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Gracilioethers E–J, new oxygenated polyketides from the marine sponge *Plakinastrella mamillaris*

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ABSTRACT

Purification of the apolar extracts of the marine sponge *Plakinastrella mamillaris* afforded the isolation of six new oxygenated polyketides, gracilioethers E–J, featuring an unprecedented oxidation pattern of the polycyclic core. On a common tricyclic core, gracilioethers E, G, and J feature the furanyliden methyl ester substructure and gracilioethers F, H, and I display a γ-lactone substructure. The new structures were elucidated on the basis of extensive spectroscopic (^{1}H and ^{13}C NMR, COSY, HSQC, HMBC, and ROESY), MS, computational and chemical methods. Pharmacological analysis demonstrated that gracilioether H is endowed with in vitro antiplasmodial activity against a chloroquine resistant strain.

1. Introduction

Marine sponges of the family Plakinidae have been reported to be a rich source of oxygenated polyketides, with large structural diversity, many of which exhibiting antimicrobial, antitumor, antifungal, and antiparasitic activities. Particularly interesting are compounds containing six-membered peroxide rings (1,2-dioxane), recently found to possess antiparasitic activity and considered promising drug leads against tropical diseases, such as malaria, African sleeping sickness, or leishmaniasis.[1–8]

In the course of our continuing search for bioactive compounds from South Pacific marine invertebrates, we undertook the study of the sponge *Plakinastrella mamillaris* collected at Fiji Islands. The analysis of the sponge extracts afforded the previously reported oxygenated polyketides methyl esters **1**[9] and **2**,[10] the γ-lactone **3**[9] and gracilioethers A (**4**)-D[11,12] (Fig. 1), together with seven new monocyclic polyketides, some of them were disclosed as new PPARγ ligands.[12]

Further investigation of the sponge extracts led to the isolation of five new tricyclic gracilioethers E–I (**5**–**9**) featuring an unprecedented oxidation pattern of the polycyclic core and gracilioether J (**10**) with a truncated carbon skeleton (Fig. 2). Gracilioethers E (**5**) and G (**7**) share with the parent gracilioether A (**4**) a common carbon skeleton and the furanyliden methyl ester substructure, and, likely, similar oxidation pathways led to the biosynthesis of gracilioethers A (**4**), E (**5**), G (**7**), and J (**10**) starting from the plausible precursor methyl ester **1**. On the other hand, following the same oxidation pathways, gracilioethers F (**6**) and H (**8**), could be biosynthetized from g-lactone 3.

Here we describe the isolation and structure elucidation of the new derivatives **5**–**10** and the evaluation of their antiprotozan activity, as parasitic diseases (especially malaria and leishmaniasis) are leading health concern all around the world.

2. Results and discussion

Gracilioether E (**5**) had a molecular formula of $C_{17}H_{24}O_5$, which was established by HRESIMS analysis [*m*/*z* 331.1533 $(M+Na)^+$]. Analysis of ^{1}H and ^{13}C NMR data (Table 1) in conjunction with the HSQC spectrum revealed the presence of three ethyls, three methines, one methylene, one methoxy group, two oxygen-bearing quaternary carbons, one protonated sp^2 carbon, and one oxygenated non-protonated sp^2 carbon, and two ester carbonyls. Although NMR data of gracilioether E (**5**) revealed some analogies with the furanoester members of the *Plakortis* metabolites, such as cladacrocin[13] and gracilioethers,[11] the gross structure of **5** was quite

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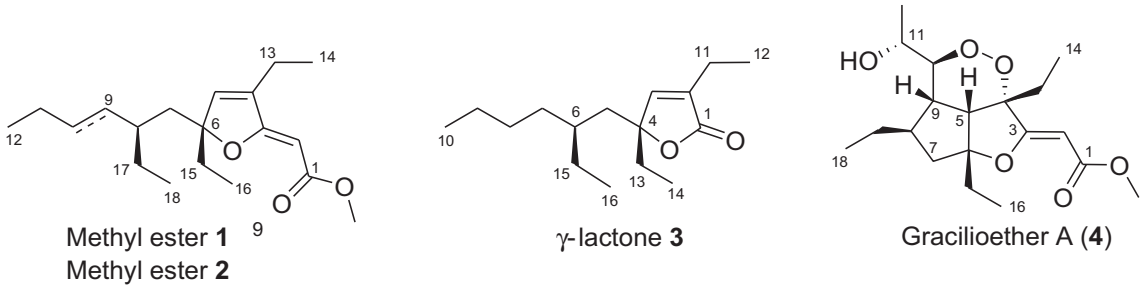

Fig. 1. Biogenetically related polyketides previously reported from *Plakinastrella mamillaris*.

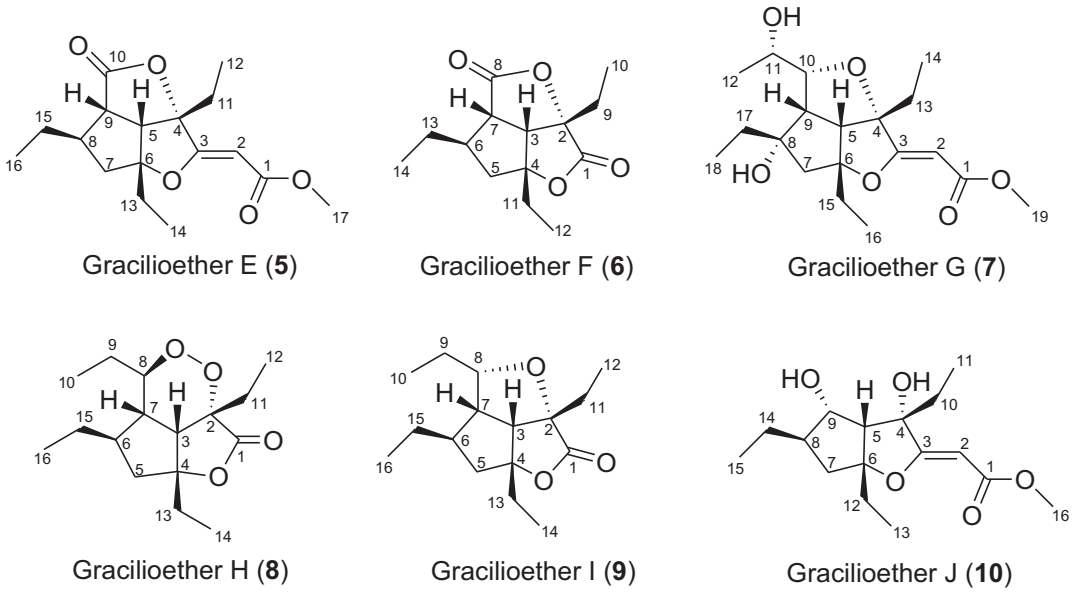

Fig. 2. New gracilioethers from *Plakinastrella mamillaris*.

Table 1
^{1}H and ^{13}C NMR data (CD_3OD) of gracilioethers E–F (**5**–**6**) and H–I (**8**–**9**)

	5[a]		**6**[a]		**8**[b]		**9**[b]	
	δ_H[c]	δ_C	δ_H[c]	δ_C	δ_H[c]	δ_C	δ_H[c]	δ_C
1	—	168.2	—	174.9	—	177.7	—	177.2
2	5.02 s	90.0	—	88.9	—	84.0	—	84.8
3	—	173.0	3.31 d (10.2)	53.8	2.74 d (9.8)	42.9	2.82 d (10.3)	47.1
4	—	96.0	—	96.9	—	95.8	—	94.5
5	3.24 d (10.1)	54.6	1.64 dd (11.7, 13.9)	44.8	1.35 dd (12.2, 13.5)	42.2	2.32 dd (5.8, 13.9)	43.5
			2.34 dd (5.8, 13.9)		2.25 dd (5.2, 13.5)		1.43 dd (12.6, 13.9)	
6	—	104.6	2.11 m	47.0	1.82 ovl	43.9	1.95 ovl	39.3
7	1.62 dd (11.7, 13.6)	45.5	2.97 t (9.7)	52.6	1.77 dd (10.1, 11.0)	46.0	2.10 dt (3.5, 10.3)	46.3
	2.42 dd (5.9, 13.6)							
8	2.08 m	47.0	—	177.9	3.69 dd (5.5, 8.9)	81.8	4.20 dt (3.5, 10.3)	84.6
9	2.90 t (9.7)	53.3	1.88 m	28.8	1.55 m	27.3	1.43 m	25.1
			2.07 m		1.96 ovl		1.55 m	
10	—	178.3	1.03 ovl	7.2	1.00 t (7.5)	10.3	1.02 t (7.3)	11.3
11	1.88 m	31.2	1.85 m	32.2	1.74 ovl	31.4	1.75 m	30.6
	2.04 m				1.85 ovl		1.83 quint (7.4)	
12	1.01 t (7.1)	8.1	1.02 ovl	8.8	0.99 t (7.5)	7.2	0.97 t (7.4)	7.1
13	1.83 m	32.0	1.49 m	27.8	1.73 ovl	32.9	1.74 m	32.5
			1.81 m		1.94 ovl		1.95 ovl	
14	1.02 t (7.4)	9.3	0.98 t (7.4)	12.2	1.04 t (7.5)	8.9	1.04 t (7.6)	8.9
15	1.45 m	28.3			1.16 m	26.4	1.17 m	28.9
	1.80 m				1.66 m		1.81 quint (7.5)	
16	0.98 t (7.4)	12.8			0.94 t (7.5)	12.6	0.91 t (7.5)	12.3
17	3.68 s	51.2						

Ovl: overlapped signals.
[a] Measured at 400 and 100 MHz.
[b] Measured at 500 and 125 MHz.
[c] Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, HSQC and HMBC experiments.

different on the basis of detailed analysis of 2D NMR spectra. A COSY spectrum allowed us to identify three spin systems: two ethyl groups linked to quaternary carbons, and a CH(5)CH(9) CH(8)(CH_2CH_3)CH_2(7) spin system (Fig. 3) reminiscent of that found in gracilioether A (**4**).[11] Moreover, correlation peaks in HSQC and HMBC spectra of **5** (Fig. 3) allowed assignment of all the carbon resonances and to connect all the identified substructures of the new molecule. In particular, correlations H-12/C-4 and H-14/C-6 connected two ethyl units at C-4 and C-6. A methyl β-oxygenated α,β-unsaturated ester was suggested by ^{13}C NMR chemical shift considerations and long range correlations H-17/C-1, H-2/C-3 and a five-member ether bridge between C-3 and C-6 was closed by mass consideration and by analogy with cladacrocin A[13] and gracilioether A (**4**).[11] The second *ortho*-condensed five-member ring was deduced from diagnostic HMBC correlations H-5/C-11, H-7/C-6 and H-7/C-13 (Fig. 3).

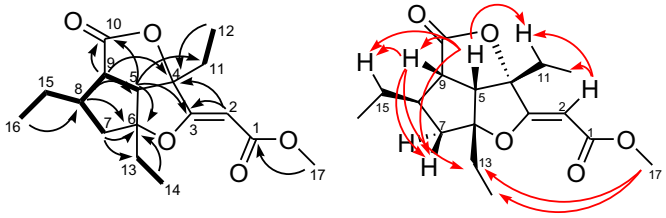

Fig. 3. COSY connectivities (bold bonds), HMBC (back arrows) and ROE (red arrows) correlations for gracilioether E (**5**).

Having defined the methyl 2-(3,6a-diethyl-hexahydrocyclopenta[*b*]furan-2-ylidene)acetate substructure, common to gracilioether A (**4**), ^{13}C NMR and mass data showed one additional ester carbonyl resonance at δ_C 178.3 (C-10). This latter was connected to C-9 on the basis of HMBC correlations H-5/C-10 and H-9/C-10. To fulfill the unsaturation and the molecular formula, a γ-lactone ring was closed between C-10 and C-4, as confirmed by the chemical shift of C-4 (δ_C 96.0). The relative stereochemistry of gracilioether E (**5**) was deduced from several key dipolar couplings evidenced by ROESY spectrum (Fig. 3). ROESY correlations H-17/H-13, H-14, and H-2/H-11, H-12 pointed to a *Z* configuration of the Δ^2 double bond.

Further, dipolar couplings H-5/H-9, H-7a, H-11, H-13 and H-9/H-15, H-7a suggested that the three ethyl groups, H-5, H-9 and H-7a were on the same face of the ring.

Taken together, these data indicated the planar structure of **5** as shown in Fig. 3.

In order to propose the absolute configuration for gracilioether E (**5**) we followed an approach relying on the comparison between experimental and calculated circular dichroism spectra. A preliminary conformational analysis by means of Molecular Dynamics (MD) calculation was performed to locate the minimum energy conformers falling in a range of ca. 10 kcal/mol. The obtained minimum energy conformers for each structure were further optimized at Density Function Theory (DFT) level, using the mPW1PW91 functional and the 6-31G(d) basis set. Finally, Boltzmann weighted averaged spectra were obtained using the Time-Dependent Density Functional Theory (TDDFT), using the mPW1PW91 functional and the 6-31G(d,p) basis set. The calculated CD spectrum of gracilioether E with the absolute configuration depicted in **5**, is in fair accordance with the experimental spectrum (Fig. 4),[14] even though the latter is broader under most of the conditions used in our experiments. These findings, together with the plausible biogenetic correlation with gracilioether A, allowed us to propose the absolute stereochemistry of gracilioether E as depicted in Fig. 2.

The molecular formula of gracilioether F (**6**) was determined as $C_{14}H_{20}O_4$ on the basis of HRESIMS [m/z 275.1263, $(M+Na)^+$].

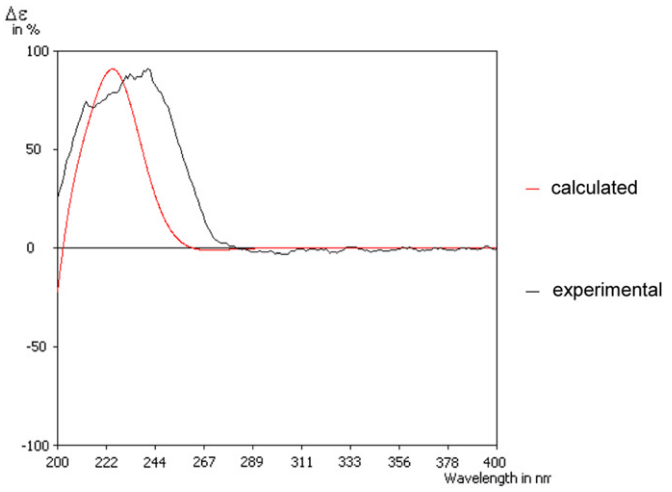

Fig. 4. Elucidation of the absolute configuration of gracilioether E (**5**) by comparison of its experimentally measured CD spectrum with the TDDFT-predicted curves.

Comparison of the ^1H NMR spectrum of **6** (Table 1) with that of furanylidenic methyl ester **5** showed the spectra to be quite similar except that gracilioether F (**6**) lacked the methyl ester signal at δ_H 3.68 and the C-2 proton signal at δ_H 5.02. Analysis of 2D NMR and MS data clearly indicated that **6** differs from compound **5** for the replacement of methoxycarbonylmethylidene group with one oxygen carbonyl. The complete matching of the chemical shifts and coupling constants values (Table 1) of all nuclei around the cyclopentane unit in **6** as compared with those of gracilioether E (**5**) allowed us to proposed the absolute stereochemistry of gracilioether F (**6**) as indicated in Fig. 2.

Gracilioether G (**7**) with the molecular formula $C_{19}H_{30}O_6$, was isomeric with gracilioether A (**4**).[11] Analysis of ^1H and ^{13}C NMR data of **7** revealed similar structural features to those of gracilioether A except for the loss of the methine ($\delta_{H/C}$ 1.82/43.6) assigned at C-8 in gracilioether A and the presence of one more quaternary oxygenated carbon (δ_C 81.8) in **7**. The ^1H NMR spectrum, recorded in DMSO-d_6, evidenced two exchangeable protons (δ_H 4.35 s and 4.60 br s) confirming the presence of one secondary and one tertiary alcoholic function (Table 2). Analysis of COSY data allowed the building of the four spin systems indicated in bold in Fig. 5, while a series of HMBC correlations allowed the connection of these subunits.

In particular, correlations H-14/C-4, H-18/C-8 and H-16/C-6 connected three ethyl units to C-4, C-6, and C-8, respectively. The same methyl-β-oxygenated-α,β-unsaturated ester as in gracilioethers A–D was easily inferred by chemical shift considerations and long range correlations H-19/C-1, H-2/C-3, H-5/C-3 and finally several ^2J and ^3J heteronuclear correlations exhibited by H-5 and H_2-7 protons allowed to close the 8-hydroxy-C5-C9 cyclopentane ring (Fig. 5). Even if no HMBC correlation was observed between H-10 and C-4, C-10 was connected to C-4 via an ether linkage on the basis of mass considerations and the ROESY data, described below and indicated in Fig. 5.

The geometry of the Δ^2-double bond was determined as *Z* by ROESY correlation H-2/H-13 and confirmed by comparison of the chemical shift of H-2 (δ_H 4.71) with literature data (δ_H 4.85 for *Z*, δ_H 5.23 for *E*).[15]

The relative configuration of the tricyclic system in **7** was established from ROESY spatial correlations as shown in Fig. 5. The correlations between H-5/H-9, –13, –14, –15, –16 and H-9/H-18, –16, indicated that H-5, H-9 and the three ethyl groups at C-4, –6, –8 were on the same face of the rings. The dipolar couplings between H-10 and H-13 and H-14, indicated the β-orientation of H-10 and confirmed the presence of an ether linkage between C-10 and C-4.

Table 2
^{1}H and ^{13}C NMR data (DMSO-d_6) of gracilioethers G and J (**7** and **10**)

7[a]			**10**[b]		
Position	δ_H[c]	δ_C	Position	δ_H[c]	δ_C
1	—	165.3	1	—	165.4
2	4.71 s	86.5	2	4.74 s	84.5
3	—	173.8	3	—	177.5
4	—	94.1	4	—	81.2
5	3.11 d (9.3)	56.1	5	2.11 s	59.9
6	—	99.1	6	—	101.3
7	1.83 d (13.9) 2.14 d (13.9)	46.7	7	1.63 ovl 1.80 m	40.3
8	—	81.8	8	1.62 ovl	43.7
9	2.87 dd (5.6, 9.3)	60.1	9	4.13 br d (2.4)	72.3
10	3.72 dd (5.6, 9.1)	88.8	10	1.54 dq (7.3, 13.9) 1.60 ovl	35.5
11	4.04 m	65.2	11	0.91 t (7.3)	7.6
12	1.10 d (6.0)	22.9	12	1.66 ovl 1.73 dq (7.5, 14.0)	32.1
13	1.60 m 1.80 ovl	28.5	13	0.93 t (7.5)	8.7
14	0.85 t (7.4)	8.8	14	1.30 dq (7.5, 13.9) 1.40 dq (7.5, 13.9)	20.9
15	1.73 m	31.9	15	0.85 t (7.5)	12.5
16	0.89 t (7.3)	8.6	16	3.51 s	49.9
17	1.78 ovl	29.2	OH-9	4.43 br s	
18	0.83 t (7.2)	9.2			
19	3.51 s	50.1			
OH-8	4.35 s				
OH-11	4.60 br s				

Ovl: overlapped signals.
[a] Spectra measured in DMSO-d_6 at 700 and 175 MHz.
[b] Spectra measured in DMSO-d_6 at 500 and 125 MHz.
[c] Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, HSQC and HMBC experiments.

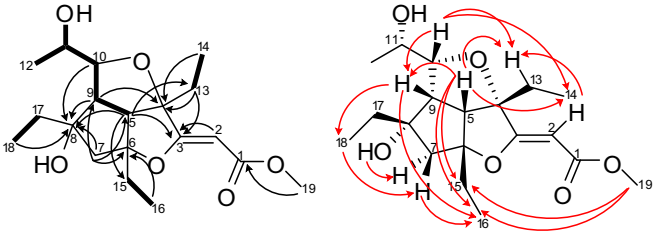

Fig. 5. COSY connectivities (bold bonds), HMBC (back arrows) and ROE (red arrows) correlations for gracilioether G (**7**).

The absolute stereochemistry at C-11 was determined as *S* based by Mosher analysis.[16]

Despite the structural resemblance, gracilioether G (**7**) has an epimeric configuration with respect to gracilioether A at both C-10 and C-11 carbons and this configurational change had a profound impact on the chemical shift of all nuclei and on the vicinal coupling between H-9 and H-10 (5.6 Hz in **7**, 0 Hz in gracilioether A).

Gracilioether H (**8**) had a molecular formula of $C_{16}H_{26}O_4$. Analysis of ^{1}H and ^{13}C NMR spectra (Table 1) disclosed that, as in gracilioether F (**6**), the C1–C3-α,β-unsaturated ester substructure of gracilioethers was replaced by a γ-lactone system. The remaining spin systems are close reminiscent in term of chemical shift values and connectivities to the corresponding ones in gracilioether A (**4**). The only difference is the presence of one unfunctionalized ethyl group at C8, easily inferred by COSY analysis and diagnostic HMBC H-8/C-9. The closure of 1,2 dioxetane ring between C-8 and C-2 followed by chemical shift and MS considerations.

The close analogy of chemical shift values, coupling constants pattern (Table 1) and ROESY data suggested that the relative stereochemistry of gracilioether H (**8**) is the same as in gracilioether A (**4**).

When gracilioether H (**8**) was treated with Zn/AcOH the formation of diol **8a** was observed (Fig. 6), thus confirming the

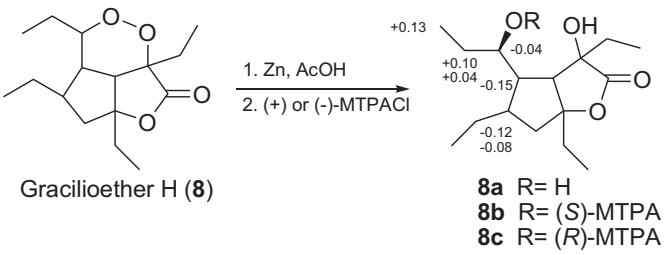

Fig. 6. Preparation of diol **8a** and $\Delta\delta_{S-R}$ values (ppm) of MTPA esters **8b** and **8c**.

presence of the peroxide linkage in the molecule as well as allowing the preparation of the MTPA esters **8b** and **8c**. As shown in Fig. 6, the $\Delta\delta$ value distribution pattern clearly indicated the 8*R* configuration.

Gracilioether I (**9**) was considered to have the molecular formula of $C_{16}H_{26}O_3$ based on the HRESIMS of the molecular ion peak at *m*/*z* 289.1791 $[M+Na]^+$, indicating that **9** possessed one less oxygen atom than gracilioether H (**8**). The ^{1}H and ^{13}C NMR spectra of **9** (Table 1) were very similar to those of **8**, and the connectivities inferred by COSY and HMBC spectra were exactly the same as in gracilioether H. Therefore to fulfill the unsaturation and the molecular formula, C-8 and C-2 should be connected via an ether linkage to form a five-membered ring, a structural feature also found in gracilioether G (**7**).

The pattern of coupling constants was found to be similar to that of gracilioether G (**7**) (Table 2), with a coupling constant of 3.5 Hz between H-7 and H-8 whereas in gracilioethers A (**4**) and H (**8**) no coupling was observed between the corresponding protons. The β-orientation of H-8, suggested by scalar coupling (Table 1), was also confirmed by the dipolar couplings in the ROESY spectrum. In particular, ROESY correlations H-3/H-7, H-8, H-11, H-13, and H-7/H-16 indicated that the ethyl groups at C-2, C-4 and C-6, H-3, H-7, and H-8 were on the same face of the molecule.

Gracilioether J (**10**) showed a molecular formula of $C_{16}H_{26}O_5$ as deduced by the sodiated-molecular peak at *m*/*z* 321.1685 in the HRESIMS spectrum. The ^{1}H NMR spectrum, recorded in DMSO-d_6, evidenced one exchangeable proton at δ_H 4.43, br s, indicating the presence of an alcoholic function in the molecule (Table 2). The carbon resonances at δ_C 165.4 (C-1), 84.5 (C-2), and 177.5 (C-3) were indicative of a furano α,β-unsaturated ester.[9] By interpretation of COSY correlations, it was possible to establish three structures of consecutive proton systems, H-10/H-11, H-12/H-13, H5/H9(OH)/H8(H14/H15)/H7, whose connectivities were further established by the HMBC correlations (Fig. 7).

The conjunction of C-10 and C-4 was elucidated on the basis of the HMBC correlations from H-11 and H-10 to C-4. Moreover, the HMBC correlations from H-13 and H-12 to C-6 indicated the attachment of the second ethyl group to C6. The HMBC correlations observed from H-9 to C-6, and from H-5 to C-12 indicated the connection of C-5 to C-6. The HMBC correlations of H-16/C-1, H-2 and H-5 to C-3 and C-4 further confirmed the existence of a furanylidenic methyl ester. To satisfy the molecular formula the oxymethine at C-4 had to be substituted with a hydroxyl group. ROE correlation between H-2 and H-10 indicated the *Z* geometry for Δ^2 double bond (Fig. 7). With all this information, the planar structure of **10** was determined as shown.

ROESY correlations (H-5/H-10, −11, and −12 and H-9/H-10, −11 and H-15) suggested that the three ethyl groups, H-5, and H-9 were on the same face of the ring in a similar situation of all members of this family of marine oxygenated polyketides. Furthermore, the $\Delta\delta$ value distribution pattern of MTPA esters **10a** and **10b** clearly indicated the 9*S* configuration (Fig. 7).

All new gracilioethers were tested for their antiplasmodial activity in vitro against chloroquine resistant CR FC29 strain (Table 3).

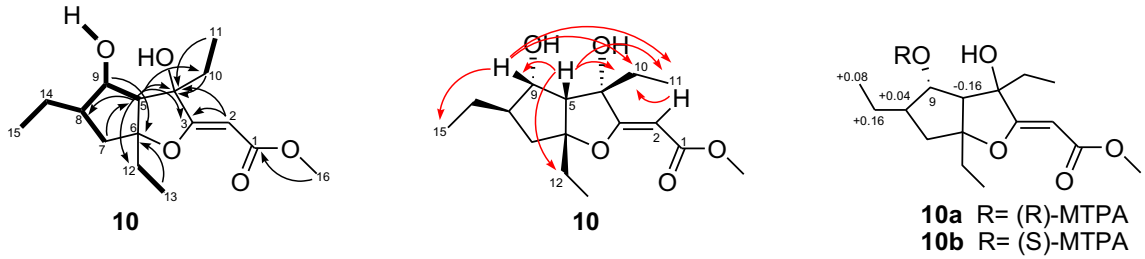

Fig. 7. COSY connectivities (bold bonds), HMBC (back arrows) and ROE (red arrows) correlations for gracilioether J (**10**). $\Delta\delta_{S-R}$ values (ppm) of MTPA esters **10b**, **10c**.

Table 3
Antimalarial and antileishmanial activity of compounds **5**–**10**

Compounds	IC_{50} μM FC29	IC_{50} μM *Leishmania infantum*	DT_{50} μM Vero cells
5	>32.5	159.3	994.2
6	>39.7	396.8	1187.5
7	>28.2	136.8	366.5
8	3.3	78.8	323.8
9	>37.6	64.1	502.1
10	>33.6	320.4	579.4

The only active compound (IC_{50} 3.26 μM) was found to be gracilioether H (**8**), confirming the importance of endoperoxide ring for the activity.[17] Interestingly, it also showed very low toxicity on Vero cells, giving a manifest selectivity index (DT_{50}/IC_{50}) of almost 100.

When tested for their antileishmanial activity, all compounds were devoid of significant activity.

In conclusion the study of the sponge *P. mamillaris* afforded, along with the previously reported oxygenated polyketides methyl esters **1** and **2**, γ-lactone **3** and gracilioether A (**4**), six new tricyclic gracilioethers, with a peculiar oxidation pattern of the cyclic core. Among these, gracilioether H (**8**) showed antimalarial activity against a chloroquine resistant strain.

3. Experimental section

3.1. General procedures

Specific rotations were measured on a Perkin–Elmer 243 B polarimeter. UV spectra were measured on a JASCO 530 spectrophotometer. The CD spectra were recorded in MeOH from 200 to 350 nm on a JASCO 715 spectropolarimeter equipped with a Peltier-type temperature control system (model PTC-348WI) using a cell with 0.1 cm path length at room temperature. High-resolution ESI-MS spectra were performed with a Micromass QTOF Micromass spectrometer. ESI-MS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 700 MHz spectrometer (^1H at 700 MHz, ^{13}C at 175 MHz, respectively) and Varian Inova 500 NMR spectrometer (^1H at 500 MHz, ^{13}C at 125 MHz, respectively) equipped with a Sun hardware, δ (parts per million), J in hertz, spectra referred to CD_3OD (δ_H 3.31, δ_C 49.0) and DMSO-d_6 (δ_H 2.50, δ_C 39.5) as internal standards. HPLC was performed using a Waters Model 510 pump equipped with Waters Rheodyne injector and a differential refractometer, model 401.

Through-space ^1H connectivities were evidenced using a ROESY experiment with mixing times of 150, 200, and 250 ms, respectively.

Silica gel (200–400 mesh) from Macherey-Nagel Company was used for flash chromatography.

The purities of compounds were determined to be greater than 95% by HPLC.

3.2. Sponge material and separation of individual compounds

P. mamillaris Kirkpatrick, 1900 (order Homosclerophorida, family Plakinidae) was collected at Fiji Islands, in May 2007.

The sample was frozen immediately after collection and lyophilized to yield 171 g of dry mass. The sponge was identified by Dr. John Hooper, Queensland Museum, Brisbane, Australia, where a voucher specimen is deposited under the accessing number G322695.

The lyophilized material (171 g) was extracted with methanol (3×1.5 L) at room temperature and the crude methanolic extract (40 g) was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane to give 17.3 g of the crude extract. The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$ to give 16.6 g of the crude extract. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH (2.4 g of crude extract).

The *n*-hexane extract (8 g) was fractionated by silica gel MPLC using a solvent gradient system from *n*-hexane to EtOAc.

Fraction eluted with hexane/EtOAc 98:2 (995 mg) was further purified by HPLC on a Nucleodur 100-5 C18 (5 μm; 10 mm i.d. ×250 mm) with 90% MeOH/H_2O as eluent (flow rate 5 mL/min) to give 12.5 mg of γ-lactone **3** (t_R=5.4 min) and 3.5 mg of methyl ester **2** (t_R=8.6 min).

The $CHCl_3$ extract (5.1 g) was chromatographed by silica gel MPLC using a solvent gradient system from CH_2Cl_2 to CH_2Cl_2/MeOH 1:1.

Fractions eluted with CH_2Cl_2/MeOH 99:1 (456 mg) were further purified by HPLC on a Nucleodur 100-5 C18 (5 μm; 10 mm i.d. ×250 mm) with 70% MeOH/H_2O as eluent (flow rate 3.5 mL/min) to give methyl ester **1** as major component (241 mg), 4.0 mg of gracilioether F (**6**) (t_R=9.6 min), 7.4 mg of gracilioether E (**5**) (t_R=11.1 min), 11.0 mg of gracilioether A (**4**) (t_R=26 min), 1.5 mg of gracilioether I (**9**) (t_R=27.9 min) and 2.8 mg of gracilioether H (**8**) (t_R=30.3 min).

Fractions eluted with CH_2Cl_2/MeOH 98:2 (326 mg) were further purified by HPLC on a Nucleodur 100-5 C18 (5 μm; 10 mm i.d. ×250 mm) with 60% MeOH/H_2O as eluent (flow rate 3.5 mL/min) to give methyl ester **1** as major component (121 mg), 5.2 mg of gracilioether J (**10**) (t_R=12.6 min) and 10 mg of gracilioether G (**7**) (t_R=21.9 min).

3.3. Characteristic data for each compound

Gracilioether E (**5**): Colorless solid; $[\alpha]_D^{25}$ −6.8 (*c* 0.11, MeOH); UV (MeOH) 244 nm (ε 4566); ^1H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 331.1 $[M+Na]^+$. HRMS (ESI): calcd for $C_{17}H_{24}NaO_5$: 331.1521; found 331.1533 $[M+Na]^+$.

Gracilioether F (**6**): Colorless solid; $[\alpha]_D^{25}$ −8.2 (*c* 0.26, MeOH); ^1H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 275.1 $[M+Na]^+$. HRMS (ESI): calcd for $C_{14}H_{20}NaO_4$: 275.1259; found 275.1263 $[M+Na]^+$.

Gracilioether G (**7**): White amorphous solid; $[\alpha]_D^{25}$ −8.7 (*c* 0.26, MeOH); ^{1}H and ^{13}C NMR data in DMSO-d_6 and CD_3OD given in Table 1; ESI-MS: *m*/*z* 377.2 $[M+Na]^+$. HRMS (ESI): calcd for $C_{19}H_{30}NaO_6$: 377.1940; found 377.1946 $[M+Na]^+$.

Gracilioether H (**8**): White amorphous solid; $[\alpha]_D^{25}$ +59.7 (*c* 0.11, MeOH); ^{1}H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 305.2 $[M+Na]^+$. HRMS (ESI): calcd for $C_{16}H_{26}NaO_4$: 305.1729; found 305.1737 $[M+Na]^+$.

Gracilioether I (**9**): White amorphous solid; $[\alpha]_D^{25}$ +39.7 (*c* 0.15, MeOH); ^{1}H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 289.2 $[M+Na]^+$. HRMS (ESI): calcd for $C_{16}H_{26}NaO_3$: 289.1780; found 289.1791 $[M+Na]^+$.

Gracilioether J (**10**): colorless solid; $[\alpha]_D^{25}$ −24 (*c* 0.14, MeOH); ^{1}H and ^{13}C NMR data in DMSO-d_6 and CD_3OD given in Table 1; ESI-MS: *m*/*z* 321.2 $[M+Na]^+$. HRMS (ESI): calcd for $C_{16}H_{26}NaO_5$: 321.1678; found 321.1685 $[M+Na]^+$.

3.4. Preparation of diol 8a

Gracilioether H (**8**, 1.1 mg) was reacted with Zn powder (51 mg) in Et_2O (1 mL) containing AcOH (70 μL) and stirred overnight at rt. The mixture was filtered and separated by reversed-phase HPLC (Nucleodur 100-5 C18, 5 μm; 4.6 mm i.d. ×250 mm) with 70% MeOH/H_2O as eluent to give diol **8a**. ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 0.92 (t, *J*=7.2 Hz, H_3-16), 0.95 (t, *J*=7.3 Hz, H_3-12), 0.98 (t, *J*=7.4 Hz, H_3-10), 1.00 (t, *J*=7.4 Hz, H_3-14), 1.44 (dd, *J*=13.4, 9.9 Hz, H-5b), 1.56 (m, H-9b), 1.65 (m, H-9a), 1.72 (m, H_2-15), 1.76 (m, H-11b), 1.81 (ovl, H_2-13), 1.83 (ovl, H-7), 1.84 (ovl, H-11a), 2.13 (m, H-6), 2.20 (dd, *J*=13.4, 7.4 Hz, H-5a), 2.67 (d, *J*=7.6 Hz, H-3), 3.73 (m, H-8). ^{13}C NMR (125 MHz, CD_3OD): 8.4 (C-12), 8.4 (C-14), 11.0 (C-10), 12.2 (C-16), 26.7 (C-15), 30.2 (C-9), 33.8 (C-11), 33.9 (C-13), 41.1 (C-6), 43.7 (C-5), 52.3 (C-3), 54.7 (C-7), 70.9 (C-8), 78.6 (C-2), 93.8 (C-4), 180.1 (C-1).

3.5. General procedure for the preparation of MTPA esters (7a,b, 8b,c, and 10a,b)

0.5 mg samples were dissolved in freshly distilled CH_2Cl_2 and treated with triethylamine (10 μL), (*R*)- or (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) (5 μL) and a catalytic amount of 4-(dimethylamino)pyridine to obtain, respectively, (*S*)- or (*R*)-MTPA esters. The mixture was left to stand at room temperature for 1 h, with the resulting mixture purified by HPLC on a Luna 5 μ Silica(2) column (5 μm; 4.6 mm i.d. ×250 mm) with 80% hexane/EtOAc as eluent (flow rate 1.0 mL/min).

[(*S*)-MTPA ester **7a**]: selected ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.30 (d, *J*=6.3 Hz, H_3-12), 1.89 (d, *J*=14.2 Hz, H-7a), 2.46 (d, *J*=14.2 Hz, H-7b), 2.93 (dd, *J*=5.1, 9.3 Hz, H-9), 3.18 (d, *J*=9.3 Hz, H-5), 4.05 (dd, *J*=5.1, 8.8 Hz, H-10), 5.72 (m, H-11).

[(*R*)-MTPA ester **7b**]: selected ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.42 (d, *J*=6.1 Hz, H_3-12), 1.86 (d, *J*=14.4 Hz, H-7a), 2.40 (d, *J*=14.4 Hz, H-7b), 2.85 (dd, *J*=5.2, 9.3 Hz, H-9), 3.19 (d, *J*=9.3 Hz, H-5), 4.06 (dd, *J*=5.1, 9.1 Hz, H-10), 5.74 (m, H-11).

[(*S*)-MTPA ester **8b**]: selected ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 0.94 (t, *J*=7.5 Hz, H_3-10), 1.33 (m, H-5a), 1.71 (m, H-9a), 1.85 (m, H-7), 1.99 (t, *J*=7.5 Hz, H-6), 2.11 (m, H-9b), 2.14 (m, H-5b), 5.41 (m, H-8).

[(*R*)-MTPA ester **8c**]: selected ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 0.81 (t, *J*=7.5 Hz, H_3-10), 1.45 (dd, *J*=13.4, 9.9 Hz, H-5a), 1.67 (m, H-9a), 2.00 (ovl, H-6), 2.00 (ovl, H-7), 2.02 (m, H-9b), 2.22 (m, H-5b), 5.45 (m, H-8).

[(*S*)-MTPA ester **10a**]: selected ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 0.92 (t, *J*=7.5 Hz, H_3-15), 1.40 (m, H-14), 2.04 (m, H-8), 2.15 (s, H-5), 5.57 (br d, *J*=3.7, H-9).

[(*R*)-MTPA ester of **10b**]: selected ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 0.84 (t, *J*=7.5 Hz, H_3-15), 1.24 (m, H-14), 2.00 (m, H-8), 2.31 (s, H-5), 5.65 (br d, *J*=3.7, H-9).

3.6. In vitro antiplasmodial activity

The *Plasmodium falciparum* FcM29 chloroquine resistant strain was cultured according to Trager and Jensen,[18] with modifications.[19] Antiplasmodial activity was determined following the [^{3}H]-hypoxanthine (Amersham-France) incorporation method.[20]

3.7. In vitro cytotoxicity

Toxicity was estimated using Vero cells (normal monkey kidney cells) cultured in the same conditions as *P. falciparum*, except for the replacement of 5% human serum with 10% fetal calf serum. After the addition of drugs to be tested at increasing concentrations, cell growth was estimated by [^{3}H]-hypoxanthine incorporation following a 48 h incubation and was compared with a control sample.[21]

3.8. In vitro antileishmanial assay

Leishmanicidal activity was determined on axenic cultures of *Leishmania infantum* amastigotes. To estimate the 50% inhibitory concentration (IC_{50}) of the drugs, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) micro-method was used as previously described.[22]

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was calculated from the equation $[\theta]=[\theta]\text{obs}/10\times l\times C$ ($[\theta]$obs=ellipticity in mdeg, C=molar concentration, and l=optical path length of the cell in cm).
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