Enol-Mediated Delivery of H_2Se from γ -Keto Selenides: Mechanistic Insight and Evaluation

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I. General

Commercial reagents were used without further purification unless stated otherwise. All glassware was oven or flame-dried and reactions were performed under an $N_2(q)$ atmosphere. Dichloromethane (DCM) and tetrahydrofuran (THF) were dried over a column of alumina. Flash chromatography was performed with columns of 40-63 Å silica from Silicycle (Québec City, Canada). Thin-layer chromatography (TLC) was performed on plates of EMD 250-µm silica 60-F254. Preparative thin-layer chromatography (PTLC) was performed on Whatman silica gel (500- μ m) F254 glass plates. The term "concentrated under reduced pressure" refers to removal of solvents and other volatile materials using a rotary evaporator while maintaining the water-bath temperature below 40 °C. Residual solvent was removed from samples at high vacuum (<0.1 torr) using an Edwards RV5 pump. All NMR spectra were acquired at ambient temperature with a Bruker AscendTM 400 MHz spectrometer and referenced to TMS or residual protic solvent. High resolution mass spectra were acquired using a Thermo Orbitrap LTQ XL (ESI). Adjustments in pH/pD for buffered solutions were accomplished with a Fisher Scientific Accumet AE150 pH meter. Cell viability was assessed spectrophotometrically using a BioTek SYNERGY-HTX multi-mode 96-well plate reader.

II. Safety and Handling

Hydrogen selenide is highly toxic and proper safety precautions must be in place when handling selenide salts and synthetic donors. All reactions and assays were performed in a well-ventilated fume hood and solutions containing donor we immediately quenched with silver nitrate prior to their disposal.

III. Synthesis



To a solution of sodium borohydride (40 mg, 1.1 mmol) in a 1:1 solution of degassed ethanol and DI water (20 mL) was added selenium powder (39 mg, 0.5 mmol). The mixture was then heated to 50 °C under an atmosphere of nitrogen for 30 minutes, at which point the solution turned colorless and the selenium had completely dissolved. Next, 3-chloropropiophenone (168 mg, 1.0 mmol) was added and the reaction continued to stir at 50 °C until the starting material was observed to be consumed by TLC. The reaction was then cooled to room temperature and extracted with dichloromethane (50 mL). The organic layer was isolated, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The desired product was isolated by silica gel chromatography (gradient of 100% hexanes to 10% ethyl acetate in hexanes) to yield **1** as a white solid (158 mg, 91% yield).

¹H NMR (400 MHz, CDCl₃): δ = 8.00–7.93 (m, 4H), 7.61–7.54 (m, 2H), 7.51–7.43 (m, 4H), 3.43 (t, *J* = 7.2 Hz, 4H), 2.99 (t, *J* = 7.2 Hz, 4H); ¹³C NMR (101 MHz, CDCl₃) δ = 198.7, 136.6, 133.3, 128.7, 128.1, 39.9, 17.5; ⁷⁷Se NMR (76 MHz, CDCl₃) δ = 200.0; HRMS calculated for [C₁₈H₁₉O₂Se⁺] (M+H⁺) requires *m*/*z* = 347.0544, found 347.0535.



3,3'-selenodipropionic acid (320 mg, 1.4 mmol)¹, EDCI hydrochloride (600 mg, 3.1 mmol), HOBt (97 mg, 0.7 mmol), and aniline (286 μ L, 3.1 mmol) were combined in dry dichloromethane (10 mL) and allowed to stir at ambient temperature under an N₂(g) atmosphere overnight. The mixture was further diluted with dichloromethane (100 mL) and washed with DI water (50 mL). The organic layer was collected, dried over anhydrous sodium sulfate, concentrated under reduced pressure, and purified by silica gel chromatography (gradient of 100% dichloromethane to 95:5 dichlormethane/methanol). The ensuing mixture was further triturated with dichloromethane (10 mL) to yield **3** as a white solid (100 mg, 19% yield).

¹H NMR (400 MHz, DMSO): δ = 9.96 (s, 2H), 7.63–7.55 (m, 4H), 7.35–7.25 (m, 4H), 7.09– 6.99 (m, 2H), 2.84 (td, *J* = 7.1, 1.2 Hz, 4H), 2.74 (td, *J* = 7.0, 1.2 Hz, 4H); ¹³C NMR (101 MHz, DMSO) δ = 170.4, 139.6, 129.2, 123.6, 119.5, 38.1, 18.7; ⁷⁷Se NMR (76 MHz, DMSO) δ = 186.3; HRMS calculated for [C₁₈H₂₁N₂O₂Se⁺] (M+H⁺) requires *m*/*z* = 377.0762, found 377.0764.



Phosphorous tribromide (475 μ L, 5.0 mmol) was added dropwise to a cooled solution (using an ice bath) of 4-hydroxybutan-2-one (861 μ L, 10.0 mmol) in dry dichloromethane (10 mL). After two hours, consumption of the starting material was inferred by TLC, and the mixture was directly concentrated under reduced pressure via rotary evaporation. The resultant oil was loaded onto a silica gel column and eluted (using a gradient of 100% hexanes to 25% ethyl acetate in hexanes) to afford 4-bromobutan-2-one as an oil (1.40 g, 93% yield).

¹H NMR (400 MHz, CDCl₃): δ =3.55 (t, J = 6.8 Hz, 2H), 3.04 (t, J = 6.7 Hz, 2H), 2.19 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ = 205.3, 46.0, 30.2, 25.2.



To a round bottom flask containing sodium borohydride (79 mg, 2.1 mmol) in a 1:1 solution of degassed ethanol and DI water (20 mL) was added selenium powder (78 mg, 1.0 mmol). The reaction mixture was heated to 50 °C under a nitrogen atmosphere for 30 minutes, at which point the solution turned colorless and the selenium had dissolved. Next, 4-bromobutan-2-one (300 mg, 2.0 mmol) was added, and the reaction was allowed to stir at 50 °C until the starting material was deemed to be consumed by TLC. The reaction was cooled to room temperature, diluted with DI water (20 mL) and extracted with dichloromethane (20 mL). The organic layer was dried over anhydrous sodium sulfate, concentrated under reduced pressure, and the resultant residue was purified by silica gel chromatography (gradient of 100% hexanes to 25% ethyl acetate in hexanes) to yield **4** as an oil (123 mg, 56% yield).

¹H NMR (400 MHz, CDCl₃): δ = 2.93–2.81 (m, 4H), 2.81–2.69 (m, 4H), 2.17 (s, 6H); ¹³C NMR (101 MHz, CDCl₃) δ = 207.1, 44.5, 30.1, 16.8; ⁷⁷Se NMR (76 MHz, CDCl₃) δ = 197.4; HRMS calculated for [C₈H₁₅O₂Se⁺] (M+H⁺) requires *m*/*z* = 223.0231, found 223.0233.



To a solution of 4,4,4-trifluorobutane-1,3-diol² (641 mg, 4.4 mmol) in dry dichloromethane (20 mL) was added *p*-toluenesulfonic anhydride—freshly recrystallized from hot hexanes—(1.83 g, 5.6 mmol),³ and pyridine (1.8 mL, 22.3 mmol), and the resultant mixture was allowed to stir at room temperature overnight. Upon confirming the consumption of starting material by TLC, the crude mixture was concentrated under reduced pressure and purified by silica gel chromatography (slow gradient of 100% hexanes to 25% ethyl acetate in hexanes) to afford 4,4,4-trifluoro-3-hydroxybutyl 4-methylbenzenesulfonate as a yellow oil (1.03 g, 79%).

¹H NMR (400 MHz, CDCl₃): δ = 7.85 – 7.77 (m, 2H), 7.42 – 7.33 (m, 2H), 4.33 (td, *J* = 10.4, 3.7 Hz, 1H), 4.20 – 4.13 (m, 2H), 2.59 (d, *J* = 23.3 Hz, 1H), 2.46 (s, 3H), 2.15 – 2.05 (m, 1H), 1.86 (ddt, *J* = 14.6, 10.7, 3.8 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ = 145.3, 132.6, 130.0, 127.9, 126.2 (q, *J* = 190.6), 66.7 (q, *J* = 31.86), 65.5, 29.1, 21.7; ¹⁹F NMR (376 MHz, CDCl₃): δ = -80.1 (d, *J* = 6.6 Hz); HRMS calculated for [C₁₁H₁₄F₃O₄S⁺] (M+H⁺) requires *m*/*z* = 299.0559, found 299.0554.



To a solution of 4,4,4-trifluoro-3-hydroxybutyl 4-methylbenzenesulfonate (260 mg, 0.9 mmol) in dry dichloromethane (10 mL) was added Dess-Martin periodinane (559 mg, 1.3 mmol). After stirring overnight, the reaction was concentrated under reduced pressure, and purified by silica gel chromatography (gradient of 100% hexanes to 50% ethyl acetate in hexanes) to afford an oily mixture of both ketone and hydrate. Further evaporation under high vacuum provided the ketone (4,4,4-trifluoro-3-oxobutyl 4-methylbenzenesulfonate) as a white solid (178 mg, 67% yield).

¹H NMR (400 MHz, CDCl₃): δ = 7.86–7.75 (m, 2H), 7.41–7.32 (m, 2H), 4.34 (t, *J* = 6.0 Hz, 2H), 3.13 (t, *J* = 6.0 Hz, 2H), 2.47 (s, 3H); ¹³C NMR (101 MHz, CDCl₃) δ = 187.6 (q, *J* = 37.0 Hz), 145.4, 132.3, 130.0, 128.1, 115.1 (q, *J* = 291.2 Hz), 62.4, 35.9, 21.7; ¹⁹F NMR (376 MHz, CDCl₃): δ = -79.5; HRMS calculated for [C₁₁H₁₂F₃O₄S⁺] (M+H⁺) requires *m/z* =297.0402, found 297.0402.



To a round bottom flask containing sodium borohydride (11 mg, 0.3 mmol) in a 1:1 solution of degassed ethanol and DI water (10 mL) was added selenium powder (9 mg, 0.1 mmol). Once the vigorous bubbling had subsided, the mixture was heated to 50 °C under nitrogen until the reaction turned colorless and the selenium had completely dissolved. At this point, the reaction was cooled to room temperature using a water bath and acetic acid (200 μ L) and 4,4,4-trifluoro-3-oxobutyl 4-methylbenzenesulfonate (78 mg, 0.26 mmol in degassed ethanol (3 mL) were both quickly added. After stirring for an additional hour, the mixture was extracted with dichloromethane (20 mL) and the organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The crude product was quickly purified via a silica gel plug (50% ethyl acetate in hexanes) and, after further evaporation under high vacuum, afforded **5** (15 mg, 45% yield). Analytically pure samples of **5** for kinetics and cell testing were obtained by preparative thin layer chromatography (1:3 hexanes:ethyl acetate) immediately prior to use.

¹H NMR (400 MHz, CDCl₃): $\delta = 3.21-3.16$ (m, 4H), 2.87 (t, J = 6.9 Hz, 4H); ¹³C NMR (101 MHz, CDCl₃) $\delta = 190.3$ (q, J = 35.9 Hz), 113.9 (q, J = 291.7 Hz), 38.0, 15.3; ¹⁹F NMR (376 MHz, CDCl₃): $\delta = -79.3$; ⁷⁷Se NMR (76 MHz, CDCl₃) $\delta = 218.2$; HRMS calculated for [C₁₀H₁₇F₆O₄Se⁺] (M+H⁺ of the methanol *bis*-hemihydrate) requires *m*/*z* = 393.0199, found 393.0042.



To a solution of sodium borohydride (76 mg, 2.0 mmol) in degassed DI water (5 mL) and ethanol (5 mL) was added selenium powder (80 mg, 1.0 mmol). The mixture was then heated to 50 °C under nitrogen for 30 minutes, at which point the solution turned colorless and the selenium had completely dissolved. Next, neat benzyl bromide (283 μ L, 2.4 mmol) was introduced and the reaction continued to stir at 50 °C until the starting material was observed to be consumed by TLC. The reaction was then cooled to room temperature and extracted with dichloromethane (30 mL). The organic layer was collected, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The product was isolated by silica gel chromatography (gradient of 100% hexanes to 10% ethyl acetate in hexanes) to yield **6** as a yellow oil (247 mg, 95% yield).

¹H NMR (400 MHz, CDCl₃): δ = 7.36–7.15 (m, 10H), 3.72 (s, 4H); ¹³C NMR (101 MHz, CDCl₃) δ = 139.2, 129.0, 128.5, 126.7, 27.6; ⁷⁷Se NMR (76 MHz, CDCl₃) δ = 333.4; HRMS calculated for [C₁₄H₁₅Se⁺] (M+H⁺) requires *m/z* = 263.0333, found 263.0333.



To a solution of sodium borohydride (40 mg, 1.1 mmol) in a 1:1 solution of degassed ethanol and DI water (20 mL) was added selenium powder (40 mg, 0.5 mmol). The mixture was then heated to 50 °C under nitrogen for 30 minutes, at which point the solution turned colorless and the selenium had completely dissolved. A second portion of selenium powder (40 mg, 0.5 mmol) was then delivered, and the mixture was allowed to react at 50 °C for an additional 30 minutes. At this point, the reaction was cooled to room temperature using a water bath and acetic acid (60 μ L) and 3-chloropropiophenone (168 mg, 1.0 mmol) in degassed THF (5 mL) were both quickly added. The reaction was then left to stir at room temperature until the starting material was deemed to have been consumed by TLC. The reaction was then diluted with DI water (20 mL) and extracted with ethyl acetate (20 mL). The organic layer was isolated, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The resultant crude product was

purified by silica gel chromatography (gradient of 100% hexanes to 10% ethyl acetate in hexanes) to yield **9** as a yellow solid (67 mg, 32% yield).

¹H NMR (400 MHz, CDCl₃): $\delta = 7.93-7.87$ (m, 4H), 7.54–7.47 (m, 2H), 7.44–7.36 (m, 4H), 3.44 (t, J = 7.0 Hz, 4H), 3.19 (t, J = 7.0 Hz, 4H); ¹³C NMR (101 MHz, CDCl₃) $\delta = 198.5$, 136.5, 133.4, 128.7, 128.1, 40.2, 22.8; ⁷⁷Se NMR (76 MHz, CDCl₃) $\delta = 329.0$; HRMS calculated for [C₁₈H₁₉O₂Se₂⁺] (M+H⁺) requires *m/z* 426.9711 =, found 426.9706.



To a solution of sodium borohydride (79 mg, 2.1 mmol) in degassed DI water (5 mL) was added a portion selenium powder (83 mg, 1.05 mmol). The mixture was then heated to 50 °C under nitrogen for 30 minutes, at which point the solution turned colorless and the selenium had completely dissolved. A second portion of selenium powder (83 mg, 1.05 mmol) was then delivered, and the mixture was allowed to react at 50 °C for an additional 30 minutes. Benzyl bromide (238 μ L, 2.0 mmol) in THF (10 mL) was then added and the reaction was allowed to stir overnight at 50 °C. Upon cooling to room temperature, the reaction mixture was diluted with dichloromethane (20 mL) and washed with DI water (20 mL). The organic layer was isolated, dried over anhydrous sodium sulfate, and concentrated under the reduced pressure. The resultant oil was purified by silica gel chromatography (100% hexanes) to afford **10** as a yellow oil (201 mg, 59% yield).

¹H NMR (400 MHz, CDCI₃): δ = 7.34 – 7.18 (m, 10H), 3.83 (s, 4H); ¹³C NMR (101 MHz, CDCI₃) δ = 139.0, 129.0, 128.5, 127.1, 32.6; ⁷⁷Se NMR (76 MHz, CDCI₃) δ = 402.7; HRMS calculated for [C₁₄H₁₈NSe₂⁺] (M+NH₄⁺) requires *m*/*z* =359.9764, found 359.9769 (infused with Ammonium bicarbonate)



4-hydroxybutan-2-one (861 μ L, 10.0 mmol) was added neat to a round bottom flask with *p*-toluenesulfonic acid monohydrate (48 mg, 0.25 mmol) and heated to 90 °C under argon overnight. The mixture was cooled to room temperature, diluted with diethyl ether (40 mL) and washed with deionized water (40 mL). The organic layer was isolated, dried over anhydrous sodium sulfate, and concentrated under the reduced pressure. The resultant oil was purified by silica gel chromatography (gradient of 100% hexanes to 50% diethyl ether in hexanes) to yield **4EC** as an oil (169 mg, 21% yield).

¹H NMR (400 MHz, CDCl₃): δ = 3.69 (t, *J* = 6.2 Hz, 4H), 2.66 (t, *J* = 6.2 Hz, 4H), 2.17 (s, 6H); ¹³C NMR (101 MHz, CDCl₃) δ = 207.2, 65.9, 43.6, 30.4; HRMS calculated for [C₈H₁₄NaO₃⁺] (M+Na⁺) requires *m*/*z* =181.0841, found 181.0840.

IV. Kinetics Studies

Prior to the start of the experiment, deuterated buffer was prepared from a 50 mM solution of Na₂HPO₄ in D₂O and the respective pD values (8.5, 7.4, 6) were achieved with the addition of deuterium chloride (DCl). A standard solution of 1,4-dioxane was prepared at 31.25 mM in D₂O. Donor stock solution (**1** or **9**) was prepared at 75 mM in deuterated acetonitrile (acetonitrile-d3). For each experiment, acetonitrile-d3 (350 μ L) was added to a conical tube followed by the dioxane standard solution (15 μ L) and donor stock solution (25 μ L). Deuterated phosphate buffer (50 mM, 360 μ L) was then added to bring the final concentration of **1** (or **9**) to 2.5 mM and the dioxane standard to 0.625 mM. At this point, the solution was immediately transferred to an NMR tube and the cap was fastened with a small strip of parafilm to prevent evaporation. Experiments were run at ambient temperature and for 64 scans at each respective timepoint. To monitor reaction progress, the integration value for 1,4-dioxane (3.75 ppm) was normalized to 1 proton in each spectrum.



Figure S1. Time-course for the conversion of ketone **1** (2.5 mM) into enone **2** at ambient temperature and in a 1:1 mixture of CD_3CN and deuterated phosphate buffer (50 mM, pD 7.4). Reaction progress was monitored by ¹H NMR using 1,4-dioxane as an internal standard.



Figure S2. Time-course for the conversion of ketone **9** (2.5 mM) to enone **2** at ambient temperature and in a 1:1 mixture of CD_3CN and deuterated phosphate buffer (50 mM, pD 7.4). Reaction progress was monitored by ¹H NMR with 1,4 -dioxane being used as an internal standard to determine the concentration of **9** and **2** at different timepoints.

V. Trapping Experiments with Benzyl Bromide

Acetonitrile-d3 (321 μ L) was added to a conical tube followed by a standard solution of 1,4-dioxane (31.25 mM) in D₂O (15 μ L). Next, a stock solution of donor (**1**, **3**, **4**, or **5**, 75 mM, 10 μ L) in acetonitrile-d3 was added. Immediately prior to the start of the experiment, of a 75 mM stock solution of benzyl bromide in acetonitrile-d3 (44 μ L, 75 mM) was introduced followed by deuterated phosphate buffer (360 μ L, 50 mM, pD of 7.4), bringing the final concentration of donor (**1**, **3**, **4**, or **5**) to 1 mM, benzyl bromide to 4.4 mM, and 1,4-dioxane to 0.625 mM. The solution was immediately transferred to an NMR tube and the cap was fastened with a small strip of parafilm to prevent evaporation. Experiments were run at ambient temperature and for 64 scans at each respective timepoint. To monitor reaction progress, the integration value for 1,4-dioxane (3.75 ppm) was normalized to 1 proton in each spectrum.

To confirm the identity of trapped selenium products, standards of benzyl monoselenide (6) and benzyl diselenide (10) were prepared (as described above) and their ¹H NMRs in a 1:1 mixture of acetonitrile-d3 and deuterated phosphate buffer (50 mM, pD 7.4) were referenced to 1,4-dioxane (3.75 ppm) (Figure S3a).



(b)









Figure S3. (a) Representative ¹H NMR spectra of **10** and **6** in a 1:1 mixture of CD_3CN and deuterated phosphate buffer (50 mM, pD 7.4), referenced to 1,4-dioxane (3.75 ppm). (b–e) Representative ¹H NMR spectra of donors **1** (b), **3** (c), **4** (d), and **5** (e) (1.0 mM) treated with benzyl bromide (4.4 mM) resulting in the formation of monoselenide **6** (for donors **1**, **3**, **4**, **5**) and diselenide **10** (donor **5**). Reactions were run at ambient temperature in a 1:1 mixture of CD_3CN and deuterated phosphate buffer (50 mM, pD 7.4). 1,4-dioxane (3.75 ppm) was used as an internal standard to determine the concentrations compounds at different timepoints.

VI. Confirmation of Se⁰ Formation with Triphenylphosphine

When kinetic experiments were deemed complete by ¹H NMR, solvent was decanted from the elemental selenium (Se⁰), and the NMR tube was placed in an oven at 115 °C for approximately 30 minutes. After cooling to room temperature, a solution of triphenylphosphine in deuterated chloroform (700 μ L, 5 mM) was then added to the NMR tube and allowed to react for 12 hours prior to analysis by ³¹P NMR (Figure 3c).

To confirm the above results, 10 mg of elemental selenium (Alfa Aesar, 325 mesh, 99.5%) was added to an NMR tube and 700 μ L of triphenylphosphine solution (5 mM) in deuterated chloroform was added. The reaction was allowed to progress for 6 hours prior to analysis by ³¹P NMR (Figure S4).



Figure S4. Resultant ³¹P NMR spectrum from the test reaction between elemental selenium (Se⁰) with triphenylphosphine. Triphenylphosphine selenide: 35.28 ppm, triphenylphosphine oxide: 29.17 ppm, triphenylphosphine: -5.41 ppm.

VII. Formation of Selenodiglutathione

To a 0.1 M solution of ammonium bicarbonate buffer (pH 7.4, 1455 μ L) was added donor 1 (15 μ L of a 10 mM solution in ethanol), glutathione (27 μ L of a 10 mM solution in DI water) and glutathione disulfide (3 μ L of a 10 mM solution in DI water) to give a final concentration of 100 μ M 1, 180 μ M glutathione, and 20 μ M glutathione disulfide. The reaction was warmed to 37 °C and, after reacting for 30 min, a 250 μ L aliquot was removed and diluted with 750 μ L of methanol with 0.1% formic acid. The resultant solution was analyzed via direct infusion mass spectrometry to observe the selenodiglutathione sodium adduct (Figure 6).

VIII: H₂Se Gas Trapping Experiments

Adopted procedure from Newton et al.⁴ Donor **1** (1.0 g) was placed in a 15 mL Falcon® tube and dissolved in a mixture of THF (1 mL), deionized water (1 mL), and 4M HCl in 1,4-dioxane (10 mL). In a second tube, iodoacetamide (186 mg, 1 mmol) was dissolved in 10 mL of ammonium bicarbonate buffer (1.0 M, pH 8.5). Both tubes were then capped with septa and wrapped with parafilm. A vent needle was inserted into the septa

containing the trapping solution and two 4" cannula needles were used to connect the two tubes, ensuring that the tips of both needles remained in the headspace at all times. The first tube containing donor **1** was then pressurized with argon using a disposable needle, and the reaction was heated to 37 °C for three days. Aliquots of the trapping solution were monitored by mass spectrometry to confirm formation of trapped hydrogen selenide with iodoacetamide.



Figure S5. High-resolution mass spectrum of volatilized hydrogen selenide gas trapped with iodoacetamide.

IX. Cell Culturing and Cytotoxicity Studies

Cell culturing

HeLa and HCT116 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 1% L-glutamine, and 1% penicillin-streptomycin. Cells were incubated at 37 °C in an atmosphere of 5% CO₂ and 95% humidified air and split at 70–80% confluency every 2–3 days.

Cytotoxicity Studies

H₂Se donors (1, 3, 4, and 5) and control (4EC) were tested for cell growth inhibition in HeLa (human cervical cancer) and HCT116 (human colorectal cancer) cells in culture. A population of cells (>1 x 10⁶ cells/mL as determined with a hemocytometer) was diluted in DMEM, containing 10% fetal bovine serum, to give a final concentration of 50,000 cells/mL. To each well of a 96-well plate (Corning Costar) was added 100 µL of cell media solution to afford 5,000 cells/well. The plates were then incubated at 37 °C under an atmosphere of 5% CO₂ and 95% humidified air for 24 h. Fresh culture media (100 µL) containing 10% FBS was then added to each well (resulting in 200 µL per well) followed by the addition of test compounds (1, 3, 4, 5, or 4EC) at various concentrations (delivered from an initial 40 mM stock solution in biological grade DMSO resulting in no more than 0.5% DMSO per well). Following the addition of donor compounds, cultures were incubated for an additional 24 h. A phosphatase assay was used to establish IC₅₀ values as follows: The media was removed from each well and treated with 100 µL of phosphatase solution (100 mg of phosphatase substrate in 30 mL of 0.1 M NaOAc, pH 5.5, 0.1% Triton X-100 buffer). After an incubation period of 30 min, 50 µL of an NaOH solution (0.1 N) was added to each well and the absorbance at 405 nm was recorded. The percent cell viability at each donor concentration was calculated as follows:

% Viable Cells =
$$\frac{(abs_{sample}-abs_{blank})}{(abs_{control}-abs_{blank})} \times 100$$

X. References

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XI. NMR Spectra







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