

**Demethyl mutactimycins, new anthracycline antibiotics from *Nocardia* and *Streptomyces* strains**

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New anthracycline antibiotics 3'-O-demethyl mutactimycin (**3**) and 4-O,3'-O-didemethyl mutactimycin (**4**) were isolated from two *Actinomyces* strains, *Nocardia pseudobrasiliensis* and *Streptomyces* sp. GW 60/1571. The chemical structures were elucidated by mass spectrometry and NMR spectroscopy. Antibiotic **3** displays moderate antimicrobial activity against Gram-positive bacteria and cytotoxicity against P388, L1210 and HeLa tumor cells (IC<sub>50</sub> 9.6, > 25 and 20 µg/ml, respectively).

Human nocardioses are caused by members of the *Nocardia asteroides* complex (*N. asteroides sensu stricto*, *N. farcinica* and *N. nova*), *N. brasiliensis*, *N. otitidiscaviarum* and *N. transvalensis*<sup>1,2,3</sup>). Recently we isolated and reported several new bioactive compounds from clinical isolates of pathogenic *Nocardia*

strains<sup>4,5,6,7</sup>. Our studies of the productivity of pathogenic *Nocardia* strains suggested that metabolite formation is limited to *Nocardia brasiliensis*.

Recently we investigated a series of *Nocardia* strains belonging to other species than *N. brasiliensis*. One of these strains from the National Institute of Health Bangkok (Thailand) identified as *Nocardia transvalensis* was found to produce several antibacterial coloured substances. During our collaborative research work another producer of the same compounds was discovered by use of chromatographic and mass spectrometric techniques. This latter strain was taxonomically identified as a representative of the genus *Streptomyces*.

In this paper we report the fermentation of both strains, isolation of two new anthracycline-type compounds, their structural characteristics and biological properties.

## Material and Methods

### Producing organisms

*Nocardia transvalensis* IFM 0456 (NIH 34-43-6) was an isolate from a clinical specimen collected by the National Institute of Health, Bangkok, Thailand. The strain has been maintained on 2 % glucose brain heart infusion agar (BHI, Difco, Detroit).

*Streptomyces sp.* GW 60/1571 was obtained from the strain collection of the laboratory for soil microbiology Lohra-Kirchvers, Germany. Cells of strain GW 60/1571 were aerobic, Gram-positive, non-acid fast, filamentous, and differentiated into substrate and aerial mycelia.

The strain formed an extensive substrate mycelium and aerial hyphae with long hooked or open-coiled chains of spores (*Retinaculiaperti* category). Fragmentation of the substrate mycelium, sporangia or sclerotium-like structures, and flagellated spores were not observed.

The color of the aerial mycelium was pale red, and the reverse was orange to brown. A soluble orange red pigment was produced on glycerol-asparagine agar and yeast extract- malt extract agar. The strain did not produce melanoid pigments on tyrosine agar or peptone iron agar.

Whole-cell hydrolysates contained the L,L-diaminopimelic acid and traces of glycine (wall chemotype I). No characteristic sugars were detected. Strain GW 60/1571 thus displays chemotaxonomic properties, growth characteristics and morphology consistent with the genus *Streptomyces*.

### Fermentation

*Nocardia transvalensis*: The seed broth of *Nocardia transvalensis* was prepared by inoculating mycelial fragments of the producing strain IFM 0624 grown on 20 g/l glucose BHI agar into 10 ml Erlenmeyer flasks containing 3 ml of a medium consisting of (g/l) glucose 10, glycerol 10, polypepton 10 and meat extract 5 (pH 7.0). The bacterial suspension was then seeded onto plastic plate containing 20 ml of BHI agar medium with 10 g/l glucose and 10 g/l glycerol, and was cultured at 30 °C for seven days.

*Streptomyces sp. GW 60/1571*: The culture medium consisted of (g/l) malt extract 10, yeast extract 4, glucose 4. The pH was adjusted to 7.8 prior to autoclaving. Ten 1 l Erlenmeyer flasks, each containing 200 ml of this medium were inoculated with agar pieces grown with *Streptomyces GW 60/1571* (culture medium containing 18 g/l agar). Fermentation was carried out at 28 °C on a rotary shaker for 72 hours.

### Analytical procedures

UV-VIS and IR spectra were recorded on a Beckman DU-640 spectrometer and Shimadzu FT-IR instrument, respectively. High-resolution electron impact (EI) and FAB mass spectra (HRFAB-MS; 3-nitrobenzylalcohol as matrix) were recorded on a AMD-402 instrument with BE geometry equipped with direct inlet system (AMD Intectra Harpstedt, Germany). Electrospray mass spectra and CID-MS/MS spectra were obtained with a triple quadrupole mass spectrometer Quattro (VG Micromass, Altrincham, England). NMR spectra were recorded in [D<sub>6</sub>]DMSO on a Bruker Avance DRX 500 and Varian VXR 500 (499.8 MHz) instruments, respectively.

### Biological activities

Antimicrobial activities were determined by microbroth dilution method using BHI broth. Cytotoxic activities were determined by the method described using L1210 and P388 leukemia and HeLa cells<sup>8)</sup>.

## **Results and Discussion**

Isolation and purification of **3** and **4** from cultures of *N. transvalensis*

After 7 days of incubation, 1 l of methanol was added to the mycelial cake collected from 40 agar plate cultures (BHI agar; 10 cm diameter) and further

incubated for 3 hours to kill the *Nocardia* and extract the active components. The extract was dried and concentrated *in vacuo*. The crude residue was subjected to silica gel chromatography using  $\text{CHCl}_3$ . The combined active fraction was purified by preparative TLC (silica gel, Merck) by use of  $\text{CHCl}_3$ /methanol (10:1 v/v) as eluent. The compounds were further purified using HPLC (Soken Pack-ODS, 20 x 250 mm; 35 %  $\text{CH}_3\text{CN}$  with 0.15 % TFA). Major active components from this culture were SO-075R1 (**1**) and mutactimycin A (**2**). The production of **3** and **4** was low and the recoveries from 40 agar plate cultures were 3 mg and 1.5 mg, respectively.

#### Isolation and purification of **3** and **4** from cultures of *Streptomyces sp.* GW 60/1571

Mycelia and liquid phase were extracted seven times with one volume of ethyl acetate. The combined organic phases were concentrated *in vacuo* to yield an orange to reddish solid. This was dissolved in methanol (150 ml) and extracted with cyclohexane (2 x 50 ml). The methanol fraction was concentrated and applied to a Sephadex LH-20 column (methanol). The orange fractions were combined and concentrated *in vacuo* to yield 150 mg of an orange solid. This was further purified by HPLC (acetonitrile/water 42/58) to yield 102 mg of mutactimycin (**2**)<sup>9</sup>, 5.7 mg of **3** and 1.7 mg of **4**.

#### Structure elucidation

Shown in table **1** are the physicochemical properties of the new anthracyclines **3** and **4** (Fig. 1) which are produced by both strains of *Nocardia* and *Streptomyces*. Their structures were elucidated by detailed UV-, IR-, mass spectrometric and NMR-spectroscopic measurements.

The presence of a *peri*-hydroxy quinone chromophore in **3** and **4** was suggested by  $\lambda_{\text{max}}$  460 nm in their UV-VIS spectra, as well as by a typical color change with diluted sodium hydroxide and  $\nu_{\text{max}}$  1673  $\text{cm}^{-1}$  and 1677  $\text{cm}^{-1}$ , respectively, in the FT-IR spectrum.

In the electrospray mass spectrum (ESI-MS) of **3**, positive pseudomolecular ions at  $m/z$  553.4 ( $[\text{M}+\text{Na}]^+$ ),  $m/z$  1083.4 ( $[\text{2M}+\text{Na}]^+$ ) and  $m/z$  1612.5 ( $[\text{3M}+\text{Na}]^+$ ) were visible. In the negative ESI-MS of **3**,  $m/z$  529.6 ( $[\text{M}-\text{H}]^-$ ) also suggested a molecular weight of 530. Collision-induced decomposition of  $m/z$  529.6 ( $[\text{M}-\text{H}]^-$ ) afforded  $m/z$  365.5 (aglycone+O;  $-\text{H}_2\text{O}$ ) as a diagnostic daughter ion. ESI-MS of **4** displayed in the positive ion mode  $m/z$  539.3 ( $[\text{M}+\text{Na}]^+$ ) and  $m/z$  1054.6 ( $[\text{2M}+\text{H}]^+$ ) but  $m/z$  515.0 ( $[\text{M}-\text{H}]^-$ ) in the negative ion mode.

The chemical formulas of **3** (C<sub>27</sub>H<sub>30</sub>O<sub>11</sub>) and **4** (C<sub>26</sub>H<sub>28</sub>O<sub>11</sub>) were calculated from the high-resolution FAB mass spectra (HRFAB-MS) showing for **3**  $m/z$  553.1723 ([M+Na]<sup>+</sup>; calcd. 553.1726) and  $m/z$  531.1888 ([M+H]<sup>+</sup>; calcd. 531.1909 for C<sub>27</sub>H<sub>31</sub>O<sub>11</sub>) but for **4**  $m/z$  539.1634 ([M+Na]<sup>+</sup>; calcd. 539.1714) and  $m/z$  517.1807 ([M+H]<sup>+</sup>; calcd. 517.1870 for C<sub>26</sub>H<sub>29</sub>O<sub>11</sub>). Moreover, the electron impact (EI) mass spectrum of **3** shows  $m/z$  368.1286 ([aglycone-O-sugar-H]<sup>+</sup>; calcd. 368.1306 for C<sub>21</sub>H<sub>20</sub>O<sub>6</sub>) and  $m/z$  348.1006 ([aglycone-H<sub>2</sub>O,-OH-sugar]<sup>+</sup>; calcd. 348.1013 for C<sub>21</sub>H<sub>16</sub>O<sub>5</sub>) as diagnostic aglycone fragments. The fragment ion with  $m/z$  334.0858 ([aglycone-H<sub>2</sub>O,-OH,-sugar]<sup>+</sup>; calcd. 334.0877 for C<sub>20</sub>H<sub>14</sub>O<sub>5</sub>) in the EI mass spectrum of **4** suggested that its aglycone is different from that of **3** by a missing CH<sub>2</sub> equivalent.

The structure of **3** and **4** as shown in Fig. 1 were assigned by one- and two-dimensional NMR experiments (<sup>1</sup>H, <sup>13</sup>C, DEPT, COSY, NOESY, HSQC, HMBC). According to the <sup>1</sup>H and <sup>13</sup>C chemical shift and coupling data (Table 2) compound **3** contains a methoxyl instead of a hydroxyl group at 4-C as compared with **4**.

Analysis of the <sup>13</sup>C and DEPT spectra of **3** revealed the presence of 4 CH<sub>3</sub>, two CH<sub>2</sub>, 8 CH groups and 13 quaternary carbon atoms. The assignment of the hexanose spin system was easily performed by analysis of the COSY spectrum. The stereochemistry of the anomeric proton was settled by its small vicinal coupling constant with 2'-H ( $J_{1',2'} = 1.4$  Hz) which is only explainable by an equatorial position. Moreover, a NOE effect between 3'-H and 5'-H, visible in the NOESY spectrum of **3**, suggested their synaxial orientation. The orientation of 4'-H was proven by its large antiperiplanar coupling to 5'-H ( $J_{4',5'} = 9.1$  Hz). Comparison of the chemical shifts and coupling constants of the sugar unit of **3** with the published data of **1** and **2** resulted in only minor differences, suggesting the identity of the sugar units in these molecules. Apart from the sugar only a small aliphatic spin system CH(O)CH<sub>2</sub> and two *ortho*-coupled aromatic protons gave rise to cross peaks in the COSY spectrum of **3**.

<sup>3</sup>J<sub>C,H</sub> long-range couplings as detectable in the HMBC spectra of **3** and **4** (Fig. 2) were of pivotal importance for the assignment of their chemical constitutions<sup>2)</sup>.

The attachment of the sugar moiety at C-7 of the aglycon was confirmed by the HMBC cross peak of 7-H to C-1' and *vice versa*. Heteronuclear long-range correlations of 14-CH<sub>3</sub> to C-8 and C-10 as well as of 7-H and 10-CH<sub>2</sub> to C-6, C-6a, C-10a and C-11 proved the molecular frame in the eastern part of the aglycon. Accordingly, the HMBC cross peaks of 1-H to the quinone carbonyl C-12, C-4a and C-12a were key correlations in the Western part of the molecule. No long range correlation was visible to C-5, but comparison of the <sup>13</sup>C NMR

chemical shifts of **3** with **1** and **2**, respectively, settled unambiguously the identity of its aglycon. Due to the similar values of the coupling constants  $^3J_{7H,8H_A}$  and  $^3J_{7H,8H_B}$  (5.8 - 6 Hz) the relative configuration of the substituent at C-7, respectively, could not be proposed from the proton coupling data. The only information which is compatible with the relative configuration of **3** as shown in Fig. 1 concerns the observed strong Nuclear Overhauser effect between 3'-H and 14-CH<sub>3</sub> and the absence of an NOE between 7-H and 14-CH<sub>3</sub>. The same feature was observed with coproduced mutactimycin (**2**) and 4-O,3'-O-didemethyl mutactimycin (**4**) from *Streptomyces sp.* attesting to the adequate relative stereochemistry of these anthracycline metabolites (see c.f. Fig. 1).

The structures of **3** and **4** thus are related to SO-75R1 (**1**)<sup>10,11</sup> and mutactimycin (**2**)<sup>9</sup>. These anthracycline-type antibiotics are discernible from the new compounds **3** and **4** by one and, respectively, two additional methyl substituents (Fig. 1).

It appears as a remarkable fact that different representatives of the *Actinomycetes* belonging to the genus *Nocardia* and *Streptomyces* are capable of producing the same anthracycline antibiotics.

### Biological properties

*In vitro* antibacterial activities of 3'-O-demethyl mutactimycin (**3**) against 5 species of bacteria in comparison with those of SO-075R1 (**1**) and mutactimycin (**2**) are shown in Table 3. **3** was only moderately active against Gram-positive bacteria, but inactive against Gram-negative bacteria, but the activity was less than that of SO-075R1 (**1**)<sup>10</sup> and mutactimycin A (**2**)<sup>9</sup> as shown in Table 1.

Most of the other Gram-positive bacteria tested were inhibited at the concentration between 12.5 and 50.0 µg/ml.

*In vitro* cytotoxic activities of **3** were studied against P388 and L1210 leukemias and HeLA cells. **3** displayed moderate activity against these three cell lines and the IC<sub>50</sub> values were 9.6, > 25.0 and 20.0 µg/ml, respectively.

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**Figure legends**

Fig. 1: Chemical constitutions of SO-75R (**1**), mutactimycin (**2**), 3'-O-demethyl mutactimycin (**3**) and 4-O,3'-O-didemethyl mutactimycin (**4**)

Fig. 2: Diagnostic  $^1\text{H}$ ,  $^{13}\text{C}$ -long range and NOE couplings of **3** as shown by the HMBC and NOESY spectra ( $\rightarrow$  HMBC;  $\leftrightarrow$ NOESY)

Table 1: Physico-chemical properties of **3** and **4**

	<b>3</b>	<b>4</b>
Appearance	red micro crystals	red micro crystals
Molecular weight	530	516
Chemical formula	C <sub>27</sub> H <sub>30</sub> O <sub>11</sub>	C <sub>26</sub> H <sub>28</sub> O <sub>11</sub>
HRFAB-MS [M+H] <sup>+</sup>	<i>m/z</i> 531.1888 (calcd. 531.1908)	<i>m/z</i> 517.1807 (calcd. 517.1860)
UV-VIS ( $\lambda_{\max}$ ; MeOH; nm)	460	460
IR (in KBr, $\lambda_{\max}$ , cm <sup>-1</sup> )	983, 1044, 1133, 1205, 1236, 1270, 1363, 1406, 1442, 1573, 1614, 1673 (CO), 2965, 3420 (OH)	980, 1047, 1070, 1093, 1131, 1185, 1206, 1290, 1314, 1359, 1400, 1428, 1625, 1649, 1677 (CO), 2970, 3430 (OH)
$[\alpha]_D$ (c = 0.29, MeOH)	+ 44.4	n.d.*
TLC (R <sub>f</sub> ; CHCl <sub>3</sub> /MeOH 9/1)	0.19	0.19
HPLC t <sub>ret</sub> (min, RP <sub>18</sub> , 5 $\mu$ m, acetonitrile/H <sub>2</sub> O; 35:65)	7.10	10.40

\* not determined

**Table 2:** Assignment of  $^1\text{H}$  and  $^{13}\text{C}$  data of **3** and **4** (in DMSO; chemical shifts ( $\delta$ ) in ppm relative to internal TMS; multiplicity in parentheses; coupling constants in Hz). Abbreviations: s: singlet, d: doublet, t: triplet, m: multiplet, q: quarter, br: broad)

Carbon atom no.	3		4	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$
1	122.8	8.03 (d; 7.8)	118.5	7.74 (d; 7.6)
2	137.2	7.83 (d; 7.8)	137.6	7.74 (d; 7.6)
3	141.7	-	135.0	-
4	159.3	-	160.1	-
4a	125.4	-	126.0	-
5	186.5	-	186.1	-
5a	110.5	-	110.4	-
6	156.9	-	157.0	-
6a	136.2	-	138.8	-
7	72.4	4.9 (t; 5.8)	72.3	4.9 (t, 5.8)
8	42.5 H <sub>A</sub> H <sub>B</sub>	2.14 (dd; 13.2;6.0) 1.94 (dd; 13.2;5.4)	42.6 H <sub>A</sub> H <sub>B</sub>	2.14 (dd; 13.5;6.0) 1.94 (dd; 13.5;5.4)
9	66.9	-	67.0	-
10	37.5 H <sub>A</sub> H <sub>B</sub>	2.67 (d; 17.9) 2.84 (d; 17.9)	37.6 H <sub>A</sub> H <sub>B</sub>	2.67 (d; 18.1) 2.83 (d; 18.1)
10a	137.2	-	137.6	-
11	157.7	-	157.7	-
11a	110.8	-	109.9	-
12	186.0	-	187.0	-
12a	133.0	-	133.0	-
13	16.3	2.39 (s)	15.8	2.32 (s)
14	28.9	1.3 (s)	29.0	1.30 (s)
15	60.7	3.84 (s)	-	-
1'	103.7	5.07 (d; 1.4)	103.5	5.05 (d; 1.5)
2'	70.54	3.62 (m,br)	70.5	3.62 (m,br)
3'	70.65	3.29 (m,br)	70.7	3.31 (m,br)
4'	71.8	3.23 (m,br)	71.8	3.13 (m,br)
5'	69.4	3.59 (dq; 9.1; 6.2)	69.6	3.58 (dq; 9.0;6.2)
6'	17.8	1.18 (d; 6.2)	17.8	1.18 (d; 6.2)
4-OH	-	-	-	12.41
6-OH	-	13.30	-	13.50
11-OH	-	14.02	-	12.91
3'-OH	-	4.77 (br)	-	4.77 (br)
4'-OH	-	4.73 (d; 5.3)	-	4.73 (d; 5.3)

**Table 3:** Antimicrobial activity of **3**, SO-075R1 (**1**) and mutactimycin (**2**)  
Brain heart infusion broth was used

Test organisms	MIC (mg/ml)		
	<b>1</b>	<b>2</b>	<b>3</b>
<i>Micrococcus luteus</i> IFM2066	3.13	3.13	25.0
<i>Staphylococcus aureus</i> 209P	1.56	3.13	25.0
<i>Escherichia coli</i> NIH JC2	> 100.0	100.0	> 100.0
<i>Bacillus subtilis</i> PCI189	3.13	6.25	50.0
<i>Corynebacterium xerosis</i> IFM	156	313	125