CYANOCYCLINE A, A NEW ANTIBIOTIC

TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION, ISOLATION AND CHARACTERIZATION

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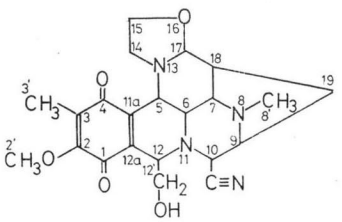
A new antibiotic, cyanocycline A, was isolated from the fermentation broth of *Streptomyces flavogriseus* strain No. 49, a soil isolate. The molecular formula of cyanocycline A was determined to be $C_{22}H_{26}N_4O_5$. The antibiotic has a cyano group and a *N*-heterocyclic quinone moiety in its structure. Cyanocycline A was found to have broad spectrum antimicrobial and antitumor activity.

During the course of screening for antibiotics produced by strains of *Actinomycetes,* a strain of *Streptomyces* was isolated from a soil sample collected at Tama City, Tokyo. Cultures of this microorganism were found to produce marked antimicrobial and antitumor activity. The antibiotic was isolated in an orange-red crystalline form from the culture filtrate of the streptomycete grown in submerged aerated liquid culture.[1,2] The IR, UV and NMR spectra suggested that the antibiotic has a cyano group and a benzoquinone moiety. The structure elucidated by X-ray crystallography revealed that the antibiotic belongs to the group of *N*-heterocyclic quinones.[3,4] At first the antibiotic was called antibiotic No. 49; it was renamed cyanocycline A, when it was found that its structure has a cyano group and heterocyclic ring. The present paper deals with the taxonomy of the producing strain, and the isolation, characterization and biological properties of cyanocycline A.

Fig. 1. The structure of cyanocycline A.



Taxonomic Studies of Strain No. 49

Taxonomic studies were performed according to the procedures and criteria recommended by SHIRLING and GOTTLIEB[5] as well as by PRIDHAM and TRESNER,[6] and WAKSMAN.[7]

Strain No. 49 shows the following characteristics:

(1) Morphological Characteristics

Morphological characteristics of strain No. 49 were observed after 1~3 weeks incubation at 28°C on the ISP (International Streptomyces Project) media.[8,9] The substrate mycelia had branched aerial mycelia, and the top of mycelia were of the form rectus-flexibilis. The spore forming chains were short, and the surface of the spores was smooth. Sclerotia-like structure were not observed.

It is likely that this strain belongs to Section *Rectiflexibilis* of the genus *Streptomyces.*

(2) Cultural Characteristics

Cultural characteristics of strain No. 49 on media for taxonomic studies are reported in Table 1.

Table 1. Cultural characteristics of strain No. 49 on various media.

Medium	Growth	Color of aerial mycelium	Substrate mycelium	Soluble pigment
Yeast - malt extract agar (ISP-2)	good flat	grayish-brown to light brownish gray	brown	none
Oatmeal agar (ISP-3)	moderate flat, powder	light gray	brownish-white to brownish-gray	none
Starch - inorganic salt agar (ISP-4)	good flat, powder	light gray	colorless	none
Glycerin - asparagine agar (ISP-5)	moderate flat, powder	white	grayish olive	none
Peptone - yeast extract iron agar (ISP-6)	moderate wrinkled	colorless	pale yellow brown	none
Tyrosine agar (ISP-7)	good, flat center raised	gray	dark brown gray	none
Sucrose nitrate agar	moderate flat	yellowish gray	yellowish gray	none
Glucose - asparagine agar	moderate flat	light gray	grayish yellow brown	none
Nutrient agar	moderate flat	light gray	pale yellowish brown	none
Gelatin medium	moderate	—	—	none
Skim milk medium	precipitate	—	—	none

The observations were made after 3 weeks of incubation at 28°C. The color of the aerial and substrate mycelia was determined by reference to "Guide to Color Standard", a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

Growth was abundant to fair on most media. The aerial mycelia were grayish, the substrate mycelium was pale yellow, brown or gray. No soluble pigment was formed on the media tested.

(3) Physiological Characteristics

Physiological properties and utilization of carbon sources of strain No. 49 are summarized in Tables 2 and 3. Coagulation and peptonization of milk are strong. Liquefaction of gelatin and hydrolysis of starch are positive. Tyrosinase and production of hydrogen sulfide are negative. Utilization of carbon sources was examined using Pridham-Gottlieb basal medium (ISP-2). Sucrose, inositol, raffinose and cellulose were not utilized for growth. These taxonomic characters were compared with the descriptions of known *Streptomyces* species.

Consequently, strain No. 49 is thought to belong to the Gray series, to which also belong *Streptomyces flavogriseus, Streptomyces griseolus* and *Actinomyces flavoviridis*. The pattern of carbon source utilization indicates that strain No. 49 belongs to the species *Streptomyces flavogriseus*.

Table 2. Physiological properties of strain No. 49.

Parameter observed	Results
Hydrolysis of starch	Positive
Tyrosinase	Negative
Liquefaction of gelatin	Positive
Peptonization and coagulation of milk	Positive
Production of hydrogen sulfide	Negative

Table 3. Utilization of various carbon compounds by strain No. 49.

Carbon sources	Growth	Carbon sources	Growth
Glucose	+	Mannitol	+
Arabinose	+	Fructose	+
Sucrose	—	Raffinose	—
Xylose	+	Cellulose	—
Inositol	—	Rhamnose	+

+: assimilation, —: no assimilation (21 days).

Strain No. 49 was deposited in the Fermentation Research Institute Collection, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces flavogriseus* No. 49 and the accession No. FERM-P 4400.

Fermentation

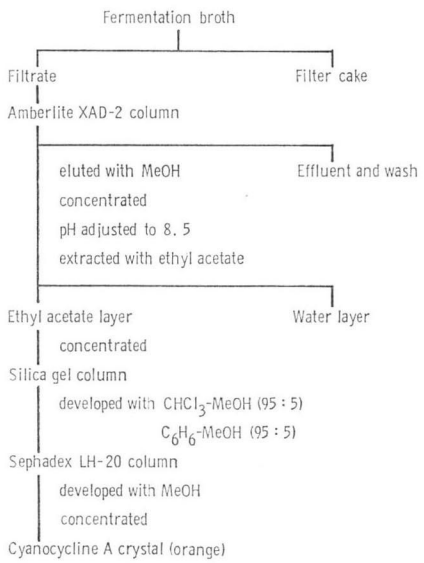
A stock culture of *S. flavogriseus* No. 49 was used to inoculate 100 ml of the seed medium in a 500-ml flask; and incubation was at 28°C on a rotary shaker. A 3-day culture (1 liter) was transferred into 150 liters of the production medium in a 200-liter fermenter and fermentation was carried for 72 hours under the following conditions: temperature 28°C, aeration 75 liters/minute, and agitation 300 rpm. The composition of the seed medium was: 1.0% glucose, 2.0% soluble starch, 1.0% soybean meal, 0.5% corn steep liquor, 0.2% $CaCO_3$ (pH 6.5 before sterilization). The production medium consisted of 1.0% glucose, 2.0% soluble starch, 0.5% corn steep liquor, 1.0% dried yeast, 0.05% KH_2PO_4, 0.001% $CoSO_4 \cdot 7H_2O$, 0.2% $CaCO_3$ and Adekanol (antifoam agent, Asahidenka Co.).

The production of cyanocycline A was determined by the paper disk method employing *Micrococcus luteus* A or *Pseudomonas aeruginosa* M57740 as test organism.

Isolation Procedure

The isolation of cyanocycline A is outlined in Fig. 2. A 72-hour culture broth (150 liters) of *S. flavogriseus* No. 49 was adjusted to pH 4.5 with 6 N HCl solution, and filtered through a filter aid (Radiolite No. 900: Showa Kagaku Co.). The culture filtrate (130 liters) was percolated at the rate of 15 liters/hour through a column (15×80 cm) of Amberlite XAD-2 (Rohm and Haas Co.), and the eluate discarded. The column was successively washed with water (30 liters) and 20% methanol (40 liters). Then, the adsorbed antibiotic was eluted with methanol (15 liters) at the flow rate of 6 liters/hour. The active eluate (6 liters) was concentrated *in vacuo* and the concentrate carefully brought to pH 8.5 with 1 N NaOH. Then, the active principle was repeatedly extracted with ethyl acetate (3 liters). The combined extract was dried with Na_2SO_4 and the solvent removed under reduced pressure to dryness. The dark reddish brown residue was dissolved in a small amount of chloroform and poured on the top of a column packed with silica gel (Wakogel C-200). The column was developed with a solvent mixture consisting of chloroform - methanol (95: 5). The fractions containing cyanocycline A were collected and concentrated under reduced pressure to dryness. The residue was dissolved in a small amount of methanol, and again purified by silica gel column chromatography (Lowber column No. 10401, Merck Co.) with a solvent mixture made of benzene - methanol (95: 5). The antibiotic fractions were pooled and evaporation of the solvent under vacuum to dryness yielded crude cyanocycline A. Further purification was carried out on a Sephadex LH-20 column using methanol as the eluent. The fractions containing cyanocycline A were combined and concentrated *in vacuo* to dry-

Fig. 2. Isolation procedure for cyanocycline A.



ness. The residue was dissolved in a small amount of chloroform, by addition of 10 volumes acetone, the solution became cloudy. The resultant mixture was kept at room temperature overnight, yielding crystalline cyanocycline A depositing on the walls of the vessel. The crystals were collected on a filter and washed with cold acetone (crude cyanocycline A 65 mg/150 liters broth). Recrystallization from acetone yielded about 50 mg of orange-red crystalline cyanocycline A.

Physical and Chemical Properties of Cyanocycline A

Cyanocycline A is basic and appears as orange-red needles. It gradually becomes brown at 163°C, and decomposes at 168~170°C. It is soluble in chloroform, ethyl acetate and alcohol, hardly soluble in water, acetone and petroleum ether. Its specific rotation is $[\alpha]_D^{25}$ +82° (*c* 1, $CHCl_3$). The elementary analysis gave the following data, C 61.89, H 6.10, N 13.05 (%). The values were consistent with the molecular formula $C_{22}H_{26}N_4O_5$; C 62.01, H 6.14, N 13.14 (%). The molecular formula was supported by mass spectrum in which the molecular ion appeared at *m/z* 426. Cyanocycline A gave a UV spectrum with absorption maxima at 268 nm (ε 10,990) in MeOH, 263 nm (ε 9,457) in 0.1 N HCl - MeOH and 283 nm (ε 10,437) in 0.1 N NaOH - MeOH as shown in Fig. 3. IR spectrum in KBr disk showed characteristic absorption bands at 3440, 3380, 3240, 2220, 1670, 1655, 1636, 1619, 1228, 1130 and 910 cm^{-1} (Fig. 4). The weak band at 2220 cm^{-1} is due to the cyano group; strong bands at 1670, 1655 and 1636 cm^{-1} and the UV spectrum indicated the presence of benzoquinone moiety. The proton nuclear magnetic resonance spectrum of cyanocycline A in $CDCl_3$ is shown in Fig. 5. Two singlets at 1.96 ppm (aromatic CH_3) and 4.18 ppm (aromatic OCH_3), and the absence of aromatic proton

Fig. 3. UV spectrum of cyanocycline A.

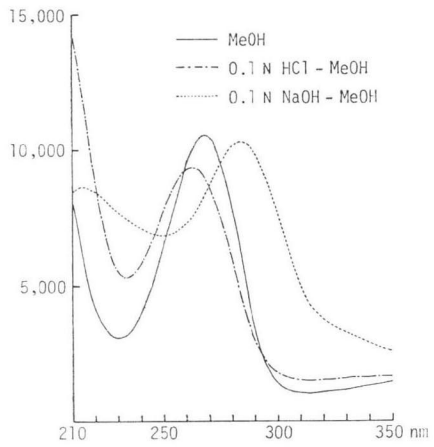


Table 4. Chromatographic properties of cyanocycline A on silica gel TLC.

Solvent	Rf
$CHCl_3$ - MeOH (9: 1)	0.83
C_6H_6 - MeOH (9: 1)	0.33
$CHCl_3$ - ethyl acetate (2: 1)	0.70

Silica gel TLC was carried out on a precoated Merck 60 F_{254} TLC plate (0.25 mm thick). The spot of antibiotic was detected with UV or ninhydrin.

Fig. 4. IR spectrum of cyanocycline A (KBr disk).

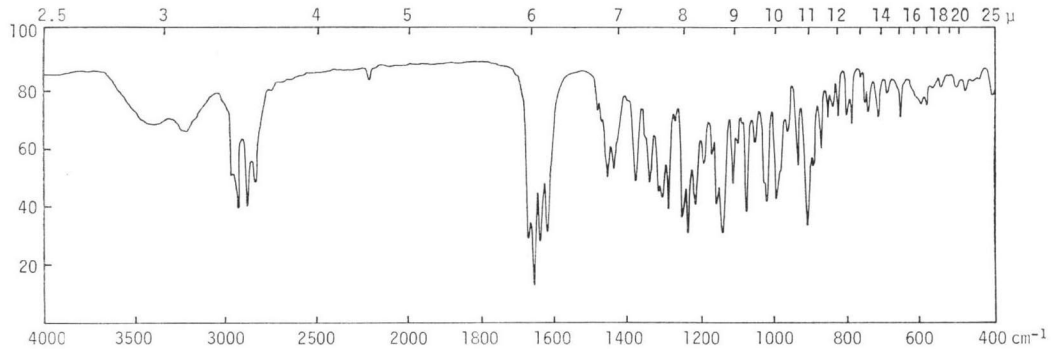
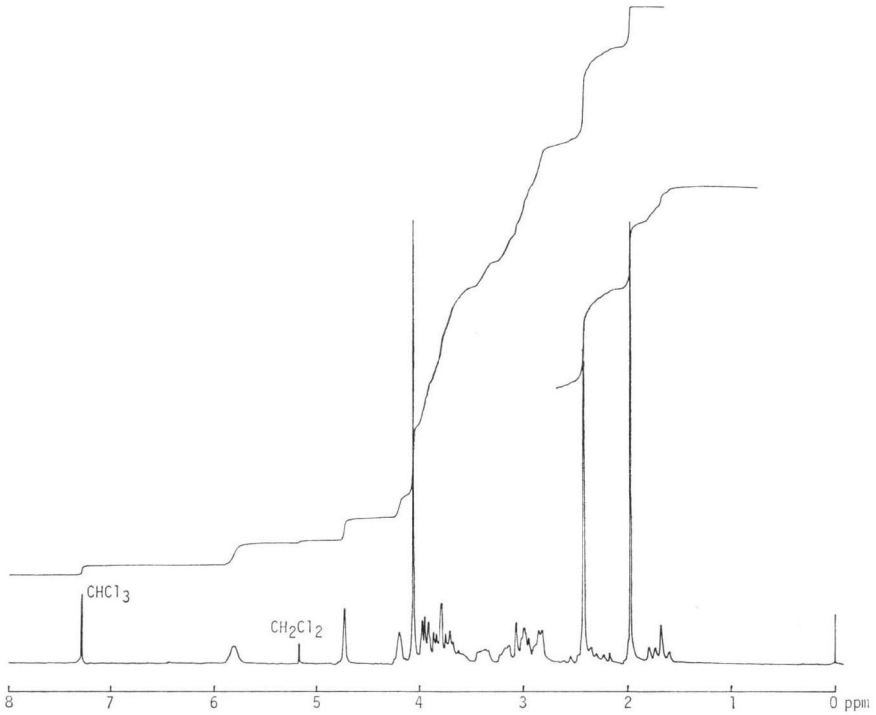


Fig. 5. ^1H NMR spectrum of cyanocycline A ($CDCl_3$).



suggest a fully substituted quinoid chromophore, such as that of mitomycins[10] and naphthyridinomycin.[3]

Rf value in thin-layer chromatography on silica gel plate (pre-coated silica gel 60 F_{254} Merck Co.) is shown in Table 4. The antibiotic gave an orange-yellowish spot, which is detectable with UV light and ninhydrin reagent. We have elucidated the stereochemical structure of cyanocycline A (Fig. 1) with the use of physico-chemical properties and X-ray crystallography. The result will be published elsewhere.[11]

Biological Activity of Cyanocycline A

Cyanocycline A is a broad spectrum antibiotic. The minimum inhibitory concentration (MIC) was determined by the broth-dilution method against selected strains of Gram-positive and Gram-negative bacteria (Table 5). The antibiotic exhibited significant activity against *Micrococcus luteus* B (2.5 ng/ml) and *Staphylococcus aureus* 209P (5.0 ng/ml). It was also very active against Gram-negative bacteria and anaerobic bacteria, but less active against yeast and fungi. Cyanocycline A exhibited strong *in vitro* cytotoxicity against Meth A tumor cell originated from BALB/c mice, and it showed antitumor activity *in vivo* against the ascites type of Meth A. Acute intraperitoneal toxicity (LD_{50}) in mice was 10 mg/kg.

Discussion

The structure of the new antibiotic, cyanocycline A, was elucidated by both spectroscopy and X-ray diffractometry.[11] This compound contains a cyano and a 2-methoxy-3-methyl-*p*-benzoquinone moiety, and belongs to the group of *N*-heterocyclic quinones which includes antibiotics such as mitomycin

Table 5. Antimicrobial activity of cyanocycline A.

Test organisms	MIC (μg/ml)	Test organisms	MIC (μg/ml)
Bacillus subtilis PCI-219	0.005	*Proteus mirabilis* 1287	0.31
Bacillus cereus T-1	0.62	*Proteus mirabilis* 9′	0.31
Micrococcus luteus B	0.0025	*Serratia marcescens* TO-50	0.62
Staphylococcus epidermidis TO-3	0.005	*Serratia marcescens* FU-111	0.62
Staphylococcus aureus 209P	0.005	*Pseudomonas aeruginosa* J-272	0.62
Escherichia coli NIHJ	0.08	*Pseudomonas aeruginosa* NGB75	0.62
Escherichia coli 11	0.15	*Pseudomonas aeruginosa* M-57740	0.31
Salmonella enteritidis T-1	0.08	*Pseudomonas aeruginosa* J-162	0.31
Salmonella typhi Tanaka	0.04	*Pseudomonas aeruginosa* Ps-4	0.15
Klebsiella pneumoniae 3K25	0.31	*Candida albicans* IAM4888	50
Klebsiella pneumoniae 15C	0.31	*Saccharomyces cerevisiae* IAM4804	50
Shigella flexneri 2b TO-1	0.08	*Penicillium chrysogenum* IAM7305	50
Shigella sonnei TO-1	0.15	*Aspergillus niger* IAM2020	100

Media: Antibiotic No. 3 agar medium (Difco) for bacteria, Czapek Dox agar for yeast and fungi.

C and saframycins.[12] Several antibiotics including saframycin A,[4] toyocamycin,[13] borreldin,[14] and kinamycin A~D[15] are known to possess a cyano group in their structure, but cyanocycline A is structurally different from those antibiotics. Moreover, the structure is closely related to that of naphthyridinomycin[3] and of the semisynthetic antibiotic, naphthocyanidine.[16] Cyanocycline A has a cyano group in place of the hydroxyl group present in naphthyridinomycin and has a methoxy group in place of the hydroxyl group in the benzoquinone moiety of naphthocyanidine.

Cyanocycline A exhibits significant antimicrobial activity against Gram-positive and -negative bacteria. This compound also exhibits marked cytotoxicity against cultured tumor cells and ascites tumors in mice. Evaluation of the antitumor activity is now underway.

Note Added in Proof

After this paper had been submitted for publication, M. J. ZMIJEWSKI, Jr. and M. GOEBEL reported cyanonaphthyridinomycin which was synthesized from naphthyridinomycin and sodium cyanide.[17] The physico-chemical data of cyanocycline A are identical to those of cyanonaphthyridinomycin.

Acknowledgement

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