ANTIBIOTIC YA 56, A NEW FAMILY OF PHLEOMYCIN-BLEOMYCIN GROUP ANTIBIOTICS*

Sir:

A new family of phleomycin-bleomycin group antibiotics, named YA 56, was isolated from the culture broth of a variant of *Streptomyces humidus* (strain No. MCRL-0387).

YA 56 is produced by fermentation of the strain for 5~7 days at 27°C in a liquid medium containing glucose, glycerol, dextrine (each 1 %), soybean meal (1.5 %), NaCl (0.5 %), $CaCO_3$ (0.2 %) and $CuSO_4$ (0.002 %) (pH: not adjusted). The active principle in the filtered broth is isolated as a bluish powder by the following procedures: (1) adsorption on Duolite S-30 resin, (2) elution with 0.1 N HCl - acetone (2 : 8), (3) alumina chromatography of the lyophilized eluate (development with H_2O) and (4) Sephadex LH-20 gel filtration. On an ascending paper chromatogram (Toyo No. 51 A; solvent system: *n*-BuOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12); bioautography with *Bacillus subtilis* and *Klebsiella pneumoniae*), YA 56 is found to be mainly composed of two active components called X (Rf 0.30) and Y (Rf 0.45). Both components were separated by repeated gel filtration with acid pre-treated Sephadex G-15[1] and LH-20 respectively as a chromatographically homogeneous bluish powder containing cupric ion as a chelating metal. By treating with H_2S, each component was obtained as a white copper-free powder.

Physicochemical properties of YA 56-X and YA 56-Y are shown in Table 1, together with those of their Cu-complexes. YA 56-X and YA 56-Y were positive to MOLISCH, anthrone, DRAGENDORFF, EHRLICH and PAULY color reactions, while they were negative to ninhydrin and ELSON-MORGAN reactions. BIURET and SAKAGUCHI's reactions were doubtful. Both were soluble in water, methanol, dimethylformamide and dimethyl sulfoxide, slightly soluble in ethanol and insoluble in propanol, butanol, acetone, ethyl acetate, chloroform, benzene, ether, petroleum ether and dioxane.

The antimicrobial activities of YA 56 are shown in Table 2. YA 56-X and YA 56-Y show similar activity against *Escherichia coli*, but, against Gram positive bacteria, Y is more active than X. Mice tolerated the intravenous injection of 50 mg/kg of each component. Intraperitoneal administration of 3.2 mg/kg of the X or Y components for 5 successive days completely inhibited the growth of EHRLICH ascites carcinoma (ascitic form) in mice.

Judging from the above physicochemical and biological properties, the antibiotic seems to be related to the previously reported phleomycin[2] and bleomycin.[3] According to UMEZAWA *et al.*[3], the ratio of UV absorbancies at the two absorption maxima, 244~246 and 295~297 nm, of phleomycin-Cu and bleomycin-Cu complexes is of great use in differentiating antibiotics of this group. The ratio 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. In this respect, YA 56 resembles phleomycin D_1, E, G, H and I, since the ratio is 2.78 in X and 2.85 in Y. To compare YA 56 with the phleomycin complex, chromatographic elution patterns on a CM-Sephadex column were examined according to IKEKAWA *et al.*[2] As illustrated in Fig. 1, YA 56-X and YA 56-Y were eluted with 0.2 M ammonium formate buffer, similarly to phleomycin D_1. Therefore, for comparison, the phleomycin D_1 fraction was recovered from the eluates and compared with YA 56 by descending paper chromatography using *n*-BuOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12) as the solvent system. After 17-hour development, YA 56-X, YA 56-Y and phleomycin D_1 run respectively 9.4, 15 and 13 cm from the origin. Thus, YA 56-X and YA 56-Y were clearly differentiated from each component of phleomycin and bleomycin.

Structural constituents of YA 56-X and YA 56-Y are now under study. However, the following data allow definite conclusions as to the novelty of YA 56. T.L.C. (Fig. 2)

* Presented at the 180 th meeting of Japan Antibiotics Research Association (July 23, 1971).

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

	YA 56-X		YA 56-Y	
	Cu-complex	Cu-free	Cu-complex	Cu-free
Melting point	195~200°C (dec.)	198~202°C (dec.)	190~197°C (dec.)	188~197°C (dec.)
Elementary analysis (%)	C 42.19 H 5.88 N 15.59 S 4.24 Cl 1.34 Cu 3.10	C 42.93 H 5.73 N 16.14 S 4.85 Cl 2.75	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 —
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 (177.5) & 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
IR: ν_{max}^{nujol} (cm^{-1})	3300, 1700 (sh), 1645, 1550, 1410, 1150, 1108, 1065, 1020 & 995	3300, 1700 (sh), 1645, 1545, 1410, 1140, 1113, 1065, 1030 & 995	3300, 1700 (sh), 1640, 1555, 1410, 1158, 1110, 1065, 1020 & 1000	3230, 1690 (sh), 1640, 1545, 1410, 1155, 1110, 1065, 1025 & 1000
Rf* 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

* PPC: Toyo No. 51A, ascending. Bioautography with *B. subtilis*.
Solvent system; I: water-saturated 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).

Table 2. Antimicrobial spectra of YA 56-X and YA 56-Y (Serial agar dilution method)

Test organism	Medium	M.I.C. (mcg/ml) X	Y
Staphylococcus aureus FDA 209 P	I	1.5	0.1
Staphylococcus aureus SMITH	I	1.5	<0.1
Bacillus subtilis PCI 219	I	0.1	<0.1
Escherichia coli NIHJ J_c-2	I	0.4	0.4
Klebsiella pneumoniae	I	0.4	1.6
Mycobacterium tuberculosis $H_{37}R_V$	II	0.4	0.2

Medium; I: Heart infusion agar (Eiken Kagaku Co.) (48 hours).
II: KIRCHNER's agar with 10% horse serum (2 weeks).

and liquid chromatography* on the acid hydrolysates of YA 56 suggested the presence of β-hydroxyhistidine[4], β-aminoalanine[5] and β-amino-β-(4-amino-6-carboxy-5-methylpyrimidine-2-yl)-propionic acid[6]. β-Hydroxyhistidine and β-aminoalanine were isolated and identified. These studies denied the existence of threonine and 2'-(2-aminoethyl)-2, 4'-bithiazole-4-carboxylic acid, both present in bleomycin[7]. T.L.C. (Fig. 3) and G.L.C.** analyses of the metha-

* Measured with Hitachi-O 34 Liquid Chromatograph.

** Determined as T.M.S. derivatives.

Fig. 1. Chromatographic elution pattern of YA 56 and phleomycin complex.

Column : CM Sephadex C-25 (1.5×15 cm)
Sample : 5 mg each
Flow rate : 85 ml/hr
Temp. : 20℃
Elution : $HCOONH_4$ buffer, stepwise

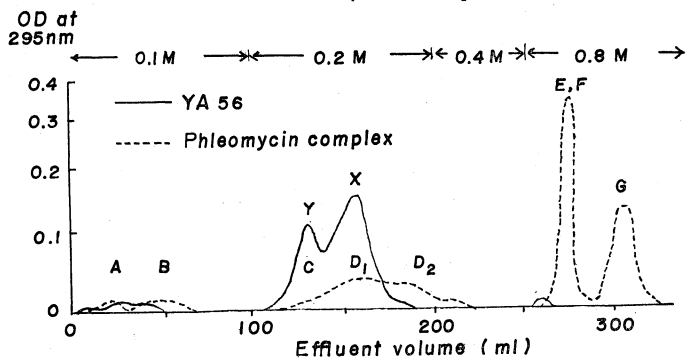
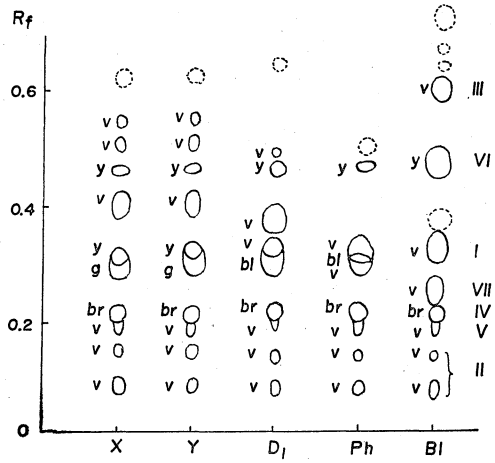
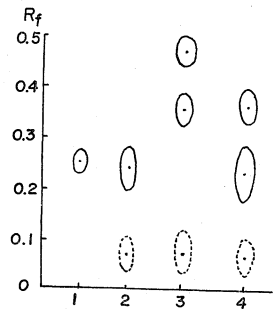


Fig. 2. Thin-layer chromatography of the acid hydrolysates of YA 56-X and YA 56-Y, phleomycin D_1, phleomycin (Ph) and bleomycin (BL) complexes.
(T.L.C. on cellulose (Merck) plate)



Hydrolysis by 6 N HCl at 105℃, 20 hours in a sealed tube. Solvent system : *n*-BuOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12). Coloration by ninhydrin spray. (v : violet, br : brown, g : green, bl : blue, y : yellow)
Assignment (Dr. T. TAKITA) ; I : L-threonine, II : β-amino-β-(4-amino-6-carboxy-5-methylpyrimidine-4-yl)-propionic acid, III : 4-amino-3-hydroxy-2-methyl-*n*-valeric acid, IV : β-hydroxyhistidine, V : L-β-aminoalanine, VI : 2′-(2-aminoethyl)-2, 4′-bithiazole-4-carboxylic acid, VII : 3-aminopropyl-dimethylsulfonium salt.

Fig. 3. T.L.C. of methanolysis products of YA 56 and bleomycin complex.

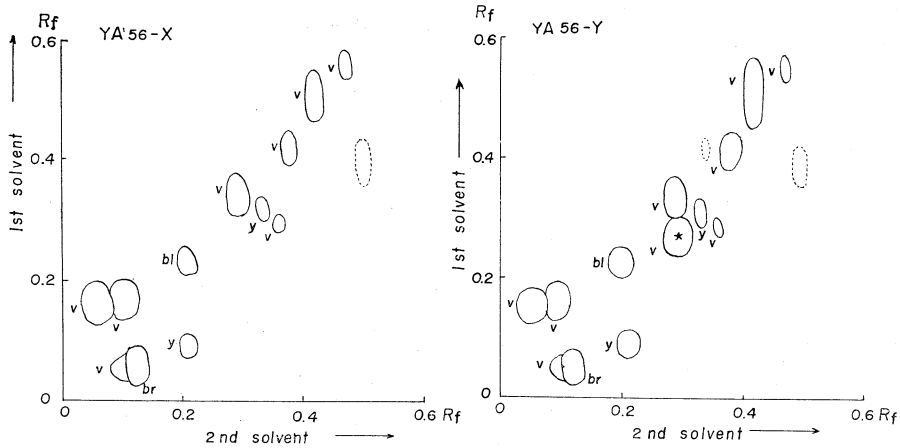


Methanolysis by Amberlyst 15 in MeOH, reflux overnight.
T.L.C. : Silica gel G
Detection : *p*-Anisaldehyde-H_2SO_4
Sample : 1. Methyl-D-mannopyranoside
2. Methyl-D-gulopyranoside
3. Methanolysis product of YA 56-X and YA 56-Y complex
4. Methanolysis product of bleomycin complex

nolysis products of YA 56 strongly suggested the existence of carbamoyl mannose, previously reported as a constituent of bleomycins.[8] In addition, another unidentified carbohydrate was detected. However, gulose, the second constitutional carbohydrate of bleomycin, was not detected. Two dimensional T.L.C. chromatograms (Fig. 4) of the acid hydrolysates of the X and Y components showed that both components were composed of common ninhydrin-positive substances except one asterisked in the figure.

Structural constituents of phleomycin have not been reported yet, but T.L.C. of the acid hydrolysate of authentic phleomycin suggested the presence of threonine in the antibiotic (Fig. 3). According to the personal communication of Drs. T. TAKITA and S. KONDO, every component of phleomycin contains threonine. This was confirmed by us on phleomycin D_1 isolated by CM-Sephadex column chromatography described

Fig. 4. Two-dimentional thin-layer chromatography of the acid hydrolysate of YA 56-X and YA 56-Y.
(T.L.C. on cellulose (Merck) plate)



1st Solvent: *n*-BuOH - AcOH - H_2O (4 : 1 : 2), 2nd Solvent: *n*-BuOH - pyridine - AcOH - H_2O (15 : 10 : 3 : 12).
Coloration by ninhydrin spray (v : violet, br : brown, bl : blue, y : yellow).

above. Thus, YA 56 is concluded to be a new family of phleomycin-bleomycin group antibiotics which is characterized by the absence of threonine as a constituent.

Acknowledgement

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Addendum: After we had contributed the present paper, the researchers of the Upjohn Co. reported three antibotics named zorbamycin, zorbonomycins B and C which were likely new members of phleomycin and bleomycin group of antibiotics (This journal, 24 : 543~557, 1971). Among these antibiotics, zorbonomycins B and C were readily distinguished from YA 56. However, zorbamycin which gave the UV absorption curve and the CM-Sephadex elution pattern of phleomycin D_1 type seemed to be somewhat similar to YA 56. YA 56-X, YA 56-Y and phleomycin complex (Lot A 9311-648) showed the following Rf-values on T.L.C. using cellulose sheets (E. Merck: DC-Fertigplatten Cellulose); detection by bioautography on *B. subtilis* seeded agar): 1) YA 56-X 0.74, YA 56-Y 0.51, phleomycin 0.3~0.1 with 0.1 M aqueous NH_4Cl (Rf reported: zorbamycin 0.48, phleomycin 0.25~0.1). 2) YA 56-X 0.9, YA 56-Y 0.6, phleomycin 0.3~0.1 with sodium citrate (0.05 M) pH 6.9 buffer (Rf reported: zorbamycin 0.4, phleomycin 0.25~0.05). Even if it was taken into consideration that reported Rf-values of zorbamycin were obtained on the Brinkman MN-polygram CEL-300 sheets, YA 56-X and YA 56-Y seemed to be different from zorbamycin. However, to our experience, comparison with data in the literature does not always give the definite conclusion to the identification of the substances. Direct comparison of YA 56 with zorbamycin or elucidation of the constituents of

zorbamycin would give the definite answer on this matter (Sept. 11, 1971)*.

References

1) Goodson, J. M. & V. Distefano: Improvement of the chromatographic properties of Sephadex G-15. J. Chromatography 45 : 139~142, 1969
2) Ikekawa, T.; F. Iwai, H. Hiranaka & H. Umezawa: Separation of phleomycin components and their properties. J. Antibiotics 17 : 194~199, 1964
3) Umezawa, H.; Y. Suhara, T. Takita & K. Maeda: Purification of bleomycins. J. Antibiotics 19 : 210~215, 1966
4) Takita, T.; T. Yoshioka, Y. Muraoka, K. Maeda & H. Umezawa: Chemistry of bleomycin. V. Revised structure of an amine component of bleomycin A_2. J. Antibiotics (in press)
5) Takita, T.; Y. Muraoka, K. Maeda & H. Umezawa: Chemical studies on bleomycins. I. The acid hydrolysis products of bleomycin A_2. J. Antibiotics 21 : 79~80, 1968
6) Muraoka, Y.; T. Takita, K. Maeda & H. Umezawa: Chemistry of bleomycin. IV. The structure of amine component II of bleomycin A_2. J. Antibiotics 22 : 252~253, 1970
7) Koyama, G.; H. Nakamura, Y. Muraoka, T. Takita, K. Maeda, H. Umezawa & Y. Iitaka: The chemistry of a sulfur-containing chromophoric amino acid. Tetrahedron Letters 1968-44 : 4635~4638, 1968
8) Takita, T.; K. Maeda, H. Umezawa, S. Omoto & S. Umezawa: Chemistry of bleomycin. III. The sugar moieties of bleomycin A_2. J. Antibiotics 22 : 237~239, 1969

* The lot number of the phleomycin sample used here was coincidentally the same as that of the phleomycin complex used by the Upjohn's group. Phleomycin D_1 and D_2 contained in this sample were hardly detected by bioautography with *B. subtilis*. Phleomycin D_1 and D_2 which were readily detected with *K. pneumoniae* run faster than the detected part of phleomycin mentioned above.