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Studies on the Biosynthesis of the Erythromycins. I. Isolation and Structure of an Intermediate Glycoside, 3-α-L-Mycarosylerythronolide B*

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ABSTRACT: A neutral glycoside, elaborated in large quantities, has been isolated from the fermentation beers of a blocked mutant of *Streptomyces erythreus*. Recovery and purification of the substance can be easily effected by solvent extraction and crystallization. Cofermentation and feeding of the glycoside to a second blocked mutant established that the compound is an intermediate in the erythromycin biosynthetic pathway. The structure of the material has been determined to be 3-α-L-mycarosylerythronolide B.

The isolation and chemistry of the erythromycins, antibiotic glycosides produced by fermentation of *Streptomyces erythreus*, has been well documented (Wiley *et al.*, 1957, and previous papers in this series by investigators of Eli Lilly and Co.). Although some progress has been made toward elucidating the biosynthetic pathway, most of the process is still unknown. Various investigators have reported that *S. erythreus* incorporates ^{14}C-labeled propionate without randomization into the macrocyclic lactone portion of the antibiotic (Kaneda *et al.*, 1962; Corcoran *et al.*, 1960). Studies by Corcoran (1964) suggest that the intact carbon chain of D-glucose is the precursor of the deoxy sugars, desosamine and cladinose, found in the erythromycins. Corcoran (1961) also reported that methionine was involved in the *C*- and *O*-methylation of cladinose. Tardrew and Nyman (1964) isolated erythronolide B, and Hung *et al.* (1965) established that the compound, the aglycone of erythromycin B, was an intermediate in the biosynthesis of erythromycins A–C. In this communication we wish to report the isolation and structure of an erythromycin biosynthetic intermediate, 3-α-L-mycarosylerythronolide B.

Experimental and Results Section[1]

Fermentation Organism. The strain employed in this investigation was *S. erythreus* (Abbott 8EI57). This variant was derived by treatment of a high erythromycin-yielding strain with ethylenimine followed by ultraviolet irradiation. The mutant has a complete block in the erythromycin biosynthetic pathway and accumulates large quantities of the intermediate glycoside in fermentation beers. The compound is devoid of antibiotic activity against *Bacillus subtilis*.

Fermentation Procedures. Seed cultures of variant 8EI57 were prepared in a medium consisting of (in grams per liter) glucose monohydrate (Cerelose), 15.0; soybean meal, 15.0; and $CaCO_3$, 1.0. The cultures were incubated at 32° for 72 hr on a rotary shaker. The seed was added at a level of 3–5% (v/v) into 500-ml erlenmyer flasks containing 50 ml of a chemically defined fermentation medium consisting of the following components (in grams per liter): glucose monohydrate (Cerelose), 10.0; corn starch, 40.0; glycine, 7.5; L-tyrosine, 0.9; triolein, 2.5; NaCl, 2.0; K_2HPO_4, 1.56; KH_2PO_4, 0.78; $MgSO_4 \cdot 7H_2O$, 0.50; $CoCl_2 \cdot 6H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.02; $MnCl_2 \cdot 4H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.05; and $CaCO_3$, 3.0. The potassium phosphate salts were sterilized and added separately. The fermentation flasks were incubated at 32° on a rotary shaker (280 rpm) for 168 hr.

Recovery from Fermentation Beer. Fermentation beer (2650 ml) was centrifuged to sediment the mycelium and the supernatant was recovered. To the supernatant was added with stirring an equal volume of an aqueous

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[1] Nuclear magnetic resonance spectra were determined as 10% solutions in deuteriochloroform with a Varian A-60 spectrometer. Infrared spectra were determined as chloroform solutions.

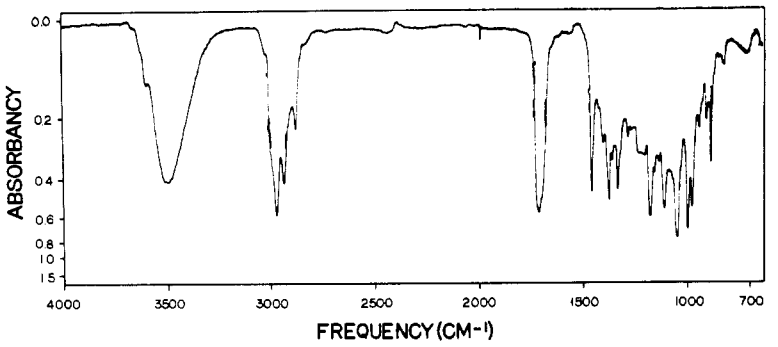


FIGURE 1: Infrared spectrum of 3-α-L-mycarosylerythronolide B (in chloroform solution).

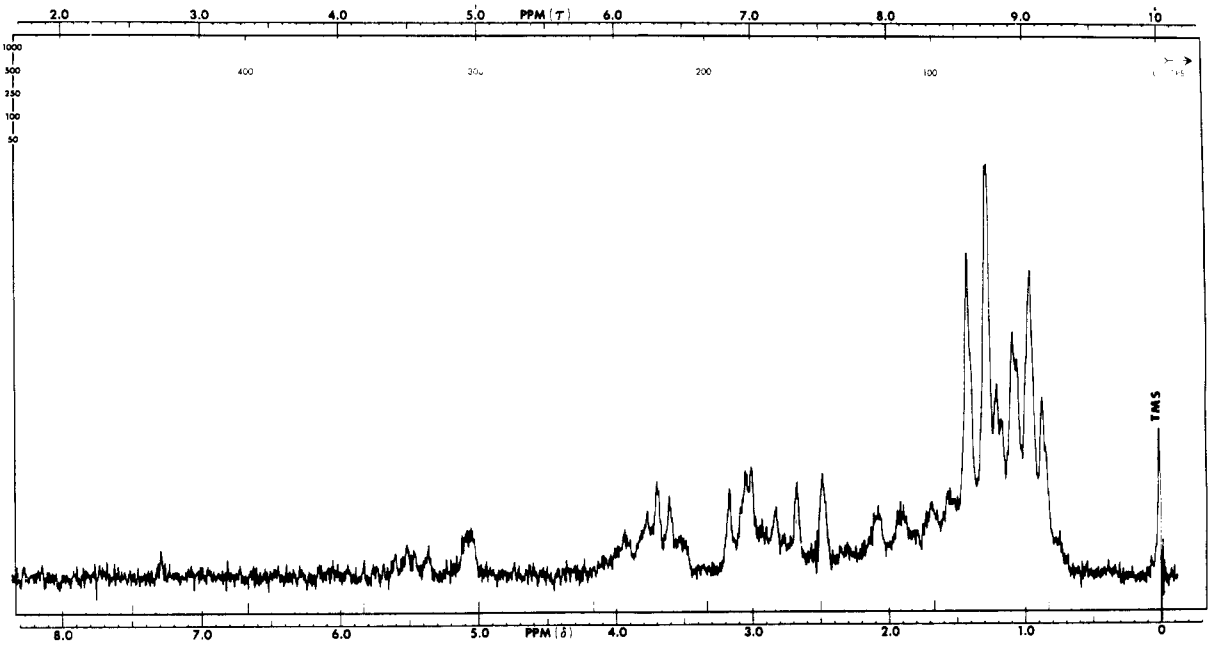


FIGURE 2: Nuclear magnetic resonance spectrum of 3-α-L-mycarosylerythronolide B (in deuteriochloroform solution).

solution of 10% zinc sulfate followed by an equal volume of 0.5 N sodium hydroxide. A filter aid, Dicalite, was added and the mixture was stirred for 5 min. The mixture was filtered and the clear filtrate of pH 6.6 was collected. The filtrate was extracted twice with equal volumes of ethyl acetate. The combined ethyl acetate extract was dried over anhydrous sodium sulfate and concentrated under reduced pressure to a small volume. Hexane was added and the material was allowed to crystallize. The crystalline material was collected by filtration and recrystallized twice from ethyl acetate–hexane and once from diethyl ether. The yield was 2.1 g of colorless, fine needles, mp 201–203°, $[\alpha]^{26}D$ − 72° (*c* 1.0, chloroform), $[\alpha]^{23}D$ −101° (*c* 0.54, methanol).

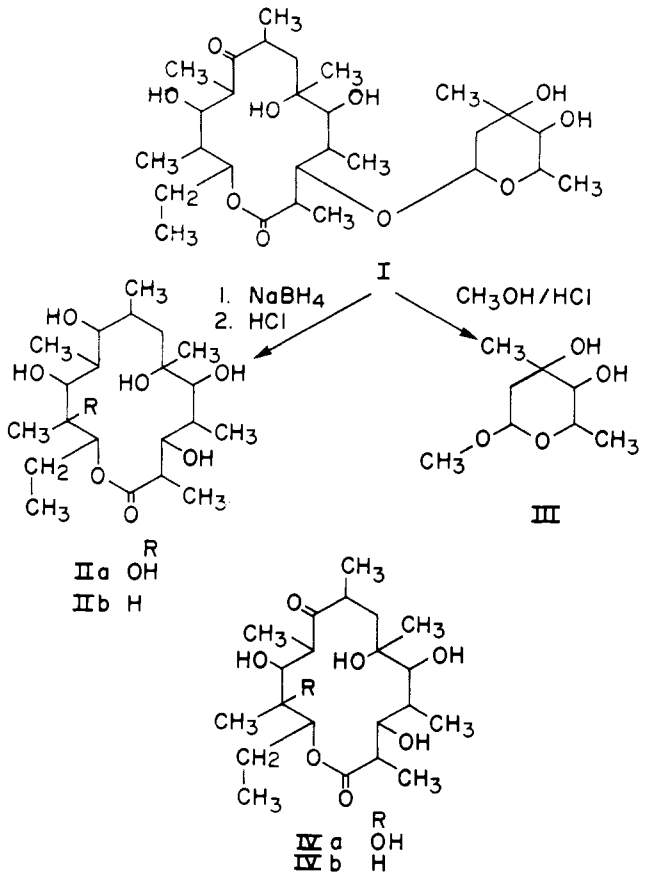
Physical Properties of the Glycoside I. The infrared spectrum (Figure 1) showed absorption at the following frequencies: 3600, 3500, 2975, 2940, 2880, 1720, 1700, 1460, 1430, 1410, 1380, 1360, 1340, 1290, 1180, 1160, 1140, 1115, 1055, 1005, 980, 950, 915, and 895 cm^{-1}. The ultraviolet absorption of the compound in ethanol had a maximum at 287 mμ and gave a molar extinction coefficient of 36. The nmr[2] spectrum (Figure 2) is similar to that of erythronolide B (IVb) and integrates for 48–50 protons. The addition of deuterated water indicated the presence of at least three exchangeable hydrogens.

Anal. Calcd for $C_{28}H_{50}O_{10}$: C, 61.51; H, 9.22; O, 29.27. Found: C, 61.61; H, 9.23; O, 29.51.

Reduction and Hydrolysis of the Glycoside. ISOLATION OF DIHYDROERYTHRONOLIDE B (IIB) (Scheme I). The glycoside (500 mg) was dissolved in 25 ml of methanol. Sodium borohydride (250 mg) in 25 ml of methanol was added and the solution was stirred for 50 min at room temperature. The reaction mixture was neutralized

[2] Abbreviation used: nmr, nuclear magnetic resonance.

SCHEME I



with 1 N hydrochloric acid and 25 ml of water was added. The solution was extracted twice with equal volumes of chloroform; the chloroform extracts were washed with water and saturated sodium chloride solution. The chloroform extract was dried over anhydrous magnesium sulfate and evaporated to dryness under reduced pressure to give 450 mg of white solid, mp 185–190°. The solid was dissolved in 10 ml of aqueous methanol containing 0.1 N hydrochloric acid. The solution was refluxed for 1 hr, cooled, and allowed to stand for 24 hr. Chloroform was added and the solution was shaken with a solution of 10% sodium carbonate. The sodium carbonate wash was extracted with chloroform and the chloroform extracts were combined and dried over anhydrous magnesium sulfate. The dried chloroform extract was evaporated to dryness yielding a viscous yellow oil which crystallized on the addition of acetonitrile. Recrystallization from acetonitrile gave 250 mg of crystals, mp 180–182°. The material was identified as dihydroerythronolide B by comparison with authentic material (Gerzon *et al.*, 1956). The mixture melting point with authentic dihydroerythronolide B was not depressed and the infrared spectrum was identical. Thin layer chromatography [silica gel G developed with methylene chloride–methanol–benzene–formamide, 80:10:20:1, v/v; spots were located with the arsenomolybdate reagent of Nelson (1944)] with authentic dihydroerythronolide A (IIa) and B gave an R_F value identical with that of dihydroerythronolide B.[3]

Anal. Calcd for $C_{21}H_{40}O_7$: C, 62.35; H, 9.97. Found: C, 62.34; H, 10.00.

Methanolysis of the Glycoside. ISOLATION OF METHYL MYCAROSIDE (III). The glycoside (4.2 g) was dissolved in 250 ml of methanol containing 1% hydrogen chloride. The reaction mixture was allowed to stand for 22 hr at room temperature and then poured into 75 ml of water containing 5.75 g of sodium bicarbonate. The solution was concentrated under reduced pressure to remove the methanol. The aqueous solution was extracted three times with 15-ml portions of chloroform. The chloroform extracts were combined, washed with 50 ml of water, and concentrated to dryness under reduced pressure leaving a viscous yellow oil. Distillation of the oil at 48–60° (0.3 mm) yielded a white solid. Sublimation of the solid gave 164 mg of white crystals, mp 54–56°, $[\alpha]^{25}D$ −141° (*c* 1.0, chloroform), $[\alpha]^{23}D$ −146° (*c* 0.89, methanol). The reported melting point of the crystalline anomer of methyl mycaroside is 60.5–61.0°, $[\alpha]^{25}D$ −141° (Regna *et al.*, 1953).

Anal. Calcd for $C_8H_{16}O_4$: C, 54.53; H, 9.15; O, 36.32. Found: C, 54.59; H, 9.26; O, 36.05.

Conversion of the Glycoside to Erythromycin by a Blocked Mutant of S. erythreus. The glycoside is readily converted to erythromycin when added to the fermentation medium of a blocked mutant (Abbott 2NU153) of *S. erythreus* or when strain 8EI57 is cofermented with strain 2NU153. Mutant 2NU153, derived from a high erythromycin-producing strain of *S. erythreus* by treatment with nitrogen mustard followed by ultraviolet irradiation, has a complete block early in the erythromycin biosynthetic pathway and is unable to synthesize erythromycin unless fed an intermediate. When 25 mg of powdered glycoside was added to 50 ml of a 24-hr culture of 2NU153 in synthetic fermentation medium, the beer assayed at 168 hr, by the agar diffusion method, 458 μg/ml of antibiotic activity against *B. subtilis* using erythromycin A as a standard. If erythronolide B is fed to 2NU153, 3-α-L-mycarosylerythronolide B can be isolated from fermentation beers 24 hr after addition of the aglycone.

The nature of the antibiotic produced was established by paper chromatography and bioautography of the developed chromatogram with *B. subtilis* as the test organism and by isolation and comparison of the substance with authentic erythromycin A. Ascending chromatography of fermentation beers of 2NU153 to which the glycoside had been added was performed on Eaton-Dikeman 916 paper with the ammonium hydroxide saturated methyl isobutyl ketone system of Hung *et al.* (1965) and on Whatman No. 3MM paper using 2% potassium phosphate (dibasic) in water as the solvent system. The R_F value of the

[3] This hydrolysis was repeated with the nonreduced glycoside to give a product (isolated in 50% yield) which was shown to be mainly erythronolide B by the infrared spectrum and by thin layer chromatographic comparison with authentic erythronolide B.

antibiotic substance was identical in both systems with authentic erythromycin A.

Crystalline erythromycin A was isolated from 168-hr fermentation beer of 2NU153 to which the glycoside had been added at a level of 500 μg/ml at 24 hr. Fermentation beer (1100 ml) was filtered and the filtrate was extracted with 1 volume of chloroform after the pH had been adjusted to 9.5. The chloroform extract was extracted twice with equal volumes of 0.1 M phosphate buffer of pH 4.5. The combined buffer extract was adjusted to pH 9.5 with sodium hydroxide and extracted with an equal volume of methylene chloride. The organic extract was washed with water and dried over anhydrous sodium sulfate. The methylene chloride was removed under reduced pressure yielding 515 mg of solid. Crystallization from methylene chloride afforded 252 mg of white crystalline antibiotic. The infrared spectrum of the isolated antibiotic was identical with that of erythromycin A.

Discussion

3-α-L-Mycarosylerythronolide B (I), an erythromycin biosynthetic intermediate, was recovered from fermentation beer of a blocked mutant of *S. erythreus* by extraction and crystallization. The compound is a neutral macrolide with an infrared spectrum that resembles that reported for erythronolide B (Tardrew and Nyman, 1964), the aglycone of erythromycin B. The compound was demonstrated to be an intermediate between erythronolide B and the erythromycins, suggesting that the compound was either the hypothetical intermediate erythronolide A (IVa) or a neutral glycoside derivative of erythronolide A or B. The nmr spectrum of the compound was similar to that of erythronolide B but had additional resonance peaks. This indicated that the substance was not erythronolide A but probably a macrolide lactone with a sugar moiety. The nmr spectrum indicated that a methoxy group was not present showing that cladinose was absent.

Analytical data suggest the molecular formula, $C_{28}H_{50}O_{10}$, mol wt 546.69. The compound consumed 1.8 moles of periodate/mole indicating the presence of two 1,2-diol groups. Reduction with sodium borohydride and hydrolysis of the resulting substance yielded a sugar-free compound identical in all respects with dihydroerythronolide B. The isolation of the dihydro aglycone does not conclusively establish the position of the ketone function, but since the compound is a biosynthetic intermediate, it follows that the aglycone portion of the compound must be erythronolide B.

Methanolysis of the glycoside yielded a yellow oil containing a mixture of anomeric sugar glycosides. Fractional distillation of the mixture afforded one of the isomers as a crystalline solid. The nmr spectrum of this isomer was essentially identical with the reported spectrum of α-L-methyl mycaroside (Hofheinz *et al.*, 1962; Korte *et al.*, 1964). The identity was confirmed by comparing the melting point and optical rotation of the compound with those of the reported crystalline methyl mycaroside.

Combination of the information derived from nmr spectra, analytical, infrared, and specific rotation data, and degradation studies suggests 3-α-L-mycarosylerythronolide B as the structure of the erythromycin intermediate. The glycosidic bond at C-3 was not established by chemical means but is a logical assumption based on the direct biological conversion of the compound to erythromycin.[4]

The glycosidic linkage at C-3 was assigned the α configuration based on the nmr and molecular rotation methods discussed and exemplified by Celmer (1965b). The nmr spectrum of 3-α-L-mycarosylerythronolide B contains a broadened doublet at τ 4.95 ($J_{1,2} \simeq 2.0/<1$ cps) which is assigned to the proton on the anomeric carbon. The splitting pattern and J values are characteristic for α-glycosidic linkages in macrolide antibiotics (Celmer, 1965a).

Molecular rotations[5] of the macrolides and their sugar and aglycone moieties can be used to predict the configuration of the glycosidic bond when the difference between the molecular rotations of the α and β sugars are sufficiently large (Celmer, 1965b; Klyne, 1950). The difference in molecular rotation between the intact macrolide and its aglycone should approximate the molecular rotation of the correct sugar anomer. The $[M]_D$ of 3-α-L-mycarosylerythronolide B is $-552°$ and the $[M]_D$ of erythronolide B is -268.[6] The difference between these two ($-284°$) approximates the $[M]_D$ of α-methyl mycaroside ($-257°$) and is vastly different from that of the β anomer ($\sim+86°$). These data supported by the nmr spectrum indicate that the mycaroside linkage has the α configuration.

The isolation of 3-α-L-mycarosylerythronolide B allows certain conclusions to be drawn concerning the biosynthesis of the erythromycins. Thus, if there is only one pathway of erythromycin biosynthesis and if 3-α-L-mycarosylerythronolide B is a normal intermediate the following are established. (1) The aglycone erythronolide B and not the hypothetical intermediate erythronolide A accepts two sugars from unknown donors to become an antibiotic glycoside. (2) The sequence of sugar attachment is first the neutral sugar followed by the amino sugar. (3) Mycarose and not cladinose is the neutral sugar accepted by the aglycone.

The first point is contrary to the conclusions arrived at by Hung *et al.* (1956). These authors speculated that hydroxylation of erythronolide B in the 12 position occurs prior to glycosylation of the macrocyclic aglycone. It seems likely, however, that at least in some strains, glycosylation occurs before ring hydroxylation and 3-α-L-mycarosylerythronolide B is indeed an intermediate on the erythromycin biosynthetic pathway. Four mutants of *S. erythreus*, either totally or partially

[4] The possibility that the glycoside is attached at C-5 can be eliminated by the periodate oxidation results which indicate the presence of the C-5,C-6 diol group.

[5] Molecular rotations were obtained as methanol solutions.

[6] $[\alpha]^{22}D$ $-66.5°$ (c 1.0, methanol).

blocked, which accumulate the glycoside, have been isolated in this laboratory (J. R. Martin, unpublished observations).

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