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ARTICLE *in* JOURNAL OF APPLIED PHYCOLOGY · OCTOBER 2013

Impact Factor: 2.56 · DOI: 10.1007/s10811-013-0185-2

J Appl Phycol
DOI 10.1007/s10811-013-0185-2

Anti-microfouling properties of compounds isolated from several Mediterranean *Dictyota* spp.

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Received: 26 July 2013 / Revised and accepted: 10 October 2013

Abstract Brown algae of the genus *Dictyota* are widespread around the world and are common along the coasts of the Mediterranean Sea. These marine organisms keep their surface relatively free from biofouling and are known for their ability to produce a wide array of bioactive compounds, mostly diterpenes, whose ecological functions are not clearly defined. In this study, an evaluation of the chemodiversity of the *Dictyota* genus was conducted on three samples, harvested on both NW and SW Mediterranean coasts (France and Algeria, respectively). Ten compounds were purified from the organic extracts of these samples; their chemical structures were elucidated by 1D and 2D NMR spectroscopy and were compared with literature data. Among them, three new diterpenes [one dolabellane (**1**), one xenicane (**2**), and one prenylated guaiane (**3**)] were characterized together with five previously described compounds [3,4-epoxy-14-oxo-7,18-dolabelladiene (**4**), acetoxycrenulide (**5**), dictyol E (**6**), 10, 18-dihydroxydolabella-2,7-diene (**7**), and 10-acetoxy-18-hydroxydolabella-2,7-diene (**8**)]. In addition, the occurrence of two known glycerol derivatives [1-*O*-octadecenoylglycerol (**9**) and sn-3-*O*-(geranylgeranyl)glycerol (**10**)] was also determined. Some of the isolated compounds (**4**–**6** and **8**–**10**) were screened for their potential to prevent the adhesion of three bacterial strains isolated from marine biofilms in comparison with four commercial antifoulants (TBTO, Zineb, ZnPT, and CuPT): those bearing a glycerol moiety (compounds **9** and **10**) exhibited the strongest anti-adhesion effects, whatever the strain, and with a moderate toxicity. Thus, these chemical structures should be further explored for both their putative involvement in keeping the algal surface free of biofouling and the development of effective and environmentally benign antifoulants.

Keywords Antifouling · Phaeophyceae · *Dictyota* sp · Diterpenes · Structural characterization · Biofilm · Anti-adhesion bioassays

Introduction

Antifouling (AF) coatings based on organotin compounds, such as tributyltin oxide (TBTO), have been used extensively on marine constructions including ship hulls (Yebra et al. 2004). Unfortunately, these chemical substances are prejudicial to the marine environment, especially for nontarget organisms. This fact has led to their worldwide ban by the International Maritime Organization (IMO) in 2008. Consequently, particular attention is being paid to the exploration for nontoxic antifoulants.

Marine biodiversity is an inexhaustible source of bioactive molecules: seaweeds, sponges, tunicates, bryozoans, or soft corals have repeatedly been considered as potential producers of natural AF metabolites. Several reviews show that, until this day, purification of active compounds from marine organisms has provided to around 200 molecules with variable degrees of AF activities against a wide spectrum of marine fouling organisms (Fusetani 2011, 2004). Furthermore, AF compounds

Electronic supplementary material The online version of this article (doi:10.1007/s10811-013-0185-2) contains supplementary material, which is available to authorized users.

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Published online: 26 October 2013

described in the literature include a wide range of biological activities from growth inhibition to anti-settlement potential. In addition, the species targeted are as different as bacteria and barnacles (Briand 2009). Considering the constraint for new antifoulants which have to inhibit the settlement without toxic effect on nontarget marine species, the number of compounds with a real AF potential dramatically decreases.

Seaweeds produce a high number of secondary metabolites that exhibit a broad spectrum of bioactivity. The first studies dealing with the search for natural antifoulants from macroalgae were mainly conducted on crude extracts or fractions, but in recent years, particular attention has been paid to the characterization of pure algal compounds (Bhadury and Wright 2004; Fusetani 2011, 2004). Specifically, brown algae belonging to the Dictyotaceae family (e.g. *Dictyota* spp.) have been the most deeply studied for their chemical composition and for the description of biological activities and ecological functions of their metabolites. With more than 400 diterpenes described from at least 35 species sampled all over the world (MarinLit 2013), the Dictyotaceae are particularly rich in bioactive terpenes, some of them displaying a variety of AF activities (Barbosa et al. 2007; Kim et al. 2006; Schmitt et al. 1995; Schmitt et al. 1998; Viano et al. 2009).

To extend this field of study, the present work aimed to (1) investigate and compare the chemodiversity of several *Dictyota* spp. collected on two French and Algerian Mediterranean coastal sites and (2) evaluate the AF capacity of their major metabolites using bacterial anti-adhesion potential against three marine strains (*Pseudoalteromonas* sp. D41, *Paracoccus* sp. 4M6, and *Polaribacter* sp. TC5) isolated from biofilms developed on artificial surfaces.

Materials and methods

Nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker Avance spectrometer. Silica gel (Si60, 40–63 μm, Merck) was used for column chromatography (CC). Flash chromatography experiments were carried out on a Spot system from Armen Instrument (France). Semi-preparative high-performance liquid chromatography (HPLC) purifications were performed on a Prostar 210 Varian system equipped with a differential refractometer detector (Varian, Model 350 RI). All solvents were of HPLC grade.

Algal material

Three morphologically distinct *Dictyota* species were studied. Algae samples were collected from two sites: (1) a southern site on the Mediterranean coasts of Algeria (Tipaza; 36°37′32.91″N, 2°24′20.61″E) at two different dates, in August 2008 and June 2009 (samples A and B, respectively) and (2) one northern site in France (Carqueiranne 43°5′12.41″N, 6°5′3.26″E, sample C, April 2011). The Algerian samples were identified to the genus level by Dr H. Seridi, and voucher specimens are kept in the herbarium of Laboratoire de Biologie Marine (USTHB, Algeria). Samples collected from Carqueiranne (French Mediterranean coast) were authenticated as belonging to the genus *Dictyota* (*Dictyota implexa*) by Pr O. De Clerck (Ghent University, Belgium), and a voucher sample is kept in Laboratoire MAPIEM (Université de Toulon, France).

Isolation of compounds

Once in the laboratory, algae were cleaned manually from epiphytic organisms and air-dried before being ground:

1. *Sample A*. The algal thalli (290 g) were extracted with a mixture of MeOH/$CHCl_3$ (1:1 (*v*/*v*); 3×750 mL) at room temperature and led, after filtration and solvent evaporation, to a dark brown lipophilic extract (26 g). A part of this extract (11 g) was fractionated by open CC on silica gel. A stepwise solvent gradient (from *n*-hexane/EtOAc (9:1 (*v*/*v*)) to EtOAc and then from EtOAc to MeOH) was applied, and 42 fractions were obtained. Fraction 9 (176 mg), eluted with *n*-hexane/EtOAc (3:2 (*v*/*v*)), was submitted to repeated semi-preparative reversed-phase HPLC (column: Merck Purospher Star RP-18e 5 μm, 10×250 mm; eluent: MeCN/H_2O (9:1 (*v*/*v*)); flow rate 3 mL min^{-1}) to give compounds **2** (1.3 mg), **6** (dictyol E, 62 mg), and **8** (10-acetoxy-18-hydroxydolabella-2,7-diene, 21 mg). Compounds **3** (3.1 mg) and **7** (10,18-dihydroxydolabella-2,7-diene, 2.1 mg) were obtained from fraction 10 (216 mg) eluted on CC with the same mobile phase as fraction 9. These compounds were then purified by reversed-phase HPLC with MeCN/H_2O (7:3 (*v*/*v*)) as eluent. Compound **9** (1-*O*-octadecenoylglycerol, 1.3 mg) was recovered from fraction 17 (161 mg), eluted on CC with *n*-hexane/EtOAc (3:7 (*v*/*v*)), and it was purified by further HPLC purification in the same conditions as for compounds **2**, **6**, and **8**.
2. *Sample B*. The air-dried algal material (248 g) was extracted with MeOH/$CHCl_3$ (1:1; 3×500 mL), yielding a dark green oil (18.7 g) after solvent removal. A part of this crude extract (2.5 g) was submitted to flash chromatography (UV detection 205 nm; flow rate 20 mL min^{-1}) on a reversed-phase C18 column (Merck, SVFD26-RP18 model, 25–40 μm, 31 g). A linear gradient elution (from H_2O to MeOH and then from MeOH to CH_2Cl_2) was applied and led to 40 fractions. Fraction 17 (147 mg) eluted with MeOH/CH_2Cl_2 (95:5 (*v*/*v*)) gave pure compound **10** [(sn-3-*O*-(geranylgeranyl)glycerol, 37 mg] after repeated

purification by semi-preparative reversed-phase HPLC with MeCN/H_2O (9:1 (*v*/*v*)) as eluent.

3. *Sample C*. The air-dried thalli (140 g) were extracted with a mixture of MeOH/CH_2Cl_2 (1:1 (*v*/*v*); 3×500 mL) to give a dark green oily crude extract (14.5 g). This step was followed by the fractionation of a part of this crude extract (8.5 g) by column chromatography on silica gel. A gradient elution (from cyclohexane/AcOEt (9:1 (*v*/*v*)) to AcOEt and then from AcOEt to MeOH) was used and allowed the recovery of 32 fractions. Fraction 7 (1.1 g) eluted with cyclohexane/AcOEt (1:1 (*v*/*v*)) was further subjected to reversed-phase HPLC using mixtures of H_2O/MeCN as eluents and afforded compounds **1** (21.8 mg), **4** (3,4-epoxy-14-oxo-7,18-dolabelladiene, 36.8 mg), **5** (acetoxycrenulide, 80.1 mg), and **6** (dictyol E, 131.3 mg).

Structural elucidation of isolated compounds

The chemical structure of the pure compounds was elucidated on the basis of their spectral data (one-dimensional (1D) and two-dimensional (2D) NMR) and by comparison with literature values. Compounds were solubilized in $CDCl_3$ (Sigma-Aldrich, France): chemical shifts were given in ppm and were referenced to the residual solvent peaks (δ_H 7.26, δ_C 77.16).

Compound 1. ^1H and ^{13}C NMR data as well as ^1H-^1H COSY, ^1H-^1H NOESY, and heteronuclear multiple bond correlation (HMBC) correlations are reported in Table 1. All 1D and 2D NMR spectra are given in the Supporting Information.

Compound 2. ^1H and ^{13}C NMR data are reported in Table 2. All 1D and 2D NMR spectra are given in the Supporting Information.

Compound 3. ^1H and ^{13}C NMR data are reported in Table 2. All 1D and 2D NMR spectra are given in the Supporting Information.

Compound 4. ^1H and ^{13}C NMR data were identical to previously reported data (Ioannou et al. 2011). ^1H and ^{13}C spectra are given in the Supporting Information.

Compound 5. ^1H and ^{13}C NMR data were identical to previously reported data (Sun et al. 1983; Wang et al. 1996). ^1H and ^{13}C spectra are given in the Supporting Information.

Compound 6. ^1H and ^{13}C NMR data were identical to previously reported data (Sun et al. 1983; Wang et al. 1996). ^1H and ^{13}C spectra are given in the Supporting Information.

Compound 7. ^1H and ^{13}C NMR data were identical to previously reported data (Ioannou et al. 2011; Ireland and Faulkner 1977; Piattelli et al. 1995). ^1H and ^{13}C spectra are given in the Supporting Information.

Compound 8. ^1H and ^{13}C NMR data were identical to previously reported data (Ioannou et al. 2011; Ireland and Faulkner 1977). ^1H and ^{13}C spectra are given in the Supporting Information.

Compound 9. ^1H and ^{13}C NMR data were identical to previously reported data (Hirao et al. 2012). ^1H and ^{13}C spectra are given in the Supporting Information.

Compound 10. ^1H and ^{13}C NMR data were identical to previously reported data (Amico et al. 1977; Zhang et al. 2006). ^1H and ^{13}C spectra are given in the Supporting Information.

Bioassays

Chemicals

Four commercial biocides were used. Bis(tri-*n*-butyltin) oxide (TBTO) was obtained from Acros (Fisher Scientific, France) and was directly solubilized in MeOH. Zinc pyrithione (ZnPT) came from Janssen Pharmaceutica (Belgium), and copper pyrithione (CuPT) came from Arch UK Biocides (UK), while zinc ethylene-bis-dithiocarbamate (Zineb) was provided by Agrica S.A. (Bulgaria). These biocides were dissolved in DMSO.

Target organisms

Three marine bacterial strains *Pseudoalteromonas* sp. (D41), *Paracoccus* sp. (4M6), and *Polaribacter* sp. (TC5) were chosen for the bioassays. D41 was isolated in November 1998 in Brest Bay (Atlantic Ocean, France) (Leroy et al. 2007). 4M6 was isolated in March 2000 in the Morbihan Gulf (Atlantic Ocean, France) (Grasland et al. 2003). TC5 was isolated in February 2008 in Toulon Bay (Mediterranean Sea, France) (Camps et al. 2011).

Anti-adhesion assay

Anti-adhesion assay was adapted from Camps et al. (2011). Bacterial strains were grown on Vaatanen Nine Salt Solution (VNSS) (Holmstrom et al. 1998) and collected at the stationary phase. After centrifugation, cells were suspended in sterile artificial sea water (ASW) and introduced into microtiter plates (sterile black PS; Nunc, Fisher Scientific, France) with tested compounds (at eight concentrations in three replicates) in the presence of three controls: (1)

Table 1 NMR data of compound **1** ($CDCl_3$, 400 MHz)

	1					
	δ_C	DEPT	δ_H mult. (*J* in Hz)	HMBC	^1H–^1H COSY	^1H–^1H NOESY
1	45.9	C	–	–	–	–
2	42.8	CH_2	a: 1.85 dd (12.5, 4.5)	a: C-1, C-3, C-4, C-11, C-14, C-15	a: H_b-2, H-3	a: H_b-2
			b: 2.13 br t (12.5)	b: C-1, C-3, C-4, C-11, C-14, C-15	b: H_a-2, H-3	b: H_a-2, H-3, H_3-16
3	125.3	CH	5.48 ddq (11.5, 4.5, 1.5)	C-2, C-5, C-16	$H_{a,b}$-2, H_3-16	H_b-2, H_b-5, H_b-6, H_3-15
4	136.3	C	–	–	–	–
5	35.1	CH_2	a: 1.91 dd (13.5, 4.0)	a: C-3, C-4, C-6, C-7, C-16	a: H_b-5, $H_{a,b}$-6	a: H_b-5
			b: 2.63 dt (13.5, 4.0)	b: C-3, C-4, C-6, C-7, C-16	b: H_a-5, $H_{a,b}$-6	b: H-3, H_a-5
6	32.7	CH_2	a: 2.47 dq (13.0, 4.0)	a: C-4, C-5, C-7, C-8	a: $H_{a,b}$-5, H_b-6, H-7	a: H_b-6, H-7, H_3-22
			b: 2.90 qd (13.0, 4.0)	b: C-4, C-5, C-7, C-8	b: $H_{a,b}$-5a, H_a-6, H-7	b: H-3, H_a-6, H-9
7	158.9	CH	6.73 dd (12.5, 5.0)	C-5, C-6, C-8, C-9, C-17	$H_{a,b}$-6	H_a-6, H-17
8	139.2	C	–	–	–	–
9	69.3	CH	5.62 br d (12.5)	C-7, C-8, C-10, C-17, C-21	$H_{a,b}$-10	H_b-6, H_3-15
10	28.4	CH_2	a: 1.43 m	a: Overlapped	a: H-9	a: –
			b: 2.24 t (13.0)	b: C-1, C-8, C-9, C-11, C-12	b: H9, H-11	b: –
11	39.4	CH	1.43 m	Overlapped	H_b-10, H-12	
12	52.1	CH	2.38 m	C-10, C-11, C-13, C-14, C-18, C-19, C-20	H-11, H_2-13, H_a-20	H_3-19
13	28.3	CH_2	1.54 m	C-1, C-11, C-12	H-12, H_a-14	
14	43.3	CH_2	a: 1.42 m	a: Overlapped	a: H-13	a: –
			b: 1.52 m	b: C-1, C-11, C-12, C-13, C-15	b: –	b: –
15	23.9	CH_3	1.16 s	C-1, C-2, C-11, C-14	–	H-3, H-9
16	20.6	CH_3	1.57 s	C-3, C-4, C-5	H-3	H_b-2
17	193.5	CH	9.44 d (1.5)	C-7, C-8, C-9	–	H-7
18	145.3	C	–	–	–	–
19	23.4	CH_3	1.47 s	C-12, C-18, C-20	H_3-20	–
20	111.6	CH_2	a: 4.63 s	a: C-12, C-18, C-19	a: H-12, H_3-19, H_b-20	a: H-12
			b: 4.86 s	b: C-12, C-18, C-19	b: H_3-19, H_a-20	b: –
21	170.6	C	–	–	–	–
22	21.3	CH_3	1.97 s	C-9, C-21	–	H_a-6

nonspecific staining control, (2) adhesion control, and (3) positive control. In the particular case of natural compounds **4**–**6** and **8**–**10**, they were solubilized in MeOH and added in the wells. After incubation during an optimized adhesion time, the non-adhered bacteria were eliminated and the adhered cells were quantified using SYTO 61 (1 μM, λ_{exc}=628 nm, λ_{em}=645 nm). A percent of adhesion was calculated per well:

$$(\mathrm{FIi}-\mathrm{nsCi})/(\mathrm{Mean\,FIc}-\mathrm{Mean\,B}) \times 100$$

with FIi as the fluorescence intensity in a treated well (tested compound + bacteria + SYTO 61), FIc as the fluorescence intensity in a control well (bacteria + SYTO 61), nsCi as the nonspecific control (tested compound without bacteria + SYTO 61), and *B* as the blank, i.e., stain control (only SYTO 61).

Finally, a sigmoid dose–response curve was obtained when the plotted percentage of adhesion with the log of compound concentrations, after mean (n=3) and standard deviation (SD) calculation per triplicate for each concentration. EC_{50} values were then calculated for each compound.

Toxicity assay

Toxicity assay was adapted from Camps et al. (2011). For this assay, bacterial strains were picked up during the exponential phase. The microtiter plates (sterile transparent PS; Nunc, Fisher Scientific) were filled as described in the protocol of the anti-adhesion assay but using VNSS instead of ASW to allow bacterial growth. Turbidity ($OD_{600\ nm}$) was measured every hour for 8 h. When the stationary phase was reached, resazurin (50 μM, Sigma-Aldrich) was added on all the wells, and fluorescence was measured after 2 h.

The growth rate μ (h^{-1}) was calculated during the exponential phase for each strain, at each concentration: $B = B_0\, e^{\mu t}$, where B is the bacterial density at time t, expressed as

Table 2 ^{1}H and ^{13}C NMR data of compounds **2** and **3** ($CDCl_3$, 400 MHz)

	2			**3**		
	δ_C	DEPT	δ_H mult. (J in Hz)	δ_C	DEPT	δ_H mult. (J in Hz)
1	133.7	C	–	39.4	CH	2.41 td (10.5, 7.0)
2	52.0	CH	2.52 s	32.7	CH_2	a: 1.81 m
						b: 2.08 m
3	46.8	CH	1.68 m	83.6	CH	4.87 dd (10.5, 6.0)
4	30.3	CH_2	a: 1.66 m	79.7	C	–
			b: 1.76 m			
5	40.2	CH_2	a: 1.97 m	58.8	CH	1.78 dd (10.5, 9.0)
			b: 2.27 br d (11.5)			
6	136.8	C	–	72.5	CH	4.28 dd (9.0, 4.0)
7	123.0	CH	5.36 dd (11.5, 3.5)	46.8	CH	1.53 m
8	29.2	CH_2	a: 2.93 ddd (17.5, 7.5, 4.5)	23.8	CH_2	a: 1.52 m
			b: 3.16 dddd (17.5, 11.5, 2.0, 2.0)			b: 1.62 m
9	142.8	CH	7.03 dt (7.5, 2.0)	38.9	CH_2	a: 2.09 m
						b: 2.56 ddd (14.5, 5.5, 3.5)
10	33.0	CH	1.61 m	151.7	C	–
11	37.7	CH_2	1.21 m	35.2	CH	1.23 m
12	26.0	CH_2	1.91 m	34.6	CH_2	1.62 m
13	124.1	CH	5.02 br t (7.0)	25.9	CH_2	a: 1.92 m
						b: 2.04 m
14	132.0	C	–	125.0	CH	5.12 tt (7.0, 1.0)
15	17.8	CH_3	1.57 s	131.4	C	–
16	25.8	CH_3	1.66 s	25.9	CH_3	1.68 s
17	17.8	CH_3	0.95 d (6.5)	17.8	CH_3	1.60 s
18	97.7	CH	5.92 s	17.7	CH_3	0.97 d (6.5)
19	172.2	C	–	107.2	CH_2	a: 4.65 s
						b: 4.72 s
20	17.5	CH_3	1.75 s	26.4	CH_3	1.24 s
21				172.6	C	–
22				21.2	CH_3	2.12 s

the $OD_{600\ nm}$, and B_0 is the density of the inoculum. A percent of growth inhibition was calculated as follows:

$$\left(\mu_i - \mu_0/\mu_0\right) \times 100$$

with μ_i as the growth rate of the bacteria for a compound at a particular concentration and μ_0 as the growth rate of the bacteria without any compound.

Finally, after mean and SD calculation per triplicate for each concentration, a sigmoid dose–response curve was obtained when the plotted percentage of growth inhibition with the log of compound concentration and IC_{50} (inhibitory concentration for 50 % of the bacteria) was determined. For viability, the same methodology used with SYTO 61 was applied to calculate a LC_{50} (lethal concentration for 50 % of the bacteria) using resazurin FI.

Statistical analyses

The sigmoidal dose–response curve fitting and the determination of EC_{50}, IC_{50}, and LC_{50} for each compound were achieved using GraphPad Prism® (GraphPad Software, USA). Two-way ANOVA and Bonferroni post tests were applied to EC_{50} using the same software.

Results and discussion

Structural characterization of the isolated compounds

A previous study conducted in our laboratory on the chemical composition of a Mediterranean species of the genus *Dictyota* collected in Le Brusc Lagoon (French coast) led to the isolation of eleven compounds, including dictyol E (**6**) and

10-acetoxy-18-hydroxydolabella-2,7-diene (**8**) (Viano et al. 2009). Herein, we explored the chemical composition of three other batches of *Dictyota* species. From the organic extracts of these samples, ten compounds (**1**–**10**, Fig. 1) were isolated and characterized. Three of them (**1**–**3**) were found to be new, and their chemical structure was elucidated based on analysis of their 1D and 2D NMR data (Tables 1 and 2).

The 22 signals displayed on its ^{13}C NMR spectrum suggested that compound **1** was an acetylated diterpene (Table 1). This hypothesis was further supported by characteristic signals of an acetyl group on the ^{1}H and ^{13}C spectra of **1** (δ_C 170.6, C-21; δ_C 21.3, δ_H 1.97, s, CH_3-22). The other ^{13}C NMR signals were defined, with the help of distortionless enhancement by polarization transfer (DEPT) and heteronuclear single-quantum coherence (HSQC) experiments, as three methyl, seven methylene (six sp^3 and one sp^2), six methine (three sp^3, one of which being an oxygen-bearing carbon, two olefinic, and one aldehyde) and four quaternary (one sp^3 and three olefinic) carbons. The ^{1}H NMR spectrum of **1** showed signals for an aldehyde proton (δ_H 9.44, d, J=1.5 Hz, H-17), three deshielded methines (δ_H 6.73, dd, J=12.5 and 5.0 Hz, H-7; 5.62, br d, J=12.5 Hz, H-9; 5.48, ddq, J=11.5, 4.5, and 1.5 Hz, H-3), one olefinic methylene (δ_H 4.63, s, and 4.86, s, $CH_{a,b}$-20), three singlet methyls (δ_H 1.16, CH_3-15; 1.47, CH_3-19; 1.57, CH_3-16), and several complex signals attributed to methine and methylene protons, which were observed between δ_H 1.40 and 3.00. An isoprenyl group fixed at C-12 was deduced, thanks to COSY correlations between $H_{a,b}$-20/H_3-19 and H_a-20/H-12, and HMBC cross peaks from $H_{a,b}$-20 to C-12, C-18, and C-19, and from H_3-19 to C-12, C-18, and C-20. As defined in Fig. 2, additional couplings of H-11/H-12, H-12/H_2-13 and H_2-13/H_a-14 observed in the ^{1}H-^{1}H COSY spectrum of **1**, together with HMBC correlations of H_2-13 with C-11 and C-1, H_b-14 with C-1 and C-11, and H_3-15 with C-1, C-11 and C-14, allowed the identification of a five-membered ring bearing a methyl at C-1. Moreover, the presence of an 11-membered ring was supported by the homonuclear coupling systems $H_{a,b}$-2/H-3/H_3-16, $H_{a,b}$-5/$H_{a,b}$-6/H-7, and H-9/$H_{a,b}$-10/H-11 defined by COSY cross peaks and with the help of HMBC correlations from H_3-16 to C-3, C-4, and C-5, and from H-17 to C-7, C-8, and C-9. Finally, cross peaks observed in the HMBC spectrum between H-9/C-21 and H_3-22/C-9 placed the acetate group at C-9. In terms of stereochemistry, the configuration of the Δ^3 and Δ^7 double bonds have been determined as *E* on the basis of NOESY cross peaks between

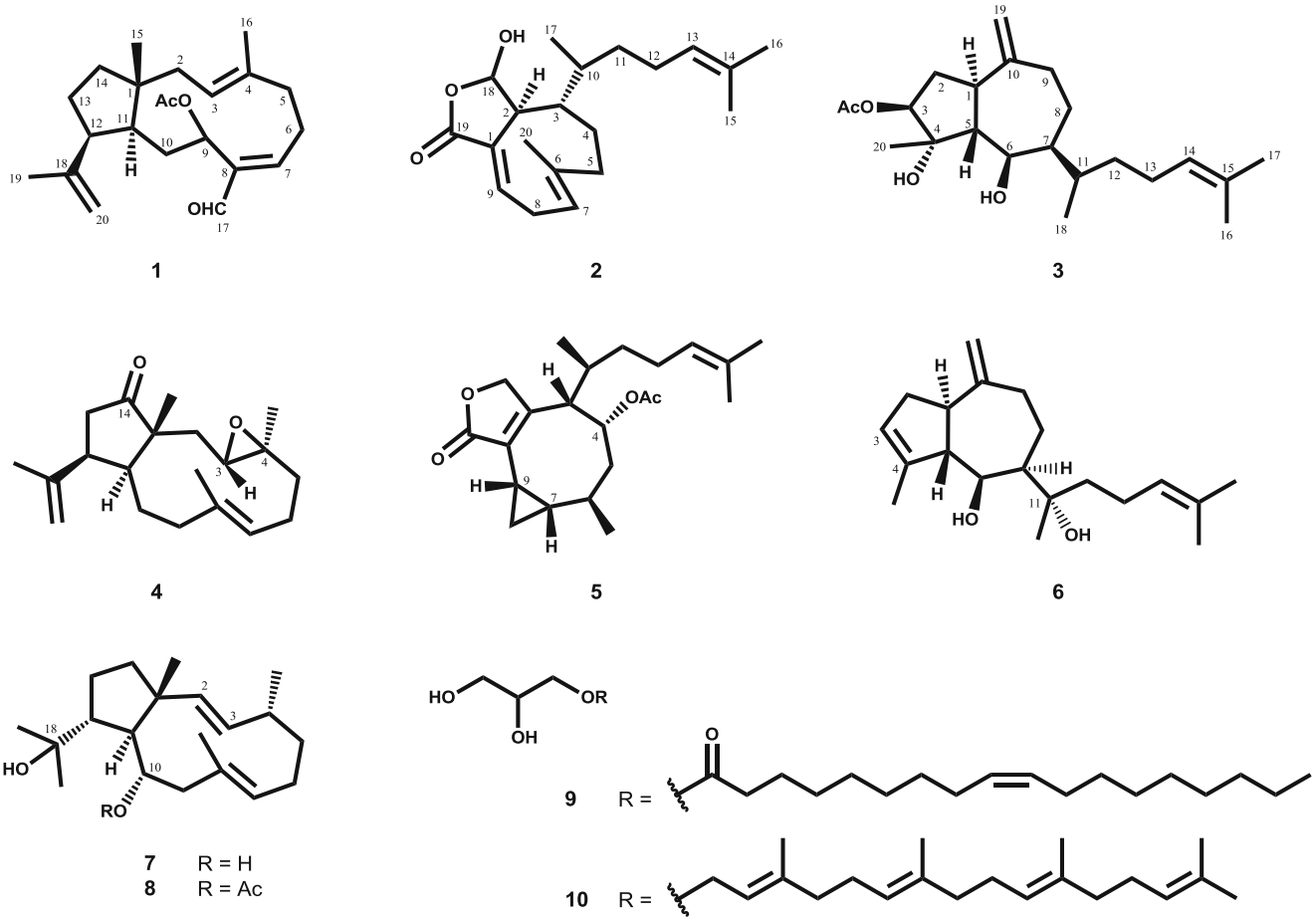

Fig. 1 Chemical structures of compounds **1**–**10**

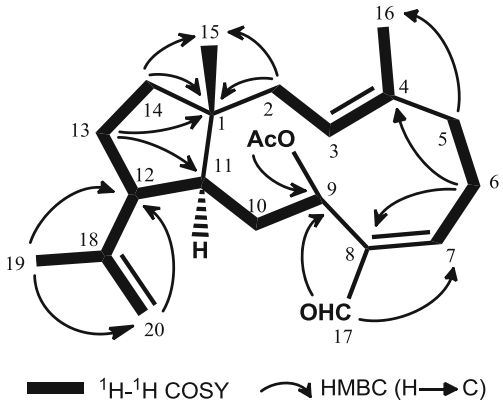

Fig. 2 Key NMR correlations for compound **1**

H-3/H_b-5, H_b-2/H_3-16, H-7/H-17, and H_b-6/H-9. The relative configuration of the stereocenters at C-1, C-11, and C-12 was deduced by comparison with the data of previously described dolabellanes bearing an isoprenyl group: in particular, the value of the ^{13}C chemical shift of C-19 (δ_C 23.4) was in accordance with a *cis* orientation of H-12 with H-11 and a *trans* orientation of these two protons with methyl CH_3-15 (Ioannou et al. 2011). Further NOESY correlations between H-3/H_b-6, H-3/H_3-15, H-9/H_b-6, and H-9/H_3-15, on one hand, and between H-7/H_a-6, and H_a-6/H_3-22, on the other hand, seemed to suggest *cis*- and *trans*-orientations for H-9 relative to H_3-15 and H-17, respectively, even if, due to the flexibility of the macrocycle, it was difficult to assert this proposition. Thus, compound **1** was identified as (1*R**, 3*E*, 7*E*, 11*S**, 12*S**) 9-acetoxy-3,7,18-dolabellatrien-17-al.

The ^{13}C NMR spectrum of compound **2** revealed 20 carbon signals (Table 2) assigned by HSQC and DEPT experiments as four methyl, five sp^3 methylene, seven methine (four sp^3 and three sp^2), and four quaternary (three olefinic sp^2 and one carbonyl) carbons. More precisely, these data evidenced the occurrence of three olefinic double bonds (δ_C 123.0, CH-7; 124.1, CH-13; 132.0, C-14; 136.8, C-6; 133.7, C-1; 142.8, CH-9) and a carbonyl group (δ_C 172.2, C-19). Moreover, characteristic signals of four methyl [three singlets (δ_H 1.57, CH_3-15; 1.66, CH_3-16; 1.75, CH_3-20) and a doublet (δ_H 0.95, J=6.5 Hz, CH_3-17)], five methylene [δ_H 1.21, m, CH_2-11; 1.66, m, and 1.76, m, $CH_{a,b}$-4; 1.91, m, CH_2-12; 1.97, m, and 2.27, d, J=11.5 Hz, $CH_{a,b}$-5; 2.93, ddd, J=17.5, 7.5, and 4.5 Hz, and 3.16, dddd, J=17.5, 11.5, 2.0, and 2.0 Hz, $CH_{a,b}$-8], and seven methine (δ_H 1.61, m, CH-10; 1.68, m, CH-3; 2.52, s, CH-2; 5.02, t, J=7.0 Hz, CH-13; 5.36, dd, J=11.5 and 3.5 Hz, CH-7; 5.92, s, CH-18; 7.03, dt, J=7.5 and 2.0 Hz, CH-9) protons were also observed on the ^{1}H NMR spectrum of **2**. Comparison of these NMR spectra with the literature data, together with analysis of ^{1}H-^{1}H COSY and HMBC spectra of **2**, allowed the identification of a 6-methyl-5-hepten-2-yl side chain and a nine-membered ring which are typical features of xenicane diterpenes. NMR data of compound **2** were very similar to those of isodictyohemiacetal (Enoki et al. 1982) and dictyolactone (Finer et al. 1979; Kim et al. 2006), two xenicanes previously isolated from dictyotacean brown algae. The main differences of **2** with these two compounds were the occurrence of NMR signals, instead of those of oxygen-bearing methylenes for (1) a lactone carbonyl group (δ_C 172.2, C-19) when **2** is compared to isodictyohemiacetal and (2) an acetal group (δ_C 97.7 and δ_H 5.92, s, CH-18) by comparison of **2** with dictyolactone. The configuration of the Δ^6 and Δ^9 double bonds and the relative configuration at C-2, C-3, and C-10 were deduced by nOes and/or by comparison of NMR data with those of similar compounds previously described from related organisms (Ovenden et al. 2012; Viano et al. 2009). Due to the small amounts and the instability of compound **2**, the configuration of the acetal carbon C-18 has not been determined.

Analysis of the ^{13}C NMR spectrum of compound **3** revealed signals for 22 carbons (Table 2) and, thus, was in accordance, in the case of metabolites isolated from dictyotacean algae, with the occurrence of an acetylated diterpene. This hypothesis was supported by typical signals of an acetate group (δ_C 172.6, C-21; δ_C 21.2 and δ_H 2.12, s, CH_3-22) observed in the ^{1}H and ^{13}C NMR spectra of **3**. Further analysis of these data allowed the identification of a vinyl methylene group (δ_C 107.2, δ_H 4.65, s, and 4.72, s, $CH_{a,b}$-19) and a 6-methyl-5-hepten-2-yl side chain. Among the dictyotacean metabolites, these two groups have been only found in bicyclic diterpenoids bearing a pachydictyane skeleton, and obviously, NMR data of **3** were found to be similar to those of one of these compounds, dictyotatriol A (Duran et al. 1997). The differences between the two ^{1}H NMR data sets were the presence of an additional methyl singlet signal at δ_H 2.12 and the downfield shift of the H-3 resonance (δ_H 4.87 in **3** compared to δ_H 3.97 in dictyotatriol A). In comparison with dictyotatriol A, the ^{13}C NMR spectral data of **3** showed additional signals characteristic of an acetate group, and the signals associated with C-2, C-3, and C-4 were shifted (from δ_C 32.7, 83.6 and 79.7 in **3** to δ_C 34.4, 79.6, and 80.5 in dictyotatriol A, respectively). All these data were consistent with **3** being the 3-acetoxyl derivative of dictyotatriol A.

The other compounds were already described, and their spectral data were identical to those previously reported in the literature. Compound **4** (3,4-epoxy-14-oxo-7,18-dolabelladiene) was initially isolated from *Dictyota dichotoma* (Amico et al. 1980; Piattelli et al. 1995), and its stereochemistry was recently revised when this metabolite was found in the organic extracts of the dictyotacean alga *Dilophus spiralis*, currently recognized as *Dictyota spiralis* (Ioannou et al. 2011). This compound was also isolated from *Dilophus ligulatus*, which was also regarded as *Dictyota spiralis* (Bouaicha et al. 1993). Compound **5** (acetoxycrenulide) was first isolated from *Dictyota crenulata* (Sun et al. 1983) and from the digestive glands of the sea hare *Aplysia vaccaria* (Midland et al. 1983), which is known to

feed on these algae. This compound was later described from various samples of *D. dichotoma* (Kolesnikova et al. 2009; Siamopoulou et al. 2004), and from *Dictyota volubilis* (Wright et al. 1990), and *Dilophus ligulatus* (Bouaicha et al. 1993). Compound **6** (dictyol E) appeared to be a common metabolite of members of the genus *Dictyota* since it was previously found in *D. dichotoma* (Amico et al. 1980; Blount et al. 1982; Siamopoulou et al. 2004), *Dictyota menstrualis* (Cronin et al. 1995; Schmitt et al. 1995), *Dictyota ciliolata* (Cronin et al. 1995), *Dictyota guineensis* (De Paula et al. 2012), *Dictyota fasciola* (Ioannou et al. 2009), *Dictyota* sp. (Viano et al. 2009), but also in *D. ligulatus* (Danise et al. 1977), *Dilophus mediterraneus*, (currently regarded as *Dictyota mediterranea* (Goez et al. 1994)), *Pachydictyon coriaceum* (currently recognized as *Dictyota coriacea* (Choi et al. 2011)), *Glossophora galapagensis* (renamed *Dictyota galapagensis* (Sun and Fenical 1979)), and in a *Halimeda stuposa–Dictyota* sp. assemblage (Ovenden et al. 2012). Two previous studies reported the isolation of compound **7** (10,18-dihydroxydolabella-2,7-diene) from the digestive gland of the opisthobranch mollusk *Dolabella californica* (Ireland and Faulkner 1977) but also from *D. dichotoma* (Amico et al. 1980; Duran et al. 1997; Piattelli et al. 1995), *Dictyota linearis* (Siamopoulou et al. 2004), *D. mediterraneus* (Goez et al. 1994) and *G. galapagensis* (Sun and Fenical 1979). Compound **8** (10-acetoxy-18-hydroxydolabella-2,7-diene), which was the 10-acetoxyl derivative of **7**, was firstly isolated from the sea hare *D. californica* (Ireland and Faulkner 1977), and it was also previously reported from organic extracts of several Dictyotaceae: *D. dichotoma* (Amico et al. 1980; Duran et al. 1997; Siamopoulou et al. 2004), *Dictyota* sp. (Viano et al. 2009), *D. linearis* (Siamopoulou et al. 2004), *Dilophus okamurai* (Suzuki et al. 2002), *Dilophus spiralis* (Ioannou et al. 2011), *D. mediterraneus* (Goez et al. 1994), and *G. galapagensis* (Sun and Fenical 1979). Compound **9** is a monoacylglycerol already characterized from the marine brown alga *Ishige sinicola* (Hirao et al. 2012). Compound **10** [sn-3-*O*-(geranylgeranyl)glycerol] was first isolated from *Dilophus fasciola* [currently regarded as *D. fasciola* (Amico et al. 1977)] and after that from the dictyotacean brown alga *Taonia lacheana* (Tringali et al. 1995).

The main constituents of the crude extracts were not the same for the different samples A, B, and C. These variations may be mainly related to their taxonomic positions but could be also due to the extraction and fractionation methods, or sampling sites, in relation to environmental and ecological associated factors. Two samples studied in this work (A and C) afforded diverse cyclic diterpenes. Interestingly, dictyol E (**6**) was found in these two samples. This compound was present in relatively high amounts in the corresponding crude extracts (around 1 % of crude extract mass). This result was already observed in previous studies which described dictyol E as a major metabolite in several dictyotacean species (Choi et al. 2011; Schlenk and Gerwick 1987; Viano et al. 2009). On the contrary, no cyclic diterpene has been found in sample B: the only compound isolated, in addition to simple lipids, was sn-3-*O*-(geranylgeranyl)glycerol (**10**), a metabolite previously described from two other Dictyotaceae.

However, chemical analysis of algae belonging to the Dictyotaceae requires caution in the interpretation of the results. Some studies have demonstrated that qualitative and quantitative variations in the chemical composition occurred in the case of a same species sampled in different areas (Teixeira et al. 1990). Moreover, in the case of the *Dictyota* species, their identification is a challenging task due to the lack of easily defined species-discriminating characters (De Clerck et al. 2001). Consequently, "intraspecific" variation in their chemical composition could be due to the presence of several species. Thus, it would be necessary to conduct extensive research based not only on morphological traits but also on phylogenetic analysis. Chemical fingerprinting of Dictyotaceae may be also a good additional tool to species discrimination as has been already reported in other brown algae (Jégou et al. 2010).

Anti-microfouling activity

The capacity of natural products and four standard biocides (TBTO, Zineb, ZnPT, and CuPT) to inhibit the settlement of *Pseudoalteromonas* sp. D41, *Paracoccus* sp. 4M6, and *Polaribacter* sp. TC5 was compared. EC_{50} values (Table 3) were dependent on both the strains and the assayed compounds (two-way ANOVA, $p<0.0001$). Both strains D41 and 4M6 were highly sensitive to TBTO showing EC_{50} lower than 5 μM, while a higher concentration (23 μM) was needed to inhibit the adhesion of 50 % of TC5 cells ($p<0.01$). These results were consistent with those previously reported (Camps et al. 2011). All the strains showed similar sensitivity to Zineb (EC_{50} between 20 and 60 μM, $p>0.05$). Likewise, ZnPT and CuPT, which are known to be effective AF agents (Faimali et al. 2003; Myers et al. 2006; Zhou et al. 2006), showed no significant differences between the strains ($p>0.05$) with very high activities ($EC_{50}<10$ μM). No EC_{50} related to anti-adhesion potential was ever reported in the literature for these three last biocides.

Natural products appeared to have less effect in the inhibition of biofilm formation, but compounds **9** and **10** were markedly active with low EC_{50} values (between 20 and 60 μM), similar to those observed for Zineb. EC_{50} of compound **9** against TC5 was not determined, as this compound was not isolated in sufficient amounts. It is interesting to point out that both effective natural compounds (**9** and **10**) beard a glycerol moiety. This would suggest that this chemical group could be involved in their anti-adhesion activity, which has never been reported before.

Table 3 Anti-adhesion activity (EC_{50}), growth inhibition (IC_{50}), and mortality (LC_{50}) on three marine bacterial strains (D41, 4M6, and TC5) for compounds **4**–**6**, **8**–**10**, and four commercial biocides

Compound	D41				4M6				TC5			
	EC_{50} (μM)	IC_{50} (μM)	LC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	LC_{50} (μM)	SI	EC_{50} (μM)	IC_{50} (μM)	LC_{50} (μM)	SI
4	164±4, R^2=0.76	ND	ND		152[a], R^2=0.93	ND	ND		172±62, R^2=0.78	ND	ND	
5	82±28, R^2=0.87	ND	ND		69±17, R^2=0.85	ND	ND		154±20, R^2=0.76	ND	ND	
6	100[a], R^2=0.99	ND	ND		133±3, R^2=0.83	ND	ND		92[a], R^2=0.83	ND	ND	
8	330[a], R^2=0.84	ND	ND		71±27, R^2=0.85	ND	ND		85±12, R^2=0.89	ND	ND	
9	54±4, R^2=0.85	69, R^2=0.88	95, R^2=0.77	1.8	26[a], R^2=0.76	ND	ND		ND	ND	ND	
10	34±12, R^2=0.86	68, R^2=0.76	80, R^2=0.86	2.3	35±31, R^2=0.88	137, R^2=0.76	97, R^2=0.97	2.8	50±11, R^2=0.95	>100	>100	>2
TBTO	4±1, R^2=0.98	2.5, R^2=0.90	7.8, R^2=0.86	1.9	4±3, R^2=0.93	0.9, R^2=0.90	1.1, R^2=0.63	0.25	23±12, R^2=0.92	8.4, R^2=0.98	6.3, R^2=0.96	0.3
Zineb®	31±5, R^2=0.93	87, R^2=0.89	55, R^2=0.97	1.8	23±10, R^2=0.92	52, R^2=0.80	31, R^2=0.99	1.3	53±12, R^2=0.93	6.6, R^2=0.95	23, R^2=0.86	0.4
ZnPT	4±2, R^2=0.84	4.5, R^2=0.66	1.9, R^2=0.99	0.5	2±1, R^2=0.95	0.8, R^2=0.99	0.7, R^2=0.99	0.4	5±2, R^2=0.90	0.7, R^2=0.94	0.7, R^2=0.99	0.1
CuPT	6±1, R^2=0.88	4.1, R^2=0.89	2.4, R^2=0.99	0.4	4±1, R^2=0.93	1.2, R^2=0.75	0.7, R^2=0.97	0.2	5±3, R^2=0.88	0.5, R^2=0.96	0.4, R^2=0.99	0.1

EC_{50} concentration corresponding to 50 % of the bacterial inhibition (values indicated are the averages of at least three independent measurements except some values measured one time as molecule quantities were not sufficient), *IC_{50}* expressed as the concentration corresponding to 50 % of the growth inhibition, *LC_{50}* expressed as the lethal concentration for 50 % of the bacteria, *SI* selectivity index, also called "therapeutic ratio" (LC_{50}/EC_{50}), R^2 goodness of fit of the sigmoidal dose–response curve, *ND* non-determined data

[a] Values measured one time

Compound **4** showed a low efficacy against the three strains (EC_{50}>150 μM). Compound **5** exhibited no significant differences between 4M6 and D41 with good activities (EC_{50} between 50 and 100 μM, respectively). On the contrary, its anti-adhesion activity on TC5 was approximately twice lower (p<0.05). Both compounds **6** and **8** have already been tested against the D41 strain (Viano et al. 2009). Herein, their activity was assessed against two additional strains (4M6 and TC5). All the strains exhibited similar moderate sensitivity when exposed to dictyol E (EC_{50} around 100 μM). The same compound was demonstrated to have an AF effect against the bryozoan *Bugula neritina* larvae; a lethal effect to larvae at 5 μg mL^{-1} was also reported (Schmitt et al. 1995). Compound **8** was inactive against D41 (EC_{50}>300 μM) but showed good activity against 4M6 and TC5 with EC_{50} values between 50 and 100 μM, respectively. Similar higher sensitivity values for 4M6 and TC5 were already noticed (Camps et al. 2011; Pénez et al. 2011). Unfortunately, the other compounds, namely **1**, **2**, **3** and **7**, were not assessed in this study as they have been found to be highly degradable.

Overall, among the natural products isolated in this study, glycerol derivatives [1-*O*-octadecenoylglycerol (**9**) and sn-3-*O*-(geranylgeranyl)glycerol (**10**)] showed the highest anti-adhesion activity. These two compounds were derived from Algerian specimens and collected at the same location but on different dates (August 2008 and June 2009). Comparatively, on the Mediterranean French coasts, the cyclic diterpene dictyol C was found to be the most active compound inhibiting the biofilm-forming bacteria (D41) with an EC_{50} of 30 μM (Viano et al. 2009). Consequently, the molecule effectiveness depends not only on the species but also on the geographical location where numbers and types of biofoulers may vary in response to ecological conditions. The release of metabolites by the host, in the surrounding environment, is considered as a strategy of defense. A recent study reported that the population structure as well as the density of a biofilm differed with sites (Goecke et al. 2010), and water quality may play a role in this distribution (Briand et al. 2012).

Some other algal metabolites belonging to various chemical classes have demonstrated AF properties. For example, three cyclic diterpenes isolated from the Brazilian Dictyotaceae *Canistrocarpus cervicornis* strongly inhibited the fixation of the brown mussel *Perna perna* (Bianco et al. 2009) and pachydictyol A afforded by the brown alga *D. menstrualis* was found to inhibit the settlement of the *B. neritina* larvae (Schmitt et al. 1995). Dictyolactone and sanadaol, two cyclic diterpenes isolated from *D. dichotoma*, exhibited algicidal activity against three phytoplankton species (Kim et al. 2006). The adhesion of brown mussel larvae (*P. perna*) was inhibited by a dolabellane diterpene from *Dictyota pfaffii* (Barbosa et al. 2007). Some meroditerpenoids isolated from Sargassaceae showed AF properties against several fouling

species (Cho 2013; Culioli et al. 2008; Mokrini et al. 2008). Glycerolipids from *Ishige sinicola*, including compound **9**, have algicidal properties (Hirao et al. 2012) while similar compounds described in *Undaria pinnatifida* and *Costaria costata* exhibited repellent effect against the blue mussel, *Mytilus edulis* (Katsuoka et al. 1990).

Toxicity of effective compounds

Toxicity assays were carried out only with effective compounds, namely the four commercial biocides (TBTO, Zineb, ZnPT, and CuPT), and compounds **9** and **10**. These assays were used to clarify whether the effectiveness of the compounds comes from their toxicity or their capacity to specifically inhibit bacterial adhesion. The same strains (D41, 4M6, and TC5) were used.

As shown in Table 3, the commercial biocides TBTO, ZnPT, and CuPT not only inhibited strongly the growth of bacteria (IC_{50} between 0.5 and 10 μM) but also exhibited a high toxicity against the three strains (LC_{50} between 0.1 and 10 μM). In comparison with our results, ZnPT and CuPT were found to be still more toxic against another marine bacterium, *Vibrio fischeri*, with IC_{50} values lower than 0.5 μM (Zhou et al. 2006). In contrast, Zineb showed a lower growth inhibitory effect (IC_{50}>50 μM except against TC5) and lower toxicity (LC_{50}>20 μM). D41 cell growth was inhibited by a relatively high concentration of both compounds **9** and **10** (IC_{50} between 50 and 75 μM), with similar toxicities (LC_{50} between 75 and 100 μM). Due to insufficient available amounts, neither IC_{50} nor LC_{50} were achieved for compound **9** in the case of TC5 and 4M6 strains. The growth inhibitory effect and the toxicity of compound **10** were much lower on TC5 and 4M6 than on D41 (IC_{50} and LC_{50} near or above 100 μM).

To assess the interest of these compounds, the selectivity index (SI), sometimes called "therapeutic ratio" (LC_{50}/EC_{50}) was determined. Generally, a compound with a LC_{50}/EC_{50} ratio >10 is considered as a nontoxic antifoulant. Nevertheless, a relatively toxic compound with a low LC_{50}/EC_{50} ratio may still be considered if it can be easily degraded in the natural environment (Qian et al. 2010). Based on this, all compounds were considered as toxic antifoulants since all the LC_{50}/EC_{50} ratios were lower than 3 and the LC_{50} values were often lower than the EC_{50} ones. However, it is noteworthy that natural compounds (**9** and **10**) always showed SI values higher than 1.5 while, in most cases, commercial antifoulants demonstrated very low SI with values below 0.5.

Conclusion

Ten compounds (**1**–**10**) have been isolated from three different samples (A–C) of *Dictyota* spp. harvested along the NW and SW Mediterranean coasts. Among them, three (**1**–**3**) were characterized here for the first time, and their chemical structure was fully elucidated by 1D and 2D NMR data. In terms of chemodiversity, these samples could be classified into two chemical groups: samples A and C in a group constituted by species producing mainly cyclic diterpenes and sample B in a group where species do not produce such compounds. Some of the isolated compounds (**4**–**6** and **8**–**10**), together with four commercial antifoulants (TBTO, Zineb, ZnPT, and CuPT), were screened for their anti-adhesion properties against three marine bacterial strains. Not surprisingly, TBTO, ZnPT, and CuPT were found to be the most active, but their toxicities confirm the need for the search of new green antifoulants. Thus, natural compounds **9** and **10** bearing a glycerol moiety showed interesting anti-adhesion effects, similar to those of Zineb but with a better selectivity index.

Further investigations concerning the assessment of the AF activity of the natural compounds against macrofoulers or the determination of minor components of the crude extracts are needed. Moreover, the characterization of the surface metabolome of algae deserves to be studied since the coupling between chemistry and ecology has become of primary importance to understand how living organisms could control foulers at their surface. In addition, when bacteria appear not to be targeted by surface molecules, their activity against macrofouler propagules like algae spores could be assessed.

Acknowledgments This work was supported by the Ministry of Higher Education and Scientific Research of Tunisia (A.O. PhD grant) and the French "Provence-Alpes-Côte d'Azur (PACA)" regional council (Y.V. PhD grant). Funds were also provided by the CMEP-PHC Tassili (N° 06 MDU 680) and the Arcus-Ceres MAEE/PACA research programs. The authors wish to thank D. Bonhomme for the technical assistance during the isolation of pure compounds from Algerian samples and Pr. O. De Clerck (Ghent University) for taxonomic identification of the algal material collected at Carqueiranne (France). C. Compère (IFREMER Brest, France) and D. Haras (LBCM Université de Bretagne Sud, France) were acknowledged for the supply of the D41 and 4M6 strains, respectively.

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