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Supporting Information

A Boronic Acid Conjugate of Angiogenin that Shows ROS-Responsive Neuroprotective Activity

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Experimental Procedures

Materials. All chemicals and reagents were from Sigma–Aldrich (St. Louis, MO) unless indicated otherwise, and were used without further purification. All glassware was flame-dried, and all synthetic chemical reactions were performed under N_2(g). Dichloromethane (DCM), tetrahydrofuran (THF), triethylamine (TEA), and dimethylformamide (DMF) were dried with columns of alumina. Removal of solvents "under reduced pressure" refers to the use of a rotary evaporator with water-aspirator pressure (<20 torr) and a water bath at <40 °C. Flash column chromatography was performed with 40–63 Å silica (230–400 mesh) from Silicycle (Québec City, Canada); thin-layer chromatography (TLC) was performed with EMD Millipore 250-μm silica gel 60 F_{254} plates from Sigma–Aldrich. Poly(cytidylic acid) was from Sigma–Aldrich; 6-FAM–dArUdAdA–6-TAMRA was from IDT (Coralville, IA).

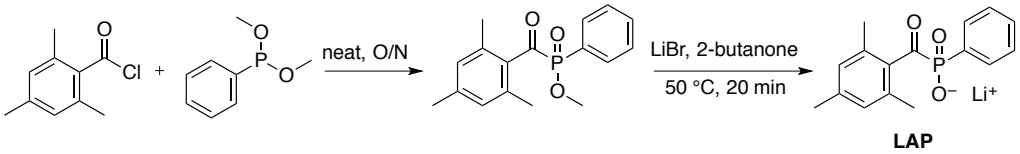
Conditions. All procedures were performed in air at ambient temperature (~22 °C) and pressure (1.0 atm) unless indicated otherwise.

Instrumentation. ^{1}H and ^{13}C NMR spectra were acquired with a Bruker Avance III 500i spectrometer at the National Magnetic Resonance Facility at Madison and referenced to residual protic solvent. Mass spectrometry was performed with a Micromass LCT (electrospray ionization, ESI) instrument at the Mass Spectrometry Facility in the Department of Chemistry at the University of Wisconsin–Madison. Fluorescence and absorbance measurements were made with an M1000 fluorescence plate reader from Tecan (Männedorf, Switzerland).

Statistics. Statistical analyses were performed with Prism 5 from GraphPad Software (La Jolla, CA). All data are the mean ± standard error (SE).

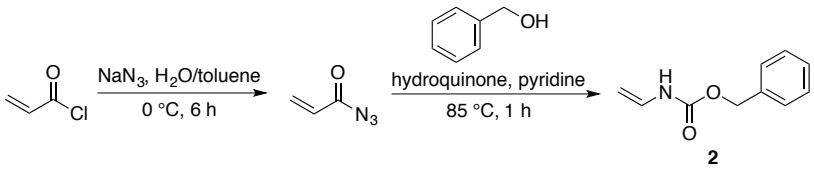
Cell culture. Human cells were from Lonza (Walkersville, MD) and were maintained according to recommended procedures. Medium and added components, trypsin (0.25% w/v), and Dulbecco's phosphate-buffered saline (PBS) were the Gibco® brand from Thermo Fisher Scientific (Waltham, MA). Cells were grown in flat-bottomed culture flasks in a cell-culture

incubator at 37 °C under CO_2(g) (5% v/v). Human umbilical vein endothelial cells (HUVEC) were grown in EGM™-2; human astrocytes were grown in AGM™. The Corning 96-well microplates used in experiments were from Sigma–Aldrich.



LAP

Synthesis of LAP. The water-soluble photoactivatable radical initiator, lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP), was synthesized as reported previously.[1] Briefly, dimethyl phenylphosphonite (0.186 mL, 1.17 mmol) was added to neat 2,4,6-trimethylbenzoyl chloride, and the reaction mixture was stirred overnight. A solution of LiBr (0.406 g, 4.68 mmol) in 2-butanone (10 mL) was added to the reaction mixture, which was then heated to 50 °C for 20 min as a white precipitate formed. The reaction mixture was cooled and filtered to afford LAP as a white solid (0.325 g, 97%). **^{1}H NMR** (500 MHz, D_2O, δ): 1.97 (s, 6H), 2.26 (s, 3H), 6.81 (s, 2H), 7.39–7.42 (t, J = 7.65 Hz, 2H), 7.48–7.51 (t, J = 7.20 Hz, 1H), 7.66–7.68 (d, J = 11.25 Hz, 1H), 7.67–7.70 (d, J = 11.23 Hz, 1H); **^{13}C NMR** (125 MHz, D_2O, δ): 21.16, 22.77, 130.72, 130.97, 131.07, 134.70, 134.73, 134.75, 134.83, 134.91, 135.77, 136.31, 140.26, 140.57, 142.53; **HRMS–ESI** (m/z): $[M - H]^-$ calcd for $C_{16}H_{16}O_3P$, 287.0843; found, 287.0841.



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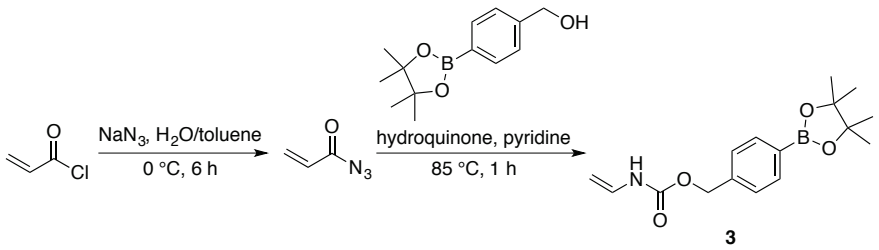
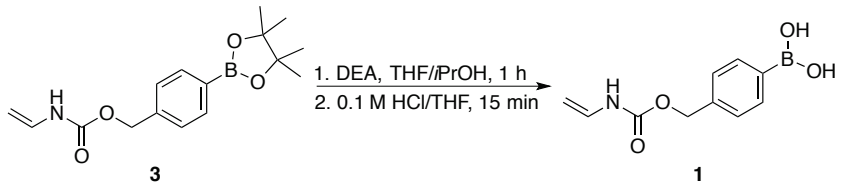
Synthesis of compound 2. Carbamate **2** was accessed via a Curtius rearrangement as reported previously.[2] Briefly, sodium azide (0.430 g, 5.52 mmol) was dissolved in water (3.75 mL), and the resulting solution was cooled to 0 °C. A solution of acryloyl chloride (0.500 mL, 5.52 mmol) in toluene (2.25 mL) was then added dropwise to the reaction mixture, which was stirred at 0 °C for 6 h. The layers were separated quickly, and the organic layer was washed with Na_2CO_3 (2 × 3 mL) and dried with $MgSO_4$(s). The reaction mixture was then filtered and added dropwise to a stirring solution of benzyl alcohol (0.776 g, 7.17 mmol), pyridine (0.218 g, 2.76 mmol), and hydroquinone (0.031 g, 0.286 mmol) at 85 °C. The reaction mixture was stirred at 85 °C for 1 h. The reaction mixture was then washed with saturated aqueous Na_2CO_3 and dried with Na_2SO_4(s). The solvent was removed under reduced pressure, and the crude product was purified with by flash chromatography (30% v/v EtOAc in hexanes) to afford compound **2** as a white solid (0.610 g, 62% yield). **^{1}H NMR** (500 MHz, $CDCl_3$, δ): 4.30–4.31 (d, J = 8.78 Hz, 1 H), 4.47–4.50 (d, J = 15.83 Hz, 1H), 5.15 (s, 2H), 6.44 (bs, 1 H), 6.68–6.76 (m, 1 H), 7.26–7.38 (m, 5H); **^{13}C NMR** (125 MHz, $CDCl_3$, δ): 67.35, 93.51, 128.42, 128.54, 128.75, 129.88, 135.98, 153.55; **HRMS–ESI** (m/z): $[M + H]^+$ calcd for $C_{10}H_{11}NO_2$, 178.0863; found, 178.0860.



Synthesis of compound 3. Pinacol-protected boronated carbamate **3** was accessed in the same manner as was carbamate **2**. Briefly, sodium azide (0.430 g, 5.52 mmol) was dissolved in water (3.75 mL), and the resulting solution was cooled to 0 °C. A solution of acryloyl chloride (0.500 mL, 5.52 mmol) in toluene (2.25 mL) was added dropwise, and the reaction mixture was stirred at 0 °C for 6 h. The layers were separated quickly, and the organic layer was washed with Na_2CO_3 (2 × 3 mL) and dried with $MgSO_4$(s). The reaction mixture was then filtered and added dropwise to a stirring solution of 4-(hydroxymethyl)phenylboronic acid pinacol ester (1.60 g, 7.17 mmol), pyridine (0.218 g, 2.76 mmol), and hydroquinone (0.031 g, 0.286 mmol) at 85 °C. The reaction mixture was stirred at 85 °C for 1 h. The reaction mixture was then washed with saturated aqueous Na_2CO_3 and dried with Na_2SO_4(s). The solvent was removed under reduced pressure, and the crude product was purified by flash chromatography (30% v/v EtOAc in hexanes) to afford compound **3** as a white solid (0.742 g, 44% yield). **^{1}H NMR** (500 MHz, $CDCl_3$, δ): 1.34 (s, 12 H), 4.30–4.32 (d, J = 8.76 Hz, 1 H), 4.47–4.50 (d, J = 15.72 Hz, 1H), 5.16 (s, 2H), 6.46 (bs, 1 H), 6.68–6.75 (m, 1 H), 7.35–7.36 (d, J = 7.67 Hz, 2 H), 7.80–7.82 (d, J = 7.77 Hz, 2H); **^{13}C NMR** (125 MHz, $CDCl_3$, δ): 24.84, 67.02, 83.87, 93.40, 127.24, 129.71, 135.02, 138.83, 153.33; **HRMS–ESI** (m/z): calcd for $C_{16}H_{22}BNO_4$, 320.2016; found, 320.2014.



Synthesis of compound 1. Boronated carbamate **1** was prepared from pinacol-protected boronated carbamate **3** by using a procedure modified from one reported previously.[3] Compound **3** (0.050 g, 0.164 mmol) was dissolved in a 4:1 THF/*i*PrOH (1.60 mL). Diethanolamine (0.019 g, 0.180 mmol) was added dropwise, and the reaction mixture was stirred for 1 h until formation of a white precipitate. The reaction mixture was then filtered, and the precipitate was collected and resuspended in 1.6 mL of 1:4 0.1 M HCl/THF. The reaction mixture was stirred for 10 min, and reaction progress was monitored with TLC. (Note: significant product degradation was observed at longer reaction times.) The reaction mixture was then diluted with water, and the organic solvent was removed under reduced pressure until a white precipitate formed. The resulting precipitate was filtered and washed with chloroform to afford compound **1** as a white solid (0.026 g, 73% yield). **^{1}H NMR** (500 MHz, CD_3OD, δ): 4.23–4.24 (d, J = 8.87 Hz, 1H), 4.53–5.56 (d, J = 15.88 Hz, 1H), 5.13 (s, 2H), 6.62–6.67 (dd, J = 15.90, 8.92 Hz, 1H), 7.35–7.37 (d, J = 7.65 Hz, 2H), 7.60–7.62 (d, J = 7.60 Hz, 2H); **^{13}C NMR** (125 MHz, CD_3OD, δ): 67.62, 93.77, 127.96, 131.48, 134.72, 139.20, 156.14; **HRMS–ESI**

(*m*/*z*): $[M + NH_4]^+$ calcd for the single methyl boronic ester $C_{11}H_{14}BNO_4$, 252.1390; found, 252.1390.

Preparation of ANG, its variants, and their conjugates. ANG and its Q117, K40C, and K40C/Q117G variants were produced in *Escherichia coli* by recombinant DNA methods. These methods used a synthetic gene that encodes ANG with an inconsequential N-terminal methionine residue[4] This gene was synthesized from synthetic DNA oligonucleotides with the Gibson Assembly® cloning kit from New England Biolabs (Ipswich, MA) and inserted into the pET-22b(+) expression vector from Addgene (Cambridge, MA).

```
5' atg cag gac aac tct cgt tac acc cac ttc ctg acc cag cac tac gac gcg
    M   Q   D   N   S   R   Y   T   H   F   L   T   Q   H   Y   D   A
   aaa ccg cag ggt cgt gac gac cgt tac tgc gaa tcg atc atg cgt cgc cgt
    K   P   Q   G   R   D   D   R   Y   C   E   S   I   M   R   R   R
   ggt ctg acg tct ccg tgc aaa gac atc aac acc ttc atc cat ggt aac aaa
    G   L   T   S   P   C   K   D   I   N   T   F   I   H   G   N   K
   cgt tct atc aaa gcg atc tgc gaa aac aaa aac ggt aac ccg cac cgt gaa
    R   S   I   K   A   I   C   E   N   K   N   G   N   P   H   R   E
   aac ctg cgt atc tct aaa tct tct ttc cag gtt act acc tgt aag ctt cac
    N   L   R   I   S   K   S   S   F   Q   V   T   T   C   K   L   H
   ggt ggt tct cca tgg cct cct tgt cag tat cgt gct acc gcg ggt ttc cgt
    G   G   S   P   W   P   P   C   Q   Y   R   A   T   A   G   F   R
   aac gtt gta gtt gcg tgc gaa aac ggt ctg ccg gtt cac ctg gac cag tct
    N   V   V   V   A   C   E   N   G   L   P   V   H   L   D   Q   S
   atc ttc cgt cgt ccg taa 3'
    I   F   R   R   P   —
```

The K40C and Q117G substitutions (red) in the ANG gene were made by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit from Agilent Technologies (Santa Clara, CA) and the following synthetic DNA oligonucleotides.

K40C substitution

Forward primer: 5' ACG TCT CCG TGC TGT GAC ATC AAC ACC 3'
Reverse primer: 5' GGT GTT GAT GTC ACA GCA CGG AGA CGT 3'

Q117G substitution

Forward primer: 5' GGT TCA CCT GGA CGG GTC TAT CTT CCG 3'
Reverse primer: 5' CGG AAG ATA GAC CCG TCC AGG TGA ACC 3'

ANG and its Q117, K40C, and K40C/Q117G were purified as described previously.[4]

The B-thiaK40 ANG, P-thiaK40 ANG, B-thiaK40/Q117G ANG, and P-thiaK40/Q117G ANG conjugates were prepared by reaction of K40C ANG or K40C/Q117G ANG (9 mg) with a 50-fold molar excess of compound **1** in 10% v/v DMSO or compound **2** in 10% v/v acetonitrile, and a 10-fold molar excess of LAP in 50 mM HEPES–KOH buffer, pH 8.0, containing reduced glutathione (20 mM). Samples were placed in a XL-1500 UV Spectrolinker from Spectronics (Westbury, NY) and irradiated with 365-nm light for 30 min. Conjugates were purified by chromatography using a HiTrap SP HP cation-exchange column (GE Healthcare). The molecular mass of each ANG conjugate was confirmed by LC–MS/MS. Protein concentration was

determined by using a bicinchoninic acid assay kit from Pierce Chemical (Rockford, IL). This procedure typically afforded 2–3 mg of conjugate.

Unmasking of ANG conjugates *in vitro*. P-thiaK40 ANG, B-thiaK40 ANG, and their Q117G variants (3.0 mg/mL) were incubated with H_2O_2 (1.0 mM) for 3 h at 37 °C, followed by dialysis against PBS to remove excess H_2O_2. The unmasked proteins were then characterized by LC-MS/MS, and used for assays of enzymatic activity *in vitro* and effects on cell proliferation.

Zymogram assay of ribonucleolytic activity. Solutions of wild-type ANG, its variants, and that conjugates (40 ng) were diluted 1:1 with 2× Laemmli buffer from Bio-Rad (Hercules, CA), and the resulting solutions were loaded on to a polyacrylamide gel (15% w/v) containing poly(cytidylic acid). The loaded gel was subjected to electrophoresis for 1.5 h at 100 V. Subsequent washing, refolding and staining with toluidine blue were performed as described previously.[5] Zymograms were quantified by scanning the stained gel with an ImageQuantLAS4000 instrument from GE Healthcare Bio-Sciences (Pittsburgh, PA) and using the program ImageJ from the National Institutes of Health (Bethesda, MD).

Fluorescence-based assay of ribonucleolytic activity. The ribonucleolytic activity of wild-type ANG, its variants, and their conjugates (100 nM) was determined by measuring the initial velocity of cleavage of 6-FAM–dArUdAdA–6-TAMRA[6] (200 nM) in 100 mM Tris–HCl buffer, pH 7.5, containing NaCl (100 mM). Assays were performed in the wells of a 96-well plate.

Assay of endothelial cell proliferation. HUVEC cells in EGM™-2 were plated at 5,000 cells per well in a 96-well plate. After 24 h, cells were transferred to EBM-2 containing wild-type ANG, its variants, and their conjugates (1 μg/mL). In some experiments, the conjugates were incubated with H_2O_2 (1.0 mM) for 3 h at 37 °C, followed by dialysis against PBS to remove excess H_2O_2. At known times, growth medium was removed and cells were incubated with a solution of CyQUANT® NF fluorescent dye from Invitrogen (Carlsbad, CA). Fluorescence intensity was measured with excitation at 485 nm and emission detection at 530 nm, and is reported as cell proliferation in arbitrary units (AU).

Measurement of intracellular ROS levels. The ROS levels within untreated, PMA-treated, and H_2O_2-treated cells were measured by staining with chloromethyl-2′,7′-dichlorofluorescein diacetate (CM–DCFDA). Human astrocytes were counted by using a CyQUANT® NF assay from Thermo Fischer Scientific. Astrocytes were plated at 10,000 cells per well in a 96-well plate. After 24 h, the astrocytes were treated with various concentrations of PMA or H_2O_2 for 1 h before incubating with CM–DCFDA for ROS analysis. Fluorescence intensity was measured with excitation at 492 nm and emission detection at 520 nm, and is reported in arbitrary units (AU).

Assay of astrocyte survival. Human astrocytes were plated at 10,000 cells per well in a 96-well plate. Wild-type ANG, its variants, their conjugates, and vehicle were added to the cells and incubated for 24 h. Cells were washed with AGM™ medium and treated with various concentrations of PMA or H_2O_2. After 24 h, the medium was removed, and cells were incubated with CellTiter 96 MTS reagent from Promega (Madison, WI) for 3 h before detection.

References

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[2] A. Kamatani, L. E. Overman, *J. Org. Chem.* **1999**, *64*, 8743–8744.
[3] J. Sun, M. T. Perfetti, W. L. Santos, *J. Org. Chem.* **2011**, *76*, 3571–3573.
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[5] J. Bravo, E. Fernández, M. Ribó, R. de Llorens, C. M. Cuchillo, *Anal. Biochem.* **1994**, *219*, 82–86.
[6] B. R. Kelemen, T. A. Klink, M. A. Behlke, S. R. Eubanks, P. A. Leland, R. T. Raines, *Nucleic Acids Res.* **1999**, *27*, 3696–3701.

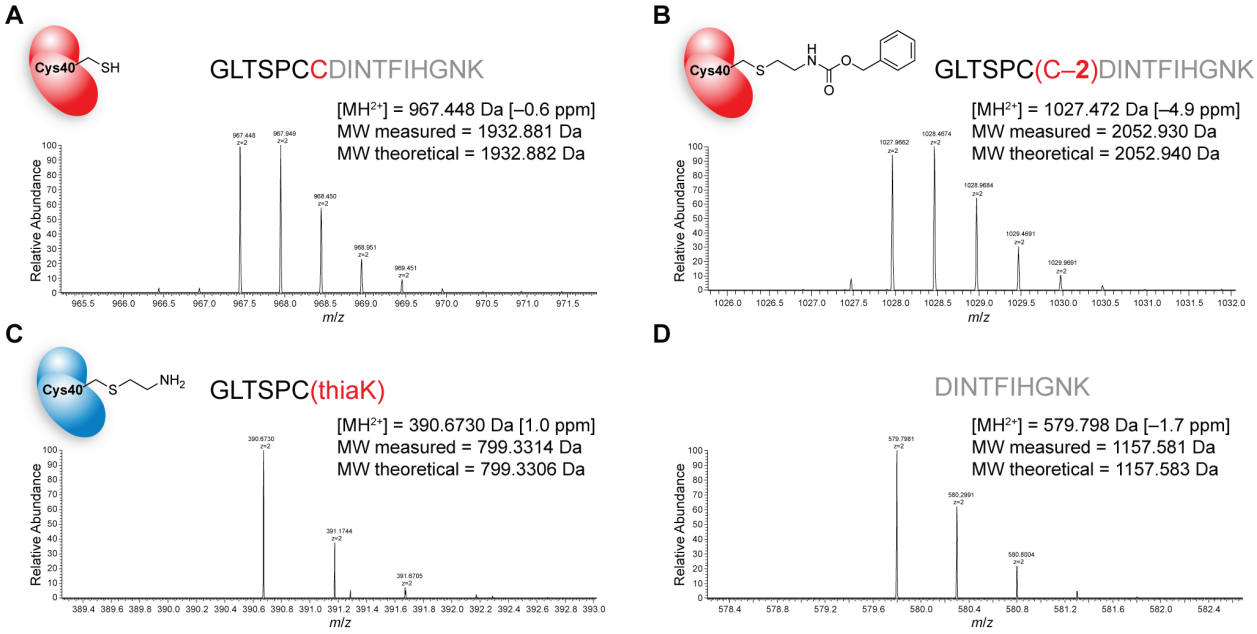


Figure S1. MS/MS mass spectra of trypsin digests. (A) K40C ANG, (B) P-thiaK40 ANG variant after treatment with H_2O_2, and (C,D) B-thiaK40 ANG variant after treatment with H_2O_2. The ensuing γ-thialysine residue is cleaved by trypsin, generating two fragments.

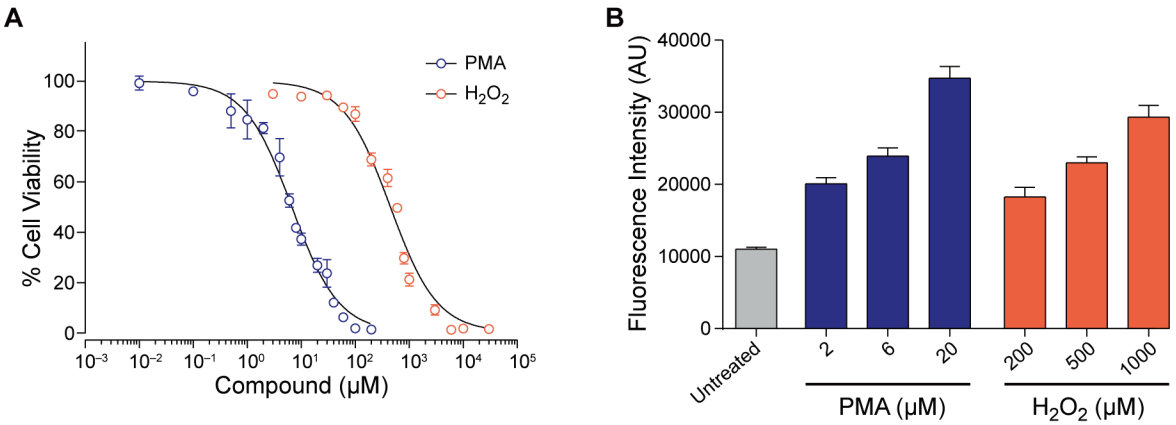


Figure S2. H_2O_2 and PMA provoke an increase in intracellular ROS in human astrocytes. (A) Human astrocytes were treated with PMA and H_2O_2 at increasing concentrations for 24 h, after which the toxicity of these agents was evaluated by using an MTS assay. (B) Accumulation of intracellular ROS in human astrocytes was dose dependent. Cells were treated with PMA and H_2O_2 at three doses for 1 h, after which the ROS concentration was measured by staining with CM–DCFDA. Values are the mean ± SE (n = 3, technical replicates).

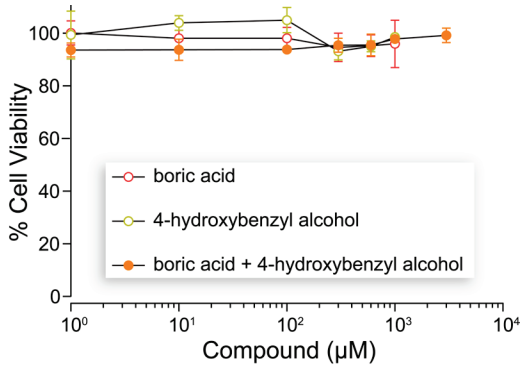
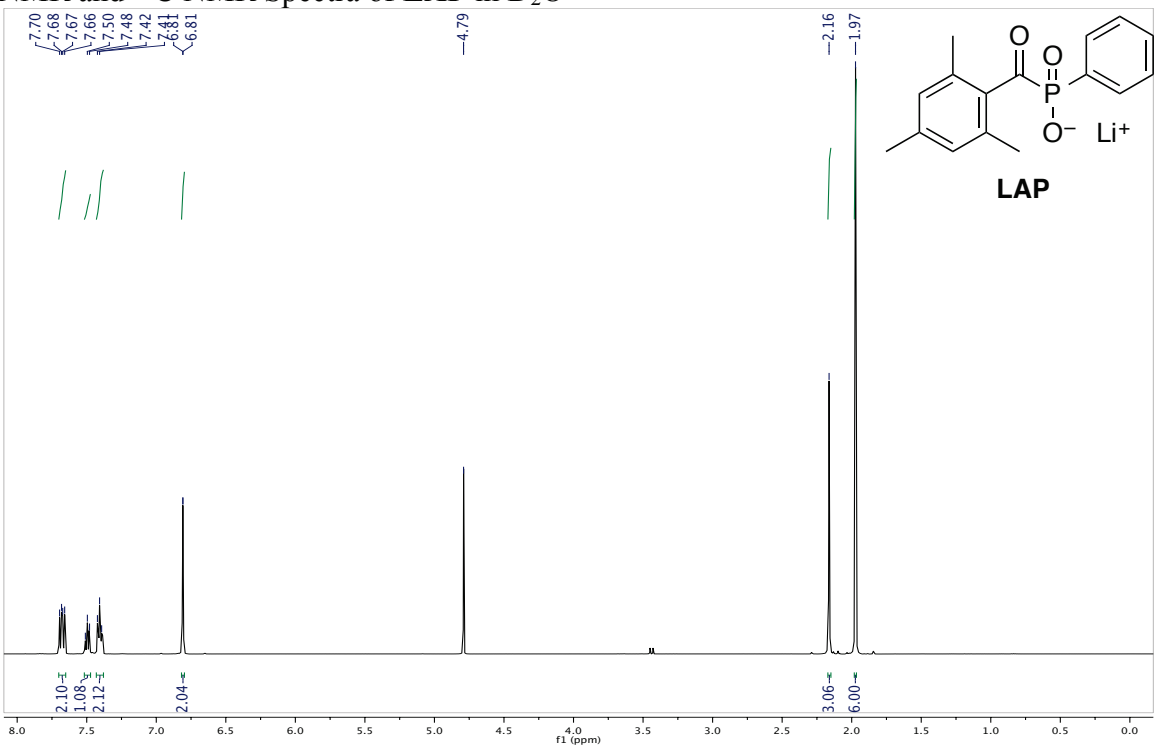
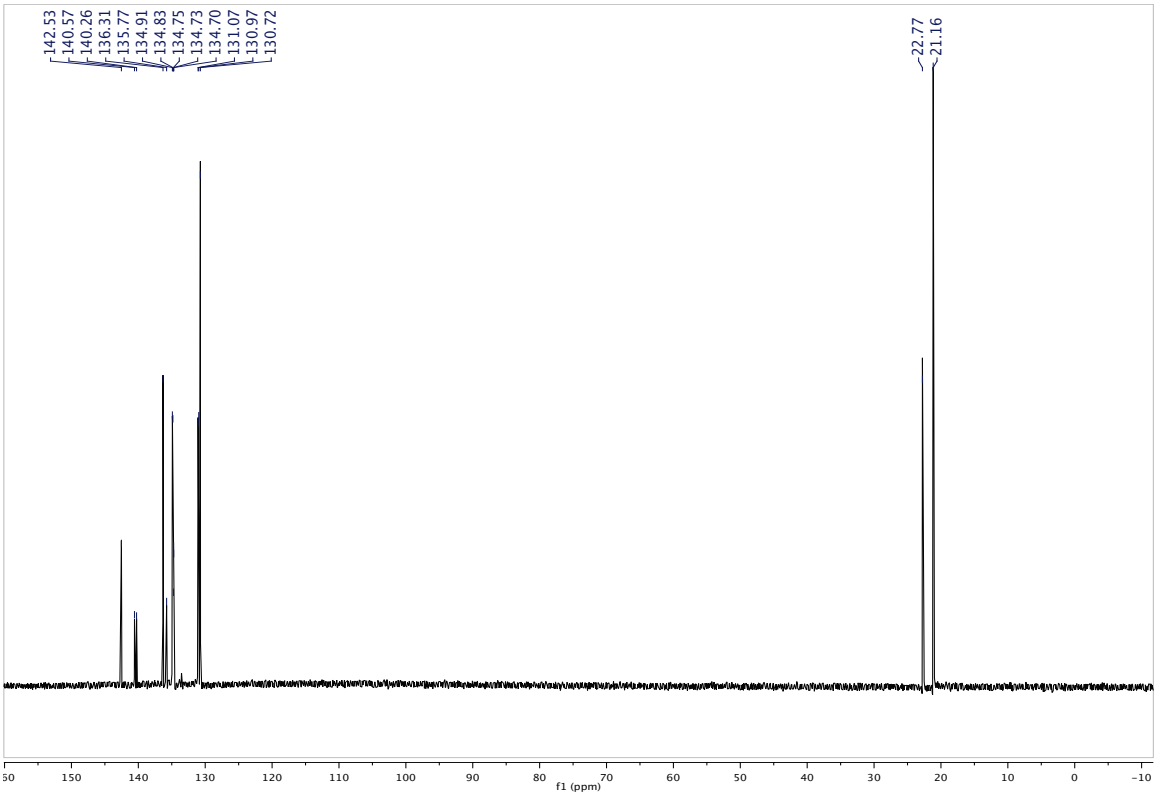


Figure S3. Byproducts of the unmasking of B-thiaK40 ANG and B-thiaK40/Q117G ANG and are not toxic to human astrocytes. Graph of cell viability indicating that treating human astrocytes with boric acid or 4-hydroxybenzyl alcohol (or both) for 48 h results in no cytotoxicity, even at millimolar concentrations. Values are the mean ± SE (n = 3, biological replicates).

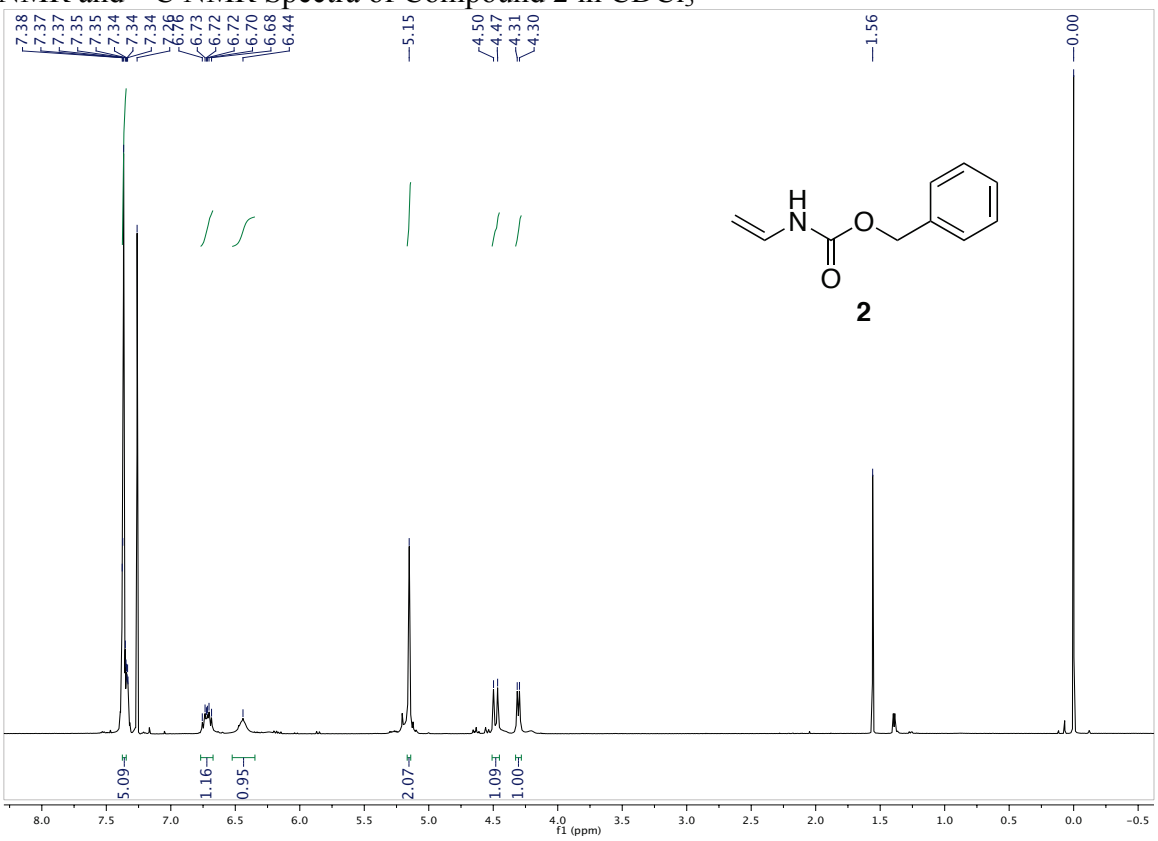
NMR Spectra

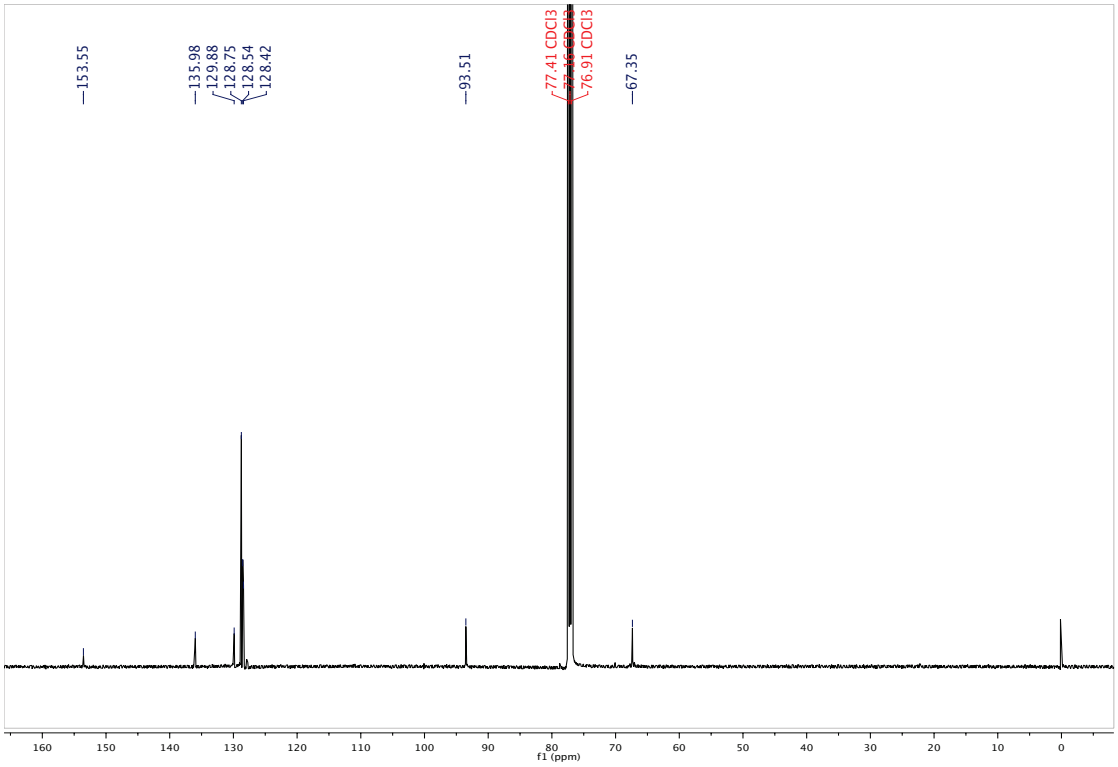
^{1}H NMR and ^{13}C NMR Spectra of **LAP** in D_2O



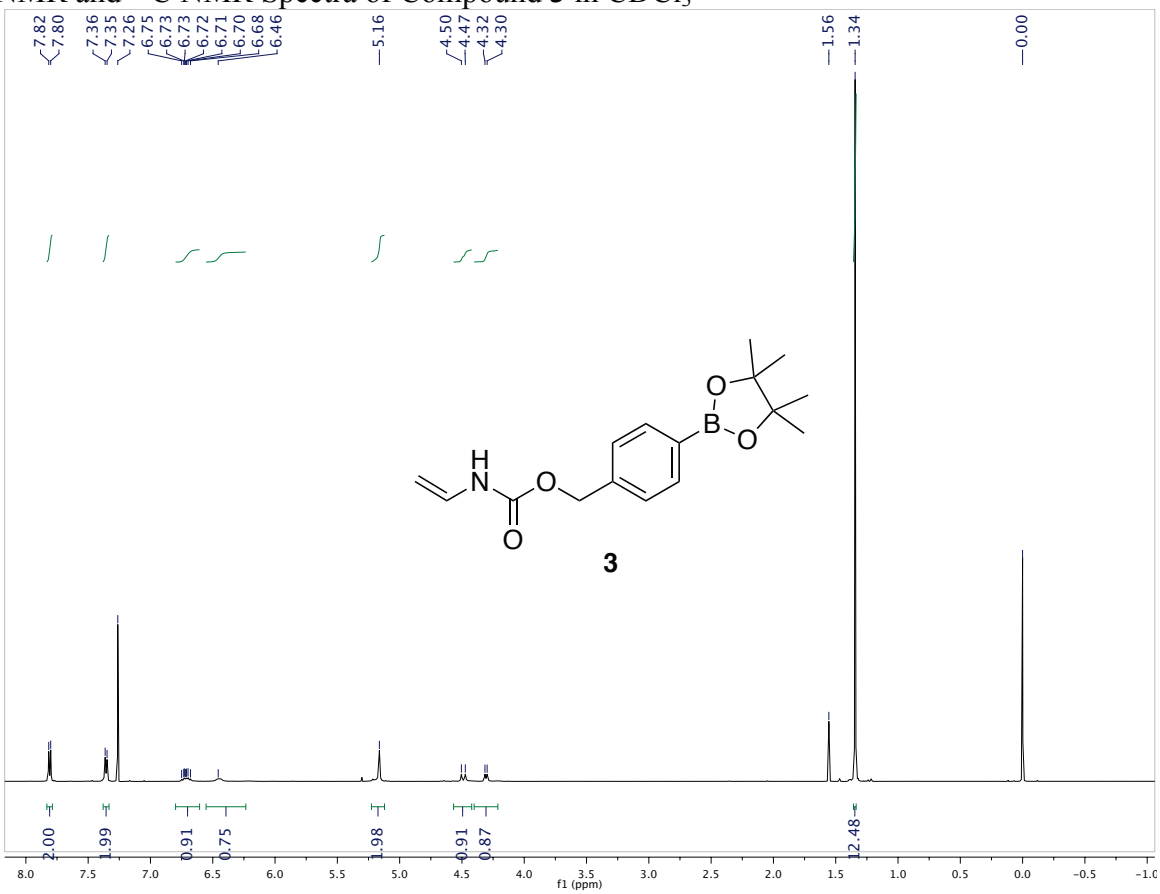


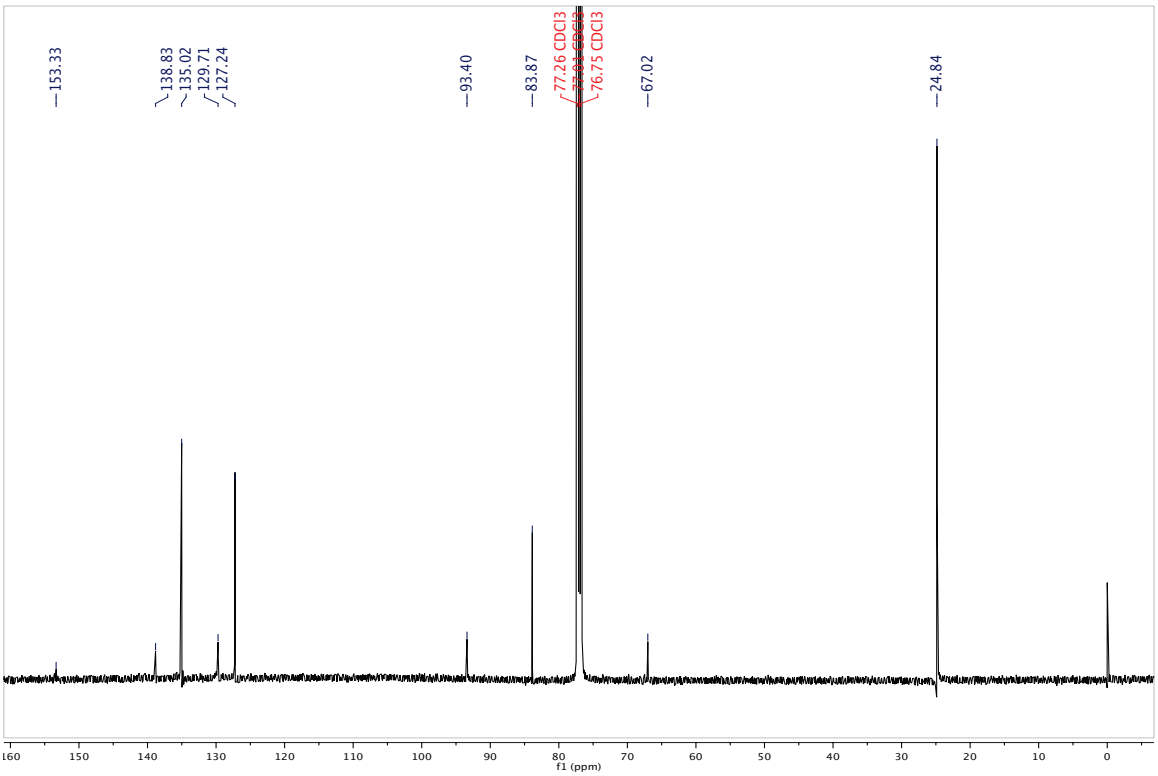
^{1}H NMR and ^{13}C NMR Spectra of Compound **2** in $CDCl_3$





^{1}H NMR and ^{13}C NMR Spectra of Compound **3** in $CDCl_3$





^{1}H NMR and ^{13}C NMR of Compound **1** in CD_3OD