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# 8-*EPI*-ERYTHRONOLIDE B AND ITS METABOLIC FATE IN FERMENTATIONS OF *STREPTOMYCES ERYTHREUS*

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**Abstract** 8-*epi*-erythronolide B (4) has been isolated as a product formed from the acid treatment of erythronolide B (3). When this compound was fed to a blocked mutant of the erythromycin producing organism, *Streptomycetes erythreus* (Abbott 2NU153), nearly quantitative yields of 3-*O*-(α-L-mycarosyl)-8-*epi*-erythronolide B (10) were obtained. The much desired 8-*epi*-erythromycins (2) were not realized.

Recent years have seen an expansion of studies on the macrolide group of antibiotics as part of an increasing interest in altering the biological activity of this family of natural products. The modification of the erythromycin group of macrolide antibiotics (1) has been under study in our laboratory for several years. We are fortunate in having a ready supply of several biogenetic precursors and a number of point blocked mutants of the erythromycin producing organism *Streptomyces erythreus*, which do not synthesize lactone precursors but are capable of converting biogenetic intermediates into erythromycins. One approach we have used to attempt to produce new novel macrolide antibiotics has involved the feeding of modified biogenetic precursors to these blocked mutants.

The report of Celmer[1] that 8,8a-deoxy-8-*epi*-oleandomycin, a 14-membered ring macrolide antibiotic similar to erythromycin, had considerable more activity against erythromycin resistant strains of *Staphylococcus aureus* than the opposite C-8 configurational isomer increased our interest in synthesizing the 8-*epi*-erythromycins (2).* Early work by Perun[2] suggested that under suitable conditions the acid catalyzed transformation of the aglycone of erythromycin B, erythronolide B (3),† might lead to formation of 8-*epi*-erythronolide B (4), a possible substrate for transformation by blocked mutants. This paper is concerned with the formation, isolation, and identification of 8-*epi*-erythronolide B (4) and its metabolic fate in fermentations of blocked mutants of *S. erythreus*.

Perun[2] observed that the treatment of erythronolide B (3) with methanolic hydrochloric acid results in a complex mixture of products. Modification of the early conditions gave a crystalline material which when examined by TLC showed the presence of three major components and a fast moving trace substance. Comparison with authentic samples enabled the identification of starting material (3) and 8,9-anhydroerythronolide B 6,9-hemiacetal (5).[4] The trace component, although not isolated, was thought to be the dienol ether 6. The substance that migrated slightly ahead of erythronolide B (3) remained unidentified.

The unidentified material, $C_{21}H_{36}O_7$, isolated by chromatography, showed UV absorption at 283 nm (ε47). The compound, later found to be 8-*epi*-erythronolide B (4), gave an IR spectrum which showed OH (3480 and 3575 cm$^{-1}$) and CO (1705 cm$^{-1}$) absorption. The work of Perun[2] and the identification of 8,9-anhydroerythronolide B 6,9-hemiacetal (5) in the acid catalyzed reaction mixture suggested that the new substance might be epimeric to 3 at C-8. Epimerization of C-8 would be easily accommodated *via* the enol ether 5. Treatment of 4 with glacial acetic acid gave 8,9-anhydroerythronolide B 6,9-hemiacetal (5) and starting material in addition to smaller quantities of erythronolide B (3). These results verify the early supposition that the new compound probably differs from 3 at C-8.

The NMR spectrum of 4 in pyridine-$d_5$ (Fig 1) was typical of those obtained for erythromycin aglycone derivatives and very similar to that of erythronolide B (3). The C-Me resonances consist of five doublets, one triplet, and one singlet, and the resonances of four ring protons (H-3, H-5, H-11 and H-13) are observed downfield of 4·0 ppm. These observations clearly indicate that the

*After completion of this work, a subsequent publication by Celmer[1a] reversed early configurational assignments and thereby attributed the increased activity against resistant strains to an isomer with natural C-8 configuration.

†The configurational notation of macrolides used in this paper is different from that used previously at positions 3, 6, 10 and 13. This change at the inward directed bonds has been made to conform to the notation used by Celmer.[1a]

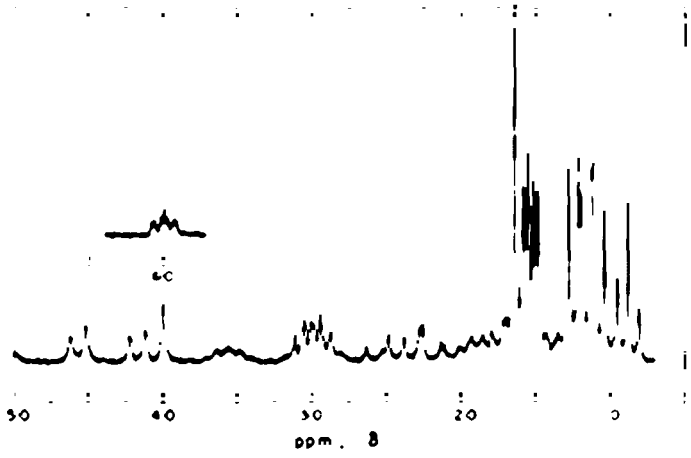



Fig. 1. NMR spectrum of 8-*epi*-erythronolide B (4) in pyridine-$d_5$ solution.

aglycone carbon skeleton has been unchanged and the acid catalyzed degradations previously encountered with erythronolide B[3] have not occurred.

A comparison of the chemical shifts and coupling constants of the ring protons of 4 with those of erythronolide B (3) (Table 1) shows that both aglycones have the same number of ring protons and the same substitution. For example, the doublet H-5 resonances indicate that C-6 must be tertiary in both compounds. The chemical shift of resonances attributable to H-8 and H-10 indicate that a C-9 ketone is present, and the chemical shifts of H-2 and H-13 offer evidence that the lactone CO is unchanged and lactonization is maintained at C-13.

The above results rule out any major structural modification of the aglycone ring and lead logically to the conclusion that 4 and 3 differ only in the configuration of one or more asymmetric centers. Examination of Table 1 reveals a number of

Table 1. NMR parameters of erythronolide B (3) and 8-*epi*-erythronolide B (4)

| | Chemical shifts* ppm | | Coupling constants Hz | | |
|---|---|---|---|---|---|
| | 3 | 4 | | 3 | 4 |
| H-2 | 3.08 | 3.03 | $J_{2,3}$ | 10.4 | 10.5 |
| H-3 | 4.18 | 4.17 | $J_{3,4}$ | 1.3 | 1 |
| H-4 | 2.66 | 1.89 | $J_{4,5}$ | 2.9 | 1 |
| H-5 | 4.22 | 4.00 | $J_{5,6,7a}$ | 14.2 | 14.2 |
| H-7a | 2.38 | 2.51 | $J_{7a,7e}$ | 7.4 | 10.2 |
| H-7e | 1.81 | 2.19 | $J_{7e,8}$ | 6.4 | 2.1 |
| H-8 | 3.1 | 3.56 | $J_{8,10}$ | 2.0 | 1 |
| H-10 | 3.20 | 2.91 | $J_{10,11}$ | 10.0 | 10.0 |
| H-11 | 4.48 | 4.57 | $J_{11,12}$ | 1.2 | 2.2 |
| H-12 | 1.? | 1.? | | | |
| H-13 | 5.92 | 5.99 | | | |

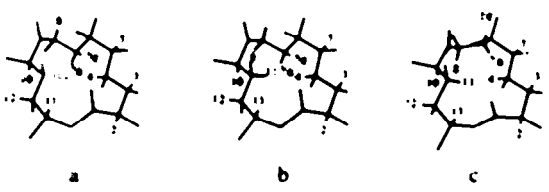
*Determined from $C_5D_5N$ solution at ambient probe temperature after addition of sufficient $D_2O$ to remove OH resonances

significant changes in chemical shifts and coupling constants which could conceivably arise from epimerization. However, since these differences can arise from both configurational and conformational changes, both factors must be considered before any conclusions can be reached.

The magnitude of $J_{2,3}$ and $J_{11,12}$ remain large indicating that the *anti*-periplanar relationships between the interacting protons known to be present in 3[3] have not been altered in 4. This suggests that the configurations of C-2, C-3, C-11, and C-12 are also unchanged. The consistency of the chemical shifts of these protons confirms that the configurations of these centers have been unaltered and no significant change in conformation has occurred in the corresponding ring segment. Numerous differences are seen in the C-4 to C-10 ring segment localizing the epimerization(s) in this portion of the aglycone.

The asymmetric centers $\alpha$ to the C-9 CO group are the most likely sites of epimerization under the experimental conditions employed and of these C-8 is indicated through the possible intermediacy of 8,9-anhydroerythronolide B 6,9-hemiacetal (5).[3,4] The chemical shift and coupling constant data for 4 compared to 3 confirm this supposition and also reveal a conformational change following epimerization.

The conformation of erythronolide B (3) which has been determined by NMR and CD studies[3] (Fig. 2a) places the C-8 Me group in an orientation which does not suffer any conformationally unfavorable interactions. However, epimerization of C-8 without concomitant conformational reorganization (Fig. 2b) places this Me into a *syn*-periplanar relationship with the 6-Me group. This unfavorable relationship can be removed by conformational reorganizations which involve rotations of the C-6 substituents either "inward" or "outward" from the center of the aglycone ring with



a b c

Fig 2 Photographs of Framework Molecular Model constructions of aglycone conformations Solid lines represent C–C and C–H bonds while outlined lines represent C–O bonds. The position of ring protons is given by the appropriate numbers Protons associated with methyl and hydroxyl groups are not shown for clarity (a) The solution conformation of erythronolide B (3) (b) The equivalent conformation of 8-*epi*-erythronolide B (4) (c) The solution conformation of 4 incorporating a conformational reorganization

corresponding opposite rotation of the 7-methylene group These rotations twist the C-6 to C-9 segment of the ring and remove the *syn-periplanar* relationship between the C-6 and C-8 Me groups.

A conformational reorganization involving the C-6 to C-9 ring segment has previously been encountered in the case of 11-acetylerythronolide B (7)[3] In this compound the reorganization has been shown to involve the "inward" rotation of the C-6 OH group with a corresponding "outward" rotation of the 7-methylene group resulting in a flattening of the C-6 to C-9 ring segment.

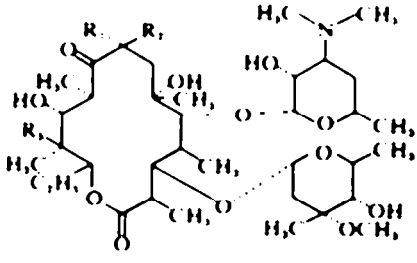
A similar reorganization in the case of 4 (Fig 2c) is consistent with the NMR data (although these data do not rigorously rule out an alternate reorganization) This reorganization offers good evidence that C-8 epimerization has occurred and supplied the necessary driving force The couplings of H-8 in 4 with the lowfield and highfield 7-methylene protons are 10 2 and 2 1 Hz respectively and are those which would be predicted from Fig 2c These couplings are, however, the opposite of those observed with the 11-acetate 7 (3 2 and 11 6 Hz respectively) which also assumes a conformation analogous to Fig 2c The reversal of the magnitudes of the two couplings is totally consistent with C-8 epimerization. This interpretation requires the assumption that the downfield 7-methylene proton resonance in the spectra of both compounds (Table 1) arises from the same proton (H-7a).

The significant change in the chemical shift of H-4 in 4 compared to 3 can be attributed to a decrease in the steric deshielding arising from the 7-methylene group when this group is rotated away from the center of the ring. An analogous upfield shift of H-4 was observed in the spectra of 7 but was of a smaller magnitude suggesting that C-8 epimerization also has a significant effect. A decrease in the steric deshielding of H-10 by the 8-Me group of 4 is responsible for the upfield shift of this ring proton Changes in the remaining ring proton coupling constants are minor and a result of additional slight reorganizations required to accommodate the flattening of the C-6 to C-9 ring segment.

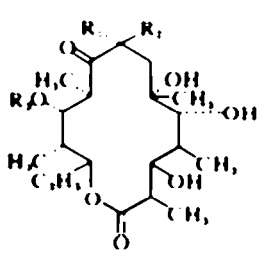
Finally chemical evidence has been obtained to rigorously eliminate the possibility of epimerization at centers other than C-8 It has been suggested[2] that there is a definite possibility of an interconversion of 5 to 8 *via* a prototropic shift thus accommodating a possible C-10 epimerization. Since acetic acid treatment of 3 and 4 results in the isolation of the same enol ether 5 as evidenced by identical TLC mobility, IR and NMR spectra, C-10 epimerization in 4 can be ruled out.

The addition of 8-*epi*-erythronolide B (4) to fermentations of early blocked mutants of *S. erythreus*, capable of converting known erythromycin macrolide progenitors to the complete antibiotic, did not result in significant antimicrobial activity in whole fermentation broths The examination of the broths indicated that the added 4 was missing and that a new, major faster moving compound was present along with several very minor components usually found in normal broths.

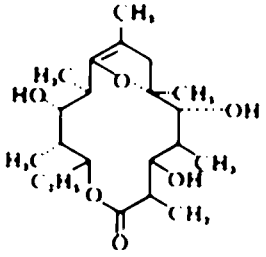
The new compound was easily isolated by column chromatography of the crude yellow oil obtained by ethyl acetate extraction of the clarified broth. The isolated white crystalline substance gave an elemental analysis that indicated $C_{37}H_{66}O_{10}$ as the



| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 1a | $CH_3$ | H | OH |
| 1b | $CH_3$ | H | H |
| 2a | H | $CH_3$ | OH |
| 2b | H | $CH_3$ | H |



| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| 3 | $CH_3$ | H | H |
| 4 | H | $CH_3$ | H |
| 7 | $CH_3$ | H | $CH_3CO$ |



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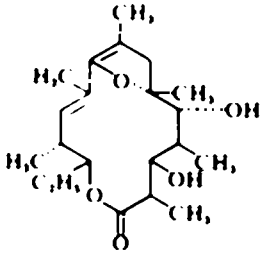

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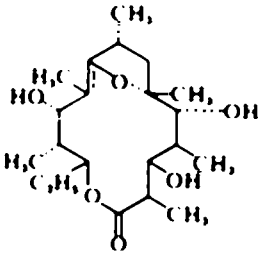

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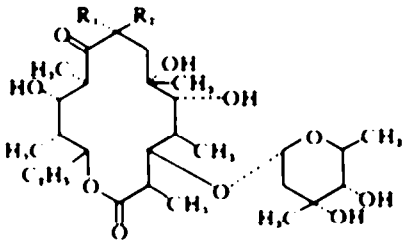

| | $R_1$ | $R_2$ |
|---|---|---|
| 9 | $CH_3$ | H |
| 10 | H | $CH_3$ |

molecular formula. This was substantiated by the mass spectra which showed a small molecular ion peak at *m/e* 546 that was verified by metastable defocussing. In the IR spectrum the lactone band at 1708 $cm^{-1}$ was consistent with the presence of the intact macrolide ring.

Examination by TLC of the products formed by mild acidic methanolysis of the compound showed the presence of 8-*epi*-erythronolide B (4), erythronolide B (3), 8,9-anhydroerythronolide B 6,9-hemiacetal (5), and the $\alpha$ and $\beta$ anomers of 1-methylmycaroside. Treatment of 3-O($\alpha$-L-mycarosyl)erythronolide B (9), which was available from a previous study,[6] under the same conditions gave an identical spectrum of products although in different ratios. At this point it was obvious that the 8-*epi*-erythronolide B (4) added to the *S. erythreus* fermentations had been glycosylated with mycarose. From biogenetic considerations this was not unexpected and further suggests that the C-3 OH group of 4 was the probable point of sugar attachment.

Comparison of the NMR spectrum of the metabolite (10) with that of 3-O($\alpha$-L-mycarosyl)erythronolide B (9) clearly indicated the addition of the mycarose moiety. Most conclusive was the observation of a resonance at 5.03 ppm attributable to the anomeric H-1″ proton of mycarose (5.05 ppm in 9). In addition, resonances of sugar ring protons H-4″ and H-5″ were present at 2.97 and 3.95 ppm, respectively. Unfortunately severe resonance overlap of the aglycone ring protons prevented clear observation of the resonances of H-8 and the 7-methylene protons which would be required to determine the C-8 stereochemistry. Although the spectrum is very similar to that of 9, there are subtle changes in the chemical shifts of observable protons consistent with the expectation that the metabolite is 3-O($\alpha$-L-mycarosyl)-8-*epi*-erythronolide B (10).

Molecular rotation differences between glycosides and their aglycones have been useful in determining glycosidic bond configurations in the macrolide series.[6,7] The $[M]_D$ difference between 3-O-($\alpha$-L-mycarosyl)8-*epi*-erythronolide B and 8-*epi*-erythronolide B is −289°. This is very close to the $[M]_D$ difference (−284°)[6] between 3-O-($\alpha$-L-mycarosyl)-erythronolide B and erythronolide B and provides evidence that the anomeric configuration of 10 is $\alpha$. The NMR coupling constants for the anomeric proton ($J_{1''-2''}$ = 3·5, $J_{1''-2''}$ = 1) confirm the $\alpha$ configuration.

The conversion of 4 to 10 involves the addition, *via* a glycosidic linkage, of mycarose to the C-3 OH of the macrolide ring. The participation of mycarose instead of cladinose is reasonable since this mimics the reaction sequence occurring during normal erythromycin biosynthesis.[6,7] The blocked mutant *S. erythreus* (Abbott 2NU153) will normally convert 25 mg of erythronolide B (3) in 100 ml of fermentation medium to erythromycin A (1a) in 120 hr. TLC examination of fermentation broths of this same strain fed 8-*epi*-erythronolide B (4) indicates approximately the same mycarosyl glycosidation rate. It would appear from the high yields achieved in the conversion of 4 to 10 that the enzyme(s) responsible for attachment of mycarose do not recognize 8-*epi*-erythronolide B (4) as

an unnatural substrate. By analogy we assume that the enzyme(s) mediating the attachment of the amino sugar moiety desosamine have much more restricted substrate specificity requirements.

It should also be pointed out, however, that one must also be cognizant of the possibility that small quantities of 8-*epi*-erythromycin B (2) may have been produced but that the material is inactive or has very low activity against the test microorganism employed. In any case, the apparent strict substrate requirement for complete glycosidation may preclude the general use of blocked mutants to prepare modified erythromycins *via* a chemical-biological route using chemically modified erythronolides. However, these results do not rule out the possibility of preparing interesting mycarosyl glycosides from chemical or biological derived modified macrolide aglycones.

## EXPERIMENTAL

*General*—M ps, determined with a Thomas-Hoover Uni-Melt, are corrected. UV spectra were recorded for 95% EtOH solns with a Cary Model 11 spectrophotometer. Optical rotations were measured in MeOH with a Hilger and Watts polarimeter. IR spectra were recorded on $CDCl_3$ solns with a Perkin-Elmer Model 521 instrument. NMR spectra were obtained at 100 MHz using a Varian Associates HA-100 spectrometer. Chemical shifts are reported in ppm ($\delta$) downfield from internal TMS. Coupling constants were obtained by direct measurement and are reported in Hz. NMR parameters were determined from first order analysis and chemical shift and coupling constants assignments were confirmed by appropriate spin decoupling experiments whenever possible. TLC was performed on Merck Silica Gel G after Stahl using 95% EtOH–$CHCl_3$ 1:10 as the developing solvent unless noted otherwise. Compounds were visualized by spraying with the arsenomolybdate reagent of Nelson.[9] Silica gel for column chromatography was that of Merck, 70–230 mesh.

*8-epi-Erythronolide B* (4). A soln of 3 (2.0 g) in 55 ml EtOH and 27.5 ml 0.1 N HCl was allowed to stand at ambient air temp for 8–10 days. Evaporation under reduced pressure gave a colorless, crystalline residue (1.7 g) which by TLC showed starting material (*Rf* 0.27–0.33) and two other major substances (*Rf* 0.35–0.40, 0.47–0.55) of nearly equal quantities as adjudged by their intensity with Nelson reagent. Also observed in some preparations was a fast moving trace component (*Rf* 0.63–0.69) thought to be the dienol ether 6. The component with *Rf* 0.47–0.55 was found to be 5 by comparison with an authentic sample.[4] The only unidentified material in the mixture (*Rf* 0.35–0.40) was isolated by chromatography on a silica gel column (3.5 × 35 cm) prepared in $C_6H_6$ and eluted with increasing concentrations of MeOH in $C_6H_6$. Although most of 3 and 4 were eluted together a number of fractions contained only 4. Concentration and recrystallization of these fractions from MeOH–$H_2O$ gave 279 mg of prisms of broad mp 170–177°. $[\alpha]_D^{25}$ 81° (c 1.0 MeOH). NMR see Table 1. The UV absorption spectrum had a single peak, $\lambda$ max 283 m$\mu$, $\epsilon$ 47. The IR spectrum showed bonded and free absorption at 3480 and 3575 $cm^{-1}$ and CO absorption at 1705 $cm^{-1}$ (Found: C, 62.39; H, 9.57. Calc for $C_{21}H_{38}O_7$: C, 62.66; H, 9.52%).

*8,9-Anhydroerythronolide B 6,9-hemiacetal* (5) *from 8-epi-erythronolide B* (4). Compound 5 was prepared from 4 by modification of the conditions employed by Kurath and Egan[4] for the preparation of 5 from 3. A soln of 265 mg of 4 in glacial AcOH (2 ml) was allowed to stand at ambient temp for 6 hr. The mixture was slowly added to 50 ml of cold excess $NaHCO_3$ aq and then extracted with $CHCl_3$. The $CHCl_3$ extract was washed, dried ($MgSO_4$), and evaporated leaving a pale yellow froth (258 mg). Examination of the material by TLC and comparison with authentic samples showed the presence of starting material (4), 5 and a trace of 3. The froth was chromatographed on a silica gel column (2.5 × 32 cm) prepared in $CHCl_3$. Elution with increasing concentrations of MeOH in $CHCl_3$ gave 5 an oil (42 mg), identical with an authentic sample prepared by Kurath[4] (IR and NMR). Further elution yielded starting material (4) as needles (51 mg) m.p. 167–175°.

*Biological conversion of 8-epi-erythronolide B* (4) *to 3-O-(α-L-mycarosyl)-8-epi-erythronolide B* (10). The general procedures and fermentation conditions have been previously described.[4,10] In a typical experiment finely divided 4 (200 mg) was equally distributed into 8 500 ml Erlenmeyer fermentation flasks each containing 50 ml of a 48 hr culture of the blocked mutant *S. erythreus* (Abbott 2NU153). The characteristics of this strain have been previously described.[4] Incubation with shaking was continued for 144 hr, then the fermentation broth was clarified as usual.[4] The clarified broth of pH 7.5 was extracted two times with 1/2 volumes of EtOAc. The combined extracts were washed two times with water and dried ($MgSO_4$). Concentrations gave 246 mg of viscous yellow oil. Examination of the oil by TLC showed absence of starting material 4 and presence of a major fast moving substance of *Rf* 0.33–0.40. The oil was chromatographed on a column of silica gel (2.0 × 35 cm) prepared in $CHCl_3$. Elution with increasing concentrations of MeOH in $CHCl_3$ eluted fractions containing the component with *Rf* 0.33–0.40. These fractions were concentrated to dryness yielding 181 mg of pale yellow oil. Crystallization from EtOAc–hexane gave 142 mg of **10** as colorless needles, mp 198–200°, $[\alpha]_D^{25}$ −112° (c 1.0 MeOH). NMR ($CDCl_3$, 55°) $\delta$ 5.50 (H-13, $J_{13,14}$ = 1, $J_{13,14'}$ = 5.5 and 9.5), 5.03 (H-1', $J_{1',2'}$ = 3.5, $J_{1',3'}$ = 1), 3.95 (H-5', $J_{4',5'}$ = 9.5), 3.76 (H-3 and H-11, $J_{2,3}$ = $J_{11,12}$ = 10, $J_{3,4}$ = $J_{10,11}$ = 1), 3.48 (H-5, $J_{4,5}$ = 1), 2.97 (H-4'), 2.7–2.9 (H-2, H-8, H-10), 2.32 (H-4), 1.37 (5'-$CH_3$), 1.35 (6-$CH_3$), 1.27 (3'-$CH_3$).

*Methanolysis of 3-O-(α-L-mycarosyl)-8-epi-erythronolide B* (10). The glycoside **10** (1 mg) was treated for 3 hr with 1% HCl in MeOH (0.2 ml) and the products compared by TLC with authentic samples of 3, 4, 5, and α- and β-L-methylmycaroside. A similar quantity of **9** was treated and examined in a like manner. The TLC was carried out on Merck Silica Gel G using the following solvent systems: $CHCl_3$–95% EtOH, 10:1, $CHCl_3$–$C_6H_6$–MeOH–$NH_4$, 80:20:5:5, $CH_2Cl_2$–95% aqueous MeOH–$NH_4$, 90:10:1. Methanolysis of **9** and **10** both gave an identical array of products as listed above. As expected, the only difference noted was the ratio of the individual hydrolytic components.

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