Brasilicardin A; a New Terpenoid Antibiotic Produced by *Nocardia brasiliensis*

Hisayuki Komaki^{1,2*}, Akira Nemoto², Yasushi Tanaka^{1,2}, Katsukiyo Yazawa¹, Takashi Tojo², Hiroaki Takagi², Kiyoshi Kadowaki², Yuzuru Mikami¹, Hideyuki Shigemori³ and Jun'ichi Kobayashi³

¹ Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8673, Japan

² Research Laboratory, Higeta Shoyu Co., Ltd., 2-8, Chuo-cho, Choshi, Chiba 288-8680, Japan

³ Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

(Received August 25,1998)

Our recent search for bioactive compounds from pathogenic *Nocardia* resulted in the isolation of a new antibiotic, brasilicardin A, from *Nocardia brasiliensis* IFM 0406, a clinical isolate from a lung nocardiosis patient. Brasilicardin A is a novel tricyclic diterpenoid containing an amino acid moiety, 3-hydroxybenzoate, a rhamnose and an *N*-acetylglucosamine unit. The antibiotic exhibited immunosuppressive activity in a mouse mixed lymphocyte reaction (MLR) assay system and its IC₅₀ value was 0.057 µg/ml. Its immunosuppressive mechanism was considered to be different from that of the reference drug, cyclosporin A (CyA), because brasilicardin A has no inhibitory activity against IL-2 production in mouse MLR assay. Also new bioactive substances which were recently isolated by our research groups from pathogenic *Nocardia* are reviewed.

Nocardia is an aerobic Gram-positive, filamentous and partially acid-fast bacterium which belongs to the pathogenic actinomycetes¹⁾. Among *Nocardia* species, *N. asteroides* complex (*N. asteroides* sensu stricto, *N. farcinica* and *N. nova*), *N. brasiliensis*, *N. otitidiscaviarum* and *N. transvalensis* cause human infections²⁾. It has been reported that the infection caused by *Nocardia* is uncommon, however, coupled with the increasing number of the immunocompromised patients treated with corticosteroids, AIDS and organ transplant patients, nocardiosis is thought not to be rare^{2,3,4)}. During the course of our screening program for new bioactive substances from pathogenic *Nocardia*, *N. brasiliensis* IFM 0406 was found to produce a new immunosuppressive terpenoid antibiotic named brasilicardin A.

In this paper we report on the immunosuppressive terpenoid brasilicardin A produced by *N. brasiliensis*. We also review the bioactive compounds produced by pathogenic *Nocardia* and reported by our research groups.

Fig. 1. Bioactive compounds produced by *Nocardia*.

Table 1. List of *N. brasiliensis* strains which showed antibacterial activity.

IFM No.	Brasilidine A	EDDS
0276	+	—
0358	+	—
0415	+	—
0466	+	—
0581	+	—
0641	+	—
0653	+	—
0666	+	—
0667	+	—
0321	—	+
0355	—	+
0568	—	+
0596	—	+
0326*	—	—
0370*	—	—
0378*	—	—
0381*	—	—
0383*	—	—
0386*	—	—
0407*	—	—
0493*	—	—
0642*	—	—
Total	9 / 22	4 / 22

*The chemical nature of these bioactive compound(s) was not determined.

1. Bioactive compounds produced by *Nocardia*

Bioactive compounds obtained through our screening of pathogenic *Nocardia* are shown in Fig. 1. *N. brasiliensis* IFM 0089 produced new cytotoxic antibiotics named brasiliquinones A-C and brasilidine A^{5,6,7}. Brasiliquinones are unique benz[a]anthraquinone antibiotics with an ethyl group at C-3. Furthermore, brasiliquinone A is the first antibiotic discovered possessing ristosamine as a sugar moiety. Brasilidine A is a new indole alkaloid possessing a unique triene unit conjugated with an isonitrile group. In addition to the cytotoxic activity, brasilidine A has antifungal activity against *Aspergillus niger*. *N. brasiliensis* IFM 0321 produced a chelating compound, *N,N'*-ethylenediaminedisuccinic acid (EDDS). Although EDDS itself was already reported to be a microbial product with phospholipase C inhibitory activity⁸, we could demonstrate its antimicrobial activity against

bacteria and fungi such as *Bacillus subtilis* and *Cryptococcus neoformans*⁹.

In 1996 Ruimy et al. proposed a new taxon, *N. pseudobrasiliensis*¹⁰. New anthracycline antibiotics named nocardicyclins A and B were isolated from the type strain, *N. pseudobrasiliensis* IFM 0624, whose structures were characterized by 1- and 8-methoxy groups, a 10-carbonyl group and a novel carbon-methylated aminosugar constituent called brasilinose¹¹. Nocardicyclin A exhibited potent antitumor activity *in vitro* and *in vivo*, and the antibiotic was less toxic than other anthracycline group antibiotics such as adriamycin. The LD₅₀ value of nocardicyclin A was >100 mg/kg (i.p.) in mice¹². Other new anthracycline antibiotics were also obtained from *N. transvalensis* IFM 0456 strain which was isolated from AIDS patient in Thailand. They were 3'-*O*-demethyl-mutactimycin and 4-*O*, 3'-*O*-didemethyl-mutactimycin¹³.

Since the most of present bioactive substances were produced by strains of *N. brasiliensis*, we were interested in the productivity of antimicrobial substance by clinical isolates of *N. brasiliensis*. Our survey on the productivity of 76 clinical isolates of *N. brasiliensis* showed that antimicrobial substances were produced in methanol extracts of 18 strains and culture filtrates of 4 strains (Table 1). Among 22 strains with antimicrobial activity, 9 strains were found to produce brasilidine A. Four strains with antimicrobial activity were found to be producers of EDDS⁹. However the chemical nature of the compounds in the remaining 9 extracts was not determined.

These studies suggest the potential usefulness of pathogenic *Nocardia* as sources of new bioactive substances, although much attention should be paid to handling of these pathogenic *Nocardia* since pathogenic *Nocardia* belongs to class the 2 category in biohazard ranks.

2. A new immunosuppressive terpenoid, brasiliardin A

Strain IFM 0406, which was tentatively identified as *Nocardia* sp., was found to produce immunosuppressive agents in our screening using mouse MLR assay system. The mycelium of the IFM 0406 strain was rudimentary branched, and primitive aerial mycelia were observed. Long time cultivation made the mycelium fragment into bacillary elements. The analysis of cell wall hydrolysates

Fig. 2. Purification scheme of brasilicardin A.

Fig. 3. Chemical structure of brasilicardin A.

revealed the presence of *meso*-diaminopimelic acid, and arabinose and galactose as major sugar constituents. Therefore, the cell wall type was considered to belong to chemotype IV¹¹. The analysis of mycolic acid showed the presence of *Nocardia* or *Rhodococcus* type one. Menaquinone composition of this strain was MK-8H₄(cycle) as the major and MK-8H₂ and MK-9H₄ as minor. Casein, hypoxanthine and tyrosine were decomposed, and acid was produced from galactose and inositol. These characteristics and the results of the susceptibility tests to antimicrobial agents¹⁴) suggested that strain IFM 0406 belongs to *N. brasiliensis*¹⁵).

When the time course of the brasilicardin A production during the cultivation in a 20 liter jar fermenter was monitored, the production was found to begin at the middle to late log-phase and it reached a maximum at 70 to 80 hours cultivation. At this time, about 60 mg/liter of brasilicardin A was produced in culture filtrate.

The purification procedure of brasilicardin A

is summarized in Fig. 2. From 15 liter culture filtrate, 158 mg of brasilicardin A was purified by successive chromatography.

The physico-chemical properties of brasilicardin A are summarized in Table 2. Structure elucidation was established on the basis of spectroscopic data, chemical means and X-ray analysis. Brasilicardin A is a tricyclic diterpenoid antibiotic containing an amino acid moiety, 3-hydroxybenzoate, a rhamnose and an *N*-acetylglucosamine unit (Fig. 3). Detailed structural studies are reported elsewhere¹⁶). Only a few terpenoid compounds have been reported as secondary metabolites from actinomycetes, although a wide variety of terpenoid compounds are produced by fungi and plants. To our knowledge, this is the first report of the natural product containing such anti/syn/anti-perhydrophenanthrene structure from actinomycetes.

Biological activities of brasilicardin A were studied. The effect of brasilicardin A on mouse MLR was examined in comparison with that of CyA. Brasilicardin A showed suppressive activity against the proliferative response of mouse lymphocytes to alloantigen stimulation. The IC₅₀ values of brasilicardin A and CyA on mouse MLR were 0.057 and 0.15 µg/ml, respectively. CyA is widely used as an immunosuppressive agents for clinical organ transplantation¹⁷). It has been reported that CyA inhibits the IL-2 production from T helper cells, and the inhibition triggers suppression of the immune system, such as allograft rejection, by blocking T-cell activation¹⁸). To determine whether the suppressive activity of brasilicardin A is due to inhibition of the IL-2 production, the effects of brasilicardin A and CyA on IL-2 production in mouse MLR were studied. CyA inhibited the IL-2 production in dose-dependent manner, however, the inhibition was not confirmed with brasilicardin A. These results indicate brasilicardin A has a different immunosuppressive mechanism from that of CyA.

The effects of brasilicardin A on the growth of human cultured cell lines *in vitro* were tested using Jurkat, Molt4, CCRF-CEM, HeLa and HEK293. Brasilicardin A exhibited relatively higher IC₅₀ values for various cell lines, and the IC₅₀ values for these cultured cell lines were more than 100 times greater than that for mouse MLR. *In vivo* toxicity test using mice indicated that single intravenous administration of

Table 2. Physico-chemical properties of brasilicardin A.

Appearance	White powder
Molecular weight	892
Molecular formula	C ₄₅ H ₆₈ N ₂ O ₁₆
HRFAB-MS(m/z)	
Found	893.4646(M+H) ⁺
Calcd.	893.4647(for C ₄₅ H ₆₉ N ₂ O ₁₆)
[α] _D ³⁰	+15.0° (c 0.5, MeOH)
IR(KBr) $\nu_{\max}$ (cm ⁻¹)	3432, 2934, 1676, 1454, 1378, 1291, 1203, 1075, 893, 839, 801, 755, 722, 570
UV(MeOH) $\lambda_{\max}$	212(ϵ 15000), 239(ϵ 5200), 300(ϵ 1900) nm
Solubility	Soluble : H ₂ O, MeOH, EtOH Insoluble : CHCl ₃ , EtOAc, Me ₂ CO, diethyl ether

brasilicardin A showed no sign of toxicity at a dose of 100 mg/kg (manuscript in preparation).

Thus, brasilicardin A produced by *N. brasiliensis* IFM 0406 is a novel terpenoid immunosuppressant with low toxicity *in vitro* and *in vivo*. Therefore, it is anticipated that brasilicardin A may be worthy of investigations in clinical application as a useful immuno-suppressive agent.

REFERENCES

- 1) Goodfellow, M. : The family Nocardiaceae. In "The Prokaryotes, 2nd ed." (ed. A. Balows et al.), pp 1188-1213, Springer Verlag, Berlin, 1992.
- 2) Beaman, B. L. & L. Beaman : *Nocardia* species: Host-parasite relationships. Clin. Microbiol. Rev. 7 : 213-264, 1994.
- 3) Schiff, T. A.; M. Sanchez, J. Moy, D. Krilsfeld, M. F. McNeil & J. M. Brown : Cutaneous nocardiosis caused by *Nocardia nova* occurring in an HIV-infected individual: A case report and review of the literature. AIDS 6 : 849-954, 1993.
- 4) Poowan, N.; M. Kusum, Y. Mikami, K. Yazawa, Y. Tanaka, T. Gono, S. Hasegawa & K. Konyama : Pathogenic *Nocardia* isolated from clinical specimens including those of AIDS patients in Thailand. Eur. J. Epidemiol. 11 : 507-512, 1995.
- 5) Nemoto, A.; Y. Tanaka, Y. Karasaki, H. Komaki, K. Yazawa, Y. Mikami, T. Tojo, K. Kadowaki, M. Tsuda & J. Kobayashi : Brasiliquinones A, B and C, new benz[a]anthraquinone antibiotics from *Nocardia brasiliensis*. I. Producing strain, isolation and biological activities of the antibiotics. J. Antibiotics 50 : 18-21, 1997.
- 6) Tsuda, M.; H. Sato, Y. Tanaka, K. Yazawa, Y. Mikami, T. Sasaki & J. Kobayashi : Brasiliquinones A-C, new cytotoxic benz[a]anthraquinone with an ethyl group at C-3 from actinomycete *Nocardia brasiliensis*. J. Chem. Soc., Perkins Trans. 1 : 1773-1775, 1996.
- 7) Kobayashi, J. ; M. Tsuda, A. Nemoto, Y. Tanaka, K. Yazawa & Y. Mikami : Brasilidine A, a new cytotoxic isonitrile-containing indole alkaloid from the actinomycete *Nocardia brasiliensis*. J. Nat. Prod. 60 : 719-720, 1997.
- 8) Nishikawa, T.; A. Okuyama, H. Naganawa, T. Takita, M. Hamada, T. Takeuchi, T. Aoyagi & H. Umezawa : Production by actinomycetes of N,N'-ethylenediaminedisuccinic acid, an inhibitor of phospholipase C. J. Antibiotics 37 : 426-427, 1984.
- 9) Imai, T. ; K. Yazawa, Y. Tanaka, Y. Mikami, T. Kudo, K. Suzuki, A. Ando, Y. Nagata & U. Graefe : Productivity of antimicrobial substance in pathogenic actinomycetes *Nocardia brasiliensis*. Microbiol. Cult. Coll. 13 : 103-108, 1997.
- 10) Ruimy, R.; P. Riegel, A. Carlotti, P. Boiron, G. Bernardin & R. J. Wallace Jr. : *Nocardia pseudobrasiliensis* sp. nov., a new species of *Nocardia* which groups bacterial strains previously identified as *Nocardia brasiliensis* and associated with invasive diseases. Int. J. Syst. Bacteriol. 46 : 259-264, 1996.
- 11) Tanaka, Y. ; U. Graefe, K. Yazawa, Y. Mikami & M. Ritzau : Nocardicyclins A and B : new anthracycline antibiotics produced by *Nocardia pseudobrasiliensis*. J. Antibiotics 50 : 822-827, 1997.
- 12) Tanaka, Y.; U. Graefe, K. Yazawa & Y. Mikami : Production of nocardicyclins by clinical isolates of *Nocardia pseudobrasiliensis* and *in vitro* antitumor activity of the antibiotics. J. Antibiotics 51 : 589-591, 1998.
- 13) Speitling, M. : N. Poonwan, K. Yazawa, Y. Mikami, I. Grun-Wollny, M. Ritzau, H. Laatsch & U. Graefe : Demethyl mutactimycins, new

- anthracycline antibiotics from *Nocardia* and *Streptomyces* strains. *J. Antibiotics*, **51**: 693-698, 1998.
- 14) Mikami, Y. & K. Yazawa : Susceptibility patterns of pathogenic *Nocardia* to some selected antimicrobial agent and their usefulness in the identification work in a clinical laboratory. *Bulletin of the Japan Federation for Culture Collections* **5**: 89-95, 1989.
- 15) Tanaka, Y.; H. Komaki, K. Yazawa, Y. Mikami A. Nemoto, T. Tojyo, K. Kadowaki, H. Shigemori & J. Kobayashi : Brasilinolide A, a new macrolide antibiotic produced by *Nocardia brasiliensis* : producing strain, isolation and biological activity. *J. Antibiotics* **50** : 1036-1041, 1997.
- 16) Shigemori, H.; H. Komaki, K. Yazawa, Y. Mikami, A. Nemoto, Y. Tanaka, T. Sasaki, Y. In, T. Ishida & J. Kobayashi: Brasilicardin A, a novel tricyclic metabolite with potent immunosuppressive activity from actinomycete *Nocardia brasiliensis*. *J. Org. Chem.* **63**: 6900-6904, 1998.
- 17) Borel, J. F. : History of cyclosporin A and its significance in immunology. In "Cyclosporin A" (ed. J. F. Borel), pp. 5-17, Elsevier Biochemical Press, Amsterdam, 1982.
- 18) Liu, J. ; J. D. Farmer Jr., W. S. Lane, J. Freedman, I. Weissman & S. L. Schreiber : Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66** : 807-815, 1991.