European Journal of Medicinal Chemistry 46 (2011) 3388–3397

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis, activity and pharmacokinetics of novel antibacterial 15-membered ring macrolones

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ARTICLE INFO

Article history:
Received 11 February 2011
Received in revised form
19 April 2011
Accepted 2 May 2011
Available online 10 May 2011

Keywords:
Macrolide
Resistance
Antibacterial activity
Pharmacokinetics
Oral bioavailability

ABSTRACT

Synthesis, antibacterial activity and pharmacokinetic properties of a novel class of macrolide antibiotics—macrolones—derived from azithromycin, comprising oxygen atom(s) in the linker and either free or esterified quinolone 3-carboxylic group, are reported. Selected compounds showed excellent antibacterial potency towards key erythromycin resistant respiratory pathogens. However, the majority of compounds lacked good bioavailability. The isopropyl ester, compound **35**, and a macrolone derivative with an elongated linker **29** showed the best oral bioavailability in rats, both accompanied with an excellent overall microbiology profile addressing inducible and constitutive MLSb as well as efflux mediated macrolide resistance in streptococci, while compound **29** is more potent against staphylococci.

1. Introduction

A continuous increase in the number of infections caused by bacteria resistant to one or multiple antibiotic classes poses a significant threat [1,2] as it may lead to treatment failures and complication. A spread of resistance among common respiratory pathogens, including *Streptococcus pneumoniae*, is recognized by Infectious Diseases Society of America as one of the three major areas of concern that creates a need for new antibiotics [2].

One of the main driving forces for development and spread of resistance is high antibiotic consumption, reflected in resistance rates directly correlating with the prescription of antimicrobial drugs [3].

Therefore, considerable differences are reported throughout the world and levels of *S. pneumoniae* resistance to the two most frequently used antibiotic classes—beta-lactams and macrolides—vary substantially in Europe, ranging from <10% penicillin non-susceptible *S. pneumoniae* (PNSP) and erythromycin resistant pneumococci (ERP) isolates in northern countries to >25% in the south [4]. Levels of macrolide resistance and underlying resistance mechanisms in different parts of the world differ, with efflux tending to be more prevalent in North America while *erm*(B) is most common in many parts of Europe (South-eastern in particular) [4,5] and Asia [6–8]. Among macrolide resistant strains in Asia, there is a high proportion of isolates having both *erm*(B) and *mef*(E) [6,7]. Generally, demographics play an important role in incidence of both penicillin and macrolide resistance, with a higher frequency of resistance consistently found among children [9].

To overcome resistance, in addition to prudent use of available drugs, a constant effort to discover and develop new agents with an

Abbreviations: EDCxHCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DBU, 1,8-Diazabicyclo[5.4.0] undec-7-ene; AZM, azithromycin; TEL, telithromycin; eryS, erythromycin sensitive; MLSb, macrolide, licosamide and streptogramin (MLSb) antibiotics; iMLSb, inducible resistance to macrolide, lincosamide and streptogramin antibiotics; iMcLSb, inducible resistance to macrolide and constitutive resistance to lincosamide antibiotics; cMLSb, constitutive MLSb resistance; M, efflux mediated macrolide resistance; PK, pharmacokinetics; IV, intravenous; PO, per os; CL, systemic clearance; CLi, intrinsic clearance; Vd, volume of distribution; LBF, liver blood flow; oral F, oral bioavailability.

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doi:10.1016/j.ejmech.2011.05.002

improved spectrum of activity is required. A variety of chemical modifications were carried out so far around the macrolide backbone (core). Among 14-membered derivatives, cethromycin [10–13], a 3-keto derivative, seems to exhibit antibacterial potency against resistant pathogens comparable/exceeding to telithromycin. In the past decade, we have explored 15-membered macrolide cores, including modifications on the N-9 position of aglycone ring [14–16], 3-decladinosyl derivatives [17,18] and acyl derivatives on 4″-position of cladinose [19] without achieving desirable antimicrobial profile.

Macrolide compounds modified at the 4″-position present a considerable opportunity for the development of novel antibiotics to effectively address the growing problem of macrolide resistance. We reported on the first 4″-*O*-quinolone derivative on 6-*O*-methyl-8a-aza-8a-homoerythromycin, with improved activity against macrolide resistant *S. pneumoniae* (with both inducible and constitutive *erm*(B) expression), iMLSb *Streptococcus pyogenes* and *Haemophilus influenzae*, compared to golden standard of macrolide therapy, azithromycin [20]. Subsequently we explored 4″-*O*-(quinolylamino-alkylamino) propionyl derivatives on various macrolide scaffolds, 8a-aza-8a-homoerythromycin, clarithromycin and azithromycin [21]. From SAR trends observed in this series, azithromycin compounds with quinolone containing cyclopropyl on N(1) position had the most favorable antibacterial profiles. Here we describe a derivatization stream followed in order to investigate the influence of linker features on biological properties, both antibacterial and pharmacological. Therefore, we introduced one or two oxygen atoms in the linker, retaining similar length or elongating it. Additional modification of quinolone carboxylic acid by esterification was performed in order to optimize pharmacokinetic (PK) properties.

2. Results and discussion

2.1. Chemistry

Synthesis of quionolone-3-carboxylic intermediates **18**–**23** can be approached by route outlined in Scheme 1.

Synthesis is based on the commercially available 6,7-disubstituted quinolone precursors **2**–**5**. The electronic effects of the other two groups on the benzene unit in quinolones **2**–**5** tend to diminish the inherent difference in the reactivity of these two halogen atoms in nucleophilic substitution. The vinilogous-amide type nitrogen, present in the *para* position to fluorine, lowers its nucleofugal aptitude, whereas carbonyl group, *para*-situated to chlorine enhances its nucleofugal property [22]. The net synthetic result of this leveling is the formation of mixtures of 6- and 7-substituted quinolones **6**–**11** (Scheme 1). On varying the solvent and temperature, the ratio of the two substitution products **6a**–**11a**/**6b**–**11b** remained in the range 45:55 ± 5%. In spite of this undesired ratio, this reaction step became workable when an effective, non-chromatographic separation process for **6a**–**11a** and **6b**–**11b** was developed (see Experimental). This protocol has allowed isolation of pure 6-chloro-7-substituted derivatives **6a**–**11a** and their transformation to the target structures **18**–**23**.

Synthesis of final macrolones **24**–**46** is outlined in Scheme 2.

In the first step, site-selective acylation at 4″–OH of 2′-*O*-acetyl azithromycin **1** was performed with an excess of quinolone diacids **18**–**23**. Several condensation procedures were investigated, e.g. mixed anhydride of diacids with pivaloyl chloride, DCC, EDC, and the most suitable reagent was EDCxHCl/DMAP. Determination of pK_a values of quinolone diacid **18** has shown remarkable differences in acid strengths of two carboxylic groups in the molecule (Fig. 1) which explains regioselectivity in the esterification reaction [23,24].

Subsequent deprotection in methanol and purification either by column chromatography or precipitation from ethyl acetate with hexane yielded final quinolone-macrolide derivatives **24**–**29**.

In order to determine the influence of halogen atom on antimicrobial activity, a series of C(7)-dechloro derivatives **30**–**33** have been synthesized in high yield and purity by catalytic hydrogenolysis of **24**–**26** and **29** (Scheme 2).

In order to improve *in vivo* biological properties, ester derivatives on quinolone-3-carboxylic acid **34**–**46** have been prepared by alkylation of macrolone acids **24**, **25**, **30** and **31** with alkyl iodide in presence of K_2CO_3 as base (Scheme 2).

Structures of new molecules were confirmed by the high-resolution mass spectra (HRMS) and NMR. NMR spectra showed two sets of signals, one corresponding to the macrolide, the other to the quinolone part of the molecule, as well as NMR signals of methylene groups of the linker. The proton on the 4″-position of the cladinose sugar revealed a shift from around 2.90 ppm in azithromycin to around 4.70 ppm in final macrolone compounds. The long-range coupling of 4″-proton with new carbonyl signal at around 171 ppm provided additional evidence that a new ester function was introduced at C(4″)-position of macrolide. ^{1}H NMR spectra of C(7)-dechloro compounds **30**–**33** showed a new proton in aromatic part of spectra at around 7.20 ppm and in ^{13}C NMR spectra an upfield shift of C(7) signal for 5 ppm is noticed.

Although C-6, C-7, and N-1 positions in the quinolone part of target molecules should correctly be C-6‴, C-7‴, and N-1‴, the designation used in this article are C(6), C(7), and N(1) because of the clearness.

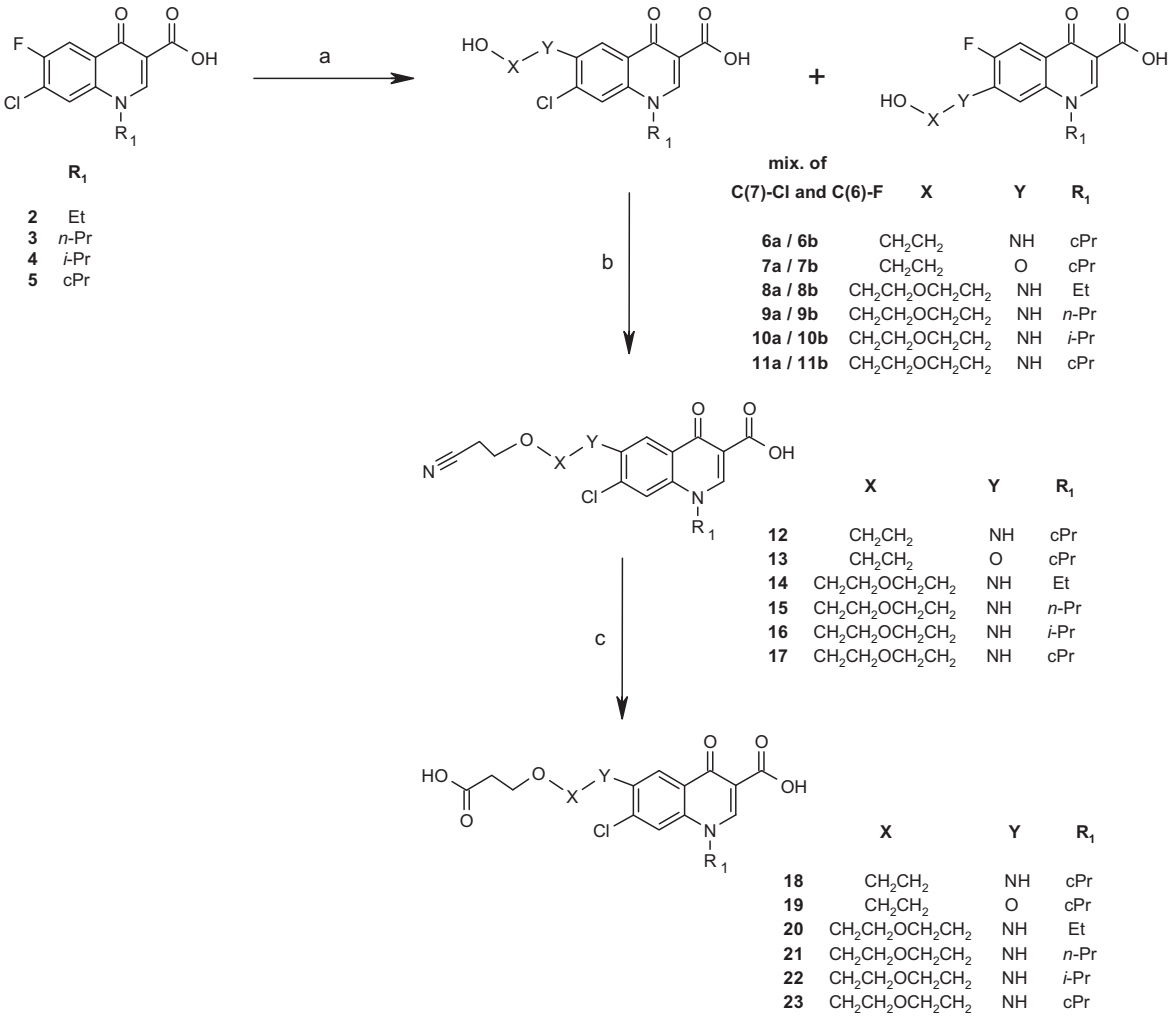
2.2. X-ray crystal structure

The described synthetic procedures provided compound **29** as an amorphous solid. Significant efforts were made to obtain crystals of **29** from some organic solvents as solvates. High quality crystals were obtained from solvent mixture cyclohexane:*sec*-butyl acetate = 1:1. Single crystal analyses reveal that the compound crystallizes with two independent molecules in asymmetric unit (Fig. 2) with significant conformational differences in orientation of cladinose substituent (Fig. 3). Orientation of C30 and C30B methyl groups into region between desosamine and cladinose sugars indicates typical “folded-in” conformation of aglycone rings. Compound crystallizes as solvate with unidentified numbers of *sec*-butyl acetate and water molecules. Molecules of compound **29** occupy about 85% of unit cell volume while the rest is filled with solvent molecules of which positions cannot be clearly defined (Fig. 4). This assumption is confirmed also with low value of calculated crystal density (1.01 mg/m^3).

Molecules of compound **29** are connected by hydrogen bonds making layers within unit cell, while solvent molecules are situated in holes between the layers and do not participate in hydrogen bonding.

2.3. In vitro antibacterial activity

The antibacterial activity of all novel compounds was determined by a standard broth microdilution method [25] and the data is expressed as minimum inhibitory concentrations (MICs) in units of μg/mL. The organisms studied represent relevant Gram-positive (*Streptococcus pneumoniae*, *S. pyogenes* and *Staphylococcus aureus*) and Gram-negative (*H. influenzae* and *Moraxella catarrhalis*) respiratory tract pathogens, and are either sensitive or resistant to macrolide antibiotics. Underlying resistant phenotypes are two major mechanisms – production of efflux pumps (M), or ribosome modification by methylation, where methylase expression is inducible (iMLSb) or constitutive (cMLSb).

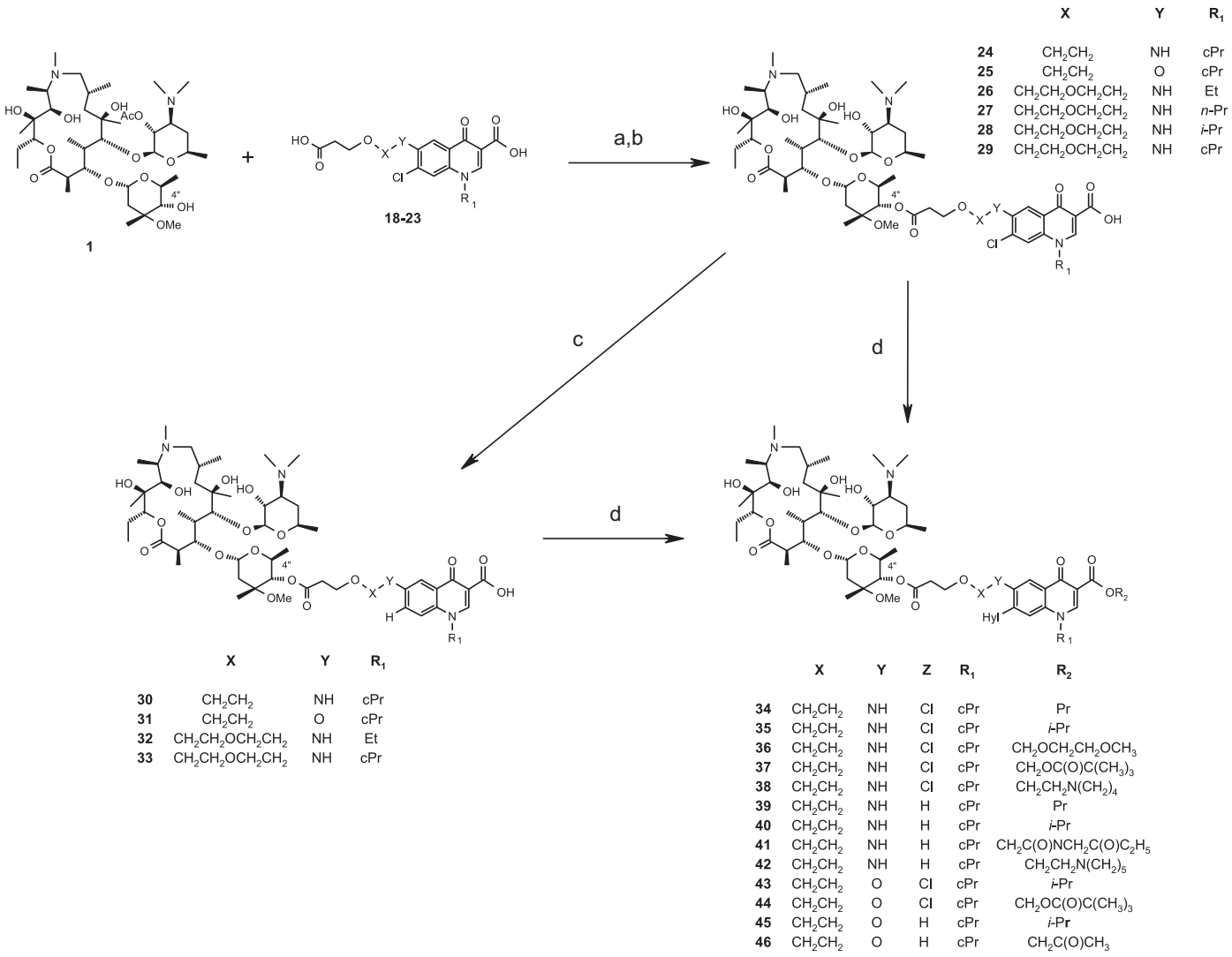


Scheme 1. Reagents and conditions: (a) 2-amino-ethanol or 2-(2-aminoethoxy)-ethanol/ethylene glycol, 1-methyl-2-pyrolidone/DMSO, no base/K_2CO_3, 90–100 °C, 20 h/6 h; (b) acrylonitrile, DBU, 70 °C, 16 h; (c) H_2SO_4:H_2O (1:1), 70 °C, 5 h.

Compared to compounds A and B [21] with two nitrogen atoms in the linker, replacing one or both nitrogens by an oxygen atom in compounds (**24**, **25**, **30** and **31**), having a free carboxylic group on the quinolone moiety, did not significantly influence the antibacterial potency and compounds retained excellent activity profiles with MIC values against *H. influenzae* in the range of azithromycin and telithromycin (Table 1). Dehalogenation of **24** and **25**, resulting in **30** and **31**, respectively, did not affect the antibacterial activity. The overall potency of all these compounds was excellent, providing full coverage of the relevant respiratory tract pathogens, regardless of the underlying resistance mechanism. The only exception was the cMLSb *S. aureus* strain, where some meager signs of activity (MIC 16–32 μg/mL) are observed. This type of resistance in *S. aureus*, however, cannot be overcome also by any of the newer macrolide or ketolide antimicrobials (telithromycin or cethromycin) [13]. Additionally, telithromycin does not provide adequate coverage of cMLSb and *erm*(B) harboring iMLSb *S. pyogenes* strains [13]. Therefore, exceptional activity of tested macrolones (MIC $<$ 0.125 μg/mL for majority of compounds, none exceeding 1 μg/mL) against cMLSb *S. pyogenes* strain clearly distinguishes them from telithromycin (MIC 16 μg/mL against tested strain). Compounds **24** (containing an ON linker) and **25** (containing an OO linker) where chosen for pharmacokinetic studies in rats (Table 2). As was observed with compounds A and B [21] (data not shown), compounds **24** and **25** also had a very low oral bioavailability of 3 and $<$1%, respectively. Thus, we tried to enhance their oral bioavailability by esterification of the 3-quinolone carboxylic acid. Based on reviews of marketed prodrugs, it is evident that one of the most successful prodrug approaches is the addition of simple esters to polar molecules to improve oral bioavailability [26–28]. Our strategy was to synthesize simple esters, due to the large molecular weight of the parent compounds, that would either be metabolically stable, providing they had adequate antibacterial potency, or rapidly cleavable after delivering compound into the systemic circulation.

We prepared a series of macrolones with different esters at 3-quinolone carboxylic (simple aliphatic units - compounds **34**, **35**, **39**, **40**, **43** and **45**, units with incorporated oxygen or nitrogen atom - **36**, **38** and **42**, and amide or carbonyl containing units - **37**, **41** and **46**). Compared to macrolone acid analogs, the antibacterial potency of esters was decreased, mainly against *H. influenzae*, iMLSb and efflux resistant *S. aureus* strains. Some of the esters still had acceptable antibacterial profiles, such as the compound **39**, **a** propyl derivative of **30**, as well as the isopropyl **35** and the pivaloyl ester **37** of compound **24**.

Macrolone acid compounds **26**, **28** and **29** with linker elongated by additional two CH_2 units and one oxygen retain superior antibacterial potency as well as their dehalogenated analoques (**26** *vs.*



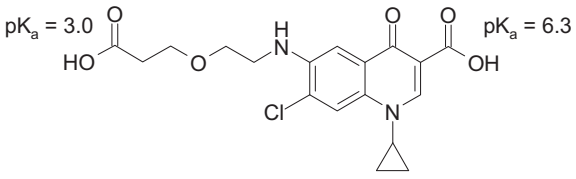
Scheme 2. Reagents and conditions: (a) EDCxHCl, DMAP, DCM, DMF, 0 °C-rt, 24 h; (b) MeOH, rt, 24 h; (c) H_2, Pd/C, MeOH, 2 barr, rt, 16 h; (d) R_1I, K_2CO_3, DMF, rt, 48 h.

32 and **29** *vs.* **33**). A decrease in potency, however, was observed in compound **27** having a propyl chain at position N(1) on the quinolone moiety (MIC of 32 and 4 μg/mL on iMLSb *S. aureus* and *H. influenzae*, respectively).

2.4. Pharmacokinetic studies

Pharmacokinetic studies were carried out in rats with several compounds, selected based on their *in vitro* antimicrobial activity and metabolic stability. The metabolic stability was evaluated in rat liver microsomes and is summarized in Table 2. Compounds **24**, **25**, **29**, **31** and **35** were found to be stable in rat liver microsomes (CLi<0.6 mL/min/g liver), however, the pivaloyl ester compound **37**, was hydrolyzed in rat liver microsomes. Pharmacokinetic parameters following intravenous and oral administration to rats are summarized in Table 2.

Compound **24**, containing an oxygen and nitrogen atom in the linker, has a moderate systemic clearance (CL) of approximately 26% of liver blood flow (LBF), a large volume of distribution (Vd) (19.2 L/kg), long half-life ($t_{1/2}$ 8.4 h) and low oral bioavailability of ca 3%, most likely as a result of poor gastrointestinal absorption. In order to investigate to what extent gastrointestinal stability affects the oral bioavailability, the compound was administered intraduodenally which led to an increase in bioavailability to ca. 9% (data not shown). Introduction of an isopropyl ester on the quinolone moiety (compound **35**), resulted in a reduction of CLs (ca. 9 LBF), an increase in Vd and a prolonged $t_{1/2}$ (26.6 h) and increase in oral bioavailability (ca 11%). In comparison to compound **35**, the pivaloyl ester derivative **37** did not improve the oral bioavailability (1.4%) with respect to the acid analog **24**, likely due to ester hydrolysis. Substitution of the nitrogen atom in the linker with an oxygen atom (compound **25**), gave a slightly lower CL (19%), very large Vd (16.7 L/kg) and long $t_{1/2}$ (10.4 h), however, did not improve the oral F (<1%). Dehalogenation did not result in specific improvement on *in vitro* antimicrobial profile or oral bioavailability (<1%) of compound **31** *vs.* its halogenated analog compound **25**, despite the reduction in CLs (10% LBF), large Vd (11 L/kg) and long $t_{1/2}$ (15 h). Lengthening of the linker by incorporating two oxygen atoms and a nitrogen atom, compound **29**, gave an improved antimicrobial activity and oral bioavailability (10.7%) in comparison to other tested non-ester compounds. These results are in line with previously reported PK data for azithromycin derivatives of the same linker length [29]. Compound **29** is characterized by a low CLs (13% LBF), a large Vd (10.7 L/kg) and long $t_{1/2}$ (9.1 h). Following intraduodenal administration (data not shown) the oral bioavailability of compound **29** increased up to 21%, indicating some gastric instability and potential for further optimization.



18

Fig. 1. pK$_a$ values of quinolone diacid **18**.

3. Conclusions

Compared to previously published data [21], insertion of an oxygen atom into the linker of macrolone acid compounds resulted in improved antibacterial potency, in particular against *H. influenzae*. However, their oral bioavailability in rats was poor. In an attempt to improve the oral bioavailability, a series of esters on quinolone-3-carboxylic acid was prepared and the isopropyl ester derivative **35** showed improved oral bioavailability, while still retaining acceptable antimicrobial activity. Furthermore, elongation of the linker in macrolone acid derivative **29** improved the oral bioavailability and the compound retained superior antibacterial profile.

It seems that quinolone-3-carboxylic acid is an important moiety for superior antibacterial activity as esterification causes an increase of MIC for iMLSb *S. aureus* and *H. influenzae*. Although esterification is a valid strategy to improve the oral bioavailability, it does not appear to be an optimal solution for this class of compounds. On the contrary, changing the linker lengths a chemical strategy resulted with both benefits – maintenance of excellent potency and achievement of acceptable oral bioavailability.

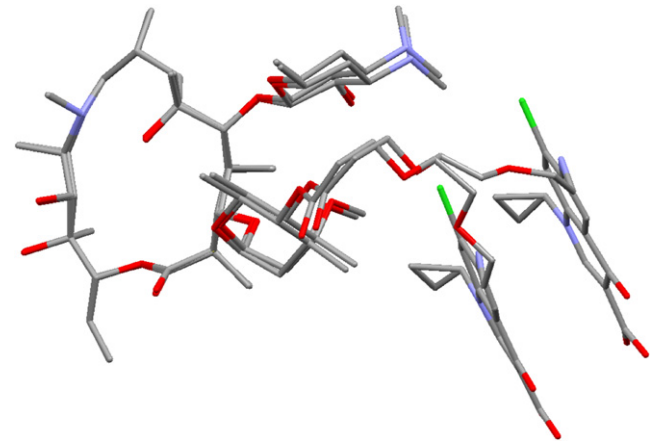


Fig. 3. Overlay of two independent molecules of compound **29**.

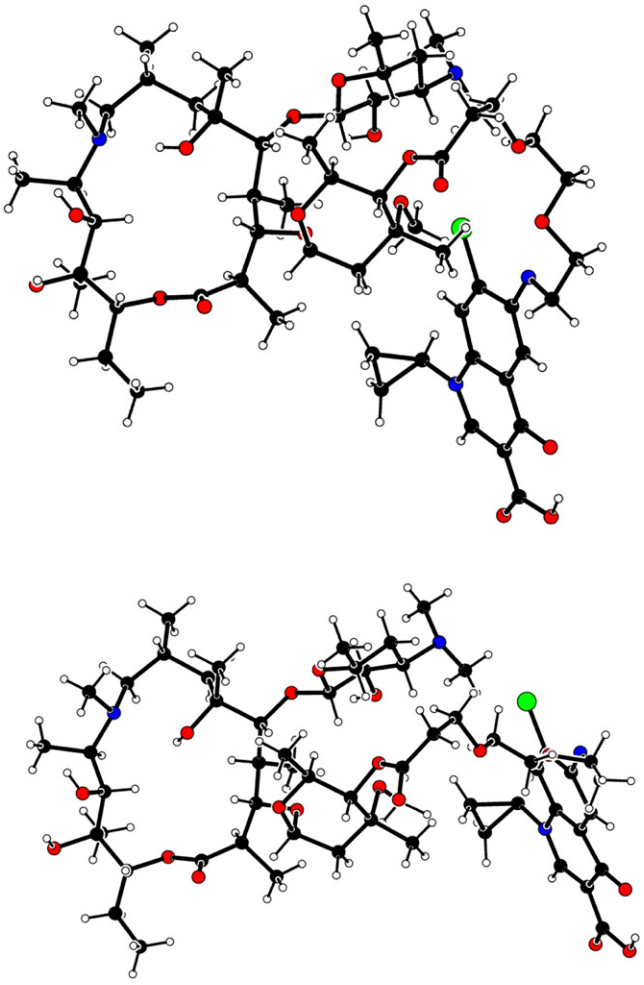


Fig. 2. Two crystallographically independent molecules of compound **29** (colored by atom; black: carbon, colorless: hydrogen, red: oxygen, blue: nitrogen, green: chlorine). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

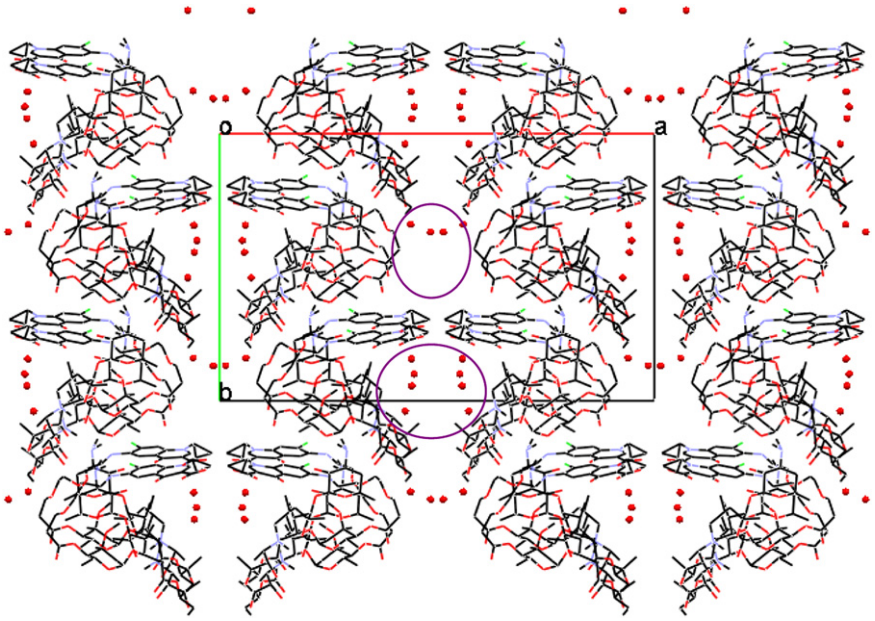


Fig. 4. A projection of the structure of compound **29** on the *ab* plane showing the formation of cavities between molecules (violet circles). Hydrogen atoms have been omitted for clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Experimental

4.1. In vitro antibacterial activity

Minimum inhibitory concentrations (MICs) were determined by the broth microdilution method according to guidelines of the Clinical Laboratory Standards Institute [25], except that for *Streptococcus* medium, lysed blood was substituted with 5% horse serum. Double dilutions of tested compounds in 96-well microtitre plates were prepared using TECAN Genesis 150. Bacteria were grown on appropriate agar plates (by Becton Dickinson, USA) - Columbia agar with 5% sheep blood for streptococci, Mueller–Hinton chocolate agar for *H. influenzae* and Mueller–Hinton agar for staphylococci.

4.2. Intrinsic clearance (CLi) assay

Intrinsic clearance (CLi) values were determined in rat and human liver microsomes. Test compounds (1.0 μM) were incubated at 37 °C for 60 min in 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 mg microsomal protein/mL. The reaction was started by addition of cofactor (NADPH generating system). The final concentration of solvent was 1% of the final volume. At 0, 10, 20, 30, 45, and 60 min, an aliquot (100 μL) was taken, quenched with acetonitrile containing an appropriate internal standard, and analyzed by HPLC MS/MS. The intrinsic clearance (CLi) was determined from the first-order elimination constant by nonlinear regression, corrected for the volume of the incubation and assuming 52.5 mg microsomal protein/g liver for all species. Values for CLi were expressed as mL/min/g liver.

4.3. In vivo pharmacokinetic studies

Male Wistar Han rats, weighing 300–350 g, were purchased from IFFA CREADO, Lyon, France. The rats were maintained in an air-conditioned animal quarter at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%. Water and food (Laboratory Rodent Chow, Nanjing, China) were allowed *ad libitum*.

4.4. Intravenous and oral administration

Rats were divided into groups of 5 and the pharmacokinetic studies including intravenous (IV) and oral (PO) dosing was performed in a crossover design including a 2 day washout period. Each compound was administered intravenously at 10 mg/kg, followed by a 2 day washout period and orally at 30 mg/kg equivalent base. Prior to oral administration animals were fasted overnight. For intravenous dosing, compounds were formulated in 1% DMF/Phosphate buffer and blood samples (50 μL) were collected at 0.17, 0.33, 0.67, 1, 2, 4, 6, 8 and 24 h. Following oral administration compounds were dissolved in 1% DMF/20% Encapsin (w/v) and saline and blood samples (50 μL) were collected at 0, 0.25, 0.5, 1, 1.5, 2, 4, 6, 8 and 24 h. Blood samples were collected serially from the tail vein, haemolysed with deionized water in a 1:2 ratio and frozen at −20 °C until analysis.

4.5. Sample preparation and bioanalysis

Haemolysed blood samples (150 μL) in Eppendorf test tubes were treated by protein precipitation with the addition of two volumes of a mixture MeCN/MeOH (1:2), containing internal standard. A 1 mg/mL stock solution of each compound was prepared in DMSO, diluted in water and spiked into rat blank blood to prepare duplicate standards ranging from 5 to 25,000 ng/mL. One set of standards was analyzed at the beginning and one set at the end of each sample batch. The mixtures were centrifuged at 4000 rpm at 4 °C for 10 min and aliquots (0.01 mL) of the resulting supernatant fractions were transferred to a 96-well plate. Samples were analyzed with either a Sciex API 3000 or Sciex API 2000 Triple Quadrupole Mass Spectrometer (Sciex, Division of MDS Inc., Toronto, Canada) coupled to an HP HPLC System (HP1100, Hewlett–Packard) and an HTS PAL CTC Autosampler (CTC). Samples (5 μL) were injected onto an HPLC column (3 μm Phenomenex Luna C18(2), 2.0 × 50 mm) and eluted with a gradient at room temperature. The chromatographic conditions consisted of mobile phase A (1000:1 MeCN/formic acid, v/v) and mobile phase B (1000:1 water/formic acid, v/v) that was run over a 6 min gradient at a flow rate of 0.3 mL/min. A positive ion

Table 1
Antibacterial activity (MIC) of compounds **24**–**46** (μg/mL).

	S. aureus ATCC 13709	*S. pneumoniae* SP030	*S. pyogenes* 3565	*S. aureus* PK1	*S. pneumoniae* Ci137	*S. pyogenes* 2 Finland	*S. aureus* 90256	*S. pneumoniae* 134 GR M	*S. pyogenes* Finland 11	*S. aureus* PK2	*S. pneumoniae* 58 Spain	*S. pyogenes* 166 GR-Micro	*H. influenzae* ATCC 49247	*M. catarrhalis* ATCC 23246
	eryS	eryS	eryS	M	M	M	iMLSb	iMcLSb	iMLSb	cMLSb	cMLSb	cMLSb		
AZM	0.5	≤0.125	≤0.125	32	8	8	>64	>64	16	>64	>64	>64	1	≤0.125
TEL	0.125	≤0.125	≤0.125	≤0.125	0.25	0.5	≤0.125	0.25	0.06	>64	0.25	16	2	≤0.125
A[a]	0.25	≤0.125	≤0.125	0.25	≤0.125	≤0.125	0,5	≤0.125	≤0.125	4	≤0.125	≤0.125	1	1
B[b]	1	≤0.125	≤0.125	1	≤0.125	≤0.125	1	≤0.125	≤0.125	8	≤0.125	≤0.125	4	1
24	≤0.125	≤0.125	≤0.125	0.25	≤0.125	≤0.125	0.25	≤0.125	≤0.125	32	≤0.125	≤0.125	0.5	0.5
25	0.25	≤0.125	≤0.125	0.5	≤0.125	≤0.125	0.5	0.25	≤0.125	>64	0.5	0.25	1	1
26	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	16	≤0.125	0.25	1	0.5
27	0.25	≤0.125	≤0.125	0.25	≤0.125	≤0.125	32	1	≤0.125	>64	≤0.125	1	4	0.5
28	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25	0.25	≤0.125	64	≤0.125	1	1	1
29	0.25	≤0.125	≤0.125	0.25	≤0.125	≤0.125	0.25	≤0.125	≤0.125	16	≤0.125	≤0.125	0.5	≤0.125
30	0.5	≤0.125	≤0.125	1	≤0.125	≤0.125	0.5	0.25	≤0.125	32	≤0.125	≤0.125	1	0.5
31	≤0.125	≤0.125	≤0.125	0.25	≤0.125	≤0.125	1	≤0.125	≤0.125	32	≤0.125	0.25	0.5	0.5
32	0.25	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.25	1	≤0.125	64	≤0.125	≤0.125	2	2
33	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	0.5	≤0.125	≤0.125	16	≤0.125	≤0.125	0.5	0.25
34	1	≤0.125	≤0.125	1	≤0.125	≤0.125	1	≤0.125	≤0.125	>64	≤0.125	≤0.125	4	2
35	0.5	≤0.125	≤0.125	2	≤0.125	≤0.125	2	≤0.125	≤0.125	>64	≤0.125	≤0.125	2	nd
36	1	≤0.125	≤0.125	2	≤0.125	≤0.125	2	≤0.125	≤0.125	>64	0.25	≤0.125	8	8
37	1	≤0.125	≤0.125	2	≤0.125	≤0.125	4	≤0.125	≤0.125	>64	0.5	≤0.125	1	2
38	0.25	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125	1	≤0.125	≤0.125	>64	≤0.125	≤0.125	4	≤0.125
39	0.5	≤0.125	≤0.125	2	≤0.125	≤0.125	4	≤0.125	≤0.125	>64	≤0.125	≤0.125	2	nd
40	0.5	≤0.125	≤0.125	8	≤0.125	≤0.125	16	≤0.125	≤0.125	>64	≤0.125	≤0.125	4	4
41	1	≤0.125	≤0.125	8	≤0.125	≤0.125	16	≤0.125	≤0.125	>64	≤0.125	≤0.125	4	nd
42	0.25	≤0.125	≤0.125	1	≤0.125	≤0.125	4	≤0.125	≤0.125	>64	≤0.125	≤0.125	8	1
43	1	≤0.125	≤0.125	4	≤0.125	≤0.125	4	0.5	≤0.125	>64	0.25	0.5	2	0.5
44	1	≤0.125	≤0.125	2	≤0.125	≤0.125	4	0.25	≤0.125	>64	≤0.125	≤0.125	4	4
45	1	≤0.125	≤0.125	16	≤0.125	≤0.125	32	≤0.125	≤0.125	>64	≤0.125	≤0.125	4	2
46	0.5	≤0.125	≤0.125	1	0.25	≤0.125	2	0.25	≤0.125	64	0.5	≤0.125	2	1

[a] Compound No. **48** in reference [21].
[b] Compound No. **54** in reference [21].

Table 2
PK parameters in blood following IV and oral administration in rats of selected compounds and their ester derivatives.

	Metabolic stability	IV administration[a]			PO administration[b]
	CLi mL/min/g liver	CL mL/min/kg	Vd L/kg	$t_{1/2}$ h	Oral F%
24	<0.6	26.3 ± 4.6	19.2 ± 3.9	8.4 ± 0.3	3.1 ± 1.2
25	<0.6	18.6 ± 3.3	16.7 ± 1.5	10.4 ± 1.1	<1
29	<0.6	13.2 ± 4.3	10.7 ± 4.8	9.1 ± 2.4	10.7 ± 5.0
31	<0.6	8.5 ± 3.4	11.0 ± 1.8	15.0 ± 6.0	<1
35	<0.6	9 ± 2.1	20.7 ± 1.6	26.6 ± 5.6	10.7 ± 1.0
a	Hydrolyzed	13.7 ± 5.3	12.2 ± 2.9	10.3 ± 2.6	1.4 ± 0.3

[a] Doses for compounds **24**, **25**, **31**, **35** and **37** 10 mg/kg IV and 50 mg/kg PO.
[b] Compound **29** 10 mg/kg IV and 30 mg/kg PO.

mode with turbo spray, an ion source temperature of 400–450 °C and a dwell time of 300–400 ms were utilised for mass spectrometric detection. Quantitation was performed using multiple reaction monitoring (MRM) at the specific transitions for each compound. Linear regression plots of compounds to internal standard peak area ratios versus drug concentrations were derived with $1/x$ or $1/x^2$ weighting. The dynamic range for the blood assay ranged from 0.005 μg/mL to 10 μg/mL.

4.6. Pharmacokinetic analysis

Noncompartmental analysis for all compounds was performed using WinNonlin Professional, version 4.0.1 (Pharsight, Mountain View, CA). Individual blood concentrations and sample times for each animal were used in the analysis. Following IV bolus administration the terminal elimination half-life (t), total area under the curve, concentration–time curve extrapolated to infinity, systemic blood clearance and steady-state volume of distribution were calculated by standard methods. After oral administration the peak plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were taken directly from individual profiles and the areas under the curve and oral bioavailabilities were determined. Summarised pharmacokinetic parameters were reported as mean values ±SD both after intravenous and oral administration.

4.7. X-ray crystal structure elucidation

Single crystal structure data were collected on the Bruker Nonius FR591 diffractometer equipped with Kappa CCD detector at 100 K using CuKα radiation. Data were corrected for Lorentz/polarization factors and also for absorption using DENZO [30] and SCALEPACK [30] programs.

The structure was solved by direct methods using SHELXS86 [31] program. It was refined by SHELXL97 [31]. Structure was refined with isotropic thermal parameters for all non-hydrogen atoms. Hydrogen atoms on carbon atoms were calculated in their ideal positions and refined using a riding model. Molecular graphic was done using PLATON 2000 [32] and Mercury 2.4 [33] programs.

Compound **29** (5 mg) was weighed out into vial and dissolved by heating in solvent mixture cyclohexane:*sec*-butyl acetate = 1:1 (200 μL). The resulting solution was cooled to room temperature and allowed solvents to slow evaporate yielding *sec*-butyl acetate solvate single crystals.

4.8. Chemistry

All commercial reagents (Merck, Sigma–Aldrich) were used as provided unless otherwise indicated, and all solvents are of the highest purity unless otherwise noted. The purity of final compounds was assessed by analytical LC–MS method and found to be ≥95% unless otherwise stated. The LC–MS analyses were performed using Waters Acquity UPLC instrument equipped with diode-array detector and MS detector, Waters SQD, using the following method: column, Waters Acquity UPLC BEH C18, 2.1 × 50 mm, 1.7 μm particles; mobile phase A, 0.1% HCOOH in water, mobile phase B, 0.1% HCOOH in CH_3CN, isocratic 5% B in 1.5 min, then gradient 5–80% B in 7.25 min followed by 1.25 min at 90% B.

Mass spectra were obtained on a Waters Micromass ZQ mass spectrometer for ES^+-MS. Electrospray positive ion mass spectra were acquired using a Micromass Q-Tof2 hybrid quadrupole time-of-flight mass spectrometer, equipped with a Z-spray interface, over a mass range of 100–2000 Da, with a scan time of 1.5 s and an interscan delay of 0.1 s in a continuum mode. Reserpine was used as the external mass calibrant lock mass ($[M + H]^+ = 609.2812$ Da). The elemental composition was calculated using a MassLynx v4.1 for the $[M + H]^+$ and the mass error quoted within ±5 ppm range. NMR spectra were recorded on a Bruker Avance DRX500 or Bruker Avance DPX300 spectrometer in CDCl3 or DMSO and chemical shifts are reported in ppm using TMS as an internal standard. In synthetic procedures, column chromatography was carried out over Merck Kieselgel 60 (230–400 mesh) or on SPE cartridge with average size silica 50 μm. Thin layer chromatography was performed on 0.24 mm silica gel plates Merck TLC 60F254. The eluent used was indicated and solvent ratios refer to volume. In general, organic solutions were dried with anhydrous Na_2SO_4 or K_2CO_3, evaporation and concentration were carried out under reduced pressure below 40 °C, unless otherwise noted.

All final compounds were isolated as amorphous solid except by compound **29**.

4.8.1. General procedure for aminolysis

To a solution of compounds **2**–**5** (1 eq) in 1-methyl-2-pyrolidone, 2-amino-ethanol or 2-(2-aminoethoxy)-ethanol (2 eq) were added. The reaction mixture was warmed up to 110 °C, and stirred at this temperature over 20 h. Then it was cooled to 20–25 °C, water was added and pH was adjusted to 12. The resulting solution was extracted with DCM, then pH value of water solution was corrected to 6, and after 10 min crystalline product was collected on filter affording pure products **6a**, **8a**–**11a**.

*4.8.1.1. 7-Chloro-1-cyclopropyl-1,4-dihydro-6-[(2-hydroxyethyl)amino]-4-oxo-3-quinolincarboxylic acid (**6a**).* According described procedure starting from intermediate **5** (50.0 g, 177.51 mmol) product **6a** (19.8 g, $Y = 35\%$) as yellow powder was obtained; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.57 (s, 1H), 8.24 (s, 1H), 7.41 (s, 1H), 3.83 (qv, 1H), 3.65 (m, 2H), 3.33 (m, 2H), 1.29 (m, 2H), 1.15 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 176.56, 166.10, 145.67, 143.01, 132.06, 126.33, 125.46, 118.95, 106.16, 102.66, 58.67, 45.32, 35.76, 7.32; MS (ESI) m/z calcd for $C_{15}H_{16}ClN_2O_4$ $[M + H]^+$ 323.0799; found 323.0795.

4.8.2. General procedure for Michael addition

To acrylonitrile solution of product **6a**–**11a** (1 eq) at 0 °C DBU (0.5 eq) was added and the reaction mixture was warmed up to 70 °C and stirred at this temperature for 16 h. The acrylonitrile was evaporated and the residue dissolved in *i*-PrOH. Water was added and the pH adjusted to 4. Obtained product was filtered, and then triturated with MeOH yielding pure products **12**–**17**.

*4.8.2.1. 7-Chloro-6-{2-[(2-cyanoethyl)oxy]ethylamino}-1-cyclopropyl-4-oxo-1,4-dihydro-3-quinolincarboxylic acid (**12**).* According described procedure starting from intermediate **6a** (7.0 g, 21.68 mmol) product **12** (5.4 g, $Y = 66\%$) as yellow powder was obtained; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.56 (s, 1H), 8.23 (s, 1H), 7.40 (s, 1H), 5.95 (t,

NH), 3.83 (qv, 1H), 3.72 (t, 2H), 3.67 (t, 2H), 3.46 (q, 2H), 2.79 (t, 2H), 1.30 (q, 2H), 1.18 (q, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 176.52, 166.09, 145.72, 142.72, 132.17, 126.37, 125.38, 119.15, 118.99, 106.14, 102.76, 67.93, 65.05, 42.40, 35.77, 18.01, 7.32; MS (ESI) *m/z* calcd for $C_{18}H_{18}ClN_3O_4$ $[M + H]^+$ 376.1064; found 376.1068.

4.8.3. General procedure for hydrolysis of cyano group

Products **12**–**17** were dissolved in H_2SO_4/H_2O (1:1) and stirred at 70 °C for 24 h. Reaction mixture was poured on ice water. Obtained precipitate was filtered off and dried under vacuum affording products **18**–**23**.

*4.8.3.1. 6-{2-[(2-Carboxyethyl)oxy]ethylamino}-7-chloro-1-cyclopropyl-1,4-dihydro-4-oxo-3-quinolincarboxylic acid (**18**).* According described procedure starting from intermediate **12** (4.70 g, 12.51 mmol) product **18** (3.1 g, $Y = 60\%$) as yellow powder was obtained; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.56 (s, 1H), 8.23 (s, 1H), 7.39 (s, 1H), 3.82 (m, 1H), 3.66 (q, 2H+2H), 3.42 (t, 2H), 2.49 (t, 2H), 1.30 (q, 2H), 1.18 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 178.70, 174.73, 168.28, 147.89, 144.93, 134.34, 128.55, 127.56, 121.15, 118.99, 108.32, 104.90, 69.98, 68.16, 44.59, 37.95, 36.74, 9.50; MS (ESI) *m/z* calcd $C_{18}H_{20}ClN_2O_6$ $[M + H]^+$ 395.1010; found 395.1001.

4.8.4. General procedure for acylation of macrolides

To a solution of quinolonic diacids **18**–**23** (2 eq) in dry DMF cooled at 0 °C, EDCxHCl (2 eq), suspension of 2′-*O*-acetyl azithromycin **1** (1 eq) in DCM and DMAP (3 eq) were added and stirred for 24 h. Into the reaction mixture EtOAc was added and organic solution extracted with water (3 × 20 mL). Organic layer was washed with brine and evaporated yielding crude 2′-*O*-acetyl derivatives which were dissolved in MeOH, stirred at rt for 24 h, and then solvent evaporated under reduced pressure. The crude products were purified by chromatography on a silica gel column using DCM/MeOH/NH_4OH (90:5:0.5) as an eluent to afford products **24**–**29**.

*4.8.4.1. 4″-O-(3-{[2-(3-carboxy-7-chloro-1-cyclopropyl-1,4-dihydro-4-oxoquinolin-6-yl)ethylamino]oxy}propanoyl)-azithromycin (**24**).* According described procedure starting from intermediate **18** (0.90 g, 2.28 mmol) product **14** (0.34 g, $Y = 48\%$) as white solid was obtained; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.73 (s, 1H), 8.06 (s, 1H), 7.53 (s, 1H), 5.20 (d, 1H), 5.03 (t, NH), 4.72 (d, 1H), 4.70 (t, 1H), 4.56 (d, 1H), 4.41 (m, 1H), 4.25 (dd, 1H), 3.80 (m, 2H), 3.79 (m, 1H), 3.78 (m, 2H), 3.68 (d, 1H), 3.59 (m, 1H), 3.58 (m, 1H), 3.47 (m, 2H), 3.31 (s, 3H), 3.29 (m, 1H), 2.75 (m, 1H), 2.68 (m, 2H), 2.65 (m, 1H), 2.55 (d, 1H), 2.40 (m, 1H), 2.39 (s, 6H), 2.32 (m, 1H), 2.31 (s, 3H), 2.07 (m, 1H), 1.99 (m, 1H), 1.90 (m, 1H), 1.75 (d, 1H), 1.64 (dd, 1H), 1.46 (m, 1H), 1.40 (m, 2H), 1.27 (s, 3H), 1.25 (m, 1H), 1.21 (m, 2H), 1.20 (d, 3H), 1.19 (m, 3H), 1.13 (d, 3H), 1.11 (s, 3H), 1.10 (m, 3H), 1.09 (s, 3H), 1.04 (d, 3H), 0.91 (m, 3H), 0.90 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.87, 177.55, 171.18, 167.32, 145.93, 142.95, 132.72, 127.65, 126.28, 118.14, 107.66, 104.52, 102.19, 94.68, 83.22, 79.02, 77.72, 77.49, 74.28, 73.67, 73.61, 72.96, 70.97, 70.06, 68.75, 67.74, 66.32, 65.61, 62.98, 62.54, 49.48, 45.17, 43.30, 42.22, 42.08, 40.43, 36.30, 35.38, 35.99, 27.50, 26.78, 21.99, 21.79, 21.31, 21.24, 17.77, 16.21, 14.59, 11.29, 9.15, 8.14, 8.10, 7.45; MS (ESI) *m/z* calcd for $C_{56}H_{90}ClN_4O_{17}$ $[M + H]^+$ 1125.5984; found 1125.6016.

4.8.5. General method for catalytic dehalogenation

To a solution of compounds **24**–**26** and **29** in MeOH (30 mL) 10% Pd/C (10% wt) was added and the reaction mixture was hydrogenated at 2 bar pressure for 16 h at room temperature. The catalyst was filtered off, washed with MeOH and the mother liquor concentrated to dryness. To the reaction mixture water (10 mL) was added and the reaction mixture was extracted at pH 9 with DCM (2 × 10 mL). The combined organic extracts were concentrated to dryness. The residue was dissolved in EtOAc (5 mL) and treated with hexane (20 mL). The resulting suspension was filtered yielding compounds **30**–**33** as white solid.

*4.8.5.1. 4″-O-{3-[(2-[(3-carboxy-1-cyclopropyl-1,4-dihydro-4-oxoquinolin-6-yl)amino]ethyl)oxy]propanoyl}-azithromycin (**30**).* According described procedure starting from compound **24** (0.55 g, 0.49 mmol) product **30** (0.40 g, $Y = 74\%$) as white solid was obtained; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.72 (s, 1H), 7.92 (d, 1H), 7.46 (d, 1H), 7.17 (dd, 1H), 5.17 (d, 1H), 4.75 (d, 1H), 4.73 (m, 1H), 4.56 (d, 1H), 4.24 (m, 1H), 4.27 (dd, 1H), 3.80 (m, 2H), 3.79 (m, 1H), 3.75 (t, 2H), 3.68 (d, 1H), 3.61 (m, 1H), 3.60 (m, 1H), 3.39 (q, 2H), 3.32 (s, 3H), 3.26 (m, 1H), 2.78 (m, 1H), 2.70 (m, 1H), 2.64 (m, 2H), 2.60 (m, 1H), 2.52 (m, 1H), 2.41 (d, 1H), 2.36 (s, 6H), 2.32 (s, 3H), 2.00 (m, 1H+1H), 1.90 (m, 1H), 1.76 (d, 1H), 1.65 (dd, 1H), 1.48 (m, 1H), 1.37 (m, 2H), 1.27 (s, 3H), 1.21 (m, 3H), 1.20 (m, 3H), 1.19 (m, 2H), 1.16 (d, 3H), 1.12 (s, 3H), 1.10 (m, 3H), 1.09 (s, 3H), 1.05 (d, 3H), 0.90 (m, 3H), 0.89 (t, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.77, 177.93, 171.67, 167.73, 145.93, 145.17, 133.40, 127.62, 122.54, 118.44, 107.43, 104.05, 102.22, 94.85, 83.28, 79.17, 77.99, 77.52, 74.33, 74.04, 73.59, 72.93, 71.00, 70.07, 68.89, 67.78, 66.16, 65.56, 62.95, 62.37, 49.50, 45.08, 43.24, 42.26, 41.72, 40.39, 36.41, 35.40, 35.09, 35.00, 27.43, 26.77, 21.98, 21.80, 21.26, 21.24, 17.90, 16.20, 14.82, 11.27, 9.24, 8.10, 8.08, 7.58; MS (ESI) *m/z* calcd for $C_{56}H_{91}N_4O_{17}$ $[M + H]^+$ 1091.6373; found 1091.6379.

4.8.6. General procedure for synthesis of ester derivatives 34–46

To a solution of compounds **24**, **25**, **30** and **31** (1 eq) in dry DMF (5 mL) potassium carbonate (3.5 eq) and appropriate halogenide (2 eq) were added and the reaction mixture was stirred at room temperature for two days. Into the reaction mixture water was added and the reaction was extracted with EtOAc. Combined organic layers were evaporated under reduced pressure. The crude products were purified by chromatography on a silica gel column using DCM/MeOH/NH_4OH (90:5:0.5) as an eluent to afford products **34**–**46**.

*4.8.6.1. 4″-O-{3-[(2-[(7-chloro-1-cyclopropyl-1,4-dihydro-4-oxoquinolin-3-[(propyloxy)carbonyl]-6-yl)amino]ethyl)oxy]propanoyl}-azithromycin (**34**).* According to general procedure starting from macrolide **24** (0.20 g, 0.18 mmol) in reaction with iodopropane (0.035 mL, 0.36 mmol) compound **34** (0.058 g, $Y = 28\%$) as white powder was obtained; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.49 (s, 1H), 7.89 (s, 1H), 7.63 (s, 1H), 7.28 (s, 1H), 5.22 (d, 1H), 4.74 (d, 1H), 4.71 (d, 1H), 4.55 (d, 1H), 0.43 (m, 1H), 4.28 (t, 2H), 4.25 (s, 1H), 3.89 (t, 1H+2H), 3.75 (t, 2H), 3.68 (s, 1H), 3.60 (d, 1H), 3.44 (m, 1H+2H), 3.31 (s, 3H), 3.26 (m, 1H), 2.74 (dd, 1H), 2.70 (m, 1H), 2.69 (m, 1H), 2.65 (m, 1H), 2.62 (m, 1H), 2.55 (d, 1H), 2.41 (d, 1H), 2.35 (s, 6H), 2.31 (s, 3H), 2.07 (m, 1H), 2.04 (m, 1H), 2.00 (m, 1H), 1.92 (m, 1H), 1.82 (k, 2H), 1.78 (d, 1H), 1.73 (d, 1H), 1.64 (dd, 1H), 1.46 (m, 1H), 1.32 (d, 2H), 1.29 (s, 3H), 1.20 (d, 3H), 1.18 (d, 3H), 1.17 (d, 3H), 1.13 (m, 2H), 1.11 (s, 3H), 1.10 (s, 3H), 1.09 (d, 3H), 1.05 (t, 3H), 1.04 (d, 3H), 0.90 (d, 3H), 0.89 (t, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.97, 173.62, 171.15, 166.25, 146.91, 142.03, 132.18, 128.79, 125.80, 117.40, 109.69, 106.51, 102.27, 94.60, 83.11, 78.95, 77.64, 74.21, 73.65, 73.45, 72.94, 70.93, 70.07, 68.84, 67.77, 66.38, 66.29, 65.61, 63.02, 62.62, 49.47, 45.25, 43.31, 42.35, 42.16, 40.35, 36.19, 35.02, 34.92, 34.47, 29.26, 27.55, 26.77, 22.17, 21.97, 21.81, 21.35, 21.23, 17.76, 16.23, 14.46, 11.27, 10.63, 9.02, 7.99, 7.28; MS (ESI) *m/z* calcd for $C_{59}H_{96}ClN_4O_{17}$ $[M + H]^+$ 1167.6454; found 1167.6472.

Acknowledgment

The authors thank Ana Čikoš, Biserka Metelko, Dubravka Gembarovski and Bill Leavens for their supports and services in structure analysis, and Branka Tavčar and Lara Mikac for providing

bioanalytical support. We gratefully acknowledge Dragica Muhvić, Božica Škrinjar, Jadranka Ivetić and Višnja Majzel for their excellent technical assistance.

Appendix. Supplementary data

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.ejmech.2011.05.002.

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