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Synthesis and antibacterial evaluation of amino acid–antibiotic conjugates

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ABSTRACT

Amino acid conjugates of quinolone, metronidazole and sulfadiazine antibiotics were synthesized in good yields using benzotriazole methodology. All the conjugates were screened for their antibacterial activity using methods adapted from the Clinical and Laboratory Standards Institute. Antibiotic conjugates were tested for activity in four medically relevant organisms; *Staphylococcus aureus* (RN4220), *Escherichia coli* (DH5α), *Pseudomonas aeruginosa* (PAO1), and *Bacillus subtilis* (168). Several antibiotic conjugates show promising results against several of the strains screened.

The increasing incidence of infection caused by the rapid onset of bacterial resistance to available antibiotics is a serious health problem.[1] While many factors may cause mutations in microbial genomes, it has been demonstrated that the incorrect use of antibiotics can greatly increase the development of resistant genotypes.[2] As multidrug-resistant bacterial strains proliferate, the necessity for effective therapy has stimulated research into the design and synthesis of novel antimicrobial molecules. Many versatile bioactive molecules are peptides and many peptide hormones and analogous shorter peptides exert their action by binding to membrane receptors.[3] Peptide derivatives can exhibit antimicrobial,[4] antiviral,[5] anticancer activity[6] etc. and can open up new perspectives in drug design as highly specific and non-toxic pharmaceuticals. In recent years, these synthesis-based derivatives have received considerable attention.[7,8] Currently there is much interest in conjugates of amino acid or peptide residues with bioactive heterocyclic motifs in the field of biomedical research taking advantage of the low toxicity, biocompatibility and structural diversity of amino acids.[9]

Cell-permeating antimicrobial agents can potentially play an important role in eliminating infections by intracellular pathogens. Unfortunately, many antibiotic classes do not penetrate the plasma membrane effectively (for example C/E ratio of fluoroquinolones is 4–10; β-Lactams is <1; metronidazole is 1).

Prodrugs serve to improve drug physicochemical properties that in turn increase drug concentration at an active site and hence prolong the effect, while decreasing, toxicity and side effects. A prodrug should be stable in the stomach and in the small intestine, nontoxic, biodegradable and biocompatible, whether it has low molecular weight (amino acid, carbohydrate) or is a macromolecule (polymers).[10]

Prodrugs formed from quinolone acids and amino acid esters are more lipophilic than the parent drugs[11,12] and can show enhanced in vivo antibacterial properties[13–15] with pronounced therapeutic effects against *Pseudomonas aeruginosa*,[16,17] *Escherichia coli*,[18] *Staphylococcus aureus*[19] and *Salmonella typhi*.[15]

The antibiotics chosen for chemical modification have a wide range of activity. All but Metronidazole are considered broad-spectrum antibiotics with activity against Gram-(+) and Gram-(−) bacteria. Metronidazole, a nitro-imidazole derivative, acts through DNA inhibition and affects both protozoa and bacteria. Unlike other antibiotics, metronidazole is primarily active against anaerobic bacteria though there are some reports of effects on aerobic bacteria.[20] The fluoroquinolones chosen in this study include ciprofloxacin, a second-generation fluoroquinolone that inhibits topoisomerase, and norfloxacin, a second-generation

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fluoroquinolone and a synthetic chemotherapeutic antibacterial agent that targets DNA gyrase and topoisomerase IV. Pipemedic acid, a pyridopyrimidine, is a first generation quinolone that targets topoisomerase and is reportedly active against *P. aeruginosa* as well as several Gram positive pathogens.[21] Fluoroquinolones can cause adverse reactions in the central nervous system, skin and gastrointestinal tract.[22] Sulfadiazine, a sulfonamide, acts through inhibition of purine metabolism and prevents DNA and RNA synthesis. Peptide derivatives may decrease arbitrary degradation of antibiotic compounds thus increasing concentration at a target site; these derivatives may maintain or improve antibacterial activity while diminishing undesirable side effects because the initial dosage may be lowered if more of the antibiotic is reaching the target.

Staphylococcus aureus (RN4220), *Escherichia coli* (DH5**α**), and *Pseudomonas aeruginosa* (PAO1) were selected for antibiotic conjugate screening because of their physiological relevance and close relation to pathogenic strains which cause disease in humans. Additionally, we chose to evaluate their antibiotic activity on *B. subtilis*, a common gut commensal bacterium.[23]

N-Acylbenzotriazoles[19] are efficient reagents for N-, O-, S- and C-acylation[24] and when prepared from *N*-protected α-amino acids have been utilized for the synthesis of di-, tripeptides.[25]

We now report syntheses of diverse classes of antibiotics-amino acid conjugates by coupling ciprofloxacin **3**, pipemidic acid **5**, norfloxacin **7**, metronidazole **9** and sulfadiazine **11** with Cbz-*N*-(aminoacyl)benzotriazoles **2a**–**e**.

The coupling of ciprofloxacin (Cip) **3**, pipemidic acid (Pip) **5** and norfloxacin **7** with Cbz-*N*-(aminoacyl)benzotriazoles **2a**–**e** (prepared by our reported procedures frorm Cbz-protected amino acids **1a**–**e**) in aqueous MeCN in the presence of Et_3N for 3 h resulted in the formation of conjugates: amino acid–ciprofloxacin **4a**–**c** (68–77%), amino acid–pipemidic acid **6a**–**e** (51–82%) and amino acid–norfloxacin (75–86%)[26] (Scheme 1, Table 1).

Compounds **2a**–**e** were reacted with metronidazole **9** in the presence of a catalytic amount of dimethylaminopyridine (DMAP) under microwave irradiations at 60 °C and 50 W for 1 h to afford novel amino acid–metronidazole conjugates **10a**–**e** in good yields (72–85%)[27] (Scheme 2, Table 2).

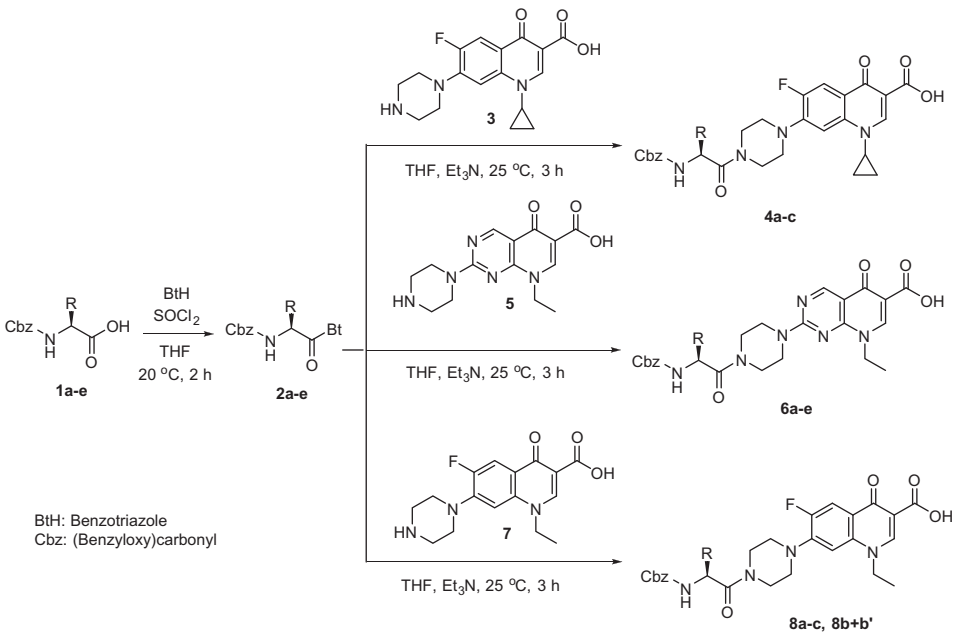
The coupling of sulfadiazine (Sul) **11** with Cbz-protected amino acids in THF in the presence of *N*-methylmorpholine and isobutyl chloroformate at room temperature for 2 h resulted in the formation of amino acid–sulfadiazine conjugates (**12a**–**c**)[28] (Scheme 3, Table 3).

It is believed that the strong lipophilic character of a drug plays an essential role in producing an antimicrobial effect. This property is related to membrane permeation in biological systems. Many of the processes of drug disposition depend on the ability to cross cellular membranes and hence there is a high correlation with lipophilicity. Hydrophobic drugs with high partition coefficients are preferentially distributed to hydrophobic compartments such as lipid bilayers of cells while hydrophilic drugs (low partition coefficients) preferentially are found in hydrophilic compartments such as blood serum.

Hydrophobicity/lipophilicity plays a major role in determining where drugs are distributed within the body after adsorption and as a consequence, in how rapidly they are metabolized and excreted. In this context, the presence of a hydrophobic moiety is important for activity. Moreover, many of the proteins involved in drug disposition have hydrophobic binding sites thus adding to the importance of lipophilicity.[29,30]

The lipophilicity of the compounds, expressed as log*P*, is the main predictor of the activity. The octanol/water partition coefficient *C*log*P* is a measure of hydrophobicity/lipophilicity and was calculated using ChemDraw Ultra 13.0 software integrated with Cambridgesoft Software (Cambridgesoft Corporation). The results are given in Table 4. The calculated values of log*P* for conjugates are higher than for the corresponding parent antibiotic.

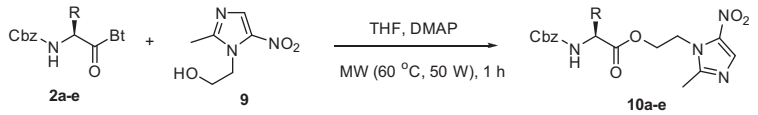
Growth inhibition was determined by comparing treated cell cultures to untreated control cultures. The cell density of the samples which were treated with the parent antibiotic or conjugate antibiotics along with the control cultures were determined by analyzing 300 μL samples in a spectrophotometer. The OD600 of the control cultures was considered to be maximum cell growth. The optical density of treated cultures was compared to control cultures to determine percent growth inhibition using the following equation:



Scheme 1. Synthesis of amino acid–ciprofloxacin (**4a**–**c**), amino acid–pipemidic acid (**6a**–**e**) and amino acid–norfloxacin conjugates (**8a**–**c**, **8b+b′**).

Table 1
Preparation of amino acid–quinolone antibiotic conjugates **4a–c**, **6a–e** and **8a–c**

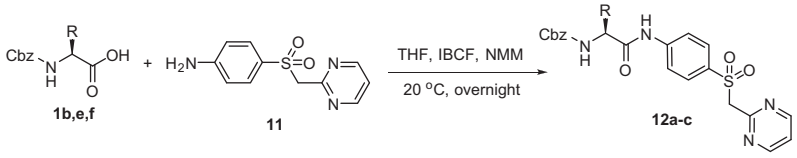
Entry	Reactant 2	Product	Yield (%)	Mp (°C)
1	Cbz-Gly-Bt **2a**	Cbz-Gly-Cip-OH **4a**	68	150–152
2	Cbz-L-Ala-Bt **2b**	Cbz-L-Ala-Cip-OH **4b**	72	128–130
3	Cbz-L-Lys(Cbz)-Bt **2c**	Cbz-L-Lys(Cbz)-Cip-OH **4c**	77	135–137
4	Cbz-Gly-Bt **2a**	Cbz-Gly-Pip-OH **6a**	82	>300
5	Cbz-L-Ala-Bt **2b**	Cbz-L-Ala-Pip-OH **6b**	51	>300
6	Cbz-L-Val-Bt **2d**	Cbz-L-Val-Pip-OH **6c**	68	>300
7	Cbz-L-Phe-Bt **2e**	Cbz-L-Phe-Pip-OH **6d**	64	>300
8	Cbz-L-Lys(Cbz)-Bt **2c**	Cbz-L-Lys(Cbz)-Pip-OH **6e**	80	140–142
9	Cbz-Gly-Bt **2a**	Cbz-Gly-Nor-OH **8a**	82	214–216
10	Cbz-L-Ala-Bt **2b**	Cbz-L-Ala-Nor-OH **8b**	79	192–194
11	Cbz-DL-Ala-Bt **2b+2b′**	Cbz-DL-Ala-Nor-OH **8b+8b′**	75	210–212
12	Cbz-L-Phe-Bt **2e**	Cbz-L-Phe-Nor-OH **8c**	86	195–197



Scheme 2. Synthesis of amino acid–metronidazole conjugates **10a–e**.

Table 2
Preparation of amino acid–metronidazole conjugates **10a–e**

Entry	Reactant **2**	Product **10**	Yield (%)	Mp (°C)
1	Cbz-Gly-Bt **2a**	Cbz-Gly-Met-OH **10a**	82	Oil
2	Cbz-L-Ala-Bt **2b**	Cbz-L-Ala-Met-OH **10b**	85	Oil
3	Cbz-L-Val-Bt **2d**	Cbz-L-Val-Met-OH **10c**	83	Oil
4	Cbz-L-Phe-Bt **2e**	Cbz-L-Phe-Met-OH **10d**	82	Oil
5	Cbz-L-Lys(Cbz)-Bt **2c**	Cbz-L-Lys(Cbz)-Met-OH **10e**	72	Oil



Scheme 3. Preparation of amino acid–sulfadiazine conjugates **12a–c**.

Table 3
Preparation of amino acid–sulfadiazine conjugates **12a–c**

Entry	Reactant **1**	Product **12**	Yield (%)	Mp (°C)
1	Cbz-L-Ala-OH **1b**	Cbz-L-Ala-Sul-OH **12a**	65	Oil
2	Cbz-L-Phe-OH **1e**	Cbz-L-Phe-Sul-OH **12b**	70	Oil
3	Cbz-L-Asp(Bzl)-OH **1f**	Cbz-L-Asp(Bzl)-Sul-OH **12c**	72	Oil

$[OD600_{control} - OD600_{treated}] * 100/[D600_{control}]$. Control and conjugate family antibiotics were tested in parallel using biological duplicates and three concentrations per compound. Tables 5–8 display antibiotic conjugates which had comparable or greater growth inhibition than the unconjugated parent drug. Antibiotic conjugates that showed little or no growth inhibition can be found in Supplemental Tables S2–S16.

Some conjugate drugs show greater inhibitory activity than the parent drug, which may be a result of several independent or combined mechanisms. The conjugate tag may increase the concentration of the compound inside the cell; the conjugate tag may also block sites on the compound that interact with antibacterial resistance proteins, thus preventing the inactivation of the drug. Similarly, the peptide tag may prevent arbitrary breakdown of the compound by enzymatic activity of catabolic enzymes in the cytosol or periplasmic space. Alternatively these modifications may support a more stable interaction between the compound and the target site. Further testing will be needed to identify the mechanism of this improved activity.

Ciprofloxacin and norfloxacin conjugates, the only two fluoroquinolones derivatives were screened and showed growth inhibition in all strains. Ciprofloxacin, however, tested at much lower concentrations than any of other antibiotics and therefore can be considered a much more potent antibiotic at equivalent concentrations. Ciprofloxacin and its' conjugate family **4a–c** are of considerable interest. Unfortunately this conjugate family shows variable results among strains, where **4a** and **4b** inhibit over 90% of growth in *B. subtilis*, **4b** does not show significant inhibition in any other strain and **4a** only inhibits 50.26% of growth in *S. aureus* (Table 5–8).

Table 4
Calculated log*P* and molar refractivity of antibiotic conjugates **4a–c**, **6a–e**, **8a–c**, **10a–e** and **12a–c**

Entry	Compound	*C*log*P*
1	**4a**	3.188
2	**4b**	3.497
3	**4c**	5.470
4	**6a**	1.436
5	**6b**	1.745
6	**6c**	2.672
7	**6d**	3.163
8	**6e**	3.719
9	**8a**	3.133
10	**8b**	3.442
11	**8c**	4.860
12	**10a**	1.805
13	**10b**	2.114
14	**10c**	3.042
15	**10d**	3.532
16	**10e**	4.088
17	**12a**	0.966
18	**12b**	2.384
19	**12c**	2.428
20	Ciprofloxacin	−0.725
21	Pipemidic acid	−2.477
22	Norfloxacin	−0.780
23	Metronidazole	−0.457
24	Sulfadiazine	−0.912

Table 5
Percent growth inhibition of effective concentrations of drug derivatives tested against *S. aureus*

Concentration (μg/mL)	Compound	% Growth inhibition	Std. Dev.
4	Ciprofloxacin	63.93	0.63
4	**4a**	50.26	1.03
4	**4c**	51.57	0.36
200	Pipemidic acid	61.84	0.72
200	**6a**	57.38	1.12
200	**6c**	70.60	2.75
200	**6d**	72.87	5.84
200	**6e**	52.37	5.44
60	Norfloxacin	81.02	0.61
60	**8a**	88.53	2.43
60	**8b**	90.99	3.89
60	**8b+b**	86.09	1.08
60	**8c**	92.66	5.85
300	Metronidazole	16.43	4.46
300	**10b**	45.86	3.65
300	**10e**	67.96	1.78
240	Sulfadiazine	18.13	8.57
240	**12b**	29.05	8.88

Table 6
Percent growth inhibition in minimum inhibitory concentrations of effective concentrations of drug derivatives tested against *P. aeruginosa*

Concentration (μg/mL)	Compound	% Growth inhibition	Std. Dev.
10	Ciprofloxacin	91.79104	1.256409
10	**4c**	87.06468	1.005127
40	Norfloxacin	89.05797	3.381815
40	**8a**	81.52174	0.512396

Norfloxacin inhibits the growth of all screened strains but the strength of its conjugates are variable among the strains. At 60 μg/mL, conjugates **8a**, **8b**, **8b+b′**, and **8c** inhibited a higher percentage of *S. aureus* growth than Norfloxacin, the parent antibiotic. In *P. aeruginosa*, at 40 μg/mL only **8a** shows activity.

Pipemidic acid and some of the conjugates within its family are active only against *S. aureus* and *B. subtilis*, both Gram-(+) bacteria. *B. subtilis* shows high sensitivity to all the pipemidic conjugates at a concentration of 40 μg/mL with **6a** and **6e** having higher growth inhibition than the parent antibiotic. At 200 μg/mL, *S. aureus* showed greatest sensitivity to conjugates **6c** and **6d**.

Table 7
Percent growth inhibition in minimum inhibitory concentrations of effective drug derivatives tested against *B. subtilis*

Concentration (μg/mL)	Compound	% Growth inhibition	Std. Dev.
0.4	Ciprofloxacin	94.32	0.00
0.4	**4a**	94.19	0.18
0.4	**4b**	91.86	0.09
40	Pipemidic acid	79.78	16.98
40	**6a**	91.71	0.31
40	**6b**	60.06	2.45
40	**6c**	78.15	1.33
40	**6d**	76.96	7.29
40	**6e**	89.91	1.02
5	Norfloxacin	90.65	1.93
5	**8a**	91.56	0.05
5	**8b**	92.19	0.25
5	**8b+b′**	92.26	0.35

Table 8
Percent growth inhibition in minimum inhibitory concentrations of effective concentrations of drug derivatives tested against *E. coli*

Concentration (μg/mL)	Compound	% Growth inhibition	Std. Dev.
0.5	Ciprofloxacin	76.95	1.90
0.5	**4c**	38.85	1.10
3	Norfloxacin	80.84	1.30
100	Metronidazole	14.96	1.41
100	**10e**	70.05	0.38

Metronidazole conjugates did not effectively inhibit the growth of any of the strains. Most literature reports indicate that metronidazole is active primarily against anaerobic bacteria; whereas all strains in this study were grown in aerobic conditions, it is unclear whether the anaerobic pathways that metronidazole targets were present. *S. aureus*, a facultative anaerobe and *E. coli*, a facultative anaerobe were not heavily affected by metronidazole treatment. They did, however, have a much greater response to **10e**, with growth inhibition being three times greater than that of the parent antibiotic. We can assume that *B. subtilis*, a facultative aerobe, and *P. aeruginosa*, an aerobic bacterium, have a different metabolism than a facultative anaerobe and would not be inhibited similarly.

Sulfadiazine did not show a significant growth inhibition against any strains screened, Supplemental Tables S2–S16.

In conclusion, we have reported convenient benzotriazole-mediated efficient syntheses of chirally pure antibiotic conjugates with amino acids. Many antibiotic conjugates show promising preliminary results and may be equivalent or more effective than the original parent drug. These modifications may provide options for treatment of bacterial-resistant strains, with the benefit of enhanced drug uptake and/or diminished adverse side effects.

Acknowledgments

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Supplementary data

Supplementary data (synthetic procedure, analysis data, antibacterial assay) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.01.065.

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26. *Experimental section*: Melting points were determined on a capillary point apparatus equipped with a digital thermometer. NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on Mercury NMR spectrometers operating at 300 MHz for ^1H (with TMS as an internal standard) and 75 MHz for ^{13}C. Microwave assisted reaction was carried out with a single mode cavity Discover Microwave Synthesizer (CEM Corporation, NC). The reaction mixtures were transferred into a 10 mL glass pressure microwave tube equipped with a magnetic stir bar. The tube was closed with a silicon septum and the reaction mixture was subjected to microwave irradiation (Discover mode; run time: 60 s; PowerMax-cooling mode. Mass spectrometry was done with 6220 Agilent (Santa Clara, CA) TOF in electrospray ionization (ESI) mode with positive and negative ESI-MS method in both Profile and Centroid mode.
General procedure for preparation of ciprofloxacin bioconjugates. ***4a–c***. A solution of Cbz-amino acid-Bt (1.386 mmol) in tetrahydrofuran (5 mL) was added to a suspension of ciprofoloxacin (1.524 mmol) and triethylamine (3.048 mmol) in water (2 mL). The mixture was stirred for 3 h. at room temperature. The solvent was evaporated under reduced pressure then a solution of 2 N HCl was added to the residue and stirred for 15 min. to give precipitate which was filtered then washed several times with 2 N HCl to give the desired product.
7-(4-(2-(Benzyloxycarbonylamino)acetyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Cbz-Gly-Cip-OH, ***4a****):* White microcrystals (68%); mp 150–152 °C; ^1H NMR ($CDCl_3$) δ 8.63 (s, 1H), 7.86 (d, J = 12.7 Hz, 1H), 7.36–7.29 (m, 6H), 5.89 (s, 1H), 5.13 (s, 2H), 4.13–4.09 (m, 2H), 3.91–3.85 (m, 2H), 3.71–3.53 (m, 3H), 3.44–3.28 (m, 3H), 1.41–1.21 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 176.8, 166.8, 166.7, 156.3, 151.8, 147.5, 145.2, 138.9, 136.4, 128.6, 128.2, 128.0, 112.4, 107.9, 105.4, 67.0, 49.7, 49.4, 44.3, 42.7, 41.7, 35.5, 8.4; Anal. calcd for $C_{27}H_{27}FN_4O_6 \cdot 1/3H_2O$: C, 61.36; H, 5.28; N, 10.60; found: C, 61.46; H, 4.98; N, 10.59.
7-(4-(2-(Benzyloxycarbonylamino)propanoyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Cbz-L-Ala-Cip-OH, ***4b****):* White microcrystals (72%); mp 128–130 °C; ^1H NMR ($CDCl_3$) δ 8.70 (s, 1H), 7.96 (d, J = 12.9 Hz, 1H), 7.35–7.27 (m, 6H), 5.82 (d, J = 7.7 Hz, 1H), 5.11 (s, 2H), 4.74 (t, J = 7.2 Hz, 1H), 4.04–3.70 (m, 4H), 3.44–3.27 (m, 4H), 1.48–1.21 (m, 8H); ^{13}C NMR ($CDCl_3$) δ 177.1, 171.2, 155.8, 152.1, 147.7, 145.5, 141.9, 139.1, 136.5, 128.7, 128.3, 128.1, 112.8, 112.5, 108.3, 105.4, 67.0, 50.2, 49.7, 49.6, 46.8, 45.4, 42.0, 35.5, 19.4, 8.4; HRMS (-ESI-TOF) m/z for $C_{28}H_{29}FN_4O_6$ $[M-H]^-$ calcd 535.1998, found 535.2001.
(S)-1-Cyclopropyl-7-(4-(3,11-dioxo-1,13-diphenyl-2,12-dioxa-4,10-diazatridecanecarbonyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Cbz-L-Lys(Cbz)-Cip-OH, ***4c****):* White microcrystals (77%); mp 135–137 °C; ^1H NMR (DMSO-d_6) δ 8.66 (s, 1H), 7.93 (d, J = 13.2 Hz, 1H), 7.62–7.53 (m, 2H), 7.35–7.25 (m, 11H), 5.02 (s, 2H), 4.98 (s, 2H), 4.47–4.43 (m, 1H), 3.81–3.72 (m, 4H), 3.58–3.54 (m, 2H), 3.09–2.95 (m, 4H), 1.62–1.51 (m, 2H), 1.40–1.29 (m, 6H), 1.21–1.16 (m, 4H); ^{13}C NMR (DMSO-d_6)δ 176.2, 170.3, 165.8, 156.1, 155.9, 154.4, 151.2, 147.9, 144.7, 139.0, 137.2, 137.0, 128.3, 127.7, 127.8, 127.6, 118.7, 111.1, 110.8, 106.7, 106.5, 65.4, 65.1, 50.6, 45.4, 44.6, 42.5, 41.2, 35.9, 30.9, 29.2, 22.6, 8.5, 7.6. HRMS (-ESI-TOF) m/z for $C_{39}H_{42}FN_5O_8$ $[M-H]^-$ calcd 726.2943, found 726.2945.
General procedure for preparation of pipemidic acid bioconjugates ***6a–e****:* A solution of Cbz-amino acid-Bt (1.513 mmol) in tetrahydrofuran (5 mL) was added to a solution of pipemidic acid (1.664 mmol) and triethylamine (3.026 mmol) in water (2 mL). The mixture was stirred for 3 h. at room temperature. The solvent was evaporated under reduced pressure then a solution of 2 N HCl was added with stirring to the residue to give precipitate which was filtered, and washed several times with 2 N HCl to give the desired product.
2-(4-(2-(Benzyloxycarbonylamino)acetyl)piperazin-1-yl)-8-ethyl-5-oxo-5,8-dihydro pyrido[2,3-d]pyrimidine-6-carboxylic acid (Cbz-Gly-Pip-OH, ***6a****):* White microcrystals (82%); mp >300 °C; ^1H NMR (DMSO-d_6) δ 9.23 (s, 1H), 8.98 (s, 1H), 7.37–7.30 (m, 6H), 5.04 (s, 2H), 4.44–4.37 (m, 2H), 4.02–3.89 (m, 6H), 3.64–3.59 (m, 4H), 1.37 (t, J = 7 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 177.1, 167.5, 165.2, 160.7, 160.2, 156.5, 155.1, 150.8, 137.1, 128.3, 127.8, 127.7, 109.6, 108.7, 65.4, 45.9, 43.8, 43.5, 42.1, 14.4. HRMS (+ESI-TOF) m/z for $C_{24}H_{26}N_6O_6$ $[M+H]^+$ calcd 495.1987, found 495.1989.
2-(4-(2-(Benzyloxycarbonylamino)propanoyl)piperazin-1-yl)-8-ethyl-5-oxo-5,8-dihydro pyrido[2,3-d]pyrimidine-6-carboxylic acid (Cbz-L-Ala-Pip-OH, ***6b****):* White microcrystals (51%); mp >300 °C; ^1H NMR ($CDCl_3$) δ 9.33 (s, 1H), 8.71 (s, 1H), 7.36–7.30 (m, 5H), 5.91–5.89 (m, 1H), 5.11 (s, 2H), 4.77 (t, J = 7.5 Hz, 1H), 4.42–3.60 (m, 10H), 1.52–1.46 (m, 4H), 1.39 (d, J = 6.8 Hz, 2H); ^{13}C NMR ($CDCl_3$)δ 177.8, 175.9, 171.7, 166.5, 161.1, 155.9, 149.4, 136.4, 128.7, 128.1, 126.5, 115.1, 111.3, 109.9, 67.2, 49.6, 46.9, 46.7, 44.3, 44.0, 19.4, 18.7, 14.9. HRMS (-ESI-TOF) m/z for $C_{25}H_{28}N_6O_6$ $[M-H]^-$ calcd 507.1991, found 507.1998.
2-(4-(2-(Benzyloxycarbonylamino)-3-methylbutanoyl)piperazin-1-yl)-8-ethyl-5-oxo-5,8-dihydropyrido[2,3-d]pyrimidine-6-carboxylic acid (Cbz-L-Val-Pip-OH, ***6c****):* White microcrystals (68%); mp >300 °C; ^1H NMR ($CDCl_3$) δ 9.32 (s, 1H), 8.66 (s, 1H), 7.34–7.31 (m, 5H), 5.55 (d, J = 9 Hz, 1H), 5.08 (s, 2H), 4.56–4.50 (m, 1H), 4.32 (q, J = 7.2 Hz, 2H), 4.18–3.59 (m, 8H), 2.03–1.94 (m, 1H), 1.48 (t, J = 7.2 Hz, 3H), 0.99 (d, J = 6.7 Hz, 3H), 0.92 (d, J = 6.7 Hz, 3H). HRMS (+ESI-TOF) m/z for $C_{27}H_{32}N_6O_6$ $[M+H]^+$ calcd 537.2451, found 537.2452.
2-(4-(2-(Benzyloxycarbonylamino)-3-phenylpropanoyl)piperazin-1-yl)-8-ethyl-5-oxo-5,8-dihydropyrido[2,3-d]pyrimidine-6-carboxylic acid (Cbz-L-Phe-Pip-OH, ***6d****):* White microcrystals (64%); mp >300 °C; ^1H NMR (DMSO-d_6) δ 9.23 (s, 1H), 8.99 (s, 1H), 7.33–7.19 (m, 10H), 4.96 (s, 2H), 4.75–4.67 (m, 1H), 4.40 (q, J = 6.4 Hz, 2H), 4.15–3.56 (m, 5H), 3.22–2.80 (m, 5H), 1.36 (t, J = 7.3 Hz, 3H). HRMS (+ESI-TOF) m/z for $C_{31}H_{32}N_6O_6$ $[M+H]^+$ calcd 585.2434, found 585.2436.
(S)-2-(4-(3,11-Dioxo-1,13-diphenyl-2,12-dioxa-4,10-diazatridecanecarbonyl)-piperazin-1-yl)-8-ethyl-5-oxo-5,8-dihydropyrido[2,3-d]pyrimidine-6-carboxylic acid (Cbz-L-Lys(Cbz)-Pip-OH, ***6e****):* White microcrystals (80%); mp 140–142 °C; ^1H NMR (DMSO-d_6) δ 10.19 (br s, 1H), 9.21 (s, 1H), 8.97 (s, 1H), 7.58–7.52 (m, 1H), 7.38–7.28 (m, 11H), 5.02 (s, 2H), 4.98 (s, 2H), 4.48–4.36 (m, 2H), 4.05–3.88 (m, 3H), 3.72–3.54 (m, 3H), 3.09–2.97 (m, 5H), 1.61–1.53 (m, 1H), 1.36 (t, J = 6.9 Hz, 4H), 1.19 (t, J = 7.3 Hz, 4H); ^{13}C NMR (DMSO-d_6) δ 177.1, 170.6, 165.2, 160.6, 160.2, 156.1, 155.9, 155.0, 150.7, 137.2, 137.0, 128.3, 127.8, 127.7, 127.7, 127.6, 109.6, 108.7, 65.4, 65.1, 50.7, 45.9, 45.4, 44.0, 43.6, 30.9, 29.2, 22.6, 14.4, 8.5. HRMS (+ESI-TOF) m/z for $C_{36}H_{41}N_7O_8$ $[M+H]^+$ calcd 700.3083, found 700.3087.
General procedure for preparation of norfloxacin bioconjugates ***8a–c****:* A solution of Cbz-amino acid-Bt (0.999 mmol) in tetrahydrofuran (5 mL) was added to a suspension of norfoloxacin (1.099 mmol) and triethylamine (1.998 mmol) in water (2 mL). The mixture was stirred for 3 h. at room temperature. The solvent was evaporated under reduced pressure and a solution of 2 N HCl was added to the residue and stirred for 15 min. to give precipitate which filtered then washed several times with 2 N HCl to give the desired product.
7-(4-(2-(Benzyloxycarbonylamino)acetyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Cbz-Gly-Nor-OH, ***8a****):* White microcrystals (82%); mp 214–216 °C; ^1H NMR (DMSO-d_6) δ 8.92 (s, 1H), 7.87 (d, J = 13.2 Hz, 1H), 7.37–7.31 (m, 6H), 7.16 (d, J = 7.3 Hz, 1H), 5.04 (s, 2H), 4.57 (q, J = 7.1 Hz, 2H), 3.96 (d, J = 5.9 Hz, 2H), 3.69–3.63 (m, 4H), 3.35–3.29 (m, 4H), 1.41 (t, J = 7.1 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 176.1, 167.4, 166.1, 156.5, 154.4, 151.1, 148.5, 145.1, 137.1, 128.3, 127.7, 119.4, 111.3, 111.0, 107.1, 106.1, 65.4, 49.5, 49.2, 49.1, 43.6, 42.0, 41.1, 14.4. Anal. calcd for $C_{26}H_{27}FN_4O_6 \cdot H_2O$: C, 59.08; H, 5.53; N, 10.60; found: C, 59.10; H, 5.25; N, 10.91.
(S)-7-(4-(2-(Benzyloxycarbonylamino)propanoyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Cbz-L-Ala-Nor-OH, ***8b****):* White microcrystals (79%); mp 192–194 °C; ^1H NMR (DMSO-d_6) δ 8.94 (s, 1H), 7.90 (d, J = 12.9 Hz, 1H), 7.34–7.18 (m, 6H), 5.01 (s, 2H), 4.62–4.55 (m, 3H), 3.72–3.61 (m, 4H), 3.34–3.25 (m, 4H), 1.40 (t, J = 7 Hz, 3H), 1.21 (d, J = 6.9 Hz, 3H); ^{13}C NMR (DMSO-d_6) δ 176.0, 170.6, 166.0, 155.5, 154.4, 151.1, 148.4, 145.0, 137.0, 128.2, 127.6, 125.3, 119.5, 114.8, 111.3, 107.1, 106.1, 65.3, 49.7, 49.3, 49.1, 44.4, 41.3, 17.6, 14.4. Anal. calcd for $C_{27}H_{29}FN_4O_6 \cdot H_2O$: C, 59.77; H, 5.76; N, 10.33; found: C, 60.07; H, 5.54; N, 10.16.
7-(4-(2-(Benzyloxycarbonylamino)propanoyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (Cbz-DL-Ala-Nor-OH, ***8b+8b′****):* White microcrystals (75%); mp 210–212 °C; ^1H NMR ($CDCl_3$) δ 8.65 (s, 1H), 8.02 (d, J = 12.6 Hz, 1H), 7.37–7.24 (m, 5H), 6.85–6.82 (m, 1H), 5.82 (d, J = 7.5 Hz, 1H), 5.08 (s, 2H), 4.75–4.68 (m, 2H), 4.75–4.68 (m, 1H), 4.41–4.26 (m, 2H), 4.08–3.10 (m, 8H), 1.73–1.56 (m, 3H), 1.45–1.32 (m, 3H); ^{13}C NMR ($CDCl_3$) δ 177.1, 175.1, 171.4, 167.4, 155.9, 147.6, 145.7, 145.6, 137.2, 136.5,

128.7, 128.4, 128.2, 113.4, 113.1, 104.6, 67.1, 50.3, 50.0, 49.8, 49.7, 46.8, 46.4, 45.4, 42.0, 19.4, 18.8, 14.7. Anal. calcd for $C_{27}H_{29}FN_4O_6{\cdot}H_2O$: C, 59.77; H, 5.76; N, 10.33; found: C, 59.91; H, 5.45; N, 10.13.

(S)-7-(4-(2-(Benzyloxycarbonylamino)-3-phenylpropanoyl)piperazin-1-yl)-1-ethyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid. (Cbz-L-Phe-Nor-OH, ***8c****):* White microcrystals (86%); mp 195–197 °C; ^{1}H NMR ($CDCl_3$) δ 8.66 (s, 1H), 8.02 (d, J = 12.8 Hz, 1H), 7.46–7.26 (m, 10H), 6.72 (br s, 1H), 5.75 (d, J = 8.8 Hz, 1H), 5.10 (d, J = 3.7 Hz, 2H), 4.98–4.92 (m, 1H), 4.41–4.26 (m, 2H), 3.85–3.69 (m, 1H), 3.61–3.51 (m, 1H), 3.25–3.00 (m, 6H), 2.61–2.52 (m, 1H), 1.59 (t, J = 7.1 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 177.1, 170.2, 167.2, 155.8, 147.4, 145.6, 137.1, 136.3, 129.8, 128.9, 128.7, 128.3, 128.1, 127.3, 121.3, 121.2, 113.2, 112.9, 108.6, 104.3, 67.2, 51.6, 50.0, 49.6, 45.4, 41.8, 40.5, 14.7. Anal. calcd for $C_{33}H_{33}FN_4O_6$: C, 65.99; H, 5.54; N, 9.33; found: C, 65.63; H, 5.45; N, 9.00.

27. *General procedure for preparation of metronidazole bioconjugates* ***10a–e****.* A solution of Cbz-protected-(aminoacyl)benzotriazole (0.497 mmol), metronidazole (0.497 mmol) and DMAP (0.248 mmol) in tetrahydrofuran (5 mL) was stirred under microwave conditions (60 °C, 50 W) for 1 h. The solvent was evaporated under reduced pressure and the residue was purified by column chromatography to give the desired product.

Benzyl 4-(2-methyl-5-nitro-1H-imidazol-1-yl)-2-oxobutylcarbamate (Cbz-Gly-Met, ***10a****).* Oil (82%); ^{1}H NMR ($CDCl_3$) δ 7.94 (s, 1H), 7.37–7.27 (m, 5H), 5.44 (t, J = 6.7 Hz, 1H), 5.11 (s, 2H), 4.58–4.55 (m, 2H), 4.49–4.46 (m, 2H), 3.91 (d, J = 5.6 Hz, 2H), 2.48 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 169.8, 156.5, 151.1, 138.6, 136.2, 133.4, 128.7, 128.4, 128.2, 67.4, 63.5, 45.0, 42.7, 14.4. HRMS (+ESI-TOF) m/z for $C_{16}H_{18}N_4O_5$ $[M+H]^+$ calcd 385.1299, found 385.1115..

Benzyl 5-(2-methyl-5-nitro-1H-imidazol-1-yl)-3-oxopentan-2-ylcarbamate (Cbz-L-Ala-Met, ***10b****).* Oil (85%); ^{1}H NMR ($CDCl_3$) δ 7.90 (s, 1H), 7.35–7.27 (m, 5H), 5.71 (d, J = 7.5 Hz, 1H), 5.06 (s, 2H), 4.54–4.26 (m, 5H), 2.47 (s, 3H), 1.30 (d, J = 7.2 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 172.6, 155.7, 151.0, 138.3, 136.1, 133.0, 128.4, 128.0, 66.9, 63.4, 49.5, 44.8, 17.9, 14.2. HRMS (+ESI-TOF) m/z for $C_{17}H_{20}N_4O_5$ $[M+H]^+$ calcd 377.1456, found 377.14.

Benzyl 2-methyl-6-(2-methyl-5-nitro-1H-imidazol-1-yl)-4-oxohexan-3-ylcarbamate (Cbz-L-Val-Met, ***10c****):* Oil (83%); ^{1}H NMR ($CDCl_3$) δ 7.94 (s, 1H), 7.34–7.28(m, 5H), 5.39 (d, J = 8.7 Hz, 1H), 5.09 (s, 2H), 4.59–4.18 (m, 5H), 2.49 (s, 3H), 2.06–1.98 (m, 1H), 0.90 (d, J = 6.6 Hz, 3H), 0.81 (d, J = 6.6 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 171.8, 156.2, 151.1, 138.3, 136.1, 133.2, 128.6, 128.3, 128.2, 67.2, 63.3, 59.2, 44.9, 31.0, 19.1, 17.6, 14.4. HRMS (+ESI-TOF) m/z for $C_{19}H_{24}N_4O_5$ $[M+H]^+$ calcd 405.1769, found 405.1771.

Benzyl 5-(2-methyl-5-nitro-1H-imidazol-1-yl)-3-oxo-1-phenylpentan-2-ylcarbamate (Cbz-L-Phe-Met, ***10d****):* Oil (82%); ^{1}H NMR ($CDCl_3$) δ 7.91 (s, 1H), 7.36–7.21(m, 8H), 7.03–7.01 (m, 2H), 5.35 (d, J = 7.9 Hz, 1H), 5.06 (s, 2H), 4.55–4.50 (m, 1H), 4.44 (br s, 2H), 4.30–4.24 (m, 1H), 3.99 (d, J = 6.4 Hz, 2H), 2.38 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 171.4, 155.7, 151.1, 138.4, 136.2, 135.4, 133.3, 129.1, 128.8, 128.6, 128.4, 128.2, 127.5, 67.2, 63.6, 55.2, 44.8, 38.3, 14.3. HRMS (+ESI-TOF) m/z for $C_{23}H_{24}N_4O_5$ $[M+H]^+$ calcd 453.1767, found 453.1769.

(S)-2-(2-Methyl-4-nitro-1H-imidazol-1-yl)ethyl 3,12-dioxo-1,13-diphenyl-2,11-dioxa-4,10-diazatridecane-5-carboxylate (Cbz-L-Lys(Cbz)-Met, ***10e****):* Oil (72%); ^{1}H NMR ($CDCl_3$) δ 7.93 (s, 1H), 7.42–7.25 (m, 10H), 5.62–5.60 (m, 1H), 5.16 (br s, 1H), 5.06 (br s, 4H), 4.54–4.22 (m, 5H), 4.44 (br s, 2H), 3.13 (br s, 2H), 2.47 (s, 3H), 1.72–1.26 (m, 7H); ^{13}C NMR ($CDCl_3$) δ 172.2, 156.8, 156.1, 151.1, 138.8, 138.6, 136.8, 136.3, 133.3, 133.2, 128.6, 128.3, 128.2, 128.1, 67.2, 66.8, 63.4, 54.0, 45.0, 40.4, 31.7, 29.5, 22.4, 14.3. HRMS (+ESI-TOF) m/z for $C_{28}H_{33}N_5O_8$ $[M+H]^+$ calcd 568.2402, found 568.2395.

28. *General procedure for preparation of sulphadiazine bioconjugates* ***12a–c****:* A solution of sulphadiazine (0.668 mmol) and morpholine (0.668 mmol) in anhydrous DMF (1 mL) was added to a solution of Cbz-amino acid (0.668 mmol), isobutyl chloroformate (0.668 mmol) and morpholine (0.668 mmol) in anhydrous tetrahydrofuran (5 mL). The mixture was stirred overnight at room temperature. The solvent was evaporated under reduced pressure and the residue was triturated by ethyl acetate and extracted by saturated Na_2CO_3 solution, brine water and 2 N HCl. The organic solvent was evaporated and purified by column chromatography to give the desired product.

Benzyl 1-oxo-1-(4-(N-pyrimidin-2-ylsulfamoyl)phenylamino) propan-2-ylcarbamate (Cbz-L-Ala-Sul, ***12a****):* Oil (65%); ^{1}H NMR ($CDCl_3$) δ 9.14 (s, 1H), 8.62 (d, J = 5.1 Hz, 2H), 7.80 (d, J = 8.3 Hz, 2H), 7.52 (d, J = 8.8 Hz, 2H), 7.31–7.23 (m, 6H), 6.97 (t, J = 5.0 Hz, 1H), 5.81 (d, J = 7.5 Hz, 1H), 5.15–5.00 (m, 2H), 4.47–4.41 (m, 1H), 1.42 (d, J = 6.6 Hz, 3H); ^{13}C NMR ($CDCl_3$) δ 171.7, 158.9, 156.9, 142.3, 135.9, 134.3, 129.5, 128.7, 128.5, 128.2, 128.1, 119.3, 116.1, 67.6, 51.6, 18.0. HRMS (+ESI-TOF) m/z for $C_{21}H_{21}N_5O_5SNa$ $[M+Na]^+$ calcd 478.1156, found 478.1162.

Benzyl 1-oxo-3-phenyl-1-(4-(N-pyrimidin-2-ylsulfamoyl)phenylamino)propan-2-ylcarbamate (Cbz-L-Phe-Sul, ***12b****):* Oil (70%); ^{1}H NMR (DMSO-d_6) δ 10.53 (s, 1H), 8.50 (d, J = 4.8 Hz, 2H), 7.94 (d, J = 8.8 Hz, 2H), 7.78 (t, J = 8.8 Hz, 3H), 7.40–7.15 (m, 10H), 7.04 (t, J = 4.8 Hz, 1H), 4.96 (s, 2H), 4.44–4.39 (m, 1H), 3.01 (dd, J = 14.2, 4.1 Hz, 1H), 2.83 (dd, J = 14.0, 10.6 Hz, 1H); ^{13}C NMR (DMSO-d_6) δ 171.1, 158.1, 156.9, 155.9, 142.6, 137.5, 136.8, 136.8, 134.3, 129.1, 128.7, 128.1, 128.0, 127.6, 127.4, 126.3, 118.6, 115.6, 65.3, 56.9, 37.2. HRMS (+ESI-TOF) m/z for $C_{27}H_{25}N_5O_5S$ $[M+H]^+$ calcd 554.1469, found 554.1462.

Benzyl 3-(benzyloxycarbonylamino)-4-oxo-4-(4-(N-pyrimidin-2-ylsulfamoyl)-phenyl amino)butanoate (Cbz-L-Asp(Bzl)-Sul, ***12c****):* Oil (72%); 8.90 (br s, 1H), 8.63 (d, J = 4.0 Hz, 2H), 7.95 (d, J = 8.1 Hz, 2H), 7.55 (d, J = 8.1 Hz, 2H), 7.36–7.22 (m, 10H), 6.99–6.95 (m, 1H), 6.20–6.17 (m, 1H), 5.10 (br s, 4H), 4.74 (br s, 1H), 3.03 (d, J = 15.0 Hz, 1H), 2.83 (d, J = 17.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 171.6, 169.2, 158.8, 156.9, 156.6, 142.0, 141.9, 135.8, 135.2, 134.6, 130.1, 129.8, 128.8, 128.6, 128.4, 128.3, 119.4, 116.0, 67.9, 67.4, 52.1, 36.0. Anal. calcd for $C_{29}H_{27}N_5O_7S$: C, 59.07; H, 4.62; N, 11.88; found: C, 59.26; H, 4.51; N, 11.75.

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