DEEPOXIDATION OF 16-MEMBERED EPOXYENONE MACROLIDE ANTIBIOTICS

I. MICROBIAL DEEPOXIDATION AND SUBSEQUENT ISOMERIZATION OF DELTAMYCINS A_1, A_2, A_3, A_4 (CARBOMYCIN A) AND X

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(Received for publication June 29, 1982)

Carbomycin A (deltamycin A_4) was deepoxidized to carbomycin A Pl by *Streptomyces halstedii* subsp. *deltae* (a deltamycins producer), favorably under anaerobic conditions. Carbomycin A P1 was spontaneously converted to geometric isomers designated carbomycins A P2 and A P3. This type of deepoxidation and subsequent isomerization was not limited to carbomycin A, but generally occurrable in other 16-membered epoxyenone macrolide compounds. Many bacteria and actinomycetes were also found to have an ability to deepoxidize deltamycins reductively. The chemical structures of carbomycins A P1, A P2 and A P3 were elucidated as shown in Fig. 3.

Among naturally-occurring 16-membered macrolide antibiotics, a family of compounds having an epoxyenone partial structure in the macrolactone ring are collectively called epoxyenone macrolides. These include angolamycin, carbomycin A, chalcomycin, cirramycin A_1, compound B-58941, deltamycins A_1, A_2, A_3 and X, juvenimicins A_2, A_3 and A_4, mycinamicins I and II, neutramycin, rosamicin and staphcoccomycin. The epoxyenone macrolides have found no clinical utility in the treatment of bacterial infections, although they are as highly antibacterial *in vitro* as non-epoxyenone macrolides. One of the major reasons for the poor therapeutic utility of this family of antibiotics is found in the low blood level attained after enteral or parenteral administration. As the characteristic epoxyenone structure may be responsible for the tight binding of these macrolides to blood cells, resulting in the unexpectedly poor serum levels, we attempted to improve the *in vivo* performance of the epoxyenone macrolides by deepoxidation.

Streptomyces halstedii subsp. *deltae* produces deltamycins A_1, A_2, A_3, A_4 (carbomycin A) and X, all of which belong to the epoxyenone family[1~8]. In this paper, we describe the reductive deepoxidation of carbomycin A by *S. halstedii* subsp. *deltae* to give an enol-type derivative of carbomycin A designated carbomycin A P1, which is then spontaneously converted to geometric isomers designated carbomycins A P2 and A P3. The chemical structures of these deepoxidation products were determined spectroscopically to be as presented in Fig. 3.

This type of deepoxidation and subsequent isomerization was specific for the epoxyenone structure and was not affected by structural differences in other regions such as sugar moieties. Biosynthetically speaking, it is very likely that the reductive deepoxidation is involved at least in the interconversion of carbomycins A and B, and more generally in the biosynthesis of 16-membered epoxyenone macrolide

antibiotics.

Materials and Methods

Microorganisms and Cultivation

Streptomyces halstedii subsp. *deltae* (FERM-P 2504)[1] was grown at 27°C for 2 weeks on an ISP-2 agar slant. One loopful of the spores were transferred into a 500-ml Erlenmeyer flask containing 100 ml of medium ML-5 (glucose 3.0%, corn steep liquor 1.0, dry yeast 0.3, NaCl 0.5, $CaCO_3$ 0.35) and shake-cultured at 28°C for 2 days on a rotary shaker (throw 35 mm, 210 rpm). Three flasks of the culture was inoculated into a 20-liter jar fermentor containing 10 liters of medium ML-5 and cultivated at 28°C for 3 days at 300 rpm under forced aeration of 10 liters/minute. Mycelia were harvested by filtration, washed in physiological saline, and suspended in 2.5 liters of 0.1 M phosphate buffer, pH 6.8.

Other actinomycetes from our culture collection were grown in 100 ml of medium ML-5 under the above-described conditions.

Bacteria were shake-cultured at 28°C for 2~3 days in 100 ml of medium A (peptone 2.0%, beef extract 1.4, glucose 2.0, NaCl 0.6).

For small-scale deepoxidation tests, microbial cells were suspended in 0.1 M phosphate buffer, pH 6.8, at a cell density of 250 mg (wet weight)/ml.

Antibiotics

Deltamycins A_1, A_2, A_3, A_4 (carbomycin A) and X were produced by fermentation of *S. halstedii* subsp. *deltae* (FERM-P 2504) and purified in our laboratories[2,3].

Deepoxidation of Carbomycin A to Carbomycin A P1 by *S. halstedii* subsp. *deltae*

One gram of carbomycin A in 100 ml of acidic water (pH 2.5) was added to 10 liters of the said mycelium suspension of *S. halstedii* subsp. *deltae* and one liter each of the mixture was distributed in ten 2-liter Erlenmeyer flasks. After nitrogen gas was bubbled thoroughly into the mixture, each flask was plugged with a rubber stopper and sealed air-tightly with Parafilm MR (American Can Co.) so that no nitrogen gas might escape from the flask during incubation. Deepoxidation of carbomycin A was completed in 2~3 hours at 28°C under shaking.

Mycelia were separated by filtration and washed with water. The filtrate and the water wash were combined and adjusted to pH 7.8 with 1 N NaOH. Macrolide compounds were extracted three times at pH 7.8 with 5 liters each of ethyl acetate. The ethyl acetate extracts were combined and concentrated to dryness under reduced pressure to give 760 mg of a syrup which was estimated to be 40% pure for carbomycin A P1 by silica gel thin-layer chromatography.

The syrup was dissolved in a small volume of methanol and charged on a Sephadex LH-20 column (30×250 mm; Pharmacia Fine Chemicals AB). Under monitoring by silica gel thin-layer chromatography, the column was developed with methanol. Evaporation of the methanol from the combined active fractions yielded 350 mg of an 80% pure powder of carbomycin A P1. Final purification was performed by quick column chromatography on silica gel (18×275 mm; silica gel for column chromatography, 230~400 mesh; E. Merck, Darmstadt) using acetone - benzene mixtures (1:10, 1:4 and 1:2). Active fractions were collected, combined, and concentrated to dryness in a rotary evaporator to give 240 mg of a 95% pure preparation of carbomycin A P1. This preparation was dissolved in a small volume of benzene and was subjected to forced precipitation with *n*-hexane to provide 195 mg of carbomycin A P1 (more than 98% pure).

From the final silica gel column described above, carbomycins A P2 (35 mg) and A P3 (30 mg) were recovered. These two compounds were confirmed to be absent in the ethyl acetate extracts.

Conversion of Carbomycin A P1 to Carbomycins A P2 and A P3

Carbomycin A P1 (400 mg) in a mixture of 5 ml dry benzene and 1 ml triethylamine was stirred overnight at room temperature. The reaction mixture was concentrated to dryness under reduced pressure and the residue was dissolved in a small volume of ethyl ether. After insoluble matter was filtered off, the filtrate was concentrated to dryness *in vacuo*. The residue containing carbomycins A P2 and A P3

was dissolved in a small amount of a 10: 1 mixture of benzene and methanol and was charged on a silica gel column (10×150 mm). Under monitoring by silica gel thin-layer chromatography, carbomycin A P3 was eluted with a 9: 1 mixture of benzene and acetone, and then carbomycin A P2 with a 87: 13 mixture of benzene and acetone. Solvent evaporation followed by forced precipitation in benzene with *n*-hexane gave 115 mg of carbomycin A P2 and 84 mg of carbomycin A P3.

Silica Gel Thin-layer Chromatographic Analysis

Macrolide compounds in ethyl acetate were spotted on a silica gel thin-layer plate (pre-coated thin-layer chromatographic plate silica gel F-254; E. Merck, Darmstadt) and developed in a solvent system of benzene and acetone (2:3) or of chloroform and methanol (20:1). After the solvent was evaporated off, the silica gel thin-layer plate was dipped in 10% sulfuric acid and heated at 110°C for 5 minutes. Color intensity of a macrolide spot was measured at 540 nm in a Shimadzu dual-wavelength thin-layer chromatographic scanner CS900.

General Methodology

Melting points were measured on a Yazawa micro-hot stage and are uncorrected. For determination of optical rotations, a Jasco digital polarimeter was used. IR, UV, ^{1}H NMR and mass spectra were recorded with a Hitachi 260-30 infrared spectrometer, a Hitachi 200-20 ultraviolet/visible spectrophotometer, a JEOL PS-100 NMR spectrometer (internal standard, tetramethylsilane) and a Hitachi RMU-7 mass spectrometer, respectively.

Results

Some Conditions for Microbial Deepoxidation of Carbomycin A to Carbomycin A P1 by *S. halstedii* subsp. *deltae*

Oxygen

When carbomycin A was aerobically incubated with cells of *S. halstedii* subsp. *deltae* under shaking, no substrate was deepoxidized. On standing incubation, the deepoxidation of carbomycin A to carbomycin A P1 was very slow. Under anaerobic conditions, in contrast, the substrate was quantitatively deepoxidized favorably under shaking.

pH

The relative deepoxidation yield in the pH range of 5.0~9.0 showed that pH 6.0 was optimum (percent yield: 73.0% at pH 5.0; 75.2 at pH 6.0; 68.0 at pH 7.0; 51.4 at pH 8.0; 43.0 at pH 9.0).

Temperature

Relative yields of deepoxidation of carbomycin A were examined at temperatures of 4, 20, 28, 35, 40 and 60°C. Although the best yield occurred at 35°C, no significant difference was noted among 28, 35 and 40°C. The deepoxidation activity of the cells was abolished by boiling for 30 seconds.

Age and Viability of Mycelium

S. halstedii subsp. *deltae* possessed a similar deepoxidizing activity in the age range of 2~6 days. In addition, no loss of the deepoxidizing activity was detected even after one week of frozen storage at −28°C.

Promoters

At 0.5 μmol/ml, flavine mononucleotide, flavine-adenine dinucleotide, reduced nicotinamide-adenine dinucleotide and reduced nicotinamide-adenine dinucleotide phosphate were found to promote the deepoxidation 2~3-folds, indicating the involvement of electron transfer in the reaction.

Inhibitors

Dicoumarol and 2,4-dibromophenol which are reported to be uncouplers[4] completely inhibited the

deepoxidation of carbomycin A at a final concentration of 1 ~ 10 nmol/ml even in the presence of reduced nicotinamide-adenine dinucleotide.

Deepoxidation of Carbomycin A by Bacteria and Other Actinomycetes

As it was biochemically interesting to determine whether the deepoxidation ability was limited to

Table 1. Screening of bacteria and actinomycetes for carbomycin A-deepoxidizing activity.

Microorganism	Relative activity	Product (antibiotic)
Alcaligenes denitrificans ATCC 15173	15%	
〃 *faecalis* B-326	0	
Arthrobacter viscosus ATCC 15294	10	
Bacillus subtilis ATCC 6633	50	
Comamonas terrigena IFO 12685	10	
Escherichia coli K-12	0	
Micrococcus luteus A9852	35	
Serratia marcescens A20019	80	
Staphylococcus aureus FDA 209P	0	
Streptomyces albireticuli IFO 12737	5	Eurocidin, carbomycins
〃 *aureofaciens* IFO 12843	75	Tetracyclines
〃 *bikiniensis* IAM 0019	65	Streptomycin
〃 *cirratus* ATCC 14699	65	Cirramycins
〃 *eurythermus* IFO 12764	35	Angolamycins
〃 *griseus* NRRL 3851	70	Carbomycins
〃 *halstedii* NRRL 2331	80	Carbomycins
〃 *lavendulae* ATCC 8664	75	Streptothricins
〃 *tendae* IFO 12822	70	Carbomycins
Streptoverticillium eurocidicum IFO 13491	25	Eurocidin, leucomycins
Streptomyces halstedii subsp. *deltae*	100	Deltamycins

Fig. 1. IR spectra (KBr) of carbomycin A (substrate) and carbomycins A P1, A P2 and A P3.

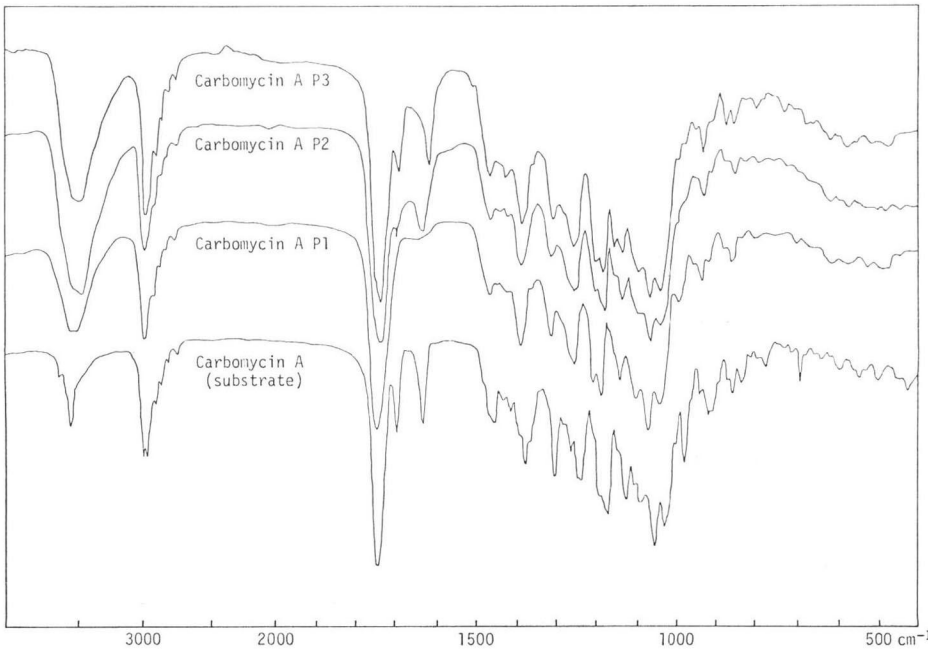


Fig. 2. ^1H NMR spectra of carbomycin A (substrate) and carbomycins A P1, A P2 and A P3.

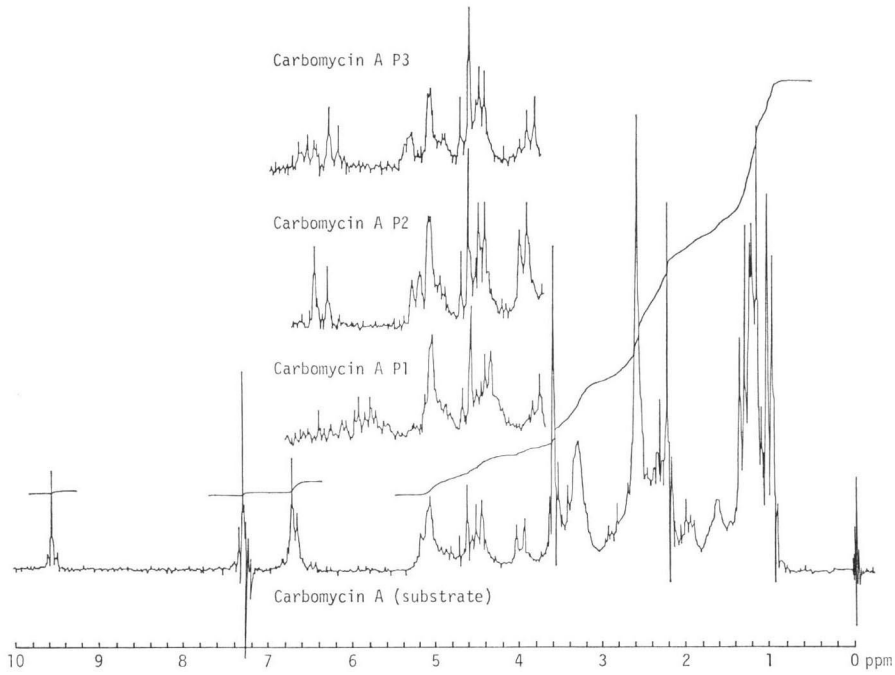


Table 2. Physico-chemical properties of carbomycin A (substrate) and carbomycins A P1, A P2 and A P3.

	Carbomycin A	Carbomycin A P1	Carbomycin A P2	Carbomycin A P3
Formula	$C_{42}H_{67}NO_{16}$	$C_{42}H_{69}NO_{16}$	$C_{42}H_{69}NO_{16}$	$C_{42}H_{69}NO_{16}$
Elementary analysis (%)				
Found (calcd) C		59.71 (59.77)	59.43 (59.77)	59.89 (59.77)
H		8.16 (8.24)	8.38 (8.24)	8.07 (8.24)
N		1.42 (1.66)	1.54 (1.66)	1.46 (1.66)
MW (MS)	841	843	843	843
Mp (°C)	211~214	117~121	105~107	115~117
$[\alpha]_D^{24}$ in $CHCl_3$	−60.5° (*c* 0.25)	−30.5° (*c* 0.10)	−52.5° (*c* 0.10)	+18.8° (*c* 0.10)
λ_{max}^{MeOH} nm (log ε)	240 (4.20)	End absorption	232.5 (3.94)	234 (3.92)
Color (H_2SO_4 treatment)	Blueish purple	Brownish purple	Yellow	Yellow
Rf (SiO_2 TLC)				
Benzene - acetone, 2: 3	0.68	0.57	0.63	0.67
$CHCl_3$ - MeOH, 20: 1	0.66	0.34	0.47	0.61
$CHCl_3$ - MeOH, 10: 1	0.69	0.49	0.49	0.65
BuOH - EtOH - H_2O, 4: 1: 1	0.61	0.62	0.69	0.71

S. halstedii subsp. *deltae*, carbomycin A was anaerobically treated with cells of bacteria and actinomycetes. Comparative deepoxidation activities of the microorganisms are summarized in Table 1, in terms of the activity of *S. halstedii* subsp. *deltae* as 100.

The results in Table 1 clearly show that the deepoxidation ability is not restricted to *S. halstedii* subsp. *deltae*, but is generally observable in many microorganisms and appears independent of their ability to form epoxyenone macrolides.

Physico-chemical Properties and Chemical Structures of Carbomycins A P1, A P2 and A P3

Carbomycins A P1, A P2 and A P3 are basic compounds. They are soluble in methanol, ethanol, acetone, methyl ethyl ketone, methyl isobutyl ketone, ethyl acetate, benzene, chloroform, toluene, pyridine and tetrahydrofuran; and hardly soluble in *n*-hexane, petroleum ether and water. These deepoxidation products gave positive reactions to the anthrone, sulfuric acid and DRAGENDORFF reagents; and negative reactions to biuret, ninhydrin, MILLON and EHRLICH reagents. For ease of comparison, the IR and ^{1}H NMR spectra of carbomycin A (substrate) and carbomycins A P1, A P2 and A P3 (deepoxidation products) are reproduced in the same charts, respectively (Figs. 1 and 2). Other physicochemical properties of the substrate and the products are presented in Table 2.

The chemical structures of carbomycins A P1, A P2 and A P3 were deduced as shown in Fig. 3, based on the following findings:

(1) Carbomycin A P1 was spontaneously converted to carbomycins A P2 and A P3 which were interconvertible.

(2) The three deepoxidation products gave the same molecular ion peak of 843 which was two mass units larger than that of the substrate.

(3) Spectrometric analysis revealed that the conjugated enone structure was lost in carbomycin A P1; and was reestablished in carbomycin A P2 (λ 232.5 nm; ν 1620, 1690 cm^{-1}; δ 6.34, 7.22 ppm) and carbomycin A P3 (λ 234 nm; ν 1600, 1680 cm^{-1}; δ 6.17, 6.52 ppm).

(4) Silica gel thin-layer chromatography indicated that the three deepoxidation products were more hydrophilic than the substrate; and that, on visualization with sulfuric acid, the brownish purple color of carbomycin A P1 suggested an intermediate structure between the dienol (*e.g.* leucomycins red-

Fig. 3. Chemical structures of carbomycin A (substrate) and carbomycins A P1, A P2 and A P3.

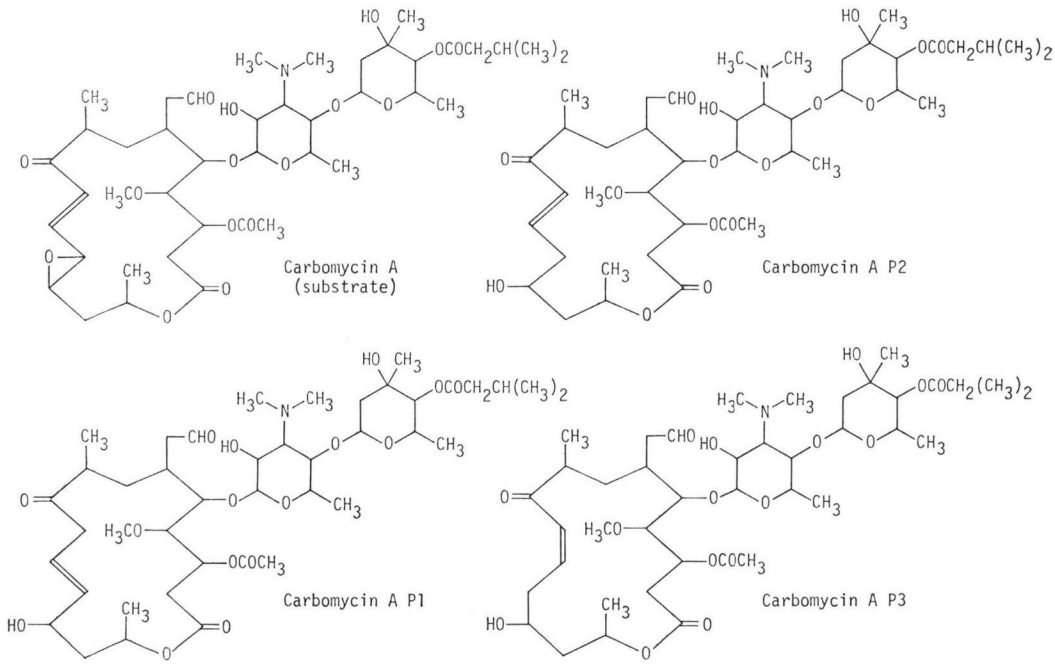


Table 3. Silica gel thin-layer chromatographic properties of deepoxidation products P1, P2 and P3 derived from deltamycins A_1, A_2, A_3 and X.

		Rf			
		Substrate	Product P1	Product P2	Product P3
Benzene - acetone, 2: 3	Deltamycin A_1	0.57	0.42	0.45	0.57
	A_2	0.61	0.47	0.50	0.55
	A_3	0.64	0.51	0.52	0.57
	X	0.24	0.16	0.18	0.26
Chloroform - methanol, 20: 1	Deltamycin A_1	0.54	0.26	0.35	0.50
	A_2	0.57	0.28	0.39	0.51
	A_3	0.61	0.31	0.42	0.55
	X	0.08	0.04	0.05	0.10
Butanol - ethanol - water, 4: 1: 1	Deltamycin A_1	0.38	0.41	0.44	0.50

Colors on visualization with sulfuric acid: Substrates, blueish purple; Products P1, brownish purple; Products P2 and P3, yellow.

dish purple ~ brownish purple) and dienone (*e.g.* carbomycin B brown ~ reddish brown) families, while the yellow color of carbomycins A P2 and A P3 did the enone structure (*e.g.* methymycin).

(5) The structural difference between carbomycins A P2 and A P3 is clearly seen in Fig. 2. The vinyl protons of the enone structure in carbomycin A P2 revealed the *trans* configuration (δ 6.34, d, J= 16.0 Hz; 7.22, m), whereas those in carbomycin A P3 did the *cis* configuration (δ 6.17, d, J=10.0 Hz; 6.52, m)[5].

Microbial Deepoxidation of Other 16-Membered Epoxyenone and Epoxyenol Macrolide Compounds

Deltamycins A_1, A_2, A_3 and X which differed from carbomycin A in the 4″-acyl group were subjected to the microbial deepoxidation by *S. halstedii* subsp. *deltae* under the above-specified reaction conditions. Table 3 shows the results of analysis of the reaction mixtures by silica gel thin-layer chromatography.

Angolamycin and rosamicin were similarly deepoxidized to P1 by the streptomycete.

It is noteworthy that none of the maridomycins, having an epoxyenol structure in the macrolactone ring, could be deepoxidized by *S. halstedii* subsp. *deltae* and other microorganisms.

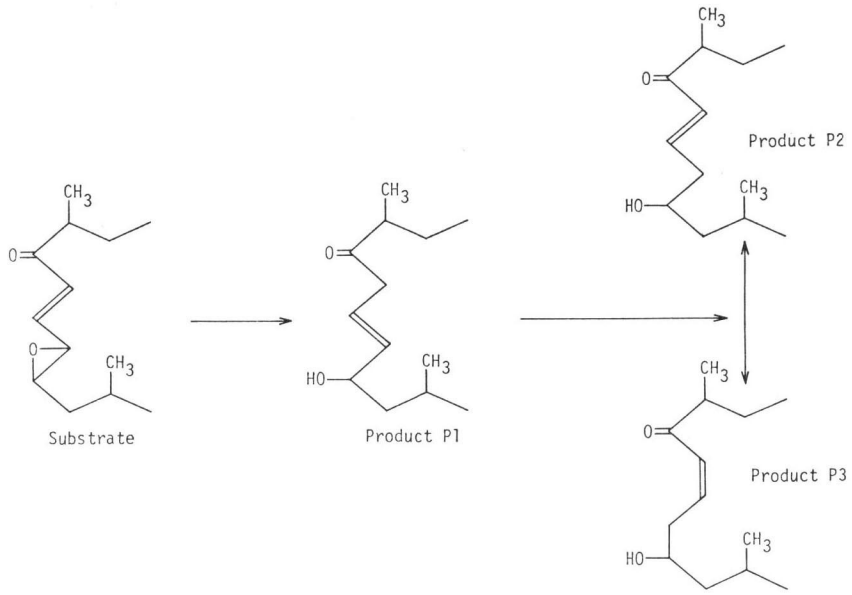
Discussion

As deltamycins A_1, A_2, A_3, A_4 (carbomycin A) and X were all deepoxidized by microorganisms and isomerized spontaneously, the general sequence of the deepoxidation and subsequent isomerization can be presented as shown in Fig. 4, with particular reference to the epoxyenone partial structure in the macrolactone ring.

There are two types of deepoxidation known in biological systems. The hydrolytic cleavage of the epoxide ring leading to the formation of a diol is the type which is seen in the reactions of epoxide hydrolase (=epoxide hydratase or arene-oxide hydratase; EC 3.3.2.3)[6] and *trans*-epoxy-succinate hydratase (=*meso*-tartarate hydrolyase; EC 4.2.1.37)[7]. The other type is the reductive opening of the epoxide ring which is catalysed by phylloquinone epoxide reductase (=vitamin K epoxide reductase)[8]. The deepoxidation of 16-membered epoxyenone macrolides is reductive, and thus belongs to the latter type of reaction.

Phylloquinone epoxide reductase catalyses the conversion of vitamin K 2,3-epoxide to vitamin K (=phylloquinone), and an intermediate that corresponds to product P1[9] can be postulated between the two vitamin K derivatives. Accordingly the reaction of phylloquinone epoxide reductase and the

Fig. 4. Deepoxidation and subsequent isomerization of 16-membered epoxyenone macrolide antibiotics.



deepoxidation of carbomycin A share some common natures. In a selective assay system for phylloquinone epoxide reductase, WHITLON *et al.*[4] stressed the necessity of replacement of oxygen with nitrogen in the atmosphere of incubation, which also held true in the deepoxidation of carbomycin A. Phylloquinone epoxide reductase is supported more effectively by dithiothreitol than by mercaptoethanol, but not by NADH. By contrast, the formation of carbomycin A P1 was promoted by NADH, NADPH and FMN and somewhat inhibited by dithiothreitol, mercaptoethanol and reduced glutathione. The inhibition of phylloquinone epoxide reductase by coumarin derivatives is well documented and the epoxide cleavage of carbomycin A by *S. halstedii* subsp. *deltae* was similarly suppressed by dicoumarol and 2,4-dibromophenol.

SUZUKI *et al.*[10] assumed that carbomycin B was the biosynthetic precursor for carbomycin A; and that the latter could not be converted to the former. Although we have not yet succeeded in solubilizing a responsible enzyme system for deepoxidation of carbomycin A, the indirect evidence described in the text seems to support the possibility that carbomycins A and B are biosynthetically interconvertible.

Acknowledgment

The authors express their deep thanks to Prof. Y. YAMADA, Tokyo College of Pharmacy, for his valuable advice and help in the chemical work.

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