Notes

ANISOMYCIN AND NEW CONGENERS ACTIVE AGAINST HUMAN TUMOR CELL LINES

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Anisomycin was first reported in 1954 as an antibiotic which was notably active against certain pathogenic protozoa, such as *Trichmonas vaginalis*.[1] It inhibits protein synthesis in eukaryotic cells, and also exhibits cytotoxicity to HeLa cells.[2] While several other reports on anisomycin or its analogs were concerned with antiprotozoal or herbicidal activities,[3,4] we rediscovered anisomycin in the course of our antitumor screening using human tumor cell lines. Here we report that anisomycin and some new relatives were isolated as antitumor substances showing cytotoxicity against human tumor cell lines *in vitro*.

The active substances named 3097-compounds were obtained from a fermentation broth of *Streptomyces* strain SA3097 isolated from a soil sample collected from a sub-marine deposit on the coast of Miura City, Kanagawa, Japan. For fermentation, the strain SA3097 was inoculated into 500 ml volume Erlenmeyer flasks each containing 100 ml of the following medium; Jamarin S 25% v/v (Jamarin Laboratories), glycerol 0.2%, Bacto-soytone 1% (Difco) and $CaCO_3$ 0.2%. The pH of the medium was adjusted to 7.4 with 0.4 N NaOH before sterilization. After fermentation for 3 days at 27°C, 180 rpm on a rotary shaker, 2 ml of this culture was transferred to 500 ml Erlenmeyer flasks each containing the same fresh medium. The fermentation was carried out on a rotary shaker at 27°C and 180 rpm. After 4 days incubation, the cultured broths were filtered by suction and the resulting filtrates (9.5 liters in total) were pooled and passed through 500 ml of Diaion HP-20 resin (Mistubishi Kasei). The column was washed with 1.5 liters of deionized water and eluted with 2 liters of acetone - 0.05 N HCl (1 : 1). The eluate was concentrated to 200 ml under reduced pressure and applied to 100 ml of CHP-20P resin (Mitsubishi Kasei) column. The adsorbed material was eluted with a gradient mixture of 500 ml of 0.01 N HCl and an equal volume of acetone - 0.01 N HCl (2 : 8). After the active fractions were collected and evaporated to dryness under reduced pressure, the resulting residue was dissolved in MeOH and subjected to silica gel column chromatography (Kieselgel 60 Merck), which was developed with $CHCl_3$ - MeOH (8 : 2). Compounds 3097-A (prolyl-tyrosine diketo-piperazine), B_1 and C_1 (anisomycin), were mainly

Table 1. Physico-chemical properties of 3097 compounds.

	3097-B_1	B_2	C_2
Appearance	White powder	Colorless oil	Colorless oil
FAB-MS *m/z* (M+H)	280	266	224
UV λ_{max}^{MeOH} nm	282, 276, 223	277, 225	277, 225
Color reaction			
Mo-H_2SO_4	+Yellow	+Yellow	+Yellow
Chlorine-Tridine	+	+	+
Ninhydrin	+Orange	+Orange	+Orange
Rf value on TLC[a]			
$CHCl_3$ - MeOH (8 : 2)	0.37	0.15	0.01
$CHCl_3$ - MeOH - conc NH_4OH (4 : 1 : 0.1)	0.74	0.47	0.15
Rm value[b]	0.88		
Soluble	MeOH, water	MeOH, water	MeOH, water

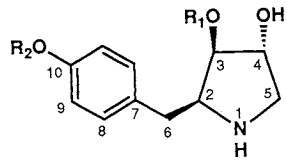
[a] Merck Art No. 5715.
[b] Relative to DL-Alanine, Formic acid - AcOH - H_2O (1 : 3 : 36), 3,000 V, 20 minutes.

Table 2. ^{1}H NMR assignments (δ) of 3097 compounds (400 MHz).

	3097-B_1	B_2	C_2
2	3.79 dt (4.2, 11.4)	3.50 dt (4.0, 8.0)	3.48 dt
3	4.90 br d (3.4)	4.77 br d (4.0)	4.75 d (5.0)
3-$OCOCH_3$			2.10 s
3-$OCOCH_2CH_3$	2.45 m (4.0, 7.2),	2.42 m (3.0, 8.0).	—
	1.19 t (7.2)	1.15 t (8.0)	—
4	4.19 br d (5.0)	4.09 ddd (2.0, 3.0, 6.2)	4.11 d (2.4)
5a	3.43 dd (5.6, 13.0)	3.32 dt (4.0, 8.0)	3.32 dt (4.0, 8.2)
5b	2.91 d (13.0)	2.69 dd (4.0, 12.8)	2.68 dd (4.0, 12.8)
6	2.87 dq (12.2, 6.6)	2.73 d (7.0)	2.73 d (7.0)
8	7.15 d (8.4)	7.00 d (8.4)	7.01 d (8.4)
9	6.86 d (8.4)	6.69 d (8.4)	6.69 d (8.4)
10-OCH_3	3.70 s	—	—

Solvents: CD_3OD.
Reference: TMS (0.0 ppm).

Fig. 1. Structures of 3097 compounds.



3097-B_1: $R_1=COCH_2CH_3$ $R_2=CH_3$
B_2: $R_1=COCH_2CH_3$ $R_2=H$
C_1: $R_1=COCH_3$ $R_2=CH_3$ (anisomycin)
C_2: $R_1=COCH_3$ $R_2=H$
D_1: $R_1=H$ $R_2=CH_3$ (deacetylanisomycin)
D_2: $R_1=H$ $R_2=H$ (deacetyldemethylanisomycin)

isolated in this manner. The fractions containing a mixture of 3097-B_2, C_2, D_1 and D_2 with Rf values of 0.2 or less on silica gel TLC were pooled and evaporated to dryness. The resultant residue was redissolved in MeOH, applied on preparative TLC and developed with $CHCl_3$ - MeOH - conc NH_4OH (4:1:0.1). The final yields of the 6 purified compounds were 15.55 mg for 3097-A, 32.15 mg for B_1, 197.04 mg for C_1, 29.68 mg for B_2, 22.54 mg for C_2, 24.82 mg for D_1 and 20.88 mg for D_2.

Physico-chemical properties of 3097-B_1, B_2 and C_2 are summarized in Table 1. They were easily distinguishable on TLC plates since anisomycin group compounds showed a characteristic yellow color on heating with Mo-H_2SO_4. The UV spectrum of 3097-B_1 was quite similar to that of anisomycin, and the others all resembled each other.

The structures were determined by ^{1}H and ^{13}C NMR spectra using ^{1}H-^{1}H COSY and HMBC experiments. 3097-C_1 was determined as anisomycin according to the physico-chemical properties including the specific rotation ($[\alpha]_D$ −23°C, lit −30°C).[1] ^{1}H NMR spectrum of B_1 showed the substitution of the propionyl group instead of acetyl group of anisomycin. The position of the propionyloxyl group in the pyrrolidine ring was assigned on the basis of chemical shifts of ^{1}H NMR (Table 2). The 3-H of anisomycin should shift downfield due to the acetoxyl group in comparison to 4-H. Such downfield-shift of 3-H also appeared in B_1 as well as anisomycin, so that 3097-B_1 was determined as an anisomycin rather than an isoanisomycin.[5] Similar features were also observed with compounds B_2 and C_2, and their structures were subsequently determined. D_1 and D_2 had no acylation in the pyrrolidine ring. The structures of 3097 compounds are shown in Fig. 1; three of the six compounds obtained turned out to be known ones; C_1 was anisomycin,[1,5,6] D_1 was deacetylanisomycin[5,6] and D_2 was 2-(*p*-hydroxyphenyl)methyl-3,4-dihydroxypyrrolidine (deacetyldemethylanisomycin) resulting from successive hydrolysis of anisomycin.[5] The rest were new congeners of anisomycin.

The activity of the three new congeners against

Table 3. Cytotoxic activity of 3097 compounds.

	IC_{50} ($M \times 10^{-8}$)	
	LU99	MCF7
3097-B_1	6.5	9.0
B_2	8.5	12.9
C_2	5.0	9.5
Anisomycin (C_1)	8.2	7.4
Deacetylanisomycin (D_1)	23	34
Demethyldeacetylanisomycin (D_2)	15,300	17,500

LU99: Human lung carcinoma.
MCF7: Human breast cancer cell.
The cells were suspended in RPMI 1640 medium containing 10% fetal calf serum (2×10^{-4} cells/ml). The cell suspension was inoculated on a 96 well microplate in 100 μl/well followed by culturing at 37°C for a day. After 10 μl of sample was added, culture was continued for further 3 days. The viable cells were counted by reduction with MTT reagent. The sample was prepared by suspending 2 mg/ml of the test compound in methanol and then diluting with medium.

two human tumor cell lines (LU99, MCF7) is listed in Table 3. A structure-activity relationship can be partly discussed as follows. Cytotoxic activity of 3097-B_1 was almost similar to that of anisomycin. Since there is no significant difference in the activity of B and C series of compounds, the difference between acetyl and propionyl groups on the pyrrolidine ring seemed to have little effect on the cytotoxicity. In addition, demethylation at the aromatic ring has no effect as long as the pyrrolidine ring preserves its acyl group. Deacylation of the pyrrolidine ring (D_1) resulted in considerable reduction of activity. The additional demethylation in the aromatic ring (D_2) caused complete loss of activity. Although the acylation of the pyrrolidine ring is considered to be important for antitumor activity, this acetyl group of anisomycin can easily be hydrolyzed under protic condition. BUTLER[5] has discussed that the pyrrolidine ring of anisomycin is conformationally twisted and has an interaction of the nitrogen atom with the carbonyl of the acetyl group. Therefore, the acetyl function can readily form a hydrogen bond followed by hydrolysis which results in reduction of the activity. This kind of deacylation in the pyrrilidine ring has similarly been observed with 3097-B_1.

Several studies on the mechanism of action of anisomycin have been reported and have indicated that the inhibition of protein synthesis was a major cause of cytotoxicity against HeLa cells.[7] With respect to the inhibition activity of protein synthesis, GROLLMAN *et al.*[8] have compared the synthesized deacetyldemethylanisomycin and anisomycin in a cell free system. It turned out that the deacetyldemethylated congener had far lower activity than that of anisomycin. This observation is in agreement with the cytotoxic activity of anisomycin congeners that we examined in this study.

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