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Novel settlement inhibition oligopeptides containing β-amino acids†

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Efficient syntheses of tripeptides containing β-amino acids and their settlement inhibition activities toward two main foulants, the barnacle *Amphibalanus amphitrite* and the blue mussel *Mytilus galloprovincialis*, are described. The tripeptide design was inspired by a tripeptide fragment of dolastatin 16, a depsipeptide isolated from the sea hare *Dolabella auricularia*. Tripeptide with only α-amino acids did not exhibit settlement inhibition, while β-amino acid-containing tripeptides and dipeptides effectively prevented settlement. Made from inexpensive amino acids, these peptides are promising candidates for cost-effective and eco-friendly antifouling additives.

Sustainability spotlight

Toward a sustainable society, reducing carbon dioxide is well known to help maintain or lower the current Earth's temperature. Ships are one of the major sources of carbon dioxide emissions from fuel, although they are very important means of transporting large quantities of goods. One factor that wastes fuel is fouling organisms on the hull of ships, which can induce up to a 40% increase in fuel consumption. To avoid biofouling, toxic materials such as copper compounds and organic compounds have been used. These materials are known as biocides. We demonstrate easy synthesis of settlement inhibition peptide with very low toxicity. Our work will contribute to the development of new green antifouling materials for the preservation of the marine ecosystem.

Introduction

Shipping is essential for fisheries, transporting mineral resources and industrial products, and leisure activities. While ships are anchored, fouling organisms[1,2] such as barnacles and mussels accumulate, increasing fuel waste (up to 40%)[3] and contributing to increased CO_2 emissions. Huge economical costs are also incurred for the removal of these organisms. To prevent the accumulation of fouling organisms, antifouling materials are applied on the bottoms of ships. $CuSO_4$ has been widely used since the nineteenth century. Compounds containing Sn, Hg and Pb, as well as organic compounds like DDT and PCB, have also been applied as antifouling agents.[4] However, the toxicities of these compounds toward marine organisms were reported, thus resulting in the prohibition of their use in antifouling paints.[5–13] Although less toxic organic compounds, such as Zn-pyrithione, Cu-pyrithione, sea-nine 211 and triphenylborane-pyridine complex are currently used,[14] they are not only biocide antifoulants[15,16] but also have harmful environmental effects.[17]

As concerns about the environmental impact of these marine antifoulants increase, industry stakeholders are focusing more on developing eco-friendly antifouling alternatives. Marine natural products have been isolated and screened for their settlement inhibiting properties.[18–25] Additionally, organic compounds inspired by these natural products have been designed and synthesized in a few steps.[26–35] Our groups investigated the following natural products: (1) 10-isocyano-4-cadinene[36–39] (50% effective concentration (EC_{50}) = 0.14 μg mL^{-1} and 50% lethal concentration (LC_{50}) > 10 μg mL^{-1} against the cypris larvae of the barnacle *Amphibalanus amphitrite*) from nudibranchs of the family Phyllidiidae, (2) omaezallene[40,41] (EC_{50} = 0.22 μg mL^{-1} and LC_{50} = 4.8 μg mL^{-1}) from the red alga *Laurencia* sp., and (3) dolastatin 16 [42–46] (EC_{50} = 0.003 μg mL^{-1} and LC_{50} = 20 μg mL^{-1}) from the sea hare *Dolabella auricularia* as antifouling candidates (Fig. 1). These compounds were synthesized and subjected to structure–activity relationship studies using their fragments and derivatives. Among them, synthetic fragments **1** and **2** of dolastatin 16 were found to exhibit settlement inhibition activity (EC_{50} = 0.90 μg mL^{-1} and EC_{50} = 0.79 μg mL^{-1} respectively). Tripeptide fragment **2b** (EC_{50} = 0.79 μg mL^{-1}) was of particular interest as it is composed of only three amino acids: proline and two unusual amino acids, dolaphenvaline (Dpv) and dolamethylleuine (Dml). Since both unusual amino acids require a four-step synthesis, it is difficult

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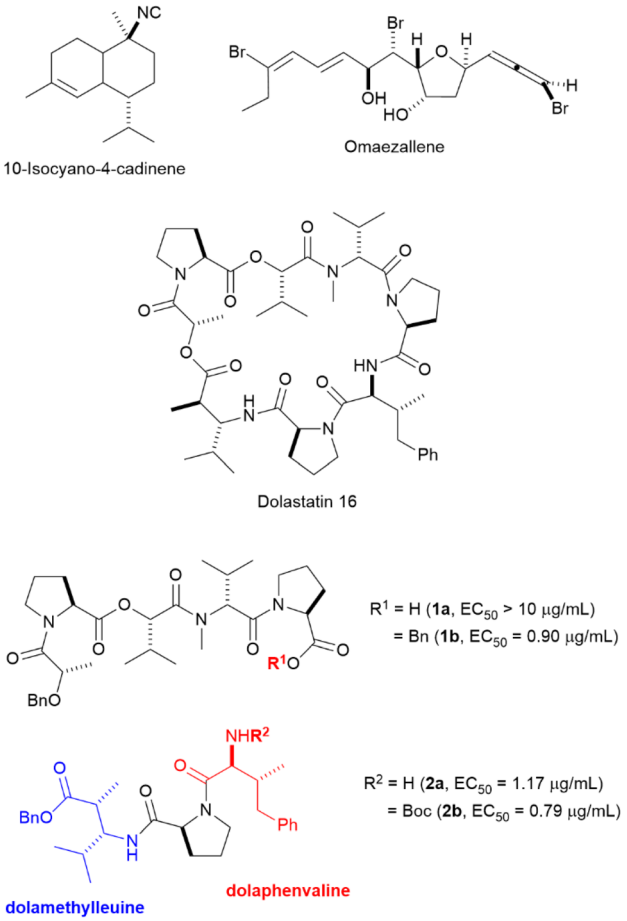

Fig. 1 Settlement inhibition natural products and fragments of dolastatin 16.

to obtain a large and effective supply of **2**. Thus, we would replace the unusual amino acids with commercially available amino acids then evaluated the resulting peptides for their settlement inhibition properties.

Herein, we describe the syntheses of simple tri- and dipeptides and their settlement inhibition activities against the cypris larvae of the barnacle *Amphibalanus amphitrite* and the blue mussel *Mytilus galloprovincialis*.

Results and discussion

L-Phenylalanine was used to replace Dpv due to their similar structures (Fig. 2). Various amino acids with alkyl groups at the α- or β-positions were then evaluated as replacements for the Dml moiety: L-alanine (Ala) ethyl ester (**3a**), L-leucine (Leu) ethyl (**3b**) and benzyl (**3c**) esters, leucine carboxylic acid (**3d**), β-alanine (β-Ala) ethyl ester (**3e**), and γ-amino butyric acid (GABA) ethyl ester (**3f**) (Fig. 3). Tripeptides **3a–c**, **3e** and **3f** were prepared in high yields by condensation reactions between dipeptide **4** and the corresponding amino acid esters in ammonium salt using PyBrop.[47] Tripeptide **3d** was prepared by hydrogenolysis of **3c** to confirm the effect of the carboxyl group on the settlement inhibition activity. Compounds **3a–3f** were evaluated for

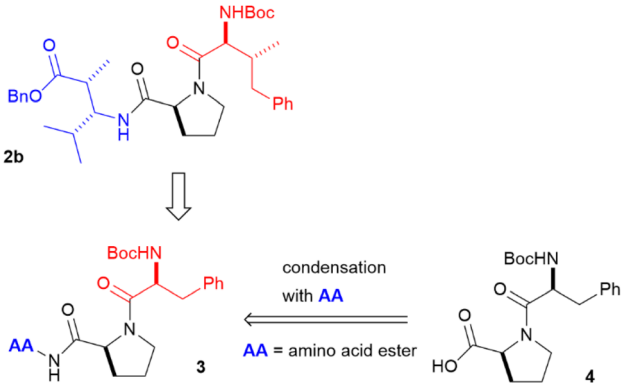

Fig. 2 Design of tripeptide.

their EC_{50} (50% effective concentration) against the cypris larvae of the barnacle *A. amphitrite* by exposing the larvae to each compound for 48 hours. All compounds did not exhibit antifouling activity (EC_{50} > 10 μg mL^{-1}) nor lethal effects on the cypris larvae (LC_{50} > 10 μg mL^{-1}).

We next prepared tripeptide **3g** to assess the effect of Dml, a β-amino acid with methyl and isopropyl groups, on

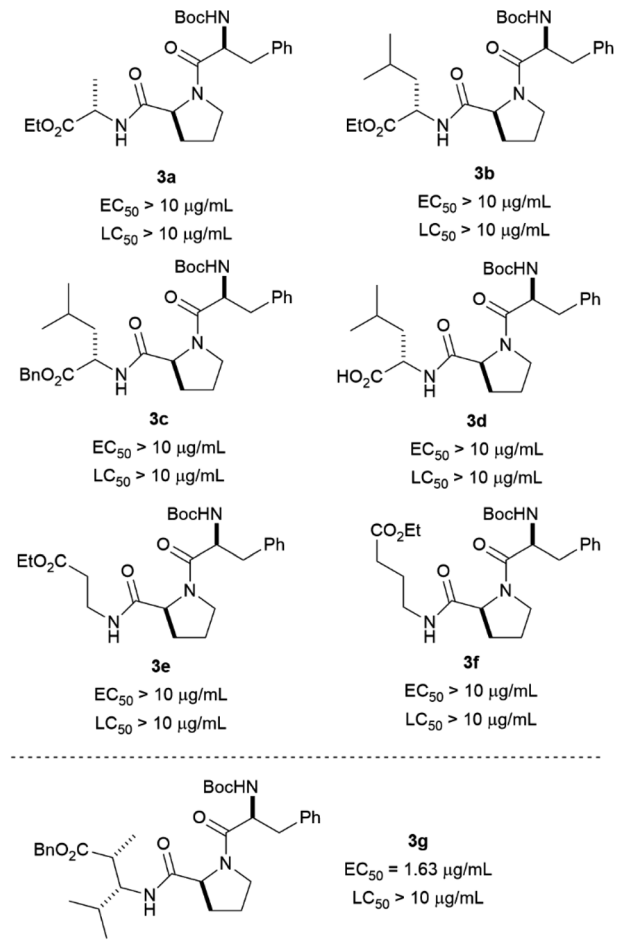

Fig. 3 Prepared tripeptides, settlement inhibition activities and toxicities toward the cypris larvae of the barnacle *Amphibalanus amphitrite*.

antifouling activity. Moderate activity was observed (EC_{50} = 1.63 μg mL^{-1}), indicating that the alkyl group containing β amino acid moiety enhances settlement inhibition. Thus, commercially available alternatives (3*R*)-methyl-β-alanine [(β-Me)βAla], (3*S*)-phenyl-β-alanine [(β-Ph)βAla], (3*R*)-isobutyl-β-alanine [(β-*i*Bu)βAla] and L-aspartic acid (Asp) were subsequently investigated. Tripeptides **3h–m**, with benzyl or ethyl esters of the amino acid, were prepared from Boc-protected amino acids in 3 steps through esterification, deprotection of the Boc group and condensation with **4** in the presence of PyBrop (Fig. 4). Among the tripeptides tested, tripeptides with ethyl esters and aspartic acid (**3h–j**) did not exhibit settlement inhibition activity (EC_{50} > 10 μg mL^{-1}). Moderate activity was found in benzyl esters (**3k–m**) (EC_{50} = 1.13–6.76 μg mL^{-1}). These results clearly suggest that the benzyl ester has significantly more effective antifouling activity than the ethyl ester. Furthermore, an alkyl or aromatic group at the β-position is essential for the benzyl ester to exhibit antifouling properties.

Next, we investigated Boc-homoPhe-Pro-(β-*i*Bu)βAla-OBn (**3n**), which incorporates the inexpensive L-homophenylalanine (homoPhe), which has the same linear carbon number as Dpv. The synthesis was achieved as follows: (1) condensation between Boc-homoPhe-OH and H-Pro-OBn, (2) deprotection of benzyl ester under hydrogenolysis conditions and (3) condensation reaction of the resulting acid and β-amino acid ester. To our surprise, **3n** showed the highest settlement inhibition activity (EC_{50} = 0.49 μg mL^{-1}, LC_{50} > 10 μg mL^{-1}), outperforming the original southern fragment **2b**, which was prepared in 15 steps from commercially available materials (Fig. 1). The LC_{50}/EC_{50} value of **3n** (>20) also suggests that this tripeptide is a non-toxic settlement inhibitor.[48] Finally, dipeptide derivatives, Boc-Pro-(β-Ph)βAla-OBn (**5a**) and Boc-Pro-Ant-OBn (**5b**), were synthesized and evaluated for their antifouling activities (Fig. 5). Amino benzoate derivatives are regarded as β-amino acids although the carbonyl and amino groups are attached to sp^2 carbon atoms. Dipeptide derivatives **5a** and **5b** exhibited moderate to high settlement inhibition activities. Notably, **5b** (EC_{50} = 0.84 μg mL^{-1}) can be synthesized in only one step from commercially available reagents, Boc-Pro and benzyl anthranilate.

The settlement inhibition activity of some of these peptides toward another fouling organism, the blue mussel *M. galloprovincialis*, was also examined. Table 1 shows the percentage (%) of *M. galloprovincialis* that attached after 72 hours exposure to the test solutions: 3.0 and 10 μg mL^{-1} of synthetic peptides (**2b**, **3h**, **3k–n**, **5a-b**), positive control (1.0 μg mL^{-1} $CuSO_4$) and negative control (no additive, 0 μg mL^{-1}). Lower values indicate more potent settlement inhibition activity. The original tripeptide **2b** having Dml and Dpv moieties showed moderate antifouling activities at 10 μg mL^{-1} (entry 1) against *M. galloprovincialis*. The synthesized tripeptides, entries 2–8 exhibited weak to moderate activities. Although there isn't a clear correlation between the antifouling activities against cypris larvae of the barnacle *A. amphitrite* and *M. galloprovincialis*, compounds with lower EC_{50} values tended to show reduced settlement of *M. galloprovincialis*, particularly for **3m** (EC_{50} = 1.13 μg mL^{-1} and 42% settlement at 3.0 μg mL^{-1}).

The structure–activity relationship study also offered some insights into the molecular mechanism of settlement inhibition. For example, peptide **3m** showed more settlement inhibition activity than **3c** despite being similar molecular formulas that only differ in their α- or β-amino acid configuration. Similarly, the anthranilate in **5b** is also classified as a β-amino acid due to the 1,2-disubstituted relationship between the alkoxycarbonyl and the amino groups. Based on these results, it is hypothesized that this 1,2-arrangement plays a crucial role in the settlement inhibition mechanism. Incorporating

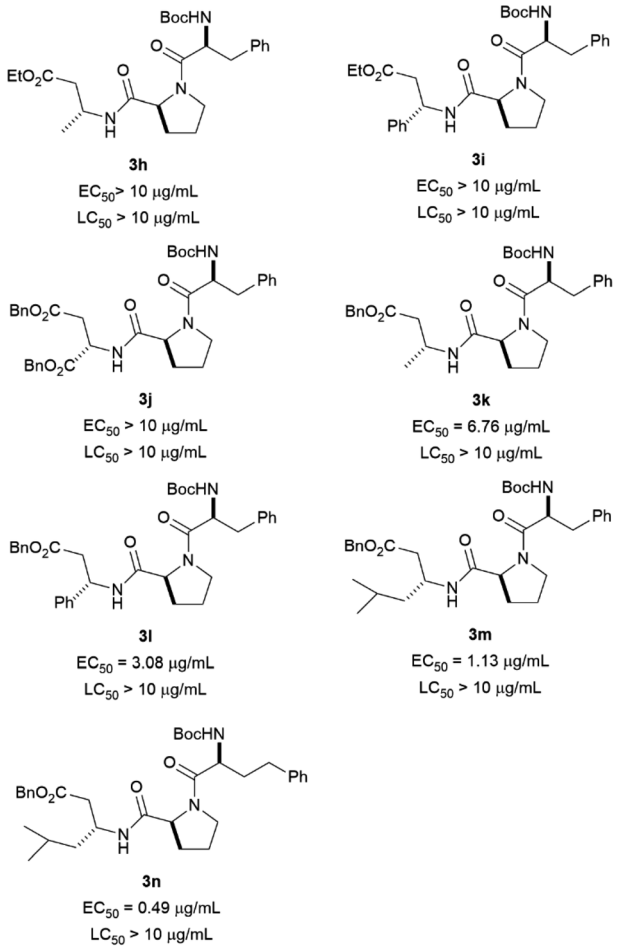

Fig. 4 Prepared tripeptides, settlement inhibition activities and toxicities toward the cypris larvae of the barnacle *Amphibalanus amphitrite*.

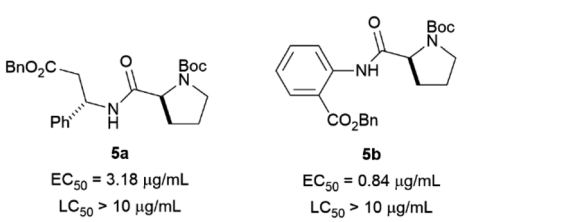

Fig. 5 Prepared dipeptide derivatives, settlement inhibition activities and toxicities toward the cypris larvae of the barnacle *Amphibalanus amphitrite*.

Table 1 Settlement inhibition test toward *Mytilus galloprovincialis*[a]

Entry	Compound	3.0 μg mL^{-1} (%)	10 μg mL^{-1} (%)	1.0 μg mL^{-1} (%)	0 μg mL^{-1} (%)	EC_{50} [b] (μg mL^{-1})
1	**2b**	67	42	—	—	0.79
2	**3h**	92	92	—	—	>10
3	**3k**	83	67	—	—	6.76
4	**3l**	100	84	—	—	3.08
5	**3m**	42	17	—	—	1.13
6	**3n**	83	75	—	—	0.49
7	**5a**	58	91	—	—	3.18
8	**5b**	67	67	—	—	0.84
9	$CuSO_4$[c]	—	—	0	—	0.29
10	None[d]	—	—	—	100	—

[a] Ratio (%) of attaching *Mytilus galloprovincialis* after 72 h is shown. [b] EC_{50} values toward the cypris larvae of the barnacle *Amphibalanus amphitrite*. [c] Positive control. [d] Negative control.

fluorescent markers to these peptides can provide deeper insights into this mechanism and facilitate the design of more effective peptide-based settlement inhibitors.

Conclusions

In summary, we have described simple tri- and dipeptides that show settlement inhibition activities toward the cypris larvae of the barnacle *Amphibalanus amphitrite* and the blue mussel *Mytilus galloprovincialis*. The EC_{50} value of compound **3n** is more potent than the original tripeptide **2b** and similar to that of $CuSO_4$ (EC_{50} = 0.29 μg mL^{-1}). These peptides could be synthesized in just a few steps from commercially available amino acids, offering an efficient and cost-effective route for large-scale production.

Experimental

General procedure for synthesis of tripeptide 3

To Boc-β-amino acid ester (1 equivalent) was added TFA/DCM (1 : 4 v/v, 0.10 M). After 1 h of stirring at room temperature, the mixture was concentrated *in vacuo*. To the residual TFA salt was added 0.5 M NaOH aq., then the mixture was extracted with DCM, washed with brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to afford the crude amine, which was used in the next step without further purification.

To a solution of crude amine in MeCN (0.30 M) was added 4 M HCl in dioxane (1.0 equivalent) under Ar atmosphere. After 10 min of stirring at room temperature, **4** (1.0 equivalent) in MeCN (0.30 M), PyBrop (1.3 equivalent) and *i*Pr_2NEt (3.0 equivalent) were added to the mixture. The reaction was stirred for 24 h, quenched with diluted NaOH, extracted with EtOAc, washed with diluted HCl and brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude product was purified by column chromatography (Hexane : AcOEt = 70 : 30) to afford tripeptide **3** (chemical yield is indicated in ESI†).

Settlement inhibition assay with *Amphibalanus amphitrite*

Settlement inhibition assay against larvae of the barnacle *A. amphitrite* and statistical analysis were conducted according to the previous literature.[45,46] The adult barnacles, *A. amphitrite*, obtained from oyster farms in Lake Hamana and a pier of Shimizu Bay, Shizuoka, were kept in an aquarium at 20 °C and were fed on *Artemia* sp. nauplii. Broods were released as I-II stage nauplii upon immersion in seawater after drying overnight. The nauplii (1.0–3.0 indiv. per mL) thus obtained were cultured in 2.0 L filtered (0.2 μm) natural seawater (diluted by DW: salinity 28) containing penicillin G (20 μg mL^{-1}) and streptomycin sulfate (30 μg mL^{-1}) at 25 °C and were fed on the diatom *Chaetoceros gracilis* at concentrations of 40 × 10^4 cells per mL. Larvae reached the cyprid stage in 5 days. The cyprids were collected, then stored at 4 °C until use (0 days-old).

The test compounds were dissolved in ethanol and aliquots of the solution (20 μL) were transferred to wells of a 24-well polystyrene culture plate and then air-dried for 3 h at room temperature. $CuSO_4$ was used as a positive control. Four wells were used for each concentration (0.03, 0.1, 0.3, 1.0, 3.0, 10.0 μg mL^{-1}). To each well were added filtered (0.2 mm) natural seawater (2.0 mL, salinity 28) and six 2 days-old cyprids. The plates were kept in the dark at 25 °C for 48 h. The numbers of cyprids that attached, metamorphosed, died, or did not settle were counted under a microscope. Three or four trials were performed for each concentration. Normality of distribution of results was verified with Shapiro–Wilk's test; in some case, the percentages of settled and dead larval were analyzed after arcsine-transformed. Dunnett's comparison test was used for multiple comparisons of treatment means with a control. The data presented in the figures are not transformed. Settlement inhibition activity (EC_{50}) indicates the concentration reducing the larval settlement to 50% of the control (non-treatment) by Probit analysis. Toxicities of compounds were expressed as LC_{50} values, which indicate the concentration showing 50% mortality estimated by Probit analysis. If the mortality rate was not over 50% at the highest concentration (10.0 μg mL^{-1}), the LC_{50} value was indicated as over 10.0 μg mL^{-1}.

Settlement inhibition assay with *Mytilus galloprovincialis*

From adult *Mytilus galloprovincialis* collected off the coast of Mega Fishing Port in Himeji, fertilized eggs were obtained. These eggs were cultured to obtain juvenile *M. galloprovincialis*.

Individuals with shell length of 1.5–2 mm exhibiting crawling behavior were selected for the experiment.

The test compounds were dissolved in ethanol to prepare 1.0 mg mL^{-1} solutions. Aliquots of the solution (30 and 100 μL) were transferred to 20 mL beakers and then air-dried overnight at room temperature. $CuSO_4$ was used as a positive control. To the beakers were added filtered (0.45 μm) natural seawater (10 mL to reach 3.0 and 10.0 μg mL^{-1}) and twelve of the juveniles. The plates were kept in the dark at 15 °C for 72 h. The numbers of juveniles that attached were counted under a microscope.

Data availability

The datasets supporting this article have been uploaded as part of the ESI.†

Author contributions

Taiki Umezawa: supervision, syntheses, characterizations and writing – original draft; Ira Novita Sari: syntheses and characterizations; Erina Yoshimura: settlement inhibition evaluations with the cypris larvae of the barnacle *A. amphitrite*; Yasuyuki Nogata: settlement inhibition evaluations, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

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