ORIGINAL ARTICLE

Cytotoxic rearranged angucycline glycosides from deep sea-derived *Streptomyces lusitanus* SCSIO LR32

Xiangcheng Zhu¹, Yanwen Duan¹, Zhaomeng Cui², Zhen Wang², Zengxia Li², Yun Zhang³, Jianhua Ju³ and Hongbo Huang³

Two new rearranged linear angucycline glycosides, designated grincamycins G and H (1 and 2), together with three known congeners P-1894B (vineomycin A₁, 3), saquayamycin B (4) and vineomycin B₂ (5), were obtained from marine-derived actinomycete *Streptomyces lusitanus* SCSIO LR32. The structures of 1 and 2 were elucidated by MS, 1D and 2D NMR techniques. Compounds 2–5 showed significant inhibitory effect on Jurkat T-cell proliferation with IC₅₀ values of 3.0, 0.011, 0.037 and 0.3 μ M, respectively.

The Journal of Antibiotics (2017) 70, 819–822; doi:10.1038/ja.2017.17; published online 22 February 2017

INTRODUCTION

Angucycline is a large group of secondary metabolites originating from the fermentation extract of different *actinomycetes*, mostly from the species of *Streptomyces*.¹ Angucycline compounds possess various biological and pharmacological properties, including cytotoxicity and antitumor, antibacterial and antiviral activities, as well as enzyme-inhibition activities.^{1,2} Structurally, classic angucyclines contain an angular benz[a]anthracene scaffold and non-classic congeners with rearranged linear tetracyclic or tricyclic core units, and most of angucyclines exist as glycosides in nature. The observed carbohydrates in angucycline glycoside usually are deoxy sugars, such as D-olivose, L- or D-rhodinose, L-aulose, L-cinerulose A, L-cinerulose B, D-kerriose and L-amicetose.^{1–3} Moreover, the typical O-glycoside and C-glycoside with different types of sugars or different numbers of sugar chains have brought broad chemical diversities to natural angucycline compounds.

During our efforts to screen for novel antitumor and antimicrobial antibiotics, we reported non-classic angucycline glycosides designated as grincamycins (GCNs) B–E from deep sea-derived actinomycete *Streptomyces lusitanus* SCSIO LR32.⁴ Subsequently, the biosynthetic gene cluster of GCN cloned from *S. lusitanus* SCSIO LR32 was heterologously expressed in *Streptomyces coelicolor* M512, resulting in the isolation of P-1894B as a predominant product.⁵ The structural difference between P-1894B and GCN is that the terminal sugars L-cinerulose A in GCN were replaced by L-aulose units in P-1894B. In addition, the LC-MS analyses of metabolite derived from the strain SCSIO LR32 cultivated in a modified AM₂ medium have detected two peaks with the molecular weight of 934.4, which were 4 units fewer

than that of grincamycin and grincamycin B (molecular weight 938.4), which presumably could be P-1894B and vineomycin B₂. Based on these preliminary data, strain SCSIO LR32 was further fermented on an 80 l scale. Subsequent purification of culture extract resulted in identification of five angucycline glycosides (1–5) containing L-aulose as terminal on the sugar chain (Figure 1). Herein we report the fermentation, isolation, structure elucidation and cytotoxic activities of these compounds.

RESULTS

Structure elucidation

Compound 1 was isolated as a yellowish powder. (–)HR-ESI-MS of 1 revealed a quasimolecular ion peak at 801.2776, in combination with ¹H and ¹³C NMR data, establishing the molecular formula as C₄₃H₄₆O₁₅. The UV spectrum of 1 showed characteristic absorption bands at 214, 243, 265 and 443 nm. The ¹³C NMR spectrum disclosed five carbonyls at δ_C 209.5, 198.2, 197.0, 189.9 and 189.8, 14 aromatic carbons in the downfield sp² region and 24 aliphatic carbons in the upfield area (Table 1). The ¹H NMR spectroscopic signals at δ_H 13.10 (2H, br s, 1-OH and 5-OH) suggested two hydrogen-bonded hydroxy groups. A pair of *ortho*-coupled aromatic protons at δ_H 8.02 (d, 8.0 Hz, H-8) and 7.90 (d, 8.0 Hz, H-7) and a single aromatic proton at δ_H 8.34 (s, H-12) were observed, which were almost consistent with those in grincamycin E⁴; inferred 1 had a linear tetracyclic anthraquinone aglycone. The HMBC correlations of H-7/C-6, C-9, C-10a, H-8/C-9, C-10, C-6a, H-12/C-1, C-4a, C-5a, C-11 and of H-2/C-1, C-4 confirmed the existence of the linear tetracyclic anthraquinone unit (Figure 2). The Me-13 was attached at

¹Xiangya International Academy of Translational Medicine, Central South University, National Engineering Research Center of Combinatorial Biosynthesis for Drug Discovery, Changsha, China; ²Key Laboratory of Metabolism and Molecular Medicine, the Ministry of Education, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai, China and ³CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China

Correspondence: Dr H Huang, CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China.

E-mail: huanghb@scsio.ac.cn

Received 18 August 2016; revised 21 December 2016; accepted 18 January 2017; published online 22 February 2017

Figure 1 Chemical structures of compounds 1–5 from *Streptomyces lusitanus* SCSIO LR32.

C-3 based on the HMBC correlations of H₃-13/C-2, C-3, C-4. Additionally, four doublet methyl signals at δ_{H} 1.34, δ_{C} 18.4 (CH₃-6'), δ_{H} 1.28, δ_{C} 17.4 (CH₃-6''), δ_{H} 0.64, δ_{C} 18.4 (CH₃-6''') and δ_{H} 1.21, δ_{C} 16.4 (CH₃-6'''), as well as four anomeric methine signals at δ_{H} 5.07 (d, 9.5), δ_{C} 73.3 (CH-1'), δ_{H} 5.26 (d, 3.0), δ_{C} 93.2 (CH-1''), δ_{H} 5.17 (br s), δ_{C} 91.9 (CH-1''') and δ_{H} 5.13 (d, 3.4), δ_{C} 96.9 (CH-1'''), indicated the presence of four deoxysugars in 1. Detailed comparison showed that the ¹H and ¹³C NMR spectroscopic data of the sugar units were similar to those of grincamycin D,⁴ except that the signals of two methines in cinerulose A disappeared, while a set of signals ascribed to a double bond at δ_{H} 6.93 (10.3, 3.4 Hz), δ_{C} 146.0 (CH-2'''), δ_{H} 5.97 (10.0 Hz) and δ_{C} 128.1 (CH-3''') was observed. Moreover, the ¹³C NMR resonance of the carbonyl in cinerulose A was shifted upfield from δ_{C} 210.9 in grincamycin D to δ_{C} 198.2 in 1, indicating that the cinerulose A unit in grincamycin D was replaced by an aculose in 1. The HMBC correlations of H-1'''/C-4''' confirmed the existence of an aculose-(1→4)-rhodinosyl unit. The relative configurations of the anomeric carbons were defined as β for olivose and α for cinerulose B, rhodinosyl and aculose on the basis of the large coupling constant ($J=9.5$ Hz) of H-1' and the small coupling constants of H-1'', H-1''' and H-1''', respectively. Being similar to the conversion of grincamycin into grincamycin E under UV irradiation,⁴ saquayamycin B (4) could be transformed to 1 in the same manner (Supplementary Figure S15). Therefore, the absolute configuration of C-3 was established to be *R*, and the sugars were elucidated as β -D-olivose, α -L-cinerulose B, α -L-rhodinosyl and α -L-aculose, which were identical with those of saquayamycin B (4).⁶ Compound 1 was named grincamycin G.

Compound 2 was isolated as a dark-red powder. Its molecular formula was determined to be C₃₇H₃₈O₁₂ by analyses of (–)HR-ESI-MS, as well as ¹H and ¹³C NMR spectra, indicating 19 degrees of unsaturation. Detailed analyses of the ¹H and ¹³C NMR spectroscopic data showed that the structure of 2 was similar to those of

quanolirones and galtamycinone,^{7–9} which contained 1,5,10-trihydroxy-naphthacenequinone scaffold. The HMBC spectrum of 2 confirmed the position of a pair of *ortho*-coupled protons at δ_{H} 7.79 (d, 7.9 Hz, H-7) and 7.85 (d, 7.9 Hz, H-8) and three singlet protons at δ_{H} 6.97 (H-2), 7.55 (H-4) and 8.45 (H-12) (Figure 2). A methyl group (δ_{H} 2.43, δ_{C} 21.8, Me-13) was attached at C-3 based on the HMBC correlations from the methyl protons to C-2 (δ_{C} 116.1), C-3 (δ_{C} 141.6) and C-4 (δ_{C} 114.2). The ¹H and ¹³C NMR spectroscopic data also revealed three anomeric carbon signals at δ_{H} 4.80 (d, 11.9) and δ_{C} 70.5 (CH-1'), δ_{H} 4.94 (br s) and δ_{C} 97.3 (CH-1''), δ_{H} 5.35 (d, 3.1) and δ_{C} 94.3 (CH-1'''), three methyl groups at δ_{H} 1.30 and δ_{C} 18.3 (Me-6'), δ_{H} 1.10 and δ_{C} 16.7 (Me-6''), δ_{H} 1.24 and δ_{C} 14.9 (Me-6'''), as well as (*Z*)- α , β -unsaturated carbonyl at δ_{H} 7.08 (10.2, 3.1 Hz) and δ_{C} 144.7 (=CH-2'''), δ_{H} 6.08 (10.2 Hz) and δ_{C} 126.1 (=CH-3''') and δ_{C} 196.6 (C-4'''), indicating the presence of a trisaccharide comprising three deoxysugars. Detailed analyses of the HMBC associations (Figure 2) revealed an α -aculose-(1→4)- α -rhodinosyl-(1→4)- β -olivose linked at C-9 of the naphthacenequinone to form a C-glycoside (Figure 2).¹⁰ The absolute configurations of the three deoxysugars were presumed to be identical with those in vineomycin B₂ (5), which were α -L-aculose, α -L-rhodinosyl and β -D-olivose based on the ¹³C NMR data for the trisaccharide in 2 being almost consistent with those in vineomycin B₂ (5) (Supplementary Table S1), as well as that 2 and 5 shared a biosynthesis pathway. Compound 2 was designated grincamycin H.

The known compounds, P-1894B (vineomycin A₁, 3),^{11,12} saquayamycin B (4)^{6,13} and vineomycin B₂ (5)^{10,14} were identified on the basis of comparisons of MS, ¹H and ¹³C NMR spectroscopic data with the compounds previously reported.

Cytotoxic activities

In the *in vitro* cytotoxic assay, the known compounds P-1894B (vineomycin A₁, 3) and saquayamycin B (4) exhibited significant

Table 1 ^1H (500 MHz) and ^{13}C (125 MHz) NMR data of grincamycins G (**1**) and H (**2**)

Position	Grincamycin G (1) ^a		Grincamycin H (2) ^b	
	δ_{C} type	δ_{H} mult. (J in Hz)	δ_{C} type	δ_{H} mult. (J in Hz)
1	197.0, C		155.9, C	
2	52.5, CH ₂	2.56, dd (17.3, 1.6) 2.90, overlapped	116.1, CH	6.97, s
3	77.7, C		141.6, C	
4	35.6, CH ₂	3.76, d (18.6) 3.00, d (18.6, 1.6)	114.2, CH	7.55, s
4a	139.4, C		128.2, C	
5	163.4, C		162.8, C	
5a	119.1, C		108.6, C	
6	189.9, C		186.3, C	
6a	133.9, C		132.7, C	
7	121.2, CH	7.90, d (8.0)	118.7, CH	7.79, d (7.9)
8	135.6, CH	8.02, d (8.0)	132.1, CH	7.85, d (7.9)
9	140.5, C		137.3, C	
10	160.8, C		158.4, C	
10a	117.6, C		116.6, C	
11	189.8, C		187.3, C	
11a	132.6, C		125.0, C	
12	117.4, CH	8.34, s	116.8, CH	8.45, s
12a	140.2, C		124.1, C	
13	27.2, CH ₃	1.56, s	21.8, CH ₃	2.43, s
1'	73.3, CH	5.07, d (9.5)	70.5, CH	4.80, d (11.9)
2'	38.3, CH ₂	2.40, m; 1.63, m	38.4, CH ₂	2.40, m; 1.36, m
3'	78.4, CH	3.91, m	70.2, CH	3.68, m
4'	76.1, CH	3.60, m	84.3, CH	3.12, t (8.8)
5'	76.1, CH	3.64, q (6.2)	74.9, CH	3.50, q (6.5)
6'	18.4, CH ₃	1.34, d (6.2)	18.3, CH ₃	1.30, d (6.5)
1''	93.2, CH	5.26, d (3.0)	97.3, CH	4.94, br s
2''	73.1, CH	4.44, dd (6.0, 3.0)	24.1, CH ₂	1.99, m; 1.56, m
3''	41.6, CH	2.84, m; 2.56, m	24.3, CH ₂	2.28, m; 1.90, m
4''	209.5, C		75.0, CH	3.68, m
5''	79.2, CH	4.79, q (6.2)	66.1, CH	4.33, q (6.5)
6''	17.4, CH ₃	1.28, d (6.2)	16.7, CH ₃	1.10, d (6.5)
1'''	91.9, CH	5.17, br s	94.3, CH	5.35, d (3.1)
2'''	26.6, CH ₂	2.00, m; 1.60, m	144.7, CH	7.08, dd (10.2, 3.1)
3'''	25.8, CH ₂	2.40, m	126.1, CH	6.08, d (10.2)
4'''	77.7, CH	3.64, m	196.6, C	
5'''	68.6, CH	3.18, q (7.0)	69.6, CH	4.55, q (6.7)
6'''	18.4, CH ₃	0.64, d (7.0)	14.9, CH ₃	1.24, d (6.7)
1''''	96.9, CH	5.13, d (3.4)		
2''''	146.0, CH	6.93, dd (10.0, 3.4)		
3''''	128.1, CH	5.97, d (10.0)		
4''''	198.2, C			
5''''	71.9, CH	4.49, q (6.2)		
6''''	16.4, CH ₃	1.21, d (6.2)		

^aRecorded in acetone-*d*₆.^bRecorded in DMSO-*d*₆/CDCl₃ (5:1).

inhibition to Jurkat T-cell proliferation with IC₅₀ values of 11 and 37 nM, respectively, which were more or nearly equally efficient than that of the positive control, doxorubicin. Vineomycin B₂ (**5**) displayed powerful inhibitory effect to Jurkat T cells with an IC₅₀ value of 0.3 μM . The new compound **2** showed cytotoxicity against Jurkat T cells with an IC₅₀ value of 3.0 μM , while new compound **1** showed no cytotoxic activity at the concentration of 20 μM (Table 2).

DISCUSSION

Following the evidence of heterologous expression of GCN gene cluster, five angucycline glycosides containing L-aculose at the end of sugar chains, which was different from the terminal sugar unit L-cinerulose A in our previously reported GCNs, were identified from marine-derived actinomycete *S. lusitanus* SCSIO LR32.⁴ The core skeleton of the new compounds **1** and **2** is the rearranged galtamycin-type non-classic aglycone of angucycline.¹ However, compounds **1** and **2** showed lower cytotoxicity on Jurkat T cells than the known compounds **3** and **4**, which possess a classic aquayamycin-type aglycone.¹ Compound **5** has a tricyclic non-classic aglycone,¹ which exhibited moderate cytotoxicity among the five compounds. These results indicated that the chemical and biological diversity of angucyclines derived not only from the sugar unit but also from the aglycone moiety.

MATERIALS AND METHODS

General experimental procedures

Column chromatography (CC) was performed by using silica gel (100–200 mesh; Jiangpeng Silica Gel Development, Inc., Yantai, China) or Sephadex LH-20 (40–70 μm ; GE Healthcare, Uppsala, Sweden). TLC was conducted with precoated glass plates (0.1–0.2 mm; silica gel GF₂₅₄, 10–40 nm, Jiangpeng). HPLC analyses were performed with a 1260 infinity system (Agilent, Santa Clara, CA, USA) using a Phenomenex Prodigy ODS (2) column (150 $\times$ 4.6 mm, 5 μm ; Torrance, CA, USA). Semipreparative HPLC was performed with a Primaide 1110 solvent delivery module equipped with a 1430 photodiode array detector (Hitachi, Japan), using a YMC-Pack ODS-A column (250 $\times$ 10 mm, 5 μm ; Kyoto, Japan). UV spectra were recorded on a U-2910 spectrometer (Shimadzu, Tokyo, Japan); IR spectra were obtained on an IRAffinity-1 spectrophotometer (Shimadzu). High-resolution mass spectral data were obtained on a MaXis Q-TOF mass spectrometer (Bruker, Raleigh, NC, USA). Optical rotations were recorded with an MCP-500 polarimeter (Anton Paar, Graz, Austria). NMR spectra were recorded on an Avance 500 spectrometer (Bruker) at 500 MHz for ^1H nuclei and 125 MHz for ^{13}C nuclei. Chemical shifts (δ) were given with reference to tetramethyl silane (TMS) or solvent residue signal. Coupling constants (*J*) were given in Hz.

Fermentation

The isolation and characterization of strain SCSIO LR32 have been described previously.⁴ A portion of SCSIO LR32 spore suspension containing about 2.16×10^7 spores was inoculated into 100 ml modified AM₂ medium (0.5% soluble starch, 0.5% yeast extract, 0.2% peptone, 2.0% glucose, 0.05% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.4% NaCl, 0.2% CaCO₃, 3.0% sea salt, pH 7.2 before sterilization) in a 250 ml Erlenmeyer flask and incubated at 30 °C for 48 h on a rotary shaker at 200 r.p.m. Then 600 ml of the seed culture from six flasks was transferred into 8 l modified AM₂ medium in a 15 l fermentor, with the agitation speed of 200 r.p.m. and the aeration rate of 5 l min⁻¹. After 24 h cultivation, the entire 8 l seed culture was further transferred into a 150 l fermentor containing 80 l production medium (0.5% corn flour, 2% soluble starch, 0.5% yeast extract, 1% malt extract, 1% glucose, 1% maltose, 0.2% CaCO₃, 3.0% sea salt, pH 7.2 before sterilization) and cultivated at 30 °C. The dissolved oxygen of broth was maintained above 50% by adjusting the agitation speed and aeration rate. Broth sample was withdrawn every 24 h and centrifuged; the resulting supernatant was applied for the residual sugar measurement, and the mycelium pellets were extracted with methanol for further HPLC analysis. The fermentation was terminated after 7 days of cultivation. The entire 80 l broth was harvested and centrifuged to yield a mycelial cake and a liquid broth.

Extraction and isolation

Each 500 g mycelial cake was extracted with 1 l acetone three times. The acetone layers were combined and vacuum dried to yield a residue. The residue was subjected to a SiO₂ CC using gradient elution with CHCl₃ and MeOH mixtures (100:0, 99:1, 97:3, 95:5, 90:10, 80:20 and 50:50, v/v) to give seven fractions (Fr.A1 ~ Fr.A7). Fr.A1 and Fr.A2 were combined after HPLC analysis and

Figure 2 Key HMBC (arrows) and COSY (bold lines) correlations of grincamycins G (1) and H (2).

Table 2 Cytotoxicities (IC_{50} values, μM) of compounds 1–5 against Jurkat T cells

	1	2	3	4	5	Doxorubicin ^a
IC_{50}	>20.0	3.0	0.011	0.037	0.3	0.034

^aPositive control.

purified by SiO_2 CC eluting with petroleum ether and EtOAc mixtures (100:0, 90:10, 80:20, 70:30, 60:40, 40:60, 20:80, 0:100, v/v) to give eight fractions (Fr.B1~Fr.B8). Fr.B3 was purified by semipreparative HPLC with an ODS column eluted with CH_3CN/H_2O (30:70 to 100:0 over 28 min and then held for 7 min 2.5 ml min^{-1}) to afford **2** (8 mg) at t_R 32 min. Fr.B4 was subjected to Sephadex LH-20 CC eluted with $CHCl_3/MeOH$ (1:1) to obtain **1** (6 mg) and **4** (12 mg). Fr.B5 and Fr.B6 were combined and further purified by semipreparative HPLC with an ODS column using the same elution procedure to afford **3** (220 mg) at t_R 26 min and **5** (7 mg) at t_R 30 min.

Grincamycin G (1): Yellowish powder; $[\alpha]_D^{20} = +53$ (c 0.09, acetone); UV (MeOH) λ_{max} (log ϵ) 214 (4.32), 243 (4.26), 265 (4.30), 443 (3.86); 1H NMR (500 MHz, acetone- d_6) and ^{13}C NMR (125 MHz, acetone- d_6) data are listed in Table 1; (–)HR-ESI-MS m/z 801.2776 ($[M-H]^-$, calcd for $C_{43}H_{45}O_{15}$, 801.2764).

Grincamycin G (2): Dark-red powder; $[\alpha]_D^{20} = +65$ (c 0.10, acetone); UV (MeOH) λ_{max} (log ϵ) 230 (4.39), 258 (4.15), 294 (3.68), 492 (3.79); 1H NMR (500 MHz, DMSO- $d_6/CDCl_3$) and ^{13}C NMR (125 MHz, DMSO- $d_6/CDCl_3$) data are listed in Table 1; (–)HR-ESI-MS m/z 673.2311 ($[M-H]^-$, calcd for $C_{37}H_{37}O_{12}$, 673.2291).

Cytotoxic activity assay

Proliferation-inhibition assay against Jurkat T cells was conducted according to the previously reported method.¹⁵ Jurkat T cells (2×10^4 cells per well) were maintained in RPMI1640 medium with 10% fetal bovine serum at 37 °C, in 5% CO_2 . For cytotoxic activity assay, Jurkat T cells were seeded in a 96-well plate and treated with compounds as indicated and then incubated at 37 °C for 72 h. The viability of cells were measured by adding alamarblue dye (1:10) to each well, and the plate was incubated at 37 °C for 4 h before fluorescence intensity was obtained by Flexstation3 (Molecular Devices, Sunnyvale, CA, USA). All data were processed with Prism6 (La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported in part by the National Science Foundation of China (41476133) and the projects of Guangdong Provincial Oceanic and Fishery Administration (A201401C05). We express our thanks to the analytical facility center (Ms Sun, Ms Zhang, Mr Li and Ms Xiao) of the South China Sea Institute of Oceanology for measuring MS and NMR data.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)