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Gracilioethers A−C, Antimalarial Metabolites from the Marine Sponge *Agelas gracilis*

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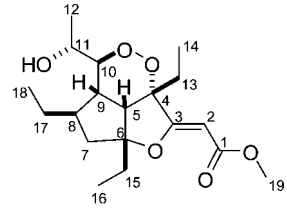
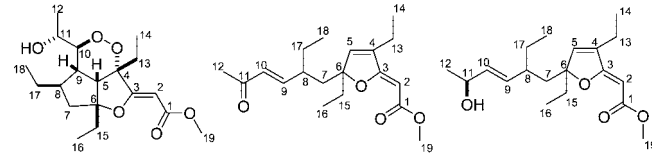


gracilioether A (**1**)

Three new antiprotozoan compounds, gracilioethers A−C (**1**−**3**), have been isolated from the marine sponge *Agelas gracilis*. Their structures were elucidated on the basis of spectroscopic and chemical methods. Gracilioethers A−C showed antimalarial activity against *Plasmodium falciparum* with IC_{50} values of 0.5−10 μg/mL, whereas gracilioether B (**2**) also showed antileishmanial activity.

Introduction

Protozoan infection is increasingly becoming a threat to human beings; for example 300−500 million people are infected by malaria worldwide each year and one to three million die.[1] Numberous attempts including use of the malaria vaccine have been made to control this disease, but the administration of antimalarial drugs is still most effective at this moment. Because of increasing resistance to existing antimalarial drugs, there is an urgent need for the development of antimalarial drugs with new structures and modes of action. Marine natural products have been explored for this purpose, which resulted in the discovery of several drug candidates, including manzamines[2] and cyclic peroxides.[3] In the course of our continuing search for drug leads from Japanese marine invertebrates, we found that the deep-sea sponge *Agelas gracilis* collected in southern Japan showed considerable antimalarial activity in the lipophilic extract. Bioassay-guided fractionation of the extract afforded three new compounds of the plakortin family named gracilioethers A−C.



gracilioether A (**1**) gracilioether B (**2**) gracilioether C (**3**)

Results and Discussion

The $CHCl_3$ soluble materials of the MeOH extract of the sponge were fractionated by the modified Kupchan procedure[4] to yield hexane, $CHCl_3$, and 60% MeOH layers, the last of which was combined with the *n*-BuOH extract of the water-soluble portion of the MeOH extract and sequentially separated by ODS flash chromatography, gel-filtration, and silica gel open column chromatography. The active fraction was finally purified by

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TABLE 1. ^{1}H and ^{13}C NMR Data for Gracilioethers A (1), B (2), and C (3) in CD_3OD

	1		2		3	
no.	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)	^{1}H (ppm)	^{13}C (ppm)
1		169.0		168.8		169.0
2	4.86 s	86.8	4.85 s	84.3	4.82 s	83.8
3		174.6		174.1		174.3
4		89.4		142.1		140.7
5	2.60 (d, 10.7)	43.6	6.40 s	142.1	6.44 s	142.8
6		103.0		99.0		99.6
7	1.38 (dd, 13.4, 12.4)	43.1	1.94 m	43.6	1.78 m	44.3
	2.34 (dd, 13.4, 5.5)		2.08 m		1.94 (d, 10.3)	
8	1.82 m	43.6	2.08 m	41.4	1.78 m	40.7
9	2.13 (dd, 11.0, 10.7)	42.0	6.62 (dd, 15.8, 8.9)	155.3	5.29 m	135.7
10	3.37 (d, 8.0)	84.5	5.82 (d, 15.8)	131.7	5.29 m	135.9
11	3.92 (dq, 8.0, 6.5)	68.1		204.2	4.16 (quint, 5.5, 6.2)	69.3
12	1.29 (d, 6.5)	20.3	2.23 s	26.8	1.19 (d, 6.2)	23.7
13	1.74 (2H, q, 7.6)	34.0	2.05 m	19.4	2.18 m	19.5
			2.13 m			
14	0.93 (t, 7.6)	8.0	1.10 (t, 7.6)	12.0	1.17 (t, 7.2)	12.4
15	1.72 (dq, 13.7, 7.3)	32.7	1.78 (quint 7.6)	33.0	1.78 m	32.8
	1.91 (dq, 13.7, 7.3)		1.83 (quint 7.6)		1.83 (m, 7.2)	
16	1.05 (t, 7.3)	9.5	0.75 (t, 7.6)	8.1	0.75 (t, 7.2)	8.1
17	1.12 (qdd, 7.6, 13.5, 8.8)	26.2	1.32 m	29.5	1.21 m	30.2
	1.64 (qdd, 7.6, 13.5, 3.3)		1.50 m		1.39 m	
18	0.93 (t, 7.6)	12.7	0.82 (t, 7.6)	11.8	0.78 (t, 7.6)	11.9
19	3.65 s	51.3	3.65 s	51.1	3.65 s	51.1

repetitive reversed phase HPLC to yield gracilioethers A (**1**) and C (**3**). The $CHCl_3$ layer was processed to afford gracilioether B (**2**).

Gracilioether A (**1**) had a molecular formula of $C_{19}H_{30}O_6$, which was established by HR-ESIMS analysis [*m*/*z* 377.1942, $(M + Na)^+$, $\Delta + 0.2$ mmu]. Analysis of the ^{1}H NMR data in conjunction with the HSQC[5] spectrum revealed the presence of three ethyls, one methoxyl, one methyl, five methines (including two oxymethines), one methylene, and one protonated sp^2 carbon (Table 1). The ^{13}C NMR spectrum further showed the presence of one carbonyl, one oxygenated nonprotonated sp^2 carbon, and two oxygenated quaternary carbons (Table 1). Although gracilioether A (**1**) was suggested to be related to cladocrocin A by comparing the ^{1}H and ^{13}C NMR data,[6] the gross structure of **1** was quite different on the basis of a detailed analysis of 2D NMR data including COSY, HOHAHA,[7] HSQC, and HMBC[8] spectra. Four partial structures **a**−**d** were deduced from COSY and HOHAHA analysis (Figure 1). Assignment of C-5 and C-8 was difficult due to overlapped carbon signals at δ 43.6. However, mutual HMBC correlations between CH-5 and CH-10 as well as correlations from H-9 to C-10 and C-11 could connect two partial structural units **c** and **d**. The connection of the partial structures **a** to **d** was unambiguously established by HMBC data: (1) correlations H-14/C-4 and H-16/C-6 connected two ethyl units at C-4 and C-6; (2) cross peaks observed for H-5/C-13 could connect **a** to **c** via C-4, the terminal methoxy group was a part of α,β-unsaturated ester (correlations for H-19/C-1 and H-2/C-3), and correlations for H-7/C-6 and 15 indicated that **b** was connected to **c** via C-6; and (3) cross peaks observed for H-5/C-3 suggested that the α,β-unsaturated ester was connected to **c** via C-4 (Figure 1). The remaining two deshielded carbons [δ 89.4 (C-4) and δ 84.5 (C-10)] could be assigned to those of a 1,2-dioxetane ring. To fulfill the unsaturation and the molecular formula, C-3 and C-6 should be connected via an ether linkage to form a five-membered ring, which was the common structural feature to that of cladocrocin A. With all this information, the planar structure of **1** was determined as shown. The geometry of the Δ^2-double bonds was determined as *Z* by comparison of the chemical shift of H-2 (δ_H 4.86) with literature data (δ for *Z* 4.85, δ for *E* 5.23).[9]

The relative stereochemistry of **1** was elucidated on the basis of NOESY data (Figure 2). NOESY correlations (H-5/H-9, -11, -14, -15, and -16, H-9/H-17 and -18, H-10/H-8, and H-11/H-17 and -18) suggested that three ethyl groups (H-5, H-9, and a 2-oxygenated ethyl group) were on the same face of the ring. Since the relative stereochemistry of C-10 and C-11 could not be determined by NOESY analysis, NOE analysis was carried out for the acetonide **1a** that was prepared according to Scheme 1. One of the acetonide methyls at δ 1.31 showed NOE's to H-10, while the other acetonide methyl at δ 1.41 showed NOE's to H-12, indicating that **1a** was a 1,2-*syn*-acetonide, revealing the relative stereochemistry of **1** as shown in Figure 2.

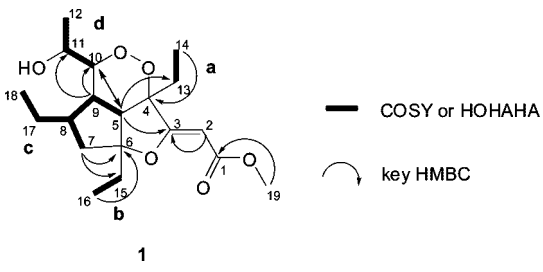


FIGURE 1. Key correlations and partial structures of **1**.

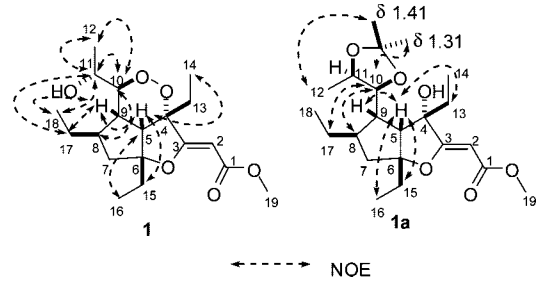
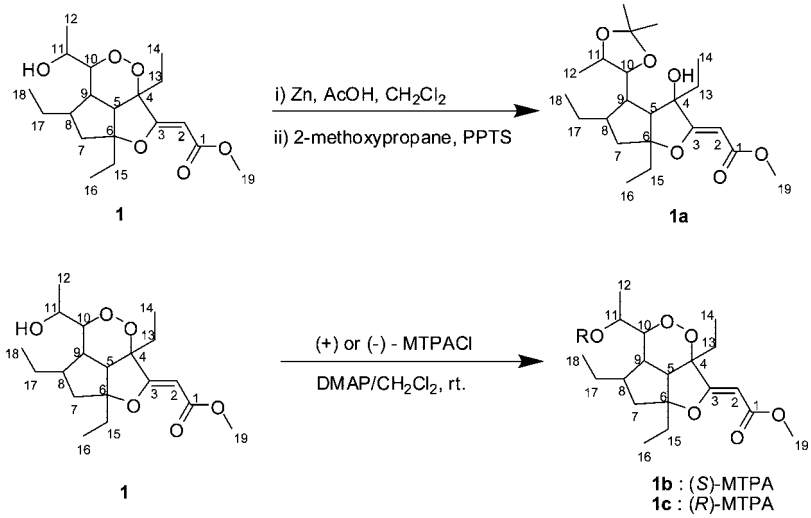


FIGURE 2. Selected NOE's for **1** and **1a**.

SCHEME 1



The absolute stereochemistry of **1** was determined by application of the modified Mosher's method to the secondary hydroxy group at C-11. Treatment of **1** with *R*-(−)- or *S*-(+)-MTPACl yielded *S*-(−)- and *R*-(+)-MTPA esters **1b** and **1c**, respectively. The $\Delta\delta$ value distribution pattern clearly indicated 11*R* configuration (Figure 3).[10]

Gracilioether B (**2**) had a molecular formula of $C_{19}H_{28}O_4$ as established by HR-ESIMS [*m*/*z* 343.1887, $(M + Na)^+$, Δ +0.2 mmu]. Three partial structures **a**−**c** deduced from COSY and HOHAHA data were connected by key HMBC correlations as follows: (1) correlations for H-12 and 10/C-11 indicated that the terminal methyl ketone was conjugated with the double bond between C-9 and 10; (2) the terminal methoxy group at the other side of the molecule was a part of the $\alpha,\beta,\gamma,\delta$-unsaturated ester (correlations for H-19/C-1 and H-2 and H-5/C-3); (3) crosspeaks observed for H-14/C-4 and H-16/C-6 connected two ethyl units at C-4 and C-6, respectively; and (4) the H-8 and H-5/C-6 correlations connected C-6 to C-5, -7, and -15 (Figure 4). To fulfill the molecular formula, C-3 was connected to C-6 via an ether linkage. Thus, the planar structure of **2** was assigned as shown. The 2*Z*-geometry was deduced from the ^{1}H chemical shift values,[9] whereas the 9*E*-geometry was indicated on the basis of a large coupling constant of 15.8 Hz.

The molecular formula of gracilioether C (**3**) was determined as $C_{19}H_{30}O_4$ on the basis of HR-ESIMS [*m*/*z* 345.2057, $(M + Na)^+$, Δ + 1.5 mmu], suggesting that **3** was a dihydro-derivative of **2**. Reduction of the C-11 conjugated ketone was straightforward from the NMR data (δ_H 4.16; δ_C 69.3) assigned as 11-dihydrogracilioether B. The geometry of Δ^2 was determined as *Z* on the basis of ^{1}H chemical shift values[9] as well. Though the coupling constant between H-9 and H-10 could not be determined due to the overlapped signals, the large coupling constant (J = 15.1 Hz) observed for the corresponding protons in MTPA ester **3a** indicated the 9*E*-geometry (Figure 5). The absolute stereochemistry at C-11 was determined as *S* based on Mosher analysis.[1]

Gracilioethers A−C showed promising antimalarial activities (IC_{50} 0.5−10 μg/mL) against *Plasmodium falciparum* as shown in Table 2, while gracilioether B inhibited growth of *Leishmania major* (68% at 10 μg/mL). Gracilioethers B and

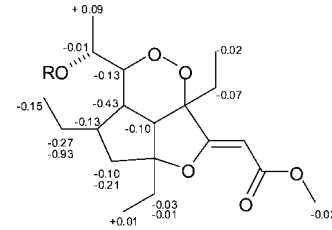


FIGURE 3. $\Delta\delta_{S-R}$ values (ppm) of MTPA esters **1b** and **1c**.

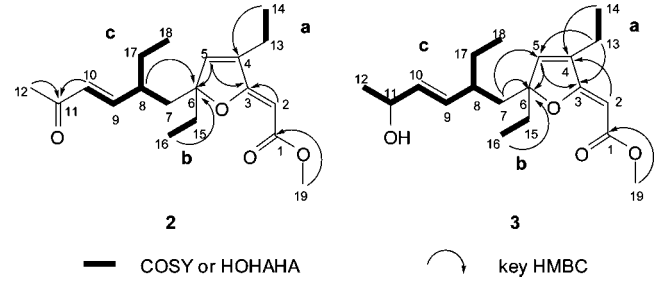


FIGURE 4. Key correlations and partial structures of **2** and **3**.

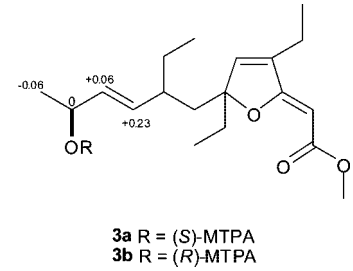


FIGURE 5. $\Delta\delta_{S-R}$ values (ppm) of MTPA esters **3a** and **3b**.

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TABLE 2. IC_{50} Values of Compounds 1, 2, and 3 (μg/mL)

	1	2	3
Plasmodium falciparum	10	0.5	10
B16F10		0.1	1.4
P388	1.2	0.05	0.7
HeLa		0.5	3
EA-hy926	5.2	0.2	1.4

C exhibited moderate cytotoxicity, while A showed less cytotoxicity.

Conclusion

Gracilioether A (**1**), a new member of the plakortin family, was isolated from the deep-sea sponge *Agelas gracilis* along with two analogues, gracilioethers B (**2**) and C (**3**). Gracilioethers A−C (**1**−**3**) showed antimalarial activity; **2** was the most active. Gracilioether B (**2**) was also antiprotozoan against *Leishmania major*. Gracilioether A (**1**) seems to derive from a common biosynthetic precursor of **1**−**3**. From this hypothesis, **2** and **3** are likely to retain the same absolute stereochemistry at C-6 and C-8.

Experimental Section

Assay for the Cytotoxicity against P388 Cells. P388 murine leukemia cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 μg/mL of kanamycin, and 10 μg/mL of 2-hydroxyethyl disulfide at 37 °C under an atmosphere of 5% CO_2. To each well of the 96-well microplate containing 100 μL of tumor cell suspension (1×10^4 cells/mL) was added 100 μL of test solution dissolved in RPMI-1640 medium then the plate was incubated in a CO_2 incubator at 37 °C for 96 h. After addition of 50 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) saline solution (1 mg/mL) to each well, the plate was incubated for 3 h under the same condition to stain live cells. After the incubation, the plate was centrifuged and the supernatants were removed and cells were dissolved in 150 μL of DMSO to determine the IC_{50} values.

Assay for the Cytotoxicity against HeLa Cells. HeLa cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 2 μg/mL of gentamycin, 2 μg/mL of antibiotic-antimicotic, and 0.3 M $NaHCO_3$ (adjusted to pH 7.0−7.4 with 2 M HCl) at 37 °C under an atmosphere of 5% CO_2. To each well of the 96-well microplate containing 200 μL of tumor cell suspension (1×10^4 cells/mL) was added test solution after the 24 h preincubation and the plate was incubated for 72 h. To determine the IC_{50} values, the plate was processed as described for P388 cells.

Antimalarial Assay. *Plasmodium falciparum* ItG strain was cultured in a suspension of 5% (v/v) type 0 (+) human red blood cells. The culture medium consisted of RPMI 1640 supplemented with 25 mM Hepes, 0.3 mM hypoxanthine, 16 mM $NaHCO_3$, and 10% type 0 (+) human plasma. Compounds were dissolved in MeOH before use. Each solution was diluted to the desired concentration with culture medium. Control medium contained MeOH in quantities equal to those used for experimental cultures. To each well of the 96-well plates containing 38 μL of *P. falciparum* ItG suspension (parasitemia: 5%) was added 2 μL of test solution, then the plates were incubated at 37 °C for 48 h. To determine the IC_{50} value, Giemsa-stained smears were made after 48 h of incubation and parasite numbers were counted.

Antileishmanial Assay. Fluorescence signals of *Lm/egfp* promastigotes cultured in 199 medium supplemented with 10% fetal bovine serum and 25 mM HEPES buffer in 96-well plates at 25 °C were measured by fluorescence microplate reader with excitation at 485 nm and emission at 538 nm. To each well of the 96-well plates which contained 100 μL of *Lm/egfp* suspension (1×10^6 cells/mL) was added 100 μL of test solution (sample dissolved in MeOH) then the plates were incubated in a low temperature incubator at 25 °C for 72 h. To determine the growth inhibitory activity of **2** at 10 μg/mL, the fluorescent signals were measured after 72 h of incubation.

Extraction and Isolation. The sponge was collected by dredging at a depth of 150 m on a seamount named Oshima-Shinsone (28°52′40″N, 129°33′19″E) near Amami-oshima Island, southern Japan and identified as *Agelas gracilis* (voucher specimen ZMAPOR19857 was deposited at the Zoological Museum, University of Amsterdam). The frozen sample (900 g) was extracted with MeOH (3 × 3 L) and concentrated in vacuo. The extract was suspended in H_2O (1 L) and extracted with $CHCl_3$ (2 × 1 L) and *n*-BuOH (2 × 1 L). The $CHCl_3$ extract was partitioned between 90% MeOH and *n*-hexane. The 90% MeOH layer was adjusted to 60% MeOH by addition of H_2O and extracted with $CHCl_3$. The $CHCl_3$ layer was concentrated and separated by ODS flash chromatography with aq MeOH to give six fractions (A−F). Fraction B (70% MeOH) was gel-filtered with MeOH to give 11 fractions. The first fraction was separated by silica gel open column chromatography with $CHCl_3$/MeOH/CH_3COOH (95:5:1) and the active fraction was further purified by reversed-phase HPLC (C_{18}-stationary phase, 10 × 250 mm) with 70% MeOH to yield 0.6 mg of gracilioether B (**2**). The *n*-BuOH and 60% MeOH layers were combined and similarly separated by ODS flash chromatography. The 70% MeOH and 70% MeCN eluates and fraction C (70% MeCN) from the $CHCl_3$ layer were combined and gel-filtered with MeOH. The fast eluting fractions were collected and separated by silica gel open column chromatography with $CHCl_3$/MeOH/CH_3COOH (95:5:1), followed by reversed-phase HPLC (C_{18}-stationary phase, 20 × 250 mm) with 70% MeOH to afford fractions A′ and B′. The fraction A′ was purified by reversed-phase HPLC (C_{18}-stationary phase, 10 × 250 mm) with 70% MeOH to give 0.3 mg of gracilioether C (**3**). The fraction B′ was purified by reversed-phase HPLC (C_{18}-stationary phase, 10 × 250 mm) with 50% MeCN giving 0.5 mg of gracilioether A (**1**).

Gracilioether A (1): colorless solid; $[\alpha]^{34.6}_D$ +20 (*c* 0.03, MeOH); UV (MeOH) 248.5 nm (ϵ 9200); IR (film) 1697, 1540 cm^{-1}; HRESIMS *m*/*z*377.1942 $(M + Na)^+$, $C_{19}H_{30}O_6Na$, (Δ + 0.2 mmu); ^1H NMR (CD_3OD) and ^{13}C NMR (CD_3OD), see Table 1.

Gracilioether B (2): colorless solid; $[\alpha]^{34.6}_D$ −120 (*c* 0.03, MeOH); UV (MeOH) 287.5 nm (ϵ 2900); IR (film) 1624 cm^{-1}; HRESIMS *m*/*z* 343.1887 $(M + Na)^+$, $C_{19}H_{28}O_4Na$, (Δ + 0.2 mmu); ^1H NMR (CD_3OD) and ^{13}C NMR (CD_3OD), see Table 1.

Gracilioether C (3): colorless solid; $[\alpha]^{31.7}_D$ −24 (*c* 0.02, MeOH); UV (MeOH) 287.0 nm (ϵ 7000); IR (film) 1697, 1539 cm^{-1}; HRESIMS *m*/*z*345.2057 $(M + Na)^+$, $C_{19}H_{30}O_4Na$, (Δ + 1.5 mmu); ^1H NMR (CD_3OD) and ^{13}C NMR (CD_3OD), see Table 1.

Preparation of Acetonide 1a. Gracilioether A (**1**; 0.2 mg) was reacted with Zn powder (4.0 mg) in Et_2O (100 μL) containing AcOH (7 μL) and stirred overnight at rt. The mixture was filtered and separated by reversed-phase HPLC (C_{18}-stationary phase, 10 × 250 mm; with 50% MeCN) to give triol **1**. Triol **1** was dissolved in 160 μL of CH_2Cl_2 and 40 μL of 2,2-dimethoxypropane containing catalytic amounts of PPTS, then stirred overnight at rt, followed by reversed-phase HPLC separation (C_{18}-stationary phase, 10 × 250 mm; with 70−100% MeOH) to yield acetonide **1a**.

Preparation of MTPA Esters 1b and 1c. Gracilioether A (**1**; 100 μg and 50 μg, respectively) was reacted with *R*-(−)- or *S*-(+)-MTPACl (5 μL) in 100 μL of CH_2Cl_2 containing 1 mg of DMAP for 5 min, respectively. The mixtures were partitioned between 0.1 M $NaHCO_3$ and $CHCl_3$. The $CHCl_3$ layers were washed with 0.1 M HCl and H_2O, then the organic layers were concentrated and

separated by reversed-phase HPLC (C_{18}-stationary phase, 10 × 50 mm; with 70−100% MeOH) to afford *S*-(−)- and *R*-(+)-MTPA esters **1b** and **1c**, respectively.

Preparation of MTPA Esters 3a and 3b. Gracilioether C (**3**; 100 μg each) was similarly processed to give *S*-(−)- and *R*-(+)-MTPA esters **3a** and **3b**, respectively.

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Supporting Information Available: NMR spectra for compounds **1**, **2**, **3**, **1a**−**c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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