Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2017

www.elsevier.com/locate/jbiosc

Nanaomycin H: A new nanaomycin analog

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Received 13 December 2016; accepted 17 January 2017
Available online xxx

Physicochemical screening identified a new nanaomycin analog, nanaomycin H, which was isolated from a culture broth of *Streptomyces rosa* subsp. *notoensis* OS-3966. This microorganism is already known to produce seven nanaomycin compounds, (nanaomycin A to G). Structural elucidation of nanaomycin H showed it to be a pyranonaphthoquinone with a mycothiol moiety. A *N*-acetylcysteine *S*-conjugate of nanaomycin H, without α-glucosamine linked to *myo*-inositol moiety, mercapturic acid derivative, was also detected in the same culture broth. Mercapturic acid derivatives of secondary metabolites are known to be produced for xenobiotic metabolism outside microbial cells. Mycothiol acts as a detoxifier to help prevent cell damage from factors such as oxidative stress. The production of $O_2^{-}\cdot$ generated by reduction of nanaomycin A is correlated with antibacterial activity. Mycothiol-containing nanaomycin H proved to be markedly decreased in $O_2^{-}\cdot$ and did not express any notable antimicrobial activity. It is suggested that nanaomycin H is produced in the detoxification process.

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[**Key words:** Nanaomycin H; Physicochemical screening; *Streptomyces* spp.; New nanaomycin analog]

Nanaomycins A–E were discovered from a culture broth of *Streptomyces rosa* subsp. *notoensis* OS-3966 at the Kitasato Institute in 1975 (1–4). Nanaomycin A displays potent antimicrobial activity, especially, against *Trichophyton* spp. and *Mycoplasma* spp. (1). Production of the superoxide anion (O_2^{-}) generated by the reduction of nanaomycin A is known to inhibit the growth of bacteria (5). The therapeutic effect of nanaomycin A on dermatophytosis (also known as ringworm) was established using a guinea pig model (6) and the compound has been used as a topical therapeutic drug for animal dermatophytosis as it improves lesions and prevents fungal growth in infected tissues. Two further analogs, nanaomycin F and G, were recently discovered by physicochemical (PC) screening (7). The structure of nanaomycin F was found to be a 4a-hydroxyl analog of nanaomycin B, while nanaomycin G possesses a skeleton with 1-indanone infused with a tetrahydropyran ring. We found a new analog with a mycothiol moiety, nanaomycin H, in the same culture broth producing nanaomycins F and G. Here, we report the fermentation, isolation, structural elucidation of nanaomycin H and an initial assay of the compound's bioactivity.

MATERIALS AND METHODS

General experimental procedures Nuclear magnetic resonance (NMR) spectra were measured using a Varian XL-400 and INOVA 600 (Varian, Palo Alto, CA, USA), with ^{1}H-NMR at 400 or 600 MHz and ^{13}C-NMR at 100, 125 or 150 MHz in CD_3OD. The chemical shifts are expressed in ppm and are referenced to residual CD_2HOD (3.31 ppm) in the ^{1}H-NMR spectra and CD_3OD (49 ppm) in the ^{13}C-NMR spectra. IR spectra (KBr) were taken on a Horiba FT-710 Fourier transform IR spectrometer (Horiba Ltd., Kyoto, Japan). UV spectra were measured with a Hitachi U-2810 spectrophotometer (Hitachi, Tokyo, Japan). Optical rotation was measured on a JASCO model DIP-1000 polarimeter (Jasco, Tokyo, Japan).

Nanaomycin H producing fermentation The *S. rosa* subsp. *notoensis* OS-3966 strain was cultured and maintained on agar slants, consisting of 1.0% starch, 0.3% NZ amine, 0.1% yeast extract, 0.1% meat extract, 1.2% agar and 0.3% $CaCO_3$. A loop of spores of strain OS-3966 was inoculated into 100 ml of the seed medium, consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% $CaCO_3$ (adjusted to pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (210 rpm) at 27°C for 3 days. A 1-ml portion of the seed culture was transferred to Erlenmeyer flasks (total 50) each containing 100 ml of starch medium, consisting of 2% soluble starch, 0.5% glycerol, 1.0% defatted wheat germ, 0.3% meat extract, 0.3% dry yeast and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization) and fermentation was carried out on a rotary shaker (210 rpm) at 27°C for 6 days.

Purification of nanaomycin H Nanaomycin H was included in the same fraction as nanaomycin F and G (7). Isolation of nanaomycin H was guided by physico-chemical properties such as its *m/z* 805 and UV spectrum (λ_{max} 231, 270 and 353 nm), using liquid chromatography/UV (LC/UV) and liquid chromatography/mass spectrometry (LC/MS) equipment (Fig. 1). The 6-day-old culture broth (5 L) of strain OS-3966 was added to an equivalent amount of EtOH. The EtOH extract was concentrated *in vacuo* and suspended with water. The sample was passed through a column of Diaion HP-20 (100 i.d. × 250 mm; Mitsubishi Chemical, Tokyo, Japan), previously equilibrated with water. After washing with water, the fraction containing nanaomycin H was eluted with 100% MeOH. The whole eluate was concentrated *in vacuo*. This material (4.7 g) was subjected to column chromatography on silica gel FL100D (60 i.d. × 200 mm; Fuji Silysia Chemical, Aichi, Japan), and eluted with a stepwise gradient of $CHCl_3$-MeOH (100:0, 100:1, 50:1, 10:1, 1:1 and 0:100 (v/v)), to give six fractions. The 1:1 and 0:100 fractions were combined and concentrated *in vacuo*. The material (1774 mg) was subjected on an ODS column (40 i.d. × 150 mm; Senshu Scientific, Tokyo, Japan) previously equilibrated with water. After washing with water, the

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http://dx.doi.org/10.1016/j.jbiosc.2017.01.011

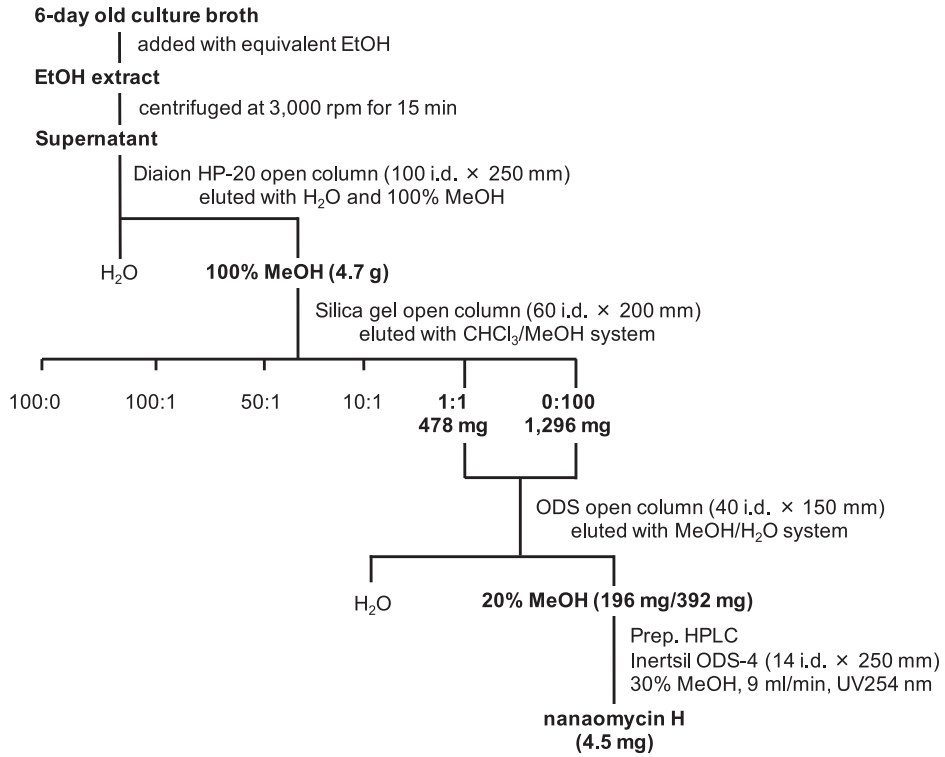

FIG. 1. Purification scheme for nanaomycin H.

fraction containing nanaomycin H was eluted with 20% MeOH and concentrated *in vacuo* to yield 392 mg. Half of this fraction (196 mg) was dissolved in a small amount of MeOH, and the compound was purified by preparative HPLC on an Inertsil ODS-4 column (14 i.d. × 250 mm; GL Sciences, Tokyo, Japan) with 30% MeOH at 9.0 ml/min and subsequently detected at UV 254 nm. The yield of nanaomycin H was 4.5 mg.

MS/MS analysis The resultant sample was injected into the electrospray ion source of a QSTAR Elite ESI quadruple time-of-flight mass spectrometer (AB Sciex, Framingham, MA, USA) coupled to an Agilent 1200 series (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was undertaken on an Inertsil ODS-4 (3.0 × 250 mm; GL Sciences) at 40°C. With regard to gradient elution, solvent A was water with 2 mM ammonium acetate, and solvent B was methanol with 2 mM ammonium acetate. Gradient elution was 0–30 min and 5–100% B. Flow rate was 0.5 ml/min, the injection volume was 5 μl, and UV detection was carried out by a photodiode array detector. ESI-MS was recorded for 30 min in the *m/z* region from 100 to 2000 Da as follows: ion spray capillary voltage of 5500 V, curtain gas flow rate 30 L/min, nebulizer gas flow rate 50 L/min, de-clustering potential of 50 V, focusing potential of 250 V, temperature of 450°C, and detector voltage of 2300 V. LC-MS analysis was undertaken by high-resolution ESI-MS ($R \geq 10{,}000$; tolerance for mass accuracy: 5 ppm). The collision energy was swept at 20, 35 and 50 eV for MS/MS analysis.

TABLE 1. Physico-chemical properties of nanaomycin H.

		Nanaomycin H
Appearance		Brown powder
Molecular formula		$C_{33}H_{44}N_2O_{19}S$
Molecular weight		804.7700
ESI-MS (*m/z*)	Calcd.	805.2337
	Found	805.2307
$[\alpha]_D^{25.7}$ (*c* 0.1, MeOH)		−76.75
UV λ_{max}^{MeOH} nm (ε)		231 (40200), 270 (16643, sh), 353 (13346)
IR ν (KBr) cm^{-1}		3412, 1637
Soluble		Water
		Methanol
		Ethanol
Insoluble		Acetone
		Hexane
Melting point		190–192°C (decomp.)

TABLE 2. NMR spectroscopic data (CD_3OD, ^1H; 400 MHz, ^{13}C; 100 MHz) of nanaomycin H.

	δ_C, *mult.*		δ_H (int., mult., *J* in Hz)	HMBC
1	75.5	CH	4.220 (1H, q, 8.0)	C-3, C-4a, C-10a, C-11
3	64.6	CH	4.700 (1H, ddd, 6.0, 6.0, 9.5)	C-4a, C-12, C-13
4	31.5	CH_2	2.053 (1H, d, 12.0)	C-4a, C-10a
			2.278 (1H, dd, 9.5, 12.0)	C-3, C-4a, C-5, C-12
4a	60.1	C		
5	190.6	C		
5a	134.7	C		
6	120.0	CH	7.570 (1H, dd, 1.0, 8.0)	C-5, C-8, C-9a, (C-10)
7	137.9	CH	7.698 (1H, dd, 8.0, 8.5)	C-5a, (C-5), C-9
8	124.6	CH	7.242 (1H, dd, 1.0, 8.5)	C-6, C-9, C-9a, (C-10)
9	162.8	C		
9a	116.0	C		
10	200.5	C		
10a	77.5	C		
11	16.2	CH_3	1.752 (3H, d, 8.0)	C-1, C-10a
12	41.5	CH_2	2.612 (2H, d, 6.0)	C-3, C-4, C-13
13	175.6	C		
1′	172.0	C		
2′	54.2	CH	4.440 (1H, dd, 5.0, 8.5)	C-1′, C-3′, C-5′
3′	33.6	CH_2	2.433 (1H, dd, 8.5, 12.5)	C-1′, C-2′, C-4a
			2.808 (1H, dd, 5.0, 12.5)	C-1′, C-2′, C-4a
5′	173.6	C		
6′	22.6	CH_3	1.907 (3H, s)	C-5′
1″	100.1	CH	5.042 (1H, d, 3.5)	C-5″, C-3″, C-1‴
2″	55.6	CH	3.850 (1H, dd, 3.5, 10.0)	C-1′, C-1″
3″	72.8	CH	3.719 (1H, dd, 9.5, 10.0)	C-2″, C-5″
4″	72.3	CH	3.309 (1H, dd, 9.5, 10.0)	C-3″, C-5″, C-6″
5″	74.4	CH	3.819 (1H, ddd, 2.5, 7.0, 10.0)	C-1″, C-3″, C-6″
6″	62.8	CH_2	3.645 (1H, dd, 7.0, 12.0)	C-5″
			3.850 (1H, dd, 2.5, 12.0)	C-5″
1‴	80.4	CH	3.430 (1H, dd, 3.0, 10.0)	C-1″, C-6‴
2‴	73.4	CH	4.118 (1H, dd, 2.5, 3.0)	C-1‴
3‴	73.2	CH	3.340 (1H, dd, 2.5, 9.5)	
4‴	74.0	CH	3.592 (1H, dd, 9.5, 9.5)	C-2‴, C-5‴
5‴	76.4	CH	3.151 (1H, dd, 9.5, 9.5)	C-4‴
6‴	74.1	CH	3.772 (1H, dd, 9.5, 10.0)	C-2‴

Parentheses of HMBC means ^4J coupling.

Please cite this article in press as: Nakashima, T., et al., Nanaomycin H: A new nanaomycin analog, J. Biosci. Bioeng., (2017), http://dx.doi.org/10.1016/j.jbiosc.2017.01.011

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