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BRIEF COMMUNICATION

Quinomycins H1 and H2, new cytotoxic antibiotics from *Streptomyces* sp. RAL404

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Abstract

Two new cytotoxic antibiotics designated quinomycins H1 (**2**) and H2 (**3**) were isolated from the culture broth of *Streptomyces* sp. RAL404. The molecular formula of both compounds was established as $C_{52}H_{65}N_{11}O_{13}S_2$ by electrospray ionization mass spectrometry (ESI-MS). Their structures were determined as echinomycin (**1**) derivatives containing a 3-hydoxyquinaldic acid molecule in place of one of the two quinoxaline-2-carboxylic acid chromophores. Quinomycins H1 (**2**) and H2 (**3**) showed selective cytotoxicity against RG-E1-4 cells bearing the adenovirus oncogenes with IC_{50}s of 11 nM and 12 nM, respectively.

The retinoblastoma tumor suppressor protein (pRB) is known to be inactivated in a wide variety of human cancers and to play a significant role in cell-cycle and apoptosis control [1, 2]. Adenovirus oncogene-transformed cells such as RG-E1-4 cells [3] are an appropriate model for pRB-inactivated cancers, because the E1A gene inactivates pRB and the E1B gene encodes two apoptosis suppressor proteins [4, 5]. In the course of our screening for microbial metabolites, an actinomycete strain RAL404 was found to produce three compounds with selective cytotoxicity against RG-E1-4 cells. One of the metabolites was identified as echinomycin (**1**, Fig. 1a) by its ^{1}H and ^{13}C NMR spectra [6]. The other two were new compounds and named quinomycins H1 (**2**) and H2 (**3**) (Fig. 1a).

Strain RAL404 was isolated from a soil sample collected at Kazo, Saitama Prefecture, Japan. The 16S rRNA gene fragment was amplified by PCR and sequenced [7]. The sequence displayed high similarity to *Streptomyces cyaneus* NRRL B-2296 (99.3%) and *Streptomyces indigocolor* NRRL B-12366 (99.3%). Accordingly, strain RAL404 was identified as a member of the genus *Streptomyces* and named *Streptomyces* sp. RAL404. The 16S rRNA gene sequence of *Streptomyces* sp. RAL404 reported here has been deposited in the GenBank, DDBJ, and EMBL databases under accession number LC383798.

The producing organism was cultivated in 500-mL Erlenmeyer flasks containing 100 mL of a medium (pH 6.2 before autoclaving) consisting of 2.5% glucose, 1.5% soybean meal, 0.2% dry yeast, and 0.4% calcium carbonate. The fermentation was carried out at 27 °C for 6 days on a rotary shaker.

The whole broth (2L) was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate. The extract was applied to a silica gel column, which was washed with chloroform and eluted with chloroform-methanol (100:1). The eluate was subjected to HPLC on an ODS column (PEGASIL ODS SP100, 20 i.d. × 250 mm) with 75% aqueous methanol to obtain three active fractions. The major component was further purified by ODS-HPLC using the same column with 70% aqueous methanol to yield 7.0 mg of echinomycin (**1**). The two minor fractions were separately purified by ODS-HPLC using the same column with 75% aqueous methanol. The isolation from 26-L fermentation yielded pale yellow powders of **2** (21.0 mg) and **3** (14.6 mg).

The physico-chemical properties were as follows. Quinomycin H1 (**2**): M.P. 197-200 °C; $[\alpha]_D^{22}$-262 (*c* 1.01, chloroform); ESI-MS *m/z* 1138.40886 ($[M + Na]^+$, calcd.

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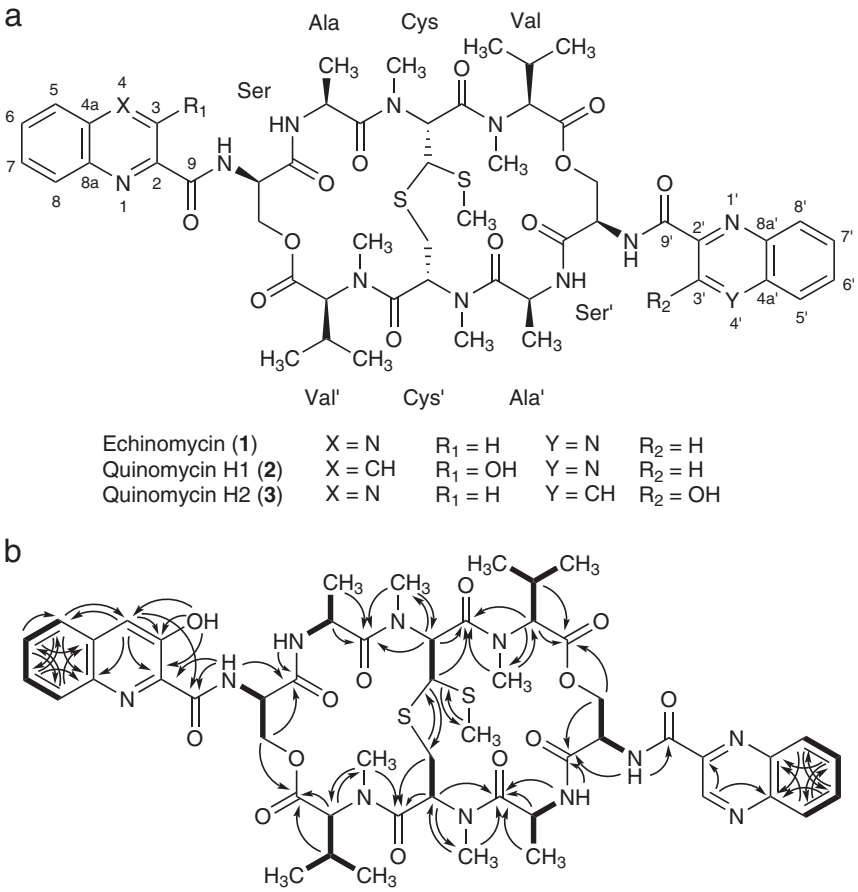

Fig. 1 **a** Structures of echinomycin (**1**) and quinomycins H1 (**2**) and H2 (**3**). **b** NMR analysis of quinomycin H1 (**2**). Bold lines show ^{1}H–^{1}H spin networks and arrows indicate ^{1}H–^{13}C long-range correlations

for $C_{52}H_{65}N_{11}O_{13}S_2$, 1138.40970); UV-$\lambda_{max}$ nm (ε) 233 (51200), 241 (50500), 312 (8600), 355 (4800) in MeOH; IR ν_{max} 3375, 1740, 1647 cm^{-1}. Quinomycin H2 (**3**): M.P. 205–208 °C; $[\alpha]_D^{23}$-261 (*c* 0.706, chloroform); ESI-MS *m/z* 1138.40775 ($[M + Na]^+$, calcd. for $C_{52}H_{65}N_{11}O_{13}S_2$, 1138.40970); UV-$\lambda_{max}$ nm (ε) 233 (51700), 241 (48700), 312 (8600), 355 (5000) in methanol; IR ν_{max} 3386, 1739, and 1651 cm^{-1}.

The molecular formula of both **2** and **3** was established as $C_{52}H_{65}N_{11}O_{13}S_2$ by ESI-MS. The ^{13}C and ^{1}H NMR data in $CDCl_3$ for **2** and **3** are summarized in Table 1. All one-bond ^{1}H-^{13}C connectivities were confirmed by an HMQC experiment.

COSY and HMBC analyses of **2** identified eight amino acid residues including serine (Ser and Ser′), alanine (Ala and Ala′), *N*,*S*-dimethylcysteine (Cys), *N*-methylcysteine (Cys′) and *N*-methylvaline (Val and Val′) as shown in Fig. 1b. The chemical shift similarity between **1** and **2** indicated the same stereochemistry and the same peptide structure linked with six amide, two ester and one thioeter bonds, which were confirmed by ^{1}H-^{13}C long-range correlations (Fig. 1b). Echinomycin (**1**) contains two molecules of quinoxaline-2-carboxylic acid (QXA) as chromophores. NMR data identified one of the chromophores in **2** as QXA (Table 1 and Fig. 1b). In the remaining part, four *ortho*-coupled aromatic protons (H-5, H-6, H-7, and H-8) exhibited long-range couplings to six aromatic carbons (C-4a, C-5, C-6, C-7, C-8, and C-8a) to construct an *ortho*-disubstituted benzene ring. This substructure was extended to a fused aromatic ring consisting of seven carbons by long-range correlations from an aromatic proton (H-4) to C-2, C-3, C-5, and C-8a. A phenolic hydroxy proton (δ 11.29) located on C-3 (δ 153.4) revealed the sequence of C-2, C-3, and C-4 with long-range correlations. A nitrogen atom was required to be placed between C-2 and C-8a due to their chemical shifts (δ 141.2 and 133.5) and the molecular formula. The remaining carbonyl carbon (C-9) displayed a four-bond coupling with H-4 to identify a 3-hydroxyquinaldic acid (HQA) moiety as the second chromophore, which was consistent with a UV bathochromic shift (from 233 to 245 nm) in alkaline solution of **2**. Amide bonds were formed between HQA and Ser and between QXA and Ser′ by ^{1}H-^{13}C long-range couplings from the NH protons to establish the structure of **2** as shown in Fig. 1a.

The structure of **3** was similarly determined and the two chromophores are exchanged between **2** and **3** (Fig. 1a). Thiocoraline [8], UK-63,052 complex [9], SW-163D and SW-163E [10] have been reported as quinomycin group

Table 1 ^{13}C (100 MHz) and ^{1}H (400 MHz) NMR data for **2** and **3** in $CDCl_3$

No.		2 δ_C	δ_H (J in Hz)	No.		3 δ_C	δ_H (J in Hz)
HQA	2	133.5		HQA	2′	133.5	
	3	153.4			3′	153.4	
	4	120.9	7.61 s		4′	120.8	7.56 s
	4a	132.1			4a′	132.1	
	5	126.5	7.66 m		5′	126.5	7.63 m
	6	128.8	7.44 t (7.0)		6′	128.7	7.40 m
	7	127.4	7.40 t (7.0)		7′	127.3	7.39 m
	8	128.8	7.64 m		8′	128.9	7.66 m
	8a	141.2			8a′	141.2	
	9	168.9			9′	169.0	
	3-OH		11.29 br s		3′-OH		11.27 br s
Ser	1	167.3		Ser	1	167.5	
	2	51.9	4.92 m		2	52.2	4.98 m
	3	64.3	4.74 m, 4.64 m		3	64.5	4.76 m, 4.66 m
	2-NH		8.99 d (8.0)		2-NH		8.57 d (8.0)
Ala	1	173.4		Ala	1	173.6	
	2	46.5	4.84 m		2	46.5	4.83 dq (7.5, 7.0)
	3	17.1	1.39 d (7.0)		3	17.0	1.38 d (7.0)
	2-NH		6.80 br d (7.5)		2-NH		6.85 br d (7.5)
Cys	1	168.8		Cys	1	168.9	
	2	59.8	6.51 d (9.0)		2	60.0	6.48 d (9.0)
	3	51.6	4.91 d (9.0)		3	51.6	4.91 d (9.0)
	S-Me	15.3	2.09 s		*S*-Me	15.3	2.09 s
	N-Me	32.1	3.00 s		*N*-Me	32.1	3.00 s
Val	1	171.1		Val	1	171.1	
	2	62.6	5.20 d (10.0)		2	62.8	5.18 d (10.0)
	3	27.7	2.36 m		3	27.9	2.37 m
	4	20.4	1.09 d (6.0)		4	20.4	1.09 d (7.0)
	5	19.0	0.91 d (7.0)		5	19.2	0.92 d (7.0)
	N-Me	31.0	3.13 s		*N*-Me	31.1	3.16 s
QXA	2′	142.3		QXA	2	142.2	
	3′	143.5	9.58 s		3	143.5	9.58 s
	4a′	144.0			4a	144.0	
	5′	129.6	8.14 d (8.5)		5	129.6	8.18 d (8.5)
	6′	131.8	7.78 t (8.0)		6	131.9	7.77 t (7.5)
	7′	130.8	7.67 m		7	130.9	7.62 m
	8′	129.2	7.83 d (8.0)		8	128.9	7.67 m
	8a′	139.9			8a	139.8	
	9′	163.9			9	163.8	
Ser′	1	167.3		Ser′	1	166.9	
	2	53.4	4.84 m		2	53.3	4.76 m
	3	64.9	4.72 m, 4.69 m		3	64.7	4.76 m, 4.67 m
	2-NH		8.78 d (7.0)		2-NH		9.14 d (6.0)
Ala′	1	173.2		Ala′	1	173.2	
	2	46.2	4.95 dq (7.5, 6.5)		2	46.1	4.98 m
	3	18.1	1.41 d (6.5)		3	18.2	1.42 d (7.5)
	2-NH		7.01 d (7.5)		2-NH		7.06 d (8.0)
Cys′	1	170.3		Cys′	1	170.2	
	2	53.5	6.08 br d (11.0)		2	53.4	6.08 br d (10.5)
	3	27.3	3.43 dd (16.0, 2.0), 2.86 dd (16.0, 11.0)		3	27.2	3.42 dd (16.0, 2.0), 2.86 dd (16.0, 11.0)
	N-Me	29.8	3.01 s		*N*-Me	29.8	3.01 s
Val′	1	170.7		Val′	1	170.8	
	2	62.0	5.12 d (10.0)		2	61.9	5.14 d (10.0)
	3	27.9	2.30 m		3	27.9	2.29 m
	4	20.3	1.08 d (6.0)		4	20.4	1.08 d (7.0)
	5	18.9	0.87 d (7.0)		5	18.8	0.88 d (7.0)
	N-Me	31.6	3.22 s		*N*-Me	31.6	3.21 s

antibiotics with 3-hydoxyquinaldic acid chromophores, although they possess two molecules of 3-hydoxyquinaldic acid. Quinomycins H1 (**2**) and H2 (**3**) are the first example of the quinomycin family containing both quinoxaline-2-carboxylic acid and 3-hydoxyquinaldic acid chromophores, although biosynthetic quinomycins produced by feeding experiments have been reported to possess quinaldic acid or 6-methylquinaldic acid in place of one of the quinoxaline-
2-carboxylic acid chromophores [11].

The cytotoxic activities of the echinomycin derivatives from *Streptomyces* sp. RAL404 were evaluated by the MTT method [12] using RG-E1-4 transformed rat glia cells and 3Y1 normal rat fibroblasts. The cells were plated and incubated for 72 h with various concentrations of samples. After the cells were treated with 0.5 mg/mL of 3-(4′5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 h at 37 °C, the relative cell number was measured as absorbance at 540 nm. The IC_{50} values against RG-E1-4 and 3Y1 cells were 16 and 160 nM for **1**, 11 and 44 nM for **2**, and 12 and 49 nM for **3**, respectively. Interestingly, echinomycin was more selective than the other derivatives, although the three metabolites showed similar cytotoxicity against transformed cells. This result suggests that the structures of chromophores influence tumor selectivity. This structure-activity relationship might contribute to design more effective quinomycin analogs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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