THE DIUMYCINS

NEW MEMBERS OF AN ANTIBIOTIC FAMILY HAVING PROLONGED *IN VIVO* ACTIVITY

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The diumycins are new members of a group of phosphorus-containing antibiotics that provide prolonged protection to mice infected with *Streptococcus pyogenes*. The diumycins are differentiated from other group members by their amino sugar composition.

Diumycin, a new antibiotic with a remarkable prophylactic activity, is a member of a group of phosphorus-containing, antibacterial antibiotics. This family of compounds includes prasinomycin[1], moenomycin[2] and 11,837 R. P.[3] Other phosphorus-containing antibiotics, 8036 R. P. and 19,402 R. P., described in the patent literature, (South Africa 65/6204 and Netherlands 68,02093 respectively) are probably related. This report describes the isolation and results of some preliminary studies with diumycin (*diu*, Latin, a long time).

Production and Isolation

The antibiotic is produced by a streptomycete isolated from a soil sample obtained in North Carolina. The organism was identified as *Streptomyces umbrinus*[4], a member of the olive-buff spore color series of PRIDHAM. Culture maintenance, fermentation and assay techniques used for the production of diumycin were the same as those previously described for prasinomycin[1].

Although the activity was present in both the mycelium and filtrate at harvest, all the activity was recovered with the mycelial cake upon acidification of the whole culture broth to pH 3 before filtration. The filter cake was extracted with methanol, the extract neutralized to pH 7, and concentrated. The resulting aqueous suspension (pH 7) was washed with butanol, acidified to pH 3, and then extracted with butanol to obtain the acidic antibiotic. The antibiotic was recovered from the butanol phase by re-extraction with a small volume of water adjusted to pH 7 with sodium hydroxide. The aqueous phase was dialyzed to remove impurities, since the antibiotic in aqueous solution does not pass through a semi-permeable (cellophane) membrane. The crude sodium salt of diumycin was recovered from the retained solution by freeze-drying. Further purification was effected by chromatography on Sephadex 75 using 0.1 N aqueous ammonium hydroxide for elution. The material recovered

from the active fractions was chromatographed on acid washed silica gel with the system, *n*-propanol - 2 N aqueous ammonium hydroxide (8 : 2). Paper chromatography, using the system, *n*-propanol - *n*-butanol - 0.5 N ammonia (1 : 3 : 4), resolved the mixture into two bioactive components, diumycin A (Rf 0.32) and diumycin B (Rf 0.45) that were detected by bioautography[6].

Diumycins A and B were subsequently isolated in pure form by countercurrent distribution of the antibiotic mixture in a nitrogen atmosphere using the system, *n*-propanol - *n*-butanol - 0.5 N ammonia (2 : 3 : 4 by volume). After five hundred transfers, tubes 65~90, which contained a single bioactive component, were combined to give diumycin A and tubes 130~160 yielded diumycin B. The free acids of these antibiotics were obtained by treating their aqueous solutions with 'Dowex 50' ion exchange resin. After filtering off the resin, the solutions were freeze-dried and the residue precipitated from methanol by the addition of ethyl acetate. The yields of diumycins A and B were usually obtained in the ratio of 9 : 1.

Chemical Properties

For the analyses the free acids were converted to their sodium salts by neutralizing aqueous solutions to pH 8 with sodium hydroxide and precipitating the salts by the addition of acetone. The salts rapidly formed hydrates on exposure to the atmosphere. The analytical data (Table 1) were obtained on the anhydrous forms after drying at 100°C under vacuum. The elemental analysis indicates that the sodium salts of the diumycins have empirical formulae in the range ($C_{68\sim72}H_{93\sim107}N_5$-$O_{38\sim40}Na_3P$).

Like the prasinomycins[1] and moenomycins[2], diumycins A and B aggregate in

Table 1. Analyses [a]

Compound	Found (per cent)						Neutral equiv. [b]	Molecular Wt. (ultracentrifuge)	
	C	H	N	P	Na	N-OAc		90 % Ethanol [c]	Buffer [d]
Diumycin A	48.65	5.42	4.00	1.91	4.08	5.15	564	1,700±250	31,000±2,000
Diumycin B	48.05	6.28	4.11	1.89	4.02	5.18	575	1,600±250	31,000±2,000

a) All analyses for anhydrous sodium salts
b) By titration with perchloric acid in acetic acid
c) 90 % ethanol-10 % aqueous 0.2 molar sodium chloride-0.2 molar phosphate buffer (pH 6.5)
d) Aqueous 0.2 molar sodium-0.2 molar phosphate (pH 6.85)

Table 2. Physical properties of the diumycins [a]

Compound	$[\alpha]_D^{H_2O}$	M. P. (°C)	Ultraviolet, mμ ($E^{1\%}$)		Rf [b]	Distribution coefficient [c]
			0.1 N HCl	0.1 N KOH		
Diumycin A	+8.0	251~257 d.	246 (78)	257 (120)	0.32	0.19
Diumycin B	+9.6	247~251 d.	—	—	0.45	0.38

a) Optical rotation and m. p. on sodium salts
b) *n*-Propanol, *n*-butanol, 0.5 N ammonia (1 : 3 : 4 by volume)
c) *n*-Propanol, *n*-butanol, 0.5 N ammonia (2 : 3 : 4 by volume)
Countercurrent distribution run in a nitrogen atmosphere

aqueous solution and exhibit molecular weights of about 30,000 when determined by the analytical ultracentrifuge method[7]. When the molecular weights were determined in 90 % ethanol, values of 1,600~1,700 were found for these antibitics (Table 1). Neutral equivalents determined by perchloric acid titration of the sodium salts in acetic acid indicated that three acidic groups were present in both diumycins A and B. Diumycin A showed strong absorption at 257 mμ (sodium salt) whereas diumycin B showed only end absorption below 200 mμ (Table 2). The infrared spectra of diumycins A and B were similar and both exhibit strong absorption bands at 3.0 μ (OH, NH), 5.82 μ (-C(=O)-), 5.95~6.15 μ (-C(=O)-NH-), and 6.45 μ (N-H). The amide functions are apparently present as N-acetyl groups since acid hydrolysis of the antibiotics liberated two equivalents of acetic acid.

Both diumycins A and B yielded two equivalents of glucosamine and approximately three equivalents of ammonia on acid hydrolysis (4 N HCl at 105°C for 6 hours). No other ninhydrin-positive fragments were observed. The diumycins are thus easily distinguished from the related antibiotics, prasinomycin and moenomycin. In contrast to diumycin, which contains a single amino sugar, the latter antibiotics both yield one equivalent of glucosamine and 6-deoxyglucosamine under the same hydrolytic conditions[1,2]. Quantitative analyses of the acid hydrolysates for the amino sugars and ammonium ion content were conveniently done using a conventional amino-acid analyzer.

Biological Properties

The antibiotic activity of diumycin closely resembles that of prasinomycin[1] in that it is active *in vitro* against gram-positive bacteria and *Mycobacterium bovis* BCG. Diumycin shows relatively little activity against gram-negative bacteria and yeasts (Table 3). The similarity of the biological action of diumycin and prasinomycin is further supported by their cross-resistance, as shown in Table 4. *In vivo*, diumycin, like prasinomycin, confers a remarkable,

Table 3. *In vitro* spectrum of diumycin [a]

Organism	Minimum inhibitory concentration (μg/ml)
Staphylococcus aureus 209P	0.06
Streptococcus pyogenes C203	< 0.002
Bacillus subtilis ATCC 6633	0.14
Sarcina lutea ATCC 9341	>25.0
Diplococcus pneumoniae Type 3 ATCC 6303	0.30
Mycobacterium bovis BCG 5516 [b]	3.1
Klebsiella pneumoniae ATCC 9997	>25.0
Escherichia coli ATCC 10536	>25.0
Salmonella schottmuelleri SC 3850 [b]	>25.0
Pseudomonas aeruginosa SC 3840 [b]	>25.0
Candida albicans CBS 35H	>25.0

a) The diumycin mixture contained approximately 85 % A and 15 % B. The individual pure components exhibited essentially the same antibacterial activity.
b) Squibb Culture Collection.

Table 4. Cross resistance of diumycin and prasinomycin

Organism	Minimum inhibitory concentration (μg/ml)	
	Diumycin	Prasinomycin
Staphylococcus aureus 209P	0.06	0.06
SC 5410*	2.3	2.3
SC 5411*	6.3	6.3
SC 5412*	>50.0	>50.0
SC 5413*	18.7	9.4

* The four resistant cultures are variants of *Staphylococcus aureus* 209P produced by serial passage in increasing concentrations of prasinomycin.

prolonged protective action with a single subcutaneous dose. Thus, 6.7 mg/kg of diumycin A was able to protect 50 % of the mice infected with a highly lethal dose (1,000 LD_{50}) of *Streptococcus pyogenes* C_{203} when the antibiotic was administered subcutaneously 14 days prior to challenge.

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