Studies on Alkaloids of *Streptomyces*

Part II. Pharmacological Activity and Isolation of Nigrifactin and Taxonomical Studies of *Streptomyces* Strain No. FFD-101

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In the course of screening research on alkaloids among metabolites of microorganisms, *Streptomyces* strain No. FFD-101 which was isolated from a soil sample collected in Bangkok of Thailand, was found to produce a new alkaloid, nigrifactin both in its culture filtrate and mycelium. This alkaloid possessed strong inhibitory activity against histamine and caused a transient fall of blood pressure. From the results of taxonomical study on the strain No. FFD-101, it was found that this is a variant of *Streptomyces nigrifaciens*, to which the name, *Streptomyces nigrifaciens* var. FFD-101 was proposed. Examination of the alkaloid productivity with media in various compositions indicated that the medium containing glycerol 3% as carbon source and casamino acid 0.35% as nitrogen source was most suitable for the production.

In the previous paper¹⁾ of this series, we reported that *Streptomyces* strain No. FFD-101 which was isolated from a soil sample collected in Bangkok of Thailand, was found to produce an alkaloid in its culture filtrate. Furthermore this alkaloid hydrochloride possesses a strong inhibitory activity against histamine and causes a transient drop of blood pressure. In the course of further investigations on alkaloids of *Streptomyces*, we have succeeded in isolating a new alkaloid, which we named nigrifactin, from the culture filtrate and mycelium of strain No. FFD-101. The present paper deals with the pharmacological activity and isolation of nigrifactin and taxonomical studies of the strain No. FFD-101.

MATERIALS AND METHODS

Taxonomical study. *Streptomyces* strain No. FFD-101

1) T. Terashima, Y. Kuroda and Y. Kaneko, *Agr. Biol. Chem.*, **34**, 747 (1969).

2) H. Nishimura and K. Tawara, *J. Antibiotics*, **10A**, 82 (1957).

was cultivated on each medium conventionally being used for the classification of *Streptomyces* at 27°C and/or 37°C and observed during a period of four weeks for the changes in growth, aerial hyphae and soluble pigment. The morphological properties of the strain were observed on organic media such as potato-glucose agar (2% glucose, 1.5% agar in boiled extract of 500 g potato with 1 liter tap water) and Bennett's agar with agar-cylinder culture method.²⁾ The structure of spore was observed under electron microscope. Utilization of carbon sources was determined by cultivation on Pridham and Gottlieb's³⁾ medium which contain 1% of various carbon compounds.

Detection of pharmacological activity. The crystalline picrate of the alkaloid was dissolved in 1N NaOH solution and extracted with chloroform. The chloroform layer was extracted with 0.1N HCl solution and then the acidic solution was extracted with chloroform until free picric acid was perfectly removed. The aqueous solution of the alkaloid hydrochloride thus prepared from the picrate was neutralized to pH 6~7 by using Amberlite IR-4B and employed for the

3) T. G. Pridham, C. W. Hesseltine and D. Gottlieb, *J. Bacteriol.*, **56**, 107 (1948).

following pharmacological test. The pharmacological actions of the alkaloid on the serosa of the small intestine *in vitro* were observed with Magnus method. Choline, atropine, adrenaline, histamine and nor-adrenaline were used as stimulants at the final concentration of 5×10^{-5} , 1×10^{-5} , 1×10^{-5} , 6×10^{-6} and 1×10^{-5} M, respectively. The rabbits were anesthetized with 1.0 g/kg (intraperitoneal injection) of urethan. The mean blood pressure of the left carotid artery was recorded on a smoked paper via an arterial cannula and a mercury manometer. Spontaneous respiration was recorded on a smoked paper by means of rubber tambour connected to a tracheal cannula, simultaneously with the arterial blood pressure. The alkaloid was injected into the vein.

Cultural condition of the alkaloid productivity on various compositions of media. Examination of the alkaloid productivity on various compositions of media was carried out using basal medium as shown in Table I.

TABLE I. COMPOSITION OF BASAL MEDIUM

Carbon sources ^{a)}	30 g/liter
Nitrogen sources ^{a)}	3.5
K ₂ HPO ₄	1.0
KCl	0.5
MgSO ₄ ·7H ₂ O	0.5
FeSO ₄ ·7H ₂ O	0.01 pH 7.0

^{a)} Carbon and nitrogen sources employed for this experiment were listed in Table V.

The strain grown in 2% glucose-potato agar slant was transferred into test tubes containing 10 ml of the each medium described above and incubated for 2 days at 30°C. Then, 10 ml of this culture broth was transferred into 500 ml shaking flasks containing 100 ml of the same medium. The fermentation was carried out at 30°C on a reciprocal shaker. The amount of nigrifactin produced was measured at daily intervals according to the following method. The culture filtrate was rendered alkaline with 1N NaOH and extracted with chloroform. The chloroform layer was extracted with 0.1N HCl and then modified Dragendorff's reagent⁴⁾ was added to the acidic solution. Turbidity formed was expressed as +, ++, +++ and ++++ scale. The mycelia were harvested

by filtration from the culture broth at various times during growth and then dried at 100°C for one hour and weighed.

Antimicrobial activity. Antimicrobial activities of the alkaloid were examined against *Staphylococcus aureus* 209 P, *Escherichia coli*, *Candida albicans*, *Aspergillus niger* and *Penicillium chrysogenum* Q 176 at the concentration of 650 ppm according to the usual procedures.

Toxicity to mammals. The toxicity of the alkaloid to mammals was examined using mice by intraperitoneal administration. The alkaloid was diluted with water in series, if necessary. Groups of six dd-mice were used which were fed with a controlled diet for 3 days before the test.

FIG. 1. Aerial Hyphae of *Streptomyces* Strain No. FFD-101 ($\times 600$) (photomicrograph).

RESULTS AND DISCUSSION

Taxonomical identification of Streptomyces strain No. FFD-101

The sporophores are branched with tendency to curl and spirals (Fig. 1). The conidia are grown in chain of more than ten spores,

4) B. T. Cromwell and S. D. Rennie, *Biochem. J.*, **58**, 318 (1954).

TABLE II. CULTURAL CHARACTERISTICS OF *Streptomyces* STRAIN No. FFD-101

Medium	Growth	Aerial mycelium	Soluble pigment
Glucose-Czapek agar	good, dark brown	white to dark brown	brown
Sucrose-Czapek "	scant, pinkish gray	white to gray	none
Glucose-asparagine "	good, cream-colored	gray	yellow
Ca-malate "	moderate, brownish	white	faint brown
Tyrosine "	moderate, white to pale brown	pale brown	dark brown
Bennett's "	good, bacteria-like, cream-colored	white	brown
Nutrient "	good, bacteria-like	none	brown
Glucose peptone "	good, bacteria-like, pale yellow	white	grayish brown
Peptone-iron "	good, pale yellow	none	dark brown
Glucose-potato "	good, white to pale brown	white	brown
Bouillon broth	surface: moderate bottom: palp-like growth	white	redish brown
Gelatin stab (18°C)	good, white	white	brown
Egg	faint, whitish gray	none	darkish brown
Carrot plug	faint, pale yellowish brown	none	none
Potato plug	moderate, pale yellow, bacteria-like	none	dark brown
Loeffler's serum	scant, brownish	none	none

the shape of which are oval to elliptical, $0.6\sim 0.7\mu\times 0.4\sim 0.5\mu$, with smooth surface (Fig. 2).

The cultural and physiological characteristics of the strain is listed in Tables II and III. The utilization of carbon sources of the

strain is shown in Table IV. From the results of these studies, the cultural and physiological characteristics of the strain No. FFD-101 are summarized as follows: Growth type on various organic media such as glucose peptone, nutrient, Bennett's and potato agar

TABLE III. BIOCHEMICAL AND PHYSIOLOGICAL PROPERTIES OF *Streptomyces* STRAIN No. FFD-101

Melanin formation	positive
Nitrate reduction	negative
Utilization of cellulose	negative
Milk coagulation	positive (weak)
Milk peptonization	positive (weak)
Liquefaction of gelatin	positive
Tyrosinase reaction	positive
Hydrolysis of starch	positive (strong)
pH range for growth (bouillon)	5.0~9.0 (opt. pH 6.0~8.0)
Temperature for growth (bouillon)	22~42°C (opt. temp. 37°C)
Relation to oxygen	aerobic
Thermal death point	60°C

FIG. 2. Conidia of *Streptomyces* Strain No. FFD-101 (electronmicrograph).

TABLE IV. UTILIZATION OF CARBON SOURCES

Carbon source	Utilization ^{a)}
Arabinose	†
Xylose	†
Raffinose	†
Rhamnose	†
Fructose	†
Galactose	†
Mannose	†
Maltose	†
Lactose	†
Dextrin	†
Glycerol	†
Glucose	†
Mannitol	†
Inositol	†
Salicin	†
Sucrose	+
Sorbitol	—
Inulin	—

^{a)} †, good growth +, faint growth —, no growth

was bacteria-like accompanying no or little quantities of aerial hyphae. Color of aerial mycelium is white to gray or dark brown on synthetic media. Color of aerial mycelium is white and sometimes white to brown, soluble pigment is blackish brown (chromogenic) on various proteinous media. Melanoid pigment and tyrosinase reaction are positive. Among many species of *Streptomyces* described in "Bergey's Manual of Determinative Bacteriology," 7th Ed.⁵⁾ and "The Actinomycetes," Vol. II,⁶⁾ the strain No. FFD-101 appears to be most closely related to *S. nigrifaciens* from morphological properties, color of aerial mycelium on synthetic agar and some of physiological characteristics. However, in the following respects of morphological and physiological properties, the strain No. FFD-101 was found to be not com-

pletely identical with *S. nigrifaciens* described so far.

1) *S.* strain No. FFD-101 clearly forms spirals, although *S. nigrifaciens* did not.

2) *S.* strain No. FFD-101 exhibited strong starch hydrolysis, but *S. nigrifaciens* showed weak hydrolysis.

3) NO₂ formation by *S.* strain No. FFD-101 is negative, but that by *S. nigrifaciens* is positive.

4) *S.* strain No. FFD-101 utilizes many carbon sources as shown in Table IV.

Therefore *S.* strain No. FFD-101 should be assigned to a variant of *S. nigrifaciens* and designated as *Streptomyces nigrifaciens* var. FFD-101.

TABLE V. EFFECT OF CARBON AND NITROGEN SOURCES ON THE PRODUCTION OF THE ALKALOID

Carbon source	Time (hr)			Max. value of the alkaloid	Growth
	96	120	144		
Soluble starch	††	†	†	††	†
Dextrin	††	††	†	††	†
Sucrose	††	†	†	††	+
Glycerol	††	††	††	††	†
Glucose	†	†	†	†	+
Nitrogen source					
NaNO ₃ +vitamine mix. ^{a)}	—	—	—	—	+
NH ₄ NO ₃ + "	—	—	—	—	+
Urea+ "	—	—	—	—	±
NaNO ₃ +0.2% yeast ext.	†	†	†	†	†
NH ₄ NO ₃ + "	†	†	†	†	†
NZ amine	†	††	††	††	†
Peptone	†	††	††	††	††
Tryptone	†	††	††	††	††
Casamino acid	††	††	††	††	†

—, +, †, †† and ††† indicate relative quantities of precipitate formed.

^{a)} Vitamine mixture: riboflavin (2 mg), thiamine (1 mg, as HCl salt), *p*-aminobenzonic acid (1 mg), pyridoxine (1 mg, as HCl salt), pyridoxal (0.2 mg), Capantothenate (1 mg), nicotinic acid (1 mg), biotin (10 ppm), folic acid (10 ppm), cyanocobalamin (1 ppm), inositol (5 mg).

5) R. S. Breed, E. G. D. Murray and N. R. Smith, "Bergey's Manual of Determinative Bacteriology," 7th Ed., Williams and Wilkins Co., 1957.

6) S. A. Waksman, "The Actinomycetes," Vol. II, Williams and Wilkins Co., 1961.

Productivity of nigrifactin on various media

Effect of carbon source. Glycerol, glucose, sucrose, soluble starch and dextrin was added to the basal medium containing 0.35 % casamino acid as nitrogen source to a concentration of 3 %. The yield of nigrifactin produced by shaking culture is shown in Table V. A maximum production was obtained at 114 hr with the medium containing 3% glycerol. Glucose, dextrin, sucrose and soluble starch were inferior to glycerol for the production of nigrifactin.

Effect of nitrogen source. Various nitrogen sources including NaNO_3 , NH_4NO_3 , urea, NZ amine, peptone and tryptone were added to the basal medium containing 3 % glycerol as a carbon source to a concentration of 0.35 %. As represented in Table V, the organic nitrogen sources were generally better for the production of nigrifactin than the inorganic nitrogen. Particularly, casamino acid was the most suitable for its production. NZ amine and peptone were somewhat inferior. With the inorganic nitrogen sources, no measurable amount of nigrifactin was produced. However, when 0.2 % yeast extract was added to the medium containing inorganic nitrogen, considerable amount of nigrifactin was obtained.

Fermentation process

The fermentation process was revealed in Fig. 3, in terms of the alkaloid production, pH and mycelium. The amount of nigrifactin attained a maximum peak at 120 hr.

Isolation of nigrifactin

When strain No. FFD-101 was cultivated in about 10 liters of medium containing 3 % of glycerol, 0.35 % of casamino acid, 0.1 % of K_2HPO_4 , 0.05 % of KCl , 0.05 % of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 % of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at 30°C for 5 days by jar fermentor, it accumulated maximum amount of nigrifactin in the culture fluid.

A schematic outline of isolation procedure

FIG. 3. Fermentation Process of Nigrifactin in a Jar Fermentor.

Cultural condition: aeration rate 1 vvm, agitation 300 rpm seed 3 %, incubation temperature 30°C .

Medium: glycerol 3 %, casamino acid 0.35, K_2HPO_4 0.1, KCl 0.05, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001, pH 7.0.

Symbols: ●—● growth
 ■—■ pH
 ▲—▲ nigrifactin

is shown in Fig. 4. The broth (about 10 liters, pH 5.0) was filtered, concentrated *in vacuo* to 2 liters under the inert gas and was treated at pH 4.0 twice with an equal volume of ethyl acetate each time to remove yellow pigment, acidic and neutral materials and lipids. The aqueous layer was then adjusted to pH 10 with 1N NaOH and extracted three times with an equal volume of *n*-hexane each time. The combined *n*-hexane layer was washed with water to eliminate yellow pigment. Thereafter the *n*-hexane layer was extracted three times with 200 ml portions of 0.1N HCl. The aqueous solution was again made alkaline and the above extraction procedure was repeated to give 150 ml of 0.1N HCl extract of nigrifactin.

As the free alkaloid was very labile and easily oxidized to a brown syrup when standing even under the inert gas, it could not be obtained in a crystalline state. In order to get the alkaloid in a pure state, it

FIG. 4. Isolation Procedure of Nigrifactin.

could not be obtained in a crystalline state. In order to get the alkaloid in a pure state, it was converted into its crystalline salts of organic acids, such as picric, oxalic and flavianic acids. A saturated aqueous solution of picric acid was added dropwise to the above mentioned acidic solution of the alkaloid until no more precipitate was formed. The crude picrate (950 mg) thus obtained was recrystallized several times from mixed solvent of water-methanol (4:1) to afford 750 mg of lemon-yellow needles. The picrate melts at 175.5~176°C under decomposition. In preliminary experiments, various kinds of solvents systems and carriers were tested. Since two solvent system, *i. e.*, butanol: acetic acid:

water (4:1:1, $R_f=0.43$), acetone: methanol (9:1, $R_f=0.27$) on Kiesel gel G plate and three solvent system, *i. e.*, butanol: acetic acid: water (4:1:1, $R_f=0.93$), cyclohexane: chloroform: methanol (15:15:1, $R_f=0.87$), isopropanol: 5% ammonia (10:1, $R_f=0.81$) on aluminium oxide G (Merk) plate gave the most satisfactory results, they were used for confirmation of purity of the alkaloid. The picrate is soluble in methanol, chloroform, ethanol, acetone and dimethyl sulfoxide. It is slightly soluble in 0.1N NaOH and insoluble in hexane, benzene and ether.

Pharmacological activity of nigrifactin

The effect of nigrifactin on blood pressure

FIG. 5. Effect of Nigrifactin on the Blood Pressure of Anaesthetized Rabbit.

Res.: Respiration

B.P.: Blood pressure

FIG. 6. Antihistaminic Activity of Nigrifactin by Magnus Method with Isolated Guinea Pig Intestine.

1: Histamine 6×10^{-6} M

2: The alkaloid 8×10^{-4} M

are shown in Fig. 5. Intravenous injection of 6 mg/kg of nigrifactin caused a transient fall of blood pressure of rabbit, but did not antagonize against the response of 0.2 mg/kg of adrenaline.

The inhibition of histamine response (6×10^{-6} M) by nigrifactin (8×10^{-4} M) was

about 60 % (Fig. 6). Nigrifactin (8×10^{-4} M) inhibited about 20~25 % of choline response (5×10^{-5} M), but did not antagonize against other stimulants, *i. e.*, atropine, adrenaline and nor-adrenaline.

Toxicity to mammals

It was little toxic to mice showing no significant symptom by intraperitoneal injection of 195 mg/kg doses.

Antimicrobial activity

The alkaloid was not effective against five test organisms, *i. e.*, *Staphylococcus aureus* 209 P, *Escherichia coli*, *Candida albicans*, *Aspergillus niger* and *Penicillium chrysogenum* Q 176 at the concentration of 650 ppm.

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