

Antifouling Activity of Celastroids Isolated from *Maytenus* Species, Natural and Sustainable Alternatives for Marine Coatings

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ABSTRACT: A group of celastroids, quinone-methide nortriterpenes isolated from *Maytenus vitis-idaea* and *Maytenus spinosa* were assayed for their antifouling activity. Toxicity assays were performed on *Balanus amphitrite* nauplii, and the most promising compounds were then incorporated in soluble-matrix antifouling paints, which were tested in the ocean. The results obtained after a 45 day-field trial of the paints indicated in all cases promising antifouling potencies. Although all compounds showed antifouling activity on a wide range of organisms, tingenone and celastrol were the most effective inhibitors of the settlement of fouling organisms. The effect of these substances on nauplii in laboratory tests was temporary instead of toxic, with a high recovery rate, which may avert a potentially adverse ecological damage on the benthic community. These results may provide a more environmentally friendly alternative for the control of biofouling, replacing toxic additives actually in use in marine paints.

■ INTRODUCTION

Marine biofouling describes the settlement and growth of a community of organisms on the surface of submerged or semisubmerged objects, both natural and artificial.¹ Any material immersed in aqueous environments is equally prone to biofouling and biodegradation.² Biofouling has been recognized as a widespread problem in the design and operation of waterborne structures, with high associated economic costs, for example in ship's hulls, oil platforms, pipes of cooling systems for power plants, and nets or cages used for aquaculture. In the shipping industry, the economic effects of biofouling are the most dramatic, since fouled hulls produce additional frictional resistance which leads to an increase of up to 40% in fuel consumption.³ Additionally, an increasing number of marine and estuarine invasive species, have been introduced to new environments via fouling of ships as a consequence of the increasing maritime global trade, and pose serious ecological problems.⁴ Chemicals currently used in antifouling coatings, such as the actually forbidden TBT or CuO can be toxic to other marine organisms.⁵ For these reasons, there is increased research in the field of ecologically "friendly" antifouling additives of natural origin.⁶

In the marine environment, the competition for space and substrate in the benthic community is intense, and fouling can have deleterious effects on these organisms. Consequently, benthic organisms have, over time, developed several strategies to confront these problems. One of the most successful is the production of antifouling secondary metabolites in what has been called a "chemical arms race".⁷ During the last 40 years, more than 16 000 new secondary metabolites have been isolated from marine organisms, mainly for their pharmacological properties.⁸ However, it is highly probable that most of

these compounds in reality play an ecological role, mainly as antifeedants or antifoulants.^{9–11}

Incorporation of bioactive compounds derived from marine organisms in matrix paints was then considered a promising strategy for the development of environmentally friendly antifouling coatings.^{12,13} However, the production of natural antifoulants from marine sources on a large scale is a big challenge, mainly for the ecological consequences of large-scale collection of marine organisms, and the lack of reproducibility which may arise from the fact that, in many cases, the bioactive compounds are really biosynthesized by symbiotic microorganisms. For these reasons, nowadays, marine organisms are considered more as a source of inspiration for the development of natural product-like antifouling compounds than a sustainable source of natural antifoulants.

A careful literature review of marine natural antifouling compounds was performed, to get clues on the presence of key structural fragments which may be involved in the observed bioactivity. One of these frequent structural motifs in bioactive marine natural products is a quinone, either attached to a terpenoid or forming part of the terpenoid skeleton itself. Several marine quinones exhibit interesting biological properties.¹⁴ In particular, avarone, a classic marine quinone, together with several semisynthetic derivatives, displayed promising activity against barnacle larvae and several strains of marine bacteria.¹⁵ Other interesting examples are puupehenone and its derivatives, isolated from sponges of the genus *Hyrtios* (order

Received: October 7, 2013

Revised: February 20, 2014

Accepted: April 8, 2014

Published: April 8, 2014

Verongida), which have cytotoxic, antimicrobial, and immunomodulatory activities which could interfere with microfouling settlement.¹⁶ Structurally speaking, puupehenone differs from other typical natural sesquiterpene quinones by having a quinonemethide system that is responsible for its unique chemical as well as biological behavior. This information was then used to search for other natural products having the same structural features, which could be accessible from alternative nonmarine sources, and the celastroids emerged as a logical choice. Celastroids are a family of nortriterpene quinonemethides with highly conjugated systems usually comprising rings A and B in a 14-nor-D:A-friedo-oleanane skeleton, which are typically isolated from the root bark of plants of the Celastraceae and Hippocrataceae families.¹⁷ In a previous work, several compounds of this family were isolated from *Maytenus vitis-idaea* and *M. spinosa*, two species collected in Salta (Argentina).¹⁸ Some of these compounds were tested for toxicity against nauplii of the cosmopolitan macrofouler *Balanus amphitrite* (Cirripedia, Balanidae). The selected compounds were then incorporated in the formulations of soluble-matrix antifouling paints, and their effect on the fouling community was studied after field trials at Mar del Plata Harbor.

MATERIALS AND METHODS

General Spectroscopic and Chromatographic Techniques. NMR experiments were performed on a Bruker Avance 2 (500 MHz) instrument at 500.13 MHz for ¹H and 125.13 MHz for ¹³C. All spectra were recorded in CDCl₃ using TMS as internal standard. HREIMS Electron-impact mass spectra were determined on a VG Auto Speccon mass spectrometer at IUBO-AG, Universidad de La Laguna, Tenerife, Spain. Optical rotations were measured on a PerkinElmer 343 polarimeter. UV spectra were obtained on a Hewlett-Packard 8453 spectrophotometer and IR spectra were recorded on a Nicolet Magna 550 spectrophotometer. Vacuum flash chromatography was carried out on reversed-phase silica gel (Aldrich Chemical Co). All solvents were distilled prior to use. HPLC separations were performed using a Thermo Separations SpectraSeries P100 pump, a Thermo Separations Refractomonitor IV RI detector and a Thermo Separations SpectraSeries UV 100 UV detector, HPLC grade solvents and YMC RP-18 (5 μm, 20 × 250 mm²; 5 μm, 10 × 250 mm²) columns. UV detection was performed at 220 nm. Sephadex LH-20 was obtained from Pharmacia Inc.; TLCs were carried out on Merck Silicagel 60 F₂₅₄ plates, using CH₂Cl₂/EtOAc mixtures as mobile phase. TLC plates were sprayed with 2% vanillin in concentrated H₂SO₄.

Plant Material. Root samples were collected at Osma, Salta (Argentina), and identified by Prof. Lázaro Novoa (Universidad Nacional de Salta, Argentina). The material was deposited at the herbarium (UNSA, Salta, Argentina) and voucher numbers are 12001 for *M. vitis-idaea* and 12281 for *M. spinosa*.

Extraction and Isolation of Compounds from *M. vitis-idaea*. Dried roots of *M. vitis-idaea* (375 g) were diced and extracted with a mixture of Et₂O/cyclohexane (1:1, 2 × 1 L) for 2 days at room temperature. The combined extracts were concentrated under reduced pressure and then triturated with CH₂Cl₂ in order to separate an insoluble latex (0.7 g). The supernatant was evaporated to dryness, giving a crude extract (2.9 g). The latter was subjected to vacuum flash chromatography on silica gel using a cyclohexane/EtOAc gradient ranging from 100% cyclohexane to 100% EtOAc, and seven fractions

(MV1–MV7) were obtained. Purification of fraction MV3 by reversed-phase HPLC with CH₃CN/H₂O (85:15) as eluant yielded compounds 1, 2 and 4. Fraction MV5 was permeated through a Sephadex LH-20 column using MeOH as eluant, and 14 fractions were collected. Fractions 6 and 7 were combined and then subjected to reversed-phase HPLC with CH₃CN/H₂O (85:15) as eluant to yield compound 3. The compounds were identified by comparison of their spectroscopic properties with literature data.^{17,19,20}

Extraction and Isolation of Compounds from *M. spinosa*. Dried roots of *M. spinosa* (400 g) were diced and extracted with a mixture of Et₂O/cyclohexane (1:1, 2 × 1 L) for 2 days at room temperature, which yielded, after evaporation at reduced pressure, a crude extract (2.7 g). Reversed-phase vacuum chromatography was used for fractionation of the crude extract, using a H₂O/MeOH gradient ranging from 50% MeOH to 100% MeOH, and four fractions (M1–M4) were obtained. Fraction M2 (600 mg, eluted with H₂O/MeOH (40:60)) was permeated through a Sephadex LH-20 column (60 × 2 cm²) using MeOH as eluant, and 30 fractions (20 mL) were collected. TLC inspection and combination resulted in nine fractions, S1–S9. Reversed-phase HPLC of fraction S8 with CH₃CN/H₂O (85:15) as eluant yielded compounds 1 and 2. Fraction M3 was in turn permeated through a Sephadex LH-20 column using MeOH as eluant. Reversed-phase HPLC (eluant: MeOH/H₂O (98:2)) afforded compound 5. The compounds were identified by comparison of their spectroscopic properties with literature data.²¹

Laboratory Tests. Adults of the barnacle *Balanus amphitrite* used for brood stock were collected from rocks and piers at Mar del Plata Harbor (Argentina, 38°02'30"S–57°32'00"W). In the laboratory, barnacle adults were maintained at 22 °C with a 12:12 h, light: dark cycle, constant aeration, and fed on a daily diet of *Artemia salina* nauplii (7 nauplii/mL). The released nauplii I were attracted to a cold light source and transferred to a beaker containing filtered seawater. Newly moulted nauplii II (approximately 1 h after release) were used for the experiments.²²

Solutions of the purified compounds were prepared to perform screening toxicity tests. Briefly, stock solutions were done by dissolving each compound in DMSO. Then, a volume was picked out in order to obtain final concentrations of 20, 30, and 40 μg/mL in seawater. The organisms were exposed to solutions for 24 h, after which the numbers of dead and living nauplii were counted. The lethal concentration for 50% of nauplii (LC₅₀) was determined.

To study the “refreshing effect”, larvae were removed from the test solutions and placed in vessels with artificial seawater. The refreshing effect was determined by observations of the organisms’ recovery of swimming movements and ability to continue their development.

Paint Preparation. For soluble matrix antifouling paints, only those compounds which demonstrated >90% toxicity on nauplii were chosen. Colophony (WW rosin) was used as binder and oleic acid as plasticizer. The paint was prepared in a laboratory scale ball mill (3.3 L jars); the operating conditions of the ball mill were chosen so as to achieve an efficient dispersion. Antifouling paints were prepared by dissolution of colophony and oleic acid in a xylene/methyl isobutyl ketone mixture (1:1) using a high-speed disperser; the ball mill was then loaded with this mixture (“vehicle”) and pigments (zinc oxide and calcium carbonate), and dispersed for 24 h. Subsequently, paints were filtered and fractionated in portions,

one of which was used as a negative control and the remaining as treatments.

For treatments, pure compounds (scutione, tingenone, celastrol, or pristimerine), previously dissolved in 1 mL of MeOH, were incorporated into the matrix paints at 0.02% Wt, based on the small amount of sample available. Finally, paints were dispersed during 1h (Table 1).

Table 1. Paint Composition

components	% w/w
zinc oxide	43.9
calcium carbonate	14.1
colophony	17.1
oleic acid	2.9
xylene/MBK (1:1)	22.0

Field Trials. Antifouling paints were applied on acrylic tiles ($4 \times 12 \text{ cm}^2$), which were previously sandblasted and degreased with toluene. Four layers of paint were applied leaving a drying time of 24 h between each coat to obtain a final dry thickness of $75 \pm 5 \mu\text{m}$. Coated panels were submerged in a marina in Mar del Plata harbor (Argentina) ($38^\circ 02' 30'' \text{S} - 57^\circ 32' 00'' \text{W}$). Controls consisted of uncoated plates and plates coated with paint matrix only. Field experiments were evaluated after 45 days exposure in the sea during the summer season. The settlement of fouling organisms was estimated as percentage cover on paints using a dot grid estimate method.²³ All tests were performed in triplicate.

Statistical Analysis. All statistical analyses were performed with IBM SPSS Statistics. Calculations of LC_{50} with 95% confidence intervals were done with Probit analysis.²⁴ Data from field trials were transformed to $1/x$. The normality assumption was verified with the Shapiro-Wilk's test.²⁵ The differences between treatment and control were determined by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

The group of available celastroids for the present study included scutione (1), tingenone (2), celastrol (3), pristimerine (4), and 7,8-dihydroisoxuxuarine E α (5) (Figure 1). Compounds 1–4 are the most common and widespread examples of this class, and have been isolated from a great number of species of *Maytenus*. In the present work, (1) and (2) were isolated from both *M. spinosa* and *M. vitis-idaea*, while compounds 3 and 4 were isolated only from the latter species. However, 5 is a less frequently isolated dimeric compound, which was first reported from *M. chuchuhuasca*, and in this case was isolated from the roots of *M. spinosa*.

Laboratory tests clearly revealed a toxic effect of *Maytenus* compounds on *B. amphitrite* nauplii II. The LC_{50} values of the five types of pure compounds indicated that naupliar inactivity occurs at concentrations lower than $20 \mu\text{g/mL}$ (Table 2). In order to determine if the effect of these compounds was temporary or permanent, nauplii exposed to solutions of the tested compounds were transferred to clean artificial seawater. Results demonstrated that larvae could recover and continue with their development, i.e., the effect of *Maytenus* compounds was temporary or narcotic. It is important to remark that recovery percentages were higher than 80% in all cases.

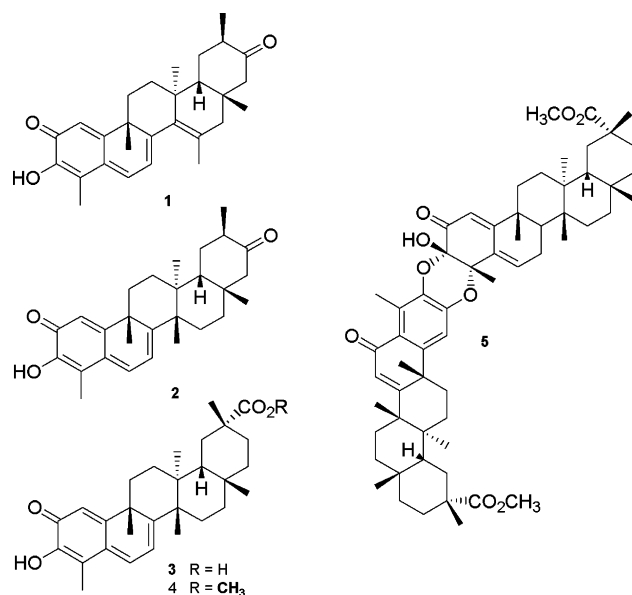


Figure 1. Celastroid structures: (1) scutione, (2) tingenone, (3) celastrol, (4) pristimerine, and (5) 7,8-dihydroisoxuxuarine E α .

Table 2. LC_{50} Values for *B. amphitrite* Nauplii II

compounds	LC_{50} ($\mu\text{g/mL}$)
scutione	13.83
pristimerine	14.20
celastrol	13.97
tingenone	12.70
7,8-dihydroisoxuxuarine E α	19.85

Since scutione, pristimerine, celastrol, and tingenone (1–4) all showed high naupliar inhibition activities at low concentrations, they were selected for field tests. Compound 5, 7,8-dihydroisoxuxuarine E α , was discarded because its larval inhibition was considerably lower than 90%. This difference in bioactivity may be explained considering that 5 is a dimeric compound with a completely different and more complex structure compared to the other four substances. Antifouling paints were prepared with the addition of one of the tested compounds; acrylic tiles were then painted and tested in the sea.

Field trials, are the most realistic assay for coating antifouling efficacy, since the abiotic and biotic factors are not regulated, and the test surfaces are offered to a diverse fouling community, which has various settlement trends.^{26,27} Settlement of foulers on the experimental paints occurs under natural conditions of flow and diffusion being exposed to a natural supply of larvae and spores of algae.

The results obtained after a 45 day-field trial of the antifouling paints incorporating compounds 1–4 indicated in all cases promising antifouling potencies (Figure 2). After the exposure period in the sea, significant differences were observed between treatments and controls. All the paints were able to inhibit the main soft-bodied and hard-shelled fouling organisms, i.e., algae (*Enteromorpha intestinalis* and *Ectocarpus* sp.), soft corals (*Antothoe chilensis*), tube-worms (*Hydroides elegans*), sandtube-builders (*Polydora ligni*), bryozoans (*Bugula stolonifera*), and colonial ascidians (*Botryllus* sp.) ($p < 0.05$) (Figure 3). Although all the compounds showed antifouling activity on a wide range of organisms, tingenone (2) and celastrol (3) were the most effective inhibitors of the settlement

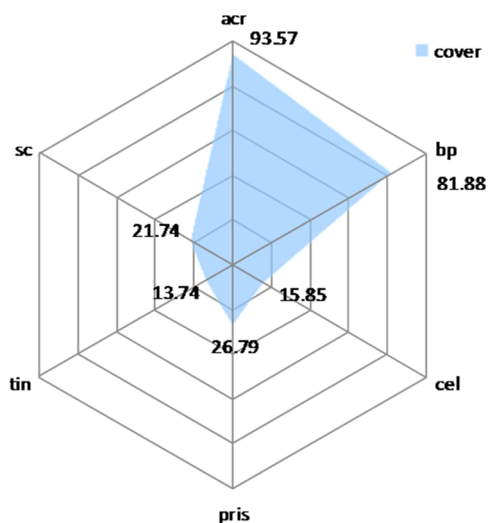


Figure 2. Fouling cover percentage on paints (45 days immersion). acr = acrylic, bp = base paint, cel = celastrol, pris = pristimerine, tin = tingenone, and sc = scutone.

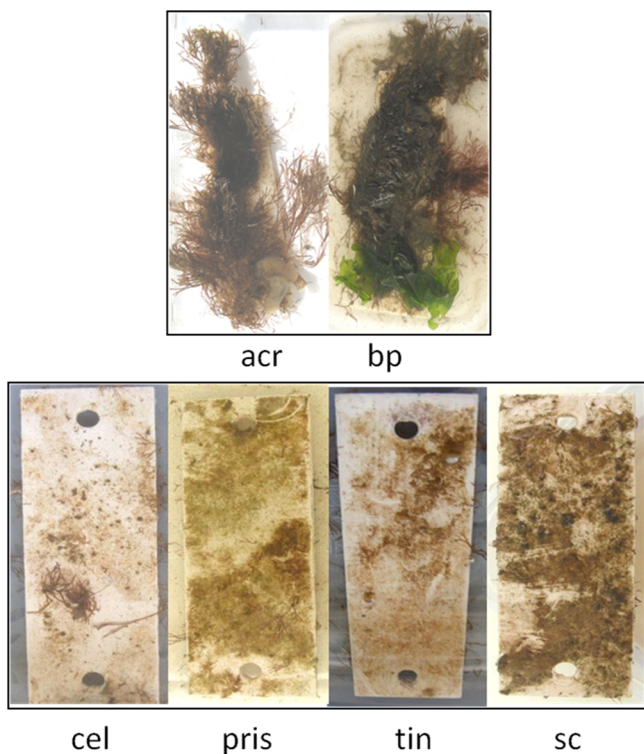


Figure 3. Painted panels after 45 days immersion. acr = acrylic, bp = base paint, cel = celastrol, pris = pristimerine, tin = tingenone, and sc = scutone.

of fouling organisms. Tingenone (2) completely inhibited the settlement of algae and the bryozoan *B. stolonifera*. In a recent paper, a similar algacide effect of tingenone has been described.²⁸ However, celastrol was more effective against the colonial ascidian *Botryllus* sp. (Figure 4). This last species was the least affected of the fouling community by the tested celastroids; however, in all cases, its inhibition was >50%.

The presence of a quinonemethide moiety in different classes of compounds has been found to be involved in several biological activities related to the growth inhibition of microorganisms or cells, such as antibacterial,^{29,30} antifun-

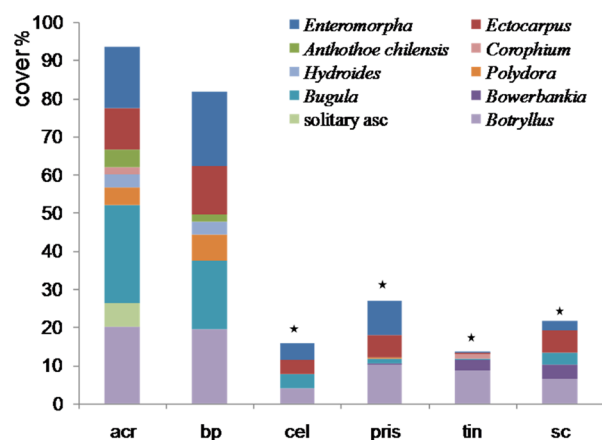


Figure 4. Fouling cover percentage on painted panels by species (45 days immersion). acr = acrylic, bp = base paint, cel = celastrol, pris = pristimerine, tin = tingenone, and sc = scutone. (*) significant differences, $p < 0.05$.

gal,^{30,31} and cytotoxic activities,^{32,33} all of which may be directly related to a possible antifouling effect. In this context, the presence of a quinonemethide moiety in the celastroid structure may play a significant role in the fouling inhibition exhibited by these compounds. However, since the observed activities were in general very good, with only a slight species-dependence, no additional structure–activity relationships can be inferred at this point. It would be also interesting to test in future experiments, combinations of different celastroids to try to extend the efficacy of these compounds against a larger diversity of fouling species.

These preliminary results indicate that the use of celastroids isolated from *Maytenus* spp. in marine coatings can be a new promising alternative to traditional cuprous oxide antifouling paints, not only for the shipping industry, but also for submerged aquaculture structures. The fact that the effect of these substances on barnacle larvae in laboratory tests were temporary or narcotic instead of toxic, with a high recovery rate, may avert a potentially adverse ecological damage on the benthic community. Besides, the final concentrations of celastroids used in the paints were very low.

These results represent a new biological activity for the celastroids, and opens up new possibilities in terms of exploring the biological activity of simpler synthetic analogues. However, the discovery of new, nontimber uses of natural resources may give support to the preservation efforts for these and other endangered plant species, and help promote their cultivation and sustainable use. However, the final sustainability of the use of celastroids for biotechnological applications will depend on a careful management of local natural resources. In this context, modern micropropagation protocols have been established for several *Maytenus* species, which may provide new plants for reforestation,³⁴ while cell tissue cultures aimed at the production of bioactive quinonemethides have also been established.³⁵ The fact that the tested compounds are produced by most of the 225 worldwide reported *Maytenus* spp. offers a large species-flexibility for these possible developments.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Comisión de Investigaciones Científicas de la provincia de Buenos Aires (CIC) Universidad Nacional de La Plata (Argentina), and Universidad de Buenos Aires for their economic support. We would also wish to thank the Club de Motonáutica of Mar del Plata for permission to use their marine testing site, and Prof. Angel Gutiérrez Ravelo (IUBO, Universidad de La Laguna, Tenerife, Spain) for mass spectra, reference NMR spectra, and helpful suggestions. M.T.R.d.A. thanks CAPES (Brazil) for a doctoral fellowship.

REFERENCES

- (1) Lewis, J. Marine biofouling and its prevention on underwater surfaces. *Mater. Forum.* **1998**, *22*, 41–61.
- (2) Gu, J. Microbiological deterioration and degradation of synthetic polymeric materials: Recent research advances. *Int. Biodeterior. Biodegrad.* **2003**, *52*, 69–91.
- (3) Yebra, D.; Kiil, S.; Dam-Johansen, K. Antifouling technology-past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Prog. Org. Coat.* **2004**, *50*, 75–104.
- (4) Molnar, J.; Gamboa, R.; Revenga, C.; Spalding, M. Assessing the global threat of invasive species to marine biodiversity. *Front. Ecol. Environ.* **2008**, *6*, 485.
- (5) Costello, M.; Grant, A.; Davies, I.; Cecchini, S.; Papoutsoglou, S.; Quigley, D.; Saroglia, M. The control of chemicals used in aquaculture in Europe. *J. Appl. Ichthyol.* **2001**, *17*, 173–180.
- (6) Chambers, L.; Stokes, K.; Walsh, F.; Wood, R. Modern approaches to marine antifouling coatings. *Surf. Coat. Technol.* **2006**, *201*, 3642–3652.
- (7) Krug, P. Defense of Benthic Invertebrates against Surface Colonization by Larvae: A Chemical Arms Race. *Marine Molecular Biotechnology*; Springer: Berlin, 2006; Vol 2.
- (8) Blunt, J.; Copp, B.; Keyzers, R.; Munro, M.; Prinsep, M. Marine natural products. *Nat. Prod. Rep.* **2012**, *29*, 144–222.
- (9) Culioli, G.; Ortalo-Magne, A.; Valls, R.; Hellio, C.; Clare, A.; Piovetti, L. Antifouling activity of meroditerpenoids from the marine brown alga *Halidrys siliquosa*. *J. Nat. Prod.* **2008**, *71*, 1121–1126.
- (10) Hellio, C.; Tsoukatou, M.; Marechal, J.; Aldred, N.; Beaupoil, C.; Clare, A.; Vagias, C.; Roussis, V. Inhibitory effects of Mediterranean sponge extracts and metabolites on larval settlement of the barnacle *Balanus amphitrite*. *Mar. Biotechnol.* **2005**, *7*, 297–305.
- (11) Kwong, T.; Miao, L.; Li, X.; Qian, P. Novel antifouling and antimicrobial compound from a marine-derived fungus *Ampelomyces* sp. *Mar. Biotechnol.* **2006**, *8*, 634–640.
- (12) Hellio, C.; De La Broise, D.; Dufossé, L.; Le Gal, Y.; Bourgougnon, N. Inhibition of marine bacteria by extracts of macroalgae: Potential use for environmentally friendly antifouling paints. *Mar. Environ. Res.* **2001**, *52*, 231–247.
- (13) Melander, C.; Moeller, P.; Ballard, T.; Richards, J.; Huigens, R.; Cavanagh, J. Evaluation of dihydrooroidin as an antifouling additive in marine paint. *Int. Biodeterior. Biodegrad.* **2009**, *63*, 529–532.
- (14) Gordaliza, M. Cytotoxic terpene quinones from marine sponges. *Mar. Drugs* **2010**, *8*, 2849–2870.
- (15) Tsoukatou, M.; Maréchal, J.; Hellio, C.; Novaković, I.; Tufegdžić, S.; Sladić, D.; Gašić, M.; Clare, A.; Vagias, C.; Roussis, V. Evaluation of the activity of the sponge metabolites avanol and avaronol

and their synthetic derivatives against fouling micro- and macro-organisms. *Molecules* **2007**, *12*, 1022–1034.

- (16) El Sayed, K.; Bartyzel, P.; Shen, X.; Perry, T.; Zjawiony, J.; Hamann, M. Marine natural products as antituberculosis agents. *Tetrahedron.* **2000**, *56*, 949–953.

- (17) Gunatilaka, A. Triterpenoids Quinonemethides and Related Compounds (Celastruloids). In *Progress in the Chemistry of Organic Natural Products*; Springer: Wien/NY, 1996; Vol. 67.

- (18) Rojo de Almeida, M.; Ríos-Luci, C.; Padrón, J.; Palermo, J. Antiproliferative terpenoids and alkaloids from the roots of *Maytenus vitis-idaea* and *Maytenus spinosa*. *Phytochemistry* **2010**, *71*, 1741–1748.

- (19) Gamlath, C.; Gunaherath, K.; Gunatilaka, A. Studies on terpenoids and steroids. Part 10: Structures of four new natural phenolic D: A-Friedo-24-noroleanane triterpenoids. *J. Chem. Soc. Perkin Trans. I* **1987**, 2849–2853.

- (20) González, A.; Alvarenga, N.; Ravelo, A.; Jiménez, I.; Bazzocchi, I.; Canela, N.; Moujir, L. Antibiotic phenol nor-triterpenes from *Maytenus canariensis*. *Phytochemistry* **1996**, *43*, 129–132.

- (21) Shiota, O.; Morita, H.; Takeya, K.; Itokawa, H. New geometric and stereoisomeric triterpene dimers from *Maytenus chuchuhuasca*. *Chem. Pharm. Bull.* **1998**, *46*, 102–106.

- (22) Hellio, C.; Marechal, J.; Veron, B.; Bremer, G.; Clare, A.; Le Gal, Y. Seasonal variation of antifouling activities of marine algae from the Brittany coast (France). *Mar. Biotechnol.* **2004**, *6*, 67–82.

- (23) Foster, M.; Harrold, C.; Hardin, D. Points versus photo quadrat estimates of the cover of sessile marine organisms. *J. Exp. Mar. Biol. Ecol.* **1991**, *146*, 193–203.

- (24) Finney, D. *Probit Analysis*, third ed.; Cambridge University Press: Cambridge, 1971.

- (25) Shapiro, S.; Wilk, M. An analysis of variance test for normality (complete samples). *Biometrika* **1965**, *52*, 591–611.

- (26) Dahms, H.; Hellio, C. Laboratory Bio-Assays for Screening Marine Antifouling Compounds. In *Advances in Marine Antifouling Coatings and Technologies*; Woodhead Publishing: Cambridge, 2009; pp 275–307.

- (27) Da Gama, B.; Pereira, R.; Soares, A.; Teixeira, V.; Yoneshigue-Valentin, Y. Is the mussel test a good indicator of antifouling activity? A comparison between laboratory and field assays. *Biofouling* **2003**, *19*, 161–169.

- (28) Silva, F.; Guedes, F.; Franco, M.; Barbosa, F.; Marra, C.; Duarte, L.; Silva, G.; Vieira-Filho, S. Algistatic effect of a quinonemethide triterpene on *Microcystis novacekii*. *J. Appl. Phycol.* **2013**, 1–6.

- (29) Williams, D.; Shamim, K. Efforts toward the total synthesis of (–)-Kendomycin. *Org. Lett.* **2005**, *7*, 4161–4164.

- (30) Cao, Y.; Chen, J.; Tan, N.; Oberer, L.; Wagner, T.; Wud, Y.; Zeng, G.; Yan, H.; Wang, Q. Antimicrobial selaginellin derivatives from *Selaginella pulvinata*. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2456–2460.

- (31) He, G.; Matsuura, H.; Takushi, T.; Kawano, S.; Yoshihara, T. A new antifungal metabolite from *Penicillium expansum*. *J. Nat. Prod.* **2004**, *67*, 1084–1087.

- (32) Yang, C.; Shao, Y.; Li, K.; Xia, W. Bioactive selaginellins from *Selaginella tamariscina* (Beauv.). *Beilstein J. Org. Chem.* **2012**, *8*, 1884–1889.

- (33) Satish, K.; Vishnuvardhan, M.; Lakshma Nayak, V.; Srihari, G.; Subrahmanyam, M.; Prabhakar Rao, T.; Ramakrishna, S.; Ravikumar, K.; Sridhard, B.; Marthanda Murthy, M. Cytotoxic diterpenoid quinonemethides from the roots of *Pygmaepremna herbacea* K. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4581–4584.

- (34) Gutiérrez-Nicolás, F.; Ravelo, Á.; Zárata, R. Seed germination and in vitro propagation of *Maytenus canariensis* through regeneration of adventitious shoots from axillary and apical buds. *Biol. Plant.* **2008**, *52*, 173–176.

- (35) Buffa Filho, W.; da Silva Bolzani, V.; Furlan, M.; Vaz Pereira, S.; Soares Pereira, A.; Castro França, S. In vitro propagation of *Maytenus ilicifolia* (Celastraceae) as potential source for antitumoral and antioxidant quinonemethide triterpenes production. A rapid quantitative method for their analysis by reverse-phase high-performance liquid chromatography. *Arquivoc* **2004**, 137–146.