ELSAMICINS†, NEW ANTITUMOR ANTIBIOTICS RELATED TO CHARTREUSIN

I. PRODUCTION, ISOLATION, CHARACTERIZATION AND ANTITUMOR ACTIVITY

MASATAKA KONISHI, KOKO SUGAWARA, FUMIO KOFU, YUJI NISHIYAMA, KOJI TOMITA, TAKEO MIYAKI and HIROSHI KAWAGUCHI

Bristol-Myers Research Institute, Ltd., Tokyo Research Center, 2-9-3, Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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New antitumor antibiotics, elsamicins A and B, were isolated from the culture broth of an unidentified actinomycete strain J907-21 (ATCC 39417). They are structurally related to chartreusin, containing the common aglycone, chartarin, but contain different sugar moieties. Elsamicin A, the major component, has an amino sugar in the molecule which makes the antibiotic much more water-soluble than chartreusin. Elsamicin A exhibits strong inhibitory activity against various murine tumors including leukemia P388, leukemia L1210, and melanoma B16 but elsamicin B which lacks the amino sugar showed only marginal activity. The potency of elsamicin A was 10~30 times more potent than that of chartreusin in terms of minimum effective dose.

In the course of a systematic search for microbial metabolites effective against tumors, an unidentified actinomycete strain J907-21 (ATCC 39417) isolated from a soil sample of El Salvador was found to produce a novel antitumor antibiotic complex designated as elsamicin. The complex was recovered from the fermentation broth by extraction with butanol and separated into a major component, elsamicin A, and a minor, elsamicin B, by a series of chromatographies. Both elsamicins A and B showed antibacterial activity against aerobic Gram-positive bacteria and anaerobic organisms. The activity of elsamicin A was 4~8 times higher than that of elsamicin B and chartreusin. Elsamicin A induced a significant prolongation of survival time in mice bearing leukemia P388, leukemia L1210 or melanoma B16. Elsamicin B was practically devoid of antitumor activity. This paper presents the taxonomy of the producing strain, and details of the fermentation, isolation, and chemical and biological properties of elsamicins A and B. The structure determination of elsamicin will be reported in a separate paper[3].

Producing Organism

Unidentified actinomycete strain No. J907-21 (ATCC 39417) forms well-branched, non-fragmenting vegetative mycelia, but lacks true aerial mycelia. Examination to date indicates that it is asporogenic. Since it contains *meso*-diaminopimelic acid in the cell wall and madurose in the whole cell hydrolysate, strain J907-21 belongs to cell wall type IIIB. Strain J907-21 does not bear any morphologically important bodies such as spore chain and sporangium. Thus, strain J907-21 can only be classified as an actinomycete strain of non-*Streptomyces* type. A brief description of strain J907-21 is shown in Table 1. A detailed description of the morphological, cultural and physiological characteristics, cell-wall composition and taxonomy will be published elsewhere.

† This antibiotic was originally called BBM-2478[1] or BMY-28090[2].

Table 1. Producing strain J907-21 (ATCC 39417).

Morphology	
Aerial mycelium:	None or rudimentary formation
Substrate mycelium:	Long, branched and non-fragmenting
Sporulation:	Not observed
Cultural and physiological characteristics	
Color of colony:	Yellow, brown or red
Melanoid pigment:	None
Growth temperature:	15~43°C, no growth at 45°C
Cellular composition	
Cell wall:	Type IIIB (*meso*-diaminopimelic acid and madurose)

Fermentation of Elsamicins

A well-grown slant culture of strain No. J907-21 was used to inoculate a vegetative medium consisting of soluble starch 3%, Bacto-liver (Difco) 1%, fish meal 0.5%, NaCl 0.3%, $(NH_4)_2SO_4$ 0.1% and $CaCO_3$ 0.6%, the pH being adjusted to 7.0 before sterilization. The vegetative medium was incubated at 28°C for 72 hours on a rotary shaker (250 rpm) and 5 ml of the culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of a fermentation medium having the same composition of the vegetative medium. The fermentation was carried out on a rotary shaker at 28°C for 7 to 10 days. The antibiotic activity in the fermentation broth was determined by the paper-disc agar diffusion method using *Micrococcus luteus* PCI 1001 as the test organism. Antibiotic productivity reached a maximum potency of 150 μg/ml after 6 to 7 days. Fermentation studies were also carried out in 200-liter fermentors. In one of the experimental fermentations, a seed culture was shaken on a rotary shaker for 4 days at 32°C in 500-ml Erlenmeyer flasks and used to inoculate 10 liters of the vegetative medium composed of the same composition as the above, in a 20-liter jar fermentor which was run at 250 rpm at 28°C with aeration of 10 liters/minute for 100 hours. The seed culture was then transferred into a 200-liter pilot tank containing 120 liters of the production medium consisting of soluble starch 3%, linseed meal 2.5%, NaCl 0.3%, $(NH_4)_2SO_4$ 0.1% and $CaCO_3$ 0.6%. The fermentor was operated at 250 rpm at 28°C with an aeration rate of 120 liters/minute. The antibiotic production reached a maximum potency of 460 μg/ml after 185 hours fermentation.

Isolation and Purification

Harvested broth (20 liters, pH 6.8) was separated to mycelial cake and supernate by using a Sharpless-type centrifuge (Kokusan No. 4A). The mycelial cake was extracted three times with 5 liter volumes of methanol. After removal of the insolubles by filtration, the methanolic extracts were combined and concentrated *in vacuo* to an aqueous solution. The supernate of the fermentation broth was extracted with butanol (20 liters) and the extract evaporated *in vacuo* to an aqueous solution. The two aqueous concentrates were combined and applied on a column of Diaion HP-20 (Mitsubishi Chem. Industries, Tokyo, 5.5×60 cm) which was developed successively with water (5 liters), 50% aqueous methanol (5 liters) and 80% aqueous methanol (6 liters). The fractions containing the elsamicin complex were monitored by paper disc assay using *M. luteus* PCI 1001 as the test organism. The active fractions eluting with 80% aqueous methanol were pooled, evaporated under reduced pressure and freeze-dried to give 4.5 g of crude elsamicin complex as a yellow solid. The crude complex was applied on a column of silica gel (3.5×55 cm) which was pre-washed with chloroform, and the activity eluted

Table 2. TLC of elsamicins A, B and chartreusin.

	Rf*	
	$CHCl_3$ - MeOH (7: 3)	EtOAc - MeOH (1: 1)
Elsamicin A	0.37	0.16
Elsamicin B	0.78	0.57
Chartreusin	0.65	0.48

* TLC: SiO_2.

Table 3. Physico-chemical properties of elsamicins A and B.

	Elsamicin A	Elsamicin B
Nature	Yellow rods	Yellow rods
MP (°C)	225~226	271~272 (dec)
$[\alpha]_D^{26}$ (c 0.5, pyridine)	+124°	−8°
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	236 (590), 266 (550), 333 (100), 378 (132), 398 (205), 422 (225)	236 (740), 266 (700), 333 (118), 378 (169), 398 (255), 422 (290)
Anal Calcd for:	$C_{33}H_{35}NO_{13}\cdot CH_3OH$	$C_{26}H_{22}O_{10}$
	C 59.55, H 5.73, N 2.04	C 63.16, H 4.48
Found:	C 59.28, H 5.40, N 2.06	C 63.16, H 4.51

with a chloroform - methanol mixture with stepwise increase of the methanol concentration (5~10%). The first active fractions, eluted by 5% methanol, were collected, concentrated *in vacuo* and lyophilized to afford elsamicin B (72 mg). The second active fractions, eluted by 10% methanol, were similarly worked up to give semi-pure solid of elsamicin A (2.51 g). The latter solid was further chromatographed on silica gel using medium pressure liquid chromatography (column: Kiriyama 11×500 mm; pump: FMI Lab pump, pressure 5.6~6.3 kg/cm^2). Elution with chloroform - methanol (97: 3) gave active fractions which, upon concentration *in vacuo*, afforded homogeneous solid of elsamicin A (1.30 g). This solid was crystallized from methanol yielding yellowish orange rods of elsamicin A monomethanol adduct.

Physico-chemical Properties

Elsamicins A and B were obtained as yellowish orange crystalline solids. Both components of elsamicin are distinguishable from chartreusin[4] by TLC as shown in Table 2. Elsamicin A is readily soluble in dimethyl sulfoxide, dimethylformamide, dioxane and acidic water, slightly soluble in methanol, ethanol and chloroform but insoluble in other organic solvents. The solubility of elsamicin B is similar to that of elsamicin A except that elsamicin B is insoluble in acidic water. Elsamicins A and B were positive towards ferric chloride and anthrone reagents. Elsamicin A showed positive reaction to ninhydrin, while elsamicin B was negative in the test. Tollens and Sakaguchi reactions were negative with both components. The physico-chemical properties of elsamicins A and B are summarized in Table 3. The UV spectra of the two components were similar, showing maxima at 236, 266, 398 and 422 nm in neutral and acidic solution and at 240, 268 and 435 nm in alkaline solution. These spectra are closely related to that of chartreusin[4]. The IR spectra of elsamicins A and B are given in Figs. 1 and 2, respectively. The ^1H NMR and ^{13}C NMR of elsamicin A are shown in Figs. 3 and 4.

Antibacterial Activity

The minimum inhibitory concentration (MIC) of elsamicins A and B was determined comparatively with chartreusin against various Gram-positive and Gram-negative bacteria and fungi, as well as

Fig. 1. IR spectrum of elsamicin A (KBr).

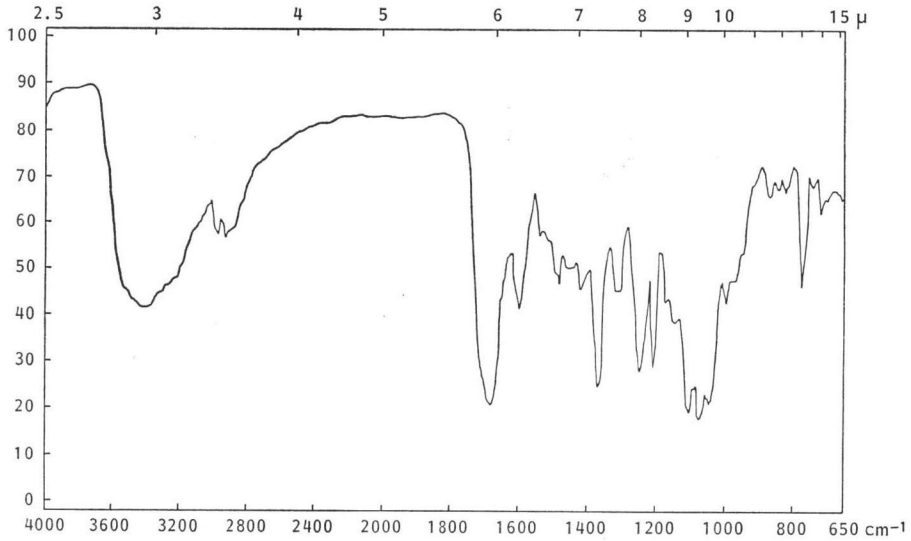
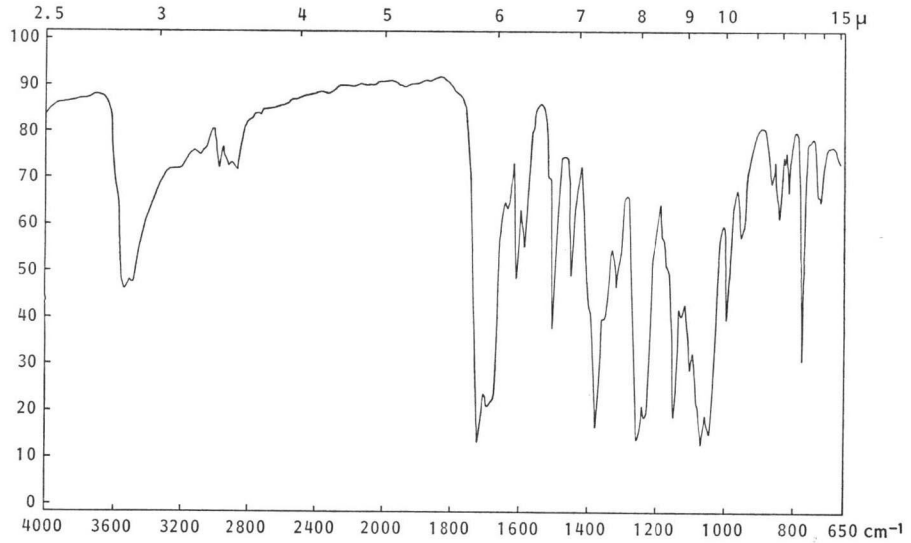


Fig. 2. IR spectrum of elsamicin B (KBr).



some anaerobic organisms, by the serial two-fold agar dilution method. Nutrient agar medium was used for Gram-positive and Gram-negative bacteria, GAM agar medium for anaerobes and Sabouraud agar medium for fungi. As shown in Table 4, elsamicins A and B, and chartreusin showed similar antibacterial spectra against Gram-positive bacteria and anaerobes, while they were inactive against Gram-negative bacteria and fungi. The anti-staphylococcal activity of elsamicin A was 2~4 times higher than that of elsamicin B and eight times higher than that of chartreusin.

Antitumor Activity of Elsamicin A

The antitumor activity of elsamicin A was examined in mice comparatively with chartreusin against leukemia P388, lymphoid leukemia L1210 and melanoma B16. Leukemia P388 was inoculated intra-

Fig. 3. ^{1}H NMR spectrum of elsamicin A in CD_3OD (80 MHz).

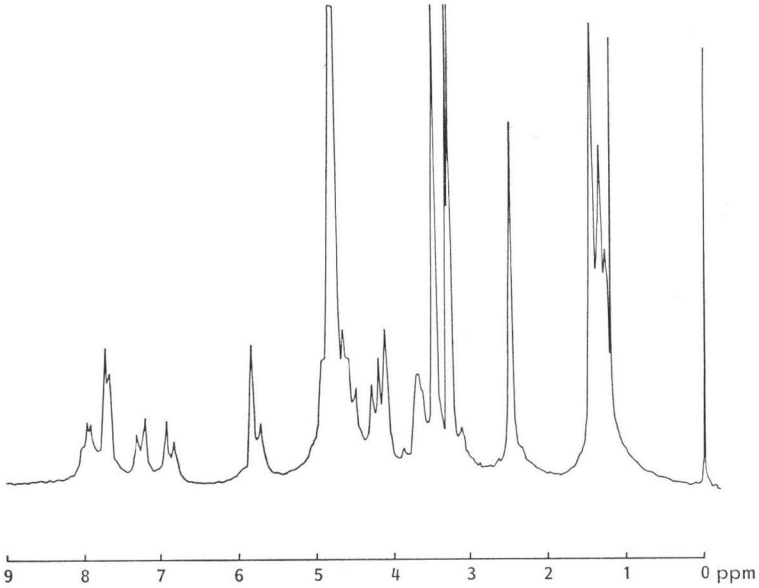
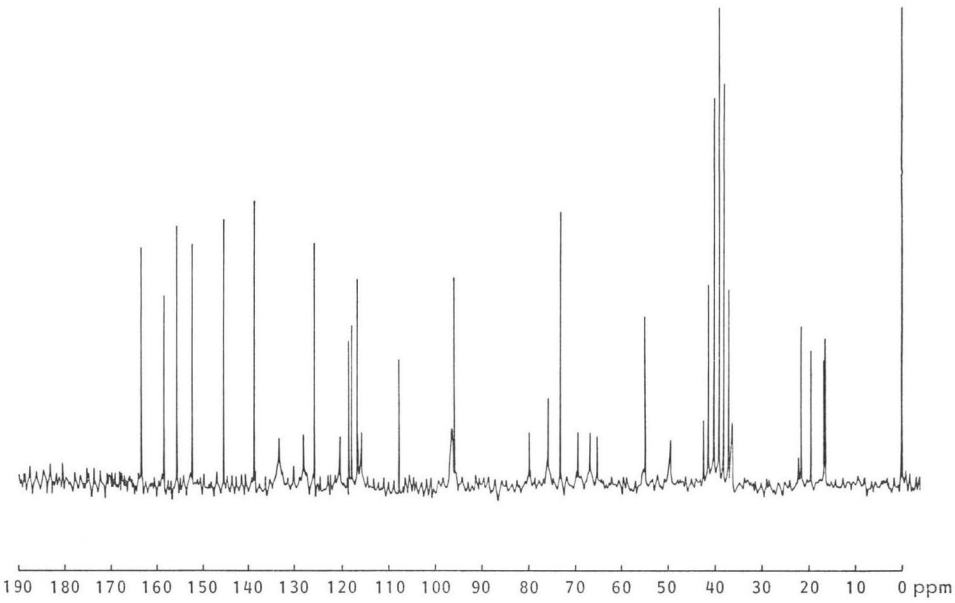


Fig. 4. ^{13}C NMR spectrum of elsamicin A in DMSO-d_6 (20 MHz).



peritoneally into CDF_1 or BDF_1 mice at 10^6 cells per mouse and leukemia L1210 and melanoma B16 were implanted into BDF_1 mice at 10^5 and 10^6 cells per mouse, respectively. Test compounds were dissolved in 0.9% saline containing 10% dimethyl sulfoxide, and graded doses of the antibiotic were administered intraperitoneally on days 1, 4 and 7 (q3d×3, CDF_1 mice) or once daily for 9 days (qd 1→9, BDF_1 mice) after tumor implantation. Death or survival of the treated and non-treated (vehicle) animals was recorded daily during the observation period of 45 days after the tumor implan-

Table 4. Antibacterial activity of elsamicins and chartreusin.

Test organism	MIC (μg/ml)		
	Elsamicin A	Elsamicin B	Chartreusin
Staphylococcus aureus 209P	0.4	1.6	3.1
S. aureus Smith	0.8	1.6	6.3
Bacillus subtilis PCI 219	0.8	0.4	0.8
Micrococcus luteus PCI 1001	<0.05	0.8	0.4
M. flavus D12	0.8	0.8	0.4
Escherichia coli NIHJ	>100	>100	>100
Klebsiella pneumoniae D11	25	>100	>100
Pseudomonas aeruginosa D15	100	>100	>100
Candida albicans IAM 4888	>100	>100	>100
Cryptococcus neoformans D49	>100	>100	>100
Aspergillus fumigatus IAM 2530	>100	>100	>100
Trichophyton mentagrophytes D155	>100	>100	>100
Bacteroides fragilis A22035	12.5	25	12.5
Clostridium difficile A21675	12.5	25	12.5
C. perfringens A9635	6.3	3.1	6.3
Propionibacterium acnes A21933	6.3	12.5	6.3

Table 5. Effect of elsamicins A and B and chartreusin on leukemia P388 (ip).

	Dose, ip (mg/kg/day)	MST (days)	T/C (%)	Average weight change on day 5 (g)
q3d ×3				
Elsamicin A	30	Toxic	Toxic	—
	10	20.5	205	−0.5
	3	18.0	180	+1.3
	1	17.0	170	+1.3
	0.3	14.0	140	+1.3
	0.1	12.5	125	+2.0
Elsamicin B	10	10.0	100	+1.8
	3	10.5	105	+1.8
	1	10.0	100	+1.8
	0.3	10.5	105	+2.0
Vehicle	—	10.0	—	+1.5
qd 1→9				
Elsamicin A	3	20.0	222	−1.0
	1	21.5	239	+0.8
	0.3	17.0	189	+1.8
	0.1	14.5	161	+1.2
	0.03	10.0	111	+2.3
Chartreusin	10	19.5	217	+1.3
	3	15.0	167	+1.5
	1	14.0	156	+1.3
	0.3	10.5	117	+2.0
Vehicle	—	9.0	—	+2.4

tation, and the median survival time (MST) was calculated for each of the test and control groups. The results are shown in Tables 5, 6 and 7. Elsamicin A showed a life prolongation effect on mice bearing leukemia P388 both by q3d×3 and qd 1→9 dose schedules, while elsamicin B did not show antitumor activity against this tumor (Table 5). Elsamicin A was also active against leukemia L1210

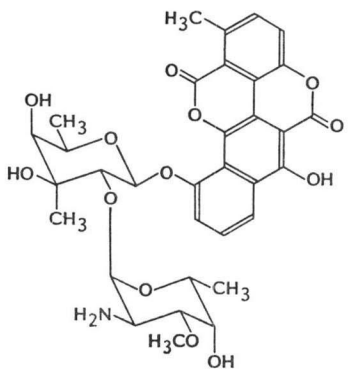
Table 6. Effect of elsamicin A and chartreusin on leukemia L1210 (ip).

	Dose qd 1→9, ip (mg/kg/day)	MST (days)	T/C (%)	Average weight change on day 5 (g)
Elsamicin A	3	12.0	150	−0.1
	1	11.0	138	+0.8
	0.3	10.5	131	+1.3
	0.1	8.0	100	+2.6
	0.03	8.0	100	+2.9
	0.01	8.0	100	+3.2
Chartreusin	10	11.5	144	+1.3
	3	9.0	113	+1.4
	1	9.0	113	+1.9
	0.3	8.0	100	+3.2
	0.1	8.0	100	+3.1
Vehicle	—	8.0	—	+2.8

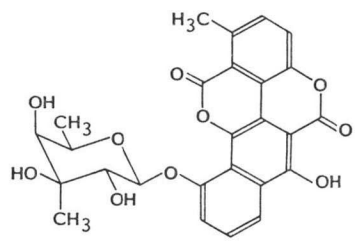
Table 7. Effect of elsamicin A and chartreusin on melanoma B16 (ip).

	Dose qd 1→9, ip (mg/kg/day)	MST (days)	T/C (%)	Average weight change on day 5 (g)
Elsamicin A	3	41.5	296	−1.5
	1	34.5	246	+2.2
	0.3	25.5	182	+2.5
	0.1	18.5	132	+2.5
	0.03	16.0	114	+2.0
Chartreusin	10	25.0	179	+1.7
	3	20.5	146	+2.3
	1	16.0	114	+2.3
	0.3	14.0	100	+1.8
Vehicle	—	14.0	—	+2.3

Fig. 5. Structures of elsamicins A and B.



Elsamicin A



Elsamicin B

and melanoma B16 (Tables 6 and 7) and was approximately 10 to 30 times more potent than chartreusin in terms of minimum effective dose. Elsamicin A achieved T/C values superior to those of chartreusin against all of the tumors tested. The acute toxicity of elsamicin A was determined in mice (*dd*Y strain) by single intraperitoneal administration, the LD_{50} being 38 mg/kg.

Discussion

Elsamicins A and B are novel antitumor antibiotics produced by an unidentified actinomycete strain, J907-21. They are yellow crystalline compounds having UV spectra quite similar to that of the antibiotic chartreusin. Structural studies[3] disclosed that elsamicins A, B and chartreusin contained the common aglycone, chartarin[5], but they were different from each other in the sugar moiety. The structures of elsamicins A and B are shown in Fig. 5. Elsamicin A which contains both a neutral sugar and an amino sugar is remarkably more water-soluble than chartreusin especially under acidic conditions, while both elsamicin B and chartreusin[6] which lack the amino sugar are nearly insoluble in water. Elsamicins A and B showed antibacterial activity against both Gram-positive bacteria and anaerobes, with elsamicin A being 2~4 times more potent than elsamicin B. Elsamicin A displayed strong antitumor activity against leukemia P388, leukemia L1210 and melanoma B16 with the T/C values over 200%. Elsamicin A was 10~30 times more potent than chartreusin in terms of minimum effective doses. Elsamicin B was practically devoid of antitumor activity. As will be reported in a separate paper, elsamicin A showed pharmacokinetics significantly different from those of chartreusin. It is particularly noteworthy that introduction of an amino sugar in the side chain moiety of chartreusin greatly increased water-solubility and antitumor activity and substantially effected the pharmacokinetics of the antibiotic.

References

1) KONISHI, M.; K. SUGAWARA, T. MIYAKI & H. KAWAGUCHI (Bristol-Myers): BBM-2478 antibiotic complex. U.S. 4,518,589 May 21, 1985
2) KONISHI, M.; K. SUGAWARA, M. TSUNAKAWA, Y. NISHIYAMA, T. MIYAKI & H. KAWAGUCHI: BMY-28090 a new antitumor antibiotic related to chartreusin: Isolation, chemistry and activity. Abstracts of 14th Int. Congr. Chemother., P-17-13, p. 320, Kyoto, June 23~28, 1985
3) SUGAWARA, K.; M. TSUNAKAWA, M. KONISHI, H. KAWAGUCHI, B. KRISHNAN, H. CHUN-HENG & J. CLARDY: Elsamicins A and B, new antitumor antibiotics related to chartreusin. II. Structures of elsamicins A and B. in preparation
4) LEACH, B. E.; K. M. CALHOUN, L. E. JOHNSON, C. M. TEETERS & W. G. JACKSON: Chartreusin, a new antibiotic produced by *Streptomyces chartreusis*, a new species. J. Am. Chem. Soc. 75: 4011~4012, 1953
5) SIMONITSH, E.; W. EISENHUTH, O. A. STAMM & H. SCHMID: Über die Struktur des Chartreusins I. Helv. Chim. Acta 47: 1459~1484, 1964
6) BEISLER, J. A.: Chartreusin, a glycoside antitumor antibiotic from *Streptomyces*. Prog. Med. Chem. 19: 247~268, 1982