Antifungal Amphidinol 18 and Its 7-Sulfate Derivative from the Marine Dinoflagellate Amphidinium carterae

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · JUNE 2014

Impact Factor: 3.8 · DOI: 10.1021/np500275x · Source: PubMed

4 AUTHORS:

Genoveffa Nuzzo
Italian National Research Council
15 PUBLICATIONS **71** CITATIONS
SEE PROFILE

Adele Cutignano
Italian National Research Council
67 PUBLICATIONS **996** CITATIONS
SEE PROFILE

Angela Sardo
CNR-ICB Pozzuoli
7 PUBLICATIONS **22** CITATIONS
SEE PROFILE

Angelo Fontana
Italian National Research Council
156 PUBLICATIONS **3,616** CITATIONS
SEE PROFILE

JOURNAL OF NATURAL PRODUCTS

Note

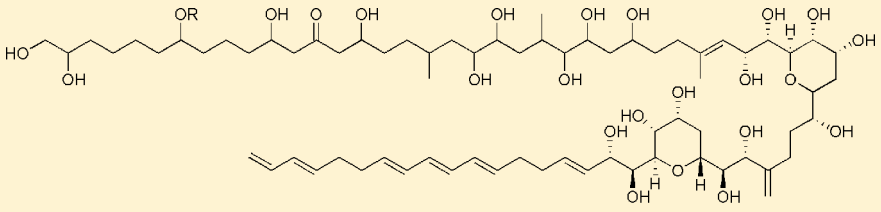
pubs.acs.org/jnp

Antifungal Amphidinol 18 and Its 7-Sulfate Derivative from the Marine Dinoflagellate *Amphidinium carterae*

Genoveffa Nuzzo, Adele Cutignano,* Angela Sardo, and Angelo Fontana

CNR, Istituto di Chimica Biomolecolare, Via Campi Flegrei 34, 80078, Pozzuoli, Naples, Italy

Supporting Information



1. R=H Amphidinol 18 (AM18)
2. R=SO_3Na Amphidinol 19 (AM19)

ABSTRACT: Two new polyketides of the amphidinol family, amphidinol 18 (AM18, **1**) and its corresponding 7-sulfate derivative (AM19, **2**), have been isolated from the MeOH extract of the dinoflagellate *Amphidinium carterae*. Structure elucidation of the two polyoxygenated molecules has been accomplished by extensive use of spectroscopic and spectrometric techniques. AM18 exhibited antifungal activity against *Candida albicans* at 9 μg/mL.

The family of amphidinols embraces a number of antifungal and hemolytic polyhydroxy-polyene polyketides typical of dinoflagellates of the genus *Amphidinium*. The first member of the series was isolated from *Amphidinium klebsii* in 1991,[1] and since then, several congeners have been reported.[2−18] These natural products share a common feature represented by two tetrahydropyran rings separated by a short C_6 chain, whose hairpin-shaped conformation possibly accounts for the antifungal activity.[8,19,20] Structural modifications occur at the two ends that contain respectively a polyunsaturated alkyl chain and a long irregular polyhydroxy arm.

In the last years we started assembling a collection of marine protists, which includes dinoflagellates obtained from commercial sources or by isolation from the coasts and the brackish waters in the gulf of Naples. We have recently examined a commercial strain of *A. carterae* (CCMP121, Provasoli-Guillard NCMA), whose MeOH extract showed antifungal activity against *Candida albicans* (32 μg/mL). The aim of this work is to describe the isolation and identification of bioactive compounds that exemplify the activity we are assaying in this fascinating group of organisms.

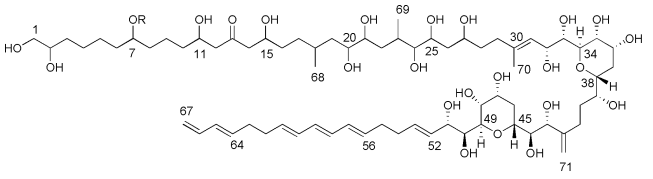
The cell pellet (3.6 g, wet weight) obtained from centrifugation of an 8 L culture of *A. carterae* was extracted with MeOH. The resulting organic material (466 mg) was separated into five fractions (A−E) on a Chromabond C18-Hydra stationary phase by using a MeOH/H_2O gradient. Fractions eluting with 50% and 75% MeOH (fractions C and D, respectively) gave a significant antifungal activity (32 and 4 μg/mL) against *C. albicans*. These fractions were further purified by RP-HPLC, yielding pure amphidinols 18 (AM18, **1**) and 19 (AM19, **2**) together with some minor congeners still under characterization.

HRESIMS of **1** (6.3 mg) on a sodium adduct ion $[M + Na]^+$ at m/z 1381.825 75 revealed a molecular formula of $C_{71}H_{122}O_{24}$ accounting for 11 formal unsaturations, while the UV λ_{max} at 260, 270, and 282 nm suggested the occurrence of a conjugated triene. NMR spectra (CD_3OD/C_5D_5N, 2:1, Table S1) revealed 71 carbon signals including a ketone resonating at 211.3 ppm and 16 sp^2 carbons (2 CH_2, 12 CH, and 2 quaternary carbons) assigned to eight double bonds that fulfilled nine out of the 11 degrees of the required unsaturations and suggested the presence of two cycles. Along with three methyl, 24 methylene, and two aliphatic methine groups, the carbon spectra showed 25 oxygenated carbons in the region between 67.3 and 80.6 ppm. According to the deuterium-induced shifts observed in ^{13}C NMR spectra acquired in CD_3OD and CD_3OH, 21 of these signals could be assigned to hydroxy groups, whereas the remaining four oxygenated methine carbons were part of ethereal bonds of two tetrahydropyran rings. In consideration of previous reports,[1−14] these data identified AM18 as a polyketide member of the amphidinol family.

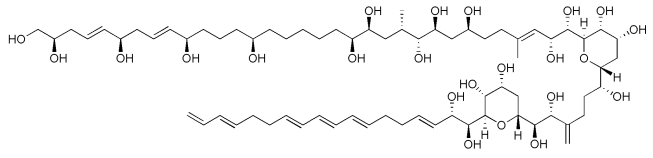
The planar structure of the polyoxygenated metabolite was elucidated by combining information from bidimensional NMR experiments and tandem mass spectrometry. In particular, a careful analysis of homonuclear (COSY and TOCSY) and heteronuclear (edHSQC, HMBC, H2BC, HSQC-TOCSY) data allowed the identification of five spin systems, A (C-1/C-

Received: March 27, 2014
Published: June 13, 2014

dx.doi.org/10.1021/np500275x | *J. Nat. Prod.* 2014, 77, 1524−1527



1. R=H Amphidinol 18 (AM18)
2. R=SO_3Na Amphidinol 19 (AM19)



3. Amphidinol 3 (AM3)

4), B (C-10/C-12), C (C-14/C-29), D (C-31/C-41), and E (C-43/C-67), as depicted in Figure 1.

HMBC correlations of signals at δ 4.18/211.3 (H-11/C-13) and 4.13/211.3 (H-15/C-13) allowed us to join the two fragments B and C through the keto group at C-13. Analogously, the connection between the substructures C and D was secured on the basis of the H2BC correlations among the methylene protons at δ 2.12 and 2.26 (H_2-29) and the quaternary sp^2 carbon at 138.6 ppm (C-30) of a trisubstituted double bond (H-31, δ 5.67 and Me-70 δ 1.75). Furthermore, cross-peaks of the exomethylene function at 151.8 and 112.4 ppm (C-42 and CH_2-71, respectively) with both the oxymethine proton at δ 4.42 (H-43) and the methylene at δ 2.27 and 2.65 (H_2-41) allowed us to join the two fragments D and E. This latter part of the molecule was characterized by six double bonds, including the conjugated triene (C-56/C-61) and a terminal diene (C-64/C-67), that were separated by two ethylene bridges. The geometries of all the double bonds of the molecule were inferred as *E* on the basis of the coupling constants (H-52/H-53, J = 15.6 Hz) and the chemical shift values of the allylic carbons (all above 30 ppm) or of the vinylic methyl group C-70 (below 20 ppm).

With the confirmation of the presence of the two tetrahydropyran rings on the basis of two diagnostic long-range correlations between H-34/C-38 and H-45/C-49, the assignment was completed by the identification of the last hydroxy function (δ 3.58/72.0 ppm) and four aliphatic methylene groups that could be predicted on the basis of the molecular formula of **1**. A key HMBC correlation between the carbinolic proton at δ 4.18 (H-11) and the methylene carbon at 22.9 ppm (C-9) led us to locate this latter carbon next to substructure B. On the other hand, inspection of the MS/MS spectra revealed two sodiated ions at m/z 169.1 and 139.1 corresponding to the fragments $C_7H_{14}O_3$ and $C_6H_{12}O_2$. This fragmentation was consistent with α cleavage at both sides of an oxygenated secondary function at C-7 according to the structure depicted. MS/MS spectra (Supporting Information) also contained a series of more intense ions that largely support the structure assignment (Figure 2).

The relative configurations of the pyran rings C-34/C-38 and C-45/C-49 were determined on the basis of proton/proton couplings and NOE effects (Figure 3). In particular, NOESY correlations observed between H-33/H-36, H-36/H-38, and H-37ax and H-39 suggested a chair conformation for the first ring with H-36 and H-38 in a 1,3-diaxial orientation. A small J coupling (1.9 Hz) measured between protons at δ 4.27 and 4.34 indicated equatorial orientations for H-34 and H-35.

Analogously, the NOE correlation observed for H-45/H-51, along with the size of the coupling constants for the coupled protons H-46ax/H-47 (12.1 Hz), H-45/H-46ax (12.1 Hz), and H-48/H-49 (1.5 Hz) suggested a chair conformation for the second ring, having protons H-45 and H-47 in axial orientations.

The more polar compound **2** showed NMR data very similar to those recorded for **1**. In fact, the NMR spectra of the two compounds were almost superimposable except for the downfield shift of the oxygenated methine (C-7, 80.6 ppm; H-7, δ 4.39) in **2**. This effect was ascribed to the presence of a sulfate ester at C-7 on the basis of the MS data that indicated a molecular formula of $C_{71}H_{122}O_{27}S$ and the presence of diagnostic fragments in the MS/MS spectra of the molecular ion at m/z 96.9587 (HSO_4^-) and 142.9385 ($HSO_4Na_2^+$) in negative and positive ion mode, respectively. Additional mass fragments and carbon chemical shifts of the region around the sulfate group were in good agreement with the depicted structure of **2**. This assignment was further confirmed by acidic hydrolysis, which gave the desulfated product, whose data were identical to those of **1** (see Supporting Information).

Chemical shift values in the region C-25/C-52 and the conformations of the two pyran rings of **1** are identical to those reported for AM3 (**3**),[3] whose absolute configuration has been determined by *J*-based configuration analysis[21] and synthesis.[22−26] Thus, we suggest the same configuration for the central core of **1** and **2** even if an enantiomeric configuration of the bis-tetrahydropyran ring fragment has been recently reported for karlotoxin-2 from *Karlodinium veneficum*.[16] Severe overlapping of the signals prevented any additional NMR analysis, thus leaving unassigned the relative configuration of the segment C-1/C-27. It is worth noting that the position of the sulfate ester at C-7 of **2** is unusual, as sulfation of amphidinols has been hitherto reported only at C-1 or C-2. AM18 (**1**) showed antifungal activity against *Candida albicans* (9 μg/mL). Interestingly, the activity of the purified product was lower than that of the original fraction D, thus suggesting

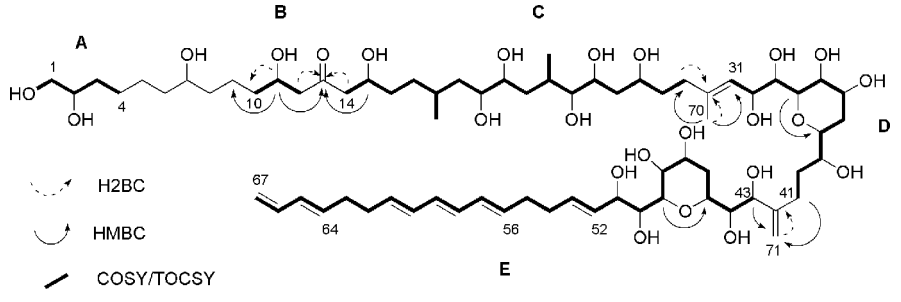


Figure 1. Spin systems A−E identified by COSY/TOCSY correlations and their connection by key H2BC/HMBC cross-peaks.

dx.doi.org/10.1021/np500275x | *J. Nat. Prod.* 2014, 77, 1524−1527

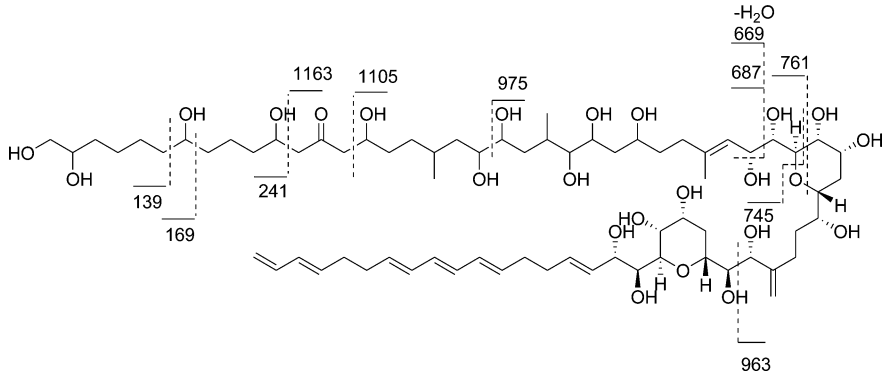


Figure 2. Positive ESIMS/MS fragmentation of the sodium adduct ion $[M + Na]^+$ of AM18 at m/z 1381.825 75.

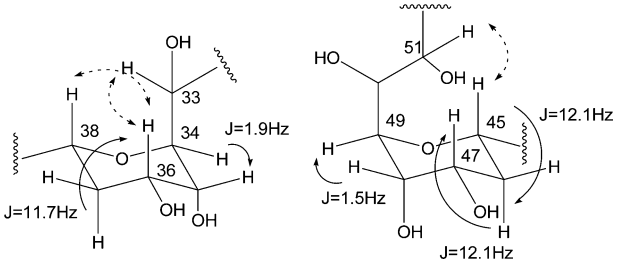


Figure 3. Relative configurations of the tetrahydropyran rings of AM-18 (**1**). Plain arrows denote coupling constants; dashed arrows denote NOE effects.

the presence of other, uncharacterized bioactive compounds. On the other hand, the absence of antifungal activity for **2** confirms that the sulfation affects negatively the anti-infective properties of amphidinols regardless of the position of esterification.[7] AM18 (**1**) and its 7-sulfate ester AM19 (**2**) are two new members of the growing family of amphidinols, bioactive polyketides showing antifungal and hemolytic activity.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco P2000 digital polarimeter. UV spectra were acquired on a Jasco V-650 spectrophotometer; NMR spectra were recorded on a Bruker Avance DRX 600 equipped with a cryoprobe operating at 600 MHz for proton. Chemical shifts values are reported in ppm (δ) and referenced to internal signals of residual protons (CD_3OD ^1H δ 3.34, ^{13}C 49.0 ppm; CD_3OH 49.15 ppm). High-resolution mass spectra and LC-MS/MS were acquired on a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) coupled with a UPLC Infinity 1290 apparatus (Agilent Technologies); HPLC analyses have been performed on a Jasco system (PU-2089 Plus-Quaternary gradient pump equipped with a Jasco MD-2018 Plus photodiode array detector).

Biological Material. *Amphidinium carterae* was purchased from Provasoli-Guillard NCMA (strain CCMP121). The dinoflagellate was cultured in K medium[27] at 22.0 ± 0.5 °C, under a 14:10 light/dark regime and at 100 μmol m^{-2} s^{-1}. The cells were mass-cultivated in eight sterile 1.8 L glass Fernbach bottles, each containing 1 L of culture. The initial cell density was around 8000 cells/mL. During the exponential phase, each subculture was halved and refreshed with 0.5 L of K medium. In the stationary phase (final cell density: 230 000 cells/mL), the 8 L of culture was harvested in a swing-out centrifuge (Allegra 12-XR), for 10 min at 4 °C at 2300*g*. The cell pellet (3.6 g) was stored at −80 °C until analysis.

Extraction and Isolation of 1 and 2. The cell pellet of *A. carterae* (3.6 g) obtained as described above was extracted with MeOH (3 × 10 mL), sonicated, and centrifuged to remove cell debris. The MeOH phase was filtered through paper and concentrated under vacuum. The extract (466 mg) was fractionated on a C-18 Hydra column (Chromabond, 20 g of dry resin) by using the following stepwise (A–E) elution protocol: A (100% H_2O, 125 mL), B (25% MeOH, 125 mL), C (50% MeOH, 125 mL), D (75% MeOH, 125 mL), and E (100% MeOH, 150 mL). Fraction D (30 mg) was further purified on an RP-HPLC column (C18-Luna, Phenomenex, 5 μm 100A 250 × 10 mm) by a MeOH/H_2O linear gradient from 65% to 80% MeOH in 20 min (flow 3 mL/min), monitoring UV absorbance at 270 nm, giving 6.4 mg of pure **1**. Compound **2** (2.5 mg) was purified from fraction C (7.0 mg) by HPLC by using an RP-HPLC column (C18-Luna, Phenomenex, 5 μm 100A 250 × 4.6 mm) and a linear gradient of MeOH/H_2O/0.2% formic acid from 65% to 80% MeOH in 20 min.

Desulfation of 2. A solution of *p*-toluenesulfonic acid monohydrate (1 mg) in 50 μL of dioxane was added to 0.1 mg of compound **2** in 100 μL of dioxane. The reaction was kept at room temperature under stirring for 30 min, then diluted with water. After neutralization by 10% Na_2CO_3, the reaction mixture was extracted with EtOAc (2 × 1 mL). The organic layer was dried at reduced pressure, and the resulting material was dissolved in 1 mL of MeOH for ESI^+-LC-MS/MS analysis (see Supporting Information).

Amphidinol 18 (AM18, 1): pale yellow, amorphous solid; $[\alpha]^{25}_D$ +3 (*c* 0.44, MeOH); UV (MeOH) λ_{max} (log ε) 280 (4.12), 270 (4.23), 261 (4.13) nm; NMR data, see Tables S1 and S2 in the Supporting Information; HRESIMS m/z 1381.82575 $[M + Na]^+$ (calcd for $C_{71}H_{122}O_{24}Na$, 1381.82237).

Amphidinol 19 (AM19, 2): pale yellow, amorphous solid; $[\alpha]^{25}_D$ +1 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 281 (4.17), 270 (4.28), 261 (4.16) nm; NMR data, see Table S1 in the Supporting Information; HRESIMS m/z 1437.78594 $[M - H]^-$ (calcd for $C_{71}H_{121}O_{27}S$, 1437.78214); m/z 1483.75757 $[M - H + 2Na]^+$ (calcd for $C_{71}H_{121}Na_2O_{27}S$, 1483.76059).

Antifungal Assay. Antifungal activity was tested against *Candida albicans* with the broth microdilution liquid growth inhibition method in sterile 96-well plates. The final fungal cell concentration was 1×10^4 cfu/mL. Plates were incubated at 37 °C for 48 h. Results have been reported as MIC (minimum inhibitory concentration) in μg/mL.

ASSOCIATED CONTENT

Supporting Information

1D and 2D NMR spectra for **1** and **2**; NMR data in CD_3OD for **1** and **2** and in CD_3OD/C_5D_5N for **1**; HRESIMS/MS spectra for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: adele.cutignano@icb.cnr.it. Phone: +39 081 8675313. Fax: +39 081 8041770.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors are grateful to Servizio NMR at ICB-CNR for recording spectra and to D. Jabes (NeED Pharmaceuticals Srl) and T. Croci (Sanofi-Aventis SpA) for antifungal tests. Funding was provided by the Consiglio Nazionale delle Ricerche of Italy (CNR) (project number PM 01.020.002) and PON 00117.

■ REFERENCES

(1) Satake, M.; Murata, M.; Yasumoto, T.; Fujita, T.; Naoki, H. *J. Am. Chem. Soc.* **1991**, *113*, 9859−9861.
(2) Paul, G. K.; Matsumori, N.; Murata, M.; Tachibana, K. *Tetrahedron Lett.* **1995**, *36*, 6279−6282.
(3) Paul, G. K.; Matsumori, N.; Konoki, K.; Sasaki, M.; Murata, M.; Tachibana, K. In *Harmful and Toxic Algal Blooms*; Yasumoto, T.; Oshima, Y.; Fukuyo, Y., Eds.; I.O.C. of UNESCO: Paris, 1996; pp 503−506.
(4) Houdai, T.; Matsuoka, S.; Murata, M.; Satake, M.; Ota, S.; Oshima, Y.; Rhodes, L. L. *Tetrahedron* **2001**, *57*, 5551−5555.
(5) Paul, G. K.; Matsumori, N.; Konoki, K.; Murata, M.; Tachibana, K. *J. Mar. Biotechnol.* **1997**, *5*, 124−128.
(6) Morsy, N.; Matsuoka, S.; Houndai, T.; Matsumori, N.; Adachi, S.; Murata, M.; Iwashita, T.; Fujita, T. *Tetrahedron* **2005**, *61*, 8606−8610.
(7) Echigoya, R.; Rhodes, L.; Oshima, Y.; Satake, M. *Harmful Algae* **2005**, *4*, 383−389.
(8) Morsy, N.; Houdai, T.; Matsuoka, S.; Matsumori, N.; Adachi, S.; Oishi, T.; Murata, M.; Iwashita, T.; Fujita, T. *Bioorg. Med. Chem.* **2006**, *14*, 6548−6554.
(9) Meng, Y.; Van Wagoner, R. M.; Misner, I.; Tomas, C.; Wright, J. L. C. *J. Nat. Prod.* **2010**, *73*, 409−415.
(10) Huang, S.; Kuo, C.; Lin, Y.; Chen, Y.; Lu, C. *Tetrahedron Lett.* **2009**, *50*, 2512−2515.
(11) Huang, X.; Zhao, D.; Guo, Y.; Wu, H.; Lin, L.; Wang, Z.; Ding, J.; Lin, Y. *Biorg. Med. Chem. Lett.* **2004**, *14*, 3117−3120.
(12) Huang, X.; Zhao, D.; Guo, Y.; Wu, H.; Trivellone, E.; Cimino, G. *Tetrahedron Lett.* **2004**, *45*, 5501−5504.
(13) Hanif, N.; Ohno, O.; Kitamura, M.; Yamada, K.; Uemura, D. *J. Nat. Prod.* **2010**, *73*, 1318−1322.
(14) Washisa, K.; Koyama, T.; Yamada, K.; Kita, M.; Uemura, D. *Tetrahedron Lett.* **2006**, *47*, 2521−2525.
(15) Van Wagoner, R.; Deeds, J. R.; Satake, M.; Ribeiro, A. A.; Place, A. R.; Wright, J. L. C. *Tetrahedron Lett.* **2008**, *49*, 6457−6461.
(16) Peng, J.; Place, A. R.; Yoshida, W.; Anklin, C.; Hamann, M. T. *J. Am. Chem. Soc.* **2010**, *132*, 3277−3279.
(17) Kubota, T.; Takahashi, A.; Tsuda, M.; Kobayashi, J. *Mar. Drugs* **2005**, *3*, 113−118.
(18) Inuzuka, T.; Yamamoto, Y.; Yamada, K.; Uemura, D. *Tetrahedron Lett.* **2012**, *53*, 239−242.
(19) Swasono, R. T.; Mouri, R.; Morsy, N.; Matsumori, N.; Oishi, T.; Murata, M. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2215−2218.
(20) Houdai, T.; Matsuoka, S.; Morsy, N.; Matsumori, N.; Satake, M.; Murata, M. *Tetrahedron* **2005**, *61*, 2795−2802.
(21) Murata, M.; Matsuoka, S.; Matsumori, N.; Paul, G. K.; Tachibana, K. *J. Am. Chem. Soc.* **1999**, *121*, 870−871.
(22) Oishi, T.; Kanemoto, M.; Swasono, R.; Matsumori, N.; Murata, M. *Org. Lett.* **2008**, *10*, 5203−5206.
(23) Crimmins, M. T.; Martin, T. J.; Martinot, T. A. *Org. Lett.* **2010**, *12*, 3890−3893.
(24) Manabe, Y.; Ebine, M.; Matsumori, N.; Murata, M.; Oishi, T. *J. Nat. Prod.* **2012**, *75*, 2003−2006.
(25) Ebine, M.; Kanemoto, M.; Manabe, Y.; Konno, Y.; Sakai, K.; Matsumori, N.; Murata, M. *Org. Lett.* **2013**, *15*, 2846−2849.
(26) Rival, N.; Hanquet, G.; Bensoussan; Reymond, S.; Cossy, J.; Colobert, F. *Org. Biomol. Chem.* **2013**, *11*, 6829−6840.
(27) Keller, D. M.; Selvin, C. R.; Claus, W.; Guillard, R. L. R. *J. Phycol.* **1987**, *23*, 633−638.