

SUPPORTING INFORMATION

Enhanced Ratiometric Fluorescent Indicators for Magnesium Based on Azoles of the Heavier Chalcogens

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1. Comparison of fluorescence excitation and emission spectra of Mag-Fura-2 and new Mag-S and Mag-Se sensors

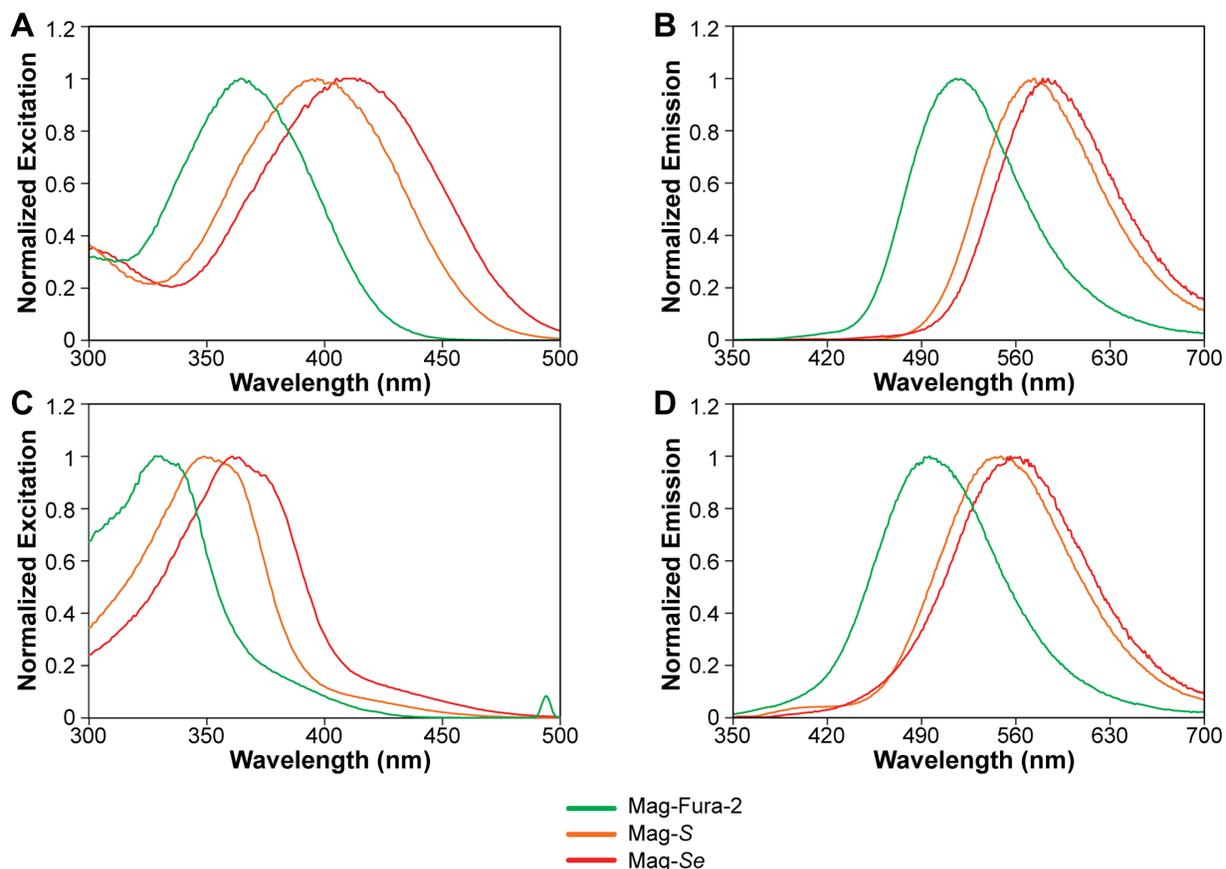


Figure S1. Comparison of excitation and emission spectra of Mag-Fura-2 and new sensors Mag-S and Mag-Se. Spectra were collected for 2 μM solutions of the sensors in aqueous PIPES buffer (50 mM PIPES, 100 mM KCl, pH 7.0) at 25 $^{\circ}\text{C}$ in the presence or absence of 50 mM of MgCl_2 . (A) Normalized fluorescence excitation, (B) normalized fluorescence emission, (C) normalized fluorescence excitation in the presence of Mg^{2+} , and (D) normalized emission in the presence of Mg^{2+} .

2. Metal selectivity data

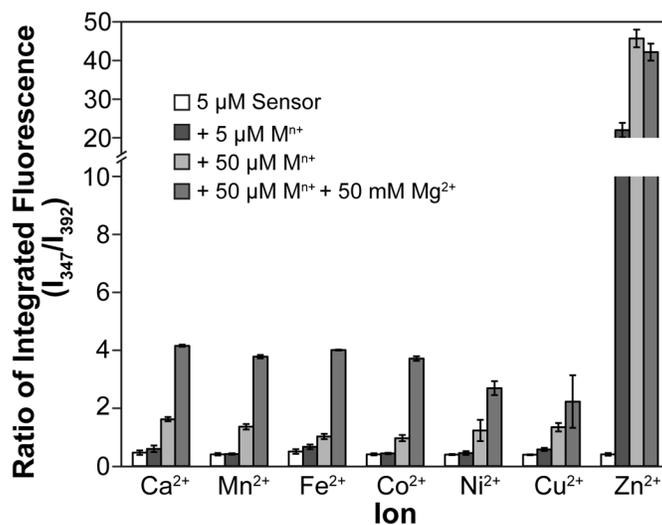


Figure S2. Metal selectivity plot for Mag-S in aqueous buffer at pH 7.0. Ratios plotted correspond to the average of three independent measurements.

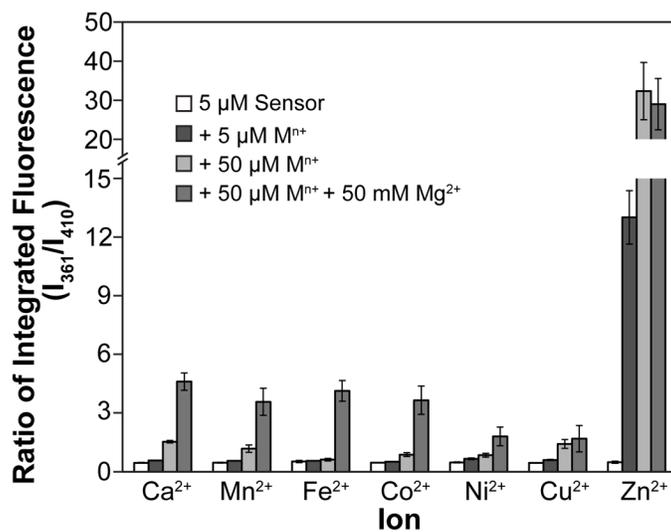


Figure S3. Metal selectivity plot for Mag-Se in aqueous buffer at pH 7.0. Ratios plotted correspond to the average of three independent measurements.

3. Magnesium binding properties of Mag-S and Mag-Se

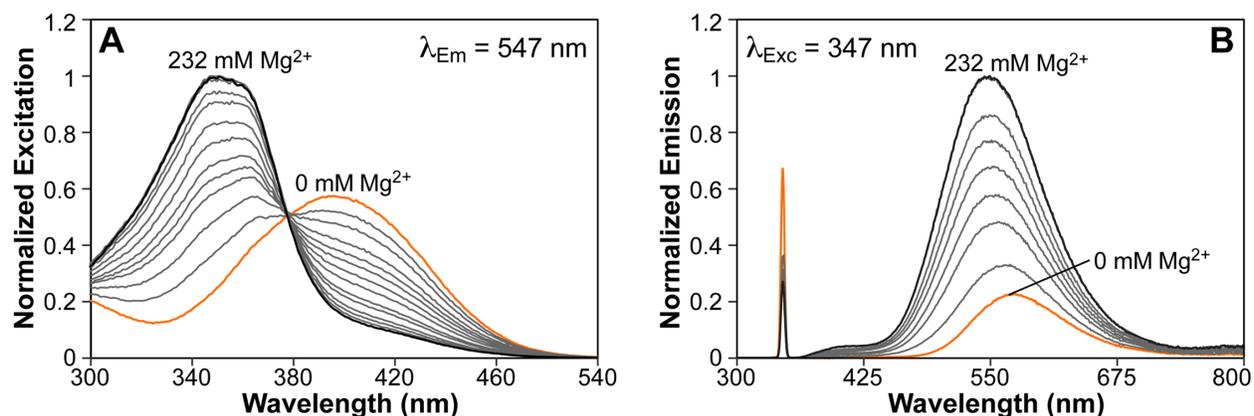


Figure S4. Representative fluorescence excitation (A) and emission (B) spectrum of a 2 μ M solution of Mag-S in aqueous buffer at pH 7.0, 25 $^{\circ}$ C, with increasing magnesium concentration.

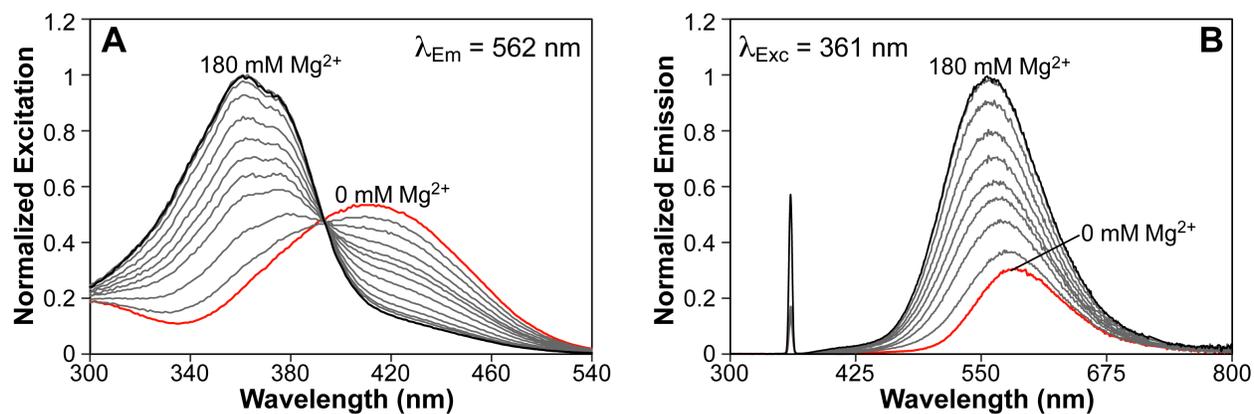


Figure S5. Representative fluorescence excitation (A) and emission (B) spectrum of a 2 μ M solution of Mag-Se in aqueous buffer at pH 7.0, 25 $^{\circ}$ C, with increasing magnesium concentration.

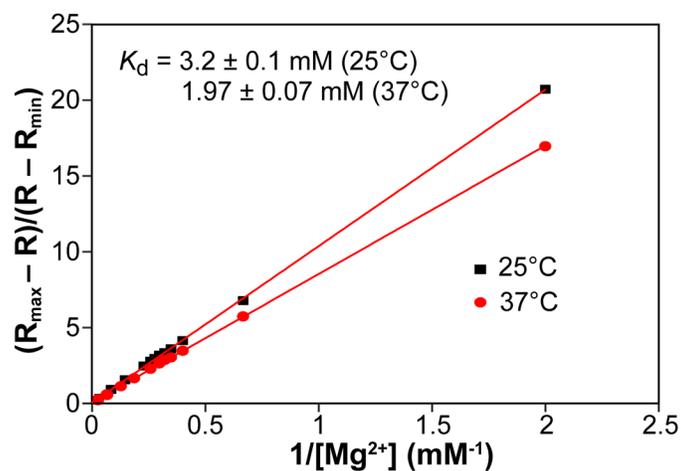


Figure S6. Double reciprocal plot for the changes in fluorescence of a 2 μM solution of Mag-S as a function of magnesium concentration in aqueous buffer at pH 7.0, at 25 and 37 $^\circ\text{C}$. Excitation wavelengths: $\lambda_1 = 347 \text{ nm}$, $\lambda_2 = 392 \text{ nm}$. Dissociation constants correspond to the average of three independent titrations.

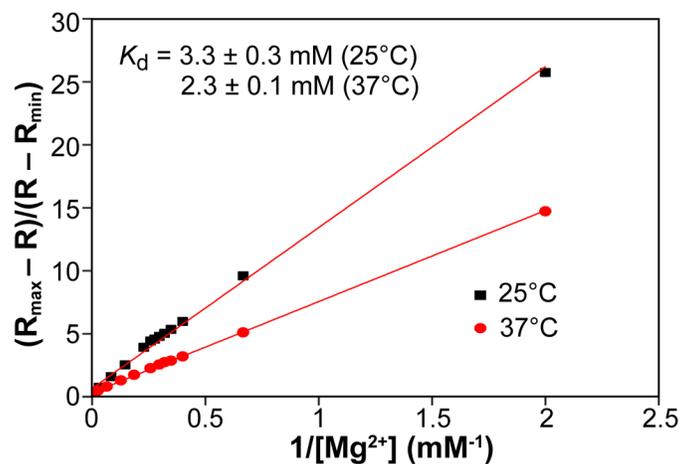


Figure S7. Double reciprocal plot for the changes in fluorescence of a 2 μM solution of Mag-Se as a function of magnesium concentration in aqueous buffer at pH 7.0, at 25 and 37 $^\circ\text{C}$. Excitation wavelengths: $\lambda_1 = 361 \text{ nm}$, $\lambda_2 = 410 \text{ nm}$. Dissociation constants correspond to the average of three independent titrations.

4. Calcium binding properties of Mag-S and Mag-Se

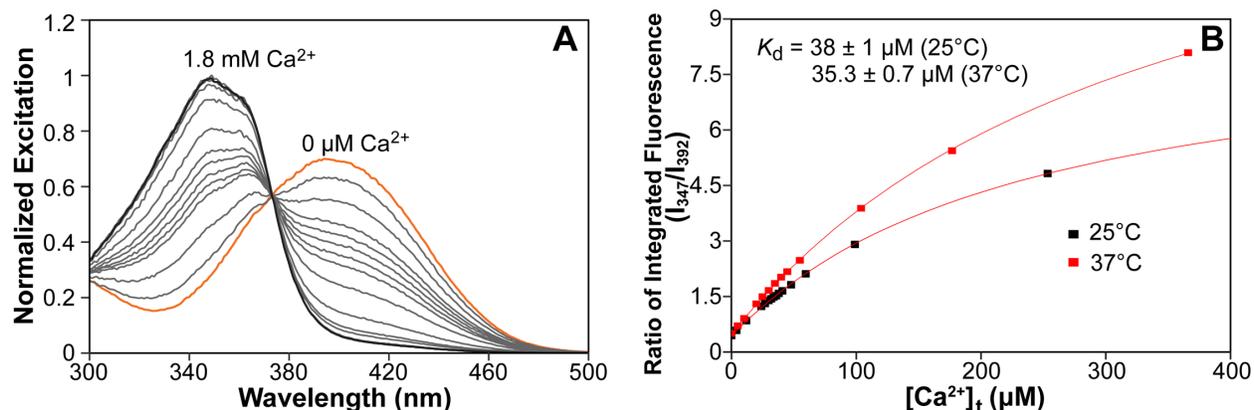


Figure S8. (A) Representative fluorescence excitation spectrum of a 2 μM solution of Mag-S in aqueous buffer at pH 7.0, 25 $^{\circ}\text{C}$, as a function of calcium concentration. (B) Representative isotherms for the binding of calcium to Mag-S in aqueous buffer at pH 7.0 at 25 and 37 $^{\circ}\text{C}$. Reported dissociation constants are the average of three independent titrations.

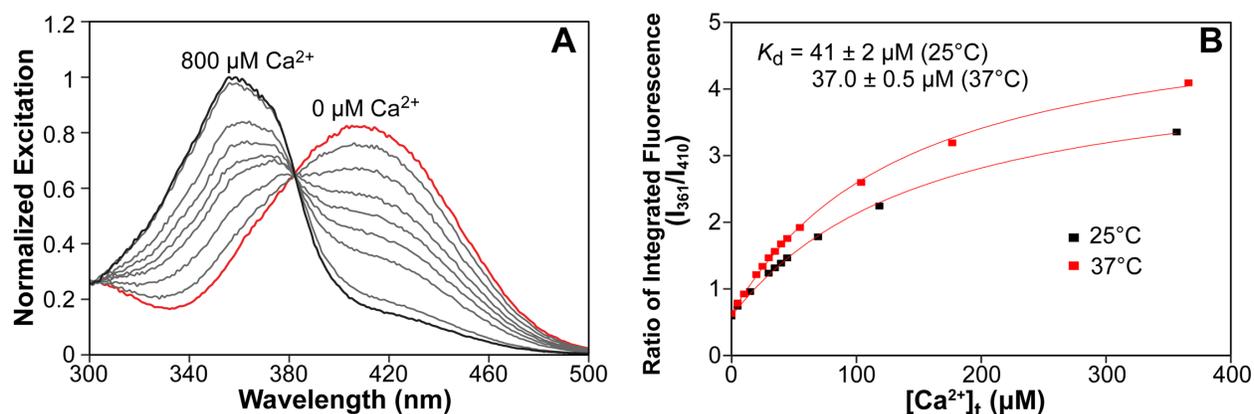


Figure S9. (A) Representative fluorescence excitation spectrum of a 2 μM solution of Mag-Se in aqueous buffer at pH 7.0, 25 $^{\circ}\text{C}$, as a function of calcium concentration. (B) Representative isotherms for the binding of calcium to Mag-Se in aqueous buffer at pH 7.0 at 25 and 37 $^{\circ}\text{C}$. Reported dissociation constants are the average of three independent titrations.

5. Zinc binding properties of Mag-S and Mag-Se

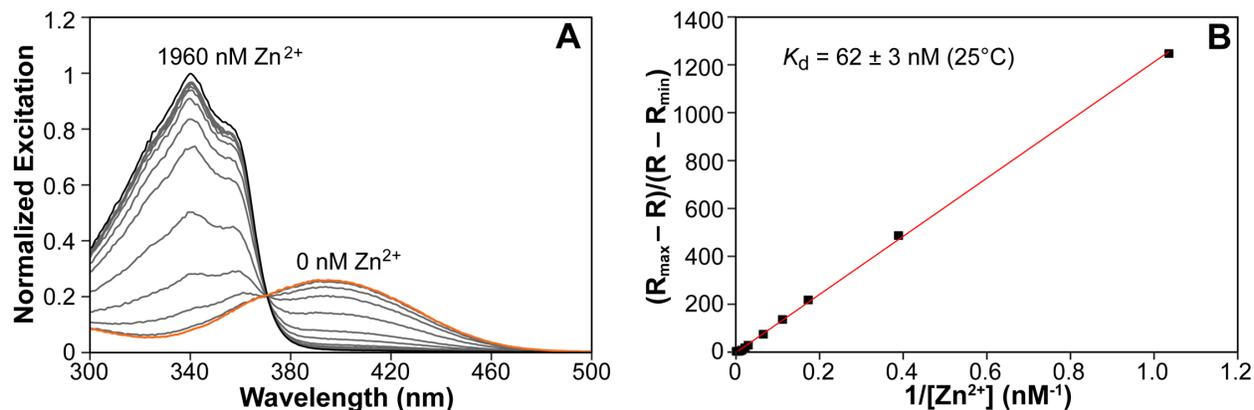


Figure S10. (A) Representative fluorescence excitation spectrum of a 1 μ M solution of Mag-S in aqueous buffer at pH 7.0, 25 °C, as a function of zinc concentration. (B) Representative isotherm for the binding of zinc to Mag-S in aqueous buffer at pH 7.0 at 25 °C; excitation wavelengths: $\lambda_1= 339$ nm, $\lambda_2= 392$ nm. Reported dissociation constants are the average of two independent titrations.

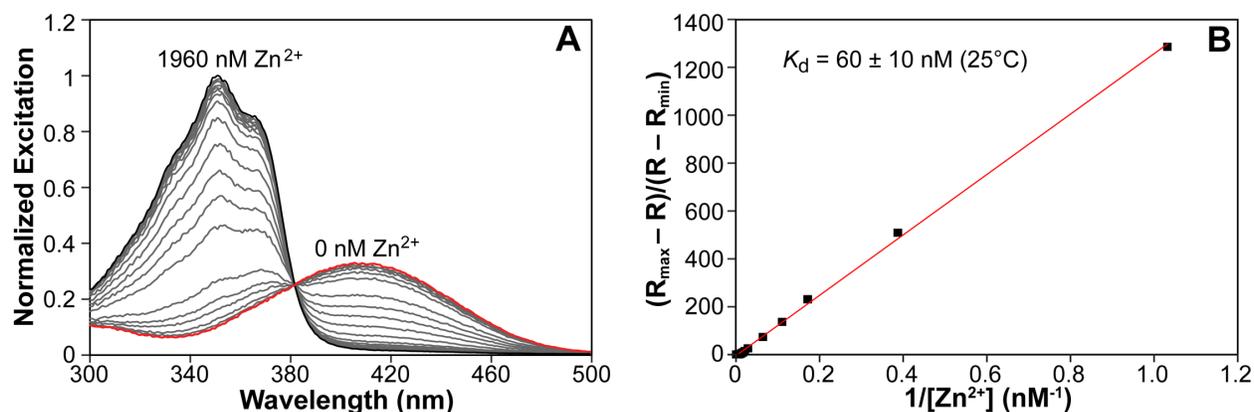


Figure S11. (A) Representative fluorescence excitation spectrum of a 1 μ M solution of Mag-Se in aqueous buffer at pH 7.0, 25 °C, as a function of zinc concentration. (B) Representative isotherm for the binding of zinc to Mag-S in aqueous buffer at pH 7.0 at 25 °C; excitation wavelengths: $\lambda_1= 351$ nm, $\lambda_2= 410$ nm. Reported dissociation constants are the average of three independent titrations.

6. Crystallographic data for compounds 3a and 3b

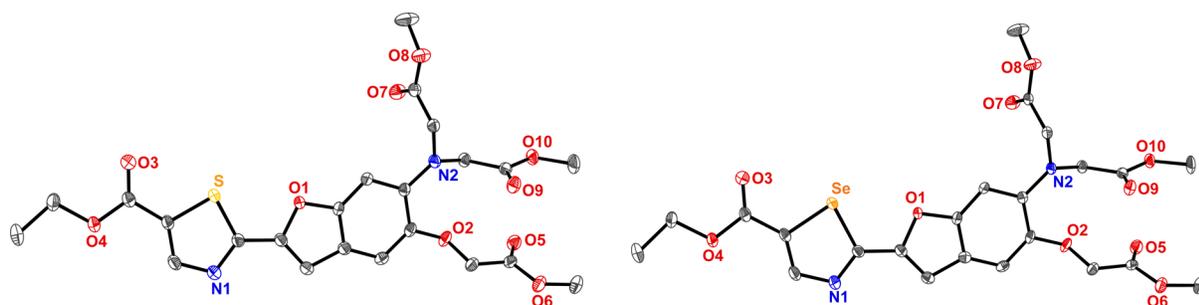


Figure S12. Molecular structure of Mag-S ester **3a** (left) and Mag-Se ester **3b** (right). ORTEP with 50% probability thermal ellipsoids.

Table S1. Crystal intensity, collection and refinement data

	Mag-S ester, 3a	Mag-Se ester, 3b
Crystal Lattice	Triclinic	Triclinic
Formula	C ₂₃ H ₂₄ N ₂ O ₁₀ S	C ₂₃ H ₂₄ N ₂ O ₁₀ Se
Formula weight	520.50	567.40
Space group	P $\bar{1}$	P $\bar{1}$
<i>a</i> /Å	10.0940(14)	10.0929(6)
<i>b</i> /Å	11.5565(16)	11.5641(7)
<i>c</i> /Å	11.7894(17)	11.7886(7)
α /°	67.376(2)	67.9280(10)
β /°	77.489(2)	77.7760(10)
γ /°	67.165(2)	67.5130(10)
<i>V</i> /Å ³	1166.3(3)	1174.40(12)
<i>Z</i>	2	2
Temperature (K)	100(2)	100(2)
Radiation (λ , Å)	0.71073	0.71073
ρ (calcd.), g cm ⁻³	1.482	1.605
μ (Mo K α), mm ⁻¹	0.202	1.661
θ max, deg.	28.44	26.37
Completeness to θ (%)	99.8	100.0
No. of data	5877	4797
No. of restraints	0	0
No. of parameters	325	325
<i>R</i> ₁ [<i>I</i> > 2 σ (<i>I</i>)]	0.0419	0.0341
<i>wR</i> ₂ [<i>I</i> > 2 σ (<i>I</i>)]	0.1060	0.0759
GOF	1.036	1.046

7. Fluorescence imaging of HeLa cells treated with Mag-S AM ester

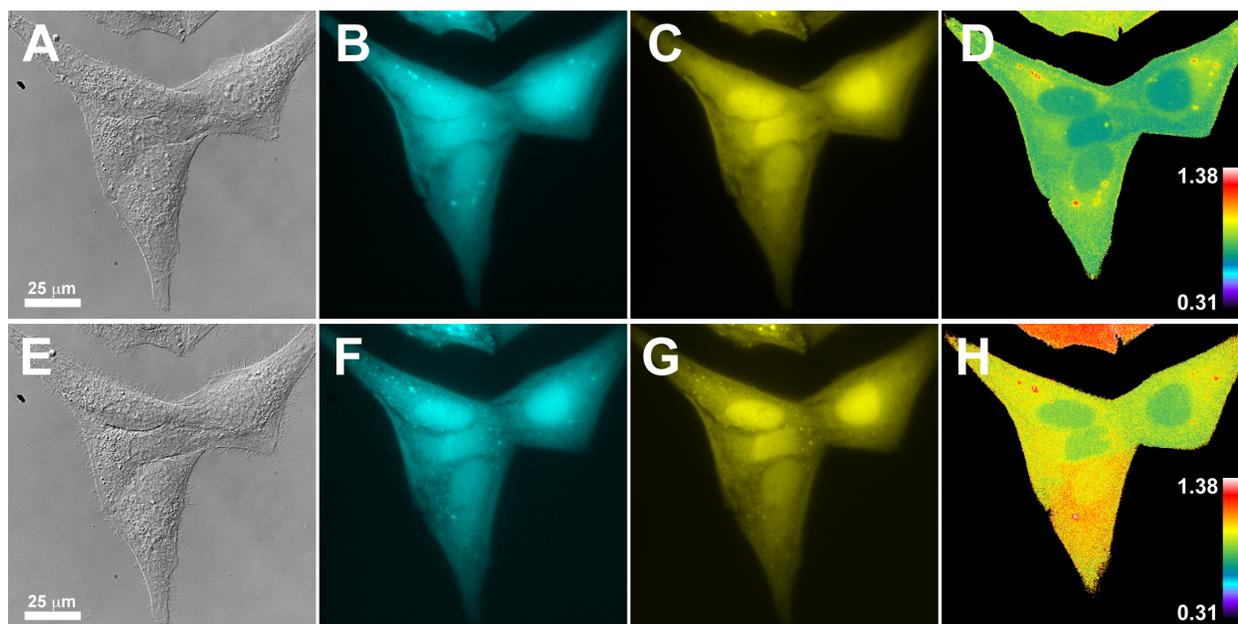


Figure S13. Fluorescence microscopy images of live HeLa cells treated with 2 μM Mag-S acetoxymethyl ester before (A-D) and after (E-H) 60 min incubation with 2.5 μM magnesium ionophore 4-bromo-A-23187 and 30 mM exogenous MgCl_2 . (A, E) DIC images; (B, F) fluorescence images from excitation of the metal-bound form of the indicator; (C, G) fluorescence images from excitation of the metal-free form of the indicator; (D, H) fluorescence ratio images, with scale bars representing uncalibrated fluorescence ratio.

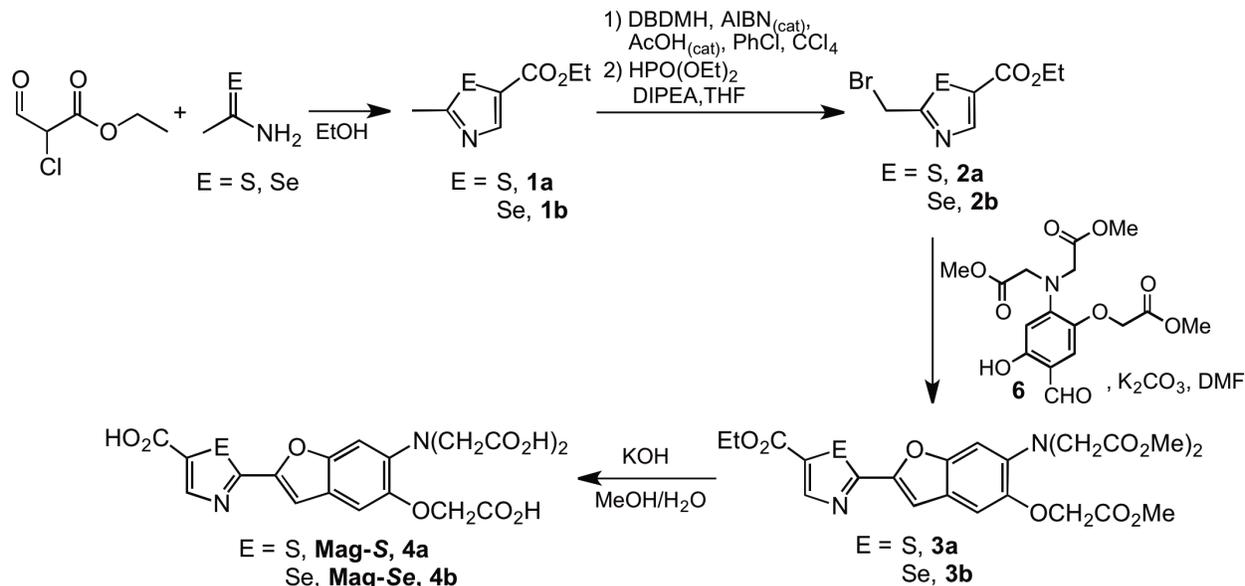
8. Experimental procedures

General Materials and Synthetic Methods

Selenoacetamide,¹ ethyl-2-methylthiazole-5-carboxylate,² and 2-amino-4-hydroxy-5-formylphenol-(*N,N,O*-triacetic acid methyl ester) (compound **6**),³ were synthesized using reported procedures. All other reagents were purchased from commercial sources and used as received. Solvents were purified and degassed by standard procedures. Analytical thin layer chromatography (TLC) was conducted on SorbTech polyester-backed 200 μm silica gel sheets. Preparative TLC was performed on SorbTech 1000 μm silica gel plates. NMR spectra were acquired on Bruker Avance 400, 500, and Avance III 600 MHz spectrometers. ^1H NMR chemical shifts are reported in ppm relative to SiMe_4 ($\delta = 0$) and were referenced internally with respect to residual protio impurity in the solvent ($\delta = 7.26$ for CHCl_3 , 4.79 for D_2O). ^{13}C NMR chemical shifts are reported in ppm relative to SiMe_4 ($\delta = 0$) and were referenced internally with respect to the solvent signal ($\delta = 77.16$ for CDCl_3). ^{13}C NMR spectra collected in D_2O were referenced using a sample of acetonitrile in water ($\delta = 1.47$ for methyl signal) as an external standard. ^{77}Se NMR chemical shifts are reported relative to neat Me_2Se ($\delta = 0$) and were referenced using Ph_2Se_2 in CDCl_3 as external standard ($\delta = 460$).⁴ Coupling constants are reported in Hz. Melting points were determined on Büchi 510 melting point apparatus. Low-resolution mass spectrometry was conducted on an Agilent 1100 Series Capillary LCMSD Trap XCT system or an Agilent 1100 Series LCMSD VL system with single quadrupole detector. High-resolution mass spectra (HRMS) were acquired on an Agilent 6224 Accurate-Mass TOF LC/MS using ES ionization.

Reverse-phased HPLC analyses were conducted on an Agilent 1260 system with UV-Vis and Fluorescence detection, using a Poroshell C18 reverse phase column (4.6×50 mm, 2.7 μm particle size) and a gradient of 10% to 100% acetonitrile/water (+ 0.1% trifluoroacetic acid) over 7 min.

Scheme 1



Synthesis of ethyl-2-bromomethylthiazole-5-carboxylate, **2a**

A mixture of ethyl-2-methylthiazole-5-carboxylate **1a** (900 mg, 5.26 mmol), 1,3-dibromo-5,5-dimethylhydantoin (3.00 g, 10.5 mmol) and azobisisobutyronitrile (AIBN, 285 mg, 1.74 mmol) in chlorobenzene (100 mL) and CCl₄ (100 mL), was treated with catalytic amount of glacial acetic acid (300 μL) and heated to 75 °C for 5 h. The hot reaction mixture was washed with water at 60 °C (5×60 mL), brine (60 mL) and then dried over Na₂SO₄. The solution was concentrated in vacuo and the residue was passed through a short column of silica gel eluting with 1:1 pentane/Et₂O. The eluate was concentrated in vacuo to a brown-yellow oil, containing a mixture of the product ethyl-2-bromomethylthiazole-5-carboxylate **2a** (R_f = 0.70 in 1:1 Et₂O/pentane) and ethyl-2-(dibromomethyl)-thiazole-5-carboxylate (R_f = 0.85 in 1:1 Et₂O/pentane). The mixture was redissolved in dry THF (4 mL) under inert atmosphere, cooled to 0 °C, and treated with diethylphosphite and diisopropylethylamine (1.5 mol equiv. each, based on the amount of dibrominated species present in the mixture, as determined by ¹H NMR spectroscopy). The solution was stirred at 0 °C until complete disappearance of the dibrominated species by TLC (~5 h). At this point the reaction was treated with water (10 mL) and extracted with Et₂O (3×40 mL). The combined extracts were washed with 1 M HCl, brine, dried over Na₂SO₄, and evaporated in vacuo. The product ethyl-2-bromomethylthiazole-5-carboxylate **2a** was obtained as a yellow oil after purification by flash chromatography on silica gel, using 1:2 Et₂O/pentane (260 mg, 32% from ethyl-2-methylthiazole-5-carboxylate). ¹H NMR (600 MHz, CDCl₃, δ): 8.31 (s, 1H), 4.71 (s, 2H, -CH₂Br), 4.37 (q, ³J = 7 Hz, 2H, -CO₂CH₂CH₃), 1.38 (t, ³J = 7 Hz, 3H, -CO₂CH₂CH₃). ¹³C{¹H} NMR (CDCl₃, δ): 171.3, 161.0, 148.3, 131.7, 62.0, 26.3, 14.4. ESI-MS (m/z): [M+H⁺] calcd for C₇H₈BrNO₂S, 250.0; found 250.0

Synthesis of Mag-S methyl ester, **3a**

A mixture of compound **6** (200 mg, 0.542 mmol), ethyl-2-bromomethylthiazole-5-carboxylate **2a** (217 mg, 0.867 mmol), and potassium carbonate (337 mg, 2.44 mmol) in dry dimethylformamide

(2.0 mL) was heated to 100°C under inert atmosphere for 1.5 h. After allowing to cool to room temperature, the reaction was poured into water (~30 mL), acidified with cold 1 M HCl, and extracted with 1:1 Et₂O/EtOAc (4×50 mL). The combined extracts were dried over Na₂SO₄ and concentrated *in vacuo*. The product ester **3a** (182 mg, 65 %) was obtained as a yellow solid after purification by flash chromatography on silica gel, using 1:1 hexanes/EtOAc. *Note*: on occasion, small amounts of residual compound **6** co-eluted during chromatography. This material may be removed by repeated washing of a methylene chloride solution of the compound with cold aqueous 0.1 M NaOH. Mp 166-169 °C. ¹H NMR (500 MHz, CDCl₃, δ): 8.41 (s, 1H), 7.34 (s, 1H), 7.10 (s, 1H), 7.05 (s, 1H), 4.70 (s, 2H, -OCH₂CO₂Me), 4.40 (q, ³J = 7 Hz, 2H, -CO₂CH₂CH₃), 4.27 (s, 4H, -NCH₂CO₂Me), 3.80 (s, 3H, -CO₂CH₃), 3.75 (s, 6H, -CO₂CH₃), 1.41 (t, ³J = 7 Hz, 3H, -CO₂CH₂CH₃). ¹³C{¹H} NMR (CDCl₃, δ): 171.5, 169.3, 162.7, 161.4, 151.92, 149.5, 149.4, 148.2, 140.6, 128.9, 122.3, 107.3, 107.1, 102.9, 67.0, 61.9, 54.0, 52.3, 52.1, 14.4. HR-TOF-MS (*m/z*): [M+H⁺] calcd for C₂₃H₂₄N₂O₁₀S, 521.1224; found 521.1244. Anal. calcd for C₂₃H₂₄N₂O₁₀S: C, 53.07; H, 4.65; N, 5.38. Found: C, 52.94; H, 4.38; N, 5.34.

Synthesis of Mag-S acid, **4a**

Compound **3a** (10.0 mg, 0.019 mmol) in methanol (200 μL) was treated with aqueous potassium hydroxide (200 μL, 1.2 M, 0.24 mmol) and stirred at room temperature for 24 h. The reaction solution was cooled in ice and treated with cold, aqueous hydrochloric acid (200 μL, 2.0 M, 0.4 mmol). The resulting precipitate was collected by centrifugal filtration, washed with cold 9:1 methanol:water (1×100 μL) followed by cold methanol (1×100 μL), and dried under high vacuum. The product acid **4a** was isolated as a yellow-orange solid (7.8 mg, 90%). ¹H NMR (500 MHz, D₂O, 5% NaOD, δ): 7.53 (s, 1H), 6.89 (s, 1H), 6.77 (s, 1H), 6.55 (s, 1H), 4.13 (s, 2H, -OCH₂CO₂H), 3.50 (s, 4H, -NCH₂CO₂H). ¹³C{¹H} NMR (D₂O, 5% NaOD, δ): 179.2, 176.8, 167.9, 160.9, 150.6, 148.54, 148.58, 145.7, 140.8, 136.9, 122.9, 107.3, 104.0, 102.6, 67.4, 58.4. ESI-MS (*m/z*): [M-H⁻] calcd for C₁₈H₁₄N₂O₁₀S, 449.0; found for C₁₈H₁₄N₂O₁₀S, 449.1.

Preparation of Mag-S quantitative stock solutions for fluorescence spectroscopy

A sample of Mag-S methyl ester **3a** (5.21 mg, 0.0100 mmol) in methanol (100 μL) was treated with aqueous potassium hydroxide (100 μL, 1.2 M, 0.12 mmol) and stirred at room temperature for 24 h. The solution was transferred quantitatively to a volumetric flask and diluted with buffer at pH 7.0 (50 mM PIPES, 100 mM KCl) to a final volume of 5 mL. The stock solution was divided into smaller aliquots, flash frozen in liquid nitrogen, and stored below -20 °C.

Synthesis of ethyl-2-methylselenazole-5-carboxylate, **1b**

A mixture of ethyl-2-chloro-3-oxopropanoate (5.28 g, 35.1 mmol) and freshly prepared selenoacetamide (5.35 g, 43.8 mmol) in anhydrous acetonitrile (23 mL) was stirred for 15 min and then heated to reflux for 6 h. After cooling, the resulting suspension was concentrated *in vacuo* to a red-brown oil and mixed with water and Et₂O (~40 mL each). The mixture was treated with solid Na₂CO₃ until pH 8 was reached and then filtered to remove small amounts of solids. The organic phase was separated and the aqueous phase was extracted three times with portions of Et₂O. The combined extracts were dried over Na₂SO₄, and concentrated *in vacuo*. The product ethyl-2-methylselenazole-5-carboxylate **1b** was obtained as a light brown oil after purification by column chromatography on silica gel, using 1:1 Et₂O/pentane (2.02 g, 26% yield, R_f = 0.45 in 1:1 Et₂O/pentane). ¹H NMR (500 MHz, CDCl₃, δ): 8.25 (s, 1H), 4.34 (q, ³J = 7 Hz, 2H, -CO₂CH₂CH₃), 2.75 (s, 3H, -Me), 1.36 (t, ³J = 7 Hz, 3H, -CO₂CH₂CH₃). ¹³C{¹H} NMR (CDCl₃, δ): 180.1, 163.1, 148.5, 135.4, 61.7, 23.8, 14.4. ⁷⁷Se NMR (CDCl₃, δ): 739.14 (qd, ³J_{CH} = 8 Hz, ³J_{CH} = 2 Hz). ESI-MS (*m/z*): [M+H⁺] calcd for C₇H₉NO₂Se, 220.0; found 220.0 (isotopic pattern matches predicted)

Synthesis of ethyl-2-bromomethylselenazole-5-carboxylate, **2b**

A mixture of ethyl-2-methylselenazole-5-carboxylate **1b** (600 mg, 2.75 mmol), 1,3-dibromo-5,5-dimethylhydantoin (1.57 g, 5.49 mmol) and AIBN (150 mg, 0.91 mmol) in chlorobenzene (28 mL) and CCl₄ (28 mL) was heated to 75 °C for 3 h. The hot reaction mixture was washed with water at 60 °C (5×60 mL), brine (60 mL) and then dried over Na₂SO₄. The solution was concentrated in vacuo and the residue was passed through a short column of silica gel eluting with 1:1 pentane/Et₂O. The eluate was concentrated in vacuo to a brown-yellow oil, containing a mixture of the product ethyl-2-bromomethylselenazole-5-carboxylate **2b** (R_f = 0.70 in 1:1 Et₂O/pentane) and ethyl-2-(dibromomethyl)-selenazole-5-carboxylate (R_f = 0.85 in 1:1 Et₂O/pentane). The mixture was redissolved in dry THF (4 mL) under inert atmosphere, cooled to 0 °C, and treated with diethylphosphite and diisopropylethylamine (1.2 mol equiv. each, based on the amount the dibrominated species present in the mixture as determined by ¹H NMR spectroscopy). The solution was stirred at 0 °C until complete disappearance of the dibrominated species by TLC (~5 h). At this point the reaction was treated with water (10 mL) and extracted with Et₂O (3×40 mL). The combined extracts were washed with 1 M HCl, brine, dried over Na₂SO₄, and evaporated in vacuo. The product ethyl-2-bromomethylselenazole-5-carboxylate **2b** was obtained as a yellow oil after purification by flash chromatography on silica gel, using 1:2 Et₂O/pentane (260 mg, 32% from ethyl-2-methylselenazole-5-carboxylate, R_f = 0.70 in 1:1 Et₂O/pentane). ¹H NMR (500 MHz, CDCl₃, δ): 8.34 (s, 1H), 4.68 (s, 2H, -CH₂Br), 4.35 (q, ³J = 7 Hz, 2H, -CO₂CH₂CH₃), 1.38 (t, ³J = 7 Hz, 3H, -CO₂CH₂CH₃). ¹³C{¹H} NMR (CDCl₃, δ): 180.1, 162.8, 148.7, 138.2, 62.1, 30.4, 14.4. ⁷⁷Se NMR (CDCl₃, δ): 749.4 (t, ³J = 7 Hz). ESI-MS (*m/z*): [M+Na⁺] calcd for C₇H₈BrNO₂Se, 319.9; found 320.1.

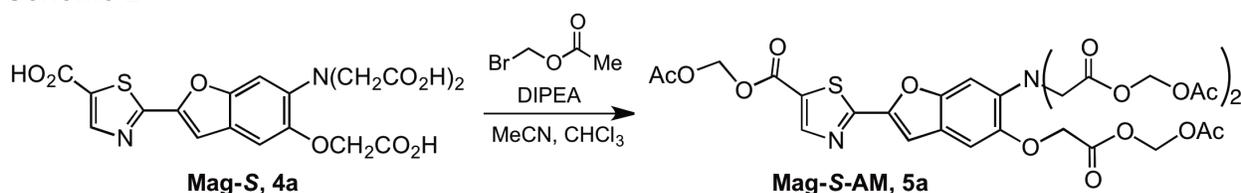
Synthesis of Mag-Se methyl ester, **3b**

A mixture of compound **6** (56.7 mg, 0.15 mmol), ethyl-2-bromomethylselenazole-5-carboxylate **2b** (57.0 mg, 0.19 mmol) and potassium carbonate (96.0 mg, 0.69 mmol) in dry dimethylformamide (1 mL) was heated to 100 °C under inert atmosphere for 2 h. After cooling to room temperature, the reaction was poured into ice-water (~30 mL), acidified with cold 1 M HCl, and extracted with 1:1 Et₂O/EtOAc (3×50 mL). The combined extracts were dried over Na₂SO₄, and concentrated *in vacuo*. The product ester **3b** was obtained as a yellow solid after purification by flash chromatography on silica gel, using 1:1 hexanes/EtOAc (17.0 mg, 20%, R_f = 0.38 in 1:1 hexanes/EtOAc). *Note*: on occasions, small amounts of residual compound **6** co-eluted during chromatography. This compound may be removed by repeated washing of a methylene chloride solution of the product with cold aqueous 0.1 M NaOH. ¹H NMR (500 MHz, CDCl₃, δ): 8.46 (s, 1H), 7.36 (s, 1H), 7.09 (s, 1H), 7.05 (s, 1H), 4.70 (s, 2H, -OCH₂CO₂Me), 4.38 (q, ³J = 7 Hz, 2H, -CO₂CH₂CH₃), 4.27 (s, 4H, -NCH₂CO₂Me), 3.80 (s, 3H, -CO₂CH₃), 3.75 (s, 6H, -CO₂CH₃), 1.40 (t, ³J = 7 Hz, 3H, -CO₂CH₂CH₃). ¹³C{¹H} NMR (CDCl₃, δ): 171.5, 169.3, 169.1, 163.1, 152.13, 152.17, 150.3, 148.3, 140.7, 134.5, 122.6, 107.1, 106.5, 102.9, 67.0, 62.0, 54.0, 52.4, 52.1, 14.5. ⁷⁷Se NMR (CDCl₃, δ): 707.7 (s). HR-TOF-MS (*m/z*): [M+H⁺] calcd for C₂₃H₂₄N₂O₁₀Se, 569.0671; found 569.0653. Anal. calcd for C₂₃H₂₄N₂O₁₀Se: C, 48.69; H, 4.26; N, 4.94. Found: C, 48.62; H, 3.98; N, 4.90.

Preparation of Mag-Se acid, **4b**, quantitative stock solutions for fluorescence spectroscopy

A sample of Mag-Se methyl ester **3b** (5.67 mg, 0.0100 mmol) in methanol (100 μL) was treated with aqueous potassium hydroxide (100 μL, 1.2 M, 0.12 mmol) and stirred at room temperature for 24 h. The solution was quantitatively transferred to a volumetric flask and diluted with buffer at pH 7.0 (50 mM PIPES, 100 mM KCl) to a final volume of 5 mL. The stock solution was divided into small aliquots, flash frozen in liquid nitrogen, and stored below -20 °C.

Scheme 2



Synthesis of Mag-S acetoxymethyl ester, 5a

A mixture of compound Mag-S acid **4a** (25 mg, 0.056 mmol) in 1:2 acetonitrile:chloroform (1.3 mL) was treated with diisopropyl ethylamine (100 μ L, 0.57 mmol) and bromomethylacetate (65 μ L, 0.66 mmol) and stirred at room temperature for 48 h protected from light. The mixture was concentrated in vacuo and the residue was taken up in dichloromethane, transferred to a preparative TLC plate, and eluted with 1:2 mixture of hexanes/EtOAc. The product acetoxymethyl ester **5a** (R_f = 0.26 in 1:1 hexanes/EtOAc) was isolated as a yellow solid (17 mg, 41% yield). ^1H NMR (600 MHz, CDCl_3 , δ): 8.48 (s, 1H), 7.39 (s, 1H), 7.09 (s, 1H), 7.07 (s, 1H), 5.97 (s, 2H), 5.83 (s, 2H), 5.80 (s, 4H), 4.74 (s, 2H), 4.30 (s, 4H), 2.16 (s, 3H), 2.11 (s, 9H). $^{13}\text{C}\{^1\text{H}\}$ NMR (CDCl_3 , δ): 169.8, 169.67, 169.64, 169.58, 167.7, 163.6, 160.1, 152.0, 150.9, 149.4, 148.2, 140.3, 127.3, 122.6, 107.8, 107.5, 103.2, 79.7, 79.6, 79.5, 66.7, 53.9, 20.88, 20.81, 20.76. HR-TOF-MS (m/z): $[\text{M}+\text{H}^+]$ calcd for $\text{C}_{30}\text{H}_{30}\text{N}_2\text{O}_{18}\text{S}$, 736.1287; found 739.1276. $[\text{M}+\text{Na}^+]$ calcd 761.1107; found 761.1099.

X-ray Data Collection and Structure Refinement

Crystals of esters **3a** and **3b** were grown by slow diffusion of pentane into solutions of the compounds in dichloromethane and diethylether. Single crystals suitable for X-ray analysis were coated with Paratone-N oil and mounted on fiber loops for analysis. Diffraction data were collected at 100(2) K on a Bruker APEXII CCD X-ray diffractometer performing ϕ scans using graphite-monochromated Mo $\text{K}\alpha$ radiation. Structures were solved by direct methods and standard difference map techniques, and were refined by full-matrix least-squares procedures on F^2 with SHELXTL (version 6.12). Crystallographic data collection and refinement parameters are presented in Table S1.

General Spectroscopic Methods

All aqueous solutions were prepared using de-ionized water having a resistivity of 18 $\text{M}\Omega/\text{cm}$. Other solvents were supplied by commercial vendors and used as received. Piperazine-*N,N*-bis(2-ethanesulfonic acid) (PIPES), 99.999% KCl, 99.999% MgCl_2 , 99.999% ZnCl_2 , 99.99% CaCl_2 , and high-purity 25% HCl, 45% KOH, and 50% NaOH were purchased from Sigma Aldrich. Stock solutions of the sensors in their acid form were stored at -20°C in 100-200 μL aliquots, and thawed immediately before each experiment. Measurements at pH 7.0 were conducted in aqueous buffer containing 50 mM PIPES and 100 mM KCl. Buffers were treated with Chelex resin (Bio-Rad) according to the manufacturer's protocol, to remove adventitious metal ions unless otherwise noted. Measurements of pH were conducted using a Mettler Toledo FE20 with glass electrode. UV-visible spectra were acquired on a Cary 100 spectrophotometer using quartz cuvettes from Starna (1 cm path length). Fluorescence spectra were acquired on a QuantaMaster 40 Photon Technology International spectrofluorometer equipped with xenon lamp source, emission and excitation monochromators, excitation correction unit, and PMT detector. Emission spectra were corrected for the detector wavelength-dependent response. The excitation spectra were corrected for the wavelength-dependent lamp intensity. Measurements were conducted at $25.0 \pm 0.1^\circ\text{C}$ or $37.0 \pm 0.1^\circ\text{C}$. Extinction coefficients were determined in the 0-11 μM range in aqueous buffer at pH 7.0, in the absence or presence of 100 mM Mg^{2+} for the metal-free and -bound forms of the sensor, respectively. Fluorescence

quantum yields were determined using 0.2-1.0 μM solutions of the sensor in aqueous buffer at pH 7.0, exciting at the reported excitation maxima for each compound. Solutions of quinine in 0.5 M aqueous sulfuric acid, with a reported quantum yield of 0.546 upon excitation at 347 nm, were used as standards. Fluorescence emission spectra were integrated from 360 to 680 nm.

Determination of metal dissociation constants

Fluorescence titrations with Mg^{2+} and Ca^{2+} were conducted using 2 μM solutions of the sensor in aqueous buffer at pH 7.0, covering a range of 0-375 mM for $[\text{Mg}^{2+}]_t$ and 0-50 mM for $[\text{Ca}^{2+}]_t$. Excess metal was employed to confirm the saturation value. Magnesium was added from a 1000 mM MgCl_2 stock solution. Calcium was added from a 10 mM and then 1000 mM CaCl_2 stock solution. For each titration, the metal stock solution was treated with an appropriate amount of the sensor to match the concentration in the cuvette and prevent sensor dilution throughout the experiment. Fluorescence titrations with Zn^{2+} were conducted using 1 μM solutions of the sensor in aqueous buffer at pH 7.0. Metal concentration was adjusted using a $\text{Zn}^{2+}/\text{EGTA}$ buffered system, with 1 mM EGTA and total concentration of Zn^{2+} (from ZnCl_2) ranging from 0 to 1 mM.

Apparent K_d values for magnesium dissociation were obtained using equation S1,⁵ where R is the ratio of integrated fluorescence intensity upon excitation at two wavelengths ($R=I_{\lambda_1}/I_{\lambda_2}$), R_{\min} is the fluorescence ratio for the metal-free sensor, R_{\max} is the fluorescence ratio of the metal-saturated sensor, and S_{f2} and S_{b2} are proportionality coefficients for the fluorescence of the metal-free and -bound forms of the sensor, respectively, upon excitation at λ_2 .

$$[\text{Mg}^{2+}] = K_d \frac{R - R_{\min} S_{f2}}{R_{\max} - R S_{b2}} \quad (\text{S1})$$

Linear fits of plots of $(R_{\max}-R)/(R-R_{\min})$ vs. $1/[\text{Mg}^{2+}]$ were employed, using the approximation $[\text{Mg}^{2+}] \approx [\text{Mg}^{2+}]_t$. Reported K_d values correspond to averages of three independent titrations at each temperature.

Apparent K_d values for calcium dissociation were obtained from non-linear plots of the fluorescence ratio R versus total calcium concentration according to equation S2, with values of $[\text{Ca}^{2+}]$ obtained by solving simultaneously equation S3.⁶ Reported K_d values correspond to averages of three independent titrations at each temperature.

$$R = \frac{R_{\max}[\text{Ca}^{2+}] + R_{\min}(K_d S_{f2} / S_{b2})}{[\text{Ca}^{2+}] + (K_d S_{f2} / S_{b2})} \quad (\text{S2})$$

$$[\text{Ca}^{2+}] + ([\text{Sensor}]_t - [\text{Ca}^{2+}]_t + K_d)[\text{Ca}^{2+}] - K_d[\text{Ca}^{2+}]_t = 0 \quad (\text{S3})$$

Apparent K_d values for zinc dissociation were obtained using equation S4,⁵ from linear regression using $(R_{\max}-R)/(R-R_{\min})$ as a function of $1/[\text{Zn}^{2+}]$. Free concentrations of zinc were calculated from values of total zinc concentration using tabulated $\text{p}K_a$ values and zinc stability constants for EGTA⁷ at 25 °C and 0.1 ionic strength.

$$[\text{Zn}^{2+}] = K_d \frac{R - R_{\min} S_{f2}}{R_{\max} - R S_{b2}} \quad (\text{S4})$$

Metal selectivity studies

Metal selectivity measurements were performed using 5 μM sensor solution in aqueous buffer at pH 7.0, treated with either CaCl_2 , MnCl_2 , $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, CoCl_2 , NiCl_2 , CuCl_2 , or ZnCl_2 in aqueous buffer for a final concentration of 5 μM $\text{M}^{\text{n}+}$, 50 μM $\text{M}^{\text{n}+}$, or 50 μM $\text{M}^{\text{n}+}$ and 50 mM MgCl_2 .

Cell culture and staining protocols

HeLa cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in 5% CO_2 humidified atmosphere. Cells were seeded in 35 mm glass bottom cell culture dishes and allowed to grow to 40-60% confluence prior to fluorescence imaging. A solution of the sensor in its acetoxymethyl ester form (2 mM in DMSO) was diluted 1:1 by volume with the non-ionic surfactant Pluronic F127 (20% solution in DMSO, Molecular Probes) immediately before use. For sensor loading, cells were washed with FBS-free DMEM (1 x 2 mL) and then incubated at 25 °C for 25 min in FBS-free DMEM (2 mL) containing the desired concentration of the sensor (2 μM). After loading, the medium was replaced with fresh FBS-free DMEM (2 mL) and allowed to stand for an additional 25 minutes at 25 °C to complete de-esterification of the sensor. Prior to imaging, the cells were washed with Hank's balanced salt solution (HBSS) without calcium and magnesium (1 x 2 mL) and bathed in HBSS without calcium and magnesium (1 mL) for image acquisition. To confirm the sensor's response to magnesium, the imaging medium was supplemented with MgCl_2 and the non-fluorescent ionophore 4-bromo A-23187 (Molecular Probes) for a total concentration of 30 mM and 2.5 μM , respectively.

Fluorescence Imaging

Fluorescence imaging experiments were performed on a Leica DMI6000B inverted fluorescence microscope equipped with a Hamamatsu ORCA-Flash 4.0 CCD camera, scanning stage, high-speed filter wheel for excitation filters and a mercury metal halide external light source. For ratiometric imaging of Mag-S sensor, a custom filter set consisting of 340/12 and 387/15 excitation filters, 425 nm dichroic, and 500 nm long pass emission filter (Chroma), was employed. The microscope was operated with Leica LAS AF software. The exposure time for acquisition of fluorescence images was kept constant among series of images. Image processing was performed with Metamorph 7.7.0.0 software. For fluorescence ratio images, background subtraction and threshold was applied on individual channel images prior ratio calculation. For comparison, images before and after treatment with excess magnesium and ionophore were acquired and processed under identical conditions, and are shown on the same uncalibrated ratio scale.

9. NMR spectroscopy and chromatographic data for new compounds

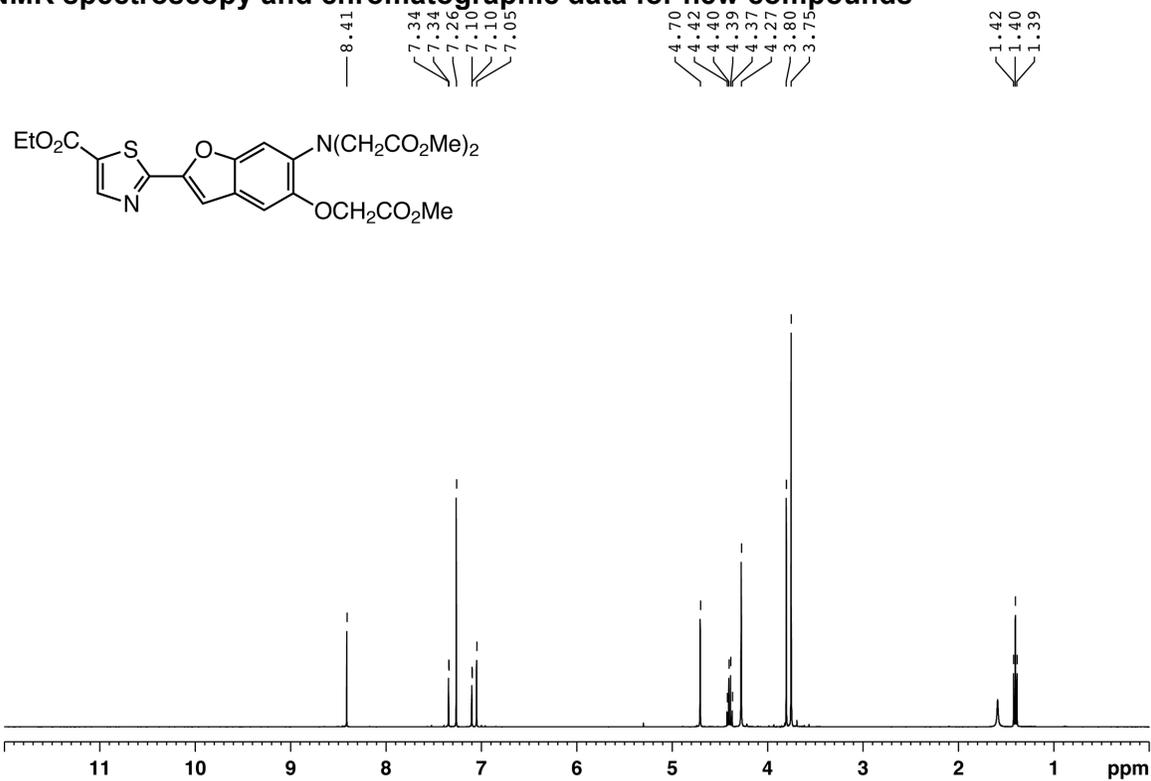


Figure S14. ¹H NMR spectrum of Mag-S methyl ester, 3a, in CDCl₃.

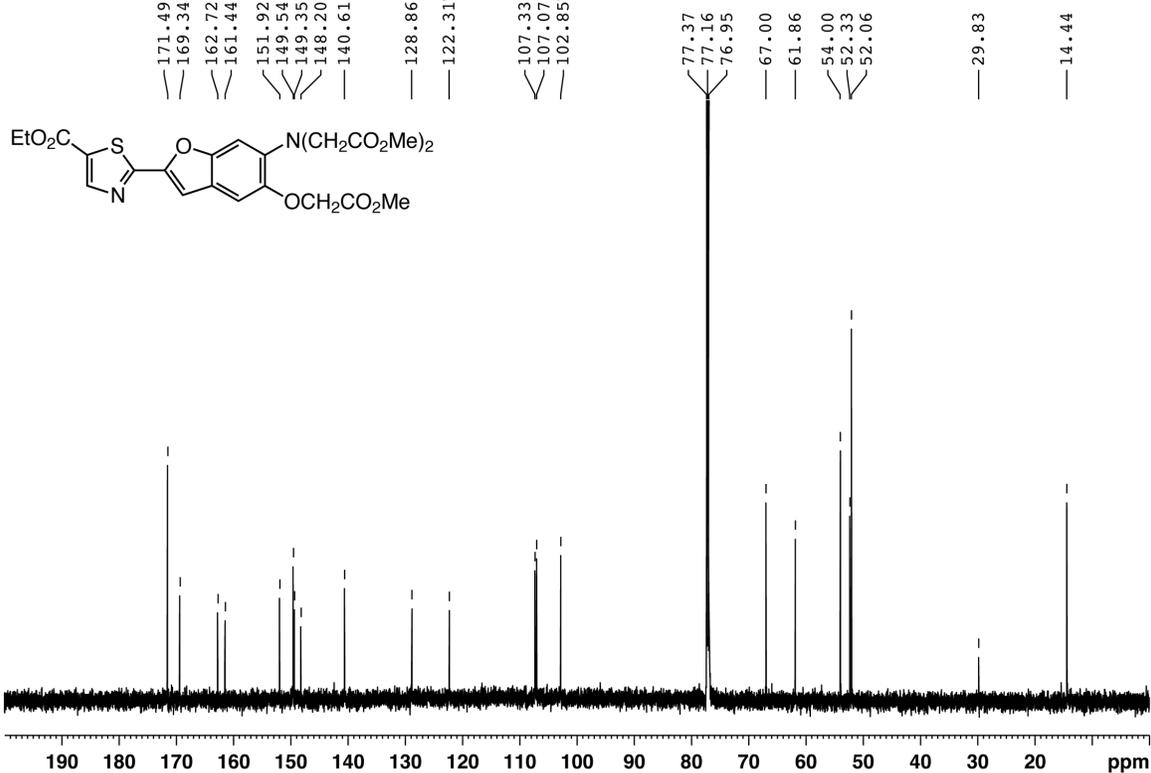


Figure S15. ¹³C{¹H} NMR spectrum of Mag-S methyl ester, 3a, in CDCl₃.

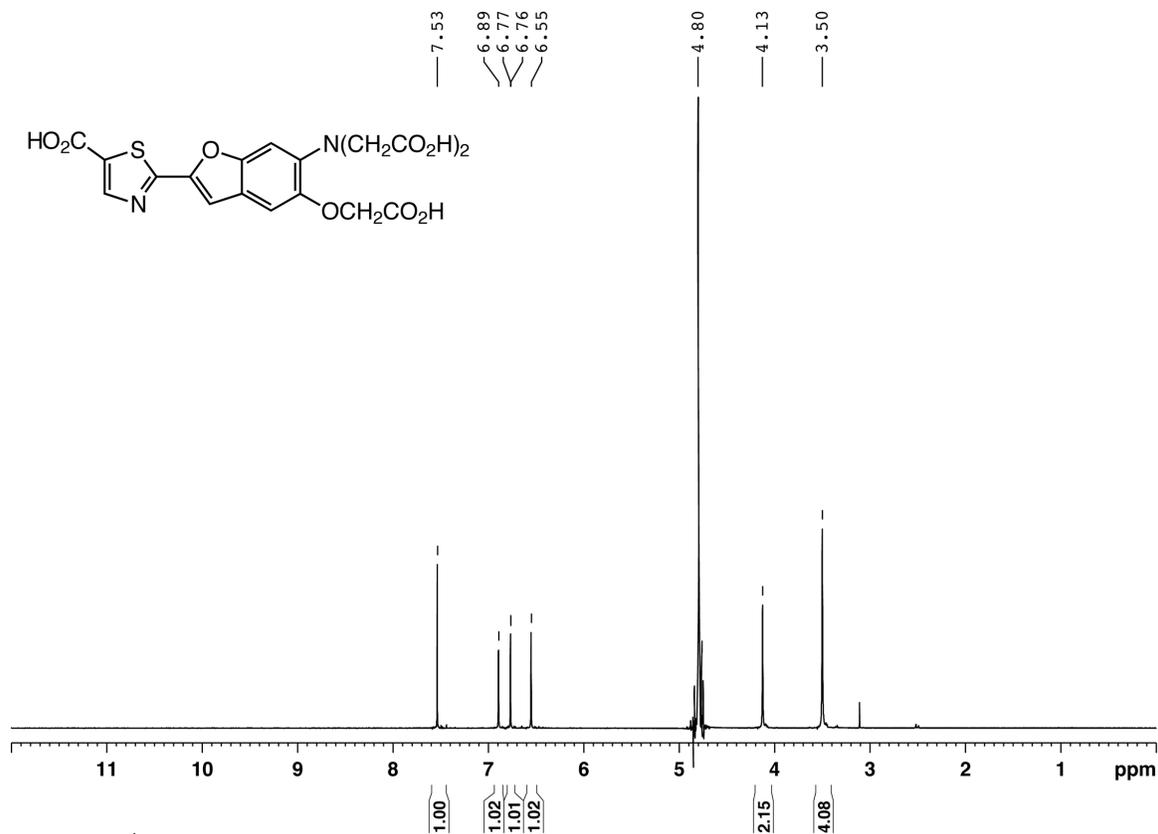


Figure S16. ¹H NMR spectrum of Mag-S in D₂O.

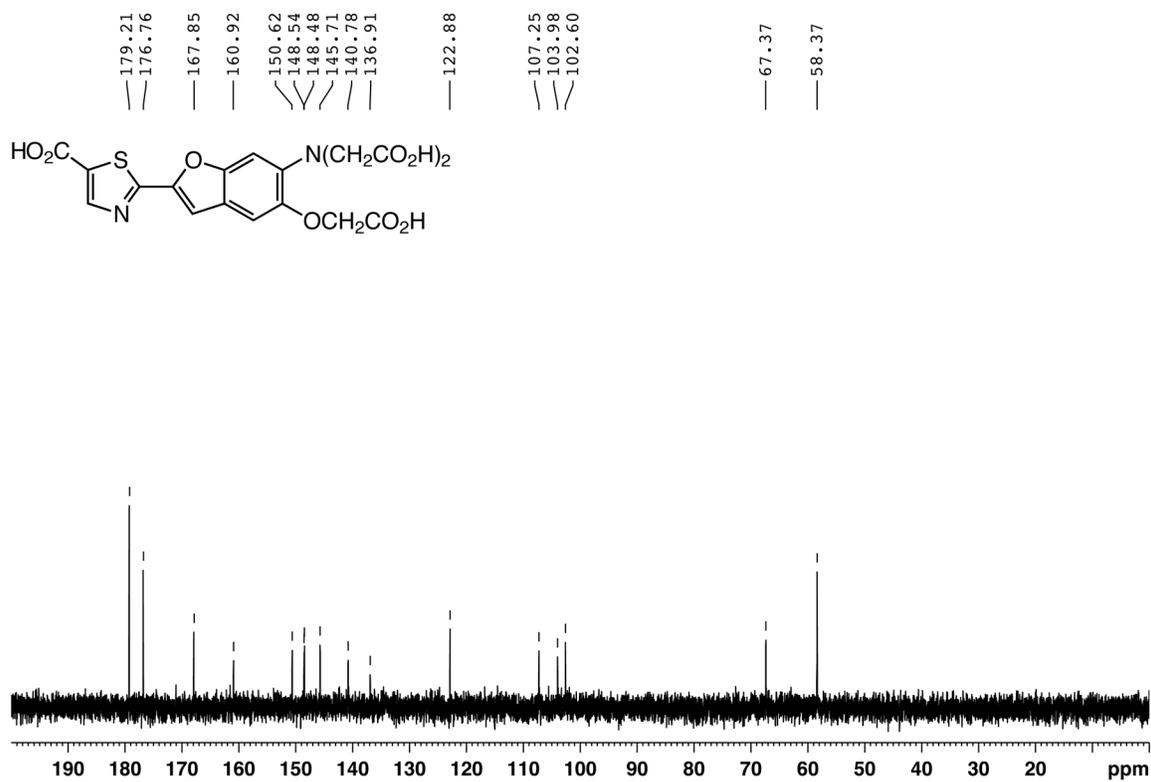


Figure S17. ¹³C{¹H} NMR spectrum of Mag-S in D₂O.

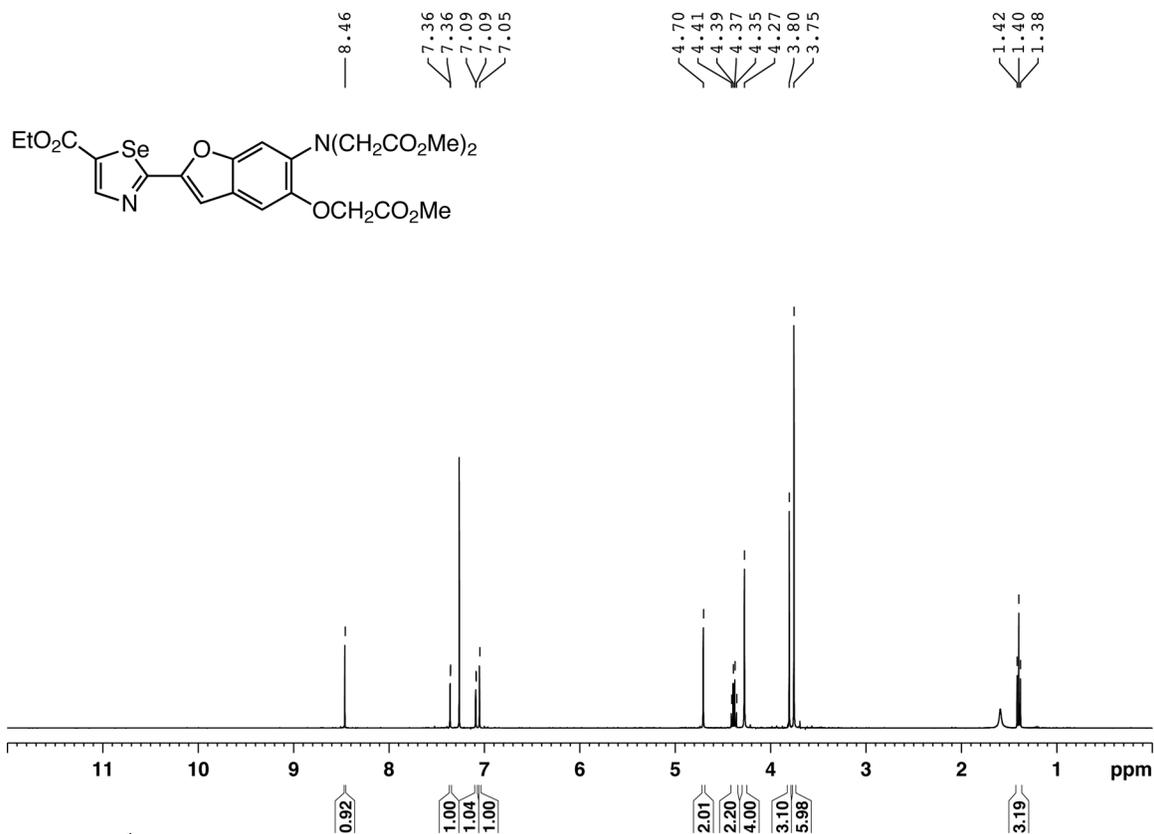


Figure S18. ¹H NMR spectrum of Mag-Se methyl ester, compound 3b, in CDCl₃.

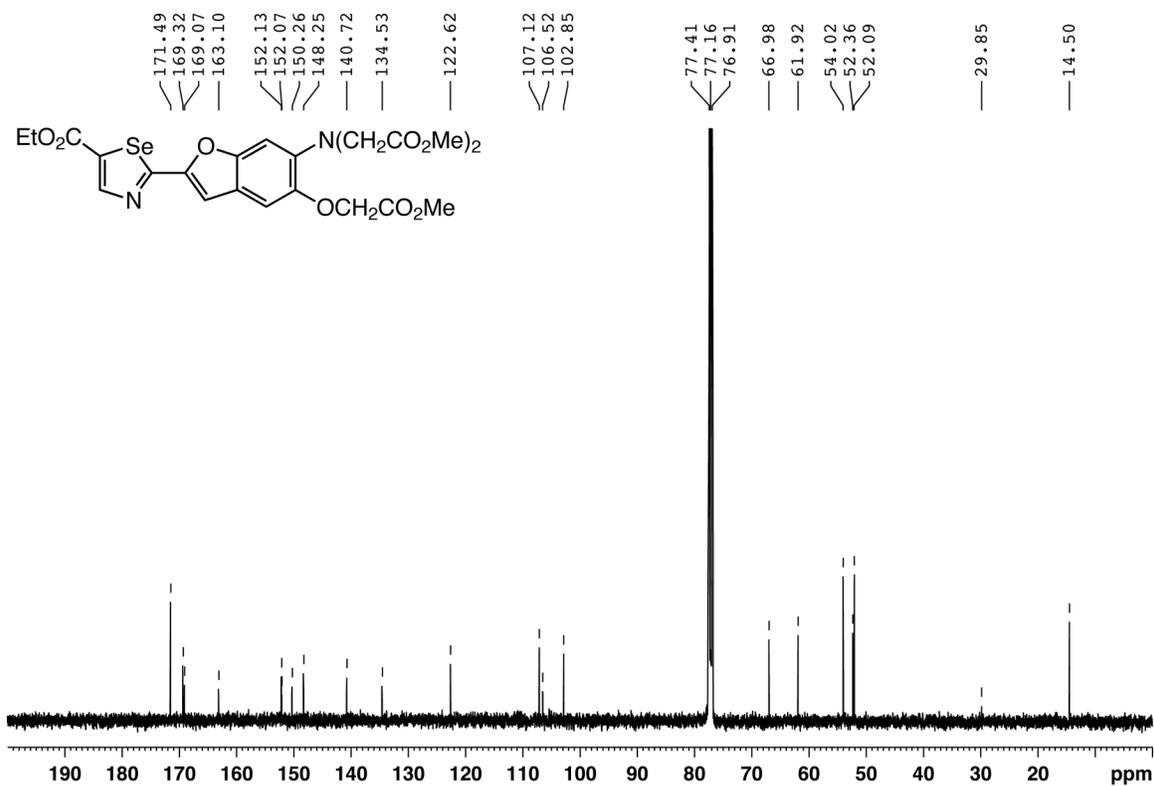


Figure S19. ¹³C{¹H} NMR spectrum of Mag-Se methyl ester, compound 3b, in CDCl₃.

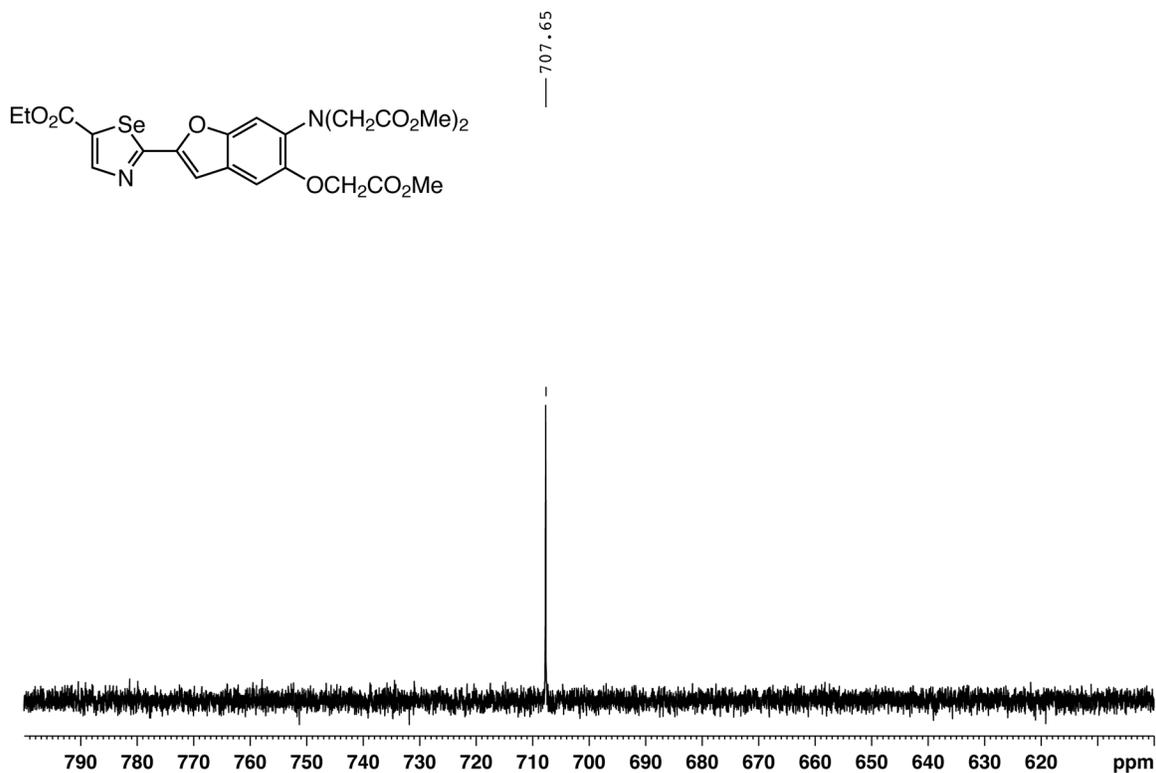


Figure S20. ⁷⁷Se NMR spectrum of Mag-Se methyl ester, compound 3b, in CDCl₃.

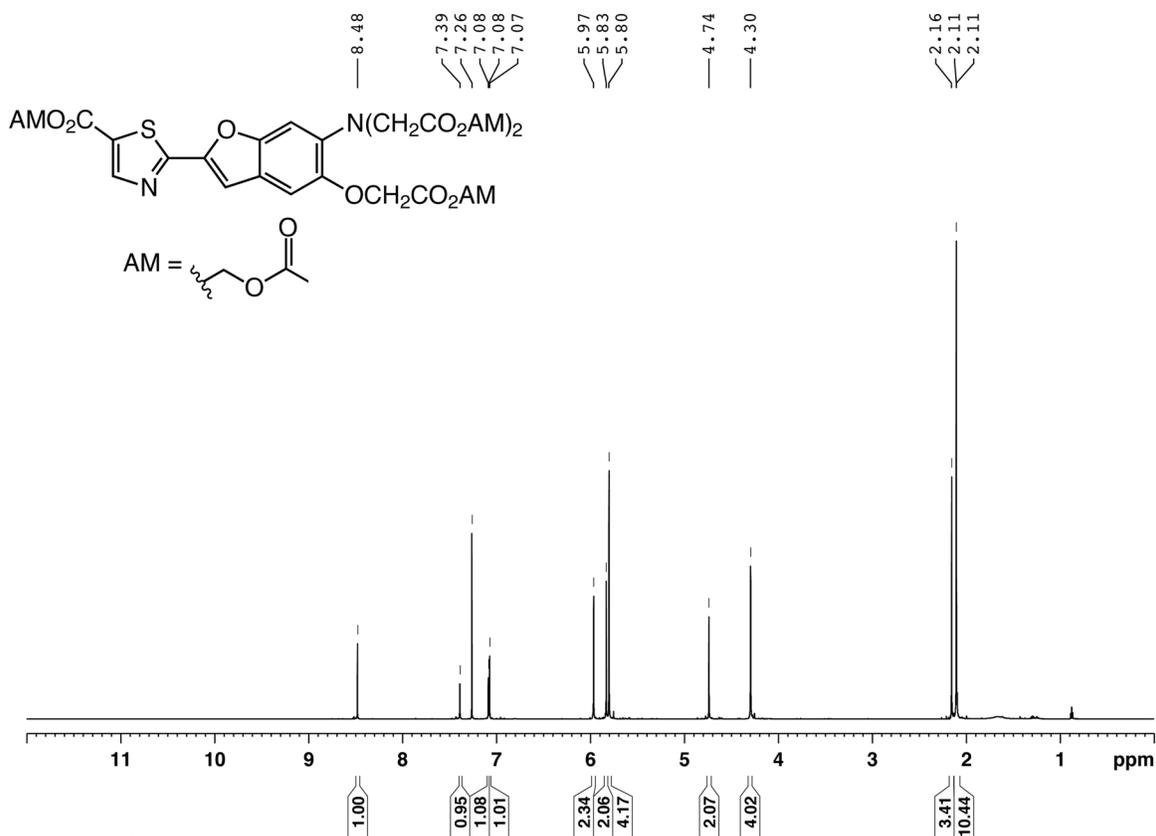


Figure S21. ¹H NMR spectrum of Mag-S acetoxymethyl ester, compound 5a, in CDCl₃.

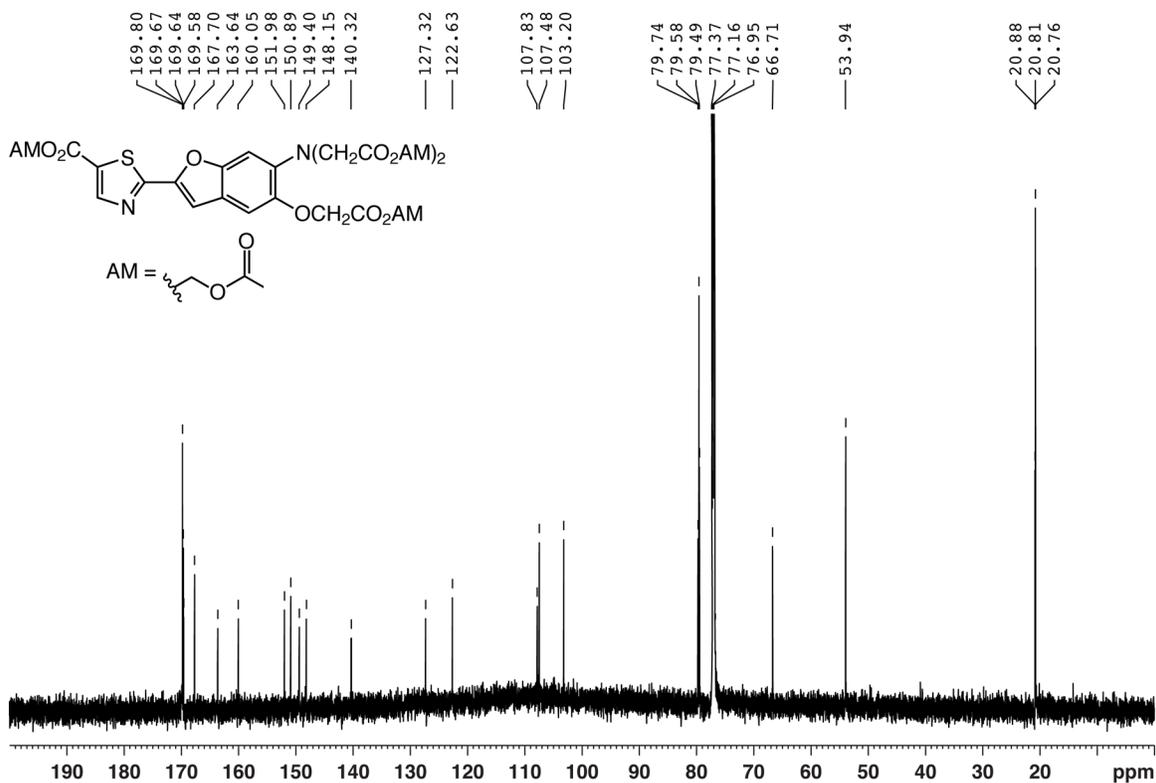


Figure S22. ¹³C{¹H} NMR spectrum of Mag-S acetoxymethyl ester, compound 5a, in CDCl₃.

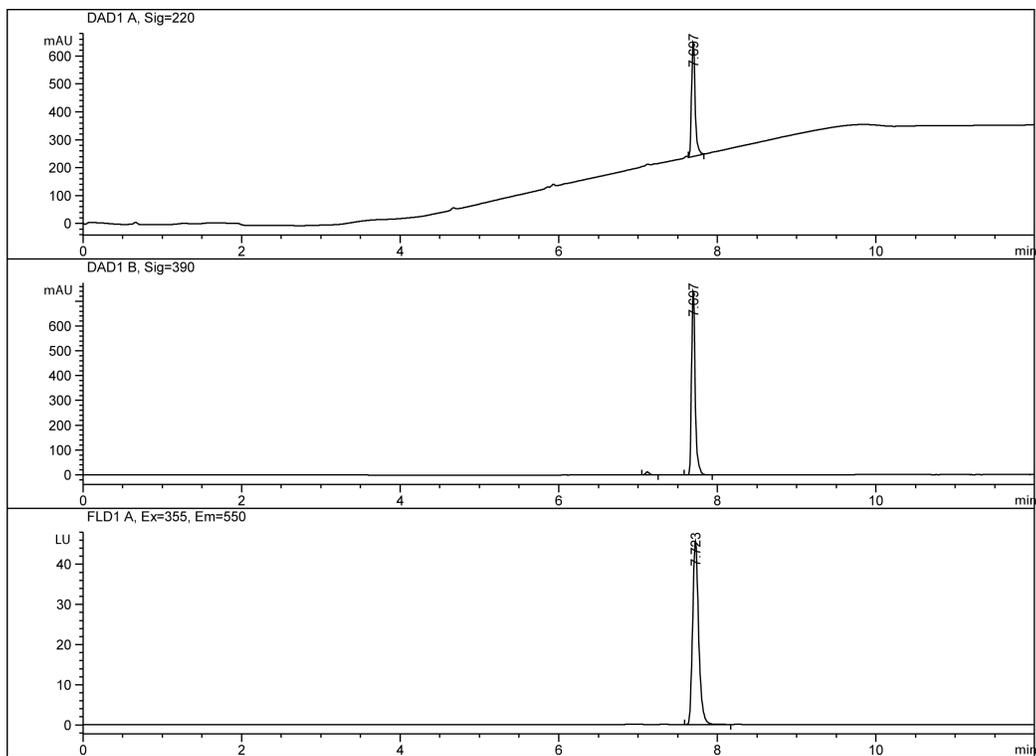


Figure S23. Reverse phase HPLC chromatogram of Mag-S methyl ester, compound 3a, eluted with an acetonitrile/water (+0.1% TFA) gradient.

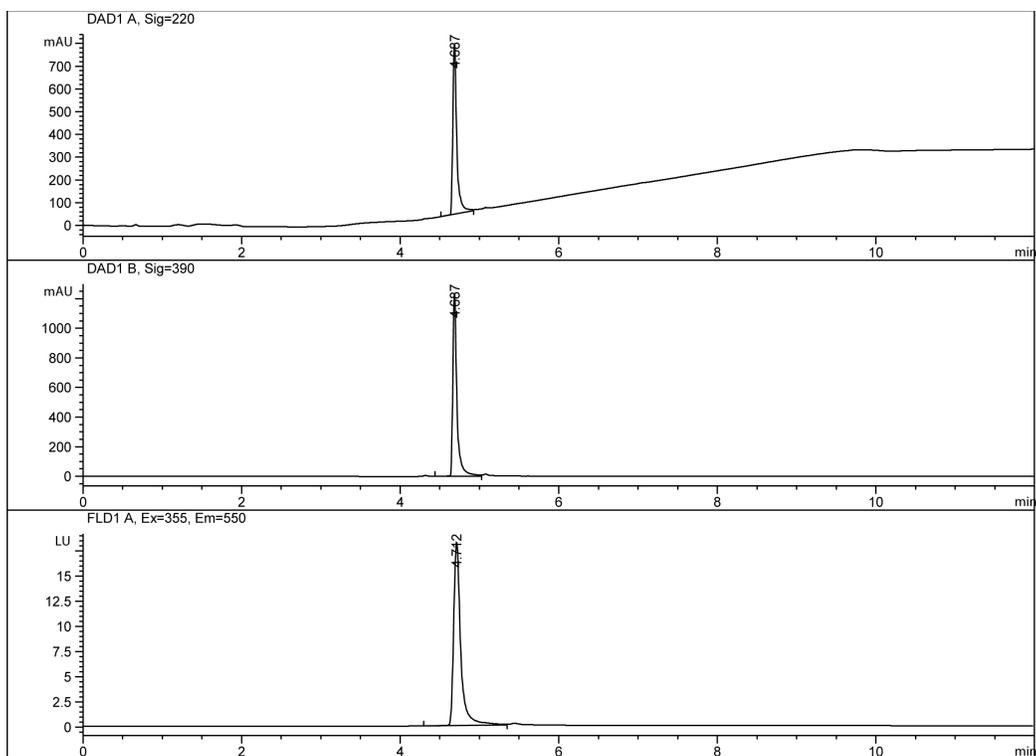


Figure S24. Reverse phase HPLC chromatogram of Mag-S acid, compound 4a, eluted with an acetonitrile/water (+0.1% TFA) gradient.

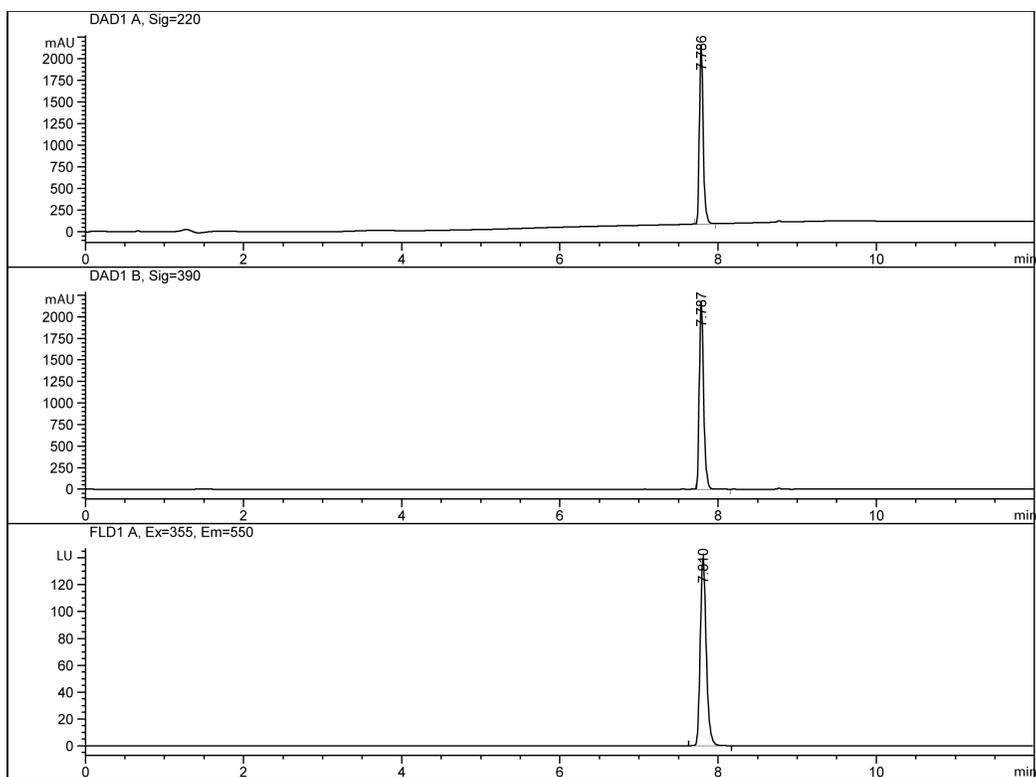


Figure S25. Reverse phase HPLC chromatogram of Mag-Se methyl ester, compound 3b, eluted with an acetonitrile/water (+0.1% TFA) gradient.

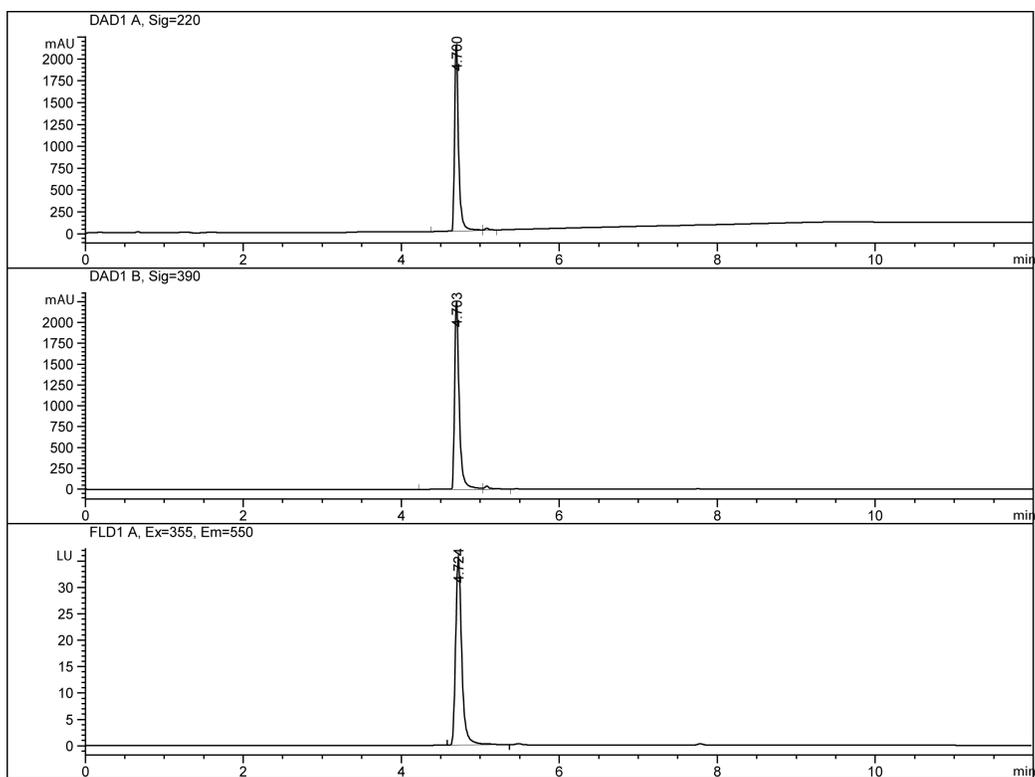


Figure S26. Reverse phase HPLC chromatogram of Mag-Se acid, compound 4b, eluted with an acetonitrile/water (+0.1% TFA) gradient.

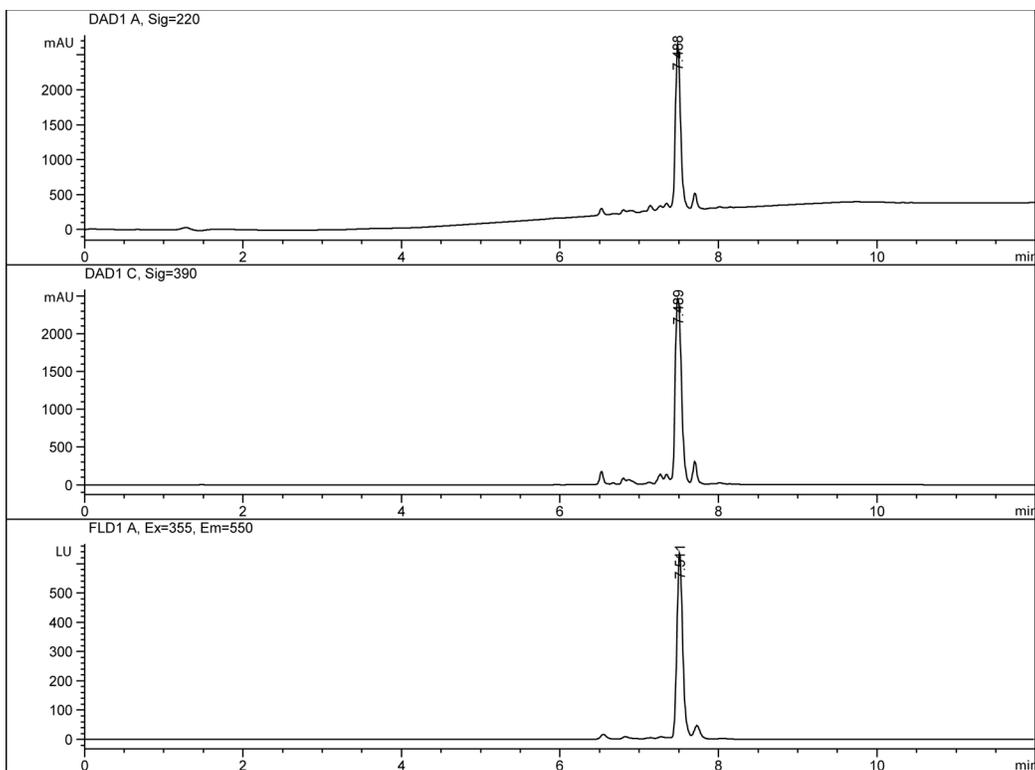


Figure S27. Reverse phase HPLC chromatogram of Mag-S acetoxymethyl ester, compound 5a, eluted with an acetonitrile/water (+0.1% TFA) gradient.

10. References

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