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Supporting Information

A novel cyan-emitting fluorescent α-amino acid: synthesis, photophysical characterization and live-cell imaging properties

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S1. Synthesis and characterization of 4-dibenzothiophen-4-yl-*L*-phenylalanine (DBT-FAA)

Step 1: Synthesis of N-(tert-Butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester intermediate (Boc-Phe(4-I)-OMe) (2)

The N-(*tert*-Butoxycarbonyl)-4-iodo-*L*-phenylalanine (**1**) (502 mg, 1.28 mmol) and $NaHCO_3$ (324 mg, 3.86 mmol) were transferred to a 100 mL RBF. The flask was then vacuumed and purged with Argon gas three times repeatedly. To this, 12 mL of DMF and then methyl iodide (~400 µL, 6.4 mmol) was added to the flask through septum with the help of syringe and allowed to reflux at 64℃ for 22 hrs. The reaction mixture was neutralized with 3N HCl and then extracted with ethyl acetate and water and finally dried with 10 mL brine solution. The extracted fraction was dried over Na_2SO_4 to completely remove the traces of water. The product was concentrated on Roto-Vap and purified by silica gel flash chromatography using hexane: ethyl acetate solvent gradient of 9:1-8:2, yielding white fibrous solid (471 mg) product (N-(*tert*-Butoxycarbonyl)-4-iodo-*L*-phenylalanine methyl ester) (**2**). **HRMS (ESI):** *m/z* calculated for $[C_{15}H_{20}INO_4 + H]^+$: 406.0437 found: *m/z*: 406.0325

Step 2: Cross-coupling reaction of N-(tert-Butoxycarbonyl)-4-iodo-L-phenylalanine methyl ester (2) with 4-Dibenzothiophenyl boronic acid (3) to form N-(Boc)-4-(4-dibenzothiophenyl)-L-phenylalanine methyl ester (4)

The catalyst $Pd(PPh_3)_2Cl_2$ (~18 mg, approx. 10 mol% of Boc-Phe(4-I)-OMe) was transferred to a 100 mL RBF, which was vacuum and then purged with Argon gas three times repeatedly. Then, Boc-Phe(4-I)-OMe (102 mg, 0.251 mmol) dissolved in 4 mL of DMF was added and allowed to react at 58℃ for 1.5 hrs. After this, 4-dibenzothiophenyl boronic acid (114

mg, 0.503 mmol) dissolved in 4 mL of DMF was added to the flask and allowed to react for 10-12 minutes. Finally, to this mixture Cs_2CO_3 (164 mg, 0.503 mmol) dissolved in 4 mL of H_2O was added. The solution changed from yellow to brownish black. The temperature was then elevated to 80℃ and allowed to react for 21 hrs under the flow of Argon gas. The reaction mixture was neutralized with 3 N HCl and then extracted with ethyl acetate and water and finally dried with 5 mL of brine solution. The product was dried over Na_2SO_4 and concentrated on a Roto-Vap. This crude product was directly employed to de-protection in Step 3 without further purification. Here, the DMF showed progressively a better solvent than 1, 4-dioxane towards Suzuki cross-coupling reaction, as the reaction was monitored on TLC after completion, offering no additional side product which is also supported by high yield of the final product. **HRMS (ESI):** *m/z* calculated for $[C_{27}H_{27}NO_4S + H]^+$: 461.1661 found: *m/z*: 461.1069

Step 3: De-protection of Boc and methyl protecting groups of N-(Boc)-4-(4-dibenzothiophenyl)-L-phenylalanine methyl ester (4) to synthesize the final product (5)

Here, first the methyl moiety was de-protected using methanolic KOH solution where 2 mL of 2 N alcoholic KOH solution was added to the crude coupled product and allowed to stir at 37℃ for 8 hrs. The solvent mixture in the crude product was evaporated in a Roto-Vap, cooled on ice-water bath and the remaining KOH was neutralized with 3 N HCl until the pH on litmus paper was indicated to acidic side. The crude product was then extracted with ethyl acetate and water and finally with 5 mL of brine solution. Then, it was dried over Na_2SO_4 to completely remove the traces of water, and the product was concentrated using a Roto-Vap. In the second de-protection step, the Boc protection group was removed using TFA. Here, 2 mL of TFA:DCM (1:1) was added to the crude product flask and allowed to stir for 4 hours at 28℃. The solvents were removed in Roto-Vap and the crude product was then purified by silica gel flash

chromatography using hexane: ethyl acetate (80:20) solvent gradient to water:methanol:acetic acid (3:1:1) solvent gradient system. The collected product was neutralized with 10% aqueous $NaHCO_3$ to remove the acetic acid. The product was dried in vacuum filtration to get the purified product (yield: 47 mg, ~54 %). Here, the product was purified over the silica column without the need of a previously used preparatory HPLC, further simplifying the synthetic methodology. **HRMS (ESI)**: *m/z* calculated for $[C_{21}H_{17}NO_2S + H]^+$: 348.0980 found: *m/z*: 348.0461 and a dimer at m/z 695.1710) (**Fig. S3**)

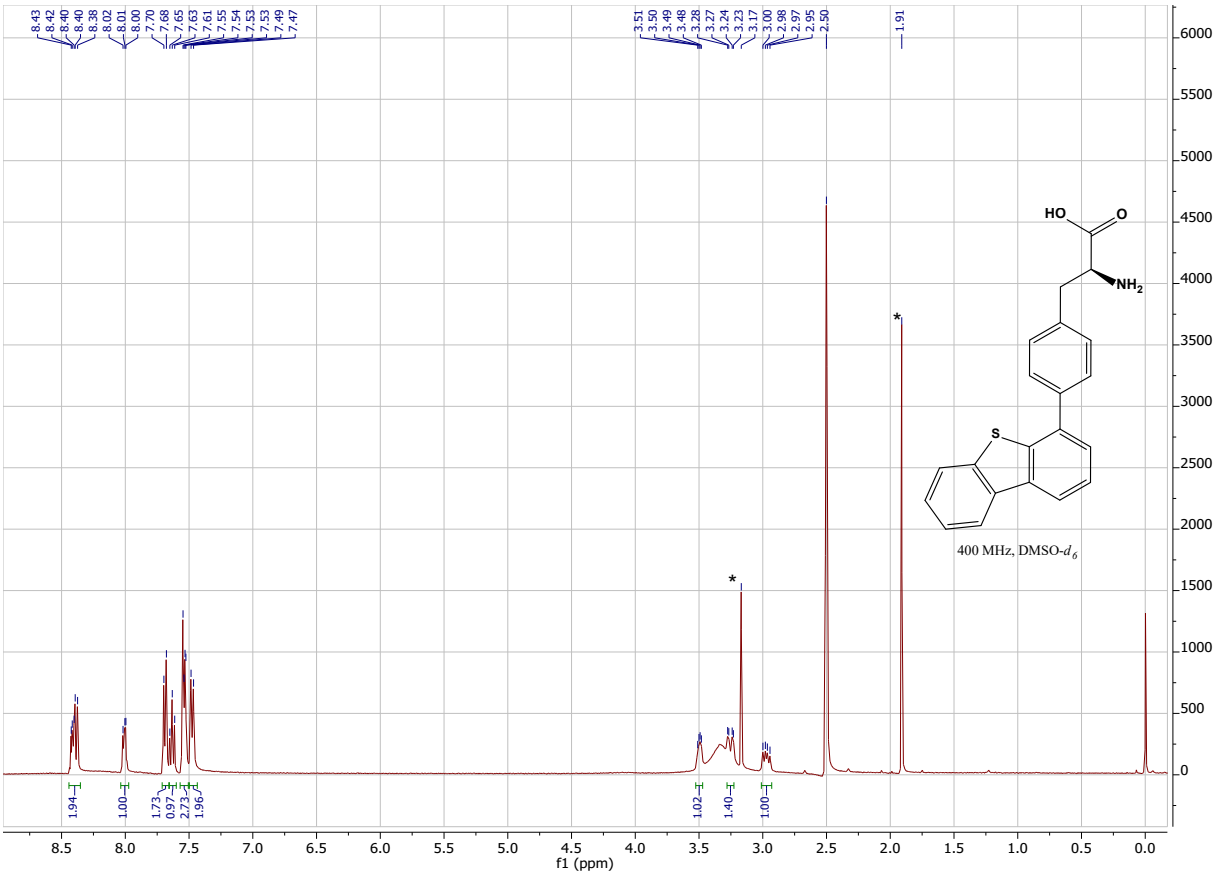


Figure S1. ^{1}H NMR spectrum of 4-dibenzothiophen-4-yl-L-phenylalanine {(S)-2-amino-3-(4-(dibenzo[b,d]thiophen-4-yl)phenyl) propanoic acid}(**5**)

^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ 8.44 – 8.35 (m, 2H), 8.04 – 7.98 (m, 1H), 7.69 (d, J = 7.9 Hz, 2H), 7.63 (t, J = 7.6 Hz, 1H), 7.55-7.543 (m, 3H), 7.48 (d, J = 7.9 Hz, 2H), 3.50 (m, 1H), 3.26 (dd, J = 14.3, 3.7 Hz, 1H), 2.97 (dd, J = 14.2, 7.9 Hz, 1H).

The chemical shifts values from δ 8.44 - δ 7.48 represent the aromatic protons of fluorescent 4-dibenzothiophen-4-yl-L-phenylalanine (**5**). Similarly, the chemical shift at δ 3.5 represents –C**H**-(COOH)-NH$_2$ and while chemical shifts at δ 3.26 and δ 2.97 represent two H–C–**H**- protons at different electronic environment of the 4-dibenzothiophen-4-yl-L-phenylalanine (**5**). The chemical shift at δ 2.50 represents DMSO-*d*$_6$ solvent peak whereas chemical shifts at δ 1.91

and δ 3.17 represents acetic acid and methanol respectively as trace solvent residue peaks (*)[1,2] as methanol and acetic acid were used in the final step of chromatographic purification.

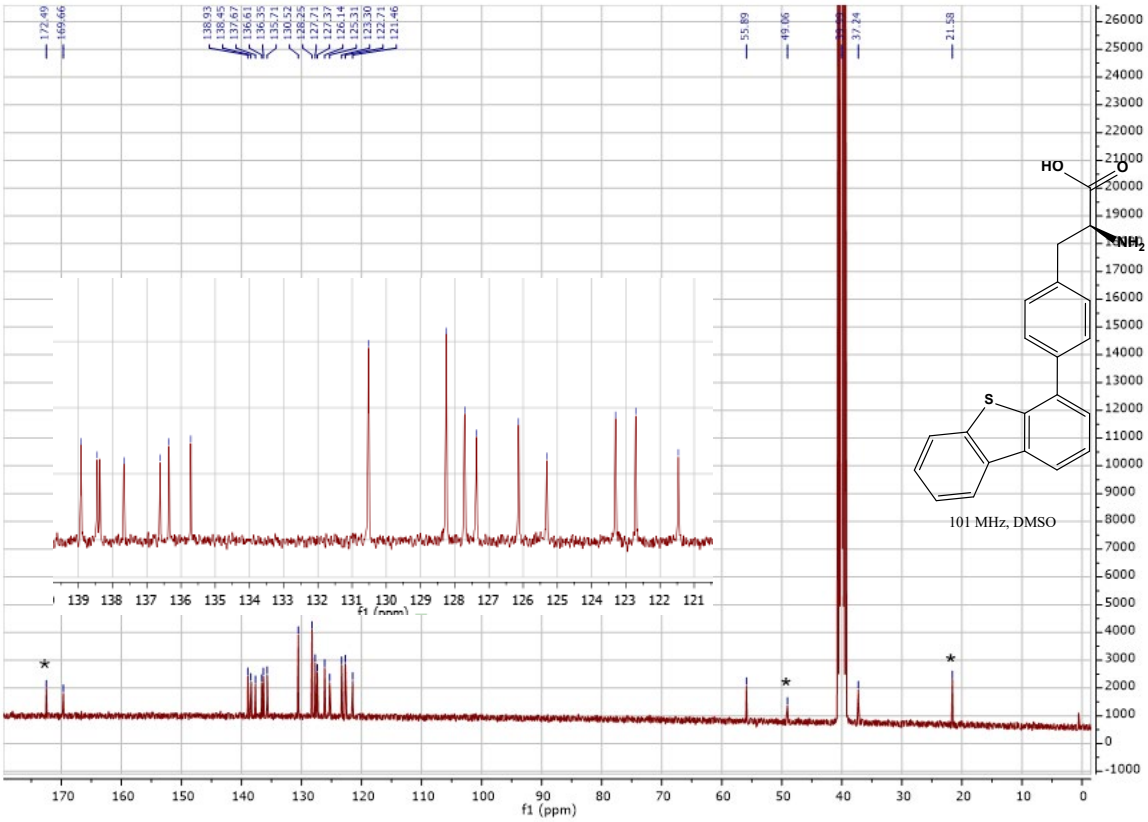


Figure S2. ^{13}C NMR spectrum of 4-dibenzothiophen-4-yl-L-phenylalanine {(S)-2-amino-3-(4-(dibenzo[b,d]thiophen-4-yl)phenyl) propanoic acid}(**5**)

^{13}C NMR (101 MHz, DMSO): δ 172.49(*), 169.66, 138.93, 138.45, 137.67, 136.61, 136.35, 135.71, 130.52, 128.25, 127.71, 127.37, 126.14, 125.31, 123.30, 122.71, 121.46, 55.89, 49.06(*), 39.99, 37.24, 21.58(*).

The chemical shift at δ 169.66 in ^{13}C NMR represents the –**C**OOH group of the fluorescent 4-dibenzothiophen-4-yl-L-phenylalanine (**5**). The chemical shifts from δ 138.93- δ 121.46 represents aromatic carbons, chemical shift at δ 55.89 represents –**C**H-(COOH)-NH$_2$ while δ 37.24

represents –**C**H$_2$– of 4-dibenzothiophen-4-yl-L-phenylalanine (**5**). The chemical shift at δ 39.99 represents DMSO solvent peak. The trace solvent residue peaks (*) of acetic acid and methanol which were seen in ^{1}H NMR, were also observed in ^{13}C NMR chemical shifts at δ 21.58, δ 172.49 (acetic acid) and δ 49.06 (methanol), respectively.[1,2]

Notes

1. G. R. Fulmer, A. J. M. Miller, N. H. Sherden, H. E. Gottileb, A. Nudelman, B. M. Stoltz, J. E. Bercaw, and K. I. Goldberg, *Organometallics* 2010, **29**, 2176.
2. N. R. Babij, E. O. McCusker, G. T. Whiteker, B. Canturk, N. Choy, L. C. Creemer, C. V. De Amicis, N. M. Hewlett, P. L. Johnson, J. A. Knobelsdorf, F. Li, B. A. Lorsbach, B. M. Nugent, S. J. Ryan, M. R. Smith, and Q. Yang, *Org. Process Res. Dev.* 2016, **20**, 661.

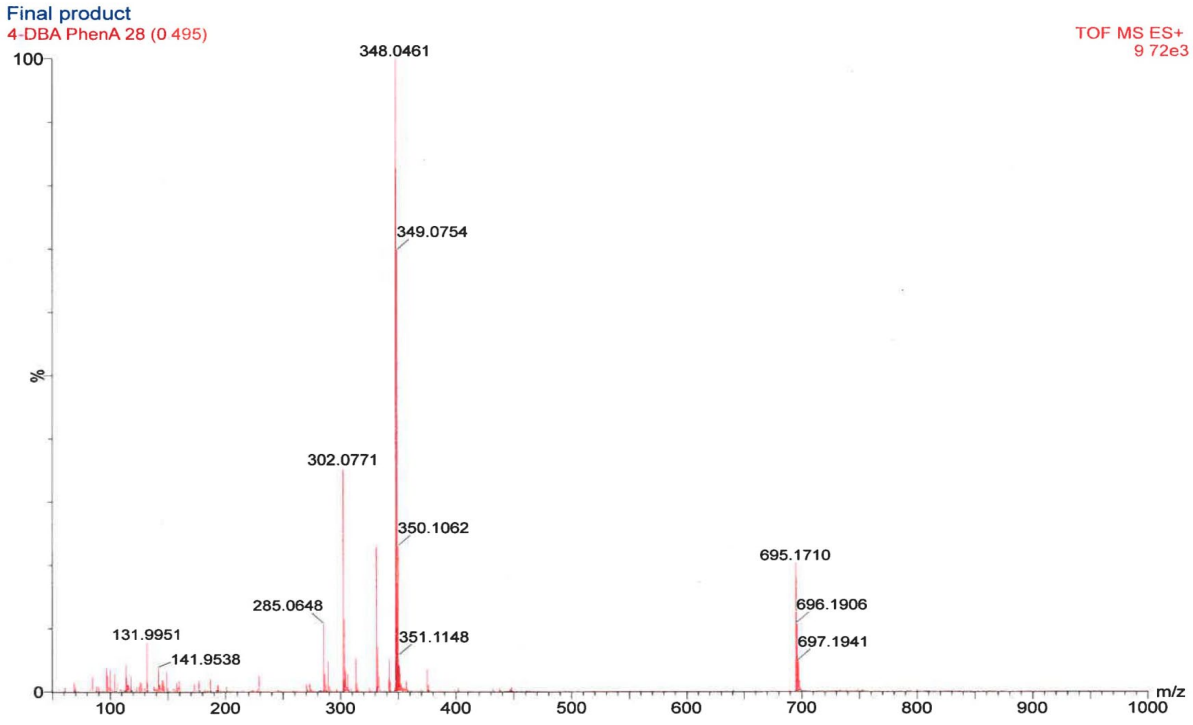


Figure S3. HRMS (ESI) spectrum of 4-dibenzothiophen-4-yl-L-phenylalanine.

S.2 Quantum yield

The quantum yield of DBT-FAA was determined using William's comparative method. Rhodamine B in ethanol with a known quantum yield (Φ_{ST} = 0.68) was used as the reference standard. Solutions for the fluorescent amino acid 4-dibenzothiophen-4-yl-L-phenylalanine (**5**) (DBT-FAA) were prepared in dimethyl sulfoxide (DMSO).

Fluorescence emission spectra for Rhodamine B were carried out with excitation and emission wavelengths set at 420 nm and 450-800 nm, respectively, and while excitation and emission wavelengths set at 380 nm and 400-800 nm, respectively, were used for 4-dibenzothiophen-4-yl-*L*-phenylalanine. The plots of the baseline corrected integrated fluorescence intensity vs. the absorbance for each concentration of both samples were plotted and the gradients (positive slopes) of the linear fit were determined. The gradients obtained from the integrated fluorescent intensity data and refractive index (η) of the solvents were used to calculate quantum yield of the unknown DBT-FAA using the equation given below. The quantum yield of -dibenzothiophen-4-yl-*L*-phenylalanine (Φx) was found to be 0.74.

$\Phi_X = \Phi_{ST} (Grad_X / Grad_{ST}) (\eta_x^2 / \eta_{ST}^2)$

where, Grad*x* is the gradient for the unknown (DBT-FAA),

$Grad_{ST}$ is the gradient for the Rhodamine B standard,

η_x is the refractive index of the dimethyl sulfoxide (1.479), &

η_{ST} is the refractive index of the ethanol (1.363)

$= 0.68 (34.769 / 37.923) (1.479^2 / 1.363^2)$

$= 0.7368$

$\Phi_X = 0.74$

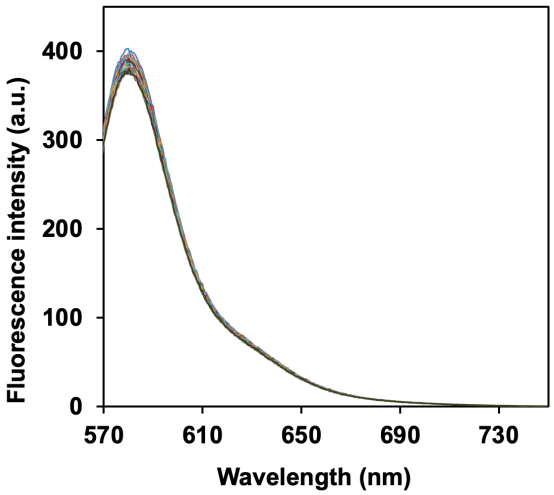


Figure S4. Repeated scans of 100 fluorescence emission spectra (λ_{ex}=550 nm, emission range = 570-750 nm, excitation slit = 15 nm, emission slit = 2.5 nm, scan speed = 250 nm/min) were recorded for Rhodamine B (0.2 μM) over a 5-hour period in DMSO-SPB buffer mixture (1:1, pH 7.0, 0.05 M). Only a minimal decrease in fluorescence intensity was observed, with an approximately 6.08% reduction at 580 nm over the 5-hour duration.

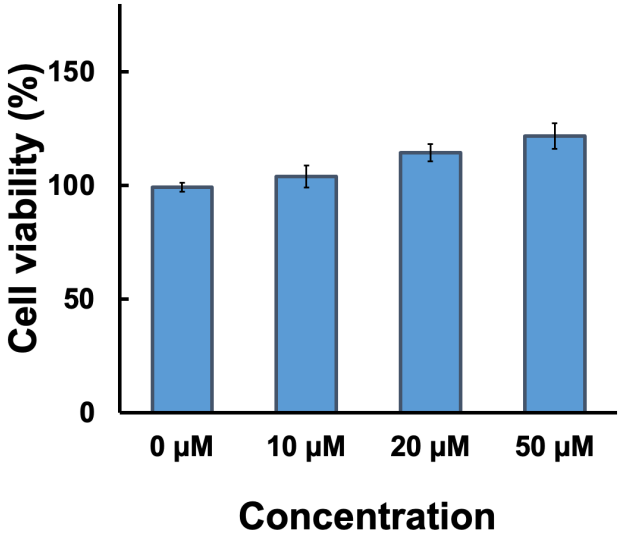


Figure S5. Cell viability of WS1 cells treated with DBT-FAA assessed by an MTT assay. WS1 cells were exposed to DBT-FAA (0–50 μM; 0.5% DMSO) for 24 h. Absorbance was measured at 570 nm with background correction at 630 nm, and signals from cell-free wells were subtracted. Cell viability was normalized to the 0 μM control (100%). Data are presented as mean ± SD (n = 3).

Table S1 A summary of the fluorescent properties of DBT-FAA in different solvents

#	Solvent	Excitation/ emission slits (nm)	λ_{Ex} (nm)	λ_{Em} maximum (nm)	λ_{Em} shoulders (nm)	Quantum yield
1	DMSO	15/ 2.5	380	425	452, 485, 540	0.74
2	Tris–HCl buffer (pH 8.8)	15/ 2.5	380	445	520, 582	
3	DMSO-SPB buffer (1:1, pH 7.0,	15/ 2.5	380	424	403, 448, 475, 513, 540	
4	0.01 mM NaOH (pH 9.0)	15/ 2.5	380	432	454, 484, 550, 582	
5	0.02 mM NaOH (pH 9.3)	15/ 2.5	380	431	454, 484, 550, 583	
6	water	15/ 2.5	380	433	460, 496, 580	
7	isopropanol	15/ 2.5	380	433	484, 555	
8	methanol	15/ 2.5	380	433	484, 550	
9	acetone	15/ 2.5	380	433	484, 532	
10	DMF	15/ 2.5	380	434	484, 548	

S.3 Additional cell imaging data

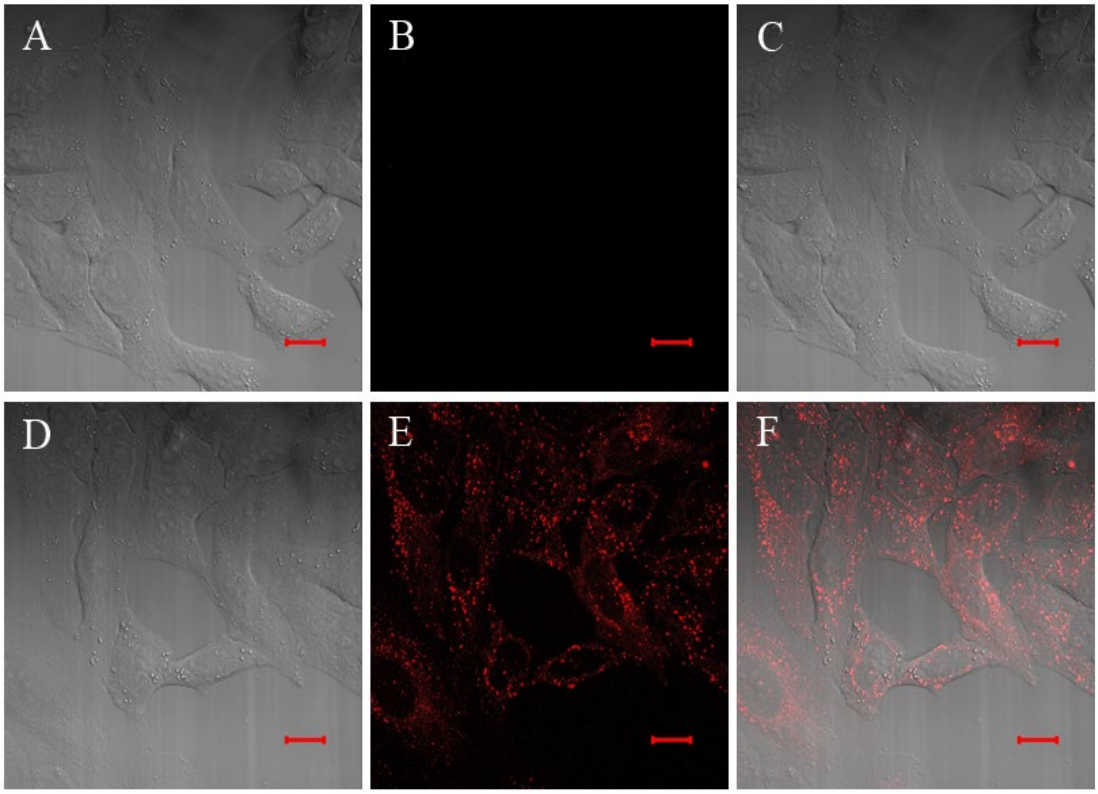


Figure S6 Confocal images of Hella cells treated or untreated with DBT-FAA. (A–C) Control Hela cells without dye treatment. (D–F) Hela cells treated with DBT-FAA at a final concentration of 10 μM. (A) and (D) were bright field images; (B) and (E) were fluorescent images after excitation at 405 nm and emission collected between 410-587 nm; (C) and (F) were merged bright-field and fluorescence signals. The fluorescence signals (red) in (E) indicate that DBT-FAA readily permeates live cells and exhibits strong intracellular fluorescence. Scale bar: 20 μm.

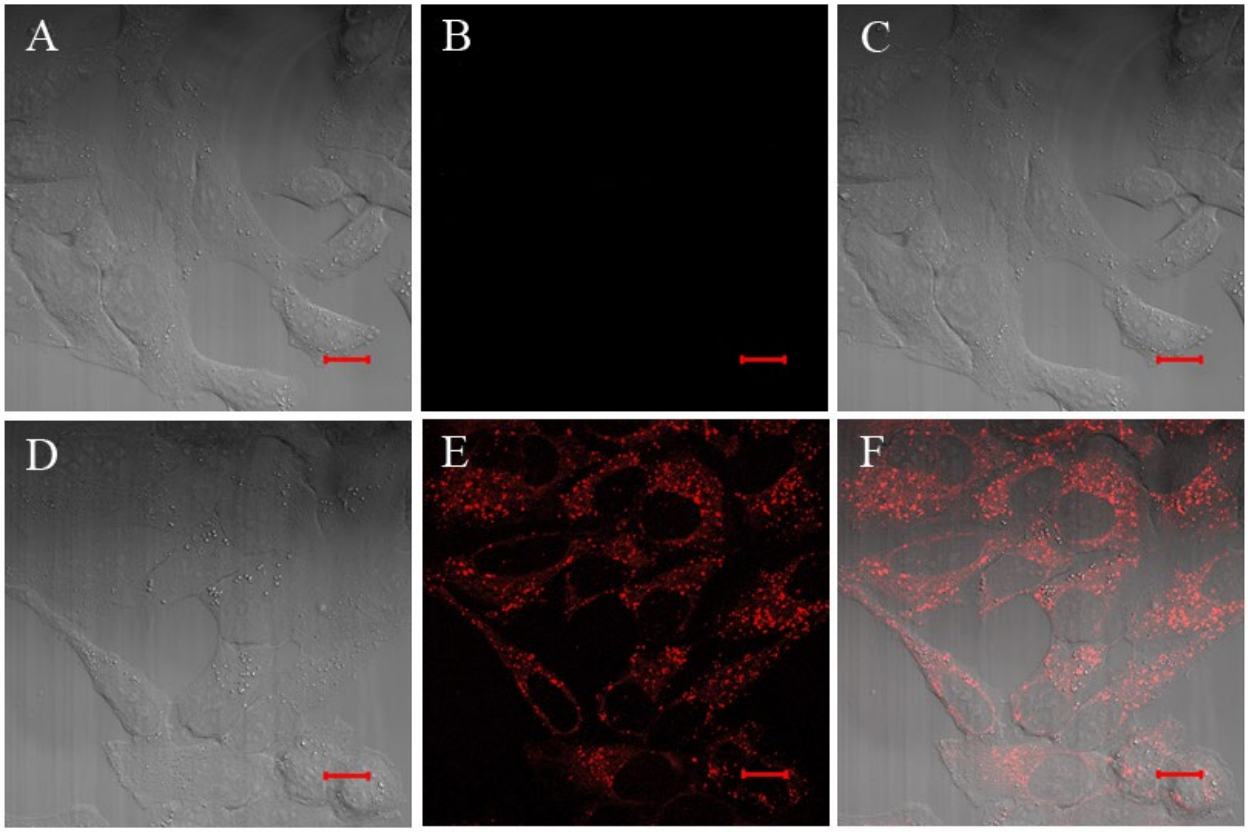


Figure S7 Confocal images of Hella cells treated or untreated with DBT-FAA. (A–C) Control Hela cells without dye treatment. (D–F) Hela cells treated with DBT-FAA at a final concentration of 50 μM. (A) and (D) were bright field images; (B) and (E) were fluorescent images after excitation at 405 nm and emission collected between 410-587 nm; (C) and (F) were merged bright-field and fluorescence signals. The fluorescence signals (red) in (E) indicates that DBT-FAA readily permeates live cells and exhibits strong intracellular fluorescence. Scale bar: 20 μm.

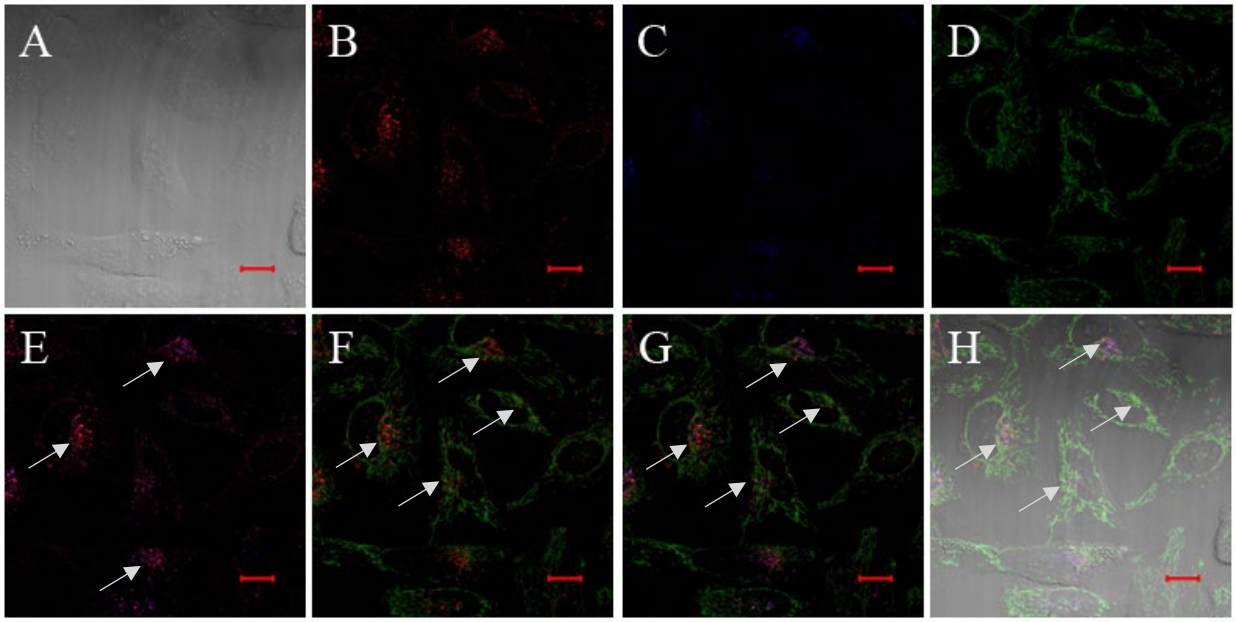


Figure S8 Confocal images of Hela cells treated with DBT-FAA (red) for 90 min, followed by incubation with mitoTracker (green) and lysoTracker (blue) for 30 min prior to imaging. (A) Bright-field image of cells; (B) fluorescence image of mitochondria (excitation at 488 nm. emission 493-620 nm); (C) fluorescent image of lysosomes (excitation at 543 nm, emission 566-690 nm); (D) fluorescent images of DBT-FAA (excitation at 405 nm, emission 410-587 nm). (E, F) Co-localization of DBT-FAA with mitochondrial and lysosomal fluorescent signals, respectively; (G) merged fluorescent image showing DBT-FAA, mitochondria, and lysosomes; (H) merged fluorescent image of DBT-FAA, mitochondria, and lysosomes overlaid with the bright-field view. Scale bar: 20 μm.

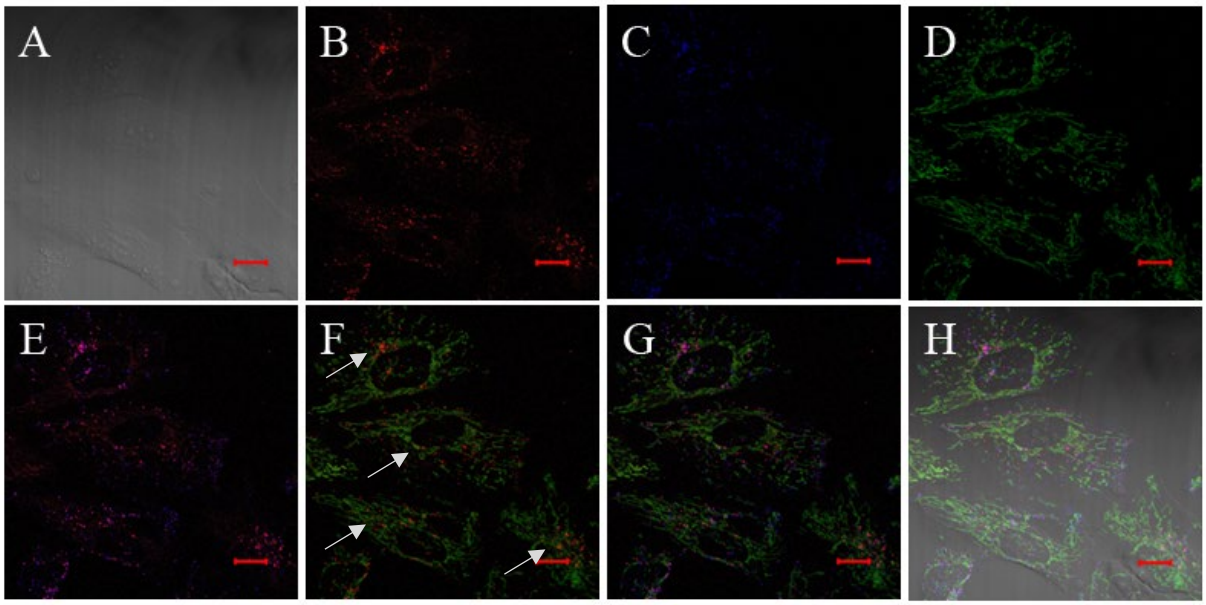


Figure S9 Confocal images of Hela cells treated with DBT-FAA (red) for 6 h, followed by incubation with mitoTracker (green) and lysoTracker (blue) for 30 min prior to imaging. (A) Bright-field image of cells; (B) fluorescence image of mitochondria (excitation at 488 nm. emission 493-620 nm); (C) fluorescent image of lysosomes (excitation at 543 nm, emission 566-690 nm); (D) fluorescent images of DBT-FAA (excitation at 405 nm, emission 410-587 nm). (E, F) Co-localization of DBT-FAA with mitochondrial and lysosomal fluorescent signals, respectively; (G) merged fluorescent image showing DBT-FAA, mitochondria, and lysosomes; (H) merged fluorescent image of DBT-FAA, mitochondria, and lysosomes overlaid with the bright-field view. Scale bar: 20 μm.

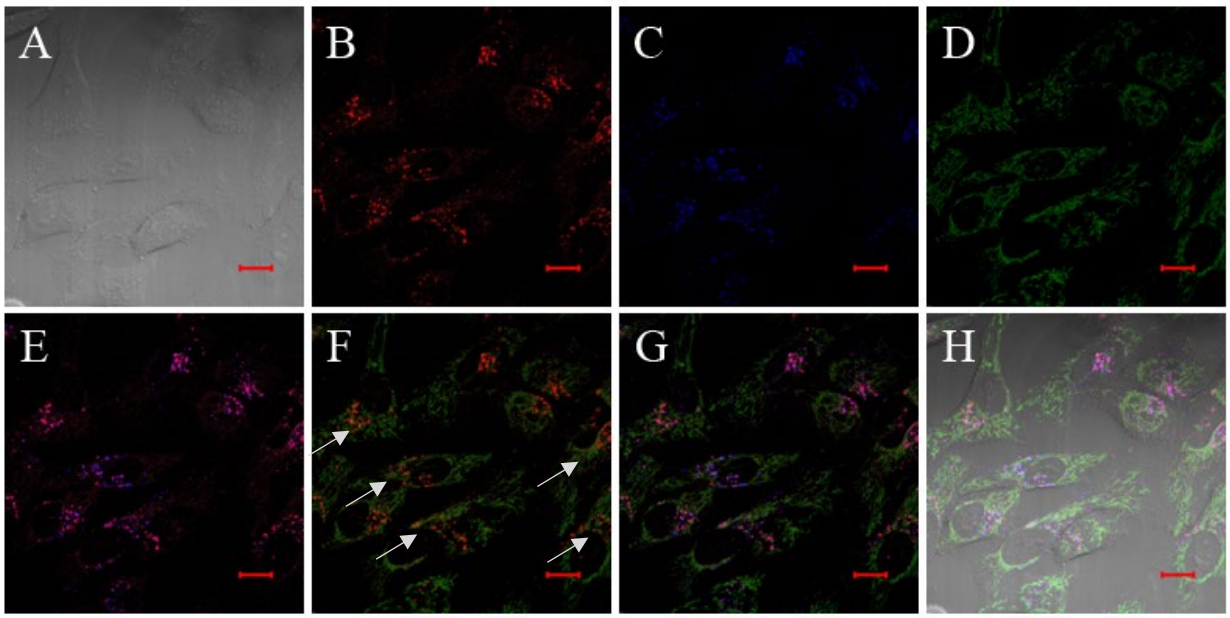


Figure S10 Confocal images of Hela cells treated with DBT-FAA (red) for 12 h, followed by incubation with mitoTracker (green) and lysoTracker (blue) for 30 min prior to imaging. (A) Bright-field image of cells; (B) fluorescence image of mitochondria (excitation at 488 nm. emission 493-620 nm); (C) fluorescent image of lysosomes (excitation at 543 nm, emission 566-690 nm); (D) fluorescent images of DBT-FAA (excitation at 405 nm, emission 410-587 nm). (E, F) Co-localization of DBT-FAA with mitochondrial and lysosomal fluorescent signals, respectively; (G) merged fluorescent image showing DBT-FAA, mitochondria, and lysosomes; (H) merged fluorescent image of DBT-FAA, mitochondria, and lysosomes overlaid with the bright-field view. Scale bar: 20 μm.

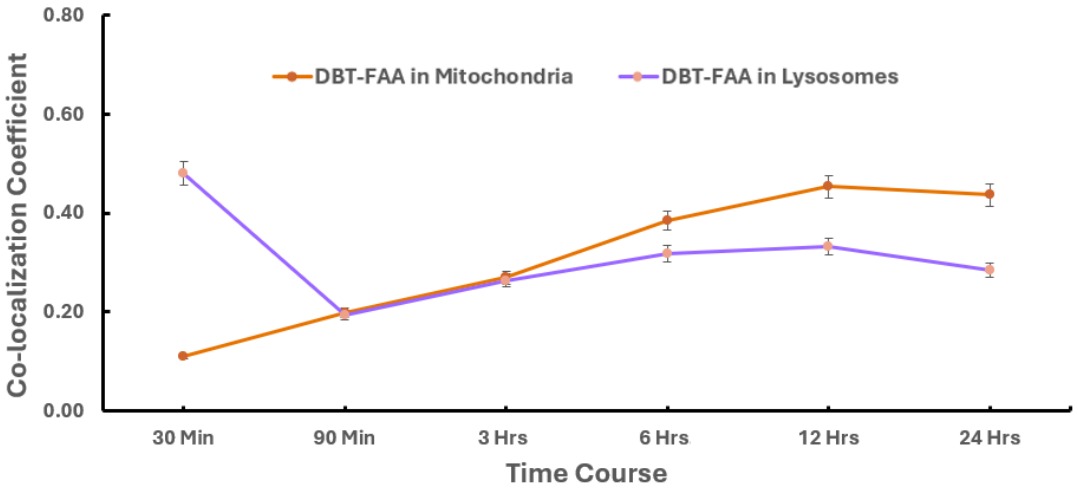
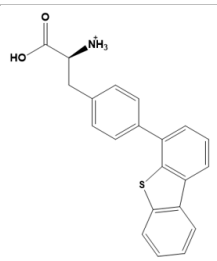
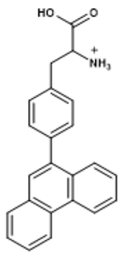
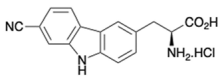
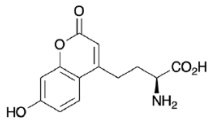
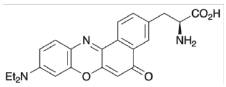
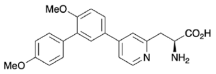
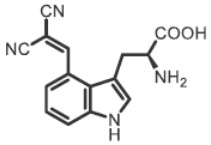
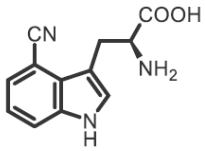
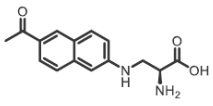
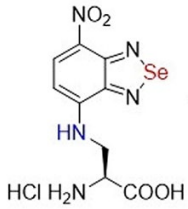


Figure S11 Quantitative analysis of the colocalization signals in the confocal images showing the distributions of DBT-FAA in mitochondria and lysosomes over time (from 30 min to 24 hours).

Table S2. Comparison of the photophysical properties and applications of representative visible-emitting fluorescent amino acids with DBT-FAA

#	FAA structure	Name or abbreviation	Absorption (nm)/extinction coefficient (cm^{-1} M^{-1})	Excitation /Emission wavelength (nm)	Quantum yield	Bioimaging applications	Reference
1		4-dibenzothiophen-4-yl-*L*-phenylalanine (DBT-FAA)	λ_{ab}=261(45700), λ_{ab}=301(14100), λ_{ab}=334(1000), λ_{ab}=352(79600), λ_{ab}=382(169)	λ_{ex}=380, λ_{em}=425	0.74 (DMSO)	Hela cell imaging	This work
2		4-phenanthracen-9-yl-Lphenylalanine (Phen-AA)	λ_{ab}=258(62100), λ_{ab}=301(15600), λ_{ab}=334(785), λ_{ab}=351(551)	λ_{ex}=334, λ_{em}=359, 375, 400, 445 (shoulder)	0.75 (DMSO)	Hela cell imaging	1
3		7-cyanocarbazole amino acid	λ_{ab}=361(19600)	λ_{em} =419	0.37 (H_2O)	incorporation into peptide monitor binding to a WW domain protein	2
4		L-(7-hydroxycoumarin-4-yl) ethylglycine (CouAA)	λ_{ab}=360 (17000)	λ_{em} =456	0.63 (phenolate form in sodium phosphate buffer)	genetically encoded protein incorporation in *E. coli*	3

5		Alared	λ_{ab}=552 λ_{ab}=596	λ_{em} =638 λ_{em} =665	0.59 (DMSO) 0.06 (H_2O)	*in vitro* characterization of biomolecular interactions, cellular ratiometric confocal imaging	4
6		(2S)-2-Amino-3-[4'-(3''-(naphthalen-2'''-yl)-4''-methoxyphenyl)pyridin-2'-yl]propanoic acid	λ_{ab}=304(19304)	λ_{em} =445	0.13 (MeOH)	viscosity probe	5
7		4-dicyanovinyltrytophan	λ_{ab}=426	λ_{em} =580	0.03 (H_2O)	none	6
8		4-cyanotryptophan	λ_{ab}=325 (6000)	λ_{em} =420	0.88 (H_2O)	incorporated into peptides and applied in cell imaging	7
9		3-((6-acetylnaphthalen-2-yl)amino)-2-aminopropanoic acid (ANAP)	λ_{ab}=360(17500)	λ_{em} =490	0.48 (H_2O)	genetically encoded fluorescent probe	8

10		benzoselenadiazole amino acid	λ_{ab}=488(4480)	λ_{em} =601	0.19 (DMSO)	solid-phase synthesis of fluorescent peptides and peptide-PAINT imaging	9

References

1 Gupta, A., Garreffi, B. P. & Guo, M. Facile synthesis of a novel genetically encodable fluorescent α-amino acid emitting greenish blue light. *Chemical Communications* **56**, 12578-12581 (2020). https://doi.org/10.1039/D0CC03643A

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