Cytotoxic Angucycline Class Glycosides from the Deep Sea Actinomycete Streptomyces lusitanus SCSIO LR32

Hongbo Huang,† Tingting Yang,‡ Xiangmei Ren,§ Jing Liu,§ Yongxiang Song,† Aijun Sun,† Junying Ma,† Bo Wang, Yun Zhang,§ Caiguo Huang,† Changsheng Zhang,† and Jianhua Ju*†

†CAS Key Laboratory of Marine Bio-resources Sustainable Utilization, Guangdong Key Laboratory of Marine Materia Medica, RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou S10301, People’s Republic of China
‡Department of Biochemistry and Molecular Biology, The Second Military Medical University, 800 Xiangyin Road, Shanghai 200433, People’s Republic of China
§Graduate University of the Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, People’s Republic of China

Supporting Information

ABSTRACT: Five new C-glycoside angucyclines, named grincamycins B−F (1−5), and a known angucycline antibiotic, grincamycin (6), were isolated from Streptomyces lusitanus SCSIO LR32, an actinomycete of deep sea origin. The structures of these compounds were elucidated on the basis of extensive spectroscopic analyses, including MS and 1D and 2D NMR experiments. All compounds except grincamycin F (5) exhibited in vitro cytotoxicities against the human cancer cell lines HepG2, SW-1990, HeLa, NCI-H460, and MCF-7 and the mouse melanoma cell line B16, with IC50 values ranging from 1.1 to 31 μM.

In the past decade, marine actinomycetes have emerged as an exciting new source for the discovery of novel bioactive natural products, especially in the anti-infective and anticancer areas.1−5 Of the 240 established genera of actinomycetes, 50, including 12 new ones, have been isolated from marine sources.5 During our efforts to identify new anticancer and anti-infective natural products from marine actinomycetes originating from the South China Sea, we reported the discovery of cytotoxic and antibacterial pseudonocardians and lobophorins from Pseudonocardia sp. SCSIO 012996 and Streptomyces sp. SCSIO 011227, respectively, and the antimalarial marinocarboline and indolactam alkaloids from Marinactinospora thermotolerans SCSIO 00652.8

The strain SCSIO LR32 was isolated from a South China Sea deep ocean sediment and identified as Streptomyces lusitanus based on 16S rDNA sequence analysis. To explore the chemical and biological capabilities of this strain, we fermented the microbe using several different media. Previous work had shown that this strain, when fermented using JNP1 medium, produced predominantly four aromatic amide compounds identified as toluene-2,4-dicarboxylic acid dimethyl ester, toluene-2,4-dicarboxylic acid diethyl ester, toluene-2,6-dicarboxylic acid dimethyl ester, and toluene-2,6-dicarboxylic acid diethyl ester.9 We found that when this strain was fermented using modified-RA medium and the extract was analyzed by HPLC-DAD-UV, an array of secondary metabolites with UV spectra characteristic of the angucycline class of antibiotics was observed.10,11 In this paper, we report the fermentation, isolation, and structure elucidation of five new angucycline C-glycosides, grincamycins B−F (1−5), from S. lusitanus SCSIO LR32 along with the previously reported grincamycin (6). Moreover, we report the cytotoxicities of compounds 1−6 against the human cancer cell lines HepG2, SW-1990, HeLa, NCI-H460, and MCF-7 and the mouse melanoma cell line B16.

RESULTS AND DISCUSSION

Strain SCSIO LR32 was cultivated on an 8 L scale using modified-RA medium in 2 L shake flasks. Silica gel, Sephadex LH-20, and semipreparative HPLC column chromatography of the fermentation extract yielded 6 as a major product and 1−5 as minor metabolites. Compound 6 was detected and isolated as a pale red powder. Positive and negative ESI MS data indicated that 6 has a molecular weight of 938. The 1H and 13C NMR spectroscopic data for 6 (Supporting Information, Table S1) suggested the presence of a typical angucycline and tetrangomycin skeleton,12,13 with the 3-position C-glycosylated and 9-position O-glycosylated. The 1H and 13C NMR spectra were characterized by resonances corresponding to five doublet methyl groups, nine methylenes, 14 methines, and two carbyl moieties arising from sugar residues, subsequently identified as a trisaccharide, consisting of β-olivose, α-rhodinose, and α-cinerulose A, and a disaccharide containing α-rhodinose and α-cinerulose B.
α-cinerulose A through detailed interpretation of both 1D and 2D NMR data. Structural assignments for compound 6 were validated by comparison of the 1H and 13C NMR and MS spectroscopic data with data previously reported for grincamycin, a known angucycline antibiotic from *Streptomyces griseoincarnatus* with inhibitory activity against murine leukemia P388 cells.14

Compound 1 was isolated as a red, amorphous solid. Negative mode ESI-MS afforded a quasi-molecular ion peak at *m/z* 937 [M−H]+, and subsequent HRESIMS revealed the molecular formula of 1 to be C43H50O16. The IR spectrum of 1 displayed characteristic absorptions of hydroxy (3416 cm−1), carbonyl (1731, 1703, and 1628 cm−1), and benzene ring (1604 and 1583 cm−1) functionalities. A full set of 1D (1H and 13C) and 2D (HMQC, HMBC, and NOESY) NMR spectra for 1 were acquired, allowing the complete assignment of its 1H and 13C NMR signals (Table 1). The 1H and 13C NMR spectra were rich in aliphatic signals with additional aromatic signals composed of D-olivose, L-rhodinose, and L-cinerulose A sugars. This new compound was accordingly named grincamycin B.

Compound 2 was isolated as a red, amorphous solid. Its molecular formula, C17H24O16, was determined by HRESIMS. Particularly striking was the signal at *m/z* 711.2649 [M−H]−, which is less than that of 1 by a C12H18O4 fragment. Comparisons of the 1H and 13C NMR spectra for 2 with those obtained with 1 revealed an absence of sugar resonances in 2. Only three anomic methines at δH 4.75 and δC 71.0 (CH-1′), at 4.99 and 99.1 (CH-1",), and at 5.08 and 99.5 (CH-1‴) were observed. The 13C NMR signal of the oxygen-bearing carbon C-12 was shifted upfield, from δC 77.2 in 1 to δC 71.5 in 2, suggesting that the C-12 is not O-glycosylated. Furthermore, the 1H and 13C NMR data of 2 could be thoroughly assigned by comparing with spectroscopic data for grincamycin B (1) and previously reported congeners.19,20 Consequently, the structure of 2 was solved and named grincamycin C.

Compound 3 was obtained as a brown-yellow, amorphous solid, and its molecular formula determined to be C43H24O16 on the basis of HRESIMS. Similarities in the 1H and 13C NMR spectroscopic data for 3 and 1 indicated a close structural relationship between the two compounds. Compounds 3 and 1 were found to be structurally differentiated only by the identity of the C-6 glycoside. In a fashion similar to that observed with compounds 1 and 2, a characteristic anomic methine at δH 4.97 (br d, J = 10.5 Hz) and δC 71.5 suggested the presence of β-olivose in 3. The COSY coupling of H-1′/H-2′/H-3′ revealed a fragment of C-1′−C-2′−C-3′ with the aid of HMBC data (Figure 3). Further long-range couplings from H-3′ to the C-4′ carbonyl, from H-1′ to an oxygen-bearing methine (C-5′), and from H-3′-5′ to C-5′ and C-4′ in the HMBC
The signals were assigned with the aid of HMQC and HMBC data. To satisfy the molecular formula, another linkage, C-1″−O−C-4′, which was confirmed by an HMBC correlation of H-1″/C-4′. To satisfy the molecular formula, another linkage,
C-2″−O−C-3′ was proposed to complete the planar structure elucidation of 3. The small coupling constant (J = 2.5 Hz) indicated an αx-equatorial orientation between H-1′ and H-2′. The NOESY correlations of H-1′H-2′H-3′H-1″H-5′′ and of H-5′′H-4′ permitted elucidation of the disaccharide relative configuration as shown in Figure 3. The 1H and 13C NMR spectroscopic data for the α-cinerulose B-(1→4, 2→3)-β-olivosyl unit were in good agreement with the data previously reported for compounds with the same sugar moieties. The carbonyl is conjugated with the anthraquinone. Further HMBC correlations involving rings A and B confirmed the structure of the tetracyclic anthraquinone aglycone as shown in Figure 4. Additionally, 1H and 13C NMR resonances attributable to the saccharide moieties of 4 were very similar to those previously noted for 6, suggesting that both compounds share the same sugar moieties. The C-glycosyl bond between C-9 and C-1′′ was determined by the HMBC correlations from H-1′ to C-9, C-10 and from H-8 to C-1′. The connection of C-3−O−C-1′′ was determined by the HMBC correlation of H-1′′/C-3. Furthermore, we found that 6 could be converted into 4 under UV irradiation (Supporting Information, Figure S35). The transformation yield is about 50% after UV irradiation for 24 h. This photochemical transformation established the 3R configuration of 4 as well as the configurations of the sugars. Thus, the structure of 4 was named grincamycin E.

Compound 5 was isolated as a dark red powder. HRESIMS of 5 revealed a prominent peak at m/z 1069.4076 [M – H]−, establishing the molecular formula as C57H66O20. From the characteristic absorption bands at 222, 302, and 511 nm in the UV spectrum, as well as the established molecular formula, 5 was determined to be a member of the angucycline class of antibiotics. The aglycone in 5 was found to be identical to that of urdamycin C on the basis of comparisons of the 1H and 13C NMR data for 5 and those previously reported for urdamycin C, an enlarged chromophore (Supporting Information, Table S3), possessing an angular tetracyclic ring system and the unique structural element (p-OH-phenyl)-pyranone. The 1H and 13C NMR spectra of 5 also revealed a set of sugar signals that were nearly identical to those of the disaccharide and trisaccharide in 1. One key difference was that the 13C NMR signals of C-3′ (δc 76.0) and C-4′ (δc 75.1) on β-olivose were different from those previously reported for grincamycin (C-3′: δc 71.4, C-4′: δc 86.6 in 1), thus defining the C-3′−O−C-1″ system as the glycosidic linkage connecting β-olivose and α-rhodinose. The CH-3′ and CH-1″ correlated to each other in the HMBC and NOESY spectra, thus verifying this linkage (Figures 5 and 6). This new compound was named grincamycin F.

Of the many angucycline antibiotics discovered thus far, only two distinct mechanisms for angucycline core formation have been reported. Feeding experiments revealed that two angucycline antibiotics, PD117198 and BE-7585A, are biosynthesized through a linear fused tetracyclic anthracycline intermediate. Most angucycline antibiotics have been shown by feeding experiments to be biosynthesized in a straightforward manner to directly form the angucycline core through a deca ketide intermediate; examples include urdamycin A9,26 vineomycin A16, and PD116740.29 However, the angucycline antibiotics suffer rearrangements to afford linear derivatives upon exposure to light, heat, or acids.35,17 For instance, aquayamycin can be rearranged into a linear tetracyclic product with light, and a tricyclic product can be formed upon acidic treatment in methanol.23 Because grincamycin (6) and aquayamycin have identical aglycones, 6 might be expected to undergo similar rearrangements. Compounds 1−3 belong to a category of nonclassically rearranged angucycline C-glycosides. The aglycones of 1−3 share a side chain (C-11−C-15) that derives from cleavage of the C-1/C-12b bond of ring A in 6 and was concluded to be identical with that of fridamycins A−D and vineomycin B2.17 Although the minor metabolites 1−5 were simultaneously detected from the fermentation of strain SCSIO LR32, compounds 1−4 may actually result from rearrangement processes that occur during fermentation.

Figure 1. HMBC correlations of grincamycin B (1).

Figure 2. Key NOESY correlation (double-headed arrow) of the α-cinerulose A-(1→4)-α-rhodinoseyl (1→4)-β-olivosyl unit (I) in grincamycins B, C, and E (1, 2, and 4) and grincamycin (6).

Figure 3. 2D NMR (COSY, HMBC, NOESY) correlations of the α-cinerulose B-(1→4, 2→3)-β-olivosyl unit (III) in grincamycin D (3).
Table 2. Summary of $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR Spectroscopic Data for Grincamycins E (4) and F (5)

<table>
<thead>
<tr>
<th>Position</th>
<th>δ$_C$ (in ppm)</th>
<th>δ$_H$ (in Hz)</th>
<th>δ$_C$ (in ppm)</th>
<th>δ$_H$ (in Hz)</th>
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<tr>
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<td>195.8 C</td>
<td>266.8 C</td>
<td>1</td>
<td>195.8 C</td>
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<td>76.8–77.3 C</td>
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<tr>
<td>4</td>
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<td>3.61, d (18.6)</td>
<td>43.7, CH$_2$</td>
<td>1.73$^{d,e,f,g}$</td>
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<td>71.0, CH</td>
<td>71.0, CH</td>
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</table>

$^a$Recorded in CDCl$_3$/CD$_3$OD; the signals were assigned with the aid of $^1$H–$^1$H COSY, HMQC, and HMBC data. $^b$Recorded in CDCl$_3$/CD$_3$OD; the signals were assigned with the aid of HMQC and HMBC data. Obscured by solvent. $^{d,e,f,g}$These signals under the same superscript and within the same column were overlapped.

Figure 4. COSY (bold) and HMBC (arrow) correlations for grincamycin E (4).

Figure 5. Key HMBC correlations for grincamycin F (5).

Compounds 1–6 were tested for their in vitro cytotoxic activities against the human hepatoma cell line HepG2, human pancreatic cell line SW-1990, human cervical cancer cell line HeLa, human lung cancer cell line NCI-H460, human breast cancer cell line MCF-7, and the mouse melanoma cell line B16 using previously described MTT-based methods. As depicted in Table 3, the known grincamycin (6) was the most effective compound evaluated. It displayed antiproliferative activities with the selected panel of cells; IC$_{50}$ values ranged from 1.1 to 11 µM. Using a similar approach, the new grincamycins B–E (1–4) showed cytotoxicity toward these cell lines with IC$_{50}$ values ranging from 2.1 to 31 µM. It is worth noting that grincamycin F (5) differs from 6 primarily in the structure of its enlarged aglycone, which contains a six-membered lactone ring and a hydroxybenzene in addition to the typical angucycline
**EXPERIMENTAL SECTION**

**General Experimental Procedures.** Optical rotations were determined with an MCP 300 (Anton Paar) polarimeter at 25 °C. UV spectra were recorded on a U-3910 spectrometer (Hitachi). IR spectra were obtained on a 5DX-FTIR spectrophotometer (Nicolet). NMR spectra were recorded with an Avance 500 spectrometer (Bruker) at 500 MHz for 1H nucleus and 125 MHz for 13C nucleus in CDCl₃ or CDCl₃/CD3OD. Chemical shifts (δ) are given with reference to TMS. Coupling constants (J) are given in Hz. ESI-MS spectra were detected with an Esquire 3000 plus spectrometer (Bruker). HRESI-MS data were acquired with a Xevo G2 TOF mass spectrometer (Waters) and a micro-TOF-QQ mass spectrometer (Bruker). Column chromatography (CC) was performed using silica gel (100–200 mesh; Qingdao Marine Chemicals) and Sephadex LH-20 (Amersham Pharmaica). HPLC was performed with a 210 solvent delivery module equipped with a 335 photodiode array detector (Varian) and using a Prodigy ODS (2) column (150 × 4.6 mm, 5 μm; Phenomenex).

**Bacterial Materials.** The strain SCSIO LR32 was isolated using 16S rDNA sequence analysis. The DNA sequence has been deposited in GenBank (accession no. JQ315184). The strain has been preserved at the RNAM Center for Marine Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences.

**Fermentation and Extraction.** The strain was maintained on modified ISP-4 medium plates. A spore and mycelial suspension was inoculated into each of the 250 mL Erlenmeyer flasks containing 50 mL of modified AM2 medium. The flasks were incubated at 28 °C on a rotary shaker (200 rpm) for eight days. After fermentation, the culture (8 L) was centrifuged to yield supernatant and a mycelial cake. The supernatant was extracted with equal volumes of butanone three times, and then samples were evaporated to dryness. The mycelial cake was extracted with 2 L of acetone three times, and the solvent was then evaporated to dryness. The two organic extracts were finally combined to give 8.3 g of residue.

**Modified AM2 medium:** 1.0% soybean flour; 2.0% soluble starch; 0.5% yeast extract; 0.2% peptone; 0.2% CaCO₃; 0.4% sea salt; pH 7.2–7.4.

**Modified RA medium:** 1.0% glucose; 2.0% soluble starch; 1.0% malt meal, 1.0% maltose, 0.5% corn steep liquor, 3.0% sea salt, trace element solution (0.1 mL/L), pH 7.2–7.4.

**Isolation.** The extract was subjected to silica gel CC using gradient elution with a CHCl₃–MeOH mixture (100:0, 99:1, 98:2, 95:5, 90:10, 80:20, v/v) to give six fractions (Fr.1–Fr.6), correspondingly. Fr.2 was applied to ODS CC eluting with a linear gradient of 20% to 100% MeOH over 80 min by medium-pressure chromatography (MPLC). The subfractions were analyzed by HPLC and grouped into four fractions (Fr.2.1–Fr.2.4). Fr.2.1 was purified by Sephadex LH-20 CC eluting with CHCl₃–MeOH (1:1) to give 6 (49 mg) and Fr.2.1-2. The latter was chromatographed on silica gel CC repeatedly, eluting with CHCl₃–MeOH (98:2), and then the subfraction was further purified by Sephadex LH-20 CC eluting with CHCl₃–MeOH (1:1) to give 2 (5 mg). Fr.2.4 was subjected to silica gel CC eluting with petroleum ether–EtOAc mixture (100:0, 98:2, 95:5, 90:10, 80:20, 50:50, v/v) to give six fractions (Fr.2.4-1–Fr.2.4-6). Fr.2.4-2 was further separated by silica gel CC eluting with CHCl₃–MeOH (97:3) to obtain 4 (12 mg). Fr.2.4-3 was subjected to ODS CC eluting with a linear gradient of 20% to 100% MeOH over 80 min by MPLC to give 1 (10 mg) and 3 (13 mg).

**Grincamycin B (1):** red solid; [α]25D −18 (c 0.40, CHCl3); UV (MeOH) λmax (log ε) 230 (4.86), 258 (4.65), 294 (4.11), 442 (4.36) nm; IR (KBr) νmax 1341, 2976, 2973, 1731, 1703, 1628, 1584, 1431, 1375, 1260 cm⁻¹; 1H and 13C NMR spectroscopic data, see Table 1; (+)-ESIMS m/z 937.7 [M − H]⁺; (+)-HRESIMS m/z 937.3853 [M − H]⁺ (calcld for C41H39O16, 937.3863). [481]

**Grincamycin C (2):** red, amorphous solid; [α]25D −41 (c 0.73, CHCl₃); UV (MeOH) λmax (log ε) 230 (4.85), 258 (4.64), 294 (4.13), 442 (4.36) nm; IR (KBr) νmax 1341, 2925, 2854, 1732, 1627, 1605, 1581, 1431, 1374, 1260 cm⁻¹; H and 13C NMR spectroscopic data, see Table 1; (+)-ESIMS m/z 735.0 [M + Na]⁺, (−)-ESIMS m/z 711.6 [M − H]⁺, 1462.0 [2M + K − H]⁺; (−)-HRESIMS m/z 711.2649 [M − H]⁺ (calcld for C38H38O14, 711.2658).

**Grincamycin D (3):** brown-yellow, amorphous solid; [α]25D +70 (c 0.55, CHCl₃); UV (MeOH) λmax (log ε) 230 (4.86), 258 (4.64), 294 (4.14), 442 (4.35) nm; IR (KBr) νmax 3433, 2978, 2936, 2885, 1734, 1628, 1605, 1583, 1431, 1334, 1259 cm⁻¹; 1H and 13C NMR spectroscopic data, see Table 1; (+)-ESIMS m/z 845.2 [M + Na]⁺, (−)-ESIMS m/z 821.33 [M − H]⁺; (−)-HRESIMS m/z 821.3002 [M − H]⁺ (calcld for C40H38O16, 821.3026).

**Grincamycin E (4):** yellowish powder; [α]25D −112 (c 0.17, CHCl₃); UV (MeOH) λmax (log ε) 214 (5.18), 262 (4.48), 442 (4.45) nm; IR (KBr) νmax 3404, 2975, 2934, 2855, 1732, 1700, 1630, 1608, 1577, 1423, 1316, 1240 cm⁻¹; H and 13C NMR spectroscopic data, see Table 2; (−)-ESIMS m/z 943.4 [M + Na]⁺, 959.3 [M + K]⁺, (−)-ESIMS m/z 919.5 [M − H]⁺, 951.6 [M + MeOH − H]⁺, (−)-HRESIMS m/z 919.3763 [M − H]⁺ (calcld for C41H39O16, 919.3758).

**Grincamycin F (5):** dark red powder; [α]25D +110 (c 0.09, CHCl₃–MeOH, 9:1); UV (MeOH) λmax (log ε) 222 (5.16), 302 (4.77), 511 (4.74) nm; IR (KBr) νmax 3420, 2970, 2924, 2853, 1729, 1638, 1404, 1282 cm⁻¹; 1H and 13C NMR spectroscopic data, see Table 2; (−)-ESIMS m/z 1093.6 [M + Na]⁺, 1125.4 [M + Na + MeOH]⁺, (−)-ESIMS m/z 1069.7 [M − H]⁺; (−)-HRESIMS m/z 1069.4076 [M − H]⁺ (calcld for C52H50O34, 1069.4075).

**Grincamycin G (6):** pale red powder; [α]25D +61 (c 0.33, CHCl₃) [lit. [α]25D +48 (c 0.1, CHCl₃)]; 1H and 13C NMR spectroscopic data, see Table SI.

**Photochemical Transformation of 6 into 4.** A solution of grincamycin in MeOH (2.0 mg/mL) was irradiated under UV254 and 365 nm for 24 h at room temperature. During irradiation, MeOH was replaced with 16S rDNA sequence analysis.
using the previously published MTT method. Briefly, the cancer cell lines NCI-H460, and MCF-7 and the mouse cell line B16 were determined for compounds grincamycin E (1 mg/mL) and grincamycin E (6 mg/mL) were used as reference compounds.

Cytotoxicity Assay. The cell growth inhibitory activities of compounds 1–6 against the human cell lines HepG2, SW-1990, HeLa, NCI-H460, and MCF-7 and the mouse cell line B16 were determined using the previously published MTT method. Briefly, the cancer cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum to maintain the volume. The product was occasionally supplied to maintain the volume. The product was analyzed by HPLC with a Prodigy ODS (2) column (150 × 4.6 mm, 5 μm; Phenomenex). The solvent system comprised solvent A (0.1% AcOH/15%MeCN in H₂O) and solvent B (0.1%AcOH/85%MeCN in H₂O). Elution was performed using a linear gradient of 20% to 100% solvent B over the course of 20 min, followed by holding at 100% solvent B for 15 min. The flow rate was 1.0 mL/min, and UV detection was at 220 nm. The analysis was performed using a linear gradient of 20% to 100% solvent B over the course of 20 min, followed by holding at 100% solvent B for 15 min. The flow rate was 1.0 mL/min, and UV detection was at 440 nm. Grincamycin (6) (1 mg/mL) and grincamycin E (4) (1 mg/mL) were used as reference compounds.

ASSOCIATED CONTENT

HRESIMS and 1D and 2D NMR spectra of compounds 1–6 were analyzed by HPLC with a Prodigy ODS (2) column (150 × 4.6 mm, 5 μm; Phenomenex). The solvent system comprised solvent A (0.1% AcOH/15%MeCN in H₂O) and solvent B (0.1%AcOH/85%MeCN in H₂O). Elution was performed using a linear gradient of 20% to 100% solvent B over the course of 20 min, followed by holding at 100% solvent B for 15 min. The flow rate was 1.0 mL/min, and UV detection was at 440 nm. Grincamycin (6) (1 mg/mL) and grincamycin E (4) (1 mg/mL) were used as reference compounds.

Notes

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Corresponding Author

*Tel/Fax: +86-20-89023028. E-mail: jju@scsio.ac.cn.

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