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Synthesis of novel fluorescent 1,3,5-trisubstituted triazine derivatives and photophysical property evaluation of fluorophores and their BSA conjugates

Abstract: Cyanuric chloride was allowed to react with *N,N*-diethylaniline to obtain 4-(4,6-dichloro-1,3,5-triazin-2-yl)-*N,N*-diethylaniline, which was converted into six novel 1,3,5-trisubstituted triazine derivatives on reaction with different amino acids. These compounds had UV absorption in the range 352–379 nm, accompanied by intense single emission in the range 420–497 nm with fairly good quantum yield (0.106–0.383). The new compounds were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectral, and elemental analyses. These fluorophores were conjugated with protein bovine serum albumin through carbodiimide chemistry between the negatively charged carboxylate groups (-COO-) of the fluorophore and the surface terminated positively charged amino groups ($-NH_3^+$) of the protein. The interaction between functionalized amino acids with protein molecules was investigated using fluorescence spectroscopy showing fluorescence enhancement or quenching of the fluorophore after conjugation.

Keywords: α-amino acid; bioconjugation; bovine serum albumin (BSA); fluorescence; 1,3,5-triazine.

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Introduction

Fluorescent probes are useful tools in analytical techniques (Mason, 1993), such as immunofluorescence and immunofluorometric assays (Gosling, 1990; Diamandis, 1993), protein conformation studies (Sytnik and Kasha, 1994; Sytnik et al., 1994; Sytnik and Litvinyuk, 1996; Parul et al., 2000; Talavera et al., 2000; Timtcheva et al., 2000), detection of compounds by HPLC (Liebes et al., 2001), capillary electrophoresis (CE) (Flanagan et al., 1995; Hegaard et al., 1998; Krylov, 2000), as well as coupling CE with laser-induced fluorescence detection (Chiu et al., 1998; Zhang et al., 2001). They also find application for determination of non-protein cysteine in human serum (Lu et al., 2011), monitoring of glycolipid uptake by cells (Cheng et al., 2011), specific protein labeling of living cells (Arai et al., 2011), red-shifted voltage-sensitive fluorescence (Perron et al., 2009), and targeting β-amyloid plaques (Parhi et al., 2008). Several fluorescence probes have been used to investigate biological processes through fluorescence measurements (Fuller et al., 1998; Birch, 2001; Okerberg and Shear, 2001; Duan et al., 2009; Han and Burgess, 2010; Wang et al., 2011). These are coumarin derivatives (Cao et al., 2011), fluorescein isothiocyanates (Sun et al., 1998; Zhang et al., 2001), anthracene derivatives (DiCesare and Lakowicz, 2001), and β-naphthol derivatives (Sartor et al., 2001).

The spectral changes observed on binding of fluorophores with proteins are an important tool for investigations of the topology of binding sites, conformational changes, and characterization of substrate to ligand binding (Song et al., 1996; Moreno et al., 1999). In addition, protein quantification in biological liquids is of great importance in biology and medicine (Kessler and Wolfbeis, 1992), and fluorescent probes are successfully applied for this approach (Haughland, 1996).

An effective fluorophore for biological studies has to show a good luminescent intensity and emission spectra free of interferences due to the emitting substances present in the analyzed matrix. A high Stoke's shift is a desired characteristic for a fluorescent probe that permits an improved separation of the light inherent to the matrix and the light dispersed by the sample (Haughland, 1992).

2-Aryl-4,6-disubstituted triazine derivatives have been reported as fluorescent probes (Cowley et al., 1991) but

they are not biocompatible. As a part of ongoing research to develop novel materials for high-tech applications (Sekar et al., 2010, 2011; Gupta et al., 2011; Padalkar et al., 2012; Patil et al., 2012), here we report the synthesis, characterization, and photophysical properties of novel fluorescent biocompatible fluorophores and their binding study with protein. The novel triazine derivatives were prepared from 4-(4,6-dichloro-1,3,5-triazin-2-yl)-*N,N*-diethylaniline and different α-amino acids.

Results and discussion

Chemistry

The reaction of cyanuric chloride with *N,N*-diethylaniline yielded dichloro-1,3,5-triazine **3** (Scheme 1). Then compound **3** was allowed to react with different α-amino acids in methanol at room temperature to give the desired fluorophores **5a–f**. A summary of the synthesized derivatives **5a–f** is given in Table 1. All compounds were characterized by FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectral, and elemental analyses. The synthesized novel triazine derivatives **5a**–**f** are fluorescent in solution when irradiated with UV light, and these compounds contain biocompatible free carboxylic acid groups, which were further explored for labeling with protein bovine serum albumin (BSA) in the presence of the coupling agent *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride. To warrant an efficacious label, the unbound fluorophore was efficiently removed by dialysis in phosphate buffer solution at pH 7. Protein conjugates were analyzed by using fluorescence analysis.

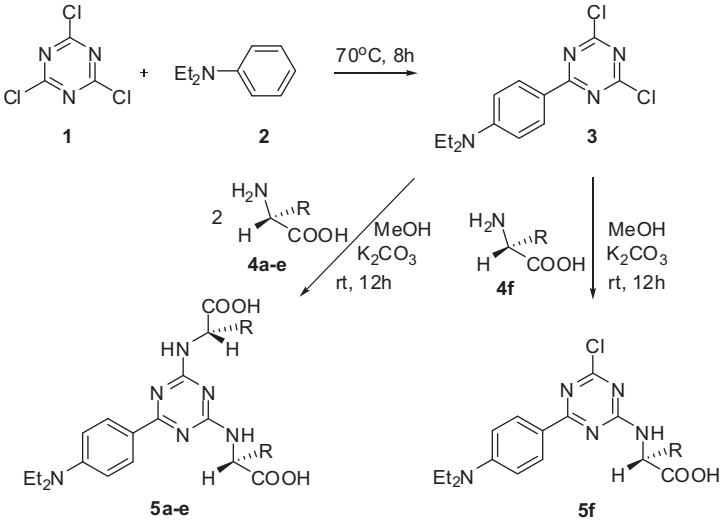
Photophysical properties

The quantum yields of compounds **5a–f** were determined using anthracene as standard. Samples were prepared in 1-cm path length quartz cells with absorbance <0.1 at excitation and all emission wavelengths to uniformly illuminate across the sample, and to avoid the inner-filter effect. Absorption and emission characteristics of the standard as well as the new compounds were measured at different concentrations (0.1–1.4 ppm). Absorbance intensity values were plotted against emission intensity values, and a linear plot was obtained. Gradients were calculated for each compound. All measurements were done by keeping the parameters such as solvent and slit width constant. Relative quantum yield of all synthesized derivatives **5a**–**f** were calculated by using Eq. (1), and for emission measurements compounds were excited at their corresponding absorption maxima.

$$\Phi_X = \Phi_{ST} (Grad_X/Grad_{ST}) (\eta^2_X/\eta^2_{ST}) \quad (1)$$

where Φ_X = quantum yield of synthesized sample, Φ_{ST} = quantum yield of standard used, $Grad_X$ = gradient of synthesized sample, $Grad_{ST}$ = gradient of standard used, η^2_{ST} = refractive index of solvent for standard sample.

Gradient of standard (Figure 1), gradient of compound **5c** (Figure 2), and quantum yield calculation of **5c** (Table 2) are shown for illustration.



Scheme 1 Synthesis of amino acid substituted 1,3,5-triazine derivatives.

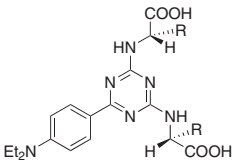
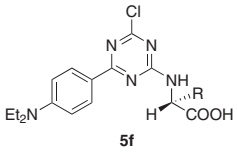
Compound		R	Absorption λ_{max} (nm)	Emission λ_{em} (nm)	Stoke's shift ($\Delta\lambda$)	Quantum yield
 5a-e	**5a**	H-	376	440	64	0.106
	5b	$HSCH_2$-	376	497	121	0.376
	5c	$PhCH_2$-	379	487	108	0.346
	5d	*p*-OH-C_6H_4-	352	464	112	0.383
	5e		379	452	112	0.289
 5f	**5f**		352	420	68	0.170

Table 1 Absorption, emission, Stoke's shift, and quantum yield of compounds **5a–f**[a].
[a]Absorption and emission analyses were carried out at room temperature; solvent used was DMSO/H_2O (1 mL:9 mL); concentration of samples: 1×10^{-6} µL; DMSO: dimethyl sulfoxide.

By putting values of gradients in Eq. (1), quantum yield was calculated for each compound by taking anthracene as standard (quantum yield of anthracene in ethanol solution is 0.27).

The fluorescence quantum yields of **5a–f** were measured in ethanol at room temperature. The quantum yields of **5b** and **5d** are much higher than those for **5a, 5c, 5e**, and **5f** (Table 1). The higher quantum yields of **5b** and **5d** are presumably due to presence of -OH and -SH groups in fluorophore. Furthermore, it was observed that except for fluorophore **5a** all compounds show similar fluorescence behavior with respect to absorption, emission, and Stoke's shift. The UV-Vis absorption and emission spectra of triazine derivatives **5a–f** were recorded in DMSO/H_2O (1:9) at room temperature using concentration 1×10^{-6} µL. The absorption and emission spectra of compounds **5a–f** are given in Figures 3 and 4, respectively. Absorption and emission wavelength range of all compounds are similar, which means that only the triazine and *N,N*-diethyl aniline groups are responsible for the delocalization of electrons from the electron donating terminus (*N,N*-diethyl) to the electron acceptor unit (1,3,5-triazine). Absorption, emission maxima, and Stoke's shift value are summarized in Table 1.

Protein labeling with fluorophores 5a–f

Synthesized fluorophores have good quantum yields, high fluorescence intensity, and contain biocompatible carboxylic acid groups which can be easily conjugated, so we explored fluorescence properties of new compounds after

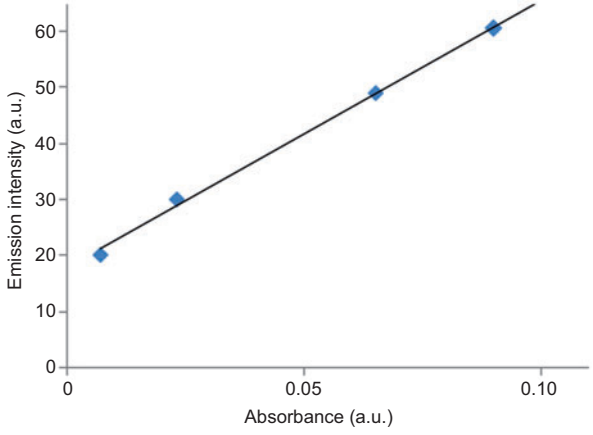


Figure 1 Graph of emission intensity vs. absorbance of standard anthracene for gradient calculation.

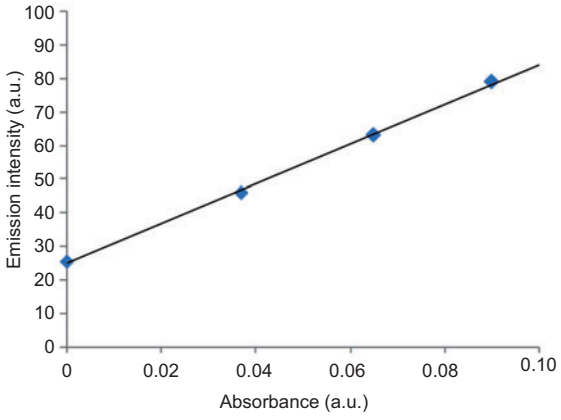


Figure 2 Graph of emission intensity vs. absorbance of compound **5c** for gradient calculation.

Gradient of anthracene	Gradient of compound 5c
$Gd_{ST} = Y_2 - Y_1/X_2 - X_1$	$Gd_{5c} = Y_2 - Y_1/X_2 - X_1$
$Gd_{ST} = (60.56–20.12)/(0.09–0.007)$	$Gd_{5c} = (79.12–46.10)/(0.09–0.037)$
$Gd_{ST} = 40.446/0.083$	$Gd_{5c} = 33.1/0.053$
$Gd_{ST} = 487.30$	$Gd_{5c} = 624.52$
$\Phi_{5c} = (624.52/487.30)\,\Phi_{ST}$	
$\Phi_{5c} = (1.2815)\,(0.27)$	
$\Phi_{5c} = 0.346$	

Table 2 Quantum yield calculation of compound **5c**.

conjugation with BSA. The extent of conjugation of BSA with fluorophores **5a–f** at various fluorophore/protein ratios was studied.

For protein labeling, the stock solution of protein (1 mg/1 mL) was made in water and stock solutions of fluorophores (different concentration: 0.1–1.4 ppm) were made in a DMSO/water mixture (0.5 mL DMSO/3.5 mL water). The stock solution of fluorophore was treated with 2 molar equivalents of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and *N*-hydroxysuccinimide and the mixture was stirred for 12 h at room temperature. After activation of fluorophore by carbodiimide chemistry, corresponding protein stock solution was added and the mixture was stirred for 12 h for conjugation with fluorophore. After 12 h of stirring the mixture was dialyzed in phosphate buffer (pH 7) to remove unconjugated fluorophore from the conjugated derivative. The amount of fluorophores **5a–f** needed for complete binding with available amino groups of BSA was optimized by varying concentration of fluorophore and BSA. At higher concentrations of fluorophore, complete binding takes place and graph of intensity against concentration of fluorophore becomes steady. This was observed before conjugation as well as after conjugation with BSA (Figure 5).

Before conjugation with BSA the fluorescence intensity increases as fluorophore concentration increases and becomes steady at particular concentration (Figure 6). After conjugation fluorescence decreases gradually and becomes steady at particular fluorophore concentration. A steady point in a graph indicates the amount of fluorophore required for complete binding of fluorophore with a certain amount of protein. The decrease in fluorescence is due to a change in microenvironment of the fluorophore. Only one carboxylic group in **5a–e** is likely to bind with the protein. Comparison of results of bioconjugation of mono-carboxylic **5f** and di-carboxylic compounds shows no difference in their trends in photophysical properties.

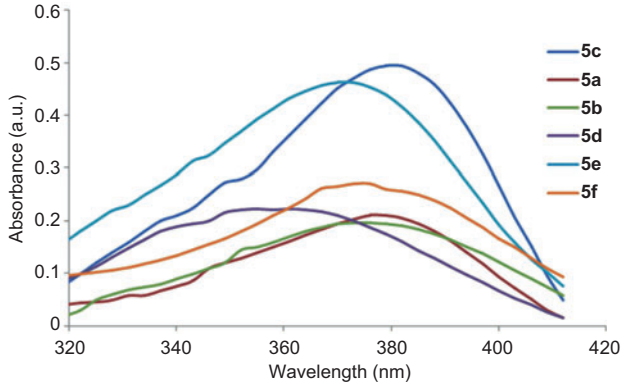


Figure 3 UV-Visible absorption spectra of compounds **5a–f**.

Conclusion

Six novel triazine-based fluorophores **5a**–**f** were synthesized, purified, and characterized by ^{1}H NMR, ^{13}C NMR, IR, UV-VIS, mass spectral, and elemental analyses. Fluorescence emission properties were studied. These fluorescent compounds are extremely fluorescent when irradiated under UV light and have potential use as fluorescent probes for protein labeling. Binding of the fluorophores with BSA is reported here.

Experimental

Biological and chemical materials

All commercial reagents and solvents were used without purification. BSA was purchased from Sigma Aldrich. Column chromatography was performed using silica gel 60–120 mm mesh size. Synthetic reactions were monitored by TLC on 0.25-mm silica gel 60 F_{254} precoated plates (Merck), which were visualized with UV light.

Instruments

The FT-IR spectra were recorded on a Perkin-Elmer 257 spectrometer using KBr discs. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian VXR 400-MHz and a 100-MHz instrument, respectively, using TMS as internal standard. Mass spectra (EI) were recorded on a Finnigan

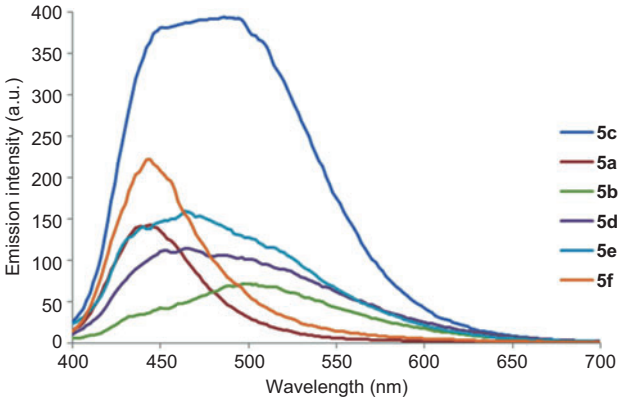


Figure 4 Fluorescence emission spectra of compounds **5a–f**.

instrument. Absorption spectra of the compounds were recorded on a Spectronic Genesys 2 UV-Visible spectrometer. Emission spectra were measured at room temperature on a Varian Cary Eclipse spectrofluorometer in a standard 1-cm quartz cell. All dyes were excited at the absorption maximum with an excitation slit width of 5 nm and emission slit width of 5 nm. The spectra were corrected. All concentrations of the fluorophores were between 0.1 ppm and 1.4 ppm. Elemental analysis was carried out by using a FLASH EA 1112 series instrument of Thermo Finnigan make.

Synthesis of 4-(4,6-dichloro-1,3,5-triazin-2-yl)-*N*,*N*-diethylaniline (3)

A mixture of *N*,*N*-diethylaniline (**2**) (27.0 g, 0.2 mol) and cyanuric chloride (**1**) (18.4 g, 0.1 mol) was heated at 70°C for 8 h under a slow stream of dry nitrogen. After completion of the reaction monitored by TLC, the mixture was extracted with hot chloroform (200 mL) and the white crystalline *N*,*N*-diethylaniline hydrochloride was removed by filtration. Slow cooling and evaporation of the chloroform extract to a volume of 50 mL yielded crystals of **3**. The product was crystallized twice from acetone (Padalkar, 2011) to give yellow crystalline solid; yield 11.7 g (40%); mp 156°C; IR: ν 3411, 2967, 1610, 1515, 1232, 1164, 839, 824, 715, 567 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ 8.29–8.33 (dd, 2H, J = 9.2, 2.8 Hz), 6.65–6.69 (dd, 2H, J = 9.2, 2.8 Hz), 3.44–3.46 (m, 4H), 1.22–1.25 (m, 6H); ^{13}C NMR

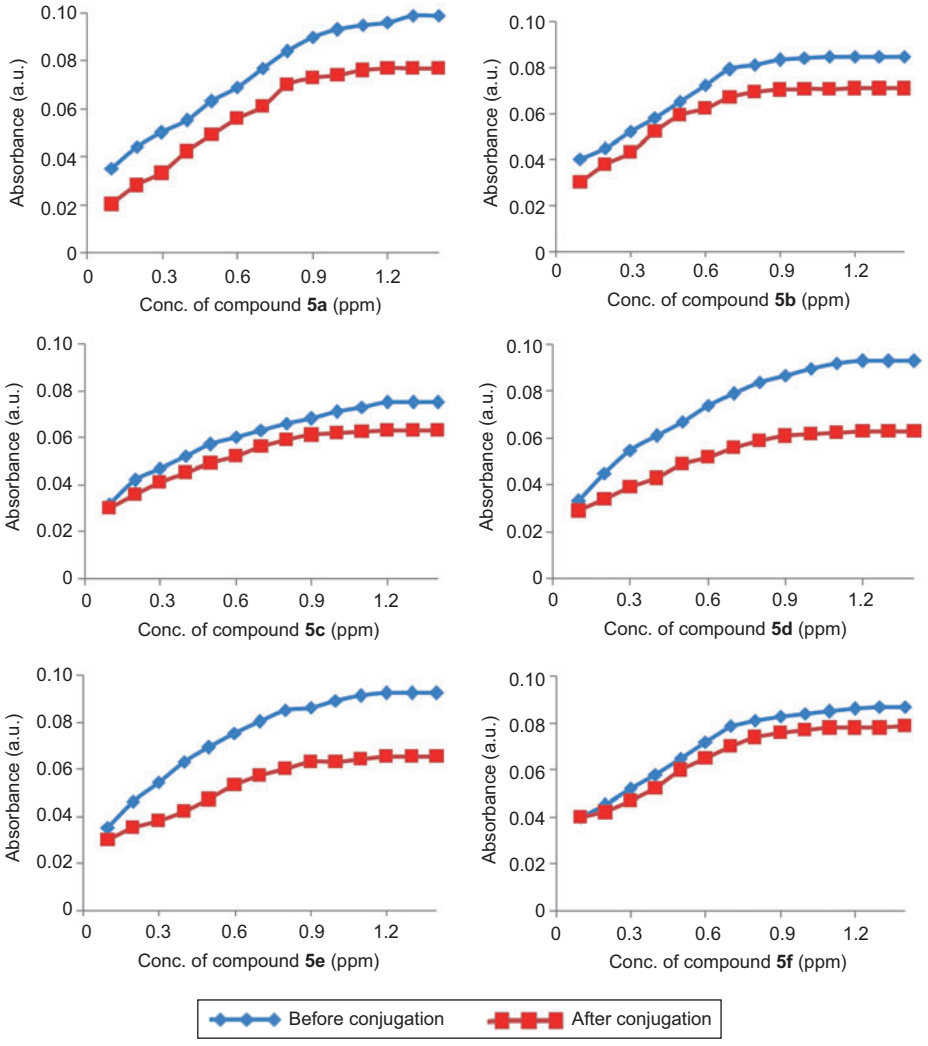


Figure 5 Absorption intensity graph of compounds **5a–f** before and after conjugation.

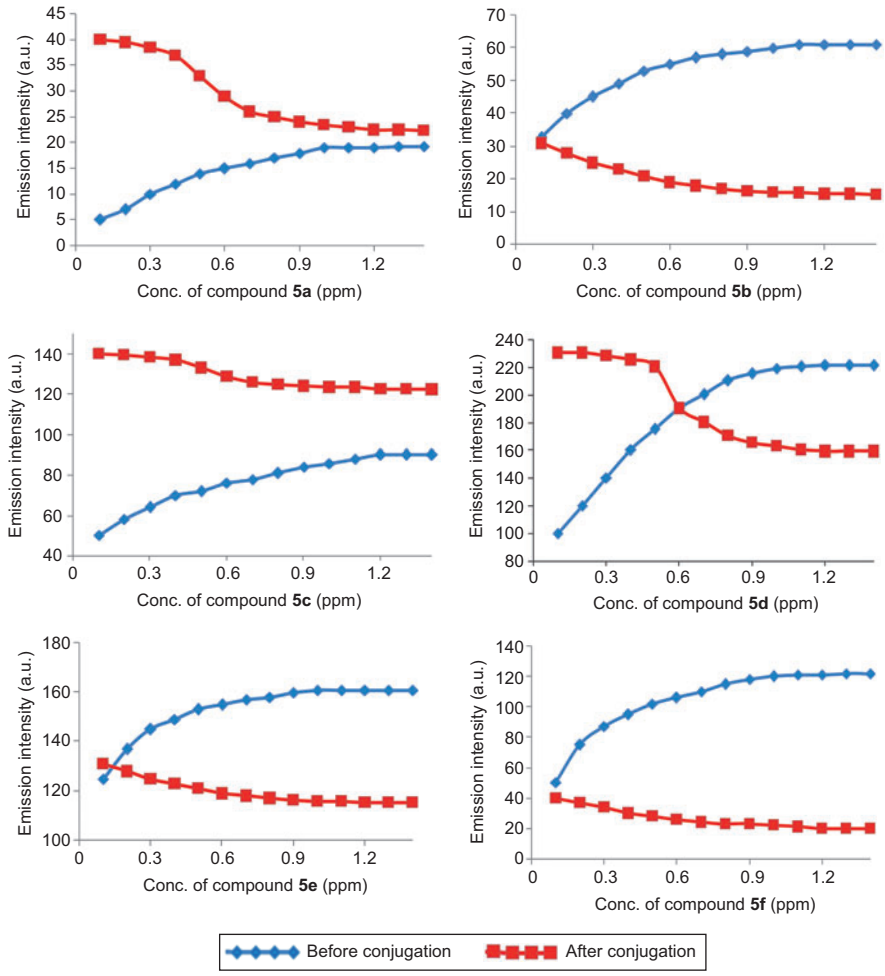


Figure 6 Fluorescence emission of compounds **5a–f** before and after conjugation.

($CDCl_3$): δ 179.5, 172.3, 154.2, 130.7, 125.6, 114.6, 49.0, 15.6; MS: m/z (%) 298 (34), 299 (100), 161 (13), 105 (11).

General procedure for synthesis of fluorophores 5a–f

A solution of α-amino acid **4a–f** (20 mmol) in methanol (30 mL) was treated with K_2CO_3 (20 mmol) and the mixture stirred at room temperature for 30 min. A solution of **3** (10 mmol) in methanol (10 mL) was added dropwise over the period of 15 min and the mixture was stirred at room temperature until TLC analysis indicated disappearance of **3**. Then the mixture was concentrated and the residue was purified by column chromatography eluting with MeOH/EtOAc, 1:9 to afford the product **5a–f**.

Dye 5a Yield 0.85 g (62%); dark yellow crystalline solid; mp 234°C; IR: ν 3398, 3305, 1733, 1644, 1570, 1509, 1415, 1212, 1149, 1078, 828, 783 cm^{-1}. ^1H NMR (DMSO-d_6): δ 12.59 (bs, 2H, -OH), 8.15 (bs, 2H, -NH), 8.07 (dd, 2H, J = 7.7, 1.5 Hz), 6.72 (dd, 2H, J = 7.7, 1.5 Hz), 3.66 (s, 4H), 3.46 (m, 4H), 1.15 (m, 6H); ^{13}C NMR (DMSO-d_6): δ 172.7, 165.0, 161.2, 148.9. 128.7, 125.2, 115.0, 49.3, 46.4, 15.6; MS: m/z (%) 375 (M+1, 67), 374 (100), 389 (49), 321 (23), 256 (43). Anal. Calcd for $C_{17}H_{22}N_6O_4$: C, 54.54; H, 5.92; N, 22.45. Found: C, 54.59; H, 5.89; N, 22.42.

Dye 5b Yield 1.147 g (67%); pale yellow solid; mp 220°C (dec.); IR: ν 3286, 1645, 1515, 1337, 1267, 1186, 786 cm^{-1}; ^1H NMR (DMSO-d_6): δ 12.12 (bs, 2H), 8.10 (dd, 2H, J = 12.4, 8.8 Hz), 6.68 (dd, 2H, J = 12.4, 8.8 Hz), 3.40 (q, 4H), 2.03 (m, 4H), 1.99 (m, 2H), 1.78 (bs, 2H), 1.86 (bs, 2H), 1.10 (m, 6H); ^{13}C NMR (DMSO-d_6): δ 175.5, 170.3, 161.9, 149.3, 129.0, 120.3, 112.4, 74.9, 49.0, 28.3, 15.6; MS: m/z = 467 (M+1, 38), 466 (100), 412 (48), 388 (72), 258 (38). Anal. Calcd for $C_{19}H_{26}N_6O_4S_2$: C, 48.91; H, 5.62; N, 18.01; S, 13.74. Found: C, 48.92; H, 5.67; N, 18.02; S, 13.75.

Dye 5c Yield 1.16 g (57%); yellow solid; mp 226–228°C; IR: ν 3286, 2928, 1650, 1518, 1337, 1267, 1191, 1148, 788, 698 cm^{-1}; ^1H NMR (DMSO-d_6): δ 12.13 (bs, 2H), 8.05 (dd, 2H, J = 9.2, 8.8 Hz), 7.20–7.35 (m, 10H), 6.68 (dd, 2H, J = 9.2, 8.8 Hz), 4.60 (bs, 2H), 3.12 (m, 4H), 2.67 (m, 2H), 2.48 (m, 4H), 1.05 (m, 6H); ^{13}C NMR (DMSO-d_6): δ 174.6, 164.8, 161.2, 149.6, 140.3, 136.9, 128.7, 127.8, 125.9, 124.6, 114.6, 72.6, 48.0, 35.7, 15.6; MS: m/z (%) 556 (M+1, 25), 557 (20), 278 (18), 256 (100), 225.3 (30). Anal. Calcd for $C_{31}H_{34}N_6O_4$: C, 67.13; H, 6.18; N, 12.12. Found: C, 67.20; H, 6.19; N, 12.08.

Dye 5d Yield 1.29 g (60%); yellow amorphous powder; mp 270°C (dec.); IR: ν 3277, 1684, 1340, 1269, 1194, 1076, 787, 740 cm^{-1}; ^1H NMR

(DMSO-d_6): δ 12.13 (bs, 2H), 9.90 (bs, 2H), 8.05 (dd, 2H, J = 7.4, 1.8 Hz), 7.12 (dd, 2H, J = 7.4, 2.4 Hz), 7.03 (m, 4H), 6.69 (m, 4H), 6.64 (dd, 2H, J = 9.2, 8.8 Hz), 4.72 (bs, 2H), 3.40 (m, 4H), 3.34 (m, 4H), 1.12 (m, 6H); ^{13}C NMR (DMSO-d_6): δ 171.6, 165.4, 162.2, 152.3, 148.7, 138.7, 130.0, 128.9, 124.6, 117.0, 113.1, 72.8, 64.3, 47.5, 15.1. MS: m/z (%) 587 (M+1, 56), 586 (100), 448 (34), 321 (23). Anal. Calcd for $C_{31}H_{34}N_6O_6$: C, 63.47; H, 5.84; N, 14.33. Found: C, 63.52; H, 5.87; N, 14.32.

Dye 5e Yield 1.94 g (84%); yellow solid; mp 263°C (dec.); IR: ν 3728, 2968, 1652, 1540, 1520, 1339, 1269, 1194, 1009, 784, 740, 699 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 12.42 (bs, 2H), 10.82 (m, 2H), 8.03 (dd, 2H, J = 8.0, 2.4 Hz), 7.30 (m, 2H), 7.18 (dd, 2H, J = 8.0, 2.4 Hz), 7.03 (m, 4H), 6.96 (dd, 2H, J = 7.4, 3.4 Hz), 6.63 (dd, 2H, J = 9.0, 2.2 Hz), 4.65 (s, 2H), 3.38 (d, 4H), 3.36 (m, 4H), 3.12 (m, 2H), 1.09 (m, 6H); ^{13}C NMR (DMSO-d_6): δ 175.3, 165.0, 160.1, 150.6, 143.4, 139.0, 137.1, 128.9, 125.6, 122.2, 120.6, 118.6, 112.9, 111.3, 99.7, 73.0, 48.8, 30.1, 14.9; MS: m/z (%) 633 (M+1, 40), 633 (100%), 451 (30). Anal. Calcd for $C_{35}H_{36}N_8O_4$: C, 64.44; H, 5.73; N, 17.71. Found: C, 64.80; H, 5.77; N, 17.69.

Dye 5f Yield 1.43 g (73%); brown-yellow solid; mp 208–210°C (dec.); IR: ν 3380, 2965, 1566, 1395, 1350, 1268, 1186, 1078, 923, 808 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 12.21 (s, 1H), 8.06 (s, 1H), 7.89 (s, 1H), 7.31 (s, 1H), 7.03 (dd, 2H, J = 7.3, 2.4 Hz), 6.63 (dd, 2H, J = 7.3, 2.4 Hz), 4.06 (s, 1H), 3.97 (m, 1H), 3.46 (m, 4H), 3.34 (m, 2H), 1.09 (m, 6H); ^{13}C NMR (DMSO-d_6): δ 177.5, 174.3, 169.0, 162.0, 150.2, 138.0, 132.4, 130.7, 125.6, 118.5, 113.8, 74.0, 48.0, 30.1, 15.4; MS: m/z (%) 414 (M+1, 43), 413 (78), 357 (100), 248 (45), 198.3 (32). Anal. Calcd for $C_{19}H_{22}ClN_7O_2$: C, 54.87; H, 5.33; N, 23.58. Found: C, 54.92; H, 5.37; N, 23.52.

General procedure for activation of fluorophore and labeling of protein

Protein labeling was carried out using *N*-hydroxysuccinimide and 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride. Stock solutions of fluorophores (different concentrations) were prepared in 0.5 mL DMF and 4.5 mL water. A mixture of **5a–f**, 2 molar equivalents of *N*-hydroxysuccinimide and 2 molar equivalents of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were stirred at room temperature for 12 h. Then, BSA (1 mg) was added to corresponding activated dye solution, and the mixture was stirred for an additional 12 h, after which time the labeled protein was separated from free fluorophore by dialysis in phosphate buffer of pH 7 (Singh et al., 2004).

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