MICROBIAL TRANSFORMATION OF 6-*O*-METHYLERYTHROMYCIN DERIVATIVES

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Mucor circinelloides f. *griseo-cyanus* IFO 4563 was found to convert 6-*O*-methylerythromycin A (TE-031, A-56268) to (14*R*)-14-hydroxy-6-*O*-methylerythromycin A ((14*R*)-14-hydroxy TE-031). The TLC and spectral data of the conversion product were perfectly identical with those of an active major metabolite of TE-031 in humans (M-5).

A related antibiotic, 6-*O*-methylerythromycin B (TB-010), was able to be similarly transformed to its C-14 hydroxy analogue ((14*R*)-14-hydroxy-6-*O*-methylerythromycin B, (14*R*)-14-hydroxy TB-010).

The MICs of (14*R*)-14-hydroxy-6-*O*-methylerythromycin B against some Gram-positive bacteria were almost equal to those of 6-*O*-methylerythromycin B. It is suggested that the hydroxylation at C-14 of 6-*O*-methylerythromycins A and B scarcely reduces their *in vitro* activity.

6-*O*-Methylerythromycin A (TE-031)[1], a newly semisynthesized and promising macrolide antibiotic, has an antimicrobial spectrum similar to that of erythromycin A, and its *in vitro* activity is equal to or superior to that of erythromycin A. Furthermore, it shows remarkably higher activity *in vivo* than erythromycin A due to its improved acid stability and pharmacokinetic characteristics[2,3].

In the course of phase 1 clinical trials with TE-031, an active major metabolite (M-5), which was found in human urine, was isolated and identified as (14*R*)-14-hydroxy-6-*O*-methylerythromycin A ((14*R*)-14-hydroxy TE-031)[4~6].

M-5 shows potent antimicrobial activity, and its MICs against some Gram-positive bacteria are equal to or 2-fold more than those of TE-031. The *in vivo* activity of M-5 against systemic infections in mice with *Staphylococcus aureus* and *Streptococcus pneumoniae* is remarkably more potent than that of TE-031[5].

Accordingly, it has become important to obtain a significant quantity of (14*R*)-14-hydroxy TE-031 in order to extend studies of the pharmacology, toxicology and pharmacokinetics of TE-031.

Since a chemical partial synthesis of (14*R*)-14-hydroxy TE-031 by introduction of a hydroxy group at C-14 of TE-031 seemed to be difficult, the microbial transformation of TE-031 was tried, and some microorganisms were found that convert TE-031 to (14*R*)-14-hydroxy TE-031.

The present paper describes the microbial transformation of TE-031 to (14*R*)-14-hydroxy TE-031, and that of the related compound 6-*O*-methylerythromycin B (TB-010)[7] to (14*R*)-14-hydroxy-6-*O*-methylerythromycin B ((14*R*)-14-hydroxy TB-010), and also deals with the antibacterial activity of these macrolide antibiotics and their transformation products.

Materials and Methods

Macrolide Antibiotics

TE-031 and TB-010 were prepared in our laboratory[1~7]. (14*R*)-14-Hydroxy TE-031 isolated

from human urine[5,6] was used as an authentic reference compound.

Spectrometry[8]

IR spectra were recorded on a Jasco Model DS 70G spectrometer. Mass spectra were obtained with a Shimadzu LKB 9000 spectrometer. ^{1}H and ^{13}C NMR spectra were taken on a Jeol GX-400 (^{1}H 400 MHz, ^{13}C 100.4 MHz) spectrometer.

Screening Methods

Fungal strains were isolated from soil samples and obtained from various culture collections. They were inoculated into ϕ 25 mm test tubes containing 10 ml of a medium (medium A) consisting of sucrose 3%, peptone 0.2%, K_2HPO_4 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and yeast extract 0.1% in deionized water (pH 7.0). The test tubes were incubated on a reciprocal shaker for from 96 to 120 hours at 28°C. To the primary phase culture 50 μg/ml of TE-031 (2% ethanolic solution) was added. Incubation without addition of TE-031 was also carried out as a control experiment. The culture broth was sampled every 24 hours and was adjusted to pH 9.0 with 10% $NaHCO_3$ aqueous solution. It was extracted with ethyl acetate. The solution was examined by TLC (Kiesel gel G plate; Merck: chloroform - methanol - 25% aqueous ammonia, 90:10:1). The detection was carried out by spraying anisaldehyde - H_2SO_4 or 2% $CeSO_4$ in 2 N H_2SO_4 onto the plate and heating it at 105°C for 3 minutes. The conversion rate was measured by a Shimadzu High Speed TLC Scanner CS-920.

Isolation of the Transformation Product from TE-031

Mucor circinelloides f. *griseo-cyanus* IFO 4563 was cultured in a 200-liter tank fermentor containing 100 liters of medium A at 30°C with aeration (200 liters air/minute) and agitation (200 rpm) for 4 days. At the start of fermentation, a solution of 10 g of TE-031 in 2 liters of ethanol was added. The broth was sampled every 24 hours in the course of fermentation.

After the completion of fermentation, the culture broth was filtered to separate the mycelium and the filtrate. The filtrate was adsorbed on 4 liters of Diaion HP-20 (Mitsubishi Chemical Industries Limited) and eluted with 10 liters of acetone. The eluate was concentrated to give crude crystals of a mixture of TE-031 and the conversion products. The crude crystals were dissolved in a mixture of chloroform, methanol and 25% aqueous ammonia (200:10:1) and were subjected to silica gel column chromatography (Lobar column type C, Merck: 37×440 mm) equilibrated with the same solvent. The column chromatography was developed with 400 ml of the same solvent. The fractions containing (14*R*)-14-hydroxy TE-031 were collected, concentrated to dryness and crystallized from ethanol to give 583 mg of (14*R*)-14-hydroxy TE-031, as white needles, mp 214.5~216.5°C.

Antibacterial Activity

The test organisms in the present study were selected from the culture collections in our laboratory. The MICs of the macrolide antibiotics and their conversion products were determined by the 2-fold serial agar plate dilution method using Sensitivity Test Agar (Eiken). The final inoculum size in the test medium was approximately 10^6 cfu/ml. The test agar plates containing antibiotics were inoculated with the microbes and incubated at 37°C for 18 hours. The MIC was defined as the lowest concentration of antibiotics where no visible cell growth occurred.

Results

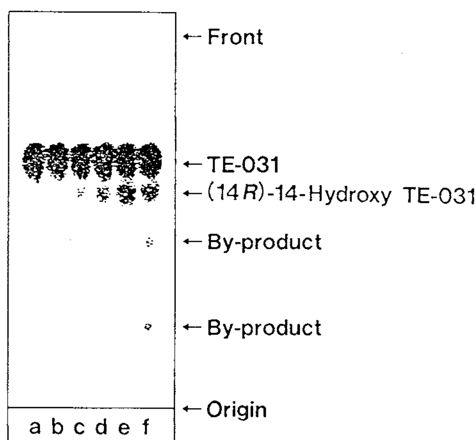
Microbial Transformation of TE-031 to (14*R*)-14-Hydroxy TE-031

Ninety-six fungal strains were screened for their ability to convert TE-031 to (14*R*)-14-hydroxy TE-031. Results showed *Mucor circinelloides* f. *griseo-cyanus* IFO 4563, *Cunninghamella echinulata* var. *elegans* IFO 6156 and *Beauveria bassiana* ATCC 7159 were capable of converting TE-031 to (14*R*)-14-hydroxy TE-031. IFO 4563 could carry out this transformation more efficiently than the other strains, and was used for subsequent work.

As shown in Fig. 1, (14*R*)-14-hydroxy TE-031 began to be visible on the TLC plate in the culture broth incubated for 72 hours. When the fermentation period reached 96 hours, the conversion rate of (14*R*)-14-hydroxy TE-031 was maximum reaching 13%. Other products also appeared with the prolongation of the fermentation period (*e.g.* 120 hours), while the spot of (14*R*)-14-hydroxy TE-031 diminished with the prolongation of fermentation. The structures and activities of these by-products will be described elsewhere.

The spectral data and physico-chemical properties of (14*R*)-14-hydroxy TE-031 biosynthesized by IFO 4563 were compared with those of (14*R*)-14-hydroxy TE-031 isolated from human urine. They were identical with each other. It was thus concluded that IFO 4563 can stereoselectively introduce a hydroxyl group at C-14 of TE-031.

Fig. 1. TLC analysis of microbial transformation of TE-031.



TE-031 (100 µg/ml final concentration) was added at the start of fermentation by the strain IFO 4563. Samples were taken at indicated intervals. Length of incubation (hours): a=0, b=24, c=48, d=72, e=96 and f=120.

Application of IFO 4563 for Related Compounds

As substrates of IFO 4563, TE-010 and erythromycins A and B were examined using the same method as for TE-031. The conversion products were analyzed by TLC and mass spectrometry. Whereas an active product (product "X") was found in the culture broth containing TB-010 (Fig. 2), no active conversion products were found in the broth using erythromycins A and B as the substrate. Product "X" was different from the original contaminant of TB-010. ATCC 7159 and IFO 6156 were also able to convert TB-010 to product "X", but IFO 4563 was superior to them, as was the case with TE-031.

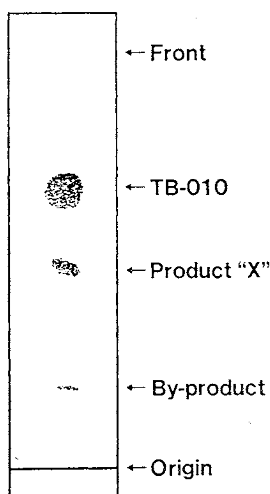
Microbial Transformation of TB-010 to (14*R*)-14-Hydroxy TB-010

Biotransformation of TB-010 using IFO 4563 was carried out in 500-ml Erlenmeyer flasks each containing 100 ml of medium A (TB-010, 100 µg/ml) at 30°C for 10 days. The culture broth was sampled by ethyl acetate extraction and examined by TLC at 24, 48, 72, 96, 144 and 240 hours.

The time course of the biotransformation of TB-010 is shown in Fig. 3. Transformation was essentially complete in from 120 to 144 hours, with IFO 4563 converting 36.4% of TB-010 to the product "X". At the same time, trace amounts of by-products were also visible on the TLC plate. The conversion product "X" of TB-010 has been isolated as white crystals from the culture broth by the same procedure as used for in (14*R*)-14-hydroxy TE-031.

The conversion product "X" of TB-010 was similar to TB-010 in its physico-chemical properties. As depicted in Table 1, elemental analysis and its molecular ion peak at m/z 748 ($(M+H)^+$) revealed that product "X" has one more oxygen atom than TB-010. This suggests that product "X" is a hydroxylated derivative of TB-010. In order to obtain further structural information, the ^{13}C NMR

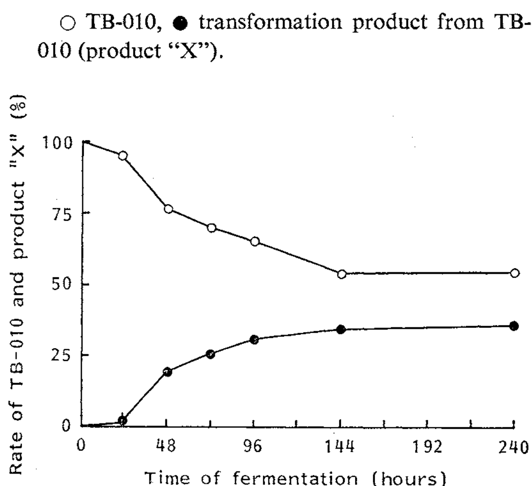
Fig. 2. TLC analysis of transformation products of TB-010 by IFO 4563.



Sample was taken at 96 hours incubation of TB-010.

Fig. 3. Time course of microbial transformation of TB-010.

○ TB-010, ● transformation product from TB-010 (product "X").



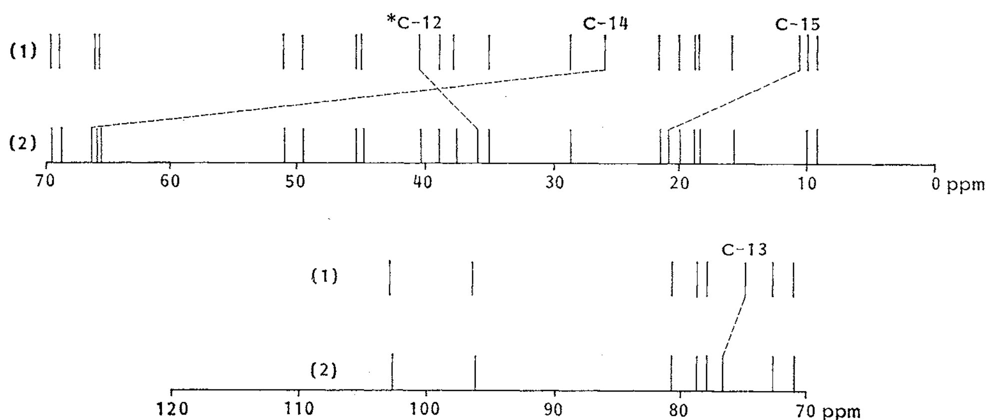
TB-010 (100 μg/ml final concentration) was added at the start of fermentation by the strain IFO 4563. Samples were taken at indicated intervals.

Table 1. Physico-chemical properties of (14*R*)-14-hydroxy TB-010 and TB-010.

	(14*R*)-14-Hydroxy TB-010	TB-010
Appearance	White needles	White needles
MP (°C)	222~224	219~220
Elemental analysis		
Calcd for:	$C_{38}H_{69}NO_{13}$	$C_{38}H_{69}NO_{12}$
	C 61.02, H 9.30, N 1.87.	C 62.36, H 9.50, N 1.91.
Found:	C 60.92, H 9.33, N 2.49.	C 62.70, H 9.40, N 1.74.
FAB-MS (*m*/*z*)	748 $(M+H)^+$	732 $(M+H)^+$
IR ν_{max}^{KBr} cm^{-1}	3470, 1734, 1688	3460, 1732, 1694

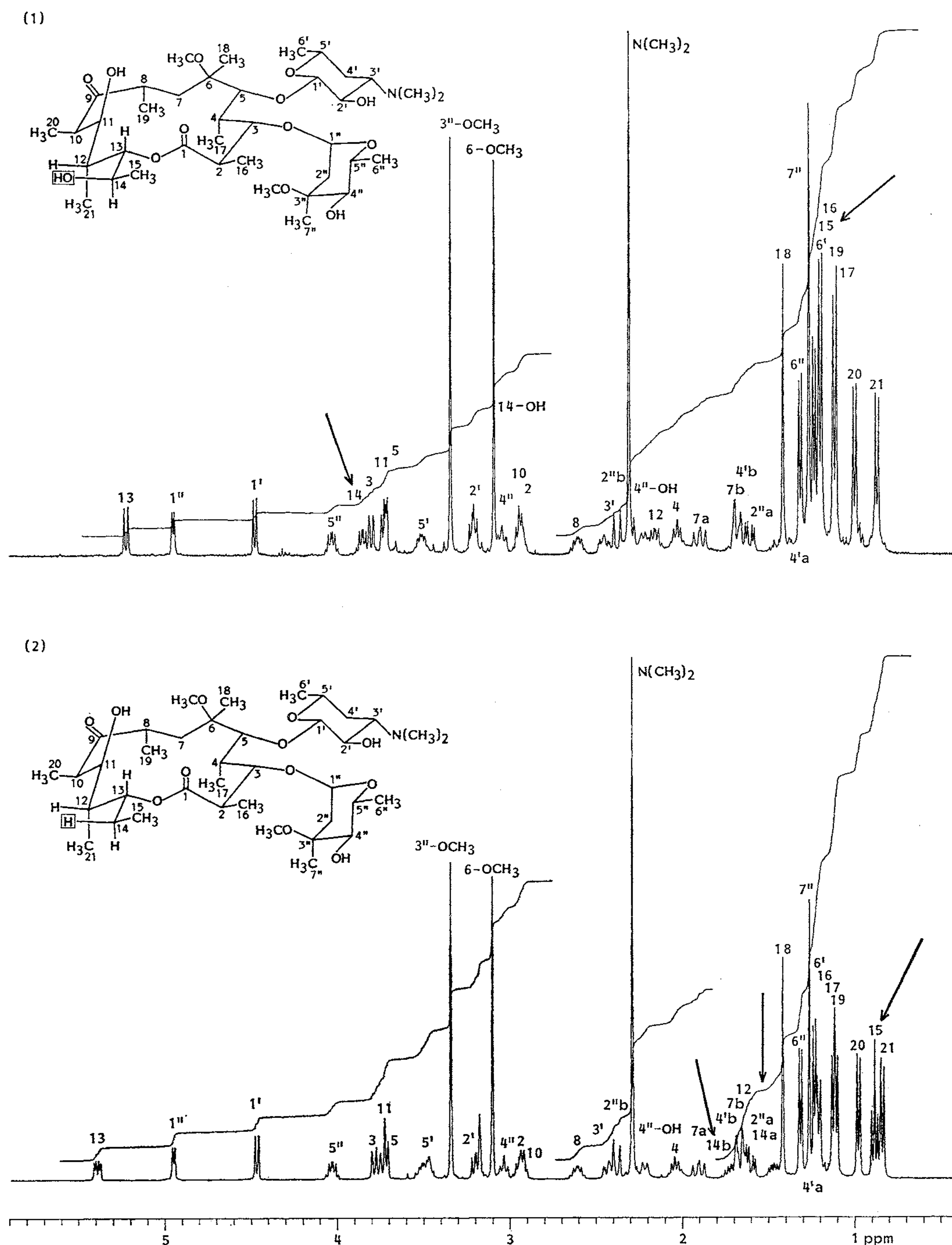
FAB-MS: Fast atom bombardment mass spectra.

Fig. 4. The ^{13}C NMR spectra of TB-010 (1) and (14*R*)-14-hydroxy TB-010 (2).



* Carbons of dimethylamino group are overlapped with C-12.

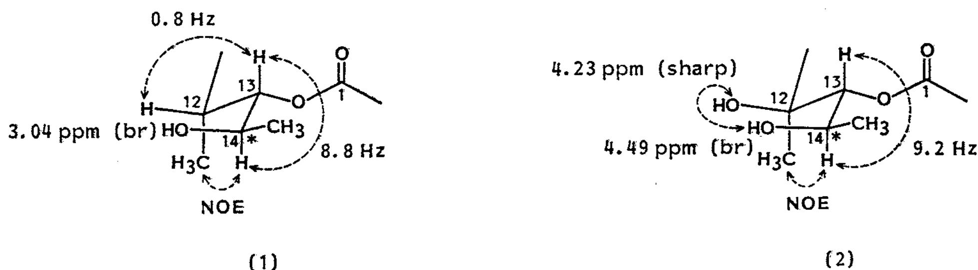
Fig. 5. The ^{1}H NMR spectra of (14*R*)-14-hydroxy TB-010 (1) and TB-010 (2).



The allow markers designated the 14-H and 15-H positions of respective compounds.

spectra were compared. The ^{13}C NMR spectrum of product "X" showed a similar spectrum to that of TB-010 except for the following signals, as depicted in Fig. 4; (1) the disappearance of the methylene line at 25.73 ppm for C-14 in TB-010, (2) the appearance of an oxymethine resonance at 66.31 ppm,

Fig. 6. The stereochemistry at C-14 of (14*R*)-14-hydroxy TB-010 (1) and (14*R*)-14-hydroxy TE-031 (M-5) (2).



Hydrogen bond between 12-OH and 14-OH.

Table 2. *In vitro* antibacterial activity of (14*R*)-14-hydroxy TE-031, TE-031, (14*R*)-14-hydroxy TB-010 and TB-010.

Microorganism	MIC (μg/ml)			
	(14*R*)-14-Hydroxy TE-031	TE-031	(14*R*)-14-Hydroxy TB-010	TB-010
Staphylococcus aureus 209P-JC	0.10	0.10	0.20	0.20
S. aureus BB	0.20	0.10	0.20	0.20
S. aureus Smith 4	0.20	0.10	0.39	0.39
S. aureus J-109*	>100	>100	>100	>100
S. aureus B1*	>100	>100	>100	>100
S. aureus C1*	1.56	0.78	1.56	0.78
S. epidermidis sp-al-1	0.20	0.10	0.78	0.39
Enterococcus faecalis ATCC 8043	0.05	0.025	0.05	0.05
Bacillus subtilis ATCC 6633	0.05	0.025	0.20	0.10
Micrococcus luteus ATCC 9341	0.025	0.012	0.05	0.05
M. luteus NIHJ	0.05	0.025	0.05	0.05
Escherichia coli NIHJ JC-2	100	50	100	>100
E. coli K-12	12.5	12.5	25	50
Klebsiella pneumoniae IFO 3317	25	50	12.5	25

Inoculum size: 10^6 cfu/ml.
Medium: Sensitivity Test Agar (Eiken).
* Resistant strain against erythromycin A.

(3) the downfield shift of the methyl peak at C-15 by 10.96 ppm, (4) the downfield shift of the oxymethine signal at C-13 by 1.77 ppm, (5) the upfield shift of the methine peak at C-12 by 2.85 ppm.

Taking into consideration these data, together with its elemental analysis and molecular ion peak, the conversion product was determined to be 14-hydroxy TB-010. This structure was confirmed by ^{1}H NMR spectrum analysis. The spectrum of the product "X" also resembled that of TB-010, except for the following differences (Fig. 5): The triplet methyl signal at 0.88 ppm due to 15-H of TB-010 disappeared and shifted at 1.19 ppm as a doublet. Furthermore, the new methine line of a doublet of quartets resonated at 3.84 ppm.

These spectral differences demonstrated that an oxymethine group was substituted for a methylene group adjacent to a methyl group in TB-010. Therefore the hydroxyl substituent must be attached at C-14.

The remaining problem was to assign the stereochemistry at C-14. The coupling constant value (8.8 Hz) between 13-H and 14-H demonstrated that 13-H was related to an *anti*-staggered conforma-

tion with 14-H. The nuclear Overhauser effect (NOE) was observed with 21-H on irradiation at 14-H, whereas the signal intensity of 12-H was not enhanced by irradiation at 15-H. Thus it was concluded that the stereochemistry at C-14 is the same as that of (14*R*)-14-hydroxy TE-031, as shown in Fig. 6.

In Vitro Antibacterial Activity

The MICs of TE-031, TB-010, (14*R*)-14-hydroxy TE-031 and (14*R*)-14-hydroxy TB-010 are shown in Table 2. The MICs of (14*R*)-14-hydroxy TE-031 and (14*R*)-14-hydroxy TB-010 against some Gram-positive bacteria such as *S. aureus* 209P-JC, *Staphylococcus epidermidis* sp-al-1 and *Micrococcus luteus* ATCC 9341 were equal to or 2-fold more than those of TE-031 and TB-010, respectively.

Discussion

The result of the microbial transformation of TE-031 by using *M. circinelloides* f. *griseo-cyanus* IFO 4563 has enabled us to easily obtain (14*R*)-14-hydroxy TE-031, a major human metabolite (M-5) of TE-031, in desirable amounts[9,10]. The success of this transformation will lead us to examine the character and the function of M-5 in preclinical studies of TE-031 such as pharmacology, toxicology and pharmacokinetics[10].

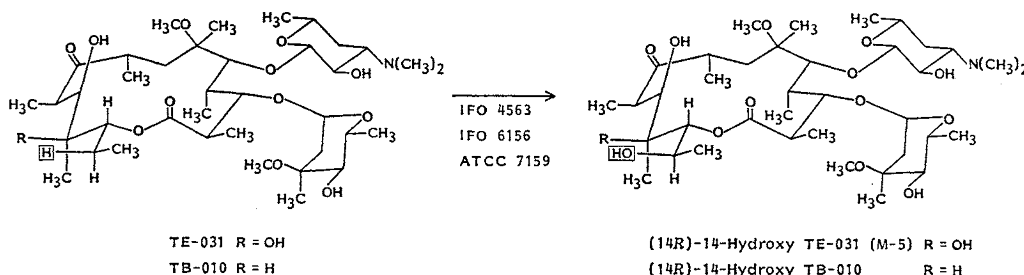
In addition, the finding of enzymatic hydroxylation at C-14 of TE-031 has aroused our interest to apply it to other macrolide antibiotics. As a first step, the compounds related to TE-031, such as TB-010, erythromycins A and B were examined to introduce a hydroxyl group at C-14. The results showed that only TB-010 could be transformed to its 14-hydroxylated derivative (Fig. 7). This suggests that the introduction of an *O*-methyl group at C-6 of erythromycins may give the substrate specificity for C-14 hydroxylation enzyme of strain IFO 4563.

On the other hand, 14-hydroxylated compounds of erythromycins A and B could not be detected in the conversion broth, although erythromycins A and B were added under adequate conditions for the 14-hydroxylation of TE-031. These results have been inconsistent with a suggestion that 14-hydroxy metabolites of erythromycin A were found tentatively by CORCORAN *et al.*[11], and it is supposed that the conditions for the 14-hydroxylation of 6-*O*-methylerythromycins A and B are not necessarily appropriate for providing 14-hydroxylated compounds of erythromycins A and B. Besides, 14-hydroxylated products of erythromycins A and B may be unstable, and the detection also hard. So, the finding out the corresponding products possibly require improvements of the reacting conditions and the analytical methods.

Another point of interest in the transformation is its stereoselectivity. 14-Hydroxylation of TE-031 and TB-010 using IFO 4563 gave the corresponding (14*R*)-14-hydroxylated compounds as major products of the transformation. This result means that IFO 4563 has stereoselectivity for hydroxylation at C-14 of 6-*O*-methylerythromycins A and B. Thus, microbial transformation seems to be an effective means to introduce a hydroxyl group to an objective compound stereoselectively.

With regard to the antibacterial activity (Table 2), (14*R*)-14-hydroxy TB-010 is equal to or 2-fold less active than TB-010 *in vitro*. The structure-antibacterial activity relationship between TE-031

Fig. 7. Microbial hydroxylation at C-14 of 6-*O*-methylerythromycin derivatives.



and (14*R*)-14-hydroxy TE-031 was similar to that of TB-010 and (14*R*)-14-hydroxy TB-010. This suggests that (14*R*)-14-hydroxylation of 6-*O*-methylerythromycins A and B hardly reduces their *in vitro* activity.

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