BIOLOGICAL CONVERSION OF ERYTHRONOLIDE B, AN INTERMEDIATE OF ERYTHROMYCIN BIOGENESIS, INTO NEW "HYBRID" MACROLIDE ANTIBIOTICS

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Transformation of erythronolide B to new antibiotics was attempted by feeding this compound during the fermentation of *Streptomyces antibioticus* ATCC 31771, a blocked mutant of an oleandomycin producing strain. As a result, four new active compounds were obtained with hybrid structures between erythromycin and oleandomycin. They were identified as 3-*O*-oleandrosyl-5-*O*-desosaminyl-15-hydroxyerythronolide B (**I**), 3-*O*-oleandrosyl-5-*O*-desosaminylerythronolide B (**II**), 3-*O*-oleandrosyl-5-*O*-desosaminyl-(8*S*)-8-hydroxyerythronolide B (**III**) and 3-*O*-oleandrosyl-5-*O*-desosaminyl-(8*R*)-8,19-epoxyerythronolide B (**IV**). They were found to be less active, but more stable to acid, than erythromycin A.

From their relative biogenetical relationship together with the structure elucidated some hypotheses about late stages of oleandomycin biosynthesis are inferred too.

In recent years there has been a considerable interest in the search of biological routes[1,2] to develop new antibiotics. Since a number of macrolide aglycones has been isolated, these compounds were found to be useful not only for the elucidation of biosynthetic pathways, but also as a temptative starting material (either as such or chemically modified)[2~6] to obtain new molecules with antibiotic activity. Because of the structural analogies between the two most important 14-membered macrolides, erythromycin and oleandomycin, which have been suggested to reflect closely related biosynthetic pathways[5,7], we assumed that new "hybrid" structures could be obtained by adding erythronolide B, an intermediate of erythromycin biogenesis[8], in the fermentation medium of *Streptomyces antibioticus* ATCC 31771, a blocked mutant of an oleandomycin producing strain. Four new antibiotics **I**, **II**, **III**, **IV** (Scheme 1) were succesfully derived by such an approach. In this paper the preparation, structure determination and properties of these compounds are described.

Materials and Methods

Analytical

Elemental analyses were performed by Alfred Bernhardt Microanalytical Laboratories, Elbach über Engelskirchen, West Germany. All melting points were taken in open capillary tubes using a Tottoli apparatus (N. Büchi, Flawil, Switzerland) and are uncorrected. Optical rotations were determined at 24~25°C in 1% methanol solutions with a Schmidt-Haentsch polarimeter. UV spectra were measured in methanol using a Varian Cary 210 spectrophotometer. IR spectra were obtained on a Perkin-Elmer 577 spectrophotometer for KBr disks (0.001 g of substance in 0.2 g of KBr). ^{1}H NMR

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spectra were obtained on a Varian T-60A spectrometer at room temperature in pyridine-d_5 (*c* 0.05 g/ml). Chemical shifts are reported in ppm from tetramethylsilane (TMS) as internal reference and coupling constants are reported in Hz. PFT ^{13}C NMR spectra were recorded with a Varian XL-200-FT spectrometer at 50.3 MHz in proton decoupled conditions at room temperature. Samples were dissolved in deuteriochloroform (*c* 0.2 g/ml) containing tetramethylsilane (TMS) as internal reference.

Thin-layer chromatography (TLC) was carried out on precoated silica gel 60 F-254 plates (Merck) using acetone - chloroform - methanol - 10% ammonium hydroxide (50: 50: 1.4: 2, v/v) as developing solvent system (three runs). Compounds were visualized by spraying the plates with anisaldehyde - acetic acid - methanol - sulfuric acid (1: 5: 90: 2, v/v). Colors developed after a few minutes at 80°C. Antibiotics were also detected by bioautography on *Micrococcus luteus* ATCC 9341 seeded agar. High performance liquid chromatography (HPLC) analyses were carried out according to a modification of a described procedure[9]. A Hewlett-Packard 1084 B liquid chromatograph equipped with a variable-wavelength detector at 210 nm and a Lichrosorb RP8 10 μm stainless steel column, 250×4.6 mm i.d., was used. Flow rate of the mobile phase was 2.0 ml/minute and the column was operated at 40°C. Two mobile phases were employed: mobile phase 1 consisted of acetonitrile - 0.01 M phosphate pH 7.0 (40: 60, v/v); mobile phase 2 consisted of acetonitrile - 0.01 M phosphate pH 7.0 (64: 36, v/v). Gas liquid chromatographies (GLC) were carried out on a Perkin Elmer Model 900 B equipped with a glass column, 2,500×2 mm i.d., packed with 4.3% Silicone OV 25 on 100~120 mesh HP Chromosorb W and heated from 100°C to 160°C (6°C/minute). Nitrogen gas was used as a carrier at 30 ml/minute and 2.8 kg/cm^2 inlet pressure.

Column Chromatography

Partition column chromatographies were carried out in conformity with a reported method[10] using Silica gel 60, 70~230 mesh (Merck). Fifteen-milliliter fractions were collected at a flow rate of 1.0 ml per minute. Residual buffer salts were removed from products by washing their chloroform solutions with water. Sephadex LH-20 (Pharmacia Fine Chemicals), particle size 25~100 μm, was refluxed for 30 minutes three times in a mixture of chloroform and methanol (1: 1, v/v), filtered, and dried at 40°C before being used for column chromatography[11].

Materials

Erythronolide B was isolated from the fermentation broth of *Streptomyces erythraeus* LMC 1192, a completely blocked mutant which accumulates large quantities of this compound. Isolation and fermentation procedures were as reported elsewhere[8].

Erythromycin B was prepared from crude erythromycin A as described previously[12]. Oleandomycin was purified from a commercial product by crystallization from methanol - water.

Mutagenesis

S. antibioticus spore cells from yeast malt extract (YME) slant culture were suspended in 10 ml of saline containing 0.1% Tween 80 and dispersed by vortexing with glass beads. After filtration through Whatman No. 1 paper, the spores were centrifuged and resuspended at a density of 1~5×10^8 cells/ml in 10 ml of saline in Petri dish, to be irradiated by UV light (emission maximum at 254 nm). The dosage was such as to kill 99.6% of the spores (about 3,000 erg/cm^2). Survivors were plated on YME agar and incubated for 5 days at 33°C.

Antibacterial Activity

Minimal inhibitory concentrations (MIC) were determined by a standard agar-diffusion well technique. The MICs were the lowest concentrations of antibiotics in a two-fold serial dilution which still showed inhibitory activity.

Experiments and Results

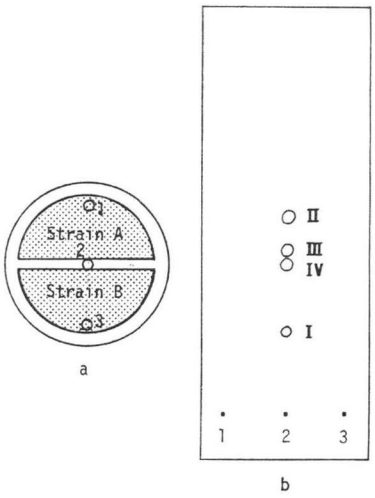
Isolation of the Converting Strain

Survivors of the UV mutagenic treatment of an industrial oleandomycin-producing strain were subjected to a two steps screening program, first employing the agar piece method[13] for selection of

completely blocked mutants, which thereafter were tested for antibiotic cosynthesis on YME agar[14] together with *S. erythraeus* LMC 1192 as the erythronolide B producing strain. When the completely blocked mutant *S. antibioticus* LMC 1163 (registered as ATCC 31771) was tested in these conditions, antibiotic activity was found in the middle region of the plate. A small agar disk from this region was applied to a TLC plate according to a previously described agar-chromatographic technique[15], and four different active compounds were detected by bioautography of the developed plate (Fig. 1).

Fig. 1. Bioautographic detection of the new antibiotics produced by agar cosynthesis.

S. erythraeus LMC 1192 (strain A), erythronolide B producer, and *S. antibioticus* ATCC 31771 (strain B) were streaked over opposite halves of a Petri dish, 1~2 mm apart, on YME agar (a). Following incubation for 5 days at 33°C, agar cylinders were picked up with a cork borer and applied on a TLC plate[15]. Results of further development and bioautography of the plate are shown in (b). Samples 1, 2, 3 came from the corresponding regions in the Petri dish. See Analyticals for details of TLC.



Conversion Experiment in Liquid Culture

In order to obtain appreciable quantities of the new antibiotics, transformation of crystalline erythronolide B was attempted with a shaken flask culture of *S. antibioticus* ATCC 31771.

Matured spores were inoculated into a 500-ml Erlenmeyer flask containing 50 ml of the following seed medium (in g/liter): glucose 15.0, soybean meal 30.0, $MgSO_4 \cdot 7H_2O$ 1.0, yeast autolysate 1.0, calcium carbonate 10.0, soybean oil 6.0 (deionized water, pH adjusted to 7.0).

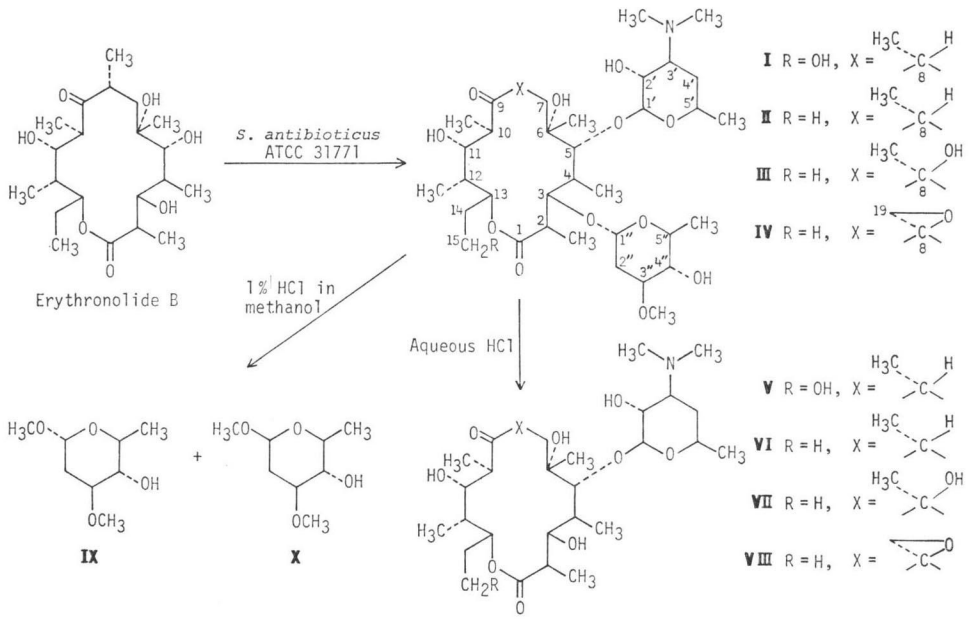
After inoculation, the strain was incubated at 28°C for 24 hours on a rotary shaker and then 1.5 ml of this culture was transferred into the same medium and incubated under the same conditions for further 16 hours; 0.9 ml of this culture inoculated into a 250-ml Erlenmeyer flask containing 30 ml of fermentation medium consisting of glucose 50.0 g, soybean meal 20.0 g, corn meal 3.0 g, brewing yeast 2.0 g, calcium carbonate 20.0 g per liter, and incubated at 28°C on a rotary shaker (250 rpm). After 32 hours of incubation, 15 mg of a finely powdered erythronolide B were added to the flask and the incubation was continued for further 68 hours. The fermentation process was monitored by HPLC, TLC and bioautography. Samples of the fermentation culture were centrifuged, the pH was adjusted to 9.8 with sodium hydroxide and the supernatant extracted with ethyl acetate. After concentration of the organic layer the residue was dissolved in acetonitrile, then injected into a HPLC column or applied on TLC plates. The rapid disappearance of added erythronolide B from the culture supernatant was noticed after 16 hours since the addition (HPLC, mobile phase 1). Corresponding production of compounds **I, II, III, IV** (Scheme 1) was noted soon after (HPLC, mobile phase 2).

At harvest, percentage content of antibiotics **I, II, III, IV** was evaluated by HPLC to be approximately 65, 5, 13 and 17% respectively. No antibiotic activity was produced in a control fermentation culture with no added erythronolide B.

Isolation of Fermentation Products

After 100-hour fermentation, the broth pooled from 130 flasks was filtered through Celite, and

Scheme 1.



the filtrate was extracted five times with methylene chloride at pH 9.0. The organic layers were separately monitored using TLC and HPLC. Because of its higher polarity, compound **I** was singularly present in the last two extracts. These two were combined and evaporated *in vacuo* to yield 1.15 g of crude 3-*O*-oleandrosyl-5-*O*-desosaminyl-15-hydroxyerythronolide B (**I**) which resisted all attempts at crystallization, $[\alpha]_D^{20}-55°$; UV 288 nm (ε 42); ^{1}H NMR δ 2.25 (s, 6, NMe_2), 3.35 (s, 3, OMe), 3.45~3.95 (m, 2, C-15 H); IR 3460, 1715, 1700 (shoulder), 1455, 1375, 1330, 1170 cm^{-1}. The compound was characterized as hydrochloride: mp 160~163°C (acetone).

Anal. Calcd. for $C_{36}H_{65}NO_{13}\cdot HCl$: C 57.17, H 8.80, Cl 4.69, N 1.85.
Found: C 56.92, H 8.77, Cl 4.59, N 1.91.

The other extracts, which contained compounds **II**, **III** and **IV**, together with small amounts of **I**, were evaporated *in vacuo* to yield 0.975 g of a white foam. This residue was chromatographed on a partition column (740×24 mm i.d.) to yield 0.085 g of pure 3-*O*-oleandrosyl-5-*O*-desosaminylerythronolide B (**II**) in the early fractions (fractions 52~110). The compound **II** had $[\alpha]_D^{20}-68.2°$; UV 290 nm (ε 59.8); ^{1}H NMR δ 2.20 (s, 6, NMe_2), 3.40 (s, 3, OMe); IR 3490, 1720, 1700 (shoulder), 1460, 1380, 1335, 1180 cm^{-1}. An analytical sample was prepared by further chromatography on Sephadex LH-20 in chloroform - hexane (1 : 1, v/v) followed by crystallization from acetone - hexane: mp 130~132°C.

Anal. Calcd. for $C_{36}H_{65}NO_{12}$: C 61.43, H 9.30, N 1.99.
Found: C 61.34, H 9.38, N 2.05.

The melting point of its hydrochloride was 179~180°C (acetone). Further elution of the column and evaporation *in vacuo* of the fractions 130~185 resulted in the isolation of 0.175 g of pure 3-*O*-oleandrosyl-5-*O*-desosaminyl-(8*S*)-8-hydroxyerythronolide B (**III**). This product was recrystallized from acetone: mp 204~206°C, $[\alpha]_D^{20}-48.35°$; UV 280 nm (ε 39.2); ^{1}H NMR δ 2.25 (s, 6, NMe_2), 3.40 (s, 3, OMe); IR 3600~3200 (broad, irregular), 1725, 1455, 1380, 1305, 1175 cm^{-1}.

Anal. Calcd. for $C_{36}H_{65}NO_{13}$: C 60.06, H 9.10, N 1.94.
Found: C 60.12, H 8.93, N 1.92.

3-*O*-Oleandrosyl-5-*O*-desosaminyl-(8*R*)-8,19-epoxyerythronolide B (**IV**) was eluted last. Evaporation *in vacuo* of the fractions 260~450 and crystallization from acetone - hexane gave 0.185 g of the analytical sample: mp 125~130°C, $[\alpha]_D^{20}$ −27.7°; UV 294 nm (ε 32.7); ^{1}H NMR δ 2.15 (s, 6, NMe_2), 2.65 (d, 1, C-19 H, $J_{19',19}$=10.0), 2.75 (d, 1, C-19 H′, $J_{19',19}$=10.0). IR 3485, 1720, 1460, 1380, 1330, 1170 cm^{-1}.

Anal. Calcd. for $C_{38}H_{63}NO_{13}$: C 60.23, H 8.85, N 1.95.
Found: C 60.12, H 8.75, N 1.79.

Structure Determination of the New Compounds

The structures of the new antibiotics present in the fermentation broth were derived mainly from their behavior on acid-catalyzed hydrolysis and comparison with erythromycin B, (8*S*)-8-hydroxyerythromycin B[12,16] and oleandomycin under the same conditions (Scheme 1). By treatment with dilute hydrochloric acid[17], compound **II** and erythromycin B afforded a common monoglycoside, 5-*O*-desosaminylerythronolide B (**VI**)[17], while both compound **III** and (8*S*)-8-hydroxyerythromycin B yielded 5-*O*-desosaminyl-(8*S*)-8-hydroxyerythronolide B (**VII**), as detected by HPLC (mobile phase 2). In an analogous way, acid hydrolysis of the compounds **I** and **IV** gave one product each (not isolated).

The four new compounds and oleandomycin produced the same complementary mixture of the α- and β- anomers[15] (**IX, X**) by acid catalyzed methanolysis[18] when compared by GLC. These and previously reported results[5] suggested that the compounds **II** and **III** were indeed 3-*O*-oleandrosyl-5-*O*-desosaminylerythronolide B and 3-*O*-oleandrosyl-5-*O*-desosaminyl-(8*S*)-8-hydroxyerythronolide B respectively. The ^{13}C NMR chemical shifts of these structures, compared to the corresponding chemical shifts of erythromycin B, oleandomycin and to those reported[19] for (8*S*)-8-hydroxyerythromycin B, supported the assigned structures. Further chemical and spectroscopic data were necessary to prove the structures of the compounds **I** and **IV**. As for erythromycin B[20], a solution of compound **I** in glacial acetic acid was allowed to stand at room temperature for two hours and processed as already reported[21] to give a white foam, whose UV absorption was compatible with the previously assigned "8,9-anhydro-6,9-hemiketal" structure[20]. Treatment of compound **I** with acetic anhydride in pyridine[22] yielded a tetraacetyl derivative. Moreover, the mild acetylation of **I** smoothly produced a monoacetyl ester from which the parent was regenerated by mild treatment with methanol[23]. These acetates were characterized by their ^{1}H NMR spectra.

Comparison of UV, IR and ^{1}H NMR spectra of compounds **I** and **II** clearly showed their similarity. The ^{1}H NMR spectrum of **I** revealed additional resonances attributable to methylene protons, but chemical shift and complex spin pattern made the analysis by inspection no longer possible. However, by comparing the ^{13}C NMR chemical shifts of compound **II** and erythromycin B with the corresponding chemical shifts of compound **I**, oxidation of the 14-CH_3 group was clearly evidenced. In fact, the carbon resonance at 10.4 ppm, attributed to 14-CH_3 both in erythromycin B and compound **II**, was absent in the spectrum of compound **I**. The signal at 58.9 ppm was assigned to the C-15 methylene group. A substantial downfield α-shift (48.5 ppm) of the carbon previously part of the 14-CH_3 group, and a modest downfield β-shift (10.3 ppm) of the C-14 carbon were observed. These shift magnitudes were consistent with the published substituent effects of replacement of a proton by a primary hydroxyl group[24]. Upfield γ-shift of 4.6 ppm (when compared with the spectrum of erythromycin B) was also observed for C-13. Other small changes between chemical shifts of compounds **I** and **II** were likely a consequence of both altered structure and slight conformational difference. All available spectroscopic data sup-

ported that the structure of compound **I** was 3-*O*-oleandrosyl-5-*O*-desosaminyl-15-hydroxyerythronolide B.

As for oleandomycin[25], treatment of compound **IV** with anhydrous hydrochloric acid yielded a chlorohydrin (mp 169~170°C from ethyl acetate.

Anal. Calcd. for $C_{29}H_{52}ClNO_{10}\cdot HCl$: C 53.87, H 8.26, Cl 10.97, N 2.17.
Found: C 53.72, H 8.09, Cl 11.13, N 2.23.),

which by addition of two equivalents of sodium hydroxide was converted to the same product obtained by hydrolysis of **IV** in dilute hydrochloric acid. This reaction was monitored by HPLC (mobile phase 2). In analogy to oleandomycin, compound **IV** did not show any reaction when treated with glacial acetic acid at room temperature for two hours. Treatment of compound **IV** with acetic anhydride in

Table 1. ^{13}C Chemical shifts[a] of compounds **I, II, III, IV**, erythromycin B[19,26,27] and oleandomycin[27].

	I	II	III	IV	Erythromycin B	Oleandomycin
C-1	176.8	176.2	176.0	176.6	176.1	176.2
C-2	44.9[d]	45.0[d]	45.8	45.0	44.9	44.9
C-3	81.2	81.5	81.5	81.1	80.2	81.3
C-4	40.2	39.8[e]	40.3	44.0	39.5	44.8[b]
C-5	84.4	84.2	87.1	84.0	83.6	84.0
C-6	75.2[b]	75.2[b]	75.1[b]	75.4[b]	74.9	30.5
C-7	38.2	38.3[e]	43.3	37.2	38.1	30.5
C-8	44.4[d]	44.7[d]	78.8	62.2	44.8	62.5
C-9	220.1	219.9	217.9	209.6	219.8	208.1
C-10	39.8	39.4[e]	40.3	40.4	39.0	43.0[b]
C-11	69.5[c]	69.5[c]	69.3[c]	69.1[c]	69.4	70.3
C-12	40.2	39.9[e]	39.6	39.5	39.8	41.7[b]
C-13	70.9[c]	75.5[b]	75.3[b]	75.7[b]	75.3	69.2
C-14	35.8	25.5	25.4	25.4	25.5	18.4[d]
2-CH_3	15.2	15.5	16.2	15.3	15.6	14.6[d]
4-CH_3	9.3[f]	9.2[g]	9.4[d]	9.2[e]	9.2	9.5[c]
6-CH_3	27.1	27.4	28.6	28.2[d]	27.3	19.9
8-CH_3	18.6[e]	18.5[f]	28.6	50.1	18.6	48.8
10-CH_3	9.2[f]	9.1[g]	9.1[d]	8.6[e]	9.2	6.8[c]
12-CH_3	9.1[f]	9.1[g]	9.8[d]	9.1[e]	9.2	9.9[c]
14-CH_3	58.9	10.4	10.3	10.4	10.4	—
C-1′	103.8	103.8	104.4	103.6	103.2	104.4
C-2′	70.9[c]	70.9[c]	70.8[c]	70.8[c]	70.9	70.5
C-3′	65.3	65.3	65.0	65.2	65.6	65.5
C-4′	28.6	28.6	28.6	28.5[d]	28.6	28.5
C-5′	68.8[c]	68.8[c]	69.0[c]	68.8[c]	68.9	69.2
5′-CH_3	21.3	21.4	21.3	21.3	21.4	21.1
$N(CH_3)_2$	40.2	40.2	40.3	40.2	40.2	40.2
C-1″	98.7	98.8	99.1	98.5	96.6	99.6
C-2″	33.5	33.5	33.5	33.5	35.0	33.9
C-3″	78.0	78.0	78.1	78.0	72.6	77.9
C-4″	75.7[b]	75.4[b]	75.6[b]	75.9[b]	77.9	76.0
C-5″	69.1[c]	69.1[c]	69.2[c]	68.8[c]	65.5	68.7
5″-CH_3	18.2[e]	18.2[f]	18.1	18.2	18.5	17.8
OCH_3	56.3	56.2	56.3	56.3	49.4	56.3
3″-CH_3	—	—	—	—	21.4	—

[a] Chemical shifts are reported in ppm downfield from internal TMS.
[b~g] These assignments may be interconverted in each column.

pyridine yielded a triacetyl derivative, and mild acetylation produced a monoacetyl ester from which the parent was regenerated by treatment with methanol. These two acetates were characterized by their ^{1}H NMR spectra. Signals due to epoxide protons were clearly observed with ^{1}H NMR analysis and confirmed by comparison with oleandomycin ^{1}H NMR spectrum. The occurrence of two ^{13}C NMR signals with chemical shifts similar to those of the oleandomycin epoxidic carbons, together with the results of chemical reactions, ^{1}H NMR spectral analysis and similarity of UV absorption to that one of oleandomycin[25], offered strong evidence that the structure of compound **IV** was 3-*O*-oleandrosyl-5-*O*-desosaminyl-(8*R*)-8,19-epoxyerythronolide B. In light of the presented structures of **I** and **IV**, we deduced that the compounds obtained by their dilute hydrochloric acid hydrolysis were 5-*O*-desosaminyl-15-hydroxyerythronolide B (**V**) and 5-*O*-desosaminyl-(8*R*)-8,19-epoxyerythronolide B (**VIII**) respectively.

^{13}C NMR chemical shifts and assignments for compounds **I**, **II**, **III**, **IV**, erythromycin B and oleandomycin are shown in Table 1.

Acid Stability

The acid stability at 25°C of compounds **I**, **II**, **III**, **IV** and related erythromycin A were compared by HPLC analysis (mobile phase 2) using 0.1% solutions in buffers at pH 2, 3, 4, as described in Table 2. In contrast to erythromycin A which rapidly decomposes in acid solution to yield the biologically inactive anhydroerythromycin[28], the new compounds **I**, **II**, **III**, **IV** are remarkably stable in the acid environment.

Table 2. Acid stability at 25°C.

Compounds	pH 2	pH 3	pH 4
	$t_{1/2}$*	$t_{1/2}$*	$t_{1/2}$*
I	>100	>100	>100
II	18	>100	>100
III	27	>100	>100
IV	65	>100	>100
Erythromycin A	0.05	0.1	2

* $t_{1/2}$: The half-life in hours.

Biological Activity

The activity of the compounds **I**, **II**, **III**, **IV** was established against a variety of bacteria; erythromycin A, erythromycin B and oleandomycin were used as control compounds (Table 3). All the new compounds are less active than erythromycin A and erythromycin B. Compound **II** has an activity comparable to oleandomycin, but it is more active against *Haemophilus influenzae*. The activity of compounds **I** and **III** is similar to that of oleandomycin against the Streptococci, 4~8 fold less active against *Staphylococcus aureus*. Compound **IV** has an intermediate activity between erythromycin A and oleandomycin over the whole spectrum of aerobic microorganisms.

Biogenetic Relationships Among the New Compounds

Following the purification and the structure determination of compounds **I**, **II**, **III** and **IV** we tested their relative biosynthetic relationships by conversion experiments in Erlenmeyer flasks. In these experiments **II**, **III** and **IV** were separately added to shaken flask cultures of *S. antibioticus* ATCC 31771, after 48 hours of incubation. After 72 hours of further incubation, HPLC analysis of the broths showed that **II** was converted to **I**, **III** and **IV**, and that **III** was converted to **IV**. No any other conversion was observed.

Discussion

Four new antibiotics, whose structures are reported in Scheme 1, were obtained by feeding ery-

Table 3. Antibacterial activity *in vitro* of compounds **I, II, III, IV** compared to that of erythromycin A, erythromycin B and oleandomycin.

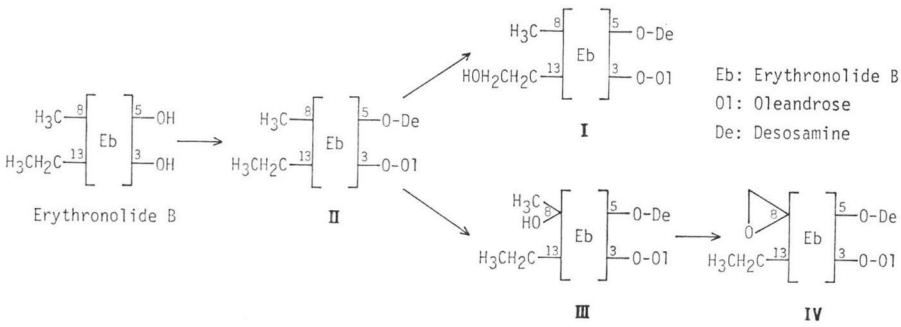
Organism	Minimum inhibitory concentration (μg/ml)						
	I	II	III	IV	Erythromycin A	Erythromycin B	Oleandomycin
1. Aerobes							
Gram-positives							
Staphylococcus aureus ATCC 6538 P	3.12	0.78	1.56	0.195	0.049	0.097	0.39
Staphylococcus aureus ATCC 14154*	>25	>25	>25	>25	>25	>25	>25
Streptococcus pneumoniae ATCC 6303	0.195	0.097	0.195	0.049	0.012	0.024	0.195
Streptococcus pyogenes ATCC 8668	0.097	0.097	0.195	0.049	0.012	0.024	0.195
Corynebacterium diphteriae PRL 24	0.097	0.049	0.097	0.024	0.006	0.012	0.097
Micrococcus luteus ATCC 9341	0.195	0.097	0.049	0.024	0.006	0.006	0.049
Micrococcus luteus ATCC 15957*	>25	>25	>25	>25	>25	>25	>25
Bacillus subtilis PRL 25	3.12	0.39	0.78	0.195	0.049	0.049	0.39
Gram-negatives							
Haemophilus influenzae ATCC 19418	>25	6.25	>25	12.5	3.12	6.25	>25
Neisseria gonorrhoeae ATCC 19424	0.195	0.195	1.56	0.195	0.049	0.097	0.195
Escherichia coli PRL 50	>25	>25	>25	25	6.25	25	>25
Klebsiella pneumoniae PRL 54	>25	>25	>25	>25	25	25	>25
Proteus vulgaris ATCC 6380	>25	>25	>25	>25	>25	>25	>25
Pseudomonas aeruginosa PRL 9	>25	>25	>25	>25	>25	>25	>25
Salmonella typhi PRL 8	25	>25	25	25	12.5	25	>25
Shigella sonnei PRL 5	>25	>25	>25	>25	12.5	>25	>25
Acholeplasma laidlawii ATCC 23206	6.25	12.5	6.25	1.56	0.097	0.097	6.25
Mycoplasma hominis 1 ATCC 14027	>25	>25	>25	>25	>25	>25	>25
2. Anaerobes							
Clostridium perfringens ATCC 3624	6.25	12.5	>25	3.12	1.56	1.56	3.12
Bacteroides fragilis ATCC 23745	>25	3.12	6.25	6.25	0.195	0.195	0.39
Fusobacterium necrophorum ATCC 27852	>25	>25	>25	25	1.56	6.25	6.25

* Erythromycin resistant.

thronolide B, a biosynthetic precursor of the erythromycins, to *S. antibioticus* ATCC 31771, a blocked mutant of an oleandomycin producing strain. This is a further evidence that biosynthesis of erythromycin A and oleandomycin are closely related. All the new antibiotics contain oleandrose, three of them receiving further modifications on the aglycone moiety.

The bioconversion experiments with these antibiotics suggested some precursor-product relationships. Compound **II** is the common precursor which, by different competing routes, can be hydro-

Fig. 2. Suggested pathway for the production of the new antibiotics. Brackets denote the erythromycin aglycone. Numbers indicate lactone carbons.



xylated at C-15 to yield compound **I**, or converted, *via* compound **III**, to compound **IV** (Fig. 2). The former pathway is not induced unless glucose is present in the medium (R. SPAGNOLI, unpublished results). The latter route suggests that a corresponding sequence could lead to epoxide formation in the still largely unknown oleandomycin biosynthesis. The hypothesis[29] that the insertion of the "extra" hydroxyl groups in macrolide aglycones biosynthesis, such as that at C-8 of oleandomycin, is a late biochemical event occurring with retention of the original stereochemistry, is confirmed by our results. The use of unnatural substrates could be an interesting tool in elucidating biosynthetic pathways, inasmuch as they introduce some rate limitations in the sequence of enzymatic reactions. As a consequence, intermediate products may accumulate which would otherwise remain undetected.

On the other hand we lack an adequate explanation for compound **I** production, as no other methyl-hydroxylation in a corresponding position is known to occur for 14-membered macrolides. We excluded that this compound was obtained due to biochemical abnormality of the employed mutant, because many other independently selected mutants also produced it (R. SPAGNOLI, unpublished results). Furthermore, the hypothesis[30] of product formation by non-specific enzymes released by old lysing mycelia was not consistent with the early appearance of compound **I**, and its quite large quantity in the fermentation broth. This new C-15 hydroxyl substituent, however obtained, offers opportunities for further chemical modifications of the corresponding antibiotic aimed to improve the microbiological activity. Activity *in vitro* was lower than erythromycins A and B for all four new antibiotics, as expected with oleandrose substituting cladinose[5]. The better antibacterial activity of compound **IV** as compared to oleandomycin indicates that C-6 hydroxyl and C-13 ethyl groups may be important for activity in the erythromycin series. Comparison between compounds **II** and **III** shows that the antimicrobial activity is negatively affected when hydrogen at C-8 is replaced by a hydroxyl group, as previously reported for erythromycins A[31,32] and B[12,16].

A very pronounced acid stability, which can be related to the neutral sugar[5] and possibly also to the aglycone structure, is the main feature of these new antibiotics which encourages us to further investigate them *in vivo*.

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