IDENTIFICATION OF NOVEL ERYTHROMYCIN DERIVATIVES IN MOTHER LIQUOR CONCENTRATES OF *STREPTOMYCES ERYTHRAEUS*

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The identification of five novel compounds, pseudo-erythromycin A-6,9-hemiketal, 8,9-anhydro-pseudo-erythromycin A-6,9-hemiketal, 8,9-anhydro-pseudo-*N*-demethylerythromycin A-6,9-hemiketal, 5-*O*-β-D-desosaminylerythronolide A and 15-nor-erythromycin C, in mother liquor concentrates of *Streptomyces erythraeus* is described. The pseudo-erythromycin derivatives are characterized by a 12-membered macrocyclic ring as a result of $C_{13}\rightarrow C_{11}$ trans-lactonization. The five compounds have very little antimicrobial activity.

In a study[1] dealing with the isolation and the identification of compounds present in the mother liquor concentrates from commercial crystallization of erythromycin, a number of compounds were obtained. Some of these compounds were identified as erythromycins A, B, C and D, erythromycin A enol ether, anhydroerythromycin A, anhydroerythromycin C and anhydro-*N*-demethylerythromycin A. Five other compounds denoted M1 to M5 were not identified. The determination of the structure of the latter group of compounds is the subject of this paper.

Results and Discussion

Three of the compounds were found to be derivatives of ring-contracted or so-called pseudo-erythromycin, in which the macrolide ring was contracted by a $C_{13}\rightarrow C_{11}$ trans-lactonization. Two of these compounds, *i.e.*, M3, pseudo-erythromycin A-6,9-hemiketal (**3**) and M2, 8,9-anhydro-pseudo-erythromycin A-6,9-hemiketal (pseudo-erythromycin A enol ether) (**4**), are directly derived from erythromycin A (**1**). Details of their synthesis from **1** and of their structural assignment are described elsewhere[2].

The third pseudo-erythromycin derivative, M5, was found to be 8,9-anhydro-pseudo-*N*-demethyl-erythromycin A-6,9-hemiketal (pseudo-*N*-demethylerythromycin A enol ether) (**5**). The structure was established by comparison of its ^{13}C NMR chemical shifts with those of **4** (Table 1), and by chemical transformation. The chemical shifts of the aglycon and cladinosyl carbons of **4** and **5** are essentially the same, and the upfield shift of the β-carbons (C-3′ and NCH_3) and the downfield shift of the γ-carbons (C-2′ and C-4′) are fully in accordance with the absence of one of the *N*-methyl groups in the desosamine moiety of **5**. This is also confirmed by the ^1H NMR spectrum where the singlet at δ 2.38

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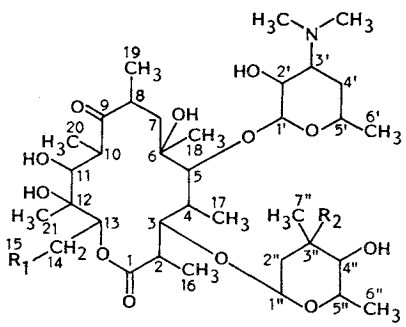

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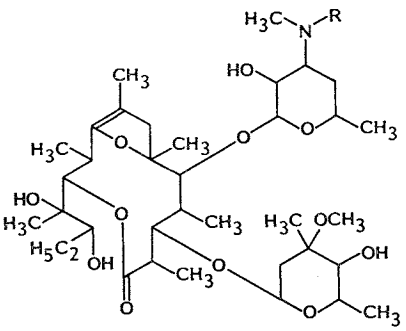
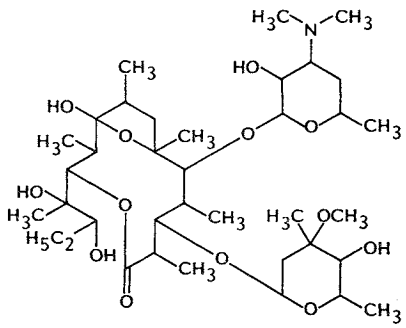
2 $R_1 = CH_3$ $R_2 = OH$
7 $R_1 = H$ $R_2 = OH$



4 R = CH_3
5 R = H



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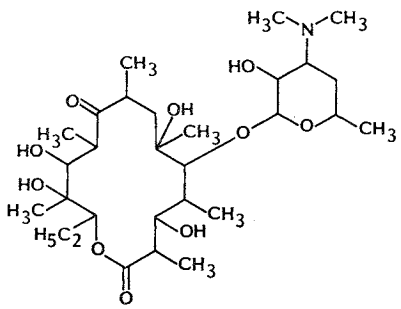

6

integrates for only 3H instead of 6H. Compound **5** has a molecular ion at *m/z* 701 and a base peak at *m/z* 144 indicating that it is an *N*-demethylated derivative of **4**. Compound **4** has a molecular ion *m/z* 715 and a base peak at *m/z* 158 (desosaminyl). Compound **5** is readily prepared from *N*-demethylerythromycin A by heating a solution of the latter in a 3 : 1 mixture of pyridine - acetic acid at 50°C for 14 days or at 70°C for 1 day. A similar procedure was used for the preparation of **4** from **1**[2]. *N*-Methylation of **5** with formaldehyde and cyanoborohydride by the procedure of BORCH and HASSID[3] gives **4**.

The structure of the two other compounds M1 and M4 was established as 5-*O*-desosaminylerythronolide A (**6**) and 15-nor-erythromycin C (**7**) respectively.

5-*O*-Desosaminylerythronolide A (**6**) gives a mass spectrum with a molecular ion at *m/z* 575 suggesting the decladinosyl analog of **1**. The presence of erythronolide A (aglycone) is inferred from the fragment ion at *m/z* 383[4] and a weak UV absorption at 285 nm. The presence of desosamine is shown by a fragment ion at *m/z* 158, and by the characteristic $N(CH_3)_2$ signal at δ 2.28 in its ^1H NMR spectrum. Absence of the neutral sugar is indicated by the lack of typical fragment ions at *m/z* 127 and 115[1], and also by the absence of the corresponding absorptions in the ^1H NMR spectrum (*e.g.* no OCH_3-signal at about δ 3.30) and in the ^{13}C NMR spectrum (Table 1). This spectrum also shows a δ value of 93.2 ppm for C-5 compared to 83.5 ppm for **1** which may be explained by hydrogen bonding of the oxygen at C-5 with the free hydroxyl group on C-3. When **6** (Rf 0.39) was dissolved in 0.75 N

Table 1. ^{13}C NMR chemical shifts of erythromycin C (**2**) and of compounds **4** to **7***.

Carbon No.	2	4	5	6	7
1	175.4	175.7	175.8	177.8	175.0
2	44.8	46.8	46.8	42.3	44.9
3	85.2	80.6	80.3	78.5	84.4
4	39.3	38.8	38.6	40.8	40.4
5	83.2	81.6	81.6	93.2	82.5
6	75.0	86.0	86.0	74.7	75.5
7	38.6	43.4	43.3	40.5	38.7
8	44.9	101.2	101.2	44.7	44.9
9	221.3	149.5	149.6	216.2	221.5
10	37.9	31.7	31.6	38.4	38.4
11	69.3	77.5	77.4	69.9	69.5
12	74.7	76.6	76.5	74.7	74.4
13	77.3	76.8	76.5	77.6	72.0
14	21.0	22.5	22.5	21.1	13.7 (q)
15	10.5	11.7	11.8	10.2	A
16	15.9	15.0	15.0	15.7	15.5
17	9.1	9.3	9.8	7.5	9.3
18	26.7	26.7	26.6	27.1	27.1
19	18.3	10.8	10.9	16.6	18.1
20	11.8	11.1	11.1	10.7	11.5
21	16.1	16.7	16.6	16.0	14.8
1′	104.8	104.0	103.3	106.1	104.6
2′	70.9	71.1	74.7	70.4	70.7
3′	65.4	65.4	60.1	65.4	65.5
4′	28.5	29.0	37.2	28.0	28.6
5′	68.7	68.9	68.6	69.9	68.9
5′-CH_3	21.1	21.1	20.9	20.9	21.2
$N(CH_3)_2$	40.0	40.1	33.0 ($NHCH_3$)	40.1	40.1
1″	98.6	97.5	97.4	A	98.9
2″	40.5	35.3	35.1	A	40.4
3″	69.3	72.4	72.4	A	69.5
4″	76.4	78.1	77.9	A	76.4
5″	65.9	65.4	65.3	A	66.3
3″-CH_3 (7″)	25.5	21.4	21.3	A	25.5
5″-CH_3 (6″)	18.3	18.3	18.3	A	18.3
3″-OCH_3	A	49.2	49.2	A	A

A: Absent.

q: Quadruplet instead of normal triplet in off-resonance.

* ^{13}C NMR spectra were measured at 22.53 MHz in $CDCl_3$ with a Jeol FX 90 Q spectrometer. Unequivocal assignments for carbons of **4** were based on heteronuclear shift correlated two-dimensional NMR as described in ref 2. All ^{13}C NMR spectra were recorded at ambient temperature. Chemical shifts are expressed as δ values in ppm, measured relative to $CDCl_3$ which was set at 76.9 ppm relative to tetramethylsilane.

hydrochloric acid and kept at room temperature for 20 hours, it was transformed into a compound with Rf 0.33. The compound was isolated by preparative TLC and shown, by UV, IR and MS, to be identical with authentic erythralosamine obtained by acid degradation of **1**[5]. As final proof, a sample of 5-*O*-desosaminylerythronolide A was prepared from **1** according to the procedure of LEMAHIEU *et al.*[6]. The IR, UV and MS spectra are identical with those obtained for **6**.

15-Nor-erythromycin C (**7**) gives a mass spectrum with a molecular ion at *m/z* 705 and fragment

Table 2. *In vitro* antibacterial activity of compounds **1** to **7**.

Organism	MIC (μg/ml)						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Bacillus subtilis ATCC 6633	0.03	0.06	16	64	64	64	2
Micrococcus luteus ATCC 9341	0.03	0.03	8	64	64	64	0.5
Staphylococcus aureus ATCC 25923	0.06	0.25	32	64	64	64	2
S. epidermidis ATCC 12228	0.06	0.25	32	64	64	64	1
Streptococcus faecalis ATCC 19433	0.5	0.5	64	64	64	64	4
S. pyogenes ATCC 19615	0.03	0.06	32	0.25	64	4	1
Enterobacter cloacae ATCC 23355	64	64	64	64	64	64	64
Escherichia coli ATCC 25922	32	64	64	64	64	64	64
Klebsiella pneumoniae ATCC 13883	16	64	16	64	64	64	64
Pseudomonas aeruginosa ATCC 27853	32	64	64	64	64	64	64
Salmonella typhimurium ATCC 14028	64	64	64	64	64	64	64
Shigella sonnei ATCC 25931	64	64	64	64	64	64	64

MIC: Minimum inhibitory concentration.

ions at *m/z* 158 and 145. The latter two fragment ions, together with the 6H-singlet at δ 2.29 and the lack of the OCH_3-signal at about 3.30 ppm in the ^1H NMR spectrum, point to desosamine and mycarose respectively, suggesting that the compound corresponds to erythromycin C (**2**) lacking a methyl group (14 amu) in the aglycon. This is confirmed by the presence of a fragment ion at *m/z* 369, being the analog of the ion at *m/z* 383 in **1** and **2**. The presence of a fragment ion at *m/z* 634, which is also present in the mass spectrum of **2** and which arises from elimination of the C-12~C-15 fragment, indicates the absence of the methyl group in the lost fragment. The structure was further established by comparing the ^{13}C NMR chemical shifts of **2** and **7** (Table 1). The chemical shifts are almost identical except for C-13 of **7** which shows an upfield shift of 5.3 ppm indicating the loss of a methyl substituent in a β-position. It was further observed by off-resonance spin decoupling experiments that the triplet of C-14 in **2** is absent in the spectrum of **7** and is replaced by a quadruplet signal (CH_3) at 13.7 ppm ($\Delta = -7.3$ ppm, α-effect), thus confirming the absence of the C-15 methyl group.

The antibacterial activity of compounds **1** to **7** was determined against various bacteria by the plate dilution method in a Mueller-Hinton medium containing 5% of lysed blood (pH 7.4) (Table 2). The compounds **3** to **7** are much less active than either **1** or even **2**.

The presence of pseudo-erythromycin A-6,9-hemiketal (**3**), pseudo-erythromycin A enol ether (**4**) and pseudo-*N*-demethylerythromycin A enol ether (**5**) in the mother liquor concentrates is probably due to transformation of erythromycin A (for **3** and **4**) or of *N*-demethylerythromycin A (for **5**). Their preparation from **1** or from *N*-demethylerythromycin A supports this view. The presence of small amounts of *N*-demethylerythromycin A in commercial samples[7] and of anhydro-*N*-demethylerythromycin A in mother liquor concentrates[1] has been demonstrated. The identification of **5** therefore is further evidence for the occurrence of *N*-demethylerythromycin A in fermentation broths of *Streptomyces erythraeus*.

The presence of 5-*O*-desosaminylerythronolide A (**6**) does not fit in the biosynthetic scheme proposed for erythromycins[4,8]. Thus there are two possibilities, first that the compound is a metabolite of **1** produced by *S. erythraeus* and second that the biosynthesis of erythromycins may proceed through a pathway involving this compound. The latter possibility is not excluded since it has been demonstrated, contrary to prior held beliefs, that erythronolide A is glycosylated to erythromycin A[9].

The presence of 15-nor-erythromycin C can be explained as follows: Erythromycin aglycon is

formed by condensation of seven propionate units[10]. One propionate is used as a starter unit, at C-13, C-14 and C-15, to which are condensed six propionates as chain extending units[11]. The biosynthesis of 15-nor-erythromycin C (**7**) may be easily rationalized within the proposed biosynthetic routes[4,8] if an acetate instead of propionate is incorporated as the starter unit. This scheme corresponds to the biosynthesis of oleandomycin. It should be mentioned that 8,8a-deoxyoleandolide has been isolated from a mutant of *Streptomyces erythraeus*[12].

Experimental

General

TLC was performed on precoated Silica gel 60 F_{254} plates (E. Merck) with CH_2Cl_2 - MeOH - 25% NH_4OH (90 : 9 : 1.5). Spots were visualized by spraying with 4-methoxybenzaldehyde - H_2SO_4 - EtOH (1 : 1 : 9) followed by heating at 110°C for 1 minute. Melting points were determined in open capillary tubes using a Büchi apparatus. Optical rotations were obtained in MeOH with a Thorn-NPL automatic polarimeter Type 243 (Thorn Automation, U.K.). IR spectra were recorded with a Perkin-Elmer model 197 spectrophotometer. UV spectra were obtained in MeOH with a Beckman model 25 spectrophotometer. Mass spectra were obtained on an AEI MS-12 mass spectrometer operated at accelerating voltage 8 kV, trap current 100 μA, ionization energy 70 eV and ion source temperature 150~170°C. Samples were introduced with the direct insertion probe. ^{1}H NMR spectra (90 MHz) were taken in $CDCl_3$-solution with a Jeol FX90Q spectrometer, and ^{13}C NMR spectra were recorded as noted in Table 1.

Isolation of Compounds

The compounds were isolated from mother liquor concentrates of the commercial production of erythromycin, by preparative HPLC on silica gel. The details of the isolation have been reported[1]. The compounds therein designated as M1 to M5 are 5-*O*-desosaminylerythronolide A (**6**) as M1, pseudo-erythromycin A enol ether (**4**) as M2, pseudo-erythromycin A-6,9-hemiketal (**3**) as M3, 15-nor-erythromycin C (**7**) as M4 and pseudo-*N*-demethylerythromycin A enol ether (**5**) as M5.

Pseudo-*N*-demethylerythromycin A Enol Ether (**5**)

The compound was isolated as a white foam: Rf 0.18; IR (KBr) cm^{-1} 3430 (OH), 2970, 1715 (lactone C=O), 1465, 1385, 1270 and 1175; UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 215 (97.5), no ketone absorption (280 nm); MS *m/z* (relative intensity %) 701 (20.0, M, $C_{36}H_{63}NO_{12}$), 643 (0.9, M−CH_3CH_2CHO), 642 (0.7, M−CH_3CH_2CHOH), 542 (5.3, M−cladinosyl), 382 (6.3, M−[cladinosyl and *N*-demethyldesosaminyl]oxy), 159 (6.7, cladinosyl), 144 (100, *N*-demethyldesosaminyl); ^{13}C NMR is given in Table 1; ^{1}H NMR (90 MHz, $CDCl_3$) δ 3.28 (OCH_3), 2.38 (3H, s, $NHCH_3$).

Preparation of **5** from *N*-Demethylerythromycin A

N-Demethylerythromycin A (2.0 g), obtained from erythromycin A by the method of FLYNN *et al.*[13], was dissolved in 30 ml of a 3 : 1 mixture of pyridine - AcOH and heated at 50°C for 14 days. The mixture was cooled, alkalinized with saturated Na_2CO_3 solution and extracted with CH_2Cl_2. The organic layers were dried over anhydrous Na_2SO_4 and evaporated to dryness. Crystallization from CH_3CN afforded 1.1 g (57% yield) of **5**: MP 193~195°C; $[\alpha]_D^{23}$ −38.8° (*c* 1.0, MeOH). Spectral and chromatographic data are identical with those of **5**, isolated from the mother liquor concentrate.

5-*O*-Desosaminylerythronolide A (**6**)

The compound was obtained as white crystals from CH_3CN: Rf 0.39; mp 135~136.5°C, after resolidification 185~187°C (literature[6] mp 188~190°C): $[\alpha]_D^{22}$ −24° (*c* 1.0, MeOH) (literature[6] $[\alpha]_D^{25}$ −24°); IR (KBr) cm^{-1} 3450 (OH), 2970, 1715 (lactone C=O), 1705 (ketone C=O), 1455, 1375 and 1165; UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 285 (0.54) (ketone); MS *m/z* (relative intensity %), 575 (6.9, M, $C_{29}H_{53}NO_{10}$), 490 (7.6, M+H−$CH_3CH_2CHC(OH)CH_3$), 383 (1.1, M−H_2O−desosaminyloxy), 158 (100, desosaminyl); ^{13}C NMR see Table 1; ^{1}H NMR (90 MHz, $CDCl_3$) δ 2.28 (6H, s, $N(CH_3)_2$), OCH_3-signal at about 3.30 ppm is absent.

15-Nor-erythromycin C (7)

The compound was obtained as a white foam: Rf 0.33; IR (KBr) cm^{-1} 3430 (OH), 2970, 1730 (lactone C=O), 1710 (ketone C=O), 1450, 1375, 1340 and 1165; UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 280 (0.5) (ketone); MS *m/z* (relative intensity %), 705 (0.6, M, $C_{35}H_{63}NO_{13}$), 634 (1.0, M+H−$CH_3CHC(OH)CH_3$), 560 (1.1, M−mycarosyl), 369 (1.5, M+H−H_2O−[mycarosyl and desosaminyl]oxy), 158 (100, desosaminyl), 145 (9.8, mycarosyl); ^{13}C NMR see Table 1; ^1H NMR (90 MHz, $CDCl_3$) δ 2.29 (6H, s, $N(CH_3)_2$), OCH_3-signal at about 3.30 ppm is absent.

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