CHRYMUTASINS: NOVEL-AGLYCONE ANTITUMOR ANTIBIOTICS FROM A MUTANT OF *Streptomyces chartreusis*

I. TAXONOMY, MUTATION, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Three novel antibiotics, named chrymutasins A, B and C, were isolated from the fermentation products of a mutant strain obtained by NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) treatment. The mutant strain produced the chrymutasins, which differed in the aglycone moiety from chartreusin, and related compounds. The production of these compounds needed a characteristically long fermentation period. The antitumor activity of chrymutasin A is better *in vivo* than that of chartreusin, the cytotoxic activity against cell lines *in vitro* is equivalent, and the antimicrobial spectrum is narrower.

As part of our microbial screening program for antitumor antibiotics, we have taken *Streptomyces chartreusis* D329 (a chartreusin (**1**)[1] producer), and tried to mutate this organism to obtain novel antibiotics related to **1**. Compound **1**, which was isolated from *S. chartreusis*, is a glycosidic antitumor antibiotics. Though the compound itself was not clinically useful, some related compounds have recently attracted attention. For example, the elsamicins[2,3] (natural products) are expected to be useful based on their antitumor activities and unique structures.

From an analysis of the products from about 3500 colonies treated with NTG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine), we found a mutant strain which produced demethylchartreusin (**2**)[4] and the D329C compound (**3**)[5]. These two compounds had different sugars as compared to **1**. As a result of continued analysis of the fermentation broth of the mutant strain, further three compounds named chrymutasin A (**4**), B (**5**) and C (**6**), with a novel aglycone (named chrymutin), were isolated[6] (Fig. 1). Details of the structure elucidation, including chemical shift assignments, incorporation studies of labeled compounds and the characterization of the compounds are described in the following paper[7].

Materials and Methods

Taxonomic Studies

The parent strain D329 was isolated from a soil sample collected in Saitama Prefecture, Japan. Morphological and physiological properties were examined according to SHIRLING and GOTTLIEB[8]. Cell wall analysis was performed by the methods of BECKER *et al.*[9] and YAMAGUCHI[10]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[11].

Mutation

NTG was used as a mutagen. NTG treatment was carried out in 0.05 M Tris-HCl buffer (pH 9). A

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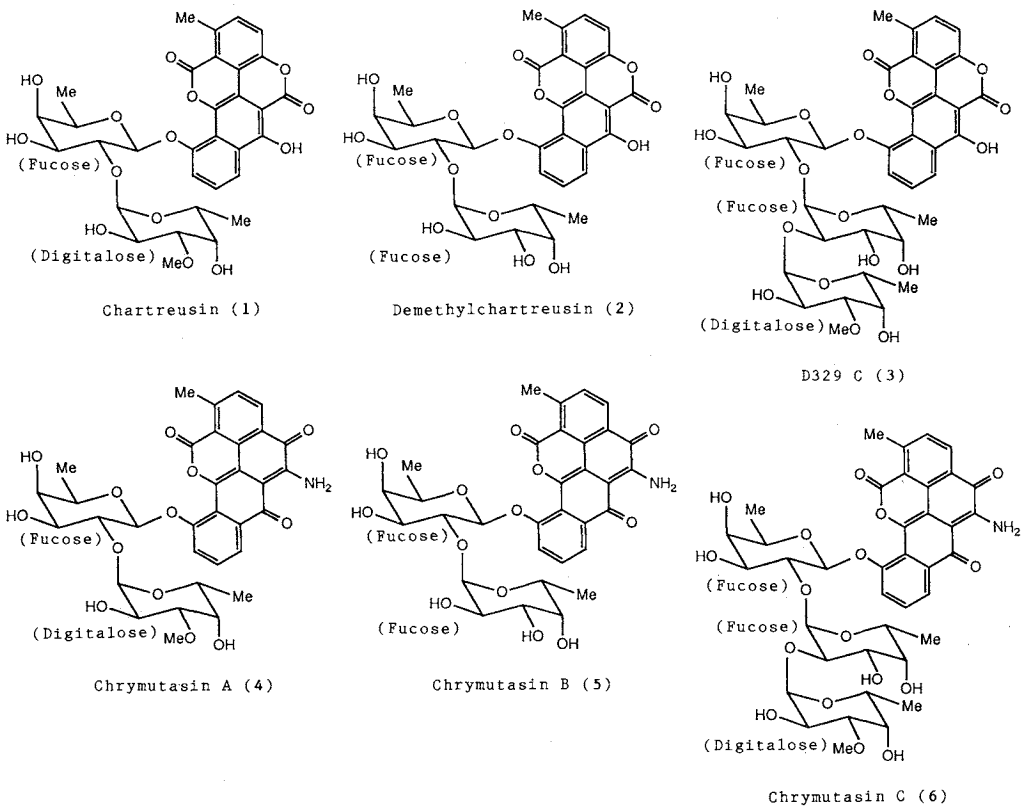
Fig. 1. Structures of chrymutasins, chartreusin and related compounds.



NTG concentration of 2 mg/ml and treatment time at room temperature of about 2 hours gave a kill of more than 90%.

Fermentation

The *S. chartreusis* D329-185 strain was cultivated in 3-liter flasks containing 600 ml of fermentation medium (dry yeast 1.0%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1%, mannitol 2.0%, $FeSO_4 \cdot 2H_2O$ 0.00001%, $MnCl_2 \cdot 4H_2O$ 0.00001%, $ZnSO_4 \cdot 7H_2O$ 0.00001%, $CuSO_4 \cdot 5H_2O$ 0.00001%, $CoCl_2 \cdot 6H_2O$ 0.00001%, adjusted to pH 8.0). The fermentation was carried out on a rotary shaker at 30°C for 19 days.

Cytotoxicities

For *in vitro* cytotoxicity, the following cell lines were used; P388 murine leukemia, Meth A murine fibrosarcoma, A549 human lung adenocarcinoma, MKN45 human gastric carcinoma and WiDr human colon adenocarcinoma. All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cytotoxicity was measured using the MTT method[12]. The IC_{50} value was calculated using PROBIT's method.

Antitumor Activities

The antitumor activity of these compounds was evaluated in an experimental tumor system in mice bearing P388 and Meth A. The cells were implanted intraperitonially into CDF1 mice with an inoculum size of 1×10^6 cells per mouse on day 0. The compounds were given ip daily from day 1 to 4. Each group consisted of 6 mice. Negative control mice and positive ones received physiological saline and 20 mg/kg of 5-fluorouracil, respectively. Antitumor activity was expressed as % T/C value of median survival time (MST of sample group/MST of negative control one $\times 100$).

Animals

Female CDF1 mice at 5~6 weeks age were purchased from Charles River Japan Inc., Atugi-city, Japan.

Antimicrobial Activities

Compounds **4**, **5** and **6** were tested for antimicrobial activity against several bacteria and yeasts. MIC values were determined using the agar dilution method. Heart infusion agar was used for bacteria and potato dextrose agar was used for the yeasts.

Results and Discussion

Taxonomic Studies of the Producing Strain

The parent strain D329 was cultured in various ISP media and the characteristics are summarized in Table 1. Substrate mycelia were well developed in the media. Aerial mycelia were abundantly formed in various media and were blue to gray. A spore chain with 10 to 50 spores was observed to be spiral. Spores were cylindrical in shape with a size of 0.7~0.9 × 1.1 μm and their surface was spiny. Sclerotium, sporangium and zoospores were not observed. The physiological characteristics and the utilization of carbohydrates observed after cultivation at 28°C for 14 days are shown in Table 2. Melanin was produced on peptone-yeast

Table 1. Cultural characteristics of strain D329 and *Streptomyces chartreusis* JCM4570.

Medium		Strain D329	*Streptomyces chartreusis* JCM4570
Yeast extract-malt extract agar ISP No. 2	Growth	Good	Good
	Aerial mass color	Pale blue (185)	Bluish gray (191)
	Diffusible pigment	Light olive brown (94)	None
Oatmeal agar ISP No. 3	Growth	Good	Good
	Aerial mass color	Pale blue (185)	Grayish blue (186)
	Diffusible pigment	Moderate olive brown (95)	Dark grayish yellow (91)
Inorganic salts-starch agar ISP No. 4	Growth	Good	Good
	Aerial mass color	Bluish gray (191)	Pale blue (185)
	Diffusible pigment	Moderate greenish yellow (102)	None
Peptone-yeast extract-iron agar ISP No. 6	Growth	Moderate	Good
	Aerial mass color	None	None
	Diffusible pigment	Dark yellowish brown (78)	Deep yellowish brown (75)
Tyrosine agar ISP No. 7	Growth	Good	Good
	Aerial mass color	Pale blue (185)	White (263)
	Diffusible pigment	Moderate olive brown (95)	None

The color name index in the parenthesis ( ) is accordance with ISCC-NBS Color Name Chart and represents the results of observation on the second week at 28°C on each medium.

Table 2. Taxonomical characteristics of strain D329.

	Strain D329
Cell wall type	I
Spore chain	Spiral (10~50 spores)
Spore surface	Spiny
Aerial mass color	Blue~Gray color series
Growth temperature	15~45°C
Gelatin liquefaction	+
Nitrate reduction	+
Milk coagulation	+
Milk peptonization	+
Starch hydrolysis	+
Utilizable carbon	L-Arabinose, D-Xylose, L-Rhamnose, D-Glucose, D-Galactose, D-Fructose, Sucrose, Raffinose, Inositol, D-Mannitol, Salicin

Table 3. Characteristics of parent and mutant strain.

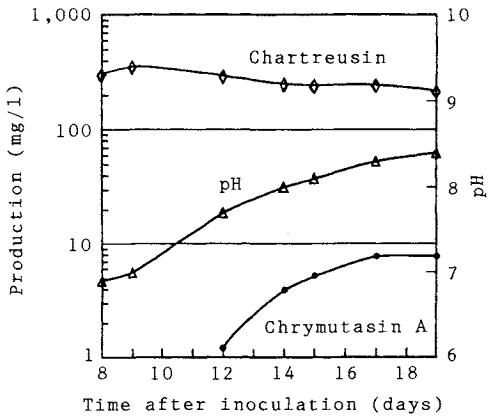
	Products	Auxotrophy
Parent strain	Chartreusin	None
Mutant strain	Chartreusin, demethylchartreusin, D329C chrymutasins A, B and C	Methionine, cysteine

extract agar (ISP 6). Whole cell hydrolysates of the strain D329 contained L,L-diaminopimelic acid and no characteristic sugars. The strain D329 was placed in the type I cell wall group.

The results of these chemotaxonomic, morphological and physiological studies indicate that the strain D329 belongs to the genus *Streptomyces*. The strain D329 is very similar to *S. chartreusis* JCM4570 except for the production of soluble pigment (chartreusin), therefore, this strain was identified as *S. chartreusis* D329.

The mutant strain D329-185, obtained by NTG treatment, showed an auxotrophic character which demanded methionine or cysteine. However, except for methionine (or cysteine) auxotrophy, the taxonomical characters of the mutant strain D329-185 were the same as those of the parent strain D329 (Table 3).

Fig. 2. Fermentation profile of the mutant strain D329-185.



The mutant strain, named *S. chartreusis* D329-185, was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrical Science and Technology, Japan, as No. FERM BP-3269.

Fermentation

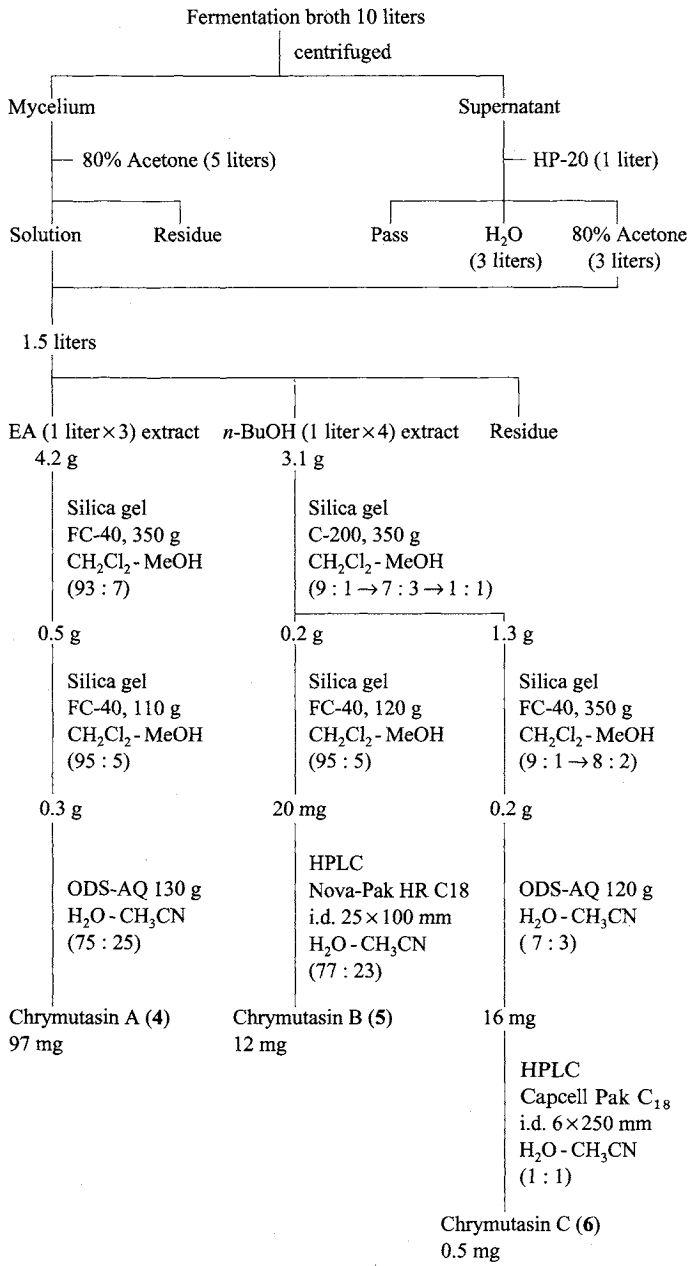
The production of chrymutasins was carried out as described in the Materials and Methods section. A typical time course of the fermentation is shown in Fig. 2. The monitoring of chrymutasin production was performed using HPLC analysis. The production of **4** reached a maximum about 19 days after inoculation. On the other hand, **1** reached a maximum on about 9 days.

Isolation

The procedure for isolation of **4**, **5** and **6** is shown in Scheme 1. The culture supernatant obtained after 19 days of fermentation was adsorbed on Diaion HP-20 (Mitsubishi Chemical Industries) column (1 liter). The compounds were eluted with 80% acetone (3 liters) after washing with water (3 liters) to remove aqueous impurities. The mycelium was extracted with aqueous acetone (5 liters). The HP-20 elution and the mycelium extract were combined and concentrated to about 1.5 liters under reduced pressure. The concentrate was extracted with EtOAc (1 liter × 3 times) and *n*-BuOH (1 liter × 4 times). Each organic layer was concentrated under reduced pressure to give an oily residue.

The EtOAc extract (4.2 g) was chromatographed on a column of silica gel (Wakogel FC-40, Wako Pure Chemical Industries, Ltd., 350 g) with CH_2Cl_2-MeOH (93:7). The fractions (0.5 g) containing **4** were further separated on a FC-40 (110 g) column with CH_2Cl_2-MeOH (95:5). After further purification of the crude **4** (0.3 g) on a ODS (ODS-AQ, YMC Co., Ltd., 130 g) column with H_2O-CH_3CN (75:25), pure

Scheme 1. Isolation procedure for chrymutasins.



4 (97 mg) was obtained as a violet powder.

The *n*-BuOH extract (3.1 g) was separated on a silica gel (Wakogel C-200, Wako Pure Chemical Industries, Ltd., 350 g) column with CH_2Cl_2-MeOH (9 : 1→7 : 3→1 : 1). The fractions (0.2 g) including **5** were further chromatographed on a FC-40 (120 g) column with CH_2Cl_2-MeOH (95 : 5). Purification of **5** was achieved with HPLC (column: Nova-Pak HR C18, Waters, 60 Å, 6 μm, for semi-preparative, i.d. 25×100 mm) eluted with H_2O-CH_3CN (77 : 23). Pure **5** (12 mg) was given after removing the mobile phase under vacuum.

Table 4. Cytotoxic activities of chrymutasins, chartreusin and related compounds.

Compounds	Cell lines (IC_{50}, μg/ml)				
	P388	Meth A	A549	MKN45	WiDr
Chrymutasin A (**4**)	0.76	1.55	4.25	1.35	1.56
Chrymutasin B (**5**)	3.25	5.83	16.66	3.92	5.54
Chrymutasin C (**6**)	>30	>30	>30	>30	>30
Chartreusin (**1**)	1.99	0.89	7.93	2.93	6.01
Demethylchartreusin (**2**)	4.31	2.37	>30	11.34	28.19
D329C (**3**)	>30	>30	>30	>30	>30

Table 5. Antitumor activities of chrymutasin A and chartreusin.

Tumor/schedule	Dose (mg/kg/day)	T/C (%)	
		Chrymutasin A (**4**)	Chartreusin (**1**)
P388/ip-ip, Q1D×4	20	136	121
Meth A/ip-ip, Q1D×4	20	173	150

Table 6. Antimicrobial spectra of chrymutasin A and chartreusin.

Organisms	MIC (μg/ml)	
	Chrymutasin A (**4**)	Chartreusin (**1**)
Bacillus subtilis M45 (Rec^-)[a]	100	3.2
B. subtilis H17 (Rec^+)	>100	6.4
Staphylococcus aureus JCM2151	>100	100
S. epidermidis JCM2414	>100	25
Micrococcus luteus JCM1464	3.2	1.6
Escherichia coli JCM1649	>100	>100
Klebsiella pneumoniae JCM1662	>100	>100
Proteus vulgaris JCM1668	>100	>100
Xanthomonas maltophilia JCM1975	3.2	12.5
Salmonella typhimurium TA1535	>100	>100
Candida albicans JCM1542	>100	>100
Saccharomyces cerevisiae JCM1499	>100	>100

[a] Recombination deficient mutant.

The fraction (1.3 g) that showed higher polarity than **5** from the *n*-BuOH extract was separated on a FC-40 (350 g) column by eluting with CH_2Cl_2 - MeOH (9 : 1→8 : 2). The fractions containing **6** were charged on the ODS-AQ (120 g), which was developed with H_2O - CH_3CN (7 : 3), to give crude **6** (16 mg). Final purification was carried out with HPLC (column: Capcell Pak C_{18}, Shiseido Co., Ltd., SG120 Å, 5 μm, i.d. 6 × 250 mm) eluted with H_2O - CH_3CN (1 : 1). Evaporation of the relevant fraction gave pure **6** (0.5 mg).

Antitumor Activities

In vitro cytotoxicities of **1**, **2**, **3**, **4**, **5** and **6** against various cell lines are shown in Table 4.

The strongest cytotoxic compound among the chrymutin-type aglycone compounds (**4**, **5** and **6**) was **4**, which corresponded to **1** among the chartarin[13]-type aglycone compounds (**1**, **2** and **3**). Therefore, *in vivo* antitumor activities of **4** and **1** against P388 and Meth A were tested.

The results are shown in Table 5. Compound **4** showed stronger antitumor activities in comparison with those of **1**.

Antimicrobial Activities

The MICs of **4**, which showed the strongest cytotoxic activities among the chrymutasins, are shown in Table 6 in comparison with those of **1**. Compound **4** inhibited the growth of *Micrococcus luteus* and *Xanthomonas maltophilia* but overall was active against less bacteria than **1**.

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