Structure revision of the lomaiviticins.

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ABSTRACT: The lomaiviticins are dimeric genotoxic bacterial metabolites that contain unusual diazocyclopentadiene functional groups and 2–4 deoxyglycoside residues. Because only 6 of 19 carbon atoms in the monomeric aglycon unit are proton-attached, their structure determination by NMR spectroscopic analysis is non-trivial. Prior structure elucidation efforts established that the two halves of the lomaiviticins are joined by a single carbon–carbon bond appended to an oxidized cyclohexenone ring. This ring was believed to comprise a 4,5-dihydroxycyclohex-2-en-1-one. The bridging bond was positioned at C6. This structure proposal has not been tested because none of the lomaiviticins have been prepared by total chemical synthesis or (to the best of our knowledge) successfully analyzed by X-ray crystallography. Here we disclose microED studies which establish that (–)-lomaiviticin C contains a 4,6-dihydroxy-cyclohex-2-en-1-one residue, that the bridging carbon–carbon bond is located at C5, and that the orientation of the cyclohexenone ring and configuration of the secondary glycoside are reversed, relative to their original assignment. High-field (800 MHz) NMR analysis supports the revised assignment and suggests earlier efforts were misled by a fortuitous combination of a near-zero $^3J_{\text{H4,H5}}$ coupling constant and a 4-bond HMBC correlation that was interpreted as a 3-bond coupling. DFT calculations of the expected ^{13}C chemical shifts of the original and revised structures of the aglycon and (–)-lomaiviticin B provide further robust support for the structure revision. Because the interconversion of lomaiviticins A, B, and C has been demonstrated, these findings apply to each isolate. These studies clarify the structures of this family of metabolites and underscore the power of microED analysis in natural products structure determination.

Introduction. The lomaiviticins are cytotoxic bacterial metabolites characterized by the presence of the deoxyglycosides β -*N,N*-dimethyl-L-pyrrolosamine and α -L-oleandrose, one or two diazotetrahydro[*b*]benzofluorene (diazofluorene) residues, and a single carbon–carbon bond linking two highly oxygenated cyclohexene rings (Fig. 1).¹ The C_2 -symmetric derivatives known as (–)-lomaiviticins A and B were first reported in 2001. The structures **1a** and **2a** were advanced by He and co-workers based on HRMS and extensive NMR analysis (Fig. 1a).² The relative stereochemistry (including anomeric configuration) of the carbohydrates was determined by analysis of $^3J_{\text{H-H}}$ coupling constants and confirmed by comparison to other natural glycosides.³ The presence of the diazofluorene was inferred from the kinamycins (e.g. kinamycin F (**4**)), monomeric diazofluorenes whose structures are known with certainty.⁴ The structure elucidation of the 2,2'-bicyclohexyl core was more challenging. In aggregate, only 6 of 19 carbon atoms in the monomeric aglycon unit are proton-attached, and two of these carbon atoms (C8, C9) are separated from the core by the diazofluorene. Thus, C2, C4, C12, and C13 constitute the only proton-attached centers to guide NMR analysis.

The structures **1a** and **2a** were based on NOESY, HMQC, and HMBC analysis. The data points from the initial report² that are germane to the work below are as follows. H2 and H4 were observed as singlets in the ^1H NMR spectra (500 MHz) of (–)-lomaiviticins A and B, but were correlated in the

COSY spectrum of (–)-lomaiviticin A. This correlation was attributed to W-plane coupling, thus forming the basis for assignment of a *syn*-facial arrangement of the C2 and C4 hydrogens, and the number of bonds separating them (four). A weak HMBC correlation between H4 and the diazo carbon (C5) in (–)-lomaiviticin A was reportedly observed; this correlation was attributed to three-bond coupling and used to anchor the location of the aminosugar with respect to the diazocyclopentadiene. Finally, a correlation between H2 and C2 was observed in both the HMQC and HMBC spectra of (–)-lomaiviticin A. These correlations supported assignment of the location of the bridging carbon–carbon bond as that shown. While the biosynthetic relationship between (–)-lomaiviticins A and B has not been rigorously established, the 2,6-dideoxy α -L-oleandrose residues of (–)-lomaiviticin A are acid-labile. Hydrolytic cleavage of these residues, followed by two-fold hemiketal formation, converts natural (–)-lomaiviticin A to semisynthetic (–)-lomaiviticin B.⁵

In 2012 the Herzon laboratory reported the isolation of (–)-lomaiviticin C, the product of formal hydrodediazotization of (–)-lomaiviticin A.⁶ The C_1 -symmetric structure **3a** was assigned to (–)-lomaiviticin C based upon the doubling of many signals in the NMR spectra, a reduction in 26 mass units relative to (–)-lomaiviticin A, and the successful conversion of natural (–)-lomaiviticin C to semisynthetic (–)-lomaiviticin A. New signals observed at 6.72 and 120.9 ppm in the ^1H and ^{13}C

Fig. 1. **a.** Original structures advanced for (–)-lomaiviticins A, B, and C (**1a**, **2a**, and **3a**, respectively), and the structures of (–)-kinamycin F (**4**), the synthetic hydroxyfulvene **5**, and the aglycon **6a**, which was first prepared in 2011. **b.** Revised structures of (–)-lomaiviticins A, B, and C (**1b**, **2b**, and **3b**, respectively) based on the data reported herein. The assignments that are exchanged in the revised structures are shown in orange in **1b**. **c.** The structural homology between (–)-lomaiviticins A–C has been established by the pathway shown.^{5,6}

spectra, respectively, of (–)-lomaiviticin C were attributed to the hydroxyfulvene shown in red. These shifts are in agreement with fully synthetic hydroxyfulvenes, such as **5** ($\delta \text{H} = 6.74$, $\delta \text{C} = 120.0$). Acidic digestion of (–)-lomaiviticin C allowed for isolation of the carbohydrate residues. Their absolute stereochemistry was determined by comparison of optical rotation data to synthetic standards (both were found to be of the L-form). ROESY analysis was then used to relay this assignment to the cyclohexane core. However, to the best of our knowledge, single crystal X-ray crystallographic data has not been obtained for any lomaiviticin. Moreover, none of the structures **1a–3a** have yet been prepared by chemical synthesis.^{5,7} The Herzon laboratory reported a synthesis of the aglycon structure **6a** in 2011.^{5,7j} Unfortunately, attempted hydrolytic cleavage of the carbohydrate residues in natural (–)-lomaiviticin A could not be effected without accompanying decomposition of the aglycon.^{5,8} Thus, a definitive link between

fully synthetic and natural lomaiviticins has not been established in the 20 years since their initial disclosure.

Here we report microED analysis of (–)-lomaiviticin C. This analysis indicates the structure of (–)-lomaiviticin C is that shown as **3b** (Fig. 1b). In this structure, the C1/C4 and C2/C3 positions are exchanged, and the configuration of C4 is inverted, relative to **3a**. Since structural homology between (–)-lomaiviticins A, B, and C has been definitively established (vide supra, see also Fig. 1c) these findings indicate the structures of (–)-lomaiviticins A and B are correctly depicted as **1b** and **2b**, respectively. High-field (800 MHz) NMR analysis of (–)-lomaiviticin C supports the structure **3b** and indicates that earlier efforts may have been misled by a fortuitous combination of a near-zero $^3J_{\text{H4,H5}}$ coupling constant and a 4-bond (H4,C5) HMBC correlation that was interpreted as a 3-bond coupling.

Results. We chose (–)-lomaiviticin C for study because the isolate is more abundant (liquid fermentation yields of (–)-lomaiviticins A and C are ~0.5–1 and 37–62 mg per liter, respectively), it is more stable than (–)-lomaiviticin A,⁹ and it is >2 orders of magnitude less cytotoxic.⁶ Additionally, the C₁-symmetry of (–)-lomaiviticin C provides a nearly 2-fold increase in the number of NMR resonances, and allows for detection of correlations between the two halves of the isolate, thereby greatly amplifying the utility of NMR analysis. We envisioned that microED, an emerging cryoEM method that allows for structural characterization of complex molecules from crystals that fall below the size required for common crystallographic techniques,¹⁰ might be uniquely effective in providing crystallographic data for (–)-lomaiviticin C.

In our initial studies, we found that deposition of 2.0 μ L of a solution of (–)-lomaiviticin C (~200 ng) in tetrahydrofuran–acetonitrile (~0.1 mg/mL) onto a carbon TEM grid, followed by room temperature evaporation, yielded microcrystalline domains that diffracted to >2 Å. While insufficient for ab initio structural determination, we were encouraged by these initial findings. Ultimately, after extensive screening of sample preparation conditions, it was found that slow evaporation (30 d) of this solution at –20 °C led to microcrystals that diffracted to 1.05 Å (complete crystallographic parameters are presented in Table S1). Cryogenic data collection was required to mitigate radiation damage during collection of rotation data. Merging data from four crystals afforded an ab initio solution (R1 = 11.9%, refined anisotropically; Fig. 2, see also Fig. S1). From the preliminary structure, which was obtained without any human input other than the molecular formula, it was apparent that there was extensive structural variation from previous reports that relied exclusively on NMR analysis. Notably, the C1/C4 (C1'/C4') and C2/C3 (C2'/C3') positions were exchanged, and the configurations of C4 and C4' were inverted. The positions of the heteroatoms in the aminosugar residues could not be confidently assigned using the microED data alone. These heteroatoms were manually introduced to the locations established by earlier chemical degradation studies.⁶ The experimental and calculated powder diffraction data for structure **3b** were in agreement (Fig. S2).

Fig. 2. Preliminary (left) and fully refined (right) microED structures of (–)-lomaiviticin C (**3b**).

In light of these findings, we acquired high-field (800 MHz) NMR spectroscopic data for (–)-lomaiviticin C in acetone-*d*₆, which was found to provide the optimal balance of solubility and consistency of shimming among a number of solvents examined. A Monte Carlo molecular dynamics simulation¹¹ was carried out on structures **3a** and **3b**; the lowest energy conformers are shown in Fig. 3 (the full collection of low-energy conformers for **3a** and **3b** are included as Supporting Information). Notably, the calculated geometry of the lowest-

Fig. 3. Lowest-energy conformers (top) and line structures (bottom) of structures **3a** and **3b**. For clarity, the naphthoquinone residues are shaded in the lowest-energy conformers and omitted from the line structures.

energy conformer of **3b** was in qualitative agreement with the geometry obtained by microED analysis (compare Figs. 2 and 3). Additionally, the lowest-energy conformer of **3a** has an equatorial disposition of H2 and H4, which is consistent with the conformational analysis in the original structural studies.²

NMR signals that were used to distinguish between the structures **3a** and **3b** are discussed below and presented in Fig. 3 (for complete data, see Table S2). ¹H NMR analysis revealed H2 and H2' each as a doublet of doublets at δ 3.95 (J = 4.2, 1.5 Hz) and δ 3.85 (J = 4.1, 1.3 Hz), respectively. These were clearly coupled to each other and to H4 and H4' in the COSY spectrum. H4 and H4' were observed as narrow doublets at 5.51 (J = 1.1 Hz) and 5.33 (J = 1.3 Hz), respectively. A TOCSY experiment showed correlations between (H2, H4') and (H2', H4), thereby confirming these protons are part of the same spin system (see the Supporting Information).

2D-ROESY analysis supported the assignment **3b** but not **3a** (Fig. 4a, b). We observed a clear correlation between (H2, H4) and (H2', H4') (Fig. 4a). These correlations are consistent with structure **3b** wherein the pairs of protons are vicinal to each other. The structure **3a** necessitates that H2, H4, H2', and H4' are equatorially-disposed to account for the putative W-plane coupling discussed above. However this spatial arrangement would not be expected to give rise to the ROESY correlation shown in Fig. 4a. Fig. 4a also shows weaker but definitive correlations between (H2', H4) and (H2, H4'). These correlations across the monomeric units are accommodated by the structure **3b**, but would not be anticipated in structure **3a**, wherein the pairs of protons are oriented on opposing sides of each cyclohexane ring. We also observed a ROESY correlation between (H5', H4) (Fig. 4b). This is readily-accommodated by the position and configuration of H4 in **3b** but not the alternate

Fig. 4. Diagnostic ROESY and HMBC correlations in the NMR spectra of (–)-lomaiviticin C supporting structure **3b**. **a.** ROESY correlations between (H4, H2), (H4', H2'), (H4, H2') and (H4', H2). **b.** ROESY correlation between (H4, H5'). **c.** HMBC correlations between (H4, C2'), (H4, C2), (H4', C2') and (H4', C2). **d.** HMBC correlations between (H12, C1) and (H12', C1').

configuration found in structure **3a**. Additional ROESY correlations support the revised structure assignment (see the Supporting Information)

HMBC analysis (using a ${}^nJ_{C,H}$ threshold of 8.0 Hz) provided further support for the structure **3b**. We observed correlations between (H4, C2) and (H4', C2'), which are consistent with either structure **3a** or **3b** (${}^3J_{C,H}$ coupling in **3a**, ${}^2J_{C,H}$ coupling in **3b**; Fig. 4c). However, we detected additional correlations between (H4, C2') and (H4', C2). These latter correlations require ${}^3J_{C,H}$ coupling in structure **3b**, but would necessitate a ${}^4J_{C,H}$ coupling in structure **3a**. However, a ${}^4J_{C,H}$ coupling is inconsistent with the ROESY data shown in Fig. 4a. Finally, correlations between (H12, C1) and (H12', C1') were observed (Fig. 4d). Interpretation of these correlations as ${}^3J_{C,H}$ couplings support the location of the carbonyl as that shown in **3b**, but would necessitate invoking a ${}^4J_{C,H}$ coupling through an aliphatic system to achieve consistency with structure **3a**. On reducing the ${}^nJ_{C,H}$ threshold to 3.0 Hz we detected a faint HMBC correlation between (H4, C5), as noted in the original characterization of (–)-lomaiviticin A,² but not between (H4', C5') or (H5', C4') (see the Supporting Information).

To provide further support for the structure revision, we carried out computational modeling of ${}^{13}\text{C}$ NMR chemical shifts according to the method of Hehre et al.¹² We focused on lomaiviticin aglycon and (–)-lomaiviticin B because their caged structures significantly decrease the conformational flexibility of the molecules, thereby reducing the computational cost. To benchmark the computational approach we first calculated the expected ${}^{13}\text{C}$ NMR chemical shifts of the aglycon structure **6a** (shown in Fig. 1a), for which experimental ${}^{13}\text{C}$ shifts have been recorded.^{5,7} Fig 5a. depicts the absolute difference between the experimental and calculated chemical shifts as a function of position. We observed good agreement between the calculations and experiment, with all but three positions (C4, C11, and

Fig. 5. **a.** Absolute difference between calculated and experimental ${}^{13}\text{C}$ chemical shifts of the aglycon structure **6a**. RMS = 3.39. **b.** Absolute difference between experimental ${}^{13}\text{C}$ chemical shifts of the aglycon structure **6a** and natural (–)-lomaiviticin B. RMS = 5.82. **c.** Absolute difference between calculated and experimental ${}^{13}\text{C}$ chemical shifts of the aglycon structure **6b** and (–)-lomaiviticin B, respectively. RMS = 1.99.

C11a) within 5 ppm of experimental values, and an overall root mean square (RMS) = 3.39. These results support this computational approach as a viable strategy to determine the expected ${}^{13}\text{C}$ NMR spectra of the lomaiviticins.

We then compared the experimental ${}^{13}\text{C}$ chemical shifts of the aglycon structure **6a** with natural (–)-lomaiviticin B (Fig. 5b). The experimental shifts were in poor agreement (RMS = 5.82). A 17.9 ppm difference was observed between the bridging carbon atoms (C2) and large differences were also observed at the all-substituted C3 stereocenter ($\Delta\delta$ = 10.6 ppm) and the center bearing the aminosugar residue (C4; $\Delta\delta$ = 9.6 ppm). We then determined the expected ${}^{13}\text{C}$ chemical shifts of the revised aglycon structure **6b** and compared these to the experimental chemical shifts of natural (–)-lomaiviticin B (Fig. 5c). Among the three data sets shown in Fig. 5, these data were in closest agreement, with an overall RMS = 1.99. All experimental chemical shifts of (–)-lomaiviticin B were within 5 ppm of the values calculated for the structure **6b**.

Fig. 6. Absolute difference between calculated ^{13}C chemical shifts of structures **2a** and **2b** and natural (–)-lomaiviticin B. Grey series: **2a** (RMS = 6.63); blue series: **2b** (RMS = 2.83).

The data above leave little doubt as to the structure of the lomaiviticins. To further support this conclusion, we calculated the expected ^{13}C NMR chemical shifts of the structures **2a** and **2b** and compared these to the experimental chemical shifts of natural (–)-lomaiviticin B (Fig. 6). With the exception of C4, all chemical shifts calculated for **2b** were within 5 ppm of the corresponding shifts of natural (–)-lomaiviticin B (RMS = 2.83). By comparison, the structure **2a** showed large discrepancies in the critical C2, C3, and C4 positions of the cyclohexene ring, at two adjacent carbon atoms (C11b, C12), and at the hemiketal carbon (C1; RMS = 6.63).

Discussion. The lomaiviticins are complex cytotoxic bacterial metabolites that were first disclosed in 2001. They contain an unusual diazotetrahydrobenzo[*b*]fluorene functional group and the deoxyglycosides L-oleandrose and *N,N*-dimethyl-L-pyrrolidine. The high degree of unsaturation of the lomaiviticins makes their *de novo* structure assignment exceedingly difficult using NMR analysis alone. In the 20 years since their initial disclosure, substantial efforts have been devoted toward their chemical synthesis. Yet, because none of the proposed structures **1a–3a** have been synthesized, a comparison of spectroscopic data derived from synthetic material to that of the natural samples has not been possible. Moreover, to the best of our knowledge, single crystal X-ray crystallographic data of any lomaiviticin has not been disclosed.

Here we used microED analysis of (–)-lomaiviticin C to revise the core structure of this family of metabolites. The results of the microED studies are supported by high-field NMR spectroscopy and computational modeling. The central issue at hand is the orientation of the cyclohexenone ring with respect to the diazofluorene residue. To the best of our knowledge, the original positional assignments in (–)-lomaiviticins A and B (as in structures **1a** and **1b**) rested on two observations. First, it was noted that a weak correlation was detected from H4 to C5 in the HMBC spectrum of (–)-lomaiviticin A (see Fig. 7). At the time this was quite reasonably interpreted as a 3-bond coupling, and this served to anchor the position of the secondary ether (bearing the aminosugar) with respect to the diazofluorene. In addition, both H2 and H4 were observed as singlets in the 500 MHz ^1H NMR spectrum of (–)-lomaiviticin A, but a COSY correlation was observed between these two protons. This COSY correlation was attributed to a *syn*, diequatorial disposition of H2 and H4 (placing the aminosugar and bridging bonds in an axial orientation) giving rise to a 4-bond W-plane coupling. This coupling, in turn served to orient the bridging carbon atom (C2), both in position and stereochemistry, relative to C4, and the remaining substituents could be placed logically within the ring. It is worth noting that X-ray analysis of a 6,6'-bicyclohexenone structurally-related to that found in (–)-

Fig. 7. Original (left) and revised (right) core structures of the lomaiviticins.

lomaiviticin A revealed an axial disposition of the conjoining bond.¹³ In our analysis of natural (–)-lomaiviticin C, we did not observe an HMBC correlation from H4 to C5 using a $^nJ_{\text{C,H}}$ threshold of 8 Hz; a faint coupling was observed when the $^nJ_{\text{C,H}}$ threshold was reduced to 3 Hz. We attribute this correlation to a weak $^4J_{\text{C,H}}$ coupling in the structure **3b**, rather than a $^3J_{\text{C,H}}$ coupling as originally-proposed in structure **1a**. Such four-bond couplings in unsaturated systems are well-precedented.¹⁴ Our molecular dynamics simulations indicate that the dihedral angles between (H2, H4) and (H2', H4') in (–)-lomaiviticin C are 78° and 86°, respectively. Thus, one would anticipate a small coupling between these protons, potentially visible in the COSY but not the one-dimensional ^1H NMR spectrum. Thus, the original assignment appears to have gone awry due to the combination of two unusual spin-coupling effects, and the absence of any additional data points to the contrary.

Because the interconversion of lomaiviticins A–C has been demonstrated (Fig. 1c), the findings here apply to each of these metabolites. The oxidation pattern found in the revised structures **1b**, **2b**, and **3b** has been identified in other isolates, such as nenestatin A (Fig. 8),¹⁵ and in intermediates in kinamycin biosynthesis.^{4c} Interestingly, biosynthetic precursors to nenestatin A were found to undergo nucleophilic addition and dimerization,^{15b} a pathway that may have implications for lomaiviticin biosynthesis. The lomaiviticin biosynthetic gene cluster has been identified,¹⁶ but the mechanism of dimerization remains poorly understood.

Fig. 8. Structure of nenestatin A.

Studies from the Herzon laboratory have established that the cytotoxic effects of (–)-lomaiviticin A derive from the induction of double-strand breaks (DSBs) in DNA.¹⁷ Because these studies were conducted using semisynthetic samples of (–)-lomaiviticin A, derived from diazotransfer to natural (–)-lomaiviticin C, the conclusions of these studies hold. However, in light of this structure revision, the mode of interaction of (–)-lomaiviticin A with DNA¹⁸ will need to be re-interpreted.

Conclusion. In conclusion, we have used a combination of microED, NMR, and computational analysis to advance revised structures for the lomaiviticins. In the revised structures **1b**, **2b**,

and **3b** the positions of the ketone and aminosugar (C1/C4), and the bridging carbon–carbon bond and the center bearing the oleandrose residues (C2/C3) are transposed. The configuration of the secondary ether bearing the aminosugar residue is also inverted. These studies highlight the power of the microED method to elucidate natural product structure in instances where NMR spectroscopy alone may be insufficient.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Procedures for microED analysis, computational methods, and graphical reproductions of NMR spectroscopic data (PDF)

Compiled NMR spectroscopic data for (–)-lomaiviticin C (.mnova)

Molecular dynamics search results for structures **3a** and **3b** (.spar-tan)

Electron crystallographic data for **3b** has been deposited to the Cambridge Crystallographic Data Center (CCDC 2062671)

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