Stitching of Tyrosine and 10H-Acrdin-9-one: *Turn-ON* Fluorescence in the Narrow pH range 7.4 – 8.5 and Intracellular Labelling of Cancer Cells

Palwinder Singh*, Arun Kumar, Sukhmeet Kaur, Amrinder Singh, Muskan Gupta,
Gurcharan Kaur

*aDepartment of Chemistry and Centre for Advanced Studies, University with Potential for Excellence – Guru Nanak Dev University, Amritsar-143005, India

bDepartment of Biotechnology, Guru Nanak Dev University, Amritsar-143005, India

Supporting Information

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General Note

$^1$H and $^{13}$C NMR spectra were recorded on Bruker 500 MHz NMR spectrometer using DMSO-d$_6$ and DMSO-d$_6$ – H$_2$O as the solvent. Chemical shifts are given in ppm with TMS as an internal reference. $J$ values are given in Hertz. Mass spectra were recorded on Bruker micrOTOF Q II Mass spectrometer. Reactions were monitored by thin layer chromatography (TLC) on glass plates coated with silica gel GF-254. The fluorescence spectra were run on BIOTEK Synergy H1 Hybrid Reader spectrofluorophotometer as per the standard procedure. The percentage purity (99%) of the all the compounds was checked with qNMR (quantitative NMR) whereas optical purity (100%) was checked by using chiral Chirobiotic® T 10 µm chiral HPLC column (25 cm x 4.6 mm). Acetonitrile-water (1:1), each charged with 0.1% formic acid, was used as eluent. 2 µL of sample (injection volume) was loaded to the column, flow rate was kept 0.2 ml and absorbance was set at 200, 220 and 254 nm. Sodium formate was used as internal calibrant.

Procedure for the fluorescence study of compounds at various pH

20 ml solution of compound (10 µM) in DMSO-HEPES buffer (1:9) was taken in a 50 ml conical flask. The electrode of the pH meter was dipped in the solution of compound to measure the pH. The pH of the above solution of compound was changed by the addition of HCl (5 N) and NaOH (5 M) solution. Fluorescence of the solution was recorded at Biotek synergy H1 hybrid reader fluorescence spectrophotometer.

Procedure for the NMR study of the compounds at various pH

0.6 ml solution of compound (1 M) in DMSO-H$_2$O (2:1) was taken in NMR sample tube. Micro electrode of the pH meter was dipped into the NMR sample tube containing solution of compound to measure the pH. The pH of the solution was changed by the addition of HCl (5 N) and NaOH (5 M) solution. NMR of the sample was recorded at 500 MHz NMR spectrometer.
Quantum Yield Measurements

Absorption and fluorescence spectra were recorded on BIOTEK synergy H1 hybrid reader spectrophotometer. Relative quantum yield of compound 2 (10⁻⁵ M) at pH 7.6 was calculated (Φ = 0.63 in DMSO) by using anthracene as standard.

Φₓ = Φₛ(Fₓ/Fₛ)(Aₛ/Aₓ)(λₓₓ/λₛₛ)(nₛ/nₓ)²

Where, Φₓ = quantum yield of unknown sample

Φₛ = quantum yield of standard

Fₓ = area under the emission spectrum of unknown sample

Fₛ = area under the emission spectrum of standard

λₓₓ = excitation wavelength of unknown sample

λₛₛ = excitation wavelength of standard

n = refractive index of solvent

Synthesis

The reaction mixture containing 2-chlorobenzoic acid (63.86 mmol), anthranillic acid (64 mmol), K₂CO₃ (96 mmol) and CuO (3.2 mmol) in 75 ml iso-amyl alcohol was stirred at 160 °C for 36 h (Scheme S1). After the work up of the reaction mixture (as described in the experimental section), the resulting precipitates were filtered and further dissolved in conc H₂SO₄ and heated at 90-100 °C for 2 h to obtain compound 3. Acridone 3 was treated with L-tyrosine methyl ester salt in the presence of 3 ml triethyl amine and 2 ml ethyl chloroformate at 0 °C for 1 h. After completion of reaction (TLC monitoring), the reaction mixture was extracted with chloroform. The organic phase was dried over anhydrous Na₂SO₄ and the residue was column chromatographed using ethyl acetate and hexane as eluents to isolate pure compound 4. Compound 4 was treated with LiOH to obtain compound 2. Likewise, as per the protocol shown in scheme S1, other compounds were synthesized.
Scheme S1
**Synthesis of compound 3 (Scheme S1).** The reaction mixture containing 2-chlorobenzoic acid (10 g, 63.86 mmol), anthranillic acid (8.75 g, 64 mmol), K$_2$CO$_3$ (1.32 g, 96 mmol) and CuO (253 mg, 3.2 mmol) in 75 ml iso-amyl alcohol was stirred at 160 °C for 36 h (Scheme 2). After the completion of reaction, iso-amyl alcohol was evaporated under the reduced pressure and the reaction mixture was washed with hot water. Reaction mixture was acidified to obtain the precipitates. The precipitates were filtered and further dissolved in conc H$_2$SO$_4$ and heated at 90-100 °C for 2 h. After 2 h, the reaction mixture was poured in water. The resulting precipitates were filtered to isolate compound 3. Yellowish green solid, mp >300 °C; $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 7.26 (2H, m, ArH), 7.55 (1H, d, $J$ 8.4 Hz, ArH) 7.69 (1H, m, ArH), 8.12 (1H, d, $J$ 9.2 Hz, ArH), 8.32-8.34 (1H, m, ArH), 8.39-8.41 (1H, m, ArH), 11.85 (1H, b, NH). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 177.41, 169.59, 141.39, 140.10, 137.42, 134.76, 132.59, 126.21, 122.87, 121.63, 120.77, 120.65, 118.71, 115.48. HRMS (ESI, m/z): Calculated for C$_{14}$H$_9$NO$_3$ [M-H]$^{-}$ 238.0499, found 238.0515.

**Methyl 3-(4-hydroxyphenyl)-2-(9-oxo-9,10-dihydroacridine-4-carboxamido)propanoate (4).**

Acridone 3 (2 g) was treated with L-tyrosine methyl ester salt (2.5 g) in the presence of 3 ml triethyl amine and 2 ml ethyl chloroformate at 0 °C for 1 h. After completion of reaction (TLC monitoring) the reaction mixture was extracted with chloroform. The organic phase was dried over anhydrous Na$_2$SO$_4$ and the residue was column chromatographed using ethyl acetate and hexane as eluents to isolate pure compound. (78%), mp 160 °C, [α]$_D$ +40° (c 1.00, MeOH). $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta$ 3.02-3.15 (2H, m, CH$_2$), 3.68 (3H, s, OCH$_3$), 4.72-4.76 (1H, m, CH), 6.67 (2H, d, $J$ 8.4 Hz, ArH), 7.13 (2H, d, $J$ 8.3 Hz, ArH) 7.29-7.39 (2H, m, ArH), 7.71-7.74 (2H, m, ArH), 8.21-8.32 (2H, m, ArH), 8.45-8.47 (1H, m, ArH), 9.25-9.32 (2H, b, 1×OH+1×NH), 12.09 (1H, b, NH). $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta$ 176.96, 172.49, 168.31,
156.49, 140.71, 134.46, 133.89, 131.09, 130.72, 127.87, 126.29, 122.52, 120.92, 120.39, 118.95, 118.16, 115.59, 52.10, 52.60, 35.97. IR (KBr, thin film) ν_{max} (cm\(^{-1}\)): 3648 (NH), 3628 (NH), 3287 (OH), 1747 (CO), 1616 (CO), 1578 (CO). HRMS-ESI (m/z): [M+H]\(^+\) calculated for C\(_{24}\)H\(_{20}\)N\(_2\)O\(_5\), 417.1444; found 417.1448.

3-(4-Hydroxyphenyl)-2-(9-oxo-9,10-dihydroacridine-4-carboxamido)propanoic acid (2).

Compound 4 (1.5 g) was treated with LiOH to obtain compound 2. (80%), mp 165 °C, [α]\(_D\) +20° (c 1.00, MeOH). \(^1\)H NMR (500 MHz, DMSO-\(d_6\)): δ 2.99-3.17 (2H, m, CH\(_2\)), 4.67-4.71 (1H, m, CH), 6.66 (2H, d, J 8.3 Hz, ArH), 7.14 (2H, d, J 8.3 Hz, ArH), 7.30-7.38 (2H, m, ArH), 7.71-7.74 (2H, m, ArH), 8.21-8.27 (2H, m, ArH), 8.45 (1H, d, J 7.8 Hz, ArH), 9.19 (2H, b, 1×OH+1×NH), 12.13 (1H, b, NH), 12.95-12.97 (1H, b, OH). \(^1\)C NMR (125 MHz, DMSO-\(d_6\)): δ 176.98, 173.62, 168.22, 156.37, 140.63, 134.46, 133.74, 130.46, 128.40, 126.31, 122.47, 122.02, 120.90, 120.41, 118.86, 118.66, 115.53, 55.09, 35.92. IR (KBr, thin film) ν_{max} (cm\(^{-1}\)): 3653 (NH), 3624 (NH), 3286 (OH), 3228 (OH), 1741 (CO), 1616 (CO), 1513 (CO). HRMS-ESI (m/z): [M+H]\(^+\) calculated for C\(_{23}\)H\(_{18}\)N\(_2\)O\(_5\), 403.1288; found 403.1201.

Compound 6. Compound 6 was prepared from compound 3 through the same procedure as for compound 2 but using D-Tyr methyl ester hydrochloride. [α]\(_D\) -18° (c 1.00, MeOH). HRMS-ESI (m/z): [M+H]\(^+\) calculated for C\(_{23}\)H\(_{18}\)N\(_2\)O\(_5\), 403.1288; found 403.1279.

Compound 5. Compound 5 was obtained by the reaction of 3 with L-Phe methyl ester hydrochloride using the same procedure as for the preparation of compound 2. [α]\(_D\) +16° (c 1.00, MeOH). HRMS-ESI (m/z): [M+H]\(^+\) calculated for C\(_{23}\)H\(_{18}\)N\(_2\)O\(_4\), 387.1279; found 387.1272.

Compound 7. Using the same coupling procedure as for preparation of 2 from 3, coupling of Gly and L-Tyr with 3 provided compound 7 (55%), mp 177 °C, [α]\(_D\) +17° (c 1.00, MeOH). \(^1\)H
NMR (500 MHz, DMSO-\textit{d}_6): \(\delta 2.81-3.0\) (2H, m, \textit{CH}_2), 3.96-4.08 (2H, m, \textit{CH}_2), 4.44-4.48 (1H, m, \textit{CH}), 6.64 (2H, d, \textit{J} 8.4 Hz, Ar\textit{H}), 7.05 (2H, d, \textit{J} 8.4 Hz, Ar\textit{H}), 7.31-7.37 (2H, m, Ar\textit{H}), 7.73-7.76 (2H, m, Ar\textit{H}), 8.16-8.25 (2H, m, Ar\textit{H}), 8.41-8.45 (2H, b, 1×Ar\textit{H}+1×OH) 9.20 (2H, b, 2×NH), 12.32 (1H, b, NH), 12.80 (1H, b, OH). 13C NMR (125 MHz, DMSO-\textit{d}_6): \(\delta 177.02, 173.36, 169.53, 156.44, 140.69, 140.22, 134.44, 133.34, 130.57, 130.24, 127.84, 126.35, 121.92, 120.87, 120.54, 120.49, 118.73, 115.48, 54.51, 42.56, 36.54\). HRMS-ESI (m/z): [M+H]\(^+\) calculated for C\textsubscript{25}H\textsubscript{21}N\textsubscript{3}O\textsubscript{6}, 460.1503; found 460.1517.

**Compound 8.** Using the same coupling procedure as for preparation of 2 from 3, coupling of Gly with 2 provided compound 8 (58%); mp 182 °C, \([\alpha]_D^\text{20} +18^\circ\) (c 1.00, MeOH). \(^1\)H NMR (500 MHz, DMSO-\textit{d}_6): \(\delta 2.88-3.17\) (2H, m, \textit{CH}_2), 3.86-3.88 (2H, m, \textit{CH}_2), 4.80 (1H, m, \textit{CH}), 6.64 (2H, d, \textit{J} 8.4 Hz, Ar\textit{H}), 7.19 (2H, d, \textit{J} 8.4 Hz, Ar\textit{H}), 7.29-7.35 (2H, m, Ar\textit{H}), 7.69-7.76 (2H, m, Ar\textit{H}), 8.19-8.23 (2H, m, Ar\textit{H}), 8.41-8.43 (1H, m, Ar\textit{H}), 8.61-8.63 (1H, b, OH), 9.07-9.16 (2H, b, 2×NH), 12.13 (1H, b, NH), 12.74 (1H, b, OH). 13C NMR (125 MHz, DMSO-\textit{d}_6): \(\delta 176.99, 172.44, 171.60, 168.04, 156.23, 140.54, 140.33, 134.43, 133.65, 130.53, 128.76, 126.30, 122.38, 121.87, 120.83, 120.41, 119.83, 118.80, 115.40, 55.67, 41.37, 36.64\). HRMS-ESI (m/z): [M+H]\(^+\) calculated for C\textsubscript{25}H\textsubscript{21}N\textsubscript{3}O\textsubscript{6}, 460.1503; found 460.1850.
Figure S1. $^1$H NMR spectrum of compound 3.

Figure S2. $^{13}$C NMR spectrum of compound 3.
Figure S3. $^1$H NMR spectrum of compound 5.

Figure S4. $^{13}$C NMR spectrum of compound 5.
Figure S5. $^1$H NMR spectrum of Compound 4.

Figure S6. $^{13}$C NMR spectrum of Compound 4.
Figure S7. DEPT-135 NMR spectrum of Compound 4.

Figure S8. $^1$H NMR spectrum of compound 2.
Figure S9. $^{13}$C NMR spectrum of compound 2.

Figure S10. DEPT-135 NMR spectrum of compound 2.
Figure S11. An overlay of HSQC (red contours) and HMBC (blue contours) NMR spectrum of compound 2.
Figure S12. HMBC NMR spectrum of compound 2.

Figure S13. HSQC NMR spectrum of compound 2.
Figure S14. $^1$H-$^1$H NOESY NMR spectrum of compound 2.

Figure S15. $^1$H-$^1$H TOCSY NMR spectrum of compound 2.
Figure S16. NMR assignments to proton and carbon of compound 2. Red: $^1$H, Blue: $^{13}$C, TOCSY cross peaks of amidic NH with CH and CH$_2$ (green arrows).

Figure S17. $^1$H NMR spectrum of compound 7.
Figure S18. $^{13}$C NMR spectrum of compound 7.

Figure S19. DEPT-135 NMR spectrum of compound 7.
Figure S20. $^1$H NMR spectrum of compound 8.

Figure S21. $^{13}$C NMR spectrum of compound 8.
Figure S22. Mass spectrum of acridone 3 (calcd m/z 238.0499 [M-H]).

Figure S23. HRMS of compound 4.
Figure S24. HRMS of compound 2.

Figure S25. Mass spectrum of compound 7 (calcd m/z for C_{25}H_{21}N_{3}O_{6} is 460.1503, found 460.1850).

Figure S26. Mass spectrum of compound 8 calcd m/z for C_{25}H_{21}N_{3}O_{6} is 460.1503, found 460.1517.
Figure S27. UV-Vis. Spectrum of compound 2 (10\(^{-5}\) M) in DMSO-HEPES buffer (1:9).

Table S1. Extinction coefficient of probe 2 at different pH.

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<th>pH</th>
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<tr>
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</tr>
<tr>
<td>5</td>
<td>5.90\times10^4</td>
</tr>
<tr>
<td>7</td>
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<tr>
<td>9</td>
<td>6.56\times10^4</td>
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Figure S28. \(^1\)H NMR titration of compound 2 at various pH.
Figure S29a. $^1$H NMR spectrum of compound 2 at pH 3.5.

Figure S29b. $^1$H NMR spectrum of compound 2 at pH 6.5.
Figure S29c. $^1$H NMR spectrum of compound 2 at pH 7.5.

Figure S29d. $^1$H NMR spectrum of compound 2 at pH 9.0.
Figure S29e. $^1$H NMR spectrum of compound 2 at pH 10.5.

Figure S30. Structures of compound 2 at different pH obtained from LigPrep module of Schrodinger software.
Figure S31. Protonation-deprotonation and ICT in 2 at different pH. Charge development in the acceptor and donor part of the molecule significantly affects ICT. Inset: HOMO, LUMO of acceptor and donor units of (A) 2a, (B) 2b. Energy gap between HOMO of 2b(A) and 2b(D) is decreased than that of 2a(A) and 2a(D). 2a/b(A): acceptor part of 2a/2b, 2a/b(D): donor part of 2a/2b.
**Figure S32.** Fluorescence intensity does not change with time at pH 7.5 indicating no photobleaching of the probe.

**Figure S33a.** Fluorescence spectra of acridone 3 (10 μM) in DMSO-HEPES buffer (1:9) at different pH.
**Figure 33b.** Fluorescence spectrum of compound 4 (10^-5 M) in DMSO-H_2O (1:9) at different pH value.

**Figure S33c.** Fluorescence spectra of acridone 5 (10 μM) in DMSO-HEPES buffer (1:9) at different pH.
Figure S33d. Fluorescence spectrum of compound 6 (10^{-5} M) in DMSO-water (1:9) at various pH.

Figure S33e. Fluorescence spectrum of compound 7 (10^{-5} M) in DMSO-water (1:9) at various pH.
Figure S34. Fluorescence spectrum of compound 2 (10^{-5} M) in different solvents supporting photoinduced intramolecular charge transfer.

Figure S35. Viability of C6 glioma cells in the presence of compound 2 as measured by using MTT assay. The cells were incubated with compound 2 for 24 h.
**Figure S36.** (A) Confocal microscopic images of compound 2 treated C6 glioma cells after: (a) 15 min, (b) 30 min, (c) 45 min and (d) 60 min of first image indicating the photostability of the probe. (B) Confocal images of cancerous and RA differentiated C6 glioma cells treated with equimolar concentration of the DMSO. Cells observed in DIC channel were live and non-fluorescent in 488 nm channel indicating the activity of the probe 2. This data demonstrates that DMSO alone treated cells were healthy, live and non-fluorescent, which clearly indicates that the increase in fluorescence intensity because of pH change in these cells was due to the function of probe and not because of DMSO which was used as vehicle to dissolve the probe.

**Density functional theory (TD-DFT) calculations**

DFT calculations were performed on Ligprep-3.2, Jaguar-8.6 modules of Schrodinger Suite 2014-4. The ground (S0) state geometries were fully optimized with the Becke’s three-parameter exchange functional along with the Lee Yang Parr’s correlation functional (B3LYP) using 6-31G (d) basis sets. These optimized structures were further characterized by comparison of harmonic vibration frequency with experimental one. The vertical excitation energies at the ground state
equilibrium geometries were calculated with TD-DFT. The low-lying first singlet excited state (S1) of each conformer was relaxed using the Tamm Dancoff Approximation to obtain its minimum energy geometry and emission wavelength. All the computations were performed by considering the effect of water as solvent using the Polarizable Continuum Model (PCM).

The pK$_a$ of the compounds were calculated by using Schrodinger Suite 2014-4 with Jaguar 8.6 module.

**Table S2.** Molecular orbitals of compound 2 at different pH values.

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Cell culture and treatments
C6 glioma cells were obtained from National Center for Cell Sciences, Pune, India. Cells were maintained in DMEM supplemented with 1X PSN (GIBCO), 10% FBS (Biological Industries) at 37 °C and humid environment containing 5% CO₂.

Cytotoxicity or cell viability assay
Compound 2 was tested for its cytotoxicity on the C6 glioma cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The cell viability was quantified by the conversion of yellow MTT by Mitochondrial dehydrogenases of living cells to purple MTT formazan crystals which were dissolved in DMSO and read at 595 nm wavelength. For this, C6 glioma cells were seeded in 96 well plates and after 24 h of seeding, the cells were treated with compound 2 at a concentrations ranging from 10 μM to 1000 μM.

For Fluorescence Detection: For this purpose, two groups were tested: I. Undifferentiated cancerous C6 glioma cells incubated with compound 2, II. Differentiated cells for which undifferentiated C6 glioma cells were treated with 10 µM RA for 5 days to induce differentiation thereafter cells were exposed to 30 μM of compound 2 for 24 h.

After exposure to the compounds, cells were washed with 1X PBS thrice for 5 minutes each to completely remove any extracellular probe and then fixed with acetone: methanol in 1:1 ratio for 10 min. Cells were then washed with 1X PBS for 5 min followed by mounting on the slides using antifading medium. Images were taken with A1R Nikon Laser Scanning Confocal Microscope.

Statistical analysis
Values are expressed as mean ± SEM. The SigmaStat for Windows (version 3.5) was adopted to analyze the results by One Way ANOVA test in order to determine the statistical significance of the means. Values of p< 0.05 were considered as statistically significant.

Observations
Cytotoxicity assay for compound 2 did not show any cell death. Based on this preliminary data 30 µM concentration was selected for further experiments. Further, it was observed that fluorescent intensity of compound 2 decreases in the cell sample obtained by treating the glioma cells with retinoic acid.