed by determining the diameter of the oil displacement area.[12] For this assay, 10 μL of crude oil was added to the surface of 40 mL of distilled H_2O in a 15 cm glass Petri dish to form a thin oil layer. The crude oil used in this experiment was manufactured by Tokyo Chemical Industry Co., LTD (S0432). Extracted samples were dissolved in H_2O for hydrophilic fractions and EtOH for lipophilic fractions to make a final concentration of 10 mg/mL. Then, 10 μL of extracted samples was gently placed on the center of the oil layer. If biosurfactant was present in the sample, the oil was displaced and a clearing zone was formed. The clearing zone was observed by light, and the diameter of this zone was measured with a vernier caliper. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity.

Surface Tension Measurement. The surface tension of sample solutions was determined by the ring method using a Du Noüy tensiometer (ITOH, No. 4334) equipped with a platinum ring at 25 °C, and measurements were done in triplicate. Calibration of the instrument was done by measuring the surface tension of the pure H_2O before each set of experiments. The CMCs for pure compounds were determined from the breakpoint of the surface tension versus the log of the bulk concentration curve.[29]

Cytotoxicity Assay. The cytotoxicities of all compounds were determined by the method previously reported by Lopez et al. (2016).[30]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00164.

Details of 16S rRNA gene sequencing and phylogenetic analysis; 1D and 2D NMR and LC-MS spectra of columbamides F−H (**1**−**3**), synthesis of *N*,*O*-dimethylserinol standard, as well as chiral-phase HPLC chromatograms (PDF)

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Notes

The authors declare no competing financial interest.

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