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Chromomycin SA analogs from a marine-derived *Streptomyces* sp.

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ABSTRACT

Two chromomycin SA analogs, chromomycin SA_3 and chromomycin SA_2, along with deacetylchromomycin A_3 and five previously reported chromomycin analogs were isolated from a marine-derived *Streptomyces* sp. The structures of the new compounds were determined by spectroscopic methods including 1D and 2D NMR techniques, HRMS and chemical methods. Chromomycin SA_3 and chromomycin SA_2 are the first naturally occuring chromomycin analogs with truncated side-chains. Biological evaluation of chromomycin analogs for cytotoxicity against two non-small cell lung cancer (NSCLC) cell-lines, A549 and HCC44, demonstrated a decrease in cytotoxicity for the truncated sides chain chromomycin analogs.

1. Introduction

Chromomycin A_3 (**1**) is a glycosylated polyketide that belongs to the class of anti-tumor compounds called aureolic acids, which are produced by a number of terrestrial bacteria of the genus *Streptomyces*.[1] The chromomycins, mithramycins and other aureolic acid family members are effective against many types of human tumors, including brain and testicular carcinomas.[2,3] Mithramycin A in particular has been used for the treatment of Paget's disease and other bone growth disorders.[4] Due to the severe hepatic, renal and gastrointestinal toxicity associated with the use of this family of compounds, their clinical use has been limited.[5] Chromomycin A_3 and aureolic acid natural products interact with the minor groove of DNA in regions of high GC content in a non-intercalative manner, via cross-linking of DNA through a 2:1 complex of the natural product to Mg^{2+}.[6] The interaction of **1** with DNA causes a DNA-dependent inhibition of RNA synthesis, rendering the drugs potent anti-tumor characteristic.[7] Through intensive NMR and X-ray crystallographic studies it was determined the deoxysugars are necessary for stabilizing the complex with DNA[8] and that acylation of the sugars increases DNA-sequence specificity by rigidifying the oligosaccharides.[9] The C-3 polyoxygenated pentyl side-chain interacts directly with the phosphate backbone of DNA, and it has been proposed that modifications of the side-chain can be exploited for modulation of the biological activity of mithramycin.[10,11] We report here the isolation and biological activity of three new chromomycin analogs that have structural modifications in the C-3 ketide side-chains and/or in the acylation of the oligosaccharides.

As part of our efforts to identify natural products with selective cytotoxicity against cancer cells, we screened a library of 1500 natural products fractions against a panel of cancer cell lines. From this screen we obtained a series of fractions from the marine-derived *Streptomyces* sp. (strain SNB-005) that exhibited modest selectivity and potency for the glioblastoma derived cell-line T98G and the non-small cell lung cancer line A549. Analysis of the active fractions by LC–UV-MS showed the common UV profile indicative of aureolic acid, with λ_{max} = 410, 320, 280 and 235 nm. More detailed analysis by MS and NMR revealed the presence of three previously uncharacterized members of the natural product family. Specifically, chromomycin SA_3 (**2**) and chromomycin SA_2 (**3**) have an abbreviated two carbon side-chain at C-3, while deacetyl chromomycin A_3 (**4**) has a unique acylation pattern on the deoxysugars. In addition to the new chromomycin analogs, we identified the known compounds chromomycin SA (**5**),[12] chromomycin A_3 (**1**),[1c] chromomycin 02-3G (**6**),[13] aburamycin (**7**)[14] and chromomycin A_2 (**8**).[15]

2. Results and discussion

Marine bacterium SNB-005 was isolated from a sediment sample collected from a hypersaline lake at East Plana Cay, Bahamas. Bacterial spores were collected via stepwise centrifugation and isolated on a humic acid media. Analysis of 16S rRNA revealed 99% identity to *Streptomyces* sp. and is highly related to *Streptomyces* species that produce aureolic acid natural products. A large-scale

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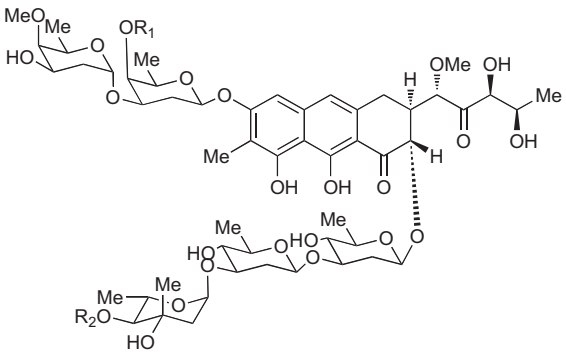
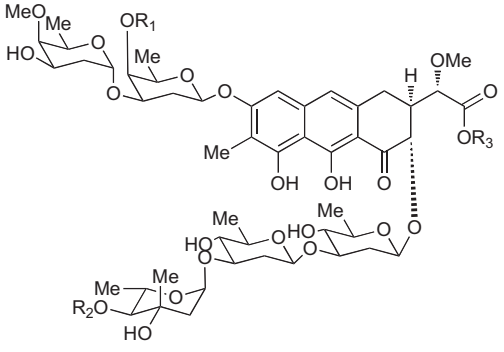
regrow (20 L) by shake fermentation was carried out to obtain sufficient material for full chemical and biological analysis of the new analogs. The excreted metabolites were collected using XAD-7 resin and the resulting crude extract purified using flash reversed phase chromatography (C_{18}) to give fractions enriched in aureolic acid containing metabolites. Final purification by gradient reversed phase HPLC gave pure compounds **1**–**8** (Fig. 1).

Chromomycin SA_3 (**2**) was isolated as a yellow amorphous powder. The molecular ions identified in negative ESI MS at *m/z* 1081 $[M-H]^-$ and in the positive ESI-MS at *m/z* 1105 $[M+Na]^+$ allowed the deduction of its molecular weight of 1082 Da, 100 Da less than **1**, indicating a more substantial structural change than the loss of an acetyl group on the oligosaccharide. High-resolution ESI-MS gave an $[M+Na]^+$ at *m/z* 1105.4439 consistent with a molecular formula of $C_{52}H_{74}O_{24}$ (calcd for $C_{52}H_{74}O_{24}Na$). The ^1H NMR spectrum of **2** closely resembled that of the chromomycin family of compounds, with two distinctive aromatic protons at δ 6.76 and 6.62 ppm and anomeric protons from δ 4.6 to 5.5 ppm (Table 1). More detailed analysis of the ^1H NMR spectra of **2** compared to **1** revealed the absence of the characteristic doublet at δ 4.12 ppm and the dq at 4.20 ppm, representative of H3′ and H4′ on the pentyl side-chain.[16] COSY correlations from H3 (δ 2.55 ppm) to H1′ (δ 4.27 ppm) confirmed the existence of a side-chain, but a lack of HMBC correlations limited the structural assignment.[17] Based on the difference in molecular formula and the lack of ^1H NMR signals for H3′–H5′, we deduced that the pentyl side-chain had been truncated to two carbons, terminating in a carboxylic acid. The carboxylic acid was confirmed by conversion of **2** to the chromomycin SA_3 methyl ester (**2a**) with TMS-CH_2N_2 and analysis by ^1H NMR showed a new –OMe signal at δ 3.74 ppm. Methyl ester **2a** provided much cleaner NMR data (Tables 1 and 2) and allowed for unambiguous assignments of the aglycon and the two-carbon side-chain. The key HMBC correlations to establish the side-chain were from H1′ (δ 4.32 ppm) and from the newly formed methyl ester singlet (δ 3.74 ppm) to a carbonyl carbon at δ 172.8 (Fig. 2). The truncated two-carbon side-chain of **2** has been previously reported for chromomycin SA (**5**), which was isolated from a *Streptomyces griseus* strain that had been genetically altered in the chromomycin biosynthetic gene cluster, specifically the inactivation of ketoreductase gene *cmmWI* (discussed below).[12] Chromomycin SA_3 (**2**) is the first example of a naturally occurring chromomycin analog that contains the two-carbon side-chain at C-3.



1: $R_1 = R_2 = Ac$
4: $R_1 = Ac\ R_2 = H$
6: $R_1 = R_2 = H$
7: $R_1 =H, R_2 = (CH_3)_2CHCO$
8: $R_1 = Ac, R_2 = (CH_3)_2CHCO$



2: $R_1 = Ac, R_2 = H, R_3 = H$
2a: $R_1 = Ac, R_2 = H, R_3 = CH_3$
3: $R_1 = Ac, R_2 = (CH_3)_2CHCO, R_3 = H$
3a: $R_1 = Ac, R_2 = (CH_3)_2CHCO, R_3 = CH_3$
5: $R_1 = R_2 = Ac, R_3 = H$

Figure 1. Chromomycin analogs.

The remaining signals in the ^1H and ^{13}C NMR of **2a** closely resembled those found in **1** (Table 2), however the molecular formula and a detailed analysis of the ^1H and ^{13}C NMR revealed that **2a** only contained a single acetyl group in the saccharide portion of the molecule. Detailed analysis of the COSY, HSQC, and HMBC spectra of **2a** showed the disaccharide portion of 4-*O*-acetyl-D-oliose (sugar A) and 4-*O*-methyl-D-oliose (sugar B) was fully intact, with key HMBC correlations from the anomeric proton H-A1 (δ 5.19 ppm) to C6 of the aglycon (δ 159.3 ppm) and from H-A4 (δ 5.12 ppm) to the carbonyl carbon of the acetate (δ 171.1 ppm). The trisaccharide portion of **2a** was assigned to be D-olivose (sugar C), D-olivose (sugar D), and L-chromose (sugar E). The key HMBC correlations for **2a** were from H-C1 (δ 4.98 ppm) to C2 of the aglycon (δ 76.2 ppm), H-D1 (δ 4.56 ppm) to C3 (δ 81.9 ppm) and H-E1 (δ 4.92 ppm) to D4 (δ 75.0 ppm). In comparison to **1**, the trisaccharide moiety of **2** lacks the 4-*O*-acetyl on the L-chromose residue. A table with 2D-correlations is included in Supplementary Table S1.

Chromomycin SA_2 (**3**) was also obtained as a yellow amorphous powder. HRESIMS of **3** showed a $[M+Na]^+$ ion peak at *m/z* 1175.4874, indicating the molecular formula of **3** was $C_{56}H_{80}O_{25}$. The ^1H NMR spectrum of **3** closely resembled that of chromomycin SA_3 (**2**), showing two aromatic protons that could be attributed to the aglycon (Table 1).[16] As in **2**, the characteristic signals of the pentyl side-chain were lacking. As we had difficulty in the interpretation of the NMR data of the free carboxylic acid of **2**, we immediately converted **3** to the methyl ester (**3a**) with TMS–CH_2N_2. Analysis of ^1H NMR of **3a** showed a new methyl singlet at δ 3.76 ppm, confirming the presence of a carboxylic acid. Detailed analysis of the COSY, HSQC, and HMBC spectra of **3a** once again confirmed the disaccharide moiety to be 4-*O*-acetyl-D-oliose (sugar A) and 4-*O*-methyl-D-oliose (sugar B), attached to the aglycon at C-6. COSY and HMBC correlations established the trisaccharide as D-olivose (sugar C), D-olivose (sugar D), and L-chromose B (sugar E). There were two remaining signals in the ^1H NMR spectra, a large methyl doublet at δ 1.17 ppm that integrated to six protons and a methine at δ 2.57 ppm, suggestive of an isopropyl ester, which was conformed by HMBC correlations from the methyl doublet and H-E4 to a carbonyl carbon at δ 177.3 ppm. The acetate substitution pattern on **3** resembles that found in chromomycin A_2 (**8**). A table with 2D-correlations is included in Supplementary Table S2.

We have assigned the relative and absolute configuration of **2** and **3** as shown, based on a combination of coupling constant analysis and biosynthetic rational. The relative configuration of the aglycon of **2** and **3**, specifically, the anti configuration of H2/H3 could be assigned based on a large 11.6 Hz coupling constant, consistent with the chromomycin family of compounds. The relative configuration of the sugar moieties was determined to be consistent with all previously isolated members of the chromomycin family of analogs via a thorough analysis of coupling constants (Table 1) and comparison to data for existing members of the

Table 1
^{1}H NMR data of chromomycin SA analogs **1–3, 2a, 3a**[a]

Position	1	2	2a	3	3a
2	4.64, d (11.8)	4.63, d (11.6)	4.64, d (11.9)	4.66, d (11.8)	4.65, d (11.6)
3	2.54, m	2.55, m, overlap	2.55, m	2.59, m, overlap	2.55, m
4	3.00, d (15.0)	3.10, d (15.4)	3.10, d (15.2)	3.10, d (16.3)	3.10, d (16.2)
4	2.57, m	2.62, m, overlap	2.64, overlap	2.74, dd (16.3, 3.4)	2.66, dd (16.2, 3.5)
5	6.59, s	6.62, s	6.63, s	6.62, s	6.65, s
10	6.72, s	6.76, s	6.78, s	6.77, s	6.79, s
7-CH_3	2.10, s	2.13, s	2.14, s	2.15, s	2.15, s
1′	4.69, br s	4.27, br s	4.32, d (1.6)	4.29, br s	4.33, d (1.9)
3′ (2′-OCH_3)	4.12, d (2.1)		3.74, s	—	3.76, s
4′	4.20, qd (6.4, 6.4)	—	—	—	—
5′	1.25, d (6.4)	—	—	—	—
1′-OCH_3	3.41, s	3.47, s	3.46, s	3.48, s	3.48, s
A1	5.19, dd (9.8, 2.2)	5.20, dd (9.6, 2.0)	5.19, dd (10.4, 2.5)	5.22, dd (9.8, 2.0)	5.21, dd (10.1, 2.3)
A2	2.13, m	2.15, m, overlap	2.16, m, overlap	2.17, m	2.18, m, overlap
	2.01, dd (10.5, 4.0)	2.01, dd (10.6, 4.7)	2.01, dd (11.7, 3.5)	2.03, dd (10.2, 4.7)	2.04, m
A3	3.91, m	3.94, m	3.94, ddd (9.1, 3.5, 2.6)	3.98, m	3.94, ddd (9.1, 3.9, 2.5)
A4	5.10, d (2.6)	5.11, d (1.9)	5.12, d (2.6)	5.13, d (2.8)	5.13, d (2.5)
A5	3.78, q (6.6)	3.78, q (5.9)	3.79, q (6.3)	3.82, q (6.4)	3.79, q (6.5)
A6	1.20, d (6.6)	1.24, d (5.9)	1.25, d (6.3)	1.26, d (6.4)	1.26, d (6.5)
CH_3-CO	2.09, s	2.12, s	2.12, s	2.15, s	2.14, s
B1	5.05, br s	5.06, d (3.3)	5.06, d (3.5)	5.08, d (4.0)	5.08, d (4.0)
B2	1.76, dt (12.2, 3.8)	1.75, dt (12.1, 3.3)	1.75, dt (11.9, 3.2)	1.78, dt (12.8, 4.0)	1.77, dt (12.8, 4.0)
	1.64, m	1.67, dd (12.1, 4.8)	1.67, dd (11.9, 5.2)	1.67, dd (12.8, 5.3)	1.66, dd (12.4, 5.3)
B3	3.89, m	3.90, m	3.91, dt (12.0, 3.6)	3.93, m	3.92, m
B4	3.15, d (2.6)	3.16, br s	3.17, br s	3.15, overlap	3.18, d (3.1)
B5	3.81, q (6.5)	3.82, q (6.5)	3.83, q (6.6)	3.84, q (6.6)	3.84, q (6.6)
B6	1.15, d (6.5)	1.22, d (6.5)	1.22, d (6.6)	1.24, d (6.6)	1.24, d (6.6)
B4-OCH_3	3.53, s	3.54, s	3.55, s	3.56, s	3.56, s
C1	5.01, dd (9.9, 1.7)	4.98, d (9.4)	4.98, dd (9.4, 1.4)	5.00, dd (10.1, 1.6)	4.99, dd (9.1, 1.8)
C2	2.46, dd (12.0, 5.0)	2.43, overlap	2.43, dd (12.9, 5.7)	2.46, dd (12.7, 6.8)	2.43, overlap
	1.64, dd (12.0, 12.0)	1.64, m	1.64, m	1.65, m	1.66, m
C3	3.57, m	3.56, m	3.57, m	3.60, m	3.58, m
C4	3.05, dd (8.5, 8.5)	3.05, dd (8.4, 8.4)	3.05, dd (8.4, 8.4)	3.05, dd (8.9, 8.9)	3.07, dd (8.7, 8.7)
C5	3.29, m	3.25, m	3.25, m	3.26, dq (8.9, 6.1)	3.26, dq (8.7, 6.5)
C6	1.28, d (6.2)	1.30, d (6.0)	1.31, d (6.5)	1.32, d (6.1)	1.32, d (6.5)
D1	4.56, dd (9.2, 1.9)	4.55, d (10.1)	4.56, dd (9.8, 1.9)	4.58, d (9.5, 1.8)	4.58, dd (9.9, 2.3)
D2	2.22, dd (12.0, 5.0)	2.21, dd (11.6, 5.6)	2.22, dd (12.3, 5.5)	2.26, dd (12.2, 5.1)	2.25, ddd (12.7, 4.7, 2.3)
	1.56, d (12.0)	1.59, d (11.6)	1.56, dd (12.2, 1.9)	1.60, m	1.56, m
D3	3.45, m	3.45, m	3.45, m	3.50, m	3.48, m
D4	3.04, dd (8.5, 8.5)	3.05, dd (8.4, 8.4)	3.05, dd (8.6, 8.6)	3.06, dd (8.9, 8.9)	3.08, dd (8.8., 8.8)
D5	3.32, m	3.32, m, overlap	3.32, m	3.36, dq (8.9, 6.1)	3.35, qd (6.1, 8.8)
D6	1.32, d (6.1)	1.33, d (6.0)	1.33,d (6.2)	1.34, d (6.1)	1.35, d (6.1)
E1	4.93, t (2.4)	4.92, dd (3.8, 0.8)	4.92, dd (3.8, 0.8)	4.99, d (4.3, 2.6)	4.92, dd (4.1, 1.8)
E2	1.91, m	1.92, d (13.3)	1.91, d (13.6)	1.96, dd (13.8, 2.6)	1.99, dd (13.7, 1.8)
		1.84, dd (13.6, 3.8)	1.84, dd (13.6, 3.8)	1.92, dd (13.8, 4.3)	1.96, dd (13.7, 4.3)
E4	4.56, d (9.2)	3.18, d (9.1)	3.18, d (9.3)	4.57, d (9.2)	4.57, d (9.4)
E5	3.94, m	3.76, dq (10.1, 5.8)	3.78, dq (9.3, 6.0)	3.97, dq (9.2, 6.2)	3.97, dq (9.0, 6.4)
E6	1.23, d (6.4)	1.25, d (5.8)	1.26, d (6.0)	1.18, d (6.2)	1.18, d (6.4)
E3-CH_3	1.30, s	1.31, s	1.31, s	1.32, s	1.32, s
(*CH_3*)$_2$CH		—	—	1.16, d (7.0)	1.17, d (7.0)
(CH_3)$_2$*CH*		—	—	2.59, m	2.57, m
CH_3-CO	2.04, s	—	—	—	—

[a] NMR spectra were recorded at 600 MHz in 10:1 $CDCl_3$:CD_3OD.

chromomycin family of compounds (Supplementary Table S4). We have not independently verified the absolute configuration of **2** and **3**, however these compounds share a biosynthetic intermediate with known chromomycin analogs **1** and **8** (which were also present in our fermentation), leading us to the same stereochemical assignment. The optical rotation for our isolated chromomycin A_3 (**1**), $[\alpha]_D$ −24 (*c* 0.1, EtOH), is consistent with literature reports.[1d]

In addition to the two side-chain truncated analogs of chromomycin, we isolated a number of analogs with the pentyl side-chain intact. These included the known compounds chromomycin A_3 (**1**), chromomycin 02-3G (**6**), aburamycin (**7**), chromomycin A_2 (8) and a previously uncharacterized deacylated chromomycin analog (**4**). Analog **4** was obtained as a yellow amorphous powder and the molecular formula was determined as $C_{55}H_{80}O_{25}$ from HRESIMS based on the $[M+Na]^+$ ion peak at *m/z* 1163.4937 (calcd for $C_{55}H_{80}O_{25}Na$). Comparison of the NMR (Supplementary Table S3) and MS data for **4** with those for chromomycin A_3 suggested there was one less acetyl group in **4** than in chromomycin A_3. HMBC correlations from H-A4 (δ 5.10 ppm) and the acetyl methyl singlet (δ 2.11 ppm) to the carbonyl carbon at δ 171.1 ppm indicated that the acetyl group in **4** was located at position 4 of sugar A. Although this compound has not been isolated from a bacterial fermentation, there is a previous report of conversion of **1** to **4** under basic conditions or by biotransformation.[18]

Biological evaluation of the new chromomycin analogs for cytotoxicity against the two non-small cell lung cancer (NSCLC) cell-lines, A549 and HCC44, demonstrated a decrease in cytotoxicity for the truncated side-chains of chromomycin analogs **2** and **3** (Table 3), with IC_{50} values >33.3 μM for **2** and 0.780 μM for **3** against NSCLC cell-line HCC44. A direct comparison of **3** with chromomycin A_2 (**8**), which have the same acylation pattern, decreases the IC_{50} from 260 pM for **8** to 0.780 μM for **3** against HCC44. Mechanistically, the role of the pentyl side-chain has been attributed to direct interactions of the hydroxyl and carbonyl groups with the

Table 2
^{13}C NMR data of chromomycin analogs (**2a, 3, 3a** and **4**)

Position	2a	3	3a	4[a]	Position	2a	3	3a	4[a]
Aglycon	–	–	–	–	B1	95.2	94.5	95.2	95.2
1	203.0	[b]	[b]	202.2	B2	32.9	32.3	33.0	32.9
2	76.2	76.6	76.3	76.3	B3	65.6	65.7	65.7	65.8
3	43.7	44.0	43.8	43.0	B4	81.3	81.2	81.3	81.4
4	27.3	27.3	27.2	27.0	B5	66.9	66.8	66.9	66.9
5	100.7	100.1	100.7	100.7	B6	17.1	16.7	17.1	17.1
6	159.3	159.1	159.3	159.3	B4-OCH_3	62.1	61.5	62.1	62.1
7	111.4	110.8	111.6	111.6	Sugar C	–	–	–	–
8	155.8	155.1	155.6	155.6	C1	100.3	100.2	100.3	100.3
9	[b]	[b]	[b]	164.2	C2	37.2	37.0	37.2	37.3
10	116.8	117.0	116.9	116.7	C3	81.9	81.2	81.9	81.7
4a	134.6	[b]	134.7	134.7	C4	75.0	74.7	75.0	74.9
8a	107.9	[b]	108	108	C5	72.0	71.8	72.1	72.0
9a	107.9	[b]	108	108	C6	18.0	17.7	17.9	18.0
10a	138.4	[b]	138.2	138.2	Sugar D	–	–	–	–
7-CH_3	8.1	8.1	8.1	8.3	D1	99.4	99.1	99.4	99.4
1′	77.6	77.6	77.6	81.5	D2	36.9	36.2	36.9	36.8
2′	172.8	[b]	172.7	211.3	D3	79.4	79.9	79.8	78.7
3′ (2′-OCH_3)	52.0	–	52.0	78.2	D4	75.0	74.7	75.0	74.9
4′	–	–	–	67.8	D5	72.1	72.2	72.1	72.2
5′	–	–	–	19.8	D6	17.6	17.4	17.6	17.8
1′-OCH_3	59.0	58.4	59.1	59.2	Sugar E	–	–	–	–
Sugar A					E1	96.9	94.8	96.8	96.2
A1	97.3	96.6	97.3	97.2	E2	42.9	43.2	43.6	42.9
A2	32.8	32.6	32.7	32.9	E3	70.7	70.0	70.7	70.7
A3	69.9	69.3	69.9	69.9	E4	78.8	78.5	78.9	78.9
A4	67.3	67.2	67.3	67.5	E5	67.8	66.1	66.7	67.7
A5	69.6	69.3	69.7	69.6	E6	17.9	17.2	17.7	18.0
A6	16.7	16.4	16.7	16.7	E3-CH3	21.7	22.1	22.6	21.8
CH_3-CO	20.7	20.4	20.7	20.8	$(CH_3)_2$CH-CO	–	18.5	18.8	–
CH_3-*CO*	171.1	171.2	171.1	171.1	$(CH_3)_2$*CH*-CO	–	34.0	34.2	–
Sugar B					$(CH_3)_2$CH-*CO*	–	176.9	177.3	–

[a] NMR spectra were acquired at 150 MHz in 10:1 $CDCl_3$:CD_3OD.
[b] Signal missing due to limited material.

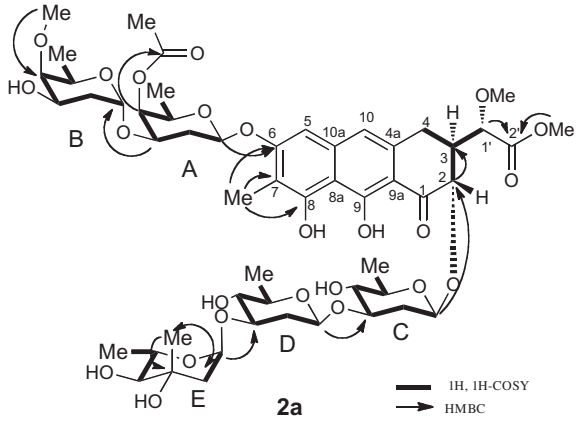


Figure 2. HMBC and COSY correlations for assignment of **2** and **2a**.

phosphate backbone of DNA, providing enhanced rigidity to the chromomycin-DNA complex.[10,19] The carboxylic acid moiety of **2** and **3** contain carbonyl groups at the terminus and the resulting negative charge could impede hydrogen-bonding interactions with the polyphosphate chains. Evaluation of the methyl esters **2a** and **3a** are ideal analogs to test this hypothesis. The methyl ester **3a** had an IC_{50} of 0.056 μM against HCC44. Although, this does not fully restore the activity found for chromomycin A_2, it is clear that the carboxylic acid moiety is deleterious to the cytotoxicity. Although it is possible that the reduced potency of the carboxylates is due to poor solubility or permeability.

Previous biological studies by Rohr and co-workers have demonstrated considerable improvement in the cytotoxicity and selectivity profiles of side-chain analogs of mithramycin and chromomycin.[11,12] One analog in particular, mithramycin SDK, showed enhanced potency against all tumor types, with particular improvement against melanoma, CNS-tumors and leukemia.[11] Mithramycin SDK also showed reduced cytotoxicity to 3T3 fibroblast cells (non-tumor) with an IC_{50} >20 μM, 1500-fold less toxic than mithramycin against the non-tumor cells. The continued chemical and biological study of chromomycin and mithramycin analogs with modified side-chains could prove valuable in further development of therapeutic analogs of this family of compounds.[20]

As mentioned previously, chromomycin and mithramycin analogs with modified ketide side-chains have been previously isolated from genetically modified strains of *Streptomyces*.[11,12] Investigation of the biosynthetic gene cluster of chromomycin A_3 identified two genes responsible for generating the pentyl side-chain. The gene *cmmOIV*, encodes for the FAD-dependent monooxygenase CmmOIV, responsible for oxidative ring opening of the

Table 3
Cytotoxicity of chromomycin analogs[a]

Compound	Cytotoxicity, IC_{50} (μM)	
	HCC44	A549
1	0.001	0.042
2	>33.3	>50
2a	3.4	–
3	0.78	1.5
3a	0.056	–
4	0.67	1.2
5	0.21	0.56
6	>50	>50
8	0.00026	0.00044

[a] IC_{50} values are means of three experiments.

fourth ring of premithramycin B (**9**) to give the tricyclic chromomycin core with the pentyl side-chain at C-3 (Fig. 3).[21,22] The gene *cmmWI* was identified to encode a ketoreductase, CmmWI, responsible for reduction of the C4′ ketone. Mutation of *cmmWI* blocked the ability of *S. griseus* to produce chromomycin A_3, however, it did lead to the accumulation of three new products, including chromomycin SA (**5**).[12] It has been proposed that **5** is a by-product of addition of H_2O to β-diketone **10** to give intermediate **11**, followed by a base-catalyzed retro-aldol to give the two-carbon side-chain product **5**.[21] We believe that the generation of **2** and **3** may be an enzymatic process, as we took extreme care to ensure that the fermentation and isolation was carried out using nearly neutral conditions. (pH 7.5). Efforts to identify β-diketo compounds in our large-scale fermentations have so far been unsuccessful, however they have been isolated from the previously mentioned mutation studies.[20]

3. Experimental

3.1. General experimental procedures

The optical rotations were recorded with an AUTOPOL® AP IV-6W polarimeter equipped with a halogen lamp (589 nm). UV spectra were recorded on a Shimadzu UV-1601 UV–vis spectrophotometer. ^{1}H and 2D NMR spectral data were recorded at 600 MHz in $CDCl_3/CD_3OD$ (10:1) solution on Varian System spectrometer, and chemical shifts were referenced to the corresponding solvent ($CDCl_3$) residual signal. ^{13}C NMR spectra were acquired at 150 MHz on a Varian System spectrometer. High resolution ESI-TOF mass spectra were provided by The Scripps Research Institute, La Jolla, CA. Low-resolution LC/ESI-MS data were measured using an Agilent 1200 series LC/MS system with a reversed-phase C_{18} column (Phenomenex Luna, 150 mm × 4.6 mm, 5 μm) at a flow rate of 0.7 mL/min. Preparative HPLC was performed on an Agilent 1200 series instrument with a DAD detector, using a Phenyl-Hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μm). ODS (50 μm, Merck) were used for column chromatography.

3.2. Collection and phylogenetic analysis of strain SNB-005

The marine-derived actinomycete, strain SNB-005, was isolated from a sediment sample collected from a hypersaline lake at East Plana Cay, Bahamas (N 22° 36′33″, W 73° 33′24″ W). Bacterial spores were collected via stepwise centrifugation as follows: 2 g of sediment was dried over 24 h in an incubator at 35 °C and the resulting sediment added to 10 mL sH_2O containing 0.05% Tween 20. After a vigorous vortex for 10 min, the sediment was centrifuged at 2500 rpm for 5 min (4 °C). The supernatant was removed and transferred into a new tube and centrifuged at 18,000 rpm for 25 min (4 °C) and the resulting spore pellet collected. The resuspended spore pellet (4 mL sH_2O) was plated on a humic acid media, giving rise to individual colonies of SNB-005 after two weeks. Analysis of the 16S rRNA sequence of SNB-005 revealed 99% identity to *Streptomyces* sp.

3.3. Cultivation and extraction

Bacterium SNB-005 was cultured in 20 2.8 L Fernbach flasks each containing 1 L of a seawater based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3{\cdot}4H_2O$, 100 mg KBr) and shaken at 200 rpm at 27 °C. After seven days of cultivation, sterilized XAD-7-HP resin (20 g/L) was added to adsorb the organic products, and the culture and resin were shaken at 200 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The acetone soluble fraction was dried in vacuo to yield 1.5 g of extract.

3.4. Isolation of compounds 1–8

The extract (1.5 g) was partitioned with *n*-hexane, $CHCl_3$, EtOAc, and MeOH/H_2O. The $CHCl_3$ and EtOAc soluble layers were combined (800 mg) and fractionated by flash column chromatography on ODS (50 μm, 50 g), eluting with a step gradient of MeOH and H_2O (30:70–100:0), and 15 fractions (Fr.1–Fr.15) were collected. Fractions 10–13 were enriched in aureolic acid-containing metabolites, as indicated by LC–MS. Fraction 10 was purified by reversed phase HPLC (Phenomenex Luna, Phenyl-Hexyl, 250 × 10.0 mm, 2.5 mL/min, 5 μm, UV = 254 nm) using a gradient solvent system from 30% to 80% CH_3CN (0.01% Formic acid) over 30 min to afford chromomycin SA_3 (**2**, 5.5 mg, t_R = 15.7 min), chromomycin 02-3G (**6**, 2.9 mg, t_R = 11.2 min), and chromomycin SA (**5**, 2.3 mg, t_R = 19.8 min). Fraction 11 was purified with the same method to afford chromomycin SA_2 (**3**, 4.5 mg, 20.5 min). Fraction 12 was purified with the same HPLC column using a gradient from 30% to 80% CH_3CN (0.01% formic acid) over 28 min to afford monodeacetylchromomycin A_3 (**4**, 12.0 mg, t_R = 14.8 min), aburamycin C (**7**, 3.6 mg, 19.0 min) and chromomycin A_3 (**1**, 4.5 mg, 17.8 min).

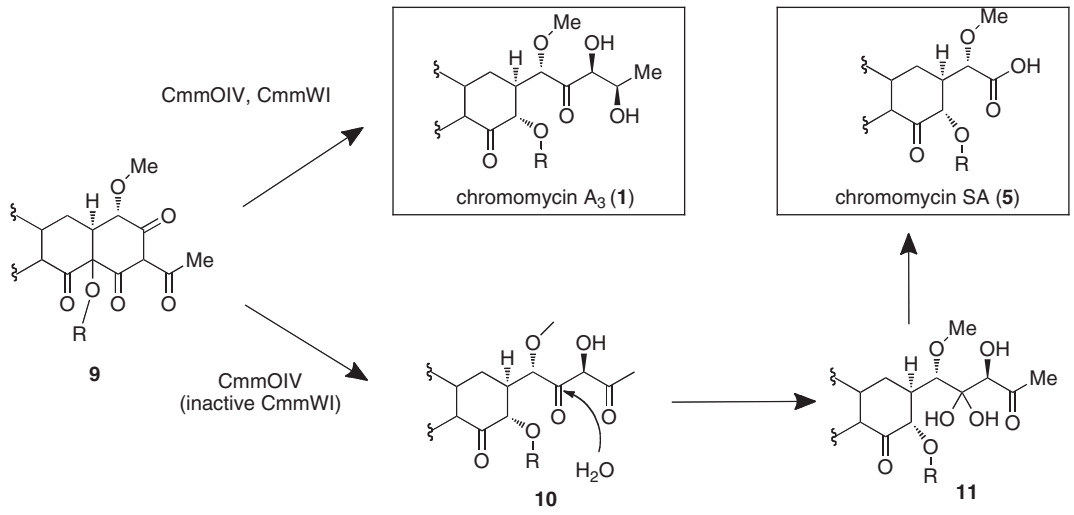


Figure 3. Proposed biosynthesis of truncated chromomycin analogs.

Chromomycin A_2 (**8**, 15.0 mg) was obtained from fraction 13 through HPLC separation by using the same method as for fraction 12.

3.5. Chromomycin SA_3 (2, 5.5 mg)

Yellow solid; $[\alpha]_D^{20}$ +9.2 (*c* 0.1 MeOH); (MeOH) $\lambda_{max}(\log \varepsilon)$ 229 (3.9), 280 (4.1), 317 (3.4), 332 (3.4), 413 (3.4); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table 1. ESI-MS $[M-H]^-$ *m/z* 1081.4, $[M+Na]^+$ *m/z* 1105.4. HRESIMS $[M+Na]^+$ *m/z* 1105.4439 ($C_{52}H_{74}O_{24}Na$, calcd 1105.4468).

3.6. Chromomycin SA_2 (3, 4.5 mg)

Yellow solid; $[\alpha]_D^{20}$+13 (*c* 0.1 MeOH); (MeOH) $\lambda_{max}(\log \varepsilon)$ 229 (4.3), 278 (4.5), 317 (3.8), 411 (3.8); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table 1, ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table 2; ESI-MS $[M-H]^-$ *m/z* 1151.4, $[M+Na]^+$ *m/z* 1175.4. HRESIMS $[M+Na]^+$ *m/z* 1175.4874 ($C_{56}H_{80}O_{25}Na$, calcd 1175.4887).

3.7. Monodeacetylchromomycin A_3 (4, 12.0 mg)

Yellow solid; $[\alpha]_D^{20}$ + 6 (*c* 0.1 MeOH); UV (MeOH) λ_{max} ($\log \varepsilon$) 230 (4.4), 276 (4.5), 317 (3.9), 331 (3.8), 409 (4.0); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table S1 and ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table 2; ESI-MS $[M-H]^-$ *m/z* 1139.4, $[M+Na]^+$ *m/z* 1163.4. HRESIMS $[M+Na]^+$ *m/z* 1163.4937 ($C_{55}H_{80}O_{25}Na$, calcd 1163.4887).

3.8. Methyl esters of compounds 2 and 3

Solutions of **2** (2.0 mg) and **3** (1.5 mg) in dry methanol (0.5 mL) were individually treated with 10 μL trimethylsilyldiazomethane (2 M in diethyl ether) under room temperature for 0.5 h. Each reaction mixture was analyzed by LC–MS and purified by RP-HPLC (Phenomenex Luna, Phenyl-Hexyl, 250 × 10.0 mm, 2.5 mL/min, 5 μm, UV = 254 nm) using a gradient solvent system from 30% to 90% CH_3CN over 30 min to afford methyl-chromomycin SA_3 (**2a**, 1.0 mg, t_R = 16.8 min) and methyl-chromomycin SA_2 (**3a**, 0.9 mg, t_R = 17.2 min), respectively.

3.9. Methyl-chromomycin SA_3 (2a)

Yellow solid; UV (MeOH) λ_{max} ($\log \varepsilon$) 228 (4.5), 278 (4.5), 317 (4.0), 332 (4.0), 419 (4.1); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table 1, ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$ = 10:1) see table 2; ESI-MS $[M-H]^-$ *m/z* 1095.3, $[M+Na]^+$ *m/z* 1119.3.

3.10. Methyl-chromomycin SA_2 (3a)

Yellow solid; UV (MeOH) λ_{max} ($\log \varepsilon$) 229 (4.4), 278 (4.5), 317 (3.9), 331 (3.8), 414 (4.0); ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table 1, ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$ = 10:1) see Table 2; ESI-MS $[M-H]^+$ *m/z* 1065.3, $[M+Na]^+$ *m/z* 1189.3.

3.11. Cytotoxicity assays

Cell lines were cultured in 10 cm dishes (Corning, Inc.) in NSCLC cell-culture medium: RPMI/L-glutamine medium (Invitrogen, Inc.), 1000 U/ml penicillin (Invitrogen, Inc.), 1 mg/ml streptomycin (Invitrogen, Inc.), and 5% fetal bovine serum (Atlanta Biologicals, Inc.). Cell lines were grown in a humidified environment in the presence of 5% CO2 at 37 °C. For cell viability assays, A549 and HCC44 cells (60 μL) were plated individually at a density of 750 and 500 cells/well, respectively, in 384 well microtiter assay plates (Bio-one; Greiner, Inc.). After incubating the assay plates overnight under the growth conditions described above, purified compounds were dissolved and diluted in DMSO and subsequently added to each plate with final compound concentrations ranging from 1 μM to 2 pM and a final DMSO concentration of 0.5%. After an incubation of 96 h under growth conditions, Cell Titer GloTM reagent (Promega, Inc.) was added to each well (10 uL of a 1:2 dilution in NSCLC culture medium) and mixed. Plates were incubated for 10 min at room temperature and luminescence was determined for each well using an Envision multi-modal plate reader (Perkin-Elmer, Inc.). Relative luminescence units were normalized to the untreated control wells (cells plus DMSO only).

Acknowledgments

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Supplementary data

Supplementary data (1D and 2D NMR spectra and NMR tables for all new compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.013.

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16. The ^{1}H NMR spectra of **2** in $CDCl_3$ or $CDCl_3$:CD_3OD (10:1) were directly compared to the tabulated NMR data for chromomycin SA (**1**) reported in ref. 15 as well as the data we acquired for chromomycin A_3 in $CDCl_3$:CD_3OD (10:1) listed in Table 1.
17. NMR spectra for compounds **2** and **3** were sensitive to concentration and temperature, resulting in multiple conformations. We believe that the free carboxylic acids form an aggregation in solution leading to these problems.

Ultimately, a 10:1 ratio of $CDCl_3$:CD_3OD gave interpretable data. Unfortunately we were unable to obtain an assignable ^{13}C spectrum for **2**.

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