# ISOLATION, PURIFICATION AND PRELIMINARY CHARACTERIZATION OF MEGALOMICIN

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A new antibiotic complex named megalomicin, which is a member of the macrolide group of antibiotics, has been isolated from the fermentation broth of *Micromonospora megalomicea*. Details of the isolation of the megalomicin complex and separation and purification of its components are given. Evidence is also presented indicating the presence of desosamine in the individual antibiotic components.

This paper reports the isolation, purification and properties of the components of a new solvent extractable antibiotic complex, megalomicin, which is produced by *Micromonospora megalomicea* as described by WEINSTEIN *et al.*[1,2,3,4]

## Isolation and Purification

Megalomicin is an antibiotic mixture most readily isolated from the fermentation broth by adjusting the whole broth to a pH of 9.5 with dilute aqueous sodium hydroxide and extracting with approximately 2 volumes of ethyl acetate for each volume of broth. The solvent phase was separated and concentrated under vacuum to an oily residue. The biological activity of a 1 : 20 dilution of this residue gave a zone of inhibition diameter of approximately 25 mm against *Staphylococcus aureus* ATCC 6538P.

This oily residue was purified by column chromatography on LH 20 Sephadex, an alkylated crosslinked dextran (Pharmacia Fine Chemicals, Inc., Upsala, Sweden) suspended in 95% aqueous ethanol. The eluted fractions were combined according to their antibacterial activities determined by agar disc testing against *S. aureus*. Fractions containing peak activity were pooled, dried, and dissolved in a small amount of acetone which was then poured into an excess volume of petroleum ether (b. p. 30~60°C). The resulting precipitate was separated by filtration and the mother liquor concentrated to obtain the purified megalomicin complex. The megalomicin complex so produced has an assigned potency of 1,000 units/mg when assayed according to a modification of the cylinder cup technique for erythromycin[5].

Bioautography on thin-layer silica gel plates following chromatography in a

solvent system consisting of chloroform and methanol in a ratio of 3:2 indicated that the megalomicin complex consisted of at least four antibacterial components designated as A, B, $C_1$ and $C_2$. This was confirmed by visualization of the components with 50% sulfuric acid in methanol. Figs. 1 and 2 illustrate the chromatographic pattern of the megalomicin components in that system.

An improved procedure for the isolation of the megalomicins was required for further work. For this purpose the ethyl acetate extract of the fermentation broth was concentrated and then extracted with 0.1 N sulfuric acid. The acid extracts were adjusted to pH 8.5~9.0 with dilute sodium hydroxide solution, and the megalomicin base was repartitioned into ethyl acetate. The organic phase was concentrated under reduced pressure, affording a glassy residue of the megalomicin complex. At this point the material consisted largely of megalomicins B, $C_1$ and $C_2$ with a small amount of megalomicin A, as shown by thin-layer chromatography as described above.

A solid megalomicin C fraction ($C_1$ and $C_2$) was obtained by taking advantage of the differential water solubility of the C components *vs* A and B as well as of the observation that the megalomicins were considerably more water-soluble in the cold than at room temperature. The megalomicin complex was dissolved in 4 parts of acetone, and the solution poured into 40 times its volume of ice water. The megalomicin C fraction was isolated by filtration after warming the aqueous suspension to room temperature. The solid obtained in this manner consisted largely of megalomicin $C_1$ with some $C_2$; megalomicins A and B were retained in the aqueous liquors, together with some C material.

The components of the megalomicin complex were separated on a silicic acid column by elution with a solvent system consisting of chloroform and methanol in a ratio of 3:2. The antibiotic complex was dissolved in acetone, and the solution mixed with silicic acid and evaporated under vacuum; the dry mixture was added to the top of the column. A 13 g sample required a column approximately 105 cm in length by 7.5 cm in diameter and was run at the rate of 100 ml/hour; 200 ml fractions were collected. The column was monitored by disc testing each fraction against *S. aureus*. The active fractions were chromatographed on silica gel thin-layer plates for 1.5 hours in the same solvent system used for the column. The patterns of the antibiotic fractions were detected by the two methods described in Figs. 1 and 2 and fractions combined according to these patterns as shown in Table 1. By this method component $C_2$ of the megalomicin complex was separated and

Fig. 1. Bioautograph of the megalomicin complex. Chromatogram run on thin-layer silica gel GF plates using the chloroform - methanol (3 : 2) system and plated against *Staphylococcus aureus*.

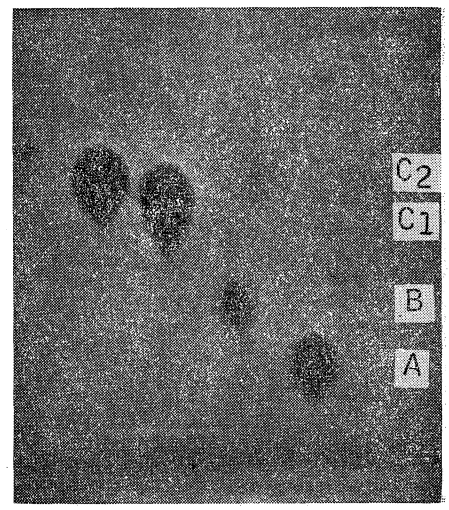


Fig. 2. Sulfuric acid - methanol sprayed chromatograms of the megalomicin complex run on thin-layer silica gel GF plates using the chloroform - methanol (3 : 2) system.

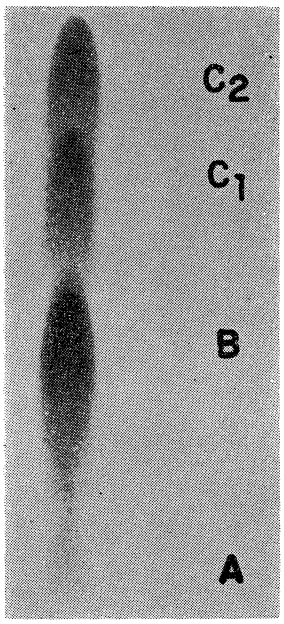



Table 1. Results of column separations

| Megalomicin fraction | Column cut | Weight | TLC Rf Chloroform - methanol (3 : 2) | Potency units/mg* |
|---|---|---|---|---|
| | | Silicic acid column | | |
| $C_2$ | 28～48 | 1.20 g | 0.65 | 4,800 |
| $C_1$ (+$C_2$) | 56～58 | 0.62 g | 0.52+(0.65) | 3,800 |
| | | Florisil column | | |
| B | 164～227 (30 % acetone in ethyl acetate) | 540 mg | 0.38 | 305 |
| A | 370～397 (80 % acetone in ethyl acetate) | 283 mg | 0.19 | 625 |

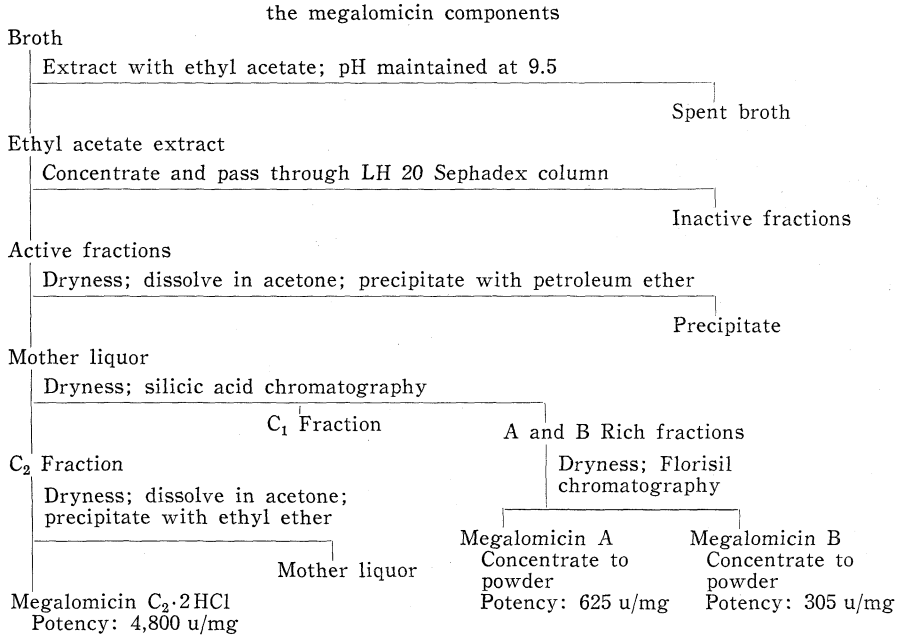
* Activity compared with the megalomicin complex which has been assigned a potency of 1,000 units/mg.

isolated as a white powder by dissolving the combined fractions in acetone and precipitating with 10 volumes of ethyl ether. It is of interest that the megalomicin $C_2$ prepared in this way was obtained as a dihydrochloride salt which was formed in the process of column chromatography. The chloroform used in the solvent system may decompose to give rise to hydrogen chloride which can react with megalomicin $C_2$ free base resulting in the hydrochloride derivative. The salt was readily converted to the free base by solution in water, addition of dilute sodium hydroxide and isolation by filtration. Fraction $C_2$ at this stage exhibited a potency of 4,800 units/mg.

Later fractions collected from the column were combined in order to isolate a white powder, rich in megalomicin $C_1$. The remaining active fractions eluted from the silicic acid column were combined and resolution of A and B was effected by Florisil (Floridan Company, Tallahassee, Florida) column chromatography. The column was prepared by activating Florisil at 100°C for 16 hours, fixing in hexane and slurrying into a glass column. A 2.8 g sample of material rich in A and B required a column approximately 105 cm in length and 3.8 cm in diameter and was run at a flow rate of 200 ml/hour, collecting 100 ml fractions. The A and B mixture was dissolved in 10 ml of methylene chloride, loaded on the column, and the latter developed progressively with 700 ml of hexane, 700 ml of ether, 700 ml of ethyl acetate, and increasing amounts of acetone in ethyl acetate. This separation technique was monitored in the same way as was the silicic acid column; *i.e.* testing of the fractions against *S. aureus* followed by thin-layer chromatography.

By combining fractions as indicated in Table 1, megalomicin A was separated from megalomicin B. Both antibiotics were isolated as off-white powders by concentrating combined fractions to dryness. At this stage they displayed potencies of

Fig. 3. Procedure for the isolation and separation of the megalomicin components




about 625 and 305 units/mg respectively in the cylinder cup assay previously mentioned. Each isolated pure component base was assigned a potency of 1,000 mcg/mg. The flow chart in Fig. 3 summarizes the procedures described above.

The megalomicin C fraction, isolated as indicated before, was chromatographed on silicic acid as described above. This chromatogram afforded substantially pure megalomicins $C_1$ and $C_2$, with the latter again being obtained as a dihydrochloride salt. They did retain minor impurities which were not removed by the purification procedures.

The extraction procedures outlined previously permitted an alternative method for the isolation of megalomicin B. After removal of most of the megalomicin complex from the alkalinized acid extract, the latter was further extracted with ethyl acetate. This yielded an extract enriched in megalomicin B which was purified by column chromatography on silica gel. Elution with ether and 1 : 1 acetone-ether, followed by combination of appropriate fractions and crystallization from aqueous acetone gave essentially pure megalomicin B.

## Properties

The megalomicin components were soluble in the usualorg anic solvents such as ethanol, acetone, chloroform and ethyl acetate. Megalomicin A showed slight water solubility, which was considerably enhanced at temperatures near the freezing point. This behavior was also shown by megalomicin B, though to a lesser extent. Megalomicins $C_1$ and $C_2$ showed minimal water solubility.

Acid salts of the megalomicins were freely soluble in water. For example, a water soluble ditartrate salt was prepared by adding a solution of approximately two molar equivalents of tartaric acid in ethanol to an ethereal solution of the megalo-

Table 2. Physical properties of the megalomicins

| | Megalomicin A | Megalomicin B | Megalomicin C fraction | Megalomicin $C_1$ | Megalomicin $C_2$ | Megalomicin $C_2$·2HCl |
|---|---|---|---|---|---|---|
| Melting point (°C) | 255~259° dec. | 135~140° | 225~230° | 238~242° dec. | 146~150° | 174~177° |
| Rotation $[\alpha]_D^{25}$ (EtOH) | −90° | −92° | −104° | −102° | −102° | −84° |
| Molecular weight (benzene) ebullioscopic | 868 | | 913 | 914 | | |
| Microanalysis | Average of 4 | | | Average of 2 | | Average of 3 |
| C | 59.82 | 58.73 | 59.62 | 59.28 | 58.93 | 52.23 |
| H | 9.19 | 8.98 | 8.61 | 8.66 | 8.73 | 7.83 |
| N | 3.31 | 2.74 | 2.92 | 2.90 | 2.88 | 2.62 |
| Cl | | | | | | 5.99 |

micin C fraction and isolating the resulting precipitate by filtration. Similarly a water-soluble ditartrate salt was prepared from megalomicin A. The dihydrochloride salt of megalomicin $C_2$ described above was also readily soluble in water.

Some physical data on the megalomicins are shown in Table 2.

Megalomicins A and B showed a loss of activity at pH 2~4 at room temperature against *Bacillus subtilis*, but were stable from pH 6 to 10 at temperatures up to 100°C. Megalomicin $C_2$ was stable at room temperature for at least 6 hours from pH 2~10 against *B. subtilis*. It was stable at up to 100°C for at least 30 minutes in a range of pH 4~10.

Desosamine was isolated after drastic acid hydrolysis of the megalomicin complex as described by FLYNN *et al.*[6] Desosamine hydrochloride was identified by a comparison with an authentic sample. The presence of desosamine in each of the megalomicin components, A, B, and $C_2$, was shown by a paper chromatographic comparison of their acid hydrolysis products with an authentic sample of desosamine in a butanol-pyridine-acetic acid-water system in a ratio of 6:4:1:3.

Acknowledgements

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