Electronic Supplementary Material (ESI) for Chemical Science.
This journal is © The Royal Society of Chemistry 2021

SUPPORTING INFORMATION

Adapting decarbonylation chemistry for the development of prodrugs capable of *in-vivo* delivery of carbon monoxide utilizing sweeteners as carrier molecules

Ladie Kimberly De La Cruz,[a†] Xiaoxiao Yang,[a†] Anna Menshikh,[b] Maya Brewer,[b] Wen Lu,[a] Minjia Wang,[c] Siming Wang,[a] Xingyue Ji,[a] Alyssa Cachuela,[a] Haichun Yang,[d] David Gallo,[e] Chalet Tan,[c] Leo Otterbein,[e] Mark de Caestecker,[b] and Binghe Wang*[a]

[a]Department of Chemistry, Georgia State University, Atlanta, GA, 30303; [b]Department of Medicine, Division of Nephrology, Vanderbilt University Medical Center, Nashville, TN, 37232; [c]Department of Pharmaceutics and Drug Delivery, University of Mississippi, University, MS, 38677; [d]Department of Pathology Microbiology and Immunology, Vanderbilt University, Nashville, TN, 37232; [e]Transplant Institute, Department of Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

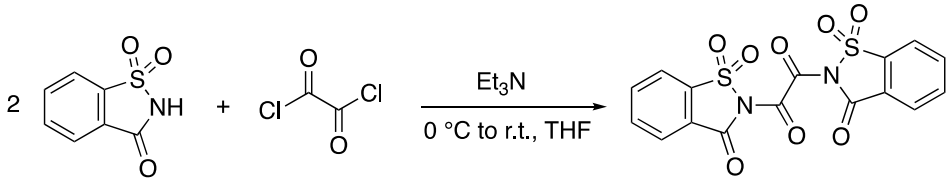
*E-mail: wang@gsu.edu

Table of Contents

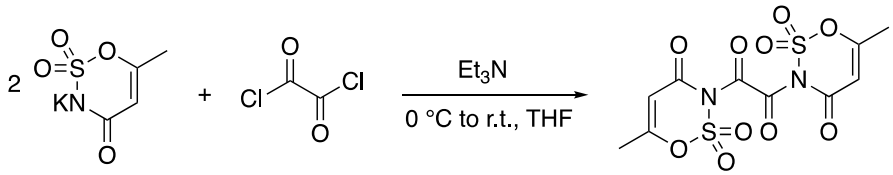
General experimental details

All reactions were carried out under nitrogen atmosphere using glassware that was previously oven-dried overnight with magnetic stirring unless otherwise indicated. Unless otherwise noted, all reagents were obtained from commercial suppliers (Sigma Aldrich, VWR International, and Oakwood Chemicals) and used without further purification. Thin layer chromatography was performed on glass-backed silica gel TLC plates (250 μm) using mixtures of hexanes/ethyl acetate as eluent, and using either UV light, iodine powder, or potassium permanganate stain for visualization. Column chromatography was done using Silica Flash P60 silica gel (230-400 mesh). ^{1}H and ^{13}C NMR spectra were recorded on Bruker-400 spectrometers (400 MHz and 100 MHz, respectively). Chemical shifts were reported in ppm relative to residual solvent peaks (δ7.26 for ^{1}H, 77.1 for ^{13}C, $CHCl_3/CDCl_3$) and (δ 2.49 for ^{1}H, and 39.1 for ^{13}C, DMSO/DMSO-d_6). Data are reported as follows: bs= broad singlet, s= singlet, d= doublet, t= triplet, q= quartet, m= multiplet, dd= doublet of doublets, ddd= doublet of doublet of doublets, ddt= doublet of doublets of triplets, td= triplet of doublets; coupling constants in Hz; integration. Accurate mass measurements were acquired at the Mass Spectrometry Facilities at Georgia State University. For spectrophotometric studies, Shimadzu PharmaSpec UV-1700 was used as UV-Vis spectrophotometer; while Shimadzu RF-5301PC fluorimeter was used for fluorescent studies. Agilent 7820A with TCD detector was used for CO and CO_2 quantification.

Synthesis of CO prodrugs



BW-CO-306

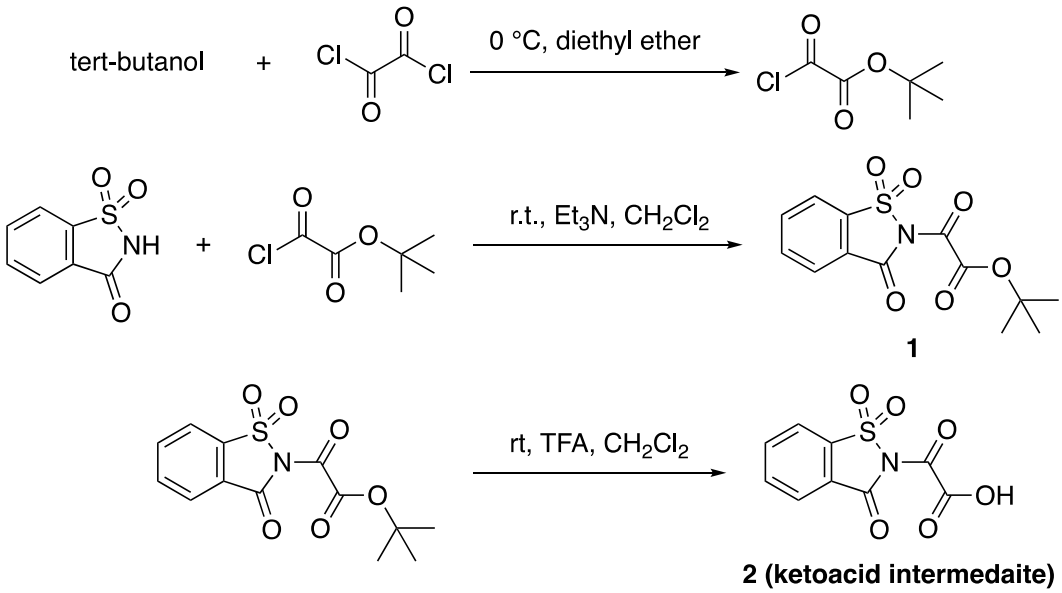


BW-CO-307

Scheme S1. One step synthesis of oxalyl-based CO prodrugs

BW-CO-306:[1] To a solution of saccharin (1g, 5.46 mmol) and triethylamine (766 µL, 5.46 mmol) in THF was added dropwise oxalyl chloride (190 µL, 2.73 mmol) dissolved in THF at 0 °C. After addition of oxalyl chloride, the reaction mixture was stirred at room temperature for 3 h. Then THF was removed by rotary evaporation. The residue was re-dissolved in dichloromethane, washed with water (2 x 10 mL), saturated $NaHCO_3$ (3 x 10 mL), and brine. The organic layer was then concentrated in the rotary evaporator at <30 °C to give a white crude solid. Purification via recrystallization using acetonitrile yielded a white solid (779 mg, 68%). ^{1}H NMR (CD_3CN): 8.13-8.22 (6H, m), 8.01-8.05 (2H, m). ^{13}C NMR (CD_3CN): 159.5, 156.8, 139.8, 139.6, 137.4, 128.4, 125.2, 123.3. HRMS (ESI) calculated for $C_{16}H_8N_2O_8S_2$ $[M+Na]^+$: m/z, 442.9620 found 442.9626.

BW-CO-307: To a suspension of acesulfame K (3 g, 4.97 mmol) in THF (20 mL) stirring at 0 °C was added dropwise oxalyl chloride (519 µL, 2.49 mmol) dissolved in THF. The reaction mixture was stirred in ice-bath for 10 min and then stirred at room temperature for 5 h. Then, the reaction mixture was concentrated in the rotavap. The crude residue was dissolved in DCM and washed with water (2 x 20 mL), sat $NaHCO_3$ (2 x 20 mL), and brine. The organic layer was dried over Na_2SO_4 and concentrated in the rotavap to give a yellowish solid which was suspended in diethyl ether and filtered to yield a white crystalline solid (200 mg, 7%). ^{1}H NMR (CD_3CN) 6.12 (m, 1H), 2.31 (m, 3H). ^{13}C NMR (CD_3CN) 167.9, 160.8, 156.4, 104.4, 20.4. HRMS (ESI) calculated for $C_{10}H_8N_2O_{10}S_2$ $[M+Na]^+$: m/z 402.9518, found 402.9521.



Scheme S2. Synthesis of saccharin ketoacid intermediate.

Compound 1: To a solution of oxalyl chloride (1.8 mL) in diethyl ether stirring at 0 °C, was slowly added a solution of *tert*-butanol (2.0 mL) dissolved in diethyl ether. The mixture was allowed to warm to room temperature and stirred further for 20 h. Then, the mixture was concentrated in the rotavap to give a clear, colorless liquid, which was directly used in the next step without further purification. To a solution of saccharin (1300 mg) and triethylamine (3.0 mL) in dichloromethane was added slowly a solution of *tert*-butyl 2-chloro-2-oxoacetate dissolved in dichloromethane. After 3 h stirring, the reaction mixture was diluted with dichloromethane and then washed with saturated $NaHCO_3$. The organic layer was dried in the rotavap and suspended in diethyl ether. The solid was isolated by filtration to yield a white powder (1489 mg, 67%). ^{1}H NMR ($CDCl_3$) 8.16-8.16 (1H, m), 7.94-8.06 (3H, m), 1.62 (9H, s). ^{13}C NMR ($CDCl_3$) 157.1, 156.8, 156.8, 138.9, 137.3, 135.5, 126.7, 124.5, 121.7, 87.2, 27.9. HRMS (ESI) calculated for $C_{13}H_{13}NNaO_6S$ $[M+Na]^+$: m/z 334.0361, found 334.0365.

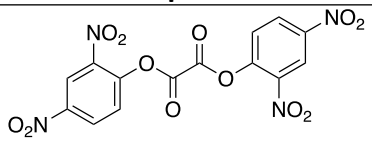
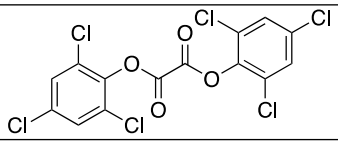
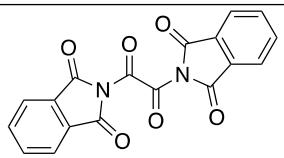
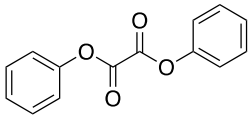
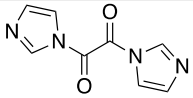
Compound 2: To a solution of compound **4** (503 mg) in dichloromethane while stirring at 0 °C was added trifluoroacetic acid (7 mL). Then the reaction was stirred at room temperature. After 5 h, the reaction mixture was filtered to give a white solid (236 mg, 57%). ^{1}H NMR (CD_3CN) 8.09-

8.12 (3H, m), 8.00-8.05 (1H, m). ^{13}C NMR (CD_3CN) 159.8, 159.1, 158.1, 139.1, 138.8, 136.8, 127.7, 125.5, 122.6. HRMS (ESI) calculated for $C_9H_5NNaO_6S$ $[M+Na]^+$: m/z 277.9741, found 277.9735.

Survey of CO-releasing capacity: effect of pKa of leaving group

To study the effect of pK_a, various commercially available oxalyl derivatives were procured from either Sigma Aldrich or vWr Chemicals. To a solution of CO probe COP-1 (0.5 µM) in 3% DMSO in PBS, a solution of the oxalyl derivatives dissolved in DMSO was added to make a final concentration of 100 µM. The resulting mixture was incubated at 37 °C at different time points, and the fluorescence emission intensity was recorded from 490-630 nm with an excitation wavelength of 475 nm.

Table S1. Effect of pK_a of leaving group on the CO releasing capability of oxalyl-based CO prodrugs

Compounds	pKa	COP-1 turn on?
	4.09	Yes, 6-fold
	6.23	No
	8.3	No
	10	No
	14.5	Yes, 0.1 fold

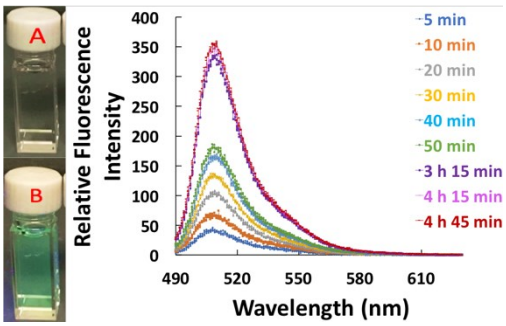


Fig S1. Fluorescent probe, COP-1 (1 μM) for CO detection: A – COP-1 only, B – COP-1 + CO prodrug **BW-CO-306** (100 μM), C – time-dependent increase in fluorescence of COP-1 probe in the presence of CO prodrug **BW-CO-306**.

Qualitative CO detection

Qualitative CO detection was done using COP-1, commercially available CO detector, and the CO myoglobin assay. Both **BW-CO-306** and **BW-CO-307** gave positive results to these tests.

Using a household CO detector

CO formation from CO prodrugs was detected in a qualitative manner using commercially available CO detector. Briefly, the prodrugs were dissolved in organic solvent in a round bottom flask and sealed with a rubber septum before heating to 37 °C. Then, a commercially available CO detector hooked to a syringe and needle was inserted to the rubber septum. After addition of water via a syringe, an increase in ppm levels were observed via the CO detector, indicating substantial CO release.

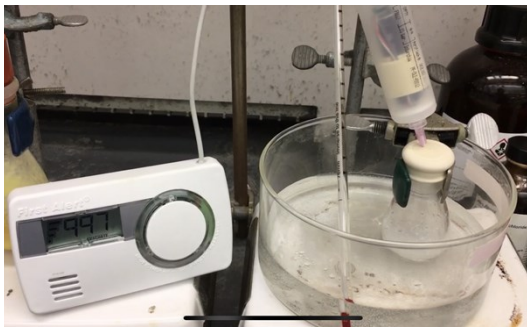


Fig S2. Experimental set-up for the use of modified of household CO detector for the qualitative detection of CO from **BW-CO-306** and **BW-CO-307**.

CO myoglobin assay

Direct detection of carbon monoxide release was done through a “two-compartment” Mb-CO assay. The set-up was assembled by putting a small vial inside a bigger vial and sealing the system with a septum. An aqueous solution of deoxy-Mb was placed in the bigger vial while a solution of CO prodrug in acetonitrile was placed in the smaller vial. The deoxy-Mb solution was prepared by degassing a solution of myoglobin in PBS (1 mg/mL, pH = 7.4) with nitrogen for at least 30 min, and then converted to deoxy-Mb by adding freshly prepared solution of sodium dithionite (1 mL, 22 mg/mL). To initiate CO release, PBS was injected into the smaller vial containing the acetonitrile solution of **BW-CO-306** and **BW-CO-307**. The whole set-up was then incubated at 37 °C. At the end of 1 h, the set-up was cooled in an ice-bath for 10 min to increase the solubility of CO gas, after which, the incubated solution was immediately transferred into a cuvette for UV-Vis spectral measurements.

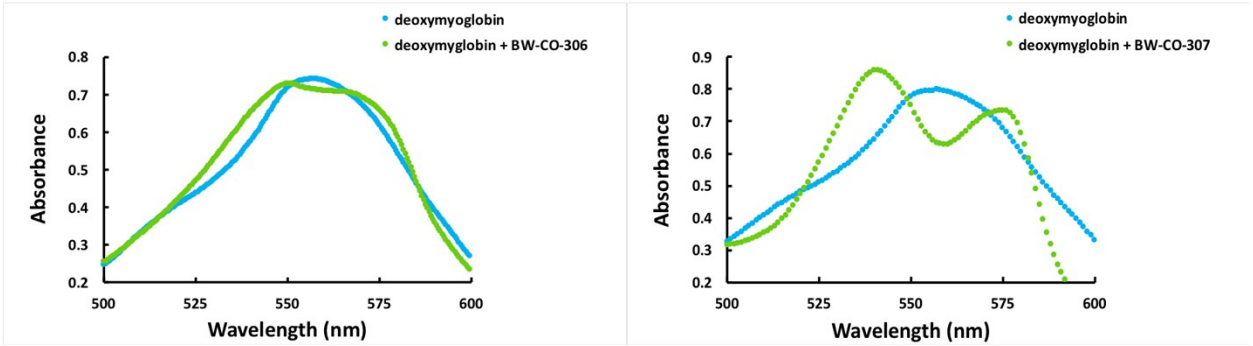


Fig S3. Characteristic spectral change attributed to the binding of CO to myoglobin – left: **BW-CO-306** right: **BW-CO-307**.

Quantitative CO Analysis

An Agilent 7820A GC System equipped with a thermal conductivity detector was used to detect and quantify CO release yield of the CO prodrugs. Using a gas tight syringe, specific volumes of the headspace of 6-mL headspace vials were sampled and transferred to the injector port maintained at 125 °C. Helium was used as the carrier gas with a flow rate of 30 mL/min. Gaseous components of the headspace were separated by passing through a packed column with 60/80 Carboxen-1000 matrix support, L × O.D. × I.D. 15.0 ft (4.6 m) × 1/8 in. × 2.1 mm (Supelco). The column was heated 35 °C for 5 min then 225 °C at 20 °C/min while the detector was held at 125 °C. Under these conditions, CO had an elution time of around 7.4 minutes while CO_2 eluted at 13.4 minutes.

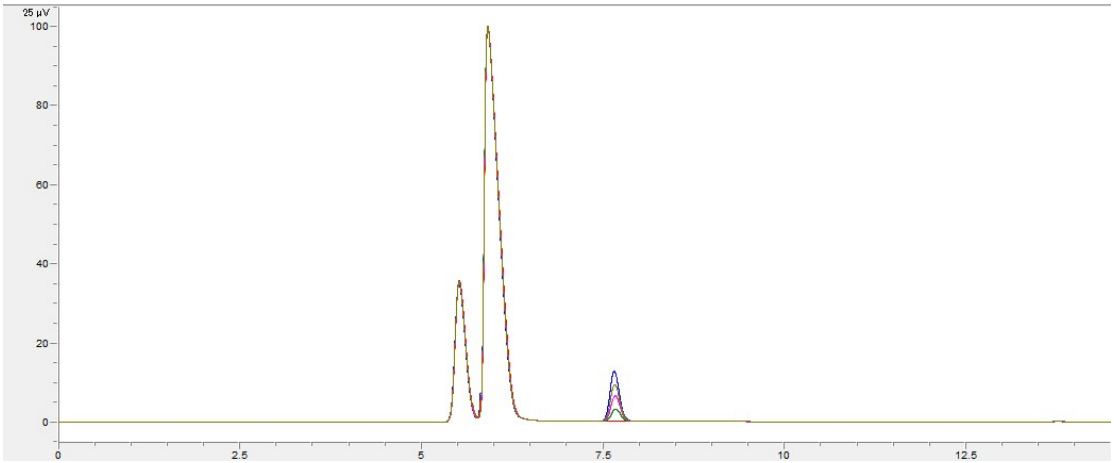


Fig S4. Sample chromatogram for CO calibration curve (0 – 20 µmol).

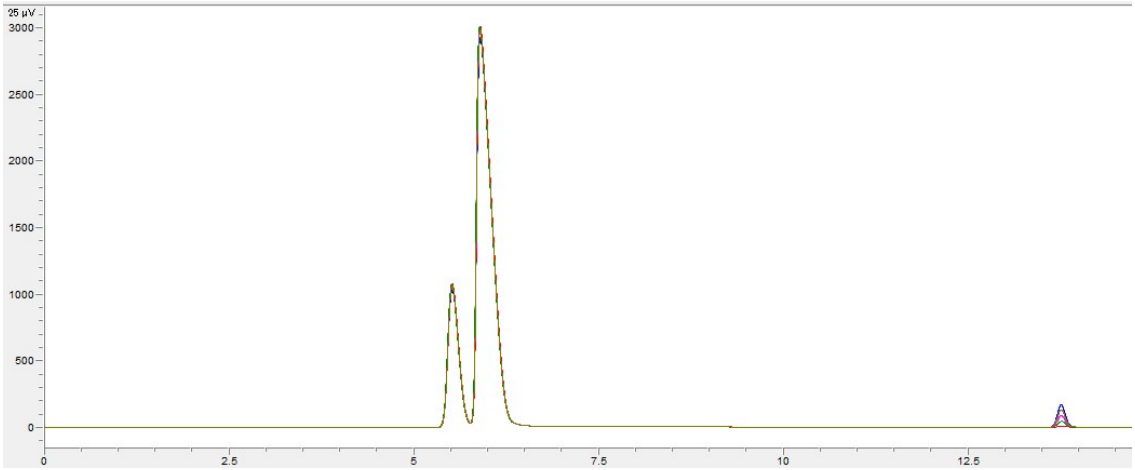


Fig S5. Sample chromatogram for CO_2 calibration curve (0 – 20 µmol).

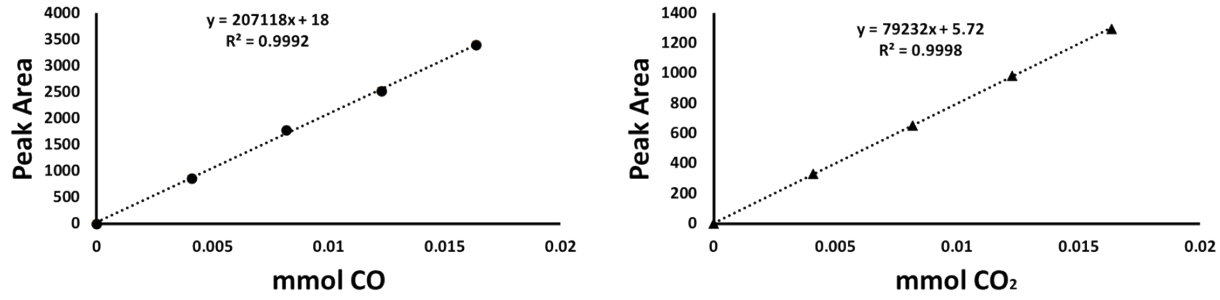


Fig **S6**. Calibration curve for CO and CO_2.

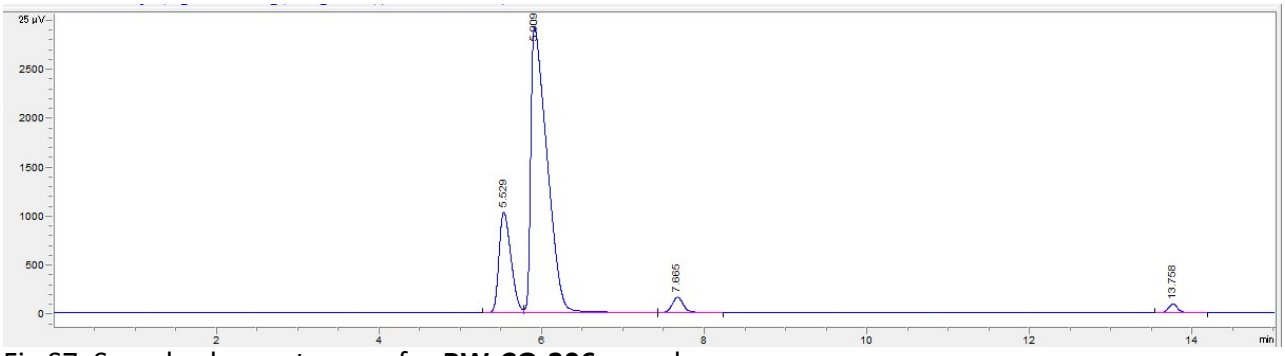
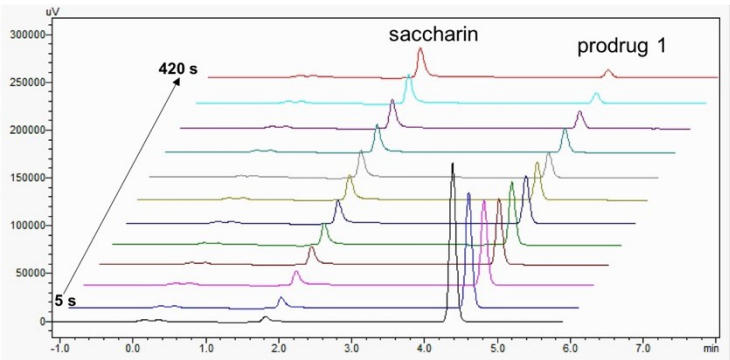


Fig S7. Sample chromatogram for **BW-CO-306** sample.

<u>HPLC Kinetics Experiment</u>

A 200 µL aliquot of incubated sample was placed inside a 0.5 mL vial followed by dilution with 200 µL of acetonitrile. The study was performed using the Shimadzu Prominence UFLC with a reversed-phase analytical column (Waters C18 3.5 µM, 4.6 x 100 mm) at 25 °C. The flow rate was set at 1 mL/min. Gradient elution using acetonitrile and deionized water with 0.05% TFA was used to elute out the components of the sample. Elution conditions: 0-7 min 50% to 70% acetonitrile; injection volume of 10 µL; detection wavelength of 254 and 280 nm.



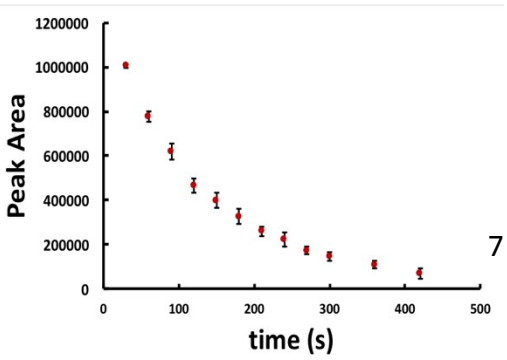


Fig S8. Decomposition profile of prodrug **BW-CO-306** in 60% PBS.

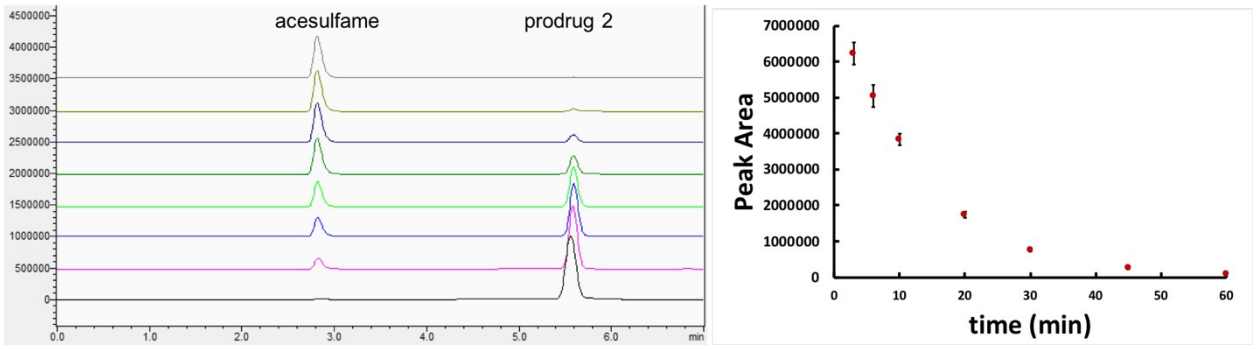


Fig S9. Decomposition profile of prodrug **BW-CO-307** in 60% PBS.

NMR Studies

Around 5 mg of prodrug was dissolved in 450 µL of deuterated acetonitrile. The ^{1}H and ^{13}C NMR spectra of this solution served as the initial time point. Both prodrugs were stable in acetonitrile as no spectroscopic changes were observed even after 24 h of incubation at room temperature. Upon addition of 250 µL of deuterated water, the prodrug peaks disappeared accompanied by the formation of peaks corresponding to the leaving groups only.

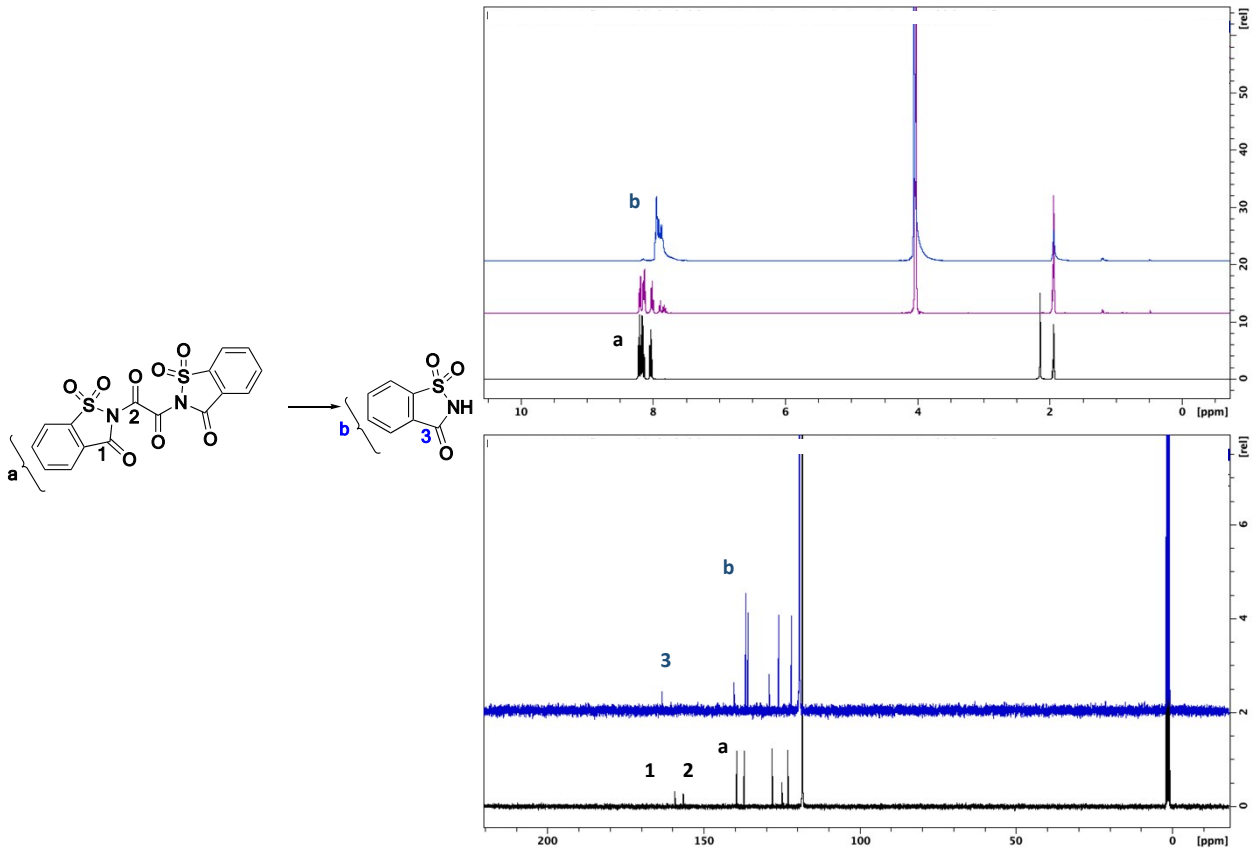


Fig S10. Decomposition profile of prodrug **BW-CO-306** as monitored by NMR.

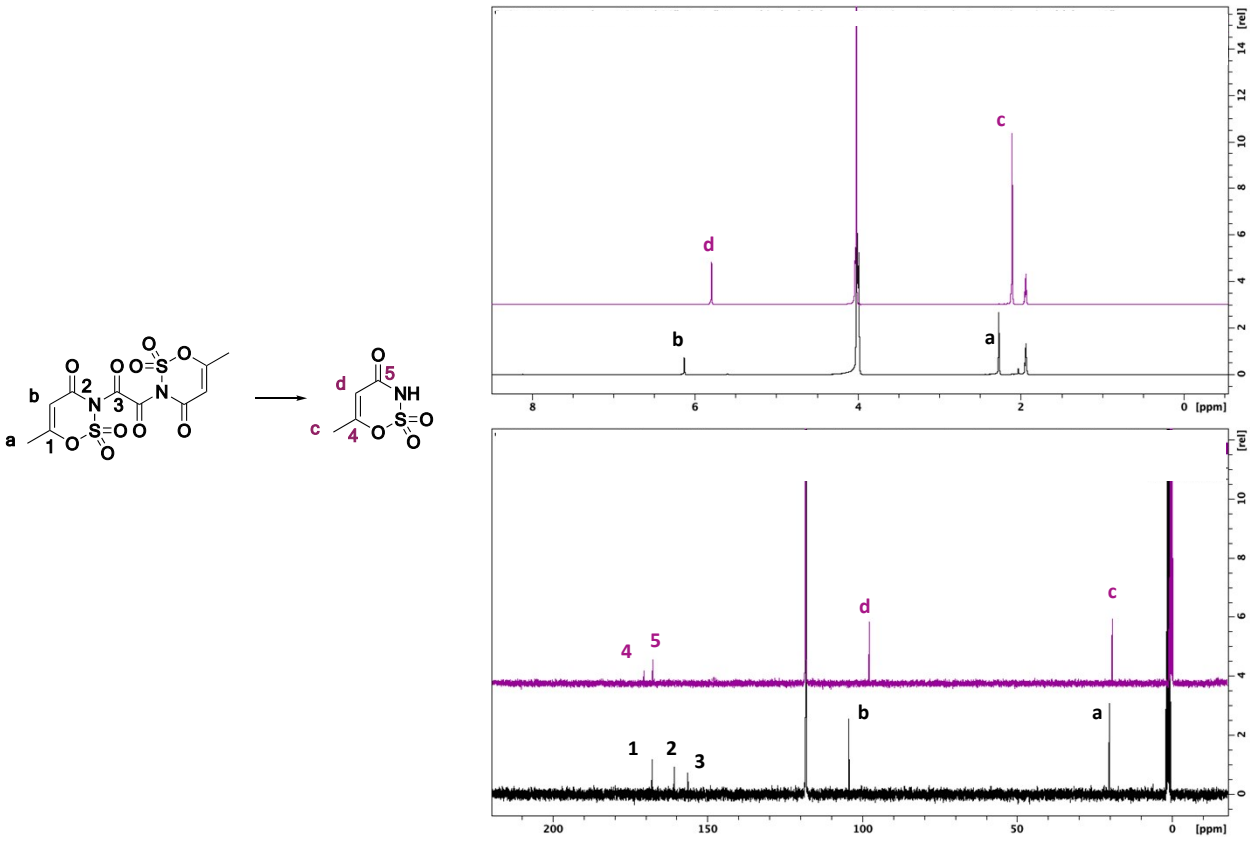


Fig S11. Decomposition profile of prodrug **BW-CO-307** as monitored by NMR.

Oxalic acid quantification (LC-MS)

The LC-MS analysis was performed on an Agilent 1200 HPLC and 3200 API triple quadrupole mass spectrometer with electrospray ionization source in a negative mode. Selected ion mode was used for the detection of ions at m/z 182 for saccharin and m/z 89 for oxalic acid. A 5 µL of sample solution was directly delivered to the mass spectrometer ionization source with an Agilent autosampler and HPLC by mixing with 1% ACN in water mobile phase at 200 µL/min flow rate. No column was used. Three minutes were recorded and the peak area was integrated and the peak are ratio of PA(Oxalic acid)/PA(Saccharin) vs Concentration ratio (C(Saccharin)/C(Oxalic acid) was plotted as standard curve. The linear regression equation was generated for the calculation of the ratio of unknowns.

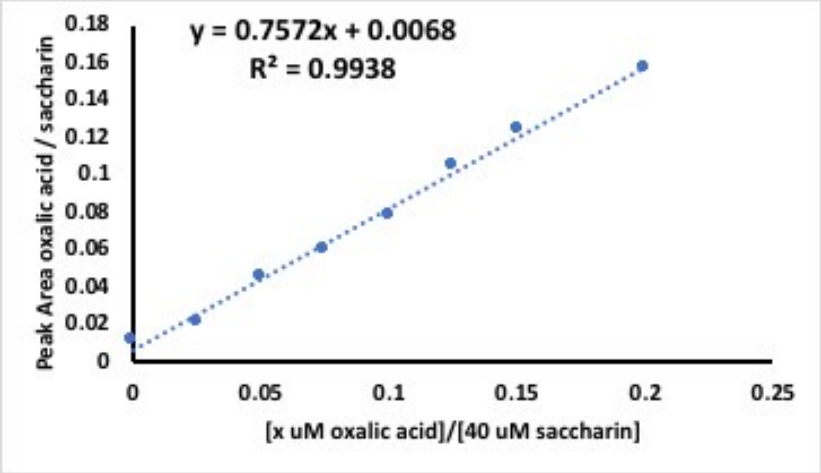


Fig S12. Standard curves for LC-MS determination of oxalic acid generated from prodrug **BW-CO-306**.

Cytotoxicity Assay

For the CCK-8 assay, Hela cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (MidSci; S01520HI) and 1% penicillin-streptomycin (Sigma-Aldrich; P4333) at 37 °C with 5% CO2. Fresh medium was replenished every other day. The cells were treated with the compounds (0–100 μM) using 1% DMSO in DMEM for 24 h.

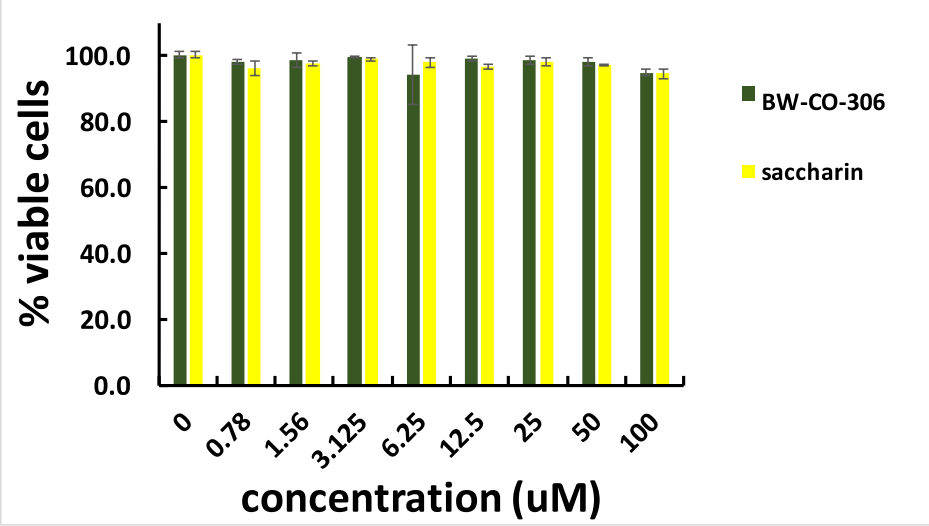


Fig S13. Cytotoxicity profile of prodrug **BW-CO-306** against HeLa cells.

ELISA assay for TNF-alpha

Anti-inflammatory effect of **BW-CO-306** was tested in RAW246.7 cell by repeated addition (5x) with 1 hour interval in 5 hours. **BW-CO-307** was only administered once. After 5 hours, 1 μg/ml of LPS was added to the cell culture medium and incubated for 1 hour. TNF-α concentration in the supernatant culture medium was then determined with ELISA kit (Biolegend, USA) according to the manufacture's protocol.

Pharmacokinetic Studies - Mouse COHb level determination

CO prodrugs were dissolved in dimethylacetamide (DMA) and diluted to 20% in PEG400. The drug-loaded formulation or the blank vehicle was administered in fed mice ($n \geq 3$) via oral gavage (p.o.) administration. At pre-determined time points post administration, blood samples were collected by retro-orbital bleeding. Circulating CO was monitored by measuring the COHb level in the whole blood using a CO-oximeter (AVOXimeter 4000, Avox Systems, New York, NY, USA). The CO-oximetry measurement strictly followed the manufacturer's protocol and was also validated by performing quality control.The baseline COHb level of each mouse was measured before administration.

Kidney Injury Animal Studies

Mice were purchased from Charles River (Wilmington, MA). Blood was collected for determination of blood urea nitrogen (BUN), and tissue harvested for histopathology and quantitative RT-PCR for injury markers. All animal experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Rhabdomyolysis-induced AKI

Male BALB/c mice aged 13 weeks were water restricted for 18 hours and underwent intramuscular (IM) injection of 5.4 ml 50% glycerol (Sigma) in sterile water into both anterior thighs while anesthetized with a ketamine-xylazine mixture (120-150 mg/kg ketamine and 12-15 mg/kg xylazine) intraperitoneally, with glycerol dose split equally between the two hindlimbs. Pain relief (0.015 mg buprenorphine subcutaneously (S/C)) was provided every 12 h for 24 h starting immediately after IM injections. Mice were given 0.5 ml of 0.9% sterile saline S/C at the time of injection and after 24 h. BUN was assessed at baseline and 1 day after glycerol injection, and tissues harvested after 3 days. Mice were treated with 50 mg/kg BW-CO-306, or the release product BW-CP-306, in activated charcoal suspension by oral gavage, starting 24 h before glycerol injection, then 2, 24 and 48 h after glycerol injection.

Assessment of renal function and kidney injury

Blood urea nitrogen (BUN).

Blood was collected by submandibular vein into lithium-heparin-coated microcuvette tubes. Four microliters of plasma were collected for blood urea nitrogen (BUN) measurement, and measured in duplicate, according to the manufacturer's instructions (Infinity Urea, Thermo Scientific, Waltham, MA).

Tubular injury score.

Five-micrometer formalin-fixed, paraffin embedded (FFPE) sections were generated as described above and stained using a periodic acid-Schiff (PAS) staining kit (Sigma-Aldrich, St Louis, MO), as

described by the manufacturer. More than 20 images of the cortex and outer stripe of the outer medulla (OSOM) were captured with light microscopy at X400 magnification. Tubular injury was scored by a pathologist (H. Yang) blinded to the injury conditions. Tubular injury was based on the following scoring system on a scale of 0 to 4: 0-no injury, 1-1–25% of area injured, 2-26–50% of area injured, 3-51–75% of area injured, and 4-76–100% of area injured. Acute tubulointerstitial injury was defined as interstitial edema with loss of the brush border, shedding of both necrotic and viable epithelial cells into the tubular lumen, intratubular cast formation, tubular dilation, or naked tubular basement membrane.

Immunofluorescence staining and quantification

FFPE sections underwent citrate antigen retrieval (Target Retrieval Solution, Citrate pH6.1, Agilent). Sections were incubated for 5 minutes with 50 mM glycine to reduce autofluorescence; blocked with Avidin/Biotin Blocking kit (Vector labs), according to the manufacturer's instructions, and incubated with 1X Universal Blocking Reagent (Power Block Universal Blocking Reagent (BioGenex) at room temperature for 30 minutes. Rat monoclonal anti-Kim1 (R&D systems, MAB1817), and biotinylated Lotus Tetraglonolobus Lectin (LTL) were incubated at 1:500 dilution in Antibody Diluent Reagent Solution (Thermo Fisher) overnight at 4^{o}C. After washing in PBS, sections were incubated with donkey anti-rat-Cy5 conjugated secondary antibody (Jackson Labs, 712-175-153) at 1:500 dilution in Antibody Diluent Reagent for 60 minutes at room temperature. After washing in PBS, sections were incubated with Neutravidin-DyLight 488 at 1:250 dilution in PBS at room temperature for 30 minutes, before washing, mounting, and slides scanned with a Zeiss Axio Scan Z1 epi-fluorescence microscope. Image analysis was performed by an observer (M.B.) who was blinded to the treatment groups. Digital CZI image files of Kim1/LTL-stained sections were then loaded into QuPath (v0.2), and a boundary drawn around the outer stripe of the outer medulla (OSOM). The selected area was then transferred to ImageJ for analysis. Once in ImageJ, a threshold was found that only depicted positive staining for Kim1 and LTL. The software calculated the positive surface area and divided that by the total surface area of the OSOM to produce the percent positive.

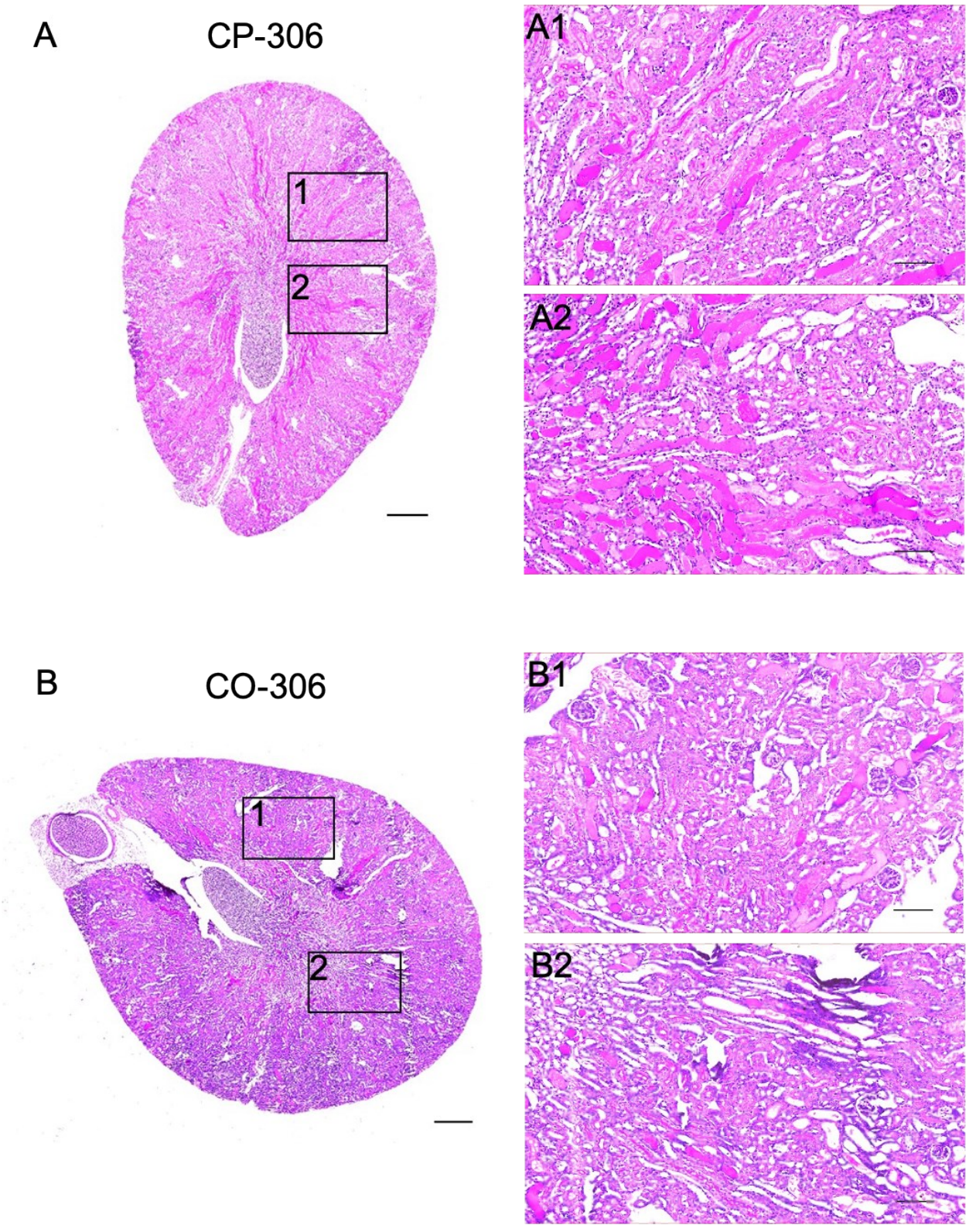


Fig S14**.** Representative PAS-stained section from **BW-CP-306** (A) and **BW-CO-306** (B) treated mouse kidneys three days after rhabdomyolysis-induced AKI showing a marked decrease in

tubular casts in **BW-CO-306** treated mouse kidneys. Scale bars, 500 µM. Insets show higher magnification images A/B, 1 and 2, as indicated. Scale bars, 100 µM.

Quantitative RT-PCR.
A transverse section of the middle of the kidney was snap frozen in liquid nitrogen, and RNA was extracted, cDNA was synthesized, and quantitative RT-PCR was performed using *Kim1* mRNA and *Gapdh* primer sets, as described.[2]

Experimental Model of Renal IRI
Mice were anesthetized with Ketamine-Xylazine, a midline incision was made, and both renal pedicles were cross-clamped for 45 min. During the procedure, animals were kept well hydrated with saline and at a constant temperature (~37 °C) through a heating pad device. Subsequently, microsurgery clamps were removed, the abdomen closed, and animals placed in single cages, warmed by indirect light until completely recovered from anesthesia. Animals were kept under adjustable conditions until sacrifice 24 h after renal reperfusion. BW-306 was dosed at 50 mg/kg, p.o., 1 h before ischemia. Serum creatinine was measured 24 h after reperfusion was started and results represent mean ± SD of 4 mice/group.

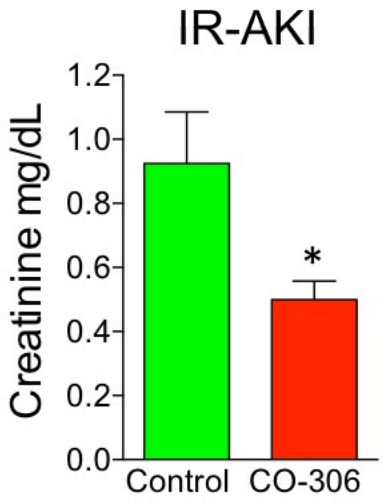
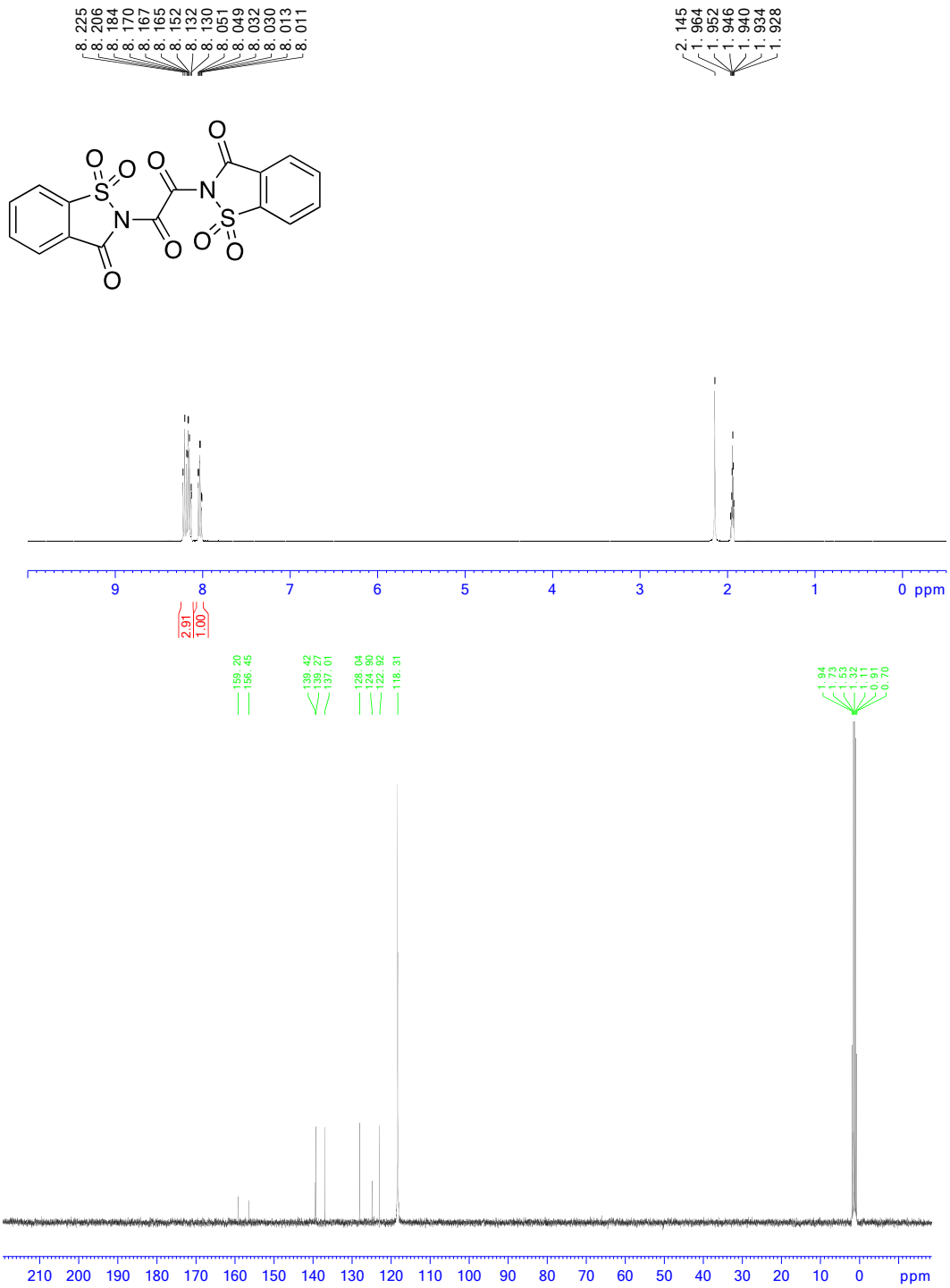


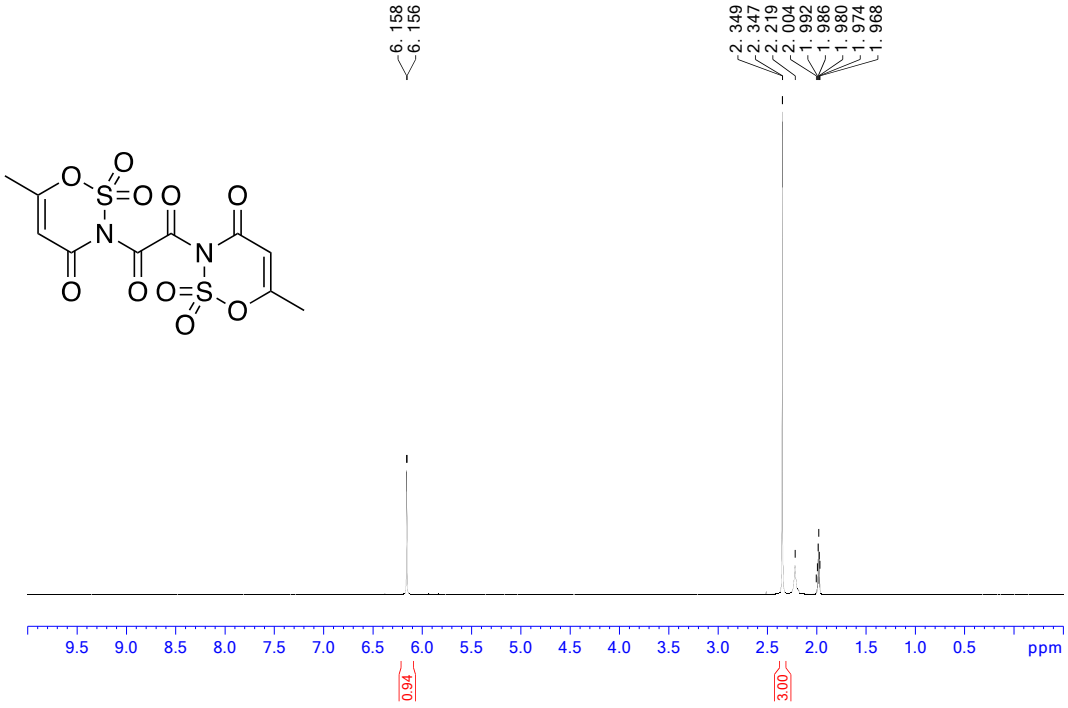
Fig S15. **BW-CO-306** (50 mg/kg) exhibits protection against renal IRI.

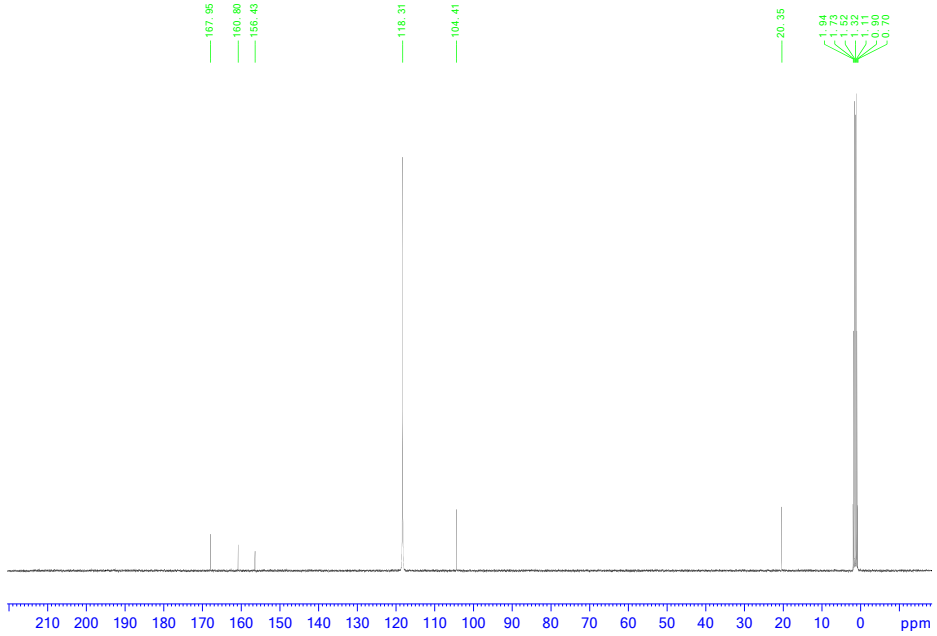
NMR Spectra

BW-CO-306:

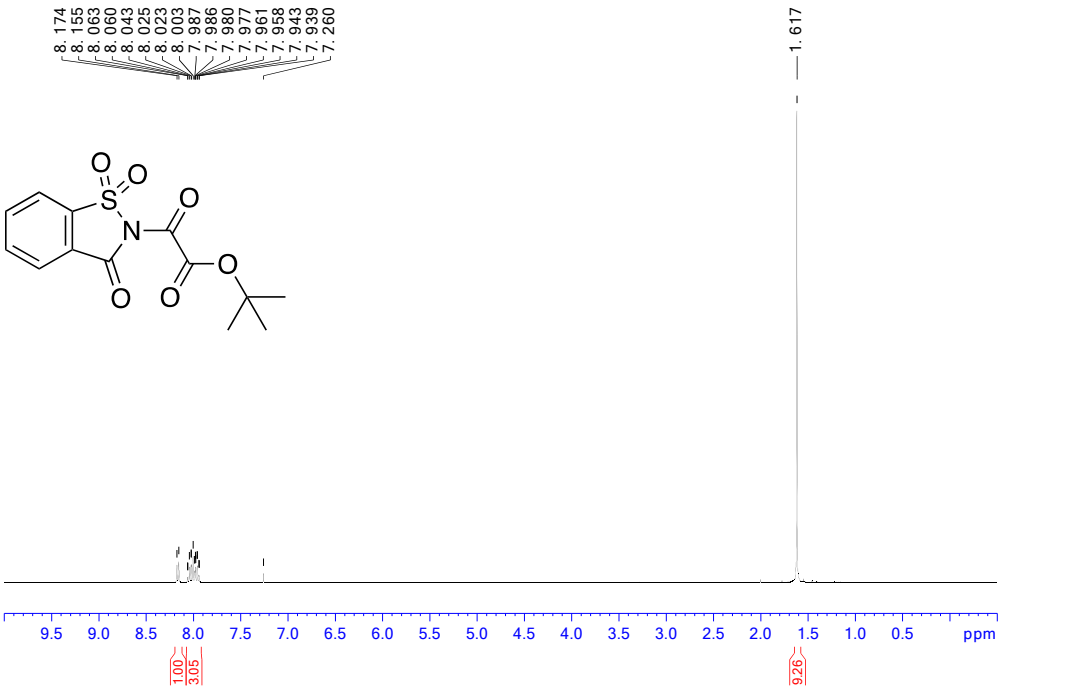


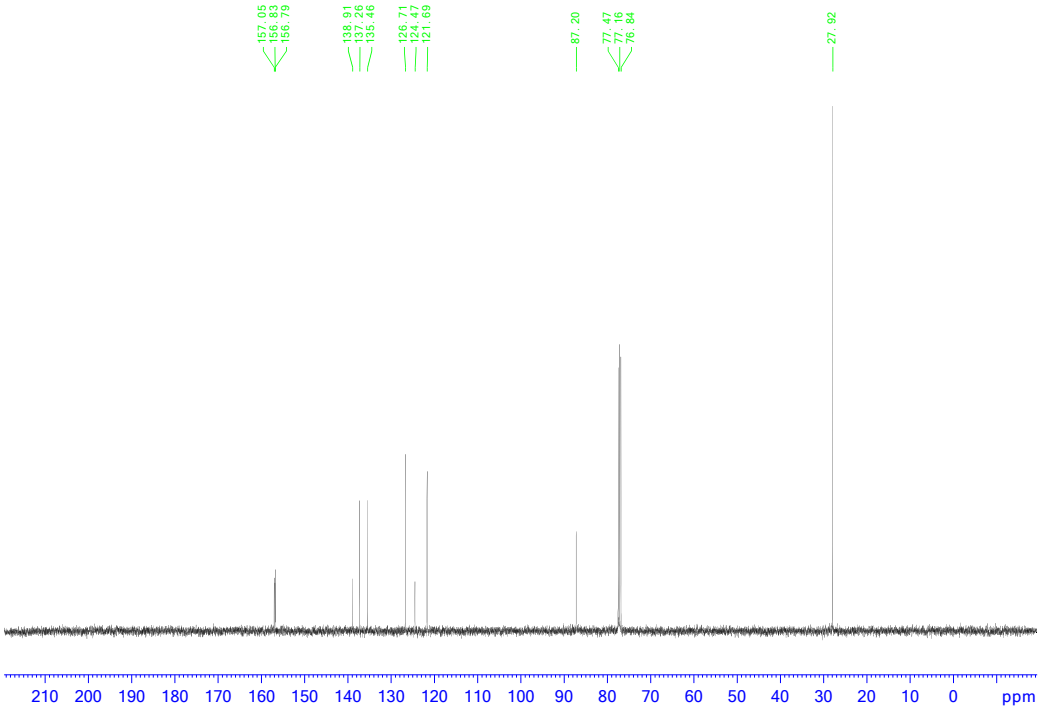
BW-CO-307:


6.158
6.156
2.349
2.347
2.219
2.004
1.992
1.986
1.980
1.974
1.968
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
0.94
3.00

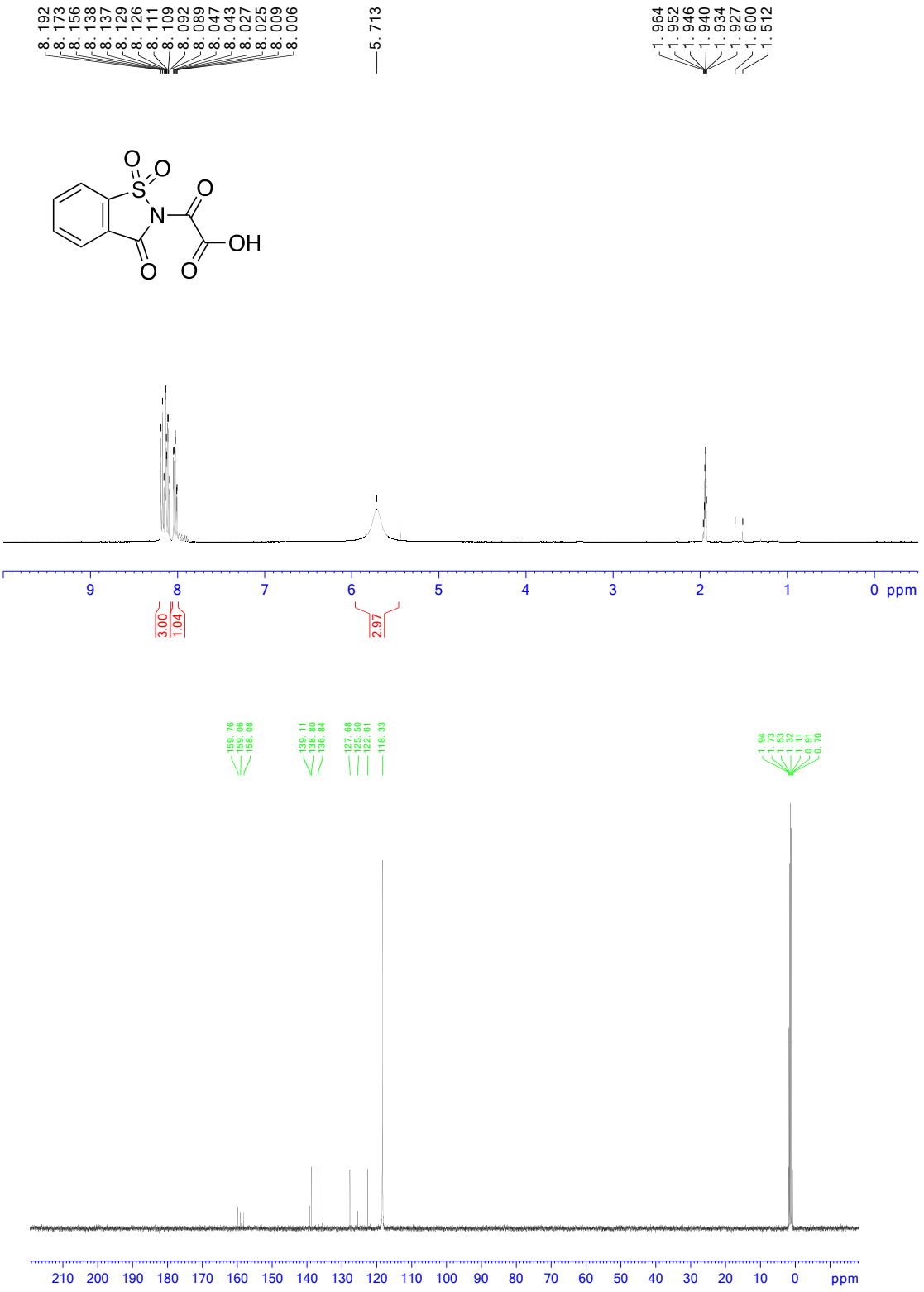

167.95
160.80
156.43
118.31
104.41
20.35
1.94
1.73
1.52
1.32
1.11
0.90
0.70
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Compound 1:


8.174
8.155
8.063
8.060
8.043
8.025
8.023
8.003
7.987
7.986
7.980
7.977
7.961
7.958
7.943
7.939
7.260
1.617
O
O
S
N
O
O
O
O
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 ppm
1.00
3.05
9.26


157.05
156.83
156.79
138.91
137.26
135.46
126.71
124.47
121.69
87.20
77.47
77.16
76.84
27.92
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 ppm

Compound 2:


8.192
8.173
8.156
8.138
8.137
8.129
8.126
8.111
8.109
8.092
8.089
8.047
8.043
8.027
8.025
8.009
8.006
5.713
1.964
1.952
1.946
1.940
1.934
1.927
1.600
1.512
O
O
S
N
O
OH
O
O
9
8
7
6
5
4
3
2
1
0 ppm
3.00
1.04
2.97
159.76
159.06
158.08
139.11
138.80
136.84
127.68
125.50
122.61
118.33
1.94
1.73
1.53
1.32
1.11
0.91
0.70
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
ppm

References:

1. T. Kitagawa, H. Kuroda, K. Iida, M. Ito and M. Nakamura, *Chem. Pharm. Bull.*, 1989, **37**, 3225.
2. C. Cianciolo Cosentino, N. I. Skrypnyk, L. L. Brilli, T. Chiba, T. Novitskaya, C. Woods, J. West, V. N. Korotchenko, L. McDermott, B. W. Day, A. J. Davidson, R. C. Harris, M. P. de Caestecker and N. A. Hukriede, *J. Am. Soc. Nephrol.*, 2013, **24**, 943.