PAULOMYCINS* A AND B

ISOLATION AND CHARACTERIZATION

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Paulomycin A, $C_{34}H_{46}N_2O_{17}S$ and paulomycin B, $C_{33}H_{44}N_2O_{17}S$ are two antibiotics produced by *Streptomyces paulus* strain 273 (UC 5142). Both antibiotics, which are mainly active against a variety of Gram-positive bacteria, contain an isothiocyanate group and in this respect they are related to senfolomycins A and B and proceomycin.

The production of paulomycin or antibiotic U-43120 by *Streptomyces paulus* was reported by WILEY[1]. In the course of our screening program we isolated a new strain of *Streptomyces paulus*, *Streptomyces paulus* strain 273, which produces increased amounts of paulomycin[2]. This finding permitted the isolation of large quantities of paulomycin which, subsequently, was found to consist of several chemically related bioactive components.

This paper describes the isolation of paulomycin complex from fermentations of *Streptomyces paulus* strain 273 and the separation and characterization of the main components of the mixture which have been designated paulomycins A and B. Information on the other components will be reported when it becomes available.

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 production of paulomycin was followed by thin-layer chromatography on silica gel G using chloroform - ethanol - water (25: 30: 5, v/v) or chloroform - methanol (90: 10, v/v) as the solvent system. The antibiotics present in the fermentation or in preparations obtained during purification were detected by bioautography on *M. luteus* seeded trays.

Spectroscopic Methods

Proton magnetic resonance 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 dimethylsulfoxide-d_6 or acetone-d_6 were used. Carbon magnetic resonance spectra were recorded on a Varian CFT-20 spectrometer operating at 20.0 MHz. PMR and CMR chemical shifts are reported as ppm relative to tetramethylsilane. High resolution mass spectra were obtained on a Varian MAT-731 double-focusing high resolution mass spectrometer using the field desorption probe for inbeam introduction of the sample in electron impact mode.

High Performance Liquid Chromatography (HPLC)

All HPLC chromatography was carried out on a Hewlett-Packard Model 1084B (Hewlett-Packard, Avondale, Calif.) instrument equipped with an HP model 79875A variable wave length detector and operating in the dual pump mode. A Brownlee 10 cm × 4.6 mm stainless steel column packed with C_{18}

* Previously described as volonomycins.[2]

(10 μ) reverse phase was used with a mobile phase composed of 38% acetonitrile (Burdick & Jackson, Muskegon, MI) and 62% 0.5 M pH 7 potassium phosphate buffer. The aqueous buffer was filtered through a 0.45 μ filter prior to use. The following instrumental conditions were established:

Flow	2.00	Oven temperature	30
%B	38.0	Wavl S: R	320: 430
S-Temperature A	30	Chart speed	0.30
S-Temperature B	30	Attention 2↑	8

Production of Paulomycin

The fermentation conditions, used for the production of the paulomycin complex by *Streptomyces paulus* strain 273 (UC® 5142), were identical to those described by MARSHALL, LITTLE and JOHNSON[2].

Isolation of Paulomycin

Filtration and Extraction with Ethyl Acetate: The fermentation broth (*ca.* 4,000 liters) was adjusted to pH 2.8 with sulfuric acid, cooled to 16°C and filtered with the aid of diatomaceous earth. The clear filtrate contained *ca.* 9% of the total bioactivity and was discarded. The filter cake was slurried with 2,000 liters of ethyl acetate and the mixture was then filtered through a filter press. The filtrate (ethyl acetate extract containing paulomycin) was concentrated to a volume of *ca.* 65 liters. This concentrate was mixed with 17.5 kg of Harborlite 2,000 filter aid (Harborlite Corporation) and the wet mixture was dried *in vacuo* at 40°C.

Purification. Harborlite Leaching Column Chromatography: The dried Harborlite-paulomycin mixture, obtained as described above, was poured into a column partially filled with dried heptane and packed under atmospheric pressure. The column was eluted at a rate of 1.5 liters per minute. The following fractions were collected. Fractions 6~14 were combined and concentrated to dryness *in vacuo*. Crystalline paulomycin complex was obtained as follows.

Fraction number	Eluting solvent		Volume (liters)	% of bioactivity of starting material
1	Heptane		107	4
2	Heptane - Ethyl acetate	(97: 3)	103	1
3	〃	(94: 6)	93	1
4	〃	(91: 9)	93	3
5	〃	(88: 12)	93	7
6	〃	(85: 15)	93	15
7	〃	〃	93	17
8	〃	〃	93	13
9	Heptane - Ethyl acetate	(85: 15)	93	17
10	〃	〃	93	15
11	〃	〃	93	11
12	〃	〃	93	10
13	〃	〃	93	3
14	〃	〃	93	1
15	〃	〃	93	<1

Crystallization of Paulomycin: A solution of the residue in 12.6 liters of methylene chloride was stirred while adding 37.8 liters of heptane. The supernatant was decanted from an oily precipitate and cooled at 5°C for 20 hours. The yield of crude crystals isolated by filtration and drying was 138.9 g.

Crude crystalline paulomycin, 138.9 g, isolated as described above, was dissolved in 6.5 liters of hot chloroform. Ethyl ether (3.25 liters) and heptane (3.25 liters) were added and the resulting cloudy solution was left at room temperature for 2 hours, then at 5°C for 20 hours. Slightly colored needles, 110.6 g, were isolated by filtration. Recrystallization by the same procedure yielded 86.2 g of colorless crystalline paulomycin.

Isolation of Paulomycin A

Partition Chromatography: Four hundred grams of Dicalite-diatomite was slurried with upper phase of the system consisting of dioxane - cyclohexane - pH 7.0, 0.1 M phosphate buffer (35: 65: 8, v/v). Lower phase (160 ml) was added and the mixture was stirred for 20 minutes. The slurry was then poured into a column and packed to a constant height. One gram of crude crystalline paulomycin complex was dissolved in 5 ml of lower phase and 25 ml of upper phase, mixed with 15 g of dicalite, and added

to the top of the column. The column was eluted with the upper phase. Eluate fractions containing, by TLC, paulomycin A were combined and concentrated to dryness. The residue was crystallized from a mixture of 5 ml of chloroform and 5 ml of ether to yield 220 mg of colorless needles.

Anal. Calcd. for $C_{34}H_{46}N_2O_{17}S$: C 51.90, H 5.85, N 3.56, S 4.07, O 34.60.
Found: C 49.77, H 5.73, N 3.29, S 4.39.

Analysis by HPLC (see experimental above) indicated that the obtained crystals contained paulomycin A only.

Isolation of Paulomycin B

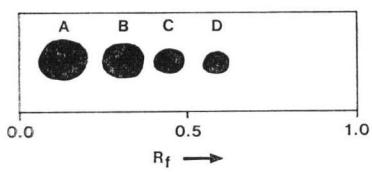
Countercurrent Distribution: Crude crystalline paulomycin complex (3.0 g) was dissolved in 40 ml of lower and 40 ml of upper phase of the solvent system consisting of acetone - ethyl acetate - hexane - pH 7.0, 0.1 M phosphate buffer (6: 1: 6: 2, v/v). The solution was introduced in an all-glass 10 ml/phase countercurrent distribution apparatus. After 800 transfers the distribution was analyzed by testing for bioactivity and by TLC. Fractions 210 ~ 273 containing paulomycin B only were combined. The upper phase was separated and the lower phase was adjusted to pH 5.5 with 1 N hydrochloric acid and extracted 3 times with one third (v/v) ethyl acetate portions each time. The ethyl acetate extracts were combined with the upper phase and this solution was concentrated to dryness *in vacuo*. The residue was crystallized from a mixture of 15 ml of chloroform, 7.5 ml of ether and 7.5 ml of heptane; yield 290 mg of colorless needles containing 99.6% paulomycin B as determined by HPLC.

Anal. Calcd. for $C_{33}H_{44}N_2O_{17}S$: C 51.29, H 5.69, N 3.62, S 4.14, O 35.23.
Found: C 50.82, H 5.71, N 3.51, S 4.14, O 35.82.

Results and Discussion

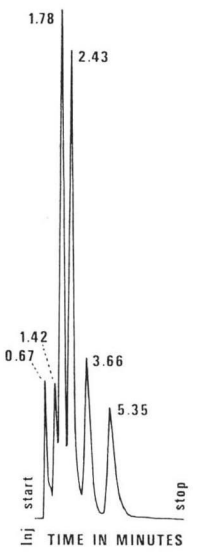
Streptomyces paulus strain 273 produces paulomycin, a mixture of chemically related antibiotics, when grown either in agar or in submerged culture. The isolation of the paulomycin mixture was followed with bioassays and thin-layer chromatography on silica gel plates using chloroform - ethanol - water (25: 30: 5, v/v) as the solvent system. The paulomycin complex appeared as one zone of bioactivity (Rf ~0.8) when thin-layer chromatograms were developed by bioautography on *Micrococcus luteus*. However, the paulomycin components were separated by thin-layer chromatography on cellulose sheets. Two major and two minor zones of bioactivity were detected and designated as paulomycin A (Rf, 0.14), paulo-

Fig. 1. Thin-layer chromatography* of paulomycins A, B, C, and D.



* Cellulose (Polygram CEL 300) pre-coated sheets; pH 7.0, 0.1 M phosphate buffer. Antibiotics were detected by bioautography on *M. luteus*-seeded agar.

Fig. 2. HPLC of paulomycins*.



* Retention times: Paulomycin A, 5.35 minutes; paulomycin B, 3.66 minutes; paulomycin C, 2.43 minutes; paulomycin D, 1.78 minutes. The components with retention times of 0.67 and 1.42 minutes have not been designated yet.

mycin B (Rf, 0.32), paulomycin C (Rf, 0.43) and paulomycin D (Rf, 0.58) (Fig. 1). The relative concentrations of the paulomycins in preparations obtained during purification were determined using the high performance liquid chromatographic system (HPLC) described earlier (see Experimental). Detection at 320 nm gave excellent sensitivity to the various paulomycins while reducing to a minimum the appearance of interfering substances. A separation of paulomycins A, B, C and D is shown in Fig. 2. Using the stop-flow mode of the detector the ultraviolet spectra of the four components were obtained. They were identical to the UV spectrum shown in Fig. 4 indicating the presence of the same chromophoric system in all four antibiotics. A typical fermentation produces paulomycin complex containing 50~55% of paulomycin A and 45~50% of paulomycin B. Paulomycins C and D are produced in small amounts and no quantitation of their production has been attempted.

Isolation of Paulomycins

The whole broth was cooled (16°C), adjusted to pH 2.8 and filtered. The cake containing most (*ca.* (91%) of the bioactivity was slurried with ethyl acetate. The ethyl acetate extract was concentrated to *ca.* 3~5% of its original volume and mixed with Harborlite (diatomaceus earth) to give, after drying, a free-flowing powder containing all paulomycins. The Harborlite-paulomycin mixture was then loaded on a column and eluted with solvents consisting of heptane and increasing amounts of ethyl acetate. Fractions containing paulomycin were concentrated to dryness. Crude crystalline paulomycin was obtained by crystallization from methylene chloride - heptane. Colorless needles of paulomycin were obtained by recrystallization from chloroform - ethyl ether - heptane (2: 1: 1, v/v).

Isolation of Paulomycins A and B

Crystalline paulomycin A, free of the other related antibiotics, was isolated by partition chromatography over diatomaceus earth using the upper phase of the system consisting of dioxane - cyclohexane - pH 7.0, 0.1 M phosphate buffer (35: 65: 8, v/v) as the eluting solvent. Paulomycin A was eluted first followed by a mixture of paulomycins A and B, paulomycin B, and a mixture of paulomycins C and D.

Colorless needles of pure paulomycin B were obtained by countercurrent distribution using the solvent system consisting of acetone - ethyl acetate - hexane - pH 7.0, 0.1 M phosphate buffer (6: 1: 6: 2, v/v). Fractions containing paulomycin B (K~0.43) yielded crystalline material upon concentration to dryness.

Preparations enriched in paulomycins C and D were obtained during the isolation of paulomycin A or B. However, neither paulomycin C nor paulomycin D has been isolated pure in amount sufficient for complete characterization.

Characterization of Paulomycins A and B

Paulomycins A and B are colorless crystalline (needles) compounds readily soluble in benzene, chloroform, methylene chloride, lower alcohols, ethyl and butyl acetates, acetone and most other common organic solvents. The antibiotics are less soluble in ether and water and quite insoluble in saturated hydrocarbon solvents.

The physical and chemical properties of paulomycins A and B are listed in Table 1. Both antibiotics melt with decomposition over a broad range of temperature; they are weak acids with *pKa'* of 7.4 as determined by titration with KOH in 60% aqueous ethanol and by their electrophoretic mobility. Paulomycins A and B are dextrorotatory when the specific rotation was determined in chloroform and levorotatory when methanol was used as the solvent.

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

	Paulomycin A	Paulomycin B
Molecular formula	$C_{34}H_{46}N_2O_{17}S$[a]	$C_{33}H_{44}N_2O_{17}S$[b]
Molecular weight		
Found	786.2522[a]	—
Calculated	786.2517	772[b]
Equivalent weight		
Found	808[c]	791[c]
Calculated	786	772
Titration (in 60% aq ethanol)	Weak acid; *pKa'*, 7.4	Weak acid; *pKa'*, 7.4
Melting point	95~105°C (dec)	105~143°C (dec)
$[\alpha]_D^{25}$ (*c* 0.95, $CHCl_3$)	+27°	+19°
$[\alpha]_D^{25}$ (*c* 0.95, CH_3OH)	−22°	−28°
UV [λ_{max} (ε); 95% ethanol]	236 nm (16.17×10^3), 276 nm (10.82×10^3), 322 nm (9.69×10^3)	236 nm (15.66×10^3), 276 nm (10.42×10^3), 322 nm (9.24×10^3)
IR (Nujol)	3356, 3230, 3070, 2050, 1735, 1695 cm^{-1}	3365, 3233, 3070, 2047, 1735, 1696 cm^{-1}

[a] By high resolution mass spectroscopy.
[b] From analytical data and comparison of C-13 and proton magnetic resonance spectra of paulomycins A and B (see text).
[c] By titration in 60% aqueous ethanol using KOH as titrant.

Fig. 3. Infrared spectra of paulomycins A and B (Nujol mull).

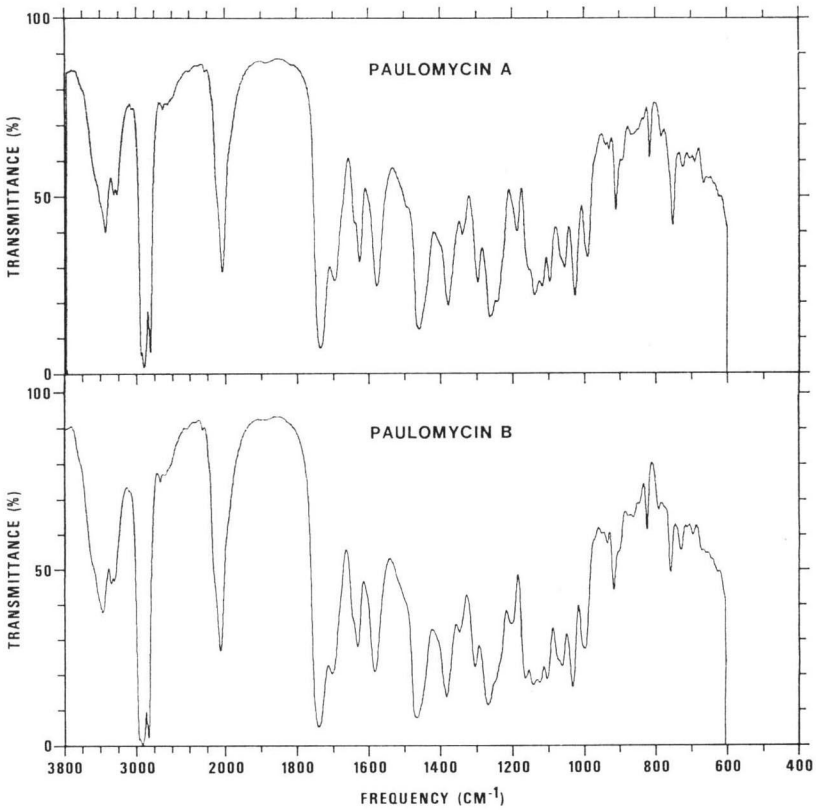


Fig. 4. UV Spectrum of paulomycins A and B (in 95% ethanol).

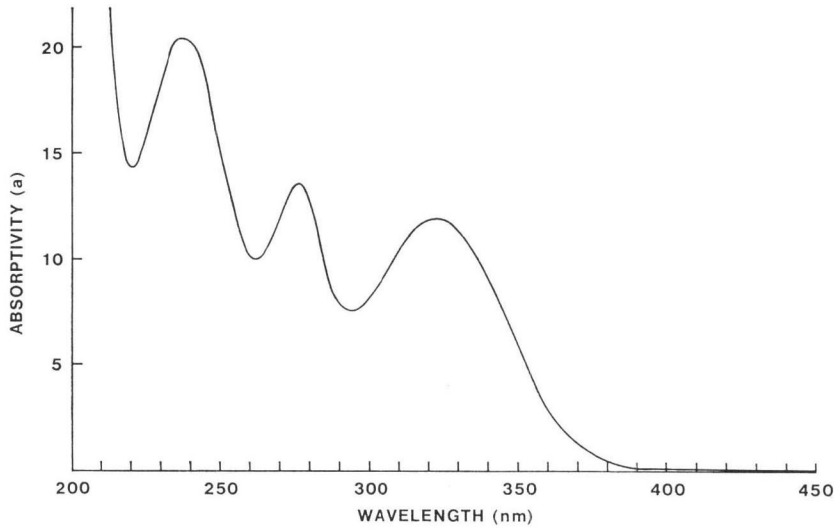


Fig. 5. Carbon-13 magnetic resonance spectra of paulomycins A and B.

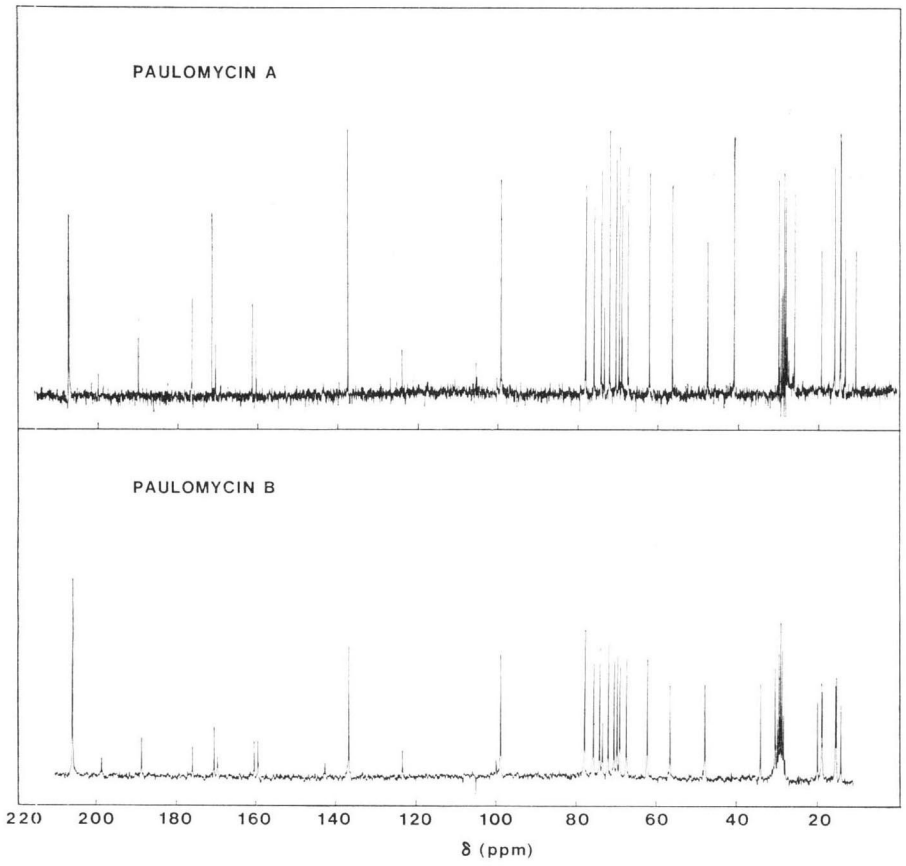
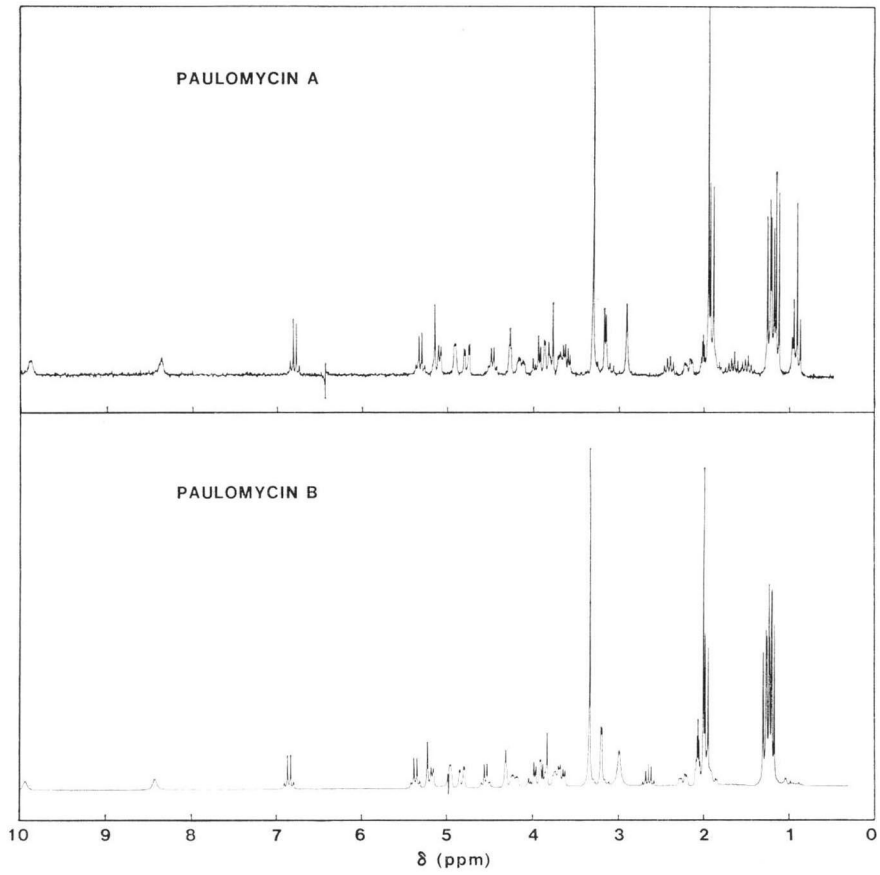


Fig. 6. Proton magnetic resonance spectra of paulomycins A and B.



The molecular formula of paulomycin A was established as $C_{34}H_{46}N_2O_{17}S$ by elemental analyses and by high resolution mass spectrometry. The molecular weight (786.2522), found by HRMS, compares well with the equivalent weight (808) determined by titration. The molecular formula of paulomycin B, $C_{33}H_{44}N_2O_{17}S$, mol. weight 772, was determined by elemental analyses and by comparison of the C-13 and PMR spectra of paulomycins A and B (see below). The equivalent weight, 791, found for paulomycin B is in agreement with this formula.

The IR spectra of paulomycins A and B, shown in Fig. 3, are nearly identical and are characterized by an absorption band at *ca.* 2050 cm^{-1} attributable to an isothiocyanate group[1]. The IR spectrum also shows the presence of carbonyl group(s) at 1735 cm^{-1}. The UV spectrum of paulomycins A or B, shown in Fig. 4, contains three peaks at 236, 276 and 322 nm. The maximum at 322 nm has been extremely useful in the isolation work and stability studies of both antibiotics. The carbon-13 and proton magnetic resonance spectra of paulomycins A and B are shown in Figs. 5 and 6, respectively. A list of the chemical shifts of carbons and corresponding protons observed in the NMR spectra of paulomycins A and B is presented in Table 2. C-13 and proton magnetic resonance spectra of paulomycin A show the presence of 34 carbons, 40 hydrogens attached to carbon and 6 exchangeable hydrogens for a total of $C_{34}H_{46}$ in agreement with the molecular formula obtained by HRMS. Similarly the spectra of paulomycin B indicate the presence of 33 carbons, 38 hydrogens attached to carbon and 6 exchangeable hydrogens

Table 2. Chemical shifts of carbons and corresponding protons observed in the NMR spectra of paulomycins A and B.

Paulomycin A			Paulomycin B		
Carbon number	CMR[b] Chemical shift (δ), multiplicity[a]	PMR[b] Chemical shift (δ) multiplicity[a]	Carbon number	CMR[b] Chemical shift (δ), multiplicity[a]	PMR[b] Chemical shift (δ), multiplicity[a]
1	198.50, s	—	1	198.37, s	—
2	188.39, s	—	2	188.40, s	—
3	175.15, s	—	3	175.71, s	—
4	170.18, s	—	4	170.18, s	—
5	169.35, s	—	5	169.38, s	—
6	160.25, s	—	6	160.25, s	—
7	159.37, s	—	7	159.36, s	—
8	142.64, s	—	8	142.54, s	—
9	136.64, d	6.83, q	9	136.66, d	6.88, q
10	123.36, s	—	10	123.32, s	—
11	100.72, s	—	11	100.14, s	—
12	99.04, d	4.95, dd	12	98.94, d	4.93, dd
13	78.26, d	3.83, d	13	78.14, d	3.86, d
14	78.20, s	—	14	78.14, s	—
15	76.18, d	4.31, dd	15	75.91, d	4.28, dd
16	74.40, d	3.65, dd	16	74.38, d	3.65, dd
17	73.62, s	—	17	73.66, s	—
18	72.29, d	4.18, ddd	18	72.21, d	4.21, ddd
19	70.73, d	4.82, dd	19	70.73, d	4.81, dd
20	69.93, d	5.36, q	20	69.96, d	5.39, q
21	69.20, d	3.70, ddd	21	69.28, d	3.70, ddd
22	67.78, d	4.52, q	22	67.65, d	4.58, q
23	62.30, t	3.88, 3.99, ABX	23	62.23, t	3.86, 3.99, ABX
24	56.62, q	3.33, s	24	56.59, q	3.33, s
25	48.01, t	3.16, 3.23, AB	25	47.95, t	3.15, 3.35, AB
26	41.51, d	2.45, ddq	26	34.15, d	2.65, ddq
27	30.56, t	1.90, 2.22, ABMX	27	30.33, t	1.90, 2.23, ABMX
28	26.65, t	1.50, 1.70, $ABMX_3$	—	—	—
29	19.99, q	1.98, s	28	19.98, q	2.02, s
30	16.73, q	1.17, d	29	18.93, q	1.17, d
31	15.39, q	1.22, d	30	18.77, q	1.19, d
32	15.28, q	1.27 or, d	31	15.44, q	1.19, d
33	14.11, q	1.97 or, d	32	15.23, q	1.28 or, d
34	11.39, q	0.94, t	33	14.13, q	1.97 or, d
—	—	14.01, s, exch; 9.92, broad s, exch; 8.41, broad s, exch; 5.13, d, exch; 5.20, s, exch; additional exch, unknown δ.	—	—	14.33, s, exch; 9.96, broad s, exch; 9.20, broad s, exch; 6.02, d, exch; 5.72, s, exch; 4.18, s, exch.

[a] Multiplicity; q=quartet; t=triplet; d=doublet; s=singlet; dd=doublet of doublets; ddd=double of doublet of doublets (6 lines); AB=4 lines; ABX=8 lines for the methylene protons; ABMX=16 lines for the methylene protons; $ABMX_3$=32 lines for the methylene protons.

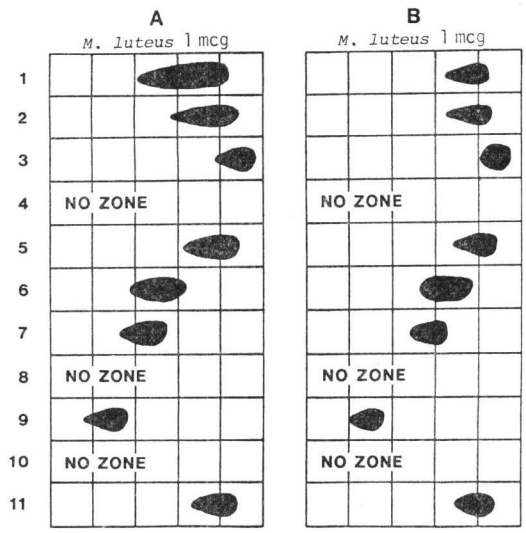
[b] Acetone-d_6 was used as solvent.

for a total of $C_{33}H_{44}$. Detailed analysis of the CMR and PMR spectra (to be discussed in another publication), and specific proton decoupling indicate the presence of an CH_3CH_2-$\underset{\displaystyle CH_3}{\underset{|}{CH}}$COO- ester grouping in the molecule of paulomycin A. This group has been replaced by a $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CHCOO$- ester group in paulomycin B. The remainder of the paulomycin A and B molecules are identical. Both antibiotics have two additional $-CHCH_3$, one $-OCH_3$, one $-OCOCH_3$, one $-N=C=S$, one amide carbonyl and two unsaturated systems:

$$\begin{matrix} CH_3 \\ H \end{matrix}\!\!>\!C=C\!<\quad \text{and} \quad -\overset{\displaystyle OH}{\overset{|}{C}}=C-\overset{\displaystyle O}{\overset{\|}{C}}-.$$

The paper chromatographic mobilities of paulomycins A and B are presented in Fig. 7. No zones of bioactivity were observed in systems 4, 8 and 10 which are either strongly alkaline or strongly acidic. This is in line with the observed loss of antibacterial activity at extreme pH's in aqueous solutions. The antibiotics are most stable at pH 5~7 and in solutions in aprotic solvents. Solutions of paulomycins in lower alcohols quickly lose their antibacterial activity with simultaneous loss of the UV maximum at 320 nm.

Fig. 7. Paper chromatographic mobilities of paulomycins A and B.



Solvent systems: 1) 1-butanol - water (84: 16); 2) 1-butanol - water (84: 16) and 0.25% *p*-toluenesulfonic acid; 3) 1-butanol - acetic acid - water (2: 1: 1); 4) 2% piperidine (v/v) in 1-butanol - water (84: 16); 5) 1-butanol - water (4: 96); 6) 1-butanol - water (4: 96)+0.25% *p*-toluene-sulfonic acid; 7) 0.5 M phosphate buffer pH 7.0; 8) 0.075 M NH_4OH saturated with methyl isobutyl ketone, lower phase; 9) benzene - methanol - water (1: 1: 2); 10) 1-butanol - water (84: 16) and 2% *p*-toluenesulfonic acid; 11) methanol - 15% aqueous sodium chloride (4: 1).

The paper used is impregnated with 0.1 M Na_2SO_4.

Biological Properties of Paulomycins A and B

The results of testing of paulomycins A and B in several *in vitro* and *in vivo* systems will be reported in a subsequent communication. Table 3 presents the *in vitro* antibacterial spectrum of paulomycins A and B against selected organisms. Both antibiotics are mainly active against Gram-positive organisms especially against *S. aureus* including strains resistant to penicillin, streptomycin, neomycin, macrolide and lincosaminide antibiotics. Paulomycin A was more active than paulomycin B against all the organisms tested. Paulomycin complex containing *ca.* 55%, paulomycin A and 45% paulomycin B was effective parenterally in the treatment of experimental infections in mice caused by *Streptococcus hemolyticus* (CD_{50} 3.54 mg/kg), *Staphylococcus aureus* (CD_{50} 10.0 mg/kg), *Streptococcus pneumoniae* I (CD_{50} 42.4 mg/kg) and *Streptococcus pneumoniae* III (CD_{50} 48.7 mg/kg). No toxic effects were observed at doses up to 500 mg/kg when the antibiotic mixture was administered subcutaneously.

Relation of Paulomycins A and B to Other Antibiotics

Paulomycins A and B, like paulomycin (U-43120)[1] have physical properties (IR, UV, elemental composition) similar to those of proceomycin[3] and senfolomycins A and B[4]. Very little has been re-

Table 3. Antibacterial spectra of paulomycins A and B.

Test organism	Minimum inhibitory concentration (mcg/ml)	
	Paulomycin A	Paulomycin B
Staphylococcus aureus UC 76	0.125	0.50
Staphylococcus aureus UC 6685	0.25	0.50
Staphylococcus aureus UC 6690	0.25	2
Streptococcus faecalis UC 694	15.6	125
Enterococcus sp. UC 701	1.0	31.2
Escherichia coli UC 45	125	250
Klebsiella pneumoniae UC 58	125	250
Pseudomonas aeruginosa UC 95	125	250
Salmonella schottmuelleri UC 126	125	500
Serratia marcescens UC 131	62.5	250
Shigella flexneri UC 143	62.5	250
Providencia stuartii UC 6570	62.5	250
Enterobacter cloacae UC 6783	250	500

Test method: Agar dilution, pH 6.0.

ported concerning the structures of the senfolomycins and proceomycin. These antibiotics, like paulomycins A and B have been believed to contain an isothiocyanate group on the basis of the infrared absorption band as *ca.* 2050 cm^{-1} and the presence of sulfur. Furthermore the C-13 NMR spectrum of paulomycins has a broad absorption peak at *ca.* δ 142.5 which is being assigned to an –N=C=S grouping on the basis of reported* C-13 chemical shifts for the isothiocyanate carbon. In view of the close similarities of the UV, IR, elemental composition and molecular size of the five antibiotics it seems certain that they are chemically quite similar and make up a small family of antibiotics.

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* Reported for phenyl isothiocyanate, broad singlet δ 135.8; *n*-heptyl isothiocyanate, broad singlet δ 130.4.