Peer-reviewed version available at *Mar. Drugs* **2018**, *16*, 470; doi:10.3390/md16120470

Article

Angucycline Glycosides from an Intertidal Sediments Strain *Streptomyces* sp. and Their Cytotoxic Activity against Hepatoma Carcinoma Cells

Aihong Peng[1,2], Xinying Qu[1], Fangyuan Liu[1], Xia Li[1], Erwei Li[2,*] and Weidong Xie[1,*]

[1] Department of Pharmacy, College of Marine Science, Shandong University at Weihai, Weihai 264209, China; e-mails: 1357139326@qq.com (A.P.); 1156665986@qq.com (X.Q.); 461762904@qq.com (F.L.); xiali@sdu.edu.cn (X.L.)

[2] State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China

* Correspondence: wdxie@sdu.edu.cn (W.X.); liew@im.ac.cn (E.L.); Tel.: +86-631-568-8303 (W.X.); +86-10-6480-6141 (E.L.)

Abstract: Four angucycline glycosides including three new compounds landomycin N (**1**), galtamycin C (**2**) and vineomycin D (**3**), and a known homologue saquayamycin B (**4**), along with two alkaloids 1-acetyl-β-carboline (**5**) and indole-3-acetic acid (**6**), were identified from the fermentation broth of an intertidal sediments derived *Streptomyces* sp. Their structures were mainly established by IR, HR-ESI-MS, 1D and 2D NMR techniques. Among the isolated angucyclines, saquayamycin B displayed potent cytotoxic activity against hepatoma carcinoma cells HepG-2, SMMC-7721 and plc-prf-5, with IC_{50} values 0.135, 0.033 and 0.244 μM respectively, superior to positive drug doxorubicin. Saquayamycin B treatment to SMMC-7721 cells led to the typical morphological signs of apoptosis in 4',6-diamidino-2-phenylindole (DAPI) staining experiment.

Keywords: *Streptomyces*; angucycline; saquayamycin; cytotoxicity

1. Introduction

Angucycline is a group of aromatic polyketides containing a benz[a]anthraquinone framework of the aglycone which is mostly attached with C-glycosidic moiety [1]. Naturally occurring angucyclines are exclusively produced by terrestrial and marine actinomycetes, especially *Streptomycetes* species, in which a decaketide initially derived from acetyl-CoA is catalytically cyclized to four-ring core of angucycline by polyketide cyclase [2]. The structures of angucycline glycosides always vary in the oxidation degree of aglycones along with the number and position of diverse deoxy sugars [1-4]. In some cases, e.g. galtamycin B [5], grincamycin B [6] and vineomycin B_2 [7], the angular four-ring of typical angucycline is rearranged to linear tetracyclic or tricyclic system by enzymatic or non-enzymatic modification. Although firstly discovered in half a century ago and possessing potent antibacterial, antiproliferative and cytotoxic activities [6-11], so far none of angucycline compounds has been successfully developed into clinical drug due to toxicity or solubility issues, which is unlike to its biogenetic relatives tetracycline and anthracycline groups [2]. Recent researches on this group mainly concentrated on the understanding of biosynthetic mechanisms, so as to obtain modified analogues with medicinal potentiality through genetic manipulation [12-14].

Intertidal ecosystems are significantly different from that of seafloor. Regular tide immersion and emersion result in the dissolution of more organic carbon as well as oxygen and sulfate into intertidal sediments, which is beneficial to microbes' survival, particularly to aerobic actinomycetes. Both metagenomes and culture-dependent isolation have verified the abundance and diversity of Actinobacteria in intertidal sediments [15]. Thus, we exploited the Actinobacteria resources from the intertidal sediments of Xiaoshi Island in Weihai, China, to screen for new antitumor agents. As a

result, a *Streptomyces* sp. designated OC1610.4 was obtained, and its 16S rRNA nucleotide sequence (Accession no. MK045847) shared only 81.8 % and 81.6 % similarity, respectively, with those of *Streptomyces chromofuscus* (FJ486284) and *Streptomyces lannensis* (KM370050) in GenBank. The TLC analysis of its EtOAc extracts of liquid culture medium displayed several yellow and brown spots presumably presented by aromatic polyketides. Subsequent large-scale fermentation and chromatographic isolation led to the identification of four angucycline glycosides including three new compounds namely landomycin N (**1**), galtamycin C (**2**) and vineomycin D (**3**), and one known compound saquayamycin B (**4**) (Figure 1), along with two alkaloids 1-acetyl-β-carboline (**5**) and indole-3-acetic acid (**6**) [16,17]. Saquayamycin B displayed potent cytotoxic activity against hepatoma carcinoma cells HepG-2, SMMC-7721 and plc-prf-5, and it could cause SMMC-7721 cells to apoptosis.

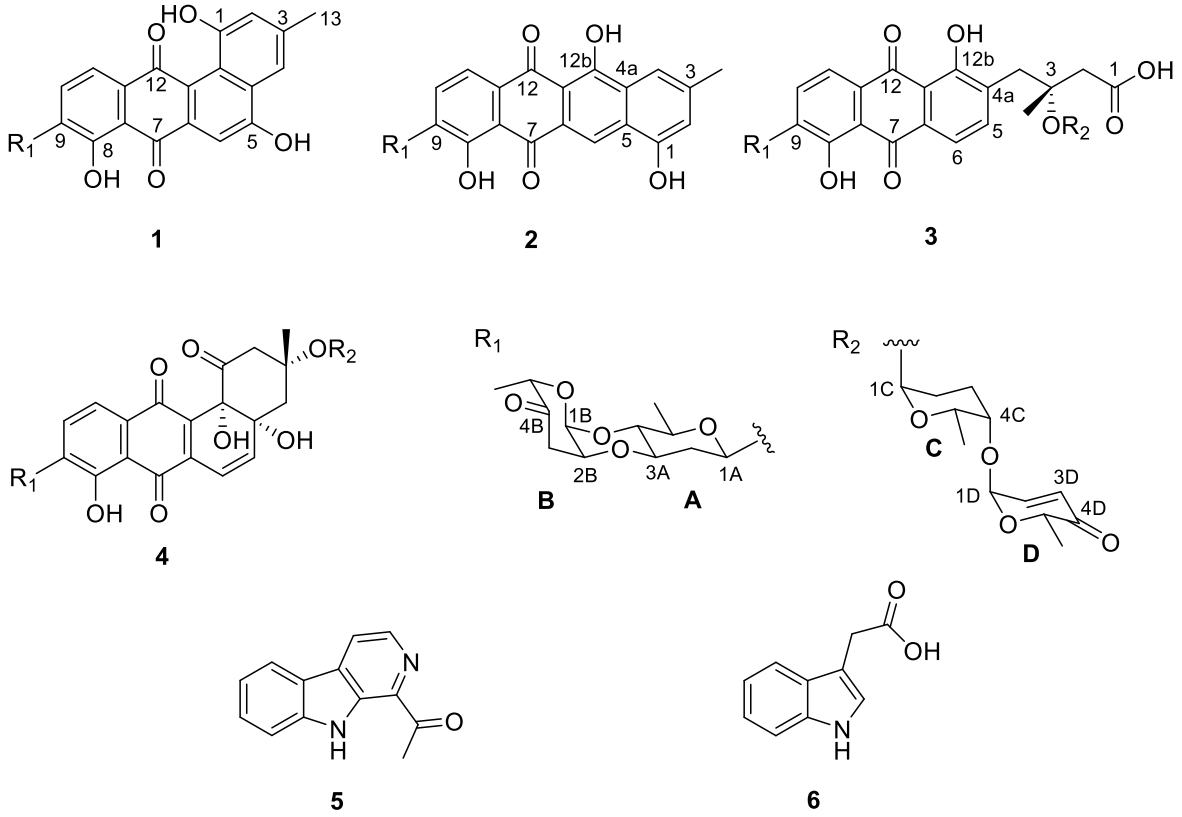


Figure 1. Structures of compounds **1**-**6**.

2. Results and Discussion

From 30 L liquid fermentation broth of strain *Streptomyces* sp. OC1610.4 cultured for 9 days, 4.6 g EtOAc extracts was obtained. After ordinary column chromatography partition and preparative HPLC purification, six yellow or brown amorphous powders were acquired from crude extracts. The major constituent in extracts was firstly purified and has a molecular formula $C_{43}H_{48}O_{16}$ deduced by the HR-ESI-MS data at *m*/*z* 838.3298 ($[M + NH_4]^+$, calcd for $C_{43}H_{52}NO_{16}$, 838.3286) and *m*/*z* 843.2842 ($[M + Na]^+$, calcd for $C_{43}H_{48}NaO_{16}$, 843.2840) (Figure S1). Its ^1H spectrum displayed complex signals including three pairs of coupling aromatic or olefinic protons ranging from δ_H 7.91 to 6.06 ppm, more than a dozen of coupling methylene and methine protons ranging from δ_H 5.39 to 1.40 ppm and five methyl groups in upfield (Figure S2). The four oxygenated methine proton signals betweem δ_H 5.40 and 5.01 ppm corresponding carbons signals at δ_C 96.0, 92.8, 92.1 and 72.0 ppm (Figure S3), along with four doublets of methyl groups are the characteristic of four deoxy sugar groups, one of which probably formed a C-glycoside owing to its anomeric carbon resonating at δ_C 72.0 ppm

[18,19]. These data especially the signals of deoxy sugar C-glycosidic moiety prompted the structure of angucycline glycoside [1]. Detailed comparison of ^{1}H and ^{13}C NMR data with those reported in references and further confirmed by 2D NMR sprectra (Figures S5-8), this compound was identified as saquayamycin B (**4**), in which four deoxy sugars β-D-olivose, α-L-cinerulose B, α-L-rhodinose and α-L-aculose are present [3,18].

Landomycin N (**1**) was a minor constituent in crude extract. Its molecular formula $C_{31}H_{28}O_{10}$ was deduced by protonated molecule ion at *m/z* 561.1753 ([M + H]$^{+}$, calcd for $C_{31}H_{29}O_{10}$, 561.1761) in HR-ESI-MS. The IR spectrum showed the absorption band of hydroxyl group (3203 cm^{-1}), carbonyl groups (1726, 1629 cm^{-1}) and aromatic ring (1607, 1578 cm^{-1}). The ^{1}H NMR of **1** displayed the signals of five aromatic protons, seven oxygenated methines, two methylenes and three methyl groups. The chemical shifts and coupling patterns of aliphatic proton signals of **1** were very similar to partial signals of saquayamycin B (**4**) (Table 1). Thus compound **1** is also supposed to be an angucycline glycoside. The aromatic protons at δ_H 7.84 (d, *J* = 7.9 Hz), 7.72 (d, *J* = 7.9 Hz), 7.62 (brs), 7.46 (s) and 6.96 (brs) ppm, similar chemical shifts to that of urdamycin N4 [4], could be assigned to three aromatic rings of angucycline aglycone. The ^{1}H-^{1}H COSY, HMQC and HMBC correlations associated with five aromatic protons and methyl group at δ_H 2.40 ppm could exactly assign the signals of each proton and carbon in angucycline aglycone which is identical to that of urdamycin N4 (Table 1) [4]. In ^{1}H-^{1}H COSY spectrum, the correlations of δ_H 7.84 (H-10)/δ_H 7.72 (H-11) and δ_H 7.62 (H-2)/δ_H 6.96 (H-4) were observed (Figure 2). The HMBC correlations of CH_3 (δ_H 2.40) to C-2 (δ_C 119.4), C-3 (δ_C 139.0), C-4 (δ_C 114.2), H-2 (δ_H 6.96) to C-1 (δ_C 155.4), C-4 (δ_C 114.2) and C-12b (δ_C 122.1), and H-4 (δ_H 7.62) to C-2 (δ_C 119.4), C-4a (δ_C 130.6) and C-12b (δ_C 122.1) confirmed the A ring of angucycline and the attachment of hydroxyl group at C-1 (δ_C 155.4) (Figure 2). The D ring and the attachment of hydroxy group at C-8 were deduced by the HMBC correlations of H-10 (δ_H 7.84) to C-8 (δ_C 156.9) and C-11a (δ_C 134.7), H-11 (δ_H 7.72) to C-9 (δ_C 135.0) and C-7a (δ_C 114.1), and 8-OH (δ_H 12.53) to C-7a (δ_C 114.1), C-8 (δ_C 156.9) and C-9 (δ_C 135.0). In ^{13}C NMR spectrum, the signal of quinone carbonyl carbon at C-12 was not observed, though, it can be deduced to be δ_C 182.6 ppm by the HMBC correlation δ_H 7.72 (H-11)/δ_C 182.6. The ^{1}H NMR singlet of aromatic proton at δ_H 7.46 ppm was assigned to H-6 in B ring, and it displayed HMBC correlations with C-4a (δ_C 130.6), C-7 (δ_C 188.9) and C-12a (δ_C 119.6). The chemical shift of C-5 (δ_C 166.4) along with the HMBC correlation of H-4 (δ_H 7.62) to C-5 (δ_C 166.4) suggested the presence of hydroxyl group at C-5.

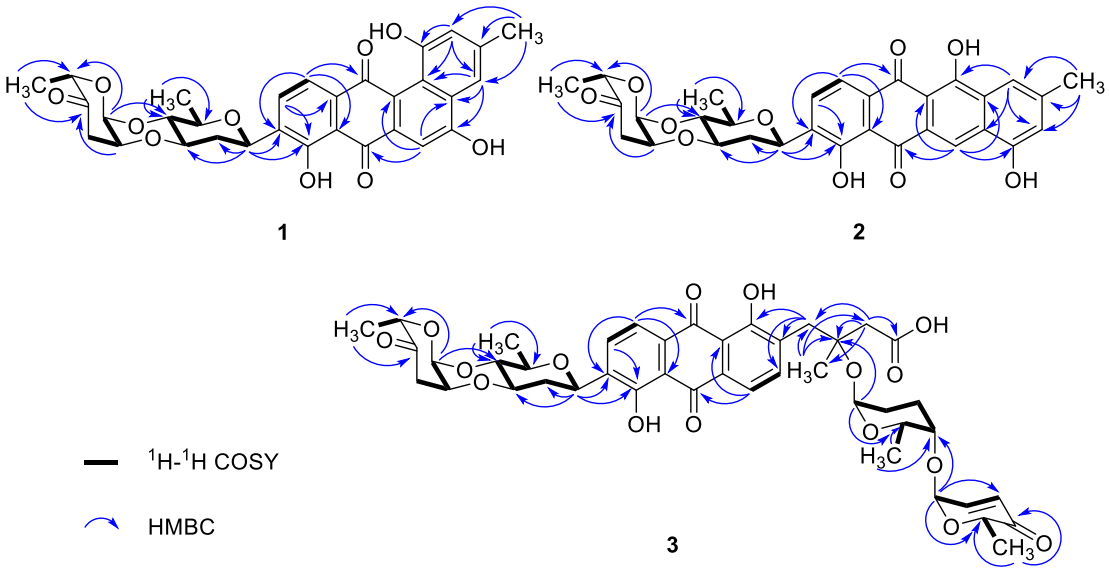


Figure 2. The ^{1}H-^{1}H COSY and selected HMBC correlations for compounds **1**-**3**.

Table 1. The ^{1}H and ^{13}C NMR data of compounds **1-3** [δ_H (500 MHz) and δ_C (125 MHz) in ppm, *J* in Hz][a].

No.	1[b]		2[b]		3[c]	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	155.4	-	155.9	-	172.1	-
2	119.4	6.96, brs	116.2	6.95, brs	44.6	2.63,d (15.0) 2.72, d (15.0)
3	139.0	-	141.8	-	78.0	-
4	114.2	7.26, brs	114.2	7.52, brs	39.0	3.19, d (13.4) 3.23, d (13.4)
4a	130.6	-	128.2	-	136.4	-
5	166.4	-	124.1	-	140.9	7.84, d (7.8)
6	106.4	7.46, s	116.7	8.39, s	119.2	7.75, d (7.8)
6a	137.3	-	125.1	-	132.5	-
7	188.9	-	187.3	-	189.1	-
7a	114.1	-	116.2	-	116.3	-
8	156.9	-	158.4	-	159.6	-
9	135.0	-	136.3	-	138.8	-
10	133.7	7.84, d (7.9)	133.2	7.87, d (7.8)	134.3	7.94, d (7.8)
11	119.6	7.72, d (7.9)	118.4	7.73, d (7.8)	119.9	7.80, d (7.8)
11a	134.7	-	132.4	-	133.0	-
12	182.6	-	186.3	-	189.2	-
12a	119.6	-	108.8	-	116.4	-
12b	122.1	-	162.1	-	162.4	-
13	20.9	2.40, s	21.9	2.40, s	23.5	1.43, s
OH	-	12.53, brs	-	14.40, brs	-	13.14, brs
OH	-	12.08, brs	-	13.41, brs	-	13.10, brs
OH	-		-	10.92, brs	-	
Sugar A, β-D-olivose						
1A	70.4	4.97, brd (10.5)	70.5	4.96, brd (10.8)	72.1	5.01, brd (10.9)
2A	35.9	2.22, m 1.63, ddd (11.6, 11.6, 10.5)	35.8	2.24, m 1.61, ddd (11.7, 11.7, 10.8)	37.4	2.40, m 1.60, ddd (11.6, 11.6, 10.9)
3A	75.7	3.85, ddd (11.6, 9.0, 4.4)	75.7	3.86, ddd (11.7, 9.0, 4.3)	77.4	3.88, ddd (11.6, 8.9, 4.4)
4A	73.6	3.51, dd (9.0, 9.0)	73.6	3.51, dd (9.0, 9.0)	75.1	3.58, dd (8.9, 8.9)
5A	73.5	3.59, m	73.5	3.60, m	75.1	3.62, m
6A	17.4	1.26, d (6.0)	17.4	1.27, d (6.0)	17.9	1.34, d (5.8)
Sugar B, α-L-cinerulose B						
1B	90.5	5.22, d (2.6)	90.2	5.23, d (2.4)	92.2	5.26, d (2.8)
2B	70.8	4.34, m	70.8	4.35, m	72.3	4.33, m
3B	39.6	2.90, dd (17.4, 2.6) 2.47, dd (17.4, 2.6)	39.8	2.91, dd (17.4, 2.6) 2.47, dd (17.3, 3.4)	40.6	2.84, dd (17.3, 2.7) 2.53, dd (17.3, 3.6)
4B	208.7	-	208.7	-	208.5	-
5B	76.9	4.72, q (6.6)	76.9	4.72, q (6.6)	78.2	4.76, q (6.8)
6B	16.0	1.24, d (6.6)	16.0	1.25, d (6.6)	16.5	1.26, d (6.8)
Sugar C, α-L-rhodinose						
1C					92.0	5.20, brs
2C					26.2	1.40, m
3C					25.3	1.95, m
4C					77.4	2.10, m 1.90, m
5C					67.0	4.09, m
6C					17.5	1.10, d (4.6)
Sugar D, α-L-aculose						
1D					96.0	5.31, d (3.5)
2D					145.2	7.03, dd (10.2, 3.5)
3D					127.2	6.02, d (10.2)
4D					197.3	-
5D					71.0	4.56, q (6.8)
6D					15.5	1.27, d (6.8)

[a] Residual signals of solvent as reference. [b] Measured in DMSO-d_6. [c] Measured in acetone-d_6.

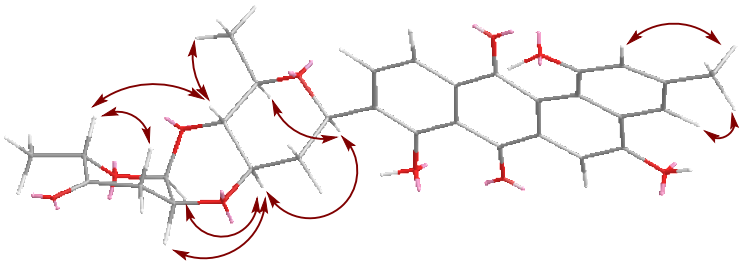


Figure 3. Key NOESY correlations for compound **1**.

Apart from the signals of angucycline aglycone, the chemical shifts of left protons and carbons of **1** were very similar to that of marangucycline B which has a disaccharide composed of β-D-olivose and α-L-cinerulose B [20]. The observed ^{1}H-^{1}H COSY correlations through H-1A (δ_H 4.97), H-2A (δ_H 2.22,1.63), H-3A (δ_H 3.85), H-4A (δ_H 3.51), H-5A (δ_H 3.59) to H-6A (δ_H 1.26) confirmed the presence of an olivose (Figure 2). The ^{1}H-^{1}H COSY correlations among H-1B (δ_H 5.22), H-2B (δ_H 4.34) and H-3B (δ_H 2.47,2.90), along with the HMBC correlations from CH_3-6B (δ_H 1.24) to C-4B (δ_C 208.7) and C-5B (δ_C 76.9), H-1B (δ_H 5.22) to C-5B (δ_C 76.9), and H-2B (δ_H 4.34) to C-4B (δ_C 208.7) confirmed the structure of cinerulose B. The interlinkage pattern of two deoxy sugars was deduced by the HMBC correlations from H-1B to C-4A, and the NOESY correlation between H-2B to H-3A (Figure 3). The relative configurations of both deoxy sugar moieties were identified as β-D-olivose and α-L-cinerulose B, respectively, owing to the observed NOESY correlations H-1A/H-5A,3A, H-3A/H-1B,2B, and H-4A/H-6A,5B in the most stable conformation (Figure 3). Based on the HMBC correlations of H-1A/C-8, H-1A/C-9 and H-1A/C-10, the disaccharide group was confirmed to connect with the aglycone at C-9 through β-D-olivose C-glycosidic bond. Thus, the structure of **1** was established and named as landomycin N according to the structural classification code of angucycline initially proposed by Rohr et al. [1] (Figure 1).

Galtamycin C (**2**) is an isomer of **1**, and its protonated molecule ion appeared at *m*/*z* 561.1752 ([M + H]$^+$, calcd for $C_{31}H_{29}O_{10}$, 561.1761) in HR-ESI-MS. The aliphatic signals in ^{1}H and ^{13}C NMR of **2** is almost same as that of **1**, suggesting the presence of disaccharide α-L-cinerulose B-(1→4, 2→3)-β-D-olivosyl moiety (Table 1). The ^{1}H NMR of **2** also showed five aromatic proton signals at δ_H 8.39 (s), 7.87 (d, *J* = 7.8 Hz), 7.73 (d, *J* = 7.8 Hz), 7.52 (brs) and 6.95 (brs) ppm, in which the singlet at δ_H 8.39 (s) is in more downfield than the corresponding singlet of **1**. In the ^{13}C NMR spectrum (Table 1), the chemical shifts of sixteen aromatic carbons ranging from δ_C 108.8 to 162.1 ppm and two quinone carbonyl carbons at δ_C 187.3 and 186.3 ppm were similar to that of rearranged linear angucycline glycosides, galtamycinone, grincamycin and grincamycin H [7,21]. Hence, compound **2** was supposed to possess a linear tetracyclic system. After entirely assigned the proton and carbon signals, the structure including the relative configurations of two deoxy sugar groups was detailed confirmed by ^{1}H-^{1}H COSY, HMBC and NOESY correlations (Figures 2 and 4), and named as galtamycin C (Figure 1).

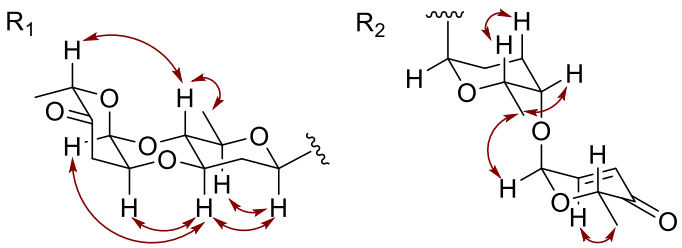


Figure 4. Key NOESY correlations in the sugar moiety of compounds **2** and **3**.

Vineomycin D (**3**) was isolated as yellow powder. Its HR-ESI-MS displayed the quisimolecule ions at *m*/*z* 838.3292 ([M + NH_4]$^+$, calcd for $C_{43}H_{52}NO_{16}$, 838.3286) and *m*/*z* 843.2838 ([M + Na]$^+$, calcd

for $C_{43}H_{48}NaO_{16}$, 843.2840), indicating the identical molecular formula $C_{43}H_{48}O_{16}$ with saquayamycin B (**4**). Similar to that of saquayamycin B, the ^1H NMR of **3** also showed two pairs of *ortho*-coupled aromatic protons signals at δ_H 7.94 (d, J = 7.8 Hz, H-10) and 7.80 (d, J = 7.8 Hz, H-11), and δ_H 7.84 (d, J = 7.8 Hz, H-5) and 7.75 (d, J = 7.8 Hz, H-6), along with a pair of olefinic protons signals of α,β-conjugated carbonyl group at δ_H 7.03 (dd, J = 10.2, 3.5 Hz, H-2D) and 6.02 (d, J = 10.2 Hz, H-3D) (Table 1). The ^1H and ^{13}C NMR spectroscopic data also revealed three O-glycosidic anomeric proton and carbon signals at δ_H 5.31 (d, J = 3.5 Hz) and δ_C 96.0 (CH-1D), δ_H 5.26 (d, J = 2.8 Hz) and δ_C 92.2 (CH-1B), and δ_H 5.20 (brs) and δ_C 92.0 (CH-1C), and one C-glycosidic anomeric proton and carbon signals at δ_H 5.01 (brd, J = 10.9 Hz) and δ_C 72.1 (CH-1A). The most obvious difference in ^{13}C NMR spectra of **3** and **4** is the disappearance of a signal above δ_C 200 ppm in **3**, and meanwhile the appearance of a signal at δ_C 172.2 ppm characteristic of a carboxylic acid or ester group. Accordingly, compound **3** was deduced to have a tricyclic system with a side chain, as a result of A-ring opening of saquayamycin B (**4**) [6,7,22]. The key HMBC correlations associated with four aromatic protons, two methylene groups appearing as two couple of AB system [δ_H 3.32 (d, J = 13.4 Hz, H-2) and 3.19 (d, J = 13.4 Hz, H-2), δ_H 2.78 (d, J = 15.0 Hz, H-4) and 2.63 (d, J = 15.0 Hz, H-4)], along with methyl group at δ_H 1.43 ppm could confirm the tricyclic system and the attached side chain (Figure 2). The presence of two disaccharides α-L-cinerulose B-(1→4, 2→3)-β-D-olivosyl and α-L-aculose-(1→4)-α-L-rhodinosyl groups were further deduced by ^1H-^1H COSY, HMBC and NOESY correlations (Figures 2 and 4). The HMBC correlations of H-1A (δ_H 5.01) to C-8 (δ_C 159.6), C-9 (δ_C 138.2) and C-10 (δ_C 134.3) suggested the α-L-cinerulose B-(1→4, 2→3)-β-D-olivosyl group was attached with C-9 through a C-glycosidic bond. The HMBC correlation between H-3A (δ_H 5.20) and C-3 (δ_C 78.0) indicated the α-L-aculose-(1→4)-α-L-rhodinosyl group was attached with C-3. In general, tricyclic angucyclines are derived from typical angucyclines with the same tetracyclic core structure under acidic conditions [1]. Accordingly, the configuration of C-3 is proposed to same as that of saquayamycin B (**4**) and other tricyclic angucyclines, e.g. grincamycin B, vineomycin B_2 and fridamycin D [6,7,22]. Thus, the structure of compound **3** was established and named as vineomycin D (Figure 1).

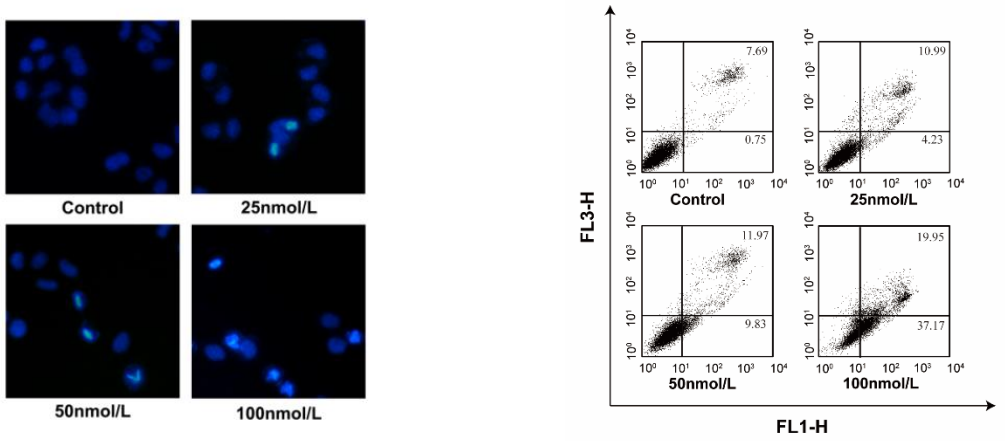
There's a few of anguclines, such as saquayamycin B, landomycin E, vineomycin A_1 etc., have been reported to exhibit remarkable antitumor activity against a series of tumor cell lines [3,7,10]. Though, the distinct *in vivo* toxicity restricted the further development of these compounds. Recently, an atypical angucycline, lomaiviticin A, was reported under preclinical evaluation for antitumor treatment due to its prominent cytotoxicity and effects of inducing double-strand breaks in DNA [14,23]. In this research, compounds **1**-**4** was assayed the cytotoxic activity against normal liver cell LO_2 and hepatoma carcinoma cells HepG-2, SMMC-7721 and plc-prf-5 by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Table 2). At the concentrations of 40 μM, compounds **1**-**3** displayed no cytotoxicity against any tested cells. Saquayamycin B (**4**) displyed potent cytotoxic activity against HepG-2, SMMC-7721 and plc-prf-5 cells, with IC_{50} values 0.135, 0.033 and 0.244 μM respectively, superior to positive drug doxorubicin. Meanwhile, treated with saquayamycin B at concentrations ranging from 0.025 to 0.100 μM for 24 hours, SMMC-7721 cells exhibited chromatin dispersion and formation of apoptosis body in DAPI staining test (Figure 5, **a**). The apoptotic ratio of SMMC-7721 cells was dependent on the concentrations of saquayamycin B (Figure 5, **b**).

Table2. Cytotoxicity of **1**-**4** against LO_2, HepG-2, SMMC-7721 and plc-prf-5 cells (IC_{50}, μM).

Cell lines	LO_2	HepG-2	SMMC-7721	plc-prf-5
1	> 40	> 40	> 40	> 40
2	> 40	> 40	> 40	> 40
3	> 40	> 40	> 40	> 40
4	0.343 ± 0.081	0.135 ± 0.056	0.033 ± 0.005	0.244 ± 0.001
Doxorubicin	2.26 ± 0.16	0.919 ± 0.599	0.706 ± 0.004	1.03 ± 0.99

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 6 November 2018 doi:10.20944/preprints201811.0137.v1

Peer-reviewed version available at Mar. Drugs 2018, 16, 470; doi:10.3390/md16120470



(**a**) (**b**)

Figure 5. (**a**) Fluorescence micrographs of untreated and saquayamycin B-treated SMMC-7721 cells (24 h) stained with DAPI, Magnification: 100×; (**b**) Quantification of saquayamycin B-induced apoptosis in SMMC-7721 cell using flow cytometric analysis. $^{**}p < 0.01$ versus saquayamycin B 0 μM group.

3. Materials and Methods

3.1. General Experimental Procedures

Optical rotations were measured with an Anton Paar MCP 200 polarimeter with a sodium lamp (589 nm) (Anton Paar GmbH, Graz, Austria). UV spectra were obtained on Genesys 10S UV-Vis spectrometer (Thermo Fisher Scientific Ltd, Waltham, USA); IR spectra were acquired with a Nicolet IS5 FT-IR spectrometer (Thermo Fisher Scientific, Waltham, USA); NMR spectra were recorded on Bruker AVANCE III 500 spectrometer (Bruker Inc., Karlsruhe, Germany). HPLC-MS were acquired on Agilent 1200HPLC/6520QTOFMS (Agilent Technologies Inc., Santa Clara, USA). Semi-preparative HPLC isolation was performed on Agilent 1260 Infinity II (Agilent Technologies Inc., Santa Clara, USA) with an ODS column (YMC-Triart C18, 10×250 mm, YMC Co. Ltd. Tokyo, Japan). Silica gel (200-300 and 300-400 mesh) used in column chromatography (CC) and silica gel GF_{254} (10-40 μm) used in thin layer chromatography (TLC) were supplied by Qingdao Marine Chemical Factory in China.

3.2. Actinomycetes Strain

The intertidal sediments were collected after tide falling in Xiaoshi Island, Weihai, China in September 2016. The strain OC1610.4 was isolated from this sediments using Gause's synthetic medium (20 g/L amylogen, 1 g/L KNO_3, 0.5 g/L NaCl, 0.5 g/L $K_2HPO_4{\cdot}H_2O$, 0.5 g/L $MgSO_4{\cdot}H_2O$, 0.01 g/L $FeSO_4{\cdot}H_2O$, and 3.0 % sea salt) containing potassium dichromate (6 μg/mL) and nalidixic acid (20 μg/mL) as antifungal and antibacterial agents. The procedures of DNA extraction and PCR amplification of 16S rRNA were same as described in reference [24]. The nucleotide sequence of OC1610.4 strain was sequenced at the Shanghai Sangon Biotech Co., China, and deposited to GenBank (Accession no. MK045847). Voucher strain (No. OC1610.4) was deposited at Laboratory of Natural Products Chemistry, Department of Pharmacy, Shandong University at Weihai.

3.3. Fermentation, Extraction and Isolation

The spore and mycelia suspension of strain OC1610.4 was inoculated in Erlenmeyer flasks (500 mL) each of which contains 100 mL S-medium (10 g/L glucose, 4 g/L yeast extract, 4 g/L K_2HPO_4, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 3.0 % sea salt). Total 30 L medium was shaking-cultured at 140 rpm and 28 °C for 9 days. The fermentation broth including mycelia was extracted with equal volume of EtOAc for five times to give 4.6 g crude extracts. The extracts was subjected to silica gel CC (60 g, 200-300 mesh) eluting with n-hexane-acetone (10:1, 5:1, 2:1 and acetone) to give four fractions F_1-F_4. Part (72 mg) of fraction F_1 (n-hexane-acetone 10:1) was isolated by semi-preparative HPLC eluting with CH_3OH-H_2O (70:30, v/v) to give **5** (5.6 mg). Fraction F_2 (n-hexane-acetone 5:1, 267mg) was further purified by silica gel CC (1 g, 300-400 mesh) eluting with n-hexane-acetone (10:1)

to give sub-fractions F_{2a} and F_{2b}. Sub-fractions F_{2a} (67 mg) was purified by semi-preparative HPLC eluting with CH_3OH-H_2O (38:62, v/v) to give **6** (4.6 mg). The sub-fractions F_{2b} (26 mg) was a mixture presenting two brown spots on TLC, and was isolated by semi-preparative HPLC eluting with CH_3CN-H_2O (70:30, v/v) to give **1** (4.2 mg) and **2** (3.4 mg). Fraction F_3 (n-hexane-acetone 2:1, 670 mg) was subjected to a silica gel CC (10 g, 200-300 mesh) eluting with CH_3Cl-CH_3OH (20:1) to give two subfractions F_{3a} and F_{3b}. From F_{3a} (220mg), compound **4** (18 mg) was purified using a low pressure silica gel CC (1 g, 300-400 mesh) eluting with n-hexane-acetone (4:1). Subfractions F_{3b} (67 mg) was isolated by semi-preparative HPLC eluting with CH_3CN-H_2O (65:35, v/v) to give **3** (5 mg).

Landomycin N (**1**): brown amorphous powder; $[\alpha]^{25}_D$ +92 (*c* 0.002, MeOH); UV (MeOH) λ_{max} (log ε) 225 (2.99), 327 (2.65) nm; IR (KBr) ν_{max} 3203, 2974, 2916, 1726, 1629, 1607, 1578, 1433, 1295, 1111, 1075, 852, 791 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, Table 1; HR-ESI-MS *m/z* 561.1753 ($[M + H]^+$, calcd for $C_{31}H_{29}O_{10}$, 561.1761).

Galtamycin C (**2**): reddish-brown amorphous powder; $[\alpha]^{25}_D$ +285 (*c* 0.003, MeOH); UV (MeOH) λ_{max} (log ε) 265 (2.40), 340 (2.07) nm; IR (KBr) ν_{max} 3383, 2917, 2879, 1727, 1657, 1608, 1584, 1525, 1471, 1286, 1247, 1108, 1017, 872, 836, 716 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data, Table 1; HR-ESI-MS *m/z* 561.1752 ($[M + H]^+$, calcd for $C_{31}H_{29}O_{10}$, 561.1761).

Vineomycin D (**3**): yellow amorphous powder; $[\alpha]^{25}_D$ +69 (*c* 0.050, MeOH); UV (MeOH) λ_{max} (log ε) 230 (3.56), 259 (3.28), 295 (2.83) nm; IR (KBr) ν_{max} 3557, 2978, 2935, 1731, 1702, 1625, 1581, 1431, 1259, 1080, 1014, 899, 808 cm^{-1}; ^1H NMR (500 MHz, acetone-d_6) and ^{13}C NMR (125 MHz, acetone-d_6) data, Table 1; HR-ESI-MS *m/z* 838.3292 ($[M + NH_4]^+$, calcd for $C_{43}H_{52}NO_{16}$, 838.3286) and *m/z* 843.2838 ($[M + Na]^+$, calcd for $C_{43}H_{48}NaO_{16}$, 843.2840).

3.4. Cytotoxicity Assays, DAPI Staining Test and Flow Cytometric Analysis

The cytotoxicity evaluations of **1**-**4** against normal liver cell and hepatoma carcinoma cells were carried out using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Doxorubicin was used as positive control drug and deionized H_2O with the same DMSO concentration was used as parallel control. DAPI staining test was employed to qualitatively observe apoptosis, and the apoptotic ratio was measured by flow cytometric analysis (Becton Dickinson FACScan, CA, USA). These tests were conducted using the methods as previously described [25,26].

4. Conclusions

Four angucycline glycosides including landomycin N (**1**), galtamycin C (**2**), vineomycin D (**3**) and saquayamycin (**4**), along with two alkaloids 1-acetyl-β-carboline (**5**) and indole-3-acetic acid (**6**), were characterized from the fermentation broth of intertidal sediments strain *Streptomyces* sp. OC1610.4. Galtamycin C (**2**) and vineomycin D (**3**) are rearranged angucycline derivatives respectively possessing a linear tetracyclic and a tricyclic framework of angucycline. Among the isolated angucycline glycosides, saquayamycin B (**4**) displayed the most cytotoxic activity against hepatoma carcinoma cells HepG-2, SMMC-7721 and plc-prf-5. Vineomycin D (**3**) and saquayamycin B (**4**) are isomers, comprising the same two disaccharide groups in structures. Though, vineomycin D showed no cytotoxicity at the concentration 40 μM, indicating that the intact A-ring or the two angular hydroxyl groups in angucycline aglycone is probably essential to its cytotoxicity. Saquayamycin B could lead to apoptosis of SMMC-7721 cell. The more definite antineoplastic mechanisms of saquayamycin B is necessary to further investigate.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. This section includes the HR-ESI-MS, 1D and 2D NMR spectra for compounds **1**-**4**.

Author Contributions: A.P. conducted the main experiments, including the isolation and culture of strain, the isolation and structural elucidation of compounds. X.Q performed the large-scale fermentation. F.L. conducted the antitumor assay. X.L. guided the antitumor assay. E.L. guided the HPLC isolation and NMR measurement. W.X supervised the whole work and wrote the manuscript. All authors have read the manuscript and approved the final manuscript for submission.

Funding: This research was funded by the Natural Science Foundation of Shandong Province, China (ZR2014HM018) and National Natural Science Foundation of China (81872771).

Acknowledgments: We are grateful to Hong-Bo Zheng from Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Science, Shandong University, Jinan 250012, China, for recording HR-ESI-MS.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rohr, J.; Thiericke, R. Angucycline group antibiotics. *Nat. Prod. Rep.* **1992**, *9*, 103-137.
2. Kharel, M.K.; Pahari, P.; Shepherd, M.D.; Tibrewal, N.; Nybo, S.E.; Shaaban, K.A.; Rohr, J. Angucyclines: biosynthesis, mode-of-action, new natural products, and synthesis. *Nat. Prod. Rep.* **2012**, *29*, 264-325.
3. Shaaban, K.A.; Ahmed, T.A.; Leggas, M.; Rohr, J.; Saquayamycins G-K, cytotoxic angucyclines from *Streptomyces* sp. including two analogues bearing the aminosugar rednose. *J. Nat. Prod.* **2012**, *75*, 1383-1392.
4. Gui, C.; Liu, Y.N.; Zhou, Z.B.; Zhang, S.W.; Hu, Y.F.; Gu, Y.C.; Huang, H.B.; Ju, J.H. Angucycline glycosides from mangrove-derived *Streptomyces diastaticus* subsp. SCSIO GJ056. *Mar. Drugs* **2018**, *16*, 185.
5. Erb, A.; Luzhetskyy, A.; Hardter, U.; Bechthold, A. Cloning and sequencing of the biosynthetic gene cluster for saquayamycin Z and galtamycin B and the elucidation of the assembly of their saccharide chains. *ChemBioChem* **2009**, *10*, 1392-1401.
6. Huang, H.B.; Yang, T.T.; Ren, X.M.; Liu, J.; Song, Y.X.; Sun, A.J.; Ma, J.Y.; Wang, B.; Zhang, Y.; Huang, C.G.; Zhang, C.S.; Ju, J.H. Cytotoxic angucycline class glycosides from the deep sea actinomycete *Streptomyces lusitanus* SCSIO LR32. *J. Nat. Prod.* **2012**, *75*, 202-208.
7. Zhu, X.C.; Duan, Y.W.; Cui, Z.M.; Wang, Z.; Li, Z.X.; Zhang, Y.; Ju, J.H.; Huang, H.B. Cytotoxic rearranged angucycline glycosides from deep sea-derived *Streptomyces lusitanus* SCSIO LR32. *J. Antibiot.* **2017**, *70*, 819-822.
8. Helaly, S.E.; Goodfellow, M.; Zinecker, H.; Imhoff, J.F.; Sussmuth, R.D.; Fiedler, H.P. Warkmycin, a novel angucycline antibiotic produced by *Streptomyces* sp Acta 2930. *J. Antibiot.* **2013**, *66*, 669-674.
9. Nakagawa, K.; Hara, C.; Tokuyama, S.; Takada, K.; Imamura, N. Saprolmycins A-E, new angucycline antibiotics active against *Saprolegnia parasitica*. *J. Antibiot.* **2012**, *65*, 599-607.
10. Panchuk, R.R.; Lehka, L.V.; Terenzi, A.; Matselyukh, B.P.; Rohr, J.; Jha, A.K.; Downey, T.; Kril, I.J.; Herbacek, I.; van Schoonhoven, S.; Heffeter, P.; Stoika, R.S.; Berger, W. Rapid generation of hydrogen peroxide contributes to the complex cell death induction by the angucycline antibiotic landomycin E. *Free Radical Bio. Med.* **2017**, *106*, 134-147.
11. Korynevska, A.; Heffeter, P.; Matselyukh, B.; Elbling, L.; Micksche, M.; Stoika, R.; Berger, W. Mechanisms underlying the anticancer activities of the angucycline landomycin E. *Biochem. Pharmacol.* **2007**, *74*, 1713-1726.
12. Fidan, O.; Yan, R.M.; Gladstone, G.; Zhou, T.; Zhu, D.; Zhan, J.X. New insights into the glycosylation steps in the biosynthesis of Sch47554 and Sch47555. *ChemBioChem* **2018**, *19*, 1424-1432.
13. Salem, S.M.; Weidenbach, S.; Rohr, J. Two cooperative glycosyltransferases are responsible for the sugar diversity of saquayamycins isolated from *Streptomyces* sp KY 40-1. *Acs Chem. Biol.* **2017**, *12*, 2529-2534.
14. Huang, C.S.; Yang, C.F.; Zhang, W.J.; Zhang, L.P.; De, B.C.; Zhu, Y.G.; Jiang, X.D.; Fang, C.Y.; Zhang, Q.B.; Yuan, C.S.; Liu, H.W.; Zhang, C.S. Molecular basis of dimer formation during the biosynthesis of benzofluorene-containing atypical angucyclines. *Nat. Commun.* **2018**, *9*, 2088.
15. Jose, P.A.; Jha, B. Intertidal marine sediment harbours Actinobacteria with promising bioactive and biosynthetic potential. *Sci. Rep.* **2017**, *7*, 10041.
16. Zhou, T.S.; Ye, W.C.; Wang, Z.T.; Che, C.T.; Zhou, R.H.; Xu, G.J.; Xu, L.S. β-Carboline alkaloids from *Hypodematium squamuloso-pilosum*. *Phytochemistry* **1998**, *49*, 1807-1809.

17. Han, X.; Hou,L.K.; Hou, J.; Zhang, Y.Y.; Li, H.Y.; Li, W.L. Heterologous expression of a VioA variant activates cryptic compounds in a marine-derived *Brevibacterium* strain. *Mar. Drugs* **2018**, *16*, 191.
18. Uchida, T.; Imoto, M.; Watanabe, Y.; Miura, K.; Dobashi, T.; Matsuda, N.; Sawa, T.; Naganawa, H.; Hamada, M.; Takeuchi, T.; Umezawa, H. Saquayamycins, new aquayamycin-group antibiotics. *J. Antibiot.* **1985**, *38*, 1171-1181.
19. Feng, Z.M.; He, J.; Jiang, J.S.; Chen, Z.; Yang, Y.N.; Zhang, P.C. NMR solution structure study of the representative component hydroxysafflor yellow A and other quinochalcone C-glycosides from *Carthamus tinctorius*. *J. Nat. Prod.* **2013**, *76*, 270-274.
20. Song, Y.X.; Liu, G.F.; Li, J.; Huang, H.B.; Zhang, X.; Zhang, H.; Ju, J.H. Cytotoxic and antibacterial angucycline- and prodigiosin-analogues from the deep-sea derived *Streptomyces* sp. SCSIO 11594. *Mar. Drugs* **2015**, *13*, 1304-1316.
21. Stroch, K.; Zeeck, A.; Antal, N.; Fiedler, H.P. Retymicin, galtamycin B, saquayamycin Z and ribofuranosyllumichrome, novel secondary metabolites from *Micromonospora* sp Tu 6368 - II. Structure elucidation. *J. Antibiot.* **2005**, *58*, 103-110.
22. Maskey, R.P.; Helmke, E.; Laatsch, H. Himalomycin A and B: isolation and structure elucidation of new fridamycin type antibiotics from a marine *Streptomyces* isolate. *J. Antibiot.* **2003**, *56*, 942-949.
23. Herzon, S.B. The mechanism of action of (-)-lomaiviticin A. *Accounts Chem. Res.* **2017**, *50*, 2577-2588.
24. Zhang, X.M.; Liu, X.; Wang, Z.; Tian, Z.H.; Xie, W.D. Viridobrunnines A and B, antimicrobial phenoxazinone alkaloids from a soil associated *Streptomyces* sp. *Heterocycles* **2015**, *91*, 1809-1814.
25. Liu, S.S.; Wang, Y.F.; Ma, L.S.; Zheng, B.B.; Li, L.; Xie, W.D.; Li, X. 1-Oxoeudesm-11(13)-eno-12,8a-lactone induces G_2/M arrest and apoptosis of human glioblastoma cells *in vitro*. *Acta Pharmacol. Sin.* **2013**, *34*, 271-281.
26. Zheng, B.B.; Wu, L.H.; Ma, L.S.; Liu, S.S.; Li, L.; Xie, W.D.; Li, X. Telekin induces apoptosis associated with the mitochondria-mediated pathway in human hepatocellular carcinoma cells. *Biol. Pharm. Bull.* **2013**, *36*, 1118-1125.