Antibiotic Glycosides. 8. Erythromycin D, a New Macrolide Antibiotic[1]

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Abstract: Erythromycin D, isolated from mother liquors after crystallization of erythromycin A, has the lactone moiety (erythronolide B) of erythromycin B and the sugars (L-mycarose and D-desosamine) of erythromycin C. Erythromycin D is structurally related to both erythromycins B and C and is a key intermediate in their biosynthesis. A transmethylase from *Streptomyces erythreus* converts erythromycin D to erythromycin B, a transformation that supports both the structural assignment and the biogenetic role proposed for erythromycin D. The antibacterial activity of erythromycin D is about a half that of erythromycin A with *Bacillus subtilis* 168 as test organism.

Erythromycin A,[3-9] B,[10-12] C,[13] and E[14] were characterized previously. The former three erythromycins are composed of two different 14-membered lactones (erythronolides A or B) combined with a common basic sugar, D-desosamine, and either of two neutral branch-chain sugars, L-mycarose[15] or L-cladinose[16] (Figure 1). The latter is the 3-*O*-methyl ether of the former, and enzymatic studies have shown that the methylation of L-mycarose only occurs when the sugar is linked glycosidically to erythronolide A as a part of erythromycin C.[17] Erythromycin E contains an ortho ester grouping and represents a later stage of the erythromycin biosynthetic pathway.

Biogenetic considerations[18,19] suggested strongly that another member of the erythromycin family must exist, a structure with the simpler B form of the lactone and the demethylated neutral sugar, L-mycarose. This missing structure, named erythromycin D, would be the mandatory precursor of both erythromycins B and C, and it would be expected to be the first diglycoside produced in the erythromycin pathway.

Experience gained in the fractionation of trace metabolites of various species of the genus *Streptomyces* producing monoglycosides of the macrolide class,[20] encouraged our use of Sephadex LH-20 in searching for erythromycin D in a concentrate of mother liquors. This concentrate, containing erythromycins A and B, was enriched with a substantial amount of erythromycin C, and the spiroketals (6 → 9:12 → 9) of erythromycins A and C.[8] In addition, a number of unidentified components were present. The properties (polarity, relative elution volume, etc.) predicted for erythromycin D focused our attention on compounds similar in chromatographic behavior to erythromycin A. An unknown substance which was eluted from Sephadex LH-20 column with nearly the same relative elution volume as erythromycin A proved to have the properties expected for erythromycin D.

Experimental Section

General. Antibiotics and authentic derivatives were gifts of Abbott Laboratories (North Chicago) and Eli Lilly and Co. (Indianapolis). When derivatives were prepared from these starting materials, their properties were compared with those in the literature and, when possible, with the authentic reference substances.

Solvents used for column chromatography were Analytical Reagent Grade and redistilled before use. Thin-layer plates were a commercial type coated with silica gel (EM Chemicals, layer thickness 0.25 or 0.5 mm). Sephadex LH-20 (Pharmacia Fine Chemicals), particle size 25–100 μ was refluxed for 30 min 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.[21]

Instrumentation. The following equipment was used: Perkin-Elmer IR spectrophotometer, 60 MHz Hitachi Perkin-Elmer R 20B NMR spectrometer, Varian Associates (Nicolet Technology XL-100) TT-100 NMR spectrometer, A.E.I. MS-902 mass spectrometer.

Sample Preparation. IR spectra were recorded from KBr disks (1 mg of substance in 200 mg of KBr). Mass spectra were obtained with an ionization energy of 70 eV. Samples were introduced into the source by a direct inlet system and determined under several conditions designed to enhance molecular ion formation. ^{1}H NMR spectra were measured at 55 °C in $CDCl_3$ on the above system using pulse Fourier transform spectroscopy. Spectra were obtained using a 1 KHz sweep width over an 8K data table. A 3.0-mg sample of erythromycin D required 500 acquisitions using a 30° tip pulse.

Chromatography. Column Chromatography. A column (2.0 × 120 cm, bed volume 300 ml) of Sephadex LH-20 in chloroform/hexane (1:1, v/v) was loaded with approximately 250 mg of sample in the elution solvent. Fractions of 5 ml were collected at a flow rate of 0.1 ml/cm^2/min. Values for the relative elution volume (REV) are expressed as a ratio of the elution volume V_e over the total (effective) volume V_t of the column.

Thin-Layer Chromatography. The individual fractions from column chromatography were examined using a solvent mixture of ethyl acetate, 2-propanol, 15% ammonium acetate (aqueous, w/v adjusted to pH 9.6), 9:7:8 (upper phase).[22] Anisaldehyde and concentrated sulfuric acid in ethanol (1:1:9, v/v/v) served as a spray detection reagent. Colors developed after a few minutes at 80 °C. The R_f values relative to erythromycin A (R_{EaDC} = 1.0) were 1.05 for erythromycin B, 0.92 for erythromycin D, and 0.87 for erythromycin C.

Chemical Procedures. The conditions employed for the acid-catalyzed hydrolysis of the four erythromycins were those described for erythromycin A.[5] The only modification involved the use of Amberlite MB-3 in removing the inorganic ions from the mildly alkaline aqueous layer after the extraction of the respective basic monoglycosides. All degradation products, i.e., erythralosamine, 5-*O*-desosaminylerythronolide B, L-cladinose, and L-mycarose, were identical with the authentic specimens. While the amounts for erythromycin A, B, and C were 16 mg, 1.6 mg of erythromycin D was sufficient for TLC and mass spectral analysis.

Bioassay. To evaluate antimicrobial activity, the "sensitive" strain of *Bacillus subtilis* 168 was used as the test organism.[23] An overnight inoculum (Antibiotic 3 Medium Difco) was transferred into a series of test tubes containing a range of concentrations of the antibiotic. After incubation for 75 min at 37 °C, absorbance was measured at 540 nm. The individual points of the dose response curves were calculated according to the following formula:

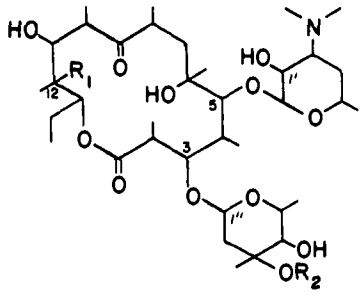
$$\% \text{ inhibition} = \frac{(C_t - C_0) - (E_t - E_0)}{(C_t - C_0)} \times 100$$

where $C_t = A_{540}$ of the control at a given time, $C_0 = A_{540}$ of the control at zero time, $E_t = A_{540}$ of the experimental at the same time as C_t, $E_0 = A_{540}$ of the experimental at zero time and arbitrarily equals C_0. In terms of ID_{50} values the activities for the four erythromycins were as follows: A:B:C:D = 0.025:0.026:0:078:0:053 μ/ml.

Results and Discussion

Column chromatography of the mixture of compounds present in the mother liquors after commercial purification (Abbott Laboratories) of erythromycin A using Sephadex LH-20 yielded a number of substances. Erythromycin B was eluted first (REV 0.8) and was closely followed by erythromycins A and C (REV 0.9 and 0.95, respectively). TLC analysis of the fractions showed one substance nearly cochromatographing with erythromycin A. The unknown compound was separated from erythromycin A by preparative thin-layer chromatography and was distinguishable from it by the color of its reaction with an anisaldehyde-sulfuric acid spray reagent. The unknown material yielded the same bluish color as erythromycin B, while both erythromycin A and C gave a greyish color.

Proof of the structure of the unknown substance followed mainly from its behavior on acid-catalyzed hydrolysis and comparison with other erythromycins under the same conditinos. Two major products were detected from each antibiotic on treatment with dilute hydrochloric acid; however, none of the antibiotics yielded the same two products. When the reactions were carried out on a larger scale and the products isolated, it was found that erythromycin B (II) and the unknown structure afforded a common monoglycoside, 5-*O*-desosaminylerythronolide B (VII), while erythromycins A (I) and C (III) yielded erythralosamine (V), a 10,11-dehydrated spiroketal derivative of erythronolide A with the 5-*O*-desosaminyl substituent. Complementarily, the unknown compound and erythromycin C produced the same second (neutral) component, and it proved to be L-mycarose (VIII), whereas erythromycins A and B yielded L-cladinose (VI) as the second hydrolysis product. Though these results suggested that the unknown compound was indeed erythromycin D (IV) (Scheme I), it was necessary to establish whether the L-mycarosyl residue was at C-3 of the aglycone to make a definite structural assignment. The obvious possibilities for the site of attachment of the L-mycarosyl residue in the unknown structure were C-3



		R_1	R_2
Erythromycin	A	OH	Me
	B	H	Me
	C	OH	H
	D	H	H

Figure 1.

and C-11, but the chemical evidence from acid-catalyzed hydrolysis could not differentiate between them. Additional physical evidence was obtained that supports the assignment at C-3.

The infrared spectrum of the unknown compound was similar to those of the known erythromycins: maxima at 3470 cm^{-1} (hydroxyl), 1740 cm^{-1} (lactone), 1710 cm^{-1} (ketone). As with erythromycin C, there was no C-H stretching absorption of the *O*-methyl group[24] at 2830 cm^{-1}, a band which is present in both erythromycin A and B and has been assigned the *O*-methyl group present in the 3-*O*-cladinosyl substituent.

The mass spectrum of the compound was consistent with the formula $C_{36}H_{65}NO_{12}$ based on M^+ 703.4485 (calcd 703.4507). The fragmentation pattern showed the presence of peaks at *m/e* 685 (M − 18), and *m/e* 558 and 542 account-

Scheme I

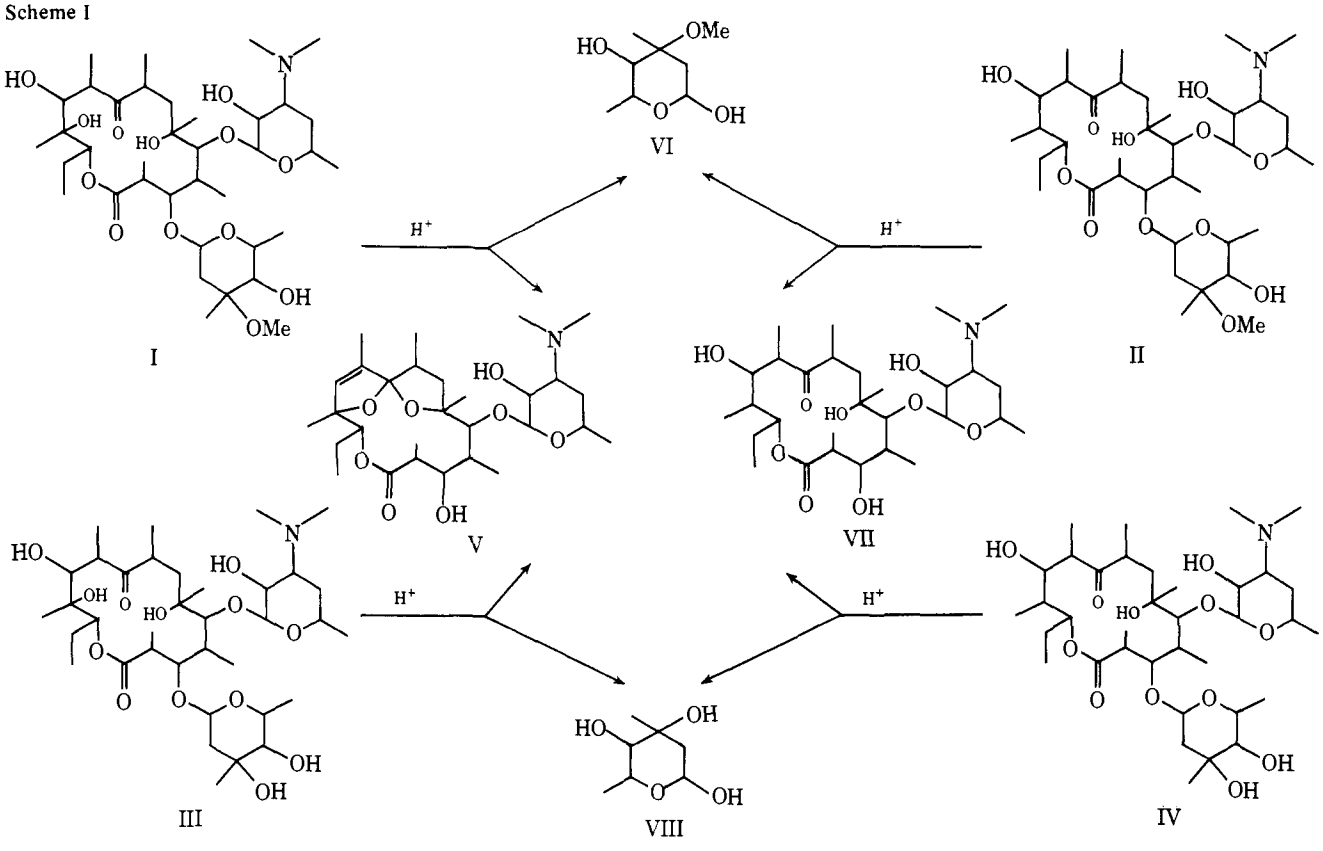


Table I. ^{1}H NMR Parameters of Erythromycins A–D

Erythromycin	Chemical shifts A	B	C	D	Erythromycin	Coupling constants A	B	C	D
H-3	4.00	4.04	4.14	4.23	$J_{2,3}$	9.0	8.5	9.8	8.5
H-5	3.56	3.58	3.51	3.52	$J_{3,4}$	2.0	~1	~1	~1
H-11	3.86	3.85	3.85	3.76	$J_{4,5}$	7.5	7.2	8.0	8.0
H-13	5.07	5.38	5.04	5.32	$J_{10,11}$	1.5	~1	~1	~1
					$J_{11,12}$		10.0		9.5
H-1′	4.41	4.44	4.27	4.28	$J_{12,13}$		1.2		~1
					$J_{13,14a}$	10.0	8.5	10.0	8.5
H-1″	4.92	4.92	5.04	4.99	$J_{13,14e}$	3.0	5.8	3.0	5.0
H-4″	2.99	2.98	2.96	2.99					
H-5″	4.04	4.06	3.85	3.89	$J_{1',2'}$	7.0	7.0	7.0	7.0
6-CH_3	1.47	1.46	1.51	1.49	$J_{1'',2a''}$	4.5	4.5	4.5	4.0
					$J_{1'',2e''}$	~1	~1	~1	~1
OCH_3	3.32	3.32			$J_{4'',5''}$	9.0	9.0	9.8	9.8
$N(CH_3)_2$	2.29	2.29	2.26	2.29					

ing for the removal of mycarose with or without the glycosidic oxygen atom, respectively. The aglycone fragment devoid of both the sugars gave rise to the peaks with mass *m/e* 385 and 367. The fragments at *m/e* 174 and 158 indicated the presence of desosamine[25] while those at *m/e* 145, 127, and 109 were characteristic for mycarose.

A comparison of the chemical shifts of diagnostically important ring protons and methyl groups of erythromycins A, B, and C with those of erythromycin D fully supported the structural assignment of the latter (cf. Table I). The absence of a 3.32 ppm OCH_3 resonance and the observation of resonances due to H-1″, H-4″, and H-5″ confirmed the presence of mycarose. The observed similarity of chemical shifts and coupling constants required that the same relative stereochemistry be present in all four compounds. The low-field chemical shift of H-13 in erythromycin B and D as well as the presence of a large $J_{11,12}$ coupling constant proved that both of those compounds are 12-deoxy derivatives. A singlet methyl resonance at 1.49 ppm indicated that erythromycin D has a 6-hydroxyl group as present in the other natural erythromycins. The similarity of the remainder of ring proton coupling constants confirmed that no further structural or stereochemical changes were present.

Although chemical and physical evidence clearly showed that the unknown substance was the antibiotic with the structure proposed for erythromycin D, one additional line of evidence was obtained that confirmed not only the structural assignment but the important biogenetic role also proposed for it. A partially purified particulate enzyme preparation from *S. erythreus* (CA 340, Abbott) which was capable of transforming erythromycin C into erythromycin A (erythromycin C: *S*-adenosyl-L-methionine transmethylase)[26] was used to determine whether erythromycin D could substitute for erythromycin C as a substrate. The *S*-adenosyl-L-methionine was radioactive in its *S*-methyl group, and after incubation of erythromycin D with this reagent and the enzyme a new radioactive product was isolated. It proved to be erythromycin B, and all of the radioactivity was shown to reside in the *O*-methyl group of the L-cladinose derived from the erythromycin B. Thus, erythromycin D as well as erythromycin C was a good substrate for the transmethylation reactions leading to erythromycin B and A, respectively, under the catalytic action of erythromycin C: *S*-adenosyl-L-methionine transmethylase.[27]

In light of the presented evidence we deduced that the new compound, which we call erythromycin D, has the chemical structure IV.

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