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Kanamienamide, an Enamide with an Enol Ether from the Marine Cyanobacterium *Moorea bouillonii*

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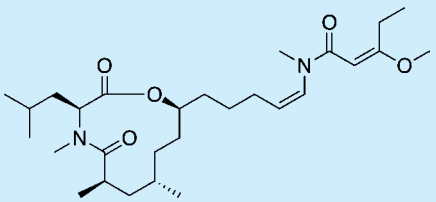
Supporting Information

ABSTRACT: Kanamienamide, an enamide with an enol ether, was isolated from the marine cyanobacterium *Moorea bouillonii*. The gross structure was established by spectroscopic analyses, and the relative stereochemistry was elucidated on the basis of the analyses of NOESY correlations and $^1H-^1H$ coupling constants. The absolute configuration was determined on the basis of the chiral HPLC analysis of the *N*-Me-Leu derived from kanamienamide. This is the first report of a natural product that possesses an *N*-Me-enamide adjacent to an enol ether. Kanamienamide showed growth-inhibitory activity toward HeLa cells with an IC_{50} value of 2.5 μM and induced apoptosis-like cell death.

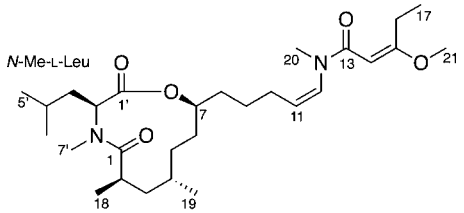


kanamienamide (**1**)

Microorganisms produce various lipopeptides that possess a wide range of bioactivities. Many of these bioactive compounds are produced by nonribosomal peptide synthetase/polyketide synthase (NRPS/PKS) hybrid pathways. Recently, a comparative genomic study revealed that a large number of NRPS/PKS are widespread in late-branching cyanobacterial lineages (e.g., *Anabaena*, *Nostoc*, *Microcystis*, and *Lyngbya*) and suggested that cyanobacterial NRPS/PKS clusters contained specific tailoring enzymes.[1] Previous reports have shown that tailoring enzymes are critical for structural diversification in natural products.[2,3] Moreover, to support drug discovery, large libraries have been generated by the insertion and inactivation of tailoring enzymes through combinatorial biosynthesis.[4] Therefore, the discovery of compounds with interesting structures and novel tailoring enzymes is important for drug development by combinatorial biosynthetic approaches.

Here, we report the isolation, structure elucidation, and preliminary biological characterization of kanamienamide (**1**), a novel enamide containing an *N*-Me-enamide group adjacent to an enol ether moiety. To date, *N*-Me-enamide- or enol ether-containing natural products, such as laingolide,[5] palmyrolide A,[6] madangolide,[7] malyngamide A,[8] barbamide,[9] jamaicamide A,[10] and lyngbyapeptins B and C,[11] have been isolated from marine cyanobacteria. Kanamienamide (**1**) is the first discovered compound that possesses an *N*-Me-enamide adjacent to an enol ether.

The marine cyanobacterium *Moorea bouillonii* (1000 g, wet weight) was collected at the shore of Kanami, Kagoshima. The collected cyanobacterium was extracted with MeOH. The extract was filtered, concentrated, and partitioned between EtOAc and H_2O. The EtOAc layer was further partitioned between 90% aqueous MeOH and hexane. The 90% aqueous MeOH layer was subjected to fractionation guided by growth-inhibitory activity against HeLa cells. The fractionation was performed by reversed-phase open column chromatography (ODS silica gel, MeOH−H_2O) and reversed-phase HPLC (Cosmosil 5C_{18} AR-II; MeOH−H_2O) to give kanamienamide (**1**) (9.2 mg) as a colorless oil.



kanamienamide (**1**)

The molecular formula of kanamienamide (**1**) was determined to be $C_{28}H_{48}N_2O_5$ by HRESIMS (*m*/*z* 493.3622, calcd for $C_{28}H_{49}N_2O_5$ $[M + H]^+$ 493.3641). The NMR data for **1** are summarized in Table 1. The ^1H NMR spectrum of **1** showed the presence of eight methyl groups: five *C*-methyl (triplet at δ_H 1.32, doublets at δ_H 0.74, 0.76, 0.91, 1.04), two *N*-methyl (singlets at δ_H 2.81 and 3.02), and one *O*-methyl (singlet at δ_H 3.14). The ^1H NMR spectrum also indicated the presence of an α-methine of amino acid (δ_H 4.40) and olefinic protons (δ_H 5.92, 5.21, 4.88). Partial structures of **1** were determined using a series of 1D and 2D NMR experiments. COSY correlations revealed the connectivity of C2′ to C6′: H-2′ (δ_H 4.40)−H-3a′ and b′ (δ_H 1.90 and 1.67)−H-4′ (δ_H 1.36)−H-5′ and 6′ (δ_H 0.74 and 0.76) (Figure 1). Furthermore, the HMBC spectrum showed two correlations: H-7′ (δ_H 2.81)/C2′ (δ_C 58.4) and H-2′ (δ_H 4.40)/C1′ (δ_C 172.7). On the basis

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Table 1. ^{1}H and ^{13}C NMR Data for 1 in C_6D_6

position	δ_C [a]	δ_H [b] (J in Hz)
1′	172.7, C	
2′	58.4, CH	4.40, dd (4.2, 11.6)
3a′	38.5, CH_2	1.90, ddd (4.2, 10.9, 14.8)
3b′		1.67, ddd (3.6, 11.6, 14.8)
4′	24.7, CH	1.36, m
5′	20.8, CH_3	0.74, d (6.9)
6′	23.3, CH_3	0.76, d (7.1)
7′	28.7, CH_3	2.81, s
1	177.8, C	
2	33.7, CH	2.60, dqd (3.1, 6.8, 11.6)
3a	43.6, CH_2	2.03, ddd (1.9, 11.6, 13.2)
3b		0.94, ddd (3.1, 11.1, 13.2)
4	34.6, CH	1.36, m
5a	30.8, CH_2	0.99, dddd (1.4, 2.1, 9.0, 14.5)
5b		0.80, dddd (1.0, 9.5, 10.4, 14.5)
6a	31.8, CH_2	1.49, dddd (1.4, 5.2, 9.5, 14.6)
6b		1.13, dddd (1.0, 9.0, 10.8, 14.6)
7	76.8, CH	4.96, dddd (5.2, 6.9, 10.8, 12.0)
8a	35.4, CH_2	1.24, m
8b		1.39, m
9	25.0, CH_2	1.20, m
10	27.1, CH_2	1.87, m
11	125.9, CH	4.88, td (7.5, 7.5)
12	131.0, CH	5.92, br
13	166.6, C	
14	91.4, CH	5.21, s
15	174.7, C	
16	25.9, CH_2	3.12, q (7.6)
17	12.6, CH_3	1.32, t (7.6)
18	19.1, CH_3	1.04, d (6.8)
19	22.6, CH_3	0.91, d (6.6)
20	34.9, CH_3	3.02, s
21	54.5, CH_3	3.14, s

[a]Measured at 100 MHz. [b]Measured at 400 MHz.

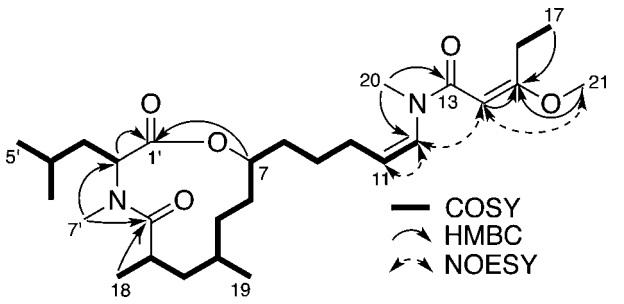

Figure 1. Gross structure of kanamienamide (**1**) based on 2D NMR correlations.

of these data, the presence of an *N*-methylleucine residue was clarified. Additionally, the other *N*-methyl protons (H-20, δ_H 3.02) were correlated with an amide carbonyl (C13, δ_C 166.6) and an olefinic carbon (C12, δ_C 131.0) on the HMBC spectrum. These data indicated the presence of an *N*-methylenamide moiety. COSY correlations revealed that a methyl group (δ_H 1.32) was located adjacent to a methylene (δ_H 3.12). Moreover, the following correlations were observed in the HMBC spectrum: H-17 (δ_H 1.32)/C15 (δ_C 174.7), H-21 (δ_H 3.14)/C15 (δ_C 174.7), and H-14 (δ_H 5.21)/C15 (δ_C 174.7). These data indicated the presence of a methyl enol ether moiety, and this was also supported by comparison of the NMR data of **1** with those of known enol ether-containing compounds.[9−11] Furthermore, detailed analyses of the ^{1}H NMR, ^{13}C NMR, COSY, HMQC and HMBC spectra revealed that **1** is composed of three partial structures as follows: *N*-methylleucine, 7-hydroxy-2,4-dimethyl-12-(methylamino)-dodec-11-enoic acid, and 3-methoxy-2-pentenoic acid. The geometries of the two olefins, C11−C12 and C14−C15, were determined to be *Z* and *E*, respectively, based on the two NOESY correlations: H-11/H-12 and H-14/H-21. The coupling constant of H11−H12 (7.5 Hz) was smaller than the typical value of *Z* olefinic protons. However, a similar value has been reported for madangolide,[7] which contains a *Z* enamide moiety.

The sequences of these partial structures were determined on the basis of HMBC and NOESY data (Figure 1 and Table S1). Three HMBC correlations (H-7′/C1, H-18/C1, and H-7/C1′) revealed an 11-membered ring structure. Furthermore, two HMBC correlations (H-20/C-12 and H-20/C-13) and one NOESY correlation (H-12/H-14) allowed us to elucidate the gross structure of **1** as shown in Figure 1.

The relative stereochemistry of **1** was elucidated based on the analyses of NOESY and coupling constants as shown in Figure 2. $^{1}H-^{1}H$ coupling constants were determined based on the

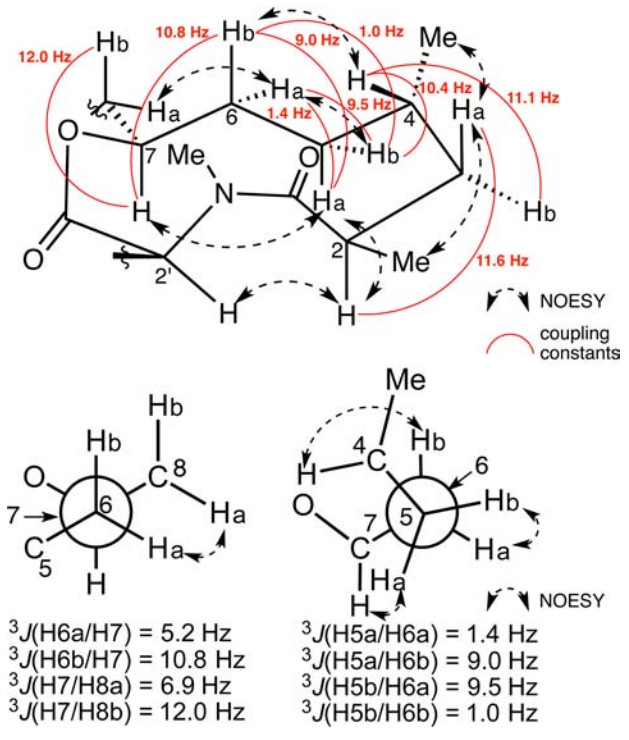

Figure 2. Configuration and conformation for **1** established on the basis of selected $^{3}J_{H,H}$ and NOESY spectra.

analyses of ^{1}H NMR, *J*-resolved, and E.COSY spectra (Figure 2, Table S1). NOESY correlations (H-2′/H-2, H-2/H-5a, and H-5a/H-7) were observed, and these data indicated that the four protons (H-2′, H-2, H-5a, and H-7) were oriented in the same face of the molecule. The large coupling constants (J_{H6b-H7} = 10.8 Hz and J_{H7-H8b} = 12.0 Hz) and NOESY correlation (H-6a/H-8a) indicated that both H-6a/O-7 and H-6b/H-7 were located in antirelationships (Figure 2). The orientation between H-5b and H-6a was determined to be synperiplanar based on the coupling constants ($J_{H5a-H6a}$ = 1.4 Hz, $J_{H5a-H6b}$ = 9.0 Hz,

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$J_{H5b-H6a}$ = 9.5 Hz, $J_{H5b-H6b}$ = 1.0 Hz) and NOESY correlations (H-4/H-6b and H-5a/H-7) (Figure 2). This conformation was supported by strong NOESY correlation between H-5b and H-6a. The large coupling constants (J_{H2-H3a} = 11.6 Hz, J_{H3b-H4} = 11.1 Hz, J_{H4-H5b} = 10.4 Hz) and NOESY correlations (H-2/H-5a, H-3a/H-18, and H-3a/H-19) indicated that H-5a/Me-4, H-5b/H-4, and H-4/H-3b were in anti orientations. These data revealed that H-2, H-3a, H-5a, H-6b, and H-7 were in the axial positions as shown in Figure 2. Furthermore, on the basis of the analyses described above, we established the relative stereochemistry of **1** to be 2′*S**,2*R**,4*S**,7*S** (Figure 2).

The absolute configuration of the *N*-Me-Leu moiety was determined by the chiral-phase HPLC analysis. Kanamienamide (**1**) was treated with 6 M HCl at 60 °C for 3 h. The *N*-Me-Leu was isolated from the acid hydrolysate, and its retention time in the chiral-phase HPLC was compared with those of D and L amino acid standards. As a result, the retention time of *N*-Me-Leu in **1** matched that of the *N*-Me-L-Leu standard (see the SI). Therefore, the absolute stereochemistry of C-2′ in kanamienamide was determined to be *S*, and the absolute configuration of kanamienamide was established to be 2′*S*,2*R*,4*S*,7*S*, as shown in **1**.[12]

To evaluate the biological activity of **1**, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was conducted with HeLa human cervical cancer cells. The cells were treated with various concentrations (0.1−20 μM) of **1** in a 96-well plate. After 72 h of incubation, kanamienamide (**1**) inhibited the growth of HeLa cells with an IC_{50} value of 2.5 μM. A trypan blue dye exclusion assay revealed that **1** induced cell death in HeLa cells (Figure 3A), and the cell death was

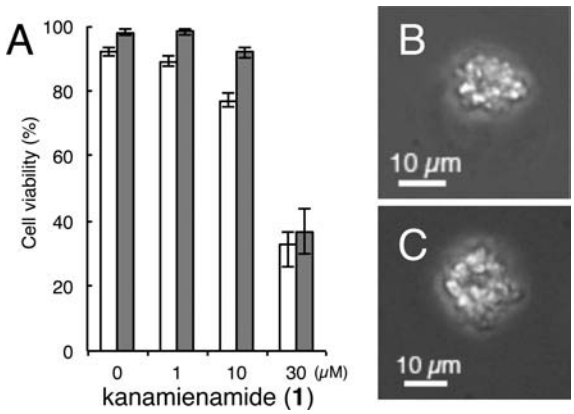

Figure 3. Induction of apoptosis-like cell death by kanamienamide (**1**) in HeLa cells: (A) HeLa cells were preincubated in the presence (gray column) or absence (white column) of 50 μM of Z-VAD-FMK and then treated with the indicated concentrations of **1**. After further incubation for 48 h, cell viability was determined on the basis of trypan blue dye exclusion. Values are the mean ± SD of quadruplicate determinations. (B) Light microscopy of apoptosis-like cell death induced by 30 μM of **1**. (C) Light microscopy of apoptosis-like cell death induced by 30 μM of **1** in the presence of Z-VAD-FMK.

morphologically similar to apoptosis (Figure 3B), severe shrinkage, rounding, and blebbing of highly refringent cells.[13] Therefore, we investigated whether the cell death was apoptosis, and this was confirmed by an evaluation of this suppression in the presence of Z-VAD-FMK, an inhibitor of caspases. As a result, cell death was slightly suppressed by Z-VAD-FMK at a low concentration of **1** (<10 μM), but not at a high concentration of **1** (30 μM) (Figure 3A). Moreover, apoptosis-like cell death was also observed even in the presence of Z-VAD-FMK (Figure 3C). These results show that **1** may have at least two target molecules for inducing apoptosis-like cell death, upstream of caspases and downstream of caspases and/or other pathways. However, further studies will be needed to elucidate the biological activities.

To date, some methyl enol ether containing compounds have been isolated from marine cyanobacteria, such as barbamide[9] and jamaicamide A.[10] The biosynthetic pathways that have been proposed to explain the formation of methyl enol ether have the same mechanism,[10,14] i.e., methylation of the enol form of the β-keto intermediate. Furthermore, the biosynthetic gene clusters for barbamide and jamaicamide A were identified in *Moorea producens*,[10,14] and kanamienamide (**1**) was isolated from a cyanobacterium in the same genus, *M. bouillonii*. Therefore, biosynthesis of the methyl enol ether moiety in **1** may involve enol formation catalyzed by a similar methyltransferase. Biosynthetic pathways for *N*-Me enamide moieties have been reported for peptides and peptidyl nucleosides, such as microcystin[15] and pacidamycin.[16] In the biosynthetic pathway for microcystin, *N*-Me-enamide is thought to be produced by dehydration of a seryl side chain.[15] In the biosynthetic pathway for pacidamycin, the enamide moiety is produced by the oxidation of uridine followed by dehydration, transamination, and condensation with peptide.[16] However, the microcystin and pacidamycin pathways for the formation of enamide are inconsistent with the structure of **1**. Furthermore, while a few enamide-containing polyketides have been isolated from cyanobacteria,[5−7,17] the biosynthetic pathways have not yet been reported. Interestingly, relatively many enamide-containing polyketides have been isolated from the marine cyanobacterium *M. bouillonii*.[5,7,17] This suggests that *M. bouillonii* is suitable for clarifying the biosynthetic pathway of the *N*-Me-enamide structure in polyketides. To clarify the biosynthetic pathway for formation of the *N*-Me-enamide moiety, we hope to perform a genome analysis of a kanamienamide (**1**) producing cyanobacterium.

In conclusion, kanamienamide (**1**), a novel enamide with an enol ether, was isolated from the marine cyanobacterium *M. bouillonii*. Kanamienamide (**1**) is the first discovered natural product that contains an *N*-Me-enamide group adjacent to an enol ether moiety. In addition, kanamienamide (**1**) showed growth-inhibitory activity in HeLa cells with an IC_{50} value of 2.5 μM and induced apoptosis-like cell death in HeLa cells. This apoptosis-like cell death was not inhibited by Z-VAD-FMK at a high concentration of **1**, while it was inhibited by Z-VAD-FMK at a low concentration of **1**. This suggests that kanamienamide (**1**) may have at least two target molecules for inducing apoptosis-like cell death, upstream of caspases and downstream of caspases and/or other pathways. Further biological investigation of **1** is ongoing in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02364.

^{1}H, ^{13}C, COSY, NOESY, HMQC, HMBC, *J*-resolved, and E.COSY NMR spectra in C_6D_6 for kanamienamide (**1**); HPLC chromatograms for determination of the absolute configurations; detailed experimental procedures (PDF)

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Notes

The authors declare no competing financial interest.

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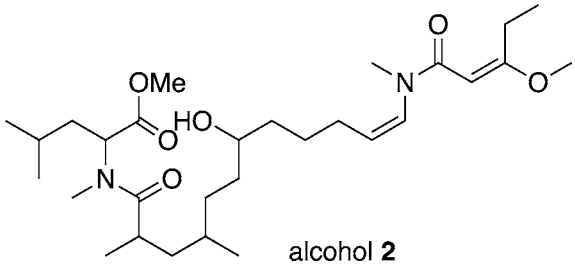
(12) To confirm the absolute configuration of **1**, we synthesized alcohol **2** by base hydrolysis of **1** followed by methylation. We then tried to derivatize **2** to the MTPA ester. However, this reaction failed, probably due to instability of the substrate.



alcohol **2**

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