WF-3405, A NOVEL ANTITUMOR ANTIBIOTIC

TAXONOMY, ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL PROPERTIES

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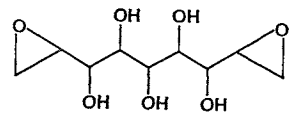
A new antitumor antibiotic, WF-3405 was isolated from the culture of *Amauroascus aureus* F-3405. The structure has been determined as 1,5-dioxiranyl-1,2,3,4,5-pentanepentanol on the basis of spectroscopic and chemical evidence.

WF-3405 exhibits strong inhibitory activity against various murine tumors including leukemia P388, leukemia L1210 and Lewis lung carcinoma.

In the course of a new antitumor screening program for microbial fermentation products with no antibacterial activity, WF-3405[1],†† was discovered in the culture broth of fungus strain F-3405. WF-3405 has a unique chemical structure shown in Fig. 1.

The present paper describes the taxonomy of producing strain, fermentation, isolation procedures and physico-chemical and biological properties and also structure elucidation of WF-3405.

Fig. 1. Chemical structure of WF-3405 (1), 1,5-dioxiranyl-1,2,3,4,5-pentanepentanol.



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Taxonomy of Strain F-3405

We identified the producing strain, freshly isolated from a soil sample collected at the foot of Mt. Mitoku, Tottori Prefecture, Japan, as *Amauroascus aureus* (Eidam) von ARX[2]. The strain's characteristics were as follows.

Cultures on YpSs agar attained 3.5 cm in diameter after two weeks at 25°C. The colony surface was plain, felty and pale yellow to yellowish grey. The reverse was pale yellow. After one month, ascomata were formed on the surface.

The ascomata were globose or subglobose, yellow and 500 to 1,000 μm in diameter. They were often aggregated and formed stroma-like masses, measuring more than 3,000 μm diameter. The peridium consisted of the interwoven hyphae, whose tips were sinuate or spiral. The asci bore irregularly in the ascomata and developed on firmly coiled gametangia in clusters (Fig. 2). They were unitunicate, evanescent, eight-spored, obovoid to pyriform with a short stalk, 13~15.5 μm long and 9~10 μm thick. The ascospores were unicellular, globose, yellow, echinulate but finally reticulate, and 4~5 μm

†† WF-3405 is identical with FR-68504[1].

Fig. 2. Photomicrograph of strain F-3405 showing the ascospores in asci, 600×.

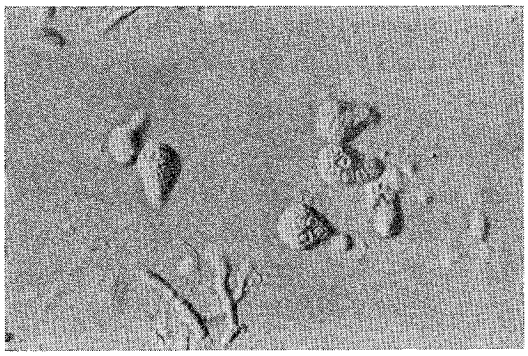
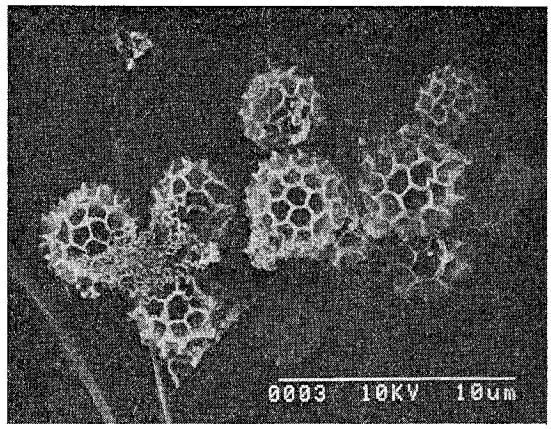


Fig. 3. Scanning electron micrograph of ascospores with reticulate ornamentations of strain F-3405.



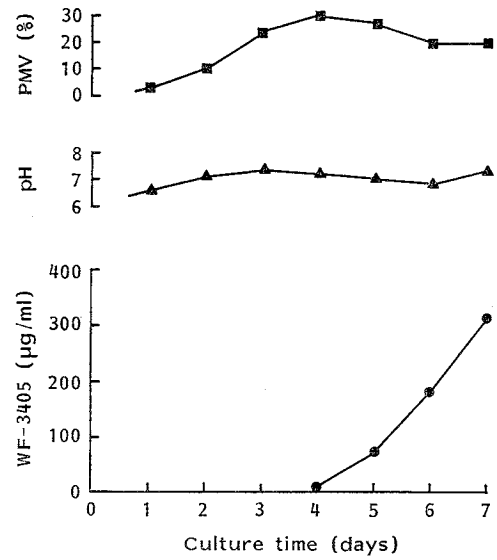
in diameter (Fig. 3). At the tips or in intercalary positions of vegetative hyphae, arthroconidia were produced. They were unicellular, hyaline, smooth, cylindrical or ovoid with truncate base, 8~18 μm long and 4~6 μm thick.

We named the strain *Amauroascus aureus* F-3405, and deposited it in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM P-8008.

Fermentation

A loopful of well-grown agar slant culture of *Amauroascus aureus* F-3405 was inoculated into a seed medium (80 ml) containing soluble starch 1%, corn starch 1%, glucose 1%, cotton seed meal 1%, dried yeast 1%, peptone 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% (pH 6.0) in a 250-ml baffled Erlenmeyer flask, and cultured at 25°C on a rotary shaker with 7.6 cm-throw at 200 rpm for 120 hours.

Fig. 4. Time course of WF-3405 production in a 200-liter jar fermentor.
● WF-3405, ▲ pH, ■ packed mycelium volume (PMV).



The seed culture was then transferred at the rate of 2% to 150 liters of the production medium in a 200-liter jar fermentor and cultivation was carried out for 144 hours at 25°C under aeration of 150 liters/minute, agitation of 200 rpm and inner pressure of 1.0 kg/cm².

The production medium contained glucose 2%, peptone 0.5%, dried yeast 1% and $CaCO_3$ 0.2% (pH 6.5).

The antitumor activity level in the culture filtrate was assayed by cytotoxic activity against P388 murine leukemia cells in tissue culture.

A maximum titer (about 350 μg/ml as WF-3405) was achieved after a 7-day fermentation (Fig. 4).

Isolation and Purification

The fermentation broth (150 liters) was filtered through a bed of filter-aid (Radiolite, 3 kg). The

filtrate (140 liters) was adjusted to pH 6.5 with hydrochloric acid and passed through a column filled with an activated carbon (40 liters). The column was washed with water (200 liters) and eluted with 10% aqueous methanol (160 liters). The active eluate was concentrated *in vacuo* to a volume of 400 ml to yield crude crystals (7 g). Recrystallization from methanol - water gave 4 g of pure colorless crystals.

Physico-chemical Properties and Structural Elucidation

The physico-chemical properties of WF-3405 (**1**) are summarized in Table 1. The neutral substance **1**, which is soluble in water slightly soluble in methyl alcohol, ethyl alcohol and acetone, and insoluble in ethyl acetate and chloroform, showed a positive reaction to ceric sulfate reagent, though negative to iodine, ninhydrin, ferric chloride and Dragendorff reagents. In the ^{1}H NMR spectrum (Jeol JNM-FX270, 270 MHz) in D_2O (Table 1), **1** showed eleven proton resonances in the region of 3.95

Table 1. Physico-chemical properties of WF-3405.

Appearance	Colorless prisms
MP	157~159°C
Specific rotation	$[\alpha]_D^{23}$ −5.8° (*c* 1.0, H_2O)
IR ν_{max}^{Nujol} cm^{-1}	3400, 3250, 1300, 1100, 1030, 950, 850
UV (MeOH)	End absorption
SI-MS	*m/z* 237 $(M+H)^+$
Elementary analysis	Calcd for $C_9H_{16}O_7$: C 45.76, H 6.83.
	Found: C 45.65, H 6.62.
^{1}H NMR (D_2O, TMSP) δ	3.95~3.90 (2H, m), 3.88~3.79 (2H, m), 3.57 (1H, dd, *J*=9.2, 5.3 Hz), 3.36~3.25 (2H, m), 3.01~2.28 (4H, m)

SI-MS: Secondary ion mass spectra.

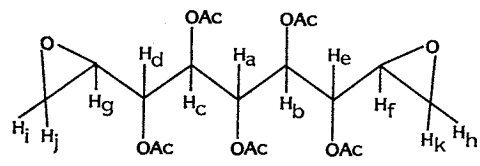
Table 2. ^{13}C NMR data of WF-3405 in D_2O.

Chemical sift[a]	Multiplicity	$^1J_{C-H}$[b]	Assignment
70.9	d	144	CHOH
70.6	d	141	CHOH
70.3	d	142	CHOH
69.6	d	144	CHOH
68.6	d	143	CHOH
55.1	d	177	
53.5	d	176	
46.6	t	177	
46.6	t	177	

[a] In ppm downfield from TMS.
[b] Determined by ^{1}H-coupled ^{13}C NMR spectra with nuclear Overhauser effect (NOE).

Table 3. ^{1}H NMR spectral data of **2** in $CDCl_3$ (400 MHz).

Proton	Chemical shift (ppm)	Multiplicity	*J* in Hz
a	5.49	dd	J_{ac}=10.2, J_{ab}=2.0
b	5.42	dd	J_{be}=9.3, J_{ba}=2.0
c	5.38	dd	J_{ca}=10.2, J_{cd}=2.0
d	4.80	dd	J_{dg}=5.4, J_{dc}=2.0
e	4.69	dd	J_{eb}=9.3, J_{ef}=6.3
f	3.06	ddd	J_{fe}=6.3, J_{fh}=3.9, J_{fk}=2.4
g	2.89	ddd	J_{gd}=5.4, J_{gj}=3.4, J_{gi}=2.4
h	2.78	dd	J_{hk}=4.9, J_{hf}=3.9
i	2.72	dd	J_{ij}=5.4, J_{ig}=2.4
j	2.69	dd	J_{ji}=5.4, J_{jg}=3.4
k	2.59	dd	J_{kh}=4.9, J_{kf}=2.4



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to 2.28 ppm, most signals of which overlaps to prevent the examination of their multiplicities. The ^{13}C NMR spectrum (Jeol JNM-FX270, 67.8 MHz, D_2O) of **1** showed nine carbon signals in the sp^3-carbon region (Table 2), which include five methine signals attributable to secondary alcohol carbons (70.9 (d), 70.6 (d), 70.3 (d), 69.6 (d) and 68.6 (d) ppm). Two methine signals at 55.1 (d) and 53.5 (d) ppm and the remaining two methylene signals at 46.6 (t) ppm $\times 2$ are assignable to two terminal oxirane ring carbons since their $^1J_{C-H}$ values are all quite large (177 or 176 Hz, Table 2).

Acetylation of **1** with Ac_2O in pyridine gave the penta-acetyl derivative **2** (field desorption (FD)-MS, m/z 447 ($M^+ + 1$)). Analysis of the ^1H NMR spectrum (Jeol JNM-GX400, 400 MHz) of **2** with the aid of decoupling experiments led to full assignments of all the proton signals (Table 3) and hence revealed the structure. The following ^{13}C NMR spectral data (Jeol JNM-FX270, 67.8 MHz, $CDCl_3$) of this penta-acetate are also consistent with the structure **2**: 169.9 (s), 169.9 (s), 169.7 (s), 169.6 (s), 169.6 (s), 70.2 (d), 68.8 (d), 68.1 (d), 67.8 (d), 66.3 (d), 51.0 (d), 49.2 (d), 44.9 (t), 44.9 (t), 20.8 (q), 20.6 (q), 20.6 (q), 20.6 (q), 20.6 (q). Consequently, the structure of WF-3405 was determined as being **1**. The study on the stereochemistry and absolute configuration of **1** are the subjects of future work.

Antimicrobial Activity

Antimicrobial activity of WF-3405 was determined by a serial broth dilution method in bouillon medium for bacteria and in Sabouraud medium for fungi and yeasts. The minimum inhibitory concentration (MIC) was expressed in terms of μg/ml after over-night incubation at 37°C and 48~72 hours incubation at 28°C for fungi and yeasts. WF-3405 had no antimicrobial activity against all the bacteria (*Staphylococcus aureus* 209P, *Bacillus subtilis* ATCC 6633, *Escherichia coli* NIHJ JC-2 and *Pseudomonas aeruginosa* NCTC 10490), fungi and yeasts (*Aspergillus oryzae*, *Aureobasidium pullulans* IFO 4466, *Candida albicans*) tested at maximum dose of 1,000 μg/ml.

Antitumor Activity

The antitumor activities of WF-3405 was determined in experimental murine tumor systems in accordance with modified protocols described by U.S. National Cancer Institute[3]. Lymphocytic leukemia P388 and lymphoid leukemia L1210 were implanted intraperitoneally and Lewis lung carcinoma was implanted intradermally into BDF_1 mice (female, 8 weeks old) at an inoculum size of 1×10^6, 5×10^5 and 2×10^6 cells per mouse, respectively. Twenty-four hours after the implantation of tumor cells, graded dose of the antibiotics were administered to mice intraperitoneally. Drug treatments were given intraperitoneally once daily from day 1 to day 4 (qd 1~4). Five mice were used in each test group. WF-3405 was suspended in physiological saline solution (0.9% saline). Control mice received intraperitoneal doses of physiological saline solution. The injection volume was 0.2 ml in all experiments. Doxorubicin hydrochloride (Adriacin-Kyowa) and cisplatin(*cis*-diammine-dichloroplatinum (II) (Randa-Nippon Kayaku)) were suspended in physiological saline solution and comparatively tested simultaneously as reference compounds.

Antitumor activity was evaluated by the mean survival time of a group and also expressed by the T/C % valve (mean survival time of treated group/mean survival time of control group, $\times 100$).

The antitumor activity of WF-3405 was determined comparatively against P388 leukemia. As shown in Table 4, WF-3405 is quite active against P388. Doses between 2~16 mg/kg on the qd$\times$4 scheduled, resulted in significant increase in life span in mice. Doxorubicin and cisplatin are also active against P388 at doses between 0.04~2.5 mg/kg, 1.25~5 mg/kg on the same schedule, respectively.

Table 4. Antitumor activity against P388 leukemia (treatment on days 1, 2, 3 and 4).

Drug	Dose (mg/kg/day)	Mean survival time (days)	T/C (%)
WF-3405	16	25.5	214 (3/5)*
	8	22.3	187
	4	17.7	149
	2	15.9	134
	1	13.5	113
Doxorubicin	2.5	24.9	209 (2/5)*
	0.6	21.9	184
	0.15	16.3	137
	0.04	15.1	127
Cisplatin	5	30.5	256 (5/5)*
	2.5	21.9	184
	1.25	21.5	181
Control	—	11.9	100

* Numbers of survivors at day 30/total mice.

Table 5. Antitumor activity against L1210 leukemia (treatment on days 1, 2, 3 and 4).

Drug	Dose (mg/kg/day)	Mean survival time (days)	T/C (%)
WF-3405	20	11.3	128
	10	13.7	155
	5	10.5	118
	2.5	9.5	107
	1.25	8.9	100
Doxorubicin	2.5	13.1	148
	0.6	10.7	121
	0.15	9.1	103
Cisplatin	2.5	14.7	166
	0.6	11.5	130
	0.15	9.1	103
Control	—	8.9	100

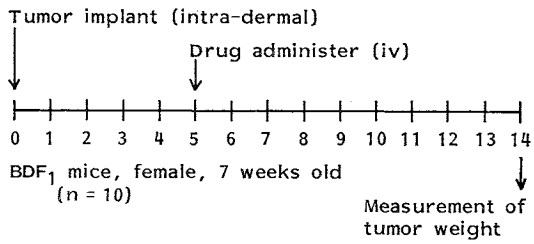
Table 6. Antitumor activity against Lewis lung carcinoma (treatment on days 1, 2, 3 and 4).

Drug	Dose (mg/kg/day)	Mean survival time (days)	T/C (%)
WF-3405	10	10.2	Toxic
	8	25.5	126
	6	32.5	160
	4	27.5	135
	2	25.7	127
	1	27.1	133
Doxorubicin	2.5	28.9	142
Cisplatin	5	12.1	Toxic
	1.25	27.1	133
	0.3	22.3	110
Control	—	20.3	100

Table 7. Antitumor activity against mouse reticulosarcoma M5076.

Drug	Dose (mg/kg)	Average tumor weight (mg)	Tumor inhibition (%)
WF-3405	60	507±131.0	73.5
	40	742±63.7	61.3
	20	1,199±56.0	37.4
	10	1,444±156.6	24.6
	5	1,658±67.3	13.4
	2.5	1,681±49.4	12.2
Cisplatin	10	968±119.8	49.3
	5	1,449±91.1	24.3
	2.5	1,600±93.2	16.4
	1.25	1,654±78.7	13.0
Vehicle		1,915±138.3	

The antitumor activity of WF-3405 was further determined against L1210 leukemia and Lewis lung carcinoma. Doxorubicin and cisplatin were used as reference compounds. As shown in Tables 5 and 6, WF-3405 was quite active in these tumor systems including L1210 and Lewis lung carcinoma.



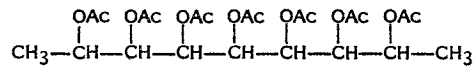
Furthermore, WF-3405 showed antitumor activity against reticulosarcoma M5076 (Table 7). M5076 was implanted intra-dermally into BDF_1 mice (female, 7 weeks old) at an inoculum size of 2×10^6 cells per mouse. The treatment was given in once on day 5. On day 14, the tumor nodules were excised and weighed individually. Ten mice were used in each test group. Cisplatin was suspended in physiological saline solution and comparatively simultaneously as a reference compound. The highest dose of WF-3405 and the reference drugs had high anti-M5076 activity; WF-3405 (60 mg/kg) and cisplatin (10 mg/kg) inhibited tumor growth by 73.5 and 49.3%, respectively. WF-3405

Table 8. Cytotoxicity of WF-3405.

Cell lines	IC_{50} (μg/ml)
P388 (mouse leukemia)	5
L1210 (mouse leukemia)	5
FM3A (mouse mammary carcinoma)	5
3LL (mouse lung carcinoma)	5
B16 (mouse melanoma)	20
PC-3 (human prostate adenocarcinoma)	40
MCF-7 (human mammary carcinoma)	150
BHK-21 (hamster kidney)	150
Bone marrow (BDF_1 mouse)	150

Table 9. Cytotoxicity of **1**, **2** and **3** against P388 leukemia cells.

Compound	IC_{50} (μg/ml)
1	5
2	5
3	700



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exhibited significant anti-M5076 activity.

Cytotoxic Activity

The cytotoxic activity of WF-3405 to 9 cell lines in tissue culture was shown in Table 8.

From these results, WF-3405 exhibited a wide (at least 30-fold) range of activity against the various cell lines.

Acute Toxicity

The acute toxicity of WF-3405 was determined in *dd*Y mice (5 weeks old, female) by a single intraperitoneal injection of graded dose of test compound into 5 mice. The LD_{50} was 50 mg/kg.

Discussion

WF-3405 is highly potent unique epoxide antibiotic representing a novel class of antitumor agent. WF-3405 is structurally related to dianhydrogalactitol (DAG), a hexitol derivative which is effective in clinical trials[4,5]. The DAG has been regarded as alkylating agent acting through its epoxides.

Catalytic hydrogenation of **2** followed by acetylation gave hepta-acetyl compound **3** (electron impact (EI)-MS, m/z 534 (M^+)). **1** and **2** as well as **3** were tested for cytotoxicity with P388 cell cultures. The results are given in Table 9. The cytotoxicity of **3** was reduced remarkably in comparison with those of **1** and **2**. Therefore antitumor activity of WF-3405 is due to the bifunctional epoxide.

The detail mechanism of this antitumor activity is being studied and will be described in a future report.

References

1) KIYOTO, S.; H. MURAI, Y. TSURUMI, H. TERANO & M. KOHSAKA (Fijisawa): A new compound FR-68504, production thereof and use thereof. Eur. Pat. Appl. 0187528, Jan. 31, 1986 [Jpn. Kokai 205491('86), Dec. 18, 1985]
2) ARX, J. A. VON; On *Arachriotus* and related genera of the Gymnoascaceae. Persoonia 6: 371~380, 1971
3) GERAN, I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOTT: Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemother. Rep. 3: 1~103, 1972
4) CHIUTEN, D. F.; M. ROZENCWEING, D. D. VON HOFF & F. M. MUGGIA: Clinical trials with the hexitol derivatives in the U.S. Cancer 47: 442~451, 1981
5) HORVÁTH, I. P.; J. KRALOVÁNSZKY, I. ELEKES, S. ECKHARDT & C. SELLEI: Studies on the mechanism of action of cytostatic hexitol derivatives. *In* Advances in Antimicrobial and Antineoplastic Chemotherapy. Vol. II. Antineoplastic Chemotherapy. Progress in Research and Clinical Application. Proceedings of the VIIIth International Congress of Chemotherapy Pragure, 1971. *Ed.*, M. S. PRAGUE *et al.*, pp. 27~29, Urban & Schwarzenberg, Berlin, 1972