Electronic Supporting Information

for

Reactive Oxygen Species (ROS)-Activatable Prodrug for Selective Activation of ATF6 After Ischemia-Reperfusion Injury

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General Methods

All air- and moisture-sensitive reactions were carried out in glassware that was oven-dried (>130°C) and cooled under nitrogen (N_2) gas. Reaction vessels were sealed with rubber septa and maintained in an inert environment under a positive pressure of anhydrous N_2. Stirring was accomplished via magnetic, Teflon-coated stir bars. Solid reagents were measured on a Mettler Toledo MS204TS balance. Air- and moisture-sensitive liquids were transferred via syringe under an atmosphere of N_2. Reaction temperatures refer to the bath temperature in which the reaction vessel was partially immersed. Room temperature indicates an external temperature of 20-25°C. Elevated temperatures were achieved by the use of a mineral oil bath heated by a VWR 620-HPS hot plate/stirrer. Temperatures of 0°C were maintained with ice/water mixtures. The term *in vacuo* refers to the use of a rotary evaporator with an attached vacuum membrane pump. Residual solvents were removed using a Welch vacuum pump held at <1.0 Torr. Analytical thin layer chromatography (TLC) was performed using 0.20 mm glass-backed silica gel 60F254-coated plates from Sigma Aldrich and monitored at 254 nm. Chromatographic compound separations were done by column chromatography, either by hand or with a Teledyne CombiFlash Chromatography system.

Materials

Unless otherwise noted, all commercial solvents and reagents were purchased from Millipore-Sigma USA and used as received. Anhydrous solvents including *N,N*-dimethylformamide (DMF, anhydrous), methylene chloride (CH_2Cl_2, anhydrous, 99.8%), tetrahydrofuran (THF, anhydrous, 99.8%) and dioxane (anhydrous, 99.8%) were also purchased from Millipore-Sigma USA and used as received. Cytrochome P450 Baculsomes Plus Reagents (CYP1A2 and Control) were purchased from ThermoFisher Scientific. Hydrogen peroxide solutions were standardized by titration with potassium permanganate prior to experimentation.[1] Water was obtained from a Barnstead Nanopure Infinity water system (18 MΩ cm). Silica gel was purchased from Silicycle, Inc. Deuterated solvents were purchased from Millipore-Sigma USA ($CDCl_3$ with 0.03% v/v TMS, acetone-d6).

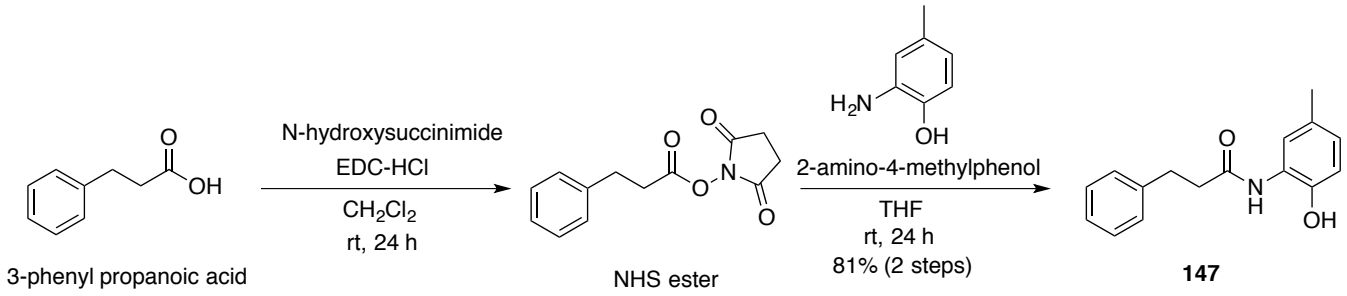
Physical and Spectroscopic Measurements

Nuclear magnetic resonance (NMR) spectra were collected on a Varian Inova 500 (^{1}H at 500 MHz, ^{13}C at 125 MHz) magnetic resonance spectrometer. Data for ^{1}H NMR spectra are reported as follows: chemical shift, multiplicity (bs = broad singlet, s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet and m = multiplet), coupling constant (Hz) and integration. Chemical shifts are reported in ppm (δ). NMR spectra were referenced to residual solvent peaks or trimethylsilane (TMS) additives.

Mass spectra were acquired using an Agilent 6230 TOF LC/MS mass spectrometer with an electrospray ion source in the positive ion mode. Liquid chromatography was performed on the Agilent 6230 TOF LC/MS or on a stand-alone Agilent 1260 HPLC system with diode array and fluorescence detection. Separations were performed on C18 columns with acetonitrile:water solvent systems spiked with 0.1% trifluoroacetic acid or formic acid.

Small Molecule Synthesis and Characterization

Synthesis of ***147***



3-phenylpropanoic acid (100.0 mg, 0.67 mmol), N-hydroxysucinamide (115.1 mg, 1.0 mmol) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl, 191.7 mg, 1.0 mmol) were dissolved in 2.5 mL CH_2Cl_2 and stirred for 24 hours. The solution was then extracted thrice with 10 mL ethyl acetate and 10 mL H_2O. The aqueous layers were combined and back-extracted twice with 10 mL ethyl acetate. The ethyl acetate layers were combined, washed with brine and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the crude solid NHS ester was taken onto the next step without further purification.

The crude NHS ester and 2-amino-4-methylphenol (90.8 mg, 0.67 mmol) were dissolved in 2.5 mL THF and stirred for 12 hours. The solvent was removed under reduced pressure. The crude solid was purified by column chromatography with silica gel (CH_2Cl_2) and product containing fractions were combined and concentrated *in vacuo* to yield **147** (107.8 mg, 81%) as a yellow-white solid. The compound was chromatographically homogeneous by TLC and analytical HPLC.

Data for 147:

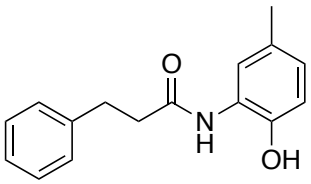
mp = 120°C

TLC: Rf = 0.21 (CH_2Cl_2)

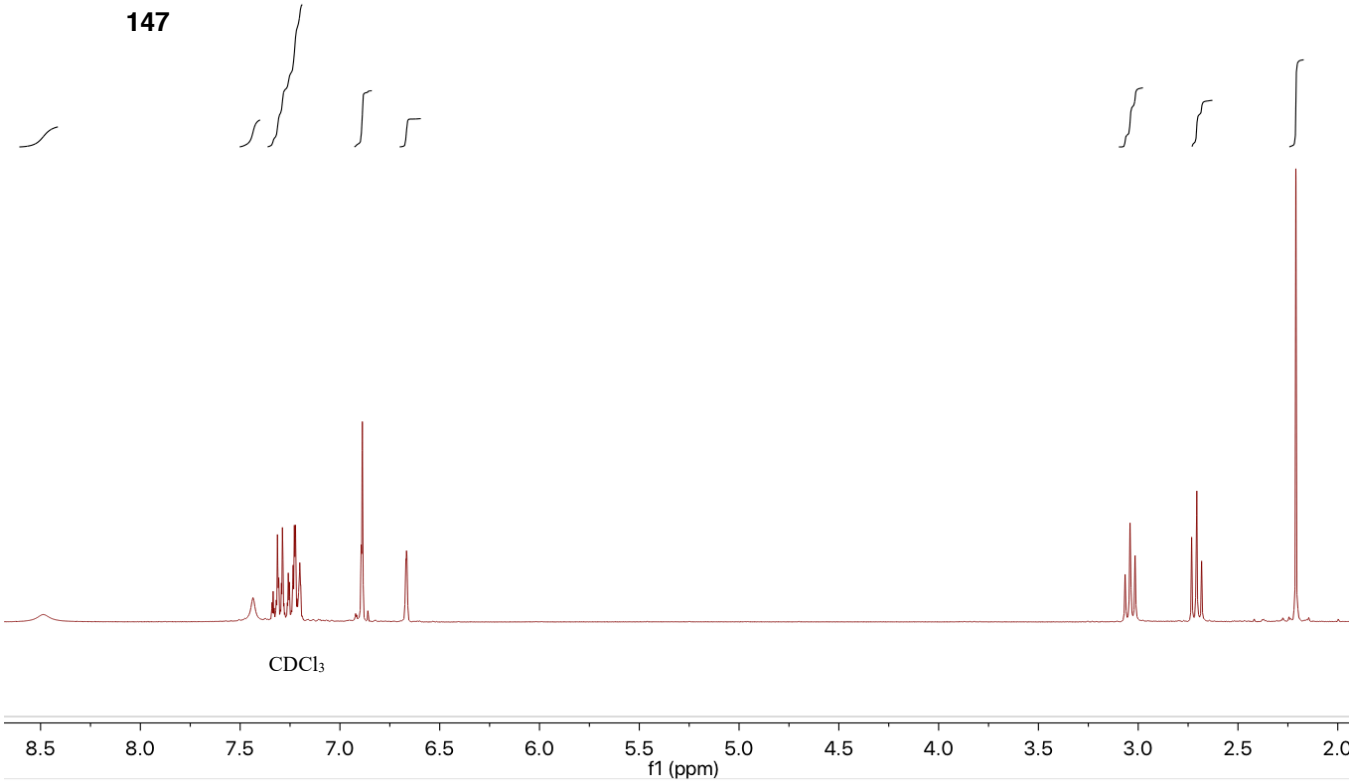
^{1}H NMR (500 MHz, $CDCl_3$): δ 8.49 (s, 1H), 7.44 (s, 1H), 7.31 (m, 2H), 7.23 (m, 3H), 6.89 (t, *J* = 0.6 Hz, 2H), 6.66 (t, *J* = 0.6 Hz, 1H), 3.04 (t, *J* = 7.8 Hz, 2H), 2.71 (t, *J* = 7.2 Hz, 2H), 2.21 (s, 3H) ppm.

^{13}C NMR (500 MHz, $CDCl_3$): δ = 170.11, 144.78, 140.64, 139.59, 135.15, 131.10, 128.54, 128.34, 127.65, 126.63, 126.23, 123.79, 120.54, 111.62, 83.917, 71.01, 39.60, 31.44, 24.87, 21.03 ppm.

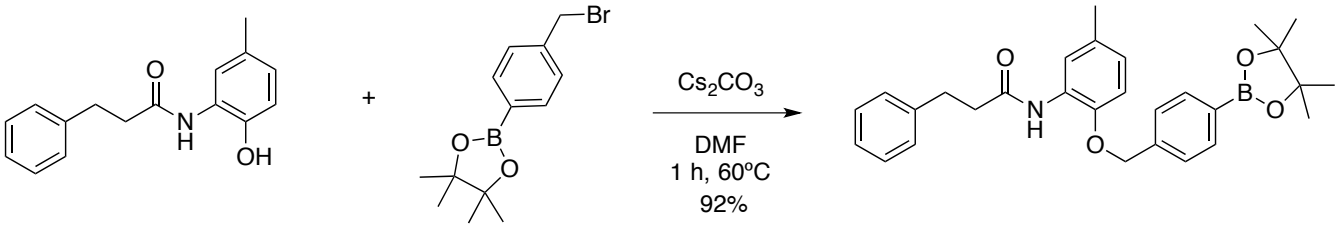
HRMS (m/z): [M+H$^+$] Calculated for $C_{16}H_{17}NO_2$, 256.1332; Found, 256.1333; mass accuracy, 0.39 ppm.



147



*Synthesis of ROS-Activatable Prodrug **1***



147 **3** **1**

147 (32.3 mg, 0.127 mmol) and cesium carbonate (45.5 mg, 0.140 mmol) were dissolved in 2 mL DMF and heated to 60ºC. 4-bromomethylphenylboronic acid pinacol ester **3** (41.3 mg, 0.140 mmol) was added to the solution and stirred for 1 hour. The reaction was quenched with 0.5 mL 10% HCl (w/v) and extracted thrice with 10 mL ethyl acetate and 10 mL H_2O. The aqueous layers were combined and back extracted thrice with 10 mL ethyl acetate. The organic layers were combined, rinsed with brine and dried over Na_2SO_4. The solvent was removed under reduced pressure. The crude solid was purified by column chromatography with silica gel (20% ethyl acetate, hexanes). The product containing fractions were combined and the solvent removed *in vacuo* to yield prodrug **1** (48.4 mg, 92%) as a white solid. The compound was chromatographically homogeneous by TLC and analytical HPLC.

Data for 1:

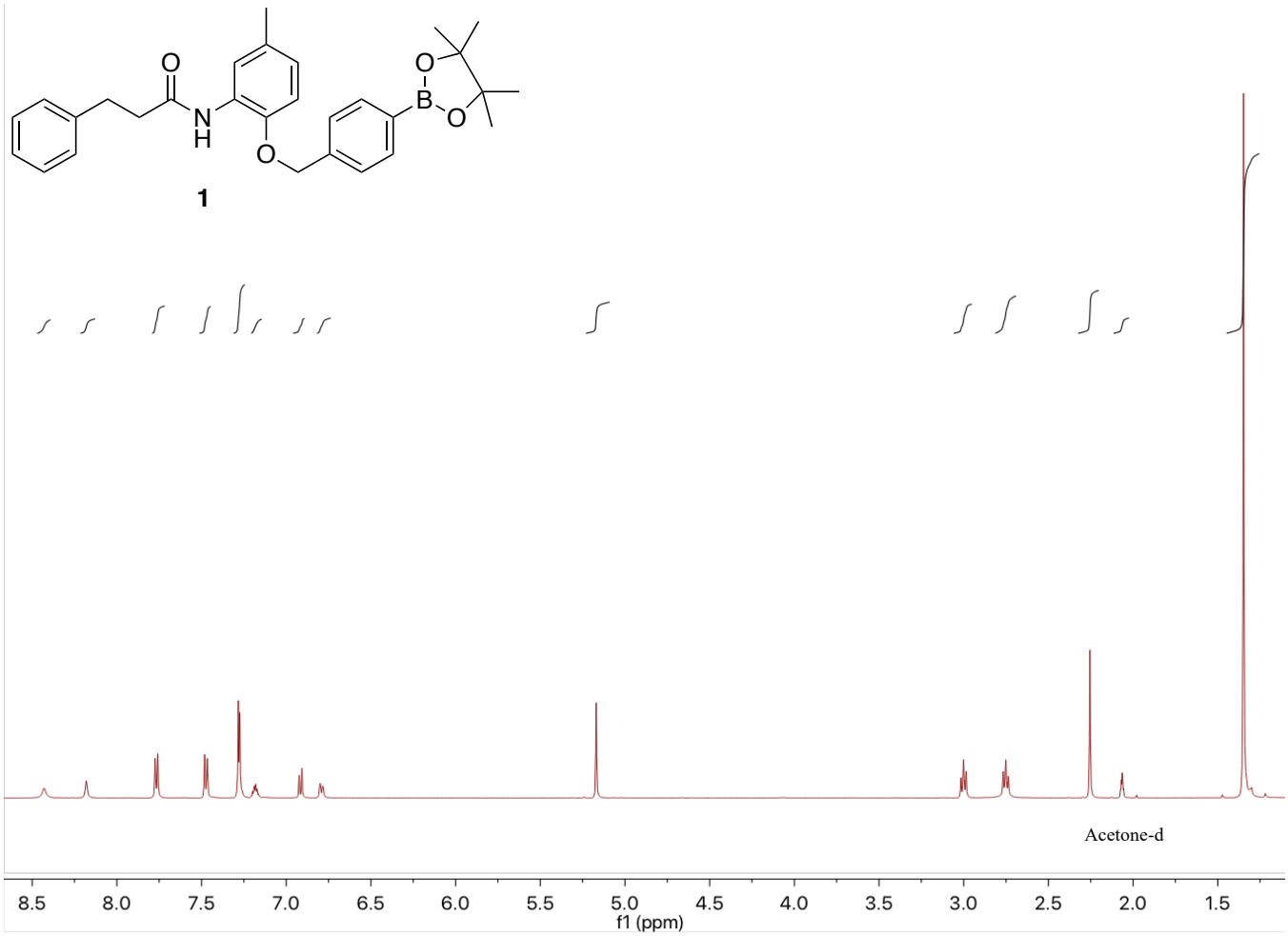
mp = 114°C

TLC: Rf = 0.21 (1:1 Ethyl Acetate:Hexanes)

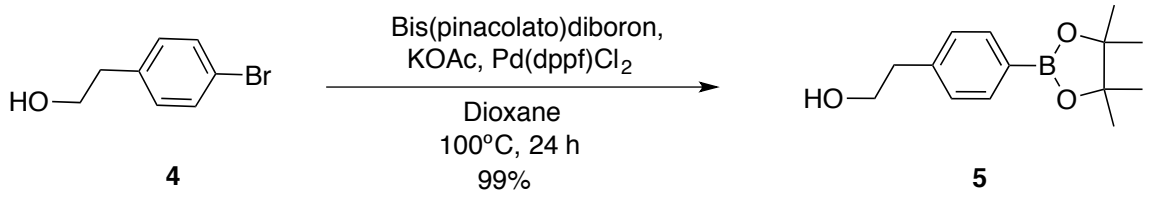
^{1}H NMR (500 MHz, acetone-d6) δ 8.43 (s, 1H), 8.18 (s, 1H), 7.77 (d, *J* = 8 Hz, 2H), 7.47 (d, *J* = 8 Hz, 2H), 7.28 (d, *J* = 4.5 Hz, 4H), 7.18 (m, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 6.79 (d, *J* = 8 Hz, 1H), 5.17 (s, 2H), 3.00 (t, *J* = 7.5 Hz, 2H), 2.75 (t, *J* = 7.5 Hz, 2H), 2.26 (s, 3H), 1.35 (s, 12H) ppm.

^{13}C NMR (500 MHz, $CDCl_3$) δ = 170.11, 144.78, 140.64, 139.59, 135.15, 131.10, 128.54, 128.34, 127.65, 126.63, 126.23, 123.79, 120.54, 111.62, 83.917, 71.01, 39.60, 31.44, 24.87, 21.03 ppm.

HRMS (m/z): [M+Na^+] Calculated for $C_{29}H_{34}BNO_4$, 494.2479; Found, 494.2490; mass accuracy, 2.22 ppm.


O
N
H
O
O
B
O
1
Acetone-d
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
f1 (ppm)

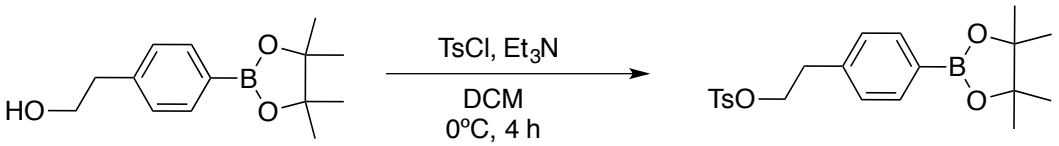
*Synthesis of Boronic Ester Alcohol **5***



4 **5**

Potassium acetate (435.3 mg, 4.437 mmol) was oven-dried in a flask with a condenser for 1 hour. The flask and condenser were cooled to room temperature under N_2. Bis(pinacolato)diboron (454.3 mg, 1.789 mmol) and $Pd(dppf)Cl_2$ (54.6 mg, 0.075 mmol) were added and dissolved with 6 mL anhydrous dioxane. The solution was sparged with N_2 for 20 minutes. 4-bromo-phenylethanol (0.21 mL, 1.491 mmol) was added and the mixture was stirred under reflux at 100ºC for 24 hours. The mixture was cooled to room temperature and filtered over a pad of celite. The solvent was removed under reduced pressure. The crude solid was purified by column chromatography with silica gel (30%-70% ethyl acetate, hexanes) and concentrated *in vacuo* to yield boronic ester alcohol **5** (369.4 mg, 99%) as a clear liquid. The compound was chromatographically homogeneous by TLC and exhibited spectral characterization consistent with literature.[2]

*Synthesis of Boronic Ester Tosylate **6***



5 **6**

2-Benzyl-boronic-ester-ethanol (417.7 mg, 1.68 mmol, 1.0 eq) was dissolved in 8.0 mL of dichloromethane. Triethylamine (341 mg, 3.37 mmol, 2.0 eq) and 4-toluenesulfonyl chloride (481.3 mg, 2.52 mmol 1.5 eq) were added after the solution was cooled to 0°C. The reaction was held at 0°C for 4 hours. The contents of the reaction were extracted 3x with dichloromethane and deionized water, then the aqueous layer was back extracted 3x with the same solvents. Organic layers were combined, washed with brine, and dried over sodium sulfate. The crude product was purified by column chromatography with silica gel (30% ethyl acetate, hexanes) and concentrated *in vacuo* to yield tosylated 2-benzyl-boronic-ester-ethanol **6** (480.8 mg, 71%) as a dark amber, amorphous solid. The compound was chromatographically homogeneous (one spot) by TLC.

Data for 6:

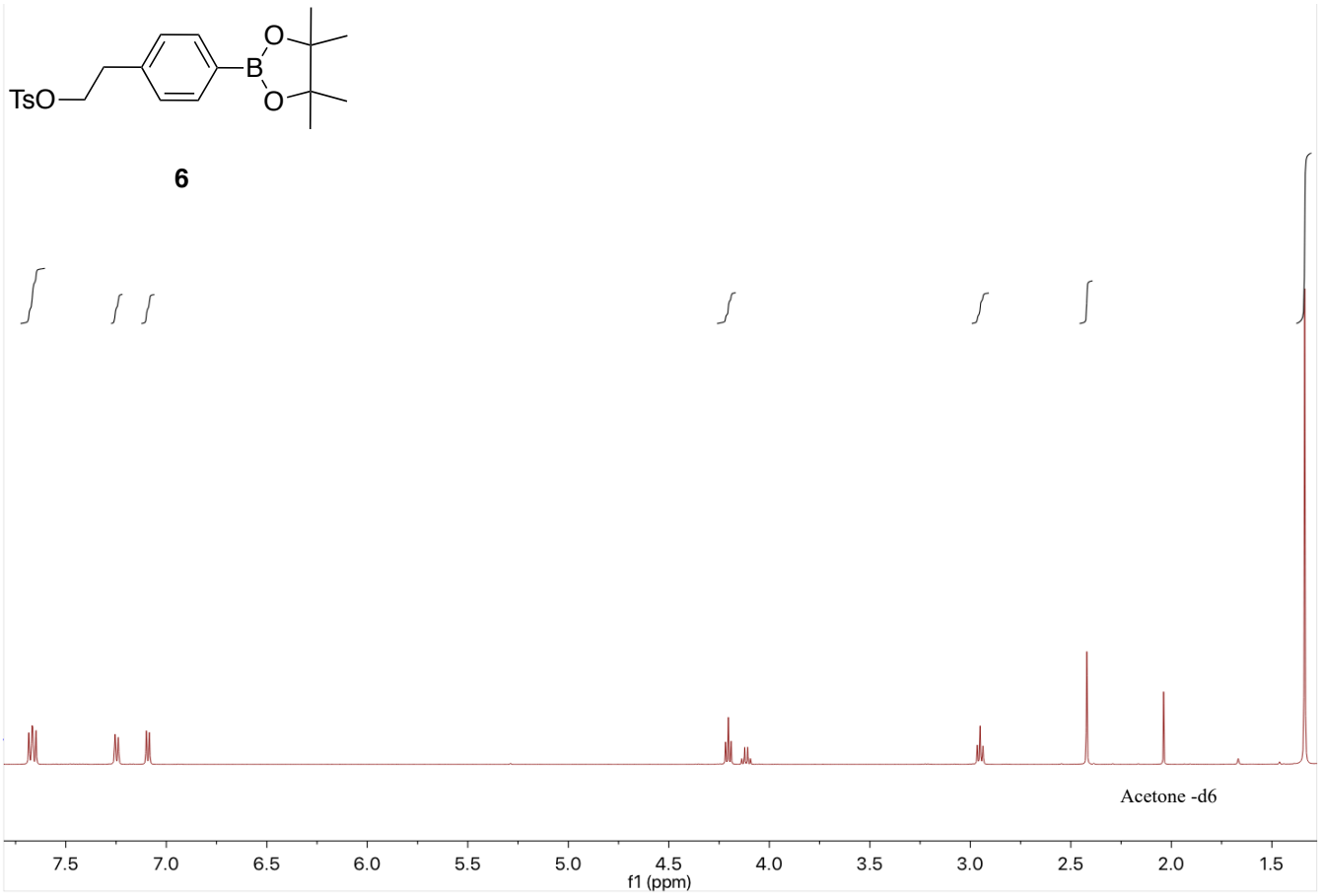
mp: amorphous solid at RT

TLC: Rf = 0.49 (1:4 EtOAc:hexanes)

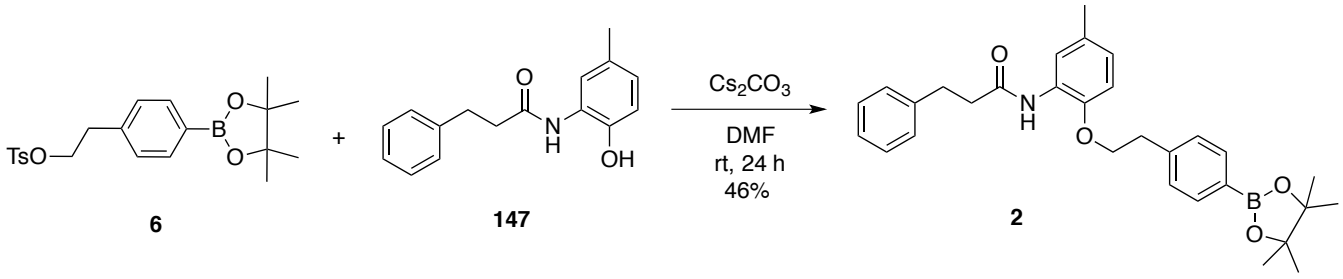
^{1}H NMR (500 MHz, acetone-d6): δ 7.67 (m, 4H), 7.25 (d, J = 8.0, 2H), 7.09 (d, J = 7.5, 2H), 4.21 (t, J = 5.0, 2H), 2.95 (t, J = 7.0, 2H), 2.42 (s, 3H), 1.34 (s, 12H) ppm.

^{13}C NMR (500 MHz, $CDCl_3$): δ 144.62, 142.88, 139.41, 137.40, 135.04, 132.83, 131.59, 130.58, 129.74, 129.57, 128.25, 127.79, 127.02, 83.77, 70.36, 41.98, 35.48, 24.85, 21.61, 14.14 ppm.

HRMS (m/z): [M] Calculated for $C_{21}H_{27}BO_4S$, 402.1672; Found, 402.1696; mass accuracy, 5.97 ppm.


TsO
O
B
O
6
Acetone -d6
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
f1 (ppm)

*Synthesis of Negative Control **2***



Tosylated 2-Benzyl-boronic-ester-ethanol **6** (99.5 mg, 0.247 mmol, 1.0 eq) was dissolved in 16.0 mL of *N,N*-dimethylformamide. **147** (127 mg, 0.494 mmol, 2.0 eq) and cesium carbonate (161 mg, 0.494 mmol, 2.0 eq) were then added. The reaction was held at room temperature for 24 hours in a nitrogen gas environment. The product was then extracted 3x with CH_2Cl_2 and deionized water, then the aqueous layer was back extracted 3x with CH_2Cl_2. Organic layers were combined, washed with brine, and dried over sodium sulfate. The crude product was purified by column chromatography with silica gel (15% ethyl acetate, hexanes) and concentrated *in vacuo* to yield negative control compound **2** (55.5 mg, 46%) as a white solid. The compound was chromatographically homogeneous by TLC and analytical HPLC.

Data for 2:

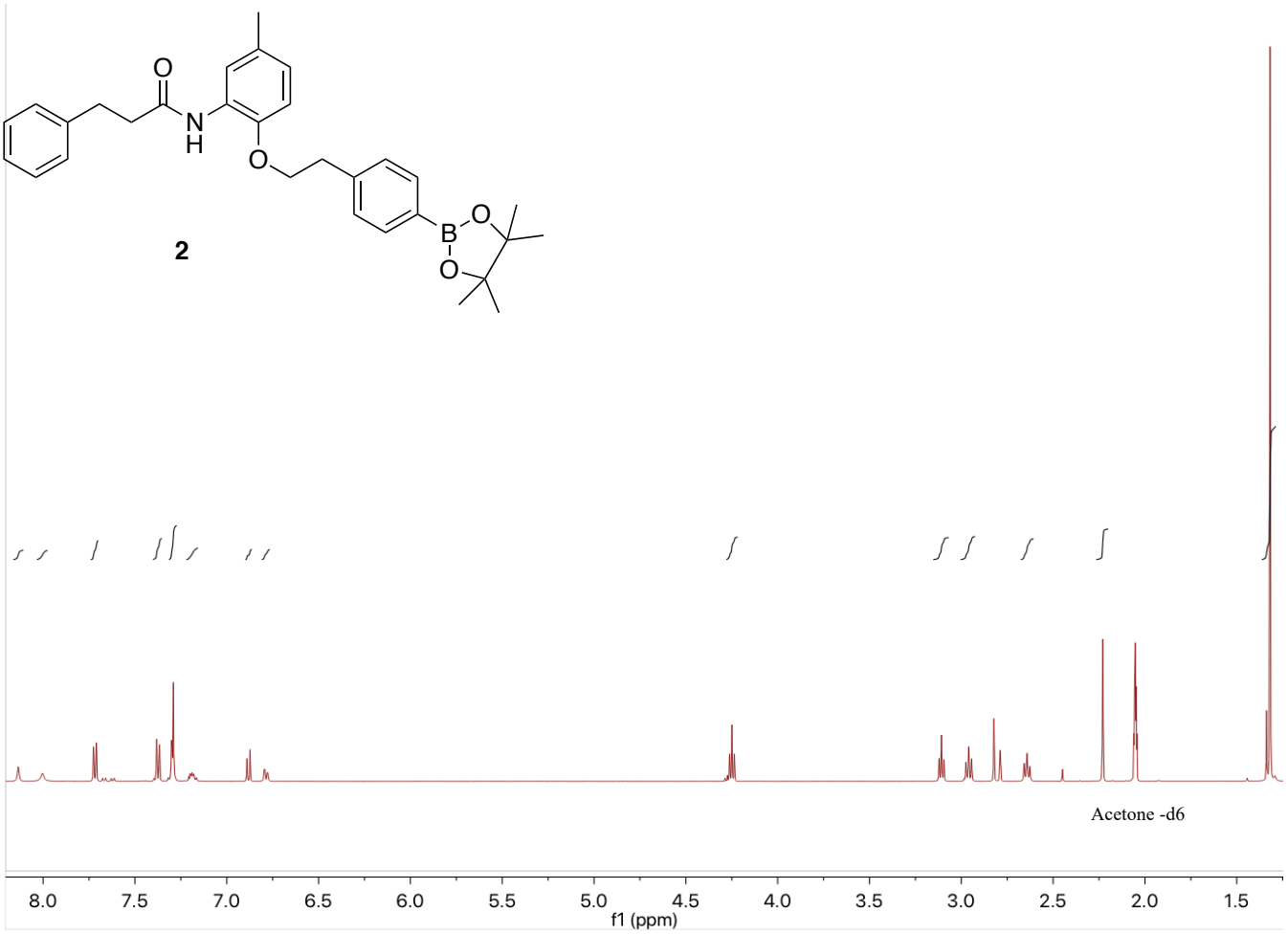
mp = 55°C

TLC: Rf = 0.36 (1:3 EtOAc:hexanes)

^{1}H NMR (500 MHz, acetone-d6): δ 8.14 (s, 1H), 8.00 (s, 1H), 7.72 (d, J = 8.0, 2H), 7.37 (d, J = 8.0, 2H), 7.29 (m, 3H), 7.18 (m, 1H), 6.88 (d, J = 8.0, 1H), 6.78 (d, J = 8.0, 1H), 4.26 (t, J = 6.5, 2H), 3.11 (t, J = 6.5, 2H), 2.97 (t, J = 7.5, 2H), 2.65 (t, J = 7.5, 2H), 2.23 (s, 3H), 1.32 (s, 12H) ppm.

^{13}C NMR (500 MHz, $CDCl_3$): δ 170.00, 144.58, 141.43, 140.75, 135.15, 130.88, 128.54, 128.39, 128.24, 127.67, 126.18, 123.68, 120.41, 111.17, 83.78, 68.96, 39.48, 35.83, 31.39, 24.85, 20.98 ppm.

HRMS (m/z): [M+Na^+] Calculated for $C_{30}H_{36}BNO_4$, 508.2635; Found, 508.2653; mass accuracy, 3.54 ppm.


O
N
H
O
B
O
O
2
Acetone -d6
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
f1 (ppm)

Stability and Peroxide Release Assays

Compounds **1**, **2**, or **147** were added to Eppendorf tubes in DMSO (20 μL of 10 mM DMSO solution) containing 170 μL HEPES buffered saline (HBS) pH 7.3 and 20 μL of a 1-napthalene methanol stock solution (5.4 mg/mL in methanol) as an HPLC internal standard. Compounds were 1 mM final concentration in a solution of 81% HBS, 9.5% DMSO and 9.5% MeOH. The tubes were vortexed for 2-5 seconds and parafilmed before placement in a 37ºC water bath. At various time points, 20 μL aliquots were withdrawn and added to 80 μL of a 1% TFA / 49% H_2O, 49% CH_3CN solution and directly analyzed by analytical HPLC or LC/MS. Half-life was calculated by an exponential fit to a curve of the decomposition of the compound (stability) or an intermediate (release) relative to the internal standard, 1-napthalene methanol. For the peroxide release assays the same protocol was followed except 160 μL of HBS was added to each Eppendorf tube and the remaining 10 μL contained a solution of hydrogen peroxide in water at various concentrations to achieve a 0.1-200 mM final peroxide concentration.

Cytochrome P450 Oxidation Experiments

Compound **147** or prodrug **2** (from 10 mM DMSO stock solution) was individually mixed with Cyp1A2 or control baculosomes in 50 mM potassium phosphate buffer (pH 7.55). A pre-mixed solution of NADPH-generating reagents in phosphate buffer (minus $NADP^+$) was added and the reactions pre-incubated for 5 min at 37 ºC in a water bath. Reactions were then initiated by the addition of $NADP^+$ in phosphate buffer to give a final volume of 200 μL and incubated at 37 ºC for 24 h. The final reaction mixture contained 200 μM compound (with 4 μL DMSO), 100 pmol/mL Cyp1A2 or control, 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phsophate dehydrogenase, and 3.3 mM magnesium chloride in 50 mM phosphate buffer (pH 7.55). Following the 24 h incubation, reactions were quenched by the addition of 30 μL trichloroacetic acid (10% in H_2O). Samples were centrifuged at 10,000*g* for 10 min at rt to pellet the precipitated protein, and supernatents were subjected to LC-MS for direct analysis of metabolites.

To trap reactive metabolites formed, 1 mM glutathione (GSH, from a stock solution in 50 mM phosphate buffer) was added to the reaction mixtures and incubations and analysis were performed as described.

Cardiomyocyte Experimental Protocols

Cardiomyocyte Isolation, Culture and H_2O_2 Toxicity Experiments

Neonatal rat ventricular myocytes (NRVM) were isolated via enzymatic digestion of 1 to 3 day-old Sprague-Dawley rat hearts, purified by Percoll density gradient centrifugation, and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) on plastic culture plates that had been pre-treated with 5 µg/ml fibronectin, as previously described.[3] Plating density was maintained at 3.0×10^5 cells/well on 12-well plates or 0.75×10^5 cells/well on 48-well plates. Cell viability was measured using an MTT assay (Cell Proliferation Kit I, Roche Diagnostics), according to the manufacturer's protocol. Sixteen hours after plating NRVM allotted to the "pretreatment experimental paradigm" were treated with vehicle, control compound **2**, **147**, or prodrug **1** (all concentrations at 10 µM) for 24 hours in DMEM/F12 supplemented with bovine serum albumin (BSA) (1 mg/ml) for NRVM and again in combination with H_2O_2 (50 µM, cat# 216763, Sigma-Aldrich) for an additional 8 hours.

Quantitative Real-Time PCR (qRT-PCR)

NRVM were treated with either vehicle, control **2**, **147** or prodrug **1** (all compounds 10 µM) for 16 hours before being lysed for RNA extraction. To "pre-activate" control **2** or prodrug **1**, 10 µM of respective compounds were incubated with 50 µM H_2O_2 for 8 hours in the absence of cells. After this period, this media was used to treat NRVM for an additional 16 hours. Total RNA was isolated from NRVM using Quick-RNA MiniPrep kit (Zymo Research, Irvine, CA). cDNA synthesis was performed using SuperScript III First-Strand Synthesis System (Thermo Fisher). qRT-PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix in a StepOnePlus RT-PCR System (Thermo Fisher). The following primers were used:

Rat	Forward 5’ to 3’	Reverse 5’ to 3’
Gapdh	AGACAGCCGCATCTTCTTGT	CTTGCCGTGGGTAGAGTCAT
Cat	CTTTGAGGTCACCCACGATATT	GTGGGTTTCTCTTCTGGCTATG
Grp78	CCAAGGATGCTGGCACTATTG	ACCCAGGTCAAACACGAGGAT

Simulated Ischemia/Reperfusion Experiments

For *in vitro* ischemia/reperfusion (I/R), ischemia was simulated by replacing all culture media with 0.5 ml of glucose-free DMEM containing 2% dialyzed FBS with either control **2**, **147** or prodrug **1** (all compounds 10 μM), then incubated at 0.1% O_2 in a hypoxia chamber with an oxygen controller (ProOx P110 oxygen controller, Biospherix, Parish, NY) for 8 hours. Reperfusion was simulated by replacing culture media with DMEM/F12 supplemented with BSA (1 mg/ml) and incubating at 21% O_2 for an additional 24 hours. NRVM reperfusion media were supplemented with control **2**, **147** or prodrug **1** (all compounds 10 μM) throughout the duration of the reperfusion period. See accompanying procedural figure (**Figure S6a**). Viability was determined by assaying the media for lactate dehydrogenase activity (LDH Activity Assay Kit, Sigma Aldrich) or by number of calcein-AM labeled NRVM using calcein-AM green (Thermo Fisher). Images were obtained with an IX70 fluorescence microscope (Olympus, Melville, NY). Numbers of viable, calcein-AM green-positive cells were counted using ImageJ or Image-Pro Plus software (Medium Cybernetics, Rockville, MD). Statistical analysis to calculate p values was conducted by one way ANOVA followed by Tukey’s post-hoc analysis.

Supplemental Figures

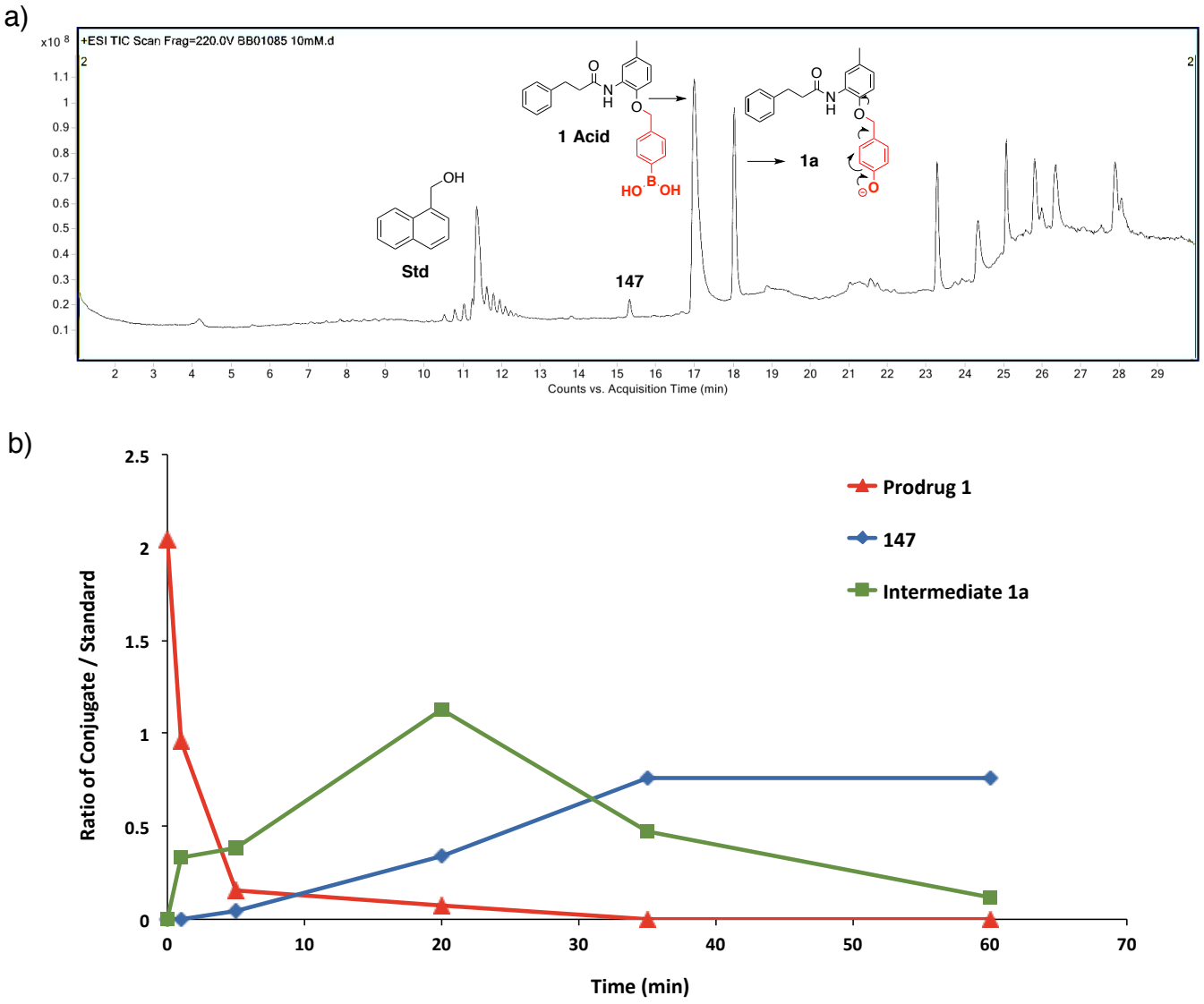
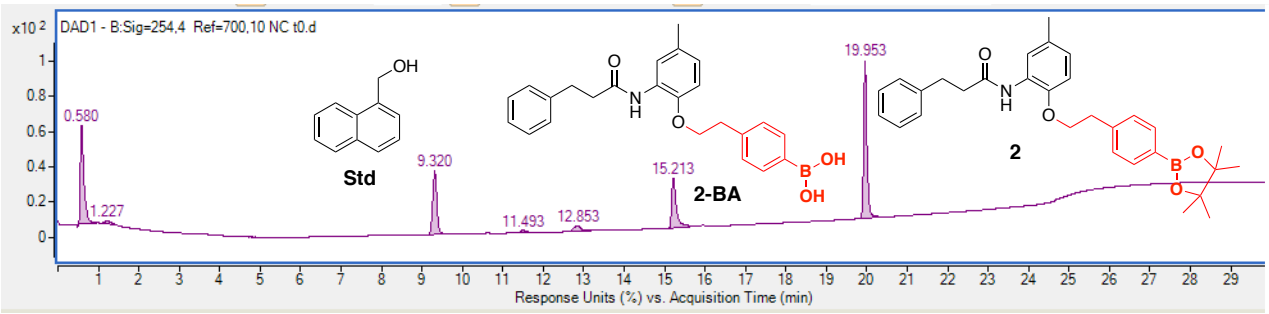
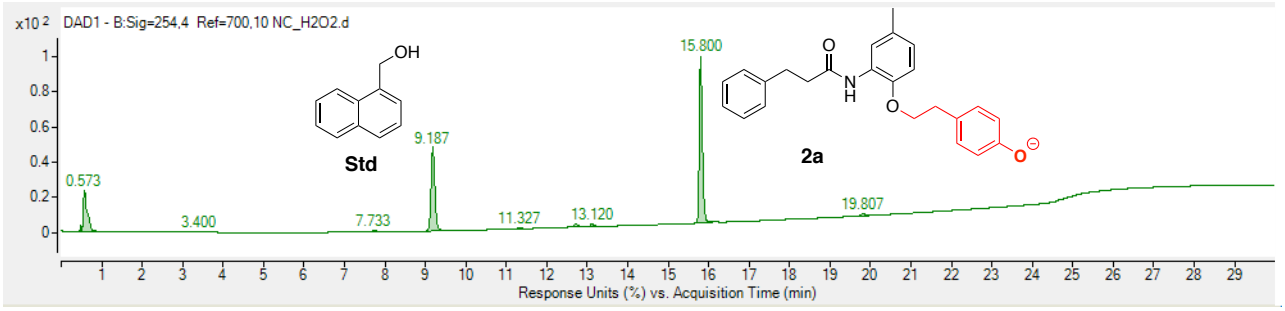


Figure S1. Prodrug **1** Releases Free **147** In Presence of Peroxide Through Intermediate **1a**.
a) Mass spectrometry chromatogram of prodrug **1** following a 10 minute incubation with 10 equivalents of hydrogen peroxide in HBS pH 7.3, 37ºC with naphthalene methanol as internal standard run on 5-90% CH_3CN:H_2O gradient with 0.1% formic acid. Mass spectral hits were verified for all labeled species. b) Graph of normalized integrations (prodrug **1**, **147**, or intermediate **1a** relative to naphthalene methanol internal standard) in 254 nm chromatogram from reaction of prodrug **1** with 10 equivalents of hydrogen peroxide in HBS pH 7.3, 37ºC.

a)



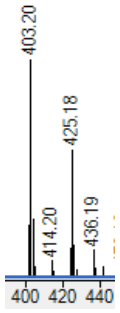
b)



c)

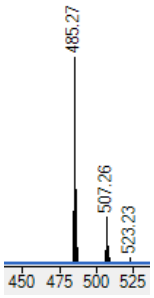
MS of **2-BA** (15.21 min)

Calculated: 403.20



MS of **2** (19.95 min)

Calculated: 485.27



MS of **2a** (15.80 min)

Calculated (M+H): 375.18

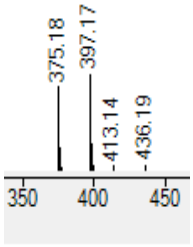


Figure S2. Negative control **2** does not release **147** upon treatment with hydrogen peroxide, but converts to detectable intermediate **2a** by LC-MS. a) UV chromatogram (254 nm) of negative control **2**, in HBS pH 7.3 with naphthalene methanol as internal standard run on 5-90% CH_3CN:H_2O gradient with 0.1% formic acid. Some conversion from the boronic ester to the boronic acid (**2-BA**) is observed on the column. b) UV chromatogram (254 nm) of negative control **2** following a 24 h incubation with 20 equivalents H_2O_2 in HBS pH 7.3, 37ºC. c) Deconvoluted mass spectra for each labeled peak in a) and b) above for verification.

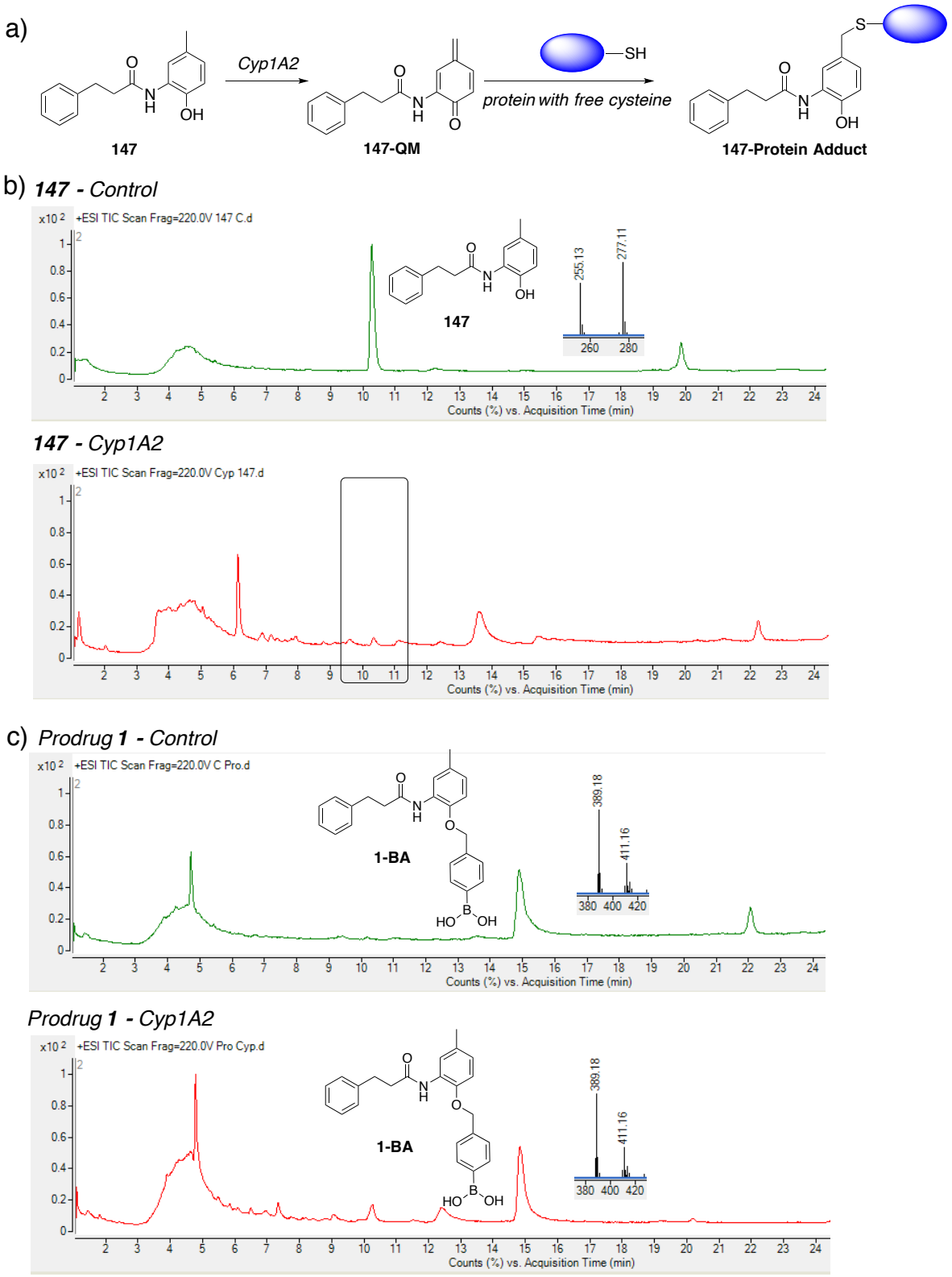


Figure S3. 147, but not prodrug **1**, is metabolically activated by Cyp1A2. a) Schematic of proposed mechanism of action of **147**. b) Mass spectrometry chromatogram of **147** incubated with control or Cyp1A2 baculosomes for 24 h at 37°C. Mass spectrum of **147** peak is inset for reference. c) Mass spectrometry chromatogram of prodrug **1** incubated with control or Cyp1A2 baculosomes for 24 h at 37°C. **1-BA** indicates the boronic acid of prodrug **1**. Mass spectrum of **1-BA** peak is inset for reference.

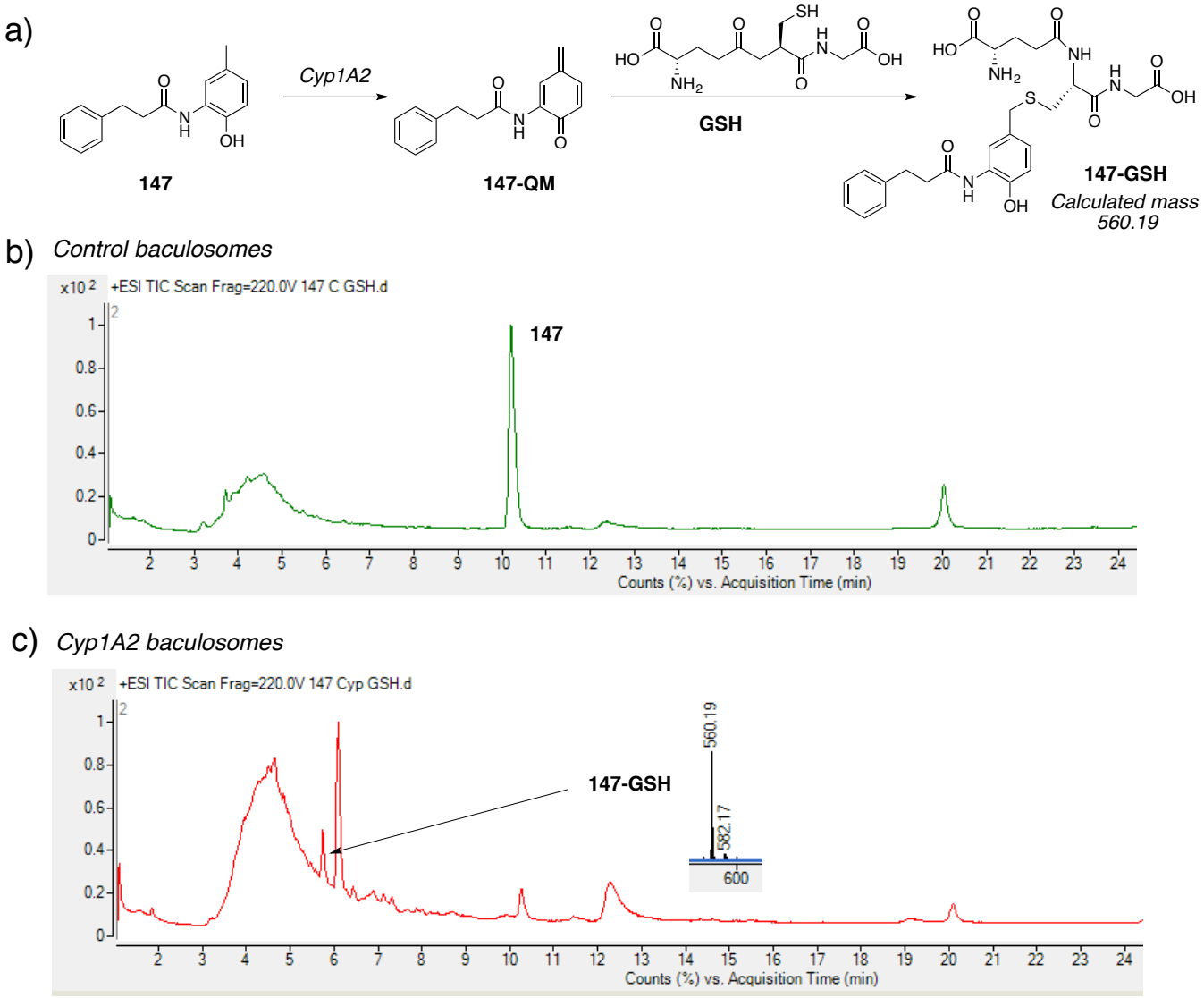


Figure S4. 147 is metabolically activated by Cyp1A2 to a quinone methide, **147-QM**, which can be trapped by glutathione. a) Schematic of **147** metabolic oxidation and GSH trapping. b) Mass spectrometry chromatogram of **147** incubated for 24 h with control baculosomes following a 24 h incubation with 1 mM GSH and NADPH regenerating solution in potassium phosphate buffer, 37ºC. c) Mass spectrometry chromatogram of **147** incubated for 24 h with Cyp1A2 baculosomes following a 24 h incubation with 1 mM GSH and NADPH regenerating solution in potassium phosphate buffer, 37ºC. The mass spectrum shown is of the indicated peak representing the **147-GSH** adduct.

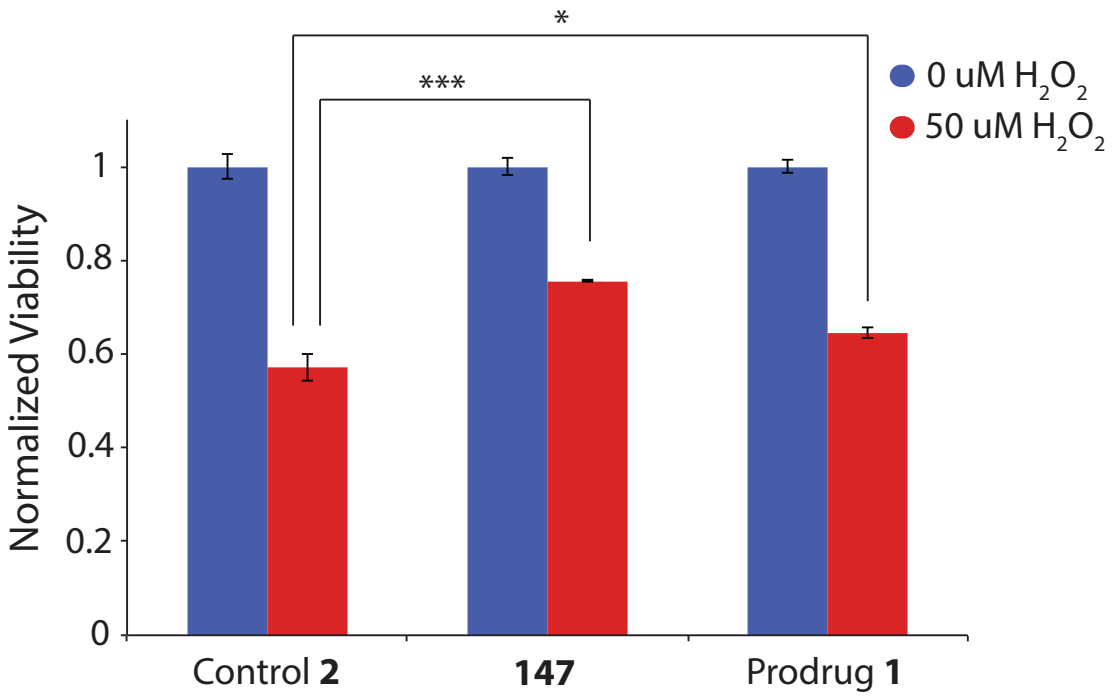


Figure S5. 147 and Prodrug **1** protect primary cardiomyocytes from peroxide-mediated toxicity. Graph of normalized viability by MTT assay in NVRM following a 24 h pretreatment with 10 μM compound, then 8 h treatment of compound in the presence or absence of 50 μM peroxide. Error bars represent the SEM for n = 6 trials per condition. * $p < 0.05$, *** $p < 0.001$ by ANOVA and Tukey post-hoc analysis.

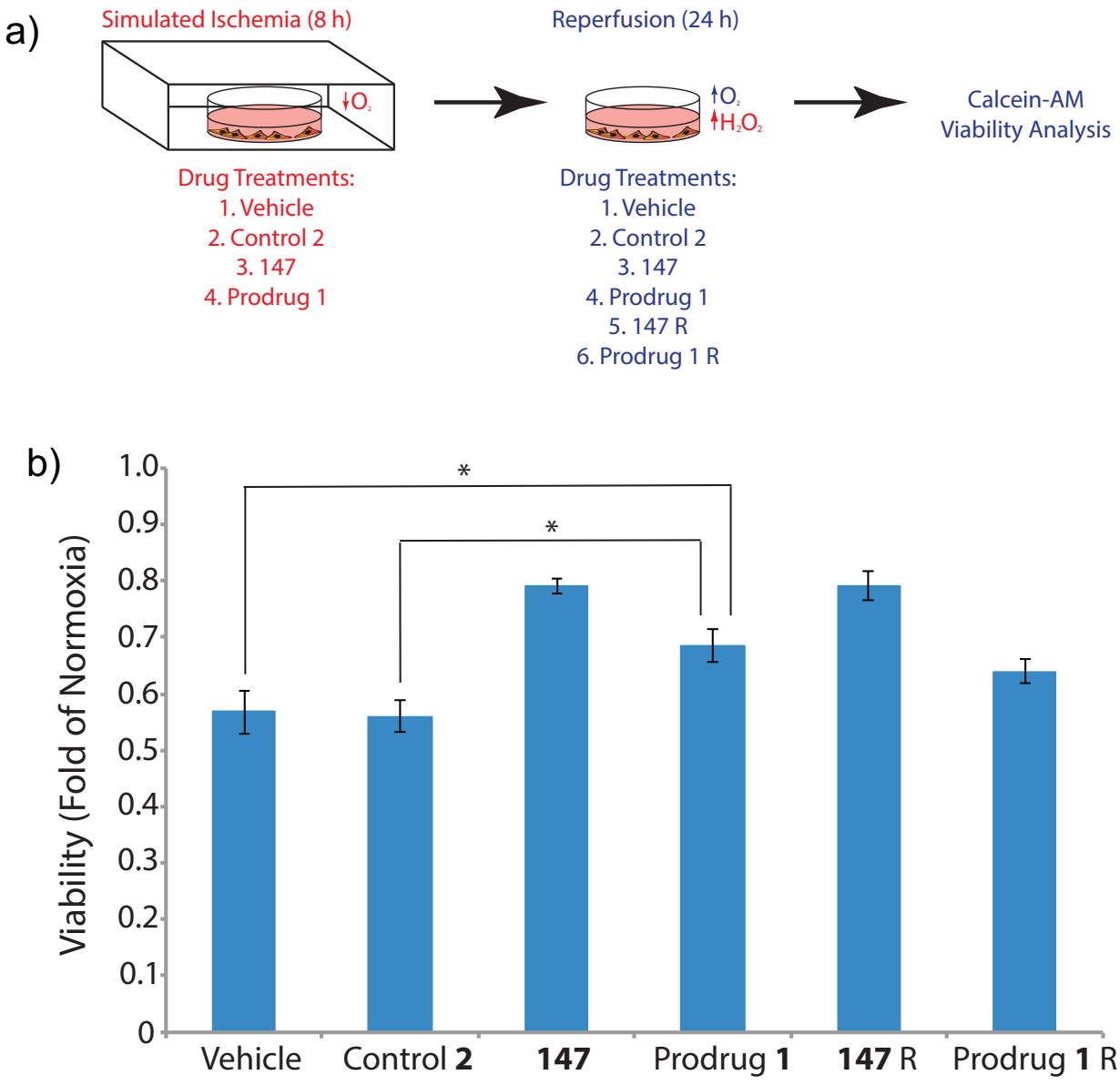


Figure S6. 147 and prodrug **1** protect cardiomyocytes from I/R injury. a) Schematic of simulated ischemia and reperfusion protocol in NVRM cells. b) Viability of NVRM determined by calcein-AM labeling following the protocol scheme in (a). Vehicle, negative control **2**, **147**, or prodrug **1** (10 μM) were added to cells during the entirety of the experiment or at the reperfusion step only (**147** R, Prodrug **1** R). Error bars represent SEM of n = 18 trials. * $p < 0.05$ by ANOVA and Tukey post-hoc analysis.

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