Supplementary Material

Synthesis and characterization of fluorescent amino acid dimethylaminoacridonylalanine

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General Information

**Materials.** *E. coli* BL21 DE3 cells were purchased from Agilent (Santa Clara, CA, USA). Milli-Q filtered (18 MΩ) water was used for all solutions (Millipore; Billerica, MA, USA). Methyl 2-((tert-butoxycarbonyl)amino)-3-((trifluoromethyl)sulfonyl)oxy)phenyl)propanoate was synthesized as previously described using Boc-L-tyrosine methyl ester, purchased from ChemImpex (Wood Dale, IL, USA), and BrettPhos Pd G1 methyl t-butyl ether adduct, purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma-Aldrich unless otherwise specified.

**Instruments.** Low resolution electrospray ionization mass spectra (LRMS) were obtained on a Waters Acquity Ultra Performance LC connected to a single quadrupole detector (SQD) mass spectrometer (Milford, MA, USA). High resolution electrospray ionization mass spectra (HRMS) were collected with a Waters LCT Premier XE liquid chromatograph/mass spectrometer. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX 500 MHz instrument (Billerica, MA, USA). Absorbance measurements were performed on a ThermoScientific Genesys 150 UV-Vis spectrometer (Waltham, MA, USA). Fluorescence readings for the incorporation assay and molar absorptivity measurements were made on a Tecan M1000 plate reader (Mannedorf, Switzerland). Amino acid excitation and emission spectra and time correlated single photon counting (TCSPC) fluorescence lifetime measurements were made using a Photon Technology International (PTI) QuantaMaster™ 40 fluorescence spectrometer (currently, Horiba; Piscataway, NJ, USA). Quantum yield (QY) measurements were performed using a Jasco FP-8300 fluorimeter with an ILF-835 integrating sphere attachment (Easton, MD, USA).
Photophysical Properties of 7-(dimethylamino)acridon-2-ylalanine (Dad)

Molar Absorptivity Measurements. The core chromophore 2-(dimethylamino)acridone (Dad’) was dissolved to a 2 mM starting stock in DMSO. Samples were individually diluted to the desired concentration using 50:50 CH₃CN/phosphate buffered saline (PBS) in triplicate. This solvent was chosen to mimic the conditions previously reported for corresponding measurements of 2-aminoacridone (Aad’) and 7-aminoacridon-2-ylalanine (Aad).² The absorbance at 425 nm was measured on a Tecan M1000 plate reader. As standards to account for pathlength, the molar absorptivities for acridone (420 nm) and Aad’ (386 nm) were also measured. The molar absorptivity measurements were confirmed by measurement of a separate batch of Dad’ and the values fitted to a line in Graphpad Prism to yield $\varepsilon_{425} = 5415 \pm 59 \text{ M}^{-1}\text{cm}^{-1}$.

Figure S1: Molar absorptivity data for Dad’ in 50:50 CH₃CN/PBS.
Absorption and Fluorescence Measurements. 7-(Dimethylamino)acridon-2-ylalanine (Dad) absorption measurements were performed either on a Tecan M1000 plate reader or a ThermoScientific Genesys 150 UV-Vis spectrometer. The pH dependent measurements were in Citric acid BIS-TRIS propane (CBTP) buffer. The buffer system relies of varying ratios of citric acid to BIS-TRIS propane to achieve buffers with predictable pH’s. The buffer pH’s were also confirmed using a pH meter. Fluorescence measurements were performed on 5 μM samples on either a Tecan M1000 plate reader or a Photon Technology International (PTI) QuantaMaster™ 40 fluorescence spectrometer.

Figure S2. Absorption and emission spectra of Dad at various pHs and in various solvents. A) Absorption spectra in CBTP at various pHs, B) Absorption spectra in various solvents, C) Emission spectra in CH₃CN/CBTP at various pHs (λₑₓ = 425 nm).

Quantum Yield Measurements. The JASCO ILF-835 integrating sphere calculates quantum yield (QY) by comparing the integrated intensity of the incident (excitation) beam to the fluorescence emission of a sample. These values are compared in the presence and absence of a fluorophore. Within each replicate, the incident excitation light spectrum was collected in the presence of 2 mL of solvent. After measuring the incident light intensity, 5, 10, or 15 μL of a 5 mM dye stock was added to the solvent and the new excitation and emission spectra were collected.
Figure S3. Representative QY acquisitions for Dad in various solvents. All had identical excitation (425 nm, excitation bandwidth 5 nm) and spectral collection (400-800 nm, emission bandwidth 5 nm, detector sensitivity: low, scan speed: 1000nm/min) parameters.
Figure S4. Representative QY acquisitions for Aad in various solvents. All had identical excitation (425 nm, excitation bandwidth 5 nm) and spectral collection (400-800 nm, emission bandwidth 5 nm, detector sensitivity: low, scan speed: 1000nm/min) parameters.
Fluorescence lifetime measurements. TCSPC measurements of fluorescence lifetime decays for 5 µM samples of Dad and Aad were collected with the PTI Quantamaster™ 40 using a pulsed LED with a maximum emission at 486 nm and with a 480 nm short pass filter in the excitation beam path. Fluorescence emission was collected at 525 or 535 nm with 20 nm slit widths. The instrument response function (IRF) was collected under identical conditions. Data analysis was performed with FluoFit software (PicoQuant GmbH; Berlin, Germany) using an exponential decay model.

Table S1. Fluorescence lifetime (in ns) of Dad at concentrations in DMSO

|       | 10 µM | 100 µM | 1 mM  |
|-------|-------|--------|-------|
| Dad   | 19.33 ± 0.03 | 19.61 ± 0.03 | 19.54 ± 0.03 |

Figure S5. Fluorescence lifetime decays (bottom) and residuals after fitting (top) for Dad in various solvents. TCSPC decay curves (pale colors) collected with the emission monochromator set to 555 nm and fits to single exponential decay models (dark lines) are shown along with the corresponding residuals in like colors. All $\chi^2$ values for fits ranged from 0.934 to 1.037.
**Figure S6.** Fluorescence lifetime decays (bottom) and residuals after fitting (top) for Dad at various concentrations in DMSO. TCSPC decay curves (pale colors) collected with the emission monochromator set to 555 nm and fits to single exponential decay models (dark lines) are shown along with the corresponding residuals in like colors. All $\chi^2$ values for fits ranged from 0.9996 to 1.334.

**Figure S7.** Fluorescence lifetime decays (bottom) and residuals after fitting (top) for Aad in various solvents. TCSPC decay curves (pale colors) collected with the emission monochromator set to 535 nm and fits to single exponential decay models (dark lines) are shown along with the corresponding residuals in like colors. All $\chi^2$ values for fits ranged from 0.953 to 1.184.
Electronic Structure Analysis

Quantum Mechanical Calculations. Ab initio electronic structure calculations of the absorption and emission spectra for 2-aminoacridone (Aad') and 2-(dimethylamino)acridone (Dad') employed the APF-D density functional as implemented in the Gaussian16™ suite of programs with the 6-311+G(2d,p) basis set. The APF-D/6-311+G(2d,p) optimized geometry input files for these calculations and the corresponding vertical transitions are provided on the following pages. We combined Franck-Condon integral calculations with vibrational calculations of the ground and first excited states [Gaussian16 keyword(options) Freq=(ReadFC,FranckCondon,ReadFCHT)] as described in Foresman and Frisch, pages 364 to 371, to generate a vibronic spectrum representing the Aad' emission spectrum in aqueous solution. These spectra as well as the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) for Aad' are shown in Figure S8. We included the differential solvation of the ground state and first excited state by employing the procedure described in Foresman and Frisch, pages 371 to 379. Unfortunately, the geometry changes between the ground and excited states for Dad' prevented completion of Franck-Condon calculations. Therefore, we approximated the Dad' solution emission spectrum by calculating the difference between the Dad' and Aad' vertical emissions ($\Delta\lambda_{Ex} = +22.58$). We shifted the Aad' emission spectrum by these values to give the calculated spectrum shown in Figure 4.

Figure S8. Comparison of experimental and computed Aad spectra. Experimental absorption and emission spectra for Aad were determined in CH$_3$CN/PBS. Computed spectra were determined from Aad' APF-D/6-311+G(2d,p) vertical excitation and emission calculations with Franck-Condon integral calculations to generate the vibronic emission spectrum. Excitation spectra are shown as dashed lines, emission spectra are shown as solid lines. Individual calculated state-to-state transitions are shown as black vertical lines.
2-(Dimethylamino)acridone (Dad')

1-A Ground State in H2O

| Wavelength (nm) | Oscillator Strength |
|-----------------|---------------------|
| 436.21          | 0.0784              |
| 324.56          | 0.0003              |
| 316.23          | 0.0228              |
| 295.18          | 0.5344              |
| 287.62          | 0.0906              |
| 259.66          | 0.0857              |
| 255.50          | 0.6060              |
| 251.81          | 0.0482              |
| 246.90          | 0.1139              |
| 240.61          | 0.0680              |
| 236.71          | 0.0128              |
| 233.10          | 0.0008              |
| 224.92          | 0.0102              |
| 224.20          | 0.0280              |
| 222.96          | 0.0152              |
| 222.13          | 0.1083              |
| 217.72          | 0.0003              |
| 216.17          | 0.0082              |
| 213.28          | 0.0299              |
| 211.15          | 0.0004              |
| 205.65          | 0.0144              |
| 203.21          | 0.2800              |
| 202.29          | 0.0238              |
| 200.91          | 0.0865              |
| 200.77          | 0.0199              |
2-(Dimethylamino)acridone (Dad')

Vertical Emission Transitions

| Wavelength (nm) | Oscillator Strength |
|-----------------|---------------------|
| 499.79          | 0.0865              |
| 337.97          | 0.0000              |
| 328.79          | 0.0366              |
| 307.70          | 0.1290              |
| 304.26          | 0.4071              |
| 273.44          | 0.0138              |
2-Aminoacridone (Aad')
1-A Ground State in H2O

| Wavelength (nm) | Oscillator Strength |
|-----------------|---------------------|
| 406.70          | 0.2800              |
| 325.35          | 0.0238              |
| 303.34          | 0.0228              |
| 281.14          | 0.0344              |
| 276.74          | 0.0906              |
| 252.89          | 0.0857              |
| 251.06          | 0.0600              |
| 247.17          | 0.0482              |
| 239.25          | 0.1139              |
| 234.35          | 0.0680              |
| 226.01          | 0.0128              |
| 225.14          | 0.0008              |
| 223.30          | 0.0102              |
| 215.24          | 0.0280              |
| 213.10          | 0.0152              |
| 211.73          | 0.1083              |
| 209.40          | 0.0003              |
| 208.16          | 0.0082              |
| 202.95          | 0.0299              |
| 202.27          | 0.0004              |
| 200.45          | 0.0144              |
| 406.70          | 0.2800              |
| 325.35          | 0.0238              |
| 303.34          | 0.0228              |
| 281.14          | 0.0344              |
2-Aminoacridione (Aad')

1-A Excited State in H2O

C  -0.01041000  3.52527000  -1.25778000
C  -0.00811000  2.25445000  -1.86106000
C  -0.00116000  1.11713000  -1.06939000
C   0.00372000  1.20326000   0.34182000
C   0.00130000  2.48051000   0.91816000
C  -0.00571000  3.63051000   0.12214000
C   0.01098000  0.00078000   1.15549000
C   0.01289000 -1.27082000   0.43820000
C   0.00780000 -1.31108000  -0.98295000
C   0.00965000 -2.53750000  -1.66813000
H   0.00565000 -2.53657000  -2.75384000
C   0.01647000 -3.73671000  -0.97974000
C   0.02158000 -3.71443000   0.43037000
C   0.01969000 -2.47311000   1.12041000
H  -0.00247000 -0.17227000  -2.67138000
H   0.01585000  4.41335000  -1.88008000
H  -0.01174000  2.15869000  -2.94265000
H   0.00502000  2.55077000   1.99953000
H  -0.00746000  4.60895000   0.59227000
H   0.01788000 -4.68175000  -1.51014000
H   0.02361000 -2.45286000   2.20507000
O   0.01545000  0.03919000   2.41200000
N   0.00112000 -0.13737000  -1.66098000
N   0.02828000 -4.85485000   1.14243000
H   0.03194000 -4.84048000   2.14971000
H   0.02985000 -5.75528000   0.69056000

Vertical Emission Transitions

| Wavelength (nm) | Oscillator Strength |
|-----------------|---------------------|
| 477.94          | 0.0947              |
| 337.96          | 0.0000              |
| 319.24          | 0.0409              |
| 301.57          | 0.0206              |
| 293.50          | 0.4467              |
| 265.82          | 0.0003              |
Screening of Aminoacyl tRNA Synthetases for Dad Activity. BL21-DE3 E. coli cells were transformed with a pBad plasmid encoding superfolder GFP (sfGFP) with a TAG mutation at position 140 (sfGFP\textsubscript{TAG140}, Addgene #85483) and a pDule2 plasmid encoding one of three (G2, G11, or A9) previously evolved \textit{Methanocaldococcus jannachii} (\textit{Mj}) tyrosine aminoacyl tRNA synthetases (aaRSs).\textsuperscript{5} Cells were grown with Acd, Dad or no unnatural amino acid (Uaa) added as described in the main text. Each culture was diluted 10-fold with MilliQ water and the sfGFP fluorescence intensity was quantified using a Tecan M1000 plate reader ($\lambda_{\text{ex}}$: 488 nm, $\lambda_{\text{em}}$: 509 nm). Additionally, the optical density was measured at 600 nM (OD\textsubscript{600}) using a ThermoScientific Genesys 150 UV-Vis spectrometer. Raw data from triplicate experiments are shown in Figure S9. These were used to generate the graph of normalized fluorescence in Figure 5.

![Graph of normalized fluorescence](image1.png)

**Figure S9.** Comparison of sfGFP fluorescence intensity and cell density for cultures grown with three \textit{Mj} aaRSs to investigate Dad incorporation ability.

![Graph of OD\textsubscript{600}](image2.png)
Figure 10. $^1$H (Top) and $^{13}$C (Bottom) NMR of 3 (Dad).
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