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Design, synthesis, and biological evaluation of a novel class of fluorescein-based *N*-glycosylamines

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ABSTRACT

A series of fluorescein-based *N*-glycosylamines was synthesized from the corresponding fluorescein amine and a partially protected D-glucose. The physiochemical investigation of these compounds by spectral and morphological studies reveals their gelation potential. The exclusive localization of fluorescence in the cytoplasm through cell imaging studies reveals the anti-cancer potentials of *N*-glycosylamines.

1. Introduction

In recent years, fluorescein derivatives have played an important role in the field of drug discovery and gene delivery systems, cancer,[1] neurodegenerative diseases,[2] biosensors,[3–9] bioimaging,[10–12] and absorption studies of protein-based indicators.[13,14] Fluorescein was first developed in 19th century and has become widely known as a highly fluorescent molecule that emits longer wavelength light upon excitation at around 500 nm in aqueous media. These derivatives have been used as fluorescent tags for many biological molecules, such as proteins and DNA, as well as serving as a platform for many kinds of fluorescence probes.[15] In addition, disulfide-linked bioconjugate[16] and aldehyde-based cyclized flourescein[12] have been shown to specifically target intracellular glutathione by undergoing chemical modification to end up with the original unmodified drug as the product. Before binding, all these probes are almost non-fluorescent, but upon binding with target molecules, tagging or binding, these compounds become highly fluorescent. Thus, the current research is mainly focused on the identification of nonspecific but equally effective water-soluble, thiol-targeting probes that are membrane-permeable and form less toxic end products. Herein, we report the synthesis, characterization, gelation, and cell imaging studies of novel class of fluorescein-based *N*-glycosylamines. Generally, fluorescein-based organic molecules are synthesized in multistep reaction sequences that result in only poor yields,[17] whereas the current study reports, a facile synthesis of fluorescein-based *N*-glycoslamine derivatives carried out using fluorescein-based amines and partially protected D-glucose that results in good yields of fluorescent products.

2. Results and discussion

2.1. Synthesis of fluorescein-based *N*-glycosylamines

Fluorescein (**1a**),[18] 4′,5′-dimethylfluorescein (**1b**),[19] 4,6-*O*-butylidene-D-glucopyranose (**BGP**),[20,21] 4,6-*O*-ethylidene-D-glucopyranose (**EGP**),[22] and 4,6-*O*-benzylidene-D-glucopyranose (**BzGP**)[23] were synthesized by adopting literature procedures. The products were characterized by spectral techniques. Fluorescein (**1a**), and 4′,5′-dimethylfluorescein (**1b**) were reacted with *p*-nitrobenzoyl chloride and *p*-nitrobenzyl bromide to give the corresponding etherified **2a** and **2b**, and esterified **3a** and **3b** fluorescein nitro derivatives in 48–60% yields, respectively.[24–27] The nitro group of etherified and esterified fluorescein derivatives was reduced using Pd/C (10% activated) in H_2(g) to give the corresponding fluorescein-based etherified **4a** and **4b**, and esterified amine derivatives **5a** and **5b** in 60–80% yields, respectively. **BGP**, **EGP,** and **BzGP** were further reacted with the fluorescein amine derivatives to give the corresponding

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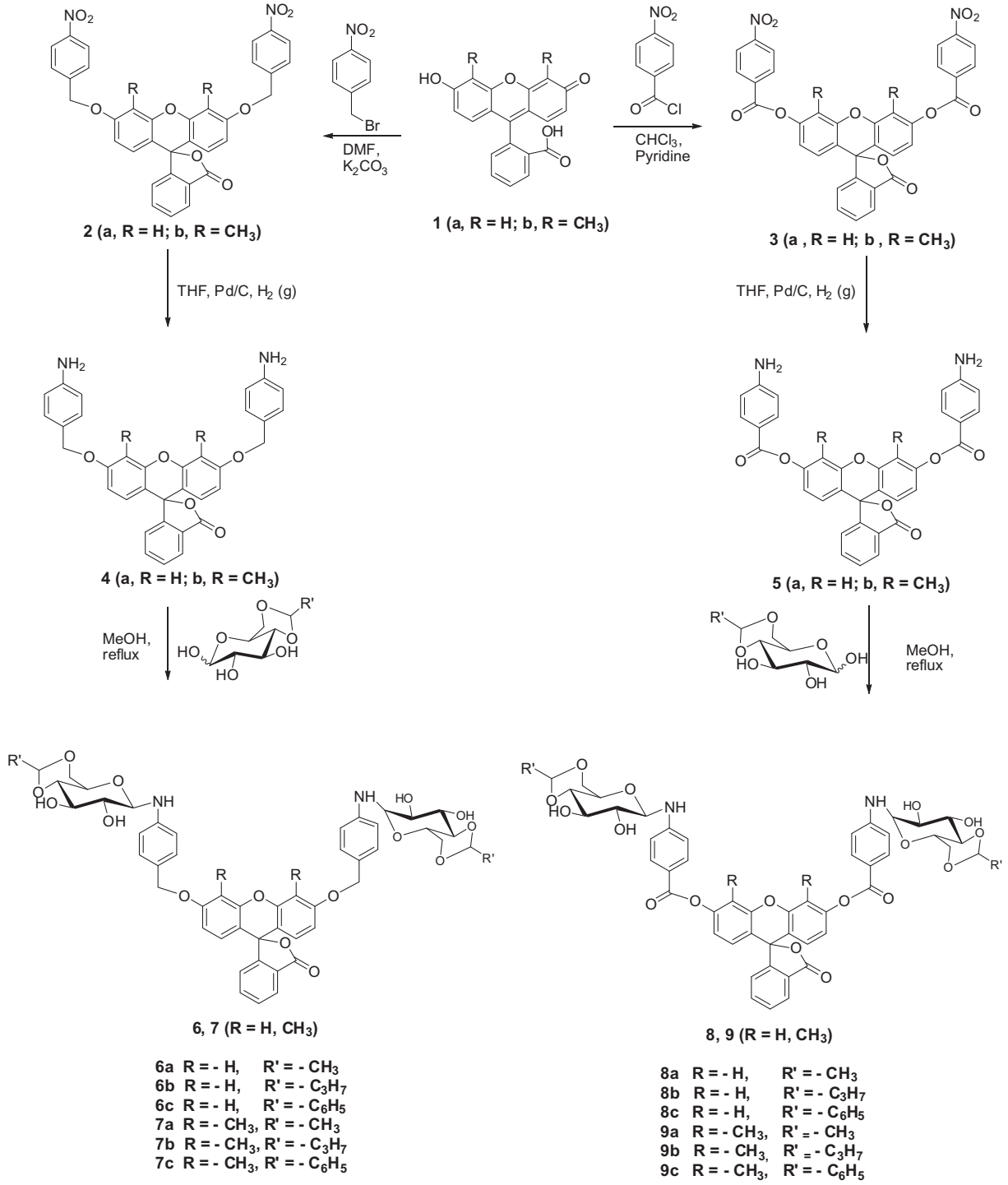
fluorescein-based N-glycosylated products **6**(**a**–**c**), **7**(**a**–**c**), **8**(**a**–**c**), and **9**(**a**–**c**) in 44–59% yield (Scheme 1). The ^{1}H NMR spectra of N-glycosylated products **6**(**a**–**c**), **7**(**a**–**c**), **8**(**a**–**c**), and **9**(**a**–**c**) showed the glycosylic-NH resonance at ~4.5–6.0 ppm, and the anomeric proton at ~4.5 ppm. The chemical shift position of the acetal protons change with respect to the protecting group (**BGP**: δ 4.62–4.71, **EGP**: δ 4.48–4.51, **BzGP**: δ 5.52–5.57 ppm), and the existence of the β-anomeric proton at ~4.5 ppm in both the esterified and etherified fluorescein-based *N*-glycosylamine derivatives were identified from the corresponding coupling constant values (J = 4.9–9.1 Hz) (Table 1).

2.2. Absorption and emission studies

Fluorescein-based *N*-glycosylamines **8**(**a**–**c**)–**9**(**a**–**c**) show characteristic absorption and emission bands at around ~490 nm and ~550 nm, respectively. The band that appeared between 440 and 490 nm in these N-glycosylated fluorescein derivatives is due to a π–π^* transition. Upon irradiation of the N-glycosylated product at 490 nm, an emission band was observed at ~550 nm. Moreover, a marginal shift of ~5 nm has been observed among the different N-glycosylated fluorescein derivatives **6**(**a**–**c**)–**9**(**a**–**c**). However, the methyl-substituted fluorescein-based *N*-glycosylamines **9**



Scheme 1. Synthesis of fluorescein-based *N*-glycosylamines.

Table 1
Synthesis and spectral data of fluorescein-based *N*-glycosylamines **6**(**a**–**c**)–**9**(**a**–**c**)

Compounds	*t* (h)	NMR data δ H-1, $^{3}J_{H1,H2}$/Hz	Yield (%)	CGC (%) g mL^{-1}
6a	8	4.66, 7.8	59	1.5
6b	8	4.55, 5.8	53	1.0
6c	6	4.61, 7.8	52	1.0
7a	8	4.61, 6.9	44	1.0
7b	10	4.56, 5.0	54	1.0
7c	8	4.68, 6.9	46	1.5
8a	8	4.71, 4.5	56	-
8b	8	4.57, 4.6	55	-
8c	6	4.65, 9.1	46	-
9a	8	4.76, 5.1	53	-
9b	10	4.55, 4.9	50	-
9c	8	4.62, 6.8	58	-

(**a**–**c**) show a bathochromic shift of ~15 nm, which may be due to the effect of substituent. Among the six different fluorescein-based *N*-glycosylamine derivatives studied, methyl substituted 4,6-*O*-benzylidene derivative (**9c**) showed a maximum shift of ~15 nm, which may be due to the presence of substituent on the aromatic fluorescein ring (Fig. 1).

2.3. Gelation studies

Recently, we have reported the gelating abilities of different classes of sugar derivatives[28] and have observed that *N*-glycosylamines are better candidates for gel formation (CGC: 1%). These findings prompted us to investigate the gelating ability of fluorescein-based *N*-glycosylamines. The presence of more aromatic moiety favors π–π stacking of the fluorescein-based N-glycosylated molecules, which is a pre-requisite for gelating ability. All gel samples were prepared by dissolving the gelator in a solvent in such a way that it forms a homogenous solution. The solution was allowed to cool down to room temperature, whereby the gel is formed. The gelation ability of fluorescein-based *N*-glycosylamines **6**(**a**–**c**)–**9**(**a**–**c**) has been assessed by using 'stable to inversion of the container' method.[29] The study includes twelve different ether-**6**(**a**–**c**) and **7**(**a**–**c**) and ester-based **8**(**a**–**c**) and **9**(**a**–**c**) fluorescein *N*-glycosylamines in 21 different solvents [see Supplementary data for more details], and the results of gelation are summarized in Table 2.

However, formation of gel has been observed with ether-based fluorescein *N*-glycosylamines **6**(**a**–**c**) and **7**(**a**–**c**). In general, protecting groups, such as, ethylidene, butylidene, and benzylidene present

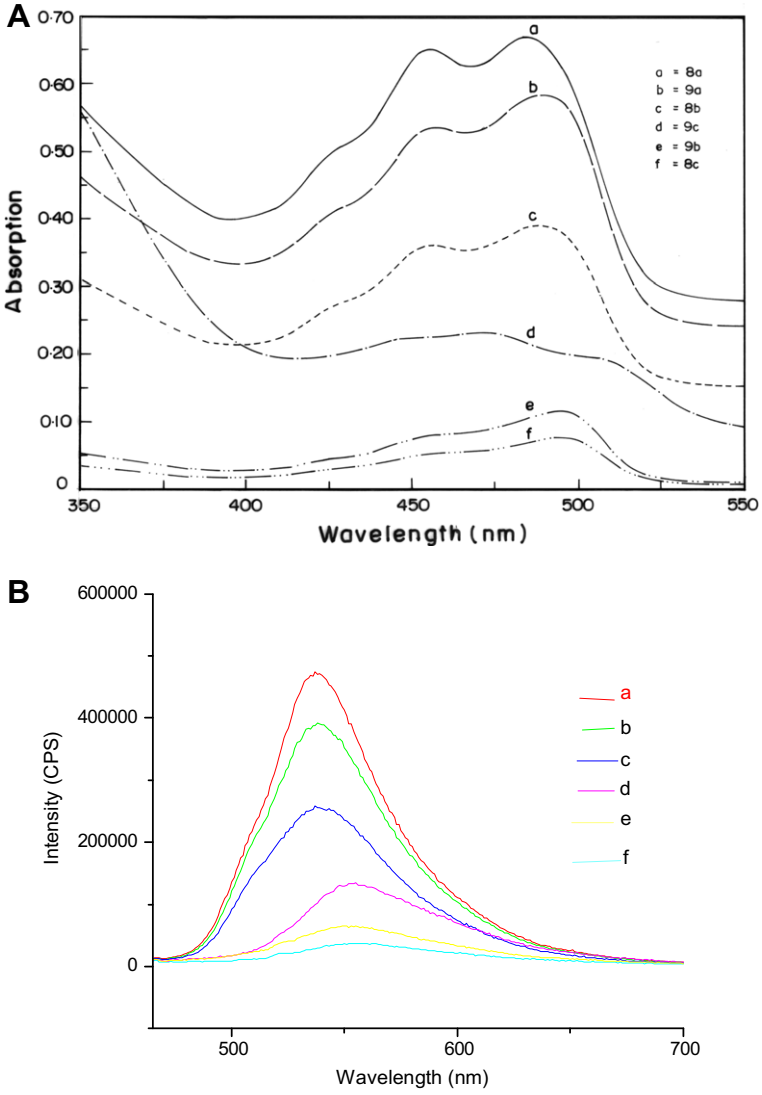


Figure 1. (A) Absorption spectra of fluorescein-based *N*-glycosylamines: (a) **8a**; (b) **9a**; (c) **8b**; (d) **9c**; (e) **9b**, and (f) **8c**; (B) emission spectra of fluorescein-based *N*-glycosylamines, (a) **8a**; (b) **9c**; (c) **8c**; (d) **9a**; (e) **8b**, and (f) **9b** recorded at 2.5×10^{-4} M concentration in DMSO at 298 K (λ_{ex} = 490 nm).

on the D-glucose moiety and also the substituents present on the aromatic ring showed remarkable changes in the gelating ability. Among the various polar and nonpolar solvents used for gelation, aromatic solvents, such as 1,2-dichlorobenzene (1,2-DCB), *o*-xylene, *m*-xylene, benzene, and toluene were found to be the best solvents, which may be attributed to a strong solute–solvent interaction and the corresponding critical gelation concentrations (CGC) of the gelators **6**(**a**–**c**)–**9**(**a**–**c**) are given in Table 1. Among the six fluorescein-based *N*-glycosylamine derivatives studied, *N*-glycosylamines **6b**, **6c**, **7a,** and **7b** act as efficient organogelators and gelate at lower concentrations (CGC = 1%). Thus, the gelation ability of the *N*-glycosylamine-based organogelator has a significant dependence on the protecting group, and also the substituents present on the aromatic ring as we have reported in the case of sugar–heterocyclic derivatives.[30–34] The greater gelation ability of 4,6-*O*-butylidene-D-glucopyranose (BGP), and 4,6-*O*-ethylidene-D-glucopyranose (EGP) derivatives are due to higher London dispersion forces that exist between the alkyl chain groups. However, in case of 4,6-*O*-benzylidene-D-glucopyranose (BzGP) derivatives, the π–π interactions seem to be largely responsible for the gelation properties. In addition, anisotropic interactions through H-bonding, CH–π, π–π stacking, and dipole–dipole interactions are also responsible for the linear dipole–dipole interactions of low molecular weight organogelators that allow gel formation.

Table 2
Gelation studies of ether-based fluorescein *N*-glycosylamines **6**(**a**–**c**)–**7**(**a**–**c**)

Solvent/compounds	6a	6b	6c	7a	7b	7c
C_6H_6	G	S	G	G	G	G
$NO_2C_6H_5$	G	S	G	G	G	G
$CH_3C_6H_5$	G	S	G	G	G	G
$(Cl)_2C_6H_4$	G	G	G	G	G	G
p-Xylene	PG	G	G	G	G	G
m-Xylene	G	PG	G	G	G	G
o-Xylene	G	PG	G	G	G	G

Note: G = good gelators; PG = partial gelators; S = solution.

2.4. Morphological studies

Scanning electron microscopy (SEM) images show the presence of elongated nanofibers, which give visual insight into the aggregation modes. SEM images of etherified fluorescein-based *N*-glycosylamines **6**(**a**–**c**) and **7**(**a**–**c**) show well-defined 3D fibrous networks as well as bundles of thin flakes (Fig. 2a–f). Morphological images of gels derived from *N*-glycosylamines **6**(**a**–**c**), **7a,** and **7b** appeared as smooth sheets or ribbons (Fig. 2a–e). The fibrous assemblies also been observed, along with the unique flower-shaped morphologies in case of *N*-glycosylamine **6c** and **7a**

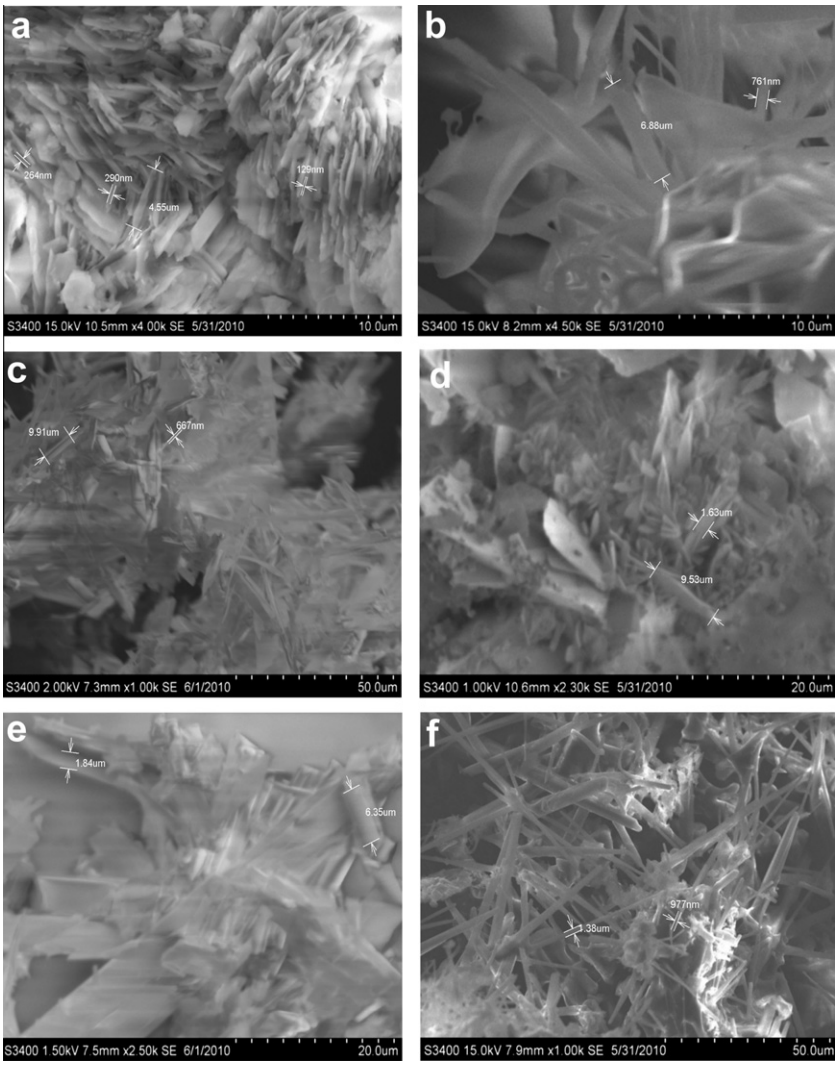


Figure 2. SEM images of organogel (pore labeled) formed from, (a) **6a** in 1,2-DCB; (b) **6b** in 1,2-DCB (flower); (c) **6c** in 1,2-DCB; (d) **7a** in benzene; (e) **7b** in benzene (fiber), and (f) **7c** in benzene.

(Fig. 2c and d). The formation of more flat sheets or ribbon-type features has been observed with *N*-glycosylamine **6b**. However, all of these are composed of fibrous strands that are longer than 5 μm length and with diameter in the range of 20–60 nm (Fig. 2a–e). From the SEM images, the pore size of **7c** (CGC = 1%) is greater than that of **6a–c**, **7a,** and **7b** (CGC = 1.5%); hence, compounds **6c** and **7**(**a**–**c**) act as good gelators, compared to the other derivatives. Three-dimensional fibrous networks hold the solvent molecules together, which is due to the existence of surface tension in the gels. The SEM images of all the compounds are shown in Figure 2.

2.5. Thermal analysis

To study the thermal properties of the organogelators, and gels, Differential Scanning calorimetry (DSC) experiments for two different etherified fluorescein-based *N*-glycosylamines **6a** and **6c** were performed. The melting point and enthalpy of organogelator **6a** in the solid phase are $\Delta H = 9.5\ J\ g^{-1}$ and 125 °C, and in gel phase the values are $\Delta H = 190\ J\ g^{-1}$ and 110 °C. The organogelator **6c** shows enthalpy values and the melting point peaks for $\Delta H = 7.5\ J\ g^{-1}$ and 120 °C in the solid phase, and $\Delta H = 280\ J\ g^{-1}$ and 95 °C in the gel phase, respectively (Fig. 3).

The peak appearing at ~112 °C ($\Delta H = 138\ J\ g^{-1}$) is due to the liberation of moisture. These results indicate the thermal stabilities of both gel and organogelator. DSC studies further reveal that in the gel phase, the molecules are loosely packed and the system moves from the most ordered state to a less ordered state.

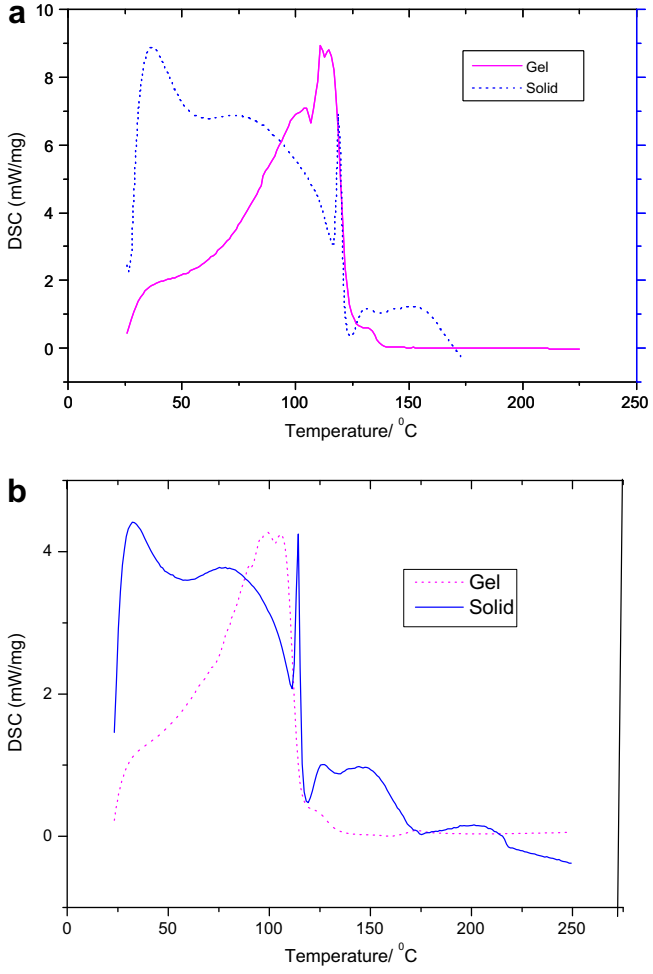


Figure 3. DSC spectra of fluorescein-based *N*-glycosylamines: (a) **6a** (gel formed from benzene); (b) **6c** (gel formed from 1,2-DCB).

Table 3
Thermodynamic parameters for sol–gel transition of fluorescein-based *N*-glycosylamines **6a** and **6c**

Compounds	T_{gs} (°C)	ΔH (J g^{-1})
6a	110	190
6c	280	95

The phase-transition temperature of compounds **6a**, and **6c** and their corresponding gels are given in Table 3. The melting temperatures of the gelator and gel are obtained from DSC experiments.

2.6. Cell imaging studies

Incubation of HT-29 cells with 40 μM fluorescein (**1a**) showed weak fluorescence at the emission wavelength of 520 nm. In contrast, 40 μM of esterified fluorescein-based *N*-glycosylamine **8b** showed a bright fluorescence at its emission wavelength of 550 nm. The bright fluorescence localized exclusively in cytoplasmic region of cells but not in the cell membrane. The background fluorescence was remarkably lower, and staining was seen profusely in cancer cells. This observation reveals that these molecules have strong potential to act as anti-cancer agents. In addition to fluorescein, sugar-based compounds have also been used in specifically targeting cancer cells.[35–37] 2-Deoxy- or 2-substituted amino sugar derivatives are precursors for synthesis of pharmaceutically and biologically important 2-deoxy-*N*-glycopeptides, which are normally used in the treatment of cancer and acquired immune deficiency syndrome (AIDS).

Recently, Rawal et al.[38] reported that 2-deoxy-*N*-glycopeptides have strong anticancer activity (IC_{50} 22 μM) against U-87 malignant glioblastoma (brain tumor) when compared to the normal human embryonic kidney (Hek) cell line. The preferential staining of the cytoplasm may most likely reflect binding to the cellular thiol-containing molecules like cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), which are concentrated more in the cytoplasm of cells. This notion is supported by the fact that fluorescein also shows a similar staining pattern, although at very low intensity. Rhodamine,[16] derived from the same parent fluorescein, has been shown to preferentially bind glutathione. It is possible to perturb the amount of cellular glutathione (GSH) by pre-incubating the cells with the known thiol-blocking agent, diethyl maleate (DEM). To prove the hypothesis that esterified fluorescein-based *N*-glycosylamine **8b** targets cellular glutathione, we incubated HT-29 cells with 0.5 mM DEM, a concentration effective to block GSH in hepatocytes,[39] followed by esterified fluorescein-based *N*-glycosylamine **8b**, and measured the cellular fluorescence (Fig. 4).

The level of cellular fluorescence was dramatically lower following pre-treatment with DEM, indicating that esterified fluorescein-based *N*-glycosylamine **8b** is responsive to intracellular GSH concentrations. Hence, the ability of esterified fluorescein-based *N*-glycosylamine **8b** probes to image endogenous thiols offer new promise in significant applications to evaluate oxidative stress in biological systems during which glutathione levels are frequently altered. Esterified fluorescein-based *N*-glycosylamine **8b** can produce discernable change, and specific cytoplasmic localization even though the parent compound fluorescein has relatively weak fluorescence.

In summary, a novel class of fluorescein-based *N*-glycosylamines have been synthesized and characterized using different spectral techniques. The etherified fluorescein-based *N*-glycosylamines are found to be good organogelators and are able to gelate even at CGC 1 w/v %. Morphological and thermal studies show the various modes of aggregation and stability of gels, respectively, which de-

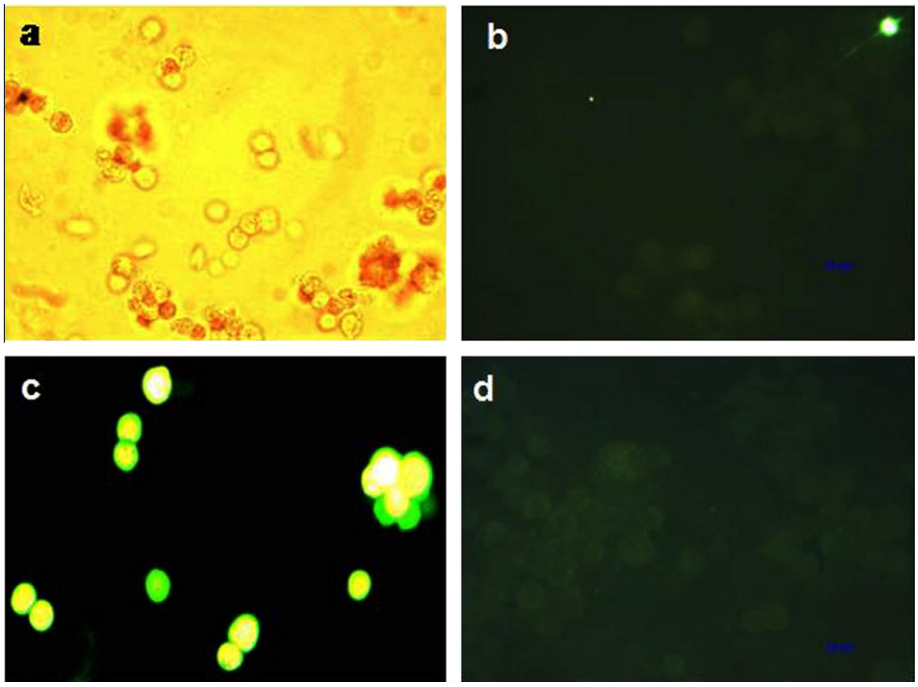


Figure 4. Fluorescence images of live HT-29 cells, (a) phase-contrast image of HT-29 cells; (b) HT-29 cells treated with 40 μM fluorescein; (c) HT-29 cells treated with 40 μM esterified fluorescein-based *N*-glycosylamine (**8b**); (d) HT-29 cells pre-incubated with 500 μM DEM and treated with 40 μM esterified fluorescein based *N*-glycosylamine (**8b**).

pend on the protecting groups and also on the substituents in the fluorescein moiety. The exclusive localization of fluorescence in the cytoplasm through cell imaging studies reveals the anti-cancer potential of *N*-glycosylamines.

3. Experimental

D-Glucose, resorcinol, 2-methylresorcinol, and phthalic anhydride were purchased from Sd-fine, India. *p*-Nitrobenzoyl chloride and *p*-nitrobenzyl bromide were purchased from Sigma–Aldrich Chemical Co., USA and were of high purity. Paraldehyde, *n*-butyraldehyde, catalyst Pd/C, pyridine, potassium carbonate, and zinc chloride were purchased from SRL, India. Chloroform, methanol, DMF, and THF were used after distillation. Column chromatography was performed on silica gel (100–200 mesh). NMR spectra were recorded on Bruker DRX 300 MHz, and JEOL GSX 400 MHz spectrometers. Elemental analysis was performed by using Perkin–Elmer 2400 series CHNS/O analyser. ESI mass spectra were recorded on JEOL JMS-D-300 spectrometer with an ionization potential of 70 eV, and ES mass spectra were determined on a Micromass Quattro-II instrument. The gels were imaged with a HITACHI-S-3400 W scanning electron microscope. All absorption spectra were obtained with a UV-1600 UV/vis spectrometer (Shimadzu). All fluorescence spectra were obtained with an F4500 fluorescence spectrometer (Hitachi) and optical rotations were performed using a Rudolph Autopol II digital polarimeter.

3.1. Synthesis of gels

The gelation ability of fluorescein-based *N*-glycosylamines was determined by the 'stable to inversion of the container' method.[29] The gelation properties have been tested with 21 different solvents as follows: gelator (1.5 mg) was mixed in a close-capped test tube with 1 mL of solvent to result in a concentration of 1.5% (g mL^{-1}), and the mixture was heated until the solid was dissolved. By this procedure, the solvent's boiling point became higher than that under standard atmospheric pressure. The sample vial was cooled in air to 25 °C, left for 12 h at this temperature, and then turned upside down. When the gelator formed a clear or slightly opaque gel by immobilizing the solvent at this stage, it was denoted by 'G'. Partial gel is denoted as PG, insoluble is as I. Some samples remained as solutions and are denoted as S. Those that precipitated were denoted as P.

3.2. Cell preparation

HT-29 cells were grown in 25 cm^2 UV-sterilized flasks to 70–80% confluence at 37 °C in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen catalog No. 31053-028) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen). For labeling, the cells were washed once with DMSO supplemented with 2 g/L D-glucose and 20 mM HEPES buffer pH 7.4, then incubated with 40 μM fluorophore in DMSO at 37 °C for 4 h. One set of cells were pre-incubated with 500 μM DEM, followed by incubation with 40 μM fluorophore in DMSO taken for imaging studies. Prior to imaging, the labeling solution was aspirated, and the cells were washed three times with phosphate-buffered saline (PBS).

3.3. Cell Imaging

Cells were imaged on a Carl Zeiss fluorescent microscope. Excitation light was provided by a 150 W Xe arc lamp transmitted through a 465–495 nm excitation filter and a 505 nm long-pass dichroic mirror, and fluorescence was measured after passage through a 513–558 nm emission filter.

3.4. General procedure for the synthesis of fluorescein dinitrobenzyl ether 2a and 2b

To a solution of fluorescein (1 mmol), and *p*-nitrobenzyl bromide (2 mmol) were added K_2CO_3 in DMF. The reaction mixture was stirred at reflux temperature for 18 h and then cooled to room temperature. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography.

3.4.1. Synthesis of fluorescein dinitrobenzyl ether 2a

Compound **2a** was obtained by the reaction of fluorescein **1a** (1 mmol, 0.33 g), and *p*-nitrobenzyl bromide (2 mmol, 0.43 g) as a brown solid. Yield: 0.31 g (51%); mp 144–145 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.00 (s, 2H, $-CH_2$), 5.18 (s, 2H, $-CH_2$), 6.59–6.88 (m, 6H, Ar-H), 7.03–7.31 (m, 3H, Ar-H), 7.58–7.74 (m, 4H, Ar-H), 8.02 (d, 3H, J = 7.5 Hz, Ar-H), 8.25–8.31 (t, 2H, J = 7.2 Hz, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 62.8, 68.6, 82.6, 101.8, 111.7, 111.9, 123.2, 123.6, 123.7, 124.7, 126.3, 126.7, 127.5, 129.0, 129.8, 135.1, 143.6, 146.6, 147.3, 149.9, 152.1, 152.4, 159.6, 169.0. Anal. Calcd for $C_{34}H_{22}N_2O_9$: C, 67.77; H, 3.68; N, 4.65. Found: C, 67.52; H, 3.59; N, 4.56.

3.4.2. Synthesis of 4′,5′-dimethylfluorescein dinitrobenzyl ether 2b

Compound **2b** was obtained by the reaction of 4′,5′-dimethylfluorescein **1b** (1 mmol, 0.36 g), and *p*-nitrobenzyl bromide (2 mmol, 0.43 g) as a brown solid. Yield: 0.33 g (53%); mp 158–160 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.28 (s, 6H, $-CH_3$), 5.01–5.26 (m, 4H, $-CH_2$), 6.61–6.78 (m, 5H, Ar-H), 6.89–7.17 (m, 4H, Ar-H), 7.30 (d, 1H, J = 7.2 Hz, Ar-H), 7.57–7.75 (m, 2H, Ar-H), 8.03 (d, 2H, J = 8.4 Hz, Ar-H), 8.21–8.30 (m, 2H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 81.3, 96.0, 109.1, 110.1, 110.4, 113.1, 113.5, 117.0, 117.7, 123.7, 123.9, 125.7, 126.4, 128.7, 129.1, 129.4, 130.0, 130.7, 131.3, 132.6, 134.2, 134.3, 135.7, 150.9, 151.5, 151.7, 152.0, 154.9, 157.4, 159.8, 162.6, 168.8, 169.1, 171.1. Anal. Calcd for $C_{36}H_{26}N_2O_9$: C, 68.57; H, 4.16; N, 4.44. Found: C, 68.81; H, 4.07; N, 4.73.

3.5. General procedure for the synthesis of fluorescein dinitrobenzoyl ester 3a and 3b

To a solution of fluorescein (1 mmol), and *p*-nitrobenzoyl chloride (2 mmol) were added pyridine in chloroform. The reaction mixture was stirred at reflux temperature for 12 h and then cooled to room temperature. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography.

3.5.1. Synthesis of fluorescein dinitrobenzoyl ester 3a

Compound **3a** was obtained by the reaction of fluorescein **1a** (1 mmol, 0.33 g), and *p*-nitrobenzoyl chloride (2 mmol, 0.35 g) as a colorless solid. Yield: 0.38 g (60%); mp 136–138 °C; (lit.[27] 140–142 °C); ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.92–7.01 (m, 3H, Ar-H), 7.26–7.28 (m. 3H, Ar-H), 7.69–7.75 (m, 2H, Ar-H), 8.08 (d, 1H, J = 7.5 Hz, Ar-H), 8.27–8.31 (d, 1H, J = 1.5 Hz, Ar-H), 8.38 (s, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 81.5, 103.1, 110.3, 110.5, 117.0, 117.8, 123.6, 123.8, 124.0, 125.4, 126.0, 129.3, 130.0, 130.3, 130.7, 131.1, 131.4, 134.3, 135.3, 135.5, 151.0, 151.6, 151.8, 152.1, 152.7, 162.8, 169.2. Anal. Calcd for $C_{34}H_{18}N_2O_{11}$: C, 64.77; H, 2.88; N, 4.44. Found: C, 64.90; H, 2.79; N, 4.59.

3.5.2. Synthesis of 4′,5′-dimethylfluorescein dinitrobenzoyl ester 3b

Compound **3b** was obtained by the reaction of 4′,5′-dimethylfluorescein **1b** (1 mmol, 0.36 g) and *p*-nitrobenzoyl chloride (2 mmol, 0.33 g) as a colorless solid. Yield: 0.31 g (48%); mp 122–124 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.41 (s, 6H, $-CH_3$), 6.78 (d, 2H, J = 8.7 Hz, Ar-H), 6.93 (d, 2H, J = 8.7 Hz, Ar-H), 7.26 (s, 1H, Ar-H), 7.65–7.77 (m, 2H, Ar-H), 8.07 (d, 1H, J = 7.2 Hz, Ar-H), 8.41 (d, 8H, J = 7.6 Hz, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 24.7, 106.1, 111.7, 115.6, 118.6, 122.8, 123.3, 125.2, 128.9, 130.2, 130.9, 131.0, 136.2, 149.7, 155.7, 156.0, 165.9, 168.5. Anal. Calcd for $C_{36}H_{22}N_2O_{11}$: C, 65.66; H, 3.37; N, 4.25. Found: C, 65.42; H, 3.62; N, 4.08.

3.6. General procedure for the synthesis of fluorescein diaminobenzyl ethers 4a, 4b, 5a and 5b

A flask containing Pd/C (10% activated 180 mg) was placed in a flask purged with argon. A portion of **2a** and **2b** and **3a** and **3b** (1 mmol) which was dissolved in 25 mL of dry THF was added, and a balloon filled with H_2 was attached. The reaction mixture was stirred vigorously under H_2 for 25 h, then filtered through Celite; the solvent was evaporated under reduced pressure. The crude product was purified by column chromatography.

3.6.1. Synthesis of fluorescein diaminobenzyl ether 4a

Compound **4a** was obtained by the reaction of fluorescein dinitrobenzyl ether **2a** (1 mmol, 0.60 g), and Pd/C (10% activated 180 mg) as a yellow solid. Yield: 0.38 g (70%); mp 150–152 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.96 (s, 4H, $-NH_2$), 4.96–5.25 (m, 4H, $-CH_2$), 6.60–7.00 (m, 6H, Ar-H), 7.09–7.17 (q, 2H, J = 7.2 Hz, Ar-H), 7.28–7.78 (m, 4H, Ar-H), 8.02–8.31 (m, 4H, Ar-H), 8.25–8.31 (t, 2H, J = 8.4 Hz, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 80.9, 102.3, 109.5, 110.0, 112.3, 116.5, 117.6, 122.9, 123.4, 123.6, 124.8, 125.4, 128.5, 128.7, 130.1, 130.4,131.0, 133.8, 135.4, 150.4, 150.9, 151.5, 151.8, 152.1, 162.2, 168.3. Anal. Calcd for $C_{34}H_{26}N_2O_5$: C, 75.26; H, 4.83; N, 5.16. Found: C, 74.94; H, 4.98; N, 4.94.

3.6.2. Synthesis of 4′,5′-dimethylfluorescein diaminobenzyl ether 4b

Compound **4b** was obtained by the reaction of 4′,5′-dimethylfluorescein dinitrobenzyl ether **2b** (1 mmol, 0.63 g), and Pd/C (10% activated 180 mg) as a yellow solid. Yield: 0.29 g (50%); mp 144–146 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (s, 3H, $-CH_3$), 1.84–1.88 (t, 3H, $-CH_3$), 3.82 (s, 4H, $-NH_2$), 5.02–5.25 (m, 4H, $-CH_2$), 6.73–7.33 (m, 8H, Ar-H), 7.64–7.74 (m, 3H, Ar-H), 8.04 (d, 3H, J = 7.8 Hz, Ar-H), 8.29 (d, 2H, J = 7.8 Hz, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.6, 30.3, 65.9, 67.9, 103.7, 114.8, 122.1, 123.8, 123.9, 124.0, 127.6, 127.9, 128.7, 128.9, 129.7, 129.9, 130.4, 130.5, 131.4, 133.0, 134.1, 141.3, 142.5, 147.8, 154.8, 157.4, 165.0, 175.4. Anal. Calcd for $C_{36}H_{30}N_2O_5$: C, 75.77; H, 5.30; N, 4.91. Found: C, 75.92; H, 5.48; N, 4.67.

3.6.3. Synthesis of fluorescein diaminobenzoyl ester 5a

Compound **5a** was obtained by the reaction of fluorescein dinitrobenzoyl ester **3a** (1 mmol, 0.63 g), and Pd/C (10% activated 180 mg) as a yellow solid. Yield: 0.28 g (60%); mp 134–136 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.73 (s, 4H, $-NH_2$), 6.85–7.01 (m, 4H, Ar-H), 7.26–7.28 (t, 3H, J = 7.2 Hz, Ar-H), 7.64–7.77 (m, 2H, Ar-H), 8.03–8.09 (m, 1H, Ar-H), 8.38 (s, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 81.5, 110.5, 117.1, 117.7, 123.8, 124.1, 125.4,126.1, 129.3, 130.3, 131.4, 134.3, 135.3, 135.5, 151.0, 151.6, 151.8, 152.8, 162.8, 169.6. Anal. Calcd for $C_{34}H_{22}N_2O_7$: C, 71.57; H, 3.89; N, 4.91. Found: C, 71.82; H, 3.96; N, 5.08.

3.6.4. Synthesis of 4′,5′-dimethylfluorescein diaminobenzoyl ester (5b)

Compound **5b** was obtained by the reaction of 4′,5′-dimethylfluorescein dinitrobenzoyl ester **3b** (1 mmol, 0.64 g), and Pd/C (10% activated 180 mg) as a yellow solid. Yield: 0.47 g (77%); mp 138–140 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.41 (s, 6H, $-CH_3$), 3.75 (s, 2H, $-NH_2$), 6.78 (d, 2H, J = 8.4 Hz, Ar-H), 6.93 (d, 2H, J = 8.7 Hz, Ar-H), 7.29 (s, 2H, Ar-H), 7.67–7.74 (q, 2H, J = 8.4 Hz, Ar-H), 8.06 (d, 2H, J = 7.2 Hz, Ar-H), 8.40 (s, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 29.7, 82.5, 117.0, 117.7, 119.3, 120.1, 123.9, 124.1, 125.4, 125.8, 126.3, 127.0, 130.2, 131.4, 134.3 135.3, 150.2, 151.1, 152.6, 162.7, 169.3. Anal. Calcd for $C_{36}H_{26}N_2O_7$: C, 72.23; H, 4.38; N, 4.68. Found: C, 72.47; H, 4.51; N, 4.81.

3.7. General procedure for the synthesis of fluorescein-based *N*-glycosylamines

To a solution of diamine **4a**, **4b** and **5a**, **5b** (1 mmol) in dry MeOH and 4,6-*O*-protected-D-glucopyranose (BGP, EGP, BzGP) (2 mmol) were added. After stirring at room temperature for given period of time, the reaction mixture was evaporated under reduced pressure. The crude product was slurried with silica gel and purified by column chromatography. For details (reaction time and yields of products) see Table 1.

3.7.1. Synthesis of fluorescein-di-(4,6-*O*-ethylidene-β-D-glucopyranosylamine) benzyl ether (6a)

Compound **6a** was obtained by the reaction of 4,6-*O*-ethylidene-β-D-glucopyranose (2 mmol, 0.41 g) and diamine **4a** (1 mmol, 0.54 g) as a yellow solid. Yield: 0.63 g (59%); mp 168–170 °C; $[\alpha]_D^{23}$ −30.0 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 1.35 (d, 6H, *J* 5.1 Hz, $-CH_3$), 3.20–3.34 (m, 3H, Sac-H), 3.43–3.49 (m, 2H, Sac-H), 3.55–3.61 (m, 2H, Sac-H), 3.79–3.86 (m, 2H, Sac-H), 4.01–4.06 (m, 2H, Sac-H), 4.09 (d, 1H, *J* = 4.2 Hz, Sac-H), 4.12 (d, 1H, *J* = 3.9 Hz, Sac-H), 4.66 (d, 2H, *J* = 7.8 Hz, Ano-H), 4.65 (s, 2H, Sac-OH), 4.71–4.76 (q, 5H, Sac-H), 5.10 (s, 1H, Sac-H), 5.14 (t, 1H, *J* = 3.3 Hz, Sac-H), 5.30 (s, 1H, –NH), 5.36 (s, 1H, –NH), 6.49–6.99 (m, 4H, Ar-H), 7.22 (t, 3H, Ar-H), 7.37 (t, 2H, Ar-H), 7.68–7.84 (m, 4H, Ar-H), 7.99–8.09 (m, 3H, Ar-H), 8.29–8.33 (m, 2H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 12.9, 16.3, 35.1, 61.2, 65.4, 67.1, 67.5, 69.3, 72.2, 75.0, 79.4, 80.1, 92.0, 96.5, 101.0, 109.4, 116.0, 117.0, 122.5, 122.7, 127.8, 128.1, 128.7, 129.4, 130.4, 133.2, 149.8, 150.4, 150.8, 161.7. ESIMS: calcd for 608.1 (100%), 333.3 (50%). Anal. Calcd for $C_{50}H_{50}N_2O_{15}$: C, 65.35; H, 5.48; N, 3.05. Found: C, 65.11; H, 5.35; N, 3.17.

3.7.2. Synthesis of fluorescein-di-(4,6-*O*-butylidene-β-D-glucopyranosylamine) benzyl ether (6b)

Compound **6b** was obtained by the reaction of 4,6-*O*-butylidene-β-D-glucopyranose (2 mmol, 0.46 g), and diamine **4a** (1 mmol, 0.54 g) as a yellow solid. Yield: 0.53 g (53%); mp 162–164 °C; $[\alpha]_D^{23}$ −18.2 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 0.93 (t, 6H, $-CH_3$), 1.38–1.47 (m, 4H, $-CH_2$), 1.56–1.64 (m, 4H, $-CH_2$), 3.16–3.34 (m, 6H, Sac-H), 3.73–3.87 (m, 3H, Sac-H), 3.98–4.13 (m, 1H, Sac-H), 4.55 (t, 5H, *J* = 5.8 Hz, Ano-H), 5.09 (s, 2H, Sac-H), 5.12 (d, 2H, *J* = 3.9 Hz, Sac-H), 5.28 (s, 1H, –NH), 5.35 (s, 1H, –NH), 6.55–7.35 (m, 9H, Ar-H), 7.63–7.85 (m, 4H, Ar-H), 7.98–8.11 (m, 2H, Ar-H), 8.21–8.31 (m, 2H, Ar-H), 8.41 (s, 6H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 18.9, 22.2, 34.5, 35.7, 41.1, 67.1, 71.2, 73.0, 73.4, 75.1, 78.1, 80.8, 98.0, 102.5, 106.8, 115.4, 123.0, 128.7, 133.7, 134.1, 134.9, 135.1, 136.4, 139.1, 140.8, 155.7, 156.2, 156.8, 167.6, 173.5. ESIMS: calcd for 333.3 (100%), 409.1 (60%). Anal. Calcd for $C_{54}H_{58}N_2O_{15}$: C, 66.52; H, 6.00; N, 2.87. Found: C, 66.30; H, 6.20; N, 2.99.

3.7.3. Synthesis of fluorescein-di-(4,6-*O*-benzylidene-β-D-glucopyranosylamine) benzyl ether (6c)

Compound **6c** was obtained by the reaction of 4,6-*O*-benzyl idene-β-D-glucopyranose (2 mmol, 0.53 g), and diamine **4a** (1 mmol, 0.57 g) as a yellow solid. Yield: 0.57 g (52%); mp 179–181 °C; $[\alpha]_D^{23}$ −37.1 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 3.30 (t, 2H, *J* = 8.5 Hz, Sac-H), 3.41–3.46 (m, 2H, Sac-H), 3.51–3.56 (m, 3H, Sac-H), 3.61–3.77 (m, 5H, Sac-H), 3.88 (t, 1H, *J* = 9.3 Hz, Sac-H), 3.96–4.04 (ddd, 1H, *J* = 4.8 Hz, *J* = 9.5 Hz, *J* = 12.0 Hz, Sac-H), 4.17–4.29 (m, 1H, Sac-H), 4.61 (d, 2H, *J* = 7.8 Hz, Ano-H), 5.09 (d, 2H, *J* = 8.4 Hz, Sac-H), 5.17 (d, 2H, *J* = 3.6 Hz, Sac-H), 5.26 (s, 1H, –NH), 5.33 (s, 1H, –NH), 5.52 (s, 3H, Ace-H), 6.55–6.67 (m, 4H, Ar-H), 6.82–7.19 (m, 6H, Ar-H), 7.32–7.48 (m, 10H, Ar-H), 7.67–7.80 (m, 2H, Ar-H), 8.01–8.06 (m, 3H, Ar-H), 8.23–8.31 (m, 3H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 27.0, 67.0, 71.1, 73.5, 73.9, 75.3, 78.0, 78.6, 80.7, 98.0, 102.4, 106.4, 115.4, 117.9, 128.5, 128.8, 130.0, 131.3, 132.9, 133.8, 135.6, 135.8, 136.2, 136.9, 140.4, 142.3, 156.4, 157.6, 167.4, 169.9, 173.8. ESIMS: calcd for 333.3 (100%). Anal. Calcd for $C_{60}H_{54}N_2O_{15}$: C, 69.09; H, 5.22; N, 2.69. Found: C, 68.91; H, 5.35; N, 2.61.

3.7.4. Synthesis of 4′,5′-dimethylfluorescein-di-(4,6-*O*-ethylidene-β-D-glucopyranosylamine) benzyl ether (7a)

Compound **7a** was obtained by the reaction of 4,6-*O*-ethyl idene-β-D-glucopyranose (2 mmol, 0.54 g) and diamine **4b** (1 mmol, 0.57 g) as a yellow solid. Yield: 0.48 g (44%); mp 167–169 °C; $[\alpha]_D^{23}$ -9.1 (*c* 1.0, EtOH); ^{1}H (300 MHz, $CDCl_3$ + DMSO-d_6): δ 1.36 (t, 6H, *J* = 4.8 Hz, $-CH_3$), 2.17 (s, 1H, Sac-H), 2.26 (s, 1H, Sac-H), 3.34–3.39 (m, 6H, Sac-H), 3.46–3.55 (q, 3H, Sac-H), 3.73 (t, 5H, *J* = 6.0 Hz, Sac-H), 3.85–3.94 (m, 3H, Sac-H), 4.04–4.13 (m, 2H, Sac-H), 4.61 (d, 2H, *J* = 6.9 Hz, Ano-H), 4.70–4.46 (q, 2H, Sac-H), 5.04 (s, 2H, –NH), 5.23 (t, 2H, *J* = 13.2 Hz, Sac-H), 6.56 (t, 3H, *J* = 9.0 Hz, Ar-H), 6.68–6.82 (m, 2H, Ar-H), 7.09 (d, 1H, *J* = 8.1 Hz Ar-H), 7.16 (t, 1H, *J* = 6.0 Hz, Ar-H), 7.31 (d, 3H, *J* = 6.9 Hz, Ar-H), 7.62–7.78 (m, 4H, Ar-H), 8.04–8.31 (m, 5H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 20.2, 29.5, 30.2, 62.0, 65.7, 66.3, 68.1, 68.5, 70.6, 73.2, 73.3, 75.9, 80.1, 80.7, 92.9, 97.3, 99.3, 103.8, 106.7, 123.6, 123.7, 123.8, 128.7, 129.6, 130.4, 132.9, 134.1, 141.4, 159.4, 161.5, 165.2. Anal. Calcd for $C_{52}H_{54}N_2O_{15}$: C, 65.95; H, 5.75; N, 2.96. Found: C, 66.29; H, 5.53; N, 2.81.

3.7.5. Synthesis of 4′,5′-dimethylfluorescein-di-(4,6-*O*-butylidene-β-D-glucopyranosylamine) benzyl ether (7b)

Compound **7b** was obtained by the reaction of 4,6-*O*-butylidene-β-D-glucopyranose (2 mmol, 0.46 g) and diamine **4b** (1 mmol, 0.57 g) as a yellow solid. Yield: 0.54 g (54%); mp 171–173 °C; $[\alpha]_D^{23}$ −20.5 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 0.93 (t, 6H, $-CH_3$), 1.25 (m, 4H, $-CH_2$), 1.43–1.46 (m, 4H, $-CH_2$), 1.59–1.63 (m, 6H, $-CH_3$), 3.24–3.53 (m, 6H, Sac-H), 3.63–4.15 (m, 8H, Sac-H), 4.56 (t, 2H, *J* = 5.0 Hz, Ano-H), 4.62 (d, 2H, *J* = 7.2 Hz, Sac-H), 5.04 (s, 1H, Sac-H), 5.19 (d, 2H, J = 3.6 Hz, Sac-H), 5.23 (s, 1H, –NH), 5.28 (s, 1H, –NH), 6.54–6.87 (m, 6H, Ar-H), 7.07–7.19 (m, 3H, Ar-H), 7.30 (d, 3H, *J* = 6.3 Hz, Ar-H), 7.62–7.78 (m, 3H, Ar-H), 8.02–8.31 (m, 4H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 17.2, 29.5, 30.2, 36.2, 62.2, 65.7, 66.5, 68.2, 68.6, 68.7, 70.8, 73.3, 76.0, 80.2, 80.7, 92.9, 97.4, 101.8, 102.2, 102.8, 103.8, 111.8, 113.0, 123.5, 123.7, 123.8, 123.9, 124.7, 127.6, 127.8, 128.6, 128.7, 128.9, 129.0, 129.1, 129.6, 129.8, 130.4, 131.2, 132.9, 134.1, 134.9, 142.7, 143.8, 147.4, 147.6, 152.5, 159.6, 169.3. ESIMS: calcd for 597.1 (100%), 668.0 (98%). Anal. Calcd for $C_{56}H_{62}N_2O_{15}$: C, 67.05; H, 6.23; N, 2.79. Found: C, 66.75; H, 6.44; N, 2.96.

3.7.6. Synthesis of 4′,5′-dimethylfluorescein-di-(4,6-*O*-benzylidene-β-D-glucopyranosylamine) benzyl ether (7c)

Compound **7c** was obtained by the reaction of 4,6-*O*-benzyl idene-β-D-glucopyranose (2 mmol, 0.53 g), and diamine **4b** (1 mmol, 0.57 g) as a yellow solid. Yield: 0.51 g (46%); mp 167–169 °C; $[\alpha]_D^{23}$ −25.6 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 2.17 (s, 6H, $-CH_3$), 3.39–3.58 (m, 3H, Sac-H), 3.68–3.80 (m, 3H, Sac-H), 3.92–4.10 (m, 2H, Sac-H), 4.21–4.38 (m, 3H, Sac-H), 4.53 (s, 1H, Sac-H), 4.68 (d, 2H, *J* = 6.9 Hz, Ano-H), 5.01 (s, 2H, Sac-OH), 5.05 (s, 2H, $-CH_2$), 5.11 (s, 1H, Sac-OH), 5.16 (s, 1H, Sac-OH), 5.23 (s, 2H, $-CH_2$), 5.28 (s, 1H, Sac-H), 5.53 (s, 2H, Ace-H), 6.10 (s, 2H, –NH), 6.53–6.87 (m, 8H, Ar-H), 7.08–7.50 (m, 9H, Ar-H), 7.62–7.79 (m, 4H, Ar-H), 8.04–8.31 (m, 4H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 29.4, 30.2, 30.8, 62.0, 65.7, 66.3, 68.6, 69.0, 70.6, 73.2, 75.8, 77.6, 80.8, 81.4, 93.0, 97.4, 101.5, 101.6, 111.7, 123.5, 123.6, 123.7, 124.6, 126.3, 127.6, 127.8, 127.9, 128.6, 128.7, 128.8, 129.1, 129.6, 130.4, 131.2, 132.8, 137.3, 137.4 158.4, 164.8, 165.0. ESIMS: calcd for 333.3

(100%). Anal. Calcd for $C_{62}H_{58}N_2O_{15}$: C, 69.52; H, 5.46; N, 2.62. Found: C, 69.70; H, 5.36; N, 2.49.

3.7.7. Synthesis of fluorescein-di-(4,6-*O*-ethylidene-β-D-glucopyranosylamine) benzoyl ester (8a)

Compound **8a** was obtained by the reaction of 4,6-*O*-ethylidene-β-D-glucopyranose (2 mmol, 0.41 g) and diamine **5a** (1 mmol, 0.57 g) as a yellow solid. Yield: 0.51 g (56%); mp 178–180 °C; $[\alpha]_D^{23}$ −12.5 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 1.35–1.37 (q, 6H, –CH_3), 3.23–3.36 (m, 4H, Sac-H), 3.49–3.52 (m, 2H, Sac-H), 3.63–3.69 (t, 1H, Sac-H), 3.85–3.90 (m, 3H, Sac-H), 4.04–4.11 (m, 5H, Sac-H), 4.26 (s, 1H, Sac-OH), 4.45 (s, 1H, Sac-OH), 4.62 (d, 1H, *J* = 6.9 Hz, –NH), 4.71–4.76 (q, 2H, *J* = 4.5 Hz, *J* = 9.9 Hz, Ano-H), 5.19 (s, 1H, –NH), 5.97 (s, 2H, Sac-OH), 6.52–7.03 (m, 3H, Ar-H), 7.22–7.30 (m, 3H, Ar-H), 7.66–7.80 (m, 2H, Ar-H), 8.01–8.08 (q, 1H, Ar-H), 8.39 (s, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 20.3, 62.1, 66.4, 68.2, 68.6, 70.8, 73.2, 73.3, 80.1, 80.7, 81.4, 93.0, 97.4, 99.4, 102.9, 109.4, 110.2, 110.4, 113.1, 117.0, 117.4, 117.7, 123.7, 124.0, 124.9, 125.3, 126.0, 126.5, 129.0, 129.2 129.9, 130.3, 131.4, 134.2, 135.2, 135.5, 150.9, 151.5, 151.8, 152.0, 152.1 152.5, 152.7, 159.7, 162.7, 168.9. ESIMS: calcd for 640.0 (100%), 333.2 (98%), 493.2 (92%). Anal. Calcd for $C_{50}H_{46}N_2O_{17}$: C, 65.42; H, 4.90; N, 2.96. Found: C, 63.70; H, 5.09; N, 2.85.

3.7.8. Synthesis of fluorescein-di-(4,6-*O*-butylidene-β-D-glucopyranosylamine) benzoyl ester (8b)

Compound **8b** was obtained by the reaction of 4,6-*O*-butylidene-β-D-glucopyranose (2 mmol, 0.46 g), and diamine **5a** (1 mmol, 0.57 g) as a yellow solid. Yield: 0.57 g (55%); mp 192–194 °C; $[\alpha]_D^{23}$ −17.1 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 0.93 (t, 6H, –CH_3), 1.38–1.50 (m, 4H, –CH_2), 1.60–1.68 (m, 4H, –CH_2), 3.21–3.35 (m, 4H, Sac-H), 3.49–3.89 (m, 5H, Sac-H), 4.06–4.16 (m, 3H, Sac-H), 4.44 (s, 1H, Sac-OH), 4.57 (t, 2H, *J* = 4.6 Hz, Ano-H), 4.63 (s, 2H, Sac-OH), 5.18 (s, 1H, Sac-H), 6.22 (s, 2H, –NH), 6.62–7.06 (m, 5H, Ar-H), 7.25–7.33 (m, 3H, Ar-H), 7.67–7.84 (m, 2H, Ar-H), 8.03–8.10 (q, 2H, Ar-H), 8.42 (s, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 18.7, 22.1, 41.0, 67.0, 71.3, 73.0, 73.4, 75.4, 78.1, 80.7, 85.1, 85.7, 86.2, 97.8, 102.2, 106.9, 107.7, 115.0, 115.2, 117.9, 121.7, 122.5, 128.5, 128.8, 129.7, 130.1, 130.7, 131.3, 133.7, 133.9, 134.8, 135.1, 136.2, 139.0, 140.1, 140.4, 155.7, 156.3, 156.6, 156.8, 157.1, 167.5, 173.7. ESIMS: calcd for 668.2 (100%), 333.2 (60%), 817.0 (40%), 1003.2 (30%). Anal. Calcd for $C_{54}H_{54}N_2O_{17}$: C, 64.66; H, 5.43; N, 2.79. Found: C, 64.90; H, 5.19; N, 2.65.

3.7.9. Synthesis of fluorescein-di-(4,6-*O*-benzylidene-β-D-glucopyranosylamine) benzoyl ester (8c)

Compound **8c** was obtained by the reaction of 4,6-*O*-benzylidene-β-D-glucopyranose (2 mmol, 0.53 g) and diamine **5a** (1 mmol, 0.57 g) as a yellow solid. Yield: 0.47 g (46%); mp 148–150 °C; $[\alpha]_D^{23}$ −40.5 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 3.35–3.57 (m, 4H, Sac-H), 3.71–3.76 (q, 2H, Sac-H), 3.92–4.09 (m, 3H, Sac-H), 4.21–4.32 (m, 2H, Sac-H), 4.61 (d, 1H, *J* = 9.0 Hz, Sac-H), 4.65–4.71 (t, 3H, *J* = 9.1 Hz, Ano-H), 5.23 (s, 1H,–NH), 5.53 (s, 4H, Sac-OH), 6.21 (s, 1H, –NH), 6.61–7.04 (m, 5H, Ar-H), 7.22–7.35 (m, 7H, Ar-H), 7.47–7.51 (m, 5H, Ar-H), 7.64–7.81 (m, 3H, Ar-H), 8.01–8.09 (q, 1H, Ar-H), 8.39 (s, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 67.0, 71.1, 73.5, 73.9, 75.4, 78.0, 78.0, 80.7, 85.7, 86.2, 86.3, 97.9, 102.3, 106.4, 106.5, 115.0, 115.2, 121.7, 122.6, 128.6, 128.8, 129.7, 130.1, 130.7, 131.2, 131.2, 132.9, 133.7, 133.9, 134.7, 135.1, 136.1, 139.0, 139.1, 140.0, 140.4, 142.2, 142.3, 155.7, 156.3, 156.6, 156.9, 157.7, 167.5. ESIMS: calcd for 702.2 (100%), 493.3 (98%), 851.0 (85%). Anal. Calcd for $C_{60}H_{50}N_2O_{17}$: C, 67.28; H, 4.71; N, 2.62. Found: C, 67.70; H, 4.19; N, 2.35.

3.7.10. Synthesis of 4′,5′-dimethylfluorescein-di-(4,6-*O*-ethylidene-β-D-glucopyranosylamine) benzoyl ester (9a)

Compound **9a** was obtained by the reaction of 4,6-*O*-ethylidene-β-D-glucopyranose (2 mmol, 0.54 g), and diamine **5b** (1 mmol, 0.59 g) as a yellow solid. Yield: 0.61 g (53%); mp 156–160 °C; $[\alpha]_D^{23}$ −30.5 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 1.35 (d, 6H, *J* = 5.1 Hz, –CH_3), 2.42 (s, 6H, –CH_3), 3.24–3.88 (m, 9H, Sac-H), 4.02–4.13 (m, 5H, Sac-H), 4.55 (d, 1H, *J* = 3.0 Hz, Sac-H), 4.60 (d, 1H, *J* = 6.9 Hz, Sac-H), 4.68 (d, 1H, *J* = 3.3 Hz, Sac-H), 4.76 (q, 2H, *J* = 5.1 Hz, *J* = 10.2 Hz, Ano-H), 4.79 (d, 1H, *J* = 3.3 Hz, Sac-H), 5.16 (s, 2H, –NH), 6.23 (d, 1H, *J* = 3.6 Hz, Ar-H), 6.77 (d, 2H, *J* = 8.7 Hz, Ar-H), 6.97 (d, 2H, *J* = 8.7 Hz, Ar-H), 7.30 (d, 1H, *J* = 7.5 Hz, Ar-H), 7.69–7.81 (m, 2H, Ar-H), 8.05 (d, 1H, *J* = 7.5 Hz, Ar-H), 8.38–8.45 (m, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 14.3, 25.6, 66.9, 71.2, 73.6, 73.9, 75.3, 78.0, 80.7, 85.8, 86.4, 98.0, 102.4, 106.4, 120.9, 122.6, 124.0, 129.8, 130.1, 131.2, 132.9, 133.8, 140.1, 155.2, 173.6. Anal. Calcd for $C_{52}H_{54}N_2O_{15}$: C, 65.95; H, 5.75; N, 2.96. Found: C, 66.29; H, 5.53; N, 2.81.

3.7.11. Synthesis of 4′,5′-dimethylfluorescein-di-(4,6-*O*-butylidene-β-D-glucopyranosylamine) benzoyl ester (9b)

Compound **9b** was obtained by the reaction of 4,6-*O*-butylidene-β-D-glucopyranose (2 mmol, 0.46 g) and diamine **5b** (1 mmol, 0.59 g) as a yellow solid. Yield: 0.53 g (50%); mp 165–167 °C; $[\alpha]_D^{23}$ −19.5 (*c* 1.0, EtOH); ^{1}H NMR (300 MHz, $CDCl_3$ + DMSO-d_6): δ 0.88–0.93 (t, 6H, –CH_3), 1.39–1.46 (q, 4H, –CH_2), 1.58–1.64 (q, 4H, –CH_2), 1.83–1.87 (q, 6H, –CH_3), 3.19–3.30 (m, 4H, Sac-H), 3.43–3.50 (t, 3H, Sac-H), 3.84–4.10 (m, 5H, Sac-H), 4.36 (d, 1H, *J* = 3.6 Hz, Sac-H), 4.52 (d, 1H, *J* = 3.0 Hz, Sac-H), 4.55 (t, 2H, *J* = 4.9 Hz, Ano-H), 4.61 (d, 1H, *J* = 5.1 Hz, Sac-H), 5.15 (d, 1H, *J* = 3.9 Hz, Sac-H), 6.19 (d, 1H, *J* = 4.2 Hz, Sac-H), 6.77 (d, 2H, *J* = 8.7 Hz, Ar-H), 6.97 (d, 3H, *J* = 8.7 Hz, Ar-H), 7.30 (d, 1H, *J* = 7.2 Hz, Ar-H), 7.69–7.81 (m, 3H, Ar-H), 8.05 (d, 2H, *J* = 7.2 Hz, Ar-H), 8.42 (s, 8H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$ + DMSO-d_6): δ 12.8, 16.0, 29.6, 34.9, 38.1, 61.1, 65.1, 66.8, 67.3, 68.9, 72.1, 74.6, 76.9, 79.2, 80.1, 91.9, 96.3, 100.6, 109.2, 116.8, 122.6, 127.6, 127.9, 128.8, 129.9, 130.2, 132.9, 134.6, 149.6, 150.1, 150.6, 161.4, 167.4. Anal. Calcd for $C_{56}H_{62}N_2O_{15}$: C, 67.05; H, 6.23; N, 2.79. Found: C, 66.75; H, 6.44; N, 2.96.

3.7.12. Synthesis of 4′,5′-dimethylfluorescein-di-(4,6-*O*-benzylidene-β-D-glucopyranosylamine) benzoyl ester (9c)

Compound **9c** was obtained by the reaction of 4,6-*O*-benzylidene-β-D-glucopyranose (2 mmol, 0.53 g), and diamine **5b** (1 mmol, 0.59 g) as a yellow solid. Yield: 0.61 g (58%); mp 188–190 °C; $[\alpha]_D^{23}$ −12.5 (*c* 1.0, EtOH); ^{1}H NMR (500 MHz, $CDCl_3$ + DMSO-d_6): δ 2.41 (d, 6H, *J* = 12.1 Hz, –CH_3), 3.22–3.24 (m, 4H, Sac-H), 3.43–3.49 (m, 2H, Sac-H), 3.69–3.70 (m, 5H, Sac-H), 3.89–4.02 (m, 3H, Sac-H), 4.22–4.26 (m, 2H, Sac-H), 4.62 (t, 1H, *J* = 6.8 Hz, Ano-H), 4.85 (d, 1H, *J* = 3.8 Hz, Sac-H), 4.91 (s, 1H, Sac-OH), 5.05 (s, 1H, Sac-OH), 5.17 (s, 1H, –NH), 5.52 (s, 1H, –NH), 6.43 (d, 1H, *J* = 4.6 Hz, Ar-H), 6.77 (d, 2H, *J* = 8.4 Hz, Ar-H), 6.93 (d, 1H, *J* = 6.8 Hz, Ar-H), 6.98 (d, 2H, *J* = 9.1 Hz, Ar-H), 7.34 (t, 4H, *J* = 2.6 Hz, Ar-H), 7.48 (d, 2H, *J* = 4.8 Hz, Ar-H), 7.70–7.87 (m, 3H, Ar-H), 8.05 (d, 1H, *J* = 7.6 Hz, Ar-H), 8.40–8.45 (q, 10H, Ar-H). ^{13}C NMR (125 MHz, $CDCl_3$ + DMSO-d_6): δ 10.7, 60.2, 67.6, 68.2, 73.4, 79.3, 79.5, 92.9, 102.1, 130.2, 105.1, 111.4, 115.0, 117.9, 124. 4, 125.4, 128.0, 130.9, 140.0, 145.2, 163.2, 165.1. Anal. Calcd for $C_{62}H_{54}N_2O_{17}$: C, 67.75; H, 4.95; N, 2.55. Found: C, 67.61; H, 5.07; N, 2.41.

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Supplementary data

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