SCREENING OF ANTIBIOTICS PREFERENTIALLY ACTIVE AGAINST *ras* ONCOGENE-EXPRESSED CELLS

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During the course of *in vitro* screening of agents which are preferentially active against *ras* oncogene-expressed cells, a new anthracycline (identified as 2-demethylsteffimycin D) and a heptaene (possibly a new member of partricins) were isolated from microbial fermentation broths. Among known compounds tested, 5-fluorouracil, 5-fluorodeoxyuridine and oxanosine showed high selectivity towards *ras* oncogene-expressed cells.

An ideal antitumor drug should be one that is active against tumor cells but not active against normal cells. Therefore, it is desirable to determine effects of a potential antitumor compound on appropriate pairs of tumor cells and normal cells early in a screening program. It is known that malfunctioning of cellular oncogenes is a cause of human cancer[1,2], and many human cancers appear to be associated with the expression of activated *ras* genes[3]. We, therefore, have been screening *in vitro* microbial products which are preferentially active against *ras* oncogene-expressed cells. Two pairs of oncogene-expressed (ras^+) and unexpressed (ras^-) types of cells are used in our screening: (1) a rat kidney cell line integrating temperature sensitive v-K-*ras* (ras^{ts}NRK), grown at 33°C (ras^+) and at 39°C (ras^-), and (2) another rat kidney cell line integrating the wild type v-K-*ras* (*ras*NRK, ras^+) and any of the two nontumorous rat kidney cell lines (either NRK-52E or NRK-49F, ras^-). The cell lines of (2) were grown at an ordinary temperature, 37°C. With these cell lines, two types of screening were conducted: One was to find antibiotics which would preferentially inhibit the growth of ras^+ cells (Effect A). The other was to find antibiotics which would alter the tumor cell morphology of ras^+ cells to the normal cell morphology of ras^- cells (Effect B). In our screening study, we have isolated a new anthracycline with Effects A and B and possibly a new heptaene with Effect A from microbial products. Various known compounds were also tested for their possible effects in respect of Effects A and/or B. This paper reports results of these studies.

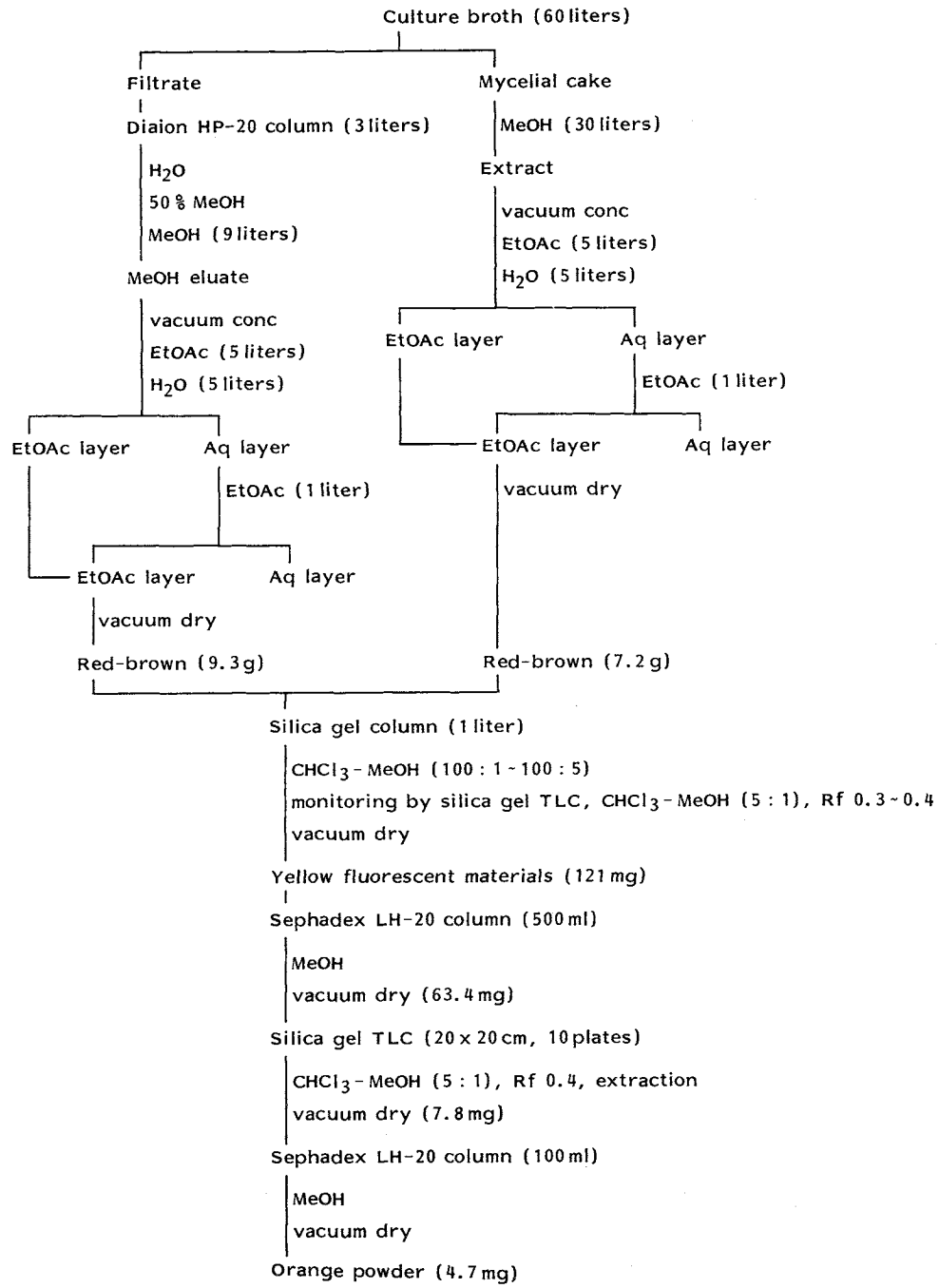
Materials and Methods

Cell Lines and Culture Conditions

A nontumorous rat kidney cell line (NRK) infected with ts371 Kirsten murine sarcoma virus (ras^{ts}NRK)[4] was a gift of Dr. T. Y. SHIH, NIH, Bethesda, MD. The following cell lines were obtained from ATCC: NRK infected with the wild type Kirsten murine sarcoma virus (*ras*NRK) (strain CRL-1569) and nontumorous NRK's (NRK-49F and NRK-52E) (strains CRL-1570 and CRL-1571, respectively).

Cells were grown in DULBECCO's modified Eagle medium (DMEM; Nissui Pharm. Co., Tokyo) supplemented with 100 μg/ml kanamycin (Meiji Seika Kaisha, Ltd., Tokyo) and 2~5% heat-inactivated calf serum (Gibco Lab., Grand Island, NY) at 33°C or 39°C for *ras*^{ts}NRK or at 37°C for the other cell lines, in 5% CO_2-containg humidified air. Cells were seeded at a density of $1 \sim 2 \times 10^4$ cells/2 ml/well in Costar 12-well tissue culture clusters (day 0). The cells received test samples on day 1 and were incubated further until day 3. During the culture period, the cells were photographed, if necessary, on a Nikon

Fig. 1. Isolation procedure of demethylsteffimycin D.



phase-contrast microscope (Diaphot-TMD) using Fuji Neopan SS films. Cell growth was determined as follows; cells were counted in a Coulter counter (a) or cells were stained with crystal violet (0.1% (w/v) in 20% methanol, 0.5 ml/well) followed by a brief wash with tap water, dried, and the dye was extracted into 0.001 N HCl-30% ethanol (2 ml/well) and the absorbance was determined at 570 nm (b). Both determinations produced closely paralleled result. Relative extent of growth in a test run was calculated based on the following equation:

$$\frac{\text{Cell number in a test run on day 3} - \text{Cell number on day 1}}{\text{Cell number in the control run on day 3} - \text{Cell number on day 1}} \times 100\ (\%)$$

Spectroscopy

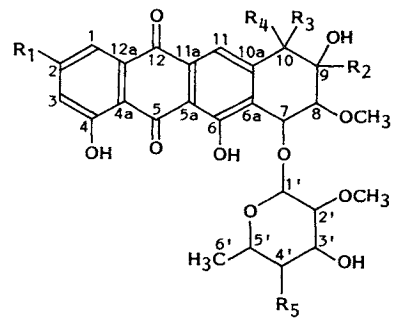
Mass spectra were recorded on a Jeol JMS-HX110 and Hitachi M-80 H. NMR spectra were recorded on a Jeol JUM-GX400 spectrometer.

Results and Discussion

Isolation and Physico-chemical Properties of 2-Demethylsteffimycin D, a New Member of Anthracyclines

The producer strain, *Streptomyces* sp. MH 538-30F7, was cultured after an inoculation of 3% of a seed culture broth at 27°C for 7 days in jar fermenters (Marubishi MSJ-U 301) each containing 15 liters of the production medium (see below), under aeration of 15 liters/minute/jar and stirring at 200 rpm. The production medium consisted of corn steep liquor 1.0%, Polypeptone 0.5%, yeast extract 0.2%, NaCl 0.1%, glucose 1.0%, potato starch 1.0%, glycerol 1.0% and $CaCO_3$ 0.3%, with the pH adjusted to 7.4 before autoclaving. Isolation procedures were as shown in Fig. 1. The purified preparation (orange powder) was soluble in MeOH, acetone, acetonitril and EtOAc, sparingly soluble in $CHCl_3$, and insoluble in water and *n*-hexane. FD-MS showed a molecular peak of *m/z* 547 $(M+H)^+$. The optical absorption maxima of the purified preparation (given in nm with ε values in parentheses) in MeOH were 223 (19,500), 255 (12,100), 290 (12,100) and 440 (6,300), in MeOH - 0.01 N HCl were 225 (24,200), 255 (11,600), 266 (12,100), 288 (11,600) and 440 (6,300), and in MeOH - 0.01 N NaOH were 260 (11,600), 295 (12,600) and 530 (4,200). Both the acidic and alkaline shifts of the optical absorption spectra were reversible. The optical absorption, especially in MeOH - 0.01 N HCl, closely resembled those of steffimycins (STFs) C and D, the position 10 of each is methylene, while different from those of STF and STF-B which have an oxo group at the corresponding position[5] (Fig. 2). STF-D has recently been isolated as an inhibitor of collagenase and identified as 4'-demethyl derivative of STF-C[6]. ^1H and ^{13}C NMR data in comparison to those of STF-D are shown in Tables 1 and 2, respectively. It should be noted, in Table 1, that (1) A, B quartets ($J=16.6$ Hz) around 3 ppm, common to STF-C (data not shown) and STF-D, suggested the methylene structure at position 10, (2) the two singlets around 3.5 ppm, as in STF (data not shown) and STF-D, suggested the aliphatic methoxyl

Fig. 2. Structure of steffimycins.



Compounds	R_1	R_2	R_3	R_4	R_5
STF	OCH_3	CH_3	=O		OH
STF-B	OCH_3	CH_3	=O		OCH_3
STF-C	OCH_3	CH_3	H	H	OCH_3
STF-D	OCH_3	CH_3	H	H	OH
dmSTF-D[a]	OH	CH_3	H	H	OH

[a] 2-Demethylsteffimycin D.

Table 1. ^{1}H NMR chemical shifts (400 MHz, acetone-d_6).

Position	Steffimycin D		2-Demethylsteffimycin D	
	δ_H (ppm)	Multiplicity	δ_H (ppm)	Multiplicity
11-H	7.51	s	7.46	s
1-H	7.17	d (2.4)[a]	7.23	d (2.4)[a]
3-H	6.72	d (2.4)	6.57	d (2.4)
7-H	4.98	d (2.0)	5.00	d (2.0)
8-H	~3.5		~3.5	
10-H	3.12, 2.80	ABq (16.6)	3.10, 2.80	ABq (16.6)
14-H	1.31	s	1.31	s
Aromatic OMe	3.98	s		
1'-H	5.48	d (1.5)	5.49	d (1.5)
5'-H	3.79	dq (6.4, 9.2)	3.78	dq (6.4, 9.0)
3'-H	~3.5		~3.5	
2'-H	3.44	br s (1.5, 2.9)	3.42	dd (1.5, 3.6)
4'-H	3.40	dt (4.4, 8.8, 8.8)	3.38	t (9.0, 9.0)
6'-H	1.32	d (6.4)	1.32	d (6.4)
OMe	3.60	s	3.58	s
OMe	3.49	s	3.49	s
OH	12.08	s	~12.4	br
OH	12.79	s	~13.2	br

[a] Coupling constant (J=Hz).

Table 2. ^{13}C NMR chemical shifts (100 MHz, acetone-d_6).

Position	STF-D	dmSTF-D	Position	STF-D	dmSTF-D
	δ_C (ppm)	δ_C (ppm)		δ_C (ppm)	δ_C (ppm)
C-5	191.8	190.6	C-7	74.7	74.7
C-12	181.6	182.5	C-8	86.3	86.3
C-2	167.8[a]	170.2[a]	C-9	69.9	69.9
C-4	166.2[a]	166.7[a]	C-10	41.4	41.4
C-6	163.5	163.4	2-OCH_3	56.8	—
C-10a	147.6	146.7	8-OCH_3	59.2	59.1
C-11a	136.1	136.4	9-CH_3	24.7	24.7
C-12a	133.1	133.3	C-1'	102.2	102.1
C-6a	130.2	130.0	C-2'	81.8	81.8
C-5a	114.5	114.9	C-3'	72.4	72.4
C-11	120.9	120.5	C-4'	73.9	73.9
C-4a	110.8	109.0	C-5'	70.8	70.7
C-1	108.9	111.8	5'-CH_3	18.2	18.2
C-3	107.2	108.9	2'-CH_3O	59.2	59.2

[a] Assignments may be interchanged.

structure at positions 8 and 2' and (3) the lack of 3.98 ppm signal in the new compound, as opposed to STF-D, indicated the absence of aromatic methoxyl. As shown in Table 2, ^{13}C NMR chemical shifts of the new compound agreed well with those of STF-D except that the former lacked 56.8 ppm peak of methoxyl and that there were some differences for C-2 and its neighbors, C-1 and C-3. All these data indicated that the newly isolated compound was 2-demethylsteffimycin D (dmSTF-D, Fig. 2).

Biological Activities of dmSTF-D

dmSTF-D inhibited *in vitro* the growth of *ras*NRK more strongly than inhibited that of NRK-49F.

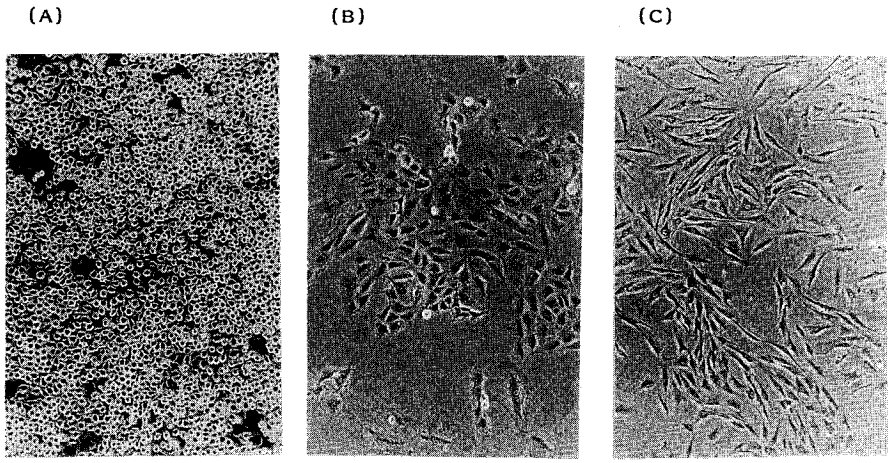
Table 3. Effects of various compounds on *in vitro* growth of *ras*-expressed and -unexpressed cells.

	ras^{ts}NRK			*ras*NRK (c)	NRK-52E (d) or (NRK-49F)	(d)/(c)
	33°C (a)	39°C (b)	(b)/(a)			
5-FU	0.15	0.15	1.0	0.036	0.17	4.6
5-Fluorouridine	0.0092	0.0084	0.9			
5-FUdR	0.00030	0.000094	0.3	0.00027	0.0012	4.4
Cytosine arabinoside	0.33	0.24	0.7			
5-Azacytidine	3.6	3.4	0.9			
Methotrexate	0.084	0.0023	0.3	0.032	0.01>	0.3>
Mitomycin C	0.17	0.1>	0.6>			
Chromomycin A_3	0.17	0.15	0.9			
Doxorubicin	0.14	0.068	0.5	0.22	(0.043)	0.2
Aclarubicin	0.14	0.13	0.9			
Cisplatin	0.63	0.40	0.6	0.25	0.33	1.3
Trichomycin	0.0013[a]	0.0035[a]	2.7	0.014[a]	0.015[a]	1.1
Amphotericin B	0.66	0.60	0.9	0.9	2.25	2.5
Filipin	16	17	1.1	38	23	0.6
Nystatin	50	55	1.1			
Oxanosine	0.3[b]	3.0	10	4.9	>40	>8
Mycophenolic acid	0.38[b]	1.3	3.3			

Values represent μg/ml except. [a] OD at 382 nm. [b] Cell morphology altered.

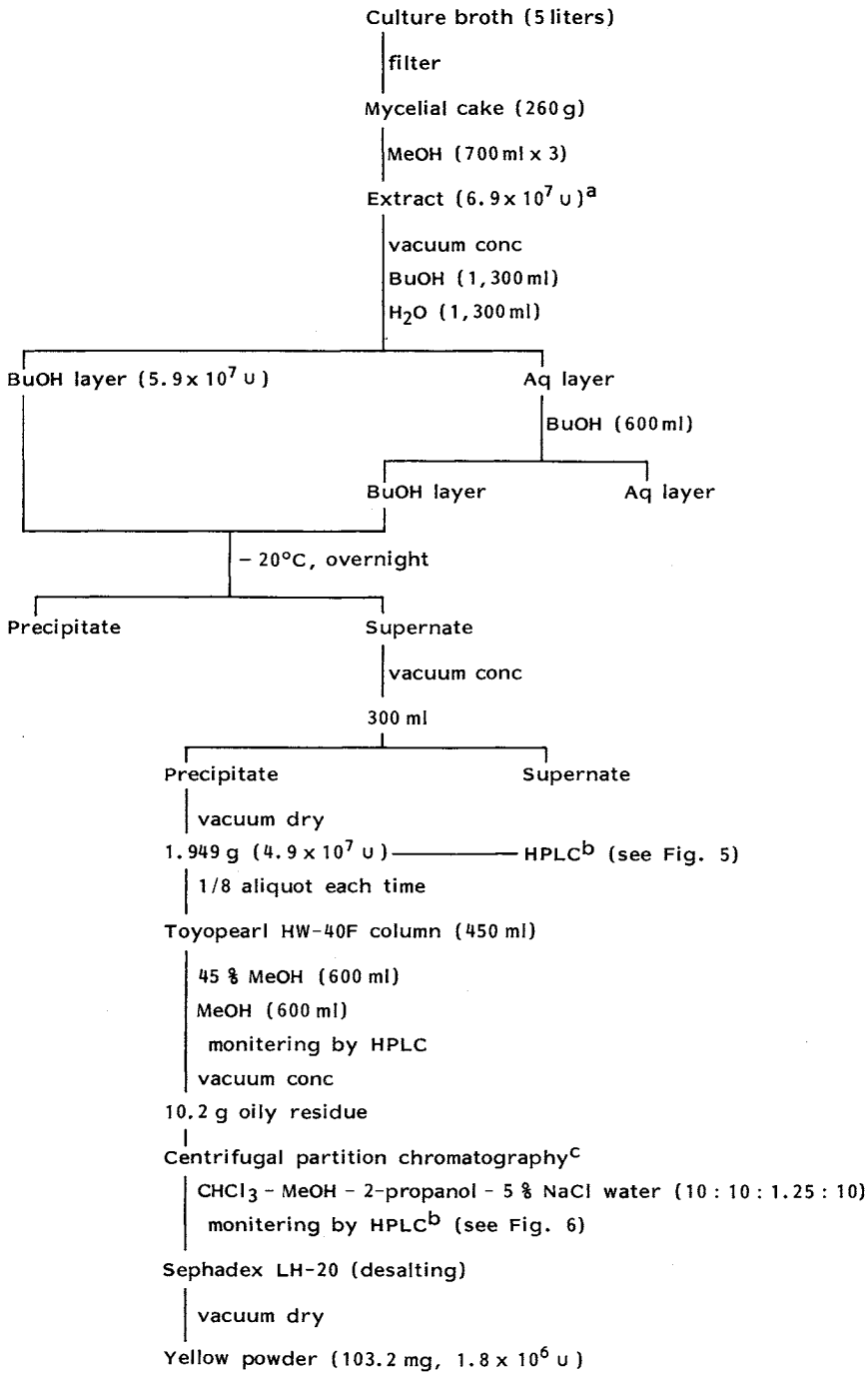
Fig. 3. Effect of dmSTF-D on the morphology of *ras*-expressed cells.

(A) *ras*NRK. (B) *ras*NRK treated with the fermentation broth of *Streptomyces* sp. MH 538-30F7 (10 μl/2 ml/well for 2 days). dmSTF-D at 1 μg/ml showed similar effect. (C) NRK-49F.



IC_{50} (the concentration of a drug required for 50% growth inhibition) for *ras*NRK and NRK-49F were 0.73 μg/ml and 5.8 μg/ml, respectively. No such selectivity was observed with STF-D and doxorubicin and the latter was somewhat more toxic to NRK-49F than to *ras*NRK (Table 3). Another characteristic of dmSTF-D activity towards *ras*$^+$ cells was its ability to alter cell morphology from "transformed" to "normal-like" at concentrations somewhat higher than IC_{50} (Fig. 3). No other anthracyclines showed this activity. dmSTF-D did not alter significantly the cell morphology of an NRK cell line expressing v-*src* even at concentrations totally inhibiting cell growth. Antibacterial activity of dmSTF-D was similar in

Fig. 4. Isolation procedure of a patricin-like heptaene.



[a] One unit was defined as an enough amount (μl or μg) of any preparation to cause 50% inhibition of growth of *ras*^{ts}NRK at 33°C in the assay system described under methods.

[b] μ-Bondapack sphere 5 μm C_{18}-100A, 3.9 mm × 15 cm (Waters Co.) eluted with 0.05 M sodium citrate-35% CH_3CN at 1 ml/minute.

[c] CPC apparatus model NMF (Sanki Engineering Co.).

Fig. 5. HPLC profile of the crude preparation (see Fig. 4).

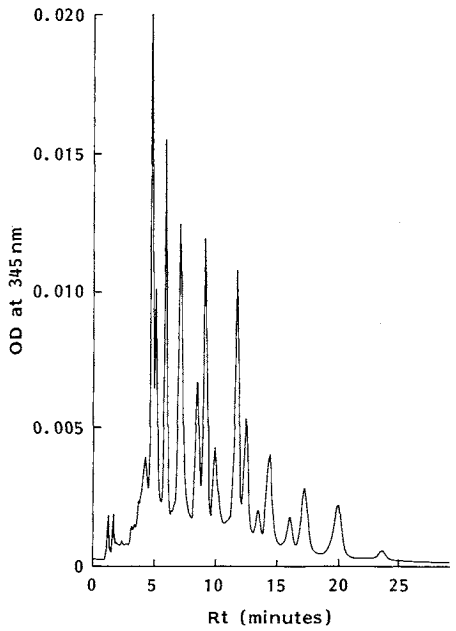
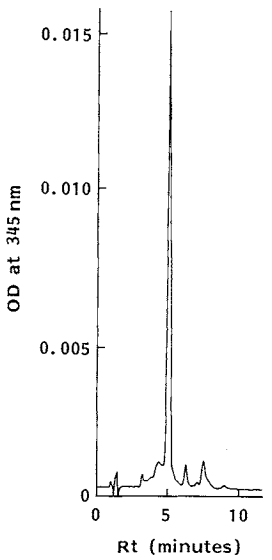


Fig. 6. HPLC profile of the purified preparation (see Fig. 4).



spectrum to those of other steffimycins[5] but only 1/4 to 1/8 as active as, for example, STF-B. The low yield of pure dmSTF-D from fermentation broths has made further studies difficult.

Isolation and Physico-chemical Properties of a Heptaene, Possibly a New Member of Partricins

The producer strain, *Streptomyces* sp. SA-1795, was cultured upon an inoculation of 1% of a seed culture broth at 27°C for 5 days on a rotary shaker (180 rpm) in 500-ml baffled Erlenmeyer flasks each containing 110 ml of a production medium consisting of glycerol 2.5%, beef extract 0.5%, Polypeptone 0.5%, yeast extract 1.0%, NaCl 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, KH_2PO_4 0.05%, and $CaCO_3$ 0.03% with the pH adjusted to 7.4 before autoclaving. The isolation procedure is shown in Fig. 4. The optical absorption maxima of the purified preparation in MeOH (given in nm with ε values in parentheses) were 345 (19,800), 360 (31,500), 380 (49,000) and 405 (41,000). Because of the instability of the purified preparation, exact identification with known heptaenes has not been possible. The ^1H and ^{13}C NMR analysis, however, strongly suggested its close resemblance to partricins[7] (data not shown). A FAB-MS experiment gave clear parent peaks of 1,097 $(M-H)^-$ and 1,099 $(M+H)^+$ daltons, suggesting that this compound is a new member of partricins because no partricins has been reported to have this MW (1,098 daltons).

Biological Activities of the Newly Isolated Heptaene

The purified preparation inhibited the growth of *ras*^{ts}NRK at 33°C more strongly than inhibited at 39°C; IC_{50} for the 33°C (*ras*$^+$) and 39°C (*ras*$^-$) cultures were 0.04 μg/ml and 0.12 μg/ml, respectively. This heptaene failed to show such selectivity towards *ras*-expressed cells when tested with the pair of *ras*NRK (*ras*$^+$) and NRK-52E (*ras*$^-$); IC_{50} for the former and the latter were 0.12 μg/ml and 0.13 μg/ml, respectively. The antibacterial spectrum of the newly isolated heptaene was like those of known polyenes;

MIC (μg/ml) values by the agar dilution method were *Candida albicans* IMC F-4 3147 (6.25), *Saccharomyces cerevisiae* IMC F-7 (3.12), *Cryptococcus neoformans* IMC F-10 (0.78), *etc.*

Specific Effects of Some known Compounds on *ras*-Expressed Cells

As described above, an attempt to find possible inhibitors of *ras* functions in fermentation broths of microorganisms led to isolation of 2-demethylsteffimycin D and a possibly new member of partricins as candidates. The molecular mechanisms underlying their effects have not been studied yet. In this connection, we have found that oxanosine, a guanosine analog, alters the transformed morphology of *ras*^{ts}NRK to the normal morphology by depleting guanine nucleotides and destabilizing p21[8] in the cells. Known antibiotics of various structures were tested for possible effect on either cell morphology or cell growth and the results are summarized in Table 3. 5-Fluorouracil (5FU) and 5-fluorodeoxyuridine (5FUdR) evidently inhibited growth of *ras*NRK (*ras*$^+$) more strongly than inhibited that of NRK-52E (*ras*$^-$), although no such selectivity was observed with *ras*^{ts}NRK grown at 33°C (*ras*$^+$) and 39°C (*ras*$^-$). The reason for the inconsistency remains unelucidated. Oxanosine and mycophenolic acid, both are inhibitors of inosine monophosphate (IMP) dehydrogenase, showed significant selectivity in inhibiting growth of *ras*$^+$. Expression of an oncogene(s) may alter IMP dehydrogenase to make it more susceptible to inhibitors, as we have shown using v-*src* expressed cells[9,10]. The cell growth inhibition by the two compounds accompanied alteration of cell morphology from "transformed" to "normal", as described above. No other compounds tested were found to alter cell morphology. Amphotericin B and trichomycin showed some selectivity with respect to cell growth inhibition. Mitomycin C, doxorubicin and cisplatin lacked the selectivity, suggesting that they may not be suitable for use in chemotherapy of *ras*-expressed tumors. 5FU and oxanosine in combination are being tested with mice inoculated with an NIH/3T3 strain integrating activated *ras*.

References

1) Duesberg, P. H.: Activated proto-onc genes: sufficient or necessary for cancer? Science 228: 669~677, 1985
2) Klein, G. & E. Klein: Evolution of tumours and the impact of molecular oncology. Nature 315: 190~195, 1985
3) Barbacid, M.: *ras* Genes. Annu. Rev. Biochem. 56: 779~827, 1987
4) Shih, T. Y.; M. O. Weeks, H. A. Young & E. M. Scolnick: p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. 31: 546~556, 1979
5) Brodasky, T. F.; S. Mizsak & J. R. Hoffstetter: Steffimycin C, a new member of the steffimycin anthracyclines. Isolation and structural characterization. J. Antibiotics 38: 849~855, 1985
6) Ogawa, K.; H. Iinuma, H. Naganawa, T. Takeuchi, T. Aoyagi & H. Umezawa: Steffimycin D, a novel inhibitor of collagenase. J. Antibiotics, in preparation
7) Tweit, R. C.; R. C. Pandey & K. L. Rinehart, Jr.: Characterization of the antifungal and antiprotozoal antibiotic partricin and structural studies on partricins A and B. J. Antibiotics 35: 997~1012, 1982
8) Itoh, O.; S. Kuroiwa, S. Atsumi, K. Umezawa, T. Takeuchi & M. Hori: Induction by the guanosine analogue oxanosine of reversion toward the normal phenotype of K-*ras*-transformed rat kidney cells. Cancer Res. 49: 996~1000, 1989
9) Uehara, Y.; M. Hasegawa, M. Hori & H. Umezawa: Differential sensitivity of RSV^{ts} (temperature-sensitive Rous sarcoma virus)-infected rat kidney cells to nucleoside antibiotics at permissive and non-permissive temperatures. Biochem. J. 232: 825~831, 1985
10) Uehara, Y.; M. Hasegawa, M. Hori & H. Umezawa: Increased sensitivity to oxanosine, a novel nucleoside antibiotic, of rat kidney cells upon expression of the integrated viral *src* gene. Cancer Res. 45: 5230~5234, 1985