Nocardicyclins A and B: New Anthracycline Antibiotics Produced by *Nocardia pseudobrasiliensis*

YASUSHI TANAKA, UDO GRÄFE†, KATSUKIYO YAZAWA and YUZURU MIKAMI*

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

MICHAEL RITZAU

Hans-Knoll-Institute of Natural Product Research e.V., D-07745 Jena, Beutenbergstr. 11, Germany

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Nocardicyclins A (**1**) and B (**2**), new anthracycline antibiotics have been isolated from the mycelial cake of *Nocardia pseudobrasiliensis* IFM 0624 (JCM 9894). The molecular formulae of **1** and **2** have been determined as $C_{30}H_{35}NO_{11}$ and $C_{32}H_{37}NO_{12}$, respectively, and the structures were characterized by 1- and 8-methoxyl groups, a 10-carbonyl group and a novel carbon-methylated aminosugar constituent. Nocardicyclin A (**1**) exerts cytotoxic activity against L1210 and P388 leukemia. Nocardicyclins A (**1**) and B (**2**) are active against Gram-positive bacteria including *Mycobacterium* spp. and *Nocardia* spp., but inactive against Gram-negative bacteria.

We recently isolated and described new bioactive metabolites from pathogenic *Nocardia* strains[1~4]. Although six species of pathogenic *Nocardia* have been reported as human pathogens[5], those metabolites were mainly isolated from *Nocardia brasiliensis*[4]. During the course of our screening program to find new bioactive metabolites from pathogenic *Nocardia*, two were isolated from the mycelial cake of *Nocardia pseudobrasiliens* IFM 0624 (JCM 9894) and were called nocardicyclins A (**1**) and B (**2**). Structural elucidation suggested that **1** and **2** are new anthracycline group of antibiotics (Fig. 1). In this paper, we report their fermentation, isolation, physico-chemical properties, structural characteristics and biological activities of the antibiotics.

Materials and Methods

Producing Organisms

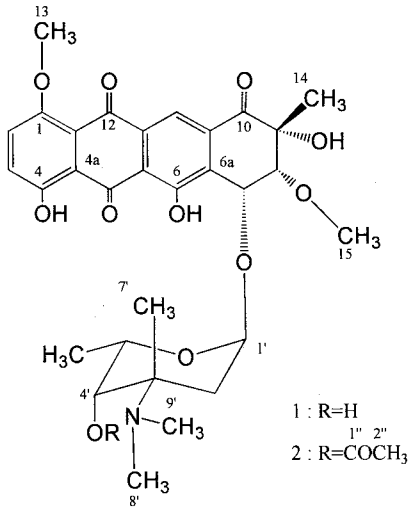
N. pseudobrasiliensis IFM 0624 (JCM 9894) was used for the isolation of **1** and **2**. The strain had been maintained on 2% glucose brain heart infusion agar (BHI, Difco, Detroit) medium in our culture collection.

Fermentation

The seed broth was prepared by inoculating mycelial fragments of the producing strain IFM 0624 grown on 2% glucose BHI agar into 500-ml Erlenmeyer flasks containing 150 ml of a medium consisting of glucose 1.0%, glycerol 1.0%, polypeptone 1.0% and meat extract 0.5% (pH 7.0). This was 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 15 liters of the same medium and the fermenter was stirred at 300 rpm with aeration at 15 liters/minute at 32°C for 5 days.

Production of **1** was monitored by HPLC (Lichrospher RP-18e, 4.6 × 150 mm, Merck) using 22% CH_3CN

Fig. 1. Structures of nocardicyclins A (**1**) and B (**2**).



† Present address: Hans-Knoll-Institute of Natural Product Research e.V., D-07745 Jena, Beutenbergstr. 11, Germany.

with 0.2% TFA as an eluent.

Spectral Analysis

Electrospray mass spectra were recorded on a triple quadruple instrument Quattro 400 (Fisons VG Biotech, Altrincham, U.K.). FAB mass spectra were recorded on a high-resolution mass spectrometer AMD-402 (AMD Intectra, Harpstedt, Germany; *m*-nitrobenzyl alcohol as matrix; direct inlet system). Polarimetry was carried out using a Propal Instrument (Dr. KERNCHEN OPTIK, Seelze, Germany). UV-VIS spectra were recorded in methanol on a Beckman DU 640 spectro-photometer. IR spectra were recorded on a Shimadzu-470 spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer operating at 500 MHz and 125 MHz, respectively. Chemical shifts are given in δ (ppm) using TMS as the internal standard.

Biological Activities

Antimicrobial activities were determined by microbroth dilution method using BHI and Sabouraud dextrose media[4]. Cytotoxic activities were determined by the method described[6] using L1210, P388, P388/ADR[7], KB and CHO cell lines.

Results and Discussion

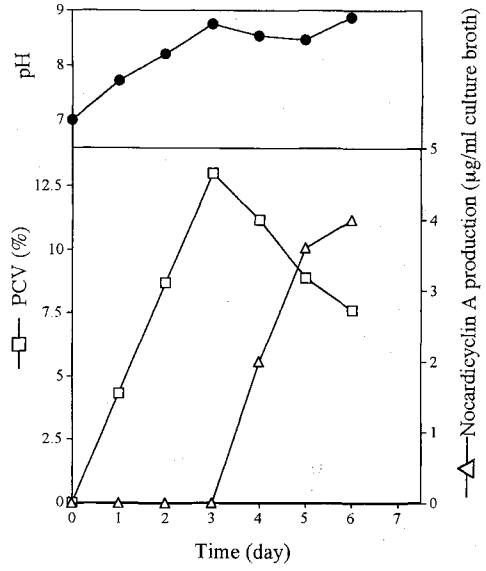
Fermentation

The fermentation of strain *N. pseudobrasiliensis* producing **1** and **2** was carried out as described in Materials and Methods. The strain IFM 0624 was fermented using a 20-liter fermenter. A typical fermentation diagram of *N. pseudobrasiliensis* IFM 0624 is shown in Fig. 2. The maximum growth was obtained on day 3; thereafter, the mycelia decreased with time. The pH of culture increased coupled with the growth until day 3, and the maximum pH value was observed on day 6. The production of **1** started on day 3, and reached a maximum (4 μg/ml) on day 6. The production time course of **2** was similar to that of **1** (data not shown).

Isolation and Purification

After 5 days of incubation, 15 liters of methanol was added to the 15 liters of culture broth and further incubated for 3 hours to kill the *Nocardia* and extract the active components. Then the broth was filtered and evaporated under reduced pressure to one third of the original quantity, and the active fractions were extracted with the same volume of ethyl acetate. The extract was concentrated *in vacuo* and the crude residue was subjected to silica gel column chromatography using a mixture of $CHCl_3$ and methanol (10:1) for fraction A (nocardicyclin A fraction) and (3:1) for fraction B (nocardicyclin B fraction) as the eluent. Each of the active fractions was combined and further purified by preparative HPLC (Soken Pack-ODS, 20 × 250 mm) using 22% CH_3CN for fraction A or 26% CH_3CN for fraction B with 0.2% TFA as the eluent (Flow rate, 10 ml/minute, detection at 238 nm). Finally 10 and 5 mg of purified **1** and **2** were obtained, respectively.

Fig. 2. Fermentation time course of nocardicyclin A (**1**) production.



Growth was determined by PCV (packed cell volume) method and the antibiotic production was monitored by HPLC method (see Materials and Method).

Structure Elucidation

The presence of a quinoid chromophore in both molecules was shown by λ_{max} 490 nm in the UV-VIS spectra of **1** and **2**. In the IR spectrum (in KBr) of **1** two carbonyl absorptions with λ_{max} 1616 cm^{-1} (CO) and 1677 cm^{-1} (*p*-quinone) were visible but the spectrum of **2** displayed three carbonyl bands at 1619 cm^{-1} (CO), 1669 cm^{-1} (*p*-quinone) and 1742 cm^{-1} ($COCH_3$).

The molecular weight of the new anthracyclines **1** and **2** was readily inferred from the mass spectrometric data. The positive ion electrospray mass spectra displayed for **1** *m/z* 586.6 $[M+H]^+$ and for **2** *m/z* 628.2 $[M+H]^+$. The molecular formulae of **1** and **2** were determined to be $C_{30}H_{35}NO_{11}$ and $C_{32}H_{37}NO_{12}$ on the basis of HRFAB-MS measurements (*m/z* found 586.23028, calcd.

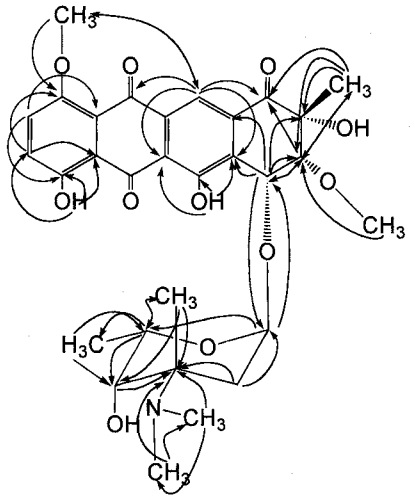
Table 1. Physico-chemical properties of nocardicyclins A (**1**) and B (**2**).

	1	**2**
Appearance	Reddish microcrystals	Reddish microcrystals
Molecular formula	$C_{30}H_{35}NO_{11}$	$C_{32}H_{37}NO_{12}$
Molecular weight	585	627
ES-MS (*m/z*)	586.6 $(M+H)^+$	628.2 $(M+H)^+$
HR-FAB-MS (*m/z*)		
found	586.23028 $(M+H)^+$	628.24170 $(M+H)^+$
calcd	586.23013 ($C_{30}H_{36}NO_{11}$)	628.24147 ($C_{32}H_{38}NO_{12}$)
UV-VIS (λ_{max}) nm	490 (broad absorption)	490 (broad absorption)
$[\alpha]_D^{25}$	$-157.9°$ (*c* 0.05 in MeOH)	Not determined
IR (λ_{max}) cm^{-1}	719, 796, 831, 957, 991, 1022, 1131, 1203, 1205, 1378, 1407, 1434, 1462, 1536, 1616, 1677, 2990, 3425	716, 760, 795, 831, 988, 1024, 1033, 1101, 1131, 1180, 1206, 1285, 1375, 1402, 1436, 1466, 1619, 1669, 1742, 2930, 3405

586.23013 for $C_{30}H_{36}NO_{11}$ $[M+H]^+$) and (*m/z* found 628.24170, calcd. 628.24147 for $C_{32}H_{38}NO_{12}$, $[M+H]^+$), respectively.

The structures of both new anthracyclines (Fig. 1) were assigned unambiguously by detailed one and two-dimensional ^1H and ^{13}C NMR measurements (DEPT, COSY, NOESY, HSQC, HMBC). The spectra were recorded in CD_3OD and additionally in DMSO to visualize the phenolic protons and their long-range ^1H, ^{13}C-connectivities. Analysis of the proton-, carbon-, DEPT and HSQC spectra of **1** revealed the presence of one ketone carbonyl, two quinone carbonyls, nine aromatic and two aliphatic quaternary carbons, three aromatic CH, one O–CH–O, four CH–O, one CH_2, two CH_3–O, two CH_3–N, and three CH_3–C units. **1** and **2** showed each two exchangeable protons with chemical shifts between 12 and 13 ppm, as expected for phenolic protons of a naphthoquinone. Compound **2** showed additionally the signals of a methyl and an ester carbonyl group. In the COSY spectra of **1** and **2** four short spin systems, namely two ortho coupled ($J=9.5$ Hz) aromatic protons and the fragments CH_3–CH–O, O–CH–CH–O and O–CH(–O)–CH_2– were easily detectable. The HMBC spectrum of **1** (Fig. 3) enabled the assignment of most of the fragments, carbons and protons except for C-5 carbonyl. As far as the sugar moiety is concerned the correlation from the anomeric proton at 5.76 ppm to the carbons at 66.1 and 66.3 ppm, respectively, were of particular assistance. Support to this contention was given by the correlations of 4′-H, 7′-CH_3 and the dimethylamino group as shown in Fig. 3. The unusual attachment of a methyl group at C-3′ is suggested by the heteronuclear long range couplings of 6′-CH_3 with C-2′, C-3′ and C-4′ and confirmed independently by the synaxial NOE cross peak with 5′-H. The connection of

Fig. 3. Selected heteronuclear long-range couplings and NOE effects as detected in the HMBC and NOESY spectra of nocardicyclin A (**1**).



the sugar to C-7 of the aglycon was elucidated by the correlation of 1′-H (5.76 ppm) to C-7 (74.0 ppm) as well as the corresponding correlation from H-7 (5.15 ppm) to C-1′ (102.2 ppm) in HMBC spectrum of **1**. Regarding the framework of the aglycone, the observed NOE between 11-H and 13-CH_3 was of special value, because the position of the two quinone-neighboured rings could be clarified only by this effect. Long range correlation from 11-H and 14-CH_3 to C-10 (199.8 ppm) gave evidence for the location of this α,β-unsaturated ketone carbonyl within the exceptional ring A of the anthracycline aglycone. Further instructive long range correlations are summarized in Fig. 3. Assignment of the relative stereochemistry of substituents in the aglycone and the sugar moieties as shown in Fig. 3 was supported

Table 2. Assignment of ^1H and ^{13}C chemical shifts of nocardicyclin A (**1**) (in CD_3OD and DMSO[b]) and nocardicyclin B (**2**) (in DMSO).

Carbon atom No.	Nocardicyclin A (**1**)		Nocardicyclin B (**2**)	
	^1H[a]	^{13}C[a]	^1H	^{13}C
	δ_H	δ_C	δ_H	δ_C
1		156.6		154.5
2	7.65 (d, 9.5)	126.6	7.74 (d, 9.6)	125.8
3	7.47 (d, 9.5)	128.7	7.47 (d, 9.6)	126.9
4	12.08[b] (s)	158.8	12.07 (s)	156.4
4a		116.5		115.6
5		193.5		191.8
5a		119.5		134.9
6	12.51[b] (s)	162.9	12.52 (s)	160.8
6a		133.1		131.0
7	5.15 (d, 2.2)	74.0	5.03 (d, 2.2)	72.0
8	3.72 (d, 2.2)	87.9	3.54 (d, 2.2)	86.3
9		77.6		75.9
10		199.8		198.7
10a		137.4		135.7
11	8.10 (s)	117.0	8.01 (s)	114.9
11a		136.3		118.4
12		180.8		178.9
12a		119.1		118.1
13	3.99 (s)	57.3	3.93 (s)	56.8
14	1.50 (s)	24.0	1.39 (s)	23.5
15	3.56 (s)	60.2	3.43 (s)	59.6
1′	5.76 (d, 4.1)	102.2	5.76 (d, 4.2)	99.6
2′	2.11 (d, 13.4, 4.1)	34.0	2.02 (dd, 13.5, 4.2)	32.9
	2.28 (d, 13.4)		2.15 (d, 13.5)	
3′		66.1		62.7
4′	3.66 (s)	69.5	5.14 (s)	69.4
5′	4.23 (q, 6.6)	66.3	4.27 (q, 6.5)	64.2
6′	1.41 (d, 6.6)	17.4	1.14 (d, 6.5)	17.1
7′	1.45 (s)	14.9	1.43 (s)	14.5
8′	2.71 (s)	37.2	2.68 (d, 4.4)[c]	37.1
9′	2.76 (s)	36.3	2.55 (d, 4.5)[c]	36.0
1″				170.6
2″			2.29 (s)	20.8

[a] Multiplicity in parentheses (s: singlet, d: doublet, q: quartet)-coupling constants in Hz.
[b] Chemical shifts of the proton of 4 and 5 positions in DMSO.
[c] Due to the coupling with the quaternary NH proton at 9.01 ppm which is present in **2**.

by other NOE effects as well as by several coupling constants (Fig. 3). A strong NOE was observed in the NOESY spectrum of **1** between 8-H, 14-CH_3 and 7-H, respectively. The corresponding NOE between 15-CH_3, 14-CH_3 and 7-H, respectively, was much smaller, suggesting the given stereochemistry at C-7 to C-9. Moreover, the given relative stereochemistry of the aglycone was settled by the NOE between the axial 7-H and 14-CH_3 as well as the small vicinal coupling constant between 7-H and 8-H of 2.2 Hz which is not compatible with the bisaxial position of these protons. With regard to the sugar moiety of **1**, the NOE between 7′-CH_3 and 5′-H proved the axial position of both substituents. In addition, the extremely small coupling constant (< 1 Hz) between 4′-H (3.66; (s)) and 5′-H, which does not lead to a cross peak in the COSY spectrum proves the equatorial position of 4-H. Hexapyranoses with equatorial 4-H and axial 5-H such as galactose typically show this small coupling constant due to their gauche orientation[8]. The stereochemistry of the anomeric position was concluded to be a due to the presence of two small coupling constants between 1-H and 2-H (Table 2) which are not in agreement with an antiperiplanar orientation. Moreover, the lack of a NOE between 1-H and the axial substituents 5-H and 3-CH_3 proves that these protons are not in synaxial position. Nocardicyclin B (**2**) was isolated as a salt with protonated sugar nitrogen ($\delta = 9.01$ Hz; broad) which couples with both N–CH_3 groups (Table 2). Thus the NMR data confirm the molecular formulae $C_{30}H_{35}NO_{11}$ for **1** and $C_{32}H_{37}NO_{12}$ for **2**.

Nocardicyclins A (**1**) and B (**2**) thus appear as new

Table 3. Antimicrobial activity of nocardicyclins A (**1**) and B (**2**).

Test organisms	MIC (μg/ml)	
	1	**2**
Micrococcus luteus IFM2066	1.56	12.5
Staphylococcus aureus 209P	3.13	100
Escherichia coli NIH JC2	>100	>100
Bacillus subtilis PCI189	6.25	3.13
Nocardia transvalensis IFM0333	3.13	6.25
N. pseudobrasiliensis IFM0624	25	25
N. brasiliensis IFM 0236	6.25	>100
N. otitidiscaviarum IFM0239	1.56	>100
N. nova IFM0290	0.78	1.56
N. asteroides IFM0319	1.56	3.13
N. farcinica IFM0284	3.13	12.5
Mycobacterium smegmatis ATCC607	1.56	3.13
M. phlei ATCC11758	0.78	3.13
M. flavescens ATCC14474	3.13	12.5
Aspergillus niger ATCC40606	>100	>100
Candida albicans ATCC90028	>100	>100

Mueller Hinton II broth (Becton Dickinson, USA) and Sabouraud dextrose broth (Difco, USA) were used for bacteria and fungi, respectively.

members of the anthracycline family of antibiotics. They are distinguishable from the already known representatives, even from the products of *Nocardia* sp.[9,10] by their unusual structural pattern of 1- and 8-methoxyl groups, a 10-carbonyl group, and a novel carbon-methylated aminosugar constituent called brasiliose, which is additionally *O*-acetylated in **2**.

Biological Pproperties

In vitro antibacterial activities of **1** and **2** are shown in Table 3. Nocardicyclins A (**1**) and B (**2**) were active against Gram-positive bacteria, but inactive against Gram-negative bacteria and fungi. Most of the bacteria tested were inhibited at the concentration between 0.78 and 12.5 μg/ml. *N. nova* IFM 0290 and *M. phlei* ATCC 11758 were the most susceptible group of microorganisms among those tested. As expected, *N. pseudobrasiliensis* IFM 0624, the producer itself, was not susceptible to the antibiotic, and was inhibited at the concentration of 25 μg/ml. The MIC values of **1** and **2** varied depending on the bacteria tested and the former was more active than the latter against the Gram-positive bacteria such as *Staphylococcus aureus* 209P, *N. brasiliensis* IFM 0236 and *N. otitidiscaviarum* IFM 0239. Since the acetylated compound **2** was less active, it is reasonable to assume that the 4′-OH group plays an important role in the exhibition of antimicrobial activity in certain Gram-positive bacteria. It will be interesting to know whether the acetylation of the 4′-OH group is associated with the self-resistance mechanism of the producer itself against the antibiotics, although the susceptibility of producer against the two antibiotics was the same.

Table 4. *In vitro* cytotoxic activities of nocardicyclin A (**1**) against various cultured cell lines in comparison with those of doxorubicin.

Cell line	IC_{50} (μg/ml)	
	1	Doxorubicin
L1210	0.15	0.09
P388	0.47	0.09
P388/ADR	0.97	0.45
KB	1.06	0.97
CHO	12.3	0.01

In vitro cytotoxic activities of **1** against L1210, P388, P388/ADR, KB and CHO cell lines are shown in Table 4 in comparison with those of doxorubicin. Nocardicyclin A (**1**) was active against these cell lines and the IC_{50} values were around 0.15 to 1.06 μg/ml. However, **1** was less active than doxorubicin against all tested cell lines under the assay conditions used. The low productivity of **2** did not allow us to test the cytotoxic activity of the antibiotic. Optimization studies on the production of **2** are now in progress for further comparative *in vitro* and *in vivo* antitumor tests of the antibiotic.

The majority of human nocardiosis are caused by members of the *N. asteroides* complex, which includes three species (*N. asteroides* sensu stricto, *N. farcinica* and *N. nova*). In contrast to *N. asteroides*, the other human pathogens, including *N. brasiliensis*, *N. otitidiscaviarum* and *N. transvalensis*, are usually considered homogenous[5]. However, STEINGRUBE *et al.*[11] and RUIMY *et al.*[12] reported a new taxon (*N. pseudobrasiliensis*) for some *N. brasiliensis* based on taxonomic characteristics like the decomposition of adenine, reduction of nitrate, and susceptibility to some antimicrobial agents like minocycline[11]. Our preliminary investigation on the productivity of **1** and **2** suggested that another reference strain, *N. pseudobrasiliensis* IFM 0623 (JCM 9893) is also forming these antibiotics. We are now planning to test this productivity using several strains of this species of *Nocardia* and the results will be of interest from a chemo-taxonomic point of view regarding pathogenic *Nocardia*.

Acknowledgments

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