The cytotoxicity of (–)-lomaiviticin A arises from induction of double-strand breaks in DNA.

.

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Supporting Information

Index

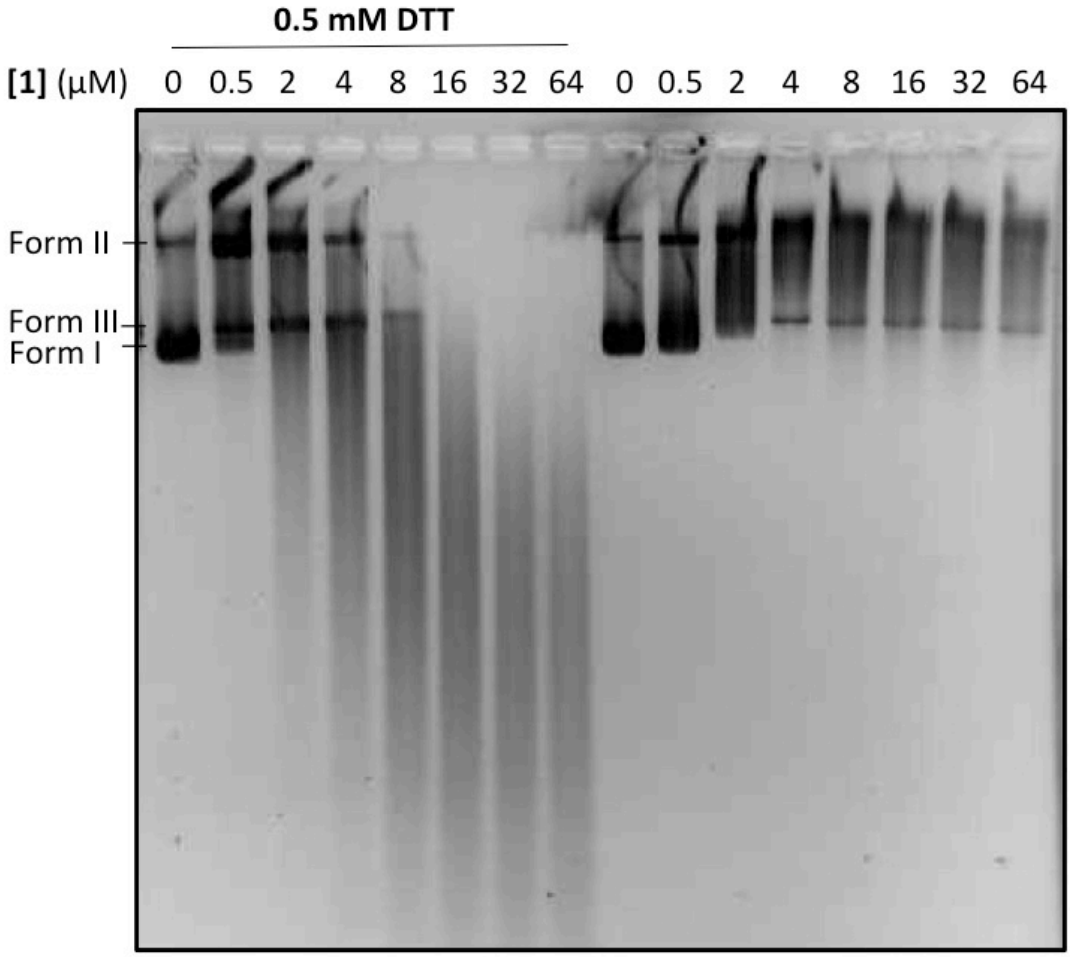


Figure S1. Analysis of nicking and cleavage of plasmid pBR322 DNA by (–)-lomaiviticin A (**1**) at varying concentrations and in the presence or absence of DTT. [**1**] = 0.5–64 μM, pH 8.0, [DTT] = 0 or 0.5 mM, 37 °C, 16 h. Form III DNA was observed as early as 1 h.

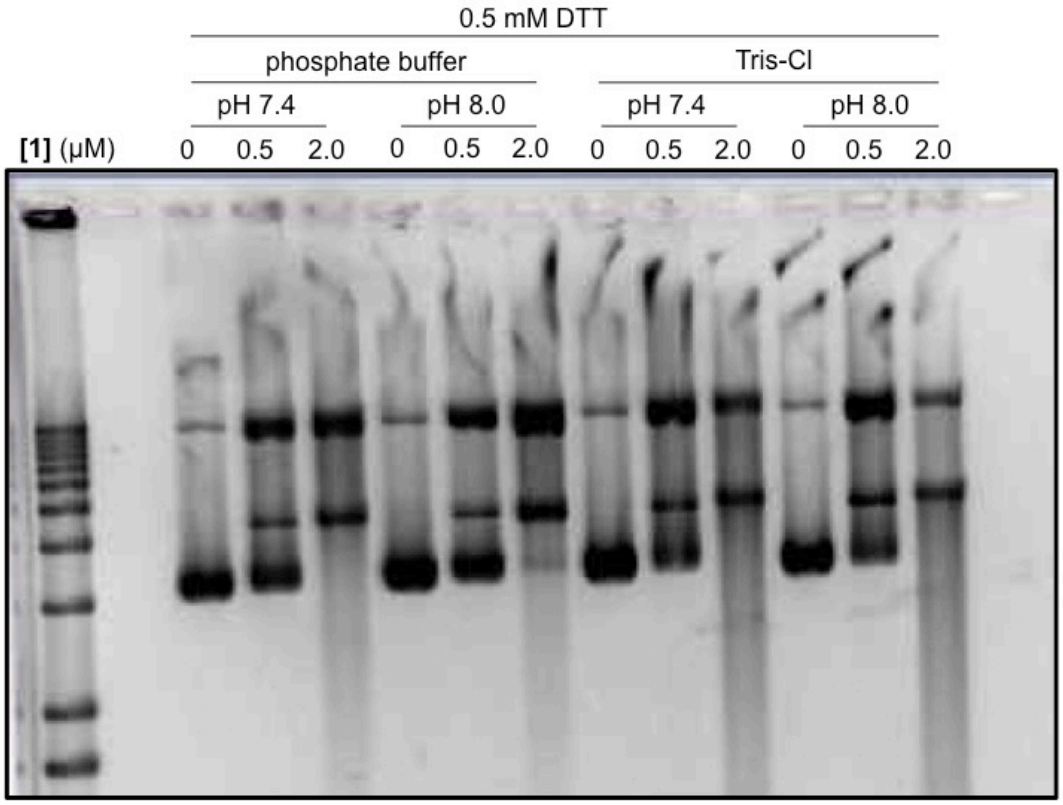
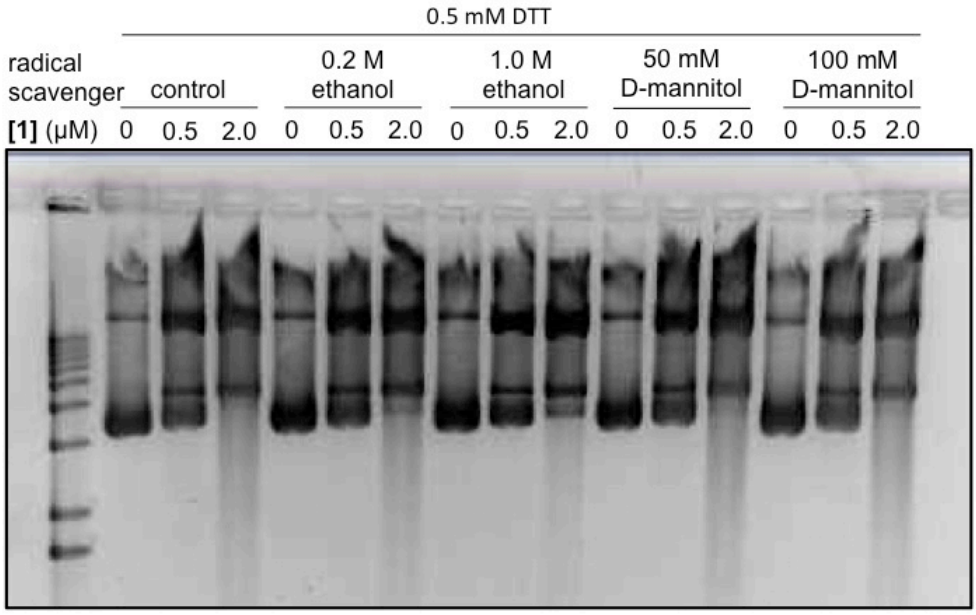


Figure S2. The DNA damaging activity of (–)-lomaiviticin A (**1**) is slightly enhanced in 10 mM Tris-Cl buffer compared to 10 mM sodium phosphate buffer. [**1**] = 0.5 or 2.0 μM, pH = 7.4 or 8.0, [DTT] = 0.5 mM, 37 °C, 16 h. Buffers: 10 mM aqueous sodium phosphate buffer/50 mM NaCl or 10 mM Tris-Cl buffer/50 mM NaCl.

A.



B.

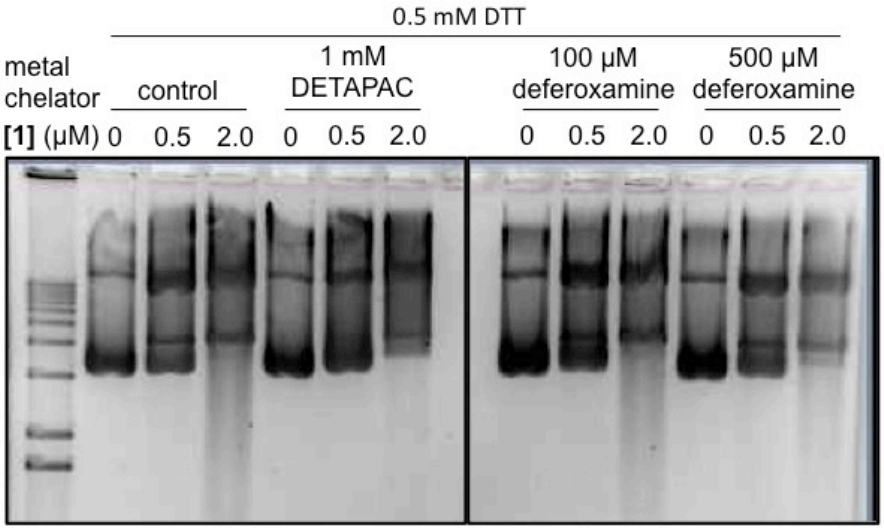
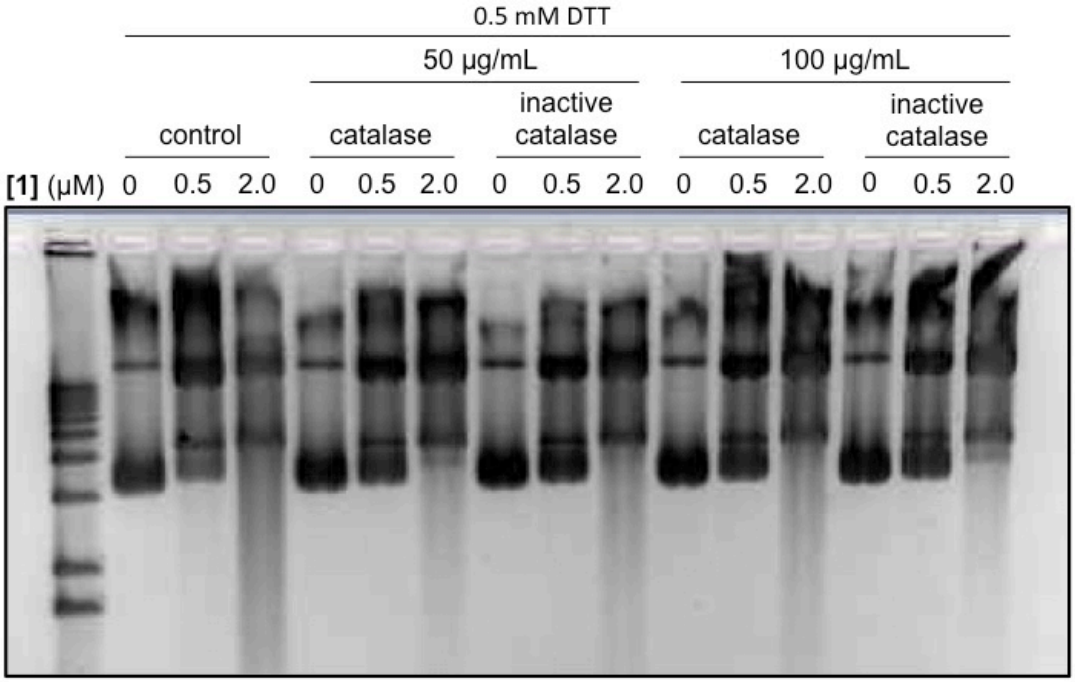


Figure S3. The DNA damaging activity of (–)-lomaiviticin A (**1**) was not measurably influenced by the presence of the hydroxyl radical scavengers ethanol and mannitol (**A**) or the metal complexing agents deferoxamine and diethylenetriaminepentaacetic acid (DETAPAC) (**B**). [**1**] = 0.5 or 2.0 μM, pH 8.0, [DTT] = 0.5 mM, scavenger, 37 °C, 16 h. The scavenger was pre-incubated with DNA and buffer for 30 min at 37 ˚C before addition of **1** and DTT, in that order.

A.



B.

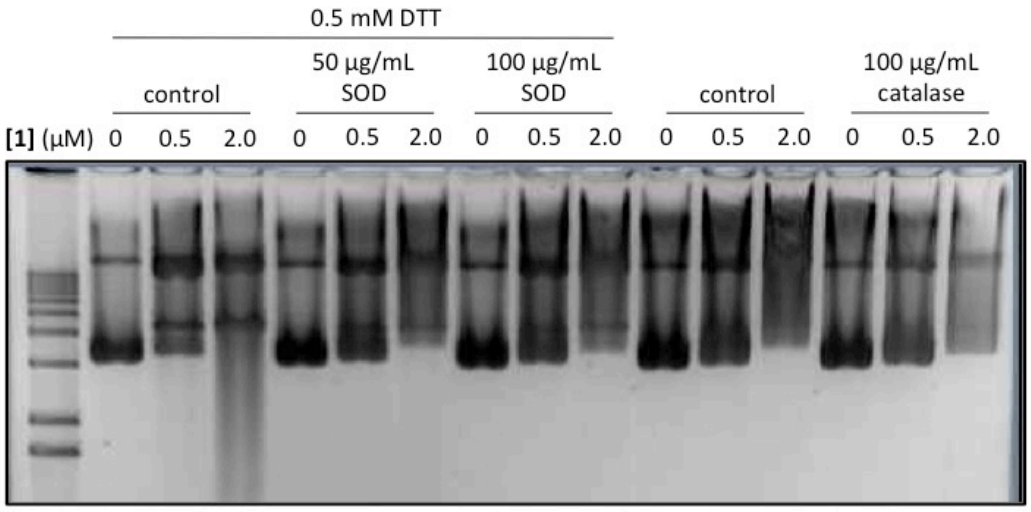


Figure S4. Plasmid DNA damaging activity of (–)-lomaiviticin A (**1**) is not mediated by hydrogen peroxide (**A**) or superoxide radical anion (**B**). [**1**] = 0.5 or 2.0 µM, pH 8.0, [DTT] = 0.5 mM (except final six lanes, Figure S4B, where no DTT was employed), catalase (active or inactive), or superoxide dismutase (SOD), 37 °C, 16 h. The enzyme was added immediately to the prior to addition of **1**, DNA in buffer and DTT, in that order. Inactive catalase was prepared by boiling catalase for 5 min.

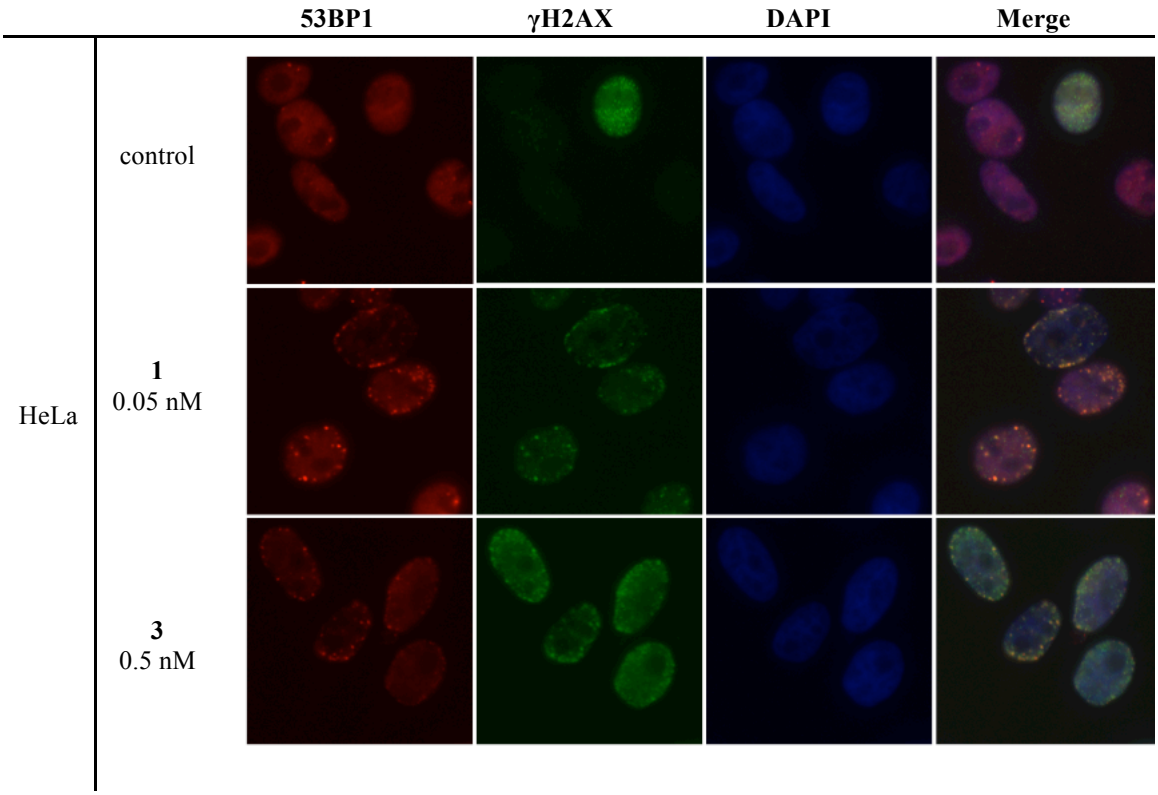
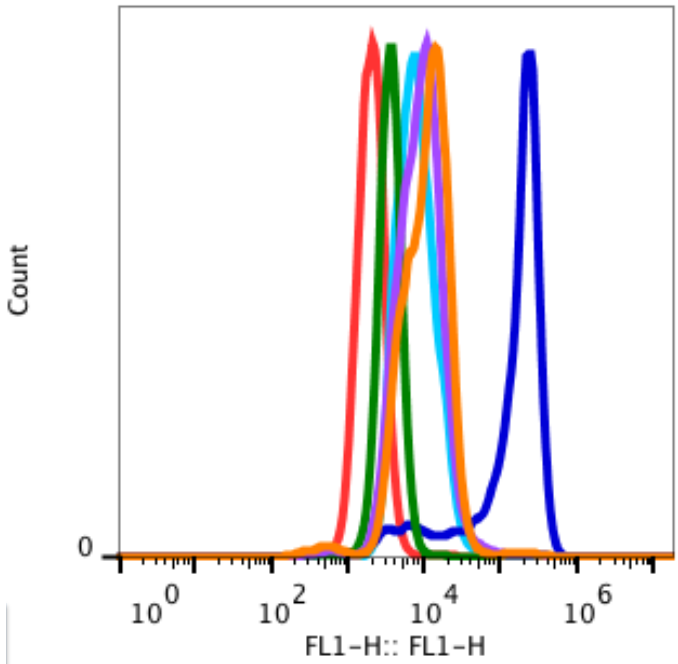


Figure S5. γH2AX and 53BP1 foci are induced by (–)-lomaiviticin A (**1**) and colocalize in HeLa cells. Columns (left to right), 53BP1 (red), γH2AX (green), DAPI (blue), merge. Rows (top to bottom): control, 0.05 nM **1**, 0.5 nM **1**. HeLa cells in exponential growth phase were incubated with 0.05 or 0.5 nM **1** for 4 h. Immunological detection was performed using a primary antibody [rabbit polyclonal anti-53BP1 antibody (Novus Biologicals) and mouse monoclonal anti-phospho-histone H2AX (SER139) antibody (Upstate)] and visualized with Alexa 488 (goat-anti-mouse IgG) and Alexa 594 (goat-anti-rabbit IgG) (Molecular Probes). Mounting medium contained DAPI to visualize nuclear DNA.



Key	mean	% increase
1	104058	1300%
2	8261	11%
3	9512	28%
anti-γH2AX AB	7433	
mouse IgG AB	3329	
no AB	1897	

Figure S6. (–)-Lomaiviticin A (**1**) induces phosphorylation of histone H2AX in K562 cells after treatment for 4 h at 37 ˚C. Cells treated with (–)-lomaiviticin C (**2**) and (–)-kinamycin C (**3**) did not induce formation of γH2AX. Cells were harvested 4 h following treatment with 312 nM **1**, **2**, or **3**, fixed, permeabilized, and stained with FITC-conjugated mouse monoclonal anti-phospho-histone H2A.X (SER139) (Upstate) or FITC-conjugated normal mouse IgG as negative control (Upstate). Sample analysis (10,000 cells) was performed on an Accuri flow cytometer using a 488 nm excitation laser. Emission detected with the filter for FITC.

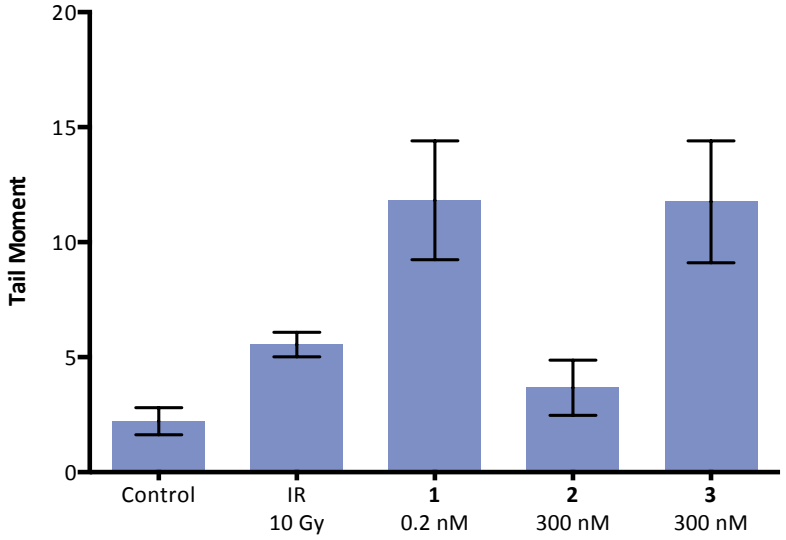


Figure S7. (–)-Lomaiviticin A (**1**) induces DNA dsb formation in PEO1 C4-2 cells at 0.2 nM concentrations, while (–)-lomaiviticin C (**2**) induces minimal production of DNA dsbs at 300 nM concentration. Tail moment obtained in a neutral comet unwinding assay employing (–)-lomaiviticin A (**1**, 0.2 nM), (–)-lomaiviticin C (**2**, 300 nM), or (–)-kinamycin C (**3**, 300 nM), and PEO1 C4-2 cells. Drug exposure was 24 h. Bars represent mean tail moment (208–263 cells), error bars represent standard error of the mean.

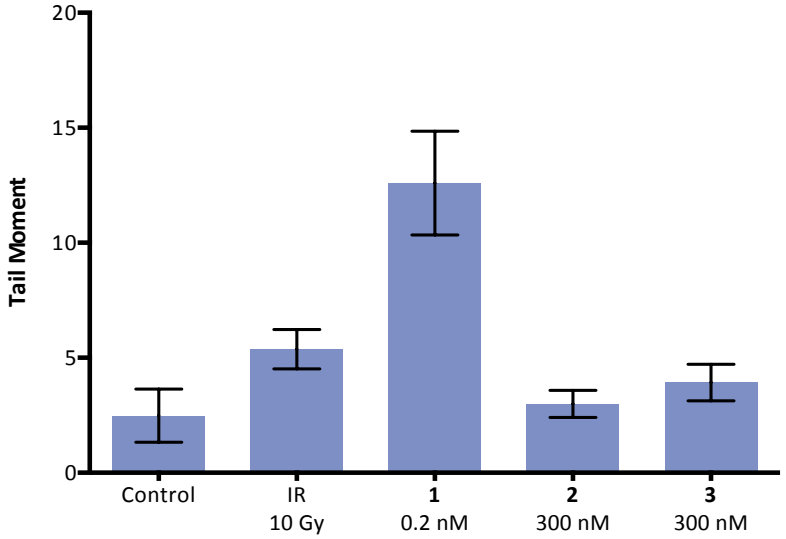


Figure S8. (–)-Lomaiviticin A (**1**) induces DNA dsb formation in PEO1 cells at 0.2 nM concentrations, while (–)-lomaiviticin C (**2**) and (–)-kinamycin C (**3**) are less active at 300 nM concentrations. Tail moment obtained in a neutral comet unwinding assay employing (–)-lomaiviticin A (**1**, 0.2 nM), (–)-lomaiviticin C (**2**, 300 nM), or (–)-kinamycin C (**3**, 300 nM), and PEO1 cells. Drug exposure was 24 h. Bars represent mean tail moment (172–227 cells), error bars represent standard error of the mean.

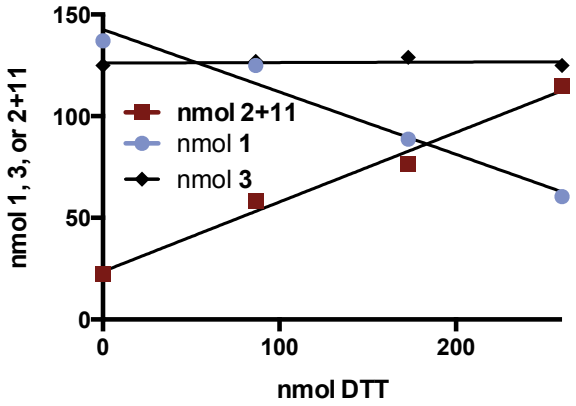


Figure S9. Competition hydrodediazotization experiment between (–)-lomaiviticin A (**1**) and (–)-kinamycin C (**3**). Sample of (–)-lomaiviticin A (**1**) contained 14% (–)-lomaiviticin C (**2**). 1/m = –0.0188, 0.01688, and 2.481 for **1**, **2**, and **3**, respectively. Conditions: **1** (137 nmol), **3** (125 nmol), DTT (4 × 86.6 nmol), methanol-d_4 (400 μL), 21 °C.

General Chemical Procedures. All reactions were performed in 1-dram vials fitted with a Teflon-coated cap under a positive pressure of nitrogen, unless otherwise noted. Organic solutions were concentrated by rotary evaporation at 30–33 °C. Normal or reverse phase flash-column chromatography was performed as described by Still, et al.[1] Normal phase purifications employ silica gel (60 Å, 40−63 μm particle size) purchased from Sorbent Technologies (Atlanta, GA). Reverse phase purifications employ C_{18}-labeled silica gel (125 Å, 55–105 μm particle size) purchased from Waters Corporation (Milford, MA).

Chemical Materials. Commercial solvents and reagents were used as received, with the following exceptions. Methanol-d_4 was sparged with dinitrogen for 10 min before use. (–)-Lomaiviticins A (**1**) and C (**3**) were prepared according to the method of Herzon and co-workers.[2] Standard solutions of **1** and **3** in dimethyl sulfoxide were stored at –80 °C.

Instrumentation. Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded at 400 or 500 MHz at 24 °C, unless otherwise noted. Chemical shifts are expressed in parts per million (ppm, δ scale) downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent (CHD_2OD, 3.31, HDO, δ 4.80). Data are represented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, m = multiplet and/or multiple resonances, br = broad, app = apparent), integration, coupling constant in Hertz, and assignment. Analytical ultra high-performance liquid chromatography/mass spectrometry (UPLC/MS) was performed on a Waters UPLC/MS instrument equipped with a reverse-phase C_{18} column (1.7 μm particle size, 2.1 × 50 mm), dual atmospheric pressure chemical ionization (API)/electrospray (ESI) mass spectrometry detector, and photodiode array detector. Samples were eluted with a linear gradient of 20% acetonitrile–water containing 0.1% formic acid→100% acetonitrile containing 0.1% formic acid over 3 min, followed by 100% acetonitrile containing 0.1% formic acid for 1 min, at a flow rate of 800 μL/min. Flow cytometry was performed on an Accuri C6 flow cytometer using a 488 nm excitation laser. Emission was detected with the 450/40 filter/bandpass for FITC. DNA in the gel was visualized by its fluorescence on a ChemiDoc XRS apparatus (Bio-Rad) equipped with a 302 nm trans-UV illuminator and a CCD camera. The fluorochromes in immunostained cell samples were visualized with a Zeiss Axiovert 200 M epifluorescence microscope equipped with a 63×/1.40 Plan-Apochromat oil immersion objective. Fluorescence illumination was initiated using an EXFO X-cite Series 120 Hg arc lamp. Filters for DAPI, GFP and Cy3 were used to acquire images. Images were captured with Zeiss Axiocam Mrm camera and AxioVision software.

Cell Culture. K562 cells were obtained from Professor David Spiegel (Yale University). HeLa cells were obtained from Professor Craig M. Crews (Yale University). All cell culture reagents were obtained from Invitrogen. K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. HeLa cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. MCF7 cells were obtained from the ATCC (Manassas, VA) and grown in RPMI with L-glutamine supplemented with 10% fetal bovine serum. PEO1 and PEO1 C4-2 ovarian cancer cells were provided by Dr. Toshiyasu Taniguchi (Fred Hutchinson Cancer Research Center, Seattle, WA). VC8 and VC8+BRCA2 Chinese hamster ovary (CHO) cells were provided by Dr. Martin Brown (Stanford University, Stanford, CA). Both cell sets were grown in high glucose DMEM supplemented with 10% fetal bovine serum. All cell lines were maintained at 37 ºC under an atmosphere of 5% CO_2 and constant humidity.

Plasmid DNA Cleavage Assays.

General Conditions:
Reactions using supercoiled pBR322 plasmid DNA (New England Biolab) were performed in 20.0 μL of 10 mM sodium phosphate buffer/50 mM NaCl, pH 8.0, unless otherwise noted. The DNA concentration was 38 μM in DNA bp. In a typical assay, DTT (5 mM, 2.0 μL) was added to a mixture of **1** in DMSO (2.0 μL of solution in DMSO), water (to make up it up to 20.0 μL), buffer (100 mM sodium phosphate solution, pH 8.0, 2.0 μL), salt (500 mM NaCl, 2.0 μL) and pBR322 DNA (0.25 μg/μL in water, 2.0 μL), mixed in that order. The solution was thoroughly mixed by pipetting and incubated at 37˚C for 16 h. Solutions of **1** were prepared immediately before use. The final reaction contained 10% DMSO by volume.

Survey of Buffer Conditions:
(–)-Lomaiviticin A (**1**) (5 or 20 μM, 2.0 μL) was allowed to react with supercoiled pBR322 plasmid DNA in the presence of DTT (5 mM, 2.0 μL) over a pH range of 5.7 to 8.0. The buffers that were tested include: 10 mM Tris-Cl buffer, pH 7.4 and 8.0; 10 mM sodium phosphate buffer, pH 5.8, 7.4 and 8.0; 10 mM sodium citrate-phosphate buffer, pH 5.8 and 7.4. The reaction was performed using the method described above.

Influence of Hydroxyl Radical Scavengers and Iron Chleating Agents:
The additives ethanol (2.0 and 10.0 M, 2.0 μL), D-mannitol (0.25 and 0.50 M, 4.0 μL), to trap hydroxyl radicals, and desferoxamine (1.0 and 5.0 mM, 2.0 μL) or DETAPAC (10.0 mM, 2.0 μL) to complex adventitious iron, were preincubated for 30 min at 37˚C with water, buffer, salt and DNA. After preincubation, the mixture was added to **1** in DMSO. DTT (5 mM, 2.0 μL) was then added last to the reaction. The solution was thoroughly mixed by pipetting and incubated at 37˚C for 16 h.

Influence of Catalase and Superoxide Dismutase:
To test the dependance of DNA cleavage on hydrogen peroxide or superoxide radical anion, active catalase (500 μg/mL and 1000 μg/mL, 2.0 μL), inactivated catalase (500 μg/mL and 1000 μg/mL, 2.0 μL), or superoxide dismutase (500 μg/mL and 1000 μg/mL, 2.0 μL), was added immediately prior to addition of **1**, followed by water, buffer, salt and DNA mixture. DTT (5 mM, 2.0 μL) was then added last to the reaction. The solution was thoroughly mixed by pipetting and incubated at 37˚C for 16 h. Inactive catalase was prepared by boiling catalase for 5 min.

*Analysis of Plasmid DNA Damage by (–)-Lomaiviticin C (**2**):*
(–)-Lomaiviticin C (**2**) (0.020, 1.0, 5.0, 10.0 mM, 10.0 μL) in DMSO was allowed to react with supercoiled pBR322 plasmid DNA (0.25 μg/μL, 10.0 μL) in a 100 μL reaction containing 10 mM sodium phosphate buffer/50 mM NaCl, pH 7.4. The reaction was performed with or without DTT (5 mM, 10.0 μL). The solution was thoroughly mixed by pipetting and incubated at 37˚C for 16 h. Following incubation, **2** was extracted from 80 μL of the reaction mixture with equal volume of phenol/chlorofom/isoamyl alcohol. The DNA was recovered by precipitation with ethanol and dissolved in TE buffer (10 mM Tris-Cl and 1mM EDTA, 20 μL), pH 7.4. After quantitation of the recovered DNA, 0.5 μg was prepared for agarose gel electrophoresis and DNA cleavage quantitation was performed as described below.

*Analysis of Plasmid DNA Damage by (–)-Kinamycin C (**3**):*
To test the DNA cleaving activity of (–)-kinamycin C (**3**), DTT (5 mM, 2.0 μL) was added to a mixture of **3** (0.020, 1.0, 5.0, 10.0 mM, 2.0 μL) in DMSO, pBR322 DNA (0.25 μg/μL, 2.0 μL), and 10 mM sodium phosphate buffer/50 mM NaCl (pH 7.4). Water (to make up it up to 20.0 μL) was added to reactions under non-reducing condition instead of DTT. The solution was thoroughly mixed by pipetting and incubated at 37˚C for 16 h.

Ratio of DNA Double-Strand Breaks to Single-Strand Breaks:
Using the plasmid DNA cleavage assay method described above, the ratio of dsbs to ssbs was obtained by titrating (–)-lomaiviticin A (**1**) over a concentration range of 0 to 1.0 μM with pBR322 plasmid DNA (0.25 μg/μL, 2μL). Forms I (supercoiled), II (nicked), and III (linearized) plasmid DNA in the reaction mixture was separated by agarose gel (1%, w/v) electrophoresis and quantified as described in the method below.

The ratio of DNA dsbs/ssbs was calculated according to the equations:[3-5]

$$f_{III} = n_2 e^{-n_2}$$

$$f_I = e^{-(n_1+n_2)}$$

where:

f_{III} and f_I represent the fractions of Form III and Form I DNA, respectively, after treatment
n_1 represents the number of ssbs/molecule of DNA
n_2 represents the number of dsbs/molecule of DNA

The first equation was solved for n_2 by a seventh-order Taylor approximation.

Agarose Gel Electrophoresis and Quantitation of DNA Cleavage:
Following incubation, 6X DNA loading dye (10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA, 3.5 μL) was added to the 20.0 μL DNA cleavage mixture. The resulting solution was mixed thoroughly, spun quickly on a microcentrifuge, and loaded immediately onto a 1X TBE agarose gel (1.0%, w/v). Forms I, II, and III DNA were separated by electrophoresis (2.5 h at 6 V/cm) in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3), followed by staining with 1X SYBR Green I solution in 1X TBE for 30 min. Immediately after staining, the DNA in the gel was visualized by its fluorescence on a ChemiDoc XRS apparatus (Bio-Rad) equipped with a 302 nm trans-UV illuminator and a CCD camera. The amount of Forms I, II, or III DNA were quantitated using Quantity One 1-D analysis software (Table S1).

Table S1. Quantification and standard deviation of Fom I, Form II, and Form III plasmid DNA.

[1], (μM)	% Form I	STDEV	% Form II	STDEV	% Form III	STDEV
0.000	73.013	0.153	22.032	0.737	4.955	0.890
0.010	68.893	0.258	25.101	0.006	6.006	0.264
0.050	54.852	0.457	35.571	2.173	9.576	2.630
0.100	53.175	4.500	36.606	2.401	10.219	2.099
0.200	37.935	1.126	48.702	1.598	13.363	2.724
0.300	32.476	1.594	52.575	4.404	14.949	2.811
0.400	24.436	2.351	58.845	6.027	16.720	3.676
0.500	22.100	4.702	59.528	0.142	18.372	4.560
0.600	14.419	0.649	64.594	3.335	20.987	3.984
0.700	13.119	0.574	65.857	3.009	21.023	3.583

Immunofluorescence.

HeLa cells and K562 cells were grown on glass coverlips and poly-L-lysine coated glass coverslips (BD Biosciences), respectively. Cells were treated with various doses of (–)-lomaiviticin A (**1**, 0.05, or 0.5 nM), (–)-lomaiviticin C (**2**, 300 nM), or (–)-kinamycin C (**3**, 300 nM) for 4 h at 37 ˚C and then washed with cold PBS. Prior to immunostaining, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized on ice for 20 min in 0.25% Triton X-100, and blocked with 3% bovine serum albumin in TBS for at least 5 minutes at room temperature.

The following primary antibodies were employed: mouse monoclonal anti-phospho-histone H2AX (Ser139) (Upstate) and rabbit polyclonal anti-53BP1 (Novus Biologicals).

Coverslips were incubated with primary antibodies (1:100) for 1 h at 37˚C, washed with PBS, and incubated with secondary antibodies conjugated to Alexa 488 (goat-anti-mouse IgG, 1:100) and Alexa 594 (goat-anti-rabbit IgG, 1:200) (Molecular Probes) for 1 h at 37˚C. After washing with PBS, the coverslips were mounted on glass slides using Vectashield mounting medium containing DAPI (Vector Laboratories) to counterstain nuclear DNA. The fluorochromes were visualized with a Zeiss Axiovert 200 M epifluorescence microscope equipped with a 63×/1.40 Plan-Apochromat oil immersion objective. Fluorescence illumination was initiated using an EXFO X-cite Series 120 Hg arc lamp. Filters for DAPI, GFP and Cy3 were used to acquire images. Images were captured with Zeiss Axiocam Mrm camera and AxioVision software.

Flow Cytometry Assay.

Flow cytometry analysis was performed using an H2A.X phosphorylation Assay Kit (Upstate Lake Placid, NY). K562 cells (7.05×10^5 cells/mL) were incubated with (–)-lomaiviticin A (**1**), (–)-lomaiviticin C (**2**), or (–)-kinamycin C (**3**) (312 nM of each) for 4 h at 37 °C. The cells were transferred into eppendorf tubes in 2 × 1.0 mL aliquots and washed with PBS (2 × 1.0 mL). The cells were treated with 1X fixation solution for 20 min at 0 °C. The fixed cells were washed with PBS (2 × 0.5 mL) and centrifuged. The cells were treated sequentially with ice-cold 1X permeabilization solution, 1.4 M sodium chloride, 25 mM calcium chloride, 100 mM HEPES, pH 7.4, 100 μL), and anti-phospho-Histone H2A.X (Ser139)–FITC conjugate (4.0 μL) for 20 min at 0 °C. Wash solution (1% saponin in PBS, 200 μL) was added to each tube, and the cells were centrifuged. The cells were washed with PBS (2 × 1.0 mL), and the washed cells were centrifuged. The centrifuged cells were suspended in PBS (300 μL), and the cells were analyzed by flow cytometry. A minimum of 10,000 cells were analyzed within the gated population.

Neutral Comet Assay and Analysis.

K562 Cells:
A comet assay reagent kit containing lysis solution, comet LM Agarose, 2-well cometslide, and 200 mM EDTA was purchased from Trevigen and used as per the manufacturer's instructions with modifications. K562 cells were seeded on T-25 flasks and treated with IR (40 Gy, X-ray), H_2O_2 (100 μM), **1** (0.5, 5.0 and 50.0 nM), **2** (300 nM) or **3** (300 nM). Cells treated with IR were incubated on ice for 30 min before irradiation at room temperature using X-RAD 320 at a dose rate of 1.79 Gy/min. Cells treated with hydrogen peroxide and diazofluorenes were incubated for 30 min at 4 ˚C and 37 ˚C, respectively. Untreated and treated cells were immediately placed on ice to ensure no enzymatic-induced DNA damage nor repair could occur after treatment.

Following treatment, the cells were removed from the tissue culture flasks or dishes, centrifuged (1000 rpm, 5 min at 4˚C), and washed once with cold PBS. The cells were resuspended in cold PBS at a concentration of 3×10^5 cells/mL. An aliquot of this cell suspension (6.0 μL) was mixed with pre-warmed (37˚C) LM Agarose (60.0 μL) and immediately pipetted onto a pre-warmed comet slide. Comet slides were allowed to gel for about 20 minutes at 4 ˚C. Slides were then carefully immersed in cold lysing solution and incubated for 1 h at 4 ˚C, followed by incubation in cold 1X neutral electrophoresis buffer (100 mM Tris, 300 mM sodium acetate, pH 9.0) for 30 min at 4˚C. Next, the slides were placed in a Comet Assay ES Tank (Trevigen) containing prechilled 1X neutral electrophoresis buffer (950 mL). Electrophoresis was carried out for 30 min at 21V (~1V/cm) at 4 ˚C. Following electrophoresis, excess buffer was drained from the slides, the slides were immersed in DNA precipitation solution (1M ammonium acetate and 86% ethanol, 50 mL), and incubated for 30 min at room temperature. DNA was further precipitated with 70% ethanol (50 mL) for 30 min at room temperature after which the slides were dried in an incubator set at 37˚C for 30 min.

DNA was stained by adding SYBR Green I solution (1X, 100 μL) in TE buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA) on each well in slide, followed by incubation for 30 min at room temperature in the dark. Individual cells were scored on a two well slide to give a total of at least 50 cells per individual experiment. Comets were analyzed using a Zeiss Axiovert 200 M epifluorescence microscope at 10X magnification and the CometScore software. Tail moment was used as an index of DNA damage which combines a measure of length of the comet tail and the proportion of DNA to migrate into the tail.

PEO1 C4-2 and PEO1 Cells:
PEO1 C4-2 and PEO1 cells were seeded in 100 mm tissue culture dishes and treated the next day with IR at a dose rate of 2.366 Gy/min (10 Gy, X-ray), **1** (0.2 nM), **2** (300 nM) and **3** (300 nM). IR-treated cells were incubated at 37˚C for 30 min. Compound-treated cells were incubated at 37˚C for 24 h. After incubation, the cells were subjected to the neutral comet assay and analyzed as described above.

Clonogenic Survival Assays.

BRCA2-proficient and BRCA2-deficient VC-8 and PEO1 cell lines were seeded at 500 cells per well in 6-well dishes and treated with various concentrations of (–)-lomaiviticin A (**1**, 5.0, 10.0, 15.0, 20.0 pM) or (–)-lomaiviticin C (**2**, 20.0, 40.0, 60.0, 80.0, 100 nM) in triplicate for 24 hours, after which the media containing inhibitor was removed and replaced with fresh media. Colonies were fixed with 0.9% saline solution and stained with crystal violet 8-14 days later. Colonies consisting of greater than 50 cells were counted.

Western Blotting.

MCF-7 cells were seeded into 6-well dishes and treated with various doses of (–)-lomaiviticin A (**1**, 10.0 and 20.0 pM), in the absence and presence of IR at a dose rate of 2.4295 Gy/min (2 Gy, X-ray) for 24 h. After treatment, the cells were harvested for lysis in AZ lysis buffer (50 mM Tris, 250 mM NaCl, 1% Igepal, 0.1% SDS, 5 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF) containing protease and phosphatase inhibitors. Western blotting was performed as previously described.[6]

Primary antibodies used were: mouse monoclonal anti-Ser1981-pATM (10H11.E12, Millipore, Temecula, CA), mouse monoclonal anti-vinculin (SPM227, Abcam, Cambridge, MA), rabbit polyclonal anti-Ser428-pATR (Cell Signaling, Danvers, MA), rabbit polyclonal anti-Thr68-pCHK2 (Cell Signaling), rabbit monoclonal anti-CHK2 (D9C6, Cell Signaling), rabbit monoclonal anti-Ser345-pCHK1 (Cell Signaling), and rabbit monoclonal anti-CHK1 (E250, Millipore).

Proteins were visualized with horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G (Thermo Scientific, Rockford, IL) and the SuperSignal West Pico Chemiluminescent Substrate detection system (Thermo Scientific).

In vitro Reactivity Studies.

*Competition Hydrodediazotization Between (–)-Lomaiviticin A (**1**) and (–)-Kinamycin C (**3**):*
A solution of (–)-lomaiviticin A (**1**) in methanol [nominally 2.2 μM, 100 μL, containing approximately 14% (–)-lomaiviticin C (**2**)] and a solution of (–)-kinamycin C (**3**) in methanol (nominally 2.0 μM, 100 μL) were combined in a J-Young NMR tube. The mixture was concentrated to dryness. The residue obtained was dissolved in methanol-d_4 (100 μL) and the resulting solution was concentrated to dryness. This procedure was repeated. The residue obtained was dissolved in methanol-d_4 (400 μL). A solution of 1,3,5-trimethoxybenzene in methanol-d_4 (20.0 mM, 20.0 μL, 403 nmol) was added.

The resulting solution was analyzed by ^{1}H NMR spectroscopy. The molar quantity of each species in solution was determined by integrating resolved resonances of **1**, **2**, and **3** [**1**: δ 6.25 (s, 2H); **2**: δ 5.43 (s, 1H); **3**: δ 5.72 (s, 1H)] against the aryl resonance of 1,3,5-trimethoxybenzene [δ 6.07 (s, 3H)]. This analysis revealed the presence of 137 nmol **1**, 22.2 nmol **2**, and 125 nmol **3** (Table S1).

A solution of DTT in methanol-d_4 (4.3 mM, 20.0 μL, 86.6 nmol) was then added, and the resulting mixture was analyzed by ^{1}H NMR spectroscopy, as described above. This process was repeated twice.

Table S1. Competition hydrodediazotization between (–)-lomaiviticin A (**1**) and (–)-kinamycin C (**3**).

entry	DTT (nmol)	**1** (nmol)	**3** (nmol)	**2** + **11** (nmol)
1	0	137	125	22.2
2	86.6	125	127	58.4
3	173	88.7	129	76.6
4	260	60.4	125	115

*Competition Hydrodediazotization Between (–)-Lomaiviticin A (**1**) and (–)-Lomaiviticin C (**2**):*
A solution of (–)-lomaiviticin A (**1**) in methanol (nominally 2.2 μM, 100 μL) and a solution of (–)-lomaiviticin C (**2**) in methanol (nominally 4.4 μM, 100 μL) were combined in a J-Young NMR tube. The mixture was concentrated to dryness. The residue obtained was dissolved in methanol-d_4 (100 μL) and the resulting solution was concentrated to dryness. This procedure was repeated. The residue obtained was dissolved in methanol-d_4 (400 μL). A solution of 1,3,5-trimethoxybenzene in methanol-d_4 (20 mM, 20.0 μL, 403 nmol) was added.

The resulting solution was analyzed by ^{1}H NMR spectroscopy. The molar quantity of each species in solution was determined by integrating resolved resonances of **1** and **2** [**1**: δ 6.25 (s, 2H); **2**: δ 6.07 (s, 1H)] against the aryl resonance of 1,3,5-trimethoxybenzene [δ 5.43 (s, 3H)]. This analysis revealed the presence of 202 nmol **1** and 421 nmol **2** (Table S2).

A solution of DTT in methanol-d_4 (5.8 mM, 20.0 μL, 117 nmol) was then added, and the resulting mixture was analyzed by ^{1}H NMR spectroscopy. This process was repeated twice.

Table S2. Competition hydrodediazotization between (–)-lomaiviticin A (**1**) and (–)-lomaiviticin C (**2**).

entry	DTT (nmol)	**1** (nmol)	**2** + **11** (nmol)
1	0	202	421
2	117	149	435
3	234	104	490
4	351	74.6	550

*Hydrodediazotization of (–)-Lomaiviticin A (**1**) in the Presence of Calf Thymus DNA and Dithiothreitol:*

Calf thymus DNA in D_2O [44.6 μL, 6.85 mM/base pairs, 116 equiv, azeotroped from D_2O (3 × 500 μL)] was added to a solution of (–)-lomaiviticin A (**1**, 3.6 mg, 2.64 μmol, 1 equiv) in 10% *N,N*-dimethylformamide-d_7–D_2O (200 μL). Dithiothreitol in D_2O (1 mM, 2.64 μL, 2.64 μmol, 1.00 equiv) was added, and the resulting mixture was stirred gently for 48 h at 37 °C. The crude solution was purified by reverse-phase flash-column chromatography (eluting with 10% methanol–water initially, grading to 80% methanol–water, seven steps) to afford separately a mixture of (–)-lomaiviticin C (**2**) and *d*-lomaiviticin C (**11**, burgundy solid, 1.2 mg, 34%) and the double hydrodediazotization product **12** (orange solid, 1.1 mg, 37%). ^{1}H NMR analysis showed 67% proton incorporation at the vinylic position for **2** and **11**, and 39% total hydrogen atom incorporation at the vinylic position of **12**.

*Hydrodediazotization of (–)-Lomaiviticin A (**1**) in the Presence of Calf Thymus DNA:*

Calf thymus DNA in D_2O [44.6 μL, 6.85 mM/base pairs, 107 equiv, azeotroped from D_2O (3 × 500 μL)] was added to a solution of (–)-lomaiviticin A (**1**, 3.9 mg, 2.85 μmol, 1 equiv) in 10% *N,N*-dimethylformamide-d_7–D_2O (200 μL). The resulting mixture was stirred gently for 4 d at 37 °C. The crude solution was purified by reverse-phase flash-column chromatography (eluting with 10% methanol–water initially, grading to 80% methanol–water, seven steps) to afford separately a mixture of (–)-lomaiviticin C (**2**) and *d*-lomaiviticin C (**11**, burgundy solid, 3.1 mg, 81%) and the double hydrodediazotization product **12** (orange solid, 0.7 mg, 18%). ^{1}H NMR analysis showed 88% proton incorporation at the vinylic position for **2** and **11**, and 60% total hydrogen atom incorporation at the vinylic position of **12**.

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