Sub-lethal activity of small molecules from natural sources and their synthetic derivatives against biofilm forming nosocomial pathogens

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ABSTRACT

Nowadays, the patient safety is seriously jeopardized by the emergence and spread of nosocomial pathogens in the form of biofilm that is resistant to traditional and affordable antimicrobials. Although advances in organic synthesis have extended the lifetime of classic antibiotics through synthetic modifications, the search of innovative antibiofilm compounds from natural sources can provide new templates, novel targets and unique mechanisms that should have advantages over known antimicrobial agents. Testing sub-lethal concentrations of crude extracts and/or isolated compounds from plants and microorganisms is critical to acting on mechanisms subtler than the killing activity, e.g. those influencing the multicellular behavior, offering an elegant way to develop novel antimicrobial-free antibiofilm strategies.

Herein we discussed the search and biological activity of small molecules from natural sources and their synthetic derivatives able to modulate biofilm genesis of nosocomial pathogens through non-microbicidal mechanisms (sub-lethal concentrations). The present work offers an overview about the approaches applied to the discovery of lead small molecules including i) conventional drug design methods like screening of chemical compounds obtained from nature and ii) computer-aided drug design approaches. Finally, a classification (not exhaustive but representative) based on the natural origin of small molecules and their synthetic derivatives was reported.

The information presented in this review should be of interest to a broad range of disciplines and represents an effort to summarize experimental research and advances in this field.

KEYWORDS: biofilm-based infections; natural compounds; sub-lethal concentrations; computer-aided drug design

RUNNING TITLE: Sub-lethal activity of small molecules against biofilms

INTRODUCTION

With Fleming's discovery of the penicillin in 1929, and Domagk's discovery in 1935 of synthetic chemicals (sulfonamides), the 20th century opened with the promise of the eradication of most infectious diseases in global host populations. At present, it is becoming more and more evident that the last century closed with the specter of re-emergence of many deadly infectious diseases as the miracle of the antimicrobial chemotherapy has been eroded by the emergence and increasing prevalence of microbial strains in form of biofilm that are resistant to available treatments. This is readily seen in the continuous escalation of healthcare-associated infections (formally called nosocomial infections) at an alarming rate. In American hospitals alone, nosocomial infections account for an estimated 1.7 million infections and 99,000 associated deaths each year [1]. The European Centre for Disease Prevention and Control (ECDC) declared that every year approximately 4.1 million people in EU Countries catch an infectious disease associated with healthcare system and that around 40,000 die as direct consequence of these infections [2].

Well-known clinical examples of recalcitrant nosocomial infections include *Pseudomonas aeruginosa* that is responsible for many infections including those associated with ventilator-associated pneumonia, bacterial keratitis, otitis, burns, ischemic osteomyelitis and lungs of patients with cystic fibrosis, and *Escherichia coli* that causes urinary and gastrointestinal tract infections and septicemia [3-5]. *Acinetobacter baumannii* is an opportunistic pathogen able to persist in the hospital environment allowing contact with susceptible patients and causing outbreaks of ventilator-associated pneumonia, meningitis, bacteremia, and urinary tract and wound infections [6]. Important etiologic agents of systemic and nosocomial infections have been detected in the microbiota of subgingival biofilm, including the causative agents of periodontitis and caries like *Streptococcus* spp., *Lactobacillus* spp., and *Candida albicans* [7]. Furthermore, tuberculous granulomas that contain latent *Mycobacterium tuberculosis* and *Staphylococcus aureus* in chronic wounds represent challenges for modern medicine [8]. The same above-mentioned microorganisms are able to colonize indwelling medical devices (IMD) like ocular/cochlear implants, orthopedic devices, catheters, and heart valves, providing the conversion from an acute infection to one that is persistent, chronic, and recurrent, most often requiring device removal in order to eliminate the infection [9]. Bacterial biofilms also underlie the persistent colonization of hospital facilities therefore driving and sustaining nosocomial infections, which place a billions of euros burden on the worldwide healthcare system annually [10]. Recently, microbial biofilms have been identified in a case of intra-amniotic infection [11] and several scientists linked the presence of unwanted biofilm to Lyme disease, autism, and chronic immune dysfunction [12, 13]. The spread of recalcitrant infections is readily stated by the Centers for Disease Control and Prevention (CDC) and the National Institute of Health (NIH) that estimate 65-80 % of microbial infections currently treated by physicians in the developed world biofilm-mediated [4, 14, 15]. However, this percentage is

destined to increase as the scenario of biofilm-related diseases is continuously growing up as well as our concerns to loose powerful weapons against infectious diseases.

Biofilm represents a surface-associated multicellular society embedded in a self-produced polymeric matrix and based on members that communicate with each other. Mounting evidence indicates that biofilm microorganisms undergo processes of cell specialization enabling coordinated and efficient survival strategies against the innate immune system and antimicrobial therapies [16]. The resistance of biofilms is clearly not caused by a single mechanism but by several factors acting in concert like diffusion limitation and non-genetic phenotypic heterogeneity [17-19]. Unsurprisingly, biofilm antibiotic susceptibility has been the subject of intense research and has been the focus of several excellent reviews [20-22].

Thus, new antimicrobial-free strategies might be designed to reduce consumption of antimicrobial agents, ensuring the prudent use of these fragile strategies, protecting and empowering antibiotics that are critically important for human and animal health and wellbeing. The antibiotics that are available today are primarily variations on a single theme and focus on microbial eradication based on different modes of action at the molecular level. Some target cell-wall biosynthesis, whereas others inhibit protein synthesis or DNA replication. More recently, fatty-acid biosynthesis has been proposed as a viable bactericidal target [23]. Lysine analogues have also been identified that target lysine riboswitches and inhibit bacterial growth [24]. However, all these strategies shut down or subvert essential cellular functions that are crucial for microbial survival, promoting the development of drug-resistance.

A new trend is to develop antibiofilm strategies functioning by mechanisms more subtle than the simple killing activities like those influencing the multicellular behavior, by manipulating the expression of specific phenotypes that represent microbial virulence traits (e.g. binding ability, motility, cell-to-cell communication, yeast–hyphae-dimorphism) [25, 26]. This can be achieved by using a sub-lethal concentration of antibiofilm agents with novel targets, unique modes of action and proprieties that are different from those of the currently used antimicrobials. The rule is ‘disarm microorganisms instead of killing them’, as depriving microorganisms of their virulence properties without threatening their existence offers a reduced selection pressure for drug-resistant mutations, providing compounds with longer shelf-lives [25, 27]. Observing the processes of biofilm formation it is reasonable to expect that interfering with the key-steps that orchestrate genesis of virtually every biofilm could be a way for new preventive strategies that do not necessarily exert lethal effects on cells but rather sabotage their propensity for a sessile lifestyle [26]. Three key steps are now considered promising targets for developing innovative antibiofilm strategies. The first step is to avoid microbial adhesion, interacting with the surface sensing process to repel pioneering cells keeping them in a planktonic form. The second step is the destruction of biofilm organization, mystifying intercellular and damaging the biofilm matrix destabilizing the physical integrity of the biofilms. Another interesting target is biofilm dispersal by forcing the

planktonic state, restoring the efficacy of antibiotics and the immune system [28, 29]. All these strategies are promising, as might be potentially used for co-dosing newly developed antibiofilm compounds that possess the above-mentioned characteristics with conventional antibiotics to eradicate biofilm-related infections.

Natural products provide very interesting classes of biologically active, low-molecular-mass (< 5 kDa) compounds that possess a plethora of valuable biological activities far from killing activities (Table **1**). In addition, natural products offer unmatched chemical diversity and such unexplored scaffolds represent promising new starting point to generate libraries of natural products analogs, which might have enhanced biological activities and drug-like properties (e.g. pharmacokinetics, solubility). Thus, organisms of terrestrial and marine origin are sources from which to draw inspiration for developing new antimicrobial-free antibiofilm strategies. We should keep in mind that the killing activity of natural compounds, like antibiotics, is a concentration-dependent process and primarily a laboratory property, since the levels of these agents available in nature would be insufficient to exert their lethal effects [26, 30, 31]. Currently used antimicrobial agents derived by screening natural products and compound libraries against whole organisms. The research myopia to see over a biocidal activity is limiting the recognition of new perspectives offered by sub-lethal concentrations of natural-derived compounds and their capacity to guide and improve the discovery, development and approval of innovative antimicrobial-free antibiofilm strategies.

This review seeks to discern the scientific literature reporting natural compounds and their recent derivatives that possess the ability to modulate biofilm development through non-microbicidal mechanisms (sub-lethal concentrations) from their counterparts. The present work offers an overview about the approaches applied to the discovery of lead small molecules including i) conventional drug design methods like screening of chemical compounds obtained from nature and ii) computer-aided drug design approaches. Finally, a classification (not exhaustive but merely representative), based on the natural origin of small molecules and their synthetic derivatives was reported.

Table 1: Partial list of nosocomial pathogens and small molecules from natural sources and their synthetic derivatives effective against biofilm associated diseases.

Nosocomial pathogen	**Biofilm associated disease**	**Active compounds**	**Target**	**Reference***
Pseudomonas aeruginosa	Cystic fibrosis lung infection, ventilator-associated pneumonia, bacterial keratitis, otitis, burns, ischemic osteomyelitis	Baicalein, Salicylic acid, nifuroxazide, chlorzoxazone, rosmarinic acid, naringin, chlorogenic acid, morin, mangiferin, furanones and their analogues	QS	42, 43, 46, 61, 65, 66
		Methanolic extract of *Capparis spinosa*	Motility and EPS production	52
		TAGE, CAGE, Oroidin derivates	Dispersion	68, 69, 70,

				73
		(*Z*)-2-decenoic acid	Dispersion	100
Acinetobacter baumannii	Burn wound, trauma infection, ventilator-associated pneumonia, meningitis, bacteremia, and urinary tract infections	Dihydrosventrin and oroidin derivates	QS and dispersion	71, 72, 74, 75, 76, 77, 78
		Ethyl N-(2-phenetyl) carbamate derivates	Unknown	97
Escherichia coli	Urinary, gastrointestinal infection and septicemia	Ursolic acid	Chemotaxi and motility	32
		Methanolic extract of *Capparis spinosa*	Motility and EPS production	52
		Furanones	QS	63, 64
		Zosteric acid	Motility, QS	26
		Oroidin derivates	QS	79, 80
		Desformylflustrabromine derivates	Unknown	94, 95
		Ethyl N-(2-phenetyl) carbamate derivates	Unknown	98
		(*Z*)-2-decenoic acid	Dispersion	100
Staphylococci	Burn wound, lung infection, sepsis, osteomyelitis and infections associated with foreign body material	Esculetin and Fisetin	QS	44
		Diterpenoid 7-(2-oxohexyl)-taxodione	Unknown	54
		Tannic acid	Influenced bacterial cell surface hydrophobicity	55
		Oroidin derivates	QS	86
		Desformylflustrabromine derivates	Unknown	95
		Guanidine and biguanide derivatives	Unknown	99
Streptococci	Infections of respiratory tract, bloodstream and central nervous system	Ellagic acid, Esculetin, Fisetin, Benzyl (6*Z*,9*Z*,12*Z*)-6,9,12-octadecatrienoate, 3-benzyloxy-1-nitro-butan-2-ol and 1,3 cyclohexane dicarbohydrazide	QS	44, 50
		2-isopropyl-5-methyl-phenol	Expression of the virulence genes *gtfB, gtfC, brpA* and *spaP* and EPS matrix	48
		Epigallocatechin gallate, Melanoidine	Attachment through suppression of *gtf* gene expression	49, 51

Candida albicans	Genital and catheter-related bloodstream infections	Zosteric acid	Unknown	39
		Muscari comosum extract	Dispersion	40
		(*Z*)-2-decenoic acid	Dispersion	100
Vibrio cholerae	Nosocomial cholera	Quinazoline-2,4-diamino analogue	Motility	35
		Analogues of mefloquine	Unknown	36
Stenotrophomonas maltophilia	Respiratory, bloodstream, and urinary infections	Emodin	QS	45
Serrantia marcescens	Catheter-associated bacteremia, urinary tract infections and wound infections	Methanolic extract of *Capparis spinosa*	Motility and EPS production	52
Proteus mirabilis	Urinary tract infections	Methanolic extract of *Capparis spinosa*	Motility and EPS production	52
		(*Z*)-2-decenoic acid	Dispersion	100
Salmonella Typhimurium	Gastrointestinal illness	7-methoxy-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazi-nyl]-5H-pyrimido[5,4-b]indole	Unknown	37
		Oroidin derivates	QS	83, 84, 86
Klebsiella pneumoniae	Pneumonia, bloodstream infections, meningitis, wound and surgical site infections	(*Z*)-2-decenoic acid	Dispersion	100

* See the numbered reference in the text.

SEARCH FOR NEW BIOFILM MODULATORS

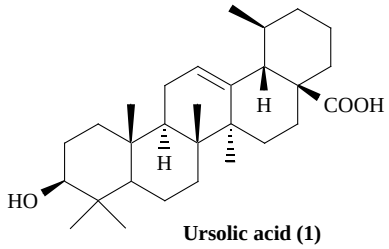
Recent technological advances and the development of new methods have revolutionized the screening of natural compounds and offer a unique opportunity to establish natural products as a major source of antibiofilm leads at sub-lethal concentrations. At present, biochemical and cell-based assays are amenable to natural products screening with the possibility to couple them with high throughput screening (HTS) methods. Traditional and virtual HTS technologies are discussed in the following sections.

High throughput technology for the screening of natural products

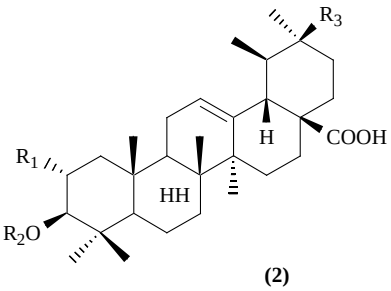
Due to the scarcity of known molecular scaffolds able to modulate microbial biofilms at sub-lethal concentrations, HTS of compound libraries has been used as potent and valuable molecular tool for the identification of novel antibiofilm agents at sub-lethal concentrations. Thus, the workhorse platform for antibiofilm compounds discovery is an HTS approach using crude extract or small molecule compound libraries isolated from natural sources to identify candidates that mediate biofilm genesis.

Ren and colleagues [32] carried out pioneering work by screening 13,000 plant fractions with the final goal to identify novel small molecules that possess antibiofilm activity. They identified the promising compound Ursolic acid (1), (Fig. **1**) which effectively inhibited *E. coli* biofilm formation at concentrations as low as 22 μM without affecting growth. To investigate the mechanism of this non-toxic inhibition on a global genetic basis, DNA microarrays were used to study the gene expression profiles of *E. coli* grown with or without ursolic acid. Transcriptome analyses demonstrated that ursolic acid had no effects on the quorum-sensing systems autoinducer-1 (AI-1) and autoinducer-2 (AI-2), but it induced chemotaxis and motility genes in *E. coli*, suggesting that ursolic acid may function as a cue that tells cells to remain too motile for a proper biofilm formation [32]. Among the naturally occurring ursolic acid derivatives (general structure 2, Fig. **1**), compound 3 showed antibiofilm activity at sub-lethal concentrations against the bacterial biofilm *P. aeruginosa* PA01, with an inhibition of 62% at 10 μg/ml [33].

Figure 1: Structures of Ursolic acid and derivatives.



Ursolic acid (1)



(2)

R_1= H, OH
R_2= H, *cis-p*-coumaroyl, *trans-p*-coumaroyl, *trans*-feruloyl
R_3= H, OH

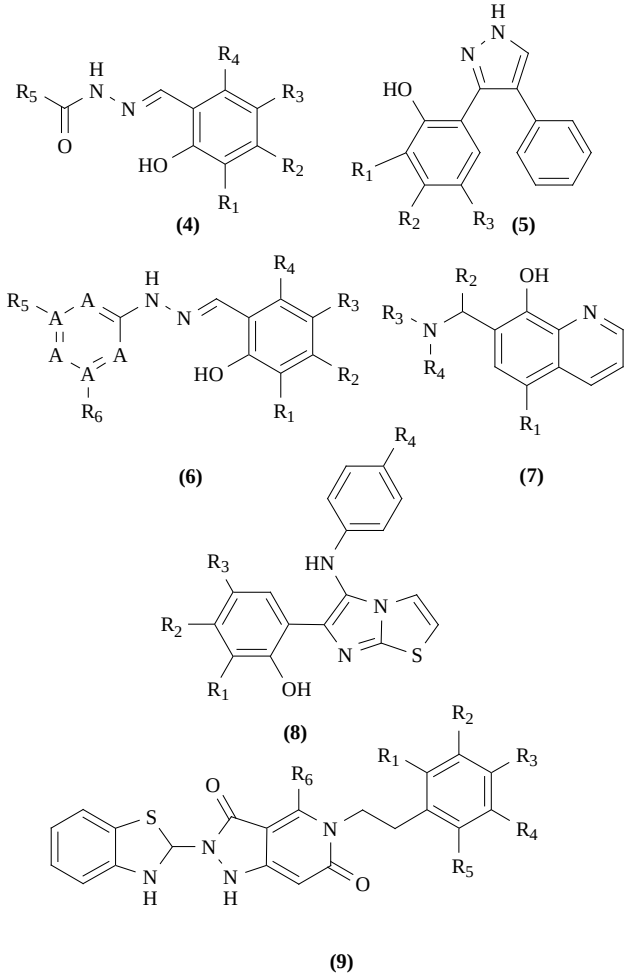
(3)

R_1= H,
R_2 *cis-p*-coumaroyl,
R_3=OH

Junker and Clardy [34] developed a luminescence-based biofilm test for both attachment and detachment to identify small molecules that disrupt biofilm development by *P. aeruginosa* in an HTS format without the use of a lethal selection pressure. Among 66,095 molecules derived from libraries containing known bioactive compounds, natural products, and commercially available entities, 61 possessed notable activities against cell attachment and of these, 30

fell into six structural classes (general formulas of six structural classes 4-9 Fig. **2**) as biofilm attachment inhibitors with EC_{50} of less than 20 μM. The active compounds and the general scaffold classes they represent do not have any known bacterial targets or mechanisms of action. However, the author speculated that the chemical nature of some of the compounds might lead them to be metal chelators.

Figure 2: General formulas of the six structural classes.

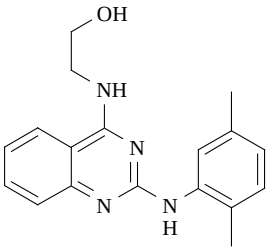


An HTS of an 8,000-compound structurally diverse chemical library for small-molecule inhibitors of bacterial motility using *Vibrio cholerae* identified several compounds that inhibited motility without exhibiting toxicity [35]. The lead compound, a quinazoline-2,4-diamino analogue (10) (Fig. **3**), showed inhibition of biofilm formation by 1.6 and 1.9-fold at 18 h and 30 h respectively, at the sub-lethal concentration of 10 μg/ml.

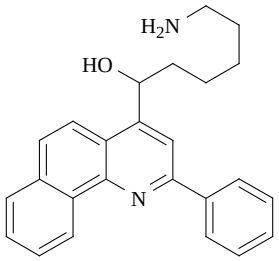
Recently, Peach et al. [36] developed high-throughput image-based 384-well format system for the identification of small molecule inhibitors of biofilm formation in *V. cholerae*. Application of this method led to the discovery of 29 compounds many of which inhibited biofilm formation without altering bacterial cell viability. In particular, two lead compounds, which are analogues of the antimalarial drug mefloquine (11, 12) (Fig. **3**), resulted effective by reducing in

biofilm coverage of 0.5 and 1.8% respectively (compared to coverage of 20% in untreated wells) with comparable bacterial growth to control wells.

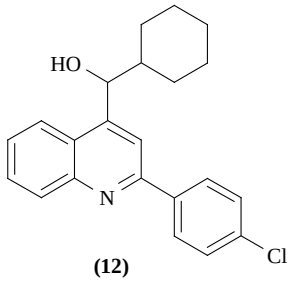
Figure 3: Structures of quinazoline-2,4-diammino analogue and mefloquine analogues.



Q24DA (10)



(11)

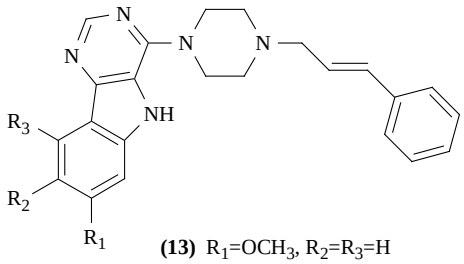


(12)

Robijns and co-workers [37] performed an HTS of 20,014 small molecules to search of non-toxic *Salmonella* biofilm inhibitors with a broad temperature activity. Out of the 20,014 compounds screened at 16 and 37 °C, 140 hits (0.7%) were identified. After characterization of the most promising hits at a broader set of temperatures, 7-methoxy-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazi-nyl]-5H-pyrimido[5,4-b]indole (13) (Fig. **4**) was identified as an interesting preventive antibiofilm compound. Structure–activity relationship (SAR) studies indicated that the phenylpropenyl residue is essential for the activity of the compound, and the shortening of the linker between the phenyl and piperazinyl moiety, made the agent ineffective. The indole moiety, part of the pyrimidoindole scaffold, was determined as a second essential feature because removing or replacing the indole group abolished the antibiofilm activity. Also, methylation of the indole nitrogen rendered the compound inactive. Nonetheless certain substitutions at the R1, R2 or R3 position of the pyrimidoindole scaffold improved the activity. Structure-Activity Relationship (SAR) studies revealed 8-fluoro-4-

[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-5H-pyrimido [5,4-b]indole (14) (Fig. **4**) a promising analogue in the prevention of *Salmonella* biofilm.

Figure 4: Structures of indole derivatives.



(13) R_1=OCH_3, R_2=R_3=H
(14) R_1=R_3=H, R_2=F

HTS of 42,865 compounds was performed to identify compounds that inhibit formation of *Staphylococcus epidermidis* RP62a biofilms without killing them [38]. After completing the three tiers (primary screening, hit confirmation, and dose-response curves), 352 compounds were selected as confirmed hit compounds from the HTS assay and among them, only 16 had an active concentration (AC_{50}) lower than 10 µM.

Despite the encouraging results, shortcomings of HTS have also been recognized and researchers begun looking at new methodological approaches for developing compounds better suited to become non-toxic antibiofilm compounds. Mounting evidences suggest that sub-lethal concentrations of naturally-derived compounds act in a concentration-dependent manner, where upper and lower threshold concentrations trigger the formation of a biofilm, making the daunting task of developing new antimicrobial-free strategies even more challenging [39, 40]. Thus, if screening tests are conducted at appropriate concentrations, there is the possibility to obtain several orders of magnitude more compounds with the desired activity. In the context of antibiofilm researches, this means screening a wide range of sub-lethal concentrations at frequent intervals in order to identify the experimental space with the maximum antibiofilm activity. However, the efforts of industrial, academic, governmental actors are made to reduce time and costs of research programs by testing few concentrations at standard conditions, demanding carefully designed experiments to explore in details and at reasonable cost the low-dose response and the cellular behavior in complex scenarios. Recently, Villa and colleagues [39, 40] successfully used the modeling tool Design of Experiment (DoE) to find the best working concentrations of plant-derived compounds just performing few experiments. The approach consists in defining a fixed range of variation for each variable (e.g. the variable concentration) then perform a series of experiments where all the variables vary within their respective ranges. When established a wide range of concentrations, the experiments are performed at the extreme points of the experimental region, which are most likely to be different from one another, and also the center-point to get information of what occurs inside the region. The

obtained dataset is analyzed by multivariate data analysis to obtain a polynomial equation predicting the performance of the compound under different conditions within the chosen domain. Thus, an area where a proper DoE set up could reduce the experimental workload is the optimization of biological assays in the multi-well framework for HTS. Novel experimental designs specifically developed to rationalize the design-making process are set up in a format that is compatible with the use of multi-pipettes and automated systems for preparation of multi-well plates [41]. The application of rational DoE could make the discovery of novel compounds more efficient and less time-consuming.

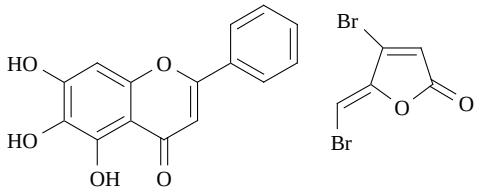
In silico methods for virtual screening of natural products

Computer-aided approaches are gaining popularity and have become an integral part of the drug discovery process in recent years. Virtual screening (VS) is increasingly used as a cost-effective complement to HTS and employs a range of methods to prioritize the selection and testing of large chemical datasets. This ensures that the most promising compounds are tested first in a lead discovery program.

In the field of antibiofilm compounds, chemoinformatics approaches have not been fully exploited yet. The main drawback relies in the lacking of knowledge about molecular targets of the most promising antibiofilm compounds. However, a significant amount of available information from the scientific literature on antibiofilm compounds active at sub-lethal concentrations is tied to research involving the quorum sensing (QS), one particular form of cell-to-cell communication involving the production, release and detection of small signaling molecules (autoinducers). Computer-aided drug design techniques, especially structure-based virtual screening (SB-VS), have also been employed in the search for new biofilm modulators. One such study involved the application of the automated docking program DOCK 5.3.0 to screening for QS inhibitors of *P. aeruginosa* from a database containing 51 active components of Traditional Chinese Medicines (TCMs) with antibacterial activity [42]. By virtually determining if any of the target compounds exhibited similar docking characteristics to a known inhibitor of the *Agrobacterium tumefaciens* quorum sensing transcriptional regulator protein TraR, the authors hypothesized that hits in the virtual screen would translate into compounds also displaying antibiofilm activity against *P. aeruginosa*. The computer-based virtual screening revealed five potential QS inhibitors. The most active compound was Baicalein (15) (Fig. **5**), which had no noticeable effect on bacterial growth. However, it significantly inhibited biofilm formation of the bacteria at a lower concentration of 20 μM. Interestingly, Baicalein and ampicillin showed synergistic activity against *P. aeruginosa*.

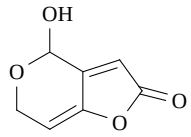
In a recent study, 147 recognized drugs and natural compounds were selected from the SuperNatural and SuperDrug databases on the basis of their two-dimensional (2D) structural similarity to the known quorum-sensing inhibitors furanone C30 (16), Patulin (17), the *P. aeruginosa* LasR natural ligand (3-oxo-C12-AHL (18)) and a known quorum-sensing receptor agonist TP-1 (19) (Fig. **5**) [43]. Six top-ranking drugs were acquired and tested for biological activity.

Figure 5: Structures of Baicalein, furanone C30, Patulin, 3-oxo-C12-AHL and TP-1.

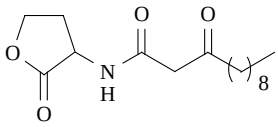


Baicalein (15)

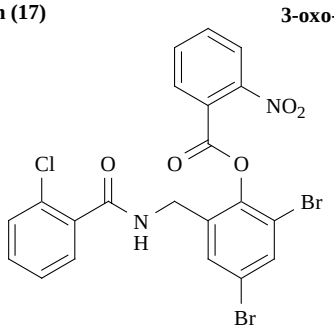
Furanone C30 (16)



Patulin (17)



3-oxo-C12-AHL (18)



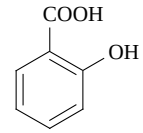
TP-1 (19)

This screen led to the discovery of three compounds, Salicylic acid (20), nifuroxazide (21), and chlorzoxazone (22) (Fig. **6**), that showed significant inhibition of QS-regulated gene expression and related phenotypes at concentrations at which they did not affect bacterial growth. These compounds were also found to alter biofilm formation by *P. aeruginosa* PAO1. Treated biofilms were thinner and less structured than untreated ones and total biofilm mass was reduced in the presence of these quorum sensing inhibitors.

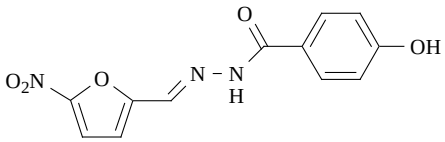
The SB-VS was used for the identification of natural inhibitors of bacterial biofilm formation on a compilation of 57,346 compounds from plants largely used in TCMs [44]. At the core of similarity-based virtual screening lies the resemblance between the reference structure and each of the structures in the compound library. The researchers found that Ellagic acid (23) (Fig. **7**) (present in green tea) significantly inhibited biofilm formation of *Streptococcus dysgalactiae*. Based on Ellagic acid, they predicted a 2nd-generation list of compounds with similar characteristics. Compounds identified by virtual screening were subsequently selected for antibiofilm activity. Among them, Esculetin (24) (Fig. **7**), proved to be the most efficient in preventing biofilm formation by *S. aureus*. To improve the efficacy and the range of affected organisms, a second round of virtual screening of Chinese Natural Product Database was performed using Esculetin as query. One of the predicted compounds, Fisetin (25) (Fig. **7**), was even better to abolish biofilm formation than the two parent compounds preventing biofilm formation by *S. dysgalactiae* NCTC 4671 and

ATTC 27957 and *S. aureus* at 16 µg/ml. The secondary metabolites Esculetin and Fisetin significantly inhibited biofilm formation at concentrations at which they did not affect bacterial growth.

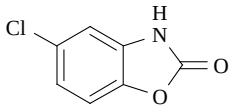
Figure 6: Structures of Salicilyc acid, nifuroxazide and choloxazone.



Salicylic acid (20)

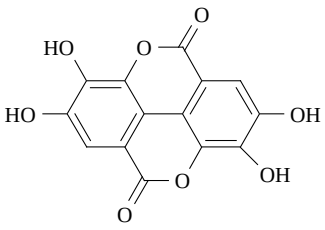


Nifuroxazide (21)

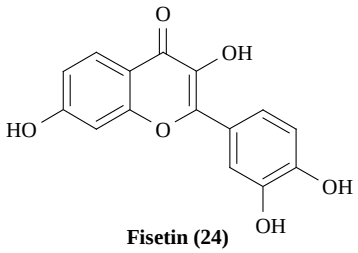


Chlorzoxazone (22)

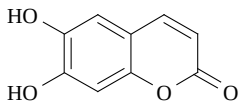
Figure 7: Structures of Ellagic acid, Esculetin and Fisetin.



Ellagic acid (23)



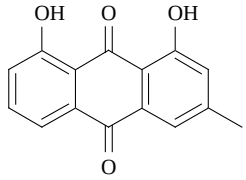
Fisetin (24)



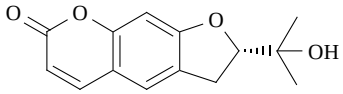
Esculetin (25)

The DOCK 5.3.0 program was applied by Ding and colleagues [45] to screen for putative novel QS inhibitors of *A. tumefaciens* TraR from the database of compounds found in TCMs. Six out of 46 active components found in TCMs were identified as putative QS inhibitor based on docking scores. Of these, three compounds (26-28) (Fig. **8**) inhibited biofilm formation by *P. aeruginosa* and *Stenotrophomonas maltophilia* at 200 µM, a concentration lower than the minimum inhibitory concentration (MIC). The fourth compound, emodin (29) (Fig. **8**), significantly inhibited biofilm formation at 20 µM and induced proteolysis of the quorum-sensing signal receptor TraR in *E. coli* at a concentration of 3–30 mM. Emodin also increased the activity of ampicillin against *P. aeruginosa*.

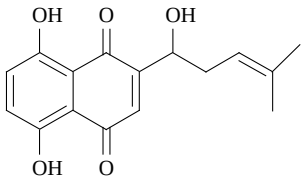
Figure 8: Structures of Chrysophanol, Nodakenetin, Shikonin and Emodin.



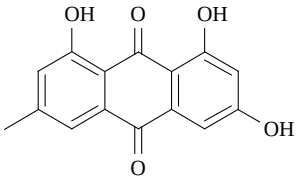
Chrysophanol (26)



Nodakenetin (27)



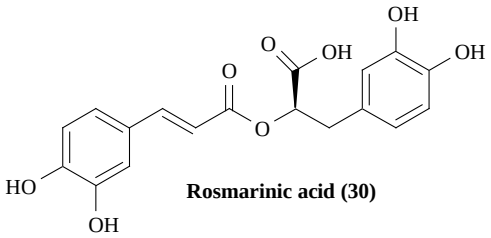
Shikonin (28)



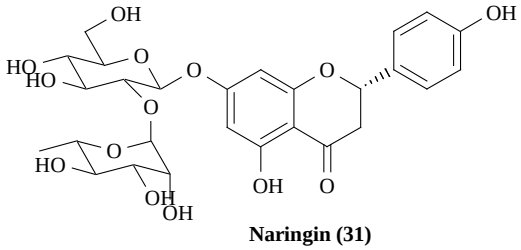
Emodin (29)

Annapoorani and colleagues [46] applied the virtual screening program Glide version 5.5 to screen 1,920 natural compounds against two QS receptor proteins (LasR and RhlR) of *P. aeruginosa*. Based on the docking scores, five top ranking compounds namely rosmarinic acid (30), naringin (31), chlorogenic acid (32) (Fig. **9**), morin (33) and mangiferin (34) (Fig. **10**) were subjected to in vitro bioassays against laboratory strain PAO1 and two more antibiotic resistant clinical isolates, *P. aeruginosa* AS1 (GU447237) and *P. aeruginosa* AS2 (GU447238). All the five compounds were found to inhibit QS dependent virulence factors production and biofilm formation in both laboratory as well as clinical strains of *P. aeruginosa* without affecting growth. In addition, protein degradation assay clearly evidenced that the selected compounds did not degrade virulence enzymes, showing that inhibition is mediated through QS.

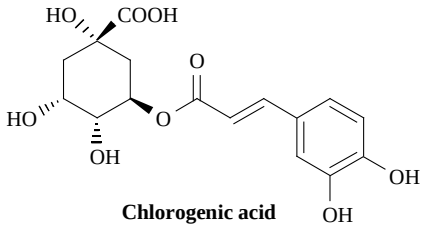
Figure 9: Structures of Rosmarinic acid and Naringin and Chlorogenic acid.



Rosmarinic acid (30)

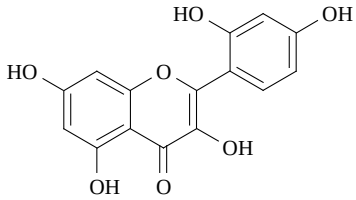


Naringin (31)

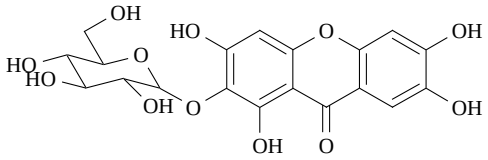


Chlorogenic acid (32)

Figure 10: Structures of Morin and Mangiferin.



Morin (33)



Mangiferin (34)

NATURAL PRODUCTS AND THEIR ANALOGUES

Creation of libraries of crude extracts and/or compounds from plants and microorganisms is the typical process by which natural product samples are prepared and stored for screening purpose. However, the creation of a large library of complex mixture or individually isolated chemical compounds from natural products sources is a complex and costly

process, and it is not usually the exclusive approach taken by scientists engaged in the discovery of antimicrobial-free antibiofilm agents. In addition, in-silico screening is hindered by the lacking of knowledge about the molecular scaffolds able to modulate bacterial biofilms and the molecular targets of the most promising isolated antibiofilm compounds.

Rather than employing conventional and virtual HTP screening programs, the exploration of smart terrestrial or marine metabolites on the basis of biologically and ecologically sound observations and experiments is gaining support [47]. For instance, the discovery of furanone compounds capable of inhibiting bacterial quorum-sensing systems isolated from the marine red alga *Delisea pulchra*, or 2-decenoic acid produced during *P. aeruginosa* growth. Thus, biodiscovery programs based on sound biological rationales, or on the ethnobotanical data can provide a direct route to the discovery of useful non-toxic antibiofilm compounds.

In addition, as the attention has now shifted to small high-quality libraries, natural product leads that possess antibiofilm activities at sub-lethal concentrations are legitimate starting templates for chemical manipulation to obtain analogues with increased activities. Novel biologically active analogs with potent and selective activities and improved solubilization and/or pharmacokinetic properties can be discovered and successfully used for the industrial scale up.

This section is organized around structured class and origins of small molecules of natural origin and their synthetic derivatives, and it summarizes recent researches and highlights the advance in this field. Based on their origin, small molecules of natural origin and their synthetic derivatives can be grouped in:

I) Plant extracts

II) Marine products and derivatives

a) halogenated furanones (HF) and their analogues.

b) pyrrole-imidazole alkaloids.

c) terpene derivatives.

d) indolic alkaloids derivatives

III) Bacterial products and derivatives

a) bacterial metabolites and their analogues.

b) derivatives guanidinic and biguanidic from norspermidine.

c) fatty acid messenger.

I) Plant extract

Extracts of *Trachyspermum ammi* (Ajowan) from the Apiaceae family are reported in literature for their antimicrobial and antioxidant properties. It was demonstrated their activity as bronchodilator in asthmatic patients and their analgesic

effect. Recently Khan et al. [48] analyzed the composition of the crude extract (CR) and its petroleum ether (PE) fraction by gas chromatography-mass spectrometry (GC-MS) and identified 2-isopropyl-5-methyl-phenol (34) (Fig. **11**) as the main component of each extract (52.05% vs. 83.65%). The MIC determined for CR and PE was 320 μg/ml and 40 μg/ml respectively on *Streptococcus mutans* ATCC 700610 involved in formation of dental bacterial plaque and recognized as responsible of cariogenicity effect on teeth. The reduction in the ability to form biofilm was dose dependent and did not affect *S. mutans* growth but only its aptitude to form biofilm. The authors proposed a mechanism of action that involved a modulation of the expression of specific virulence genes (*gtfB, gtfC, brpA* and *spaP*) by *S. mutans* and on the accumulation and structural organization of extracellular polysaccharides (EPS). Finally, a docking study was performed on models of four *S. mutans* proteins (Gtf B-C, BrpA and SpaP) to investigate their mode of interaction in presence of the 2-isopropyl-5-methyl-phenol. These results were helpful to design new drug candidates in order to inhibit virulence in *S. mutans*.

Tea (infusion of dried leaves of *Camelia sinensis*) is the most popular beverage all over the world today. The polyphenolic components of this extract possess a large number of pharmacological properties such as antimicrobial, antidiabetic, anti-inflammatory. It was also demonstrated its anticariogenicity in humans and experimental animals [49]. The main component of black tea is Epigallocatechin gallate (EGCG) (35) (Fig. **11**) that exhibited a wide range of pharmacological effects on *S. mutans* UA159 virulence factors related to its acidogenicity and acidurity. The EGCG inhibited biofilm formation at sub-lethal concentration (15.6 μg/ml) affecting the initial attachment of *S. mutans* to the surface through a mechanism that involved the suppression of *gtf* gene expression. It was demonstrated that this concentration was easily reached in oral cavity and did not influence the growth of oral bacteria.

Salvadora persica is a desert plant miswak used in Saudi Arabia as a natural toothbrush. It was demonstrated that aqueous extract from this plant inhibited the growth of several microorganisms. In their work Murugan et al. [50] showed the antibiofilm activity of methanol, ethanol, chloroform, acetone and aqueous extracts. The methanolic ones was selected based on high activity on biofilm forming (87.92% inhibition) cariogenic isolate *S. mutans* SMS09 and then analyzed with GC-MS. Among the components identified, benzyl (6*Z*,9*Z*,12*Z*)-6,9,12-octadecatrienoate (36) (Fig. **11**), 3-benzyloxy-1-nitro-butan-2-ol (37) (Fig. **11**) and 1,3 cyclohexane dicarbohydrazide (38) (Fig. **11**) interfered with the QS. Their binding mode was investigated performing docking studies utilizing the crystal structure of quorum sensing response regulator (Luxr family) disposable in the Protein Data Bank (PDB ID:1NXO). This study demonstrated that extracts of *S. persica* might offer an interesting source of compounds able to reduce the dental caries inhibiting the initial adhesion and the following biofilm formation by cariogenic bacteria [50].

Barley coffee (BC), derived from roasted barley, limits the adsorption of *S. mutans* on hydroxyapatite, the main constituent of surface teeth. The BC components were isolated through dialysis and gel filtration chromatography that

divided the low molecular mass fraction (polyphenols, zinc and fluoride ions) from the high molecular melanoidine mass fraction.

The latter formed during the roasted process was devoid of antibacterial activities but displayed strong antiadhesion properties towards *S. mutans* ATCC25175. The antibiofilm activity of the high molecular mass fraction seemed to be related with the sucrose-dependent and -independent adhesion mechanism interfering with the enzyme Gtf and the QS system [51].

Methanolic extract of *Capparis spinosa* showed an interesting activity on AHL mediated-quorum sensing in a dose dependent manner (0.5-2 mg/mL) without affecting bacterial growth of *Serrantia marcescens* FJ584421, *E. coli* ATCC10536, *Proteus mirabilis* ATCC7002 and *P. aeruginosa* PAO1. The extract was able to inhibit swimming and swarming motility of the bacterial patogens, EPS production and thus biofilm formation. One of the isolated components of the methanolic extract was homovanillic acid (39) (Fig. **11**) that might possibly act as an analogue to the AHL molecule [52].

Sub-MIC concentration of extracts from the following Indian medicinal plants seemed to act as direct or indirect inhibitors of QS of *P. aeruginosa* PAO1: *Hemidesmus indicus* (L.) Schult (root), *Holarrhena antidysenterica* (Roth) A.DC. (bark), *Mangifera indica* (L.) (seed), *Punica granatum* L. (pericarp), and *Psoralea corylifolia* L. (seed) [53].

An n-exane extract of *Salvia austriaca* hairy roots containing the diterpenoid 7-(2-oxohexyl)-taxodione (40) (Fig. **11**) decreased adhesion of staphylococci to abiotic surfaces at 1.25-2.5 μg/ml concentration, which in turn caused a reduction in *S. aureus* ATCC29213 (MRSA methicillin – resistant strain) biofilm formation [54].

Tannic acid (41) (Fig. **12**), the main component of the extract of *Quercus infectoria* G. Olivier nutgalls, a traditional Thai medicinal plant, showed the same MIC (0.13-0.50 mg/ml) of the whole extract against methicillin-resistant *S. aureus* suggesting that it might be the active component of the herb extract that influenced bacterial cell surface hydrophobicity that is involved in antibiofilm mechanisms. At sub-MIC only antibiofilm acitivity and not bacterial growth was inhibited [55].

Figure 11: Structures of Tymol, Epigallocatechin gallate, benzyl (6Z,9Z,12Z)-6,9,12-octadecatrienoate, 3-benzyloxy-1-nitro-butan-2-ol, 1,3 cyclohexane dicarbohydrazide, homovanillic acid and 7-(2-oxohexyl)-taxodione.

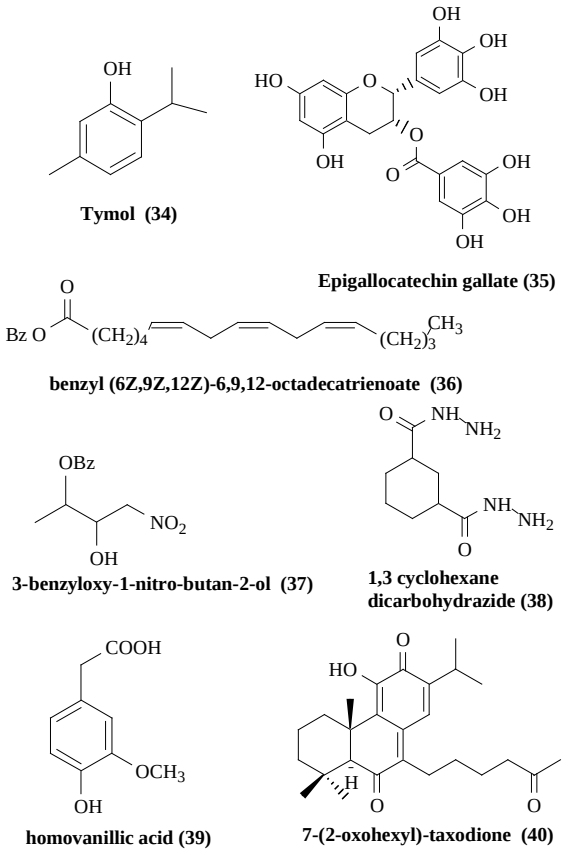
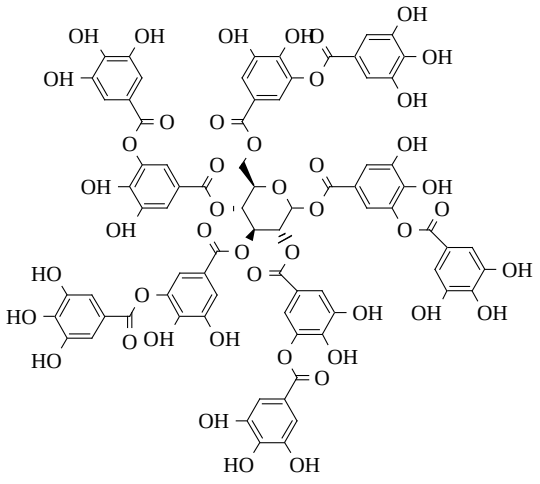


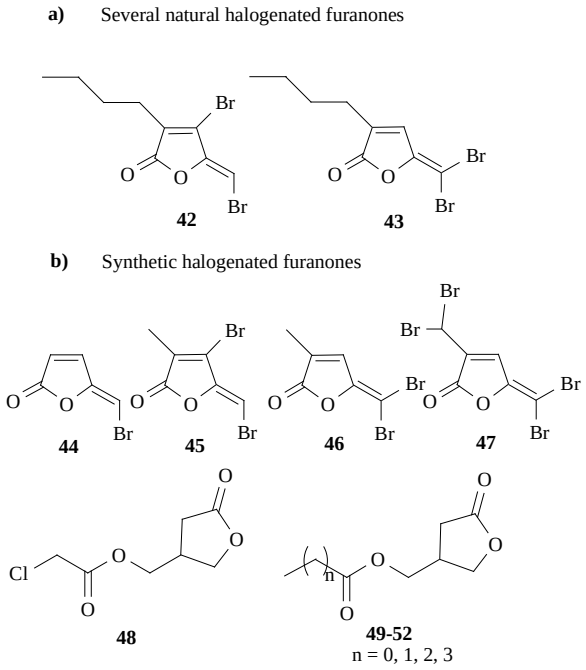
Figure 12: Structure of tannic acid.



II) Marine products and derivatives

a) Halogenated furanones (HFs) are small molecules (e.g. Compounds 42, 43) (Fig. **13**) produced by the marine red algae *Delisea pulchra*, as defence mechanism, well known for their effect on bacterial QS [56, 57]. It is possible to evidence several structural similarities between HFs and AHLs such as the presence of a non-polar aliphatic carbon 'tail' linked to a relatively polar 'head', besides to the most significant structural difference as the presence of bromine atoms. It has been shown that specific furanones affect several AHL-dependent activities through the binding with AHLs target receptors (LuxR protein homologues) favouring their degradation by proteolytic cleavage [58]. HFs were able to prevent biofilm formation and swarming in *E. coli* and *B. subtilis* [57,59] and they affected the expression of 93 genes in *P. aeruginosa* [60].

Figure 13: Structures of natural halogenated furanones (a), synthetic halogenated furanones (b).



Some furanones, as 5-bromomethylene-5H-furan-2-one (44) (Fig. **13**), lacking a side chain but bearing a vinyl bromide moiety, were able to penetrate *P. aeruginosa* biofilm and interfere with QS without any associated microbicidal properties [61]. Moreover, 44 showed activity against some Gram-positive oral *Streptococcus* biofilms. Particularly, it caused 63% and 76% reduction in biofilm growth, in polystyrene wells, of *S. mutans* and *Streptococcus intermedius* respectively, at 60 µM concentration [62].

Starting from the natural furanone 43 (enhancing biofilm formation of *S. epidermidis* and *S. aureus* at concentration of 10 to 20% of the MIC) [63], the influence of the number and the position of bromine atoms on the activity were explored. Compounds 45 and 46 at 224 µM concentration reduced *E. coli* biofilm formation by 75% and 63%

respectively, while the tetrabromo derivative 47 at 141 μM concentration caused a reduction of biofilm formation by 80% [64]. Nevertheless, the other synthetic furanones 48-52, lacking bromine atoms but bearing an exocyclic ester, inhibited *P. aeruginosa* biofilm formation in flow cells confocal microscopy experiments (Fig. **13**) [65]. By molecular modelling studies the binding energies of the synthetic furanones to the LasR receptor, compared with the known AHL signalling molecule 3-oxo-C12-AHL 18, were evaluated. Moreover docking studies highlighted that, in the binding pocket, the furanone scaffold overlapped with the lactone moiety of AHL, supporting the activity data.

Recently, with the aim to target LasR and LuxR receptors simultaneously (multitarget approach) and to increase the potency of 3,4-dihalo-5H-furan-2-one by introduction of a substituted aromatic moiety, a set of 5-substituted 3,4-dihalo-5H-furan-2-ones (*erythro/threo* 5-(aryl-1'-hydroxy-methyl)-3,4-dihalo-5H-furan-2-one 53a-53h and 5-(aryl-2-methylene)- 3,4-dihalo-5H-furan-2.one 54a-b, 54f-i) (Fig. **14**) were designed and synthesized. Their biofilm formation inhibitory activities against *P. aeruginosa* were studied by MIC assay, quantitative analysis of biofilm inhibition, and observation of biofilm formation with the scanning electron microscopy (SEM). Among these derivatives compound 54i showed the best activity: 41.3%, 71.8% and 38.9% of biofilm formation inhibition against ATCC 27853, ATCC 9027 and PAOA respectively, at 64 μM concentration. By molecular docking studies the binding mode between the inhibitors and the LasR receptor was identified. In contrast, the covalent linkage between the bromine atoms and the LuxS protein was not appropriate for the docking studies. The key structural features were the hydrogen bonds among the compound, the receptor protein and the aromatic ring substituted (especially with the hydroxyl group) as side chain. Therefore the highest potency of *Z*-54i could be related to the stabilization by hydrogen bonds and lipophilic electron-donating aromatic ring (Fig. **14**) [66].

b) Oroidin 55 and Bromoageliferin 56 are alkaloids produced by the marine sponge *Agelasidae* as chemical anti-feeding defence mechanism against predator [67]. They are structurally characterized by the presence of 2-aminoimidazole (2-AI) subunits, the supposed key pharmacophore.

The simplification of bromoageliferin structure led to the couple of analogues TAGE (*trans*- bromoageliferin) 57 and CAGE (*cis*-bromoageliferin) 58 showing a biofilm formation inhibition against *P. aeruginosa* PA01 and PA14. In details, TAGE inhibited PA01 and PA14 biofilm formation with IC_{50} values of 100 μM and 190 μM respectively, similarly to CAGE (IC_{50} values of 100 μM and 180 μM respectively) [68]. Moreover, TAGE was also able to disperse established *P. aeruginosa* biofilms with EC_{50} values of 82 μM and 114 μM against PA01 and PA14 respectively. The introduction of di-bromo substituted acylpyrrole moiety on TAGE scaffold (by analogy with bromoageliferin) led to several compounds including 59. 59 showed activity against *P. aeruginosa* PA01 and PA14 biofilm formation with IC_{50} values of 1.77 μM and 12.0 μM respectively and against the mucoid variant isolated from patients affected by cystic

fibrosis with an IC_{50} value of 2.47 µM. Nevertheless, the acylated analogues were less efficient to disperse the pre-formed *P. aeruginosa* biofilm in comparison with TAGE (Fig. **15**) [69].

Figure 14: Structures of 5-substituted 3,4-dihalo-5H-furan-2-ones (erythro/threo 5-(aryl-1'-hydroxy-methyl)-3,4-dihalo-5H-furan-2-one) (a) and 5-(aryl-2-methylene)- 3,4-dihalo-5H-furan-2.one (b).

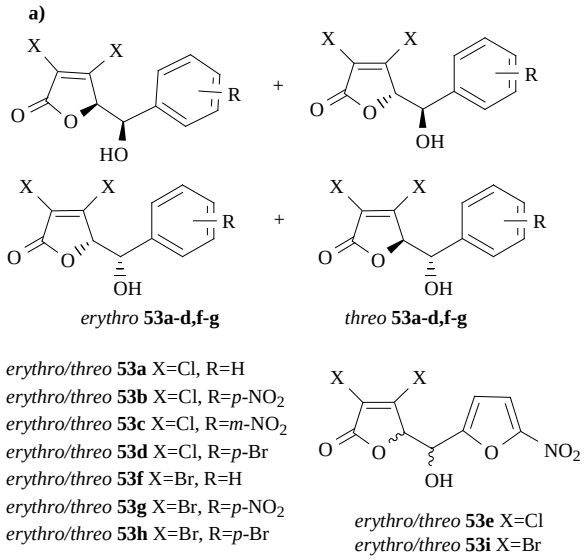
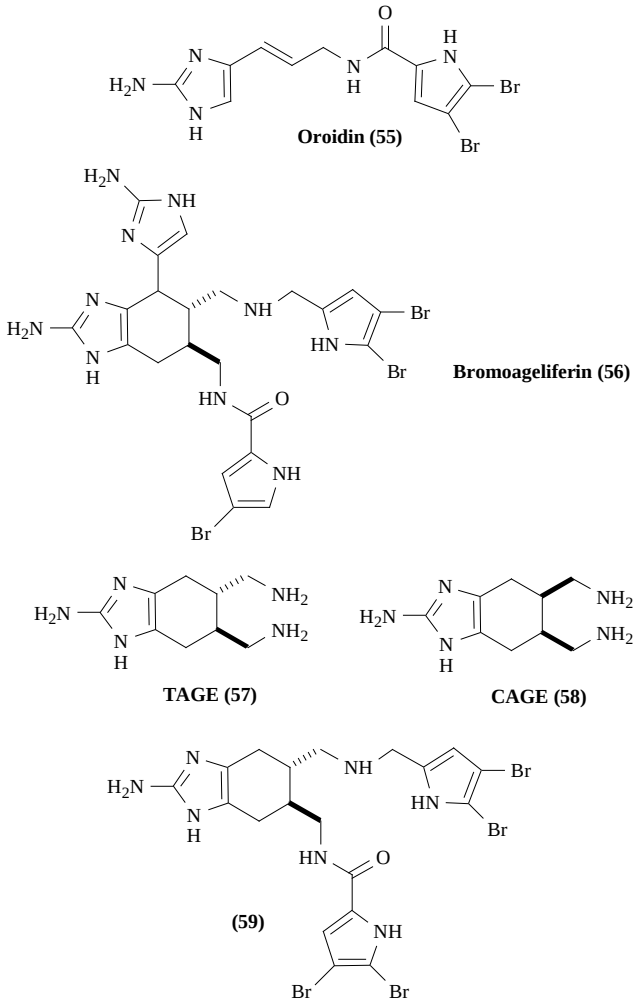
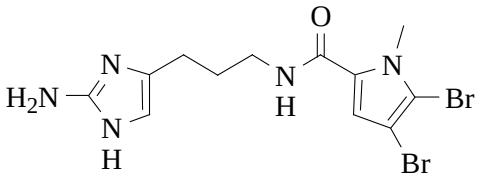


Figure 15: Structures of Orodin, Bromoageliferine and its derivatives.

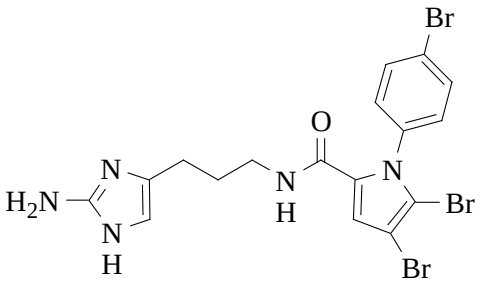


Since Oroidin showed a similar activity to TAGE and CAGE on *P. aeruginosa* (IC_{50} values of 190 µM and 166 µM against PA01 and PA14 respectively) a 50-member library based on Oroidin scaffold/template was prepared [70]. The scaffold was divided into three sections: the AI-2 head group, the linker chain, and the pyrrole tail group and their modification were explored to define the structural requirements essential for the activity (Fig. **16**). SAR analyses highlighted that the modifications to the 2-AI moiety led to inactive compounds. Nevertheless, the compound 60 dihydrosventrin (DHS), an analogue of the natural alkaloid sventrin produced by the marine sponge *Agelasidae*, exhibited an antibiofilm activity against PA01, PA14, PD0300, *A. baumannii* and *Bordatella bronchiseptica* strain RB50 (with IC_{50} value of 51 µM, 111 µM, 115 µM, 110 µM and 238 µM) [71]. Belonging to the second-generation derivatives, compound 61, bearing a *para*-bromo phenyl substituent at nitrogen of the pyrrole ring, showed the highest activity against *A. baumannii* (IC_{50} value 27 µM and EC_{50} value 41 µM) (Fig. **16**) [72].

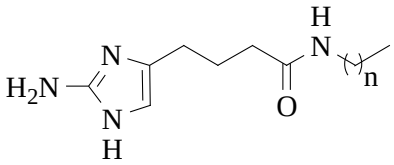
Figure 16: Structures of dihydrosventrin (DHS) and 2-AI derivatives.



Dihtdrosventrin (DHS) (60)



(61)



(62), **(63)** as HCl salt
n=11, 12

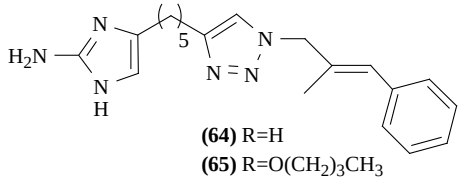
Among the chemical modifications on the Oroidin template, also derivatives presenting the reversed amide bond were synthesized [73]. In particular, compounds bearing an aliphatic chain showed a low micromolar inhibition of biofilm development by PA01 and PA14 (better than Oroidin) and their potency was directly related to chain length. The most active compound, 62, exhibited the highest dispersion property (IC_{50} values 2.84 and 2.26 µM against PA01 and PA14 EC_{50} values 33 and 21 µM against PA01 and PA14 respectively), although compound 63 showed an IC_{50} value of 729 nM against PA14. The latter was grafted into a methacrylate polymer by a triazole linker, to develop new antibiofilm material for medical devices inhibiting the biofilm formation [74].

Moreover, the introduction of a triazole moiety on Oroidin scaffold was explored [75]. The most active 2-aminoimidazole-triazole (2-AIT) obtained presented a triazole bearing, by an unsatured chain, an aryl group. In particular, compound 64 showed a broad spectrum inhibitory activity against both Gram-positive and Gram-negative bacteria (IC_{50} values of 5.6 µM, 530 nM, 980 nM and 810 nM against PA01 and PA14, *A. baumannii* and *S. aureus* respectively), dispersal properties, a synergistic effect with several antibiotics in the dispersion of pre-established biofilm of various bacterial strains, and re-sensitized planktonic bacteria of drug resistant strains of *S. aureus* and *A. baumannii* [76]. By introduction of substituents on phenyl ring, several dispersion agents against *A. baumannii* were

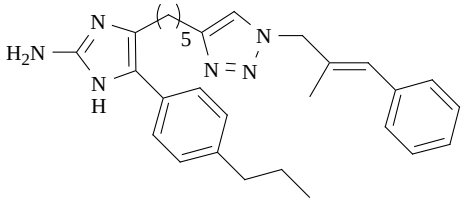
obtained and, in particular, 65 exhibited the best EC_{50} value (44.7 μM) respect to 64 (120 μM) [77]. The presence of further substituents on 2-AI moiety such as the phenyl ring led to a library of compounds and among them the most potent was 66 (Fig. **17**) that showed an IC_{50} value of 1.42 μM against MRSA. Although 66 was less potent of the parent compound 64 against *A. baumannii* (IC_{50} value of 11.28 μM), it exhibited the property to disperse their pre-formed biofilm (EC_{50} values of 44.6 μM) [78].

The introduction of various alkyl-linked amides in substitution of the unsaturated aryl moiety led to the identification of compounds showing low micromolar biofilm inhibition against various Gram-positive and Gram-negative bacterial strains as well as fungal antibiofilm activity and dispersal property. The most interesting compounds presented *para*-alkyl substituents on phenyl ring and the alkyl chain length modulated the activity. Although derivative 67 was the most active against MRSA (IC_{50} value of 0.7 μM and EC_{50} values of 0.9 μM), compound 68 inhibited biofilm formation by *E. coli* and a multi-drug resistant strain of *A. baumannii* with IC_{50} value of 11.2 μM and 1.9 μM respectively and EC_{50} values of 23.4 μM and 7.9 μM. Furthermore 68 showed a high activity also against Gram-positive bacteria such as vancomycin-resistant enterococci (VRE) (IC_{50} value of 0.5 μM and EC_{50} values of 0.7 μM) and *S. epidermidis* (IC_{50} value of 0.6 μM and EC_{50} values of 28.1 μM) [79, 80].

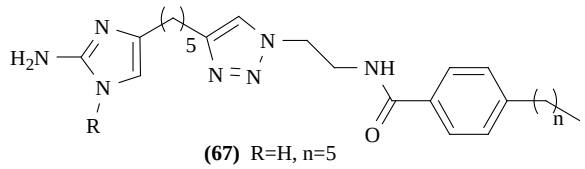
Figure 17: Structures of 2-AIT derivatives.



(64) R=H
(65) R=$O(CH_2)_3CH_3$



(66)



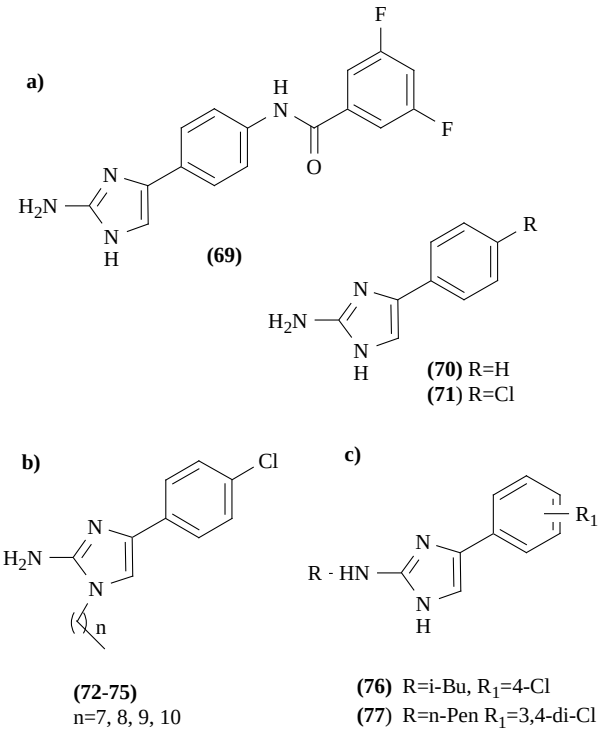
(67) R=H, n=5
(68) R=H, n=6

The replacement of the triazole by a substituted phenyl ring, directly bonded to 2-AI moiety, led to a 30-member library. Among them compound 69 inhibited *E. coli* biofilm formation with an IC_{50} value of 5.2 μM (Fig. **17**) [81]. The parent compound 70, belonging to a related series of aryl 2-AI derivatives, showed a moderate activity against biofilm

formation by *Salmonella* Typhimurium (IC_{50} value of 130 µM at 25°C) and *P. aeruginosa* (IC_{50} value of 72.6 µM at 25°C), while its *para*-chloro substituted derivative 71, the most active compound, inhibited biofilm formation by *S.* Typhimurium and *P. aeruginosa* with IC_{50} values of 16.0 µM and 3.5 at 25°C respectively (Fig. **18**).

The presence of alkyl substituents at 1-position of the 2-AI moiety (Fig. 18) tuned the activity: the derivatives bearing an intermediate length chain were most active against *P. aeruginosa* biofilm formation as compound 72 (IC_{50} value of 2.6 µM) while longer alkyl chains led to compounds as 73-75 exhibiting activity against *S.* Typhimurium (IC_{50} values around 4 µM at 25°C) [82]. The introduction of a substituent also at position 2 of 2-AI ring led to a 2,4-disubstituted 2-amino imidazole series and the most active compounds 76 and 77 showed antibiofilm activity against *S.* Typhimurium with IC_{50} values of 2.0 µM and 2.2 µM respectively and against *P. aeruginosa* with IC_{50} values of 0.9 µM and 0.7 µM respectively (Fig. **18**) [83].

Figure 18: Structures of 2-AI 4-phenyl substituted derivatives (a), 2-AI 5-substituted derivatives (b) and 2-substituted AI derivatives (c).



Among the 1,4,5-substituted series, the most active compounds 78 and 79 inhibited biofilm formation by *S.* Typhimurium with IC_{50} values of 10.3 µM and 18.3 µM respectively and by *P. aeruginosa* with IC_{50} values of 27.4 µM and 17.4 µM respectively (Fig. **19**) [84].

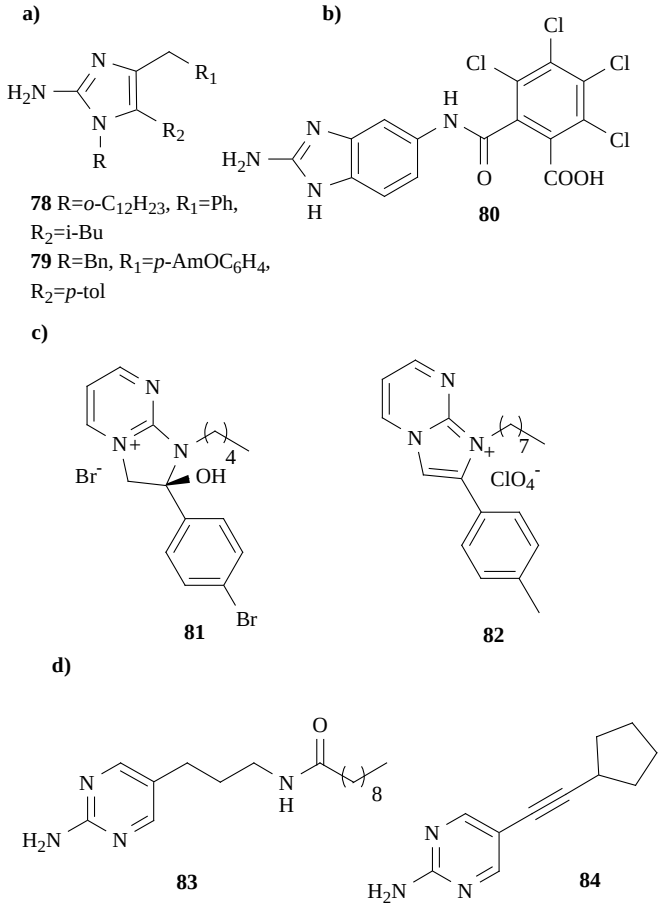
The antibiofilm properties of scaffolds related to 2-AI were evaluated. Concerning the 2-aminobenzoimidazole (2-ABI) derivatives, the most active compound, 80, showed activity (possibly related to directly bind to Zn(II)) against the

biofilm formed by MRSA with IC_{50} value of 890 nM and EC_{50} value of 2.9 µM, by VRE with IC_{50} value of 1.4 µM and EC_{50} value of 75 µM, and by *S. epidermidis* with IC_{50} value of 570 nM and EC_{50} value of 7.3 µM [85].

Considering the pyrimidinium scaffold, among the series of 2-hydroxy-2-aryl-2,3- dihydroimidazo-pyrimidinium salts (intermediates in the synthesis of 2,4,5-trisubstituted 2-AI), compound 81 inhibited biofilm formation by *S.* Typhimurium with IC_{50} value of 1.7 µM and by *P. aeruginosa* with IC_{50} value of 4.2 µM [86]. Moreover, compound 82**,** belonging to a series of imidazo- [1,2α]pyrimidinium salts, showed antibiofilm formation activity against *S.* Typhimurium with an IC_{50} value of 1.5 µM and against *P. aeruginosa* with an IC_{50} value of 1.0 µM. It has been suggested that activity of these compounds derived from their *in vivo* cleavage to the substituted 2-AI [82, 83].

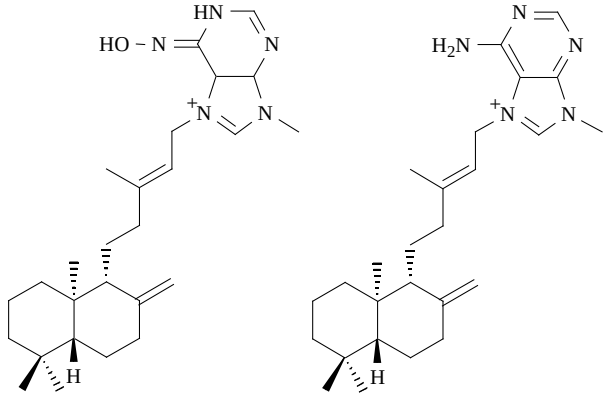
Also the 2-aminopyrimidine (2-AP) scaffold was investigated and the obtained derivatives exhibited a lower activity in comparison with 2-AI compounds (in particular against the Gram-negative bacteria) and were not able to disperse preformed biofilm. Among the most active derivatives, 83 inhibited biofilm formation by MRSA (with an IC_{50} value of 72 µM) and 84 inhibited the formation of methicillin sensitive strain of *S. aureus* biofilm (with an IC_{50} value of 67 µM) (Fig. **19**) [86].

Figure 19: Structures of 1,4,5 substituted 2-AI derivatives (a), 2-ABI derivative (b), dihydroimidazo-pyrimidinium salts (c), 2-AP derivatives (d).



c) Among the diterpene alkaloids (Fig. **20**), isolated as metabolites from the marine sponge *Agelas nakamura*, the adenine salts (structurally constituted by cyclic diterpene linked to 9-methyladeninium moiety), (-) Ageloxime D 85 (an oxime derivative) inhibited biofilm formation by *S. epidermidis* without interfering with the planktonic growth on the contrary of (-) Agelasine D 86, the corresponding amine (MIC < 0.0877 μM) [87-88].

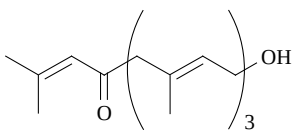
Figure 20: Structure of (-)Ageloxime D and (-)Agelasine D.



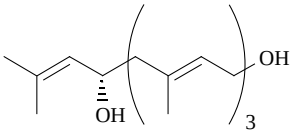
(-)Ageloxime D (85) **(-)Agelasine D (86)**

Recently, a terpenoid-like library has been designed and a new class of 1,4-disubstituted-(1*H*)-1,2,3-triazole derivatives exhibiting antibiofilm activity identified. These compounds were obtained by the bioconjugation of natural terpenic framework (derived by linear diterpenes as Eleganolone 87 and Eleganediol 88 from the brown alga *Bifurcaria bifurcata*), meroditerpenes related to Geranylgeranyltoluquinol 89 (from the brown alga *Halidrys siliquosa*) and 3-(4'-geranyloxy-3-methoxyphenyl)-2-*trans* propenoic acid 90 (from *Acronychia baueri* Schott)) (Fig. **21**) and a synthetic moiety (related to the aromatic part of meroditerpenes), through a triazole linker via click chemistry methodologies. A first polyprenyl-type library (A) was constituted by 1,4-disubstituted triazoles 91 (Fig. **22**) and these compounds were obtained as *Z/E* mixtures, while the second generation library (B) derivatives, presenting an oxygen bridge, were obtained as pure *E*-isomers 92, due to their easier preparation. By molecular modelling studies, performed on 4-methoxy derivatives of each library, the comparison between the two libraries scaffolds highlighted that in the scaffold B the size of the triazolic linker, due to the presence of the oxygen bridge, is longer of 1.76 A, cLog(P) is lower and an additional H-bonding is located on the oxygen atom. SAR confirmed that, due to their similarities of the two series, the nature of the linker tune the activity without relevant variations in the biological response. The antibiofilm activity was evaluated against *Pseudoalteromonas* sp. strain and the most active compounds 92a and 10 92b, belonging to the library B, inhibited biofilm formation with EC_{50} values of 103 μM and 74 μM respectively (Fig. **22**) [89].

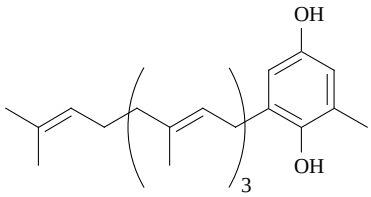
Figure 21: Structures of Eleganolone, Eleganediol, Geranylgeranyltoluquinol and 3-(4'-geranyloxy-3-methoxyphenyl)-2-trans propenoic acid.



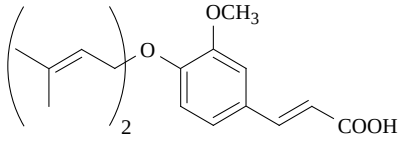
Eleganolone (87)



Elegandiol (88)

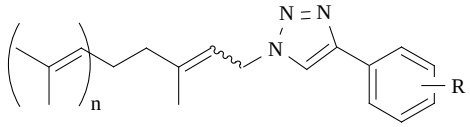


Geranylgeranyltoluquinol (89)

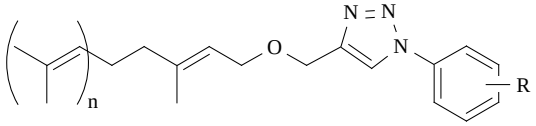


3-(4'-geranyloxy-3-methoxyphenyl)-2-trans propenoic acid (90)

Figure 22: Structures of 1,4-disubstituted triazole derivatives.



Library A, first generation of analogues (*Z*/*E* mixture) R=OCH_3, OH; n=1, 2 **(91)**



Library B, second generation of analogues (*E* isomers) R=OCH_3, OH, COOH; n=1, 2 **(92)**

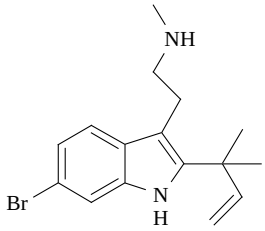
92a n=1, R=3-OCH_3
92b n=1, R=3-COOH

d) Flustramines are indole derived secondary metabolites [90, 91] produced by the bryozoan *Flustra foliacea* found in the northern Atlantic Ocean as chemical defence system, tuning the indole signaling pathway. Indole, along with AI-2, is considered a putative universal signal [92] since it is produced by 85 species of bacteria and it modulates biofilm formation [93], antibiotic resistance, virulence etc.

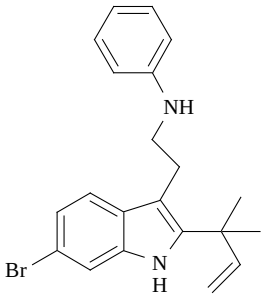
Flustramines is a family consisting of eleven secondary metabolites: six pyrroloindolines and five indolic alkaloids. Among the latter, Desformylflustrabromine (dFBr) 93 (Fig. **23**) inhibited *E. coli* and *S. aureus* biofilm formation with IC_{50} values of 174 µM and 70 µM respectively, although by a toxic mechanism against *E. coli* [94].

With the aim to identify non-toxic modulators of bacterial behaviour, considering dFBr as template, new indole derivatives were developed. The dFBr scaffold was subdivided into four areas (bromine region, prenyl group, indole nitrogen, aliphatic nitrogen region) and SAR studies were performed [95]. The most potent analogue was 94 (Fig. **23**) showing IC_{50} values of 5.9 µM and 53 µM against *S. aureus* and *E. coli* respectively [95]. Furthermore 94 is 10 - 1000 times more active than indole itself to inhibit biofilm formation.

Figure 23: Structures of Desformylflustrabromine and its derivative.



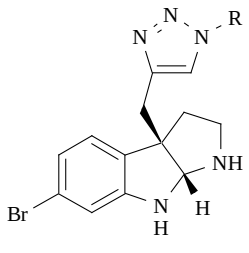
Desformylflustrabromine (dFBr) (93)



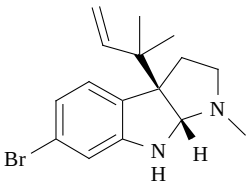
(94)

Recently, several interesting compounds, belonging to a pyrroloindoline triazole amide library (general formula 95) (Fig. **24**), derived from Flustramine C and Dihydroflustramine C 96 (Fig. **14**), have been identified as modulators of biofilm formation at low micromolar IC_{50} values against Gram-positive and Gram-negative bacteria strains [96].

Figure 24: General formula of pyrroloindoline triazole amide library and Dihydroflustramine C structure.



(95)



Dihydroflustramine C (96)

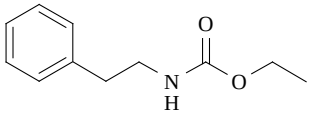
III) Bacterial products

a) The bacterial metabolite, ethyl N-(2-phenetyl) carbamate 97 (Fig. **25**) isolated from the cultured medium of the marine bacterium SCRC3P79 (*Cytophaga* sp.) showed a moderate biofilm formation inhibition of *S. epidermidis*

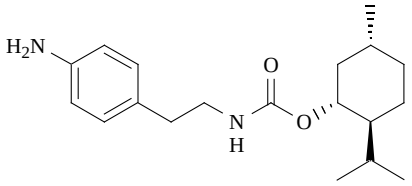
MRSA, VRE, MDRAB (multi-drug resistant *A. baumannii*) and *E. coli*. A library of analogues was synthesized obtaining compounds endowed with increased activity. Among them the most active were the menthyl derivatives 98-100 (Fig. **25**) inhibiting biofilm formation by *S. aureus* with IC_{50} values ranging from the mid to low molar [97].

In particular, compound 99 showed antibiofilm activity against MRSA (IC_{50} value of 4.87 µM) and *E. coli* (IC_{50} value of 34.6 µM) [98].

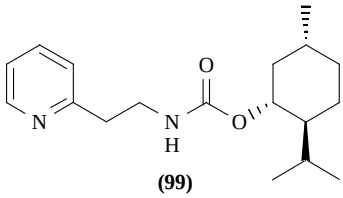
Figure 25: Structures of ethyl N-(2-phenetyl) carbamate and its menthyl derivatives.



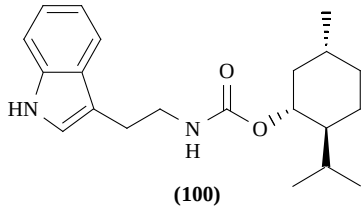
ethyl N-(2-phenetyl) carbamate (97)



(98)



(99)



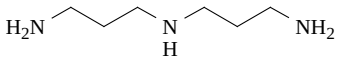
(100)

b) Considering Norspermidine 101 (Fig. **26**) (self-produced factor of *B. subtilis*), a natural key disruptor of the polymeric component of EPS, a focused library of biomimetic compounds, guanidine 102 and biguanide 103 derivatives (Fig. **26**) was developed. Some of these analogues exhibited 5-20 fold increased potency in preventing biofilm formation and >8 fold increased activity against respect to Norspermidine. In terms of minimum biofilm inhibitory concentration (MBIC), the most active inhibitors of biofilm formation were compounds 102a (2 µM) and 102b (30 µM) against *B. subtilis* and compounds 102aa (55 µM) and 102b (20 µM) against *S. aureus*. By SAR a common structural motif useful to inhibit both biofilm species was identified, although the composition and structure of the two biofilms were different. Therefore the influence of the correct spacing of multiple amino or guanidine groups, during the binding of polyamine-based inhibitors with the exopolymer, was confirmed. In addition, studies on

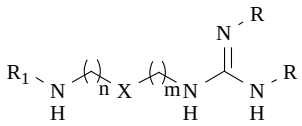
protonation constants and crystal structure data revealed that the spacing of positively charged groups and the total charge at physiological pH played a determinant role in the antibiofilm activity [99].

Figure 26: Structures of Norspermidine (a) and its guanidine (b) and biguanide (c) derivatives.

a)



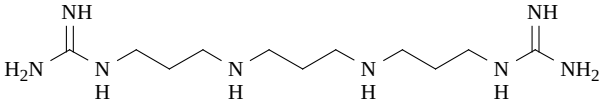
Norspermidine (101)



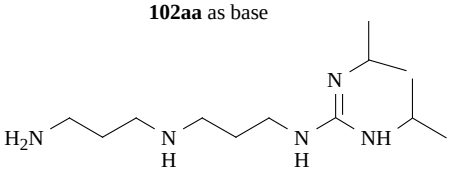
(102)

X=NH, $NH(CH_2)_3NH$, N-C(=NR)NHR
R=H, i-Pr; R_1=H, $C(=NH)NH_2$, C(=NiPr)=NHiPr
m=0, 2, 3; n=2, 3

b)

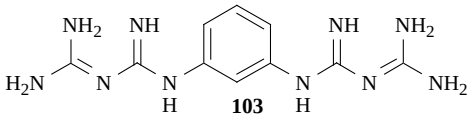


102a as H_2SO_4 salt
102aa as base



102b as HCOOH salt

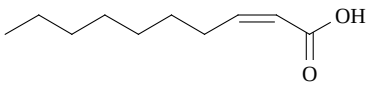
c)



103

c) (*Z*)-2-decenoic acid 104 (Fig. **27**), produced by *P. aeruginosa* during growth, appeared to be functionally and structurally related to the class of short-chain fatty acid signalling molecules such as diffusible signal factor. Compound 104 has also been identified as a potent biofilm-dispersion inducer, since, when added exogenously to *P. aeruginosa* PA01 microcolonies, it induced the complete biofilm dispersion at a native concentration of 2.5 nM. Moreover, 104 was shown to induce dispersion of biofilms formed by *E. coli*, *K. pneumoniae*, *P. mirabilis*, *S. pyogenes*, *B. subtilis*, *S. aureus*, and the yeast *Candida albicans* [100].

Figure 27: Structure of (Z)-2-decenoic acid.



(*Z*)-2-decenoic acid (104)

CONCLUSION

Healthcare-associated infections are a major problem that clinicians must deal with. Nowadays, the patient safety is seriously jeopardized by the emergence and spread of nosocomial pathogens in form of biofilm that are resistant to traditional and affordable antimicrobials. Considering that infectious disease is the second leading cause of death worldwide and that we are now entering the post-antibiotic era, limited treatment options for many nosocomial infections are available. In the light of the previously considerations, the development of novel antibiofilm compounds able to interfere, at sub-lethal doses, with the pathogenic biofilm cascade, represents an ideal preventive strategy to pursue. Depriving microorganisms of their virulence properties without affecting their existence may also apply a milder evolutionary pressure for the development of resistance, as most virulence traits are not essential for bacterial survival, restoring the efficacy of traditional antimicrobial agents. Thus, new antibiofilm templates with novel targets and unique mechanisms of action are urgently required.

Nature has been proven to be an outstanding source for new and innovative antibiofilm compounds active at sub-lethal concentrations. Plants and microbes of terrestrial and marine origin are known to produce small molecules offering unmatched chemical diversity with structural complexity and biological potency. Some of these molecules exhibit novel biological activities that make them indispensable tools in biomedical research and unique prototypes for the development of innovative therapies.

Now, the question is how to re-establish the leadership of natural products as a major source of leads for the development of antibiofilm compound active at sub-lethal concentrations. The first obstacle to overcome in the development of non-toxic antibiofilm agents is setting an appropriate screening method. Since biofilm formation is a very complex process, which is regulated by an interplay between many cellular systems, a ‘top-down’ approach, like screening for prevention of the biofilm as a whole, as compared to a target-based screening (‘bottom-up’), which depends on knowledge of biofilm targets already identified, is preferred. In addition, by subsequently studying the mode of action of potential biofilm modulators identified in a ‘top-down’ screening, possible new important biofilm targets can be identified providing the springboard to apply elegant computer-aided drug design approaches. Recently, the development of new technologies has revolutionized the screening of natural products offering a unique opportunity to re-establish natural products as a major source for antibiofilm compounds discovery.

The diverse spectrum of biological activities these molecules possess in addition to their fascinating chemical structures makes them of particular interest for target-oriented synthesis. After identifying natural product leads, applying new synthetic methodologies would generate a large number of novels, structurally diverse analogs that can be screened for improved activities and properties at sub-lethal concentrations. We currently have the technology to prepare analogs and to explore the structure-activity relationships to truly harness the potential of these compounds.

Since the scientific community advances knowledge of how to discover, design and improve potent antibiofilm molecules, several issues emerge. First, we need to understand what are the exact mechanisms by which these molecules exert their effects at sub-lethal concentrations. Second, as with any drug development strategy, there still remain multiple technical challenges that need to be overcome before small molecule modulators can successfully transition into the clinic practice. Researchers will need to assess such parameters as compound toxicity, pharmacokinetics and pharmacodynamics, and validation in animal models. As a therapeutic strategy, these compounds will mostly likely serve as adjuvants to conventional antibiotics, and there are dosing and pharmacokinetic/pharmacodynamic issues that must be optimized between the antibiotic and antibiofilm agent. Third, new molecular classes should be investigated to expand our repertoire of antibiofilm agents. Fourth, various in vitro biofilm assays need to be compared to in vivo outcomes. Finally, further toxicity tests need to be established, especially with regards to the effects of antibiofilm compounds on commensal bacteria in their native host.

The study of small molecules from natural products represents a cornerstone of medicinal chemistry and such compounds remain a fascinating group, not least because of the remarkable array of intriguing structures that continue to be isolated from almost every conceivable source showing antibiofilm activity at sub-lethal concentrations. However, much remains to be achieved as far as understanding how small molecules of natural origin are able to inhibit biofilm formation in diverse organisms and which are the compounds responsible of the activity. Despite these questions, it is exciting to imagine the potential of combinatorial therapy, such as the coupling of these molecules at sub-lethal doses with improved antibiotics, for more successful eradication of detrimental nosocomial infections. Future anti-infective treatments will most likely be comprised of combination therapies that produce additive or synergistic effects to target key processes in both the pathogen and the host. The overall promise of discovering novel antibiofilm compounds working at non-microbicidal manner has generated great hope in the biomedical community for discovery of new countermeasures against nosocomial infections.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was partially supported by Fondazione Cariplo, grant no. 2011-0277.

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