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Synthesis of citrate–ciprofloxacin conjugates

Siti R Md-Saleh [a], Emily C. Chilvers [a], Kevin G. Kerr [b,c], Stephen J. Milner [a], Anna M. Snelling [c], Jan P. Weber [d], Gavin H. Thomas [d], Anne-Kathrin Duhme-Klair [a,*], Anne Routledge [a,*]

[a] Department of Chemistry, University of York, Heslington, York, YO10 5DD, UK
[b] Department of Microbiology, Fewston Wing, Harrogate District Hospital, Lancaster Park Road, Harrogate, HG2 7SX, UK
[c] Bradford Infection Group, University of Bradford, Bradford BD7 1DP, UK
[d] Department of Biology (Area 10), University of York, Heslington, York, YO10 5YW, UK

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ABSTRACT

Two regioisomeric citrate-functionalized ciprofloxacin conjugates have been synthesized and their antimicrobial activities against a panel of clinically-relevant bacteria have been determined. Cellular uptake mechanisms were investigated using wild-type and *ompF* deletion strains of *Escherichia coli* K-12.

Fluoroquinolones are broad spectrum antibiotics used to treat a wide variety of both Gram-negative and Gram-positive bacterial infections.[1] The mechanism of action of this class of antibiotics is via inhibition of cytoplasmic DNA gyrase or topoisomerase IV[2] and bacterial resistance arises primarily by amino acid substitutions within these target enzymes.[3] Efflux pumps can contribute to resistance, and newly emerging resistance mechanisms include the Qnr protective proteins and a variant aminoglycoside acetyltransferase enzyme capable of modifying ciprofloxacin.[4]

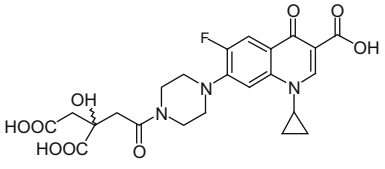
A secondary resistance mechanism in Gram-negative bacteria involves decreasing permeability of the outer membrane by a reduction in the expression of key outer membrane porins.[5] The outer membrane porin OmpF is important in the movement of fluoroquinolones into the cell[6] and reduced levels of OmpF result in reduced permeability, leading to lower intracellular concentrations.[7]

In order to increase the intracellular concentration of fluoroquinolones in bacterial isolates in which OmpF is no longer synthesized, citrate modified analogs of ciprofloxacin were designed, with the aim of increasing intracellular concentration by exploiting the iron citrate uptake pathway.[8,9] Citrate is used by *E. coli* as an exogenous siderophore[10], although citrate has a relatively low affinity for Fe^{III} [11,12], compared to enterobactin, the native tris(catecholate) siderophore of *E. coli*. It has, however, been demonstrated that even siderophores with low affinity for Fe^{III}, such as monocatecholates, can mediate the transport of antibiotics across the bacterial outer membrane through the siderophore uptake mechanism.[13]

In fluoroquinolones, it has been demonstrated that chemical modification of the secondary amine in the piperazinyl ring can result in a conjugate that retains comparable activity to the parent fluoroquinolone[14] without the need for intracellular cleavage and release of the antibiotic. In other examples intracellular release has been shown to be necessary for maximum activity. For example, norfloxacin has been conjugated to pyoverdin[15] and pyochelin[16] to target *Pseudomonas aeruginosa*. These conjugates displayed greatest activity with a chemically labile linkage.

Herein we describe the synthesis of two regioisomeric citrate–ciprofloxacin conjugates **1** and **2** with the aim of exploring their antimicrobial activity and cellular uptake mechanism.

Our aim was to conjugate a monocitrate unit to the piperazinyl ring of ciprofloxacin via a chemically stable amide bond.



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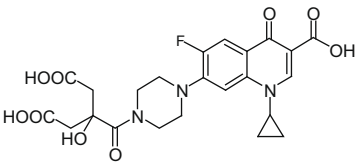

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* Corresponding authors.
E-mail address: ar30@york.ac.uk (A. Routledge).

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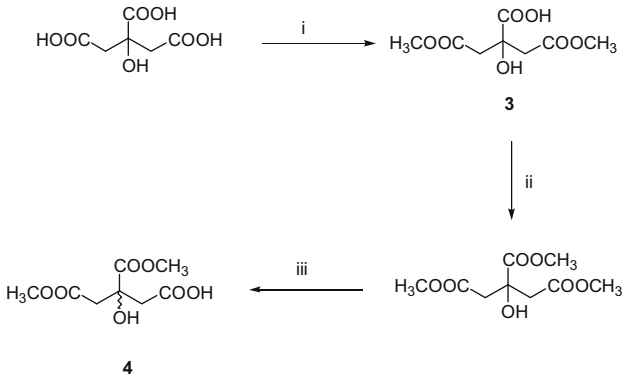
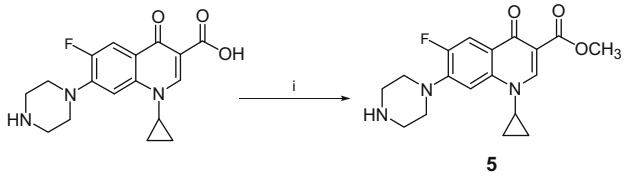


Scheme 1. Synthesis of citrate esters **3** and **4**. Reagents and conditions: (i) H_2SO_4, MeOH, reflux, 1 h, 32%; (ii) H_2SO_4, 2,2-dimethoxypropane, reflux, 7 h, 62%; (iii) 0.1 M NaOH, MeOH: H_2O (1:1), 20 °C, 2 h, 99% based on conversion of trimethyl citrate.

Two regioisomeric methyl esters of citric acid **3**[17] and **4**[18] were prepared, as shown in Scheme 1. Both have one free carboxylic acid moiety allowing direct conjugation to ciprofloxacin.

In order to conjugate via amidation of the piperazinyl ring, ciprofloxacin was esterified using a thionyl chloride-mediated methylation[19] to give the methyl ester of ciprofloxacin **5** in good yield (Scheme 2).



Scheme 2. Synthesis of ciprofloxacin methanoate. Reagents and conditions: (i) $SOCl_2$, MeOH, reflux, 24 h, 86%.

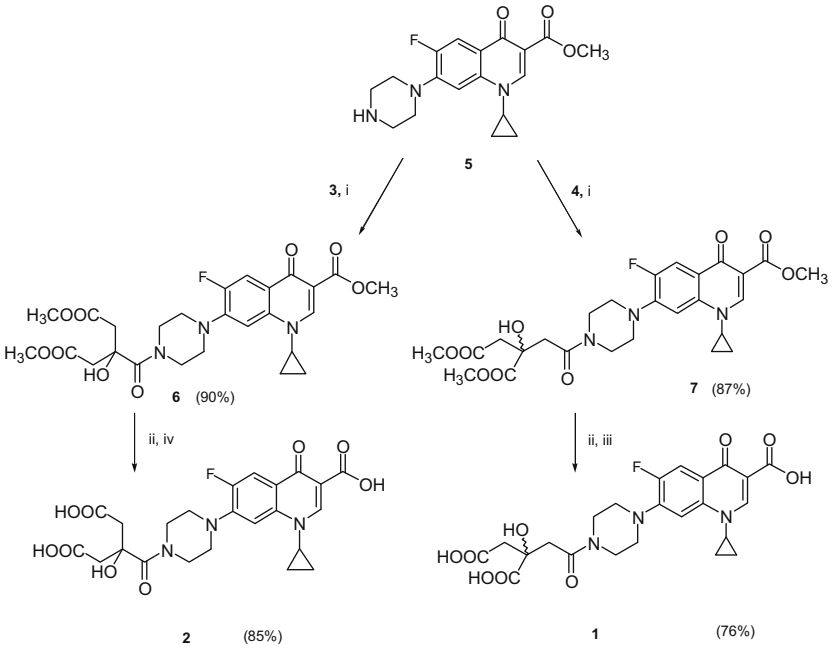
EDCI-mediated coupling to citrates **3** and **4**, then subsequent deprotection, furnished conjugates **1** and **2**[20] (Scheme 3).

As a preliminary evaluation to assess the influence of the citrate moiety on biological activity, the two conjugates were screened against a collection of reference and clinical isolates of bacteria associated with infection in humans (Table 1).[21] No activity was observed against ciprofloxacin resistant bacteria but **1** and **2** retained activity against strains that were susceptible to ciprofloxacin, as indicated by the zones of inhibition around discs impregnated with 5 μg of the conjugates having similar diameters to those observed with a 5 μg ciprofloxacin disc. These results suggest that both **1** and **2** are able to reach the target enzymes within the cytoplasm and can still effectively inhibit these enzymes. In addition, regiochemistry of the citrate moiety does not appear to influence activity of the conjugate.

In order to explore the cell membrane permeability of conjugate **1** it was tested against wild-type (BW25113) and an isogenic *ompF* mutant strain of *E. coli*[23] (Table 2).[24]

The decrease in activity for both ciprofloxacin and conjugate **1** in the *ompF* mutant is comparable; this indicates that OmpF provides a key uptake pathway for both compounds and that for conjugate **1**, there is no significant additional uptake via the iron–citrate pathway. Similarly, when using a *fepC* mutant of *E. coli*, that lacks the high-affinity enterobactin uptake system and has an increased dependence on the Fec system for iron acquisition, we observed no difference in the ability of the free ciprofloxacin and conjugate **1** to inhibit growth of *E. coli* K-12 (data not shown). This suggests that the citrate-conjugates are not recognized by FecA or that they are not able to compete successfully with either native siderophores or citrate present in the growth medium.

The formation of conjugates **1** and **2** involves the conversion of one of the carboxylate functionalities of citrate into an amide. The conjugates are thus expected to have a lower affinity for Fe^{III} than citrate itself since carbonyl oxygen donors in amides bind less strongly to hard M^{III}-cations than the oxygen donors in carboxylates.[25] More competitive carboxylate siderophores, such as rhizoferrin or staphyloferrin, however, also rely on amide-linked citrates, but utilize two citrate moieties to chelate the Fe^{III}. That the corresponding increase



Scheme 3. Synthesis of regioisomeric citrate–ciprofloxacin conjugates. Reagents and conditions: (i) EDCI, HOBt, DIPEA, DMF, ambient temperature, 18 h; (ii) 0.25 M NaOH, H_2O, ambient temperature, 3 h for **7**, 48 h for **6**; (iii) 0.1 M HCl. (iv) conc. HCl.

Table 1
Activity of conjugates **1** and **2** in a disc diffusion assay

Bacterial strain	Zone of inhibition (cm)		
	Ciprofloxacin[a]	**1**[a]	**2**[a]
Staphylococcus aureus (Oxford) NCTC 6571	2.4	1.7	2.0
Staphylococcus aureus NCTC 10399	2.7	2.0	2.3
Staphylococcus aureus HG-1[b,c] (methicillin-resistant)	R	R	R
Staphylococcus aureus NCTC 13142[b] (EMRSA-15; methicillin-resistant)	2.2	–	2.1
Staphylococcus aureus NCTC 13143[b,c] (EMRSA-16, methicillin- resistant)	R	–	R
Staphylococcus epidermidis NCTC 11047	2.5	2.8	2.1
Staphylococcus epidermidis NCTC 2749	2.9	2.8	2.4
Staphylococcus haemolyticus NCTC 11042	2.5	3.0	2.0
Staphylococcus aureus BIG 0052[b,c] (methicillin-resistant)	R	R	R
Pseudomonas aeruginosa NCTC 6749	3.5	2.3	-
Pseudomonas aeruginosa BIG 0039(Environmental)	2.7	1.8	-
Pseudomonas aeruginosa BIG 0037 (clinical)	3.1	2.5	2.4
Pseudomonas aeruginosa NCTC 10662	3.1	2.3	–
Pseudomonas aeruginosa BIG 0063	3.2	–	2.4
Serratia marcescens NCTC 1377	3.0	2.6	2.5
Burkholderia cepacia NCTC 10744	2.2	1.8	1.8
Escherichia coli BIG 0046[c]	R	R	R
Escherichia coli NCTC 10418	3.2	3.0	2.7
Escherichia coli BIG 0051[c]	R	R	R

R, resistant; zone of inhibition = 0 cm.
[a] Five micrograms discs used in assay.
[b] Methicillin-resistant *S. aureus* (MRSA).
[c] Strains of clinical origin defined as resistant to ciprofloxacin by CLSI criteria.[22]

Table 2
Activity of conjugate **1** against *E. coli*

E. coli strain	Zone of inhibition (cm)	
	Ciprofloxacin	**1**
BW25113 (10^{-6} M Fe^{III})	1.7	1.1
BW25113 (10^{-3} M Fe^{III})	1.7	1.3
BW25113 *ompF* (10^{-6} M Fe^{III})	1.0	0.6
BW25113 *ompF* (10^{-3} M Fe^{III})	1.1	0.8

in ligand denticity can counteract the loss of Fe-affinity due to amide bond formation is reflected in the corresponding pFe values: 17.7 for citrate and 19.7 for rhizoferrin.[26] The pFe values indicate the free Fe^{III} concentration at a total ligand concentration of 10^{-5} M and a total iron concentration of 10^{-5} M at pH 7.4 and therefore allow a direct comparison of the different siderophores.

Work is in progress to synthesize conjugates containing citrate-based siderophores with increased iron affinity by using similar design principles.

Acknowledgments

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References and notes

1. Schmitz, F-J.; Verhoef, J.; Fluit, A. C. *Int. J. Antimicrob. Agents* **1999**, *12*, 311.
2. Drlica, K.; Zhao, X. *Microbiol. Mol. Biol. Rev.* **1997**, 377.
3. Ruiz, J. *J. Antimicrob. Chemother.* **2003**, *51*, 1109.
4. Robicsek, A.; Jacoby, G. A.; Hooper, D. C. *Lancet Infect. Dis.* **2006**, *6*, 629.
5. Hooper, D. C.; Wolfson, J. S.; Souza, K. S.; Ng, E. Y.; McHugh, G. L.; Swartz, M. N. *Antimicrob. Agents Chemother.* **1989**, 283.
6. Fernandes, F.; Neves, P.; Gameiro, P.; Loura, L. M. S.; Prieto, M. *Biochim. Biophys. Acta* **2007**, *1768*, 2822.
7. Hirai, H.; Aoyama, H.; Irikura, T.; Iyobe, S.; Mitsuhashi, S. *Antimicrob. Agents Chemother.* **1986**, 535.
8. Ferguson, A. D.; Chakraborty, R.; Smith, B. S.; Esser, D.; van der Helm, D.; Deisenhofer, J. *Science* **2002**, *295*, 1658.
9. Braun, V.; Herrmann, C. *J. Bacteriol.* **2007**, 6918.
10. Wagegg, W.; Braun, V. *J. Bacteriol.* **1981**, *145*, 156.
11. Hussein, S.; Hantke, K.; Braun, V. *Eur. J. Biochem.* **1981**, *117*, 431.
12. Gautier-Luneau, I.; Merle, C.; Phanon, D.; Lebrun, C.; Biaso, F.; Serratrice, G.; Pierre, J.-L. *Chem. Eur. J.* **2005**, *11*, 2207.
13. Nikaido, H.; Rosenberg, E. Y. *J. Bacteriol.* **1990**, *172*, 1361.
14. Siddiqui, R.; Sultana, N.; Khan, K. M.; Akbar, N.; Ali, M.; Arayne, S. *J. Chin. Clinc. Med.* **2007**, *2*, 188.
15. Hennard, C.; Truong, Q. C.; Desnottes, J.-F.; Paris, J.-M.; Moreau, N. J.; Abdallah, M. A. *J. Med. Chem.* **2001**, *44*, 2139.
16. Rivault, F.; Liebert, C.; Burger, A.; Hoegy, F.; Abdallah, M. A.; Schalk, I. J.; Mislin, G. L. A. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 640.
17. Guo, H.; Naser, S. A.; Ghobrial, G.; Phanstiel, O., IV *J. Med. Chem.* **2002**, *45*, 2056.
18. Hirota, K.; Kitagawa, H.; Shimamura, M.; Ohmori, S. *Org. Lett.* **1980**, 191.
19. Koga, H.; Itoh, A.; Murayama, S.; Suzue, S.; Irikura, T. *J. Med. Chem.* **1980**, *23*, 1358.
20. Procedure for the synthesis of **1**.To a stirred solution of **5** (0.222 g, 0.64 mmol), **4** (0.141 g, 0.64 mmol), HOBt·H_2O (0.087 g, 0.64 mmol) and DIPEA (111.5 μL, 0.62 mmol) in dry DMF (18 mL) was added, in portions over 15 min, EDCI (0.123 g, 0.64 mmol). The resultant mixture was stirred at ambient temperature for 18 h. The mixture was diluted with water (60 mL) and extracted with DCM (3 × 80 mL). The organic extract was washed with water (2 × 50 mL) and brine (2 × 50 mL) then dried over $MgSO_4$. Removal of the solvent in vacuo afforded **6** (0.30 g, 87%).To a stirred solution of aqueous NaOH (10 mL, 0.25 M) was added **6** (0.109 g, 0.20 mmol). The mixture was stirred at ambient temperature for 1 h. The pH of the mixture was adjusted to 2 with HCl (0.1 M). The resulting precipitate was filtered, washed with water (2 mL) and dried to give **1** as a pale yellow powder (0.08 g, 76%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H 8.66 (1 H, s, H-2), 7.91 (1H, d, $^3J_{H-F}$ = 13.4 Hz, H-5), 7.57 (1H, d, $^4J_{H-F}$ = 7.3 Hz, H-8), 3.84–3.79 (1H, m, cyclopropyl ring N1—C*H*), 3.73–3.68 (4H, m, piperazine CH_2), 3.02–2.99 (4H, m, piperazine CH_2), 3.01–2.87 (2H, m, citrate CH_2), 2.76–2.62 (2H, m, citrate CH_2), 1.33–1.31 (2H, m, cyclopropyl ring CH_2), 1.22–1.18 (2H, m, cyclopropyl ring CH_2). ^{13}C NMR (DMSO-d_6, 100 MHz) δ_C 176.3 (C═O, C-4), 174.9 (C═O), 171.4 (C═O), 168.4 (C═O), 165.9 (C═O, C3—*C*OOH), 154.4 (d, $^1J_{C-F}$ = 249 Hz, C6), 148.0 (C—H, C-2), 144.4 (d, $^2J_{C-F}$ = 10 Hz, C-7), 139.1 (C-8a), 118.7 (d, $^3J_{C-F}$ = 7.7 Hz, C-4a), 111.5 (d, $^2J_{C-F}$ = 23 Hz, C-5), 106.6 (d, $^3J_{C-F}$ = 24 Hz, C-8), 73.1 (C—OH), 49.6 (CH_2), 49.2 (CH_2), 45.1 (citrate CH_2), 43.2 (citrate CH_2), 40.7 (CH_2), 40.2 (CH_2), 35.9 (N1—*C*H), 7.6 (cyclopropyl CH_2). HRMS (ESI) Calcd for $C_{23}H_{24}N_3O_9F$ 504.1418. Found 504.1424.
21. Strains were obtained from the culture collection of the Bradford Infection Group. Bacteria were harvested from overnight plates and suspended in sterile saline to McFarland turbidity standard 0.5. Suspensions were used to make confluent lawns of each strain on Iso-Sensitest agar (Oxoid Ltd). Sterile filter paper discs (0.6 cm diameter) were impregnated with 5 μg of ciprofloxacin stock or conjugate. Commercially available 5 μg ciprofloxacin discs (Oxoid) were also used to determine if strains were ciprofloxacin resistant, as defined by CLSI criteria. Discs were placed on the agar surface, and plates incubated for 18–20 h at 37 °C, and the diameter of zones of inhibition were then recorded.
22. Clinical and Laboratory Standards Institute. 2006. Performance standards for antimicrobial susceptibility testing; 16th informational supplement. CLSI M100-S16. Clinical and Laboratory Standards Institute, Wayne, PA.
23. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K. A.; Tomita, M.; Wanner, B. L.; Mori, H. *Mol. Syst. Biol.* **2006**, *2*, 2006.0008.
24. Cultures of BW25113 and its isogenic *ompF* mutant grown aerobically in M9 minimal medium with either 1 mM or 1 μM ferric citrate were subcultured into 20 ml M9 medium with either 1 mM or 1 μM ferric nitrate and grown to mid-log phase, then harvested by centrifugation, and washed three times in M9. 120 μL of each culture was mixed with 7 mL bacteriological agar which was poured as an overlay on a bottom layer of M9 agar. 0.1 μg of ciprofloxacin or 0.15 μg of **1** were spotted on the surface of the plates and then the plates were incubated at 37 °C overnight before measuring the diameter of the zone of inhibition.
25. Selvin, P. R.; Jancarik, J.; Hung, L.-W. *Inorg. Chem.* **1996**, *35*, 700.
26. Carrano, C. J.; Drechsel, H.; Kaiser, D.; Jung, G.; Matzanke, B.; Winkelmann, G.; Rochel, N.; Albrecht-Gary, A. M. *Inorg. Chem.* **1996**, *35*, 6429.