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Isolation of Lomaiviticins C–E, Transformation of Lomaiviticin C to Lomaiviticin A, Complete Structure Elucidation of Lomaiviticin A, and Structure–Activity Analyses

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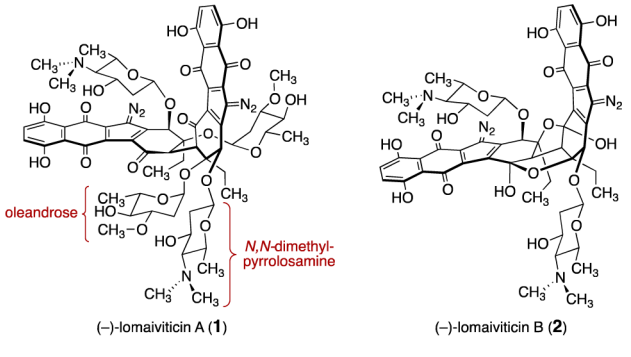
Supporting Information

ABSTRACT: We describe the isolation of (−)-lomaiviticins C–E (**6**–**8**), elucidation of the complete absolute and relative stereochemistry of (−)-lomaiviticin A (**1**), the synthetic conversion of (−)-lomaiviticin C (**6**) to (−)-lomaiviticin A (**1**), and the first evidence that the dimeric diazofluorene of (−)-lomaiviticin A (**1**) plays a defining and critical role in antiproliferative activity.

Lomaiviticins A (**1**) and B (**2**) are complex C_2-symmetric bacterial metabolites that contain a dimeric diazotetrahydrobenzo[*b*]fluorene (diazofluorene) skeleton bearing 2–4 dideoxyglycoside residues, oleandrose and *N*,*N*-dimethylpyrrolosamine (Figure 1).[1] They are structurally related to the monomeric diazofluorenes known as the kinamycins (**3**–**5**), which were first isolated by Ōmura and co-workers.[2] The constitution and relative stereochemistry of the carbohydrate and aglycon substructures of **1** and **2** were elucidated by extensive 1D and 2D NMR, IR, and high-resolution mass spectrometry (HRMS) analyses. The absolute stereochemistry of the aglycon residue was not rigorously established but was assigned by analogy to that of the kinamycins; the absolute stereochemistry of the carbohydrates was not defined.[3]



kinamycin	R_1	R_2	R_3	R_4
(−)-A (**3**)	H	Ac	Ac	Ac
(−)-B (**4**)	H	Ac	H	H
(−)-C (**5**)	Ac	H	Ac	Ac

Figure 1. Structures of lomaiviticins A (**1**) and B (**2**) and kinamycins A–C (**3**–**5**).

Lomaiviticins A (**1**) and B (**2**) are powerful antibiotics, with minimum inhibitory concentrations (MICs) of 6–25 ng/spot, and **1** is an exceptionally potent anticancer agent, with half-maximal inhibitory concentration (IC_{50}) values in the 72 nM–7.3 pM range against 24 cancer cell lines.[1a,4] Evidence suggests that **1** may operate by a novel mechanism of action, but studies of **1** and **2** have been impossible to initiate because of the absence of a synthetic route to either target and their low levels of natural production (reported fermentation yields: 1 mg/L for **1** and 0.17 mg/L for **2**).[1a]

As part of our synthetic investigations,[3,5] we sought to reisolate lomaiviticin A (**1**). Accordingly, "*Salinispora pacifica*" strain DPJ-0019 was acquired from the USDA Agricultural Research Service as NRRL 50168. Careful examination of the concentrated fermentation extracts revealed the presence of **1** and three novel metabolites, which we have named lomaiviticins C–E (**6**–**8**). Lomaiviticin B (**2**) was not detected [ultra high-performance liquid chromatography (UPLC)/MS analysis]. We describe herein the structure elucidation of (−)-lomaiviticins C–E (**6**–**8**), the direct conversion of (−)-lomaiviticin C (**6**) to (−)-lomaiviticin A (**1**), and the first evidence implicating the dimeric diazofluorene structure of (−)-lomaiviticin A (**1**) as underlying its potent antiproliferative effects. We have also elucidated the complete relative and absolute stereochemistry of **1**, which has remained an unresolved issue for over a decade. These latter data bear particular significance to synthetic studies,[3,5,6] since both D- and L-oleandrose are found in nature[7] and the absolute stereochemistry of pyrrolosamine[8] has never been determined.

"*S. pacifica*" DPJ-0019 was fermented in SPYESS medium in the presence of Dianion HP-20 resin. The resin was collected and extracted with methanol, and the concentrated extract was purified by reversed-phase chromatography. Lomaiviticins C–E (**6**–**8**) were isolated by mass-spectrometry-guided fractionation[9] in yields of 37–62, 10–19, and 1.6–3.6 mg/L, respectively, over several fermentations.

Lomaiviticin C (**6**) was obtained as a burgundy amorphous solid. HRMS analysis indicated a molecular formula of $C_{68}H_{82}N_4O_{24}$ (*m*/*z* 670.2724 $[M + 2H]^{2+}$, error = 1.2 ppm).

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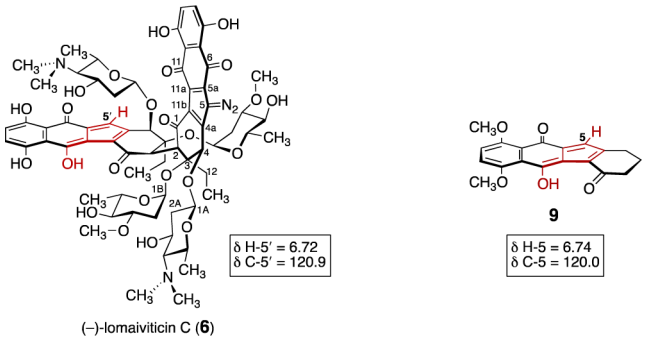

Figure 2. Structures of (−)-lomaiviticin C (**6**) and the synthetic hydroxyfulvene **9**.

Table 1. Selected NMR data for (−)-Lomaiviticin C (6)[a]

position	δ_H (ppm)	δ_C (ppm)	HMBC (H → C)
1		198.9	
2	3.88, d, J = 3.2 Hz	47.8	C-1, -3, -2′, -11b
3		83.6	
4	5.38, s	67.4	C-2, -3, -4a, -11b, -1A
4a		131.9	
5		78.3	
5a/11a		134.6/128.9	
6/11		185.1/185.0	
11b		137.0	
1A	4.47, d, J = 9.2 Hz	95.8	C-4, -2A
1′		200.9	
2′	3.80[b]	47.1	C-2, -4′, -3′, -11b′, -1′
3′		83.6	
4′	5.27, s	68.6	C-2′, -3′, -4a′, -11b′, -1A′
4a′		129.8	
5′	6.72, s	120.9	
5a′/11a′		122.0/127.6	
6′/11′		185.5/182.6	
11b′		135.3	
1A′	4.55, d, J = 9.2 Hz	95.6	C-4′, -2A′

[a]NMR spectra (500 MHz) were recorded in methanol-d_4 at 24 °C. Positions 1−1A correspond to the half of **6** containing the diazofluorene. Positions 1′−1A′ correspond to the half of **6** containing the hydroxyfulvene. Full spectroscopic data are presented in the Supporting Information. [b]Obscured by an overlapping resonance.

The structure of **6** (Figure 2) was elucidated by 1D and 2D NMR analysis and IR spectroscopy. Selected NMR data are shown in Table 1.[10] These data revealed that **6** shows some homology to **1**, including oleandrose and *N*,*N*-dimethylpyrrolosamine residues, but more surprisingly that **6** is C_1-symmetric. The carbon atoms C-2 and C-2′ were observed at 47.8 and 47.1 ppm, respectively, in good agreement with those of **1** (47.6 ppm). A rotating-frame nuclear Overhauser effect (ROE) interaction between H-2 (3.88 ppm) and H-2′ (3.80 ppm), a ^3J coupling (3.2 Hz) between these same protons, and a heteronuclear multiple-bond correlation (HMBC) confirmed C-2/C-2′ as the conjoining bond between the monomeric units. 2D nuclear Overhauser effect spectroscopy (NOESY) analysis revealed contacts between H-2 and H-4 and between H-2′ and H-4′, supporting a 1,3-cis stereorelationship and consistent with the observation of W-plane couplings between H-2 and H-4 and between H-2′ and H-4′ in the correlation spectroscopy (COSY) spectrum of **6**. ROE contacts between H-1A and H-4 and between H-1A′ and H-4′, as well as correlations between H-4 and C-1A, H-1A and C-4, H-4′ and C-1A′, and H-1A′ and C-4′ in the HMBC spectrum of **6**, secured the location of the pyrrolosamine residues. ROE contacts between H-1B and H-12 and between H-1B′ and H-12′ established the location of the oleandrose residues. Distinct signals in the ^{1}H and ^{13}C NMR spectra of **6** [δ_H = 6.72 ppm (s, 1H); δ_C = 120.9 ppm] that were conspicuously absent in the spectra of **1** were also observed. A heteronuclear multiple-quantum coherence (HMQC) experiment established the one-bond connectivity of these atoms. These ^{1}H and ^{13}C resonances correspond well to those of hydroxyfulvenes such as **9** (Figure 2) that have been previously synthesized in this laboratory ($\delta_{H\text{-}5}$ = 6.74 ppm, $\delta_{C\text{-}5}$ = 120.0 ppm, chloroform-*d*).[5a] The presence of a hydroxyfulvene rather than two diazofluorenes is consistent with the HRMS data for **6** and accommodates the C_1 symmetry of the metabolite. Carbon C-5 of **6**, which bears the single remaining diazo group, was observed at 78.3 ppm (78.8 ppm in **1**). A strong IR absorbance at 2141 cm^{-1} also supported the presence of a diazo group.

Lomaiviticins D (**7**) and E (**8**) were isolated as burgundy amorphous solids. HRMS of **7** and **8** suggested molecular formulas of $C_{69}H_{84}N_4O_{24}$ (*m*/*z* 677.2818 [M + 2H]$^{2+}$, error = 6.3 ppm) and $C_{70}H_{86}N_4O_{24}$ (*m*/*z* 684.2886 [M + 2H]$^{2+}$, error = 1.1 ppm), respectively, corresponding to addition of one or two methylene units to **6**. The NMR spectroscopic data for **7** and **8** revealed many similarities to **6**, with the following exceptions. First, in the ^{1}H NMR spectrum of **7**, two additional singlet resonances at 3.57 and 3.56 ppm were detected, each apparently integrating for 1.5H, and doubling of many other resonances was observed. In the ^{1}H NMR spectrum of **8**, two additional singlets resonating at 3.57 and 3.56 ppm and integrating for 3H each were observed. Analysis of the MS/MS data for **7** revealed mass fragments with differences of *m*/*z* 144, 158, and 302 units from the parent ion. MS/MS analysis of **8** revealed mass fragments with differences of *m*/*z* 158 and 316 from the parent ion. Collectively, these data suggest that **7** and **8** are derivatives of **6** bearing an additional *O*-methyl substituent on one (for **7**) or both (for **8**) oleandrose residues. (−)-Lomaiviticin D (**7**) is an inseparable mixture of isomers in which the methylation is proximal or distal to the diazofluorene (Figure 3).

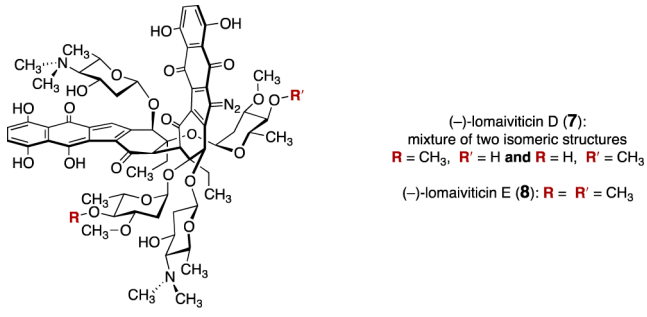

Figure 3. Structures of (−)-lomaiviticins D (**7**) and E (**8**).

Acidic digestion of (−)-lomaiviticin C (**6**) effected the hydrolysis of both oleandrose residues and one pyrrolosamine residue.[11] Isolation and analysis by optical rotation revealed that both carbohydrates are of the L form. The absolute stereochemistry of the pyrrolosamine residue was used to elucidate the stereochemistry of the aglycon substructure in **6**: observation of an NOE interaction between H-1A and H-4

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established the C-1A−H-1A and C4−O bonds as synperiplanar, and an additional NOE between H-2A$_{eq}$ and H-12 necessitated that the ethyl substituent face the 2-position of the aminosugar. These NOE interactions are uniquely accommodated by the diastereomer shown.

Treatment of **6** with excess trifluoromethanesulfonyl azide and trifluoroacetic acid formed semisynthetic (−)-lomaiviticin A (**1**) (43%), which was indistinguishable (by ^{1}H NMR, HMQC, IR, HMRS, optical rotation, and HPLC retention time) from natural material (Scheme 1).[1a] We previously reported diazo transfers to synthetic hydroxyfulvenes such as **9** using triflyl azide in the presence of base.[5] Here, trifluoroacetic acid significantly enhanced the yield, apparently by stabilizing the product **1** without suppressing the diazo transfer. In our hands, fermentation yields of (−)-lomaiviticin A (**1**) have never exceeded 0.5 mg/L,[12] while the yields of (−)-lomaiviticin C (**6**) are as high as 62 mg/L. Thus, this sequence represents a >50-fold increase in lomaiviticin A production. Perhaps more importantly, this chemistry provides an unequivocal link between lomaiviticins A (**1**) and C (**6**) and establishes the complete structure of (−)-lomaiviticin A (**1**) for the first time.

Scheme 1. Conversion of Natural (−)-Lomaiviticin C (6) to (−)-Lomaiviticin A (1)

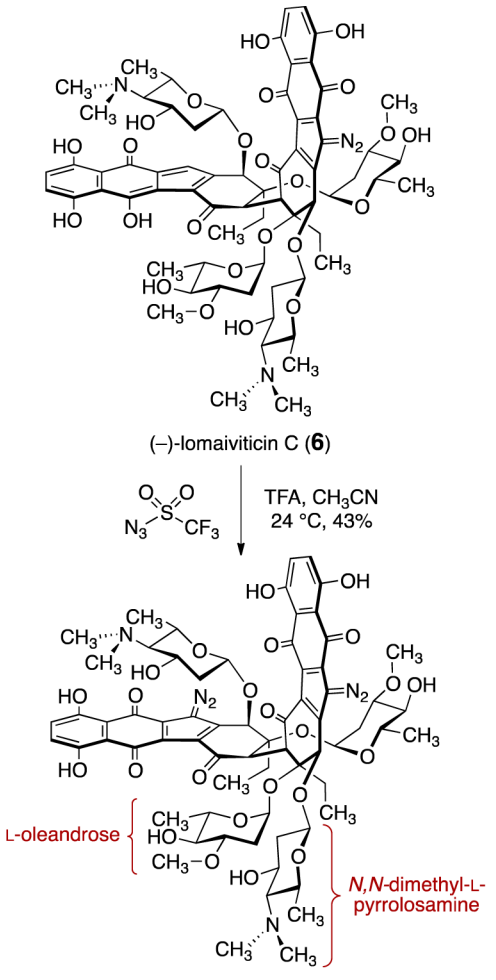

Table 2. IC$_{50}$ Values (in nM) of (−)-Lomaiviticins A (1) and C−E (6−8) and (−)-Kinamycin C (5)

	cell line			
compound	K562	LNCaP	HCT-116	HeLa
1	11	2	2	7
6	472	332	223	589
7	197	196	167	161
8	469	964	255	292
5	72	116	274	517

Cell viability studies revealed that lomaiviticins C−E (**6**−**8**) are submicromolar antiproliferative agents (Table 2). The most striking result of these assays is the large (up to 166-fold) difference between the potencies observed for **1** and **6**. In view of the nearly identical structures of the two metabolites, these data establish that the dimeric diazofluorene structure of **1** is critical for potent activity. A large body of evidence suggests that **1** is reductively activated,[13] and we have observed that dimeric diazofluorenes undergo reduction at rates several times faster than related monomeric diazofluorenes.[14] It is possible that a similar rate enhancement is manifested in the reactivity of **1** and underlies its cytotoxicity.

We have previously presented evidence that the structure **6** (e.g., lomaiviticin C) forms from (−)-lomaiviticin A (**1**) under reducing conditions,[11] and it is possible that **6** is a product of degradation of **1** during the fermentation process. However, if this is the case, the transformation of **1** to **6** in the medium must be rapid, as **1** does not accumulate in concentrations detectable by UPLC/MS analysis prior to concentration. Regardless, the synthetic transformation of (−)-lomaiviticin C (**6**) to (−)-lomaiviticin A (**1**) provides a means to reestablish cytotoxic activity and access quantities of **1** required for chemical biological studies. The complete structure elucidation of (−)-lomaiviticin A (**1**) will additionally facilitate its eventual chemical synthesis.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and detailed characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(3) In the reference that follows, the absolute stereochemistries of the carbohydrates of **1** and **2** were inferred on the basis of their graphical representation in ref 1a. We have subsequently learned that this representation was intended to show only relative, and not absolute, stereochemistry (Ellestad, G. A., Columbia University, New York, NY. Personal communication, 2011). See: Gholap, S. L.; Woo, C. M.; Ravikumar, P. C.; Herzon, S. B. *Org. Lett.* **2009**, *11*, 4322.

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(9) The mass spectra of **6**–**8** exhibited a prominent daughter ion (m/z = 176) corresponding to free *N*,*N*-dimethylpyrrolosamine, which facilitated their initial detection.

(10) Complete spectroscopic data are presented in the Supporting Information.

(11) The pyrrolosamine adjacent to the hydroxyfulvene substructure may preferentially cleave by an acid-catalyzed elimination pathway. See: Mulcahy, S. P.; Woo, C. M.; Ding, W. D.; Ellestad, G. A.; Herzon, S. B. *Chem. Sci.* **2012**, *3*, 1070.

(12) This is an estimated value obtained by integration of the UV absorbances of **1** and **6** in concentrated extracts. This value is likely to overstate the actual production of **1**, as we have previously shown that the ratio of extinction coefficients (254 nm) for hydroxyfulvenes and diazofluorenes structurally related to **1** and **6** is >5:1 (see ref 11).

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