Journal of Experimental Marine Biology and Ecology 445 (2013) 88–92

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Bio-organic and anti-barnacle studies of fluorescence-labeled probe compounds against cyprids of barnacles

Shoko Fujiwara [a,1], Chika Akima [a,1], Yasuyuki Nogata [b], Erina Yoshimura [c], Kazuhiro Chiba [a], Yoshikazu Kitano [a,*]

[a] Laboratory of Bio-organic Chemistry, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu-shi, Tokyo 183-8509, Japan
[b] Environmental Science Research Laboratory, Central Research Institute of Electric Power Industry, 1646 Abiko, Abiko-shi, Chiba 270-1194, Japan
[c] CERES Inc., 1-6-1 Ogawa-cho, Kanda, Chiyoda-ku, Tokyo 101-0052, Japan

ARTICLE INFO

Article history:
Received 23 November 2012
Received in revised form 22 March 2013
Accepted 10 April 2013
Available online 11 May 2013

Keywords:
Antifouling activity
Barnacle
Cypris larva
Fluorescently-labeled probe
Oil cell

ABSTRACT

The mechanism of antifouling activity was investigated by synthesizing 11 fluorescence-labeled probe compounds and using them in bio-organic studies of barnacle cyprids. Dansyl-labeled fluorescent probes with a variety of functional groups were synthesized, and their antifouling activity against barnacle cyprids was evaluated. Interactions between the synthesized probes and the barnacle cyprids were monitored under a fluorescence microscope. Probes containing ether or alkene groups showed more potent antifouling activity than other synthesized probes. After treatment with probes that had strong antifouling activity, strong fluorescence was observed in the oil cell region in barnacle cyprid. The results suggest that the action of a compound in the oil cell region in barnacle cyprid may be a factor in efficient antifouling activity.

1. Introduction

Marine fouling organisms, such as barnacles, mussels, and hydroids, cause serious economic and ecological problems by settling on ship hulls, the pipes of cooling systems for power plants, fishing nets, and aquaculture cages (Richmond and Seed, 1991; Townsin, 2003). Antifouling paints that contain a wide range of substances have been used to limit damage caused by marine fouling organisms. For example, organotin compounds, such as tributyltin (TBT), are very effective anti-fouling agents that gained widespread use in the 1960s and continued to be used for the following three decades. However, numerous reports of environmental contamination due to these compounds have brought the use of these metal-based compounds to the attention of the public. The negative environmental effects of compounds like TBT have led to regulations banning or restricting the use of metal-based antifouling agents in several countries (Clare et al., 1992; Ellis, 1991; Evans and Clarkson, 1993; Rittschof, 2001). The International Maritime Organization (IMO) passed a complete ban on the use of tin-based antifouling paints in September 2008. In recent years, Cu_2O-based marine coatings and various other booster biocides have been widely used instead of organotin compounds. However, some researchers have suggested that these compounds also have negative impacts on the environment (Armstrong et al., 2000; Bellas, 2006; Konstantinou and Albanis, 2004; Negri et al., 2002; Omae, 2003; Thomas and Brooks, 2010). Therefore, there is an urgent demand for antifouling compounds and technologies that are environmentally friendly and are non-toxic or have low toxicity.

The creation of potent antifouling agents requires detailed structure–activity relationship studies of antifouling activity and toxicity. In addition to the production of new active compounds with antifouling properties, it is necessary to understand the mechanisms of action of the compounds used in the development of antifouling agents. The use of fluorescence probes is one of the most suitable methods for investigating such mechanisms because it allows the site of action for antifouling compounds to be visualized. As a part of the search for new antifouling agents, we recently conducted a detailed structure–activity relationship study of anti-barnacle activity by using a variety of synthesized compounds. Among the compounds studied, those with isocyano functional groups had potent antifouling activity. We then synthesized several additional isocyano compounds, all of which demonstrated antifouling activity (Kitano et al., 2002, 2003, 2004, 2011; Nogata et al., 2004). In addition, we used a fluorescently-labeled probe compound that contained a dansyl group to investigate the specific mechanism of action of one of these isocyano compounds. Strong fluorescence was observed in the oil cells of barnacles and the isocyano compound affected the oil cell region in barnacle cyprids (Kitano et al., 2005). The results also showed that there might be a correlation between antifouling activity and fluorescence intensity in the oil cells of cyprids. However, the synthesized fluorescently-labeled probes were limited to two compounds: isocyanide and alkane. Detailed studies of ways in which fluorescence

* Corresponding author. Tel./fax: +81 42 367 5700.
E-mail address: kitayo@cc.tuat.ac.jp (Y. Kitano).
[1] These authors contributed equally to this work.

0022-0981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jembe.2013.04.004

probes with other functional groups act on barnacle cyprids have yet to be completed. Therefore, we synthesized additional fluorescently-labeled probes and observed the interaction between the synthesized probes and the cyprids because compounds with a wide variety of structures are reported to have antifouling properties (Fusetani, 2004; Qian et al., 2010; Rittschof, 2001). In the present study, eleven fluorescently-labeled probes, each with a different functional group, including isocyano, hydroxy, ester, halogen, ether, alkene, amide, and amine groups were synthesized. The antifouling activity of the synthesized probe compounds were evaluated against barnacle cyprids, and a fluorescence observation study of the cyprids treated with the probes was conducted in order to examine correlations between antifouling activity and fluorescence intensity.

2. Materials and methods

2.1. Cyprids

Adult barnacles, *Balanus amphitrite*, attached to bamboo poles, were procured from oyster farms in Lake Hamana, Shizuoka, Japan. Barnacles were maintained in an aquarium kept at 20 ± 1 °C and were fed with brine shrimp, *Artemia salina* nauplii. The barnacles were dried at room temperature for one day and were then immersed in seawater. After immersion, broods released I–II stage nauplii. The nauplii thus obtained were cultured in 2-L glass beakers at 25 °C at a density of 3 larvae mL^{-1}. Nauplii fed on the diatom *Chaetoceros gracilis* (initial concentration of 3.0×10^5 cells mL^{-1}). Cultures were maintained in mildly aerated 0.22-μm-filtered seawater (with salinity adjusted to 28 with deionized water) containing streptomycin (30 mg L^{-1}) and penicillin G (20 mg L^{-1}). The nauplii were cultured for four days and *C. gracilis* was added daily ($0.5–1.0 \times 10^5$ cells mL^{-1}). Larvae reached the cyprid stage in 5 days. Cyprids were stored in the dark at 5 °C until used. The day that newly transformed cyprids were collected was designated as Day 0 (Satuito et al., 1996).

2.2. Synthesis of fluorescence probes

Eleven different fluorescently-labeled probes were synthesized according to the procedure described in Supplementary material. All probes had a dansyl group as a fluorescent moiety, and each compound had a different functional group, including isocyano, hydroxy, ester, halogen, ether, alkene, amide, and amine groups. In addition, there were 12-carbon chains between a terminal functional group and the dansyl group in all probes. In this study, we synthesized isocyanide **1** because it has the same number of carbon chains as the other probes. However, in a previous study, we synthesized a fluorescently-labeled isocyanide that had an 11-carbon chain (Kitano et al., 2005).

2.3. Antifouling assay

The fluorescence probes were dissolved in MeOH. The solution was further diluted in MeOH to create the following range of fluorescence-probe concentrations: 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, or 100 μg mL^{-1}. 2 mL solution was then pipette into the wells of 24-well polystyrene tissue culture plates and air dried. 2 mL of seawater diluted to 80% with deionized water (80% filtered seawater) and six 2- or 3-day-old cyprids were then added to each well. Four wells were used for each experiment. The plates were maintained in the dark at 25 °C. After 120 h, the numbers of larvae that attached, metamorphosed, died, or did not settle were counted under a microscope (Fusetani et al., 1996; Rittschof et al., 1992). Cyprids that did not move, had extended appendages, and did not respond after a light touch by a metal probe, were counted as dead. The experiments were repeated three to five times with different batches of larvae. Settlement inhibition activity (EC_{50}) at 120 h and mortality (LC_{50}) values at 120 h were calculated according to a previously reported method (Nogata et al., 2004). Cyprids still swimming at the end of the antifouling assay were immediately fixed in 10% formalin. Fixed cyprids were observed under a fluorescence microscope that was equipped with a filter set (e.g., 340–380 nm, Br. 420 nm) in order to monitor interactions between the probes and the cyprids.

2.4. Observation of fluorescence

Fluorescence observations were performed every 24 h. Swimming cyprids were retrieved from the test plate, fixed with 10% formalin, and observed immediately under a Leica MZ FLIII microscope with a UV filter set (Ex. 340–380 nm, Br. 420 nm) equipped with an Olympus DP50 CCD color camera head. The fluorescence intensity of the oil cells area (the anterior part of the cyprid, see Supplementary material) was quantitated as the average brightness of the oil cells in each individual cyprid. The overall brightness for each compound was computed as the average fluorescence intensity for 10–20 cyprids. Average brightness was obtained using a computerized image analysis system (Win ROOF; Mitani Corp, Fukui, Japan). Digitalized images were converted to 256-gray-scale images and their average brightness was determined using Win ROOF. The digital brightness values ranged from 0 to 256, and the image background was defined as 0.

3. Results

3.1. Antifouling activity of the synthesized probe compounds and fluorescence study

The structure of the synthesized probes and their EC_{50} or LC_{50} values for cyprids, and fluorescence micrographs of cyprids treated with the probes are shown in Fig. 1. Synthesized probes **1**–**8** showed anti-barnacle activity and had EC_{50} of 0.25–12.90 μg mL^{-1} without significant toxicity ($LD_{50} > 100$ μg mL^{-1}). On the other hand, probes **9** and **10** did not show effective antifouling activity ($EC_{50} > 100$ μg mL^{-1}) and probe **11** resulted in high mortality at a low concentration ($LD_{50} = 2.23$ μg mL^{-1}).

Cyprids exposed to 10 μg mL^{-1} fluorescence probes were used for fluorescent observation because at this concentration, nearly all of the cyprids in the wells remained alive, and fewer than half of the cyprids settled (with the exception of amide probes **9** and **10**, and amine probe **11**). In addition, at concentrations higher than 10 μg mL^{-1}, the average brightness for all compounds was too high for effective comparison. At concentrations lower than 10 μg mL^{-1}, almost all of the cyprids settled and, therefore, could not be observed effectively. Because of the poor antifouling activity and high toxicity of acetamide probe **9**, formamide probe **10**, and amine probe **11**, images for these probes are not included in this report. Almost of all the cyprids used in the bioassay settled when treated with 10 μg mL^{-1} acetamide probe **9** or formamide probe **10**. All cyprids treated with 10 μg mL^{-1} amine probe **11** died.

3.2. Quantitative analyses of fluorescence intensity

As shown in Fig. 1, fluorescence was observed in the oil cell region in barnacle cyprids (cyprid fluorescence) after treatment with probes **1**–**8**. The average brightness of cyprid fluorescence at 24-h intervals over a 120-h period and the standard deviation for the final measurements are presented in Table 1. As analyzed by Win Roof, cyprid fluorescence was relatively strong in cyprids treated with alcohol probe **2**, chloride probe **5**, ether probe **7**, and alkene probe **8**; was moderate in cyprids treated with isocyanide probe **1**, acetate probe **3**, and fluoride probe **4**; and was weak in cyprids treated with bromide probe **6**. Qualitative visual observations matched the results of the computer analysis.