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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

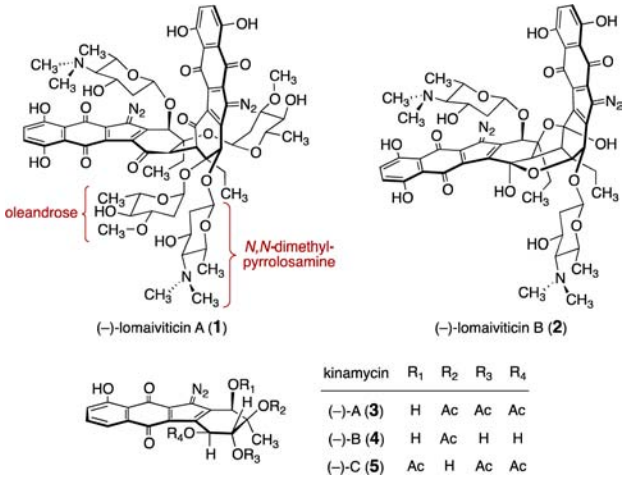

Figure 1. Structures of lomaiviticins A (**1**) and B (**2**) and kinamycins A–C (**3**–**5**).

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]

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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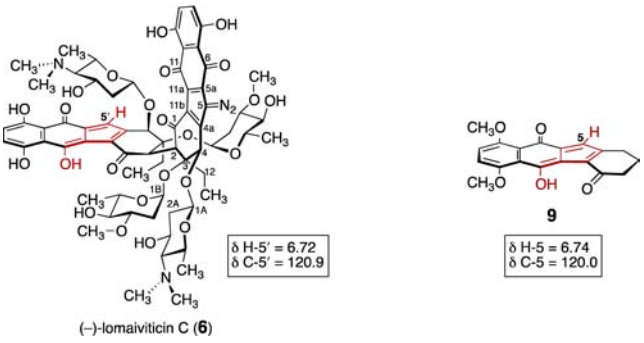

Figure 2. Structures of (−)-lomaiviticin C (**6**) and the synthetic hydroxyfulvene **9**.

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).

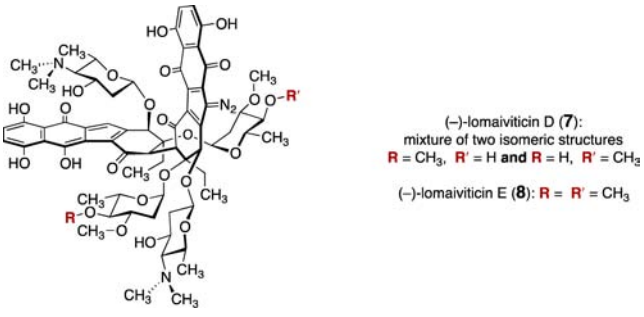

Figure 3. Structures of (−)-lomaiviticins D (**7**) and E (**8**).

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)

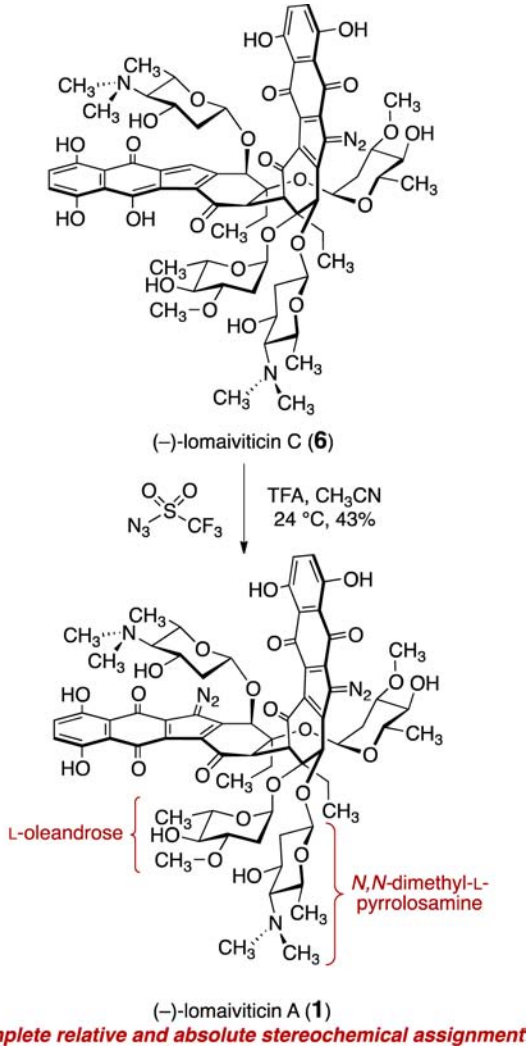

Table 2. IC$_{50}$ Values (in nM) of (−)-Lomaiviticins A (1) and C−E (6−8) and (−)-Kinamycin C (5)

	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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(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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