THREE NEW REDUCED ANTHRACYCLINE RELATED COMPOUNDS FROM PATHOGENIC *Nocardia brasiliensis*

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Three new metabolites were isolated from a pathogenic bacterium, *Nocardia brasiliensis* IFM 0075 strain, a producer of a new anthracycline antibiotic (SO-075R1) and its mutant strain (IFM 0075-13-1). The structural studies showed that they are reduced anthracyline related compounds. Some biosynthetic routes of these matabolites were discussed.

During our investigations on bioactive substances from pathogenic actinomycetes, we have reported that *Nocardia brasiliensis* IFM 0075 strain produced a new mutactimycin type antibiotic, SO-075R1[1-3]. Our further studies on the minor components of the strain resulted in the isolation of three novel components from the strain and its mutant strain (IFM 0075-13-1) which was obtained by UV irradiation. In this paper, the fermentation, isolation and structural studies of these are described.

Materials and Methods

Organism and Isolation of Mutant

N. brasiliensis IFM 0075 and its orange-pigmented mutant strain (IFM 0075-13-1) obtained by UV irradiation were used in this experiment.

Fermentation

One loopful of spores or mycelial fragments from slant cultures of *N. brasiliensis* IFM 0075 strain were inoculated into a 100-ml Erlenmeyer shake flask containing 20 ml of brain heart infusion (Difco) medium with 1% glucose. The flasks were shaken at 250 rpm for 4 days at 27°C. One hundred ml of the matured seed culture was used to inoculate a 2.0-liter fermenter containing 1.0 liter of the production medium composed of meat extract 0.5%, peptone 0.5% and glucose 2.0%, supplemented with antifoam (Adecanol LG-109, Asahidenka Co., Ltd, Japan) 0.005%[4]. The pH was adjusted to 7.4. The fermenter was stirred at 500 rpm with aeration at 1.0 liter/minute at 30°C for 4 days.

Physico-chemical Characteristics

^{1}H and ^{13}C NMR spectra were measured in $CDCl_3$ on a JEOL ALPHA-500 NMR spectometer at 500 and 125 MHz, respcectively. Chemical shifts of ^{1}H and ^{13}C NMR were recorded in δ units relative

to internal teteramethylsilane ($\delta=0$). GFAB-MS and HRFAB-MSs were measured on a JEOL HX110 instrument.

Results

Isolation of M-3 and M-4

After incubation, one and a half volumes of methanol was added to the culture broth and further incubated for 3 hours to kill the *Nocardia*. Then the broth was filtered and evaporated under reduced pressure to the original volume and extracted with a half volume of methylene chloride. The extract was washed with water, dried and evaporated to dryness. Antifoam-separated fraction (4.253 g) obtained by *n*-hexane treatment was purified by silica gel column chromatography (ethyl acetate - toluene 2 : 9). The first moving fraction was subjected to Sephadex LH-20 column chromatography with methanol and crystalyzed from THF-methanol to yield 3.5 mg of M-3. The slow moving fraction was further purified by Sephadex LH-20 and 4.3 mg of M-4 was obtained by silica gel column chromatography (toluene - THF 15 : 2) and crystalization (solvent mixture of methanol - ether).

Isolation of M-13-1

Antifoam separated fraction (2.5 g) obtained by *n*-hexane treatment of the mycelial extract (IFM 0075-13-1 strain) was purified by silica gel column chromatography ($CHCl_3$) and following preparative TLC (benzene - ethyl acetate 1 : 1). M-13-1 was purified by an HPLC column (Hiber, Cica-Merck, E. Merck) using a mixture of CH_3CN - H_2O as the eluent and finally, 100 mg of crystalized M-13-1 from 1-liter culture broth was obtained from chloroform solution.

Physico-chemical Properties and Structure Determination

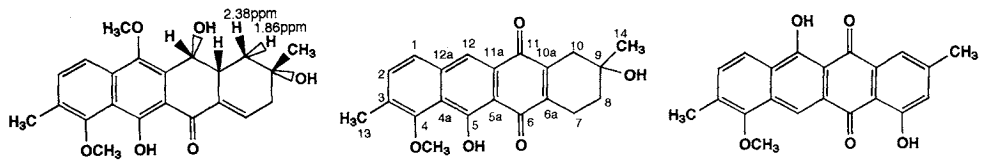
M-3: M-3 (**1**) was obtained as yellow crystals and is optically active, $[\alpha]_D^{23}$ +58 (*c* 0.06, $CHCl_3$). M-3 is soluble in methanol, ethyl acetate, chloroform and other organic solvents, and also in water. The UV absorption spectrum (ν_{max} nm (ε)) in methanol showed maxima at 231 (21,500), 285 (22,700), 312 (9,200) and 426 (6,100). The main absorption bands in the IR spectrum (film) were observed at following wavelength: 3525, 2950, 1635, 1600, 1370, 1280 and 1080 cm^{-1}. The molecular formula of M-3 was determined as $C_{22}H_{24}O_5$ by HRFAB-MS (*m/z* 385.1660, $(M+H)^+$, 0.9 mmu).

DEPT and ^{13}C-^1H COSY spectra showed the presence of two methyl (15.9 ppm and 27.5 ppm), two methoxyl (61.5 ppm and 61.6 ppm), one quaternary oxy-carbon (69.2 ppm), one aliphatic oxy-methine (71.6 ppm), three olefinic methine (117.1 ppm, 134.9 ppm and 138.2 ppm) and ten olefinic quaternary carbons including one carbonyl (190.8 ppm) in M-3 (**1**). Furthermore these results and the molecular formula indicated the presence of three hydroxyl groups. Interpretation of ^1H-^1H COSY, HMBC and COLOC spectra disclosed the planar structure of M-3 except for the carbonyl group which showed no relationship between any protons. However, it was obvious that the carbonyl group was located at C-6, as in Fig. 1, to satisfy the molecular formula. The hydrogen bonding between the carbonyl and C-5 hydroxyl group makes the hydroxyl proton shift to low field (15.78 ppm). The irradiation of H-7 (7.20 ppm) changed a triplet (dd, 3.7 Hz, 3.7 Hz) of H-8 protons to a broad doublet (3.3 Hz). Thus the absence of the expected geminal couplings (*ca.* 10 Hz) can be explained by AB type second order coupling H-8 protons (their chemical shifts are quite close). On NOESY spectrum, NOEs between 10-Ha and 10a-H, 10a-H and 14-H_3, 10a-H and 11-H, and 10-Hb and 11-H were observed. These results clarified the relative structure of M-3 as in Fig. 1.

Table 1. ^{1}H and ^{13}C NMR chemical shifts (δ, ppm) of M-3, M-4 and M-13-1 in $CDCl_3$.

Position	M-3		M-4		M-13-1	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	7.68 (d, 8.3)	117.1	7.61 (d, 8.2)	126.8	8.17 (d, 8.5)	120.5
2	7.56 (d, 8.3)	134.9	7.53 (d, 8.2)	134.7	7.51 (d, 8.5)	132.9
3		129.7		133.1		133.4
4		157.4		157.3		156.1
4-OMe	3.89 (3H, s)	61.6	3.92 (3H, s)	61.8	3.97 (s)	62.1
4a		120.3		108.9		131.0
5		163.5		163.8	8.54 (s)	116.1
5-OH	15.78 (s)		14.95 (s)			
5a		108.9		122.0		127.6
6		190.8		188.5		187.4
6a		132.9		145.4		115.2
7	7.20 (ddd, 2.4, 4.1, 4.1)	138.2	2.84 (2H, m)	21.0		163.3
7-OH					12.94 (s)	
8	2.50 (2H, dd, 3.7, 3.7)	40.8	(a) 1.94 (m) (b) 1.69 (ddd, 7.1, 9.3, 13.5)	33.8	7.09 (d, 1.4)	124.2
9		69.2		67.9		148.5
10	(a) 2.38 (dd, 5.9, 12.9) (b) 1.86 (dd, 9.3, 12.9)	40.5	(a) 2.82 (m) (b) 2.62 (ddd, 2.5, 2.5, 20.1)	37.5	7.70 (d, 1.4)	120.2
10a	2.87 (dddd, 2.7, 6.1, 9.5, 10.3)	41.8		144.3		133.8
11	5.02 (d, 10.5)			183.5		187.2
11-OH	5.83 (s)					
11a		127.7		127.1		109.0
12		143.9	7.99 (s)	122.0		164.1
12-OMe	3.88 (3H, s)	61.5				
12a		133.4		137.1		128.3
12-OH					14.59 (s)	
13	2.46 (3H, s)	15.9	2.48 (3H, s)	16.3	2.51 (s)	16.4
14	1.31 (3H, s)	27.5	1.43 (3H, s)	29.7	2.48 (s)	22.3

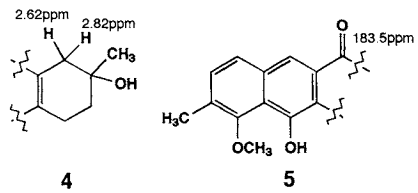
Fig. 1. The structures of M-3 (**1**), M-4 (**2**) and M-13-1 (**3**).



M-4: It (**2**) was obtained as red crystals and is soluble in most organic solvents. M-4 is optically active ($[\alpha]_D^{23}$ +28 (*c* 0.15, $CHCl_3$), IR (film) spectrum showed 3525, 29550, 1635, 1600, 1370, 1280 and 1080 cm^{-1}; UV ν_{max} nm (ε) 245 (34,800), 287 (9,200) and 472 (5,600). The molecular formula was determined to be $C_{21}H_{20}O_5$ on the basis of HRFAB-MS (*m/z* 353.1389 $(M+H)^+$, O). The ^{13}C NMR spectrum (183.5 ppm and 188.5 ppm) suggested the presence of carbonyl functions. DEPT and ^{13}C-^{1}H NMR spectra also showed the presence of two methyl (16.3 ppm and 29.7 ppm), one methoxyl (61.8 ppm), three methylene (21.0 ppm,

33.8 ppm and 37.5 ppm), and three olefinic methine (122.0 ppm, 126.8 ppm and 134.7 ppm) groups. These results and the molecular formula indicate the existence of two hydroxyl groups. The partial structure (**4** in Fig. 4) was deduced from ^{1}H-^{1}H COSY and HMBC correlations. The structure (**5** in Fig. 5) was mainly obtained by interpretation of ^{1}H-^{1}H COSY, HMBC and NOE difference spectra.

Fig. 2. Partial structures of M-4.



4 **5**

NOEs were observed between 1-H and 12-H, 1-H and 2-H, 2H-and 13-H_3, 13-H_3 and CH_3O-H_3, and CH_3O-H_3 and 5-OH. The carbonyl carbon (188.5 ppm) showed no cross peak with either proton on HMBC experiments. Considering the partial structures (**4**, **5**) and the molecular formula, the carbon (188.5 ppm) was concluded to be a quinone. Although it was not clear whether the quinone is an orthoquinone or a paraquinone. Therefore an LSPD experiment was conducted. Irradiation at 2.62 ppm (10-Hb) made a broad doublet at 183.5 ppm (C-11) evidently sharp, and a broad singlet at 188.5 ppm (C6) did not change. These results clarified the connection between C-10 at C-11 through C-10a. Thus the structure of M-4 was determined as in Fig. 1. The down field shifts of the quinone carbon (188.5 ppm) and 5-OH proton (14.95 ppm) were caused by a hydrogen bonding between each other (peri position). M-4 is the first example of 7,12-deoxyanthracyclinone from actinomycetes although some synthetically[5] or enzymatically[6] derived 7,12-deoxyanthracyclinones have been reported.

M-13-1 (**3**) is an orange powder and is optically inactive. It is slighty soluble in methanol, chloroform and ethyl acetate and soluble in water. The other physico-chemical properties of M-13-1 are as follows: The IR absorption bands (film) ν_{max} cm^{-1} 2975, 1610, 1500, 1485, 1460, 1395, 1350, 1265 and 1010. The UV absorption spectrum λ_{max} nm (ε) in methanol: 245 (22,600), 263 (20,200), 288 (11,100), 301 (10,400) and 458 (7,700). The molecular formula was determined to be $C_{21}H_{16}O_5$ on the basis of HREI-MS (*m/z* 348.1009, M$^+$, 1.1). DEPT and ^{13}C-^{1}H COSY spectrum showed the presence of two methyl (16.4 ppm and 22.3 ppm), one methoxyl (62.1 ppm), five olefinic methines (116.1 ppm, 120.2 ppm, 120.5 ppm, 124.2 ppm and 132.9 ppm), and thirteen olefinic quaternary carbons including two carbonyls (187.2 ppm and 187.4 ppm). The existence of two hydroxyl groups was also suggested. The gross structure of M-13-1 (Fig.4) was elucidated on the basis of ^{1}H NMR, HMBC and NOESY investigations. Carbon assignments of C-5a and C-10a were established by the comparison with reference values[6,7].

Discussion

Recently three new antibiotics from pathogenic *Nocardia*[8-10] have been isolated, although such studies were very rare. Interestingly, it was also found the producers are restricted to two species of pathogenic *Nocardia*, *i.e.*, *N. brasiliensis* and *N. otitidiscaviarum*, although there are five pathogenic *Nocardia* species. Since few studies on the secondary metabolites from pathogenic *Nocardia* has been conducted, we are interested in the minor components produced by *N. brasiliensis* IFM 0075 strain, SO-075R1 producer. In the present studies, we also tried to isolate anthracycline related compounds from its mutant strain. Our studies resulted in the isolation of three anthracycline related compounds, M-3 (**1**), M-4 (**2**) and M-13-1 (**3**). Structral studies indicated that they have C-14 and C-13 methyl functional groups in the skeleton. Since C-13 and C-14 methyl functions are characteristic to SO-075R1 and mutactimycin A group antibiotics, M-3, M-4 and M-13-1 might be derived by bioreduction of SO-075R1 or its anthracyclinone (**7**). Some reductive transformation of anthracycline and 7-deoxy anthracyclinone have been

Fig. 3. Structures of SO-075R1 (**6**) and its anthracylinone (**7**).

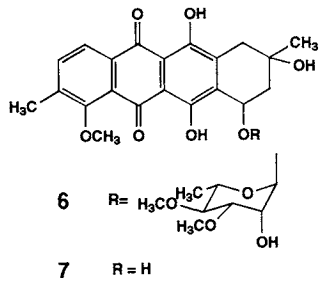


Fig. 4. Structures of quinone methide (**8**).

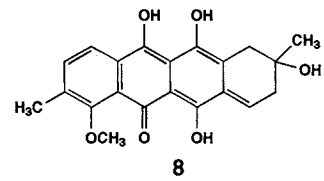
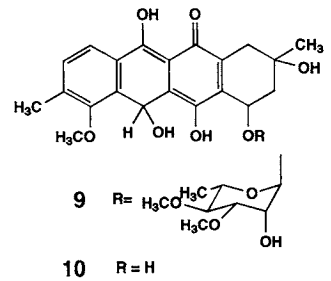


Fig. 5. Plausible intermediate compound to M-13-1.



considered[11-13]. On M-13-1 (**3**), the presence of a C-7 hydroxyl group in the molecule may suggests that this compound would not be biosynthesized via quinone methide (**8**). Our present studies also suggested that hydroquinones (**9**, **10**) are intermediates of a bioreductive pathway from S0-75R1 (**6**) or its anthracyclinone (**7**) to M-13-1. Also M-3 and M-4 were considered to be produced via quinone methide intermediate from SO-075R1, although further detail studies are necessary. Further detail biosynthetic studies of M-13-1 are of interest since the productivity of M-13-1 was 20 to 50 times higher than that of M-3 and M-4.

SULIKOWSKI *et al.*[11] speculated on the basis of chemical synthesis data that reductive tautomerization of daunomycin was not always triggered by the expulsion of the glycosyloxy group at C-7 accompanied with the formation of quinone methide. Our present intermediates, M-3, M-4 and M-13-1 isolated from the mycelial extract did not show further infomation to these speculation. Therefore, we tried to isolate other biosynthetic intermediates which may provide more positive information about elimination of glycosyloxy group, we could not succeed in any other intermediates.

With regards to *in vivo* reduction of anthracycline antibiotics, few bioreduced compounds have been characterized[12]. Therefore, M-3, M-4 and M-13-1 were considered to be rare representatives of *in vivo* reduced anthracyclines or anthracyclinones.

Although M-3, M-4 and M-13-1 did not show antimicrobial activities at the concentration of 100 μg/ml against *Micococcus luteus* and *Bacillus subtilis* by the broth dilution method, these anthracylcine related compounds with C-13 and C-14 methyl functions may give an interesting parent compound which lead to the less toxic antitumor agents because SO-075R1 was less toxic[1,2] compared to other antracycline group antibiotics.

Acknowledgments

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