HYDRAMYCIN†, A NEW ANTITUMOR ANTIBIOTIC

TAXONOMY, ISOLATION, PHYSICO-CHEMICAL PROPERTIES, STRUCTURE AND BIOLOGICAL ACTIVITY

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A new antitumor antibiotic hydramycin was isolated from the fermentation broth of *Streptomyces violaceus* P950-4 (ATCC 53807). It showed potent antibacterial and cytotoxic activity and increased the survival time of mice inoculated with P388 leukemia. A new structure related to the pluramycin group antibiotics was assigned by its spectroscopic experiments.

In the course of our antitumor screening program for microbial metabolites, we found an actinomycete strain No. P950-4 produced a novel antitumor antibiotic. The antibiotic designated hydramycin was recovered from the cultured broth by solvent extraction and purified by a series of chromatographies. It was yellowish orange crystals having characteristic UV absorption and was shown to have a novel structure related to the chromophore moiety of the pluramycin group antibiotics. In addition to its strong antibacterial activity against Gram-positive bacteria, hydramycin exhibited potent cytotoxicity to B16 melanoma and Moser cells and induced a significant prolongation of survival time of mice bearing P388 leukemia. This paper describes the isolation, physico-chemical properties, structure determination and biological activity of hydramycin.

Taxonomy

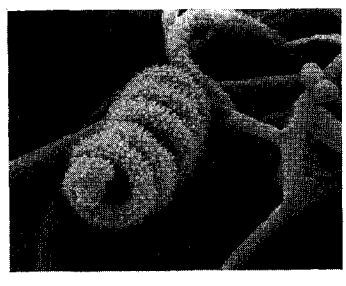
Source of Strain P950-4

A soil sample, collected in Hyderābād, Andhra Pradesh State, India.

Morphology

Strain P950-4 formed both substrate and aerial mycelia, which were long, well-branched and not fragmented into short elements. Chains of spores were formed on the monopodially branched aerial hyphae. The spore chains were loop or compact spiral in shape, and contained 10 to 50 spores per chain. The spores were spherical to oval ($0.6 \times 0.6 \sim 0.9\,\mu m$) and had spiny surface. Motile spores and sporangium-like bodies were not observed.

Fig. 1. Scanning electron micrographs of spore chain of strain P950-4 grown on glycerol-asparagine agar (ISP No. 5) at 28°C for 2 weeks.



† Hydramycin was originally called BU-3839T or BMY-28689.

Cultural Characteristics (Table 1)

The aerial mycelium was well formed on most agar media, but poorly on glucose-asparagine agar and not on ISP medium No. 6. The color of aerial mycelium was light grayish pink with sporulation. Melanin was produced distinctly in ISP medium No. 6, poorly in ISP No. 1, but not in ISP No. 7. A reddish orange to purplish red pigment, which was more or less diffusible, was formed in ISP media Nos. 2 and 7, and CZAPEK's sucrose-nitrate agar. The pigment was pH-sensitive (yellowish orange in acid and violet in alkali).

Physiological Characteristics (Table 2)

The growth was observed between 17°C and 40°C, but not at 15°C and 43°C. Tyrosinase reaction was positive. All of eleven diagnostic sugars were utilized for growth.

Table 1. Cultural characteristics of strain P950-4.

Medium	Growth Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Good Moderate; white (263)	Light yellowish brown (76) to vivid dark purplish red (260)	Brownish orange (54)
Tryptone-yeast extract broth (ISP No. 1)	Moderate; not turbid	Colorless	Deep brown (56)
Yeast extract-malt extract agar (ISP No. 2)	Good Good; grayish yellowish pink (32)	Pale yellow (89) to brownish orange (54)	Strong yellowish brown (74)
Oatmeal agar (ISP No. 3)	Moderate Moderate; grayish pink (8)	Pale yellow (89)	None
Inorganic salts-starch agar (ISP No. 4)	Moderate Moderate; grayish pink (8)	Deep yellow (85)	None
Glycerol-asparagine agar (ISP No. 5)	Moderate Moderate; grayish pink (8)	Strong yellowish brown (74)	Moderate yellow (87)
Peptone-yeast extract-iron agar (ISP No. 6)	Moderate None	Colorless	Brownish black (65)
Tyrosine agar (ISP No. 7)	Moderate Moderate; yellowish white (92)	Light olive brown (94)	Dark grayish yellow (91)
Glucose-asparagine agar	Poor Scant; white (263)	Pale yellow (89)	None

Observation after incubation at 28°C for 3 weeks.
Color name used: ISCC-NBS Color-Name Charts.

Table 2. Physiological characteristics of strain P950-4.

Positive reaction:
Hydrolysis of gelatin and starch; coagulation and peptonization of milk; production of nitrate reductase and tyrosinase; tolerance to 7% NaCl; utilization of glycerol, L-arabinose, D-xylose, D-ribose, L-rhamnose, D-glucose, D-galactose, D-fructose, D-mannose, sucrose, lactose, cellobiose, melibiose, trehalose, raffinose, D-melezitose, soluble starch, inositol, and D-mannitol. Weekly positive utilization of D-sorbitol and salicin.

Negative reaction:
Tolerance to 0.01% lysozyme and 8% NaCl; utilization of D-arabinose, L-sorbose, cellulose and dulcitol.

Basal medium for carbon utilization: PRIDHAM-GOTTLIEB's inorganic medium (=ISP No. 9).

Cell Chemistry

The whole cell hydrolysate contained LL-diaminopimelic acid, glucose, and ribose, and hence the cell wall belongs to type I and the sugar pattern NC. The phospholipid contained diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol, therefore is placed in type P-II.

Taxonomic Position

The morphology, cultural and physiological characteristics and cell chemistry revealed that strain P950-4 is a species of *Streptomyces*. According to the descriptions of PRIDHAM and TRESNER[1], and WILLIAMS *et al.*[2], the major characteristics of strain P950-4 are as follows: A) aerial mycelium, red (R), B) spore chain, *Spira* (S), C) melanoid pigments, positive (C^+), D) spore surface echinulate (Spiny). According to the descriptions of SHIRLING and GOTTLIEB[3], among the nine species showing the above properties[1], strain P950-4 is closely related to the type strain (ATCC 15888) of *Streptomyces violaceus* (Rossi Doria) Waksman in the medium specificity of melanoid formation, the pH-indicative property of a reddish pigment, and the sugar utilization pattern. Thus, strain P950-4 was identified as *S. violaceus*. It has been deposited with the American Type Culture Collection, Washington D.C., under accession No. ATCC 53807.

Extraction and Purification

The whole harvested broth (50 liters, pH 8.4) was extracted with butanol (25 liters) under vigorous stirring. The separated butanol phase was evaporated under reduced pressure to an aqueous solution (1 liter) which was extracted three times with 1 liter each of ethyl acetate. The organic extracts were combined and concentrated *in vacuo* to 200 ml of the solution. The solution was added dropwise to 2.3 liters of *n*-hexane under stirring. The precipitates deposited were collected by filtration and dried *in vacuo* to yield the crude solid of hydramycin (6.9 g).

The solid was dissolved in methylene chloride (50 ml) and applied on a column of silica gel (5.0 × 50 cm) which had been pre-washed with methylene chloride. The elution was carried out with methylene chloride and increasing amount of methanol (99.5 : 0.5 ~ 98 : 2). The eluate was checked by the bioassay using *Bacillus subtilis* M45 (Rec^- mutant) and the active fractions eluted with methylene chloride - methanol (99 : 1 ~ 98 : 2) were pooled and concentrated *in vacuo*. Upon standing in a cold room, the concentrate deposited yellowish orange needles of pure hydramycin (109 mg). Rechromatography of the mother liquor and the subsequent active eluate afforded further amount of pure hydramycin (120 mg).

Physico-chemical Properties

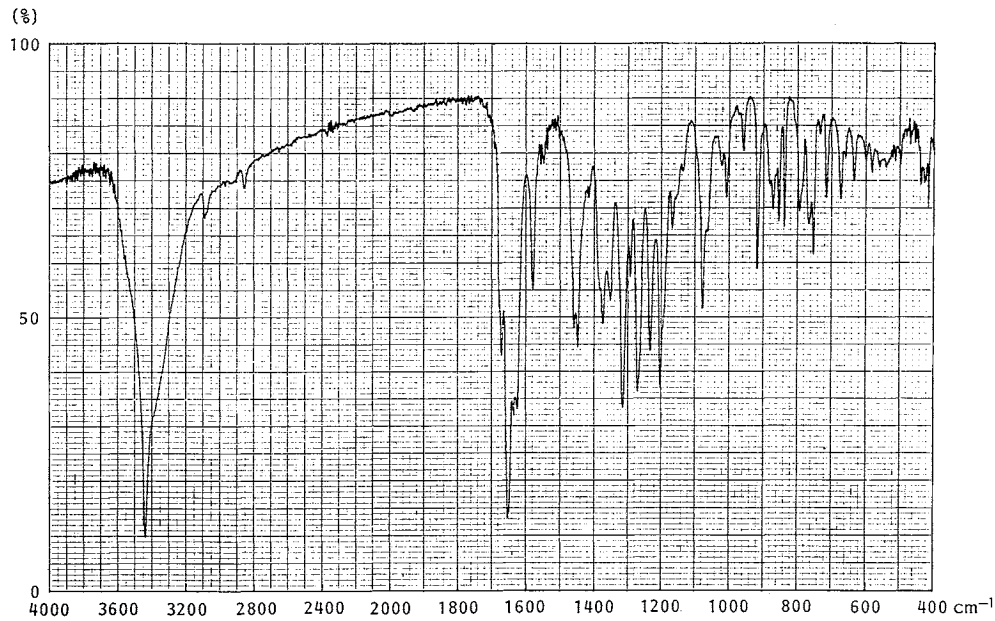
Hydramycin was obtained as yellowish orange needles which melted at 255°C with decomposition. It was soluble in dimethyl sulfoxide and *N*,*N*-dimethylformamide, slightly soluble in chloroform, methylene chloride and alkaline water and almost insoluble in other organic solvents and water. It gave positive response to ferric chloride reagent but gave no coloration with ninhydrin, anthrone and Sakaguchi tests. The physico-chemical properties of hydramycin are summarized in Table 3. The molecular formula of the antibiotic was established as $C_{22}H_{16}O_8$ by the mass spectrum (M^+ *m/z* 408) and microanalysis. Hydramycin exhibited the UV absorption maxima at 240, 267, 286 (sh) and 417 nm in methanol and acidic methanol which shifted to 243, 282 (sh), 324 and 518 nm in alkaline solution. The IR spectrum in KBr (Fig. 2) showed characteristic absorptions at 1670, 1650, 1640, 1630 and 1580 cm^{-1} suggesting a quinone moiety. The ^1H and ^{13}C NMR spectra are shown in Figs. 3 and 4, respectively.

Table 3. Physico-chemical properties.

Nature	Yellowish orange powder	
MP	255°C (dec)	
$[\alpha]_D^{25}$	+26° (*c* 0.25, DMF)	
UV λ_{max} nm (ε)		
in MeOH	240 (36,900), 267 (19,900), 286 (sh), 417 (6,800)	
in 0.1 N HCl-MeOH	240 (32,200), 267 (17,500), 286 (sh), 417 (6,200)	
in 0.1 N NaOH-MeOH	243 (35,700), 282 (sh), 324 (8,000), 518 (5,500)	
Analysis	Calcd for $C_{22}H_{16}O_8$:	Found:
	C 64.71	C 64.86
	H 3.95	H 3.93
EI-MS (*m/z*)	408 (M^+)	
TLC, SiO_2	Rf 0.51[a]	
	Rf 0.63[b]	

[a] CH_2Cl_2-MeOH (9:1).
[b] EtOAc-MeOH (4:1).

Fig. 2. IR spectrum of hydramycin.



Structure Determination

The UV absorptions and observed pH shifts of hydramycin suggested a close resemblance to those of the pluramycin group antibiotics. The ^1H NMR spectrum revealed the presence of a total of 16 protons which were analyzed as 5 aromatic protons and 1 methyl, 2 methylene, 1 methine and 3 hydroxy groups. The presence of these functionalities were ascertained by the ^{13}C NMR spectrum (Fig. 4). The spectrum exhibited 3 carbonyl (178.0, 181.2, 186.6 ppm), 14 sp^2 (with proton $\times 5$, 111.3, 118.6, 124.6 $\times 2$, 136.5 ppm and without proton $\times 9$, 116.7, 119.7, 125.7, 132.0, 135.6, 148.2, 155.5, 161.3, 168.9 ppm) and 1 quarternary carbon (74.2 ppm) in addition to 1 methyl (23.1 ppm), 2 methylene (41.8, 65.1 ppm) and 1 methine carbon (53.2 ppm) signals. Unlike those of the common pluramycin group antibiotics, the ^1H and ^{13}C NMR spectra of hydramycin did not show the signals assignable to amino or neutral sugar. The molecular

Fig. 3. ^{1}H NMR spectrum of hydramycin.

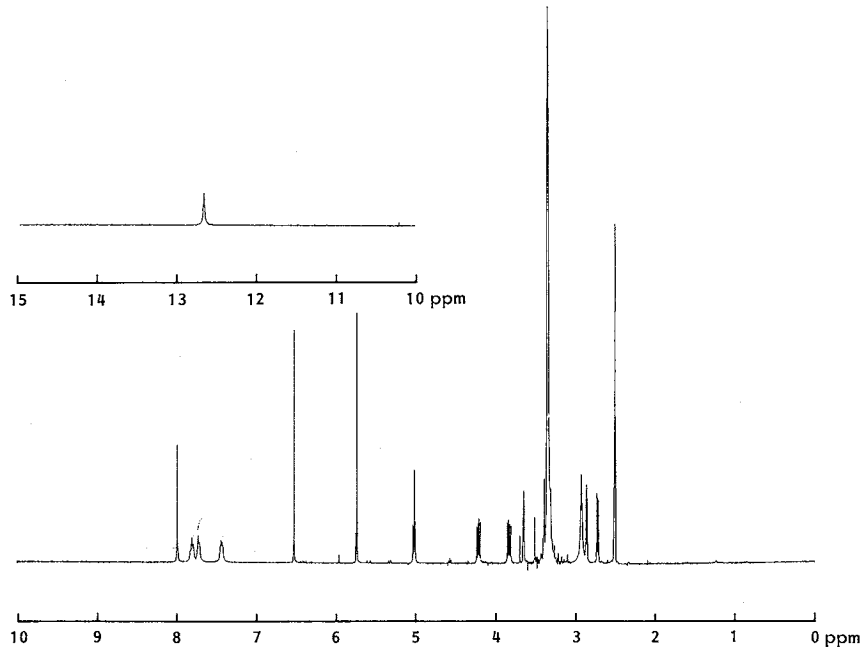
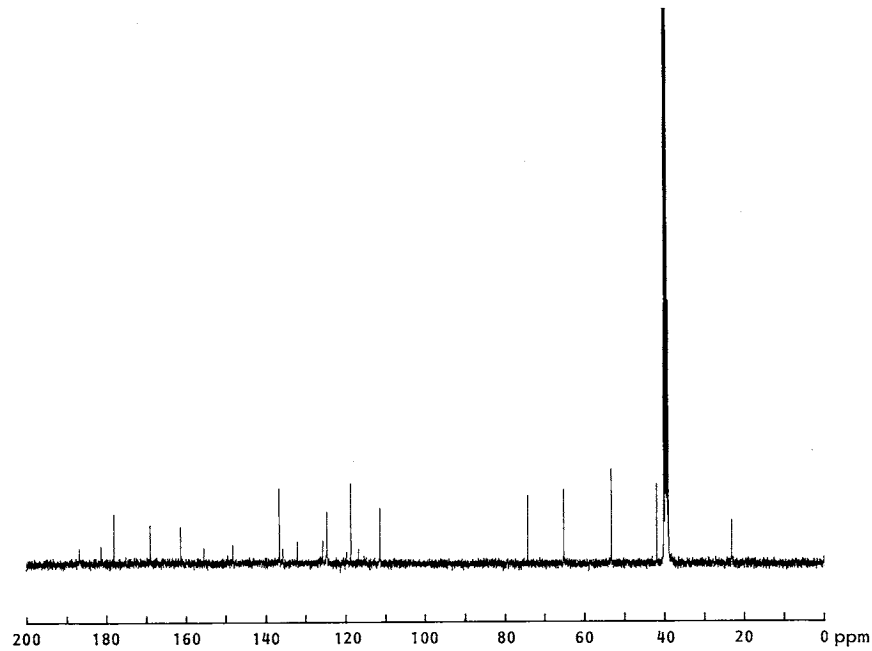
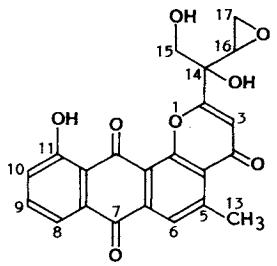


Fig. 4. ^{13}C NMR spectrum of hydramycin.



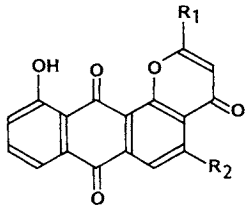
formula assigned to the antibiotic ($C_{22}H_{16}O_8$) also showed the absence of amino functionality in the molecule. The combined information, thus, suggested that hydramycin had a chromophore structure of the pluramycin family of antibiotics. Three antibiotics with such type of structure, indomycinones[4],

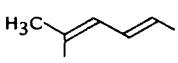
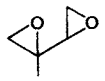
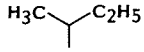
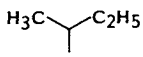
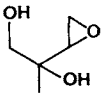
Table 4. ^{1}H NMR data of hydramycin (400 MHz, in DMSO-d_6).



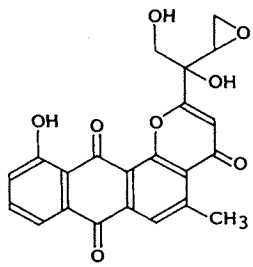
Chemical shift δ (ppm)	Proton	Multiplicity (J=Hz)	Assignments
2.72	1H	dd (4.0, 5.6)	17-H
2.86	1H	dd (2.8, 5.6)	17-H
2.91	3H	s	13-H
3.64	1H	dd (2.8, 4.0)	16-H
3.82	1H	dd (6.1, 11.3)	15-H
4.21	1H	dd (6.1, 11.3)	15-H
5.02	1H	t (6.1)	C-15 OH
5.72	1H	s	C-14 OH
6.52	1H	s	3-H
7.42	1H	d (8.1)	10-H
7.72	1H	d (7.3)	8-H
7.79	1H	dd (7.3, 8.1)	9-H
7.97	1H	s	6-H
12.64	1H	s	C-11 OH

Fig. 5. Structures of the antibiotics related to hydramycin.



	R_1	R_2
α-Indomycinone		$-CH_3$
SF-2330		$-CH_3$
SS43405D		$-CH_2-COOH$
SS43405E		$-CH_3$
Hydramycin		$-CH_3$

SF-2330[5] and SS43405D[6] and E[7] (Fig. 5) have been reported. The ^{1}H and ^{13}C NMR data described for SF-2330 were nearly identical with those of hydramycin in the chromophore moiety and the differences appeared to reside only in the alkyl side chain of 4-carbon unit (^{13}C NMR: 41.8 t, 53.2 d, 65.1 t and 74.2 s). The behavior of UV maxima of hydramycin and SF-2330 was consistent at different pHs. The ^{1}H NMR spectrum (in DMSO-d_6) of the side chain moiety showed 7 protons, which were analyzed at two ABX's (2.72, 2.86, 3.64 ppm and 3.82, 4.21, 5.02 ppm, Table 4) and an isolated hydroxyl proton (5.72 ppm). Upon D_2O addition, the hydroxyl proton and one of the lower-field ABX proton (5.02 ppm) disappeared with concomitant collapsing of the ABX to a ABq. The spectroscopic results taken together with the earlier physico-chemical data indicated the following structure for hydramycin.



Antimicrobial Activity

Antimicrobial activity of hydramycin was tested against bacteria and fungi by 2-fold serial dilution in agar media. As described in Table 5, hydramycin exhibited remarkable inhibitory activity against Gram-positive bacteria but relatively weak activity against Gram-negative bacteria and anaerobic bacteria. Yeast and fungi were not susceptible to the antibiotic.

Table 5. Antimicrobial activity.

Test organism	Medium[a]	MIC (μg/ml)	Test organism	Medium[a]	MIC (μg/ml)
Staphylococcus aureus FDA 209P	NA	0.05	*Proteus vulgalis* A9436	NA	6.3
S. aureus Smith	NA	0.05	*P. mirabilis* A9554	NA	>50
S. aureus A20239	NA	0.05	*Bacteroides fragilis* A22035	GAM	0.1
S. epidermidis D153	NA	0.1	*Clostridium difficile* A21675	GAM	0.4
Streptococcus faecalis A9612	NA	0.4	*C. perfringens* A9635	GAM	0.4
S. pyogenes A20201	NA	0.1	*Propionibacterium acnes* A21933	GAM	0.8
Micrococcus luteus No. 1001	NA	0.025	*Candida albicans* IAM 4888	SDA	>50
Bacillus subtillis PCI 219	NA	0.05	*Cryptococcus neoformans* D49	SDA	>50
Escherichia coli NIHJ	NA	>50	*Aspergillus fumigatus* IAM 2530	SDA	>50
Klebsiella pneumoniae D11	NA	6.3	*Trichophyton mentagrophytes* D155	SDA	>50
Pseudomonas aeruginosa A9930	NA	>50			

[a] NA: Nutrient agar, GAM: Gifu anaerobic medium agar, SDA: Sabouraud dextrose agar.

Table 6. *In vitro* cytotoxicity against murine and human tumor cells.

Compound	IC_{50} (μg/ml)		
	B16-F10	Moser	HCT-116
Hydramycin	0.0009	0.0046	0.0026
Mitomycin C	0.50	1.2	0.80

Table 7. Inhibition of macromolecule synthesis in L1210 leukemia cells.

Compound	IC_{50} (μg/ml)		
	DNA	RNA	Protein
Hydramycin	0.013	0.029	0.039
Mitomycin C	1.7	>100	>100

Table 8. Antitumor activity against P388 leukemia (ip).

Compound	Dose[a] (mg/kg/day)	MST (day)	T/C (%)	Body weight change on day 4 (g)	Compound	Dose[a] (mg/kg/day)	MST (day)	T/C (%)	Body weight change on day 4 (g)
Hydramycin	1.0	7.0	70	−2.5	Hydramycin	0.008	11.0	110	+1.0
	0.5	14.5	145[b]	−2.3	Mitomycin C	2	20.0	200[b]	−1.8
	0.25	12.5	125[b]	−1.8		1	14.5	145[b]	−0.3
	0.13	14.0	140[b]	−0.5		0.5	15.0	150[b]	−0.5
	0.063	13.0	130[b]	+0.8		0.25	13.0	130[b]	+1.5
	0.031	12.5	125[b]	+1.3	Vehicle	—	10.0	—	+1.4
	0.016	11.5	115	+1.0					

[a] QD × 3, ip.
[b] Significant antitumor effect (T/C ≧ 125%).

Antitumor Activity

Hydramycin was tested for *in vitro* cytotoxicity against murine and human tumor cells, for inhibition of macromolecular synthesis and for *in vivo* antitumor activity in mice using mitomycin C as a reference. B16-F10 (murine melanoma), Moser (human colon carcinoma) and HCT-116 (human colon carcinoma) cells were grown as described previously[8].

The cytotoxic activities against the above tumor cell lines were determined colorimetrically at 540 nm after staining viable cells with neutral red. The results were summarized in Table 6. Cytotoxicities of hydramycin were quite potent against these tumor cells and were approximately 300 ~ 600 times superior to those of mitomycin C in terms of IC_{50} value. Inhibitory effects of hydramycin on macromolecule (DNA, RNA and protein) synthesis were determined *in vitro*. Cultured L1210 murine leukemia cells (5×10^5 cells/ml) were incubated with test materials at 37°C for 15 minutes. Labeled precursor, [^{3}H]thymidine,

[^{14}C]uridine or [^{3}H]leucine was added into the cultured mixtures and further incubated for 60 minutes. After washing with chilled 5% trichloroacetic acid solution, the radioactivity incorporated into the acid-insoluble fraction of the tumor cells was determined in a liquid scintillation counter. As shown in Table 7, hydramycin inhibited DNA, RNA and protein synthesis non-specifically. *In vivo* antitumor activity of hydramycin was tested in the experimental mouse tumor system. Female CDF_1 mice were intraperitoneally inoculated with 0.4 ml of diluted ascitic fluid containing 10^6 lymphocytic leukemia P388 cells. Test compounds were administered intraperitoneally once a day on days 1, 2, 3 (QD × 3). As shown in Table 8, hydramycin demonstrated relatively broad and moderate chemotherapeutic activity against P388 leukemia with maximum T/C value of 145%.

Discussion

A new antitumor antibiotic hydramycin was isolated from the cultured broth of an actinomycete strain P950-4. It was inhibitory for Gram-positive bacteria, some Gram-negative bacteria and anaerobes. In addition to the antimicrobial activity, hydramycin was strongly cytotoxic to the tumor cells tested and exhibited life prolongation of mice implanted with P388 leukemia. The structural study disclosed that hydramycin was structurally related to α-indomycinone, SF-2330 and SS43405E having the common chromophore, 11-hydroxy-5-methyl-4*H*-anthra[1,2-*b*]pyran-4,7,12-trione. These antibiotics and SS43405D were differentiated from the pluramycin group antibiotics by the absence of aminosugar attached to the chromophore by *C*-glycoside linkage. Hydramycin differs from the above three related antibiotics in the alkyl side chain at C-2. α-Indomycinone was reported to have no antimicrobial activity, while SF-2330 and SS43405D were shown to have antibacterial activity. No antitumor activity was so far reported on these groups of antibiotics. It is very interesting that hydramycin, an alkyl side chain analog of the above known antibiotics, showed potent antimicrobial and antitumor activity.

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