Supporting Information

for

Two Pyrenylalanines in Dihydrofolate Reductase Form an Excimer Enabling the Study of Protein Dynamics

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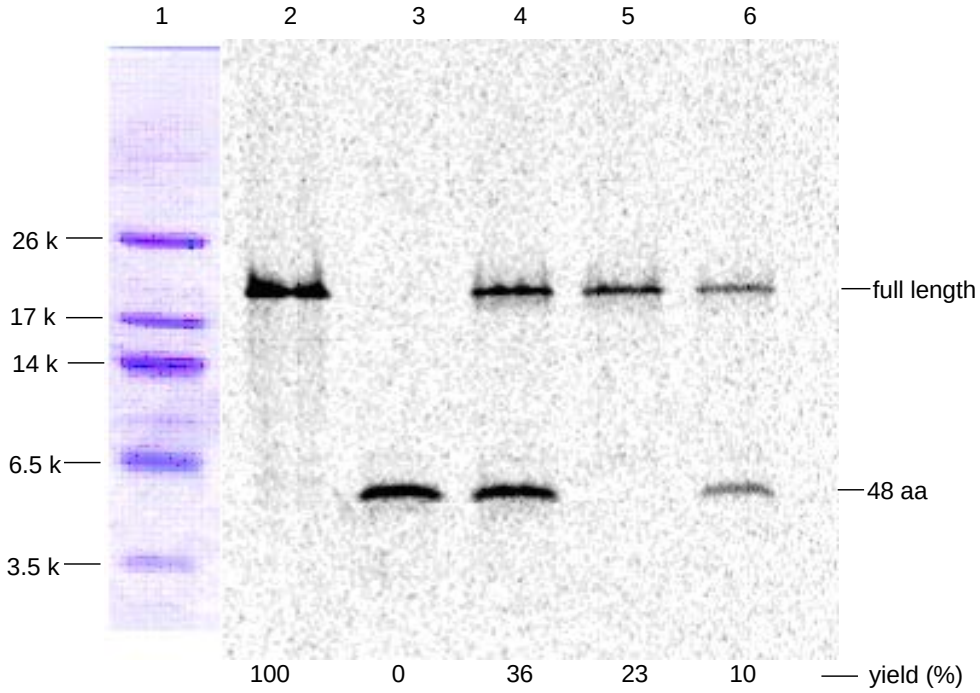


Figure S1. Expression of DHFRs containing one or two pyren-1-ylalanine moieties. Lane 1, molecular weight markers; lane 2, wild-type DHFR; lane 3, modified DHFR mRNA (codon 49 UAG) translated in the presence of unacylated tRNA-C_{OH}; lane 4, modified DHFR mRNA (codon 49 UAG) translated in the presence of pyren-1-ylalanyl-$tRNA_{CUA}$; lane 5, modified DHFR mRNA (codon 16 UAG) translated in the presence of pyren-1-ylalanyl-$tRNA_{CUA}$; lane 6, modified DHFR mRNA (codons 16 and 49 UAG) translated in the presence of pyren-1-ylalanyl-$tRNA_{CUA}$.

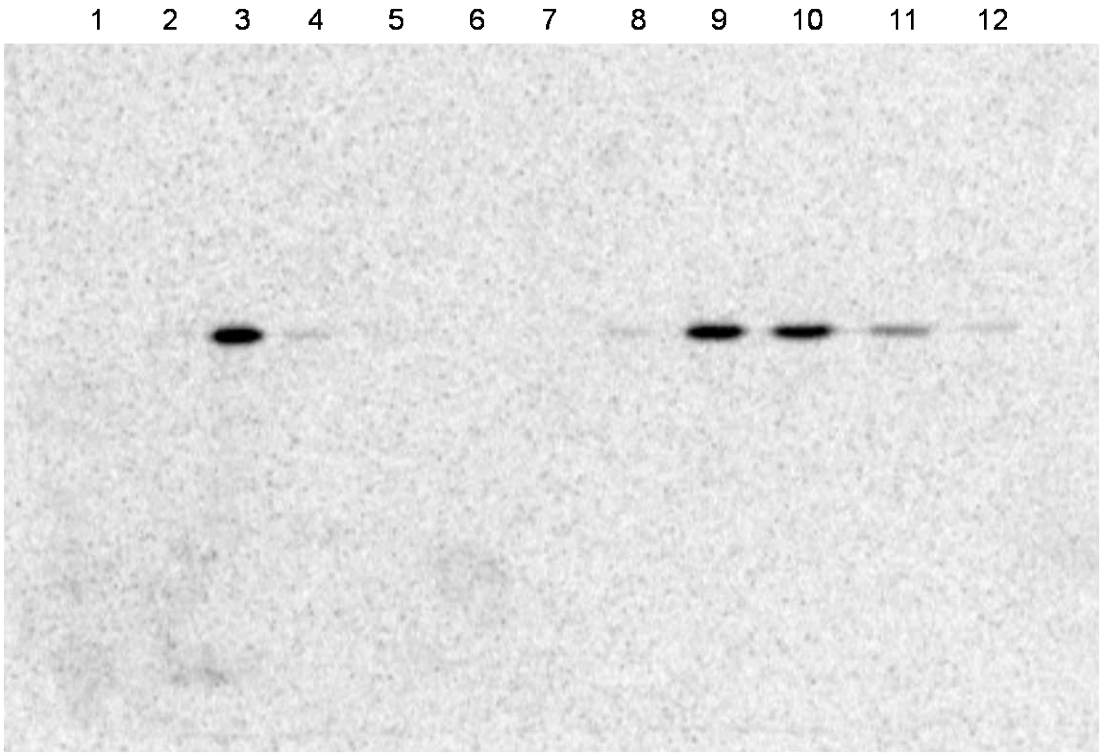


Figure S2. Purification of the modified DHFR containing pyren-1-ylalanine at positions 16 and 49 using successive Ni-NTA and DEAE-Sepharose CL-6B columns. Lane 1, flow through from Ni-NTA column; lane 2, elution with 20 mM imidazole; lane 3, first elution with 150 mM imidazole (200 μL); lane 4, second elution with 150 mM imidazole; lane 5, third elution with 150 mM imidazole; lane 6, flow through from DEAE-Sepharose CL-6B column; lane 7, elution with 100 mM NaCl; lane 8, elution with 200 mM NaCl; lane 9, first elution with 300 mM NaCl (300 μL); lane 10, second elution with 300 mM NaCl; lane 11, third elution with 300 mM NaCl; lane 12, elution with 400 mM NaCl.

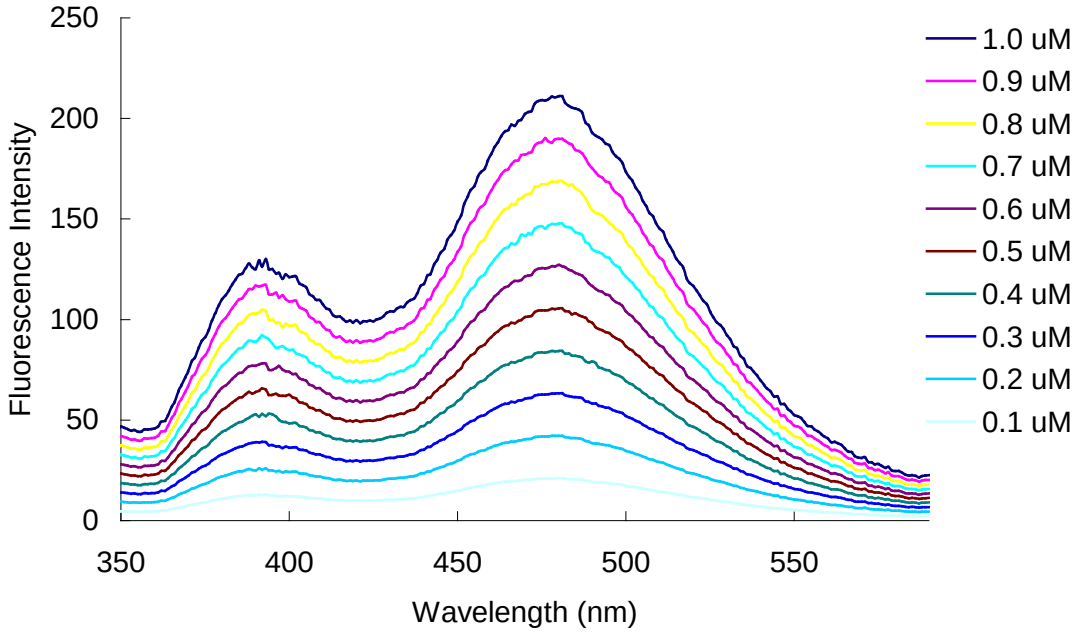


Figure S3. Fluorescence spectra of bis-(pyren-1-yl)-DHFR at different concentrations. The fluorescence emission was monitored from 350–580 nm following irradiation at 310 nm.

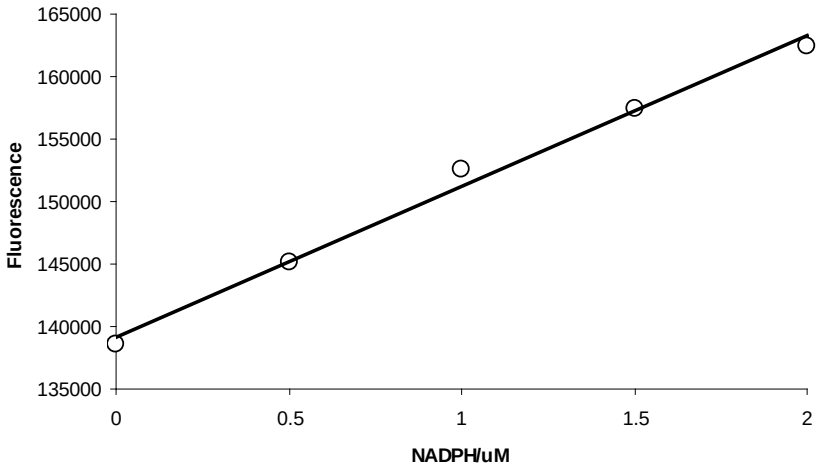


Figure S4. Fluorescence intensity versus NADPH concentration in the presence of bis-(pyren-1-yl)-DHFR (pyrene moieties at positions 16 and 49) at pH 7.0 in MTEN buffer. Excitation was at 360 nm with a 450 nm cutoff filter for emission. The data (black circles) were fitted to a straight line.

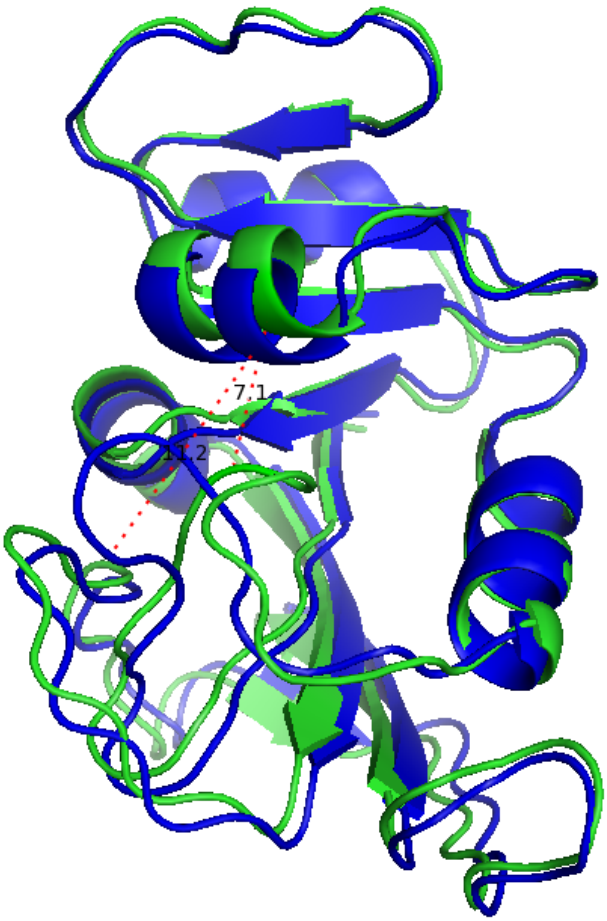
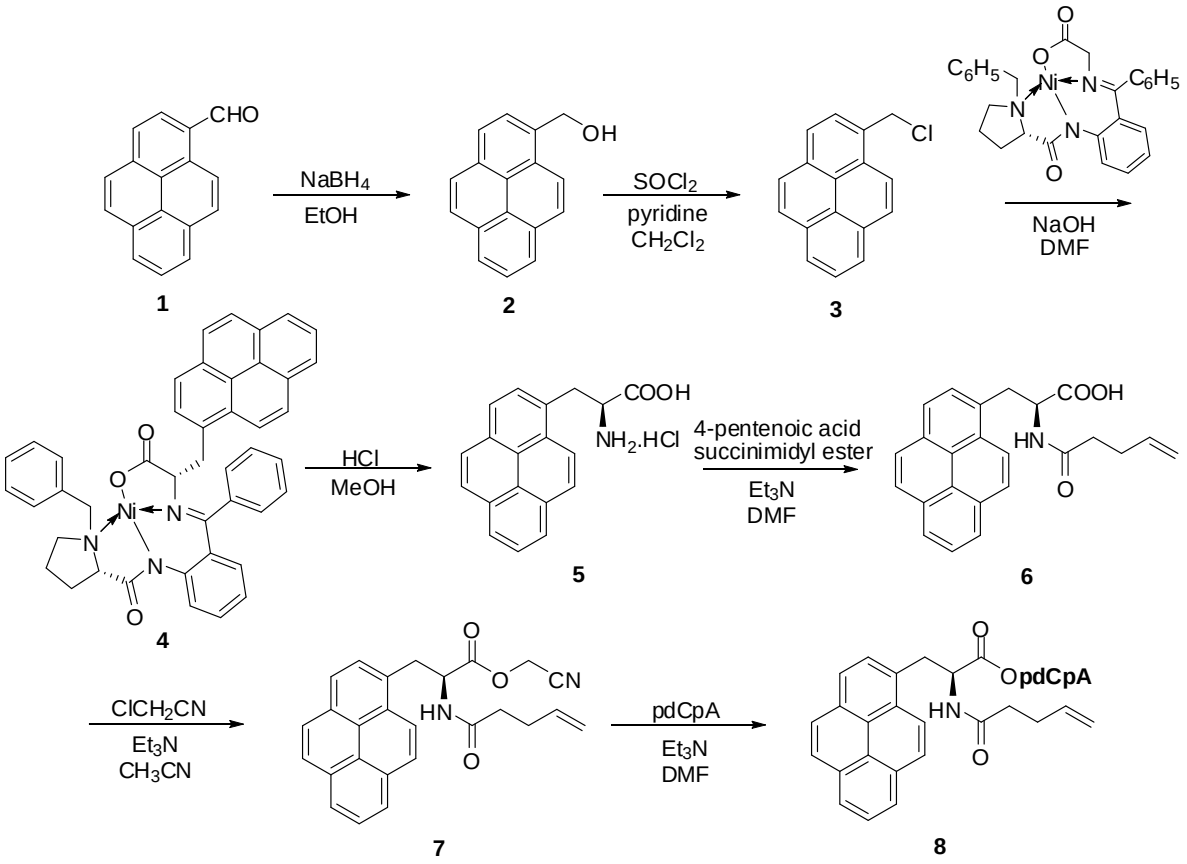


Figure S5. Superposition of the backbone structures of the E:NADPH:ddTHF (5,10-dideazatetrahydrofolate; PDB 1rx6, green) and E:NADPH:methotrexate (PDB1rx3, blue) complexes of *E. coli* DHFR. The distances between α-carbons of Met 16 and Ser 49 were measured as 11.2Å and 7.1 Å, respectively using Pymol ver. 1.3.

Scheme S1. Synthesis of *N*-pentenoylpyren-1-ylalanyl-pdCpA



Scheme S2. Simplified kinetic scheme for bis-(pyren-1-yl)-DHFR catalysis. E, S and P represent enzyme, substrate (DHF) and product (THF), respectively. EP* and EP** designate different conformations of product–enzyme complexes (DHFR•$NADP^+$•H_4F). The kinetic steps involved are highlighted in the red box. In the scheme, k_f is the rate of the conformational change monitored by the excimer after hydride transfer (k_H). The value of k_f was determined to be 2.6±0.5/s by burst experiments.

Experimental

Ni-NTA agarose was ordered from Qiagen Inc. DNA oligonucleotides were purchased 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 from PerkinElmer Inc. Protease inhibitor (complete, EDTA-free) was obtained from Boehringer Mannheim Corp. T4 RNA ligase and T4 polynucleotide kinase were obtained 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.

Synthesis of *N*-Pentenoylpyren-1-ylalanyl-pdCpA

1-Hydroxymethylpyrene (2)[1]

To a suspension of 5.00 g (21.7 mmol) of pyrene-1-carboxaldehyde (**1**) in 25 mL of EtOH was added 1.03 g (27.3 mmol) of $NaBH_4$ and the reaction mixture was stirred at room temperature for 5 h. Fifty mL of water was added and the mixture was extracted with two 30-mL portions of Et_2O. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The light brown residue was crystallized from EtOAc to give a first fraction of alcohol **2** (2.44 g). The filtrate from this crystallization was concentrated and the residue was again crystallized from 80 mL of 1:1 hexane – CH_2Cl_2 to give a second fraction (1.46 g). 1-Hydroxymethylpyrene (**2**) was obtained as a light brown solid: yield 3.90 g (77%); mp 124-126 °C (lit.[1] 123-124 °C); ^{1}H NMR ($CDCl_3$) δ 5,34 (s, 2H), 7.98-8.05 (m 4H), 8.08-8.11 (m, 2H), 8.17 (d, 2H, J = 8.0 Hz) and 8.30 (d, 1H, J = 9.2 Hz); ^{13}C NMR ($CDCl_3$) δ 63.9, 123.1, 124.8, 124.9, 125.0, 125.3, 125.4, 126.1, 127.5, 127.6, 128.0, 128.8, 130.9, 131.3 and 133.8; mass spectrum (MALDI), *m/z* 232.1 $(M)^+$ (theoretical *m/z* 232.1).

1-Chloromethylpyrene (3)

To a cooled (-10 °C) solution containing 0.50 g (2.15 mmol) of 1-hydroxymethylpyrene **(2)** in 15 mL of anhydrous CH_2Cl_2 and 0.52 mL (0.51 g, 6.45 mmol) of pyridine was added dropwise over 10 min 0.47 mL (0.77 g, 6.45 mmol) of $SOCl_2$. The reaction mixture was left to warm slowly to room temperature and stirred for 16 h. Thirty mL of a 5% aq $NaHCO_3$ solution was added carefully and the mixture was stirred for another 30 min. The organic layer was separated, washed with 25 mL of 5% aq $NaHCO_3$, dried ($MgSO_4$) and concentrated under diminished pressure. 1-Chloromethylpyrene **(3)** was obtained as a light green solid: yield 487 mg and was used directly in the next step without further purification; mass spectrum (MALDI), *m/z* 250.0 $(M)^+$ (theoretical *m/z* 250.1) and *m/z* 215.1 $(M\text{-}Cl)^+$ (theoretical *m/z* 215.1).

Ni (II) Complex of the Imine Formed from (*S*)-Pyren-1-ylalanine and 2-[(*N*-Benzyl-(*S*)-prolinyl)amino]benzophenone (4)

To a suspension of 535 mg (1.07 mmol) of the Ni (II) complex of the imine formed from glycine and 2-[(*N*-benzyl-(*S*)-prolinyl)amino]benzophenone[2] and 100 mg (2.50 mmol) of powdered NaOH in 5 mL of DMF was added a solution containing 487 mg (1.92 mmol) of the crude 1-chloromethylpyrene **(3)** in 3 mL of DMF. The reaction mixture was stirred at room temperature under argon for 45 min then diluted with 15 mL of EtOAc. The organic layer was washed with 25 mL of 0.1 N HCl, 15 mL of brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified on a silica gel column (20 × 3 cm) using a stepwise gradient of acetone in $CHCl_3$ (10:1 → 5:1). The product **4** was obtained as a dark red foam: yield 605 mg (78%); ^{1}H NMR ($CDCl_3$) δ 1.45 (m, 1H), 1.81 (m, 1H), 2.07 (m, 1H), 2.15 (m, 1H), 2.28 (m, 1H), 2.87 (m, 1H), 3.25 (dd, 1H, *J* = 10.2 and 7.0 Hz), 3.35 (d, 1H, *J* = 12.8 Hz), 3.94 (dd, 1H, *J* = 14.2 and 5.0 Hz), 4.23 (d, 1H, *J* = 12.8 Hz), 4.31 (dd, 1H, *J* = 14.4 and 7.2 Hz), 4.60 (dd, 1H, *J* = 6.8 and 5.2 Hz), 5.96 (d, 1H, *J* = 8.0 Hz), 6.48 (m, 1H), 6.59 (m, 1H), 7.04-7.13 (m, 3H), 7.20 (d, 1H, *J* = 7.6 Hz), 7.26 (m, 3H), 7.35 (t, 1H, *J* = 7.2 Hz), 7.85 (m, 2H) and 7.93-8.20 (m, 10H); mass spectrum (APCI), *m/z* 712.2121 $(M+H)^+$ ($C_{44}H_{36}N_3NiO_3$ requires 712.2110).

(*S*)-Pyren-1-ylalanine (5)

To a solution containing 483 mg (0.67 mmol) of the glycine–nickel complex **4** in 11 mL of MeOH was added 1 mL of 6 N HCl. The reaction mixture was stirred at reflux for 30 min, or until a clear yellow solution was obtained. The solvent was concentrated under diminished pressure and the residue was purified on a silica gel column (20 × 2 cm), eluting with 6:2:1 iPrOH−MeOH−NH_4OH. The fractions containing the desired product were combined and concentrated to give the product as an off-white solid. The product was triturated with two 5-mL portions of acetone and filtered. Pyren-1-ylalanine ammonium salt was obtained as an off-white solid: yield 195 mg (93%). A portion of the product (95 mg) was suspended in 1 mL of methanol and 1 mL of 1 N HCl and heated until most of the material dissolved. The solvent was concentrated and the residue was triturated with 2 mL of water and filtered, then washed with two 1-mL portions of water. The resulting hydrochloride salt **5** was dried under vacuum and was obtained as an off-white solid: 62 mg; ^{1}H NMR (DMSO-d_6) δ 3.80 (dd, 1H, *J* = 14.0 and 8.0 Hz), 3.97 (dd, 1H *J* = 14.0 and 6.6 Hz), 4.19 (m, 1H), 7.98 (d, 1H, *J* = 8.0 Hz), 8.08 (t, 1H, *J* = 7.6 Hz), 8.16 (s, 2H), 8.23-8.33 (m, 4H) and 8.47 (d, 1H, *J* = 9.6 Hz); ^{13}C NMR (DMSO-d_6) δ 33.4, 53.2, 122.8, 123.6, 123.9, 124.6, 124.9, 125.0, 126.0, 126.9, 127.0, 127.5, 128.4, 128.5, 129.1, 129.9, 130.0, 130.4 and 169.9; mass spectrum (APCI), *m/z* 290.1187 $(M+H)^+$ ($C_{19}H_{16}NO_2$ requires 290.1181).

***N*-(4-Pentenoyl)-(*S*)-pyren-1-ylalanine (6)**

To a suspension of 62.0 mg (0.19 mmol) of (*S*)-pyren-1-ylalanine hydrochloride **(5)** in 5 mL of DMF was added was added 132 μL (95.0 mg, 0.94 mmol) of triethylamine followed by 56.0 mg (0.28 mmol) of 4-pentenoic acid succinimidyl ester. The rection mixture was stirred at room temperature for 18 h, acidified to pH ~ 2-3 with 1 N HCl and

extracted with two 10-mL portions of EtOAc. The combined organic layer was washed with 5 mL of brine, dried ($MgSO_4$) and concentrated. The residue was purified on a silica gel column (25 × 2 cm), eluting with 9:1:0.05 CH_2Cl_2−MeOH−AcOH. *N*-(4-Pentenoyl)-(*S*)-pyren-1-ylalanine **(6)** was obtained as an off-white solid: yield 52 mg (74%); ^{1}H NMR (CD_3OD) δ 2.04-2.61 (m, 4H), 3.56 (dd, 1H, *J* = 14.0 and 9.2 Hz), 3.97 (dd, 1H, *J* = 14.0 and 5.0 Hz), 4.68-4.82 (m, 2H), 4.92 (m, 1H, hidden by HDO peak), 5.52 (m, 1H), 7.86 (d, 1H, *J* = 7.6 Hz), 7.93 (t, 1H, *J* = 7.6 Hz), 7.96 (s, 2H), 8.04 (d, 2H, *J* = 8.0 Hz), 8.07 (d, 1H, *J* = 9.2 Hz), 8.11 (dd, 2H, *J* = 8.0 and 2.6 Hz) and 8.32 (d, 1H, *J* = 9.2 Hz); ^{13}C NMR (CD_3OD) δ 26.2, 30.5, 36.1, 36.2, 55.4, 115.5, 124.1, 125.6, 125.91, 125.94, 126.1, 127.0, 128.0, 128.4, 128.6, 129.3, 130.4, 131.8, 132.1, 132.6, 132.7, 138.0, 174.9 and 175.1; mass spectrum (APCI), *m/z* 372.1587 $(M+H)^+$ ($C_{24}H_{22}NO_3$ requires 372.1600).

***N*-(4-Pentenoyl)-(*S*)-pyren-1-ylalanine Cyanomethyl Ester (7)**

To a solution containing 44.0 mg (0.11 mmol) of *N*-(4-pentenoyl)-(*S*)-pyren-1-ylalanine **(6)** in 3 mL of anhydrous DMF was added 18.0 mg (0.17 mmol) of Na_2CO_3 followed by 35.0 μL (42.0 mg, 0.55 mmol) of chloroacetonitrile. The reaction mixture was stirred under argon at room temperature for 24 h. The mixture was acidified to pH ~ 3 with 1 N $NaHSO_4$ and extracted with 10 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 (17 × 2 cm), eluting with a stepwise gradient of EtOAc in hexanes (20 → 50%). *N*-(4-Pentenoyl)-(*S*)-pyren-1-ylalanine cyanomethyl ester **(7)** was obtained as a colorless solid: yield 23 mg (47%); silica gel TLC R_f 0.20 (3:2 hexanes–ethyl acetate); ^{1}H NMR (acetone-d_6) δ 2.21 (m, 4H), 3.81 (dd, 1H, *J* = 14.2 and 8.6 Hz), 3.98 (dd, 1H, *J* = 14.0 and 6.0 Hz), 4.80-4.94 (m, 2H), 4.97 (s, 2H), 5.04 (m, 1H), 5.69 (m, 1H), 7.69 (d, 1H, *J* = 7.6 Hz), 7.97 (d, 1H, *J* = 8.0 Hz), 8.05 (t, 1H, *J* = 7.8 Hz), 8.12 (s, 2H), 8.21 (t, 1H, *J* = 7.8 Hz), 8.25-8.29 (m, 3H), 8.42 (d, 1H, *J* = 9.6 Hz); ^{13}C NMR (CD_3CN) δ 29.9, 35.3, 35.5, 50.3, 54.5, 115.4, 115.9, 124.0, 125.4, 125.6, 125.8, 126.1, 126.2, 127.2, 128.1, 128.4, 128.7, 129.4, 130.2, 131.5, 131.7, 131.9, 132.3, 138.3, 171.6 and 173.1; mass spectrum (APCI), *m/z* 411.1715 $(M)^+$ ($C_{26}H_{23}N_2O_3$ requires 411.1709).

***N*-(4-Pentenoyl)-(*S*)-pyren-1-ylalanine pdCpA Ester (8)**

To a conical vial containing a solution of 9.40 mg (22.8 μmol) of *N*-(4-pentenoyl)-(*S*)-pyren-1-ylalanine cyanomethyl ester **(7)** in 60 μL of anhydrous DMF was added a solution of 5.60 mg (4.11 μmol) of the tris-(tetrabutylammonium) salt of pdCpA[3] in 40 μL of anhydrous DMF, followed by 5 μL of triethylamine. The reaction mixture was stirred at room temperature under argon for 22 h. A 2-μL aliquot of the reaction mixture was diluted with 58 μL of 1:1 CH_3CN/50 mM NH_4OAc at 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 600 μL with 2:1 CH_3CN–50 mM NH_4OAc, pH 4.5, and purified using the same C_{18} reversed-phase column. *N*-(4-Pentenoyl)-(*S*)-pyren-1-ylalanine pdCpA ester **(8)** (retention time 32-33 min) was recovered from the appropriate fractions as a white solid by lyophilization: yield 1.7 mg; mass spectrum (ESI), *m/z* 990.2569 $(M+H)^+$ ($C_{43}H_{46}N_9O_{15}P_2$ requires 990.2589).

Ligation of Suppressor $tRNA^{Phe}_{CUA}$-C_{OH} to *N*-pentenoylpyren-1-ylalanyl-pdCpA. The yeast suppressor $tRNA^{Phe}_{CUA}$ was prepared as reported previously.[4] Suppressor $tRNA^{Phe}_{CUA}$-C_{OH} ligation to an aminoacylated pdCpA derivative 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 *N*-pentenoyl-pyren-1-ylalanyl-pdCpA, 15% DMSO and 200 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 suspension was maintained at –20 °C for 30 min, then centrifuged at 15,000 × g at 4 °C for 30 min. The supernatant was carefully decanted 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 in 5 mM aq I_2 solution at room temperature for 10 min.

***In vitro* Translation of Modified DHFRs.[4]** The modified DHFR plasmids were obtained by site-directed mutagenesis as described as previously[5] using the wild-type DHFR plasmid as the template. The DNA primer for the mutation at position 16 was 5'-GCGGTAGATCGCGTTATCGGCTAGGAAAACGCCATGCCGTGGAAC-3'; and the primer for the mutation at position 49 was 5'-GGCCGCCATACCTGGGAATAGATCGGTCGTCCGTTGCCAGG -3'. The *in vitro* expression mixture (300 μL total volume) contained 30 μg of modified DHFR plasmid DNA (TAG at position 16, 49 or both), 120 μL of premix (35 mM Tris-acetate, pH 7.0, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM dithiothreitol, 11 mM magnesium 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 $tRNA_{CUA}$ 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 Modified DHFRs. The modified 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. The mixture was then applied to a column and washed with 600 μL of 50 mM Tris-HCl, pH 8.0, containing 300 mM NaCl and 20 mM imidazole. Finally, the column 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 elutions from the Ni-NTA column were combined and applied to a 200-μL DEAE-Sepharose column. The column was washed 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.

Catalytic Activities of Wild-type and Modified DHFRs. The enzymatic activities of wild-type and bis-(pyren-1-yl)-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 100 ng of protein. The reaction 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.

Kinetics Measurements of Bis-(pyren-1-yl)-DHFR. The enzyme was dialyzed against 50 mM sodium phosphate, pH 7.0, containing 1 mM DTT and 10% glycerol, and concentrated before use. All kinetic measurements were carried out at 25°C in 50 mM 2-morpholinoethane, 25 mM ethanolamine, 100 mM NaCl, and 1 mM DTT (MTEN). The steady-state turnover number (k_{cat}) was determined on a Cary 100 Bio by monitoring the change in absorbance intensity at 340 nm (NADPH absorbance maximum, ε 132,200 $M^{-1}cm^{-1}$) upon mixing the DHFR, which had been pre-equilibrated with 0.2 mM NADPH, with 0.2 mM DHF. Further experiments were performed under both multiple and single turnover conditions by using a stopped-flow instrument (Applied Photophysis Ltd., Leatherhead, U.K.). In the single turnover experiments, approximately 8 μM DHFR was preincubated with 1.25 μM NADPH in MTEN buffer, pH 7.0, and the reaction was initiated by rapid mixing with 0.2 mM DHF. The hydride transfer rate (k_H) was determined by following the change in NADPH absorbance at 340 nm. The excimer complex was excited at 360 nm and monitored through a 450 nm cutoff filter. The fluorescence traces collected were fitted to a single-exponential equation.

References

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