Brasilinolide A, a New Macrolide Antibiotic Produced by *Nocardia brasiliensis*: Producing Strain, Isolation and Biological Activity

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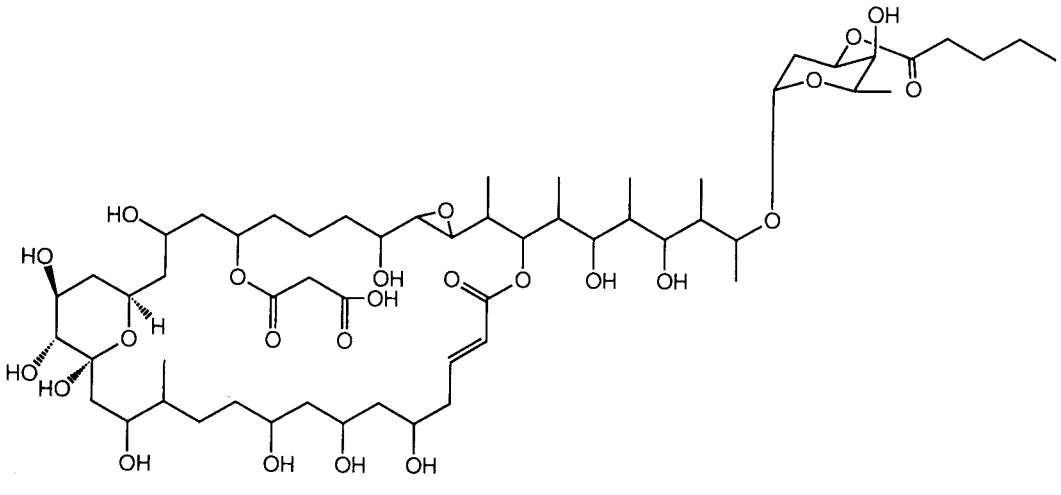
A new 32-membered macrolide antibiotic, brasilinolide A was isolated from the fermentation broth of *Nocardia* sp. IFM 0406. The producer was identified as *Nocardia brasiliensis*. The antibiotic was only active against *Aspergillus niger*, but not active against other fungi including yeasts as well as other filamentous like fungi and bacteria. Brasilinolide A exerted an immunosuppressive activity in the assay system of a mixed lymphocyte reaction (MLR).

Immunosuppressive agents which attack specific target sites of immunocompetent cells are good candidate for immunotherapy. The examples of useful drugs of this type are cyclosporin A[1] and FK-506[2]. We had reported that some opportunistic pathogenic *Nocardia*, such as *Nocardia otitidiscaviarum* and *Nocardia brasiliensis*, produce novel bioactive substances[3~5]. In the course of continuing search for bioactive substances including immunosuppressive agents from *Nocardia*, we found that IFM 0406 strain produces a novel 32-membered macrolide antibiotic with immunosuppressive activity (Fig. 1)[6]. This paper describes the taxonomy of the producing strain, isolation and biological activities of the antibiotic, which we have named brasilinolide A.

Fig. 1. Structure of brasilinolide A.



Materials and Methods

Producing Strain

The IFM 0406 strain was an isolate from a patient of lung nocardiosis and had been kept in our center. This strain has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Ibaragi, Japan, as *Nocardia* sp. IFM 0406 with the accession number FERM BP-5498 under the Budapest treaty.

Cultural and Physiological Characteristics of the Producer

The media and procedures used for culture and physiological characteristics of the IFM 0406 strain were those of described by GOODFELLOW[7], SCHAAL[8] and MIKAMI and YAZAWA[9].

Cell Chemistry

The IFM 0406 strain was grown in brain heart infusion (BHI medium, Difco) broth containing 1% glucose at 32°C for 7 days on a rotary shaker. The cultures were killed by 1% (final concentration) of formaldehyde. The washed and packed mycelial cakes were freeze-dried as a whole-cell preparation. Cell walls were prepared as described by SCHAAL[8]. For the determination of diaminopimelic isomers (DAP) and sugar components, cell wall hydrolysates were developed by thin layer chromatography (TLC) plate and the type of DAP and sugar components were determined in comparison with those of standard compounds[8].

For the determination of mycolic acid patterns, a mixture of methanol-toluene-sulfuric acid (30:15:1, v/v) were added to the dried cells. The methanolysates were applied to TLC plates and the mycolic acid patterns were determined[8].

Menaquinone fractions were purified by preparative thin layer chromatography (Pre-TLC). The fraction was monitored by HPLC using following conditions: column, Lichro cart RP-18 (Merck, 4.6×150 mm), flow rate; 1 ml/minute, detection; UV 235 nm, mobile phase; methanol: *iso*-propanol (2:1). Components were identified by comparison of retention time with those of standard mixtures[10].

DNA Base Composition and DNA-DNA Hybridization

Level of DNA reassociation were determined by using photobiotin (Vectra Laboratories, Inc., Burlingame, Calf.) labeling probes in microplate wells as described by EZAKI *et al.*[11] and TAKAGI *et al.*[12].

Fermentation

A loopful of the slant culture of the IFM 0406 strain was inoculated into a 500-ml Erlenmeyer flask containing 150 ml of 1% glucose BHI seed medium. The seed culture was transferred to 20 liters of a production medium contained of glycerol 2%, polypeton 1%, meat extract 0.5%, Adekanol (Asahi Denka Co., Ltd.) 0.1%, pH 8.0 before sterilization. Fermentation was carried out at 30°C for 7 days under aeration of 15 liters/minute and agitation at 200 rpm.

MLR Test

The immunosuppressive activity of the compound was assessed with two-way mixed lymphocyte reactions as described by MCALPINE *et al.*[13]. Briefly, BALB/c and C57BL/6 mice (female, 17~18 g) were sacrificed and spleens were aseptically removed. Spleens were homogenized and contaminating erythrocytes were lysed by suspension in EDTA-ammonium chloride solution. The BALB/c and C57BL/6 spleen cell suspensions were diluted to 2.5×10^6 viable cells/ml and pooled, before mixed cultures were established in 96-well microtiter plates. After 72 hours incubations at 37°C in a humidified atmosphere of 5% CO_2 in air, increases in proliferation were assessed by cellular uptake of tritiated thymidine. The IC_{50} values of immunosuppressive compound were graphically determined after incorporation of thymidine was corrected for incorporation in unstimulated cultures and normalized as a percent of incorporation in stimulated cultures.

Ascomycin (FR-900520)[14] and cyclosporin A[15] were used as reference immunsuppressive drugs.

Biological Activity

Antimicrobial activities were determined by microbroth dilution method using BHI broth (Difco).

Cytotoxic activities were also determined by the method described in our previous paper[16].

Results and Discussion

Taxonomic Studies

The isolates contained the *meso*-isomer of DAP, as well as galactose and arabinose. These results indicated that the IFM 0406 strain belongs to chemotype IV in the classification system of LECHEVALIER and LECHEVALIER[17]. When the mycolic acids of the isolate were determined by TLC patterns and HPLC profiles, it was found that

they contained mycolic acid type of *Nocardia* or *Rhodococcus*[18]. Our studies on the menaquinone compositions of the isolate indicated that they have MK-8H_4 (cycl.) as the major menaquinone composition and MK-8H_2 and MK-9H_4 as minor menaquinones.

IFM 0406 strain decomposed, casein, hypoxanthine and tyrosine, but did not decompose adenine and xanthine. The organisms also hydrolyzed urea after growth on Christensen urea agar at 27°C for 3 weeks. The strain could grow at 45°C on Sabouraud dextrose agar, and Na-citrate was utilized as a carbon source. β-Lactamase production of IFM 0406 strain was confirmed by a cefinase test. (Table 1). These data indicated that IFM 0406 strain is closely related to those of *N. brasiliensis*. We had reported that pathogenic *Nocardia* showed species-specific drug susceptibility patterns[9]. When the susceptibility of the present strain against imipenem, 5-fluorouracil, tobramycin and kanamycin was tested, this strain was found to show the similar susceptibility pattern against the first three drugs to that of the type species, but showed different susceptibility to kanamycin. Except for the failure to decompose urea and for the different susceptibility to kanamycin as shown in Table 1, three test results were consistent with identification of the isolate as *N. brasiliensis*. In general, the acid production patterns from various carbon sources indicated that the isolate belongs to *N. brasiliensis*, although acid production from erythritol was not demonstrated for IFM 0406 strain.

Table 1. Physiological characteristic of brasilinolide A producer.

Characteristics	IFM 0406	*N. brasiliensis*[a]
Acid fastness	±[b]	±
Decomposition of:		
Adenine	−	−
Casein	+	+
Hypoxanthine	+	+
Tyrosine	+	+
Urea	−	+
Xanthine	−	−
Acid from:		
Adonitol	−	−
Arabinose	−	−
Erythritol	−	+
Galactose	+	+
Glucose	+	+
Inositol	+	+
Maltose	−	−
Mannose	−	−
Rhamnose	−	−
Sorbitol	−	−
Utilization of citrate	−	−
Growth at 45°C	−	−
Production of β-lactamase	+	+
Susceptibility to:		
Imipenem	−	−[c]
Tobramycin	+	+
5-Fluorouracil	−	−
Kanamycin	−	+

[a] Data were obtained from ref. 18.
[b] Partially acid fastness.
[c] Susceptibility was determined by the method of ref. 9.

As shown in Table 2, the GC contents of *Nocardia* sp. IFM 0406 was 68.0% and the level of relatedness among the DNAs of IFM 0406, *N. brasiliensis* IFM 0236^T DNA and *N. transvalensis* IFM 0333^T were 94 and 8%, respectively. These results indicated that the IFM 0406 strain should belongs to the species of *N. brasiliensis*[18~20].

Fermentation

A typical time course of the brasilinolide A production is shown in Fig. 2. The antibiotic production began at 25 hours and reached to maximum after 70 hours incubation, coupled with the growth. The pH of culture broth increase until 20 to 25 hours after the inoculation and then decreased to pH 5.0. After 100 hours' incubation, the pH again began to increase and attain to 7.0 to 8.0 at 120 hours.

Isolation and Purification

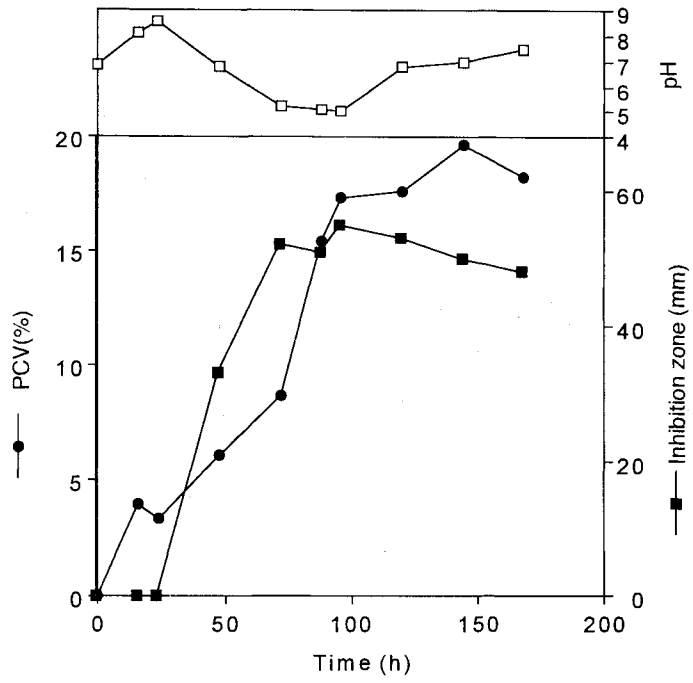
The culture (20 liters) was killed by the addition of 2 volume of methanol and evaporated to the original volume. The filtrate was subjected to a Diaion HP-20 column (30 × 30 cm) and washed with 2 M NaCl (4

Table 2. DNA base composition of *Nocardia* sp. IFM 0406 strain and levels of relatedness to *N. brasiliensis* IFM 0236^T or *N. transvalensis* IFM 0333^T.

Strain	G+C content (mol%)	% Reassociation with DNA from: *N. brasiliensis* IFM 0236^T	*N. transvalensis* IFM 0333^T	*Nocardia* sp. IFM 0406
N. brasiliensis IFM 0236^T	68.1	100	NT[a]	100
N. transvalensis IFM 0333^T	69.0	NT	100	25
Nocardia sp. IFM 0406	68.0	94	8	100

[a] Not tested.

Fig. 2. Time course of brasilinolide A production.



The growth was determined by packed cell volume (PCV) method. The activity was determined by the inhibition zone against *Aspergillus niger* IFM 40606.

liters) and then distilled water (4 liters). The active fractions against *A. niger* were eluted with MeOH - H_2O (1 : 4, 2 liters) and then MeOH (2 liters), and lyophilized. The dried fractions (20 g) were chromatographed on a silica gel column (3 × 30 cm) and eluted with $CHCl_3$ - MeOH (4 : 1). The combined active fractions were further purified by reverse-phase HPLC (Capcell pack C18 SG120, Shiseido Co. Ltd., 5 × 25 cm, flow rate; 20 ml/minutes, MeCN - H_2O, 1 : 19) to give brasilinolide A (100 mg).

Physico-chemical Properties

Physico-chemical properties of brasilinolide A summarized in Table 3. Brasilinolide A is a colorless amorphous solid. Brasilinolide A is soluble in water and methanol, but insoluble in most of the organic solvents such as chloroform, EtOAc and acetone. $[\alpha]_D^{28}$ −27.4 (*c*, 1.0, MeOH); IR (KBr) γ_{max} 3400, 1740 (sh), 1640, and 1589 (cm^{-1}); UV (MeOH) λ_{max} 214 (ε 14900) nm; FAB-MS (positive, *m*-nitrobenzylalcohol matrix) *m/z* 1189 $(M+Na)^+$; HRFAB-MS *m/z* 1189.6403 $(M+Na)^+$, calcd. for $C_{57}H_{98}O_{24}Na$, 1189.6460. Brasilinolide A was found to be a new 32-membered macrolide with a tetrahydropyran ring and a 2-deoxyfucopyranose[6]. On the basis of above information, the structure was determined as shown in Fig. 1, and the structural studies were repoted[6].

Table 3. Physico-chemical properties of brasilinolide A.

Appearance	Colorless amorphous solid
Molecular weight	1166
Molecular formula	$C_{57}H_{98}O_{24}$
HRFAB-MS (*m/z*)	
Found	1189.6403 $(M+Na)^+$
Calcd	1189.6460 (for $C_{57}H_{98}O_{24}Na$)
$[\alpha]_D^{28}$	−27.4° (*c* 1.0, MeOH)
IR (KBr) ν_{max} (cm^{-1})	3400, 1740 (sh), 1700, 1640, 1580
UV (MeOH) λ_{max}	214 (ε 14900) nm
Solubility	Soluble: H_2O, MeOH, EtOH, DMSO
	Insoluble: $CHCl_3$, EtOAc, *n*-hexane, acetone

Antimicrobial and Antitumor Activities

Brasilinolide A showed moderate antifungal activity against *Aspergillus niger* IFM 40406 and it's MIC value was 3.13 μg/ml (Table 4). The antibiotic was not active against any other bacteria and fungi tested at the concentration of 100 μg/ml. It is well-known that FK-506 and cyclosporin A show antifungal activity. Therefore,

Table 4. *In vitro* antimicrobial activity of brasilinolide A.

Test organism	MIC (μg/ml)
Aspergillus niger IFM 40406	3.13
Aspergillus fumigatus IFM 41994	>100
Candida albicans ATCC 90029	>100
Candida glabrata ATCC 90030	>100
Candida krusei IFM 40081	>100
Cryptococcus neoformans ATCC 90112	>100
Staphylococcus aureus IFM 2014	>100
Micrococcus luteus IFM 2066	>100
Bacillus subtilis PCI 219	>100
Escherichia coli NIHJ-2	>100

the detail mode of action of brasilinolide A against *A. niger* is of interest because brasilinolide A was only active against *A. niger*.

Cytotoxicity

Comparison of the toxicity of brasilinolide A with ascomycin and cyclosporin A are shown in Table 5. When IC_{50} values were compared, brasilinolide A was less toxic than ascomycin and cyclosporin A against 9 tumor cell-lines tested. Generally cyclosporin A was more toxic than ascomycin against the cells tested. Interestingly, intravenous administration of brasilinolide A showed no sign of toxicity in the dose of 500 mg/kg in mice. It was reported that LD_{50} values of FK-506[2,14] and cyclosporin A[15] were 25 mg/kg and 107 mg/kg in mice by the same administration route, respectively. In the present experiments, we used ascomycin (FK-90052) as one of the reference drugs. The structural difference between ascomycin and FK-506 are in their side chain, and the side chain in ascomycin is ethyl group instead of the allyl group in FK-506. Although it is reported that ascomycin is less toxic than FK-506[14], our preliminary studies showed that cytotoxicity of FK-506 and ascomycin were similar. Detailed comparative studies in their toxicity are in progress in our laboratory.

Suppression of MLR

The suppressive effect of various concentration of brasilinolide A, ascomycin and cyclosporin A on the mouse MLR are shown in Table 6. The IC_{50} values (the concentration causing 50% inhibition) under the present conditions, were 0.625, 0.016 and 0.040 μg/ml, respectively. Although brasilinolide A was less effective than cyclosporin A and ascomycin, the antibiotic found to be less toxic against cultured cells as shown in Table 5. Comparison of the acute toxicity data of FK-506 and cyclosporin A which had been reported showed that brasilinolide A was less toxic than those of two reference drugs. Furthermore, brasilinolide A is a water soluble macrolide and anticipated to show different pharmacokinetic behaviors from cyclosporin A and FK-506. Therefore, brasilinolide A is considered worthy of further evaluation as an immunotherapeutic agent.

Table 5. Comparison of cytotoxic activities of brasilinolide A, cyclosporin A and ascomycin against 9 tumor cell-lines.

Cell-line	IC_{50} (μg/ml)		
	Brasilinolide A	Cyclosporin A	Ascomycin
L1210	>100	3	34
MOLT3	>100	3.3	20
MOLT4	>100	3.4	30
Jurkat	>100	40	50
P815	>100	25	40
P388	>100	5.2	28
HEK-297	>100	3.8	14
HeLa	>100	53	54
Cos-1	>100	15	54

Table 6. Effect of brasilinolide A, ascomycin and cyclosporin A on mouse MLR.

Compound	IC_{50} (μg/ml)
Brasilinolide A	0.625
Ascomycin	0.016
Cyclosporin A	0.040

Acknowledgments

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