Arvoredol – an unusual chlorinated and biofilm inhibiting polyketide from a marine *Penicillium* sp. of the Brazilian coast

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Abstract

Penicillium sp. F37 has been isolated from the marine sponge *Axinella corrugata* and shown to be closely related to *Penicillium maximae*. From the culture of *Penicillium* sp. F37 arvoredol, a novel chlorinated polyketide with 6,7-dihydro-4(5H)-benzofuranone moiety has been isolated and characterized by spectroscopic methods. Arvoredol prevented biofilm formation of the human pathogen *Staphylococcus epidermidis* at a concentration of 125 µg mL^{-1} by 40%. It was also active against colorectal carcinoma HCT116 cells with a MIC of 7.9 µg mL^{-1}.

Keywords: marine sponge, *Penicillium* sp., biofilm, biofilm dispersion, polyketide, *Staphylococcus epidermidis*

Microorganisms, especially bacteria, can attach to inert or living surfaces, producing a matrix of extracellular polymers where they form structured and dynamic microbial communities, known as biofilms. Biofilms are well protected against the environment including noxious agent, e. g. antibiotics, and can involve a single microbial species or microbial communities[1,2]. Pathogenic bacteria inhabiting biofilms are associated with several chronic diseases and the results of this colonization are persistent infections that are hard or even impossible to treat, leading to severe health complications and longer hospital stays[3].

All higher organisms, especially those living in wet habitats, are at risk of biofilm formation and we hypothesized that most of them developed weapons to protect themselves against biofilm infections. In our search for novel metabolites able to inhibit biofilms we focused on sponges which do not have an adaptive immune system. Many of them harbor a number of microorganisms on their surface and we speculated that some of these microorganisms have a mutualistic relationship with their sponge and may produce biofilm dispersing metabolites.

To assess the potential of fungi isolated from marine sponges collected at the coast of the Arvoredo Island in the Arvoredo Biological Marine Reserve (Santa Catarina state, Brazil), several isolates were screened against bacterial pathogenic biofilms[4]. Among these fungi, the ascomycete *Penicillium* sp. F37, isolated from the sponge *Axinella corrugata,* was selected for further investigation of compounds against *Staphylococcus epidermidis* biofilms. The isolation and characterization of the unusual chlorinated polyketide arvoredol from the marine *Penicillium* sp. F37 is report here, displaying antistaphylococcal biofilm activity. Its structure was characterized by extensive 2D NMR and MS spectrometry. Antibiofilm and antibacterial activities were determined applying the crystal violet method and turbidimetric assay.

The fungal strain was identified by comparison of its ITS region with that of related type species and the sequence was deposited (HE608803). According to the sequence of the ITS region, *Penicillium* sp. F37 belongs to the subgenus *Aspergilloides* and is closely related to *Penicillium maximae* (Figure 1). The strain was cultivated in 5 L Sabouraud broth, at 25°C for 7 days. The cultures were filtered through cheesecloth, lyophilized and partitioned between distilled water and ethyl acetate. The organic phase was separated and the solvent evaporated to yield a red-orange residue (350 mg). The extract showed anti-biofilm activity against *Staphylococcus epidermidis* strains. The extract was fractioned by semi-preparative HPLC on a RP-C18 column and eluted by a gradient of (A) water/methanol (9:1) and (B) water/methanol (1:9), starting isocratically at 80:20 for 5 min, increasing to 100% B during 30

min, and holding isocratically for 10 min. The purified fraction (rt = 21.0 min) was obtained as orange crystals (7.4 mg).

Compound **1**, named arvoredol referring to the habitat of the producing fungus, showed in the HRESI mass spectrum a $[M+H]^+$ ion of 429.1672 in the positive mode which led to a molecular formula of $C_{21}H_{29}ClO_7$. Such a molecular composition requires seven double bond equivalents. The ^{13}C NMR spectra displayed the resonances of two carbonyls and three double bonds which required two rings to fulfill the molecular formula. ^{1}H COSY NMR together with HMBC spectra led (Table 1) to the elucidation of a 3,4-dihydroxy-3,5-dimethyl-*trans*-1-heptenyl side chain. The trans-configuration is mandatory because of J=16 Hz between the olefinic protons H-1' and H-2'. This side chain it attached at C-2 to a 5,6,7,8-tetrahydro-4-keto-benzofuran moiety. The observed chemical shifts of C-8 ($\delta^{13}C$ 145.8) and C-9 ($\delta^{13}C$ 119.4) only allowed a 4-position of the keto-function in the tetrahydro-benzofuran group. The cyclohexane ring is heavily substituted bearing a double substitution at C-5, acetoxy at C-6 and a hydroxymethyl group at C-7, all well confirmed by the corresponding resonances in the HMBC spectra. Because of the large coupling of J=10 Hz between H-6 and H-7 a di-axial position of the two protons is required. The methyl group at C-5 has to be *trans* to H-6 because no resonance between these two moieties can be seen in the ROESY spectra. The carbon atom to which the chlorine atom is attached should be less deshielded than a similar one bearing a hydroxyl group. In **1** the chlorine is located at C-5 which is in agreement with the chemical shift of C-5 compared to the similar situation in the napyradiomycins[5] **2** (Figure 2).

The 1,3-dimethyl-pentyl side chain is unusual and only known from fungi. It was found in obionin A **3** obtained from the marine fungus *Leptosphaeria obiones*[6], asperfuranone **4** from *Aspergillus nidulans*[7], 07H239-A **5** isolated from the marine fungus LL-07H239 of the *Xylariaceae*[8], tenellin **6** from *Beauveria bassiana*[9], militarinones from *Paecilomyces militaris*[10] or epolactaene from an unidentified *Penicillium* species[11]. The biosynthesis of arvoredol probably comprises a number of different biosynthetic routes including a polyketide intermediate. In the case of tenellin **6** it has been shown that it is formed from a polyketide precursor which is twice methylated[12]. In the case of the related fusarin C an iterative type I polyketide synthase produces the required poly-unsaturated methylated heptaketide[13]. We did not study the biosynthesis of arvoredol but assume that a related biosynthetic pathway involving an iterative polyketide synthase produces arvoredol.

Because *Staphylococcus epidermidis* is an important pathogen in the clinic which is difficult to control due to biofilm formation and the resulting resistance to antibiotics, the ability of arvoredol to suppress biofilm formation of *Staphylococcus epidermidis* ATCC 35984 was determined. *S. epidermidis* ATCC 35984 wase used in biofilm inhibition assays in 96-well plates (Falcon® Micro Test™). After overnight incubation at 37°C, bacteria were inoculated in CASO broth with the active compound (1000 µg mL $^{-1}$ to 62.5 µg mL $^{-1}$, MeOH as control). Biofilms were quantified with crystal violet[14].

Arvoredol was found to suppress biofilm formation of this pathogen by 62% at a concentration of 0.125 mg mL^{-1} and by 80% at 1 mg mL^{-1}. These concentrations did not suppress bacterial growth considerably indicating that arvoredol was not acting as an antibiotic. To shed some light on the mechanism of action, biofilms treated with arvoredol were analyzed by scanning electron microscopy and compared with untreated biofilms. As expected treated biofilms were highly fragmented not forming a continuous biofilm but isolated clusters of small cell aggregates. It was obvious that treated cells were much smoother than the control indicating a strong reduction in the formation of the extracellular matrix (Figure 3). Furthermore, arvoredol showed considerable cytotoxicity against cancer cells with 7.9 µg mL^{-1} against colorectal carcinoma HCT116 cells.

The biodiversity of fungi is high and one cause for the broad spectrum of bioactive secondary metabolites[15]. Especially fungi isolated from various marine sources proved to be a rich source for novel bioactive compounds[16] and many screening programs for antibacterial and antifungal compounds focused on this group of microorganisms. Interestingly, a compilation of publications on antimicrobials from marine fungi over the last 5 years came to the conclusion that fungi from sponges may be overestimated in their potential for the production of bioactive compounds[17]. These searches were facilitated by several highly sophisticated techniques for cultivation, dereplication and structure elucidation[18].

One of the main producers of a broad spectrum of secondary metabolites are marine-derived isolates of the genus *Penicillium*[19]. From a marine-derived *Penicillium* species a number of secondary metabolites with quorum-quenching activities have been isolated and the most active of these metabolites was aspergillumarin B[20]. The extract of *Penicillium chrysogenum*, obtained from the marine sponge *Tedania anhelans*, showed antimycobacterial activity but the active compound has not been characterized[21]. Peniciadametizine A and B were characterized from the marine-sponge derived *Penicillium adamatzioides* and possess

activities against the pathogenic fungus *Alternaria brassicae*[22]. Another sponge-associated *Penicillium* sp. produced citrinin which has antibacterial and cytotoxic properties[23].

Although for a number of secondary metabolites from various sources interference with biofilm formation has been shown, their number from fungi is still rather small[24],[25]. Screening of 75 fungal isolates from marine sources identified *Sarocladium*, *Fusarium*, *Epicoccum* and *Khuskia* species as producers of quorum-quenching compounds but the responsible metabolites were not identified[26]. From terrestrial *Penicillium* species both penicillic acid and patulin have been identified interfering with homoserine lactone signaling in *Pseudomonas aeruginosa*[27]. From *Penicillium* sp. F37, the fungus reported here to produce arvoredol, *cis*-cyclo(leucyl-tyrosyl) have been characterized which inhibited biofilm formation of several clinical isolates of *Staphylococcus epidermidisFehler: Verweis nicht gefunden*. During a search for bioactive metabolites of *Penicillium commune* lovastatin and *cis*-cyclo(leucyl-proline) have been detected. Lovastatin at 100 μg mL^{-1} was shown to inhibit biofilm formation of *Pseudomonas aeruginosa* by 53 % and 100 μg mL^{-1} *cis*-cyclo(leucyl-proline) of unresolved stereochemistry prevented biofilm formation of *Staphylococcus aureus* by 90 %[28].

In conclusion, arvoredol, a novel chlorinated polyketide, has been characterized from a *Penicillium* sp. which colonizes the marine sponge *Axinella corrugata*. Arvoredol bears a 6,7-dihydro-4(5H)-benzofuranone moiety and inhibits biofilm formation of the human pathogen *Staphylococcus epidermidis* without acting as antibiotic. The activity is moderate and both activity and selectivity should be further improved by chemical derivatization. Nevertheless, the novel skeleton represented by arvoredol opens a new window into the diversity of biofilm preventing compounds which makes it a valuable compound for the search of novel targets and studies of structure-activity relationships. Its finding demonstrates once more the great potential of marine organisms as sources for new bioactive compounds.

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Table 1: NMR of arvoredol 1 in $CDCl_3$

	C	H	HMBC
2	161.9	-	6.50; 6.23; 6.06; 4.31
3	101.9	6.06 (1H, s(br))	6.23
4	188.0	-	1.36
5	74.6	-	1.36
6	73.2	4.93 (1H, d, J=10 Hz)	1.36
7	35.4	3.39 (1H, ddd, J=12, 10, 5 Hz)	6.06; 4.93; 4.31
8	145.8	-	4.31; 3.77; 3.39
9	119.4	-	6.06; 3.39
1'	122.4	6.23 (1H, d, J=16 Hz	6.50; 6.06
2'	144.9	6.50 (1H, d, J=16 Hz)	1.28
3'	75.9	-	6.50; 6.23; 1.28
4'	78.4	3.45 (1H, s(br))	1.28; 0.96
5'	35.3	1.67 (1H, tq(br), J=7, 7 Hz)	3.45; 1.34; 0.96; 0.89
6'	28.6	1.34 (2H, m)	3.45; 0.96; 0.89
7'	11.9	0.89 (3H, t, J=Hz)	1.34
8'	13.	0.96 (3H, d, J=7 Hz)	3.45; 1.34
9'	23.6	1.28 (3H, s)	6.50
1''	20.3	1.36 (3H, s)	4.93
2''	170.4	-	4.93; 2.19
3''	20.6	2.19 (3H, s)	
4''	67.8	4.31 (1H, dd, J=9, 5 Hz) 3.77 (1H, dd, J=12, 9 Hz)	4.93

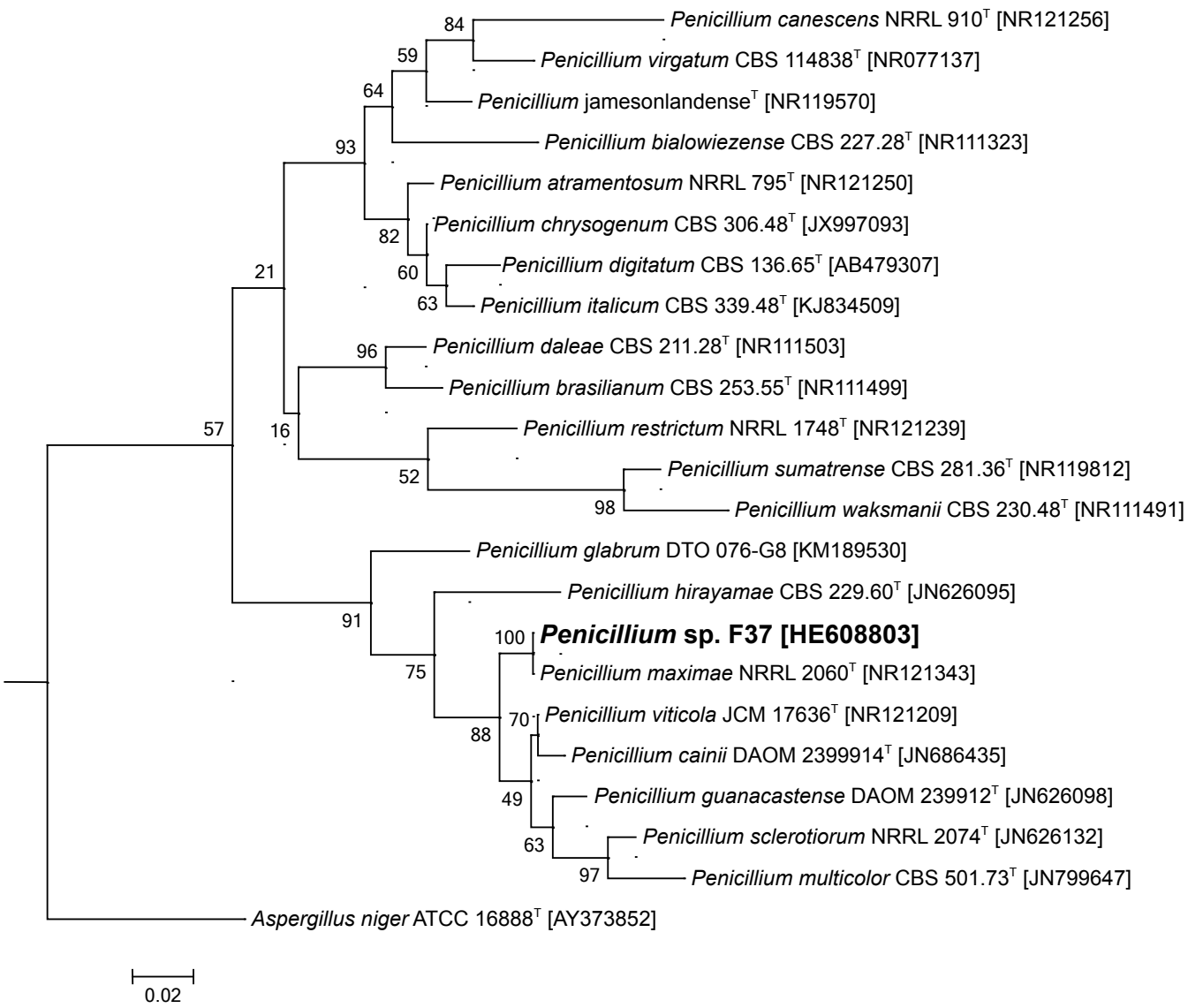


Figure 1: Phylogenetic tree of *Penicillium* sp. F37 and its nearest relatives based on their ITS sequences. The Maximum-Likelihood algorithm with 100 bootstrap replications and *Agaricus campestris* [DQ182533] was used as outgroup.

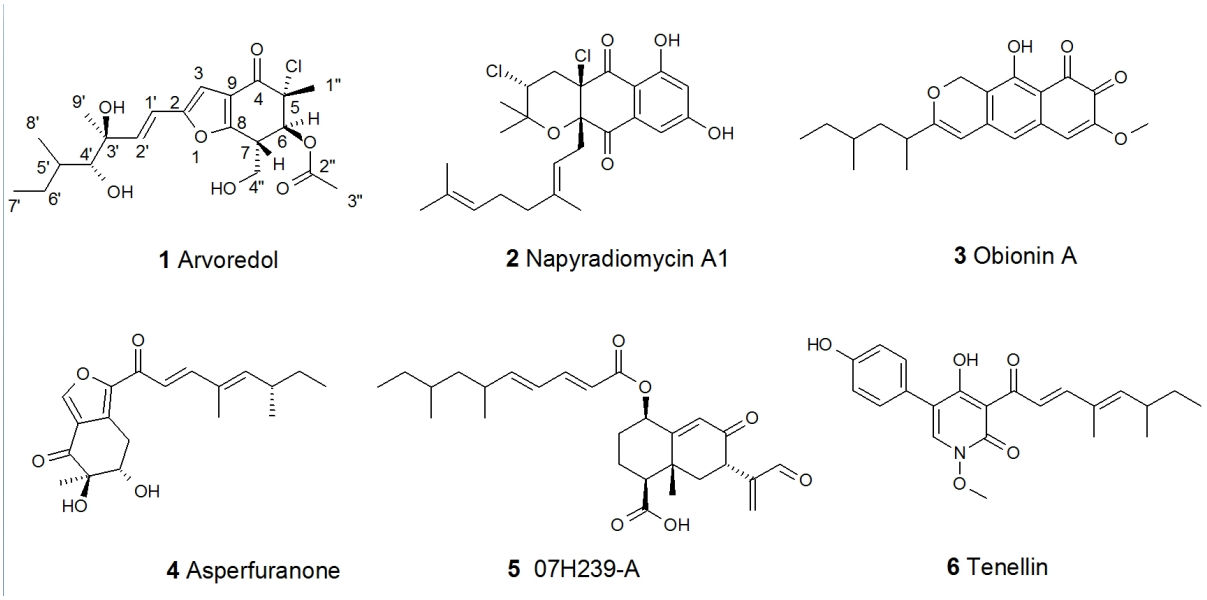


Figure 2: Structures of arvoredol **1** and related compounds

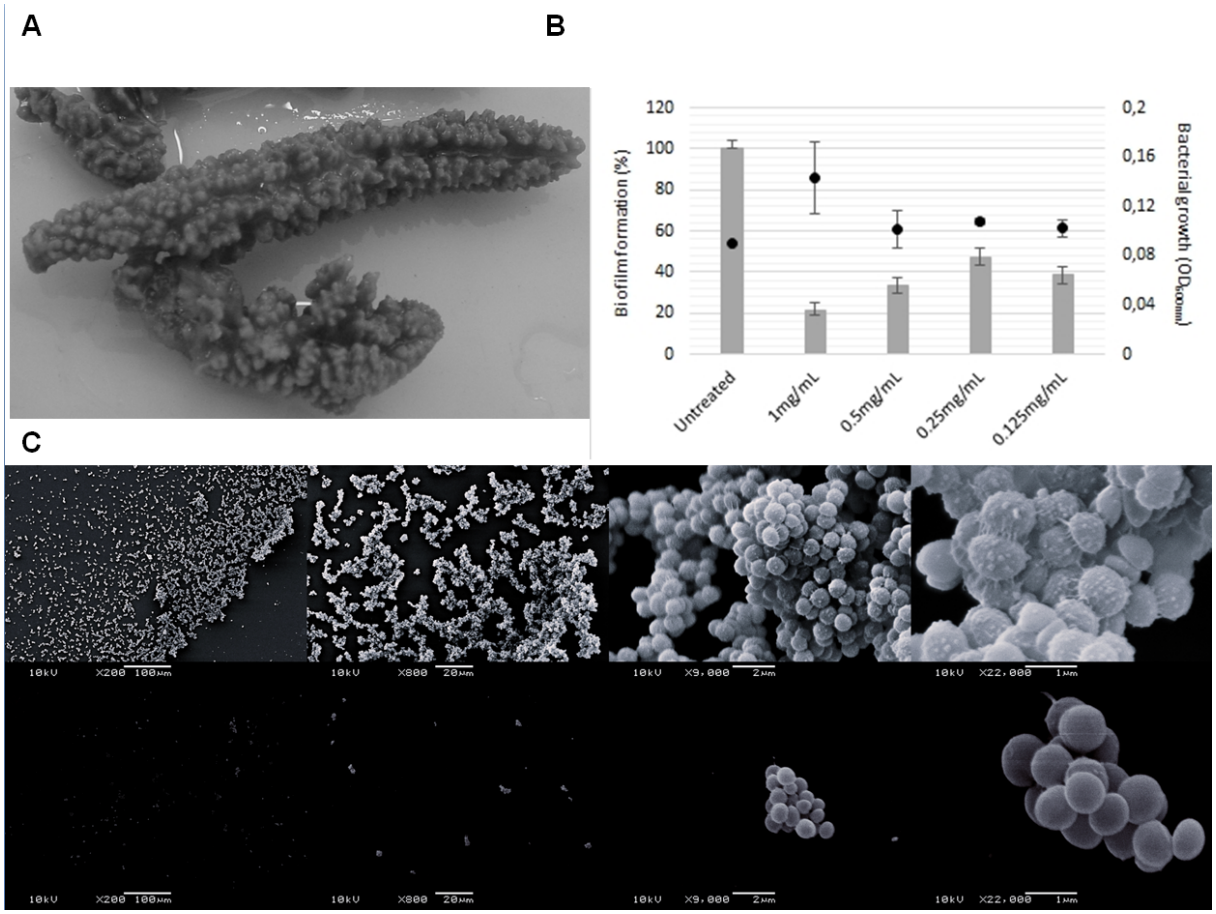
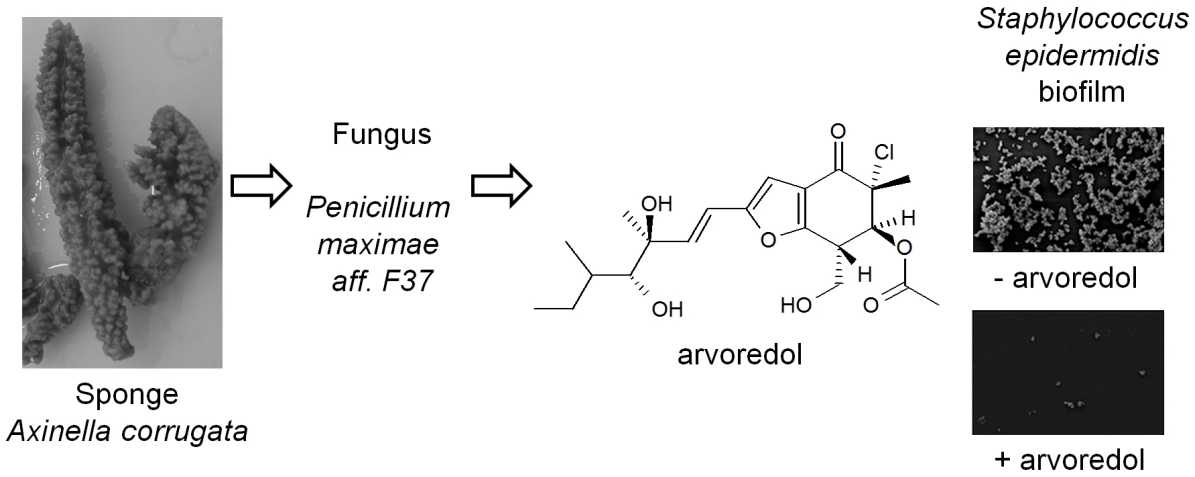


Figure 3: A) The sponge *Axinella corrugata* from where *Penicillium* sp. F37 has been isolated; B) Biofilm formation (columns) and growth (dots) of *S. epidermidis* under different concentrations of arvoredol based on three replicates; C) Architecture of *Staphylococcus epidermidis* biofilms treated with 1 mg mL^{-1} arvoredol (lower row) and compared with untreated biofilms (upper row) in different magnifications. The smoother texture seen on the arvoredol treated cells indicates a strong reduction of extracellular polymeric substance.

Graphical abstract:



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