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Brasilicardin A, a New Terpenoid Antibiotic from Pathogenic *Nocardia brasiliensis*: Fermentation, Isolation and Biological Activity

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A novel tricyclic diterpenoid antibiotic, brasilicardin A, was isolated from the culture broth of *Nocardia brasiliensis* IFM 0406. The antibiotic exhibited immunosuppressive activity in a mouse mixed lymphocyte reaction (MLR) assay system and its IC_{50} value was 0.057 μg/ml. Although the inhibitory activity of cyclosporin A (CyA) against IL-2 production was confirmed in the MLR assay system, brasilicardin A did not have the activity. The results of *in vitro* toxicity testing of brasilicardin A against various human cell lines were compared with those of CyA.

During our continuing search for bioactive metabolites from pathogenic *Nocardia*, we isolated and reported new antibiotics, brasiliquinones A~C[1,2], brasilidine A[3] and nocardicyclins A, B[4]. Recently we also reported a new 32-membered macrolide antibiotic, brasilinolide A, with immunosuppressive activity from *N. brasiliensis* IFM 0406[5,6]. Further studies on the active metabolites from the culture broth of strain IFM 0406 led to the isolation of a new terpenoid antibiotic, brasilicardin A.

In this paper we report on the fermentation, isolation and biological activity of brasilicardin A.

Materials and Methods

Producing Strain

N. brasiliensis IFM 0406 (deposit No. FERM BP-5498)[5] was used for the isolation of brasilicardin A. The strain had been maintained in the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

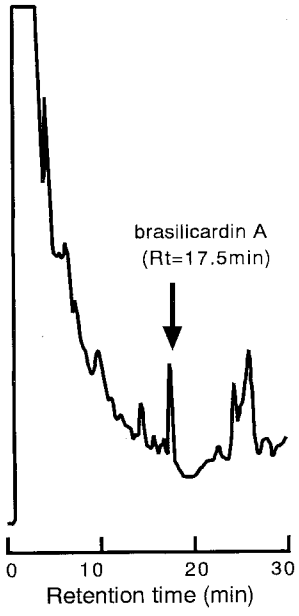
Fermentation

The seed culture was prepared by inoculating mycelial fragments of strain IFM 0406 into a 500 ml-Erlenmeyer flask containing 150 ml of brain heart infusion medium (BHI, Difco) supplemented with 1% glucose and 1% glycerol. The culture was performed at 32°C for 96 hours on a rotary shaker at 300 rpm. The seed culture (150 ml) was then inoculated into a 20 liter jar fermenter containing the production medium (15 liters) consisting of 2% glycerol, 1% polypeptone and 0.5% meat extract in distilled water. The pH was adjusted to 7.0 with 1 M NaOH before sterilization. Adekanol (Asahi Denka Co., Ltd.) was added as the antifoam. Fermentation was carried out at 32°C for 3 days under aeration of 15 liters/minute and agitation at 200 rpm.

HPLC Analysis of Brasilicardin A

The amount of brasilicardin A in the culture broth was estimated by HPLC analysis using a reverse phase column (LiChrospher 100 RP-18 endcapped, 4 mm × 125 mm, Merck). The analysis was carried out as follows:

Fig. 1. HPLC chromatogram of cultured broth of *N. brasiliensis* IFM 0406.



a linear gradient from 18% to 50% of MeCN containing TFA 0.15% for 30 minutes with a flow rate of 1 ml/minute using a 220 nm UV detector. Brasilicardin A was eluted at 17.5 minutes under these conditions as shown in Fig. 1.

Cell Culture and Medium

RPMI1640, Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Dainippon Pharmaceuticals Co., Ltd. Jurkat, MOLT-4 and CCRF-CEM leukemias were maintained in RPMI1640 medium. HeLa and HEK293 cells were maintained as adherent cells by serial passage in DMEM using trypsinization (0.05% trypsin).

Cytotoxicity Assay

Cytotoxicity assay was performed in a 96-well flat-bottom microtest plate; each well contained 10^4 cells and a variable amount of test compound in 0.2 ml of medium. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2-95% air for 96 hours. The cell growth was measured by MTT colorimetric assay[7].

Mouse MLR

The immunosuppressive activity of brasilicardin A was assessed with one-way mouse mixed lymphocyte reaction (MLR) as described by HATANAKA *et al.*[8]. The spleens obtained from BALB/C and C57BL/6 mice (female, 6~7 weeks old) were homogenized into single cell suspensions. Ammonium chloride buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) was added to the cell suspension in order to lyse erythrocytes followed by washing three times with RPMI1640 medium. The erythrocyte-free cell preparation was then resuspended in RPMI1640 complete medium (supplemented with 10% FCS and 50 μM 2-mercaptoethanol). The mouse MLR was performed in a 96-well round-bottom microtest plate with each well containing 5×10^5 C57BL/6 spleen cells (responder cells, H-2^b) and 5×10^5 mitomycin C-treated (25 μg/ml of mitomycin C at 37°C for 30 minutes and washed three times with RPMI1640 medium) BALB/C spleen cells (stimulator cells, H-2^d), and various amounts of test compound in 0.2 ml RPMI1640 complete medium. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2-95% air. After 92 hours of cultivation, cells were pulse-labeled with 0.5 μCi of [^{3}H]thymidine (^{3}H-TdR) for 4 hours at 37°C and harvested using a multiple cell harvester. The radioactivity incorporated into the cells was measured with a liquid scintillation counter. Results were expressed as IC_{50} values.

IL-2 Quantitative Analysis

The amount of IL-2 in mouse MLR supernatant was measured by a mouse IL-2 ELISA kit (Amersham).

Peptidyl-prolyl *cis-trans* Isomerase (PPIase) Assay

PPIase activities of human FK-506 binding protein (hFKBP12) and CyA binding protein (cyclophilin A; hCyP-A) were measured as described by HARRISON *et al.*[9] and TAKAHASHI *et al.*[10].

Antimicrobial Activity

MIC values were determined by microbroth dilution method[11,12] using BHI medium (Difco, supplemented with 1% glucose) for antibacterial testing and RPMI1640 medium (supplemented with 0.165 M MOPS and 2 g/liter $NaHCO_3$, pH 7.0) for antifungal testing.

Results

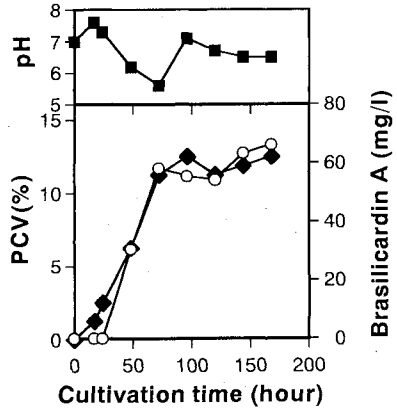
Fermentation

N. brasiliensis IFM 0406, a producer of the 32-membered macrolide antibiotic brasilinolide A, was used. Immunosuppressive activity in the culture broth was determined using the mouse MLR assay system. A typical HPLC profile of brasilicardin A in the culture broth of *N. brasiliensis* IFM 0406 is shown in Fig. 1. The

time course of the brasilicardin A production, growth and pH changes in a 20 liter jar fermenter are shown in Fig. 2. Antibiotic production began at the middle to late log-phase and reached maximum at 70 to 80 hours after incubation. About 60 mg/liter of brasilicardin A was obtained under these conditions.

Fig. 2. Time course of brasilicardin A production by *N. brasiliensis* IFM 0406.

Cell growth (◆) was expressed by PCV (packed cell volume) and the antibiotic production (○) was monitored by HPLC analysis.



Isolation and Purification of Brasilicardin A

Brasilicardin A was purified from the culture broth of the strain IFM 0406 as shown in Fig. 3. The compound was traced by both HPLC and the antimicrobial activity checked using the paper disk method with *Nocardia asteroides* as a test organism. After 72 hours of cultivation, 30 liters of methanol was added to the culture broth to inactivate the producing organism. The culture filtrate was concentrated to the original volume by evaporation, then applied to a Diaion HP-20 column (5 cm × 30 cm) and washed with 50% methanol (4 liters). The active fraction was eluted with methanol (2 liters) and evaporated to dryness *in vacuo*. The dry residue (5 g) was subjected to chromatography using a DEAE Toyopearl 650M column (2.5 × 10 cm) with 20 mM Tris-HCl buffer, pH 8.0. The combined active fractions were rechromatographed on a CM Toyopearl 650M column (2.5 × 10 cm) with 20 mM sodium acetate buffer, pH 4.0 as the mobile phase. The active fractions were further purified by reverse-phase HPLC (column; Capcell Pak SG120 C18, Shiseido Co., Ltd., 5 × 25 cm, elution; linear gradient, 18 to 42% of acetonitrile with TFA 0.15% for 60 minutes, flow rate; 30 ml/minute, detection; UV 220 nm) and evaporated to give brasilicardin A (158 mg). In HPLC and TLC analyses (TLC plate silica

Fig. 3. Isolation and purification scheme of brasilicardin A.

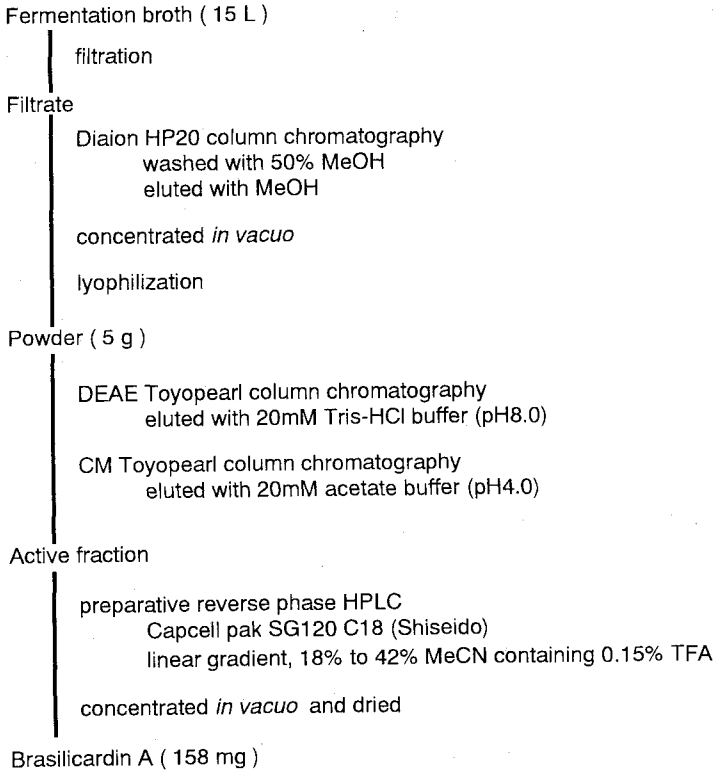
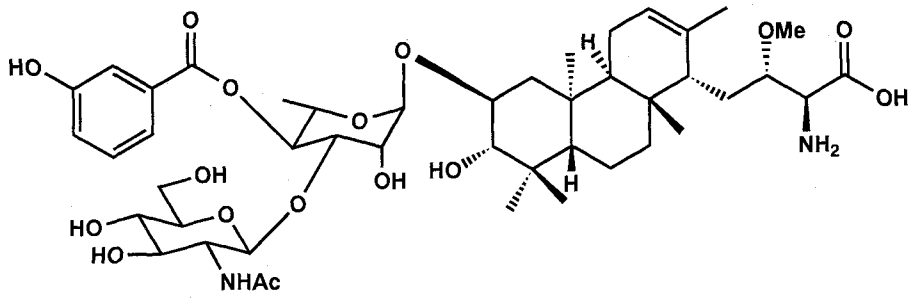


Table 1. Physico-chemical properties of brasilicardin A.

Appearance	White powder
Molecular weight	892
Molecular formula	$C_{45}H_{68}N_2O_{16}$
HRFAB-MS(m/z)	
Found	893.4646 $(M+H)^+$
Calcd.	893.4647 (for $C_{45}H_{69}N_2O_{16}$)
$[\alpha]_D^{30}$	+15.0° (*c* 0.5, MeOH)
IR(KBr) ν max(cm^{-1})	3432, 2934, 1676, 1454, 1378, 1291, 1203, 1075, 893, 839, 801, 755, 722, 570
UV(MeOH) λ max	212(ε15000), 239(ε5200), 300(ε1900) nm
Solubility	Soluble : H_2O, MeOH, EtOH Insoluble : $CHCl_3$, EtOAc, Me_2CO, diethyl ether

Fig. 4. Chemical structure of brasilicardin A.



gel 60 F254 pre-coated, Merck, $CHCl_3$ - methanol - 25% ammonia water, 6:5:0.5) the retention time and Rf values of the purified brasilicardin A sample were 17.5 minutes and 0.17, respectively.

Physico-chemical Properties

The physico-chemical properties of brasilicardin A are summarized in Table 1. Brasilicardin A was white powder, soluble in water, methanol and ethanol, but insoluble in chloroform, ethyl acetate, acetone and diethylether. $[\alpha]_D^{30}$ +15.0° (*c* 0.5, MeOH); FT-IR(KBr) ν_{max} 3432, 2934, 1676, 1454, 1378, 1291, 1203, 1075, 893, 839, 801, 755, 722, 570 cm^{-1}; UV (MeOH) λ_{max} 212 (ε15000), 239 (ε5200), 300 (ε1900) nm; HRFAB-MS (positive, glycerol matrix) *m/z* 893.4646 $(M+H)^+$ calcd. for $C_{45}H_{69}N_2O_{16}$. The chemical structure of brasilicardin A shown in Fig. 4 is characterized as having a tricyclic diterpenoid antibiotic containing an amino acid moiety, 3-hydroxybenzoate, a rhamnose and an *N*-acetylglucosamine unit. Detail of the structure has been reported[13].

Table 2. Effect of brasilicardin A and cyclosporin A on mouse MLR.

Compound	IC_{50}(μg/ml)
Brasilicardin A	0.057
Cyclosporin A	0.15

Biological Activities of Brasilicardin A

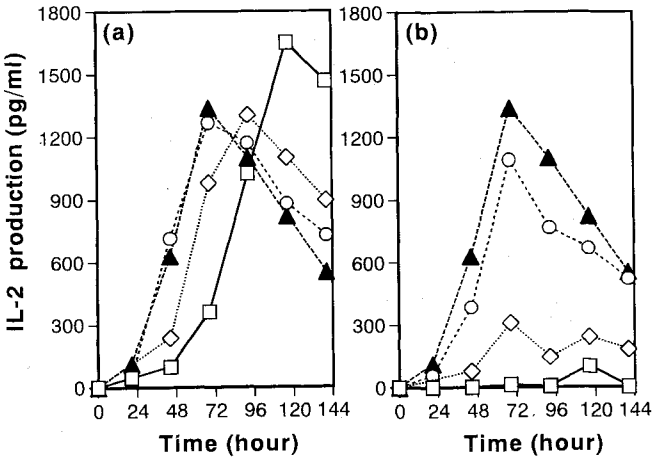
Effect of Brasilicardin A on Mouse MLR

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. As shown in Table 2, the IC_{50} values of brasilicardin A and CyA on mouse MLR were 0.057 and 0.15 μg/ml, respectively.

To determine whether these suppressive activities were due to inhibition of IL-2 production, the effects of brasilicardin A and CyA on IL-2 production in mouse MLR were studied. Brasilicardin A and CyA were added

Fig. 5. Effects of brasilicardin A and cyclosporin A on IL-2 production in mouse MLR.

(a) brasilicardin A, (b) cyclosporin A.
□; 0.25 μg/ml, ◇; 0.063 μg/ml, ○; 0.016 μg/ml, ▲; control.



separately to mouse MLR at the initiation of culture, and the amount of IL-2 in the supernatant was determined by ELISA. As shown in Fig. 5, CyA inhibited IL-2 production in dose-depended manner. However, brasilicardin A only delayed the production of IL-2 in dose-dependent manner, and the production was not inhibited at the concentration of 0.016 μg to 0.25 μg/ml. Moreover, enhancement of IL-2 production was observed at the concentration of 0.25 μg/ml of brasilicardin A. When we tested the effect of brasilicardin A on ^{3}H-TdR uptake, inhibition was observed, but there was no enhancement of ^{3}H-TdR uptake in this condition.

Effects of Brasilicardin A on Immunophilins

The effects of brasilicardin A on the PPIase activity of immunophilins (hFKBP12 and hCyP-A) were compared with those of CyA and FK-506. CyA completely inhibited the PPIase activity of hCyP-A at the concentration of 10^{-7} M (0.12 μg/ml), and FK-506 inhibited that of hFKBP12 at 10^{-7} M (0.082 μg/ml). However, when 10^{-5} M (8.9 μg/ml) of brasilicardin A was used, no inhibitory activities on either PPIase was noted. (data not shown).

In Vitro and *In Vivo* Toxicity of Brasilicardin A

Comparison of the effects of brasilicardin A on the growth of human cultured cell lines *in vitro* with those of CyA showed that the IC_{50} values of the former varied depending on the cell line tested, ranging from 5.6 to 100 μg/ml (Table 3). Intravenous administration of brasilicardin A caused no sign of toxicity at a dose of 100 mg/kg in mice.

Table 3. *In vitro* cytotoxic activities of brasilicardin A in comparison with those of cyclosporin A for human cell lines.

Cell lines	IC_{50}(μg/ml)	
	Brasilicardin A	Cyclosporin A
Jurkat	5.6	9.0
Molt4	29	16
CCRF-CEM	25	13
HeLa	100	100
HEK293	6.3	7.2

Antimicrobial Activity

The antimicrobial activities of brasilicardin A against Gram-positive and negative bacteria, yeasts and filamentous fungi such as *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Mycobacterium smegmatis*, *Aspergillus niger*, *Candida albicans* and *Cryptococcus neoformans* were examined. No antimicrobial activity was observed and the MIC value of brasilicardin A against these microorganisms was >100 μg/ml. However the incomplete inhibition zone around the paper disk of brasilicardin A (30 μg/disk) was noted when *N. asteroides* IFM 0319 (type strain) was used as a test organism (data not shown).

Discussion

Brasilicardin A is a novel tricyclic diterpenoid antibiotic containing an amino acid moiety, 3-hydroxybenzoate, a rhamnose and an *N*-acetylglucosamine unit (Fig. 4). Only a few terpenoid compounds have been reported as secondary metabolites from actinomycetes, although a wide variety of terpenoid compounds are produced by fungi[14]. To our knowledge, this is the first report of a natural product containing such an anti/syn/anti-perhydrophenanthrene structure from actinomycetes.

Brasilicardin A was not active against the bacteria or fungi tested but displayed a characteristic incomplete (partial) inhibition against *N. asteroides* IFM 0319. Since the inhibition was partial or incomplete around the paper disk, the MIC value could not be determined, however, this activity was quite useful for monitoring the active

principle in the culture broth. We were also interested in the activity of brasilicardin A against other *Nocardia* species, including each type strain of *N. brasiliensis*, *N. otitidiscaviarum*, *N. farcinica*, *N. nova*, *N. pseudobrasiliensis* and *N. transvalensis*. Our preliminary studies suggested that among these 6 species of *Nocardia*, *N. otitidiscaviarum* was also susceptible to brasilicardin A. Confirmation of these *Nocardia* species-specific antimicrobial activities using many strains of *N. asteroides* and *N. otitidiscaviarum* are in progress in our laboratory.

Brasilicardin A showed a potent inhibitory activity against mouse MLR. The MLR used here and the assay of alloreactive cytotoxic T lymphocyte (CTL) *in vivo* have been thought to be the correlative model for allograft rejection[15]. Our preliminary experiments also revealed that brasilicardin A has an inhibitory activity against human MLR and mouse CTL induction.

CyA and FK-506 are widely used as immunosuppressive agents for organ transplantation[16~18]. Both drugs have been reported to suppress the immune system by blocking T-cell activation, binding to intracellular binding protein hCyP-A and hFKBP12, respectively[19,20]. These binding proteins, called immunophilins catalyze PPIase activity. Although the inhibition of PPIase does not completely account for the immunosuppressive action, both drugs have been reported to be potent PPIase inhibitors. The inhibition of Ca^{2+}-calmodulin dependent serine-threonine specific protein phosphatase, calcineurin, by FK-506-hFKBP12 complex or CyA-hCyP-A complex also reportedly triggers the inhibition of IL-2 production from T helper cells followed by the suppression of immune response such as allograft rejection[16,21,22]. Brasilicardin A is structurally different from these reference compounds. Although the existence of binding proteins such as hFKBP12 and hCyP-A for brasilicardin A has not yet been demonstrated, it is reasonable to consider that the immunosuppressive mechanism of brasilicardian A is different from those of FK-506 and CyA because it did not inhibit PPIase. Our present experiments showed that brasilicardin A did not inhibit IL-2 production in mouse MLR. These results indicate brasilicardin A has a different immunosuppressive mechanism from CyA and FK-506.

When cytotoxicities for cultured cell lines were examined, brasilicardin A exhibited relatively higher IC_{50} values for various cell lines, and these values were more than 100 times greater than that for mouse MLR. Therefore, the inhibitory activity of brasilicardin A on mouse MLR is not likely due to a non-specific direct cytotoxic effect. The LD_{50} value of CyA was reported to be 107 mg/kg[23], and brasilicardin A did not show any sign of toxicity at a dose of 100 mg/kg body weight in mice. Our studies, therefore indicate that brasilicardin A is less toxic than CyA and has a similar level of toxicity to FK-506 *in vitro* and *in vivo*, although further detailed comparative studies with those of FK-506 are necessary. Although CyA and FK-506 are effective immunosuppressive agents, their clinical use is limited by the occurrence of renal dysfunction and other side effects[24,25]. Brasilicardin A is a water soluble compound, while CyA and FK-506 are not soluble in water. It is thus anticipated that brasilicardin A will show different pharmacokinetic behaviors from these reference drugs and may be worthy of investigation for clinical application as useful immunosuppressive agent.

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