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Supporting Information

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Supporting Information

for

Selective Antifolates for Chemically Labeling Proteins in Mammalian Cells

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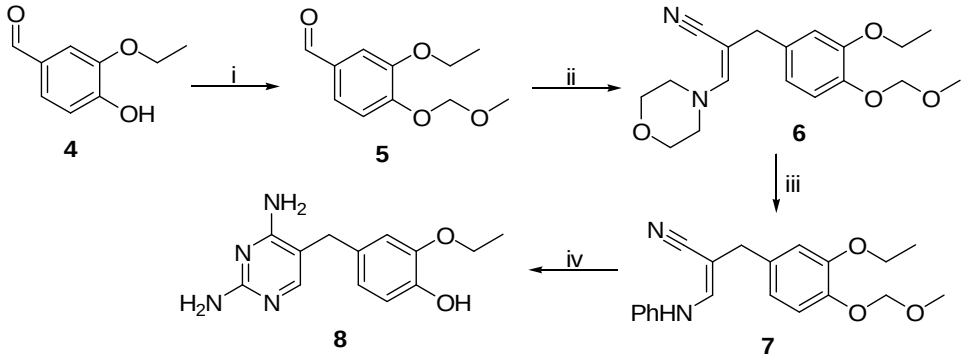
Materials. QuickChange Multi Site- Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Dulbeccos modified eagle medium (DMEM), Dulbecco's phosphate buffered saline (PBS), fetal bovine serum, and Lipofectamine™ 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA). Plamid vectors containing DNA for *P. falciparum* DHFR soluble domain and *P. carinii* DHFR were were kind gifts from Prof. Yongyuth Yuthavong and Prof. Victoria Cody, respectively. Cloning services were provided by Genscript, Inc. (Piscataway, NJ) NIH 3T3 cells were obtained as a gift from Prof. Wonhwa Cho. All chemicals were obtained from Sigma Aldrich, Inc. (Milwaukee, WI).

Plasmid vector construction. Site directed mutagenesis (QuickChange™ Multi-site-directed mutagenesis kit, manufacturers instructions) was used to introduce mutations into plasmid pfDHFR(K27E)-GFP (C172T, T319A, C320A, G321T) to yield DNA encoding pfDHFR (K27E, C59R, S108N)-GFP. The genes for pfDHFR (K27E C59R S108N, 693bp) and pcDHFR (615bp) were inserted between the AgeI and XbaI restriction sites of the vector pLL1-NLS (Active Motif, Inc., Carlsbad, CA), yielding expression vectors that constitutively express pfDHFR (K27E, C59R, S108N) and pcDHFR as C-terminal fusions to three copies of the simian virus 40 large T-antigen nuclear localization sequence (DPKKKRKV).

Syntheses of compounds 2a, 2b, 3a, 3b. The general scheme for preparation of the antifolate anlogs in this study first entailed aldol condensation of an appropriately substituted benzaldehyde with 3-morpholinopropanenitrile, followed by replacement

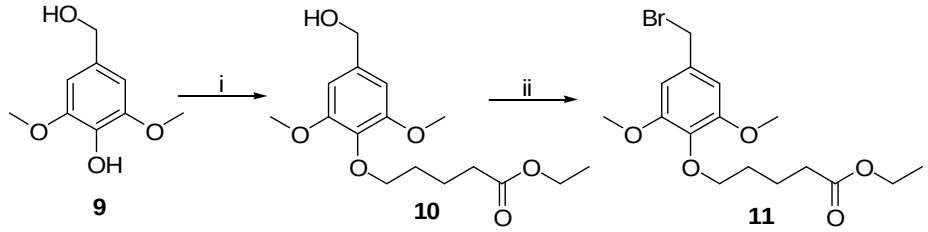
of the morpholine leaving group with aniline and subsequent cyclization with guanidine to yield 5-benzyl pyrimidine scaffolds. (**Schemes 1, 4**). Further substitutions yielded analogs **2a, 3a**, which could be conjugated to commercially available 5(6)-carboxyfluorescein diacetate to yield **2b, 3b** (**Schemes 3, 5**).

Scheme 1.



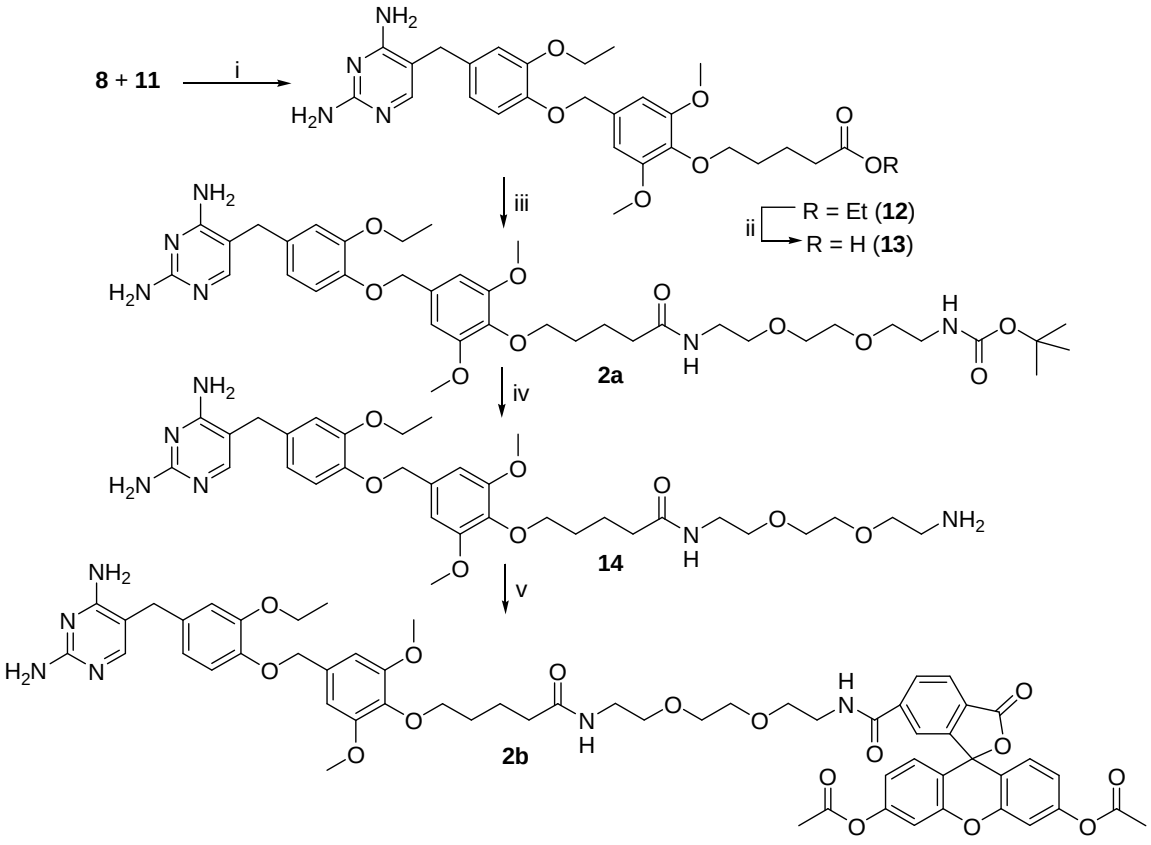
Reagents: i) MOM-Cl, DIPEA, DMAP, DCM; ii) 3-morpholinopropanenitrile, NaOMe, DMSO; iii) aniline hydrochloride, EtOH; iv) guanidine hydrochloride, NaOEt, EtOH.

Scheme 2.



Reagents: i) ethyl-5-bromovalerate, DBU, DMSO; ii) PBr_3, DCM.

Scheme 3.



Reagents: i) DBU, DMSO; ii) 2N NaOH, EtOH; iii) *tert*-butyl-2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate, EDCI, HOBt, DMF; iv) TFA, DCM; v) NEt_3, DMF, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester.

Synthesis of 2a, 2b: 3-Ethoxy-4-(methoxymethoxy)benzaldehyde (**5**): A magnetically stirred mixture of 3-ethoxy-4-hydroxybenzaldehyde (**4**, 4 gm, 24.1 mmol), DMAP (500 mg, 4.1 mmol), and DIPEA (3.73 gm, 28.91 mmol) in DCM (60 mL) maintained at 0 ^{o}C (ice-water bath) under an atmosphere of nitrogen was treated, dropwise, with MOM-Cl (2.31 gm, 28.9 mmol). The ensuing reaction mixture was allowed to warm to 18 ^{o}C, stirred at this temperature for 18 h and then poured into cold HCl (140 mL of a 0.1N aqueous solution). The separated aqueous layer was extracted with DCM (3x50 mL) and combined organic extracts washed with water (60 mL) and brine (60 mL) and then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting solid was subjected to flash chromatography (3:7 ethyl acetate/hexane elution) to afford, after concentration of appropriate fractions 3-ethoxy-4-(methoxymethoxy)benzaldehyde (4.02 gm, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.46-1.60 (m, 3H), 3.52 (s, 3H), 4.15-4.18 (m, 2H), 5.31 (s, 2H), 7.25-7.27 (m, 1H), 7.39-7.42 (m, 2H), 9.85 (s, 1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 14.6, 56.4, 64.4, 95, 110.8, 115.3, 126.1, 131.1, 149, 152.5; ESMS^{+} (*m/z*) 211 $[M+H]^{+}$.

*4-((2,4-Diaminopyrimidin-5-yl)methyl)-2-ethoxyphenol (**8**)*: *Step 1*. A solution of NaOMe was prepared by dissolving clean metallic Na (120 mg, 5.22 mmol) in anhydrous MeOH (10 mL). The solvent was evaporated under reduced pressure, and the solid was taken up in DMSO (15 mL), and to the solution was added 3-morpholinopropanenitrile (**24**) (2.20 gm, 15.71 mmol) at 65 ^{o}C. The mixture was heated to 80 ^{o}C for 45 min, followed by the addition of 3-ethoxy-4-(methoxymethoxy)benzaldehyde (**5**), 3 gm, 14.28 mmol) in 15 mL of DMSO over a 45 min period. After heating for 2.5 h, the reaction mixture was cooled and partitioned between EtOAc and H_2O that had been slightly acidified with dilute aqueous citric acid to prevent the formation of an emulsion and extracted with EtOAc. The combined organic extracts washed with brine and then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (1:1 EtOAc/hexane elution) to afford the pure 2-(3-ethoxy-4-(methoxymethoxy)benzyl)-3-morpholinoacrylonitrile (**6**) (1.2 gm, 25%) as a yellow gum. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.41-1.45 (t, 3H, *J* = 4 Hz), 3.30 (s, 2H), 3.40-3.47 (m, 4H), 3.51 (s, 3H), 3.60-3.72 (m, 4H), 4.06-4.13 (q, 2H, *J* = 8, 16 Hz), 5.18 (s, 2H), 6.21 (s, 1H), 6.65-7.07 (m, 3H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.8, 38.9, 49.4, 56.1, 64.4, 66.2, 75.4, 95.8, 113.7, 117.6, 120.4, 121.7, 134, 145.4, 148.8, 149.4.

Step 2. A solution of 2-(3-ethoxy-4-(methoxymethoxy)benzyl)-3-morpholinoacrylonitrile (**6**, 1.2 gm, 3.61 mmol) and aniline hydrochloride (698 mg, 5.41 mmol) in anhydrous EtOH (15 mL) was refluxed for 1 h. In a separate flask, guanidine hydrochloride (1.71 gm, 18 mmol) was added to a solution of NaOEt prepared by dissolving clean metallic Na (415 mg, 18.04 mmol) in anhydrous EtOH (20 mL), and the flask was swirled manually for 10 min. The entire contents of the second flask (includeing the NaCl) were added to the first, and the combined mixture was refluxed for 20 h and then filtered while hot. Flash chromatography with 9:1 EtOAc/MeOH as the eluent afforded the pure product (500 mg, 53%). ^{1}H NMR (500 MHz, CD_3OD) δ 1.36-1.40 (t, 3H, *J* = 5.0 Hz), 3.59 (s, 2H), 3.90-4.10 (m, 2H), 6.60-6.65 (m, 1H), 6.70-6.80 (m,2H), 7.26 (s, 1H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 15.24, 32.64, 64.30, 106.86, 114.77, 115.83, 121.13, 131.10, 145.43, 146.88, 155.46, 162.39, 162.69; $ESMS^{+}$ (*m/z*) 261 $[M+H]^{+}$.

*Ethyl 5-(4-(hydroxymethyl)-2,6-dimethoxyphenoxy)pentanoate (**10**)*: To a well stirred solution of 4-(hydroxymethyl)-2,6-dimethoxyphenol (**9**), 500 mg, 2.72 mmol) in DMSO (4 mL), was added DBU (496 mg, 3.26 mmol) dropwise at room temperature. After 30 min, ethyl-5-bromovalerate (681 mg, 3.26 mmol) was added slowly to the reaction mixture and stirred for 12 h. Water (~50 ml) was added and the product was extracted with EtOAc (3 x 50 mL). The combined EtOAc solution was washed with ~100 mL water, dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The crude product was subjected to flash chromatography (3:7 EtOAc/ hexane) to afford the pure product (704 mg, 83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.22-1.26 (m, 3H), 1.79-1.83 (m, 5H), 2.36-2.37 (m, 2H), 3.83 (s, 6H), 3.93-3.96 (m, 2H), 4.10-4.20 (m, 2H), 4.61-4.62 (m, 2H), 6.57 (s, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 14.2, 21.4, 29.4, 33.9, 56, 60.1, 65.5, 72.6, 103.8, 136.4, 153.5, 174.

*Ethyl-5-(4-(bromomethyl)-2,6-dimethoxyphenoxy)pentanoate (**11**)*: Phosphorous tribromide (835 mg, 3.09 mmol) was added to a solution of ethyl-5-(4-(hydroxymethyl)-2,6-dimethoxyphenoxy)pentanoate (**10**) (2.68 gm, 8.59 mmol) in dry DCM (60 mL) at 0 ^{o}C. The mixture was stirred at room temperature for 1 h before it was treated with cold water (30 mL). The layers were separated and the water phase extracted with DCM (3x50 mL). The combined organic layers were washed with water (50 mL), a saturated $NaHCO_3$ solution (50 mL), a saturated NaCl solution (50 mL), dried over $MgSO_4$, filtered, concentrated in vacuo and purified by column chromatography (2:8 EtOAc/hexane) to afford the pure bromide (**11**) (1.84 gm, 57%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.23-1.26 (m, 3H), 1.78-1.90 (m, 5H), 2.35-2.42 (m, 2H), 3.84 (s, 6H), 3.92-3.98 (m, 2H), 4.10-4.20 (m, 2H), 4.45 (m, 2H), 6.60 (s, 2H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 14.2, 21.4, 29.4, 33.9, 34.3, 56.1, 60.1, 72.7, 106.2, 132.9, 137.4, 153.4, 173.6; ESMS$^+$ (*m/z*) 397 [*M*+Na]$^+$.

*Ethyl-5-(4-((4-((2,4-diaminopyrimidin-5-yl)methyl)-2-ethoxyphenoxy)methyl)-2,6-dimethoxyphenoxy)pentanoate (**12**)*: To a well stirred solution of 4-((2,4-diaminopyrimidin-5-yl)methyl)-2-ethoxyphenol (**8**) (460 mg, 1.77 mmol) in DMSO (5 mL), was added DBU (323 mg, 2.13 mmol) dropwise at room temperature. After 30 min, ethyl 5-(4-(bromomethyl)-2,6-dimethoxyphenoxy)pentanoate (**11**) (665 mg, 1.77 mmol) was added slowly to the reaction mixture and stirred for 12 h. Water (~50 mL) was added and the product was extracted with EtOAc (4 x 50 mL). The combined EtOAc solution was washed with ~100 mL water, dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The crude product was subjected to flash chro-

matography (9:1 EtOAc/MeOH) to afford the pure product (390 mg, 40%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.22-1.26 (m, 3H), 1.36-1.40 (m, 3H), 1.68-1.90 (m, 4H), 2.30-2.2.45 (m, 2H), 3.95-4.20 (m, 14H), 5.01 (s, 2H), 6.45-7.05 (m, 5H), 6.60 (s, 2H); 7.40 (s, 1H); $ESMS^+$ (*m/z*) 555 $[M+H]^+$.

*5-(4-((4-((2,4-Diaminopyrimidin-5-yl)methyl)-2-ethoxyphenoxy)methyl)-2,6-dimethoxyphenoxy)pentanoic acid (**13**)*: To a stirred solution of ethyl-5-(4-((4-((2,4-diaminopyrimidin-5-yl)methyl)-2-ethoxyphenoxy)methyl)-2,6-dimethoxyphenoxy)pentanoate (**12**) (390 mg, 0.70 mmol) in EtOH (10 mL) 2 N NaOH aqueous solution (1.5 mL) was added dropwise and the reaction mixture was stirred at room temperature for 10 h. Then the EtOH was removed under reduced pressure and the crude reaction mixture was adjusted to pH 4 with 1N HCl and extracted with EtOAc (6x50 mL). The combined organic extracts washed with brine and then dried over $MgSO_4$, filtered, and concentrated under reduced pressure (100 mg, 27%). The acid was used directly for the next step.

*Synthesis of compound **2a***: To a well stirred solution of 5-(4-((4-((2,4-diaminopyrimidin-5-yl)methyl)-2-ethoxyphenoxy)methyl)-2,6-dimethoxyphenoxy)pentanoic acid (**13**) (100 mg, 0.19 mmol) in DMF (3 mL) were added EDCI (43.55 mg, 0.23 mmol), HOBt (30.78 mg, 0.23 mmol) and tert-Butyl-2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (**25**) (56.58 mg, 0.23 mmol) and the mixture was stirred for 24 h at room temperature. The DMF was evaporated in vacuum, the residue was dissolved in ethyl acetate and washed with saturated sodium bicarbonate solution and then with brine solution. The combined organic phases were dried over $MgSO_4$; the solvent was removed under reduced pressure and purified by column chromatography (9:1 EtOAc/MeOH) to afford the pure compound (50 mg, 35%). ^{1}H NMR (400 MHz, CD_3OD) δ 1.30-1.41 (m, 12H), 1.65-1.80 (m, 4H), 2.20-2.35 (m, 2H), 3.18-3.22 (m, 2H), 3.25-3.65 (m, 8H), 3.75 (s, 6H), 3.85 (s, 2H), 3.90-4.08 (m, 4H), 5.02 (s, 2H), 6.55-6.95 (m, 5H), 7.38 (1H); ^{13}C NMR (125.7 MHz, CD_3OD) δ 13.9, 22.2, 27.3, 29, 32.2, 35.2, 38.8, 39.8, 55.2, 64.4, 69.2, 69.6, 69.8, 71.1, 72.5, 104.5, 107.6, 114.3, 115.3, 120.6, 131.7, 133.3, 147.1, 149.3, 150.2, 153.3, 157.5, 160, 163.4, 174.8; $ESMS^+$ (*m/z*) 757 $[M+H]^+$.

*Synthesis of compound **14***: Trifluoroacetic acid (0.1 mL) was added to a solution of compound **2a** (15 mg, 0.02 mmol) in dichloromethane (4 mL) at 0 ^{o}C. The reaction mixture was stirred for 12 h at room temperature. After the reaction was complete,

the solvent was removed under reduced pressure at room temperature and used directly in the next step.

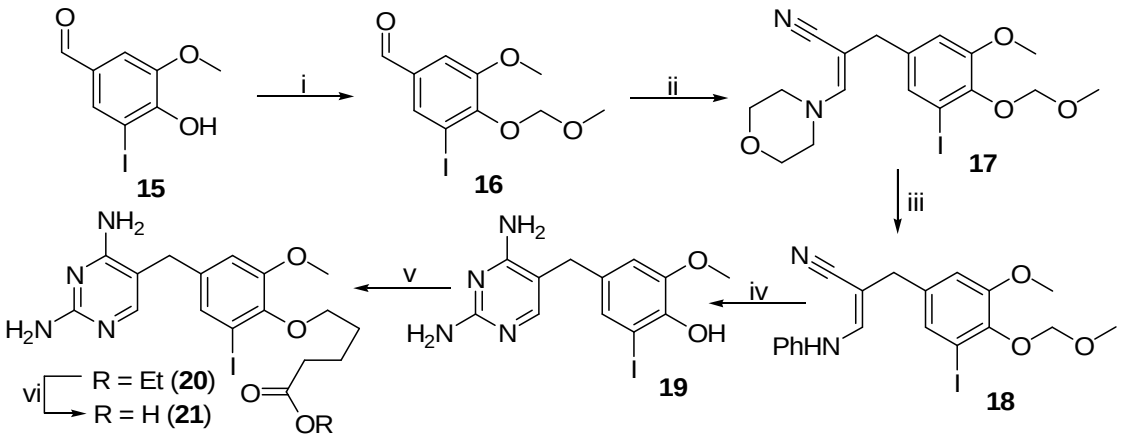
*Synthesis of compound **2b***: To a well stirred suspension of amine **14** (12 mg, 0.02 mmol) in DMF (2 mL), two drops of triethylamine was added. After 30 min, 5-carboxy-fluorescein diacetate N-succinimidyl ester (12 mg, 0.02 mmol) was added and stirred the reaction mixture for 3 h at room temperature. The solvent was removed under reduced pressure and extracted with ethyl acetate (4x30 mL). The combined organic phases were dried over $MgSO_4$, filtered; the solvent was removed under reduced pressure and purified by column chromatography (9:1 EtOAc/MeOH) to afford the pure compound (8 mg, 40%). $ESMS^+$ (*m/z*) 1117 $[M+H_2O+H]^+$.

*3-Morpholinopropanenitrile (**24**)*: A mixture of morpholine (1 gm, 11.49 mmol) and acrylonitrile (609 mg, 11.49 mmol) was stirred at room temperature as a neat mixture without any solvent and catalyst for 3 h. The reaction mixture was then straightway subjected to short column chromatography over silica gel (3:7 EtOAc/hexane) to provide the pure 3-morpholinopropanenitrile (1.2 gm, 75%). ^1H NMR (400 MHz, $CDCl_3$) δ 2.47-2.52 (m, 6H), 2.64-2.68 (m, 2H), 3.69-3.71 (m, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 15.7, 53, 53.6, 66.7, 118.6.

tert-*Butyl-2-(2-(2-aminoethoxy)ethoxy)ethylcarbamate (**25**)*: 2-(2-(2-Aminoethoxy) ethoxy) ethanamine (6 gm, 40.54 mmol) was dissolved in a solution of triethyl amine methanol (10% TEA in MeOH, 130 mL). A solution of di-tert-butyl dicarbonate (2.95 gm, 13.53 mmol) in methanol (10 mL) was added to this mixture with vigorous stirring. The mixture was refluxed for 2 h and left to stir at room temperature overnight. The excess methanol and TEA were removed in vacuo to yield an oily residue that was dissolved in dichloromethane and washed with 10% aqueous sodium carbonate. The organic layer was separated, dried over $MgSO_4$ and filtered, and the solvent was removed in vacuo. The oily residue was filtered through a bed of silica and used directly in the next step.

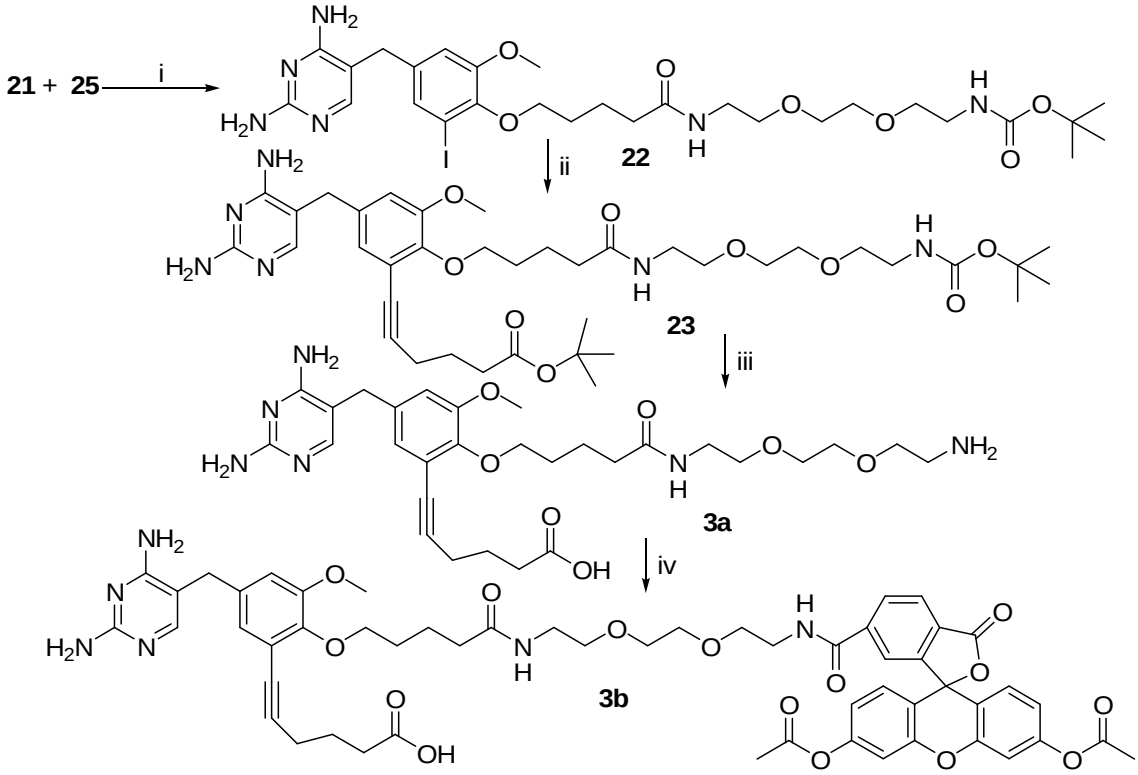
Synthesis of 3a, 3b.

Scheme 4.



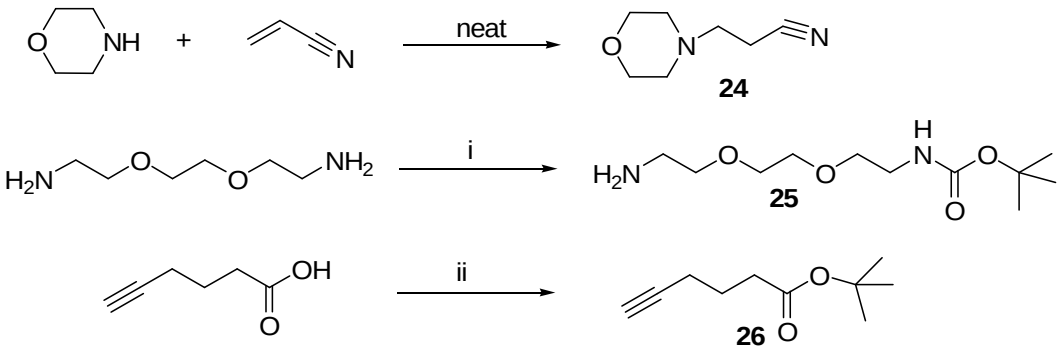
Reagents: i) MOM-Cl, DIPEA, DMAP, DCM; ii) **24**, NaOMe, DMSO; iii) aniline hydrochloride, EtOH; iv) guanidine hydrochloride, NaOEt, EtOH; v) ethyl-5-bromovalerate, DBU, DMSO; vi) 2N NaOH, EtOH.

Scheme 5.



Reagents: i) EDCI, HOBt, DMF; ii) **26**, $(Ph_3P)_2PdCl_2$, $(Ph_3P)CuBr$, NEt_3, DMF; iii) TFA, DCM; iv) NEt_3, DMF, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester.

Scheme 6.



Reagents: i) di-*tert*-butyl dicarbonate, 10% TEA/MeOH; ii) trifluoroacetic anhydride, THF, *t*-butanol.

*3-Iodo-5-methoxy-4-(methoxymethoxy)benzaldehyde (**16**)*: A magnetically stirred mixture of 5-iodovanillin(**15**) (3 gm, 10.79 mmol), DMAP (658 mg, 5.39 mmol), and DIPEA (1.67 gm, 12.95 mmol) in DCM (60 mL) maintained at 0 ^{o}C (ice-water bath) under an atmosphere of nitrogen was treated, dropwise, with MOM-Cl (1.04 gm, 13 mmol). The ensuing reaction mixture was allowed to warm to 18 ^{o}C, stirred at this temperature for 18 h and then poured into cold HCl (120 mL of a 0.1 N aqueous solution). The separated aqueous layer was extracted with DCM (3 x 50 ML) and combined organic extracts washed with water (60 mL) and brine (60 mL) and then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The resulting solid was subjected to flash chromatography (3:7 ethyl acetate/hexane elution) to afford, after concentration of appropriate fractions 3-iodo-5-methoxy-4-(methoxy-methoxy)benzaldehyde (2.80 gm, 81%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.66 (s, 3H), 3.97 (s, 3H), 6.68 (S, 2H), 7.37 (s, 1H), 7.81 (s, 1H), 9.77 (s, 1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 56.5, 80.4, 98, 108.6, 131, 136, 146.4, 151.4, 189.4.

*4-((2,4-Diaminopyrimidin-5-yl)methyl)-2-iodo-6-methoxyphenol (**19**)*: Step 1. A solution of NaOMe was prepared by dissolving clean metallic Na (120 mg, 5.22 mmol) in anhydrous MeOH (10 mL). The solvent was evaporated under reduced pressure, and the solid was taken up in DMSO (15 mL), and to the solution was added 3-morpholinopropanenitrile (**24**) (1.43 gm, 10.21 mmol) at 65 ^{o}C. The mixture was heated to 80 ^{o}C for 45 min, followed by the addition of 3-iodo-5-methoxy-4-(methoxymethoxy)-benzaldehyde (**16**) (3 gm, 9.32 mmol) in 15 mL of DMSO over a 45 min period. After heating for 2.5 h, the reaction mixture was cooled and partitioned between EtOAc and H_2O that had been slightly acidified with dilute aqueous citric acid to prevent the

formation of an emulsion and extracted with EtOAc. The combined organic extracts washed with brine and then dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (1:1 EtOAc/hexane elution) to afford the pure 2-(3-iodo-5-methoxy-4-(methoxymethoxy)-benzyl)-3-morpholinoacrylonitrile (**17**) (900 mg, 22%) as a yellow gum. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.24 (s, 2H), 3.46-3.48 (m, 4H), 3.65 (s, 3H), 3.68-3.71 (m, 4H), 3.82 (s, 3H), 5.12 (s, 2H), 6.23 (s, 1H), 6.76 (s, 1H), 7.18 (s, 1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 38.5, 49.4, 56, 58.3, 66.2, 74.2, 92.6, 98.6, 113.1, 121.4, 130.2, 137.6, 145, 149, 152.1; ESMS$^+$ (*m/z*) 445 $[M+H]^+$, 462 $[M+NH_3+H]^+$, 467 $[M+Na]^+$.

Step 2. A solution of 2-(3-iodo-5-methoxy-4-(methoxymethoxy)benzyl)-3-morpholinoacrylonitrile (**17**) (900 mg, 2.03 mmol) and aniline hydrochloride (393 mg, 3.05 mmol) in anhydrous EtOH (15 mL) was refluxed for 1 h. In a separate flask, guanidine hydrochloride (964 mg, 10.15 mmol) was added to a solution of NaOEt prepared by dissolving clean metallic Na (233 mg, 10.13 mmol) in anhydrous EtOH (15 mL), and the flask was swirled manually for 10 min. The entire contents of the second flask (including the NaCl) were added to the first, and the combined mixture was refluxed for 20 h and then filtered while hot. Flash chromatography with 9:1 EtOAc/MeOH as the eluent afforded the pure product (400 mg, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.58 (s, 2H), 3.82 (s, 3H), 6.80 (s, 1H), 7.10 (s, 1H), 7.28 (s,1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 32, 56.4, 84.8, 106.2, 113, 129.6, 133.6, 144.7, 147.3, 156.1, 162.5, 162.6; ESMS$^+$ (*m/z*) 373 $[M+H]^+$.

*Ethyl-5-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2-iodo-6-methoxyphenoxy)pentanoate (**20**)*: To a well stirred solution of 4-((2,4-diaminopyrimidin-5-yl)methyl)-2-iodo-6-methoxyphenol (**19**) in DMSO (5 mL), was added DBU (196 mg, 1.29 mmol) dropwise at room temperature. After 30 min, ethyl-5-bromovalerate (269 mg, 1.29 mmol) was added slowly to the reaction mixture and stirred for 12 h. Water (~50 ml) was added and the product was extracted with EtOAc (4 x 50 mL). The combined EtOAc solution was washed with ~100 mL water, dried over $MgSO_4$, filtered and the solvent was evaporated under reduced pressure. The crude product was subjected to flash chromatography (9:1 EtOAc/MeOH) to afford the pure product (450 mg, 83%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.23-1.26 (t, *J* = 5 Hz, 3H), 1.80-1.87 (m, 4H), 2.40-2.43 (t, *J* = 10 Hz, 4H), 3.61 (s, 2H), 3.79 (s, 3H), 3.91-3.93 (t, *J* = 5 Hz, 2H), 4.09-4.13 (q, *J* = 5, 10 Hz, 2H), 6.86 (s, 1H), 7.16 (s, 1H), 7.50 (s, 1H); ^{13}C NMR (125.7 MHz, $CDCl_3$) δ

13.1, 21.4, 29.1, 31.8, 33.5, 54.9, 59.9, 71.9, 91.7, 106.1, 113, 129.7, 137.1, 146.5, 152.5, 154.3, 161.7, 163, 174; $ESMS^+$ (*m/z*) 501 $[M+H]^+$.

*5-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2-iodo-6-methoxyphenoxy)pentanoic acid (**21**)*: To a stirred solution of ethyl-5-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2-iodo-6-methoxyphenoxy)pentanoate (**20**) (500 mg, 1 mmol) in EtOH (10 mL) 2 N NaOH aqueous solution (2 mL) was added dropwise and the reaction mixture was stirred at room temperature for 10 h. Then the EtOH was removed under reduced pressure and the crude reaction mixture was adjusted to pH 4 with 1 N HCl and extracted with EtOAc (6x50 mL). The combined organic extracts washed with brine and then dried over $MgSO_4$, filtered, and concentrated under reduced pressure (300 mg, 64%). The acid was used directly for the next step.

*Synthesis of compound **22***: To a well-stirred solution of 5-(4-((2,4-diaminopyrimidin-5-yl)methyl)-2-iodo-6-methoxyphenoxy)pentanoic acid (**21**) (360 mg, 0.76 mmol) in DMF (6 mL) were added EDCI (174 mg, 0.91 mmol), HOBt (123 mg, 0.91 mmol) and compound **25** (189 mg, 0.76 mmol) and the mixture was stirred for 24 h at room temperature. The DMF was evaporated in vacuum, the residue was dissolved in ethyl acetate and washed with saturated sodium bicarbonate solution and then with brine solution. The combined organic phases were dried over $MgSO_4$, the solvent was removed under reduced pressure and purified by column chromatography (9:1 EtOAc/MeOH) to afford the pure compound (300 mg, 56%). ^{13}C NMR (125.7 MHz, $CDCl_3$) δ 22.4, 27.4, 29.3, 31.6, 35.3, 38.9, 39.8, 55.1, 69.2, 69.6, 69.8, 72.2, 78.7, 92, 107.2, 113.2, 129.8, 136.1, 146.8, 148.2, 152.6, 163.6, 174.7; $ESMS^+$ (*m/z*) 703 $[M+H]^+$, $ESMS^+$ (*m/z*) 725 $[M+Na]^+$.

*Synthesis of compound **23***: A stirred mixture of **26** (14.31 mg, 0.08 mmol), iodide (**22**) (50 mg, 0.07 mmol), $(Ph_3P)_2PdCl_2$ (10 mg), $(Ph_3P)_3CuBr$ (1 mg), and NEt_3 (1 mL) in dry DMF (4 mL) was heated at 60 ^oC for 72 h. The solvent was removed in vacuum and extracted with ethyl acetate (4x50 mL). The combined organic phases were dried over $MgSO_4$, the solvent was removed under reduced pressure and purified by column chromatography (1:9 EtOAc/MeOH) to afford the pure compound (20 mg, 38%). $ESMS^+$ (*m/z*) 743 $[M+H]^+$, $ESMS^+$ (*m/z*) 765 $[M+Na]^+$.

*Synthesis of compound **3a***: Trifluoroacetic acid (4 mL) was added to a solution of the *tert*-butyl ester **23** (20 mg, 0.03 mmol) in dichloromethane (1 mL) at 0 ℃. The reaction mixture was stirred for 12 h at room temperature. After the reaction was com-

plete, the solvent was removed under reduced pressure at room temperature and used directly in the next step. $ESMS^+$ (*m/z*) 587 $[M+H]^+$, $ESMS^+$ (*m/z*) 609 $[M+Na]^+$.

*Synthesis of compound **3b***: To a well stirred suspension of **3a** (20 mg, 0.03 mmol) in DMF (2 mL), three drops of triethylamine was added. After 30 min, 5-carboxy-fluorescein diacetate *N*-succinimidyl ester (20.05 mg, 0.04 mmol) was added and stirred the reaction mixture for 8 h at room temperature. The solvent was removed under reduced pressure and filtered, washed with diethyl ether, dichloromethane and cold ethyl acetate. The solid was collected and dried in vacuum, and purified by preparative TLC (1:9 MeOH/EtOAc). $ESMS^+$ (*m/z*) 1029 $[M+H]^+$, $ESMS^+$ (*m/z*) 1047 $[M+H_3O]^+$.

*Hex-5-ynoic acid tert-butyl ester (**26**)*: A dry flask was charged with hexynoic acid (1 gm, 8.93 mmol) and purged with nitrogen. THF (40 mL) was added, and the solution was cooled to 0 ℃. Trifluoroacetic anhydride (2.72 mL, 19.60 mmol) was added drop wise. The reaction was stirred at 0 ℃ for 2.5 h, then *t*-butanol (3 mL) was added slowly. After 1 h, the reaction was warmed to room temperature. The reaction was stirred for an additional 17 h, quenched with water (50 mL) and extracted with ether (4 x 50 mL). The combined organic layers were dried with $MgSO_4$, filtered and concentrated. The resulting oil was purified by flash chromatography (3% EtOAc in hexane) yielding 14 (1.2 gm, 80 %) as a clear oil. ^1H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 9H), 1.80 (pentet, *J* = 7.2 Hz, 2H), 1.96 (t, *J* = 2.7 Hz, 1H), 2.24 (dt, *J* = 2.7, 7.0 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 2H); ^{13}C NMR (125.6 MHz, $CDCl_3$) δ 18.0, 24.0, 28.3, 34.4, 69.1, 80.5, 83.7, 172.7.

Fluorescent labeling of pfDHFR fusion constructs: NIH3T3 fibroblast cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (FBS; 10%), L-glutamine (2 mM), penicillin (100 IU μL^{-1}), streptomycin (100 mg mL^{-1}), HEPES (15 mM), and incubated in a humidified atmosphere at 37 °C and 5% CO_2. Cells (ca. 80,000) were seeded into 6-well plates, and transient transfection was performed by using Lipofectamine2000™ reagent according to the manufacturer's protocol (2 µg DNA 6 µL Lipofectamine2000™). After ca. 6 h, the transfected cells were trypsinized and aliquoted (ca. 14,000 cells/well) into 8-well chambered coverslips (Nunc, Lab-Tek) and allowed to incubate another 12-18 h. For imaging, fluorescein conjugates (**2b**, or **3b**) were diluted (500 nM) in culture medium

and incubated with the cells for ca. 15 min. at 37 °C. The cells were then washed 2X with PBS, and indicator-free DMEM without small molecule was added to the cells.

Microscopy. Epi-fluorescent microscopy of adherent live cells was performed using a Zeiss Axiovert 200 equipped with a 63X EC Plan Neofluar oil immersion objective (NA = 1.25). Excitation illumination was provided by a 100W Hg lamp. Excitation and emission light were selected by appropriate band-pass filters (Chroma Technologies, Inc. HQ480/40 (ex.), HQ535/50 (em.)). Images were detected using a Zeiss Axiocam MRM CCD camera, and captured with Zeiss Axiovision 4.6 software. Images were adjusted for brightness and contrast using NIH Image J and prepared for publication using Adobe Photoshop 5.5.

Enzyme inhibition assay. Compounds **2a** and **3a** were screened for their activity against a panel of purified DHFRs using an absorption-based inhbition assays.[1] The assay was based on measurement of the change in absorbance at 340 nm when di-hydrofolate is reduced to tetrahydrofolate in the presence of NADPH.

***E. coli* growth inihibition assay.** *E. coli* (strain DH5α) was streaked onto Luria broth/agar plates containing varying concentrations of compounds **1** (TMP) **2a** or **3a**, and the plates were incubated at 37 °C for 24 hrs. The minimal inhibitory concentration (MIC) was reported as the lowest concentration at which no colonies formed.

References:

[1] A. Rosowsky, V. Cody, N. Galitsky, H. Fu, A. T. Papoulis, S. F. Queener, *J. Med. Chem*. **1999**, *42*, 4853-4860.