Supplementary Materials: Inhibitory Effects of Imidazole Dipeptides and 2-Oxo-Imidazole Dipeptides on Intracellular ROS Generation and Degradation of Protein and DNA

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Synthesis of 2-oxo-Ans and 2-oxo-Bal

The reaction mixtures (5 mL) containing 10 mM Ans or Bal, 200 mM phosphate buffer (pH 7.2), 200 mM ascorbate, and 2 mM $CuSO_4$ were incubated at room temperature for 30 min under bubbling O_2. The peak position derived from 2-oxo-Ans and 2-oxo-Bal was identified using the following conditions: a COSMOSIL 3PBr column (4.6 mm I.D. × 250 mm, particle size: 3 μm, Nacalai Tesque, Inc., Kyoto, Japan) in an isocratic mode with 100 mM phosphate buffer (pH 7.0) at a flow rate of 1.0 mL/min at 30°C. UV detection was performed at 220 nm. The chemical structures of 2-oxo-Ans and 2-oxo-Bal were determined by MS spectrum analysis using LC–MS (Figure S5A, B). For the LC–MS measurements, the nebulizing gas flow was set to 2.0 L/min, the drying gas flow was 5.0 L/min, the heating gas flow was 7.0 L/min, and the desolvation temperature was maintained at 450°C. Data acquisition was achieved in positive ion mode with a detector voltage of 1.0 kV.

Synthesis of 5,6-carboxyfluorescein conjugated Car

Flu-Car (Car conjugated with 5,6-carboxyfluorescein) was synthesized manually on Wang resin (100 μmol, mmol/g) using the DIPCI method (10 eq., *N*,*N*′-diisopropylcarbodiimide)-DMAP (0.1 eq., *N*,*N*-dimethyl-4-aminopyridine) for the first residue [10 eq., Fmoc-His(Trt)-OH, Watanabe Chemical Industries, Ltd., Hiroshima, Japan], and Fmoc solid phase peptide synthesis with HATU (4 eq., Oakwood Chemical, Inc., Estill, SC, USA) and DIPEA (8 eq., Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) for subsequent residues (4 eq., Fmoc-*β*Ala-OH, Watanabe Chemical Industries, and 4 eq., 5,6-carboxyfluorescein, Merck, Darmstadt, Germany). Fmoc deprotection was achieved using 20% piperidine in NMP (Nacalai Tesque). After synthesis, the peptidyl resin was washed with chloroform and dried under vacuum. Flu-Car was cleaved from the resin, and the side-chain was deprotected by incubating the peptides for 1 h in TFA/H_2O/triisopropylsilane/thioanisol (47/1/1/1, v/v/v/v). The peptide was precipitated by the addition of cold diethyl ether (Nacalai Tesque) and collected by centrifugation. Synthesized Flu-Car was purified by RP-HPLC (Figure S14A) and characterized by LC–MS (Figure S14B).

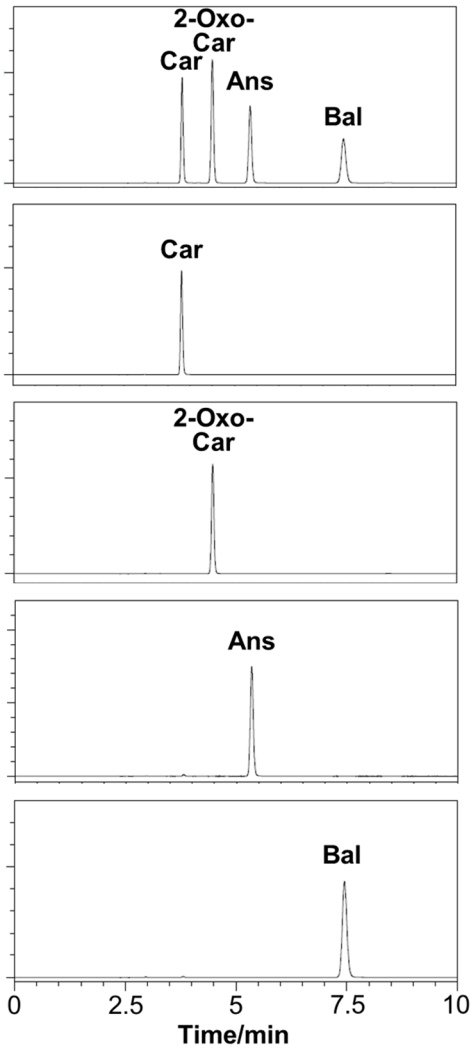


Figure S1. HPLC chromatograms of Car, Ans, Bal, and 2-oxo-Car (0.25 mg/mL). The analysis was conducted using a COSMOSIL 3PBr (4.6 mm I.D. × 250 mm, particle size: 3 μm). A 100 mM phosphate buffer (pH 7.0) was used as the mobile phase in isocratic mode for 10 min at a flow rate of 1 mL/min and a column temperature of 30°C. The injection volume was 1 μL, and UV detection was performed at 220 nm.

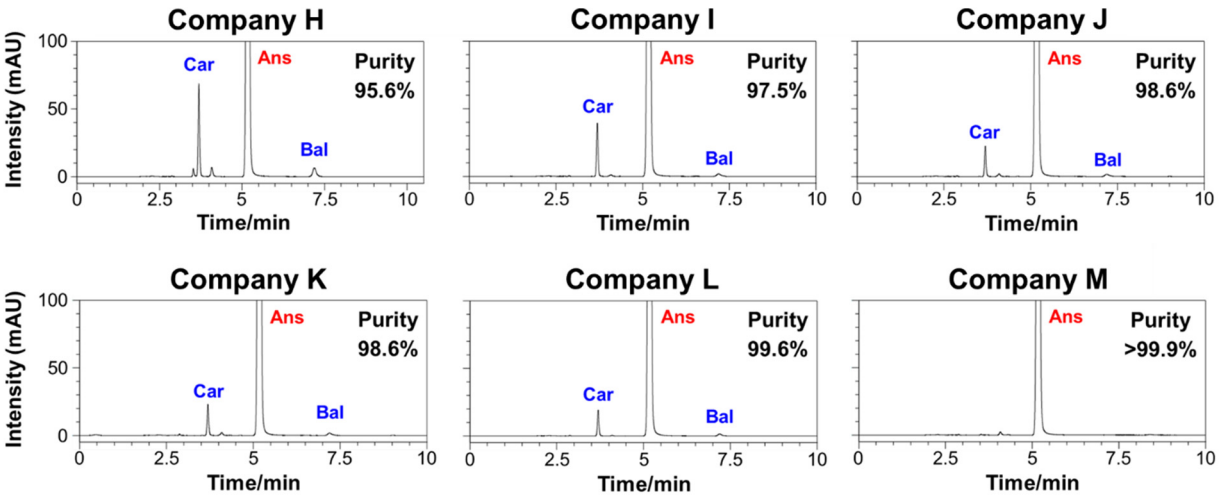


Figure S2. HPLC chromatograms of Ans (20 mM) from companies H–M. The analysis was performed using a COSMOSIL 3PBr (4.6 mm I.D. × 250 mm, particle size: 3 μm). A 100 mM phosphate buffer (pH 7.0) was used as the mobile phase in isocratic mode for 10 min at a flow rate of 1 mL/min and a column temperature of 30°C. The injection volume was 1 μL, and UV detection was performed at 220 nm.

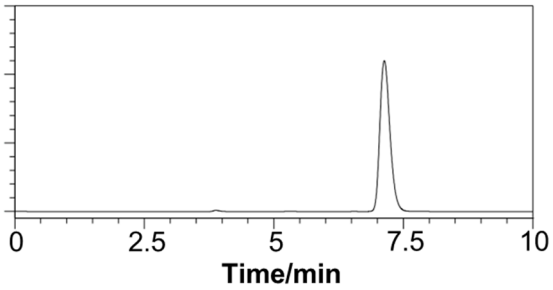


Figure S3. HPLC chromatograms of Bal (20 mg/mL). The analysis was conducted using a COSMOSIL 3PBr (4.6 mm I.D. × 250 mm, particle size: 3 μm). A 100 mM phosphate buffer (pH 7.0) was used as the mobile phase in isocratic mode for 10 min at a flow rate of 1 mL/min and a column temperature of 30°C. The injection volume was 1 μL, and UV detection was performed at 220 nm.

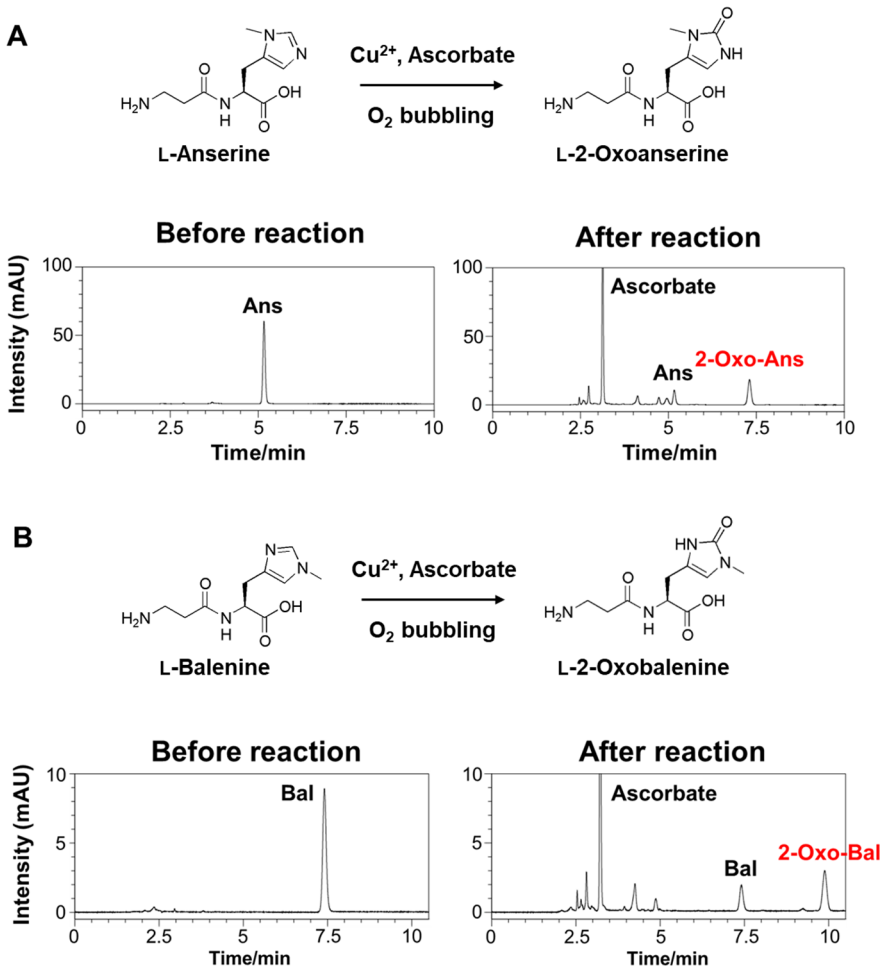


Figure S4. HPLC chromatograms of (A) 2-oxo-Ans synthesized from Ans and (B) 2-oxo-Bal synthesized from Bal. The analysis was performed using a COSMOSIL 3PBr (4.6 mm I.D. × 250 mm, particle size: 3 μm). A 100 mM phosphate buffer (pH 7.0) was used as the mobile phase in isocratic mode for 10 min at a flow rate of 1 mL/min and a column temperature of 30°C. The injection volume was 1 μL, and UV detection was performed at 220 nm.

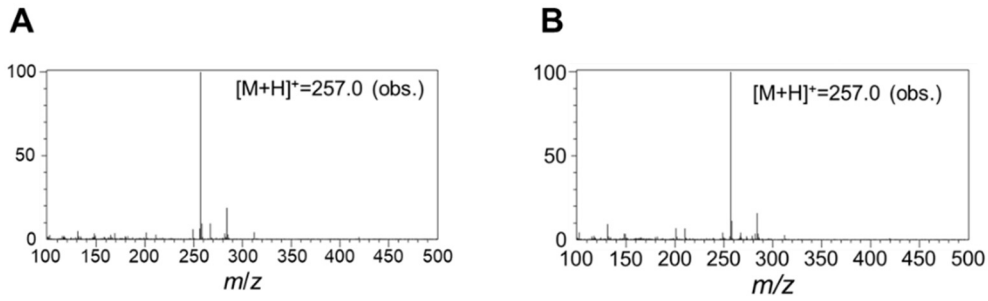


Figure S5. MS spectra of (A) 2-oxo-Ans and (B) 2-oxo-Bal purified by HPLC using a PBr column.

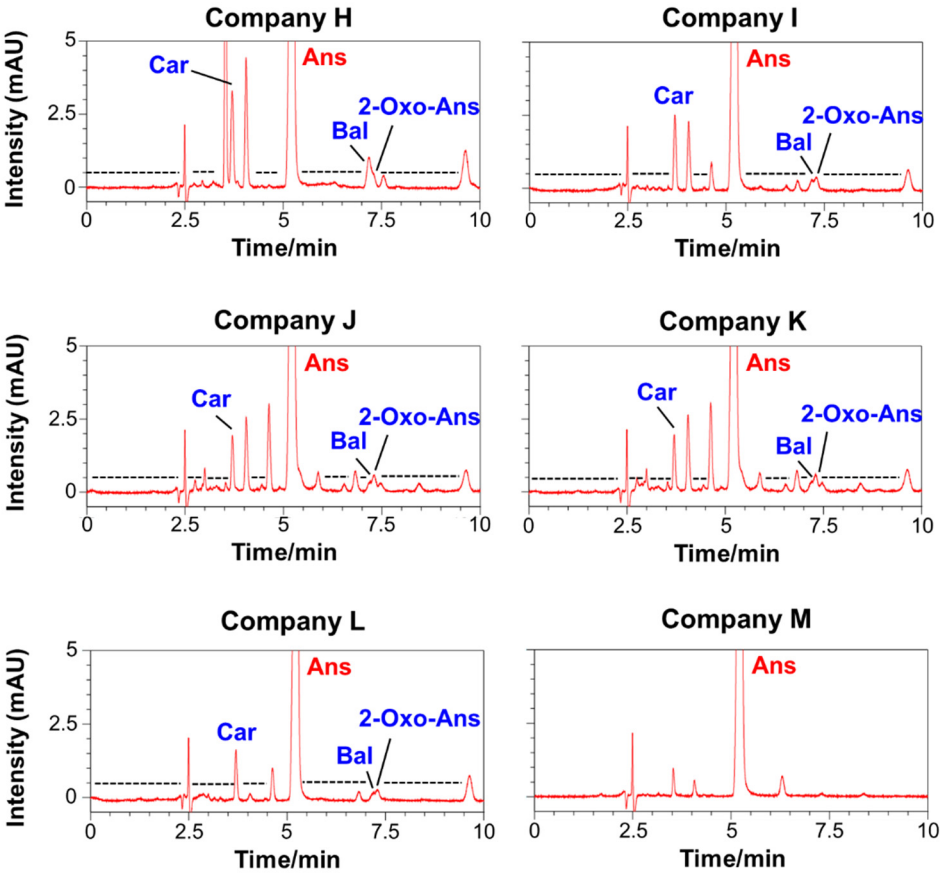


Figure S6. HPLC chromatograms of Ans (50 mM) from companies H – M. The analysis was conducted using a COSMOSIL 3PBr (4.6 mm I.D. × 250 mm, particle size: 3 μm). A 100 mM phosphate buffer (pH 7.0) was used as the mobile phase in isocratic mode for 10 min at a flow rate of 1 mL/min and a column temperature of 30°C. The injection volume was 10 μL, and UV detection was performed at 250 nm.

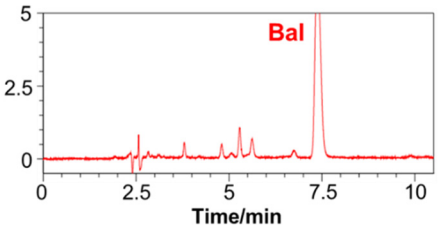


Figure S7. HPLC chromatograms of Bal (20 mM). The analysis was conducted using a COSMOSIL 3PBr (4.6 mm I.D. × 250 mm, particle size: 3 μm). A 100 mM phosphate buffer (pH 7.0) was used as the mobile phase in isocratic mode for 10 min at a flow rate of 1 mL/min and a column temperature of 30°C. The injection volume was 5 μL, and UV detection was performed at 250 nm.

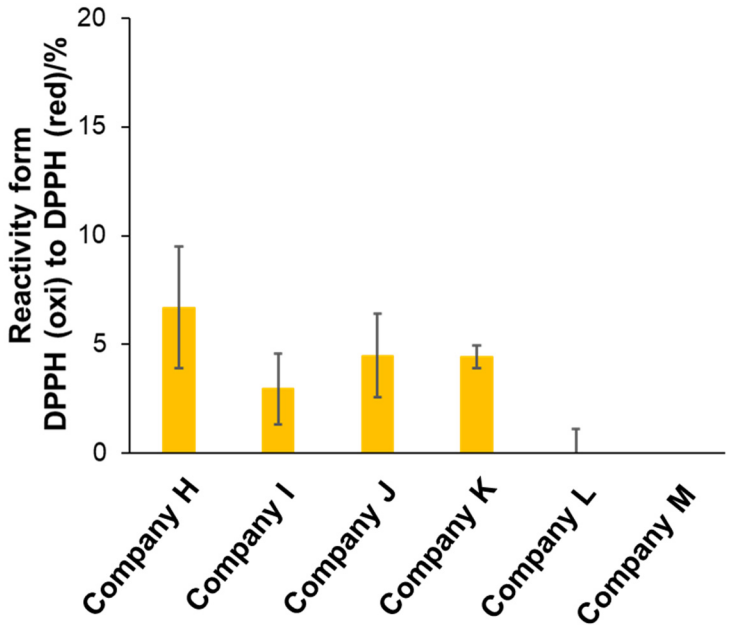


Figure S8. Reactivity of DPPH in Ans from companies H–M.

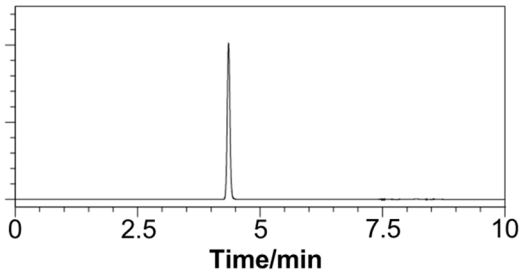


Figure S9. HPLC chromatograms of 2-oxo-Car (20 mM). The analysis was conducted using a COSMOSIL 3PBr (4.6 mm I.D. × 250 mm, particle size: 3 μm). A 100 mM phosphate buffer (pH 7.0) was used as the mobile phase in isocratic mode for 10 min at a flow rate of 1 mL/min and a column temperature of 30°C. The injection volume was 1 μL, and UV detection was performed at 220 nm.

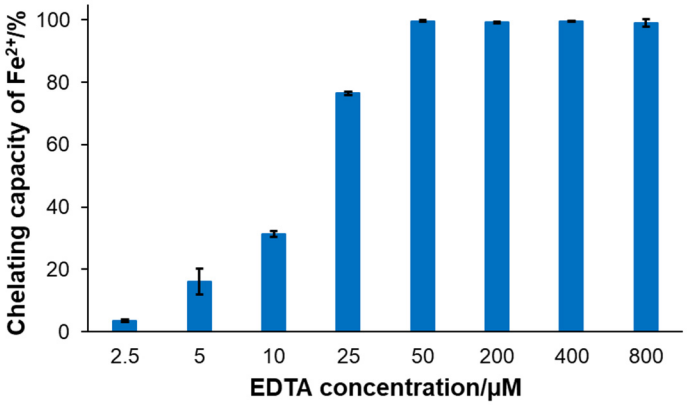


Figure S10. Chelating capacity of Fe^{2+} ion of EDTA.

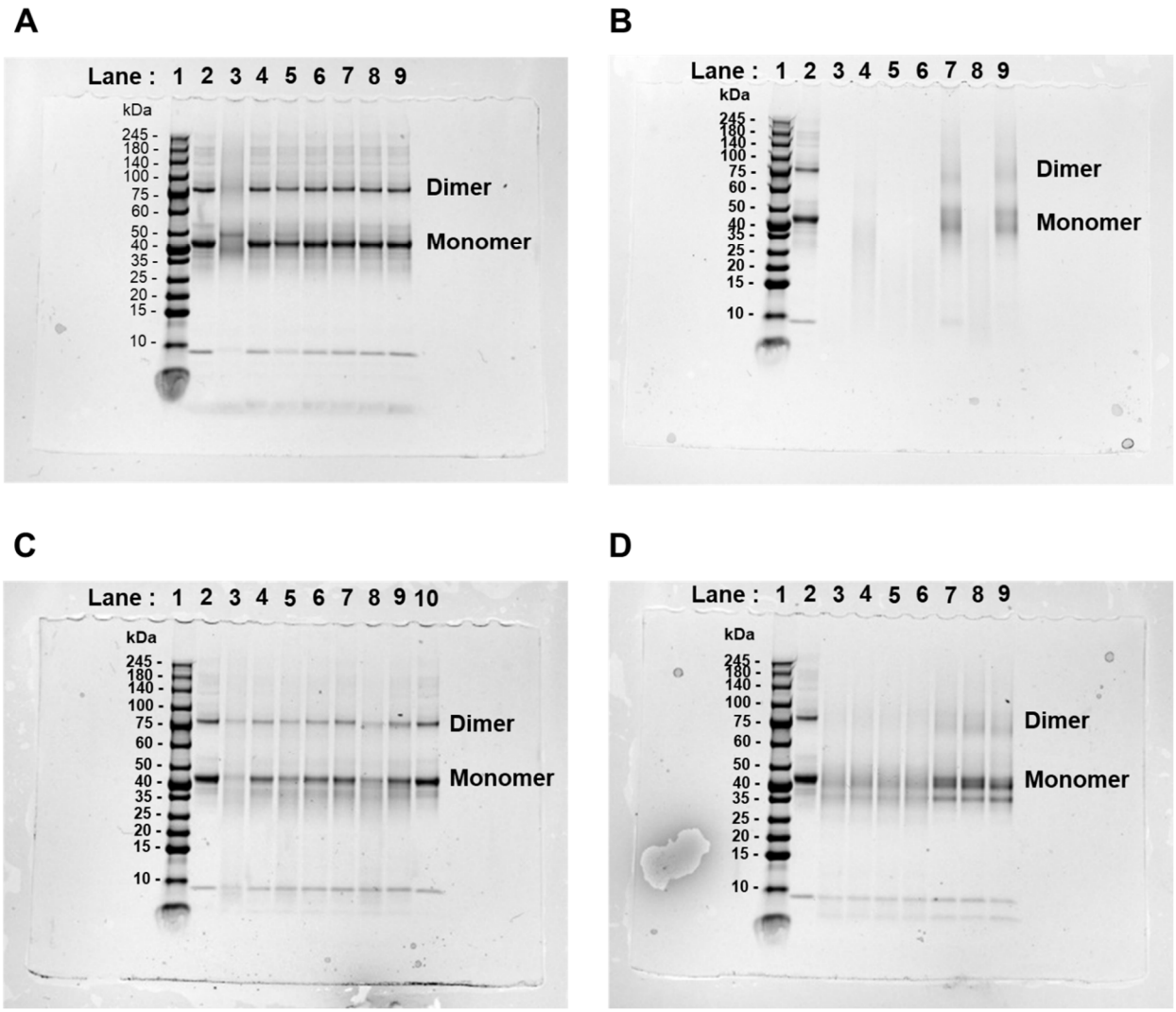


Figure S11. Electrophoresis images showing the inhibition of ovalbumin degradation by (A) and (B) ClO^- (low conc. 0.5 mM and high conc. 5 mM), (C) $OH\cdot$ (10 mM), and (D) $ONOO^-$ (2 mM) using IDPs, 2-oxo-Car, VC, and GSH. 1, Marker; 2, reference protein (without ROS); 3, control (without antioxidant); 4, Car; 5, Ans; 6, Bal; 7, 2-oxo-Car; 8, VC; 9, GSH; 10, samples not related to this study.

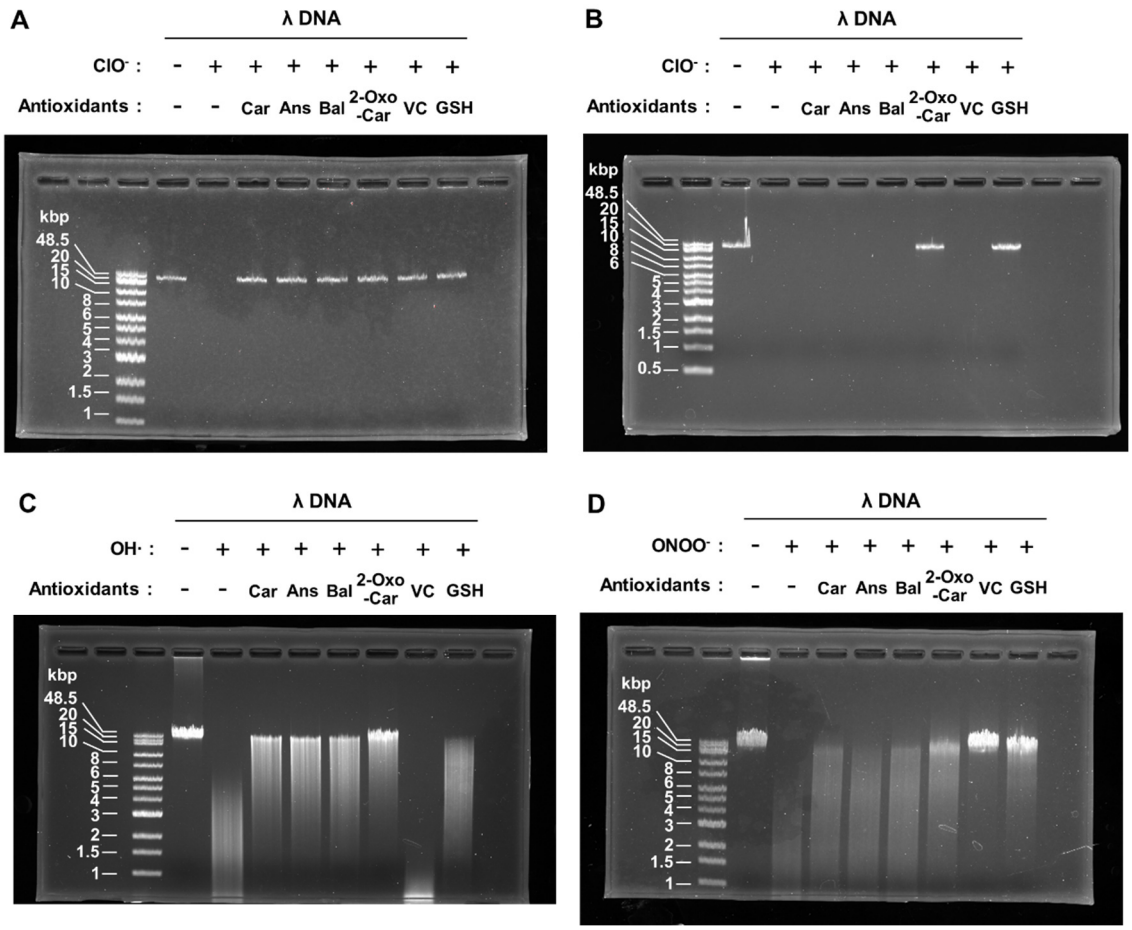


Figure S12. Electrophoresis images showing the inhibition of λ DNA degradation by (A) and (B) ClO^- (low conc. 0.5 mM and high conc. 5 mM), (C) OH· (5 mM), and (D) $ONOO^-$ (1 mM) using IDPs, 2-oxo-Car, VC, and GSH.

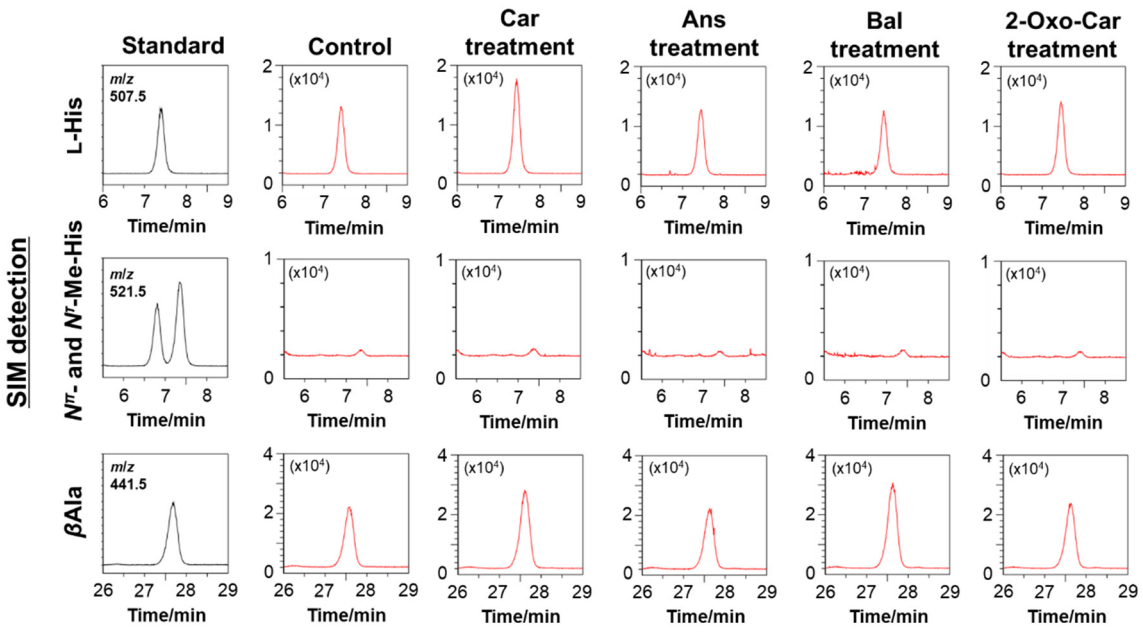


Figure S13. LC-MS chromatograms of the lysate samples from C2C12 cells treated with each IDP and 2-oxo-Car obtained from an analysis using a PBr column after labeling with L-FDVDA.

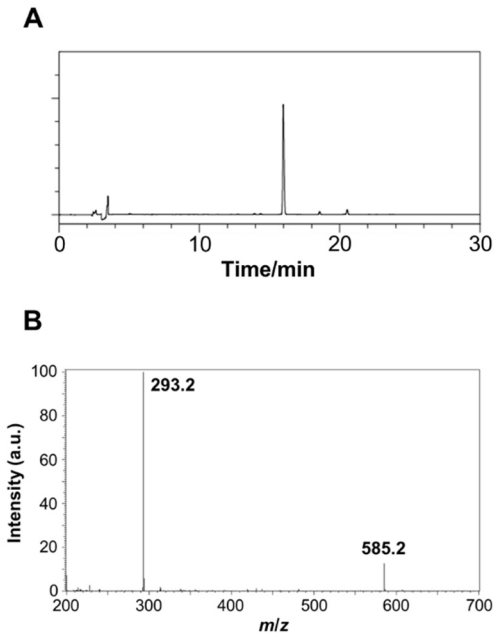


Figure S14. (A) HPLC chromatograms of Flu-Car (250 μM). The analysis was conducted using a COSMOSIL 5C_{18}-AR-II (4.6 mm I.D. × 250 mm, particle size: 5 μm) with ultrapure water containing 0.1% TFA as solvent A and a gradient from 17.5% to 37.5% (0–30 min), and acetonitrile containing 0.08% TFA as solvent B for 30 min at a flow rate of 1 mL/min at 30°C. The injection volume was 10 μL, and UV detection was performed at 220 nm. (B) MS spectrum of Flu-Car purified by HPLC.

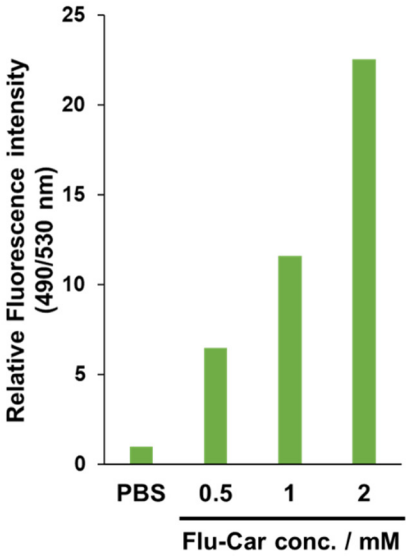


Figure S15. Relative fluoresce intensity of C2C12 cells treated with Flu-Car.