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Synthesis and characterization of novel 4-(1-(4-(4-(4-aminophenyl)-*1H*-pyrazol-1-yl)-6-(4-(diethylamino)phenyl)-1,3,5-triazin-2-yl)-*1H*-pyrazol-4-yl)benzenamine fluorescent dye for protein binding

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ABSTRACT

Novel 4-(1-(4-(4-(4-aminophenyl)-1H-pyrazol-1-yl)-6-(4-(diethylamino)phenyl)-1,3,5-triazin-2-yl)-1H-pyrazol-4-yl)benzenamine fluorescent dye was synthesized by multistep synthesis from cyanuric chloride and phenyl acetonitrile. It has absorption at 360 nm with single emission at 497 nm having fairly good quantum yield (0.379). The intermediates and the dye were characterized by FT-IR, 1H NMR, 13C NMR and Mass spectral analysis. Its utility as biocompatible fluorescent dye was explained by conjugation with bovine serum albumin. The method is based on direct fluorescence detection of fluorophore labelled protein before and after conjugation. Purified fluorescent conjugates were subsequently analyzed by fluorimetry. The analysis showed that the tested conjugation reaction yielded fluorescent conjugates of the dye through carbodiimide chemistry.

1. Introduction

Protein recognition by synthetic molecules is a challenging endeavour, since these materials must bind to a large, relatively flat surface domain and recognize a unique distribution of amino acid residues of varying charges, sizes and shapes[1]. Identification and quantification of specific proteins is an important issue in medical and clinical research as many diseases have specific changes in protein expression[2]. The most commonly applied method in clinics is enzyme-linked immunosorbent assay (ELISA), which needs specific storage of active enzymes and tedious protein modifications[3]. Various strategies have been proposed to simplify the detection procedure, which include specific metal coordination, epitope-docking on miniature proteins, aptamer selection, non natural peptide isosteres, functionalized platforms, secondary structure mimetics, molecular imprinting and receptors

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embedded in lipid layers. Recognition of protein binding or change in the structure is determined with use of fluorescence [4], electrochemistry[5], raman spectroscopy[6], chemiluminescence[7], flow cytometry[8] and micro fluidic methods[9]. Nevertheless, most of these techniques need sophisticated instrumentation and proficient manipulation, which highly create necessary motivation for the development of simple and reliable protein detection systems.

The preferred fluorescent labels normally ought to maintain high fluorescence quantum yields and keep the biological activities of the parent unlabeled biomolecules. A fluorescent dye is normally attached to a peptide at a specific point through a covalent bond based on the sequence of peptide. The relationship between dye and peptide is a covalent bond, which is stable and not destructive under most biological circumstances. In special cases, a functional linker is used between dye and peptide for minimization the alteration of biological activity of peptide. For all the peptide labeling, the dye requires to be attached at a defined position: N-terminus, C-terminus, or in the middle of sequence. AAT Bioquest provides a variety of fluorescent labeling reagents for facilitating the conjugation of dyes to peptides those are used for a variety of biological studies[10].

Various fluorescent probes have been reported to study biological process through fluorescence measurements [11-13]. For the use as reporter molecule in biological systems many organic dyes have been investigated, like coumarin derivatives[14], fluorescein isothiocyanates [15,16], anthracene derivatives [17] and β-naphthol [18].

Dyes containing amino groups are used to modify peptides using water-soluble carbodiimides (such as EDC or DCC) to convert the carboxyl group of the peptide into amide group. Either NHS or NHSS may be used to improve the coupling efficiency of EDC-mediated protein–carboxylic acid conjugations. A substantially excess amount of the amine-containing dyes is normally implemented for EDC-mediated bioconjugations in concentrated large peptide solutions at low pH to reduce intra- and inter-protein coupling residues, a common side reaction.

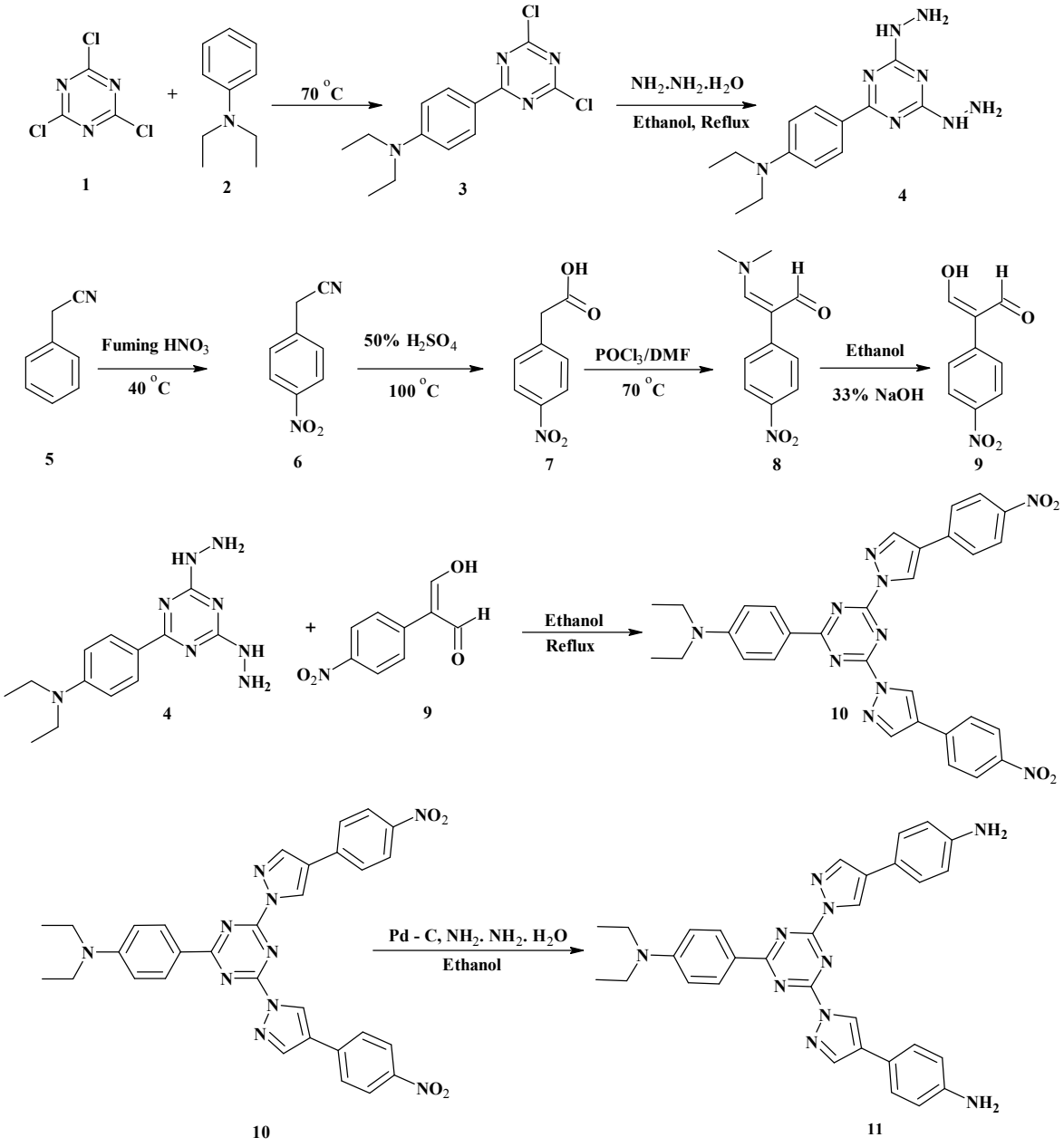
The spectral changes observed on the binding of fluorophores with proteins serve as important tool for the investigations of the topology of binding sites, conformational changes and characterization of substrate to ligand binding. In addition, determination of protein quantity in biological liquids plays essential role in biology and medicine where fluorescent probes are successfully applied for this approach [19].

2-Aryl-4,6-disubstituted fluorescent triazine derivatives are reported in literature as fluorescent probes [20], but they are not biocompatible, and have less photostability. As a part of our ongoing research to develop novel materials for high tech applications [21-27], here we report the synthesis, characterization and photophysical properties of novel fluorescent biocompatible fluorescence probe for protein binding contains a 1,3,5-triazine skeleton. The novel fluorophore was prepared from 4-(4,6-dichloro-1,3,5-triazin-2-yl)-*N*,*N*-diethylaniline and *Z*-2-(4'-nitrophenyl)-3-hydroxypropenal, **Scheme 1** and proposed mechanism as shown in **Scheme 2.**

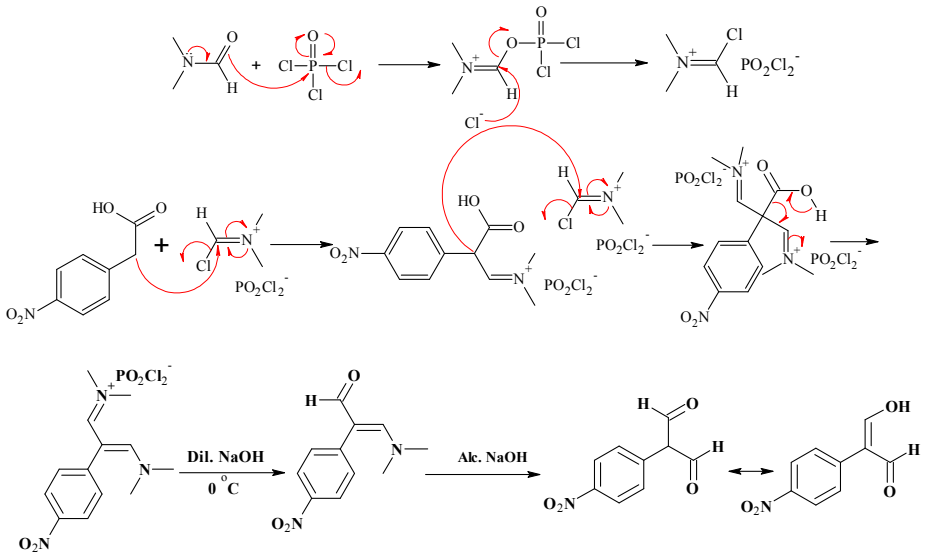
2. Results and discussion

The reaction of cyanuric chloride with *N,N*-diethyl aniline yielded the desired dichloro 1,3,5-triazine **3** which was further converted into 4-(4,6-dichloro-1,3,5-triazin-2-yl)-*N*,*N*-diethylaniline **4** by reaction with hydrazine hydrate in ethanol**.** 4-(4,6-Dichloro-1,3,5-triazin-2-yl)-*N*,*N*-diethylaniline **4** was further reacted with Z-2-(4'-nitrophenyl)-3-hydroxypropenals **9** in ethanol at reflux temperature for appropriate time to gave desired disubstituted triazine fluorophore **10** containing nitro group which is not biocompatible. The basic requirement of fluorophore to be good candidate is that it should contain biocompatible group for binding and high photostability with good quantum yield. Dinitropyrazoles triazine **10** was further reduced to diamine pyrazole triazine **11** by using Pd/C and hydrazine hydrate as shown in **Scheme 1,** and mechanism for formation of intermediate **9** is shown in

Scheme 2. The structures of the intermediates and product were confirmed by FT-IR, ^{1}H NMR and Mass spectral analysis. The synthesized novel fluorophore **11** is fluorescent in solution when irradiated with UV-light and framework contain biocompatible free amino group which was further explored for labeling with the protein, bovine serum albumin in presence of coupling agent *N*-hydroxy succinamide and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride**.** In order to warrant an efficacious label the unbound fluorophore was efficiently removed by dialysis in phosphate buffer solution at pH 7. Conjugated protein with fluorophore was further analyzed by using fluorescence emission analysis.



Scheme 1. Synthesis of 4-(1-(4-(4-(4-aminophenyl)-1H-pyrazol-1-yl)-6-(4-(diethylamino) phenyl)-1,3,5-triazin-2-yl)-1H-pyrazol-4-yl)benzenamine



Scheme 2. Proposed Mechanism for Intermediate **9**

2.1. Photophysical Properties

An effective fluorescent fluorophore for biological application has to present a good fluorescent intensity and high quantum yield with high photostability. Quantum yield of fluorophore **11** was determined by using anthracene as standard. The absorption and emission characteristics of standard as well as compound **11** were measured at different concentration of unknown samples and standard at (2 ppm, 4 ppm, 6 ppm, 8 ppm and 10 ppm level). Absorbance intensity values were plotted against emission intensity values. A linear plot was obtained. Gradients were calculated for compound **11** and for standard. All the measurements were done by keeping the parameters constant such as same solvent and constant slit width. Relative quantum yield of synthesized fluorophore **11** was calculated by using **Equation 1**[28]. The fluorescence quantum yield of **11** was recorded in ethanol at room temperature. Quantum yield of dye **11** is 0.379 in ethanol.

Equation 1: Relative fluorescence quantum yield

$$\Phi x = \Phi_{ST} (Gradx / Grad_{ST}) (\eta^2 x / \eta^2 ST)$$

where:

Φ_X = Quantum yield of unknown sample
Φ_{ST} = Quantum yield of standard used
$Grad_X$ = Gradient of unknown sample
$Grad_X$ = Gradient of standard used
η^2_{ST} = Refractive index of solvent for standard sample
η^2_X = Refractive index of solvent for sample

Gradient of standard and unknown; quantum yield calculation of novel compound **11** is described in **Figs. 1, 2** and **Table 1,** respectively.

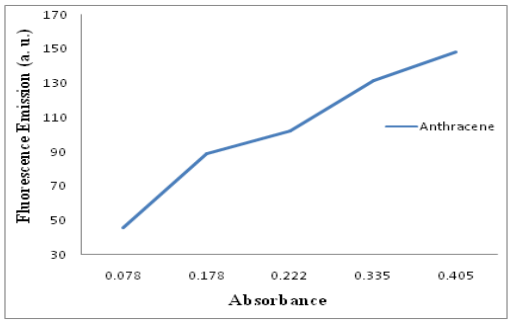


Fig. 1. Graph of emission intensity Vs absorbance of standard anthracene for gradient calculation

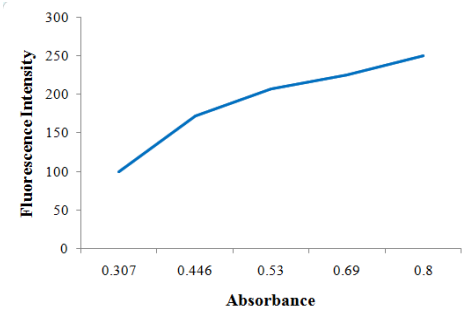


Fig. 2. Graph of emission intensity Vs absorbance of compound 11 for gradient calculation

Table 1 Quantum Yield Calculation of compound **11**

Gradient Anthracene	**Gradient of Compound 11**
$Gd_{ST}=Y_2 - Y_1/ X_2 - X_1$	$Gd_{16}=Y_2 - Y_1/ X_2 - X_1$
Gd_{ST} = (148.08 – 45.85) / (0.405 – 0.078)	Gd_{16}= (207.42 – 110.00) / (0.530- 0.307)
Gd_{ST} = 102.23 / 0.327	Gd_{16} = 97.47 / 0.223
Gd_{ST} = 312. 62	***Gd_{16} = 435.06***
Φ_{11} = (437.06 / 312.6) ϕ_{ST}	
Φ_{11} = (1.402) (0.27)	
ϕ_{16} = 0.379	

By putting value of gradients in **Equation 1**, quantum yield was calculated for dye by taking anthracene as standard (Quantum yield of anthracene in ethanol solution is 0.27).

*2.2. Protein binding with fluorophore **11***

Binding of dye **11** with protein was confirmed by change in the fluorescence wavelength before and after conjugation. Observed difference between fluorescence emission after conjugation and before conjugation is 37 nm. Measurement was performed at very low concentration of dye (0.001 mg, 10 ppm concentration)**.** The change in emission wavelength after conjugation is due to formation of covalent linkage between carboxyl group of protein and amino group of dye. This dye **11** was proposed to bind with carboxylate group on protein surface. Accordingly, the binding number of dye per protein molecules depends on the accessible number of carboxylate groups on each type of protein. The conjugation extent of bovine serum albumin with fluorophore **11** at various concentration of dye was studied. The amount of dye bound to protein increased gradually as the dye concentration increases (0.001 to 0.022 mg/10 mL). At higher dye concentrations the fluorescence intensity became steady, probably due to saturated binding between dye and protein **Fig. 3**

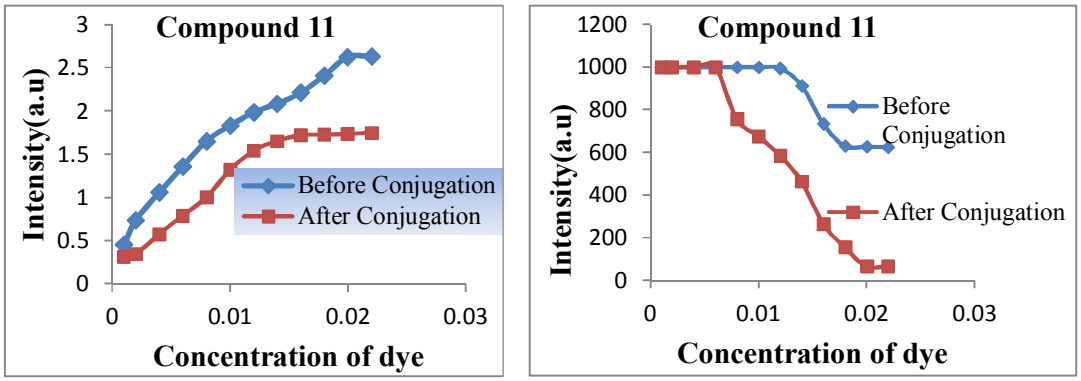


Fig. 3. Fluorescence absorption and emission intensity of fluorophore **11** before and after conjugation with protein bovine serum albumin

3. Conclusion

Fluorescent diamine pyrazole triazine fluorophore was synthesized, purified up to optical purity grade and characterized by ^{1}H-NMR, FT-IR and Mass spectral analysis. This fluorescent compound is extremely fluorescent when irradiated with UV-light and have a potential use for protein conjugation. The method is based on the direct fluorescence detection of protein-labelled with triazine fluorophores after dialysis. The more evident fluorescent signal was obtained at very little concentration of fluorophore (0.001 mg/10 mL) and easily detectable in near visible region which indicates an important application for this compound as fluorophore for protein assay.

Acknowledgement: Authors are thankful to Indian Institute of Technology, Mumbai for recording NMR and Mass spectra.

4. Experimental Procedure

4.1. Material and Methods

All reagents and solvents were purchased from s. d. fine chemicals (India) and were used without purification. Bovine serum albumin (BSA) was purchased from Sigma Aldrich. Column chromatography was performed using silica gel 60-120 mesh size. The reaction was monitored by Thin Layer Chromatography (TLC) using on 0.25 mm E-Merck silica gel 60 F_{254} precoated plates, which were visualized with UV light.

4.2. Absorption Spectra

Absorption spectra were measured at room temperature on a Perkin Spectronic Genesys 2 UV-Visible spectrophotometer. Absorption maxima were recorded with an accuracy of 0.5 nm.

4.3. Emission Spectra

Emission spectrum of the dye and dye-BSA was measured at room temperature on a Varian Cary Eclipse spectrofluorometer in a standard 1 cm quartz cell. Dye was excited with an excitation slit width of 5 nm and emission slit width of 5 nm. The spectra were corrected. All concentrations of the fluorophore were chosen to be between 0.001 and 0.022 μM.

4.4. General Procedure for activation of protein and labeling with fluorophore ***11***

Protein binding reactions were carried out using coupling reagent *N*-hydroxy succinamide (NHS) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). A stock solution of 2 mg dye was prepared in 1 ml DMF and 9 mL water. Various amounts of dye from the stock solution were used for protein binding at room temperature. To a solution of protein, excess of *N*-hydroxy succinamide and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride was added and mixture shake on shaker (150 rpm) at room temperature for 12 h. After activation of protein, different concentration of fluorophore **11** in dimethyl formamide: water was added in activated protein at room temperature for binding of protein and stirred for 12 h (**Fig. 4**). After 12 h, labelled protein was separated from free fluorophore by dialysis technique (**Fig. 5**), labelled protein contain fluorophore was dialyzed in phosphate buffer (pH: 7) to separate out unconjugated fluorophore from conjugated one. Dialyzed labelled protein was further analyzed by using fluorescence spectrophotometry to confirmed binding of fluorophore with protein.

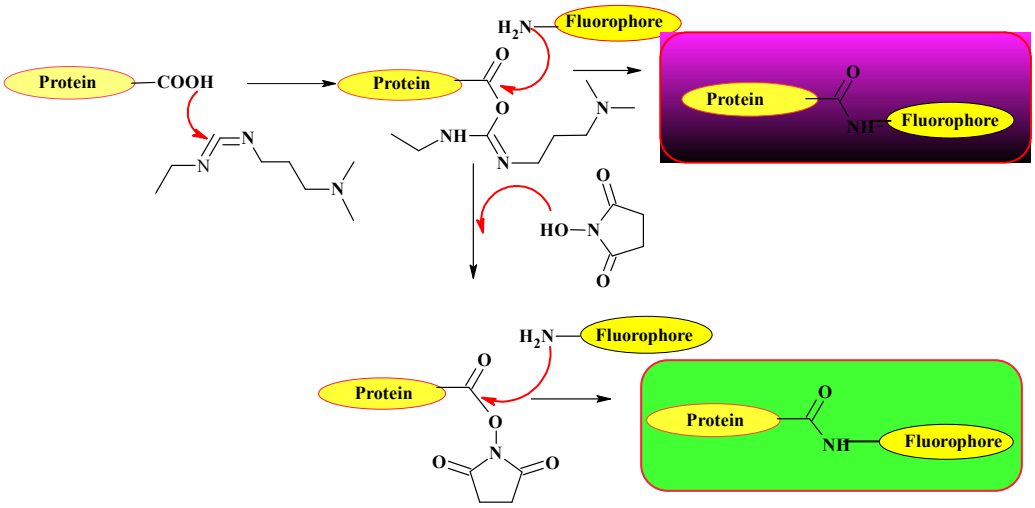


Fig. 4. Activation of protein for binding

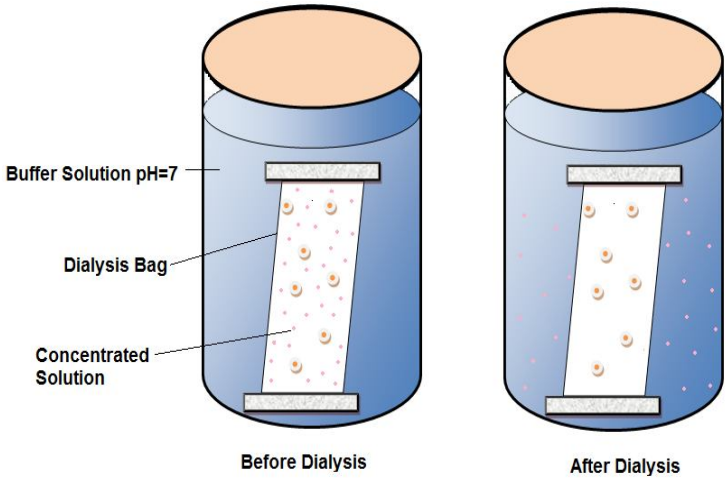


Fig. 5. Dialysis of conjugated protein

4.5. Instruments

The FT-IR spectra were recorded on Perkins-Elmer 257 spectrometer using KBr discs. ^{1}H NMR spectra were recorded on VXR 400-MHz instrument using TMS as an internal standard. Mass spectra were recorded on Finnigan mass spectrometer. The visible absorption spectra of the compounds were recorded on a Spectronic Genesys 2 UV-Visible spectrometer. Fluorescence emission analysis and quantum yield were carried out on Cary-Eclipse fluorescence spectrophotometer (Varian, Australia). Simultaneous DSC-TGA measurements were performed out on SDT Q 600 v 8.2 Build 100 model of TA instruments Waters (India) Pvt. Ltd.

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

A mixture of *N,N*-diethylaniline **1** (27.0 g, 0.2 mol) and cyanuric chloride **2** (18.4 g, 0.1 mol) was heated at 70 ºC for 8 h under a slow stream of dry nitrogen, after completion of reaction as monitored by TLC, the mixture was extracted with hot chloroform (200 mL) and the white crystalline hydrochloride salt of *N,N*-diethylaniline was removed by filtration. Slow cooling and evaporation of the chloroform extract to a volume of 50 mL yielded good crystals of **3**. The product was recrystallized two times from acetone.

Yield: 11.68 g, 40%; m.p.: 156 ºC; lit. 156 ºC [29] (Recrystallized from acetone).
FT-IR (KBr), υ max (cm^{-1}): 567, 715, 824, 839, 1164, 1232, 1515, 1610, 2967, 3411
^{1}H NMR (400 MHz) (δ, ppm): 1.22-1.25 (t, 6H, -CH_3), 3.44-3.46 (q, 4H, -CH_2), 6.65-6.69 (dd, 2H, *J* = 9.2, 2.8 Hz, Ar-H), 8.29-8.33 (dd, 2H, *J* = 9.2, 2.8 Hz, Ar-H).
^{13}C NMR (100 MHz) (δ, ppm): 15.6, 49.0, 114.6, 125.6, 130.7, 154.2, 172.3, 179.5.
Mass: m/e = 298 (M^+ + 1), 299 (M^+ + 2)

*4.7. 4-(4, 6-Dihydrazinyl-1,3,5-triazin-2-yl)-N,N-diethylaniline **4***

A mixture of 4-(4,6-dichloro-1,3,5-triazin-2-yl)-*N,N*-diethylaniline **3** (2.0 g, 0.0067 mol), hydrazine hydrate (0.65 g, 0.013 mol) and ethanol (20 mL) was refluxed for 1h, cooled, filtered and washed with ethanol to yield **4**.

Yield: 0.93 g, 96 %; m.p.: 265-268 ºC (Recrystallized from ethyl alcohol).
FT-IR (KBr) υ max (cm^{-1}): 567, 715, 824, 839, 1164, 1232, 1515, 1610, 2967, 3411 cm^{-1}.
^{1}HNMR (400 MHz) (δ, ppm): 1.13 (t, 6H, -CH_3), 3.44 (q, 4H, -CH_2), 4.31 (bs, 4H, -NH_2), 6.84 (d, 2H, Ar-H, *J* = 9.2 Hz), 8.14-8.34 (d, 2H, Ar-H, *J* = 9.2 Hz), 9.72 (bs, 2H, -NH).
Elemental Analysis: Calculated: C 54.15, H 6.99, N 38.86; Observed: C 54.19, H 6.97, N 38. 83

*4.8. 4-Nitrophenyl acetonitrile **6***

Concentrate nitric acid (specific gravity 1.42) (138 mL, 2.15 mol) was cooled to 0-10 ºC and to it was added phenylacetonitrile **5** (50 g, 0.425 mol) at such a rate that the temperature remained at about 10 ºC and did not exceed 20 ºC during addition. After the addition of phenyl acetonitrile the ice-cold bath was removed and the reaction mixture warmed at 40 ºC for 2h. From the reaction mass poured on crushed ice (500 g) separated a yellow solid. The solid was filtered, washed separately with ice-cold water to remove the trace of acid and dried to yield **6.**

Yield: 55 g, 79 %; m.p.: 114ºC (115-116 ºC lit [29]).

4.9. 4-Nitrophenylacetic acid 7

Concentrated sulfuric acid (150 mL, 2.75 mol) was added to water (150 mL) and to this mixture, 4-nitrophenylacetonitrile **6** (50 g, 0.31 mol) was added. The reaction mixture was refluxed for 30 min, diluted with 150 mL of water and cooled to 0 ^{o}C when colourless crystalline solid separated. The solid was filtered off, washed with ice-cold water to remove the trace of acid and dried to yield **7.**

Yield: 53 g, 95%; m.p.150 ^{o}C (151-152 ^{o}C lit [29]).

*4.10. Z-2-(4'-Nitrophenyl)-3-N,N-dimethylaminopropenal **8***

To a vigorously stirred solution of DMF (2.9 mL) at 0 ^{o}C, $POCl_3$ (2.8 mL) was added dropwise. After 5 min, the 4-nitrophenylacetic acid **7** (1.81g, 0.01mol) in solution of DMF (5 mL) was carefully added. The reaction mixture was warmed to 70 ^{o}C for 12h and then poured on ice, after neutralization by K_2CO_3, a solution of NaOH 50% (12 mL) was added and a precipitate was obtained upon cooling at 0^{o}C. The precipitate was dissolved in dichloromethane and recrystallized from diethyl ether to give yellow powder **8**.

Yield: 1.43 g, 65%, m.p. 131^{o}C (131^{o}C lit[29]), (Recrystallized from diethyl ether).
^{1}H NMR (400 MHz) (δ, ppm): 2.87 (s, 6H, HN $(CH_3)_2$), 6.97 (s, 1H), 7.35-7.38 (d, 2H, *J* = 8.7 Hz, Ar-H), 8.19-8.22 (d, 2H, *J* = 8.7 Hz, Ar-H), 9.12 (s, H, CHO).

*4.11. Z-2-(4'-Nitrophenyl)-3-hydroxypropenals **9***

33% NaOH (20 mL) was added to a solution of Z-2-(4'-nitrophenyl)-3-*N,N*-dimethylaminopropenal **8** (2.2 g, 0.01 mol) in ethanol (20 mL) and the reaction mixture was stirred at reflux for 3h. Ethanol was removed under reduced pressure and the residue was cooled down with ice to give a powder which was filtered and washed with CH_2Cl_2, then taken up in water and acidified by 6 N HCl to give precipitate, the solid was dissolved in acetone and recrystallized from ether to give **5** as a light brown powder **9**.

Yield: 1.37 g, 71%, m.p.:220 ^{o}C (223 ^{o}C lit [29]) (Recrystallized from diethyl ether).
^{1}H NMR (400 MHz) (δ, ppm): 7.82-7.85 (d, 2H, *J* = 8.8 Hz, Ar-H), 8.18-8.23 (d, 2H, *J* = 8.8 Hz, Ar-H), 8.64 (s, 2H, CHO), 14.45 (s, 1H, OH).

*4.12. Synthesis of 4-(4, 6-Bis (3-(4-nitrophenyl)-1H-pyrazol-1-yl)-1,3,5-triazin-2-yl)-N,N-diethylaniline **10***

A mixture of Z-2-(4'-nitrophenyl)-3-hydroxypropenals **9** (3.86 g, 0.02 mol), 4-(4,6-dihydrazinyl-1,3,5-triazin-2-yl)-*N,N*-diethylaniline **4** (2.98 g, 0.01 mol) and ethanol (40 mL) was refluxed for 5h, cooled and poured into ice water (100 mL) to yield **10.**

Yield: 4.92 g, 79 %, m. p.>300 ^{o}C (Recrystallized from ethyl alcohol)
FT- IR (KBr) υ max (cm^{-1}): 571, 715, 834, 839, 1164, 1427, 1534, 1643, 2967, 3426 cm^{-1}.
^{1}H NMR (400 MHz) (δ, ppm): 2.49 (t, 6H, $-CH_3$), 3.84 (q, 4H, $-CH_2$) 6.74-6.88 (d, 3H, *J* = 7.7 Hz), 6.90-7.12 (d, 3H, *J* = 7.8 Hz), 7.72 (d, 1H), 8.00-8.02 (d, 3H, *J* = 8.8 Hz), 8.24 – 8.27 (d, 2H, *J* = 8.0 Hz, Ar-H), 8.47 (d,1H), 9.09-9.11 (d, 2H), 9.19 (d, 1H).
Mass m/z: (M+1) 603.

*4.13. 4-(1-(4-(4-(4-Aminophenyl)-1H-pyrazol-1-yl)-6-(4-(diethylamino) phenyl)-1,3,5-triazin-2-yl)-1H-pyrazol-4-yl)benzene amine **11***

Palladium-carbon catalyst (0.01g, 10 %) was added portion-wise during 5-10 min to a hot solution of **10** (2.0 g, 0.003 mol) in ethanol (50 mL) containing hydrazine hydrate (1.16 g 0.023 mol). The mixture was heated under reflux for 1h. The hot solution was filtered through a whatmanpaper to remove Pd and further filtrate was filtered through silica gel (10 g) and the solvent was evaporated. Pure product was obtained.

Yield: 1.60 g, 89 %, m. p. > 300 ºC (Recrystallized from ethyl alcohol).
FT- IR (KBr) υ max (cm^{-1}): 804, 950, 1074, 1191, 1267, 1398, 1507, 1562, 2971 cm^{-1}.
^{1}H NMR (400MHz) (δ, ppm): 1.25 (t, 6H, $-CH_3$), 3.51 (q, 4H, $-CH_2$), 6.77-6.79 (t, 1H, *J* = 6.4, 7.8 Hz, Ar-H), 6.88-6.90 (d, 1H, *J* =7.2, 8.2 Hz, Ar-H), 7.01-7.09 (t, 1H, *J* = 7.8 Hz, Ar-H), 7.12 (d, 1H, *J* = 8.2 Hz, Ar-H), 7.74-7.75 (t, 1H, *J* = 4.8, Ar-H), 8.00-8.02 (d, 2H, *J* = 8.7 Hz), 8.24-8.27 (d, 2H, *J* = 8.7 Hz, Ar-H), 8.47 (t, 1H, *J* = 1.8, 7.8 Hz, Ar-H), 8.60 (d, 1H, *J* = 7.8 Hz, Ar-H), 8.75 (t, 1H, *J* = 3.4, 8.8 Hz, Ar-H), 9.10 - 9.17 (d, 2H), 9.19 (s, 1H).
Mass: m/z: (M+1) 542.4 (100 %), 544.0 (10 %).

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