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ScienceDirect
European Journal of Medicinal Chemistry 42 (2007) 138–145
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

Azithromycin–sulfonamide conjugates as inhibitors of resistant *Streptococcus pyogenes* strains

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Received 13 April 2006; received in revised form 13 July 2006; accepted 13 August 2006
Available online 28 September 2006

Abstract

Novel hybrid compounds **6a–6d**, conjugates of 15-membered azalides and sulfonamides, *i.e.* unsubstituted, 4-aryl- and 4-heteroaryl-aminosulfonyl derivatives of 9a-[*N*′-(phenylcarbamoyl)]-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A were synthesized and characterized by IR, one- and two-dimensional NMR spectroscopies and MALDI-TOF and MS/MS mass spectrometry.

The new compounds were evaluated *in vitro* against a panel of sensitive and resistant Gram-positive and Gram-negative bacterial strains. 9a-{*N*′-[4-(Aminosulfonyl)phenyl]carbamoyl} – (**6a**) and 9a-{*N*′-[4-(phenylaminosulfonyl)phenyl]carbamoyl} – (**6b**) derivatives showed improvements in activity against inducible resistant *Streptococcus pyogenes* in comparison with macrolide antibiotic azithromycin and starting material 9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (**2**). In addition, the synthesized azithromycin–sulfonamide conjugates **6a–6d** showed good antibacterial activity against sensitive *S. pyogenes* and *Streptococcus pneumoniae* strains.

The kinetics of degradation in the artificial gastric juice showed that the most active compounds, **6a** and **6b**, exhibited azithromycin like stability. The cleavage of the cladinose sugar was found to be the main decomposition pathway leading to inactive **7a** and **7b**, prepared also as analytical standards by the alternative synthetic route together with **7c** and **7d**.

Keywords: 15-Membered azalides; Azithromycin–sulfonamide conjugates; Antimicrobial activity; Structural elucidation; Acid stability

1. Introduction

Macrolide antibiotic azithromycin **1** (Fig. 1), the first representative of the azalide class, is well-established antimicrobial agent that has been widely prescribed for the treatment of respiratory tract infections owing to its high efficacy and safety [1,2]. Over the past decade, the increasing incidence of bacterial resistance to a large number of antibacterial agents is becoming a major threat to successful treatment of infectious diseases. Due to a rapid emergence of macrolide-resistant *Streptococcus pneumoniae* and *Streptococcus pyogenes* among clinical isolates [3,4] both in hospital settings and in the community, significant efforts have been devoted to chemical modifications of the existing macrolides. One of the main types of bacterial resistance is connected to methylases which are responsible for developing macrolide, lincosamide and streptogramin (MLS) resistance mechanisms of the inducible (iMLS) or constitutive (cMLS) types.

As part of our continuous synthetic efforts focused on a preparation of 9a-substituted azalide derivatives [5,6] we have recently reported the synthesis of novel sulfonylureas of 15-membered azalides which showed significant activity against inducible resistant *S. pyogenes* strains [7]. Those results prompted us to further explore modifications at position 9a of an azalide lactone ring, especially by the carbamoyl group linked sulfonamides, such as sulfanilamide, sulfabenz, sulfapyridine

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doi:10.1016/j.ejmech.2006.08.008

and sulfamethoxazole (Fig. 1, Scheme 1). Macrolide antibiotics exert their antibacterial activity by binding to ribosomal 23S RNA and thus blocking the bacterial protein synthesis [8,9]. Strategies which involved macrolide conjugates incorporating heteroaromatic rings [10], as well as macrolide–nucleoside and macrolide–nucleobase conjugates [11] have already been introduced and these compounds showed an increased affinity for the ribosome. Similarly, we expect that by introducing novel interactive groups into the azalide backbone further improvements in activity might be achieved. Therefore, the present paper describes the synthesis, structure elucidation and antibacterial *in vitro* evaluation of novel hybrid compounds, conjugates of 15-membered azalides and sulfonamides, *e.g.* the 4-aminosulfonyl – (**6a**), 4-phenylaminosulfonyl – (**6b**), 4-[(pyridyl-2-yl)aminosulfonyl] – (**6c**), and 4-[(5-methylisoxazol-3-yl)aminosulfonyl] – (**6d**), derivatives of 9a-[*N*′-(phenylcarbamoyl)]-9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (Scheme 1).

In order to study the stability of the most active compounds, an acid hydrolysis was performed and degradation products were analyzed and biologically evaluated.

2. Results and discussion

2.1. Chemistry

The compounds **6a–6d** were prepared by the reaction of 9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (**2**), the last intermediate in the azithromycin (**1**) synthesis [12,13] with 4-(chlorosulfonyl)phenylisocyanate in toluene at 0–5 °C (Scheme 1). The smoothly formed 9a-{[4-(chlorosulfonyl)-phenyl]carbamoyl} – derivative **4** was transformed without the isolation into the compounds **6a–6d**, by the reaction with 2 equiv of ammonia, aniline, 2-aminopyridine and 5-methyl-3-aminoisoxasole, respectively. The regioselective hydrolysis of **6a** and **6b** in 1 M hydrochloric acid at room temperature, furnished the decladinosyl derivatives **7a** and **7b**, alternatively prepared as the analytical standards, by the same type of reactions as mentioned above, *i.e. via* **2** → **3** → **5** → **7a–d** pathway (Scheme 1).

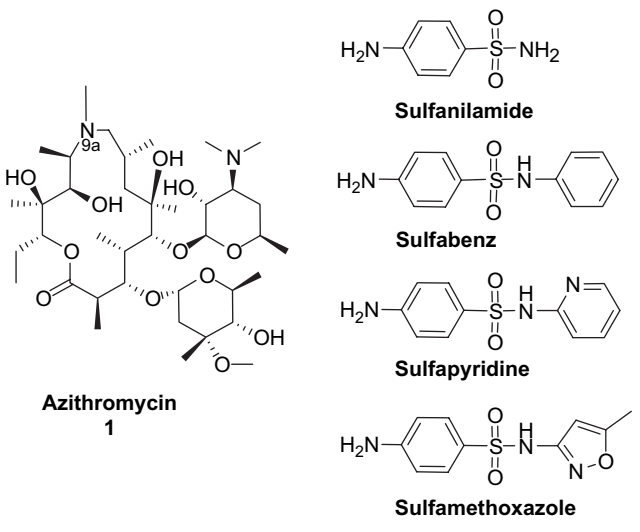


Fig. 1. Structures of azithromycin, sulfanilamide, sulfabenz, sulfapyridine and sulfamethoxazole.

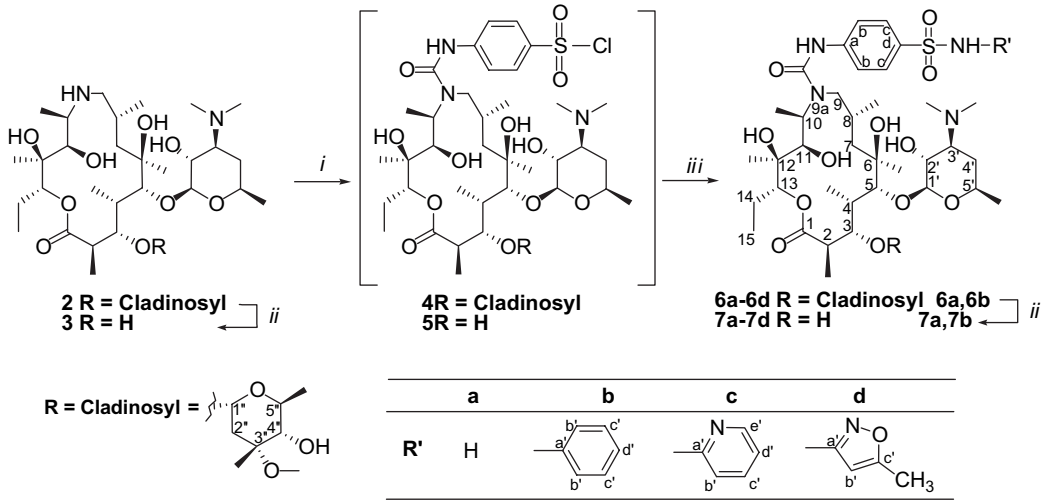
2.2. Structural characterization

Structures of all synthesized compounds were determined by IR and NMR spectroscopies and mass spectrometry (MALDI-TOF MS and MS/MS). The assignments of proton and carbon chemical shifts were made by the combined use of one- (^{1}H and APT) and two-dimensional (gCOSY, gHSQC and gHMBC) NMR spectra. With respect to **2** and **3** several new resonances were observed in NMR spectra of compounds **6a–6d** and **7a–7d**. The additional singlet at approximately 155 ppm in the ^{13}C NMR spectra was observed reflecting the presence of a carbamoyl carbon atom. Strong bands found at approximately 1660–1634 cm^{-1} in IR spectra were assigned to carbamoyl C=O stretching vibrations. The proton and carbon chemical shifts in the regions 6.8–8.0 ppm (DMSO-d_6), 7.9–8.1 ppm (pyridine-d_5) and 6.1–8.3 ($CDCl_3$) for protons and 116–170 ppm, for carbons were diagnostic for substituted benzene, pyridine and isoxazole rings. Carbon and proton chemical shifts of all compounds are given in Section 4. The mass spectra exhibited precursor ions that are consistent with masses of the respective compounds **6a–6d** and **7a–7d** as shown in Scheme 1. Elemental composition was determined by accurate mass measurements in the mass range *m/z* 749.5613–1046.5423 with the observed mass accuracy better than 4 ppm. Accurately measured masses together with calculated molecular formulas and IR bands of compounds **6a–6d** and **7a–7d** are displayed in Table 1. A typical fragmentation pattern of **6b** with theoretically calculated masses is shown in Fig. 2. Accurately measured masses of **6b** product ions revealed predominantly ions produced after a loss of benzensulfonamido moiety at the position 9a, *e.g. m/z* 735.4954. Other product ions were formed by a loss of desosamine sugar and one water molecule (*m/z* 851.4437 and *m/z* 833.4339), or by a loss of both sugars and one or two water molecules (*m/z* 694.3341, *m/z* 676.3221 and *m/z* 658.3160). Also, the mass spectra exhibited product ions formed by a simultaneous carbamoyl and two glycosidic bond cleavages (desosamine and cladinose) giving *m/z* 420.2959, *m/z* 402.2843 and *m/z* 384.2741, respectively. Similar fragmentation was observed for other compounds too.

2.3. Acid stability

It is known that in aqueous acidic media 14-membered erythromycin derivatives underwent intramolecular cyclization reactions [14,15] to form inactive by-products with unfavourable side effects. On the other hand 15-membered azithromycin derivatives were found to be more stable with no cyclization products. The main decomposition pathway for azithromycin included hydrolysis of the cladinose sugar glycosidic bond leading to an inactive decladinosyl derivative [16].

The acidic stability studies of the most active compounds **6a** and **6b** were carried out in 0.01 M HCl aqueous



Scheme 1. Synthesis of azithromycin–sulfonamide conjugates **6a–6d** and **7a–7d**. Reagents and conditions: i, 4-(chlorosulfonyl)phenylisocyanate, toluene, 0–5 °C, 1 h; ii, 1 M hydrochloric acid, r.t., 12 h; iii, 2 equiv of different amines: ammonia, aniline, 2-aminopyridine and 5-methyl-3-aminoisoxasole.

hydrochloric acid at 37 °C, as a model of artificial gastric juice. We were particularly interested to see whether the hydrolysis of the cladinose glycosidic bond was the main degradation pathway for **6a** and **6b** or would decomposition include also cleavages of 9a-carbamoyl and sulfonamide bonds.

A combined use of LC/UV and LC/MS methods to study kinetics of hydrolysis for parent compounds and their degradation products was employed. In the LC/UV chromatogram, peaks at retention times 9.41 min, 8.05 min, 11.41 min and 10.45 min were attributed to **6a**, **7a**, **6b** and **7b**, respectively. The mass spectra of the reaction mixtures after 10 h showed only precursor ions at *m/z* 775.42 and *m/z* 851.49, which stemmed from the cleavage of the cladinose sugars giving decladinosyl compounds **7a** and **7b**, respectively. No further degradation products were observed under the studied conditions.

The hydrolytic degradation of **6a** and **6b** was shown to follow pseudo first-order kinetics as was also observed for azithromycin [16]. Reaction rate constants (k) were determined from the exponential fit analysis of the plot of the reactant concentration (**6a** and **6b**) versus time. The calculated rate constants for hydrolytic degradation at different temperatures and times for 50% decay ($t_{1/2}$) are listed in Table 2.

The activation energies of hydrolysis of glycosidic bonds for **6a** and **6b** were determined next, and were found to be 90.5 kJ/mol (21.8 kcal/mol) and 89.9 kJ/mol (21.6 kcal/mol), respectively (Fig. 3). These values were similar to that obtained for azithromycin which amounted to 105.9 kJ/mol (25.3 kcal/mol) [16].

To confirm the structures of degradation products **7a** and **7b** and to support the proposed hydrolytic pathway their isolation and characterization were subsequently performed.

2.4. In vitro antimicrobial activity

The antibacterial screening of azithromycin–sulfonamide conjugates **6a–6d** and **7a–7d** was performed by a standard dilution assay for the determination of minimal inhibitory concentrations (MICs). MIC values for all compounds (Table 3) were determined in comparison with azithromycin (**1**) and 9-deoxo-9-dihydro-9a-aza-9a-homoerythromycin A (**2**) on a panel of sensitive and resistant Gram-positive bacterial strains (*Staphylococcus aureus*, *S. pneumoniae* and *S. pyogenes*) and on Gram-negative strains (*Escherichia coli*, *Haemophilus influenzae* and *Moraxella catarrhalis*).

In Table 3 it is clearly seen that **6a** and **6b** possess two to three times better activity against iMLS resistant *S. pyogenes* strain (MIC 2 μg/ml) when compared to both azithromycin **1** and starting cyclic amine **2**. These activities are comparable to those observed for azalide sulfonylureas reported previously [7]. The introduction of 4-aryl- and 4-heteroaryl-aminosulfonyl groups in **6b–6d** does not improve the activity against iMLS resistant *S. pyogenes* in comparison with **6a** and azalide sulfonylureas [7]. Compounds **6a–6d** are found to be inactive against pathogens with an efflux gene (*mef*) and against pathogens with a constitutively resistant *S. pyogenes*. New azithromycin–sulfonamide conjugates **6a** and **6b** exhibit somewhat lower activity than azithromycin (**1**) and starting amine **2** against sensitive *S. pneumonia* (MIC 0.5 and 1 μg/ml) and *S. pyogenes* (MIC 2 μg/ml) strains. Furthermore, **6c** and **6d** showed in general lower activity against most of the tested bacterial strains except for sensitive *S. aureus* and *M. catarrhalis* where better activity was observed in comparison with **6a** and **6b** analogs.

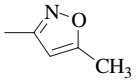
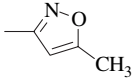
All synthesized azithromycin–sulfonamide conjugates **6a–6d** are inactive against Gram-negative *H. influenzae* and *E. coli* strains.

The decladinosyl derivatives **7a–7d** didn't show any antibacterial activity at all against tested bacterial strains, as already observed for the related macrolides [7].

3. Conclusion

The coupling of a benzenesulfonamido moiety to the 9a position of 15-membered azalide scaffold via carbamoyl linker has proven to be a promising method to tackle the resistance

Table 1
Spectroscopic data for the synthesized compounds **6a–6d** and **7a–7d**

Compound number	R	R′	Molecular formula	IR (cm^{-1})	Calculated monoisotopic *m/z* $[M+H]^+$	Measured monoisotopic *m/z* $[M+H]^+$
6a	Cladinosyl	H	$C_{44}H_{76}N_4O_{15}S$	3439, 2974, 2937, 1722, 1644, 1591, 1520, 1462, 1333, 1160, 834	933.5106	933.5129
6b	Cladinosyl		$C_{50}H_{80}N_4O_{15}S$	3418, 2973, 2937, 1725, 1650, 1591, 1523, 1498, 1158, 1464, 836, 756	1009.5419	1009.5433
6c	Cladinosyl		$C_{49}H_{79}N_5O_{15}S$	3444, 2972, 2935, 1728, 1634, 1592, 1525, 1464, 1383, 1165, 836, 775	1010.5371	1010.5410
6d	Cladinosyl		$C_{48}H_{79}N_5O_{16}S$	3434, 2961, 2932, 1728, 1642, 1591, 1523, 1466, 1272, 1165, 947, 836, 751	1014.5321	1014.5355
7a	H	H	$C_{36}H_{62}N_4O_{12}S$	3444, 2975, 2937, 1723, 1651, 1592, 1538, 1456, 1328, 1160, 835, 756	775.4163	775.4168
7b	H		$C_{42}H_{66}N_4O_{12}S$	3444, 2975, 2937, 1723, 1660, 1591, 1525, 1497, 1159, 836, 756	851.4476	851.4490
7c	H		$C_{41}H_{65}N_5O_{12}S$	3444, 2969, 2935, 1724, 1634, 1592, 1526, 1462, 1385, 1285, 1164, 836, 775	852.4428	852.4433
7d	H		$C_{40}H_{65}N_5O_{13}S$	3444, 2972, 2934, 1725, 1651, 1592, 1520, 1464, 1269, 1165, 944, 836, 754	856.4378	856.4391

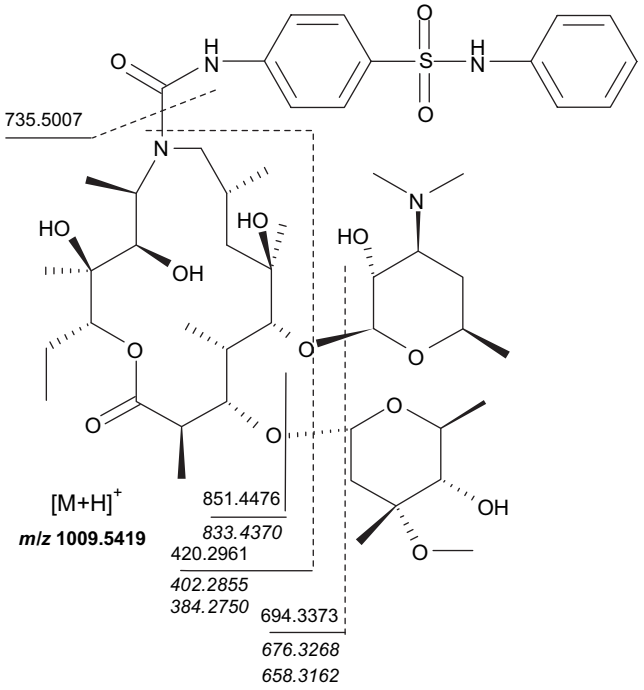


Fig. 2. Proposed fragmentation pattern of the compound **6b** based on accurate mass MS/MS experiments with theoretically calculated masses.

problems. Hence, newly synthesized azalides **6a** and **6b** displayed a strong antibacterial activity against inducible resistant *S. pyogenes*.

In acidic conditions **6a** and **6b** showed azithromycin like stability giving only inactive decladinosyl degradation products.

The results presented here together with those previously reported for azalide sulfonylureas [7] can serve as a good platform for further derivatization and development of antimicrobial agents with improved activity against resistant *S. pyogenes* strains. Next steps in this direction should also include characterization of binding modes and interactions with ribosome by using biochemical and structural methods.

4. Experimental

4.1. Chemistry

Analytical TLC was performed on Merck 60 F254 plates using CH_2Cl_2:CH_3OH:NH_3(25%) = 90:9:1.5 as eluents. Column chromatography was performed on a Merck Silica gel 60 (0.043–0.060 mm). The purity of the isolated compounds was better than 90% according to LC/MS measurements. MALDI-TOF MS spectra in reflectron mode were obtained on an Applied Biosystems Voyager DE STR instrument (Foster City, CA). The laser wavelength was 337 nm (N_2 laser) and laser frequency amounted to 20 Hz. Desorbed ions were accelerated to 2.0 kV. Delayed extraction was 150 ns. Calibrant and analyte spectra were obtained in positive ion mode. Calibration type was internal with calibrants azithromycin and angiotensin II dissolved in α-cyano-4-hydroxycinnamic acid matrix in the mass range *m/z* 749.5613–1046.5423. Accurately measured MS/MS analyses were performed with a quadrupole-time of flight mass spectrometer Q-TOF Micro equipped with the lock-spray (Micromass, Manchester, UK). Azithromycin was used as an internal lock-mass standard. NMR experiments were carried out on a Bruker Avance DRX500 spectrometer operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C, respectively, with a 5 mm diameter inverse detection probe and a *z*-axis gradient coil. Standard spectral conditions were used. The solvents were pyridine-d_5, DMSO-d_6 and $CDCl_3$ and all experiments were performed at ambient temperature. TMS was used as the internal standard. IR spectra were recorded in KBr pellets on a Nicolet Magna 760 FT-IR spectrometer. The number of scans was 16 and the resolution was 4 cm^{-1}.

Table 2
Pseudo first-order reaction rate constants and $t_{1/2}$ values for **6a** and **6b**

Comp.	**6a**		**6b**	
t/°C	k^b/min^{-1}	$t^a_{1/2}$/min	k^b/min^{-1}	$t^a_{1/2}$/min
25	2.29×10^{-3}	303	1.88×10^{-3}	369
30	3.77×10^{-3}	184	3.21×10^{-3}	216
37	8.31×10^{-3}	83	7.10×10^{-3}	98
40	1.39×10^{-2}	50	1.12×10^{-2}	62

[a] Calculated according to $t_{1/2} = \ln 2/k$.
[b] Standard deviation was better than 0.6×10^{-3} min^{-1} in all experiments.

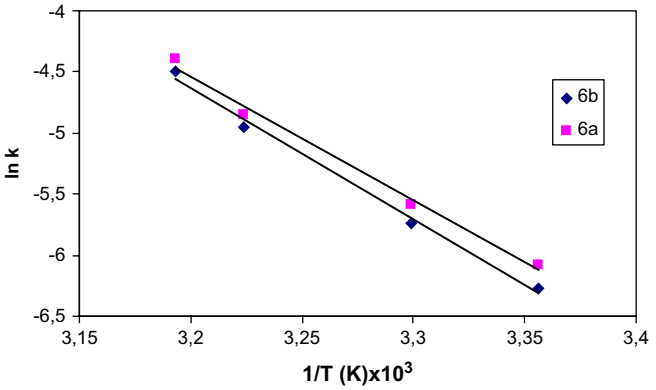


Fig. 3. Arrhenius plot of temperature versus pseudo first-order reaction rates of **6a** and **6b**.

4.1.1. General procedure for the preparation of *6a–6d* and *7a–7d*

The solution of **2** or **3** (1.84 mmol) and 4-(chlorosulfonyl)-phenyl isocyanate (1.84 mmol) in dry toluene (30 ml) was stirred for about 2 h at temperature 0–5 °C. In the reaction mixture amine (23% water solution of ammonia (30.0 mmol), aniline (4.6 mmol), 2-aminopyridine (5.2 mmol) or 5-methyl-3-aminoisoxasole (5.8 mmol) was added and the reaction mixture was stirred for about 2 h at room temperature. The crude product was filtered, wherefrom by column chromatography on silica gel using solvent system CH_2Cl_2:CH_3OH = 9:1 pure compound was obtained.

*4.1.1.1. 9-Deoxo-9-dihydro-9a-{N′-[4-(aminosulfonyl)phenyl]-carbamoyl}-9a-aza-9a-homoerythromycin A (**6a**).* Yield 86.2%. ^{1}H NMR (pyridine) δ 3.12 (1H, H-2), 4.67 (1H, H-3), 2.45 (1H, H-4), 4.05 (1H, H-5), 2.23, 1.91 (2H, H-7),

Table 3
In vitro antibacterial activity of novel azithromycin–sulfonamide conjugates **6a–6d** and their decladinosyl analogs **7a–7d**

Test compound	Strain/MIC (μg/ml)								
	S. aureus Ery-S	*S. pneumoniae* Ery-S	*S. pyogenes* Ery-S	*S. pyogenes* iMLS *erm*TR	*S. pyogenes* cMLS	*S. pyogenes* M *mef*A	*M. catarrhalis* ATCC-0324	*H. influenzae* ATCC-0529	*E. coli* ATCC-0001
1	0.5	≤0.125	≤0.125	8	64	2	≤0.125	0.25	1
2	2	0.25	0.25	16	>64	16	NT	1	4
6a	64	1	2	4	>64	>64	>64	32	>64
6b	16	0.5	2	2	>64	32	64	32	>64
6c	8	4	2	>64	>64	>64	4	64	>64
6d	32	16	8	>64	>64	>64	8	>64	>64
7a–7d	>64	>64	>64	>64	>64	>64	>64	>64	>64

iMLS: inducible resistance to macrolide, lincosamide and streptogramin (MLS) antibiotics; cMLS: constitutive MLS resistance; M: efflux mediated macrolide resistance; NT: not tested.

2.85 (1H, H-8), 4.34 (1H, H-11), 5.75 (1H, H-13), 2.23, 1.7 (2H, H-14) 0.99 (3H, H-15), 4.92 (1H, H-1′), 3.44 (1H, H-2′), 2.68 (1H, H-3′), 2.23 (6H, 3′ NMe_2), 1.42, 1.2 (2H, H-4′), 3.98 (1H, H-5′), 5.12 (1H, H-1″), 2.39, 1.5 (2H, H-2″), 3.44 (3H, 3″ OMe), 3.23 (1H, H-4″), 4.51 (1H, H-5″), 1.35 (3H, 2 Me), 1.64 (3H, 4 Me), 2.01 (3H, 6 Me), 1.08 (1H, H-8 Me), 1.29 (3H, 12 Me), 1.29 (3H, 5′ Me), 1.29 (3H, 3″ Me), 1.49 (3H, 5″ Me), 8.63 (2H, phenyl, b), 8.19 (2H, phenyl, c), 8.01 (1H, phenyl, d); ^{13}C NMR (pyridine) δ 176.9 (C-1), 45.7 (C-2), 79.9 (C-3), 40.9 (C-4), 85.6 (C-5), 73.8 (C-6), 40 (C-7), 28.5 (C-8), 56.2 (C-9), 75.0 (C-11), 73.8 (C-12), 77.5 (C-13), 22.9 (C-14), 11.8 (C-15), 103.1 (C-1′), 71.3 (C-2′), 65.3 (C-3′), 40.2 (3′ NMe_2), 29.6 (C-4′), 68.2 (C-5′), 96.5 (C-1″), 35.6 (C-2″), 74.8 (C-3″), 49.6 (3″ OMe), 78.6 (C-4″), 66.1 (C-5″), 13.8 (2 Me), 10.0 (4 Me), 30.2 (6 Me), 20.7 (8 Me), 10 (10 Me), 16.2 (12 Me), 21.9 (5′ Me), 21.6 (3″ Me), 19.2 (5″ Me), 156.7 (N*C*ONH), 145.0 (phenyl, a), 128.4 (phenyl, b), 118.5 (phenyl, c), 138.1 (phenyl, d).

*4.1.1.2. 9-Deoxo-9-dihydro-9a-{N′-[4-(phenylaminosulfonyl)-phenyl]carbamoyl}-9a-aza-9a-homoerythromycin A (**6b**).* Yield 41.0%. ^{1}H NMHR (DMSO) δ 2.54 (1H, H-2), 4.01 (1H, H-3), 2.05 (1H, H-4), 3.38 (1H, H-5), 2.05 (1H, H-8), 4.01 (1H, H-11), 5.03 (1H, H-13), 1.76, 1.28 (2H, H-14), 0.79 (3H, H-15), 4.45 (1H, H-1′), 3.45 (1H, H-2′), 2.5 (1H, H-3′), 2.50 (6H, 3′ NMe_2), 1.46, 1.11 (2H, H-4′), 3.67 (1H, H-5′), 4.76 (1H, H-1″), 2.26, 1.48 (2H, H-2″), 3.22 (3H, 3″ OMe), 2.90 (1H, H-4″), 4.01 (1H, H-5″), 1.06 (3H, 2 Me), 0.89 (3H, 4 Me), 1.27 (3H, 6 Me), 0.88 (1H, H-8 Me), 0.88 (3H, 12 Me), 1.11 (3H, 5′ Me), 1.14 (3H, 3″ Me), 1.06 (3H, 5″ Me), 10.1 (SO_2NH), 7.57 (2H, phenyl, b), 7.44 (2H, phenyl, c), 7.20 (2H, phenyl, c′), 7.07 (2H, phenyl, b′), 6.99 (1H, phenyl, d′); ^{13}C NMR (DMSO) δ 175.9 (C-1), 78.2 (C-3), 85 (C-5), 73.7 (C-6), 27.9 (C-8), 75 (C-11), 75.1 (C-12), 76.1 (C-13), 21.8 (C-14), 11.3 (C-15), 101.6 (C-1′), 71 (C-2′), 64.8 (C-3′), 40.2 (3′ NMe_2), 29.9 (C-4′), 66.7 (C-5′), 96.2 (C-1″), 34.8 (C-2″), 72.8 (C-3″), 49.0 (3″ OMe), 77.4 (C-4″), 65.0 (C-5″), 15.4 (2 Me), 10.9 (4 Me), 27.9 (6 Me), 20.4 (8 Me), 9.2 (10 Me), 14.8 (12 Me), 21.3 (5′ Me), 21.1 (3″ Me), 18.5 (5″ Me), 155.5 (N*C*ONH), 145.1 (phenyl, a), 138.1 (phenyl, a′), 131.2 (phenyl, d), 128.8 (phenyl, c′), 127.5 (phenyl, b), 123.9 (phenyl, d′), 120.1 (phenyl, b′).

*4.1.1.3. 9-Deoxo-9-dihydro-9a-{N′-[4-(2-pyridylaminosulfonyl)-phenyl]carbamoyl}-9a-aza-9a-homoerythromycin A (**6c**).* Yield 42.1%. ^{1}H NMR (DMSO) δ 2.61 (1H, H-2), 4.00 (1H, H-3), 2.50 (1H, H-4), 3.39 (1H, H-5), 2.08 (1H, H-8), 3.65 (1H, H-10), 3.34 (1H, H-11), 5.03 (1H, H-13), 1.76, 1.34 (2H, H-14), 0.79 (3H, H-15), 4.41 (1H, H-1′), 3.00 (1H, H-2′), 2.5 (1H, H-3′), 2.24 (6H, 3′ NMe_2), 1.57, 1.15 (2H, H-4′), 3.64 (1H, H-5′), 4.75 (1H, H-1″), 2.27, 1.48 (2H, H-2″), 3.21 (3H, 3″ OMe), 2.89 (1H, H-4″), 4.02 (1H, H-5″), 1.07 (3H, 2 Me), 0.88 (3H, 4 Me), 1.28 (3H, 6 Me), 0.90 (1H, H-8 Me), 0.88 (3H, 12 Me), 1.11 (3H, 5′ Me), 1.13 (3H, 3″ Me), 1.06 (3H, 5″ Me), 8.03 (1H, pyridyl, e′), 7.70 (2H, phenyl, b), 7.66 (1H, pyridyl, c′), 7.43 (2H, phenyl, c), 7.10 (1H, pyridyl, b′), 6.86 (1H, pyridyl, d′); ^{13}C NMR (DMSO) δ 175.6 (C-1), 44.6 (C-2), 78.2 (C-3), 84.0 (C-5), 73.6 (C-6), 27.6 (C-8), 75 (C-12), 76 (C-13), 21.6 (C-14), 11.1 (C-15), 101.9 (C-1′), 70.5 (C-2′), 64.5 (C-3′), 40.1 (3′ NMe_2), 29.4 (C-4′), 67.0 (C-5′), 95.1 (C-1″), 34.7 (C-2″), 72.6 (C-3″), 48.8 (3″ OMe), 77.3 (C-4″), 64.8 (C-5″), 15.4 (2 Me), 9.11 (4 Me), 28.3 (6 Me), 19.9 (8 Me), 9.1 (10 Me), 14.2 (12 Me), 21.3 (5′ Me), 20.8 (3″ Me), 18.4 (5″ Me), 155.4 (N*C*ONH), 152.7 (pyridyl, a′), 144.5 (pyridyl, e′), 144.4 (phenyl, a), 139.4 (pyridyl, c′), 133.4 (phenyl, d), 127.2 (phenyl, b), 116.0 (pyridyl, d′), 113.0 (pyridyl, b′).

*4.1.1.4. 9-Deoxo-9-dihydro-9a-{N′-[4-(5-methyl-3-isoxazolylaminosulfonyl)phenyl]carbamoyl}-9a-aza-9a-homoerythromycin A (**6d**).* Yield 61.7%. ^{1}H NMR (DMSO) δ 2.62 (1H, H-2), 4.01 (1H, H-3), 1.74 (1H, H-4), 3.44 (1H, H-5), 2.08 (1H, H-8), 3.44 (1H, H-11), 5.04 (1H, H-13), 1.66, 1.34 (2H, H-14), 0.79 (3H, H-15), 4.42 (1H, H-1′), 3.09 (1H, H-2′), 2.77 (1H, H-3′), 2.43 (6H, 3′ NMe_2), 1.77, 1.52 (2H, H-4′), 3.66 (1H, H-5′), 4.75 (1H, H-1″), 2.24, 1.49 (2H, H-2″), 3.20 (3H, 3″ OMe), 2.89 (1H, H-4″), 3.99 (1H, H-5″), 1.08 (3H, 2 Me), 0.87 (3H, 4 Me), 1.28 (3H, 6 Me), 0.88 (1H, 8 Me), 0.87 (3H, 12 Me), 1.09 (3H, 5′ Me), 1.12 (3H, 3″ Me), 1.09 (3H, 5″ Me), 7.62 (2H, phenyl, b), 7.46 (2H, phenyl, c), 6.05 (1H, isoxazolyl, b′), 2.24 (3H, isoxazolyl, Me); ^{13}C NMR (DMSO) δ 175.8 (C-1), 44.6 (C-2), 78.2 (C-3), 39.9 (C-4),

84 (C-5), 73.7 (C-6), 27.8 (C-8), 78.1 (C-11), 75 (C-12), 78.1 (C-13), 21.8 (C-14), 11.3 (C-15), 101.7 (C-1′), 70.0 (C-2′), 64.8 (C-3′), 40.1 (3′ NMe_2), 30.1 (C-4′), 66.8 (C-5′), 95.8 (C-1″), 34.9 (C-2″), 72.8 (C-3″), 48.9 (3″ OMe), 77.4 (C-4″), 65.0 (C-5″), 14.6 (2 Me), 8.2 (4 Me), 28 (6 Me), 20.2 (8 Me), 13.6 (12 Me), 21.3 (5′ Me), 21.1 (3″ Me), 18.5 (5″ Me), 155.6 (N*C*ONH), 170 (isoxazolyl, c′), 160 (isoxazolyl, a′), 14.0 (isoxazolyl, Me), 144.7 (phenyl, a), 132.0 (phenyl, d), 127.2 (phenyl, b), 119 (phenyl, c).

*4.1.1.5. 5-O-Desosaminyl-9-deoxo-9-dihydro-9a-{N′-[4-(aminosulfonyl)phenyl]carbamoyl}-9a-aza-9a-homoerythronolide A (**7a**).* Yield 28.8%. ^{1}H NMR (pyridine) δ 3.02 (1H, H-2), 4.30 (1H, H-3), 1.69 (1H, H-4), 4.41 (1H, H-5), 2.2 (1H, H-8), 4.3 (1H, H-11), 5.7 (1H, H-13), 2.36, 1.77 (2H, H-14), 1.01 (3H, H-15), 5.1 (1H, H-1′), 3.61 (1H, H-2′), 2.62 (1H, H-3′), 2.21 (6H, 3′ NMe_2), 1.53, 1.19 (2H, H-4′), 3.49 (1H, H-5′), 1.58 (3H, 2 Me), 1.26 (3H, 4 Me), 1.76 (3H, 6 Me), 1.15 (1H, H-8 Me), 1.40 (3H, 12 Me), 1.16 (3H, 5′ Me), 8.16 (2H, phenyl, b), 7.92 (2H, phenyl, c); ^{13}C NMR (pyridine) δ 176 (C-1), 45.6 (C-2), 77 (C-3), 39.1 (C-4), 92.8 (C-5), 74 (C-6), 40.3 (C-7), 28 (C-8), 75 (C-12), 78 (C-13), 23.1 (C-14), 11.4 (C-15), 103.3 (C-1′), 69.9 (C-2′), 65.4 (C-3′), 40.3 (3′ NMe_2), 29.2 (C-4′), 69.2 (C-5′), 16.7 (2 Me), 8.9 (4 Me), 27 (6 Me), 21.1 (8 Me), 19.9 (12 Me), 21.5 (5′ Me), 156.7 (N*C*ONH), 138 (phenyl, a), 127.6 (phenyl, b), 118 (phenyl, c), 144.8 (phenyl, d).

*4.1.1.6. 5-O-Desosaminyl-9-deoxo-9-dihydro-9a-N-{N′-[4-(phenylaminosulfonyl)phenyl]-carbamoyl}-9a-aza-9a-homoerythronolide A (**7b**).* Yield 20.0%. ^{1}H NMR ($CDCl_3$) δ 2.67 (1H, H-2), 3.86 (1H, H-3), 3.57 (1H, H-5), 1.9 (1H, H-8), 4.59 (1H, H-13), 1.96, 1.56 (2H, H-14), 0.93 (3H, H-15), 4.35 (1H, H-1′), 3.31 (1H, H-2′), 2.55 (1H, H-3′), 2.30 (6H, 3′ NMe_2), 1.70, 1.30 (2H, H-4′), 3.57 (1H, H-5′), 1.30 (3H, 2 Me), 0.94 (3H, 4 Me), 1.31 (3H, 6 Me), 1.07 (1H, 8 Me), 1.13 (3H, 12 Me), 1.20 (3H, 5′ Me), 7.57 (2H, phenyl, b), 7.37 (2H, phenyl, c), 7.21 (2H, phenyl, c′), 7.09 (2H, phenyl, b′), 7.06 (1H, phenyl, d′); ^{13}C NMR ($CDCl_3$) δ 178.2 (C-1), 44.7 (C-2), 75.4 (C-3), 38.1 (C-4), 90 (C-5), 74.1 (C-6), 58 (C-9), 27.6 (C-8), 74.1 (C-12), 78.4 (C-13), 20.9 (C-14), 10.4 (C-15), 105.3 (C-1′), 68.9 (C-2′), 64.6 (C-3′), 39.6 (3′ NMe_2), 27.6 (C-4′), 69.8 (C-5′), 15.5 (2 Me), 7.0 (4 Me), 27 (6 Me), 19.6 (8 Me), 16.3 (12 Me), 20.4 (5′ Me), 155.8 (N*C*ONH), 152.8 (phenyl, a′), 143.3 (phenyl, a), 136.3 (phenyl, d), 128.7 (phenyl, c′), 127.8 (phenyl, b), 124.5 (phenyl, d′), 120.9 (phenyl, b′), 117.8 (phenyl, c).

*4.1.1.7. 5-O-Desosaminyl-9-deoxo-9-dihydro-9a-N-{N′-[4-(2-pyridylaminosulfonyl)phenyl]-carbamoyl}-9a-aza-9a-homoerythronolide A (**7c**).* Yield 47.0%. ^{1}H NMR ($CDCl_3$) δ 2.63 (1H, H-2), 3.84 (1H, H-3), 3.65 (1H, H-5), 1.8 (1H, H-8), 3.5 (1H, H-11), 4.61 (1H, H-13), 1.94, 1.55 (2H, H-14), 0.92 (3H, H-15), 4.34 (1H, H-1′), 3.31 (1H, H-2′), 2.6 (1H, H-3′), 2.29 (6H, 3′ NMe_2), 1.71, 1.26 (2H, H-4′), 3.59 (1H, H-5′), 1.29 (3H, 2 Me), 0.93 (3H, 4 Me), 1.29 (3H, 6 Me), 1.07 (1H, 8 Me), 1.12 (3H, 12 Me), 1.22 (3H, 5′ Me), 8.30 (1H, pyridyl, e′), 7.74 (2H, phenyl, b), 7.64 (1H, phenyl, c′), 7.42 (2H, phenyl, c), 7.38 (1H, pyridyl, b′), 6.80 (1H, pyridyl, d′); ^{13}C NMR ($CDCl_3$) δ 176 (C-1), 44.7 (C-2), 75.3 (C-3), 38.1 (C-4), 90 (C-5), 74.1 (C-6), 29.1 (C-8), 74.1 (C-12), 78.4 (C-13), 20.9 (C-14), 10.4 (C-15), 105.4 (C-1′), 68.9 (C-2′), 64.6 (C-3′), 39.6 (3′ NMe_2), 27.4 (C-4′), 69.9 (C-5′), 15.5 (2 Me), 7.0 (4 Me), 27 (6 Me), 19 (8 Me), 16.3 (12 Me), 20.2 (5′ Me), 155.8 (N*C*ONH), 142.8 (phenyl, a), 141.5 (pyridyl, e′), 140.8 (pyridyl, c′), 133.3 (phenyl, d), 127.4 (phenyl, b), 117.9 (phenyl, c), 114.5 (pyridyl, d′), 114.1 (pyridyl, b′).

*4.1.1.8. 5-O-Desosaminyl-9-deoxo-9-dihydro-9a-N-{N′-[4-(5-methyl-3-isoxazolylaminosulfonyl)phenyl]carbamoyl}-9a-aza-9a-homoerythronolide A (**7d**).* Yield 54.4%. ^{1}H NMR ($CDCl_3$) δ 2.66 (1H, H-2), 3.87 (1H, H-3), 3.47 (1H, H-5), 1.8 (1H, H-8), 4.60 (1H, H-13), 1.95, 1.59 (2H, H-14), 0.92 (3H, H-15), 4.36 (1H, H-1′), 3.32 (1H, H-2′), 2.47 (1H, H-3′), 2.33 (6H, 3′ NMe_2), 1.71, 1.33 (2H, H-4′), 3.56 (1H, H-5′), 1.30 (3H, 2 Me), 0.93 (3H, 4 Me), 1.31 (3H, 6 Me), 1.06 (1H, H-8 Me), 1.13 (3H, 12 Me), 1.20 (3H, 5′ Me), 7.64 (2H, phenyl, b), 7.40 (2H, phenyl, c), 6.16 (1H, isoxazolyl, c′), 2.32 (3H, isoxazolyl, c′-Me); ^{13}C NMR ($CDCl_3$) δ 176 (C-1), 44 (C-2), 74.6 (C-3), 38.6 (C-4), 90 (C-5), 74.1 (C-6), 29 (C-8), 74 (C-12), 77.1 (C-13), 21.4 (C-14), 10.8 (C-15), 105.6 (C-1′), 69.3 (C-2′), 64 (C-3′), 40.1 (3′ NMe_2), 28.8 (C-4′), 69 (C-5′), 16.0 (2 Me), 7.4 (4 Me), 26 (6 Me), 19 (8 Me), 16.7 (12 Me), 20.8 (5′ Me), 156.1 (N*C*ONH), 170.6 (isoxazolyl, c′), 157.6 (isoxazolyl, a′), 142 (phenyl, a), 133 (phenyl, d), 128.1 (phenyl, b), 117.9 (phenyl, c), 95.5 (isoxazolyl, b'), 12.6 (isoxazolyl, c'-Me).

*4.1.2. General procedure for the hydrolysis of **6a** and **6b***

The suspension of **6a** or **6b** (0.55 mmol) in 1 M hydrochloric acid (5 ml) was stirred for 12 h at room temperature; the pH was adjusted to 9.5–10 by adding 1 M NaOH and was extracted with CH_2Cl_2 (3 × 4 ml). The combined organic layers were washed with water, dried over anhydrous Na_2SO_4, evaporated to dryness under reduced pressure to give crude product wherefrom by chromatography on silica gel column using CH_2Cl_2:CH_3OH:NH_3(25%) = 90:9:1.5 as eluents pure **7a** or **7b** was obtained. The spectroscopic data of so obtained compound were identical to the spectroscopic data of **7a** or **7b** obtained by the general procedure for preparation of **6a–6d** and **7a–7d**.

*4.2. Acid stability of **6a** and **6b***

*4.2.1. Preparation of **6a** hydrolyzate in 0.01 M HCl*

Solution of **6a** in 0.01 M HCl of concentration 1 mg/ml (100 ml) was stirred for 10 h at room temperature. After isolation and purification procedures, described in Section 4.1.2, the pure **7a** was obtained. Its ^{1}H and ^{13}C NMR spectra were identical to the spectra of the **7a** authentic sample.

4.2.2. HPLC assay and decay data

The solution of **6a** or **6b** in 0.01 M HCl aqueous hydrochloric acid (concentration of 1 mg/ml) was held at 25 °C,

30 °C, 37 °C and 40 °C for 10 h. The sample of the solution (20 μl) was taken each 20 min, and the course of reaction was monitored by the reverse phase HPLC. Each experiment was repeated three times, and displayed data represent average values.

An Agilent 1100 Series LC/MSD trap, SL model with an electrospray interface (ESI), a binary pump, degasser, autosampler, thermostatted column compartment and diode array detector were used. The analysis was carried out with UV detection at 240 nm on a YMC Pack Pro C_{18} column, 150 mm × 4.6 mm i.d., 3 μm particle size with a mobile phase consisted of channel A 40 mM ammonium acetate buffer solution (pH = 5) and channel B acetonitrile. Gradient elution at flow rate 1.0 ml/min started with 10% of channel B and ended after 15 min with 90% of channel B. The ESI source was operated in the positive ion mode. Nitrogen was used as nebulizing and drying gas at 350 °C. The *m/z* range scanned in the MS measurement was 200–1200. Complete system control, data acquisition and processing were done using the ChemStation for LC/MSD version 4.2 from Agilent.

Reaction rate constants were determined by using exponential fit method available within Excel program. The activation energies for hydrolysis of the glycosidic bond in **6a** and **6b** were calculated according to the Arrhenius equation (1).

$$\ln k = \ln A - E_a/RT \quad (1)$$

where A is the temperature factor, E_a is the activation energy, R is the ideal gas constant (8.3184 J K^{-1} mol^{-1}) and T is the thermodynamic temperature.

4.3. In vitro antimicrobial activity

Antibacterial activity data given in Table 3 were obtained by microdilution test in Mueller–Hinton media. Test substances and standards were dissolved in DMF (Merck) at concentration 5 mg/ml. Solutions of the substances were prepared in Mueller–Hinton broth media with final concentration ranging from 64 to 0.125 μg/ml. After 24 h incubation, optical density was detected by measuring absorbance at 600 nm. MIC is defined as the concentration that shows 90% growth inhibition, and was determined by broth dilution methods, National Committee for Clinical Laboratory Standards (NCCLS, M7-A2 protocols). All screening procedures were performed on a Tecan Genesis 150 robot unit.

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