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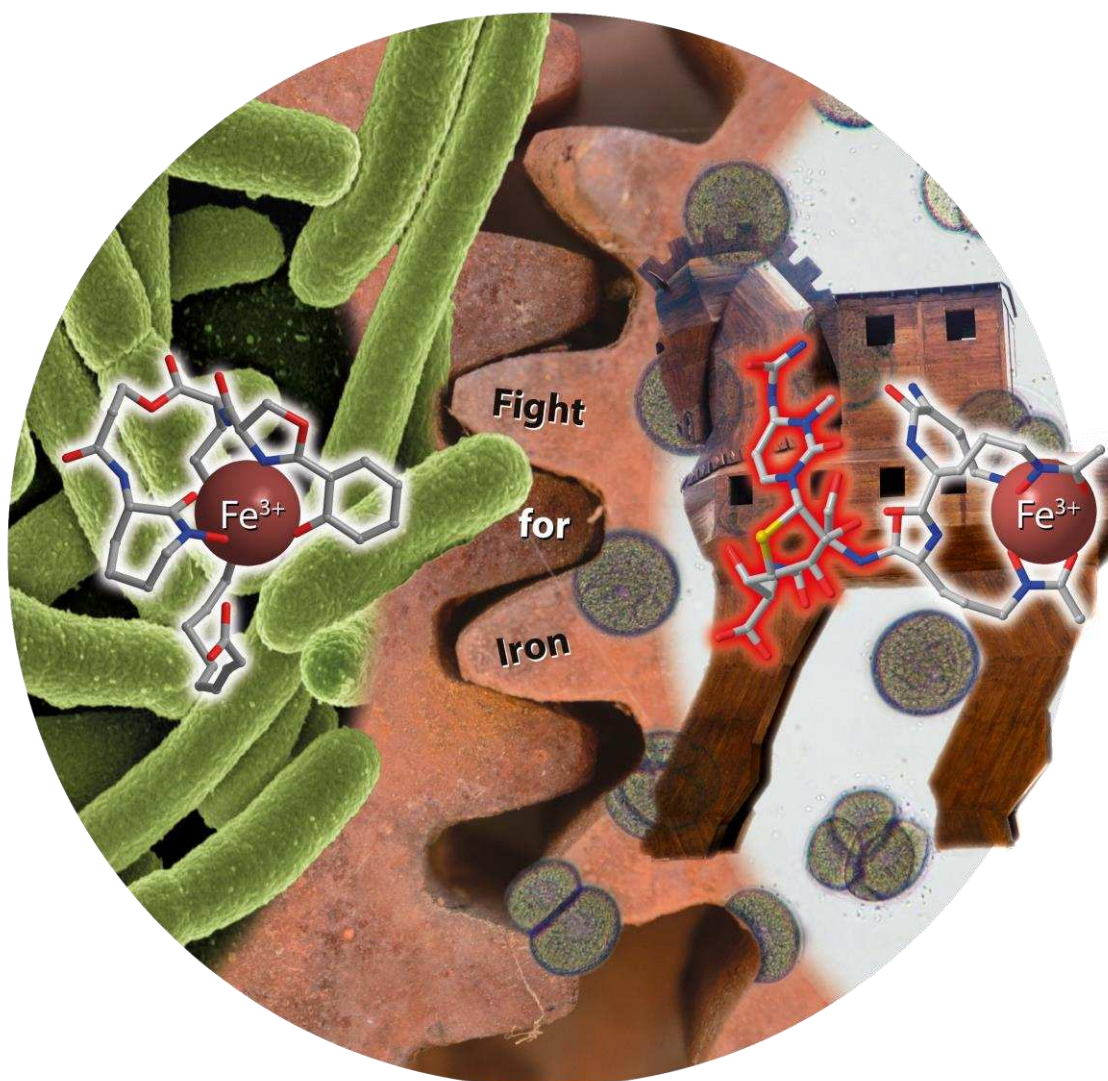
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Chemical and Biological Aspects of Nutritional Immunity – Perspectives for New Anti-infectives Targeting Iron Uptake Systems



Accepted Manuscript

Chemical and Biological Aspects of Nutritional Immunity – Perspectives for New Anti-infectives Targeting Iron Uptake Systems

Ursula Bilitewski, Joshua A. V. Blodgett, Anne-Kathrin Duhme-Klair, Sabrina Dallavalle, Sabine Laschat, Anne Routledge, and Rainer Schobert*

Dedicated to Prof. Gerhard Erker on the occasion of his 70th birthday.

Upon bacterial infection one of the defense mechanisms of the host is the withdrawal of essential metal ions, in particular iron leading to “nutritional immunity”. However, bacteria have evolved strategies to overcome iron starvation, e.g. by stealing iron from the host or other bacteria by specific iron chelators with high binding affinity. Fortunately, these complex interactions between host and pathogen regarding metal homeostasis provide several opportunities for interception and thus allow the development of novel antibacterials. The current review will give an overview with a major focus on iron, discuss recent highlights and will give some future perspectives, which are relevant in the fight against antibiotic resistance.

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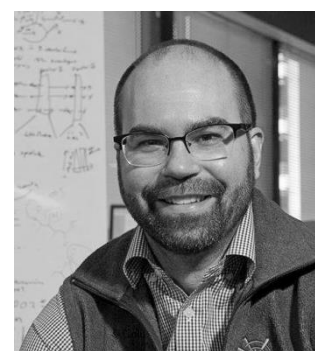
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1. Introduction

As recently emphasized by the WHO^[1] and the PEW Charitable Trust in their “Scientific Roadmap for Antibiotic Discovery”^[2] the development of novel strategies to fight bacterial infections is one of the most urgent goals for preserving public health. This is mainly due to the increasing number of bacterial strains which are resistant to a wide spectrum of antibiotics.^[3] On the other hand many pharmaceutical companies worldwide have almost abandoned the development of novel anti-infectives.^[4] Among the current research efforts the activities exploiting host–pathogen–interactions and aiming at strengthening the host immune system are particularly promising.

Infections are established when pathogens grow, proliferate or replicate within a host without limit. This is true for all types of pathogens, but in this article we focus our discussion on pathogenic bacteria.

A prerequisite for unlimited proliferation of bacteria is the adequate supply with nutrients. Thus, the first line of defense of the host against infections is the withdrawal of nutrients from the pathogen, a phenomenon called “nutritional immunity”. Whereas pathogens can adapt central metabolic pathways to the respective available carbon and nitrogen sources, hosts have developed efficient means to limit the availability of essential metal ions. This is reflected in *in vivo* transcriptomic studies of bacteria from infection sites of animal models showing a stress response to metal ion limitation.^[5–7] The best studied example is the dependence of pathogens on iron^[8] so that most research approaches aiming at the exploitation of nutritional immunity as therapeutic target address the iron acquisition systems. That is why in the current review mainly iron acquisition systems and respective chemical inhibitors are described, but where possible we also include chemical approaches to influence the uptake of other metal ions. For various other aspects related to nutritional immunity, the recent reviews on microbiological aspects,^[9,10] coordination chemistry of siderophores,^[11] environmental applications of siderophores,^[12] zinc homeostasis^[9,13] and chemical synthesis of antibacterials^[14] are highly recommended. More specific reviews can be found in the following chapters.

2. Iron Acquisition, one Aspect of Nutritional Immunity and of Host-Pathogen Interactions

Almost all organisms depend on iron due to its favorable properties as redox and as complexing agent. Thus, iron ions are present as different species and the $\text{Fe}^{2+/3+}$ redox couple is involved in electron transfer reactions of the energy metabolism, but also in stabilizing protein structures and transportation of oxygen. In response to the iron - deficient situation in the host bacteria have developed various iron uptake systems^[8] which are specific for the available iron species (Figure 1).

Within a vertebrate host the most abundant iron species is heme (approximately 70% of the total iron). Heme is a cofactor of intracellular proteins, for example catalyzing electron transfer reactions in cytochromes and peroxidases or allowing oxygen transport by hemoglobin. Thus heme can be used as an iron source only by intracellular pathogens or by pathogens, which produce cytolysins or hemolysins to disrupt host cells and lead to the release of hemoproteins. Though heme is essential also for host cells, free heme becomes

negative bacteria is an energy consuming step, which is enabled by coupling to the membrane potential of the inner membrane via the transperiplasmic protein TonB and the proteins of the inner membrane ExbB and ExbD, similar to the transport of siderophores (see below) (Figure 1). Direct heme transport through the periplasm and the peptidoglycan layer respectively occurs via binding proteins, e.g. PhuT and IsdC. Usually expression of these protein systems is regulated in response to iron limitation via the Fur (ferric uptake regulator) repressor.

Extracellular iron would be more easily accessible to invading bacteria. However, due to the aerobic conditions in body fluids (serum, tears, sweat) extracellular iron usually is present as Fe^{3+} and is predominantly complexed by transport proteins, such as transferrin (Tf) and lactoferrin (Lf).^[16] These high-affinity protein complexes contribute to the iron-limited environment experienced by bacteria invading a host. Pathogens have developed different systems to get access also to these iron species: bacteria from the *Neisseriaceae* and *Pasteurellaceae* families bind the iron-loaded host proteins via surface receptors, extract the Fe^{3+} -ions and channel them through the bacterial cell membranes.^[17,18]

More common, however, are low-molecular weight Fe^{3+} -chelating compounds (typically <1 kDa), the siderophores, which are secreted by microorganisms and steal Fe^{3+} from the host proteins due to higher affinities. To date more than 250 different siderophores are structurally characterized and grouped according to the functional groups which are involved in Fe^{3+} -coordination. The most common motifs are catecholates, hydroxamates, α -hydroxycarboxylates and phenolates (examples are shown in Figures 3–5), which can also be combined in “mixed type” siderophores.^[19] Binding constants for Fe^{3+} vary over more than 30 orders of magnitude. As only deprotonated donor atoms (O and N) are effective, binding constants are dependent on pH and the competitive advantages of the different siderophore types depends on the body niche and its physico-chemical properties.

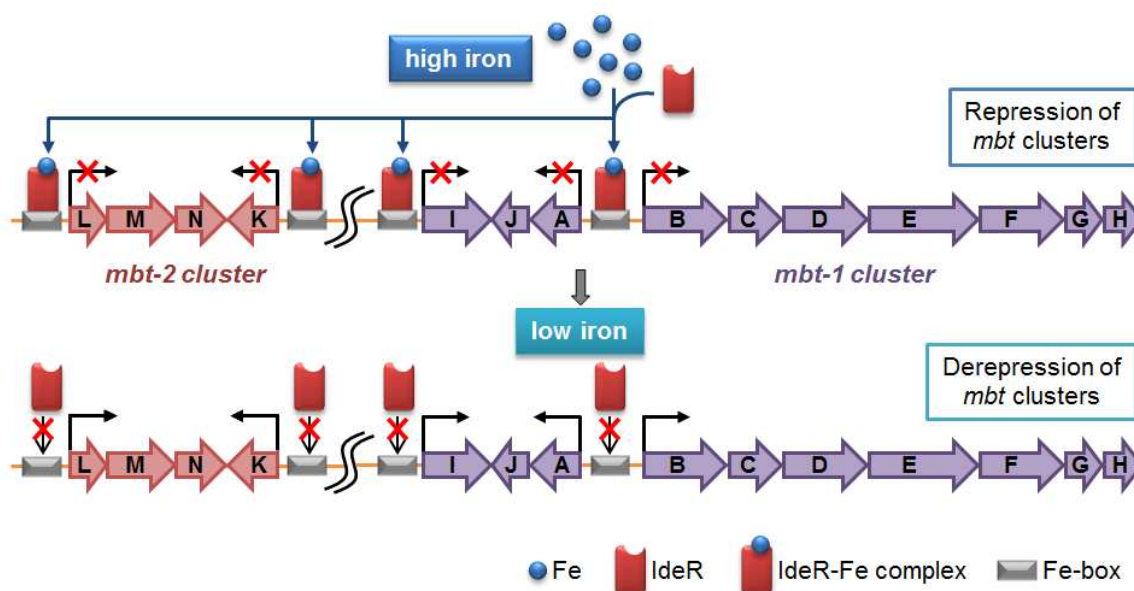


Figure 2. Control of gene expression via IdeR by Fe^{2+} concentration. In the presence of high iron concentrations in *Mycobacterium tuberculosis* biosynthesis of siderophores is repressed, as the transcriptional regulator IdeR (iron dependent regulator) is complexed with iron. This IdeR-Fe-complex is bound to DNA and represses expression of genes, which have a Fe-box in their promoter regions, such as the mbt gene clusters for the synthesis of the siderophore mycobactin (see also Fig. 3). Under low iron conditions iron dissociates from the IdeR-Fe-complex, which induces the relief of the repression and induces expression of the respective genes (redrawn from Ref. [21]).

Biosynthesis of siderophores is regulated via intracellular iron concentrations following the principle of induced gene expression under low intracellular iron concentrations, as shown for *Mycobacterium tuberculosis* in Figure 2. Other bacteria use the Fur transcriptional regulator instead of IdeR (iron dependent regulator). A comprehensive description of Fur regulation can be found in a recent review.^[20]

Two different synthesis principles are known, either via the protein complexes of non-ribosomal peptide synthetases (NRPS) (Figure 3) or via NRPS-independent siderophore (NIS) synthesis. NRPS incorporate aryl and amino acid building blocks, whereas NIS synthesis is based on amide or ester bond formation between dicarboxylic acids, diamines, amino alcohols or alcohols. Siderophore biosynthetic pathways have been reviewed by Challis.^[22]

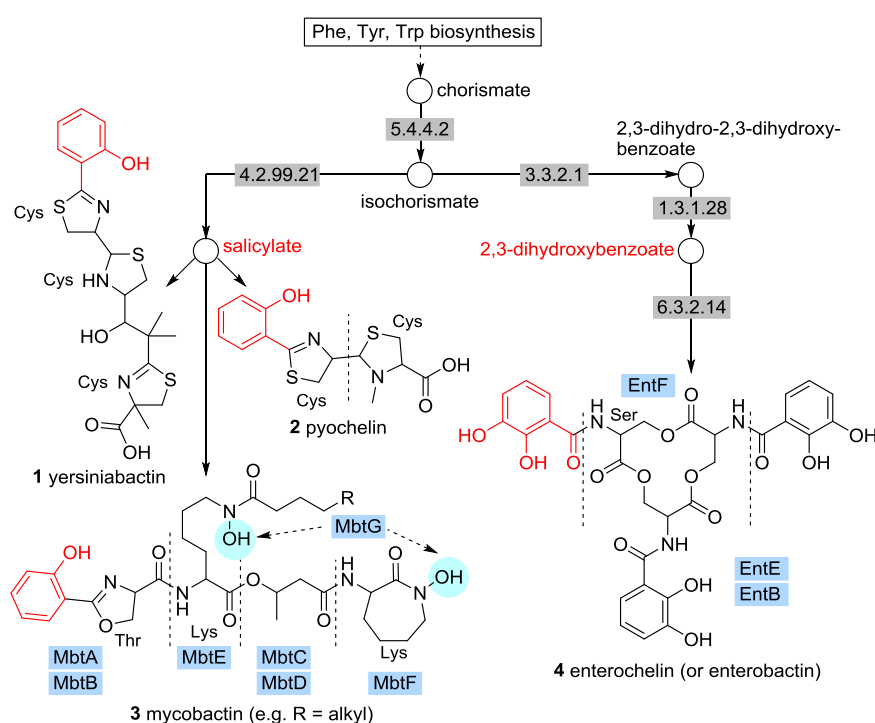


Figure 3. NRPS-dependent siderophore biosynthesis showing the pathways for example to the phenolate siderophores yersiniabactin **1**, pyochelin **2** and mycobactin **3** (see chapter 3.2.) and the catecholate siderophore enterochelin **4**. EC: 5.4.4.2 Isochorismate synthase; 3.3.2.1 bifunctional isochorismate lyase/acyl carrier protein; 4.2.99.21 isochorismate pyruvate lyase; 1.3.1.28 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase; 6.3.2.14 enterobactin synthase; Source: KEGG-database.

After synthesis, siderophores are secreted from the cell via export systems and get loaded with Fe^{3+} in the extracellular space.^[23] Fe^{3+} -loaded siderophores are bound by specific cell-surface receptors, e.g. HtsA from *Staphylococcus aureus* binds the carboxylate siderophore staphyloferrin A, FepA from *E. coli* the catecholate siderophore enterochelin **4**. In Gram-negative bacteria internalization into the cytoplasm is achieved via energy-consuming TonB-dependent transport through the outer membrane (Figure 1), shuttling by periplasmic binding proteins, e.g. FepB from *E. coli*, and transportation into the cytosol via ATPases, e.g. FepCDG. In the cytosol Fe^{3+} is extracted from the siderophore and is reduced to Fe^{2+} (Figure 1).

In response to secretion of the siderophore enterochelin **4**, nowadays better known as enterobactin (Ent), by Gram-negative bacteria, neutrophils and host mucosal cells secrete the

Ent-binding protein lipocalin 2 (Lcn2), thus disrupting the bacterial iron acquisition system by neutralizing the siderophores and preventing their re-uptake by the bacteria. As a countermeasure pathogens produce alternative siderophores, which are not bound by lipocalin 2, for example yersiniabactin 1 or glycosylated Ent, also called salmochelin.^[24]

Thus, bacteria possess several, at least partly redundant iron acquisition systems, which allow adaptation to different environmental niches according to the respective available iron source. As pathogens are highly dependent on iron acquisition, these systems are attractive targets for therapeutic intervention or vaccination strategies. Due to its almost universal presence the siderophore biosynthesis and uptake systems are most frequently targeted either by inhibitors or by siderophore-antibiotic conjugates, so-called “Trojan horses”, which utilize the siderophore transport proteins to facilitate transportation of antibiotics into the cells.^[25] However, some pathogens, such as *Staphylococcus aureus* rely on heme utilization and inhibition of the access to intracellular heme proteins could also be a valuable therapeutic target.^[26,27]

3. Inhibition of the Acquisition of Extracellular Iron

3.1. Regulation of Iron Uptake Systems

As iron is an essential but in too high concentrations also a toxic metal ion, its uptake is carefully regulated by the availability of $\text{Fe}^{2+/3+}$. Thus, only when intracellular Fe^{2+} concentrations are low, the genes for siderophore biosynthesis, siderophore or heme receptors and binding proteins are expressed. This regulation is largely dependent on DNA binding proteins, such as Fur and IdeR (Figure 2), which bind Fe^{2+} , but also Fe^{3+} and other divalent metal ions, such as Zn^{2+} , Mn^{2+} or Co^{2+} . Metal binding increases the stability of a Fur-dimer and the affinity of Fur to its DNA binding site, which represses expression of genes involved in iron uptake. Decreasing intracellular metal ion concentrations leads to dissociation of the metal ion from the protein and as a consequence to the dissociation of the protein from the DNA, and thus to the induction of gene expression. It is expected that any interference with this regulatory system will affect bacterial viability and physiology, as inhibition of DNA binding will lead to uncontrolled uptake of $\text{Fe}^{2+/3+}$ and thus to the toxic effects from high Fe^{2+} concentrations, and activation of DNA binding even in the absence of Fe^{2+} will further decrease intracellular Fe^{2+} concentrations.

Inhibition of IdeR binding to DNA was already described in 2007 by Monfelli and Beeson.^[28] They claimed that they had identified small peptides, which modulated IdeR activity. However, no peptide sequences were given. This approach was followed by Michaud-Soret and Crouzy,^[29] who identified peptide aptamers, which inhibited the activity of Fur in the μM range. However, these peptides were tested only in cell-free *in vitro* systems so that the final proof of concept is still pending.

Klemm made use of the higher affinity of Fur for Zn^{2+} and Co^{2+} and added 500 μM Zn^{2+} to the medium during biofilm formation by *Escherichia coli* and *Klebsiella pneumoniae* under nonlimiting iron conditions.^[30] They observed significantly reduced biofilm formation in microtiter plates and also on urinary catheters, which they took as a proof that iron uptake is important for biofilm formation and that interference with these uptake systems may be an effective way to eradicate biofilms from pathogenic strains.

Zn^{2+} also is an essential trace element, which is toxic at high concentrations, requiring regulated uptake systems, as described for Fe^{2+} . Indeed, high-affinity uptake systems are described for example for the Gram-negative bacteria *Escherichia coli*^[31a] and *Acinetobacter baumannii*,^[31b] the expression of which is regulated by the DNA-binding protein Zur (zinc uptake regulator), which has high homology to the iron uptake regulator Fur and whose activity is also dependent on dimerization. However, until now no chemical inhibitors of Zur activity are described.

3.2. Biosynthesis of Siderophores

Under aerobic conditions iron usually exists in the Fe^{3+} oxidation state. Due to its low solubility, this form is hardly bioavailable, which has led to the evolution of biosynthetic pathways for small molecules with high binding affinity for Fe^{3+} called siderophores. Such molecules are broadly employed by microbes for iron acquisition, including pathogens and non-pathogens.

For environmental microorganisms the ability to block neighboring strains from producing siderophores, thus restricting critical iron uptake, is a competitive advantage. Screens to discover such inhibitors of two pathogen associated NIS biosynthetic enzymes^[32] led to the discovery of baulamycin A & B **5a,b**. Baulamycin A (**5a**) is the most active of the pair, inhibiting AsbA and SbnE with IC_{50} values at 180 μM and 4.8 μM , respectively (Figure 4). These enzymes conjugate spermidine **9** or *L*-2,3-diaminopropionic acid **10** with citrate **8** in an ATP dependent fashion during staphyloferrin **6** and petrobactin **7** siderophore biosynthesis.

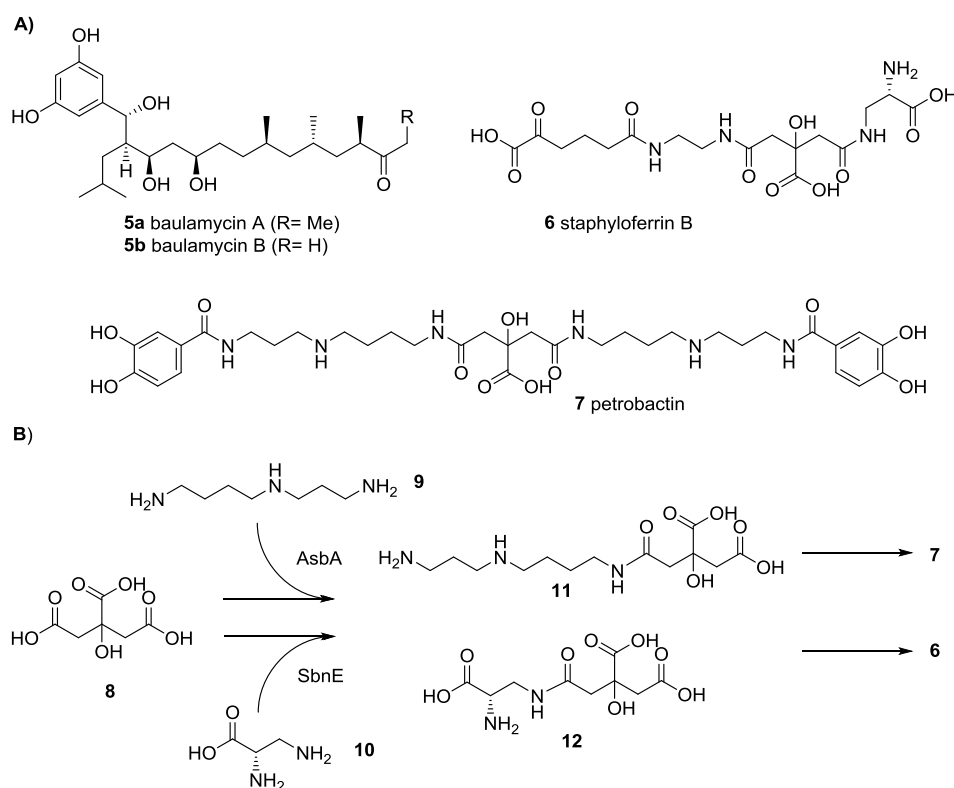


Figure 4. **A)** Baulamycin A **5a**, a natural product discovered in screens for staphyloferrin and petrobactin-family siderophore inhibitors. **B)** Baulamycins inhibit the AsbA-catalyzed condensations of citrate **8** with spermidine **9** in petrobactin **7** biosynthesis, and the analogous SbnE-catalyzed condensation of **8** and diaminopropionic acid **10** in staphyloferrin **6** biosynthesis.

These siderophores are important for *Bacillus anthracis* and *Staphylococcus aureus* virulence,^[33] and baulamycins **5** are antibacterial when assayed against these bacteria. However, **5a** causes growth inhibition under both iron-limiting and iron-replete conditions at similar concentrations, suggesting the antibiosis of these compounds unlikely stems from siderophore inhibition alone. Despite the uncertainty surrounding baulamycin targets *in vivo*, **5** rank among the only natural siderophore biosynthetic inhibitors discovered thus far. Molecules that act analogously to the baulamycins **5**, but inhibit other siderophore biosynthesis pathways likely exist in nature.

The knowledge of siderophore biosynthesis also provides the opportunity for targeted design of synthetic small molecule inhibitors which have the overall effect of withholding essential iron from the microorganism, inducing nutritional immunity. A comprehensive overview of siderophore biosynthesis inhibitors was recently published.^[34] Thus, we focus on a survey of current research (2015/2016); the majority of the work in this period has been targeting siderophore biosynthetic pathways of *Mycobacterium tuberculosis*, the causing agent of tuberculosis, specifically adenylation inhibitors. Although this target was covered in the Lamb review our review focuses on the most recent work in the area. Two publications in the period surveyed inhibitors that target other siderophore biosynthetic pathways. Tan's α -hydroxyacyl-adenosine 5'-*O*-monosulfamate derivatives to probe inhibition of HMWP2 via inhibition of the cysteine adenylation domain and subsequent yersiniabactin **1** production.^[35] Kirienko's use of 5-fluorouridine to inhibit pyoverdine biosynthesis in *P. aeruginosa*^[36] via RNA disruption are included here for completeness.

M. tuberculosis is exposed to the iron-limited intracellular environment of macrophages during host infection. Intracellular survival of the bacteria depends on the production of mycobactin-class siderophores **3** (e.g. **3a**, Figure 5), making the pathogen's iron uptake system an attractive target for therapeutic intervention.

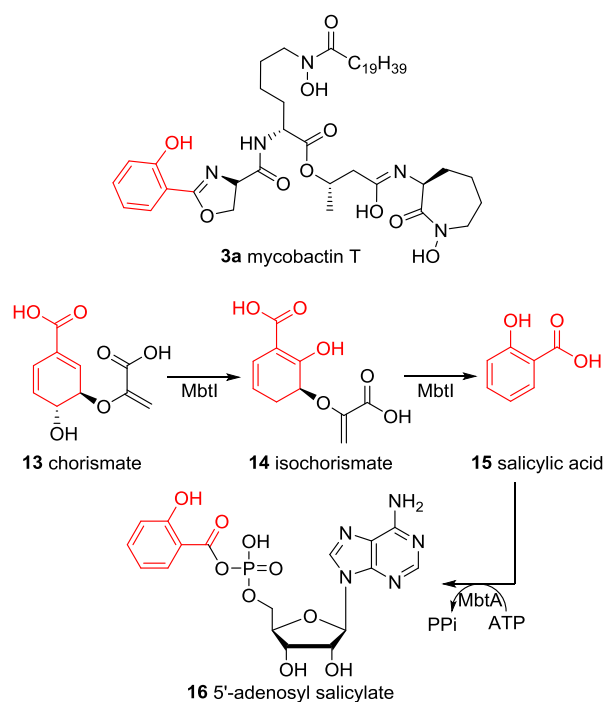


Figure 5. Biosynthesis of 5'-adenosyl salicylate **16**, a precursor of the siderophores **1** and **3**.

Mycobactins **3** have salicylic acid **15** as a structural substituent, which is also common to a number of other pathogen siderophores, including yersiniabactin **1**, produced by Gram-negative *Yersinia* sp.. Siderophores containing **15** are particularly suitable for drug design because **15** is an important metabolite in some plants and bacteria, while humans do not produce the compound minimizing toxicity risk after dosing.

In *M. tuberculosis*, **15** is elaborated from the central metabolite chorismate **13** by MtbI, a salicylate synthase that acts via an isochorismate **14** intermediate. After MbtI produces **15**, it is activated for NRPS incorporation in a reaction catalysed by MbtA, a representative of the aryl acid adenylating enzymes (AAAE). These enzymes are present in a number of pathogenic bacteria and are essential for NRPS-dependent siderophore biosynthesis.^[37] The reaction requires ATP and releases inorganic pyrophosphate and the salicyl adenylate **16**, which is subsequently loaded on the NRPS assembly line finally leading to **3**. MbtI and MbtA have been targeted with biosynthetic intermediate analogs, as noted below.

The nucleoside antibiotic 5'-O-[N-(salicyl)sulfamoyl]adenosine (SAL-AMS **17**) is a prototype for a new class of antibiotics that inhibit AAAE.^[37,38] Numerous analogs have been synthesized (covered in the Lamb review)^[34] but more recently, Aldrich synthesized 2'- and 3'-fluorine analogs of a sulfamide isostere **18**^[39] with the sugars adopting the northern (C3'-endo, C2'-exo) or southern (C2'-endo, C3'-exo) pucker (Figure 6).

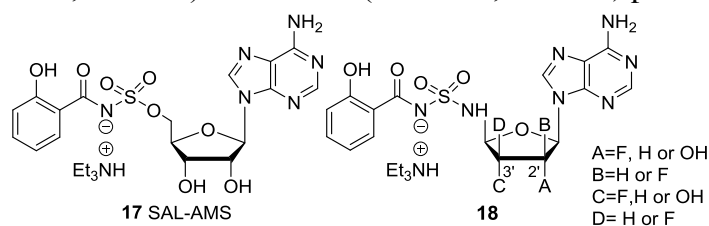


Figure 6. Structure of nucleoside antibiotic SAL-AMS **17** and modified derivatives **18**.

The six synthesized compounds were screened for enzyme inhibition against MbtA, the molecular target for **17** in *M. tuberculosis*. The results showed a bias for the C3'-endo for activity (C3' fluoro 0.60 μ M, C2' fluoro 2.3 μ M against *M. tuberculosis* H37Rv). It was also found that C2'-endo fluorination improved pharmacokinetic (PK) parameters in *i.v.* and *p.o.* measurements when compared to the C3' fluoro analog.

Aldrich further probed structure-activity relationship of SAL-AMS analogs **19**, with ten compounds encompassing a variety of structural modifications (Figure 7),^[40] again concluding C2' fluorination (R^4) being the most successful modification, maintaining activity against MbtA whilst improving PK properties. Other modifications, enhancement of lipophilicity and prodrug approaches, e.g. ester prodrugs of C2'-fluorosulfamide isostere **21** vs. parent compound **20** led to inferior PK data (plasma stability at $t_{1/2}$ typically lower than **20**).^[41] An inherent limitation of SAL-AMS **17** is degradation via cyclonucleoside formation.^[42] Although this does not occur in 8-aza-3-deaza adenines (e.g. **22**) as they lack the correctly positioned N required for intramolecular cyclisation, bioactivity against whole-cell *M. tuberculosis* H37Rv was limited and only revealed under iron deficient conditions for all synthesized analogs (e.g. R^1 = Ph MIC 25 μ M).^[43] In contrast to adenosine derivatives, inhibition of the transition state of MbtI-catalyzed reaction of **13** to isochorismate **14** by compounds such as **23** was found to be less promising (<10% inhibition of MbtI at 100 μ M).^[44]

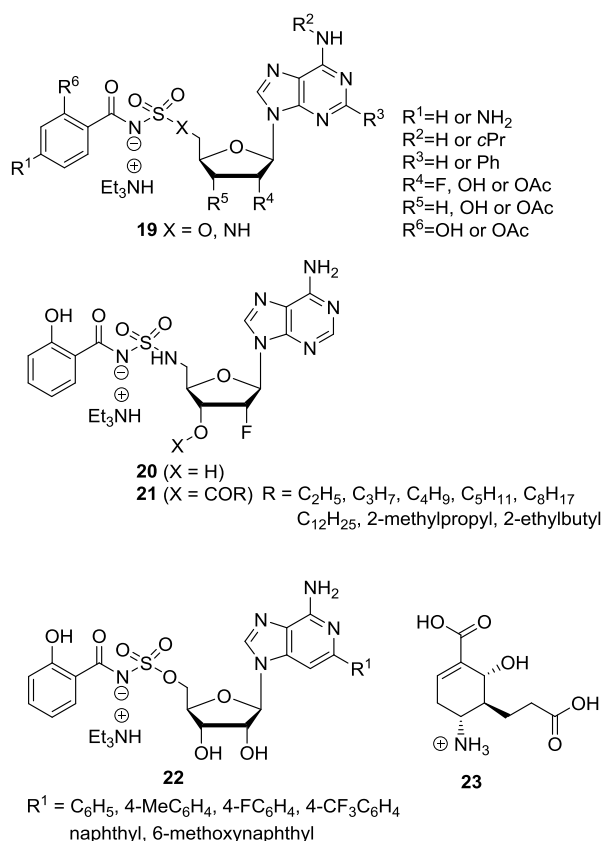


Figure 7. Additional SAL-AMS analogs synthesized to improve PK properties.

3.3. Regulation of Siderophore Biosynthesis via Quorum Sensing Systems

Bacteria are able to adapt the expression of genes in response to cell density via intercellular communication. The “language” of this communication are small signalling molecules (autoinducers), which are secreted into the surrounding medium.^[45] The phenomenon of quorum sensing (QS) uses the principle that at low cell densities the concentrations of these autoinducers are too low to bind to their receptors. Only when the concentration of the signal has reached a critical threshold the autoinducers bind to specific cognate receptor proteins, which then start to regulate transcription by activating (or repressing) the expression of genes under QS control (Figure 8).^[46]

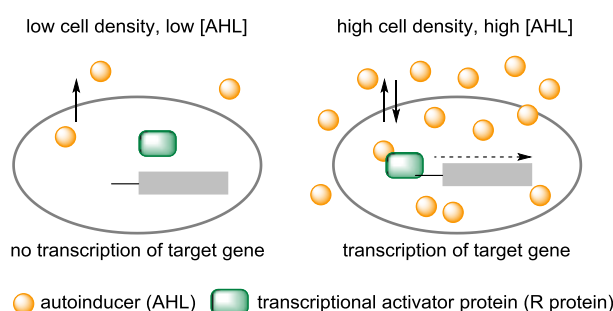


Figure 8. Principle of quorum sensing. N-acyl homoserine lactones (AHL) are the best known bacterial auto-inducers; R is the autoinducer receptor protein, usually coupled to a transcriptional regulator (redrawn from Ref. [47]).

A large variety of physiological processes, among which are biofilm formation and the expression of key virulence factors such as proteases and siderophores, are regulated by QS.

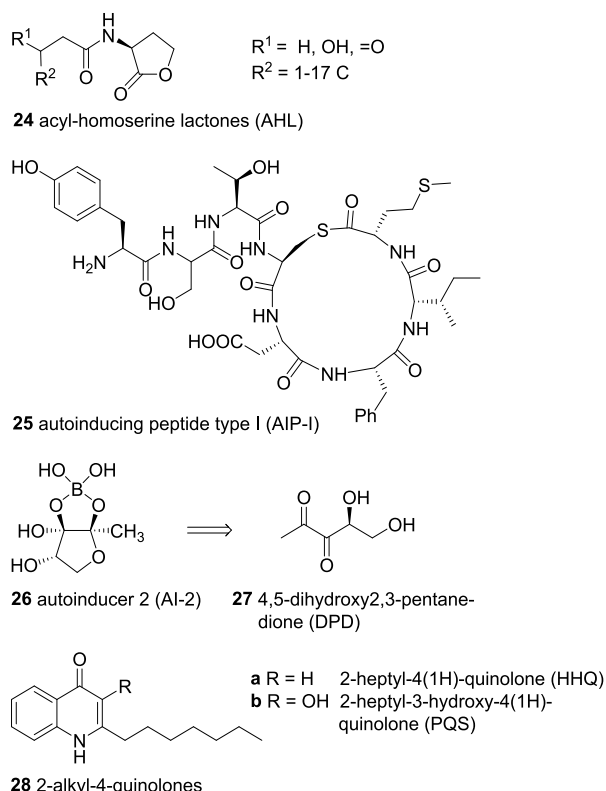


Figure 9. QS signalling molecules.

Mediators of QS signalling can be broadly divided into four major classes:^[4b,48] i) N-acyl homoserine lactones (AHLs) **24**, produced by many Gram-negative bacteria; ii) oligopeptides (e.g. autoinducing peptides (AIPs) **25**), made of 5–20 residues, generally produced by Gram-positive bacteria; iii) autoinducer-2 (AI-2) **26**, a group of furanones derived from 4,5-dihydroxy-2,3-pentanedione (DPD) **27**, produced by both Gram-negative and Gram-positive bacteria; iv) 2-alkyl-4-quinolones **28** including 2-heptyl-4(1H)-quinolone **28a** (HHQ) and the pseudomonas quinolone signal (PQS) 2-heptyl-3-hydroxy-4(1H)-quinolone **28b** (Figure 9).^[49,50]

QS based systems may play different roles in controlling siderophore production. The AHL-mediated quorum sensing-disrupter **29** (Figure 10) from the marine red alga *Delisea pulchra* was found to inhibit the formation of the siderophore produced by *P. putida* F1 in a concentration-dependent manner (the addition of **29** decreased siderophore concentration by 1.7 fold at 20 $\mu\text{g mL}^{-1}$ and 40 $\mu\text{g mL}^{-1}$ and 2.3 fold at 100 $\mu\text{g mL}^{-1}$). On the contrary, siderophore synthesis in *P. aeruginosa* PAO1 was stimulated (it induced siderophore production by 3.5-fold at 100 $\mu\text{g mL}^{-1}$).^[51]

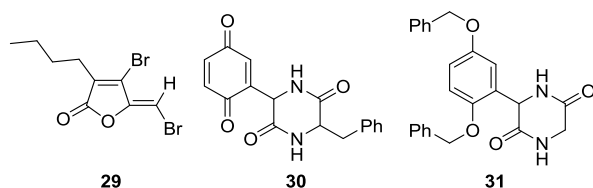


Figure 10. Selected compounds interfering with QS systems based on **24**.

Scoffone aimed at specifically inhibiting the *Burkholderia cenocepacia* QS-system, which comprises the receptor CepR and the synthase CepI catalysing synthesis of *N*-octanoyl-L-homoserine lactone.^[52] Out of ten different compounds assayed against recombinant CepI, four were effective inhibitors, with IC₅₀ values in the micromolar range (e.g. **30**, IC₅₀ = 5 μM and **31**, IC₅₀ = 30 μM, Figure 10). The best compounds interfered with protease and siderophore production and with biofilm formation, and showed good *in vivo* activity in a *Caenorhabditis elegans* infection model. These compounds, when tested on human cells, showed very low toxicity (significant effects on HeLa cell growth were observed only after 48h treatment at a concentration of 100 μM).

P. aeruginosa possess a complex QS network, which comprises representatives of the compound classes **24** and **28** and which is involved in the regulation of a number of virulence phenotypes. In particular the compound **28b** plays multifunctional roles in iron entrapment.^[53] **28b** not only is involved in the regulation of the biosynthesis of the siderophores pyoverdinin and pyochelin **2** but it also forms iron complexes, which are not siderophores but instead appear to trap iron at the cell surface and facilitate siderophore-mediated iron uptake. Moreover, they control the expression of iron-regulated and iron-independent genes.^[53] Thus, inhibition of the **28b** signalling cascades leads to inhibited production of QS-controlled virulence factors and of the multi-level iron acquisition systems of *P. aeruginosa*. In the following some examples are mentioned.

Several studies have focused on the discovery of inhibitors of the receptor of **28**, PqsR. The synthesis of various PqsR-antagonists was described, which had high affinity for the PqsR ligand-binding domain and strongly inhibited PqsR activity in a heterologous *E. coli*-based or *P. aeruginosa* reporter system.^[54–56] Examples are **28a** analogs (compound **32**; IC₅₀ = 51 nM),^[54] the hydroxamic acid **33** (IC₅₀ = 12.5 μM)^[55] and 2-aminooxadiazole **34** (EC₅₀ = 7.5 μM) (Figure 11).^[56] In *P. aeruginosa* reduced production of the redox-active phenazine antibiotic and of the virulence factor pyocyanin **35** was observed. **35** reduces Fe³⁺ to bioavailable Fe²⁺, leads to damage of host cells, alters immune responses and contributes to the persistence of *P. aeruginosa* in the lung of cystic fibrosis patients.^[57]

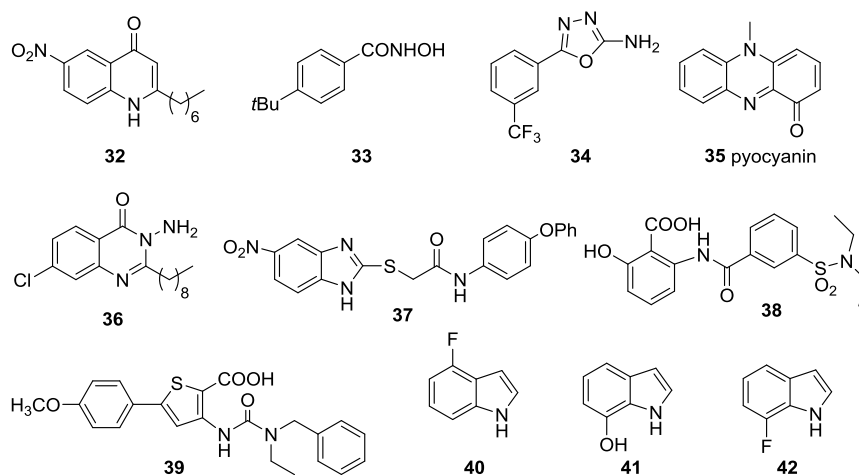


Figure 11. Selected compounds interfering with the QS system based on **28b** and structure of pyocyanin **35**.

Recently, the structure of the PqsR co-inducer binding domain has been solved.^[58] This structural information was used to guide the synthesis of a series of quinazolinone analogs as

possible PqsR antagonists. Compound **36** was identified as a potent inhibitor of the **28b**-dependent QS system ($IC_{50} = 5 \mu M$), strongly reducing pyocyanin production, lectin expression, and biofilm development. Notably, also the crystal structure of PqsR bound to **36** was determined, providing valuable data for further development. Benzimidazole **37** was among the first compounds to reduce formation of antibiotic-tolerant persister cells.^[59]

Elucidations of the **28** biosynthesis mechanisms^[60–62] have allowed a rational design of QS biosynthesis inhibitors, such as the 2-benzamidobenzoic acid derivatives **38** (IC_{50} PqsD = $1.2 \mu M$),^[63] 5-aryl-ureidothiophene-2-carboxylic acids **39** (IC_{50} PqsD = $6 \mu M$)^[64] and indoles **40–42**. These latter have been shown to inhibit **28b**, pyocyanin **35**, pyoverdine, and rhamnolipid production in *P. aeruginosa*.^[65,66]

Thus, though these compounds do not directly target siderophore biosynthesis, they indirectly inhibit iron acquisition systems, as they interfere with the regulation of their expression.

When QS regulation, and thus the signaling molecules **24**, are no longer needed, they are converted to the corresponding 3-acyltetramic acids (3ATA), for example the autoinducer N-dodecanoyl-L-homoserine lactone of *P. aeruginosa* is converted to 3-decanoyl-5-(β -hydroxyethyl)pyrrolidine-2,4-dione **43a** through an irreversible, nonenzymatic Dieckmann reaction (Figure 12).^[67,68]

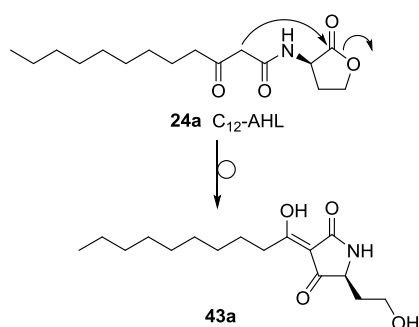


Figure 12. Conversion of an autoinducer **24a** to an antibacterial 3ATA.

This conversion may even be induced by metal chelation. The 5-(β -hydroxyethyl) substituted 3ATA are potent antibacterial agents acting by dissipating both the membrane potential and the pH gradient of Gram-positive bacteria.^[69] They also elicit a negative feedback to biofilm formation by withdrawing the necessary iron.

Williams proved that synthetic 5-(β -hydroxyethyl) substituted 3ATA may also inhibit QS in *S. aureus* by acting as negative allosteric modulators of the autoinducer receptor resulting in vastly reduced bacterial virulence. The concentration window between the desirable reduction of the virulence and the undesirable inhibition of the growth of bacteria can be enlarged by adjusting the length of the 3-acyl side-chain.^[70]

3.4. Competition for Iron

3.4.1. Siderophore Cheating

The concept of cheating is common in bacterial iron acquisition,^[71] where cheating cells unable to produce siderophores rely on neighboring organisms for production. In recent experiments examining co-cultivated assemblages of environmental bacteria, certain isolates were found to be completely dependent on neighboring bacteria for growth.^[72,73] Chemical

dissection of this dependency confirmed siderophores have a critical role, supporting the idea that siderophore piracy is a potentially common and essential survival strategy for some bacteria. Certain pathogenic bacteria appear to engage in siderophore piracy, even among nearly clonal strains. *P. aeruginosa* populations accumulate siderophore (pyoverdine)-negative mutations during host infections, and the surviving mutants presumably acquire iron by scavenging iron-pyoverdine complexes produced by neighboring cells.^[74] Growing evidence suggests that a number of bacterial pathogens cheat not only by intercepting intact siderophores, but also by utilizing siderophore degradation products. Structural and biophysical studies on *Campylobacter jejuni*, an organism not known to produce their own siderophores revealed how CeuE, a periplasmic siderophore binding protein can scavenge ‘second-hand’ enterobactin hydrolysis fragments with high affinity.^[75] This ability contrasts starkly with more typical Fe^{3+} uptake involving intact hexadentate siderophores.

Iron piracy is also documented in organisms that can produce their own siderophores. Siderophore piracy is genomically evident in *Rhodopseudomonas palustris*, where siderophore receptors far outnumber siderophore biosynthetic loci.^[76] In the clinic, a number of pathogenic Staphylococci are documented to utilize hydroxamate and catecholate siderophores, molecules they lack biosynthetic genes for.^[33] Complex siderophore piracy phenotypes have emerged during studies on differentiation and stress response in actinomycete bacteria. Actinobacterial competition screens, historically detecting siderophores^[77] and ionophores,^[78] recently led to the discovery of the potent mixed-ligand siderophore amyachelin. Further characterization revealed *Amycolatopsis* sp AA4-produced amyachelin controls co-cultivated *Streptomyces coelicolor* bacterial development in part by direct siderophore-mediated iron competition.^[79] But *Amycolatopsis* also affects *S. coelicolor* by scavenging *S. coelicolor*’s ferrioxamine siderophores, further depriving the co-cultivated strain of iron.

In studies on food-safe bacteria having antibacterial properties, a series of human gut associated *Bifidobacterium* strains were discovered that strongly inhibits the growth of Gram-negative and Gram-positive bacteria in an iron dependent manner suggestive of siderophore competition.^[80] Of note, *Bifidobacterium* RecB1 inhibited the common lab bacteria *Escherichia coli* K-12 and *Micrococcus luteus* in addition to the serious gut pathogens *Clostridium perfringens* and *Clostridium difficile*. Although incomplete siderophore-linked bacterial growth inhibition was noted above, the clear broad spectrum-antibiosis demonstrated by these *Bifidobacteria* is striking. Unfortunately the siderophores responsible remain uncharacterized.

3.4.2. Synthetic Iron Chelators

Synthetic high-affinity iron chelators can have a biostatic effect on pathogens when they decrease the amount of Fe^{3+} that can be utilised by a pathogen. This biostatic effect relies on the competition, both thermodynamically and kinetically, between natural microbial siderophores and the added synthetic iron chelator. In the design of synthetic iron chelators it is essential to ensure that the iron complexes formed cannot be taken up and utilised by a pathogen, as this could lead to growth promotion rather than growth inhibition. Since coordinating groups that are commonly found in natural siderophores are likely to be recognised by microbial transporters, these groups should be avoided in the design of

synthetic antimicrobial iron chelators. Synthetic chelate ligands, such as polyaminocarboxylates, organophosphonates, 8-hydroxyquinolines and related compounds have long been known to possess bacteriostatic properties.^[81,82] Also hydroxypyridinones (HOPOs) **44–46** fulfil this requirement and hence have attracted interest as alternative iron-binding units (Figure 13).^[81,83–88]

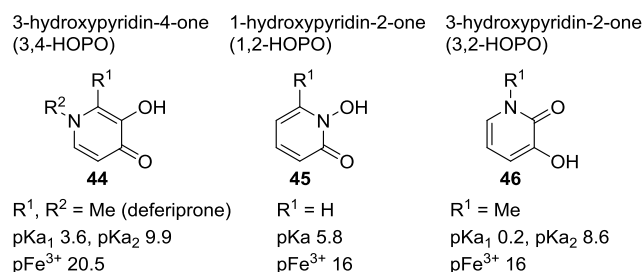


Figure 13. Structures, pK_a and pFe values of selected hydroxypyridinones (pFe³⁺ = -log[Fe³⁺] when [Fe³⁺]_{total} = 10⁻⁶ M and [ligand]_{total} = 10⁻⁴ M at pH 7.4).^[83]

In their deprotonated form, these ligands provide adjacent oxygen-donors that can form 5-membered chelate rings and are able to compete thermodynamically with the chelating units commonly found in natural siderophores, such as catecholates, hydroxamates and hydroxycarboxylates. Since HOPO groups are rarely found in siderophores, their iron complexes were expected not to be taken up by receptor-mediated bacterial transport. Of the three types of metal-chelating HOPOs (Figure 13), the 3,4-HOPOs have the highest affinity for Fe³⁺, as reflected by their high pFe³⁺ values, a property that led to their application in chelation therapy. Whilst bidentate ligands, such as deferiprone, are now established drugs in the treatment of iron overload, the development of antimicrobial iron chelators has moved on from bidentate to hexadentate ligands.^[83,84] Since size restrictions do not apply to extracellularly-acting antimicrobial metal chelators, the higher thermodynamic and kinetic stability of the Fe³⁺ complexes of hexadentate ligands is of advantage in the competition with natural siderophores, especially at low concentrations.

Hexadentate ligands of type **47** (Figure 14) for example, have a higher affinity for Fe³⁺ and higher antimicrobial activity than diethylenetriaminepentaacetic acid (DTPA **51**)^[84,85] (Figure 15) and their Fe³⁺ complexes are not taken up by *E. coli* or *S. aureus*.^[83] Hexadentate 3,4-HOPOs **48** have pFe³⁺ values of over 30 and were found to inhibit the growth of both Gram-positive and Gram-negative bacteria, including methicillin-resistant *S. aureus* and clinical isolates of *P. aeruginosa*.^[86,87]

Even though 1,2-HOPOs contain weaker Fe³⁺-coordinating groups, a number of 1,2-HOPOs was recently shown to act as antimicrobial agents.^[88] Their lower pK_a values, typically around 6 (Figure 13), ensure deprotonation at physiological pH and the resulting negatively charged ligands are less likely to penetrate host cells. Of the series of 1,2-HOPOs tested,^[88] the hexadentate ligand **49**^[89] showed the most promising biostatic effect against a range of pathogenic bacteria, whilst HOPO ligand **50** was somewhat less effective. These observations confirm that the amide linkers and/or flexibility of the backbone are important design criteria to be considered in future studies.

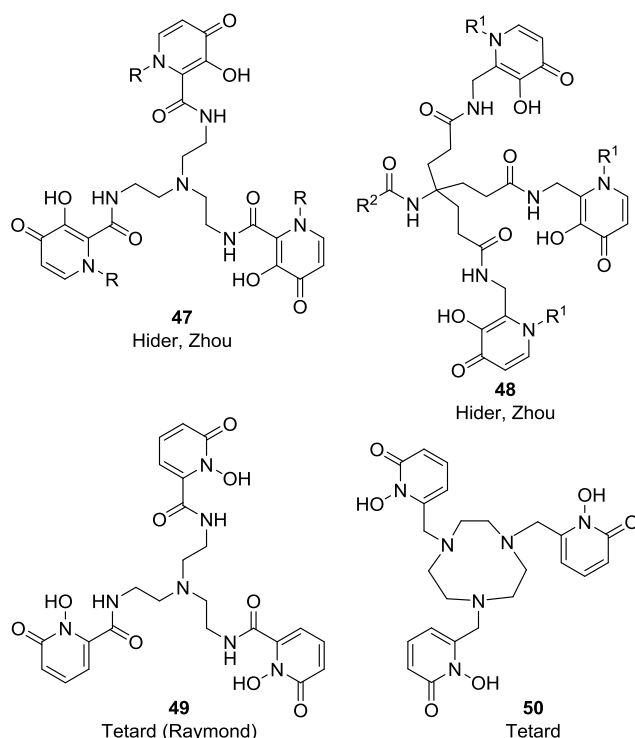


Figure 14. Structures of selected hexadentate siderophore mimics.

The antimicrobial effect of ethylenediaminetetraacetic acid (EDTA) was documented as early as 1954.^[90] Today the hexadentate ligand is used in many domestic, industrial and medicinal applications, in particular disinfection and surface cleaning. The bacteriostatic properties of aminocarboxylate ligands, such as EDTA and DTPA **51**, are associated with their ability to strongly chelate Fe^{3+} , however, these ligands also bind a range of other metal ions. The chelation of Ca^{2+} and Mg^{2+} , for example, disrupts and permeabilizes the outer membrane of Gram-negative bacteria, thereby enhancing the susceptibility of the cells to antibiotics. Further advantages of EDTA are its low toxicity.

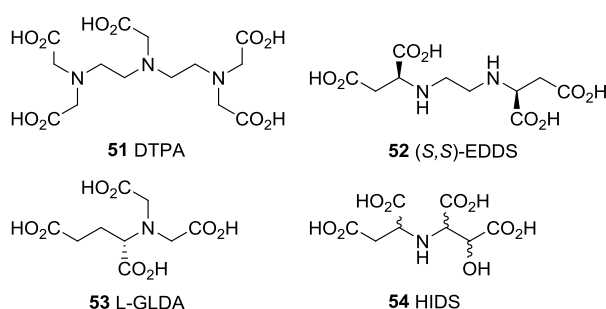


Figure 15. Examples of aminocarboxylate-based metal chelators.

The widespread use of EDTA and DTPA **51** combined with their low biodegradability has led to their accumulation in wastewater and may cause long-term changes in environmental metal speciation.^[82] The urgent need for biodegradable alternatives has led to the development of substitutes, such as **52–54**. The antimicrobial activity of **53** is slightly higher than that of EDTA. In addition, **53** is also more potent than EDTA in enhancing the effectiveness of antibacterial agents by increasing the permeability of the bacterial membrane.^[91]

Another group of metal chelators is based on 3-acyltetramic acids (3ATA) **43**. They occur in nature as metabolites of bacteria, cyanobacteria, molds, fungi, and sponges.^[92] They are comprised at the minimum of polyketides and α -amino acids and normally exist as mixtures of interconverting rotamers and tautomers. 3ATA frequently show antibacterial, antiprotozoal, antifungal, herbicidal, or antitumoral activity. These biological effects are thought to originate from the accuracy of fit of their polar tris- β -keto unit into the active sites of kinases and phosphatases, from their amphiphilic detergent-like nature which allows them to interfere with plasma membranes and to act as ionophores, and from their high metal affinity leading to an accumulation or sequestration of biologically relevant metals. 3ATA form chelate complexes with a range of metal ions. The two possible complex isomers *E*-**55** and *Z*-**55** are far less polar than the free ligands, they do not normally interconvert under biological conditions, they are both synthetically accessible and readily distinguishable via IR spectroscopy,^[93,94] and they may have different biological properties (Figure 16).

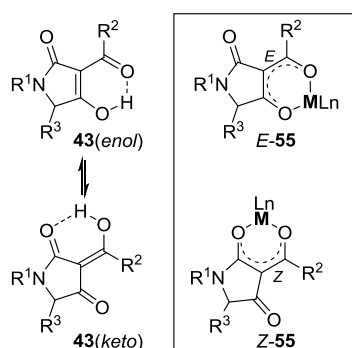


Figure 16. Tautomers and metal chelate complexes of 3ATA. M = metal ion; L_n = number of auxiliary ligands.

Metal chelation seems to be crucial for the transport of certain natural tetramic acids and most of them were actually isolated as metal chelate complexes.^[92e,95] Tenuazonic acid (**43b**: $R^1 = H$, $R^2 = Me$, $R^3 = CHMeEt$), a toxic L-isoleucin-derived metabolite of *Alternaria tenuis*, was isolated as a 10.5 : 2 : 1.5 mixture of its chelates with Mg^{2+} , Ca^{2+} , and Na^+ , aside of trace amounts of Zn^{2+} and K^+ chelate complexes, which reflects the affinity of tenuazonic acid for these metals.^[96] However, synthetic 3ATA chelates of other transition metal ions were also reported, e.g. of Cu^{2+} ,^[97] Ni^{2+} ,^[93a] and Mn^{2+} .^[98] Such chelate complexes often show biological effects different from those of the parent 3ATA. The tendency of 3ATA to form defined metal chelate complexes with a conformationally rigidified planar geometry was even utilized for their synthesis (Figure 17).^[99,100]

The overall formation constants for the complexation of Fe^{3+} by 3ATA are similar to those of typical siderophores such as pyoverdine.^[67] However, unless applied in high concentrations, 3ATA are inferior to the latter in terms of iron chelation at physiological pH values due to their statistic disadvantage as merely bidentate ligands and because of their relatively high acidity which favours stable complexes only at relatively low pH. As a consequence $Fe^{3+}(3ATA-H)_3$ complexes have pM values distinctly lower than those of ferrisiderophores. It is worth noting that the capacity of 3ATA to form iron complexes and so sequester iron in bacteria is not necessarily causative for their growth inhibition or cytotoxic effect. This was shown by Lebrun by treating bacteria with tenuazonic acid and its octahedral (3:1) Fe^{3+} complex only to find them equally cytotoxic.^[88]

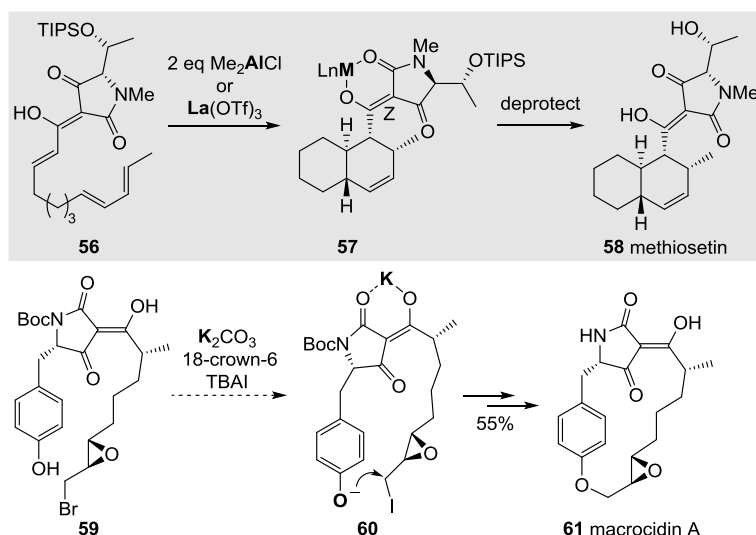


Figure 17. Metal chelate assisted syntheses of methioisetin **58** and macrocidin A **61**. TIPS = tris(isopropyl)silyl; OTf = CF₃SO₃; TBAI = tetrabutylammonium iodide; M = Al or La; L_n = number of auxiliary ligands.

Quite a few other 3ATA with proven affinity for iron have not even been tested for antibacterial activity, e.g. macrocidin A **61** which was lately shown to interfere with vital enzymes of the photosystem II and of the chlorophyll and carotinoid biosynthesis by sequestering iron and magnesium.^[101,102]

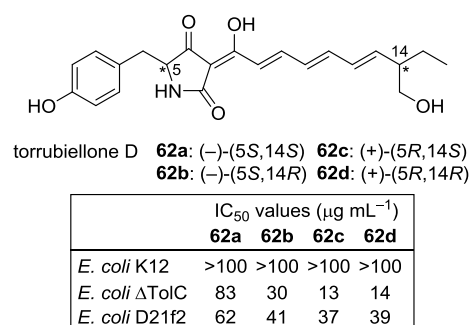


Figure 18. Antibacterial activity of stereoisomers of torrubiellone D **62** against *E. coli* strains. *E. coli* K12: wildtype; *E. coli* ΔTolC: mutant lacking the ArcAB–TolC efflux system; *E. coli* D21f2: supersusceptible mutant with truncated lipopolysaccharide core.

It was also shown that synthetic analogs and precursors of the macrocyclic 3ATA aburatubolactam and macrocidin A **61** can have antibacterial activities.^[103,104] An antibacterial screening of the fungus metabolite torrubiellone D **62** revealed distinctly different activities of its four stereoisomers against drug-susceptible *E. coli* strains (Figure 18).^[105] The influence of metal ion concentrations, in particular of Fe³⁺, was not yet studied.

Growth inhibiting effects of the removal of metal ions were also observed when Zn²⁺-specific chelators are present. For example, the endogenous protection of the human skin from *Escherichia coli* infection was attributed to the Zn²⁺-chelating activity of the protein psoriasin, which is secreted by keratinocytes, i.e. the cells of the upper skin layers.^[106] Also synthetic Zn²⁺-chelators, such as *N,N,N',N'*-Tetrakis(2-pyridylmethyl)ethylene diamine (**63**, TPEN) and dithiolopyrrolones, such as reduced holomycin **64** (Figure 19), are reported to inhibit growth rates of Gram-positive bacteria, such as *Streptococcus pyogenes*,^[107] and Gram-negative bacteria, e.g. *Escherichia coli*.^[108] The reduced holomycin particularly inhibited the activity of

zinc-dependent metalloenzymes, such as class II fructose biphosphate aldolase (85–90% inhibition at 25 μM), an essential enzyme for *E. coli* growth, and NDM-1 ($\text{IC}_{50} = 0.15 \mu\text{M}$), the New Delhi metallo- β -lactamase, which is responsible for resistance to all types of β -lactam antibiotics. As both **63** and **64** are membrane-permeable it was speculated that their activity is mainly due to competition with intracellular proteins for Zn^{2+} .

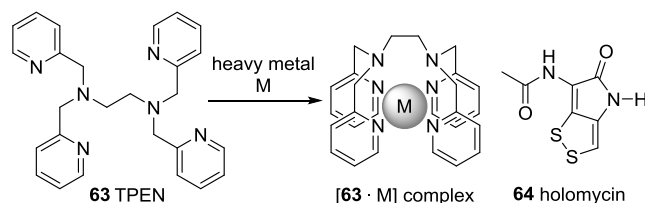


Figure 19. Synthetic Zn^{2+} -chelators.

The application of metal ion chelators enhances nutrient stress for the pathogen, so that affected microorganisms have to compete with the added synthetic chelator. In the case of iron starvation this leads to increased production of high-affinity siderophores and this has a metabolic cost associated with it. It is not yet proven, whether a similar metallopeptide production is also observed in response to limitations of other metal ions. In addition, synthetic ion chelators can work synergistically with existing antibiotics. It is likely that the antimicrobial effect of metal ion chelators will vary with the type of pathogen to be targeted, depending for example on the set of siderophores it relies on. However, synthetic iron chelators are already used in topical applications, for example in surface cleaning, wound healing and in the treatment of nail and eye infections. The development of antimicrobial metal ion chelators for systemic use, however, is facing additional challenges. In particular, interferences with the host's metalloenzymes and immunodefence system have to be avoided.

3.5. The “Trojan Horse” Strategy: Utilization of the Siderophore Transport Machinery

3.5.1. Trojan Horses from Nature

Hybrid compounds comprised of a siderophore fused to an antibacterial component, so called “Trojan horses”, have long been known to the literature, in fact predating the discovery of siderophores themselves.^[109,110] They depend on recipient cell siderophore transporters for active transport into the cytosol, where subsequent cleavage releases the free antibiotic to kill susceptible cells.

Two major classes of siderophore-linked antibiotics are documented in the literature, categorized by producing bacteria and siderophore moiety (Recently reviewed by Johnstone and Nolan^[111]). The sideromycins (including salmycins, albomycins and ferrimycins) are produced by Gram-positive soil actinomycetes. Typical examples are shown in Figure 20. These Trojan horses are characterized by a ferrioxamine class siderophore linked to an antibiotic.^[112] The antibiotic moiety found in each varies by sideromycin: whereas salmycin **65** carries an aminoglycoside as warhead, the warhead of albomycin δ_2 **66** is the seryl adenylate **67** analog and inhibitor of seryl-tRNA synthetase SB-217452 **68**.^[113]

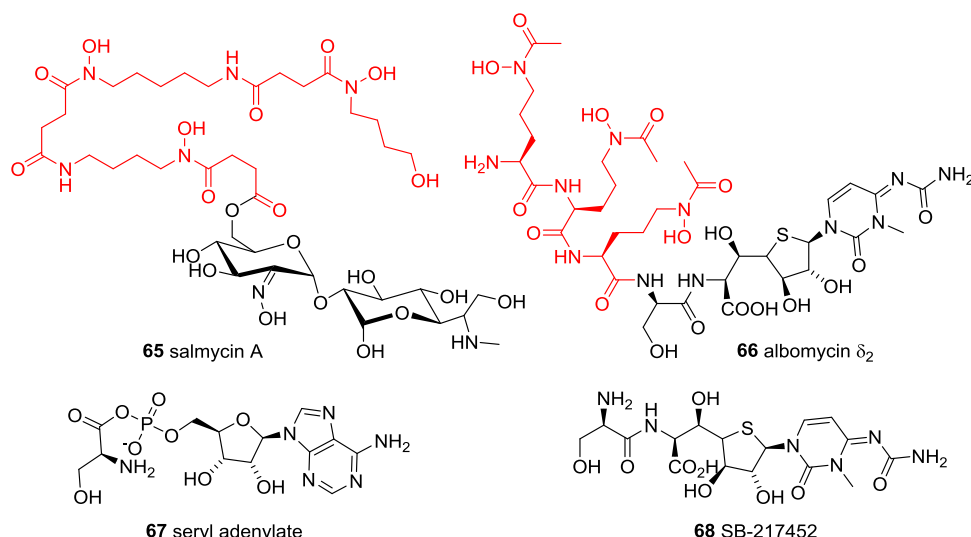


Figure 20. Sideromycins, salmycin A **65** and albomycin δ_2 **66**. The latter contains **68**, a seryl adenylate analog as warhead. Structurally diverse ferrioximine siderophore substructures are highlighted in red.

In contrast to the sideromycins, microcin-class siderophore conjugates **69**, e.g. MccE492 **69a**, MccM **69c**, and MccH47 **69d** are sourced from Gram-negative enterobacteria.^[114] These highly similar compounds share a salmochelin-type siderophore,^[115] and the bioactive warhead conjugates are short antimicrobial peptides (Figure 21).

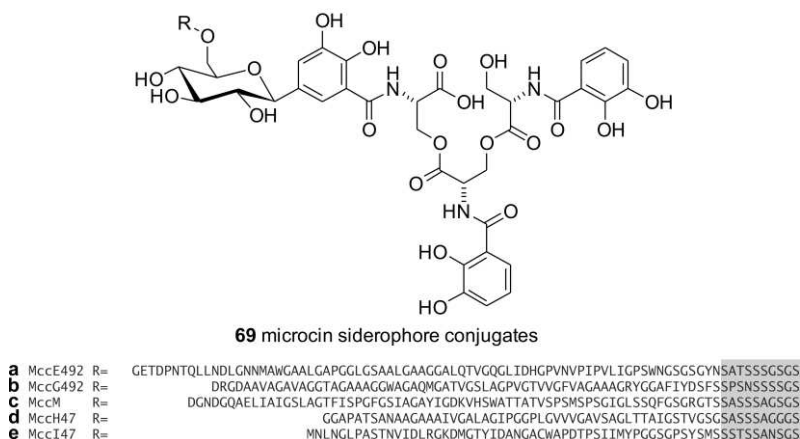


Figure 21. The invariant siderophore moiety of microcin siderophores **69** is linked to various antimicrobial peptides with little overall identity, excepting a semi-conserved poly-serine enriched motif and strictly conserved carboxy terminal serine where the peptides are linked to the siderophore glycosyl moiety by MceIJ^[114,118,119] (grey box).

The bioactivity of microcin siderophores is limited to certain Gram-negative enterobacteria, but their incorporation of salmochelin makes them active against bacteria FepA, Cir and Fiu catecholate siderophore transporters.^[116,117]

Bacterial resistance to the sideromycins has been documented, often arising from mutations in siderophore transporters.^[109] These mutants seem to be less viable than their wildtype counterparts,^[109] perhaps because iron acquisition is perturbed during the selection of resistant mutants. This phenomenon, where a measurable fitness cost is revealed in resistant mutants, may indicate hidden value for drug development in sideromycin-type antibiotic leads. One clinically utilized antibiotic with similar resistance attributes is fosfomycin (trade name

Monurol), used to treat non-complicated urinary tract *E. coli* infections. Clinical resistance is scarce, but commonly encountered under laboratory conditions.^[120] Fosfomycin resistance maps to a number of genes encoding transporters required for drug uptake. Mutants in these genes appear to suffer in growth rate, imposing a fitness cost on surviving bacteria and mutants are more easily cleared in a bladder infection model. Thus molecules having novel mechanisms of action, but suffering from rapid resistance development could still have clinical value if mutants are reduced in virulence or other fitness parameters critical *in vivo*.

3.5.2. Genome Mining for new Siderophore Antibiotics

A vast literature exists supporting the genomics-guided discovery of biosynthetically common and long studied families of molecules such as non-ribosomal peptides and polyketides. Far fewer studies have addressed the gene clusters of siderophore-microcins and sideromycins biosynthesis, and likewise, few genome mining efforts for siderophore antibiotics have been undertaken. This is changing, and the siderophore-microcins MccM **69c** and MccH47 **69d** were recently discovered after comparing the genomes of an array of microcin producing *Klebsiella* and *Escherichia* strains.^[121] A bioassay testing MccE492 **69a**, MccM **69c** and MccH47 **69d** against a panel of gram negative pathogens including *E. coli*, *K. pneumonia*, *Enterobacter cloacae*, and *Salmonella enterica* serovars Typhimurium and Enteritidis revealed E492 is generally more active than M or H47. Despite these newer microcins having less desirable activity profiles than E492, genomic data indicate additional microcin siderophores await discovery, including MccI47 **69e** and MccG492 **69b**. Spectrometric data consistent with MccI47 **69e** detection have been obtained, but low-level production has impeded further characterization and MccG492 **69b** remains chemically undiscovered. Engineering work is likely required to increase MccI47 **69e** and MccG492 **69b** production for activity testing.

Genome mining for new microcin siderophore conjugates is fairly straightforward and will likely lead to new bioactive members in the future. Biosynthetic loci encoding these conjugates are easily recognized in genomes by the presence of genes similar to *mceIJ*. Enzymes encoded by these genes link the C-terminal serine of peptide E492 to its cognate siderophore glycosyl moiety.^[119] As expected, orthologs of these enzymes are encoded in the MccE492 **69a**, MccM **69c** and MccH47 **69d** biosynthetic loci, consequently making *mceIJ* genes useful bioinformatic hallmarks for siderophore-microcin locus identification. Further, the discovery of MccM **69c**, MccH47 **69d**, MccI47 **69e** and the prediction of MccG492 **69b** are the products of a single work.^[121] Drastically increasing the number of class IIb microcins available for study, these results illustrate the discovery potential inherent to genomics-guided natural products discovery.

In contrast to the emerging body of biochemical and genetic data used to support genome mining for microcin-siderophores, biosynthetic data for actinobacterial sideromycin production is nearly non-existent. A single study revealing the albomycin δ_2 biosynthetic locus^[122] is the only such inquiry thus far. This is surprising considering the growing availability of sequenced actinomycete genomes and the long recognized drug interest in the sideromycins. To discover the albomycin biosynthetic locus in *Streptomyces* sp ATCC700974 in the absence of albomycin-specific genes to guide genomic searches, Zeng searched for ornithine N-hydroxylase homologs commonly associated with actinomycete

ferrioxamine/ferrichrome siderophore loci. Siderophores of this type are substructures of the sideromycins. Once the albomycin warhead genes were identified, homology searches in other bacteria led to the identification of a potentially novel, yet chemically undiscovered sideromycin encoded in the genome of *Streptomyces* sp. C.^[122] Aside from this single predicted molecule, a genomics guided path to discover additional sideromycins remains unclear. In contrast to the microcin siderophores, where specialized locus-specific enzymes (*mceIJ*) can help guide genomic discovery, the sideromycins have structurally dissimilar warhead moieties, and share sparingly few substructural elements from their incorporation of variant ferrioxamine or ferrichrome type siderophores.^[111] While a large scale Orn N-hydroxylase-based search for new sideromycins in other actinomycete genomes could be attempted, such a strategy is unfavorable. This is because N^5 -OH-Orn is a common intermediate in many actinomycete siderophore and other secondary metabolite pathways.^[123] Searches using Orn N-hydroxylase to survey genomes would identify far more non-sideromycin loci compared to desirable sideromycin hits. Considering this, our ability to genomically recognize new sideromycin loci is significantly hampered. Should additional sideromycin-producing organisms be sequenced in the future, new data enabling more efficient genomic discovery may emerge.

3.5.3. Synthetic Trojan Horses

Inspired by the sideromycins a range of synthetic Trojan horse conjugates were developed over the past 50 years, with the aim of exploiting active bacterial nutrient uptake, thereby evading resistance associated with a reduction of membrane permeability or an increase in efflux (for recent reviews see ref.^[124–127]).

Uptake studies with various synthetic tris-bidentate chelators with different combinations of catechol, hydroxamate, and (*o*-hydroxy)phenacyl ligands and receptor-defective *E. coli* mutants showed that their corresponding Fe^{3+} complexes were actively internalized only via catecholate rather than via hydroxamate receptors. This holds also true for their conjugates with β -lactam antibiotics, e.g. conjugates **70** (Figure 22).^[128] It was also shown that simplified mimics of the natural siderophores parabactin and agrobactin, carrying an *o*-hydroxybenzamide instead of the 2-(*o*-hydroxyphenyl)oxazoline ligand, unlike the originals, were fully functional siderophores in *E. coli*.^[129]

Of the many siderophore- β -lactam conjugates reported to date, at least three have undergone further development by the pharmaceutical industry (Figure 22):^[130] MC-1 **72**,^[131] BAL30072 **71**^[132] and S-649266 (Cefiderocol) **73**.^[133] The latter is currently in phase 2/3 clinical trials. Interestingly, all three conjugates rely on bidentate iron-chelating units that resemble the unusual 3,4-dihydroxybenzoyl unit found in petrobactin, a stealth siderophore that evades capture by the human immune system protein siderocalin.^[134]

In addition, glycosylated enterobactin derivatives that were inspired by stealth siderophores of the salmochelin type were linked to β -lactams and shown to evade siderocalin binding.^[135]

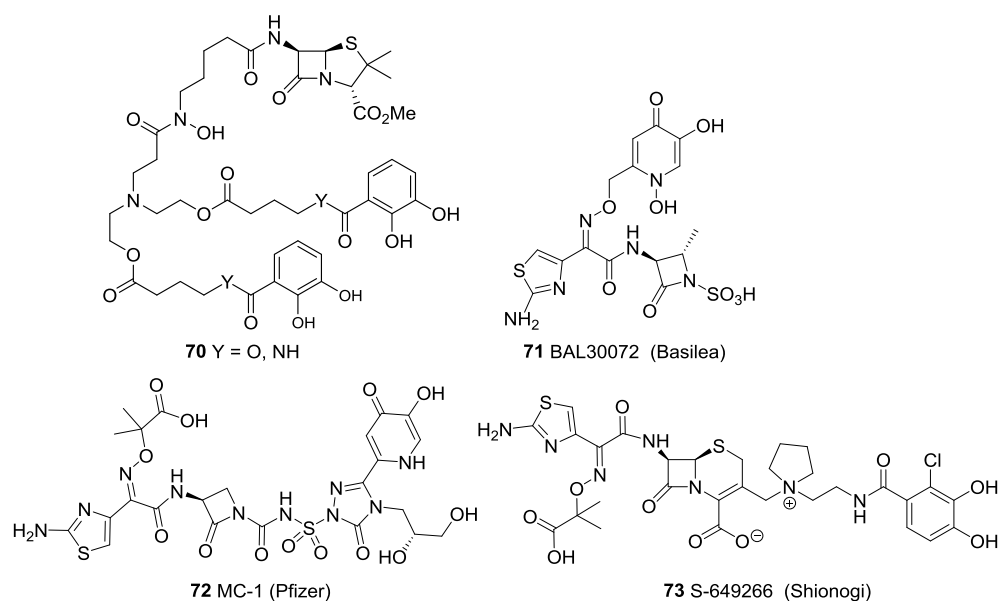


Figure 22. Siderophore- β -lactam-conjugates as Trojan horses for antibiotics.

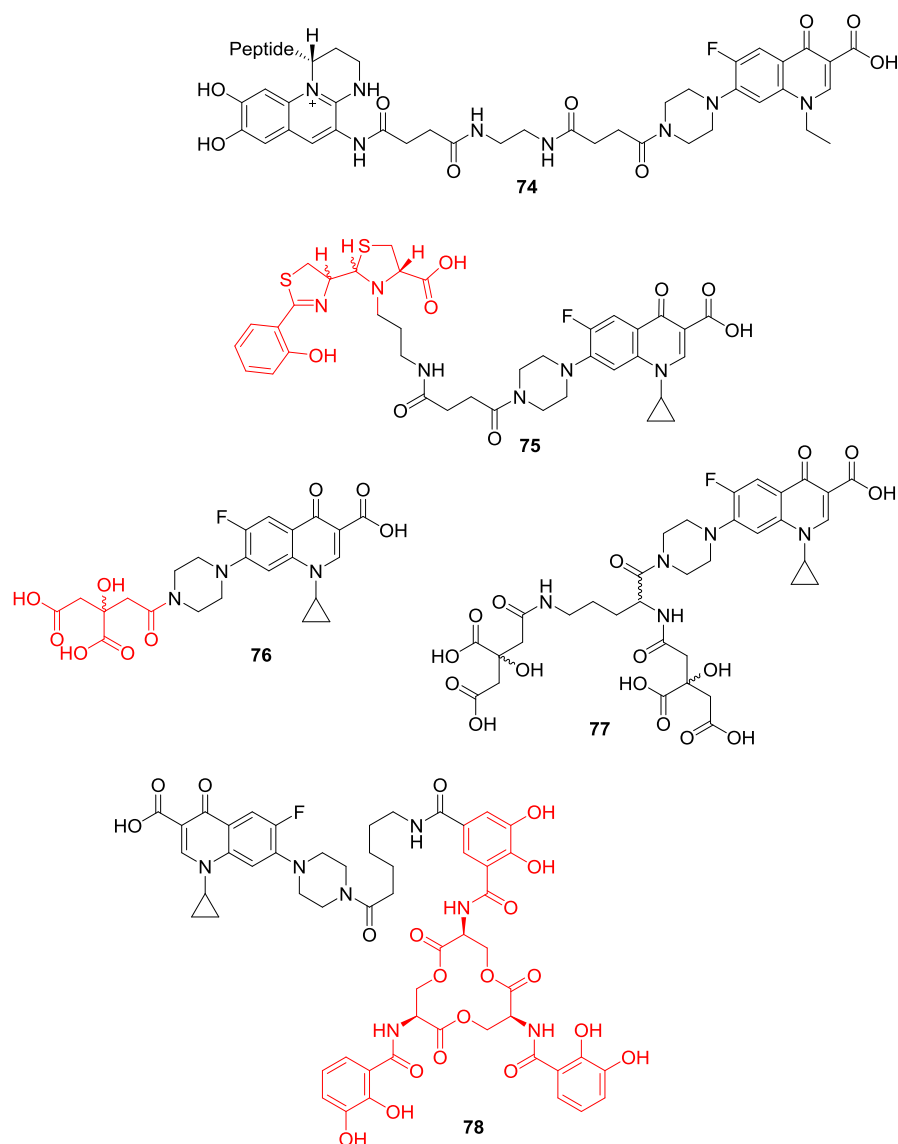


Figure 23. Examples of fluoroquinolone conjugates.

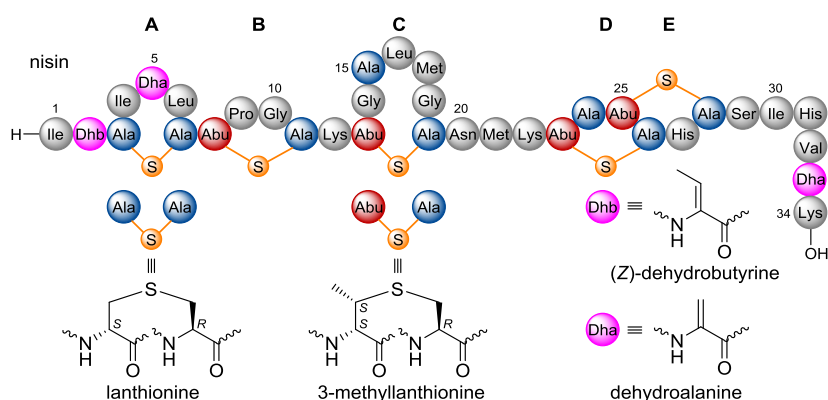
The inhibition of cytoplasmic drug targets has also been attempted. Studies with fluoroquinolone antimicrobials investigated a variety of derivatised siderophore components (Figure 23), including pyoverdin (**74**),^[136] pyochelin derivative **75**,^[137] citric acid derivative **76**,^[138] staphyloferrin (**77**)^[139] and enterobactin derivative **78**,^[140] and highlighted the necessity of biolabile linkers that allow the release of the antimicrobial inside the bacterial cell. Very recently, Miller has patented a conjugate of an *Acinetobacter baumannii* specific siderophore with daptomycin, which expanded the scope from strictly Gram-positive to Gram-negative bacteria.^[141]

Finally, also conjugates with membrane-targeting compounds, such as ionophors and lantibiotics, were synthesized. Ionophors recognize specific ions even in complex mixtures and facilitate transmembrane transport.^[142] Typical examples are synthetic crown ethers, azacrowns and the like as well as natural polyether antibiotics, e.g. lasalocid A **79**.^[143] It is well known that crown ethers and related macrocycles display pronounced antimicrobial properties, which is due to the formation of cation-transporting pores in bacterial cell membranes thereby irreversibly disturbing the ion gradient.^[144,145] In addition, crown ether derivatives may also function as siderophore mimics thus competing with the bacterial iron scavenging system.^[146] Ramazanov reported that azamacrocyclic **80** inhibited the growth of *Staphylococcus aureus* (MIC 1 $\mu\text{g mL}^{-1}$) (Figure 24), but did not affect *Escherichia coli* and *Klebsiella sp.*^[146] However, when magnetite nanoparticles were functionalized with azacrown **81** via non-covalent interactions, the resulting hybrid material inhibited both *E. coli* (MIC 0.5 $\mu\text{g mL}^{-1}$) and *Klebsiella sp.* (MIC 0.5 $\mu\text{g mL}^{-1}$). The complementary activity profile was rationalized by the formation of a Fe^{3+} complex, which is shuttled inside the bacterial cell through the siderophore uptake system in a Trojan horse manner. Recent work by Gokel revealed that shuttling by lariat ethers **81** enhanced the potency of antibiotics rifampicin and tetracycline against *E. coli* 20fold upon co-administration with **81** in a synergistic fashion.^[147,148]

The combination of pore-forming crown ethers with siderophores provides an additional strategy to increase the transport of Fe^{3+} through the bacterial cell membrane and thus interfere with bacterial iron homeostasis. Following this approach Crumbliss studied extensively the supramolecular interaction of lariat ether carboxylic acids, i.e. crown ethers carrying a peripheral alkyl carboxylate arm (as hosts) with the natural siderophore desferrioxamine B (DFB) (as guest).^[149] The corresponding Fe^{3+} complex ferrioxamine B FeHDFB^+ forms a ternary second sphere complex **82** with the lariat ether via the terminal ammonium unit, while the carboxylate arm further contributes to the complex stability via electrostatic interactions (Figure 24). Complex **82** was compared with the corresponding host-guest assemblies derived from lasalocid A **79** and ferrioxamine B^[150,151] and carrier-facilitated bulk liquid membrane transport of the Fe^{3+} siderophore was achieved.^[152,153] Based on this precedent Orcutt developed liposomes containing **80** in the bilayer membrane, which selectively recognized and extracted FeHDFB^+ preferably with respect to Ca^{2+} , Na^+ and K^+ from the surrounding aqueous phase.^[153] Although initially intended as chemical sensor for monitoring of Fe^{3+} availability in seawater, this liposome-based nanodevice for sequestering siderophore-bound Fe^{3+} might be useful to study host-pathogen interactions.



Lantibiotics also lead to the formation of pore complexes, which severely disturb the intra- and extracellular ion concentrations leading to bacterial cell death. An attractive feature of lantibiotics is their low tendency to develop resistance.^[154,155] They are small polycyclic peptides (< 10 kDa) produced by prokaryotes, which belong to the class of endogenous host defensive peptides. They are an important part of the innate bacterial immune system to fight other pathogenic bacteria.^[156,157] A prominent member of this family is nisin (Figure 25) produced by *Lactococcus lactis*. Nisin contains several structural features unique for this type of peptides, namely the amino acids lanthionine (Lan), 3-methyllanthionine (MeLan), (Z)-dehydrobutyrine (Dhb) and dehydroalanine (Dha) as well as thioether bridges.^[158,159]



Recent work by Rijkers showed that modification of nisin did neither compromise antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* nor the membrane permeabilization.^[160] To enhance the transport through the outer membranes of Gram-negative bacteria Vederas synthesized conjugates **83–85** of the lantibiotic gallidermin with the three different siderophores pyochelin **2**, agrobactin and desferrioxamin B, respectively, employing squaric acid methylester as a bifunctional linker (Figure 26).^[161]

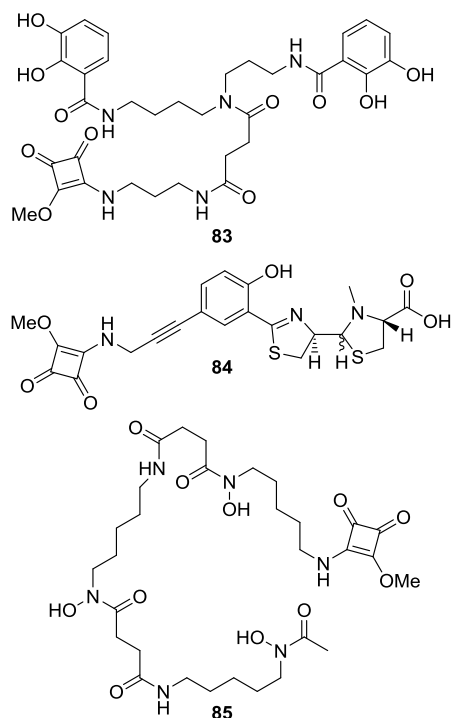


Figure 26. Siderophore-monomethylsquarate esters **83–85**.^[161]

While the conjugates retained at least some antibacterial activity of gallidermin against the indicator organism *Lactococcus lactis* subsp. *cremoris* HP, an unexpected growth promotion rather than antibacterial activity was observed against *Pseudomonas aeruginosa* suggesting that the conjugates kept their siderophore activity but failed to permeabilize the inner membrane.

These results indicate that the synthesis of Trojan horses requires careful choice of the siderophore moiety, the linker and the antibiotic.

3.5.4 Siderophore quenching

Siderophores are chelators showing specificity for Fe^{3+} , but are able to also complex other metal ions though with different affinities.^[162] Ga^{3+} is structurally rather similar to Fe^{3+} so that in almost all complexes Fe^{3+} can be replaced by Ga^{3+} . Thus, bacterial siderophores can be pre-loaded with Ga^{3+} and transport Ga^{3+} into the cells, where it replaces Fe^{3+} also in proteins without having the redox properties of Fe^{3+} . Resulting antibacterial effects are based on the combination of the utilization of the siderophore transport machinery *via* competitive binding of Ga^{3+} to siderophores with Fe^{3+} depletion. The area has recently been reviewed.^[163] Gallium citrate, for example, shows activity against a range of Gram-negative bacteria, with MICs ranging from ~1 to 5 $\mu\text{g/ml}$. Importantly, in comparison with conventional antibiotics, resistance to the toxic effects of Ga^{3+} is slower to develop and Ga^{3+} -containing compounds remain active even against bacteria that grow in biofilms.^[164] Phase 1 clinical trials with an intravenous gallium nitrate-citrate formulation have shown promising results in cystic fibrosis-associated lung infections and in January 2017 Aridis Pharmaceuticals announced plans for a phase 2a clinical trial with gallium citrate (PanaecinTM), supported by an award from the Cystic Fibrosis Foundation. [<http://www.aridispharma.com/panaecin.html>]

3.6. Siderophore Transport into the Cell

Fe³⁺-loaded siderophores are recognized by receptors in the outer membrane of Gram-negative bacteria and are transported in an energy-consuming step into the periplasm. The required energy originates from the proton motive force across the bacterial inner membrane and is coupled to the siderophore receptor via the TonB system (Figure 1). The exact mechanism, how the energy is transduced to the outer membrane is not yet elucidated and several models are discussed. They include also periplasmic binding proteins, such as FhuD, which interacts with an inner membrane transporter, FhuBC, to carry the siderophore to the cytoplasm.^[165,166]

To counteract all iron acquisition systems of uropathogenic *E. coli* (UPEC) a whole-cell growth-based screen was performed using iron-limiting conditions, and a counterscreen under normal cultivation conditions. 16 out of a library of approximately 150.000 compounds were found to specifically inhibit UPEC under iron-limiting conditions with IC₅₀ values in the range from 0.8–48 μM. Mode of action studies with loss of function mutants revealed that for at least 2 of these compounds probably the TonB-system is the target (**86**, **87**, Figure 27).^[166] Further studies of these compounds with respect to *in vivo* applicability are required, in particular as only 4 out of the 16 compounds were active on a wild-type strain and not only on the ΔTolC mutant, which lacks the export pump TolC.

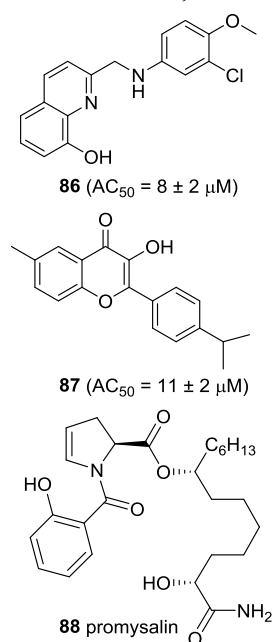


Figure 27. Structures and inhibitory constants of the TonB inhibitors^[166] and promysalin **88**.

Promysalin **88** (Figure 27), a salicylate-containing *Pseudomonas putida* antibiotic, promotes swarming and biofilm formation and selectively targets other *Pseudomonas* sp. including the opportunistic pathogen *P. aeruginosa*.^[167] It disperses established biofilms and inhibits pyoverdine production.^[168] At concentrations ranging from 6 to 100 mM it shows Fe³⁺ chelation properties, albeit with reduced affinity when compared to the known iron chelator EDTA. Thus, it was proposed that **88** inhibited siderophore transport pathways thereby severely limiting or inhibiting the organism's ability to acquire iron. However, the mechanism was not elucidated in detail. The total synthesis of promysalin **88** and a series of analogs and the evaluation of their antimicrobial activity have been recently reported.^[168–171]

In Gram-positive bacteria, such as *Staphylococcus aureus*, transport systems for essential metal ions typically rely on substrate binding proteins (SBP) and an ABC transporter (Figure 1). The Mn^{2+} uptake system comprising the SBP MntC and the membrane importer MntB is essential in *S. aureus*, as Mn^{2+} is a cofactor of enzymes involved in detoxification of reactive oxygen species. Until now no small molecule inhibitors of this pathway are described, but an antibody fragment was developed, which complexes MntC and prevents its interaction with MntB.^[172] As a proof of concept it was shown that this antibody fragment ($100\ \mu\text{g mL}^{-1}$) reduced viable cell counts of *S. aureus* 12fold when exposed to reactive oxygen species. Thus, the potential of metal ion import systems as antibacterial targets may not yet be fully exploited.

4. Iron Acquisition from Heme

Heme is the most abundant iron species in the human body, but is present only intracellularly. Thus, heme uptake systems have evolved and are utilized by pathogens to various degrees depending on the type of the pathogen and the site of infection. Some Gram-positive bacteria, such as *Staphylococcus aureus*^[173] and *Corynebacterium diphtheria*, rely on heme uptake during pathogenesis, and also for *P. aeruginosa* the relevance during chronic lung infections of cystic fibrosis patients was shown.^[174] A prerequisite for iron acquisition from heme is the secretion of hemolysins or cytolysins, usually proteins, which lead to lysis of host cells and thus to the release of intracellular proteins. As these mechanisms are best studied for *S. aureus*, the following description is focused on the heme uptake system of this pathogen.

S. aureus produces several cytolysins, such as α -toxin (α -hemolysin), γ -toxin and bi-component leucocidins, pore-forming toxins, which were shown to be relevant for the severity of diseases, such as pneumonia or sepsis.^[175,176] The expression of these toxins and of the heme uptake system is induced by low iron concentrations via the Fur repressor, and further regulated by the activity of virulence-associated transcriptional regulators^[177,178] so that they are only produced when needed. Lysis of host cells makes intracellular proteins, such as hemoglobin and other heme proteins, available to the pathogen, and *S. aureus* possess surface proteins providing capture of heme from hemoglobin and myoglobin, transportation through the membrane, and in the cytoplasm heme-degrading monooxygenases make Fe^{2+} available for bacterial processes.^[179]

Until now inhibitors of heme–iron acquisition target the expression or the activity of α -toxin and some examples will be described in the following:

Expression of the hemolysins is, together with the expression of other virulence factors, under the control of the accessory gene regulatory (agr) quorum sensing two-component system, in addition to the mgrA global transcriptional regulator and the Fur repressor. Due to their general relevance for virulence, screening campaigns for inhibitors of Agr and MgrA were performed.^[180] The small molecule savarin **89** and mimetics of the quorum sensing compounds **25** decreased agr activity and significantly reduced the hemolytic activity of *S. aureus*. However, the therapeutic value is not unambiguous, as Agr-dysfunction has also been associated with persistent rather than resolving bacteremia, and mortality.^[180] Reduced hemolytic activity and reduced virulence in a mouse model were also observed, when MgrA was inhibited directly or via phosphorylation by a serine–threonine phosphatase (stp1).^[178]

Screening resulted in the identification of 5,5-methylenedisalicylic acid (**90**, MDSA) and the related compound aurintricarboxylic acid (**91**, ATA) (Figure 28) as inhibitors with activities in the μM range.

Whereas these studies still await further exploitation, the activity of α -hemolysin is inhibited by several small molecules and also by a monoclonal antibody. α -Hemolysin is secreted by *S. aureus* as monomer. Contact of the protein to host cells leads to oligomerization and the formation of heptameric pores, a process which is favored by a protein receptor (ADAM10) but also by lipid components. β -Cyclodextrin derivatives **93** inhibited α -hemolysin activity probably by blocking the pore and thus preventing loss of ions and small molecules from the cytosol.^[181] A similar mechanism is suggested for the isatin-Schiff copper(II) complex **92** (Figure 28).^[182]

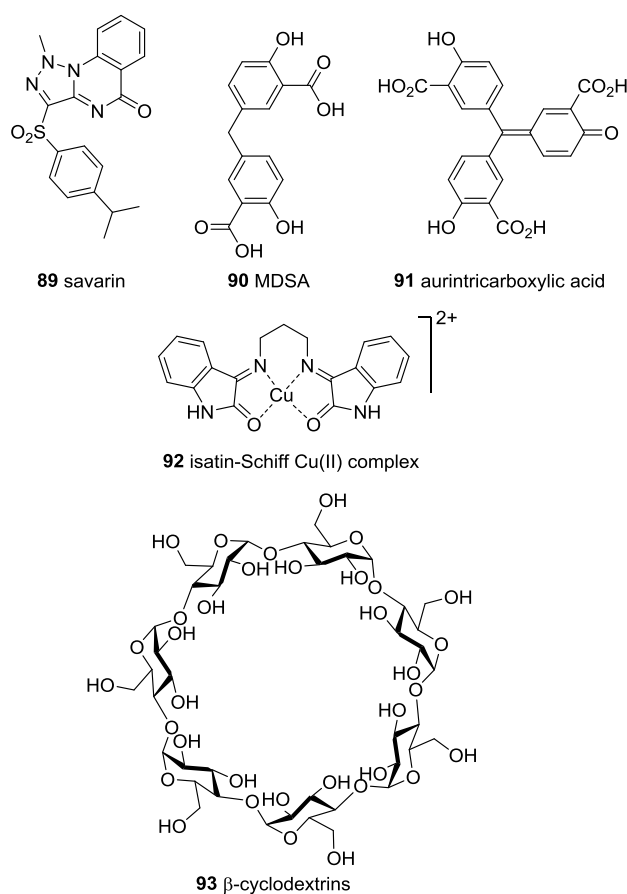


Figure 28. Inhibitors of α -hemolysin expression (**89–91**) and of the permeability of the α -hemolysin pore (**92**, **93**).

The phenolic and flavonoid inhibitors baicalin **94**,^[183] oroxylin A **96**,^[184] cyrtominetin **95**^[185] and curcumin **97**^[186] (Figure 29) are suspected to prevent assembly of the monomers to the heptamer. The most advanced agent, however, is the monoclonal antibody MEDI4893, which has proven efficacy in murine *S. aureus* pneumonia models, when used in a passive immunization strategy. This antibody is now evaluated in clinical studies.^[187,188]

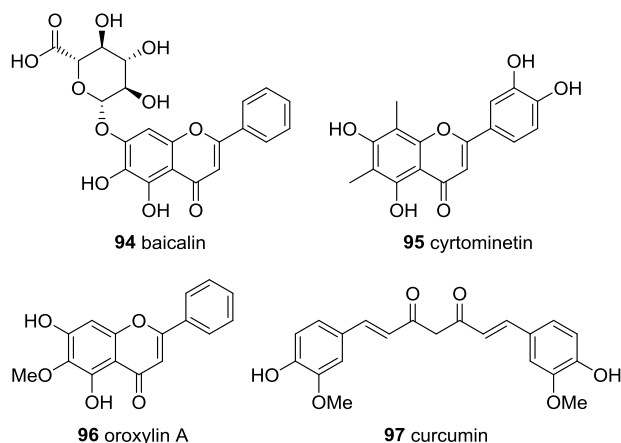


Figure 29. Phenolic and flavonoid inhibitors of the assembly of α -hemolysin monomers to a functional pore.

Inhibition of heme-iron acquisition systems of other bacteria have not yet gained as much interest as the system of *S. aureus*, though iron-regulated expression of hemolysin is also reported for other bacteria, such as *Bacillus cereus*, *E. coli* and *Vibrio cholera*.^[189,190] Among the rare examples of inhibition of heme uptake is the structure-guided development of synthetic metal complexes, which replace heme in its binding site on the heme acquisition system A (HasA) protein. HasA is secreted by *P. aeruginosa*, binds heme “like a pair of tweezers” and transfers the heme to the hemophore-specific outer membrane receptor HasR.^[191] From the crystal structure of the heme-loaded HasA it was concluded that also other metal complexes could fit into the binding site, which was experimentally proven by ESI-TOF-MS. The Fe-phthalocyanine complex Fe-Pc completely inhibited growth of *P. aeruginosa* with a half-maximal inhibitory concentration of approximately 24 nM even in the presence of heme-bound HasA_p.^[191]

5. Conclusion and Outlook

As iron is essential for viability and growth of most organisms, sophisticated uptake systems have evolved in bacteria, which allow them to circumvent the limitations of low available iron concentrations imposed by an aerobic environment and by the additional withdrawal mechanisms of a vertebrate host. The relevance of the different uptake systems varies with bacterial species and with the particular conditions in the colonized niche. High affinity iron uptake systems are synthesized only on demand, i.e. bacteria regulate the expression of the respective proteins and the activity of enzymes in response to intracellular iron concentrations. In addition, the drop of the iron concentration is frequently taken as signal for the presence of host cells, leading to the expression of additional virulence factors. Thus, generally, bacterial iron uptake could be exploited for the treatment of infectious diseases by targeting a wide range of different proteins, resulting in decreased growth rates and virulence. However, a valuable target for one specific infectious disease may be useless for another. Moreover, the complexity of biological systems may lead to competition and interactions among different metal ions and thus, to side effects and loss of efficacy. Thus, the biological target and the inhibitory strategy have to be carefully chosen and a particular solution may be not applicable as broad-range therapy.

These may be the reasons why until now only some agents are the topic of *in vivo* or of even clinical studies. Animal models vary in their degree of clinical relevance depending on the animal, the infection route and the infection dose. For example, the infection of mice via a single dose of pathogens into the bloodstream will lead to a systemic infection, which may reflect the situation of sepsis, but this model is hardly indicative for infections of the gut, the lung or the skin. However, the relevance of specific iron uptake pathways may differ between infectious diseases as the available iron concentration and iron species differ between organs. Thus, application of inhibitors of a specific iron uptake pathway to a single animal model may not be sufficient to comprehensively evaluate the *in vivo* potency of the compound, which increases the efforts to obtain valid preclinical data.

Thus, it is not surprising that until now mainly broad-spectrum reagents were evaluated in clinical studies: The siderophore – cephalosporin conjugate (Trojan horse) S-649266 **73** (cefiderocol, Figure 22) from Shionogi & Co. Ltd (Japan) was applied to patients with complicated urinary tract infections (cUTI) with Gram-negative bacteria. Due to the success of the study cefiderocol **73** is eligible for the fast-track approval status of the FDA. A phase 3 study with patients carrying carbapenem-resistant Gram-negative bacteria is ongoing. Gallium citrate (PanaecinTM) from Aridis Pharmaceuticals is in a phase 2a clinical study, which enrolls cystitis fibrosis (CF) patients who are chronically infected with *Pseudomonas aeruginosa*. Due to the broad antimicrobial activity of PanaecinTM an extension of the application range is expected. The development stage of the other β -lactam antibiotic – siderophore conjugates BAL 30072 **71** (Basilea) and MC-1 **72** (Pfizer) (Figure 22) is unclear, as they do not appear on the lists of antibiotics in clinical phases anymore. The *in vitro* development of resistant strains was reported for these two Trojan horse – antibiotics, where reduced susceptibility correlated with increased expression of drug efflux pumps (BAL 30072) or siderophore receptors (MC-1 and MB-1). However, the resistance against MC-1 was not relevant *in vivo*, perhaps *in vivo* the loss of activity of a single receptor can be compensated by another one. Moreover, loss of activity of all siderophore receptors will also prevent the uptake of siderophores, which, however, is required for survival under iron-limiting conditions. As discussed above (Chap. 3.5.1) this may lead to fitness costs which may prevent the viability of resistant mutants in an infected host. The same is to be expected for PanaecinTM, as Ga³⁺-loaded siderophores are taken up via the same pathways as Fe³⁺-loaded siderophores. Thus, any mutation, which leads to resistance due to inhibited uptake, will also lead to reduced uptake of the essential metal ion Fe³⁺ and thus, to reduced fitness and survival in the host.

Whereas the present compounds in clinical phases are antibiotics, of which the uptake mechanism is optimized to prevent resistance development, the monoclonal antibody against α -hemolysin of *S. aureus* does not target an essential bacterial protein but a virulence factor, which is active on host cells. As viability of *S. aureus* is not directly affected by the antibody, there is no selection pressure leading to resistant strains. On the other hand the risk of failure is high when the pathogen is able to use alternative toxins to get access to heme-iron.

As discussed in this review the response of bacterial pathogens to metal ion stress in an infected host offers a wide range of very different targets, which could be the basis for novel anti-infective therapeutic approaches. The optimal future drug target may lead to the correlation between reduced susceptibility of strains to the drug and reduced viability in the infected host. However, compounds resulting from respective screening campaigns may be

only applicable to defined infections, which originate from a specific pathogen and affect a specific organ. This correlates to the trends of individualized medicine in other disease areas (e.g. cancer) and may lead to a more focused application of drugs thus preventing the rapid development of resistances, but requires additional efforts to establish suitable models which allow validation of the efficacy of lead compounds.

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Keywords: antibiotics • iron • heme proteins • inhibitors • siderophores

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