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Exploiting bacterial iron acquisition: siderophore conjugates

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Abstract

Siderophores are chelators synthesized by bacteria and fungi to sequester iron, which is essential for virulence and pathogenicity. Since the process involves active transport, which is highly regulated, remarkably efficient and often microbially selective, it has been exploited as a Trojan Horse method for development of microbe-selective antibiotics. Siderophores also have significant potential for the development of imaging contrast agents and diagnostics for pathogen-selective detection. These promising results demonstrate the versatility of natural and synthetic microbial iron chelators and their potential therapeutic applications.

Iron is one of the most abundant elements on Earth, yet its bioavailability is limited by the formation of highly insoluble salts in the presence of oxygen and aqueous solutions. Within biological systems, iron-pools are tightly controlled by protein-based metal-chelators, in order to ensure their proper use and to protect the host against oxidative damage. Most organisms require iron, since it is an essential element used as a cofactor in enzymes, involved in the transport of oxygen, production of energy and other vital metabolic processes [1]. Bacteria also require iron, and ensuring its acquisition is a fundamental step during the establishment of an infectious cycle [2]. Although microorganisms possess an array of methods for the acquisition of iron, one of the most widespread mechanisms is the biosynthesis of low-molecular mass, high affinity Fe^{3+}-chelators called **siderophores**, which are secreted to the extracellular environment, where they gather iron from diverse sources. The resulting complexes are assimilated using an active transport system by the bacteria (Figure 1) [3–7].

Since the need for iron is critical, microorganisms have developed receptors to recognize and transport siderophore–iron complexes from other species (xenosiderophores) in order to gain a competitive growth advantage (Figure 1) [8]. To counter this siderophore-thievery, some microorganisms synthesize siderophore–drug conjugates called **sideromycins** that consist of a modified chelator with a covalently linked drug-like molecule (Figure 2). Acting as a Trojan Horse, a sideromycin enters the competing organism cell via the corresponding siderophore uptake pathway to deliver the drug moiety, which eventually leads to cell death (Figure 1) [9]. Long ago, the potential of exploiting siderophore–drug conjugates was

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recognized by many laboratories and synthetic analogues of these molecules have been reported in order to better understand their mode of action, therapeutic potential and limitations [10]. Although studies of **siderophore conjugates** have primarily focused on the area of antibiotic delivery, other applications have also been considered including the generation and use of related metal complexes to disrupt iron metabolism, use as imaging elements, delivery of fluorescent probes and even as diagnostic agents for detection of microorganisms. Herein, we provide an overview of siderophore conjugate studies reported since the year 2000, with the objective of better grasping the enormous potential of these molecules.

Structurally, a sideromycin consists of a metal–binding portion, a spacer or linker, and a drug moiety. Albomycins are among the most extensively studied, naturally occurring siderophore-drug conjugates (Figure 2) [9]. They consist of a trihydroxamate-based siderophore portion and a thioribosyl-pyrimidine moiety, which is a seryl-*t*-RNA synthetase inhibitor. When actively acquired by non-producing strains of bacteria, the drug-moiety in albomycin is cleaved by the action of a serine peptidase N. The drug-release process is essential for biological activity, microorganisms lacking the ability to recognize and transport the Fe^{3+} complex, or without the serine peptidase N to cleave the drug, are not sensitive to the antibiotic activity of the albomycins [11,12]. Microcins are siderophore conjugates containing polypeptide antibiotics attached to the siderophore enterobactin by means of a glucose linker [13,14]. The delivery of large molecules, as exemplified by these microcins, demonstrates the ability to introduce almost any imaginable compound by exploiting bacterial iron uptake. Based on their general structural features, synthetic analogues of these sideromycins can be designed in a similar fashion.

Synthesis & biological activity of desferridanoxamine–antibiotic conjugates

As another example of a sideromycin, salmycin B was isolated from the fermentation broth of *Streptomyces violaceus* DSM 8286 in 1995 by Vértesy and co-workers [15]. Salmycin B, along with other congeners (A, C and D), was found to be highly active against both *Staphylococci* and *Streptococci*, including resistant strains of these pathogens. The structure of salmycin B comprises the known siderophore danoxamine and an unusual amino-disaccharide antibiotic. After completing the assembly of danoxamine [16], Dong *et al.* reported the first total synthesis of desferrisalmycin B [17], which revealed that the heptopyranose component in the disaccharide has a D-glycero-D-gluco configuration. As expected, the synthetic desferrisalmycin B was found to have comparable antibiotic activity to that of the naturally occurring salmycins [18], demonstrating the feasibility of building these types of molecules by complete chemical synthesis. Wencewicz *et al.* further explored the use of desferridanoxamine as a delivery agent of other antibiotics by preparing siderophore–drug conjugates 1–3 (Figure 3) using loracarbef, ciprofloxacin and triclosan as the drugs of choice [19].

The antibacterial activity of the synthetic desferridanoxamine–drug conjugates 1–3 was studied by performing growth inhibition assays in agar media. All three drug conjugates were active against an array of different bacteria and even the drug-free desferridanoxamine itself inhibited the growth of *Escherichia coli* and *Mycobacterium vaccae,* probably due to

induction of iron starvation at high concentrations (2 mM). In all cases but triclosan conjugate 3, the siderophore-drug conjugate had diminished antibacterial activity compared with the parent drug, suggesting either the need for an intracellular drug release process or that the drug did not reach its target or, if it did, had a lower affinity for the target due to the chemical modification of the drug. In the case of triclosan conjugate 3, the observed inhibitory activity was equal or higher than the drug itself. To probe if drug release by hydrolysis occurred in the agar medium, the synthetic precursor of conjugate **3** (*O*-benzyl protected hydroxamates) was screened for antibacterial activity and found to be inactive, suggesting that triclosan was not being released extracellularly under the conditions tested.

Desketoneoenactin–siderophore conjugates for *Candida*

In 2005, Bernier *et al.* reported the use of siderophore–drug conjugates and their efficacy, based on the specificity of the yeast iron transport systems in *Candida* spp [20]. A series of natural and synthetic siderophores including ferrichrome (FCH), ferrioxamine B, isocyanurate-based hydroxamate, ornithine-based trihydroxamate (ORNI) and rhodotorulic acid were screened to determine their potential as growth promoters in yeast. FCH was found to be most effective, followed by its acyclic trihydroxamate-based siderophore compontent (ORNI). Based on these observations, three siderophore-conjugates were prepared and screened against a panel of *Candida* spp. (Figure 4).

Conjugation of antifungal agent 13C-Desketoneoenact in (DE) **4** to hydroxamate-based siderophores significantly reduced the activity relative to the drug (DE) itself (Figure 4). However, the activity of the siderophore–conjugate did improve when tested under iron-deficient conditions, possibly due to an over-expression of the FCH receptor, which is responsible for the acquisition of trihydrox-amate-based siderophores. The observed activity increase induced by iron deficiency was most evident when screened against strains that were able to use ORNI siderophores as growth factors. Interestingly, the DE-monohydroxamate conjugate (DE-mono) **6** was the most active compound of the synthesized panel and its activity was the most influenced by the presence of iron. However, the presence of an additional hydroxamate group in DE itself, makes **6** a *bis*-hydroxamate overall, which could enhance its metal binding capability. Detailed studies related to whether conjugation of DE with siderophores might interfere with drug recognition and/or whether drug release is necessary for this class of antibiotics would be beneficial.

Pyoverdin & pyochelin antibiotic conjugates targeting *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous Gram-negative bacterium and has emerged as an important nosocomial pathogen during the past several decades. Cystic fibrosis patients [21] or patients having a weakened immune system, such as those with burns and those having AIDS [22], are especially susceptible to fatal pseudomonal infections. Treatment is generally compromised since *P. aeruginosa* is highly resistant to most of the existing antibiotics and multidrug resistance is becoming more prevalent.

One major problem of antibiotic resistance in Gram-negative bacteria such as *Pseudomonas* is the low permeability of their outer membrane which serves as a barrier to prevent antibiotic uptake by passive diffusion [23]. Thus, exploitation of the pseudomonal iron transport system to promote active uptake of antibiotics is of considerable interest and, as described below, has proved to be effective.

The primary siderophore of *P. aeruginosa* is the fluorescent yellow–green pigment pyoverdin, although, like many other types of bacteria, it can also use a number of xenosiderophores. Different strains of *P. aeruginosa* very selectively recognize and utilize one of three pyoverdins (Types I, II and III) that are structural variants (Figure 5) [24]. This specificity of pyoverdin recognition makes *P. aeruginosa* an ideal candidate for sideophore-mediated drug delivery.

Two pyoverdin–ampicillin conjugates were synthesized by Budzikiewicz *et al.* by selectively modifying the Lys residue in both type I pyoverdin analog and type II pyoverdin with a dicarboxylic acid linker (Figure 6) [25]. This long, flexible spacer was chosen to avoid any potential steric hindrance between the extensively modified ampicillin and its targeted bacterial transpeptidase as well as not to affect the outer membrane recognition by close attachment of the drug to the siderophore.

Conjugate **8** and **9** showed significant antibacterial activity against the corresponding pyoverdin producing *P. aeruginosa* strain. The minimum inhibitory concentration (MIC) values are 0.39 μM for **8** and 0.024 μM for **9**, while *P. aeruginosa* is resistant to ampicillin alone. Detailed kinetic growth curve studies of conjugates **8** and **9** showed that the two conjugates could delay bacteria growth for approximately 16 h and eventually resulted in induction or selection of resistant mutants that were no longer susceptible to the conjugates. The conjugate-resistant mutants have impaired pyoverdin uptake systems and are expected to be less virulent since they are more prone to iron starvation. Similar phenomena were observed in studies of *E. coli* exposed to catechol-based siderophore–loracarbef conjugates [26].

A type I pyoverdin–cephalexin conjugate was reported by the same group [27]. The siderophore and the β-lactam were crosslinked by condensing the guanidine group of Arg residue with β-diketone **10** to form a pyrimidine ring that served as the ultimate linker (Figure 7). However, the conjugate had no antibacterial activity against the pyoverdin producing strain. Instead, it was actually a growth promoter, indicating that site of or mode of drug attachment to the siderophore may be crucial for conjugate activity. Since earlier studies indicate that β-lactam antibiotics do not need to be released from their respective siderophore conjugates to be active [25], these results imply that the chemical modification of the siderophore backbone used to prepare this conjugate interfered with siderophore recognition and transport.

Hennard *et al.* reported syntheses and antibacterial assays of type I pyoverdin conjugates formed by attaching two quinolones, norfloxacin and benzonaphthyridone, with a non-labile simple amide linker and separately with a methylenedioxy-containing linker, which was anticipated to be susceptible to non-specific esterases or just randomly hydrolyzable (Figure

8) [28]. Use of the potentially releasable linker was expected to negate potential steric hindrance due to the presence of the pyoverdin moiety when the drugs interacted with their intracellular DNA gyrase target. The transport assays using ^{55}Fe showed that all four conjugates were actively transported into the bacterial cells almost as efficiently as pyoverdin itself. Thus, modification of the natural succinyl group that is peripheral to the essential chromophore of the pyoverdins did not appear to affect the siderophore recognition and transport process. Furthermore, inhibition assays with the isolated gyrase of *E. coli* showed that the quinolone moiety of the conjugate inhibited the enzyme without being released from the pyoverdin. However, the conjugates were up to 50-times less active compared with parent quinonlones in the assay, probably due to steric hindrance caused by the presence of pyoverdin in the conjugates. Therefore, release of the antibiotic moiety was still highly preferred.

In whole-cell assays with the *P. aeruginosa* strain ATCC 15692, conjugates **15** and **16** were found to be less active than the parent drug, most likely because of the decreased target recognition mentioned earlier or because the antibiotic actually did not reach its cellular target before pyoverdin was effluxed. Only conjugate **18** with a labile linker showed improved inhibition activity. However, the studies did not determine if the quinolone had been prematurely released due to the randomly hydrolyzable nature of the labile linker. Improvement of activity might require incorporation of a linker that could release the attached antibiotic from the conjugates in a controlled fashion by an intracellularly specific hydrolysis.

Another siderophore that *P. aeruginosa* synthesizes is pyochelin. Unlike pyoverdins, which are strain selective, pyochelin is used by all *P. aeruginosa* strains as a common sideophore, though it is responsible for only 5–10% of iron transport in the bacteria [29]. Therefore, conjugates using pyochelin as an antibiotic vector were anticipated to have broader antipseudomonal activity. A series of pyochelin–norfloxacin conjugates was synthesized and their bactericidal activity profile was found to be similar to the pyoverdin–norfloxacin conjugates **15** and **17** [30]. Growth curve studies indicated that only conjugates with the hydrolyzable linker **20** and **22** showed activity comparable to norfloxacin itself against *P. aeruginosa* while conjugates **19** and **21** with non-hydrolyzable linkers were inactive. Other than pyoverdin and pyochelin, using citrate as an exogenous siderophore to deliver antibiotic into *P. aeruginosa* and other Gram-negative bacteria such as *E. coli* has proved to be less effective, presumably because of the decreased iron affinity of the modified citrate [31].

Catecholate–aminopenicillin conjugates targeting Gram-negative bacteria

To initiate one of the most comprehensive studies of siderophore-based Trojan Horse antibiotics, Möllmann's group at the Han-Knoell Institute (HKI, Jena, Germany) prepared a large number of penicillin conjugates with totally synthetic biomimetic siderophore analogs [32–34] (Figure 9). These unnatural, but rationally designed, iron-chelators containing biscatecholate, triscatecholate and/or substituted hydroxamates were first tested for their siderophore activity. The most effective growth promoters were selected as active transport vectors and coupled with various β-lactams such as ampicillin and amoxicillin.

A clever novel feature of these conjugates was use of protected catecholates that were expected to serve as prodrugs to the required iron binding catechols while circumventing potential methylation by a catechol *O*-methyltransferase that would lead to a permanent loss of effective iron binding ability necessary for siderophore activity [35]. Impressively, preliminary testing against laboratory reference *P. aeruginosa* strains showed that conjugates **23**–**27** were ≥1000-fold more active relative to ampicillin! Further testing against clinically isolated aminopenicillin resistant strains of *P. aeruginosa* revealed that these unique conjugates were remarkably active, with a typical MIC below 0.25 μg/ml. Furthermore, unlike β-lactams, the conjugates were equally active against wild type and efflux pump overproducing strains like MexAB-OprM, MexCD-OprJ or MexEF-OprN. Thus, the conjugates circumvented efflux, a major cause of antibiotic resistance. Finally, *in vivo* studies showed that conjugates **23**–**27** effectively cured *P. aeruginosa* infections in mice with ED_{50} values comparable to the quinolone ofloxacin and low toxicity was observed. Taken together, the design, syntheses and combined *in vitro* and *in vivo* studies of these novel conjugates clearly demonstrate the tremendous potential of sideromycins for development of much needed microbe-selective antibiotic therapy.

Synthesis & biological studies of siderophore–lantibiotic conjugates

All of the siderophore conjugates described earlier incorporated relatively low MW antibiotics, that most often were independently active and capable of being assimilated by passive diffusion. No systematic study has determined the size limitations of siderophore conjugates. The isolation and structure determination of microcins, natural sideromycins containing antibiotic peptides that are significantly larger (~84 mer) than the siderophore core, suggest tremendous potential for active transport of many types of biomolecules, regardless of size. To test this potential versatility, the Vederas group prepared conjugates of the lantibiotic gallidermin to target Gram-negative bacteria [36]. Lantibiotics are a class of post-translationally modified antimicrobial peptides produced by some Gram-positive bacteria with potent antibacterial activity against other Gram-positive bacteria [37,38]. They are active in very low concentrations by either inhibiting peptidoglycan biosynthesis or by forming cell membrane pores that lead to leakage and cell death. However, the inability to penetrate the outer membranes of Gram-negative bacteria significantly limits their potential. Syntheses and studies of conjugates of lantibiotics with Gram-negative selected siderophores were anticipated to not only test size limits of iron transport-mediated drug delivery in Gram-negative bacteria, but also if recognition of the lantibiotic by its target would tolerate the incorporated siderophore component.

The lysine constituents of the lantibiotic gallidermin were separately attached to pyochelin **28**, agrobactin **29** and desferrioxamine (DFO) B **30** by dimethylsquarate linkages (Figure 10). The resulting conjugates, **31**–**35**, retained their activity against several Gram-positive indicator strains. However, they were not active against Gram-negative bacteria and actually promoted the growth of some strains. The growth promotion observed suggests that either the conjugates were able to penetrate the outer membrane of the cells, but modified lantibiotic was not active or that the conjugates served to solubilize iron, which then was exchanged with the naturally produced siderophores. More studies to determine the cause of inactivity would be of interest.

Synthesis & studies of a mycobactin–artemisinin conjugate that is selectively potent against both tuberculosis & malaria

In 2011, Miller *et al.* reported the synthesis of a mycobactin–artemisinin conjugate **36** with potent anti-tuberculosis (TB) and antimalaria activity (Figure 11) [39]. Mycobactin T, the intracellular siderophore of *M. tuberculosis* does not have a site for structural modification. However, chemical synthesis allowed the incorporation of an amino moiety for derivatization. Artemisinin, a naturally-derived drug, and its semi-synthetic analogs have been used for the treatment of malaria. However, artemisinin displays no anti-TB activity by itself. It was hypothesized that if artemisinin could be sequestered by *M. tuberculosis,* the redox chemistry associated with reductive removal of the iron from the mycobacterial siderophore might initiate Fenton chemistry to intracellularly produce free radicals that would be lethal to the assimilating bacteria. Despite the apparently labile structure of this drug, the molecule is robust enough for chemical modification and a conjugate was able to be synthesized to test this intriguing hypothesis. Gratifyingly, the synthetic mycobactin–artemisinin conjugate was found to be a potent anti-TB agent with an MIC of 0.39 μg/ml against *Mtb* H37Rv. More extensive testing revealed remarkable activity against various multidrug-resistant strains (MIC = 1.25–0.16 μg/ml and even extensive drug resistant strains (MIC = 0.625–0.078 μg/ml). To determine if conjugation of the artemsinin to the mycobactin would eliminate antimalarial activity, it was tested against several strains of *P. falciparum* (malaria) and found to essentially retain the same low nanomolar activity of artemisinin itself. Since the conjugate was designed to be selective for *Mtb* because of the exquisite recognition of the mycobactin component, it was tested against a broad panel of bacteria and, as expected, no other activity was observed. This example of using a conjugate with a drug that had no anti-TB activity itself, but with a potential target if it could be assimilated by using a microbe-selective delivery agent provided a clear example of the tremendous potential of siderophore–drug conjugates for generation of bacteria-specific antibiotic delivery agents. To re-emphasize this remarkable selectivity, all the other synthetic intermediates, and artemisinin itself, did not have any anti-TB activity. When an *O*-benzyl-protected hydroxamate intermediate was tested, the antimalarial activity decreased between 20–100-fold and all anti-TB activity was lost, stressing the importance of iron-binding. In a comparative study, the corresponding DFO–artemisinin conjugate was prepared and shown to retain some antimalarial activity, albeit lower than artemisinin itself, but it had no anti-TB activity. This was expected since *Mtb* does not use DFO as a siderophore. Physicochemical assays showed that, as hypothesized, the mycobactin conjugate did undergo the expected Fenton chemistry and whole-cell studies with live *Mtb* demonstrated that it did induce formation of reactive oxygen species intracellularly. Thus, a single conjugate was successfully and rationally designed to inhibit proliferation of two of the most significant diseases known.

Siderophore–monobactam conjugates are active against MDR Gram-negative bacteria

Natural and synthetic siderophore analogs most often contain three bidentate ligands for stoichiometric binding of iron (III). Attempts to reduce this inherent complexity have

encouraged syntheses and studies of lower valent conjugates, including incorporation of a single bidentate ligand, usually a catechol, a hydroxypyridone or a hydroxamic acid, which is covalently attached to a drug. A large number of such simplified conjugates have been synthesized since the 1980s and, as with other conjugates, most incorporate β-lactam antibiotics [10,40,41]. Syntheses of monobactams with hydroxypyridone iron chelating groups such as MC-1 **(37)** [42,43] and BAL30072 **(39)** [44–46] were recently reported and their antibacterial activities were extensively studied (Figure 12). Monobactams such as aztreonam **40** have advantages over classical penicillins and cephalosporins since they are highly resistant to hydrolysis by class B metallo-β-lactamases and are potent inhibitors of class C β-lactamases [47,48]. Hydroxypyridones are isosteres of catechols and they are specifically recognized by the Cir and Fiu outer membrane proteins of bacteria after binding iron. Unlike catechols, they are not substrates for catechol-*O*-methyl-transferase, so the use of prodrugs previously mentioned to circumvent this deactivating process was not anticipated to be necessary. Since monobactams tolerate extensive peripheral modification, attachment of siderophore components was expected to promote penetration of the outer membrane of Gram-negative bacteria and increase drug concentration in the periplasm.

MC-1 **(37)** was developed based on the previously reported Upjohn monobactam U-78608 **(38)**. Both **37** and **38** exhibited excellent activity against Gram-negative bacteria including clinical isolates of *P. aeruginosa,* with MIC_{90} values 2 and 0.5 μg/ml, respectively. Crystallographic studies revealed that **37** binds to PBP3 efficiently in *P. aeruginosa* [43]. However, with a polar diol side chain on the triazalone, **37** showed a significant reduction of plasma protein binding compared with **38**, thus, increasing its concentration in tissue. MC-1 **(37)** exhibited superior protection in an *in vivo* model of respiratory tract infection with *P. aeruginosa* 1091–05, with PD_{50} values of 20.6 versus >150 mg/kg for U-78608 **(38)**. This is important because lungs are frequently the sites of infection of some nosocomial Gram-negative pathogens such as *P. aeruginosa.*

Similar to MC-1, BAL30072 **(39)** is a sulfactam with a dihydropyridone moiety, which was designed to promote conjugate influx across the Gram-negative outer membrane. BAL30072 displayed potent activity against multidrug-resistant (MDR) *P. aeruginosa* and *Acinetobacter* spp. isolates, including many carbapenem-resistant strains. The MIC_{90} values were 4 μg/ml for MDR *Acinetobacter* spp. and 8 μg/ml for MDR *P. aeruginosa,* while the MIC_{90} of meropenem for the same sets of isolates was >32 μg/ml. Further study found that in a time-kill study against MDR *Acinetobacter baumannii*, combining BAL30072 with meropenem lowered the bactericidal concentrations two- to eightfold, compared with using meropenem or BAL30072 alone.

The potential of a DFO–gallium complex as an anti-pseudomonal therapeutic agent

Non-iron siderophore–metal complexes have also been explored as potential Trojan Horses. Rogers *et al.* reported the use of scandium and indium complexes of enterobactin to inhibit growth of *E. coli* [49]. Kaneko *et al.* reported that $Ga(NO_3)_3$ inhibited growth of *P. aeruginosa in vitro* and as biofilm, presumably by disrupting the iron metabolism of the

pathogen [50]. More recently, Banin *et al.* expanded this idea by reporting the use of the siderophore DFO, to deliver gallium rather than iron to *P. aeruginosa* and observe its effect [51]. The rationale behind their design being that *Pseudomonas* is believed to possess two pathways for uptake of the DFO–Fe^{3+} complex, thus, DFO might effectively be used as a Trojan Horse to deliver the Ga^{3+} ion and induce iron starvation. Their results demonstrated that both the DFO–Ga^{3+} complex and $GaCl_3$ inhibited growth of the organism, while DFO alone did not, even when tested at a maximum concentration of 1 mM. When compared with antibiotic gentamicin, the DFO–Ga^{3+} complex was 10–100-times more effective, and when gentamicin was combined with DFO–Ga^{3+}, the growth was synergistically decreased by sixfold. Growth studies using siderophore-transporter mutants revealed that as long as one of the DFO–Fe^{3+} uptake paths was available to the organism, the Ga^{3+} complex remained effective, and only when both uptake mechanisms were eliminated, was a reduced sensitivity observed. Also when these agents were tested at sub-MIC levels, only DFO–Ga^{3+} and $GaCl_3$ (0.001 mM) were capable of inhibiting growth, whereas neither DFO nor Gm alone did. DFO–Ga^{3+} was also effective in blocking biofilm formation and killing mature preformed biofilm. The gallium complex was also tested against 15 clinical isolates of *Pseudomonas* and all were found to be sensitive to DFO–Ga^{3+}. Considering the organism has two uptake methods for this siderophore, development of resistance was expected to possibly be more complicated. The details about the mode of action of the gallium complexes are still being investigated.

Siderophore conjugates as potential diagnostic agents for prostate cancer

In 2007, Ding *et al.* reported the syntheses of inhibitors of *N*-acetylated α-linked acidic dipeptidase (NAALADase), a protease involved in the release of glutamate in the brain [52]. A protein having NAALADase activity, the prostate specific membrane antigen (PSMA), is expressed very early in prostate cancer. Conjugation of PSMA inhibitors with siderophores is of particular interest since the conjugates could be employed as contrast agents for prostate-derived cancer cells detection by means of MRI. A NAALADase inhibitor, 2-PMPA, was used as a template to develop the trihydroxamate-based siderophore–PMPA conjugate **41** (Figure 13). Conjugate **41** displayed strong NAALADase inhibitory activity with IC_{50} = 5 nM, with abilities in formation of Fe^{3+} or Gd^{3+} complexes for further MRI experiments. In 2008, Ding *et al.* reported the synthesis of a modified siderophore-conjugate **42**, which incorporated a linker to prevent interaction of the carboxylic acids in the PSMA inhibitors and the metal binding siderophore moiety. The inhibitory activity of this conjugate was retained at IC_{50} = 4 nM [53].

A novel near-IR fluorescent integrin-targeted DFO analog

Another use of siderophores is in combination with various fluorescent molecules for *in vivo* and *in vitro* imaging. Fluorescent probes based on bidentate iron chelators have been applied in detection of labile iron in primary hepatocytes [54]. More recently, Ye *et al.* reported the development of an integrin-targeted near-IR (NIR) fluorescent probe in conjugation with the siderophore DFO as an optical contrast agent [55]. The authors evaluated the use of cypate (Figure 14), a NIR active compound, previously reported to have excellent spectral properties and employed to label diverse peptides as well as other bioactive molecules [56],

and cyclo[RGDfK(~)], a small cyclic peptide that targets the cell surface receptor (integrin αVβ3) ABIR with high affinity and selectivity. The over-expression of ABIR has been correlated with tumor growth, invasion and metastasis and, therefore, constitutes an attractive molecular target for specific imaging, diagnosis and therapy. Three conjugates were assembled, DFO-cypate-cyclo[RGDfK(~)] **43** (Figure 14), cypate-cyclo[RGDfK(~)] **44** and cypate-DFO, and studied to determine the ability of cyclo[RGDfK(~)] to transport DFO into the tumors, the effect of the siderophore on the biological activity of the conjugates and the potential modulation of the fluorescent NIR probe due to the chelating capabilities of DFO. Metal binding studies were carried out using 13 different metal ions, among which the fully elaborated conjugate **43** showed high affinity for Al^{3+}, Fe^{3+} and Ga^{3+}, indicating that chelation by the siderophore remained viable after the assembly.

With the exception of Fe^{2+}, Fe^{3+} and Cu^{2+}, addition of metals did not change the probe's green color. And based on the affinity results, the Fe complexes were presumed to quench fluorescence by a photo-induced electron transfer mechanism, while the Cu^{2+} complex did so by interaction with cypate. Ga^{3+} seemed to enhance the light emission of the conjugate, as expected since it has been used in other nuclear imaging studies.

ABIR binding studies showed that incorporation of DFO in **43** diminished the receptor affinity of the reference compound **44** by a twofold factor, but the resulting affinity was comparable to that of the cyclo [RGDfK(~)] peptide alone. Cell internalization experiments showed that both **43** and **44** internalized rapidly into ABIR-positive cells within 1 h post-incubation and remained in the intracellular space for up to 10 h. In contrast, DFO itself had difficulty crossing cell membranes without a mechanism of penetration.

Use of siderophore–protein carrier conjugates in the development of bacterial vaccines

Bergeron and co-workers realized that covalently linking siderophore analogues to siderophore bacterial outer-membrane receptors represents a credible target for bacterial vaccine development [57]. Therefore, vibriobactin (VIB), a siderophore responsible for iron utilization in *Vibrio Cholerae,* fixed to large carrier proteins ovalbumin (OVA) and bovine serum albumin, respectively, were synthesized with a thiol tether (Figure 15). The resulting OVA–VIB conjugate **45** was successfully used as an antigen to raise antibodies in mice. Further studies showed that the antigenic determinants of the antibody were associated with the VIB rather than with the carrier proteins. IgG monoclonal antibodies specific to the VIB fragment of the bovine serum albumin and OVA conjugates were also isolated. Furthermore, studies to evaluate the antibodies' potential in a vibrio-infected animal model would be of great interest.

Use of siderophores for pathogen detection & diagnostics

Diagnostic processes are desperately needed for rapid and effective identification of bacteria in infections to facilitate proper treatment. Since many siderophores are microbe selective, their use as affinity probes for selective bacteria is under consideration. Covalent attachment of metal-bound siderophores to surfaces or absorbents with appropriate linkers may allow

recognition of bacterial outer membrane siderophore receptors and with consequent selective absorbance of the targeted bacteria. The immobilized bacteria might then be observed and identified by microscopic techniques or detected by other devices. The result could be a faster, cheaper, more portable and accurate method for pathogen diagnosis relative to traditional microbiological methods, which often take several days to complete.

Low *et al.* developed such a device for detection of *P. aeruginosa* by coating pyoverdin onto a gold-plated glass chip (Figure 16) [58]. After exposure of the chip to a *P. aeruginosa* containing solution, the bacteria were visualized by darkfield microscopy. The detection limit was as low as 10^2 bacteria/ml and at higher bacteria concentration, the captured bacteria could be observed within 1 min. As a demonstration of the selectivity of this siderophore-based detection process, other bacteria such as *E. coli* and *Y. enterocolitica* were not able to be captured on the pseudomonal selective chip. Immobilization of *Microbacterium flavescens* with an artificial hydroxamate siderophore on a gold electrode was also reported [59]. The bacteria absorption was observed by optical, scanning electron and atomic force microscopy as well as quartz crystal microbalance method.

Future perspective

Siderophores are an important class of natural products that have received wide attention for many years due to their unique properties as iron chelators and potential use in drug delivery, imaging and, most recently, in diagnostics. Significant advances have been made in the past decade, but additional studies and applications show promise for extended utility. Further appropriately designed studies will demonstrate the scope and limitations of the use of siderophores as active transport vehicles that may circumvent cell permeability and/or drug efflux problems associated with drug-resistant strains of bacteria. Advances in X-ray crystallography have revealed structures of a number of outer membrane receptors and will certainly have an impact on the field of siderophore research by, for example, determination of the proper modification sites on siderophores for drug attachment to ensure active transport [36]. Further attention in the development of controlled drug release processes from siderophore–antibiotic conjugates will expand the drug profiles and types of intracellular targets that can be considered. In addition, siderophore metal complexes and conjugates have considerable potential for use as targeted contrast agents and new pathogen detection devices. Successful development of siderophore–drug conjugates and siderophore-based diagnostic device will facilitate microbe selective, personalized antibiotic therapies that will slow the inevitable development of antibiotic resistance and help keep us one step ahead in the never ending microbial war.

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Key Terms

Siderophore

Low MW iron chelators synthesized and secreted by microbes to sequester and solubilize iron from the environment.

Sideromycins

Siderophore conjugates consisting of a metal-binding portion, a spacer or linker, and a drug moiety.

Siderophore conjugates

Combinations of various functionalized molecules (e.g., drug or fluorescent probe) ligated onto siderophores to achieve the designed purpose (e.g., microbial drug delivery, bacterial detection or diagnosis, cancer cell recognition).

Executive summary

- Iron acquisition has been proven to play an essential role in microbial virulence and pathogenicity. Investigation of microbial iron transport system led to the invention of siderophore–drug conjugates as a promising ‘Trojan Horse’ approach that uses active iron-transport processes to deliver antimicrobial agents.
- Studies on the use of siderophores for development of imaging agents and pathogen detection devices have provided great opportunities for future application of these versatile microbial iron chelators.

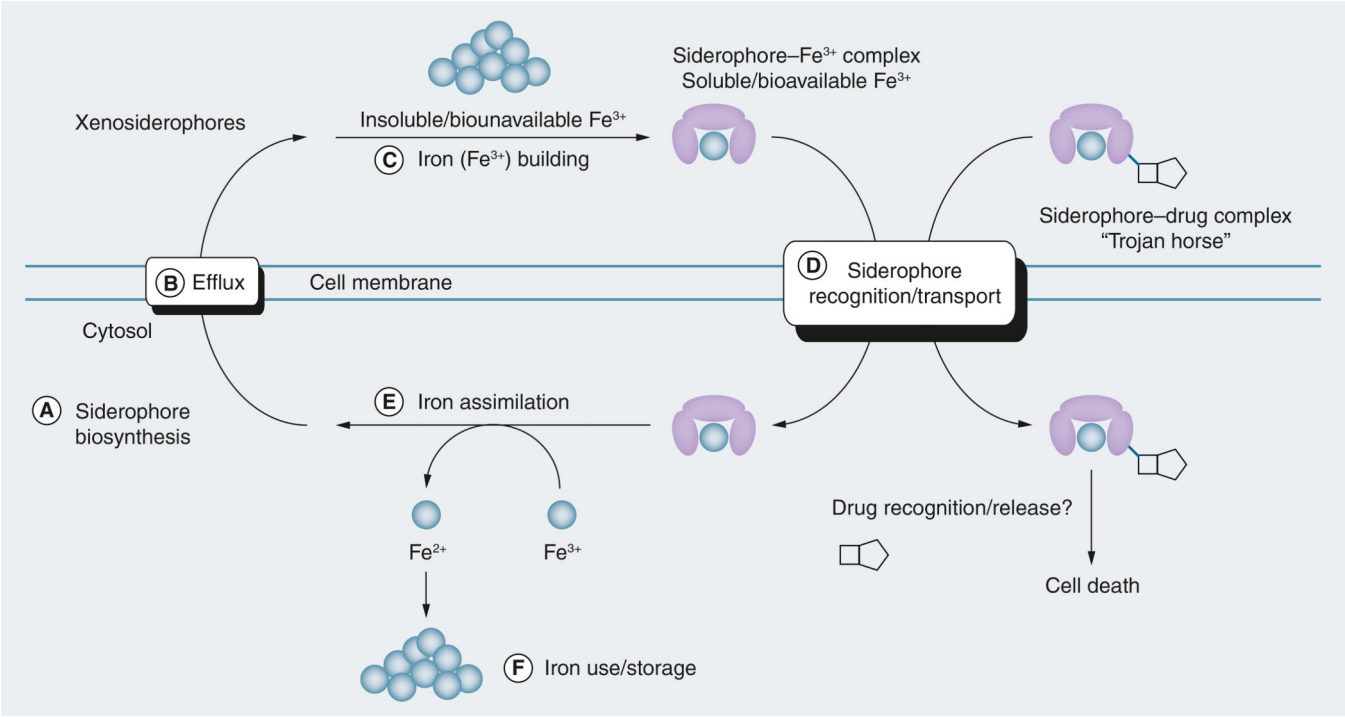

Figure 1. Siderophore-mediated iron acquisition and its exploitation by 'Trojan horses'.

(A) Siderophore biosynthesis; **(B)** siderophore efflux to extracellular environment; **(C)** iron(III) binding; **(D)** siderophore–iron(III) complex recognition and transport into the cell; the siderophore–drug conjugate enters the competing organism cell via the corresponding siderophore uptake pathway to deliver the drug moiety, which eventually leads to cell death; **(E)** iron assimilation from the siderophore–iron(III) complex; **(F)** iron use/storage [8].

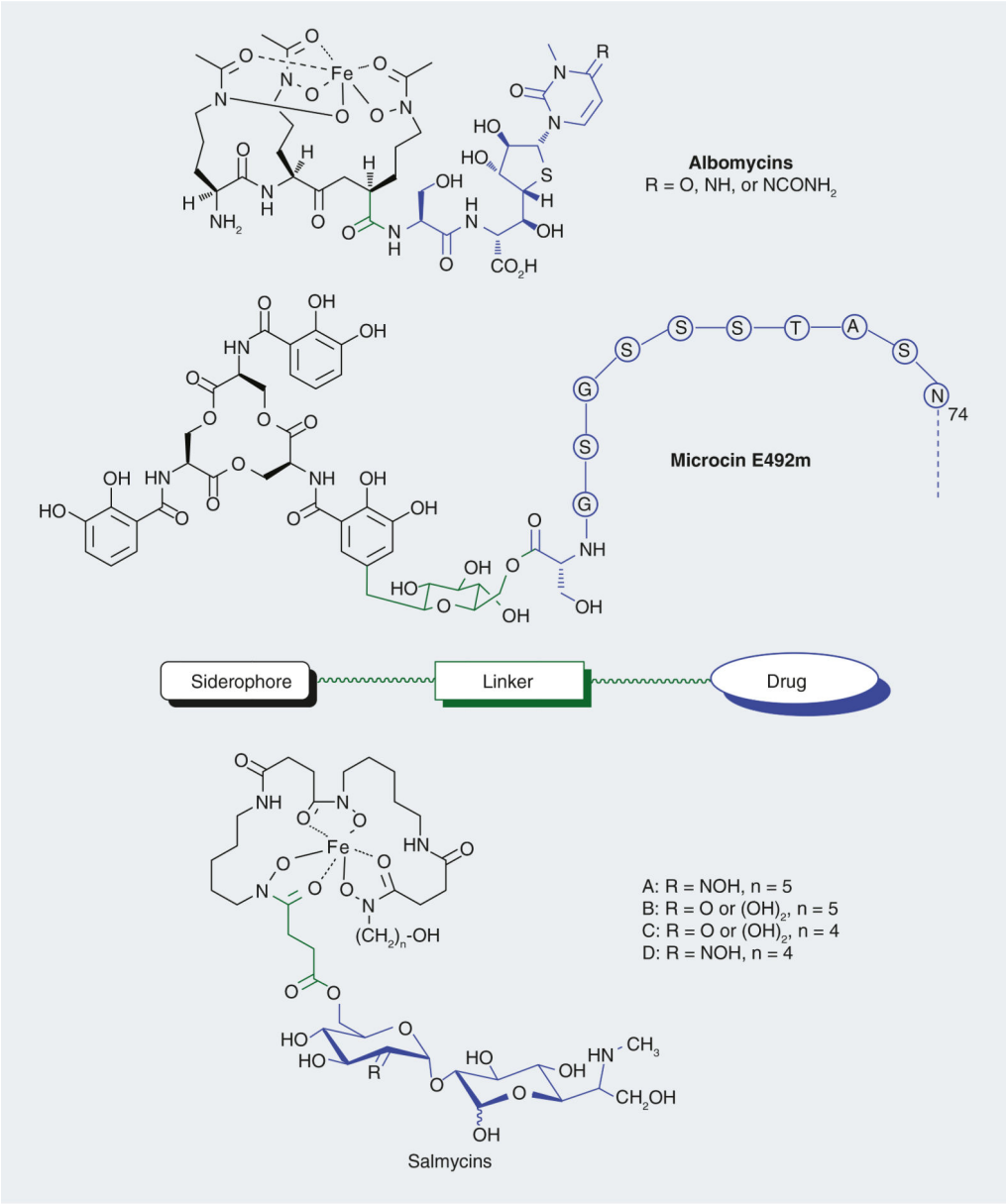

Figure 2.
Examples of naturally occurring siderophore-drug conjugates (sideromycins).

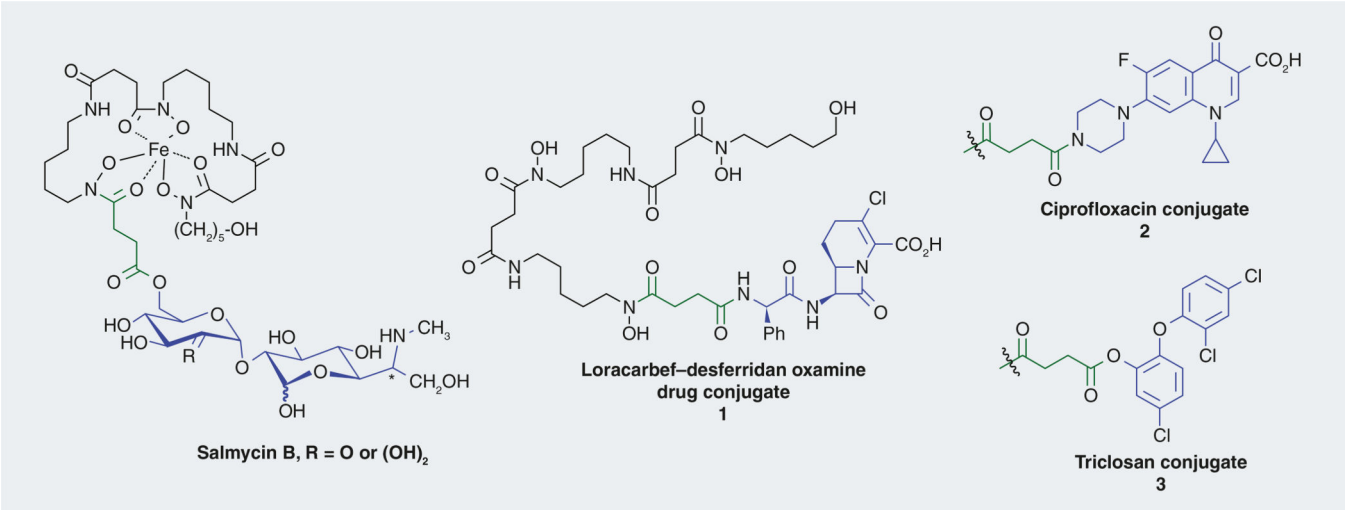

Figure 3.
Naturally occurring salmycins and synthetic desferridanoxamine-drug conjugates.

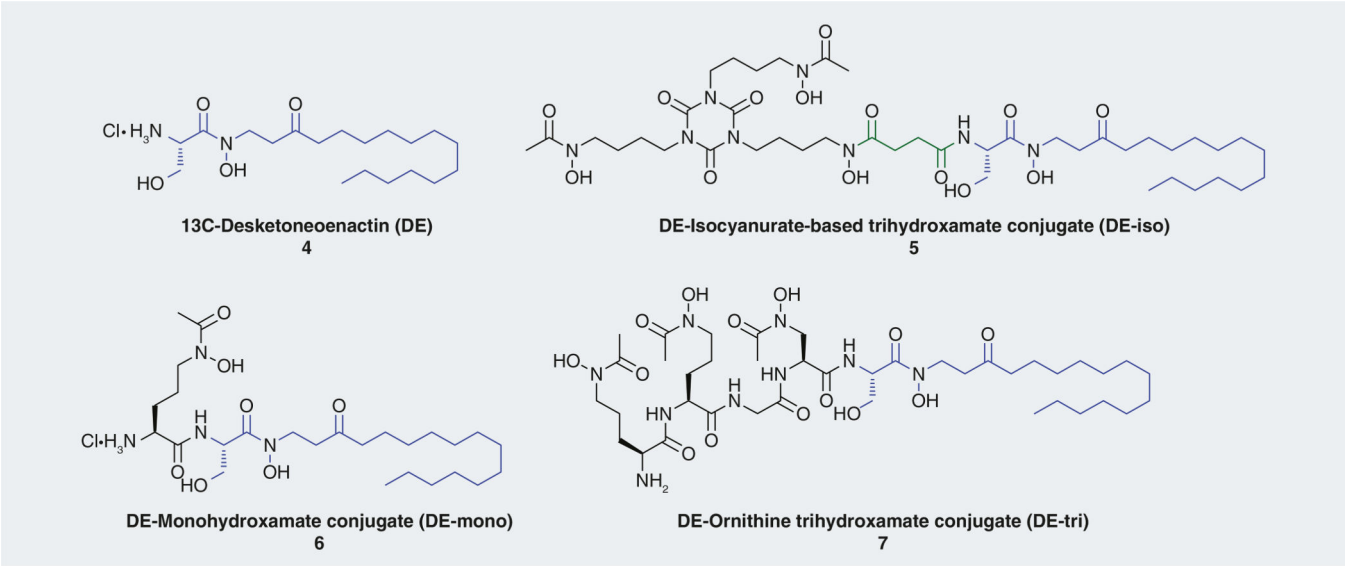

Figure 4.
Siderophore–drug conjugates against *Candida* spp.

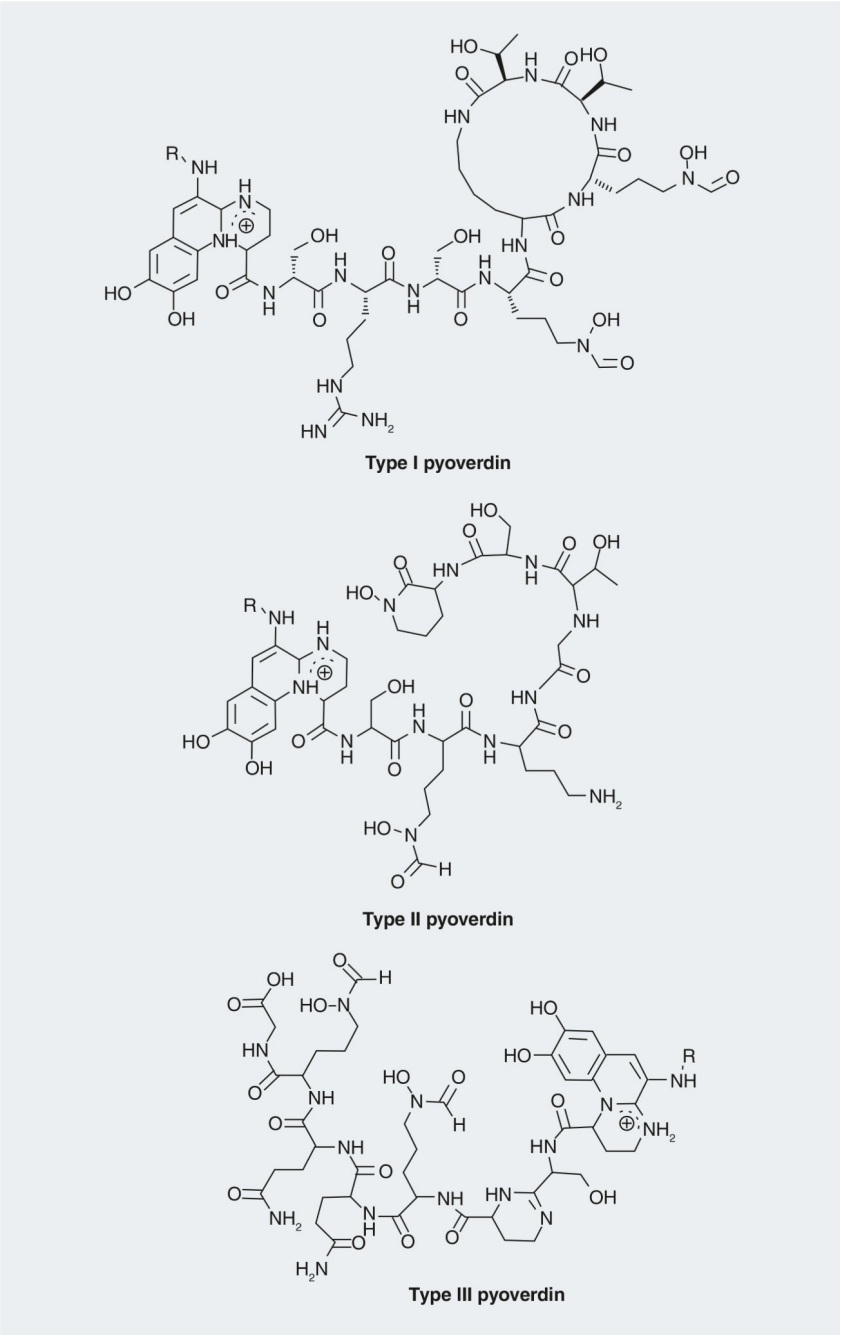

Figure 5.
The three types of pyoverdin of *Pseudomonas aeruginosa.*

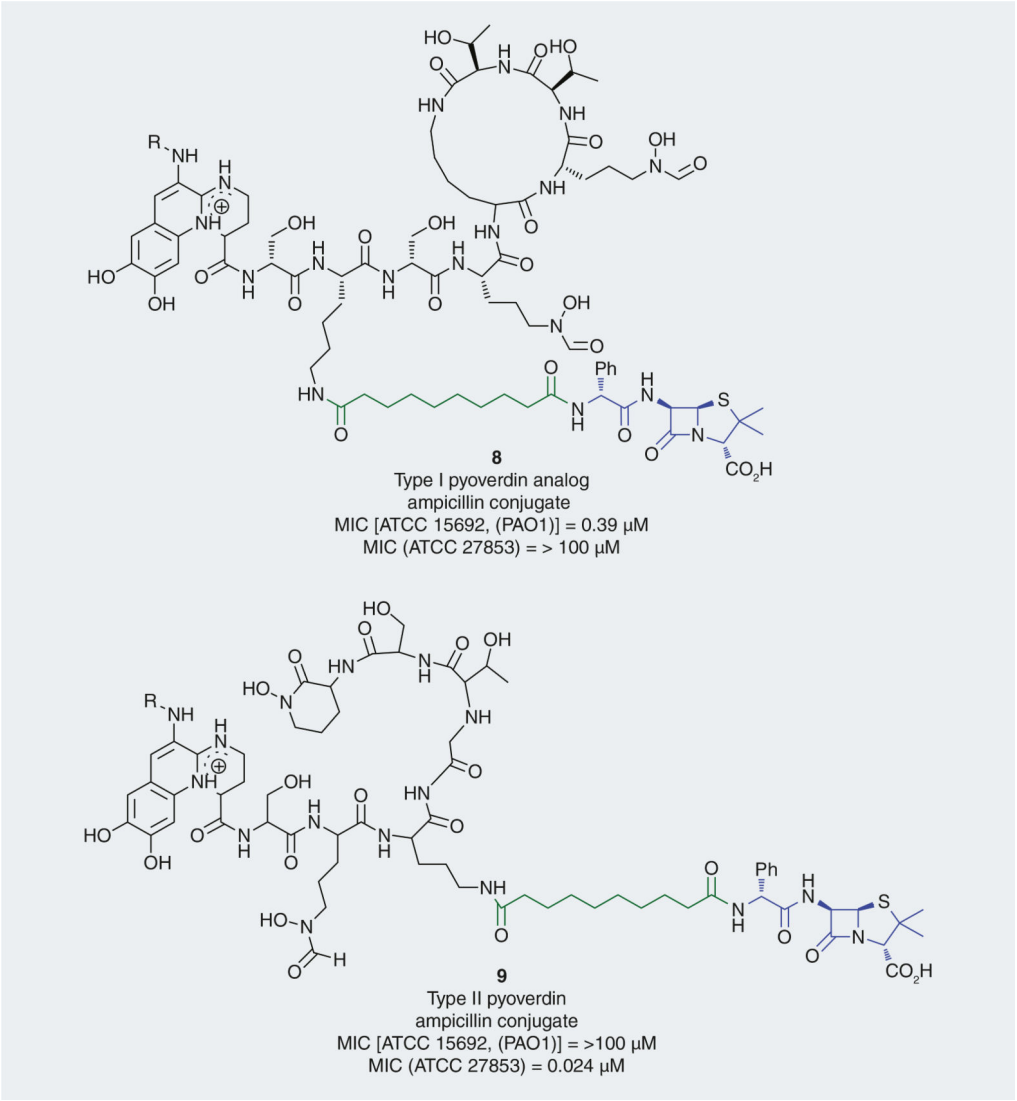

Figure 6.
Type I and II pyoverdin ampicillin conjugates.

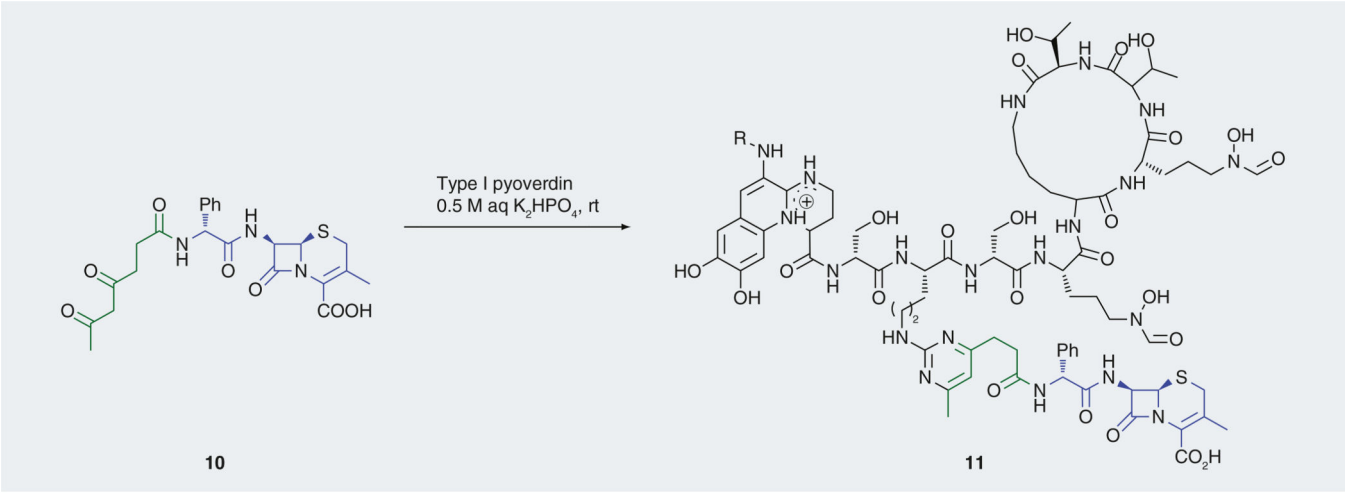

Figure 7.
Cross-linking reaction of type I pyoverdin with cephalexin.

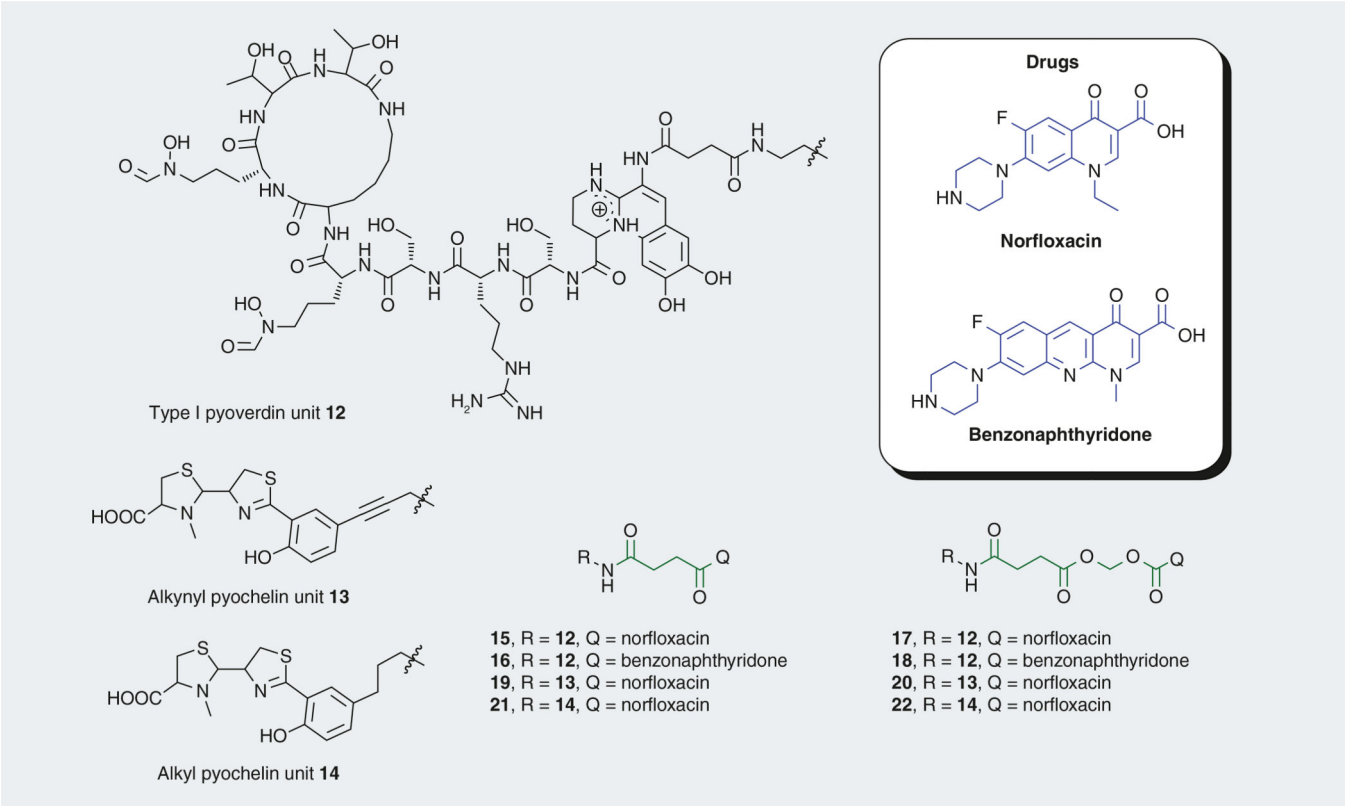

Figure 8.
Pyoverdin and pyochelin-fluoroquinolone conjugates with stable or labile linkers.

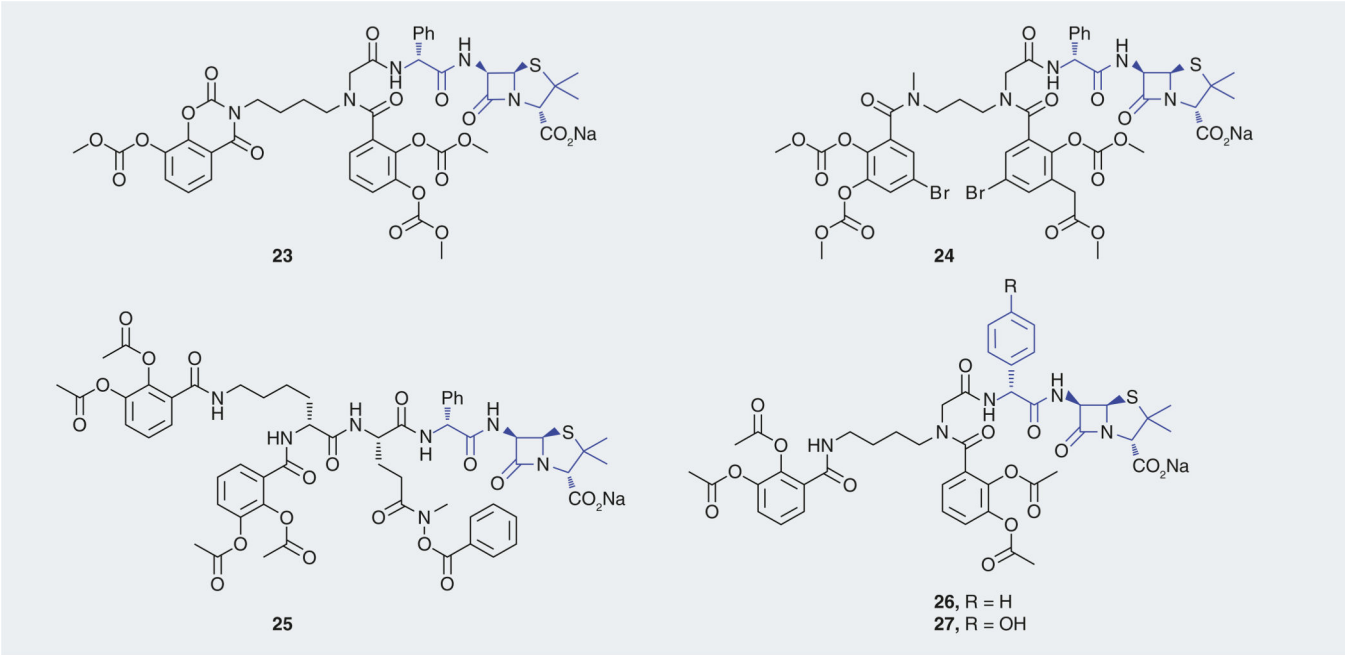

Figure 9.
Siderophore–aminopenicillin conjugates.

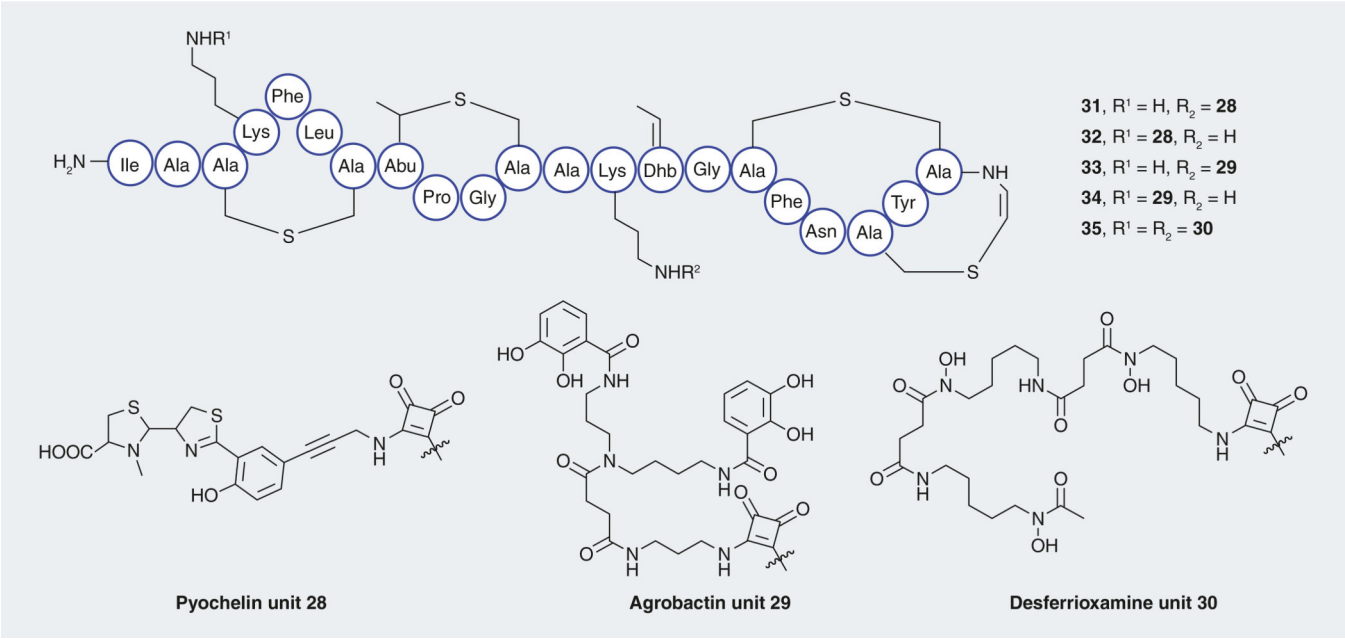

Figure 10.
Lantibiotic gallidermin–siderophore conjugates.

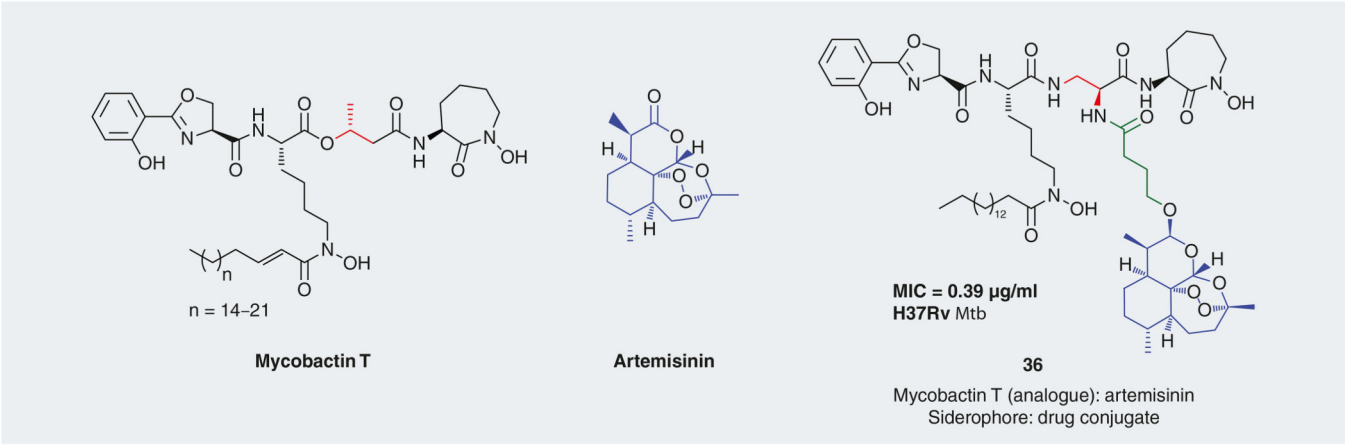

Figure 11.
Mycobactin–artemisinin conjugate.

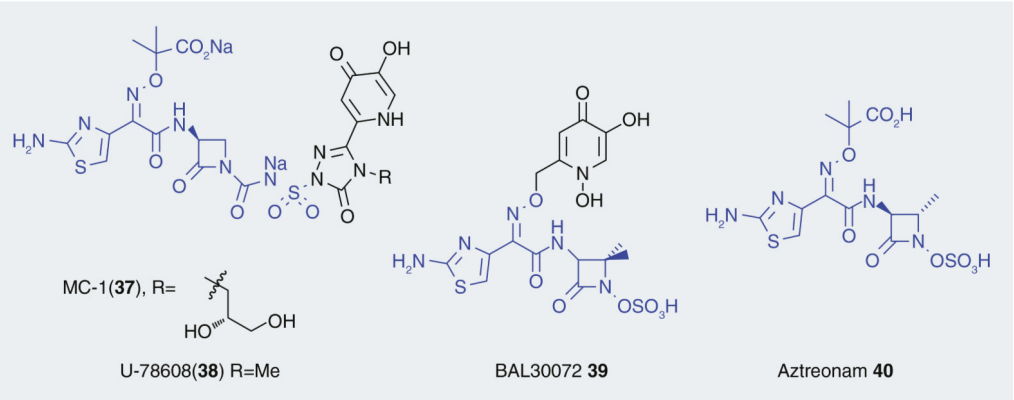

Figure 12.
Monobactams MC-1, U-78608, BAL30072 and aztreonam.

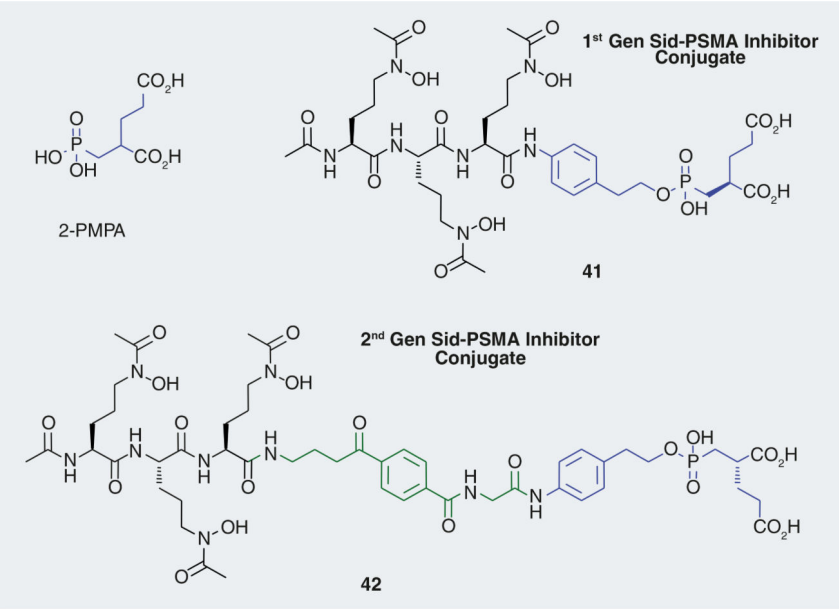

Figure 13.
Siderophore conjugate: potential diagnostic agent for prostate cancer.

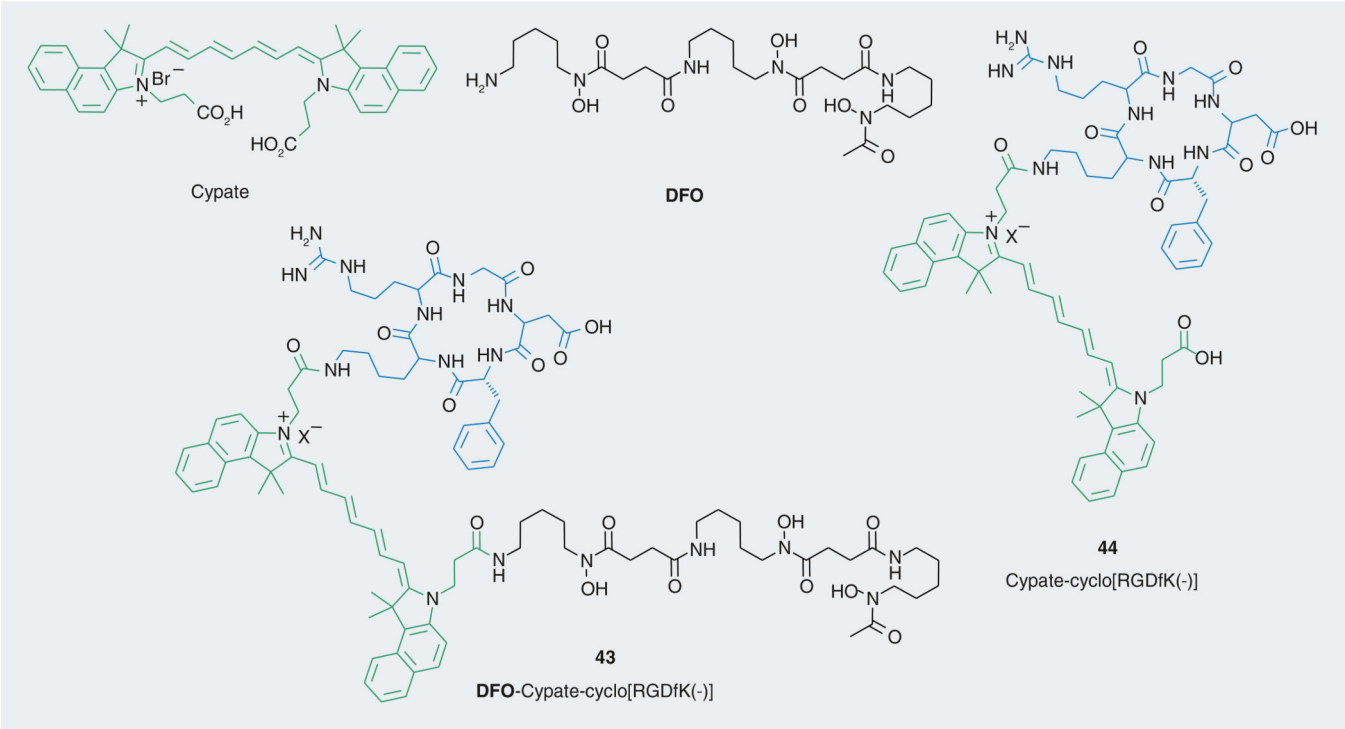

Figure 14.
Near-IR fluorescent integrin-targeted siderophore conjugate.

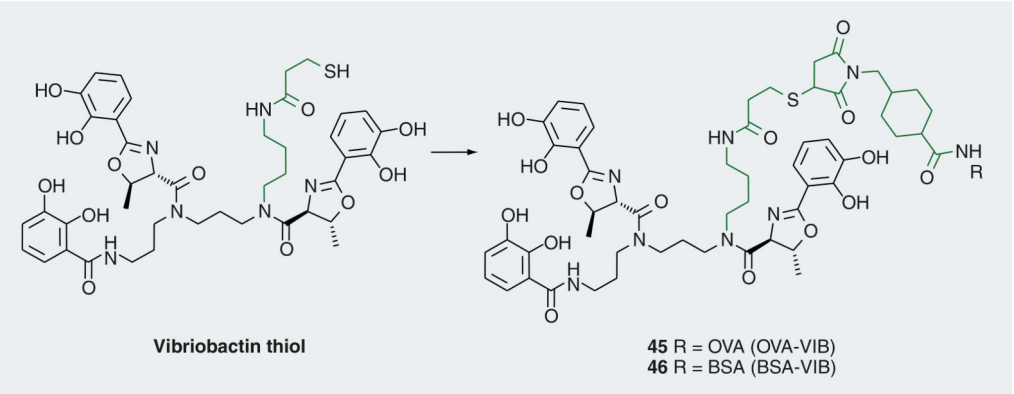

Figure 15.

Vibriobactin–ovalbumin and vibrobactin–bovine serum-albumin conjugates from vibriobactin thiol.

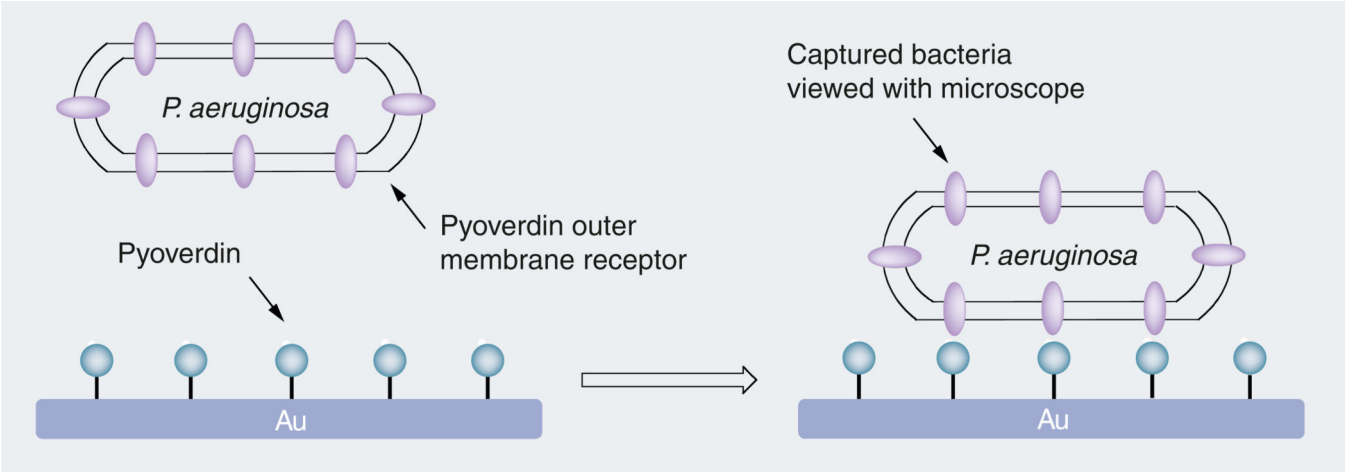

Figure 16.
Pseudomonas aeruginosa detection by pyoverdin-coated chips.