ISOLATION OF THREE MAIN COMPONENTS, F_3, F_4 AND F_5, FROM AZALOMYCIN F-COMPLEX

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Isolation of azalomycins F_3, F_4 and F_5 from azalomycin F-complex crystals was successfully conducted. F_3 was less soluble in methanol than other components and was isolated by fractional crystallization of F-complex from 80~100 % methanol in water. F_4 and F_5 were separated by P-cellulose column chromatography, from which F_5 and F_4 were eluted with 60 % aqueous methanol and 0.01 M ammonium acetate in 60 % aqueous methanol, respectively. Determination of physico-chemical as well as biological properties pointed to a very close resemblance of the structures of these components.

As described in a previous paper[1], azalomycin F, which behaved as a single compound on paper chromatography[2], was found to be a complex of at least 5 components being separated on thin-layer chromatography (TLC).

The present report describes a satisfactory isolation of three main components, F_3, F_4, and F_5, from the azalomycin F-complex by fractional crystallization and by P-cellulose column chromatography. Physico-chemcal and biological properties of the isolated components are also presented.

Experimental

Separation of azalomycin F components on TLC: Thin-layer plates were prepared in the usual manner with Silica Gel G (E. Merck AG, Darmstadt). Azalomycin F samples were dissolved in 80 % aqueous methanol (10 mg/ml) and 2~5 μl of the solutions were applied to the plates. The plates were developed for 3 hours at 20°C by the ascending method using the upper phase of a solvent system of *sec*-butanol - 0.1 M phosphate buffer, pH 6.0, (2 : 1). For detection of azalomycin F by charring, the plates were sprayed with 6 N sulfuric acid and heated at 120°C. Quantitative determination of each component on TLC was performed by densitometry. The instrument used to scan the plates was an Atago Densitometer Model 80-2 with a slit 0.5 mm wide and 6 mm long. Bioautography of the chromatoplates after removal of the solvent at 60°C was effected by burying them into agar plates seeded with *Candida albicans* YU 1200.

Isolation of F_3 by fractional crystallization: Azalomycin F-complex, Lot AL-19-1 375 g, was dissolved in a mixed solvent of 750 ml of methanol and 1,000 ml of ethylcellosolve at 60°C. By standing at room temperature azalomycin F, mainly composed of components F_3 and F_4, was crystallized. After five times repeated crystallizations of this mixture from 1,000~5,000 ml portions of 70~90 % aqueous methanol, 77 g of azalomycin F crystals containing 85.7 % of the component F_3 were obtained. The balance of approximately 14 % was represented by F_4. For further purification 10 g of this product were recrystallized from 300 ml of 90 % aqueous methanol followed by further two successive recrystallizations from 2,000 ml of methanol. Two grams of crystals, giving a single spot of F_3 on

TLC, were obtained by concentration *in vacuo* of this methanol solution to 200 ml.

Isolation of F_4 and F_5 on a P-cellulose column: The column (2×62 cm) was made up using a slurry of P-cellulose (Serva, 0.79 meq/g, H^+ form) in 60 % aqueous methanol. A solution of 10.8 g azalomycin F-complex, Lot AS-7, in 500 ml of 60 % aqueous methanol, was applied to the column and elution was carried out at a flow rate of 0.28 ml/min with 60 % methanol, followed by 0.01 M ammonium acetate in the same solvent. Fractions, each 10 ml, were collected. All operations were performed at 26°C. The amount of azalomycin F in the effluent was quantitatively determined by measurement of the characteristic UV-absorbancy of azalomycin F at 240 mμ. The components of each fraction were detected on TLC. Fractions mainly containing F_4 or F_5 were combined and reduced in volume to approximately $^2/_3$ at 60°C *in vacuo* to crystallize F_4 or F_5 respectively. Both components were recrystallized from 40 % aqueous methanol to give materials with a single spot on TLC.

Solubility of azalomycin F components in methanol: Twenty milligrams of powdered crystals of the F-complex and of each single component were suspended in graded concentrations of aqueous methanol and vigorously stirred for 2 minutes. Insoluble residues were precipitated by centrifugation, and the azalomycin F content in the supernatants was determined by UV-absorbancy.

Antimicrobial spectra: An agar or liquid dilution technique was used to determine the minimal inhibitory concentrations (MIC) of azalomycin F components against various microorganisms. The antibiotics were dissolved in 50 % aqueous acetone and diluted with water or 5 % aqueous acetone to prepare serial 2-fold dilutions. The final concentration of acetone in the medium was adjusted to 0.5 %. The test organisms were previously grown at 25°C for 10 days (fungi), at 30°C for 24 hours (yeasts), or at 37°C for 1~4 days (bacteria). After inoculation the plates were incubated for 4 days at 26°C for fungi and yeasts and for 2 days at 37°C for bacteria. *Trichomonas vaginalis* grown in V.F. bouillon at 37°C for 3 days was inoculated (4 % inoculum size) into ASAMI's medium with azalomycin F and incubated at 37°C for 3 days. The lowest concentration of azalomycin F at which no moving cell was observed under the microscope was designated as MIC.

Stability of azalomycin F components: The heat stability of azalomycin F components was tested in buffer solution with varying pH values. To each sample, 1,280 μg/ml, in 50 % *iso*-propanol was added the same volume of MCILVAINE's buffer, pH 2.6~8.0, or M/50 Na_2CO_3-$NaHCO_3$ buffer, pH 10.0. After heating at 28°C or 60°C for 1 hour or at 98°C for 30 minutes, the pH of the solutions was readjusted to 6.0 with M/25 Na_2HPO_4 or M/50 citric acid in 25 % *iso*-propanol followed by dilution with 25 % *iso*-propanol in MCILVAINE's buffer, pH 6.0, to prepare solutions of the antibiotic containing 80 and 20 μg/ml (estimated) for determination of the remaining activity by the cylinder-plate method. *C. albicans* YU 1200 was used as the test organism.

Effect of serum on the antibiotic activity: The effect of serum on the antibiotic activity was examined by turbidimetry using *C. albicans* YU 1200 as the test organism. Each 1 ml of horse serum and of 2-fold dilutions of azalomycin F samples in 2.5 % aqueous acetone (range over 0.9~125 μg/ml) were added to 3 ml of SABOURAUD's medium seeded with 3 % of a culture previously grown at 37°C for 18 hours (optical density at 530 mμ was 0.022). After incubation at 30°C for 18 hours the optical density at 530 mμ was recorded.

Results and Discussion

Solubilities of azalomycin F components in various concentrations of methanol in water are shown in Fig. 1. In general, a marked difference in solubility was noted at methanol concentration between 40 and 60 %. This difference in solubility was therefore applied to the recrystallization of azalomycin F components from methanol.

Fig. 1. Solubility of azalomycin F-compelx (AS-3) and components in methanol-water.

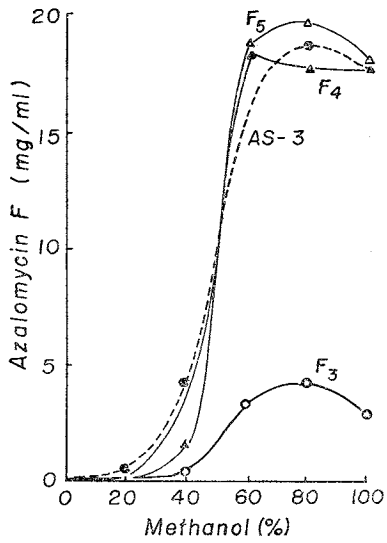
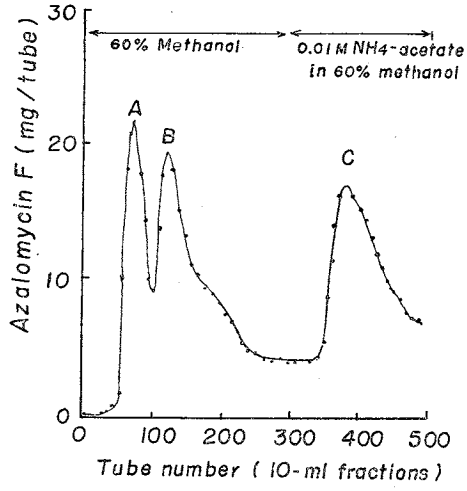
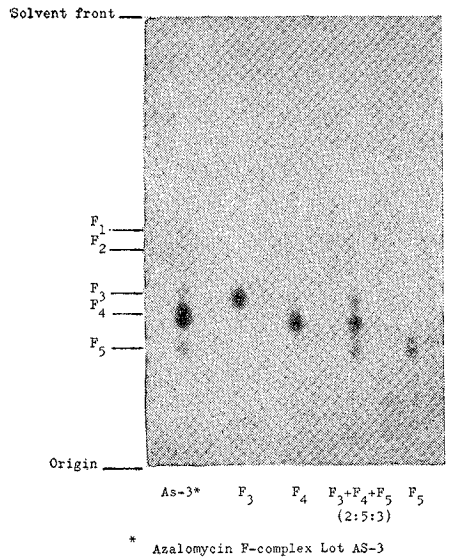


Fig. 2. Elution of azalomycins F_4 and F_5 from a P-cellulose column.



Among three main components the solubility of F_3 in 60~100 % methanol was approximately $^1/_5$ of that of the other two components, which made it possible to crystallize component F_3 preferentially from a 80~100 % methanol solution of the azalomycin F-complex.

Fig. 3. TLC of azamloycin F-complex* (AS-3) and components



Although the chemical structure of azalomycin F is not yet revealed, it was known to be a weakly basic antibiotic being adsorbed on strongly acidic ion-exchangers such as Dowex-50 but not on weakly acidic ones such as IRC-50 (both in the H^+ form). Since a degradation of azalomycin F was observed on its elution from Dowex-50, P-cellulose, a cation exchanger not so strongly acidic was chosen to separate the azalomycin F-complex. But even in this case a slight degradation of azalomycin F could not be avoided on prolonged elution with 0.01 M ammonium acetate in 60 % aqueous methanol. However, separation of F_4 and F_5 was successfully achieved when an excess of F-complex was applied to the column, on which components F_1 to F_4 were adsorbed and F_5 and a part of F_4 were passed through. Fig. 2 shows one of the elution patterns described above.

Section A represents material readily eluted from the column and was identified as F_5 on TLC. The fractions of tube No. 51 to 80 were combined and concentrated *in vacuo*. Recrystallization from 40 % aqueous mathanol yielded 148 mg of F_5. F_4 was prominent in section B but its separation from F_5, contaminating in these frac-

tions, could not readily be achieved merely by recrystallization from methanol. Section C, the eluate with 0.01 M ammonium acetate, was mainly composed of F_4 and 200 mg of F_4 were obtained by concentration of fractions, tube No. 361~410, followed by recrystallization from 40 % methanol. By further elution with the same solvent most of the azalomycin F was recovered in the effluent, but separation of components F_1 to F_3 in these fractions was not successful because of partial degradation of the antibiotic. Separation of each component of azalomycin F-complex on TLC is illustrated in Fig. 3. By quantitative determination of each component of different fermentation lots of azalomycin F-complex by TLC densitometry, the ratio of each component in the F-complex was found to be fairly constant, giving 2~3 : 5~6 : 1~3 for the three main components, F_3, F_4 and F_5. Bioautography of TLC plates revealed the existence of antimicrobial activity of all five components.

Physico-chemical properties of the isolated components are summarized in Table 1. No distinct difference was observed among F_3, F_4 and F_5 in these properties, pointing to a close resemblance of the structures of these three components. Fig. 4 shows infrared spectra of the F_3, F_4 and F_5 components, in which some discrepancies were demonstrated especially within the range of 1610~1730 cm^{-1}. Slight differences were observed in absorption intensity of three bands at 1670, 1710 and 1730 cm^{-1} which might be attributed to carbonyl or double-bond vibrations.

The antimicrobial activity of F_3 against *C. albicans* YU 1200, determined by the cylinder-plate method, proved to be slightly stronger than that of the other two

Fig. 4. Infrared spectra of azalomycins F_3, F_4 and F_5 (KBr).

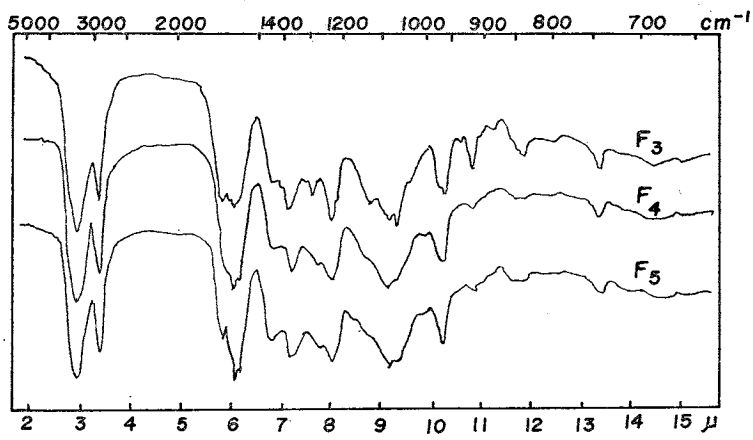


Table 1. Physico-chemical properties of azalomycin F components.

	M. P. (dec.)	$[\alpha]_D^{27}$ **	Elementary analysis (%)			UV-absorption ($E_{1cm}^{1\%}$)		
			C	H	N	240 mμ	258 mμ	268 mμ
AS-3*	125~126°C	+35°	60.88	8.88	3.46	385	211	229
F_3	132~133	+35	60.41	8.80	3.54	372	204	219
F_4	131~132	+39	61.51	8.90	3.75	395	209	228
F_5	125~126	+44	62.27	8.94	3.88	387	203	224

* Azalomycin F-complex Lot AS-3. ** C=1 in 60 % methanol.

Fig. 5. Stability of azalomycin F-complex (AS-3) and components.

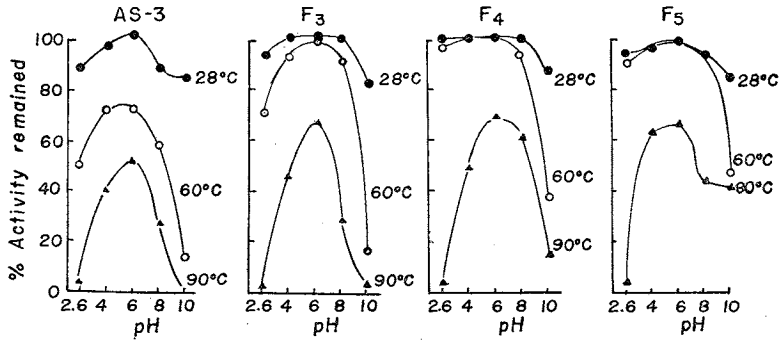


Table 2. Minimal inhibitory concentrations (MIC) of azalomycin F components.

Test organism	Medium	MIC μg/ml			
		AS-3*	F_3	F_4	F_5
Bacillus subtilis PCI 219	GB	6.25	12.5	12.5	12.5
Staphylococcus aureus 209P		12.5	6.25～12.5	25.0	12.5～25.0
Sarcina lutea		6.25	6.25	12.5	12.5
Corynebacterium xerosis		6.25	6.25	12.5	12.5
Mycobacterium smegmatis ATCC 607		12.5～25.0	25.0	25.0	25.0
Escherichia coli NIHJ		>100	>100	>100	>100
Pseudomonas aeruginosa		>100	>100	>100	>100
Candida albicans YU 1200	S (PS)	1.56～3.12	1.56	1.56～3.12	1.56～3.12
Saccharomyces cerevisiae		6.25	1.56～3.12	1.56	6.25
Torula utilis		3.12～6.25	1.56～3.12	3.12～6.25	6.25
Cryptococcus neoformans		1.56	1.56	1.56	1.56～3.12
Kloeckera africana		1.56	1.56	1.56	1.56
Trichophyton asteroides		3.12 (0.78)	3.12 (1.56)	3.12 (0.78)	3.12 (1.56)
Trichophyton interdigitale		6.25	1.56	1.56～3.12	1.56～6.25
Aspergillus oryzae		12.5	6.25	12.5	12.5
Aspergillus niger		12.5	6.25～12.5	12.5～25.0	12.5～25.0
Penicillium notatum		3.12～6.25	1.56～3.12	6.25	6.25
Piricularia oryzae	PS	0.78～1.56	0.78～1.56	0.78～3.12	0.78～3.12
Ophioborus miyabeanus		0.78	0.78	0.78	1.56
Alternaria kikuchiana		3.12	1.56	1.56	3.12
Sclerotinia libertiana		0.78～1.56	0.78～1.56	0.78～1.56	0.78～1.56
Fusarium lycopersici		1.56～3.12	3.12	3.12	3.12
Fusarium lini		1.56～3.12	1.56～3.12	1.56～6.25	1.56～6.25
Ceratostomella fimbriata		3.12～6.25	1.56	1.56	3.12
Trichomonas vaginalis	A	25.0	12.5	12.5	25.0

* Azalomycin F-complex Lot AS-3.
GB : Glycerol-bouillon, S : SABOURAUD's dextrose agar, PS : Potato-sucrose agar, A : ASAMI's medium.

components. Assuming a unit antibiotic activity of the F-complex against *C. albicans* as 100 %, the relative potencies of the components F_3, F_4 and F_5 were 155, 90.5 and 92.8 %. In addition, an admixture of F_3, F_4 and F_5 at a ratio of 1 : 2 : 1 revealed 108 % activity of the F-complex being in agreement with the value calculated from the relative activities of these components, $(155\,\% \times 1 + 90.5\,\% \times 2 + 92.8\,\% \times 1) \times {}^1/_4 = 107\,\%$. From

this result it is concluded that the action of these components is additive, rather than synergistic or antagonistic.

A comparison of the minimal inhibitory concentrations of F_3, F_4 and F_5 components with the MIC of the F-complex is shown in Table 2. Similar to the results described for the azalomycin F-complex in the previous papers[1,2], all three components were also effective against bacteria, yeasts, fungi and protozoa. The MICs of each component against these organisms were almost the same as those of the F-complex. However, the antibiotic activity of F_3 against some organisms proved to be slightly stronger than that of F_4 and F_5.

As shown in Fig. 5, F_3, F_4 and F_5 as well as the F-complex were stable at 28°C within the pH range of 2.6~10.0. The antibiotics were also heat stable at neutral reaction but rather labile in acidic and alkaline solutions, especially in the latter. More than 90 % of the initial activity of all three components still remained even after heating at 60°C for 1 hour at pH 4 to 8. It is of interest to note that either of the three single components was slightly more stable than the F-complex.

For a comparison of the effect of serum on the activity of each component, we determined the concentration of the antibiotics necessary to reduce the optical density of *C. albicans* cultures to a value of 0.150 because in this region of optical density we found a linear relationship between the logarithm of azalomycin F concentration and the growth of the test organism. Without the addition of serum, the concentrations of the F-complex, F_3, F_4 and F_5 necessary to get an optical density of 0.150 were 1.23, 0.64, 1.15 and 1.40 μg/ml. In the presence of serum, concentrations of 13.0, 14.0, 18.0 and 14.0 μg/ml of these components were required to get the same growth retardation of *C. albicans*. This indicates that the antibiotic activities of all azalomycin F components are similarly affected by serum.

In conclusion, the results of our experiments demonstrate that azalomycins F_3, F_4 and F_5 are very closely related in their physico-chemical and biological properties, even though they can be separated on TLC.

References

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