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Mechanism of Nucleophilic Activation of (−)-Lomaiviticin A

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Supporting Information

ABSTRACT: (−)-Lomaiviticin A (**1**) is a C_2-symmetric cytotoxin that contains two diazofluorene functional groups and which induces double-strand breaks (DSBs) in DNA. Evidence suggests DNA cleavage is initiated by hydrogen atom abstraction from the deoxyribose backbone. Here we demonstrate the formation of the vinyl radicals **1**• and **2**• from **1** by 1,7-addition of thiols to the diazofluorenes. These radicals can affect hydrogen atom abstraction from methanol and acetone. The first addition of thiol to **1** proceeds at a much greater rate than the second. The diazosulfide **5** formed en route to **1**• has been detected at −50 °C and undergoes decomposition to **1**• with a half-life of 110 min at −20 °C under air. These data, which constitute the first direct evidence for the generation of **1**• and **2**• from **1**, provide insights into the mechanism of DNA cleavage by **1**.

The C_2-symmetric bacterial metabolite (−)-lomaiviticin A (**1**, Scheme 1) induces double-strand breaks (DSBs) in DNA[1] and is undergoing preclinical evaluation as a combination[2] and monotherapy[1e] for the treatment of DNA DSB repair-deficient tumors.[3] **1** binds DNA by a mode of association involving penetration of both diazotetrahydrobenzo[*b*]fluorene (diazofluorene) residues into the duplex.[4] The related metabolite (−)-lomaiviticin C (**2**)[1b,c] contains only one diazo substituent and does not induce DNA DSBs.[1d] In vitro reactivity studies of synthetic diazofluorene analogs[5] led to the hypothesis that carbon-centered radical intermediates form from the diazofluorene. It was later proposed that **1** is transformed to the sp^2-radicals **1**• and **2**• in tissue culture and that these affect strand cleavage[6] by hydrogen atom abstraction from the deoxyribose backbone.[1d] A single hydrodediazotization of **1** generates **2**; 2-fold reaction of **1** forms the double hydrodediazotization product **3**.

Here we provide the first direct experimental evidence for the formation of **1**• and **2**• from **1** by 1,7-addition of thiol-based nucleophiles to the diazofluorene. Our studies lead to the unexpected observation that the rate of the first thiol addition (**1**→**2**) vastly exceeds that of the second (**2**→**3**). The radicals **1**• and **2**• affect hydrogen atom abstraction from methanol and acetone (BDE = 95 and 93.9 kcal/mol, respectively).[7]

We first studied the reactivity of **1** toward *N*-acetyl-L-cysteine methyl ester (NACME) in methanol-d_4 and acetone-d_6. Each experiment was conducted under air and argon. In the absence of base, mixtures of (−)-lomaiviticin A (**1**) bis(trifluoroacetate) (1.22 mM) and NACME (40 equiv) remained unchanged after at least 8 h at 25 °C.[8] When triethylamine (TEA, 40 equiv) was added to a solution of **1** (1.22 mM) and NACME (40 equiv) in methanol-d_4 at 25 °C under air, an instantaneous color change from vivid red to dark-brown red was observed. Immediate (<5 min) analysis by ^{1}H NMR spectroscopy revealed the formation of **2-*d*** (94%, entry 1, Table 1). Upon aging, the solution of **2-*d*** transformed to **3-d_2**, with a half-life of 49 min (83%). Under argon, the yield of **2-*d*** was 97%, and the rate of conversion to **3-d_2** was faster ($t_{1/2}$ = 5 h at 5 °C, entry 2). (−)-Lomaiviticin A (**1**) was also instantaneously converted to **2-*d*** in acetone-d_6 (84% and 96% yield under air and argon, entries 3, and 4, respectively) but **3-d_2** was not observed. Instead, **2-*d*** slowly transformed to unidentified decomposition products. This sequence was readily followed by ^{1}H NMR spectroscopy as transformation of the C_2-symmetric structure **1** to the C_1-symmetric structure **2-*d*** results in doubling of most signals (Figure 1). Loss of the remaining diazo substituent restores C_2-symmetry (as **3-d_2**), leading to simplification of the spectra. These experiments reveal that the rate of 1,7-addition to the first diazofluorene of **1** is faster than the remaining diazofluorene in **2**.

The source of deuterium at the vinylic position of **2-*d*** was elucidated by repeating the experiments separately in methanol and methanol-d_4, and analyzing the products by LC/HRMS. (−)-Lomaiviticin C (**2**) ionizes by ejection of the aminosugar residue proximal to the hydroxyfulvene, leading to a prominent daughter ion corresponding to **4** upon MS analysis (Figure 2A).[1b,9] LC/HRMS analysis of the first hydrodediazotization of **1** in methanol indicated formation of **4** ($[M]^+ = C_{60}H_{66}N_3O_{21}^+$: calculated, =1164.4183; observed = 1164.4169; error = 1.20 ppm), whereas the same experiment conducted in methanol-d_4 provided **4-*d*** ($[M]^+ = C_{60}DH_{65}N_3O_{21}^+$: calculated = 1165.4246; observed = 1165.4220; error = 2.23 ppm, Figure 2B). To identify the site of bond cleavage in methanol (e.g., C−H/D or O−H/D), and to remove any potential complications arising from O−H/D exchange, we conducted additional experiments in CH_3OD and CD_3OH. Mass spectral analysis of the hydrodediazotization of **1** in CH_3OD revealed generation of **4** (observed = 1164.4162; error = 1.80 ppm), and analysis of the same experiment in CD_3OH indicated generation of **4-*d*** (observed = 1165.4222; error = 2.06 ppm). Strictly analogous results were obtained when the 2-fold hydrodediazotization of **1** was conducted in CH_3OD or CD_3OH. Reaction in the former solvent formed the protiated product **3** whereas reaction in the latter solvent generated **3-d_2** (Figure S1). These results indicate that the newly formed C−H/D bonds in **2** and **3**

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Scheme 1. Proposed Pathway for the Conversion of (−)-Lomaiviticin A (1) to (−)-Lomaiviticin C (2) and the Double Hydrodediazotization Product 3 via the Vinyl Radical Intermediates 1· and 2·

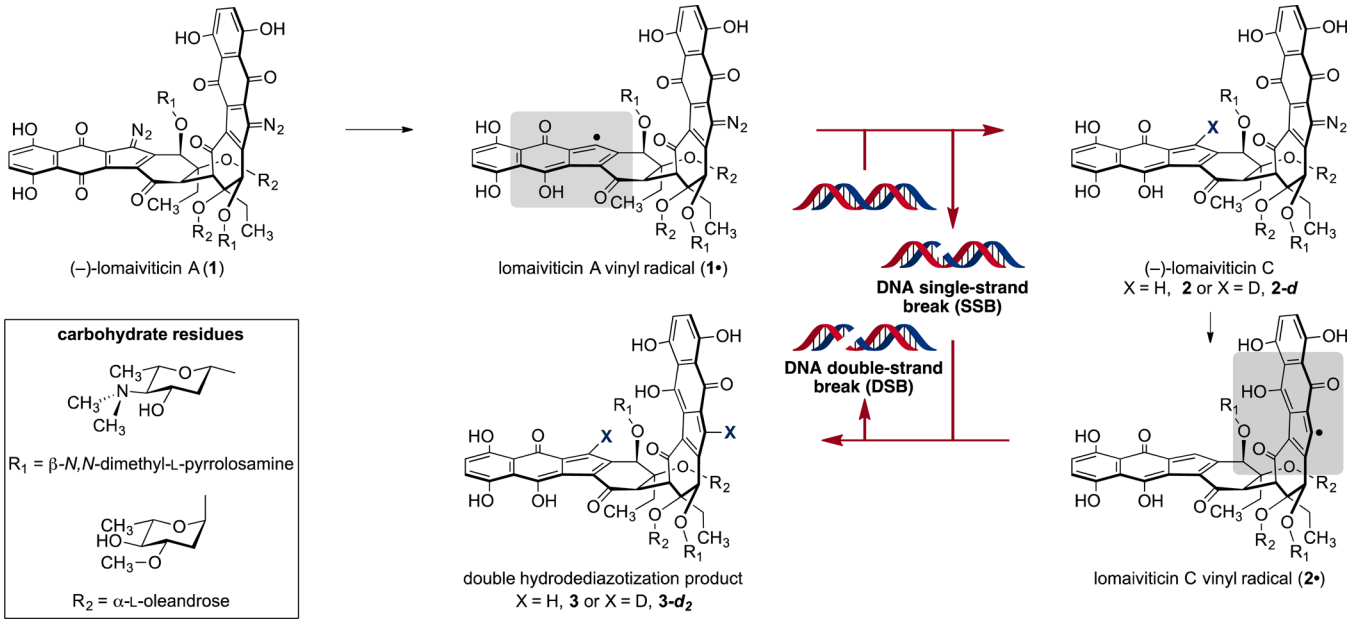
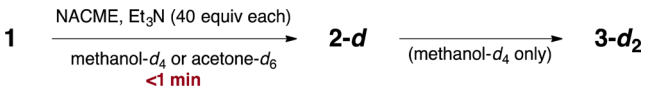


Table 1. Hydrodediazotization Studies of 1



entry	atmos.	solvent	yield 2-*d*[a]	yield 3-d_2[a] ($t_{1/2}$, T)
1	air	CD_3OD	94%	83% (49 min, 25 °C)
2	argon	CD_3OD	97%	87% (5 h, 5 °C)
3	air	acetone-d_6	84%	n/d[b]
4	argon	acetone-d_6	96%	n/d[b]

[a]Yields were determined by ^{1}H NMR spectroscopy using 1,4-dicyanobenzene as an internal standard and are based on **1**. [b]n/d = not detected.

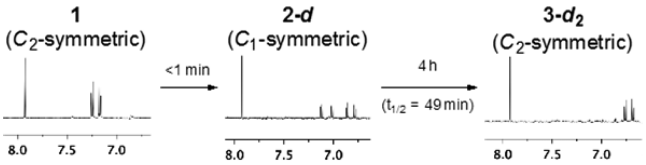


Figure 1. Aryl region of the ^{1}H NMR spectra of **1**, **2-*d***, and **3-d_2**. Conditions: **1** (1.22 mM), TEA (40 equiv), NACME (40 equiv), methanol-d_4, air, 25 °C.

derive from C−H/D bond cleavage in methanol and provide compelling evidence for the intermediacy of the sp^2 radicals **1·** and **2·**.

The requirement for base in the conversion of **1** to **2** and **3** is consistent with 1,7-addition of thiolate to generate a diazosulfide intermediate, followed by loss of dinitrogen and thiyl radical (Scheme 2). To probe this, we monitored the reactivity of **1** toward benzylthiol in the presence of triethylamine at low temperature.[10] Addition of benzylthiol (10 equiv) and triethylamine (10 equiv) to a solution of **1** (1.22 mM) in acetone-d_6 at −50 °C under air instantaneously formed the diazosulfide **5** (81%, Scheme 3). The diazosulfide **5** was generated as a ∼1:1 mixture of *E*:*Z* isomers that converted to a 3:1 mixture (presumably, *E*:*Z*) after standing for 1 h at −50 °C.

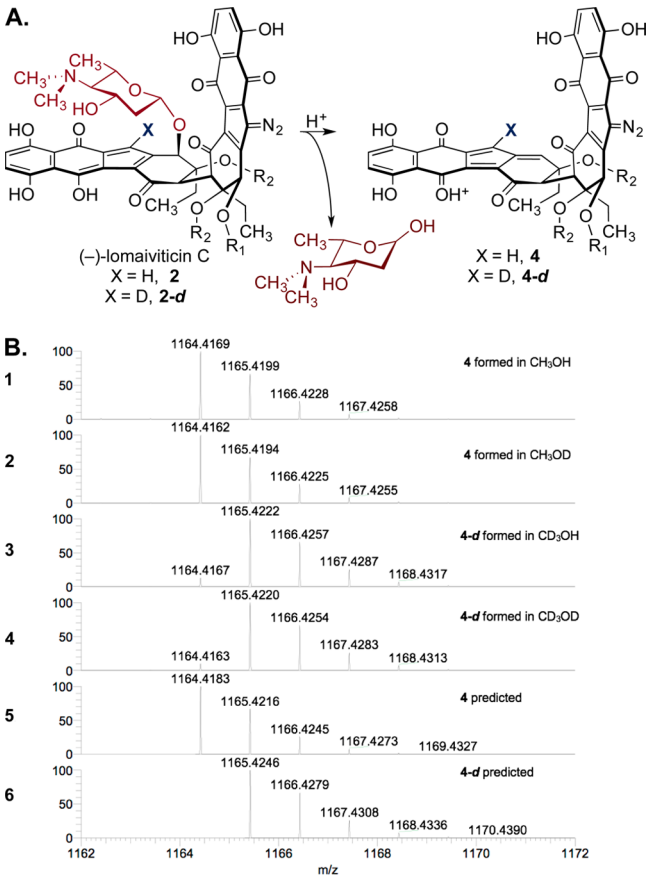


Figure 2. (A) Ionization of **2** or **2-*d*** leads to ejection of the aminosugar residue and observation of the elimination products **4** or **4-*d*** by HRMS analysis.[1b,9] (B) 1, 2: Selected region of the HRMS spectrum of **4**, generated by hydrodediazotization of **1** in CH_3OH or CH_3OD, respectively. 3, 4: Selected region of the HRMS spectrum of **4-*d***, generated by hydrodediazotization of **1** in CD_3OH or CD_3OD, respectively. 5, 6: Predicted isotope distribution of **4** and **4-*d***, respectively.

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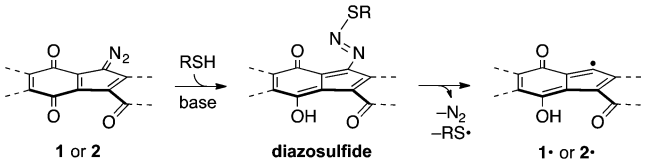
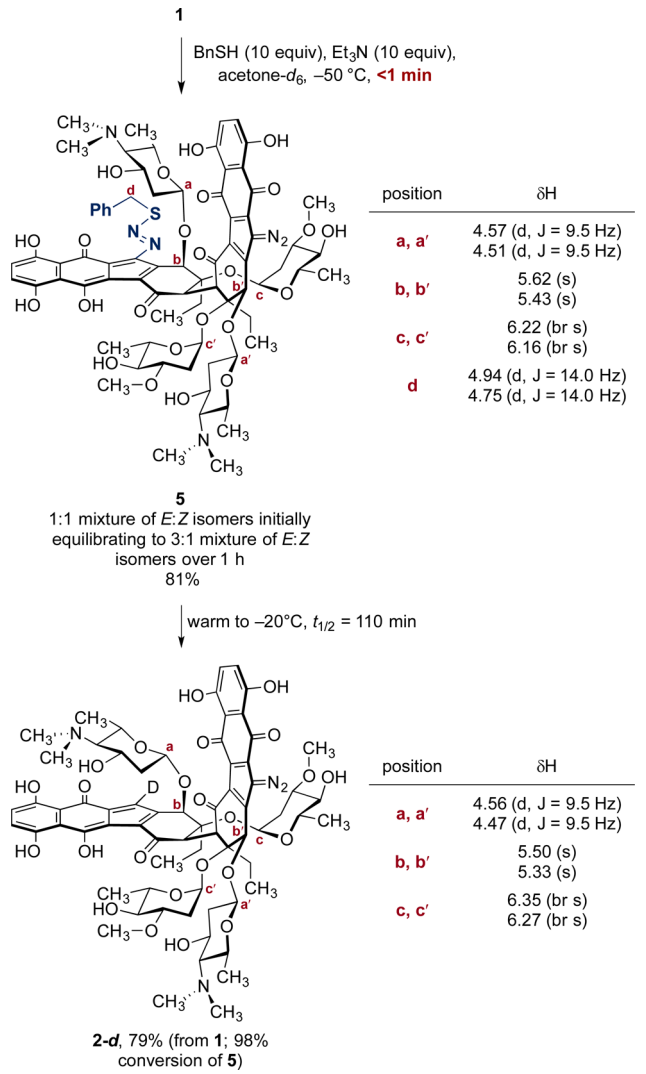
Scheme 2. Postulated Pathway for the Formation of 1· and 2· via Nucleophilic Addition of Thiol



Scheme 3. Generation and Decomposition of the Diazosulfide 5.[a]



[a]Spectroscopic data shown corresponds to the major diazosulfide isomer. Reaction was run under air. ^{1}H NMR data were acquired at −20 °C.

The 3:1 mixture of diazosulfides **5** was stable for at least 12 h at −50 °C and was characterized by ^{1}H, HSQC, and HMBC NMR analysis. The protons α to sulfur in the major isomer of **5** appeared as two distinct doublets (J = 14.0 Hz) centered at 4.94 and 4.75 ppm. These were correlated to the same carbon atom (36.5 ppm; 28.4 ppm in free benzylthiol) in the HSQC spectrum and to the quaternary carbon of the phenyl ring in **5** (130.5 ppm; 128.8 in free benzylthiol) in the HMBC spectrum. Warming to −20 °C induced transformation of **5** to **2-*d***, with a half-life of 110 min (79% yield from **1** at 98% conversion of **5**). Under argon, the diazosulfide **5** was formed in quantitative yield and transformed to **2-*d*** with a half-life of 49 min (>99%). No intermediates were detected when the conversion of **2-*d*** to **3-d_2** was monitored carefully by NMR spectroscopy, suggesting decomposition of the putative diazosulfide derived from **2-*d*** is faster than its formation.

DFT calculations were employed to gain insight into the relative rates of addition to **1** and **2**.[1d] The optimized structure of **1** using the B3LYP 6-31G(d) level of theory and an aqueous solvent model is shown in Figure 3 and indicates that the distance from the diazo carbon to the opposing diazofluorene is 3.8 Å. We propose that the developing anionic charge in the transition state for addition to **1** is stabilized by a through-space interaction with the adjacent electron-deficient diazofluorene. The transformation of **1** to **2** converts a diazofluorene to an electron-rich hydroxyfulvene, and the transition state for the second addition may not benefit from the same stabilization. It is also possible that the hydroxyfulvene in **2** is strongly hydrogen-bound (or deprotonated) under these conditions, which would further decrease electrophilicity. The structure shown in Figure 3 parallels of **1** bound to DNA,[4] suggesting these transannular interactions are relevant in tissue culture.

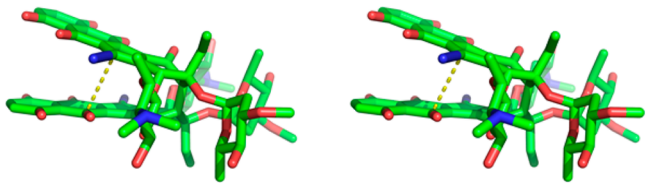


Figure 3. Stereoview of DFT-minimized structure of **1** in water [B3LYP 6-31G(d)]. Hydrogen atoms are omitted for clarity.

These data provide several insights into the mechanism of DNA cleavage by **1**. First, these experiments show that the radicals **1·** and **2·** can be formed from **1** by nucleophilic addition, and that these are competent to cleave relatively strong C−H bonds, providing support for DSB induction by a hydrogen atom abstraction mechanism. Second, the faster rate of nucleophilic addition to **1** than **2** may provide an explanation for the greater proportion of single-strand breaks than DSBs produced by **1** (in an in vitro plasmid cleavage assay, this ratio was ∼5:1[1d]): dissociation of **2** from the duplex may be competitive with the formation of **2●**. Finally, the facile conversion of **1** to **2** suggests that natural **2** derives from hydrodediazotization of **1** during bacterial growth. As **2** is several orders of magnitude less potent,[1b] this reactivity may constitute a fortuitous detoxification pathway for the producing strain. A question currently unresolved is the nature of the nucleophile in the presence of DNA. Given the short lifetimes of sp^2 radicals, we hypothesize that **1·** and **2·** are generated after binding, potentially by direct addition of a nucleotide[11] to the coordinated metabolite.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b09657.

Detailed experimental procedures and complete spectroscopic data for all new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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