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Construction and screening of 2-aryl benzimidazole library identifies a new antifouling and antifungal agent†

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Biofouling is the undesirable growth of organisms on artificial and natural structures immersed in either seawater or freshwater. It causes huge economic loss and also the global prohibition on known antifouling agents has led to an increased search for safe and effective antifouling agents. In the past, marine natural products have shown tremendous potential by providing new skeletons that could be used as eco-friendly antifouling agents. The library of the 2-aryl benzimidazole core inspired from marine natural products (oroidin and bromoageliferin) was identified and synthesized to explore the antifouling/antifungal properties for the first time. Twelve 2-aryl benzimidazole derivatives were synthesized and evaluated for their antifouling performance against 10 strains of marine biofilm forming bacteria developed on copper panels exposed for 14 days at Dona Paula, Arabian Sea, India. These compounds were also evaluated for their antibacterial and antifungal activities. Two compounds, *i.e.* **4j** and **4l**, showed a broad spectrum of antifouling activities against nine marine fouling species, whereas 2-(furan-2-yl)-1*H*-benzo[d]imidazole **4g** showed strong antifungal activity against the clinical pathogen *Aspergillus niger*. Our results reveal that the 2-aryl substituent on the benzimidazole core had strong impact on their biological profile. Moreover, here we report the first study of the benzimidazole library as a target in 10 representative fouling strains.

Introduction

Biofouling is a natural process of the marine ecosystem caused by the surface colonization and development of micro- and macro-foulers on submerged natural or man-made marine structures, leading to huge economic losses. From the 1960s, tributyltin (TBT) and its derivatives were found to be the best candidates to solve the fouling problem. However, increasing environmental concerns led to legislation that put an end to the regime of TBT.[1] The well-known alternative to toxic antifoulant is natural product antifoulant (NPA). Most of the marine organisms, such as tropical sponges and octocorals (are rich resources of novel secondary metabolites), do not foul when they are alive as the organisms in question secrete antifouling substances.[2,3] Fouling is a widespread phenomenon and some organisms may be heavily fouled, whereas others can be totally fouling-free. This has generated high interest in identifying the biological metabolites that might repel or inhibit fouling organisms. Thus, natural antifouling agents are active substances found in marine flora, fauna, and in terrestrial plants. They prevent the settlement of microorganisms and glaze formation on the surface of their structures, and they are believed to function as a natural chemical defense against fouling.[4,5] Generally, these chemical families include steroids, terpenes, phenolics, brominated hydrocarbons, brominated tyrosine derivatives and saponins (Fig. 1a).

The fouling species are known to "communicate" by quorum sensing. Smit and co-workers demonstrated that if the micro-organisms are prevented from quorum sensing, the tendency to foul diminishes.[6] In general, halogenated furanones are the most popular compounds undergoing investigations of quorum sensing inhibition, and their effects on bacterial biofilm have been observed in a wide range of Gram-negative bacteria.[6] The potential of quorum sensing inhibition is not only tested in terms of antifouling perspective, but are interesting even in the medical industry as a substitute for other antibacterial agents.[7] To date, the mechanism of NPAs in biofouling inhibition is documented in the literature and was found to depend on the structural features of each chemical. The two known biochemical pathways to explain the role of NPAs against bacterial attachment is depicted in Fig. 1b–d. The first involves the interference of NPAs containing special chemicals with the

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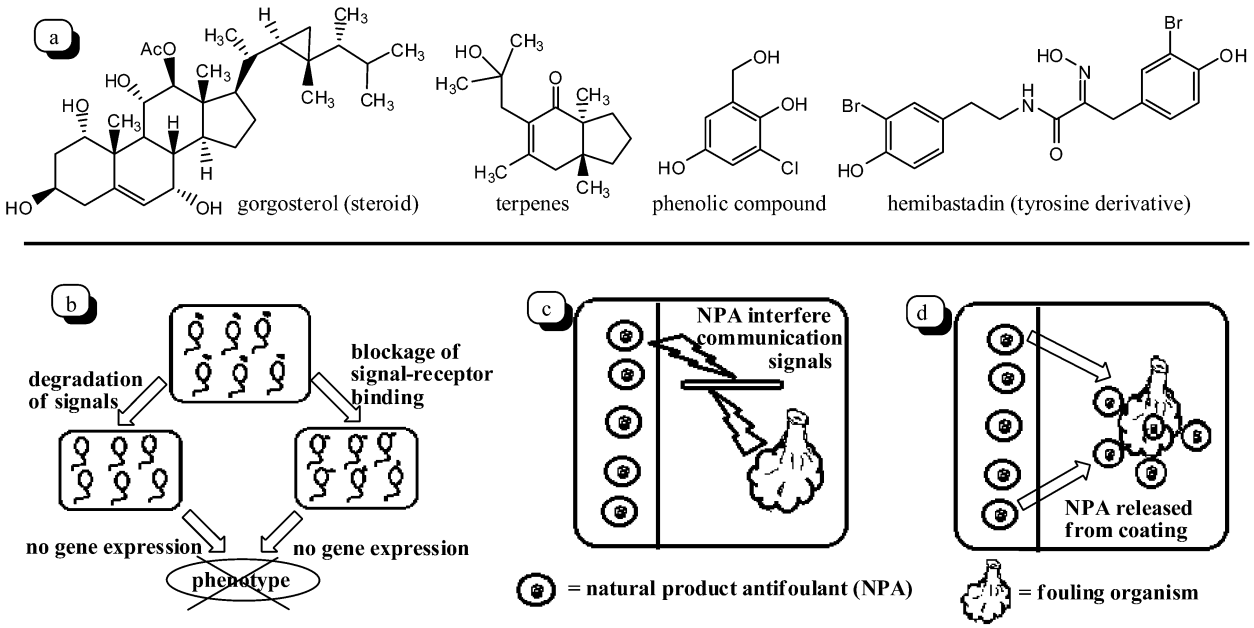

Fig. 1 Biochemical pathway of biofouling inhibition using NPAs: (a) some examples of natural product antifoulants (NPAs); (b) quorum sensing (QS)-regulated phenotype inhibition; (c) NPA chemical interferes with the communication signals between fouling organisms; (d) release of NPA molecule to block signals.

communication signals between the bacteria to prevent the biofilm formation.[8] In the second approach, the NPA molecules are released from the coating. As a result, these molecules bind to the specific binding sites on the surface of the target organism and block the specific interaction between the organism and the surface (Fig. 1d).[9]

In 1990, oroidin and bromoageliferin **1** (2-aminoimidazole core) metabolites from the marine sponge *Agelas* were reported to show antifouling activity against the Gram-negative marine bacterium *Rhodothalassium salexigens*, representing the first report on marine biofilm inhibitors.[10] Bromoageliferin **1** has a propeller-like structure constituted by the connection of three heteroaromatic arms onto a reduced form of benzimidazole at the centre called TAGE (*trans*-bromoageliferin, **2**) in short (Fig. 2). In this context, Melander's group showed that the 2-aminobenzimidazole derivatives **3** (synthesized analogue TAGE **2**) have significant biofilm inhibitory activity against several biofilm forming Gram-negative bacteria.[11] Melander and co-workers[12] also constructed aryl-2-aminoimidazole (2-AI) libraries for evaluation of biofilm inhibitory activity against Gram-negative *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. More recently, Blackwell and co-workers[13] identified 2-aminobenzimidazole derivatives as the most active against *P. aeruginosa* biofilm modulators. Inspired by these facts, we envisaged that 2-aryl (or substituted as cinnamoyl) benzimidazoles (**4**) could also be potential candidates as biofilm modulators. Benzimidazole derivatives have recently drawn a major research focus due to their broad range of biological functions[14] and pharmacological applications.[15] In this regard, the broad utility of benzimidazole scaffolds has prompted significant efforts towards their synthesis.[16] The traditional method for the preparation of benzimidazole involves the condensation of an *o*-diaminoarene with carboxylic acid under harsh conditions.[17] Recently, several eco-friendly methods have been reported for the synthesis of 1,2-disubstituted benzimidazole, in contrast, safe and "green" protocols

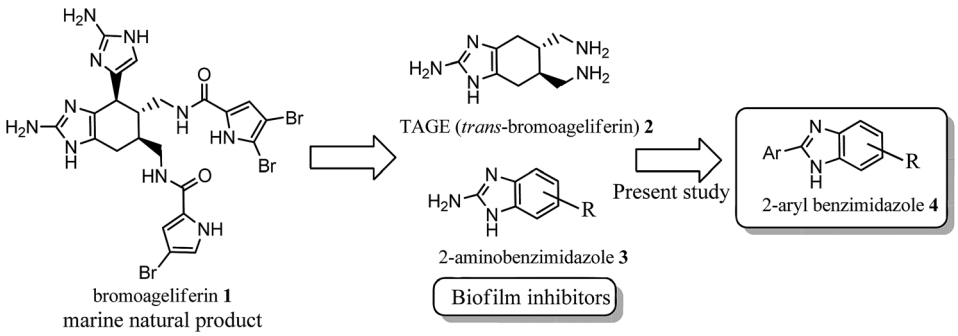

Fig. 2 Rational approach towards 2-aryl benzimidazoles.

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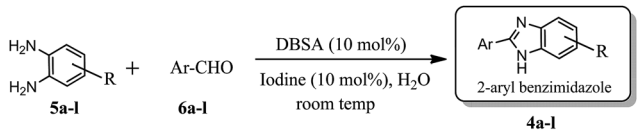

Entry	4a-l	R	Ar	Yield (%)
1	**a**	H	Ph	92
2	**b**	H	4-$NO_2C_6H_4$	94
3	**c**	4-CH_3	4-$NO_2C_6H_4$	93
4	**d**	4-Cl	4-$NO_2C_6H_4$	90
5	**e**	4-NO_2	4-$OCH_3C_6H_4$	92
6	**f**	H	4-BrC_6H_4	87
7	**g**	H	Furan-2-yl	80
8	**h**	4-CH_3	Furan-2-yl	81
9	**i**	4-NO_2	Furan-2-yl	83
10	**j**	4-NO_2	Pyrrole-2-yl	88
11	**k**	4-NO_2	Thiophene-2-yl	86
12	**l**	H	C_6H_5CH=CH-	86

Scheme 1 Synthesis of 2-aryl benzimidazole library. Each compound was characterized by ^{1}H NMR, ^{13}C NMR and HRMS prior to screening.

available for the chemoselective synthesis of 2-substituted benzimidazole are rare. In this regard, Banerjee and co-workers developed[18] a chemoselective "greener" approach for the preparation of 2-substituted benzimidazoles, which we adopted for the syntheses of our targeted 2-substituted benzimidazoles derivatives. Therefore, as a part of our continuous research interest on the design, synthesis and evaluation of the bioactivities of marine natural products,[19] we report the potential antifouling activity of 2-aryl benzimidazoles **4** for the first time. The rationale of choosing 2-aryl benzimidazoles for the present study is schematically presented in Fig. 2.

Results and discussion

Synthesis of 2-aryl benzimidazole library

A general synthesis of 2-aryl benzimidazoles is described in Scheme 1. In our previous study,[18] we demonstrated that the acidic nature of DBSA and slow reaction rate are helpful for the formation of 2-substituted benzimidazoles with high chemoselectivity along with a small amount of undesired 1,2-disubstituted benzimidazoles. We also found that an oxidizing agent, such as I_2, H_2O_2, *p*-benzoquinone, and oxone, may act as a co-catalyst to expedite the rate of the reaction preserving or improving the chemoselectivity aspect. Thus, based on the observed selectivity and considering the fact that iodine is milder and easier to handle, it was selected as the co-catalyst for further study. The results obtained were discussed in detail in our previous communication.[18] Here, the optimized condition (*i.e.* both DBSA and I_2; 10 mol% each) was applied for the syntheses of all desired 2-substituted benzimidazoles (**4a–l**) from corresponding aromatic aldehydes **6** and *o*-diaminoarenes **5** in water at room temperature. The aldehydes **6** with electron-donating and electron-withdrawing groups gave uniform results with a good yield (86–94%) of the target product **4** in a chemoselective manner. However, a sensitive substrate like furfuraldehyde **6g** produced the desired product in slightly lower yield (80–83%, entries 7–9). Overall, we prepared 12 2-substituted benzimidazole derivatives in excellent yields, which have been utilized further in biological studies.

Biological assay results

Initially, each benzimidazole at concentration of 100 μg per disc was screened to check their ability to inhibit marine fouling

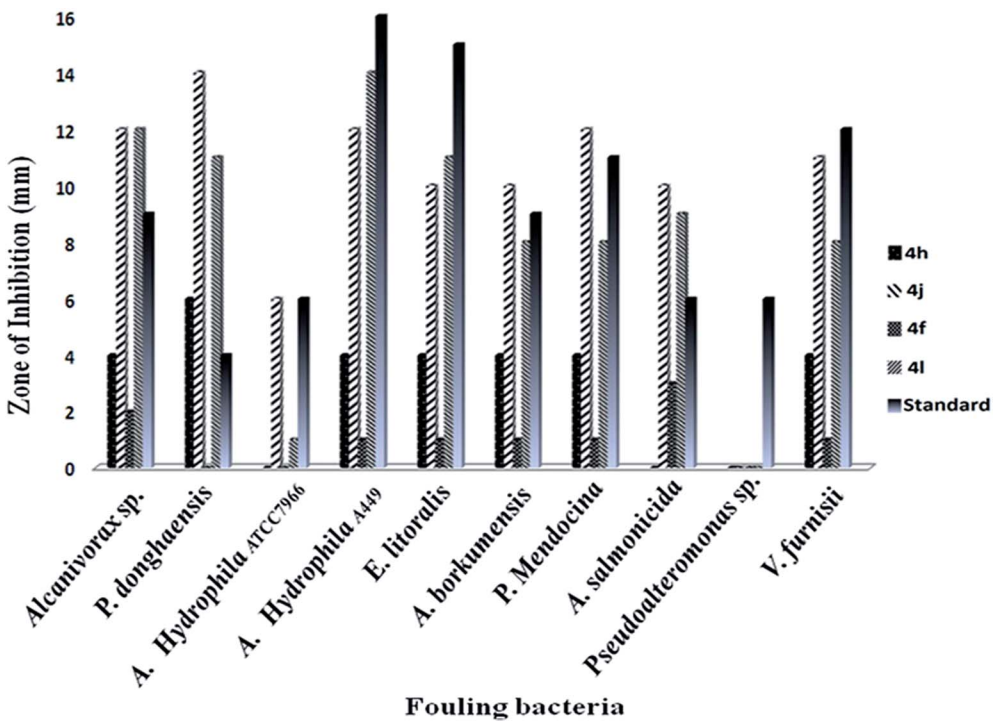

Fig. 3 Antifouling activities of the selected 2-aryl benzimidazoles (the standard is gentamycin).

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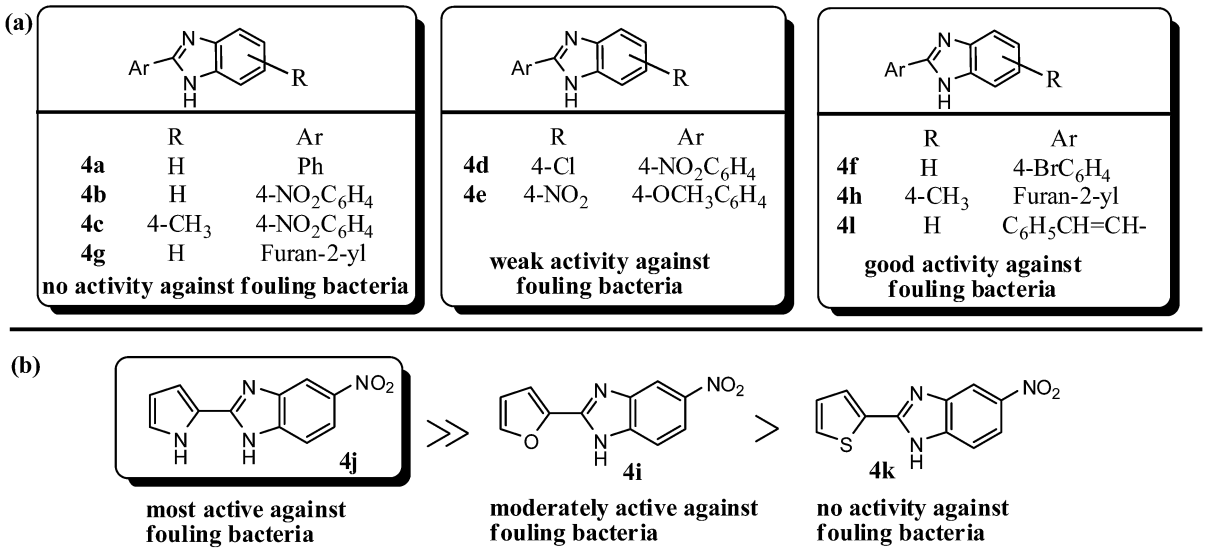

Fig. 4 (a) Comparison of the effect of benzimidazole substituents (R and Ar) on the biological activity; (b) effect of the heteroatom aromatic moiety of benzimidazole on the antifouling activity.

bacteria. The activities were monitored under static conditions using the Kirby–Bauer disc diffusion method. Among the family of 2-phenyl substituted benzimidazole (**4a–f**, entry 1–6), 2-phenylbenzimidazole **4a**, **4b** (4-nitro substituted) and **4c** (4-nitro-6-methyl substituted) were found to be inactive against all the fouling bacteria.[20] This study addressed the effect of electron-withdrawing (NO_2) and electron-donating substituents (H, CH_3) of the phenyl moiety on the antifouling activity. It is interesting to note that the introduction of a chlorine atom at the 6-position of 2-phenyl benzimidazole enhanced the activity as seen in compound **4d** (Cl substituted). It showed weak activity against *Aeromonas salmonicida A449*. Furthermore, the introduction of a 4-methoxyphenyl moiety on benzimidazole led to a significant reduction in antifouling activity of **4e**. The presence of a bromine atom in the structure of many antifouling agents[21] inspired us to study the impact of bromine substituent upon the antifouling properties of benzimidazole. It has been recognized that the introduction of a 4-bromophenyl ring at the 2-position of benzimidazole resulted in increased activity as seen in **4f**. It was found to be moderately active against *Alcanivorax* spp. and *Alivibrio salmonicida*, and it showed weak activity against *Aeromonas salmonicida A449*, *Erythrobacter litoralis*, *Alcanivorax borkumensis* and *Pseudomonas mendocina* (Fig. 3 and 4a).

After screening the 2-phenyl benzimidazole (**4a–f**), we next tested the potential of the 2-heteroaromatic substituted benzimidazole (**4g–k**) to understand the effects of the heteroaromatic substituents. The introduction of a furan ring at the 2-

Table 1 Table showing the zones of inhibition values[a] of **4j** against fouling bacteria

	Concentration in μg						
	25	50	75	100	125	150	50
Fouling bacteria	Inhibition diameter in mm						Std
Alcanivorax spp.	8	10	11	12	13	16	9
Planococcus donghaensis	9	11	12	14	16	16	4
Aeromonas hydrophila subsp. *hydrophila ATCC 7966*	3	4	5	6	5	7	6
Aeromonas hydrophila subsp. *salmonicida A449*	6	11	9	12	13	8	16
Erythrobacter litoralis	7	8	9	10	13	14	15
Alcanivorax borkumensis	5	8	7	10	11	14	9
Pseudomonas mendocina	7	9	11	12	13	17	11
Alivibrio salmonicida	6	8	9	10	11	14	6
Pseudoalteromonas spp.	—	—	—	—	—	—	6
Vibrio furnisii	9	9	10	11	12	14	12

[a] The data are expressed as a measure of the inhibition zones (mm) at concentration varying from 25 to 150 μg per disc; the standard in the present study was gentamycin; and the data given are the mean of three replicates.

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position of benzimidazole led to the complete loss of activity, whereas the presence of an electron-donating group/electron-withdrawing group at the 6-position of furan-2-yl benzimidazole showed an effect on the activity profile (**4h–i**). The next modification involved the replacement of a furan ring by pyrrole or thiophene at the 2-position of benzimidazole (**4j–k**). In this case, the **4j** containing pyrrole moiety showed intensification in the antifouling activity and was found to have better functionality compared to **4i** and **4k**. This suggests that the presence of pyrrole core at the 2-position of benzimidazole is necessary for the enhancement of activity. The study reveals that compound **4j** is more active than **4i** and **4k** (Fig. 4b). Furthermore, compound **4l** with the stilbene functionality at the C-2 position of benzimidazole showed good activity against all the tested fouling bacteria.

Among the libraries of synthesized 2-aryl benzimidazole, **4j** is the most promising lead compound with the maximum antifouling properties and hence was selected for further studies. The dose response studies were performed on compound **4j** against 10 strains of Gram-positive and Gram-negative fouling bacteria with concentrations ranging from 25 to 150 μg per disc (Table 1). Overall, benzimidazole **4j** containing the pyrrole moiety is identified as the most potent antifouling agent showing a broad spectrum of activity against fouling bacteria. Moreover, an antifungal potential of benzimidazole library was explored in this study.[23] Benzimidazole **4g** showed strong antifungal activity against *Aspergillus niger*, whereas **4h** exhibited moderate active against *Aspergillus niger* and *Cryptococcus neoformans*. Compounds, **4i** and **4j** were found to possess moderate activity against the fungal strain *Candida albicans* at 100 μg per disc.

Conclusions

We have disclosed herein a comprehensive evaluation of the benzimidazole library for the antifouling activities against marine fouling bacteria. Moreover, we demonstrated the use and application of our recently developed green approach towards the synthesis of 2-aryl benzimidazole. The results obtained of these preliminary experiments are quite encouraging and reveals that compounds **4j** and **4l** possess a broad spectrum of antibacterial activity against fouling bacteria and may be a potential candidate for the development of new antifouling agents. Given the promising antifouling/antifungal activity displayed by these benzimidazoles, we are continuing to develop methodologies to access further functionalized libraries based on the benzimidazole core motif.

Experimental section

Synthesis of compounds

General methods. ^{1}H NMR spectra ($CDCl_3$) were recorded on Bruker Avance 500 MHz spectrometer. Chemical shifts are reported in parts per million (δ). The mass spectra were recorded on Agilent Technologies 6220 Accurate-Mass TOF LC/MS spectrometer. The reactions were monitored with TLC (Merck precoated $60F_{254}$ plates), and the spots were detected by viewing under UV light and spraying with acidic *p*-anisaldehyde. Column chromatography was performed on silica gel (60–120 mesh, Merck). The reagents were purchased from Aldrich chemical company. All solvents were obtained from local suppliers.

General procedure for the syntheses of 2-substituted benzimidazoles

To a solution of DBSA (0.05 mmol) in H_2O (2 ml) were added amine **5** (0.5 mmol) and iodine (0.05 mmol). An aldehyde **6** (0.5 mmol) was added portionwise and the reaction mixture was stirred at room temperature until completion (the progress of the reaction was monitored by TLC). The aqueous layer was decanted and the organic part was taken in ethyl acetate, washed successively with saturated $NaHCO_3$, water, brine, and then dried over anhydrous Na_2SO_4. The organic layer was filtered, concentrated and purified by silica gel chromatography (EtOAc–hexane). The physical data of the synthesized compounds are provided in our previous communication.[18] A representative example of the data for one compound is given below: 2-(furan-2-yl)-5-nitro-1*H*-benzo[d]imidazole (Table 1, entry 9): almond color solid, mp 222–223 °C; ^{1}H NMR (DMSO-d_6): δ 6.75 (dd, J = 1.5 Hz, 3.4 Hz, 1H), 7.31 (d, J = 3.4 Hz, 1H), 7.67 (d, J = 8.9 Hz, 1H), 8.00 (s, 1H), 8.07 (dd, J = 2.1 Hz, 8.9 Hz, 1H), 8.38 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 113.3, 118.6, 143.3, 145.0, 146.4, 148.1, 148.3; HRMS (ESI): *m*/*z* calcd for $C_{11}H_8N_3O_3$ $[M + H]^+$ 230.0566, found 230.0564.

Antifouling assay

10 strains of marine fouling bacteria were tested to determine the anti-fouling capacity of the compounds: Gram-positive bacteria (*Planococcus donghaensis*) & Gram-negative bacteria (*Alcanivorax* spp., *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966, *Aeromonas hydrophila* subsp. *salmonicida* A449, *Erythrobacter litoralis*, *Pseudomonas mendocina*, *Alcanivorax borkumensis*, *Alivibrio salmonicida*, *Pseudoalteromonas* spp., and *Vibrio furnisii*). The strains were isolated and identified using the methods of Allegrucci and Sauer,[22] Dalton *et al.*,[23] Bollet *et al.*,[24] and Weisburg *et al.*[25] from natural biofilms that were allowed to develop on steel and copper panels for 14 days, which were exposed at Dona Paula, Arabian Sea (15° 27′17″N and 73°48′17″E). Exposure was done at a temperature of 15–20 °C at a salinity of 35 psu. The cultures were preserved in 30% glycerol at −80 °C, and prior to the assay, subcultured in Zobell's marine broth (having 1% peptone and 0.1% yeast extract) at 28 °C until they attained a turbidity comparable to the 0.5 McFarland turbidity standards containing approximately 1–2 × 10^8 CFU ml^{-1} for *E. coli* ATCC 25922. The Kirby–Bauer disc diffusion method[26] was used to conduct the assay. The compounds to be tested were dissolved in 5% DMSO and pipetted onto sterile paper discs (Whatman no. 1, diameter = 6 mm) at various concentrations. Control discs with 5% DMSO and copper sulphate were used as controls at concentrations of 2–100 μg per disc as additional controls. The discs were dried aseptically at room temperature. For the assay, 0.1 ml of each fouling strain suspension with approximately 10^8 CFU ml^{-1} was spread plated

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onto Mueller–Hinton agar plates and dry paper discs with either the test or the control compounds were aseptically laid on the agar surface. The plates were then incubated at 28 °C for 24 h until bacterial matt growth was observed on the agar surface. The zones of growth inhibition surrounding the discs were measured up to 0.5 mm. The compounds were tested in triplicate with different concentrations to determine the minimum inhibitory concentration (MIC) required to inhibit the test bacteria.

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