# ZORBAMYCIN AND RELATED ANTIBIOTICS

## I. PRODUCTION, ISOLATION AND CHARACTERIZATION

A. D. ARGOUDELIS, M. E. BERGY and T. R. PYKE

Research Laboratories, The Upjohn Company,
Kalamazoo, Michigan, U.S.A.

(Received for publication April 5, 1971)

Zorbamycin and its related antibiotics, zorbonomycin B and zorbonomycin C, are three new antibiotics isolated from fermentations of *Streptomyces bikiniensis* var. *zorbonensis*. All three antibiotics are active against a variety of Gram-positive and Gram-negative bacteria and various fungi. Zorbamycin as well as zorbonomycins B and C appear to belong to the phleomycin and bleomycin families of antibiotics and to be most closely related to the phleomycins. The three antibiotics described in this paper show differences from the reported phleomycins and are likely new members of this group.

Culture of *Streptomyces bikiniensis* var. *zorbonensis**, grown in a complex medium, were found to contain a mixture of antibacterial agents which were characterized by their thin-layer chromatographic behavior. Since the bioactivity designated IV (Fig. 1) appeared to be one of the main components of the fermentation, we concentrated our efforts in isolating this material. Component IV was subsequently found to consist of three bioactivities. The main antibiotic was designated zorbamycin (U-30604E)** while the two minor activities were named zorbonomycin B (U-32166E) and zorbonomycin C (U-33046E). The present communication deals with the production, isolation and chemical characterization of the three antibiotics. Results of preliminary *in vitro* testing of zorbamycin and both zorbonomycins B and C are also included in this paper. However, detailed discussion of the biological properties of these antibiotics will be the subject of subsequent communications.

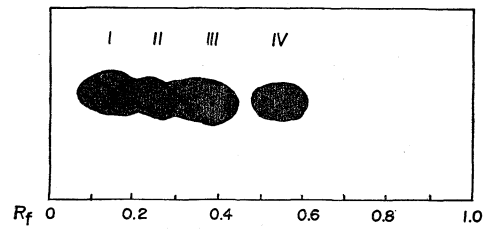



Fig. 1. Thin-layer chromatography* of fermentations of *S. bikiniensis*.

* Brinkman MN-Polygram CEL-300 sheets were employed as support; solvent system, sodium citrate (0.05 M) pH 6.9 buffer. Antibiotics were detected by bioautography on *B. subtilis* seeded agar.

### Experimental

#### Fermentation Procedures

Seed cultures of *S. bikiniensis* var. *zorbonensis* were prepared in a medium consisting of glucose monohydrate, 25 g/liter and Pharmamedia, 25 g/liter (Trader's Oil Mill Co.,

* Taxonomic studies were done by Miss ALMA DIETZ of The Upjohn Company; *see* also Ref. 9.

** The U-numbers refer to the dihydrochloride salts of the antibiotics.

Fort Worth, Texas, U.S.A.); seed medium pre-sterilization pH 7.2. The cultures were incubated at 28°C for 72 hours on a rotary shaker. Fermentation medium consisting of glucose monohydrate, 10 g/liter; cornstarch, 20 g/liter; calcium carbonate, 5 g/liter; ammonium sulfate, 3 g/liter; Distiller's solubles, 15 g/liter (Brown-Forman Distillers Co., P. O. Box 1080, Louisville, Ky. 40201); corn oil, 2 ml/liter and Ucon LB 625, 2 mg/liter, (Union Carbide Co., Chemical Division, 10421 West 7 Mile Road, Detroit, Michigan, 48221, U.S.A.) was inoculated at a rate of 5 per cent (v/v) with the 72-hour seed medium. Fermentations were incubated at 28°C on a rotary shaker and beers were harvested after total fermentation times of 120~144 hours.

## Assay Procedures

Antibiotic titers were measured by a disc plate assay using *Bacillus subtilis*, *Klebsiella pneumoniae* or *Staphylococcus aureus* as assay organisms. A biounit of activity is defined as that quantity of antibioitic necessary to produce a 20-mm zone of inhibition from a 12.7 mm paper disc containing 0.08 ml of test solution.

## Chromatographic Analysis

Clear filtrates were analyzed by paper chromatography or thin-layer chromatography using Brinkman MN-Polygram CEL 300 (Brinkman Instruments, Inc., Westbury, New York 11590) as chromatographic support. Sodium citrate (0.05 M) pH 6.9 buffer was used as the solvent system. Antibiotics were detected by bioautography on agar trays seeded with *B. subtilis*, *K. pneumoniae* or *S. aureus*.

Preparations obtained during the purification work described below, were analyzed by thin-layer chromatography using MN-Polygram CEL-300 and aqueous ammonium chloride solutions (adjusted to pH 7.5 with aqueous ammonium hydroxide) as the developing solvent. Antibiotics were detected by bioautography as above.

## Extraction of Antibiotics from Fermentation Broth

Adsorption of Amberlite XAD-2: Fermentation broth (5,000 liters) was filtered with the aid of diatomaceous earth. The filter cake was washed with water and the aqueous wash was combined with the clear filtrate. This soluiton was passed over a column containing 120 liters of Amberlite XAD-2 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) at a flow rate of 13 liters per minute. The spent beer was discarded. The column was washed first with water and then with 440 liters of 5% aqueous acetone. Both the aqueous and the aqueous acetone fractions were found bio-inactive and were discarded. The column was then eluted with 400 liters of 50% aqueous acetone. This acetone eluate was adjusted to pH 8.5, with sodium hydroxide and concentrated to an aqueous solution which was then freeze-dried to yield 3.17 kg of material containing (by TLC) all the bioactive components present in the fermentation broth.

## Purification

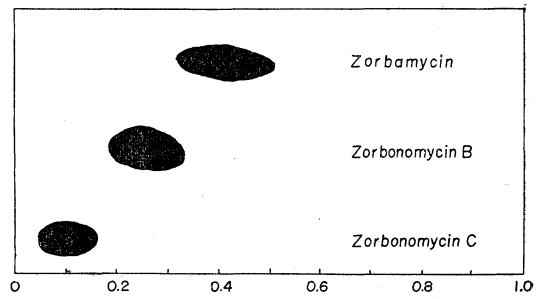
I. Methanol-Acetone Precipitation: The above freeze-dried preparation was suspended in 45 liters of absolute methanol. The mixture was filtered using filter aid. The filtrate was concentrated to a volume of 12 liters, adjusted to pH 6.2 with aqueous hydrochloric acid and then concentrated to a volume of 3 liters. This concentrate was mixed with 1.5 liters of acetone. The precipitated material was removed by filtration and discarded. The filtrate was mixed with 30 liters of acetone and the mixture was allowed to stand at 5°C for 12 hours. The precipitated material was then isolated by filtration, washed with 8 liters of acetone and dried *in vacuo* to give 520 g of a highly purified preparation which contained all of the antibiotics present in the original fermentation broth.

II. IRC-50 Chromatography: The column was prepared from 7 liters of IRC-50-cation exchange resin (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) in the ammonium form. Five hundred grams of the preparation, obtained by methanol-acetone precipitation as described in the previous section, was dissolved in 25 liters of water and 10 liters of

methanol. The solution was adjusted to pH 6.0 with aqueous hydrochloric acid. This solution was then passed through the column at a flow rate of 150 ml/minute. The effluent was discarded. The column was then washed with 60 liters of water followed by 16 liters of aqueous ammonium hydroxide solution, prepared by mixing one volume of concentrated ammonium hydroxide with 19 volumes of water. The column was finally eluted with 30 liters of 5 % aqueous ammonium chloride solution. The effluent was collected in 4-liter fractions which were assayed for bioactivity and analyzed by thin-layer chromatography. Fractions containing bioactive materials were combined and processed as described in the next section.

Fig. 2. Thin-layer chromatography* of zorbamycin, zorbonomycin B and zorbonomycin C.

* Support: Brinkman MN-Polygram CEL-300. Solvent: 0.1 M aqueous ammonium chloride. Antibiotics were detected by bioautography on *B. subtilis* seeded agar.




III. Amberlite XAD-2 Chromatography-Removal of Ammonium Chloride: A column was prepared from 2 kg of Amberlite XAD-2 resin packed in water. The 5 % aqueous ammonium chloride eluate from the IRC-50 chromatography, described above, was passed through the column. The effluent was found bio-inactive and was discarded. The column was then eluted with 6 liters of water which was collected as twelve 500-ml fractions numbered 1 through 12. TLC on Brinkman MN-Polygram CEL-300 using sodium citrate (0.05 M, pH 6.9) buffer as the solvent showed that fractions 5 to 12 contained component IV (Fig. 1). These fractions were then combined and freeze-dried to yield 4.9 g of a blue-colored amorphous material (Prep I). Further TLC using the same support and 0.1 M aqueous ammonium chloride as the solvent indicated the presence of three bioactivities in Prep I. The relative mobilities of these antibiotics which have been designated zorbamycin (main component) and zorbonomycins B and C (minor components) are presented in Fig. 2. Separation of these materials is described in the next section.

Separation of Zorbamycin from Zorbonomycin B and Zorbonomycin C

I. CM-Sephadex Chromatography: CM-Sephadex, C-25 (40~120 $\mu$, Pharmacia Fine Chemicals, Inc., Uppsala, Sweden), 175 g, was mixed with 2 liters of 0.05 M aqueous ammonium chloride and adjusted to pH 7.3 with aqueous ammonium hydroxide. The gel was allowed to swell for 12 hours and then packed into a chromatographic column (4.7 cm internal diameter) to a constant height of 79 cm by flowing 0.05 M aqueous ammonium chloride, pH 7.3.

Prep. I (4.35 g) (obtained as described in the previous section), was dissolved in 5 ml of 0.05 M aqueous ammonium chloride (pH 7.5) solution and applied on the top of the column. The column was eluted at a flow rate of 5 ml per minute. Fractions 1~65 (*ca* 18 ml each) were obtained by eluting the column with 0.05 M aqueous ammonium chloride (adjusted to pH 7.5 with ammonium hydroxide). Fractions 66 to 586 were obtained by eluting the column with 0.15 M aqueous ammonium chloride (pH 7.5) and finally fractions 587~886 were obtained by elution with 0.3 M aqueous ammonium chloride (pH 7.5). Selected fractions were tested for bioactivity and analyzed by thin-layer chromatography. Fractions 401~494, containing zorbamycin, were combined and designated Pool I. Fractions 505~535, found to contain zorbonomycin B were also combined and designated Pool II. Fractions 850~885, contained zorbonomycin C (Pool III). The antibiotics were isolated pure, free of ammonium chloride by Amberlite XAD-2 chromatography as described below.

II. Isolation of Pure Zorbamycin, Zorbonomycin B and Zorbonomycin C by Amberlite XAD-2-Chromatography:

A. Isolation of Zorbamycin. Pool I, obtained as described above, was passed through a column containing 200 ml of Amberlite XAD-2 at a flow rate of 8 ml per minute. Zorbamycin was absorbed in the resin and the effluent containing ammonium chloride was discarded. The column was washed with 180 ml of water and then eluted with 250 ml of acetone - water (1 : 1, v/v). The eluate was concentrated to an aqueous solution and this solution was then freeze-dried to give 2.3 g of blue-colored amorphous zorbamycin.

B. Isolation of Zorbonomycin B. Pool II, from the CM-Sephadex chromatography described previously, was passed over a column containing 50 ml of Amberlite XAD-2 at a flow rate of 2 ml per minute. The ammonium chloride effluent was discarded. The column was washed with water and zorbonomycin B was then eluted with 250 ml of acetone - water (1 : 1, v/v). This eluate was concentrated to an aqueous solution and freeze-dried to yield 450 mg of zorbonomycin B as a blue-colored amorphous material.

C. Isolation of Zorbonomycin C. Pool III (fractions 850~855 of the CM-Sephadex chromatography described previously) was passed over a column containing 9 ml of Amberlite XAD-2. The effluent was discarded. The column was washed with water and zorbonomycin C was isolated by elution with 40 ml of acetone - water (1 : 1, v/v). A total of 125 mg of the blue-colored amorphous antibiotic was isolated by concentration of the aqueous acetone eluate to an aqueous solution followed by freeze-drying.

Copper-free Zorbamycin

Zorbamycin, 500 mg, was dissolved in 50 ml of absolute methanol. Hydrogen sulfide was then introduced into this solution until the blue color had been replaced by the dark brown color of the precipitated copper sulfide. The precipitated material was separated by filtration and discarded. The filtrate was concentrated to a volume of 10 ml. This solution was then mixed with 1 liter of ether. The precipitated, colorless, copper-free zorbamycin was isolated by filtration and dried ; yield, 371 mg.

Copper-free Zorbonomycin B

Zorbonomycin B, 1.0 g, was dissolved in 100 ml of absolute methanol. Hydrogen sulfide was introduced into the solution until the dark brown color of the precipitated copper sulfide prevailed. The precipitate was removed by filtration. The slightly yellow filtrate was then mixed with 1 liter of ether. Colorless copper-free zorbonomycin B was then precipitated, isolated by filtration and dried ; yield, 800 mg.

## Results and Discussion

Bio-activities Produced by *S. bikiniensis* var. *zorbonensis*

*S. bikiniensis* var. *zorbonensis* produces a mixture of antibiotics when grown either on agar or in submerged culture. The antibiotic components produced were determined by ascending thin-layer chromatography of the clear filtrates on Brinkman MN-Polygram CEL-300 sheets (see experimental). Four major zones of bioactivity are indicated when the thin-layer chromatograms were plated on *B. subtilis* (Fig. 1). The components labelled I, II and III are very active against *B. subtilis* and *S. aureus* and moderately active against *K. pneumoniae*. Work on the isolation and characterization of these three components is continuing and will be reported when completed. The component designated IV (Fig. 1) is highly active *versus K. pneumoniae* and *Escherichia coli*, and moderately active against *B. subtilis*. This material, isolated practically free of components I, II and III, was found to be a mixture of a major and two minor antibiotics (Fig. 2). As already mentioned, the major activity designated zorbamycin and the two minor constituents named zorbonomycin B and zorbonomycin C were separated by CM-Sephadex chromatography. Paper and thin-layer chromato-

graphy of each of the three antibiotics in several solvent systems showed the presence of one bioactive component. In addition, the use of chemical detecting systems (sulfuric acid, permanganate-periodate, iodine) indicated the absence of extraneous materials.

## Characterization of Zorbamycin and Zorbonomycins B and C

I. Zorbamycin. This antibiotic, as already mentioned, was isolated as the blue-colored hydrochloride salt. Careful neutralization of an aqueous solution of zorbamycin hydrochloride with silver carbonate afforded zorbamycin which did not contain chlorine indicating the absence of non-ionic halogen in the antibiotic molecule. Analytical data obtained on the hydrochloride salt (Table 1) suggested the empirical formula $C_{56}H_{100}N_{18}O_{24}S_2Cu \cdot 2HCl$ (calcd. empirical weight, 1,610). Since we have been unable to obtain a meaningful molecular weight value by either mass spectrometry, potentiometric titration*, or vapor pressure osmometry*, we propose the above formula containing one copper atom per molecule as the molecular formula for zorbamycin hydrochloride. This would indicate the presence of two basic groups in zorbamycin, *i.e.*, the isolated material is zorbamycin dihydrochloride and the molecular formula of zorbamycin would be $C_{56}H_{100}N_{18}O_{24}S_2Cu$.

Zorbamycin dihydrochloride is highly soluble in water. However, at pH 3.8 the material precipitates out of solution and re-dissolves when the pH is lowered to *ca* 1.0. This behavior suggests the presence of acidic group(s) in the zorbamycin molecule and the formation of a zwitter ion of limited solubility at the indicated pH. Zorbamycin dihydrochloride is also soluble in methanol, slightly soluble in ethanol and insoluble in higher alcohols, ketones, and aromatic, saturated or chlorinated hydrocarbon solvents.

The IR spectrum of zorbamycin dihydrochloride, shown in Fig. 3, indicates the

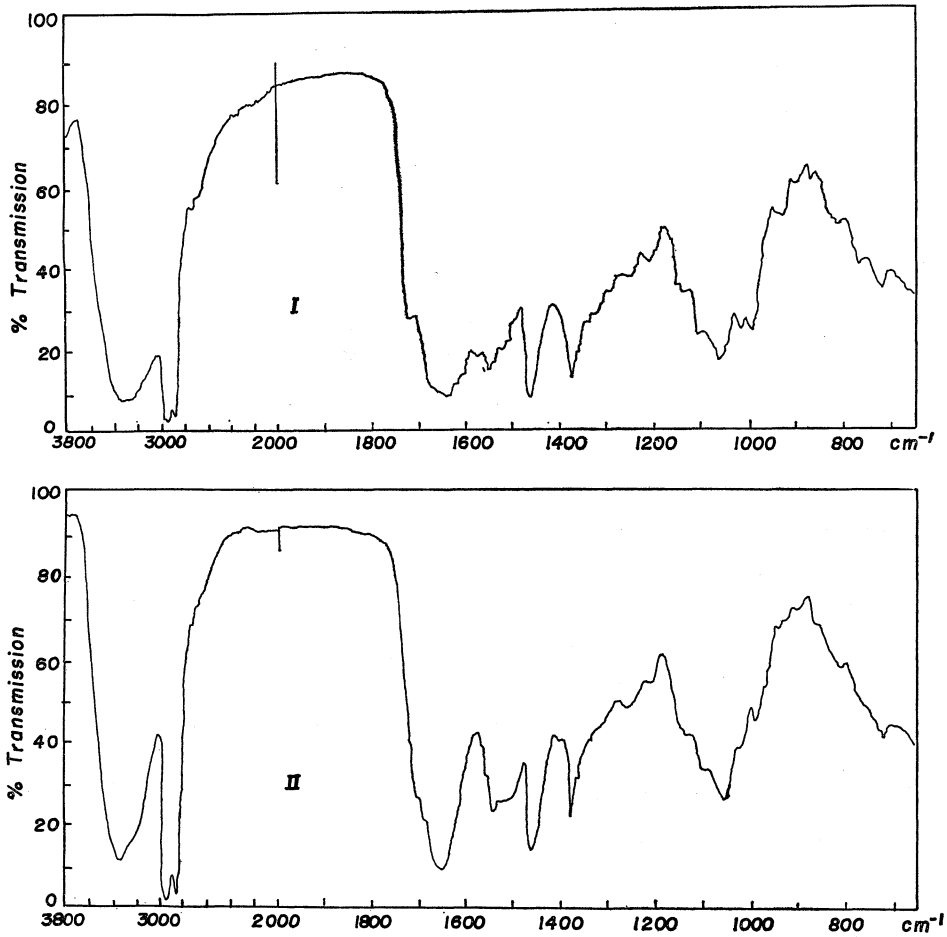
Table 1. Characterization data

| Anal. data | Zorbamycin[3] | Copper-free[4] zorbamycin | Zorbonomycin B[5] | Copper-free[6] zorbonomycin B |
|---|---|---|---|---|
| C | 42.48 | 43.22 | 42.26 | 43.73 |
| H | 5.92 | 6.55 | 5.43 | 5.71 |
| N | 16.16 | 16.11 | 16.06 | 16.20 |
| S | 3.81 | 4.13 | 3.75 | 4.26 |
| Cl | 4.26 | 4.14 | 4.32 | 4.74 |
| Cu | 3.86 | 0.006 | 3.81 | 0.12 |
| O | 23.51 (diff) | 24.45 | 24.37 (diff) | 25.24 (diff) |
| Molecular weight[1] | 677 | 543 | — | — |
| Equivalent weight[2] | 595 | 628 | 860 | 818 |

1) Molecular weight was determined by vapor pressure osmometry in methanol.
2) Equivalent weight was determined by potentiometric titration in glacial acetic acid using perchloric acid as the titrant.
3) Calcd. for $C_{56}H_{100}N_{18}O_{24}S_2Cu \cdot 2HCl$: C 41.78, H 6.39, N 15.66, O 23.85, S 3.98, Cl 4.40, Cu, 3.94, M.W. 1610.
4) Calcd. for $C_{56}H_{100}N_{18}O_{24}S_2 \cdot 2HCl$: C 43.49, H 6.65, N 16.30, O 24.83, S 4.15, Cl 4.58, M.W., 1546.
5) Calcd. for $C_{58}H_{90}N_{18}O_{24}S_2Cu \cdot 2HCl$: C 42.90, H 5.71, N 15.53, S 3.95, Cl 4.37, Cu 3.91, M.W., 1624.
6) Calcd. for $C_{58}H_{90}N_{18}O_{24}S_2 \cdot 2HCl$: C 44.64, H 5.94, N 16.16, S 4.11, Cl 4.54, M.W., 1560.

* Potentiometric titration in glacial acetic acid using perchloric acid as the titrant gave an equivalent weight of 595. Since the number of titratable groups is not known, the molecular weight cannot be calculated. Determination of the molecular weight by vapor pressure measurements in methanol as the solvent gave a value of 677. However, this method depends on the number of particles (ionic or neutral species) present in solution. Since zorbamycin is expected to dissociate in solution, the obtained value is the average value of the molecular weights of the species present in the solution.

Fig. 3. Infrared spectra of : I. Zorbamycin dihydrochloride.
II. Copper-free zorbamycin dihydrochloride (in nujol mull).




presence of OH/NH at 3400~3200 $cm^{-1}$, carbonyl absorptions at 1720 (shoulder) and amide carbonyl at 1650 (amide I) and 1550 (amide II) $cm^{-1}$. The UV spectrum of zorbamycin dihydrochloride in methanol, presented in Fig. 4, showed absorption at 244 (a=16.61), 290 (shoulder, a=5.52), 298 (a=5.73), 309 (shoulder, a=5.39) and 600 (a=0.08) nm. The optical rotation of zorbamycin dihydrochloride was found to be, $[\alpha]_D^{25}$ +247° (*c* 0.6, water). The optical rotatory dispersion curves in water and methanol are presented in Figs. 5 and 6, respectively. The ORD curves are discussed later in connection with the comparison of the phleomycin and bleomycin antibiotics to zorbamycin. Thin-layer chromatographic behavior of zorbamycin in different systems is presented in Figs. 1, 2, 11 and 12.

II. Copper-free Zorbamycin. This compound was isolated as the colorless amorphous hydrochloride salt by treatment of zorbamycin dihydrochloride with hydrogen sulfide (see experimental). Analytical data (Table 1) are in agreement with the empirical formula of $C_{56}H_{100}N_{18}O_{24}S_2 \cdot 2HCl$. The molecular weight, as determined by vapor pressure osmometry in methanol, was found to be 543. Potentiometric titration

in glacial acetic acid using perchloric acid as titrant gave equivalent weight of 628. Since these values are close to the corresponding values obtained for zorbamycin dihydrochloride, we propose* the above formula as the molecular formula for copper-free zorbamycin dihydrochloride. This material has the same solubility properties as the copper-containing material. The IR spectrum of copper-fee zorbamycin dihydrochloride (Fig. 3) is similar to that of zorbamycin dihydrochloride.

Fig. 5. Optical rotatory dispersion of zorbamycin dihydrochloride (in water).

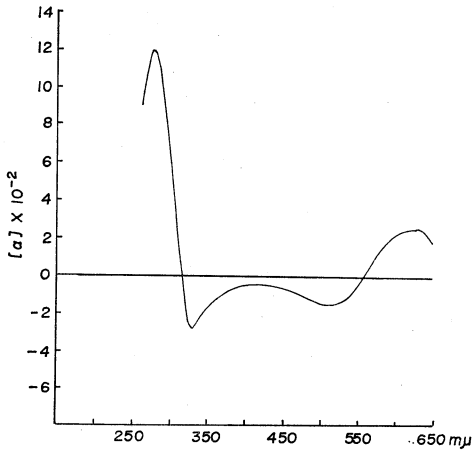



Fig. 6. Optical rotatory dispersion of zorbamycin dihydrochloride (in methanol).

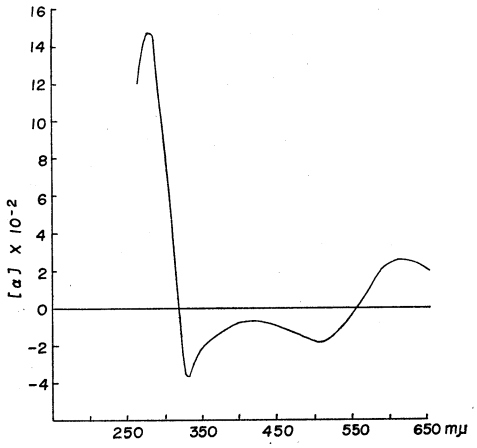



Fig. 4. Ultraviolet absorption spectrum of zorbamycin dihydrochloride (in methanol).

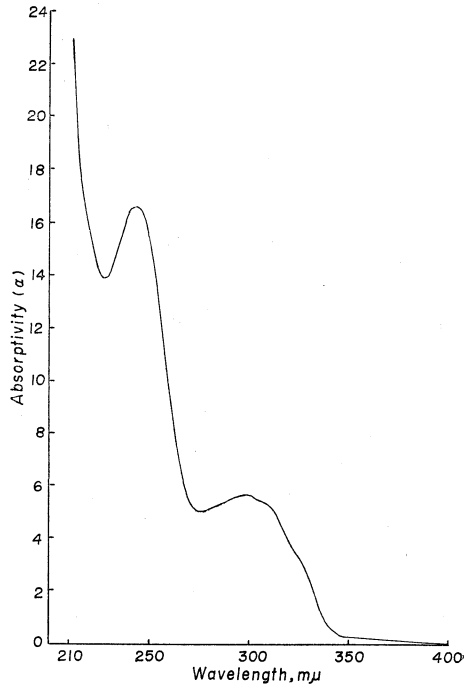
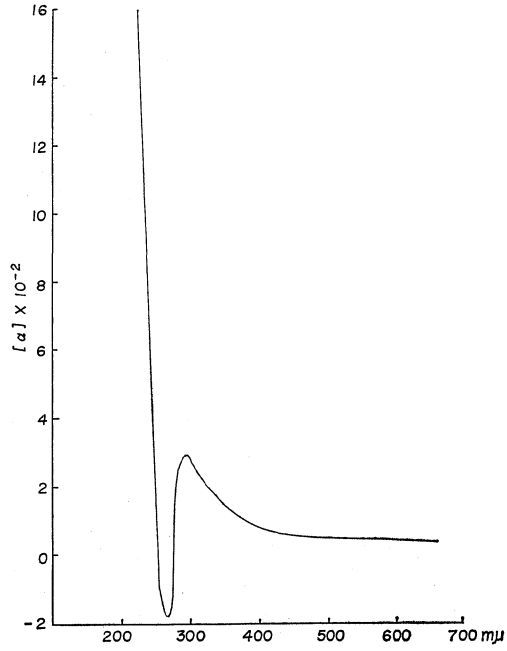


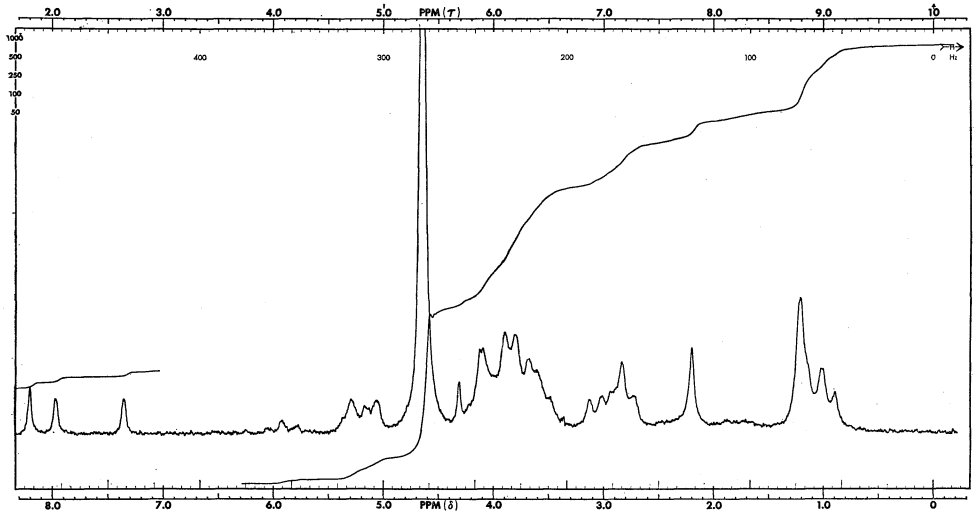

Fig. 7. Optical rotatory dispersion of copper-free zorbamycin dihydrochloride (in methanol).




* See discussion related to the molecular formula of zorbamycin.

Fig. 8. Nuclear magnetic resonance spectrum* of copper-free zorbamycin dihydrochloride.

* NMR spectra were observed with a Varian A-60 spectrometer on solutions (*ca* 0.4 ml, *ca* 0.25 M) of the compound in deuterium oxide.

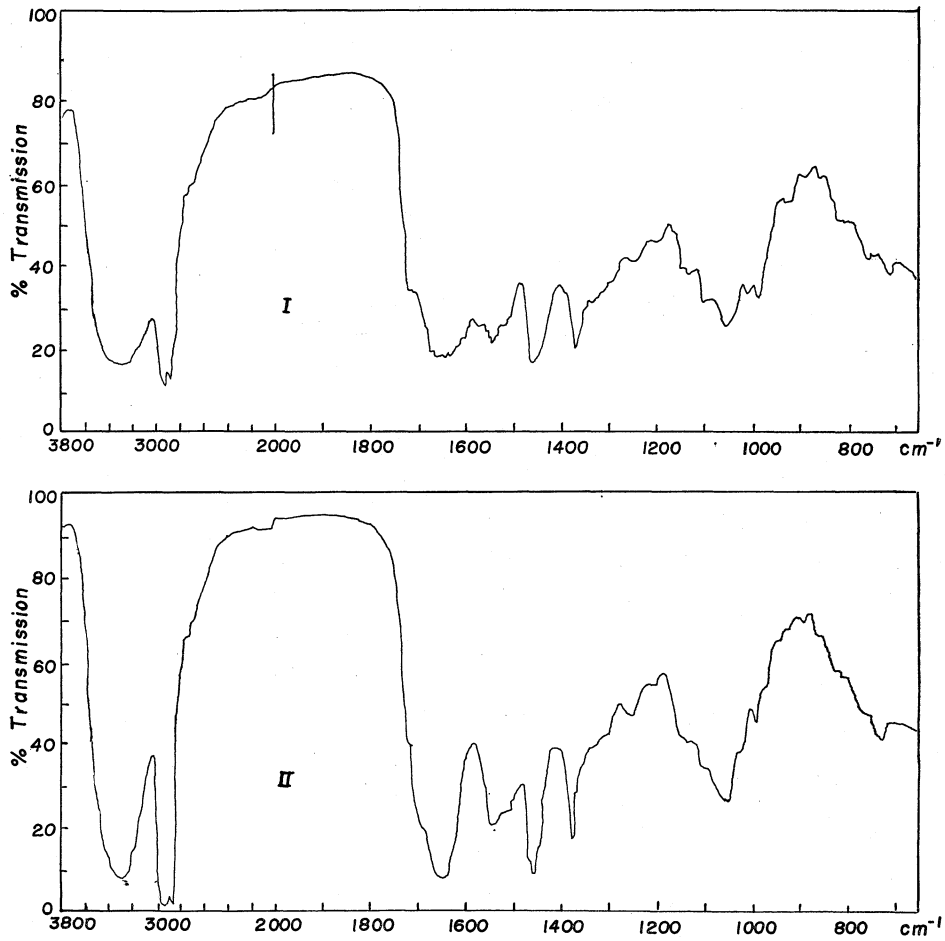



The UV spectrum showed absorptions at 234 (a=16.97) and 298 (a=3.20) nm. The specific rotation, $[\alpha]_D^{25}$ was found to be +19° (*c* 0.98, water). The ORD curve is presented in Fig. 7. The presence of copper in zorbamycin dihydrochloride prevented the obtaining of the NMR spectrum of the antibiotic. Copper-free zorbamycin dihydrochloride gave a rather complex NMR spectrum* (Fig. 8). The spectrum shows absorption at $\delta$ 0.85 to 1.3 ppm indicating the presence of several methyl groups attached on saturated carbon systems [$-\underset{|}{\text{CH}}-\text{CH}_3$, $-\underset{|}{\underset{\text{O}-}{\text{CH}}}-\text{CH}_3$ or $-\underset{|}{\underset{\text{N}-}{\text{CH}}}-\text{CH}_3$]. Singlet absorption at $\delta$ 2.2 ppm can be assigned to methyl(s) on aromatic or unsaturated systems or on sulfur. The presence of olefinic or anomeric hydrogens is indicated by absorptions at *ca* $\delta$ 5.0 and 6.0. Finally, the absorptions from $\delta$ 7.0 to 8.3 can be assigned to aromatic or olefinic hydrogens.

III. Zorbonomycin B. This zorbamycin-related antibiotic has been characterized as the blue-colored dihydrochloride amorphous salt. Analytical data (Table 1) indicate the presence of copper in zorbonomycin B. The molecular formula of $C_{58}H_{90}N_{18}O_{24}S_2Cu \cdot 2HCl$ is proposed for this antibiotic assuming the presence of one copper atom in the zorbonomycin B dihydrochloride molecule. Potentiometric titration in glacial acetic acid using perchloric acid as the titrant showed an equivalent weight of 860. Solubilities of zorbonomycin B are identical to those of zorbamycin. The IR spectrum of zorbonomycin B dihydrochloride (Fig. 9) is indistinguishable from the spectrum of

* Assuming that the singlet absorption at $\delta$ 2.2 ppm is due to a single methyl group then it appears that the total number of hydrogens in the copper-free zorbamycin molecule is about 50~55. This suggests a molecular formula of $C_{28}H_{51}N_9O_{12}S$ for copper-free zorbamycin. Zorbamycin then can be pictured as a copper complex of the type $[C_{28}H_{51}N_9O_{12}S]$-Cu-$[C_{28}H_{51}N_9O_{12}S]$ in which the metal is complexed to two $C_{28}$ units. However, final conclusion on the molecular formula of both zorbamycin and copper-free zorbamycin has to await work on the structure of the antibiotic.

Fig. 9. Infrared spectra of : I. Zorbonomycin B dihydrochloride.
II. Copper-free zorbonomycin B dihydrochloride (in nujol mull).

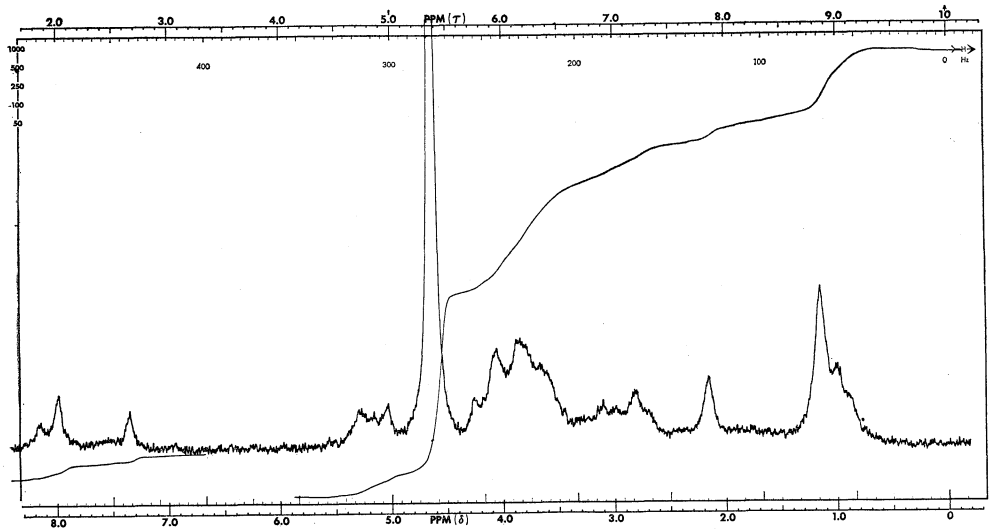



zorbamycin dihydrochloride (Fig. 3) showing OH/NH absorption, carbonyl absorption at *ca* 1720 $cm^{-1}$ and amide I and II absorptions at 1650 and 1550 $cm^{-1}$, respectively. The UV spectrum of zorbonomycin B dihydrochloride in methanol showed maxima at 243 (a=15.29), 293 (a=12.63), 308 (slight shoulder, a=9.59) and 600 nm (a=0.08). The UV spectrum and specifically the ratio of the intensities of the absorptions at 243~244 and at 293~295 nm (1.21 for zorbonomycin B dihydrochloride and 2.91 for zorbamycin dihydrochloride), differentiates the two antibiotics. Another differentiating property is the behavior of these antibiotics in thin-layer chromatography under the conditions described in Fig. 2. Additional TLC data on zorbonomycin B are presented in Figs. 14 and 15.

IV. Copper-free Zorbonomycin B. Colorless, amorphous, copper-free zorbonomycin B dihydrochloride is obtained by treatment of zorbonomycin B dihydrochloride with hydrogen sulfide. On the basis of the analytical data (Table 1) and the similarity to copper-free zorbamycin dihydrochloride, we propose the molecular formula of $C_{58}H_{90}N_{18}O_{24}S_2 \cdot 2HCl$ for copper-free zorbonomycin B. However, the

Fig. 10. Nuclear magnetic resonance spectrum* of copper-free zorbonomycin B dihydrochloride.

* NMR spectra were observed with a Varian A-60 spectrometer in solutions (*ca* 0.4 ml, *ca* 0.25 M) of the compound in deuterium oxide.




arguments regarding the molecular formula of zorbamycin (*see* footnote, page 477), based on NMR spectral data, can be used in the case of the molecular formula of zorbonomycin B since the NMR spectrum of copper-free zorbonomycin B (Fig. 10) is almost identical to that of copper-free zorbamycin (Fig. 8). As expected, the IR spectra of both copper-free antibiotics are indistinguishable (*see* Figs. 3 and 9). The UV spectrum of copper-free zorbonomycin B dihydrochloride in methanol, which shows absorptions at 235 (slight shoulder, a=6.40), 293 (a=4.28) and 306 nm (slight shoulder, a=3.22) is different from the UV spectrum of copper-free zorbamycin.

V. Zorbonomycin C. This antibiotic has been isolated in small amounts as a blue-colored amorphous material which has not been completely characterized. Analytical data are not available, but since this material has been obtained by ion exchange procedures, it is assumed that zorbonomycin C, like zorbamycin and zorbonomycin B, has been obtained as the hydrochloride salt. It is also assumed that this antibiotic contains copper because of its bright blue color. The UV spectrum of zorbonomycin C showed absorption at 244 (a=18.6) and 295 (a=6.7) nm. The UV spectrum differs from the spectra of both zorbamycin and zorbonomycin B, being closer to that of zorbamycin. Thin-layer chromatographic behavior of zorbonomycin C is presented in Figs. 2, 16 and 17.

## Biological Properties of Zorbamycin and Zorbonomycins B and C

The results obtained in testing zorbamycin and its related antibiotics in several *in vitro* and *in vivo* systems will be reported in a subsequent communication (C. LEWIS and T. R. PYKE, paper in preparation). Table 2 presents the *in vitro* antibacterial spectrum of zorbamycin and zorbonomycin B and C dihydrochlorides tested under identical conditions. As shown in Table 2, zorbamycin and zorbonomycin B were equally active against the organisms listed. Zorbonomycin C hydrochloride

Table 2. *In vitro* antibacterial spectrum* of zorbamycin and zorbonomycins B and C

| Test organism | Minimum inhibitory concentration (mcg/ml) | | |
|---|---|---|---|
| | Zorbamycin dihydrochloride | Zorbonomycin B dihydrochloride | Zorbonomycin C hydrochloride |
| *Escherichia coli* UC 51 | 0.2 | 0.1 | 1.56 |
| *Klebsiella pneumoniae* UC 57 | 0.05 | 0.025 | 0.78 |
| *Pseudomonas aeruginosa* UC 95 | 200.0 | 200.0 | 200.0 |
| *Proteus vulgaris* UC 93 | 6.25 | 6.25 | 25.0 |
| *Proteus mirabilis* UC 3123 | 25.0 | 12.5 | 200.0 |
| *Staphylococcus aureus* UC 76 | 0.39 | 0.2 | 1.56 |
| *Staphylococcus aureus* UC 70 | 0.78 | 0.2 | 1.56 |
| *Staphylococcus aureus* UC 552 | 0.78 | 0.2 | 3.12 |
| *Streptococcus hemolyticus* UC 152 | 100.0 | >200.0 | >200.0 |
| *Streptococcus faecalis* UC 3235 | >200.0 | >200.0 | >200.0 |
| *Diplococcus pneumoniae* UC 41 | 100.0 | 50.0 | >200.0 |
| *Sarcina lutea* UC 130 | 12.5 | 25.0 | 25.0 |
| *Salmonella typhosa* UC 215 | 0.1 | 0.05 | 0.78 |
| *Salmonella paratyphi B* UC 263 | 0.2 | 0.2 | 3.12 |
| *Salmonella gallinarum* UC 265 | 0.1 | 0.05 | 0.78 |
| *Aerobacter aerogenes* UC 3 | 0.78 | 0.39 | 6.25 |
| *Shigella sonnei* UC 933 | 0.39 | 0.39 | 12.5 |

* Test method: Two-fold dilution endpoints in brain-heart infusion broth; read at 20 hours.

Table 3. Comparison of antibacterial activity* of copper-containing and copper-free antibiotics

| Test organism | Minimum inhibitory concentration (mcg/ml) | | | |
|---|---|---|---|---|
| | Zorbamycin dihydrochloride | Copper-free zorbamycin dihydrochloride | Zorbonomycin B dihydrochloride | Copper-free zorbonomycin B dihydrochloride |
| *Escherichia coli* UC 51 | 0.2 | 0.19 | 0.2 | 0.1 |
| *Klebsiella pneumoniae* UC 57 | 0.05 | 0.025 | 0.025 | 0.0125 |
| *Pseudomonas aeruginosa* UC 95 | 200.0 | >200.0 | >200.0 | >200.0 |
| *Proteus vulgaris* UC 93 | 6.25 | 12.5 | 3.12 | 1.56 |
| *Proteus mirabilis* UC 3123 | 25.0 | >200.0 | 6.25 | 12.5 |
| *Staphylococcus aureus* UC 76 | 0.39 | 0.78 | 0.2 | 0.2 |
| *Staphylococcus aureus* UC 70 | 0.78 | 0.78 | 0.78 | 0.39 |
| *Staphylococcus aureus* UC 552 | 0.78 | 0.39 | 0.39 | 0.39 |
| *Streptococcus hemolyticus* UC 152 | 100.0 | — | >200.0 | >200.0 |
| *Streptococcus faecalis* UC 3235 | >200.0 | — | >200.0 | >200.0 |
| *Sarcina lutea* UC 130 | 12.5 | 12.5 | 25.0 | 6.25 |
| *Salmonella typhosa* UC 215 | 0.1 | 0.19 | 0.05 | 0.025 |
| *Salmonella paratyphi B* UC 263 | 0.2 | 0.39 | 0.2 | 0.2 |
| *Salmonella gallinarum* UC 265 | 0.1 | 0.05 | 0.1 | 0.025 |
| *Aerobacter aerogenes* UC 3 | 0.78 | 0.39 | 0.39 | 0.39 |
| *Shigella sonnei* UC 933 | 0.39 | 0.39 | 0.2 | 0.10 |

* Test method: Two-fold dilution endpoints in brain-heart infusion broth; read at 20 hours.

was less active than zorbamycin against most of the organisms tested. Comparison of the *in vitro* antibacterial spectra of zorbamycin, copper-free zorbamycin, zorbonomycin B and copper-free zorbonomycin B is presented in Table 3. In general, the copper-free materials were as active as the copper-containing antibiotics against the listed organisms.

## Comparison of Zorbamycin and Zorbonomycins B and C to Other Antibiotics

The physical, chemical and biological properties described in the previous section help to differentiate zorbamycin and the zorbonomycins from most antibiotics reported in the literature. The same data however indicated similarities of the three antibiotics described in this paper to the phleomycin and bleomycin families of antibiotics. The discussion which follows shows that zorbamycin and zorbonomycins B and C are new antibiotics, most probably related to the phleomycins.

### I. Comparison of Zorbamycin to Phleomycin and Bleomycin Antibiotics.

Phleomycin was reported in 1956 by MAEDA and his coworkers[1]. Further studies established the presence of copper in the phleomycin molecule[2] and the existence of several phleomycins which were separated by chromatography[3]. In 1966 UMEZAWA, *et al.*[4] reported the isolation of bleomycins A and B from fermentations of *Streptomyces verticillus* (producer of phleomycins). Purification of bleomycins[5] resulted in isolation of several components. A total of fourteen phleomycins* (A, B, C, $D_1$, $D_2$, E, F, $G_1$, $G_2$, H, I, J, K,

* However A, B, J, K, L obtained in small amounts were not characterized at all. Therefore, comparison of the antibiotics reported in this paper to phleomycins A, B, J, K, or L is impossible.

Fig. 11. T.L.C. comparison of zorbamycin and bleomycin-complex.
1. Zorbamycin; 2. Mixture of zorbamycin and bleomycin complex; 3. Bleomycin complex.
Support: Brinkman MN-Polygram CEL-300.
Solvent: 0.1 M aqueous ammonium chloride.
Antibiotics were detected by bioautography on *B. subtilis* seeded agar.

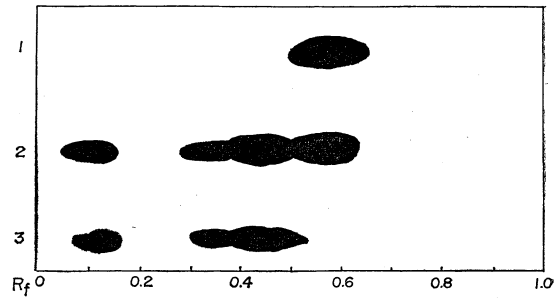



Fig. 12. T.L.C. comparison of zorbamycin and phleomycin-complex.
1. Zorbamycin; 2. Mixture of zorbamycin and phleomycin complex; 3. Phleomycin complex.
Support: Brinkman MN-Polygram CEL-300.
Solvent: Sodium citrate (0.05 M) pH 6.9 buffer.
Antibiotics were detected by bioautography on *B. subtilis* seeded agar.

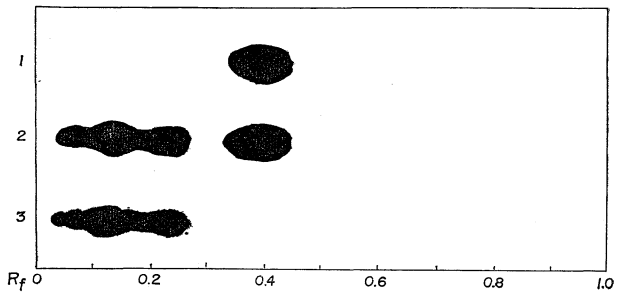
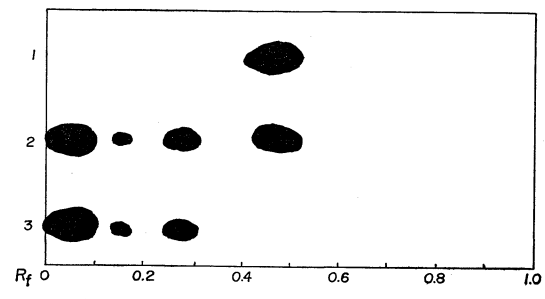



Fig. 13. T.L.C. comparison of zorbamycin and phleomycin-complex.
1. Zorbamycin; 2. Mixture of zorbamycin and phleomycin complex; 3. Phleomycin complex.
Support: Brinkman MN-Polygram CEL-300.
Solvent: 0.1 M Aqueous ammonium chloride.
Antibiotics were detected by bioautography on *B. subtilis* seeded agar.



L) and eleven bleomycins (Cu-$At_1$ to Cu-$At_6$ and Cu-$Bt_1$ to Cu-$Bt_5$) have been reported. Zorbamycin can be differentiated from all bleomycins (Cu-At or Cu-Bt) by UV data and specifically by the relative absorptivities (a) at the lower (*ca* 244 nm) and the higher (*ca* 295 nm) wavelengths. In addition, zorbamycin has been compared by TLC with the bleomycin complex (commercial bleomycin, Lot No. B-8, Nippon Kayaku Co., Japan). As shown in Fig. 11, zorbamycin (run in parallel and in mixture with bleomycin complex) separates from the bioactive components present in the bleomycin sample. Similarly, on the basis of UV spectra, zorbamycin can be differentiated from phleomycins C, $D_2$ and F. Of the remaining phleomycins ($D_1$, E, $G_1$, $G_2$, H and I) for which data are available, phleomycins G ($G_1$ and $G_2$), H and I can be easily eliminated from consideration since they are reported[6] to be practically inactive against *E. coli* (MICs of *ca* 50 mcg/ml) as compared to their activity against *B. subtilis* (MICs of 0.2~0.8 mcg/ml). Zorbamycin is highly active against *E. coli* (MIC of 0.2 mcg/ml) and was found to protect *E. coli* infected mice with a $CD_{50}$ of *ca* 3.0 mg/kg (C. LEWIS, personal communicatcn) when administered subcutaneously. Phleomycins $D_1$ and E could also be eliminated from consideration on the basis of the reported molecular formulas, $C_{36}H_{69}N_{13}SO_{19}Cu$

Fig. 14. T.L.C. comparison of zorbonomycin B and phleomycin-complex.
1. Zorbonomycin B; 2. Mixture of zorbonomycin B and phleomycin complex; 3. Phleomycin complex.

Support: Brinkman MN-Polygram CEL 300.
Solvent: 0.2 M aqueous ammonium chloride.
Antibiotics were detected by bioautography on *B. subtilis* seeded agar.

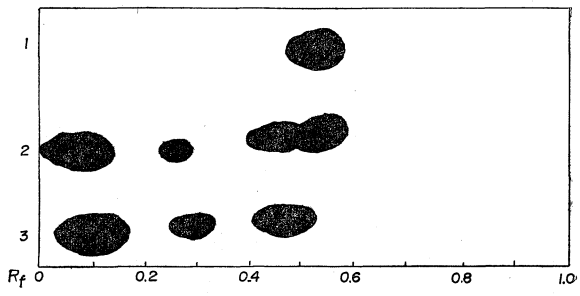



Fig. 15. T.L.C. comparison of zorbonomycin B and bleomycin complex.
1. Zorbonomycin B; 2. Mixture of zorbonomycin B and bleomycin complex; 3. Bleomycin complex.

Support: Brinkman MN-Polygram CEL-300.
Solvent: 0.2 M aqueous ammonium chloride.
Antibiotics were detected by bioautography on *B. subtilis* seeded agar.

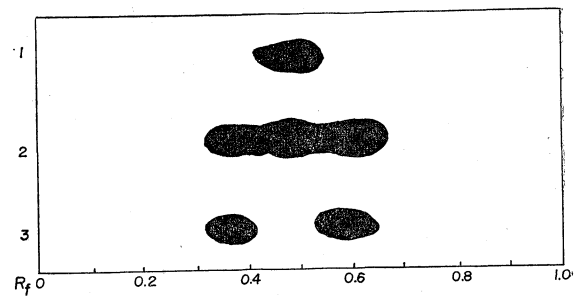
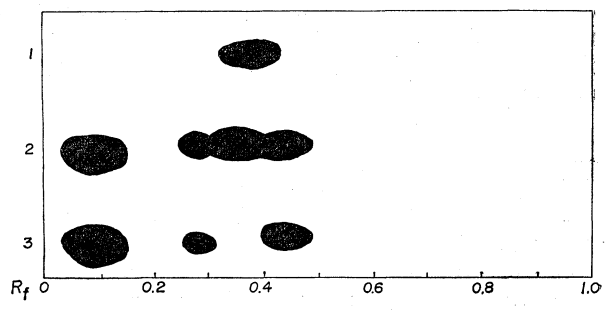


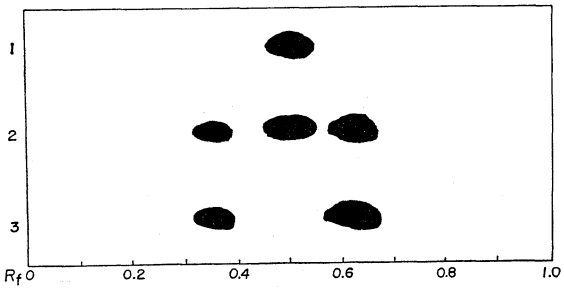

Fig. 16. T.L.C. comparison of zorbonomycin C and phleomycin complex.
1. Zorbonomycin C; 2. Mixture of zorbonomycin C and phleomycin complex; 3. Phleomycin complex.

Support: Brinkman MN-Polygram CEL-300.
Solvent: 0.2 M aqueous ammonium chloride.
Antibiotics were detected by bioautography on *B. subtilts* seeded agar.



for phleomycin $D_1$ and $C_{42}H_{74}N_{16}SO_{21}Cu$ for phleomycin E[3]. On the other hand zorbamycin and zorbonomycin B have the formulas of $C_{56}H_{100}N_{18}O_{24}S_2Cu$ and $C_{58}H_{90}N_{18}O_{24}S_2Cu$ respectively. Furthermore, zorbamycin was compared by TLC with the phleomycin complex* in two different solvent systems. As shown in Figs. 12 and 13, zorbamycin separates from all activities present in the phleomycin samples. In addition, the optical rotatory dispersion curves of zorbamycin in water or methanol (Figs. 6 and 7, respectively) show positive COTTON effect at 615 nm. The ORD curve of phleomycins[3], though similar in shape to that of zorbamycin, shows a positive COTTON effect at 654 nm.

Fig. 17. T.L.C. comparison of zorbonomycin C and bleomycin complex.
1. Zorbonomycin C; 2. Mixture of zorbonomycin C and bleomycin complex; 3. Bleomycin complex.
Support: Brinkman MN-Polygram CEL-300.
Solvent: 0.2 M aqueous ammonium chloride.
Antibiotics were detected by bioautography on *B. subtilis* seeded agar.




II. Comparison of Zorbonomycins B and C to Phleomycin and Bleomycin Antibiotics.

The UV spectrum of zorbonomycin B differentiates this antibiotic from phleomycins $D_1$, E, $G_1$, $G_2$, H, and I. The spectrum, though different, is closer to the UV spectra of bleomycins (Cu-At or Cu-Bt) and phleomycins C, $D_2$ and F. TLC comparison of zorbonomycin B to phleomycin (Fig. 14) and the bleomycin-complex (Fig. 15) indicated that this antibiotic is different from the activities present in the reference samples.

Zorbonomycin C has a UV spectrum similar to the spectra of either phleomycin or bleomycin antibiotics, however, the absorptivities at both lower (245 nm) and higher (295 nm) wavelengths are different. Comparison by TLC of zorbonomycin C to the phleomycin (Fig. 16) and to the bleomycin (Fig. 17) reference sample showed that zorbonomycin C is also different from the bioactive components present in these antibiotic-mixtures.**

Acknowledgements

The authors wish to express their appreciation to various members of The Upjohn Company who contributed to this work. In particular, the authors wish to thank Mr. W. H. DEVRIES for his expert adaptation of the extraction procedures to large scale fermentations and members of the Physical and Analytical Chemistry Department for spectral and analytical data. The technical assistance of Mr. K. J. GEIPEL and Mr. D. R. WAIT is greatly appreciated.

* Phleomycin-complex samples were obtained from Bristol Laboratories (Lot A 9331-648) and from Cancer Chemotherapy National Service Center (Lot NSC 61586).

** Further TLC work has shown that not only zorbamycin, zorbonomycin B and zorbonomycin C but also components I, II and III (see Fig. 1) produced by *S. bikiniensis* have been separated from the bioactivities present in the phleomycin and bleomycin reference samples.

## References


1) MAEDA, K.; H. KOSAKA, K. YAGISHITA & H. UMEZAWA: A new antibiotic, phleomycin. J. Antibiotics, Ser. A 9 : 82~85, 1956
2) TAKITA, T.: Studies on purification and properties of phleomycin. J. Antibiotics, Ser. A 12 : 285~289, 1959
3) IKEKAWA, T.; F. IWAMI, H. HIRANAKA & H. UMEZAWA: Separation of phleomycin components and their properties. J. Antibiotics, Ser. A 17 : 194~199, 1964
4) UMEZAWA, H.; K. MAEDA, T. TAKEUCHI & Y. OKAMI: New antibiotics, bleomycin A and B. J. Antibiotics, Ser. A 19 : 200~209, 1966
5) UMEZAWA, H.; Y. SUHARA, T. TAKITA & K. MAEDA: Purification of bleomycins. J. Antibiotics, Ser. A 19 : 210~215, 1966
6) ISHIZUKA, M.; H. TAKAYAMA, T. TAKEUCHI & H. UMEZAWA: Studies on antitumor activity, antimicrobial activity and toxicity of phleomycin. J. Antibiotics, Ser. A 19 : 260~271, 1966
7) COATS, J. H. & J. ROESSER: Genetic recombination in *Streptomyces bikiniensis* var. *zorbonensis*. J. Bact. (in press)