Brasiliquinones A, B and C, New Benz[*a*]anthraquinone Antibiotics from *Nocardia brasiliensis*

I. Producing Strain, Isolation and Biological Activities of the Antibiotics

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New benz[*a*]anthraquinone antibiotics (brasiliquinones A, B and C) with an ethyl group at C-3 were isolated. The producer was identified as *Nocardia brasiliensis*. The antibiotics were active against Gram-positive bacteria including *Mycobacterium* sp., but not active against Gram-negative bacteria or fungi. They were also active against multiple drug-resistant P388/ADR tumor cells.

During our studies on the host parasite relationships of *Nocardia* infection, we found and reported that the pathogenic *Nocardia* strains *Nocardia otitidiscaviarum* and *Nocardia brasiliensis* produce the cytotoxic substances HS-6[1] and SO-75R1[2,3] against such cultured tumor cells as L1210. Recently we also reported a new 32-membered macrolide antibiotic (brasilinolide A) which was produced by *N. brasiliensis*[4]. Our further survey on the bioactive substances from pathogenic *Nocardia* indicated that strain IFM 0089 produces new benz[*a*]anthraquinones called brasiliquinones A, B and C (Fig. 1)[5]. This paper describes the taxonomy of the producing strain, and the isolation and biological activities of the antibiotics.

Materials and Methods

Brasiliquinones-producing Strain

Nocardia sp. strain IFM 0089 which had been kept on brain heart infusion (BHI, Difco, Detroit) medium in our culture collection was used.

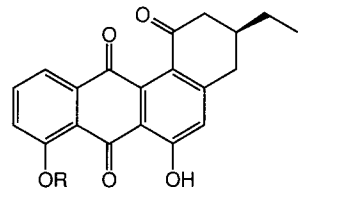
Cell Chemistry

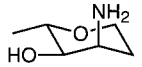
The strain IFM 0089 was grown in BHI medium containing 1% glucose at 32°C for 7 days on a rotary shaker. The cultures were killed by 1% (final concentration) of formaldehyde. The washed and packed mycelial cakes were freeze-dried as a whole-cell preparation. For the determination of diaminopimelic (DAP) isomer and sugar components, the whole-cell hydrolysates were developed by thin layer chromatography (TLC) and each component was identified by comparing with those of standard compounds[6,7].

The mycolic acid patterns were determined by comparing with those from reference strains as described by SCHAAL[6].

Menaquinone fractions were purified by preparative TLC. The fraction was monitored by HPLC and each component of the menaquinones was determined by

Fig. 1.



Brasiliquinone A R= 

Brasiliquinone B R= H

Brasiliquinone C R= CH_3

comparison of retention time with those of standard mixtures as described earlier[8].

Cultural and Physiological Characteristics

The media and procedures used for cultural and physiological characteristics of the strain IFM 0089 were those described by GOODFELLOW[7], SCHAAL[6] and MIKAMI and YAZAWA[9].

Biological Activity

Antimicrobial activities were determined by micro-broth dilution method using BHI medium.

Cytotoxic activities were determined by the method described[10] using L1210 and P388 tumor cell lines[11].

Results and Discussion

Taxonomic Studies

The isolates contained the *meso*-isomer of DAP as well as galactose and arabinose. These results indicate that the strain IFM 0089 belongs to chemotype IV using the classification system of LECHEVALIER and LECHEVALIER[12]. Determination of the mycolic acid of the strain IFM 0089 by TLC patterns and HPLC profiles showed a pattern characteristic of the type strain of *Nocardia* or *Rhodococcus* spp.

Our studies on the menaquinone compositions of the isolate indicated that the present strain has MK-8H_4 (cycl.) as its major menaquinone component.

The strain IFM 0089 decomposed, casein, hypoxanthine and tyrosine, but did not decomposed adenine or xanthine (Table 1). The organism also hydrolyzed urea after growth on Christensen urea agar at 27°C for 3 weeks (Table 1). The strain was able to grow at 45°C on Sabouraud dextrose agar, and Na-citrate was not utilized as a carbon source. β-Lactamase production was confirmed by cefinase test[9]. Comparison of these physiological and biochemical characteristics of strain IFM 0089 with those of *Nocardia brasiliensis* type strain revealed the two are closely related. We reported earlier that pathogenic *Nocardia* showed species-specific drug susceptibility patterns[9]. The strain IFM 0089 showed the same patterns against imipenem, gentamicin and 5-fluorouracil, the only exception being its susceptibility to kanamycin. These results seem to confirm that the strain IFM 0089 was *N. brasiliensis*.

The results of acid production from various carbon sources showed that acid was not produced from adonitol, arabinose, erythritol, maltose, mannose, rhamnose and sorbitol, but was produced from glucose, galactose and inositol. These production patterns were very similar to that of *N. brasiliensis* (Table 1)[12].

Fermentation

For the isolation of brasiliquinones, a spore of the producing strain was inoculated into 500-ml of Erlenmeyer flasks containing 70 ml of a medium consisting of glucose 2%, polypeptone 1.0%, meat extract 0.5% and NaCl 0.5% (pH 7.0) and cultured at 32°C for 96 hours on a rotary shaker at 300 rpm. The seed culture (5.0%) was then inoculated into a 20-liter jar fermenter containing the same medium and the fermenter was stirred at 300 rpm with aeration at 20 liters/minute at 32°C for 4 days.

Isolation and Purification

The culture was killed by the addition of 2 volumes of methanol. After concentration *in vacuo* to the original volume, the culture broth was extracted with the same volume of EtOAc. The EtOAc layer was concentrated *in vacuo* to dryness, the residue was extracted with EtOAc and the EtOAc soluble materials were dissolved in hexane (2 liters). The hexane-soluble materials were evaporated *in vacuo* and subjected to a silica gel column (hexane : EtOAc = 5 : 1) and silica gel HPLC (YMC Pack SIL-06, 20 × 250 mm, eluent, hexane : EtOAc = 8 : 1; flow

Table 1. Physiological and biochemical characteristics of brasiliquinones producer.

Characteristic	IFM0089	*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[c]:		
imipenem	−	−
tobramycin	+	+
5-fluorouracil	−	−
kanamycin	−	+

[a] Data were obtained from ref. 13.
[b] Partially acid fastness.
[c] Susceptibility was determined by the method of ref. 9.

Table 2. Physico-chemical properties of brasiliquinones A, B, and C.

	Brasiliquinones		
	A	B	C
Appearance	Red powder	Yellow powder	Yellow powder
Molecular weight	466	336	350
Molecular formula	$C_{26}H_{27}NO_7$	$C_{20}H_{16}O_5$	$C_{21}H_{18}O_5$
HR-FAB-MS (*m/z*)			
found	468.2052 $(M+2+H)^+$		
calcd	468.2022 (for $C_{26}H_{27}NO_7$)		
HR-EI-MS (*m/z*)			
found		336.0988 $(M)^+$	350.1175 $(M)^+$
calcd		336.0997 (for $C_{20}H_{16}O_5$)	350.1154 (for $C_{21}H_{18}O_5$)
$[\alpha]_D$	+130° (*c* 0.03, $CHCl_3$, 25°C)	+42° (*c* 0.1, $CHCl_3$, 23°C)	+69° (*c* 0.14, $CHCl_3$, 23°C)
IR (film) ν_{max} (cm^{-1})	3350, 3150, 1670, 1630	3440, 1690, 1675, 1640	3440, 1690, 1675, 1640
UV(MeOH) λ	412, 266, 228	428, 267, 228	406, 266, 227
Melting point (°C)	132~135	187~190	215~217

Table 3. Antimicrobial activities of brasiliquinones A, B and C.

Test organisms	MIC values (μg/ml)		
	Bras A	Bras B	Bras C
Staphylococcus aureus 209P	25.0	25.0	25.0
Staphylococcus aureus MRSA IFM 62971	50.0	25.0	12.5
Mycobacterium smegmatis ATCC 607	0.39	0.78	12.5
Micrococcus luteus IFM 2066	6.25	3.13	3.13
Escherichia coli NIH JC2	>100	>100	>100
Aspergillus niger ATCC 40606	>100	>100	>100
Candida albicans ATCC 90028	>100	>100	>100

Brain heart infusion agar and Sabouraud dextrose agar were used for bacteria and fungi, respectively.

rate. 30 ml/minute; UV detection, 380 nm) to afford brasiliquinones B (1.4 mg, 12.6 minutes) and C (1.0 mg, 6.0 minutes).

The hexane-insoluble materials of the extract were subjected to a silica gel column (benzene : EtOAc to acetone) chromatography. The active fraction eluted with acetone was further purified using a Sephadex LH-20 column ($CHCl_3$: MeOH = 1 : 1) and C_{18} HPLC (Shiseido Capcel Pack C18, 50 × 250 mm; eluent, isocratic gradient, from 32% MeCN with 0.1% TFA; flow rate 20 ml/minute; UV detection, 380 nm) to afford brasiliquinone A (2.0 mg).

Physico-chemical Properties

The physico-chemical properties of brasiliquinones A, B and C are summarized in Table 2. The antibiotics were soluble in methanol, chloroform, acetone and ethyl acetate, but insoluble in water. The molecular formula of brasiliquinones A, B and C was determined as $C_{26}H_{27}NO_7$, $C_{20}H_{16}O_5$ and $C_{21}H_{18}O_5$, respectively, by HRFAB-MS.

The NMR spectral data showed the brasiliquinones to be new benz[*a*]anthraquinone antibiotics isolated from *N. brasiliensis*. All benz[*a*]anthraquinone antibiotics hitherto reported possessed a C1 unit at the C-3 position, while brasiliquinones have an ethyl group at C-3. Interestingly brasiliquinone A has ristosamine as a sugar moiety, making it the first benz[*a*]anthraquinone antibiotic possessing ristosamine as an *O*-glycoside moiety and an ethyl group at C-3 position. (Fig. 1)[14]. Studies on the biogenetic origins of the ethyl group at C-3 position are now in progress in our laboratory.

Antimicrobial and Antitumor Activities

Among the bacteria tested, *M. smegmatis* and *Micrococcus luteus* were relatively susceptible (Table 3); the MIC values of brasiliquinones A, B and C against *M. smegmatis* were 0.39, 0.78 and 12.5 μg/ml. They also showed moderate activity against *S. aureus* 209 P and *S. aureus* MRSA strains at the concentration of 12.5 to

Table 4. *In vitro* antitumor activity of brasiliquinones A, B and C.

Test drug	IC_{50} (μg/ml)		
	L1210	P388	P388/ADR[a]
Brasiliquinone A	7.0	3.3	3.5
Brasiliquinone B	3.3	3.0	3.0
Brasiliquinone C	2.9	3.2	3.8

[a] Multiple-drug resistant strain of P388 leukemia (see ref. 11).

50.0 μg/ml. The antibiotics were inactive against Gram-negative bacteria and fungi tested.

In vitro antitumor activity of brasiliquinones against L1210 and P388 cells shown in Table 4. Brasiliquinones B and C were more effective than brasiliquinone A against L1210 tumor cells. IC_{50} values of the antibiotics against L1210 and P388 tumor cells ranged from 2.9 to 7.0 μg/ml. Since it has been reported that benz[*a*]anthraquinone antibiotics were active against multidrug-resistant tumor cells[15], the antitumor activity of brasiliquinones against drug resistant P388/ADR tumor cells was tested. As expected, brasiliquinones were active against P388/ADR as well as P388 cells. These antibiotics thus appeared to overcome the multidrug-resistance of P388 cells, although further detail studies are necessary.

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