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Effective natural antifouling compounds from the plant *Nerium oleander* and testing

Hui Liu[a,b], Si-Yu Chen[a,b], Jia-Ying Guo[a], Pei Su[a,b], Ying-Kun Qiu[c], Cai-Huan Ke[a,b], Dan-Qing Feng[a,b,*]

[a] State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, College of Ocean & Earth Sciences, Xiamen University, Xiamen 361000, PR China
[b] Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, Xiamen University, Xiamen 361000, PR China
[c] School of Pharmaceutical Sciences, Xiamen University, Xiamen 361000, PR China

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ABSTRACT

One major challenge to commercialization of natural antifoulants is to find the effective antifouling substances from natural flora and fauna with sufficient amount available. In this study, four cardenolides, odoroside A, digitoxigenin, oleandrin and odoroside H, were isolated from a widely distributed plant *Nerium oleander* L. These four compounds and their eight analogues were then evaluated for antifouling activity against the barnacle *Balanus albicostatus* cyprids. All of the tested compounds showed a strong inhibitory activity against barnacle settlement, with EC_{50} values ranging from 0.58 to 230.67 ng ml^{-1}. Additionally, evaluation of their lethality against a non-target organism *Artemia salina* L., revealed LC_{50} values of 17.23 to above 100 μg ml^{-1}, indicating moderate to low toxicity towards *A. salina*. Furthermore, investigation of the field antifouling performance of three *N. oleander* extracts containing cardenolides by incorporation into coatings revealed significant antifouling efficiency in marine water for 30 days. These findings indicate the commercial potential for these natural antifouling products from *N. oleander* as natural antifoulants.

1. Introduction

Marine biofouling poses serious global economic problems (e.g., reduced ship speed and increased fuel consumption) and environmental risks (e.g., increased emission of greenhouse gases and dissemination of invasive foreign species) (Fletcher, 1988; Schultz, 2007; Hellio, 2010; Poloczanska and Butler, 2010; Maréchal and Hellio, 2009; Chan et al., 2014). Antifouling biocides such as organotin, copper oxide and some herbicides have been widely applied to control marine biofouling. However, there are currently bans and regulations on the use of these antifoulants due to their negative environmental impacts (Burgess et al., 2003; Bellas, 2006; Callow and Willingham, 1996; Thomas and Brooks, 2010). Hence, there is an urgent need for environmentally friendly antifouling agents. As a promising source of such alternatives, natural antifouling products have received a lot of attention.

Studies of natural antifouling products have focused on isolating antifouling active secondary metabolites from marine organisms, including marine bacteria, fungi, algae, sponges, corals, bryozoans and ascidians (Clare, 1996; Omae, 2003; Qian et al., 2009; Chen et al., 2013; Almeida and Vasconcelos, 2015). Many marine natural products with antifouling activity have been found and identified as terpenoids, steroids, saponins, alkaloids, fatty acids, amino acids, polyketides and polyphenolics (Fusetani, 2004, 2011; Omae, 2006; Qian et al., 2015). However, these are usually difficult to produce on a large-scale for commercial because they are generally not available in sufficient quantities from marine organisms and difficult to chemically synthesize at a low cost (Raveendran and Mol, 2009; Feng et al., 2009a; Peérez et al., 2014b; Qian et al., 2015). When compared with marine organisms, many terrestrial plants can be easily harvested on a commercial scale because of their wide distribution and/or mass cultivation (Feng et al., 2009b; Pérez et al., 2014a). Moreover, a few studies have confirmed the antifouling activity of extracts and compounds from terrestrial plants. *Trans*-6-, 8- and 10-shogaols, isolated from the roots of the ginger *Zingiber officinale* Roscoe, inhibited attachment of the blue mussel *Mytilus edulis galloprovincialis*, while *trans*-8-shogaol showed antifouling activity in the field (Etoh et al., 2002). Bioassay-guided isolation of acetone extract from the stem of the betel *Piper betle* led to the discovery of four piperamides with antifouling activity, and one of their synthesized analogues, 1-[1-oxo-7-(3′,4′-methylenedioxyphenyl)-6E-heptenyl]- piperidine, exhibited inhibitory activity against barnacle

* Corresponding author. State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, College of Ocean & Earth Sciences, Xiamen University, Xiamen 361000, PR China.
E-mail address: dqfeng@xmu.edu.cn (D.-Q. Feng).

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settlement (Huang et al., 2014). Incorporation of secochiliolide acid isolated from the ethanol extract of the Patagonian shrub, *Nardophyllum bryoides*, into paint inhibited settlement of macrofouling organisms for over 45 days in the sea (Pérez et al., 2014b). Taken together, these findings indicate that terrestrial plants are a valuable source of natural antifouling products.

In the reviews conducted by Rittschof (2000) and Omae (2003), the compound bufalin, isolated from the toad skin, was suggested as the most potent natural product antifoulant. In the original report by Gerhart et al. (1993), three bufadienolides (bufalin, cinobufagin, cinobufotalin) and a cardenolide (digoxin) were found to be highly antifouling active against settlement of the barnacle *Balanus amphitrite*. However, after Gerhart et al. (1993), Rittschof (2000) and Omae (2003), no studies have investigated application of these compounds in antifouling paints or antifouling activity of other analogues. One important obstacle to their application is probably their production on a large scale. Cardenolides and bufadienolides are actually well known for their cardiac activity. The backbone structure of cardenolides has a steroid nucleus and a five-membered lactone ring at C-17 (together referred to as aglycone or genin). The sugar moiety is usually attached to the C-3-OH group. Bufadienolides are different from cardenolides in structure only in that bufadienolides are characterized by a six-membered lactone ring at C-17 (Agrawal et al., 2012). Cardenolides and bufadienolides have been found in many plants, especially in Apocynaceae, including *Nerium*, *Asclepias*, and *Digitalis* (Isman, 1977; Siddiqui et al., 1997; Afolabi et al., 2011), as well as in some animals such as toads, insects and snakes (Oycke et al., 1987; Krenn and Kopp, 1998).

Nerium oleander L. (Syn, *N. indicum* Mill; *N. odorum* Soland) (Apocynaceae) is an evergreen shrub (or small tree) distributed in many subtropical and tropical areas of the world. In China, it is widely used as an ornamental plant and a medicinal plant. In the present study, we isolated four cardenolides from this plant and evaluated their antifouling activity against settlement of *B. albicostatus* cyprids. Additionally, eight analogues were also tested against *B. albicostatus* to obtain information regarding structure-activity relationships. All the compounds were evaluated for toxicity toward a non-target organism, *Artemia salina*. Furthermore, field tests of the *N. oleander* extracts containing cardenolides were conducted to confirm their antifouling potency in the marine environment.

2. Materials and methods

2.1. Extraction, isolation and identification of active compounds from N. oleander

2.1.1. Extraction and isolation

The stems of *N. oleander* were collected from Dadeng Island, Fujian Province, China in July 2013. The species was identified by Professor Z.J. Li, School of Life Sciences, Xiamen University. The *N. oleander* stems (7.25 kg) were air-dried, powdered mechanically and extracted with methanol three times. The combined extracts (258 g) were then suspended in 90% methanol (CH_3OH, 1 l) and further extracted with petroleum ether (1 l). The remaining solution was evaporated under reduced pressure and resuspended in distilled water (1 l), after which it was successively partitioned with equal volumes of dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc) and n-butanol. The resultant five fractions, including the residual aqueous fraction, were dried under reduced pressure and then tested for antifouling activity. The active CH_2Cl_2 fraction (44.85 g) was separated on a silica gel column eluted with petroleum ether-EtOAc (5:1 then 4:1 then 3:1) to produce 11 fractions (F1 − F11). The active fractions F7 and F9 were further subjected to column chromatography. F7 (1.73 g) was isolated by silica gel column chromatography and elution with $CHCl_3$-CH_3OH (60:1 followed by 40:1, 20:1 and then 10:1) to give eight sub-fractions (F7.1 − F7.8). F7.4 (112.5 mg) was subjected to a Sephadex LH-20 column and eluted with methanol to give two fractions (F7.4.1 − F7.4.2). The fraction F7.4.1 (60.0 mg) was purified by high performance liquid chromatography (HPLC) using a C18 column (5 μm, 10 × 250 mm, ThermoScientific, USA), then eluted by a gradient of CH_3OH-H_2O (3:7–8:2) at a flow rate of 2.0 ml min^{-1} to yield compound **1** (30.0 mg, white powder), and the same method was used to purify F7.4.2 (24.3 mg) to yield compound **2** (5.8 mg, white powder). Another subfraction, F7.5 (1032.0 mg), was subjected to chromatography on a silica gel column and eluted with petroleum ether-EtOAc (4:7 followed by 4:9, 1:3 and then 1:5) before being further purified by the same HPLC C18 column as mentioned above and gradient elution with CH_3OH-H_2O (4:6–8:2) to yield compound **3** (7.4 mg, white powder). Fraction F9 (230 mg) was subjected to column chromatography using silica gel eluted with $CHCl_3$-CH_3OH (50:1 followed by 20:1, 5:1 and then 1:1) before being further purified on a silica gel column eluted with n-hexane-acetone (2:3 followed by 1:2, 1:5 and then 1:9) to obtain compound **4** (13.8 mg, white powder).

2.1.2. Identification of compounds

The structures of the compounds were identified based on NMR and MS spectral data. The NMR spectra were obtained in deuterochloroform ($CDCl_3$) or deuteromethanol (CD_3OD) on a Bruker Avance II 400 instrument (^{1}H-NMR, 400 MHz; ^{13}C-NMR, 100 MHz) with tetramethylsilane (TMS) as the internal standard. The MS data were measured in positive ion mode on a Bruker ESI-Q-TOF mass spectrometer.

2.2. Bioassay for antifouling activity against the barnacle Balanus albicostatus

B. albicostatus adults were collected from the intertidal zone in Xiamen, Fujian Province, China. Upon immersion in seawater, the adults released the naupliar larvae, which were cultured to cyprids as previously described by Feng et al. (2009a). The assay against settlement of barnacle cyprids was conducted as described by Hellio et al. (2005) and Kitano et al. (2004). The compounds isolated from *N. oleander* were dissolved in methanol and applied to glass Petri dishes (6 cm diameter). After complete evaporation of the solution, 10 ml of filtered (0.22 μm) seawater (FSW) and 30 cyprids were added to each dish. There were three replicates for each treatment and the FSW control. The Petri dishes were incubated in the dark at 25 °C for 72 h, after which the number of cyprids that had settled, died or were still swimming was counted under a stereomicroscope.

2.3. Bioassay for toxicity against the brine shrimp Artemia salina

The bioassay for toxicity against *A. salina* was conducted using the Artoxkit M procedure (Artoxkit, 1990), with slight modification. *Artemia* cysts were purchased from Wudi Aijia Pet Aquarium Co., Ltd. (Binzhou, China) and incubated in FSW with continuous illumination (3000–4000 lux) and aeration at 25 °C. After 24 h, the hatched larvae were transferred to fresh FSW and incubated for another 24 h. Next, *Artemia* nauplii of instar stages II–III were collected for use in the bioassay. The bioassay was conducted using 24-well plates. The compounds were dissolved in dimethyl sulfoxide (DMSO). A volume of 10 μl of each compound solution, 1.99 ml FSW and 10–20 nauplii were added into each well of a 24-well plate. Wells containing 0.5% DMSO in FSW (*v/v*) served as controls. There were six replicates for each treatment and the solvent control. After 24 h of incubation in darkness at 25 °C, the dead nauplii in each well were counted.

2.4. Evaluation of antifouling activity and toxicity of eight analogues

In this study, the antifouling activity and toxicity of eight analogues of the compounds we isolated from *N. oleander* (four cardenolide compounds) were tested. Seven bufadienolides (cinobufagin, resibufogenin, gamabufotalin, arenobufagin, telocinobufagin, bufotalin