Bioorganic Chemistry 54 (2014) 89–95

Contents lists available at ScienceDirect

Bioorganic Chemistry

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

Design and synthesis of marine natural product-based 1*H*-indole-2,3-dione scaffold as a new antifouling/antibacterial agent against fouling bacteria

Mahesh S. Majik *, Cheryl Rodrigues, Stacey Mascarenhas, Lisette D'Souza

Bio-Organic Chemistry Laboratory, CSIR-National Institute of Oceanography, Dona-Paula Goa 403 004, India

ARTICLE INFO

Article history:
Received 23 January 2014
Available online 10 May 2014

Keywords:
Biofilm
Biofouling
Secondary metabolites
Isatin
Marine natural products
Structure activity relationships

ABSTRACT

Marine organisms such as seaweeds, sponges and corals protect their own surfaces from fouling by their high anesthetic, repellant, and settlement inhibition properties. Within the marine ecosystem, evolution has allowed for the development of certain antifouling properties. Isatin is a biologically active chemical produced by an *Alteromonas* sp. strain inhibiting the surface of embryos of the cardiean shrimp *Palaemon macrodectylus*, which protect them from the pathogenic fungus *Lagenidium callinectes.* In present study, an antibacterial activity of isatin and its synthetic analogues were evaluated against different fouling bacteria in order to explore the structure activity relationships for the first time. The synthesized compounds along with parent isatin were tested against different ecologically relevant marine microorganisms by using the Kirby–Bauer disc diffusion method. Few synthetically modified isatin exhibited potent inhibitory activity at concentration of 2 μg/disc against *Planococcus donghaensis, Erythrobacter litoralis, Alivibrio salmonicida, Vibrio furnisii.* Overall, the modified analogues showed stronger activity than the parent marine natural product (isatin) and hence 1*H*-indole-2,3-dione scaffold has immense potential as future antibacterial/antifouling candidate.

1. Introduction

Marine organisms are highly prolific sources of biologically active metabolites, many of which assumed key roles in elucidation of cellular mechanisms or as lead structure for drug discovery [1]. Marine sessile organisms such as barnacles, mussels, and hydroids also known as fouling organisms cause serious global damages in marine systems by settling on ship bulls, fishing equipment, aquaculture cages and also the cooling systems of power stations. And hence, biofouling emerged as a problem many centuries ago and likely to be continued for long as mankind will sail the oceans [2]. Apart from creating technical and economical challenges for marine organisms, biofouling also generates many other environmental problems such as spread of invasive species [3]. Corresponding to these facts, various antifouling technologies have been developed over past several decades to protect submerged surfaces by deterring the settlement of colonizing stages of fouling organisms [4]. These include UV irradiations, ultrasound, foul-release polymeric coating and antifouling agents (biocides) [5]. In the past, the most widely used and best performing AF-compounds such as organotin (TBT) was recently prohibited for application to ships, due to the severely toxic biocides and lack of degradation in the natural environment [6]. Subsequently, marine coating industries have been actively developing alternative AF biocides including copper (Cu) supplemented by booster biocides (irgarol 1051, diuron, zinc pyrithione, copper pyrithione, chlorothalonil and SeaNine 211) [7]. However, many of the booster biocides used worldwide cause serious damage to the marine environment and are found to accumulate in coastal water at levels that are deleterious for marine organism [8]. Therefore, discovery of new environmentally friendly antifouling agents is desired. It is well recognized that marine organisms produced diverse natural products to protect themselves from the harmful process of biofouling and protect the surface of their bodies without causing serious environmental problems [9]. On the basis of a literature survey, various potential antifouling marine natural products such as terpenes, acetylenes, steroids, phenols, isothiocyanates, nitrogen-containing compounds, glycerol derivatives, and higher fatty acids are derived from sponges, algae, and cnidarians and exhibit not only toxins but also anesthetics, growth-inhibiting, attachment-inhibiting, metamorphosis-inhibiting, and repelling properties [10].

The chemistry behind the sessile, unfouled surfaces of marine organisms is largely being investigated. In the course of search

* Corresponding author. Fax: +91 832 2450607.
E-mail addresses: majikms@gmail.com, mmajik@nio.org (M.S. Majik).

http://dx.doi.org/10.1016/j.bioorg.2014.05.001
0045-2068/

for compounds involved in defense mechanisms of crustacean eggs, Fenical and co-workers demonstrated that marine bacteria consistently isolated from healthy embryos of the caridean shrimp *Palaemon macrodectylus* (*altermonas* sp.) produced isatin and showed inhibitory activity against the phycomycetous fungus *Lagenidium callinectes* (a pathogen of many crustaceans) [11] (Fig. 1). Recently, Sakata et al. had identified isatin as an algicidal substance produced by the marine bacterium *Pseudomonas* sp. C55a-2 isolated from coastal sea water Kagoshima Bay, Japan. Moreover, many strains belonging to genera *Pseudomonas*, *Alteromonas* and *Pseudoalteromonas* spp. were reported to use an indirect attack in the form of extracellular agents wherein, they produce extracellular algicidal or growth inhibiting substances [12]. These species are reported to produce isatin as a defensive compound. A variety of natural products have been isolated from marine organisms and some may be developed as antifouling agents. However, poor yield of natural compounds from natural source have hampered further developments. In order to create a potent antifouling agent, structural activity relationships must be investigated in detail with respect to inhibitory activity against fouling bacteria [13]. Realizing the importance of these facts and our research interest in marine natural products [14] has motivated us to undertake these studies to provide an efficient synthesis of natural product-based synthetic analogues of isatin and to determine therein antibacterial activities (also called as antifouling activity, as this bioassay study in conducted on fouling bacteria) in order to understand detail SAR profile. To provide an alternative molecular scaffold that inhibits the fouling organisms, we have turned to antifungal and ecologically defensive marine natural product isatin as source for structural insight to guide molecular design.

2. Materials and methods

2.1. Antibacterial assay (inhibitory studies against fouling bacteria)

Nine strains of fouling bacteria were tested to determine the antibacterial capacity of the compounds: Gram positive bacteria (*Planococcus donghaensis*) & Gram negative bacteria (*Alcanivorax* spp, *Aeromonas hydrophila* subsp *hydrophila* ATCC 7966, *A. hydrophila* subsp. *salmonicida* A449, *Erythrobacter litoralis, Pseudomonas mendocina, Alcanivorax borkumensis, Allivibrio salmonicida, Pseudoalteromonas* spp., *Vibrio furnisii*). The strains were isolated and identified by the method of Allegrucci and Sauer [15], Dalton et al. [15], Weisburg et al. [15], from natural biofilms 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 4%. 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 × 10^8 CFU/ml for *E. coli* ATCC 25922.

The Kirby–Bauer disc diffusion method [16] 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 of 2–10 μg/disc. 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/m l was spread plated onto Mueller Hinton agar plates and the dry paper discs with either the test compounds or the control compounds were aseptically laid on the agar surface. The plates were then incubated at 28 °C for 24 h. 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 concentration required to inhibit the test bacteria.

2.2. Synthesis of compounds

General methods: Melting points (Mp.) were determined by using melting point apparatus MR-VIS (LABINDIA). IR spectra were recorded on Shimadzu (IR affinity-1) FT/IR spectrometer on KBr pellets. ^{1}H NMR spectra ($CDCl_3$) were recorded on Varian Unity Inova 300 MHz. Chemical shifts are reported in parts per million (δ) units relative to the solvent peak. The ^{1}H NMR data are reported as peak multiplicities: s for singlet; d for doublet; dd for doublet of doublets; t for triplet; br s for broad singlet; and m for multiplet. Coupling constants were reported in Hertz. Mass spectra were recorded on Agilent Technologies 6220 Accurate-Mass TOF LC/MS spectrometer. Reactions were checked with TLC (Merck precoated 60F254 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. All the anhydrous solvents were distilled over CaH_2, P_2O_5, or Na/benzophenone prior to the reaction.

3. Experimental procedures

3.1. General method for alkylation of isatin [17]

The isatin **1** (1 equv.) was taken in anhyd DMF (2 mL per 0.1 mmol of isatin) and cooled on ice bath with stirring. Solid K_2CO_3 (1.2 equv.) was added in one portion and the dark coloured suspension was stirred at room temp for 2 h. The appropriate alkyl bromide (1.2 equv.) was added and reaction mixture was stirred at 60 °C for 8–12 h until isatin starting material was consumed (monitored by TLC). The reaction mixture was diluted with water

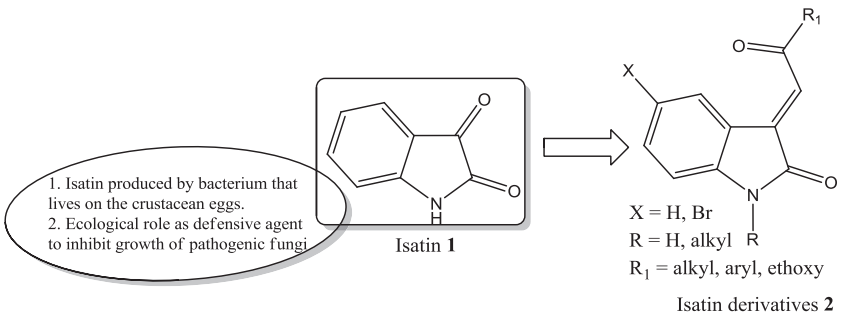


Fig. 1. Isatin **1** from *Alteromonas* sp. and chemical compounds **2** created by considering SAR in present work.

(50 mL) and extracted with EtOAc (2 × 50 mL). The combined organic extract was washed with brine (2 × 50 mL), and dried over anhyd Na_2SO_4 and filtered. The solvent was evaporated and the crude product was purified by using silica gel column chromatography (EtOAc: hexanes). This procedure is utilized in the preparation of **3a** and **3b**.

3.2. Bromination of isatin [18]

To a stirred solution of isatin **1** (2.00 g, 0.013 mol) in EtOH (10 mL) was added bromine (6.52 g, 0.041 mol) at 0 °C and the reaction mixture was stirred at room temperature for 12 h. The reaction was cooled to 0 °C, the orange solid was filtered, washed with water (3 × 50 mL) and dried under high vacuum to give orange colour solid **4**.

3.3. General procedure for Wittig reaction [19]

To a stirred solution of isatin **1** or substituted isatin (**3** & **4**) in EtOH (20 mL) was added corresponding phosphoranes **P1–P3** (prepared using the standard procedure). The reaction mixture was stirred at room temperature for 12 h. The solvent was evaporated under vaccum and the crude product was purified by using silica gel column chromatography (EtOAc: hexanes) to give orange crystalline solid. Compounds **5**, **6**, **7** and **8 (a–c)** were synthesized using this procedure.

3.4. 1H-indole–2,3-dione 1

Orange crystalline solid, mp 196–198 °C. IR (*ν* max): 3461, 1760, 1696 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 6.91 (d, *J* = 8.2 Hz, 1H), 7.11 (t, *J* = 7.7 Hz, 1 H), 7.55 (t, *J* = 7.7 Hz, 1 H), 7.60 (d, *J* = 7.8 Hz, 1 H), 8.16 (br s, 1 H).

3.5. (E)-3-(2-Oxopropylidene)indolin-2-one **5a**

Yield 70%; orange solid, mp 150–151 °C. IR (*ν* max): 3233, 1726, 1678, 1603, 1462, 1325, 1222, 746 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 2.51 (s, 3 H), 6.85 (d, *J* = 7.5 Hz, 1 H), 7.06 (t, *J* = 7.2 Hz, 1 H), 7.18 (s, 1 H), 7.33 (t, *J* = 7.8 Hz, 1 H), 7.75 (br s, 1 H), 8.53 (d, *J* = 7.8 Hz, 1 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{11}H_{10}NO_2$: 188.0712. Found: 188.0710.

3.6. (E)-Ethyl 2-(2-oxoindolin-3-ylidene)acetate **5b**

Yield 82%; orange solid, mp 152–154 °C. IR (*ν* max): 3160, 1713, 1655, 1607, 1462, 1332, 1230, 1027, 752 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 1.39 (t, *J* = 7.2 Hz, 3 H), 4.35 (q, *J* = 7.2 Hz, 14.4 Hz, 2 H), 6.87 (d, *J* = 7.5 Hz, 1 H), 6.89 (s, 1 H), 7.06 (t, *J* = 7.8 Hz, 1 H), 7.34 (t, *J* = 7.5 Hz, 1 H), 8.47 (br s, 1 H). 8.56 (d, *J* = 7.8 Hz, 1 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{12}H_{12}NO_3$: 218.0817. Found: 218.0811.

3.7. (E)-3-(2-Oxo-2-phenylethylidene)indolin-2-one **5c**

Yield 76%; orange solid, mp 174–176 °C. IR (*ν* max): 3188, 1715, 1645, 1612, 1464, 1327, 1032, 791, 754 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 6.90 (d, *J* = 7.8 Hz, 1 H), 7.04 (t, *J* = 7.8 Hz, 1 H), 7.34 (t, *J* = 7.5 Hz, 1 H), 7.55–7.66 (m, 3 H), 7.89 (s, 1 H), 8.14 (d, *J* = 7.2 Hz, 2 H), 8.33(d, *J* = 8.1 Hz, 2 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{16}H_{12}NO_2$: 250.0868. Found: 250.0852.

3.8. 1-Methylindoline-2,3-dione **3a**

Yield 86%; orange solid, mp 128–129 °C [lit. 20]. IR (*ν* max): 1732, 1724, 1606, 1470, 1327, 1032, 864, 760 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 3.27 (s, 3 H), 6.91 (d, *J* = 8.1 Hz, 1 H), 7.15 (t, *J* = 7.5 Hz, 1 H), 7.61–7.65 (m, 2 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_9H_8NO_2$: 162.0555. Found: 162.0543.

3.9. (E)-1-Methyl-3-(2-oxopropylidene)indolin-2-one **6a**

Yield 71%; orange solid, mp 120–121 °C. IR (*ν* max): 1707, 1688, 1599, 1471, 1340, 786 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 2.50 (s, 3 H), 3.25 (s, 3 H), 6.81 (d, *J* = 7.8 Hz, 1H), 7.07 (t, *J* = 7.8 Hz, 1 H), 7.21 (s, 1 H), 7.40 (t, *J* = 7.5 Hz, 1 H), 8.53 (d, *J* = 7.8 Hz, 1 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{12}H_{12}NO_2$: 202.0868. Found: 202.0853.

3.10. (E)-Ethyl 2-(1-methyl-2-oxoindolin-3-ylidene)acetate **6b**

Yield 74%; orange solid, mp 75–76 °C. IR (*ν* max): 1708, 1663, 1607, 1471, 1342, 1200, 1027, 754 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 1.39 (t, *J* = 7.2 Hz, 3 H), 3.25 (s, 3 H), 4.34 (q, *J* = 7.2 Hz, 14.1 Hz, 2 H), 6.81 (d, *J* = 7.8 Hz, 1H), 6.92 (s, 1 H), 7.05 (t, *J* = 7.8 Hz, 1 H), 7.38 (t, *J* = 7.5 Hz, 1 H), 8.53 (d, *J* = 7.8 Hz, 1 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{13}H_{14}NO_3$: 232.0974. Found: 232.0964.

3.11. (E)-1-Methyl-3-(2-oxo-2-phenylethylidene)indolin-2-one **6c**

Yield 79%; orange solid, mp 105–106 °C. IR (*ν* max): 1707, 1663, 1603, 1468, 1338, 1227, 781, 750 cm^{-1} ^1H NMR ($CDCl_3$, 300 MHz) *δ* 3.30 (s, 3 H), 6.83 (d, *J* = 8.1 Hz, 1 H), 7.05 (t, *J* = 7.8 Hz, 1 H), 7.38 (t, *J* = 7.5 Hz, 1 H), 7.55 (t, *J* = 7.8 Hz, 2 H),), 7.63 (t, *J* = 7.5 Hz, 1 H), 7.92 (s, 1 H), 8.12 (d, *J* = 7.2 Hz, 2 H). 8.53 (d, *J* = 7.5 Hz, 1 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{17}H_{14}NO_2$: 264.1025. Found: 264.1011.

3.12. 1-Butylindoline-2,3-dione **3b**

Yield 90%; orange solid, mp 40–42 °C [lit. 20]. IR (*ν* max): 1732, 1724, 1606, 1470, 1327, 1032, 864 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 0.98 (t, *J* = 7.5 Hz, 3 H), 1.39–1.46 (m, 2 H), 1.65–1.75 (m, 2 H), 3.73 (t, *J* = 7.2 Hz, 2 H), 6.91 (d, *J* = 8.4 Hz, 1 H), 7.12 (t, *J* = 7.5 Hz, 1 H), 7.57–7.62 (m, 2 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{12}H_{14}NO_2$: 204.1025. Found: 264.1013.

3.13. (E)-1-Butyl-3-(2-oxopropylidene)indolin-2-one **7a**

Yield 71%; orange solid, mp 65–66 °C. IR (*ν* max): 1707, 1680, 1603, 1466, 1362, 787 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 0.98 (t, *J* = 7.5 Hz, 3 H), 1.39–1.44 (m, 2 H), 1.62–1.72 (m, 2 H), 2.49 (s, 3 H); 3.74 (t, *J* = 7.2 Hz, 2 H), 6.81 (d, *J* = 7.8 Hz, 1 H), 7.05 (t, *J* = 7.8 Hz, 1 H), 7.20 (s, 1 H), 7.38 (t, *J* = 7.5 Hz, 1 H), 8.53 (d, *J* = 7.5 Hz, 1 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{15}H_{18}NO_2$: 244.1338. Found: 244.1326.

3.14. (E)-Ethyl 2-(1-butyl-2-oxoindolin-3-ylidene)acetate **7b**

Yield 78%; orange solid, mp 187–189 °C. IR (*ν* max): 1769, 1699, 1526, 1344, 1196, 1027, 852 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 0.97 (t, *J* = 7.5 Hz, 3 H), 1.36–1.46 (m, 4 H), 1.61–1.72 (m, 3 H), 3.74 (t, *J* = 7.2 Hz, 2 H), 4.34 (q, *J* = 7.2 Hz, 14.4 Hz, 2 H), 6.82 (d, *J* = 7.8 Hz, 1 H), 6.92 (s, 1 H), 7.06 (t, *J* = 7.8 Hz, 1 H), 7.37 (t, *J* = 7.8 Hz, 1 H), 8.58 (d, *J* = 7.8 Hz, 1 H). HRMS: *m*/*z* $[M+H]^+$ Calcd for $C_{16}H_{20}NO_3$: 274.1443. Found, 274.1431.

3.15. (E)-1-Butyl-3-(2-oxo-2-phenylethylidene)indolin-2-one **7c**

Yield 76%; orange semi solid. IR (*ν* max): 1708, 1665, 1602, 1470, 1338, 1227, 784, 752 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) *δ* 0.99 (t, *J* = 7.5 Hz, 3 H), 1.40–1.48 (m, 2 H), 1.69–1.74 (m, 2 H), 3.78 (t, *J* = 7.2 Hz, 2 H), 6.84 (d, *J* = 7.8 Hz, 1 H), 7.03 (t, *J* = 7.5 Hz,

1 H), 7.34 (d, J = 7.8 Hz, 1 H), 7.52–7.65 (m, 3 H), 7.90 (s, 1 H), 8.13 (d, J = 8.1 Hz, 2 H), 8.33 (d, J = 7.5 Hz, 1 H). HRMS: m/z $[M+H]^+$ Calcd for $C_{20}H_{20}NO_2$: 306.1494. Found: 306.1480.

3.16. 5-Bromoindoline-2,3-dione 4

Yield 66%; orange crystalline solid, mp 185–187 °C. IR (ν max): 3160, 1713, 1655, 1606, 1462, 1333, 1230, 752 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 6.84 (d, J = 8.4 Hz, 1 H), 7.71 (d, J = 8.4 Hz, 1 H), 7.76 (s, 1 H). HRMS: m/z $[M+H]^+$ Calcd for $C_8H_5BrNO_2$: 225.9504. Found: 225.9502.

3.17. (E)-5-Bromo-3-(2-oxopropylidene)indolin-2-one **8a**

Yield 72%; orange solid, mp 148–149 °C. IR (ν max): 3215, 1720, 1682, 1611, 1300, 816 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 2.52 (s, 3 H), 6.78 (d, J = 8.4 Hz, 1 H), 7.19 (s, 1 H), 7.47 (d, J = 8.1 Hz, 1 H), 8.68 (br s, 1 H), 8.70 (s, 1 H). HRMS: m/z $[M+H]^+$ Calcd for $C_{11}H_{9}BrNO_2$. 265.9817. Found: 265.9801.

3.18. (E)-Ethyl 2-(5-bromo-2-oxoindolin-3-ylidene)acetate **8b**

Yield 80%; orange solid, mp 207–208 °C. IR (ν max): 3169, 1713, 1650, 1612, 1309, 816 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 1.38 (t, J = 7.2 Hz, 3H), 4.37 (q, J = 7.2 Hz, 14.1 Hz, 2 H), 6.76 (d, J = 8.1 Hz, 1 H), 6.92 (s, 1 H), 7.47 (d, J = 8.4 Hz, 1 H), 7.74 (br s, 1 H), 8.76 (s, 1 H). HRMS: m/z $[M+H]^+$ Calcd for $C_{12}H_{11}BrNO_3$: 295.9922. Found: 295.9902.

3.19. (E)-5-Bromo-3-(2-oxo-2-phenylethylidene)indolin-2-one **8c**

Yield 72%; orange solid, mp 205–206 °C. IR (ν max): 3194, 1721, 1663, 1599, 1448, 1312, 1230, 721, 694 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) δ 6.78 (d, J = 8.4 Hz, 1 H), 7.48 (d, J = 8.1 Hz, 1 H), 7.54–7.67 (m, 3 H), 7.80 (br s, 1 H), 7.93 (s, 1 H), 8.13 (d, J = 8.4 Hz, 2 H), 8.56 (s, 1 H). HRMS: m/z $[M+H]^+$ Calcd for $C_{16}H_{11}BrNO_2$: 327.9973. Found: 327.9971.

4. Results and discussion

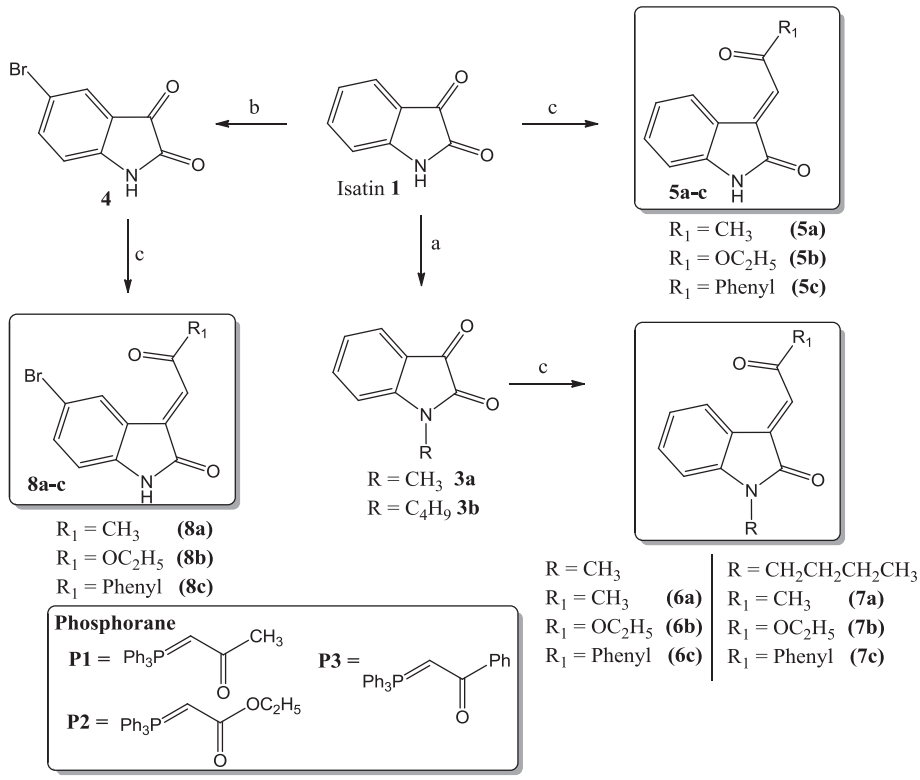
4.1. Synthesis of isatin analogues

Isatin and its derivatives are well known compounds with pharmacological properties e.g. anticonvulsants, antimicrobial, depressants of central nervous systems, antitumour agents, etc. [21]. However, there is only one report regarding the remarkable role of isatin as an allelochemical in protection of shrimp embryos from fungal infections [11]. In the present work, we have modified the 3-carbonyl functionality of isatin **1** using simple synthetic manipulation, in order to understand the importance of carbonyl function (C-3 position) of isatin for its observed inhibitory activity. Since these compounds are Michael acceptor and therefore would be expected to be biologically active. Towards this end, isatin **1** was treated with different phosphoranes (**P-1**, **P-2**, **P-3**) to furnish corresponding olefins **5a–c** (Scheme 1). The geometry around the double bond was assumed to be *trans* (*E*) by comparing the spectroscopic data with the reported compound [22]. Also the detailed study of NOE NMR spectrum of the related compound having similar structure were previously investigated by Shimazawa et al. and were found to contain *trans*-geometry of the double bond [23] (refer Supplementary information for detail 1D-NOE and 2D-NOESY NMR spectra of **5a**). The intension of using this phosphoranes is that the modification at C-3 position of isatin could give us library of isatin derivatives with different substitution at terminal carbon of olefin i.e. α, β-unsaturated ketone (methyl ketone, phenyl ketone), and α, β-unsaturated ester. The effect of this different functionality on inhibitory activity is also evaluated in this work. Furthermore, various *N*-protected derivatives **3a–3b** were prepared in 86–90% yield by treating isatin **1** with different alkylating agents using potassium carbonate as base in ethanol. The presence of bromine atom in structure of many antifouling agents [24] have inspired us to study the impact of bromination upon antibacterial activity in isatin derivatives by introducing bromine atom at 5-position of indole skeleton. Hence, 5-bromoisatin **4** was synthesized by using reported method employing bromination of isatin at room temp. With the sufficient amounts of 5-bromoisatin **4** and *N*-protected isatin **3a–3b** in our hands, these compounds were subjected to Wittig olefination to give corresponding alkenes **6**, **7**, **8** in 70–82% yield. This gives another series of compounds containing *N*-protected isatin derivatives (**6a–c** and **7a–c**) and brominated isatin derivatives (**8a–c**). All the synthesized compounds were obtained in good yield and were characterized by different spectroscopic methods like NMR and mass spectroscopy. The synthesized compounds were then screened for their inhibitory activity against marine fouling bacteria.

4.2. Antibacterial activities of isatin and its synthesized derivatives against fouling bacteria

Compounds were screened initially at concentration of 100 μg/disc to investigate their effects on fouling bacteria using *E. coli, S. aureus* etc. The antifouling activity was monitored under static condition using Kirby–Bauer disc diffusion method (refer Supplementary information for detail antifouling activity data). Initially, isatin **1**, **5a** and **4** was evaluated for their antifouling activity. This preliminary screen analyses (refer Supplementary information for detail antifouling activity data) revealed that isatin **1** is moderately active against *A. salmonicida A449* and *P. donghaensis* whereas, compound **5a** showed broad spectrum of antibacterial activity against Gram +ve and Gram −ve fouling bacteria such as *E. litoralis*, *A. salmonicida,* and *V. furnisii.* Also, bromoisatin **4** exhibited weak inhibitory activity. This observation indicated that the introduction of bromine atom at C-5 position of isatin diminishes its antibacterial property (Fig. 2).

Furthermore, we selected **5a** as our most promising lead compound sought to identify potential structure motif within **5a** that were responsible for the observed antibacterial activity to further improve its inhibitory potential. Five more derivatives of isatin were synthesized imposing modifications at C-3 position and keeping C-1 and C-2 position intact as that of parent isatin to give series of compounds **5b–5c** and **8a–8c**. Compound **5b** (with ethoxy moiety) shows weak activity whereas compound **5c** (aromatic component) loses the activity. This result suggests that, the replacement of methyl group with hydrophobic aromatic moiety led to the complete loss of inhibitory activity. However, the introduction of bromine atom at C-5 position (**8a–8c**) led to either total loss or significant reduction in activity (refer Supplementary information for detail antifouling activity data). Intrigued by these results; we next synthesized more isatin derivatives to further probe the activity of this compound and to understand the role of free NH group of isatin. Eight more derivatives **3a**, **6a–6c** and **3b**, **7a–7c** were prepared by imparting modification at *N*-substituent (*N*-methyl and *N*-butyl, instead of NH) and maintaining C-3 positions unchanged (as that of **5a–5c**). Introduction of methyl group at 1-position of isatin led to decrease in antibacterial activity (**3a**) and further increase in alkyl chain length led to the complete loss of inhibitory activity (comparison **1**, **3a** and **3c**). This suggests that the presence of NH group is necessary for the observed inhibitory activity of isatin family (Fig. 3). This was further supported by observation that the antibacterial activity of derivatives decreases in order of



Scheme 1. Synthesis of isatin derivatives. *Reagents and condition:* (a) K_2CO_3, DMF, methyl bromide or *n*-butyl bromide, 2 h, room temp (86–90%); (b) bromine, EtOH, 0 °C to room temp, 2 h (66%); (c) Phosphorane (**P1** or **P2** or **P3**), EtOH, room temp, 12 h (70–82%).

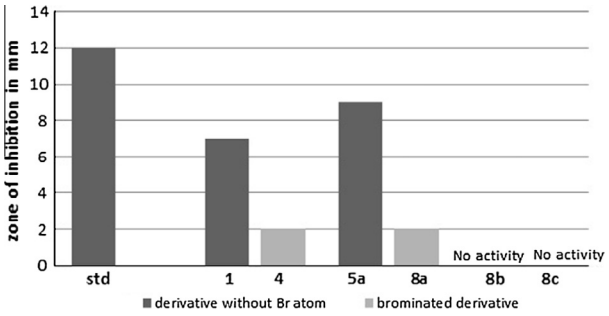


Fig. 2. Effects of bromine substitution of isatins on antibacterial activities against *A. salmonicida A449* (std is Gentamycin). *X*-axis represents synthesized derivatives and *Y*-axis represents zone of inhibition in mm at conc. of 100 μg/disc.

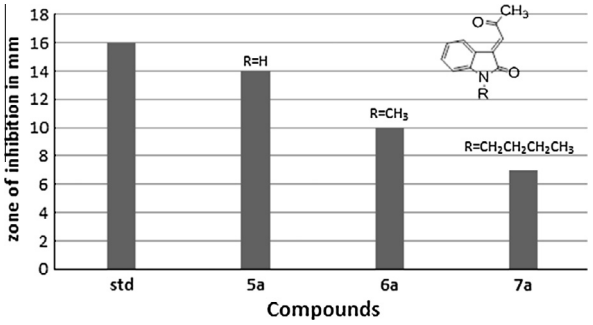


Fig. 3. Effects of *N*-protection of isatin derivatives against *E. litoralis* at conc. of 100 μg/disc. (std is Gentamycin).

5a> **6a**> **7a**> **8a**. For the compounds showing the ability to either inhibit fouling organisms, dose–response studies are subsequently performed to quantify activity (Table 1). Overall compounds **5a**, **6a**, **7a** were prominently inhibitory towards 3 Gram negative fouling bacteria *E. litoralis, A. salmonicida* and *V. furnisii* with only **5a** showing good broad spectrum of activity against Gram +ve and Gram −ve bacteria. The most potent antibacterial agent identified overall was the **5a** (2 μg/disc), which was more active than the parent marine natural product **1** (Fig. 4).

4.3. Analyses of structure activity relationship

To examine the role of different modifications that would be useful in understanding the functional components responsible for the activities of these compounds towards fouling organisms, isatin modifications has been undertaken in present project (Fig. 5) such as (a) bromine substituent at C-5 carbon of isatin ring (**8a–c**), (b) the length of alkyl chain (**6a–c & 7a–c**) relationship, (c) *N*-protections in compounds (**5, 6 & 7**).

(a) *Effect of bromine substituent at C-5 carbon of the isatin ring* (**4**, **8a–c**): From the data presented in Supplementary information for detail antifouling activity data, it has been observed that the use of bromine substituent at C-5 carbon atom in the derivatives of isatin leads to the decrease in antibacterial activity as compared with parent isatin (Fig. 2).

(b) *Effect of alkyl chain length of N-protected isatin derivatives* (**3**, **6, 7**): *N*-methyl and *N*-butyl derivatives were prepared in order to check the effect of hydrophobicity on inhibitory activity of synthesized compounds i.e. [NH (less hydrophobic), N–CH_3, N–C_4H_{10} (more hydrophobic)]. The antibacterial activity remains unchanged for derivatives for *N*-methyl and *N*-butyl iastin derivatives but their activity decreases as compared to isatin with free NH group. Hence, free NH moiety of isatin is necessary for its good inhibitory property against fouling bacteria (Fig. 3).

Table 1
Table showing zones of inhibition values[a] of **5a** and **6a** against fouling bacteria.

Sr. no.	Compounds	Concentration (μg)	*Planococcus donghaensis*	*Erythrobacter litoralis*	*Alivibrio salmonicida*	*Vibrio furnisii*
1	**5a**	2	9	10	10	7
2	**5a**	4	13	11	11	9
3	**5a**	6	13	12	11	9
4	**5a**	8	13	14	13	9
5	**5a**	10	15	15	13	10
6	**6a**	2	5	6	6	4
7	**6a**	4	6	7	5	6
8	**6a**	6	3	5	7	6
9	**6a**	8	7	7	5	5
10	**6a**	10	5	7	4	3
11	Std antibiotic	2	26	14	15	12
12	Std antibiotic	4	28	18	20	17
13	Std antibiotic	6	31	20	23	20
14	Std antibiotic	8	33	25	26	24
15	Std antibiotic	10	35	28	29	27

[a] The data are expressed as the measure of inhibition zones (mm) at concentration varying from 2 to 10 μg/disc; std antibiotic is Gentamycin; data given are the mean of three replicates.

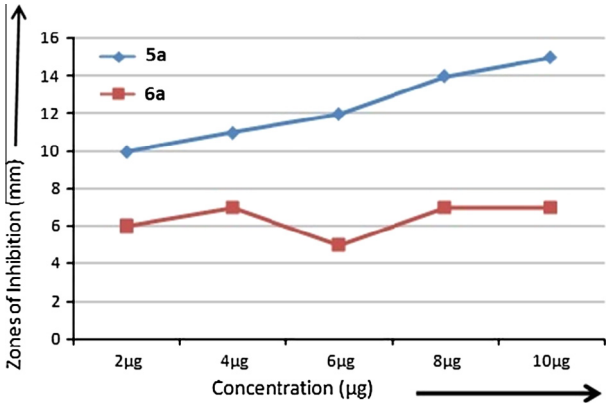


Fig. 4. Graph of concentration of **5a** & **6a** *v/s* Zones of inhibition, showing the minimum concentration required against the fouling bacterium *E. litoralis*.

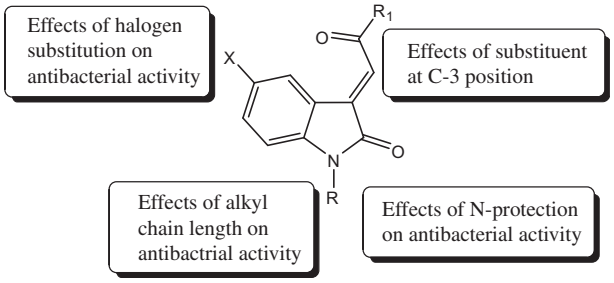


Fig. 5. Different modifications on isatin ring.

(c) *Effects of modification of C-3 positions of isatin* (**5–8**): Isatin derivatives with the extended conjugation at C-3 position (Michael acceptor) were prepared by maintaining C-2 carbonyl and 1H intact (NH). The antibacterial activity increased in this class of systems wherein acetonyl moiety is found to have better functionality compared to more extended hydrophobic benzoyl, as well as electron donating ethoxy group. Also the series of compounds **5a–c** from this group were found to possess good inhibitory effect compared to parent isatin as well as *N*-protected isatins (**6a–c** & **7a–c**) and 5-bromoisatin derivatives **8a–c**. The presence of 3-acetonylidene group and free NH moiety is a crucial factor responsible for enhancing antibacterial activity amongst isatin family **5a**.

5. Conclusions

This report represents the first studies towards the antibacterial activity of marine natural isatin and its modified compounds. The syntheses of compounds have been achieved by using simple synthetic manipulation employing easily available starting materials. The 3-acetonylidene oxindole **5a** is identified as the most potent with maximum antibacterial properties against fouling bacteria. Moreover, the modified analogues showed stronger activity than the parent marine natural product (isatin). The compounds in present study show good activity against bacteria that cause fouling, whereas to demonstrate actual antifouling activity, further antifouling assays on barnacle larvae would need to be carried out. Overall, this study suggest that the screening of natural products and its modified analogues is a promising way to find novel antifouling agents and may potentially be used in the future for antifouling and antibiofilm applications.

Acknowledgments

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.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bioorg.2014.05.001.

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