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EXTENSION OF THE ERYTHROMYCIN BIOSYNTHETIC PATHWAY

ISOLATION AND STRUCTURE OF ERYTHROMYCIN E[1]

J. R. MARTIN,* R. S. EGAN, A. W. GOLDSTEIN and P. COLLUM
Division of Antibiotics and Natural Products, Abbott Laboratories, North Chicago, IL 60064, U.S.A.

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Abstract—Erythromycin A is slowly metabolized to a new antibiotic, erythromycin E (**5**), when incubated with fermentations of certain strains of the producing organism *Streptomyces erythreus*. Erythromycin E contains a novel ortho ester grouping not previously encountered in macrolide antibiotics. The isolation of erythromycin E extends and further delineates the latter stages of the erythromycin biogenetic pathway.

The naturally occurring erythromycin macrolide antibiotics (**1–4**) consist of a multi-branched, poly-functional, 14-membered lactone ring substituted with an amino and a nitrogen-free 6-deoxy sugar. Earlier studies[2–4] have enabled us to propose a plausible scheme for the final steps in erythromycin biosynthesis. We pointed out[4] that erythromycin A (**1**), considered the final metabolic product of the erythromycin biogenetic pathway, was further converted by certain strains of the producing organism to additional antibiotics. For example, antibiotic activity decreased with time when finely divided erythromycin A was incubated with fermentations of *Streptomyces erythreus* (Abbott 2NU153), a point blocked mutant which synthesizes erythromycin only when provided with authentic biosynthetic intermediates. Examination of fermentation broths revealed that an unknown antibiotic accumulated as a major product as the antibiotic activity decreased. This compound, now designated erythromycin E (**5**), is the subject of the present communication.

Erythromycin E (**5**) was identified by chromatography and/or recovered from the fermentation broths of several point blocked mutants of *S. erythreus* incubated with erythromycins A–C. As an illustration, when erythromycin A was added to fermentations of a mutant strain capable of high conversion of known erythromycin lactone progenitors to erythromycin A, it was slowly metabolized to erythromycin E (**5**). Conversion amounted to approximately 30% after 120 hr incubation. Erythromycin A substrate (45%) was recovered intact and a further 5% was isolated as spiroanhydroerythromycin (**11**), a non-enzymatic degradation product. Several minor conversion products, amounting to approx. 20% of added substrate, were not identified.

The PMR spectrum of erythromycin E (**5**) proved most informative (Table 1, Fig. 1). The spectra of erythromycin

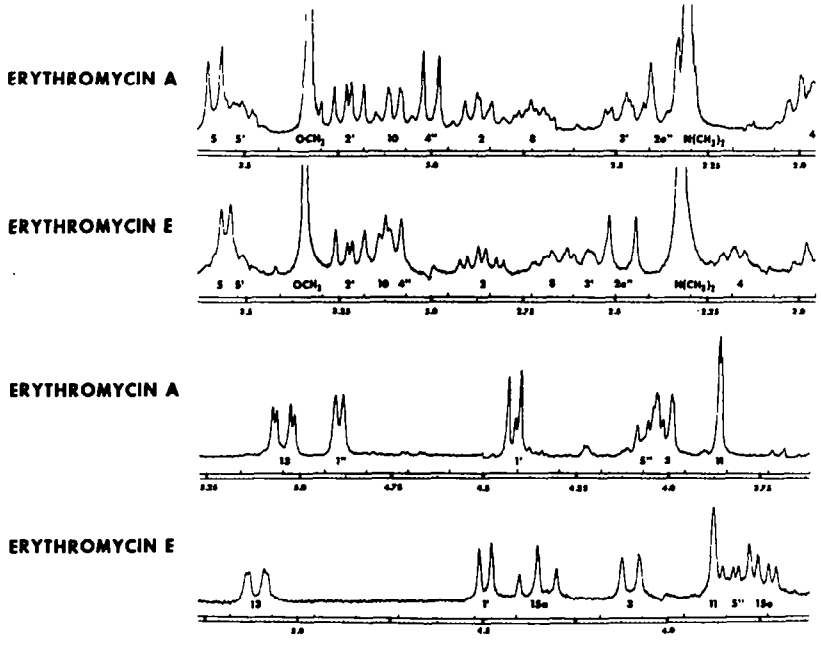


Fig. 1. The 220 MHz PMR spectra of the lactone ring protons of erythromycin A (**1**) and erythromycin E (**5**). Proton assignments are shown under the appropriate resonances.

Table 1. PMR parameters of erythromycin E (5)[a]

Chemical Shifts ppm				Coupling Constants Hz	
H-2	2.86	H-5'	3.7	$J_{2,3}$	10.5
H-3	4.10	H-2"a	1.60	$J_{2,15a}$	10.5
H-4	2.17	H-2"e	2 48	$J_{2,15e}$	4.5
H-5	3.55	H-4"	3.10	$J_{3,4}$	1
H-7a	2.15	H-5"	3.81	$J_{4,5}$	5.5
H-7e	1.64			$J_{7a,7e}$	14.5
H-8	2.68	OCH_3	3.35	$J_{7e,8}$	12.0
H-10	3.13	NMe_2	2.34	$J_{10,11}$	1
H-11	3.87			$J_{13,14a}$	1.2
H-13	5.12	CH_3-4	1.17	$J_{13,14e}$	10.5
		CH_3-8	1.15	$J_{1',2'}$	2.7
		CH_3-10	1 09	$J_{2',3'}$	7.2
H-15a[b]	4.32	CH_3-6	1.40	$J_{15a,15e}$	10.2
H-15e	3.74	CH_3-12	1.25	$J_{2"a,2"e}$	-11.0
		CH_3-14	1.15	$J_{4",5"}$	15.2
H-1'	4.49				9 5
H-2'	3.22	CH_3-5'	1.32		
H-3'	2.57				
		CH_3-5"	1.20		

[a] Obtained from 220 MHz spectra in $CDCl_3$ by direct measurement using first order rules and reported in ppm downfield from internal TMS.

[b] Notations of a and e are for convenience only and do not necessarily represent orientations.

A (1) and B (2) have been previously interpreted[5] allowing direct comparison and observation of significant similarities and differences between the various spectra. The chemical shift and multiplicity of H-13 (5·12 ppm; $J_{13,14} = 10·5$ and 2·7 Hz) and H-11 (3·87 ppm; $J_{10,11} = 1·2$ Hz) indicates that erythromycin E has a 12-OH substituent and is therefore an erythromycin A derivative. The resonance of H-3 (4·10 ppm; $J_{2,3} = 10·5$; $J_{3,4} = 1$ Hz) is only slightly shifted from its position in the spectrum of erythromycin A (4·01 ppm; $J_{2,3} = 9·0$; $J_{3,4} = 2·0$ Hz); however the resonance of H-2 which was located by spin-decoupling was shown to be significantly altered.

In erythromycin A, the resonance of H-2 appears at 2·87 ppm as a doublet of quartets of which only the four most intense lines are normally visible in absence of high gain. The resonance of the corresponding proton in **5** appears at 2·86 ppm and exhibits an unusual triplet of doublets multiplicity suggesting that H-2 is no longer coupled to a freely rotating methyl group. A spin-decoupling experiment further revealed that H-2 is coupled not only to H-3 but also to protons the resonances of which are centered at 4·32 and 3·74 ppm. The latter are designated as H-15a and H-15e ($J_{2,3} = J_{2,15a} = 10·5$ Hz; $J_{2,15e} = 4·5$ Hz). These latter protons were further shown to be coupled to each other with $J_{15a,15e} = -11·0$ Hz indicating that they might arise from protons formerly a part of the 2-CH_3 group. Their chemical shift indicates that they are attached to a carbon also substituted by an electronegative atom suggesting that the 2-CH_3 group has been oxidized. The loss of the 2-CH_3 group was confirmed by the absence of one high field methyl doublet in the 1·0–1·5 ppm region.

The remaining ring proton resonances of erythromycin E (**5**) (Table 1) are virtually unchanged from their corresponding chemical shifts and multiplicities in the spectrum of erythromycin A (**1**). The resonances associated with the amino sugar moiety, desosamine, are similarly unchanged indicating that the amino sugar is unaffected and that the aglycone is altered only by the oxidation of the 2-CH_3 group.

The PMR spectrum of **5** shows an additional significant difference in that the resonance of the anomeric proton of the neutral sugar moiety cladinose (H-1") found at 4·89 ppm for **1** is missing and no resonance at a different chemical shift can be ascribed to this proton. The resonances of the adjacent methylene protons, H-2"a and H-2"e, confirm the absence of H-1" by a loss of their vicinal coupling. No other structural changes are indicated by the remaining resonances of the neutral sugar.

The CMR spectrum (Table 2) offers additional evidence for the structure of erythromycin E (**5**) by comparison with the recently published analysis of the spectrum of erythromycin A.[6] Oxidation of the 2-CH_3 group is clearly evidenced by the large upfield γ shifts exhibited by the aglycone lactone carbonyl C-1 as well as C-3. A substantial downfield α-shift of the carbon previously part of the 2-CH_3 group is also observed. A downfield α-shift is also visible for the anomeric carbon of cladinose C-1" confirming that this center is also oxidized. Additionally, the off-resonance single-frequency decoupled spectrum reveals that C-1" is a quaternary carbon. Comparisons of other chemical shifts reveal only small changes which are likely a consequence of both altered structure and slight conformational differences which may involve the relative orientation of the sugars to each other and to the aglycone ring.

These observations can be satisfied by two different structures both involving oxidation at 2-CH_3 and C-1". One possibility involves ortho ester formation between these two centers as shown in **5** and the second involves hydroxylation of both sites as shown in **6**. Inspection of the mass spectrum of erythromycin E clearly differentiates between these structures. The highest *m/e* ion present in the high resolution mass spectrum, determined under several conditions designed to enhance molecular ion formation,[7] is at 747·4443 which corresponds to an

Table 2. CMR chemical shifts[a] of erythromycin E (**5**) and erythromycin A (**1**)[6]

	Chemical Shifts			Chemical Shifts	
	5	1		5	1
C-1	166.9	176.6	C-1'	101.5	103.7
C-2	43.5	45.4	C-2'	71.0	71.4
C-3	74.9	80.5	C-3'	65.5	66.0
C-4	35.6	39.9	C-4'	28.9	29.2
C-5	84.0	84.1	C-5'	68.9	69.3
C-6	74.9	75.3	5'-CH_3	21.1	21.8
C-7	39.0	38.9	N-$(CH_3)_2$	40.4	40.7
C-8	45.4	45.4			
C-9	222.7	222.4	C-1"	109.3	96.8
C-10	37.5	38.5	C-2"	38.4	35.4
C-11	68.9	69.3	C-3"	74.3	73.1
C-12	74.5	75.3	C-4"	77.3	78.5
C-13	77.4	77.5	C-5"	68.5	66.0
C-14	21.1	21.8	5"-CH_3	17.4	19.0
C-15	62.0	----	O-CH_3	49.4	49.9
			3"-CH_3	22.3	21.8
2-CH_3	----	16.7			
4-CH_3	8.7	9.6			
6-CH_3	27.0	27.2			
8-CH_3	11.9	12.5			
10-CH_3	16.3	16.4			
12-CH_3	18.2	18.8			
14-CH_3	10.6	11.1			

[a] Chemical shifts are reported in ppm downfield from internal TMS.

elemental composition of $C_{37}H_{65}NO_{14}$. This is consistent with structure **5** for erythromycin E.

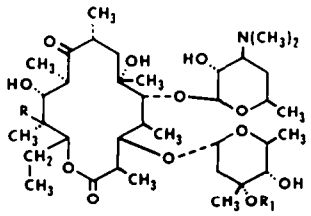
Acid catalyzed methanolysis of erythromycin A (**1**) has been shown to give erythralosamine (**7**),[8] with a molecular formula of $C_{29}H_{49}NO_8$, and the anomeric α- and β-methyl glycosides of cladinose. In accord with the structure formulated by PMR and CMR for erythromycin E, methanolysis cleanly yields the expected erythralosamine derivative 2a-hydroxyerythralosamine (**9**), with a molecular formula of $C_{29}H_{49}NO_9$, i.e. with one more O atom than that of **7**. The additional CO absorption band in **9** is ascribed to the ester CO group after H-bonding with the C-2a OH group. Erythralosamine (**7**) displays the normal single CO frequency. The two minor co-produced methanolysis products of **5**, thought to arise from the ortho ester linked cladinosyl moiety, were not isolated due to paucity of **5**.

Comparison of the PMR spectra (Table 3) of **7** and **9** clearly determines their similarity and locates the additional O atom in **9** at C-2a. The PMR spectrum of **9** revealed additional resonances attributable to the C-15 methylene protons, but chemical shift overlap and deceptively simple higher order spin patterns make complete analysis impossible.

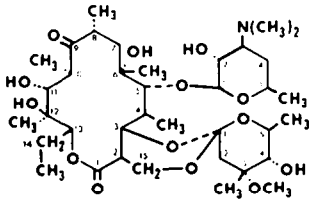
Treatment of erythralosamine (**7**) with acetic anhydride in pyridine yields the diacetyl derivative **8** (Table 3). In contrast, under the same conditions, **9** gives the expected triacetate (**10**). These labile acetates were characterized by their IR, PMR and mass spectra. A more complete PMR analysis of **10** was possible in which the coupling between the C-15 methylene protons and H-2 could be clearly observed and confirmed by decoupling experiments. These details further substantiated the constitution of **5** and **9**.

To our knowledge erythromycin E represents the first example of a naturally occurring macrolide having a sugar attached *via* an ortho ester linkage. Ortho esters have previously been encountered in the antibiotic field as exemplified by hygromycin B and derivatives.[10,11]

Recently Majer *et al.*[12] isolated and identified erythromycin D from the mother liquors of a primitive commercial production strain of *S. erythreus*. This antibiotic, previously predicted,[4] closed the last known gap in the latter stages of the biosynthetic sequence of the previous known erythromycins. The isolation of erythromycin E, a more advanced metabolite further extends the erythromycin biogenetic pathway, the latter stages of which can now be depicted as shown in Fig. 2.



	R	R_1
1	OH	CH_3
2	H	CH_3
3	OH	H
4	H	H



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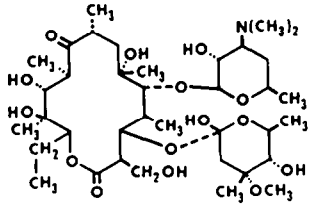

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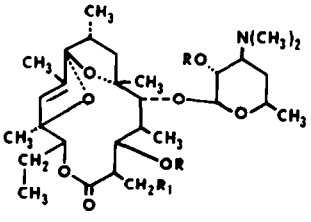

	R	R_1
7	H	H
8	$COCH_3$	H
9	H	OH
10	$COCH_3$	$OCOCH_3$

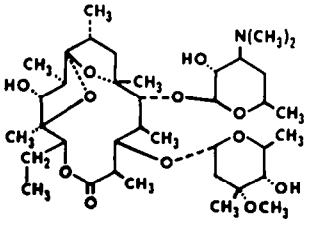

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Table 3. PMR parameters of erythralosamine (7), 3,2"-di-O-acetylerythralosamine (8), 2a-hydroxyerythralosamine (9) and 2a-acetoxyl-3,2'-di-O-acetylerythralosamine (10)[a]

	Chemical Shifts ppm					Coupling Constants Hz			
	7	8	9	10		7	8	9	10
H-2	2.79	2.5	3.4	2.92	$J_{2,3}$	4.5	2.4	3.7	2.5[b]
H-3	4.29	5.40	4.48	5.47	$J_{3,4}$	4.5	6.7	7.0	6.0[b]
H-4	2.29	2.5	2.3	2.3	$J_{4,5}$	7.5	10.0	5.2	10.0
H-5	3.45	3.52	3.49	3.51	$J_{13,14a}$	10.5	10.7	10.5	10.5
H-8	2.5	2.5	2.5	2.5	$J_{13,14e}$	3.2	3.2	3.3	3.2
H-11	5.50	5.46	5.50	5.47					
H-13	4.94	4.97	4.97	5.02	$J_{1',2'}$	7.2	7.4	7.2	7.5
H-1'	4.20	4.18	4.26	4.18	$J_{2',3'}$	10.0	10.2	10.2	10.5
H-2'	3.22	4.78	3.24	4.80					
H-3'	2.5	2.5	2.5	2.5	$J_{11,10\text{-}CH_3}$	1.2	1.5	1.5	1.5
H-5'	3.45	3.44	3.4	3.5					
$2\text{-}CH_3$	1.13	1.10			$J_{2,15a}$			c	6.0
$4\text{-}CH_3$	1.09	0.88	1.17	0.88	$J_{2,15e}$			c	9.5
$6\text{-}CH_3$	1.43	1.59	1.40	1.59	$J_{15a,15e}$			c	-10.7
$8\text{-}CH_3$	0.96	0.97	0.97	0.97					
$10\text{-}CH_3$	1.88	1.76	1.94	1.76					
$12\text{-}CH_3$	1.25	1.25	1.26	1.26					
$14\text{-}CH_3$	0.86	0.87	0.85	0.94					
$5'\text{-}CH_3$	1.21	1.21	1.22	1.20					
NMe_2	2.30	2.28	2.29	2.27					
H-15a			3.97	4.51					
H-15e			3.76	3.96					
OAc		2.03		1.97					
		2.06		2.02					
				2.05					

[a]Obtained from 100 MHz spectra in $CDCl_3$ (unless otherwise noted) by direct measurement using first order rules and reported in ppm downfield from internal TMS.

[b]Determined from C_5D_5N solution.

[c]Deceptively simple ABX pattern observed.

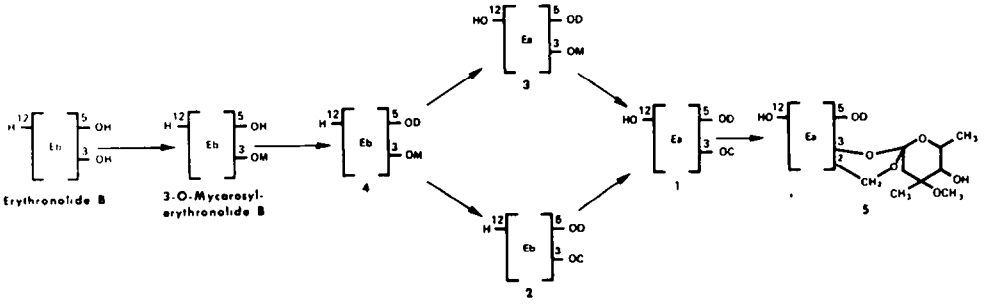

Fig. 2. Proposed pathway for the latter stages of erythromycin biosynthesis. Brackets denote the erythromycin aglycone. Abbreviations: erythronolide B, Eb; erythronolide A, Ea; mycarose, M; cladinose, C; desosamine, D. Numbers indicate lactone carbons.

Since a number of erythromycin related structures, some having antibiotic activity, remain unidentified after erythromycin A is fed to blocked mutants of the producing organism, it can be inferred that further metabolically derived erythromycins might be isolated. Due to the known chemical instability of the erythromycins, the assumption that the unknown structures are biosynthetic transformation products remain speculative. Further, it is highly possible that some or all of the minor unidentified compounds might not be true biosynthetic intermediates but instead, products formed by non-specific enzymes released by old, lysing mycelia.

In view of the low antibiotic activity of erythromycin E (Table 4), it is fortunate that erythromycin A, the medically useful member of the erythromycin group, is only slowly converted to erythromycin E in production strains.

EXPERIMENTAL

General. M.ps were determined with a Thomas-Hoover Uni-Melt apparatus. UV spectra were recorded for MeOH solns employing a Beckman Acta 5 spectrophotometer using the expanded scale. Optical rotations were measured with a Hilger and Watts polarimeter. Unless otherwise noted IR spectra were recorded on $CDCl_3$ solns with a Perkin-Elmer Model 521

Table 4. Antibacterial activity of erythromycin E (5)[a]

Organism	Minimum Inhibitory Concentration mcg/ml pH 7.4 Erythromycin A (1)	Erythromycin E (5)
Staphylococcus aureus 9144	0.2	1.56
Staphylococcus aureus Smith	0.2	1.56
Staphylococcus aureus Smith ER	100	>100
Staphylococcus aureus Wise 155	100	>100
Streptococcus faecalis 10541	0.05	0.39
Escherichia coli Juhl	50	>100
Klebsiella pneumoniae 10031	3.1	6.2
Proteus vulgaris Abbott JJ	>100	>100
Proteus mirabilis Finland No. 9	>100	>100
Salmonella typhimurium Ed. No. 9	25	>100
Shigella sonnei 9290	25	100
Pseudomonas aeruginosa BMH No. 10	50	>100

[a]Determined by an agar dilution method.

instrument. Mass spectra were recorded with an A.E.I. MS-902 mass spectrometer 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.[7] PMR spectra were obtained at 100 MHz using a Varian Associates HA-100 spectrometer or at 220 MHz with a Varian Associates HR-220. CMR spectra were recorded with a Varian Associates XL-100-15/TT-100 spectrometer system.

Partition column chromatographies were carried out by the method of Oleinick and Corcoran[13] using silica gel, 70–230 mesh (Merck, Darmstadt). Residual buffer salts were removed from products by washing chloroform solutions with water. Solvents were evaporated under reduced pressure. TLC was performed on Analtech precoated silica gel GF plates using methylene chloride, 95% aqueous MeOH, NH_4OH, 90:10:1 v/v as the developing solvent. Compounds were visualized by spraying with the arsenomolybdate reagent of Nelson.[14]

Erythromycin E (5)

Fermentation and isolation. The organism usually employed to produce erythromycin E (5) was *S. erythreus* (Abbott 2NU153). This early blocked mutant, previously described,[3] was derived from a high erythromycin producing strain and is unable to synthesize erythromycin or recognized erythromycin precursors *de novo* although this strain will readily convert known erythromycin progenitors to the complete antibiotic.

Fermentation procedures were as before.[3] In a typical experiment 1·0 g of finely divided erythromycin A (1) was equally distributed among 40, 500 ml Erlenmeyer flasks each containing 50 ml of a 48 hr fermentation. Incubation with shaking was continued for an additional 120 hr, then the flask contents were pooled and clarified as before.[2] Chloroform extraction gave 1·11 g of viscous, dark yellow-brown oil. TLC examination of the oil revealed the presence of unchanged 1 (*ca.* 45%), 5 (*ca.* 30%), 11 (*ca.* 5%) and at least 7 minor components (*ca.* 20%).

The oil was fractionated crudely by chromatography on a column (2·5 × 90 cm) of Sephadex LH-20 prepared and eluted with acetone. All fractions containing 5 were collected, evaporated to dryness and further purified by partition column chromatography. Repeated partition chromatography and treatment of the resulting product with Darco G-60 in MeOH gave 311 mg of pure 5. Crystallization from ether gave prisms of broad m.p. 160–165°; $[\alpha]_D^{23}$ −49·7 (*c* 1·0 MeOH); λ_{max} 285 mμ (ε 31); IR, 3590, 3460, 1730 and 1683 cm^{-1}; PMR see Table 1; CMR see Table 2; mass spectrum molecular ion at *m/e* 747·4443, Calc. 747·4404 for $C_{37}H_{65}NO_{14}$. (Found: C, 59·29; H, 8·82; N, 2·04. Calcd for $C_{37}H_{65}NO_{14}$: C, 59·41; H, 8·74; N, 1·85%).

Methanolysis of Erythromycin E (5)

(a) *TLC comparison with erythromycin A (1) methanolysis.* 5 (5 mg) was treated for 3 hr with 1% methanolic HCl (1 ml) and the products compared by TLC with authentic samples of α- and β-methyl-cladinoside and 7. A sample of 1 was treated and examined in a like manner. Acid catalyzed methanolysis of erythromycin A gave exclusively 7 and the α and β anomers of methylcladinoside. Methanolysis of 5 clearly gave 3 products—a slow moving major component and 2 faster moving minor compounds—none of which were identical to those obtained from 1.

(b) *Isolation of 2a-hydroxyerythralosamine (9).* (5) (564 mg) was treated with 112 ml of 1% methanolic HCl and allowed to stand at room temp for 4 hr. The resulting soln was added dropwise to a stirred suspension of excess $NaHCO_3$ in water. The major portion of the MeOH was evaporated under reduced pressure. Chloroform extraction gave 449 mg of colorless foam. Partition column chromatography of the product gave 321 mg of pure 9 as a glass which defied crystallization; $[\alpha]_D^{25}$ +50·1 (*c* 1·0 MeOH); IR, 3600, 3440, 1721 and 1700 (sh) cm^{-1}; (CCl_4) 1731 and 1700 cm^{-1}; PMR see Table 3; mass spectrum molecular ion at *m/e* 555·3395, calc 555·3408 for $C_{29}H_{49}NO_9$.

2a-*Acetoxy*-3,2′-*di*-O-*acetylerythralosamine* (10). A soln of 120 mg of 9 in 4·0 ml pyridine and 0·5 ml Ac_2O was heated on a steam bath for 6 hr and then allowed to stand at room temp for 72 hr. The mixture was poured into 50 ml cold 5% $NaHCO_3$ aq and extracted with ether. The ether extract was rapidly washed with 5% $NaHCO_3$ aq and then twice with water. Evaporation of the ether and co-distillation with benzene to remove residual pyridine gave 10 (114 mg) as a straw colored oil. TLC examination of the product displayed a single spot; IR, 1738 cm^{-1}, no OH absorption; PMR see Table 3, mass spectrum molecular ion at *m/e* 681·3751, calc 681·3724 for $C_{35}H_{55}NO_{12}$.

3,2″-*Di*-O-*acetylerythralosamine* (8). A soln of 1·0 g of 7 in 30 ml pyridine and 6·0 ml Ac_2O was heated on a steam bath for 8 hr and then allowed to stand at. room temp for 8 days. The product, 8 (1·18 g) was isolated as a colorless foam by the method employed for the isolation of 10 above after treatment with Darco G-60. The product showed a single spot on TLC examination; IR, 1737 cm^{-1}, no OH absorption; PMR see Table 2; mass spectrum molecular ion at *m/e* 623·3669, calc 623·3669 for $C_{33}H_{53}NO_{10}$.

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