VOL. 56 NO. 3, MAR. 2003 THE JOURNAL OF ANTIBIOTICS pp. 280 — 288

Isolation and Structure of a New Macrolide Antibiotic, Erythromycin G, and a Related Biosynthetic Intermediate from a Culture of *Saccharopolyspora erythraea*

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(Received for publication August 12, 2002)

The new naturally occurring erythromycin G (**4**), formally derived from erythromycin B by hydroxylation of the C-16 methyl group, and 3-*O*-mycarosylerythronolide B (**5**), an erythromycin biosynthetic intermediate previously obtained only from microorganisms blocked in erythromycin biosynthesis, were isolated from a concentrate of mother liquors derived from a culture of *Saccharopolyspora erythraea*. The structure of erythromycin G was defined by spectroscopic data and X-ray crystallographic analysis. Theoretical calculation of **4** has been performed at MM2 level, and the low-energy conformations have been compared with X-ray data: both theoretical and experimental approaches give similar three-dimensional shapes. Antibacterial activity of **4** against both Gram-positive and Gram-negative organisms has been evaluated. A simple method for the isolation of large amounts of erythromycins B (**2**) and D is provided as well.

Erythromycins are naturally occurring antibiotics of the macrolide group. This medicinally important class of natural products is characterized by a 14-membered polyhydroxyketolactone (aglycone) bearing two deoxy sugars, L-mycarose or L-cladinose (3-*O*-methylmycarose) on C-3 and D-desosamine on C-5. Erythromycin A (**1**, Fig. 1), a drug used to treat illnesses caused by Gram-positive and some Gram-negative bacteria, was obtained in 1952 from a culture of *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*)[1] and structurally inferred in 1957[2,3]. Subsequently, five other erythromycins were isolated from *S. erythraea*: erythromycins B (**2**)[4], C[5], D[6], and F (**3**)[8] were directly obtained from cultured broths of *S. erythraea* as primary biosynthetic compounds, whereas erythromycin E was slowly produced from erythromycin A incubated with a point-blocked mutant of the same microorganism[7].

Until now, many structural analogues have been obtained in order to study biosynthetic pathways[9] and their stabilities in acid[10] or basic[11] conditions.

Now, we report the new erythromycin G (**4**) and the known 3-*O*-mycarosylerythronolide B (**5**) as primary biosynthetic products of *S. erythraea* fermentation processes. The first (**4**) has been isolated by an original procedure from a concentrate of mother liquors derived from crystallization in CH_2Cl_2 of the industrially produced erythromycin A. The molecular structure of **4** was inferred by extensive 1D and 2D NMR experiments, and by X-ray crystallographic techniques. Compound **4** differs from **2** in the hydroxylation of the C-16 atom: it is 16-hydroxyerythromycin B. The second (**5**) was purified by semi-preparative HPLC from the same mother liquors and was characterized by spectroscopic means. This is the first report on isolation of **5** from non-blocked microorganisms.

Results and Discussion

During HPLC examination of mother liquors derived from a high yielding production strain of *S. erythraea* (Biochemie Ery 1898), we found a new metabolite, which was purified by a simple method. A *tert*-butyl methyl ether solution of the dried mother liquors, kept at low temperature, gave a crystalline precipitate. This, subjected

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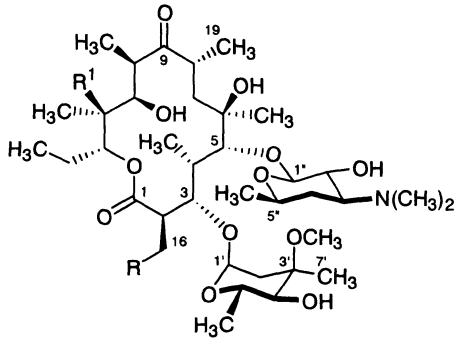
Fig. 1. Structures of the 3-*O*-mycarosylerythronolide B (**5**), the known erythromycins A (**1**), B (**2**) and F (**3**), and the new erythromycin G (**4**).

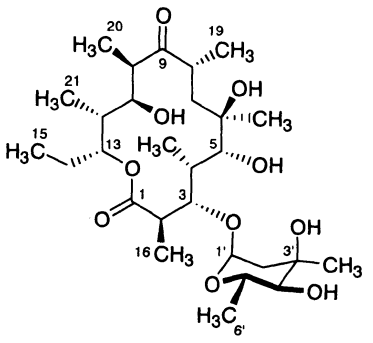


1 (Erythromycin A): R=H, R^1=OH

2 (Erythromycin B): R=R^1=H

3 (Erythromycin F): R=R^1=OH

4 (Erythromycin G): R=OH, R^1=H



5: 3-*O*-mycarosylerythronolide B

to open-column chromatography, gave **4** and a large amount of erythromycin D as pure compounds, and a lot of mixed erythromycins A and B. The latter was easily purified by crystallization in ethyl acetate.

From the same mother liquors we also have isolated 3-*O*-mycarosylerythronolide B (**5**).

Structure Elucidation of 4

Detailed spectroscopic analyses suggested a new erythromycin for compound **4**, which has thus been named erythromycin G.

The molecular formula of erythromycin G ($C_{37}H_{67}NO_{13}$) was securely set up by low and high resolution EI-MS spectra, the first showing a very weak molecular ion ($M^{+\cdot}$) at *m/z* 733.5 from which derives, by loss of H_2O, the ion at *m/z* 715.45064 ($C_{37}H_{65}NO_{12}$) recorded in the HREI-MS spectrum. This formula was also supported by ^1H and ^{13}C NMR spectra, which exhibited good dispersion of the signals using Py-d_5 as solvent, at 30°C. Especially, the hydroxyl at C-16 was firmly established on the basis of the following spectroscopic features (Table 1): (a) ^1H NMR spectrum shows a clear ABX pattern at 4.21, 4.40 and 3.69 ppm (J_{AB}=10.00, J_{AX}=3.9, J_{BX}=10.00 Hz), (b) the low- (AB part) and the high-field (X part) protons are directly (^1J) coupled with two C-atoms exhibiting absorptions at δ_c=62.45 ppm (triplet) and δ_C=55.53 ppm (doublet) respectively, and (c) all the three protons are long-range (^nJ) correlated with the ester carbonyl at 174.90 ppm (C-1, singlet). Comparison of ^1H and ^{13}C NMR data of erythromycin G with erythromycin B[12] (Table 1) evidences their structural similarities, excluding the area around C-16. In fact, the difference in ^{13}C frequency of corresponding nuclei of **4** and **2** ($\Delta\delta=\delta_C(\mathbf{4})-\delta_C(\mathbf{2})$) is smaller than 0.5 ppm, except C(1), C(2), C(3) and C(16) atoms. For these nuclei, in erythromycin G, the HO-C(16) produces a large and medium deshielding of C_α and C_β atoms ($\Delta\delta$=46.24 and 10.01 ppm for C-16 and C-2 respectively), and a small shielding of C_γ atoms ($\Delta\delta=-1.20$ and -1.61 ppm for C-1 and C-3 respectively). Moreover, the AB pattern above described replaces the doublet at 1.35 ppm of the C-16 methyl group in **2**. However, **4** shows NMR data almost identical to erythromycin F[12] in the C-16 zone (Table 1).

Complete assignment of proton and carbon frequencies (Table 1) was inferred by 1D (^1H, ^{13}C, and DEPT) and 2D (^1H-^1H COSY, ^1J ^1H-^{13}C COSY, and HMBC[13]) experiments. In the flat structure of Fig. 2, bold lines show proton correlations identified by ^1H-^1H COSY spectra: thus, the connectivity for the full C-skeleton of D-desosamine sugar, and partial correlations for both L-cladinose moiety and the macrocyclic system were established. These structural fragments have been fixed together (see arrows) by long-range hetero-correlation (HMBC) experiments

Table 1. NMR data for erythromycins B (**2**), F (**3**), and G (**4**), and for the 3-*O*-mycarosylerythronolide B (**5**).

Position	**2**[a,,b,c]		**3**[a,,b,c]		**4**[a,,b,d]			**5**[d,e]		
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	^nJ, ^1H–^{13}C[f]	δ_C	δ_H	^nJ, ^1H–^{13}C[f]
1	176.10 (s)		175.04 (s)		174.90 (s)		H-2, H-3, H-13, H_a-16, H_b-16	174.76 (s)		H-2, H-16
2	45.52 (d)	3.19[g]	55.51 (d)	3.65[g]	55.53 (d)	3.69 (ddd, 10.0, 9.9, 3.9)	H_a-16	44.19 (d)	2.76[g]	H-16
3	81.16 (d)	4.64 (d, 9.3)	79.48 (d)	4.78 (br.d, 9.6)	79.55 (d)	4.79 (d, 9.9)	H-2, H-5, H-17, H-1'	90.37 (d)	3.64 (d, 10.3)	H-5, H-16, H-17
4	40.10 (d)	2.60[g]	40.25 (d)	2.63[g]	39.69 (d)	2.73 (br.dq, 7.5, 7.3)	H-2, H-3, H-5, H-17	36.14 (d)	2.12[g]	H-17
5	84.55 (d)	4.12 (d, 7.6)	84.22 (d)	4.13 (d, 7.7)	84.33 (d)	4.13 (d, 7.5)	H-3, HO-6, H-17, H-18, H-1"	81.70 (d)	3.51 (br.s, $W_{1/2}$=9.0)	H-3, H-17, H-18
6	74.58 (s)		74.38 (s)		74.31 (s)	6.26 (br.s, OH)	H-5, H-7, H-8, H-18	75.27 (s)		Ha-7, H-18
7	39.13 (t)	1.86[g] and 2.38[g]	39.83 (t)	1.99 (dd, 15.0, 3.6)and 2.35 (dd, 15.0, 8.0)	39.03 (t)	1.90[g] and 2.40 (dd, 14.8, 7.8)	H-5, HO-6, H-8, H-18	36.75 (t)	1.42 (br.d, 14,5) and 1.87 (dd, 14.5, 11.3)	H-18, H-19
8	43.45 (d)	3.30[g]	43.65 (d)	3.26[g]	43.24 (d)	3.33 (dq, 7.7, 6.9)	H-7, H-10	45.46 (d)	2.67[g]	H-19
9	218.18 (s)		219.89 (s)		218.31 (s)		H-7, H-8, H-10	219.39 (s)		Hb-7, H-19
10	41.40 (d)	3.37 (br.q, 7.0)	40.80 (d)	3.40[g]	41.32 (d)	3.08 (br.q, 6.8)	H-11	38.78 (d)	2.94[g]	H-20
11	70.07 (d)	4.53[g]	69.90 (d)	4.64 (s)	70.09 (d)	4.60 (br.d, 9.8)	H-10, H-13, H-21	69.82 (d)	3.79 (br.d, 9.8)	H-12, H-20, H-21
12	41.10 (d)	1.80[g]	75.70 (s)		41.43 (d)	1.84[g]	H-21	39.94 (d)	1.64[g]	H-10, H-21
13	74.79 (d)	5.86 (dd, 9.4, 4.6)	77.97 (d)	5.80 (dd, 10.7, 2.2)	75.07 (d)	5.99 (dd, 9.9, 4.2)	H-11, H-15	75.16 (d)	5.44 (dd, 9.9, 4.4)	H-15
14	26.37 (t)	1.45[g] and 1.74[g]	22.28 (t)	1.5[g] and 1.7[g]	26.66 (t)	1.5[g] and 1.8[g]	H-13, H-15	25.95 (t)	1.46[g] and 1.65[g]	H-15
15	10.71 (q)	0.87 (t, 7.3)	11.28 (q)	1.15 (t, 7.4)	10.86 (q)	1.00 (t, 7.3)	H-13	10.32 (q)	0.84 (t, 7.3)	
16	16.21 (q)	1.35 (d, 7.1)	62.52 (t)	4.23 (br.dd,10.0, 3.8) and 4.27 (br.dd, 10.0, 9.8)	62.45 (t)	4.21 (dd,10.0, 3.9) and 4.40 (dd, 10.0, 10.0)	H-2	15.77 (q)	1.19 (d, 6.7)	
17	10.06 (q)	1.68 (d, 7.4)	10.01 (q)	1.75 (d, 7.5)	10.12 (q)	1.76 (d, 7.3)	H-3, H-4	8.61 (q)	0.99 (d, 7.2)	H-3
18	28.14 (q)	1.91 (s)	27.53 (q)	1.93 (s)	28.26 (q)	1.92 (s)	H-C5, HO-6, H-7	26.12 (q)	1.38 (s)	Ha-7
19	18.41 (q)	1.21 (d, 6.9)	18.64 (q)	1.22 (d, 6.9)	18.31 (q)	1.21 (d, 6.9)	H-7, H-8	18.20 (q)	1.12 (d, 7.0)	
20	9.39 (q)	1.24 (d, 6.7)	12.44 (q)	1.51 (d, 6.8)	9.26 (q)	1.25 (d, 6.8)	H-10, H-11	9.40 (q)	0.96 (d, 7.0)	
21	9.66 (q)	0.99 (d, 7.0)	17.71 (q)	1.46 (s)	9.80 (q)	1.02 (d, 7.0)	H-13	9.14 (q)	0.87 (d, 7.1)	
1'	97.19 (d)	5.06 (br.d, 4.7)	96.79 (d)	4.98 (br.d, 4.7)	96.76 (d)	4.94 (d, 4.0)	H-3, H-2'	100.86 (d)	5.03 (d, 3.3)	
2'	35.81 (t)	1.52[g] and 2.43 (d, 14.8)	35.77 (t)	1.45[g] and 2.34 (d, 14.6)	35.68 (t)	1.42[g] and 2.31 (d, 15.0)		41.01 (t)	1.79 (dd, 14.5,3.7) and 2.10[g]	
3'	73.47 (s)		73.42 (s)		73.33 (s)		H-1', H-2', H-5', H-7', H-8'	69.76 (s)		
4'	78.77 (d)	3.24[g]	78.73 (d)	3.22[g]	78.57 (d)	3.21 (d, 9.4)	H-2', H-6'	76.22 (d)	2.96[g]	
5'	66.21 (d)	4.55[g]	66.31 (d)	4.55 (dq, 9.4, 6.1)	66.19 (d)	4.54 (dq, 9.4, 6.1)	H-4', H-6'	67.03 (d)	3.90 (dq, 9.8, 6.0)	
6'	19.50 (q)	1.56 (d, 6.1)	19.50 (q)	1.55 (d, 6.1)	19.51 (q)	1.55 (d, 6.1)	H-4'	17.67 (q)	1.31 (d, 6.2)	
7'	21.54 (q)	1.31 (s)	21.50 (q)	1.27 (s)	21.40 (q)	1.26 (s)		25.43 (q)	1.23 (s)	
8'	49.66 (q)	3.46 (s)	49.64 (q)	3.42 (s)	49.57 (q)	3.40 (s)				
1"	103.81 (d)	4.91 (br.d, 7.3)	103.81 (d)	4.92 (d, 7.2)	103.61 (d)	4.92 (br.d, 7.2)	H-5, H-2"			
2"	71.94 (d)	3.60 (dd, 10.1, 7.4)	71.93 (d)	3.62[g]	71.81 (d)	3.61 (dd, 10.1, 7.2)	H-3"			
3"	65.68 (d)	2.63[g]	65.71 (d)	2.62[g]	65.45 (d)	2.61 (ddd, 12.5, 10.1, 3.9)	H-1", H-2", H-7"/8"			
4"	30.47 (t)	1.40[g] and 1.18[g]	30.55 (t)	1.4[g] and 1.2[g]	30.34 (t)	1.4[d] and 1.17 (dd, 12.5, 12.5)	H-3"			
5"	68.38 (d)	3.89 (m)	68.38 (d)	3.91 (m)	68.13 (d)	3.91 (m)				
6"	21.87 (q)	1.30 (d, 6.1)	21.89 (q)	1.30 (d, 6.0)	21.88 (q)	1.29 (d, 6.0)				
7", 8"	40.51 (q)	2.19 (s)	40.55 (q)	2.20 (s)	40.43 (q)	2.18 (s)	H-3"			

[a] In Py-d_5, at 30°C.

[b] The resonance range of the OH protons (br. singlets) is from 4.5 to 6.4 ppm.

[c] Assignments based on ^1H–^1H COSY, ^1J ^1H–^{13}C COSY experiments and literature data.

[d] Assignments based on DEPT, ^1H–^1H COSY, ^1J ^1H–^{13}C COSY, and HMBC experiments.

[e] In $CDCl_3$ at 31°C.

[f] Correlation derived from HMBC experiment: protons correlate with C atom marked by the number in the first column.

[g] Superimposed in the same column.

Fig. 2. Flat structure of erythromycin G (**4**) showing, in bold, partial fragments inferred from ^{1}H-^{1}H COSY spectrum, and their connection (arrows) stated by key correlations from HMBC experiment.

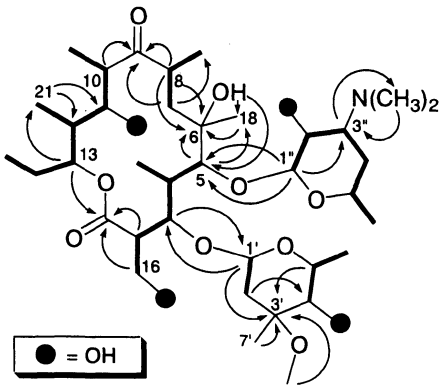
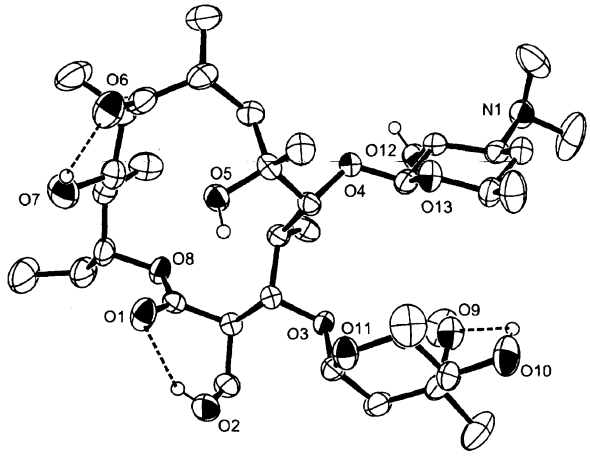


Fig. 3. ORTEP picture of erythromycin G (**4**) obtained from X-ray analysis and elaborated by ORTEP-3 for Windows program[14].



For clarity, crystallization's solvents and C-bonded hydrogen atoms have been removed, and only the hetero-nuclei are labelled.

(Fig. 2 and Table 1). In particular, C-2/C-13, C-5/C-7, and C-8/C-10 were connected through the ester group, the quaternary C-6 atom, and the carbonyl at C-9, respectively, establishing the erythronolide moiety. In addition, D-desosamine and L-cladinose structures are clearly arranged by hetero correlations involving C-3″ and C-3′ atoms. At the end, the gross structure **4** was nearly completed by H-3-C-1′, H-1′-C-3, H-5-C-1″, and H-1″-C-5 correlations, which suggest the connecting-points of the two sugar moieties to the erythronolide backbone.

Moreover, the molecular formula and chemical shift for C-6, C-11, C-16, C-2″, and C-4′ nuclei suggest that each of these C-atoms has to bear a hydroxyl group. In ^{1}H NMR spectra, recorded in deuterated pyridine, only the OH at C-6 is clearly discerned as a broad singlet at 6.26 ppm: the other hydroxyl protons resonate as very broad signals centred at 5.4 and 6.4 ppm.

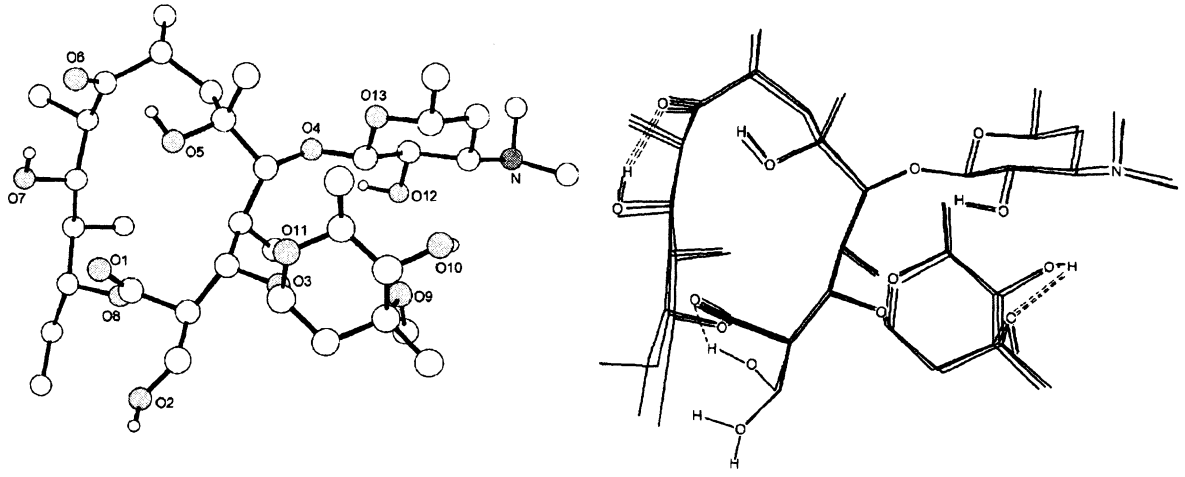
The above spectroscopic features support both the flat and the three-dimensional structure of erythromycin G. The close correlation of ^{13}C NMR data of **4** and **2** or **3** suggests the absolute stereochemistry of **4** as reported in Fig. 1. The stereochemistry is further based on X-ray crystallographic analysis of a monocrystal sample: the ORTEP picture is reported in Fig. 3, as well as the main crystal data and the refinement method are described in Material and Methods.

X-Ray crystallographic analysis of **4** pointed out also the presence of intra- and inter-molecular hydrogen bonds. On the one hand, the first originate by O2-H-O1, O7-H-O6 and O10-H-O9 electronic interactions (Fig. 3) with angles equal to 138.8°, 141.8° and 112.6°, respectively. On the other hand, intermolecular interactions derive from O5-H-O2 and O12-H-N1 hydrogen bonds (with angles equal to 161.1° and 175.9°, respectively), involve six molecules, and shape a macrocyclic system holding a solvent aggregate in the centre.

Molecular-mechanics Calculation

Theoretical analysis, made by molecular-mechanics (MM) calculations[15], identifies three low-strained conformations (Fig. 4) with energy equal to 67.2, 67.8, and 68.7 kcal mol^{-1}. On the left side of Fig. 4, the least-energy conformer is reported with ball and stick shape; furthermore, on the right side, the three conformers are compared to put in evidence the intramolecular hydrogen bonds between O7-H-O6, O10-H-O9, and O2-H-O1 (this for one structure only) with angles agreeing nicely with X-ray data. The comparison evidences their similarity, except the spatial orientation of the hydroxymethyl and ethyl groups. In fact, the high-, medium-, and low-energy conformers differ each other for having the ethyl group pointing outside, or an O2-H-O1 hydrogen bond, or the ethyl and hydroxymethyl chains in nearly parallel arrangement, respectively. The low-energy shape of **4** is just like the low-energy, "folded out" conformer of **1**,

Fig. 4. Low energy (left), and comparison of the three least strained conformers (right) of **4**.



Atoms are labelled accordingly to ORTEP sketches in Fig. 3.

previously predicted by a combination of NMR spectroscopy and molecular dynamics[16].

The calculated conformations also show a spatial atomic distribution very similar to the X-ray results: in Table 2 are reported the mean values of selected dihedral angles in comparison with the corresponding ones derived from the X-ray diffraction analysis. Significant (≥5 degrees) but not substantial differences were deduced for C1-C2-C3-C4, C7-C8-C9-C10, C9-C10-C11-C12, C4-C5-O4-C30, and C6-C5-O4-C30 dihedral angles: these involve the ketone group and the spatial orientation of the D-desosamine sugar. The differences are attributable at probable inadequate minimization parameters for the C=O function and at the disregarded inter-molecular hydrogen bonds in the MM2 computations.

Interesting structural features of erythromycin G, derived from both X-ray measures and MM2-computations, are the following: (a) all the polar functionalities (OH and C=O) of the erythronolide ring have upward orientation, (b) the polar groups of D-desosamine sugar (OH and NMe_2) are pointing downward, and (c) the mean carbon plane of the macrocyclic lactone is almost parallel or perpendicular to the ones of L-cladinose or D-desosamine moieties, respectively.

Table 2. Selected dihedral angles derived from MM2-PCMODEL simulation and X-Ray analysis of **4**.

Dihedral angle[a]	MM[b]	X-ray[c]
O8-C1-C2-C3	112.4±3.2	109.2
C1-C2-C3-C4	-62.5±4.0	-67.7
C2-C3-C4-C5	164.6±1.2	167.9
C3-C4-C5-C6	-99.8±0.3	-102
C4-C5-C6-C7	-78.7±1.2	-74.5
C5-C6-C7-C8	167.1±0.5	170.8
C6-C7-C8-C9	-73.3±1.2	-77.7
C7-C8-C9-C10	-67.8±0.9	-59.7
C8-C9-C10-C11	123.2±1.0	118.6
C9-C10-C11-C12	-163.1±1.1	-170.1
C10-C11-C12-C13	172.4±0.1	171.5
C11-C12-C13-O8	-70.4±1.7	-76.2
C12-C13-O8-C1	102.3±1.4	114.8
C13-O8-C1-C2	172.5±2.2	174.1
C2-C3-O3-C22	-89.5±0.8	-90.3
C4-C3-O3-C22	148.0±1.1	147.6
C3-O3-C22-O11	-77.2±1.1	-77.7
C4-C5-O4-C30	-105.0±0.1	-99.5
C6-C5-O4-C30	129.0±0.1	136.5
C5-O4-C30-O13	-77.2±0.6	-76.4

[a] Atoms are labelled accordingly to Figs. 3 and 4.
[b] Mean values (degrees) and standard deviations for the three low energy conformers derived from MM2-PCMODEL computation.
[c] Degrees derived from ORTEP figure[14].

Biological Properties

The antimicrobial activity of erythromycin G and of the analogues **1**, **2**, and **3** was determined against a variety of

Table 3. MIC of erythromycins A (**1**), B (**2**), F (**3**) and G (**4**) against Gram-positive and Gram-negative organisms.

Strain, reference number	MIC (μg/ml) of:			
	1	**2**	**3**	**4**
Escherichia coli, 25922[a]	64	128	> 128	> 256
E. coli, 35218[a]	32	64	> 128	256
E. coli, 10536[a]	32	64	128	128
E. coli, Δ 643[b]	128	256	> 128	> 256
Enterobacter cloacae, Δ 1090[b]	256	> 256	128	256
E. cloacae, Δ 1089[b]	256	> 256	128	256
E. cloacae, Δ 1680[b]	> 256	> 256	> 128	> 256
Enterococcus faecalis, 29212[a]	1	1	4	4
E. faecalis, 51299[a]	> 256	> 256	> 128	> 256
E. faecium, Δ 459[b]	> 256	> 256	> 128	> 256
Klebsiella pneumoniae, 10031[a]	4	4	4	8
K. edwardsii, 10896[a]	128	> 256	> 128	> 256
K. pneumoniae, 11228[a]	64	> 256	> 128	> 256
Moraxella catarrhalis, B 22[b]	< 0.125	< 0.125	1	2
M. catarrhalis, B 23[b]	< 0.125	< 0.125	1	2
Staphylococcus aureus, 10390[a]	0.25	0.5	8	8
S. aureus, 29213[a]	0.25	0.5	8	8
S. aureus, 29506[a]	0.25	0.25	8	8
S. aureus, 49951[a]	0.25	0.5	8	8
S. aureus, 9144[a]	< 0.125	0.25	4	4
Streptococcus pyogenes, 29218[a]	< 0.125	< 0.125	0.125	0.25
S. pneumoniae, 49619[a]	< 0.125	< 0.125	0.125	0.25
Pseudomonas aeruginosa, 25668[a]	> 256	> 256	> 128	> 256
P. aeruginosa, 27853[a]	> 256	> 256	> 128	> 256
MRSA[c,d], 33591[a]	> 256	> 256	> 128	> 256
MRSA[c,d], 43300[a]	> 256	> 256	> 128	> 256

[a] ATCC number.
[b] Selected from Biochemie's culture collection.
[b] MRSA=Methicillin-resistant *Staphylococcus aureus*.
[c] NaCl was not supplemented.

bacteria using a twofold dilution procedure. The tests were performed in Mueller-Hinton Agar according to NCCLS standard methods[18]. The minimum inhibitory concentrations (MICs) of **1**~**4** *vs.* both Gram-positive and Gram-negative bacteria are reported in Table 3.

The antibacterial tests show that the potency of erythromycin F is similar to erythromycin G; the latter is 2÷8 fold less active than erythromycins A and B. Moreover, all four the macrolides are practically inactive towards *Enterobacter*, *Klebsiella*, *Pseudomonas* and methycill-resistant *Staphylococcus* genera.

3-*O*-Mycarosyl-erythronolide B

Full NMR spectra of **5** have been acquired (Table 1), which now complete the poor spectroscopic data of this compound and correct wrong assignments of some methyl frequencies reported in CS_2 solvent[17]. To the best of our knowledge, this is the first isolation of 3-*O*-mycarosyl-erythronolide B from non-blocked microorganisms. In fact, it has been reported that chromosomal mutants carrying a deletion in regions containing genes for deoxysugars attachment to erythronolide B accumulate **5**[19,20]. Also the

8-epi isomer of 3-*O*-mycarosyl-erythronolide B has been obtained by incubation of 8-epi-erythronolide B with a culture of a blocked mutant of *S. erythraea*[21].

Conclusion

The isolation and complete structural characterization of the new erythromycin G has been reported. MM Calculations and ^{1}H-NMR data have demonstrated that **4** adopts, in solution, a conformation nearly equal to the one determined by X-ray diffraction in the crystalline state, and similar to the "folded out" one for eryhromycin A (previously described)[16].

The C-16 methyl of erythronolides is oxidized to hydroxymethyl group in erythromycin G; this is a structural feature previously reported only for erythromycins E[7] and F[8]. Formerly, the sequence of biogenetic events erythromycin A→erythromycin F→erythromycin E was suggested[8]. Analogously, now we propose a biosynthetic cascade with erythromycin B precursor of erythromycin G, from which 12-deoxy-erythromycin E (the equivalent orthoester of erythromycin E) should derive. We believe the latter should be another minor analogue obtainable from *S. erythraea* culture. Currently, in order to demonstrate this, we are involved in further deep studies of the mother liquors of *S. erythraea.*

The present study report also the antibacterial activity of macrolides **1**~**4** and provides a simple method for the isolation of erythromycins B and D in large amount.

Finally, this is the first time that **5** has been isolated from non-blocked microorganisms.

Material and Methods

General Experimental Procedures

NMR: ^{1}H (299.94 MHz), ^{13}C (75.43 MHz), and 2D-NMR spectra were acquired at 30°C, in deuterated pyridine (Py-d_5) using a Varian XL-300 spectrometer; chemical shifts are referenced to the residual solvent signals (C_5D_5N: δ_H= 7.21, 7.57, and 8.72 ppm; δ_C=123.5, 135.5, and 149.5 ppp); ^{1}H and ^{13}C signal-assignments were obtained by DEPT, homonuclear ^{1}H-^{1}H COSY, and one bond (^1J, ^{1}H-^{13}C COSY) and long range (HMBC) heteronuclear correlation experiments. Low- (EI-MS) and high-resolution (HREI-MS) mass spectra were performed at 70 eV by a Kratos MS80 equipment with home-built acquisition system. IR spectra were recorded by a Paragon 500 FT-IR spectrometer (Perkin-Elmer). Optical rotations were carried out by Perkin-Elmer 243 polarimeter using Na line (589 nm) as monochromatic light. Melting points were determined with a Büchi B540 apparatus.

Analytical HPLC was performed by an assembled isocratic HPLC system bringing together a Gilson 306 HPLC pump working at 1 ml/minute, a Gilson 234 autosampler equipped with a 50 μl loop, a column heater CROCO-CIL (CIL Cluzeau Info-Lab) set at 50°C, a reversed-phase column (Asahipak ODP-50 column, 250× 4.0 mm, particle size 5.0 μm, Agilent Technologies), a Gilson 119 variable wavelength UV detector set at 210 nm, and a HP 3396 series integrator; a mixture of CH_3CN-H_2O-20 mM Na_2HPO_4 at pH 10.3, (25 : 40 : 35 in volume), degassed by sonication, was used as mobile phase; the Na_2HPO_4 buffer was adjusted to the required pH adding 1.0 N sodium hydroxide; retention times (Rt) are expressed relative to elution time of **1**.

Semi-preparative HPLC was performed by a Merck-Hitachi apparatus (L-7100 pump, L-7400 UV detector, D-7500 integrator, and Rheodyne manual injector equipped with a 200 μl loop) using a reversed-phase column (Hewlett Packard, Nucleosil® 100-5 C18, 250×10 mm i.d.). The column was flushed (3.0 ml/minute flow-rate) with a mixture of CH_3CN-10 mM Na_2HPO_4 at pH 10.3 (4 : 6 in volume) and the elute was monitored by UV detection at 212 nm.

Open-column chromatography was done in a glass column (7×60 cm) packed with 2 kg of silica gel 60 F_{254}, 0.063~0.200 mm (MERCK); a mixture of ethyl acetate-hexane-triethylamine (75 : 20 : 5 in volume) was used as mobile phase.

Isolation

A concentrate of mother liquors was obtained from a high yielding strain of *S. erythraea* (Biochemie Ery 1898), after crystallisation of **1** from CH_2Cl_2. Analytical HPLC of this mixture revealed the presence of **1** (Rt=1.00, 17%), **2** (Rt=1.80, 25%), erythromycin C (Rt=0.48, 15%), erythromycin D (Rt=0.71, 0.6%), erythromycin E (Rt=0.86, 18%), **4** (Rt=0.56, 1%) and **5** (Rt=0.40, 0.2%). This mixture (220 g) was dissolved in *tert*-butyl methyl ether and kept at −20°C. After 24 hours, compounds **1** (4%), **2** (77%), erythromycin D (1%), and **4** (4%) selectively precipitated as a colourless solid (59 g), which was subjected to open-column chromatography (2.5 ml/cm^2 per minute flow-rate, 500 ml fractions, UV detection). A mixture of **1** and **2**, and non-mixed erythromycin D and compound **4** were collected at 0.8÷2.0, 2.8÷3.5 and 5.0÷ 7.5 column volumes, respectively. Finally, pure **2** (31.5 g), erythromycin D (0.4 g) and **4** (1.5 g) were obtained by

crystallization at −20°C from ethyl acetate, *tert*-butyl methyl ether and CH_3CN, respectively.

The concentrate of mother liquors (310 mg), after selective crystallisation of **1**, **2**, erythromycin D and **4** from *tert*-butyl methyl ether, subjected to semi-preparative HPLC, gave pure compound **5** at Rt=17.6 (12.6 mg).

Erythromycin G (**4**)

[3*R*-(3*R**,4*S**,5*S**,6*R**,7*R**,9*R**,11*R**,12*R**,13*S**,14*R**)]-4-[(2,6-Dideoxy-3-C-methyl-3-*O*-methyl-α-L-ribo-hexopyranosyl)oxy]-14-ethyl-7,12-dihydroxy-3-hydroxymethyl-5,7,9,11,13-pentamethyl-6-[[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy]oxacyclotetradecane-2,10-dione.

Colourless solid (220~222°C from CH_3CN); $[\alpha]_D^{25}$= −80.5° (MeOH, *c*=0.93); $IR_{max}^{(KBr)}$ cm^{-1}: significant signals at 3449, 2973, 2830, 1698, 1459, 1380, 1169 and 1010; ^1H and ^{13}C NMR (Py-d_5) data are reported in Table 1; EI-MS, *m/z* (rel int): 733.5 $[M]^+$ (0.02), 715.5 $[M-H_2O]^+$ (0.6), 557.4 (0.6), 424.3 (1.7), 423.3 (1.8), 312.2 (1.7), 223.2 (2.0), 174.1 (14.2), 158.1 (80.3), 43.0 (100); HREI-MS *m/z* calcd for $C_{37}H_{65}NO_{12}$ 715.45068 found 715.45064 $[M-H_2O]^+$.

Crystal Data of **4**

X-Ray analyses were performed on a suitable monocrystal (colourless, 0.52×0.11×0.08 mm) selected among the precipitates obtained from a $CHCl_3$-CH_2Cl_2 (1 : 1 in volume) solution kept at 20°C for two weeks. Diffraction data were collected by a Kappa CCD diffractometer with a highly oriented graphite crystal monochromator (MoK_α radiation). The structure was solved by direct methods, and the hydrogen atoms were calculated with isotropic displacement parameters 1.2 and 1.5 times higher than U_{eq} of the C and refined at O2, O5, O7, O10 and O12 atoms. The unit cell shows a monoclinic crystal system with four molecules of erythromycin G co-crystallized besides $CHCl_3$, CH_2Cl_2, and H_2O (molecular formula $C_{37}H_{67}NO_{13}\times0.25\ CHCl_3\times0.25\ CH_2Cl_2\times0.50\ H_2O$). Refinement data show 1 : 1 occupancy disorder of $CHCl_3$ and ($CH_2Cl_2\times2H_2O$), the $CHCl_3$ has also a symmetry induced 1 : 1 orientation disorder, O14 and C14 are nearby and must be refined with restrained distances to C38 atom. Further crystal data and structure refinement are the following: crystal with C_2 (No. 5) space group; a=3483.41(6) pm, b=901.5(1) pm, c=1460.1(2) pm, α=90.00°, β=110.256(4)°, γ=90.00° for geometry of the unit cell; 7722 reflections collected; refinement method by full-matrix least-squares F^2; 1.021 goodness-of-fit on F^2; R_1=0.0350, wR_2=0.0826 for final R indices [$I>2\sigma(I)$]; R_1=0.0412, wR_2=0.0866 for R indices (all data).

Molecular Mechanics Calculations of **4**

Theoretical analysis was carried out by MM2 force field implemented in PCMODEL for Windows program. The low-strained conformations were searched refining many conformers derived by rotation of selected dihedral drivers involving both the cycles and the chains. Only three high-probable conformers with 67.2, 67.8, and 68.7 kcal mol^{-1} were detected in the lower global energy range of 2.5 kcal mol^{-1}.

3-*O*-Mycarosylerythronolide B (**5**)

[3*R*-(3*R**,4*S**,5*S**,6*R**,7*R**,9*R**,11*R**,12*R**,13*S**,14*R**)]-4-[(2,6-Dideoxy-3-C-methyl-α-L-ribo-hexopyranosyl)oxy]-14-ethyl-6,7,12-trihydroxy-3,5,7,9,11,13-hexamethyl-oxacyclotetradecane-2,10-dione.

^1H and ^{13}C NMR ($CDCl_3$) data are reported in Table 1; EI-MS, *m/z* (rel int): 528.6 $[M-H_2O]^+$ (3), 223 (21), 155 (9), 153 (6), 145 (25), 127 (34), 125 (27), 101 (36), 43.0 (100); HREI-MS *m/z* calcd for $C_{28}H_{48}O_9$ 528.32983 found 528.32976 $[M-H_2O]^+$.

Biological Properties

Minimal inhibitory concentration (MIC) determination was done following NCCLS standard method M7-A4. Erythromycins **1**~**4** were dissolved in a mixture of DMSO-H_2O (1 : 4 in volume, 5 ml) and further diluted in water with a twofold progression. Each diluted solution (2 ml) was added to 18 ml of Mueller-Hinton Agar, warmed at 50°C, and then poured in Petri dish. The higher concentration tested was 128 μg/ml for erythromycin F and 256 μg/ml for **1**, **2**, and **4**. As requested, NaCl salt was not supplemented to MRSA strains. Each bacterium strain was inoculated in duplicate by a multipoint inoculator Dynatec MIC 2000 with 10000 colony-forming units (CFU) per spot. The inoculated plates were incubated at 36°C for 29 hours and then evaluated for visible growth. The results are reported in Table 3.

Acknowledgements

We like to thank Dr. KLAUS WURST for X-ray analysis, Dr. JOHANNES HILDEBRANT for antibacterial tests, Dr. INGOLF MACHER for early NMR experiments in $CDCl_3$, and Dr. ENRICO CASARETO for much helpful advice. The authors also thank Dr. THOMAS GEROLA and Dr. GERHARD FLEISSNER for having performed FT-IR spectra and early LC-MS experiments, respectively. The author A. G. is grateful to MURST (COFIN 2000) and PAT (AGRIBIO project) for financial support.

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