ENZYMIC HYDROLYSIS OF ERYTHROMYCIN BY A STRAIN OF *ESCHERICHIA COLI*

A NEW MECHANISM OF RESISTANCE

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Escherichia coli BM2195 is highly resistant to erythromycin by inactivation of the antibiotic. We have determined the structure of the modified antibiotic by physico-chemical techniques including mass spectrometry, infrared spectrophotometry, ^{13}C nuclear magnetic resonance, and circular dichroism. The results obtained indicate that *E. coli* BM2195 resists erythromycin by the production of an erythromycin esterase which hydrolyzes the lactone ring of the antibiotic.

Escherichia coli, like most Gram-negative bacteria, is resistant to "low levels" of macrolides (minimal inhibitory concentrations (MICs) ≤ 250 μg/ml). We recently described an *E. coli* strain, BM2195, resistant to high-levels (MIC >2 mg/ml) of erythromycin and demonstrated that this new resistant phenotype was due to the constitutive synthesis of a plasmid-mediated erythromycin-modifying enzyme[1].

The genetic information located on the plasmid was transferred to *E. coli* BM694, and, using this strain, the modified antibiotic was isolated and its structure was determined; the resistance involves the production of an erythromycin esterase which hydrolyzes the lactone ring of the antibiotic.

Materials and Methods

Bacterial Strains and Plasmids

E. coli BM2506 which is highly resistant to erythromycin (MIC >1 mg/ml) and does not inactivate the antibiotic was a clinical isolate, identified and kindly provided by A. ANDREMONT, Institut Gustave-Roussy, Villejuif. *E. coli* BM694, prototroph, *gyrA*[2] and *E. coli* BM694/pAT63 were from our laboratory collection. Hybrid plasmid pAT63 consists of pBR322 (Tra^-, Mob^-, Ap^R, Tc^R) with a 1,660bp *Sau*3a insert of plasmid pIP1100 DNA encoding the erythromycin esterase[3].

Media

Brain-heart infusion broth and agar (Difco) were used. Disc agar diffusion susceptibility tests were done on Mueller-Hinton agar. All incubations were at 37°C.

Antibiotic

Erythromycin base was provided by Roussel-Uclaf.

Inactivation of Erythromycin by Resting Cells

Cells from 1 liter of an exponential broth culture of *E. coli* BM694/pAT63, at approximately 3×10^9 bacteria/ml, were harvested, washed once in 0.1 M phosphate buffer (pH 7.0 or 8.0), resuspended in 1 liter of the same buffer containing 1 mg/ml of erythromycin, and incubated for 3 hours at 37°C. The pH of this suspension, which remained constant, was monitored and inactivation of erythromycin was

verified by a microbiological technique[4]. The suspension was centrifuged and the supernatant containing the modified erythromycin was filtered through 0.45 μm pore filters and stored at 4°C.

Extraction and Purification of Inactivated Erythromycin

The supernatant containing inactivated antibiotic was extracted with chloroform to eliminate the intact antibiotic and exhaustively extracted with butanol to recover the products of inactivation of erythromycin. The butanol extract was evaporated to dryness under reduced pressure and stirred with methylene chloride. After filtration, the insoluble residue was washed with methylene chloride, dried, and designated fraction A. The solution was evaporated to dryness and designated fraction B.

According to this procedure, and starting from 1 g of erythromycin, we obtained, depending upon the pH used to perform the inactivation, the following recoveries:

pH	Fraction A	Fraction B
7.0	Trace	0.893 g
8.0	0.54 g	0.381 g

Identification of the Modified Compounds

Thin-layer Chromatography: Thin-layer chromatography was performed on precoated Merck silica gel plates 60 F 254, using pure methanol as solvent. Detection was by spraying ethanolic H_2SO_4 and heating at 100°C for 15 minutes.

Mass Spectrometry: Mass spectrometry measurements were obtained by field desorption on a Varian MAT 311A apparatus.

NMR: Natural abundance of ^{13}C NMR spectra were obtained on a Brucker 250 WM apparatus.

Circular Dichroism: The curves of circular dichroism were obtained on a D. C. 3 Jobin Yvon dichrograph.

UV Spectra: UV absorption spectra were obtained on a Cary 15 spectrophotometer.

IR Spectra: IR absorption spectra were obtained on Perkin Elmer 247 or 257 apparatus.

Results and Discussion

The compounds recovered after inactivation of erythromycin by *E. coli* BM694/pAT63, fraction A and B, were white solid substances that we did not attempt to recrystallize. Their properties were as follows:

Fraction A

Thin-layer Chromatography

The Rf of fraction A was 0.30 whereas that of erythromycin is 0.13.

UV Spectrum

After dissolution of fraction A in a mixture of ethanol and water (20: 80) we observed: a shoulder at 221 nm ($E^{1\%}_{1cm}$ 2), a maximum at 255 nm ($E^{1\%}_{1cm}$ 0.6).

Circular Dichroism

We noted, after dissolution in the same solvent as that for UV spectrum determination: maximum at 210 nm ($\Delta E^{1\%}_{1cm}$ -4×10^{-3}), maximum at *ca.* 252 nm ($\Delta E^{1\%}_{1cm}$ $+0.2\times10^{-3}$), maximum at *ca.* 290 nm ($\Delta E^{1\%}_{1cm}$ -0.1×10^{-3}).

Based on the data obtained by the latter two techniques, we concluded the presence of less than 0.5% of non conjugated ketone which may represent impurities.

IR Spectrum

In Nujol we noted the absence of lactone.

^{13}C NMR

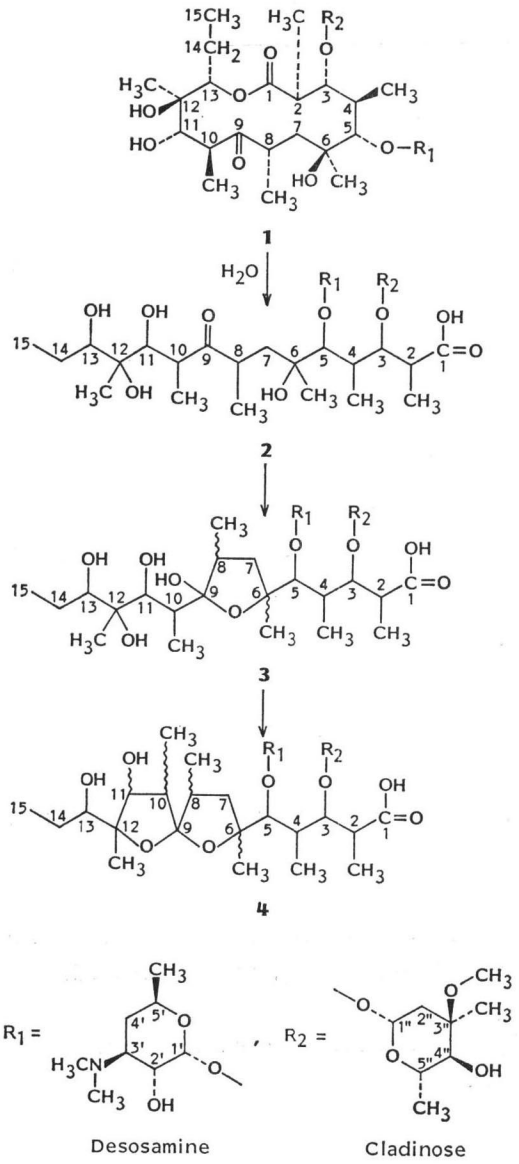
We observed: Absence of ethylenic carbons; 1 carbonyl at 186.4 ppm; 1 O–C–O at 103.6 ppm; 3 –C–O at 78.4, 76.7, 76.3 ppm; 10 >CH–O and 1 >CH–N at 105.3 (1′), 99.9 (1″), 87.8, 85.2, 80.1, 78.5, 72.7, 71.8, 68.1, 67.0 ppm; 1 OCH_3 at 52.0 ppm; 1 $N(CH_3)_2$ at 42.1 ppm; 4 –CH at 48.4, 42.8, 40.8, 37.4 ppm; 4 $>CH_2$ at 41.4, 37.9, 33.0, 23.5 or 23.4 ppm; 10 CH_3 at 23.5 or 23.4, 23.0, 22.8, 20.6, 20.3, 15.6, 14.9, 14.0, 13.7, 13.5 ppm.

The results of the different determinations are in agreement with the structure **3** shown in Fig. 1.

Fraction B

Fig. 1. Reaction catalyzed by the erythromycin esterase.

Structure of fractions A and B.



Thin-layer Chromatography

The Rf of fraction B was 0.26 whereas, as already mentioned, those of fraction A and erythromycin are 0.30 and 0.13, respectively.

UV Spectrum

After dissolution in ethanol, the UV spectrum observed for fraction B was not significantly different from that obtained with fraction A.

Circular Dichroism

Values determined in ethanol solution: maximum at 214 nm ($\Delta E^{1\%}_{1cm}$ -13×10^{-3}), maximum at 243 nm ($\Delta E^{1\%}_{1cm}$ $+0.4\times10^{-3}$), maximum at 265 nm ($\Delta E^{1\%}_{1cm}$ -0.8×10^{-3}).

Based on the data obtained by the latter two techniques, we concluded the absence of ketone and of an internal enolic ether Δ^{8-9} 9~12.

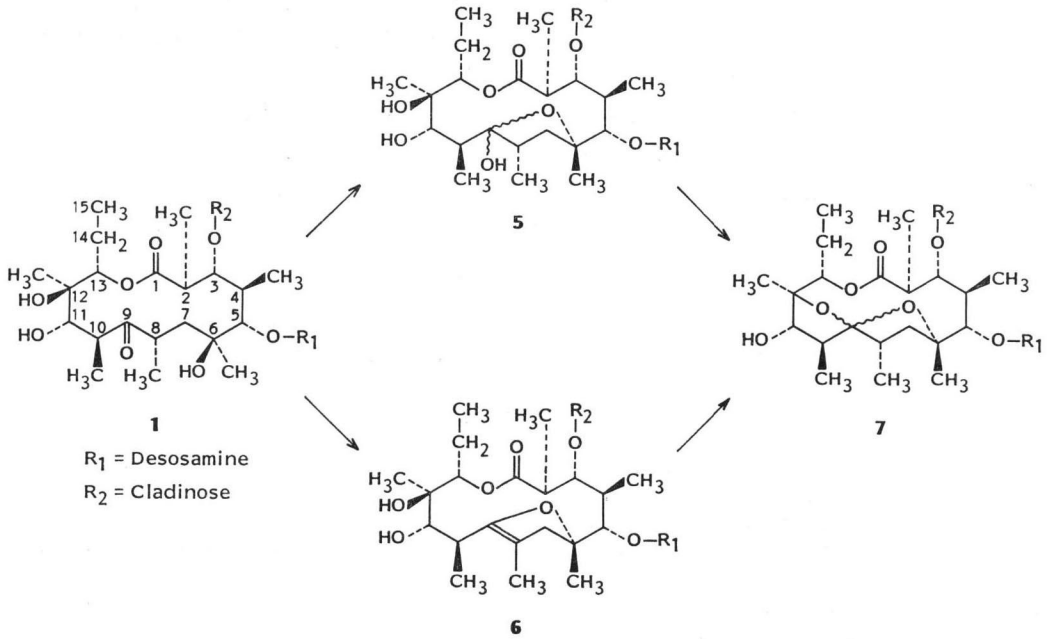
IR Spectrum

In chloroform solution we noted the absence of ketone and lactone and the presence of associated OH (3530 cm^{-1}) and very associated OH/NH (3320 cm^{-1}).

^{13}C NMR

We observed: 1 carbonyl at 186.9 ppm; 1 O–C–O at 116.9 ppm (C9); 3 –C–O at 89.2, 83.2 (C6 and C12), 76.2 (C3″) ppm; 11 >CH–O at 105.9 (C1′), 101.2 (C1″), 89.8, 88.0, 87.6, 80.0, 78.3, 71.6 (×2), 68.6, 68.4 ppm; 1 OCH_3 at 52.3 ppm; 1 $N(CH_3)_2$ at 41.9 ppm; 4 –C–H at 45.7, 44.7, 41.2, 39.3 ppm; 4 $>CH_2$ at 44.3 (C7), 38.1 (C2″), 32.2 (C4′), 25.4 (CH_2 ethyl) ppm; 10 CH_3 at 24.4, 23.7, 23.6, 22.8, 19.9, 15.1, 14.2 (×2), 13.3, 13.1 ppm.

Fig. 2. Formation of anhydroerythromycin according to STEPHENS and CONINE[5] and to KURATH *et al.*[6]



Mass Spectrometry

The mass spectrum obtained with fraction B showed a molecular peak at 734 (erythromycin 734).

The results of the different determinations are in agreement with the structure **4** shown in Fig. 1. This compound results from the dehydration at C-9 and C-12 of fraction A.

The structures of the products recovered after inactivation of erythromycin by *E. coli* strain BM-694/pAT63 indicate enzymic modification of the antibiotic. Plasmid pAT63 encodes an erythromycin esterase which cleaves the lactone group in the macrolide nucleus according to the reaction shown in Fig. 1. Similar experiments with antibiotic isolated from the culture medium of *E. coli* strain BM2506, highly resistant to erythromycin without modification of the antibiotic, indicated minimal chemical inactivation of the antibiotic. In these conditions, only a small amount of anhydroerythromycin was formed.

The stereochemistry of the new compounds were not examined, but one may assume that all the centers not involved in the reactions kept the natural erythromycin orientation.

According to Fig. 1, the inactivation products obtained, fractions A and B, differ from the product of the simple enzymic hydrolysis of the lactone **2**, because two spontaneous chemical reactions occur after the enzymic step: hemiketalization between the C-9 keto and the hydroxyl group of C-6 in the opened macrolide nucleus gives fraction A (**3**). Then dehydration between the hydroxyl groups of C-9 and C-12 of the hemiketal fraction A leads to fraction B (**4**).

Similar chemical dehydration reactions have been described by STEPHENS and CONINE[5] and by KURATH *et al.*[6] (Fig. 2). By acid treatment of the intact antibiotic **1**, these authors obtained the 9,12-anhydro 6,9-hemiketalerythromycin (**7**), either through the 6,9-hemiketalerythromycin (**5**) or the 8,9-anhydro 6,9-hemiketalerythromycin (**6**). In our case, by dissolving the hemiketal of the hydrolyzed lactone (fraction A) in diluted aqueous hydrochloric acid, we obtained a mixture of unchanged fraction

A and another compound indistinguishable in thin-layer chromatography from fraction B.

Until recently, microbial degradation of macrolide antibiotics has been detected in strains of *Streptomyces*, *Lactobacillus* and *Pseudomonas* (for a recent review, see[7]). However, the biochemical mechanisms and the genetic basis of the bacterial detoxification of macrolides has been poorly studied.

In the case of erythromycin, FELDMAN *et al.*[8] noted inactivation of the antibiotic by steroid-transforming strains of *Streptomyces*. More recently, FLICKINGER and PERLMAN[9] described a strain of *Pseudomonas* which enzymically degraded erythromycin. Starting from erythromycin A, they obtained erythronolide, erythralosamine, and two products which were not identified.

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