CHAPTER-4

DEVELOPMENT OF WATER-SOLUBLE BODIPYS

FOR BIOLOGICAL APPLICATIONS
Chapter 4: Table of Contents

4.1. Preamble ................................................................. 99
4.2. Principle of PDT ......................................................... 99
4.3. Bodipy-based PDT Agents ........................................... 100
4.4. Studies on Bodipy-based PDT Agents ............................. 101
   4.4.1. Molecular design .................................................. 102
   4.4.2. Synthesis ............................................................ 103
   4.4.3. Photophysical characteristics ................................... 114
   4.4.4. Aggregation behaviour ............................................ 115
   4.4.5. DNA binding characteristics .................................... 120
   4.4.6. PDT studies ....................................................... 122
4.5. Summary .................................................................... 134
4.6. Experimental .............................................................. 135
   4.6.1. General methods .................................................... 135
   4.6.2. Synthesis ............................................................ 136
   4.6.3. DLS studies ........................................................ 142
   4.6.4. Biological studies .................................................. 142
4.1. Preamble

Fluorescence spectroscopy, fluorescence imaging and fluorescence indicators are nowadays indispensable tools in various fields of modern science and medicine, including clinical diagnostics, biotechnology, molecular biology, and biochemistry. The major classes of currently used synthetic fluorophores include the cyanines, rhodamines, and oxazines. While these dye systems provide a host of functional groups that can be used for covalent attachment to various target molecules, many have limited biological utility due to poor photochemical stability, lengthy synthetic routes, poor solubility, and tendency for aggregation in aqueous media that surrounds a biomolecule. Instead, the bright fluorescence, high photostability, generally low toxicity and ease of synthetic make the Bodipy-type dyes particularly promising candidates for biological imaging/sensing applications. The fluorescence-based in vivo imaging has emerged as a promising real-time, non-invasive, and high-resolution technique that uses fluorescent probes to visualize normal and abnormal biological processes.

Moreover, the Bodipy derivatives are amenable to modifications by attachment of ancillary residues at the appropriate positions of their cores. This can help in attenuating their strong fluorescence, thereby generating relatively long-lived triplet states, and eventually large amount of singlet oxygen ($^{1}$O$_{2}$) that would induce selective photo-damage in regions that are illuminated. Such an approach, known as photodynamic therapy (PDT) was the one of the foci of the present investigations. Hence the concept of PDT and in particular the application of the Bodipy compounds in PDT is briefly discussed below.

4.2. Principle of PDT
PDT is a therapeutic modality wherein nontoxic light-sensitive compounds, known as photosensitizer (PS) are selectively exposed to light, preferably in the visible or near-IR regions whereupon they become toxic to the targeted malignant and other diseased cells. Depending on the nature of the PS, its photo-excitation a specific wavelength leads to a transition of its electron from the $S^0$ state to the $S^1$ state. This is followed by an inter system crossing (ISC) of the excited electron for the favourable cases where the $T^1$ state is stable. Subsequently the PS can excite $O_2$ molecules at its ground ($T^1$) state to generate $^1O_2$ molecules, which, because of its higher energy can damage important bio-macromolecules. Hence the process can be used for therapy if targeted to specific cells (tumour, microbial etc.). The light used for PDT can come from a laser or other sources that can be directed through fiber optic cables (thin fibers that transmit light) to deliver light to areas inside the body. Because the excitation wavelength determines how far the light can travel into the body, and the PS is nontoxic, PDT generally show minimal side effect. Thus, PDT combines a photosensitizer (PS), its preferential localization in target tissues (like tumors), photo-excitation to generate reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) in presence of oxygen to achieve selective cell killing irreversibly. It provides a noninvasive therapeutic modality for certain types of cancers and pre-cancerous inductions, age-related macular degeneration and actinic keratosis, and other diseases, such as localized infections, dermatological and cardiovascular illnesses and wound healing. It is particularly promising in the treatment of multidrug-resistant (MDR) tumors selectivity, as both PS and light can be effectively localized to the tumor. It offers an attractive alternative or complement to conventional therapies.
4.3. Bodipy-based PDT Agents

Despite offering these advantages its current clinical use is restricted to a few functionalized porphyrins. However, the porphyrins are not the ideal PDT drugs due to their low extinction coefficients in the therapeutic window (650–800 nm), and low absorptivity in mammalian tissues.\(^8^5\) Recognition of these disadvantages of porfimer sodium has inspired efforts to develop more effective PDT photosensitizers. Most of the Bodipy dyes have many ideal characteristics of PDT agents such as low dark toxicities, good cellular uptake, high extinction coefficients, and low quantum yields for photobleaching. These dyes can also be functionalized at different positions to enhance ISC and \(^1\)O\(_2\) generation. Spin-coupling to heavy atoms is the most common of these modifications (the “heavy atom effect”), and the one most frequently encountered is halogenation. Appropriate placing of heavy atoms on the Bodipy core promotes spin–orbit coupling, hence ISC, but not energy loss from excited states. However, Bodipys, possessing the heavy atoms, such as iodine, bromine, selenium, sulphur and certain lanthanides often show dark toxicity, as well as extended toxicity even after switching off the light. To this end, PSs containing dimeric Bodipys at an orthogonal orientation,\(^8^6^a\) or use of spin convertors, such as C60, in Bodipy-based dyads\(^8^6^b\) may help to sensitize \(^1\)O\(_2\) generation, by intramolecular energy transfer (EnT). These aspects have been adequately highlighted in several excellent reviews.\(^8^7\)

4.4. Studies on Bodipy-based PDT Agents

The existing limitations and future prospects, discussed above collectively provide a compelling rationale for the development of new Bodipy derivatives as PDT
 photosensitizers. These provided the required impetus to undertake the present project for developing some novel and efficient Bodipy sensitizers for potential use in PDT.

4.4.1. Molecular design

In terms of molecular design, the first criteria of the fluorophore is the emission range between 700-900 nm, due to the optical sensitivity associated with the ‘biological window’, including minimal interference from endogenous chromophores, reduced light scattering, increased photon penetration through tissue, and low photodamage to the cells or tissues under observation. For an enhanced biological effect in deeper tissues, it is imperative that the PS has a high extinction coefficient in the body’s therapeutic window (650–800 nm), where typical mammalian tissues show low absorptivity. This can be conveniently achieved by a base-catalyzed condensation of the 3- or 5-methyl substituents of the Bodipys with suitable aldehydes. The resultant dyes possess longer wavelength absorption (~100 nm red-shifted) to move it to 590–600 nm with intramolecular charge transfer (ICT) characteristics. Incorporation of a second styryl group would result in further red shifts in the absorption spectrum. Another factor of paramount importance is the water solubility of the PS. It should have a balance between hydrophilicity and lipophilicity, as too high lipophilicity would hamper their transport through blood vessel, while a high hydrophilicity would impede its cell membrane penetration. Earlier attempt to overcome hydrophobicity of the dye sensitizer, using micellar drug formulations have met with limited success, as the emulsifying agents often elicit anaphylactic reactions in vivo. Given that the Bodipy core is hydrophobic, attachment polar neutral or ionic moieties can impart a higher amphiphilicity. For this purpose, Senge et al. have designed four water soluble porphyrin derivatives as
piperazinium and imidazole salts and studied their comparative PDT activities on the human lung cancer A549 cells. Incorporation of the hydrophilic bases was found to potentiate the PDT property.\textsuperscript{91a} With regard to the Bodipy compounds, introduction of a number of amphiphilic triethylene glycol moieties at the \textit{meso}-aryl and/ or 3,5-distyryl groups also improved their efficacy as PDT agents.\textsuperscript{52} More recently, some unsymmetrical distyryl Bodipys were also converted to their amphiphilic analogues using both oligoethylene glycol as well as 2-ethylene glycol ammonium moieties.\textsuperscript{91b} In another report, the combination of bromination as well as pegylation of some distyryl Bodipys was used by the same group to harness the benefit of heavy atom effect, amphiphilicity and red-shifted emission.\textsuperscript{48b} The rationale of choosing the oligoethylene glycol appendages are based on the fact these moieties are biocompatible, and confer cell permeability as well as tumor targeting characteristics on the photosensitizers.\textsuperscript{92}

\subsection*{4.4.2. Synthesis}

For the present investigation, it was envisaged that the C-3/C-5 styrylation strategy, used extensively by others,\textsuperscript{48b,91b} as well as our group (presented in Chapter 2) would be easy to adopt to red-shift the emission wavelength of the Bodipy dyes. Hence the same strategy was adopted, as discussed below. However, the resultant dyes would be highly hydrophobic, as realized in the lasing studies of the red-Bodipys (\textit{vide supra} Chapter 2). It was also found that appending an ethylene glycol moiety at the B-centre of a even the mono-styryl dye 60a did not improve its water-solubility. Hence, for the present work, attachment of a glucose unit was planned to improve the hydrophilicity. Such a strategy seemed attractive for the following reasons. Carbohydrates are known as
ligands of various cell surface lectins. Intense research have been focused to develop tumor-specific imaging probes by targeting cell membrane glycoproteins, such as galectins that play significant roles in numerous types of cancer.\textsuperscript{93a} The galectins are abundant in tumor cells to promote tumor growth and development, angiogenesis and metastasis.\textsuperscript{93} Hence, the proposed carbohydrate-based fluorophores may selectively target tumors. Also, carbohydrate–protein interactions are viewed as important mechanisms for many biological processes including immune response, cell proliferation, adhesion and death, cell–cell interaction and communication. Aggregation of the carbohydrates in vivo, may enhance the protein binding affinity.\textsuperscript{94a-c}

Although the mechanism of retention of PSs by tumors is not well understood, the balance between lipophilicity and hydrophilicity is recognized as an important factor for photosensitizing efficiencies and tumor and cellular uptake. The linkages of a PS with sugar moieties are of great importance in terms of membrane interaction and specific affinity for malignant tumors. Hence, particular attention was devoted to the glycoconjugated Bodipy derivatives. It was planned to incorporate the glucose unit at the phenolic functionality present at the \textit{meso}-position or as a part of the C-3/C-5 styryl/distyryl moieties in the Bodips. Thus, three Bodipy dyes shown in 4.4.2.3 were targeted. Of these, compound 72 was devoid of any additional conjugation, while compounds 74 and 76 had additional styryl moieties.

As shown in \textbf{Scheme 4.4.2.1}, the syntheses of the parent Bodipy were straightforward. Thus, kryptopyrrole 58 was condensed with the aryl aldehydes 67/68 using trifluoroacetic acid (TFA) as the catalyst to obtain compounds 69 and 70. In general, CH$_2$Cl$_2$ is used as the solvent in the synthesis of Bodipys dyes, and the yields are
moderate with most of the aromatic aldehydes. During the synthesis of 70, it was found that the reaction became sluggish to form a dark brown mixture from which the product could be isolated in poor yield. However, minor modification of the reaction conditions such as use of THF as the solvent and addition of DDQ at 0 °C improved the yield of 70, as the reaction was clean and separation was easy. Due to the shielding effect of the C-8 aryl groups, the C-1 and C-7 methyl protons of both the compounds appeared upfield in their respective ¹H NMR spectra.

Scheme 4.4.2.1 Synthesis of the precursor Bodipys for glucosylation.

Next two types of red Bodipys viz. meso-methyl and meso-phenyl groups were synthesized. For this, compound 21 (PM567) was subjected to condensation with the aldehyde 68 in the presence of piperidine and acetic acid. This expectedly led to the
formation of the monostyryl and distyryl derivatives 71a and 71b respectively. A similar condensation 69 with 68 afforded the corresponding monostyryl and distyryl derivatives 72a and 72b respectively. The results were consistent with that observed when the condensation was carried out between 21 and 4-methoxybenzaldehyde.

The attachment of the glucose moiety to the compounds 70, 71a/71b and 72 was achieved by the improved classical Konigs-Knorr method.95a The required glycosyl donor 74 was synthesized by acetylation of glucose with acetic anhydride in the presence of NaOAc to furnish glucose pentaacetate 73. This on treatment with HBr-HAc furnished the bromide 74 via chemo-selective deacetylination of the anomeric acetyl group and bromination (Scheme 4.4.2.2.). After recrystallization from Et2O/hexane and stored in the refrigerator at -18 °C.95b-d

[Scheme 4.4.2.2 Synthesis of the glucosylating agent.]

For the glycosylation of compound 70 and 71a, the pentaacetate 73 was used as the glycosyl donor in presence of BF₃.etherate catalyst in CH₂Cl₂. Appearance of characteristics mutiplets due to carbohydrate –CH protons between δ 3.5 to 5.5 in the ¹H NMR spectrum and four carbonyl peaks at δ 170 in the ¹³C NMR spectrum confirmed formation of compounds 75 and 77 (Figure 4.2.4 and 4.2.5). However, when the same strategy was applied to glycosylate the dyes 71a and 71b, a very low yield of product from 71a and complete degradation of 71b were observed. Apparently, the reagent BF₃.etherate induced two parallel processes viz. glycosylation and removal of a F atom.
from the Bodipys, the latter causing the partial or complete degradation dyes, depending on their nature. Fortunately, the glycosylation of 72a and 72b could be successfully performed by reacting them at room temperature with 74 in 1:1 H₂O/CHCl₃ and in the presence of K₂CO₃ as the base and Bu₄NBr as the phase transfer catalyst to afford 79 and 81 respectively. Because the glycosylation process does not change the L-configuration of the anomeric carbon of sugars,⁹⁵e the structures of the products were assigned as shown in Scheme 4.4.2.3. Finally, the acetyl groups of 75, 77, 79 and 81 were removed by treating the products with NaOMe in MeOH to furnish the target Bodipys 76, 78, 80a and 80b respectively (Scheme 4.4.2.3).⁹⁵b These water-soluble Bodipy compounds were subsequently used for the spectroscopic and PDT studies. As some representative data, the ¹H and ¹³C NMR spectra of compound 76 and its precursor 75 are shown in Figures 4.2.1.2 and 4.2.1.3.
Figure 4.4.2.1 The NMR spectrum of 70 (a) $^1$H NMR, (b) $^{13}$C NMR.
Figure 4.4.2.2 The NMR spectrum of 75 (a) $^1$H NMR, (b) $^{13}$C NMR.
Figure 4.4.2.3 The NMR spectrum of 76 (a) $^1$H NMR, (b) $^{13}$C NMR.
Scheme 4.4.2.3 Synthesis of the water-soluble Bodipy-dyes.
Figure 4.4.2.4 The $^1$H NMR spectrum of compound (a) 71a, (b) 72a.
Figure 4.4.2.5 The NMR spectrum of 81 (a) $^1$H NMR, (b) $^{13}$C NMR.
4.4.3. Photophysical characteristics

The UV/Vis absorption and fluorescence spectra of Bodipy-O-glycosides 76, 78, 80a and 80b in ethanol are shown in Figures 4.4.3.1a and b and the photophysical data summarized in Table 4.4.3.1. All these Bodipy-O-glycosides exhibited typical spectral characteristics of the Bodipy core dyes with narrow $S_0 \rightarrow S_1$ absorption band, a weak and broad $S_0 \rightarrow S_2$ absorption band, high molar extinction coefficients, intense fluorescence emissions and small Stokes’ shifts. The absorption and emission spectra were almost mirror images of each other, indicating that the emitting species are similar to the absorbing ones. Compared to the non-styrylated Bodipy 76, introduction of first styryl group as in 78 and 80a induced significant bathochromic shifts in the absorption (51-63 nm) and emission maxima (50-55 nm), whereas introduction of a second styryl moiety as in 80b caused further red-shifts (123 nm) of both absorption and emission maxima. Fluorescence quantum yields of compounds 80a and 80b were significantly lower due to nonradiative loss of energy via rotation around the C-Ar bonds.96
Table 4.4.3.1: Photophysical parameters of the dyes 76, 79a, 80a, and 80b in ethanol.

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{abs}}^{[a]}$ [nm]</th>
<th>$\varepsilon_{\text{max}}^{[b]}$ [10$^4$ M$^{-1}$ cm$^{-1}$]</th>
<th>$\lambda_{\text{em}}^{[c]}$ [nm]</th>
<th>$\Phi_{\text{fl}}^{[d]}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>76</td>
<td>523</td>
<td>8.3</td>
<td>540</td>
<td>0.84$^{[e]}$</td>
</tr>
<tr>
<td>78</td>
<td>574</td>
<td>8.2</td>
<td>590</td>
<td>0.60</td>
</tr>
<tr>
<td>80a</td>
<td>586</td>
<td>8.0</td>
<td>595</td>
<td>0.52</td>
</tr>
<tr>
<td>80b$^{[f]}$</td>
<td>646</td>
<td>9.2</td>
<td>665</td>
<td>0.50</td>
</tr>
</tbody>
</table>

$^{[a]}$Error: ± 0.2 nm. $^{[b]}$Extinction coefficients for the corresponding $\lambda_{\text{max}}$. $^{[c]}$Error: ± 0.3 nm. $^{[d]}$The fluorescence quantum yields of the dyes 78, 80a and 80b are relative to that of the dye Rh101 ($\Phi_{\text{fl}} = 1$ in EtOH). $^{[e]}$The fluorescence quantum yields of 76 is relative to that of 21 ($\Phi_{\text{fl}} = 0.84$ in EtOH). $^{[f]}$Because of poor solubility of 80b, its solution in EtOH was prepared by diluting its stock solution, made in DMSO with appropriate amount of EtOH.

Figure 4.4.3.1. Spectral features of the water-soluble Bodipy dyes in EtOH. (a) Absorption spectra; (a) Emission spectra.

4.4.4. Aggregation behaviour

Fluorescent organic nanoparticles are of interest in materials science, particularly for biological imaging, and as delivery vehicles.$^{97}$ These materials offer the possibilities of intercalating multiple dyes of disparate optical properties into one-dimensional
heterostructures,\textsuperscript{98a} or promoting fluorescence resonance energy transfer (FRET) for white-light emission.\textsuperscript{98b} Further, compounds with carbohydrates as appendages are known to form organogels or hydrogels that are of potential applications in tissue engineering, and development of new materials that reversibly respond to various external stimuli.\textsuperscript{99} A large number of glucose derivatives have been reported to form hydrogels.\textsuperscript{100} The poor solubility of compound 80\textbf{b} in most of the organic solvents except DMSO and presence of two glucose moieties in it encouraged us to probe its ability to form aggregates. For this, the UV–vis absorption and fluorescence spectra of compound 82\textbf{b} (5.0 \( \mu \)M) in THF and EtOH as such, and with gradual addition of H\textsubscript{2}O were recorded. The H\textsubscript{2}O concentration was varied between 0-90\%. In pure THF and EtOH, the compound showed strong and sharp absorption maxima at 648 nm and at 646 nm respectively due to the \( S_0 \rightarrow S_1 \) transition of the monomeric Bodipy core, and showed an emission maximum at 700 nm on excitation at 580 nm.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{spectra.png}
\caption{UV–vis spectra of 80\textbf{b} (5.0 \( \mu \)M) in THF-water (a), and EtOH-water (b).}
\end{figure}

On incremental addition of H\textsubscript{2}O, a new absorption peak at 725 nm emerged, but at a considerably higher water concentration. However, the spectra retained the typical shapes of the Bodipy chromophores, while the lower wavelength peak at 370 nm
accounted for the styryl moieties. The emergence of the new peak signified aggregation of the dye that started at 60% and 52% water in THF and EtOH respectively. The absorption peak of the parent dye completely vanished in 90% water-THF and 75% of water-EtOH media (Figure 4.4.4.1). Significant color changes, dramatically reduced fluorescence color and intensities were the other distinct hallmarks of water addition. The dye fluorescence completely vanished at 95% aqueous THF. These changes signify solvent-induced formation of aggregates. The lack of any isosbestic point in the absorption spectra during the aggregation process, clearly indicated non-existence of a two-state equilibrium between the monomer and aggregates.

Furthermore, the loss of the monomer peak or the rise of the aggregates peak followed a complex kinetics rather than the first or second order kinetics (data not shown), negating the possibility of a simple reaction mechanism that did not involve intermediates. Finally, it should be noted that the changes in monomer absorbance exactly paralleled changes in fluorescence intensity, but no changes were observed in the shape of the excitation spectrum, consistent with the nonfluorescent nature of the aggregate species.\(^{101}\) Previously, almost quantitative formation of nonfluorescent Bodipy H dimers has been reported by molecular confinement of the dyes within a sodium silicate derived glass. No interference from higher-order aggregates or fluorescent J dimers was observed.\(^{102}\) Based on the previous and present observations, it is tempting to propose initial formation H dimers of 80b in aqueous THF and EtOH solvents that may stack to furnish the aggregates.

The aggregation process was also followed as a function of water concentration by \(^1\)H NMR spectra (Figure 4.4.4.2). Significant upfield and downfield shifts of various
aromatic protons as well as their signatures clearly established the complex kinetics of the process.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.4.4.2.png}
\caption{\textsuperscript{1}H NMR spectra of 80b (50 \mu M) in d\textsuperscript{4}-methanol (a) 0\% D\textsubscript{2}O; (b) 20\% D\textsubscript{2}O; (c) 30\% D\textsubscript{2}O.}
\end{figure}

Figure 4.4.4.2\textsuperscript{1}H NMR spectra of 80b (50 \mu M) in d\textsuperscript{4}-methanol (a) 0\% D\textsubscript{2}O; (b) 20\% D\textsubscript{2}O; (c) 30\% D\textsubscript{2}O.

Final evidence for the aggregation behavior of 80b in the mixed solvents was obtained from the dynamic light scattering (DLS) studies. In pure THF or EtOH, no correlation was observed indicating the absence of any aggregation. Figure 4.4.4.3a shows the intensity of the correlation function of 80b (5 \mu M) in THF-water mixtures at varying water concentration. The correlation function developed as the water content reaches 70\% and its amplitude increased progressively with the increase in water content from 72\% to 76\%. Analysis of the correlation function using the Inverse Laplace
transform software gives the size distribution of the aggregates present. Figure 4.4.4.3.b shows the corresponding size distribution obtained by analyzing the data of Figure 4.4.4.3a. The aggregates were polydisperse in nature with the size ranging from about 20 nm to 1 micron, and an intensity weighted average diameter of 260 nm. With an increase in water content, the distribution shifted to larger sizes, although the size distribution did not change much beyond 74% of water content.

![Figure 4.4.4.3.](image)

Figure 4.4.4.3. (a) The scattered intensity correlation function of 80b-aggregates (5 μM) in THF/water mixture at different water contents. (b) Size distribution of the aggregates obtained by fitting the data in Figure 4.4.4.3a (solid lines indicating the fit).

The size distribution of the nanoaggregates in EtOH-water was found to vary from 100 nm to 10 micron with an intensity-weighted average diameter of 1 micron. However, there was no further growth in size of aggregates in 52% aqueous EtOH, and the aggregates were stable even after more than one month.
Figure 4.4.4. Images of self-assemblies of 80b-aggregates, formed in THF-water system via slow evaporation, as revealed by obtained from optical microscopy.

The organogels can self-assemble into various nanoscale superstructures such as fibers, rods, ribbons, and tubes. The ability of the Bodipy derivative 80b to undergo self-association was clearly visible in the optical microscopic images (representative images shown in Figure 4.4.4). The morphology of the aggregates, obtained on slow evaporation of the solvent appeared as solid fibrillar assemblies without any tubular structure. Possibly, hydrogen bonding between the hydroxyl groups of the sugar moiety in 80b stabilized the aggregates and determined the overall morphologies. More ordered crystalline aggregates can be induced by reducing the water concentration in aqueous THF. The formation of the self-assembly is a spontaneous process, and takes place under non-equilibrium conditions. Noncovalent interactions give rise to the formation of superstructures, which subsequently entrain and immobilize the solvent inside the interstices of a three-dimensional network. Earlier, formation of Bodipy aggregates could be synthesized by incorporating long chain trialkoxyphenyl fragments and trimethylammonium head-groups. Thus, the present result of Bodipy aggregation by mere introduction of two glucose units is unique, and may be useful for soft matter chemistry.

4.4.5. DNA binding characteristics
$^{1}\text{O}_2$ is a highly reactive species but has a short apparent lifetime (ca. 2.0 $\mu$s) with a low apparent diffusion coefficient ($4 \times 10^6$ cm$^2$ s), and thus a very limited sphere of activity (about 155 nm in radius) in biological systems. This implies that the PDT agents should have good binding ability toward DNA that is one of the main targets of many anticancer drugs, including the PDT agents. Hence the DNA binding properties of the dyes 76 and 78 were studied using absorption photometric technique. Compounds 76 and 78 had strong absorption bands at 522.6 nm and 573.8 nm respectively, which could be conveniently used the spectrophotometric titrations. Incremental addition of double stranded calf thymus (CT)-DNA (0–200 $\mu$M DNA base pair) to a fixed concentration of 76 (20 $\mu$M) and 78 (50 $\mu$M) led to gradual reductions in the intensities of their respective absorption maxima (Figure 4.4.5.1), confirming their binding with DNA. Based on the site-exclusion model, the equilibrium binding constants (K) in respective cases were derived by quantitative analysis of the UV–visible data (Figure 4.4.5.2). The moderate K-values of were $3.4 \times 10^4$ M$^{-1}$ and $1.8 \times 10^4$ M$^{-1}$ for of 76 and 79a respectively, while the linear fits in regression analysis indicated a single mode binding of them with DNA. Because the DNA-intercalators generally show higher binding constants $\sim 10^5$ - $10^7$ M$^{-1}$, the lower K-values suggested an ionic binding. This was also evident from the lack of additional absorption peak for any new species. Earlier the well-known DNA intercalator, coralyne was reported to produce new species on binding with DNA. 106
The UV–vis titration of dyes with CT-DNA. (a) dye 76 (20.4 µM); (b) dye 78 (50 µM). The experiments were carried out in phosphate buffer (5 mM, 7.4 pH) at at 25 °C. The CT DNA concentrations were varied from 9.9 to 130.8 µM for 76 and 5.3 to 110.5 µM for 78, using 0.164 mM bp aliquots.

Scatchard plots of the DNA binding experiments. (a) dye 76 (20.4 µM); (b) dye 78 (50 µM).

4.4.6. PDT studies

In view of the above results, the in vitro photodynamic activities of the Bodipy-O-glycosides 76, 78 and 80a were assessed against the highly invasive and metastatic human lung cancer A549 cell lines. For comparison, the corresponding non-glycosylated precursors, 70, 71a and 72a as well as the commercial dye PM567 21 were included for
their cytotoxicities. In addition, the dark cytotoxicities of some of the compounds were also evaluated. The compounds used for the PDT studies are shown in Figure 4.4.6.1.

Figure 4.4.6.1 The chemical structures of the Bodipys, used for the PDT evaluation.

The studies were specifically targeted to lung cancer as it remains one of the most common cancer-related causes of death. This type of cancer typically develops over a period of many years, and its incidence and mortality rate increase gradually each year. Despite the rapid progress of surgery, radiotherapy, chemotherapy, and biotherapy, the long-term survival rate of patients with lung cancer remains poor, and new therapeutic strategies are urgently needed. Fortunately its early detection can improve the prognosis significantly and increase the life span of the patients. Since early lung cancer detection is now becoming feasible, PDT may play an important role in lung cancer treatment especially at an early stage. Several clinical trials have established the efficacy of PDT with superficial small tumors, while its use as a preoperative measure may reduce tumor burden and the degree of surgery for larger tumors. Presently, besides evaluating the in vitro cytotoxicity of the designated Bodipys, their intracellular localization, and the involvement of apoptosis in their PDT property were also examined, as discussed below.
4.4.6.1. Cytotoxicity. None of the test compounds showed any toxicity to the A549 cells up to 200 μM, as revealed by the MTT results at 24 h. However, all the compounds dose-dependently reduced viabilities of the A549 cells under photo-exposure, with respect to vehicle treated controls (Figure 4.4.6.2).

![Graph showing dose-dependent photo-cytotoxicities of PM57, the parent Bodipy dyes and their O-glycosides against human lung cancer A549 cells. Cells (1 × 10⁴/well), grown in 96-well plates were treated with vehicle (0.1% DMSO) or increasing concentrations of the test compounds along with photo-exposure (dose 5.5 J/cm²). The cell viability was assessed by the MTT assay after 24 h. The results are expressed in percentage survival considering that of the vehicle-treated control cells as 100. The experiments were repeated three times with similar results. All determinations were made in four replicates, and the values are means ± S. E. M. *P<0.01, **P<0.001 compared to vehicle control.](image)

Based on the MTT assay results, the growth inhibitory IC₅₀ values, defined as the concentrations of the dyes required to kill 50% of the cells were calculated and are shown
in Table 4.4.6.1. The relative potency of the test compounds was $80a > 70 > 78 > 71a = 72a = 21 > 76$. Thus, amongst those chosen, the mono- and distryryl Bodipys 78 and 80a were more potent than their non-glycosylated precursors 71a and 72a. Surprisingly, however, glycosylation reduced the potency in case of the meso-aryl containing compounds 70 and 76. The other interesting observation was the almost similar activity of 71a, 72a and 21, suggesting that red-shifting of the emission maxima had less impact on the PDT activity of the chosen Bodipys.

Table 4.4.6.1: Comparative cytotoxicities of the chosen Bodipy dyes against A549 human lung cancer cells.[a]

<table>
<thead>
<tr>
<th>Bodipys</th>
<th>IC$_{50}$ (µM)</th>
<th>Bodipys</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>6.8 ± 1.8</td>
<td>76</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>70</td>
<td>2.1 ± 0.6*</td>
<td>78</td>
<td>2.7 ± 0.8**</td>
</tr>
<tr>
<td>71a</td>
<td>6.5 ± 2.1</td>
<td>80a</td>
<td>1.8 ± 0.5**</td>
</tr>
<tr>
<td>72a</td>
<td>6.4 ± 2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] The MTT data shown above was used to calculate IC$_{50}$ values. The experiments were repeated three times with similar results. All determinations were made in four replicates, and the values are means ± S. E. M. *$P<0.001$ compared to the corresponding glycosylated Bodipy, **$P<0.001$ compared to the corresponding non-glycosylated Bodipys.

4.4.6.2. Subcellular localization. Photodynamic efficacy is principally determined by the subcellular localization of a PS.$^{110a}$ The distribution of a PS within a cell depends on its chemical nature, concentration in the culture medium and also on the incubation time.$^{110b}$ Some photosensitizers show a broad distribution, while some may localize more specifically. Hence, to explain the photocytotoxicity results, fluorescence microscopic
studies were carried out to investigate the cellular uptake and localization of these compounds. The intracellular accumulation of all the Bodipy dyes loaded on the A549 cells as displayed in the dual staining with Hoechst-33342 (nucleus specific dye) by fluorescence microscopy exhibited particular localization in the cytoplasm (Figure 4.4.6.3). However, compared to the other dyes, compound 21 showed lower fluorescence, indicating its lesser cellular uptake. Also, the patchy fluorescence in the cells, stained with 21 indicated its non-uniform presence in the cytoplasm with higher accumulation near the membrane. The other dyes had uniform florescence all over the cytoplasm of the cells. The significantly less uptake of the dye 80b was anticipated considering its tendency of aggregation in aqueous-organic media. Hence, the uptake of the acetate 79, the less polar precursor of 80b was checked. Surprisingly, the acetate displayed almost no cellular uptake (Figure 4.4.6.4) that was even less than that of 21. This also ascertained good hydrophilic-hydrophobic balance in the glycosylated dyes, selected for the present investigations. The non-glycosylated precursors (70, 71a, 72a, and 72b) showed more diffused florescence compared to their respective glycosylated dyes. This may account for the poorer efficacy of the styryl-Bodipys 71a, and 72a compared to that of the glycosylated counterparts 78, and 80a.
Figure 4.4.6.3 Subcellular localization of the glycosylated Bodipy dyes in A549 cells. The cells were incubated with the dyes (5 μM) and Hoechst 33342 (used as the nucleus tracer) for different periods and the images captured with a fluorescence microscope. Representative Hoechst 33342-stained, bright field and the corresponding superimposed images, captured at 1 h are shown in columns 2-4, respectively.
Figure 4.4.6.4 Subcellular localization of the non-glycosylated Bodipy dyes in A549 cells. The cells were incubated with the dyes (5 μM) and Hoechst 33342 (used as the nucleus tracer) for different periods and the images captured with a fluorescence microscope. Representative Hoechst 33342-stained, bright field and the corresponding superimposed images, captured at 1 h are shown in columns 2-4, respectively.
4.4.6.3. Apoptosis induction. Apoptosis is an important cellular event that plays a key role in pathogeny and therapy of many diseases. It is believed to be associated with caspase activation via two separate viz. extrinsic or cytoplasmic, and intrinsic or mitochondrial pathways. The intrinsic pathway involves perturbation of intracellular homeostasis, and is linked primarily to mitochondrial changes, directly inducing the release of cytochrome c into the cytosol and apoptosome complex formation with activation of caspase-9. The extrinsic pathway, however, is initiated by the binding of a member of the tumor necrosis-factor (TNF)-family of death-receptor ligands to their cognate receptors (TNFR or Fas). When a death stimulus triggers the pathway, the membrane-bound FasL interacts with the inactive Fas complexes and forms the death-inducing signaling complex (DISC). The DISC contains the Fas-associated death domain (FADD) and procaspase 8, which becomes autocatalytically activated, and in turn, cleaves downstream effector pro-caspase-3/-7. The effector caspases then process different substrates, leading to apoptotic cell death. Studies have demonstrated that apoptosis induction plays the most vital role in the cancer treatment. Dysregulation in apoptotic pathways has been implicated in the development and progression of malignant tumors as well as occurrence of chemoresistant phenotypes. In response to PDT, apoptosis has been found to be a prominent form of cell death for many cell lines in tissue culture.

In the present study, apoptosis induction by the Bodipys was confirmed by quantifying the sub G1 population in the cells, treated with different concentrations (0–50 μM) of 76 and 78 at 24 h. Although compound 80a was slightly more potent, the vast difference in the MTT assay results of 76 and 78 was inexplicable. In particular, it was
important to confirm that the better cytotoxicity of 78 was due to apoptosis, and not necrosis. Hence, the compounds 76 and 78 were chosen for the apoptosis studies that also included parallel assays in the absence of light. The results (Figure 4.4.6.5.) indicated that the compounds are essentially non-toxic toward the A549 cells in darkness. However, under illumination, both the compounds increased the sub-G1 cells population concentration-dependently, compared to control, and the effects were also proportional to the illumination dose up to 80 min of photo-exposure. For an illumination time of 80 min, compound 76 (10-50 μM) increased the sub-G1 phase cell population to ~2–4.8 fold, revealing induction of a robust apoptosis. But when the illumination time was reduced to 30 min, it could induce significant apoptosis only at concentrations ≥20 μM. In comparison, compound 78 was a more effective apoptosis-inducer as it (5 μM) increased the sub-G1 phase cell population to ~3.5 and 5.2 folds, compared to control at 30 and 80 min of illumination respectively. Further, its activity reached a plateau value (4 fold) at 10 μM after 30 min of photo-exposure. A similar activity trend was also noticed when the photo-exposure time was 80 min. This confirmed that the photo-toxicity of 78 to the A549 cells was due to apoptosis without any significant necrosis.
Figure 4.4.6.5 Apoptosis induction by 76 and 78 in A549 cells under dark and white light illumination, as revealed by flow cytometry. The cells were incubated with 76 and 78 (0-50 µM) for 1 h, exposed to light for 30/80 min and further incubated for 24 h. Subsequently, 20000 cells in each treatment were acquired using a flow cytometer, and the DNA content of the nuclei registered on a logarithmic scale. The Sub-G1 region (RN1) represents the percentage of cells undergoing apoptosis. The experiments were repeated three times with similar results. All determinations were made in three replicates, and the values are means ± S. E. M. *P<0.001 compared to vehicle control. A representative figure is shown.

4.4.6.4. Caspase activation. Several observations have indicated that compounds that localize within mitochondria or ER promote apoptosis, while activation of PS targeting either the plasma membrane or lysosomes can either delay or even block the apoptotic program prompting the cells to necrosis.\textsuperscript{113a,b} Because, all the chosen Bodipy compounds for the present studies were accumulated in the cytoplasm, it was necessary to confirm the apoptosis induction by other methods, besides the sub-G1 accumulation. Activation
of the caspases is perhaps the most well characterized apoptotic cascade.\textsuperscript{114a} Depending on the involvement of extrinsic or cytoplasmic pathway, the final execution of apoptosis is often mediated through caspase-8 or caspase-9 as the initiators, and caspase-3 as the effector. Assaying caspase-3 is widely used as a tool for detecting programmed cell death.\textsuperscript{114b,c} Presently the involvement of apoptosis was further ascertained by examining the activation of the above caspases by compound 78 under photo-exposure. The compound 78 was chosen because of its better activity than 76.

At 16 h, compound 78 (1.25 µM) stimulated ($P<0.001$) the activities of caspase-3 (~2.7 fold) and caspase-8 (~3.1 fold) without any increase in the caspase-9 activity, compared to the untreated control cells (Figure 4.4.6.6). Such activation of caspase-3/8 was abrogated in the presence of the respective specific caspase inhibitors (each 20 µM).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{caspase_activations.png}
\caption{Caspase activation by 78 in A549 cells under white light illumination. The cells, as such or pre-incubated with specific caspase-3 and caspase-8 inhibitors (40 µM) for 1 h were incubated with 78 (1.25 µM) for 1 h, and exposed to light for 1 h. The caspase-3 and caspase-8 activities were assayed by ELISA after 16 h. The experiments were repeated three times with similar results. All determinations were made in three replicates, and the values are means ± S. E. M. $^*P<0.001$ compared to vehicle control; $^#P<0.001$ compared to 78-treatment.}
\end{figure}

For further confirmation, the effects of the specific caspase inhibitors (each 40 µM) on the cytotoxicity of compound 78 (1.25 µM) under photo-exposure for 80 min
were assessed by the MTT assay at 24 h. Pre-incubation of the A549 cells for 1 h with specific caspase-8 and caspase-3 inhibitors increased the cell viability by 76.2% ($P<0.001$) and 22.4% ($P<0.05$) respectively, compared to the only 78-treated cells under photo-illumination (Figure 4.4.6.7). However, the caspase-9 inhibitor did not show any effect on the cell survival. Pre-treatment the cells with the pan-caspase inhibitor increased the cell survival to 2.1 fold ($P<0.001$) compared to the cells not receiving the above inhibitor. Taken together, these suggested the involvement of the extrinsic apoptotic pathway in the photo-toxicity of 78 to the A549 cells.

![Graph](image)

**Figure 4.4.6.7** Identification of the apoptotic pathway in the photo-toxicity of 78 to the A549 cells. The cells, as such or pre-incubated with specific caspases inhibitors (each 40 $\mu$M) for 1 h were incubated with 78 (1.25 $\mu$M) for 1 h, and exposed to light for 1 h. The cell survival was assayed by the MTT reduction protocol after 24 h. The experiments were repeated three times with similar results. All determinations were made in three replicates, and the values are means ± S. E. M. $^*P<0.001$ compared to vehicle control.

Current evidence suggests that the most common pathway for apoptosis in PDT-treated cells involves mitochondria. Most PSs are lipophilic, and preferentially localize in
the intracellular membrane systems, particularly mitochondria. From that perspective the cytoplasmic localization of the chosen Bodipys is interesting. However, this is not unprecedented. Few PSs photosensitizers, and with limited cell types, follow other pathways, especially through caspase-8, particularly when the dominant pathway is suppressed.\textsuperscript{113a} For example, the widely used PS, photofrin has been shown to concentrate into plasma membranes or cytoplasm upon brief incubation, and in the Golgi complex or ER upon prolonged incubation.\textsuperscript{115a} It is possible that the Bodipys 70, 76, 78, 80a or some of them can re-localize at other subcellular locations from the primary sites of their accumulation. Such time-dependent re-localization of certain PS has been reported earlier.\textsuperscript{115b,c} It has been reported that procaspase-3 localizes in the cytoplasm and that caspase-3 activation is initiated in the cytosol. The activated caspase-3 redistributes to the nuclear compartment to induce apoptosis.\textsuperscript{116a,b}

4.5. Summary

Considerable efforts have been devoted to develop PSs with better tumor selectivity and higher phototherapeutic efficiency. Although the mechanism of PS retention by tumors is not well understood, the balance between lipophilicity and hydrophilicity is recognized as an important factor for photosensitizing efficiencies and tumor and cellular uptake. The linkages of PSs with sugar moieties are of great importance because the sugar increases water solubility, membrane interaction and specific affinity for malignant tumors. Factors such as lower pH and more low-density lipoprotein (LDL) receptors in malignant tissue than normal one may explain the observed cellular specificity of the PS.\textsuperscript{117}
Keeping this in mind, in this work, a new series of Bodipy carbohydrate conjugates have been designed and synthesized by introducing glucose units to a hydroxyl phenyl subunit present at the meso- or C-3 styryl moieties of the Bodipy core. The styryl moieties were introduced so as to induce red shifts in the emission maxima. The in vitro biological investigations with the human lung cancer A549 cells revealed insignificant dark cytotoxicity of the synthesized dyes, despite high cell membrane permeability. As expected, two of the mono-styryl glycosylated dyes 78 and 80a showed better potency than their respective non-glycosylated precursors 71a and 72a. Both 78 and 80a induced apoptosis via the extrinsic pathway. Surprisingly, the dye 70 containing a meso-phenol moiety instead of any extended conjugation (lack of the C-3-styryl group) showed a similar growth inhibitory property as that of 78 and 80a, and incorporation of a glucose unit at a meso-phenol unit reduced the activity drastically.

It was also demonstrated that compound 80b, possessing the glycosylated styryl moieties at both C-3 and C-5 positions of the Bodipy core formed stable fibrillar nano-aggregates in aqueous THF and EtOH. More importantly the aggregate size could be controlled by changing the water content of the media without the need of any additional lipophilic attachment, as reported previously.\textsuperscript{104} Taken together the good phototoxicity of the red-emissive Bodipy-\textit{O}-glycosides 78 and 80a, and hydrogel formation ability of 80b make them potentially attractive materials for photodynamic therapy, and as biological imaging, and delivery vehicles respectively.

\textbf{4.6. Experimental}

\textit{4.6.1. General details}
The general details of the synthetic methodologies and spectroscopic studies have already been discussed in Chapter-2. The chemicals used for the biological studies were: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, and kits of caspases-3, -8, -9 activities (all from Sigma chemicals, St. Louis, MO); Dulbecco’s modified Eagle’s medium (DMEM, HiMedia, Mumbai); and fetal bovine serum (FBS, Gibco Life Technologies, Carlsbad, CA); Z-DEVD-FMK (pan-caspase inhibitor), Z-VAD-FMK (caspase-8 inhibitor), Z-IETD-FMK (caspase-3 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor).

4.6.2. Synthesis

2,6-Diethyl-4,4-difluoro-1,3,5,7-tetramethyl-8-phenyl-4-bora-3a,4a-diaza-s-indecene (69). As described in Chapter-2, compound 69 was synthesized using 58 (0.450 g, 3.7 mmol), 67 (0.196 g, 1.85 mmol), TFA (0.012 g, 0.10 mmol), DDQ (0.420 mg, 1.85 mmol), Et₃N (0.5 mL) and BF₃·Et₂O (0.762 mL, 4.6 mmol) in CH₂Cl₂ (10 mL). Usual work-up, followed by column chromatography (silica gel, hexane-EtOAc) furnished pure 69 as a reddish solid. Yield: 0.175 g (24.9%); orange needles (hexane); mp: 184-185 °C (lit.¹¹⁸ mp: 185-186 °C); $^1$H NMR: $\delta$ 0.97 (t, $J = 7.6$ Hz, 6H), 1.26 (s, 6H), 2.27 (q, $J = 7.6$ Hz, 4H), 2.52 (s, 6H), 7.25-7.29 (m, 2H), 7.45-7.48 (m, 3H); $^{13}$C NMR: $\delta$ 11.6, 12.5, 14.6, 17.1, 128.3, 128.7, 129.0, 130.8, 132.7, 135.8, 138.4, 140.2, 153.7; MS (m/z): 380 [M]$^+$.

2,6-Diethyl-4,4-difluoro-1,3,5,7-tetramethyl-8-(4'-hydroxyphenyl)-4-bora-3a,4a-diaza-s-indecene (70). For the synthesis of 70, compounds 58 (0.500 g, 4.07 mmol), 68 (0.226 g, 1.85 mmol), DDQ (0.420 mg, 1.85 mmol), Et₃N (1.56 mL, 11.09 mmol), BF₃·Et₂O (1.39 mL, 11.09 mmol) and THF (70 mL) were used. It was purified by column
chromatography (silica gel, hexane/EtOAc). Yield: 0.350 g (23.4%); red-orange square crystals (acetone/cyclohexane); mp: 228 °C; \(^1\)H NMR (700 MHz, (CD\(_3\))\(_2\)CO): \(\delta\) 0.98 (t, \(J = 7.7\) Hz, 6H), 1.41 (s, 6H), 2.34 (q, \(J = 7.7\) Hz, 4H), 2.48 (s, 6H), 7.04 (d, \(J = 8.4\) Hz, 2H), 7.15 (d, \(J = 8.4\) Hz, 2H), 8.71 (s, 1H); \(^{13}\)C NMR (175 MHz, (CD\(_3\))\(_2\)CO): \(\delta\) 10.2, 10.6, 13.0, 15.6, 115.0, 125.4, 128.5, 130.1, 131.5, 137.3, 140.3, 152.0, 157.1; EI-MS (m/z): 396.1 [M]+, 395.4 [M−1]+. Anal. Calcd. for C\(_{23}\)H\(_{27}\)BF\(_2\)N\(_2\): C, 69.71; H, 6.87; N, 7.07%. Found: C, 69.42; H, 6.74; N, 7.22%.

2,6-Diethyl-4,4-difluoro-3-(4'-hydroxystyryl)-1,5,7,8-tetramethyl-4-bora-3a,4a-diaza-s-indecene 71a. As described in Chapter-2, compound 71a was synthesized by condensing 21 (0.200 g, 0.629 mmol) with 68 (0.077 g, 0.629 mmol) in the presence of glacial acetic acid (0.3 mL) and piperidine (2.0 mL) in toluene (20 mL), followed by usual isolation. Yield: 0.080 g (30.1%); dark pink crystals (CH\(_2\)Cl\(_2\)/cyclohexane); mp: 225 °C; \(^1\)H NMR (700 MHz, (CD\(_3\))\(_2\)CO): \(\delta\) 1.04 (t, \(J = 7.7\) Hz, 3H), 1.19 (t, \(J = 7.7\) Hz, 3H), 2.38 (s, 3H), 2.39 (s, 3H), 2.45 (q, \(J = 7.7\) Hz, 2H), 2.49 (s, 3H), 2.69 (s, 3H), 2.72 (q, \(J = 7.7\) Hz, 2H), 6.88 (d, \(J = 8.4\) Hz, 2H), 7.18 (d, \(J = 16.8\) Hz, 1H), 7.45 (d, \(J = 8.4\) Hz, 2H), 7.56 (d, \(J = 16.8\) Hz, 1H), 8.6 (s, 1H); \(^{13}\)C NMR (125 MHz, (CD\(_3\))\(_2\)CO): \(\delta\) 12.7, 14.2, 14.6, 14.8, 15.2, 17.6, 18.8, 116.7, 118.3, 129.3, 130.2, 133.1, 133.5, 133.9, 135.2, 138.1, 140.9, 148.7, 153.6, 159.1; HRMS (m/z): Calcd.: 423.2419 [M+1]+; Found: 423.2400 [M+1]+.

2,6-Diethyl-4,4-difluoro-3-(4'-hydroxystyryl)-1,5,7-trimethyl-8-(4'-phenyl)-4-bora-3a,4a-diaza-s-indecene 72a and 2,6-Diethyl-4,4-difluoro-3,5-di(4'-hydroxystyryl)-1,7-trimethyl-8-(4'-phenyl)-4-bora-3a,4a-diaza-s-indecene 72b. As above, condensation of 69 (0.600 g, 1.579 mmol) and 68 (0.193 g, 1.579 mmol) in the presence of glacial acetic
acid (0.9 mL) and piperidine (6.0 mL) in toluene (60 mL), followed by usual isolation
give a residue, which on column chromatography (silica gel, hexane/EtOAc) furnished
72a and 72b.

**72a:** Yield: 0.160 g (20.9%); dark pink crystals (acetone/cyclohexane); mp: 228 °C; $^1$H
NMR (200 MHz, (CD$_3$)$_2$CO): δ 0.99 (t, $J$ = 7.4 Hz, 3H), 1.13 (t, $J$ = 7.4 Hz, 3H), 1.33(s,
3H), 1.34 (s, 3 H), 2.34 (q, $J$ = 7.4 Hz, 2H), 2.54 (s, 3H), 2.62 (s, $J$ = 7.4 Hz, 2H), 2.85
(s, 6H), 6.89 (d, $J$ = 8.6 Hz, 2H), 7.22 (d, $J$ = 17 Hz, 1H), 7.38-7.43 (m, 2H), 7.48 (d, $J$
= 8.6 Hz, 2H), 7.58-8.00 (m, 4H); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 11.4, 11.7, 12.7, 14.1,
14.5, 17.1, 18.3, 24.3, 115.7, 117.5, 128.4, 128.7, 129.0, 129.9, 131.7, 132.9, 133.4,
135.2, 135.8, 138.6, 138.8, 139.2, 149.8, 154.7, 156.7; EI-MS (m/z): 483.3 [M-1]$^+$. Anal.
Calcd. for C$_{30}$H$_{31}$BF$_2$N$_2$O: C, 74.39; H, 6.45; N, 5.78%; Found: C, 74.68; H, 6.31; N,
5.94%.

**72b:** Yield: 0.400 g (43.1%); dark green powder (acetone/cyclohexane); mp: >250 °C; $^1$H
NMR (300 MHz, (CD$_3$)$_2$SO): δ 1.09 (t, $J$ = 7.2 Hz, 6H), 1.29 (s, 6H), 2.50(s, 6H), 2.57 (q,
$J$ = 7.4 Hz, 4H), 6.85 (d, $J$ = 8.2 Hz, 4H), 7.19 (s, $J$ = 17 Hz, 2H), 7.40-7.57 (m,
11H);EI-MS (m/z): 587.5 [M-1]$^+$. Anal. Calcd. for C$_{37}$H$_{35}$BF$_2$N$_2$O$_2$: C, 75.51; H, 5.99; N,
4.76%; Found: C, 75.14; H, 5.83; N, 4.87%.

**Glycosylation of 70.** To a mixture of 73 (0.142 g, 0.568 mmol) and 70 (0.150 g, 0.379
mmol) in CH$_2$Cl$_2$ (20 mL), BF$_3$.Et$_2$O (47.6 µL, 0.379 mmol) was added and the resulting
mixture refluxed for 5 h. It was brought to room temperature, washed with aqueous
saturated NaHCO$_3$ (2 × 10 mL), H$_2$O (2 × 10 mL) and brine (10 mL), and dried. Removal
of solvent in vacuo followed by column chromatography of the residue (silica gel,
hexane/EtOAc) furnished 75. Yield: 0.138 g (50.2%); orange powder
(acetone/cyclohexane); mp: 216 °C; H NMR (200 MHz, CDCl3): δ 0.97 (t, J = 7.6 Hz, 6H), 1.29 (s, 6H), 2.04-2.09 (m, 12H), 2.27 (q, J = 7.6 Hz, 4H), 2.51 (s, 6H), 3.86- 3.95 (m, 1H), 4.15-4.21 (m, 1H), 4.28-4.37 (m, 1H), 5.13-5.34 (m, 4H), 7.09 (d, J = 8.6 Hz, 2H), 7.19 (d, J = 8.6 Hz, 2H); C NMR (50 MHz, CDCl3): δ 11.9, 12.5, 14.6, 17.0, 20.5, 20.6, 62.0, 68.2, 71.1, 72.2, 72.6, 99.2, 117.5, 129.7, 130.8, 130.9, 132.8, 138.2, 139.3, 153.8, 157.2, 169.2, 169.4, 170.2, 170.4; EI-MS (m/z): 727.4 [M+1]+, 725.5 [M-1]+, 707.4 [M-19]+. Anal. Calcd. for C37H45BF2N2O10: C, 61.16; H, 6.24; N, 3.86%. Found: C, 60.94; H, 6.20; N, 3.50%.

**Glycosylation of 71a.** To a mixture of 73 (0.071 g, 0.284 mmol) and 71a (0.80 g, 0.189 mmol) in CH2Cl2 (15 mL) was added BF3·Et2O (23.8 µL, 0.189 mmol), and the resulting mixture refluxed for 3 h, brought to room temperature, washed with aqueous saturated NaHCO3 (2 × 10 mL), H2O (2 × 10 mL) and brine (10 mL), and dried. Removal of solvent in vacuo followed by column chromatography of the residue (silica gel, hexane/EtOAc) furnished 77. Yield: 0.060 g (42.1%); dark pink powder (acetone/cyclohexane); mp: 186 °C; H NMR (200 MHz, CDCl3): δ 1.04 (t, J = 7.4 Hz, 3H), 1.20 (t, J = 7.4 Hz, 3H), 2.03-2.09 (m, 12H), 2.34-2.42 (m, 8H), 2.46 (s, 3H), 2.52-2.69 (m, 5H), 3.83-3.90 (m, 1H), 4.12-4.32 (m, 2H), 5.06-5.31 (m, 4H), 6.95 (d, J = 8.8 Hz, 2H), 7.03 (d, J = 16.8 Hz, 1H), 7.48-7.61 (m, 3H); C NMR (75 MHz, CDCl3): δ 13.8, 14.3, 14.6, 17.0, 18.2, 20.4, 20.5, 61.2, 61.7, 67.5, 68.2, 69.6, 70.0, 70.1, 71.9, 72.6, 91.5, 98.8, 117.0, 119.2, 128.1, 132.2, 132.4, 132.8, 133.0, 133.2, 136.2, 137.2, 139.2, 146.9, 153.7, 156.6, 169.1, 169.3, 170.0, 170.4; HRMS (m/z): Calcd.: 752.3292 [M]+; Found: 752.3255 [M]+.
Glycosylation of 72a and 72b. A mixture of Bu₄NBr (4 mmol) in 1:1 H₂O–CHCl₃ (20 mL) was stirred at 40 °C. A solution of 74 (0.02 mol) in CHCl₃ (15 mL), and another solution of 72a or 72b (0.02 mol) and K₂CO₃ (6.9 g) in water (20 mL) were simultaneously dropped into the above mixture, the reaction mixture heated to 60 °C and stirred vigorously for 6 h. It was brought to room temperature, washed with aqueous 5% NaOH (3 ×10 mL), H₂O (2 × 10 mL) and brine (10 mL), and dried. Removal of solvent in vacuo followed by column chromatography of the residue (silica gel, hexane/EtOAc) furnished the 79 and 81 respectively. 79: Yield: 0.095 g (35.3%); mp: 105 °C; HRMS (m/z): Calcd.: 837.3346 [M+Na]⁺; Found: 837.3309 [M+Na]⁺.

81: Yield: 0.180 g (28.3%); mp: 218 °C; ¹H NMR (200 MHz, CDCl₃): δ 1.14 (t, J = 7.6 Hz, 6H), 1.34 (s, 6H), 2.04-2.09 (m, 18H), 2.57 (q, J = 7.4 Hz, 4H), 3.88-3.93 (m, 2H), 4.14-4.36 (m, 4H), 5.12-5.33 (m, 8H), 6.98 (d, J = 8.6 Hz, 4H), 7.14 (d, J = 17 Hz, 2H), 7.28-7.32 (m, 2H, meso-Ph), 7.48-7.57 (m, 7H); ¹³C NMR (50 MHz, CDCl₃): δ 11.4, 13.9, 14.1, 18.3, 20.5, 20.6, 20.7, 22.6, 28.9, 29.1, 29.3, 29.4, 29.6, 31.8, 33.8, 61.8, 68.2, 71.1, 71.1, 72.0, 72.6, 98.8, 114.0, 117.1, 119.3, 128.6, 128.8, 129.0, 132.7, 133.0, 133.7, 134.7, 135.8, 138.6, 139.1, 157.0, 169.3, 169.3, 170.2, 170.6; HRMS (m/z): Calcd.: 1287.4298 [M+K]⁺; Found: 1287.4213 [M+K]⁺.

Deacetylation of the Bodipy-O-glucosides. NaOMe (0.399 mmol) was added to the solution of 77/79/81 (0.080 mmol) in MeOH (5 mL) and the resulting mixture was stirred at room temperature for 1 h. Solvent removal in vacuo followed by column chromatography (silica gel, CHCl₃/MeOH) of the residues furnished 78/80a/80b. Compound 76 was prepared in a similar manner using NaOMe (0.691 mmol) and 75 (0.100 g, 0.138 mmol) in MeOH (5 mL).
Compound 76: Yield: 0.070 g (91.1%); orange powder (CH₂Cl₂/cyclohexane); mp: 166 °C; ¹H NMR (300 MHz, (CD₃)₂CO): δ 0.97 (t, J = 7.5 Hz, 6H), 1.37 (s, 6H), 2.33 (q, J = 7.5 Hz, 4H), 2.48 (s, 6H), 2.93 (broad s, 4H), 3.49-3.56 (m, 4H), 3.70-3.75 (m, 1H), 3.88-3.92 (m, 1H), 5.05-5.08 (d, J = 7.2 Hz, 1H), 7.24-7.28 (m, 4H); ¹³C NMR (200 MHz, (CD₃)₂CO): δ 10.3, 10.7, 13.0, 15.6, 60.8, 69.5, 72.8, 75.9, 76.1, 100.3, 116.3, 128.0, 128.4, 129.9, 131.6, 137.3, 139.7, 152.3, 157.7; EI-MS (m/z): 559.4 [M+1]⁺, 558.4 [M]⁺. Anal. Calcd. for C₂₉H₃₇BF₂N₂O₆: C, 62.37; H, 6.68; N, 5.02%; Found: C, 62.63; H, 7.11; N, 4.98%.

Compound 78: Yield: 85.8%; dark pink crystals (acetone/cyclohexane); mp: 143 °C; ¹H NMR (300 MHz, (CD₃)₂CO): δ 1.06 (t, J = 9.0 Hz, 3H), 1.21 (t, J = 9.0 Hz, 3H), 2.40-2.41 (m, 6H), 2.42-2.51 (m, 5H), 2.71-2.76 (m, 5H), 2.85 (s, 3H), 3.46-3.49 (m, 4H), 3.72-3.76 (m, 1H), 3.88-3.93 (m, 1H), 5.01-5.04 (m, 1H), 7.10 (d, J = 8.6 Hz, 2H), 7.19 (d, J = 16.8 Hz, 1H), 7.52 (d, J = 8.8 Hz, 2H), 7.61 (d, J = 17 Hz, 1H); ¹³C NMR (75 MHz, (CD₃)₂CO): δ 12.7, 14.1, 14.2, 14.6, 14.7, 14.8, 14.9, 15.2, 15.3, 17.6, 18.8, 18.9, 62.7, 71.4, 74.7, 77.9, 78.0, 101.8, 117.8, 119.5, 128.8, 128.9, 132.5, 132.7, 132.9, 133.2, 134.2, 134.4, 137.9, 138.5, 140.8, 141.2, 148.0, 154.2, 159.2; HRMS (m/z): Calcd.: 585.2948 [M+1]⁺; Found: 585.2910 [M+1]⁺.

Compound 80a: Yield: 0.038 g (50.4%); dark pink powder (acetone/cyclohexane); mp: 138 °C; ¹H NMR (200 MHz, (CD₃)₂CO): δ 0.99 (t, J = 7.6 Hz, 3H), 1.14 (t, J = 7.4 Hz, 3H), 1.33 (s, 3H), 1.34 (s, 3H), 2.35 (q, J = 7.6 Hz, 2H), 2.54 (s, 3H), 2.62 (q, J = 7.6 Hz, 2H), 3.44-3.87 (m, 7H), 5.01 (d, 1H), 7.11 (d, J = 8.0 Hz, 2H), 7.24 (d, J = 17.4 Hz, 1H), 7.39-7.41 (m, 2H), 7.43-7.72 (m, 6H); HRMS (m/z): Calcd.: 669.2923 [M+Na]⁺; Found: 669.2887 [M+Na]⁺.
**Compound 80b:** Yield: 0.060 g (45.6%); dark green powder (acetone/cyclohexane); mp: >250 °C; HRMS (m/z): Calcd.: 935.3714 [M+Na]^+; Found: 935.3653 [M+Na]^+.

4.6.3. Dynamic light scattering (DLS) studies

The DLS measurements were performed using a Malvern 4800 Autosizer instrument employing a diode pumped solid state laser (532 nm, vertical polarisation) and avalanche photodiode detector, at a scattering angle of 130°. The data processing was carried out by Malvern 7132 digital correlator. The scattered intensity correlation function was analyzed by the inverse Laplace transformation algorithm, CONTIN (software supplied by Malvern Instruments, UK) to extract the size distribution data.

4.6.4. Biological studies

4.6.4.1 Cell culture: The A549 cell line, procured from National Centre for Cell Science, Pune, India were cultured in DMEM medium, supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were grown at 37 °C under an atmosphere of 5% CO₂.

4.6.4.2 MTT assay. Viabilities of the control cells and those treated with various concentrations of the test compounds were determined at 48 h by the MTT reduction assay. Briefly, cells (1 × 10⁴/well) grown in 96-well plates were incubated overnight at 37 °C under an atmosphere of 5% CO₂. Next day the cells were incubated with vehicle (0.1% DMSO) or various concentrations of the test compounds for 1 h. The cells were washed two times with PBS, DMEM (200 μL) added, and subsequently exposed to light (dose rate 7.76 Watt) for different time periods (0.5, 1, 2, 3, and 4 h) using a 20 W CFL lamp. Serum was added to the medium which was incubated for 24 h. The cells were washed once with PBS, MTT solution (0.5 mg/mL, 100 μL) was added to each well and kept at 37 °C for 6 h. The formazan crystals in the viable cells were solubilized with 0.01
N HCl (100 μL) containing 10% SDS and the absorbance at 550 nm read. Experiments were also carried out without the photo-exposure, wherein the assays were carried out at 48 h after addition of the compounds. For studying the Similar assays were also carried out by pre-incubating the cells with the caspases inhibitors (each 40 μM) prior to the addition of the test compound 78.

4.6.4.3 Flow cytometry. The hypodiploid DNA content were analyzed as a marker for apoptosis by flow cytometry, after staining with PI. The cells were incubated with 76 or 78 (0-50 μM) for 1 h, washed two times with PBS, DMEM (200 μL) added, and subsequently exposed to light (dose rate 7.76 Watt) for different time periods (30 and 80 min) using a 20 W CFL lamp. Serum was added to the medium which was incubated for 24 h. The cells were washed once with cold PBS, incubated with PI (400 μg/mL) and RNase A (200 μg/mL) in 1 mL hypotonic buffer (0.1% sodium citrate plus 0.1% Triton X-100) for 30 min at 37 °C, and analyzed with a Pertec CyFlow® Space flow cytometer using the FlowJo program. Cellular debris was excluded from analysis by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. At least 2 × 10⁴ cells of each sample were analyzed. The apoptotic nuclei appeared as broad hypodiploid DNA peaks.

4.6.4.4 Fluorescence microscopy. A549 cells seeded in 6-well plate on coverslip were loaded with the Bodipy dyes (5 μM) for varying periods (0.25, 0.5 h, 1, 2 and 3 h) at 37 °C, washed with PBS, subsequently stained with Hoechst 33342 (10 μM), washed once again with PBS, mounted with 70% glycerol, and analyzed under Axioskop II Mot plus (Zeiss) microscope (40 × optics). When overlaying blue/red images by ImageJ software, some adjustments to image stretch and tone were made to both blue and red images. This
was done only for overlay display contrast purposes so as to ensure that the blue color of the Hoechst-stained nucleus and red color of the dye are both visible.

**4.6.4.3 Caspase activity assay.** The assays were performed with a caspase-3 colorimetric kit or caspase-8 and caspase-9 fluorimetric kits according to the manufacturer’s protocol. Briefly, cells (1 × 10⁶/well), seeded in 90 mm plate were incubated with 78 (1.25 μM) for 1 h followed by 1 h photo-illumination, and the individual caspase activities were assayed at 16 h. These assays were based on spectrophotometric detection of the chromatophore, pNA after cleavage from the respective labeled substrates by the caspases. The untreated but photo-exposed cells served as the control. For the caspase inhibition studies, the cells were pre-incubated with the inhibitory peptides, Z-VAD-FMK, Z-IETD-FMK, Z-LEHD-FMK (each 40 μM), prior to the other treatments.