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Microbial Glycosylation of Erythromycin A†

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Erythromycin A (compound 1) was inactivated by *Streptomyces vendargensis* ATCC 25507 in fermentation. The inactivation product was isolated and characterized by nuclear magnetic resonance and mass spectroscopy as 2′-(*O*-[β-D-glucopyranosyl])erythromycin A (compound 2). The MICs of compounds 1 and 2 were determined. Compound 2 lacked antibiotic activity when tested against several gram-positive pathogens, as well as *S. vendargensis*.

It has been proposed that resistance genes of pathogenic bacteria originate in antibiotic-producing microorganisms (3, 5, 17). For example, isoenzymes of aminoglycoside phosphotransferases and rRNA methylases conferring resistance to macrolide-lincosamide-streptogramin B antibiotics occur in both producers and pathogens (2, 15). Recently, resistance to lincomycin by nucleotidylation was found in clinically isolated staphylococci (5). Similar nucleotidylation of lincosamide antibiotics was first detected in *Streptomyces coelicolor* Müller (NRRL 3532) (1, 11). In addition, resistance to macrolide antibiotics in *Escherichia coli* was found to be conferred through a 2′-phosphotransferase system (13, 14). A similar mechanism of macrolide inactivation was noted previously in *S. coelicolor* Müller (9, 19). Although *S. coelicolor* Müller produces neither macrolides nor lincosamides, it is closely related to producers of these antibiotics and serves as an example of a producing-type microorganism possessing antibiotic resistance mechanisms similar to those found in pathogens.

Erythromycin A (Fig. 1; structure 1) is a commercially important, naturally occurring member of the macrolide class of antibiotics. The compound is composed of a polyfunctionalized 14-membered lactone ring substituted with desosamine and cladinose sugar units. Like the other macrolide antibiotics, compound 1 can be inactivated by microorganisms through phosphorylation (9, 10, 19). In this report, we present data indicating that compound 1 is glycosylated and inactivated by *Streptomyces vendargensis* and that the product of glycosylation is 2′-(*O*-[β-D-glucopyranosyl])erythromycin A (Fig. 1; structure 2). Such a glycosylation system could protect macrolide-producing microorganisms during antibiotic biosynthesis or be a mechanism of macrolide resistance in pathogens.

MATERIALS AND METHODS

Inactivation of erythromycin by *S. vendargensis*. *S. vendargensis* UC 5315 (ATCC 25507) was stored and maintained on sterile soils in the culture collection of The Upjohn Company. The organism was inoculated into a seed medium (GS-7) which contained Cerelose (C.P.C. International) and Pharmamedia (Procter and Gamble), each added at 25 g/liter of tap water. The medium was adjusted to pH 7.2 with NH_4OH and was autoclaved for 30 min. The inoculated 100-ml volumes of GS-7 were shaken at 250 rpm in 500-ml wide-mouthed fermentation flasks for 48 h at 28°C. The mature seed cultures were used as the source of inoculum (5% seed rate) for the fermentation medium. The latter was a modification of a medium reported by Coats and Argoudelis (6) and contained KH_2PO_4, 20.4 g; glucose, 20 g; NZ-amine B (Sheffield Chemicals), 5.0 g; yeast extract (Difco Laboratories), 2.5 g; $NaNO_3$, 1.5 g; and $FeSO_4$, 10 mg/liter of deionized water. After formulation, the medium was adjusted to pH 7.2 with NH_4OH and was sterilized by autoclaving. The medium was used in the manner described above for GS-7. At 24 h of fermentation, 5 mg of erythromycin A free base (Upjohn) dissolved in 0.1 ml of dimethyl sulfoxide (DMSO) was added to each 100-ml fermentation. The fermentation process was continued for 24 h before harvest. Loss of antibacterial activity of compound 1 was measured by a disk plate assay using *Streptococcus pyogenes* UC 6055 as the assay microorganism.

Determination of MICs. The MICs of compounds 1 and 2 for *S. vendargensis* were determined by growth inhibition in broth. The organism was inoculated into GS-7 and grown under the conditions described above for 72 h. A 1-ml sample of *S. vendargensis* was transferred into 100 ml of fermentation medium, which was then incubated as the secondary seed culture for 24 h. Samples (0.05 ml) of this culture were inoculated into 5-ml volumes of the fermentation medium contained in 25-ml flasks. The sterilized macrolides were added aseptically before inoculation at final concentrations ranging from 5 to 1,000 μg/ml. Growth inhibition was determined after incubation (250 rpm, 28°C) of *S. vendargensis* for 24 h in the presence of the macrolides.

Spectroscopic methods. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-300 spectrometer operated at 300 and 75 MHz for the ^1H and ^{13}C nuclei, respectively. Spectra were run in DMSO doped with D_2O and reported as parts per million relative to tetramethylsilane. Fast-atom bombardment–mass spectrometry spectra were obtained on a MAT CH-5 spectrometer operated in positive-ion mode. The two-dimensional ^1H-^1H correlation (COSY) and ^1H-^{13}C correlation (direct and long-range) spectra were obtained by the standard sequences. The multiplicities of carbon signals were determined by distortion enhancement polarization transfer (DEPT) experiments, also using standard sequences provided by the Bruker instrument.

The assignments of protonated carbons were made by examining the two-dimensional ^1H-^{13}C correlation spectrum with subsequent comparison to the published assignments

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† This publication is dedicated to the memory of Alexander D. Argoudelis.

‡ Deceased July 1988.

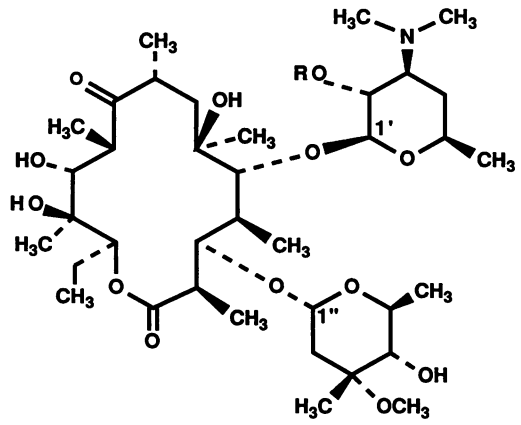

FIG. 1. Structures of compounds 1 (R = H) and 2 (R = glucose).

for compound 1 in $CDCl_3$ (7). The assignments of five quaternary carbons were based on chemical shift considerations and the results of the two-dimensional long-range C-H correlation experiment.

TLC analysis. Standards of compound 1 and samples containing the bioconverted product were assayed by thin-layer chromatography (TLC) using silica gel GF plates (Analtech) with ethyl acetate–methanol–25% (vol/vol) ammonia (75:20:5) as the mobile phase (8, 16). Detection was accomplished by spraying the plates with anisaldehyde-concentrated sulfuric acid-ethanol (1:1:18) and heating at 90 to 100°C for 5 to 10 min. R_f values of compounds 1 and 2 are 0.54 and 0.46, respectively, for this TLC system.

Isolation and purification of compound 2. The 15-liter fermentation broth containing compound 2 produced by microbial transformation of compound 1 was filtered at harvest pH using dicalite as a filter aid to obtain clear beer. The beer was passed over a column containing 500 ml of Amberlite XAD-2 (Rohm & Haas Co., Philadelphia, Pa.). The column was washed with 2 liters of water, followed by elution with 2 liters of acetone-water (70:30, vol/vol). Both the water wash and the acetone-water eluate were collected as single fractions. The eluate was concentrated to 600 ml before undergoing a triplicate extraction with 100-ml volumes of ethyl acetate, followed by a repeat triplicate extraction with 100-ml volumes of ethyl acetate and, finally, another triplicate extraction with 100-ml volumes of *n*-butyl alcohol. These three triplicate extractions yielded three pools of organic extract. Each was subsequently concentrated to dryness to give three samples of isolated material to which further purification procedures were applied, yielding compound 2 in high purity.

A lobar column (310 by 25 mm) prepacked with silica gel 60 (40 to 60 μm; EM Science, Cherry Hill, N.J.) was prepared for use by equilibration with 5 to 10 column volumes of a mobile phase consisting of ethyl acetate–methanol–25% (vol/vol) ammonia (85:10:5). The flow rate was approximately 10 ml/min. A total of 300 mg of the material isolated as described above was dissolved in 1 ml of mobile-phase solvent and loaded on the column. Isocratic elution ensued as 10-ml fractions were collected. Fractions containing compound 2, as evidenced by TLC analysis, were combined and concentrated to dryness. Repetitive runs on the lobar column were made with more starting material to obtain additional pure compound. For this to be accomplished, however, it became very important to thoroughly wash the column with 100% methanol (5 to 10 column volumes) preceding reequilibration, with the mobile-phase solvent in between runs. As a result, total yield of pure compound 2, a flaky white-to-yellow powder, was approximately 165 mg.

RESULTS AND DISCUSSION

Production and isolation of compound 2. The production of compound 2 is described in Materials and Methods. Since compound 1 spontaneously converts to its anhyro derivative at low pH, protection of compound 1 from acidity produced by *S. vendargensis* in the fermentation was necessary. This was accomplished through the addition of potassium phosphate to a medium used previously in our laboratories (19). Potassium phosphate added to a final concentration of 150 mM was found to be sufficient to maintain the fermentation pH at 6.5 throughout the transformation procedure.

Since compound 2 lacks in vitro antibacterial activity, its formation from compound 1 was monitored by measuring the loss of such antibacterial activity, as described in Materials and Methods. Isolation and purification of compound 2 was followed by TLC and the spray reagent, as described in Materials and Methods. Crude compound 2 was obtained from the beer by XAD-2 column chromatography followed by solvent extraction. Pure compound 2 was obtained from the crude preparation by repeated chromatographies using a lobar silica column, which was also described in detail in Materials and Methods. TLC of this material showed one major component.

Characterization and structure elucidation of compound 2. Compound 2 was isolated as an amorphous, colorless-to-slightly yellow material soluble in water, lower alcohols, and DMSO and slighly soluble in chloroform and ethyl acetate. Analytical data combined with molecular weight determination by positive-ion fast-atom bombardment–mass spectrometry indicated a molecular formula of $C_{43}H_{77}NO_{18}$ and molecular weight of 895. The molecular formula difference between compounds 1 and 2 suggests that compound 2 is probably a monosaccharide of compound 1. This notion was confirmed by the NMR results, since both ^1H and ^{13}C NMR spectra of compound 2 displayed patterns similar to those of compound 1. However, there were important differences. For example, the ^1H NMR spectrum of compound 2 had a doublet (J = 8 Hz) at 4.29 ppm which indicated the presence of an anomeric or anomericlike proton in compound 2. The rest of the signals in the ^1H NMR spectra were too extensively overlapped to allow detailed analysis. The ^{13}C NMR spectrum of compound 2 displayed the expected 43 signals, compared with 37 signals observed for compound 1. Furthermore, the six extra signals were made by one methylene carbon and five methine carbons, as determined by the DEPT experiment. Since the carbon signals of compound 1 were very closely spaced, it was not easy to assess which six signals were derived from the new fragment. This problem was solved by the two-dimensional NMR experiments, since the COSY results allowed the unambiguous assignment of all proton signals which, through C-H correlation experiments, further allowed the carbon assignments. Although assignments of ^1H and ^{13}C NMR signals of compound 1 in $CDCl_3$ solution were reported before (7), there were significant differences for ^1H NMR spectra, as observed in DMSO. We therefore conducted both COSY and C-H correlation experiments for compounds 1 and 2 in DMSO. The results indicated that carbons with chemical shift values 62.0 (t), 70.4 (d), 74.9 (d), 76.5 (d), 77.3 (d), and 105.5 (d) ppm were due to the new fragment. These values

TABLE 1. MICs of compounds 1 and 2[a]

Organism and UC no.	MIC (μg/ml) of compound:	
	1	2
Staphylococcus aureus 9218	0.5	>32
Staphylococcus aureus 9271	0.5	>32
Streptococcus (Enterococcus) faecalis 9217	2	>32
Streptococcus pneumoniae 41	0.06	32
Streptococcus pyogenes 152	0.03	16
Streptomyces vendargensis 5315	50	1,000

[a] MICs for *S. vendargensis* were determined as described in Materials and Methods. MICs for the remaining organisms were determined in broth by National Committee for Clinical Laboratory Standards guidelines (12).

are in excellent agreement with the reported chemical shift values of β-D-glucopyranoside (18). The size of the coupling constant (8 Hz) of the anomeric proton of the new fragment is also characteristic of the β configuration of a glucoside.

Other significant differences observed in the ^{13}C NMR data are the downfield shift of C-2′ (10 ppm) and the upfield shift (2 ppm) of the C-3′ carbon of the desosamine moiety. The size of the chemical shift change is consistent with the effect on the α carbon in going from the alcohols to the corresponding ethers (4). This result was also confirmed by a two-dimensional long-range C-H correlation experiment. It was found that the 2′-H of the desosamine moiety was long-range coupled to the C-1‴ of the glucose unit. Thus, the site of glycosylation of compound 1 has been unambiguously identified as the C-2′ position of the desosamine moiety. No other differences were observed in the ^{13}C NMR data between compounds 1 and 2, indicating that the rest of the molecule structure of compound 1 was not altered. Particularly, compound 1 did not convert to the anhydro form during isolation. The mass spectrum result also supported this notion. Therefore, the structure of compound 2 has been unambiguously demonstrated.

Bacterial growth inhibition by compounds 1 and 2. To compare the antibacterial activities of compounds 1 and 2, the MICs were determined for five gram-positive microorganisms and *S. vendargensis*. The results of these studies are presented in Table 1. As shown, glycosylation of compound 1 resulted in virtually complete loss of antibacterial activity.

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