Microbiol. Cult. Coll. Dec. 1997. p. 103-108 Vol. 13, No. 2

Productivity of Antimicrobial Substances in Pathogenic Actinomycetes *Nocardia brasiliensis*

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The productivity of antimicrobial substances by isolation from clinical specimens was studied. Among 76 clinical isolates tested, 18 methanol extracts of mycelia and 4 culture filtrates from different strains showed antimicrobial activities. Chemical studies of the antibacterial substance from 9 mycelial extracts showed it to be an indole alkaloid type antibiotic, brasilidine A, although the chemical nature of the remaining 9 extracts was not determined. The structure of hydrophilic antimicrobial substance from the culture filtrates of 4 other strains was found to be N, N′-ethylenediaminedisuccinic acid (EDDS). Concomitant production of benz[*a*]anthraquinone type antibiotics was also observed in 9 strains.

Key words : *Nocardia brasiliensis*, productivity, antimicrobial substance, indole alkaloid

INTRODUCTION

Actinomycetes are well known for their economic importance as producers of biological substances such as antibiotics, vitamins, and enzymes (4). Most of the producers belong to the restricted genera of the actinomycetes *Streptomyces, Actinoplanes, Actinomadura*, and *Micromonospora* (2, 4). However, recent extensive screening for new antibiotics has focused on the rare actinomycetes such as *Saccharopolyspora* (3) and *Dactylosporangium* (15).

Nocardia brasiliensis has become a well-recognized human pathogen (1). Typically, it causes serious infections of pulmonary or central nervous system, or subcutaneous ones in mostly immunocompetent individuals (1). Coupled with the increasing number of the immunocompromised patients treated with corticosteroids, AIDS and organ transplant patients, nocardioses have been thought not to be rare (12).

We recently found that one of the clinical isolates of *N. brasiliensis* produced a new cytotoxic and antimicrobial indole alkaloid called brasilidine A (Fig. 1) (17) containing an isonitrile group. Our preliminary studies suggested that some strains of *N. brasiliensis* produce a similar substance. Therefore, we are interested to compare the productivity of antimicrobial substances of clinical isolates of *N. brasiliensis* and, here, we report the data and chemical nature of other antimicrobial substances produced by *N. brasiliensis* as well.

MATERIALS AND METHODS

Microorganisms. Seventy-six strains of *N. brasiliensis* including the type strain (IFM 0236=JCM 3374=ATCC 19296) were cultivated. Most of the clinical strains were identified in our laboratory.

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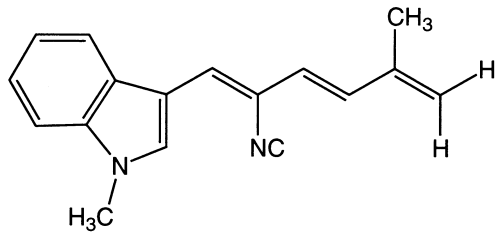

Fig. 1. Structures of an indole alkaloid antibiotic, brasilidine A

These strains have been maintained on brain heart infusion (BHI, Difco, Detroit, U.S.A.) agar medium in our culture collection. *Corynebacterium xerosis* IFM 2057 and *Micrococcus luteus* IFM 2036 were used as organisms for the susceptibility test.

Cultivation and extraction of active substances. Inocula for fermentation were prepared by growing each *Nocardia* strain in 5 m*l* of 2% glucose BHI broth in 10 m*l* Erlenmeyer flasks for 72 h at 30°C under shaking conditions (250 rpm) in a dry incubator (New Brunswick, model G-25). The bacterial suspension was placed in 500 m*l* Erlenmeyer flasks containing 100 m*l* of a medium consisting of 1.0% glucose, 1.0% glycerol, 1.0% Polypepton (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) and 0.5% meat extract (pH 7.0), and was cultured at 32°C for 96 h on a rotary shaker at 300 rpm. The culture broth was separated by centrifugation, and methanol extraction was carried out by using harvested mycelia.

Antimicrobial activities. Antimicrobial activities of methanol extracts (50 μl) and culture supernatant (50 μl) were tested by the paper disk method using BHI medium and *C. xerosis* IFM 2057 and *M. luteus* IFM 2036 as test organisms.

Identification of brasilidine A by HPLC. The methanol extracts which showed antibacterial activity were concentrated *in vacuo*, and then dissolved in 1 m*l* of *n*-hexane. The material soluble in *n*-hexane was analyzed by HPLC (YMC Pack SIL-06, 4.6×250 mm, eluent, *n*-hexane-ethyl acetate=30 : 1, flow rate, 1 m*l*/min ; UV detection, 365 nm). Brasilidine A prepared in our laboratory was used as the standard for HPLC analysis.

Detection of brasilidine A by bioautography. The hexane soluble fractions mentioned above were analyzed using a combination of silica gel TLC and bioautography (5) with *C. xerosis* IFM 2057 as a test organism. They were developed with the solvent system of *n*-hexane-ethyl acetate (4 : 1) and chloroform-methanol (3 : 1). Brasilidine A showed Rf values of 0.4 and 0.9 on the above TLC and bioautography system, respectively.

Purification of ethylenediaminedisuccinic acid (EDDS). Since the active fraction was not absorbed by active carbons (Wako Pure Chemicals), the fraction passed from the culture filtrates was absorbed by a column of Dowex-X 4 H. After washing with water, the active fraction was eluted with 0.5 N ammonia and the eluate was evaporated to dryness *in vacuo*. The dried residue was further purified by preparative TLC using a silica gel (Merck preparative plates : methanol-25%ammonia=95 : 5, Rf ; 0.6～0.7). This fraction passed through a column of an anion exchanger (BIORAD, A 63-X 4 A[Cl]) and was eluted with 5 M KCl. Desalting of the bioactive fraction was done using a cation exchanger on Dowex 50 W-4 X(H). Elution of the active fraction occurred by 0.5 N NH_4OH. It was further purified by column chromatography on Sephadex LH-20 (methanol-dist. water=1 : 1) and needle-shaped crystals of the active substance which crystallyzed from the antibacterial LH-20 fraction were obtained.

Isolation of a mutant strain with high antimicrobial activity. A seed culture of the type strain *N. brasiliensis*, IFM 0236 was inoculated in a 10 m*l* Erlenmeyer flask containing 4 m*l* of BHI broth. After growing to a stationary phase (4 days), it was spun down and suspended in 15 m*l* of saline. The suspension was irradiated by UV light. The UV irradiated suspension was diluted and plated on 1% glucose BHI agar medium (8).

RESULTS AND DISCUSSION

Brasilidine A production. During the surveying investigations of the antibacterial substances produced by 76 strains of *N. brasiliensis*, it was found that the methanol extracts of 18 strains showed antimicrobial activity against *C. xerosis* IFM 2057. The number and the history of each clinical isolate among 76 strains of *N. brasiliensis*, which released antimicrobial activities in methanol extracts or culture filtrates, are shown in Table 1. Nine samples of the methanol extracts were extractable by *n*-hexane or ethyl acetate after concentration. Bioautography of these samples

Table 1. List of *Nocardia brasiliensis* strains which shows antibacterial activity for *Corynebacterium xerosis* IFM 2057 for brasilidine A and *Micrococcus luteus* IFM 2036 for EDDS

IFM No.	Time of isolation	Place of isolation	Source of specimen	Brasilidine A[a]	EDDS[b]
0236		Type strain		−(−)[c]	−
0236-3		mutant[d]		+(+)	−
0236-7		mutant[d]		+(+)	−
0276	1987.10.23	Tokushima Univ	arm	+(+)	−
0321	1989.9.4	Hiroshima Tetsudo Hosp	foot	−(−)	+
0326	1989.12.21	Tokyo Kosei Nenkin Hosp	lymphatic gland	−(+)	−
0355	1991.6.17	Shiga Univ Med Science	femur	−(−)	+
0358	1991.11.21	Matsue Red Cross Hosp	hand abrasion	+(+)	−
0370	1992.8.5	Musashi Japan Red Cross Hosp	knee	−(+)	−
0378	1992.10.2	Keio Uniy	face exanthema	−(+)	−
0381	1992.11.19	Chiba Univ	nose	−(+)	−
0383	1993.1.27	Tokyo Kosei Nenkin Hosp	toe	−(+)	−
0386	1993.2.12	Saitama Med Sch Pediat	skin	−(+)	−
0407	1993.10.28	Kawasaki Med Univ	foot	−(+)	−
0415	1994.3.4	Kumamoto Rosai Hosp	hand pad	+(+)	−
0466	1994.9.10	Thailand NIH	pus from leg	+(+)	−
0493	1994.12.5	Kurashiki Central Hosp	femur	−(+)	−
0568	1995.9.14	Dokkyo Med Univ	femur	−(−)	+
0581	1995.12.25	Keio Univ	femur	+(+)	−
0596	1996.4.10	Chiba Univ	nose	−(−)	+
0641	1996.8.29	Nanki general Hosp	waist	+(+)	−
0642	1996.8.29	Nanki general Hosp	lung, whole body	−(+)	−
0653	1996.9.5	National Tsu Hosp	lymphatic gland	+(+)	−
0666	1996.10.21	Komagome Hosp	knee	+(+)	−
0667	1996.11.25	Komagome Hosp	skin	+(+)	−

[a] Productivity of brasilidine A

[b] Productivity of EDDS

[c] Parenthesis shows the result of antimicrobial activity test for *C. xerosis* IFM 2057

[d] see the materials and methods section

showed that one of the substances possesses similar Rf value to that of brasilidine A in the two solvent systems tested [brasilidine A ; Rf 0.4 (*n*-hexane : ethyl acetate (4 : 1)] and Rf 0.9 [chloroform : methanol (3 : 1)]. Further identification of the active principle with brasilidine A was done by HPLC using the standard sample. A typical HPLC profile of *n*-hexane extracted brasilidine A from *N. brasiliensis* IFM 0089 (9) was shown in Fig. 2. These studies confirmed that the active substance produced by the 9 strains of *N. brasiliensis* is brasilidine A.

Production of brasiliquinones. The occurrence of other active components in the bioautography of the ethyl acetate extracts suggested the production of second antibiotic in addition to brasilidine A. Since the active substances seem to be similar to benzo [*a*]anthraquinone type antibiotics reported earlier (9, 18), *N. brasiliensis* IFM 0667 was selected and the active substance was purified by the reported method (9, 18). By the ^{13}C- and ^{1}H-NMR spectral studies on the purified component, it was confirmed that the active substances were composed of a mixture of brasiliquinones A, B, and C. Identification of the active substances produced by other strains as brasiliquinones was also done by HPLC

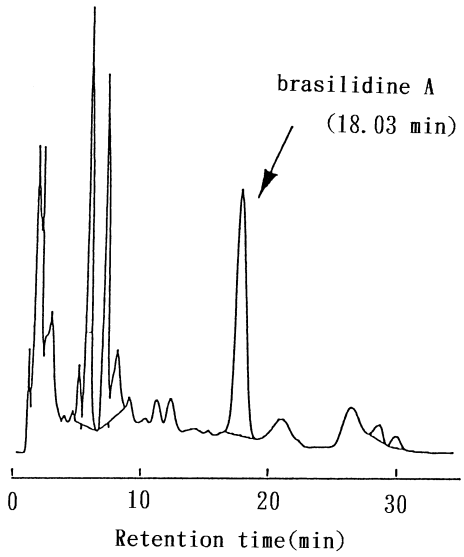

Fig. 2. HPLC patterns of brasilidine A which is extracted with *n*-hexane from the methanol extracts of *N. brasiliensis* IFM 0089 (see ref. 9)

(Lichrospher RP-18, 4.6×125 mm, eluent : 32% acetonitrile including 0.2% TFA ; flow rate, 1 m*l*/min ; UV detection, 235 nm). Thus concomitantly produced active substances from the remaining 9 strains were also found to be brasiliquinones. It is well known that some actinomycetes produce different antibiotics at the same time as satellite antibiotics (12). However, further studies on the biosyntheses of both compounds are of interest because these antimicrobial substances are structurally different from each other.

Since the production of brasilidine A and brasiliquinones was recognized in the 9 strains of *N. brasiliensis*, we were interested in the productivity of the type strain of *N. brasiliensis* IFM 0236 (= JCM 3374=ATCC 19296). When the productivity of antimicrobial substances of this strain was tested, a weak antimicrobial activity in the mycelial extracts was noted. However, their low production did not permit elucidation of their chemical nature. To obtain sufficient amounts for chemical analyses, mutant strains with high producing ability were isolated after UV-treatment. Among the 960 colonies isolated, 2 strains were selected : IFM 0236-3 and 0236-7, which showed higher activity against *C. xerosis* IFM 2057. HPLC studies showed that the major active substance produced by *N. brasiliensis* strains IFM 0236-3 and -7 is brasilidine A. Production of brasiliquinones by the two strains has not yet been confirmed.

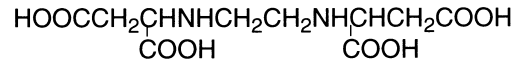

Fig. 3. Structure of N, N′-ethylenediaminedisuccinic acid (EDDS)

In addition to these 10 strains of *N. brasiliensis* including the mutant strain derived from IFM 0236 type strain, 9 other strains of *N. brasiliensis* were found to produce the antimicrobial substances in their mycelial extracts (Table 1). Although we were unable to classify their chemical nature due to their lower productivity, these substances are considered to be mixtures of brasilidine A and brasiliquinones because they displayed similar chemical characteristics of solubility in *n*-hexane and ethyl acetate. Most *N. brasiliensis* strains seemed to possess the ability to produce these compounds.

Production of N, N′-ethylenediaminedisuccinic acid (EDDS). During the test on antimicrobial substance, 4 culture filtrates of *N. brasiliensis* (IFM 0321, 0355, 0568, and 0596) were found to produce antibacterial substances with activity against *M. luteus* IFM 2036 (Table 1). Since the inhibition zone around the paper disc showed incomplete inhibition as a characteristic feature, the active substance is thought not to be identical with brasiliquinones or brasilidine A. Hence we isolated and purified the active substance by the method shown in Materials and Methods. Our mass and ^{13}C- and ^{1}H-NMR spectral studies showed unambiguously that the active component is N, N′-ethylenediaminedisuccinic acid (EDDS) (Fig. 3). EDDS was first reported (11) as a synthetic chelating compound by Neal and Rose (10). In 1984 Nishikiori et al. (11) isolated EDDS as a microbial product from actinomycetes and reported it to be an inhibitor of phospholipase C. However, its antimicrobial activity, has not yet been described. EDDS cheletes calcium and magnesium ions in a similar manner as ethylenediaminetetraacetic acid, hence the biological effects of this compound are as ascribed to this property. Our unreported results suggested that EDDS also inhibits α-glucosidases from Baker's yeast (*p*-nitrophenylglucosides as a substrate). This is the

first demonstration of antimicrobial activity of EDDS against bacteria and fungi such as *M. luteus, Aspergillus niger*, and *Cryptococcus neoformans*, and is also the first report of isolation of EDDS from pathogenic *Nocardia*.

Among pathogenic *Nocardia* species, most of the infections in man and animals are caused by the *N. asteroides* complex which includes three species, *N. asteroids* sensu stricto, *N. farcinica*, and *N. nova* (1, 13). In contrast to *N. asteroides*, the other human pathogens including *N. brasiliensis, N. otitidiscaviarum*, and *N. transvalensis* have been considered homogenous (13). Interestingly, our survey investigations of pathogenic *Nocardia* spp. showed that production of antibiotics is restricted to just two species, namely *N. brasiliensis* (7, 14, 18) and *N. otitidiscaviarum* (6). Most members of these two bacterial species produce abundantly aerial mycelium, but this character is different from that of other antibiotic-non-producing *Nocardia* species, such as the *N. asteroides* complex. Therefore, as for morphology, antibiotic-producing *Nocardia* appear more similar to *Streptomyces* as the most well-known antibiotic producer, than to these *N. asteroides* complexes. Demain and Fang (2) reported that secondary metabolisms in actinomycetes are switched on by the exhaustion of a nutrient and/or by a decrease in growth rate. These events are thought to generate signals which effect a cascade of regulatory events resulting in the onset of antibiotic production and morphological differentiation (2). Production of brasilidine A was observed in 23% of the *N. brasiliensis* strains tested when determined by the antimicrobial activity test. However, in general, assay using gene (s) of antibiotic production is considered to be a more sensitive method for the detection of antibiotic production than that of the antimicrobial susceptibility test. One person of our research group is doing cloning studies on brasilidine A production genes. Further detailed studies on the capacity of secondary metabolite production in pathogenic *Nocardia* using the antibiotic production gene detection method are of interest.

In 1996 Ruimy et al. (13) proposed a new taxon, *N. pseudobrasiliensis*, for some strains of *N. brasiliensis* based on such taxonomic characteristics as the decomposition of adenine, reduction of nitrate and susceptibility to certain antimicrobial agents like minocycline (13). Quite recently, we isolated new anthracycline antibiotics called nocardicyclins (16) from the type strain of *N. pseudobrasiliensis* IFM 0624 (=JCM 9894). The results of our preliminary studies also suggested that nocardicyclins are produced by another strain of *N. pseudobrasiliensis* IFM 0623 (=JCM 9893). In addition to these findings, the present results may indicate that the production of antibiotics in *Nocardia* can be used as one of the chemotaxonomic characteristics for species identification.

Acknowledgments. This work was partly supported by a Grant of the Ministry of Education, Sciences, Sports, and Culture of Japan.

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臨床材料より分離した病原性放線菌 *Nocardia brasiliensis* の抗微生物活性物質の生産性について

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患者より分離した76株の *Nocardia brasiliensis* の抗微生物活性物質の生産性について検討した．検討した76株の中で，18株の菌体のメタノール抽出液中に抗微生物活性が確認された．活性物質の生産が微量であり，活性物質の抽出が困難な9株を除いた残りの9株について，生産物質の抽出および化学性状の検討の結果，これら9株の生産する抗微生物物質はインドールアルカロイド系の物質 brasilidine A であることが明らかになった．さらに，これら9株では同時に，アントラキノン系の brasiliquinone の生産も確認された．一方培養液では76株中，4株において抗微生物活性が確認された．これら4株の生産物は，mass, NMR 等の化学分析の結果，N, N′-ethylenediaminedisuccinic acid (EDDS) であることが確認できた．これらの抗微生物活性物質の生産性と分類との関係を論じた．