THE DIUMYCIN COMPLEX. COMPARATIVE STUDIES ON ANTIBIOTICS FROM DIUMYCIN- AND MACARBOMYCIN-FERMENTATIONS

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Six phosphorus-containing antibiotics were isolated from both diumycin and macarbomycin fermentation products. On the basis of their chromatographic behavior and of their physico-chemical and microbiological properties it can be assumed that not only the main component but also the five minor components are the same in both antibiotic complexes. A comparison of the six components with the known classifications of diumycins and macarbomycins was made.

Diumycin[1,2] and macarbomycin[3] are members of the group of phosphorus-containing antibiotics which also includes prasinomycin,[4] moenomycin,[5,6] 8036 RP,[7] 11837 RP[8] and 19402 RP.[9] Previous investigations on the main components of the diumycin and macarbomycin complex indicated their close relationship. During the isolation of diumycin from fermentation broth in 1971 we found two minor antibiotics in addition to the active components known at that time. In 1973 TAKAHASHI *et al.*[10] reported four minor antibiotics in the macarbomycin fermentation that they isolated during their studies on strain improvement and large scale production. Recently three minor components from macarbomycin mycelial cake were described as neomacarbomycins.[11]

The present paper deals with the comparison of phosphorus-containing microbiologically active components that were isolated from diumycin fermentation broth and dried mycelial cake prepared thereof and from macarbomycin dried mycelial cake.

Production and Isolation

Diumycin was produced in shake-flask culture and 10-liter laboratory fermenters with strains of *Streptomyces umbrinus* (KV 17 and KV 21, Squibb collection; NRRL 5683 and 5764) incubated for 6 to 8 days at 27°C. The composition of the medium was (g/liter): corn starch 16, sucrose 28, soybean meal 40, K_2HPO_4 4, KNO_3 1, limestone 10, butyloleate 0.5, cod liver oil 50, pH 8.0.

The antibiotic content was determined by the agar diffusion method using *Staphylococcus aureus* FDA 209 P. A standard diumycin preparation of high purity with a microbiological activity of 97 % diumycin A and 3 % diumycin B was designated 1,000 μg*/mg.

One aliquot of the broth was adjusted to pH 6.2 with diluted sulfuric acid, centrifuged, the filtrate discarded, the mycelial cake dried at 50°C *in vacuo* and ground. Only traces of activity (18 mg*/liter) were found in the filtrate. The ground cake was twice extracted with 50 % v/v methanol for 15 minutes at 50°C.

Another aliquot of the broth was directly extracted with the same volume of methanol for

15 minutes at 50°C. The extraction was repeated with 50% v/v methanol.

Dried mycelial cake that had been prepared from macarbomycin fermentation broth (Meiji-batch 2003) was twice extracted with distilled water at pH 7.2~7.4. Upon subsequent extraction with 50% v/v methanol only traces of activity could be obtained.

The diumycin and macarbomycin extracts, methanolic or aqueous, were purified separately. They were passed through a Dowex 1×2 ion-exchange column (Cl^--form) and the column was washed consecutively with water, 5% w/v aqueous solution of NaCl, water, 1% w/v solution of formic acid in 80% v/v aqueous methanol and water. The activity was eluted with 1% w/v NaCl in 80% v/v aqueous methanol.

The eluates were concentrated to about 1/6 volume and the salt removed by gel-filtration with Sephadex G 10 or ultrafiltration with Diaflo membrane type UM-2 (Amicon Corp., Lexington, Mass., U. S. A.). The desalted solution was lyophilized and the product obtained was chromatographed on acid-washed silica gel columns (silica gel 0.063~0.2 mm, E. Merck) by developing with isopropanol - 2 N ammonia (10:2) and eluting with isopropanol - 2 N ammonia (9:2). Separation of the complex occurred during elution and was monitored by thin-layer chromatography (see below). The corresponding eluate fractions were pooled and further purified by repetitive silica gel column chromatography. Ammonia and water were removed by repeated evaporation with alcohol and the purified substances were precipitated from methanol with ether.

Physical and Chemical Properties

The substances were separated by thin-layer chromatography (tlc) on precoated silica gel 60 F-254 plates (E. Merck) and descending paper chromatography (pc) for 15 hours on Schleicher and Schüll 2040 b. Substances isolated from macarbomycin cake were denoted M, those from diumycin products D (Table 1). With tlc the substances were detected by UV-light (254 mμ) or as dark blue spots by spraying with a reagent consisting of 1% (w/v) ceric sulfate and 1.5% (w/v) molybdic acid in 10% (v/v) sulfuric acid followed by heating for 15 minutes at

Table 1. Comparative thin-layer chromatography (tlc) and paper chromatography (pc) of the purified D- and M-substances

Substance	tlc* (Rf)	tlc** (Rf)	pc*** (R_A)*****	pc**** (R_A)
D1, M1	0.30	0.27	1.16	1.12
D2, M2	0.27	0.34	1.34	1.31
D3, M3	0.25	0.24	1.00	1.00
D4, M4	0.22	0.32	1.24	1.20
D5, M5	0.20	0.22	0.87	0.90
D6, M6	0.15	0.18	0.87	0.96

* isopropanol - 2 N ammonia (7:3)
** chloroform - ethanol - water (2:3:1)
*** *n*-butanol - pyridine - water (4:1:4), upper phase (chamber saturated with lower phase)
**** *n*-butanol - *n*-propanol - 0.5 N ammonia (3:1:4), upper phase (chamber saturated with lower phase)
***** migration in relation to diumycin A=1.00

Table 2. General conditions for paper electrophoresis

pH	Electrolyte stock solution*	V	mA
5.0	26ml pyridine+20ml glacial acetic acid	300	12
7.0	26ml pyridine	300	4
9.0	26ml pyridine+5ml 0.1 N NaOH	200	20
11.0	26ml pyridine+20ml 0.1 N NaOH	250	20

* All stock solutions were made up to 100ml with distilled water.

For final electrolyte concentration 20ml of stock solution were mixed with 400ml formamide and made up to 1 liter with distilled water. For electrolyte pH 7.0 2 ml of 0.1 N NaOH had to be added.

105°C, with pc by bioautography with *S. aureus* FDA 209 P. Reference samples of diumycin A, A′, B and B′ showed the behavior of D, or M respectively, 3, 1, 4 and 2.

UV-absorption was shown by D1, M1, D3 and M3, while D5 and M5 showed only traces of UV-absorption.

Tlc systems isopropanol - water - 0.5 N borate buffer pH 9.0 (70 : 25 : 5) and chloroform - ethanol - 2 N ammonia (2:3:1) gave less satisfactory separation. However, also with these systems no differences were found between corresponding D- and M-substances.

Paper-electrophoresis was performed in four electrolyte solutions (Table 2) on Schleicher & Schüll 2040 b. The separated substances were localized by bioautography after 2-hour drying at 100°C. Duration and temperature of drying should not be exceeded to avoid inactivation of the antibiotics. Migration occurred in all four electrolyte solutions with diumycin A migrating faster to the anode than diumycin B.

The substances investigated could be divided into two groups, the one group behaving like diumycin A, the other like diumycin B. No differences were found between corresponding D- and M-substances:

Group A: D1, M1; D3, M3; D5, M5; D6, M6

Group B: D2, M2; D4, M4.

Further studies were made with D3 and M3, the main components of the two complexes, and with D6 and M6. Acid hydrolysis (2 N HCl, 105°C, 20 minutes) and extraction with chloroform gave a mixture of lipids that could be separated by tlc on silica gel (60 F-254, E. Merck) with a system benzene - chloroform - methanol (8:1:1)[5,12] into 3 components, however by successive tlc, first with benzene and after drying with petroleum ether, into 6 components. On prolonged hydrolysis (2 N HCl, 105°C, 3 hours) all four substances yielded glucose and glucosamine as indicated by tlc on cellulose (microcristalline, E. Merck) in the systems pyridine - ethylacetate-acetic acid-water (36:36:7:21) and *n*-propanol - *n*-butanol - water (4:6:3).

Table 3. Elemental analyses

Substance	Found (per cent)			
	C	H	N	P
D3	49.96	6.91	5.17	1.91
M3	50.16	6.93	5.22	1.92
D6	47.41	6.81	4.93	2.00
M6	47.13	6.70	4.77	2.10

Elemental analysis was made with the ammonium salts after 6-hour drying *in vacuo* (Table 3). There were no differences between D3 and M3 or D6 and M6. The IR-spectra of D3 and M3 potassium salt showed no differences and are in good agreement with the spectrum of diumycin A potassium salt.[14] They showed characteristic bands at 3380, 2930, 1725, 1675, 1645, 1530, 1380, 1240, 1070, 1045 and 975 cm^{-1} (Fig. 1). Also no differences were found between the spectra of D6 and M6 potassium salt. They showed characteristic bands at 3380, 2930, 1725, 1660~1605 (broad), 1380, 1330, 1240, 1070, 1045 and 975 cm^{-1} (Fig. 2).

Microbiological Properties

A modification of the agar well diffusion method with agar double layer (Difco Penassay Base Agar and Penassay Seed Agar) and with *S. aureus* FDA 209 P and *Bacillus cereus* ATCC 11778 as test organisms had been developed. Each determination of microbiological activity

Fig. 1. IR-Spectrum of substances D3 and M3 potassium salt pelleted in KBr

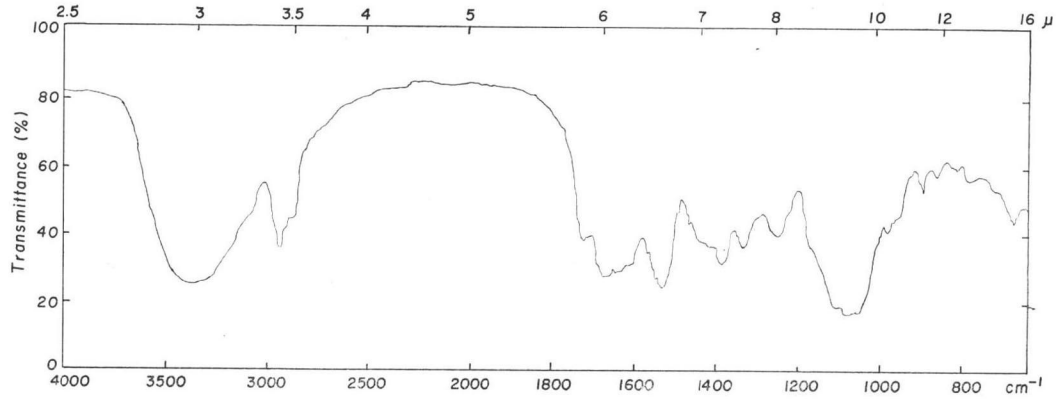
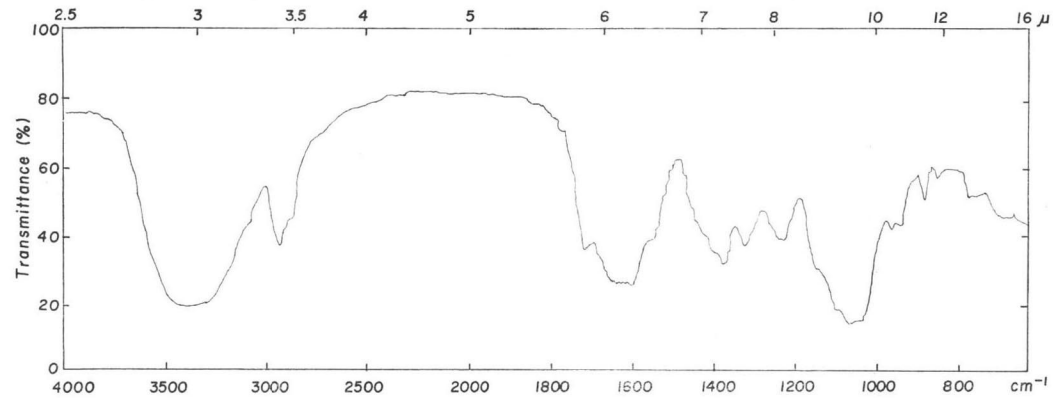


Fig. 2. IR-Spectrum of substances D6 and M6 potassium salt pelleted in KBr



consisted of 6 assays with two dilutions each per sample tested parallel to a standard solution (standard: 1,000 μg*/mg). The coefficient of variability of one determination was 5~7 %. Sample activity was computed from 4~12 replicate determinations (Table 4). Details of the method will be described elsewhere.[13]

Table 4. Microbiological activity in per cent (Standard: 100%)

Substance	Determined with	
	S. aureus FDA 209P	*B. cereus* ATCC 11778
D 3	99.6*	102.1**
M 3	99.6*	97.5**
D 6	99.6*	142.2***
M 6	99.5*	144.3***

* 12 replicate determinations
** 4 replicate determinations
*** 9 replicate determinations

Discussion

Already earlier reports had referred to the close relationship between diumycin and macarbomycin.[10,15] However, these reported comparisons were limited to the respective main component of the two complexes, diumycin A and macarbomycin. By parallel isolation from products of the diumycin and macarbomycin fermentation we were able to obtain from each antibiotic complex the same number of phosphorus-containing components. By means of chromatographic and electrophoretic methods it could be demonstrated that for each of the six components in the one complex there existed a corresponding component in the other. Comparison of the six isolated substances with diumycin reference samples and of their chromatographic behavior and UV-absorption with the data des-

Table 5. Classification of the D- and M-substances based on chromatographical behavior and UV-absorption

Substance	Designation
D1, M1	Diumycin A′, Macarbomycin III
D2, M2	Diumycin B′, —
D3, M3	Diumycin A, Macarbomycin
D4, M4	Diumycin B, Macarbomycin Ia (Neomacarbomycin Ia)
D5, M5	Diumycin B″*, Macarbomycin Ib (Neomacarbomycin Ib)
D6, M6	Diumycin U*, Macarbomycin II (Neomacarbomycin II)

* Designation by the authors

cribed by SLUSARCHYK for 4 diumycins[2] and by TAKAHASHI *et al.* for 5 macarbomycins[10] leads to a classification of diumycins and macarbomycins (Table 5). Further investigations of the physico-chemical and microbiological properties of the main component and one minor component isolated from brath complexes also showed no difference between the corresponding diumycins and macarbomycins. It is concluded therefore that the main component as well as the five minor components are not only closely related to each other but the same in both antibiotic complexes.

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