Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Facile Synthesis of a Novel Genetically Encodable Fluorescent α-Amino Acid Emitting Greenish Blue Light

Aakash Gupta,[a] Brian P. Garreffi,[a] and Maolin Guo*[ab]

a) Department of Chemistry and Biochemistry, UMass Cranberry Health Research Center, University of Massachusetts Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747 (USA), E-mail: mguo@umassd.edu.

b) Department of Chemistry, University of Massachusetts Amherst, 710 North Pleasant Street, Amherst, MA 01003 (USA), E-mail: mguo@umass.edu.

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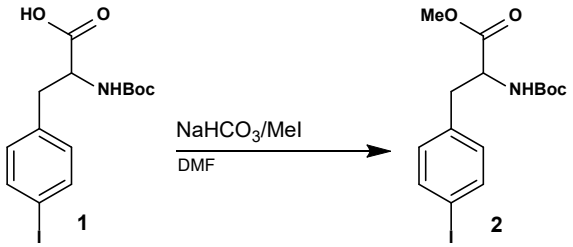
1. Experimental Section

Chemicals: N-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine, 9-Phenanthracenylboronic acid, N,N-Dimethyformamide, and Bis(triphenylphosphine) palladium (II) dichloride were purchased from Sigma-Aldrich. Methyl iodide was purchased from Alfa Aesar. Rhodamine B was purchased from Sigma-Aldrich. All chemicals were used without further purification. The reagents not mentioned as well as the solvents used in the syntheses were purchased commercially and were ACS grade.

Instrumentation and characterization: The ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker AVANCE III HD 400 MHz High-Performance Digital NMR spectrometer. Chemical shifts were analyzed on MestReNova software and reported in delta (δ) unit parts per million (ppm) downfield tetramethylsilane (TMS). The spectra were obtained in Methanol-d_4 or DMSO-d_6 olvent with 0.03 % of TMS as the internal reference. The abbreviation for splitting patterns represents, s = singlet, d = doublet, m = multiplet and coupling constants are reported in Hertz (Hz). The mass spectra were obtained on a Waters ACQUITY UPLC (Ultra-Pure Liquid chromatograph) Xevo QTOF (Quadrupole Time of Flight) high resolution mass spectrometer in a mobile phase of Acetonitrile:Water with a solvent gradient of 100% to 50% over 10 minutes of time. The product was purified by HPLC using an Agela Technologies Cheetah HP100 equiped with a 12g Flash Column. Flow rate was set to 8.0 mL min^{-1}.The mobile phase solvent system consisted of water (A)/Methanol (B) gradient system (0% B for 5 min, 0-40% for 10 min, 40-100% for 1 min, and 100% for 9 min). UV-Vis spectra were obtained on a Perkin-Elmer Lambda 25 spectrometer over wavelength range of 200-800 nm. Fluorescence spectra were recorded on a Perkin-Elmer LS55 luminescence spectrometer at 293 K. The excitation wavelengths and filters used were indicated in the figures. The relative quantum yield was measured using rhodamine B

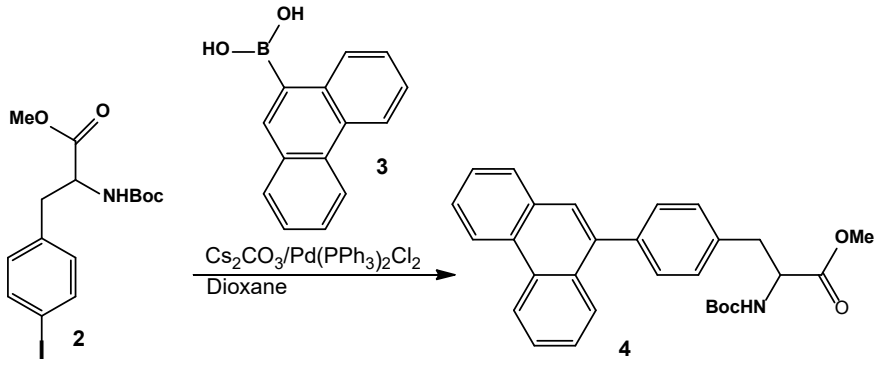
as a standard. The pH measurements were carried out on a Corning pH meter equipped with a Sigma-Aldrich micro combination electrode calibrated with standard buffer solutions. Optical rotation was measured by optical spectrophotometry using a Rudolph Research Analytical Autopol III Automatic Polarimeter. Zeiss LSM 710 laser scanning confocal microscope with excitation lasers at 405 nm, 458 nm, 488 nm, 514 nm, 543 nm, and 633 nm was used in the imaging experiments. The reuse function of the Zeiss software enables the use of previous experiment perimeter to minimize differences between different batch of experiments if needed. 35 mm glass bottom dishes (P35G-1.5-14-C) with cover slips for confocal cell experiments were purchased from Mattek.

Synthesis and Characterization



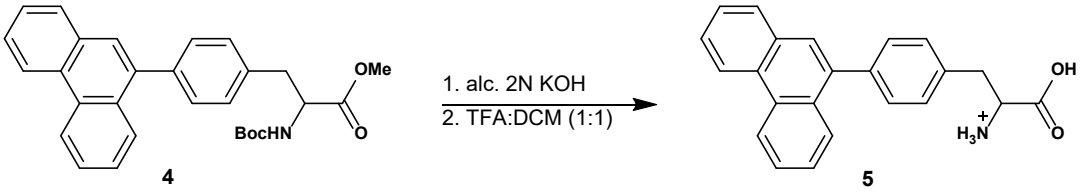
Synthesis of N-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester intermediate (Boc-Phe(4-I)-OMe) (2) (Step 1) To a 100 mL RBF containing N-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine (205 mg, 0.524 mmol) dissolved in 10 mL of DMF, $NaHCO_3$ (130 mg, 1.55 mmol) was added. The solution was degassed with argon and then methyl iodide (156 µL, 2.50 mmol) was slowly added, which caused the reaction mixture to change color from colorless to yellow, and was allowed to react for 20 hours at 80℃. The mixture was then diluted with water and extracted with DCM (3 x 20 mL). Then

the organic layers were combined and washed with a saturated brine solution (1 x 10 mL) before the addition of anhydrous $MgSO_4$ to remove any traces of water. The solvent was removed using roto-evaporation set at 40-50℃ to afford a yellow oily compound. The product was purified with silica gel flash chromatography using a Hexane: Ethyl acetate solvent gradient of 9:1. The product obtained (N-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester) (**2**) is a white fibrous solid (190 mg). HRMS (ESI): calcd for $[C_{15}H_{20}INO_4+H]^+$: 406.0437, found: *m/z*: 406.0450.



Coupling reaction of N-(*tert*-Butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester (2) with 9–Phenanthracenylboronic acid (3) to form N-(Boc)-4-(9-phenanthracenyl)-L-phenylalanine methyl ester (4) (Step 2). A 50 ml RBF, containing of $Pd(PPh_3)_2Cl_2$ (4 mg) was flushed with flow of Argon gas, followed by the addition of Boc-Phe(4-I)-OMe (61 mg, 0.151 mmol) dissolved in 2 mL dioxane. To this, Cs_2CO_3(98.40 mg, 2 equiv., 0.302 mmol) dissolved in 2 mL of water was added with continuous stirring and then 9-Phenanthracenyl boronic acid (67 mg, 2 equiv., 0.302 mmol) dissolved in 2 mL dioxane was added before the reaction was diluted with 4 mL of dioxane. This reaction was allowed to stir for 16 h at 80℃ under argon gas. The reaction mixture was then diluted with water and extracted with DCM (3 x 10 mL). The combined organic layers were then washed with

a saturated brine solution (1 x 5 mL) before the addition of anhydrous $MgSO_4$ to remove any traces of water. The product was concentrated using roto-evaporation, set at 40-50℃ which afforded the yellow oily compound. HRMS (ESI): calcd for $[C_{29}H_{29}NO_4 + H]^+$: 456.2097, found: *m/z*: 456.2057. Minor deprotected product was also observed. The crude product was directly used in the deprotection step (**step 3**) without further purification.



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De-protection of the methyl and Boc protecting groups from N-(Boc)-4-(9-phenanthracenyl)-L-phenylalanine methyl ester (4) to produce the final product (5) (Step 3). Here, 10 mL of 2N alc. KOH (KOH dissolved in methanol) solution was added to the crude coupled product and allowed to stir at 35℃ for 4 hrs. The methanol solvent was removed and the crude compound was cooled using an ice-water bath prior to the slowly addition of 6N HCl until the pH of the solution was 3-4 as indicated by Litmus paper. The reaction mixture was then diluted with water and extracted with DCM (3 x 10 mL). The organic layers were combined and washed wtih a saturated brine solution (1 x 5 mL) before the addition of anhydrous $MgSO_4$ to remove any traces of water. The solvent was removed using roto-evaporation set at 40-50℃. To this crude product, 2 mL of TFA:DCM (1:1) was added and allowed to stir for 2 hrs at room temperature. The TFA and DCM were removed using roto-evaporation set at 40-50℃ and the product was then purified by preparative HPLC using water(A):methanol(B) solvent gradient system (**Figure S1**). The solvent was removed using roto-evaporation set at 40-50℃ yielding the

final product (4-phenanthracen-9-yl-L-phenylalanine) (**5**) as a yellow solid (yield over three steps: 19 mg, ~37 %). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.94 (d, J = 8.2 Hz, 1H), 8.87 (d, J = 8.1 Hz, 1H), 8.02 (d, J = 9.0 Hz, 1H), 7.91 (d, J = 7.5 Hz, 1H), 7.75 – 7.59 (m, 5H), 7.46 (s, 4H), 3.51 (m, 1H), 3.28 (dd, J = 14.1, 1H), 2.98 (dd, J = 14.1, 1H) (**Figure S2**). ^{13}C NMR (101 MHz, DMSO- d_6): δ 170.08, 138.52, 138.46, 137.66, 131.55, 130.86, 130.64, 130.15, 129.97, 129.79, 129.12, 127.56, 127.53, 127.39, 127.25, 126.86, 123.82, 123.24, 56.14, 37.42 (**Figure S3**). HRMS (ESI): calcd for $[C_{23}H_{19}NO_2 + H]^+$: 342.1416, found: *m/z*: 342.1278, [minor dimer at m/z 683.3774] (**Figure S4**).

Optical Rotation. The specific rotation ([α]) of the the final product (4-phenanthracen-9-yl-L-phenylalanine) (**5**) (0.2 mM in DMSO) was measured by optical spectrophotometry using a Rudolph Research Analytical Autopol III Automatic Polarimeter at 589 nm wavelength, 303 K and with an optical path length 100 mm, and calculated according to [2]:

$$\text{Specific rotation } [\alpha]_{\lambda}^{T} = \frac{\alpha}{c \times l}$$

$$= \frac{observed\ optical\ rotation\ in\ deg.(\alpha)}{\text{concentration in}\frac{g}{ml}(c) \times pathlength\ in\ decimeter(l)}$$

$$= \frac{-0.0018}{0.0000682805\ g/mL \times 1}$$

$$= -26.36^{\circ}$$

Thus, the specific rotation of (4-phenanthracen-9-yl-L-phenylalanine) (**5**) $[\alpha]_{\lambda}^{T}$ = - 26.36 °. As the specific rotation of D-phenylalanine is defined as "+" and the specific rotation of L-phenylalanine is defined as "-" [1], and 99% pure L-phenylalanine has a specific rotation of – 32.7 °at 298 K in H_2O (see Sigma-Aldrich data sheet at https://www.sigmaaldrich.com/catalog/product/aldrich/p17008?lang=en&region=US) and a

value of -34.3 ° in an earlier literature [3], the (4-phenanthracen-9-yl-L-phenylalanine) (**5**) is thus in its L-form. Previous studies using similar Suzuki coupling to synthesize fluorescent unnatural amino acids have been verified that these reaction conditions didn't cause epimerization [4].

Fluorescence quantum yield measurement. Rhodamine B (0.1 M) solution was prepared by dissolving Rhodamine B (0.047 g) into ethanol (0.001 L). Rhodamine B solution (10 μM) was prepared by diluting the Rhodamine B solution (0.1 M). Rhodamine B solutions (1 μM, 0.8 μM, 0.6 μM, 0.4 μM, 0.2 μM) were prepared by diluting the Rhodamine B solution (10 μM). Solutions for the fluorescent amino acid 4-phenanthracen-9-yl-L-phenylalanine(Phen-AA) were prepared in dimethyl sulfoxide (DMSO). Each solution was mixed using a vortex. Fluorescence spectra for rhodamine B were carried out with excitation and emission wavelengths set at 420 nm and 450-800 nm, respectively, and using an excitation slit width of 15 nm and an emission slit width of 2.5 nm, while excitation and emission wavelengths set at 380 nm and 400-800 nm, respectively, were used for Phen-AA. A plot of the base-line corrected integrated fluorescence intensity vs. the absorbance for each concentration was prepared and the positive slope of the linear fit was calculated. The data were compared to the rhodamine standard using the following equation, where Φ_{ST} is the quantum yield of the rhodamine standard (0.68) [5], Grad$x$ is the slope of the absorbance vs. emission line found for each compound, $Grad_{ST}$ is the slope found for the Rhodamine standard, ηx is the refractive index of the dimethyl sulfoxide (1.479) and ηst is the refractive index of the ethanol (1.363). The quantum yield of Phen-AA (Φx) was calculated to be 0.75:

$$\Phi_X = \Phi_{ST} \frac{Gradx}{GradST} \left(\frac{\eta x2}{\eta st2}\right)$$

$$= 0.68 \left(\frac{34.638}{37.141}\right) \left(\frac{1.479^2}{1.363^2}\right)$$

$$= 0.75$$

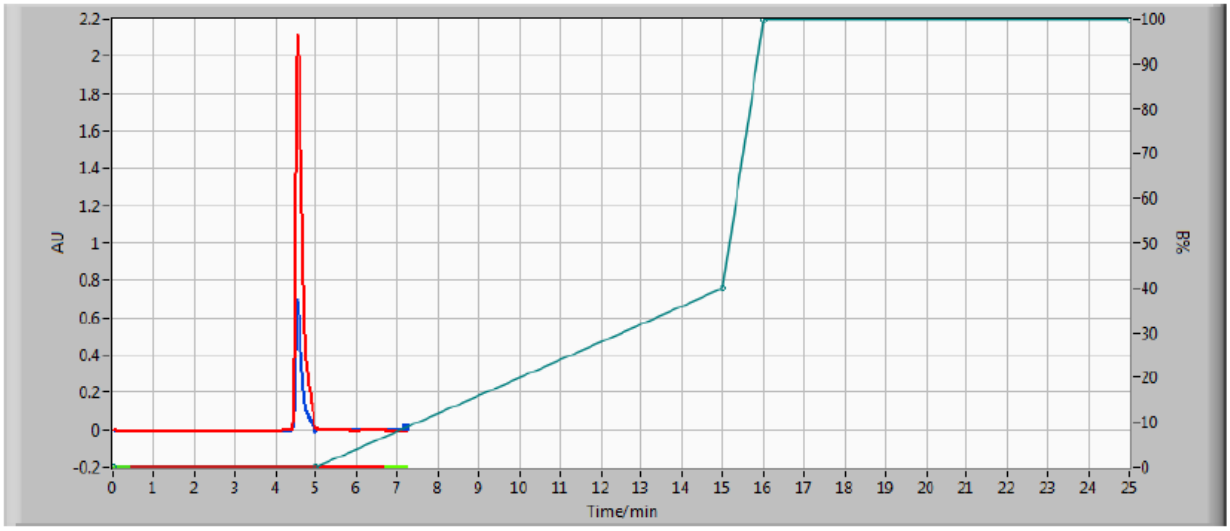
Photostability study. A series of fluorescence emission spectra (λ_{ex}=380 nm, excitation slit 15 nm and emission slit 2.5 nm) of 4-phenanthracen-9-yl-L-phenylalanine(Phen-AA) solution (0.001 M in DMSO-SPB buffer, pH 7.0, 0.05 M) was recorded at 293 K over a 5 h period. The fluorescence spectrometer was set to scan the sample and record an emission spectrum every 180 seconds. 100 spectra were recorded over 5 hours. Therefore, a spectrum was recorded 120 seconds after the previous spectrum until 100 spectra were recorded.

Solvachromatic study. Due to low solubility of Phen-AA in some solvents, stock solution of Phen-AA was prepared in DMSO and diluted 10 times in the specified solvents. Phen-AA (0.001 M) solutions were prepared in a 9:1 mixture of solvents by diluting aliquots of a Phen-AA (0.01 M) solution in DMSO, DMF, MeOH, SPB buffer (pH 7.0, 0.05 M), EtOH, acetone, and isopropanol, respectively. Each solution was mixed using a vortex. Fluorescence emission spectra (λ_{ex}=380 nm, excitation slit 15 nm and emission slit 2.5 nm) were recorded at 293 K.

Cell culture and confocal imaging. Hela cells (ATCC) were cultured and maintained with the complete growth medium composing of DMEM with supplement of 10% FBS in a humid 37˚C incubator under 5% CO_2 level. To prepare for confocal experiments, Hela cells were cultured in the flask until the confluency reached about ~70%. After the flask was rinsed with PBS twice, 2 ml 0.25% trypsin-EDTA solution was added followed by incubation in the incubator. Upon cell dissociation from the flask's wall, 2 ml complete growth medium was added to neutralize the enzyme and stop the digestion. The mixture was then transferred into a 15 mL tube and

centrifuged for 3 min at 1,000 g. The medium was discarded to remove the residual enzyme and other waste products. 5 ml of fresh complete medium was added to the tube to resuspend the cell pellet. The same amount of cell containing medium from the tube was transferred into each of the 35-mm glass bottom dishes, respectively, and 1 ml fresh complete medium was added. Change the medium when necessary or every two days to support cell maintenance and growth until experiment. To test the biocompatibility and imaging potential of the fluorescent amino acid Phen-AA in live cells, Phen-AA (0.010 mM or 0.020 mM) was added to the medium of the experimental dish with Hela cells for 1 hour. Before imaging, all dishes were washed three times with fresh warm complete medium in a period of 45 mins to remove the excessive free Phen-AA in the medium. Confocal fluorescent images of the Phen-AA incubated and control cells were then acquired and analyzed. All dishes were excited with 405 nm, and emissions were collected in the range of 410-550 nm.

2. Supplemental Figures



B%	Start	End	Duration
Equ	100.0	100.0	10.0
1	0.0	0.0	5.0
2	0.0	40.0	10.0
3	40.0	100.0	1.0
4	100.0	100.0	9.0

Figure S1: HPLC purification profile of 4-phenanthracen-9-yl-L-phenylalanine (**5**) using a reverse phase column with a water(A):Methanol(B) solvent gradient for a duration of (0-0)% of B for 5 min, (0-40)% of B for 10 min, (40-100)% of B for 1 min, (100-100)% of B for 1 min at 25℃.

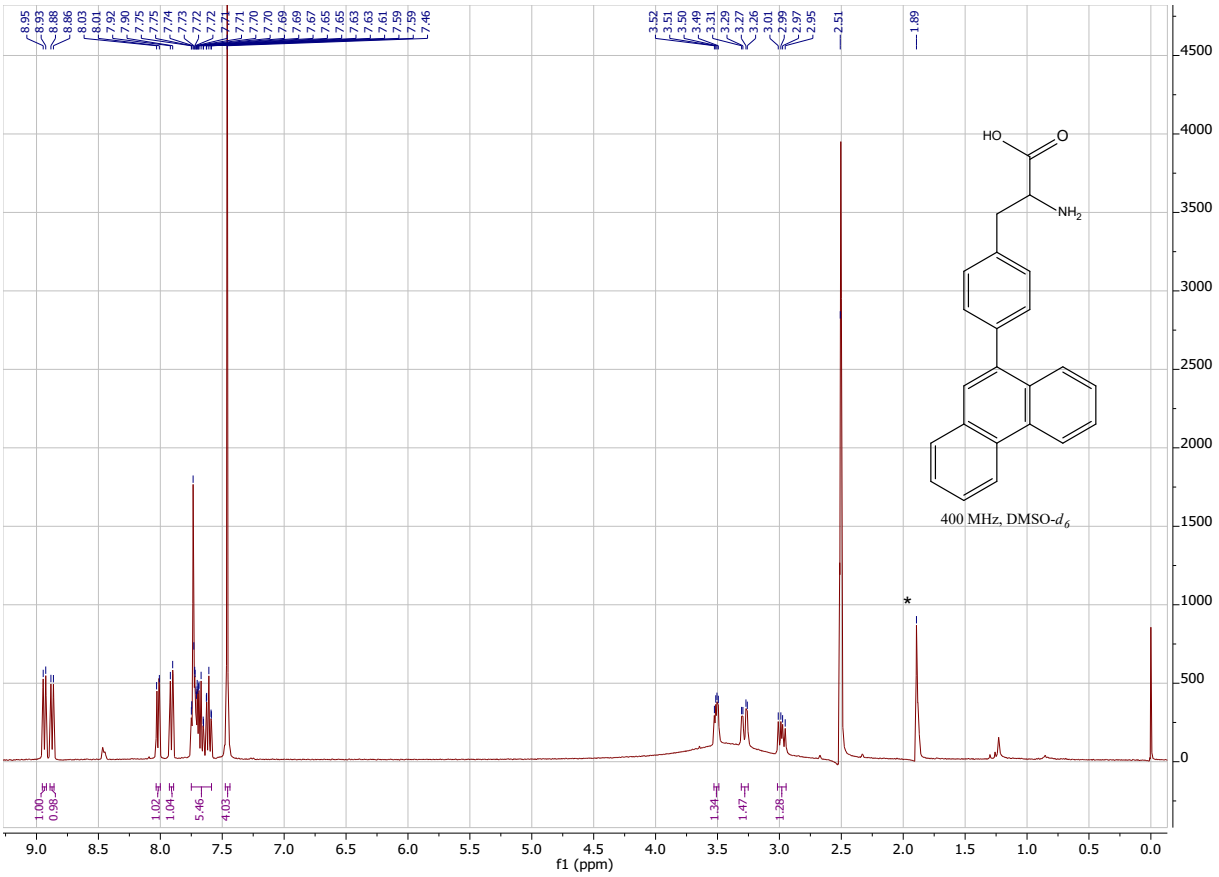


Figure S2. ^{1}H NMR spectrum of 4-phenanthracen-9-yl-L-phenylalanine {2-amino-3-(4-(phenanthren-9-yl) phenyl) propanoic acid}(**5**)

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.94 (d, J = 8.2 Hz, 1H), 8.87 (d, J = 8.1 Hz, 1H), 8.02 (d, J = 9.0 Hz, 1H), 7.91 (d, J = 7.5 Hz, 1H), 7.75 – 7.59 (m, 5H), 7.46 (s, 4H), 3.51 (m, 1H), 3.28 (dd, J = 14.1, 1H), 2.98 (dd, J = 14.1, 1H).

The peaks from δ 8.94- δ 7.58 are aromatic hydrogens from the fluorescent amino acid (**5**). The peak at δ 3.51 is from –C**H**-(COOH)-NH_2 and peaks δ 3.28 and δ 2.98 is from two H–C–**H**- protons at different electronic environment of the fluorescent amino acid (**5**). The –COOH hydrogen was not seen probably due to exchanging with deuterated solvent. The peak at δ 2.51 is DMSO-d_6 solvent residual peak and trace impurity peak (*) of acetic acid δ 1.89 is present [6,7].

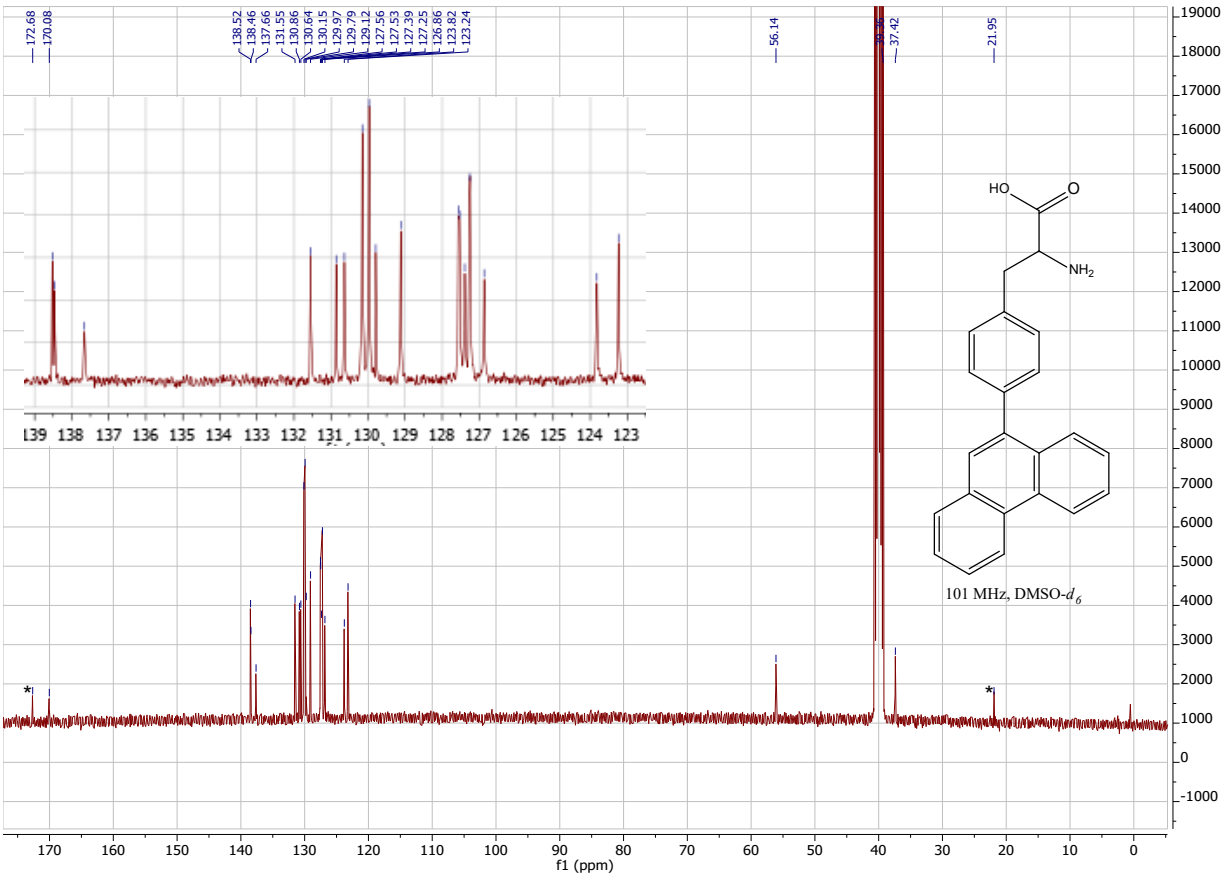


Figure S3. ^{13}C NMR spectrum of 4-phenanthracen-9-yl-L-phenylalanine {2-amino-3-(4-(phenanthren-9-yl) phenyl) propanoic acid}(**5**)

^{13}C NMR (101 MHz, DMSO- d_6): δ 172.68, 170.08, 138.52, 138.46, 137.66, 131.55, 130.86, 130.64, 130.15, 129.97, 129.79, 129.12, 127.56, 127.53, 127.39, 127.25, 126.86, 123.82, 123.24, 56.14, 39.36, 37.42, 21.95.

The weak ^{13}C NMR peak at δ170.08 is from the –COOH group of the fluorescent amino acid (**5**). The peaks from δ 138.52-123.24 are aromatic ring carbons of the fluorescent amino acid (**5**). The peak at δ 37.46 is from –**C**H$_2$– and peak at δ 56.14 is from –**C**H-(COOH)-NH$_2$ of the fluorescent amino acid. The peak at δ 39.36 is DMSO-d_6 solvent residual peak. The trace impurity peaks (*)

of acetic acid (δ 21.95 and δ 172.68)[6,7] were observed in ^{13}C NMR. The trace amount of acetic acid impurity was also seen in the ^{1}H NMR spectrum (Fig. S2).

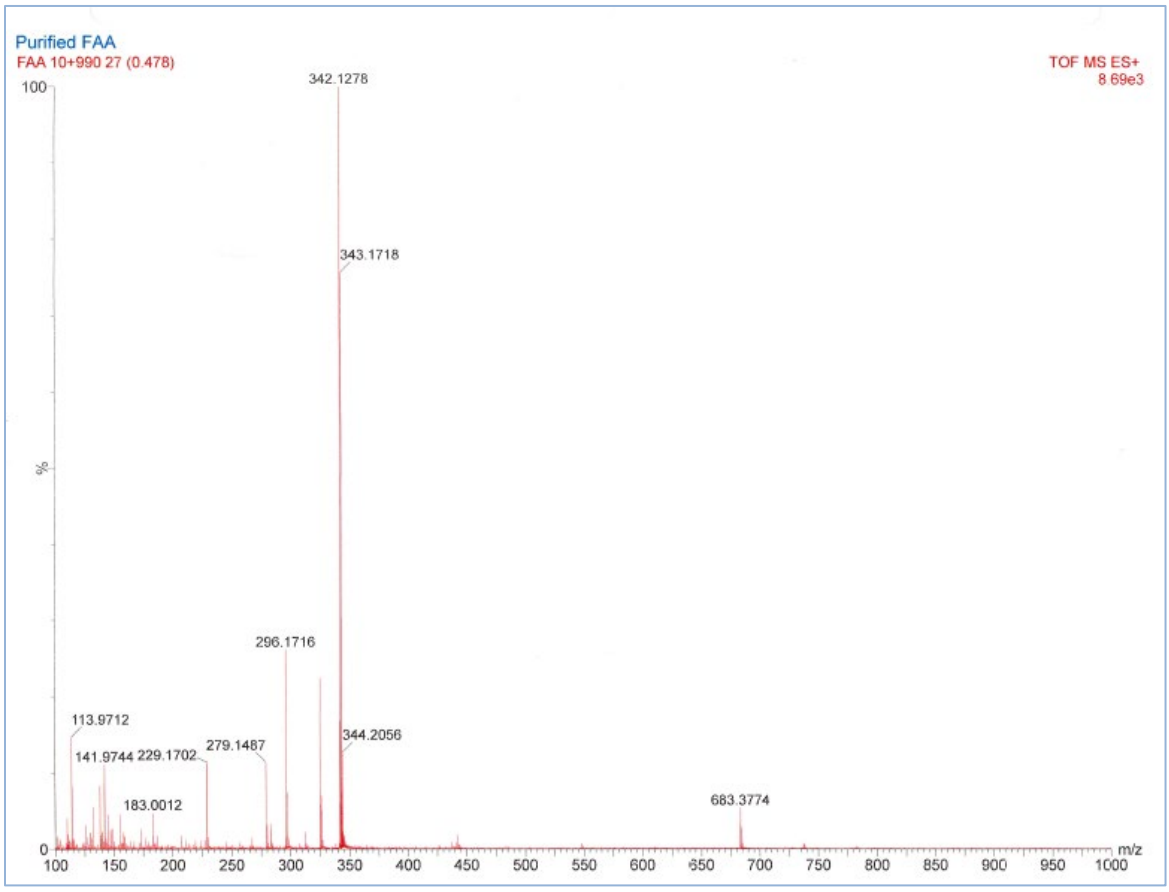


Figure S4. HRMS (ESI) spectrum of 4-phenanthracen-9-yl-L-phenylalanine {2-amino-3-(4-(phenanthren-9-yl) phenyl) propanoic acid}(**5**)

HRMS (ESI): *m/z* calculated for $[C_{23}H_{19}NO_2 + H]^+$: 342.1416 found: *m/z*: 342.1278,

[Dimer at m/z 683.3774]

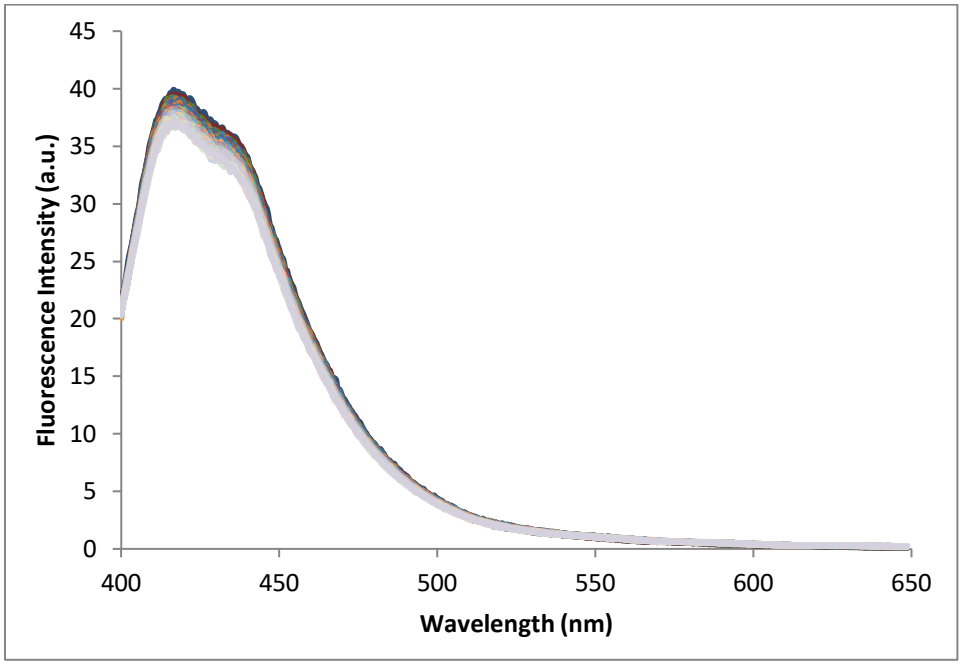


Figure S5. Repeated scan of 100 fluorescence spectra (λ_{ex}=380 nm, excitation slit 15 nm and emission slit 2.5 nm) of 4-phenanthracen-9-yl-L-phenylalanine (**5**) (0.001 M) over a 5 h period in DMSO-SPB buffer (1:1, pH 7.0, 0.05 M). Little decrease in fluorescence intensity (~ 6.7% at 421 nm peak) was observed during the 5 h period, demonstrating that the Phen-AA is resistant to photo-bleaching under the condition.

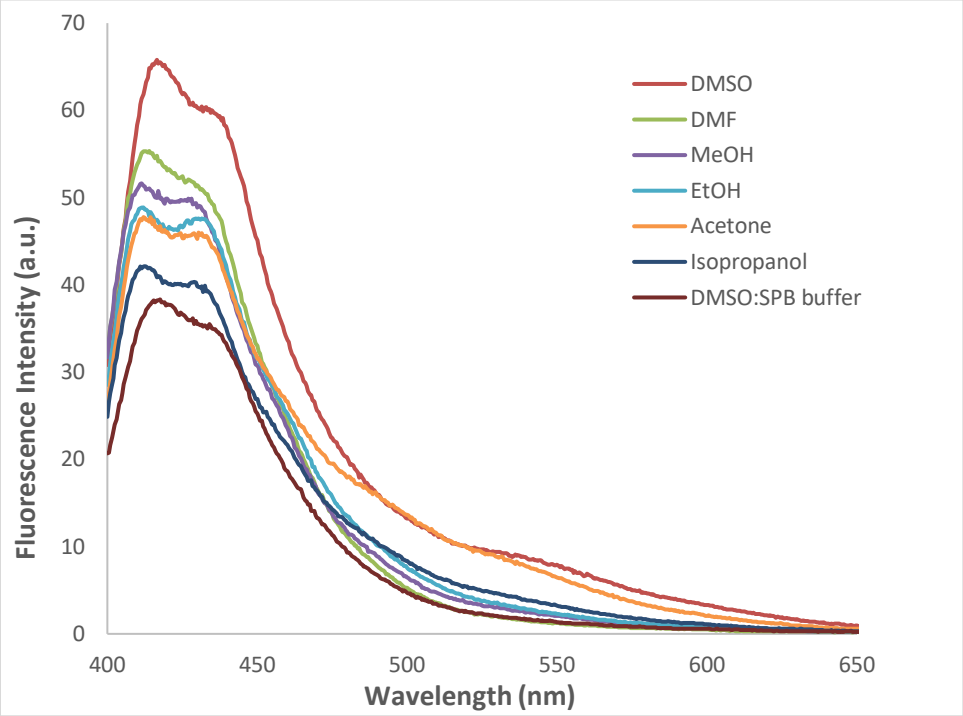


Figure S6. Fluorescence spectra (λ_{ex}=380 nm, excitation slit 15 nm and emission slit 2.5 nm) of 4-phenanthracen-9-yl-L-phenylalanine (**5**) in various solvents.

4. References

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