Supporting Information

for

Detection of DHFR Conformational Change by FRET Using Two Fluorescent Amino Acids

Shengxi Chen,[†] Nour Eddine Fahmi,[†] Lin Wang,[‡] Chandrabali Bhattacharya,[†] Stephen J. Benkovic*[,‡] and Sidney M. Hecht*[,†]

Table S1. Relative activities of DHFRs containing **1** and **2**

DHFR	relative activity (%)
Wild type	100
1 at position 17	76 ± 5
2 at position 115	23 ± 3
1 at position 17; **2** at position 115 (DHFR I)	20 ± 3
2 at position 17	105 ± 5
1 at position 115	108 ± 5
2 at position 17; **1** at position 115 (DHFR II)	50 ± 3

Table S2. Emission anisotropy of free **1**, **2** and DHFRs containing **1** and **2**

DHFR or free dyes	emission anisotropy
free **1**	0.053 ± 0.002 (λ_{ex} 280nm, λ_{em} 350nm)
1 at position 17	0.190 ± 0.004
1 at position 115	0.206 ± 0.007
free **2**	0.005 ± 0.001 (λ_{ex} 350nm, λ_{em} 450nm)
2 at position 17	0.167 ± 0.004
2 at position 115	0.164 ± 0.006

Table S3. MALDI-MS analysis of tryptic digests of wild-type and modified DHFR analogues. Analogue 1 contains biphenylphenylalanine **1** in position 17; analogue 2 contains coumarin **2** in position 17. Position 17 is denoted **in red.**

position	peptide sequence	MALDI-MS analysis, molecular mass, D					
		wild-type		analogue 1		analogue 2	
		Calc	MS	Calc	MS	Calc	MS
13- 32	VIGMENAMPWNLPADLAWFK	2304	2304.1	**2474**	**2473.9**	**2420**	**2420.3**
34-44	NTLNKPVIMGR	1242	1242.8	1242	1242.6	1242	1242.9
45-57	HTWESIGRPLPGR	1506	1505.8	1506	1505.8	1506	1506.1
59-71	NIILSSQPGTDDR	1415	1415.8	1415	1415.6	1415	1415.9
72-76	VTWVK	632	632.5	632	634.3	632	634.5
77-98	SVDEAIAACGDVPEIMVIGGGR	2159	2159.9	2159	2159.8	2159	2159.5
99-106	VYEQFLPK	1023	1023.7	1023	1023.6	1023	1023.8

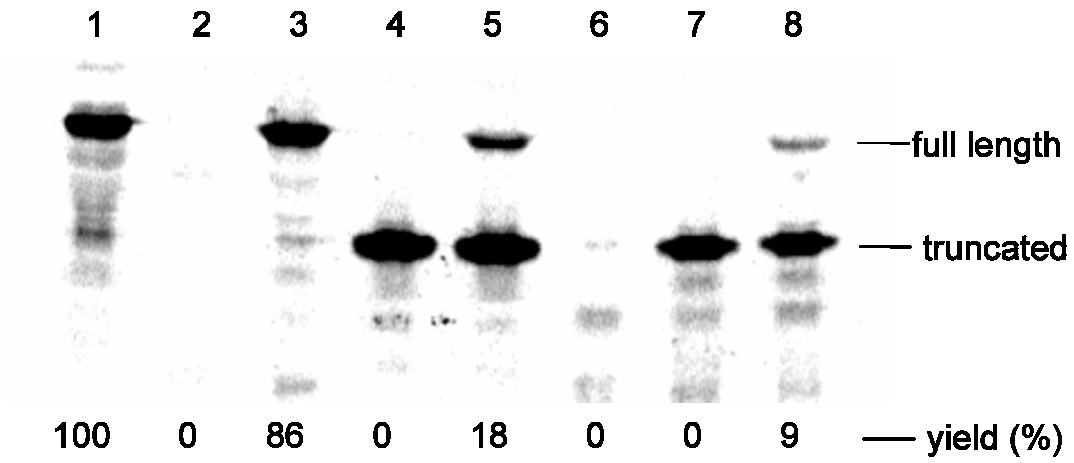


Figure S1. *In vitro* incorporation of biphenyl-phenylalanine (**1**) and L-(7-hydroxycoumarin-4-yl)ethylglycine (**2**) into DHFR I at positions 17 and 115, respectively. The protein synthesis reaction was analyzed by 15% SDS-PAGE at 100 V for 2 h. Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. Lane 1, wild-type DHFR; lane 2, suppression of an mRNA CGGG codon at DHFR position 17 with unacylated $tRNA_{CCCG}$-C_{OH}; lane 3, suppression of a mRNA CGGG codon at DHFR position 17 with biphenyl-phenylalanyl-$tRNA_{CCCG}$; lane 4, suppression of a mRNA UAG codon at DHFR position 115 with unacylated $tRNA_{CUA}$; lane 5, suppression of a mRNA UAG codon at DHFR position 115 with L-(7-hydroxycoumarin-4-yl)ethylglycyl-$tRNA_{CUA}$; lane 6, suppression of mRNA CGGG and UAG codons at DHFR positions 17 and 115 with unacylated $tRNA_{CCCG}$-C_{OH}; lane 7, suppression of mRNA CGGG and UAG codons at DHFR positions 17 and 115 with biphenyl-phenylalanyl-$tRNA_{CCCG}$; lane 8, suppression of mRNA CGGG and UAG codons at DHFR positions 17 and 115 with biphenyl-phenylalanyl-$tRNA_{CCCG}$ and L-(7-hydroxycoumarin-4-yl)-ethylglycyl-$tRNA_{CUA}$.

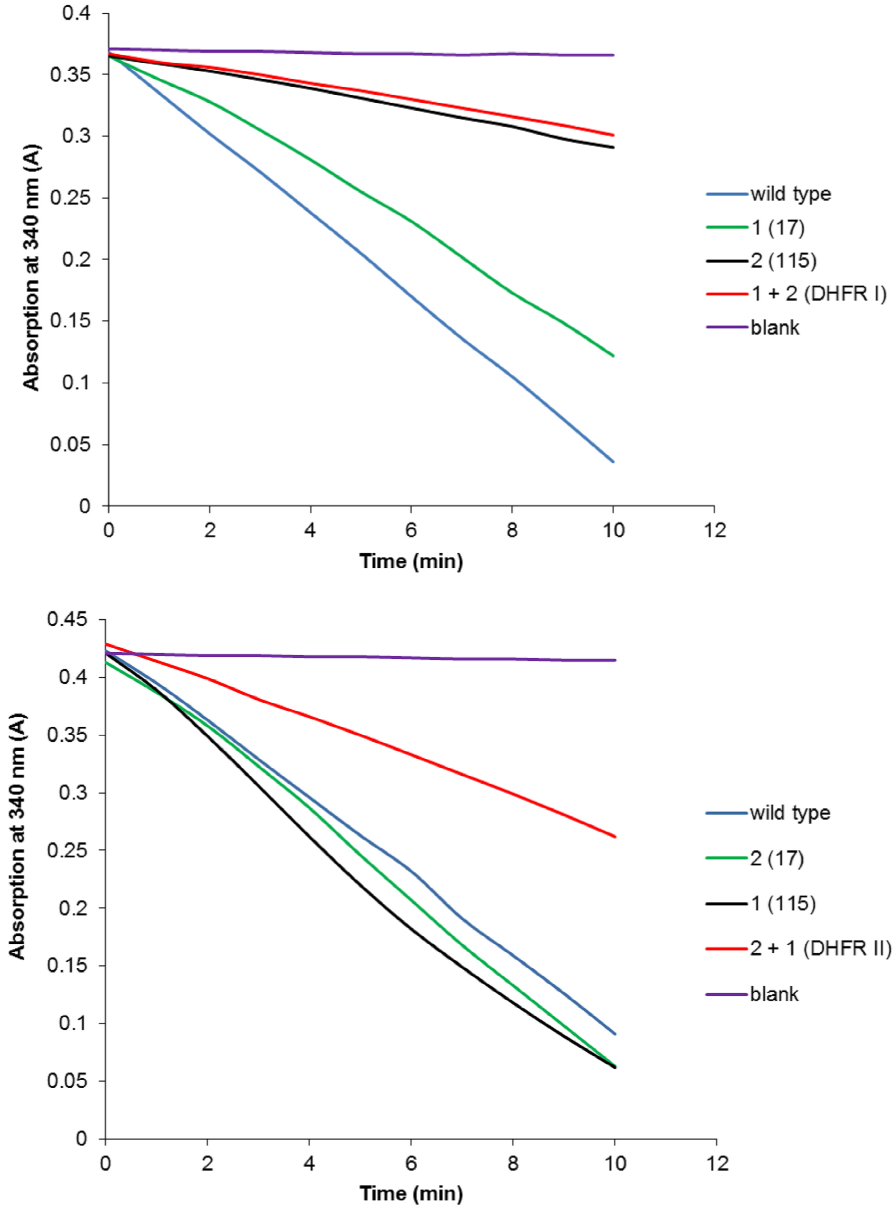


Figure S2. Enzymatic activities of wild-type and modified DHFRs (200 ng/mL reaction) in the reduction of dihydrofolate to tetrahydrofolate. Upper panel, DHFRs substituted with fluorophore **1** at position 17 and/or with fluorophore **2** at position 115. Lower panel, DHFRs substituted with fluorophore **2** at position 17 and/or fluorophore **1** at position 115.

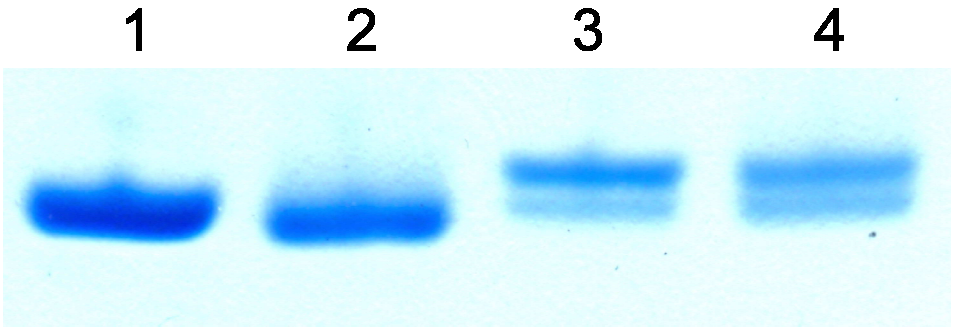


Figure S3. Ligation of suppressor tRNAs with aminoacyl-pdCpAs via the agency of T4 RNA ligase. Lane 1, abbreviated $tRNA_{CUA}$-C_{OH}; lane 2, abbreviated $tRNA_{CCCG}$-C_{OH}; lane 3, N-protected, full length biphenyl-phenylalanyl-$tRNA_{CUA}$; lane 4, N-protected, full length L-(7-hydroxycoumarin-4-yl)-ethylglycyl-$tRNA_{CCCG}$. The analysis was carried out by polyacrylamide gel electrophoresis under acidic conditions.

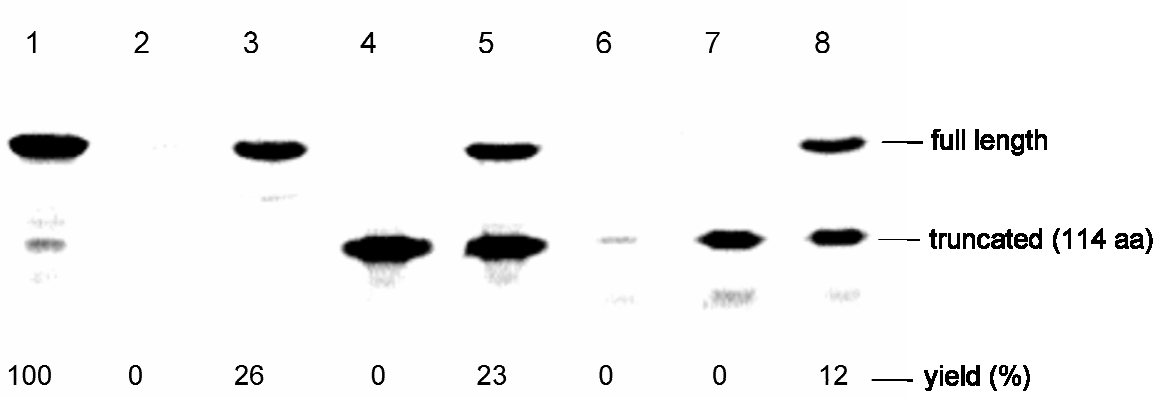


Figure S4. *In vitro* incorporation of L-(7-hydroxycoumarin-4-yl)ethylglycine (**2**) and biphenyl-phenylalanine (**1**) into DHFR II at positions 17 and 115, respectively. The protein synthesis reaction was analyzed by 15% SDS-PAGE at 100 V for 2 h. Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. Lane 1, wild-type DHFR; lane 2, suppression of an mRNA CGGG codon at DHFR position 17 with unacylated $tRNA_{CCCG}$-C_{OH}; lane 3, suppression of a mRNA CGGG codon at DHFR position 17 with L-(7-hydroxycoumarin-4-yl)ethylglycyl-$tRNA_{CCCG}$; lane 4, suppression of a mRNA UAG codon at DHFR position 115 with unacylated $tRNA_{CUA}$; lane 5, suppression of a mRNA UAG codon at DHFR position 115 with biphenyl-phenylalanyl-$tRNA_{CUA}$; lane 6, suppression of mRNA CGGG and UAG codons at DHFR positions 17 and 115 with unacylated $tRNA_{CCCG}$-C_{OH}; lane 7, suppression of mRNA CGGG and UAG codons at DHFR positions 17 and 115 with L-(7-hydroxycoumarin-4-yl)ethylglycyl-$tRNA_{CCCG}$; lane 8, suppression of mRNA CGGG and UAG codons at DHFR positions 17 and 115 with L-(7-hydroxycoumarin-4-yl)ethylglycyl-$tRNA_{CCCG}$ and biphenyl-phenylalanyl-$tRNA_{CUA}$.

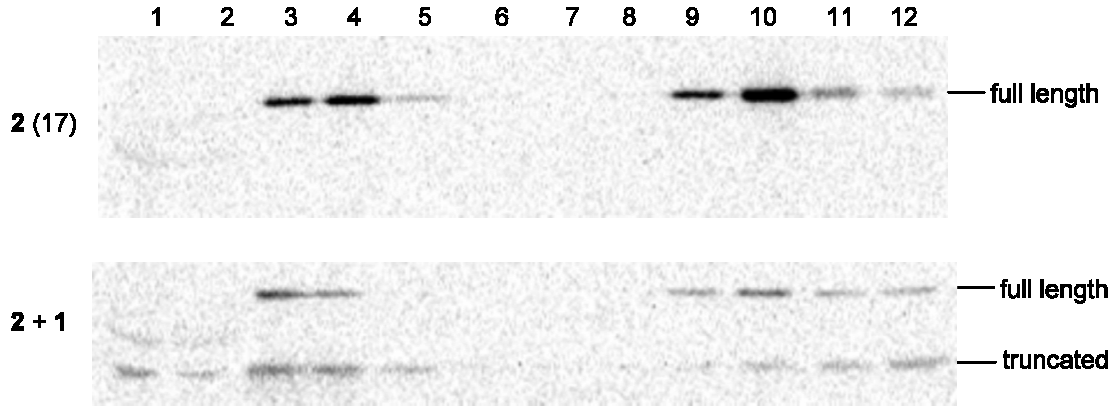


Figure S5. Autoradiogram of a 15% SDS-polyacrylamide gel illustrating the purification of singly and doubly modified DHFRs containing L-(7-hydroxycoumarin-4-yl)ethylglycine (**2**) at position 17 for the singly modified DHFR, and L-(7-hydroxycoumarin-4-yl)ethylglycine (**2**) at position 17 + biphenyl-phenylalanine (**1**) at position 115 for the doubly modified DHFR II. Purification was carried out on a Ni-NTA column, and then on a DEAE-Sepharose CL-6B column. Lane 1, flow-through from the Ni-NTA column; lane 2, 10 mM imidazole wash from the Ni-NTA column; lane 3, first elution of Ni-NTA column with 150 mM of imidazole; lane 4, second elution of Ni-NTA column with 150 mM imidazole; lane 5, third elution of Ni-NTA column with 150 mM imidazole; lane 6, flow-through from DEAE-Sepharose column; lane 7, elution of DEAE-Sepharose column with 100 mM NaCl; lane 8, elution of DEAE-Sepharose column with 200 mM NaCl; lane 9, first elution of DEAE-Sepharose column with 300 mM NaCl; lane 10, second elution of DEAE-Sepharose column with 300 mM NaCl; lane 11, third elution of DEAE-Sepharose column with 300 mM NaCl; lane 12, elution of DEAE-Sepharose column with 400 mM NaCl.

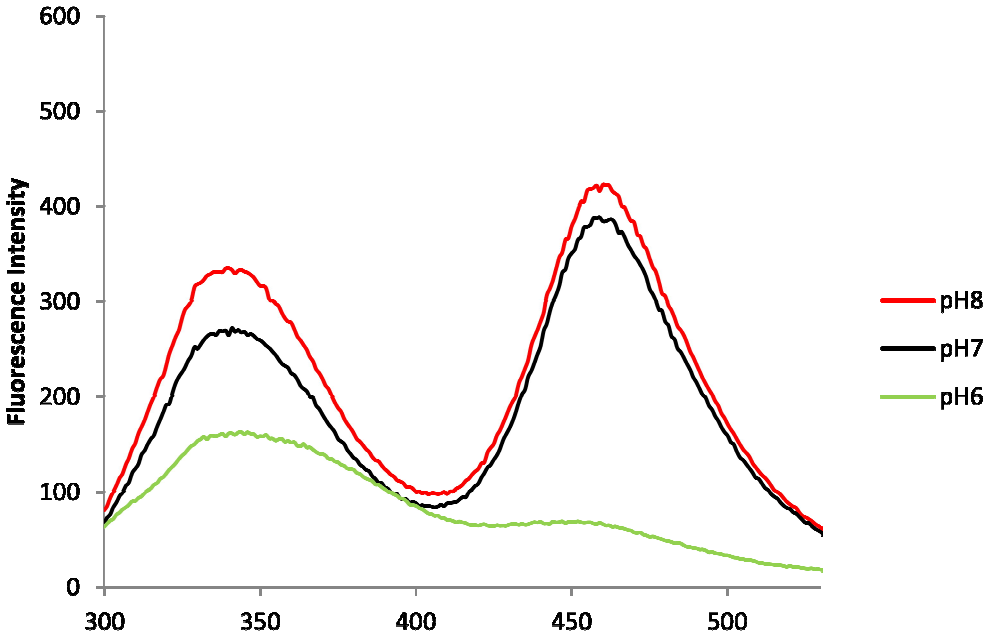


Figure S6. Fluorescence emission spectra of DHFR II measured at pH 8.0, 7.0 and 6.0, following excitation at 280 nm.

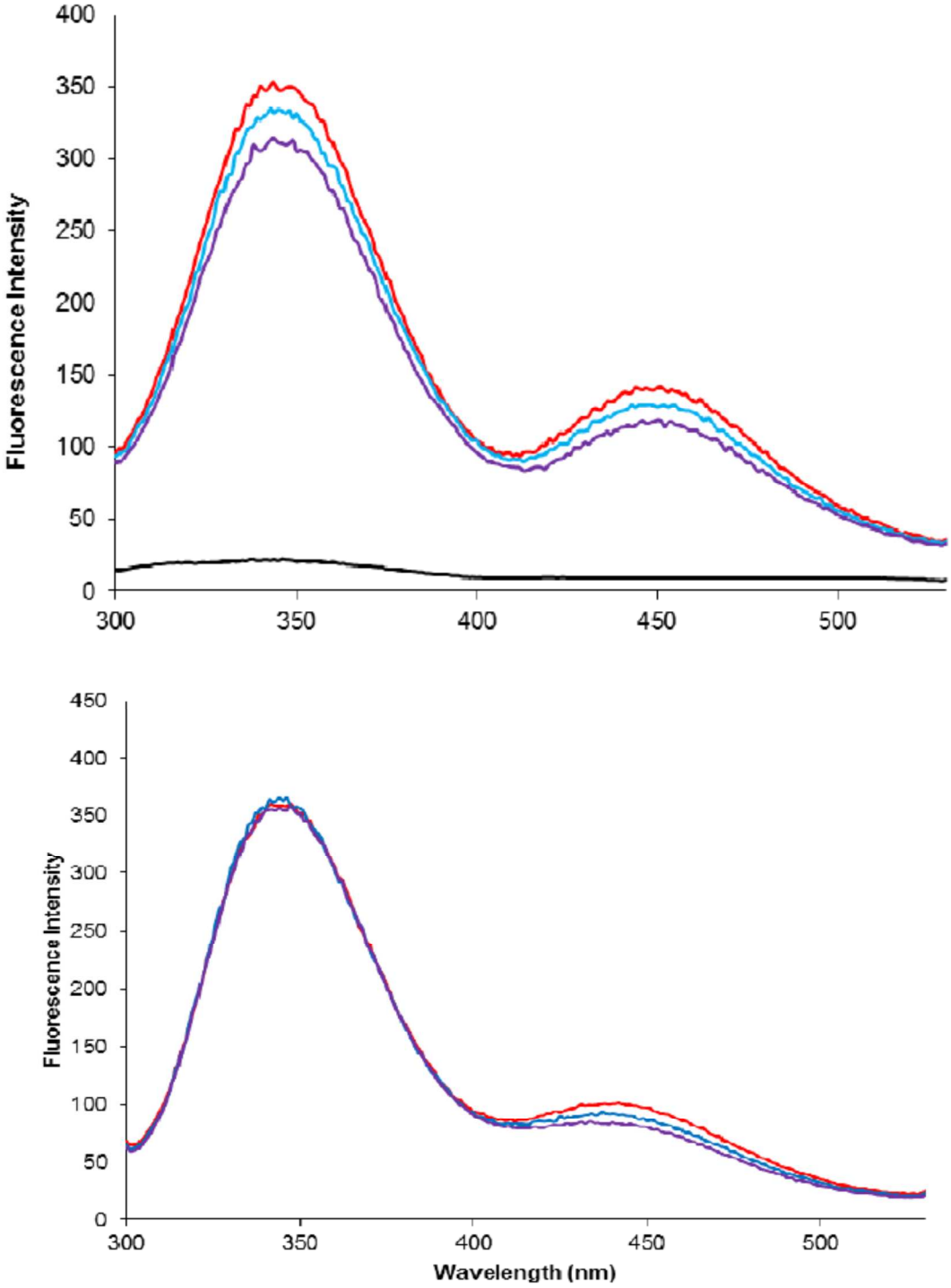


Figure S7. The upper panel shows the fluorescence emission of DHFR I in the absence of trimethoprim (red line) and in the presence of 2 μM trimethoprim (TMP) (blue line) and 4 μM TMP (purple line) following irradiation at 280 nm. The black line is 4 μM TMP alone.The lower panel shows the same measurements carried out on a modified DHFR I having phenylalanine at position 22 in place of tryptophan.

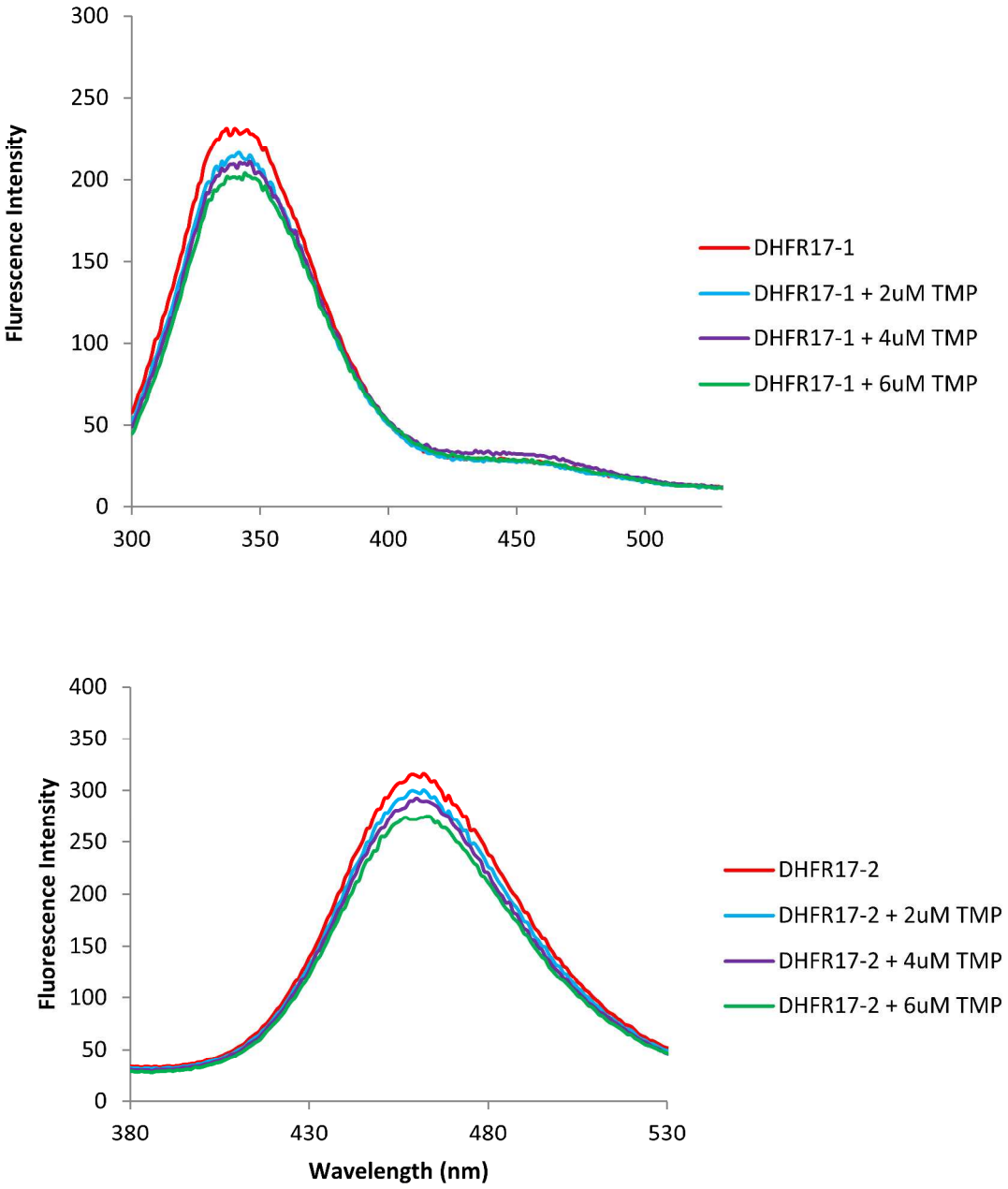


Figure S8. The upper panel shows the fluorescence emission of a DHFR containing **1** at position 17 in the absence of trimethoprim (red line) and in the presence of 2 μM trimethoprim (TMP) (blue line), 4 μM TMP (purple line) or 6 μM TMP (green line) following irradiation at 280 nm. The lower panel shows the same measurements for a DHFR containing **2** at position 17 following irradiation at 340 nm.

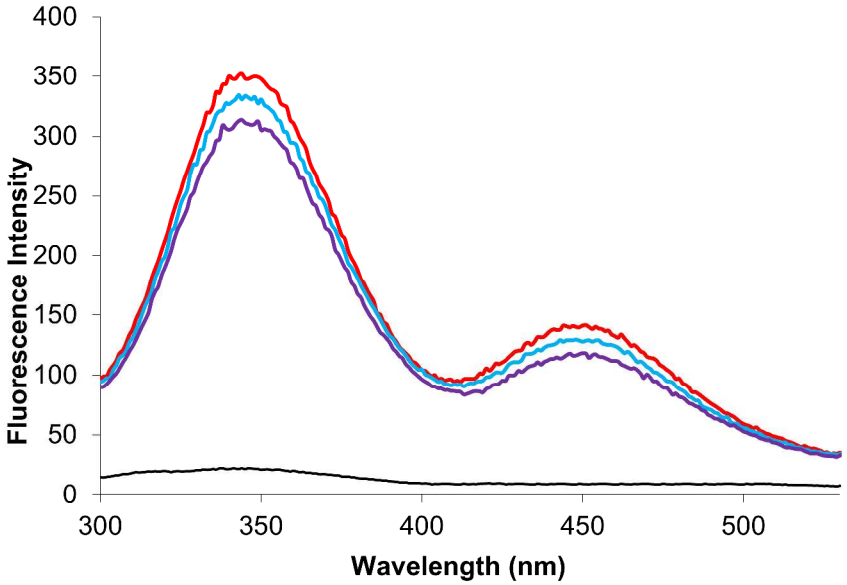


Figure S9. Effect of the DHFR inhibitor trimethoprim on the fluorescence emission spectrum of 0.5 μM modified DHFR I containing biphenyl-phenylalanine (**1**) at position 17 and L-(7-hydroxycoumarin-4-yl)ethylglycine (**2**) at position 115 following excitation at 280 nm. The red line is the emission spectrum of the modified DHFR alone; the blue and purple lines reflect the effect of 2 and 4 μM trimethoprim, respectively, on the emission spectrum of the modified DHFR. The black line is the emission spectrum of 2 μM trimethoprim alone following excitation at 280 nm.

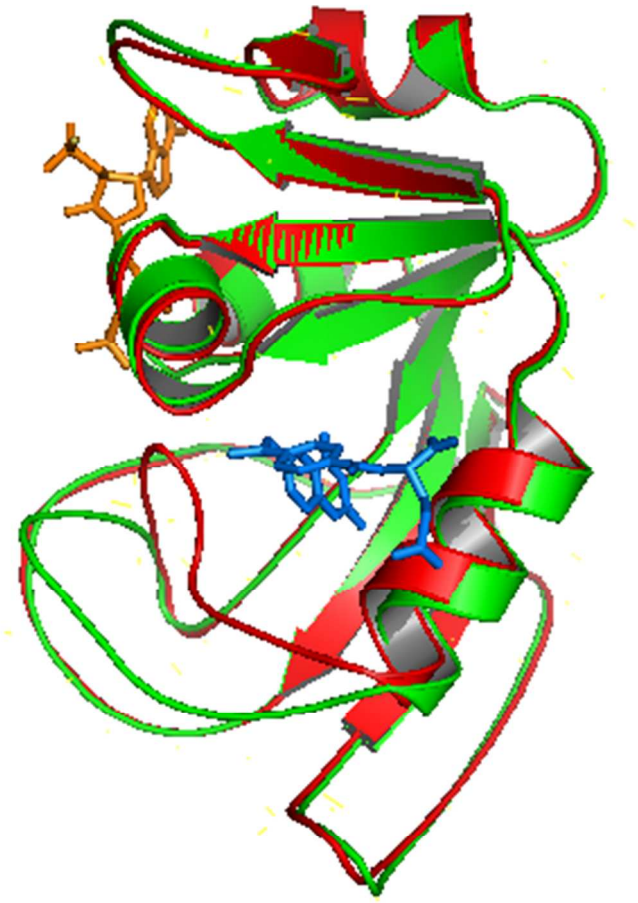
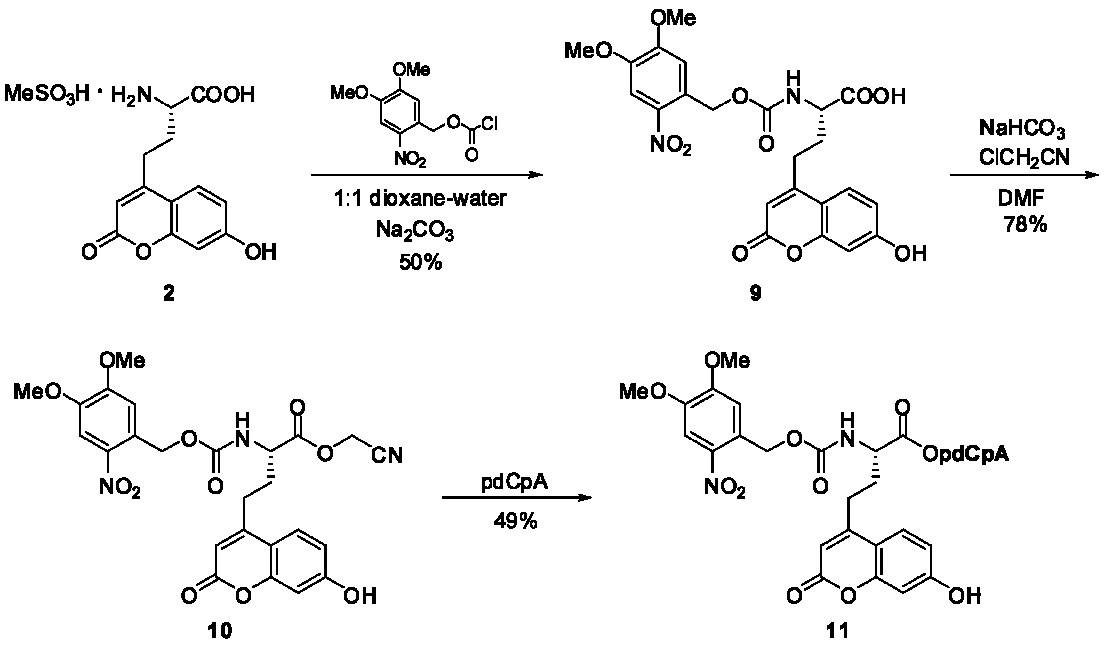


Figure S10. Comparison of the structures of DHFR-NADPH (1RA1, green, NADPH is shown in orange) and DHFR-MTX (1RG7, red, MTX is shown in blue).

Scheme S1


MeSO3H · H2N COOH
2
OMe
MeO
NO2
O
Cl
1:1 dioxane-water
Na2CO3
50%
9
NaHCO3
ClCH2CN
DMF
78%
CN
10
pdCpA
49%
OpdCpA
11
OH

Experimental Section

General Methods

Except as noted otherwise, all non-aqueous reactions were carried out in oven-dried glassware under a balloon pressure of argon or nitrogen. Reagents were commercially available and used as received; anhydrous solvents were purchased as the highest grade from Sigma-Aldrich or purified as follows: THF was distilled from Na-benzophenone, CH_2Cl_2 was distilled from CaH_2. Reactions were monitored by thin layer chromatography using 0.25 mm Silicycle or EM silica gel 60 F_{254} plates. Flash column chromatography was performed using Silicycle 40-60 mesh silica gel. Yields are reported as isolated yields of spectroscopically pure compounds. ^1H and ^{13}C NMR spectra were obtained using 400 and 500 MHz Varian spectrometers. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^1H resonance of the solvent ($CDCl_3$, 7.26 ppm; DMSO-d_6, 2.49 ppm, CD_3OD, 3.31 ppm or acetone-d_6, 2.05 ppm). ^{13}C spectra are referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77.16 ppm; DMSO-d_6, 39.52 ppm, CD_3OD, 49.00 ppm or acetone-d_6, 29.84 ppm). Splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were acquired at the Arizona State University mass spectral core facility or Michigan State University mass spectrometry facility.

Ni-NTA agarose was obtained from Qiagen Inc. DNA oligonucleotides were ordered from Integrated DNA Technologies. DEAE-Sepharose CL-6B, ammonium persulfate, acrylamide, *N*, *N´*-methylene-bis-acrylamide, acetic acid, potassium glutamate, ammonium acetate, dithiothreitol, magnesium acetate, phospho(enol)pyruvate, *Escherichia coli* tRNA, isopropyl β-D-thiogalactopyranoside (IPTG), ATP, GTP, CTP, UTP, cAMP, amino acids, rifampicin and formamide were obtained from Sigma-Aldrich. Tris and SDS were obtained from Bio-Rad Laboratories. [^{35}S]-methionine (1000 Ci/mmol, 10 μCi/μL) was purchased from PerkinElmer Inc. Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. T4 RNA ligase and T4 polynucleotide kinase were purchased from New England Biolabs Inc.

Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics. UV spectral measurements were made using a Perkin-Elmer Lamdba 20 UV/vis spectrometer. Fluorescence was monitored using a Cary Eclipse Fluorescence Spectrophotometer.

N-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine Methyl Ester (4)[1]

To a solution containing 1.00 g (2.55 mmol) of *N*-(*tert*-butoxycarbonyl)-4-iodo-L-phenylalanine (**3**) in 12 mL of dry DMF was added 282 mg (3.36 mmol) of $NaHCO_3$ followed by 0.60 mL (1.36 g, 9.60 mmol) of iodomethane. The reaction mixture was stirred at room temperature under nitrogen for 40 h. EtOAc (30 mL) was added and the organic layer washed with three 20-mL portions of water. The organic layer was dried ($MgSO_4$), filtered and concentrated under diminished pressure. The residue was purified on a silica gel column (12 x 4 cm), elution was with 1:1 hexanes–EtOAc. *N*-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester (**4**) was obtained as a colorless solid: yield 0.82 g (79%); mp 78−80 °C (lit.[1a] mp 78−80 °C and lit.[1b] 78.5−80 °C); silica gel TLC R_f 0.66 (1:1 hexanes–ethyl acetate); ^1H NMR (400 MHz, $CDCl_3$) δ 1.41 (s, 9H), 2.97 (dd, 1H, J = 13.6 and 6.0 Hz), 3.07 (dd, 1H, J = 13.8 and 5.8 Hz), 3.71 (s, 3H), 4.56 (m, 1H), 4.96 (d, 1H, J = 7.2 Hz), 6.87 (d, 2H, J = 8.0 Hz) and 7.61 (d, 2H, J = 8.4

Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.4, 38.0, 52.4, 54.3, 80.2, 92.6, 131.4, 135.9, 137.7, 155.1 and 172.2; mass spectrum (MALDI), *m/z* 427.86 $(M+Na)^+$ (theoretical 428.03).

***N*-(*tert*-Butoxycarbonyl)-4[(4';1',1")biphenyl]-L-phenylalanine Methyl Ester (5)**

To a mixture containing 200 mg (0.24 mmol) of *N*-(*tert*-butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester (**4**) and 202 mg (1.02 mmol) of 4-biphenylboronic acid in 24 mL of THF–toluene (1:1 v/v) was added a solution containing 108 mg (1.02 mmol) of Na_2CO_3 in 10 mL of water. The mixture was degassed with a stream of N_2 for 30 min. then 29.0 mg (25.0 μmol) of $Pd(PPh_3)_4$ was added. The reaction mixture was stirred vigorously at 80–85 ºC for 15 h, and the cooled reaction mixture was then diluted with 40 mL of water and extracted with three 25-mL portions of EtOAc. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified on a silica gel column (21 x 4 cm); elution was with 9:1→4:1 hexanes–EtOAc. *N*-(*tert*-Butoxycarbonyl)-4[(4';1',1")biphenyl]-L-phenylalanine methyl ester (**5**) was obtained as a colorless solid: 211 mg (99%); silica gel TLC R_f 0.22 (4:1 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 9H), 3.10 (dd, 1H, J = 13.8 and 5.8 Hz), 3.18 (dd, 1H, J = 13.6 and 5.6 Hz), 3.74 (s, 3H), 4.64 (q, 1H, J = 14.0 and 6.0 Hz), 5.02 (d, 1H, J = 8.0 Hz), 7.21 (d, 2H, J = 7.6 Hz), 7.36 (m, 1H), 7.46 (m, 2H), 7.57 (m, 2H, J = 8.0 Hz) and 7.63–7.66 (m, 6H); mass spectrum (APCI), *m/z* 432.2180 $(M+H)^+$ ($C_{27}H_{30}NO_4$ requires 432.2175).

***N*-(4-Pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine Methyl Ester (6)**

To a solution containing 75.0 mg (0.17 mmol) of *N*-(*tert*-butoxycarbonyl)-4[(4',1',1")biphenyl]-L-phenylalanine methyl ester (**5**) in 5 mL of dichloromethane was added 0.5 mL of CF_3COOH. The reaction mixture was stirred at room temperature for 5 h. An additional 0.1 mL of CF_3COOH was added and stirring was continued for another 18 h. The solvent was concentrated under diminished pressure and the residual CF_3COOH was removed by coevaporation with 3 mL of toluene. The crude product was obtained as an off-white solid and was used directly in the next step.

The crude residue was suspended in a solution containing 36.0 mg (0.34 mmol) of Na_2CO_3 in 2 mL of water. 4-Pentenoic acid succinimidyl ester (40 mg, 0.2 mmol) in 3 mL of 1,4-dioxane was added and the heterogeneous mixture was stirred at room temperature for 5 h. The mixture was diluted with 5 mL of water, acidified to pH 2-3 with 1 N $NaHSO_4$ and extracted with 10 mL of EtOAc. The organic layer was washed with 5 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column (18 x 2 cm); elution was with 4:1→7:3 hexanes–EtOAc. *N*-(4-Pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine methyl ester (**6**) was obtained as a colorless solid: yield 37 mg (51%); mp 180–182 °C; silica gel TLC R_f 0.28 (7:3 hexanes–EtOAc); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.31 (m, 2H), 2.37 (m, 2H), 3.15 (dd, 1H, J = 14.0 and 5.6 Hz), 3.21 (dd, 1H, J = 14.0 and 5.6 Hz), 3.76 (s, 3H), 4.94–5.07 (m, 3H), 5.78 (m, 1H), 5.97 (d, 1H, J = 8.0 Hz), 7.18 (d, 2H, J = 8.4 Hz), 7.36 (m, 1H), 7.46 (m, 2H), 7.57 (d, 2H, J = 8.0 Hz) and 7.63–7.69 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.5, 35.7, 37.6, 52.5, 53.1, 115.8, 127.1, 127.3, 127.4, 127.5, 127.6, 128.9, 129.9, 135.1, 136.9, 139.6, 140.3, 140.7, 172.0 and 172.2; mass spectrum (APCI), *m/z* 414.2060 $(M+H)^+$ ($C_{27}H_{28}NO_3$ requires 414.2069).

***N*-(4-Pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine Cyanomethyl Ester (7)**

To a solution containing 61.0 mg (0.14 mmol) of *N*-(4-pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine methyl ester (**6**) in 3 mL of THF was added a solution containing 18.0 mg (0.44 mmol) of $LiOH·H_2O$ in 2 mL of water. The reaction mixture was stirred at room temperature for 9 h, at which point silica gel TLC analysis, development with 1:1 hexanes–ethyl acetate, showed the reaction to be complete. The mixture was acidified to pH ~3 with 1 N $NaHSO_4$ (~1 mL) and extracted with 10 mL of EtOAc. The organic layer was washed with 5 mL of water, dried ($MgSO_4$) and concentrated under diminished pressure.

The crude acid was dissolved in 5 mL of dry acetonitrile and 97.0 μL (70.8 mg, 0.70 mmol) of triethylamine was added followed by 44.0 μL (52.8 mg, 0.70 mmol) of chloroacetonitrile. The mixture was stirred under nitrogen at room temperature for 18 h. Silica gel TLC analysis of the reaction mixture (development with 1:1 hexanes–ethyl acetate or 9:1:0.01 CH_2Cl_2–MeOH–AcOH) showed the reaction to be incomplete. An additional 44.0 μL (52.8 mg, 0.70 mmol) of chloroacetonitrile was added and stirring was continued for 4 h. More triethylamine (97.0 μL, 0.70 mmol) and chloroacetonitrile (44.0 μL, 0.70 mmol) were added and the reaction mixture was stirred for another 18 h, after which silica gel TLC analysis showed all of the acid to have been consumed. The mixture was diluted with 10 mL of dichloromethane, and washed successively with 15 mL of 1 N $NaHSO_4$ and 15 mL of water. The organic layer was dried ($MgSO_4$) and concentrated under diminished pressure. The crude residue was purified on a silica gel column (15 x 2.5 cm); elution was with 1:1 hexanes–ethyl acetate. *N*-(4-Pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine cyanomethyl ester (**7**) was obtained as a colorless solid: yield 39 mg (60%); mp 185−187 °C; silica gel TLC R_f 0.43 (1:1 hexanes–EtOAc); ^{1}H NMR (400 MHz, acetone-d_6) δ 2.27 (m, 4H), 3.11 (dd, 1H, J = 14.0 and 8.8 Hz), 3.25 (dd, 1H, J = 14.0 and 5.6 Hz), 4.82 (m, 1H), 4.94 (m, 2H), 4.99 (s, 2H), 5.77 (m, 1H), 7.38 (m, 3H), 7.48 (t, 2H, J = 7.6 Hz), 7.57 (d, 1H, J = 7.6 Hz), 7.66 (d, 2H, J = 8.0 Hz), 7.72 (m, 2H) and 7.76 (s, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.3, 29.8, 35.5, 37.4, 49.1, 52.9, 113.9, 116.1, 127.2, 127.49, 127.55, 127.6, 127.7, 129.0, 129.8, 134.2, 136.7, 139.4, 140.1, 140.4, 140.7, 170.6 and 172.3; mass spectrum (APCI), *m/z* 439.2014 $(M+H)^+$ ($C_{28}H_{27}N2O_3$ requires 439.2022).

***N*-(4-Pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine pdCpA Ester (8)**

To a conical vial containing 15.8 mg (36.0 μmol) of *N*-(4-pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine cyanomethyl ester (**7**) was added a solution of 7 mg (5.15 μmol) of the tris-(tetrabutylammonium) salt of pdCpA in 70 μL of anhydrous DMF followed by 7 μL of triethylamine. The reaction mixture was stirred at room temperature for 20 h. A 2-μL aliquot of the reaction mixture was diluted with 60 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and was analyzed by HPLC on a C_{18} reversed phase column (250 x 10 mm). The column was washed with 1 to 65% CH_3CN in 50 mM NH_4OAc at pH 4.5 over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remaining reaction mixture was diluted to a total volume of 1 mL with 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed phase column. *N*-(4-Pentenoyl)-4[(4';1',1")biphenyl]-L-phenylalanine pdCpA ester (**8**) (retention time 31.3 min) was recovered from the appropriate fractions as a colorless solid by lyophilization: yield 0.9 mg (17%); mass spectrum (ESI), *m/z* 1018.2930 $(M+H)^+$ ($C_{45}H_{50}N_9O_{15}P_2$ requires 1018.2902).

***N*-(6-Nitroveratryloxycarbonyl)-L-(7-hydroxycoumarin-4-yl)ethylglycine (9)**

To a solution of 213 mg (0.59 mmol) of L-(7-hydroxycoumarin-4-yl)ethylglycine methanesulfonic acid (**2**) in 21 mL of 1:1 dioxane–water, was added 498 mg (5.93 mmol) of

$NaHCO_3$ followed by 327 mg (1.18 mmol) of 6-nitroveratryl chloroformate. The reaction mixture was stirred at room temperature for 18 h, then acidified to pH ~ 2-3 with 1 N HCl and extracted with three 25-mL portions of EtOAc. The combined organic layer was washed with 25 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was eluted through a pad of silica gel, eluting with (1 → 2%) methanol in chloroform to remove the starting materials. *N*-(6-Nitroveratryloxycarbonyl)-L-(7-hydroxycoumarin-4-yl)ethylglycine (**9**) was obtained as a colorless solid: yield 150 mg (50 %); silica gel TLC R_f 0.26 (10:1 chloroform–methanol), and was used directly in the next step without further purification; mass spectrum (APCI), *m/z* 503.1303 $(M+H)^+$ ($C_{23}H_{23}N_2O_{11}$ requires 503.1302).

_N_-(6-Nitroveratryloxycarbonyl)-L-(7-hydroxycoumarin-4-yl)ethylglycine Cyanomethyl Ester (10)

To a solution containing 36.0 mg (0.07 mmol) of *N*-(6-nitroveratryloxycarbonyl)-L-(7-hydroxycoumarin-4-yl)ethylglycine (**9**) in 1.35 mL of anhydrous DMF was added 30.0 mg (0.36 mmol) of $NaHCO_3$ followed by 23.0 μL (27.0 mg, 0.36 mmol) of chloroacetonitrile. The reaction mixture was stirred under argon at room temperature for 16 h. The mixture was acidified to pH ~ 3 with 1 N $NaHSO_4$ and extracted with 15 mL of EtOAc. The organic layer was washed with 15 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified on a silica gel column (18 × 2 cm) eluting with 1:1 hexanes–EtOAc. *N*-(6-Nitroveratryloxycarbonyl)-L-(7-hydroxycoumarin-4-yl)ethylglycine cyanomethyl ester **(10)** was obtained as a colorless solid: yield 30 mg (78%); silica gel TLC R_f 0.16 (1:2 hexanes–ethyl acetate); ^1H NMR ($CDCl_3$, 500 MHz) δ 0.83-0.93 (m, 1H), 1.16-1.23 (m, 2H), 1.38-1.42 (m, 1H), 1.95-2.08 (m, 1H), 2.79-2.90 (m, 2H), 3.84-3.98 (m, 6H), 4.78-4.94 (m, 1H), 5.43 (s, 1H), 5.61 (s, 1H), 6.21 (s, 1H), 7.01-7.32 (m, 2H), 7.67 (s, 1H) and 7.75 (m, 1H); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 27.8, 29.8, 49.6, 56.6, 56.8, 67.7, 108.45, 108.51, 110.4, 110.7, 111.0, 113.8, 114.2, 117.0, 117.8, 125.4, 125.5, 140.1, 148.9, 152.5, 153.4, 153.9, 154.5 and 160.2; mass spectrum (APCI), *m/z* 542.1404 $(M-H)^-$ ($C_{25}H_{24}N_3O_{11}$ requires 542.1411).

_N_-(6-Nitroveratryloxycarbonyl)-L-(7-hydroxycoumarin-4-yl)ethylglycine pdCpA Ester (11)

To a conical vial containing a solution of 29.5 mg (54.4 μmol) of *N*-(6-nitroveratryloxycarbonyl)-(*L*)-7-hydroxycoumarin-4-yl-ethylglycine cyanomethyl ester (**10**) in 150 μL of anhydrous DMF was added a solution of 14.8 mg (10.9 μmol) of the tris-(tetrabutylammonium) salt of pdCpA,[2] followed by 15 μL of triethylamine. The reaction mixture was sonicated at room temperature under argon for 16 h. A 2-μL aliquot of the reaction mixture was diluted with 98 μL of 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and analyzed by HPLC on a C_{18} reversed phase column (250 × 10 mm). The column was washed with 1 → 65% CH_3CN in 50 mM NH_4OAc, pH 4.5, over a period of 45 min at a flow rate of 3.5 mL/min (monitoring at 260 nm). The remainder of the reaction mixture was diluted to a total volume of 500 μL with 1:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed phase column. *N*-(6-Nitroveratryloxycarbonyl)-L-(7-hydroxycoumarin-4-yl)ethylglycine pdCpA ester (**11**) (retention times 22.8 and 23.6 min for the positional isomers) was recovered from the appropriate fractions as a colorless solid by lyophilization: yield 6.0 mg (49%); mass spectrum (ESI), *m/z* 1119.2126 $(M-H)^-$ ($C_{42}H_{45}N_{10}O_{23}P_2$ requires 1119.2134).

Biochemical Experiments

Ligation of Suppressor $tRNA^{Phe}_{CUA}$-C_{OH} and $tRNA^{Phe}_{CCCG}$-C_{OH} with *N*-pentenoyl-biphenyl-L-phenylalanyl-pdCpA and *N*-NVOC-L-(7-hydroxycoumarin-4-yl)ethylglycyl-pdCpA.
The yeast suppressor $tRNA^{Phe}_{CUA}$ and $tRNA^{Phe}_{CCCG}$ were prepared as previously reported.[3,4] Suppressor $tRNA^{Phe}$-C_{OH} activation was carried out in 100 μL (total volume) of 100 mM Hepes buffer, pH 7.5, containing 2.0 mM ATP, 15 mM $MgCl_2$, 100 μg of suppressor tRNA-C_{OH}, 2.0 A_{260} units of aminoacyl-pdCpA, 15% DMSO and 100 units of T4 RNA ligase. After incubation at 37 °C for 1 h, the reaction was quenched by the addition of 10 μL of 3 M NaOAc, pH 5.2, followed by 300 μL of ethanol. The reaction mixture was incubated at –20 °C for 30 min, then centrifuged at 15,000 × g at 4 °C for 30 min. The supernatant was decanted carefully and the tRNA pellet was washed with 100 μL of 70% ethanol and dissolved in 80 μL of RNase free H_2O. The pentenoyl group was removed by treatment with 5 mM I_2 at room temperature for 10 min. The nitroveratryloxycarbonyl (NVOC) group was removed by exposure to high intensity UV light at 2 °C for 2 min.

In vitro Translation of Mutant DHFR Analogues.[3]
The mutant DHFR plasmids were obtained by site-directed mutation as described as previously using the wild-type DHFR plasmid as the template.[5] The DNA primer for the mutation at position 17 was
5'-GTAGATCGCGTTATCGGCATGCGGGAACGCCATGCCGTGGAACCTG-3'; and the primer for the mutation at position 115 was
5'-CTGTATCTGACGCATTAGGACGCAGAAGTGGAAGG -3'. The *in vitro* expression mixture (300 μL total volume) contained 30 μg of mutant DHFR (CGGG at position 17 and/or TAG at position 115) plasmid DNA, 120 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2.0 mM dithiothreitol, 11 mM maganesium acetate, 20 mM phospho(enol)pyruvate, 0.8 mg/mL of *E. coli* tRNA, 0.8 mM IPTG, 20 mM ATP and GTP, 5 mM CTP and UTP and 4 mM cAMP), 100 μM of each of the 20 amino acids, 30 μCi of [^{35}S]-L-methionine, 10 μg/μL rifampicin, 90 μg of deprotected misacylated $tRNA_{CUA}$s and 90 μL of S-30 extract from *E. coli* strain BL21(DE3). The reaction mixture was incubated at 37 °C for 45 min. Plasmid DNA containing the gene for wild-type DHFR was used as the positive control, and an abbreviated tRNA (tRNA-C_{OH}) lacking any amino acid was used as the negative control. An aliquot containing 2 μL of reaction mixture was removed, treated with 2 μL of loading buffer and heated at 90 °C for 2 min. This was analyzed by 15% SDS-PAGE at 100 V for 2 h.

Purification of mutant DHFRs
The mutant DHFRs containing an N-terminal hexahistidine fusion peptide were purified by Ni-NTA chromatography.[6] The *in vitro* translation reaction mixture (300 μL) was diluted with 900 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 10 mM imidazole, and mixed gently with 100 μL of a 50% slurry of Ni-NTA resin at 4 °C for 2 h. Then the mixture was loaded on a column and washed with 600 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 10 mM imidazole. Finally, the DHFR analogue was washed three times with 200 μL of 50 mM Tris-HCl, pH 8.0, containing 30 mM NaCl and 150 mM imidazole. The three eluates from the Ni-NTA column were combined and loaded on a 200 μL DEAE-Sepharose column. The column was washed successively with 300 μL of 50 mM Tris-HCl, pH 8.0, containing 100

mM NaCl, 300 μL of 50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl, three 300-μL portions of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, and then 300 μL of 50 mM Tris-HCl, pH 8.0, containing 400 mM NaCl. Aliquots of each fraction were analyzed by 15% SDS-PAGE.

Enzymatic Activities of Wild-type and modified DHFRs

The enzymatic activities of wild-type and modified DHFRs were measured in 1 mL of MTEN buffer (containing 50 mM MES, 25 mM trizma base, 25 mM ethanolamine, 100 mM NaCl, 0.1 mM EDTA and 10 mM β-mercaptoethanol, pH 7.0). MTEN buffer (0.97 mL) was mixed with 10 μL of 10 mM NADPH and 200 ng of protein. The mixture was incubated at 37 ^{o}C for 3 min. Then 20 μL of 5 mM dihydrofolate in MTEN buffer, pH 7.0, was added. The OD value at 340 nm was monitored over a period of 10 min.

Measurement of Fluorescence of DHFRs

Fluorescence measurements of the modified DHFRs were carried out in 100 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl. One-μL of 200 μM inhibitor was added for the binding experiment. Fluorescence was monitored using a Cary Eclipse Fluorescence Spectrophotometer.

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