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

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Abstract:

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 increase hunt for safe and effective antifouling agents. In the past, marine natural products have shown tremendous potential by providing new skeletons which could be used as eco-friendly antifouling agents. Library of 2-aryl benzimidazole core inspired from marine natural products (oroidin and bromoageliferin), is identified and synthesized to explore 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 antibacterial and antifungal activities. Two compounds *i.e*. **4j** and **4l** showed broad spectrum of antifouling activity against nine marine fouling species whereas, 2-(Furan-2-yl)- 1*H*-benzo[d]imidazole **4g** showed strong antifungal activity against clinical pathogen *Aspergillus niger*. Our results reveal that the 2-aryl substituent on the benzimidazole core had strong impact on their biological profile. Additionally, here we report the first study of benzimidazole library as target in 10 representative fouling strains.

Keywords: Antifouling, Biofouling, Benzimidazole, Marine natural products, Structure activity relationships.

Introduction

Biofouling is a natural process of marine ecosystem caused by the surface colonization and development of micro and macrofoulers on submerged natural or man-made marine structures, leading to huge economic forfeiture. From the 1960's, tributyltin (TBT) and their derivatives were found as the most potential candidates to solve fouling problem. However, increasing environmental concern 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 alive as the organisms in question secrete antifouling substances.[2,3] The fouling is a widespread phenomenon and some organisms may be heavily fouled, while 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 the glaze formation on the surface of their structures, and are believed to function as natural chemical defense against fouling.[4,5] Generally, these chemical families include steroids, terpenes, phenolics, brominated hydrocarbons, brominated tyrosine derivatives and saponins (Figure **1a**).

The fouling species are known to "communicate" by quorum sensing. Smit and co-workers have demonstrated that if the microorganisms 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; their effect on bacterial biofilm has 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 medical industry as substitution for other antibacterial agents.[7] So far, the mechanism of NPAs in biofouling inhibition is well documented in 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 Figure **1b-d**. The first one involves the interference of NPAs containing special chemicals with the 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 with the specific binding sites on the surface of the target organism and thus, blocking the specific interaction between organism and the surface (Figure **1d**).[9]

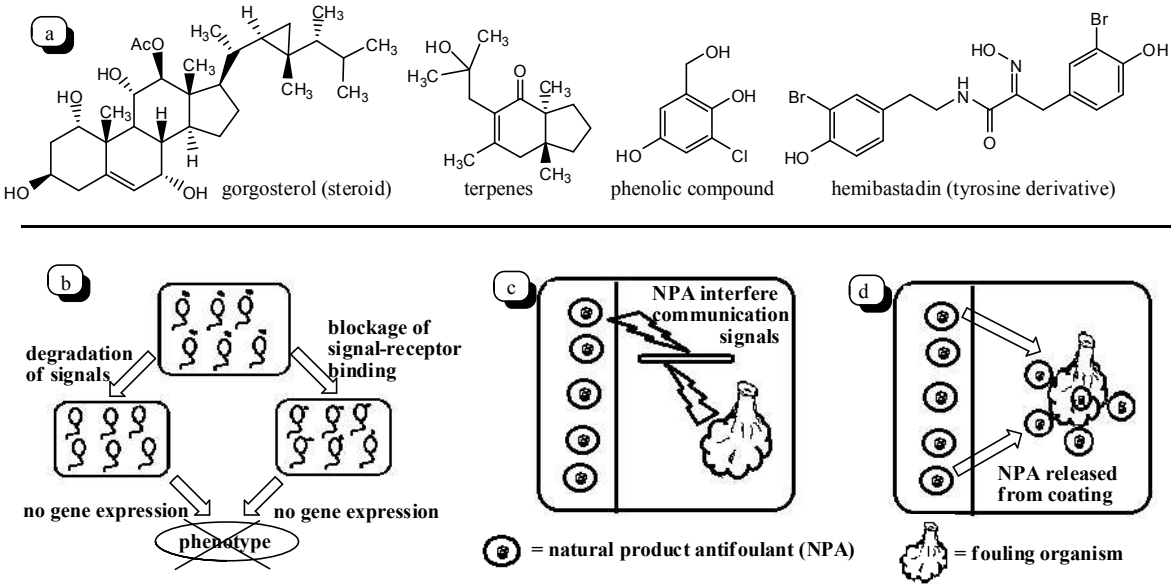


Figure 1. Biochemical pathway of biofouling inhibition using NPAs: (a) some examples of natural product antifoulants (NPAs); (b) quorum sensing (QS)-regulated phenotype inhibition; (c) NPA chemical interfere the communication signals between fouling organism; (d) release of NPA molecule to block signals.

In 1990, oroidin and bromoageliferin **1** (2-aminoimidazole core) metabolites from marine sponge *Agelas* are reported to display antifouling activity against the Gram negative marine bacterium *Rhodomalassium salexigens* representing the first report on marine biofilm inhibitors.[10] Bromoageliferin **1** is having 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 (Figure 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 2-aryl (or substituted as cinnamoyl) benzimidazoles (**4**) could also be potential candidates as biofilm modulator. Benzimidazole derivatives have recently drawn a major research focus due to their broad range of biological functions[14] and pharmacological applications.[15] In this regards, 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 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 bioactivities of marine natural products,[19] herein, 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 Figure **2**.

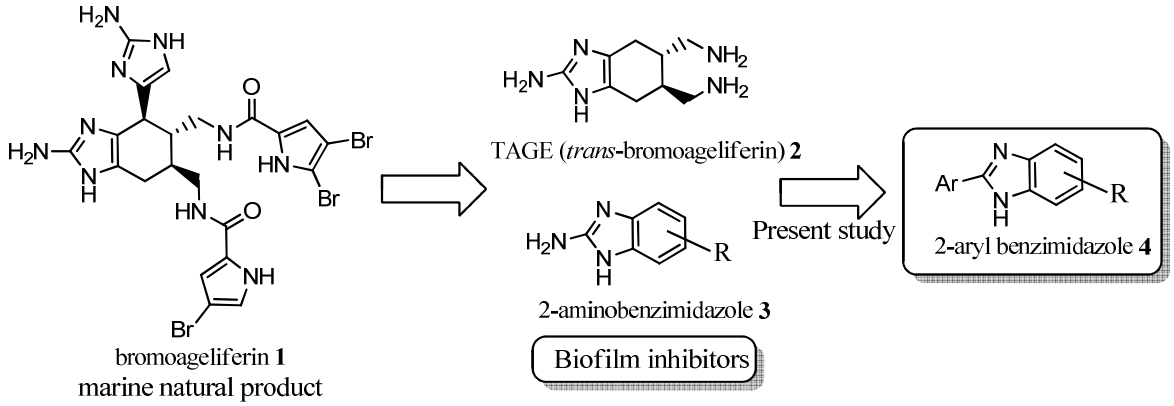


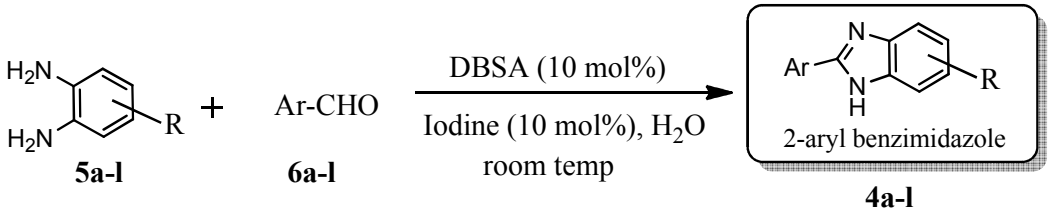
Figure 2. Rational approach towards 2-aryl benzimidazoles

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 small amount of undesired 1,2-disubstituted benzimidazoles. We also found that an oxidizing agent such as I_2, H_2O_2, *p*-benzoquinone, oxone may act as a co-catalyst to expedite the rate of the reaction preserving or bettering the chemoselectivity aspect, and 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 were discussed in detailed 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 as well as with electron withdrawing group gave uniform results with good yield (86-94%) of the target product **4** in chemoselective manner. However, sensitive substrate like furfuraldehyde **6g** produced the desired product with slightly lower yield (80-83%, entries 7-9). Overall, we had prepared twelve 2-substituted benzimidazole derivatives, in excellent yields, which have been utilized further in biological studies.



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

[a]each compounds was characterized by ^{1}H NMR, ^{13}C NMR and HRMS prior to screening

Scheme 1. Synthesis of 2-aryl benzimidazole library.[a]

Biological assay results

Initially, each benzimidazole at concentration of 100 µg/disc was screened to check their ability to inhibit marine fouling bacteria. The activities were monitored under the static condition using Kirby-Bauer disc diffusion method. Amongst the family of 2-phenyl substituted benzimidazole (**4a-f**, entry 1-6), 2-phenylbenzimidazole **4a**, **4b** (4-nitro subtituted) and **4c** (4-nitro-6-methyl subtituted) 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 phenyl moiety on the antifouling activity. It is interesting to note that the introduction of chlorine atom at 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 4-methoxyphenyl moiety on benzimidazole led to the significant reduction in antifouling activity of **4e**. The presence of bromine atom in structure of many antifouling agents[21] had inspired us to study the impact of bromine substituent upon antifouling properties of benzimidazole. It has been recognized that the introduction 4-bromophenyl ring at 2-position of benzimidazole resulted in increased activity as seen in **4f**. It was found to be moderately active against *Alcanivorax spp.* and *Allivibrio salmonicida.* It showed weak activity against *Aeromonas salmonicida A449, Erythrobacter litoralis, Alcanivorax borkumensis* and *Pseudomonas mendocina* (Figure **3** and **4a**).

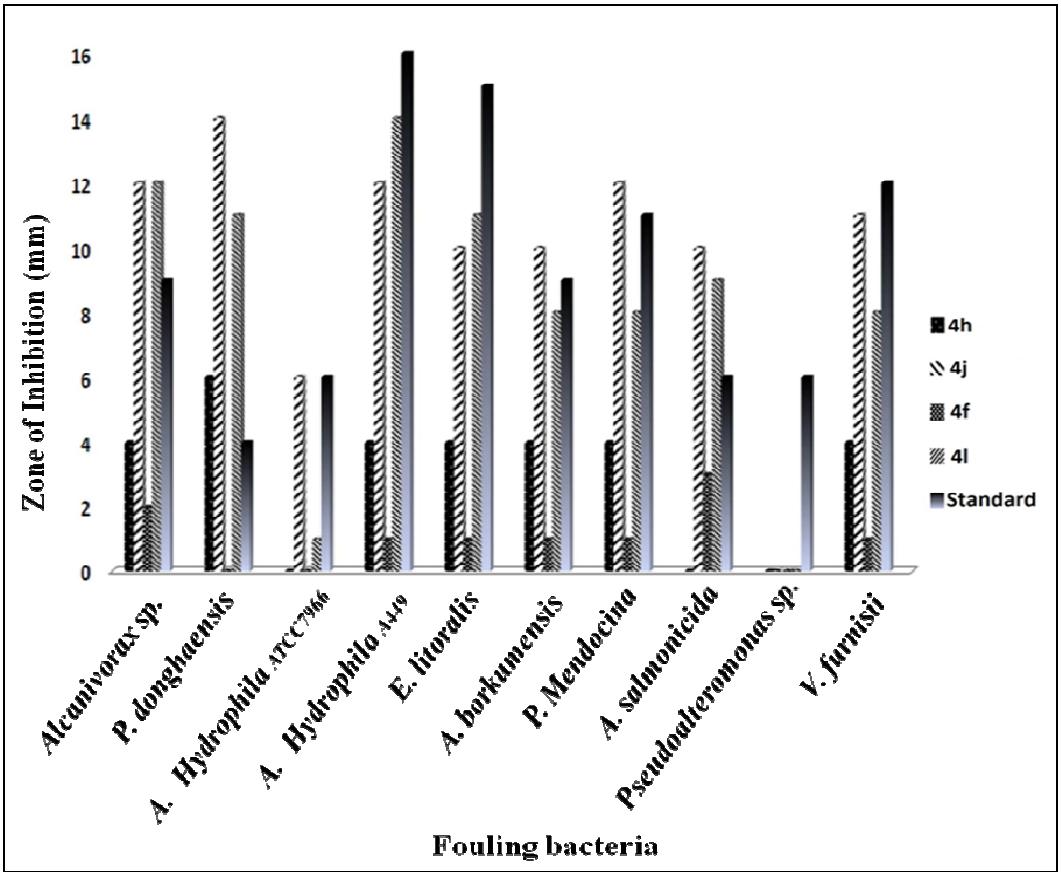


Figure 3. Antifouling activities of selected 2-aryl benzimidazole (standard is Gentamycin).

After screening the 2-phenyl benzimidazole (**4a-f**), next we had tested potential of the 2-heteroaromatic substituted benzimidazole (**4g-k**) to understand the effect of heteroaromatic substituents. Introduction of furan ring at 2-position of benzimidazole led to complete loss of activity, whereas, the presence of electron donating group/ electron withdrawing group at 6-position of furan-2-yl benzimidazole showed effect on the activity profile (**4h-i**). Next modification involved the replacement of furan ring by pyrrole or thiophene at 2-position of benzimidazole (**4j-k**). In this case, the **4j** containing pyrrole moiety showed intensification in antifouling activity and found to be better functionality compared to **4i** and **4k**. This result suggested that the presence of pyrrole core at 2-position of benzimidazole is necessary for the enhancement of activity. The study reveals that compound **4j** is most active than **4i** and **4k** (Figure **4b**). Furthermore, compound **4l** with stilbene functionality at C-2 position of benzimidazole showed good activity against all the tested fouling bacteria.

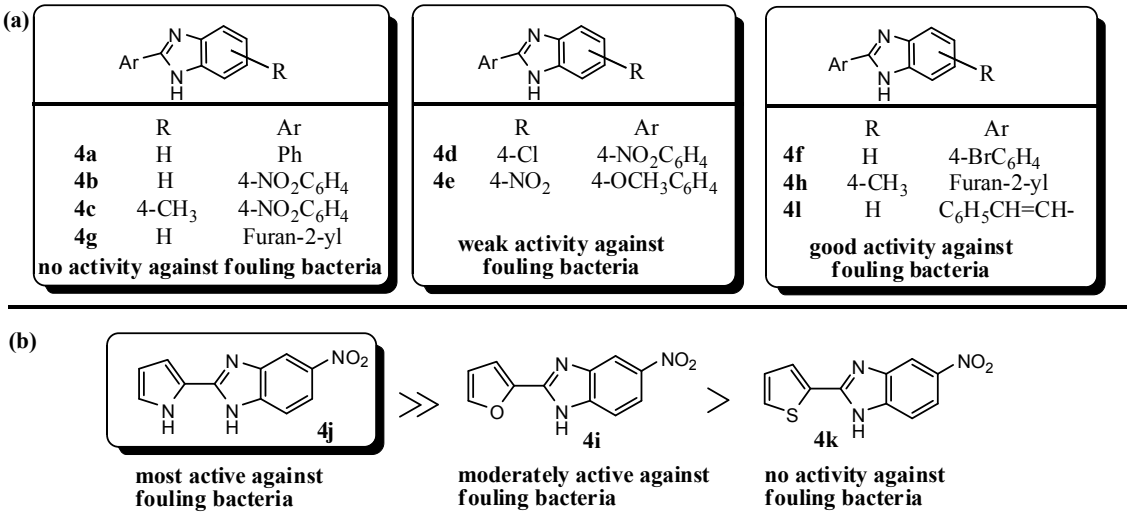


Figure 4. (a) Comparison of effect of benzimidazole substituents (R and Ar) on biological activity; (b) effect of heteroatom aromatic moiety of benzimidazole on antifouling activity.

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

Fouling Bacteria	**Concentration in µg**						
	25	**50**	**75**	**100**	**125**	**150**	**50**
	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**
Allivibrio 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 the measure of inhibition zones (mm) at concentration varying from 25-150 μg/disc; Standard in present study is Gentamycin; Data given are the mean of three replicates.

Amongst libraries of synthesized 2-aryl benzimidazole, **4j** is the most promising lead compound with 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 concentration ranging from 25-150 μg/disc (Table 1). Overall, benzimidazole **4j** containing pyrrole moiety is identified as the most potent antifouling agent showing broad spectrum of activity against fouling bacteria. Additionally, an antifungal potential of benzimidazole library has been 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 fungal strain *Candida albicans* at concentration of 100 μg/disc.

Conclusions

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

are continuing to develop methodology to access further functionalized libraries based upon benzimidazole core motif.

Experimental section

Synthesis of compounds: *General methods.* ^{1}H NMR spectra ($CDCl_3$) were recorded on Bruker Avance 500 MHz. Chemical shifts are reported in parts per million (δ). Mass spectra were recorded on Agilent Technologies 6220 Accurate-Mass TOF LC/MS spectrometer. Reactions were monitored with TLC (Merck precoated $60F_{254}$ plates) and spots were detected by viewing under a UV light and spraying with acidic *p*-anisaldehyde. Column chromatography was performed on silica gel (60-120 mesh, Merck). Reagents were purchased from Aldrich chemical company. 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 till the 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] Representative example of 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 ^oC; ^{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, Allivibrio salmonicida,*

Pseudoalteromonas spp., and *Vibrio furnisii*). The strains were isolated & identified using the method of Allegrucci & Sauer,[22] Dalton *et al.*,[23] Bollet *et al.*,[24] and Weisburg *et al.*,[25] from natural biofilms which were allowed to develop on steel and copper panels for 14 days, 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 & prior to the assay, subcultured in Zobell's marine broth (having 1% peptone & 0.1% yeast extract) at 28 °C till they attained turbidity comparable to 0.5 McFarland turbidity standards containing approximately 1-2 X 10^8 CFU/ml 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 & pipetted onto sterile paper discs (Whatman no. 1, diameter = 6 mm) at varying concentrations. Control discs with 5% DMSO & copper sulphate were used as controls at concentrations of 2-100 µg/disc as additional controls. The discs were dried aseptically at room temperature. For the assay, 0.1 ml of each fouling strain suspension having approximately 10^8 CFU/ml was spread plated onto Mueller Hinton agar plates and the 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 hrs. till a 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 triplicates (3 times) and with different concentrations to determine the minimum inhibitory concentration (MIC) required to inhibit the test bacteria.

Acknowledgements

The authors thank the Director, CSIR-National Institute of Oceanography for constant encouragement. Financial assistance provided by the OCEAN FINDER and EU-FP7-KBBE-2009-3-245137 MAREX is highly acknowledged. Author MSM is grateful to CSIR-NIO for the award of Scientist Fellow-QHS. Author M.B. is indebted to DST (India) (project No. SR/FT/CS-023/2010) for financial support.

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