Supporting Information for:

Library Synthesis, Screening and Discovery of Modified Zinc(II)-bis(dipicolylamine) Probe for Enhanced Molecular Imaging of Cell Death

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1. Experimental Details and Supplementary Figures.

Method of Measuring Zn$^{2+}$ Concentration in RED Assay. The azo dye NO$_2$-PAPS is a colorimetric indicator for Zn$^{2+}$. Upon exposure to Zn-BDPA scaffolds the absorbance maxima of NO$_2$-PAPS shifts from 492 nm to 565 nm. Addition of Zn-BDPA S1 (Chart S1) to a solution of NO$_2$-PAPS (75 µM) in TES buffer (10 mM TES, 145 mM NaCl, pH 7.4) produces a ratiometric change in the absorbance spectrum (Figure S1), and the linear response (Figure S1, B) demonstrates that NO$_2$-PAPS can be used to measure the concentration of a Zn-BDPA complex.

Chart S1. Chemical Structure of Zn-BDPA S1.

![Chart S1](image)

Figure S1. Absorbance profile for NO$_2$-PAPS (75 µM) in TES buffer (10 mM TES, 145 mM NaCl, pH 7.4) upon titration with compound S1 (A). Beer’s law plot for the addition of S2 to NO$_2$-PAPS (B).
Proof that Ratio of Zn\(^{2+}\) Concentrations Reflects Zn-BDPA Binding in RED Assay.

To validate the assumption that the concentration of Zn\(^{2+}\) is proportional to the concentration of Zn-DPA scaffold in the two compartments of the Rapid Equilibrium Dialysis (RED) apparatus, a series RED experiments were conducted using fluorescent probe 12 and PS-rich vesicles. Equilibrated samples were analyzed using the NO\(_2\)-PAPS assay to measure Zn\(^{2+}\) concentration, and the deep-red fluorescence of 12 was used to independently measure its concentration. Increasing the amount of PS-rich vesicles in the ‘source’ compartment of the RED apparatus shifts the dialysis equilibrium ratio for 12, and the data in Figure S2 shows that the ratio of Zn\(^{2+}\) concentration in the two compartments reflects the ratio of Zn-BDPA concentration.

**Figure S2.** Validation that Zn\(^{2+}\) concentration reflects Zn-BDPA scaffold concentration in a RED assay. PS-rich vesicles (10:65:25 POPS:POPC:Cholesterol) and fluorescent probe 12 were added to the ‘source’ compartment, and the RED apparatus was allowed to shake for 16 hours at 180 rpm and 37 °C. The colorimetric NO\(_2\)-PAPS method was used to measure Zn\(^{2+}\) concentration (black criss-cross pattern) and the deep-red fluorescence of 12 (blue vertical lines, \(\lambda_{\text{excite}} = 600\) nm, \(\lambda_{\text{emit}} = 658\) nm) was used to measure its concentration in each compartment.
Measuring Membrane Affinity Using the RED Assay. Quantitative RED assays were conducted as proof-of-principle studies for measuring dissociation constants of Zn-BDPA compounds with PS-rich (POPC:Cholesterol:POPS, 65:25:10) or PC (POPC:Cholesterol, 75:25) vesicles. If the concentration of Zn-BDPA scaffold is held constant between RED compartments while the concentration of vesicles in the ‘source’ compartment is varied, a saturated binding isotherm can be obtained (Figure S3). Control experiments were conducted to ensure that unbiased RED equilibration occurred in the absence of vesicles.

Figure S3. Binding isotherms (generated using the RED:NO₂-PAPS dialysis assay) for association of compound 11c (A) and compound 11k (B) to PS-rich vesicles (POPS:Cholesterol:POPC, 10:25:65, red squares) or PC vesicles (Cholesterol:POPC, 25:75, blue circles). The RED apparatus was allowed to shake for 16 hours at 180 rpm and 37 °C.
**FRET Displacement Assay and Association Constant Determination.** A FRET ensemble of energy acceptor 12 bound to the surface of PS-rich vesicles containing energy donor 14 was prepared by mixing 12 (10 µM) and PS-rich vesicles (20 µM total lipid; POPS:POPC:14, 50:49:1) in 2.0 mL of HEPES buffer (5 mM HEPES, 137 mM NaCl, 3.2 mM KCl, 1.0 mM Zn(NO₃)₂•6H₂O, pH 7.4) at 25 °C. Aliquots of lead candidates 11k or 11o were titrated into separate samples of the FRET ensemble while stirring. After a waiting period of approximately 60 seconds for equilibration to occur, the fluorescence emission spectrum was acquired (λ<sub>ex</sub> = 480 nm, λ<sub>em</sub> = 500-750 nm). Plots of fluorescence intensity (I<sub>567nm</sub>/I<sub>663nm</sub>) as a function of lead candidate concentration were generated and fit using a computer to a competitive binding model that determined 1:1 association constants. Specifically a plot of S<sub>t</sub>/P versus Q (equations 1-3) produces a linear graph that has a slope equal to $K_1/K_2$. For equations 1-3 the parameters were defined as follows: Substrate = Zn-BDPA library candidate 11k or 11o, Indicator = probe 12 (10 µM), Ligand concentration was 10 µM, the total lipid concentration in the cuvette. Parameter $K_1$ is the association constant between PS-rich vesicles and fluorescent probe 12 while parameter $K_2$ is the association constant between library candidates and PS-rich vesicles. Since $K_1$ is easily measured using a standard FRET titration assay $K_2$ can be readily obtained. FRET displacement experiments were conducted using library candidates 11k (Figure S4) and 11o (Figure S5).

$$\frac{S_t}{P} = \frac{K_1}{K_2} Q + 1$$

Equation 1

Where $S_t$ = Total substrate concentration
$K_1$ = Ligand – Indicator binding constant

$$P = L_t - \frac{1}{Q * K_1} * \frac{I_t}{Q + 1}$$

Equation 2

Where $L_t$ = Total ligand concentration
$I_t$ = Total indicator concentration

$$Q = \frac{F_i - F_I}{F_{lim} - F_n}$$

Equation 3

Where $F_I$ = Initial fluorescence intensity
$F_{lim}$ = Quenched fluorescence intensity
$F_n$ = Fluorescence intensity at titration point $n$
Figure S4. FRET displacement data for Zn-BDPA library candidate 11k and PS-rich vesicles (POPS:POPC:14, 50:49:1). Cartoon depiction of the FRET displacement titration experiment showing that energy transfer between the FRET donor 14 and the FRET acceptor 12 is lost when a Zn-DPA library candidate (either 11k or 11o) has displaced 12 from the vesicle surface (A) (copy of Figure 2A). Emission profile (B), normalized FRET response (C), table of FRET displacement results (D), and fitted data (E) for the FRET displacement induced by 11k binding to PS-rich vesicles. Chemical structure of Zn-BDPA library candidate 11k (F).
Figure S5. FRET displacement data for Zn-BDPA library candidate 11o and PS-rich vesicles (POPS:POPC:14, 50:49:1). Emission profile (A) (copy of Figure 2C), normalized FRET response (B), table of FRET displacement results (C), and fitted data (D) for the FRET displacement induced by 11o binding to PS-rich vesicles. Chemical structure of Zn-BDPA library candidate 11o (E).
**FRET Titration Assay and Association Constant Determination.** Fluorescent probes 12 and 13 were titrated separately into aqueous samples containing either PS-rich (10 µM total lipid; POPS:POPC:14, 50:49:1) or PC (10 µM total lipid; POPC:14, 99:1) vesicles in 3.0 mL HEPES buffer (5 mM HEPES, 137 mM NaCl, 3.2 mM KCl, 1.0 mM Zn(NO₃)₂•6H₂O, pH 7.4) at 25 °C while stirring. After a waiting period of approximately 60 seconds for equilibration to occur, the fluorescence emission spectrum was acquired (λ<sub>ex</sub> = 480 nm, λ<sub>em</sub> = 500-750 nm). Plots of fluorescence intensity (λ<sub>em</sub> = 567 nm) as a function of Zn-BDPA concentration were generated and the association constants were determined using a nonlinear least-squares fitting procedure adapted for fluorescence spectroscopy (Equation 4). The parameters for Equation 4 were defined as follows: The Host concentration was 10 µM, the total lipid concentration in the cuvette, Guest = Zn-BDPA probe 12 or 13. FRET titration experiments were conducted using fluorescent probes 12 and 13 (Figure S6).

\[
F = F_o + \frac{F_{lim} - F_o}{2C_o} \left\{ C_o + C_g + \frac{1}{K} - \left[ \left( C_o + C_g + \frac{1}{K} \right)^2 - 4C_oC_g \right]^{1/2} \right\}
\]

Equation 4

Where  
F = Fluorescence intensity  
F<sub>o</sub> = Initial Fluorescence intensity  
F<sub>lim</sub> = Quenched fluorescence intensity (end of titration experiment)  
C<sub>O</sub> = Total host concentration  
C<sub>G</sub> = Total guest concentration  
K = Association Constant

The association constants reported in this study formally correspond to 1:1 association of a Zn-BDPA compound and a phospholipid within a vesicle membrane. In the case of PS-rich vesicles the reported value is an apparent association constant since it is the weighted average of 1:1 association with either a PS or PC phospholipid in the membrane.
Figure S6. Schematic cartoon of FRET titration assay (A) (copy of Figure 3A). FRET profiles for fluorescent probes 12 and 13 binding to PS-rich (10 µM total lipid; POPS:POPC:14, 50:49:1) and PC (10 µM total lipid; POPC:14, 99:1) vesicles; 12 and PS-rich vesicles (B), 12 and PC vesicles (C), 13 and PS-rich vesicles (D) (copy of Figure 2D), 13 and PC vesicles (E).
Figure S7. Absorbance (red solid line) and emission (blue dashed line) profile of 13 (2 µM) in 100 % DMSO ($\lambda_{\text{ex}} = 610$ nm).

Table S1. Photophysical Properties of 12 and 13.$^a$

| Compound | Molar Absorptivity, M$^{-1}$cm$^{-1}$ | Quantum Yield$^b$ | Absorbance $\lambda_{\text{max}}$, nm | Emission $\lambda_{\text{max}}$, nm |
|----------|------------------------------------|------------------|--------------------------------------|-------------------------------|
| 12       | 1.1 x $10^5$                       | 0.13             | 656                                  | 673                           |
| 13       | 1.1 x $10^5$                       | 0.13             | 656                                  | 672                           |

$^a$ 2 µM samples in DSMO at 25 °C. $^b$ Quantum yield measured relative to commercially purchased 12 (PSVue® 643, Molecular Targeting Technologies Inc., West Chester, PA).
Figure S8. Cell viability of CHO-K1 cells (Blue crisscross) and MDA-MB-231 cells (red checkered) treated with either 12 (A) or 13 (B) for 18 hours at 37 °C.
**Figure S9.** Fluorescence micrographs showing selective targeting of probe 13 for rounded dead/dying cells over elongated healthy cells. MDA-MB-231 cells (top, A-C) and CHO-K1 cells (bottom D-F) stained with 13. Cells were treated with etoposide (15 μM) for 12 hours, incubated with probe 13 (10 μM) for 30 minutes at 37 °C, then washed with HEPES buffer. Micrographs C and F are merges of the brightfield images (A, D) and the deep-red Cy-5 fluorescence images (B, E). Scale bar = 25 μm.
Figure S10. Confocal micrographs of MDA-MB-231 cell treated with etoposide (15 µM) for 12 hours to induce cell death, then stained with probe 13 (10 µM) for 30 minutes prior to deep-red fluorescence imaging. Images are 4 µm slices (60 x magnification) taken through the cell separated by 20 µm (A – F). The micrographs indicate probe distribution throughout the cytosol.
Figure S11. Fluorescence microscopy of dead MDA-MB-231 cells stained with blue-emitting nucleic acid stain SYTOX® Blue (B) and red-emitting 13 (C). A bright field (A) and merge (D) are also shown. The images show that probe 13 stains the cytosol of dead/dying cells but not the cell nucleus. Scale bar = 25 µm.
Figure S12. Biodistribution of deep-red CyAL-5 dye, probe 12, or probe 13 in organs taken from healthy rats sacrificed at 24 hours after intravenous injection of the probe (3.0 mg/kg). Error bars are standard error of the mean (N = 4).

Figure S13. Biodistribution of deep-red CyAL-5 dye, probe 12, or probe 13 in organs taken from rats bearing a subcutaneous prostate tumor at 24 hours after intravenous injection of the probe (3.0 mg/kg). Error bars are standard error of the mean (N = 4). **P = 0.004; ****P < 0.0001.
**Synthesis – Reaction conditions.** The synthesis of library candidates 11d and 11e are shown below in Scheme S1. The synthetic route for both compounds follow the same chemical transformations; a benzylic aldehyde is reduced to a secondary alcohol which is then brominated via an Appel reaction. Treatment of alkyl bromides S1c and S2c with 2-picoylamine leads to the desired modified DPA building blocks S1d and S2d which are appended to the desired m-xylene scaffold.

**Scheme S1.** Synthesis of compounds 11d-e (Chart 2, Entries 13 & 14)\(^a\)

\(^a\)Reagents and conditions: (i) NaBH₄, CaCl₂, EtOH; (ii) PPh₃, CBr₄, K₂CO₃, DCM, 0 °C → rt; (iii) 2-Picolylamine, Na₂CO₃, MeCN; (iv) m-Xylene dibromide, CHCl₃, DIPEA.

**Chart S2.** Chemical structure of CyAL-5
2. COMPOUND CHARACTERIZATION.

6c. To a solution of 6b (20.0 mg, 62.0 µmol) in CHCl₃ (250 µL) was added butylisocyanate (70.5 µL, 630 µmol). The reaction was allowed to stir under argon for 12 hours. Solvent was removed and the crude material was purified using silica gel column chromatography with 0-2% MeOH:CHCl₃ as the eluent to yield the desired product as a white powder (26.3 mg, 81%). ¹H NMR (600 MHz, CDCl₃) δ 0.94 (t, J = 7 Hz, 6H), 1.42 (sextet, J = 7 Hz, 4H), 1.58 (p, J = 7 Hz, 4H), 3.39 (q, J = 7 Hz, 4H), 3.67 (s, 4H), 6.71 (d, J = 8 Hz, 2H), 7.08 (d, J = 7 Hz, 2H), 7.25 (t, J = 8 Hz, 1H), 7.32 (t, J = 7 Hz, 2H), 7.38 (d, J = 8 Hz, 2H), 7.54 (t, J = 8 Hz, 2H), 9.03 (br s, NH, 2H), 9.62 (br s, NH, 2H) ppm; ¹³C NMR (600 MHz, CDCl₃) δ 13.8, 20.3, 32.1, 39.5, 58.6, 59.8, 110.3, 115.2, 127.2, 128.4, 128.6, 138.5, 138.6, 153.1, 156.0, 156.4 ppm; HRMS (ESI, MeCN): m/z = 518.3232 ([M+H]+).

6d. To a solution of 6b (7.0 mg, 21.9 µmol) in CHCl₃ (2 mL) was added DIPEA (20 µL, 120 µmol) and butyric anhydride (11 µL, 67 µmol). The reaction was allowed to stir under argon for 12 hours. The reaction was quenched upon addition of H₂O (1 mL) and the crude product was extracted using chloroform. The organic layer was dried over MgSO₄ and solvent was removed. The crude material was purified using silica gel column chromatography with 0-2% MeOH:CHCl₃ as the eluent to yield the desired product as a clear oil (9.3 mg, 92%). ¹H NMR (600 MHz, CDCl₃) δ 1.00 (t, J = 7 Hz, 6H), 1.76 (sextet, J = 7 Hz, 4H), 2.36 (t, J = 7 Hz, 4H), 3.68 (s, 6H), 7.22-7.28 (m, 3H), 7.32 (t, J = 7 Hz, 2H), 7.38 (d, J = 7 Hz, 2H), 7.67 (t, J = 7 Hz, 2H), 7.91 (br s, NH, 2H), 8.07 (d, J = 7 Hz, 2H) ppm; ¹³C NMR (600 MHz, CDCl₃) δ 13.7, 18.8, 39.7, 58.4, 59.3, 112.0, 118.5, 127.2, 128.4, 128.9, 138.9, 150.6, 171.6 ppm; HRMS (ESI, MeCN): m/z = 460.2716 ([M+H]+).

10c. To a solution of 10b (15.0 mg, 49.3 µmol) in CHCl₃ (100 µL) was added butylisocyanate (50 µL, 440 µmol). The reaction was allowed to stir at room temperature for 12 hours. The crude material was purified using silica gel column chromatography with 0-3% MeOH:CHCl₃ as the eluent to yield the desired product as a clear oil (12.0 mg, 60%). ¹H NMR (600 MHz, CDCl₃) δ 0.94 (t, J = 7 Hz, 3H), 1.42 (s, J = 7 Hz, 2H), 1.58 (p, J = 7 Hz, 2H), 3.39 (q, J = 7 Hz, 2H), 3.68 (s, 4H), 3.80 (s, 2H), 6.62 (d, J = 8 Hz, 1H), 7.10 (d, J = 8 Hz, 1H), 7.14 (d of d of d, J = 1, 6, 8 Hz, 1H), 7.24 (t, J = 7 Hz, 1H), 7.32 (t, J = 7 Hz, 2H), 7.40 (d, J = 7 Hz, 2H), 7.54 (t, J = 7 Hz, 1H), 7.5 (d, J = 8 Hz, 1H), 7.65 (d of d, J = 2, 8 Hz, 1H) ppm; ¹³C NMR (600 MHz, CDCl₃) δ 13.8, 20.3, 32.1, 39.5, 58.6,
11c. To a solution of α,α'-Dibromo-m-xylene (102.7 mg, 390 µmol) in CHCl₃ (2 mL) was added DIPEA (0.17 mL, 980 µmol), and 2,2’-Dipicolylamine (0.15 mL, 830 µmol). The reaction mixture was allowed to stir at room temperature for 4 hours. The resulting solution was washed once with water and was dried over MgSO₄. The crude residue was purified by column chromatography with 0-15 % MeOH:EtOAc as the eluent to yield the desired product as a brown oil (60 mg, 31 %).

1H NMR (500 MHz, CDCl₃) δ 3.67 (s, 4H), 3.81 (s, 8H), 7.11 (m, 4H), 7.29 (m, 4H), 7.48 (s, 1H), 7.60 (m, 7H), 8.51 (d, J = 5 Hz, 4H) ppm; 13C NMR (500 MHz, CDCl₃) δ 58.5, 59.9, 122.0, 122.8, 127.7, 128.3, 129.3, 136.5, 138.8, 148.9, 159.4 ppm; HRMS (ESI, MeCN): m/z = 501.2757 ([M+H]+).

11d. To a solution of α,α'-Dibromo-m-xylene (17.8 mg, 67.4 µmol) in CHCl₃ (4 mL) was added DIPEA (50 µL, 290 µmol), and 20d (41.3 mg, 150 µmol). The reaction mixture was allowed to stir at room temperature overnight. The resulting solution was washed once with water and the organic layer was dried over MgSO₄. The crude residue was purified by column chromatography with 0-20 % MeOH:CHCl₃ as the eluent to yield the desired product as a yellow oil (41.5 mg, 93 % yield).

1H NMR (500 MHz, CDCl₃) δ 3.71 (s, 4H), 3.81 (s, 4H), 3.82 (s, 4H), 7.16-7.18 (m, 2H), 7.27-7.28 (m, 3H), 7.33 (d, J = 8 Hz, 2H), 7.47 (s, 1H), 7.50 (t, J = 8 Hz, 2H), 7.58 (t, J = 8 Hz, 4H), 7.66 (dt, J = 2, 8 Hz, 2H), 8.53-8.55 (m, 2H) ppm; 13C NMR (500 MHz, CDCl₃) δ 58.7, 59.5, 60.3, 121.6, 122.3, 123.0, 126.4, 127.9, 128.6, 129.5, 136.7, 139.0, 141.4, 149.3, 159.7, 161.9 ppm; HRMS (ESI, MeCN): m/z = 657.0976 ([M+H]+).

11e. To a solution of α,α'-Dibromo-m-xylene (11.4 mg, 43.2 µmol) in CHCl₃ (4 mL) was added DIPEA (50 µL, 290 µmol), and 21d (20.0 mg, 93.7 µmol). The reaction mixture was allowed to stir at room temperature overnight. The resulting solution was washed once with water and the organic layer was dried over MgSO₄. The crude residue was purified by column chromatography with 0-10 % MeOH:CHCl₃ as the eluent to yield the desired product as a yellow oil (7.7 mg, 34 % yield).

1H NMR (500 MHz, CDCl₃) δ 3.51 (s, 6H), 3.67 (s, 6H), 3.77 (s, 4H), 3.80 (s, 4H), 6.98 (d, J = 8 Hz, 2H), 7.11-7.14 (m, 2H), 7.26-7.30 (m, 3H), 7.44 (d, J = 8 Hz, 2H), 7.48 (s, 1H), 7.51 (t, J = 8 Hz, 2H), 7.59-7.63 (m, 4H), 8.51 (dt, J = 1, 5 Hz, 2H) ppm; 13C NMR (500 MHz, CDCl₃) δ 24.7, 58.8, 60.3, 60.4, 119.6, 121.6, 122.1, 122.9, 127.8, 128.5,
129.4, 136.6, 136.9, 139.4, 149.2, 157.7, 159.5, 160.2 ppm; HRMS (ESI, MeCN): m/z = 529.3079 ([M+H]+).

**11f.** To a solution of **11b** (15.1 mg, 28.5 µmol) in CHCl₃ (2 mL) was added acetic anhydride (40 µL, 430 µmol). The reaction mixture was allowed stir overnight. Solvent was removed under reduced pressure and the crude residue was purified by column chromatography with 0-10 % MeOH:CHCl₃ as the eluent to yield the desired product as a brown oil (10 mg, 57 % yield). $^1$H NMR (500 MHz, CDCl₃) $\delta$ 1.87 (s, 6H), 3.64 (s, 4H), 3.75 (s, 4H), 3.82 (s, 4H), 7.18-7.27 (m, 7H), 7.48, (d, J = 8 Hz, 2H), 7.58 (td, J = 2, 8 Hz, 2H), 7.71 (t, J = 8 Hz, 2H), 7.80 (s, 1H), 8.15 (d, J = 8 Hz, 2H), 8.50-8.52 (m, 2H), 9.30 (br s, NH, 2H) ppm; $^{13}$C NMR (500 MHz, CDCl₃) $\delta$ 24.4, 58.1, 59.4, 60.4, 112.8, 119.5, 122.5, 123.4, 128.2, 130.3, 136.7, 139.4, 149.1, 151.7, 169.3 ppm; HRMS (ESI, MeCN): m/z = 615.3178 ([M+H]+).

**11g.** To a solution of **11b** (19.0 mg, 35.8 µmol) in CHCl₃ (2.0 mL) at 0 °C was added freshly distilled trifluoroacetic anhydride (50 µL, 350 µmol). The reaction mixture was allowed to warm to room temperature while stirring overnight. The resulting solution was washed with water and the organic layer was dried over MgSO₄. The crude residue was purified by column chromatography with 0-10 % MeOH:CHCl₃ as the eluent to yield the desired product as a brown oil (13.4 mg, 52 % yield). $^1$H NMR (500 MHz, CDCl₃) $\delta$ 3.67 (s, 4H), 3.71 (s, 4H), 3.81 (s, 4H), 7.14-7.16 (m, 2H), 7.23-7.29 (m, 3H), 7.39 (d, J = 8 Hz, 2H), 7.47 (s, 1H), 7.51 (d, J = 8 Hz, 2H), 7.61 (td, J = 2, 8 Hz, 2H), 7.74 (t, J = 8 Hz, 2H), 7.99 (br s, 2H), 8.50-8.52 (m, 2H), 9.35 (br s, NH, 2H) ppm; $^{13}$C NMR (500 MHz, CDCl₃) $\delta$ 58.5, 59.1, 60.3, 113.3, 120.8, 122.4, 123.1, 128.2, 128.6, 129.8, 136.7, 138.4, 139.6, 149.2, 159.4 ppm; HRMS (ESI, MeCN): m/z = 723.2616 ([M+H]+).

**11h.** To a solution of **11b** (15.0 mg, 28.3 µmol) in CHCl₃ (2.0 mL) was added benzoic anhydride (63.3 mg, 280 µmol). The reaction mixture was allowed to stir overnight. Solvent was removed under reduced pressure and the crude residue was purified by column chromatography with 0-2 % MeOH:CHCl₃ as the eluent to yield the desired product as an off-white solid (18.0 mg, 86 % yield). $^1$H NMR (500 MHz, CDCl₃) $\delta$ 3.65 (s, 4H), 3.69 (s, 4H), 3.82 (s, 4H), 7.12-7.17 (m, 2H), 7.27 (s, 2H), 7.31 (d, J = 8 Hz, 2H), 7.38 (t, J = 7 Hz, 4H), 7.47-7.52 (m, 2H), 7.53-7.63 (m, 5H), 7.73 (t, J = 8 Hz, 2H), 7.81 (d, J = 8 Hz, 4H), 8.11 (d, J = 7 Hz, 1H), 8.31 (d, J = 8 Hz, 2H), 8.51-8.53 (m, 2H), 9.28 (br s, NH, 2H) ppm; $^{13}$C NMR (500 MHz, CDCl₃) $\delta$ 58.6, 59.5, 60.3, 112.7, 119.3, 122.3, 123.0, 127.4, 128.1, 128.4, 128.5, 128.8, 129.6, 130.2, 132.2,
133.1, 134.6, 136.8, 139.3, 149.0, 151.5, 158.3, 159.7, 166.4 ppm; HRMS (ESI, MeCN): m/z = 739.3526 ([M+H]^+).

**11k.** To a solution of **11b** (25.0 mg, 47.1 µmol) in CHCl₃ (3.0 mL) was added butyl isocyanate (27 µL, 240 µmol). The reaction mixture was allowed stir for 36 hours and solvent was removed under reduced pressure. The crude residue was purified by alumina column chromatography with 0-2 % MeOH:CHCl₃ as the eluent to yield the desired product as a brown oil (21.6 mg, 63 % yield). ¹H NMR (600 MHz, CDCl₃) δ 0.83 (t, J = 8 Hz, 6H), 1.31 (sextet, J = 8 Hz, 4H), 1.47 (p, J = 8 Hz, 4H), 3.28 (q, J = 8 Hz, 4H), 3.57 (s, 4H), 3.59 (s, 4H), 3.70 (s, 4H), 6.59 (d, J = 8 Hz, 2H), 7.00 (d, J = 8 Hz, 2H), 7.02 - 7.05 (m, 2H), 7.19 - 7.21 (m, 2H), 7.31 (s, 1H), 7.41 (t, J = 8 Hz, 2H), 7.44 (d, J = 8 Hz, 2H), 7.52 (dt, J = 2, 8 Hz, 2H), 8.40-8.41 (m, 2H), 8.66 (s, 2H), 9.47 (s, 2H) ppm; ¹³C NMR (600 MHz, CDCl₃) δ 13.8, 20.2, 25.7, 32.1, 39.5, 58.6, 59.8, 60.1, 110.1, 115.2, 122.0, 122.6, 127.5, 128.4, 129.0, 136.4, 138.5, 138.9, 149.0, 153.0, 156.2, 156.3, 159.6 ppm; HRMS (ESI, MeCN): m/z = 729.4350 ([M+H]^+).

**11m.** To a solution of **11b** (14.9 mg, 28.1 µmol) in CHCl₃ (3.0 mL) was added p-tolyl isocyanate (20 µL, 160 µmol). The reaction mixture was allowed stir overnight and solvent was removed under reduced pressure. The crude residue was purified by alumina column chromatography with 0-2 % MeOH:CHCl₃ as the eluent to yield the desired product as a brown oil (16.1 mg, 73 % yield). ¹H NMR (600 MHz, CDCl₃) δ 2.31 (s, 6H), 3.66 (s, 4H), 3.71 (s, 4H), 3.81 (s, 4H), 6.72 (s, 2H), 7.09-7.11 (m, 8H), 7.21-7.24 (m, 1H), 7.28 (d, J = 8 Hz, 2H), 7.41-7.43 (m, 5H), 7.49-7.56 (m, 6H), 8.48-8.49 (m, 2H), 8.74 (s, 2H), 11.94 (s, 2H) ppm; ¹³C NMR (600 MHz, CDCl₃) δ 20.9, 58.7, 59.6, 60.2, 110.4, 115.9, 120.1, 122.1, 122.9, 127.8, 128.4, 129.4, 132.7, 136.0, 136.5, 138.5, 139.0, 149.0, 152.4, 153.6, 156.3, 159.3 ppm; HRMS (ESI, MeCN): m/z = 797.4350 ([M+H]^+).

**11n.** To a solution of **11b** (30.0 mg, 56.5 µmol) in CHCl₃ (4.0 mL) was added 3,5-Bis(trifluoromethyl)phenyl isocyanate (49 µL, 280 µmol). The reaction mixture was allowed stir for overnight and solvent was removed under reduced pressure. The crude material was purified using preparatory TLC methods (alumina gel preparatory TLC plate, with 5 % MeOH:CHCl₃ as the mobile phase. The purified material was dissolved in CHCl₃, washed with water, and dried over Na₂SO₄ to yield the desired product as a white solid (15.2 mg, 26 % yield). ¹H NMR (600 MHz, CDCl₃) δ 3.65 (s, 4H), 3.69 (s, 4H), 3.82 (s, 4H), 6.80 (s, 2H), 7.04 (d, J = 8 Hz, 2H), 7.09-7.11 (m, 2H), 7.17-7.20 (m, 1H), 7.23 (d, J = 8 Hz, 1H), 7.42 (d, J = 8 Hz, 2H), 7.46 (s, 2H),
7.52-7.56 (m, 4H), 7.99 (s, 4H), 8.43-8.44 (m, 2H) ppm; $^{13}$C NMR (600 MHz, CDCl$_3$) δ 58.4, 59.2, 59.6, 110.5, 116.1, 116.7, 119.1, 120.4, 122.2, 122.9, 124.0, 125.9, 128.1, 128.4, 129.6, 131.4 (q, J = 133 Hz), 136.7, 137.6, 139.2, 140.1, 148.8, 151.8, 153.2, 156.3, 158.7 ppm; HRMS (ESI, MeCN): m/z = 1041.3249 ([M+H$^+$]).

11o. To a solution of 11b (20.0 mg, 37.7 µmol) in CHCl$_3$ (500 µL) was added 4-fluorophenethyl isocyanate (27.4 µL, 190 µmol). The reaction was allowed to stir at room temperature for 12 hours. Solvent was removed and the crude material was purified using silica gel column chromatography with 0-10 % MeOH:CHCl$_3$ as the eluent to yield the desired product as a clear oil (8.7 mg, 27 %).

$^1$H NMR (500 MHz, CDCl$_3$) δ 2.85 (t, J = 7 Hz, 4H), 3.44 (s, 4H), 3.61-3.65 (m, 8H), 3.74 (s, 4H), 6.60 (s, 2H), 6.91-6.95 (m, 4H), 7.01-7.17 (m, 8H), 7.27-7.32 (m, 3H), 7.42 (s, 1H), 7.49 (t, J = 7 Hz, 2H), 7.52 (d, J = 7 Hz, 2H), 7.60 (t of d, J = 8 Hz, J = 1 Hz, 2H), 8.12 (br s, 2H), 8.50-8.52 (m, 2H), 9.47 (br s, 2H) ppm; $^{13}$C NMR (500 MHz, CDCl$_3$) δ 35.7, 41.2, 58.9, 59.6, 60.4, 110.0, 115.5, 115.7, 122.3, 122.9, 127.8, 128.7, 129.2, 130.4, 135.3, 136.7, 139.3, 149.3, 152.7, 156.1, 158.7, 160.8, 162.7 ppm; HRMS (ESI, MeCN): m/z = 861.4184 ([M+H$^+$]).

11p. To a solution of 11b (40.0 mg, 75.4 µmol) in CHCl$_3$ (250 µL) was added 6-Isocyanato-1,4-benzodioxane (51.5 µL, 380 µmol). The reaction was allowed to stir at room temperature for 12 hours. Solvent was removed and the crude material was purified using silica gel column chromatography with 0-10 % MeOH:CHCl$_3$ as the eluent to yield the desired product as a clear oil (14.8 mg, 22 %).

$^1$H NMR (600 MHz, CDCl$_3$) δ 3.67 (s, 4H), 3.71 (s, 4H), 3.80 (s, 4H), 4.20-4.24 (m, 8H), 6.72 (s, 2H), 6.78 (d, J = 9 Hz, 2H), 6.93 (dd, J = 2, 8 Hz, 2H), 7.10 (t, J = 7 Hz, 4H), 7.20-7.23 (m, 3H), 7.27 (d, J = 8 Hz, 2H), 7.41 (s, 1H), 7.49-7.56 (m, 6H), 8.48 (d, J = 4 Hz, 2H), 8.83 (br s, 2H), 11.9 (br s, 2H) ppm; $^{13}$C NMR (600 MHz, CDCl$_3$) δ 58.7, 59.6, 60.2, 64.3, 64.5, 109.7, 110.4, 113.8, 115.8, 117.1, 122.1, 122.9, 127.8, 128.4, 132.4, 136.5, 138.6, 139.0, 139.7, 143.4, 148.9, 152.4, 153.6, 156.2, 159.3 ppm; HRMS (ESI, MeCN): m/z = 885.3846 ([M+H$^+$]).

11q. To a solution of 11b (40.0 mg, 75.4 µmol) in CHCl$_3$ (250 µL) was added 4-phenoxyphenyl isocyanate (68 µL, 380 µmol). The reaction was allowed to stir at room temperature for 12 hours. Solvent was removed and the crude material was purified using silica gel column chromatography with 0-10 % MeOH:CHCl$_3$ as the eluent to yield the desired product as a clear oil (2.6 mg, 4 %).

$^1$H NMR (600 MHz, CD$_3$OD) δ 3.54 (s, 4H), 3.62 (s, 4H), 3.67 (s,
4H), 6.75 (d, J = 8 Hz, 2H), 6.77-6.79 (m, 4H), 6.82-6.84 (m, 4H), 6.95-6.97 (m, 5H), 7.03-7.05 (m, 1H), 7.08-7.10 (m, 4H), 7.20-7.23 (m, 4H), 7.28 (s, 1H), 7.31-7.33 (m, 4H), 7.44 (d, J = 8 Hz, 2H), 7.48-7.53 (m, 3H), 8.25-8.26 (m, 2H) ppm; HRMS (ESI, MeCN): m/z = 954.4256 ([M+H]+).

11r. To a solution of 11b (40.0 mg, 75.4 µmol) in CHCl₃ (250 µL) was added allyl isocyanate (31 µL, 360 µmol). The reaction was allowed to stir at room temperature for 12 hours. Solvent was removed and the crude material was purified using silica gel column chromatography with 0-10 % MeOH:CHCl₃ as the eluent to yield the desired product as a clear oil (20.1 mg, 38 %). ¹H NMR (600 MHz, CDCl₃) δ 3.65 (s, 4H), 3.66 (s, 4H), 3.78 (s, 4H), 4.02-4.03 (m, 4H), 5.11 (dq, J = 2, 10 Hz, 2H), 5.25 (dq, J = 2, 17 Hz, 2H), 5.95 (dp, J = 5, 17 Hz, 2H), 6.69 (d, J = 8 Hz, 2H), 7.05 (d, J = 7 Hz, 2H), 7.10-7.13 (m, 2H), 7.24-7.31 (m, 3H), 7.36 (s, 1H), 7.48-7.53 (m, 4H), 7.60 (td, J = 2, 8 Hz, 2H), 8.48-8.49 (m, 4H), 8.84 (br s, NH, 2H), 9.72 (br s, NH, 2H) ppm; ¹³C NMR (600 MHz, CDCl₃) δ 42.2, 58.7, 59.6, 60.1, 110.2, 115.2, 115.5, 115.9, 122.0, 122.6, 127.5, 128.4, 129.1, 135.1, 136.5, 138.6, 138.9, 148.9, 152.9, 156.2, 159.6 ppm; HRMS (ESI, MeCN): m/z = 697.3712 ([M+H]+).

11s. To a solution of 11b (40.0 mg, 75.4 µmol) in CHCl₃ (250 µL) was added 2-(2-thienyl)ethyl isocyanate (50 µL, 380 µmol). The reaction was allowed to stir at room temperature for 12 hours. Solvent was removed and the crude material was purified using silica gel column chromatography with 0-10 % MeOH:CHCl₃ as the eluent to yield the desired product as a clear oil (37.1 mg, 59 %). ¹H NMR (600 MHz, CDCl₃) δ 3.09 (t, J = 6 Hz, 4H), 3.48 (s, 4H), 3.63 (s, 4H), 3.69 (q, J = 6 Hz, 4H), 3.75 (s, 4H), 6.66 (d, J = 8 Hz, 2H), 6.82 (dd, J = 2, 4 Hz, 2H), 6.84 (dd, J = 3, 6 Hz, 2H), 7.06 (dd, J = 1, 5 Hz, 2H), 7.09 (d, J = 8 Hz, 2H), 7.12-7.14 (m, 2H), 7.27-7.29 (m, 3H), 7.40 (s, 1H), 7.48 (t, J = 8 Hz, 2H), 7.52 (d, J = 8 Hz, 2H), 7.60 (td, J = 2, 8 Hz, 2H), 8.49-8.51 (m, 2H), 8.72 (br s, NH, 2H), 9.69 (br s, NH, 2H) ppm; ¹³C NMR (600 MHz, CDCl₃) δ 30.6, 41.1, 58.6, 59.4, 60.1, 105.0, 115.0, 122.0, 122.5, 123.7, 125.1, 127.0, 127.5, 128.4, 128.9, 136.5, 138.6, 139.0, 141.9, 149.0, 152.7, 156.2, 156.5, 159.7 ppm; HRMS (ESI, MeCN): m/z = 837.3481 ([M+H]+).

apo-12. To a solution of CyAL-5 (37 mg, 52 µmol) in anhydrous DMF (550 µL) was added triethylamine (110 µL, 780 µmol) and N,N'-Disuccinimidyl carbonate (54 mg, 210 µmol). The reaction mixture was allowed to stir under argon at room temperature for 24 hours. To the reaction mixture was added a solution of S1 (155 mg, 260 µmol, 5 eq) dissolved in DMF (250
µL). The reaction mixture was allowed to stir under argon at room temperature for 48 hours. Solvent was removed under reduced pressure and the crude material was purified using preparatory TLC methods (silica gel preparatory TLC plate, 80:20:2 CHCl₃:MeOH:NH₄OH as the mobile phase, redeveloped 3 times to achieve good separation) to yield the desired product as a blue solid (61.6 mg, 93 % Yield). ¹H NMR (600 MHz, DMSO-d₆) δ 1.25 (t, J = 6 Hz, 6H), 1.43-1.48 (m, 2H), 1.50-1.54 (m, 2H), 1.64-1.69 (m, 4H), 1.70 (s, 12H), 2.15 (t, J = 6 Hz, 2H), 2.62 (t, J = 6 Hz, 4H), 3.07-3.10 (m, 2H), 3.57 (s, 4H), 3.69 (s, 8H), 3.91 (t, J = 6 Hz, 2H), 4.17-4.21 (m, 4H), 6.18 (d, J = 12 Hz, 2H), 6.78 (s, 2H), 7.06 (s, 1H), 7.22-7.24 (m, 4H), 7.30 (d, J = 12 Hz, 2H), 7.54-7.58 (m, 4H), 7.63 (d, J = 12 Hz, 2H), 7.70-7.74 (m, 4H), 7.80-7.83 (m, 4H), 8.16 (d, J = 12 Hz, 2H), 8.45-8.49 (m, 4H); HRMS (ESI, MeCN): m/z = 1240.9654 ([M-H]⁻).

S1b. To a solution of 6-bromopicolinaldehyde (500 mg, 2.7 mmol) and CaCl₂ (600 mg, 5.4 mmol) in EtOH (25 mL) at 0 °C was added NaBH₄ (310 mg, 8.2 mmol). After stirring for 3 hours at 0 °C H₂O was added to the reaction mixture. The reaction mixture was extracted three times with chloroform and the organic layer was dried over MgSO₄. The crude residue was purified by silica gel column chromatography with 0-10 % MeOH in CHCl₃ as the eluent to yield the desired product as a colorless oil (200 mg, 40 % yield). ¹H NMR (300 MHz, CDCl₃) δ 4.23 (s, OH, 1H), 4.67 (s, 2H), 7.26-7.30 (m, 2H), 7.48 (dd, J = 7, 8 Hz, 1H) ppm; ¹³C NMR (300 MHz, CDCl₃) δ 64.3, 119.6, 126.6, 139.3, 141.2, 162.0 ppm; HRMS (ESI, MeCN): m/z = 187.9722 ([M+H]+), m/z = 209.9553 ([M+Na]+).

S1c. To a solution of S1b (200 mg, 1.1 mmol), CBr₄ (700 mg, 2.1 mmol), and K₂CO₃ (430 mg, 3.1 mmol) in anhydrous dichloromethane (10 mL) at 0°C was added by dropwise addition a solution of PPh₃ (550 mg, 2.1 mmol) in anhydrous dichloromethane (5.5 mL). The reaction mixture was allowed to warm to room temperature while stirring overnight. The reaction mixture was filtered to remove any insoluble material. The crude residue was purified by silica gel column chromatography with 0-5 % MeOH in CHCl₃ as the eluent to yield the desired product as an off-white solid (139 mg, 52 % yield). ¹H NMR (300 MHz, CDCl₃) δ 4.50 (s, 2H), 7.40-7.43 (m, 2H), 7.56 (t, J = 8 Hz, 1H) ppm; ¹³C NMR (300 MHz, CDCl₃) δ 32.7, 122.7, 127.7, 139.6, 141.7, 158.3 ppm; HRMS (ESI, MeCN): m/z = 249.8877 ([M+H]+).

S1d. To a solution of 2-picolylyamine (0.22 mL, 2.2 mmol) and Na₂CO₃ (250 mg, 2.4 mmol) in MeCN (8 mL) at 0 °C was added by dropwise addition a solution of S1c (138.6 mg, 0.55 mmol) in MeCN (12 mL). The reaction mixture was allowed to warm to room temperature
overnight while stirring. The reaction mixture was filtered to remove any insoluble material. The residue was dissolved in EtOAc (25 mL) and wash with water (5 x 25 mL). The organic layer was dried over MgSO₄ and solvent was evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography with 0-10 % MeOH in CHCl₃ as the eluent to yield the desired product as a yellow oil (41.3 mg, 27 %). ¹H NMR (500 MHz, CDCl₃) δ 2.48 (s, 1H), 3.96 (s, 2H), 3.97 (s, 2H), 7.17 (ddd, J = 1, 5, 10 Hz, 1H), 7.34-7.38 (m, 3H), 7.51 (t, J = 8 Hz, 1H), 7.657 (td, J = 2, 7 Hz, 1H), 8.56-8.58 (m, 1H) ppm; ¹³C NMR (500 MHz, CDCl₃) δ 54.4, 54.9, 121.2, 122.3, 122.6, 126.5, 136.7, 139.1, 141.9, 149.6, 159.7, 161.9 ppm; HRMS (ESI, MeCN): m/z = 278.0312 ([M+H]+).

S2b. To a solution of 6-bromopicolinaldehyde (84 mg, 700 µmol) and CaCl₂ (160 mg, 1.4 mmol) in EtOH (10 mL) at 0 °C was added NaBH₄ (190 mg, 4.9 mmol). After stirring for 3 hours at 0 °C H₂O was added to the reaction mixture. The reaction mixture was extracted three times with chloroform and the organic layer was dried over MgSO₄. The crude residue was purified by silica gel column chromatography with 1:1 Hexanes:EtOAc as the eluent to yield the desired product as a colorless oil (54.8 mg, 64 % Yield). ¹H NMR (500 MHz, CDCl₃) δ 2.49 (s, 3H), 4.69 (s, 2H), 4.83 (s, OH, 1H), 7.00 (d, J = 8 Hz, 1H), 7.09 (d, J = 8 Hz, 1H), 7.53 (t, J = 8 Hz, 1H) ppm; ¹³C NMR (500 MHz, CDCl₃) δ 24.1, 64.2, 117.8, 121.9, 137.1, 157.4, 159.0 ppm; HRMS (ESI, MeCN): m/z = 124.0760 ([M+H]+).

S2c. To a solution of S2b (600 mg, 4.9 mmol), CBr₄ (3.2 g, 9.7 mmol), and K₂CO₃, (2.0 g, 14 mmol) in anhydrous dichloromethane (40 mL) at 0°C was added by dropwise addition a solution of PPh₃ (2.5 g, 9.7 mmol) in anhydrous dichloromethane (25 mL). The reaction mixture was allowed to warm to room temperature while stirring overnight. The reaction mixture was filtered to remove any insoluble material. The crude residue was purified by silica gel column chromatography with 25 % EtOAc in hexanes as the eluent to yield the desired product as a red solid (479.5 mg, 53 % yield). ¹H NMR (300 MHz, CDCl₃) δ 2.55 (s, 3H), 4.51 (s, 2H), 7.06 (d, J = 8 Hz, 1H), 7.23 (d, J = 8 Hz, 1H), 7.56 (t, J = 8 Hz, 1H) ppm; ¹³C NMR (300 MHz, CDCl₃) δ 24.6, 34.4, 120.7, 122.9, 137.5, 156.3, 158.6 ppm; HRMS (ESI, MeCN): m/z = 185.9908([M+H]+).

S2d. To a solution of 2-picolyamine (1.0 mL, 9.7 mmol) and Na₂CO₃ (120 mg, 1.1 mmol) in MeCN (2 mL) at 0 °C was added by dropwise addition a solution of S2c (50 mg, 270 µmol) in MeCN (15 mL). The reaction mixture was allowed to warm to room temperature
overnight while stirring. The reaction mixture was filtered to remove any insoluble material. The residue was dissolved in EtOAc (25 mL) and wash with water (5 x 25 mL). The organic layer was dried over MgSO$_4$ and solvent was evaporated under reduced pressure. The crude residue was purified by silica gel column chromatography with 0-10 % MeOH in EtOAC as the eluent to yield the desired product as a dark orange oil (14.4 mg, 25 %). $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 2.54 (s, 3H), 3.02 (br s, NH, 1H), 3.95 (s, 2H), 3.99 (s, 2H), 7.02 (d, $J = 8$ Hz, 1H), 7.15-7.17 (m, 2H), 7.36 (d, $J = 8$ Hz, 1H), 7.53 (t, $J = 8$ Hz, 1H), 7.64 (d of t, $J = 2, 8$ Hz, 1H), 8.56 (d of q, $J = 1, 5$ Hz, 1H) ppm; $^{13}$C NMR (500 MHz, CDCl$_3$) $\delta$ 24.7, 54.9, 55.0, 119.3, 121.8, 122.2, 122.6, 136.7, 137.0, 149.5, 158.2, 158.9, 159.8 ppm; HRMS (ESI, MeCN): m/z = 214.1368 ([M+H]$^+$).
3. Assigned NMR Spectra of apo-12 and apo-13.
Apo-13
4. $^1$H and $^{13}$C NMR data.
8-Mono
11s
5. References.

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