VOL. 55 NO. 4, APR. 2002 THE JOURNAL OF ANTIBIOTICS pp. 371 — 376

Anicequol, a Novel Inhibitor for Anchorage-independent Growth of Tumor Cells from *Penicillium aurantiogriseum* Dierckx TP-F0213

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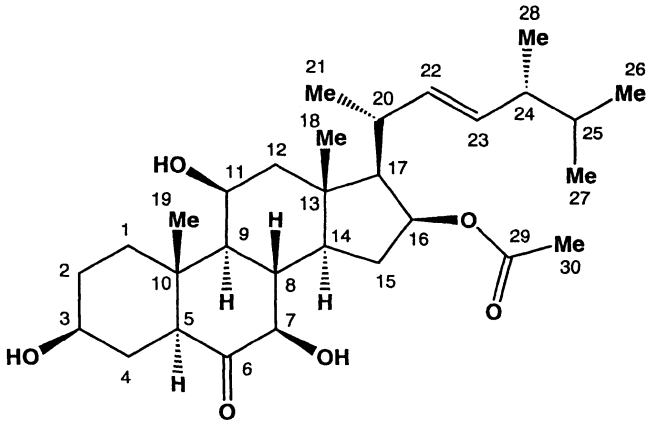
(Received for publication November 2, 2001)

A novel inhibitor for anchorage-independent growth of tumor cells was isolated from the culture broth of a fungal strain. The producing strain TP-F0213 was identified as *Penicillium aurantiogriseum* Dierckx based on the taxonomic study. The compound designated anicequol was obtained by solvent extraction, HP-20 and silica gel chromatographies and recrystallization. The planar structure was elucidated by NMR analysis to be 16-acetoxy-3,7,11-trihydroxyergost-22-en-6-one. The absolute configuration was determined by the X-ray analysis of 3,7-bis-*p*-bromobenzoyl derivative. The carbon skeleton of anicequol has the same absolute configuration as ergostane and the configurations of substituents are 3β,5α,7β,11β, 16β and 24*S*. Anicequol inhibited the anchorage-independent growth of human colon cancer DLD-1 cells with the IC_{50} of 1.2 μM whereas the IC_{50} against anchorage-dependent growth was 40 μM.

Anchorage-independent growth is one of the phenotypic characteristics of tumor cells. Although the molecular mechanism behind anchorage control of cell proliferation is not well understood, growth ability of tumor cells without the firm substrate attachment (anchorage-independence) significantly correlates with their tumorigenicity. Therefore, the development of inhibitors for anchorage-independent growth is expected to provide a unique approach to understanding anchorage-independency and hence cancer chemotherapy. UEHARA *et al.* developed a conventional 96-well microplate assay for quantitation of anchorage-independent growth of tumor cells[1,2], and isolated a novel cell cycle inhibitor NA22598 by using the assay[3,4]. In our program of screening new antitumor substances from microbial secondary metabolites, we found anicequol as an

Fig. 1. Structure of anicequol.



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inhibitor of anchorage-independent growth of human colon cancer DLD-1 cells from *Penicillium aurantiogriseum* Dierckx TP-F0213. In this screening, we tested 2,800 *n*-butanol extracts prepared by cultivating 560 strains of soil-derived actinomycetes and fungi in five kinds of media, and identified three positive extracts including strain TP-F0213.

In this paper, we describe the taxonomy of the producing strain, fermentation, isolation, physico-chemical properties, structure determination and biological properties of anicequol.

Materials and Methods

Instrumental Analysis

Melting point was determined on a Yanagimoto apparatus and is uncorrected. NMR experiments were performed on a JEOL JNM-LA400 NMR spectrometer in $CDCl_3$-CD_3OD (3 : 1) with TMS as an internal standard. MS spectrum was measured on a JEOL DX303 spectrometer. UV spectrum was recorded on a Beckman DU 640 spectrophotometer. IR spectrum was recorded on a Shimadzu FT IR-300 spectrophotometer. Optical rotation was measured on a Horiba SEPA-300 polarimeter.

X-Ray Analysis

Anicequol (5.0 mg, 10 μmol) was dissolved in pyridine (500 μl) and CH_2Cl_2 (500 μl). To this solution were added 4-dimethylaminopyridine (3.0 mg, 25 μmol) and *p*-bromobenzoyl chloride (9.0 mg, 41 μmol) at room temperature. The mixture was stirred overnight and poured into ice-water, and the solution was extracted with ethyl acetate. The organic layer was washed successively with $CuSO_4$ solution, $NaHCO_3$ solution and brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by preparative TLC to yield 3.2 mg of 3,7-bis-*p*-bromobenzoate of anicequol.

This crystalline derivative was recrystallized from methanol-CH_2Cl_2 to give colorless prisms. A colorless prismatic crystal having approximate dimensions of 0.20×0.10×0.40 mm was mounted on a glass fiber. All measurements were made on a Rigaku AFC7S diffractometer with graphite monochromated Cu-Kα radiation. Crystal data are summarized in Table 3. Of the 8791 reflections which were collected, 4451 were unique (R_{int}=0.035). The intensities of three representative reflections were measured after every 150 reflections. No decay correction was applied. The structure was solved by direct methods (SIR92)[5] and expanded using Fourier techniques (DIRDIF94)[6]. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 2998 observed reflections ($I>3.00\sigma(I)$) and 488 variable parameters and converged with unweighted and weighted agreement factors of R=0.047 and R_w=0.058. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.31 and $-0.39e^-/Å^3$, respectively. All calculations were performed using the teXstan crystallographic software package of Molecular Structure Corporation.

Biological Assay

Antitumor activity in the culture broth and fractions was monitored by measuring the anchorage-independnt growth of DLD-1 cells on poly(2-hydroxyethyl methacrylate)-(polyHEMA) coated plates as described below. Human colon cancer DLD-1 cells[7] were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100 μg/ml kanamycin at 37°C with 5% CO_2. The exponentially growing cells were trypsinized and resuspended in fresh medium and then were inoculated onto polyHEMA-coated (suspension culture) or uncoated (attached culture) plates. PolyHEMA-coated plates were prepared as described[1]. The cell growth was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) as described[1].

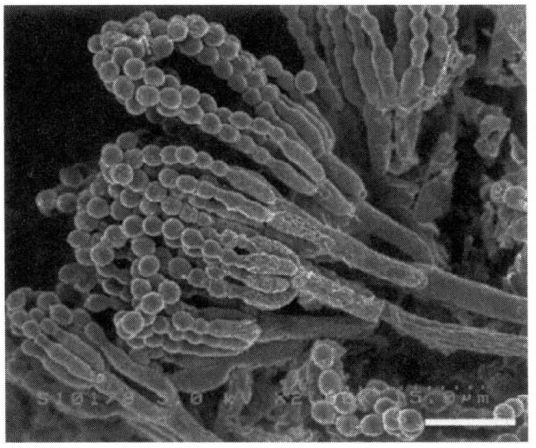
Results and Discussion

Taxonomy of the Producing Strain

The producing fungal strain TP-F0213 was isolated from a soil sample collected in Kosugi, Toyama, Japan. Identification of the producing strain was carried out following the methods of PITT[8,9] and SAMSON *et al.*[10]. Morphological properties were examined after incubation at 25°C for 7 days on Czapek yeast extract agar (CYA), malt extract agar (MEA), 25% glycerol nitrate agar (G25N), Creatine agar (CREA), and Yeast extract sucrose agar (YES). This strain grew rapidly to form blue-green velutinous colonies with a diameter of 30~40 mm on CYA and MEA. It also showed good growth on G25N. The growth on CREA was weak but creatine was decomposed to form acid within 14 days. Soluble pigment was not produced, but an earthy or moldy odor was distinct. Colonies did not grow at 5 or 37°C on CYA. A scanning electron micrograph is shown in Fig. 2. Penicilli were typically terverticillate with smooth stipes. Phialides were ampulliform (11~12×2.5~3.5 μm). Conidia were subglobose, smooth-walled, and less than 3.5 μm in diameter

(2.5~4.0×2.0~3.5 μm, average 3.0×3.0 μm). Because of the terverticillate penicilli and earthy odor, the strain was readily assignable to the *Penicilium aurantiogriseum* complex in the Subgenus *Penicillium*, to which a number of very closely related species belong. The producing strain, TP-F0213 was identified as *Penicillium aurantiogriseum* Dierckx based on the following distinct morphological and cultural characteristics: blue-green velutinous colonies and subglobose smooth-walled conidia with a diameter less than 3.5 μm. TP-F0213 showed some resemblance to *Penicillium aethiopicum* but the latter produces conidia larger than 3.5 μm in diameter and grows at 37°C. The present strain is not a typical *P. aurantiogriseum*, because it grew more rapidly.

Fig. 2. Scanning electron micrograph of *Penicillium aurantiogriseum* Dierckx TP-F0213.



Bar represents 10 μm.

Fermentation

A loopful of a mature slant culture of strain TP-F0213 was inoculated into three 500-ml K-1 flasks containing 100 ml of seed medium composed of glucose 2.0%, Protein S 1.5%, $CaCO_3$ 0.3% and NaI 0.00025% (pH 7.0). The seed culture was incubated for 3 days at 30°C on a rotary shaker (200 rpm). One-ml aliquot of the seed culture was inoculated into two hundred 500-ml K-1 flasks containing 100 ml of production medium composed of the same ingredients as the seed culture. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were incubated for 5 days at 30°C on a rotary shaker (200 rpm).

Isolation

The whole broth (20 liters) was filtered through Celite. The filtrate was discarded and the collected mycelial cake was extracted with 60% aqueous acetone (10 liters) three times. The acetone solution (30 liters) was evaporated and the resultant aqueous solution was applied on a column of HP-20 (Mitsubishi Chemical Co., 3 liters). The column was washed with water (5 liters) and eluted with 50% methanol (5 liters), 80% acetone (10 liters) and 100% acetone (5

Table 1. Physico-chemical properties of anicequol.

Appearance	Colorless needles
MP	174~175°C
$[\alpha]_D^{23}$	+67.1 (*c* 1.0, $CHCl_3$-MeOH=1:1)
HRFAB-MS	
Found:	527.3343 $[M+Na]^+$
Calcd:	527.3349 (for $C_{30}H_{48}O_6Na$)
Molecular formula	$C_{30}H_{48}O_6$
UV λ_{max} nm (log ε)	End absorption
IR ν_{max} (cm^{-1})	3440, 2940, 1720, 1640, 1550, 1385
Solubility	
soluble in	DMSO
slightly soluble in	$CHCl_3$, MeOH
TLC (Rf)[a]	0.36

[a] Silica gel TLC (Merck Art 5715): (toluene-ethyl acetate=1:3)

Table 2. ^{1}H and ^{13}C NMR data of anicequol.

Position	^{13}C	^{1}H (mult, J)
1	35.61	1.35 (m), 2.00 (dd, 2.7, 14.0 Hz)
2	29.51	1.45 (m), 1.83 (m)
3	69.31	3.55 (tt, 4.6, 11.2 Hz)
4	28.54	1.62 (dt, 12.2, 12.5 Hz), 1.80 (m)
5	54.45	2.29 (dd, 1.7, 11.7 Hz)
6	210.33	
7	78.60	3.73 (d, 9.9 Hz)
8	42.17	2.15 (ddd, 9.8, 9.8, 10.8 Hz)
9	55.84	1.35 (dd, 2.9, 12.0 Hz)
10	40.39	
11	67.20	4.36 (dt, 3.0, 1.0 Hz)
12	48.07	1.44 (m), 2.22 (dd, 2.4, 14.2 Hz)
13	42.96	
14	55.20	1.35 (m)
15	36.13	1.54 (dt, 4.6, 13.9 Hz), 2.60 (dd, 6.8, 13.9 Hz)
16	75.15	5.06 (ddd, 4.4, 7.9, 7.9 Hz)
17	59.78	1.25 (dd, 7.8, 11.0 Hz)
18	14.66	1.16 (s)
19	14.98	0.96 (s)
20	34.05	2.55 (m)
21	20.58	1.09 (d, 6.8 Hz)
22	134.85	5.17 (dd, 3.9, 15.2 Hz)
23	132.57	5.19 (dd, 3.9, 15.2 Hz)
24	42.73	1.78 (m)
25	32.72	1.40 (m)
26	19.18	0.81 (d, 6.6 Hz)
27	19.61	0.82 (d, 6.6 Hz)
28	17.60	0.87 (d, 6.8)
29	170.04	
30	21.04	2.00 (s)

liters). Activity was found in fractions eluted with 80% acetone, which was then evaporated to remove acetone. The remaining aqueous solution was extracted with ethyl acetate (1 liter) twice and the organic layer was evaporated to give a crude extract (4 g). The extract was chromatographed on a column of silica gel (100 ml) with toluene-ethyl acetate (1 : 1~1 : 3). Active fractions were combined and evaporated to dryness. The residual white solid was crystallized from methanol to give anicequol (55 mg) as colorless fine needles.

Structure Determination

Physico-chemical properties of anicequol are summarized in Table 2. The molecular formula of anicequol was determined as $C_{30}H_{48}O_6$ on the basis of HRFAB-MS, which gave a $[M+Na]^+$ ion at *m/z* 527.3343 (Δ −0.6 mmu; calcd for $C_{30}H_{48}O_6Na$), and ^{13}C-NMR spectral data. In combination with DEPT and HMQC, the ^{13}C NMR spectrum showed the presence of 30 carbon signals which were assigned to seven methyl, five methylene, twelve methine, two sp^3 quaternary carbons, two olefin carbons and an ester and ketone carbonyl

Table 3. Crystal data for 3,7-bis-*p*-bromobenzoate of anicequol.

Formula	$C_{44}H_{54}O_8Br_2$
Formula weight	870.71
Crystal color, habit	colorless, prismatic
Crystal dimensions	0.20 × 0.10 × 0.40 mm
Crystal systeme	orthorhombic
Lattice type	primitive
No. of reflections used for unit cell determination (2θ range)	20 (33.4-36.5°)
Omega scan peak width at half-height	0.23°
Lattice parameters	a=17.818(2) Å
	b=24.368(1) Å
	c=9.9120(8) Å
	V=4303.7(6) Å^3
Space group	$P2_12_12_1$ (#19)
Z value	4
D_{calc}	1.344 g/cm^3
F_{000}	1808.00
μ(CuKα)	27.93 cm^{-1}

Fig. 3. NMR analysis of anicequol.

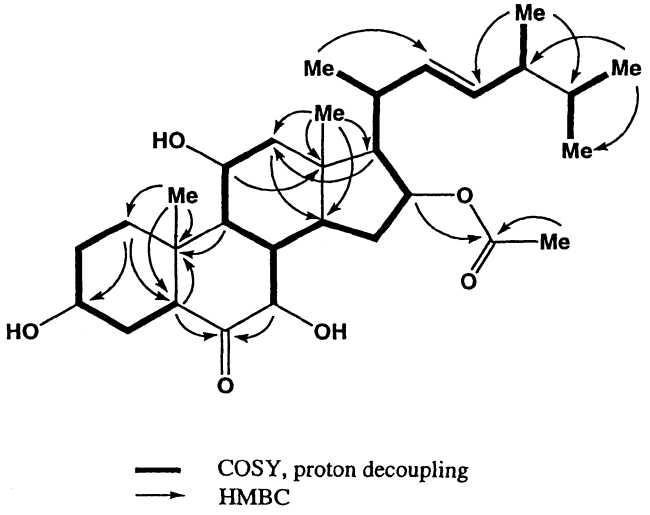
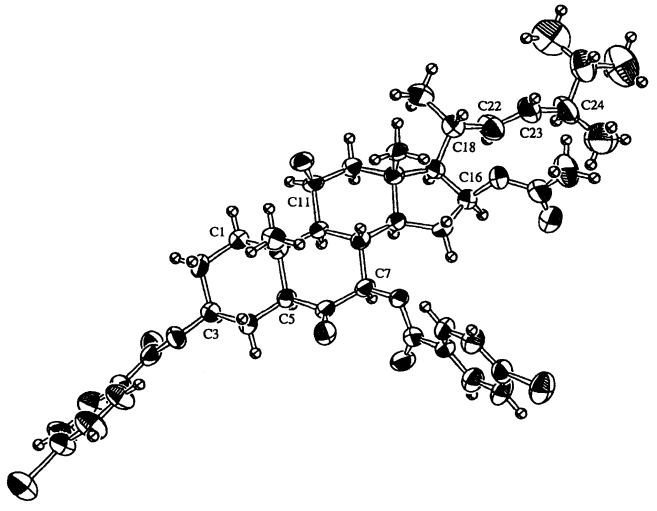


Fig. 4. Perspective view of 3,7-bis-*p*-bromobenzoyl derivative of anicequol.



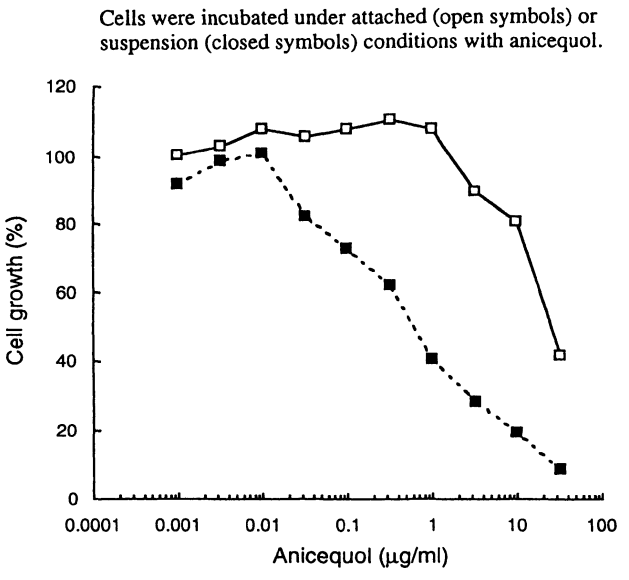
carbons. Analysis of DQF-COSY and proton-decoupling experiments allowed identification of three spin systems as shown in Fig. 3. HMBC correlations from the methyl proton at 0.96 ppm to C-1, C-5, C-9 and C-10 and from H-5 and H-7 to the carbon at 210.33 ppm formed the A-B rings. In addition, HMBC correlations from another methyl group at 1.16 ppm to C-12, C-13, C-14 and C-17 completed the C and D rings of the sterol. The connectivity between C-24 and C-25 was confirmed by the long-range couplings from H-26 to C-24 and H-28 to C-25. The presence of hydroxyl groups at C-3, C-7 and C-11 was deduced from the carbon and proton chemical shifts, and the substituent acetoxy group at C-16 was established by the HMBC correlation from H-16 and a singlet methyl at 2.00 ppm to the ester carbonyl carbon at 170.04 ppm.

In order to determine the stereochemistry, anicequol was converted to the corresponding 3,7-bis-*p*-bromobenzoate by treatment with *p*-bromobenzoyl chloride. Recrystallization of the bis-*p*-bromobenzoate from methanol-CH_2Cl_2 yielded colorless prismatic crystals. The absolute configuration of anicequol was determined by X-ray crystallography as shown in Fig. 4. Anicequol possesses the ergostane skelton, and all of the four oxygenated substituents at C-3, C-7, C-11 and C-16 have β-orientation. The structure of anicequol was thus determined as (3β,5α,7β,11β,16β,24*S*)-16-acetoxy-3,7,11-trihydroxy-ergost-22-en-6-one.

Biological Properties

Anicequol inhibited the anchorage-independent growth of DLD-1 cells on polyHEMA-coated plates with an IC_{50} of

Fig. 5. Effect of anicequol on DLD-1 cell growth.



1.2 μM whereas it did not show cytotoxicity on uncoated plates at the same concentration (Fig. 5). The IC_{50} on uncoated plates was 40 μM. Several commercially available and synthetic steroids (*e.g.* cholesterol, ergosterol, 3β,5α-dihydroxystigmast-22-en-6-one, 3β,5α-dihydroxycholestan-6-one, 3β,5α,7β-trihydroxycholestan-6-one and 3,7-diacetylanicequol) were tested for comparison, but no other steroids have been found active in this assay system so far. Studies on the biological function of anicequol will be discussed in a future paper.

Acknowledgements

The authors thank to Ms N. SAITO at National Institute of Infectious Diseases for the measurement of scanning electron microscope. This work was partly supported by the Social Institute Agency Contract Fund of the Japan Health Science Foundation.

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