STUDIES ON A NEW ANTITUMOR ANTIBIOTIC, LARGOMYCIN. II

ISOLATION, PURIFICATION AND PHYSICOCHEMICAL PROPERTIES

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Isolation and purification of largomycin from the culture filtrate of *Streptomyces pluricolorescens* MCRL-0367 is described. The crude complex of largomycin was obtained from the culture filtrate by means of precipitation with ammonium sulfate. Three active components were separated by the following procedures: gradient extraction with aqueous ammonium sulfate solution, isoelectric point precipitation, preparative electrophoresis on polyacrylamide gel, AE-collulose column chromatography, gel-filtration on Sephadex G-100 and dialysis with a cellophane membrane. As a result, the most biologically active component, largomycin F-II, was obtained, the homogeneity of which was proved by ultracentrifugation and electrophoretic analyses. Largomycin F-II proved to be a chromoprotein of high molecular weight (25,000) containing a pH-indicator chromophore in the molecule. The most unique characteristic of largomycin F-II, which differentiate it from similar antibiotics is the lack of hexosamine and cysteine in its molecule.

In the course of screening for antitumor antibiotics, a new macromolecular antibiotic complex named largomycin was isolated from the culture filtrate of *Streptomyces puricolorescens* MCRL-0367. Taxonomic studies of the antibiotic-producing strain and antibiotic production have been reported in a previous paper[1]. The present paper gives details of the isolation and purification, which made it possible to separate the largomycin complex into three active components named largomycin F-I, F-II, and F-III, and also gives the physichochemical properties of each component. Antitumor activity against transplantable animal tumors and biological characteristics of the antibiotics will be dealt with in the following paper[2].

Isolation and Purification

As will be mentioned below, largomycin and its active components are macromolecular proteinaceous substances with an unidentified chromophoric moiety, and

are relatively stable at neutral and weakly acidic pH. Considerring these properties, the isolation of largomycin from the culture broth was carried out as follows: The harvested broth was filtered with 2% Celite 545, and ammonium sulfate added to the filtrate to give 0.7 saturation. The filtrate was kept overnight at 4°C to yield an active precipitate which was collected by filtration. The precipitate thus obtained was dissolved in distilled water and dialysed against water with a cellophane membrane. The inner solution was lyophilized to yield crude largomycin complex.

As shown in Fig. 1, when the crude largomycin complex was analyzed by gel-filtration on Sephadex G-100 equilibrated with M/15 phosphate buffer, three major peaks which were named F-I, F-II and F-III were observed. It was noted that the protein and sugar contents exactly run parallelled antimicrobial, anti-HeLa cell and antitumor activities. Of the three fractions, F-II showed the highest biological activity.

The largomycin complex was successfully separated into the three components, F-I, F-II and F-III, by applying the following procedures in succession.

1) Gradient extraction with aqueous ammonium sulfate solution[3].
2) Isoelectric point precipitation
3) Gel-filtration on Sephadex G-100

Fig. 1. Gel-filtration of largomycin complex with Sephadex G-100.

Column Sephadex G-100 10×60 cm
Material Largomycin crude powder 10 g
Elution M/15 Phosphate buffer (pH 7.2)

Broken line: Optical density at 280 mμ (FOLIN's method for protein content)
Chain line: Anti-*Sarcina lutea* activity
Solid line: Optical density at 470 mμ (Phenol-sulfric acid method for sugar content)

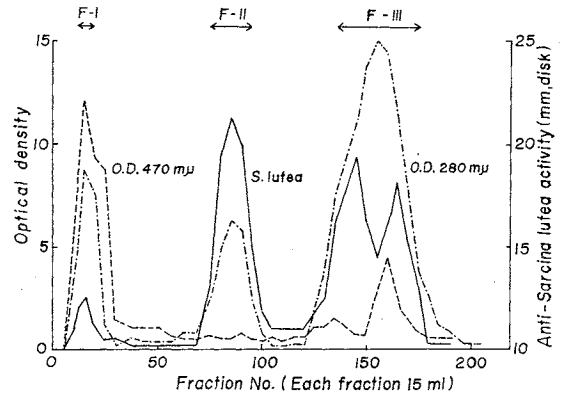


Chart 1. Isolation and purification of largomycin

Fermentation broth
↓ filtered with Celite 545 (2 %)
Filtrate, 2,000 liters
↓ added with Celite 545 (2 %)
↓ pptd. with Celite $(NH_4)_2SO_4$ (70 %)
↓ filtered
Largomycin crude complex, 60 kg
(Content of largomycin complex estimated: about 3,690 g)
↓ extracted with 0.7 satd. $(NH_4)_2SO_4$

Extract
↓ dialyzed
Inner solution
↓ lyophilized
↓ gel-filtration
Largomycin F-I powder 1.0 g

Residue
↓ extracted with 0.5 satd. $(NH_4)_2SO_4$,
↓ pptd. with $(NH_4)_2SO_4$, filtered
↓ Hyflo-super-cell
Largomycin F-II and F-III mixture
↓ washed with 0.7 satd. $(NH_4)_2SO_4$
Residue
↓ extracted with 0.53 satd. $(NH_4)_2SO_4$
Extract
↓ pptd. with $(NH_4)_2SO_4$
↓ filtered with Hyflo-super-cell
Yellowish powder
↓ extracted with 0.5 satd. $(NH_4)_2SO_4$ —M/10 acetate buffer mixture (1 : 1, pH 4.0)

Extract
↓ dialyzed
↓ lyophilized
↓ gel-filtration
Largomycin F-III powder 1.0 g

Residue
↓ extracted with 0.5 satd. $(NH_4)_2SO_4$ —M/10 acetate buffer mixture (1 : 1, pH 6.0)
Extract
↓ dialyzed, lyophilized, gel-filtration
Largomycin F-II powder 1.5 g

As shown in Chart 1, largomycin crude complex obtained from the broth filtrate was first extracted with 0.7 saturated ammonium sulfate solution* to separate the F-I component from the insoluble F-II and F-III components. Largomycin F-I was then dialyzed, lyophilized, and purified through gel-filtration. Thus, largomycin F-I powder was obtained. The F-II and F-III components, which remained undissolved in 0.5 saturated ammonium sulfate solution were extracted with 0.5~0.53 saturated ammonium sulfate solution to free them from other impurities. The yellowish powder recovered from the extract was then treated with pH 4.0 acetate buffer solution and an equal volume of 0.5 saturated ammonium sulfate. Largomycin F-III was soluble and was thus removed from the insoluble F-II component. Largomycin F-III in the extract was recovered as a brown powder by dialysis, lyophilization, and gel-filtration. Largomycin F-II was extracted with a 1 : 1 mixture of pH 6.0 acetate buffer solution and 0.5 saturated ammonium sulfate, and recovered as a light brown powder.

Sephadex G-100 gel-filtration analyses of the samples thus obtained revealed that the each component was still contaminated with small amounts of the other fractions. However, repetition of Sephadex G-100 gel-filtration of each component yielded components F-I, F-II and F-III in an almost pure state.

Chromatography utilizing an ion-exchanger was the most suitable procedure for further purification of largomycin F-II. The light brownish powder containing impure largomycin F-II (1 g) was dissolved in 0.01 M phosphate buffer (pH 7.0) and dialysed in a cellophane tube against the same buffer overnight in a cold room. The inner solution was applied to an AE-cellulose column (2.4×47 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.0). The column was first washed completely with the same buffer and then developed with a linear gradient of phosphate buffer (pH 7.0) of concentrations ranging from 0.01 to 0.2 M. Eluates were scanned at 280 mμ and the sugar content in each eluate was determined by the phenol-sulfuric acid method[5] at 470 mμ. As shown in Fig. 2, the fractions which were eluted with buffer of around 0.07 M showed a maximum absorbancy at 280 mμ and had an $O.D._{280}/O.D._{470}$ ratio of 7~10. These fractions (A_1-III) were combined and dialysed in a cellophane tube against water overnight in a cold room. The

Fig. 2. Chromatography of largomycin F-II on AE-cellulose column.

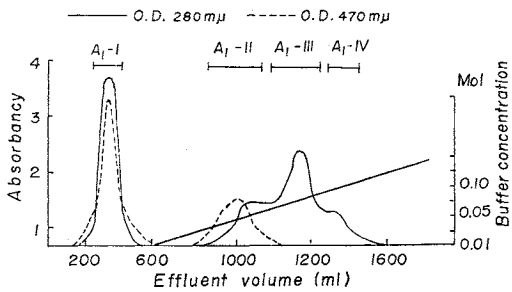
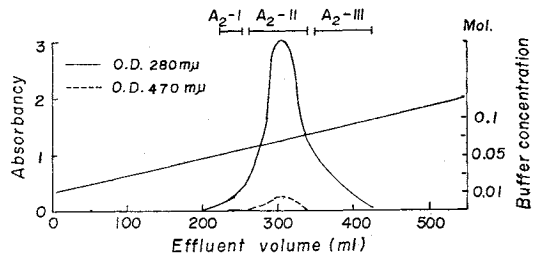


Fig. 3. Rechromatography of largomycin F-II on AE-cellulose column.



* Ammonium sulfate solutions used in the present experiment were prepared by HOFFMEISTER's method.

Fig. 4. Gel-filtration of largomycin F-II on Sephadex G-100 column.

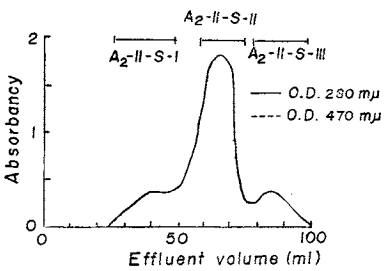


Fig. 5. Isoelectric focussing pattern of largomycin F-II.

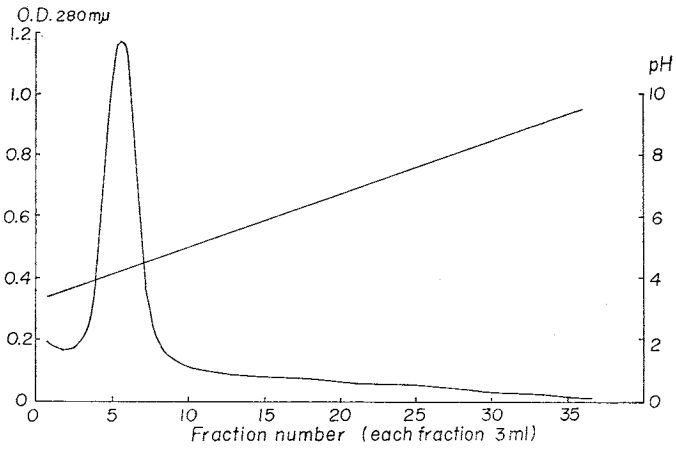
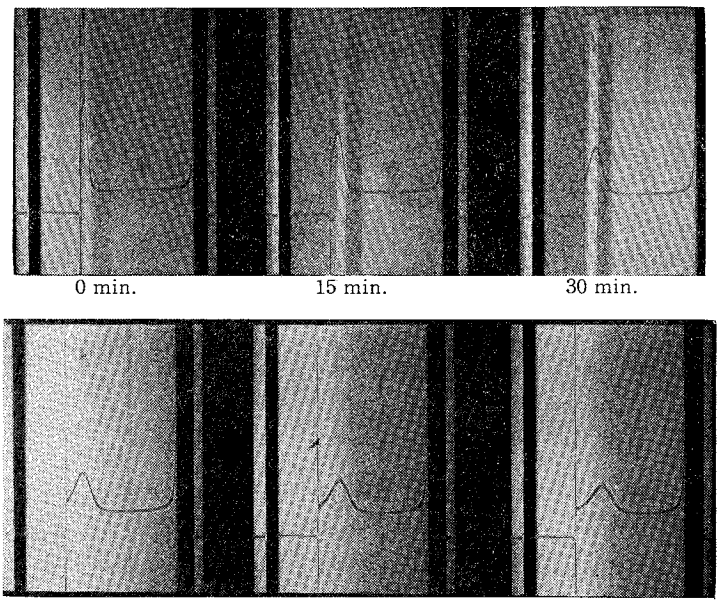


Fig. 6. Sedimentation pattern of largomycin F-II at 60,000 r.p.m. (synthetic boundary cell) *sw*. 20, 2, 2.



dialyzed solution was refractionated by the same AE-cellulose gradient chromatography (1.5×57 cm) (Fig. 3). The fractions which showed maximum absorption at 280 mμ (A_2-II) were combined and lyophilized without dialysis. The lyophilized (A_2-II) fraction was then redissolved in 5 ml of water and chromatographed with water on Sephadex G-100 in a 2.4×47 cm column (Fig. 4). Pure largomycin F-II was recovered by lyophilizng fractions from the second peak (A_2-II-S-II) with the maximum absorption at 280 mμ. A_2-II-S-I fraction was less biologically active and contained minor components which could be detected by electrophoretic analyses in polyacrylamide gel[6]. The last component, A_2-II-S-III, contained strongly bound phosphate which could not be removed by repeated gel filtration chromatography on Sephadex G-100 and G-75. The last component, however, showed no biological activity.

Further purification of largomycin F-II fraction (A_2-II-S-II) was accomplished by preparative electrophoresis using polyacrylamide gel*. The acrylamide gel column was extruded from the apparatus, and the zone containing the active principle was cut into small pieces and extracted with distilled water. The active extract was

* This apparatus was devised by one of the authors (T. KASHIDA) in our laboratory and will be described elsewhere.

dialysed against water and lyophilized to yield a powder, which was again subjected to gel filtration, dialysis and lyophilization. The final product was a pale yellow amorphous powder, the homogeneity of which was proven by electrofocussing analysis[4] and also by ultracentrifugation as shown in Figs. 5 and 6 respectively.

Physicochemical Properties

Physicochemical properties of largomycin F-I, F-II and F-III are summarized in Table 1. Whereas largomycin F-II was homogenous, largomycin F-I and F-III were still contaminated with minor impurities.

Purified largomycin F-II is an acidic protein with an isoelectric point at pH 4.2[4] (Fig. 5). The molecular weight of largomycin F-II was estimated to be about 25,000 by the ARCHIBALD method,[7,8] and from amino acid analysis. The value was also supported by the sedimentation constant of about 2.2 S and by gel-filtration utilizing ANDREW's procedure[9].

As shown in Fig. 7, the infrared spectra of largomycin components in KBr tablets were quite similar to each other and were typical of a polyamino-polyoxy compound. As shown in Fig. 8, the ultraviolet absorption spectrum of largomycin F-II showed a maximum at 278 mμ ($E^{1\%}_{1cm}$ 19.4) in 0.1 N HCl and 540 mμ ($E^{1\%}_{1cm}$ 4.0) in 0.1 N NaOH solution. The latter suggested the presence of a chromophoric moiety in largomycin F-II molecule.

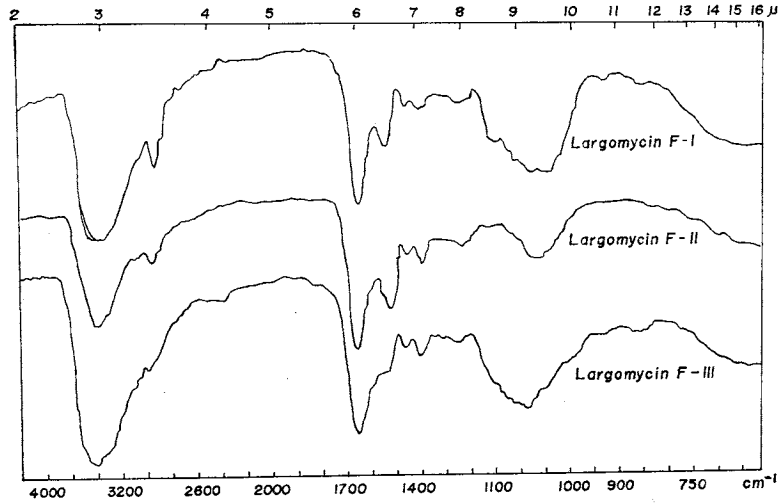
The pH-indicator constituent in largomycin F-II which could not be removed by Sephadex chromatography or dialysis in cellophane tube against distilled water was

Table 1. Physicochemical properties of largomycin

Properties	Largomycin F-I	Largomycin F-II	Largomycin F-III
Nature	Acidic, pale yellow amorphous powder	Acidic, yellow amorphous powder	Brown amorphous powder
Molecular weight	50,000*	25,000	20,000~10,000*
Melting point (decomp.)		no definite	
Isoelectric point		pH 4.2	
Elementary analysis, found (%)	C: 47.93 H: 7.46 N: 6.50 S: 0.82	47.83 8.30 13.63 0.25	46.79 6.57 8.64 1.08
UV absorption max. in 0.1 N HCl max. in 0.1 N NaOH	275 mμ	278 mμ 540 mμ	275 mμ 425 mμ
Sugar content (Phenol-H_2SO_4)	56±1 %	3±1 %	22±1 %
Sugar identified	Glucose	Glucose	Glucose
Amino acid analysis (F-II)	Lys, Arg, His, Asp. Glu, Thr, Ser, Pro, Gly, Ala, Val, Leu, Ileu, Try, Tyr, Phe, Met		
Color reaction Positive	Ninhydrin, Biuret, Xanthoprotein, SAKAGUCHI, MOLISCH, Anthrone, Phenol-H_2SO_4, FOLIN-CIOCALTEU		
Negative	ELSON-MORGAN		
Precipitation	PPtn. with 50 % $(NH_4)_2SO_4$, 50 % $MgSO_4$, 50 % $ZnCl_2$, 20 % T.C.A., 5 % rivanol		

* These values were obtained by Sephadex gel filtration method.

Fig. 7. Infrared spectra of largomycin F-I, F-II and F-III.



extracted with glacial acetic acid from F-II as in the case of plurallin[10].

The glacial acetic acid extract was dried *in vacuo* to yield pigment mixtures which were dissolved in chloroform and the chloroform solution was extracted with acidic water (pH 5.0, HCl). The aqueous layer was lyophilized to yield the crude hydrochloride of the pigment which was again dissolved into a small amount of chloroform. The chloroform solution was applied to an alumina column, developed with chloroform - methanol (95 : 5). The eluates were tested for activity against HeLa cells and those in a single peak were combined and evaporated *in vacuo* to yield a yellowish brown hydrochloride. To obtain the free base of the pigment, the aqueous solution of the hydrochloride was extracted with chloroform at pH 7.5. The chloroform layer was concentrated *in vacuo* and precipitated with petroleum ether to yield a yellowish powder of the free base. This did not show any clear melting point up to 270°C. The ultraviolet absorption spectrum showed absorption maxima at 244 mμ ($E^{1\%}_{1cm}$ 556), 275 mμ ($E^{1\%}_{1cm}$ 380) and 433 mμ ($E^{1\%}_{1cm}$ 87) in methanol or methanol - 1 N HCl (9 : 1) solution and 255 mμ ($E^{1\%}_{1cm}$ 151) and 550 mμ ($E^{1\%}_{1cm}$ 56) in methanol - 1 N NaOH (9 : 1) solution (Fig. 9). In the infrared absorption spectrum, the major bands were at 3390, 2920, 1660, 1470, 1325, 1270, 1090 and 850 cm^{-1} (Fig. 10). Elementary analysis gave following values: C 62.83, H 6.58, N 3.23%. Thin-layer chromatography on silicagel with the solvent system EtOH - NH_4OH - H_2O (8 : 1 : 1) and on aluminum oxide with the solvent system *n*-BuOH - MeOH - H_2O (4 : 1 : 2), gave Rf values of 0.95 and 0.74 respectively. The pigment showed anti-HeLa cell activity at a concentration of 0.2 mcg/ml and also antitumor activity against EHRLICH

Fig. 8. Ultraviolet absorption spectra of largomycin F-II.

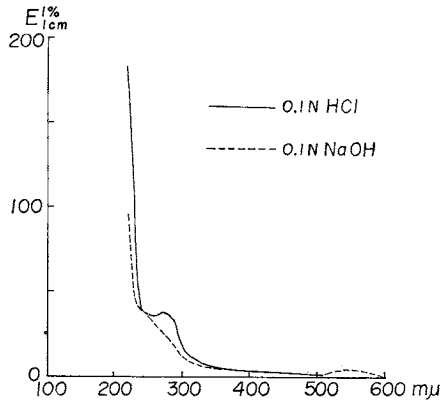


Fig. 9. Ultraviolet absorption spectra of largomycin F-II pigment.

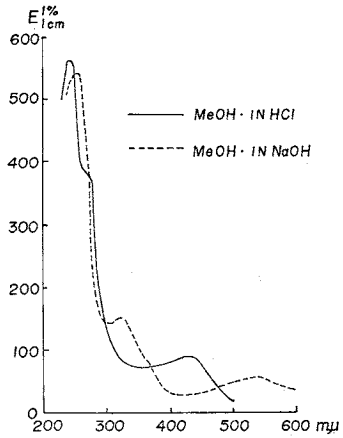
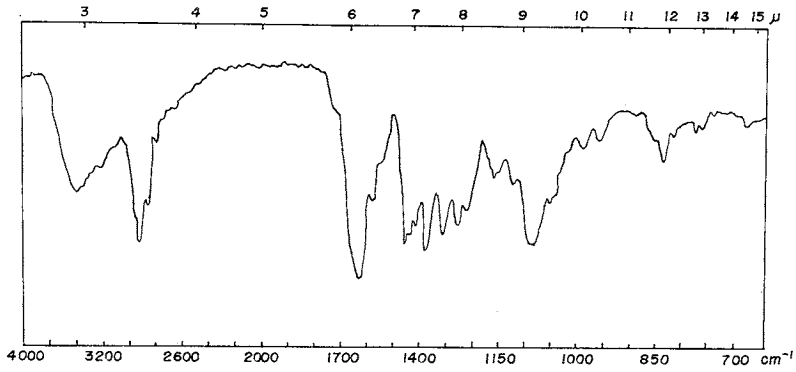


Table 2. Amino acid composition of largomycin F-II

Amino acid	mol. ratio
Lysine	6
Histidine	2
Arginine	5
Aspartic acid and asparagine	12
Threonine	8~9
Serine	7
Glutamic acid and glutamine	17
Proline	4~5
Glycine	11~12
Alanine	16
Cystine	0
Valine	10~11
Methionine	1
Isoleucine	4~5
Leucine	11~12
Tyrosine	2
Phenylalanine	5
Tryptophan*	4~5

* The content of tryptophan was determined by a colorimetric method[13] and an ultraviolet absorption method[14].

Fig. 10. Infrared spectra of largomycin F-II pigment.



ascites carcinoma at 1.6 mg/kg. In infrared and ultraviolet spectra, the chromophore pigment of largomycin F-II was similar to that of iyomycin B_1 [15]. Although some discrepancy was observed on elementary analysis, the difference between largomycin pigment and iyomycin B_1 is not certain in this moment.

Table 2 illustrates the amino acid composition of largomycin F-II determined after hydrolysis with constant boiling hydrochloric acid at 110°C for 24 hours in an evacuated tube. The content of tryptophan was determined by a colorimetric method[13] and by ultraviolet absorption[14]. It was characteristic that cysteine was not detected in largomycin F-II hydrolyzate. Amino acid analyses were also attempted on the hydrolyzates of largomycin F-I and F-III fractions. In these cases, cysteine was not detected as well.

Hydrolyses to examine for neutral sugars and amino sugars were carried out with 0.24 N hydrochloric acid at 100°C for 18 hours and 4 N hydrochloric acid at 100°C for

4 hours respectively. The sugar components in the acid hydrolysate of largomycin F-II were analysed by paper chromatography* and by gas chromatography of pertrimethylsilyl derivatives.** Glucose and traces of galactose, mannose and xylose were found. However, no amino sugar was detected.

Discussion

Three antitumor components, largomycin F-I, F-II and F-III, were obtained from the culture filtrate of *S. pluricolorescens* MCRL-0367. The most biologically active component F-II was obtained in a pure form. The physicochemical properties of largomycin F-II suggest that it is a member of the chromoprotein group of antitumor antibiotics. From published data, plurallin[10], iyomycin[11], and prunacetin[12] seem to be related to largomycin F-II that they contain a pH-indicator chromophore. However, prunacetin is apparently different from largomycin F-II in the physicochemical characteristics of its protein moiety and chromophore.

Since plurallin was isolated from the same species of Streptomyces as the largomycin-producing strain, the antibiotics were compared most carefully. They could be differentiated from each other in the following points. Unlike largomycin F-II, plurallin gave a positive ELSON-MORGAN test. After acid hydrolysis plurallin gave cysteine which was not obtained from largomycin F-II. Furthermore, the pH indicator pigment extracted from largomycin by the same method as used with plurallin had a different infrared spectrum from the indicator prosthetic group of plurallin.

Since the iyomycin A complex reported in the literature seems to be a mixture and no details are reported on the prosthetic group of this antibiotic, a precise comparison of these antibiotics is difficult at present. However, iyomycin A could be differentiated from largomycin F-II by the fact that largomycin F-II does not contain cysteine.

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* Solvent systems: BuOH - AcOH - H_2O (4 : 1 : 4), AcOEt : *n*-PrOH : H_2O (57 : 32 : 13), and AcOEt : H_2O : pyridine (4 : 4 : 2).
Spray reagents: Benzidine, ninhydrin, TTC and ELSON-MORGAN reagents.
** Apparatus: Shimazu Model GC-1C
Column: 1.5 % SE-30 on Chromosorb W, 1.5 m.
Condition: Carrier gas N_2, column temp. 250°C

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