STUDIES ON MARINE MICROORGANISMS. IV

A NEW ANTIBIOTIC SS-228 Y PRODUCED BY *CHAINIA* ISOLATED FROM SHALLOW SEA MUD

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A new antibiotic named SS-228 Y, which inhibits growth of Gram-positive bacteria, EHRLICH carcinoma in mice, and dopamine-β-hydroxylase, was obtained from a species of *Chainia* isolated from shallow sea mud in Sagami Bay. It was yellowish brown powder having the molecular formula $C_{19}H_{14}O_6$. From the physical and chemical properties, SS-228 Y was concluded to be a new antibiotic having structure of peri-hydroxyquinone moiety.

In course of a screening for antibiotics produced by actinomycetes from marine environments, a *Chainia* species has been isolated from a sea mud collected in Sagami Bay. The taxonomy of the strain SS-228, the cultural conditions, the isolation and properties of SS-228Y are reported in the present paper.

SS-228 Y Producing Strain

This actinomycete was isolated from a shallow sea mud collected at Koajiro inlet in Sagami Bay in 1972 and numbered as SS-228. The taxonomic characteristics are described below:

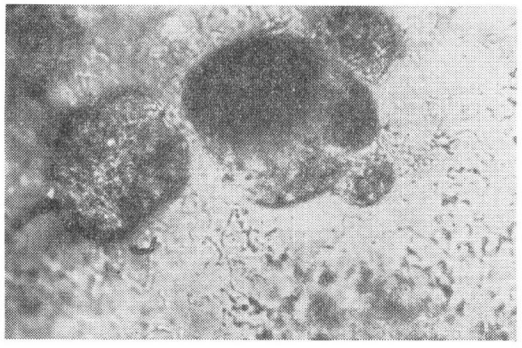
Morphology

This strain forms a sparse aerial mycelium on the growth which becomes compact and elevated lately. Typical sclerotic granules are formed as shown in Plate 1. The sclerotic granules range from 30 to 100 μ in diameter.

Cultural characteristics on various agar media after incubation for 3 weeks at 27°C

The following characteristics were observed on various media suggested by SHIRLING and GOTTLIEB[1] with addition of some special media recomended by WAKSMAN[2], and on media that had been use for isolation of marine actinomycetes which had been examined by the authors and described in the previous paper[3].

Plate 1. 14-day culture on yeast-malt agar, ×400.



On SC agar[3] (soluble starch 1.0 %, casein 0.1 %, agar 1.7 %, artificial sea water 500 ml + dist. water 500 ml, pH 7.4)

AM* : very sparse, white, if any.

G** : moderate, light brown to dark reddish brown.

DP*** : slightly, pale pink.

On GG agar[3] (glycerol 2.0 %, glycine 0.25 %, NaCl 0.1 %, K_2HPO_4 0.1 %, $FeSO_4 \cdot 7H_2O$ 0.01 %, $MgSO_4 \cdot 7H_2O$ 0.01 %, $CaCO_3$ 0.01 %, agar 1.7 %, pH 7.4)

AM : none

*; Aerial mycelium. **; Growth. ***; Diffusible pigment.

G : moderate, pale yellowish brown.
DP : none.

On MSY agar[3] (maltose 1.0 %, yeast extract 0.4 %, agar 1.7 %, pH 7.2)
AM : none.
G : fair, cream-yellow.
DP : none.

On sucrose-nitrate agar[2]
AM : none.
G : fair, reddish brown (5 ie)*.
DP : slightly, pale reddish brown (5 ge).

On glucose-asparagine agar[2]
AM : none.
G : fair, light brown (3 ic).
DP : slightly, pale reddish brown (5 gc).

On glycerol-asparagine agar[1] (ISP. Med.** No. 5)
AM : none.
G : fair, colorless with pale reddish brown (5 gc) patches.
DP : slightly, pale reddish brown (5 gc).

On inorganic salts-starch agar[1] (ISP. Med. No. 4)
AM : none.
G : fair, colorless with pale yellowish brown (1-1/2 1c) patches.
DP : none.

On tyrosine agar[1] (ISP. Med. No. 7)
AM : none.
G : fair, colorless to grayish yellow brown (3 ni).
DP : very slightly, if any, brownish gray (3 lg).

On nutrient agar[2]
AM : none.
G : fair, pale yellow (2 ea).
DP : none.

On yeast-malt agar[1] (ISP. Med. No. 2)
AM : none.
G : fair, colorless with yellowish brown (3 ne) patches.
DP : none.

On oatmeal agar[1] (ISP. Med. No. 3)
AM : none.
G : poor, colorless to yellowish brown (3 ne)
DP : none.

On peptone-yeast extract iron agar[1] (ISP. Med. No. 6)
AM : none.
G : poor, colorless.
DP : none.

On calcium malate agar[2]
AM : none.
G : colorless with pale yellow (2 ea) patches.
DP : slightly, sunlight yellow (1-1/2 ea).

Physiological properties

Utilization of carbon source was examined according to the method of PRIDHAM and

*; Designation number of color by Color Harmony Manual[4].
**; Medium employed by International Streptomyces Project[1].

GOTTLIEB,[5] and the results are shown in Table 1. Other physiological characteristics are described in Table 2. The strain is able to grow at 15°C to 45°C in SC and MYS media, though the optimal temperature is 20~27°C.

Cell-wall composition

According to the method of BECKER *et al.*,[6] the cell-wall composition was examined. The cell-wall of this strain SS-228 contains L-DAP and glycine as the major constituents *i.e.* cell-wall Type 1 according to M.P. LECHEVALIER and H.A. LECHEVALIER.[7]

Table 1. Utilization of carbon compounds by strain SS-228.

Carbon source	Utilization*
No. carbon source**	−
D-Glucose***	+
L-Arabinose	+
Sucrose	+
D-Xylose	+
i-Inositol	+
D-Mannitol	+
D-Fructose	+
Rhamnose	+
Raffinose	+

* +=Positive utilization, −= No growth
** Negative control
*** Positive control

Table 2. Physiological characteristics of strain SS-228.

Test	Results
Hydrolysis of starch	positive
Tyrosinase reaction	negative
Casein hydrolysis	positive
Solubilization of calcium malate	positive
Nitrate reduction	negative
Liquefaction of gelatine	weakly positive
Litmus milk coagulation	weakly positive
Litmus milk peptonization	weakly positive

Summarizing the above, the strain SS-228 isolated from sea mud shows characteristics of the genus *Chainia*.[8]

Among known species, *Chainia purpurogena* THIRUMALACHAR[9] resembles the strain SS-228. Although sporulation of the strain SS-228 is inferior than that of *Chainia purpurogena* on various media, the other morphological properties or the cultural and physiological characteristics are similar to each other in both strains. Therefore, the present strain SS-228 is concluded to be a strain of *Chainia purpurogena* and has been designated *Chainia purpurogena* SS-228.

Production of the Antibiotic SS-228 Y

Among cultural conditions, temperature, constitution of medium, metal iron and pH *etc.*, have been known to be important factors in productivity of secondary metabolites. This strain SS-228 produced only when it was cultured in special medium. In 20 kinds of ordinary media for antibiological screening of streptomycetes, the broths of this strain did not show any inhibition zone when shake-cultured. When it was shake-cultured in another 20 kinds of media, containing Kobu-Cha (powdered tangle sea weed, *Laminaria*) and various carbon sources, this strain showed antibiotic activity to *Staphylococcus aureus* FDA 209 P only in media Nos. 1~7 as shown in Tables 3 and 4. The strongest activity was obtained in medium No. 5, and when other nutrient sources such as yeast-extract and peptone were added to these media, any antibiotic activity was not found.

Since soil extract* media have been known to maintain the characteristics of *Chainia* sp.,[9] soil extract media were also used to examine whether the productivity of antibiotic increases

* Soil extract was prepared by the procedure described in footnote of Table 4.

Table 3. Productivity of antibiotic SS-228 Y in media containing Kobu-Cha (powdered tangle sea weed, *Laminaria*)

Med.	15°C	27°C		Med.	15°C	27°C	
	11 Days	4 Days	7 Days		11 Days	4 Days	7 Days
1	0	10.5*	12.0	11	0	0	0
2	0	15.0	19.0	12	0	0	0
3	0	15.9	11.0	13	0	0	0
4	0	13.0	0	14	0	0	0
5	0	16.5	21.0	15	0	0	0
6	0	16.0	12.0	16	0	0	0
7	0	16.0	18.8	17	0	0	0
8	0	0	0	18	0	0	0
9	0	0	0	19	0	0	0
10	0	0	0	20	0	0	0

* Inhibition diameter by cylinder plate method; *Staphylococcus aureus* FDA 209 P.

or not. As shown in Table 3, the antibiotic productivity was not found in these media.

After a survey of proper medium for the antibiotic production, a medium containing Kobu-Cha 1.0 %, glycerol 1.0 %, pH 7.4 was found to be suitable for the production. Accordingly, the strain SS-228 cultivated on an agar slant of GG medium at 27°C for 2~3 weeks was inoculated to the Kobu-Cha glycerol medium. The inoculated medium, 125 ml in a 500-ml SAKAGUCHI flask, was shaken on a reciprocating shaking machine with 8-cm amplitude, 130 strokes per minute at 27°C for 72 hours. Twenty ml of this broth were transferred to 1,250 ml of the same medium described in flask and shake-cultured in the same way as above for 120 hours. The antibiotic production was followed with a microbiological assay by cylinder plate method using *Staphylococcus aureus* FDA 209 P as the test organism.

Table 4. Composition of medium.

Medium	Components (g/liter)									pH
	Kobu-Cha	Glucose	Maltose	Soluble-starch	Glycerol	Sucrose	Yeast-ext.	Peptone	Soil ext.* (ml/liter)	
1	20									7.4
2	20	10								7.4
3	20		10							7.4
4	20			10						7.4
5	20				10					7.4
6	20					10				7.4
7	20				10				500	7.4
8	20								500	7.4
9	20	10					10			7.4
10	20		10				10			7.4
11	20			10			10			7.4
12	20				10		10			7.4
13	20					10	10			7.4
14	20				10		10		500	7.4
15	20	10						10		7.4
16	20		10					10		7.4
17	20			10				10		7.4
18	20				10			10		7.4
19	20					10		10		7.4
20	20				10			10	500	7.4

* The soil extract is prepared by treating 1 kg garden soil with 2.5 liters of tap water for 1 hour in autoclave at 15 lb pressure.[2]

Isolation and Purification

The fermented broth (pH 7.2) was adjusted to pH 5.0 with diluted hydrochloric acid and filtered to remove the mycelium. The filtrate contained 10 mcg of the antibiotic per ml. The filtrate of 20 liters was extracted twice each time with 10 liters of ethyl acetate, extracting about 85 % of the activity in the broth. The solvent layer was concentrated *in vacuo* at 40°C. Reddish brown colored crude powder (2.23 g) was obtained. This crude powder was placed on the top of a column (2.5×30 cm) filled with 60 g of silicic acid (Silicic acid A.R., 100 mesh, Mallinckrodt) moistened with chloroform. The charged column was developed with chloroform and eluted fractions having antibiological activity were collected. After the active fraction was concentrated *in vacuo* at 40°C, the powder was again applied to the column (2.5 ×30 cm) filled with 50 g of silicic acid. The active fraction developed with water-saturated butyl acetate were collected. From 55 ml of the fraction, 80 mg of yellowish orange powder designated SS-228 Y were obtained (yield about 40 %).

SS-228 Y gave a single spot on TLC developed with chloroform - methanol (9:1) Rf 0.87, benzene - methanol (97 : 3) Rf 0.32 and benzene - ethyl acetate (4:1) Rf 0.21, when detected by color, iodine and potassium permanganate.

Physico-Chemical Properties

The activity of SS-228 Y in aqueous solution at 60°C for 10 minutes at pH 2.0 or pH 7.0 did not decrease, but at pH 9.0 completely disappeared. The activity was not decreased when dissolved in methanol and kept for 3 days in dark room at room temperature. However, after exposure to direct sunlight for a day time or fluorescent light (Toshiba Co., FL 15S) for 18 hours, the activity was reduced to 32 % and for 72 hours inactivated completely. Biologically inactive and dehydrated product from SS-228 Y was designated SS-228 R and it formed red needle crystal.

SS-228 Y forms orange to reddish orange solid material having weakly acidic property and gradually turns to SS-228 R at temperatures of 100°C and higher, then decomposes at 256~266°C. The result of elemental analysis was as follows; calcd. for $C_{19}H_{14}O_6$: C 67.45, H 4.17, O 28.38 (MW 338), found: C 67.26, H 4.28, O 28.26. SS-228 Y is soluble in methanol, ethanol, acetone, ethyl acetate and chloroform. It is slightly soluble in water.

Ultraviolet and visible absorption maxima of SS-228 Y are observed as follows; 218 mμ ($E^{1\%}_{1cm}$ 655), 228 mμ (sh, $E^{1\%}_{1cm}$ 645), 415 mμ (sh, $E^{1\%}_{1cm}$ 118) and 440~460 mμ ($E^{1\%}_{1cm}$ 122) in 0.2 N HCl - 80 %methanol or 80 % methanol solution; 269 mμ ($E^{1\%}_{1cm}$ 530), 408 mμ ($E^{1\%}_{1cm}$ 190) and 552 mμ ($E^{1\%}_{1cm}$ 130) in 0.2 NNaOH - 80 %methanol solution. (Fig. 1) As shown in Fig. 2, infrared absorption spectrum of SS-228 Y shows the following characteristic bands; 1680 cm^{-1} (α, β-unsaturated carbonyl group), 1665 cm^{-1} (quinone carbonyl group) and 1630 cm^{-1} (chelated quinone carbonyl group).

The NMR spectrum of SS-228 Y (d_4-methanol) shows the bands at δ 1.93 (3H, s), δ 2.68 (2H, s), δ 6.12 (1H, m), δ 6.47, 6.90 (2H, AB quartet, J-10.0), δ 7.24 (1H, dd, J=7.5, 2.0). δ 7.45-7.85 (2H, m). The mass spectrum showed the parent peak at *m/e* 320 (M^+—18). SS-228 Y is positive in magnesium acetate test (reddish violet) and sodium dithionate test (red).[10]

Fig. 1. Ultraviolet and visible spectra of SS-228 Y and R.

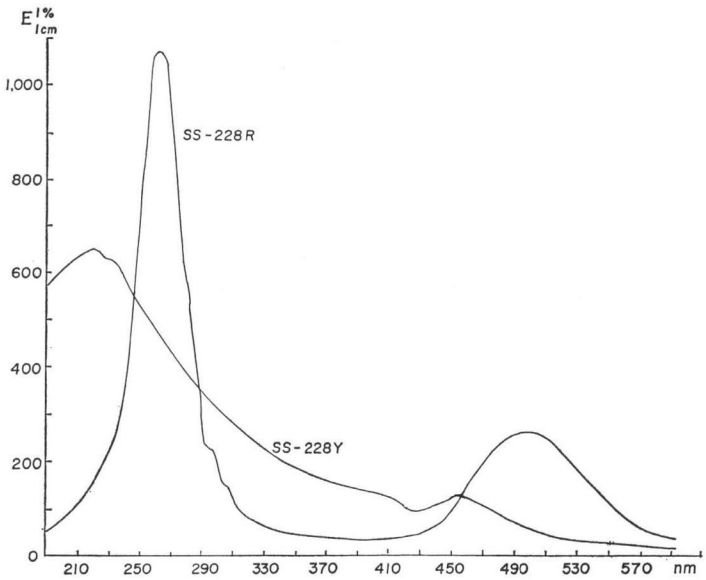
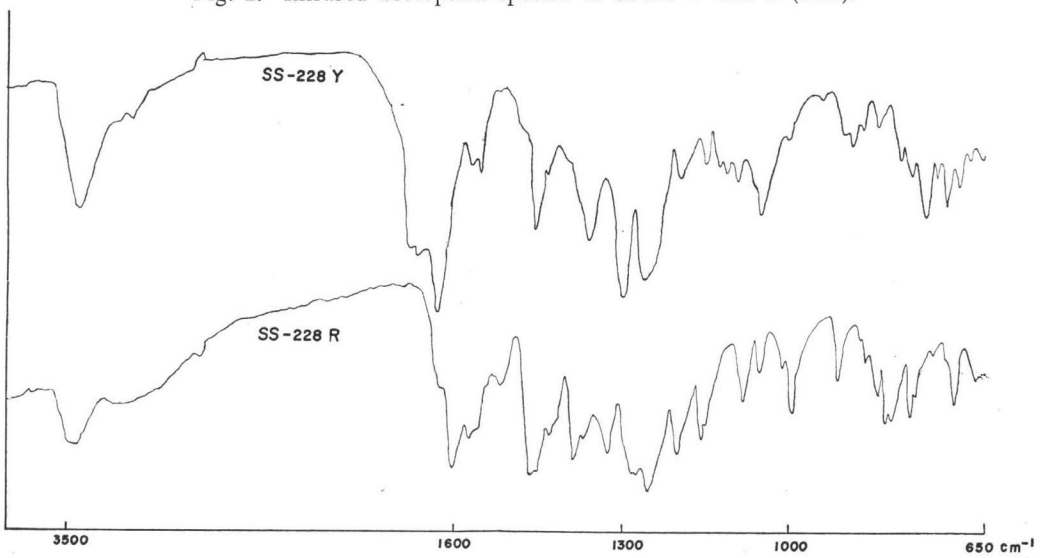


Fig. 2. Infrared absorption spectra of SS-228 Y and R (KBr).



From these results of physicochemical analysis, the structure of SS-228 should be a unusual peri-hydroxyquinone derivative.

Biological Activity

As shown in Table 5, SS-228 Y inhibits gram-positive bacteria except mycobacteria *in vitro*. When 1.56, 3.12, 6.25 and 12.5 mcg of SS-228 Y/mouse/day were administered daily to mice inoculated with EHRLICH ascites tumor for 10 days, prolongation of survival period was observed at doses of more than 1.56 mcg/mouse/day. Inhibition of dopamine β-hydroxylase by

Table 5. Inhibitory concentration of SS-228 Y.

Organism	M.I.C. (mcg/ml)*
Mycobacterium smegmatis ATCC 607	50
〃 *phlei*	25
Bacillus subtilis NRRL B-558	3.12
〃 〃 PCI 219	1.56
〃 *anthracis*	1.56
Corynebacterium bovis 1810	12.5
Micrococcus flavus FDA 16	<0.78
Sarcina lutea PCI·1001	1.56
Staphylococcus aureus FDA 209 P	3.12
〃 〃 Smith	1.56
Escherichia coli NIHJ	12.5
〃 〃 K-12	>100
〃 〃 ML 1629	>100
〃 〃 W 677	>100
〃 〃 JR 66/W 677	100
〃 *freundii* GN 346	>100
Klebsiella pneumoniae PCI 602	50
Pseudomonas aeruginosa No. 12	>100
Proteus vulgaris OX 19	50
〃 *mirabilis* IFM OM-9	50
Salmonella typhosa T-63	100
Serratia marsescens	>100
Shigella flexneri 4b JS 11811	50
Candida albicans 3147	50

* Medium: Nutrient agar dilution method for bacteria, nutrient agar contained 1% glycerol for mycobacteria and nutrient agar contained 1% glucose for yeast.

SS-228 Y was examined according to the method of NAGATSU *et al.*[11] It showed 65.2% inhibition at 0.1 mcg/ml. Acute toxicity shown by LD_{50} in mice was 1.56~6.25 mg/kg by the intraperitoneal injection.

Discussion

The abundance of terrestrial actinomycetes and their antibiotic productivity have been well known. However, investigations dealing with the marine microorganisms are extreamly few and inconclusive. Thus far we have isolated 500 strains of actinomycetes from shallow sea muds in Sagami Bay in Japan, these isolates include species of genus *Chainia*, *Nocardia*, *Micromonospora*, *Actinoplanes* and *Streptomyces*. Some of these should be of interest because of their ability to produce antibiotics.

Since antibiotic productivity has been known to be closely related with the cultural conditions, the cultural conditions relating to the environment where the organism was isolated might be one which should be employed.

Strain SS-228 showed an example for that antibiological substance was produced only in specially deviced media pertaining to the conditions in environment where the strain SS-228 was isolated. When this strain is shake-cultured in ordinary media for antibiological screening of actinomycetes, it did not show any inhibitory activity, but when it was shake-cultured in medium containing Kobu-Cha (powdered tangle sea weed, *Laminaria*), this strain showed

antibiotic activity to *Staphylococcus aureus* FDA 209 P.

Another interest was emphasized by the production of antibiotics having unusual structure derived from other genus of actinomycetes (*Chainia* in this case) than streptomycetes which have been known to be rich source of antibiotics.

Two antibiotics were hitherto isolated from genus *Chainia*; chainin was isolated from *Chainia* sp. No. 3047[12] and aburamycin, which was first isolated from *Streptomyces aburaviensis*,[13] was isolated from *Chainia minutisclerotica*,[14] as reported by THIRUMALACHAR *et al.* Antibiotic, SS-228 Y is clearly different from chainin (polyene) and aburamycin (aureolic acid). Spectroscopic properties of SS-228 Y are characteristic for peri-hydroxyquinone derivative.

Many colored antibiotics produced by actinomycetes have been known to have hydroxyquinone structure containing no nitrogen. SS-228 Y, therefore, was compared with known antibiotics lacking nitrogen and having similar ultraviolet spectra. Among known colored antibiotics, SS-228 Y is differentiated from ayamycin A_2,[15] TA-435A,[16] julimycin B-II,[17] tetrangomycin,[18] hedamycin,[19] rabelomycin[20] and aquayamycin[21] in physical and chemical properties, such as infrared spectra and elemental analysis.

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