# STRUCTURE OF A PHOSPHORYLATED DERIVATIVE OF OLEANDOMYCIN, OBTAINED BY REACTION OF OLEANDOMYCIN WITH AN EXTRACT OF AN ERYTHROMYCIN-RESISTANT STRAIN OF *ESCHERICHIA COLI*

Sir:

Strains of Enterobacteriaceae, highly resistant to erythromycin (EM), have been isolated in clinical settings, most often after therapy with EM. It has been reported that there exist two genes, *ere* A and *ere* B, which encode an EM esterase and/or the *erx* (*erm*) gene which encodes an rRNA methylase[1~7].

We investigated the inactivation of EM by EM-resistant strains clinically isolated in Japan. One of 197 strains of *Escherichia coli*, 3 of 77 strains of *Klebsiella pneumoniae* and 9 of 300 strains of *Pseudomonas aeruginosa* were selected from plates which contained 800 $\mu$g/ml of EM. The inactivation of EM by these 13 strains was investigated by the method of KONO *et al.*[8,9] with intact cells and adenosine triphosphate (ATP). It was found that an EM-resistant strain (MIC[10]: 1,600 $\mu$g/ml) of *E. coli*, designated Tf481A, inactivated EM. A crude extract (20 mg of protein[11]/ml) of enzymes from the strain was prepared by the method of O'HARA *et al.*[12]. The extract also inactivated oleandomycin (OL), midekamycin and spiramycin. The inactivation was the result of an enzymatic reaction which was dependent on ATP and magnesium ions (Table 1).

Table 1. Requirements for inactivation of OL by a crude extract of enzymes from *Escherichia coli* Tf481A.

| Reaction mixture | Inactivation (%) |
|---|---|
| Complete system[a] | 100 |
| −Crude enzyme extract | 0 |
| −ATP | 0 |
| $-Mg(CH_3COO)_2$ | 0 |
| $-Mg(CH_3COO)_2+MgCl_2$ | 100 |
| −KCl | 100 |
| Control[b] | 0 |

[a] Crude extract dialyzed against TMK buffer (387.5 $\mu$l), 1 mg/ml OL (12.5 $\mu$l) and 40 mM ATP (100 $\mu$l) were mixed and incubated together for 1 hour at 37°C.

[b] In the control, the crude extract was heated at 100°C for 3 minutes before use.

OL was also a good substrate for the inactivation, and the structure of the inactivated form of OL was determined by physico-chemical techniques, which included UV absorption and IR spectrophotometry, $^{31}P$ and $^{1}H$ NMR and mass spectrometry. Preliminary reports of these data were presented[13~15].

*E. coli* Tf481A was grown in 4 liters of nutrient broth, with shaking at 37°C, to the exponential phase. The cells were harvested by centrifugation, washed with TMK buffer (0.06 M KCl, 0.01 M magnesium acetate and 0.006 M 2-mercaptoethanol in 0.1 M Tris-HCl buffer, pH 7.8)[12] and suspended in 80 ml of the same buffer. The cells in suspension were disrupted by sonication (Ohtake Sonicator, Tokyo) at 20Kc for 15 minutes and centrifuged at 22,000×$g$ for 30 minutes at 4°C to remove cell debris. The supernatant thus obtained was used as a crude extract of enzymatic activity.

The reaction system for the inactivation of OL was as follows. 50 mg of powdered OL (phosphate salt, Sigma, St Louis, U.S.A.) was mixed with 80 ml of the crude extract and 20 ml of 80 mM ATP (disodium salt) in TMK buffer, preneutralized to pH 7.8 with $NaHCO_3$. The complete reaction mixture was incubated overnight at 37°C. The reaction was stopped by heating at 90°C for 3 minutes and the residual antibiotic activity in the reaction mixture was determined with *Bacillus subtilis* ATCC 6633[16]. The reaction mixture containing the inactivated OL was centrifuged at 22,000×$g$ for 30 minutes. The supernatant was extracted with $CHCl_3$ to remove any remaining intact antibiotic and was then exhaustively extracted with BuOH to recover the inactivated OL. The BuOH extract was evaporated to dryness under reduced pressure. The residue was dissolved in 2 ml of MeOH - $Me_2CO$ (1 : 1). This solution of inactivated OL was passed over a column of silica gel (2×15 cm). The chromatography was carried out with a MeOH - $Me_2CO$ (1 : 1) mixture as eluent, and 5 ml fractions were collected. 5 $\mu$l of each fraction were applied to a silica gel plate. Fractions containing the inactivated OL were detected by heating the silica gel plates after they had been sprayed with a *p*-anisaldehyde reagent (PAA), which consisted of 9 ml of EtOH, 0.5 ml of *p*-anisaldehyde and 0.5 ml of sulfuric acid. The fractions which showed a violet color

Table 2. Rf values of the inactivated OL by TLC.

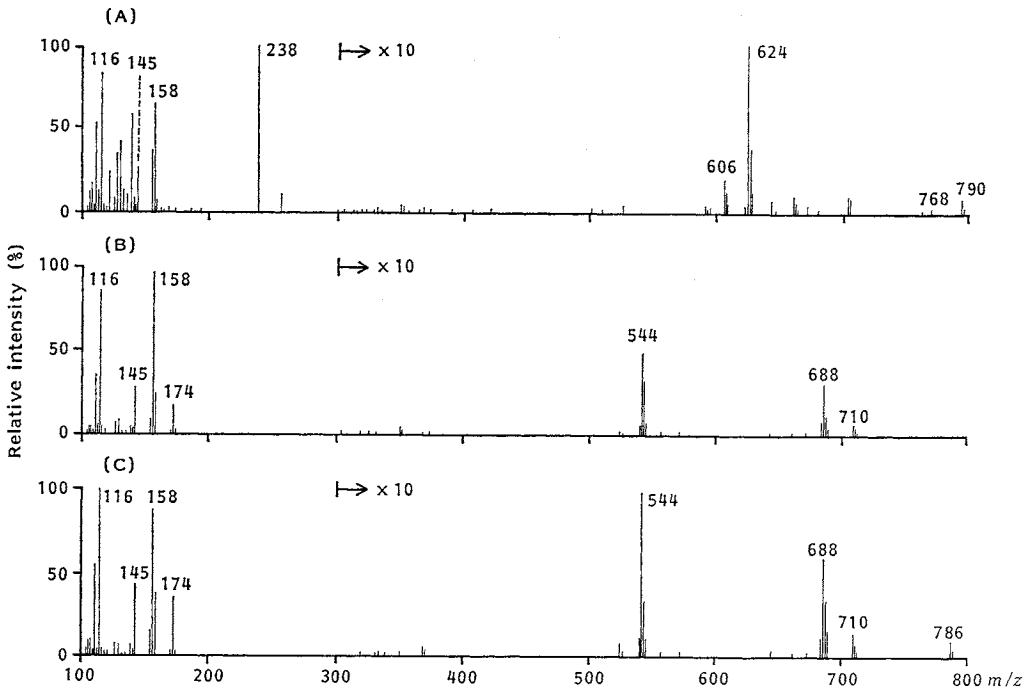
| Sample | Rf in the indicated solvent system[a] | | | | Antibiotic activity[b] |
|---|---|---|---|---|---|
| | I | II | III | IV | |
| Inactivated OL (Tf481A) | 0.18 | 0.07 | 0.40 | 0.34 | − |
| OL (W3110rif) | 0.34 | 0.34 | 0.16 | 0.34 | + |
| OL (TLC) | 0.34 | 0.34 | 0.16 | 0.34 | + |
| OL phosphate salt | 0.34 | 0.34 | 0.16 | 0.34 | + |

[a] Solvent systems were as follows: I, MeOH - $Me_2CO$ (19 : 6); II, MeOH - $Me_2CO$ (1 : 1); III, MeOH - $H_2O$ (9 : 1) and IV, MeOH. TLC was performed on precoated Merck Silica gel plates (60 F 254). Location of compounds was detected by spraying with PAA and heating at 100°C.

[b] Measured by bioautography.

Fig. 1. SI-MS of the inactivated OL and of OL.

(A) Inactivated OL, (B) OL (W3110rif), (C) OL (TLC).

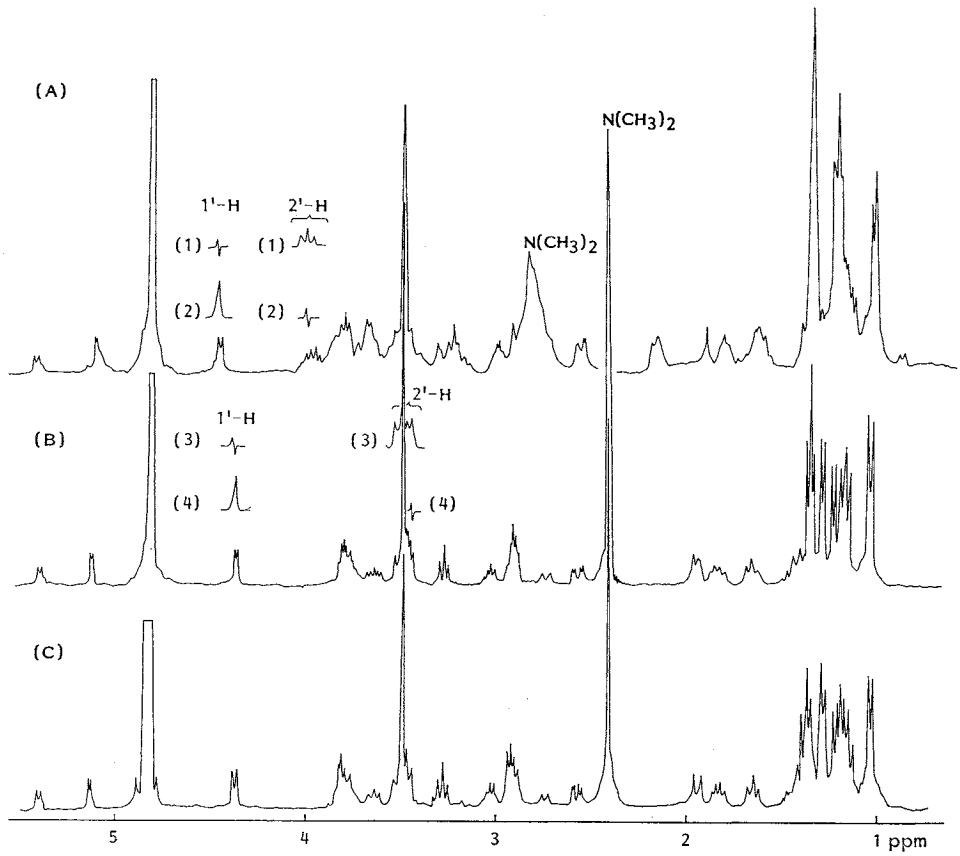



were positive for the PAA reaction and were evaporated to dryness under reduced pressure. The residue was dissolved in MeOH. The crude preparation was spotted onto a silica gel plate. The inactivated OL was purified by TLC on the silica gel plate developed with MeOH - $Me_2CO$ (19 : 6), solvent I and with MeOH - water (9 : 1). The inactivated OL was extracted from the silica gel plate with MeOH. After filtration, the solution was dried, and the residue dissolved in distilled water and lyophilized, yielding 28 mg of white powder.

On the other hand, OL purchased was also isolated by TLC on the silica gel plate as described above. And it was designated OL (TLC) and used as standard sample of OL.

Analysis by TLC of OL and inactivated OL yielded Rf values in solvent I of 0.34 and 0.18, respectively. However, in MeOH, the Rf values were identical for OL and inactivated OL and were equal to 0.34 (Table 2). A rifampicin resistant mutant of *E. coli* K12W3110, *E. coli* W3110rif[17,18], was used as internal control to check that OL was not inactivated in a susceptible strain, and that the structure of inactivated OL is not an artifact due to purification conditions. As a control, non-inactivated OL, designated OL (W3110rif), was similarly extracted

Fig. 2. $^{1}H$ NMR spectra of the inactivated OL and of OL.
(A) Inactivated OL, (B) OL (W3110rif), (C) OL (TLC).




with $CHCl_3$ after OL, ATP and a crude extract from the EM-susceptible (MIC: 100 $\mu$g/ml) strain of *E. coli* W3110rif were incubated as described above, and was isolated by chromatography on silica gel plates under the same conditions. In each case, the chromatograms were developed by the ascending technique and visualized with either PAA or 50% sulfuric acid. The inactivated OL was seen as a single spot on TLC by each reagent and UV light. The spot of OL (50 $\mu$g) on TLC plates showed antibiotic activity by bioautography and that of the inactivated OL did not (Table 2).

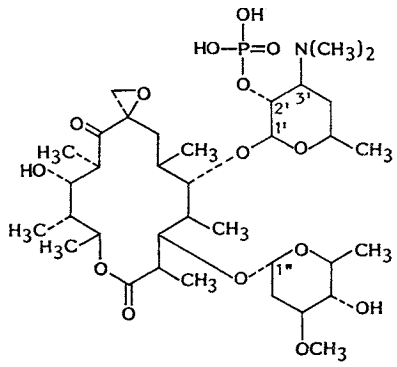
The UV spectra of OL and inactivated OL, dissolved in distilled water, were similar. The IR spectrum (KBr, Hitachi IR spectrophotometer 260-30 type) of the inactivated OL showed a phosphoric ester band at 965 $cm^{-1}$. $^{31}P$ NMR spectra were obtained on a JNM-FX200 spectrometer (Jeol) at 80.76 MHz in deuterium oxide ($D_2O$), pH 7.0. The $^{31}P$ NMR spectrum of OL showed a peak for phosphate at 1.556 ppm and the spectrum of the inactivated OL showed a peak for a phosphoric acid ester at 3.203 ppm.

The MS of the inactivated OL was compared to that of OL (Fig. 1). MS was obtained with a Hitachi M-80 Mass Spectrometer by secondary ion mass spectrometry (SI-MS). The MS of the phosphate salt of OL (MW 687 daltons) exhibited a $(M+H)^+$ ion at *m/z* 688 and $(M+H_3PO_4+H)^+$ ion at *m/z* 786.

As shown in Fig. 1B, OL (W3110rif) showed ionic peaks at *m/z* 688 and 710 $(M+Na)^+$, and OL (TLC) exhibited ionic peaks at *m/z* 688, 710 and 786 (Fig. 1C). However, the inactivated OL showed ionic peaks of $(M(687)+HPO_3(80)+H)^+$ and $(M+HPO_3+Na)^+$ at *m/z* 768 and 790, respectively (Fig. 1A). Therefore, the inactivated OL appeares to be a monophosphoryl OL (MW 767 daltons).

The $^{1}H$ NMR spectra were obtained with a Bruker AM-400 spectrometer at 400 MHz in

Fig. 3. Structure of the inactivated OL.



$D_2O$ solution, with 3-(trimethylsilyl)propionic acid-$d_4$ sodium salt (Merck) as internal reference (0 ppm) (Fig. 2). It has been reported that the signals of the C-1′ and the C-1″ anomeric protons in OL appear at about 4.26 ppm and 5.01 ppm, respectively[19,20]. The signals of the C-1′ proton in OL and the inactivated OL extracted from the silica gel plate were 4.32 ppm (Figs. 2B and 2C) and 4.40 ppm (Fig. 2A), respectively. The signals of the C-2′ proton in OL and in the inactivated OL were determined by decoupling of each of the C-1′ proton signals as shown in Fig. 2 (1)~(4). In the NMR spectrum of the inactivated OL, the C-2′ proton signal at 3.44 ppm in OL was shifted to 3.93 ppm. The signal of the C-3′-$N(CH_3)_2$ protons at 2.40 ppm (sharp singlet) in OL was shifted to 2.80 ppm (broad signal) in inactivated OL. The broad signal in the inactivated OL changed to a sharp singlet upon heating of the inactivated OL at 80°C. It is clear that phosphorylation must have occured at the 2′-OH of the desosamine moiety.

From the reported structure of OL[21,22], we have deduced the structure of the inactivated OL to be as shown in Fig. 3, namely the 2′-*O*-phosphoryl OL. Furthermore, the new macrolide(M)-inactivating enzyme from EM-resistant *E. coli* Tf481A can be identified as an M-2′-phosphotransferase.

The inactivated compound formed by reaction of OL with an extract of EM-resistant *E. coli* Tf481A is the same as that generated by *Streptomyces coelicolor* and recently reported[23]. Accordingly, it is interested in the properties of the M-2′-phosphorylating enzyme from EM-resistant *E. coli* Tf481A.

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