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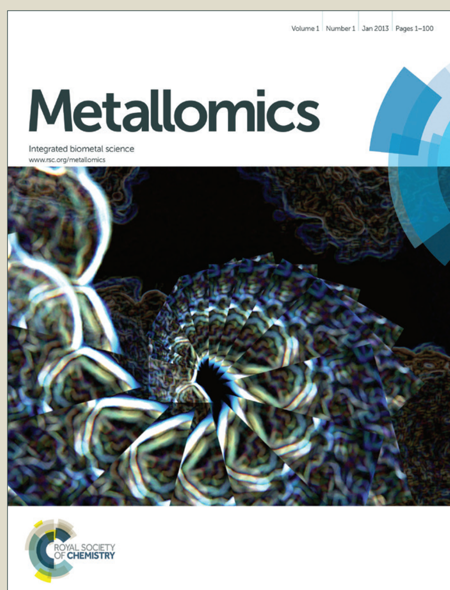

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Journal Name

RSCPublishing

ARTICLE

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Siderophore-dependent iron uptake systems as gates for antibiotic Trojan horse strategies against *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen responsible for nosocomial infections. The prevalence of antibiotic-resistant *P. aeruginosa* strains is increasing, necessitating the urgent development of new strategies to improve the control of this pathogen. Its bacterial envelope constitutes of an outer and an inner membrane enclosing the periplasm. This structure plays a key role in the resistance of the pathogen, by decreasing the penetration and the biological impact of many antibiotics. However, this barrier may also be seen as the “Achilles heel” of the bacterium as some of its functions provide opportunities for breaching bacterial defenses. Siderophore-dependent iron uptake systems act as gates in the bacterial envelope and could be used in a “Trojan horse” strategy, in which the conjugation of an antibiotic to a siderophore could significantly increase the biological activity of the antibiotic, by enhancing its transport into the bacterium. In this review, we provide an overview of the various siderophore-antibiotic conjugates that have been developed for use against *P. aeruginosa* and show that an accurate knowledge of the structural and functional features of the proteins involved in this transmembrane transport is required for the design and synthesis of effective siderophore-antibiotic Trojan horse conjugates.

Introduction

The emergence and increasing prevalence of pathogenic bacteria resistant to most of the drugs in clinical use raises fears of the end of the antibiotic era.[1] This development of bacterial resistance to antibiotics is an inevitable process that has nevertheless been greatly accelerated during recent decades by the massive use of antibiotics in human health and animal husbandry.[2] The evolution of antibiotic-resistant strains has also been accelerated by the almost complete lack of new classes of clinically relevant antibiotics reaching the market in the last few decades.[1,3] For all these reasons, there is now an urgent need for both new generations of antibiotics with new targets, and for other novel strategies for increasing the efficacy of known antibiotics.[3] This second aspect is particularly important in the struggle against *Pseudomonas aeruginosa*. This Gram-negative bacterium is an opportunistic pathogen involved in severe nosocomial infections.[4] Indeed, in the last few decades, *P. aeruginosa* has become a major threat in immunocompromised patients,[4] cystic fibrosis patients[5,6] and patients with extensive burns.[7] Infections due to *P. aeruginosa* have a frequent fatal outcome even in developed countries.[8,9] This high lethality mostly reflects the increasing resistance of this microorganism to the arsenal of antibiotics currently available.[10] This resistance is partly due to the chemical composition and diffusion limit of the bacterial envelope, greatly restricting the penetration of antibiotics from several families.[10-12] Furthermore, the expression of efficient efflux pumps in *P. aeruginosa* greatly decreases the biological impact of many antibiotics by preventing them accumulation in the bacterial cells.[13,14,15] In addition *P. aeruginosa* is able to acquire new resistance mechanisms rapidly.[16] These new mechanisms mostly involve modification of the biological target or the expression of enzymes able to inactivate antibiotics.[10,17] Finally, the communities of *P. aeruginosa* cells in the polysaccharide matrix of a biofilm are significantly less sensitive to the main classes of antibiotics.[18,19]

One way of increasing the efficacy of known antibiotics is the Trojan horse strategy, in which drugs are transported into the bacteria via nutrient uptake pathways. Indeed, membranes are physical barriers protecting the cell and transmembrane uptake systems act as gates for those molecules in possession of the appropriate “key”.[20] This strategy has been employed in therapeutic applications, but its use is most advance in anticancer,[21,22] and antibiotic applications.[23-33] In this context, the use of iron uptake systems as gates for introducing

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antibiotics into the inner compartment of the bacteria is particularly promising.[23-33] Iron is a crucial nutriment for bacterial growth, due to its role as a co-factor in important fundamental biological processes.[34] Paradoxically, the bioavailability of this metal is limited by the low solubility of iron(III) at physiological pH.[35] The human host contains substantial amounts of iron, but this crucial element is tightly associated with transport and storage proteins and is not freely available to pathogens. Moreover, during an infection, the innate immune system of the host limits the pathogens' access to iron by reducing intestinal iron absorption, through increases in the production of ferritin, and of lactoferrin by neutrophils at sites of infection, and by producing siderocalins.[36] As a result, the concentration of free iron bioavailable to pathogens in biological fluids has been estimated between 10^{-18} and 10^{-24} M,[37] whereas bacteria require iron concentrations in the micromolar range for proliferation during the infection process.[38] Microorganisms have overcome this problem of iron accessibility by developing highly efficient uptake systems for taking advantage of the iron present in the host.[39,40] The most common mechanism is mediated by low-molecular weight organic chelators (150 to 2000 Da) called siderophores.[41,42] These metabolites are synthesized by bacteria and released into the environment, where they chelate iron with an extremely high affinity. Indeed, siderophores are rich in the heteroatoms oxygen and nitrogen, which are able to form highly stable octahedral hexacoordinated complexes with iron(III),[41-43] which can compete with host proteins for iron. Bacteria recuperate their ferric-siderophores *via* specific membrane uptake machineries. Due to the essential nature of iron uptake for bacterial pathogenesis, siderophore iron uptake pathways are interesting gates for antibiotic treatment via Trojan horse strategies.

The design of promising antibiotic Trojan horse strategies against *P. aeruginosa* is thus highly dependent on our knowledge of the various biological molecular mechanisms leading to the translocation of the ferric-siderophore from the bacterial cell surface to the inner compartments of the bacteria. In this context, we aim here to provide an overview of the structural and functional data available for siderophore-dependent iron uptake systems in *P. aeruginosa* and the antibiotic Trojan horse strategies developed on the basis of siderophores excreted by *P. aeruginosa*.

Siderophores used by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa excretes two principal siderophores: pyoverdine (PVD) **1** and pyochelin (PCH) **2** (Figure 1). PVDs constitute a family of chromopeptide siderophores based on a common chromophore connected to an oligopeptide of 6 to 14 amino acids produced by all Pseudomonads. More than 100 pyoverdines have been characterized to date, and these molecules differ mostly in the number, composition and configuration of the amino acids of the oligopeptide fraction.[44-46]. Three structurally different PVDs (with different peptide chains) have been identified (PVD types I, II and III) in cultures of *P. aeruginosa* strains.[44-46] Each strain of *P. aeruginosa* produces one of these three types of PVD. In PVDI, produced by *P. aeruginosa* PAO1, iron(III) is coordinated by the catechol group of the fluorophore and by the two *N*-hydroxy formyl-ornithine moieties in the peptide sequence. Pyoverdines associate with iron(III) at high affinity ($10^{30.8}$ M^{-1} for PVDI), in 1:1 stoichiometry. The resulting ferric complexes are highly stable (pFe(III) of 27.0 for PVDI).[47]

Pyochelin **2** is a siderophore of *P. aeruginosa,*[48] but has also been shown to be excreted by other Gram-negative bacteria.[49,50] The structure of PCHs is based on a 2-hydroxyphenyl-thiazolinyl-thiazolidine scaffold. This siderophore complexes iron(III) with a stoichiometry of 2:1.[51,52] In the ferric complex, one PCH is tetradentate and binds the metal ion via the phenol oxygen, the nitrogen atoms from the thiazoline and thiazolidine rings and the oxygen atoms from the terminal carboxylate group. The second PCH involved in chelation interacts with iron(III) via the phenol oxygen and the nitrogen of the thiazoline ring.[51,52] The affinity of this siderophore for iron(III) is $10^{28.8}$ M^{-2} and with a pFe(III) of 18.0, ferric-PCH is significantly less stable than ferric-PVD.[52] The advantage to *P. aeruginosa* of excreting two siderophores — PVD, with a high affinity for iron(III), and PCH, with a lower affinity for this cation — remains unclear. However, recent findings suggest that *P. aeruginosa* synthesizes the efficient but "metabolically expensive" PVD only in conditions of severe iron starvation. The less efficient PCH seems to be the principal siderophore produced under less severe conditions of iron(III) limitation.[53] Thus, the apparent redundancy of the PVD- and PCH-dependent uptake systems may optimize bacterial survival in changing environments.[53]

P. aeruginosa can also use many of the siderophores secreted by other microorganisms (xenosiderophore):[54] pyoverdines from other pseudomonads,[55] enterobactin **4**,[56] cepabactin,[57] mycobactin and carboxymycobactin,[58] ferrichrome **3**,[59] deferrioxamines,[59,60] desferrichrysin, desferricrocin, coprogen[61] and naturally occurring chelators such as citrate.[62,63] The importance of these xenosiderophore uptake systems in infection has yet to be established. They may be involved in polymicrobial infections, with *P. aeruginosa* making use of the siderophores produced by other microorganisms. The presence of these xenosiderophores induces the expression of the corresponding uptake transporters in *P. aeruginosa*.[64] All these siderophore-dependent iron uptake pathways are, therefore, possible gates for Trojan horse strategies based on siderophore-antibiotic conjugates.

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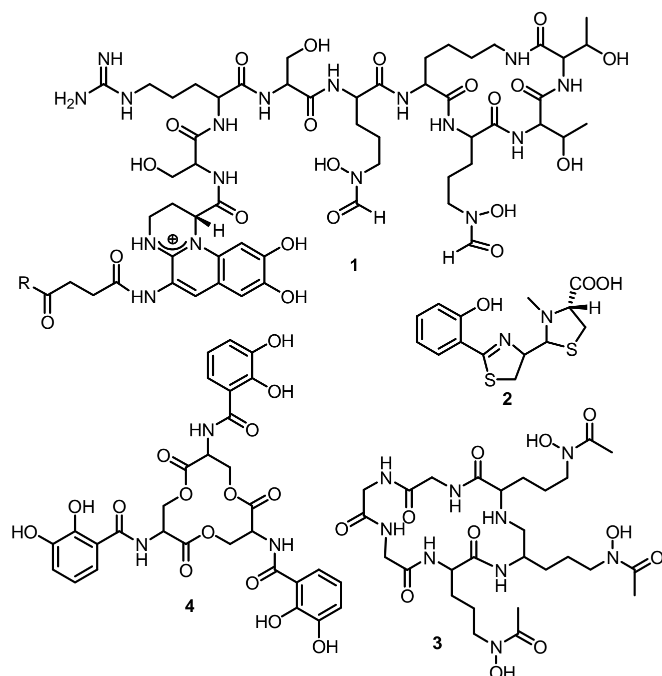

Figure 1. Structures of the two siderophores of *P. aeruginosa*, pyoverdine (Pch) **1** and pyochelin (Pch) **2** and of the two xenosiderophores ferrichrome **3** and enterobactin **4** used by *P. aeruginosa*.

Uptake of ferrisiderophore complexes into *Pseudomonas aeruginosa*

Once iron(III) has been chelated by siderophores in the environment of the bacterium, the ferrisiderophore complexes are recognized at the bacterial cell surface by specific TonB-Dependent Transporters (TBDT),[65,66] TonB being the inner membrane activator of these transporters. TBDTs are characterized by very stringent siderophore selectivity,[66] and the number of siderophores a bacterium can use is strongly correlated with the number of genes encoding TBDTs present in its genome. *In silico* analysis of the *P. aeruginosa* genome (see http://www.pseudomonas.com) has identified 32 genes encoding putative TBDTs,[67] at least 12 of which are involved in iron uptake.[58] FpvA and FpvB are the outer membrane transporters involved in PVD-Fe uptake.[68,69] FptA transports PCH-Fe[70] and FecA ferricitrate.[71] For heterologous siderophores, two transporters, PfeA and PirA, mediate the uptake of enterobactin;[72,73] two, FoxA and FiuA, are involved in the transport of ferrioxamine B and ferrichrome;[59,74] FemA is responsible for the transport of mycobactin and carboxymycobactin;[58] ChtA transports rhizobactin, aerobactin and schizokinen;[75] and FvbA is responsible for the uptake of vibriobactin.[76]

Many TBDT structures have been solved in the last 15 years.[65,66] They all consist of a 22-stranded antiparallel transmembrane β-barrel with large extracellular loops and small periplasmic loops (Figure 2). The lumen of the barrel is filled with the N-terminal globular domain (known as the plug), a mixed four-stranded β-sheet surrounded by loops and helices. The plug domain occludes the lumen of the β-barrel, thereby preventing the siderophore-iron complex from passing directly through the barrel. The plug is maintained in position within the β-barrel by 40 to 70 hydrogen bonds and two salt bridges.[77,78]

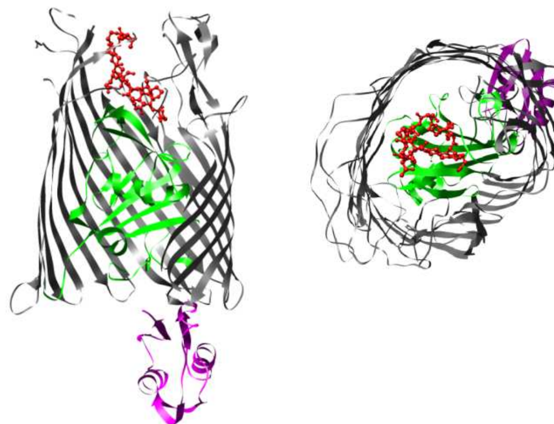

Figure 2. FpvA structure in complex with PVDI-Fe (PDB: 2IAH). Left : General description of the complex. The view is perpendicular to the barrel axis. Those strands, which form the front of the barrel, have been removed to provide an unobstructed view of the cork domain. Right : The PVDI-Fe binding site as viewed from the external environment. In both panels, the barrel domain and the plug domain are colored in grey and green, respectively. The PVDI-Fe complex in its binding site on FpvA is shown in red. FpvA has in addition to the plug domain a signaling domain (in purple) at the N-terminal end. This signaling domain is not present in all TonB-dependent transporters and is for example not present in FptA, it is involved in a signaling cascade regulating the expression of genes involved in iron metabolism.

Ferrisiderophore binding sites are highly siderophore-specific, always located on the extracellular face of the TBDT and are composed of residues of both the plug and the β-barrel domains.[66] In *P. aeruginosa*, only FpvA (PVDI-Fe TBDT) and FptA (PCH-Fe TBDT) have been crystallized and their structures solved.[66] Determinations of the structure of FpvA in complex with different PVD-Fe complexes purified from different Pseudomonads have shown that the specificity of the FpvA binding site is conferred by the structural elements common to all ferric pyoverdines — the chromophore, iron, and its chelating groups — together with the structure of the first few amino-acid residues of the PVD peptide chain.[55] The C-terminal portion of the PVDs, which is often cyclic, does not appear to make a major contribution to the interaction between the siderophore and its transporter.[55] Moreover, investigation of the mechanism of interaction of PVDI-Fe with FpvA, through the synthesis of various analogues of this siderophore and determinations of their affinity for FpvA *in vitro* and *in vivo* and their ability to transport iron, has demonstrated that the succinyl moiety linked to the chromophore of PVDI and the first amino acid of the peptide moiety can be subjected to steric hindrance, with no effect on binding or on the iron uptake properties of PVDI-Fe.[79]

PCH, the second siderophore produced by *P. aeruginosa*, has three chiral centers, in positions C4', C2" and C4", and its binding site on FptA is therefore highly stereospecific. This binding site consists mostly of hydrophobic and aromatic

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residues from the plug and the barrel, consistent with the hydrophobicity of PCH.[57,80] In the crystal structure, PCH provided tetradendate coordination of iron, the remaining bidentate coordination being provided by the ethylene glycol used for crystallization. This suggested that a single PCH molecule was sufficient for recognition by FptA.[80] In addition, the crystal structure of FptA and FetA (enantioPCH-Fe TBDT in *P. fluorescens*), binding assays and docking experiments with synthetic PCH analogues have all shown that the specific recognition of PCH-Fe by FptA was due to the configuration of the C4" and C2" chiral centers and was only weakly dependent on the configuration of the C4' carbon atom.[80-83] In addition, studies of FptA-PCH-Fe structure and binding studies carried out with PCH analogues have shown that this siderophore could experience steric hindrance on the nitrogen N3" position with no effect on the recognition of the siderophore by its transporter.[57] This high level of siderophore specificity or stereospecificity of TBDTs is a key aspect to be taken into account when designing antibiotic-siderophore conjugates: it is essential to take the structure of the corresponding TBDT into account, to determine where on the siderophore to conjugate the antibiotic moiety.

The protonmotive force of the inner membrane provides the energy required for the translocation of the ferrisiderophore through the TBDT. This energy is transferred to the outer membrane TBDTs *via* the TonB machinery, which consists of three different inner membrane proteins: TonB, ExbB and ExbD. [84] This TonB complex is required for the formation of a channel in the TBDT, to allow the uptake of ferrisiderophore complexes across the outer membrane. Several mechanisms have been proposed for channel formation in TBDTs, including: (i) a major change in the conformation of the plug domain, (ii) the movement of this domain out of the β-barrel, and (iii) a combination of these two mechanisms.[85,86] Many different experimental approaches have been used to resolve this issue, but without real success.[85,86] The data obtained with different approaches are often contradictory, making it impossible to draw any firm conclusions about the mechanism involved. Once transported across the outer membrane, the fate of the ferric siderophore complex depends on the siderophore pathway and the bacterium concerned. In most siderophore-based iron uptake systems, the ferricomplex is transported across the periplasm and inner membrane by ABC transporters or proton motive force-dependent permeases. Iron is then released from the chelator by either a combination of iron reduction and degradation or chemical modification of the siderophore.[87] The fate of ferrisiderophore complexes after transport across the outer membrane in *P. aeruginosa* has been investigated only for the PVDI, PCH and ferrichrome pathways (Figure 3).

For the iron uptake cycle involving PVDI, ferri-PVDI is transported across the outer membrane by its specific TBDT, FpvA (Figure 3).[68,88] Iron is then released from PVDI in the periplasm, by a mechanism involving iron reduction and no chemical modification of the siderophore.[89] The apo-PVDI is then recycled to the extracellular medium by the efflux pump PvdRT-OpmQ, where it can initiate a new iron uptake cycle.[90-92] Iron is further transported, across the inner membrane, by the ABC transporter FpvCDEF, which consists of two periplasmic proteins (FpvC and FpvF), a permease (FpvD) and an ATPase (FpvE).[93] More precisely, it is currently suggested but not proved that the two periplasmic binding proteins are involved somehow in the PVDI-Fe dissociation and the permease FpvD with the ATPase FpvE in the translocation of siderophore-free iron across the inner membrane into the cytoplasm.[93] In this iron uptake pathway, the siderophore PVDI never reaches the cytoplasm.

In the PCH pathway (Figure 3), ferri-PCH is transported across the outer membrane by its specific TBDT, FptA,[70] and across the inner membrane by the permease FptX.[94] Iron is released from PCH into the cytoplasm by an unknown mechanism.

The xenosiderophore ferrichrome is transported across the outer membrane in *P. aeruginosa* by the TBDTs FiuA and FoxA, and across the inner membrane by the permease FiuB (Figure 3).[59,74] Iron is released from the siderophore by a mechanism involving the acetylation of desferrichrome by FiuC and, probably, iron reduction.[74] The acetylated desferrichrome is recycled to the extracellular medium by an unknown secretion system.[74]

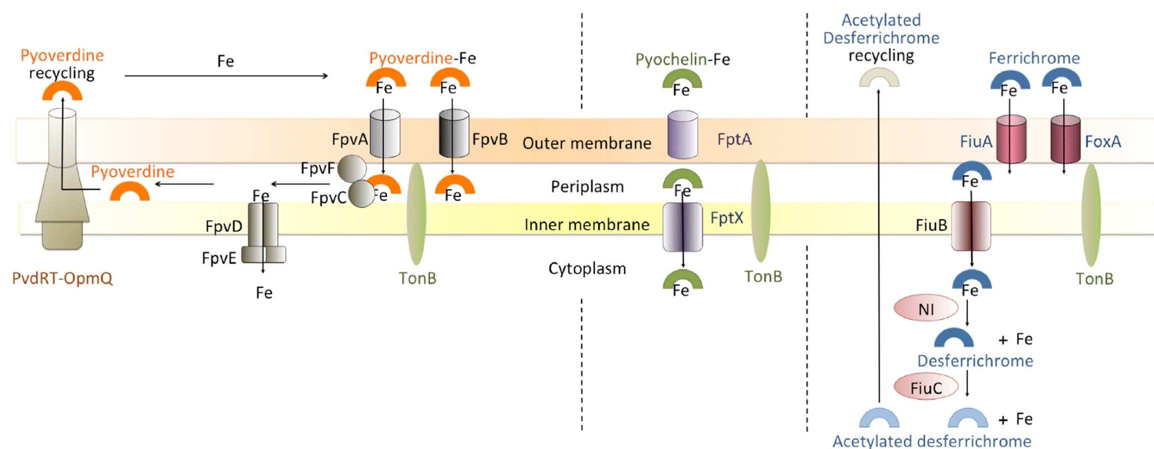

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Figure 3. Pyoverdine, pyochelin and ferrichrome-dependent uptake pathways in *P. aeruginosa*. For details and explanations, refer to the text.

Sideromycins: antibiotic Trojan horse compounds developed by microorganisms

Sideromycins are antibiotics covalently linked to siderophores, produced by various microorganisms.[95-100] The two chemical entities are generally connected by a spacer arm. Several families of sideromycins have been discovered and characterized in the last decades. They have been classified according to the siderophore moiety, the target of the antibiotic fraction and the microorganism producing them. The sideromycins identified to date include albomycins,[96] ferrimycins,[97] danomycins,[98] salmycins[99] and certain microcins.[100] These natural siderophore-antibiotic conjugates can chelate iron(III) and are then transported into the targeted bacterium via siderophore-dependent iron uptake pathways. This energy-coupled transport across the bacterial membranes greatly increases the antibiotic efficacy of sideromycins: their minimal inhibitory concentration is often at least two orders of magnitude lower than that of the antibiotic moiety without the siderophore, entering the cells by diffusion.[95b] Albomycins **5** to **7** and microcin E492 (MccE492) **9** are representative examples of this family of natural Trojan horse antibiotics (Figure 4).[96,100]

Albomycins were among the first sideromycins to be characterized.[96] These molecules, excreted by *Streptomyces* strains, are the biosynthetic results of the conjugation of a ferrichrome siderophore analogue and a thioribosyl pyrimidine antibiotic mediated by an amide group.[101] These conjugates display highly potent antibiotic activity against several Gram-negative bacteria, with a reported minimum inhibitory concentration (MIC) of 5 ng/ml for albomycin δ_2 **6** against *Escherichia coli* (MIC of 256 µg/ml for the antibiotic moiety alone),[102] for example. These conjugates are transported by the ferrichrome-dependent iron uptake mechanism in bacteria expressing this system.[102,103] After their transfer into the bacteria, an endogenous peptidase in the targeted bacterium promotes the cleavage of the amide linker and the release of the antibiotic.[104] Neither the enzyme involved in this hydrolysis, nor the compartment in which the reaction takes place has yet been clearly identified. The antibiotic released, identified as SB-217452 **9**, is a potent inhibitor of bacterial seryl-tRNA-synthetase (SerRS), a key enzyme involved in the translation process.[105]

For some sideromycins the antibiotic moieties are peptides and the most fascinating example of such a molecule is the microcin E492 (MccE492), produced by *Klebsiella pneumoniae* RYC492. This natural compound is a conjugate of an 84-amino-acids antibacterial peptide and a linearized enterobactin analogue, connected by a β-D-glucose spacer.[100] MccE492 displays strong antibiotic activity against several bacterial species (*E. coli*, *Salmonella enteritidis* and *S. typhimurium*).[100a] The effectiveness of MccE492 has been shown to be correlated with the expression of the FepA, Cir and Fiu outer membrane transporters in *E. coli*.[106] MccE492 uses the catechol siderophore-dependent iron uptake systems as a gateway, to cross the bacterial outer membrane.[106] Once inside the bacterial periplasm, the ester group connecting the sugar to the peptide is cleaved by intracellular hydrolases. The released peptide induces depolarization of the inner membrane, and interfere with the bacterial mannose metabolism (Figure 4).[107,108]

MccE492 demonstrates that the antibiotic moieties carried by sideromycins can be more bulky than the siderophore part of the molecule, suggesting that the channel formed in TBDTs during the process of uptake across the outer membrane has a large diameter and does not seem to interact in a specific manner with the compounds transported. The critical step in the transport of sideromycins by TBDT thus appears to be the recognition of the siderophore moiety by the highly specific binding site of the TBDT. Following this recognition step, TonB activates the formation of a channel, facilitating the diffusion whatever is bound to the binding site: the siderophore or a bulkier sideromycin.

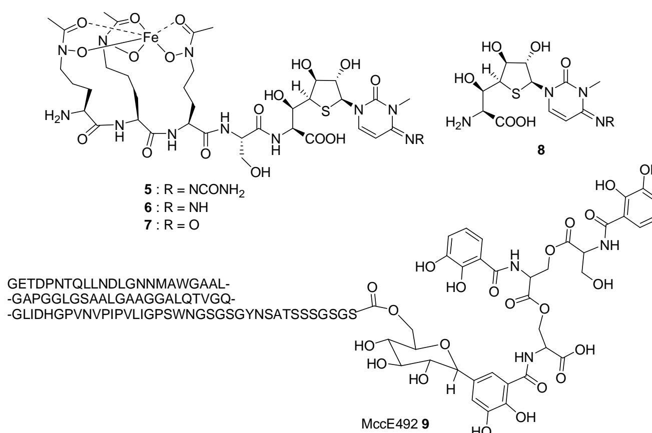

Figure 4 : Structures of albomycins **5**,**6** and **7** produced by *Streptomyces* species and of the antibiotic SB-217452 **8**. The structure of the microcin E492 **9** produced by *K. pneumonia* is also given.

The subtle *modus operandi* of natural sideromycins is often compared to the Trojan horse strategy described by Homer in

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the Odyssey. The bactericidial activity of sideromycins is driven by the specificity of recognition between a siderophore and its corresponding TBDT. Unfortunately, the natural sideromycins described to date have generally been found to have only weak bactericidal activity against *P. aeruginosa*.[29,95b] However, the impressive efficacy and selectivity of sideromycins against other Gram-negative bacteria has encouraged several groups around the world to develop bio-inspired Trojan horse approaches targeting *P. aeruginosa* more specifically. The principal strategies described to date for *P. aeruginosa* are based on the vectorization of antibiotics by the two endogenous siderophores, PVD and PCH.

Antibiotic Trojan horses based on pyoverdine

Many siderophores can be readily generated by synthetic chemistry from commercially available starting materials. However, a few, such as PVD, still constitute a challenge to organic chemists, as they have complex structures involving polycylic moiety, asymmetric centers or unnatural amino acids.[109,110] In the specific case of PVDs, the development of antibiotic Trojan horse strategies has been envisaged only by hemisynthesis, starting from chromopeptides extracted from *P. aeruginosa* culture broth. The amount of PVD produced by fluorescent *Pseudomonas* is highly strain-dependent but is generally between 200 and 500 mg per liter in iron-depleted culture conditions.[111,112] Higher productivity has been achieved in the presence of certain metal ions.[113,114] In a pioneering approach, Budzikiewicz and coworkers purified the PVDs produced by *P. aeruginosa* ATCC 27853 and *Pseudomonas fluorescens* ATCC 13525.[115] The peptide fractions of these two PVDs each contain one amino acid bearing a terminal primary amino group (ornithine and lysine, respectively). These amine groups were connected to ampicillin, a beta-lactam antibiotic, via a sebacic spacer arm.[115] The activity of the resulting conjugates, **10** (PaTSebAmp) and **11** (Pv9446SebAmp), was tested against *P. aeruginosa* ATCC 27853 and ATCC 15692 (Figure 5).

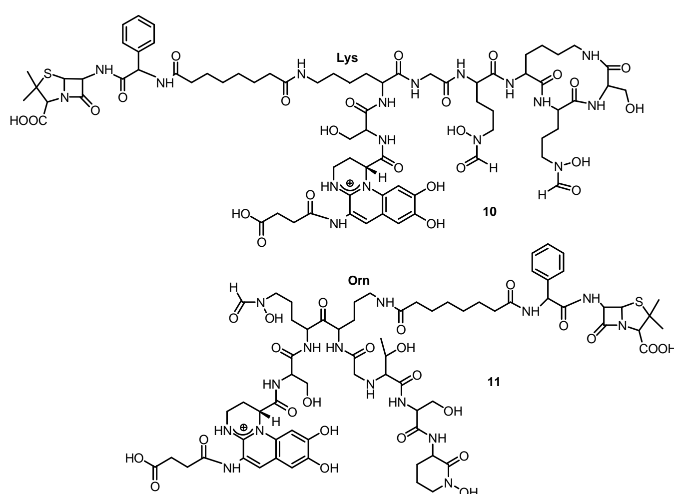

Figure 5 : Structures of PaTSebAmp **10** and Pv9446SebAmp **11**, two conjugates between pyoverdines and ampicillin.

These conjugates had high levels of antibacterial activity, with MICs of 0.04 µg/ml and 0.67 µg/ml, respectively, against strains described as resistant to free ampicillin. These PVD-beta-lactam conjugates, like the PVDI produced by *P. aeruginosa* PAO1, probably dissociated from the transported iron ion in the periplasm, with the apo-chelator accumulating in this cell compartment before recycling to the extracellular medium.[115] In this iron uptake cycle, the beta-lactam antibiotic linked to the PVD seems to be able to reach its target in the periplasm and to inhibit the synthesis of the peptidoglycan.

In a second approach, the same group purified the PVD produced by *P. aeruginosa* ATCC 15692, for the preparation of conjugates with cephalexin, another beta-lactam antibiotic.[116] For this purpose, the arginine side chain of the chromopeptide was crosslinked with *N*-4,6-dioxoheptanoyl-cephalexin in buffered conditions. The resulting pyrimidine linker was described as stable in biological medium. The PVD-cephalexin conjugate **12** (PDPyrCeph) was tested against bacteria and was found to have no activity against *P. aeruginosa* ATCC 15692 (Figure 6).[116] However, compound **12** induced bacterial growth, demonstrating that the conjugate promoted iron uptake in the targeted bacteria. The absence of antibiotic activity may be due to an inability of the antibiotic to interact with its target in the periplasm, due to steric hindrance caused by linkage to the siderophore. Indeed, the presence of an antibiotic moiety on the siderophore can affect vector recognition by the TBDT and therefore transport. Conversely, conjugation to the siderophore moiety can markedly decrease the interaction of the antibiotic with its biological targets.

Abdallah and coworkers developed conjugates between the PVD extracted from ATCC 15692 and fluoroquinolones, potent inhibitors of bacterial DNA gyrase.[117] These compounds were developed long before the molecular mechanisms involved in iron uptake by PVD were known, accounting for the choice of antibiotics with cytoplasmic targets. Two conjugates (**13** and **14**) of PVD and norfloxacin have been synthesized. In compound **13** (NPS), the siderophore and the antibiotic are connected by a succinate linker that is stable in physiological conditions. In compound **14** (NPL), PVD and norfloxacin are connected by a linker bearing a methylenedioxy group that is readily hydrolyzed *in vivo* (Figure 6).

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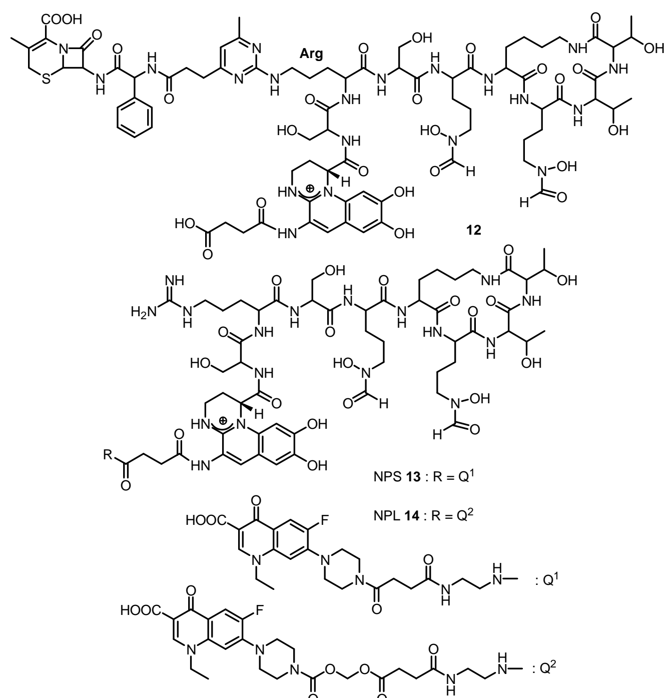

Figure 6: Structure of conjugates **12**, **13** and **14** between the pyoverdine produced by *P. aeruginosa* ATCC 15692 and two different antibiotics : cephalexin and norfloxacin.

Iron transport experiments showed that this conjugate promoted iron uptake in *P. aeruginosa* ATCC 15692. *In vitro* inhibition assays on purified *Escherichia coli* gyrase showed that the fluoroquinolone remained fairly active against its biological target, even when connected to the siderophore via a physiologically stable spacer arm. However, when tested *in vivo* on bacteria, the MIC for compounds **13** and **14** were higher than the MIC of free fluoroquinolone. Similar results were obtained whatever the strain of *P. aeruginosa* or the conditions tested (*e.g.* iron depleted succinate or Müller-Hinton medium).[117] These results are not surprising, given that PVD never reaches the cytoplasm in its iron-uptake cycle.[87,89] This provides another illustration of the need for excellent knowledge of the iron uptake systems and intracellular fate of siderophores for the development of active antibiotic-siderophore conjugates. A siderophore, which releases its complexed iron in the periplasm and therefore never reaches the cytoplasm, should only be conjugated to antibiotics with targets in this cell compartment.

Another important aspect to be taken into account in the development of PVD-antibiotic conjugates is the production by *P. aeruginosa* strains of three structurally different PVDs (with different peptide chains) — PVD types I, II and III[44-46] — with their corresponding specific outer membrane transporters, FpvAI, FpvAII and FpvAIII. Each strain of *P. aeruginosa* produces one of these three types of PVDs and the corresponding TBDT. Thus, a PVDI-antibiotic conjugate, for example, will not be active against all *P. aeruginosa* strains, thus limiting the therapeutic potential of antibiotic Trojan horse approaches based on PVD-dependent iron uptake systems. The PCH-dependent iron uptake system may therefore be more promising for the development of more general antibiotic Trojan horse strategies.

Antibiotic Trojan horses based on pyochelin

PCH is a siderophore produced by *P. aeruginosa,*[48] but also by *Burkholderia cepacia*,[50] both of which cause severe, often lethal, lung infections. The PCH-dependent iron uptake system is thus common to both bacterial threats and is therefore an interesting target in the framework of an antibiotic Trojan horse approach. The stereocontrolled synthesis of this siderophore, described in early 2000 by Abdallah and coworkers,[118] paved the way for the synthesis of functionalized analogues for use as vectors for the delivery of antibiotics to these two bacterial species. In this context, a first generation of functionalized PCHs was synthesized. In the absence of structural data for the transporter involved, the C5 position was initially selected for modification,[119] due to its accessibility for synthesis procedures. Two analogues were synthesized, one **15** bearing an alkyne extension and the second **16** with a more flexible aliphatic chain.[119] The terminal amine group of the siderophore analogues was connected to norfloxacin via the two different spacer arms previously described by the group of Abdallah and coworkers in their approach targeting the PVD-dependent iron uptake system.[120] The activity of the four corresponding conjugates, **17** to **20**, was assessed against *P. aeruginosa* ATCC 15692 cultured in iron-depleted medium (succinate medium). At a concentration of 10 µM, conjugates **17** and **19**, bearing a non-hydrolyzable succinic linker, displayed no antibiotic activity. At the same concentration, conjugates **18** and **20**, in which the siderophore was connected to the antibiotic by a hydrolyzable spacer arm, completely inhibited bacterial growth. Nevertheless, the level of activity observed was no higher than that for free norfloxacin at the same concentration (Figure 7).[120]

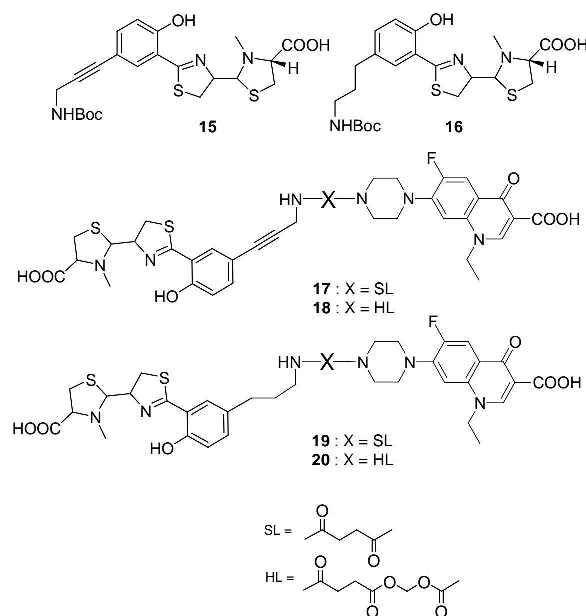

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Figure 7 : Structures of the C5 functionalized pyochelin analogues **15** and **16**, and structures of pyochelin-norfloxacin conjugates **17** to **20**.

Based on the structure of FptA, which has since been determined,[80] the C5 position on PCH used to link the antibiotic may affect siderophore recognition by the transporter: the attachment of bulky substituents in the C5 position may generate steric clashes with the protein.[121] This aspect was illustrated in a recent article, in which Vederas and coworkers reported the use of functionalized PCH **15** to vectorize gallidermin, a peptide antibiotic of the antibiotic family.[122] In conjugates **21** and **22**, the squarate linker connects the amine group of the PCH analogue **15** to the amine group of one of the two lysines side chains from the lantibiotic. The choice of this spacer arm seems to have been based exclusively on synthetic constraints and no information was provided about the stability of the squarate linker *in vivo* (Figure 8).

Conjugates **21** and **22** were tested, in their ferric form, against several bacterial species. These conjugates slightly enhanced the growth of *P. aeruginosa* ATCC 14207 and *B. cepacia* ATCC 25416, whereas neither antibacterial activity nor growth promotion was detected for *E. coli* and *Salmonella* strains. This led the authors to suggest that conjugates **21** and **22** continued to behave like siderophores, promoting iron transport in bacterial species expressing the PCH-dependent iron uptake system.[122] However, in the absence of control experiments on bacteria with appropriate mutations, the promotion of growth by iron transfer from conjugates to other endogenous siderophores (*e.g.* PVD, ornibactin or cepabactin) cannot be ruled out. The conjugates **17** to **20** and **21** and **22**, prepared by either our group or by Vederas and coworkers, involve the use of the same vector, but with different types of spacer arms connected to antibiotics from two different families with markedly different biological targets. The results obtained suggested that the weak points of this approach were probably the vector and the C5 position selected for functionalization. This example highlights the more general need for an understanding of the structure and function of the proteins and mechanisms involved in a siderophore-based Trojan horse strategy.

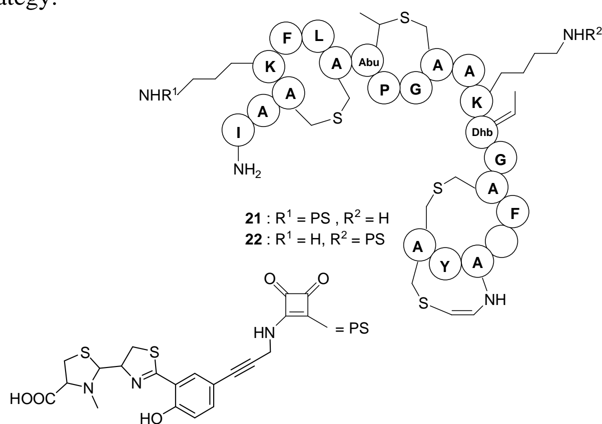

Figure 8 : Structures of pyochelin-gallidermin conjugates **21** and **22**.

In 2004, the team of Pattus and coworkers solved the three-dimensional structure of the PCH outer membrane transporter FptA.[80] The receptor was cocrystallized with one molecule of ferric-PCH, providing crucial information about the interactions between the siderophore and its specific transporter. Knowledge of this structure led to the design and synthesis of the PCH analogue **23**, with functionalization of the N3" position.[121,123] Functionalized PCH **23** had binding and iron uptake properties similar to those of the natural siderophore. The N3"-functionalized PCH was then conjugated to an NBD fluorophore as a model for the development of PCH-based Trojan horse conjugates (Figure 9). Fluorescence microscopy experiments showed that fluorescent probes **24** and **25** were recognized by FptA, the outer membrane transporter of PCH.[123]

The N3"-functionalized PCH analogue **23** thus appears to be a promising vector for the specific delivery of an antibiotic in the framework of a Trojan horse strategy. Conjugates **26** to **31** between this PCH analogue and three fluroroquinolones (ciprofloxacin, norfloxacin and *N*-desmethyl-ofloxacin) were synthesized.[121] For each antibiotic, the conjugate was synthesized either with a non-hydrolyzable succinic linker or with a spacer arm containing the methylene-dioxy moiety, which is labile *in vivo* (Figure 10).

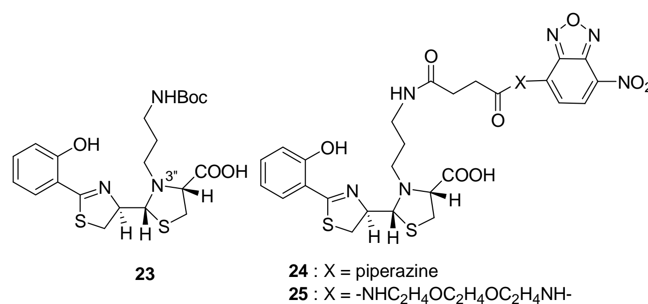

Figure 9 : Structures of the N3"-functionalized pyochelin **23** and of the pyochelin-based fluorescent probes **24** and **25**.

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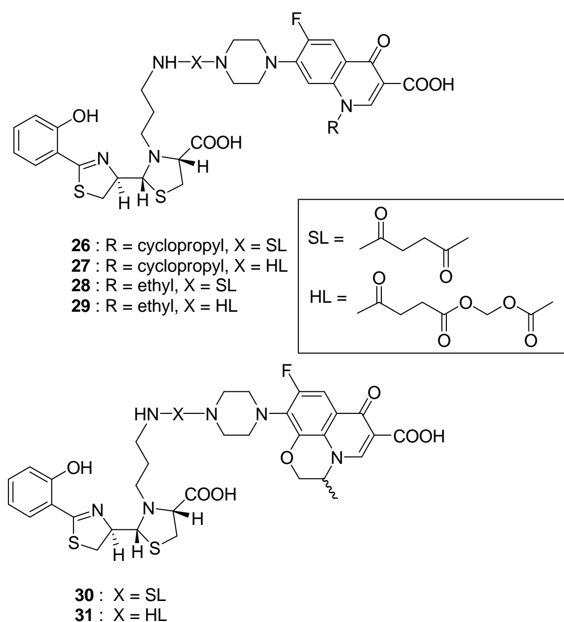

Figure 10 : Structures of pyochelin-fluoroquinolone conjugates **26** and **31**.

As expected, the unconjugated ciprofloxacin, norfloxacin and *N*-desmethyl-ofloxacin strongly inhibited the proliferation of *P. aeruginosa* strains.[121] Conjugates **26**, **28** and **30** with a non-hydrolyzable succinic linker had no antibiotic activity against *P. aeruginosa*. These results demonstrated that the antibiotics could not be released from the conjugates by hydrolysis of the succinate linker. The PCH-fluoroquinolone conjugates **27** and **29**, which have a hydrolyzable spacer arm, displayed antibiotic activity against *P. aeruginosa*. However, this activity was lower than that of unconjugated ciprofloxacin and norfloxacin, by a factor of three to 20. In the case of *N*-desmethyl-ofloxacin conjugate **31**, no antibiotic activity was observed, whatever the conditions or the strain used. Nevertheless, the MIC_{50} values obtained were probably underestimated, because all the conjugates were only weakly soluble in physiological conditions. The progressive precipitation of the conjugates in the medium during bacterial growth may greatly decrease the amounts of antibiotic available to the bacteria.[121] Moreover, conjugates **27** and **29** displayed significant activity against a *tonB* mutant of *P. aeruginosa* that cannot acquire iron(III) via the PCH-dependent iron uptake system. This observation may reflect the presence of extracellular hydrolases able to cleave the methylene-dioxy linker before the conjugates cross the bacterial envelope. The released antibiotic can then penetrate further into the *tonB* mutant of *P. aeruginosa* by diffusion. Thus, biological evaluations of conjugates **26** to **31** run into two major problems: the lack of solubility of the conjugates in physiological conditions and the hydrolysis of the spacer arm in the extracellular medium.[121] The specific example of PCH-antibiotic conjugates thus highlights more general problems faced by organic chemists in the development of a siderophore-based antibiotic Trojan horse strategy. None of the artificial sideromycins based on PCH or PVD developed to date has yet found a promising therapeutic application. Nevertheless, the results reported open up new perspectives in the development of effective artificial sideromycins for combating *P. aeruginosa*.

Criteria necessary for designing successful antibiotic-siderophore conjugates against *P. aeruginosa*.

A Trojan horse conjugate is a coherent ensemble of three components: the siderophore, the linker and the antibiotic. Each of these components should first be optimized separately and selected so as to create the precise conjugate with the best biological properties in terms of both membrane penetration and antibiotic activity. The difficulty of optimizing these aspects simultaneously explains why so many promising approaches have led to only a very small number of highly successful Trojan horse strategies to date. A review of the literature rapidly reveals that most of Trojan horse conjugates bearing an antibiotic with a cytoplasmic target are only poorly active against Gram-negative bacteria in general and *P. aeruginosa* particularly.[24,25b-e,26,28,29,117,120,121] This limitation must result principally from a difference in transport selectivity between the outer and inner membrane transporters and the release of chelated iron by some siderophores in the periplasm, such that these siderophores never reach the cytoplasm (the PVDI pathway in *P. aeruginosa* (Figure 3). Indeed, as explained above, TBDTs seem to display highly selective binding to a specific siderophore, but can transport compounds larger than the recognized siderophore, resulting in a broad transport selectivity. For siderophores transported into the cytoplasm, less is known about the ABC transporters and the permeases involved in the transport of ferri-siderophores across the inner membrane.[87] However ABC transporters form a large family of proteins involved in the transport of many different chemical compounds in prokaryotic and eukaryotic cells and our current knowledge about these transporters indicate a narrow transport specificity.[124,125] Therefore in ferri-siderophore uptake pathways, binding and especially transport selectivity may be narrower at the level of the inner membrane uptake than for translocation across the outer membrane. It seems likely that most siderophore-antibiotic conjugates will be retained in the periplasm because unable to be transported further across the inner membrane. This explains why most of the successful Trojan horse strategies described to date have been based on antibiotics with periplasmic targets, such as beta-lactam derivatives in particular.[25a,27,33b,30-32]

Another obstacle to the development of siderophore-antibiotic conjugates is the very small number of spacer arms suitable for use in Trojan horse strategies. The ideal linker should be resistant to extracellular conditions, stable during the translocation promoted by iron uptake systems but cleavable in the bacterial inner space. In addition, depending on the antibiotic vectorized, release must occur preferentially in either the periplasm or the cytoplasm. Unfortunately, the linkers described to date are far from ideal, accounting for the failure

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of so many Trojan horse approaches, and the preference for stable linkers as a model for further developments. The use of a non-hydrolyzable linker has been identified as the best choice for the vectorization of beta-lactam antibiotics. Indeed transpeptidase, the principal target of beta-lactam antibiotics, seems to be highly permissive to steric hindrance around its substrate. Other biological targets, particularly those in the cytoplasm, seem to be more sensitive to steric hindrance and the release of the antibiotic from the linker appears to be necessary for an optimal interaction.

The third component of the siderophore-antibiotic conjugates to be considered in Trojan horse approaches is the siderophore responsible for vectorization. A huge number of siderophore-antibiotic conjugates have been described to date, but the most efficient are generally based on catechol or hydroxamate siderophore analogues.[24-33] The TBDTs targeted by these strategies are transporters of enterobactin, other catechol siderophores or ferrichrome. Homologs of these receptors are present in many Gram-negative bacteria, including *P. aeruginosa*.[59,60,72-74] In this context, Miller and coworkers recently reported synthesis of tris-catechol derivatives **32** and **33**,[25a] which could be considered as prototypes of a new generation of Trojan horses designed to have the profile described above. These compounds have high levels of activity against *P. aeruginosa* and are based on an analogue of enterobactin connected to either ampicillin or amoxycillin, two beta-lactam antibiotics, via a non-hydrolyzable succinate linker (Figure 11).[25a]

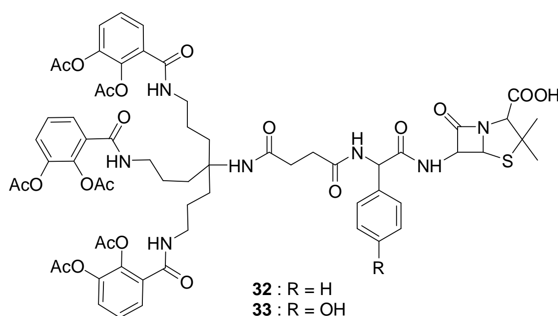

Figure 11 : Structures of ampicillin and amoxcillin Trojan horse conjugates **32** and **33**.

P. aeruginosa cannot synthesize catechol siderophores, but it can make use of such siderophores produced by other bacterial species, transporting them across its outer membrane via PfeA and PirA.[72,73] Catechol and hydroxamate siderophores are also used by other pathogens and by “human-friendly” bacteria. Thus, such Trojan horse conjugates could be seen as “antibiotics of mass destruction” of considerable interest for the treatment of acute infection or co-infections. By contrast, chronic infections with *P. aeruginosa*, particularly in patients with cystic fibrosis, require a “surgical strike” precisely targeting the microorganism concerned and closely related bacterial species. In this context, the use of endogenous siderophores as vectors remains the best choice. However, as pointed out above, each *P. aeruginosa* strain excretes and recognizes its own PVD from the three produced in these species, which differ in terms of their peptide sequences.[44-46] Furthermore, the periplasmic fate of PVDI explains why the use of this siderophore seems to be limited to the vectorization of beta-lactam antibiotics.[115,116] These two characteristics restrict the use of PVDs in the framework of Trojan horse approaches for therapeutic applications. In this context, PCH appears to be a more promising vector. PCH can reach the cytoplasm in its ferric form, and regulates the expression of its own iron uptake pathway.[126] This characteristic makes PCH an interesting candidate for the vectorization of inhibitors of both periplasmic and cytoplasmic targets. In this context, the N3"-functionalized PCH **23** appears to be the ideal vector for the design of such Trojan horse conjugates and for the transport of antibiotics, at least across the bacterial outer membrane *via* the TBDT FptA. However, there is currently no evidence to suggest that this vector can convey antibiotics across the inner membrane via the permease FptX. We know nothing of this transporter and its mechanism of interaction with PCH-Fe. Moreover, the development of dedicated hydrolyzable linkers and the exhaustive screening of linker-antibiotic combinations are required to increase the potential of PCH analogue **23** as a vector for antibiotic Trojan horse strategies against *P. aeruginosa*.

Acknowledgements

Authors warmly thank *Vaincre la Mucoviscidose* (the French cystic fibrosis association) for repeated financial support for the antibiotic Trojan horse project. We thank also the *Centre National de la Recherche Scientifique* (CNRS) and the *Agence National de la Recherche* (ANR) for general financial support. In addition, the research leading to some of these results was conducted as part of the Translocation consortium (www.translocation.com) and has received support from the Innovative Medicines Joint Undertaking under Grant Agreement n°115525, resources which are composed of financial contribution from the European Union’s seventh framework programme (FP7/2007-2013) and EFPIA companies in kind contribution.

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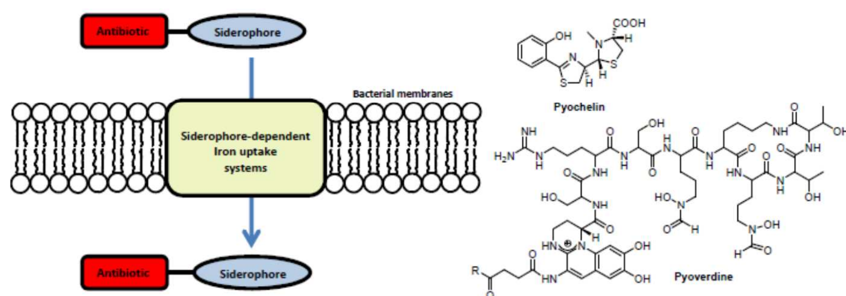

Pyoverdine and pyochelin siderophore could be used as vector for the specific delivery of antibiotics to *Pseudomonas aeruginosa* using the Trojan horse strategy.

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