

## Small molecule control of bacterial biofilms

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Bacterial biofilms are defined as a surface attached community of bacteria embedded in a matrix of extracellular polymeric substances that they have produced. When in the biofilm state, bacteria are more resistant to antibiotics and the host immune response than are their planktonic counterparts. Biofilms are increasingly recognized as being significant in human disease, accounting for 80% of bacterial infections in the body and diseases associated with bacterial biofilms include: lung infections of cystic fibrosis patients, colitis, urethritis, conjunctivitis, otitis, endocarditis and periodontitis. Additionally, biofilm infections of indwelling medical devices are of particular concern, as once the device is colonized infection is virtually impossible to eradicate. Given the prominence of biofilms in infectious diseases, there has been an increased effort toward the development of small molecules that will modulate bacterial biofilm development and maintenance. In this review, we highlight the development of small molecules that inhibit and/or disperse bacterial biofilms through non-microbicidal mechanisms. The review discusses the numerous approaches that have been applied to the discovery of lead small molecules that mediate biofilm development. These approaches are grouped into: (1) the identification and development of small molecules that target one of the bacterial signaling pathways involved in biofilm regulation, (2) chemical library screening for compounds with anti-biofilm activity, and (3) the identification of natural products that possess anti-biofilm activity, and the chemical manipulation of these natural products to obtain analogues with increased activity.

### Introduction

A significant factor contributing to the pathogenesis of, and anti-biobiotic/host immune resistance to, a number of medically important bacterial strains is the ability of the bacteria to form a biofilm. Bacterial biofilms are highly organized surface-associated communities of bacteria encased within an extracellular matrix. Bacteria within a biofilm exhibit distinct phenotypes from planktonic cells, particularly with respect to growth and gene expression.<sup>1</sup> Bacterial biofilms have become recognized as a serious threat to both the medical and industrial sectors of society within the past 20 years.<sup>2</sup> On a global scale, biofilm-related costs incur billions of dollars to the agricultural, engineering, and medical sectors of the economy.<sup>3</sup> The correlation between biofilms and infectious disease is a connection that is becoming well documented in the medical community and The National Institutes of Health (NIH) estimates that 80% of all bacterial infections occurring in the human body are biofilm related.<sup>3</sup> An estimated 17 million new biofilm infections arise each year in the U.S., which result in up to 550 000 fatalities annually. Common ailments that are driven and are perpetuated

by bacterial biofilms include, but are not limited to: lung infections of cystic fibrosis (CF) patients, burn wound infections, ear infections, catheter infections, bacterial endocarditis, chronic wound infections, and tooth decay.<sup>3,4</sup> Longer hospital stays, chronic infection, and increased fatalities as a result of biofilm-mediated infections place a significant economic burden on healthcare systems worldwide.<sup>5</sup> Bacterial biofilms also underlie the persistent colonization of hospital facilities, both driving and sustaining nosocomial infections.

Biofilms are inherently insensitive to antiseptics and microbicides that would typically eliminate their planktonic brethren and are known to be upwards of 1000-times more resistant to conventional antibiotics<sup>6</sup> and bacteria within a biofilm reach a much higher cell density ( $10^{11}$  CFU mL<sup>-1</sup>) than do planktonic bacteria ( $10^8$  CFU mL<sup>-1</sup>).<sup>7</sup> Multi-drug resistant (MDR) bacteria are becoming commonplace in the global healthcare setting and antibiotics which have previously been of last resort are being used with increased frequency in attempts to alleviate particularly aggressive infections.<sup>8</sup> Compounding this problem, only two new classes of antibiotics (oxazolidinones and lipopeptides) have been introduced into the clinic over the last 40 years.<sup>9</sup> Biofilms also underlie importunate infections of indwelling medical devices (IMDs), and it has been demonstrated that the presence of such a foreign body decreases the minimal infecting dose of *Staphylococcus aureus* by 100 000-fold.<sup>7</sup> Eradication of

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these infections is virtually impossible, requiring aggressive antibiotic therapy, removal of the indwelling device, and surgical debridement.<sup>10</sup>

Phenotypic changes brought about by the formation of a biofilm contribute to bacterial resistance to antibiotics. These changes include production of the extracellular polymeric substance (EPS) and upregulation of genes responsible for porin proteins or specialized efflux pumps to purge antibiotics from the cell. While the 3-dimensional morphology of biofilms lends itself to nutrient distribution and waste disposal, it also provides a fertile environment for the efficient transfer of genetic material.<sup>11</sup> Gene transfer rates in biofilms facilitated through the conjugation process have been reported to be up to 1000-fold higher than those found in planktonic cells.<sup>12</sup> Another problem exacerbated by bacteria occupying a biofilm state is hypermutation. This occurs when bacteria, in response to environmental pressures (such as antibiotics), mutate at higher rates to evolve resistance and alleviate the particular stress on the community.<sup>13</sup> Approximately 20% of *Pseudomonas* isolates from the lungs of cystic fibrosis patients display this phenomenon,<sup>14</sup> making the task of treatment even more challenging.

The slow growth rates of biofilm communities have also been posited as a factor in increased resistance to eradication.<sup>15</sup> Within a biofilm, there exists a heterogeneous population of cells which differ in growth rates dependant upon the area within the biofilm in which the cells are located. Bacterial cells embedded deep within the biofilm matrix grow more slowly due to lack of nutrients and oxygen.<sup>16</sup> Cells with reduced metabolic activity are also inherently more recalcitrant to antimicrobial therapies<sup>17</sup> as almost all antibiotics target five biosynthetic processes, all of which occur in actively growing bacteria: the biosynthesis of proteins, RNA, DNA, peptidoglycan and folic acid.<sup>18</sup> Therefore most antibiotics that are capable of killing growing and dividing bacterial cells tend to be very inefficient at killing non-multiplying bacteria.<sup>19</sup> This, coupled with the differential penetration distances into a biofilm that antibiotics display, explains how some drugs fail to completely eradicate biofilm bacteria.<sup>15</sup>

Our understanding of the diverse mechanisms that govern bacterial biofilm formation and maintenance is continually increasing. Recent research into the environmental cues and genetic elements that play a part in biofilm regulation, from two-component signal transduction systems, to indole-signaling, and the role of the second messenger c-di-GMP, have led to novel targets and compound scaffolds for the development of anti-biofilm agents. These new strategies, coupled with the development of antagonists of the more well studied quorum sensing pathways, have led to the development of a number of anti-biofilm molecules that have been explored for their potential to be developed into effective treatments for biofilm-mediated infections.<sup>20</sup>

As most pathogenic bacteria exist as biofilm communities, the discovery and development of agents with the ability to limit biofilm formation, or even eradicate established biofilms, will have the potential to enhance the efficacy of antibiotics that are ineffective against biofilm bacteria. Such therapeutic entities should have a profound impact on human medicine.<sup>2,7</sup> It is important to distinguish molecular scaffolds that have the ability to affect biofilm development *via* non-microbicidal mechanisms from their microbicidal counterparts, as the pressure on bacteria to evolve resistance to agents which are not microbicidal will be

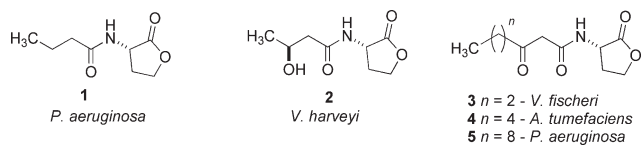
significantly reduced or even eliminated as compared to the selective pressures exerted by conventional bactericidal entities. Therefore, this review seeks to present compounds that have been shown to explicitly demonstrate anti-biofilm behavior in a non-bactericidal manner and cause phenotype shifts among bacteria commonly known to form biofilms. This is significant, as there exists the potential for co-dosing newly developed anti-biofilm compounds that possess the above-mentioned characteristics with conventional antibiotics to eliminate established biofilm infections. This review provides an overview of the numerous approaches that have been applied to the discovery of small molecules able to mediate the formation and maintenance of bacterial biofilms. These approaches are grouped into: (1) the identification and development of small molecules that target one of the bacterial signaling pathways involved in biofilm regulation, (2) chemical library screening for compounds with anti-biofilm activity, and (3) the identification of natural products that possess anti-biofilm activity, and the chemical manipulation of these natural products to obtain analogues with increased activity.

## Compounds that modulate biofilms by targeting bacterial signaling pathways

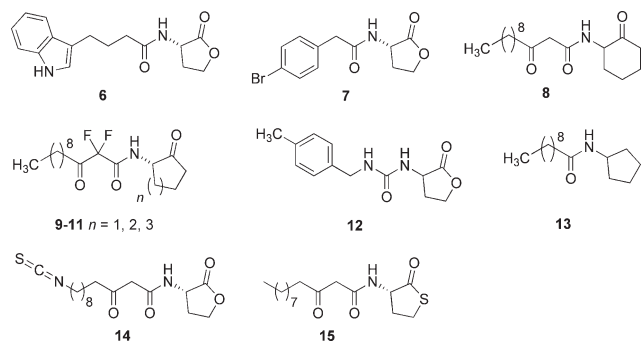
A significant amount of the current information we have regarding biofilm development is tied to research involving the phenomenon of bacterial quorum sensing (QS). QS is the term used to describe intercellular communication between bacteria that allows the community to make coordinated efforts at altering gene expression based upon population density.<sup>21</sup> It is known that QS pathways heavily influence the formation of biofilms, in addition to the production of other virulence factors. A diverse range of biomolecules serve as the facilitators for QS systems in bacteria. Therefore, extensive research in this area has produced a number of analogues with the ability to modulate QS-dependent enzymes. These molecules compose the vast majority of compounds thus far investigated for biofilm control. The understanding of complex QS-based communication systems in Gram-negative organisms is based upon the study of a variety of bacteria, including the bioluminescent marine bacterium *Vibrio fischeri* and the human pathogens *Vibrio cholerae* and *Pseudomonas aeruginosa*.<sup>22–24</sup> At its most basic level, QS in Gram-negative bacteria is effected by a set of two proteins. One protein is responsible for the production of a signaling molecule, which is typically referred to as an autoinducer (AI), while the other protein responds to the autoinducer. There are several classes of autoinducers, based upon on shared molecular features, these include acyl homoserine lactones (AHLs), autoinducing peptides (AIPs) and autoinducer-2 (AI-2).<sup>25</sup>

### Acyl homoserine lactones (AHLs)

Gram-negative bacteria predominantly employ *N*-acyl homoserine lactones (AHLs) as autoinducers, and more than 70 species of bacteria communicate *via* AHL-mediated QS with specificity mediated *via* variation in the length and oxidation state of the acyl side chain (compounds 1–5) (Fig. 1).<sup>25</sup> Native AHLs found in bacteria are *L*-isomers, and the *D*-isomers are



**Fig. 1** AHLs utilized by Gram-negative bacteria for quorum sensing.



**Fig. 2** Derivatives of the AHL scaffold that possess biological activity.

known to be essentially devoid of biological activity. In *V. fischeri*, AHL synthesis occurs when the *luxI* gene is activated to produce the AHL synthase enzyme LuxI. When the newly produced AHL reaches a threshold intracellular concentration, binding to the transcriptional activator LuxR takes place, leading to activation of the *luxR* gene set. AHLs are able to freely diffuse in and out of bacterial cells, allowing the total AHL concentration to correlate to the total bacterial concentration, thus enabling population density-based control of gene expression. This cascade of events ultimately leads to the control of gene expression resulting in the control of virulence factor production and biofilm formation and maintenance.<sup>26</sup> Thorough investigations of other Gram-negative QS pathways, most notably those belonging to *P. aeruginosa*, have shown that homologous systems to the LuxI and LuxR proteins (LasI and LasR) exist for the purpose of AHL synthesis and response.<sup>24</sup> The most important AHLs responsible for QS in *P. aeruginosa* are *N*-butanoyl-homoserine lactone **1** (C4-AHL, for the *rhl* system) and 3-oxo-C12-AHL **5** (for the *las* system).

AHLs have served as one of the primary scaffolds studied over the past thirty years for the design of potential biofilm inhibitors.<sup>27,28</sup> A considerable amount of work has been published involving the biological consequences of chemically modified AHL derivatives in a variety of QS systems. Work from the Blackwell group has documented the synthesis and identification of a number of natural and unnatural AHLs with the ability to modulate QS in *P. aeruginosa* and *Agrobacterium tumefaciens*.<sup>29</sup> They also demonstrated that two of their most active synthetic AHLs could retard biofilm formation in *P. aeruginosa* PA01. Qualitative experiments were performed, in which an engineered *P. aeruginosa* strain capable of producing green fluorescent protein (GFP) for visualization was grown in the presence of synthetic ligands **6** and **7** (Fig. 2) at 50  $\mu$ M. Significant inhibition of biofilm formation was observed for both derivatives. More recently, the Blackwell group has synthesized and presented the QS antagonist/agonist properties of diverse

synthetic AHL-based libraries that were screened against *P. aeruginosa*, *A. tumefaciens*, and *V. fischeri*.<sup>30</sup> Other research that includes the modification of AHLs to discern their effects on quorum sensing and biofilm formation in *P. aeruginosa* includes that of Suga and Spring. The work by Suga *et al.* entailed the synthesis of a 96-member library constructed through solid phase protocols to mimic AHLs by replacing the homoserine lactone moiety with a variety of functionalities.<sup>31,32</sup> A noteworthy compound identified within this study was AHL derivative **8**, which had no effect on biofilm growth, yet elicited a noticeable change in the biofilm morphology of *P. aeruginosa* PA01. Spring *et al.*'s research investigated analogues of *P. aeruginosa* AHLs in which the lactone functionality was replaced by a ketone, with additional difluorination between the  $\beta$ -keto amide positions (**9–11**).<sup>33</sup> Other analogues have been reported involving ureas (**12**),<sup>34</sup> thiolactones,<sup>35</sup> sulfonamides,<sup>36</sup> and *N*-acylated cyclopentylamides<sup>37</sup> as viable modifications of AHLs to drive QS inhibition and thereby affect biofilm formation. One specific *N*-acyl cyclopentylamide, designated as C10-CPA (**13**), was shown to completely prevent biofilm formation by *P. aeruginosa* PA01:GFP at a concentration of 250  $\mu$ M after seven days under flow conditions. These signaling molecule derivatives are particularly important because the biological activity of nearly every compound in this class is not driven by microbicidal properties.

The determination of the crystal structure of the *P. aeruginosa* transcriptional regulator LasR bound to its natural ligand (3-oxo-C12-AHL **5**) by Bottomley *et al.* gives new insight into the binding features required for the design of potent inhibitors of LasR.<sup>25,38</sup> This information was exploited in the design of covalent inhibitors of LasR in *P. aeruginosa* that were able to reduce biofilm formation. A series of inhibitors bearing electrophilic functional groups including isothiocyanates, bromoacetamides, and chloroacetamides, were designed to allow reaction with a nucleophilic cysteine residue present in the LasR binding pocket (Cys79). Isothiocyanate containing ligands were able to covalently and selectively bind Cys79 and using reporter strains were shown to inhibit quorum sensing. The lead compound, **14** (Fig. 2), was also shown to inhibit biofilm formation by wild type PA01 by almost 50% at a concentration of 50  $\mu$ M.<sup>25</sup>

Despite the potential that these molecules may possess toward potentially unlocking the ability to modulate bacterial communication on demand, there exists differing opinions on how relevant AHL treatment is to the treatment of biofilms. On one hand, modulating AHL-driven processes has great promise as bacterial strains of *P. aeruginosa* deficient in AHL production grown as planktonic or biofilm populations have been found to be similarly susceptible to biocides as wild-type populations.<sup>39</sup> However, homoserine lactones themselves are notoriously prone to hydrolysis at physiological pH and the ring-opened product is QS inactive, and they have also been reported to possess immunomodulatory activity.<sup>40</sup>

Seeking to address the problem of lactone hydrolysis inherent to native AHLs, the potential of thiolactone AHL analogues as QS agonists and antagonists in Gram-negative bacteria has been investigated.<sup>41</sup> A focused library of thiolactone analogues was examined against LuxR-type QS receptors in a number of bacteria: LasR (*P. aeruginosa*), LuxR (*V. fischeri*), and TraR (*A. tumefaciens*). Several highly active LuxR inhibitors were



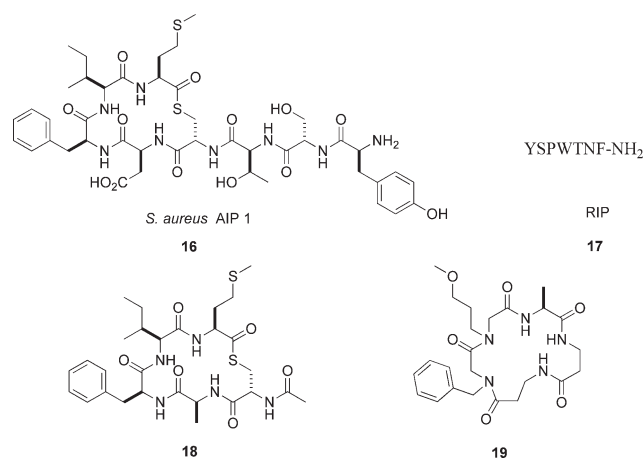


Fig. 3 Peptide derived molecules with activity against *S. aureus*.

identified and the half-life of a representative active analogue **15** (Fig. 2), was shown to be almost double that of its parent AHL in bacterial growth medium. These analogues have not yet been tested for their activity as inhibitors of biofilm formation; however, the thiolactone is a promising scaffold for the development of stable modulators of QS controlled biofilm formation.

#### Autoinducing peptides (AIPs)

QS in Gram-positive bacteria is predominantly mediated by autoinducing peptides (AIPs) (Fig. 3),<sup>42</sup> though it has been shown that Gram-positive bacteria may not exclusively utilize peptide-signaling molecules for communication and small molecules known as  $\gamma$ -butyrolactones have been identified as signaling molecules in some species of *Streptomyces*.<sup>43</sup> Many AIPs possess hydrophobic domains, which have been shown to be crucial for activity. These motifs are postulated to play an important role in the promotion of hydrophobic interactions that influence receptor activation.<sup>44</sup> The *agr* and TRAP QS systems in *S. aureus* regulate a number of virulence phenotypes, including biofilm formation. The accessory gene regulator (*agr*) operon contains the *agrD* gene, which encodes AgrD, the precursor of the *S. aureus* AIP **16**. Upon reaching threshold levels, AIP will bind AgrC leading to the expression of a small non-coding RNA known as RNA-III, which subsequently down-regulates genes that encode adhesins required for biofilm formation.<sup>45</sup> The RNA-III activating protein (RAP) activates TRAP (target of RNA-III activating peptide) *via* phosphorylation, leading to increased cell adhesion and biofilm formation, in addition to inducing expression of the *agr* operon.<sup>46</sup>

It has been demonstrated that the RNA-III inhibiting peptide (RIP) **17** inhibits phosphorylation of TRAP, leading to reduced biofilm formation. RIP has been studied in multiple animal models and has been shown to possess *in vivo* activity in preventing many types of infections including those caused by antibiotic resistant strains.<sup>47</sup> More importantly, no toxicity has been observed and no RIP-resistant strains have yet been reported.<sup>48</sup> Additional studies utilizing peptides and peptidomimetics as possible tools to probe quorum sensing have identified analogues of *S. aureus* AIP-1 that can also influence QS pathways in *S. aureus*. Truncated peptides such as **18** have been found to be

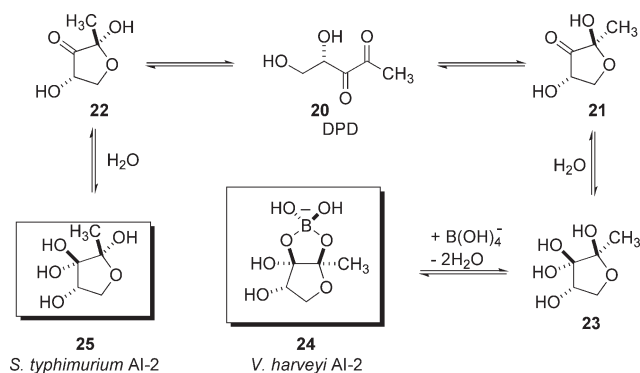


Fig. 4 Role of DPD **20** in the synthesis of AI-2 molecules isolated from *V. harveyi* and *S. typhimurium*.

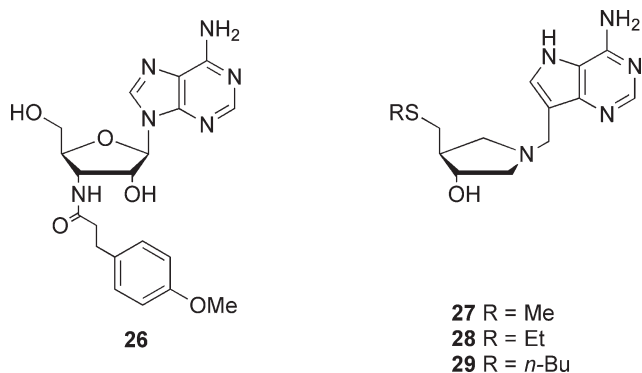
potent AgrC-I QS antagonists,<sup>49</sup> and the peptidomimetic **19** has been shown to promote biofilm formation in *S. aureus*.<sup>50</sup>

#### Autoinducer-2 (AI-2)

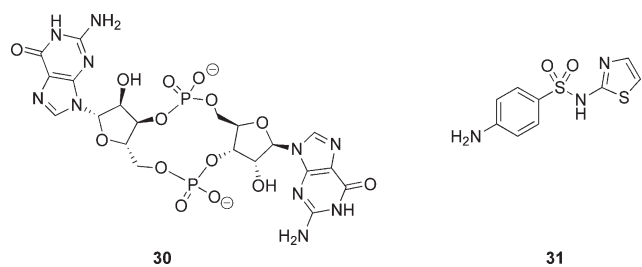
One of the putative universal quorum sensing mechanisms shared by both Gram-negative and Gram-positive bacteria involves the production of a small molecule termed autoinducer-2 (AI-2). All AI-2 molecules are derived from the precursor molecule (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) **20**, and the synthase enzyme that drives DPD production has been found to be conserved in over 55 bacterial species.<sup>51</sup> This finding has led many researchers to postulate that the AI-2 system is in fact the universal language of bacterial communication.<sup>52</sup> Two AI-2 molecules that have been isolated and characterized are shown in Fig. 4, one from *Vibrio harveyi* **24** and another from *Salmonella typhimurium* **25**. In the case of AI-2 isolated from *V. harveyi*, the DPD derivative exists as a borate diester **24**, a result of the high concentrations of boron in the natural marine water habitat of this bacterium.<sup>53</sup> DPD **20** is a highly reactive intermediate in the Activated Methyl Cycle (AMC), which recycles *S*-adenosyl-L-methionine (SAM), the primary methyl donor in bacterial and eukaryotic cells. SAM also plays a role as an intermediate in the pathway responsible for the synthesis of AHLs. LuxI synthase is known to catalyze the reaction of SAM with acylated acyl carrier proteins delivering AHLs.<sup>53</sup>

Recently Janda and co-workers as well as Sintim and co-workers have been able to synthesize and assay a small number of DPD analogues to delineate their effects on QS in *V. harveyi* and *S. typhimurium*.<sup>54–56</sup> As the biosynthesis of DPD begins from SAM, the screening of a small library of nucleoside analogues for their ability to disturb AI-2-based QS in *V. harveyi* has also been investigated.<sup>57</sup> The adenosine analogue **26** (Fig. 5) was found to block AI-2-based QS without interfering with bacterial growth. This compound was subsequently shown to affect biofilm formation in *Vibrio anguillarum*, *Vibrio vulnificus* and *V. cholerae*.<sup>57</sup>

The 5'-methylthioadenosine nucleosidase (MTAN) enzymes are directly involved in the biosynthesis of AI-2 and AHLs, catalyzing the *N*-glycosyl hydrolysis of SAM and other adenosyl derivatives.<sup>58,59</sup> Transition state analogues **27**, **28**, and **29** (Fig. 5) designed against MTANs were shown to inhibit MTAN



**Fig. 5** Nucleoside analogues that interfere with AI-2 based QS pathways.



**Fig. 6** c-di-GMP **30** and sulfathiazole **31** that inhibits DGC activity.

activity with nanomolar  $IC_{50}$  values in cell lysates of a virulent *V. cholerae* strain. These transition state analogues were also shown to inhibit QS induction in *V. harveyi* reporter strains. Analogues **27** and **29** also inhibited MTAN in *Escherichia coli* and resulted in nontoxic inhibition of AI-2 production. Compound **29** also reduced biofilm formation in *E. coli* and *V. cholerae* by 18% and 71% respectively at a concentration of 1  $\mu$ M without inhibiting planktonic growth.<sup>59</sup>

### c-di-GMP

Bis-(3'5')-cyclic di-guanylic acid (c-di-GMP) **30** (Fig. 6) is a second messenger signaling molecule that is thought to be ubiquitous in bacteria. Diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) are responsible for the synthesis and breakdown of c-di-GMP, respectively.<sup>60</sup> DGCs contain a conserved GGDEF (Gly–Gly–Asp–Glu–Phe) domain which has been observed in virtually all sequenced bacterial genomes<sup>61</sup> and PDEs contain a conserved EAL domain (enriched in Glu–Ala–Leu).<sup>60</sup> These proteins are highly regulated by various environmental and intracellular signals such as oxygen, light, and small molecules. The GGDEF domain-containing proteins effect changes in c-di-GMP levels in response to environmental and intracellular stimuli. Second messengers such as c-di-GMP can amplify the original signal into an intracellular signal capable of bringing about considerable biochemical changes within the cell.<sup>62</sup> This is accomplished by the binding of c-di-GMP to downstream proteins, which results in conformational changes that lead to either positive or negative regulation of cellular functions. There is increasing evidence that the transition between the planktonic and biofilm lifestyle in *P. aeruginosa* is regulated

via proteins with DGC or PDE activities through control of c-di-GMP levels.<sup>62</sup> It has also been observed that exopolysaccharide synthesis (and thus the exopolysaccharide-dependent formation of biofilms) is regulated by c-di-GMP in various proteobacterial species such as *V. cholera*, *P. aeruginosa*, *Pseudomonas fluorescens*, *A. tumefaciens*, *E. coli*, and *Salmonella enterica*.<sup>61</sup>

Dispersion of bacteria from a mature biofilm is another process that may be potentially regulated by c-di-GMP. A *P. aeruginosa* mutant that demonstrated elevated c-di-GMP levels exhibited defective detachment in response to environmental signals that typically elicit biofilm dispersion, such as changes in nutrient availability. It is postulated that c-di-GMP is maintained at a level high enough to promote the initial stages of biofilm formation and maturation (which require motility), but levels are then decreased to allow for full maturation of the biofilm.<sup>62</sup> As QS and c-di-GMP signaling pathways likely regulate some of the same complex processes in bacteria, (e.g. biofilm formation), it is possible that the two signaling pathways are linked. A direct connection has not yet been reported though evidence for an indirect connection has been presented.<sup>63</sup> For example, in *V. cholerae*, the QS-regulated transcription factor AphA influences the expression of genes encoding DGC and PDE. Additionally, during the QS-mediated formation of a symbiotic biofilm between the hyperthermophiles *Thermotoga maritima* and *Methanococcus jannaschii*, genes encoding DGC and PDE were upregulated and down-regulated respectively.<sup>63</sup> One of the best-studied DGC is PleD, a signaling protein from the aquatic bacterium *Caulobacter crescentus*. Analysis of the crystal structure of the complex of PleD with c-di-GMP suggests that two PleD monomers dimerize and catalyze c-di-GMP synthesis from two molecules of GTP. The DGC activity of PleD is regulated by phosphorylation, which is thought to promote dimerization.<sup>63</sup>

Interfering with c-di-GMP signaling is an attractive target for control of biofilm formation, be it with c-di-GMP analogues, or with small molecules that interfere with the synthesis or degradation of c-di-GMP.<sup>64</sup> The addition of exogenous c-di-GMP itself has been shown to inhibit biofilm formation in some bacterial species. *S. aureus* biofilm formation *in vitro* and adherence to HeLa cells was shown to be inhibited by c-di-GMP, while cyclic GMP (cGMP) and cyclic AMP (cAMP) had a much lower inhibitory effect on biofilm formation.<sup>65</sup> Studies involving the dose-dependent effects of c-di-GMP on biofilm formation on abiotic surfaces in *Streptococcus mutans* revealed that biofilm formation could effectively be reduced in a dose dependent manner while simultaneously reducing adherence to tooth surfaces.<sup>66</sup> In *P. aeruginosa* PA14, elevated intracellular c-di-GMP levels are known to be associated with increased biofilm formation and decreased swarming motility. Such behaviors can be influenced by the addition of exogenous amino acids that affect c-di-GMP levels. It has been shown that the addition of exogenous arginine results in an increase in c-di-GMP levels and concurrent stimulation of biofilm formation.<sup>67</sup>

Screening of a 1120 compound library with known biological activities against DGC identified sulfathiazole **31** (Fig. 6), as a hit, which inhibited DGC activity. Follow up biofilm inhibition assays confirmed that **31** was able to inhibit *E. coli* biofilm formation ( $IC_{50}$  = 5.8  $\mu$ M) while not significantly inhibiting bacterial growth at concentrations up to 50-fold higher than those needed to inhibit biofilm formation.<sup>68</sup>

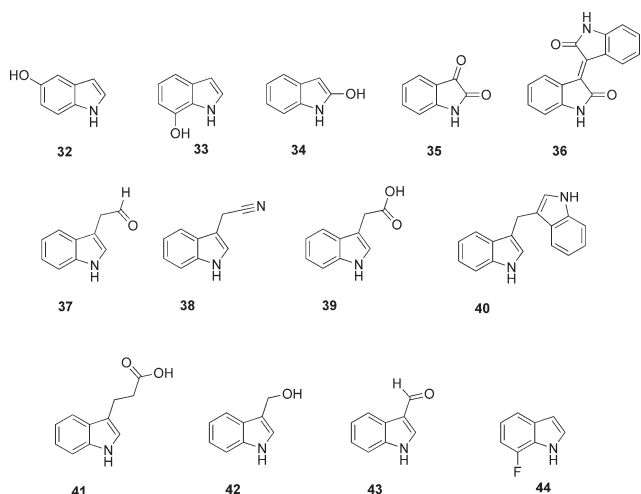


Fig. 7 Indole derivatives that affect biofilm formation.

### Indole signaling

Indole is another putative universal intercellular signal molecule amongst diverse bacteria<sup>69</sup> that plays a direct role in the control of many bacterial behaviors in a QS-dependent fashion.<sup>70</sup> Such behaviors include: antibiotic resistance,<sup>71</sup> virulence,<sup>72</sup> and biofilm formation.<sup>73</sup> Eighty-five species of bacteria have been documented to produce indole, with both indole-positive and indole-negative strains of bacteria altering various behaviors upon the extracellular presence of indole.<sup>69</sup> High concentrations (>600  $\mu\text{M}$ ) of extracellular indole are produced by *E. coli* when cultured in rich medium and indole has been shown to decrease biofilm formation in *E. coli* in a non-toxic manner.<sup>74</sup> The regulation of biofilm formation by indole in *E. coli* has been demonstrated to be temperature dependant, with a greater effect observed at lower temperatures (25  $^{\circ}\text{C}$  or 30  $^{\circ}\text{C}$  compared to 37  $^{\circ}\text{C}$ ) and is mediated by the transcriptional regulator SdiA,<sup>75</sup> which is a LuxR homologue.<sup>76</sup> The addition of exogenous indole to an *E. coli* strain unable to produce indole (lacking the tryptophanase (*tnaA*) gene) significantly affected the expression of 186 genes (more than two-fold) in biofilm cells at 30  $^{\circ}\text{C}$ , including the biofilm stress regulator *bhsA*, and *bssR* (*yliH*), which encodes a regulator of biofilm formation.<sup>75</sup> Conversely, the presence of indole increases biofilm formation by *P. aeruginosa* and *P. fluorescens* despite the fact that these pseudomonads do not themselves produce indole.<sup>74</sup>

Indole is readily converted by oxygenases found in several bacterial species to a number of oxidized indole derivatives, such as hydroxyindoles 32–34, isatin 35, and isoindigo 36, (Fig. 7). It was therefore hypothesized that these oxidized indole metabolites may also play a role in biofilm formation.<sup>70</sup> Screening of these compounds for the ability to inhibit biofilm formation by a strain of enterohemorrhagic *E. coli* (EHEC) revealed that 5-hydroxyindole 32 and 7-hydroxyindole 33 were able to inhibit biofilm formation by 11-fold and 27-fold respectively at a concentration of 1 mM. This is compared to 18-fold inhibition for indole itself at the same concentration.<sup>74</sup> 2-Hydroxyindole 34 at the same concentration had no effect on biofilm formation, while isoindigo 36 (at a concentration of 250  $\mu\text{M}$ , which was the limit of its solubility) also had no effect. Isatin 35 increased

biofilm formation by this strain of EHEC in a dose-dependant manner, with a four-fold increase effected at a concentration of 250  $\mu\text{M}$ . None of the active indole derivatives significantly affected growth of EHEC, indicating that biofilm modulation is brought about *via* a non-toxic mode of action. Against PA01, 7-hydroxyindole 33 (500  $\mu\text{M}$ ) brought about a two-fold increase in biofilm formation while 5-hydroxyindole 32, isatin 35 and indole itself increased biofilm formation by 20–30% at the same concentration.<sup>74</sup>

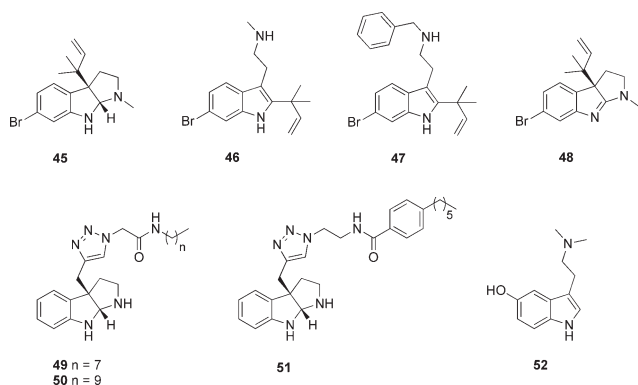
Another indole derivative, indole-3-acetaldehyde, 37, produced by the plant pathogen *Rhodococcus* sp. BFI 332 inhibited biofilm formation by enterohemorrhagic *E. coli* O157:H7 without affecting planktonic growth.<sup>77</sup> Compound 37 was shown to suppress expression of two curli operons, *csgBAC* and *csgDEFG*, and induce the expression of tryptophanase in *E. coli* O157:H7 biofilm cells. The spent medium of *Rhodococcus* sp. BFI 332, from which this compound was identified, was also shown to have an inhibitory effect on biofilm formation by two Staphylococcal species, *S. aureus* and *Staphylococcus epidermidis*.<sup>77</sup>

As many pathogenic bacteria rapidly degrade indole into some of its various metabolites depicted above, the ability of indole-derived plant secondary metabolites to modulate bacterial biofilm formation has also been investigated.<sup>78</sup> 3-Indolylacetonitrile (IAN) 38, indole 3-acetic acid (IAA) 39, 3,3'-methylene bisindole (MI) 40, indole-3-propionic acid (I3PA) 41, indole-3-carbinol (I3C) 42, and indole-3-carboxyaldehyde (I3CA) 43 (Fig. 7) were screened at 100  $\mu\text{g mL}^{-1}$  for their effects on biofilm formation by *E. coli* O157:H7 and PA01. The most active derivatives for suppression of biofilm formation by this *E. coli* strain were 38 and 43, which reduced biofilm formation by 11-fold and 24-fold respectively, compared to a three-fold reduction brought about by indole.<sup>78</sup> In contrast to indole and the hydroxyindoles, 38 and 43 also weakly inhibited biofilm formation by PA01, effecting a 1.9-fold and 2.3-fold reduction in biofilm formation, respectively. IAN 38 was not degraded by either of these two bacterial strains, and did not affect planktonic growth of the bacteria. It was also shown that IAN increased indole production, providing a partial explanation for its ability to reduce biofilm formation.<sup>78</sup>

In a follow up study to identify indole derivatives with increased anti-virulence activity compared to IAN 38, 31 natural and synthetic indole derivatives were tested for the ability to inhibit biofilm formation and hemolytic activity of *P. aeruginosa*.<sup>79</sup> The synthetic indole derivative, 7-fluoroindole (7FI) 44, was identified as the most active compound, and at a concentration of 1 mM reduced biofilm formation by four-fold and *P. aeruginosa* hemolytic activity by 14-fold. It was shown that 44 suppressed swarming motility, protease activity, and extracellular polymeric matrix production in *P. aeruginosa*. In addition, 44 reduced the production of QS-regulated virulence factors including 2-heptyl-3-hydroxy-4(1*H*)-quinolone, pyocyanin, rhamnolipid, and the two siderophores pyoverdine and pyochelin.<sup>79</sup>

The flustramines are a collection of monobrominated secondary metabolites isolated from the bryozoan *Flustra foliacea* that contain a pyrroloindoline or indolic core (Fig. 8).<sup>80</sup> Several of these compounds exhibit a host of biological activities, including antibacterial activity against several species of marine bacteria associated with the bryozoan, though they display no





**Fig. 8** Flustramine derivatives and related compounds that inhibit biofilm formation.

antibacterial activity against terrestrial bacterial strains.<sup>80</sup> Dihydroflustramine C **45**, also inhibited the production of reporter genes in an AHL-based QS reporter assay at concentrations below that at which it affected bacterial growth. It was therefore hypothesized that this class of natural products has the potential to act as a scaffold for the design of quorum sensing or indole signalling antagonists, which may have the ability to modulate biofilm formation.<sup>80</sup>

To investigate the indolic flustramine scaffold as a potential modulator of biofilm formation, desformylflustrabromine (dFBr) **46** was tested for the ability to modulate *S. aureus* and *E. coli* biofilm formation. dFBr was able to inhibit biofilm formation by both strains, exhibiting  $IC_{50}$  values of 70  $\mu$ M and 174  $\mu$ M for *E. coli* and *S. aureus*, respectively. However, this compound demonstrated microbicidal effects on planktonic growth at these concentrations.<sup>81</sup> Synthetic manipulation of four regions of the dFBr scaffold was performed with the aim of tuning the flustramine derivatives to modulate biofilm formation through a non-microbicidal mechanism. The most active compound identified, **47**, inhibited biofilm formation by *S. aureus* and *E. coli* with  $IC_{50}$  values of 5.9  $\mu$ M and 53  $\mu$ M and was successful in eliciting its effects through a non-microbicidal mechanism.<sup>81</sup> Mechanistic studies with **47** and indole itself in wild-type and knockout *E. coli* strains have shown that the activity of **47**, like that of indole, is dependent on temperature, the transcriptional regulator SdiA, and tryptophanase; suggesting that the anti-biofilm activity of **47** may be occurring through modulation of indole-based signaling pathways.<sup>81</sup>

The potential of the flustramine pyrroloindole scaffold to serve as a template for the design of compounds with anti-biofilm activity was assessed by synthesizing flustramine C, **48** and evaluating its activity against three diverse terrestrial bacterial pathogens: *Acinetobacter baumannii*, *E. coli*, and methicillin resistant *S. aureus* (MRSA). Flustramine C exhibited moderate anti-biofilm activity against the two Gram-negative strains studied, inhibiting biofilm formation by 20–30% at a concentration of 150  $\mu$ M, but did not display any anti-biofilm activity (<5% inhibition) against MRSA at the same concentration.<sup>82</sup> A library of flustramine C analogues was constructed in order to delineate the structural components required for anti-biofilm activity and identify more potent inhibitors. The library was designed to preserve the amphipathic nature of the molecule,

with the reverse prenyl group of flustramine C replaced by a propargyl group from which structural diversity could be introduced through click chemistry with a series of azide amide moieties.<sup>82</sup> Compounds from this library exhibited considerably increased activity compared to flustramine C. Compound **49** inhibited biofilm formation by *A. baumannii* with an  $IC_{50}$  value of 193  $\mu$ M, while compound **50** inhibited biofilm formation by *E. coli* with an  $IC_{50}$  value of 36  $\mu$ M. Furthermore, inhibitors of biofilm formation by Gram-positive bacteria were also identified from this library, with the most active compound **51** able to inhibit MRSA biofilm formation with a low micromolar  $IC_{50}$  value (3.4  $\mu$ M). All three compounds were shown to be acting via a non-microbicidal mechanism.<sup>82</sup>

A number of structurally related brominated tryptamine derivatives have been isolated from the Mediterranean gorgonian *Paramuricea clavata* and assessed for their anti-biofouling activity against three marine biofilm bacteria: *Pseudoalteromonas* spp D41 and TC8 and *Paracoccus* sp. 4M6.<sup>83</sup> Bufotenine **52** exhibited significant anti-adhesion activity against one of the tested strains (D41) and did not display microbicidal activity towards *V. fischeri* in the Microtox assay.<sup>83</sup> This family of natural products may therefore provide a further indole-based scaffold that could be manipulated to obtain highly active inhibitors of biofilm formation by medically relevant bacteria.

## Two-component systems

Two component signal transduction systems (TCS) are a class of regulatory systems found mainly in prokaryotes, though some have been identified in a small number of eukaryotic organisms. These regulatory systems are the predominant signaling mechanism in bacteria and allow the organism to sense and respond to changes in their environment. TCS consist of a histidine kinase and a response regulator. In response to an extracellular signal, the histidine kinase undergoes autophosphorylation at a conserved histidine residue. The histidine kinase then transfers the phosphate group to a conserved aspartate residue on the response regulator, resulting in activation of an effector domain, which then leads to the response.<sup>84</sup> This typically involves the control of gene expression by DNA binding of the phosphorylated response regulator to an upstream regulatory region. Many histidine kinases can also act as phosphatases and dephosphorylate the phosphorylated response regulator, thus reversibly controlling gene expression.<sup>85</sup> TCS are activated by a variety of environmental factors including pH, nutrient level, redox state, osmotic pressure, quorum signals, and the presence of antibiotics. TCS regulate the expression of genes that control behaviors such as cell growth, virulence, biofilm formation and maintenance, quorum sensing and antibiotic resistance.<sup>85</sup> Examples of TCS involved in biofilm regulation include, AgrAC (*S. aureus*)<sup>85</sup> LytSR (*S. aureus*),<sup>86</sup> BfmRS (*A. baumannii*)<sup>87–89</sup> BfmRS (*P. aeruginosa*),<sup>90</sup> BfiSR<sup>91</sup> (*P. aeruginosa*) GacAS (*P. aeruginosa*)<sup>92</sup> and VicRK (*S. mutans*).<sup>93</sup>

The AgrAC TCS in *S. aureus* is part of the *agr* regulatory locus described earlier and controls virulence in response to AIPs. AIPs act upon the histidine kinase AgrC, leading to autophosphorylation and subsequent phosphorylation of AgrA. Once phosphorylated AgrA binds the DNA region between the RNA

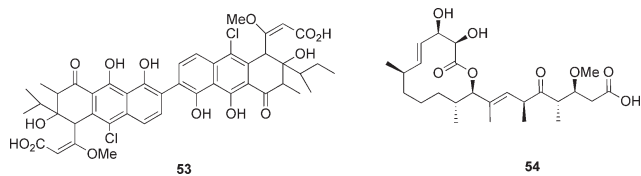


Fig. 9 TCS inhibitors walkmycin C **53** and carolacton **54**.

III and *agrB* genes, promoting transcription of RNA III.<sup>85</sup> The LytSR TCS plays an important role in *S. aureus* biofilm development as a result of its effect on the expression of the *lrgAB* operon. The *lrgAB* operon regulates cell death and lysis, along with the *cidABC* operon. The gene product of *cidA* is an effector of murein hydrolase activity and cell lysis, while *lrgA* encodes an inhibitor of these processes. One function of the *cid* and *lrg* operons is to mediate cell death and lysis during biofilm development, providing a source of extracellular genomic DNA (eDNA) for use as a component of the biofilm matrix and inhibition of cell lysis by the *lrg* gene product inhibits DNA release in the biofilm.<sup>86</sup> Disruption of *lytSR* results in altered murein (peptidoglycan) hydrolase activity in addition to spontaneous cell lysis.<sup>86</sup> Inactivation of *lytS* and *lrgAB* does not result in a murein hydrolase or lysis phenotype during planktonic growth, but produces a pronounced phenotype during biofilm growth. *LytS* mutation results in a thicker, more adherent biofilm with increased levels of eDNA in the biofilm matrix.

The BfmRS TCS in *A. baumannii* has been shown to play an important role in biofilm formation. Inactivation of the response regulator of this TCS, BfmR, in the *A. baumannii* strain ATCC 19606 results in a decreased ability of this strain to form a biofilm on abiotic surfaces, and also results in a significant change in cell morphology. BfmR is required for the expression of the Csu pili chaperone–usher assembly system and the biofilm proficiency of this strain of *A. baumannii* is dependent upon this system, which plays a role in the initial steps of biofilm formation on abiotic surfaces by enabling adherence of the bacterial cells and formation of microcolonies. Inactivation of the sensor component of this TCS, BfmS, causes only a modest reduction in biofilm forming ability, suggesting that cross-talk between other sensors and BfmR may be important.<sup>87,88</sup> It has been shown however, that mutation of *bfmS* results in a large reduction in motility (80% reduction compared to wild type).<sup>89</sup>

The role of TCS in the regulation of biofilm formation and maintenance makes them attractive potential targets for the development of anti-biofilm compounds, which have been underexploited to date. It has been shown that both the histidine kinase and response regulator of TCS are targetable by small molecules.<sup>94</sup> Walkmycin C **53** (Fig. 9) is a known histidine kinase inhibitor that was isolated from a screen for inhibitors of the WalK histidine kinase in *Bacillus subtilis*, and has previously been shown to inhibit autophosphorylation of WalK in both *B. subtilis* and *S. aureus*.<sup>95</sup> Walkmycin C targets the conserved catalytic domain of WalK and was therefore posited to potentially possess inhibitory activity against multiple histidine kinases. One such example is the VicRK TCS in *S. mutans*, an orthologue of WalRK that is involved in sucrose dependant biofilm formation. Walkmycin C was shown to inhibit the *in vitro* autophosphorylation activity of purified VicK with an IC<sub>50</sub> of

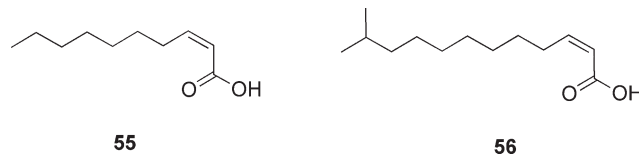


Fig. 10 Fatty acid signaling molecules that affect biofilm formation.

2.87  $\mu\text{M}$  (2.53  $\mu\text{g mL}^{-1}$ ).<sup>95</sup> Addition of walkmycin C at levels below the minimum inhibitory concentration (MIC), resulted in the formation of abnormal biofilms and a reduction in biofilm mass (37.6% of the control at 0.63  $\mu\text{g mL}^{-1}$ ), demonstrating the potential of targeting TCS with small molecules as an anti-biofilm strategy.<sup>95</sup>

The bacterial secondary metabolite carolacton **54**, affects the viability of *S. mutans* biofilms at nanomolar concentrations.<sup>93,96</sup> This compound had only minor effects on the growth of planktonic bacteria but killed bacterial cells within a biofilm state. Carolacton was subsequently shown to affect the expression of six different TCS in *S. mutans* biofilm cells: CiaRH, VicKRX, RelRS, SMU.1037c/1038c, SMU.659/660, and ComDE.<sup>93</sup> Microarray analysis identified VicKRX and ComDE as playing essential roles in the carolacton response, and known VicR controlled genes were down-regulated upon treatment with carolacton. It was shown that this response is mediated through the serine/threonine protein kinase PknB, which the authors posited could be due to phosphorylation of VicR by PknB.<sup>93</sup> Although the ability of carolacton to modulate *S. mutans* biofilms is not through a strictly non-microbicidal mechanism, the fact that this compound does not affect planktonic growth represents an alternative strategy for the small molecule control of biofilms and further validates targeting of TCS as a means of controlling bacterial biofilms.

### Other signaling molecules

Davies and Marques identified a fatty acid signaling molecule, *cis*-2-decenoic acid **55** (Fig. 10), produced by *P. aeruginosa* during growth, which was shown to inhibit biofilm development.<sup>97</sup> Another fatty acid, *cis*-11-methyl-2-dodecenoic acid **56**, known as diffusible signal factor (DSF), which was recovered from *Xanthomonas campestris*, is able to disaggregate cell flocs formed by *X. campestris*.<sup>98</sup> *cis*-2-Decenoic acid **55**, was also shown to induce the dispersion of established biofilms formed by a number of bacteria including *E. coli*, *Klebsiella pneumoniae*, *B. subtilis*, and *S. aureus* at low nanomolar concentrations, as evidenced by microcolony disaggregation and measurement of the number of cells released into the bulk biofilm culture medium.<sup>97</sup> The ability of *cis*-2-decenoic acid **55** to disperse preformed biofilms of *A. baumannii* and *S. aureus*, when investigated using a crystal violet reporter assay, which measures the biofilm mass remaining, was determined to be much lower and <10% dispersion was observed at a concentration of 400  $\mu\text{M}$ , while the compound exhibited IC<sub>50</sub> values of 121  $\mu\text{M}$  and 220  $\mu\text{M}$  against two *S. aureus* strains.<sup>99</sup>

Certain D-amino acids, which are produced by bacteria during stationary phase, are thought to be a native signal for biofilm disassembly in *B. subtilis* and were shown to inhibit biofilm



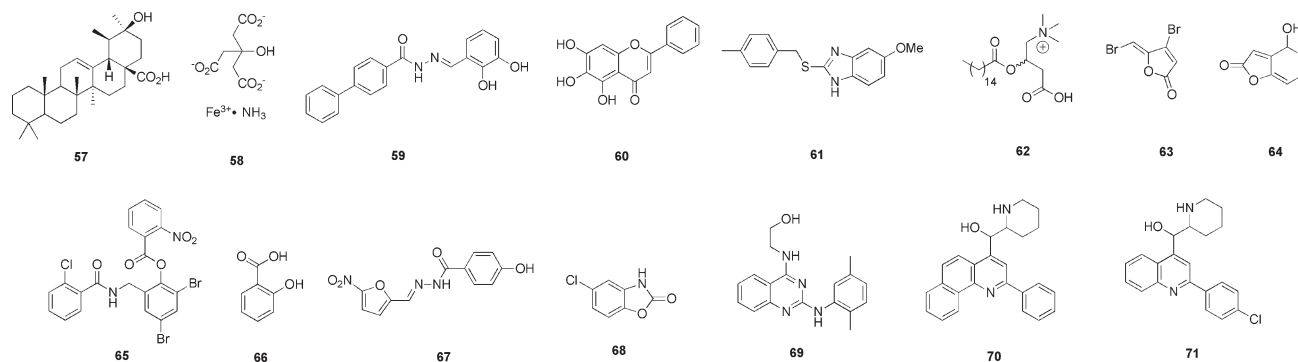


Fig. 11 Compounds with anti-biofilm activity identified through HTS.

formation in fresh cultures of *B. subtilis*, *S. aureus* and *P. aeruginosa*.<sup>100,101</sup> The inhibitory activity of D-amino acids is thought to be a consequence of disruption of the connection between an extracellular matrix protein and the bacterial cell leading to blocking of the growth of initial foci into larger assemblies of cells. Against *B. subtilis*, D-tyrosine, D-leucine, D-tryptophan, and D-methionine inhibited biofilm formation while the analogous L-amino acids, and other D-amino acids including D-alanine and D-phenylalanine were inactive. The individual amino acids showed inhibitory activity at concentrations from low micromolar (D-tyrosine) to millimolar (D-leucine); however, combination of the four amino acids resulted in markedly increased activity compared to any alone, inhibiting biofilm formation at a concentration of 10 nM.<sup>100</sup> Against *S. aureus*, the combination of D-phenylalanine, D-proline, and D-tyrosine exhibited greater biofilm inhibitory activity than the combination of D-tyrosine, D-leucine, D-tryptophan, and D-methionine.<sup>101</sup> D-amino acids did not affect planktonic bacterial growth.<sup>101</sup>

## Chemical library screening

Due to the scarcity of known molecular scaffolds that inhibit/disperse bacterial biofilms, high throughput screening (HTS) has been employed in attempts to discover leads for new anti-biofilm modulators (Fig. 11). One of the first reports detailing the screening of a large library of compounds with the objective of identifying novel small molecules that possessed anti-biofilm activity was disclosed in 2005 by the Wood group.<sup>102</sup> The screening of 13 000 compounds revealed a hit (0.08%), identified as ursolic acid **57**, which effectively inhibited *E. coli* biofilm formation at concentrations as low as 22  $\mu\text{M}$  without affecting growth. Microarray analysis of *E. coli* samples treated with compound **57** showed upregulation of genes responsible for chemotaxis, motility, and heat shock, all genes closely tied to biofilm formation.

In 2005, the Hergenrother group reported the identification of iron salts as effective non-antibiotic inhibitors and disruptors of *P. aeruginosa* biofilms from a screen of over 4500 compounds which belonged to the University of Illinois Marvel Library Compound Collection (MLCC).<sup>103</sup> Six compounds (0.13%) were found to inhibit biofilm development by at least 30% at a concentration of 50  $\mu\text{M}$  in the preliminary assays. Ferric ammonium citrate (FAC) **58** was found to be a non-toxic

inhibitor of *P. aeruginosa* PA14 biofilms, exhibiting an  $\text{IC}_{50}$  value of approximately 60  $\mu\text{M}$ . No toxicity was observed even when FAC concentrations were increased upwards of 500  $\mu\text{M}$ . Further investigation revealed that neither the ammonium or citrate ions were responsible for the observed anti-biofilm activity. The analysis of other iron salts (ferric chloride, ferric sulfate, ferrous sulfate) revealed biofilm inhibitory profiles that were comparable to that of FAC. They also demonstrated that by switching to an iron-rich growth media, established *P. aeruginosa* biofilms could be disrupted and cleared in continuous flow experiments. Additionally, analysis of various clinically isolated sputum samples from patients with cystic fibrosis showed that each genetically different strain exhibited varying degrees of susceptibility to elevated iron levels. Iron is known to be an important chemical in quorum sensing and bacterial virulence, high concentrations of iron are believed to suppress the expression of genes that encode for enzymes that scavenge excess iron, thus making the absence of these products detrimental to biofilm growth and maintenance.<sup>104</sup> This same group has also shown that various iron chelates can suppress biofilm formation in *P. aeruginosa*.<sup>105</sup>

Clardy and Junker reported a comprehensive HTS in 2007, in which they sought to identify novel small molecules that inhibited and dispersed PA01 biofilms.<sup>106</sup> A total of 66 095 unique molecules derived from libraries containing known bioactive compounds, natural products, and commercially available entities were screened using a luminescence-based assay. Of the compounds analyzed, 61 (0.09%) were shown to possess notable activities in bacterial anti-attachment assays and of these, 30 compounds were determined to exhibit  $\text{IC}_{50}$  values of less than 20  $\mu\text{M}$ . The screens also identified a number of molecules that elicited biofilm antibiotic-enhancing effects. The most active compound discovered, **59**, was shown to possess an  $\text{IC}_{50}$  value of 530 nM for biofilm inhibition. This makes **59** one of the most active biofilm modulators ever disclosed against either Gram-positive or Gram-negative bacteria. Compound **59** was also reported to exhibit an  $\text{EC}_{50}$  value for biofilm dispersion of 230  $\mu\text{M}$ . The active compounds and the general scaffold classes they represent do not have any known bacterial targets or mechanisms of action. The authors, however, state that the chemical nature of some of the compounds may lead them to be metal chelators. The chemical composition of the scaffolds identified from the screens included aromatic based hydrazines and hydrazides, quinolines, and aryl substituted pyrazoles.

Computer-aided drug design (CADD) techniques have also been employed in the search for new biofilm modulators. One such study involved the investigation of 51 active compounds derived from traditional Chinese medicines whose antibacterial properties had been previously documented.<sup>107</sup> By virtually determining if any of the target compounds exhibited similar docking characteristics in a 3-D environment to a known inhibitor of the *A. tumefaciens* quorum sensing transcriptional regulator protein TraR, the authors hypothesized that hits in the virtual screen would translate into compounds that would also display anti-biofilm activity against *P. aeruginosa*. Five compounds that performed well in the virtual screens were able to inhibit biofilm development and completely disperse *P. aeruginosa* biofilms at 200  $\mu\text{M}$ , however no  $\text{IC}_{50}$  values were determined. The most active compound, baicalein **60**, had no noticeable effect on bacterial growth. Baicalein is a flavonoid that is speculated to play a role in apoptosis and to also inhibit lipid peroxidation. The highlight of this article was the finding that ampicillin-resistant *P. aeruginosa* biofilms could be sensitized to the antibiotic in the presence of baicalein. No effects were observed when just the resistant cells were treated with only antibiotic or the anti-biofilm agent, yet when dosed together a significant synergistic effect was seen.

Screening of approximately 66 000 compounds and natural-product extracts from the Center for Chemical Genomics at the University of Michigan to identify compounds that affected induction of a *V. cholerae* c-di-GMP-inducible transcriptional fusion led to the discovery of a novel benzimidazole **61**.<sup>108</sup> This compound was examined for the ability to inhibit biofilm formation by a number of pathogenic bacterial strains. Compound **61** was shown to be a broad spectrum inhibitor of biofilm formation, significantly inhibiting biofilm formation by *P. aeruginosa* (CF-145), *K. pneumoniae*, *Erwinia amylovora*, and *Shigella boydii*, at 100  $\mu\text{M}$  and by MRSA USA300, and *S. aureus* Newman at 25  $\mu\text{M}$ , using the minimum biofilm eradication concentration (MBEC) static assay, without affecting bacterial growth.  $\text{IC}_{50}$  values, as calculated from the MBEC assay for inhibition of biofilm formation by *P. aeruginosa* and *V. cholerae* were determined to be 45.9 nM and 32.3 nM, respectively. The compound was, however, unable to disperse pre-formed biofilms.<sup>108</sup>

Examples of more focused libraries include the screening of 80 known eukaryotic protein kinase inhibitors for effects on biofilm formation.<sup>109</sup> These compounds were selected as they have the potential to interact with a broad range of targets due to their ATP-like scaffolds and the effects of such compounds had not yet been explored in microbial systems. Palmitoyl-DL-carnitine **62** was identified as an inhibitor of both PA01 and *E. coli* biofilm formation, and exhibited  $\text{IC}_{50}$  values of 13  $\mu\text{M}$  and 3.0  $\mu\text{M}$ , respectively.<sup>109</sup> This compound was also subsequently shown to inhibit biofilm formation by *Listeria monocytogenes* with an  $\text{IC}_{50}$  value of 5.9  $\mu\text{M}$ <sup>110</sup> yet was unable to disperse pre-formed biofilms. The compound was observed to inhibit expression of the *P. aeruginosa* LasR/LasI quorum sensing system, though it is thought that this system is not the main target for these compounds as evidenced by the fact that palmitoyl-DL-carnitine exhibited almost comparable biofilm inhibitory effects on *P. aeruginosa* MW1, a lasI/rhlI double mutant, which is unable to produce autoinducers. The exact mechanism of

action of palmitoyl-DL-carnitine therefore remains to be determined.<sup>109</sup>

A structure-based virtual screen (SB-VS) for the identification of putative quorum sensing inhibitors was carried out using a focused database comprising compounds that possess structural similarities to the known quorum-sensing inhibitors furanone C30 **63**, patulin **64**, the *P. aeruginosa* LasR natural ligand (3-oxo-C12-AHL **5**) and a known quorum-sensing receptor agonist TP-1, **65**.<sup>111</sup> This screen led to the discovery of three compounds, all recognized drugs: salicylic acid **66**, nifuroxazide **67**, and chlorzoxazone **68**, which were subsequently shown to significantly inhibit quorum sensing-regulated gene expression at concentrations at which they did not affect bacterial growth. In addition to affecting quorum sensing regulated virulence factor production, these compounds were shown to affect biofilm formation by PA01. Biofilms treated with these compounds appeared thinner and less structured than control biofilms, and total biofilm mass was reduced in the presence of these quorum sensing inhibitors.<sup>111</sup>

A HTS of an 8000-compound structurally diverse chemical library for small-molecule inhibitors of bacterial motility using *V. cholerae* identified several compounds that inhibited motility without exhibiting toxicity.<sup>112</sup> The lead compound, a quinazoline-2,4-diamino analogue **69**, completely suppressed motility in swarm agar plates without affecting the planktonic growth rate and was subsequently shown to inhibit biofilm formation by 1.6 and 1.9-fold at 18 h and 30 h respectively, at a concentration of 10  $\mu\text{g mL}^{-1}$ , using a crystal violet staining assay.<sup>112</sup>

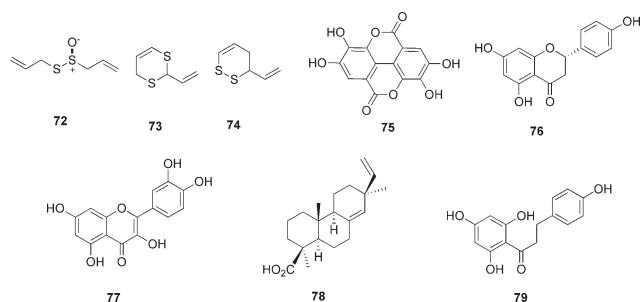
A high-throughput epifluorescence microscopy imaging based HTS has been developed for the identification of small molecule inhibitors of biofilm formation in *V. cholerae*.<sup>113</sup> A number of compounds that reduced biofilm formation without altering bacterial cell viability were identified, with the two lead compounds **70** and **71**, which are analogues of the antimalarial drug mefloquine, resulting 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. Mefloquine has known antibiotic activity against Gram-positive bacteria but has not been shown to have any antibiotic activity against Gram-negative strains.<sup>113</sup>

## Natural products and natural product analogues

Natural products provide a diverse array of chemical structures and possess a plethora of biological activities. A number of natural products that possess the ability to inhibit or disperse bacterial biofilms have been used as the starting points for medicinal chemistry programs in which synthetic manipulation of the natural product scaffold has allowed for the design of more efficacious compounds. Much of the natural product inspiration for these programs has come from compounds isolated from plants and marine organisms.

### Plant extracts

Interest in studying natural products derived from plant sources for the discovery of new biologically active compounds is not uncommon as many traditional medicines have been rooted



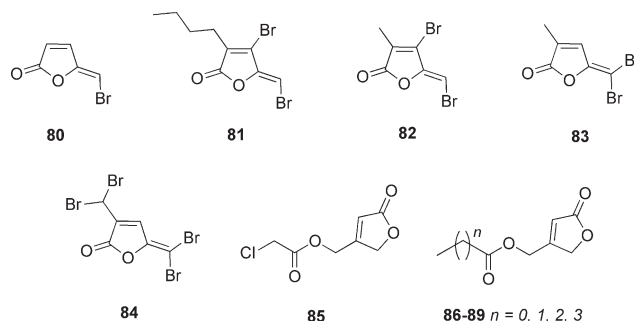
**Fig. 12** Molecules isolated from plant extracts and their derivatives that possess anti-biofilm activity.

heavily in the use of plant extracts for the treatment and cure of disease symptoms.<sup>114</sup> Garlic possesses antibiotic properties which have been traced to the compound allicin **72** (Fig. 12).<sup>115</sup> Renewed interest in garlic as a source for novel small molecules that modulate biofilms was generated with the finding that garlic extracts have shown the ability to clear pulmonary *P. aeruginosa* infections in mouse models.<sup>116</sup> This particular study also demonstrated that *P. aeruginosa* biofilms grown *in vitro* and treated with garlic extracts were more susceptible to treatment with the antibiotic tobramycin and to grazing by polymorphonuclear (PMN) leukocytes. Isolation and analysis of the compounds responsible for these activities led to the identification of a number of new compounds that possess antibiotic properties, while two particular dithianes, **73** and **74**, have shown the ability to modulate QS in LuxR systems without microbicidal activity.<sup>117</sup>

Extracts from *Rubus ulmifolius* Schott., Rosaceae (Elderberry), which are rich in the polyphenol ellagic acid **75** and glycosylated derivatives, have been shown to have an inhibitory effect on biofilm formation by a number of diverse *S. aureus* strains at concentrations well below those required to limit bacterial growth.<sup>7</sup> Ellagic acid alone was also shown to possess anti-biofilm properties.<sup>7</sup> Meanwhile, a number of flavonoids found in citrus species, including naringenin **76**, and quercetin **77**, which are antagonists of homoserine lactone and AI-2-mediated cell-cell signaling in *V. harveyi*, were able to inhibit biofilm formation by *V. harveyi* BB120 and *E. coli* O157:H7 in a dose-dependent manner.<sup>118</sup>

Another plant derived natural product that has recently been identified as a potential inhibitor of biofilm formation by oral pathogens is the resin acid 4-*epi*-pimaric acid **78**, which was isolated from *Aralia cachemirica* L. (Araliaceae). 4-*epi*-Pimaric inhibited biofilm formation by *S. mutans* on a saliva-coated surface at sub-MIC concentrations, and was shown by confocal microscopy to inhibit clumping and attachment.<sup>119</sup>

The antioxidant phloretin **79** (found in apples) has been shown to markedly reduce biofilm formation by enterohemorrhagic *E. coli* O157:H7 without affecting the growth of planktonic cells, and also without affecting commensal *E. coli* K-12 biofilms.<sup>120</sup> This is significant as the eradication of commensal bacteria by conventional antibiotics is a major problem and can result in increased susceptibility to infections. Therefore, antibacterial strategies that selectively eliminate pathogenic bacteria are much sought after. Phloretin repressed several genes including those encoding toxins, AI-2 importer genes, and curli genes in



**Fig. 13** Halogenated furanones that possess anti-biofilm activity.

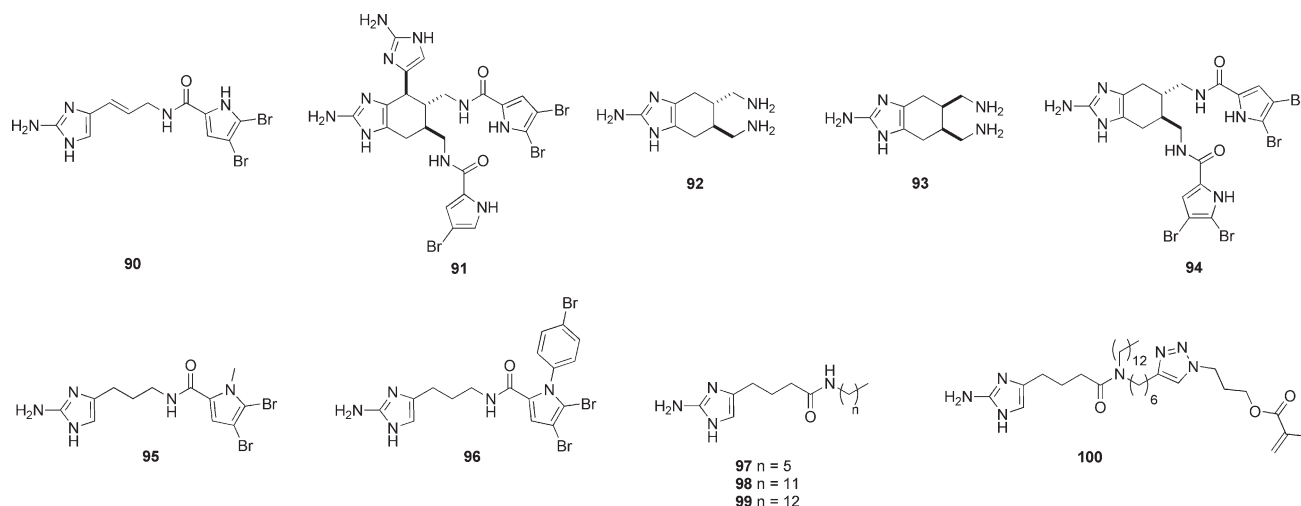
*E. coli* O157:H7 biofilm cells and it was also shown that phloretin resulted in reduced fimbria production, suggesting possible modes of action for this compound.<sup>120</sup>

### Halogenated furanones

Comprehensive research reports have detailed the biological activities and effects that halogenated furanones (HFs) have on bacterial QS.<sup>121–124</sup> These small molecules are natural products native to the marine red algae *Delisea pulchra* and primarily serve as a defense mechanism by acting as natural chemical deterrents (Fig. 13).<sup>125</sup> It has been proposed that these small molecules are an evolutionary response to the adverse effects of AHL driven colonization of the host by marine bacterial species.<sup>126</sup> Studies have shown that natural HFs isolated from the algae possess the ability to prevent biofilm formation and swarming in *E. coli* and *B. subtilis*.<sup>123,124</sup> These findings have led to an extensive effort to chemically modify the furanone scaffold in the hope of tuning biological activity. Structurally, the similarity between HFs and AHLs is apparent when considering how each possesses a non-polar aliphatic carbon “tail” attached to a relatively polar “head”. As seen in AHLs, the length of the carbon chain attached to the furanone ring is varied in addition to the presence of hydroxyl or acyl functionalities. The presence of bromine atoms in these small molecules is one of the most significant structural differences between AHLs and HFs.

The activity of HFs in bioassays designed to assess AHL inhibitory activity has been well documented and includes the ability to impede luminescence in *V. fischeri*, and to affect the expression of 93 genes in *P. aeruginosa* studied through DNA microarray analysis.<sup>127,128</sup> Most of the affected genes contribute to the production of virulence factors, notably biofilm formation. Giskov and co-workers have shown that specific furanones have the ability to affect these AHL-dependent activities by binding to LuxR protein homologues (the target receptors of AHLs) and promoting their degradation through proteolytic cleavage.<sup>129,130</sup> Furthermore, the concentration of furanone needed to elicit the biological effect is consistent with concentrations of furanones found in *D. pulchra*.<sup>131</sup> These observations have been thought to be a consequence of the strong binding affinity that comes from attack by nucleophilic residues in the binding pocket of the receptor to the electrophilic enone moiety of the HF, which results in structural changes that make the protein more prone to degradation.<sup>132</sup>





**Fig. 14** 2-Aminoimidazole alkaloids from sponges of the family *Agelasidae*: oroidin **90**, bromoageliferin **91** and structurally simplified analogues that retain anti-biofilm activity.

Studies involving the explicit use of HFAs as tools to modulate bacterial biofilm growth and maintenance have revealed that synthetic furanones possess the ability to penetrate *P. aeruginosa* biofilm matrices and interfere with bacterial QS without any associated microbicidal properties.<sup>121</sup> HF **80**, which lacks a side chain and possesses a vinyl bromide, has been reported to display such activity. When biofilms were grown in the presence of HF **80**, noticeable changes in biofilm maturation and structure were seen, similar to those observed in some *P. aeruginosa* mutants lacking the *lasI* system. While the furanone had no effect on initial bacterial attachment, bacterial detachment was more rapid resulting in a loss of more biomass from the treated sample in comparison to the untreated. Other experiments in which biofilms were grown in the presence or absence of **80** showed that there was no noticeable difference in biofilm morphology, yet the furanone-treated biofilm was sensitized to antibiotic treatment with tobramycin.<sup>86</sup>

The activity of furanones against QS pathways in Gram-positive bacteria is less known, however, the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone **81**, from *D. pulchra* has been shown to enhance biofilm formation by *S. epidermidis* and *S. aureus* at concentrations of 10 to 20% of the MIC.<sup>133</sup> This increase in biofilm formation correlated with an increase in polysaccharide intercellular adhesion (PIA) and was shown to be a result of interference with *luxS*.<sup>133</sup> Additionally, furanone **80** has also been shown to exhibit activity against a number of Gram-positive oral *Streptococcus* biofilms.<sup>134</sup> Furanone **80** at a concentration of 60  $\mu\text{M}$  resulted in a 63% and 76% reduction in biofilm growth in polystyrene wells of *S. mutans* and *Streptococcus intermedius* respectively.

The importance of the degree and pattern of bromination in furanones on the effects of biofilm formation in *E. coli* has been investigated.<sup>135</sup> Compounds **82**, **83**, and **84** were shown to reduce biofilm formation at concentrations of 224  $\mu\text{M}$  (**82** and **83**) and 141  $\mu\text{M}$  (**84**) by 75%, 63%, and 80%, respectively. In contrast, another set of five chemically synthesized furanones (**85–89**), which contained an exocyclic ester functionality attached to the furanone ring and were completely devoid of

bromination, were shown to be effective inhibitors of *P. aeruginosa* biofilm formation in flow cell confocal microscopy experiments.<sup>136</sup> Molecular modeling was then employed to estimate the binding energies of the synthetic furanones to the LasR receptor in comparison to the known *P. aeruginosa* AHL signaling molecule 3-oxo-C12-AHL **5**. Docking studies indicated that the furanone portion of the molecule overlapped with the lactone moiety of the AHL in the binding pocket, leading to docking scores that corresponded well with one another and provided a plausible explanation for the observed activity.

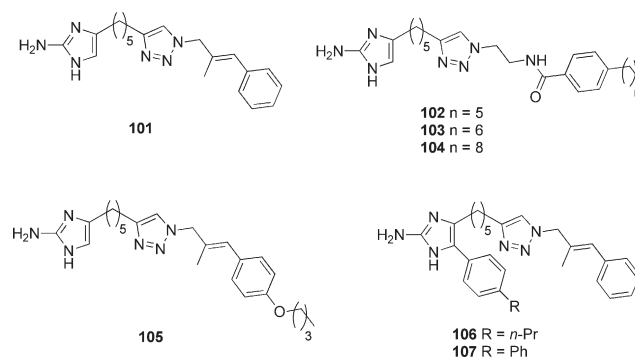
There are a number of issues that still need to be addressed to allow the use of halogenated furanones for human treatment therapies. These include toxicity, carcinogenic properties, and instability under aqueous conditions.<sup>137</sup> Future research should address these issues to help solidify this class of small molecules as important players in the development of new therapeutics to combat bacterial biofilms.

### Other marine natural products and analogues

Some of the most active anti-biofilm compounds discovered to date have been based upon the molecular scaffolds of natural products isolated from marine natural products. The oroidin family of alkaloids possess nitrogen-dense architectures characterized by the incorporation of one or more 2-aminoimidazole (2-AI) sub-units.<sup>138</sup> These molecules are found in marine sponges of the family *Agelasidae*, which reside in tropical water environments.<sup>139</sup> Their role is believed to be that of a chemical anti-feeding defense mechanism against predators.<sup>140</sup> The first report of oroidin family members inhibiting the formation of biofilms occurred in 1997 with the discovery that both oroidin **90** and bromoageliferin **91** (Fig. 14) displayed anti-biofouling activity against the Gram-negative marine  $\alpha$ -proteobacterium *Rhodospirillum salexigens*.<sup>141</sup> Bromoageliferin was reported to have an  $\text{IC}_{50}$  value of 2.43 nM while oroidin exhibited an  $\text{IC}_{50}$  value of 169  $\mu\text{M}$ . Oroidin has also been reported to possess anti-attachment activity against *V. vulnificus*.<sup>142</sup>

Based upon these findings, it was posited that the architecture embedded within these marine alkaloids could provide a basis for the development of novel small molecules with the ability to inhibit and disperse bacterial biofilms. The ubiquity of the 2-aminoimidazole motif in these compounds led to the hypothesis that it was the key pharmacophore responsible for biological activity. Indeed, initial studies with two simplified analogues of bromoageliferin, TAGE (*trans*-bromoageliferin) **92** and CAGE (*cis*-bromoageliferin) **93** (Fig. 14), showed that these simple analogues inhibited biofilm formation by PA01 and PA14 under static conditions with  $IC_{50}$  values of 100  $\mu$ M and 190  $\mu$ M against PA01 and PA14 respectively for TAGE and  $IC_{50}$  values 100  $\mu$ M and 180  $\mu$ M against PA01 and PA14 respectively for CAGE. Both compounds were shown to be acting *via* a non-microbicidal mechanism.<sup>143</sup> TAGE was also shown to possess the ability to disperse established *P. aeruginosa* biofilms ( $EC_{50}$  of 82  $\mu$ M and 114  $\mu$ M against PA01 and PA14 respectively). Confocal microscopy revealed a significant reduction in PA01 biofilm biomass after 48 hours in the presence of 100  $\mu$ M TAGE. The ratio of live *vs.* dead cells in TAGE treated *vs.* untreated cells was similar, validating that the molecule was not selectively killing biofilm bacteria. Analysis of established PA01:GFP biofilms under flow condition confirmed the results obtained under static conditions.<sup>144</sup> Installation of di-brominated acylpyrrole moieties similar to those present in bromoageliferin onto the TAGE scaffold result in compound **94**, which exhibited increased biofilm inhibitory activity against *P. aeruginosa*, including against a mucoid variant (PD0300) representative of bacteria isolated from patients with cystic fibrosis.  $IC_{50}$  values for this compound were 1.77  $\mu$ M, 12.0  $\mu$ M and 2.47  $\mu$ M against PA01, PA14 and PD0300 respectively. However, none of the acylated analogues were able to disperse pre-formed biofilms with the same efficiency as TAGE.<sup>144</sup>

Oroidin **90** exhibited similar activity to TAGE and CAGE against *P. aeruginosa*, with  $IC_{50}$  values of 190  $\mu$ M and 166  $\mu$ M against PA01 and PA14 respectively, confirming that a simpler architecture based upon the 2-AI scaffold was viable as an anti-biofilm agent.<sup>145</sup> Structure–activity–relationships (SARs) for anti-biofilm activity were investigated thorough the construction of a 50-member library of analogues based upon the oroidin template.<sup>145</sup> Three regions within the oroidin template were systematically varied in order to delineate which structural features of the molecule were required for biological activity in each section, these regions were: the pyrrole tail group, the linker chain, and the 2-AI head group. These studies revealed that any modifications to the 2-AI head of the molecule completely abolished activity. The most active molecule discovered was dihydrosventrin (DHS) **95** (Fig. 14), an analogue of the oroidin family alkaloid sventrin, which has also been isolated from marine sponges. DHS inhibited the formation of biofilms produced by PA01, PA14, PD0300, *A. baumannii*, and *Bordatella bronchiseptica* strain RB50 with  $IC_{50}$  values of 51  $\mu$ M, 111  $\mu$ M, 115  $\mu$ M, 110  $\mu$ M, and 238  $\mu$ M, respectively.<sup>146</sup> DHS was also able to disperse preformed biofilms of PA01, PA14, *A. baumannii*, and RB50. A library of second generation analogues designed to probe the effect of the *N*-alkyl substituent on the pyrrole ring on anti-biofilm activity in comparison to DHS<sup>147</sup> led to the identification of compound **96**, which possesses a *para*-bromo phenyl substituent, and exhibited greater non-



**Fig. 15** Highly active oroidin analogue **101** and derivatives that possess biofilm inhibition and dispersal activity.

microbicidal anti-biofilm activity against *A. baumannii* than DHS ( $IC_{50}$  = 27  $\mu$ M,  $EC_{50}$  = 41  $\mu$ M).

Other oroidin analogues, which could be accessed by more facile synthetic routes, included those in which the native amide bond directionality was reversed,<sup>148</sup> those in which the amide bond was removed, moved along the linker chain of the molecule, and substituted with additional aliphatic side chains.<sup>149</sup> Compounds with a reverse amide bond possessing an aliphatic tail group exhibited low micromolar inhibition of PA01 and PA14 biofilm development, with even the least active compound (**97**) (Fig. 14) possessing activity four to five times greater than that of oroidin **90**. The most active inhibitor of this first generation of reverse amide analogues (**98**) was also an extremely effective dispersion agent ( $IC_{50}/EC_{50}$  = 2.84  $\mu$ M/33  $\mu$ M against PA01,  $IC_{50}/EC_{50}$  = 2.26  $\mu$ M/21  $\mu$ M against PA14). A clear trend in which potency of the analogue was directly proportional to the length of the aliphatic chain was observed, with a break point against PA14 of a chain length of 13 carbons (**99**), for which an  $IC_{50}$  value of 729 nM was recorded. Removal of the amide bond entirely indicated that the presence of the amide bond or its respective directionality in this class of analogues is not entirely crucial for anti-biofilm activity, though these analogues exhibited lower activities than the reverse amide series.<sup>149</sup>

Covalent incorporation of one simple oroidin analogue, **99**, into a methacrylate polymer in efforts to develop new biofilm-resistant material for use on indwelling medical devices (IMD) produced a polymeric composite of 4% monomer **100**, which was observed to be resistant to biofilm colonization by *A. baumannii*.<sup>150</sup>

The incorporation of a triazole moiety to the oroidin analogues resulted in a number of highly active compounds. A number of 2-aminoimidazole–triazole (2-AIT) conjugates were generated,<sup>151</sup> with the most active compounds possessing a substituted unsaturated aryl pendant group. This series yielded a highly active compound (**101**) (Fig. 15), which possessed broad spectrum inhibitory and dispersal properties against a variety of bacterial strains. Compound **101** was active against both Gram-positive and Gram-negative bacteria with  $IC_{50}$  values of 5.6  $\mu$ M, 530 nM, 980 nM, and 810 nM and against PA01, PA14, *A. baumannii*, and *S. aureus*, respectively. This compound exhibited a synergistic effect with several antibiotics in the dispersion of pre-established biofilms of several bacterial strains, and was also

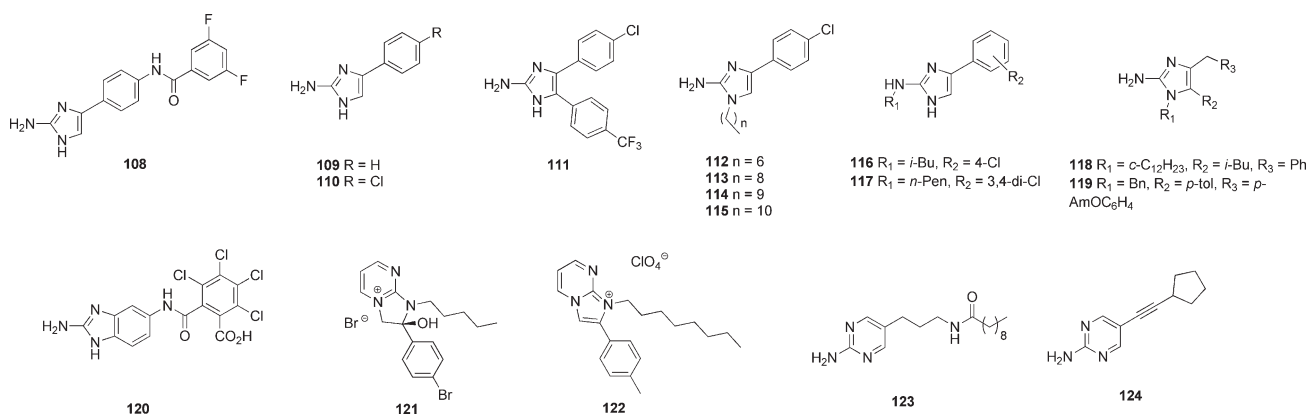


Fig. 16 Aryl 2-AI, 2-ABI, and 2-AP compounds that possess anti-biofilm activity.

shown to re-sensitize planktonic bacteria of drug-resistant strains of *S. aureus* and *A. baumannii* to the effects of conventional antibiotics.<sup>152</sup>

Replacing the unsaturated aryl appendage of **101** with a diverse array of alkyl linked amides<sup>153,154</sup> resulted in the discovery of several compounds with low micromolar biofilm inhibition and dispersal activity against a number of Gram-positive and Gram-negative bacterial strains (in addition to fungal anti-biofilm activity and activity against mixed species biofilms). The most active compounds possessed *para*-alkyl phenyl substituents and activity could again be tuned by adjusting the alkyl chain length. Compound **102**, which has a six carbon alkyl chain, exhibited IC<sub>50</sub>/EC<sub>50</sub> values of 21.4 μM/36.2 μM against *V. vulnificus* and an IC<sub>50</sub> of 8.9 μM against *R. solanigens*, making this compound significantly more active than oroidin. Compound **103**, which has a chain length of seven carbons, exhibited IC<sub>50</sub> values of 11.2 μM and 1.9 μM against *E. coli* and a multi-drug resistant strain of *A. baumannii* respectively, and respective EC<sub>50</sub> values of 23.4 μM and 7.9 μM against the same two strains. Compound **103** was highly active against Gram-positive bacteria, exhibiting IC<sub>50</sub>/EC<sub>50</sub> values of 0.5 μM/0.7 μM against vancomycin-resistant enterococci (VRE) and 0.6 μM/28.1 μM against *S. epidermidis*. The most active compound against MRSA was compound **102**, which exhibited IC<sub>50</sub>/EC<sub>50</sub> values of 0.7 μM/0.9 μM. Compound **104**, which has a nine carbon chain, exhibited increased biofilm inhibition activity against PA01 compared to **101**, with an IC<sub>50</sub> of 0.5 μM compared to 5.6 μM, and increased biofilm dispersion activity against PA14, with an EC<sub>50</sub> of 2.5 μM compared to 22 μM for **101**. All compounds were shown to be acting *via* a non-microbicidal mechanism.

Further efforts to increase the activity of **101** through modification of the aryl appendage have been carried out through the use of Suzuki–Miyaura coupling.<sup>155</sup> A number of these compounds were found to be effective biofilm dispersion agents against *A. baumannii*, with the most active compound **105** exhibiting an EC<sub>50</sub> of 44.7 μM, compared to 120 μM for the parent compound **101**.

The effect of addition of substituents to the 2-AI moiety upon the ability of **101** to modulate biofilms has been investigated through a synthetic approach involving Grignard addition to  $\alpha$ -amino Weinreb amides.<sup>99</sup> The most potent compound identified from this library (**106**) exhibited an IC<sub>50</sub> value of 1.42 μM

against MRSA. Further substitution of the newly introduced phenyl ring resulted in the identification of a compound (**107**) with improved biofilm inhibition activity against MRSA (IC<sub>50</sub> 1.34 μM). The most potent non-microbicidal inhibitor of *A. baumannii* biofilm formation from this series (**106**) was not as active as the parent compound **101**, exhibiting an IC<sub>50</sub> value of 11.28 μM, though it was able to disperse pre-formed *A. baumannii* biofilms with an EC<sub>50</sub> value of 44.6 μM.

Construction of a 30-member library based upon an aryl 2-AI scaffold (Fig. 16) lead to the identification of compound **108**, which was able to inhibit *E. coli* biofilm formation *via* a non-microbicidal mechanism, exhibiting an IC<sub>50</sub> value of 5.2 μM.<sup>156</sup> Steenackers and co-workers have subsequently reported the anti-biofilm activity of a related series of aryl 2-AI compounds.<sup>157</sup> The parent compound **109**, which possess a phenyl group at the 4-position of the 2-AI ring, displayed moderate biofilm inhibitory activity, inhibiting biofilm formation by *S. typhimurium* with an IC<sub>50</sub> of 130 μM at 25 °C and an IC<sub>50</sub> of 146 μM at 37 °C, and *P. aeruginosa* biofilm formation with IC<sub>50</sub> values of 72.6 and 22.7 μM at 25 °C and 37 °C, respectively. Substitution of the phenyl ring at the *para*-position resulted in increased biofilm inhibition activity, with the most active compound, **110**, which possesses a chlorine substituent, inhibiting biofilm formation by *S. typhimurium* with IC<sub>50</sub> values of 16.0 and 30.1 μM at 25 °C and 37 °C respectively, and *P. aeruginosa* biofilm formation with IC<sub>50</sub> values of 3.5 and 29.3 μM at 25 °C and 37 °C respectively. Introduction of a number of aryl substituents at the 5-position of the 2-AI ring resulted in a slight increase in activity relative to **110**, with the most active analogue, **111**, inhibiting biofilm formation by *S. typhimurium* at 25 °C with an IC<sub>50</sub> value of 10.8 μM. The introduction of alkyl substituents at the 1-position of the 2-AI ring also modulated activity; short chain substituents in general resulted in a decrease in activity, while the most active analogues against *P. aeruginosa* biofilm formation were those possessing an alkyl chain of intermediate length, with **112**, which has a heptyl substituent, exhibiting an IC<sub>50</sub> value of 2.6 μM.<sup>157</sup> Longer alkyl chain substituents resulted in the highest activity against *S. typhimurium*, with compounds possessing nonyl (**113**), decyl (**114**) and undecyl (**115**) chains exhibiting IC<sub>50</sub> values of ~4 μM at 25 °C. The synthesis of a related series of 2,4-disubstituted 2-aminoimidazoles also identified a number of inhibitors of biofilm formation with the most active



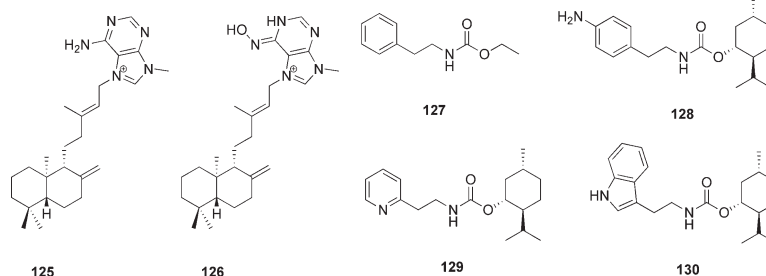


Fig. 17 (–)-Agelasine D **125**, (–)-ageloxime D **126** and marine derived carbamates that possess anti-biofilm activity.

compounds, **116** and **117**, able to inhibit biofilm formation by *S. typhimurium*, with  $IC_{50}$  values of 2.0 and 2.2  $\mu\text{M}$  respectively, and *P. aeruginosa* biofilm formation, with  $IC_{50}$  values of 0.9 and 0.7  $\mu\text{M}$  respectively at 25 °C.<sup>158</sup>

The generation of a series of 1,4,5-trisubstituted naamine alkaloids via a silver(I) mediated synthesis from secondary propargylamines produced several compounds that could inhibit biofilm formation by *S. typhimurium* and *P. aeruginosa* without significantly affecting planktonic growth. The lead compounds, **118** and **119**, exhibited  $IC_{50}$  values of 10.3  $\mu\text{M}$  (**118**) and 18.3  $\mu\text{M}$  (**119**) against *S. typhimurium* and 27.4  $\mu\text{M}$  (**118**) and 17.4  $\mu\text{M}$  (**119**) against *P. aeruginosa*.<sup>159</sup>

Scaffolds related to the 2-AI scaffold that have been investigated for anti-biofilm properties include 2-aminobenzimidazole (2-ABI)<sup>160</sup> and 2-aminopyrimidine (2-AP)<sup>161</sup> (Fig. 16). The most active non-microbicidal 2-ABI identified was compound **120**, with  $IC_{50}$  and  $EC_{50}$  values of 890 nM and 2.9  $\mu\text{M}$  (MRSA), 1.4  $\mu\text{M}$  and 75  $\mu\text{M}$  (VRE), and 570 nM and 7.3  $\mu\text{M}$  (*S. epidermidis*), respectively. The ability of compound **120** to prevent biofilm formation by these Gram-positive bacterial strains was inhibited by the presence of Zn(II), and the compound was subsequently shown to bind Zn(II) directly, providing insight into the potential mode of action of this compound.<sup>160</sup>

A series of 2-hydroxy-2-aryl-2,3-dihydroimidazopyrimidinium salts, which are intermediates in the synthesis of 2,4,5-trisubstituted 2-aminoimidazoles, included a number of compounds with low micromolar biofilm inhibition activity against *S. typhimurium* and *P. aeruginosa* at 25 °C, with the most active compound, **121**, exhibiting  $IC_{50}$  values of 1.7  $\mu\text{M}$  and 4.2  $\mu\text{M}$ .<sup>231</sup> Similarly, a series of imidazo[1,2- $\alpha$ ]pyrimidinium salts produced several active compounds, with the most active compound, **122**, exhibiting  $IC_{50}$  values of 1.5  $\mu\text{M}$  and 1.0  $\mu\text{M}$  against *S. typhimurium* and *P. aeruginosa* at 25 °C.<sup>157</sup> However, it is believed that both of these pyrimidinium scaffolds are cleaved *in vivo* to the corresponding substituted 2-aminoimidazoles, which may be responsible for the observed activity.<sup>157,158</sup>

A number of 2-aminopyrimidine derivatives that could not be cleaved to a 2-aminoimidazole were synthesized to unequivocally evaluate the 2-AP scaffold for the ability to modulate bacterial biofilms. 2-AP derived compounds were found to be less active as inhibitors of biofilm formation than their 2-AI counterparts, particularly against Gram-negative bacteria. None were able to disperse pre-formed biofilms. The most active, non-microbicidal 2-APs discovered were compounds **123**, which inhibited MRSA biofilm formation with an  $IC_{50}$  of 72  $\mu\text{M}$ , and

compound **124**, which inhibited biofilm formation by a methicillin sensitive strain of *S. aureus* with an  $IC_{50}$  of 67  $\mu\text{M}$ .<sup>161</sup>

Two diterpene alkaloids, (–)-agelasine D **125**, and the corresponding oxime derivative (–)-ageloxime D **126** (Fig. 17), were isolated from the marine sponge *Agelas nakamurai*.<sup>162</sup> (–)-Agelasine D **125** inhibited planktonic growth of *S. epidermidis* (MIC <0.0877  $\mu\text{M}$ ) but did not inhibit biofilm formation, while the converse was true for (–)-ageloxime D **126**, which inhibited biofilm formation without affecting bacterial growth. A synthetic route to (+)-agelasine D has been developed<sup>163</sup> and this scaffold has the potential to be exploited for the development of compounds with anti-biofilm activity *via* synthetic manipulation, as has been achieved for the oroidin and bromoageliferin scaffolds.<sup>162</sup>

The structurally simple bacterial metabolite ethyl *N*-(2-phenethyl) carbamate **127** (Fig. 17), which was isolated from the culture medium of the marine bacterium SCRC3P79 (*Cytophaga* sp.) has been reported to possess moderate anti-biofilm activity against *R. solis*.<sup>141</sup> This compound was also shown to be moderately active in inhibiting biofilm formation by medically relevant strains of bacteria including *S. epidermidis*, MRSA, VRE, multi-drug resistant *A. baumannii* (MDRAB), and *E. coli*.<sup>164</sup> A library of analogues based upon compound **127** was constructed, leading to the identification of a number of compounds with increased activity. The menthyl derived compounds **128–130** were the most active, inhibiting biofilm formation by several strains of *S. aureus* with  $IC_{50}$  values in the mid to low micromolar range.<sup>164</sup>

## Conclusion

Bacterial biofilms pose a serious threat to society and are responsible for the morbidity and mortality of a myriad of diseases as they are inherently insensitive to antibiotics and the host immune response. The increase in antibiotic resistance among many bacterial strains has put a tremendous pressure on the medical community to find alternative approaches for the treatment of biofilm-mediated diseases. This review describes the current standing of research into the control of bacterial biofilms using small molecules that do not act as antibiotics or microbicides, and instead elicit anti-biofilm activity through alternative mechanisms, such as interfering with the complex regulatory systems that control biofilm formation and maintenance. Molecules with such characteristics should offer the unique ability to mitigate bacterial selection pressures while simultaneously providing

insight into how bacteria communicate with one another. Agents with the ability to prevent the formation of, or even eradicate, biofilms have the potential to restore the efficacy of antibiotics that are ineffective against biofilm bacteria, and should have a profound impact upon human medicine.

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