Bioorganic & Medicinal Chemistry 21 (2013) 295–302

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis and antibacterial activity of conjugates between norfloxacin and analogues of the siderophore vanchrobactin

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ARTICLE INFO

Article history:
Received 6 July 2012
Revised 12 October 2012
Accepted 13 October 2012
Available online 29 October 2012

Keywords:
Vibrio anguillarum
Norfloxacin
Antibacterial compounds
Vanchrobactin
Siderophores
Trojan horse strategy

ABSTRACT

From synthetic functionalized analogues of vanchrobactin, a siderophore produced by the fish pathogenic bacteria *Vibrio anguillarum* serotype O2, several vanchrobactin analogues–norfloxacin conjugates were obtained and their antimicrobial activities against the wild-type and mutant strains of *Vibrio anguillarum* serotype O2 have been determined.

1. Introduction

Vibriosis is a highly fatal hemorrhagic septicaemia affecting marine and freshwater fish species throughout the world which is mainly caused by some strains of the fish pathogen *Vibrio anguillarum*. This disease results in considerable economic losses in aquaculture farming worldwide.[1,2] The abusive and indiscriminate use of broad spectrum antibiotics in aquaculture is causing serious problems such as the emergence of pathogenic bacteria resistant to most of the drugs, threats to human health via the food chain and environmental problems due to the presence of antibiotic residues in aquaculture products for human consumption. For all these reasons, the search for more effective and specific treatments against pathogenic bacteria is a priority in aquaculture.[3,4]

Iron is one of the most essential nutrients for all microorganisms. However, iron bioavailabity is limited due to the low solubility of iron(III) at physiological pH. Thus, iron concentration in the open sea is among the lowest observed in earth due to the slightly basic pH of seawater.[5] In response to the restricted access to soluble iron, bacteria evolved high-affinity iron uptake pathways comprised of ferric iron specific carriers, termed siderophores. They are excreted to the extracellular environment where they bind ferric ions to form a complex which is recognized by outer membrane receptors and transported into the cell by an intricate system of membrane-associated proteins. Finally, ferric reductases enzymes can then liberate the internalized iron.[6]

In order to develop suitable targets for antimicrobial therapies, several approaches have being applied using well known iron uptake pathways.[7–10] One of them, the siderophore-mediated drug delivery (Trojan Horse strategy) facilitates the penetration of antibiotics into the bacterial cells transporting the antimicrobial drugs across the bacterial membranes.[11,12] This methodology, inspired in the natural sideromycins,[13,14] consists in the conjugation of a known antibiotic to a siderophore analogue through a linker to enter bacterial cells via active iron uptake pathways.[15,16], In some cases, the drug must be cleaved from the siderophore moiety.[17]

In previous studies, we identified vanchrobactin (**1**)[18] as the second siderophore-mediated system of *V. anguillarum* encoded by a chromosomal gene cluster and its structure as well as a synthesis[19]/regulation[20] scheme was established. The other siderophore-mediated iron uptake system previously described in *V. anguillarum* is a plasmid (pJM1)-encoded siderophore named anguibactin which is uniquely found in pJM1-containing serotype O1 strains.[21] The synthesis of a series of vanchrobactin analogues allowed us to identify two of them, compounds **2** and **3**, which maintain the siderophore activity to *V. anguillarum* and bear an appropriate functionality (an amino group) that can be used as anchor group to attach it to other bioactive agents (Fig. 1). In this

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http://dx.doi.org/10.1016/j.bmc.2012.10.028

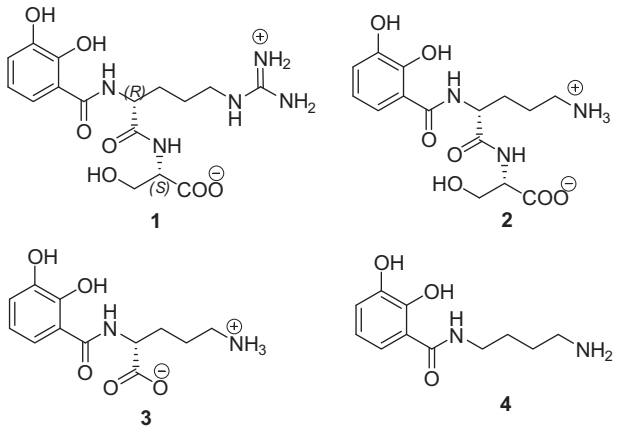


Figure 1. Structures of vanchrobactin (**1**), vanchrobactin analogues **2** and **3**, and aminochelin (**4**).

way, we have considered them as potential antibiotic vectors for the delivery of antibiotics via the bacterial iron uptake systems (Trojan horse strategy).[22]

Later on, we have found that FvtA protein is the outer membrane transporter for vanchrobactin (**1**) and its analogues **2** and **3** in *V. anguillarum*. The gene *fvtA* encoding the vanchrobactin receptor and most vanchrobactin biosynthesis genes are ubiquitous in both vanchrobactin and anguibactin producing *V. anguillarum* strains. These findings reinforced the utility of the iron scavenging system mediated by vanchrobactin as an appropriate candidate for a Trojan horse strategy to develop new drugs potentially effective against fish vibriosis.[23]

Herein we describe the synthesis of several vanchrobactin analogues–norfloxacin conjugates, compounds **5**–**8**, with the aim of exploring their antimicrobial activity against the fish pathogen *Vibrio anguillarum*.

2. Results and discussion

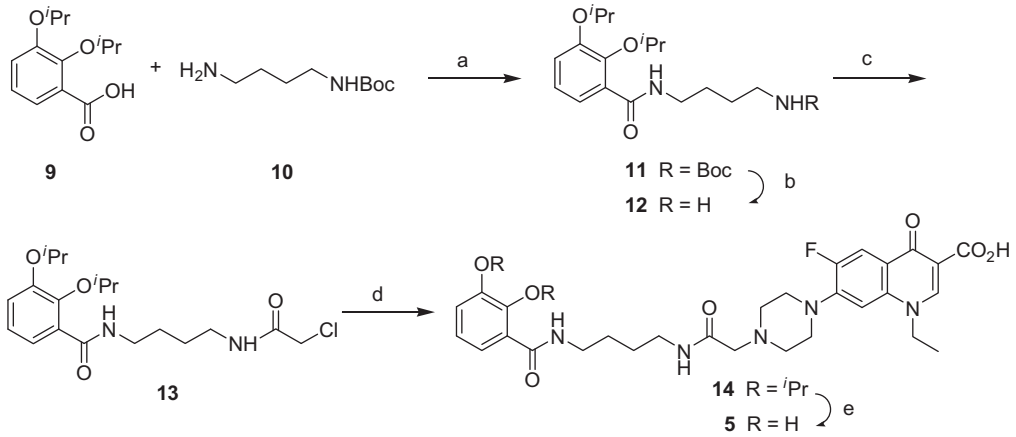
2.1. Chemistry: synthesis of vanchrobactin analogues–norfloxacin conjugates

In the preparation of the conjugates, the antibiotic norfloxacin was chosen because it showed a strong antimicrobial activity against *V. anguillarum* and it has a secondary amine function in the piperazinyl ring that facilitates its conjugation. We have selected the vanchrobactin analogues DHBA-D-ornithine (**2**) and DHBA-D-ornithine–L-serine (**3**) because they keep the siderophore activity when they were tested for growth promotion under iron deficient conditions in the wild-type *V. anguillarum* strain RV22, and also they can be anchored through the amino group to norfloxacin.[12] Secondly, compounds **2** and **3** promote the growth of *V. anguillarum* serotype O2 mutant strain containing the Δ*vabB* mutation (deletion of *vabB* prevents production of vanchrobactin in *V. anguillarum*) while they were not able to promote the growth of *V. anguillarum* serotype O2 double mutant strain containing Δ*vabB* Δ*fvtA* mutations (further deletion of Δ*fvtA* abolish production of vanchrobactin receptor in *V. anguillarum*). This last fact was indicative that FvtA is the sole route of entry for these compounds into the bacteria.[13] An acetate linker was used as space arm to attach the drug to the vanchrobactin analogues.

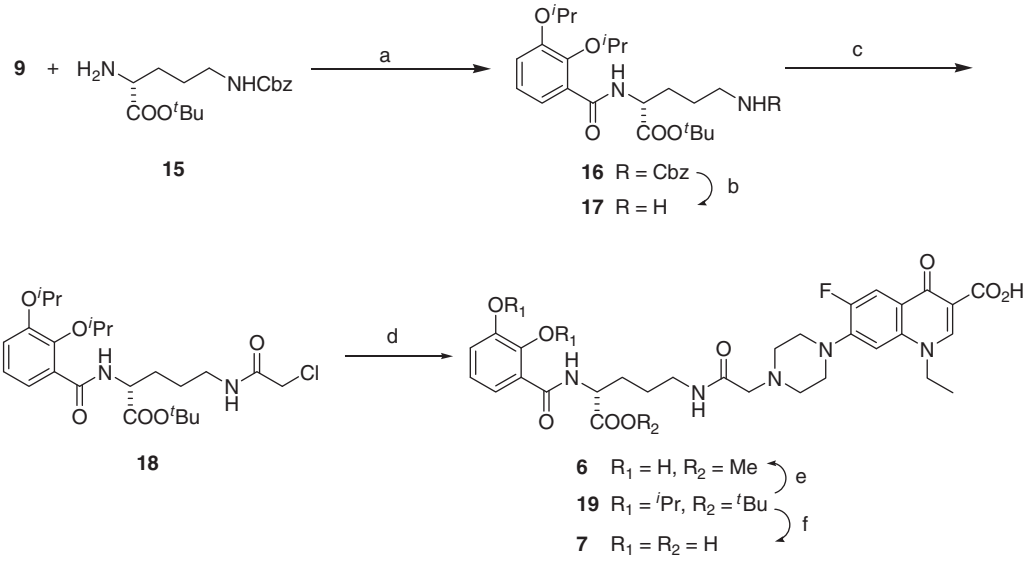
In order to find a simpler analogue of vanchrobactin, we have synthesized aminochelin (**4**), one of the siderophores produced by the Gram-negative free-living soil bacterium *Azotobacter vinelandii*.[24] Although aminochelin (**4**) displayed siderophore activity in *V. anguillarum* serotype O2, it is not using the FvtA receptor as the route of entry since it was able to promote the growth of both a Δ*vabB* mutant strain and a Δ*vabB* Δ*fvtA* double mutant strain.

The synthesis of aminochelin–norfloxacin conjugate **5** started with the coupling of 2,3-diisopropyloxybenzoic acid (**9**)[12] with *tert*-butyl(4-aminobutyl)carbamate (**10**) using TBTU as the coupling agent to give **11** in very good yield. The carbamate of **11** was cleaved quantitatively with trifluoroacetic acid (TFA), affording the free amine **12** which was coupled with chloroacetyl chloride to afford **13**. Coupling reaction between the functionalized vanchrobactin analogue **13** and norfloxacin was carried out in dry DMF in the presence of DIPEA and KI to give compound **14**. The *iso*-propyl protecting group in **14** was removed with BCl_3 in MeOH to afford desired conjugate **5** which was purified by RP-HPLC (Scheme 1).

For the synthesis of DHBA-D-ornithine-norfloxacin conjugates **6** and **7**, N^{δ}-Cbz-D-ornithine-O^{t}Bu (**15**), prepared from the commercially available N^{δ}-carboxybenzoyl-D-ornithine, was coupled with 2,3-diisopropyloxybenzoic acid (**9**)[25] using TBTU as the coupling agent to give the corresponding ester **16** in very good yield. Removal of the Cbz group of fully protecting **16** by catalytic hydrogenation gave the free amine **17**. Following the same procedure as before, the free amine **17** was coupled with chloroacetyl chloride and the resulting chloride **18** was reacted with norfloxacin to give the conjugate **19**. The catechol *iso*-propyl group and the carboxylic



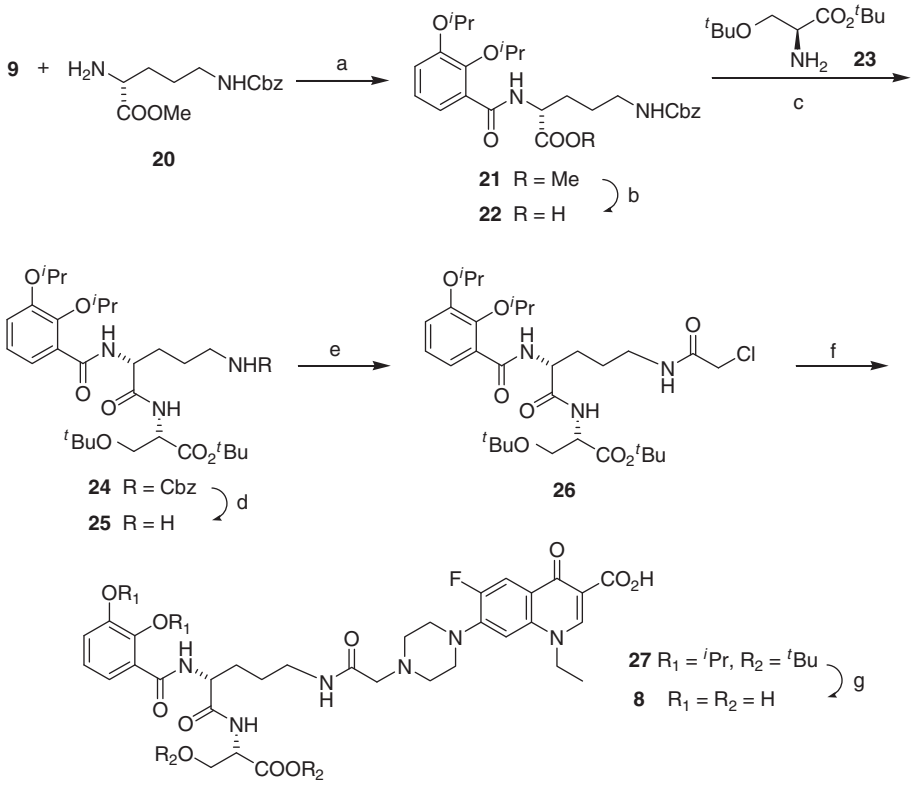
Scheme 1. Synthesis of conjugate **5**. Reagents and conditions: (a) TBTU, Et_3N, DMF, rt, 67%; (b) TFA/CH_2Cl_2 (1:2.3), 3 h, quant; (c) chloroacetyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt, 53%; (d) norfloxacin, DIPEA, KI, DMF, rt, 36 h, quant; (e) 1 M BCl_3/CH_2Cl_2 then MeOH, 43%.



Scheme 2. Synthesis of conjugates **6** and **7**. Reagents and conditions: (a) TBTU, Et_3N, DMF, rt, 53%; (b) H_2, Pd–C, MeOH, overnight, quant; (c) chloroacetyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt, 97%; (d) norfloxacin, DIPEA, KI, DMF, rt, overnight, 64%; (e) 1 M BCl_3/heptanes then MeOH, 15%; (f) 1 M BCl_3/heptanes then H_2O, 30%.

tert-butyl groups in **19** could be removed by the treatment with BCl_3 in MeOH. However, in these conditions the carboxylic acid was methylated giving the corresponding methyl ester **6**. When removal of the protecting groups in **19** was performed with BCl_3 in H_2O the expected conjugate diacid **7** was obtained. Both products were purified by RP-HPLC (Scheme 2).

The synthesis of last conjugate **8** started with coupling of 2,3-diisopropyloxybenzoic acid (**9**) and N^{δ}-Cbz-D-ornithine-OMe (**20**), which was prepared by esterification of the commercially available *N*-Cbz-D-ornithine.[12] The resulting ester **21** was hydrolyzed with barium hydroxide, giving the acid **22** in a quantitative yield. This acid **22** was coupled with *O*-*tert*-butyl-L-serine *tert*-butyl ester (**23**), which was prepared from L-serine with treatment with $HClO_4$ in AcO^tBu, to give the dipeptide **24**. Following a similar scheme as before, removal of the Cbz group giving the free amine **25**, coupling with chloroacetyl chloride to afford **26**, reaction with norfloxacin to obtain **27** and deprotection with BCl_3 in H_2O gave the conjugate **8**, which was finally purified by RP-HPLC (Scheme 3).



Scheme 3. Synthesis of conjugate **8**. Reagents and conditions: (a) TBTU, Et_3N, DMF, rt, 63%; (b) $Ba(OH)_2$, THF/H_2O, quant; (c) TBTU, Et_3N, DMF, rt, 70%; (d) H_2, Pd–C, MeOH, overnight, quant; (e) chloroacetyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt, 59%; (f) norfloxacin, DIPEA, KI, DMF, rt, overnight, 85%; (g) 1 M BCl_3/heptanes then H_2O, 13%.

Table 1
Antibiotic activities of conjugates **5**–**8** and of norfloxacin against the three *Vibrio anguillarum* O2 strains: RV22 (wild-type strain), the Δ*fvtA* mutant MB12 (non vanchrobactin-producing strain) and Δ*vabB* mutant MB84 (vanchrobactin receptor mutant). The CAS test gives negative values: higher numbers indicate higher iron chelating activity

Compound	MIC (μg/mL) RV22/MB12/MB84	CAS test
Norfloxacin	0.006	
5	0.097	−0.541
6	25	NT
7	12.5	−0.612
8	3.125	−0.562
Vanchrobactin		−0.632

NT = not tested.

2.2. Biological evaluations

The antibacterial activities (MIC expressed in μg/mL) of the four conjugates **5**–**8** were evaluated in vitro against three *Vibrio anguillarum* O2 strains: the wild-type strain (RV22), the Δ*fvtA* mutant (MB12) and Δ*vabB* mutant (MB84). As a control, the experiment was repeated with siderophore-free norfloxacin (Table 1). Although the four conjugates showed clear antibacterial activity, this activity was lower than that of the corresponding unconjugated norfloxacin. Conjugate **5** showed the highest antibiotic activity among the conjugates synthesized being less active than norfloxacin by an order of magnitude. The activity of ester conjugate **6** was lower than the corresponding acid conjugate **7**. The four conjugates displayed identical growth inhibitions against the wild-type strain as well as against the Δ*fvtA* mutant and Δ*vabB* mutant and they also were comparable in presence and absence of iron (III). Taken together these results demonstrate that conjugates **5**–**8** do not employ the siderophore uptake system (i.e. FvtA) for entry into *V. anguillarum* and thus do not operate by a Trojan Horse strategy. While disappointing these results do show that introduction of the siderophore moiety on the piperazinyl ring is tolerated since conjugate **5** possesses nearly identical activity as norfloxacin.[26] One explanation for the lack of FvtA-mediated uptake by conjugates **5**–**8** is that the terminal amino group in vanchrobactin analogues **2**–**4** may be critical for recognition by FvtA.

The relative iron complexing capacities of the conjugates were evaluated by reactivity with chrome azurol-S (CAS) assay,[27] and the results are shown in Table 1. All of them gave a positive reaction in this test with a range of values from −0.562 for conjugate **8** to −0.612 for conjugate **7** and they were similar to that of vanchrobactin (−0.632). These results suggest that the binding of the vanchrobactin analogs to norfloxacin through the amino functionality did not affect the chelating ability of these compounds. Thus, the reduced inhibitory activities of the conjugates compared to that of the norfloxacin alone, indicated that the drug conjugation interferes in their action mode as antibiotics.

3. Conclusions

In summary, four vanchrobactin analogue–norfloxacin conjugates (**5**–**8**) were synthesized using acetate as a spacer arm. The biological assays of these compounds against a wild-type strain of *Vibrio anguillarum*, a Δ*fvtA* mutant and a Δ*vabB* mutant showed that the four conjugates displayed antimicrobial activity. The lower antibiotic activity displayed by conjugates **5**–**8** in relation to unconjugated norfloxacin against the same strains indicates that they are not working as Trojan horse conjugates. The fact that the conjugate **5** shows a similar antibiotic activity as norfloxacin suggests that introduction of the siderophore moiety on the piperazinyl ring is tolerated.[28–30] Furthermore, these results seem to indicate that terminal amino group in vanchrobactin analogues **2**–**4** may be critical for recognition by FvtA.

4. Experimental

4.1. Chemistry

Nuclear magnetic resonance spectra (proton and carbon) were recorded on Bruker AC200 F, and 300 or 500 Advance spectrometers at the University of A Coruña, using $CDCl_3$ and CD_3OD as the solvents and internal standards. Multiplicities of ^{13}C signals were obtained by DEPT. Medium-pressure chromatographic separations were carried out on silica gel 60 (230–400 mesh). Optical rotations were determined on a JASCO DIP-1000 polarimeter, with Na (589 nm) lamp and filter. LREIMS and HRESIMS were measured on Applied Biosystems QSTAR Elite. HPLC separations were carried out on an Agilent HP1100 liquid chromatography system equipped with a solvent degasser, quaternary pump and an UV detector (Agilent Technologies, Waldbronn, Germany). A Scharlau HPLC column Nucleosil 120 C18 10 μm 250 × 4 mm was used in the HPLC separations.

All moisture-sensitive reactions were carried out under an atmosphere of argon in flame-dried glassware closed by rubber septum, unless otherwise noted. Solvents were distilled prior to use under argon atmosphere and dried according to standard procedures using the following desiccants: Na/benzophenone for THF and Et_2O, CaH_2 for dichloromethane, pyridine and triethylamine; magnesium for methanol and anhydrous $CaSO_4$ for acetone. DMF was distilled over CaH_2 and was kept over molecular Sieves 4 Å under argon atmosphere. Solutions and solvents were added via syringe or cannula. Thin layer chromatography was performed using sílica gel GF-254 Merck, spots were revealed employing UV light (254 nm) and/or by heating the plate pretreated with an ethanolic solution of phosphomolibdic acid, a solution of cerium sulphate or a solution of ninhydrine in BuOH–AcOH–H_2O. CRYOCOOL apparatus was used for low-temperature reactions.

4.1.1. Synthesis of conjugate 5

4.1.1.1. Compound 11. 2,3-Diisopropoxybenzoic acid (**9**) (1 g, 4.19 mmol) was coupled to *tert*-butyl(4-aminobutyl)carbamate (**10**) (0.79 g, 4.19 mmol) in DMF (25 mL) and triethylamine (1.46 mL, 10.49 mmol) using TBTU as coupling agent (2.02 g, 6.29 mmol). The solvent was removed in vacuo and the residue was pre-absorbed onto silica and purified by column chromatography (silica gel, CH_2Cl_2/MeOH 1%) to yield *tert*-butyl 4-(2,3-diisopropoxybenzamido)butylcarbamate (**11**) as a light yellow oil (1.147 g) in a 67% yield: ^1H NMR (300 MHz, $CDCl_3$) δ_H: 1.27 (d, J = 6.2 Hz, 6H, *i*-Pr); 1.34 (d, J = 6.1 Hz, 6H, *i*-Pr); 1.41 (s, 9H, *t*-Bu); 1.59 (m, 4H); 3.14 (m, 2H); 3.43 (dd, J = 12.7, 6.7 Hz, 2H); 4.53 (m, 1H, *i*-Pr); 4.64 (m, 1H, *i*-Pr); 6.97 (dd, J = 7.9, 1.7 Hz, 1H); 7.05 (t, J = 7.9 Hz, 1H); 7.63 (dd, J = 7.9, 1.7 Hz, 1H); 8.03 (br t, J = 5.1 Hz, NH). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 22.18 (2 CH_3, *i*-Pr); 22.46 (2 CH_3, *i*-Pr); 27.14 (CH_2); 27.87 (CH_2); 28.50 (CH, *t*-Bu); 39.33 (CH_2); 40.32 (CH_2); 71.20 (CH, *i*-Pr); 76.36 (CH, *i*-Pr); 118.32 (CH); 122.90 (CH); 123.81 (CH); 128.61 (CH); 146.05 (C); 150.86 (C); 156.09 (C); 162.63 (C, CO); 165.96 (C, CO). (+)-LREIMS m/z (%): 409 ([M+H]$^+$, 100).

4.1.1.2. Compound 12. Compound **11** (0.814 g, 1.994 mmol) was dissolved in anhydrous CH_2Cl_2 (14 mL), then TFA (6 mL) was added and the mixture was stirred at room temperature. After 3 h, the solvent was concentrated under reduced pressure to afford *N*-4-aminobutyl-2,3-diisopropoxybenzamide (**12**) as an orange oil (0.615 g) in a quantitative yield, which was used without further purification: ^1H NMR (500 MHz, $CDCl_3$) δ_H: 1.26 (d, J = 6.2 Hz, 6H, *i*-Pr); 1.35 (d, J = 6.1 Hz, 6H, *i*-Pr); 1.70 (m, 2H); 1.78 (m, 2H);

3.09 (br s, 2H); 3.42 (m, 2H); 4.54 (m, 1H, *i*-Pr); 4.68 (m, 1H, *i*-Pr); 7.02 (dd, *J* = 7.9, 1.6 Hz, 1H); 7.06 (t, *J* = 7.9 Hz, 3H); 7.49 (dd, *J* = 7.9, 1.6 Hz, 1H); 8.44 (br t, NH). ^{13}C NMR (126 MHz, $CDCl_3$) δ_C: 21.80 (2 CH_3, *i*-Pr); 22.08 (2 CH_3, *i*-Pr); 24.57 (CH_2); 26.43 (CH_2); 38.51 (CH_2); 38.97 (CH_2); 71.13 (CH, *i*-Pr); 76.39 (CH, *i*-Pr); 118.51 (CH); 121.96 (CH); 124.18 (CH); 127.68 (C); 145.96 (C); 150.79 (C); 166.90 (C, CO). (+)-LREIMS *m*/*z* (%): 309 ([M+H]$^+$, 100).

4.1.1.3. Compound 13. Compound **12** (0.071 g, 0.230 mmol) was dissolved in anhydrous CH_2Cl_2 (3 mL) and stirred at 0 °C, then chloroacetyl chloride (46 μL, 0.576 mmol) and Et_3N (80 μL, 0.576 mmol) were added and the mixture was stirred at this temperature during 1 h and for another 2 h at room temperature. After that, the mixture was extracted with CH_2Cl_2 and the organic layer was washed with 1 N HCl, water and brine, dried with anhydrous $MgSO_4$, filtered and concentrated under reduced pressure. The crude was purified by column chromatography (silica gel, CH_2Cl_2/MeOH 95:5) to afford *N*-(4-(2-chloroacetamido)butyl)-2,3-diisopropoxybenzamide (**13**) as a yellow oil (0.047 g) in a 53% yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.29 (d, *J* = 6.3 Hz, 6H, *i*-Pr); 1.36 (d, *J* = 6.0 Hz, 6H, *i*-Pr); 1.66 (m, 4H); 3.36 (m, 2H); 3.44 (m, 2H); 4.03 (s, 2H); 4.55 (m, 1H, *i*-Pr); 4.67 (m, 1H, *i*-Pr); 7.00 (dd, *J* = 8.0, 1.9 Hz, 1H); 7.08 (dd, *J* = 8.0, 7.8 Hz, 1H); 7.64 (dd, *J* = 7.8, 1.9 Hz, 1H); 8.10 (t, *J* = 5.1 Hz, NH); 8.19 (t, *J* = 5.1 Hz, NH).^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 22.21 (2 CH_3, *i*-Pr); 22.52 (2 CH_3, *i*-Pr); 26.92 (CH_2); 27.36 (CH_2); 39.13 (CH_2); 39.69 (CH_2); 42.79 (CH_2–Cl); 71.25 (CH, *i*-Pr); 76.48 (CH, *i*-Pr); 118.43 (CH); 122.84 (CH); 123.90 (CH); 128.45 (C); 146.06 (C); 150.90 (C); 166.14 (C, CO); 166.49 (C, CO). (+)-LREIMS *m*/*z* (%): 385 ([M+H]$^+$, 67).

4.1.1.4. Compound 14. A mixture of norfloxacin (0.048 g, 0.151 mmol) and DIPEA (0.058 g, 0.451 mmol) in DMF (2 mL) was added to a mixture of compound **13** (0.058 g, 0.151 mmol) and KI (0.006 g) also dissolved in DMF (2 mL) and stirred at room temperature over 36 h. Then, the mixture was concentrated under reduced pressure to dryness to afford 100 mg of compound **14** as yellow oil in a quantitative yield: ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.26 (d, *J* = 6.2 Hz, 6H, *i*-Pr); 1.33 (d, *J* = 6.1 Hz, 6H, *i*-Pr); 1.53 (t, *J* = 7.2 Hz, 3H, norfloxacin); 1.64 (m, 4H); 2.77 (m, 4H, norfloxacin); 3.11 (s, 2H); 3.35 (m, 4H, norfloxacin); 3.43 (m, 4H); 4.32 (q, *J* = 7.2 Hz, 2H, norfloxacin); 4.52 (m, 1H, *i*-Pr); 4.65 (m, 1H, *i*-Pr); 6.89 (d, *J* = 6.9 Hz, 1H, norfloxacin); 6.98 (dd, *J* = 7.5, 2.8 Hz, 1H); 7.04 (t, *J* = 7.5 Hz, 1H); 7.61 (br d, *J* = 7.5 Hz, 1H); 8.06 (d, *J* = 13.9 Hz, 1H, norfloxacin); 8.64 (s, 1H, norfloxacin). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 14.57 (CH_3, norfloxacin); 22.13 (2 CH_3, *i*-Pr); 22.45 (2 CH_3, *i*-Pr); 25.91 (CH_2); 27.30 (CH_2); 38.76 (CH_2); 39.62 (CH_2); 49.83 (CH_2, norf); 49.85 (2 CH_2, norf); 53.18 (2 CH_2, norf); 61.40 (CH_2); 70.72 (CH, *i*-Pr); 76.43 (CH, *i*-Pr); 104.30 (CH); 108.35 (C); 112.94 (CH); 118.08 (CH); 119.26 (C); 122.58 (CH); 123.80 (CH); 128.26 (C); 137.20 (C); 145.91 (C); 145.93 (C); 146.00 (CH); 147.24 (C); 150.87 (C); 166.06 (C, CO); 167.34 (C, CO); 169.53 (C, CO); 177.06 (CO). (+)-LREIMS *m*/*z* (%): 668 ([M+H]$^+$, 100).

4.1.1.5. Conjugate 5. To a solution of compound **14** (100 mg, 0.151 mmol) in anhydrous CH_2Cl_2 (5 mL), 1.05 mL (1.05 mmol) of 1 M BCl_3 in CH_2Cl_2 was added at −78 °C and stirred overnight. Then, the reaction was quenched by the addition of MeOH (3 mL), allowed to reach room temperature and concentrated in vacuo to afford a yellow oil residue. Part of this residue (3 mg) was purified by HPLC (Scharlau C18, flow rate 1.5 mL/min, H_2O/MeOH, 1:4 containing 0.1 % TFA, λ = 254 nm) to give 1.2 mg of conjugate **5** (retention time of 11 min) as a colorless oil (43% yield): ^{1}H NMR (300 MHz, CD_3OD) δ_H: 1.55 (t, *J* = 7.1 Hz, 3H, norfloxacin); 1.64 (m, 4H); 2.75 (m, 4H, norfloxacin); 3.11 (s, 2H); 3.17–3.47 (m, 8H); 4.52 (q, *J* = 7.1 Hz, 2H, norfloxacin); 6.68 (t, *J* = 8.0 Hz, 1H); 6.87 (d, *J* = 7.8 Hz, 1H); 7.13 (d, *J* = 7.1 Hz, 1H, norfloxacin); 7.19 (br d, *J* = 8.0 Hz, 1H); 7.99 (d, *J* = 13.3 Hz, 1H, norfloxacin); 8.89 (s, 1H, norfloxacin). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 14.72 (CH_3, norfloxacin); 27.89 (CH_2); 27.96 (CH_2); 39.63 (CH_2); 40.07 (CH_2); 50.83 (CH_2, norfloxacin); 50.87 (CH_2, norfloxacin); 50.95 (CH_2, norfloxacin); 54.20 (2 CH_2, norfloxacin); 62.24 (CH_2); 106.51 (CH); 108.46 (C); 112.89 (CH); 116.76 (C); 118.53 (CH); 119.52 (CH); 121.36 (CH); 138.95 (C); 147.37 (C); 149.45 (C); 150.32 (C); 151.90 (C); 154.07 (CH); 156.06 (C); 169.79 (C, CO); 171.55 (C, CO); 172.62 (C, CO); 178.36 (CO). (+)-LREIMS *m*/*z* (%): 584 ([M+H]$^+$, 100); (+)-HRESIMS: *m*/*z* 584.2518 [M+H]$^+$ (calcd. for $C_{29}H_{35}N_5O_7F$, 584.2515).

4.1.2. Synthesis of conjugates 6 and 7

4.1.2.1. Compound 16. 2,3-Diisopropoxybenzoic acid (**9**) (0.993 g, 4.14 mmol) was coupled to (*R*)-*tert*-butyl 2-amino-5-(((benzyloxy)carbonyl)amino)pentanoate (**15**) (1.343 g, 4.17 mmol) in DMF (37 mL) and triethylamine (1.45 mL, 10.4 mmol) using TBTU as coupling agent (2.010 g, 6.26 mmol). The solvent was removed in vacuo and the residue was pre-absorbed onto silica and purified by column chromatography (silica gel, hexanes/ethyl acetate 1:5) to yield (*R*)-*tert*-butyl 5-(((benzyloxy)carbonyl)amino)-2-(2,3-diisopropoxybenzamido)pentanoate **16** as a light yellow oil (1.251 g) in a 53% yield: $[\alpha]_D^{31}$: +1.74 ($CHCl_3$, *c* = 1.11). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.25 (d, *J* = 6.6 Hz, 3H, *i*-Pr); 1.33 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.34 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.36 (d, *J* = 6.6 Hz, 3H, *i*-Pr); 1.49 (s, 9H, *t*-Bu); 1.51–1.67 (m, 2H); 1.68–1.82 (m, 1H); 1.82–1.96 (m, 1H); 3.12–3.27 (m, 2 H); 4.52 (m, 1 H, *i*-Pr); 4.65 (dt, *J* = 6.6, 6.0 Hz, 1H); 4.77 (m, 1H, *i*-Pr); 5.05 (s, 2H, Cbz); 6.96–7.08 (m, 2H); 7.21–7.36 (m, 4H); 7.66 (dd, *J* = 7.1, 2.2 Hz, 1H); 8.68 (d, *J* = 7.7 Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 14.15; 22.03; 22.10; 22.19; 25.83; 27.98; 29.64; 30.43; 31.36; 36.41; 38.56; 40.56; 52.81; 60.32; 66.43; 71.24; 76.17; 81.85; 119.03; 123.01; 123.42; 127.70; 127.92; 128.40; 136.67; 146.52; 150.74; 156.38; 162.49; 171.24. (+)-LREIMS *m*/*z* (%): 543 ([M+H]$^+$, 75%), 565 ([M+Na]$^+$, 100%); (+)-HRESIMS: *m*/*z* 543.3067 [M+H]$^+$ (calcd. for $C_{30}H_{43}N_2O_4$, 543.3064).

4.1.2.2. Compound 17. Compound **16** (1.177 g, 2.102 mmol) was dissolved in methanol (20 mL) and the flask was flushed with argon. 10% Pd/C (100 mg) was added and the flask was again flushed with argon. The reaction mixture was flushed with hydrogen and stirred under a hydrogen atmosphere overnight. The reaction mixture was filtered through Celite and the solvent was removed in vacuo to afford (*R*)-*tert*-butyl 5-amino-2-(2,3-diisopropoxybenzamido)pentanoate (**17**) as a colorless oil (0.955 g) in a quantitative yield, which was used without further purification: $[\alpha]_D^{31}$: +7.02 ($CHCl_3$, *c* = 0.225); ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.27 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1,33 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.35 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.38 (d, *J* = 6.6 Hz, 3H, *i*-Pr); 1.47 (s, 9H, *t*-Bu); 1.47–1.64 (br s, 1H); 1.68–1.83 (m, 1H); 1.83–2.00 (m, 2H); 2.64–2.80 (br s, 2 H); 4.53 (m, 1H, *i*-Pr); 4.66 (dt, *J* = 6.0, 6.6 Hz, 1H); 4.78 (m, 1H, *i*-Pr); 6.93–7.09 (m, 2H); 7.67 (dd, *J* = 1.7, 7.7 Hz, 1H); 8.66 (d, *J* = 7.1 Hz, 1H, NH). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 21.70; 22.05; 22.13; 22.23; 28.03; 29.66; 30.38; 30.88; 38.57; 41.66; 53.03; 71.26; 76.14; 81.67; 118.99; 123.07; 123.43; 127.88; 146.50; 150.75; 165.23; 171.43. (+)-LREIMS *m*/*z* (%): 409 ([M+H]$^+$, 97); (+)-HRESIMS: *m*/*z* 409.2692 [M+H]$^+$ (calcd. for $C_{22}H_{37}N_2O_5$, 409.2696).

4.1.2.3. Compound 18. Compound **17** (0.909 g, 2.225 mmol) was dissolved in CH_2Cl_2 (20 mL) and stirred at 0 °C, then chloroacetyl chloride (0.44 mL, 5.56 mmol) and Et_3N (0.78 mL, 5.56 mmol) were added. After 4 h, the mixture was distributed between CH_2Cl_2 and aqueous 5% HCl. The organic layer was washed with water and brine, dried with $MgSO_4$, filtered and concentrated under reduced

pressure. The crude was purified by column chromatography (silica gel, CH_2Cl_2/MeOH, 96:4) to afford (*R*)-*tert*-butyl 5-(2-chloroacetamido)-2-(2,3-diisopropoxybenzamido)pentanoate (**18**) as a brown oil (1.046 g) in a 97% yield: $[\alpha]_D^{30}$: +1.55 ($CHCl_3$, *c* = 2.075). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.28 (d, *J* = 6.6 Hz, 3H, *i*-Pr); 1.37 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.38 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.41 (d, *J* = 6.6 Hz, 3H, *i*-Pr); 1.50 (s, 9H, *t*-Bu); 1.60–1.86 (m, 1H); 1.87–2.01 (m, 1H); 3.34 (m, 2H); 4.04 (s, 2H); 4.56 (m, 1H, *i*-Pr); 4.71 (q, *J* = 6.6 Hz, 1H); 4.80 (m, 1H, *i*-Pr); 6.77–6.95 (br s, 1H); 6.99–7.13 (m, 4H); 7.67 (d, *J* = 2.2 Hz, 1H, NH); 8.76 (d, *J* = 7.1 Hz, 1H, NH). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 21.69; 22.05; 22.12; 25.12; 28.01; 29.66; 30.58; 39.31; 40.97; 42.58; 52.17; 71.32; 76.33; 82.17; 119.20; 122.97; 123.52; 127.40; 146.63; 150.79; 165.65; 166.35; 169.11; 171.07. (+)-LREIMS *m*/*z* (%): 507 ($[M+Na]^+$, 27); 485 ($[M+H]^+$, 100); (+)-HRESIMS: *m*/*z* 485.2414 $[M+H]^+$ (calcd. for $C_{24}H_{38}N_2O_6Cl_{35}$, 485.2412).

4.1.2.4. Compound 19. A mixture of compound **18** (152 mg, 0.372 mmol), norfloxacin (119 mg, 0.372 mmol) and KI (186 mg, 1.12 mmol) in DMF (7 mL) was stirred at rt overnight. Then, the mixture was concentrated under reduced pressure, filtered throught a C18 cartridge, and purified by HPLC (Scharlau C18, flow rate 1.5 mL/min, H_2O/MeOH, 1:4 containing 0.05 % TFA) to afford 5 mg of compound **19** (retention time of 13.2 min) as a yellow solid (183 mg) in a 64% yield: $[\alpha]_D^{30}$: +46.57 ($CHCl_3$, *c* = 0.07). ^{1}H NMR (500 MHz, $CDCl_3$) δ_H: 1.28 (d, *J* = 6.5 Hz, 3H); 1.37 (d, *J* = 6.0 Hz, 3H); 1.36 (d, *J* = 6.5 Hz, 3H); 1.38 (d, *J* = 6.0 Hz, 3H); 1.50 (s, 9H); 1.54–1.62 (m, 2H); 1.67 (br s, 3H); 1.77–1.89 (m, 1H); 1.89–2.00 (m, 1H); 3.27 (m, 2H); 3.50–3.57 (m, 4H); 3.57–3.65 (m, 4H); 4.36 (br s, 2H); 4.55 (m, 1H); 4.61 (m, 1H); 4.80 (m, 1H); 7.01–7.09 (m, 3H); 7.64 (d, *J* = 6.4 Hz, 1H); 8.12 (d, *J* = 12.3 Hz, 1H); 8.26 (br s, 1H); 8.72 (s, 1H); 8.97 (d, *J* = 7.1 Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 14.07; 14.58; 21.72; 22.03; 22.13; 22.26; 28.06; 29.70; 38.71; 49.87; 49.91; 49.92; 49.95; 53.04; 53.22; 54.97; 71.18; 76.36; 82.21; 108.45; 113.14; 118.74; 122.59; 123.42; 123.46; 125.01; 127.48; 128.79; 130.89; 135.20; 146.51; 147.26; 150.83; 165.30; 167.17; 167.76; 171.13; 177.00. (+)-LREIMS *m*/*z* (%): 768 ($[M+H]^+$, 100); (+)-HRESIMS: *m*/*z* 768.3962 $[M+H]^+$ (calcd. for $C_{40}H_{55}N_5O_9F$, 768.3978).

4.1.2.5. Conjugate 6. To a solution of compound **19** (90 mg, 0.0391 mmol) in CH_2Cl_2 (6 mL), 1.23 mL (1.23 mmol) of 1 M BCl_3 in heptanes was added at −78 °C and allowed to reach −40 °C for 6 h. Then, the reaction was quenched by the addition of MeOH (3 mL), allowed to reach rt and concentrated in vacuo. The residue was purified by HPLC (Scharlau C18, flow rate 1.5 mL/min, H_2O/MeOH, 55:45 containing 0.05 % TFA) to give 11 mg of conjugate **6** (retention time of 37.00 min) as a white solid in a 15% yield. $[\alpha]_D^{32}$: +20.32 ($CHCl_3$, *c* = 0.37). ^{1}H NMR (500 MHz, CD_3OD) δ_H: 1.58 (t, *J* = 7.1 Hz, 3H); 1.67–1.77 (m, 2H); 1.85–1.97 (m, 1H); 2.01–2.12 (m, 1H); 3.37 (m, 2H); 3.50–3.64 (m, 4H); 3.64–3.83 (m, 4H); 3.78 (s, 3H); 4.03 (s, 2H); 4.59 (q, *J* = 7.1 Hz, 2H); 4.73 (m, 1H); 6.77 (t, *J* = 8.0 Hz, 1H); 6.97 (dd, *J* = 8.0, 1.4 Hz, 1H); 7.29 (d, *J* = 7.0 Hz, 1H); 7.35 (dd, *J* = 8.0, 1.4 Hz, 1H); 8.10 (d, *J* = 12.9 Hz, 1H); 8.94 (s, 1H). ^{13}C NMR (500 MHz, C_6D_6) δ_C: 14.57; 23.22; 29.88; 32.39; 49.82; 49.87, 49.95; 50.10; 52.48; 53.31; 53.33; 105.96; 108.92; 115.12; 115.92; 117.38; 134.93; 137.93; 142.35; 146.27; 148.31; 167.63; 167.69; 169.58; 173.45; 177.68. (+)-LREIMS *m*/*z* (%): 642 ($[M+H]^+$, 100).

4.1.2.6. Conjugate 7. To a solution of compound **19** (30 mg, 0.039 mmol) in CH_2Cl_2 (1 mL), 0.41 mL (0.41 mmol) of 1 M BCl_3 in heptanes was added at −78 °C and allowed to reach −40 °C for 6 h. Then, the reaction was quenched by the addition of H_2O (1 mL), allowed to reach rt and concentrated in vacuo. The residue was purified by HPLC (Scharlau C18, flow rate 1.5 mL/min, H_2O/MeOH, 55:45 containing 0.05% TFA) to give 7 mg of conjugate **7** (retention time of 22.13 min) as a white solid in a 30% yield: $[\alpha]_D^{30}$: +20.27 (MeOH, *c* = 0.225). ^{1}H NMR (300 MHz, D_2O) δ_H: 1.63 (t, *J* = 7.2 Hz, 3H); 1.75 - 1.94 (m, 2H); 1.94–2.11 (m, 1H); 2.11–2.23 (m, 1H); 3.49 (br t, 2H); 3.62–3.85 (m, 8H); 4.21 (s, 2H); 4.60 (q, *J* = 7.2 Hz, 2H); 4.70 (m, 1H); 6.90 (t, *J* = 8.0 Hz, 1H); 7.05 (d, *J* = 8.0 Hz, 1H); 7.23 (d, *J* = 6.9 Hz, 2H); 7.35 (d, *J* = 8.0 Hz, 1H); 7.92 (d, *J* = 12.9 Hz, 1H); 8.90 (s, 1H). ^{13}C NMR (500 MHz, D_2O) δ_C: 13.64; 24.59; 27.88; 38.79; 46.29; 46.32; 50.37; 52.01; 52.75; 56.91; 106.18; 106.41; 111.36; 115.13; 117.45; 119.17; 119.40; 119.64; 137.26; 144.26; 144.51; 146.65; 148.24; 148.27; 162.83; 164.21; 169.73; 175.69; 176.19. (−)-LREIMS *m*/*z* (%): 626 ($[M-H]^-$, 31); (−)-HRESIMS: *m*/*z* 626.2296 $[M-H]^-$ (calcd. for $C_{30}H_{33}N_5O_9F$, 626.2267).

4.1.3. Synthesis of conjugate 8

4.1.3.1. Compound 21. 2,3-Diisopropoxybenzoic acid (**9**) (0.890 g, 3.74 mmol) was coupled to (*R*)-methyl 2-amino-5-(((benzyloxy)carbonyl)amino)pentanoate (**20**) (1.119 g, 3.74 mmol) in DMF (37 mL) and triethylamine (1.3 mL, 9.35 mmol) using TBTU as coupling agent (1.804 g, 5.62 mmol). The solvent was removed under reduced pressure and the obtained residue was pre-absorbed onto silica and purified by column chromatography (silica gel, gradient of hexanes/ethyl acetate from 1:3 to 1:5) to give 1.136 g of (*R*)-methyl 5-(((benzyloxy)carbonyl)amino)-2-(2,3-diisopropoxybenzamido)pentanoate (**21**) as a light yellow oil in a 63% yield: $[\alpha]_D^{30}$: +6.1 ($CHCl_3$, *c* = 1.15). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.28 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.37 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.38 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.40 (d, *J* = 6.0 Hz, 3H, *i*-Pr); 1.54–1.73 (m, 2H); 1.73–1.89 (m, 1H); 1.89–2.06 (m, 1H); 3.25 (q, *J* = 6.0 Hz, 2H); 3.77 (s, 3H, methyl ester); 4.57 (m, 1H, *i*-Pr); 4.82 (m, 1H); 4.84 (m, 1H, *i*-Pr); 5.10 (s, 2H, Cbz); 6.99–7.11 (m, 2H, aromatic protons), 7.28–7.40 (m, 5H, aromatic protons); 7.70 (dd, *J* = 7.6, 2.0 Hz, 1 H); 8.75 (d, *J* = 7.7 Hz, 1H, NH). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 14.19; 21.60; 22.06; 22.14; 22.31; 26.03; 30.22; 40.54; 52.16; 52.28; 66.61; 71.26; 76.58; 77.20; 99.98; 119.00; 123.09; 123.54; 127.43; 128.03; 127.47; 136.58; 146.43; 150.73; 156.34; 165.39; 172.61. (+)-LRESIMS *m*/*z* (%): 501 ($[M+H]^+$, 100); (+)-HRESIMS: *m*/*z* 501.2572 $[M+H]^+$ (calcd. for $C_{27}H_{37}N_2O_7$, 501.2595).

4.1.3.2. Compound 22. To a solution of compound (*R*)-methyl 5-(((benzyloxy)carbonyl)amino)-2-(2,3-diisopropoxybenzamido)pentanoate (**21**) (1.055 g, 2.11 mmol) in THF (25 mL) and water (25 mL), 2.070 g (4.58 mmol) of barium hydroxide octahydrate was added and the mixture was stirred at 50 °C overnight. Then, DOWEX-50 W(H^+) was added to the solution to give a pH = 4 and the resin was removed by filtration. The resulting filtrate was evaporated under reduced pressure to give 1.018 g of compound (*R*)-5-(((benzyloxy)carbonyl)amino)-2-(2,3-diisopropoxybenzamido)pentanoic acid (**22**) as a brown oil in a quantitative yield: $[\alpha]_D^{31}$: +4.2 ($CHCl_3$, *c* = 0.195). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.23 (d, *J* = 6.2 Hz, 3H, *i*-Pr); 1.33 (d, *J* = 5.9 Hz, 3H, *i*-Pr); 1.35 (d, *J* = 6.2 Hz, 3H, *i*-Pr); 1.38 (d, *J* = 5.86 Hz, 3H, *i*-Pr); 1.46–1.73 (m, 2H); 1.71–2.13 (m, 2H); 3.02–3.31 (m, 2H); 4.41–4.64 (m, 1H, *i*-Pr); 4.69–4.90 (m, 2H); 5.05 (s, 2H, Cbz); 6.95–7.12 (m, 1H); 7.20–7.38 (m, 1H); 7.61–7.73 (m, 5H); 7.65 (dd, *J* = 5.5, 3.6 Hz, 1 H); 8.76–8.94 (m, 1H, NH). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 21.58; 22.04; 22.12; 22.24; 25.85; 29.87; 30.94; 40.49; 52.35; 55.23; 66.59; 71.19; 78.21; 119.03; 122.94; 126.60; 127.21; 127.98; 128.45; 136.87; 146.46; 150.70;151.65; 165.00; 172.56; 174.28. (+)-LREIMS *m*/*z* (%): 487 ($[M+H]^+$, 100); (+)-HRESIMS: *m*/*z* 487.2420 $[M+H]^+$ (calcd. for $C_{26}H_{35}N_2O_7$, 487.2438).

4.1.3.3. Compound 23. (*S*)-2-Amino-3-hydroxypropanoic acid (1 g, 9.43 mmol) was suspended in AcO^tBu (30 mL), $HClO_4$ (0.9 mL, 70% in water, 10.4 mmol) was added and the mixture

was stirred for 4 h. After that time, the solution was poured onto a saturated $NaHCO_3$ aqueous (100 mL) and stirring for 0.5 h. Then, the mixture was partitioned between water and CH_2Cl_2, the aqueous phase was extracted with CH_2Cl_2 and the organic layer was washed with brine and dried over $MgSO_4$, filtered and concentrated in vacuo to afford 1.65 g of (*S*)-*tert*-butyl 2-amino-3-*tert*-butoxypropanoate (**23**) as a colorless oil in a 80% yield: $[\alpha]_D^{31}$: −5.0 ($CHCl_3$, c = 4.71). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.15 (s, 9H); 1.45 (s, 9H); 1.97 (br s, 2H); 3.46 (br s, 1H); 3.51–3.66 (m, 2H). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 27.55; 28.20; 55.69; 63.99; 72.98; 81.12; 173.39. (+)-LREIMS m/z (%): 218 ([M+H]$^+$, 100); (+)-HRESIMS: m/z 218.1721 [M+H]$^+$ (calcd. for $C_{11}H_{24}NO_3$, 218.1756).

4.1.3.4. Compound 24. (*R*)-5-(((Benzyloxy)carbonyl)amino)-2-(2,3-diisopropoxybenzamido) pentanoic acid (**22**) (1 g, 2.13 mmol) was coupled to (*S*)-*tert*-butyl 2-amino-3-*tert*-butoxypropanoate (**23**) (0.460 g, 2.13 mmol) in anhydrous DMF (10 mL) and triethylamine (0.7 mL, 5.33 mmol) using TBTU as coupling agent (1.030 g, 3.20 mmol). The solvent was removed under reduced pressure and the residue was pre-absorbed onto silica and purified by column chromatography (silica gel, gradient of hexanes/ethyl acetate from 1:1 to 1:2) to give 1.020 g of compound **24** as a light yellow oil in a 70% yield: $[\alpha]_D^{31}$: +17.3 ($CHCl_3$, c = 3.595). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 1.08 (s, 9H); 1.26 (d, J = 6.3 Hz, 3H); 1.34 (d, J = 6.3 Hz, 3H); 1.35 (d, J = 6.0 Hz, 3H); 1. 38 (d, J = 6.0 Hz, 3H); 1.41 (s, 9H); 1.53–1.71 (m, 2H); 1.73–1.89 (m, 2H); 1.90–2.02 (m, 2H); 3.53 (dd, J = 8.8, 3.1 Hz, 1H); 3.74 (dd, J = 8.8, 2.9 Hz, 1H); 4.56 (m, 2H); 4.79 (m, 2H); 5.07 (s, 2H); 7.04 (t, J =7.3 Hz, 1H); 7.05 (dd, J = 7.3, 1.7 Hz, 1H); 7.68 (dd, J = 7.3, 2.3 Hz, 1H); 8.76 (m, 1H). ^{13}C NMR (200 MHz, $CDCl_3$) δ_C: 20.56; 21.14; 21.38; 21.58; 21.62; 21.74; 21.85; 26.82; 27.49; 30.88; 38.12; 51.94; 52.84; 59.96; 65.78; 70.77; 75.77; 79.37; 81.04; 122.34; 122.49; 122.94; 123.08; 127.39; 127.43; 127.48; 127.93; 127.96; 146.08; 150.33; 156.29; 164.90; 168.66; 170.55; 172.18. (+)-LREIMS m/z (%): 686 ([M+H]$^+$, 100).

4.1.3.5. Compound 25. Compound **24** (0.506 g, 0.738 mmol) was hydrogenated in a similar way as **16** to afford 0.407 g of compound **25** as a colorless oil in a quantitative yield, which was used without further purification: $[\alpha]_D^{31}$: +13.9 ($CHCl_3$, c = 0.245). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.15 (s, 9H); 1.31 (d, J = 6.2 Hz, 3H); 1.34 (d, J = 6.0 Hz, 3H); 1.35 (d, J = 6.0 Hz, 3H); 1.41 (d, J = 6.2 Hz, 3H); 1.47 (s, 9H); 1.52–1.68 (m, 2H); 1.87–2.02 (m, 2H); 2.67 (m, 2H); 3.53 (dd, J = 8.3, 3.6 Hz, 1H); 3.60 (dd, J = 8.3, 4.9 Hz, 1H); 4.54 (m, 2H); 4.76 (m, 2H); 7.02 (dd, J = 7.9, 1.8 Hz, 1H); 7.07 (t, J =7.9 Hz, 1H); 7.69 (dd, J = 7.9, 1.8 Hz, 1H); 8.88 (d, J = 5.6 Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 21.24; 21.51; 22.15; 22.20; 22.23; 27.44; 27.48; 28.02; 29.76; 41.93; 51.10; 55.64; 62.22; 71.50; 76.51; 80.99; 81.74; 119.49; 123.20; 123.50;128.22; 146.84; 150.90; 165.86; 170.94; 171.52. (+)-LRESIMS m/z (%): 552 ([M+H]$^+$, 100%); (+)-HRESIMS: 552.3637 [M+H]$^+$ (calcd. for $C_{29}H_{50}N_3O_7$ [M+H]$^+$, 552.3643).

4.1.3.6. Compound 26. Compound **25** (300 mg, 0544 mmol) was coupled with chloroacetyl chloride (0.11 mL, 1.36 mmol) in a similar way as **12** and **17**. The crude was purified by column chromatography (silica gel, CH_2Cl_2/MeOH 96:4) to afford 203 mg of compound **26** as brown oil in a 59% yield: $[\alpha]_D^{31}$: +15.17 ($CHCl_3$, c = 0.530). ^{1}H NMR (200 MHz, $CDCl_3$) δ_H: 1.07 (s, 9 h); 1.21–1.49 (m, 12H); FALTA 1tBu 1.57–2.04 (m, 4H); 2.24–2.95 (m, 2H); 3.17–3.56 (m, 3H); 3.66–3.85 (m, 1H); 4.02 (s, 2H); 4.44–4.63 (m, 2H); 4.65–4.88 (m, 1H); 6.83–7.16 (m, 3H); 7.61–7.71 (m, 1H); 8.70–8.80 (m, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C: 21.90; 22.03; 22.10; 22.21; 25.34; 27.28; 27.93; 30.26; 38.58; 39.18; 42.61; 52.90; 53.22; 61.98; 71.29; 73.03; 76.07; 76.35; 78.55; 81.81; 119.22; 123.07; 123.46; 127.35; 146.53; 150.70; 165.56; 166.08; 168.93; 170.86. (+)-LREIMS m/z (%): 628 ([M+H]$^+$, 100%); (+)-HRESIMS: 628.3353 ([M+H]$^+$ (calcd. for $C_{31}H_{50}Cl_{35}N_3O_8$, 628.3359).

4.1.3.7. Compound 27. Compound **26** (182 mg, 0.376 mmol) was coupled with norfloxacin (93 mg, 0.290 mmol) in a similar way as **13** and **18**. The resulting mixture was concentrated in vacuo, filtered through a C18 cartridge and purified by HPLC (Scharlau C18, flow rate 1.5 mL/min, H_2O/MeOH, 1:4 containing 0.05 % TFA) to afford compound **27** (retention time of 14.33 min) as a yellow solid (224 mg) in a 85% yield: $[\alpha]_D^{31}$: +16.30 ($CHCl_3$, c = 0.745). ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 1.08 (s, 9H); 1.27 (d, J = 6.2 Hz, 3H); 1.33 (d, J = 6.0 Hz, 9H); 1.39 (s, 9H); 1.53 (t, J = 7.2 Hz, 3H); 1.62–1.74 (m, 2H); 1.84 (m, 1H); 1.96 (m, 1H); 3.37 (m, 2H); 3.57 (dd, J = 9.0, 3.2 Hz, 1H); 3.61–3.70 (m, 4H); 3.74 (dd, J = 9.0, 3.2 Hz, 1H); 4.03 (br s, 2H); 4.36 (br s, 2H); 4.44–4.61 (m, 2H); 4.72–4.86 (m, 2H); 6.99 (m, 3H); 7.54 (t, J = 4.8 Hz, 1H); 7.95 (d, J = 12.5 Hz, 1H); 8.41 (br s, 1H); 8.66 (br s, 1H); 8.88 (d, J = 7.5 Hz, 1H). ^{13}C NMR (300 MHz, $CDCl_3$) δ_C : 14.45; 21.92; 22.00; 22.03; 22.07; 22.15; 24.64; 27.23; 27.92; 30.66; 39.06; 46.83; 46.86; 46.89; 49.85; 52.10; 53.03; 53.39; 57.41; 61.93; 71.31; 76.41; 77.22; 81.97; 105.37; 108.15; 112..73; 119.21; 121.46; 122.58; 123.43; 126.85; 137.00; 144.28; 146.60; 147.47; 150.79; 154.94; 163.65; 165.94; 167.32; 169.23; 171.21; 176.76. (+)-LREIMS m/z (%): 911 ([M+H]$^+$, 100%); (+)-HRESIMS: 911.4917 [M+H]$^+$ (calcd. for $C_{47}H_{67}FN_6O_{11}$, 911.4924).

4.1.3.8. Conjugate 8. To a solution of compound **27** (50 mg, 0.055 mmol) in CH_2Cl_2 (3 mL), 0. 768 mL (0.768 mmol) of 1 M BCl_3 in heptanes was added at −78 °C and allowed to reach −40 °C for 6 h. Then, the reaction was quenched by the addition of H_2O (3 mL), allowed to reach rt and concentrated in vacuo. The residue was purified by HPLC (Scharlau C18, flow rate 1.5 mL/min, H_2O/MeOH, 55:45 containing 0.05 % TFA) to give 5 mg of conjugate **8** (retention time of 21.43 min) as a white solid in a 13% yield: $[\alpha]_D^{32}$: +29.71 (MeOH, c = 0.105). ^{1}H NMR (500 MHz, CD_3OD) δ_H: 1.57 (t, J = 7.1 Hz, 3H), 1.64–1.79 (m, 2H), 1.79–1.95 (m, 1H), 1.95–2.12 (m, 1H), 3.45 (m, 2H), 3.60 (br s, 4H), 3.70 (br s, 4H), 3.88 (dd, J = 11.3, 3.8 Hz, 1H), 3.97 (dd, J = 11.3, 4.7 Hz, 1H), 4.04 (s, 2H), 4.53–4.63 (m, 4H), 6.78 (t, J = 7.9 Hz, 1H), 6.98 (dd, J = 7.9, 1.4 Hz, 1H), 7.27 (d, J = 7.0 Hz, 1H), 7.36 (dd, J = 7.9, 1.4 Hz, 1H), 8.05 (d, J = 12.9 Hz, 1H), 8.90 (s, 1H). ^{13}C NMR (500 MHz, CD_3OD) δ_C: 13.45; 25.13; 29.14; 38.42; 46.63; 49.72; 52.35; 52.67; 53.35; 54.83; 56.91; 56.93; 61.29; 106.16; 107.18; 111.19; 111.63; 116.01; 118.45; 118.52; 118.66; 129.34; 137.39; 145.70; 148.37; 156.16; 156.28; 164.26; 168.19; 169.34; 172.00; 172.72; 176.92. (−)-LREIMS m/z (%): 713 ([M−H]$^-$, 100%); (−)-HRESIMS: 713.2581 [M−H]$^-$ (calcd. for $C_{33}H_{39}FN_6O_{11}$, 713.2588).

4.2. Biological activity

The antibacterial activity was evaluated by determining the Minimum Inhibitory Concentration (MIC) for each compound. MIC was defined as the lowest concentration at which no visible growth was observed. MICs were calculated in a micro-well dilution assay as follows. Thirty-two serial twofold dilutions of each assayed compound were prepared to obtain a final concentration range from 100 to 9.2×10^{-8} μg/mL in 50 μL of CM9 medium. Overnight cultures of strains RV22, MB12 (non vanchrobactin-producing strain) and MB84 (vanchrobactin receptor FvtA mutant) in TSB medium were used as inocula. The cultures were diluted 1:20 in CM9 medium containing 80 μM of the iron chelator 2,2′-dipyridil to achieve iron limiting conditions for bacterial growth. Each micro-plate well contained a final volume of 100 μL of CM9 at a 40 μM of 2,2′-dipyridil. The growth assays were also performed in parallel using CM9 medium plus 10 μM $FeCl_3$ instead 2,2′-dipyridil to obtain iron repleting growth conditions. The plates

were incubated at 25 °C for 24 h. For each bacterial strain and for each condition (iron limited and iron repleting medium), three rows of eight wells in a microtiter plate were used. All tests were performed in triplicate and the MIC was expressed as the mean of the inhibition values obtained.

4.3. Siderophore activity

Iron chelating activity of each compound was measured using the CAS (chrome-azurol S) spectrophotometric assay.[27] This assay is based in the change of color of CAS dye when it is bound to Fe(III) or when it is Fe(III) free. When complexed with Fe(III) the CAS solution has a blue color, while in the absence of Fe(III) it shows an orange color. The change is measured by absorbance at 630 nm. In the presence of a strong iron chelator like a siderophore, the A_{600} values are lower. To measure the iron chelating activity the compounds were diluted 1/100 with distilled water and mixed with an equal volume of the CAS solution (prepared as described by Schwyn & Neilands).[27] The A_{600} was measured against a blank made by using equal volumes of distilled water and CAS.

Acknowledgments

This work was financially supported by Grants from the Ministry of Science and Innovation of Spain (CTQ2008-04024 and AGL2009-12266-C02-02) and Xunta de Galicia (10PXIB235157PR). We also thank to Servizos de Apoio a Investigación from University of A Coruña (SAI-UDC) for the analytical support.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.10.028.

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