A NEW ANTHRACYCLINE ANTIBIOTIC, CINERUBIN R

TAXONOMY, STRUCTURAL ELUCIDATION AND BIOLOGICAL ACTIVITY

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A novel anthracycline antibiotic, cinerubin R, was isolated from the fermentation broth of *Streptomyces eurythermus* strain H1715MY2. The structure of cinerubin R was elucidated to be 4''-aculosyl-4'-rhodinosyl-7-rhodosaminyl-ε-pyrromycinone. Cinerubin R was active against Gram-positive bacteria and inhibited the growth of divergent multi-drug-resistant cells to the same extent as their parental cells.

In the course of our screening for new anthracycline antibiotics equally active against both parental and doxorubicin-resistant cell lines, candidates were discovered in the broth filtrate of strain H1715MY2 isolated from the soil sample collected at Matsumoto city in Nagano prefecture. Five anthracycline antibiotics were isolated; four of them were identified to be cinerubins A[1], B[2], pyrraculomycin[3] and 1-hydroxysulfurmycin B[4], whereas one of them was found to be a novel cinerubin congener, being named cinerubin R.

In this paper, the isolation, structural elucidation and biological properties of the anthracyclines produced by strain H1715MY2 as well as the taxonomic studies on the producing organism are reported.

Materials and Methods

General

UV spectra were examined with a Hitachi 124 spectrophotometer, 1 cm light pass, using methanol as a solvent. ^{1}H NMR and ^{13}C NMR spectra were measured in $CDCl_3$ on a Jeol JNM-GX500 spectrometer using tetramethylsilan (TMS) as the internal reference. Mass spectra were determined on a Jeol JMS-SX102 spectrometer.

Microorganisms

Strain H1715MY2 was isolated from the soil sample collected in Matsumoto city, Nagano prefecture, Japan.

Taxonomic Studies

Growth characteristics and carbohydrate utilization were determined according to the methods of SHIRLING and GOTTLIEB[5]. Morphology of spore chains was observed after incubation on ISP media at 27°C for 14 days. The Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois) was used to identify the color of mycelia and soluble pigments. Spores were observed with a

scanning electron microscope (Model S-510, Hitachi Co., Ltd.). Diaminopimelic acid isomers of whole-cell hydrolysate were analyzed by the method of BECKER *et al.*[6].

Fermentation

Strain H1715MY2 was cultured in 500-ml Erlenmeyer flasks each containing 100 ml of a seed medium composed of maltose 1% and yeast extract 0.4% (adjusted to pH 7.0 before sterilization). After 20 hours of incubation at 27°C on a rotary shaker, 1-ml portion of the culture was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of a production medium consisting of soyameal 2%, soluble starch 1.5%, glucose 1%, Ebios 0.5% (Tanabe Seiyaku Co., Ltd.), $CaCO_3$ 0.3% and NaCl 0.25% (adjusted to pH 7.6 before sterilization). The fermentation was carried out at 27°C for 4 days on a rotary shaker (160 rpm).

Antimicrobial Activity

The *in vitro* antimicrobial activity was determined on glucose-nutrient agar by the 2-fold serial dilution method.

Cell Lines

Mouse lymphoblastoma L5178Y cells and its doxorubicin-resistant cells (L5178Y/S and L5178Y/ADM[7], respectively) were kindly donated by Prof. N. TANAKA, Institute of Applied Microbiology, The University of Tokyo. Mouse leukemia P388 cells and its doxorubicin-resistant cells (P388/S and P388/ADM[8], respectively) were supplied by Dr. M. INABA, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research. Human leukemia K562 cells (K562/S) and the doxorubicin- and vincristine-resistant cells (K562/ADM[9] and K562/VCR[10], respectively) were obtained from Institute of Applied Microbiology, The University of Tokyo. The L5178Y and K562 cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS); P388 cells were maintained in RPMI-1640 medium supplemented with 10% FCS and 5 μM hydroxyethyl disulfate. All cell lines were cultured at 37°C in an atmosphere of 5% CO_2 in air.

In Vitro Antitumor Activity

The cells were cultured in an appropriate medium at 3×10^4/ml (L5178Y and P 388 cells) or 5×10^4/ml (K562 cells) in the absence or the presence of antibiotic for 3 days. The number of cells were counted in a Coulter counter (Coulter Electronics Ltd., Beds, England). The resistance factor (RF) against individual antibiotics was defined as a ratio of IC_{50}'s for the resistant cells and the parental cells.

Results and Discussion

Taxonomy

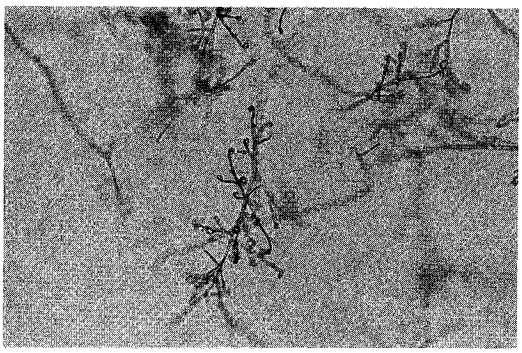
Morphological observations were made on the cultures grown at 27°C for 14 days on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar and glycerol-asparagine agar. Mature spore chains consisted of 10~20 or more spores in the forms of hook and loop (Fig. 1). The smooth-surfaced spores shaped oval and measured $0.6 \sim 0.7 \times 1.0 \sim 1.2$ μm (Fig. 2).

The whole-cell hydrolysate contained the L,L-isomer of diaminopimelic acid corresponding to cell wall type I.

The cultural and physiological characteristics are shown in Tables 1 and 2, respectively.

Based on the taxonomic properties described above, strain H1715MY2 is considered to belong to the genus *Streptomyces* and to be a strain of the gray color series of the PRIDHAM and TRESNER grouping[11]. By consulting BERGEY's Manual[12] and the literatures[13~16], *Streptomyces eurythermus* was most closely related to strain H1715MY2. These two strains were indistinguishable from each other with respect to the colors of aerial hypha on various media (gray series), spore chain morphology (section *Rectiflexibiles*), spore surface structure (smooth), melanin production and carbon utilization. Therefore, strain H1715MY2

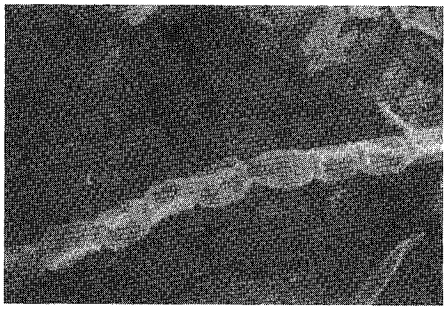
Fig. 1. Microscopic features of strain H1715MY2.



Grown on ISP medium 4 for 14 days at 27°C. (×400).

Fig. 2. Sporophore morphology of strain H1715MY2.

Bar represents 1 μm.



Grown on ISP medium 4 for 14 days at 27°C.

Table 1. Cultural characteristics of strain H1715MY2.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast extract-malt extract agar (ISP medium 2)	Dark red (6½pg)	Good Greenish gray (22ih)	Brown
Oatmeal agar (ISP medium 3)	White (a)	Moderate White (a) to light blueish gray (15fe)	None
Inorganic salts-starch agar (ISP medium 4)	Yellowish brown (5nc~5ga)	Good Greenish gray (22ih)	Pale brown
Glycerol-asparagine agar (ISP medium 5)	Reddish brown (8pe)	Good Greenish gray (22ih)	None
Sucrose-nitrate agar	White (a)	Poor White (a)	None
Glucose-asparagine agar	Light brown (5ic)	Moderate White (a)	None
Nutrient agar	White (a)	Moderate White (a)	None

Table 2. Physiological characteristics of strain H1715MY2.

Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Positive
Melanin production on	
Tyrosine agar (ISP medium 7)	Positive
Peptone-yeast extract iron agar (ISP medium 6)	Positive
Tryptone-yeast extract broth (ISP medium 1)	Positive
Gelatin liquefaction	Positive

Table 3. Carbon utilization of strain H1715MY2.

Carbon source	Utilization
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Fructose	+
Sucrose	−
L-Rhamnose	−
i-Inositol	−
D-Mannitol	+
Raffinose	±
Cellulose	±

Symbols: +, good; ±, doubtful; −, poor or none.

was designated as *S. eurythermus* H1715MY2.

Extraction

A flow diagram for the extraction of anthracyclines is shown in Fig. 3.

Fig. 3. Extraction of anthracycline mixture produced by strain H1715MY2.

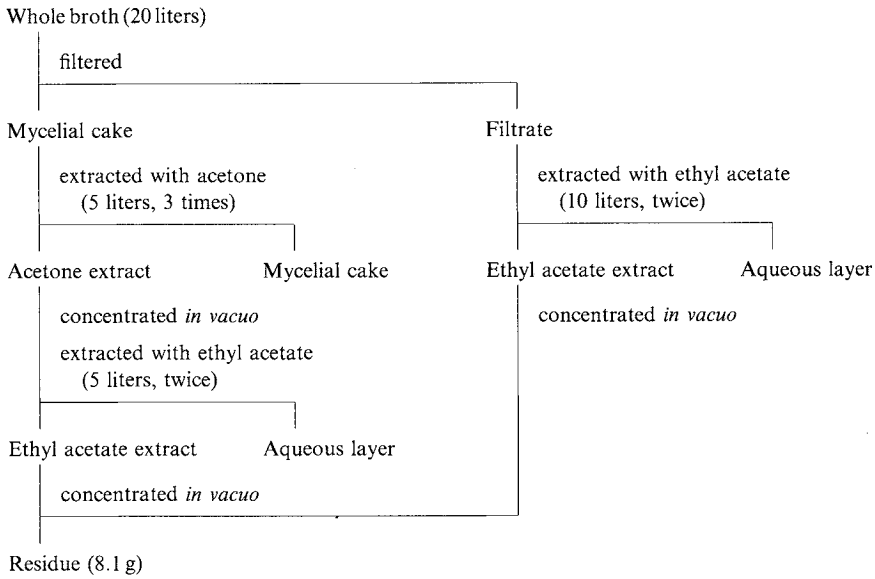
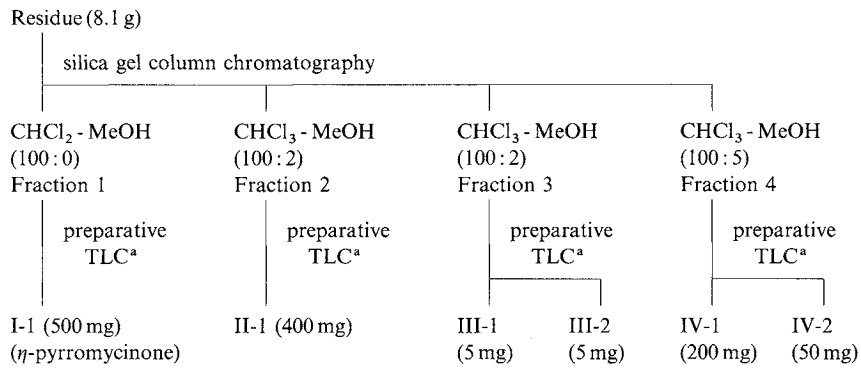


Fig. 4. Purification of anthracyclines produced by strain H1715MY2.



[a] Silica gel 60 F_{254} plate (Merck) developed with $CHCl_3$ - MeOH (20 : 1).

Isolation

Anthracyclines were purified by silica gel (Kieselgel 60, 0.063~0.200 mm, Merck Co.) column chromatography with $CHCl_3$ - MeOH mixture. For the further purification a TLC (Kieselgel 60 F_{254}, Merck Co.) with $CHCl_3$ - MeOH (20 : 1, v/v) was used (Fig. 4). Finally, η-pyrromycinone (I-1) and five anthracycline antibiotics (II-1, III-1, III-2, IV-1 and IV-2) were isolated. Based on the ^{13}C NMR spectra and the physico-chemical properties shown in Tables 4 and 5, respectively, as well as the comparison of the Rf values on silica gel TLC between H1715MY2 substances and authentic standards (data not shown), II-1, III-1, IV-1 and IV-2 were identified as cinerubin B, 1-hydroxysulfurmycin B, cinerubin A and pyrraculomycin, respectively[1~4,17,18]. All the H1715MY2 substances including III-2 contained a

Table 4. ^{13}C chemical shift assignments of anthracyclines produced by strain H1715MY2 (100 MHz, $CDCl_3$).

Position	II-1	III-1	III-2	IV-1	IV-2
1	157.8	158.0	157.9	157.8	157.9
2	129.7	129.8	129.7	129.8	129.8
3	130.1	130.2	130.1	130.1	130.1
4	158.4	158.4	158.4	158.4	158.5
4a	112.3	112.4	112.4	112.4	112.4
5	190.6	190.7	190.7	190.6	190.6
5a	114.8	115.0	114.9	114.8	114.9
6	162.3	162.3	162.3	162.3	162.3
6a	132.8	132.8	132.8	132.8	132.9
7	70.7	70.9	70.6	70.7	70.7
8	33.8	34.7	33.8	33.8	33.8
9	71.7	70.9	71.7	71.7	71.7
10	57.2	56.6	57.2	57.2	57.1
10a	142.4	141.5	142.5	142.4	142.4
11	120.4	120.5	120.4	120.4	120.4
11a	131.5	131.3	131.5	131.5	131.5
12	185.7	185.8	185.8	185.8	185.8
12a	112.5	112.6	112.6	112.6	112.6
13	32.2	50.9	32.3	32.2	32.2
14	6.7	208.2	6.7	6.7	6.7
14a		32.5			
15	171.3	170.8	171.3	171.3	171.3
16	52.5	52.7	52.5	52.5	52.6
1′	101.6	101.3	101.6	101.6	101.6
2′	29.3	29.3	29.3	29.2	29.3
3′	61.5	61.6	61.7	61.6	61.5
4′	74.1	74.1	73.9	74.0	74.1
5′	65.3	65.4	68.6	66.8	66.6
6′	17.9	18.0	17.9	17.0	17.0
NMe_2	43.2	43.2	43.3	43.3	43.2
1″	99.1	99.1	98.5	100.2	99.3
2″	27.0	26.9	24.6	34.3	34.4
3″	67.3	67.2	24.6	65.3	65.6
4″	68.3	68.2	77.0	83.0	83.9
5″	66.9	66.9	66.4	68.4	68.3
6″	16.0	16.1	17.1	17.9	20.0
1‴	91.5	91.6	95.3	99.4	95.7
2‴	63.0	62.9	143.3	27.7	142.3
3‴	39.7	39.7	127.2	33.5	127.2
4‴	208.1	208.2	197.0	210.1	195.8
5‴	77.9	77.9	70.6	71.8	77.9
6‴	16.2	16.2	15.1	14.8	15.2

Chemical shifts in ppm from TMS.

trisaccharide moiety (Table 4, Fig. 5). Since III-2, which is a novel anthracycline, shared the common aglycone ε-pyrromycinone with the cinerubin congeners II-1, IV-1 and IV-2, it was designated as cinerubin R.

Structural Elucidation of Cinerubin R

The molecular formula was established to be $C_{42}H_{51}NO_{15}$ by high resolution FAB-MS (*m/z* 810.3366 MH^+, calcd 810.3337). The ^1H NMR spectrum of cinerubin R showed signals due to three phenolic hydroxyl groups (δ 12.96, 12.80, 12.23), a singlet aromatic methine (δ 7.72), two doublet aromatic methines (δ 7.31, 7.29; $J=9.0$ Hz), a broad doublet methine (δ 5.27, $J=4.0$ Hz), a singlet methine (δ 4.12), a methoxy (δ 3.70), a methylene (δ 2.52, dd, $J=15.5$, 4.0 Hz; δ 2.30, br d, $J=15.5$ Hz) and an ethyl group (δ 1.75,

Table 5. Physico-chemical properties of anthracyclines produced by strain H1715MY2.

	II-1 Cinerubin B	III-1 1-Hydroxy-sulfurmycin B	III-2 Cinerubin R	IV-1 Cinerubin A	IV-2 Pyrraculomycin
FAB-MS (*m/z*)	828 MH^+	854 MH^+	810 MH^+	826 MH^+	826 MH^+
MP	176~177°C	118~121°C	158~162°C	157~158°C	145~150°C
Rf value[a]					
$CHCl_3$-MeOH (10:1)	0.61	0.53	0.49	0.47	0.45
$CHCl_3$-MeOH (20:1)	0.45	0.31	0.29	0.28	0.23
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	234 (523), 256 (267), 288 (105), 481 sh (145), 490 (153), 510 sh (116), 524 (95)	234 (531), 256 (285), 288 (132), 481 sh (145), 490 (152), 510 sh (118), 525 (100)	234 (553), 258 (280), 288 (115), 481 sh (133), 491 (141), 512 sh (110), 526 (93)	234 (521), 257 (263), 288 (104), 481 sh (146), 491 (156), 510 sh (120), 525 (100)	233 (523), 256 (258), 286 (100), 480 sh (147), 490 (156), 510 sh (118), 525 (99)

[a] Silica gel TLC (Merck Art. No. 5715).

m; δ 1.51, m; δ 1.09, t, 3H, J=7.5 Hz), which were assignable to ε-pyrromycinone. In addition, the presence of three anomeric protons (δ 5.54, br d, J=2.5 Hz; δ 5.23, d, J=3.5 Hz; δ 4.93, br s) with α-configurations, *cis* olefinic protons (δ 6.87, dd, J=10.5, 3.5 Hz; 6.08, d, J=10.5 Hz) and an *N*-dimethyl group (δ 2.19, br s, 6H) indicated that cinerubin R contains a trisaccharide moiety including a dehydro sugar and a dimethylamino sugar.

Hydrolysis of cinerubin R with 0.1 N HCl at 85°C for 30 minutes gave ε-pyrromycinone, rhodosamine, rhodinose and aculose. These components were identified by silica gel TLC developed with $CHCl_3$-MeOH (20:1) or BuOH-AcOH-H_2O (4:1:1) by comparison with authentic samples, which were obtained by hydrolysis of cinerubin A[1] and vineomycin A_1 (P-1894B)[19,20].

The ^{13}C NMR spectrum of cinerubin R showed signals due to ε-pyrromycinone and three sugar components. Assignments of the signals were carried out by comparison with the spectral data of mussetamycin (deoxyfucosyl-rhodosaminyl-ε-pyrromycinone)[21] and vineomycin A_1.

The FAB-MS revealed a fragment peak at *m/z* 586 corresponding to pyrromycin (rhodosaminyl-ε-pyrromycinone) derived from the loss of aculosyl-rhodinose.

Thus, the structure of cinerubin R was established to be 4″-aculosyl-4′-rhodinosyl-7-rhodosaminyl-ε-pyrromycinone.

Antimicrobial Activity

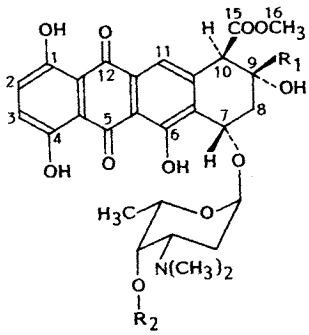
The minimum inhibitory concentrations (MICs) of the anthracyclines produced by strain H1715MY2 for a variety of organisms including bacteria, yeasts and fungi are shown in Table 6. All antibiotics inhibited the growth of Gram-positive bacteria but little or no effect was observed against Gram-negative bacteria, yeasts and fungi.

Among the cinerubin congeners, pyrraculomycin showed the highest activity against Gram-positive bacteria. Cinerubin R is less potent than pyrraculomycin, but superior to cinerubins A and B.

In Vitro Antitumor Activity

The anthracyclines produced by strain H1715MY2 were examined for their *in vitro* antitumor activity and the results are shown in Table 7. Doxorubicin (Kyowa Hakko Kogyo Co., Ltd.) and daunorubicin (Meiji Seika Co., Ltd.) served as references. L5178Y/ADM, P388/ADM, K562/ADM and K562/VCR

Fig. 5. Structures of anthracyclines produced by strain H1715MY2.



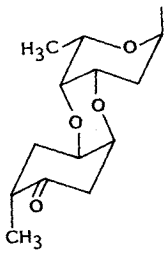
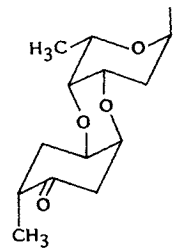
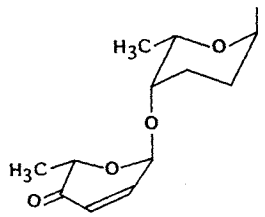
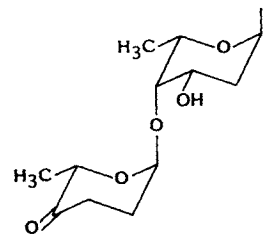
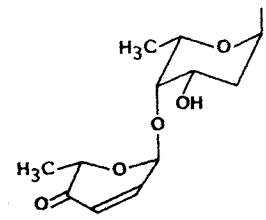
	R_1	R_2
II-1 (Cinerubin B)	$^{13}CH_2{}^{14}CH_3$	
III-1 (1-Hydroxysulfurmycin B)	$^{13}CH_2{}^{14}C(=O)^{14a}CH_3$	
III-2 (Cinerubin R)	$^{13}CH_2{}^{14}CH_3$	
IV-1 (Cinerubin A)	$^{13}CH_2{}^{14}CH_3$	
IV-2 (Pyrraculomycin)	$^{13}CH_2{}^{14}CH_3$	

Fig. 6. ^1H NMR spectrum of cinerubin R in $CDCl_3$ (500 MHz).

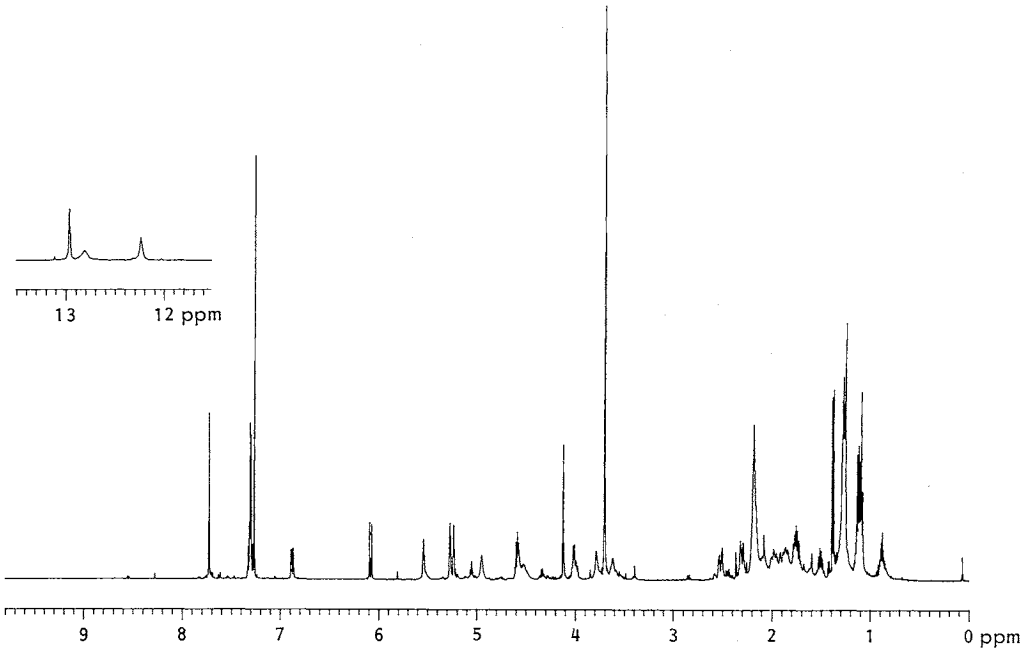


Table 6. Antimicrobial spectra of anthracyclines produced by strain H1715MY2.

Organism	MIC (μg/ml)				
	Cinerubin B	1-Hydroxy-sulfurmycin B	Cinerubin R	Cinerubin A	Pyrraculomycin
Staphylococcus aureus FDA 209P	3.13	3.13	0.78	3.13	0.39
S. aureus Smith	6.25	6.25	1.56	1.56	0.78
Micrococcus luteus PCI 1001	3.13	3.13	0.39	0.78	0.39
Bacillus subtilis PCI 219	3.13	3.13	0.78	3.13	0.39
B. anthracis	1.56	1.56	0.39	0.78	0.20
Corynebacterium bovis 1810	1.56	1.56	0.39	0.39	0.39
Escherichia coli NIHJ	>100	>50	>50	>100	50
Proteus vulgaris ox 19	100	50	>50	>100	25
Serratia marcescens	>100	>50	>50	>100	>50
Mycobacterium smegmatis ATCC 607	1.56	12.5	3.13	3.13	1.56
M. phlei	6.25	12.5	3.13	3.13	1.56
Candida tropicalis NI 7495	12.5	>50	25	3.13	12.5
C. albicans 3147	6.25	>50	25	1.56	6.25
C. krusei NI 7492	>100	>50	50	25	>50
Saccharomyces cerevisiae	25	>50	50	12.5	25
Cryptococcus neoformans	6.25	>50	3.13	6.25	3.13
Aspergillus niger F-16	12.5	>50	12.5	25	3.13

cells are known as "multi-drug-resistant (mdr) cells" being resistant to a variety of antitumor agents such as anthracyclines, vinka alkaloids, actinomycin D and etoposide. The resistance factor (*i.e.*, the ratio of IC_{50} for resistant and sensitive cells) of these mdr cells varied 16~410 for doxorubicin and 19~170 for

Table 7. Cytocidal activity of anthracyclines produced by strain H1715MY2.

	IC_{50} (μg/ml)		RF	IC_{50} (μg/ml)		RF
	L5178Y/S (*a*)	L5178Y/ADM (*b*)	(*b*/*a*)	P388/S (*c*)	P388/ADM (*d*)	(*d*/*c*)
Cinerubin B	8.3×10^{-4}	1.5×10^{-3}	1.8	8.5×10^{-4}	9.7×10^{-4}	1.1
1-Hydroxysulfurmycin B	5.0×10^{-3}	9.6×10^{-3}	1.9	4.7×10^{-3}	1.0×10^{-2}	2.1
Cinerubin R	7.0×10^{-3}	1.2×10^{-2}	1.7	4.6×10^{-3}	7.8×10^{-3}	1.7
Cinerubin A	7.2×10^{-4}	1.5×10^{-3}	2.1	1.2×10^{-3}	3.5×10^{-3}	2.9
Pyrraculomycin	1.9×10^{-3}	4.0×10^{-3}	2.1	7.8×10^{-4}	1.8×10^{-3}	2.3
Doxorubicin	3.8×10^{-2}	6.1×10^{-1}	16	2.2×10^{-2}	1.4	64
Daunorubicin	1.7×10^{-2}	3.3×10^{-1}	19	7.8×10^{-3}	1.8×10^{-2}	23

	IC_{50} (μg/ml)		RF	IC_{50} (μg/ml)	RF
	K562/S (*e*)	K562/ADM (*f*)	(*f*/*e*)	K562/VCR (*g*)	(*g*/*e*)
Cinerbin B	3.0×10^{-3}	1.7×10^{-2}	5.7	2.7×10^{-3}	0.9
1-Hydroxysulfurmycin B	5.2×10^{-3}	2.2×10^{-2}	4.2	9.3×10^{-3}	1.8
Cinerubin R	4.9×10^{-2}	5.9×10^{-2}	1.2	5.5×10^{-2}	1.1
Cinerubin A	3.4×10^{-3}	3.1×10^{-3}	0.9	4.6×10^{-3}	1.4
Pyrraculomycin	2.7×10^{-3}	1.1×10^{-2}	4.1	3.3×10^{-3}	1.2
Doxorubicin	8.7×10^{-3}	3.6	410	2.2×10^{-1}	25
Daunorubicin	5.1×10^{-3}	8.7×10^{-1}	170	5.3×10^{-2}	10

daunorubicin, whereas those for the cinerubin congeners and 1-hydroxysulfurmycin B were less than 6. The present *in vitro* results might indicate the possibility of overcoming *in vivo* resistance of mdr-phenotype tumor cells with these anthracyclines. The mechanism by which cinerubins reverse the function of P-glycoprotein which plays a central role in mdr cells will be reported elsewhere.

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