PALDIMYCINS A AND B AND ANTIBIOTICS 273a$_{2\alpha}$ AND 273a$_{2\beta}$ SYNTHESIS AND CHARACTERIZATION

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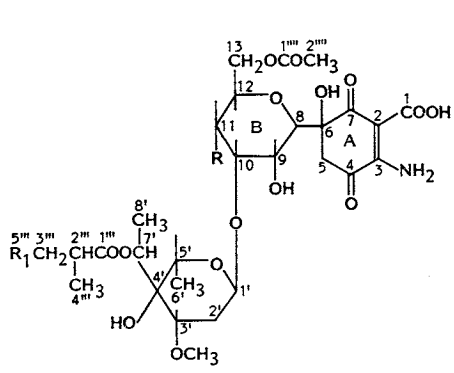
(Received for publication September 13, 1986)

Paldimycin (antibiotic 273a$_1$) and antibiotic 273a$_2$ as well as their individual components, paldimycins A (273a$_{1\alpha}$) and B (273a$_{1\beta}$) and antibiotics 273a$_{2\alpha}$ and 273a$_{2\beta}$ were synthesized from paulomycin, paulomycin A and paulomycin B, respectively, by reacting with *N*-acetyl-L-cysteine.

The semisynthetic antibiotics had chromatographic behavior (TLC, HPLC) and physical and chemical properties identical to the properties of the corresponding antibiotics produced by *Streptomyces paulus*.

The production and isolation of paldimycin (antibiotic 273a$_1$) and antibiotic 273a$_2$ from fermentations of *Streptomyces paulus* were reported in the first paper in this series.[1] Extensive chromatographic work and mass spectral analysis indicated that each of the antibiotics consisted of two closely related materials. The components present in paldimycin were designated paldimycin A (antibiotic 273a$_{1\alpha}$) and paldimycin B (antibiotic 273a$_{1\beta}$). The two components in antibiotic 273a$_2$ are known as antibiotics 273a$_{2\alpha}$ and 273a$_{2\beta}$. The information discussed in the previous paper[1] indicated that both paldimycin and antibiotic 273a$_2$ are produced by *S. paulus* from paulomycins A and B by addition of two or one molecules of *N*-acetyl-L-cysteine, respectively. In order to confirm the proposed structures[1] (Fig. 1) for these antibiotics and to produce the needed materials, we studied the synthesis of paldimycin and antibiotic 273a$_2$. This paper discusses this work and describes the chemical and physical properties of these novel antibiotics.

Fig. 1.



Paulomycin A $R_1 = CH_3$
Paulomycin B $R_1 = H$
$R = CH_3CH{=}CCOO-$ (4" 3" 2" 1"), N=C=S

273a$_{2\alpha}$ $R_1 = CH_3$
273a$_{2\beta}$ $R_1 = H$
$R = CH_3CH{=}CCOO-$ (4" 3" 2" 1"), NHCSCH$_2$CHCOOH (S), NHCOCH$_3$

Paldimycin A $R_1 = CH_3$
Paldimycin B $R_1 = H$
$R = CH_3CHCHCOO-$ (4" 3" 2" 1"), S, NHCSCH$_2$CHCOOH, CH$_2$, S, NHCOCH$_3$, CHCOOH, NHCOCH$_3$

Experimental

Assay and Testing Procedures

Antibiotic production and purification was measured by a microbiological disc-plate assay procedure with *Micrococcus luteus* as the assay organism.

Thin-layer Chromatographic Procedures

The reaction of paulomycin with *N*-acetyl-L-cysteine was followed by TLC on Silica gel G using chloroform - ethanol - water (25 : 30 : 5) or on cellulose powder using pH 7.0 phosphate buffer as the solvent systems. The antibiotics present in reaction mixtures or in preparations obtained during purification were detected by bioautography on *M. luteus*-seeded trays.

Spectroscopic Methods

^{1}H NMR spectra were recorded on a Varian XL-200 spectrometer operating at 200 MHz. Solutions (*ca.* 0.4 ml, *ca.* 0.25 M) of the compounds in dimethyl sulfoxide-d_6 or acetone-d_6 were used. ^{13}C NMR spectra were recorded on a Varian XL-200 spectrometer operating at 50.0 MHz. ^{1}H and ^{13}C NMR chemical shifts are reported as ppm relative to tetramethylsilane. Mass spectra were obtained on a ZAB-2F high resolution mass spectrometer using a fast atom bombardment (FAB) source.

Analytical High Performance Liquid Chromatography (HPLC)

All analytical HPLC chromatography of synthetic paldimycin and antibiotic 273a$_2$ was carried out on a Waters instrument equipped with a Waters 440 UV detector and operating in the dual pump mode. A Waters C-18, 30 cm, 10 μm reverse phase silica column was used. Mobile phase consisted of gradient of solvent A to solvent B in 35 minutes; flow rate, 1.2 ml/minute, 70.3 kg/cm^2. Solvent A consisted of 0.05 M K_2HPO_4 in acetonitrile - water (15 : 85) adjusted to pH 5.3. Solvent B consisted of 0.05 M K_2HPO_4 in acetonitrile - water (35 : 65) adjusted to pH 5.3. A 10 μl sample was usually injected.

Preparation of Paldimycin and Paldimycins A and B

1. Paldimycin: Paulomycin (A and B mixture), 5.98 g (7.75 mmol), was added under stirring to a solution prepared from 25.2 g (150 mmol) of *N*-acetyl-L-cysteine in 500 ml of 0.1 M phosphate buffer, pH 7.85. The solution was adjusted to pH 8.7 with aqueous sodium hydroxide and allowed to stand at room temperature for 1 hour. The pH was then adjusted to 3.0 with 2 N aqueous hydrochloric acid and the acidified mixture was extracted three times with 500 ml portions of ethyl acetate. The ethyl acetate extracts were combined, dried over sodium sulfate and concentrated to dryness, to give 14.0 g of crude paldimycin. This material contained (by TLC) paldimycin and traces of antibiotic 273a$_2$ as the only bioactive components. Crude paldimycin was dissolved in 120 ml acetone and the solution was mixed with 1.1 liters of ethyl ether. The precipitated material was isolated by filtration and dried; it was then re-dissolved in 120 ml acetone and this solution was mixed with 1.1 liters of ethyl ether. Pure paldimycin, 6.2 g was isolated by filtration and drying. Characterization of this material is described in the characterization section.

2. Paldimycin A: Paldimycin A, 0.91 g, was isolated by the above procedure by reacting 1.0 g of paulomycin A and 4.2 g of *N*-acetyl-L-cysteine. The properties of paldimycin A are described in the characterization section.

3. Paldimycin B: Paldimycin B, 1.1 g, was isolated by the procedure used for paldimycin (see above) by reacting 1.0 g of paulomycin B and 4.2 g of *N*-acetyl-L-cystein. The properties of paldimycin B are described in the characterization section.

Preparation of Antibiotic 273a$_2$

Antibiotic 273a$_2$: Paulomycin (A and B mixture), 2.0 g (2.56 mmol), was added under stirring to a solution prepared from 628 mg (3.84 mmol) of *N*-acetyl-L-cysteine in 100 ml of 0.1 M phosphate buffer, pH 7.85. This solution was adjusted to pH 8.7 and allowed to stand at room temperature for 20 minutes; it was then adjusted to pH 3.0 and extracted twice with 100 ml portions of ethyl acetate. The ethyl acetate extracts were combined, dried over sodium sulfate and concentrated to dryness to give 2.29 g of colorless amorphous material which contained (by TLC) paldimycin and antibiotic 273a$_2$ as the only bioactive components. This material was dissolved in 20 ml of acetone and the solution

was mixed with 450 ml of ethyl ether - Skellysolve B (3 : 1.5). The precipitated material was isolated by filtration and dried (1.56 g). This highly active preparation was found to be a mixture of paldimycin and antibiotic $273a_2$. Separation of these antibiotics was obtained by counter-double-current distribution as described below.

Separation of Antibiotic $273a_2$ from Paldimycin: Crude antibiotic $273a_2$ (1.56 g), obtained as described above, was dissolved in both phases (50 ml) of the solvent system consisting of cyclohexane - ethyl acetate - acetone - water (1 : 1 : 1 : 1). The solution was put in tube 15 (where the lower phase enters the machine) of a 100 - tube all-glass counter-double-current distribution machine. The distribution was analyzed by TLC after 85 transfers. Tubes 10~50 contained antibiotic $273a_2$ while paldimycin was found in tubes 80~100. Fractions containing antibiotic $273a_2$ were concentrated to dryness to give, after precipitation from chloroform - Skellysolve B, 440 mg of pure antibiotic $273a_2$. Characterization of this material is discussed later in this paper.

Antibiotic $273a_{2\alpha}$: Antibiotic $273a_{2\alpha}$, 390 mg, was prepared by a procedure identical to that described for antibiotic $273a_2$, from 1.0 g of paulomycin A (1.3 mmol) and 314 mg (1.92 mmol) of *N*-acetyl-L-cysteine. The properties of antibiotic $273a_{2\alpha}$ are described in the characterization section.

Antibiotic $273a_{2\beta}$: Antibiotic $273a_{2\beta}$, 350 mg, was prepared by the procedure used for antibiotic $273a_2$ (see above) by reacting 1.0 g of paulomycin B (1.3 mmol) and 314 mg (1.92 mmol) of *N*-acetyl-L-cysteine. Characterization of antibiotic $273a_{2\beta}$ is discussed later in this paper.

Separation of Stereoisomers of Paldimycins A and B

Semi-preparative HPLC: The following conditions were used: Column, Waters semi-prep C-18 reverse phase silica; pumps, 2 Waters 6000A; gradient programmer, Waters 500; detector, Waters 440 at 254 nm; injector, Valco with 100 μl loop; flow 4 ml/minute; solvent A, 0.05 M K_2HPO_4 in acetonitrile - water (15 : 85) adjusted to pH 5.3; solvent B, 0.05 M K_2HPO_4 in acetonitrile - water (35 : 65) adjusted to pH 5.3; gradient program from 100% solvent A to 70% solvent B in 35 minutes and hold; sample concentration, 1.1 mg of paldimycin (mixture of A and B)/100 μl of solvent A. A typical chromatogram is presented in Fig. 9. A total of 20 runs were made. Corresponding fractions in the shaded area of each of the six peaks (I~VI) observed were pooled. The solutions were acidified (pH ~3.5) and the paldimycins present were extracted with methylene chloride. Concentration of the extract to dryness and precipitation from methylene chloride - cyclohexane yielded amorphous colorless materials which were characterized as indicated in the Results and Discussion section of this paper.

Results and Discussion

Reaction of Paulomycin with *N*-Acetyl-L-cysteine

In selecting the conditions (solvent, pH, temperature) for the reaction of paulomycin and *N*-acetyl-L-cysteine we had to consider the properties of paulomycin; specifically, its weakly acidic nature (*pKa'*, 7.5), its limited solubility in water and its sensitivity to heating and to both extreme alkaline and extreme acidic environments. Work on the structure of paulomycin[2] (Fig. 1) has shown that the antibiotic is transformed easily to paulomycinone, a bio-inactive compound by dehydration of ring A. This reaction, which proceeds slowly in aqueous media at neutral pH, is accelerated at acidic pH's and in solvents like methanol or ethanol. In alkaline media, ring A appears to be reasonably stable. The main reaction which occurs under alkaline conditions is hydrolysis of the paulic acid ester and formation of paulomenol[2] which like paulomycinone is bio-inactive.

We decided, therefore, to use 0.1 M aqueous phosphate buffer, pH 8.6, as the solvent and room temperature. Paulomycin has increased solubility in the slightly alkaline solution and the main degradative reactions mentioned above occur very slowly. In order to determine the concentration of the reactants for the quantitative production of paldimycin, a constant amount of paulomycin

(200 mg, 0.256 mmol) was added under stirring to different solutions (20 ml) of *N*-acetyl-L-cysteine in 0.1 M phosphate buffer, pH 8.6, so that the molar ratio of *N*-acetyl-L-cysteine to paulomycin was 1:1 (solution A); 2:1 (solution B); 5:1 (solution C); 10:1 (solution D) and 20:1 (solution E). Reaction mixtures were allowed to stand at room temperature and analyzed at different time intervals by bioactivity determination against *M. luteus*, UV spectra and TLC. No loss of total bioactivity was observed in each solution for 2 hours. The UV spectra of solutions A, B, C and D observed at 15 minutes were similar to the spectra of paldimycins A and B (Fig. 4) and for practical purposes identical to the spectra obtained at 30, 60 or 120 minutes. Comparison of these spectra to the UV spectrum of paulomycin[3] indicates a fast transformation of paulomycin to paldimycin. Thin-layer chromatography of the reaction mixtures showed that at low *N*-acetyl-L-cysteine to paulomycin molar ratios (1:1 or 2:1), the solutions contained unreacted paulomycin even after 2 hours of reaction time. In addition to paulomycin, paldimycin (Rf ~0.15) and antibiotic 273a_2 (Rf ~0.6) were also present. At higher *N*-acetyl-L-cysteine to paulomycin ratios (5:1, 10:1, 20:1) paulomycin is converted rapidly to antibiotics 273a_1 and 273a_2. Finally, at *N*-acetyl-L-cysteine to paulomycin ratio of 20:1 all paulomycin had been transformed to paldimycin within 1 hour of reaction time.

Preparation and Isolation of Paldimycin and Antibiotic 273a_2

Paldimycin (a mixture of A and B), paldimycin A and paldimycin B were prepared by reacting paulomycin (a mixture of paulomycins A and B), paulomycin A or paulomycin B, respectively, with *N*-acetyl-L-cysteine (molar ratio 1:20). The reaction was carried out for 1 hour at room temperature in aqueous buffer, pH 8.6. Acidification of the reaction mixture followed by extraction with ethyl acetate yielded the corresponding crude paldimycin which was purified by precipitation from acetone - ether mixture.

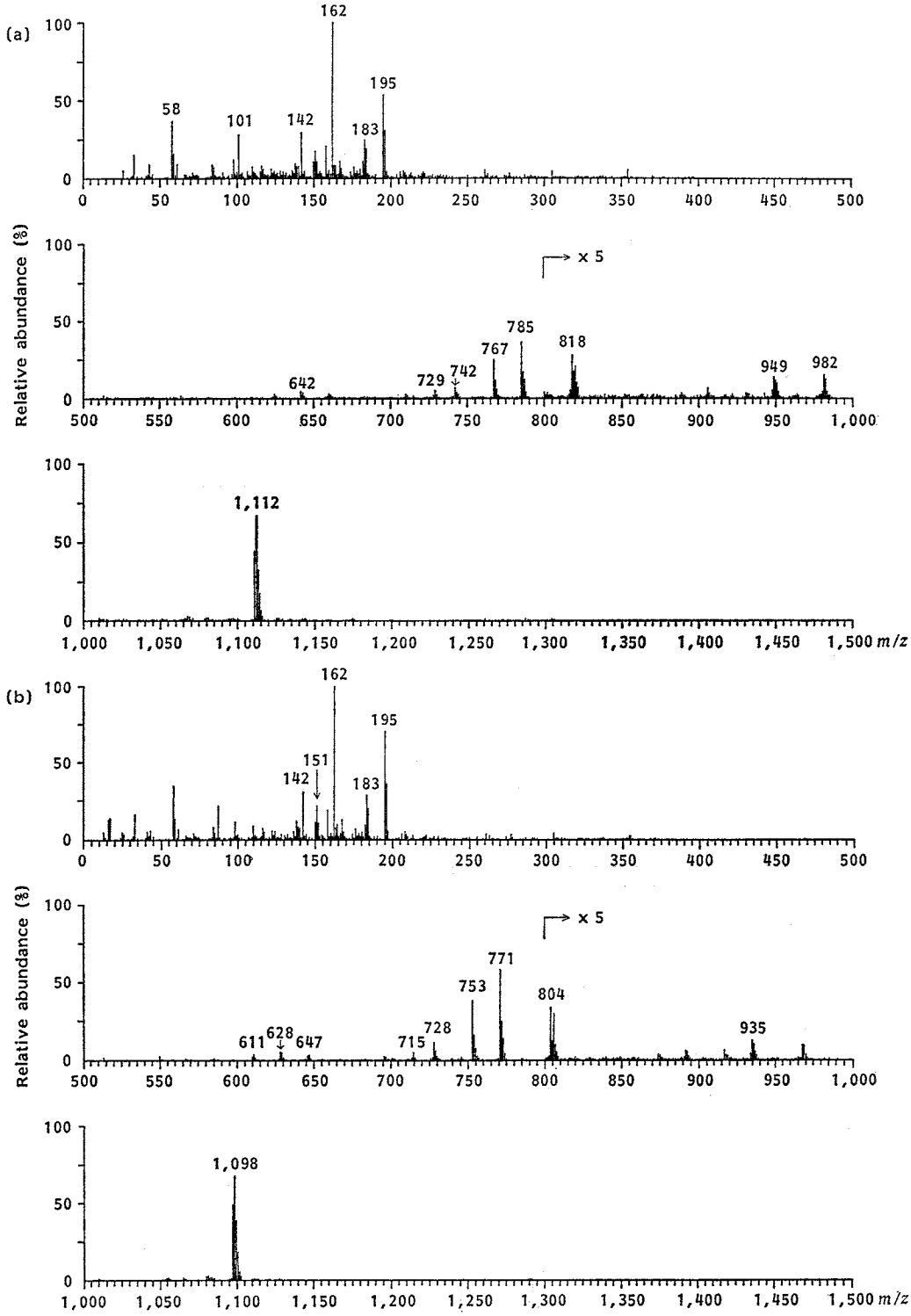
Reaction of paulomycin, paulomycin A or paulomycin B with limited amounts of *N*-acetyl-L-cysteine (molar ratio 1:1.5) for 20 minutes at room temperature in pH 8.6 buffer yielded, after acidification and extraction with ethyl acetate, a mixture of substantial amounts of the corresponding paldimycin and the corresponding 273a_2 antibiotic (paldimycin, 273a_2; paldimycin A, 273a_{2a}; paldimycin

Table 1. Physical and chemical properties of paldimycins A and B.

	Paldimycin A	Paldimycin B
Molecular formula[a,b]	$C_{44}H_{64}N_4O_{23}S_3$	$C_{43}H_{62}N_4O_{23}S_3$
Molecular weight[a] (*m/z*)	1,112	1,098
Anal (Calcd/Found)		
C	47.48/46.82	46.99/46.22
H	5.75/5.78	5.64/5.69
N	5.03/4.93	5.10/5.00
S	8.63/8.72	8.74/8.83
MP	Decomposition starts at *ca.* 120°C	Decomposition starts at *ca.* 120°C
Titration	*pKa'* 3.8~4.0	*pKa'* 3.8~4.0
(in 60% aq ethanol)[c]	Eq weight 379	Eq weight 399
$[\alpha]_D^{25}$ (*c* 0.9, methanol)	−31°	−35°
UV λ_{max} nm (ε)	248 (18.01×10^3), 274 (9.46×10^3),	248 (17.88×10^3), 274 (9.40×10^3),
in 95% ethanol	321 (8.99×10^3)	321 (9.01×10^3)
IR (Nujol) cm^{-1}	3469, 3238, 1949, 1736, 1661, 1574	3468, 3273, 1943, 1736, 1662, 1575

[a] By high resolution mass spectrometry (fast atom bombardment).
[b] From analytical data.
[c] Aqueous KOH was used as titrant.

Fig. 2. FAB-MS of paldimycin A (a) and paldimycin B (b).



B, $273a_{2\beta}$). Separation of paldimycins from antibiotics $273a_2$ was obtained by counter-double-current distribution using cyclohexane - ethyl acetate - acetone - water (1 : 1 : 1 : 1).

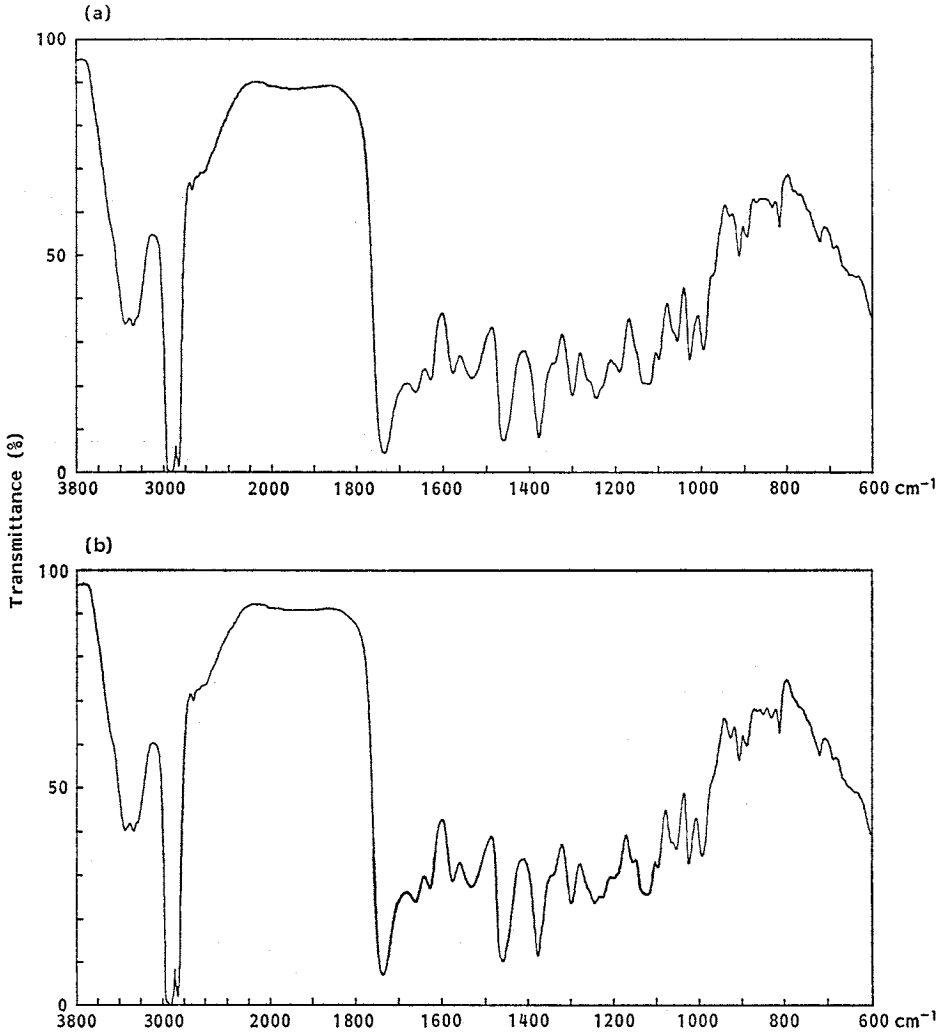
Comparison of Semisynthetic and Fermentation-produced Paldimycin and Antibiotic $273a_2$

Paldimycin and antibiotic $273a_2$ prepared by reaction of a mixture of paulomycins A and B with *N*-acetyl-L-cysteine were found identical in all respects (TLC, HPLC, IR, ^{13}C NMR, FAB-MS) to paldimycin (antibiotic $273a_1$) and antibiotic $273a_2$ isolated from fermentations of *Streptomyces paulus*.[1]

Characterization of Paldimycins A and B

Paldimycins A and B are colorless amorphous compounds readily soluble in chloroform, methylene chloride, lower alcohols, ethyl and butyl acetate, acetone and most other common organic solvents. The antibiotics have limited solubility in ether and water and are quite insoluble in saturated hydrocarbon solvents.

Fig. 3. IR spectra of paldimycins A and B.
(a) Paldimycin A, (b) paldimycin B.



The physical and chemical properties of paldimycins A and B are listed in Table 1. The antibiotics are tribasic acids and form mono-, bis- and tri-potassium, sodium or ammonium salts which are soluble in water and concentrations of *ca.* 150~200 mg/ml. Both antibiotics melt with decomposition at *ca.* 120°C. The specific rotation of paldimycin A was found to be −31°; paldimycin B had a rotation of −35°. The molecular formulas of $C_{44}H_{64}N_4O_{23}S_3$ for paldimycin A and $C_{43}H_{62}N_4O_{23}S_3$ for paldimycin B were established from analytical data and high resolution FAB-MS (Fig. 2). The IR spectra of paldimycins A and B, shown in Fig. 3, are nearly identical and characterized by the

Fig. 4. UV spectra of paldimycins A and B.
(a) Paldimycin A, (b) paldimycin B.

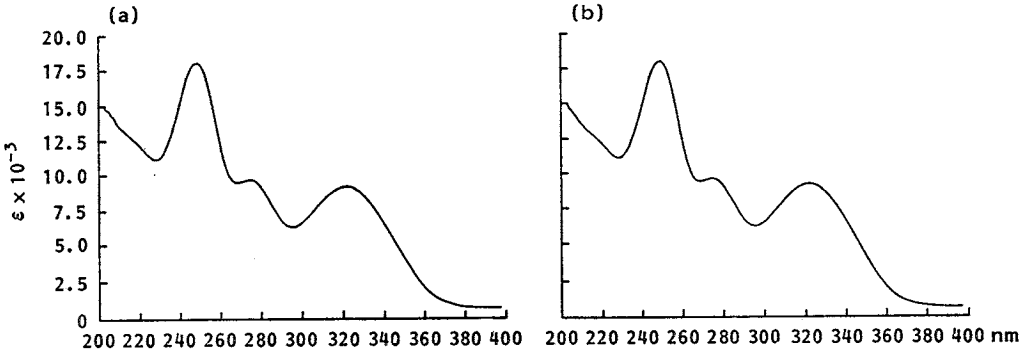


Fig. 5. ^{13}C NMR spectra of paldimycins A and B (in DMSO-d_6).
(a) Paldimycin A, (b) paldimycin B.

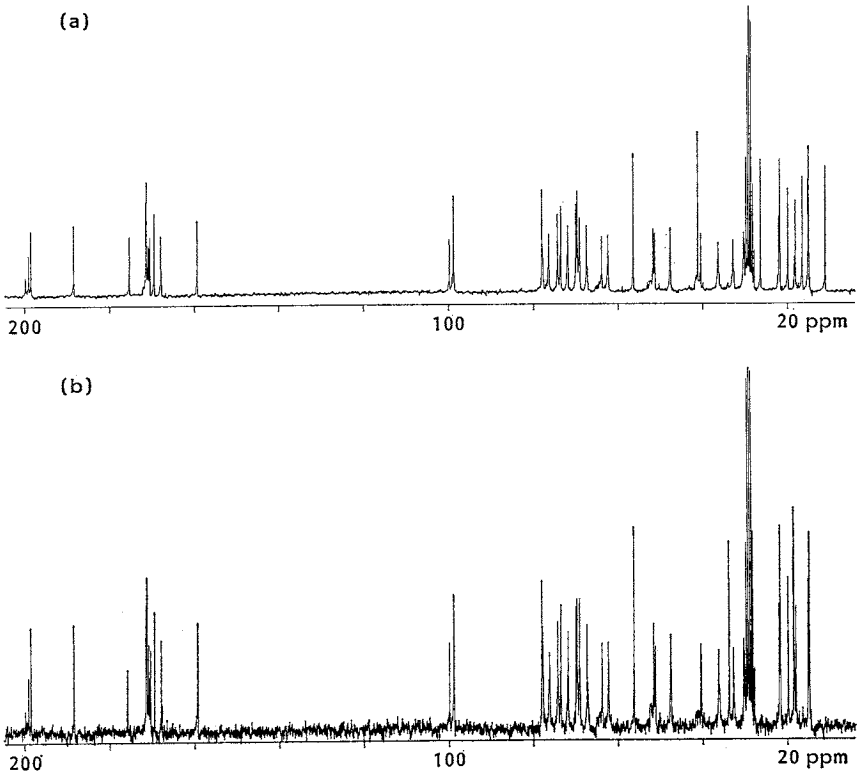


Table 2. Chemical shifts observed in the ^{13}C NMR spectra of paulomycin A, paldimycin A and antibiotic $273a_{2\alpha}$.

Carbon No.[a]	Chemical shift, δ[b] (multiplicity)[c]		
	Paulomycin A	Paldimycin A	Antibiotic $273a_{2\alpha}$
1	169.35 (s)	170.02 (s)	170.01 (s)
2	100.72 (s)	100.51 (s)	100.57 (s)
3	159.37 (s)	159.91 (s)	159.91 (s)
4	198.50 (s)	198.91 (s)	198.99 (s)
5	48.01 (t)	48.24 (t)	48.54 (t)
6	78.20 (s)	78.32 (s)	78.82 (s)
7	188.39 (s)	188.85 (s)	188.84 (s)
8	78.26 (d)	78.21 (d)	78.42 (d)
9	69.20 (d)	69.64 (d)	70.01 (d)
10	76.18 (d)	76.79 (d)	76.81 (d)
11	70.73 (d)	70.48 (d)	70.57 (d)
12	72.29 (d)	72.38 (d)	73.17 (d)
13	62.30 (t)	63.18 (t)	63.47 (t)
1′	99.04 (d)	99.56 (d)	99.21 (d)
2′	30.56 (t)	30.86 (t)	31.25 (t)
3′	74.40 (d)	74.74 (d)	75.04 (d)
4′	73.62 (s)	73.60 (s)	74.51 (s)
5′	67.78 (d)	68.02	67.81 (d)
6′	15.28[e] (q)	15.68[e] (q)	16.01[e] (q)
7′	69.73 (d)	70.19 (d)	70.34 (d)
8′	15.39[e] (q)	15.58[e] (q)	15.83[e] (q)
CH_3O	58.62 (q)	57.23 (q)	57.16 (q)
1″	160.25 (s)	168.48 (s)	163.06 (s)
2″	123.36 (s)	64.40	131.71 (s)
3″	136.64 (d)	41.25 (d)	136.95 (d)
4″	14.11 (q)	18.67 (q)	13.44 (q)
N=C=S or NHCS ‖ S	142.64 (s)	199.47 (s)	199.95 (s)
1‴	175.15 (s)	175.80 (s)	175.89 (s)
2‴	41.51 (d)	41.99 (d)	42.00 (d)
3‴	26.65 (t)	26.90 (t)	27.13 (t)
4‴	16.73 (q)	17.04 (q)	17.38 (q)
5‴	11.39 (q)	11.68 (q)	11.95 (q)
1⁗	170.18 (s)	170.97 (s)	170.92 (s)
2⁗	19.99 (q)	20.43 (q)	20.69 (q)
N-Ac-Cyst[f]			
SCH_2	—	33.37 and 36.90 (t)	37.31 (t)
CHNH	—	52.17 and 52.52 (d)	53.50 (d)
COOH	—	171.43 and 171.25 (s)	171.28 (s)
$NHCOCH_3$	—	172.10 and 171.94 (s)	171.80 (s)
$NHCOCH_3$	—	22.35 and 22.54 (q)	22.63 (q)

[a] For numbering of carbons, see Fig. 1.
[b] Relative to tetramethylsilane.
[c] Multiplicity in off-resonance decoupled spectra. s: singlet, d: doublet, t: triplet, q: quartet.
[e] Assignments are interchangeable.
[f] Abbreviation for *N*-acetyl-L-cysteine.

Table 3. Chemical shifts observed in the ^{13}C NMR spectra of paulomycin B, paldimycin B and antibiotic $273a_{2\beta}$.

Carbon No.[a]	Chemical shift, δ[b] (multiplicity)[c]		
	Paulomycin B	Paldimycin B	Antibiotic $273a_{2\beta}$
1	169.35 (s)	170.03 (s)	170.02 (s)
2	100.14 (s)	100.53 (s)	100.58 (s)
3	159.36 (s)	159.92 (s)	159.92 (s)
4	198.37 (s)	198.95 (s)	199.04 (s)
5	47.95 (t)	48.48 (t)	48.54 (t)
6	78.14 (s)	78.74 (s)	78.81 (s)
7	188.40 (s)	188.88 (s)	188.86 (s)
8	78.14 (d)	78.53 (d)	78.35 (d)
9	69.28 (d)	69.93 (d)	70.02 (d)
10	75.91 (d)	76.94 (d)	76.78 (d)
11	70.71 (d)	70.78 (d)	70.59 (d)
12	72.21 (d)	72.61 (d)	73.15 (d)
13	62.23 (t)	63.21 (t)	63.49 (t)
1′	98.94 (d)	99.52 (d)	99.20 (d)
2′	30.33 (t)	31.14 (t)	31.27 (t)
3′	74.38 (d)	75.02 (d)	75.08 (d)
4′	73.66 (s)	74.50 (s)	74.59 (s)
5′	67.65 (d)	68.19 (d)	67.76 (d)
6′	15.23[e](q)	15.79[e](q)	15.81[e](q)
7′	69.96 (d)	70.53 (d)	70.37 (d)
8′	15.44[e](q)	16.01[e](q)	16.08[e](q)
CH_3O	56.59 (q)	57.20 (q)	57.17 (q)
1″	160.25 (s)	168.48 (s)	163.10 (s)
2″	123.32 (s)	64.66 (d)	131.72 (s)
3″	136.66 (d)	41.26 (d)	136.94 (d)
4″	14.13 (q)	18.94 (q)	13.96 (q)
N=C=S or NHCS ‖ S	142.54 (s)	199.47 (s)	199.97 (s)
1‴	175.71 (s)	176.36 (s)	176.46 (s)
2‴	34.15 (d)	34.67 (d)	34.69 (d)
3‴	18.77[g](q)	19.36[g](q)	19.39[g](q)
4‴	18.93[g](q)	194.70[g](q)	19.52[g](q)
5‴	—	—	—
1⁗	170.18 (s)	170.97 (s)	171.35 (s)
2⁗	19.98 (s)	20.70 (s)	20.71 (s)
N-Ac-Cyst[f]			
SCH_2	—	33.63 and 37.18 (t)	37.34 (t)
CHNH	—	52.52 and 52.16 (d)	53.36 (d)
COOH	—	171.41 and 171.79 (s)	170.96 (s)
$NHCOCH_3$	—	171.91 and 171.98 (s)	171.09 (s)
$NHCOCH_3$	—	22.62 and 22.80 (q)	22.66 (q)

[a] For numbering of carbons, see Fig. 1.
[b] Relative to tetramethylsilane.
[c] Multiplicity in off-resonance decoupled spectra. s: singlet, d: doublet, t: triplet, q: quartet.
[e,g] Assignments are interchangeable.
[f] Abbreviation for *N*-acetyl-L-cysteine.

absence of the 2050 cm^{-1} band which is present in the spectra of paulomycins A and B and has been assigned to the isothiocyanate group present in these antibiotics. The UV spectra of paldimycin A and B (Fig. 4) are also identical and contain three peaks at 248, 274 and 321 nm. The maximum at

Fig. 6. ^1H NMR spectra of paldimycins A and B (in DMSO-d_6).
(a) Paldimycin A, (b) paldimycin B.

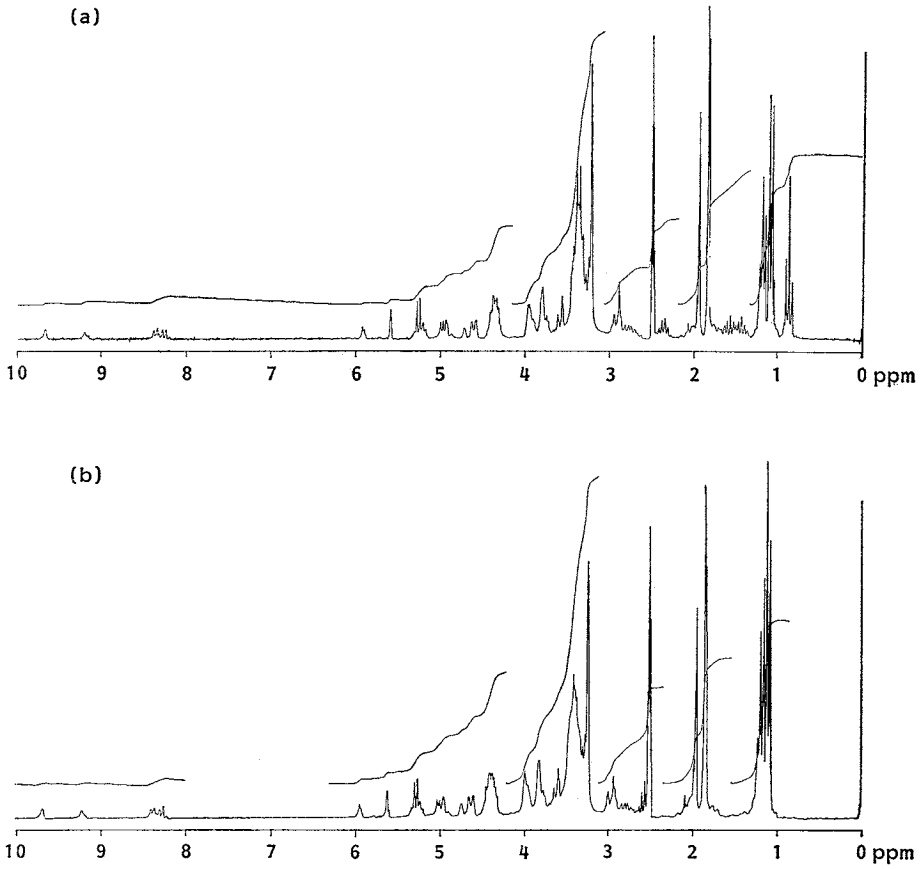


Table 4. ^1H NMR chemical shifts[a] of selected groups of paldimycins A and B.

Paldimycin A		Paldimycin B	
Chemical shift, δ (multiplicity)[b]	Assignment[c]	Chemical shift, δ (multiplicity)[b]	Assignment[c]
0.95 (3H, t)	5‴-CH_3	—	—
1.17 (3H, d)	4‴-CH_3	1.15 (6H, d)	3‴-CH_3 and 4‴-CH_3
1.20 (3H, d)	4″-CH_3	1.17 (3H, d)	4″-CH_3
1.22 (3H, d), 1.25 (3H, d)	8′-CH_3 and 6′-CH_3	1.20 (3H, d), 1.25 (3H, d)	8′-CH_3 and 6′-CH_3
1.87 (6H, 2×s)	2×CH_3CONH	1.85 (6H, 2×s)	2×CH_3CONH
1.99 (3H, s)	2⁗-CH_3COO	1.99 (3H, s)	2⁗-CH_3COO
3.30 (3H, s)	CH_3O	3.28 (3H, s)	CH_3O
3.37~3.45 (complex)	2×SCH_2	3.35~3.45 (complex)	2×SCH_2

[a] Dimethyl sulfoxide-d_6 was used as solvent.
[b] Multiplicity, s: singlet, d: doublet, t: triplet.
[c] For groups designation, see Fig. 1.

Fig. 7. TLC comparison of paulomycins, paldimycins A and B and antibiotics 273$a_{2\alpha}$ and 273$a_{2\beta}$.
Silica gel (Polygram Sil-N-HR) precoated sheets; chloroform - ethanol - water (25 : 30 : 5). Antibiotics were detected by bioautography on *Micrococcus luteus* - seeded agar.

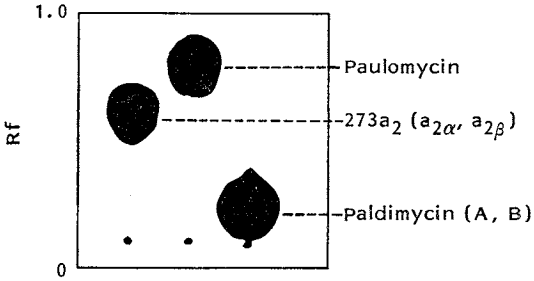
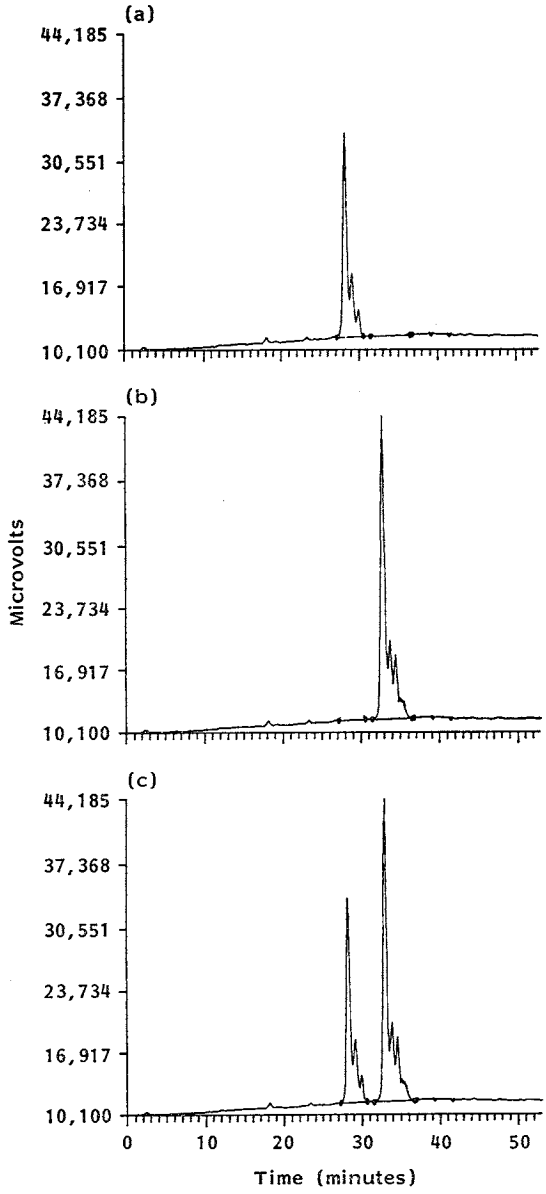


Fig. 8. HPLC of paldimycins A and B.*
(a) Paldimycin B, (b) paldimycin A, (c) paldimycins A and B (mixture).
* For conditions see Experimental.



321 nm has been extremely useful in the chromatographic studies described below and in studies related to the stability of these antibiotics. The ^{13}C NMR spectra of paldimycins A and B are shown in Fig. 5. Lists of the chemical shift of carbons of each paldimycin and the corresponding paulomycins are presented in Tables 2 and 3. The ^{13}C NMR spectra are characterized by the absence of absorptions at δ 123.36 (s), 136.64 (d), 142.64 (s) assigned to the unsaturated and isothiocyanate carbons of paulic acid ester in paulomycins[2] indicating the addition of two molecules of *N*-acetyl-L-cysteine to the paulic acid moiety as shown in Fig. 1. The ^{1}H NMR spectra of paldimycins A and B (Fig. 6) are, as expected, very similar. Since paldimycins A and B were isolated as a mixture of at least three stereoisomers (see discussion below) the ^{1}H NMR spectra are not well defined. A list of absorptions due to basic features of the two antibiotics is presented in Table 4. Fig. 7 presents a TLC of paulomycins (A and B), paldimycins (A and B) and antibiotic 273a_2 (273$a_{2\alpha}$ and 273$a_{2\beta}$). Paulomycins A and B have been separated by TLC,[3] however no TLC systems have been found which could separate efficiently paldimycins A and B or antibiotics 273$a_{2\alpha}$ and 273$a_{2\beta}$.

Paldimycins A and B are separated easily by analytical HPLC using C-18 reverse phase silica columns and mobile phases consisting of gradients of acetonitrile-phosphate buffer, pH 5.5. Fig. 8 shows a typical chromatogram of paldimycins A and B and of the paldimycin mixture. As in the case of paldimycin produced by *S. paulus*,[1] three peaks are observed in the HPLC chromatogram of

either paldimycin. This is due to the formation of two new asymmetric centers at C-2″ and C-3″ of the paldimycin molecule (Fig. 1) when two molecules of *N*-acetyl-L-cysteine are added to the paulic acid moiety of paulomycin. Four stereoisomers are theoretically possible; it appears, that at least three stereoisomers are formed or separated under the conditions used. Using a semi-preparative HPLC column, 22.2 mg of a mixture of paldimycins A and B was chromatographed. Six peaks were observed, three for each paldimycin A or paldimycin B, and designated I to VI (Fig. 9). Material from the shaded areas of each peak was isolated and analyzed by HPLC, then characterized by UV, FAB-MS, bio-TLC, and agar diffusion bioassays vs. *Micrococcus luteus*. Peaks I, II and III yielded materials which showed only one peak in HPLC with retention times identical to those shown in Fig. 9 for the respective peaks. These materials had properties identical to those of paldimycin B (MW 1,098) while the materials obtained from peaks IV, V and VI, though differentiated by HPLC, behaved like paldimycin A (MW 1,112). All material isolated from the six peaks were, within the experimental error, equally active vs. *M. luteus*.

Fig. 9. Semi-preparative separation of stereoisomers of paldimycins A and B.*

* For conditions see Experimental.

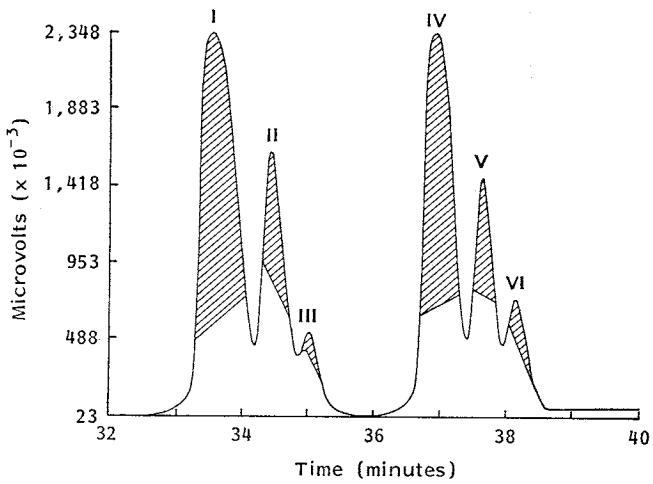


Table 5. Physical and chemical properties of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$.

	Antibiotic $273a_{2\alpha}$	Antibiotic $273a_{2\beta}$
Molecular formula[a,b]	$C_{38}H_{55}N_3O_{20}S_2$	$C_{38}H_{53}N_3O_{20}S_2$
Molecular weight[a] (*m/z*)	949	935
Anal (Calcd/Found)		
C	49.31/49.11	48.77/48.98
H	5.79/5.91	5.67/5.97
N	4.42/4.30	4.49/4.25
S	6.74/6.63	6.84/6.77
MP	119~150°C (dec)	Decomposition begins at 122°C
$[\alpha]_D^{25}$	−33° (*c* 0.89, methanol)	−34° (*c* 0.4, methanol)
UV λ_{max} nm (ε)	246 (18.6×10^3), 270 (sh,	246 (17.91×10^3), 270 (sh,
in 95% ethanol	11.24×10^3), 320 (8.87×10^3)	9.90×10^3), 320 (8.91×10^3)
IR (Nujol) cm^{-1}	3470, 3413, 3233, 2031, 1734, 1659, 1575	3468, 3412, 3236, 2030, 1734, 1660, 1574

[a] By high resolution mass spectrometry (fast atom bombardment).

[b] From analytical data.

Characterization of Antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$

Antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ have been also isolated as colorless amorphous compounds with solubilities identical to those of paldimycins A and B. The physical and chemical properties of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ are listed in Table 5. The antibiotics are di-basic acids and form salts with sodium, potassium or ammonium which are soluble in water at *ca.* 150 mg/ml. Both antibiotics melt with decomposition over a wide range of temperatures. The specific rotation of antibiotic $273a_{2\alpha}$ is $-33°$; antibiotic $273a_{2\beta}$ has a rotation of $-34°$. The molecular formula of $C_{39}H_{55}N_3O_{20}S_2$ for antibiotic $273a_{2\alpha}$ and $C_{38}H_{53}N_3O_{20}S_2$ for antibiotic $273a_{2\beta}$ were established from analytical data and high resolution FAB-MS (Fig. 10). The IR spectra of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ (Fig. 11) are, like the IR spectra of paldimycins, characterized by the absence of the 2050 cm^{-1} band which is present in the IR spectra of paulomycins A and B, and is due to the isothiocyanate group of these antibiotics. The UV spectra of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ (Fig. 12) are identical and contain absorptions at

Fig. 10. FAB-MS of antibiotics $273a_{2\alpha}$ (a) and $273a_{2\beta}$ (b).

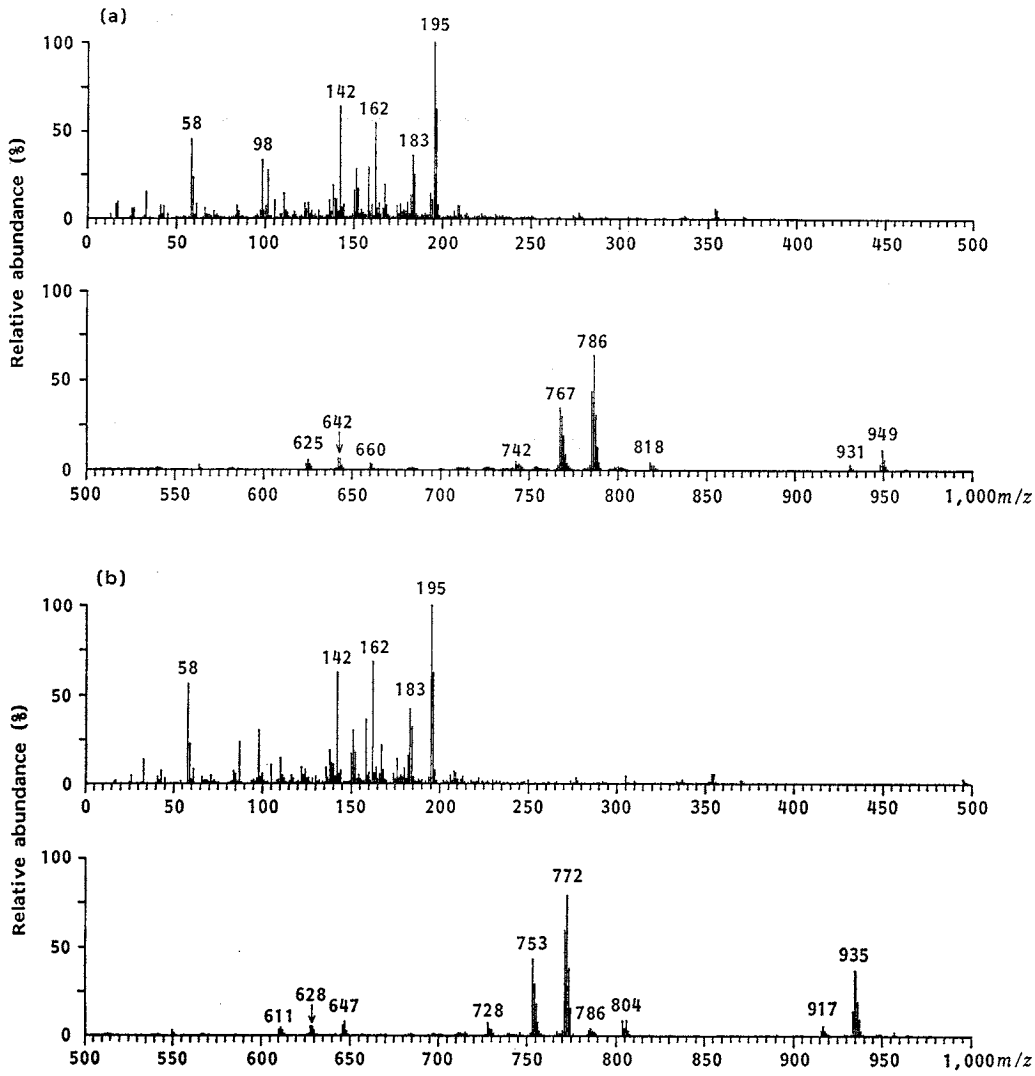


Fig. 11. IR spectra of antibiotics $273a_2$.
(a) $273a_{2\alpha}$, (b) $273a_{2\beta}$.

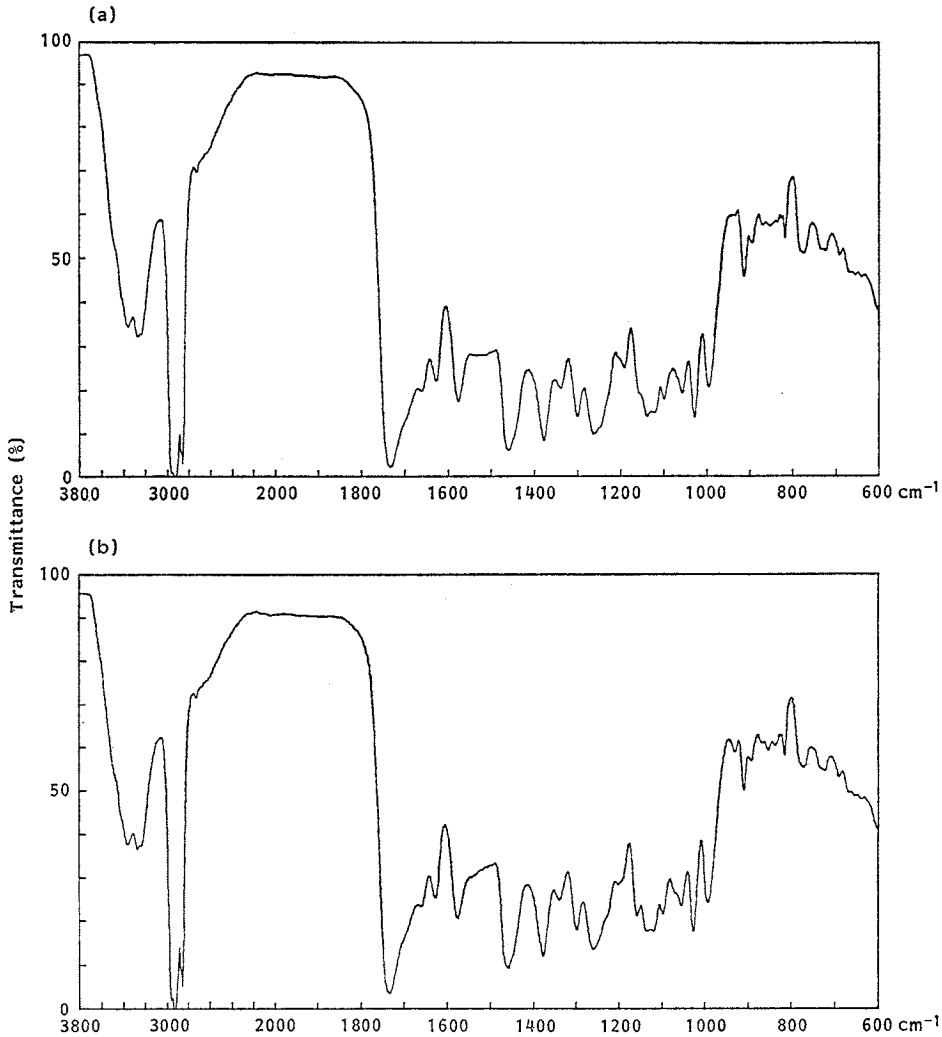
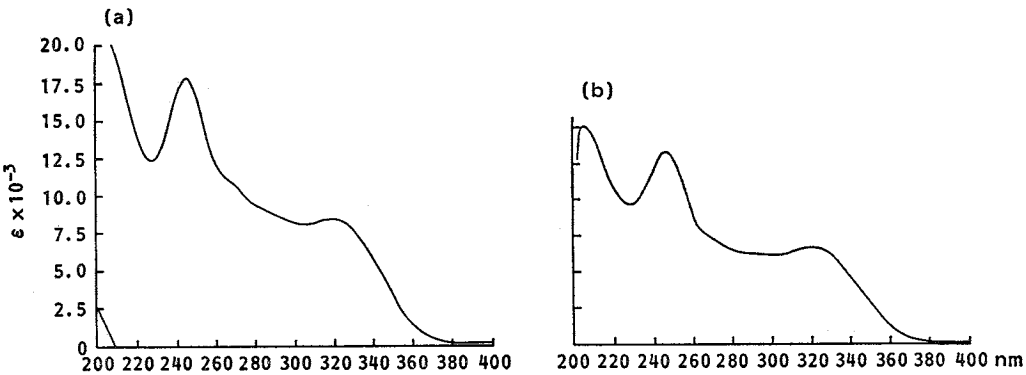


Fig. 12. UV spectra of antibiotics $273a_2$.
(a) $273a_{2\alpha}$, (b) $273a_{2\beta}$.



246, 270 and 320 nm. The ^{13}C NMR spectra of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ are shown in Fig. 13. Lists of the chemical shifts of carbons of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ and the corresponding paldimycin and paulomycins are presented in Tables 2 and 3. The ^{13}C NMR spectra of antibiotics $273a_{2\alpha}$

Fig. 13. ^{13}C NMR spectra of antibiotics $273a_2$.
(a) $273a_{2\alpha}$, (b) $273a_{2\beta}$.

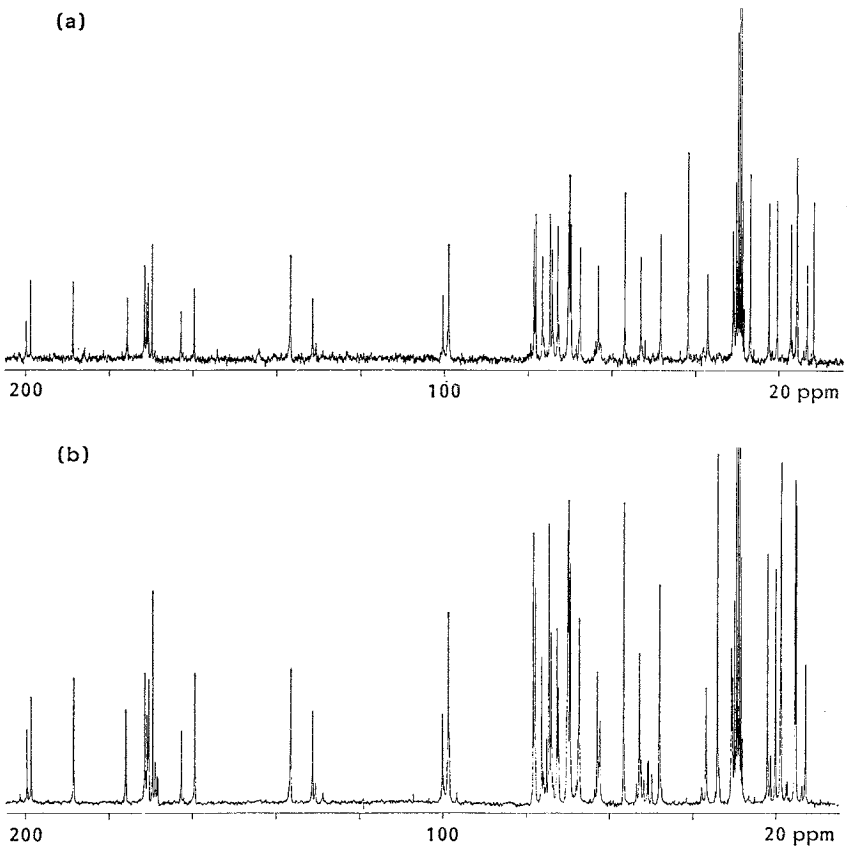


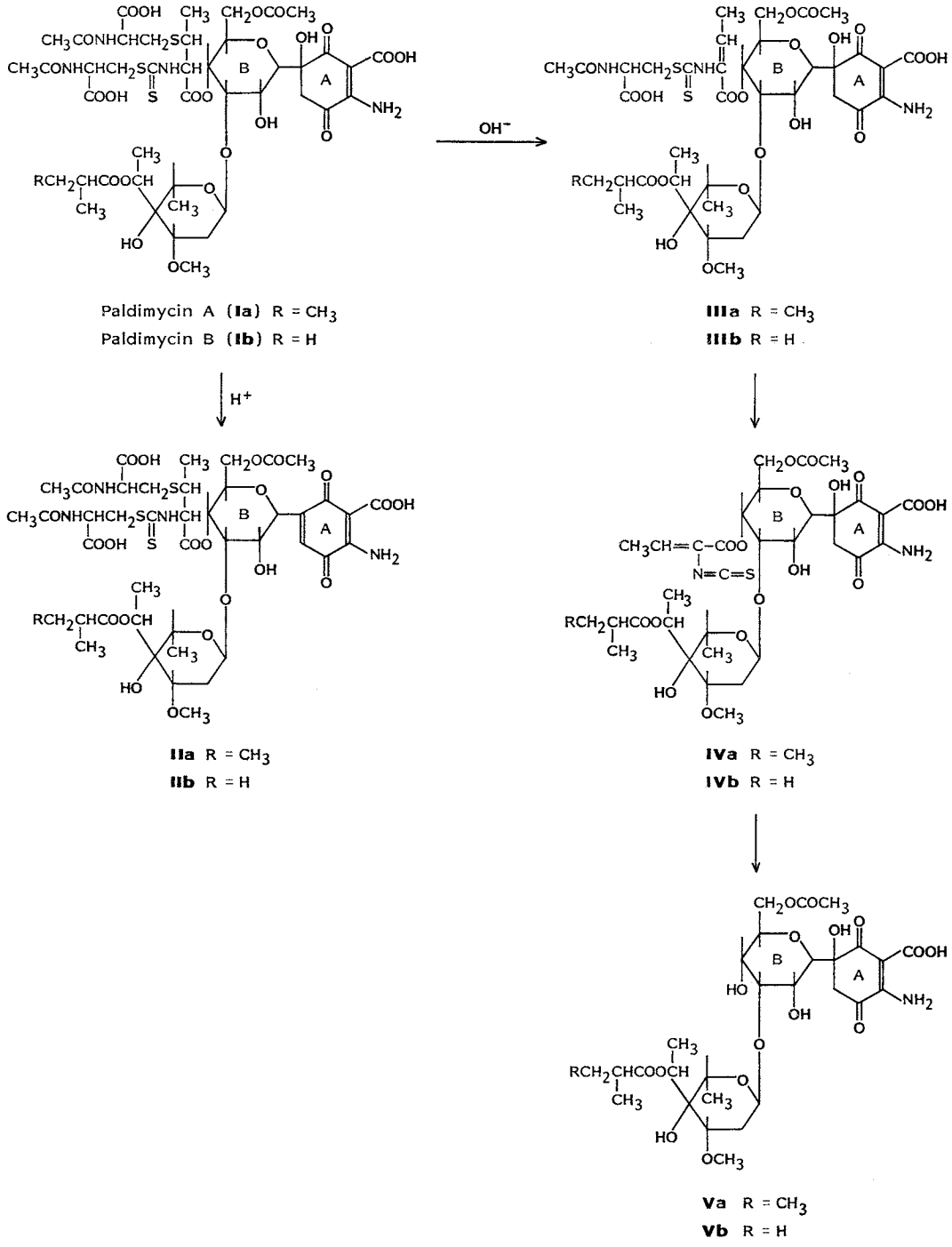
Table 6. ^1H NMR chemical shift[a] of characteristic groups of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$.

Antibiotic $273a_{2\alpha}$		Antibiotic $273a_{2\beta}$	
Chemical shift, δ (multiplicity)[b]	Assignment[c]	Chemical shift, δ (multiplicity)[b]	Assignment[c]
0.95 (3H, t)	5‴-CH_3	—	—
1.18 (3H, d)	4‴-CH_3	1.14 (6H, d)	3‴-CH_3 and 4‴-CH_3
1.23 (3H, d),	8′-CH_3 and 6′-CH_3	1.20 (3H, d),	8′-CH_3 and 6′-CH_3
1.28 (3H, d)		1.25 (3H, d)	
1.87 (3H, s)	CH_3CONH	1.90 (3H, s)	CH_3CONH
1.93 (3H, d)	4″-CH_3	1.97 (3H, d)	4″-CH_3
1.99 (3H, s)	2⁗-CH_3COO	2.01 (3H, s)	2⁗-CH_3COO
3.31 (3H, s)	CH_3O	3.30 (3H, s)	CH_3O
ca. 3.5 (complex)	SCH_2	*ca.* 3.5 (complex)	SCH_2
6.81 (1H, q)	3″-CH=	6.80 (1H, q)	3″-CH=

[a] Acetone-d_6 was used as solvent.
[b] Multiplicity, s: singlet, d: doublet, t: triplet, q: quartet.
[c] For group designation, see Fig. 1.

and $273a_{2\beta}$, like those of paulomycins A and B, show the presence of absorptions at δ 131.71 (s) and 136.95 (d) assigned to carbons C-2″ and C-3″ of the unsaturated acid present in antibiotics $273a_2$ (Fig. 1). The singlet at δ 142.64, present in the ^{13}C NMR spectrum of paulomycins and assigned to

Fig. 14. Transformation of paldimycin in aqueous solutions.



the isothiocyanate carbon,[2] is not present in the ^{13}C NMR spectra of antibiotics $273a_2$ indicating the addition of *N*-acetyl-L-cysteine on this group as shown in Fig. 1. The ^1H NMR spectra of antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$, as expected, are very close to those of paulomycins A and B. A list of absorptions due to characteristic features of these antibiotics is presented in Table 6. Thin-layer chromatographic comparison of antibiotics $273a_2$, paldimycins A and B and paulomycins A and B is presented in Fig. 7. As mentioned earlier, no TLC systems have been developed which would separate antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$, however, the antibiotics can be differentiated by the HPLC procedures described in a previous paper in this series.[1]

Stability of Paldimycins A and B

Paldimycins A and B, like the corresponding paulomycins, were found to be very sensitive to heating and to acidic or alkaline environments. Paldimycins, free acids, are reasonably stable when kept at room temperature over desiccants. In moist environments, colorless amorphous paldimycin A or B in the free acid form, are slowly transformed to a yellowish-orange colored material with corresponding loss of antibacterial activity. However, salts like the paldimycin trisodium salt (U-70,138F) are quite stable. No color formation was observed after storage at room temperature for over a year; slight (~5%) loss of bioactivity was observed during the same period.

In acidic solutions (pH <4.0) paldimycins A and B are transformed to the bio-inactive paldimycinones A and B (Fig. 14, **II**) by dehydration of ring A. This reaction which results in the formation of strongly orange-red colored solutions, proceeds very slowly at neutral pH's. At pH 3.0 and room temperature, about 22, 57, 71, 75 and 80% of paldimycins A and B are transformed to the corresponding paldimycinones in 4, 24, 48, 72 and 96 hours, respectively. In neutral or slightly alkaline solutions paldimycins A and B are transformed slowly to antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ (Fig. 14, **III**) and to paulomycins A and B (Fig. 14, **IV**), respectively. About 73, 30, 9, 4 and 2.5% of paldimycins A or B remain when solutions of the antibiotics are kept at pH 7.5 and room temperature for 4, 24, 48, 72 and 96 hours, respectively. The amounts of antibiotics $273a_2$ (α or β) and paulomycins (A or B) found at these times were: 14 and 5% (4 hours); 15 and 26% (24 hours); 9 and 47% (48 hours); 7.2 and 33% (72 hours); and 7.0 and 19.8% (96 hours), respectively. Several other degradation products were observed by HPLC analysis of which paulomenols A and B (Fig. 14, **V**) were easily

Table 7. *In vitro* biological comparison of paldimycins A and B.

Organism	UC No.	MIC[a] (μg/ml)		
		Paldimycin A	Paldimycin B	Paldimycin[b]
Staphylococcus aureus	6675	0.25	0.50	0.25
S. aureus	3665	0.25	0.50	0.25
S. aureus	6685	0.25	0.25	0.25
S. epidermidis	30031	0.25	0.50	0.25
Streptococcus faecalis	9217	1	2	2
Escherichia coli	6674	>64	>64	>64
Klebsiella pneumoniae	30090	>64	>64	>64
Serratia marcescens	30161	>64	>64	>64
Proteus vulgaris	30264	>64	>64	>64
Pseudomonas aeruginosa	6676	>64	>64	>64

[a] MIC: Minimum inhibitory concentration (μg/ml) determined by agar dilution method in Mueller-Hinton agar (pH 6.0).

[b] Paldimycin (U-70,138) consisted of 60% paldimycin A and 40% paldimycin B.

Table 8. *In vivo* activity of paldimycins A and B.

Organism	UC No.	CD_{50} determinations[a]		
		Paldimycin[b]	Paldimycin A	Paldimycin B
Streptococcus pyogenes	152	1.8	1.5	2.0
Staphylococcus aureus	6685	6.4[c]	9.6	6.7
S. aureus	9213	4.9[c]	3.8	7.9

[a] The CD_{50} is the dose in mg of drug per kg of body weight given each day that is required to protect 50% of the animals from death.

[b] Paldimycin (U-70,138) consisted of *ca.* 60% paldimycin A and 40% paldimycin B.

[c] Vancomycin gives CD_{50} values of 2.3 (1.5~3.5) against UC 6685 and 1.5 (0.9~2.2) against UC 9213. Gentamicin gives those of 13.2 (8.5~20.6) against UC 6685 and >200 against UC 9213.

recognized. These bio-inactive compounds have been isolated from alkaline hydrolysis of paulomycins A and B.[2)]

Biological Properties

The biological properties of paldimycins A and B and antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ will be reported in detail in a paper under preparation. Table 7 shows the *in vitro* activity of paldimycins A and B and paldimycin (mixture of A and B, U-70,138) against a related group of Gram-positive and Gram-negative bacteria. The antibiotics appear to be equally active against a variety of *Staphylococcus aureus* strains including those multiply-resistant to β-lactam, macrolide and lincosaminide antibiotics.* Limited results of *in vivo* testing of paldimycin and paldimycins A and B are shown in Table 8. The antibiotics were equally active against three strains of *S. aureus*. Paldimycins were found non-toxic when administered intraperitoneally. The LD_{50}'s in mice were above 3,000 mg/kg.** Antibiotics $273a_{2\alpha}$ and $273a_{2\beta}$ had biological activities identical to those of paldimycins A and B and paulomycins A and B.

Acknowledgments

The authors wish to express their appreciation to members of the Physical and Analytical Chemistry Department for spectral and analytical data. The technical assistance of Mr. K. J. GEIPEL is highly appreciated.

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* *In vitro* activity data were provided by Mr. G. E. ZURENKO of The Upjohn Company.

** *In vivo* data were provided by Dr. C. W. FORD of The Upjohn Company.