Chem Soc Rev

This article was published as part of the

2008 Chemistry–Biology Interface Issue

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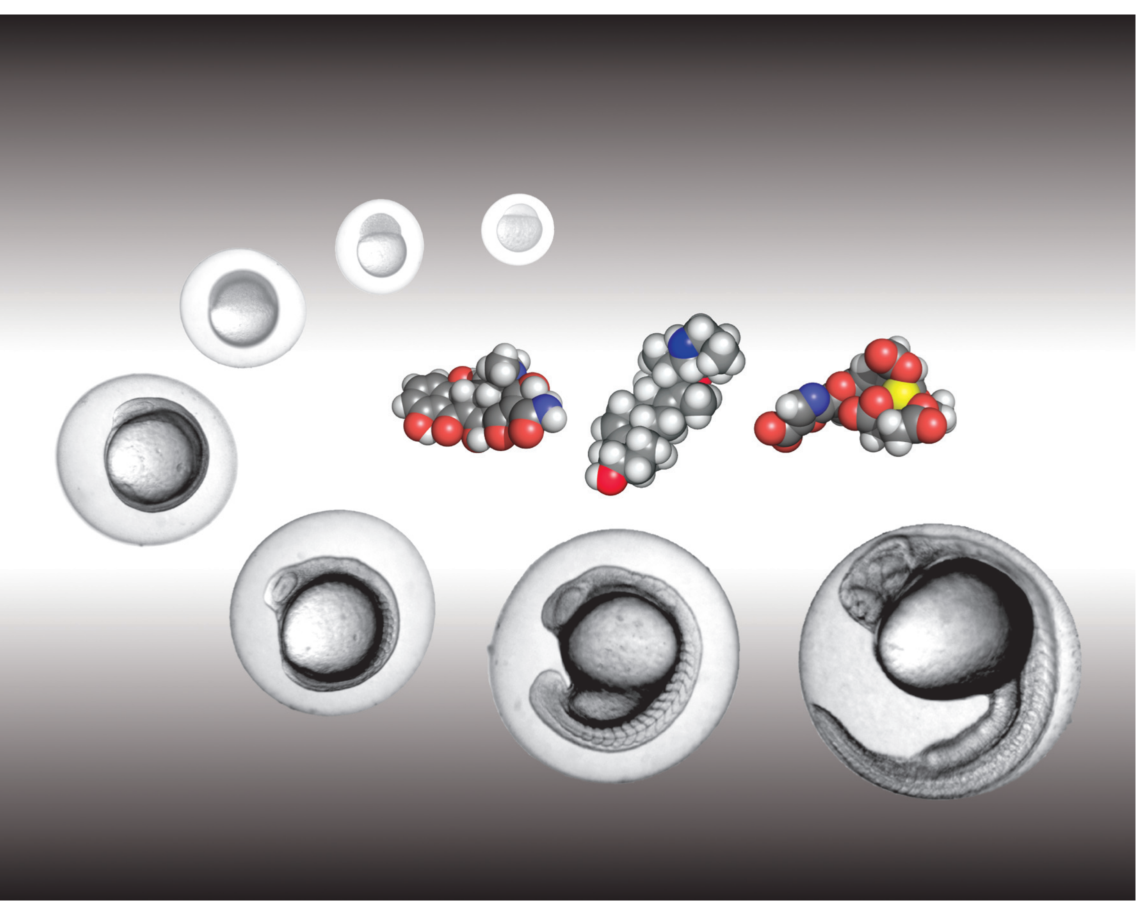

Small molecule inhibition of microbial natural product biosynthesis—an emerging antibiotic strategy†

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Received 2nd March 2008
First published as an Advance Article on the web 21st May 2008
DOI: 10.1039/b702780j

A variety of natural products modulate critical biological processes in the microorganisms that produce them. Thus, inhibition of the corresponding natural product biosynthesis pathways represents a promising avenue to develop novel antibiotics. In this *tutorial review*, we describe several recent examples of designed small molecule inhibitors of microbial natural product biosynthesis and their use in evaluating this emerging antibiotic strategy.

1. Introduction

Classically, natural products have been viewed as agents of 'microbial warfare' between microorganisms competing for limited resources. In keeping with this view, many cytotoxic and cytostatic natural products have been developed into important antiinfective and anticancer drugs.[1] However, mounting evidence points to more subtle functions of some natural products in modulating bacterial pathogenesis and communication.[2] Natural products have been shown to play key roles in critical microbial processes such as nutrient uptake, quorum sensing, biofilm formation, virulence, and commensalism. Thus, an emerging antibiotic strategy involves inhibiting the microbial biosynthetic pathways that produce these natural products. Structural and mechanistic information about enzymes involved in these pathways is often available to facilitate the rational design of these inhibitors. Notably, many of these natural products are considered virulence factors,[3] which are not essential for bacterial viability, but are considered attractive new antibiotic targets since they mediate pathogenicity in the human host. Herein, we describe recent examples of natural product biosynthesis inhibitors that target iron-chelating siderophores, virulence-conferring bacterial lipids, and quorum-sensing autoinducers. These inhibitors will allow further evaluation of this promising new antibiotic strategy.

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† Part of a thematic issue examining the interface of chemistry with biology.

2. Iron-chelating siderophores

Iron is an essential nutrient for nearly all organisms and pathogenic bacteria must acquire iron from the host to support growth and virulence.[4,5] However, the free iron concentration is extremely low in the host environment ($\approx 10^{-24}$ M) due to the low solubility of Fe^{3+} and the presence of numerous iron-sequestering host proteins. Thus, to acquire this iron, pathogenic bacteria biosynthesize iron-chelating small molecule natural products called siderophores. These siderophores are secreted into the host milieu where their high affinities for Fe^{3+} allow them to 'steal' iron from host proteins. The iron–siderophore complexes are then recognized by specific receptors and actively transported back into the bacteria, where the iron is released.

A significant number of siderophores have been identified as virulence factors in pathogenic bacteria. For example, a siderophore-deficient mutant strain of *Yersinia pestis* exhibits a >10 000-fold higher LD_{50} in mice than a corresponding siderophore-producing strain.[6] Further, a siderophore-deficient

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Mycobacterium tuberculosis mutant exhibits a significantly reduced growth rate in a macrophage-like cell line compared to a wildtype strain.[7] Thus, small molecules that inhibit siderophore biosynthesis represent an important new class of potential antibiotics.

2.1 Biosynthesis of siderophores by non-ribosomal peptide synthetases

Many siderophore biosynthetic pathways involve non-ribosomal peptide synthetases (NRPS).[8,9] These modular 'megaenzymes' assemble amino acid building blocks in a stepwise fashion and introduce a variety of chemical modifications into the polypeptide products.[10] The sequence and structure of the non-ribosomal peptide product is encoded by the order of dedicated domains within the NRPS (Fig. 1). Adenylation (Ad) domains catalyze the activation and transfer of specific amino acid building blocks onto a thiol moiety of a peptidyl carrier protein (PCP or thiolation domain). This thiol is derived from a phosphopantetheinyl group that is installed by a phosphopantetheinyl transferase enzyme. Adenylation domains most commonly accept natural amino acid substrates, but can also specify other substrates, including non-proteinogenic amino acids (*e.g.* D-alanine, 2,4-diaminobutyric acid, ornithine) and aryl acids (*e.g.* salicylic acid, dihydroxybenzoates). Adjacent aminoacyl-*S*-PCP intermediates are coupled by a condensation (C) domain to form a peptide bond. A variety of chemical modifications, including epimerization, methylation, reduction, and oxidation reactions are carried out by other NRPS domains or associated soluble enzymes. Iterative couplings catalyzed by downstream modules lead to a penultimate polypeptidyl-*S*-PCP thioester intermediate, which is then released from the NRPS machinery by a terminal thioesterase domain through hydrolysis or cyclization. NRPS may also be associated intra- or intermolecularly with related polyketide synthetases in hybrid biosynthetic pathways.[10] Notably, several siderophores have been shown recently to be biosynthesized by NRPS-independent pathways.[11]

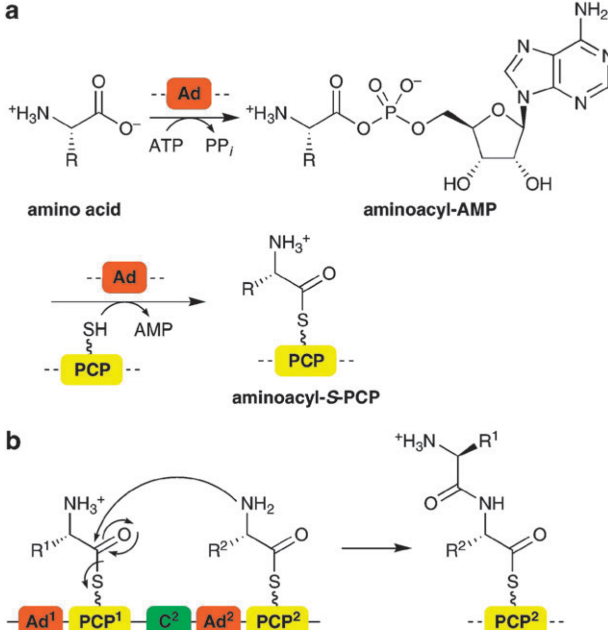

Fig. 1 Peptide assembly by a non-ribosomal peptide synthetase. (a) An adenylation (Ad) domain catalyzes the activation of a specific carboxylic acid building block and acyl transfer onto a peptidyl carrier protein (PCP) domain. (b) A condensation (C) domain catalyzes coupling of two PCP-tethered acyl units, extending the peptide chain by one residue.

Inhibitors of a variety of enzymes involved in siderophore biosynthesis have been reported recently and are described in the following sections.

2.2 Inhibition of isochorismate synthase and salicylate synthase

The first gene in the biosynthetic operons of the *Y. pestis* and *M. tuberculosis* siderophore biosynthesis gene clusters encodes a salicylate synthase (Irp9 and MbtI, respectively) that converts chorismate to salicylic acid.[8] Related enzymes convert chorismate to dihydroxybenzoic acids. These aryl acids are then accepted by NRPS adenylation domains and ultimately transformed into the 'aryl cap' seen in a variety of phenolic and catecholic siderophores (Fig. 2).[8,9]

The salicylate synthase reaction is proposed to proceed through a two-step mechanism (Fig. 3a).[12,13] First, nucleophilic addition of water to C2 of chorismate displaces the C4 hydroxyl group through a S_N2'' mechanism to generate isochorismate, which remains bound to the enzyme in a twist–boat conformation. This conformation facilitates the second step, in which a [1,5]-sigmatropic rearrangement yields pyruvate and salicylic acid.[14]

Abell and co-workers have used this mechanistic information to design a series of Irp9 inhibitors that could potentially block yersiniabactin siderophore biosynthesis.[15] These chorismate (substrate) and isochorismate (intermediate)

Fig. 2 Aryl-capped siderophores.

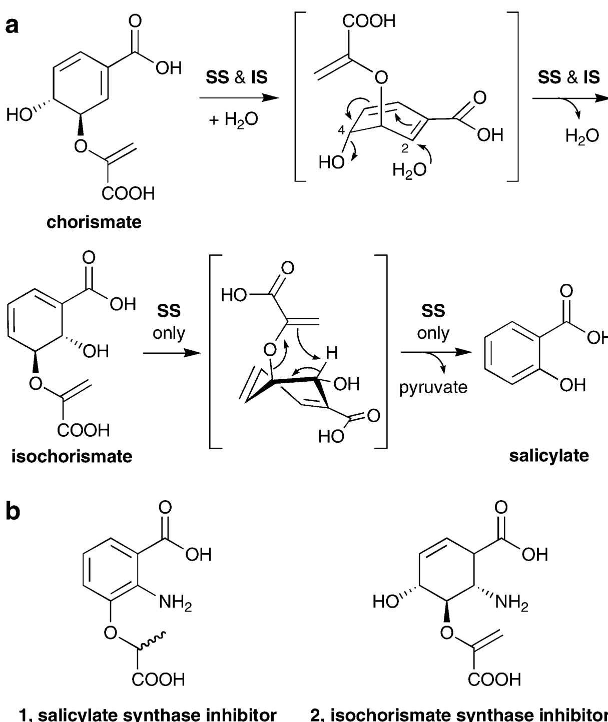

Fig. 3 (a) Reactions catalyzed by salicylate synthases (**SS**, *e.g.* Irp9) and isochorismate synthases (**IS**, *e.g.* EntC). (b) Designed inhibitors of an isochorismate synthase (*E. coli* EntC) and a salicylate synthase (*Y. enterocolitica* Irp9).

analogs were tested against purified enzyme from the gastroenteric pathogen *Y. enterocolitica* and several inhibitors with moderate activity were identified (*e.g.* **1**, Fig. 3b). These are the first reported inhibitors of a salicylate synthase and set the stage for further exploration of these inhibitor designs and the therapeutic potential of these targets.

Notably, this work was inspired by earlier studies of Bartlett and co-workers on transition state analog inhibitors of the *E. coli* isochorismate synthase EntC.[16] This enzyme has high homology to the salicylate synthase family and performs the first half-reaction of salicylate synthase to provide isochorismate (Fig. 3a). Two additional enzymes (EntB and EntA) then convert isochorismate to the 2,3-dihydroxybenzoic acid building block used in enterobactin biosynthesis.

These inhibitors were designed to mimic the EntC S_N2'' reaction transition state, which was proposed to involve a metal-coordinated structure with the nucleophile and leaving group in a *syn* orientation. Potent biochemical inhibitors were identified using this approach (*e.g.* **2**, Fig. 3b). Thus, these inhibitors may be useful for targeting biosynthetic pathways leading to aryl-capped siderophores.

The availability of these salicylate synthase and isochorismate synthase inhibitors sets the stage for their further evaluation in cellular assays for inhibition of enzymatic activity, siderophore biosynthesis, and bacterial growth. Recently reported crystal structures of two salicylate synthases, Irp9 and MbtI,[14,17,18] provide new insights into the reaction mechanism and, combined with existing structure–activity relationship (SAR) information, should facilitate the design of additional inhibitors.

2.3 Inhibition of salicylic acid adenylation enzymes and of salicylate-derived siderophore biosynthesis

The NRPS-mediated biosynthesis of aryl-capped siderophores is initiated by aryl acid adenylation enzymes, which are generally soluble proteins that are not linked covalently with the remainder of the NRPS machinery.[8,9] These enzymes select and activate aryl acid substrates and load them onto an aryl carrier protein (ArCP) domain. This process involves a two-step reaction mechanism (Fig. 4). In the first half-reaction, the aryl acid is adenylated to form an aroyl-AMP intermediate, which remains non-covalently bound to the enzyme active site. In the second half-reaction, the aroyl group is transthioesterified onto the phosphopantetheine moiety of the ArCP domain. The aryl acid is then coupled with downstream building blocks (*e.g.* amino acids), leading to the aryl-capped siderophore product.

NRPS adenylation domains and mechanistically-related adenylate-forming enzymes bind their cognate acyl-AMP intermediates 2–5 orders of magnitude more tightly than the corresponding carboxylic acid and ATP substrates.[19–21] Thus, a variety of non-hydrolyzable analogs of the acyl-AMP intermediates can be used to inhibit these enzymes.[22,23] Furthermore, the reported cocrystal structure of DhbE, a 2,3-dihydroxybenzoate adenylation enzyme, with its cognate aroyl adenylate intermediate, 2,3-dihydroxybenzoyl-AMP, can be used to facilitate inhibitor design.[24] Notably, the aroyl adenylate is bound by DhbE residues that are highly conserved across all aryl acid adenylation enzymes.

Our group, in collaboration with Quadri and co-workers, reported the first inhibitor of salicylate adenylation enzymes that was designed using this mechanistic and structural information.[25] Salicyl-AMS (**3**, Table 1) contains a comparatively stable *N*-acylsulfamate moiety in place of the acylphosphate group in the corresponding salicyl-AMP reaction intermediate. This compound was shown to be a potent inhibitor of three salicylate adenylation enzymes used in the biosynthesis of yersiniabactin (*Y. pestis* YbtE), mycobactin (*M. tuberculosis* MbtA), and pyochelin (*Pseudomonas*

Fig. 4 Two-step reaction catalyzed by aryl acid adenylation enzymes, leading to aryl-capped siderophores.

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Table 1 Representative inhibitors of aryl acid adenylation enzymes

3–7, aryl acid adenylation enzyme inhibitors

Inhibitor	Enzyme	K_i^{app}/nm	Organism	IC_{50}/μM
3	YbtE MbtA	0.3–1.1 5.1–6.6	*Y. pestis* *M. tuberculosis*	51.2 0.091–2.2
4	MbtA	3.7–3.8	*M. tuberculosis*	0.077
5	DhbE	85	*B. subtilis*	Nd[b]
6	EntE	9	*E. coli*	Nd
7	AsbC	250[a]	*B. anthracis*	Nd

[a] IC_{50} value. [b] Nd = not determined.

aeruginosa PchD) siderophores. Inhibition of YbtE was shown to be competitive with respect to ATP and non-competitive with respect to salicylate.

Salicyl-AMS also inhibited *Y. pestis* and *M. tuberculosis* growth in iron-deficient media, which mimics the host environment and where bacterial growth is known to be siderophore dependent, with IC_{50} values of 51.2 μM and 2.2 μM, respectively (Table 1). Furthermore, siderophore production was shown to be inhibited in both organisms by radiometric TLC visualization of ^{14}C-salicylate-labeled siderophores. Importantly, the growth inhibitory effects were attenuated significantly in iron-rich media, in which bacterial growth does not require siderophore production. These additional experiments provide support for the mechanism of action of salicyl-AMS. Separately, Aldrich and co-workers have also shown that this compound is non-toxic to a mammalian cell line (P388 murine leukemia) at >200 μM concentration.[26]

Aldrich and co-workers have also described a large number of salicyl-AMS analogs with variations in the sulfamate,[26,27] glycosyl,[28] and aryl acid regions,[29,30] providing a detailed SAR profile with respect to inhibition of MbtA and *M. tuberculosis* growth. Biochemical potency can be increased slightly by replacement of the sulfamate with a sulfamide (**4**, Table 1), replacement of the ribosyl ring 4′-oxygen with a carbon, or omission of either the 2′- or 3′-hydroxyl groups. Docking analyses using a homology model based on the DhbE structure suggested that maintenance of a 3′-*endo* ribose conformation is critical for binding.[26,28] Importantly, an intramolecular hydrogen bond between the phenolic hydroxyl group and sulfamate nitrogen appears to be required for salicyl-AMS to adopt an appropriate pharmacophoric conformation.[27] Along these lines, Bisseret and co-workers have reported an indolylphosphonamide analog of salicyl-AMS designed to enforce this conformation.[31]

Two analogs have been identified with slightly more potent growth inhibitory activity compared to salicyl-AMS, the sulfamide analog **4** (Table 1) and 4-fluorosalicyl-AMS (not shown).[26,30] Several other analogs have more potent or equipotent biochemical activity but exhibit greatly reduced cellular activity. Based on this information, Aldrich and co-workers have suggested that salicyl-AMS may be a substrate for an as yet unidentified transporter that mediates its uptake.[28]

The salicyl-AMS class is the first series of compounds demonstrated to inhibit siderophore biosynthesis and bacterial growth in cell culture assays. Further studies in animal infection models will be critical for evaluating the ability of these compounds to block bacterial virulence *in vivo* and will also provide key insights into the therapeutic potential of blocking siderophore biosynthesis as a new antibiotic strategy.

2.4 Inhibition of a 2,3-dihydroxybenzoic acid adenylation enzyme

Adenylation enzymes specific for 2,3-dihydroxybenzoic acid are used in the biosynthesis of a variety of catecholic siderophores known to be required for virulence in animal models, including enterobactin derivatives that are produced in several Gram-negative enteric bacteria (Fig. 2).[8,9] Two 2,3-dihydroxybenzoate adenylation enzyme inhibitors, which are aroyl-AMP mimics, have been reported (**5**, **6**, Table 1). Marahiel and co-workers showed that 2,3-dihydroxybenzoyl-AMS (**5**) is a potent inhibitor of DhbE, the adenylation enzyme from *Bacillus subtilis* bacillibactin synthetase.[32] Callahan and co-workers have also explored a series of novel *N*-acylhydroxamoyl adenylates, in which a nitrogen atom is inserted between the phosphate and acyl groups. The 2,3-dihydroxybenzoyl derivative (**6**) proved to be a potent inhibitor of EntE, the adenylation enzyme from *Escherichia coli* enterobactin synthetase.[33] The potency of this inhibitor is notable considering that the *N*-acylhydroxamoylphosphate is ≈ 2 Å longer than the acylphosphate it replaces. While cellular assays with these compounds have not yet been reported, they demonstrate that non-hydrolyzable aroyl-AMP analogs may be useful for inhibiting a variety of additional siderophore biosynthesis pathways.

2.5 Inhibition of a 3,4-dihydroxybenzoic acid adenylation enzyme

Pathogenic *B. anthracis* uses an unusual 3,4-dihydroxybenzoate adenylation enzyme, AsbC, to synthesize a second siderophore, petrobactin. Strains of *B. anthracis* that lack the *asb* locus, and, thus, the ability to biosynthesize petrobactin, have reduced virulence in mice models.[34] AsbC has homology to other NRPS-associated aryl acid adenylation enzymes,[35] but the majority of the biosynthetic pathway is actually

NRPS-independent.[11] Using the sulfamate-based inhibitor design strategy described above, Sherman and co-workers have explored 3,4-dihydroxybenzoyl-AMS (**7**, Table 1) as a small molecule inhibitor of AsbC.[35] Interestingly, this compound exhibits much weaker inhibitory activity against this enzyme compared to structurally related inhibitors of other aryl acid enzymes described above. While the molecular basis for this difference awaits further investigation, this work demonstrates that small molecule inhibition of the petrobactin is, in principle, possible and further broadens the potential therapeutic range of siderophore biosynthesis inhibitors.

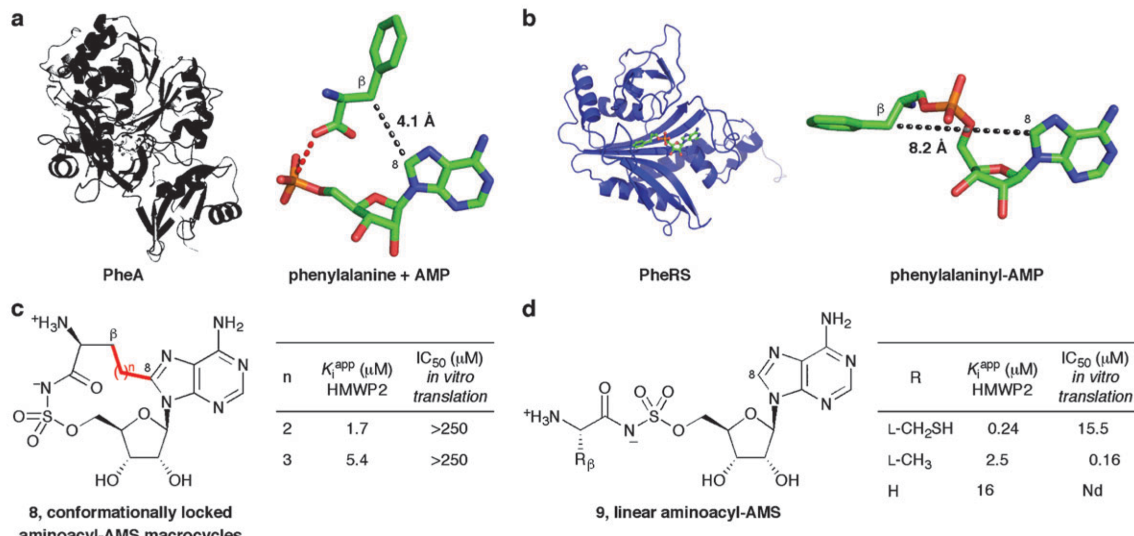
2.6 Selective inhibition of an amino acid adenylation domain

Many siderophores do not contain aryl acid-derived moieties. Indeed, this is true of most NRPS-derived natural products. However, amino acid adenylation domains are, by definition, found in all NRPS biosynthetic pathways and, as such, are attractive targets for small molecule inhibition. Indeed, Marahiel and co-workers have demonstrated that aminoacyl-AMS derivatives can be used to inhibit amino acid adenylation domains from *B. brevis* gramicidin synthetase and *B. subtilis* surfactin synthetase.[23] However, these compounds also inhibit aminoacyl-tRNA synthetases, which catalyze mechanistically identical reactions, with the PCP thiol replaced by a tRNA hydroxyl group as the final nucleophile.[21,22] As the latter enzymes are used ubiquitously in ribosomal protein translation, simple aminoacyl-AMP analogs are unsuitable as antibiotics. Two approaches to avoiding this undesired cross-reactivity for aminoacyl-tRNA synthetases can be considered. First, aminoacyl-AMP analogs derived from non-proteinogenic amino acids should only inhibit the NRPS adenylation domains since there would be no corresponding aminoacyl-tRNA synthetases. This approach has been used successfully to target a D-alanine adenylation domain and is discussed in section 3.2 below.[36] Alternatively, pronounced structural differences between amino acid adenylation domains and aminoacyl-tRNA synthetases can be exploited to design selective inhibitors. This approach has been used successfully to target a cysteine adenylation domain involved in *Y. pestis* yersiniabactin biosynthesis.[37]

Our group, in collaboration with Quadri and co-workers, recognized that, although amino acid adenylation domains and aminoacyl-tRNA synthetases catalyze mechanistically identical reactions, the requisite aminoacyl-AMP intermediates are bound in drastically different conformations in available cocrystal structures (Fig. 5). In the structure of the phenylalanine adenylation domain (PheA) of gramicidin synthetase, phenylalanine and AMP ligands are observed in an overall cisoid conformation with respect to the amino acid and adenine moieties (Fig. 5a).[38] Examination of related structures of an aryl acid adenylation enzyme,[24] long chain fatty acid synthetase,[39] and luciferase[40] suggests that this general cisoid conformation is conserved across this enzyme superfamily. In contrast, a carbonyl-reduced analog of phenylalanyl-AMP is bound in a transoid conformation in a cocrystal structure with a phenylalanyl-tRNA synthetase (Fig. 5b).[41] Indeed, similar transoid conformations are observed in all available structures of ligand-bound aminoacyl-tRNA synthetases.

Thus, we designed macrocyclic aminoacyl-AMP analogs **8** (Fig. 5c) to enforce the pharmacophoric cisoid conformation that is specific to NRPS amino acid adenylation domains.[37] These macrocycles were shown to inhibit the cysteine adenylation activity of *Y. pestis* yersiniabactin synthetase HMWP2 with affinities comparable to those observed for the corresponding linear aminoacyl-AMS inhibitors **9** (Fig. 5d). Most importantly, in contrast to the linear inhibitors, these macrocycles did not inhibit aminoacyl-tRNA synthetases, as determined by *in vitro* translation assays containing all 20 of these enzymes.

Further studies to explore the scope of adenylation domain inhibition and the cellular activity of these novel macrocycles



n	K_i^{app} (μM) HMWP2	IC_{50} (μM) *in vitro translation*
2	1.7	>250
3	5.4	>250

R	K_i^{app} (μM) HMWP2	IC_{50} (μM) *in vitro translation*
L-CH_2SH	0.24	15.5
L-CH_3	2.5	0.16
H	16	Nd

Fig. 5 (a) Crystal structure of a phenylalanine adenylation domain (PheA) and bound conformations of phenylalanine and AMP ligands. (b) Crystal structure of a phenylalanyl-tRNA synthetase (PheRS) and bound conformation of a phenylalaninyl-AMP ligand. (c,d) Macrocyclic and linear aminoacyl-AMP analogs and inhibition of a cysteine adenylation domain ($HMWP2^{1-1491}$-His_6) and *in vitro* translation in rabbit reticulocyte lysates.

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Fig. 6 MbtA adenylation enzyme-catalyzed covalent modification of the ArCP domain of MbtB using a vinyl sulfonamide analog of salicyl-AMP.

inhibitor	K_i^{app} (nM) IroB
R^1 = Br, R^2 = R^3 = H	23
R^1 = R^2 = Br, R^3 = H	7.0
R^1 = R^2 = R^3 = Br	3.3

Fig. 7 (a) The 2,3-dihydroxybenzoyl moieties of enterobactin are iteratively *C*-glucosylated by IroB to form *C*-glucosylated enterobactin derivatives. (b) Bromoenterobactin analogs are potent inhibitors of IroB.

are ongoing. Such compounds may have broad potential in inhibiting the biosynthesis of siderophores as well as other NRPS-derived natural products.

2.7 Covalent modification of an aryl carrier protein domain

Another potential set of targets for inhibition of siderophore biosynthesis are the carrier protein domains that accept acyl-AMP intermediates from adenylation enzymes/domains using a phosphopantetheine thiol nucleophile. Aldrich and co-workers have used a vinyl sulfonamide analog of salicyl-AMP (**10**, Fig. 6) to target covalent modification of this thiol in the ArCP domain of MbtB from *M. tuberculosis* mycobactin synthetase.[29] While this compound is a weak inhibitor of the salicylate adenylation enzyme MbtA, probably due to its inability to form the critical intramolecular hydrogen bond between the phenolic hydroxyl and the (carbon) α-position of the sulfonamide moiety,[27] it has an appropriately positioned electrophilic center at the β-carbon to trap the MbtB ArCP thiol nucleophile, forming a stable thioether linkage (observed by MALDI-TOF-MS at 2 μM inhibitor concentration). This adduct also stabilized the MbtA–MbtB protein–protein interaction and, as such, has the potential to block two separate components of the mycobactin biosynthetic machinery.

Notably, Burkart and co-workers have previously reported a related approach to trapping thiol nucleophiles in polyketide synthetase ketosynthase domains, using carrier proteins functionalized with electrophilic phosphopantetheine analogs.[42]

2.8 Inhibition of enterobactin *C*-glucosylation

Several Gram-negative enteric bacteria, including *Salmonella* spp., *E. coli*, and *Klebsiella pneumoniae*, produce *C*-glucosylated variants of enterobactin (salmochelins), such as diglucosyl-enterobactin (Fig. 2). Walsh and co-workers have demonstrated that this *C*-glucosylation modification allows the bacterial siderophores to evade sequestration by lipocalin 2, a protein that is secreted by mammalian cells as part of the innate immune response to infection.[43] While the parent, non-glucosylated enterobactin–iron complex is bound tightly by lipocalin 2 (K_d = 0.43 nM), the diglucosylated variant is not (K_d > 1 μM), and remains available for use in bacterial iron acquisition. The machinery for *C*-glucosylation of enterobactin and processing of the corresponding iron complexes is encoded by the *iroA* gene cluster in *E. coli*. Introduction of this gene cluster into non-pathogenic *E. coli* leads to a hypervirulent phenotype in a mouse infection model. Thus, the biosynthesis of *C*-glucosylated enterobactins represents a potential antibiotic target.

Enterobactin *C*-glucosylation is carried out by the IroB glycosyltransferase enzyme in *E. coli* (Fig. 7). Walsh and co-workers have identified several substrate analogs **11** that are potent inhibitors of this enzyme.[44] Interestingly, none of these bromoenterobactin derivatives is a substrate for IroB-catalyzed *C*-glucosylation. All three inhibitors are competitive with enterobactin and form non-covalent complexes with IroB. These inhibitors will allow further evaluation of the therapeutic potential of inhibiting enterobactin *C*-glucosylation in enteric bacteria.

3. Virulence-conferring bacterial lipids

In addition to their canonical roles in maintaining membrane integrity, various bacterial lipids have been identified as specific virulence factors. Rather than being biosynthesized by generic fatty acid synthetase pathways, these lipids are produced by specialized enzymatic pathways that often involve elements of NRPS and polyketide synthetase machinery. As such, mechanistic information about these classes of enzymes can be used to design small molecule inhibitors targeting the biosynthesis of these virulence-conferring lipids.

3.1 Inhibition of a *p*-hydroxybenzoic acid adenylation domain and of phenolic glycolipid biosynthesis

Phenolic glycolipids (PGL), which are dimycoserate esters of phenolphthiocerol, are produced by various mycobacteria,

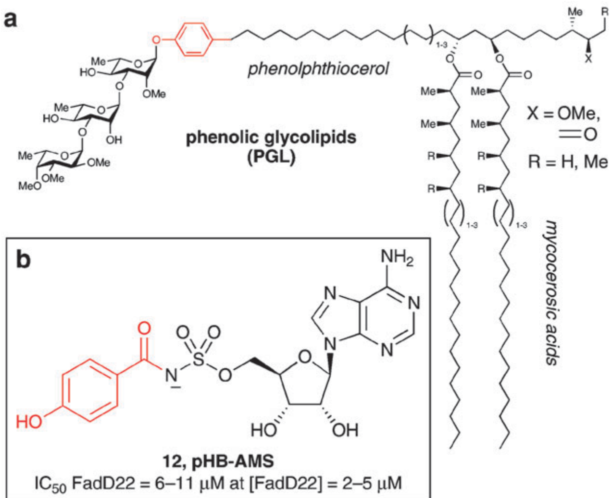

Fig. 8 (a) General structure of mycobacterial phenolic glycolipids (PGL) with *p*-hydroxybenzoic acid-derived moiety (red). (b) pHB-AMS is a tight-binding inhibitor of the *p*-hydroxybenzoic acid adenylation domain of the PGL biosynthetic enzyme FadD22.

Fig. 9 (a) Structure of a lipoteichoic acid with D-alanyl ester functionalities (red). (b) D-ala-AMS is an effective inhibitor of the D-alanine adenylation enzyme DltA.

including *M. tuberculosis* and *M. leprae*, and have been linked to hypervirulent phenotypes in animal models.[45] While their mechanisms of action are still under investigation, they have been associated with protection of the bacteria from oxidative stress and attenuation of the host immune response.

PGL are synthesized by a combination of polyketide synthetases that produce the phenolphthiocerol and mycocerosic acid components (Fig. 8a).[45] Notably, the phenolphthiocerol moiety contains a phenolic group that is derived biosynthetically from *p*-hydroxybenzoic acid. Quadri and co-workers, in collaboration with our group, recently demonstrated that this building block is incorporated into PGL by FadD22, an unusual stand-alone didomain initiation module comprised of a *p*-hydroxybenzoate adenylation domain and an ArCP domain.[46] A small molecule inhibitor, pHB-AMS (**12**, Fig. 8b), was designed to mimic the cognate *p*-hydroxybenzoyl-AMP reaction intermediate. This compound is a tight-binding inhibitor of FadD22 and blocks both *p*-hydroxybenzoic acid adenylation and *p*-hydroxybenzoylation of the ArCP domain of FadD22. Moreover, pHB-AMS was shown to inhibit PGL production specifically in several *Mycobacterium* spp. (IC_{50} = 4–12 μM), without affecting the production of related dimycoserate esters. As expected, pHB-AMS did not inhibit mycobacterial growth in cellular assays, consistent with its mechanism of action in targeting the PGL virulence factor. This sets the stage for further evaluation of this compound in *in vivo* infection models to assess the therapeutic potential of inhibiting PGL biosynthesis.

3.2 Inhibition of a D-alanine adenylation domain involved in lipoteichoic acid biosynthesis

Lipoteichoic acids (LTA) are key components of the cell envelope in Gram-positive bacteria that have been implicated in a variety of processes, including virulence and biofilm formation.[47] Most LTA are comprised of a glycolipid anchor linked to a poly(glycerolphosphate) chain (Fig. 9a). A significant fraction of the glycerol 2-hydroxyl groups are often functionalized with D-alanyl esters, which are critical to LTA structure and function. In particular, mutant strains of several bacteria, including *Staphylococcus aureus*, that lack the biosynthetic machinery to install these D-alanyl esters exhibit decreased virulence in animal models. Thus, the biosynthesis of D-alanyl ester-functionalized LTA represents an attractive potential antibiotic target.

The D-alanyl esters are installed onto LTA by unusual NRPS-related adenylation enzymes that are specific for D-alanine. Marahiel and co-workers have leveraged this information to design D-alanyl-AMS (**13**, Fig. 9b) to inhibit such D-alanine adenylation enzymes by mimicking the cognate D-alanyl-AMP intermediate.[36] Notably, this compound would not be expected to inhibit aminoacyl-tRNA synthetases involved in ribosomal protein translation, which are specific for L-amino acids. The D-alanyl-AMS compound was shown to be an effective inhibitor of DltA, the D-alanine adenylation enzyme from *B. subtilis*. Consistent with the higher sensitivity of DltA knockouts to certain antibiotics, D-alanyl-AMS (1 mM) also potentiated the activity of vancomycin (0.4 nM) against *B. subtilis*, blocking recovery of bacterial growth that was observed after treatment with vancomycin alone. These promising results support the potential therapeutic value of targeting D-alanyl ester formation in LTA virulence-conferring lipids.

4. Quorum-sensing autoinducers

A variety of processes in pathogenic bacteria, including virulence factor production and biofilm formation, are regulated by cell density through quorum sensing.[2,48] The key signaling molecules in this intercellular communication are natural products called autoinducers. These molecules are biosynthesized and secreted until a threshold level of cell density and autoinducer concentration is reached. Binding of autoinducers to bacterial receptors then initiates a signal transduction cascade, leading to altered gene expression. Several classes of autoinducers have been identified and targeted for inhibition as a new antibiotic strategy.

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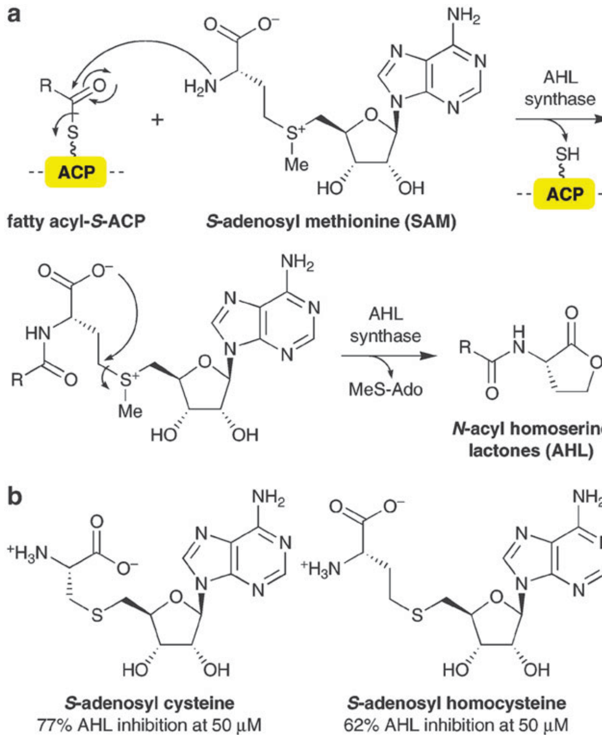

Fig. 10 (a) AHL synthase-mediated biosynthesis of acyl homoserine lactones. (b) SAM analogs inhibit the *P. aeruginosa* AHL synthase RhlI.

4.1 Inhibition of *N*-acyl homoserine lactone autoinducer biosynthesis

N-Acyl homoserine lactones (AHL) are the predominant autoinducers in Gram-positive bacteria. They are derived from *S*-adenosyl methionine (SAM) and various fatty acids loaded on acyl carrier proteins (ACP). LuxI-type AHL synthases catalyze acyl transfer to the α-amino group of SAM, followed by lactonization to form the AHL and a 5′-methylthioadenosine byproduct (Fig. 10a).

As an initial approach to developing AHL synthase inhibitors, Greenberg and co-workers tested a number of substrate and product analogs against the *P. aeruginosa* AHL synthase RhlI.[49] Several moderate inhibitors were identified, including the SAM analogs *S*-adenosyl cysteine and *S*-adenosyl homocysteine (Fig. 10b). Because SAM is a widely used cofactor, such analogs are unlikely to be effective in cellular assays. However, this study provides a basis for the development of more potent and selective AHL synthase inhibitors in the future. Two recent crystal structures of AHL synthases may facilitate the design of such inhibitors to target the biosynthesis of quorum-sensing natural products.[50,51] Furthermore, Schramm and co-workers have recently reported picomolar inhibitors of 5′-methylthioadenosine nucleosidases that are involved indirectly in regulating autoinducer biosynthetic pathways and may also be useful targets.[52]

4.2 Inhibition of quinolone autoinducer biosynthesis and of *P. aeruginosa* virulence

In addition to AHL-based quorum sensing, *P. aeruginosa* uses a second system involving quinolone autoinducers. Two key

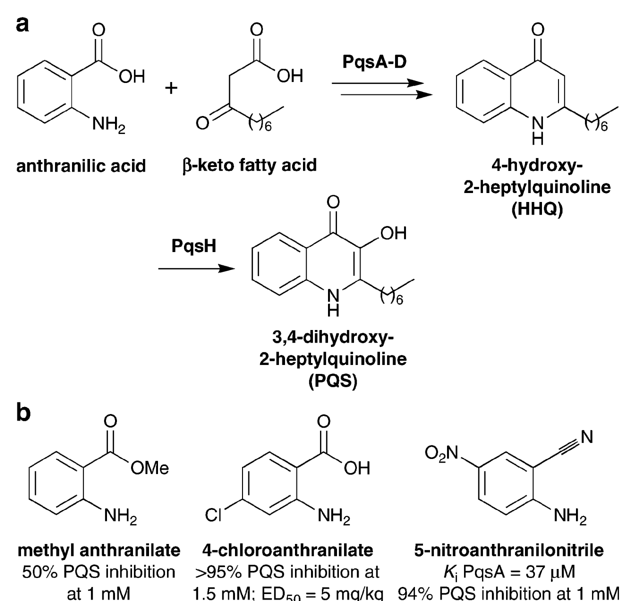

Fig. 11 (a) Biosynthesis of 4-hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (PQS). (b) Anthranilic acid derivative inhibitors of PQS and HHQ biosynthesis.

autoinducers are 4-hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (PQS, for *Pseudomonas* quinolone signal) (Fig. 11a). These autoinducers are required for the expression of several virulence factors, including pyocyanin, hydrogen cyanide, elastase, and lectins.[53] Both are known to be derived biosynthetically from anthranilic acid and a β-keto fatty acid, under the action of the *pqs* operon. HHQ is first produced, then converted to PQS.

While the exact enzymatic mechanisms of HHQ and PQS biosynthesis have not yet been elucidated, several analogs of the anthranilic acid substrate have been identified as weak inhibitors of this process. In early efforts to elucidate the biosynthetic pathway, Pesci and co-workers discovered that, at millimolar concentrations, methyl anthranilate inhibits PQS production by *P. aeruginosa*, as well as the resulting expression of elastase.[54] Recently, Rahme and co-workers have identified a number of 4- and 6-halo-anthranilic acids that also inhibit HHQ and PQS production, again at high concentrations, including 4-chloroanthranilate.[55] Importantly, this group further demonstrated that these compounds disrupt gene expression that is regulated by quinolone quorum sensing, and that they reduce the virulence of *P. aeruginosa* and mortality in a mouse infection model (5–14 mg kg^{-1} *iv*). Very recently, Pesci and co-workers have biochemically characterized PqsA as an anthranilyl-CoA ligase and have investigated a panel of anthranilic acid analogs as substrates and inhibitors of this enzyme.[56] Several moderately potent PqsA inhibitors were identified, including 5-nitroanthranilonitrile, which also inhibited PQS production in *P. aeruginosa*. Taken together, these results support the potential therapeutic value of inhibiting PQS biosynthesis and quorum sensing in *P. aeruginosa*. Increasing levels of mechanistic information on this pathway should facilitate the design of more potent inhibitors for further evaluation.

5. Conclusions and outlook

Natural product and synthetic antibiotics have been used clinically for the past 80 years to target bacterial functions that are essential for viability (*e.g.* cell wall synthesis, DNA replication, RNA transcription, protein synthesis). However, the increasing incidence of multidrug-resistant infections necessitates the investigation of new targets, such as virulence factors, which may not be essential for bacterial viability *per se*, but are required for virulence and pathogenicity in the host.[3] Pharmacological inhibition of virulence should prevent bacterial growth and damage to the host, allowing effective clearance of an infection by the host immune response. In contrast to classical bacteriocidal agents, novel antibiotics that target virulence factors may also be less prone to drive the development of resistant strains.

As described herein, a variety of natural product virulence factors have now been identified. Tremendous recent progress in elucidating the mechanistic and structural details of the corresponding biosynthetic pathways can now be leveraged to develop rationally designed inhibitors. As these new inhibitors continue to be developed, it will be imperative to advance them to animal infection models to assess the true therapeutic potential of these targets in a pharmacological context.

Thus, the role of natural products in antibiotic development is coming full circle. While many natural products, produced by microbial biosynthetic pathways, have been used successfully as antibiotics, we are now poised to use inhibitors of those same biosynthetic pathways to explore promising new therapeutic strategies to combat bacterial infections.

Acknowledgements

We thank our collaborators Prof. Luis E. N. Quadri and Dr. Julian A. Ferreras (Cornell University) for numerous stimulating discussions. D.S.T. is an Alfred P. Sloan Research Fellow. Financial support from the NIH (R01 AI068038, R21 AI063384), Northeast Biodefense Center (U54 AI057158–Lipkin), NYSTAR Watson Investigator Program, William H. Goodwin and Alice Goodwin and the Commonwealth Foundation for Cancer Research, and MSKCC Experimental Therapeutics Center is gratefully acknowledged.

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