Fluorescent Guanidinium-Azacarbazole for Oxoanion Binding in Water

Lindsey O. Calabretta, Yana D. Petri, and Ronald T. Raines*

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139, USA *Email: rtraines@mit.edu

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General Experimental

Materials. Reagents and solvents were from Sigma–Aldrich (St. Louis, MO) or Combi-Blocks (San Diego, CA) and were used without further purification. (Note: Sodium amide must be fresh and pure white to be fully active.) Water was obtained from a Milli-Q IQ 7000 purification system and had a resistivity of $18.2 \times 10^6 \Omega$ cm.

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

Solvent Removal. The phrase "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator while maintaining a water-bath temperature at 40 °C. Residual solvent was removed from samples at high vacuum (<0.1 Torr), which refers to the vacuum achieved by a mechanical belt-drive oil pump, or through lyophilization (freeze-drying) using a Labconco FreeZone.

Chromatography. Chemical reactions were monitored by thin-layer chromatography (TLC) using EMD 250 μ m silica gel 60-F₂₅₄ plates and visualization with UV-illumination or KMnO₄-staining, or by LC–MS on an ESI Agilent 6125B mass spectrometer. Flash chromatography was performed with a Biotage Isolera automated purification system using prepacked and re-packed SNAP KP silica gel columns or SNAP KP C18 columns.

Instrumentation. ¹H NMR and ¹³C NMR spectra for compound characterization were obtained with Bruker spectrometers, ESI HRMS data were obtained with an Agilent 6545 Q-ToF mass spectrometer, and FTIR spectra were obtained with a Bruker Alpha II spectrometer equipped with a Diamond Crystal Attenuated Total Reflectance (ATR) accessory, all at the Department of Chemistry Instrumentation Facility at the Massachusetts Institute of Technology. Fluorescence spectra were obtained on a QM-1 photon-counting spectrofluorometer from Photon Technology International running Felix fluorescence analysis software version 1.3. UV–Vis spectra were obtained with an Agilent Cary 60 UV–Vis spectrometer.

Chemical Synthesis



1H-Pyrrolo[2,3-c]pyridin-7-amine (1). 6-Azaindole (590 mg, 5 mmol) was placed in a pressure-rated vial and dimethylaniline (20 mL, dried over molecular sieves) was added. The mixture was sonicated until fully dissolved. Sodium amide (780 mg, 20 mmol) was added, the vial was capped, and the solution stirred at 160 °C in an aluminum heating block for 5 h. The solution was cooled and vacuum filtered, and the solids rinsed with hexanes to remove dimethylaniline. Water was added carefully to the solid to quench the sodium amide. The aqueous mixture was then extracted with EtOAc three times, the organics were combined and dried with NaSO₄(s), filtered, and concentrated under reduced pressure. The product was purified by chromatography on silica

gel with a gradient of DCM and MeOH containing TEA (1% v/v), eluting at 20% MeOH to produce a tan solid (442 mg, 66% yield).

¹**H NMR** (400 MHz, DMSO-*d*₆, δ): 10.99 (s, 1H), 7.44 (d, *J* = 5.7 Hz, 1H), 7.38 (d, *J* = 2.9 Hz, 1H), 6.76 (d, *J* = 5.7 Hz, 1H), 6.31 (d, *J* = 2.9 Hz, 1H), 5.90 (s, 2H). ¹³C{¹H} **NMR** (101 MHz, DMSO-*d*₆, δ): 146.4, 135.9, 131.5, 126.2, 120.0, 105.5, 101.2. **HRMS** *m*/*z* calcd for C₇H₈N₃ [M + H]⁺, 134.0718; found, 134.0711.



1-(4-Methoxybenzyl)-3-(1*H***-pyrrolo[2,3-c]pyridin-7-yl)thiourea (2).** 7-Amino-6-azaindole 1 (442 mg, 3.3 mmol) was dissolved in dry THF (30 mL). Triethylamine (1.37 mL, 9.9 mmol) and 4-methoxybenzyl isothiocyanate (656 mg, 3.66 mmol) were added, and the resulting solution was placed under a condenser connected to an $N_2(g)$ line and heated in an oil bath at reflux overnight. The reaction mixture was concentrated under reduced pressure. Ice-cold isopropanol was added to precipitate the product, and the solids were vacuum filtered and rinsed with cold isopropanol to obtain a gray powder. The filtrate was left to evaporate in air and the aforementioned process was repeated to obtain further product (625 mg, 61% yield).

¹**H NMR** (400 MHz, chloroform-*d*, *δ*): 13.48 (t (br), J = 5.7 Hz, 1H), 11.12 (s, 1H), 10.68 (s, 1H), 7.68 (d, J = 5.7 Hz, 1H), 7.33 (d, J = 8.6 Hz, 2H), 7.23 (d, J = 5.7 Hz, 1H), 7.10 (t (br), J = 2.7 Hz, 1H), 6.87 (d, J = 8.7 Hz, 2H), 6.47 (br, J = 3.1, 1.5 Hz, 1H), 5.10 (d, J = 5.6 Hz, 2H), 3.81 (s, 3H). ¹³C{¹H} **NMR** (101 MHz, chloroform-*d*, *δ*): 179.4, 159.1, 140.6, 134.7, 133.6, 129.6, 128.7, 128.2, 120.2, 114.3, 111.9, 102.5, 55.5, 48.8. **HRMS** *m*/*z* calcd for C₁₆H₁₇N₄OS [M + H]⁺, 313.1123; found, 313.1112.



1-(4-Methoxybenzyl)-3-(1*H***-pyrrolo[2,3-c]pyridin-7-yl)guanidine (3).** Thiourea **2** (194 mg, 0.62 mmol) was placed in a flask and dissolved in MeOH (30 mL). Concentrated ammonium hydroxide (6 mL) and 5 M *tert*-butyl hydroperoxide in decane (2.48 mL, 12.4 mmol) were added, and the resulting solution was stirred overnight. The reaction mixture was concentrated under reduced pressure and resuspended in water. The solids were separated by vacuum filtration, rinsing with water and EtOAc. The product was obtained as an orange solid. (161 mg, 87% yield).

¹**H NMR** (500 MHz, DMSO-*d*₆, *δ*): 12.23 (s, 2H), 10.99 (s, 1H), 7.75 (d, J = 5.7 Hz, 1H), 7.55 (t, J = 2.7 Hz, 1H), 7.34 (d, J = 5.8 Hz, 1H), 7.30 (d, J = 8.7 Hz, 2H), 6.86 (d, J = 8.7 Hz, 2H), 6.55 (dd, J = 2.9, 1.7 Hz, 1H), 4.51 (d, J = 5.8 Hz, 2H), 3.70 (s, 3H). ¹³C{¹H} **NMR** (126 MHz, DMSO-*d*₆, *δ*): 158.7, 154.4, 139.1, 133.9, 133.5, 129.5, 128.6, 128.5, 121.2, 114.0, 111.6, 101.9, 55.03, 43.5. **HRMS** *m*/*z* calcd for C₁₆H₁₈N₅O [M + H]⁺, 296.1511; found, 296.1502.



1-(1*H***-pyrrolo[2,3-c]pyridin-7-yl)guanidine Hydrochloride (GAI).** PMB-guanidine 4 (100 mg, 0.34 mmol) was placed in a pressure-rated vial. TFA (3 mL) and MeOH (100 μ L) were added, and the vial was sealed and stirred at 80 °C in an aluminum heating block overnight. The solution was condensed under a stream of N₂(g), then dissolved in water and EtOAc. The mixture was passed through a syringe filter, and the organic layer was extracted with water two times. The aqueous fractions were combined, concentrated under reduced pressure, and purified with reversed-phase chromatography in acetonitrile and water containing TFA (0.1% v/v). The resulting product was dissolved in water, excess HCl was added, and the resulting solution was lyophilized to produce a cream solid (17.4 mg, 29% yield).

¹**H NMR** (500 MHz, DMSO-*d*₆, δ): 12.23 (s, 1H), 8.40 (s (br), 3H), 7.73 (d, *J* = 5.6 Hz, 1H), 7.59 (d, *J* = 2.9 Hz, 1H), 7.24 (d, *J* = 5.6 Hz, 1H), 6.51 (d, *J* = 2.9 Hz, 1H). ¹³C{¹H} **NMR** (126 MHz, D₂O, δ): 158.7, 141.4, 138.4, 135.7, 133.0, 127.7, 117.8, 106.8. **HRMS** *m*/*z* calcd for C₈H₁₀N₅ [M + H]⁺, 176.0936; found, 176.0938.



9H-Pyrido[3,4-*b*]indol-1-amine (4). β -Carboline (500 mg, 3 mmol) was placed in a pressurerated vial, and dimethylaniline (12 mL, dried over molecular sieves) was added. The mixture was heated until fully dissolved. Sodium amide (468 mg, 12 mmol) was added, the vial was capped, and the solution was stirred at 180 °C in an aluminum heating block for 5 h. The solution was cooled and vacuum-filtered, and the solids rinsed with hexanes to remove the dimethylaniline. Water was then carefully added to the solid to quench the sodium amide. The aqueous mixture was then extracted with EtOAc three times, and the organics were combined and dried with NaSO₄(s), filtered, and concentrated under reduced pressure. The product was purified by chromatography of silica gel with a gradient of DCM and MeOH containing NH₃ (1 M), eluting at 12% v/v MeOH to produce a tan solid (377 mg, 69% yield). ¹**H NMR** (400 MHz, DMSO-*d*₆, δ): 11.01 (s, 1H), 8.06 (d, *J* = 7.8 Hz, 1H), 7.71 (d, *J* = 5.5 Hz, 1H), 7.58 (d, *J* = 8.2 Hz, 1H), 7.45 (t, *J* = 7.1 Hz, 1H), 7.29 (d, *J* = 5.5 Hz, 1H), 7.17 (t, *J* = 7.0 Hz, 1H), 6.06 (s, 2H). ¹³C{¹H} **NMR** (101 MHz, DMSO-*d*₆, δ): 147.1, 139.5, 137.0, 127.1, 126.9, 123.3, 122.2, 121.7, 119.4, 112.4, 105.3. **HRMS** *m*/*z* calcd for C₁₁H₁₀N₃ [M + H]⁺, 184.0875; found, 184.0866.



1-(4-Methoxybenzyl)-3-(9H-pyrido[3,4-b]indol-1-yl)thiourea (5). 7-Amino-6-azacarbazole **4** (174 mg, 0.95 mmol) was dissolved in dry THF (9 mL). Triethylamine (340 μ L, 2.85 mmol) and 4-methoxybenzyl isothiocyanate (340 mg, 1.9 mmol) were added, and the solution was placed in a pressure-rated vial and stirred at 80 °C in an aluminum heating block overnight. The solution was concentrated under reduced pressure. Ice-cold ethanol was added to precipitate the product, and the solids were vacuum-filtered and rinsed with ice-cold ethanol to obtain a gray powder (190 mg, 55% yield).

¹**H NMR** (400 MHz, DMSO-*d*₆, *δ*): 12.69 (t, J = 5.6 Hz, 1H), 11.94 (s, 1H), 10.46 (s, 1H), 8.22 (d, J = 7.9 Hz, 1H), 7.92 (d, J = 5.5 Hz, 1H), 7.85 (d, J = 5.5 Hz, 1H), 7.68 (d, J = 8.3 Hz, 1H), 7.57 (t, J = 7.7 Hz, 1H), 7.37 (d, J = 8.1 Hz, 2H), 7.28 (t, J = 7.5 Hz, 1H), 6.94 (d, J = 8.2 Hz, 2H), 4.90 (d, J = 5.5 Hz, 2H), 3.74 (s, 3H). ¹³C{¹H} **NMR** (101 MHz, DMSO-*d*₆, *δ*): 180.4, 158.9, 140.6, 140.1, 134.2, 130.6, 129.5, 129.3, 128.6, 123.1, 122.1, 121.3, 120.4, 114.4, 112.8, 111.0, 55.6, 48.0. **HRMS** *m*/*z* calcd for C₂₀H₁₉N₄OS [M + H]⁺, 363.1280; found, 363.1262.



1-(4-Methoxybenzyl)-3-(9H-pyrido[3,4-b]indol-1-yl)guanidine (6). Thiourea **5** (108 mg, 0.3 mmol) was placed in a flask and suspended in MeOH (15 mL). Concentrated ammonium hydroxide (3 mL) and 5 M *tert*-butyl hydroperoxide in decane (1.2 mL, 6 mmol) were added, and the suspension was stirred overnight. The reaction mixture was subsequently concentrated under reduced pressure and resuspended in water. The solids were separated by vacuum filtration, rinsing with water. The product was obtained as a gray solid that was used directly. **HRMS** m/z calcd for C₂₀H₂₀N₅O [M + H]⁺, 346.1668; found, 346.1661.



1-(9*H***-Pyrido[3,4-b]indol-1-yl)guanidine Hydrochloride (GAC).** PMB-guanidine **6** (88 mg, 0.26 mmol) was placed in a pressure-rated vial. TFA (2.5 mL) and MeOH (100 μ L) were added, and the vial was sealed and the solution was stirred at 80 °C in an aluminum heating block overnight. The reaction mixture was condensed under a stream of N₂(g), then dissolved in water and EtOAc. The aqueous solution was extracted with EtOAc three times, the organics were combined and dried with Na₂SO₄(s), filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel with a gradient of DCM and MeOH, eluting at 5% v/v MeOH to produce a brown solid (23 mg, 40% yield). The product was converted to the HCl salt by suspending in DCM and adding one equivalent of 0.1 M HCl in MeOH, at which point the compound fully dissolved. The product was concentrated under reduced pressure to produce a pale pink solid.

¹**H NMR** (500 MHz, DMSO-*d*₆, δ): 11.58 (s, 1H), 11.47 (s, 1H), 8.35 (s, 3H), 8.26 (d, *J* = 7.9 Hz, 1H), 8.08 (d, *J* = 5.5 Hz, 1H), 7.98 (d, *J* = 5.4 Hz, 1H), 7.69 (d, *J* = 8.2 Hz, 1H), 7.60 (t, *J* = 7.6 Hz, 1H), 7.31 (t, *J* = 7.5 Hz, 1H). ¹³C{¹H} **NMR** (151 MHz, DMSO-*d*₆, δ): 156.0, 140.4, 138.4(br), 135.2, 130.1, 129.1, 125.4, 122.3, 121.3, 120.6, 112.8, 112.2. **HRMS** *m*/*z* calcd for C₁₂H₁₂N₅ [M + H]⁺, 226.1093; found, 226.1079.



tert-Butyl(3'-chloro-[4,4'-bipyridin]-3-yl)carbamate (7). *N*-(Boc)-3-amino-4-bromopyridine (1 g, 3.66 mmol), 3-chloropyridine-4-boronic acid (1150 mg, 7.33 mmol), and Pd(dppf)Cl₂ (270 mg, 0.37 mmol) were evenly divided into two pressure-rates vials. Dioxane (18 mL) and 2 M K₂CO₃ (2.25 mL) were equally divided and added to the two vials. The vials were capped, and the solutions were stirred at 70 °C in an aluminum heating block for $1\frac{1}{2}$ -2 h. The vials were removed from heat, the contents were combined, and water and EtOAc were added. The aqueous fraction was extracted with EtOAc four times, dried with NaSO₄(s), filtered, and concentrated under reduced pressure. The crude product was purified by chromatography on silica gel with a gradient of EtOAc and hexanes, eluting at 100% v/v EtOAc to produce a cream solid (366 mg, 33% yield). The *N*-(Boc)-3-amino-4-bromopyridine was also recovered as a white solid (446 mg, 68% recovered from theoretical remaining amount).

¹**H NMR** (400 MHz, chloroform-*d*, *δ*): 9.13 (s, 1H), 8.69 (s, 1H), 8.57 (d, J = 4.9 Hz, 1H), 8.42 (d, J = 4.9 Hz, 1H), 7.25 (d, J = 4.8 Hz, 1H), 7.10 (d, J = 4.9 Hz, 1H), 6.56 (s, 1H), 1.42 (s, 9H). ¹³C{¹H} **NMR** (101 MHz, chloroform-*d*, *δ*): 152.6, 150.2, 148.2, 145.0, 142.8, 135.3, 131.9, 130.7, 125.1, 123.5, 81.5, 28.1. **HRMS** m/z calcd for C₁₅H₁₇ClN₃O₂ [M + H]⁺, 306.1009; found, 306.1000.



9H-Pyrrolo[2,3-c:5,4-c']dipyridine (8). Bipyridine **7** (1.15 g, 3.77 mmol) was added to a flask and TFA (5 mL) and MeOH (100 μ L) were added. The orange solution was stirred for 30 min, after which time the solution was dried under a stream of N₂(g). The product was dissolved in EtOAc, and 1 M NaOH was added. The aqueous fraction was extracted with EtOAc four times, dried with Na₂SO₄(s), filtered, and concentrated under reduced pressure. The deprotected bipyridine was then split into three equal parts and placed in three pressure-rated vials. XPhos Pd G2 (255 mg, 0.32 mmol) and sodium *tert*-butoxide (1.09 g, 11.3 mmol) was split evenly and added to the three vials, followed by THF (37 mL), split evenly. The vials were flushed with N₂(g) and capped, and the solution was added to dissolve the mixture fully. Silica was added, and the mixture was concentrated under reduced pressure. The crude product was purified by chromatography on silica gel with a gradient of DCM and MeOH, eluting at 15% v/v MeOH to produce a cream colored solid (552 mg, 87% yield).

¹**H NMR** (500 MHz, DMSO-*d*₆, δ): 11.98 (s, 1H), 9.06 (d, *J* = 1.2 Hz, 2H), 8.44 (d, *J* = 5.3 Hz, 2H), 8.24 (dd, *J* = 5.3, 1.2 Hz, 2H). ¹³C{¹H} **NMR** (126 MHz, DMSO-*d*₆, δ): 139.0, 136.9, 136.1, 126.2, 116.4. **HRMS** *m*/*z* calcd for C₁₀H₈N₃ [M + H]⁺, 170.0718; found, 170.0708.



9H-Pyrrolo[2,3-c:5,4-c']dipyridine-1,8-diamine (9). Diazacarbazole **8** (338 mg, 2 mmol) was placed in a pressure-rated vial, and dimethylaniline (10 mL, dried over molecular sieves) was added. The solution was stirred at 180 °C to dissolve diazacarbazole **8**. Once dissolved, the solution was cooled slightly, and sodium amide (624 mg, 16 mmol) was added. The vial was sealed, and the solution was stirred at 180 °C in an aluminum heating block for 5 h. The solution was cooled and vacuum filtered, and the solids rinsed with hexanes to remove the dimethylaniline. Water was then added carefully to the solid to quench the sodium amide. The aqueous mixture was then extracted with EtOAc four times, the organics were combined and dried with NaSO4(s), filtered, and concentrated under reduced pressure. The product was purified by chromatography on silica gel with a gradient of DCM and MeOH containing NH₃ (1 M), eluting at 20% v/v MeOH to produce a yellow solid (274 mg, 69% yield). The product could also be purified by reverse-phased

C18 chromatography in water and acetonitrile to avoid elution of silica from the highly polar solvent phase.

¹**H** NMR (500 MHz, DMSO- d_6 , δ): 11.69 (s, 1H), 7.68 (d, J = 5.7 Hz, 2H), 7.30 (d, J = 5.7 Hz, 2H), 6.37 (s, 4H). ¹³C{¹H} NMR (151 MHz, MeOD, δ): 147.2, 133.6, 125.9, 125.0, 105.4. **HRMS** m/z calcd for C₁₀H₁₀N₅ [M + H]⁺, 200.0936; found, 200.0927.

Synthesis of 1-(8-Amino-9H-pyrrolo[2,3-c:5,4-c']dipyridin-1-yl)guanidine (GADAC)



Step 1: 1-(8-Amino-9*H*-pyrrolo[2,3-*c*:5,4-*c*']dipyridin-1-yl)-3-(4-methoxybenzyl)thiourea (10). Diamine 9 (55 mg, 0.28 mmol) was placed in a pressure-rated vial and dissolved in dry THF (11 mL) and dry DMF (3 mL). Triethylamine (115 μ L, 0.83 mmol) and *para*-methoxybenzyl isothiocyanate (500 mg, 2.8 mmol) were added. The headspace was flushed with N₂(g), and the vial was capped, and the solution was stirred at 80 °C in an aluminum heating block for 5 h. The solution was removed from heat and condensed under a stream of air overnight. Hexanes were added to the resulting orange oil, and the mixture was sonicated. The mixture was then vacuum-filtered and rinsed with hexanes to produce 88.5 mg of orange powder, which was used directly. **HRMS** *m*/*z* calcd for C₁₉H₁₉N₆OS [M + H]⁺, 379.1341; found, 379.1313.



Step2:1-(8-Amino-9H-pyrrolo[2,3-c:5,4-c']dipyridin-1-yl)-3-(4-methoxybenzyl)guanidine (11). Half of the crude thiourea 10 (43 mg, ~0.11 mmol) wasdissolved in dry DMF (11 mL), and the resulting solution was cooled in an ice bath. Mercury(II)chloride (33 mg, 0.12 mmol) was added, and the solution was stirred for 5–10 min until cloudy. A0.5 M solution of ammonium in dioxane (2.2 mL, 1.1 mmol) was added dropwise, and the solutionwas stirred on ice for 15 min, then stirred at room temperature for 1 h. The gray cloudy mixturewas then filtered through celite and rinsed with EtOAc. The filtrate was condensed under a streamof N₂(g) overnight, and the resulting dark orange oil was used directly.HRMS m/z calcd for C₁₉H₂₀N₇O [M + H]⁺, 362.1729; found, 362.1704.



Step 3: 1-(8-Amino-9*H*-pyrrolo[2,3-*c*:5,4-*c*]dipyridin-1-yl)guanidine (GADAC). PMBguanidine 11 (~0.11 mmol) was dissolved in TFA (5 mL) and MeOH (100 μ L) in a pressure-rated vial, and the solution was stirred at 80 °C in an aluminum heating block for 3 h. The solution was condensed under a stream of N₂(g), then resuspended in EtOAc and 1 M NaOH. The aqueous layer was extracted with EtOAc five times, the organics were combined and concentrated under reduced pressure without drying with Na₂SO₄(s). The solid was then resuspended in water with a few drops 1 M HCl, and the resulting solution was filtered through cotton. The aqueous solution was purified by reversed-phase C18 chromatography with a gradient of acetonitrile and water containing ammonium acetate (10 mM), eluting at 30% v/v acetonitrile. The resulting fractions were concentrated under reduced pressure and lyophilized to remove excess ammonium acetate. The product was then redissolved in water, and a few drops of 1 M HCl were added. The product was lyophilized again to convert the product from the diacetate salt to the dichloride, resulting in a bright yellow solid (8.3 mg, 19% yield over three steps).

¹**H** NMR (600 MHz, D₂O, δ): 7.71 (d, J = 5.4 Hz, 1H), 7.34 (d, J = 6.6 Hz, 1H), 7.33 (d, J = 6.1 Hz, 2H), 7.13 (d, J = 6.6 Hz, 1H). ¹³C{¹H} NMR (151 MHz, D₂O, δ): 154.6, 143.7, 137.2, 136.0, 127.0, 126.5, 125.3, 124.5, 121.6, 112.2, 106.0. **HRMS** m/z calcd for C₁₁H₁₂N₇ [M + H]⁺, 242.1154; found, 242.1147.

Unsuccessful Reactions and Synthetic Routes



Carbamate Transfer During Guanidinylation with Thiourea

Figure S1. The guanidinylation of amino-azaindole **1** produces a product with a mass corresponding to a Boc addition. (A) Reaction scheme showing the conditions and major undesired product. (B) LC–MS total ion chromatogram (TIC). (C) Mass observed at the peak highlighted in the LC–MS TIC.

We attempted to convert the amino group into a guanidino group through a mercury-aided reaction with a thiourea. Attempts at this reaction resulted in very low product yield and multiple byproducts as observed by LC–MS. The main byproduct corresponded to the addition of the Boc protecting group onto the azaindole (Figure S1). This result was surprising, given that other groups have been successful at producing a guanidino compound through this method using a 2-aminopyridine.¹⁻² We have also observed a di-Boc protected guanidino group as the major product by LC–MS when reacting 2-aminopyridine with isothiourea (S1). We hypothesize that the indole N-H is able to form a hydrogen bond with the carbonyl oxygen of the carbamate, increasing its electrophilicity and leading to the amine attacking the carbonyl through a 7-endo-trig cyclization.

Subsequently, other methods were explored to append the guanidino group. Another common route is the reaction of the amine with an isothiocyanate to produce a thiourea. The most commonly

used isothiocyanates are protected with carbamate groups, typically CBZ or Fmoc. We attempted reacting Cbz-NCS, Fmoc-NCS, as well at Bz-NCS with aminoazaindole **1**, but these routes were unsuccessful because we observed addition of the amino group to the carbonyl carbon rather than the isothiocyanate carbon. The reaction with Bn-NCS was successful, but we were unable to remove the benzyl protecting group from the ensuing guanidino compound.

Pictet-Spengler Route to 2,7-Diazacarbazole

Scheme S1. Synthetic Route to 2,7-Diazacarbazole using a Pictet-Spengler Reaction



To synthesize the 2,7-diazacarbazole, we sought to use the traditional Pictet–Spengler route that has been used in the synthesis of azacarbazoles (Scheme S1).³ This route is biomimetic and typically involves the cyclization of tryptophan. In this case, we used the 6-azaindole and reacted it with methyl oxalyl chloride to produce methyloxoacetate-azaindole (S2), followed by exposure to NH₃ to produce the oxoacetamide-azaindole (S3). We were able to reduce compound S3 to form azatryptamine S4. We were, though, unsuccessful at closing the third ring upon exposure of azatryptamine S4 to formaldehyde in acidic or basic conditions. Although we observed the formation of the hemiaminal and the imine by LC–MS, the electronics of this ring structure are such that the 1-position is apparently not nucleophilic enough to attack the imine.

Methyl 2-Oxo-2-(1*H***-pyrrolo[2,3-c]pyridin-3-yl)acetate (S2).** 6-Azaindole (350 mg, 3 mmol) and aluminum chloride (2 g, 15 mmol) were added to a dry flask. A mixture of DCM (11 mL) and nitromethane (4 mL) that had been dried over molecular sieves was added, followed by methyl chlorooxoacetate (554 μ L, 6 mmol). The resulting solution was stirred for 1 h. MeOH was added to quench the solution until the solution stopped bubbling. Saturated aqueous NaHCO₃ was added until the pH reached 7. The solution was then concentrated under reduced pressure to remove the organic solvents. The aqueous solution was extracted four times with EtOAc, dried with Na₂SO₄(s), filtered, and concentrated under reduced pressure to produce a tan powder (513 mg, 84% yield).

¹**H** NMR (500 MHz, DMSO- d_6 , δ): 12.84 (s, 1H), 8.91 (d, J = 1.2 Hz, 1H), 8.66 (s, 1H), 8.38 (d, J = 5.4 Hz, 1H), 8.06 (dd, J = 5.4, 1.2 Hz, 1H), 3.91 (s, 3H). ¹³C{¹H} NMR (126 MHz, DMSO-

 d_6, δ): 179.0, 163.7, 142.2, 141.3, 136.0, 134.2, 131.2, 115.9, 112.4, 53.2. **HRMS** *m*/*z* calcd for C₁₀H₁₂N₃O₃ [M + NH₄]⁺, 222.0879; found, 222.0875.

2-Oxo-2-(1*H***-pyrrolo[2,3-c]pyridin-3-yl)acetamide (S3).** Methyloxoacetate-azaindole S2 (500 mg, 2.45 mmol) was dissolved in 7 M NH₃ in MeOH (15 mL) for 45 min. The solution was concentrated under reduced pressure to produce a tan solid (430 mg, 93% yield).

¹**H NMR** (500 MHz, DMSO-*d*₆, δ): 12.60 (s, 1H), 8.88 (s, 1H), 8.86 (s, 1H), 8.36 (d, *J* = 5.4 Hz, 1H), 8.14 (s, 1H), 8.10 (dd, *J* = 5.4, 1.1 Hz, 1H), 7.80 (s, 1H). ¹³C{¹H} **NMR** (126 MHz, DMSO-*d*₆, δ): 183.5, 165.9, 142.0, 141.1, 135.9, 133.8, 131.6, 116.0, 112.0. **HRMS** *m/z* calcd for C₉H₈N₃O₂ [M + H]⁺, 190.0617; found, 190.0609.

2-(1*H***-Pyrrolo[2,3-***c***]pyridin-3-yl)ethan-1-amine (S4).** Oxoacetamide-azaindole S3 (150 mg, 0.79 mmol) was placed in a pressure-rated vial, and dry THF (4 mL) was added. The mixture was sonicated to suspend S3, and lithium aluminum hydride (524 mg, 15.9 mmol) was added. The vial was flushed with $N_2(g)$ and capped, and the solution was stirred at 70 °C in an aluminum heating block overnight. The solution was quenched with water, 2 M NaOH, more water, and then filtered. The filtrate was purified by chromatography on silica gel with a gradient of MeOH containing NH₃ (7 M) and DCM to produce a yellow oil (58 mg, 46% yield).

¹**H NMR** (400 MHz, DMSO-*d*₆, δ): 11.31 (s, 1H), 8.68 (d, *J* = 1.1 Hz, 1H), 8.05 (d, *J* = 5.5 Hz, 1H), 7.50 (dd, *J* = 5.5, 1.1 Hz, 1H), 7.38 (s, 1H), 3.17 (s, 2H), 2.82 (dd, *J* = 7.9, 4.8 Hz, 2H), 2.76 (dd, *J* = 7.8, 4.8 Hz, 2H). ¹³C{¹H} **NMR** (101 MHz, DMSO-*d*₆, δ): 137.1, 134.3, 133.5, 131.4, 126.8, 113.2, 112.3, 42.5, 28.8. **HRMS** *m*/*z* calcd for C₉H₁₂N₃ [M + H]⁺, 162.1031; found, 162.1024.

Suzuki Conditions

$R = H, Boc$ H OH Ho^{B} CI N Ho^{B} CI N CI T					
	Conditions				
Catalysts	Pd(PPh ₃) ₂ Cl ₂	Xphos Pd G3			
	Pd(amphos)Cl ₂	Sphos Pd G3			
	Pd(dppf)Cl ₂	cataCXium Pd G3			
	Pd(DTBPF)Cl ₂ ,	phosphaadamantane Pd G3			
Bases	Na ₂ CO ₃				
	K ₃ PO ₄				
Solvents	dimethoxyethane				
	dioxane				
	dimethylformamide				
Temperatures	70°				
	100°				
	150°				

Table S1. Conditions Explored for the Suzuki Reaction to Produce Bipyridine 7.

A range of Suzuki reaction conditions were explored to produce bipyridine 7 (Table S1). The reaction conditions were attempted *ad hoc* and not as part of a screen. Reactions with the unprotected bromopyridine resulted in yields $\leq 20\%$, whereas the Boc-protected bromopyridine resulted in yields of up to 31%. In addition, whereas the unprotected bromopyridine is a less expensive reagent, reactions using this starting material suffered from more difficult purifications, and the unprotected bromopyridine was found to be highly unstable, likely undergoing an S_NAr reaction with itself.

Fluorescence Spectra



Figure S2. Normalized absorbance/emission spectra of fluorescent guanidino compounds in MeOH. (A) GAC ($\lambda_{ex} = 315 \text{ nm}$). (B) GADAC ($\lambda_{ex} = 340 \text{ nm}$).



Figure S3. Normalized absorbance/emission spectra of fluorescent guanidino compounds in water. (A) GAC ($\lambda_{ex} = 315 \text{ nm}$). (B) GADAC ($\lambda_{ex} = 340 \text{ nm}$).



Figure S4. Normalized absorbance/emission spectra of GADAC in buffers ($\lambda_{ex} = 340$ nm). (A) Spectra in 10 mM citrate buffer, pH 4.0, containing NaCl (150 mM). (B) Spectra in 10 mM sodium phosphate buffer, pH 7.4, containing NaCl (150 mM).



Figure S5. Normalized excitation/emission spectra of the 2-azacarbazole backbone compounds in MeOH ($\lambda_{ex} = 315$ nm). (A) β -Carboline. (B) Amino-azacarbazole 4. (C) GAC.



Figure S6. Normalized excitation/emission spectra of the 2,7-azacarbazole backbone compounds in MeOH, unless stated otherwise. (A) Diazacarbazole (8) ($\lambda_{ex} = 325$ nm). (B) Diaminodiazacarbazole (9) ($\lambda_{ex} = 325$ nm). (C) GADAC ($\lambda_{ex} = 340$ nm). (D) GADAC in water ($\lambda_{ex} = 340$ nm).

pH Titrations

Fluorescence pH Titration. A 50- μ L aliquot of the stock GAC or GADAC solution in isopropanol was diluted with 9.95 mL of water to produce a final concentration of GAC (4.42 μ M) or GADAC (0.685 μ M). NaCl (30 mg) was added to a cuvette along with 3 mL of the aqueous solution of GAC or GADAC. The excess chloride anions ensure that any change in fluorescence comes from changes in pH as opposed to changes in chloride binding or ionic strength upon the addition of HCl. The pH of the resulting solution was then adjusted with aqueous NaOH or HCl. Emission spectra were acquired at each pH, and the intensity of the selected wavelengths at each point was determined and plotted in triplicate. The pK_a values of each molecule were determined by fitting the data with GraphPad Prism 6 software.

Absorbance pH Titration. Sodium chloride (30 mg) was added to a cuvette along with 2.94 mL of water and a 60 μ L aliquot of an aqueous solution of GADAC (0.51 mM). The pH of the resulting solution was then adjusted with aqueous NaOH or HCl. Absorbance spectra were acquired at each pH, and the absorbance of the selected wavelengths at each point determined and plotted in triplicate. The p K_a values of GADAC were determined by fitting the data with GraphPad Prism 6 software.

¹**H NMR pH Titration.** GAI (4.2 mg, 0.02 mmol) was dissolved in 15 mL D₂O in a vial with a stir bar. While stirring, a pH probe was used to measure the pH(obs) of the solution. The pH(obs) of the solution was adjusted with a solution of NaOD or DCl in D₂O. Aliquots were taken for analysis with ¹H NMR spectroscopy at each increment, and changes in chemical shifts were plotted and fitted with GraphPad Prism 6 software. The experiment was repeated in triplicate. Because the ¹H NMR titration was performed in D₂O rather than H₂O, the observed pK_a value was adjusted by using eq S1⁴:

$$pK_a = 0.929 \times pK_a(obs) + 0.42$$
 (S1)



Figure S7. Fluorescence spectra of GAC in water at different pH values. (A) Representative fluorescence spectra of one replicate ($\lambda_{ex} = 315 \text{ nm}$). (B) Fitted titration curve from low pH values with data obtained at 390 nm, resulting in p K_{a1} of 2.57 ± 0.03. (C) Fitted titration curve from high pH values with data obtained at 390 nm, resulting in a p K_{a2} of 8.96 ± 0.05.



Figure S8. ¹H NMR spectra of GAI in D₂O at different pH values. (A) Representative ¹H NMR spectra of one replicate. (B) Fitted titration curve with data obtained from the starred signal originating at 7.56 ppm, resulting in a pK_a of 9.43 ± 0.01.



Figure S9. Fluorescence spectra of GADAC in water at different pH values. (A) Fluorescence spectra of one replicate ($\lambda_{ex} = 340 \text{ nm}$). (B) Fitted titration curve from the pH values, data obtained at 403 nm, resulting in a p K_a of 6.42 ± 0.04.



Figure S10. Absorbance spectra of GADAC in water at different pH values. (A) Representative absorbance spectra of one replicate. (B) The absorbance at 340 nm, which is the excitation wavelength for Figure 3A. (C) Fitted titration curve from low pH values with data obtained at 356 nm, resulting in a pK_a of 6.18 ± 0.10 . (D) Fitted titration curve from high pH values with data obtained at obtained at 356 nm, resulting in a pK_a of 8.92 ± 0.07 .

Extinction Coefficients

GAC. A 0.833 M stock solution of GAC was prepared in isopropanol. The concentration of this solution was confirmed by acquiring an integrated ¹H NMR spectrum of an aliquot that was spiked with a known concentration of acetonitrile. Aliquots from the stock solution were diluted into six cuvettes that each contained a total of 3.00 mL of water, producing a concentration gradient. The absorbance of each solution was obtained and normalized to a buffer-only blank. The values of concentration were plotted versus those of absorbance at λ_{max} with GraphPad Prism 6 software. The extinction coefficient was determined from a linear fit of the data, forcing the *x*-and *y*-intercept values to be zero.



Figure S11. Extinction coefficient of GAC in water. (A) Absorbance spectra of GAC at increasing concentrations. (B) Linear fit of GAC concentration versus absorbance at $\lambda_{max} = 350$ nm, giving $\varepsilon_{GAC} = 6400 \pm 30 \text{ M}^{-1} \text{cm}^{-1}$.

GADAC. A 0.51 mM stock solution of GADAC was prepared in water. The concentration of this solution was confirmed by acquiring an integrated ¹H NMR spectrum of an aliquot that was spiked with a known concentration of acetonitrile. Aliquots from the stock solution were diluted into six cuvettes that each contained a total of 3.00 mL of buffer, producing concentration gradients. The buffers were 10 mM sodium citrate buffer, pH 4.0, containing NaCl (150 mM) and 10 mM sodium phosphate buffer, pH 7.4, containing NaCl (150 mM). The absorbance of each solution was obtained and normalized to a buffer-only blank. The values of concentration were plotted versus those of absorbance at λ_{max} with GraphPad Prism 6 software. The extinction coefficient was determined from a linear fit of the data, forcing the *x*- and *y*-intercept values to be zero.



Figure S12. Extinction coefficient of GADAC in aqueous buffer. (A) Absorbance spectra of GADAC at increasing concentrations in 10 mM sodium citrate buffer, pH 4.0, containing NaCl (150 mM). (B) Absorbance of GADAC at increasing concentrations in 10 mM sodium phosphate buffer, pH 7.4, containing NaCl (150 mM). (C) Linear fits of GADAC concentration versus absorbance at $\lambda_{max} = 356$ nm giving $\varepsilon_{pH 4.0} = 13,400 \pm 20 \text{ M}^{-1} \text{cm}^{-1}$, $\varepsilon_{pH 7.4} = 12,200 \pm 40 \text{ M}^{-1} \text{cm}^{-1}$.

Quantum Yields

Absolute quantum yields (Φ) of GADAC were measured with a Quantaurus-QY spectrometer (model C11374) from Hamamatsu. This instrument uses an integrating sphere to determine photons absorbed and emitted by a sample. Measurements were carried out using dilute samples (A < 0.1) and self-absorption corrections were performed using the instrument software.⁵

For quantum yield measurements, GADAC was first dissolved in DMSO to yield a 10 mM stock solution, which was then diluted 1:1000 (final concentration: 10 μ M) or 1:2000 (final concentration: 5 μ M) in either 10 mM sodium citrate buffer, pH 4.0, containing NaCl (150 mM) or 10 mM sodium phosphate buffer, pH 7.4, containing NaCl (150 mM). Quantum yield values are averages of the values obtained from three separate experiments, two at 10 μ M GADAC and one at 5 μ M GADAC.

Anion-Binding Titrations

General Procedure for Titrations with GP, GAC, and GADAC

Bulk amounts of the three guanidino compounds were separated into many vials, and the resulting amount of guanidino compound per vial was calculated by obtaining an ¹H NMR of the vial with an internal concentration reference: GP (1 µmol per vial), GAC (0.883 µmol per vial), GADAC (0.137 µmol per vial). When a titration was performed, 1.0 mL of isopropanol was added to a chosen vial. A 100-µL aliquot of this solution was then added to 19,900 µL of a solvent (*i.e.*, isopropanol, methanol, or unbuffered water) to obtain a final concentration of GP (5 µM), GAC (4.42 µM), and GADAC (0.685 µM). The desired anion, **12**, **13**, or **14** (0.1 mmol), was then dissolved in 1.0 mL of the GP, GAC, or GADAC stock solution to make a 100 mM solution of oxoanion. The anion was serially diluted with more stock solution to make 10 mM and 1 mM solutions, as necessary.

The GP, GAC, or GADAC solution (3.0 mL) was then added to a cuvette, and a fluorescence emission scan was acquired. Aliquots of an anion solution were added sequentially, and an emission scan was acquired after each addition. Thus, the GP, GAC, or GADAC concentration was constant, and anion concentrations were calculated to account for dilution. An appropriate wavelength was chosen for each guanidino compound, and the intensity of that wavelength at each concentration of anion was determined and plotted. These curves were obtained in triplicate and analyzed in one input with the web-hosted program Bindfit on supramolecular.org. One-replicate examples of the full emission scans are shown below as well as the binding curves for the selected wavelengths. The open-source data for each titration is included as a supramolecular.org link.⁶⁻⁸

solvent	compound	acetate 12	phosphate 13	sulfate 14
isopropanol —	GP	No binding	—	_
	GAC	$(5.3 \pm 0.3) \times 10^4$	$(1.0 \pm 0.1) \times 10^5$	$(1.2 \pm 0.1) \times 10^4$
methanol	GAC	$(1.1 \pm 0.1) \times 10^3$	680 ± 30	$(1.1 \pm 0.1) \times 10^4$
water —	GAC	No binding	_	_
	GADAC	$(3.6 \pm 0.1) \times 10^4$	$(1.1 \pm 0.0) \times 10^3$	$(4.2 \pm 0.3) \times 10^3$

Table S2. Binding Affinities (M⁻¹) of GP, GAC, and GADAC for Oxoanions 12, 13, and 14

–, not measured



Figure S13.1. Binding titrations of GAC and oxoanions in isopropanol. Fluorescence spectra are shown for one replicate with acetate 12 (A), phosphate 13 (C), and sulfate 14 (E) with $\lambda_{ex} = 315$ nm. Binding curves are shown for one replicate with acetate 12 (B), phosphate 13 (D), and sulfate 14 (E). Fits can be found at the following links.

(B) http://app.supramolecular.org/bindfit/view/fb149c6f-55db-4f79-82e6-7a6227a9534c

- (D) http://app.supramolecular.org/bindfit/view/83244507-9aa8-4feb-8eae-a99e477d688c
- (F) http://app.supramolecular.org/bindfit/view/26ac45b7-f96c-46e0-9443-4a3209ebb8d1



Figure S13.2. Binding titrations of GAC and oxoanions in MeOH. Fluorescence spectra are shown for one replicate with acetate **12** (A), phosphate **13** (C), and sulfate **14** (E) with $\lambda_{ex} = 315$ nm. Binding curves are shown for one replicate with acetate **12** (B), phosphate **13** (D), and sulfate **14** (F). Fits can be found at the following links.

- (B) http://app.supramolecular.org/bindfit/view/5f1bac0d-a54a-4a32-b2eb-a52ec468ee83
- (D) http://app.supramolecular.org/bindfit/view/165c9e03-172e-458d-89a2-3e6542b0da07
- (F) http://app.supramolecular.org/bindfit/view/ea0a2b47-7769-444b-96fb-a904fe6e3bf4



Figure S13.3. Binding titrations of GADAC and oxoanions in water. Fluorescence spectra are shown for one replicate with acetate **12** (A), phosphate **13** (C), and sulfate **14** (E) with $\lambda_{ex} = 340$ nm. Binding curves are shown for one replicate with acetate **12** (B), phosphate **13** (D), and sulfate **14** (F). Fits can be found at the following links.

- (B) http://app.supramolecular.org/bindfit/view/bbb829b9-f1df-4b52-8f63-21127053995d
- (D) http://app.supramolecular.org/bindfit/view/dc29a248-7d50-4ab6-b3c7-c02a1aa461d3
- (F) http://app.supramolecular.org/bindfit/view/9acaf0b2-de07-4e13-8a00-928ad2fe93fc



Figure S13.4. Binding titration of GP and acetate **12** in isopropanol. (A) Fluorescence spectra for one replicate with $\lambda_{ex} = 305$ nm. (B) Binding curve for one replicate.



Figure S13.5. Binding titration of GAC and acetate **12** in water. (A) Fluorescence spectra for one replicate with $\lambda_{ex} = 315$ nm. (B) Binding curve for one replicate.

Lack of Reactivity Between GADAC and Sulfate 14

Oxygen and nitrogen nucleophiles can react with alkyl sulfates, such as sulfate **14**, in water.⁹ Hence, we sought to confirm that GADAC, which has more nitrogen nucleophiles than GP and GAC, forms only noncovalent complexes with sulfate **14** in our titration experiments. To do that, we incubated 1 equiv of GADAC (final concentration: 6.85μ M; $10\times$ greater than that in titration experiments) with 1000 equiv (6.85 mM) of sulfate **14** in 5% v/v isopropanol in water for 3 h at room temperature. After the indicated time, the solution was concentrated $10\times$ under reduced pressure and analyzed by injection into an Agilent 6545 Q-ToF mass spectrometer. The potential alkylation byproducts expected if GADAC reacted with sulfate **14** are shown in Scheme S2. No *N*-alkylation or *N*-sulfation products were detected by HRMS analysis (Figure S14). These results indicate that no reaction occurred between GADAC and sulfate **14** during the titration experiments.

Scheme S2. Potential Products for the Reaction of GADAC Reacted with Sulfate 14



[M + H]⁺ expected: 242.1149 [M + H]⁺ observed: 242.1151

H₂N NH H NH





[M + H]⁺ expected: 322.0717 [M + H]⁺ observed: none



[M + H]⁺ observed: none



[M + H]⁺ expected: 402.0285 [M + H]⁺ observed: none



[M + H]⁺ expected: 336.0873 [M + H]⁺ observed: none



Figure S14. HRMS analysis (direct injection) of a solution of GADAC incubated with 1000 equiv of sulfate 14 for 3 h in water at room temperature. (A) Baseline peak chromatogram. (B,C) Detected m/z within the indicated retention times. (D) Detected m/z of GADAC. No m/z of a reaction product (Scheme S2) was detected.

Lipophilicity Calculations



Figure S15. The cLog *P* and TPSA values for (A) GADAC, (B) GCP,¹⁰ and (C) SMoC¹¹ were calculated with the web-hosted software on https://www.molinspiration.com (Molinspiration Cheminformatics, Slovensky Grob, Slovakia). GADAC was found to have the highest cLog *P* and lowest TPSA values.

Microscopy

Biological Reagents, Supplies, Instrumentation, and Software. Penicillin-streptomycin solution containing 10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin (Catalog Number: 15140122) was from Thermo Fisher Scientific (Waltham, MA). DMEM, powder, high glucose (Catalog Number: 12100046) for M21 cells was from Thermo Fisher Scientific. Fetal Bovine Serum (FBS), Premium, US Sourced (Catalog Number: 45001-108) was from Corning (Corning, NY). Trypsin-EDTA (0.05%) with phenol red was from Thermo Fisher Scientific. Brightfield and fluorescent live cells images were acquired using an epifluorescent EVOS M7000 Imaging System (Catalog Number: AMF7000) from Thermo Fisher Scientific. µ-Slide 18-well chambered coverslips (Catalog Number: 81816, ibiTreat: #1.5 polymer coverslip, tissue culture treated, sterilized) or µ-Slide 8-well chambered coverslips (Catalog Number: 80826) from Ibidi (Fitchburg, WI) were used for live cell imaging. DPBS with calcium and magnesium (Catalog Number: 14040141) was from Gibco (Waltham, MA). FluoroBrite DMEM (Catalog Number: A1896701) was from Thermo Fisher Scientific. ER-ID Red assay kit (GFP-CERTIFIED) (Catalog Number: ENZ-51026-K500) and LYSO-ID Red detection kit (GFP-CERTIFIED) (Catalog Number: ENZ-51005-0100) were from Enzo Life Sciences (Farmingdale, NY). NucSpot Live 488 (Catalog Number: 40081-T) was from Biotium (Fremont, CA). MitoTracker Green FM, special packaging (Catalog Number: M7514) was from Thermo Fisher Scientific. Epifluorescent images were analyzed using the software Fiji and Pearson's correlation coefficients were calculated using the JACoP plugin.

Cell Line and Cell Culture Conditions. The human melanoma M21 cell line¹²⁻¹³ was a kind gift from Dr. Oscar Ortiz (Merck KGaA, Darmstadt, Germany). The cell line tested negative for mycoplasma using the Lonza MycoAlert Plus kit. The M21 cell line was further authenticated by short tandem repeat profiling (STR) to validate the identity of the cell line and rule out intraspecies contamination. M21 cells were grown in sterile culture flasks in a cell culture incubator at 37 °C under CO₂(g) (5% v/v). Cells were counted to determine seeding density using a Countess II FL Automated Cell Counter from Thermo Fisher Scientific. To minimize genetic drift, thawed vials were used for fewer than twenty passages. M21 cells were grown in high-glucose DMEM medium (Catalog Number: 12100046) from Thermo Fisher Scientific supplemented with 1.5 g/L sodium bicarbonate, 5–10% v/v FBS, penicillin (100 U/mL), and streptomycin (100 μ g/mL). The cells were passaged upon reaching 80% confluency with trypsin–EDTA (0.05%).

GADAC Stock Solutions. Stock solutions of GADAC (137 μ M for dose response experiments, 320 μ M for co-staining and 4 °C experiments) were prepared by dissolving 0.137 μ mol or 0.32 μ mol of GADAC, respectively, in 1 mL of water immediately before use.

Fluorescence Dose Response of GADAC (0–14 μ M) in Live M21 Cells. M21 cells in DMEM supplemented with 5% v/v FBS (100 μ L per well) were seeded at a density of 15,000 cells/well in a sterile 18-well plate. After 19 h, the cells were washed with DPBS (×2) and incubated with either DMEM supplemented with 5% v/v FBS (100 μ L per well; negative control) or GADAC (100 μ L per well; solutions of 1 μ M, 3 μ M, 8 μ M, or 14 μ M) in DMEM supplemented with 5% v/v FBS ($\leq 10\%$ v/v water) for 1 h at 37 °C. After the indicated time, the treatment medium

was removed from cells and the cells were rinsed with DPBS (\times 3) and FluoroBrite DMEM without FBS (\times 3). The cells were then placed in FluoroBrite DMEM without FBS (100 µL per well) and examined with an epifluorescent EVOS M7000 microscope (Figure S16).



Figure S16. Images of the uptake of GADAC into live M21 cells over 1 h at 37 °C in the presence of serum. DAPI channel (left) with 357/44 nm excitation and 447/60 nm emission; DIC channel (right). The DAPI channel epifluorescent images are normalized (*i.e.*, acquired at the same LED intensity, exposure, and gain parameters). Scale bars, 50 µm.

GADAC Co-Staining with an Endoplasmic Reticulum (ER)-Selective Dye. M21 cells in DMEM supplemented with 10% v/v FBS (300 µL per well) were seeded at a density of 44,000 cells/well in a sterile 8-well plate. After 24 h, the medium was removed, and the cells were incubated with either DMEM supplemented with 10% v/v FBS (200 µL per well; negative control) or GADAC (200 µL per well; solutions of 16 µM) in DMEM supplemented with 10% v/v FBS for 3 h at 37 °C. After the indicated time, the ER-ID Red assay kit was used to selectively stain the ER of the cells with a dye (that emits in the Texas Red region of the spectrum) using a modified version of the manufacturer's protocol. Briefly, the 10× assay buffer from the kit was diluted to $1 \times$ with water. To prepare a stock solution of the ER dye, 1 μ L of the ER-ID Red detection reagent was diluted with 1000 µL of the 1× assay buffer. Medium with GADAC was removed from cells, and 200 µL of the stock solution of the ER dye was added to each well. The cells were incubated with the dye for 15 min at 37 °C and subsequently rinsed with 1× assay buffer (×1), DPBS (×4), and FluoroBrite DMEM without FBS (×4). The cells were then placed in FluoroBrite DMEM without FBS (200 µL per well) and examined with an epifluorescent EVOS M7000 microscope. Co-localization analysis was performed in Fiji using the JACoP plugin. The calculated Pearson's coefficient of r = 0.7 suggests that there is a high positive correlation between the localization of GADAC and ER (Figure S17).



Figure S17. Co-localization of GADAC and an ER-selective dye in live M21 cells after a 3-h incubation at 37 °C in the presence of serum. DAPI channel: 357/44 nm excitation and 447/60 nm emission; Texas Red channel: 585/29 nm excitation and 628/32 nm emission; "Merged" refers to overlaid DAPI and Texas Red channels. The DAPI channel epifluorescent images shown in the figure are normalized (*i.e.*, acquired at the same LED intensity, exposure, and gain parameters). Scale bars, 50 µm.

GADAC Co-Staining with a Dye Selective for Mitochondria. M21 cells in DMEM supplemented with 10% v/v FBS (300 μ L per well) were seeded at a density of 44,000 cells/well in a sterile 8-well plate. After 24 h, the medium was removed, and the cells were incubated with

either DMEM supplemented with 10% v/v FBS (200 µL per well; negative control) or GADAC (200 µL per well; solutions of 16 µM) in DMEM supplemented with 10% v/v FBS (\leq 5% v/v water) for 3 h at 37 °C. After the indicated time, the MitoTracker Green FM dye was used to selectively stain mitochondria of the cells using a modified version of the manufacturer's protocol. Briefly, 50 µg of the dye was dissolved in 74 µL of DMSO to prepare a 1 mM solution. This solution was further diluted to 100 nM with FluoroBrite DMEM without FBS. FBS was not used in this co-staining experiment because the reduced form of the MitoTracker Green FM dye is susceptible to oxidases in serum. Medium with GADAC was removed from cells, and 200 µL of the diluted solution of the MitoTracker dye was added to each well. The cells were incubated with the dye for 15 min at 37 °C and rinsed with DPBS (×4) and FluoroBrite DMEM without FBS (×4). The cells were then placed in FluoroBrite DMEM without FBS (200 µL per well) and examined with an epifluorescent EVOS M7000 microscope. Co-localization analysis was performed in Fiji using the JACOP plugin. The calculated Pearson's coefficient of r = 0.68 suggests that there is a moderate positive correlation between the localization of GADAC and mitochondria (Figure S18).



Figure S18. Co-localization of GADAC and a dye selective for mitochondria in live M21 cells after a 3-h incubation at 37 °C in the presence of serum. DAPI channel: 357/44 nm excitation and 447/60 nm emission; GFP channel: 482/25 nm excitation and 524/24 nm emission; "Merged" refers to overlaid DAPI and GFP channels. The DAPI channel epifluorescent images shown in the figure are normalized (*i.e.*, acquired at the same LED intensity, exposure, and gain parameters). Scale bars, 50 µm.

GADAC Co-Staining with a Dye Selective for Nuclei. M21 cells in DMEM supplemented with 10% v/v FBS (300 µL per well) were seeded at a density of 44,000 cells/well in a sterile 8-well plate. After 24 h, the medium was removed, and the cells were incubated with either DMEM supplemented with 10% v/v FBS (200 µL per well; negative control) or GADAC (200 µL per well; solutions of 16 µM) in DMEM supplemented with 10% v/v FBS ($\leq 5\%$ v/v water) for 3 h at 37 °C. After the indicated time, NucSpot Live 488 was used to selectively stain nuclei of the cells using

a modified version of the manufacturer's protocol. Briefly, 1 μ L of 1000× NucSpot Live 488 was added to 1 mL of DMEM supplemented with 10% v/v FBS to prepare a 1× stock solution. A 10 mM solution of verapamil (dye efflux pump inhibitor) was prepared by adding 2 μ L of a 100 mM solution of verapamil in DMSO to 18 μ L of DMEM supplemented with 10% v/v FBS. Note that verapamil was added to the media during staining, washing, and imaging to improve dye retention by live cells. The 1× stock of the NucSpot dye was spiked with 1 μ L of the 10 mM verapamil solution. Medium with GADAC was removed, and the cells were rinsed with DPBS (×4) and FluoroBrite DMEM without FBS (×4). Then, each well was incubated with 200 μ L of the spiked NucSpot dye solution for 15 min at 37 °C. The staining medium was removed, and the cells were placed into FluoroBrite DMEM without FBS (200 μ L per well) containing 10 μ M verapamil. Imaging was performed using an epifluorescent EVOS M7000 microscope. Colocalization analysis was performed in Fiji using the JACoP plugin. The calculated Pearson's coefficient of r = 0.69 suggests that there is a moderate positive correlation between the localization of GADAC and nuclei (Figure S19).



Figure S19. Co-localization of GADAC and a dye selective for the nucleus in live M21 cells after a 3-h incubation at 37 °C in the presence of serum. DAPI channel: 357/44 nm excitation and 447/60 nm emission; GFP channel: 482/25 nm excitation and 524/24 nm emission; "Merged" refers to overlaid DAPI and GFP channels. The DAPI channel epifluorescent images shown in the figure are normalized (*i.e.*, acquired at the same LED intensity, exposure, and gain parameters). Scale bars, 50 µm.

GADAC Co-Staining with a Dye Selective for Acidic Organelles. M21 cells in DMEM supplemented with 10% v/v FBS (300 μ L per well) were seeded at a density of 44,000 cells/well in a sterile 8-well plate. After 24 h, the medium was removed, and the cells were incubated with either DMEM supplemented with 10% v/v FBS (200 μ L per well; negative control) or GADAC (200 μ L per well; solutions of 16 μ M) in DMEM supplemented with 10% v/v FBS for 3 h at 37 °C. After the indicated time, the LYSO-ID Red detection kit was used to selectively stain acidic

organelles of the cells (*e.g.*, lysosomes) with a dye (that emits in the Texas Red region of the spectrum) using a modified version of the manufacturer's protocol. Briefly, the 10× assay buffer from the kit was diluted to 1× with water. To prepare a stock solution of the LYSO dye, 1 μ L of the LYSO-ID Red detection reagent was diluted with 1000 μ L of the 1× assay buffer. Medium with GADAC was removed from the cells, and 200 μ L of the stock solution of the LYSO dye was added to each well. The cells were incubated with the dye for 15 min at 37 °C and subsequently rinsed with 1× assay buffer (×1), DPBS (×4), and FluoroBrite DMEM without FBS (×4). The cells were then placed in FluoroBrite DMEM without FBS (200 μ L per well) and examined with an epifluorescent EVOS M7000 microscope. Co-localization analysis was performed in Fiji using the JACoP plugin. The calculated Pearson's coefficient of *r* = 0.49 suggests that there is a low positive correlation between the localization of GADAC and acidic organelles (Figure S20).



Figure S20. Co-localization of GADAC and a dye selective for acidic organelles in live M21 cells after a 3-h incubation at 37 °C in the presence of serum. DAPI channel: 357/44 nm excitation and 447/60 nm emission; Texas Red channel: 585/29 nm excitation and 628/32 nm emission; "Merged" refers to overlaid DAPI and Texas Red channels. The DAPI channel epifluorescent images shown in the figure are normalized (*i.e.*, acquired at the same LED intensity, exposure, and gain parameters). Scale bars, $50 \,\mu\text{m}$.

GADAC Uptake Studies at 37 °C and 4 °C. M21 cells in DMEM supplemented with 10% v/v FBS (300 µL per well) were seeded at a density of 44,000 cells/well in two sterile 8-well plates (**a** and **b**). After 24 h, one of the plates (**a**) was placed on ice, along with all the solutions (*e.g.*, DMEM supplemented with 10% v/v FBS, GADAC stock, DPBS, FluoroBrite DMEM without FBS) that would come in contact with the cells. After 10 min of pre-cooling to 0 °C, the medium was removed, and the cells were incubated with either DMEM supplemented with 10% v/v FBS (200 µL per well; negative control) or GADAC (200 µL per well; solutions of 16 µM) in DMEM supplemented with 10% v/v FBS (\leq 5% v/v water). The 8-well plate (**a**) was then placed into a cold room at 4 °C for 3 h. The second 8-well plate (**b**) was treated in the same way, except dosing was

at room temperature and the incubation was done at 37 °C. After the indicated time, the chilled plate (**a**) was placed on ice again (0 °C) and rinsed with pre-cooled DPBS (×4) and FluoroBrite DMEM without FBS (200 μ L per well) (still on ice) and examined with an epifluorescent EVOS M7000 microscope. Note that the cells were held on the microscope stage and imaged for a maximum of 10 s before being returned on ice and pre-cooled before the next round of imaging. The imaging was performed rapidly to avoid the cells warming up beyond 4 °C while on the microscope stage. The second 8-well plate (**b**) was washed and imaged the same way as the first plate (**a**), except these procedures were done at room temperature. The goal of comparing GADAC images at 37 °C and 4 °C was to elucidate whether the uptake mechanism involves an energy-dependent process such as endocytosis and/or an energy-independent process such as passive diffusion or direct transduction. It has been reported that endocytosis is negligible at temperatures below 10 °C.¹⁴ As seen in Figure 5, GADAC enters cells at 4 °C approximately as efficiently as at 37 °C. These results suggest that GADAC's uptake is largely mediated by direct transduction, although a smaller contribution from endocytosis cannot be fully ruled out.

NMR Spectra

1*H*-pyrrolo[2,3-*c*]pyridin-7-amine (1)

¹H NMR, 400 MHz, DMSO-*d*₆



1-(4-Methoxybenzyl)-3-(1*H*-pyrrolo[2,3-c]pyridin-7-yl)thiourea (2)

¹H NMR, 400 MHz, chloroform-*d*



1-(4-Methoxybenzyl)-3-(1*H*-pyrrolo[2,3-c]pyridin-7-yl)guanidine (3)

¹H NMR, 500 MHz, DMSO-*d*₆



90 80 f1 (ppm)

1-(1*H*-Pyrrolo[2,3-*c*]pyridin-7-yl)guanidine hydrochloride (GAI)



¹H NMR, 500 MHz, DMSO-*d*₆

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9*H*-Pyrido[3,4-*b*]indol-1-amine (4)

¹H NMR, 400 MHz, DMSO-*d*₆



1-(4-Methoxybenzyl)-3-(9H-pyrido[3,4-b]indol-1-yl)thiourea (5)

¹H NMR, 400 MHz, DMSO-*d*₆



1-(9H-Pyrido[3,4-b]indol-1-yl)guanidine (GAC)

¹H NMR, 500 MHz, DMSO-*d*₆



¹³C{¹H} NMR, 151 MHz, DMSO-*d*₆, zoom (**GAC**)



HSQC, 600 MHz, DMSO- d_6 , zoom (**GAC**)



HMBC, 600 MHz, DMSO-*d*₆, zoom (**GAC**)



8.35 8.30 8.25 8.20 8.15 8.10 8.05 8.00 7.95 7.90 7.85 7.80 7.75 7.70 7.65 7.60 7.55 7.50 7.45 7.40 7.35 7.30 7.25 f2 (ppm)

tert-Butyl (3'-chloro-[4,4'-bipyridin]-3-yl)carbamate (7)

¹H NMR, 400 MHz, chloroform-*d*



9H-Pyrrolo[2,3-c:5,4-c']dipyridine (8)

¹H NMR, 500 MHz, DMSO-*d*₆



9H-Pyrrolo[2,3-c:5,4-c']dipyridine-1,8-diamine (9)





1-(8-Amino-9*H*-pyrrolo[2,3-*c*:5,4-*c*']dipyridin-1-yl)guanidine (GADAC)

¹H NMR, 600 MHz, D₂O





FT-IR, isopropanol (blank subtracted)

Methyl 2-oxo-2-(1*H*-pyrrolo[2,3-*c*]pyridin-3-yl)acetate (S2)

¹H NMR, 500 MHz, DMSO-*d*₆



2-Oxo-2-(1*H*-pyrrolo[2,3-*c*]pyridin-3-yl)acetamide (S3)

¹H NMR, 500 MHz, DMSO-*d*₆



2-(1*H*-Pyrrolo[2,3-*c*]pyridin-3-yl)ethan-1-amine (S4)

¹H NMR, 400 MHz, DMSO-*d*₆



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