Design, Synthesis, and Antifouling Activity of Glucosamine-Based Isocyanides

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Abstract: Biofouling, an undesirable accumulation of organisms on sea-immersed structures such as ship hulls and fishing nets, is a serious economic issue whose effects include oil wastage and clogged nets. Organotin compounds were utilized since the 1960s as an antifouling material; however, the use of such compounds was later banned by the International Maritime Organization (IMO) due to their high toxicity toward marine organisms, resulting in masculinization and imposex. Since the ban, there have been extensive efforts to develop environmentally benign antifoulants. Natural antifouling products obtained from marine creatures have been the subject of considerable attention due to their potent antifouling activity and low toxicity. These antifouling compounds often contain isocyanate groups, which are well known to have natural antifouling properties. On the basis of our previous total synthesis of natural isocyanoterpenoids, we envisaged the installation of an isocyanate functional group onto glucosamine to produce an environmentally friendly antifouling material. This paper describes an effective synthetic method for various glucosamine-based isocyanides and evaluation of their antifouling activity and toxicity against cypris larvae of the barnacle Amphibalanus amphitrite. Glucosamine isocyanides with an ether functionality at the anomeric position exhibited potent antifouling activity, with EC_{50} values below 1 μg/mL, without detectable toxicity even at a high concentration of 10 μg/mL. Two isocyanides had EC_{50} values of 0.23 and 0.25 μg/mL, comparable to that of CuSO_{4}, which is used as a fouling inhibitor (EC_{50} = 0.27 μg/mL).

Keywords: antifouling; glucosamine; isocyanide

1. Introduction

Many essential human activities, such as transportation and fishing, are carried out in the ocean, and submarine structures—seawater intakes pipes for power plants, breeding nets, and so on—are also widely used. Ocean-based activities such as offshore energy generation will continue to increase in the future [1]. Any structure immersed in the ocean, including ship hulls and fishing nets, will encounter biofouling, that is, an undesirable accumulation of organisms such as barnacles and mussels. Biofouling can have serious economic consequences [2], including fuel waste—up to 40% [3]—and clogging of pipes and nets [4]. Removal of the fouling organisms for annual maintenance must be done at huge cost [5]. Since the 1960s, organotin compounds such as tributyltin (TBT) and triphenyltin (TPT) were widely employed to prevent fouling [6]. However, organotin compounds were suspected to be toxic,
and biological consequences for marine organisms included growth inhibition, masculinization of shellfish, abnormal shell development, brittle shells, poor weight gain, and a disorder known as imposex in snails [7]. Harmful effects of TBT were also reported for marine organisms such as fish [8,9], crustaceans [10,11], and particularly mollusks [12]. These unfavorable aspects drove the International Maritime Organization (IMO) to ban the use of organotin compounds on ships worldwide in 2008 [13]. Alternative antifouling agents in current use mainly contain copper [6], and there have been some reports of negative consequences for the ocean environment and ecosystem [14,15]. The development of an environmentally friendly antifouling agent that is suitable for the ocean environment is therefore a priority.

In current research on green antifouling agents [16], natural products obtained from marine creatures have attracted much attention [17–24]. In particular, isocyanate compounds are well-known examples of natural antifouling products. For example, 10-isocyanate-4-cadinene (1), obtained from nudibranchs of the family Phyllidiidae by Okino, shows strong antifouling activity (50% effective concentration (EC50) = 0.14 µg/mL) against cypris larvae of the barnacle Amphibalanus amphitrite, comparable to CuSO4 (EC50 = 0.27 µg/mL), along with low toxicity (50% lethal concentration (LC50) > 10 µg/mL) [25], as shown in Figure 1. Our group achieved the first total synthesis of 1 and examined the biological activity of a synthetic sample against the same cypris larvae, obtaining similar activities (EC50 = 0.06 µg/mL, LC50 > 10 µg/mL) compared to those of the natural compound [26–28]. Alcohol 2 and the nitrile 3, synthetic intermediates in the total synthesis of 1, were found to have less potency than 1. Other isocyanate compounds such as 3-isocyanatotheonellin (4) also exhibited potent antifouling profiles [29–33]. These results clearly indicate that the isocyanate group confers antifouling properties. We envisaged a compound in which the isocyanate functionality was installed on a glucosamine unit (5), obtained from crustaceans such as crabs and shrimp as a cheap and abundant biomass platform, through organic synthesis, because 5 possesses an amino group, which is essential for introduction of the isocyanate group. In addition, the four hydroxy groups in 5 enable easy access to a wide variety of synthetic isocyanides. Furthermore, the compound, which is derived from a sugar-based structure, was expected to have low toxicity. In this study, glucosamine-based isocyanides (6) were designed and synthesized in short reaction sequences. The antifouling activities and toxicities of the synthetic compounds 6 against the cypris larvae of Amphibalanus amphitrite were evaluated to prove the feasibility of these compounds as alternative environmentally friendly antifouling agents, including through investigation of substituent effects at the C-1 and C-3, 4, and 6 positions.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Materials containing isocyanate groups exhibit potent antifouling properties. (a) Previously reported natural products and derivatives with antifouling properties, along with their antifouling activities. (b) Synthetic plan for glucosamine-based isocyanides.
2. Results

2.1. Compounds with Ester Groups at the C-1 Position

Initially, the tetraacetate 6a and the tetrapalmitate 6b were prepared from D-glucosamine hydrochloride (5) by a sequence of formamide formation with HCO₂Me and NaOMe [34], the acylation of hydroxy groups with Ac₂O or palmitoyl chloride (PalCl), respectively, and the dehydration of formamides 7a and 7b with POCl₃ and Et₃N [35] (Figure 2). The antifouling activities of 6a and 6b were evaluated against the cypris larvae of Amphibalanus amphitrite after a 48 h incubation period, showing moderate activity (EC₅₀ = 5.54 and 6.36 µg/mL, respectively) and very low toxicity (lethal larva was not observed at the concentration of 10 µg/mL).

![Figure 2. Synthesis and biological activity of tetraacylated glucosamine-based isocyanides. (a) Synthesis of tetraacetate 6a. (b) Synthesis of tetrapalmitate 6b.](image-url)

2.2. Compounds with Ether at the C-1 Position

Next, the effect of replacement of the C-1 ester groups of 6a and 6b with ether groups upon antifouling activity and toxicity against Amphibalanus amphitrite was examined. Phenyl (Ph), benzyl (CH₂Ph), allyl (CH₂CH = CH₂), isopropyl (CH(CH₃)₂, iPr), and 2,2,2-trifluoroethyl (CH₂CF₃) groups were chosen as the substituent R of the C-1 alkoxy group. Figure 3 summarizes the synthesis and biological activities of isocyanides 6c-g. For the synthesis of 6c-g, the commercially available D-glucosamine derivative 8 was employed as a starting compound because glycosidation reactions between formamide 7a and alcohols such as isopropyl alcohol under various conditions did not afford the desired glycosidation products. In glycosidation reactions of 8 promoted by BF₃-OEt₂ (Step A), two solvent systems (CH₂Cl₂/room temperature, and MeCN/80 °C) were employed for optimization of chemical yield. Phenyl glycoside 9c and trifluoroethyl glycoside 9g were obtained in higher yields by allowing 8 to react with phenol and 2,2,2-trifluoroethanol at room temperature in CH₂Cl₂. However, treatment of 8 with benzyl alcohol, allyl alcohol, and isopropyl alcohol at 80 °C in MeCN produced benzyl glycoside 9d, allyl glycoside 9e, and isopropyl glycoside 9f in better yields. During each of the glycosidation reactions, the β-anomer was formed as the sole glycosidation product. The β-configurations at the anomeric position (C-1) of 9c-g were determined based on coupling constant analysis. Thus, a large vicinal coupling constant, J₁₂ = 9 Hz (axial/axial relationship between the vicinal methine protons at C-1 and 2), was observed in all the¹H NMR spectra. The conversion of 9c-g into formamides 7c-g was accomplished in the following three steps (Step B): (i) removal of the phthloyl and acetyl groups with ethylenediamine (EDA); (ii) a formamide formation reaction with HCO₂Me and NaOMe; and (iii) acetylation of the three resulting hydroxyl groups with Ac₂O.
and pyridine. Isocyanides 6c–g were derived from 7c–g using the same dehydration protocol with POCl₃ and Et₃N (Step C) that was used in the preparation of 7a and 7b. The biological activities of synthetic 6c–g against the cypris larvae of *Amphibalanus amphitrite* were then evaluated to reveal enhanced potency for 6c–g (EC₅₀ = 0.23–0.71 μg/mL) relative to 6a and 6b (EC₅₀ = 5.54 and 6.36 μg/mL, respectively). Furthermore, it was noteworthy that mortality among the cypris larvae was not observed during the assay, even at a high concentration of 10 μg/mL.

![Figure 3](image)

**Figure 3.** Synthesis and biological activities of glucosamine-based isocyanides. (a) Synthesis of isocyanides 6c–g. (b) Table of reactions and biological activities.

| Compound | R     | Conditions | Yield (%) | Yield (%) for 3 steps | EC₅₀ (µg/mL) | LC₅₀ (µg/mL) |
|----------|-------|------------|-----------|-----------------------|--------------|--------------|
| 6c       | Ph    | CH₂Cl₂, rt | 33        | 10                    | 0.71         | >10          |
| 6d       | CH₃Ph | MeCN, 80 °C | 54        | 41                    | 0.70         | >10          |
| 6e       | CH₃CH=CH₂ | MeCN, 80 °C | 66        | 20                    | 0.37         | >10          |
| 6f       | iPr   | MeCN, 80 °C | 31        | 31                    | 0.23         | >10          |
| 6g       | CH₃CF₃| CH₂Cl₂, rt | 77        | 37                    | 0.36         | >10          |

2.3. Effects of Substituents at the C-3, 4, and 6 Positions

Further modifications were made by replacing the acetyl groups at C-3, 4, and 6 of the isopropyl glycoside 6f with other substituents. As illustrated in Figure 4, after removal of the phthaloyl and acetyl groups of 9f followed by formamide formation, octanoyl (CON₉C₆H₁₃), palmitoyl (CON₉C₁₅H₃₁), benzoyl (COPh), and TBS (SiMe₂Bu) groups were installed to afford isopropyl glycosides 7h–k, respectively. Each of 7h–k was transformed to the corresponding isocyanide (6h–k) under dehydration conditions that were the same as those used for the preparation of 6a–g. Isocyanides 6h–k were tested against *Amphibalanus amphitrite* and exhibited similar antifouling activities (EC₅₀ = 0.25–0.81 μg/mL) to 6f (EC₅₀ = 0.23 μg/mL). Again, none of 6h–k showed detectable toxicity (LC₅₀ > 10 μg/mL).
The results obtained in this study are promising in terms of the development of green antifouling agents for practical use. Further preparation of a wide variety of glucosamine-based isocyanides along with relevant field assay and biodegradability test will be reported in our laboratory.
4. Materials and Methods

Optical rotations were obtained using a Horiba SEPA-300 instrument (Horiba, Kyoto, Japan). IR spectra were recorded on a JASCO FT/IR 4100 spectrometer using a NaCl cell. $^1$H and $^{13}$C NMR spectra were recorded using a JNM-EX 400 (400 MHz and 100 MHz) spectrometer (see Supplementary Materials). N,N-dimethylformamide (DMF), methanol (MeOH), and acetonitrile (MeCN) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). Dichloromethane (CH$_2$Cl$_2$) was distilled from CaH$_2$. All commercially available reagents were employed as received. Analytical TLC was carried out using pre-coated silica gel plates (TLC silica gel 60 F$_254$). The silica gel used for column chromatography was Wakogel 60N 63–212 µm.

4.1. General Procedure for the Preparation of Formamides 7a and 7b

To a solution of D-glucosamine hydrochloride (5) (1.0 equiv) in MeOH (0.40 M) and HCO$_2$Me (1.0 M), NaOMe (1.4 equiv) and Et$_3$N (1.0 equiv) was added at room temperature under an Ar atmosphere. The mixture was stirred at room temperature overnight and then concentrated in vacuo. To a solution of the crude formamide in pyridine (15 equiv), Ac$_2$O (6.0 equiv) for 7a or palmitoyl chloride (6.0 equiv) for 7b was added at room temperature under an Ar atmosphere. After stirring at room temperature overnight, MeOH (6.0 equiv) was added at 0°C. The mixture was extracted with EtOAc, washed successively with 1.0 M NaOH, 3.0 M HCl, and brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane = 40:60) to yield 7a or 7b, respectively.

4.2. General Procedure for the Preparation of Isocyanides 6a–k

To a solution of each of the series of formamides 7a–k (1.0 equiv) in CH$_2$Cl$_2$ (0.20 M), Et$_3$N (9.0 equiv) and POCl$_3$ (3.0 equiv) were added dropwise at 0°C under an Ar atmosphere. After stirring at 0°C for 10 min, the mixture was warmed to room temperature, stirred at room temperature for 1 h, quenched with saturated NaHCO$_3$, and extracted with EtOAc. The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was then purified by silica gel column chromatography (EtOAc/hexane = 10:90) to yield isocyanides 6a–k, respectively.

4.3. General Procedure for the Preparation of Glycosides 9d–f

To a solution of 8 (1.0 equiv) in MeCN (0.25 M), benzyl alcohol, allyl alcohol, or isopropyl alcohol (1.0 equiv) and BF$_3$·OEt$_2$ (3.0 equiv) were added at room temperature under an Ar atmosphere. The mixture was stirred at 80°C overnight, quenched with saturated aqueous NaHCO$_3$, and extracted with EtOAc. The combined organic extracts were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was then purified by silica gel column chromatography (EtOAc/hexane = 20:80) to yield 9d–f, respectively.

4.4. General Procedure for the Preparation of Glycosides 9c and 9g

To a solution of 8 (1.0 equiv) in CH$_2$Cl$_2$ (0.20 M), phenol or 2,2,2-trifluoroethanol (2.0 equiv) and BF$_3$·OEt$_2$ (2.0 equiv) were added at room temperature under an Ar atmosphere. The mixture was stirred at room temperature overnight, quenched with saturated aqueous NaHCO$_3$, and extracted with AcOEt. The combined extracts were washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was then purified by silica gel column chromatography (EtOAc/hexane = 30:70) to yield 9c or 9g, respectively.

4.5. General Procedure for the Preparation of Formamides 7c–g from Glycosides 9c–g

To a solution of each of 9c–g (1.0 equiv) in MeCN (0.20 M), EDA (4.7 equiv) was added at room temperature under an Ar atmosphere. The mixture was stirred at 80°C overnight and concentrated in vacuo. To a solution of the crude amine in MeOH (0.40 M) and HCO$_2$Me (0.40 M), NaOMe (1.2 equiv)
was added at room temperature under an Ar atmosphere. After stirring at room temperature overnight, DOWEX was added for neutralization. The mixture was filtered through a celite pad and concentrated in vacuo. To a solution of the crude formamide in pyridine (30 equiv), Ac$_2$O (25 equiv) was added at room temperature under an Ar atmosphere. After stirring at room temperature overnight, MeOH (25 equiv) was added at 0 °C. The mixture was extracted with EtOAc, washed successively with 1.0 M NaOH (25 equiv), 3.0 M HCl, and brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc/hexane = 40:60) to yield 7c–g, respectively.

4.6. Synthesis of 7h

To a solution of crude formamide in pyridine (50 equiv) and DMAP (0.50 equiv), obtained from 9f in two steps, octanoyl chloride (6.0 equiv) was added at room temperature under an Ar atmosphere. The mixture was stirred for 24 h, quenched with 3.0 M HCl (60 equiv), diluted with AcOEt, washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc:Hexane = 5:95) to yield 7h (24% over three steps) as a white solid.

4.7. Synthesis of 7i

To a solution of crude formamide obtained from 9f in two steps, DMF (0.050 M), palmitic acid (5.0 equiv), DMAP (0.50 equiv), and EDCI (5.0 equiv) were added at room temperature under an Ar atmosphere. The mixture was stirred for 20 h, supplemented with EtOH (2.0 equiv) and EDCI (2.0 equiv), quenched with saturated aqueous NH$_4$Cl, diluted with AcOEt, washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc:Hexane = 10:90) to yield 7i (16% over three steps) as a white solid.

4.8. Synthesis of 7j

To the solution of crude formamide in pyridine (15 equiv) and DMAP (0.50 equiv), obtained from 9f in two steps, benzoyl chloride (5.0 equiv) was added at 60 °C under an Ar atmosphere. The mixture was stirred for 24 h, quenched with 3.0 M HCl (60 equiv), diluted with AcOEt, washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc:Hexane = 30:70) to yield 7j (46% over three steps) as a white solid.

4.9. Synthesis of 7k

To the solution of crude formamide, obtained from 9f in two steps, in DMF (0.050 M) and 2,6-Lutidine (15 equiv), TBSOTf (20 equiv) was added at 120 °C under an Ar atmosphere. The mixture was stirred for 1.5 h, quenched with saturated aqueous NaHCO$_3$, diluted with AcOEt, washed with brine, dried over Na$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel column chromatography (EtOAc:Hexane = 10:90) to yield 7k (43% over three steps) as a white solid.

4.10. Antifouling Assay

Adult barnacles (Amphibalanus amphitrite) procured from oyster farms at Lake Hamana and a pier at Shimizu bay, Shizuoka, were kept in an aquarium at 20 °C and fed on Artemia salina nauplii. Broods were released as I–II stage nauplii upon immersion in seawater after drying overnight. The nauplii thus obtained were cultured in filtered natural seawater (salinity 28) containing penicillin G (20 µg/mL) and streptomycin sulfate (30 µg/mL) and were fed on the diatom Chaetoceros gracilis at concentrations of 40 × 10$^4$ cells/mL. Larvae reached the cyprid stage in 5 days. The cyprids were collected and stored at 4 °C until use (0 days old).

The test compounds were dissolved in ethanol and aliquots of the solution were transferred to wells of a 24-well polystyrene culture plate and air-dried. Four wells were used for each concentration. To each well, filtered seawater (2.0 mL, salinity 28) and six 2-day-old cyprids were added. The plates
were kept in the dark at 25 °C for 48 h. The numbers of cyprids that attached, metamorphosed, died, and did not settle were counted under a microscope. Three or four trials were done for each concentration. Probit analysis was used to calculate the EC_{50} values.

**Supplementary Materials:** The following are available online at www.mdpi.com/1660-3397/15/7/203/s1; characterization of all compounds and 1H and 13C NMR data are available.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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