



Marine bisindole alkaloid 2,2-bis(6-bromo-3-indolyl)ethylamine to control and prevent fungal growth on building material: a potential antifungal agent

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Abstract

The potential antifungal activity of the marine alkaloid 2,2-bis(6-bromo-3-indolyl)ethylamine (URB 1204) was firstly assessed by minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against different fungi. Then, URB 1204 was applied to a building material experimentally contaminated with selected fungi, in single and mixed species, for determining its potential application in preventing fungal growth. In addition, the over-time protection efficacy of URB 1204 was verified, subjecting the treated building surfaces to natural fungal contamination for 6 weeks. URB 1204 showed different antifungal activity, with the lowest MIC value (16 µg/mL) observed against *Aspergillus flavus* IDRA01, *Cladosporium cladosporioides* ATCC 16022 and *Mucor circinelloides* EHS03, and the highest MIC (128 µg/mL) against the dermatophytes strains. The growth *Alternaria alternata* BC01, *Penicillium citrinum* LS1, and *C. cladosporioides* ATCC 16022 on building material treated with URB 1204 water solution (64 µg/mL) was remarkably reduced with an effect time-dependent and related to the examined fungi. In terms of over-time efficacy, the samples treated with URB 1204 showed a delay of fungal growth comparable with that of a commercial antifungal product. These findings evidenced not only the ability of 2,2-bis(6-bromo-3-indolyl)ethylamine to limit the growth of different fungal species on building material but also to provide long-term protection against mold growth and proliferation, opening new perspectives for URB 1204 as preventive agent.

Keywords Marine bisindole alkaloid · Fungal growth · Building material · Preventive agent

Introduction

Molds and decay fungi, responsible for the decomposition and recycling of organic matter, in the built environment are often related with esthetic damages (Airaksinen et al. 2004; Mensah-Attipoe et al. 2015) as well as with human health problems, such as asthma and respiratory infections (Mendell et al. 2011; Kanchongkittiphon et al. 2015). Indeed, these microorganisms possess bioactive potential

and, more importantly, have the capacity to spread spore and fragments in the surrounding environment. These events are able to induce symptoms in healthy people at concentration depending on the specific characteristics of the involved species, such as mycotoxin production and pathogenicity (Pitkäranta et al. 2011).

In the indoor environment, moisture is the limiting factor for microbial growth, but not for fungi that are more tolerant to low-moisture conditions than bacteria (Flannigan et al. 2011) and, for this, the low levels of adsorbed water may be enough to support their growth on building materials (Hyvärinen et al. 2002; Andersen et al. 2011; Nevalainen et al. 2015). Indeed, moisture may increase different exposing agents, such as volatile organic compounds produced from damp building materials and structures or during microbial growth (Jayaprakash et al. 2017). Moreover, structural factors, such as condensation, improper construction practices, water leaks, inadequate drainage, and ventilation, can lead to increased indoor humidity (Adams et al. 2016). In this direction, modern building practices have improved insulation hindering ventilation, but

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the result was often an increased area of condensation with the consequent mold's growth (Schmidt 2006). However, studies in non-wetted buildings have challenged the viewpoint that substrate composition influences the microbial community structure (Dunn et al. 2013; Adams et al. 2014). In general, wooden materials have greater fungal diversity than plaster-board or ceramics (Hyvärinen et al. 2002), while cellulose-based materials are more sensitive to fungal contamination than inorganic materials such as gypsum, mortar, and concrete (Gutarowska 2010; Hoang et al. 2010). Nevertheless, molds can also attack synthetic floor coverings, glues, paints, and textiles demonstrating a great ability to respond to a variety of conditions such as water activity, relative humidity, and pH (Schmidt 2006). Moreover, several environmental conditions (temperature, available water, cleaning chemicals and frequency, carbon sources) can create diverse selective pressures for microorganisms resulting in different survival and persistence rates (Tang 2009; Adams et al. 2016).

Cladosporium spp., *Penicillium* spp., and *Alternaria* spp. are the fungal genera more diffused in indoor environments (Flannigan et al. 2011; Barberán et al. 2015; Prussin and Marr 2015; Salem et al. 2015), responsible of the negative effect on the esthetic value of the building materials due to their pigmented mycelium and melanin production in and around the fungal hyphae. These fungi are also recognized as the main cause of allergic symptoms and respiratory diseases in individuals exposed to high levels of spores and/or allergens. Fungicides are the common agents used to control and prevent these problems, but one risk they have posed is the development of fungal resistance and the accelerated evolution of virulence in pathogenic fungi connected with human activities (Fisher 2012). In this direction, the search of naturally and environmentally friendly alternatives is still a challenge and several studies described the application of essential oils or other natural compounds to control fungal growth (Clausen and Yang 2005; Bound et al. 2016; Salem et al. 2015). The marine environment provides structurally diverse and biologically active secondary metabolites and, during the last 15 years, around 65% of natural products possessing antifungal activities have been isolated from marine organisms (El-Hossary et al. 2017; Ji et al. 2018). The marine bioactive molecules were generally produced to act in a variety of chemically mediated interactions, such as the primary protection against predators or competitors for natural sites, and against the opportunistic pathogenic microorganisms (Acevedo et al. 2013). Sponges are the most studied sessile organisms producing active secondary metabolites, and, among these, the marine alkaloid 2,2-bis(6-bromo-3-indolyl)ethylamine (URB 1204), isolated from the Californian tunicate *Didemnum candidum* and the New Caledonian sponge *Orina* spp. (Fahy et al. 1991; Bifulco et al. 1995), has recently shown different biological activities, including cytotoxicity against several tumor cell lines (Mari et al. 2014; Mantenuto et al. 2016; Salucci

et al. 2018), antibacterial and anticandidal activities (Campana et al. 2019). Indeed, some marine bisindole alkaloids derived from sponges, such as Dendridine A and Hamacanthin A (Fig. 1) sharing with URB 1204 a common two bromoindole molecular units, displayed antifungal activity (Tsuda et al. 2005; Oh et al. 2006). However, to the best of our knowledge, no studies were devoted to exploring the antifungal ability of URB 1204. For these reasons, in the present work, the antifungal activity of 2,2-bis(6-bromo-3-indolyl)ethylamine (URB 1204) was investigated. The experimental design included three different steps: (i) the preliminary determination of the minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) against different fungal species; (ii) the application of URB 1204 on building material (a plaster), experimentally contaminated with three different fungi, to verify the potential of this natural compound in preventing fungal growth in indoor environments; and (iii) the assessment of the over-time efficacy of by a prolonged exposure of building material treated with URB 1204 to natural fungal contamination.

Experimental section

Chemicals

All organic solvents and reagents were purchased from Sigma (Milan, Italy). Prior to use, acetonitrile was dried with molecular sieves with an effective pore diameter of 4 Å. Column chromatography purifications were performed under “flash” conditions using Merck 230–400 mesh silica gel (VWR, Milan, Italy). Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel plates (silica gel 60 F254) (VWR), which were visualized by exposure to ultraviolet light and an aqueous solution of cerium ammonium molybdate (CAM). Stock solution was prepared in DMSO (dimethyl sulfoxide), while work solutions were diluted in sterile water. A commercial chemical antifungal (Smuffer, Henkel, Italy) containing NaClO (5–15%) was used as internal control. Electrospray ionization-mass spectrometry (ESI-MS) spectra were recorded with a Waters Micromass ZQ spectrometer (Milford, Massachusetts, USA). ¹H NMR spectra were recorded on a Bruker AC 400 spectrometer (Billerica, Massachusetts, USA) and analyzed using the TopSpin software package. Chemical shifts were measured by using the central peak of the solvent.

Synthesis of 2,2-bis(6-bromo-3-indolyl)ethylamine

Diphenyl phosphate (0.02 mmol) was added to a solution of 6-bromoindole (0.4 mmol) and (trifluoroacetyl amino)acetaldehyde dimethyl acetal (0.2 mmol) in anhydrous acetonitrile (0.2 mL), and the resulting mixture was stirred at 80 °C for 24 h in a sealed

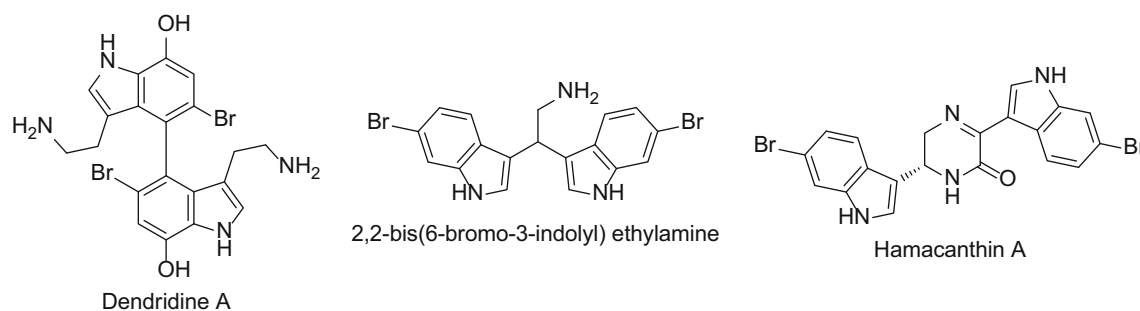


Fig. 1 2,2-Bis(6-bromo-3-indolyl)ethylamine (URB 1204) and two examples of marine bisindole alkaloids with antifungal activity having two bromoindole molecular units

tube, monitoring the progress of the reaction by TLC and high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Waters). After cooling at room temperature, saturated aqueous sodium bicarbonate (30 mL) and dichloromethane (30 mL) were added and the two phases were separated. The aqueous solution was extracted with dichloromethane (3×20 mL). After drying over dry sodium sulfate, the combined organic phases were concentrated in vacuum and the resulting crude product was utilized without further purification. A mixture of that crude trifluoroacetamide derivative and potassium carbonate (1 mmol) in methanol (1.9 mL) and water (0.15 mL) was stirred and heated at reflux for 2 h. The methanol was removed under reduced pressure and water was added (30 mL). The aqueous solution was extracted with dichloromethane (3×30 mL), and the resulting solution was dried with sodium sulfate and concentrated in vacuum. The crude material was purified by flash chromatography on neutral alumina (dichloromethane/methanol/ammonia, 98:1:1) to give the product as a white solid in 82% yield (two steps). The reaction scheme is reported in Fig. 2.

The physico-chemical data of the compound are in agreement with those reported (Mari et al. 2014). TLC: $R_f = 0.19$ (silica gel; dichloromethane/methanol/triethylamine, 90:9:1; UV, CAM). MS (ESI): m/z 430 (50), 432 (100), 434 (50) $[M-H]^-$. ^1H NMR (400 MHz, DMSO- d_6 , 298 K): $\delta = 3.20$ (d, $J = 7.0$ Hz, 2H, $CHCH_2NH_2$), 3.34 (brs, 2H, $CHCH_2NH_2$), 4.33 (dd, $J_1 = J_2 = 7.0$ Hz, 1H, $CHCH_2NH_2$), 6.98 (dd, $J_1 = 1.5$, $J_2 = 8.5$ Hz, 2H, ArH), 7.25 (d, $J = 1.5$ Hz, 2H, ArH), 7.38 (d, $J = 8.5$ Hz, 2H, ArH), 7.48 (d, $J = 1.5$ Hz, 2H, ArH), and 10.99 (brs, 2H, NH_2) ppm.

Fungal strains and inoculum preparation

The preliminary screening of the antifungal activity was performed against 15 filamentous fungi belonging to our culture collection and including *Absidia corymbifera* EHS01, *Alternaria alternata* BC01, *Aspergillus flavipes* EHS02, *Aspergillus flavus* IDRA01, *Aspergillus fumigatus* IDRAH01, *Aspergillus nidulans* IDRAH04, *Aspergillus terreus* IDRS023, *Aspergillus ustus* IDRS08, *Aspergillus versicolor* IDRA02, *Fusarium solanii* IDRS03, *Fusarium sporotrichioides* IDRS05, *Mucor circinelloides* EHS03, *Penicillium citrinum* LS1, and *Rhizopus* spp. IDRAH07. Two dermatophytes strains, *Trichophyton rubrum* EHS05 and *Trichophyton tonsurans* EHS06, were added as well as *Cladosporium cladosporioides* ATCC 16022 (Supplementary Table S1). The fungal strains were grown on Potato Dextrose Agar (PDA, Liofilchem, Roseto degli Abruzzi, Italy) at 25 °C for 5–7 days, while dermatophytes at 30 °C. Fungal suspensions were prepared as described by the National Committee for Clinical Laboratory Standards (CLSI, M38-A2). Briefly, for each strain, spores were harvested from PDA plate by adding 2 mL of sterile 0.85% saline solution supplemented with 0.05% Tween 80; the surface was scraped with a sterile cotton swab, and the suspension was transferred in a sterile tube and left at room temperature for 5 min to allow the sedimentation of hyphal fragments. Afterward, the upper homogeneous suspension was vortexed for 15 s and adjusted to an optical density (OD_{530nm}) between 0.09 and 0.4 corresponding to about 10^6 spores/mL. The quantification of each inoculum was verified with the agar plate count method on PDA (Liofilchem).

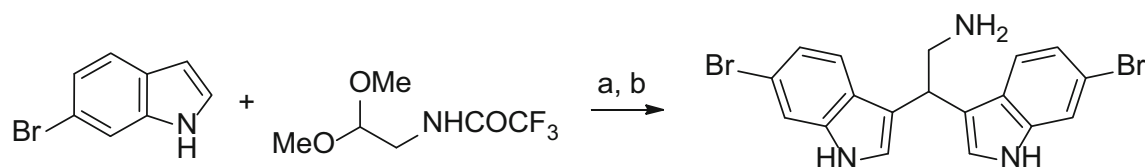


Fig. 2 Synthesis of 2,2-bis(6-bromo-3-indolyl)ethylamine. Reaction conditions: (a) diphenyl phosphate, acetonitrile, 80 °C, 24 h; and (b) potassium carbonate, methanol, reflux, 2 h

MIC and MFC determination

MICs were determined by the standard micro-dilution method according to NCCLS (M38-A) (National Committee for Clinical Laboratory Standards 2008). As first, a concentrated stock solution of URB 1204 was prepared in pure DMSO (Sigma) and used to perform the antimicrobial assay. Preliminary experiments were carried out with DMSO to exclude its possible anti-fungal activity; in any case, the volume of DMSO never exceeded 5% (v/v) of the final volume. Successively, 100 μ L of each fungal suspension, prepared as above described, was diluted 1:50 in standard RPMI 1640 medium (Sigma) and inoculated into 96-well plates together with the appropriate volumes of the test solution (256–2 μ g/mL). Two rows were left for positive control growth and negative controls (medium only), respectively. In addition, amphotericin B (64–0.25 μ g/mL) and fluconazole (128–8 μ g/mL) (Sigma) were used as internal controls. Plates were incubated at 25 °C (30 °C for dermatophytes) and examined after 72 h of incubation. MIC is defined as the lowest drug concentration that inhibits the visible growth in comparison with the control (untreated sample). In addition, the turbidity of the 96-wells plate was assessed by spectrophotometer (530 nm) (Multiskan EX, Thermo Scientific, Monza, Italy).

To determine the minimum fungicidal concentration (MFC), from each well showing complete growth inhibition (clear well), the last positive well (growth similar to that of the growth control), and the growth control, respectively, 20 μ L were removed and streaked on PDA (Liofilchem). The plates were incubated at 25 °C (30 °C for dermatophytes) for 5–7 days; for each microorganism, MFC is defined as the lowest drug concentration that showed either no growth or fewer than three colonies (approximately 99 to 99.5% killing activity). Each experiment was performed in triplicate.

Assessment of antifungal activity of URB 1204 on building material

In order to simulate a real environmental indoor condition, a finishing plaster based on lime and white or gray cement (IG 21, Fassa Bortolo, Treviso, Italy) was chosen. The product was prepared according to the manufacturer's instructions, but using sterile water and sterile utensils to limit bacterial contaminations.

The antifungal activity of URB 1204 on the chosen material was determined following the indication of ASTM G21-96 (2002), with several modifications. First, nutrient salt agar (NSA) was prepared (KH_2PO_4 0.7 g/L, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.7 g/L, NH_4NO_3 1 g/L, NaCl 0.005 g/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.002 g/L, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.002 g/L, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.001 g/L, K_2HPO_4 0.7 g/L, agar 15 g/L) (pH 6.0–6.5) and sterilized by autoclaving at 121 °C for 15 min.

The building material was prepared following the indication of the manufacturer. In brief, 100 g of IG21 was mixed

with 32 ml of sterile water for 3 min and left to repose for 10 min. At this point, about 5 g of product was gently distributed with a sterile spatula on the surface of NSA plates in order to obtain rectangular surfaces (6×4.5 cm, 5 mm of thickness). Once dried (up to 5 days in sterile condition at room temperature), the samples were sprayed with 100 μ L of compound URB 1204 water solution (64 μ g/mL) or 100 μ L of the control solution; some samples were left as negative controls (not treated) (Supplementary Fig. S1).

Selected fungal strains, *P. citrinum* LS1, *A. alternata* BC01, and *C. cladosporioides* ATCC 16022, were grown in PDA at 28 °C for 5–7 days; afterward, the inoculums were prepared as described above to obtain the final concentration of about 10^6 spores/mL. The rectangular surfaces were then covered with 75 μ L of spore suspension of every single strain as well as mixed strains, with the following scheme of inoculation:

1. Four plates were inoculated with single or mixed fungal strains (positive controls).
2. Four plates were treated with URB 1204 water solution and inoculated with single or mixed fungal strains.
3. Four plates were treated with the control solution and inoculated with single or mixed fungal strains.
4. Four plates were not inoculated (negative controls).

All the plates were incubated at 28 °C and regularly observed to check the fungal growth progression up to 28 days. The experiments were performed two times in triplicate.

Visual observation

At each time point of the incubation period (7, 14, 21, and 28 days), fungal growth was visually evaluated by the naked eye in accordance with ASTM G21-96 (2002) standard. The fungicidal activity of URB 1204 was then estimated as fungal growth delay, using the following visually determined rating (*R*): *R* = 0, no visible growth; *R* = 1, trace of growth (less than 10%); *R* = 2, light growth (ranging from 10 to 30%); *R* = 3, medium growth (ranging from 30 to 60%); and *R* = 4, heavy growth (ranging from 60% to complete coverage). All the notes about the visual fungal growth were recorded and, when necessary, microscopic observation was performed by a stereomicroscope (Stemi DV4, Zeiss, Jena, Germany).

Over-time protecting efficacy

The building material was prepared as described above and the obtained rectangular samples were treated with 100 μ L of URB 1204 water solution (64 μ g/mL) or 100 μ L of the control solution. Also in this case, some samples were left as negative controls (not treated). All the samples, in triplicate, were then subjected to natural environmental contamination leaving

them at room temperature (ranging from 18–25 °C) and away from direct light up to 6 weeks. The samples were weekly observed, and the differences in fungal growth between treated and not treated surfaces were recorded.

Results

Antifungal activity of URB 1204

The antifungal activity of URB 1204 is summarized in Table 1. As shown, the lowest MIC values of 16 µg/mL were observed for *A. flavus* IDRA01, *C. cladosporioides* ATCC 16022, and *M. circinelloides* EHS03, while the MICs of the other fungal strains ranged from 32 µg/mL (*A. corymbifera* EHS01, *A. alternata* BC01, *A. ustus* IDRS08, *A. versicolor* IDRA02, *F. sporotrichioides* IDRS05, and *Rhizopus* spp. IDRAH07) up to 64 µg/mL (*A. flavipes* EHS02, *A. fumigatus* IDRAH01, *A. nidulans* IDRAH04, *A. terreus* IDRS023, *F. solanii* IDRS03, *P. citrinum* LS1). The highest values of 128 µg/mL were observed for the dermatophytes strains, *T. rubrum* EHS05 and *T. tonsurans* EHS06. As regards the MFCs, most of the fungal strains (41.7%) were completely killed by URB 1204 at a concentration of 64 µg/mL, followed by an MFC of 32 and 128 µg/mL (35.2 and 43.5%, respectively). Also in this case, the two dermatophytes exhibited the highest MFC values. With regard to the internal controls, most of the strains (70%) were sensitive to amphotericin B with MICs of 2 µg/mL and MFCs values of

4 µg/mL; only *A. nidulans* IDRAH04, *C. cladosporioides* ATCC 16022, and *P. citrinum* LS1 showed higher MIC and MFC values. In the case of fluconazole, MICs of 16 and 64 µg/mL were observed for *T. rubrum* EHS05 and *T. tonsurans* EHS06, with MFC values of 64 and 128 µg/mL, respectively.

Assessment of antifungal activity of URB 1204 on building material

The antifungal effect of URB 1204 applied to the building material, artificially contaminated with three common fungi in single and mixed species, was assessed and compared with the related untreated and antifungal-sprayed samples. The visual observations of fungal growth at 14, 21, and 28 days are presented in Figs. 3 and 4.

As a general trend, the application of the marine alkaloid to the studied material showed remarkable effects on the mold growth, with an effect, in most cases time-dependent and related to the examined fungi. It can be noted that the fungal growth started from the edge of the plates and, during the experiment, moved to the center where are placed the building material samples. Indeed, a delay on growth was observed after 14 days of incubation with only a few colonies recovered in the case of *C. cladosporioides* ATCC 16022 (Fig. 3a); the prolongation to 21 days resulted in a more remarkable delay in the growth of *P. citrinum* LS1, *A. alternata* BC01 and *C. cladosporioides* ATCC 16022 in comparison to the related untreated samples (Fig. 3b). This trend was still visible at the

Table 1 MIC and MCF values (µg/mL) of the tested URB 1204 against selected fungal strains, assessed by broth micro-dilution method. Amphotericin B and fluconazole were used as internal controls

	URB 1204 MIC/MFC (µg/mL)	Amphotericin B	Fluconazole
<i>A. corymbifera</i> EHS01	32/32	2/4	n.d.
<i>A. alternata</i> BC01	32/32	2/4	n.d.
<i>A. flavipes</i> EHS02	64/64	2/4	n.d.
<i>A. flavus</i> IDRA01	16/64	2/4	n.d.
<i>A. fumigatus</i> IDRAH01	64/64	2/4	n.d.
<i>A. nidulans</i> IDRAH04	64/128	8/16	n.d.
<i>A. terreus</i> IDRS023	64/64	2/4	n.d.
<i>A. ustus</i> IDRS08	32/32	2/4	n.d.
<i>A. versicolor</i> IDRA02	32/64	2/4	n.d.
<i>F. solanii</i> IDRS03	64/64	2/4	n.d.
<i>F. sporotrichioides</i> IDRS05	32/64	2/4	n.d.
<i>M. circinelloides</i> EHS03	16/32	2/4	n.d.
<i>P. citrinum</i> LS1	64/128	4/8	n.d.
<i>Rhizopus</i> spp. IDRAH07	32/32	2/4	n.d.
<i>T. rubrum</i> EHS05	128/128	n.d.	16/64
<i>T. tonsurans</i> EHS06	128/128	n.d.	64/128
<i>C. cladosporioides</i> ATCC 16022	16/32	8/16	n.d.

n.d. not determined

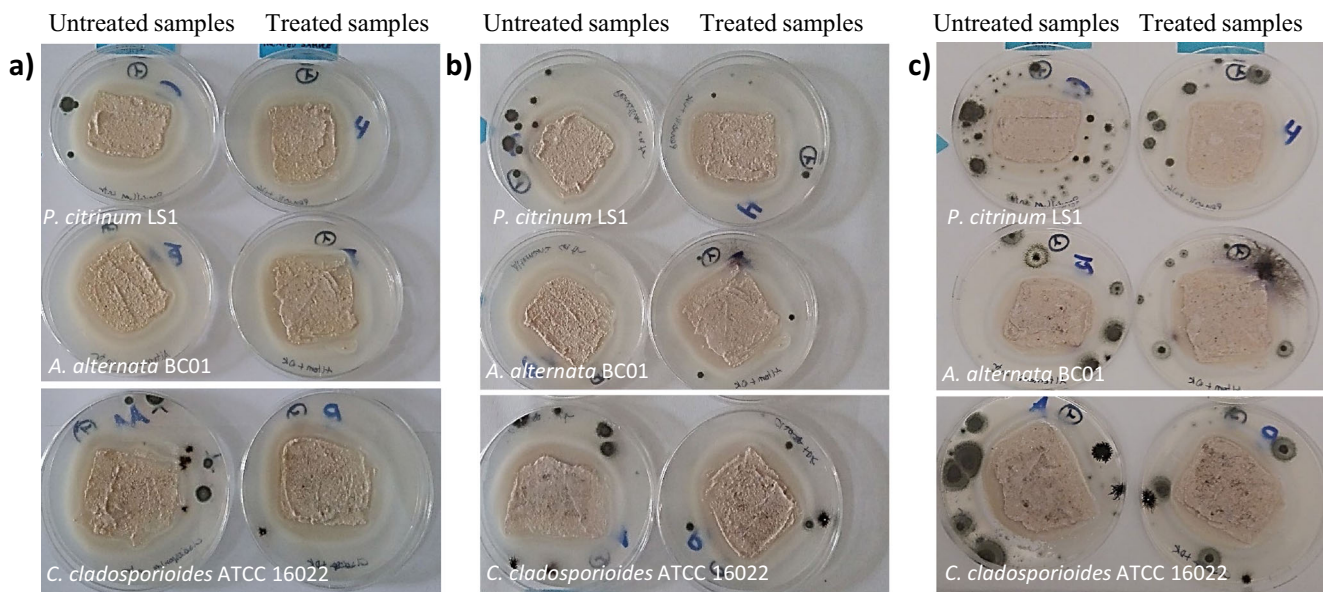


Fig. 3 Visual observations of URB 1204 effect on the growing of different fungi on the surface of IG21 (finishing plaster) after 14 days (a), 21 days (b), and 28 days (c) of incubation in comparison to the untreated samples

last time of incubation (28 days) when a reduced growth was observed on the treated surfaces for all the examined fungi (Fig. 3c). Also in the case of the mixed fungi, the effect of URB 1204 was verified as a wide delayed growth (Fig. 4). Interestingly, the effect of this molecule in protecting the surface of IG21 from the fungal growth was still evident after 28 days of incubation, with a lower number of observed

colonies compared to the negative and the untreated samples (Fig. 4c). As expected, the protecting effect was highest in samples treated with the commercial antifungal product, where no growth was observed after 28 days of incubation (Supplementary Fig. S2).

Figure 5 summarizes the visual fungicidal activity as growth delay (ranging from 0 to 3 marks). A general delay in growth of single strain and mixed species was verified. In detail, after 14 days of URB 1204 application, the complete delay of *P. citrinum* LS1, *A. alternata* BC01, and *C. cladosporioides* ATCC 16022 growth as single and mixed

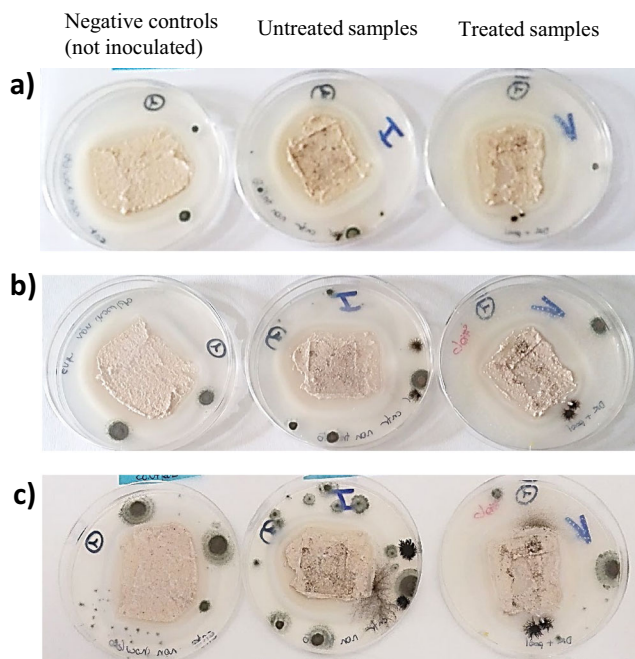


Fig. 4 Visual observations of URB 1204 effect on the growing of mixed fungal strains on surface of the IG21 (finishing plaster) after 14 days (a), 21 days (b), and 28 days (c) of incubation, in comparison to the untreated samples and negative controls

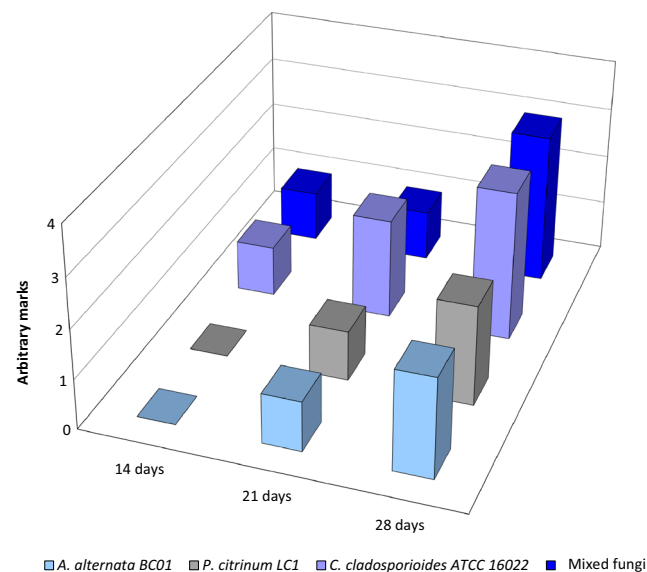


Fig. 5 Fungicidal activity of URB 1204 estimated by fungal growth delay on finishing plaster using the visually determined marks (from 0 to 4), according to G21-96 standards (2002)

species in comparison to the related untreated samples was observed, with marks ranging from 0 to 1. These delays of growth were maintained also after 21 days of incubation (mark from 1 to 2 for all the species, in single and mixed species), whilst reached higher marks after 28 days (from 2 to 3).

Assessment of URB 1204 over-time protecting activity

In these last experiments, the over-time protecting activity of the natural marine alkaloid was verified. The samples were subjected to natural fungal contamination for a long period (up to 6 weeks), and the results were weekly photographed and evaluated as fungal growth delay (Fig. 6). A total of nine samples were observed, three untreated, three treated with URB 1204, and three treated with the commercial antifungal. As shown, the initial fungal contamination started after 4 weeks; microscopic and macroscopic observations revealed that the most visible growth was related to *Penicillium* spp. followed by *Cladosporium* spp. (data not shown). In terms of over-time efficacy, the samples treated with the natural alkaloid showed a reduction of fungal growth after 4, 5, and 6 weeks comparable with that of the commercial antifungal product based on NaClO (5–15%).

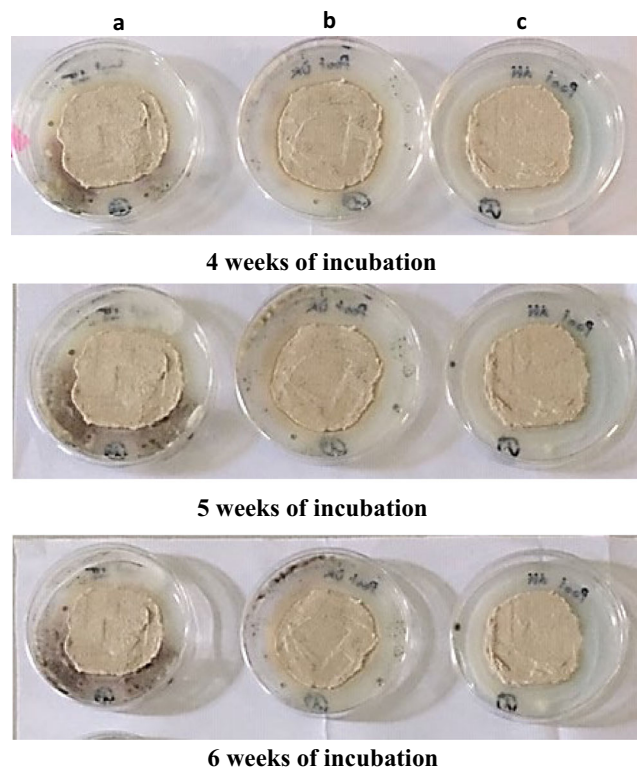


Fig. 6 Over-time protecting effect of URB 1204 against fungal growth on the surface of IG21 (finishing plaster) compared to a commercial antifungal product (containing 5–15% NaClO) after 4, 5, and 6 weeks of incubation: (a) untreated samples, (b) URB 1204 treated samples, and (c) commercial antifungal treated samples

Discussion

The information about the growth of various microorganisms on different building materials and the related human health adverse effects is currently incomplete (Hyvärinen et al. 2002; Bertron 2014; Adams et al. 2016). Some efforts were made to contrast the growth of the most common molds on building materials, but their adapting ability to adverse conditions represents the fundamental problem for every type of application. Usually, indoor surfaces with fungal contamination were removed and treated with an antifungal product; moreover, control strategies to modify the indoor environmental conditions and prevent fungal growth were recommended (Chakravarty and Kovar 2013; Rogawansamy et al. 2015). The European Communities Council Directive 89/106/EEC established specific requirements for building construction in terms of hygiene, sanitation, and biological corrosion resistance. In this direction, the use of materials, products, and building elements resistant to fungus and other forms of degradation were obligatory and building structures shall not constitute a health risk to users or the environment (Essential Requirements No. 3 1994). There are three main types of well-known coatings that can inhibit adhesion and prevent the formation of biofilms, or slowly release of biocides into paints and in the environment, or directly kill the microorganisms (Edge et al. 2001; McDaniel et al. 2006; Wales et al. 2006; Konwar et al. 2010). However, in the last decade, fungicides originated from a “natural” source are being investigated for their use in fungal contamination remediation on a different field of application (Konwar et al. 2010; Rogawansamy et al. 2015; Salem et al. 2015; Barberia-Roque et al. 2018).

In the present study, we assessed the possible application of the natural alkaloid bisindole (URB 1204) as an antifungal resource for the indoor environment. The synthesis of this alkaloid was carried out according to literature procedures (Mari et al. 2014). Moreover, the ethylamino side chain of URB 1204 might improve its water solubility, possibly enhancing the antifungal activity as described for Dendridine A (Tsuda et al. 2005). As first step, to better define the antifungal activity of URB 1204, a fungal collection including environmental isolates as well as human pathogens was screened. The obtained data, expressed as MIC/MFC, showed that URB 1204 was ineffective toward human dermatophytes, while possessed antifungal activity against the environmental fungi, with MFC values ranging from 32 to 128 µg/mL. Based on these results, three different fungal species, *A. alternata* BC01, *P. citrinum* LS1, and *C. cladosporioides* ATCC 16022, representing the most implicated genera in build indoor damages, were selected for the following step that included the application of URB 1204 water solution (64 µg/mL) to one commercial building material. The applied modified protocol has utilized single fungal strain and the three mixed species to simulate the real condition where more than

one species is able to colonize a building surface. The visual observation evidenced that the application of the marine alkaloid URB 1204 to the surface of the finishing plaster was effectively able to contrast the growth of fungi that prevalently have grown in the area around the rectangular surfaces of the plaster and not on the surfaces themselves. This effect was remarkable against the single fungal species, with a different degree of sensitivity to URB 1204, and was still evident toward the mixed fungi. It can be observed that under the unfavorable conditions of these experiments (such as the used nutrient salt agar that contains no specific nutrients but only salts), mold growth may start and proceed at different rates, depending upon the interrelationship between humidity and temperature or other factors, such as the specific microorganisms involved or the properties of the building material (Viitanen et al. 2009). The final part of the presented research was focused to determine the over-time protecting effect of the natural bisindole alkaloid (2,2-bis(6-bromo-3-indolyl)ethylamine) on the treated material. Interestingly, the obtained results appeared encouraging, since on surfaces treated with URB 1204, a fungal growth delay comparable to that evidenced on the surfaces treated with the commercial antifungal product was noticed. According to the available literature, *Penicillium* sp., the so-called first colonizer even in healthy buildings, was the dominant microorganism recovered in our samples after the first 4 weeks of experiments. During the following weeks, we observed as the secondary colonizers (such as *Cladosporium* spp.) and were able to colonize the constructing material under optimal thermal and moist conditions (Piecková et al. 2007). These data support the idea that “natural” compounds, such as essential oils, nanoparticles, or bioactive molecules (Vengurlekar et al. 2012; Ogar et al. 2015; Verma and Devi 2015), can be applied as antifungal on different types of surfaces/environments. Currently, we could not fully explain the mode of action of antimicrobial activity of URB 1204, because the molecular mechanism of bis(indole) alkaloids is still little investigated. However, a preliminary mechanism study (Zhang et al. 2018) revealed that similar heterocycle-rich compounds could depolarize fungal membrane potential and intercalate into DNA to possibly block DNA replication, thus exhibiting powerful antifungal activity.

In conclusion, the surfaces of several building materials, such as plasterboard and mortar, are generally highly porous and, in damp environments, can provide favorable support to the proliferation of microorganisms. The constant challenge to understand the interaction between microorganisms and building materials necessarily requires a multidisciplinary approach involving microbiologists, civil engineers, chemists, and process engineers. Herein, we report as the tested natural marine alkaloid URB 1204 resulted able to limit the growth of different fungal species on the selected building material and, more interestingly, provided long-term protection especially in

environments promoting fungal growth. Overall, our results, even if preliminary and semi-quantitative, could be interesting to develop preventive solutions for building surfaces and could be a starting point for future mechanistic studies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Marine bisindole alkaloid 2,2-bis(6-bromo-3-indolyl)ethylamine to control and prevent fungal growth on building material: a potential antifungal agent

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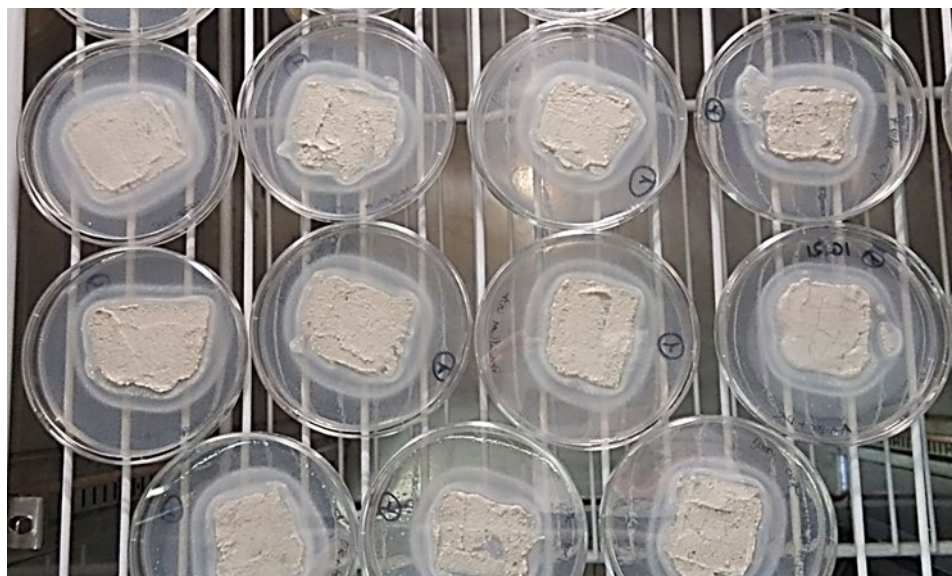
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Supplementary Table S1. List of the fungi used in this study with the related source of isolation.

Fungal species	Source of isolation
<i>A. corymbifera</i> EHS01	Hospital setting surfaces
<i>A. alternata</i> BC01	Environment
<i>A. flavipes</i> EHS02	Hospital setting surfaces
<i>A. flavus</i> IDRA01	Indoor air
<i>A. fumigatus</i> IDRAH01	Indoor air from hospital environment
<i>A. nidulans</i> IDRAH04	Indoor air from hospital environment
<i>A. terreus</i> IDRS023	Indoor setting surfaces
<i>A. versicolor</i> IDRA02	Indoor air
<i>A. ustus</i> IDRS08	Indoor setting surfaces
<i>F. solani</i> IDRS03	Indoor setting surfaces
<i>F. sporotrichioides</i> IDRS05	Indoor setting surfaces
<i>M. circinelloides</i> EHS03	Hospital setting surfaces
<i>P. citrinum</i> LC1	Environment
<i>Rhizopus</i> spp. IDRAH07	Indoor air from hospital environment
<i>T. rubrum</i> EHS05	Surfaces hospital setting
<i>T. tonsurans</i> EHS06	Surfaces hospital setting
<i>C. cladosporioides</i>	ATCC 16022

Supplementary Figure S1. Example of the building material IG21 (finishing plaster), distributed on NSA, and treated with 2,2-bis(6-bromo-3-indolyl)ethylamine (URB 1204) or commercial antifungal for the artificial contamination with different fungal strains.



Supplementary Figure S2. Representative visual observations of commercial antifungal effect on the growing of different fungi (*P. citrinum* LS1, *A. alternata* BC01 and *C. cladosporioides* ATCC 16022), alone and as mixed fungi, on the surface of IG21 (finishing plaster) after 28 days of incubation.

