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Preparation of Erythromycin Analogs Having Functional Groups at C-15

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Abstract Chemobiosynthesis has been used to prepare analogs of erythromycins having unique functional groups at the 15-position. Using diketide thioester feeding to genetically engineered *Streptomyces coelicolor*, analogs of 6-deoxyerythronolide B were prepared having 15-fluoro, 15-chloro, and 15-azido groups. Bioconversion using a genetically engineered mutant of *Saccharopolyspora erythraea* was used to produce 15-fluoroerythromycin A and 15-azidoerythromycin A. These new erythromycin analogs provide antibacterial macrolides with unique physicochemical properties and functional groups that allow for selective derivatization.

Keywords erythromycin, fluorine, azide, antibacterial, genetic engineering, polyketide

Introduction

The search for new macrolide antibacterial agents has been driven by the need to improve the spectrum, stability, and physicochemical properties of existing antibiotics. Erythromycin A (**1**) has been successfully used to treat respiratory infections for over 50 years, but suffers from marked instability, poor bioavailability, undesired gastrointestinal side effects, and an increasingly limited spectrum of activity with the rise in macrolide-lincosamide-streptogramin B MLS(B)-resistant bacteria. Chemical derivatization of erythromycin A has now been thoroughly investigated as a means of overcoming most of these limitations. For example, 6-*O*-methylation to produce clarithromycin and ring expansion of the 9-oxime to produce azithromycin have been highly successful at stabilizing the macrolide, but with no marked improvements in spectrum. Chemical conversion of **1** to the ketolides telithromycin and cethromycin has improved both the stability and spectrum of activity, although at the expense of rather complex chemistry. Replacement of the cladinose sugar with various acyl groups has yielded acylides with improved spectrum, in part due to overcoming inducible MLS(B) resistance [1]. Given the extensive medicinal chemistry that has been performed, there appear to be few remaining positions on the erythromycin scaffold that have not been modified. Further development of erythromycin thus requires radically new technology.

The production of novel erythromycins through chemobiosynthesis has been previously reported [2, 3]. In this process, synthetic starter units are introduced into the erythromycin polyketide by feeding a diketide analog to an engineered strain in which the ketosynthase domain of the initial polyketide synthase module has been inactivated. This technology has been used to make chemically rather conservative changes to the erythromycin structure, for example replacing the starting propionyl group with a butyryl group. Another technology using a polyketide synthase (PKS) having a genetically engineered load domain from the avermectin PKS has also provided erythromycins with chemically conservative changes to the starter unit, such as replacing the starting propionyl group with a cyclopentyl group [4]. These erythromycins

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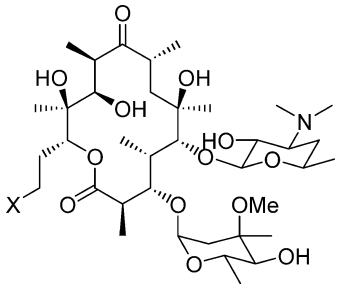


(**1**) X = H
(**2**) X = F
(**3**) X = N_3

Fig. 1 Erythromycin A and C-15 substituted erythromycins.

have generally shown similar to reduced activity relative to erythromycin A. The C-15 hydroxylation of the erythromycin derivative 6-*O*-methylerythromycin has also been reported, however 15-hydroxyerythromycin itself appears to be unreported [5, 6].

We recognized that it might be possible to use chemobiosynthesis technology to introduce functional groups that will provide erythromycin analogs having significantly altered physicochemical properties or having unique chemical handles that allow for selective derivatization. We describe here the preparation and properties of two such analogs, 15-fluoroerythromycin A (**2**) and 15-azidoerythromycin A (**3**).

Materials and Methods

General

NMR spectra were obtained in $CDCl_3$ at 298 K using a Bruker DMX-400 spectrometer fitted with a 3-mm proton–carbon probe (Nalorac). Exact masses were obtained using a Mariner time-of-flight mass spectrometer (Applied Biosystems) with electrospray ionization. Samples were introduced by flow injection using 1 : 1 MeOH/H_2O containing 5 μM NH_4OAc and three standard compounds whose *m/z* ratios were used to calibrate the exact mass. Optical rotations were measured using a Perkin-Elmer model 341 polarimeter with a 100-mm pathlength cell. IR spectra were obtained from thin films prepared from evaporation of a CH_2Cl_2 solution using attenuated total reflection on a Perkin-Elmer Spectrum One FT-IR spectrophotometer. Erythromycin A for antibacterial testing was obtained from Sigma. Bacterial strains were from the American Type Culture Collection (ATCC) as indicated in Table 1, or were clinical isolates. MIC values for reference and test compounds were determined in Mueller-Hinton broth (BBL) by the broth microdilution method according to NCCLS guidelines [7]. All fermentation medium components were from Sigma-Aldrich, with the exception of soya meal flour, which was obtained from Giusto's Specialty Foods, South San Francisco, CA, USA.

Partition Coefficients

Log D values were estimated by two methods. In the first, the individual compounds **1** and **2** were partitioned between 1-octanol and 0.1 M sodium *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES-Na^+), pH 7.4, and the amount of compound remaining in the aqueous phase was determined by HPLC analysis. In a second method, a mixture of equal amounts of **1** and **2** in 10 mM sodium *N*-2-(hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate (HEPES-Na^+), pH 7.4, was stirred vigorously with an equal volume of buffer-saturated 1-octanol for 24 hours, and the aqueous phase was analyzed by HPLC, revealing an 80 : 20 ratio of **2** to **1**.

3-Fluoropropanal (4)

Trichloroisocyanuric acid (17.0 g, 73 mmol) was added in *ca.* 1 g portions over 1 hour to a vigorously stirred mixture of 3-fluoropropanol (15.6 g, 200 mmol), $NaHCO_3$ (16.8 g, 200 mmol), and TEMPO (0.35 g, 2.2 mmol) in CH_2Cl_2 (300 ml) and water (10 ml). The temperature was kept at 20∼25°C using a water bath. After addition was complete, the mixture was stirred until the orange color faded to a pale yellow (approximately 30 minutes). The resulting solution was decanted from the gummy white residue, filtered through a pad of silica gel (5 g), and dried over $MgSO_4$ (10 g) for 30 minutes to provide a solution of **4** in CH_2Cl_2. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.82 (1H, br s, C*H*O), 4.78 (2H, t, J=46.4, 5.6 Hz, CH_2F), 2.84 (2H, dt, J=25.6, 5.6 Hz, CH_2CO).

The concentration of **4** was determined by dilution of 50 μl of the solution into 300 μl of $CDCl_3$ and recording the ^{1}H-NMR spectrum. The integral of the CH_2Cl_2 peak (δ 5.28) was taken as representing 31.2 M protons in the neat solution, and compared to the integral of the aldehyde peak (δ 9.82) such that the concentration of **4** was estimated using the formula: [**4**]=(integral of C*H*O)/(integral of CH_2Cl_2)*31.2. This procedure provided an approximately 0.5 M solution of **4** in CH_2Cl_2, which was stable for several weeks when stored at −20°C.

3-Azidopropanal (5)

Prepared according to [8]. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.81 (1H, t, J=1.0 Hz, C*H*O), 3.62 (2H, t, J=6.3 Hz, CH_2N_3), 2.73 (2H, dt, J=1.0, 6.5 Hz, CH_2CO). ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.2, 44.5, 42.8.

Table 1 *In vitro* activities of **1**, **2**, and **3** against selected pathogens

		MIC (μg/ml)			
		1[a]	**2**	**1**[b]	**3**
S. pneumoniae					
ATCC6301		0.06	0.06	0.03	0.03
ATCC49619		0.12	0.06	0.06	0.06
OC2540		≤0.015	≤0.015	≤0.015	≤0.015
OC4445		0.03	0.06	0.06	0.06
OC4821		0.06	0.06	0.06	0.03
OC4869		0.03	0.06	0.03	0.03
OC4876		0.06	0.06	0.03	0.03
	Range	≤0.015∼0.12	≤0.015∼0.06	≤0.015∼0.06	≤0.015∼0.06
	Median	0.06	0.06	0.03	0.03
OC4438	mef	8	8	4	4
OC4439	mef	2	4	2	2
OC4429	mef	4	8	4	4
OC4436	mef	2	4	2	4
OC4421	mef	4	8	4	2
OC4568	mef	2	4	1	1
	Range	1∼8*	4∼8		1∼4
	Median	2∼4	4∼8	2∼4	2∼4
OC3604	erm	>16	>16	>16	>16
OC4051	erm	>16	>16	>16	>16
OC4432	erm	>16	>16	>16	>16
OC4444	erm	4	8	2	4
	Range	2∼>16*	8∼>16		4∼>16
	Median	>16	>16	>16	>16
H. influenzae					
ATCC49247		2	2	1	0.5
ATCC49766		2	2	1	0.5
OC4881		8	8	4	2
OC4882		8	8	8	2
OC4884		4	4	4	1
OC4888		4	4	2	1
	Range	1∼8*	2∼8		0.5∼2
	Median	4	4	4	1
S. aureus					
ATCC29213		0.5	0.5	0.5	0.5
ATCC13709		0.5	0.5	0.5	0.5
OC3726		0.5	0.5	0.5	0.5
	Range	0.5	0.5	0.5	0.5
	Median	0.5	0.5	0.5	0.5

Table 1 Continued

		MIC (μg/ml)			
		1[a]	**2**	**1**[b]	**3**
S. hemolyticus					
OC3882	ermC-inducible	>16	>16	>16	>16
OC4545	ermC-constitutive	>16	>16	>16	>16
	Range	>16	>16	>16	>16
	Median	>16	>16	>16	>16

[a] Measured on same day as for **2**, [b] Measured on same day as for **3**, * Combined ranges for both days.

3-Chloropropanal (6)

A solution of acrolein (11.2 g, 0.20 mol) and dicinnamylacetone (5 mg) in 100 ml of CH_2Cl_2 was stirred and cooled on ice, and anhydrous HCl was bubbled into the solution until the color changed to red. The resulting solution was dried by addition of $MgSO_4$, then filtered to provide a solution of **6** containing <5% remaining acrolein. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.78 (1H, t, J=1.2 Hz, C*H*O), 3.80 (2H, t, J=6.4 Hz, CH_2Cl), 2.93 (2H, dt, J=0.8, 6.4 Hz, CH_2CO).

The concentration was determined as described above for **4**. This procedure provides an approximately 1.8 M solution of **6** in CH_2Cl_2.

(±)-*N*-[*u*-5-X-3-Hydroxy-2-methylpentanoyl]-2-benzoxazolones (7, 8, 9)

Aldehydes **4**~**6** were converted into their racemic *syn N*-[*u*-5-X-3-hydroxy-2-methylpentanoyl]-2-benzoxazolone aldol adducts **7**~**9**, and the aldol adducts were converted into the thioesters **10**~**12** using the procedure described by [9].

(±)-*N*-[*u*-5-Fluoro-3-hydroxy-2-methylpentanoyl]-2-benzoxazolone (7)

White needles from methyl *tert*-butyl ether (MTBE), mp 117~118°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07~8.02 (m, 1 H), 7.29~7.18 (m, 3H), 4.77~4.05 (m, 2H), 4.31 (m, 1 H), 3.96 (dq, J=7, 3 Hz, 1H), 2.00~1.79 (m, 2H), 1.34 (d, J=7 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.1, 151.1, 142.2, 127.7, 125.6, 125.0, 116.2, 110.0, 81.2 (d, J_{CF}=163 Hz), 68.0 (d, J_{CF}=4 Hz), 43.8, 34.6 (d, J_{CF}=19 Hz), 10.4.

(±)-*N*-[*u*-5-Azido-3-hydroxy-2-methylpentanoyl]-2-benzoxazolone (8)

White needles from 1 : 1 MTBE/hexane, mp 79~81°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (1H, m), 7.27 (3H, m), 4.23 (1H, ddd, J=3.2, 6.4, 9.7 Hz), 3.96 (1H, dq, J=2.9, 7.0 Hz), 3.52 (2H, dd, J=5.7, 7.5 Hz), 2.79 (1H, dd, J=1.3, 3.4 Hz), 1.83 (1H, m), 1.75 (1H, m), 1.34 (3H, d, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 176.0, 151.1, 142.2, 127.6, 125.6, 125.0, 116.3, 110.0, 68.8, 48.4, 43.8, 32.9, 10.5.

(±)-*N*-[*u*-5-Chloro-3-hydroxy-2-methylpentanoyl]-2-benzoxazolone (9)

White needles from MTBE, mp 116~117°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (1H, m), 7.27 (3H, m), 4.36 (1H, ddd, J=3.0, 5.0, 9.0 Hz), 3.97 (1H, dq, J=3.0, 7.0 Hz), 3.74 (2H, m), 2.83 (1H, br d, J=3.0 Hz), 2.06 (1H, m), 1.89 (1H, m), 1.31 (3H, d, J=7.0 Hz). ^{13}C-NMR (100 MHz, $CDCl_3$) δ 176.1, 151.1, 142.2, 127.6, 125.6, 125.0, 116.2, 110.0, 68.4, 43.7, 41.7, 36.4, 10.6.

(±)-*u*-5-Fluoro-3-hydroxy-2-methylpentanoyl *N*-Propionylcysteamine Thioester (10)

White amorphous solid, mp 42~43°C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.96 (1H, br s), 4.64 (1H, ddt, J=47, 9, 5 Hz), 4.58 (1H, ddt, J=47, 9, 5 Hz), 4.13 (1H, dt, J=9, 4 Hz), 3.50 (2H, m), 3.30 (1H, br s), 3.04 (2H, ddt, J=19.4, 14, 6 Hz), 2.75 (1H, dq, J=4, 7 Hz), 2.20 (2H, q, J=7 Hz), 1.8 (2H, m), 1.23 (3H, d, J=7 Hz), 1.13 (3H, t, J=7 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.3, 174.9, 81.5 (d, J_{CF}=163 Hz), 68.7 (d, J_{CF}=4 Hz), 54.0, 39.3, 35.3 (d, J_{CF}=19 Hz), 29.8, 29.0, 12.0, 10.2.

(±)-*u*-5-Azido-3-hydroxy-2-methylpentanoate *N*-Acetylcysteamine Thioester (11)

Pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.14 (1H, br s), 4.06 (1H, br d, J=9.6 Hz), 3.40 (4H, m), 3.18 (1H, br s), 3.02 (2H, m), 2.72 (1H, dq, J=4.4, 7.2 Hz), 1.98 (3H, s), 1.71 (2H, m), 1.24 (3H, d, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.4, 170.7, 69.4, 53.6, 48.5, 39.1,

33.2, 28.7, 23.1, 11.6.

(±)-*u*-5-Chloro-3-hydroxy-2-methylpentanoyl *N*-Acetylcysteamine Thioester (12)

Pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.07 (1H, br s), 4.17 (1H, ddd, J=3.0, 6.4, 10.0 Hz), 3.70 (2H, m), 3.47 (2H, m), 3.08 (3H, m), 2.74 (1H, dq, J=4.0, 7.2 Hz), 1.98 (3H, s), 1.96 (1H, m), 1.80 (1H, m), 1.24 (3H, d, J=7.2 Hz). ^{13}C NMR (100 MHz, $CDCl_3$) δ 203.5, 170.8, 69.0, 53.4, 41.8, 39.1, 36.8, 28.8, 23.2, 11.5.

Preparation of 15-X-6-Deoxyerythronolide B Analogs (13～15)

A seed culture of *Streptomyces coelicolor* CH999/pJRJ2 was made by inoculating 1 ml of frozen mycelium into a 250 ml baffled flask containing 50 ml of FKA medium (corn starch, 45 g/liter; corn steep liquor, 10 g/liter; dried, inactivated brewers yeast, 10 g/liter; and $CaCO_3$, 1 g/liter), 0.050 ml of 50 mg/ml thiostrepton in DMSO (filter sterilized), and 0.500 ml of 50% Antifoam B (JT Baker). The flask was incubated at 30°C with shaking at 175 rpm for 48 hours (Innova floor shaker). The culture was transferred into a 2.8-liter baffled flask containing 500 ml of FKA medium, 0.500 ml of 50 mg/ml thiostrepton in DMSO, and 5 ml of 50% Antifoam B, and the flask was incubated at 30°C with shaking at 175 rpm for 48 hours.

A 10-liter stirred tank bioreactor (B. Braun) was autoclaved, filled with 5 liters of sterile FKA medium and 5 ml of 50 mg/ml thiostrepton in DMSO, and then inoculated with 500 ml (10%) of seed culture. The bioreactor was run for 24 hours at 30°C with stirring at 600 rpm, sparged with air at 1.33 LPM, and the pH was maintained at 6.50 by automated addition of 2.5 N NaOH and 2.5 N H_2SO_4.

Three liters of the above culture were used to inoculate a 100-liter bioreactor containing 55 liters of sterile FKA medium with 2 g/liter Tastone 310 added prior to sterilization. The initial fermentor agitation rate was set at a tip speed of 2.5 m/second, the temperature was maintained at 30°C, the pH was controlled at pH 6.5 by automated addition of 2.5 N NaOH and 2.5 N H_2SO_4, and the airflow was set at 0.4 vvm. Foaming was controlled by automated addition of 50% Antifoam B. During the fermentation, the dissolved oxygen was maintained at >50% air saturation by cascade control using the agitation rate (tip speed of 2.5～3.0 m/second) and back pressure (0.1～0.4 bar) in that order. After 24 hours post-inoculation, a 400 g/liter solution of the appropriate thioester **10～12** in DMSO was added to a final concentration of 1 g/liter. Titers of **13～15** were monitored by HPLC, and the culture was harvested by centrifugation when a maximum titer was reached.

The **13～15** was isolated by solid phase extraction. The broth was clarified by centrifugation and loaded onto a column containing HP-20 resin (Rohm and Haas) at a concentration of 1 liter resin/20 g 15-X-6-deoxyerythronolide B. The column was then equilibrated with 5 column volumes of water at a flow rate of 2～4 ml/cm^2-minute. The loaded resin was washed with 2 column volumes of water followed by 2 column volumes of 30% methanol in water. The compound was eluted from the resin with methanol. The fractions containing desired compound were identified by HPLC with ELSD detection, then were pooled and the volatiles were removed under reduced pressure. The dried solids were extracted with 3～5 liters of methanol and filtered to yield a solution containing 6～10 mg/ml of the compound, which was diluted with an equal volume of water. This solution was loaded onto a column of HP20SS (1 liter resin/20 g of compound), which was then washed with 2 column volumes of 50% aqueous methanol. The compound was then eluted with 70% methanol in water, and the fractions were analyzed by HPLC. Product-containing fractions were pooled and evaporated to dryness.

15-Fluoro-6-deoxyerythronolide B (13)

Crystals from ether/hexane, mp 122～123°C. $[\alpha]_D^{20}$ −62.2 (c 0.4, CH_3CN). IR ν_{max} (thin film) cm^{-1} 1702, 1176. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.49 (1H, dd, J=1.6, 10.0 Hz, H-13), 4.39～4.61 (2H, m; H-15), 3.99 (1H, m; H-5), 3.90 (1H, d, J=10.4 Hz, H-3), 3.84 (1H, d, J=4.0 Hz, 11-OH), 3.70 (1H, ddd, J=2.4, 4.8, 10.4 Hz, H-11), 3.18 (1H, br s; 3-OH), 2.77 (2H, m; H-2+H-10), 2.61 (1H, m; H-8), 2.47 (1H, br s; 5-OH), 2.10～2.25 (1H, m; H-14a), 2.00 (1H, m; H-6), 1.92 (1H, m; H-14b), 1.85 (m, 1H; H-4), 1.72 (1H, m; H-12), 1.67 (1H, ddd, J=4.0, 10.8, 14.4 Hz, H-7a), 1.29 (3H, d, J=6.8 Hz, Me2), 1.26 (1H, m; H-7b), 1.07 (3H, d, J=7.2 Hz, Me4), 1.06 (3H, d, J=6.4 Hz, Me8), 1.05 (3H, d, J=7.6 Hz, Me6), 1.02 (3H, d, J=6.8 Hz, Me10), 0.93 (3H, d, J=6.8 Hz, Me12). ^{13}C NMR (100 MHz, $CDCl_3$) δ 213.7 (C-9), 178.0 (C-1), 80.7 (d, J_{CF}=166 Hz, C-15), 79.3 (C-3), 76.4 (C-5), 70.8 (d, J_{CF}=4 Hz, C-13), 70.7 (C-11), 43.9 (C-2), 43.3 (C-10), 41.1 (C-12), 39.5 (C-8), 37.6 (C-4), 37.5 (C-7), 35.5 (C-6), 33.3 (d, J_{CF}=20 Hz, C-14), 16.6 (Me6), 14.6 (Me2), 13.3 (Me8), 9.2 (Me12), 6.9 (Me4), 6.3 (Me10). MS Found: $(M+Na)^+$, 427.2456. $C_{21}H_{37}FO_6$ requires $(M+Na)^+$, 427.2466.

15-Azido-6-deoxyerythronolide B (14)

A mixture of **15** (19.07 g, *ca.* 88% pure), sodium iodide (5.95 g), and sodium azide (10.31 g) in 60 ml of dimethylsulfoxide was heated at 50°C for 3 days. The mixture was cooled to ambient temperature and diluted

with ethyl acetate. The solution was washed with water, and the aqueous phase was extracted with ethyl acetate. The combined organic phases were washed with brine, dried over $MgSO_4$, filtered, and evaporated. The residue was purified by silica gel chromatography using ethyl acetate/hexanes, yielding 13.96 g of 15-azido-6-dEB. Colorless needles from ether, mp 143~144°C. $[\alpha]_D^{20}$ −62.8 (*c* 0.4, CH_3CN). IR ν_{max} (thin film) cm^{-1} 2100, 1710, 1689, 1178. ^1H NMR (400 MHz, $CDCl_3$) δ 5.39 (1H, ddd, *J*=1.4, 2.9, 10.2 Hz, H-13), 3.98 (1H, m, H-5), 3.90 (1H, dd, *J*=2.8, 10.4 Hz, H-3), 3.80 (1H, d, *J*=4.0 Hz, 11-OH), 3.68 (1H, ddd, *J*=2.0, 4.4, 10.0 Hz, H-11), 3.36 (2H, m, H-15), 3.06 (1H, d, *J*=2.8 Hz, 3-OH), 2.76 (2H, m, H-2+H-10), 2.61 (1H, m, H-8), 2.29 (1H, d, *J*=3.6 Hz, OH-5), 2.12 (1H, m, H-14a), 2.00 (1H, m, H-6), 1.82 (1H, dq, *J*=1.6, 5.6 Hz, H-4), 1.70 (2H, m, H-12+H-14b), 1.65 (1H, ddd, *J*=3.6, 10.4, 14.0 Hz, H-7a), 1.30 (3H, d, *J*=6.8 Hz, Me2), 1.26 (1H, ddd, *J*=4.4, 12.8, 14.0 Hz, H-7b), 1.07 (3H, d, *J*=7.2 Hz, Me4), 1.06 (3H, d, *J*=6.0 Hz, Me8), 1.05 (3H, d, *J*=7.2 Hz, Me6), 1.02 (3H, d, *J*=6.8 Hz, Me10), 0.92 (3H, d, *J*=6.8 Hz, Me12). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 213.6 (C-9), 178.0 (C-1), 79.3 (C-3), 76.4 (C-5), 71.8 (C-13), 70.7 (C-11), 48.5 (C-15), 43.9 (C-2), 43.2 (C-10), 41.2 (C-12), 39.6 (C-8), 37.7 (C-4), 37.5 (C-7), 35.6 (C-6), 31.7 (C-14), 16.6 (Me6), 14.7 (Me2), 13.4 (Me8), 9.3 (Me12), 6.9 (Me4), 6.3 (Me10). MS Found: $(M+Na)^+$, 450.2577. $C_{21}H_{37}N_3O_6$ requires $(M+Na)^+$, 450.2575.

15-Chloro-6-deoxyerythronolide B (15)

Colorless needles from ether/hexane, mp 132~133°C. IR ν_{max} (thin film) cm^{-1} 1702, 1173. ^1H NMR (400 MHz, $CDCl_3$) δ 5.47 (1H, br d, *J*=8.8 Hz, H-13), 3.96 (1H, m; H-5), 3.88 (1H, dd, *J*=2.8, 10.4 Hz, H-3), 3.84 (1H, d, *J*=4.4 Hz, 11-OH), 3.67 (1H, m; H-11), 3.53 (2H, m; H-15), 3.24 (1H, d, *J*=2.0 Hz, 3-OH), 2.76 (2H, m; H-2+H-10), 2.75 (1H, m; H-8), 2.56 (1H, br s; 5-OH), 2.30 (1H, m; H-14a), 2.00 (1H, m; H-6), 1.88 (1H, m; H-14b), 1.70 (1H, m; H-12), 1.64 (1H, m; H-7a), 1.27 (3H, d, *J*=6.8 Hz, Me2), 1.23 (1H, ddd, *J*=4.0, 13.6, 13.6 Hz, H-7b), 1.04 (3H, d, *J*=7.2 Hz, Me4), 1.03 (3H, d, *J*=6.0 Hz, Me8), 1.02 (3H, d, *J*=7.2 Hz, Me6), 0.99 (3H, d, *J*=6.8 Hz, Me10), 0.90 (3H, d, *J*=6.8 Hz, Me12). ^{13}C NMR (100 MHz, $CDCl_3$) δ 213.7 (C-9), 178.0 (C-1), 79.3 (C-3), 76.3 (C-5), 71.6 (C-13), 70.7 (C-11), 43.8 (C-2), 43.2 (C-10), 41.0 (C-12+C-15), 39.5 (C-8), 37.6 (C-7), 37.5 (C-4), 35.4 (C-6), 35.0 (C-14), 16.6 (Me6), 14.6 (Me2), 13.3 (Me8), 9.4 (Me12), 6.9 (Me4), 6.2 (Me10). MS Found: $(M+Na)^+$, 443.2186. $C_{21}H_{37}ClO_6$ requires $(M+Na)^+$, 443.2171.

Preparation of Erythromycin A analogs (2, 3)

A seed culture of *Saccharopolyspora erythraea* K39-14V was made by inoculating a 1 ml aliquot of frozen mycelium into each of three 250 ml baffled flasks containing 50 ml of V1 medium (corn starch, 16 g/liter; corn dextrin, 10 g/liter; soya meal flour, 15 g/liter; corn steep liquor, 5 g/liter; soy bean oil, 6 g/liter; sodium chloride, 2.5 g/liter; ammonium sulfate, 1 g/liter; and $CaCO_3$, 4 g/liter) and 0.100 ml of Antifoam B. The flasks were incubated at 34°C with shaking at 175 rpm for 48 hours (Innova floor shaker). Each culture was transferred into a 2.8-liter baffled flask containing 500 ml of V1 medium and 1 ml of Antifoam B, and the flasks were incubated at 34°C with shaking at 175 rpm for 48 hours.

Three 10-liter stirred tank bioreactors (B. Braun) were autoclaved, and each filled with 10 liters of sterile 50% F1 medium (corn starch, 17.5 g/liter; corn dextrin (type 3), 16 g/liter; soy meal flour, 16.5 g/liter; calcium carbonate, 4 g/liter, corn steep liquor, 6 g/liter; soy bean oil, 3 g/liter; sodium chloride 2.5 g/liter; and ammonium sulfate, 1 g/liter). The fermentor agitation rate was set at a tip speed of 2~4 m/second, the pH was controlled at pH 7.0 by automated addition of 2.5 N NaOH and 2.5 N H_2SO_4, the temperature was maintained at 34°C, and the airflow was set at 0.15 vvm. Foaming was controlled by automated addition of 50% Antifoam B. Each fermentor was inoculated with a 500-ml seed culture prepared above. During the fermentation, the dissolved oxygen was maintained at >80% air saturation by cascade control using agitation rate (tip speed of 2~4 m/second), airflow (0.15~0.5 vvm), and oxygen enrichment in that order. After 24 hours post-inoculation, a continuous 2 g/liter/day dextrin feed (150 g/liter dextrin in deionized water) was initiated, and a 4% w/v solution of **13** or **14** was added to a final concentration of 250~300 mg/liter. Samples were analyzed by HPLC every 12 hours. After cessation of erythromycin analog production (*ca.* 60 hours), the culture was harvested by centrifugation.

The erythromycin analog was isolated by solid phase extraction. The broth was adjusted to pH 9 using 2.5 N NaOH, clarified by centrifugation, and loaded onto a column containing HP-20 resin (Rohm and Haas) at a concentration of 1 liter resin/20 g of erythromycin A analog. The column was then equilibrated with 5 column volumes of water at a flow rate of 2~4 ml/cm^2-minute. The loaded resin was washed with 2 column volumes of water. The erythromycin analog was eluted from the resin with 5 column volumes of methanol. The fractions containing erythromycin analog were identified by HPLC, pooled, and the volatiles were removed under reduced pressure. The dried solids were mixed with 800 ml of acetone and 3.2-liter of hexane for 20 minutes. The mixture was then filtered using a #4 Whatman filter paper. The solids were

extracted twice in this manner, and the filtrates were combined and evaporated.

The crude product was dissolved in methanol and diluted with an equal volume of water. This solution was loaded onto a column of HP20SS (1 liter resin/20 g of erythromycin analog), which was then washed successively with 1 column volume of 50% aqueous methanol, 3 column volumes of 3 : 2 methanol/water, 3 column volumes of 7 : 3 methanol/water, 10 column volumes of 4 : 1 methanol/water, and finally 5 column volumes of 100% methanol. The fractions were analyzed by HPLC. Product-containing fractions were pooled and evaporated to dryness.

15-Fluoroerythromycin A (2)

Crystals from CH_3CN (solvate with 1.0 equivalent of CH_3CN), mp 175~176°C. $[\alpha]_D^{20}$ −77 (*c* 1, EtOH). IR ν_{max} (thin film) cm^{-1} 1734 (s), 1720, 1699, 1165, 1052, 1012. ^1H NMR (400 MHz, $CDCl_3$) δ 5.29 (1H, dd, *J*=1.6, 10.4 Hz, H-13), 4.90 (1H, d, *J*=4.8 Hz, H-1″), 4.49 (2H, dt, *J*=47.2, 6.0 Hz, H-15), 4.40 (1H, d, *J*=7.2 Hz, H-1′), 4.00 (1H, m; H-5″), 3.98 (1H, d, *J*=9.2 Hz, H-3), 3.88 (1H, s; H-11), 3.55 (1H, d, *J*=8.0 Hz, H-5), 3.48 (1H, m; H-5′), 3.32 (3H, s; 3″-OMe), 3.23 (1H, dd, *J*=7.2, 10.0 Hz, H-2′), 3.08 (1H, q, *J*=7.2 Hz, H-10), 3.00 (1H, m; H-4″), 2.85 (1H, dq, *J*=7.2, 9.2 Hz, H-2), 2.70 (1H, m; H-8), 2.45 (1H, m; H-3′), 2.36 (1H, d, *J*=15.2 Hz, H-2″a), 2.32 (1H, m; H-14a), 2.29 (6H, s; NMe_2), 2.05~1.80 (3H, m; H-4+H-7a+H-14b), 1.73 (1H, m; H-7b), 1.68 (1H, m; H-4′a), 1.58 (1H, dd, *J*=4.8, 15.2 Hz, H-2″b), 1.47 (3H, s; Me6), 1.31 (3H, d, *J*=7.2 Hz, H-6″), 1.24 (3H, s; Me3″), 1.22 (3H, d, *J*=6.0 Hz, H-6′), 1.16 (6H, d, *J*=6.8 Hz, Me2+Me8), 1.15 (3H, s; Me12), 1.14 (3H, d, *J*=6.4 Hz, Me10), 1.11 (3H, d, *J*=7.6 Hz, Me4). ^{13}C NMR (100 MHz, $CDCl_3$) δ 221.5 (C-9), 175.5 (C-1), 103.2 (C-1′), 96.3 (C-1″), 83.4 (C-5), 83.2 (d, J_{CF}=170 Hz, C-15), 79.8 (C-3), 77.9 (C-4″), 75.1 (C-6), 74.3 (C-12), 72.6 (C-3″), 72.5 (d, J_{CF}=4 Hz, C-13), 70.9 (C-2′), 68.9 (C-5′), 68.5 (C-11), 65.6 (C-3′+C-5″), 49.5 (3″-OMe), 45.2 (C-8), 44.8 (C-2), 40.3 (NMe_2), 39.6 (C-4), 38.5 (C-7), 37.7 (C-10), 34.9 (C-2″), 29.4 (d, J_{CF}=20 Hz), 28.7 (C-4′), 27.0 (Me6), 21.5 (Me3″), 21.4 (C-6′), 18.6 (C-6″), 18.2 (Me8), 16.2 (Me12), 15.3 (Me2), 11.9 (Me10), 9.1 (Me4). MS Found: $(M+H)^+$, 752.4564. $C_{37}H_{66}FNO_{13}$ requires $(M+H)^+$, 752.4591.

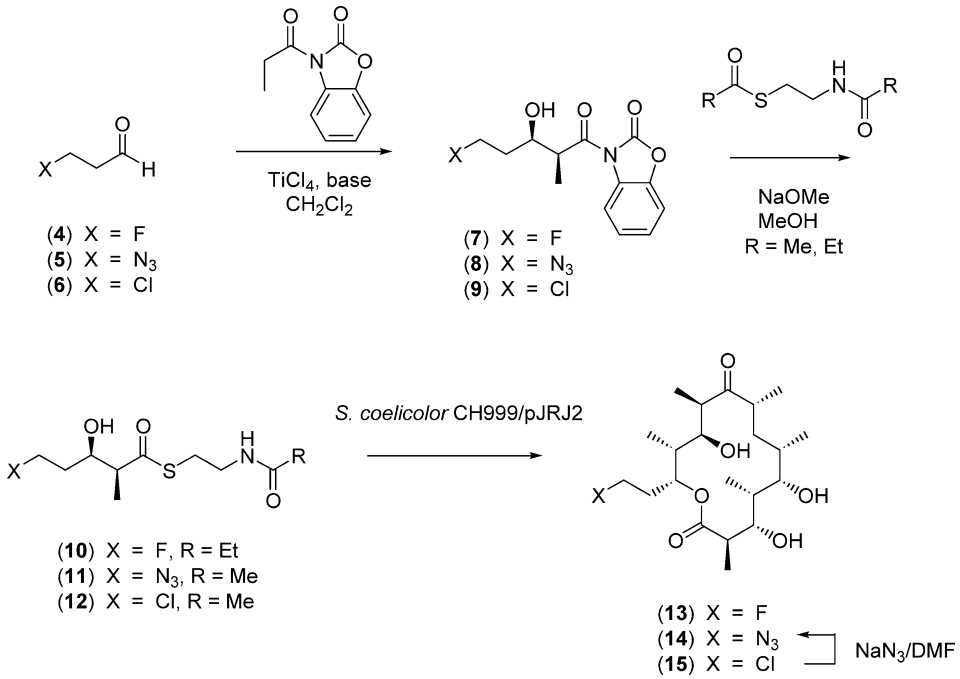
15-Azidoerythromycin A (3)

Crystals from CH_2Cl_2/hexanes, mp 135~137°C. $[\alpha]_D^{20}$ −78 (*c* 1, EtOH). IR ν_{max} (thin film) cm^{-1} 2100, 1734, 1722, 1698, 1164, 1010. ^1H NMR (400 MHz, $CDCl_3$) δ 5.17 (1H, dd, *J*=2.4, 10.4 Hz, H-13), 4.88 (1H, d, *J*=4.8 Hz, H-1″), 4.40 (1H, d, *J*=7.2 Hz, H-1′), 4.00 (2H, m; H-3+H-5″), 3.84 (1H, s; H-11), 3.57 (1H, d, *J*=8.0 Hz, H-5), 3.49 (1H, m; H-5′), 3.33 (3H, s; 3″-OMe), 3.33 (1H, m; H-15a), 3.21 (m, 2H; H-2′+H-15b), 3.08 (1H, q, *J*=6.8 Hz, H-10), 3.01 (1H, dd, *J*=9.6, 9.6; H-4″), 2.86 (1H, dq, *J*=7.2, 9.2; H-2), 2.70 (1H, m; H-8), 2.42 (1H, m; H-3′), 2.37 (1H, d, *J*=15.2 Hz, H-2″a), 2.28 (6H, s; NMe_2), 2.20 (1H, m; H-14a), 1.95 (2H, m; H-4+H-7a), 1.75 (2H, m; H-7b+H-14b), 1.68 (1H, m; H-4′a), 1.56 (1H, dd, *J*=4.8, 15.2 Hz, H-2″b), 1.47 (3H, s; Me6), 1.28 (3H, d, *J*=7 Hz, H-6″), 1.24 (3H, s; 3″-Me), 1.22 (3H, d, *J*=7 Hz, H-6′), 1.19 (3H, d, *J*=7 Hz, Me2), 1.16 (3H, d, *J*=7 Hz, Me8), 1.13 (3H, s; Me12), 1.12 (3H, d, *J*=7 Hz, Me10), 1.11 (3H, d, *J*=7 Hz, Me4). ^{13}C NMR (100 MHz, $CDCl_3$) δ 221.9 (C-9), 175.4 (C-1), 103.2 (C-1′), 96.3 (C-1″), 83.3 (C-5), 79.8 (C-3), 78.0 (C-4″), 75.0 (C-6), 74.4 (C-12), 73.0 (C-13), 72.6 (C-3″), 70.9 (C-2′), 69.0 (C-5′), 68.6 (C-11), 65.6 (C-5″), 65.6 (C-3′), 49.5 (3″-OMe), 49.1 (C-15), 45.2 (C-8), 44.7 (C-2), 40.3 (2C, NMe_2), 39.6 (C-4), 38.5 (C-7), 37.7 (C-10), 34.9 (C-2″), 28.6 (C-4′), 27.8 (C-14), 26.9 (Me6), 21.5 (3″-Me), 21.4 (C-6′), 18.7 (C-6″), 18.2 (Me8), 16.2 (Me12), 15.6 (Me2), 12.0 (Me10), 9.1 (Me4). MS Found: $(M+H)^+$, 775.4691. $C_{37}H_{66}N_4O_{13}$ requires $(M+H)^+$, 775.4699.

Results and Discussion

Diketide thioesters **10**~**12** were prepared using a titanium-mediated aldol condensation between *N*-propionyl-2-benzoxazolone and a 3-substituted propanal to give racemic *syn*-aldol adducts **7**~**9**. The 2-benzoxazolone auxiliary was readily displaced by the thiolate anion of either *N*-acetylcysteamine or *N*-propionylcysteamine to provide the racemic *syn* diketide thioester (Scheme 1, R=Me or Et) [9]. The *N*-propionylcysteamine thioester was chosen for **10** as this gave a more conveniently handled, solid material.

The required 3-azidopropanal (**5**) and 3-chloropropanal (**6**) were prepared by addition of the corresponding acids to acrolein, with the addition of HCl being titrated using dicinnamylacetone as indicator [10]. As an efficient synthesis and isolation of 3-fluoropropanal (**4**) had not been previously reported, a simple process suitable for the production of this unstable synthon in a form useable in the titanium-mediated aldol without purification was developed. Numerous conditions for oxidation of 3-fluoropropanol were examined, yet most resulted in significant decomposition of the sensitive product or the generation of byproducts that were incompatible with the subsequent aldol reaction. TEMPO-catalyzed oxidation using NaOCl resulted in significant quantities of acrolein due to base-catalyzed elimination of HF, even under buffering with $NaHCO_3$, and the large proportion of water



Scheme 1 Preparation of C-15 substituted 6-deoxyerythronolide B analogs.

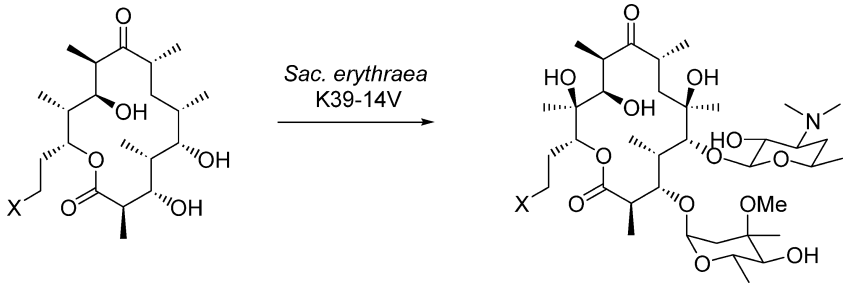
involved led to low recoveries of the relatively water-soluble **4**. We found that the basicity of the reaction was ameliorated, and the proportion of water used lessened, by using trichloroisocyanuric acid as oxidant along with a slurry of $NaHCO_3$ in a minimal volume of water in CH_2Cl_2. Thus, treatment of an equimolar mixture of 3-fluoropropanol and $NaHCO_3$ in CH_2Cl_2 containing $<$10% v/v water with catalytic TEMPO and one-third molar equivalent of trichloroisocyanuric acid, followed by decanting and drying of the resulting solution, provided a moderately stable solution of **4** in ~80% yield.

The titanium-mediated aldol condensation between *N*-propionyl-2-benzoxazolone and the base-sensitive aldehydes **4** or **6** gave improved results using *N*-methylmorpholine rather than triethylamine as base. Use of the weaker base minimized formation of the acrolein adduct. Azidopropanal **5** was efficiently condensed using triethylamine as base.

The racemic diketide thioesters **10**~**12** were converted into the corresponding 6-deoxyerythronolide B analogs **13**~**15** using *Streptomyces coelicolor* CH999/pJRJ2 as previously described for other analogs [11]. While **14** was successfully prepared by feeding of **11**, concerns over the large-scale synthesis of **5** led us to adopt an indirect route to this analog. Feeding of the chloride **12** produced **15** in good yield, demonstrating that the chemobiosynthesis process can tolerate a reactive primary alkyl halide. Conversion of **15** into azide **14** was readily accomplished by reaction with NaN_3 in DMF.

Subsequent bioconversion of **13** and **14** using *Saccharopolyspora erythraea* K39-14V, an engineered mutant strain having an inactivated polyketide synthase, provided the erythromycin A analogs **2** and **3** [12]. The presence of the functional groups at the 15-position did not adversely affect the ability of the tailoring enzymes to act on these substrates, such that **13** and **14** were predominantly bioconverted into their erythromycin A analogs. Bioconversion of **15** provided only low titers of the corresponding 15-chloroerythromycin A.

The erythromycin A analogs described here show *in vitro* antibacterial activity comparable to that of erythromycin A as shown in Table 1. (As compounds **2** and **3** were tested on separate days, Table 1 gives a comparison of the MIC values with those for **1** measured on the same day as either **2** or **3**.) The introduction of chemical handles at the 15-position is itself not deleterious to antibacterial activity. Against macrolide-susceptible pneumococci, the MIC values of **2** and **3** were similar to those of **1**. Activities against *mef*(*A*)-containing pneumococci were comparable for **1** and **3**, while MIC values for **2** were generally two-fold higher. With the exception of one pneumococcal strain, all compounds were relatively inactive against *erm*(*B*)-containing *S. pneumoniae* or *S. haemolyticus* isolates, with MIC values $>16\,\mu$g/ml. In contrast, **3** was selectively more active than the other two antibiotics against the Gram-negative respiratory pathogen *H. influenzae*, with MIC



Scheme 2 Preparation of 15-fluoroerythromycin A and 15-azidoerythromycin A.

values two- to four-fold lower than **1** or **2**.

Fluorine substitution is commonly used in medicinal chemistry to alter the lipophilicity or metabolic stability of a drug, and its inclusion often has beneficial effects on activity, protein binding, and cell penetration [13]. Flurithromycin, (8*S*)-8-fluoroerythromycin A, had higher serum and tissue levels than erythromycin at similar doses, demonstrative of the beneficial pharmacological effect of fluorination on the macrolide molecule [14]. Measurement of partitioning between 1-octanol and water at pH 7.4 indicates that **2** ($\log D \cong 0.3$) is significantly less lipophilic than **1** ($\log D \cong 0.9$). Thus, **2** is a scaffold for antibacterial development having unique pharmacological properties while retaining the fundamental bioactivity of **1**.

Another erythromycin analog, **3**, has a unique chemical handle that allows selective derivatization to create new analogs. Reduction of the azido group and subsequent functionalization of **3** has been recently described [15].

In summary, we have shown that chemobiosynthesis can provide novel analogs of erythromycin that are inaccessible by traditional chemical methods. These analogs contain unique functional groups that alter the physicochemical properties and allow for selective derivatization of the macrolide core, and so enable a new area of investigation into the generation of improved macrolide antibacterial agents.

References

1. Tanikawa T, Asaka T, Kashimura M, Misawa Y, Suzuki K, Sato M, Kameo K, Morimoto S, Nishida A. Synthesis and antibacterial activity of acylides (3-*O*-acyl-erythromycin derivatives): A novel class of macrolide antibiotics. J Med Chem 44: 4027–4030 (2001)
2. Jacobsen JR, Hutchinson CR, Cane DE, Khosla C. Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase. Science 277: 367–369 (1997)
3. Frykman S, Leaf T, Carreras C, Licari P. Precursor-directed production of erythromycin analogs by *Saccharopolyspora erythraea*. Biotechnol Bioeng 76: 303–310 (2001)
4. Brown MS, Dirlam JP, McArthur HA, McCormick EL, Morse BK, Murphy PA, O'Connell TN, Pacey M, Rescek DM, Ruddock J, Wax RG. Production of 6-deoxy-13-cyclopropyl-erythromycin B by *Saccharopolyspora erythraea* NRRL 18643. J Antibiot 52: 742–747 (1999)
5. McAlpine JB, Theriault RJ, Grebner KD, Hardy DJ, Fernandes PB. Minor products from the microbial transformation of 6-*O*-methylerythromycin A by *Mucor circinelloides*. Program and Abstracts of the 27th Intersci. Conf. on Antimicrob. Agents Chemother. No. 222, p. 134, New York, Oct. 4～7 (1987)
6. Adachi T, Sasaki J, Ōmura S. Hydroxylation and *N*-demethylation of clarithromycin (6-*O*-methylerythromycin A) by *Mucor circinelloides*. J Antibiot 42: 1433–1437 (1989)
7. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 5th Edition, Approved Standard M7-A5. National Committee for Clinical Laboratory Standards, Wayne, Pa. (2003)
8. Davies AJ, Donald ASR, Marks RE. The acid-catalysed decomposition of some β-azido-carbonyl compounds. J Chem Soc (C): 2109–2112 (1967)
9. Burlingame MA, Mendoza E, Ashley GW. *N*-Acyl-2-benzoxazolinones in titanium-mediated aldol reactions. Tetrahedron Lett 45: 2961–2964 (2004)
10. Stowell JC, Keith DR, King BT. β-Halo acetals and –ketals: 2-(2-bromoethyl)-1,3-dioxane and 2,5,5-trimethyl-2-(2-bromoethyl)-1,3-dioxane. Organic Syntheses 62: 140–148 (1984)
11. Leaf T, Burlingame M, Desai R, Regentin R, Woo E, Ashley G, Licari P. Employing racemic precursors in directed biosynthesis of 6-dEB analogs. J Chem Tech Biotech 77: 1122–1126 (2002)
12. Carreras C, Frykman S, Ou S, Cadapan L, Zavala S, Woo E, Leaf T, Carney J, Burlingame M, Patel S, Ashley G, Licari P.

Saccharopolyspora erythraea-catalyzed bioconversion of 6-deoxyerythronolide B analogs for production of novel erythromycins. J Biotechnol 92: 217–228 (2002)

13. Böhm H-J, Banner D, Bendels S, Kansy M, Kuhn B, Müller K, Obst-Sander U, Stahl M. Fluorine in medicinal chemistry. Chem Bio Chem 5: 637–643 (2004)
14. Benoni G, Cuzzolin L, Leone R, Consolo U, Ferronato G, Bertrand C, Puchetti V, Fracasso ME. Pharmacokinetics and human tissue penetration of flurithromycin. Antimicrob Agents Chemother 32: 1875–1878 (1988)
15. Shaw SJ, Abbanat D, Ashley GW, Bush K, Foleno B, Macielag M, Zhang D, Myles DC. 15-Amido erythromycins: synthesis and *in vitro* activity of a new class of macrolide antibiotics. J Antibiot 58: 167–177 (2005)