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Characteristics of Antitumorial Antibiotics B-1123 from *Aspergillus terreus*

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Aspergillus terreus 균주가 생산하는 항암항생물질 B-1123의 성상

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An antitumor antibiotic named B-1123 substance was isolated from the culture filtrate of a new isolate fungus identified as *Aspergillus terreus*. The fermentation yield reached about 23 mg per liter of the broth. The B-1123 substance, chlorine containing antibiotic, has the molecular formular of $C_{17}H_{12}O_7Cl_2$. Its structure was determined to be geodin by spectroscopic data. It is active against some Gram-positive bacteria and it prolongs the life span of mice inoculated with Ehrlich carcinoma.

Several antitumor antibiotics from natural products which have α-exo methylene-γ-lactone structure were reported (1-7). Recently, novel antitumor antibiotics such as terrecyclic acid (8), oxaspirol A(9), myrocin C(10) and methylenolactocin (11) were founded from culture filtrate of microbial sources. These compounds have an antitumor activity and reversed by glutathione addition in the microbial screening system.

During the course of screening for potential antitumor antibiotics from molds by use of a *Bacillus subtilis* IFO 12210 as a test organism, an active substance has been isolated from the culture filtrate of an *Aspergillus* sp. strain No. 1123 which was isolated from soil picked up in a farmyard of Chuncheon city, Korea. The assay for antitumor antibiotics was based on Michael addition reaction (12) and the antibiotic whose activity was reversed by glutathione addition was chosen. The chemical investigation revealed that the antibiotic was geodin. The present paper deals with the screening of producing microorganism, the taxonomy of the producing organism, fermentation, isolation, structure determination and biological characteristics of B-1123 substance.

Key words: Antitumor antibiotics, geodin, *Aspergillus terreus*,
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Materials and Methods

Screening method

Isolation of microorganisms was carried out using medium listed in Table 1. Selected colonies were transferred on slant and stored. A loopful of spore from the stock culture of selected mold strain was inoculated in a 500 m*l* Erlenmeyer flask of 100 m*l* medium. The medium composition was glucose 30g, soybean meal 2.0g, yeast extract 0.5g, KH_2PO_4 1.0g, $MgSO_4 \cdot 7H_2O$ 1.0g, NaCl 0.5g, $CaCl_2$ 0.5g, $FeCl_3 \cdot 6H_2O$ 2 mg and $ZnSO_4 \cdot 7H_2O$ 3 mg in 1,000 m*l* distilled water and it was adjusted to pH 5.5 before sterilization. Fermentation was carried out at 30°C for 4 days on a reciprocal shaker. The culture filtrate was then subjected to antitumor screening. First screening was carried out by determined the antibacterial activity against *Bacillus subtilis* IFO 12210 as test strain. The cultured broths which showed the antibacterial activity was selected. Among them, the strain which showed antibacterial activity and whose activity was reversed by glutathione addition was chosen in second

Table 1. Composition of the media for isolation.

(1)	Soluble starch	10 g/*l*
	K_2HPO_4	0.5
	NH_4Cl	0.5
	Agar	18
(2)	Sucrose	30g/ *l*
	$NaNO_3$	2
	$MgSO_4$. $7H_2O$	0.5
	K_2HPO_4	1.0
	KCl	0.5
	$FeSO_4$. $7H_2O$	0.5
	Agar	18
	pH	6.0

screening.

Taxonomy of the strain

Morphological and cultural studies were carried out using the following media.

Czapek's agar; $NaNO_3$ 3g, K_2HPO_4 1g, $MgSO_4$. $7H_2O$ 0.5g, KCl 0.5g, $FeSO_4$. $7H_2O$ 0.01g, sucrose 30g, agar 15g, distilled water 1,000 m*l*.

Malt extract agar; malt extract 20g, polypeptone 1g, glucose 20g, agar 15g, distilled water 1,000 m*l*.

MY 20 agar; Polypepton 5g, yeast extract 3g, malt extract 3g, glucose 200g, agar 15g, distilled water 1,000 m*l*.

After 10 days incubation at 28°C, observation was done with eye and microscope.

Fermentation procedure

A loopful of conidia from *Aspergillus* sp. strain B-1123 wss inoculated in 100 m*l* of the medium in a 500 m*l* Erlenmeyer flask. The medium composition was same as in screening method and it was adjusted to pH 5.5 before sterilization. Glucose and soybean meal was the best carbon and nitrogen sources, respectively, among the several carbon and nitrogen sources tested. Fermentation was carried out at 30°C for 5 days in a shaking incubator. Scale up fermentation was carried out in 2,000 m*l* of the same medium in a 5,000 m*l* Erlenmeyer flask inoculated with 50 m*l* of seed culture (1% glucose, 0.5% Polypepton, 0.5% yeast extract, 0.003% $FeSO_4$. $7H_2O$) at 30°C on a rotary shaker at 167 rpm for 5 days. Antimicrobial activity was assayed by paper-dise agar diffusion method using *B.subtilis* IFO 12210 as a test organism.

Physico-chemical measurements

The melting point was determined on a microscope hot plate and uncorrected. The IR spectrum was obtained with a Jasco IRA-2. The UV spectrum was measurd on a Shimadzu double-beam spectrometer UV-180. The low and high resolution mass spectra were obtained on a Hitachi RMU-6 M and a Jeol JMS D-300 mass spectrometer, respectively. The ^1H and ^{13}C-NMR spectra were measured on a Jeol FX-100 spectrometer.

Antimicrobial assay

The MIC of B-1123 substance was determined by agar dilution method using Bouillon agar for bacteria and Sabouraud agar for fungi and yeasts (13). Observation was made after 18 hours for bacteria and 48 hours for fungi and yeasts at 30°C following inoculation of test organisms.

Antitumor activity

To determine the antitumor effect of B-1123 substance, 2×10^6 of Ehrlich carcinoma cells were inoculated intraperitoneally into each ICR mouse (female, 5 weeks old) and 0.1 m*l*, 0.2 m*l* and 0.3 m*l* of an ethanol solution of B-1123 substance was injected into the mice intraperitoneally once every day for 10 days, starting on the day of the tumor cells inoculation. A drug solution was made by mixing one part of ethanol solution of B-1123 substance (5 mg/m*l*) and four parts of 0.5% carboxy-methoxy cellulose solution. The survival time of tumor-bearing mice were observed for 40 days and the mean survival time (days) of drug-treated mice was compared with that of the control. Six mice were used for each determination.

Results and Discussion

Results of screening test

Of about 1,000 strains isolated, 48 strains were active against test organism. Among these strains, 6 strains were reversed by glutathione and strain numbered B-1123 showed potent activity which was reversed selectively by glutathione. Thus, we took up this strain for further studies.

Taxonomy of the producing organism

Morphological characteristics: Numerous phialide type conidia were found. Conidiophores arose directly from the substrate hyphae or aerial hyphae and not branched. The top of the conidiophores bulged to become vesicles with phialide.

Conidial head; cylindrical, 30-60 × 80-250 μm in length, brown to reddish brown.

Conidiophores; 4-7 × 100-200 μm, smooth, light yellow, somewhat bended.

Vesicles; 10-15 μm in diameter, subspherical with phialide.

Conidia; globose or subspherical, 2-3 μm in diameter, smooth. Schlerotium was not observed.

Cultural characteristics: On Czapek's agar, colonies grew to 35-40 mm in diameter after 10 days incubation at 28°C. The colonies were floccose and reddish brown to brown in color; reverse was light yellow. On malt extract agar, growth occurred more slowly than on Czapek's agar (32 mm in diameter at the same condition). The colony surface was flat and become powdery to felt-like, the formation of conidia was very good. The reverse was light brown to yellow brown, no soluble pigment was formed. On MY 20 agar, growth occurred most rapidly (70 mm in diameter at the same condition) and colony surface was felt-like, others including color identical to that on malt extract agar.

The above mentioned morphological and cultural characteristics of srtrain No. 1123 indicated that it belongs to *Asp. terreus* referring to the description in the textbooks of Rafer and Fennell (14) and Udagawa *et al.* (15). Although, the species of this strain should be resolved in further studies.

Production and isolation of B-1123 substance

Fermentation was performed as described in Materials and Methods. As most of the antibiotic activity was found in the broth filtrate, the filtrate (23 liters) was adjusted to pH 3.0 with 2N-HCl and the active principals were extracted ethyl acetate with the same volume of broth. The combined ethyl acetate extract was concentrated about 700 m*l in vacuo* (at 40°C) and extracted twice with the same volume of 5% $NaHCO_3$ (pH 9.0). The organic layer was dried with anhydrous sodium sulfate and concentrated *in vacuo*. The residue (ca. 4.6g) was subjected to silica gel column chromatography (Fusica gel BW-820 MN). The column (35 × 350 mm) was developed with a solvent system of benzene-ethyl acetate. The elution was done

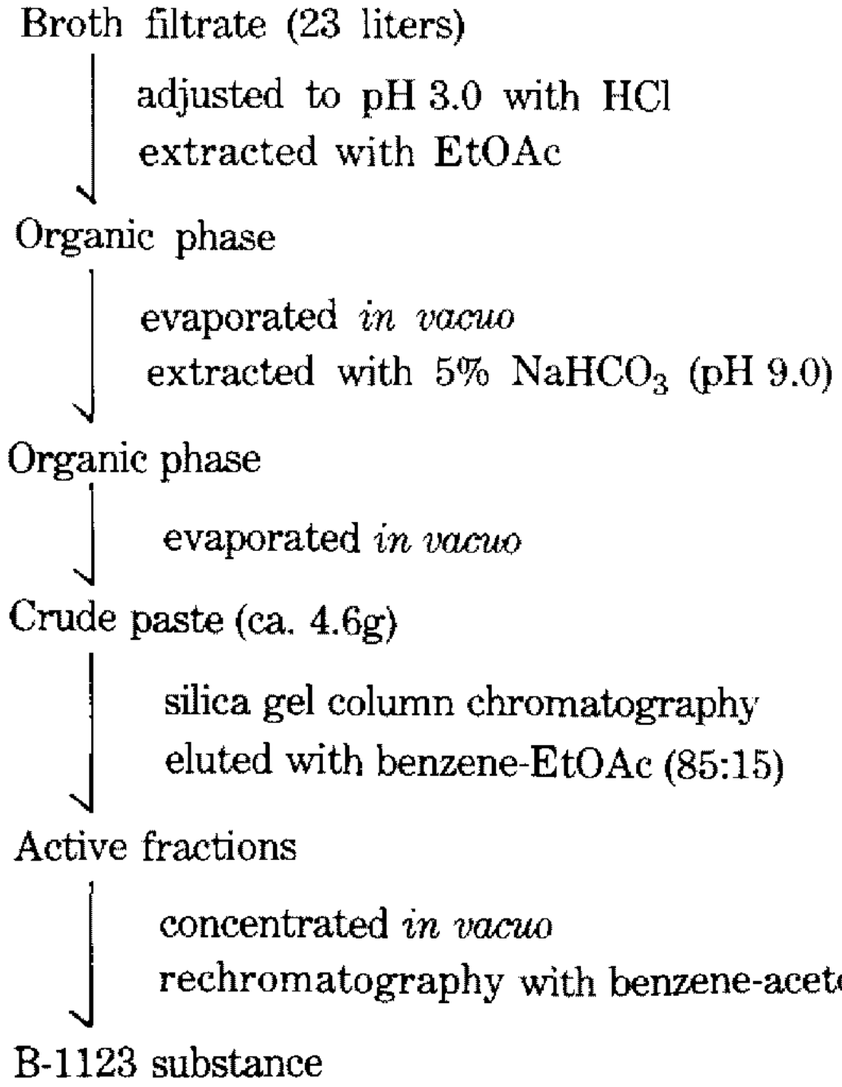

Fig. 1. Isolation procedure of B-1123 substance.

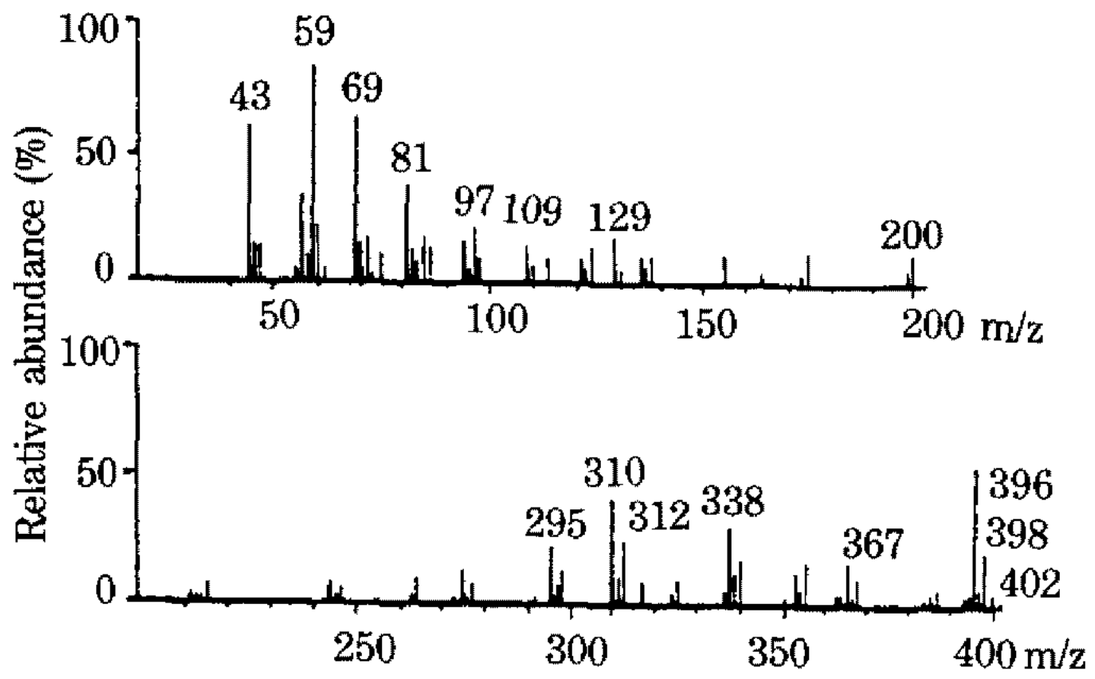

Fig. 2. Mass spectrum of B-1123 substance.

stepwise with a solvent system of benzene-ethyl acetate ratio from 95:5 to 70:30. The elution volume was 500 m*l* each and fractionized to 18 m*l* per tube. The elutions monitored by TLC and detectd under UV lamp. Active fractions were combined, concentrated *in vacuo* and rechromatographed on silica gel column with a solvent system of benzene-acetone (95:5). After recrystallization from *n*-hexane-ethyl acetate, the pure antibiotic was obtained as a yellow leaflet. Yield was about 23 mg from 1 liter of broth. Isolation procedure is shown in Fig. 1.

Physico-chemical properties of B-1123 substance

The B-1123 substance (I) was obtained by recrystallization from *n*-hexane and ethyl acetate as crystals of mp. 231-235°C. The molecular formula of

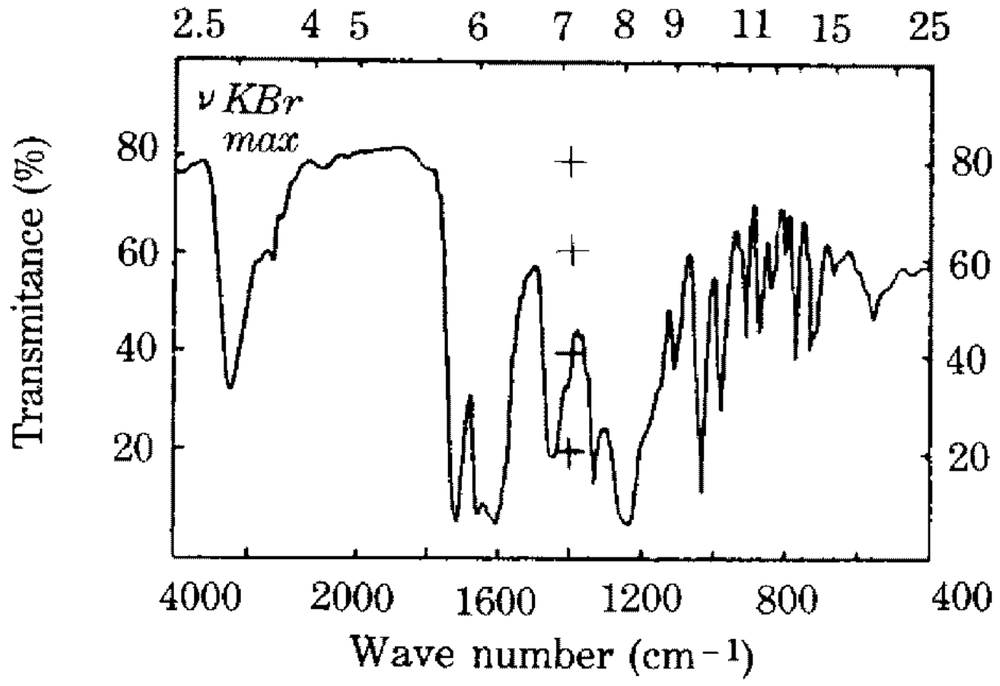

Fig. 3. IR spectrum of B-1123 substance (KBr).

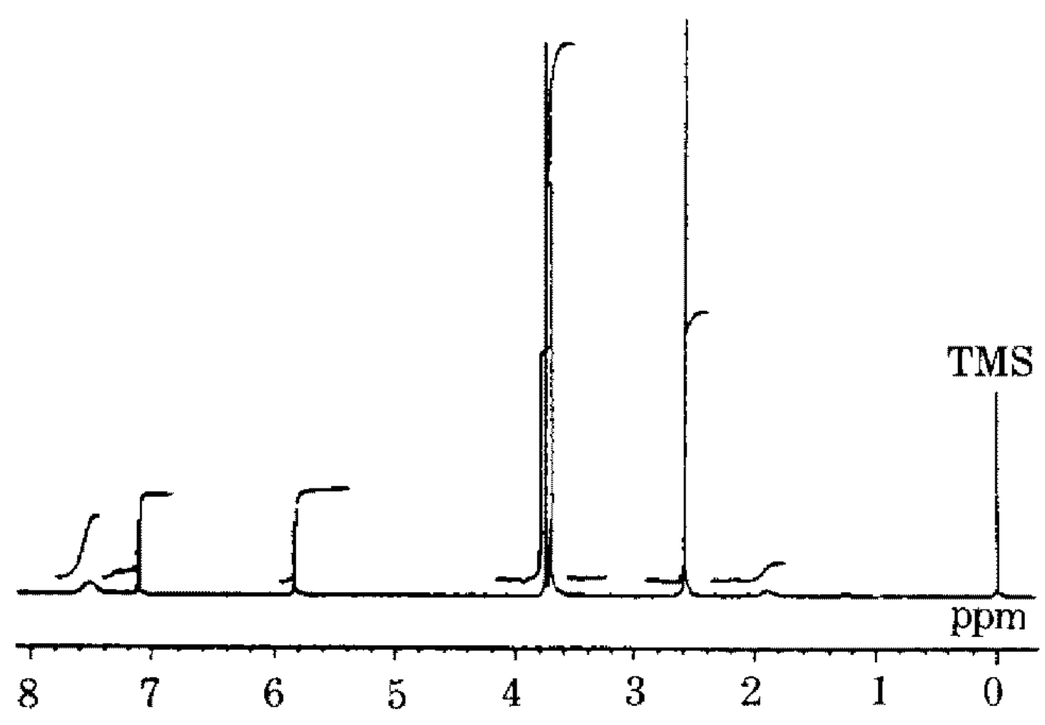

Fig. 4. ^{1}H-NMR spectrum of B-1123 substance (100 MHz, $CDCl_3$) .

I was determined to be $C_{17}H_{12}O_7Cl_2$ by mass spectrometry (Fig. 2) and elementary analysis; this was further confirmed by the high resolution mass spectrum. The IR spectrum is shown in Fig. 3. Also, the ^{1}H-NMR and ^{13}C-NMR spectra are shown in Fig. 4 and Fig. 5, respectively. The Rf values on TLC using several developing solvents are shown in Table 2 and **I** appeared a single spot on TLC plate. Physico-chemical properties including solubility and color reactions of **I** are summarized in Table 3.

Structure of B-1123 substance

The B-1123 substance (**I**) is a neutral substance and containing two chlorine judging from mass spectrum (m/z 398, 400,402 fragment peak appeared and its intensity ratio was 9:6:1). UV spectrum of **I** revealed maximum absorption at 285 and 353 nm in methanol. From data of physico-chemical properties of **I** and a fact that **I** containing chlorine, we found **I** is similar to geodin. As the results of melting point, elementary analysis and IR spectrum (16, 17), the B-1123

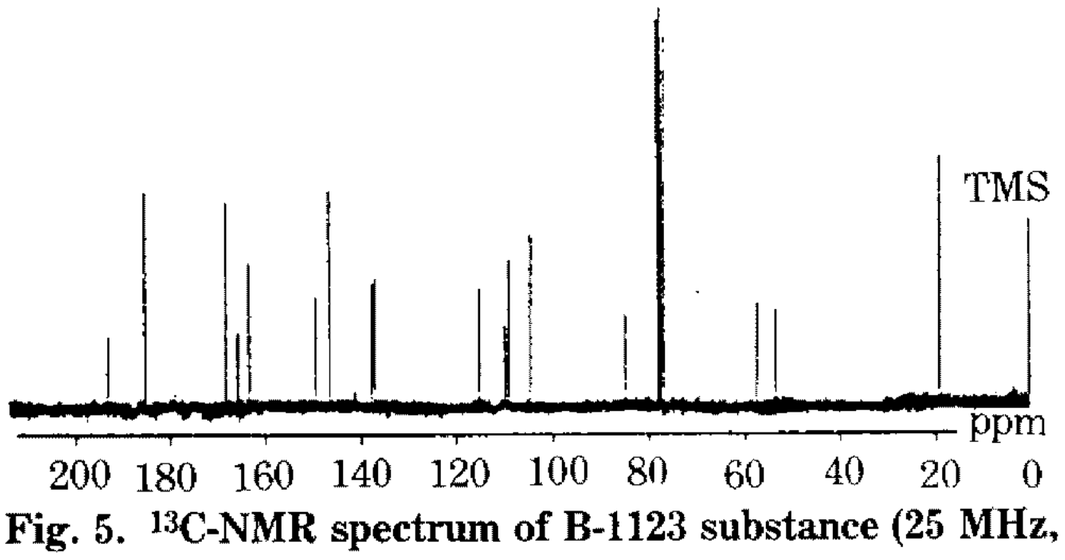

Fig. 5. ^{13}C-NMR spectrum of B-1123 substance (25 MHz, $CDCl_3$) .

Table 2. Rf values of B-1123 substance.

Solvent system	Rf
Benzene: ethyl acetate (6:4)	0.50
Benzene: acetone (6:4)	0.54
Benzene: methanol (8:2)	0.53
Chloroform: ethyl acetate (9:1)	0.51
Chloroform: acetone: ethyl acetate (35:60:5)	0.81
Ethyl acetate	0.60

TLC; Merck silica gel plate 60 GF_{254} (0.25 mm) were used and the spots were detected under UV lamp or by spraying 1% $KMnO_4$

Table 3. Physico-chemical properties of B-1123 substance .

Appearance	Neutral, yellow plate
Melting point	231-235°C (decomposition)
Elementary analysis	Found; C: 51.24, H: 3.02, Cl: 17.98 (%) Calced.; C: 51.13, H: 3.01, Cl: 17.80 (%)
Mass spectrum	*m/z*, 398 (M^+) for $C_{17}H_{12}O_7Cl_2$
UV λ_{max}^{MeOH} nm	285, 353 (sh)
IR ν_{max}^{Kbr} cm^{-1}	3400, 1720, 1660, 1610, 1440, 1330, 1225, 1040, 980,
Solubility,	Soluble; MeOH, acetone, EtOAc, chloroform, Insoluble; Ethyl ether, *n*-hexane, water,
Color reactions,	
	Positive; $KMnO_4$, I_2 vapor, 2, 4-DNPH, $FeCl_3$,
	Negative; Dragendorff, 2, 6-dichloroindophenol, ninhydrin,

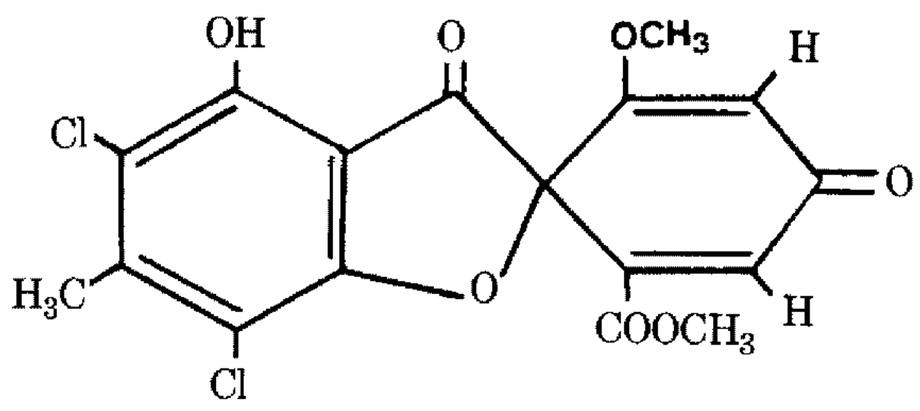

Fig. 6 Structure of geodin.

substance was identified to be geodin. In order to determine whether **I** is identical with geodin, the NMR spectra of **I** was compard with geodin (Fig. 6).

In the ^{1}H-NMR spectrum, 12 protons signal appeared and the signal at δ 2.58 ppm (3H, s) was assigned to methyl protons adjacent to benzene ring. Two signals at δ 3.70 ppm (3H, s) and δ 3.74 ppm (3H, s) were assigned to methoxy protons and methyl ester of carboxylic acid protons in the structure of geodin. It cannot be distinguished which signal is assigned to methyl ester but it may be assumed that the signal of low-field assigned to methyl ester protons.

Two signals at δ 5.83 ppm (1H, d, J = 1.8) and δ 7.15 ppm (1H, d, J = 1.8) were attributable to proton of methoxy side and proton of carboxylic acid methyl ester side in the cyclohexadiene structure, respectively. Signals of two protons at δ 5.83 and δ 7.15 ppm were appeared doublet due to its long range coupling. After irradiation of the proton at δ 5.83 ppm, the signal at δ 7.15 ppm was changed from a doublet to a singlet and also irradiation of the proton at δ 7.15 ppm, the signal at δ 5.83 ppm was changed from a doublet to a singlet. Hydroxy proton adjacent to benzene ring appeared at δ 7.55 ppm (1H, s, broad). From the spectral data described above, it was concluded that the B-1123 substance was geodin. For reference, the ^{13}C-NMR data is shown in Table 4. Presence of 17 carbons containing three methyl carbons, two methine carbons, two carbonyl carbons, one carboxy carbon and 9 singlet carbons were confirmed from the ^{13}C-NMR chart.

Table 4. Chemical shift of ^{13}C-NMR spectrum of B-1123 substance (25 MHz in $CDCl_3$).

Peak No.	ppm	Multiplicity	Peak No.	ppm	Multiplicity
1	18.7	q	10	137.5	s
2	53.1	q	11	146.5	s
3	57.0	q	12	149.3	s
4	84.5	s	13	163.3	s
5	104.4	d	14	165.5	s
6	108.8	s	15	167.9	s
7	109.4	s	16	185.0	s
8	114.7	s	17	193.1	s
9	137.0	d			

Table 5. Antibacterial spectrum of B-1123 substance.

Organisms	MIC (μg/ml)
Staphylococcus aureus IFO 3060	6
Bacillus subtilis IFO 12210	50
B. brevis IFO 3331	25
B. cereus IFO 3514	50
Micrococcus roseus IFO 3764	12
M. luteus IFO 3333	6
Corynebaterium xerosis IFO 12684	12
Escherichia coli K-12 IFO 3301	>200
E. coli B IFO 13168	>200
Pseudomonas aeruginosa IFO 3923	>200
Ps. putida IFO 3738	>200
Proteus vulgaris IFO 3851	100
Serratia marcescens IFO 12648	>200
Alcaligens faecalis IFO 13111	>200

Biological properties of B-1123 substance

Results of the examination of the antimicrobial activity of B-1123 substance are shown in Table 5. It showed selective antibacterial activity, especially against Gram-positive bacteria including *Staphylococcus, Micrococcus, Corynebacterium* and *Bacillus*. Gram-negative bacteria were insensitive to **I** except some strain of *Proteus*. Fungi and yeasts were not affected by concentration up to 200 μg/m*l*.

The antitumor activity of **I** on Ehrlich carcinoma is shown in Table 6. The intraperitoneal injection of this antibiotic at a dose of 0.3 mg per mouse caused a prolongation of the life span of the treated mice bearing tumor cells at a level of 143.8 T/C (%) (see footnote to Table 6).

During the course of screening for antitumor antibiotics from molds, an active substance named B-1123 has been isolated from the culture filtrate of an *Aspergillus* sp. strain No. 1123. The B-1123 substance was identical to geodin from the data of spectral analysis. It is regrettable that newly isolated

Table 6. Antitumor activity of B-1123 substance to mice inoculated with Ehrlich carcinoma.

Dose (mg/mouse)	MSD[a] (days)	Evaluation[b] T/C (%)
0.1	13.9	106.9
0.2	15.8	121.5
0.3	18.7	143.8
Mitomycin 0.02	32.2	247.7
Control	13.0	

2×10^6 Ehrlich carcinoma cells were inoculated intraperitoneally into each ICR mouse (female, 5 weeks old) and ethanol solution of B-1123 substance was administered intraperitoneally once every day for 10 days, starting on the day of the tumor cell inoculation.
[a]MSD; Mean survival days
[b]T/C (%)=MSD (treated)/MSD (control)×100

substance met with already known compound and could not obtain a new compound suit the purpose. But it is interesting that the antibiotic reversed by glutathione was obtained from cultural filtrate of microorganisms. For SH compounds play important roles in a living body and have an affect on the reaction of living thing (18), it can be considered that the substance concerning in a Michael addition reaction is merely toxic substance. But among them, several things such as α-methylene-γ-lactone compounds were known to have selective toxicity. Although the secondary screening is very elementary method, it is very effective method for searching a new chemical structure in the screening step.

요 약

토양에서 새로히 분리한 곰팡이를 배양하고 그의 배양액 중에 함암활성을 갖는 물질을 screening 한 결과 시험균 *B. subtilis*에 대해 항균활성을 갖고 glutathione에 의해 항균활성이 길항되는 물질을 생산하는 B-1123 균주를 얻었다. 생산균주의 형태학적, 배양학적 성상의 관찰결과 B-1123 균주는 *Aspergillus terreus*로 동정되었다.

대두분을 질소원으로 한 배지에서 30℃, 5일간 진탕배양하고 배양여액 중의 활성물질을 ethyl acetate로 추출하고 silica gel column chromatography에 의해 정제하여 연황색 판상결정을 얻었으며 B-1123 물질이라 명명했다. 수율은 1*l* 당 23 mg이였다. B-1123 물질은 융점 231°-235℃의 중성물질로 분자식 $C_{17}H_{12}O_7Cl_2$, 분자량 399였다. 이 활성물질은 IR, Mass, NMR 등의 기기분석 결과 구조적으로 geodin과 일치함을 확인했다. Gram 양성균에 강한 항균활성을 나타냈으며 암세포를 감염시킨 쥐에 대한 연명효과가 있었다.

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