Synthesis and Activities of Pyoverdin−Quinolone Adducts: A Prospective Approach to a Specific Therapy Against *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is particularly resistant to most all the antibiotics presently available, essentially because of the very low permeability of its outer membrane. To overcome this, we synthesized four siderophore-based antibiotics formed by two quinolones − norfloxacin and benzonaphthyridone − bound to the pyoverdin of *P. aeruginosa* ATCC 15692 via two types of spacer arms: one stable and the other readily hydrolyzable. From the comparison of their antibacterial properties with those of the two unbound quinolones, we reached the following conclusions: (a) The adducts inhibit *Escherichia coli*'s gyrase showing that the dissociation of the compounds is not necessary for their activity. However, the presence of the pyoverdin moiety on the molecule decreases the inhibition activity compared to the antibiotic alone. (b) They facilitate the uptake of ^{55}Fe using the specific pyoverdin-mediated iron-transport system of the bacterium. No uptake was observed either with *P. aeruginosa* ATCC 27853, which produces a structurally different pyoverdin, or with *P. aeruginosa* K690, which is a mutant of *P. aeruginosa* ATCC 15692 lacking FpvA, the outer-membrane pyoverdin receptor. (c) MIC determinations have shown that only strains *P. aeruginosa* ATCC 15692 and the derived outer-membrane receptor-producing but pyoverdin-deficient *P. aeruginosa* IA1 mutant present higher susceptibility to the pyoverdin−quinolone adducts, whereas *P. aeruginosa* ATCC 27853 and K690 are much more resistant. (d) Growth inhibition by these adducts confirmed these results and showed that the adducts with the hydrolyzable spacer arm have better activity than those with the stable one and that the labile spacer arm adducts present much higher activity than the quinolones alone. These results show clearly that the penetration of the antibiotic into the cells is favored when this latter is coupled with pyoverdin: Only the strains possessing the appropriate outer-membrane receptor present higher susceptibility to the adduct. In this case the antibiotic uses the pyoverdin-mediated iron-transport system. Furthermore, better efficiency is obtained when the spacer arm is labile and favors the antibiotic release inside the cell, allowing better inhibition of gyrase.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogenic Gram-negative bacterium, infecting primarily cystic fibrosis patients[1] or patients having a weakened immune system, such as those highly burnt and, more recently, those having AIDS at an advanced level.[2] Among these, the number of people infected by this bacterium has more than tripled between 1992 and 1994.[3] The predominance of this bacterium, particularly in hospitals, is due (a) to its high resistance against most of the existing antibiotics, (b) to its ability to mutate readily and acquire new resistances, and (c) to its particularly low outer-membrane permeability.

In Gram-negative bacteria, the outer membrane is the main barrier encountered by antibiotics especially quinolones for which the target is DNA gyrase, located in the cytoplasm. The exclusion barrier of the outer membrane of *P. aeruginosa* is in the order of 350−400 Da.[4,5] This explains the very high resistance of the bacterium against most of the antibiotics known so far.

It was shown that catechol-containing β-lactams are transported by specific proteins, Fiu and Cir, coded by a *tonB*-dependent iron-transport locus.[6,7] To facilitate the penetration of quinolones into the bacterial cells and improve the access of the antibiotics to their target, we designed adduct molecules possessing a quinolone moiety bound to pyoverdin, the main siderophore of *P. aeruginosa*, via a spacer arm. These adduct molecules should be able to cross membranes which the antibiotic alone cannot normally cross, using the pyoverdin-mediated iron-transport system of the bacteria, and reach their target.

As targets, the quinolones have two type II topoisomerases: DNA gyrase and DNA topoisomerase IV, both required for cell growth and division. These enzymes are composed of four subunits: the products of the *gyrA* and *gyrB* genes for gyrase, *parC* and *parE* for topoisomerase IV. Both use a double-strand passage mode of action.[8] The primary target of the quinolones

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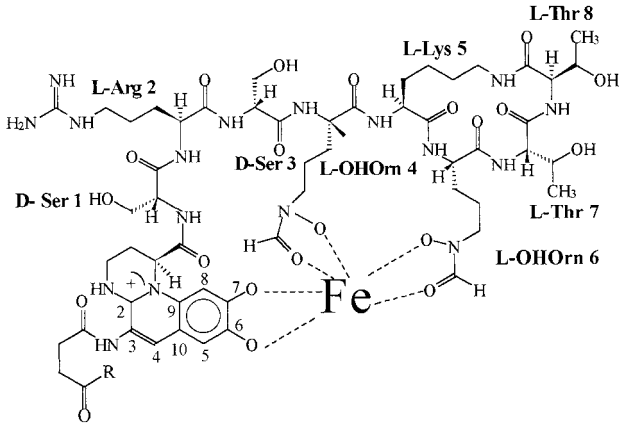


Figure 1. Structures of PaA (**1a**, R = OH) and PaA-NH_2 (**1b**, R = NH-CH_2-CH_2-NH_2) as their iron(III) complexes.

depends on the bacteria and seems to be gyrase in most Gram-negative microorganisms and topoisomerase IV in *Staphylococcus aureus* and *Streptococcus pneumoniae*. The binding of quinolones to gyrase−DNA complexes is still not completely understood. However, the role of Mg^{2+} appears to be essential.[9−11]

The wild-type strain of *P. aeruginosa* we used here is *P. aeruginosa* ATCC 15692 which is being extensively studied and for which pyoverdin-defective or pyoverdin outer-membrane receptor-defective mutants are available. As pyoverdin, we used a derivative of PaA (**1a**), namely aminopyoverdin (**1b**, PaA-NH_2)[12] (Figure 1), to carry quinolones through the outer membrane of *P. aeruginosa* in order to reach the gyrase.

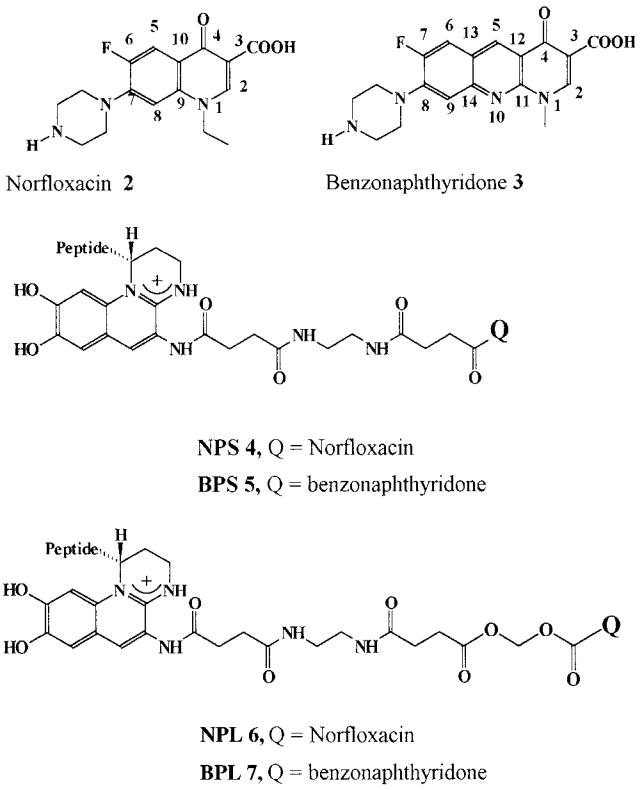
In this paper we describe the synthesis and biological activities of siderophore-based antibiotics formed by two quinolones bound to the pyoverdin of *P. aeruginosa* ATCC 15692 by means of two types of spacer arms and compare the activities of these adducts with that of gyrase. We also compare their MICs with those of the unbound quinolones in the case of the wild-type strain, the mutants deficient in the receptors involved in iron transport, and the standard reference strain ATCC 27853.

Results and Discussion

Synthesis of the Pyoverdin−Quinolone Adducts. (a) Choice of Antibiotics and Their Binding with Pyoverdin. The target of the quinolones being topoisomerases, the antibiotics must reach the cytoplasm in order to be active. Thus improving the access of the cell to the adduct should yield better antibiotic activity. Norfloxacin (**2**) usually shows good activity against *P. aeruginosa*, but its use remains limited to urinary infections. Compound **3**, on the contrary, shows weak biological activity against *P. aeruginosa*, although inhibiting the corresponding gyrase. Its weak activity is probably due to a limited penetration of the bacterial cell. If the transport of this compound through the bacterial membrane could be facilitated using a pyoverdin adduct, one can expect better biological activity.

The choice of the binding site for the siderophore must not modify the sites which are essential for the activity of the antibiotic moiety. For this reason we have performed coupling of the pyoverdin moiety on the secondary amine function of molecules **2** and **3**, at positions C-7 of norfloxacin and C-8 of benzonaphthyridone, respectively. The use of a spacer arm linking the quinolone moieties to the pyoverdin can facilitate the coupling reactions. Moreover, to form an amide bond between PaA-NH_2 (**1b**) and the antibiotic, it was necessary to modify the quinolones and introduce a carboxylic acid function on the spacer arm.

Chart 1



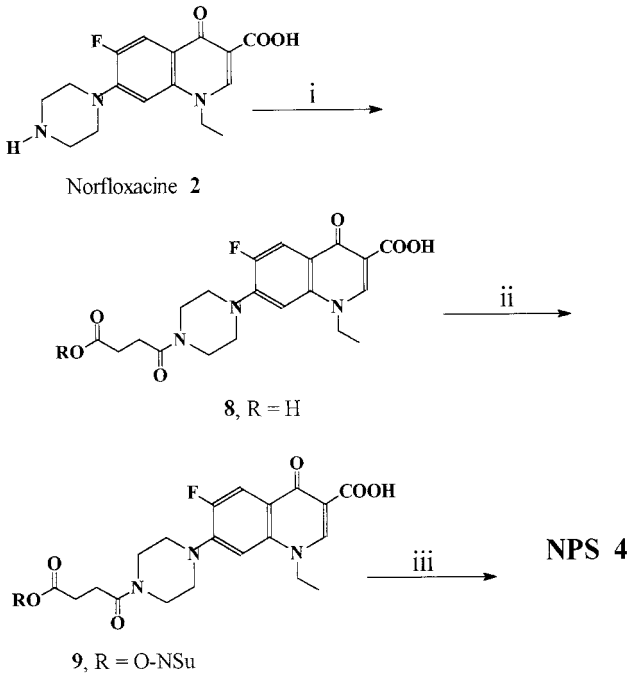
For each antibiotic we have synthesized adducts possessing spacer arms either with the fairly stable amide bonds (compounds **4** and **5**) or bearing a labile methylenedioxy function which could undergo hydrolysis catalyzed by a nonspecific esterase (compounds **6** and **7**) (Chart 1). In these two latter cases norfloxacin and benzonaphthyridone would be released after hydrolysis, and steric hindrance due to the presence of the pyoverdin moiety would be prevented during the antibiotic interaction with gyrase.

For convenience, the following abbreviations were used to define the different pyoverdin−quinolone adducts: norfloxacin−pyoverdin adduct with a stable spacer arm, NPS (**4**); benzonaphthyridone−pyoverdin with a stable spacer arm, BPS (**5**); norfloxacin−pyoverdin adduct with a labile spacer arm, NPL (**6**); and benzonaphthyridone−pyoverdin with a labile spacer arm, BPL (**7**).

(b) Synthesis of the Norfloxacin−Pyoverdin Adduct with a Stable Spacer Arm: 4. The synthesis of NPS (**4**) was performed in three steps starting from norfloxacin (Scheme 1). The quinolone was reacted with succinic anhydride in DMSO in the presence of pyridine to yield compound **8** (87%), which was treated with dicyclohexylcarbodiimide in the presence of *N*-hydroxysuccinimide to yield the activated ester **9**. The reaction of the pyoverdin derivative **1b** with this latter was achieved in situ giving NPS (**4**) with 62% yield.

(c) Synthesis of the Benzonaphthyridone−Pyoverdin Adduct with a Stable Spacer Arm: 5.

Scheme 1. Synthesis of NPS (**4**), the Norfloxacin−Pyoverdin Adduct with a Stable Spacer Arm[a]



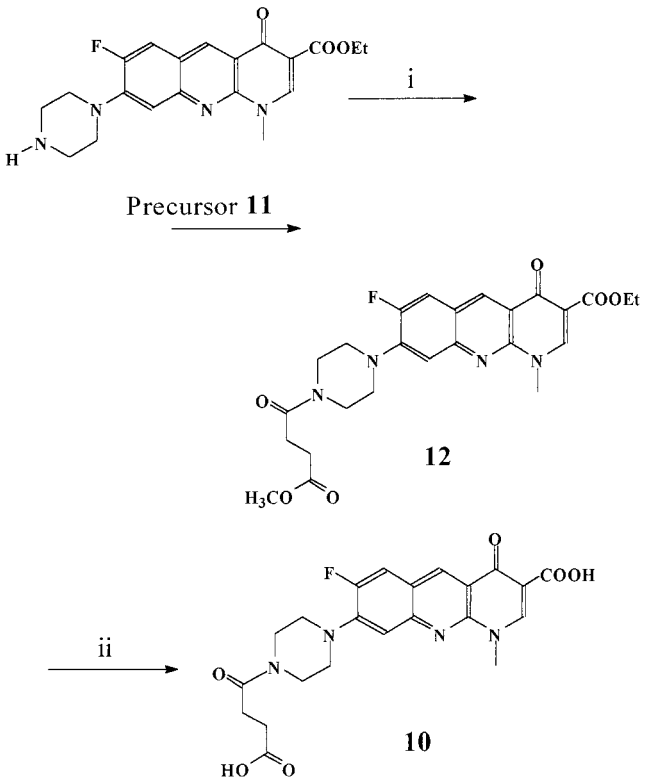
[a] (i) Succinic anhydride, anhyd pyridine, 5 h under Ar at 95 °C in DMSO; (ii) *N*-hydroxysuccinimide, DCC, 1 h at rt in DMF; (iii) Pa-NH_2, 2 h at rt.

Similarly, benzonaphthyridone (**3**) was reacted with succinic anhydride to yield 79% compound **10**. However another approach with one more step giving compounds easier to handle was devised via the methyl succinate ester (Scheme 2). Precursor **11** of benzonaphthyridone (**3**) was reacted with methyl hemisuccinate acid chloride, in the presence of pyridine at room temperature, yielding 47% of compound **12**. Diacid **10** was prepared with 74% yield after saponification of both ester functions by aqueous sodium hydroxide at room temperature.

Because PaA-NH_2 (**1b**; Figure 1) failed to react with the activated ester derivative of **10**, the method of mixed anhydrides proved successful (46% yield). The reaction was performed in two steps with the formation of mixed anhydride **13** obtained by coupling isobutyl chloroformate with acid **10**, followed by nucleophilic attack of mixed anhydride **13** with PaA-NH_2 (**1b**). No bis-coupling products were isolated.

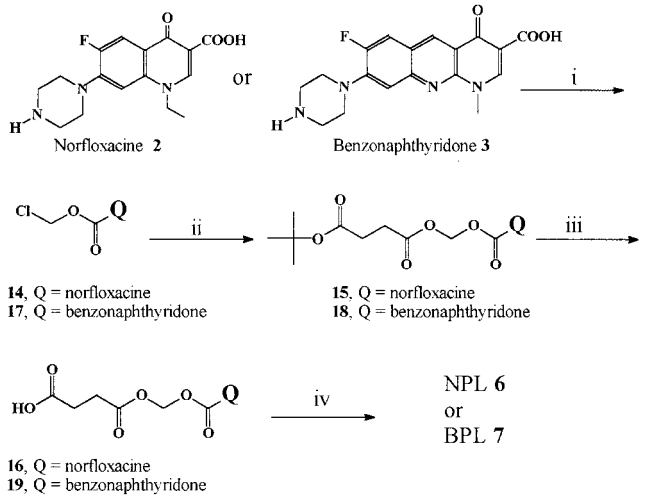
(d) Synthesis of the Norfloxacin−Pyoverdin Adduct with a Labile Spacer Arm: 6. This labile spacer arm was built up with a succinate group and a methylenedioxy function. To prepare adduct **6**, a new approach shown in Scheme 3 was devised. Norfloxacin (**2**) was reacted with chloromethyl chloroformate in the presence of 1,8-bis(*N*,*N*-dimethylamino)naphthalene at room temperature to yield 70% of compound **14**. This in turn was reacted with the carboxylate of *tert*-butyl hemisuccinate at 95 °C in DMF and yielded 58% of *tert*-butyl ester **15**. This latter was deprotected with TFA and yielded the corresponding acid **16** which could be equally coupled with PaA-NH_2 (**1b**) either by the activated ester method or by the mixed anhydride method. In both cases, NPL (**6**) was obtained with 36% yield for the last step.

Scheme 2. Synthesis of the Benzonaphthyridone Derivative **10**[a]



[a] (i) Methyloxysuccinyl chloride, pyridine, 48 h under Ar at rt in chloroform; (ii) aq sodium hydroxide, 8 h at rt in THF.

Scheme 3. Synthesis of NPL (**6**) and BPL (**7**), Norfloxacine−Pyoverdin and Benzonaphthyridone−Pyoverdin Adducts with a Labile Spacer Arm[a]



[a] (i) Chloromethyl chloroformate, 1,8-bis(*N*,*N*-dimethylamino)-naphthalene, 48 h under Ar at rt in chloroform/methylene chloride; (ii) *tert*-butyl hemisuccinate, 1,8-bis(*N*,*N*-dimethylamino)naphthalene, 24 h under Ar at 95 °C in anhyd DMF; (iii) trifluoroacetic acid, 2 h at rt in dichloromethane; (iv) isobutyl chloroformate, *N*-methylpiperidine, Pa-NH_2, 24 h at rt in anhyd DMF.

(e) Synthesis of the Benzonaphthyridone−Pyoverdin Adduct with a Labile Spacer Arm: 7. BPL (**7**) was synthesized similarly to NPL (**6**; Scheme 3). The chloro compound **17** was obtained with 77% yield. It was reacted with *tert*-butyl hemisuccinate to give the corresponding succinic ester **18** (84% yield) which was

Table 1. Values for MIC and Inhibition of Supercoiling[a]

	P. aeruginosa ATCC 15692		*P. aeruginosa* IA1		*P. aeruginosa* K690		*P. aeruginosa* ATCC 27853		*E. coli* gyrase
compd	MH	SC	MH	SC	MH	SC	MH	SC	MED
norfloxacin, **2**	0.25	0.06	1	0.03	8	4	2	0.25	1
NPS, **4**	8	2	32	4	32	8	>64	8	50
NPL, **6**	2	2	4	2	16	16	16	8	50
benzonaphthyridone, **3**	8	4	16	16	32	16	16	8	4
BPS, **5**	16	8	32	8	64	4	>64	8	25
BPL, **7**	8	1	16	1	32	8	64	4	25

[a] Tests performed in solid media on gelose prepared either in Müller-Hinton (MH) or in succinate (SC) media. MIC values are expressed in μg/mL equivalents of quinolone.

deprotected by TFA to yield acid **19**. However, the coupling of PaA-NH_2 (**1b**) to this acid was only possible when the mixed anhydride method was used and yielded BPL (**7**) in 40% yield.

Characterization of the Pyoverdin−Quinolone Adducts. (a) Purification of the Adducts. In the course of the reactions leading to the pyoverdin−quinolone adducts, PaA-NH_2 (**1b**) has been used as its iron(III) complex. The protection of the groups involved in the chelation of the metal could be thus ensured, the other amino acids forming the peptide chain remaining unmodified in the reaction conditions. Since the adducts were obtained as iron(III) complexes, their solubilities were much lower than the solubility of the native pyoverdin. Much higher ionic strength buffers were required to ensure good solubilization of these adduct complexes. Since the free ligands are more soluble than the corresponding iron(III) complexes, the adducts were first decomplexed with EDTA by a procedure devised in the laboratory:[13] Under these conditions ferric EDTA and any remaining salts could be readily removed using reverse-phase chromatography, and purity of the compounds could be checked by cellulose acetate film electrophoresis where each adduct gave one single spot.

(b) Characterization. ES-MS of the adducts as free ligands confirmed their structure: a quinolone bound to PaA-NH_2 (**1b**), without unexpected modification of the siderophore moiety.

Two-dimensional mono- and heteronuclear NMR spectroscopy confirmed that the coupling of pyoverdin to the quinolones was performed via the carboxylic acid moiety of succinate (as expected) and not the carboxylate group of the quinolone ring which is essential for antibiotic activity. The latter could actually not have reacted in the course of the coupling reactions, because of the high value of its pK_a due to the stability of the hydrogen bond formed between the carboxylic acid in position 3 and the carbonyl in position 4 of the quinolone ring(s).

ROESY homonuclear two-dimensional spectra gave evidence for a correlation between the methylene group in the vicinity of the carbonyl of the succinyl moiety and the proton of the amide bond newly formed in the adduct. Similarly the methylene group close to the nitrogen atom showed a $^1H-^{13}C$ long-distance correlation with the carbonyl of the succinyl moiety

(Figure 2). Besides no correlation involving the carboxyl function at position 3 of the quinolone ring was observed, and the presence of the signal characteristic of this group in the ^{13}C NMR spectra confirmed that it was not affected in the coupling.

(c) Differences in the Reactivity Between Norfloxacin and Benzonaphthyridone. As previously

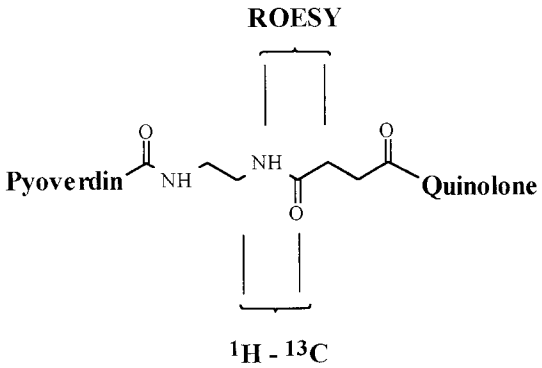


Figure 2. Two-dimensional homonuclear and heteronuclear correlations observed on the coupling moieties between PaA-NH_2 (**1b**) and the quinolones.

mentioned, the coupling reactions between norfloxacin derivatives **8** and **16** and PaA-NH_2 (**1b**) were equally possible either using the activated ester method or the mixed anhydrides method, but the former was preferred for greater convenience.

In the case of benzonaphthyridones **10** and **19**, all attempts to perform the coupling by the activated ester method failed, and the use of other carbodiimides such as the water-soluble ECDI or other compounds such as *N*-hydroxybenzotriazole were unsuccessful. Chlorination of the succinic acid residue into the corresponding acid chloride with thionyl chloride or oxalyl chloride was also attempted, but with no success as well.

Inhibition of Gyrase. To study the properties of the quinolone−pyoverdin adducts on the antibiotic target, inhibition tests against *Escherichia coli* gyrase were performed. Gyrase was chosen, since it is the target of quinolones in both *E. coli* and *P. aeruginosa*. This gyrase is considered a good model of the *P. aeruginosa* gyrase since (a) it presents high homology with this latter and (b) it has the advantage of being more readily available. The test we used in this study was inhibition of the negative supercoiling of the relaxed DNA.[14]

In the presence of circular double-stranded relaxed DNA, gyrase induces negative supercoils which are not formed in the presence of a gyrase inhibitor. This can be visualized by gel electrophoresis where the relaxed DNA shows a shorter migration distance than the more compact supercoiled DNA. Results are shown in Table 1 and are expressed as minimum effective dose (MED), the minimum amount of drug required to cause any inhibition of activity.

Inhibition starts at concentrations of 25 μg/mL equivalents of benzonaphthyridone (see note in the Experimental Section) for BPS (**5**) and BPL (**7**). For NPS (**4**) and NPL (**6**), inhibition starts at 50 μg/mL equivalents

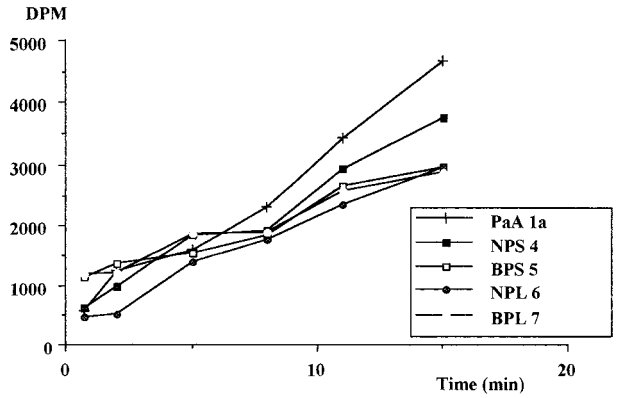


Figure 3. Incorporation of ^{55}Fe with time in *P. aeruginosa* ATCC 15692. Comparison between PaA (**1a**) and the pyoverdin−quinolone adducts **4**−**7**.

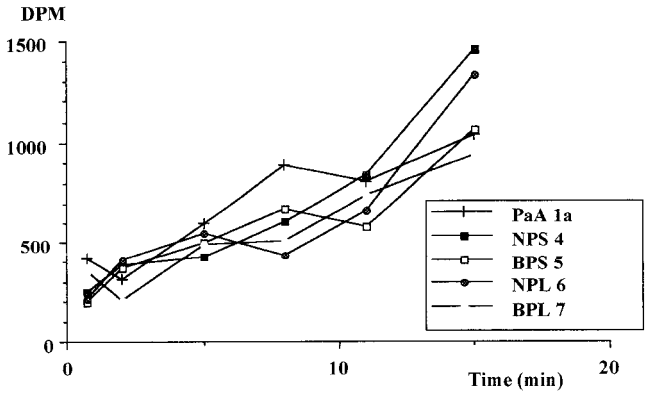


Figure 4. Incorporation of ^{55}Fe with time in *P. aeruginosa* IA1, a mutant strain unable to synthesize pyoverdin. Comparison between PaA (**1a**) and the pyoverdin−quinolone adducts **4**−**7**.

of norfloxacin. Norfloxacin (**2**) and benzonaphthyridone (**3**) alone inhibit gyrase respectively at concentrations of 1 and 4 μg/mL. Thus the pyoverdin−quinolone adducts are between 12.5 and 50 times less active than the antibiotic not bound to the siderophore. This may be due to steric hindrance caused by the presence of pyoverdin in the adducts.

Iron-Transport Mediated by the Pyoverdin−Quinolone Adducts: Results Obtained with Different Strains of *P. aeruginosa*. The transport assays with the pyoverdin−quinolone adducts **4**−**7** (respectively NPS, BPS, NPL, and BPL) on strain *P. aeruginosa* ATCC 15692 have shown that all the adducts transport iron into the bacteria cells with only slight differences (Figure 3). Similar results were obtained with strain IA1, a mutant of the wild-type strain *P. aeruginosa* ATCC 15692 unable to synthesize pyoverdin (Figure 4). This shows that the observed iron transport is not due to PaA neosynthesized by cells during the experiment. In the same conditions, the FpvA-deficient mutant strain K690, which does not produce the pyoverdin outer-membrane receptor, showed a total absence of incorporation of ^{55}Fe in the presence of the same adducts (data not shown) which demonstrates transport via this receptor. These experiments show that the ^{55}Fe incorporated is due to the siderophore-based adducts which use the FpvA receptor to transport iron like PaA (**1a**) and the adducts with quinolones and that this transport is specific.

The transport assays were also performed on strain *P. aeruginosa* ATCC 27853 which is used as a control strain for antibiotic assays. No incorporation of iron mediated by these adducts or by PaA (**1a**) was observed with this strain; only pyoverdin Pa ATCC 27853 was able to transport iron into the cells (data not shown). Conversely pyoverdin Pa ATCC 27853 was unable to transport iron into the cells of *P. aeruginosa* ATCC 15692. This result is in agreement with the results obtained by Cornelis et al.,[15] illustrating a high specificity in the recognition of pyoverdins by a given *Pseudomonas* strain, reflecting differences in the siderophore recognition sites of their outer-membrane receptors.

Growth Inhibition by the Pyoverdin−Quinolone Adducts. The antibiotic activity of a given compound with a given strain of bacterium can be assayed by growth inhibition tests, yielding the minimum inhibiting concentration (MIC, expressed in μg/mL). The MIC of an antibiotic is mostly determined either in solid media or in solution.

(a) Results in Solid Media. MIC determinations in solid media require a set of solutions at varying concentrations of the compounds to be tested which are incorporated in gelose containing the nutrient medium. The MIC of the compound tested against a given strain is the value of the lowest concentration of the compound tested to prevent the bacterial growth of that strain.

From the data presented in Table 1 it is clear that norfloxacin always shows lower MED and MIC values against gyrase than the corresponding adducts, both in iron-deficient succinate medium and in Müller-Hinton medium. The pyoverdin−norfloxacin adduct **4** shows lower MIC values in the iron-deficient medium than in Müller-Hinton medium; this holds, but less obviously for the adduct **6**.

For benzonaphthyridone and its adducts, the MIC values are lower in the iron-deficient medium than in Müller-Hinton medium, and furthermore, these MICs are lower than those of the free molecule in iron-deficient medium. This is more pronounced for strains ATCC 15692 and IA1, whereas strains K690 and ATCC 27853 were found to be less susceptible.

The pyoverdin−quinolone adducts **4**−**7** always showed lower MIC values in iron-deficient succinate media than in Müller-Hinton media, and strain ATCC 27853 was found to be more resistant than strain 15692 especially in Müller-Hinton medium. Norfloxacin (**2**), when *not bound* to pyoverdin, presented better activity than the adducts, but benzonaphthyridone (**3**), if more active than adduct **5**, has lower activity than the adduct BPL (**7**). Adducts **6** and **7** with a labile spacer arm showed better antibiotic activity than adducts **4** and **5** with a stable spacer arm. This difference was more pronounced for strains ATCC 15692 and IA1, whereas strains K690 and ATCC 27853 were found to be less susceptible (Table 2).

(b) Results in Liquid Media. The results in liquid media corroborated those in solid media confirming the lowest susceptibility of strain ATCC 27853 and showing in addition that norfloxacin (**2**) is more active than benzonaphthyridone (**3**) in solution and that the activity of this latter when bound to pyoverdin is improved against strains ATCC 15692 and IA1 (Table 2).

Table 2. Tests Performed in Liquid Media[a]

	P. aeruginosa ATCC 15692		*P. aeruginosa* IA1		*P. aeruginosa* ATCC 27853	
compd	MH	SC	MH	SC	MH	SC
norfloxacin, **2**	1	0,25	1	0.25	4	2
NPL, **6**	2	0.5	4	0.5	16	8
benzonaphthyridone, **3**	16	64	16	32	32	>64
BPL, **7**	4	2	8	2	32	32

[a] Tests performed in liquid media either in Müller-Hinton (MH) or in succinate (SC) media. MIC values are expressed in μg/mL equivalents of quinolone.

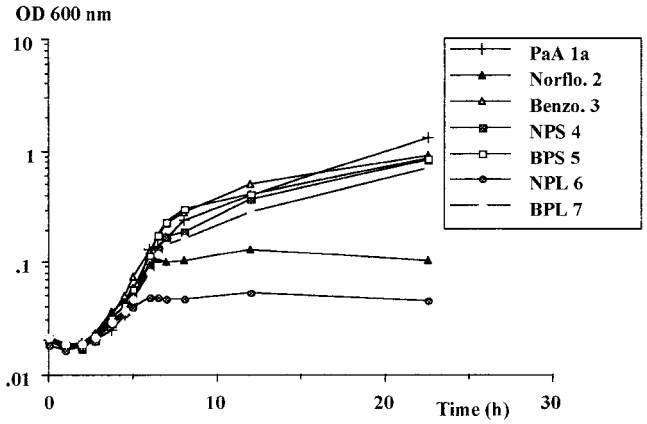


Figure 5. Growth of *P. aeruginosa* ATCC 15692 at 600 nm in the presence of added PaA (**1a**), norfloxacin (**2**), benzonaphthyridone (**3**), or pyoverdin−quinolone adducts **4**−**7**. The compounds tested were added at a concentration of 1 μM as free ligands (not bound to iron(III)).

Growth Kinetics in the Presence of the Pyoverdin−Quinolone Adducts. (a) Principles and Goals. MIC determinations in solid media may not be always as conclusive as in liquid media.This was already reported by Minnick et al.[7] who worked on other adducts involving a siderophore (a ferrichrome analogue) and β-lactams. The antibiotic activity of some of these compounds against several strains of *E. coli* susceptible to β-lactams could only be shown while monitoring the bacterial growth. The lag time was longer when the bacteria were cultivated in the presence of the antibiotic-bearing adducts, but after 20 h, the culture had reached the same growth level as the control without antibiotic (or adduct), and one single measurement performed after 18 or 24 h incubation did not give any information on the way how this growth took place. The kinetics of the bacterial growth reflected the differences related to the presence or absence of the antibiotic−siderophore adducts. The resumption of the bacterial growth was attributed to a selection of mutants, and in one case it could be shown that the mutation affected protein TonB involved in the transport of the ferrisiderophore used to carry the antibiotic into the bacterial cells.[7]

(b) Results. In the case of the *P. aeruginosa* ATCC 15692 strain, the growth curves did not show any lag time. Only for norfloxacin and its adduct NPL (**6**) could growth inhibition be observed (Figure 5). The activity of this labile adduct is much more pronounced than that of free norfloxacin or NPS (**4**).

The comparison of the bacterial growth kinetics in the presence of a 1:1 mixture of norfloxacin:pyoverdin and in the presence of the adduct NPL (**6**) showed that the observed inhibition was not due to any hydrolysis of the adduct in the medium, because this latter adduct had better inhibition activity than the mixture, and that this inhibition was not related to any synergistic effect due to the presence of the siderophore. The same experiments performed on pyoverdin−iron(III) and pyoverdin−quinolone adduct−iron(III) complexes showed a rapid increase of the growth followed by a plateau of the bacterial population. The stimulating effect of this additional iron could explain the less efficient inhibitions observed with the ferrated adducts **4**−**7** (Figure 6). In the case of the *P. aeruginosa* strain ATCC 27853, no difference was observed between the four adducts whose antibiotic activity was found to be weaker than the activity of the corresponding quinolones alone.

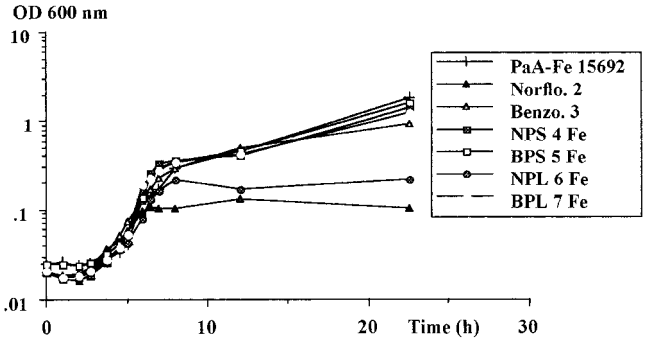


Figure 6. Growth curves of *P. aeruginosa* ATCC 15692 at 600 nm in the presence of the PaA-Fe(III) complex (**1a**) (1 μM) with added norfloxacin, benzonaphthyridone, or ferrated pyoverdin−quinolone adducts (1 μM).

Table 3. ^{1}H NMR (500 MHz) of PaA-NH_2 (**1b**) as a Free Ligand in a Mixture of H_2O/D_2O (5%)[a]

peptide	NH α	CH α	CH β	CH γ	CH δ	CH ϵ	others
Ser 1	9.68	4.42	4.05				
Arg 2	8.46	4.43	1.69	1.33	2.86		6.55 (N*H* ϵ)
Ser 3	8.18	4.32	3.62				
Orn 4	8.34	4.39	1.69	1.85	3.64		7.99 (*H*CO)
Lys 5	8.20	4.34	1.79	1.22	1.54	3.19	7.96 (N*H* ϵ)
Orn 6	8.64	4.36	1.89	1.72	3.60		8.01 (*H*CO)
Thr 7	8.20	4.30	4.49	1.28			
Thr 8	8.45	4.17	4.42	1.29			

[a] Chromophore: 8.00 (C_4H aromatic); 7.22 (C_5H aromatic); 7.23 (C_8H aromatic); 5.81 ($C_{11}H$); 3.77/3.44 ($C_{12}H$); 3.63/3.45 ($C_{13}H$); 9.86 (N_3H); 8.36 ($N_{14}H$). Succinate: 2.90 ($COCH_2$); 2.78 (CH_2CO). Ethylenediamine: 8.28 (CON*H*); 3.60 ($NHCH_2$); 3.23 (CH_2NH_2).

Table 4. ^{13}C NMR (125 MHz) of PaA-NH_2 (**1b**) as a Free Ligand in a Mixture of H_2O/D_2O (5%)[a]

peptide	C α	C β	C γ	C δ	C ϵ	CO	others
Ser 1	58.8	62.2				173.7	
Arg 2	54.7	29.1	25.7	41.8		176.0	*C*NH = 157.9
Ser 3	57.4	62.4				173.3	
Orn 4	54.6	29.2	24.6	51.2		174.3	H*C*O *cis* = 160.8 & H*C*O *trans* = 165.1
Lys 5	56.1	24.1	23.3	29.1	40.6	175.0	
Orn 6	54.7	28.9	24.0	51.2		174.8	H*C*O *cis* = 160.8 & H*C*O *trans* = 165.1
Thr 7	60.6	67.8	20.7			173.5	
Thr 8	61.3	67.7	20.8			173.1	

[a] Chromophore: 150.8 (C_2 aromatic); 119.2 (C_3 aromatic); 140.3 (C_4 aromatic); 115.6 (C_5 aromatic); 145.2 (C_6 aromatic); 152.9 (C_7 aromatic); 101.6 (C_8 aromatic); 133.0 (C_9 aromatic); 116.3 (C_{10} aromatic); 58.1 (C_{11}); 36.6 (C_{12}); 47.3 (C_{13}). Succinate: 177.8 ($COCH_2$); 32.0 ($COCH_2$); 31.6 (CH_2CO); 177.2 (CH_2CO). Ethylenediamine: 38.4 ($NHCH_2$); 40.8 (CH_2NH_2). Note: The numbering of the amino acids corresponds to their location in the peptide chain starting from the chromophore.

Conclusion

The four adducts **4**−**7** synthesized had two types of spacer arms and two different quinolones. The first type of spacer arm links the quinolone to the siderophore by means of *stable* amide bonds (compounds NPS, **4**, and BPS, **5**), whereas the second has a readily hydrolyzable methylenedioxy group (compounds NPL, **6**, and BPL, **7**) releasing very likely the free quinolone (norfloxacin, **2**, or benzonaphthyridone, **3**) inside the cell where it can act on its target without being hindered by the pyoverdin moiety.

The ^{55}Fe-transport assays with these adducts showed that the very large structural modifications introduced in pyoverdin after binding the antibiotic moieties affected only very slightly ^{55}Fe uptake kinetics. However selectivity in the transport was observed between the strains of *P. aeruginosa* ATCC 15692 and ATCC 27853. Only strain ATCC 15692, producing the pyoverdin used to synthesize the adducts, was able to recognize these adducts, and no significant difference was observed in the ^{55}Fe-transport abilities of the four adducts **4**−**7**.

The inhibition assays with the gyrase of *E. coli* showed that the quinolone moiety of the adducts can inhibit the enzyme without being necessarily dissociated from pyoverdin. However higher amounts of adducts in comparison with the free quinolones (expressed in quinolone equivalents) were required to observe a similar inhibition. This is very likely due to steric hindrance induced by the bulky pyoverdin moiety in the adduct.

The classical MIC determinations for these adducts in the presence of various strains of *P. aeruginosa* generally showed rather poor antibiotic behavior, and strain ATCC 27853 appeared to be particularly resistant. Similar results had already been obtained by Miller and co-workers for the antibiotic activities of adducts formed by β-lactams bound to synthetic siderophores derived from albomycine.[7]

Only for NPL **6** was the inhibition enhanced when the bond between the quinolone and pyoverdin was labile. The weaker activity of adducts NPS **4** and BPS **5** could be due either to steric hindrance as mentioned above or to the fact that pyoverdin is not accumulated inside the cell after release of iron; therefore, the antibiotic is not released fast enough and is partly excreted bound to the siderophore.

Our results show clearly that pyoverdins facilitate the transport of pyoverdin-bound antibiotics into the cells of the bacteria which generate the siderophore. To increase the efficiency of these adducts, it seems preferable to use a labile spacer arm to link pyoverdin to the antibiotic moiety which can thus be readily released inside the cell.

It has been shown previously that two pyoverdin−ampicillin conjugates synthesized from two pyoverdins occurring from two different strains of *P. aeruginosa* (respectively ATCC 13525 and ATCC 27853) enter the bacteria via the pyoverdin-mediated iron-uptake pathway.[16] Each conjugate shows specific antibacterial activity against the strains which recognize the corresponding pyoverdins: ATCC 13525 and ATCC 15692, the pyoverdins of which are structurally very closely related and are therefore interchangeable,[17,18] for the first and ATCC 27853 for the second. A third conjugate, where cephalexin was linked to the arginine moiety of pyoverdin Pa via a 2,4-pentanedione-derived spacer arm, showed no antibacterial activity but instead growth promoting activity.[19]

In the two former conjugates, sebacic acid, the spacer arm used, is chemically stable and bound to the pyoverdins on the peptide chain, not on the chromophoric side chain as used in this study. These results are similar to ours in the sense that the pyoverdin−ampicillin conjugates also penetrate the cell, but their targets, transpeptidases, seem to be well-inhibited despite the presence of the bulky pyoverdin.

In our study, the spacer arm with the methylenedioxy function has the disadvantage of being unspecifically hydrolyzable, and it is likely that in the course of the in vivo antibiotic assays, some esterase of the host released the quinolone from the pyoverdin before the adduct had reached the bacterial cell. Higher activities with such adducts bearing a quinolone bound to a pyoverdin via a spacer arm might be obtained if the spacer arm is hydrolyzable only inside the cell. In this respect the presence on the spacer arm of a function which can be reduced by physiological reductants inside the bacterial cell should be considered. Thus the quinolone can be released during the iron release process from ferripyoverdins. Since iron is released from the pyoverdin as iron(II) by a reductive process involving physiological reductants and a flavin reductase,[20,21] a spacer arm susceptible to reduction would make the quinolone be readily released into the target bacterial cell in the course of the iron release process. An amine-protective function possessing these characteristics has been recently described in the literature.[22]

Experimental Section

Abbreviations: PaA, pyoverdin PaA bearing a succinic acid moiety at position N-3 of the chromophore; PaA-NH_2, aminopyoverdin PaA derived from the former after coupling of 1,2-diaminoethane on the succinyl moiety; -Fe(III), the complexed form; ES-MS, electrospray mass spectrometry; ROESY, rotating frame Overhauser spectroscopy.

Chemicals. The chemicals were either from Merck (Merck, Darmstadt, Germany) or from Prolabo (Prolabo, Paris, France). Norfloxacin and benzonaphthyridone were a generous gift from Rhône Poulenc Rorer (Vitry sur Seine, France).

Strains and Cultures. The strains used in the present study were *P. aeruginosa* ATCC 15692 (PAO1 strain) and *P. aeruginosa* ATCC 27853. IA1 and K690 were respectively a pyoverdin nonproducing derivative of PAO1 and a FpvA-deficient derivative of PAO1.[23,24]

The smaller cultures were performed in a thermostated shaker (New Brunswick G76 Scientific, NJ). Cultures larger than 1 L were performed in a 20 L glass fermentor, centrifuged then lyophylized. Centrifugations for the preparation of bacteria to the transport assays were performed in a Beckman microfuge centrifuge.

Purification of the Siderophores. PaA and PaA-NH_2 were prepared and purified as described previously.[12,13,25]

HPLC was performed either on a Chromatem 380 instrument (Touzart and Matignon, Paris, France), fitted with a Rheodyne 7125 injector with a 1 mL loop, a Philips Pye Unicam 4020 UV detector and a Kipp and Zonen BD 41 recorder, or a Kontron Instruments 325 System with a Kontron diode array DAD 440 and a Rheodyne 7000 injector. UV−visible spectra were determined with a Uvikon 930 instrument (Kontron Instruments, Montigny le Bretonneux, France). The pH values were measured with a Metrohm 632 pH-meter. Electrophoreses were performed on 5.7 × 14 cm cellulose acetate films (Midifilm Cellogel) in a horizontal system tank

(Sebia, Issy-les-Moulineaux, France), at a constant voltage of 300 V provided by a Consort E 455 generator (Bioblock, Illkirch, France). The buffer used was pyridine/acetic acid (0.1 M, pH 5.0). ^{55}Fe radioactivity was measured on a LKB Wallac 1209, Rack beta counter. The scintillation liquid used was Aqua Luma Plus. Distribution of the media on the microplates was performed using a Biomek1000 distributer. For the MIC determinations in liquid media, the ELISA plates were detected on either a Dynatech MR 700 or MR 5000. Melting points (mp) were measured in a capillary tube using a Büchi SMP-20 instrument and are not corrected. FAB (Fast atom bombardment) spectra were performed using a ZAB-HF instrument (VG Analytical, Manchester, U.K.). The electrospray spectra were determined on a VG Analytical Bio-Q quadrupole instrument. Proton NMR spectra were measured on a Bruker AC-200 (200 MHz), AM-400 (400 MHz), ARX-500 (500 MHz) with gradient. Column chromatographies were performed using Merck 9385 silica gel (Darmstadt. Germany) 40−63 μm mesh. Thin layer chromatographies (TLC) were performed using silica gel analytical plates Merck 5715 (F_{254}) of 0.25 mm thickness. The detection on TLC plates was performed by UV light at 254 or 365 nm or using a spray and heating the plates. The sprays used were: 0.3% solution of ninhydrin in a mixture of butanol/acetic acid (97:3, v/v); 0.4% solution of 2.4-dinitrophenylhydrazine in 2 M hydrochloric acid; 10% solution of phosphomolybdic acid in ethanol. Anhydrous solvents were obtained by distillation from an appropriate drying agent: phosphoric anhydride for methylene chloride and dimethylformamide; calcium hydride for toluene, ether, and acetonitrile; sodium for methanol. The oxygen- or humidity-sensitive reactions were performed under argon.

*Note: The mass of the pyoverdin−quinolone adducts is much superior to the mass of the quinolones used for these systems. Since the biological activities of the antibiotics are usually expressed in mass units per volume, we decided to express the amounts of adducts in mass equivalent of quinolone present in the solution and not to take into account the mass of pyoverdin. Example: 319 mg equivalent norfloxacin represents 1853 mg of adduct NPL (**6**).*

Production of Pyoverdins. The composition of the minimum culture medium was in g/L: K_2HPO_4, 6 (Merck 5101); KH_2PO_4, 3 (Merck 7734); $(NH_4)_2SO_4$, 1 (Prolabo 21 332.296); $MgSO_4 \cdot 7H_2O$, 0.2 (Prolabo 29 763.158); succinic acid, 4 (Merck 682). The pH was adjusted to 7.0 by addition of a solution of sodium hydroxyde and measured using a pH-meter. The media were distributed in fractions of 200 mL in 1 L conical flasks, stoppered with carded cotton and sterilized at 120 °C for 20 min. For strain K690, 100 μg/mL of tetracycline was added after cooling. All the glassware used for the preparation of the media was carefully washed with a 1 N HCl solution and rinsed with distilled water. The strains were stored at −80 °C in a physiological broth containing horse serum, physiological serum and glycerol. The strains were: *P. aeruginosa* ATCC 15692, ATCC 27853, IA1 and K690. The two latter strains derive from strain *P. aeruginosa* ATCC 15692 by chromosomic mutations and were generously provided by Professor Keith Poole (Queens University, Ontario, Canada).

Preparation of PaA-NH$_2$ (1b). This derivative was prepared from PaA-Fe(III) according to a previous publication[12] but with slight modification: PaA-Fe(III) complex **1a** (76 mg, 55 μmol) and ECDI **27** (53 mg, 275 μmol) were dissolved in a mixture of DMSO (350 μL) and water (50 μL). The mixture was kept at room temperature for 2 h, then treated with freshly distilled ethylenediamine monohydrate (43 mg, 550 mmol) in DMSO (150 μL) and water (75 μL) and kept 5 h at room temperature. The solvent was removed under reduced pressure (1 mmHg), and the residue dissolved in pyridine/acetic acid buffer (1 mL, 50 mM, pH 5.0), then purified by ion-exchange chromatography on a CM Sephadex C25 column (1.5 × 20 cm), eluted with a linear gradient of pyridine/acetic acid pH 5.0 (50 mM to 1 M, 2 × 150 mL). After removal of the buffer and lyophylization pure PaA-NH$_2$ **(1b)** (69 mg, 87%) was obtained. Film electrophoresis: 30 mm migration distance in 20 min (for the iron complex). EI-MS: $M^+ = 1429.6$ (for the iron complex). ^{1}H NMR (500 MHz) (as a free ligand): see Table 3. ^{13}C NMR (125 MHz) (as a free ligand): see Table 4.

Synthesis of the Pyoverdin−Quinolone Adducts. 1. Synthesis of the Norfloxacin NPS (4) Adduct. (a) 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-succinylpiperazinyl)]-3-quinolinecarboxylic Acid (8). Norfloxacin **(2)** (320 mg, 1.0 mmol) was dissolved in DMSO (2 mL), then treated successively with anhydrous pyridine (160 mL, 2 mmol) and succinic anhydride (140 mg, 1.4 mmol). The mixture was heated at 95 °C and stirred under argon 5 h, cooled, the solvent removed under reduced pressure (1 mmHg) and the residue purified by chromatography on a silica gel column eluted with a mixture of acetone/ethyl acetate/acetic acid (80:20:4). Pure norfloxacin succinylated derivative 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-succinylpiperazinyl)]-3-quinolinecarboxylic acid **(8)** (364 mg, 87%) was obtained. Mp: 238 °C dec. R_f = 0.17 (silica gel; acetone/ethyl acetate/acetic acid, 50:48:2; bromophenol blue spray after the plate was dried). Fluorescent when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+$ = 420.1. ^{1}H NMR (200 MHz) in a 5% mixture of $CDCl_3/CD_3$-COOD: 8.55 ppm (s, 1H, C_2H); 7.84 ppm (d, 1H, J = 13 Hz, C_5H aromatic); 6.79 ppm (d, 1H, J = 7 Hz, C_8H aromatic); 4.22 ppm (q, 2H, J = 7 Hz, CH_2 ethyl); 3.61 ppm (broad s, 4H, CH_2−N piperazinyl); 3.20 ppm (broad s, 4H, CH_2N piperazinyl); 2.49 ppm (s, 4H, CH_2 succinyl); 1.38 ppm (t, 3H, J = 7 Hz, CH_3 ethyl). ^{13}C NMR (125 MHz) in DMSO-d_6: 176.0 ppm (*C*O quinolone); 174.0 (***C***OOH succinyl); 170.0 ppm (***C***ON succinyl); 166.0 ppm (C_3-***C***OOH); 152.7 ppm (d, $\boldsymbol{C}_6$−F, J = 249 Hz aromatic); 148.3 ppm ($\boldsymbol{C}_2$H); 145.1 ppm (d, $\boldsymbol{C}_7$N aromatic, J = 10 Hz); 137.1 ($\boldsymbol{C}_9$ aromatic); 119.4 ppm (d, $\boldsymbol{C}_{10}$ aromatic, J = 8 Hz); 111.1 ppm (d, $\boldsymbol{C}_5$H aromatic, J = 23 Hz); 107.2 ppm ($\boldsymbol{C}_3$−COOH); 105.9 ppm ($\boldsymbol{C}_8$H aromatic); 49.5 and 49.2 ppm ($\boldsymbol{C}$H$_2$N piperazinyl); 48.9 ppm ($\boldsymbol{C}$H$_2$ ethyl); 44.4 and 40.8 ppm ($\boldsymbol{C}$H$_2$NCO piperazinyl); 29.6 and 27.7 ($\boldsymbol{C}$H$_2$ succinyl); 14.2 ppm ($\boldsymbol{C}$H$_3$ ethyl).

(b) Norfloxacin NPS (4) Adduct. 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-succinylpiperazinyl)]-3-quinolinecarboxylic acid **(8)** (20 mg, 48 μmol) and DCC (10 mg, 48 μmol) were dissolved in DMF (300 μL) and the mixture kept 1 h at room temperature. After addition of *N*-hydroxysuccinimide (60 mg, 52 μmol) the mixture containing mainly crude *N*-hydroxysuccinimide activated ester **9** thus obtained was kept at room temperature for another 2 h, before addition of PaA-NH$_2$ **(1b)** (68 mg, 48 μmol dissolved in 600 μL DMF) and keeping the whole for another 2 h at room temperature. The solvent was removed under reduced pressure (1 mmHg), and the residue was dissolved in a 0.1 M EDTA solution (30 mL) and stirred 3 h at room temperature. The aqueous phase was extracted 3 times with dichloromethane containing acetic acid, then applied to an ODS column made up in aqueous acetic acid pH 4.0. The column was washed with a solution of acetic acid pH 4.0, then with a 1:1 mixture of 1 M pyridine/acetic acid buffer pH 5.0 and acetonitrile. After evaporation, the solid residue was dissolved in a 50 mM pyridine/acetic acid buffer pH 5.0 (1 mL) and applied on a CM-Sephadex C-25 column (0.5 × 15 cm) made up in the same buffer. The column was eluted with a linear gradient of 50 mM to 1 M pyridine/acetic acid buffer pH 5.0 (2 × 100 mL). After evaporation of the buffer and lyophylization, NPS **(4)** (53 mg, 30 μmol, 62%) was obtained as a bright yellow compound. Electrophoresis: migration distance 8 mm in 20 min. ES-MS: $M^+ = 1778.4$; $(M + H)^+/2 = 889.6$; $(M + 2H)^+/3 = 593.5$.

2. Synthesis of the Benzonaphthyridone BPS (5) Adduct. (a) 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-succinylpiperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic Acid (12). Ethyl 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-piperazinyl]-3-benzo[*b*][1,8]naphthyridinecarboxylate **(11)** (300 mg, 0.78 mmol) was dissolved in chloroform (5 mL), then methyloxy succinyl chloride (135 mg, 110 μL, 0.9 mmol) and pyridine (73 μL, 0.9 mmol) were added. The mixture was kept under argon for 48 h at room temperature, then extracted 3 times with a saturated solution of sodium bicarbonate, then 3 times with water. The organic phase was dried over sodium sulfate, filtered and evaporated under reduced

pressure. The solid residue was suspended in dichloromethane and purified by column chromatography on silica gel. The column was eluted with a mixture of toluene/tetrahydrofuran/acetic acid 36:60:4. The solvent was evaporated and the residue crystallized in a dichloromethane/hexane mixture to yield ethyl 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(methoxysuccinyl)piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylate **(12)** as yellow crystals (184 mg, 47%). Mp: 226 °C dec. $R_f = 0.54$ (silica gel, toluene/THF/acetic acid, 46:60:4; anisaldehyde spray after the plate was dried). Fluorescent when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 499.2$. ^{1}H NMR (200 MHz, $CDCl_3$): 9.12 ppm (s, 1H, C_5H aromatic); 8.71 ppm (s, 1H, C_2H); 7,61 ppm (d, 1H, $J = 13$ Hz, C_6H aromatic); 7.34 ppm (d, 1H, $J = 8$ Hz, C_9H aromatic); 4.42 ppm (q, 2H, $J = 7$ Hz, CH_2 ethyl); 4.04 ppm (s, 3H, CH_3N_1); 3.81 ppm (m doubled, 4H, $J = 23$ Hz, CH_2N piperazinyl); 3.76 ppm (s, 3H, CH_3O succinyl); 3.33 ppm (broad m, 4H, CH_2N piperazinyl); 2.72 ppm (s, 4H, CH_2 succinyl); 1,42 ppm (t, 3H, $J = 7$ Hz, CH_3 ethyl). ^{13}C NMR (50 MHz, $CDCl_3$): 174.9 ppm (***C***O quinolone); 173,3 ppm (***C***OOH succinyl); 169.7 ppm (***C***ON succinyl); 164.7 ppm (C_3-***C***OOH); 154.5 ppm (d, C_7F aromatic, $J = 252$ Hz); 151.1 ppm (C_2H); 147.8 ppm (C_{11} aromatic); 146.9 ppm (C_{14} aromatic); 145.6 ppm (d, C_8N aromatic, $J = 12$ Hz); 136.3 ppm (C_5H aromatic); 121.1 ppm (d, C_{13} aromatic, $J = 10$ Hz); 120.3 ppm (C_{12} aromatic); 114.1 ppm (C_9H aromatic); 112.5 ppm (d, C_6H aromatic, $J = 21$ Hz); 109.3 ppm (C_3COOH); 60.5 ppm (CH_2 ethyl); 51.6 ppm (CH_3O succinyl); 50.0 and 49.7 ppm (CH_2-NCO piperazinyl); 44.9 and 41.3 ppm (CH_2N piperazinyl); 39.0 ppm (CH_3N_1); 28.8 and 27.7 ppm (CH_2 succinyl); 14.2 ppm (CH_3 ethyl).

(b) 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-succinylpiperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic Acid (10). To ethyl 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(methoxysuccinyl)piperazinyl)]-3-benzo[*b*][1,8]-naphthyridinecarboxylate **(12)** (117 mg, 0.24 mmol) dissolved in THF (3 mL) was added sodium hydroxide (20.8 mg, 0.52 mmol) in water (280 μL). The mixture was kept 8 h at room temperature then evaporated and the residue suspended in a mixture of dichloromethane/acetic acid. The organic phase was extracted with water, then dried over magnesium sulfate, filtered and evaporated under reduced pressure to yield 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-succinylpiperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(10)** as a yellow solid (81 mg, 74%). Mp: 195 °C dec. $R_f = 0.16$ (silica gel; acetone/ethyl acetate/acetic acid, 50:48:2; bromophenol blue spray after the plate was dried). Fluorescent when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 457.1$. ^{1}H NMR (200 MHz) in a mixture of 5% CD_3COOD in $CDCl_3$: 9.11 ppm (s, 1H, C_5H aromatic); 8.99 ppm (s, 1H, C_2H); 7.62 ppm (d, 1H, $J = 13$ Hz, C_6H aromatic); 7.37 ppm (d, 1H, $J = 8$ Hz, C_9H aromatic); 4.09 ppm (s, 3H, CH_3N_1); 3.74 ppm (m doubled, 4H, $J = 15$ Hz, CH_2N piperazinyl) 3.28 ppm (broad m, 4H, CH_2N piperazinyl); 2.64 ppm (s, 4H, CH_2 succinyl). ^{13}C NMR (125 MHz) in DMSO-d_6: 179.0 ppm (***C***O quinolone); 173.8 ppm (***C***OOH succinyl); 169.8 ppm (***C***ON succinyl); 165.5 ppm (C_3***C***OOH); 154.4 ppm (d, C_7F aromatic, $J = 252$ Hz); 152.4 ppm (C_2H aromatic); 147.8 ppm (C_{11} aromatic); 147.7 ppm (C_{14} aromatic); 146.6 ppm (d, C_8N aromatic, $J = 12$ Hz); 145.6 ppm (C_{12} aromatic); 136.5 ppm (C_5H aromatic); 121.3 ppm (d, C_{13} aromatic, $J = 11$ Hz); 113.3 ppm (C_9H, aromatic); 113.2 ppm (d, C_6H aromatic, $J = 22$ Hz); 106.1 ppm (C_3COOH); 49.7 and 49.4 ppm (CH_2NCO piperazinyl); 44.3 and 40.8 ppm (CH_2N piperazinyl); 39.3 ppm (CH_3N_1); 28.9 and 27.4 ppm (CH_2 succinyl).

The same acid **10** was obtained with 79% yield by treatment of benzonaphthyridone (**3**) with succinic anhydride as described above for acid **8** derived from norfloxacin (**2**).

(c) Benzonaphthyridone BPS (5) Adduct. To 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-succinylpiperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(10)** (13 mg, 28 μmol) dissolved in anhydrous DMF (200 μL) were added isobutyl chloroformate (3.8 mg, 3.6 μL, 28 μmol) and *N*-methylpiperidine (5.6 mg, 7.2 μL, 60 μmol), and the mixture was kept for 30 min at 4 °C. PaA-NH_2-Fe(III) complex **(1b)** (40 mg, 28 μmol) in DMF (200 μL) was then added and the mixture was kept 3 h at room temperature. The solvent was removed under reduced pressure (1 mmHg) and the residue dissolved in a 0.1 M aqueous solution of EDTA (30 mL) and stirred 3 h at room temperature. The solution was then extracted 3 times with dichloromethane acidified with acetic acid, and the aqueous phase was applied to an ODS column made up in aqueous acetic acid pH 4.0. The column was washed first with a solution of acetic acid pH 4.0, then with a 1:1 mixture of 1 M pyridine/acetic acid buffer pH 5.0 and acetonitrile. After evaporation, the solid residue was dissolved in 50 mM pyridine/acetic acid buffer pH 5.0 (1 mL), and applied to a CM-Sephadex C-25 column (0.5 × 15 cm) made up in the same buffer. The column was eluted with a linear gradient of 50 mM to 1 M pyridine/acetic acid buffer pH 5.0 (2 × 100 mL). After evaporation of the buffer and lyophylization, BPS **5** (24 mg, 46%) was obtained as a bright yellow compound. Electrophoresis: the migration distance was 8 mm in 20 min. ES-MS: $M^+ = 1815.3$; $(M + H)^+/2 = 908.3$; $(M + 2H)^+/3 = 605.7$.

3. Synthesis of the Norfloxacin NPL (6) Adduct. (a) 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(chloromethyloxycarbonyl)piperazinyl)]-3-quinolinecarboxylic acid (14). This compound was prepared according to Alexander et al.,[26] with slight modifications: To norfloxacin methanesulfonate **(2)** (413 mg, 1.0 mmol) and 1,8-bis(*N*,*N*-dimethylamino)naphthalene (430 mg, 2.0 mmol), dissolved in chloroform (17 mL), was added chloromethyl chloroformate (0.11 mL, 1.25 mmol), and the mixture was kept under argon for 29 h. After removal of the solvent and excess reagent, the residue was dissolved in dichloromethane and extracted successively with water then with a 0.01 N solution of hydrogen chloride then washed 3 times with water. The organic phase was dried up over magnesium sulfate, filtered then evaporated to yield after crystallization in dichloromethane, 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(chloromethyloxycarbonyl)-piperazinyl)]-3-quinolinecarboxylic acid **(14)** (288 mg, 70%). Mp: 207 °C dec. $R_f = 0.29$ (silica gel; toluene/THF/acetic acid, 56:40:4; anisaldehyde spray after the plate was dried). Blue fluorescence when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 411.9$ and 413.9 with an isotopic ratio confirming the presence of chlorine on the molecule. ^{1}H NMR (200 MHz, $CDCl_3$): 8.67 ppm (s, 1H, C_2H); 8.08 ppm (d, 1H, $J = 13$ Hz, C_5H aromatic); 6.85 ppm (d, 1H, $J = 7$ Hz, C_8H aromatic); 5.82 ppm (s, 2H, CH_2−Cl); 4.32 ppm (q, 2H, $J = 7$ Hz, CH_2 ethyl); 3.77 ppm (s, 4H, CH_2N piperazinyl); 3.31 ppm (s, 4H, CH_2N piperazinyl); 1.59 ppm (t, 3H, $J = 7$ Hz, CH_3 ethyl). ^{13}C NMR (100 MHz, $CDCl_3$): 176.7 ppm (***C***O quinolone); 166.6 ppm (C_3***C***OOH); 153.1 ppm (d, C_6F aromatic, $J = 252$ Hz); 152.2 ppm (***C***ON); 146.9 ppm (C_2H aromatic); 145.3 ppm (d, C_7N aromatic, $J = 11$ Hz); 136.7 (C_9 aromatic); 121.0 ppm (d, C_{10} aromatic, $J = 8$ Hz); 112.8 ppm (d, C_5H aromatic, $J = 23$ Hz); 108.3 ppm (C_3COOH); 103.9 ppm (C_8H aromatic); 70.7 ppm (CH_2Cl); 49.4 ppm (CH_2NCO piperazinyl); 49.2 ppm (CH_2 ethyl); 43.6 and 43.4 ppm (CH_2N piperazinyl); 14.2 ppm (CH_3 ethyl).

(b) 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(*tert*-butyloxysuccinyloxymethyleneoxycarbonyl)piperazinyl)]-3-quinolinecarboxylic Acid (15). 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(chloromethyloxycarbonyl)piperazinyl)]-3-quinolinecarboxylic acid **(14)** (173 mg, 0.42 mmol), *tert*-butyl hemisuccinate (146 mg, 0.84 mmol) and 1,8-bis(*N*,*N*-dimethylamino)naphthalene (180 mg, 0.84 mmol) were dissolved in anhydrous DMF and the mixture was heated 24 h under argon at 95 °C. After removal of the solvent under reduced pressure (1 mmHg), the residue was dissolved in dichloromethane (20 mL) and extracted with water (30 mL), then at 0 °C with a 2 N solution of sulfuric acid, then with water (3 × 30 mL). The organic phase was dried over sodium sulfate, filtered and evaporated. The crude compound was purified by column chromatography on silica gel and eluted with a mixture of toluene/THF/acetic acid (75:21:4), then with another toluene/THF/acetic acid (56:40:4) mixture. After crystallization from dichloromethane/hexane, pure 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(*tert*-butyloxysuccinyloxymethyleneoxycarbonyl)-

piperazinyl)]-3-quinolinecarboxylic acid **(15)** (134 mg, 58%) was obtained. Mp: 128 °C dec. $R_f = 0.29$ (silica gel; toluene/THF/acetic acid, 56:40:4; anisaldehyde spray after the plate was dried). Blue fluorescence when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 550.1$. ^{1}H NMR (200 MHz, $CDCl_3$): 8.67 ppm (s, 1H, C_2H); 8.08 ppm (d, 1H, $J = 13$ Hz, C_5H aromatic); 6.85 ppm (d, 1H, $J = 7$ Hz, C_8H aromatic); 5.82 ppm (s, 2H, OCH_2O); 4.32 ppm (q, 2H, $J = 7$ Hz, CH_3CH_2 ethyl); 3.74 ppm (s, 4H, CH_2N piperazinyl); 3.30 ppm (s, 4H, CH_2N piperazinyl); 2.67−2.54 ppm (m, 4H, CH_2CO succinyl); 1.59 ppm (t, 3H, $J = 7$ Hz, CH_3CH_2 ethyl); 1.43 ppm (s, 9H, CH_3 *tert*-butyl). ^{13}C NMR (50 MHz, $CDCl_3$): 176.8 ppm (***C***O quinolone); 171.5 ppm (***C***O_2tBu succinyl); 171.2 ppm (***C***O_2CH_2 succinyl); 167.0 ppm (C_3***C***OOH); 153.4 ppm (d, ***C***$_6$F aromatic, $J = 252$ Hz); 153.4 ppm (***C***ON); 147.2 ppm (***C***$_2$H); 145.7 ppm (d, ***C***$_7$N aromatic, $J = 10$ Hz); 137.1 (***C***$_9$ aromatic); 120.7 ppm (d, ***C***$_{10}$ aromatic, $J = 7$ Hz); 112.6 ppm (d, ***C***$_5$H aromatic, $J = 23$ Hz); 108.2 ppm (***C***$_3$COOH); 104.4 ppm (***C***$_8$H aromatic); 80.9 ppm (***C***CH_3 *tert*-butyl); 80.5 ppm (***C***H_2O); 49.8 ppm (***C***H_2NCO piperazinyl); 49.6 ppm (***C***H_2 ethyl); 43.9 and 43.5 ppm (***C***H_2N piperazinyl); 30.0 and 29.2 ppm (***C***H_2 succinyl); 28.1 ppm (***C***H_3C *tert*-butyl); 14.5 ppm (***C***H_3 ethyl).

(c) 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(succinyloxymethyleneoxycarbonyl)piperazinyl)]-3-quinolinecarboxylic Acid (16). 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(*tert*-butyloxysuccinyloxymethyleneoxycarbonyl)-piperazinyl)]-3-quinolinecarboxylic acid **(15)** (100 mg, 0.182 mmol) was dissolved in dichloromethane (5 mL), and TFA (5 mL) was added and the solution was then kept 3 h at room temperature. The solvent was removed under reduced pressure. The residue was dissolved in dichloromethane and extracted with water then 3 times with a saturated solution of sodium bicarbonate. The aqueous phase was then acidified with acetic acid and extracted 3 times with dichloromethane. The organic phase was dried over magnesium sulfate, filtered and evaporated to yield 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(succinyloxymethyleneoxycarbonyl)piperazinyl)]-3-quinolinecarboxylic acid **(16)** (84 mg, 93%). Mp: 151−152 °C dec. $R_f = 0.37$ (silica gel; toluene/THF/acetic acid, 36:60:4; anisaldehyde spray after the plate was dried). Blue fluorescence when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 494.45$. ^{1}H NMR (200 MHz, $CDCl_3$ and trifluoroacetic anhydride): 9.07 ppm (s, 1H, C_2H); 8.11 ppm (d, 1H, $J = 13$ Hz, C_5H aromatic); 7.04 ppm (d, 1H, $J = 7$ Hz, C_8H aromatic); 5.86 ppm (s, 2H, $O-CH_2-O$); 4.59 ppm (q, 2H, $J = 7$ Hz, CH_2 ethyl); 3.79 ppm (s, 4H, CH_2N piperazinyl); 3.49 ppm (s, 4H, CH_2N piperazinyl); 3.02−2.94 ppm (m, 2H, CH_2 succinyl); 2.81−2.75 ppm (m, 2H, CH_2 succinyl); 1.64 ppm (t, 3H, $J = 7$ Hz, CH_3 ethyl). ^{13}C NMR (125 MHz) (DMSO-d_6): 176.1 ppm (***C***O quinolone); 173.6 (***C***OOH succinyl); 171.3 ppm (***C***$OOCH_2$ succinyl); 166.0 ppm (C3***C***OOH); 152.8 ppm (d, ***C***$_6$F aromatic, $J = 249$ Hz); 152.7 ppm (***C***ON piperazinyl); 148.7 ppm (***C***$_2$H); 145.2 ppm (d, ***C***$_7$N aromatic, $J = 10$ Hz); 137.1 (***C***$_9$ aromatic); 119.6 ppm (d, ***C***$_{10}$ aromatic, $J = 8$ Hz); 111.2 ppm (d, ***C***$_5$H aromatic, 34 Hz); 107.1 ppm (***C***$_3$COOH); 106.3 ppm (***C***$_8$H aromatic); 81.9 ppm (***C***H_2O); 49.5 and 49.0 ppm (***C***H_2N piperazinyl); 47.9 ppm (***C***H_2 ethyl); 43.4 and 43.0 ppm (***C***H_2-NCO piperazinyl); 28.8 and 28.6 (***C***H_2 succinyl); 14.3 ppm (***C***H_3 ethyl).

(d) Norfloxacin NPL (6) Adduct. 1-Ethyl-6-fluoro-1,4-dihydro-4-oxo-7-[1′-(4′-*N*-(succinyloxymethyleneoxycarbonyl)-piperazinyl)]-3-quinolinecarboxylic acid **(16)** (7.2 mg, 15 μmol) was dissolved in anhydrous DMF (200 μL), and *N*-methylpiperidine (3.6 μL, 30 μmol) and isobutyl chloroformate (2 μL, 15 μmol) were added; the mixture was kept 1 h at 4 °C. After addition of PaA-NH_2 **(1b)** (21 mg, 15 μmol) the mixture was kept 24 h at room temperature, then evaporated to dryness under reduced pressure (1 mmHg). The residue was dissolved in 30 mL of a 0.1 M aqueous solution of EDTA, stirred at room temperature for 3 h, extracted 3 times with a mixture of dichloromethane and acetic acid. The aqueous phase was applied to an ODS reverse-phase C_{18} column, washed with acidified water pH 4.0, then with a 1:1 mixture of 1 M pyridine/acetic acid buffer pH 5.0 and acetonitrile. The product was then purified on a CM Sephadex C-25 column eluted with a linear gradient of pyridine/acetic acid buffer pH 5.0 (50 mM to 1 M). After removal of the solvents under reduced pressure (20 mmHg) NPL **(6)** adduct was obtained as a deep yellow solid (10 mg, 36%). Electrophoresis: the migration distance was 8 mm in 20 min. ES-MS: $M^+ = 1853.2$; $(M + H)^+/2 = 927.0$; $(M + 2H)^+/3 = 618.4$.

4. Synthesis of the Benzonaphthyridone BPL (7) Adduct. (a) 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(chloromethyloxycarbonyl)piperazinyl)]-3-benzo[*b*]-[1,8]naphthyridinecarboxylic Acid (17). Benzonaphthyridone (**3**) (100 mg, 0.28 mmol) and 1,8-bis(*N*,*N*-dimethylamino)naphthalene (128 mg, 0.60 mmol) were dissolved in a 1:1 mixture of chloroform and dichloromethane (40 mL). Chloromethyl chloroformate (90 μL, 1.02 mmol) was added and the mixture was kept 48 h at room temperature under argon. After removal of the solvent and excess reagent, the residue was dissolved in dichloromethane and extracted successively with water (50 mL), with 0.2 N aqueous hydrogen chloride (50 mL), then with water (3 × 50 mL). The organic phase was dried over magnesium sulfate, filtered and evaporated yielding 137 mg of a crude chloro compound **17** which can be used as such in the next step. After crystallization from dichloromethane/hexane at 18 °C, pure 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(chloromethyloxycarbonyl)piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(17)** (97 mg, 77%) was obtained. Mp: 220−240 °C dec. $R_f = 0.34$ (silica gel; toluene/dioxane/acetic acid, 75:21:4; anisaldehyde spray after the plate was dried). Yellow fluorescence when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 449.1$ and 451.1, with an isotopic ratio confirming the presence of chlorine on the molecule. ^{1}H NMR (200 MHz, $CDCl_3$): 9.18 ppm (s, 1H, C_5H aromatic); 8.98 ppm (s, 1H, C_2H); 7.66 ppm (d, 1H, $J = 13$ Hz, C_6H aromatic); 7.44 ppm (d, 1H, $J = 8$ Hz, C_9H aromatic); 5.84 ppm (s, 2H, CH_2-O); 4.15 ppm (s, 3H, CH_3N); 3.79 ppm (s, 4H, CH_2N piperazinyl); 3.39 ppm (s, 4H, CH_2N piperazinyl). ^{13}C NMR (100 MHz, $CDCl_3$): 179.9 ppm (***C***O quinolone); 166.8 ppm (C3***C***OOH); 155.4 ppm (d, ***C***$_7$F aromatic, $J = 255$ Hz); 152.9 ppm (***C***ON piperazinyl); 151.4 ppm (***C***$_2$H); 148.3 ppm (***C***$_{11}$ aromatic); 147.2 ppm (***C***$_{14}$ aromatic); 147.0 ppm (d, ***C***$_8$N aromatic, $J = 12$ Hz); 136.9 ppm (***C***$_5$H aromatic); 121.2 ppm (d, ***C***$_{13}$ aromatic, $J = 11$ Hz); 118.8 ppm (***C***$_{12}$ aromatic); 113.8 ppm (***C***$_9$H aromatic); 113.2 ppm (d, ***C***$_6$H aromatic, $J = 21$ Hz); 107.9 ppm (***C***$_3$COOH); 71.2 ppm (***C***H_2Cl); 50.1 ppm (***C***H_2NCO piperazinyl); 43.9 and 43.7 ppm (***C***H_2N piperazinyl); 40.2 ppm (***C***H_3N).

(b) 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(*tert*-butyloxysuccinyloxymethyleneoxycarbonyl)piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic Acid (18). 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(chloromethyloxycarbonyl)piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(17)** (133 mg, 0.23 mmol), *tert*-butyl hemisuccinate (96 mg, 0.55 mmol) and 1,8-bis(*N*,*N*-dimethylamino)naphthalene (128 mg, 0.60 mmol) were dissolved in anhydrous DMF (10 mL). The mixture was heated at 95 °C for 24 h under argon, the solvent was then evaporated under reduced pressure (1 mmHg) and the residue was dissolved in dichloromethane (20 mL) and extracted successively with water (30 mL), with a 0.2 N solution of sulfuric acid (20 mL) at 0 °C, then with water (3 × 30 mL). The organic phase was dried over sodium sulfate, filtered and evaporated and yielded 172 mg of crude compound **18**, after chromatography on a silica gel column (25 g; 2 × 20 cm) and elution first with a mixture of toluene/THF/acetic acid (76:20:4), then with a mixture of toluene/THF/acetic acid (56:40:4). After removal of the solvents under reduced pressure (20 mmHg), 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(*tert*-butyloxysuccinyloxymethyleneoxycarbonyl)piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(18)** (146 mg, 84%) was obtained. Mp: 170 °C dec. $R_f = 0.32$ (silica gel; toluene/THF/acetic acid, 75:21:4; anisaldehyde spray after the plate was dried). Yellow fluorescence when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 587.3$. ^{1}H NMR (200 MHz, $CDCl_3$): 9.12 ppm (s, 1H, C_5H aromatic); 8.95 ppm (s, 1H, C_2H); 7.61 ppm (d, 1H, $J = 12$ Hz, C_6H

aromatic); 7.41 ppm (d, 1H, $J = 9$ Hz, C_9H aromatic); 5.83 ppm (s, 2H, O−CH_2−O); 4.13 ppm (s, 3H, CH_3N piperazinyl); 3.76 ppm (s, 4H, CH_2N piperazinyl); 3.38 ppm (s, 4H, CH_2N piperazinyl); 2.65−2.56 ppm (m, 4H, CH_2COO succinyl); 1.44 ppm (s, 9H, CH_3 C tBu). ^{13}C NMR (50 MHz) ($CDCl_3$): 179.5 ppm (***C***O quinolone); 171.5 ppm (***C***OO-*t*Bu succinyl); 171.2 ppm (***C***O_2CH_2 succinyl); 166.4 ppm (C3***C***OOH); 155.4 ppm (d, ***C***$_7$F aromatic, $J = 256$ Hz); 153.4 ppm (***C***ON piperazinyl); 151.0 ppm (***C***$_2$H); 148.3 ppm (***C***$_{11}$ aromatic); 147.8 ppm (***C***$_{14}$ aromatic); 147.0 ppm (d, ***C***$_8$N aromatic, $J = 11$ Hz); 136.4 ppm (***C***$_5$H aromatic); 121.8 ppm (d, ***C***$_{13}$ aromatic, $J = 11$ Hz); 118.2 ppm (***C***$_{12}$ aromatic); 114.3 ppm (***C***$_9$H aromatic); 112.8 ppm (d, ***C***$_6$H aromatic, $J = 23$ Hz); 107.4 ppm (***C***$_3$COOH); 80.9 ppm (***C***CH_3 *tert*-butyl); 80.5 ppm (***C***H_2O); 49.7 ppm (***C***H_2NCO piperazinyl); 43.8 and 43.4 ppm (***C***H_2N piperazinyl); 39.8 ppm (***C***H_3N); 30.0 and 29.1 ppm (***C***H_2 CO succinyl); 28.0 ppm (***C***H_3C *tert*-butyl).

(c) 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(succinyloxymethyleneoxycarbonyl)piperazinyl)]-3-benzo-[*b*][1,8]naphthyridinecarboxylic Acid (19). 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(*tert*-butyloxysuccinyloxymethyleneoxycarbonyl)piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(18)** (93 mg, 0.158 mmol) was dissolved in dichloromethane (4 mL) and trifluoroacetic acid was added (4 mL). The mixture was kept 2 h at room temperature, the solvent was removed under reduced pressure, and the residue was purified by column chromatography on silica gel and eluted with a mixture of toluene/THF/acetic acid (76:20:4; 400 mL), then with with a mixture of toluene/THF/acetic acid (56:40:4; 400 mL). The yield was 56 mg (66%) of 1-methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(succinyloxymethyleneoxycarbonyl)-piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(19)**. Mp: 200 °C dec. $R_f = 0.40$ (silica gel; toluene/THF/acetic acid, 56:40:4; anisaldehyde spray after the plate was dried). Yellow fluorescence when irradiated at 254 and 365 nm. FAB-MS: $(M + H)^+ = 531.2$. ^{1}H NMR (200 MHz, $CDCl_3$ and trifluoroacetic anhydride): 9.35 ppm (s, 1H, C_5H aromatic); 9.26 ppm (s, 1H, C_2H); 7.80 ppm (d, 1H, $J = 13$ Hz, C_6H aromatic); 7.54 ppm (d, 1H, $J = 8$ Hz, C_9H aromatic); 5.88 ppm (s, 2H, O−CH_2−O); 4.37 ppm (s, 3H, CH_3N); 3.84 ppm (s, 4H, CH_2N piperazinyl); 3.66 ppm (s, 4H, CH_2N piperazinyl); 2.80−2.77 ppm (m, 4H, CH_2COOH succinyl). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3COOD 5%): 179.6 ppm (***C***O quinolone); 174.7 ppm (***C***OOH succinyl); 171.2 ppm (***C***OOCH_2 succinyl); 167.1 ppm (C3***C***OOH); 155.1 ppm (d, ***C***$_7$F aromatic, $J = 255$ Hz); 153.6 ppm (O***C***ON piperazinyl); 151.7 ppm (***C***$_2$H); 148.9 ppm (***C***$_{11}$ aromatic); 147.9 ppm (***C***$_{14}$ aromatic); 147.1 ppm (d, ***C***$_8$N aromatic, $J = 12$ Hz); 136.7 ppm (***C***$_5$H aromatic); 122.0 ppm (d, ***C***$_{13}$ aromatic, $J = 9$ Hz); 118.2 ppm (***C***$_{12}$ aromatic); 114.3 ppm (***C***$_9$H aromatic); 112.9 ppm (d, ***C***$_6$H aromatic, $J = 23$ Hz); 106.9 ppm (***C***$_3$COOH); 80.2 ppm (***C***H_2O); 49.7 ppm (***C***H_2NCO piperazinyl); 43.8 and 43.5 ppm (***C***H_2N piperazinyl); 39.8 ppm (***C***H_3N); 28.6 and 28.5 ppm (***C***H_2CO succinyl).

(d) Benzonaphthyridone BPL (7) Adduct. 1-Methyl-7-fluoro-1,4-dihydro-4-oxo-8-[1′-(4′-*N*-(succinyloxymethyleneoxycarbonyl)piperazinyl)]-3-benzo[*b*][1,8]naphthyridinecarboxylic acid **(19)** (5.3 mg, 10 μmol) was dissolved in anhydrous DMF (200 μL), and *N*-methylpiperidine (2.4 μL, 20 μmol) and isobutyl chloroformate (1.3 μL, 10 μmol) were added; the mixture was kept 1 h at 4 °C. After addition of PaA-NH_2 **(1b)** (14 mg, 10 μmol) the mixture was kept 24 h at room temperature, then evaporated to dryness under reduced pressure (1 mmHg). The residue was dissolved in 30 mL of a 0.1 M aqueous solution of EDTA, stirred at room temperature for 3 h, extracted 3 times with a mixture of dichloromethane and acetic acid. The aqueous phase was applied to an ODS reverse-phase C_{18} column, washed with acidified water pH 4.0, then with a 1:1 mixture of 1 M pyridine/acetic acid buffer pH 5.0 and acetonitrile. The product was then purified on a CM Sephadex C-25 column eluted with a linear gradient of pyridine/acetic acid buffer pH 5.0 (50 mM to 1 M). The yield was 4 mg (40%) of a deep yellow BPL **7** adduct. Electrophoresis: the migration distance was 8 mm in 20 min. ES-MS: $M^+ = 1889.6$; $(M + H)^+/2 = 945.3$; $(M + 2H)^+/3 = 630.6$.

Synthons Required for the Synthesis of the Labile Spacer Arms. Isobutyl chloroformate was commercially available from Aldrich and was distilled before use. Chloromethyl chloroformate was prepared according to Kharasch and Brown;[27] methoxysuccinic acid and methoxysuccinyl chloride were prepared according to Cason;[28] *tert*-butyloxy methoxysuccinate and *tert*-butyl hemisuccinate were prepared according to Büchi and Roberts.[29]

Gyrase Inhibition Assays. (a) Preparation of the Samples To Be Assayed. The solutions containing the inhibitor at a concentration 10 times the final concentration were prepared after solubilization of the compounds in water. The concentrations of the adducts NPS **(4)**, BPS **(5)**, NPL **(6)** and BPL **(7)** are expressed in μg/mL of quinolone equivalents.

(b) Inhibition of the Supercoiling: Determination of the Amount of Gyrase Necessary for the Assay. This assay was performed according to Bazile et al.[30] In a first step, the amount of gyrase necessary for the assay was determined as follows: A circular relaxed pBR 322 DNA solution (2 μL, 150 ng) was mixed with complete buffer (5 μL) and increasing volumes of an *E. coli* gyrase solution and water (qsp 15 μL). The medium was homogenized and kept 30 min at 37 °C. The reaction was stopped by addition of 2 μL of the stock solution, and the medium is submitted to an electrophoresis on 0.8% agarose at 70 V (140 mA) for 2 h. After staining for 10 min in a BET solution the gel was irradiated at 302 nm. The first concentration of gyrase leading to complete supercoiling was chosen for inhibition assays. Then the inhibition assay was performed as above after addition of 5 μL of the inhibitor solution at various concentrations. The results were expressed as MED, the minimum effective dose (the minimum amount of drug required to cause any inhibition of activity).

(c) Inhibition Assays. They were performed as above after addition of 5 μL of the inhibitor solution at a concentration 3 times larger than the final solution for each condition. Composition of the solution solubilizing DNA: Tris, 10 mM; EDTA, 1 mM, pH 7.5. Composition of the complete buffer: 129 μL buffer I + 21 μL 60 mM aqueous ATP solution. Composition of buffer I: KCl, 75 mM; t-RNA, 0.1% w/v; HEPES, 60 mM; DTT, 12 mM; AcOMg, 24 mM; spermidine, 5.1 mM; EDTA, 1.5 mM; Tris, 6 mM; ethylene glycol, 9% w/v. Composition of the stock solution: saccharose, 40% w/v; bromophenol blue, 60% w/v. Composition of the 50 times concentrated electrophoresis buffer: Tris base, 242 g; EDTA, 0.5 M, pH 8, 100 mL; acetic acid, 57.1 mL; H_2O, qsp 1 L. Composition of the staining solution: BET, 40 μL of a 5 mg/mL solution; H_2O, 200 mL.

^{55}Fe-Transport Assays. The bacteria were cultivated in succinate medium at 25 °C and stirred vigorously. 12 mL of a bacterial suspension at the end of the exponential phase was centrifuged 4 min at 13000 rpm then suspended in 1 mL 60 mM MOPS buffer pH 7.5 and recentrifuged in the same conditions. This operation was repeated 3 times, then a bacterial suspension with an OD_{600nm} of 0.5 was prepared. 900 μL of this suspension was stirred 15 min at 29 °C in a hemolysis tube with 100 μL of a solution containing the compound to be tested as its ^{55}Fe complex. 100 μL aliquots of the suspension were withdrawn at the times indicated and filtered on a filtration membrane (Micronsep, porosity 0.45 μm) presoaked in a 0.3% solution of polyethylenimine, then rapidly washed with 2 × 2 mL 2 M pyridine/acetic acid buffer pH 5.0. Each filter was then placed in counting vials (Pony vial) containing 3 mL scintillation liquid, and the radioactivity measured after 8 h incubation. All the tests were performed in duplicate.

Preparation of the Pyoverdin and Adducts Solutions. PaA **(1a)** and adducts NPS **(4)**, BPS **(5)**, NPL **(6)** and BPL **(7)** were dissolved in water to a 1 mM concentration. To 100 μL of this solution were added 3.3 μL of a $^{55}FeCl_3$ solution and 900 μL 60 mM MOPS buffer pH 7.5. The $^{55}FeCl_3$ was prepared after dilution of 5 μL of $^{55}FeCl_3$ NEN (1 μCi/μL) solution in 95 μL 0.5 N aqueous hydrochloric acid.

Growth Inhibition Assays (MIC). (a) Antibiotic Assays in Solid Media. Each compound to be tested was solubilized in water at a concentration 20 times superior to the highest

final concentration used (generally 64 μg/mL). A range of concentrations was obtained by successively diluting by a factor of 2 this first solution. To 1 mL of each diluted solution 19 mL of agar medium (to which 25 mg/L Mg^{2+}, 50 mg/L Ca^{2+} and the nutrient medium, Müller-Hinton or succinate, were added) maintained at 50 °C was added, and the mixture was poured in a Petri dish. The dishes were inoculated with 1 μL of bacterial suspension (10^7 cells/mL) using a Denley multipoints instrument, then incubated 18 h at 37 °C.

(b) Antibiotic Assays in Liquid Media. Each compound to be tested was solubilized in water at a concentration twice as high as the highest final concentration used (generally 64 μg/mL). 200 μL of this solution was placed in the first row of an ELISA plate. The successive dilutions by a factor 20 were performed using a BIOMEK 1000 instrument (100 μL of this solution + 100 μL of the culture medium). The same instrument distributes 100 μL of a bacterial suspension of 2×10^6 cells/mL in each well of the plate. A final concentration of 10^6 cells/mL was obtained. The plates were incubated 18 h at 37 °C and monitored measuring the OD_{550nm} using a microplate head. After comparison of the measurements before and after incubation, the concentration for which the increase in the OD was less than 10% with respect to the blank without antibiotic was determined.

For the MIC assays, the concentration of adducts NPS **(4)**, BPS **(5)**, NPL **(6)** and BPL **(7)** was expressed in μg/mL of quinolone equivalents.

Growth Kinetics. Ten milliliters of bacterial suspension at the end of the exponential phase of growth was centrifuged for 4 min at 12000 rpm, then resuspended in succinate culture medium to an OD_{600nm} between 0.02 and 0.04. To 2 mL of this suspension placed in disposable spectrophotometric cuvettes (the medium devoid of bacteria being taken as the reference in the measurements) was added 20 μL of a 100 μM solution of the compound to be assayed (final concentration 1 μM), and the OD_{600nm} was measured as a function of time.

The siderophore and the adducts were obtained as iron complexes after addition of 1 equiv of $FeCl_3$ to the ligand.

All these experiments were performed in sterile conditions. The assayed compounds were previously filtered on cellulose acetate membranes of 0.2 μm porosity. The cuvettes and the stirring magnets were soaked in ethanol overnight then dried before use. The tests were all performed in duplicate.

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