A NEW SEMISYNTHETIC MACROLIDE ANTIBIOTIC 3-O-OLEANDROSYL-5-O-DESOSAMINYLERYTHRONOLIDE A OXIME

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A new antibiotic, 3-O-oleandrosyl-5-O-desosaminylerythronolide A oxime (**3**) was produced from erythronolide A oxime (**1**) by the oleandomycin-producing culture, *Streptomyces antibioticus* ATCC 11891. The structure of **3** was determined by degradative studies and confirmed by X-ray analysis. Compound **3** was found to be less active, but more stable to acid, than erythromycin A oxime.

The availability[1] of the oximes of both erythronolide A (**1**) and 5-O-desosaminylerythronolide A (**2**) provides the intriguing possibility of attaching different sugars at the C-3 and C-5 hydroxyls to produce more effective erythromycin derivatives. We now wish to report some of our efforts to that end and to describe the structure determination and properties of a new antibiotic **3** produced from **1** by the oleandomycin-producing culture, *Streptomyces antibioticus* ATCC 11891.

Experimental

I. General Methods

Melting points were taken in glass capillaries and are corrected. Ir and nmr spectra were obtained in $CHCl_3$ and $CDCl_3$ (Me_4Si) solutions respectively; for the latter a Varian A-60 or HA-100 spectrometer was used. The high-resolution mass spectrum was run on a CEC 21-110 mass spectrometer at 70 eV. The glc-ms data were obtained using a Finnigan MS 1015 connected to a Becker 741 gas chromatograph. Tlc's were carried out on Brinkmann or Merck silica gel plates and developed twice using a solvent system of CH_2Cl_2-CH_3OH-H_2O-conc·NH_4OH (90:9.5:0.5:1). Visualization was accomplished by spraying with phosphomolybdic acid (5% in EtOH) or xanthydrol (0.15% in 12:1 conc·HCl-HOAc) followed by heating at 100°C. Countercurrent distribution was carried out in an H. O. Post all-glass automatic unit with a lower (stationary) phase capacity of 40 ml.

II. Fermentation

Streptomyces antibioticus ATCC 11891 was maintained on ATCC sporulation agar number 5. Inoculum was prepared by transferring slant growth to 500 ml Erlenmeyer flasks each containing 100 ml of the following medium (in g/liter): soluble starch, 15.3; soybean meal, 15.3; corn steep solids, 4.8; sodium chloride, 4.8; and calcium carbonate, 2.9. The pH of the medium was adjusted to 6.8 before sterilization. Inoculated flasks were incubated at 28°C for 72 hours on a rotary shaker. Three hundred ml of the resulting growth was added to 10 liters of a fermentation medium, containing (in g/liter): soluble starch, 15.2; defatted soy flour, 15.2; corn

steep solids, 5; sodium chloride, 3.3; and calcium carbonate, 5.0. The pH of the medium was adjusted to 6.8 before sterilization. The culture was incubated at 28°C in a 14-liter stirred jar fermentor, aerated at 4 liters per minute and agitated at 550 rpm. Polydimethylsiloxane antifoam (SAG-4130, Union Carbide Co.) was added as needed to control frothing. After 6 hours of incubation, 1 g of erythronolide A oxime (**1**) in 100 ml of ethanol was added. Incubation was continued for an additional 55 hours. The fermentation broth was then centrifuged at 15,000×*g* in a Sharples centrifuge. The supernatant was adjusted to pH 8.5 with NaOH and extracted twice with equal volumes of CH_2Cl_2. The extract was then concentrated to a small volume *in vacuo*.

III. Isolation Procedure

From 12 stirred jar fermentations carried out as described above, the CH_2Cl_2 concentrate was extracted three times with half volumes of 0.01 M H_3PO_4. The pH of the aqueous extract was immediately adjusted to 9.3 by addition of 3 N KOH, NaCl was added to near saturation and the solution was extracted four times with 1/3 volumes of CH_2Cl_2. Concentration of the extract *in vacuo* yielded 6 g of solids. This material was purified about 5-fold by counter-current distribution in *n*-butanol-acetic acid-water (10:1:10, aqueous phase adjusted to pH 2.95 by addition of 2 N NaOH). A 100-tube distribution was carried out with 40 ml of each phase per tube. Compound **3** was shown by tlc to be in tubes 79~88 while the major contaminant, oleandomycin, was in tubes 60~67. The contents of tubes 79~88 were combined and concentrated *in vacuo* to a volume of 300 ml. Following adjustment of the pH to 9.5 with 40% NaOH, the mixture was extracted four times with half volumes of CH_2Cl_2. The combined extracts were concentrated *in vacuo* to a residue of 1.2 g. Crystallization from ether-ethanol (10:1) gave 0.30 g of solid. Tlc showed this material to be free of oleandomycin but to contain **3** along with a slower-moving impurity. Preparative tlc of 200 mg on four 2 mm thick plates gave 112 mg which was crystallized from acetone-hexane to yield 82 mg of **3**: mp 148~156°C; ir 1733 (lactone carbonyl) and 1640 cm^{-1} (C=N); nmr δ 2.28 (NMe_2) and δ 3.42 (OMe). The high resolution mass spectrum revealed the molecular ion at *m/e* 734.4545 ($C_{38}H_{66}N_2O_{13}$).

Anal. Calcd. for $C_{38}H_{66}N_2O_{13}$: C 58.83, H 9.05, N 3.81.
Found: C 58.69, H 9.26, N 3.45.

IV. Acid Stability

The acid stability of compound **3** and related antibiotics is shown in Table 1. Antibiotic activity remaining after incubation of a 200 μg/ml solution in 0.1 N HCl (pH 1.2) at 37°C was determined by an agar-diffusion bioassay *vs. Sarcina lutea* ATCC 9341. Samples were prepared for bioassay by dilution with an equal volume of 1 M K_2HPO_4 (final pH 7.6). The percent remaining antibiotic was measured by reference to appropriate standard curves.

Table 1.

Compound	Half-life (min)
Erythromycin A	<3
Erythromycin A oxime	10~ 20
3	60~ 80
Oleandomycin	240~300

V. Acid Cleavage

Basic Fragment

Compound **3** (16.5 mg) was treated with 1 ml of 1% HCl in methanol at room temperature

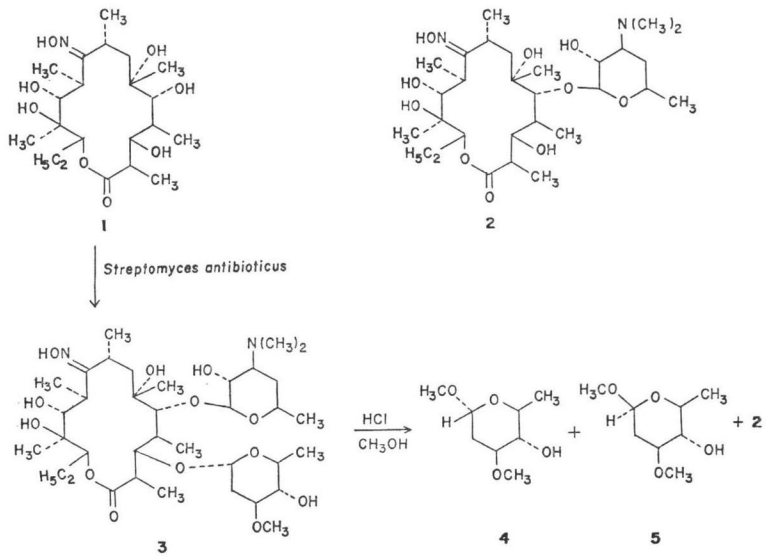
for 17 hours. Saturated $NaHCO_3$ solution (1 ml) was added and the solvent removed *in vacuo*. Saturated NaCl solution (3 ml) was added and the mixture extracted with ethyl acetate. The extract was washed with three 2 ml portions of 1 N HCl and the combined acid extract made basic and extracted with ethyl acetate. The dried ($MgSO_4$) extract was concentrated to yield 6.2 mg of a foam. Tlc analysis revealed a spot with an Rf identical to authentic 5-O-desosaminylerythronolide A oxime (**2**).[1] The low resolution mass spectrum of this material gave a molecular ion at *m/e* 590 and the fragmentation pattern was essentially identical with that of authentic **2**.[1]

Neutral Fragment

Compound **3** (20.0 mg) was treated with 1 ml of 1% HCl in methanol at room temperature for 21 hours. The total ethyl acetate extract obtained as above was dried ($MgSO_4$) and concentrated *in vacuo* to yield 20 mg of a foam. Glc on a 10% OV 101 column at 150°C showed two volatile components with retention times of 12 minutes (84%) and 14 minutes (16%). Glc-ms analysis revealed the base peak in both components at *m/e* 74 and the mass spectra were essentially identical below *m/e* 74. The highest observed mass in the major component **4** was *m/e* 145 ($M^+ - 31$) while in the minor component **5** it was *m/e* 175 ($M^+ - 1$).

Cleavage of Oleandomycin

Twenty grams of oleandomycin phosphate (Pfizer) was dissolved in 500 ml of 1% HCl in CH_3OH and left at room temperature for 4 days.[2] Saturated $NaHCO_3$ solution (250 ml) was added and the MeOH removed *in vacuo*. The mixture was extracted with $CHCl_3$ and the extract washed with 3 N HCl and 5% $NaHCO_3$ solution. After drying ($MgSO_4$), the extract was concentrated *in vacuo* to an oil. Distillation gave 1.9 g of a mixture of the α- and β-anomers of methyl L-oleandroside, bp 100~102°C/8 mm. Glc as above showed two components with retention times of 12 minutes (90%) and 14 minutes (10%). The mass spectra of the separated components, obtained by glc-ms, were identical to the spectra of **4** and **5** obtained from the cleavage of **3**. The minor component (**5**) was separated by preparative glc and its nmr spectrum:



δ 1.36 (d, J=6, C−5′CH_3), 3.39 (s, C−3′OCH_3), 3.49 (s, C−1′OCH_3) and 4.37 (dd, J=9.5, 3, C−1′H) was in agreement with that reported[3] for the β-L-anomer of methyl oleandroside.

VI. Preparation of 3 Methiodide

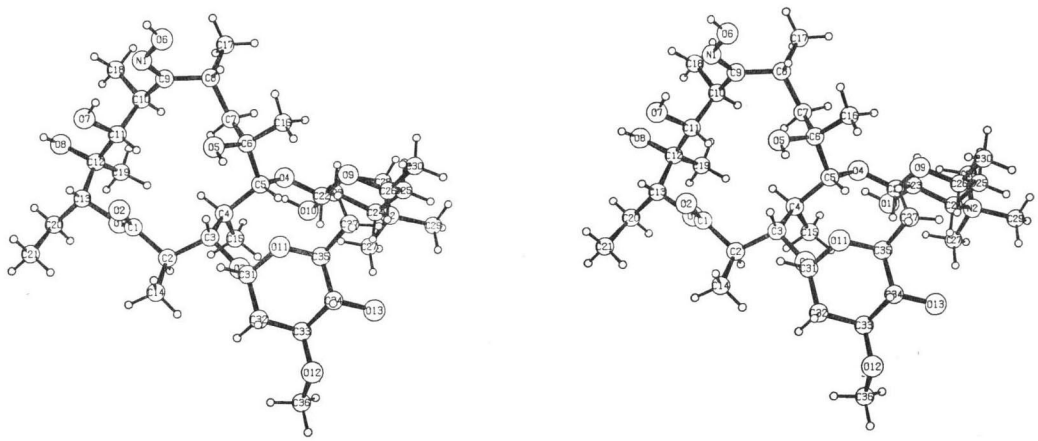
To 29.7 mg of **3** in 7 ml of ether was added 0.3 ml of methyl iodide and the solution left at room temperature for 18 hours. The resultant solid was filtered to yield 25.6 mg of methiodide. Crystals (mp 201~205°C) suitable for X-ray analysis were obtained from methanol/ether.

VII. X-ray Diffraction Analysis of 3

Crystals of **3** are orthorhombic, space group $P2_12_12_1$, with a=10.30 (1), b=18.00 (1), c=25.21 (1) Å, and d_{calc}=1.271 g cm^{-3} for Z=4. The diffraction data were measured on Hilger-Watts four-circle diffractometer ($\theta-2\theta$ scans, Ni-filtered Cu Kα radiation, pulse height discrimination). The size of the crystal used for data collection was 0.25×0.35×0.6 mm. Of the 4861 accessible reflections with 2θ <140°, 3299 had intensities which were significantly greater than background. The reflection data were corrected for absorption (μ=59.9 cm^{-1}).

The structure was solved by straightforward application of the heavy atom method. The analysis also revealed an atom of a solvent molecule, which is most likely water but possibly is methanol. This atom was treated as an oxygen atom. The initial refinement of the structure was done by full-matrix least squares. Subsequent refinements were carried out by block-diagonal least squares in which the matrix was partitioned into four blocks. In the refinements the iodine scattering curve was corrected for the real and imaginary parts of the anomalous dispersion and the known absolute stereochemistry of erythromycin A was assumed.[4] Near the end of the refinement the idealized positions of all hydrogen atoms, except those of the solvent molecule, were calculated. In the final refinement anisotropic thermal parameters were used for the heavier atoms and isotropic temperature factors for the hydrogen atoms. The hydrogen atoms were included in the structure factor calculations, but their parameters were not refined. The final discrepancy index is R=0.078 for the 3299 observed reflections. A difference FOURIER based on the final parameters has three small peaks (1.2 eA^{-3}) which are probably due to the presence of a small quantity of methanol in the crystal. A stereodrawing of **3** is shown in Fig. 1.

Fig. 1. A stereoscopic drawing of **3** showing its conformation in the crystalline state.



VIII. Biological Activity

Minimum inhibitory concentration (MIC) values obtained for **3** are given in Table 2; included for comparison are those for erythromycin A oxime, erythromycin A and oleandomycin. Compound **3** shows approximately one-tenth the molar activity of erythromycin A oxime. Binding studies[5] have shown **3** to be bound about one-eighth as well as erythromycin A and about one-third as well as erythromycin A oxime to *Escherichia coli* ribosomes. The antimicrobial spectrum of **3** is typical of the macrolide antibiotic family. Furthermore, **3** was not active against any of the six erythromycinresistant clinical isolates of *Staphylococcus aureus* tested.

The ability of **3** to induce resistance to other macrolide antibiotics was tested by the paper-disc counter-diffusion assay *vs. Staphylococcus aureus* 1206 as described by WEISBLUM and DEMOHN.[6] Erythromycin A, erythromycin A oxime and **3** are strong inducers of resistance to clindamycin and oleandomycin. Oleandomycin is a poor inducer of resistance to itself and to clindamycin. Compound **3** appears to be more like erythromycin than oleandomycin in inducing macrolide resistance. Thus, in compound **3** the erythronolide ring system seems to dominate the neutral sugar in inducing resistance.

Table 2. Antimicrobial spectra of **3** and related antibiotics

Organism	MIC* (μg/ml)			
	3	Erythromycin A oxime	Erythromycin A	Oleandomycin
Staphylococcus aureus ATCC 6538 P	6.25	1.57	0.63	12.5
Sarcina lutea ATCC 9341	0.79	0.09	0.16	1.57
Bacillus megaterium ATCC 8011	1.57	0.09	0.16	1.57
Bacillus sp. E ATCC 27859	6.25	0.79	0.31	25.0
Bacillus subtilis NRRL 558	6.25	0.79	0.63	3.13
Bacillus sp. TA ATCC 27860	12.5	0.79	0.63	3.13
Streptomyces cellulosae ATCC 3313	>100	>100	50	25
Paecilomyces varioti ATCC 26820	>100	>100	>100	>100
Candida albicans NRRL 477	>100	>100	>100	>100
Saccharomyces cerevisiae ATCC 4266	>100	>100	>100	>100
Pseudomonas aeruginosa ATCC 8709	>100	>100	>100	>100
Proteus vulgaris ATCC 6380	>100	>100	>100	>100
Escherichia coli ATCC 27856	>100	>100	>100	>100
Klebsiella pneumoniae ATCC 27858	>100	>100	>100	>100
Serratia marcescens ATCC 27857	>100	>100	100	>100
Acinetobacter calcoaceticus ATCC 10153	25	6.25	10	100

* MIC values were determined by an agar-diffusion well technique; the values reported are the lowest concentration of compound in a two-fold serial dilution which still showed inhibitory activity.

Discussion

A new antibiotic, 3-O-oleandrosyl-5-O-desosaminylerythronolide A oxime (**3**) was produced from erythronolide A oxime (**1**) by the oleandomycin-producing culture, *S. antibioticus* ATCC 11891. Spectral and analytical data as well as acid cleavage experiments leading to 5-O-desosaminylerythronolide A oxime (**2**) and a mixture of the α- and β-anomers of methyl-L-oleandroside suggested the structure **3**. This structure, which incorporates the sugars of oleando-

mycin (oleandrose and desosamine) with the aglycone of erythromycin A, was confirmed by X-ray diffraction analysis of the methiodide.

It has been shown[7] that erythronolide B is efficiently converted *via* 3-O-mycarosylerythronolide B to erythromycin A by strains of *Streptomyces erythreus*. In contrast, 5-O-desosaminylerythronolide B is only poorly converted to an unidentified antibacterial compound.[7] Mycarose apparently must be added to erythronolide B before attachment of the amino sugar. We found that *S. antibioticus* did not transform 5-O-desosaminylerythronolide A oxime (**2**) to an active antibiotic. This may be a further demonstration of the importance of the order in which the sugars are attached to the aglycone.

It has been suggested that the biosynthesis of erythromycin and oleandomycin are related.[8] A blocked mutant of the erythromycin-producing organism, *Streptomyces erythreus*, was shown to elaborate 8, 8A-deoxyoleandolide.[8] When this latter compound was added to an early blocked mutant of *S. erythreus*, a new antibiotic was formed. Although not characterized, it was suggested to contain a hydroxyl substituted oleandolide aglycone with the erythromycin sugars (cladinose and desosamine) attached at C-3 and C-5, respectively.[8] Our finding that an oleandomycin producing culture can glycosylate erythronolide A oxime is further evidence that the biosyntheses of oleandomycin and erythromycin are closely related.

The well known acid instability of erythromycin A involves formation of an internal enol ether followed by participation of the C-12 hydroxyl leading to a stable, inactive ketal.[9] With this precluded by the protection of the ketone as the oxime in erythromycin A oxime and in **3**, the acid cleavage of these compounds involves removal of the sugar from the C-3 hydroxyl group. The greater acid stability of **3** with respect to erythromycin A oxime may be related to the 3″-methoxyl group of oleandrose, which is equatorially oriented. On the other hand, the 3″-methoxyl of cladinose in erythromycin A oxime is in the axial position and the removal of cladinose would relieve the 1, 3-diaxial interaction with the glycosidic oxygen bond.

The lower antibiotic activity of **3** is probably also related to the equatorial 3″-methoxyl group of oleandrose. The axial 3″-methoxyl of cladinose in erythromycin A is essential for formation of the ribosome complex and thus for high antibacterial activity.[10] The low ribosomal binding and low *in vitro* antibacterial activity of **3** indicate that axial stereochemistry of the 3″-methoxyl is important for the high antibiotic activity of erythromycin A.

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