STUDIES ON NEPLANOCIN A, NEW ANTITUMOR ANTIBIOTIC. I.
PRODUCING ORGANISM, ISOLATION AND CHARACTERIZATION

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Neplanocin A, $C_{11}H_{13}N_5O_3$, is a novel carbocyclic analog of adenosine with cyclopentene. It was isolated from the culture filtrate of *Ampullariella regularis* A11079 by means of ion-exchange, carbon, silica gel adsorption, or partition chromatography. Neplanocin A forms crystals, and is stable at acidic or alkaline pH. Neplanocin A has cytotoxicity against L5178Y cells in culture and showed a remarkable effect on the life prolongation of mice infected with L1210 leukemia.

In the course of screening program for antitumor substances from microorganisms, a new antibiotic named neplanocin A has been obtained from the culture broth. The producing organism was isolated from a soil sample collected at Niigata Prefecture, Japan, and designated A11079. It was identified as *Ampullariella regularis* from its taxonomical characters. In preliminary screening, the fermentation broth inhibited the growth of lymphoma cell line L5178Y cells.

The taxonomic studies on the producing organism, isolation and characterization of the neplanocin A are presented in this paper.

Taxonomy of Producing Organism

Most of the taxonomic studies of the culture were carried out in accordance with methods adopted by the International Streptomyces Project (ISP)[1]. Additional media recommended by WAKSMAN[2] and other authors were also used. All tests were run at 30°C. The various media were inoculated with washed mycelial suspensions from a broth culture grown in shake flasks for 96 hours in a liquid medium (1.0% dextrin, 1.0% glucose, 0.5% yeast extract, 0.5% polypepton and 0.1% $CaCO_3$). Microscopic observations were made on cultures that were grown from 10 to 15 days on inorganic salts-starch agar. The method used of BECKER *et al.*[3] was used for whole-cell analysis of 2,6-diaminopimelic acid (DAP). The mycelium was cultivated for 5 days in the liquid medium (described above with exception of $CaCO_3$).

Morphological Characters

The mycelial colonies were colored orange yellow to yellow brown on inorganic salts-starch agar, oat meal agar, yeast extract-malt extract agar or other media. The sporangia were well formed when grown on inorganic salts-starch agar. Usually no aerial mycelium was formed, but it did form in a rudimentary non-sporulating form on inorganic salts-starch agar or oat meal agar. Microscopic observation showed that the hyphae of the substrate mycelium were well developed, straight to wavy and monopodialy branched. The hyphae had a diameter of $0.5 \sim 0.8\ \mu$ and did not fragment into bacillary or coccoid elements. The sporangia were formed on the substrate mycelium, usually on short sporangiophores (Fig. 1). The sporangia were about $5 \sim 15 \times 10 \sim 25\ \mu$ in size and cylindrical to bottle-shaped in shape. Many sporangiospores were arranged in parallel chains within the sporangium.

Fig. 1. Scanning electron micrograph of sporangia of strain A11079.
(Inorganic salts-starch agar, the bar represents 20 μ).

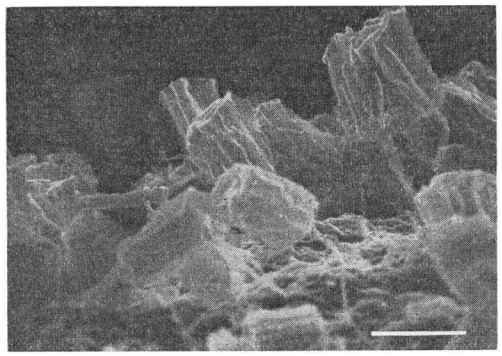


Fig. 2. Electron micrograph of sporangiospore of strain A11079.
(Inorganic salts-starch agar, the bar represents 1 μ).

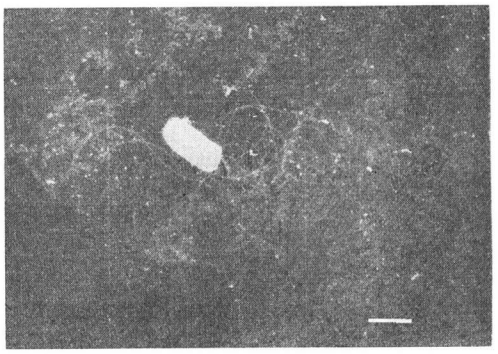


Table 1. Cultural characters of strain A11079.

Medium	Growth	Color of substrate mycelium[a]	Sporangium	Soluble pigment[a]
Sucrose-nitrate agar (WAKSMAN No. 1)	poor to moderate	pearl pink (3ca) to light melon yellow (3ea)	trace	none
Glucose-asparagine agar (WAKSMAN No. 2)	moderate to poor	bamboo (2fb) to pearl pink (3ca)	trace	none
Glycerol-asparagine agar (ISP medium 5)	poor to moderate	bamboo (2fb) to pearl pink (3ca)	none	none
Inorganic salts-starch agar (ISP medium 4)	good	amber (31c) to pastel orange (4ic)	good	none
Tyrosine agar (ISP medium 7)	poor to moderate	light amber (3ic)	none	none
Oat meal agar (ISP medium 3)	good	amber (31c) to bright maize (31a)	moderate	none
Yeast extract-malt extract agar (ISP medium 2)	good to moderate	topaz (3ne) to amber (3pe)	poor	pale amber (3pe)
Glucose-yeast extract agar (WAKSMAN No. 29)	moderate to good	cinnamon (31e)	none	golden brown (3pg)
BENNETT's agar (WAKSMAN No. 30)	good to moderate	amber (3pe) to topaz (3ne)	none	pale golden brown (3pg)
Peptone CZAPEK's agar[b]	moderate to good	light amber (3ic) to amber (31c)	trace	pale golden brown (3pg)
Yeast extract CZAPEK's agar[c]	moderate to good	amber (31c to 3nc)	trace	none
Tyrosine agar[d]	poor	light tan (3gc) to bisque (3ec)	none	clove brown (3pl)
Nutrient agar (WAKSMAN No. 14)	poor	light amber (3ic) to cinnamon (31e)	none	golden brown (3pg)
EMERSON's agar (WAKSMAN No. 28)	good to moderate	cinnamon (31e) to camel (3ie)	none	golden brown (3pg)
Peptone-yeast extract iron agar (ISP medium 6)	moderate to poor	light amber (3ic)	none	dark spice brown (4pl)
Casein agar[d]	good	camel (3ic) to cinnamon (31e)	none	clove brown (3pl)

[a] Color designation from Color Harmony Manual.
[b] J. Elisha Mitchell Sci. Soc. 79: 54 (1963).
[c] J. Virol. 3: 210 (1969).
[d] J. Bacteriol. 69: 147 (1955).

The sporangiospores were rod-shaped, about $0.5\sim1.0\times1.0\sim2.0\ \mu$ in size, and motile by a tuft of polar flagella (Fig. 2).

Whole-cell Analysis

Whole-cell DAP analysis revealed meso- and hydroxy-forms.

Cultural Characters

The cultural characters of strain A11079 shown in Table 1 were observed after 20 days of incubation on the designated media. The number in parentheses corresponds to the hue number used in the Color Harmony Manual[4].

Physiological Characters

The physiological characters of strain A11079 are shown in Table 2, and properties of carbohydrate utilization are shown in Table 3. Hydrolysis of casein or tyrosine and decomposition of xanthine or hypoxanthine and reduction of nitrate were determined on the media described by GORDON *et al.*[5,6,7] The temperature range for growth was observed on the ISP medium 2 for 2 weeks and decomposition of cellulose was observed after one month. Utilization of carbon sources was examined using ISP medium 9 as a basal medium.

Table 2. Physiological characters of strain A11079.

Formation of melanoid pigment	negative on tyrosine agar
	positive on peptone-yeast extract iron agar
Peptonization of milk	positive
Coagulation of milk	positive
Hydrolysis of starch	positive
Hydrolysis of cellulose	negative
Hydrolysis of casein	positive
Liquefaction of gelatin	positive
Decomposition of tyrosine	negative
Decomposition of xanthine	negative
Decomposition of hypoxanthine	negative
Formation of H_2S	negative
Reduction of nitrate	negative
Growth temperature	10~45°C

Table 3. Carbohydrate utilization of strain A11079.

Response	Carbohydrate
Positive	L-arabinose, D-xylose, D-glucose, D-fructose, D-mannose, D-mannitol, L-rhamnose, D-galactose, D-cellobiose, sucrose, starch, salicin, glycerol, maltose, trehalose
Negative	L-sorbose, D-ribose, D-sorbitol, α-melibiose, β-lactose, inositol, raffinose, cellulose, dulcitol, melezitose

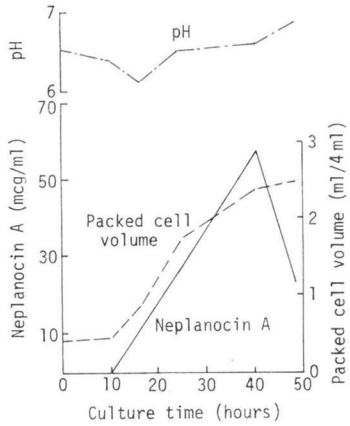
From the results of these studies, the main characters of strain A11079 were summarized as follows: The sporangia were cylindrical to bottle-shaped; the sporangiospores arranged in parallel chains within the sporangium; the sporangiospores were rod-shaped, motile by a tuft of polar flagella; whole-cell hydrolysates contain meso-DAP and hydroxy-DAP; the color of substrate mycelium was orange yellow to yellow brown; the soluble pigment was yellow brown on some media.

The above morphological characters and whole-cell analysis of strain A11079 indicate that this isolate belongs to the genus *Ampullariella* COUCH 1964[8,9,10]. When the characters of strain A11079 were compared in detail with those of *Ampullariella* species described previously, the characters of strain A11079 are in good agreement with those of *Ampullariella regularis*[8,9,10]. Therefore, strain A11079 is identified as a strain of *Ampullariella regularis*. Strain A11079 has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaragi, Japan and at the United States Department of Agriculture, Northern Utilization Research and Development Division, Peoria, Illinois where it has been assigned accession number FERM-P No. 4495 and NRRL 11451, respectively.

Fermentation and Isolation

The medium employed for fermentation of neplanocin A was as follows: 4% glucose, 1% soy bean flour, 0.4% meat extract, 0.4% peptone, 0.1% yeast extract, 0.25% NaCl and after adjustment to pH 6.5 with 6 N NaOH, 0.25% $CaCO_3$ was added. Two-hundred liters of sterilized medium in a stainless steel fermentor was inoculated with 10 liters of a seed culture. The cultivation was performed at 30°C for 40 hours with aeration (130 liters/min.) and agitation (180 rpm). Some analytical data on a large-scale fermentor production of neplanocin A are shown in Fig. 3. Progress of the fermentation was monitored by the cytotoxic activity to lymphoma cell line L5178Y cells and detection of neplanocin A on silica gel TLC using *n*-BuOH - 28% NH_4OH - H_2O (10 : 0.5 : 1) as a solvent system for development. Quantitative determination of neplanocin A was performed by high performance liquid chromatography.

Fig. 3. Time course of neplanocin A fermentation.



The production of neplanocin A is almost parallel to the increase in mycelial growth and reaches a maximum of 60 mcg/ml after 40 hours. The change in broth pH during fermentation is gradual between pH 6.2 and 6.8. Fermentation broth (200 liters) containing neplanocin A was adjusted to pH 6.0 with oxalic acid and filtered with filter aid. The filtrate (140 liters; potency, 57 mcg/ml) was passed through a column of Amberlite IR-120B (H^+, 20 liters). After the column was washed with water, neplanocin A was eluted with 4 N NH_4OH. The biological active fractions (120 liters) were collected and readsorbed on active carbon (4 liters) at pH 8.0. The carbon was washed with water and eluted with 70% aqueous methanol. The active eluates (90 liters) were combined and concentrated under reduced pressure to 300 ml. To the concentrate, 300 g of silica gel powder was added and the mixture was dried under reduced pressure. This powder was charged on a silica gel column (2 liters) packed with *n*-BuOH - 28% NH_4OH - H_2O (10: 0.2: 1). The column was developed with the same solvent. The fractions containing neplanocin A (7.8 liters) were collected and concentrated under reduced pressure. The concentrated solution kept in a refrigerator overnight gave crude crystals of neplanocin A (5.5 g, 710 mcg/mg). The crude neplanocin A was further purified by chromatography on silica gel. The crude powder (5.4 g) was dissolved in 80% hot aqueous acetone and adsorbed on a silica gel column (600 ml) packed with acetone. The column was developed with 95% aqueous acetone. The fractions containing neplanocin A were collected and concentrated under reduced pressure. The concentrate held at room temperature gave crystals of neplanocin A (3.3 g). Recrystallization from hot water gave pure neplanocin A (3.1 g) as colorless needles.

Physicochemical Properties

Neplanocin A behaved as a weakly basic substance, and was stable at acidic or alkaline pH. It was easily soluble in water, acetic acid, dimethylsulfoxide, aqueous methanol and aqueous acetone, and insoluble in most organic solvents such as chloroform, ethyl acetate and benzene. The color reactions of neplanocin A revealed that it was positive for potassium metaperiodate, but negative for MOLISCH,

anthrone, ninhydrin, FEHLING, $FeCl_3$ and EHRLICH. Potassium permanganate was immediately decolorized. By mass spectrometry, the molecular weight of neplanocin A was 263. A strong fragment ion was observed at *m/z* 136. Physical and chemical properties are summarized in Table 4. The UV and IR spectra of neplanocin A are given in Figs. 4 and 5, respectively. The ^{1}H-NMR spectrum of neplanocin A (Fig. 6) showed the presence of two aromatic protons at δ 8.16 (1H) and 8.09 (1H) as singlets and an amino group at δ 7.21 (2H) which disappeared on addition of deuterium oxide.

Table 4. Physicochemical properties of neplanocin A.

Melting point	220~222°C
Analysis	Calcd: C 50.19 H 4.98 N 26.60
	Found: C 49.96 H 5.00 N 26.43
Formula	$C_{11}H_{13}N_5O_3$
Mol. weight	263
$[\alpha]_D^{23}$	−157° (*c* 0.5, H_2O)
UV maxima	in H_2O: 262 nm ($E_{1cm}^{1\%}$ 602)
	in 0.1 N HCl: 260 nm ($E_{1cm}^{1\%}$ 566)
	in 0.1 N NaOH: 262 nm ($E_{1cm}^{1\%}$ 595)
TLC (silica gel f)	Rf 0.36 (*n*-BuOH - AcOH - H_2O, 6 : 1 : 1)
	Rf 0.26 (*n*-BuOH - NH_4OH - H_2O, 10 : 0.5 : 2)
	Rf 0.34 (Acetone - H_2O, 10 : 1)

Fig. 4. UV spectrum of neplanocin A in H_2O.

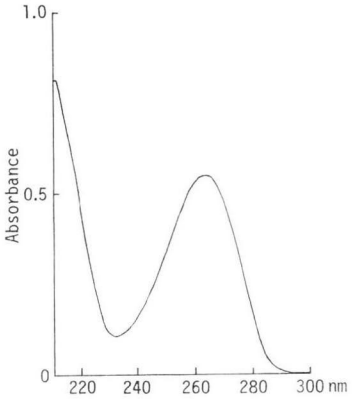


Fig. 5. IR spectrum of neplanocin A.

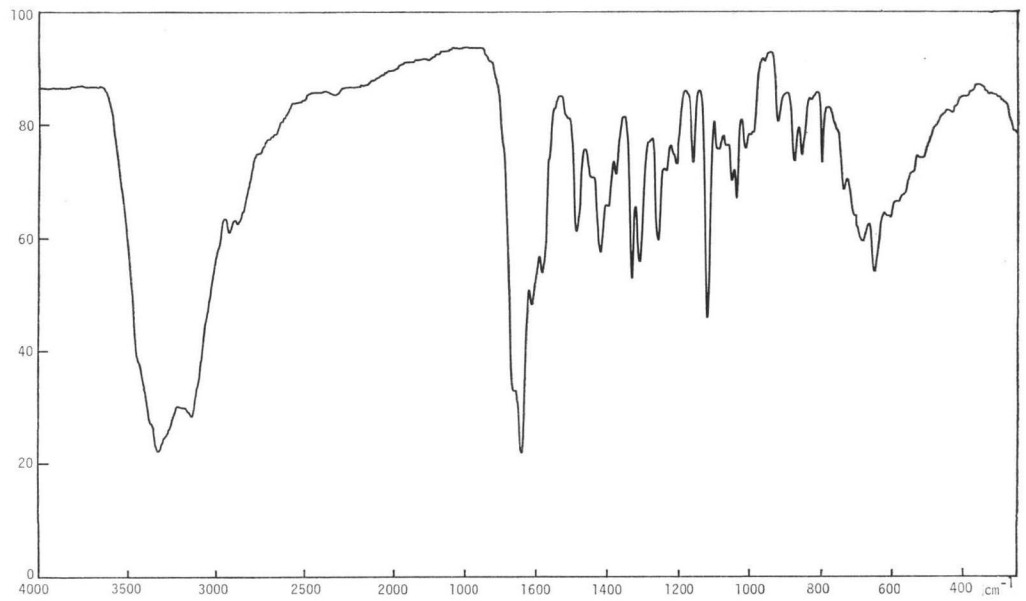
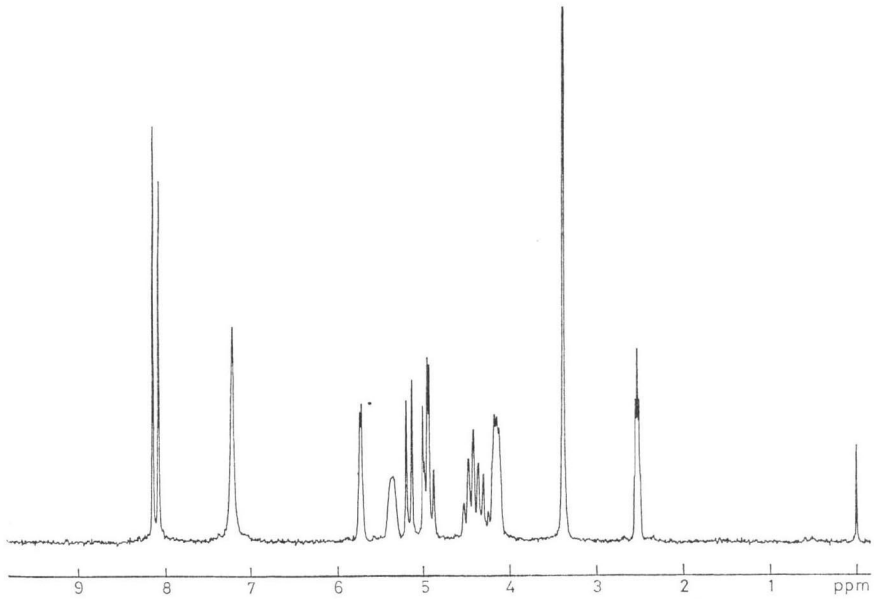


Fig. 6. NMR spectrum of neplanocin A in DMSO-d_6 (100 MHz).



Biological Properties

The antimicrobial and antifungal spectra of neplanocin A obtained by the agar diffusion method are shown in Table 5. As shown in this table, neplanocin A has very slight antibacterial or antifungal activity. But, it is cytotoxic at 0.2 mcg/ml against lymphoma cell line L5178Y cell in culture. Neplanocin A shows significant activity on the life prolongation effect in mice bearing L1210 leukemia. The acute toxicity (LD_{50}) in mice was 13.7 mg/kg when administered intraperitoneally.

Table 5. Biological properties of neplanocin A.

Antimicrobial activity[a] (diameter, mm)	*Alcaligenes faecalis*	11
	Vibrio percolans	15
	Sphaerotilus natans	17
	Bacillus subtilis	0
Antifungal activity[a] (diameter, mm)	*Helminthosporium oryzae*	32
	Alternaria kikuchiana	0
	Glomerella cingulata	0
Cytotoxic concentration[b]	0.2 mcg/ml	
Antitumor activity[c]	120 (5)	
% ILS (mg/kg per day)	58 (0.16)	

a) Determined by paper disc method using 500 mcg/ml drug solution. Agar diffusion assays performed with 7 mm paper discs, thus 8 mm result indicates no-zone observed larger than the disc itself.
b) L5178Y cells were used.
c) Increased life span examined against L1210 leukemia. Drug doses indicated in parentheses were administered for 5 days.

Discussion

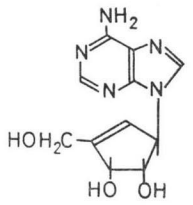
From the taxonomic studies performed it is evident that strain A11079 is classified in the genus *Ampullariella*. Reports on antibiotic production by this genus have been few. Recently, Bayer researchers[11] reported the adenine-containing antibiotic ($C_{10}H_{14}N_6O_3$), which can be considered as being the first antibiotic isolated from this genus. Now, we have also obtained a novel antibiotic named neplanocin A from the cultured broth of *Ampullariella regularis*.

The physicochemical properties, such as UV maximum at 262 nm, fragment ion at *m/z* 136 in the mass spectrum and ^1H NMR spectrum, suggested the existence of an adenine moiety in the

structure of neplanocin A. Accordingly, neplanocin A is clearly a member of the adenosine analog antibiotic group which includes antibiotics such as aristeromycin[12], angustmycin A[13] and C[14], cordycepin[15], nebularine[16], canarius[17], A-9145[18], SF-1306[19], SF-1306 B[20] and herbicidin[21]. But elemental analysis, optical rotation, melting point, IR spectrum and color reactions differentiated neplanocin A from these antibiotics. Therefore, it is concluded that neplanocin A is a new antibiotic.

When neplanocin A was refluxed with dilute sulfuric acid, there was no hydrolysis, which suggested the absence of a N-glycoside linkage. The color reactions of neplanocin A revealed that it is negative for MOLISCH and anthrone, indicating doubt as to the existence of a sugar moiety in its structure. Neplanocin A has a vicinal hydroxy group in the molecule, due to potassium metaperiodate consumption. We propose the structure shown in Fig. 7 for this antibiotic. Thus, neplanocin A is a new carbocyclic analog of adenosine with cyclopentene. Structure studies will be reported separately[22].

Fig. 7. Structure of neplanocin A.



It is known that aristeromycin and nucleoside Q[23] are naturally occurring carbocyclic analogs of nucleoside with cyclopentane and cyclopentene moieties, respectively. Nucleoside Q was discovered in the first position of the anticodon of *Escherichia coli* tRNA (Tyr, His, Asn and Asp) and it has become clear that nucleoside Q is widely distributed in tRNA's from prokaryotes to eukaryotes such as mammalian cells. Accordingly, the production of carbocyclic analogs of nucleoside with a cyclopentene moiety such as in neplanocin A, may occur widely in microorganisms, but such production may be related to growth conditions and stage of cells in a subtle way.

Neplanocin A showed a strong effect on the life prolongation in mice infected with L1210 leukemia. But, aristeromycin was not active against L1210 tumor[24].

In neplanocin A and aristeromycin, the difference in antitumor effectiveness is interesting in view of the difference in structure between cyclopentene and cyclopentane.

Detailed biological studies will be reported separately[25].

It is expected that neplanocin A will provide a new aid in the treatment of cancer.

Acknowledgements

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