DEEPOXIDATION OF 16-MEMBERED EPOXYENONE MACROLIDE ANTIBIOTICS

III. *IN VITRO* AND *IN VIVO* EVALUATION OF DEEPOXIDATION PRODUCTS OF CARBOMYCIN A, DELTAMYCIN A_1, 4″-PHENYLACETYL-DELTAMYCIN, ANGOLAMYCIN AND ROSAMICIN

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Deepoxidation products P1, P2 and P3 of carbomycin A, deltamycin A_1 and 4″-phenylacetyldeltamycin showed high *in vitro* antibacterial and antimycoplasmal activities which were comparable to those of the respective parent compounds. By contrast, the *in vitro* antimicrobial potencies of angolamycin P1 and rosamicin P1 were about ten-fold lower than those of the parent macrolides. In mice, the increase in the plasma levels of the epoxyenone macrolides due to deepoxidation was highly significant with the P1, P2 and P3 derivatives of carbomycin A and 4″-phenylacetyldeltamycin, whereas angolamycin P1 gave a moderately-improved plasma level compared with angolamycin.

In an attempt to modify the pharmacokinetically undesirable properties of the epoxyenone macrolide antibiotics, we have developed both biological and chemical methods for epoxide-ring opening of these macrolide compounds. The initial deepoxidation product P1 was spontaneously converted to geometric isomers P2 and P3, unless prevented by a methyl group at C-12 in the epoxyenone structure[1,2].

Materials and Methods

Antibiotics

The deepoxidation products P1, P2 and P3 of carbomycin A, deltamycin A_1 and 4″-phenylacetyldeltamycin were prepared as detailed in previous papers[1,2]. Angolamycin P1 and rosamicin P1 were chemically synthesized as reported in the preceding paper[2].

In minimum inhibitory concentration (MIC) tests, the macrolides were dissolved in small volumes of methanol and diluted with sterile media to give the indicated final concentrations before inoculation. In preliminary experiments, a final concentration of methanol upto 5% had been confirmed to have no additional inhibitory effect on the growth of the test organisms used.

Antibacterial Activity

The test bacteria employed in the present study were from our culture collection. The MIC's of the macrolide antibiotics were determined by the two-fold serial broth dilution method using brain-heart infusion broth (BHI "Difco") or BHI supplemented with 10% horse blood (blood-BHI). Unless stated otherwise, the final inoculum size was approximately 10^5 cells/ml test medium. Assay test tubes containing the antibiotics were inoculated with the microbes and incubated at 37°C for 18 hours. The MIC was defined as the lowest concentration of antibiotic where no visible cell growth occurred.

Antimycoplasmal Activity

This study included *Mycoplasma pneumoniae* Mac, *M. fermentans, M. hominis* PG 21 and *M. salivarium* ATCC 14277 (human mycoplasmas); *M. pulmonis* PG-22 (rodent mycoplasma); *M. gallisepticum* KP-13 and *M. gallisepticum* S-6 (avian mycoplasmas) and *M. agalactiae* PG-2 (goat mycoplasma).

Liquid medium consisted of 70 ml of 2.1% PPLO broth (Difco), 10 ml of 25% fresh dry yeast extract, 20 ml of horse serum, 50 mg of thallium acetate and 50,000 units of benzylpenicillin sodium salt in a total volume of 100 ml (pH 7.6~7.8). For easy reading, 2 mg of phenol red and 1 g of glucose were added to the liquid medium, except that glucose was replaced by 1 g of L-arginine for *M. hominis* PG 21 and *M. salivarium*. In this liquid medium, a maximum growth titer of $10^8 \sim 10^7$ colony-forming units (cfu)/ml was usually observed after 3 days of incubation at 37°C. The antimycoplasmal activity of antibiotic was determined by the two-fold serial broth dilution technique using an inoculum size of $10^5 \sim 10^6$ cfu/ml PPLO broth. After incubation for 7 days at 37°C, the MIC of antibiotic was read as the lowest concentration of the drug at which the color change of phenol red was completely inhibited.

Plasma Level of Antibiotics in Mice

The macrolide to be tested was suspended in 0.1% carboxymethylcellulose with a mortar and pestle and then diluted to the desired concentrations using physiological saline. Male *dd*Y mice (aged 5 weeks; weighing 19~21 g) in groups of 4 animals received single subcutaneous injections at a dose of 200 mg/kg. Blood samples were collected from the retroorbital sinus at the indicated time intervals with heparinized capillary tubes. The capillary tubes were sealed at one end and centrifuged at $5,000 \times g$ for 5 minutes to give plasma samples. The concentrations of the drug in plasma samples were measured by the paper disc-agar diffusion method using *Micrococcus luteus* S 19 as bioassay organism.

Experimental Therapy

The therapeutic effect of carbomycin A P2 was examined in male *dd*Y mice (aged 5 weeks; weighing 19~21 g) infected by *Staphylococcus aureus* Smith. As control drug, carbomycin A (parent macrolide) was simultaneously tested. The challenge microbe was cultured overnight in BHI broth (Difco) at 37°C and then suspended in 5% bacteriological mucin (Wilson Pharmaceutical and Chemical Company). Two hours after intraperitoneal injection of 0.5 ml of the cell suspension ($100 \times LD_{50}$), a single dose of macrolide was given subcutaneously. The mice were observed for 3 days. The therapeutic effect of the test drug was expressed in ED_{50} (mg/kg) according to the method of LITCHFIELD and WILCOXON[8].

Results and Discussion

In Vitro Antimicrobial Activity

Table 1 summarizes the *in vitro* antibacterial and antimycoplasmal activities of carbomycins A P1, A P2 and A P3. In general, the three deepoxidation products as well as the parent compound are very active against the Gram-positive bacteria and mycoplasmas. The antibacterial activity of carbomycin A seems not to be much affected by epoxide-ring opening, whereas its antimycoplasmal activity has some relation to the epoxyenone structure. For example, carbomycin A is 8-fold less active against *M. pulmonis* PG-22 than the deepoxidation products, while *M. agalactiae* is the most sensitive to carbomycin A P1 and the least sensitive to carbomycin A P3.

The antimicrobial spectra of deltamycin A_1 and deepoxidation products are presented in Table 2. It is apparent that the cleavage of the epoxide ring results in a 2~8-fold reduction of the antibiotic potency against the Gram-positive bacteria and mycoplasmas. As deltamycin A_1 differs from carbomycin A in the 4″-acyl group only, the length of the 4″-acyl group to the antibiotic effect seems to be selectively important in the significant reduction of the antimicrobial potency of the deltamycins by deepoxidation.

Table 3 shows the comparative antimicrobial activities of 4″-phenylacetyldeltamycin and deepoxidation products. Although slightly less active than the parent macrolide against some test strains, the

three new derivatives generally have higher antibiotic potencies against the bacteria and mycoplasmas. This observation supports the conclusion drawn above concerning the length of the 4''-acyl moiety and the activity of the deltamycins.

As far as carbomycin A, deltamycin A_1 and 4''-phenylacetyldeltamycin (and probably other 16-membered epoxyenone macrolides having no substituent at C-12 in the macrolactone ring) are concerned, the reductive cleavage of the epoxide ring does not lead to an unacceptable reduction of the *in vitro* antimicrobial activity.

Table 4 presents the antimicrobial activities of angolamycin P1, rosamicin P1 and the respective parent macrolides.

Contrary to our expectation, angolamycin P1 is generally far less active against the Gram-positive bacteria and mycoplasmas than angolamycin, the parent macrolide. For instance, the MIC against

Table 1. Comparative antimicrobial activities of carbomycins A P1, A P2 and A P3 and the parent macrolide (carbomycin A), (MIC in μg/ml).

Microorganism	Medium	Carbomycin A P1	Carbomycin A P2	Carbomycin A P3	Carbomycin A
Micrococcus luteus S 19	I	<0.10	<0.10	<0.10	<0.10
Staphylococcus aureus FDA 209P	I	0.20	0.39	0.39	0.39
S. aureus Smith	I	0.39	0.78	0.39	0.78
Streptococcus pneumoniae Type III	II	0.10	0.20	0.20	0.10
S. pyogenes NY 5	II	0.20	0.20	0.20	0.10
Escherichia coli K-12	III	>50	>50	>50	>50
Mycoplasma pneumoniae Mac	III	<0.002	0.004	0.008	<0.002
M. gallisepticum S-6	III	0.004	0.004	0.031	0.004
M. gallisepticum KP-13	III	0.016	0.016	0.016	0.016
M. pulmonis PG-22	III	0.04	0.04	0.04	0.31
M. fermentans	III	0.08	0.08	0.08	0.16
M. agalactiae PG-2	III	0.31	2.5	5.0	1.25

Medium I: brain-heart infusion broth (Difco).
II: brain-heart infusion broth (Difco) containing 10% horse blood.
III: PPLO broth (Difco) containing 10% horse serum.

Table 2. Comparative antimicrobial activities of deltamycins A_1 P1, A_1 P2 and A_1 P3 and the parent macrolide (deltamycin A_1), (MIC in μg/ml).

Microorganism	Medium	Deltamycin A_1 P1	Deltamycin A_1 P2	Deltamycin A_1 P3	Deltamycin A_1
Bacillus subtilis ATCC 6633	I	6.25	6.25	3.13	0.78
Micrococcus luteus S 19	I	0.20	0.20	0.20	0.05
Staphylococcus aureus FDA 209P	I	3.13	1.56	0.78	0.39
S. aureus Smith	I	6.25	3.13	3.13	0.78
Streptococcus pneumoniae Type III	II	0.78	0.78	0.78	0.20
S. pyogenes NY 5	II	0.78	0.78	0.78	0.20
Escherichia coli K-12	I	>50	>50	>50	>50
Mycoplasma pneumoniae Mac	III	0.039	0.078	0.039	<0.01
M. gallisepticum S-6	III	0.31	0.31	0.16	0.04
M. pulmonis PG-22	III	25	25	25	25
M. fermentans	III	0.78	1.56	0.78	0.20
M. hominis	III	3.13	25	6.25	0.78
M. salivarium	III	3.13	12.5	6.25	0.78

Medium I: brain-heart infusion broth (Difco).
II: brain-heart infusion broth (Difco) containing 10% horse blood.
III: PPLO broth (Difco) containing 10% horse serum.

Table 3. Comparative antimicrobial activities of 4″-phenylacetyldeltamycins P1, P2 and P3 and the parent macrolide (4″-phenylacetyldeltamycin (PAD)) (MIC in μg/ml).

Microorganism	Medium	PAD P1	PAD P2	PAD P3	PAD
Bacillus subtilis ATCC 6633	I	0.78	0.78	0.78	0.39
Micrococcus luteus S 19	I	0.10	0.10	0.10	0.05
Staphylococcus aureus FDA 209P	I	0.39	0.39	0.39	0.20
S. aureus Smith	I	1.56	0.78	0.78	0.39
Streptococcus pneumoniae Type III	II	0.10	0.10	0.10	0.05
S. pyogenes NY 5	II	0.20	0.20	0.20	0.05
Escherichia coli K-12	I	>50	>50	>50	>50
Mycoplasma pneumoniae Mac	III	0.031	0.008	0.016	0.016
M. gallisepticum S-6	III	0.125	0.031	0.016	0.016
M. gallisepticum KP-13	III	0.063	0.004	0.016	0.004
M. pulmonis PG-22	III	10	2.5	5	10
M. fermentans	III	0.31	0.04	0.16	0.08
M. hominis	III	0.31	0.16	0.31	0.63
M. salivarium	III	1.25	1.25	1.25	1.25

Medium I: brain-heart infusion broth (Difco).
II: brain-heart infusion broth (Difco) containing 10% horse blood.
III: PPLO broth (Difco) containing 10% horse serum.

Table 4. Comparative antimicrobial activities of angolamycin P1, angolamycin, rosamicin P1 and rosamicin (MIC in μg/ml).

Microorganism	Medium	Angolamycin P1	Angolamycin	Rosamicin P1	Rosamicin
Bacillus subtilis ATCC 6633	I	1.56	0.39	5.0	0.16
Micrococcus luteus S 19	I	0.39	0.05	1.25	0.08
Staphylococcus aureus FDA 209P	I	6.25	0.78	1.25	0.08
S. aureus Smith	I	12.5	1.56	5.0	0.31
Streptococcus pneumoniae Type III	II	12.5	3.13	1.25	0.08
S. pyogenes NY 5	II	12.5	0.78	5.0	0.16
Mycoplasma pneumoniae Mac	III	0.16	0.02		
M. gallisepticum S-6	III	0.31	0.08		
M. pulmonis PG-22	III	25	25		
M. fermentans	III	6.25	1.56		
M. hominis	III	25	25		
M. salivarium	III	25	25		

Medium I: brain-heart infusion broth (Difco).
II: brain-heart infusion broth (Difco) containing 10% horse blood.
III: PPLO broth (Difco) containing 10% horse serum.

Streptococcus pyogenes NY 5 is 0.78 μg/ml with angolamycin and 12.5 μg/ml with angolamycin P1. The similar reduction of the antibiotic potency appears true of rosamicin P1, too, although its antimycoplasmal activity was not measured because of unavailability of the compound. A plausible explanation for the marked difference between carbomycin A and angolamycin in the antimicrobial activity of the deepoxidation product P1 may be found in the steric hindrance of the methyl substituent at C-12.

Plasma Level and the *In Vivo* Antibacterial Activity

After subcutaneous injection in mice at a single dose of 200 mg/kg, the plasma levels of carbomycins A P1, A P2 and A P3 and the parent macrolide were followed for 150 minutes (Fig. 1).

It is clearly seen in Fig. 1 that the reductive epoxide-ring opening results in a highly significant eleva-

Fig. 1. Plasma concentrations of carbomycins A P1, A P2 and A P3 and the parent macrolide.
Dose: 200 mg/kg (sc), n=4
CRM=carbomycin

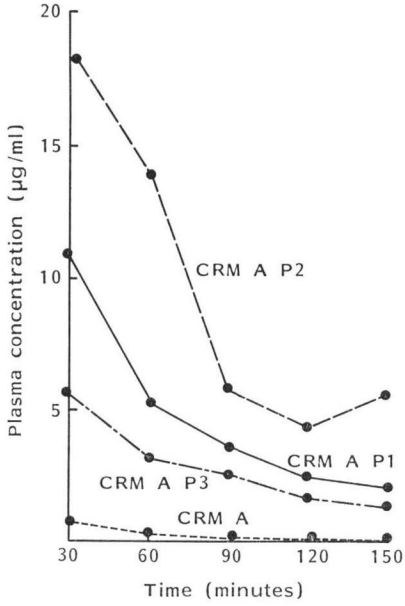

Fig. 2. Plasma concentrations of 4″-phenylacetyldeltamycins P1, P2 and P3 and the parent macrolide.
Dose: 200 mg/kg (sc), n=4
PAD=4″-phenylacetyldeltamycin

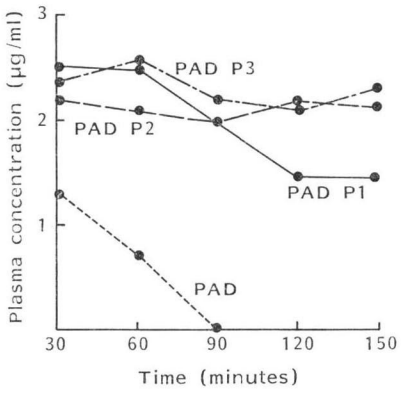

Fig. 3. Plasma concentrations of angolamycin P1 and the parent macrolide.
Dose: 200 mg/kg (sc), n=4
AGM=angolamycin

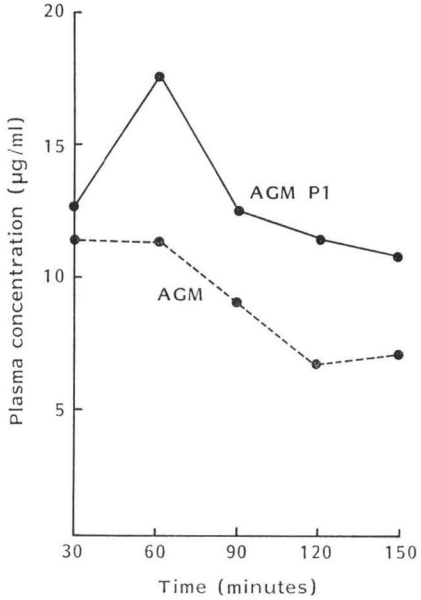

Table 5. *In vivo* effects of carbomycin A P2 and carbomycin A on staphylococcal infection.

Carbomycin A P2*		Carbomycin A**	
Dose (sc) (mg/kg)	Survived/ treated	Dose (sc) (mg/kg)	Survived/ treated
80	9 / 9	200	3 / 3
40	9 / 9	50	2 / 3
20	5 / 9	12.5	1 / 3
10	5 / 9	3.1	0 / 3
		0.8	0 / 3

* ED_{50} 10 mg/kg
** ED_{50} 30 mg/kg

tion and maintenance of the plasma level. The half-lives of carbomycins A P1, A P2 and A P3 were calculated to be 30, 44 and 70 minutes, respectively, while the parent macrolide gave a half-life of 21 minutes. The best among the three deepoxidation products is carbomycin A P2 having the conjugated enone structure in the *trans* configuration.

Under similar test conditions, 4″-phenylacetyldeltamycins P1, P2 and P3 were compared with the parent antibiotic. The results shown in Fig. 2 explicitly elucidate the improvement of the pharmacokinetic property of 4″-phenylacetyldeltamycin by deepoxidation. As the 4″-phenylacetyl group of 4″-phenylacetyldeltamycin is more lipophilic than the 4″-isovaleryl group of carbomycin A, 4″-phenylacetyldeltamycins P1, P2 and P3 produce lower, but longer-lasting serum concentrations than carbo-

mycins A P1, A P2 and A P3. It is noteworthy that the plasma levels of 4″-phenylacetyldeltamycins P1, P2 and P3 are sustained at around 2 μg/ml from the start of measurement.

Fig. 3 compares the pharmacokinetic behaviors of angolamycin P1 and the parent macrolide. Although a relative elevation of the plasma level by deepoxidation is noted, the degree of improvement is not so significant as in carbomycin A and 4″-phenylacetyldeltamycin. Angolamycin kept a plasma level as high as 12 μg/ml for 60 minutes after injection which did not drop as rapidly as that of carbomycin A, giving a plasma level of 7 μg/ml at 150 minutes. The relative importance of the epoxyenone structure in the capability of the macrolide molecule to bind with blood cells appears to be smaller in angolamycin than in carbomycin A and 4″-phenylacetyldeltamycin.

The therapeutic effects of carbomycin A P2 and the parent macrolide were compared in mice experimentally infected by *Staphylococcus aureus* Smith. The data in Table 5 apparently indicate that the improved plasma level of carbomycin A P2 was reflected in a better therapeutic efficiency.

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