Supporting Information for

Oxidative activation of leinamycin E1 triggers alkylation of guanine residues in double-stranded DNA

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Experimental Procedures

Materials and general methods. Reagents were purchased from the following suppliers and were of the highest purity available: oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Uracil DNA glycosylase (UDG), and T4 DNA polynucleotide kinase (T4 PNK) were from New England Biolabs (Ipswich, MA). [γ-^{32}P]-ATP (6000 Ci/mmol) was purchased from PerkinElmer. C-18 Sep-Pak cartridges were purchased from Waters (Milford, MA), and BS Poly prep columns were obtained from BioRad (Hercules, CA). Acrylamide/bis-acrylamide 19:1 (40% solution, electrophoresis grade) was purchased from Fisher Scientific (Waltham, MA). Quantification of radioactivity in polyacrylamide gels was carried out using a Personal Molecular Imager (BIORAD) with Quantity One software (v.4.6.5). DNA was 5'-^{32}P-labeled using and hybridized using established procedures.[1] LNM and LNM E1 were produced as described previously.[2]

Gel electrophoretic analysis of DNA alkylation by LNM E1 and LNM. Labeled DNA was treated with LNM E1 (200 μM) and various activating agents at 37 °C for various times in HEPES buffer (50 mM, pH 7 containing 100 mM NaCl and 10% acetonitrile) unless otherwise specified. The DNA was ethanol precipitated, redissolved in aqueous piperidine (50 μL of a 0.1 M solution) and incubated at 90 ˚C for 25 min (Maxam-Gilbert workup).[3] The solution was frozen on dry ice and lyophilized for 40 min in a SpeedVac Concentrator at 37 ˚C, redissolved in 20 μL of water, and evaporated again. The dried DNA fragments were dissolved in formamide loading buffer, loaded onto a 20% polyacrylamide denaturing gel, and electrophoresed at 1400 V for 5 h. The amount of radioactivity in DNA bands on the gel were quantitatively analyzed by phosphorimager analysis.

LC/MS analysis. A solution of duplex **A** (100 μM), LNM E1 (200 μM) and H_2O_2 (500 μM) were incubated for 12 h at 37 °C in HEPES buffer (50 mM, pH 7, containing 100 mM NaCl, 10% acetonitrile). The solution was heated at 90 ˚C for 30 min and extracted with *n*-butanol (2 x 100 μL). The organic layers were combined and dried in a SpeedVac Concentrator at 37 ˚C. The residue was resuspended in 20% methanol-water prior to LC-MS analysis. The LC/MS analyses were performed using a Beckmann Coulter System Gold HPLC equipped with a 168 diode array detector, a 507e auto injector and the 32 KARAT software package (Beckmann Coulter,

Fullerton, CA). coupled with the LCQ Fleet ion trap mass spectrometer from ThermoFisher, Waltham, MA. Positive ion electrospray was used for ionization. The heated inlet capillary temperature was 375 °C and electrospray needle voltage was 5 kV. Nitrogen sheath gas was supplied at 45 psi and the LC/ESI-MS analysis was performed in the positive ion mode. The analytical column was BetaBasic C18, 150 Å, 0.46 cm x 15 cm, 5 μm (ThermoFisher, Waltham, MA). The samples were eluted at a flow rate of 1 mL/min using a continuous gradient over 30 min from 10% solvent B to 50% solvent B, where solvent A is water and solvent B is acetonitrile, each containing 0.1% TFA. The analytes were detected by monitoring the UV absorbance at 320 nm.

Literature Citations

1. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Lab Manual*. Cold Spring Harbor Press: Cold Spring Harbor, NY, 1989.

2. Huang, S.-X.; Yun, B.-S.; Ma, M.; Basu, H. S.; Church, D. R.; Ingenhorst, G.; Huang, Y.; Yang, D.; Lohman, J. R.; Tang, G.-L.; Ju, J.; Liu, T.; Wilding, G.; Shen, B. Leinamycin E1 acting as an anticancer prodrug activated by reactive oxygen species. *Proc. Nat. Acad. Sci. USA* **2015,** 112, 8278-8283.

3. Maxam, A. M.; Gilbert, W. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **1980,** 65, 499-560.

Figure S1

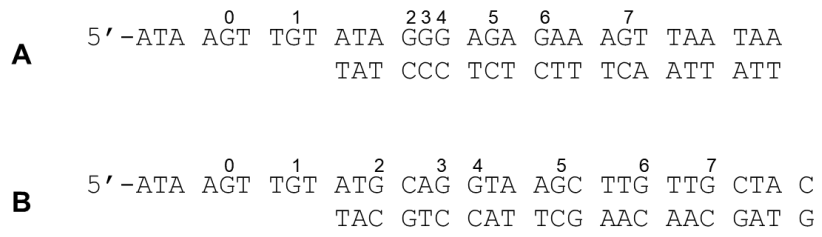


Figure S1. DNA duplexes used in these studies.

Figure S2

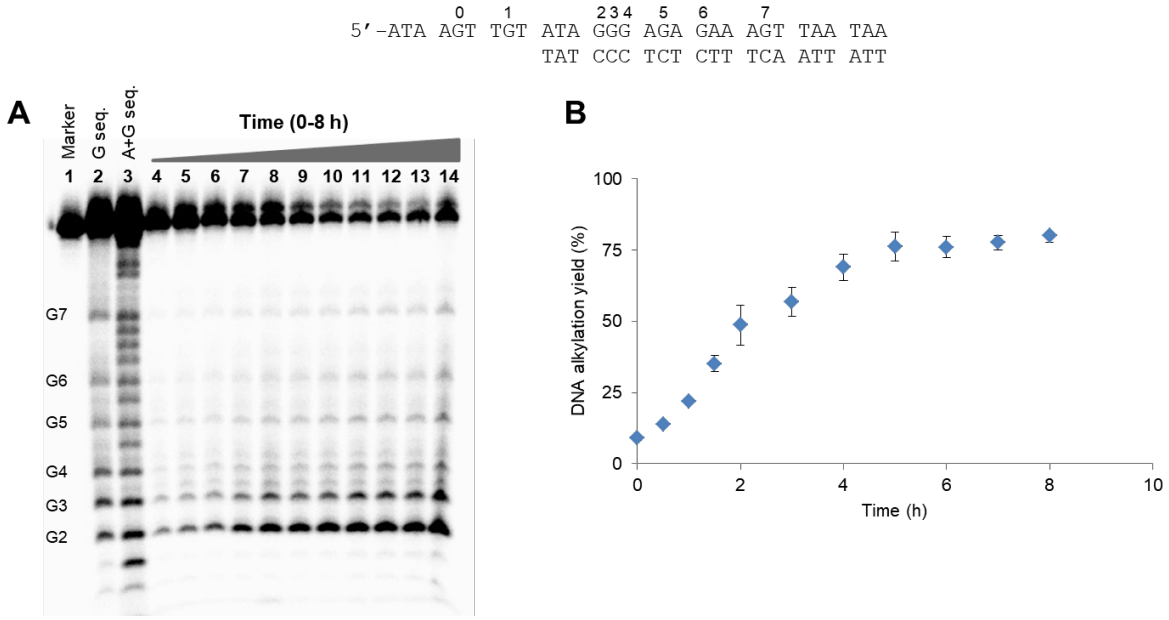


Figure S2. Time course for the alkylation of duplex **A** by LNM E1+H_2O_2. Panel A. Gel electrophoretic analysis of DNA alkylation in duplex **A**. The ^{32}P-labeled 2'-deoxyoligonucleotide duplex was incubated with LNM E1 (200 μM) and H_2O_2 (500 μM) in HEPES buffer (50 mM, pH 7 containing 100 mM NaCl and 10% acetonitrile) at 37 ˚C. At 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 h (lanes 4-14) aliquots were removed from the reaction and frozen at –20 °C prior to ethanol precipitation and piperidine workup (0.1 M, 90 ˚C, 25 min). The resulting ^{32}P-labeled 2'-deoxyoligonucleotides were resolved by denaturing 20% polyacrylamide gel electrophoresis and the radioactivity in each band quantitatively measured by phosphorimager analysis. Lane 1, untreated duplex **A**; lane 2, Maxam-Gilbert G specific cleavage reaction; lane 3, A+G reaction. Panel B. Plot of total alkylation yield versus time from the electrophoretic data shown in Panel A. The alkylation yield is the fraction of radioactivity found in the cleavage products versus the total amount of radioactivity in the lane.

Figure S3

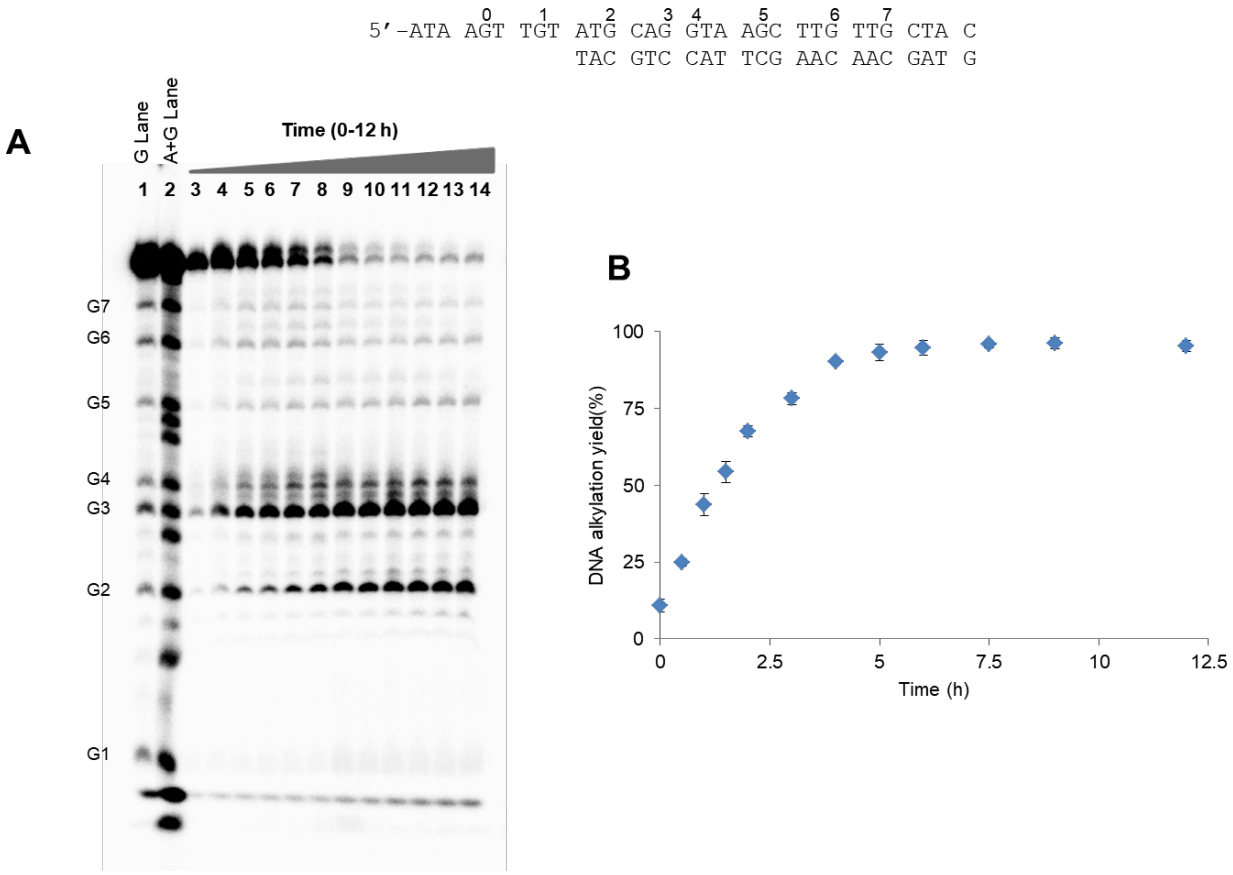


Figure S3. Time course for the alkylation of duplex **B** by LNM E1+H_2O_2. Panel A. Gel electrophoretic analysis of DNA alkylation in duplex **B**. The ^{32}P-labeled 2'-deoxyoligonucleotide duplex was incubated with LNM E1 (200 μM) and H_2O_2 (500 μM) in HEPES buffer (50 mM, pH 7 containing 100 mM NaCl and 10% acetonitrile) at 37 ˚C. At 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7.5, 9, and 12 h (lanes 3-14) aliquots were removed from the reaction and frozen at –20 °C prior to ethanol precipitation and piperidine workup (0.1 M, 90 ˚C, 25 min). The resulting ^{32}P-labeled 2'-deoxyoligonucleotides were resolved by denaturing 20% polyacrylamide gel electrophoresis and the radioactivity in each band quantitatively measured by phosphorimager analysis. Lane 1, Maxam-Gilbert G specific cleavage reaction; lane 2, A+G reaction. Panel B. Plot of total alkylation yield versus time from the electrophoretic data shown in Panel A. The alkylation yield is the fraction of radioactivity found in the cleavage products versus the total amount of radioactivity in the lane.

Figure S4

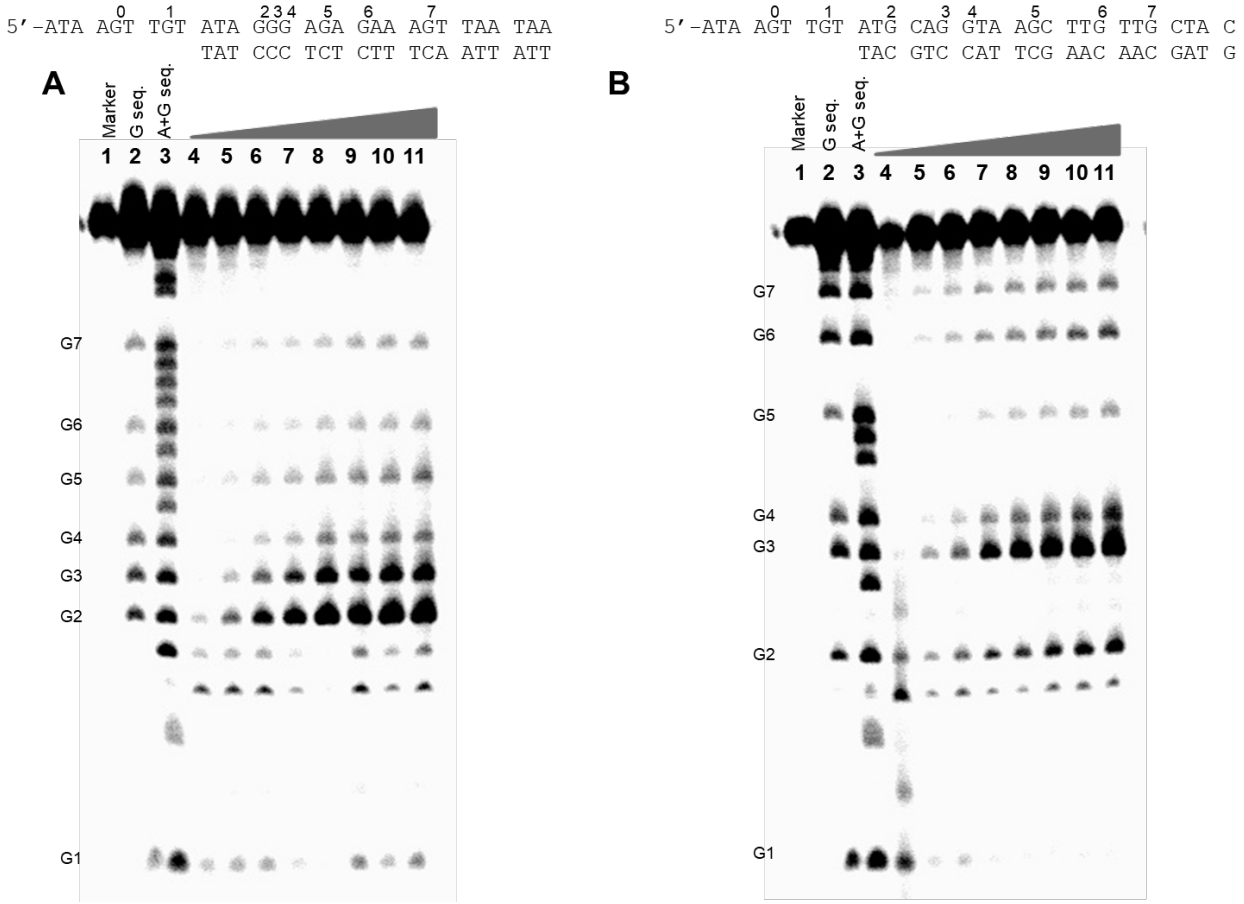


Figure S4. Concentration dependence of DNA alkylation by LNM E1+H_2O_2. Reactions were carried out as described in the Legend of Figure S1 except various concentrations of LNM E1 were incubated with a 2.5-fold molar excess of H_2O_2. After 2 h, the samples were ethanol precipitated, subjected to piperidine workup, and analyzed by 20% denaturing polyacrylamide gel electrophoretic analysis. Lane 1: untreated duplex, lane 2: Maxam-Gilbert G specific cleavage reaction, in lanes 4-11 the concentrations of LNM E1 was 1, 10, 25, 50, 75, 100, 150 and 200 μM respectively. Panel A is duplex **A** and Panel B is duplex **B**.

Figure S5

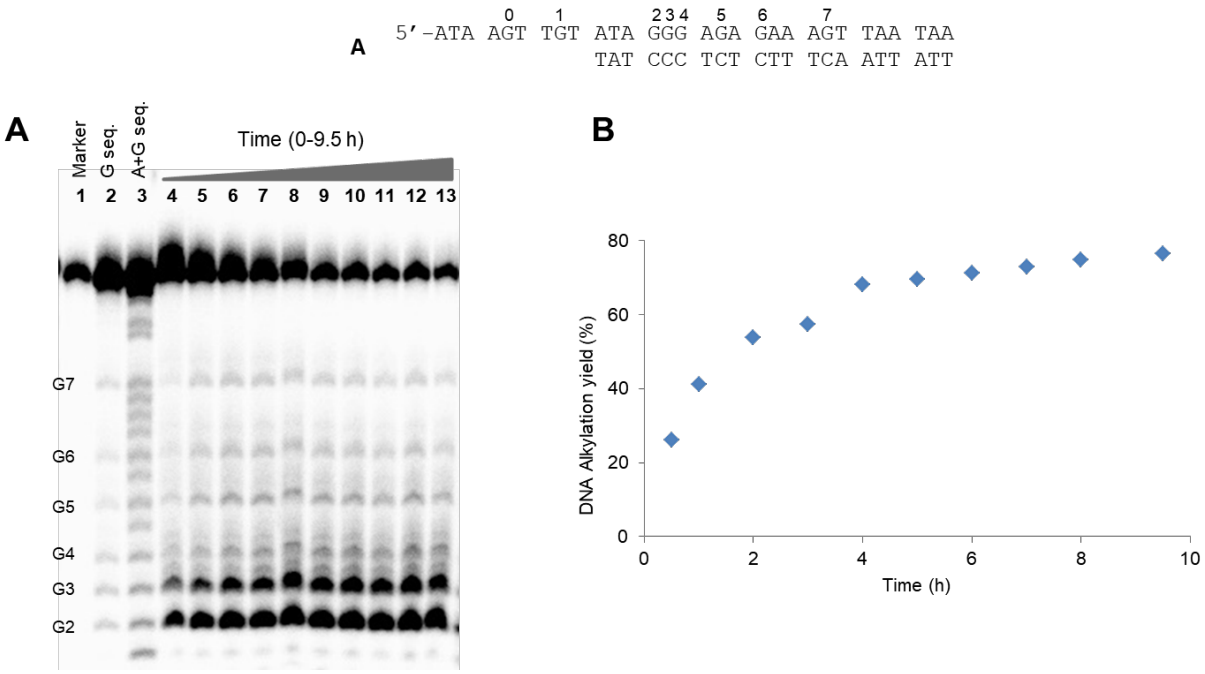


Figure S5. Time course for the alkylation of duplex **A** by LNM+2-mercaptoethanol. Panel A. Gel electrophoretic analysis of DNA alkylation in duplex **A**. The ^{32}P-labeled 2'-deoxyoligonucleotide duplex was incubated with LNM E1 (100 μM) and 2-mercaptoethanol (500 μM) in HEPES buffer (50 mM, pH 7 containing 100 mM NaCl and 10% acetonitrile) at 37 ˚C. At 0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 9.5 h (lanes 4-13) aliquots were removed from the reaction and frozen at –20 °C prior to ethanol precipitation and piperidine workup (0.1 M, 90 ˚C, 30 min). The resulting ^{32}P-labeled 2'-deoxyoligonucleotides were resolved by denaturing 20% polyacrylamide gel electrophoresis and the radioactivity in each band quantitatively measured by phosphorimager analysis. Lane 1, untreated duplex **A**; lane 2, Maxam-Gilbert G specific cleavage reaction; lane 3, A+G reaction. Panel B. Plot of total alkylation yield versus time from the electrophoretic data shown in Panel A. The alkylation yield is the fraction of radioactivity found in the cleavage products versus the total amount of radioactivity in the lane.

Figure S6

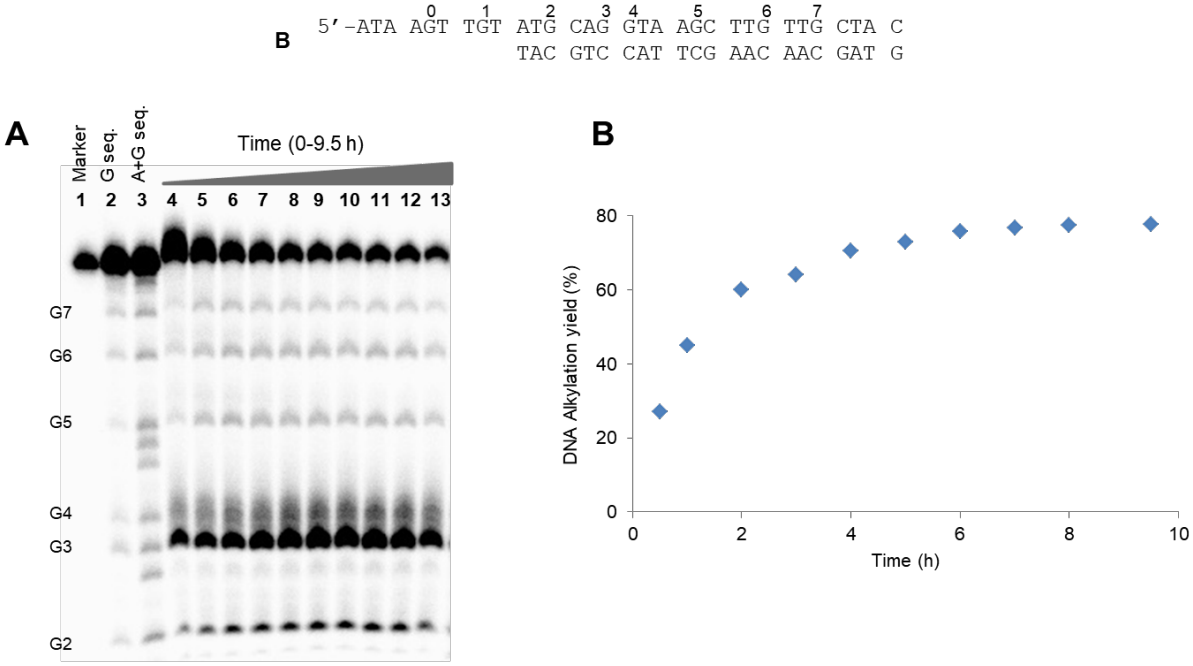


Figure S6. Time course for the alkylation of duplex **B** by LNM+2-mercaptoethanol. Panel A. Gel electrophoretic analysis of DNA alkylation in duplex **B**. The ^{32}P-labeled 2'-deoxyoligonucleotide duplex was incubated with LNM E1 (100 μM) and 2-mercaptoethanol (500 μM) in HEPES buffer (50 mM, pH 7 containing 100 mM NaCl and 10% acetonitrile) at 37 ˚C. At 0.5, 1, 2, 3, 4, 5, 6, 7, 8 and 9.5 h (lanes 4-13) aliquots were removed from the reaction and frozen at –20 °C prior to ethanol precipitation and piperidine workup (0.1 M, 90 ˚C, 25 min). The resulting ^{32}P-labeled 2'-deoxyoligonucleotides were resolved by denaturing 20% polyacrylamide gel electrophoresis and the radioactivity in each band quantitatively measured by phosphorimager analysis. Lane 1, untreated duplex **B**; lane 2, Maxam-Gilbert G specific cleavage reaction; lane 3, A+G reaction. Panel B. Plot of total alkylation yield versus time from the electrophoretic data shown in Panel A. The alkylation yield is the fraction of radioactivity found in the cleavage products versus the total amount of radioactivity in the lane.

Figure S7

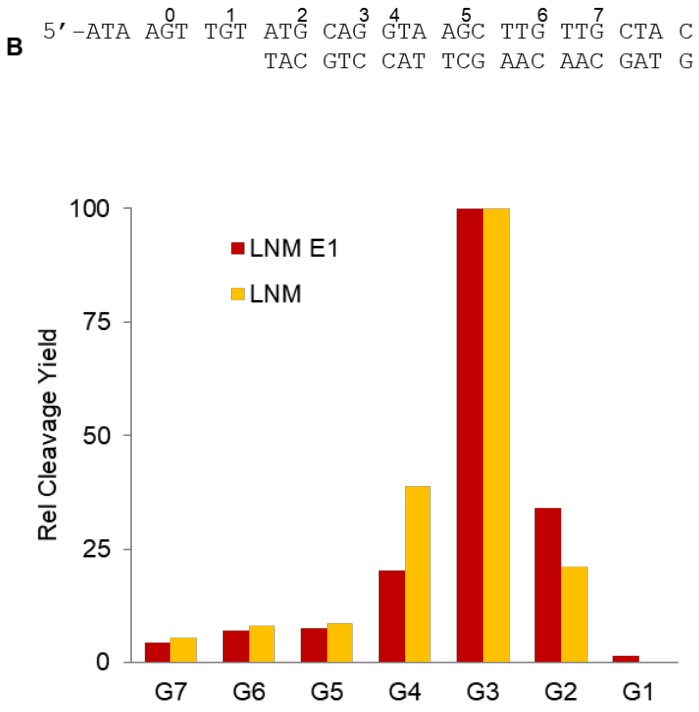


Figure S7. Sequence specificity of DNA alkylation in duplex **B** by LNM E1+H_2O_2 compared to LNM+2-mercaptoethanol. Reactions were conducted and analyzed as described in the Legends of Figures S1 and S5, with an incubation time of 12 h. The bars depict the relative amount of cleavage generated by LNM E1+H_2O_2 and LNM+2-mercaptoethanol at each guanine residue in duplex **B**. To facilitate comparison of sequence preferences displayed by LNM E1 and LNM, the amount of cleavage at G_3 was normalized to 100 in each case and background cleavage in a control lane containing either no LNM E1 or no LNM was subtracted.

Figure S8

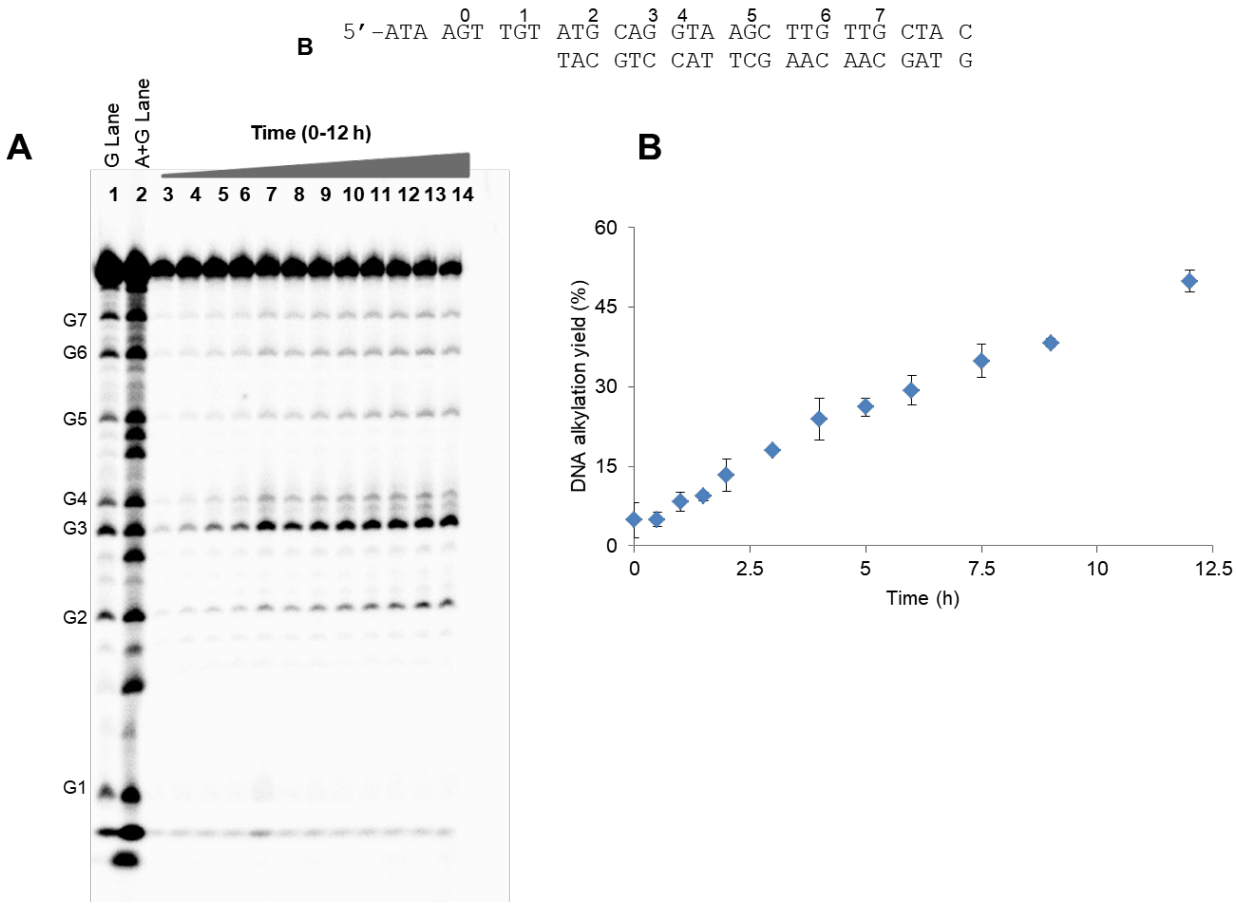
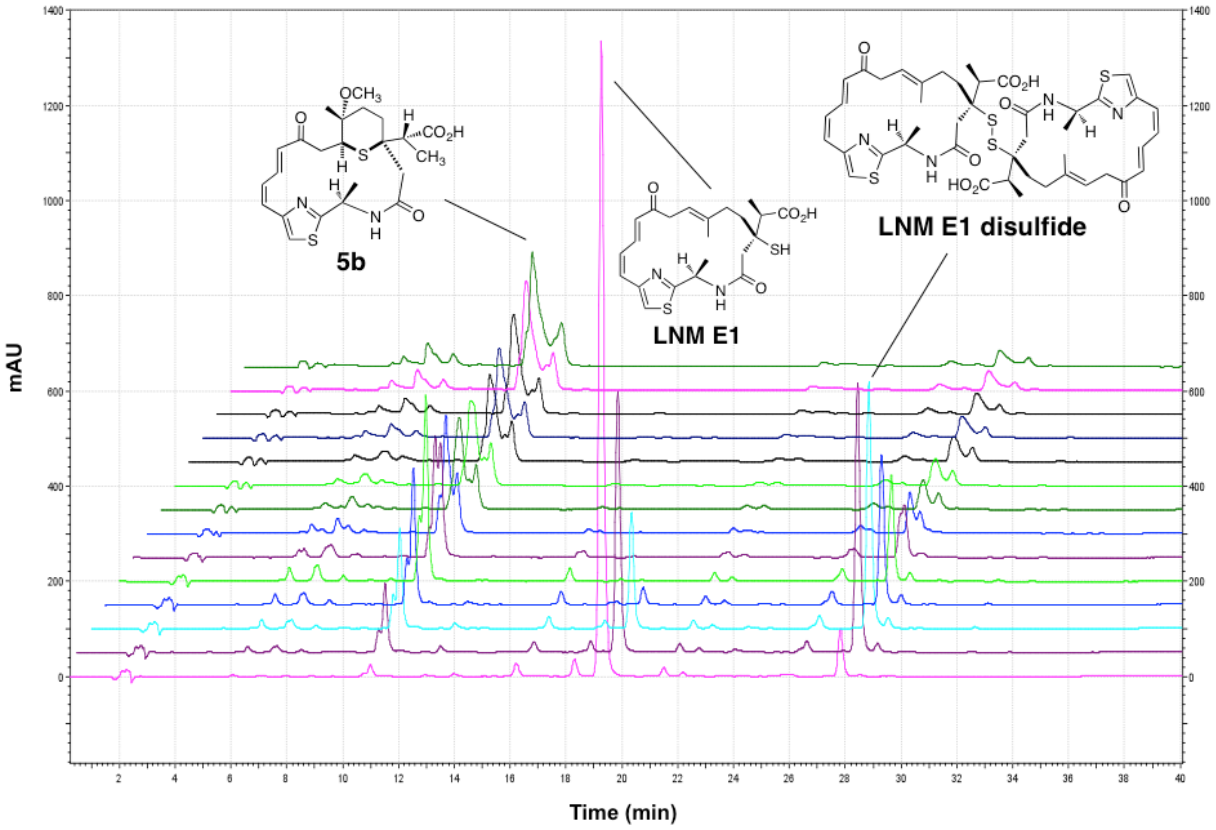


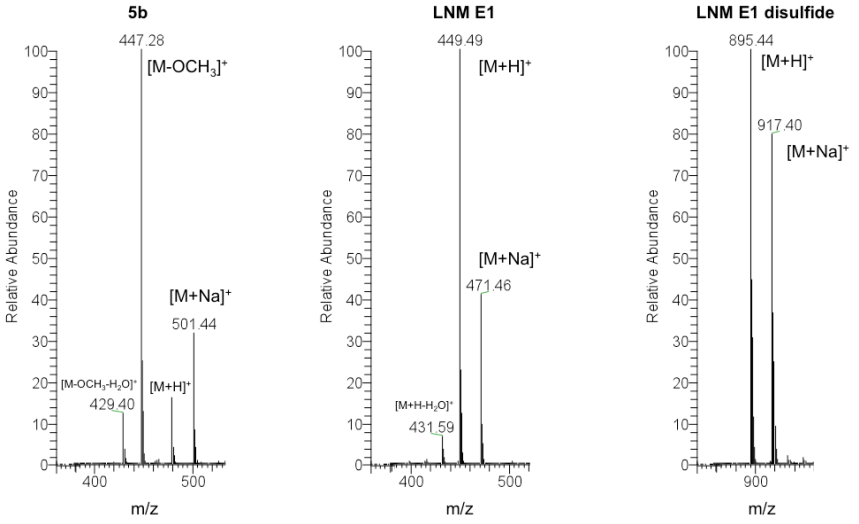
Figure S8. Time course for the alkylation of duplex **B** by LNM E1 in the presence of glutathione disulfide (GSSG). Panel A. Gel electrophoretic analysis of DNA alkylation in duplex **A**. The ^{32}P-labeled 2'-deoxyoligonucleotide duplex was incubated with LNM E1 (200 μM) and GSSG (500 μM) in HEPES buffer (50 mM, pH 7 containing 100 mM NaCl and 10% acetonitrile) at 37 ˚C. At times of 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7.5, 9, and 12 h (lanes 3-14) aliquots were removed from the reaction and frozen at –20 °C prior to ethanol precipitation and piperidine workup (0.1 M, 90 ˚C, 25 min). The resulting ^{32}P-labeled 2'-deoxyoligonucleotides were resolved by denaturing 20% polyacrylamide gel electrophoresis and the radioactivity in each band quantitatively measured by phosphorimager analysis. Lane 1, Maxam-Gilbert G specific cleavage reaction; lane 2, A+G reaction. Panel B. Plot of total alkylation yield versus time from the electrophoretic data shown in Panel A. The alkylation yield is the fraction of radioactivity found in the cleavage products versus the total amount of radioactivity in the lane.

Figure S9

A


5b
LNM E1
LNM E1 disulfide
mAU
Time (min)

B


5b
447.28
[M-OCH3]+
[M+Na]+
501.44
[M-OCH3-H2O]+
429.40
[M+H]+
LNM E1
449.49
[M+H]+
[M+Na]+
471.46
[M+H-H2O]+
431.59
LNM E1 disulfide
895.44
[M+H]+
917.40
[M+Na]+
Relative Abundance
m/z

Legend for Figure S9 (previous page). LC-MS analysis of the time course for the reaction of LNM E1 (4.5 mM) with H_2O_2 (9.8 mM) in methanol at 24 ˚C. In the display, the X axis is offset by 40 s and Y axis is offset by 10 mAU. The times for chromatographs (from bottom to top) are 0.02, 1, 2, 4, 8, 12, 18, 24, 26, 28, 36, 48, 65, 72 h. Panel **A**: HPLC analysis monitoring products at 320 nm. Panel **B**: Mass spectra of the major products generated in the reaction. The calculated m/z the $[M+Na]^+$ of **5b** is 501.6. Other products eluting near 10 min display the same m/z and likely are stereoisomers and regioisomers of **5b**. The calculated m/z for the $[M+Na]^+$ of LNM E1 is 471.6 and the calculated m/z for the $[M+H]^+$ of LNM E1 is 449.6. The calculated m/z for the $[M+Na]^+$ of LNM E1 disulfide is m/z 918.2 and the calculated m/z for the $[M+H]^+$ of LNM E1 disulfide is 896.2. Structures were assigned based on their mass spectra and the major products reported for methanolic oxidation of LNM E1 by Huang *et al*. *Proc. Nat. Acad. Sci. USA* **2015**, *112*, 8278-8283.

Figure S10

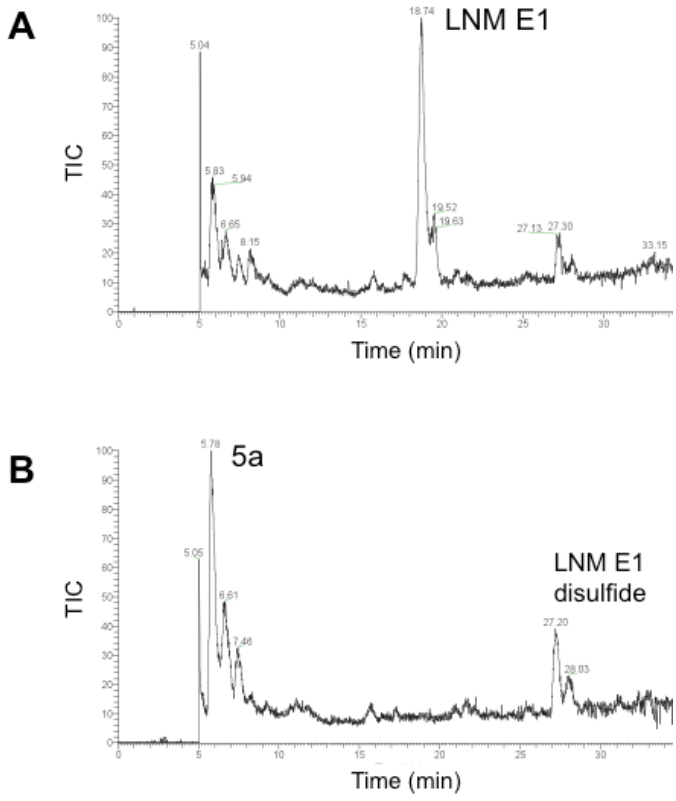


Figure S10. LC-MS analysis of the reaction between LNM E1 (4.5 mM) with H_2O_2 (9.8 mM) in 50 mM HEPES buffer (pH 7, containing 100 mM NaCl, and 10% acetonitrile at 24 ˚C). Panel A: Analysis at time = 1 min. Panel B: Analysis at time = 1 h, showing that LNM E1 is almost completely consumed.