Supplementary Information

Polyamine modification by acrolein exclusively produces 1,5-diazacyclooctanes: A previously unrecognized mechanism for acrolein-mediated oxidative stress

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Representative procedure of the reaction of a polyamine with acrolein. To a solution of spermine (**1**) (5.0 mg, 25 μmol) in either $CHCl_3$ or a PBS buffer solution (1.0 mL) was slowly added acrolein (90 %, 1.8 μL, 25 μmol) at room temperature. After stirring for 15 min at this temperature, the mixture was concentrated *in vacuo* to give **1a** as a white solid (6.0 mg). Direct NMR analysis without purification confirmed the production of **1a** (in about 80%) with byproducts, which mostly consisted of the starting polyamine and polymerized products: ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.17 (m, 2H: This acetal C-H group has ^{1}H-^{13}C HSQC-TOCSY correlations with the neighboring four protons on the eight-membered ring (protons at around 1.6, 1.8, 2.5, and 2.5 ppm) and ^{1}H-^{15}N long range correlations with two tertiary nitrogens on the six-membered ring, see structure **1a** in Supplementary Fig. S1), 3.05-3.02 (m, 4H), 2.50-2.35 (m, 14H), 1.80-1.33 (m, 36H); ^{13}C NMR (100 MHz, $CDCl_3$), δ 75.4, 75.2, 54.1 (2C), 51.9 (2C), 51.1 (2C), 50.8 (2C), 44.9 (2C), 30.9 (2C), 30.1 (2C), 28.3, 28.1, 25.4, 25.1, 24.9; HRESI-MS *m/z* calcd for $C_{26}H_{56}N_8Na$ $[M+Na]^+$ 503.4526, found 503.4506. Data for **2a**: HRESI-MS *m/z* calcd for $C_{30}H_{61}N_8O_2$ $[M+H]^+$ 565.4918, found 565.4956. Data for **3a**: HRESI-MS *m/z* calcd for $C_{20}H_{42}N_6Na$ $[M+Na]^+$ 389.3369, found 389.3355. Data for **4a**: ESI-MS *m/z* calcd for $C_{18}H_{41}N_6$ $[M+H]^+$ 341.3, found 341.3.

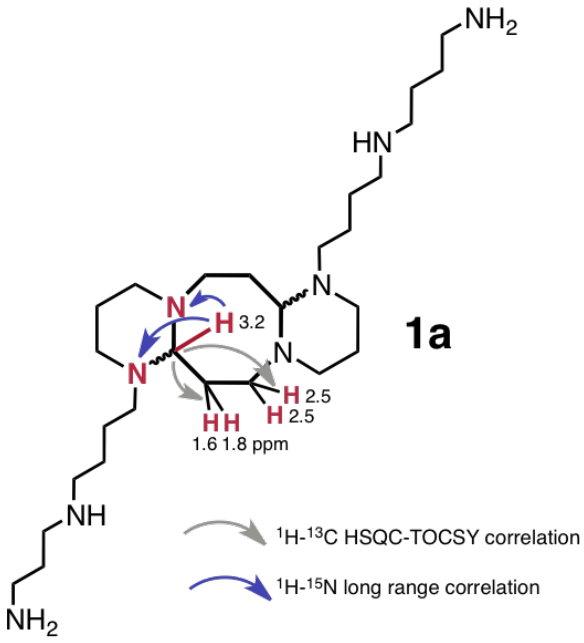

Fig. S1 ^{1}H-^{13}C HSQC-TOCSY and ^{1}H-^{15}N long range correlations in **1a** ($CDCl_3$).

NMR monitoring of the enzymatic oxidation of 1,5-diazacyclooctane 3a by amine oxidase. The substrate **3a**, plasma amine oxidase (I.U.B.: 1.4.3.6, Worthington Biochemical Corporation, bovine plasma, 19.5 μ/mgDW), and PBS buffer were freeze-dried twice from D_2O prior to use following the procedure reported by Howen and co-workers (ref. 4). The enzymatic reaction was performed at 37 °C, pH 7.2, using 3.8 units of bovine amine oxidase. The final concentration of **3a** was adjusted to 10 μM.

Cell culture and MTS assay. HeLa cells, RCB0007, were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. HeLa cells were cultured in DMEM medium (Wako) supplemented with 10% (v/v) FBS at 37 °C in a 5% CO_2-incubator. The cells were seeded on a 96-well plate (500 cells/well) in 100 μL DMEM and incubated for 18 h. Solution containing the polyamines **1**–**4**' and the acrolein-modified polyamines **1a**–**4a**' in various concentrations were prepared in advance by diluting with the PBS(-). Fresh culture medium (95 μL) was added to each well, and the compound solutions (5 μL) were added. For the amine oxidase inhibition experiments, the aminoguanidine was added to the incubation solution (final concentration was adjusting to 1 mM). The treated cells were incubated for 72 h at 37 °C under a 5% CO_2 atmosphere. After the wells in the plates were washed twice with the cultured medium, the cell cultures in each well (100 mL medium) were supplemented with a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolim (MTS) solution (20 mL, Promega, CellTiter 96^R Aqueous One solution cell Proliferation Assay) and then incubated for 3 h under a 5% CO_2 atmosphere according to the established procedure. The absorbance of each well at 450 nm was measured using a Bio-Rad Model 680 Microplate Reader.

Western Blotting. The HeLa cells (6.0×10^5) were seeded onto 100 mm tissue culture plates and

cultured for 18 h, followed by treatment with the acrolein-modified polyamines **1a**–**4a**' (60 μM) for 48 h at 37 °C. Cells were pelleted and lysed in ice cold buffer (1% Triton X-100/50 mM β-glycerophosphate/1.5 mM EGTA/0.5 mM EDTA/5% glycerol/25 mM Tris HCl, pH 7.4) in the presence of a protease inhibitor cocktail (Complete; Roche Diagnostics), and the resulting lysates were cleared by centrifugation. The resulting samples were analyzed on a 4–20% gradient SDS-PAGE and then transferred to PVDF membranes. After incubation with 5% non-fat dried milk in TBS containing 0.1% Tween 20, the membranes were incubated with the anti-HO-1 antibody (ab13248, Abcam), followed by HRP-conjugated anti-mouse IgG (GE Healthcare). Protein bands were detected by treatment with Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) using a LAS-1000P lus•MAC analyzer (Fujifilm). After treating the PVDF membranes with the Pestore TM western Blot Stripping Buffer (Thermo Scientific), an anti-GAPDH antibody (clone 6C5/MAB374, Millipore) and an HRP-conjugated anti-mouse IgG were introduced to detect GAPDH.

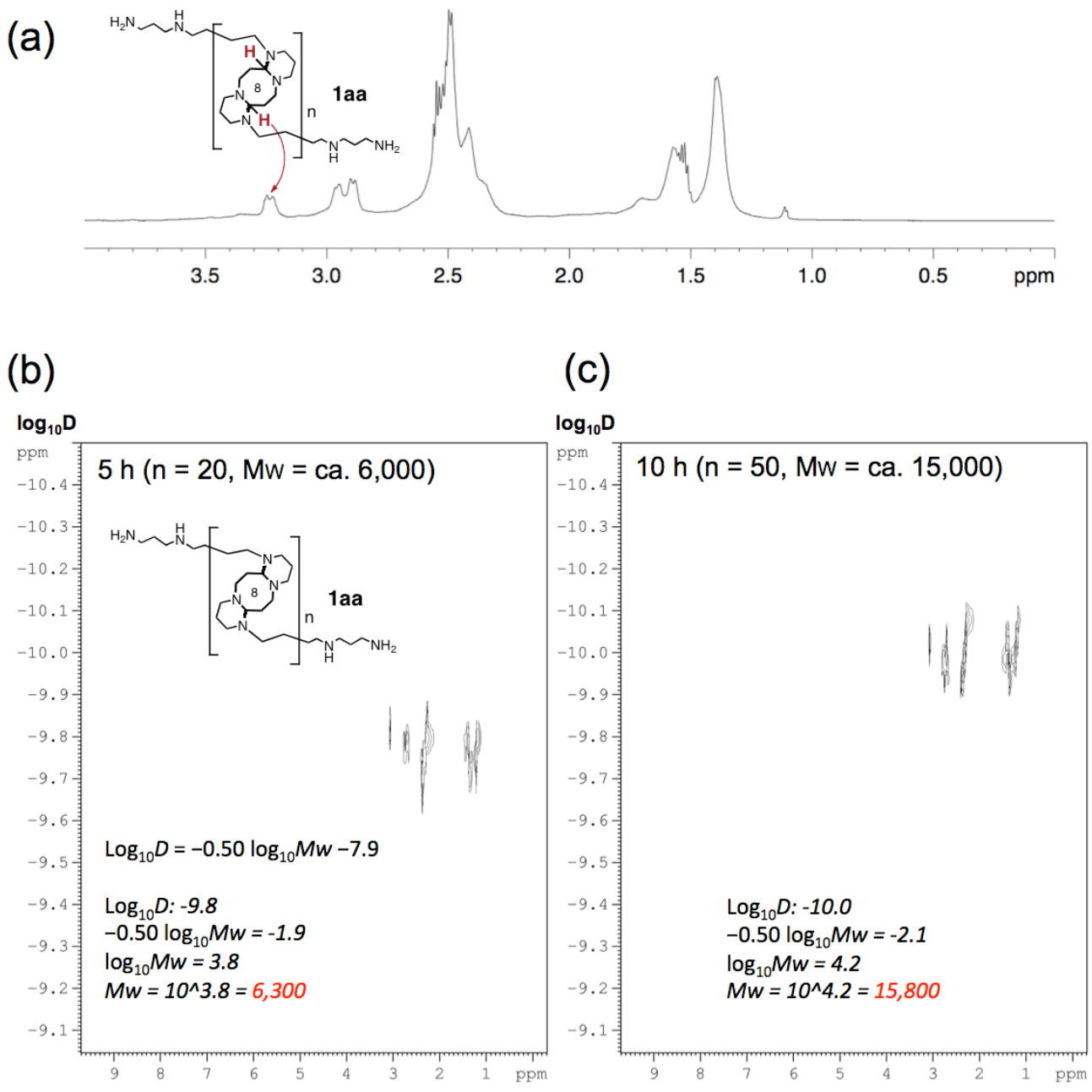

Fig. S2 Time-dependent NMR analysis of [4+4] polymerization reaction. The reaction of spermine **1** with 2 equivalents acrolein gradually produced the 1,5-diazacyclooctane polymers **1aa** at room temperature. (a) ^{1}H NMR in D_2O after 5 h. Diffusion ordered NMR spectroscopy (DOSY) technique estimated the polymers with average molecular weights of 6,000 after 5 h (b) and 15,000 after 10 h (c).

compounds	IC_{50} (μM)	IC_{50} (μM) (+ aminoguanidine: 1 mM)
spermine **1**	2.8	> 50
acrolein-spermine **1a**	3.0	> 50
N^1-Ac-spermine **2**	9.1	> 50
acrolein-N^1-Ac-spermine **2a**	16.0	> 50
spermidine **3**	8.1	> 50
acrolein-spermidine **3a**	6.8	> 50
N^1-Me-propanediamine **4'**	> 50	> 50
acrolein-N^1-Me-propanediamine **4a'**	5.7	5.8

Table S1 Cytotoxic activities of the polyamines, the 1,5-diazacyclooctanes, and the 2,6,9-triazabicyclo[3.3.1]nonane derivatives **1**–**4a'** on A549 cells. The cells were treated with the compounds **1**–**4a'** for 72 h at 37 °C, and the cytotoxicities were evaluated using the MTS method. For the amine oxidase inhibition experiments, 1 mM of the aminoguanidine was applied.