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PAPER

Synthesis and biological properties of conjugates between fluoroquinolones and a N3″-functionalized pyochelin†

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Pyochelin is a siderophore common to *Pseudomonas aeruginosa* and several other pathogenic bacteria. A pyochelin functionalized at the N3″ position with a propyl-amine extension was previously synthesized. In the present work we proved that this analog binds FptA, the pyochelin outer membrane receptor, and transports iron(III) efficiently into bacteria. This functionalized pyochelin seemed to be a good candidate for antibiotic vectorization in the framework of a Trojan horse prodrug strategy. In this context, conjugates between pyochelin and three fluoroquinolones (norfloxacin, ciprofloxacin and *N*-desmethyl-ofloxacin) were synthesized with a spacer arm that was either stable or hydrolyzable *in vivo*. Some pyochelin–fluoroquinolone conjugates had antibacterial activities in growth inhibition experiments on several *P. aeruginosa* strains. However, these activities were weaker than those of the antibiotic alone. These properties appeared to be related to both the solubility and bioavailability of conjugates and to the stability of the spacer arm used.

Introduction

The development of bacterial resistance to antibiotics is inevitable, based on the rules of Darwinian selection.[1] This process has been greatly accelerated under the strong selection pressure imposed by the use of high levels of antibiotics and the misuse of antibiotics in human health and to promote animal growth in farming. According to some specialists, the emergence of pathogenic bacteria resistant to most of the drugs in clinical use foreshadows the end of the antibiotic era.[1–3] 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 over the last decades.[2–4] Iron is a crucial element for almost all life forms. This has led to the identification of bacterial iron acquisition systems as a promising target for the design of new antibiotic strategies,[4,5] to regain the upper hand against human pathogens. Iron bioavailability is limited by the low solubility of iron(III) at physiological pH. In addition, 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. The level of free iron(III) in biological fluids is usually estimated at 10^{-9} M to 10^{-18} M, while bacteria require iron concentrations in the micromolar range for proliferation during the infection process.[6] Microorganisms have developed very efficient iron uptake systems mediated by low molecular weight molecules called siderophores, to enable them to compete with the host for this essential nutrient.[7,8] Under iron limitation conditions, microorganisms synthesize and excrete these molecules into the extracellular medium, to chelate iron(III). In Gram-negative bacteria, the ferric-siderophore is recognized by a specific outer membrane receptor. The siderophore–iron(III) complex is then translocated through the membranes into the cytoplasm, by a multiprotein system.[9,10]

The low permeability of membranes serves as the first line of defense against antibiotics in bacteria. Iron acquisition pathways, which breach this barrier, are therefore of potential interest as a means of introducing antibiotic compounds into the microorganism. This approach has been adopted by certain bacteria during evolution, as a way of competing with other microorganisms in the struggle for essential nutrients and the conquest of new biological niches. Natural sideromycins,[11] such as salmycins,[12] albomycins[13,14] and microcins,[15,16] are conjugates between an antibiotic molecule and a siderophore analog. These conjugates are recognized and transported into the targeted bacteria by the siderophore-dependent iron uptake pathways. The siderophore and antibiotic components of sideromycins are often connected by a hydrolyzable linker that can be cleaved by endogenous enzymes. The antibiotic is therefore released only after the sideromycin has been transferred across the bacterial envelope.[13] The impressive efficiency of this natural strategy led several groups around the

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† Electronic supplementary information (ESI) available: HRMS spectra of final conjugates **35**, **36**, **37**, **38**, **39** and **40**. See DOI: 10.1039/c1ob06250f

world to develop bio-inspired Trojan horse approaches, using synthetic siderophores as vectors for the delivery of antibiotics *via* the bacterial iron uptake systems.[17–27]

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium responsible for severe and often lethal lung infections, particularly in patients with cystic fibrosis.[28] The low permeability of its outer membrane accounts for the natural resistance of this bacterium to many antibiotics. The permeability of this membrane may be particularly low in some resistant *P. aeruginosa* strains.[29] In this context, the use of Trojan horse antibiotic prodrugs has been put forward as a promising strategy for the treatment of infections due to *P. aeruginosa*.[5] Most of the molecules developed to date have structures based on tris-hydroxamate or bis- or tris-catecholate scaffolds, mimicking ferrichrome, enterobactin and catecholate siderophores, respectively.[17,18,20,25–27] Some of these conjugates have been shown to inhibit the growth of *P. aeruginosa* strains efficiently, but very few strategies based on endogenous siderophores of *P. aeruginosa* have been described.[21–24] In iron starvation conditions, *P. aeruginosa* produces two principal siderophores: pyoverdin and pyochelin **1** (Fig. 1).

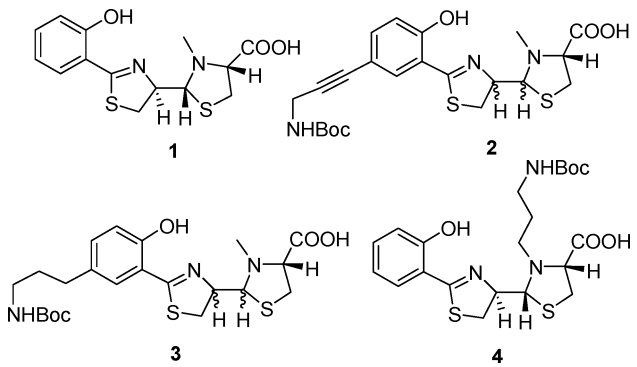


Fig. 1 Structures of pyochelin **1** and of three functionalized analogs **2**, **3** and **4**.

Pyoverdin is a chromopeptide siderophore consisting of a fluorophore conjugated to an oligopeptide of 6 to 14 amino acids.[30,31] Several examples of Trojan horse antibiotic prodrugs based on pyoverdin have been described.[22,23,25] However, each *P. aeruginosa* strain produces its own pyoverdin with a specific oligopeptide sequence and expresses a corresponding specific outer membrane transporter, FpvA, for this siderophore. Only a very small number of examples of cross-feeding have been observed,[31,32] accounting for the narrow spectrum of activity of some pyoverdin–antibiotic conjugates, which have antibacterial activity against only a subset of *P. aeruginosa* strains.[22,23] By contrast, pyochelin **1** is a siderophore common to all strains of *P. aeruginosa*,[33–34] and to many clinical strains of *Burkholderia cepacia*, a complex family of Gram-negative pathogens involved in acute infections affecting cystic fibrosis patients.[35] In all strains, pyochelin is recognized and transported by a specific outer membrane receptor: FptA.[36] Pyochelin therefore appears to be a good candidate for antibiotic delivery in the framework of a Trojan horse prodrug strategy. Adapted pyochelin–antibiotic conjugates should thus have a bactericidal activity against many *P. aeruginosa* and *B. cepacia* strains. We previously reported the synthesis of pyochelin analogs **2** and **3**, functionalized with an amine group in the C5 position of the phenol ring (Fig. 1).[37]

Based on the analogs **2** and **3**, four conjugates with norfloxacin, a fluoroquinolone, were efficiently synthesized. The resulting conjugates had only moderate antibacterial activity, below the levels generally observed for norfloxacin, used at the same concentrations.[21] Vederas and coworkers recently reported similar results for conjugates between pyochelin **2** and gallidermin, a lantibiotic.[24] Resolution of the three-dimensional structure of FptA,[38] the outer membrane receptor of pyochelin, has provided insight into the structural basis of the interaction between the siderophore and its specific transporter.[39–41] FptA is a transmembrane receptor folded into two domains: a C-terminal 22-stranded β-barrel, the channel of which is occluded by the N-terminal plug domain. The pyochelin binding pocket site is located at the extracellular face and consists mostly of hydrophobic and aromatic residues (Phe114, Leu116, Leu117) from the apical loop of the plug domain. Within the binding site, the ferric-pyochelin is stabilized by several van der Waals interactions and by one hydrogen bond between its carboxylate group and the main chain of FptA.[38,39]

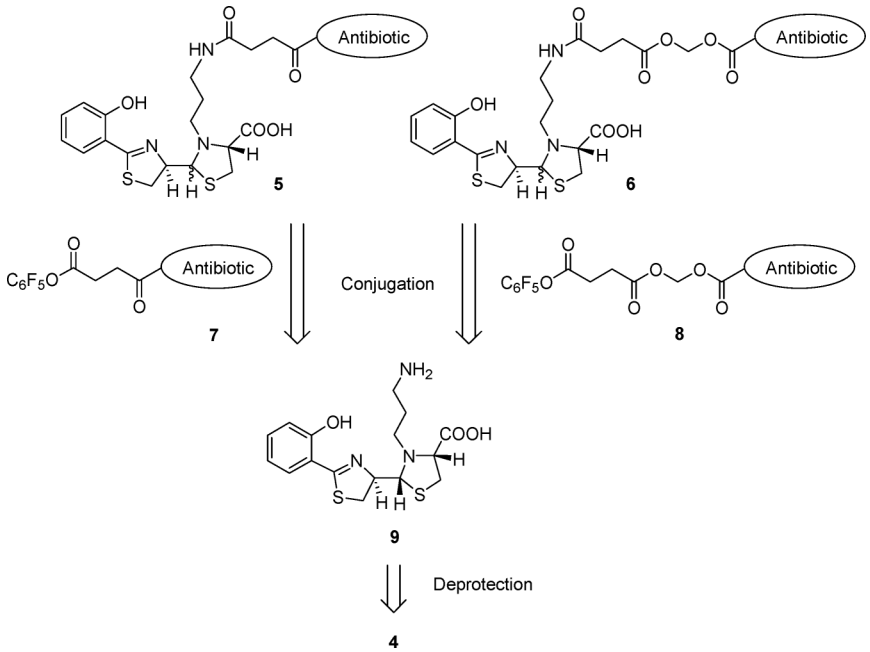
Molecular modeling, based on the crystallographic structure, suggests that analogs **2** and **3** can interact with FptA,[24] but binding and ^{55}Fe uptake experiments with *P. aeruginosa* have demonstrated that these analogs neither bind FptA nor promote iron transport into the bacterial cell. The structure of FptA and the structure–activity relationship studies inspired the design of a novel pyochelin analog **4**, functionalized in the N3″ position with a propyl-amine extension (Fig. 1).[42] In preliminary experiments, analog **4** was conjugated to a fluorescent NBD (4-nitro-benzo[1,2,5]oxadiazole) label. Fluorescence microscopy demonstrated that the conjugates were addressed selectively to *P. aeruginosa* strains expressing FptA.[42] We therefore developed the Trojan horse antibiotic conjugates **5** and **6**, based on pyochelin analog **4**. In this context, the free amine **9** resulting from the deprotection of pyochelin **4** was reacted with the activated esters of synthetic building blocks consisting of an antibiotic connected to a spacer arm. The spacer arms used to link the drug to the functionalized pyochelin were of two types: a succinic linker (building block **7**), stable in physiological conditions, and a labile linker (building block **8**) from which the antibiotic could be released by cleavage under physiological conditions (Scheme 1).

The antibiotics linked to the siderophore were three fluoroquinolones: ciprofloxacin, norfloxacin and *N*-desmethylofloxacin. These compounds are potent inhibitors of bacterial topoisomerases with demonstrated bactericidal activities against many pathogenic microorganisms, including *P. aeruginosa*.[1] We investigated the ability of N3″-functionalized pyochelin to bind FptA and to transport iron(III). We also describe the synthesis and biological properties against *P. aeruginosa* of novel conjugates between pyochelin **4** and fluoroquinolones.

Results and discussion

Binding of the N3″-functionalized analog of pyochelin to FptA

Preliminary fluorescence microscopy experiments showed that fluorescent probes prepared from the pyochelin analog **4**, functionalized on the N3″ position, selectively labeled *P. aeruginosa* strains expressing FptA, the pyochelin outer membrane transporter.[42] These results suggest that pyochelin **4** may be recognized by the pyochelin outer membrane transporter. We therefore investigated



Scheme 1 Retrosynthesis of pyochelin–antibiotic conjugates. The siderophore and the drugs are linked *via* a spacer arm that is either stable (compounds **5**) or cleavable (compounds **6**) *in vivo*.

the binding and iron uptake properties of functionalized pyochelin **4** in more detail. Iron(III) complexes of both pyochelin **1** and functionalized analog **4** were docked into the three-dimensional structure of FptA. The score obtained for Fe(III)–**4** was lower than that for Fe(III)–pyochelin **1** (55.64 *vs.* 63.83), but both molecules seem to interact with the receptor in a similar manner (Fig. 2-Table 1).

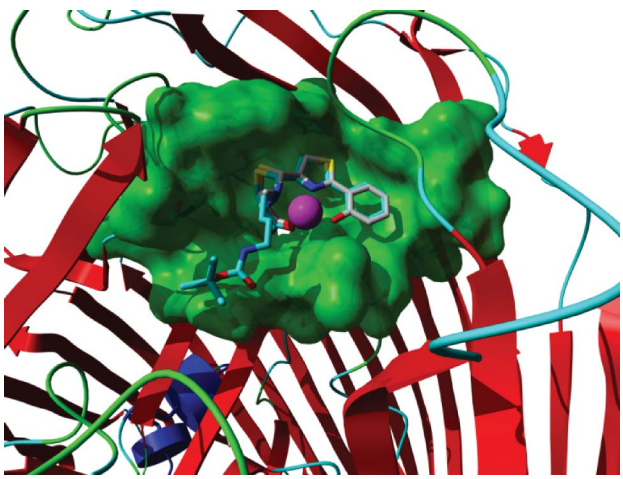


Fig. 2 Docking mode of the functionalized pyochelin analog **4** (cyan sticks) in the ferric-pyochelin binding site (green solid surface) of FptA displayed as ribbons (blue, α-helix; red, β-sheet; green, turn; cyan, random coil). Ligands are displayed according to the following color code: cyan for **4**, grey for pyochelin **1**; nitrogen, blue; oxygen, red; sulfur, yellow. Iron is shown as a magenta ball.

We also carried out binding experiments with bacterial cells. Inhibition constants (K_i) for the binding of Fe(III)–pyochelin **1** and Fe(III)–pyochelin analog **4** to the outer membrane FptA were determined for *P. aeruginosa* PAD07 cells, which are unable to produce pyochelin **1** and pyoverdin. These binding experiments were performed at 0 °C to prevent ferrisiderophore uptake. In these conditions, the K_i of Fe(III)–pyochelin analog **4** (37.4 ± 13.4 nM) is one tenth that of Fe(III)–pyochelin **1** (3.6 ± 0.5 nM), but this affinity remains within the range observed for interactions between natural siderophores and their transporters (Table 2).[39,41,43,44]

The ten-fold difference in K_i between the natural siderophore **1** and its synthetic analog **4** is consistent with the scores obtained in docking experiments and may be accounted for by the much higher flexibility of compound **4**, the binding free energy of which is penalized by a higher loss of entropy upon binding.

Table 1 Docking scores obtained by *Gold* docking

Compounds	Fitness score[a]
Pyochelin **1**	63.83
Functionalized analog **4**	55.64

[a] Goldscore fitness value.

Table 2 Inhibition constants (K_i) were determined by assessing competition with pyochelin–^{55}Fe(III). PAD07 (OD_{600} = 0.3) cells were incubated at 0 °C for 1 h in 50 mM Tris-HCl (pH 8.0), in the presence of 1 nM pyochelin–^{55}Fe(III) and various concentrations of pyochelin **1**–Fe(III) or pyochelin analog **4**–Fe(III). The experiment was carried out at 0 °C, in the presence of 200 μM CCCP, to prevent iron uptake

Siderophores	K_i (nM)[a]
Pyochelin **1**–Fe(III)	3.6 ± 0.5
Pyochelin analog **4**–Fe(III)	37.4 ± 13.4

[a] K_i values are means of independent experiments carried out in triplicate.

^{55}Fe uptake by the N3″-functionalized analogs of pyochelin

The ability of pyochelin **1** and of the N3″-functionalized analog to promote ^{55}Fe(III) uptake in *P. aeruginosa* was assessed with siderophore-deficient *P. aeruginosa* cells (PAD07), in order to prevent the ^{55}Fe(III) uptake promoted by endogenous siderophores. Unfortunately, in our experimental conditions, slight precipitation of pyochelin analog **4** and its corresponding ^{55}Fe(III) chelate was observed, resulting in a high signal/noise ratio for the radioactive signal. We overcame this problem by carrying out ^{55}Fe uptake experiments with pyochelin analog **9**. This compound was obtained as a water-soluble trifluoroacetate salt, by elimination of the protective *tert*-butyloxycarbonyl (Boc) group in the presence of trifluoroacetic acid (TFA). The siderophore and its analog were loaded with ^{55}Fe(III) and the radioactivity incorporated into bacteria was monitored after separating bacteria from the free siderophore–^{55}Fe(III) by centrifugation. The radioactivity in the bacterial pellet corresponds to the amount of ^{55}Fe(III) transported into the cells. As a control, the experiment was repeated in the presence of the protonophore CCCP at 0 °C, conditions resulting in the inhibition of iron uptake. The observed kinetics clearly demonstrate that functionalized pyochelin **9** transports ^{55}Fe into PAD07 cells at the same velocity as the natural siderophore. No ^{55}Fe uptake was observed in the presence of CCCP at 0 °C, demonstrating that the transport process is dependent on the inner membrane proton motive force rather than on the passive diffusion through bacterial porins (Fig. 3).

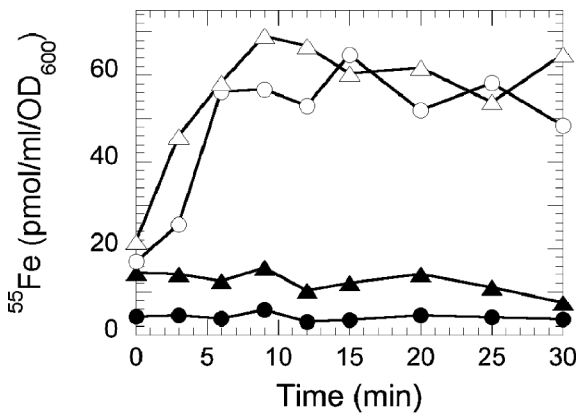
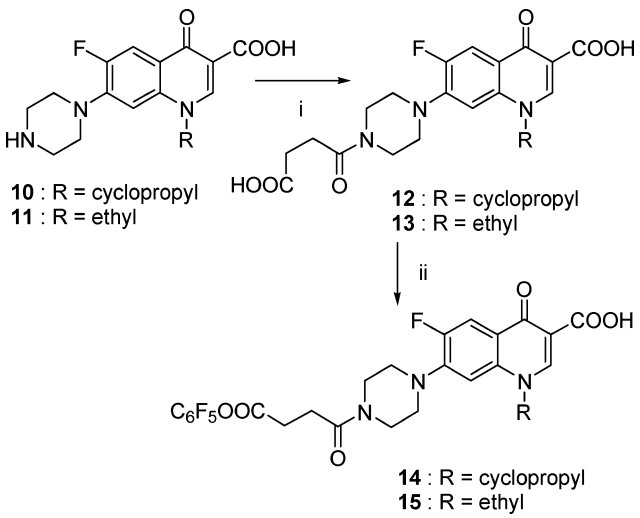


Fig. 3 Time-dependent uptake of pyochelin **1**-^{55}Fe(III) and pyochelin analog **9**-^{55}Fe(III) in *P. aeruginosa* PAD07 cells. Cells at an OD_{600} of 1 were incubated for 15 min in 50 mM Tris-HCl (pH 8.0) at 37 °C, before the initiation of transport assays by the addition of 100 nM pyochelin **1**-^{55}Fe(III) (○) or pyochelin analog **9**-^{55}Fe(III) (△) complexes. Samples (100 µL) of the suspension were removed at various times and 200 µM of ice cold Tris buffer was added and the mixture immediately centrifuged. The supernatants containing the unbound siderophore were filtered, and the radioactivity retained was counted. The experiment was repeated with the protonophore CCCP at a concentration of 100 µM (pyochelin **1**-^{55}Fe(III), ●; pyochelin analog **9**-^{55}Fe(III), ▲).

Thus, the N3″-functionalized pyochelin is recognized by FptA and is transported into the bacterial cell *via* this receptor. Moreover, the docking experiments indicated that the N3″-propylamine extension bulges out of the pyochelin-binding site on the FptA receptor (Fig. 2). This accounts for the specific recognition of analogs **4** and **9** conjugated to a bulky xenobiotic molecule by the FptA outer membrane receptor reported previously.[42] All these functional and structural data strongly confirm the suitability of N3″-functionalized pyochelin for use in Trojan horse strategies for the delivery of an antibiotic.

Synthesis of pyochelin–fluoroquinolone conjugates

With our approach, three fluoroquinolones—ciprofloxacin, norfloxacin and *N*-desmethyl-ofloxacin—were vectorized by pyochelin. We began by synthesizing the building blocks bearing the succinic linker. For this purpose, commercially available ciprofloxacin **10** and norfloxacin **11** were reacted with succinic anhydride in DMSO at 95 °C, according to a published protocol.[21,23] The yields of the resulting succinate derivatives **12** and **13** were 97% and 84%, respectively. The carboxylate groups of compounds **12** and **13** were further activated in the presence of pentafluorophenol and a carbodiimide coupling agent. A water-soluble carbodiimide (EDCI) was used in a first approach, but the resulting pentafluorophenyl-esters **14** and **15** were obtained at only low to moderate yield, often contaminated with undesirable side-products. We then tested the use of dicyclohexylcarbodiimide (DCC) as a coupling agent in these reactions. However, the presence of DCU in the crude mixtures required time-consuming purification on silica gel columns. Finally, the carboxylate groups of succinate derivatives **12** and **13** were activated efficiently in the presence of pentafluorophenol and polymer-supported DCC. In these conditions, the expected pentafluorophenyl-esters **14** and **15** were isolated with 88% and 65% yields, respectively (Scheme 2).

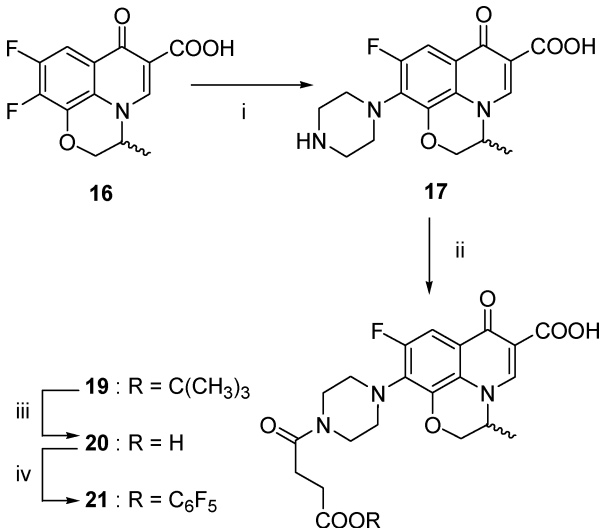


Scheme 2 Synthesis of ciprofloxacin- and norfloxacin-stable spacer arm building blocks **14** and **15**. i. Succinic anhydride, DMSO, 95 °C. ii. C_6F_5OH, polymer-supported DCC, $CHCl_3$, 45 °C.

Synthesis of the *N*-desmethyl-ofloxacin-succinate spacer arm building block required a slightly different protocol. The commercially available difluorinated precursor **16** was first treated with an excess of piperazine, generating *N*-desmethyl-ofloxacin **17** with a yield of 93%. The reaction of this compound with succinic anhydride in the conditions described above resulted in a complex mixture, from which it was difficult to purify efficiently the succinate product **20**. Alternatively, *N*-desmethyl-ofloxacin **17**

was treated with *tert*-butyl-pentafluorophenyl succinate **18**[42] to generate the *tert*-butyl succinate derivative **19**, which was isolated with a yield of 71%.

The *tert*-butyl protecting group was cleaved with TFA, and the released free carboxylate **20**, isolated with 57% yield, was further activated as described above. The preparation of the expected pentafluorophenyl-ester **21** thus had a yield of 98% (Scheme 3).



Scheme 3 Synthesis of *N*-desmethyl-ofloxacin-stable spacer arm building block **21**. i. Piperazine, DMSO, 95 °C. ii. $C_6F_5OOCCH_2CH_2COOC(CH_3)_3$ (**18**), pyridine–toluene, 20 °C. iii. TFA–CH_2Cl_2, 20 °C. iv. C_6F_5OH, polymer-supported DCC, $CHCl_3$, 45 °C.

In parallel, we synthesized the building blocks connecting the antibiotics to a hydrolyzable spacer arm. For this purpose, three fluoroquinolone antibiotics **10**, **11** and **17** were first treated with chloromethylchloroformate in the presence of 1,8-bis(dimethylamino)naphthalene (also referred to as "proton sponge"). In these conditions, the expected halogenated compounds **22**, **23** and **24**, were isolated cleanly, in high yield (77–94%). Chlorides **22**, **23** and **24** were used for an *O*-alkylation reaction of *tert*-butyl hemisuccinate **25**,[23,42] promoted by silver carbonate. The resulting diesters, **26** to **28**, were isolated with good yields (66–80%). The *tert*-butyl protecting groups of compounds **26** to **28** were then cleaved in the presence of TFA. The resulting free carboxylates, **29** to **31**, were used without further purification, and were converted into the corresponding pentafluorophenyl-esters, **32** to **34** (Scheme 4).

The Boc protecting group of functionalized analog **4** was then cleaved using TFA and the resulting free amine (compound **9**) was reacted with pentafluorophenyl-esters **14**, **15**, **21**, **32**, **33** or **34**, in the presence of DIPEA in dichloromethane, at room temperature. The resulting conjugates, esters **35** to **40**, were isolated with moderate to excellent yields (39–99%) (Scheme 5).

Biological properties of the pyochelin–fluoroquinolone conjugates

Our pyochelin–fluoroquinolone conjugates were only weakly soluble in aqueous media at physiological pH. For biological evaluations, conjugates **35** to **40** were dissolved in dimethylsulfoxide (DMSO) before dilution in bacterial growth media. We observed progressive precipitation of the conjugates in the wells during bacterial growth. The low solubility of compounds **35** to **40** probably reflects the conjugation of pyochelin, a hydrophobic siderophore, to fluoroquinolones, which are known to be soluble in aqueous media only as salts. Several attempts to convert conjugates **35** to **40** into their corresponding water-soluble salts were unsuccessful, generally leading to degradation of the pyochelin moiety.

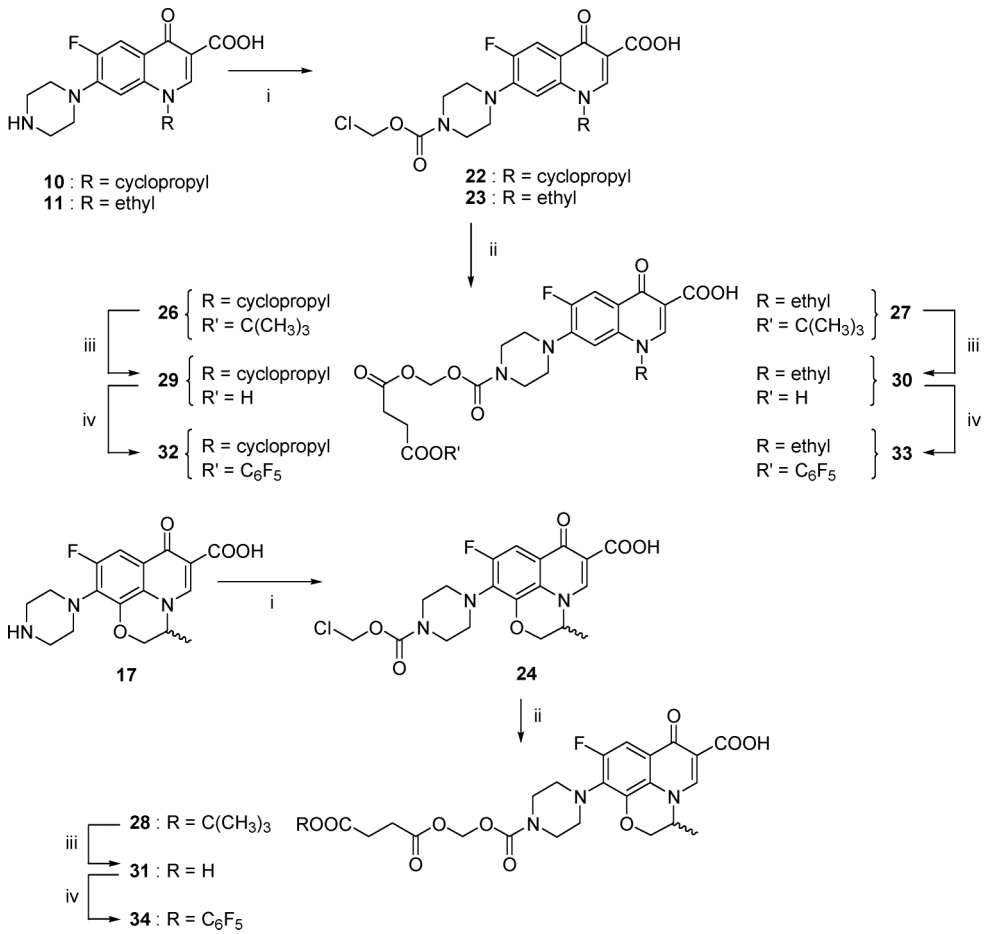
Table 3 Antibiotic activities of conjugates **35** to **40** and of ciprofloxacin **10**, norfloxacin **11** and *N*-desmethyl-ofloxacin **17** against the three *P. aeruginosa* strains: PAO1, PAD07 and PAD14

	MIC_{50} (μM)[a]					
	PAO1		PAD07		PAD14	
	−Fe(III)	+Fe(III)	−Fe(III)	+Fe(III)	−Fe(III)	+Fe(III)
Ciflox **10**	0.040	0.060	0.060	0.045	0.035	0.040
35	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
36	0.600	0.700	0.700	0.600	0.170	0.200
Norflox **11**	0.110	0.200	0.200	0.190	0.120	0.120
37	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
38	n.a.	1.000	1.000	1.000	0.450	0.550
DMO **17**	0.200	0.350	0.350	0.360	0.180	0.210
39	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
40	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

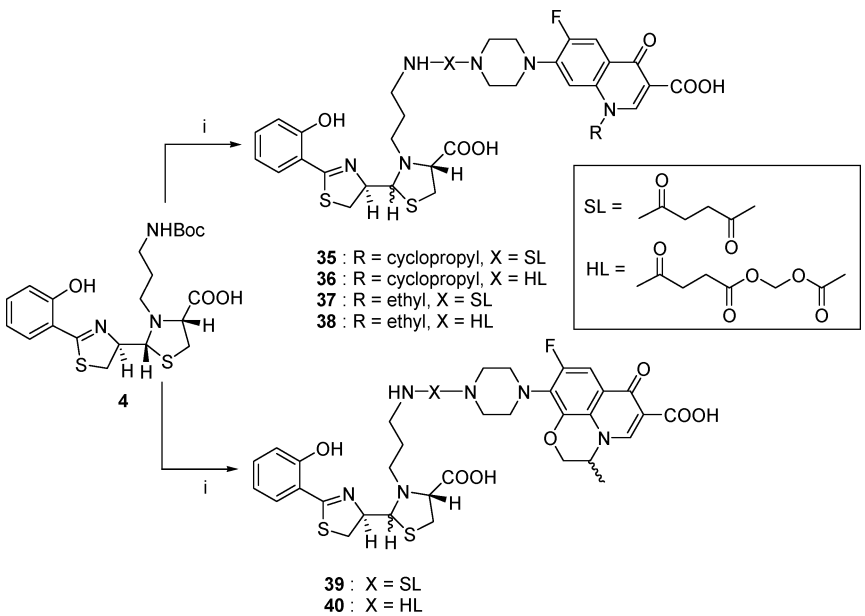
[a] MIC_{50} values are mean of a triplicate experiment. Ciflox: ciprofloxacin, Norflox: norfloxacin, DMO: *N*-desmethyl-ofloxacin. n.a.: no activity.

Despite their poor solubility, we determined the MIC_{50} of the six conjugates **35** to **40** in the presence and absence of iron(III), with three *Pseudomonas aeruginosa* strains: the wild-type strain PAO1, the pyoverdin- and pyochelin-deficient strain PAD07 and the pyoverdin- and TonB-deficient strain PAD14. TonB is a key protein in the iron assimilation process, because it couples the inner membrane proton motive force to ferric-siderophore transport *via* the outer membrane transporters. No ferrisiderophore uptake occurs in the absence of TonB. As a control, the experiment was repeated with siderophore-free ciprofloxacin **10**, norfloxacin **11** and *N*-desmethyl-ofloxacin **17** (Table 3).

In our experimental conditions, the three unconjugated antibiotics **10**, **11** and **17** inhibited strongly the proliferation of the three *P. aeruginosa* strains tested. The observed growth inhibitions were similar in the presence and absence of iron(III). The conjugates **35**, **37** and **39** with a non hydrolyzable succinic linker had no antibiotic activity, whatever the strain used for evaluation. This absence of activity probably reflects the lack of hydrolysis of the succinic linker *in vivo*, and the resulting absence of antibiotic release. Indeed, piperazine nitrogen has been shown to be crucial for the biological activity of fluoroquinolones.[1,45] Pyochelin–ciprofloxacin **36** and pyochelin–norfloxacin **38** conjugates which have a spacer arm that can be cleaved *in vivo*, displayed antibiotic activity against *P. aeruginosa*. However, this activity was lower than that measured for the corresponding unconjugated fluoroquinolones, by a factor of three to 20. In the case of *N*-desmethyl-ofloxacin conjugate **40**, no antibiotic activity was observed, whatever the conditions and the strain used in the experiments. The MIC_{50} values obtained are probably largely underestimated, because the progressive precipitation of the conjugates limits the concentration of the drug available to the bacteria. Moreover, our conjugates displayed significant activity against PAD14, a *P. aeruginosa* strain that cannot acquire iron(III) through the pyochelin-dependant iron uptake system. This control strain was therefore expected to



Scheme 4 Synthesis of ciprofloxacin-, norfloxacin- and *N*-desmethyl-ofloxacin-cleavable spacer arm building blocks **32**, **33** and **34**. i. $ClCH_2OCOCl$, proton sponge, $CHCl_3$, 20 °C. ii. $(CH_3)_3COOCCH_2CH_2COOH$ (**25**), Ag_2CO_3, DMF, 90 °C. iii. TFA–CH_2Cl_2, 20 °C. iv. C_6F_5OH, polymer-supported DCC, $CHCl_3$, 45 °C.



Scheme 5 Synthesis of six fluoroquinolone–pyochelin conjugates **35** to **40**. i. a. TFA–CH_2Cl_2, 8 : 2, 20 °C. b. Pentafluorophenyl-esters **14**, **15**, **21**, **32**, **33** or **34**, DIPEA, CH_2Cl_2, 20 °C.

display little or no sensitivity to our conjugates. Our observations suggest that the spacer arm connecting the siderophore and the antibiotic was actually hydrolyzed in the extracellular medium, rather than in the bacteria. The released antibiotic can penetrate further into the PAD14 cells by diffusion through the bacterial envelope. It therefore seems likely that extracellular hydrolases secreted into the culture medium by the bacteria promote the progressive cleavage of the spacer arm. At first glance, our conjugates more closely resemble antibiotic prodrugs than Trojan horse conjugates. Thus, the biological evaluation of conjugates **35** to **40** is confronted by two major problems: the solubility of the conjugates in physiological conditions and the hydrolysis of the spacer arm in the extracellular medium. We envisage studies in two different directions to improve these parameters and to develop further Trojan horse antibiotic conjugates vectorized by pyochelin: on one hand we plan to vectorize β-lactam or aminoside antibiotics, which should generate more soluble conjugates; on the other hand, we aim to develop new labile spacer arms, resistant to extracellular conditions but readily cleaved when exposed to the intracellular metabolism of bacteria. The Trojan horse strategy was "bio-inspired" by sideromycins. In these natural compounds, the antibiotics are connected to the siderophores through linkers that are cleaved by intracellular enzymes.[13] The linkers found in sideromycins have thus evolved for the efficient and specific intracellular release of the antibiotic. These natural linkers are difficult to synthesize, but they remain a promising alternative to the synthetic spacer arms developed to date for drug vectorization systems.

Conclusions

The pyochelin analog functionalized by a propyl-amine extension in the N3″ position bound the pyochelin outer membrane transporter FptA with high affinity. In addition, the functionalized analog **9** promoted iron(III) transport as efficiently as natural pyochelin **1** in the pyochelin-dependent iron uptake system in *P. aeruginosa*. The previously developed N3″-functionalized pyochelin analog is therefore a promising candidate vector for the specific delivery of an antibiotic in the framework of a Trojan horse strategy. Based on this observation, we efficiently synthesized six conjugates of the functionalized pyochelin and three fluoroquinolones (ciprofloxacin, norfloxacin and *N*-desmethylofloxacin), **35** to **40**. The antibiotic and the siderophore were connected by either a stable succinic linker or a spacer arm that could be hydrolyzed *in vivo*. Some of these conjugates had antibiotic activity against several *P. aeruginosa* strains, but this activity was weaker than that of the corresponding unconjugated fluoroquinolones. This weaker activity was probably due to the low solubility of the conjugates in aqueous media and hydrolysis of the spacer arm in the extracellular medium. Taking these factors into account, new conjugates based on N3″-functionalized pyochelins **4** and **9** are currently being developed in our laboratory. Once synthesized, this new generation of pyochelin–antibiotic conjugates will be tested on clinical strains of *P. aeruginosa* and on strains from the cepacia complex. This entire project is our contribution to global efforts to find innovative strategies for treating chronic infections in patients suffering from cystic fibrosis.

Experimental

General

All reactions were carried out under argon purchased from Air Liquide. Solvents used for reaction were of analytical grade purity (>99.9%). When necessary, and specified in the protocols, solvents were purchased extra-dry from Aldrich or Acros companies. Amines were distilled and stored on KOH before use. All chemicals were obtained from commercial suppliers (Aldrich, Acros or Alfa-aesar) and were used as received, unless otherwise stated. Functionalized pyochelin **4**, the *tert*-butyl-pentafluorophenyl succinate **18** and the *tert*-butyl-hemisuccinate **25** were synthesized using previously published protocols.[42] Pyochelin was also synthesized according to previously published protocols.[46,47] $^{55}FeCl_3$ was obtained from Perkin Elmer Life and Analytical Sciences (Billerica, MA, USA) with a specific activity of 93.76 Ci g^{-1}. This radioactive ^{55}Fe solution was diluted with non-radioactive $FeCl_3$ to 9.4 Ci g^{-1}. Carbenicillin disodium salt and tetracyclin hydrochloride were purchased from Euromedex. The protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and streptomycin sulfate were purchased from Sigma.

Chromatography

All reactions were monitored by thin-layer chromatography (TLC) using Merck aluminium sheets precoated with silica gel $60F^{254}$ (0.25 mm). Column chromatography purifications were performed using Merck silica gel 60 (63–200 μm). In our protocols, silica should be demetallated prior to use. For this purpose commercial silica was suspended and stirred 10 h in 1 N HCl. Silica gel was filtered and washed several times with bidistillated water, dried under reduced pressure and then in an oven for at least 72 h.[40]

Instrumentation

NMR spectra were recorded either on a Bruker Avance 300 (^1H: 300 MHz, ^{13}C: 75 MHz) or on Bruker Avance 400 (^1H: 400 MHz, ^{13}C: 100 MHz), using the residual non-deuterated solvent as reference. The chemical shifts (δ) and coupling constants (J) are expressed in ppm and Hertz respectively. Multiplicities were indicated as s (singlet), d (doublet), t (triplet), q (quadruplet) and m (multiplet). A broad signal is mentioned with br preceding the multiplicity. Mass spectra were recorded in the *Service Commun d'Analyse* (SCA) *de la Faculté de Pharmacie de l'Université de Strasbourg* and were measured after calibration in ES-TOF experiments on a Bruker Daltonic MicroTOF mass spectrometer. Counting of radioactive samples was performed on a Packard TriCarb 2100TR.

General procedure for the synthesis of diacids 12 and 13

Succinic anhydride (100 mg, 1.00 mmol) was added to a suspension of fluoroquinolone **10** or **11** (1.00 mmol) in DMSO (2.0 mL). The reaction mixture was stirred for 12 h at 95 °C. The reaction mixture was cooled down to 20 °C and the resulting precipitate was filtered, washed successively with water and Et_2O, and dried under reduced pressure. Expected diacids **12** (418 mg, 0.97 mmol, 97%) and **13** (352 mg, 0.84 mmol, 84%) were isolated as white powders.

7-[4-(3-Carboxy-propionyl)-piperazin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 12

R_f 0.20 (acetone–EtOAc–AcOH 50 : 48 : 2). ^{1}H NMR (400 MHz; $CDCl_3$): δ 14.81 (s, 1H), 8.57 (s, 1H), 7.79 (d, J = 13 Hz, 1H), 7.29 (d, J = 7 Hz, 1H), 3.70–3.80 (m, 4H), 3.53–3.56 (m, 1H), 3.24–3.33 (m, 4H), 2.54–2.60 (m, 4H), 1.35 (m, 2H), 1.15 (m, 2H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 176.8, 172.3, 170.1, 166.7, 152.2, 147.4, 145.4, 139.0, 119.8, 112.1, 107.9, 105.1, 50.0, 49.9, 49.4, 45.1, 41.3, 30.3, 27.9, 8.23. MS (ESI): m/z 431.1 [M+H$^+$].

7-[4-(3-Carboxy-propionyl)-piperazin-1-yl]-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 13

R_f 0.17 (acetone–EtOAc–AcOH 50 : 48 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.55 (s, 1H), 7.84 (d, J = 13 Hz, 1H), 6.79 (d, J = 7 Hz, 1H), 4.22 (q, J = 7 Hz, 2H), 3.55–3.65 (m, 4H), 3.15–3.25 (m, 4H), 2.49 (s, 4H), 1.38 (t, J = 7 Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 176.0, 174.0, 170.0, 166.0, 152.7, 148.3, 145.1, 137.1, 119.4, 111.1, 107.2, 105.9, 49.5, 49.2, 48.9, 44.4, 40.8, 29.6, 27.7, 14.2. MS (ESI-FABS): m/z 420.1 [M+H$^+$].

***N*-Desmethyl-ofloxacin 17**

Piperazine (184 mg, 2.14 mmol) was added to a suspension of commercial difluoro-compound **16** (100 mg, 0.35 mmol) in DMSO (1.5 mL). The suspension was stirred at 95 °C for 12 h. Cold acetone was then added to the mixture. The resulting brown precipitate was triturated in acetone to give the expected compound **17** (113 mg, 0.32 mmol, 93%) as a yellow powder. R_f 0.11 (CH_2Cl_2–EtOH 9 : 1). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.95 (s, 1H), 7.78 (d, J = 12 Hz, 1H), 4.93–4.88 (m, 1H), 4.57 (dd, J = 12, 2 Hz, 1H), 4.36 (dd, J = 12, 2 Hz, 1H), 3.38–3.25 (m, 4H), 2.45 (t, J = 4 Hz, 4H), 1.44 (d, J = 4.0 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 180.2, 172.4, 158.3, 157.2, 144.2, 140.1, 132.7, 124.6, 119.3, 108.1, 105.4, 68.4, 54.2, 50.1, 46.5, 42.6, 18.2. MS (ESI): m/z 348.1 [M+H$^+$].

9-[4-(3-*tert*-Butoxycarbonyl-propionyl)-piperazin-1-yl]-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalene-5-carboxylic acid 19

A solution of *N*-desmethyl-ofloxacine **17** (100 mg, 0.29 mmol) and diester **18** (195 mg, 0.57 mmol) in a mixture of pyridine–toluene 1 : 1 (10 mL) was stirred for 3 h at 20 °C. The volatile compounds were removed under reduced pressure and the crude product was purified by chromatography on a silica gel column (CH_2Cl_2–acetone 97 : 3) to afford compound **19** (102 mg, 0.20 mmol, 71%) as a white solid. R_f 0.58 (CH_2Cl_2–EtOH 9 : 1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62 (s, 1H), 7.61 (d, J = 12 Hz, 1H), 4.59 (br s, 1H), 4.20–4.30 (m, 2H), 3.71 (t, J = 4 Hz, 2H), 3.60 (br s, 2H), 3.37–3.23 (m, 4H), 2.59–2.57 (m, 4H), 1.57 (d, J = 4 Hz, 3H), 1.41 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.6, 172.4, 170.1, 167.5, 158.1, 155.1, 144.8, 140.2, 132.7, 124.6, 119.2, 108.4, 105.4, 80.7, 68.4, 55.6, 50.3, 46.5, 42.6, 30.9, 29.9, 28.3, 18.7. MS (ESI): m/z 504.2 [M+H$^+$].

9-[4-(3-Carboxy-propionyl)-piperazin-1-yl]-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalene-5-carboxylic acid 20

TFA (0.5 mL) was added to a solution of compound **19** (70 mg, 0.135 mmol) in CH_2Cl_2 (4.5 mL). The solution was stirred for 2 h at 20 °C. The volatile compounds were removed under reduced pressure and the residue was diluted in CH_2Cl_2. The organic layer was washed successively with water and brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure to give diacid **20** (34 mg, 0.077 mmol, 57%) as a pale yellow oil. R_f 0.18 (CH_2Cl_2–EtOH 9 : 1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.61 (s, 1H), 7.72 (d, J = 12 Hz, 1H), 4.52–4.47 (m, 1H), 4.46–4.33 (m, 2H), 3.75 (t, J = 4 Hz, 2H), 3.63 (br s, 2H), 3.36–3.29 (m, 4H), 2.67–2.64 (m, 4H), 1.60 (d, J = 4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 172.5, 170.3, 167.2, 157.4, 154.9, 145.1, 140.0, 132.5, 124.8, 121.1, 107.9, 105.2, 68.5, 55.7, 50.8, 46.3, 42.7, 30.6, 29.8, 18.5. MS (ESI): m/z 448.1 [M+H$^+$].

General procedure for the synthesis of chloride derivates 22, 23 and 24

Chloromethylchloroformate (1.45 mmol) and 1,8-bis(dimethylamino)naphthalene (2.00 mmol) were added to a suspension of fluoroquinolone **10**, **11** or **17** (1.00 mmol) in $CHCl_3$ (15 mL mmol^{-1}). The resulting solution was stirred for 5 h at 20 °C. The volatile compounds were removed under reduced pressure and the residue was dissolved in CH_2Cl_2. The organic layer was washed successively with 0.1 N HCl, water and brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by chromatography on a silica gel column (CH_2Cl_2–EtOH 98 : 2 to 97 : 3), leading to chloride derivates **22** (326 mg, 0.77 mmol, 77%), **23** (337 mg, 0.82 mmol, 82%) and **24** (412 mg, 0.94 mmol, 94%) as white powders.

7-(4-Chloromethoxycarbonyl-piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 22

R_f 0.66 (CH_2Cl_2–acetone 8 : 2). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.81 (s, 1H), 8.09 (d, J = 13 Hz, 1H), 7.39 (d, J = 7 Hz, 1H), 5.84 (s, 2H), 3.75–3.85 (m, 4H), 3.53–3.56 (m, 1H), 3.35–3.45 (m, 4H), 1.35 (m, 2H), 1.15 (m, 2H).^{13}C NMR (100 MHz, $CDCl_3$): δ 176.8, 166.7, 152.2, 147.4, 145.4, 139.0, 119.8, 112.1, 107.9, 105.1, 80.5, 50.0, 49.9, 49.4, 45.1, 41.3, 27.9, 8.23. MS (ESI): m/z 426.1 [M+H$^+$].

7-(4-Chloromethoxycarbonyl-piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 23

R_f 0.64 (CH_2Cl_2–acetone 9 : 1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.67 (s, 1H), 8.08 (d, J = 13 Hz, 1H), 6.85 (d, J = 7 Hz, 1H), 5.82 (s, 2H), 4.32 (q, J = 7 Hz, 2H), 3.77 (s, 4H), 3.31 (s, 4H), 1.59 (t, J = 7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.7, 166.6, 153.1, 152.2, 146.9, 145.3, 136.7, 121.0, 112.8, 108.3, 103.9, 70.7, 49.4, 49.2, 43.6, 43.4, 14.2. MS (ESI): m/z 413.9 [M+H$^+$].

9-(4-Chloromethoxycarbonyl-piperazin-1-yl)-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalene-5-carboxylic acid 24

R_f 0.56 (CH_2Cl_2–acetone 9 : 1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62 (s, 1H), 7.72 (d, J = 12 Hz, 1H), 5.81 (s, 2H), 4.54–4.49 (m, 1H), 4.39 (ddd, J = 25, 11, 2 Hz, 2H), 3.65 (s, 4H), 3.32 (s, 4H),

1.60 (d, J = 6 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 167.1, 152.2, 144.9, 139.8, 136.8, 132.6, 124.4, 119.3, 108.3, 105.6, 71.1, 68.5, 55.7, 50.6, 45.1, 43.8, 29.9, 18.52. MS (ESI): m/z 442.1 [M+H$^+$].

General procedure for the synthesis of *tert*-butyl succinylesters 26, 27 and 28

tert-Butyl hemisuccinate **25** (2.00 mmol) and silver carbonate (5.00 mmol) were added to a suspension of chloride derivates **22**, **23** or **24** (1.00 mmol) in DMF (10 mL). The reaction mixture was stirred at 95 °C for 12 h. The solution was cooled down to 20 °C and filtered through Celite 545. Celite was thoroughly washed with a mixture of CH_2Cl_2–MeOH 9 : 1 and the filtrate was evaporated to dryness. The residue was diluted in CH_2Cl_2 and washed successively with 1 N $NaHCO_3$, water and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by chromatography on a silica gel column (CH_2Cl_2–EtOH 99 : 1 to 98 : 2) to give the expected compounds **26** (438 mg, 0.78 mmol, 78%), **27** (362 mg, 0.66 mmol, 66%) or **28** (462 mg, 0.80 mmol, 80%) as white powders.

Succinic acid *tert*-butyl ester 4-(3-carboxy-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinolin-7-yl)-piperazine-1-carbonyloxymethyl ester 26

R_f 0.58 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.70 (s, 1H), 7.94 (d, J = 13 Hz, 1H), 7.33 (d, J = 7 Hz, 1H), 5.78 (s, 2H), 3.71 (br s, 4H), 3.52 (br s, 1H), 3.28 (br s, 4H), 2.62–2.47 (m, 4H), 1.39 (s, 9H), 1.24–1.15 (m, 4H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 177.2, 171.7, 167.2, 163.1, 153.6, 147.8, 145.7, 139.2, 120.4, 112.6, 108.3, 105.4, 81.1, 81.0, 49.7, 48.5, 44.0, 43.7, 36.8, 35.5, 30.4, 29.3, 28.2, 8.4. MS (ESI): m/z 562.2 [M+H$^+$].

Succinic acid *tert*-butyl ester 4-(3-carboxy-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-quinolin-7-yl)-piperazine-1-carbonyloxymethyl ester 27

R_f 0.74 (CH_2Cl_2–MeOH 95 : 5). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.96 (s, 1H), 7.94 (d, J = 13 Hz, 1H), 7.21 (d, J = 7 Hz, 1H), 5.73 (s, 2H), 4.59 (q, J = 7 Hz, 2H), 3.61 (br s, 4H), 3.35 (br s, 4H), 2.58–2.43 (m, 4H), 1.41 (t, J = 7 Hz, 3H), 1.37 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 176.8, 171.5, 171.2, 167.0, 153.4 (d, J = 252 Hz), 153.4, 147.2, 145.7 (d, J = 10 Hz), 137.1, 120.7 (d, J = 7 Hz), 112.6 (d, J = 23 Hz), 108.2, 104.4, 80.9, 80.5, 49.8, 49.6, 43.9, 43.5, 30.0, 29.2, 28.1, 14.5. MS (ESI): m/z 578.2 [M+H$^+$].

Succinic acid *tert*-butyl ester 4-(5-carboxy-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalen-9-yl)-piperazine-1-carbonyloxymethyl ester 28

R_f 0.65 (CH_2Cl_2–EtOH 9 : 1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.61 (s, 1H), 7.62 (d, J = 12 Hz, 1H), 5.75 (s, 2H), 4.54 (br s, 1H), 4.37 (ddd, J = 25, 11, 2 Hz, 2H), 3.71 (t, J = 4 Hz, 2H), 3.58 (br s, 2H), 3.30–3.25 (m, 4H), 2.60–2.45 (m, 4H), 1.55 (d, J = 4 Hz, 3H), 1.38 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.2, 174.4, 171.6, 167.4, 153.6, 145.1, 139.9, 124.8, 121.1, 107.9, 105.3, 81.02, 80.6, 70.4, 68.5, 65.3, 63.5, 55.7, 50.5, 44.9, 34.3, 32.1, 29.8, 28.2, 18.5. MS (ESI): m/z 562.2 [M+H$^+$].

General procedure for the synthesis of pentafluorophenyl esters 14, 15, 21, 32, 33 and 34

To a solution of *tert*-butyl ester **19**, **26**, **27** or **28** (1.00 mmol) in CH_2Cl_2 (9.0 mL) was added TFA (1.0 mL). The solution was stirred 3 h at 23 °C and the mixture was evaporated to dryness. The residue was dissolved in CH_2Cl_2 and washed successively with water and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was used without any further purification in the next step.

Diacid **12**, **13**, **20**, **29**, **30** or **31** (1.00 mmol), pentafluorophenol (2.00 mmol) and polymer supported-DCC (1.9 mmol g^{-1}) (3.00 mmol) were poured in $CHCl_3$ (10 mL) and the reaction suspension was gently stirred at 45 °C for 2 h. The resulting mixture was filtered and the resin was washed several times with CH_2Cl_2. The filtrate was evaporated under reduced pressure and was purified by chromatography on a silica gel column (CH_2Cl_2–EtOH 99 : 1 to 98 : 2) leading to activated ester **14** (525 mg, 0.88 mmol, 88%), **15** (380 mg, 0.65 mmol, 65%), **21** (601 mg, 0.98 mmol, 98%), **32** (456 mg, 0.68 mmol, 68%), **33** (264 mg, 0.40 mmol, 40%) or **34** (481 mg, 0.70 mmol, 70%) as white powders.

1-Cyclopropyl-6-fluoro-4-oxo-7-[4-(3-pentafluorophenyloxy-carbonyl-propionyl)-piperazin-1-yl]-1,4-dihydro-quinoline-3-carboxylic acid 14

R_f 0.61 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.57 (s, 1H), 7.79 (d, J = 12 Hz, 1H), 7.29 (d, J = 7 Hz, 1H), 3.86 (t, J = 5 Hz, 2H), 3.72 (t, J = 5 Hz, 2H), 3.49 (br s, 1H), 3.32 (t, J = 5 Hz, 2H), 3.24 (t, J = 5 Hz, 2H), 3.06 (t, J = 6 Hz, 2H), 2.81 (t, J = 6 Hz, 2H), 1.32–1.26 (m, 2H), 1.09–1.05 (m, 2H). ^{13}C NMR (100 MHz; $CDCl_3$): δ 176.6, 172.0, 169.4, 166.0, 152.0, 148.4, 144.5, 138.4, 123.6, 113.6, 110.4, 108.3, 105.4, 89.9, 61.3, 50.6, 49.8, 45.5, 41.38, 35.1, 29.9, 28.8, 28.0, 8.5. ^{19}F NMR (400 MHz, $CDCl_3$): δ −123.4 (s, 1F), −152.3 – −152.4 (m, 2F), −158.0 (t, J = 22 Hz, 1F), −162.2 – – 162.4 (m, 2F). MS (ESI): m/z 598.1 [M+H$^+$].

1-Ethyl-6-fluoro-4-oxo-7-[4-(3-pentafluorophenyloxycarbonyl-propionyl)-piperazin-1-yl]-1,4-dihydro-quinoline-3-carboxylic acid 15

R_f 0.64 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz; $CDCl_3$): δ 8.53 (s, 1H); 8.02 (d, J = 13 Hz, 1H), 7.29 (d, J = 7 Hz, 1H), 4.32 (q, J = 7 Hz, 2H), 3.91–3.88 (m, 2H), 3.77–3.74 (m, 2H), 3.55–3.38 (m, 2H), 3.30–3.27 (m, 2H), 3.09 (t, J = 6 Hz, 2H), 2.83 (t, J = 6. Hz, 2H), 1.59 (t, J = 7 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 177.1, 169.3, 169.0, 167.2, 153.6, 147.4, 145.7, 137.1, 121.2, 113.1, 108.5, 104.4, 50.3, 50.2, 49.9, 49.6, 49.5, 28.7, 27.9, 14.6. ^{19}F NMR (400 MHz, $CDCl_3$): δ −121.1 (s, 1F), −153.2 – – 152.8 (m, 2F), −158.0 (t, J = 24 Hz, 1F), −162.5 – – 162.7 (m, 2F). MS (ESI): m/z 586.1 [M+H$^+$].

8-Fluoro-3-methyl-6-oxo-9-[4-(3-pentafluorophenyloxy-carbonyl-propionyl)-piperazin-1-yl]-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalene-5-carboxylic acid 21

R_f 0.58 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.61 (s, 1H), 7.72 (d, J = 12 Hz, 1H), 4.52–4.47 (m, 1H), 4.46–4.33 (m, 2H), 3.75 (t, J = 4 Hz, 2H), 3.63 (br s, 2H), 3.36–3.29 (m, 4H), 2.67 (m, 4H), 1.60 (d, J = 4 Hz, 3H). ^{13}C NMR (100 MHz,

$CDCl_3$): δ 177.2, 172,5, 170.3, 167.2, 157.4, 154.9, 145.1, 140.0, 137.6, 132.5, 130.3, 129.8, 124.8, 120.3, 108.2, 105.4, 68.5, 51.3, 50.7, 45.3, 43.2, 30.4, 29.8, 18.1. ^{19}F NMR (400 MHz, $CDCl_3$): δ −122.6 (s, 1F), −152.6 – – 151.8 (m, 2F), −157.4 (t, J = 22 Hz, 1F), −162.7 – – 162.5 (m, 2F). MS (ESI): m/z 614.1 [M+H$^+$].

Succinic acid 4-(3-carboxy-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinolin-7-yl)-piperazine-1-carbonyloxymethyl ester pentafluorophenyl ester 32

R_f 0.68 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.96 (s, 1H), 7.93 (d, J = 13 Hz, 1H), 7.19 (d, J = 7 Hz, 1H), 5.76 (s, 2H), 3.60 (t, J = 6 Hz, 4H), 3.37–3.27 (m, 5H), 3.08–3.05 (m, 2H), 2.83–2.78 (m, 4H), 1.42–1.39 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.3, 171.3, 168.6, 165.8, 152.7, 148.1, 144.8, 139.1, 135.3, 129.4, 111.0, 106.7, 80.3, 49.04, 43.3, 43.0, 35.8, 28.6, 28.3, 27.8, 7.58. ^{19}F NMR (400 MHz, DMSO-d_6): δ −121.7 (s, 1F), −153.1 – – 153.2 (m, 2F), −157.9 (t, J = 25 Hz, 1F), −162.6 – – 162.8 (m, 2F). MS (ESI): m/z 672.1 [M+H$^+$].

Succinic acid 4-(3-carboxy-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-quinolin-7-yl)-piperazine-1-carbonyloxymethyl ester pentafluorophenyl ester 33

R_f 0.54 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.69 (s, 1H), 8.10 (d, J = 13 Hz, 1H), 6.83 (d, J = 7 Hz, 1H), 5.85 (s, 2H), 4.31 (q, J = 7 Hz, 2H), 3.73 (br s, 4H), 3.28 (br s, 4H), 3.03 (dd, J = 6, 5 Hz, 2H), 2.85 (dd, J = 6, 5 Hz, 2H), 1.59 (t, J = 7 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 177.1; 170.7; 168.4; 167.3; 159.9; 153.6; 153.4; 147.4; 145.8; 137.1; 121.2; 113.1; 108.5; 104.4; 80.8; 49.9; 49.7; 43.9; 43.6; 28.8; 28.2; 14.6. ^{19}F NMR ($CDCl_3$, 400 MHz): δ −121.9 (s, 1F), −153.1 – – 153.2 (m, 2F), −158.0 (t, J = 24 Hz, 1F), −162.6 – – 162.7 (m, 2F). MS (ESI): m/z 660.1 [M+H$^+$].

Succinic acid 4-(5-carboxy-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalen-9-yl)-piperazine-1-carbonyloxymethyl ester pentafluorophenyl ester 34

R_f 0.70 (CH_2Cl_2–EtOH 9 : 1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.57 (s, 1H), 7.67 (d, J = 12 Hz, 1H), 5.76 (s, 2H), 4.53–4.49 (m, 1H), 4.40 (ddd, J = 25, 11, 2 Hz, 2H), 3.66 (s, 4H), 3.32 (br s, 4H), 2.60–2.45 (m, 4H), 1.60 (d, J = 4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.4, 175.8, 169.1, 165.4, 151.7, 143.7, 138.5, 135.1, 131.1, 123.4, 121.1, 117.5, 106.4, 104.2, 83.6, 81.6, 71.1, 68.5, 55.7, 50.2, 45.3, 44.6, 44.0, 39.1, 34.3, 29.8, 26.1, 18.5. ^{19}F NMR ($CDCl_3$, 400 MHz): δ −124.2 (s, 1F), −153.1 – – 153.2 (m, 2F), −160.0 (t, J = 24 Hz, 1F), −164.2 – – 164.3 (m, 2F). MS (ESI): m/z 703.1 [M+H$^+$]

Synthesis of 3-(3-amino-propyl)-2′-(2-hydroxy-phenyl)-2,3,4,5,4′,5′-hexahydro-[2,4′]bithiazolyl-4-carboxylic acid 9

Pyochelin analog **4** (148 mg, 0.31 mmol) was dissolved in a mixture of CH_2Cl_2–TFA 8 : 2 (3 mL) and stirred 4 h at 20 °C. The mixture was evaporated to dryness under reduced pressure and then dissolved in CH_2Cl_2. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The resulting yellow oil was triturated with Et_2O to give amine **9** (116 mg, 0.31 mmol, 100%) as a yellow solid. R_f 0.16 (CH_2Cl_2–EtOH 9 : 1). ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ 7.40–7.27 (m, 2H), 6.99–6.79 (m, 2H), 5.05–4.99 (m, 1H), 4.82–4.77 (m, 3H), 4.32–4.13 (m, 2H), 3.65–3.58 (m, 1H), 3.54–3.07 (m, 4H), 1.71–1.62 (m, 2H). ^{13}C NMR (100 MHz, $(CD_3)_2CO$): δ 173.7, 159.1, 156.2, 155.6, 133.7, 130.8, 119.4, 117.4, 72.2, 66.6, 59.9, 36.7, 34.9, 32.2, 29.9. MS (ESI): m/z 368.1 [M+H$^+$].

General procedure for the synthesis of pyochelin–fluoroquinolone conjugates 35 to 40

Functionalized pyochelin **9** (1.00 mmol) and DIPEA (2.00 mmol) were successively added to a solution of pentafluorophenyl ester **14**, **15**, **21**, **32**, **33** or **34** (1.40 mmol) in CH_2Cl_2 (15 mL). The solution was stirred at 20 °C for 12 h. The mixture was washed with 0.5 N HCl, water and brine. The organic layer was collected, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by chromatography on a silica gel column (CH_2Cl_2–EtOH 99 : 1 to 98 : 2), leading to the expected pyochelin–fluoroquinolone conjugates **35** (429 mg, 0.55 mmol, 55%), **36** (333 mg, 0.39 mmol, 39%), **37** (576 mg, 0.75 mmol, 75%), **38** (522 mg, 0.62 mmol, 62%), **39** (494 mg, 0.62 mmol, 62%) and **40** (861 mg, 0.99 mmol, 99%).

7-[4-(3-{3-[4-Carboxy-2′-(2-hydroxy-phenyl)-4,5,4′,5′-tetrahydro-[2,4′]bithiazolyl-3-yl]-propylcarbamoyl}-propionyl)-piperazin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 35

R_f 0.76 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.73 (d, J = 4 Hz, 1H), 8.01 (d, J = 13 Hz, 1H), 7.37–7.30 (m, 3H), 6.93 (d, J = 8 Hz, 1H), 6.83 (br t, J = 8 Hz, 1H), 6.24 (br s, 1H), 4.83–4.77 (m, 1H), 4.25–4.12 (m, 3H), 3.82 (br s, 3H), 3.72 (br s, 3H), 3.35–3.27 (m, 9H), 2.70 (t, J = 6 Hz, 2H), 2.52 (t, J = 6 Hz, 2H), 1.92–1.84 (m, 3H), 1.40–1.31 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 186.8, 177.3, 172.6, 170.7, 167.1, 159.4, 156.8, 151.5, 147.8, 144.2, 138.9, 133.5, 130.8, 119.1, 117.3, 112.9, 108.6, 105.4, 81.7, 71.3, 71.3, 64.8, 64.5, 63.4, 50.1, 45.3, 41.6, 41.0, 37.7, 35.5, 34.5, 31.3, 29.9, 28.7, 22.9, 14.3, 8.51. MS (ESI): m/z 781.2 [M+H$^+$]. HRMS (ESI): $C_{37}H_{41}FN_6O_8S_2$: calcd 780.24113, found 780.24057.

7-[4-(3-{3-[4-Carboxy-2′-(2-hydroxy-phenyl)-4,5,4′,5′-tetrahydro-[2,4′]bithiazolyl-3-yl]-propylcarbamoyl}-propionyloxymethoxy-carbonyl)-piperazin-1-yl]-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 36

R_f 0.49 (CH_2Cl_2–EtOH 98 : 2). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.66 (s, 1H), 7.92 (d, J = 13 Hz, 1H), 7.36–7.24 (m, 3H), 6.93–6.81 (m, 2H), 6.13–6.07 (m, 1H), 5.78 (s, 2H), 4.80–4.75 (m, 1H), 4.24–4.09 (m, 3H), 3.71 (br s, 4H), 3.55–3.42 (m, 4H), 3.35–3.26 (m, 7H), 2.72–2.67 (m, 3H), 2.47 (q, J = 7 Hz, 2H), 1.88–1.82 (m, 2H), 1.37–1.25 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.1, 173.5, 172.1, 171.4, 167.1, 159.2, 153.6, 152.4, 147.7, 145.3, 139.1, 133.5, 130.8, 120.2, 119.1, 116.2, 112.6, 108.2, 105.4, 81.6, 80.8, 71.3, 64.5, 63.3, 49.8, 49.6, 44.1, 43.7, 37.9, 36.4, 35.5, 34.7, 30.8, 29.9, 29.6, 28.8, 27.1, 8.44. MS (ESI): m/z 855.2 [M+H$^+$]. HRMS (ESI): $C_{39}H_{43}FN_6O_{11}S_2$: calcd 854.24153, found 854.24150.

7-[4-(3-{3-[4-Carboxy-2′-(2-hydroxy-phenyl)-4,5,4′,5′-tetrahydro-[2,4′]bithiazolyl-3-yl]-propylcarbamoyl}-propionyl)-piperazin-1-yl]-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 37

R_f 0.68 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.01 (d, J = 3 Hz, 1H), 8.39 (d, J = 13 Hz, 1H), 7.76–7.68 (m, 1H), 7.26 (d, J = 8 Hz, 1H), 7.23 (d, J = 7 Hz, 2H), 6.98–6.93 (m, 1H), 5.21–5.17 (m, 1H), 4.75–4.59 (m, 4H), 4.22 (br s, 2H), 4.13 (br s, 2H), 3.75–3.68 (m, 8H), 3.15–3.09 (m, 3H), 2.975–2.93 (m, 3H), 2.33–2.25 (m, 2H), 1.98–1.93 (m, 3H), 1.65 (br s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.9, 172.6, 171.2, 170.7, 167.2, 159.1, 154.7, 152.3, 147.3, 145.7, 137.2, 133.5, 130.7, 120.8, 119.1, 117.2, 116.1, 112.9, 109.3, 104.4, 81.7, 72.3, 71.3, 65.6, 64.4, 63.3, 49.9, 45.2, 41.6, 41.0, 37.9, 36.1, 34.7, 31.3, 26.6, 27.0, 14.6. MS (ESI): m/z 769.2 [M+H$^+$]. HRMS (ESI): $C_{36}H_{41}FN_6O_8S_2$: calcd 768, 24113 found 768.24101.

7-[4-(3-{3-[4-Carboxy-2′-(2-hydroxy-phenyl)-4,5,4′,5′-tetrahydro-[2,4′]bithiazolyl-3-yl]-propylcarbamoyl}-propionyloxymethoxy-carbonyl)-piperazin-1-yl]-1-ethyl-6-fluoro-4-oxo-1,4-dihydro-quinoline-3-carboxylic acid 38

R_f 0.69 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.64 (s, 1H), 8.05 (d, J = 13 Hz, 1H), 7.38–7.30 (m, 2H), 6.94 (d, J = 8 Hz, 1H), 6.87–6.82 (m, 2H), 5.98 (t, J = 6 Hz, 1H), 5.79 (s, 2H), 4.83–4.78 (m, 1H), 4.30–4.14 (m, 3H), 3.71 (br s, 4H), 3.35–3.26 (m, 8H), 2.71 (q, J = 6 Hz, 2H), 2.47 (q, J = 6, 2H), 2.46 (q, J = 7 Hz, 2H), 1.89–1.84 (m, 3H), 1.54 (dd, J = 7, 2 Hz, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.1, 173.5, 172.1, 171.4, 167.3, 159.3, 154.4, 153.6, 152.4, 147.4, 145.3, 137.2, 133.6, 130.8, 120.4, 119.4, 118.6, 117.2, 113.5, 108.6, 104.5, 81.6, 80.8, 71.2, 63.3, 57.7, 50.0, 44.1, 43.8, 37.6, 36.4, 35.5, 34.7, 30.8, 29.9, 29.6, 28.8, 27.1, 14.7. MS (ESI): m/z 843.2 [M+H$^+$]. HRMS (ESI): $C_{38}H_{43}FN_6O_{11}S_2$: calcd 842.24153, found 842.24162.

9-[4-(3-{3-[4-Carboxy-2′-(2-hydroxy-phenyl)-4,5,4′,5′-tetrahydro-[2,4′]bithiazolyl-3-yl]-propylcarbamoyl}-propionyl)-piperazin-1-yl]-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalene-5-carboxylic acid 39

R_f 0.71 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.59 (s, 1H), 7.73 (d, J = 12 Hz, 1H), 7.38–7.31 (m, 2H), 6.98–6.94 (m, 1H), 6.85 (t, J = 8 Hz, 1H), 6.33–6.30 (m, 1H), 4.34–4.12 (m, 4H), 3.78–3.54 (m, 8H), 3.36–3.29 (m, 8H), 2.70 (dd, J = 12, 6 Hz, 2H), 2.53 (t, J = 6 Hz, 2H), 1.59 (d, J = 7 Hz, 3H), 1.46–1.38 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.3, 175.9, 172.7, 170.6, 167.5, 159.3, 152.6, 151.0, 147.7, 142.0, 137.7, 133.5, 130.7, 123.7, 119.8, 117.4, 115.5, 112.3, 109.8, 105.8, 73.6, 71.2, 64.4, 63.3, 52.3, 50.7, 49.2, 45.5, 43.6, 41.0, 37.9, 36.1, 34.7, 31.3, 29.9, 28.7, 18.86. MS (ESI): m/z 797.2 [M+H$^+$]. HRMS (ESI): $C_{37}H_{41}FN_6O_9S_2$: calcd 796.32605, found 796.32549.

9-[4-(3-{3-[4-Carboxy-2′-(2-hydroxy-phenyl)-4,5,4′,5′-tetrahydro-[2,4′]bithiazolyl-3-yl]-propylcarbamoyl}-propionyloxymethoxy-carbonyl)-piperazin-1-yl]-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6*H*-1-oxa-3a-aza-phenalene-5-carboxylic acid 40

R_f 0.62 (CH_2Cl_2–EtOH 95 : 5). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.62 (s, 1H), 7.71–7.66 (m, 1H), 7.37–7.32 (m, 2H), 6.96–6.82 (m, 2H), 5.82 (s, 2H), 4.75–4.70 (m, 1H), 4.39–4.25 (m, 4H), 3.58–3.55 (m, 8H), 3.37–3.22 (m, 8H), 2.70 (dd, J = 12, 6 Hz, 2H), 2.53 (t, J = 6 Hz, 2H), 1.59 (d, J = 7 Hz, 3H), 1.46–1.38 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.3, 175.9, 172.7, 170.6, 167.5, 159.3, 152.6, 151.0, 147.7, 142.0, 137.7, 133.5, 130.7, 123.7, 119.8, 117.4, 115.5, 112.3, 109.8, 105.8, 81.6, 73.6, 71.2, 64.4, 63.3, 52.3, 50.7, 49.2, 45.5, 43.6, 41.0, 37.9, 36.1, 34.7, 31.3, 29.9, 28.7, 18.86. MS (ESI): m/z 871.2 [M+H$^+$]. HRMS (ESI): $C_{39}H_{43}FN_6O_{12}S_2$: calcd 870.23644, found 870.23456.

Pseudomonas aeruginosa strains

Wild type *P. aeruginosa* strain PAO1,[48] the pyochelin- and pyoverdin-deficient *P. aeruginosa* strain PAD07,[49] and the TonB mutant and pyoverdin deficient strain PAD14 of *P. aeruginosa*,[50] have been described previously.

Culture media and growth conditions

The strains were grown overnight in succinate medium (composition in g L^{-1} was: K_2HPO_4, 6.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; sodium succinate, 4.0 and the pH was adjusted to 7.0 by addition of NaOH) at 30 °C.[51] When necessary, media were supplemented with the relevant antibiotics at the following concentrations: tetracyclin hydrochloride 50 μg mL^{-1} (PAD07, PAD14) and streptomycin sulfate 100 μg mL^{-1} (PAD07) or 500 μg mL^{-1} (PAD14).

Ligand-binding assays using ^{55}Fe

In vivo binding affinity constants (K_i) of ferric-pyochelin analogues to FptA were determined according to the following procedure: PAD07 cells were washed twice with an equal volume of fresh medium and resuspended in 50 mM Tris-HCl (pH 8.0) buffer at an OD_{600} of 0.3. The cells were then incubated for 1 h at 0 °C in the presence of 200 μM CCCP to avoid iron uptake[52] in a final volume of 500 μL with 1 nM of pyochelin–^{55}Fe(III) and various concentrations of unlabelled iron-loaded pyochelin or pyochelin analog (0 to 1 mM). The mixtures were then centrifuged at 12 000 g for 3 min and the supernatants containing the unbound siderophore (labelled or not labelled) were removed. The tubes containing the cell pellet were counted for radioactivity in scintillation cocktail. Binding affinity constants (K_i) of the siderophores were calculated from the IC_{50} values, which were determined in competition experiments, according to the equation of Cheng and Prusoff[53]: $K_i = IC_{50}/(1 + L/K_d)$ where L is the concentration of radiolabelled ligand and K_d is its equilibrium dissociation constant determined experimentally. The K_d value of pyochelin–Fe(III) for FptA is 0.54 ± 0.19 nM, as determined previously.[41]

Iron uptake experiments

Iron uptake assays were carried out as previously reported for the FptA/pyochelin system.[39] After an overnight culture (20 h), bacteria were prepared in 50 mM Tris-HCl (pH 8.0) at OD_{600} of 1 for *P. aeruginosa*, and incubated at 37 °C. Transport assays were started by adding 100 nM of pyochelin–^{55}Fe(III). Pyochelin–^{55}Fe(III) and other siderophore–^{55}Fe(III) complexes were prepared at concentrations of 10 μM of ^{55}Fe(III) with a siderophore : iron (mol : mol) ratio of 20 : 1. The solutions were prepared using a

4.4 mM solution of pyochelin (in methanol). To 45 µL of this solution were added 40 µL of a solution of $^{55}FeCl_3$ (250 microM, 9.4 Ci g^{-1} in HCl 0.5 N), obtained by dilution of the stock solution, plus 973 µL of 50 mM TrisHCl pH 8.0. To separate siderophore–^{55}Fe transported into *P. aeruginosa* cells from unbound siderophore–^{55}Fe, aliquots (100 µL) of the suspensions were removed at different times and 200 µL of ice cold Tris buffer was added and centrifuged (13 000 g for 3 min). The supernatants containing the unbound siderophore were removed and the tubes containing the cell pellets were counted for radioactivity in scintillation cocktail. As controls, the experiments were repeated with cells incubated at 0 °C in the presence of 200 µM CCCP and in the absence of cells.

Antimicrobial susceptibility assay

Evaluation of the conjugates activities was carried out in succinate medium by the two-fold serial dilution method with an inoculum of 5×10^5 cells mL^{-1}. Data were reported as MIC_{50}s, which reflects the lowest concentration of antibiotic that reduces by more than 50% visible cell growth after an 18 h incubation at 30 °C.

Ligand docking

The starting conformation of pyochelin **1** and compound **4** was obtained by converting IsisDraw (Accelrys, Inc. San Diego, CA 92121, USA) 2-D sketches into 3-D coordinates using the Corina 3.10 program (Molecular Networks GmbH, D-91052 Erlangen, Germany). Automated docking of manually ionised ligands to the X-ray FptA structure (PDB entry 1xkw) was done using 7–8 times speed-up settings of Goldv3.1 (Cambridge Crystallographic Data Centre, Cambridge, CB2 1EZ, UK.).[54] All organic molecules (water, LDA, sulfate, ethylene glycol, ferric-pyochelin) were first removed from the protein structure and all hydrogen atoms automatically added using the Biopolymer module of the SYBYL package (TRIPOS, Inc., St-Louis, MO.63144-2917, USA). The active site used for sampling the conformational space of the ligand was defined by a 12.5 Å-radius sphere centered on the center of mass of ferric-pyochelin bound to FptA. To further speed up the calculation, docking was stopped when the top three-ranked solutions were within 1.5 Å rmsd. If this criterion is met, we can assume that these top solutions represent a reproducible pose for the ligand. A maximum of 10 poses were saved for each ligand in standard mol2 format (TRIPOS, Inc., St-Louis, MO.63144-2917, USA).

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