THE ANTIBIOTIC YA-56 COMPLEX : ISOLATION, PURIFICATION AND PHYSICOCHEMICAL PROPERTIES OF THE MAIN COMPONENTS

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Isolation, purification and physicochemical properties of the antibiotic complex YA-56, a family of phleomycin-bleomycin group, are described. The antibiotic YA-56 complex was found to be composed mainly of two active components named YA-56 X and Y. These components were clearly differentiated from the reported phleomycins and bleomycins by their physicochemical properties. However, by direct comparison, YA-56 X was found to be identical with zorbamycin reported recently. YA-56 Y was recognized to be a new antibiotic.

In the course of the screening of new antibiotics, an antibiotic complex named YA-56 belonging to the phleomycin-bleomycin group antibiotics was isolated from the culture filtrate of a streptomyces. The taxonomic studies and the antibiotic production by the YA-56 producing strain, *Streptomyces humidus* var. *antitumoris*, were reported in the preceeding paper[1].

Antibiotic YA-56 was mainly composed of two active components named YA-56 X and Y, of which isolation and characterization have already been communicated briefly[2]. The present paper deals with the experimental details of the isolation, purification and physicochemical properties of YA-56 X and Y.

Examination of chemical constituents of YA-56 X and Y achieved through their degradation products will be dealt with in the succeeding paper[3]. Details of the biological properties of YA-56 X and Y will be reported later.

Preparation of YA-56 X and Y

YA-56 was recovered from the filtered broth by the procedure outlined in Chart 1. The active principle in the filtrate was first isolated as a bluish powder by an adsorption procedure on a high porous polymer (Duolite S-30) followed by alumina chromatography. By paper chromatography (Fig. 3a and 3b), the bluish powder thus obtained was found to be mainly composed of two active components named X and Y. Separation of X and Y components was effectively achieved by gel filtration on acid pre-treated Sephadex G-15[4]. After separation, YA-56 X and Y were respectively purified by Sephadex LH-20 gel filtration and chromatographically homogenous bluish powders were obtained. The bluish powders which contained cupric ion as a

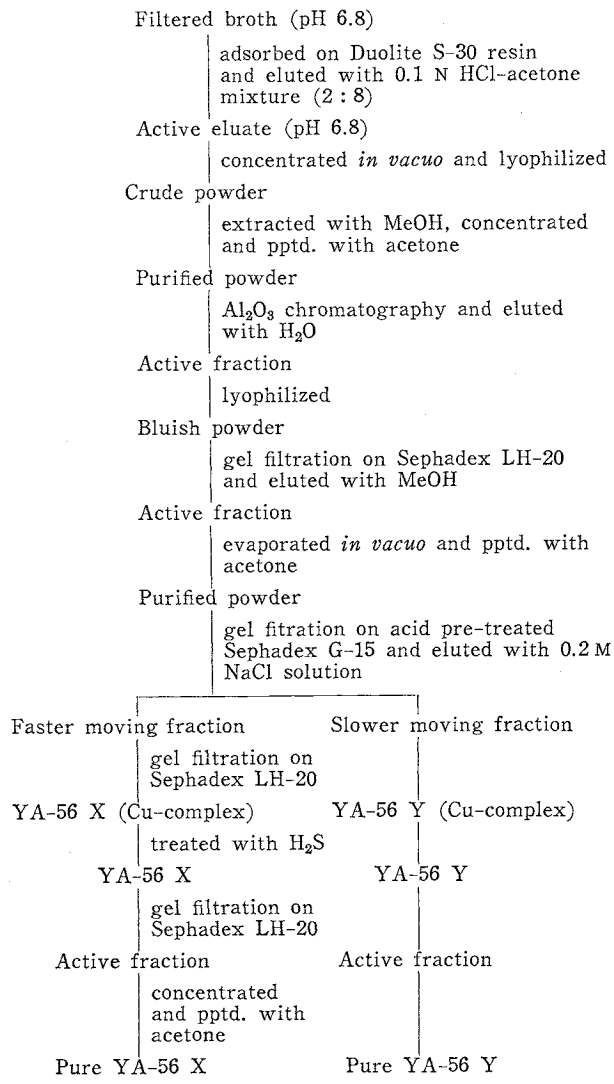
chelating metal were then treated with hydrogen sulfide. Thus, being freed from cupric ion, each component was obtained as a white amorphous powder.

Physicochemical Properties of YA-56 X and Y

Some of physicochemical properties (m.p., analytical data, UV maxima, ORD data and Rf values) of YA-56 X and Y are summarized in Table 1, together with those of their Cu-complexes. Judging from the elemental analysis data, YA-56 X and its Cu-complex must be present as their hydrochlorides.

The infrared spectra of YA-56 X and Y (Fig. 1 a) were quite similar to each other and indicated the presence of OH/NH at 3400~3200, carbonyl at 1700~1690 (shoulder) and amide carbonyl at 1645~1640 (amide I) and 1545 cm^{-1} (amide II). YA-56 X and Y Cu-complexes showed similar infrared spectra (Fig. 1 b) to those of YA-56 X and Y. The UV spectra of YA-56 X, Y and their Cu-complexes are shown in Fig. 2 a and 2 b.

Chart 1. Preparation of YA-56 X and Y.



YA-56 X and Y were positive to MOLISH and anthrone tests, indicating the presence of carbohydrate constituent(s) in the molecule. DRAGENDORFF, EHRLICH and PAULY color reactions were positive, while the ninhydrin, ELSON-MORGAN, SAKAGUCHI and biuret reactions were negative.

YA-56 X and Y, as well as their Cu-complexes, were soluble in water, methanol, dimethylformamide and dimethylsulfoxide, slightly soluble in ethanol and insoluble in propanol, butanol, acetone, ethyl acetate, chloroform, benzene, ether, petroleum ether and dioxane.

Comparison of YA-56 with Phleomycin, Bleomycin and Zorbamycin

From the above-described physicochemical properties (IR, UV, color reactions and the chelating property) and the biological properties, YA-56 X and Y seemed to be

Table 1. Physicochemical properties of YA-56 X and Y.

	YA-56 X		YA-56 Y	
	YA-56 X Hydrochloride (Cu-complex)	YA-56 X Hydrochloride	YA-56 Y (Cu-complex)	YA-56 Y
Melting point	195~200°C (dec.)	198~202°C (dec.)	190~197°C (dec.)	188~197°C (dec.)
Elementary analysis*a) (%)	C 43.24 H 4.65 N 17.28 S 4.70 Cl 4.86 Cu 4.34	C 45.41 H 5.82 N 17.60 S 4.64 Cl 4.69	C 42.22 H 5.51 N 14.64 S 3.56 Cl — Cu 2.74	C 42.85 H 5.55 N 14.64 S 3.57 Cl —
Molecular weight*b)	641	559	not measured	
UV : $\lambda_{max}^{H_2O}$ nm, ($E_{1cm}^{1\%}$)	246.5 (126.6) &300~303 (45.6)	234 (155.9) &295 (36.9)	245.5 (134.0) &302.0 (47.0)	240~241 (117.5) &297 (36.4)
ORD (H_2O)	positive cotton curve $[\alpha]_{620}$+217.6 (peak) $[\alpha]_{589}$+151.5 (D-line) $[\alpha]_{560}$ 0.0 $[\alpha]_{516}$−109.6 (trough) (*c* 0.363)	positive cotton curve $[\alpha]_{589}$+ 20 (D-line) $[\alpha]_{330}$+ 90 (peak) $[\alpha]_{296}$ 0.0 $[\alpha]_{272}$−360 (trough) (*c* 0.05)	positive cotton curve $[\alpha]_{615}$+187.5 (peak) $[\alpha]_{589}$+134.8 (D-line) $[\alpha]_{559.5}$ 0.0 $[\alpha]_{510}$−85.4 (trough) (*c* 0.328)	not measured
Rf*c) I	0.00	0.00	0.00	0.00
II	0.05	0.05	0.05	0.05
III	0.93	0.93	0.93	0.93
IV	0.10	0.10	0.10	0.10
V	0.47	0.47	0.47	0.47
VI	0.30	0.30	0.45	0.45
VII	0.23	0.23	0.31	0.31

*a) Because of hygroscopicity, analytical values of YA-56 X varied on each sample in accordance with the moisture contained. To avoid such fluctuation, the water content was determined simultaneously by a thermogravimetric-Differential Scanning Calorimeter (Rigaku Denki Kogyo Co. Standard type) on each sample and the analytical values were corrected based on the moisture content. For this reason, analytical values of YA-56 X and its Cu-complex were revised as shown. Presentation of the empirical formula is reserved until the survey of the constituents of YA-56 has been completed.

*b) Molecular weight was determined by vapor pressure osmometry in MeOH.

*c) PPC : Toyo No. 51 A paper, ascending. Bioautography with *Bacillus subtilis* PCI 219. Solvent system : I : water-saturated *n*-BuOH, II : acetone - H_2O (1 : 1), III : phenol-H_2O (3 : 1), IV : *n*-BuOH - MeOH - H_2O (4 : 1 : 2), V : *n*-BuOH - MeOH - H_2O-methyl orange (40 ml : 10 ml : 20 ml : 1.5 g), VI : *n*-BuOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12), VII : *n*-BuOH - AcOH - H_2O (4 : 1 : 5).

related to the previously reported phleomycins[5] and bleomycins[6].

According to UMEZAWA *et al.*[6] the ratio of UV absorbancies at the two absorption maxima, 244~246 and 295~297 nm, of phleomycin and bleomycin Cu-complexes is of great use in differentiating the members of this family. The ratio reported is 1.1~1.3 in bleomycins, about 2.7~2.8 in phleomycin D_1, E, G, H and I, and about 1.1~1.3 in phleomycin C, D_2 and F. With regard to UV spectra, YA-56 resembles phleomycin D_1, E, G, H and I, since the ratio is 2.78 in X and 2.8 in Y.

To compare YA-56 with the phleomycin complexes, chromatographic elution patterns on CM-Sephadex column were examined according to IKEKAWA *et al*[5]. As illustrated in the preliminary communication[2], YA-56 X and Y were eluted with 0.2 M ammonium formate buffer, similarly to phleomycin D_1. Therefore, YA-56 was directly compared with the phleomycin D_1 fraction isolated from phleomycin complex by the above-described chromatographic procedure. As shown in Fig. 3 a and 3 b, YA-56 X and Y were clearly differentiated from phleomycin D_1. Thus, the difference

Fig. 1 a. Infrared spectra of YA-56 X and Y in nujol.

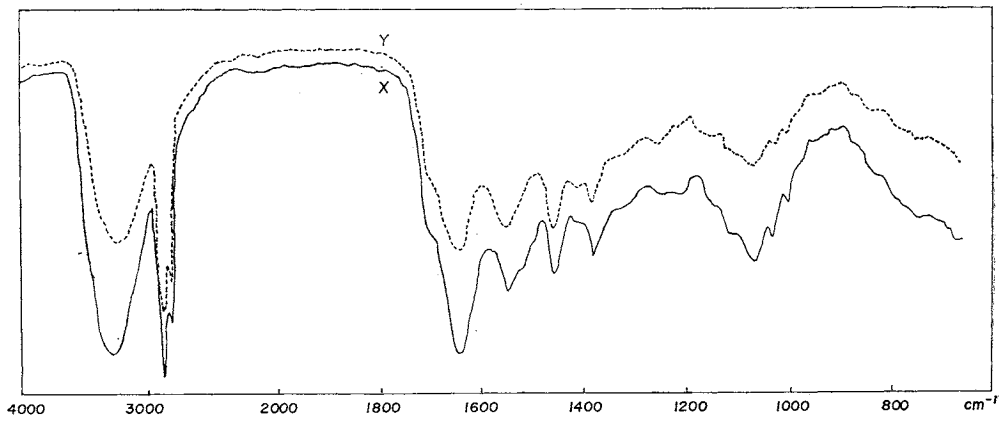
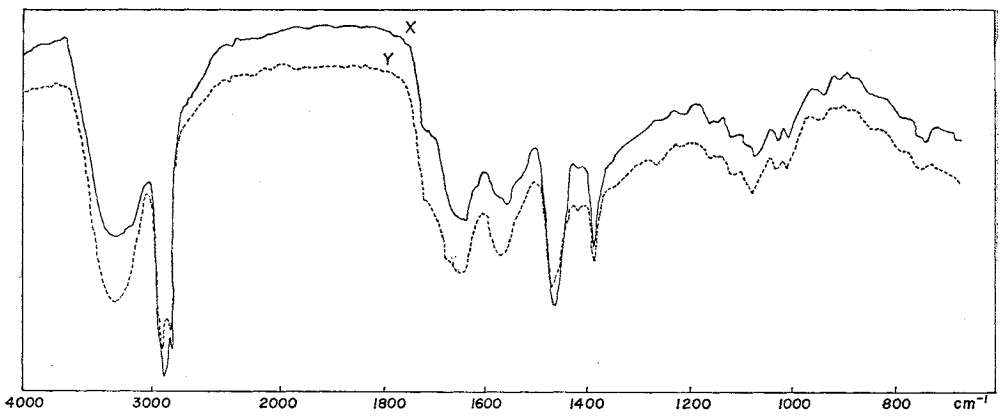


Fig. 1 b. Infrared spectra of YA-56 X and Y (Cu-complex) in nujol.



of YA-56 X and Y from each components of phleomycin and bleomycin was established. This conclusion has been strongly supported by the comparisons of the constituents of these antibiotics, which will be mentioned in the succeeding paper[3].

Recently ARGOUDELIS *et al.*[7] reported on production, isolation and characterization of zorbamycin, zorbonomycin B and zorbonomycin C, which were likely new members of phleomycin-bleomycin group antibiotics. Among these antibiotics, zorbamycin, which gave the UV absorption curve and the CM Sephadex elution pattern of phleomycin D_1 type seemed to be similar to YA-56 X. Thus, YA-56 X and Y were compared directly with the authentic sample of zorbamycin, which was kindly afforded by Dr. ARGOUDELIS. As shown in Fig. 3 a and 3 b, the chromatographic behaviors of YA-56 X and zorbamycin were the same. Moreover, YA-56 X and zorbamycin were not separable by CM Sephadex C-25 column chromatography. The NMR spectrum of zorbamycin which was published in the paper[7] appeared to be identical with that of YA-56 X. As a result, zorbamycin and YA-56 X are thought to be identical. Comparative study of the hydrolysates of YA-56 X and zorbamycin, which will be mentioned in the succeeding paper[3], supports their identity. YA-56 Y is readily

Fig. 2a. Ultraviolet absorption spectra of YA-56 X (in H_2O).

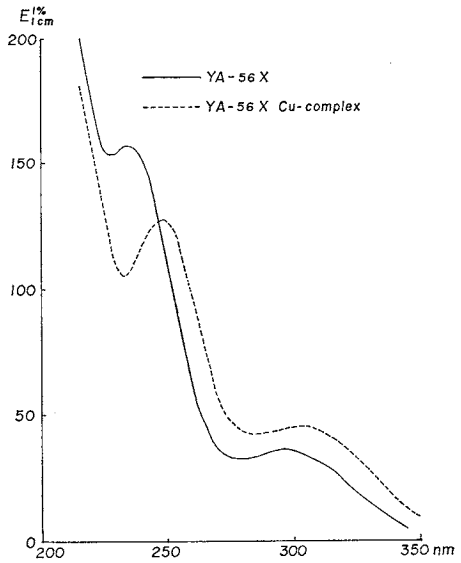


Fig. 2b. Ultraviolet absorption spectra of YA-56 Y (in H_2O).

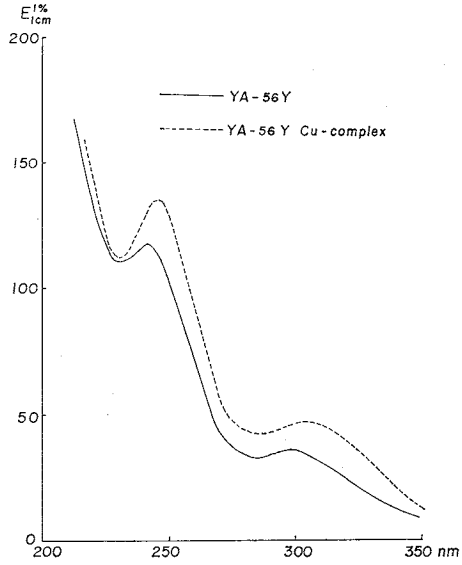


Fig. 3a. Paper chromatographic comparison of YA-56 with related antibiotics.
Paper : Toyo No. 51 A (descending)
Solvent : *n*-BuOH-pyridine-AcOH-H_2O (15 : 10 : 3 : 12)
Bioautography : *Bacillus subtilis* PCI 219, *Klebsiella pneumoniae* PCI 602.

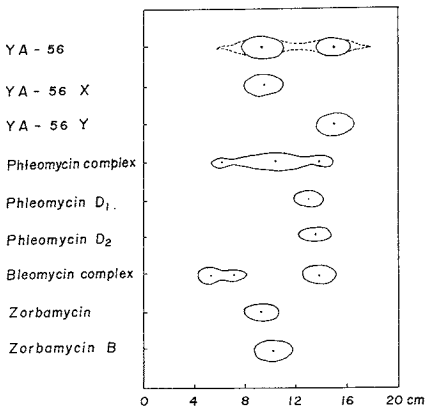
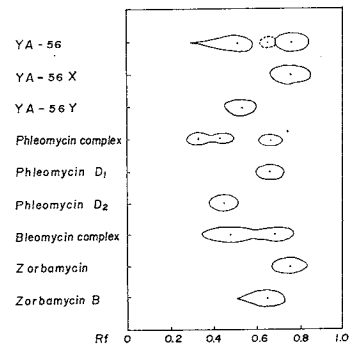


Fig. 3b. Thin-layer chromatographic comparison of YA-56 with related antibiotics.
Plate : Cellulose (E. Merck)
Solvent : 0.1 M NH_4Cl (pH 7.5)
Bioautography : *Bacillus subtilis* PCI 219, *Klebsiella pneumoniae* PCI 602.



distinguished from zorbonomycins B and C by their UV spectra and elution patterns of CM Sephadex.

It was quite surprising that zorbamycin (patent name : zorbonomycin) and YA-56 X were independently found and characterized almost at the same time[8,9].

Experimental

Isolation and Purification

In the course of purification, a cylinder-cup or pulp-disc assay method was applied using *Bacillus subtilis* PCI 219 and *Escherichia coli* NIHJ as test organisms. Column chromatographic fractions and the isolated products in all stages of the purification were also monitored by ascending paper chromatography on Toyo No. 51 A paper using solvent

system A (BuOH - pyridine - AcOH - H_2O, 15 : 10 : 3 : 12) and/or solvent system B (0.05 M NH_4Cl, pH 7.5) for development, and using *B. subtilis* PCI 219 and/or *Klebsiella pneumoniae* PCI 602 as the test organisms for bioautography. All solvent removals were carried out *in vacuo* under 40°C.

The fermentation broth was filtered with Celite 545 and adjusted to pH 6.8. The filtered broth (1,300 liters) was poured onto a column of Duolite S-30 (20 liters, Diamond Shamrock Chemical Co.) charged in a QVF glass tube (28×200 cm). After adsorption, the column was washed with water (200 liters) and 80 % aqueous acetone (50 liters), and then the column was eluted with 0.1 N hydrochloric acid - acetone mixture (2 : 8) (100 liters). The active eluate was adjusted to pH 6.5~6.8 and concentrated. The concentrate was lyophilized to give a brown colored powder (210 g). The powder was extracted with MeOH (0.5 liter×3), and the extract was concentrated to 0.8 liter, to which was added acetone (9 liters) to precipitate a brownish powder (55 g). This powder was dissolved in H_2O (100 ml) and poured onto a column (8×200 cm) of alumina (7.5 kg). The column was developed with water and the eluate was fractionated into three portions: the first fraction (Fr. I, 250 ml), the second fraction (Fr. II, 1,320 ml) and the third fraction (Fr. III, 10,730 ml). Fraction II, which was bluish in color and contained main active components, was adjusted to pH 6.4~6.8 and lyophilized to yield a bluish green powder. The powder was extracted with MeOH (125 ml) and the extract was concentrated to 50 ml. Then, the solution was applied onto a column (5×135 cm) of Sephadex LH-20. By developing the column with MeOH, the following three fractions were obtained; the first fraction (Fr. IV, 550 ml), the second fraction (Fr. V, 250 ml) and the third fraction (Fr. VI, 1,670 ml). The main active fraction (Fr. V) was concentrated to 95 ml. Acetone was added to this concentrate to yield a blue powder (1.5 g). This powder was repeatedly (2~3 times) purified by gel filtration on Sephadex LH-20 using MeOH as a solvent in the manner described above. Purified hydrochloride of YA-56 Cu-complex (500 mg) was obtained as a blue powder. The side fractions, Fr. I, III, IV and VI were combined and the pool was subjected to column chromatography on a column of alumina, Sephadex G-15 and Sephadex LH-20, successively. By treating the eluate thus obtained in the same manner as described above, additional hydrochloride of YA-56 Cu-complex (379 mg) was recovered. Total yield was 12.3 %.

Separation of YA-56 X and Y

Sephadex G-15 (2.4 liters) was boiled in 1 N HCl (2 liters) for 2 hours. After cooling, the Sephadex was washed with water until the washed solution showed pH of about 6.2. Then, the Sephadex gel was charged in a column (5×135 cm). The hydrochloride of YA-56 Cu-complex (1.78 g) was dissolved in 0.2 M aqueous NaCl solution and the solution was poured onto the column. By developing the column with the same solvent, YA-56 was observed to be separated into two blue colored bands along the column. Two main eluates were collected by continuous development with 0.2 M aqueous NaCl. The faster moving eluates containing the X component were combined and the solution (245 ml) was lyophilized to give a blue powder (1.12 g). The slower moving eluates containing the Y component were pooled and the solution (1,400 ml) was lyophilized to yield a blue powder (5.5 g). The powder containing the X component was dissolved in MeOH - H_2O (5 : 1, 18 ml) and the solution was poured onto a column of Sephadex LH-20 (2.4 liters). By developing the column with MeOH, the blue colored fractions which showed antimicrobial activity were collected and the solution was concentrated to 20 ml. Acetone (200 ml) was added to the concentrate to yield a precipitate. The precipitate was again dissolved in MeOH (5 ml) and the solution was subjected to gel filtration on a column of Sephadex LH-20, using MeOH as a solvent and the fractions showing Rf values of 0.3 on a paper chromatography (solvent system A) were combined. Concentration of the eluate followed by precipitation with acetone afforded the pure sample of the hydrochloride of YA-56 X Cu-complex as a blue powder (167 mg). For the separation of the YA-56 Y, the blue powder containing

the Y component (5.5 g) was dissolved in MeOH - H_2O (5 : 1, 30 ml) and the solution was poured onto a column (5×135 cm) of Sephadex LH-20. By developing the column with MeOH, the blue colored fractions (300 ml) showing antimicrobial activity were collected. The pool was concentrated to 30 ml. Addition of acetone (200 ml) to the solution gave a precipitate (400 mg), which was further purified by gel filtration of Sephadex LH-20 column in the same manner as described above to yield a purified sample of YA-56 Y Cu-complex (78 mg).

Preparation of YA-56 X and Y

The hydrochloride of YA-56 X Cu-complex (160 mg) was dissolved in dehydrated MeOH (16 ml) and dry hydrogen sulfide gas was introduced into the solution at room temperature. The precipitated copper sulfide was removed by centrifugation. The pale yellow supernatant was evaporated. The residue (120 mg) was dissolved in MeOH (1.5 ml) and the solution was applied on a column (1.8×90 cm) of Sephadex LH-20. By using MeOH as the developing solvent, main peak fractions showing antimicrobial activity were collected and combined. The combined solution was concentrated and precipitated with acetone to give a pure sample of the hydrochloride of YA-56 X (97.5 mg) as a white amorphous powder.

The methanolic solution of YA-56 Y Cu-complex (60 mg/ml) was treated with hydrogen sulfide in the manner described for YA-56 X. After purification by Sephadex LH-20 gel filtration, the pure sample of YA-56 Y (35 mg) was obtained as a white amorphous powder.

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