CHAPTER 4

Novel cationic fullerene derivatized s-triazine scaffolds for photoinduced DNA cleavage
Abstract

In this chapter we describe the synthesis of a series of novel cationic fullerene ($C_{60}$) derivatives conjugated to a substituted s-triazine moiety. The derivatives were synthesized by using the 1, 3 dipolar cycloaddition reaction of $C_{60}$ with azomethine ylides generated from the corresponding Schiff base of substituted s-triazine. All the synthesized compounds were characterized by elemental analysis, FT-IR, $^1$H NMR, $^{13}$C NMR and ESI-MS. The compounds $7a$, $7d$, $7e$ and $7f$ cleaved the supercoiled pBR322 DNA into nicked form efficiently upon visible light irradiation in the presence of NADH.
4.1 Introduction

Fullerene and its derivatives have shown promising potency in biological applications such as HIV-protease inhibitors [1,2], anti-bacterials [3] and anti-tuberculars [4]. Another significant application of C\textsubscript{60} is related to the easy photoexcitation of fullerenes. On photoirradiation, ground state fullerene can be excited to \(^1\text{C}_\text{60}\); which is readily converted to \(^3\text{C}_\text{60}\) via intersystem crossing. This long-lived triplet state can decay to the ground state by transferring its energy to molecular oxygen (O\textsubscript{2}). The singlet oxygen thus generated is a highly cytotoxic species.

Furthermore, the high-energy species \(^1\text{C}_\text{60}\) and \(^3\text{C}_\text{60}\) are excellent acceptors and, in the presence of a donor like guanosine residue present in DNA, can easily reduce to \(\text{C}_\text{60}^-\). In the presence of oxygen, the fullerene radical anion can transfer one electron, producing superoxide radical anion \(\text{O}_2^-\). The singlet oxygen and superoxide radical anion are well known reactive species towards DNA and hence will effectively cause DNA cleavage [5].

The selectivity of DNA cleavage by fullerenes can be improved by conjugating it with units having specific affinity for nucleic acids. Hence nucleic acid specific agents, such as acridine [6], netropsin [7], complementary oligonucleotides [8,9] and trimethoxyindole-2-carboxylate [10], have been linked to fullerenes resulting in improved selectivity in DNA binding, increased DNA-photocleavage activity and consequently enhanced cytotoxicity. Coupling of fullerene with these agents is also an effectual method to increase its hydrophilicity; since the poor solubility of fullerenes in biologically relevant media is a major deterrent towards its use as a photo dynamic therapy reagent.

In the present investigation we have chosen triazine as our nucleic acid or protein binding agent and further conjugated it with fullerene to carry out DNA cleavage.

4.1.1 Importance of s-triazine

The triazine structure is a heterocyclic ring, analogous to the six-membered benzene ring but with three carbons replaced by nitrogen. The three isomers of triazine are 1,2,3-triazine, 1,2,4-triazine, and 1,3,5-triazine. (Figure 1)
1,3,5-triazines moieties, represent an interesting class of compounds possessing a wide spectrum of biological activities such as anti-cancer, antiviral, fungicidal, insecticidal, bactericidal, herbicidal and antimicrobial, antimalarial and DNA cleavage agents. s-triazine synthon is used efficiently to prepare multidentate ligands for the preparation of supramolecular assemblies. A wide variety of sophisticated s-triazine derivatives can be easily prepared from low-cost cyanuric chloride, i.e. 2,4,6-trichloro-1,3,5-triazine. The advantage of cyanuric chloride is the differential reactivity of its three chloride atoms. They can be substituted by nucleophiles at different temperatures in the presence of a base. Compounds with a maximum of three functions can be synthesized in a good yield. Numerous s-triazine derivatives have been prepared using this synthetic route.

Figure 2 General preparation of multifunctional s-triazine derivatives

4.1.2 Triazines in DNA Cleavage

A novel fused 1,2,4-triazine aryl derivatives containing the hydrazide moiety has been reported as having antitumor and DNA cleaving activity. (Figure 3)
A 3-amino-1,2,4-benzotriazine 1,4-dioxide has been reported as a clinically promising antitumor agent that derives its biological activity from DNA cleavage[11]. It cleaves DNA due to a radical species generated by enzymatic one-electron reduction of the heterocycle [22]. A series of dicationic diaryltriazines have been reported as nucleic acid binding agents which bind in the minor groove of DNA at AT sites [23].

Hence s-triazine and related derivatives are known to be minor groove binders with specific affinity for the AT-rich sequences of DNA. (Figure 4) Hence our interest was to develop fullerene based s-triazine substituted derivatives which can act as a systematic DNA photocleaving agent. We presume that the presence of cationic fullerene substituted s-triazine containing compounds would result in better DNA cleaving ability compared to substituted s-triazine alone, due to the favourable electrostatic and ionic interactions between positively charged cationic fullerene s-triazine derivatives and negatively charged phosphate DNA backbone.
4.2 Experimental

4.2.1 Chemical and Reagents

All the chemicals and reagents were of analytical grade of BDH, Aldrich and Merck unless and otherwise specified. The solvents used for the analysis were purified by standard methods [24]. The progress of the reaction was monitored on readymade silica gel plates purchased from Merck having toluene: ethyl acetate as a solvent system.

4.2.2 Apparatus

The melting points (°C, uncorrected) were taken using MPA100 Automated Melting Point Apparatus. The FT-IR spectra were recorded on Bruker Tensor 27 FT-IR spectrometer with KBr pellets. The ESI-MS spectra were recorded on Applied Biosystems, API 2000 LC/MS/MS System. Vario Micro cube elemental analyzer was used as elemental analysis system; ¹H NMR and ¹³C NMR spectra were recorded on a Bruker Avance II 400 MHz spectrometer in DMSO-d₆ or CDCl₃ with tetramethylsilane (TMS) as an internal standard. The nanoparticle size was determined using Transmission Electron Microscope (TEM) JEOL JEM 2100 and Dynamic Light Scattering (DLS)
4.3 Synthesis and characterization

The synthetic scheme adopted for the novel cationic fullerene derivatives having substituted s-triazine units is as shown below:

**Scheme 1: Synthesis of novel cationic fullerene-s-triazine conjugates.**
The synthesis of 2,4-dichloro-6-morpholin-4-yl-1,3,5-triazine (2) was carried out by reported methods [25].

The synthesis of 4-chloro-6-morpholino-N-(substituted-phenyl-1,3,5-triazin-2-amine) (3a-f) were carried out by following a similar method reported earlier [25].

The synthesis of 4a-f was carried out as described below:

4.3.1 General procedure for the synthesis of novel 4-(3-aminophenyl)-6-morpholino-N-substituted-1,3,5-triazin-2-amine (4a):

To a mixture of 4-chloro-6-morpholino-N-(substituted-phenyl-1,3,5-triazin-2-amine 3a (1.31 g, 4.5 mmol) and tetrakis(triphenylphosphine) palladium (0.16 g, 0.13 mmol) in DME (Dimethyl ether) (10 mL) was added 3-amino phenylboronic acid (0.92 g, 6.75 mmol). This was followed by the immediate addition of aqueous Na₂CO₃ (2 M, 4.7 mL). The mixture was flushed with N₂ for 5 min and the reaction mixture was then heated under reflux for 48 h. After cooling, the reaction mixture was evaporated under reduced pressure to dryness. THF (100 mL) was added and the suspension was placed in an ultrasonic bath for a few minutes. The mixture was filtered, washed thoroughly with THF and the filtrate was evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate as eluant, to afford 4a as a white solid. The compounds 4b-f were also synthesized by following the above procedure.

4.3.1.1 4-(3-aminophenyl)-6-morpholino-N-phenyl-1,3,5-triazin-2 amine (4a):

Yield: 73 %, mp >240°C; Analysis for C₁₉H₁₈N₆O, (348.40) Calcd: % C, 65.50; H, 5.79; N, 24.12. Found: % 65.53; H, 5.76; N, 24.15. FT-IR (KBr, ν cm⁻¹): 3100-3300 (-NH), 3085 (Aromatic CH str), 1255 (C-O-C), 814 (s-triazine C-N str.); ¹H NMR (400 MHz, DMSO-d₆) δ ppm: 4.19 (s, 1H, NH), 6.11 (m, 2H, NH₂), 6.9-8.1 (m, 9H, Ar-H), 3.85 (t, 4H, J = 7.73, 2,6-CH₂ of morpholine), 3.90 (t, 4H, J = 7.77, 3,5-CH₂ of morpholine); ¹³C NMR (125 MHz, DMSO-d₆) δ ppm: 177.1, 168.3, 161.2 (s-triazine carbon 3C), 114.22, 113.42, 117.11, 116.02, 118.32, 122.24, 128.18, 129.63, 130.252, 130.24, 137.48, 146.43, (Phenyl ring carbon C), 68.40, 67.13, 48.27, 48.41 (4C of morpholine). MS: m/z 348.17 (M⁺).

4.3.1.2 4-(3-aminophenyl)-6-morpholino-N-(4-chlorophenyl)-1,3,5-triazin-2-amine (4b):

Yield: 75%, mp 212-214°C; Analysis for C₁₉H₁₇ClN₆O, (382.85) Calcd: % C, 59.61; H, 5.00; N, 21.95. Found: % 59.63; H, 5.03; N, 21.97. FT-IR (KBr, ν cm⁻¹): 3150-3320 (-NH), 3085 (Aromatic CH str), 1255 (C-O-C), 814 (s-triazine C-N str.); ¹H NMR (400 MHz, DMSO-d₆) δ ppm: 4.31 (s, 1H, NH), 5.9 (m, 2H, NH₂), 7.0-8.3 (m, 8H, Ar-H), 3.23 (t, 4H, J = 7.73, 2,
6-CH₂ of morpholine), 3.95 (t, 4H, J = 7.77, 3,5-CH₂ of morpholine); ¹³C NMR (125 MHz, DMSO-d₆) δ ppm: 178.1, 166.3, 160.2 (s-triazine carbon 3C), 113.13, 114.74, 118.50, 118.09, 119.18, 122.42, 129.41, 129.09, 130.20, 130.38, 137.41, 146.24, (Phenyl ring carbon C), 67.18, 68.33, 48.51, 47.74 (carbon of morpholine). MS: m/z 382.13 (M⁺).

4.3.1.3 4-(3-aminophenyl)-N-(m-nitrophenyl)-6-morpholino-1,3,5-triazin-2-amine (4c):

Yield: 78 %, mp 218-220°C; Analysis for C₁₉H₁₉N₇O₃, (393.40) Calcd: % C, 58.01; H, 4.87; N, 24.92. Found: % C, 58.03; H, 4.88; N, 24.90. FT-IR (KBr, v cm⁻¹): 3100-3304 (-NH), 3005 (Aromatic CH str), 1554 (N-O str), 1250 (C-O-C), 815 (s-triazine C-N str.);¹H NMR (400 MHz, DMSO-d₆) δ ppm: 10.22 (s, 1H, NH), 6.03 (m, 2H, NH₂), 4.24 (s, 1H, NH-Ar), 6.90-8.22 (m, 8H, Ar-H), 4.02 (t, 4H, J = 7.73, 2,6-CH₂ of morpholine), 3.35 (t, 4H, J = 7.77, 3,5-CH₂ of morpholine);¹³C NMR (125 MHz, DMSO-d₆) δ ppm: 178.10, 164.31, 162.23 (s-triazine carbon 3C), 113.20, 114.04, 116.19, 117.84, 118.32, 122.24, 127.10, 128.31, 130.22, 130.85, 137.42, 146.33, (Phenyl ring carbon C), 67.40, 66.32, 45.37, 47.38 (carbon of morpholine). MS: m/z 393.15 (M⁺).

4.3.1.4 4-(3-aminophenyl)-N-(o-methoxyphenyl)-6-morpholino-1,3,5-triazin-2-amine (4d):

Yield: 72 %, mp 196-198°C; Analysis for C₂₀H₂₂N₆O₂, (378.43) Calcd: % C, 63.48; H, 5.86; N, 22.21. Found: % C, 63.50; H, 5.85; N, 22.25. FT-IR (KBr, v cm⁻¹): 3200-3330 (-NH), 3012 (Aromatic CH str), 2805 (Aliphatic CH str), 1255 (C-O-C), 814 (s-triazine C-N str.);¹H NMR (400 MHz, DMSO-d₆) δ ppm: 10.19 (s, 1H, NH), 3.2 (s, 3H CH₃), 5.14 (m, 2H, NH₂), 4.24 (s, 1H, NH-Ar), 6.8-8.1 (m, 8H, Ar-H), 4.10 (t, 4H, J = 7.73, 2,6-CH₂ of morpholine), 3.63 (t, 4H, J = 7.77, 3,5-CH₂ of morpholine);¹³C NMR (125 MHz, DMSO-d₆) δ ppm: 175.10, 167.32, 162.26 (s-triazine carbon 3C), 53.20, 114.12, 114.46, 117.15, 117.26, 118.30, 122.52, 128.92, 128.30, 130.26, 130.42, 146.43, 154.28 (Phenyl ring carbon C), 67.38, 67.44, 48.77, 48.63 (carbon of morpholine). MS: m/z 378.18 (M⁺).

4.3.1.5 4-(4-(3-aminophenyl)-6-morpholino-1,3,5-triazin-2-ylamino)phenol (4e):

Yield: 77 %, mp 193-195°C; Analysis for C₁₉H₂₀N₆O, (364.40) Calcd: % C, 62.62; H, 5.53; N, 23.60. Found: % 62.63; H, 5.56; N, 23.63. FT-IR (KBr, v cm⁻¹): 3500 (-OH), 3100-3300 (-NH), 3085 (Aromatic CH str), 1255 (C-O-C), 814 (s-triazine C-N str.);¹H NMR (400 MHz, DMSO-d₆) δ ppm: 10.14 (s, 1H, NH), 5.4 (s, 1H, OH), 5.63 (m, 2H, NH₂), 4.24 (s, 1H, NH-Ar), 6.9-8.1 (m, 8H, Ar-H), 3.33 (t, 4H, J = 7.73, 2,6-CH₂ of morpholine), 3.95 (t, 4H, J = 7.77, 3,5-CH₂ of morpholine);¹³C NMR (125 MHz, DMSO-d₆) δ ppm: 177.14, 168.32, 161.28 (s-triazine carbon 3C), 114.41, 114.93, 117.12, 116.94, 118.43, 122.22, 128.1, 128.96, 130.16, 139.84, 146.36, (Phenyl ring carbon C), 68.41, 67.03, 48.74, 48.36 (carbon of morpholine). MS: m/z 364.16 (M⁺).
4.3.1.6 3-(4-(3-aminophenyl)-6-morpholino-1,3,5-triazin-2-ylamino)benzene-1,2-diol (4f):

**Yield:** 79 %, mp 198-200°C; **Analysis for C_{19}H_{20}N_{6}O_{3}, (380.40) Calcd:** % C, 59.99; H, 5.30; N, 22.09. **Found:** % C, 59.97; H, 5.32; N, 22.10. **FT-IR** (KBr, v cm⁻¹): 3500 (-OH), 3100-3300 (-NH), 3080 (Aromatic CH str), 1255 (C-O-C), 810 (s-triazine C-N str.); \(^1\)H NMR (400 MHz, DMSO-d₆) δ ppm: 10.12 (s, 1H, NH), 5.45 (m, 2H, NH₂), 5.4 (s, 1H, OH) 4.24 (s, 1H, NH-Ar), 6.9-8.1 (m, 7H, Ar-H), 4.4 (t, 4H, \(J = 7.73\), 2,6-CH₂ of morpholine), 3.85 (t, 4H, \(J = 7.77\), 3,5-CH₂ of morpholine); \(^1\)C NMR (125 MHz, DMSO-d₆) δ ppm: 177.13, 168.32, 161.42 (s-triazine carbon 3C), 114.24, 114.45, 117.71, 117.25, 118.33, 122.20, 128.21, 128.23, 130.02, 130.4, 137.4, 146.3, (Phenyl ring carbon C), 68.4, 67.3, 48.7, 48.3 (carbon of morpholine). **MS:** m/z 380.16 (M+).

4.3.2 General procedure for the (E)-4-((3-(4-morpholino-6-(substitutedphenylamino)-1,3,5-triazin-2-yl)phenylimino)methyl)benzaldehyde (5a):

To an ethanolic solution of terephthaldehyde (0.11 g, 1 mmol) was added 3-4 ml of glacial acetic acid. This mixture was refluxed for a while and in the mean time 4a (0.34 g, 1 mmol) was added portion wise slowly for a period of half an hour. The yellow product obtained was isolated and purified by column chromatography (toluene/methanol 9:1). The solvent was then distilled out to get 5a. Compounds 5b-f was synthesized by following the above procedure.

4.3.2.1 (E)-4-((3-(4-morpholino-6-(pbenylamino)-1,3,5-triazin-2-yl)phenylimino)methyl)benzaldehyde (5a):

**Yield:** 83 %, mp 221°C ; **Analysis for C_{27}H_{24}N_{6}O_{2} (464.52) Calcd:** % C: 69.81, H: 5.21, N: 18.09. **Found:** % C: 69.83, H: 5.22, N: 18.10. **FT-IR** (KBr, v cm⁻¹) 3230 (N-H), 3094 (Ar-H), 1695 (CHO), 1595 (C=N), 1050 (C-O-C); \(^1\)H NMR (400 MHz, DMSO-d₆) δ ppm: 9.97 (s, 1H, CHO), 4.20 (s, 1H, NH-Ar), 7.22-8.36 (m, 13H, Ar-H), 8.06 (s, 1H, N=CH), 3.80 (t, 4H, \(J = 7.73\), 2,6-CH₂ of morpholine), 3.65 (t, 4H, \(J = 7.77\), 3,5-CH₂ of morpholine); \(^1\)C NMR (125 MHz, DMSO-d₆) δ ppm: 191.31, 177.23, 167.21, 161.44, 153.22, 142.14, 139.42, 131.72 130.48, 129.37, 126.37, 121.21, 117.88, 68.12, 67.22, 47.42, 47.54. **ESI-MS** m/z (M+): 464.20.

4.3.2.2 (E)-4-((3-(4-(4-chlorophenylamino)-6-morpholino-1,3,5-triazin-2-yl)phenylimino)methyl)benzaldehyde (5b):

**Yield:** 81 %, mp 201°C; **Analysis for C_{27}H_{23}N_{7}O_{4} (509.52) Calcd:** % C: 63.65, H: 4.55, N: 19.24. **Found:** % C: 63.64, H: 4.53, N: 19.22. **FT-IR** (KBr, v cm⁻¹) 3240 (N-H), 3094 (Ar-H), 1695 (CHO), 1605 (C=N), 1110 (C-O-C); \(^1\)H NMR (400 MHz, DMSO-d₆) δ ppm: 10.20 (s, 1H, CHO), 4.20 (s, 1H, NH-Ar), 7.22-8.36 (m, 12H, Ar-H), 8.06 (s, 1H, N=CH), 3.81 (t, 4H, \(J = 7.77\), 2,6-CH₂ of morpholine), 3.65 (t, 4H, \(J = 7.77\), 3,5-CH₂ of morpholine).
7.73, 2,6-CH$_2$ of morpholine), 3.98 (t, 4H, $J = 7.77$, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ ppm: 190.32, 177.15, 167.42, 161.44, 153.25, 142.32, 139.32, 138.52, 131.26, 130.34, 129.76, 128.24, 126.96, 125.38, 124.26, 123.87, 122.84, 121.22, 119.18, 67.29, 66.62, 48.41, 47.35. EI-MS m/z (M$^+$) 509.18.

4.3.2.3 (E)-4-((3-(4-morpholino-6-(4-nitrophenylamino)-1,3,5-triazin-2-yl)phenylimino)methyl)benzaldehyde (5c):

Yield: 79 %, mp 197°C ; Analysis for C$_{27}$H$_{22}$ClN$_6$O$_2$ (498.96) Calcd: % C: 64.99, H: 4.65, N: 16.84. Found: % C: 65.01, H: 4.66, N:16.86. FT-IR (KBr, v cm$^{-1}$) 3240 (N-H), 3040 (Ar-H), 1693 (CHO), 1585 (C=N), 1050 (C-O-C); $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm: 9.97 (s, 1H, CHO), 4.24 (s, 1H, NH-Ar), 7.22–8.36 (m, 13H, Ar-H), 8.06 (s, 1H, N=CH), 3.85 (t, 4H, $J = 7.73$, 2,6-CH$_2$ of morpholine), 3.93 (t, 4H, $J = 7.77$, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ ppm 191.23, 177.53, 168.42, 162.42, 153.56, 147.42, 138.24, 139.34, 131.22, 130.41, 129.77, 128.16, 127.24, 126.28, 125.23, 124.24, 124.56, 121.72, 122.24, 124.19, 124.81, 124.42, 67.22, 66.61, 45.24, 46.68. EI-MS m/z (M$^+$) 498.16.

4.3.2.4 (E)-4-((3-(4-(3-methoxyphenylamino)-6-morpholino-1,3,5-triazin-2-yl)phenylimino)methyl)benzaldehyde (5d):

Yield: 79%, mp 208°C ; Analysis for C$_{28}$H$_{26}$N$_6$O$_3$ (494.54) Calcd: % C: 68.00, H: 5.30, N: 16.99. Found: % C: 68.02, H: 5.32, N: 17.01. FT-IR (KBr, v cm$^{-1}$) 3200 (N-H), 3010 (Ar-H), 2801 (Aromatic C-H), 1702 (CHO), 1601 (C=N), 1030 (C-O-C); $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm: 9.96 (s, 1H, CHO), 4.20 (s, 1H, NH-Ar), 7.22–8.36 (m, 12H, Ar-H), 8.06 (s, 1H, N=CH), 5.2 (s, 1H, OH), 3.74 (t, 4H, $J = 7.73$, 2,6-CH$_2$ of morpholine), 3.66 (t, 4H, $J = 7.77$, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ ppm: 191.24, 177.13, 167.24, 161.52, 161.76, 160.63, 153.23, 146.53, 142.23, 139.72, 131.28 130.31, 131.22, 131.28, 129.72, 129.17, 126.27, 122.39, 122.23, 121.27, 117.89, 119.32, 106.29, 67.23, 67.23 47.54, 48.13. EI-MS m/z (M$^+$) 494.21.

4.3.2.5 (E)-4-((3-(4-(4-hydroxyphenylamino)-6-morpholino-1,3,5-triazin-2-yl)phenylimino)methyl)benzaldehyde (5e):

Yield: 72%, mp 214°C ; Analysis for C$_{27}$H$_{22}$N$_6$O$_3$ (480.52) Calcd: % C: 67.49, H: 5.03, N: 17.49. Found: % C: 67.47, H: 5.06, N: 17.51. FT-IR (KBr, v cm$^{-1}$) 3210 (N-H), 3030 (Ar-H), 1700 (CHO), 1605 (C=N), 1020 (C-O-C); $^1$H NMR (400 MHz, DMSO-$d_6$) δ ppm: 9.96 (s, 1H, CHO), 4.20 (s, 1H, NH-Ar), 7.22–8.36 (m, 12H, Ar-H), 8.06 (s, 1H, N=CH), 5.2 (s, 1H, OH), 3.75 (t, 4H, $J = 7.73$, 2,6-CH$_2$ of morpholine), 3.67 (t, 4H, $J = 7.77$, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ ppm: 191.22, 177.43, 167.24, 161.62, 161.61, 160.23 153.63.
4.3.2.6 \((E)-4-((3-(4-(2,3-dihydroxyphenylamino)-6-morpholino-1,3,5-triazin-2-yl)phenylimino)me
thy)benzaldehyde (5f):

Yield: 83%, mp 210°C ; Analysis for \(C_{27}H_{24}N_6O_2\) (496.52) Calcd: % C: 65.31, H: 4.87,
N: 16.93. Found: % C: 65.32, H: 4.85, N: 16.95. FT-IR (KBr, \(\nu\) cm\(^{-1}\)) 3220 (N-H), 3093 (Ar-H),
1695 (CHO), 1595 (C=N), 1050 (C-O-C); \(^1H\) NMR (400 MHz, DMSO-d<sub>6</sub>) \(\delta\) ppm: 9.97 (s, 1H,\nCHO), 4.25 (s, 1H, NH-Ar), 7.22-8.36 (m, 11H, Ar-H), 8.06 (s, 1H, N=CH), 5.2 (s, 1H, OH), 5.1
(s, 1H, OH), 3.75 (t, 4H, \(J = 7.73\), 2,6-CH<sub>2</sub> of morpholine), 3.75 (t, 4H, \(J = 7.77\), 3,5-CH<sub>2</sub>
of morpholine); \(^13C\) NMR (125 MHz, DMSO-d<sub>6</sub>) \(\delta\) ppm: 191.30, 177.23, 167.45, 161.40, 161.62,
160.53 153.34, 149.26, 146.43, 142.38, 139.29, 131.09 131.03, 131.09, 130.83, 129.57, 128.71,
126.42, 122.32, 121.32, 117.08, 119.23, 106.24, 69.21, 65.53 48.44, 49.23.; ESI-MS m/z (M+) 496.19.

4.3.3 General procedure for the synthesis of fulleropyrrolidines (6a-f)

Schiff base 5a (46.42 mg, 0.1 mmol), N-methylglycine (5 mg) and C<sub>60</sub> (72 mg,
0.1 mmol) were refluxed in dry toluene in inert atmosphere for 6 h. The product was first
purified by column chromatography (toluene/ethyl acetate 9:1) to get pure product 6a. In
a similar way all other products 6b-f were obtained by the above procedure.

4.3.3.1 Compound 6a:

Yield: 35 %, mp >240°C; Analysis for \(C_{89}H_{29}N_7O\) (1212.23) Calcd for % C: 88.18; H: 2.41;
N: 8.09. Found % C: 88.15; H: 2.43; N: 8.11. FT-IR (KBr, \(\nu\) cm\(^{-1}\)) 3340 (N-H ), 3086 (Ar-\nH), 2890 (C-H stre), 1540 (N-H ben), 1595 (C=N), 1150 (C-O-C), 523 (organo fullerene); \(^1H\) NMR (400 MHz, DMSO-d<sub>6</sub>) \(\delta\) ppm: 4.75 (dd, 1H, \(J = 9.2\), HHC-N of the pyrrolidine ring), 4.29 (s,
1H, CH of the pyrrolidine ring), 2.24 (s, 3H, CH<sub>3</sub> linked to N of pyrrolidine ring) 4.22 (s, 1H,
NH-Ar), 7.22-8.36 (m, 13H, Ar-H), 8.02 (s, 1H, N=CH), 3.88 (t, 4H, \(J = 7.72\), 2,6-CH<sub>2</sub>
of morpholine), 3.87 (t, 4H, \(J = 7.71\), 3,5-CH<sub>2</sub> of morpholine); \(^13C\) NMR (125 MHz, DMSO-d<sub>6</sub>) : 177.14, 167.54, 161.14, (s-triazine ring) 153.42, 151.52, 150.53, 145.87, 146.49, 145.72, 144.32,
143.81, 143.71, 142.32, 141.42, 140.65, 135.73, 134.57, 132.84, 130.75, 129.34, 128.92, 128.61,
127.47, 126.77, 125.39, 124.56, 123.78, 122.48, 118.89, 115.49, 112.63, 111.12, 66.42, 65.45,
48.68, 47.61 (CH<sub>2</sub> of morpholine) 37.16 (CH<sub>3</sub> linked to N of the pyrrolidine ring, 68.43 (NCH<sub>2</sub>
of pyrrolidine ring), 83.47 (NCH of the pyrrolidine ring), 76.21, 73.23 (sp<sup>3</sup> C- of C60), 72.71,
71.34, 66.76 (sp<sup>3</sup> C-of C60); ESI m/z: 1213.20 (M+).
4.3.3.2 Compound 6b:

Yield: 36%, mp 220°C; Analysis for C$_9$H$_2$ClN$_7$O, (1246 67) Calcd for % C: 85.74; H: 2.26; N: 7.86. Found % C: 85.75; H: 2.27; N: 7.88. FT-IR (KBr, v cm$^{-1}$) 3310 (N-H), 3016 (Ar-H), 2850 (C-H stre), 1612 (N-H ben), 1595 (C=N), 1204 (C-O-C), 525 (organofullerene); $^1$H NMR (400 MHz, CHCl$_3$) $\delta$ ppm: 5.20 (dd, 1H, $J = 9.33$, HHC-N of the pyrrolidine ring), 4.39 (s, 1H, CH of the pyrrolidine ring), 2.20 (s, 3H, CH$_3$ linked to N of pyrrolidine ring) 4.19 (s, 1H, NH-Ar), 7.22–8.26 (m, 12H, Ar-H), 8.16 (s, 1H, N=CH), 3.80 (t, 4H, $J = 7.72$, 2,6-CH$_2$ of morpholine), 3.68 (t, 4H, $J = 7.71$, 3,5-CH$_2$ of morpholine).

4.3.3.3 Compound 6c:

Yield: 37%, mp >240°C; Analysis for C$_8$H$_2$N$_8$O$_3$, (1257.23) Calcd for % C: 85.02; H: 2.24; N: 8.91. Found % C: 85.04; H: 2.27; N: 8.94. FT-IR (KBr, v cm$^{-1}$) 3340 (N-H), 3080 (Ar-H), 2840 (C-H stre), 1570 (N-H ben), 1595 (C=N), 1120 (C-O-C), 527 (organofullerene); $^1$H NMR (400 MHz, CHCl$_3$) $\delta$ ppm: 5.25 (dd, 1H, $J = 9.6$, HHC-N of the pyrrolidine ring), 4.39 (s, 1H, CH of the pyrrolidine ring), 2.24 (s, 3H, CH$_3$ linked to N of pyrrolidine ring) 4.21 (s, 1H, NH-Ar), 7.26–8.12 (m, 12H, Ar-H), 8.10 (s, 1H, N=CH), 3.77 (t, 4H, $J = 7.72$, 2,6-CH$_2$ of morpholine), 3.89 (t, 4H, $J = 7.71$, 3,5-CH$_2$ of morpholine).

4.3.3.4 Compound 6d:

Yield: 36%, mp 231°C; Analysis for C$_9$H$_3$N$_7$O$_2$, (1242.25) Calcd for % C: 87.02; H: 2.52; N: 7.89. Found % C: 87.05; H: 2.49; N: 7.92. FT-IR (KBr, v cm$^{-1}$) 3314 (N-H), 3070 (Ar-
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II), 2790 (C-H stre), 1604 (N-H ben), 1560 (C=N), 1040 (C-O-C), 528 (organo fullerene); $^1$H NMR (400 MHz, CHCl$_3$) δ ppm: 5.25 (dd, 1H, J = 9.6, HHC-N of the pyrrolidine ring), 4.37 (s, 1H, CH of the pyrrolidine ring), 2.24 (s, 3H, CH$_3$ linked to N of pyrrolidine ring) 4.28 (s, 1H, NH-Ar), 7.22–8.36 (m, 12H, Ar-H), 3.52 (s, 3H, OCH$_3$), 8.16 (s, 1H, N=CH), 3.58 (t, 4H, J = 7.72, 2,6-CH$_2$ of morpholine), 3.87 (t, 4H, J = 7.71, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, CHCl$_3$) 168.42, 165.32, 162.14, (s-triazine ring) 153.12, 153.32, 149.24, 146.35, 146.87, 145.47, 144.16, 143.42, 142.21, 142.31, 139.25, 135.81, 132.72, 131.84, 130.45, 129.23, 128.10, 127.11, 126.37, 125.39, 124.16, 123.41, 122.58, 116.31, 114.31, 112.02, 110.24, 63.22, 61.43, 48.20, 46.20 (CH$_2$ of morpholine) 66.36 (NCH$_2$ of pyrrolidine ring), 83.27 (NCH of the pyrrolidine ring), 74.43, 73.22 (sp$^3$ C- of C60), 72.41, 71.61, 67.49 (sp$^3$ C-of C60); ESI m/z: 1243.40 (M+).

4.3.3.5 Compound 6e:

Yield: 39 %, mp 232°C; Analysis for C$_{89}$H$_{29}$N$_7$O$_2$ (1228.23) Calcd for % C: 87.03; H: 2.38; N: 7.98. Found % C: 87.05; H: 2.41; N: 7.99. FT-IR (KBr, ν cm$^{-1}$) 3230 (N-H), 3080 (Ar-H), 2890 (C-H stre), 1548 (N-H ben), 1610 (C=O-C), 524 (organo fullerene); $^1$H NMR (400 MHz, CHCl$_3$) δ ppm: 5.25 (dd, 1H, J = 9.6, HHC-N of the pyrrolidine ring), 5.52 (s 1H of OH), 4.38(s, 1H, CH of the pyrrolidine nng), 2.24 (s, 3H, CH$_3$ linked to N of pyrrolidine ring) 4.23 (s, 1H, NH-Ar), 7.22–8.36 (m, 13H, Ar-H), 8.16 (s, 1H, N=CH), 3.88 (t, 4H, J = 7.72, 2,6-CH$_2$ of morpholine), 3.87 (t, 4H, J = 7.71, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, CHCl$_3$) 172.34, 165.24, 162.25, (s-triazine ring) 154.32, 153.32, 149.24, 146.35, 146.87, 145.47, 144.16, 143.42, 142.21, 142.31, 139.25, 135.81, 132.72, 131.84, 130.45, 129.23, 128.10, 127.11, 126.37, 125.39, 124.16, 123.41, 122.58, 116.31, 114.31, 112.02, 110.24, 63.22, 61.43, 48.20, 46.20 (CH$_2$ of morpholine) 69.4 (NCH$_2$ of pyrrolidine ring), 83.5 (NCH of the pyrrolidine ring), 76.2, 73.50 (sp$^3$ C- of C60), 72.7, 72.4, 68.6 (sp$^3$ C- of C60); ESI m/z: 1229.40 (M+).

4.3.3.6 Compound 6f:

Yield: 40 %, mp >240°C; Analysis for C$_{90}$H$_{30}$N$_7$O$_3$ (1244.23) Calcd for % C: 85.91; H: 2.35; N: 7.88. Found % C: 85.94; H: 2.38; N: 7.86. FT-IR (KBr, ν cm$^{-1}$) 3240 (N-H), 3240 (N-H ), 3080 (Ar-H), 2895 (C-H stre), 1516 (N-H ben), 1588 (C=O-C), 1070 (C-O-C), 529 (organo fullerene); $^1$H NMR (400 MHz, CHCl$_3$) δ ppm: 5.25 (dd, 1H, J = 9.6, HHC-N of the pyrrolidine ring), 4.39 (s, 1H, CH of the pyrrolidine ring), 5.25 (s 1H of OH), 2.24 (s, 3H, CH$_3$ linked to N of pyrrolidine ring) 4.26 (s, 1H, NH-Ar), 7.22–8.36 (m, 13H, Ar-H), 8.16 (s, 1H, N=CH), 3.88 (t, 4H, J = 7.72, 2,6-CH$_2$ of morpholine), 3.77 (t, 4H, J = 7.71, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, CHCl$_3$) 171.12, 164.33, 166.54, (s-triazine ring) 154.21, 153.30, 151.22, 147.38, 146.74, 145.43,
144.13, 143.42, 142.20, 139.45, 134.31, 132.37, 131.40, 130.15, 129.23, 128.41, 127.19, 126.37, 125.49, 124.52, 123.72, 122.92, 115.31, 113.31, 111.02, 110.24, 64.24, 62.44, 47.20, 49.45 (CH$_2$ of morpholine), 42.34 (CH$_3$ linked to N of the pyrrolidine ring), 66.36 (NCH$_2$ of pyrrolidine ring), 84.27 (NCH of the pyrrolidine ring), 75.26, 73.35 (sp$^3$ C-of C60), 73.45, 71.52, 65.28 (sp$^3$ C-of C60); 69.14 (NCH$_2$ of pyrrolidine ring), 83.73 (NCH of the pyrrolidine ring), 76.71, 74.62 (sp$^3$ C-of C60), 73.17, 76.28, 68.36 (sp$^3$ C-of C60), ESI m/z: 1245.20 (M$^+$).

4.3.4 General procedure for the synthesis of fullerene-$N,N$-dimethylpyrrolidine quaternary ammonium iodide salt (Cationic fullerene-triazine conjugates) (7a-f)

A solution of fulleropyrrolidine derivative 6a (80 mg, 0.066 mmol) and iodomethane (9.3 mg, 0.066 mmol) was refluxed with stirring for 2 days under argon. Then, the residue was washed with toluene (three times) and hexane (twice). The solvent was removed under vacuum to give fullerene-$N,N$-dimethylpyrrolidine quaternary ammonium iodide salts 7a as brownish solid. In a similar way all other products 7b–f were obtained by the above procedure.

4.3.4.1 Compound 7a:

Yield: 98%, mp >240°C; Analysis for $C_{90}H_{32}IN_7O$, (1354.17) Calcd for % C: 79.82; H: 2.38; N: 7.24. Found % C: 79.84; H: 2.41; N: 7.27. FT-IR (KBr, $\nu$ cm$^{-1}$) 3340 (N-H), 3086 (Ar-H), 2890, 2850 (Ali C-H stre of CH$_3$), 1540 (N-H ben), 1595 (C=N), 1150 (C-O-C), 524 (organo fullerene); $^1$H NMR (400 MHz, CHCl$_3$) $\delta$ ppm. 4.75 (dd, 1H, $J = 9.3$, HHC-N of the pyrrolidine ring), 4.29 (s, 1H, CH of the pyrrolidine ring), 2.24 (s, 3H, CH$_3$ linked to N of pyrrolidine ring), 2.84 (s, 3H, CH$_3$ linked to N of pyrrolidine ring), 4.20 (s, 1H, NH-Ar), 7.22–8.36 (m, 13H, Ar-H), 8.06 (s, 1H, N=CH), 3.88 (t, 4H, $J = 7.72$, 2,6-CH$_2$ of morpholine), 4.03 (t, 4H, $J = 7.71$, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, CHCl$_3$) : 177.12, 167.84, 160.14, (s-triazine ring) 153.12, 151.52, 150.51, 145.97, 146.48, 145.82, 144.33, 143.91, 143.01, 142.33, 141.41, 140.75, 138.25, 137.22 133.21, 133.62 135.93, 135.11, 134.27, 132.48, 130.35, 129.33, 128.90, 128.01, 127.42, 126.57, 125.39, 124.06, 123.73, 122.78, 118.59, 115.41, 112.63, 111.10, 66.2, 65.2, 48.2, 47.1 (CH$_2$ of morpholine) 40.14, 40.32 ((CH$_3$)$_2$ linked to N of the pyrrolidine ring), 69.43 (NCH$_2$ of pyrrolidine ring), 83.27 (NCH of the pyrrolidine ring), 72.34, 66.76 (sp$^3$ C-of C60); ESI m/z: 1228.10 (M$^+$).

4.3.4.2 Compound 7b:

Yield: 97%, mp 223°C; Analysis for $C_{90}H_{31}ClIN_7O$, (1388.61) Calcd for % C: 77.84; H: 2.25; N: 7.06. Found % C: 77.87; H: 2.27; N: 7.09. FT-IR (KBr, $\nu$ cm$^{-1}$) 3310 (N–H), 3016 (Ar–H), 2850, 2810 (Ali C-H stre of CH$_3$), 1612 (N-H ben), 1595 (C=N), 1204 (C-O-C), 525 (organo…
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fullerene); $^1$H NMR (400 MHz, CHCl$_3$) δ ppm: 5.20 (dd, 1H, $J = 9.3$, HHC-N of the pyrrolidine ring), 4.22 (s, 1H, CH of the pyrrolidine ring), 2.20 (s, 3H, CH$_3$ linked to N of pyrrolidine ring), 2.71 (s, 3H, CH$_3$ linked to N of pyrrolidine ring), 4.31 (s, 1H, NH-Ar), 7.22–8.26 (m, 12H, Ar-H), 8.16 (s, 1H, N=CH), 3.80 (t, 4H, $J = 7.72$, 2,6-CH$_2$ of morpholine), 3.68 (t, 4H, $J = 7.71$, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, CHCl$_3$) 172.32, 162.84, 160.64, (s-triazine ring) 154.30, 152.22, 150.11, 147.40, 146.89, 145.47, 144.43, 143.21, 142.23, 141.31, 139.25, 138.21, 137.20 135.30, 133.62 134.37, 131.80, 130.35, 129.23, 128.50, 127.62, 127.10, 126.37, 125.39, 124.16, 123.43, 122.38, 117.31, 112.31, 111.02, 64.21, 64.24, 49.20, 47.15 (CH$_2$ of morpholine) 42.10, 41.13 ((CH$_3$)$_2$ linked to N of the pyrrolidine ring), 67.63 (NCH$_2$ of pyrrolidine ring), 81.29 (NCH of the pyrrolidine ring), 75.21, 65.27 (2C, sp$^3$ C-of C60); ESI m/z: 1262.61 (M$^+$).

4.3.4.3 Compound 7c:

Yield: 96%, mp >240°C; Analysis for C$_9$H$_{31}$IN$_8$O$_3$, (1399.17) Calcd for % C: 77.26; H: 2.23; N: 8.01. Found % C: 77.28; H: 2.26; N: 8.04. FT-IR (KBr, v cm$^{-1}$) 3340 (N-H), 3080 (Ar-H), 2840, 2796 (Ali C-H stre of CH$_3$), 1570 (N-H ben), 1595 (C=N), 1120 (C-O-C), 528 (organo fullerene); $^1$H NMR (400 MHz, CHCl$_3$) δ ppm: 5.18 (dd, 1H, $J = 9.3$, HHC-N of the pyrrolidine ring), 4.39 (s, 1H, CH of the pyrrolidine ring), 2.24 (s, 3H, CH$_3$ linked to N of pyrrolidine ring), 2.26 (s, 3H, CH$_3$ linked to N of pyrrolidine ring), 4.45 (s, 1H, NH-Ar), 7.26–8.12 (m, 12H, Ar-H), 8.10 (s, 1H, N=CH), 3.47 (t, 4H, $J = 7.72$, 2,6-CH$_2$ of morpholine), 3.89 (t, 4H, $J = 7.71$, 3,5-CH$_2$ of morpholine); $^{13}$C NMR (125 MHz, CHCl$_3$) : 173.26, 166.31, 163.24, (s-triazine ring) 152.23, 151.52, 150.32, 146.48, 145.82, 145.07, 142.13, 143.21, 142.23, 141.31, 140.25, 139.32, 139.91, 138.21, 137.20, 135.30, 135.10, 134.27, 133.68, 132.48, 133.24, 130.35, 129.23, 128.95, 127.01, 126.37, 125.39, 124.06, 123.73, 122.38, 118.31, 113.20, 111.23, 110.24, 65.21, 64.02, 49.22, 47.25 (CH$_2$ of morpholine) 42.30, 42.14 ((CH$_3$)$_2$ linked to N of the pyrrolidine ring), 68.13 (NCH$_2$ of pyrrolidine ring), 83.27 (NCH of the pyrrolidine ring), 71.14, 66.73 (2C sp$^3$ C-of C60); ESI m/z: 1273.10 (M$^+$).

4.3.4.4 Compound 7d:

Yield: 98%, mp 210°C; Analysis for C$_9$H$_{31}$IN$_8$O$_3$, (1384.19) Calcd for % C: 78.96; H: 2.48; N: 7.08. Found % C: 78.98; H: 2.51; N: 7.11. FT-IR (KBr, v cm$^{-1}$) 3314 (N-H), 3070 (Ar-H), 2790, 2812 (Ali C-H stre of CH$_3$), 1604 (N-H ben), 1560 (C-N), 1040 (C-O-C), 527 (organo fullerene); $^1$H NMR (400 MHz, CHCl$_3$) δ ppm: 4.95 (dd, 1H, $J = 9.3$, HHC-N of the pyrrolidine ring), 4.59 (s, 1H, CH of the pyrrolidine ring), 2.22(s, 3H, CH$_3$ linked to N of pyrrolidine ring), 2.71 (s, 3H, CH$_3$ linked to N of pyrrolidine ring), 4.42 (s, 1H, NH-Ar), 7.22
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8.36 (m, 12H, Ar-H), 3.52 (s, 3H, OCH3), 8.16 (s, 1H, N=CH), 3.88 (t, 4H, J = 7.72, 2,6-CH2 of morpholine), 3.87 (t, 4H, J = 7.71, 3,5-CH2 of morpholine); 13C NMR (125 MHz, CHCl3) 169.42, 164.32, 160.14, (s-triazine ring) 153.42, 153.32, 149.26, 147.35, 146.89, 145.67, 144.10, 143.42, 142.21, 142.31, 139.25, 135.81, 132.27, 131.84, 130.45, 129.23, 128.10, 127.11, 126.37, 125.39, 124.16, 123.41, 122.58, 116.31, 114.31, 112.02, 63.24, 61.44, 48.20, 46.25 (CH2 of morpholine) 43.52, 42.21 ((CH3)2 linked to N of the pyrrolidine ring), 66.36 (NCH2 of pyrrolidine ring), 83.27 (NCH of the pyrrolidine ring), 74.42, 73.12 (sp3 C- of C60), 72.41, 71.61, 67.47 (sp3 C-of C60); ESI m/z: 1258.10 (M+).

4.3.4.5 Compound 7e:

Yield: 96%, mp 218°C; Analysis for C90H34IN7O2, (1372.17) Calcd for % C: 77.87; H: 2.47; N: 7.06. Found % C: 77.91; H: 2.49; N: 7.09. FT-IR (KBr, v cm⁻¹) 3230 (N-H), 3080 (Ar-H), 2890, 2797 (Ali C-H stre of CH3), 1548 (N-H ben), 1610 (C=N), 1226 (C-O-C), 528 (organo fullerene); 1H NMR (400 MHz, CHCl3) δ ppm: 5.02 (dd, 1H, J = 9.3, HHC-N of the pyrrolidine ring), 5.32 (s 1H of OH), 4.31 (s, 1H, CH of the pyrrolidine ring), 2.26 (s, 3H, CH3 linked to N of pyrrolidine ring), 2.67 (s, 3H, CH3 linked to N of pyrrolidine ring), 4.34 (s, 1H, NH-Ar), 7.22–8.36 (m, 13H, Ar-H), 8.21 (s, 1H, N=CH), 3.88 (t, 4H, J = 7.72, 2,6-CH2 of morpholine), 3.87 (t, 4H, J = 7.71, 3,5-CH2 of morpholine); 13C NMR (125 MHz, CHCl3) 171.32, 165.23, 161.14, (s-triazine ring) 154.30, 153.22, 150.21, 147.32, 146.89, 145.47, 144.43, 143.21, 142.21, 139.05, 139.95, 138.35, 137.14, 137.90. 136.54, 135.31, 134.47, 132.37, 131.80, 130.45, 129.23, 128.50, 127.10, 127.01, 126.37, 125.39, 124.16, 123.43, 122.38, 117.31, 112.31, 111.02, 63.21, 61.43, 49.20, 48.25 (CH2 of morpholine) 43.52, 42.21 ((CH3)2 linked to N of the pyrrolidine ring), 66.36 (NCH2 of pyrrolidine ring), 83.27 (NCH of the pyrrolidine ring), 74.42, 73.12 (sp3 C- of C60), 72.41, 71.61, 67.47 (sp3 C-of C60); ESI m/z: 1258.10 (M+).

4.3.4.6 Compound 7f:

Yield: 98%, mp 239°C; Analysis for C90H32IN7O3, (1386.17) Calcd for % C: 77.98; H: 2.33; N: 7.07. Found % C: 77.96; H: 2.36; N: 7.09. FT-IR (KBr, v cm⁻¹) 3240 (N-H), 3075 (Ar-H), 2820, 2895 (Ali C-H stre of CH3), 1548 (N-H ben), 1510 (C=N), 1070 (C-O-C), 529 (organo fullerene); 1H NMR (400 MHz, CHCl3) δ ppm: 4.85 (dd, 1H, J = 9.3, HHC-N of the pyrrolidine ring), 5.52 (s 1H of OH), 4.39 (s, 1H, CH of the pyrrolidine ring), 2.24 (s, 3H, CH3 linked to N of pyrrolidine ring), 2.32 (s, 3H, CH3 linked to N of pyrrolidine ring), 4.47 (s, 1H, NH-Ar), 7.22–8.36 (m, 13H, Ar-H), 8.22 (s, 1H, N=CH), 3.88 (t, 4H, J = 7.72, 2,6-CH2 of morpholine), 3.87 (t, 4H, J = 7.71, 3,5-CH2 of morpholine); 13C NMR (125 MHz, CHCl3) 172.12, 164.23, 166.24, (s-triazine ring) 154.11, 153.29, 151.25, 147.38, 146.84, 145.42, 144.13, 143.21, 142.21, 139.25, 138.54, 138.99, 137.39, 136.01, 135.82, 135.02, 134.61, 133.37, 131.40, 130.15, 129.23,
128.40, 127.20, 127.19, 126.37, 125.49, 124.62, 123.73, 122.92, 116.31, 112.31, 111.02, 110.24, 64.21, 61.43, 47.20, 49.25 (CH$_2$ of morpholine) 43.28, 42.32 (CH$_3$ linked to N of the pyrrolidine ring), 66.36 (NCH$_2$ of pyrrolidine ring), 83.27 (NCH of the pyrrolidine ring), 75.22, 65.29 (sp$^3$ C of C60); ESI m/z: 1260.20 (M$^+$).

4.3.5 Preparation of 6a-f and 7a-f water suspensions

The aqueous suspensions of compounds 7a-f were prepared by a method reported earlier by our group [4]. 50 mg of compound 7a in 50 mL of Milli-Q water was stirred at 40 °C for 2 weeks. The suspension thus obtained was then filtered through a Whatmann filter; then through a 0.22 µm nylon membrane to remove aggregates larger than 200 nm.

The size of suspended particles in water was determined using DLS (dynamic light scattering) and (Transmission Electron Microscope) TEM. The concentration of the solution used for the measurement was $1 \times 10^{-5}$ M. TEM samples were prepared by depositing a drop of dispersed compound 7a-f particles in water on the carbon-coated Formvar films on copper grids with an electron microscope operated at 200 keV.

4.4 Biological Assay

4.4.1 DNA cleavage assay

Thirty microlitres of aqueous solution of DNA pBR322 (Plasmid pBR322 DNA; Sigma Aldrich) (0.50 µg µL$^{-1}$) was diluted by adding 270 µL of water. Typically, 10 µL of aqueous suspension of 7a-f (1.0 × 10$^{-5}$ M), 10 µL of aqueous solution of DNA pBR322, NADH (Nicotinamide adenine dinucleotide) (5 µL, 0.126 M) and 8 µL of tris-EDTA buffer (10 µL, 150 × TE, pH 8.0) were mixed in a micro test tube under dark conditions. The samples were incubated under irradiation with visible light for 3 h at 298 K, mixed with 10 µL of loading buffer (0.1% bromophenol blue and 30% glycerol in TBE buffer) and loaded onto a 1% agarose gel containing ethidium bromide (1 µg mL$^{-1}$). The gels were run at a constant voltage of 70 V for 2 h in TBE buffer, washed with distilled water, visualized under a UV transilluminator and photographed using an instant camera. The band intensity was measured using ImageJ software [26] and expressed as % of DNA cleavage.
4.5 Results and discussion

The route adopted for the synthesis of substituent s-triazine and fullerene s-triazine conjugates are as shown in Scheme 1. The synthetic intermediates, 4-(4,6-dichloro-1,3,5-triazin-2-yl) morpholine was obtained in high yield when s-triazine 1 and morpholine were reacted in dry acetone and K2CO3 using pyridine as the hydrochloride scavenger and were cooled at 0°C under anhydrous conditions for 4 h. Similarly, 4-chloro-(N-substituted phenyl) 6-morpholino-1,3,5-triazin-2-amine 3a-f were obtained by reacting 2 with substituted aromatic amine in acetone-water using pyridine as the hydrochloride scavenger. The electrophilic component of the Pd-catalyzed Suzuki coupling was the 4-chloro-N-(substituted-phenyl)-6-morpholino-1,3,5-triazin-2-amine 3a-f, and the nucleophilic component for the reaction was 3-aminoarylboronic acid using Na2CO3 as base and [Pd(pph3)4] as a catalyst. Synthesis of 4-(3-aminophenyl)-N-(substituted-phenyl)-6-morpholino-1,3,5-triazin-2-amine 4a-f resulted in moderate to excellent yields Corresponding 4-(3-aminophenyl)-N-(substituted-phenyl)-6-morpholino-1,3,5-triazin-2-amine 4a-f were treated with equimolar amounts of terephthaldehyde in ethanol and glacial acetic acid to form 4-((3-(4-(N-substitutedphenylamino)-6-morpholino-1,3,5-triazin-2-yl)phenylimino) methyl) benzaldehyde 5a-f (Scheme 1).

Compounds (5a-f) 4-((3-(4-(N-substitutedphenylamino)-6-morpholino-1,3,5-triazin-2-yl)phenylimino) methyl) benzaldehyde, N-methyl glycine and Ce60 were then allowed to reflux in toluene wherein they undergo a 1,3 dipolar cycloaddition reaction to give fulleropyrrolidines 6a-f. The cationic fullerene s-triazine derivatives 7a-f (Scheme 1) was obtained by refluxing 6a-f with stochiometric amount of methyl iodide in chloroform for 2 days.

The FT-IR spectra of the novel compounds 5a-f showed characteristic bands of aldehyde carbonyl between at 1695-1702 cm⁻¹ and compound 6a-f organo fullerences between at 523-529 cm⁻¹. The ¹H NMR showed the presence of the pyrrolidine protons as two doublets at 4.39 and 4.85 ppm with coupling constants between 9.2-9.6 Hz and a singlet of OH at 5.52 ppm.

The quaternization of the nitrogen atom on the pyrrolidine ring was confirmed from the spectral data. The ESI mass spectrum of compound 7a-f (7f a representative compound) showed molecular ion peak at m/z 1260.20, in conformity with the molecular
formula $C_{90}H_{32}IN_{7}O_{3}$. The presence of a peak in the $^1H$ NMR spectra of compound 7f at $\delta$ 8.06 to 8.22 attributed to the (N=CH) proton. The peak between 3340 and 3230 cm$^{-1}$ for N–H stretching and organo fullerenes between at 523 - 529 cm$^{-1}$ were observed in all the derivatives and also IR spectra showed that the s-triazine nitrogen has not participated in salt formation. The study of the $^1H$ NMR and $^{13}C$ NMR for the surrounding atoms of the s-triazine nitrogen also showed that it has not undergone methylation. Owing to the basic nature of the pyrrolidine nitrogen which is the most basic, methylation occurs at the nitrogen of pyrrolidine.

4.5.1 Particle size of compounds 7a-f

The compounds 7a-f were characterized using DLS and TEM. DLS measures particle size based upon the rate of fluctuation of scattered light intensity due to Brownian motion of particles in suspension. Dynamic light scattering (Representing compounds d, 7e, 7f) produced an average particle diameter of 100 nm, 102 nm and 86 nm for compounds 7d, 7e, 7f respectively. The particle size distributions are shown in Figures 5.

The fullerene suspensions were prepared for TEM by pipetting a drop of the suspension to the TEM grid and allowing it to dry. TEM produced a 2-dimensional image of the material attached to the copper support grid. TEM images (Representating compounds 7f) results shows spherical shape particle, with diameter 20 nm and 2 nm in size as shown in Figure 6.

4.5.2 Cationic fullerene s-triazine conjugates as DNA scissors:

The photoinduced DNA cleavage activity of substituted s-triazine(5a-f) and cationic fullerene s-triazine derivative (7a-f) has been studied. The DNA scission activity of these derivatives were investigated using pBR322 supercoiled plasmid (form I) in C$_{60}$ s-triazine-NADH (Nicotinamide Adenine Dinucleotide - reduced) system under visible light irradiation and its product, nicked DNA (form II) was examined using agarose gel electrophoresis (Figure 7). From the Figure 7 it is evident that compounds 7a, 7d, 7e and 7f possess DNA cleaving property (lane 3(7a), lane 12(7d), lane 15 (7e) and lane 18(7f) ) in the presence of NADH and on visible light irradiation. In the absence of NADH or visible light these molecules do not exhibit any cleaving activity. Compounds 7b and 7c
(lane 4-6 and 7-9) showed no DNA cleavage under any condition. The cleaving ability follows the order: 7f (100%) > 7e (~68.99%) > 7d (~68.63%) > 7a (~30.38%).

4.5.3 Photocleavage activity of 7a-f and its relation to molecular properties:
Substituent effects on photocleavage of cationic fullerene s-triazine conjugates can be described by the cleaving ability order: 7f (o, m-OH) > 7e (p-OH) > 7d (o-OCH3) > 7a (phenyl) > 7b (p-Cl) > 7c (m-NO2). Compounds with electron-donating groups (two hydroxyl groups in 7f) photocleaved supercoiled DNA more efficiently in the presence of NADH than 7e which has only one hydroxyl group. The presence of 2 electron-donating groups in 7f gives it stronger ability to push electrons so as to facilitate intersystem crossing to generate superoxide anions. On the other hand compounds bearing electron-withdrawing groups 7b (Cl) and 7c (NO2) will pull the electrons towards them and thereby decreases the electron density. The diminished electron density attributed to compounds 7b and 7c can be considered as one of the reasons for poor DNA cleavage activity in its initial concentration (1 x 10^-5 M) and henceforth, requires high amount (1.5 x 10^-3 M) to accomplish DNA nicking.

4.5.4 Contribution of substituted s-triazine scaffold 5a-f (intermediates) towards DNA cleavage:
There are various studies indicating compounds bearing s-triazine pharmacophore as potential DNA cleaving agents [27-28]. We examined the cleaving ability of substituted s-triazine derivatives (5a-f). No DNA cleavage was observed for compounds 5a-c under dark conditions (Figure 8; lane 1-9) or under visible-light irradiation in the presence and absence of NADH. Compound 5d-f possessed DNA scission activity in the presence of NADH under visible-light irradiation after 3 h. It should be noted that this cleaving ability was only observed when the concentration of these molecules (5d-f) was increased to 1.5 x 10^-3 M which is greater than the initial concentration of 1 x 10^-5 M. At the enhanced concentration, the cleaving ability follows the order: 5f (100% cleavage) > 5e (~81.11%) > 5d (77.35%) and can be compared to the cleaving activity of cationic fullerene s-triazine derivatives discussed in later section.
4.5.5 Effect of visible-light irradiation time on DNA cleavage:

Figure 7 illustrates the DNA cleavage efficiency of the cationic fullerene s-triazine conjugates as a function of irradiation time. It can be seen that compounds 7a, 7d, 7e and 7f are the most competent DNA cleavage agent among the other fullerene derivatives. In addition, the conversion rate of nicked DNA (form II) from supercoiled plasmid DNA (form I) gradually decreases after 3 h of irradiation and the presence of nicked DNA almost gets saturated after 6 h of irradiation.

4.5.6 Effect of cationic fullerene s-triazine derivatives and NADH concentration on DNA cleavage:

The concentration of substituted s-triazine derivatives and NADH has been examined to acquire better understanding of DNA photocleavage by cationic fullerene s-triazine conjugates. At initial concentration (1×10⁻⁵ M) of derivatized molecules, a small quantity of supercoiled plasmid DNA (form I) was converted to nicked DNA (form II) as evident from the corresponding band intensity (Figure 7) (7a, lane 3; 7d lane 12; 7e lane 15 and 7f lane 18). However, the increased concentration (1.5×10⁻³ M) drastically improved the conversion rate of form I to II and this concentration is relatively less than the reported γ-cyclodextrine-bicapped C₆₀ (2.5 × 10⁻⁵ M) [29].

The concentration of NADH also plays a vital role in DNA cleavage activity; with increase in concentration of NADH there was an increase in the rate of cleaving activity. The results obtained are consistent with our previous studies indicating that NADH is an important coagent for photoinduction of DNA damage using fullerene conjugates [3].

4.5.7 Effect of pH on DNA cleavage:

The effect of pH on DNA cleavage was also analyzed by varying the pH from 7 to 9. The DNA-cleaving efficiency was found to increase from pH 7 to 8, reached its maximum at pH 8 and then declined at pH 8–9. Therefore pH 8 is the optimum pH scale for photoinduced DNA cleavage activity 7a-f under fullerene s-triazine NADH system.
4.5.8 Mechanism of DNA cleavage:

The photoinduced DNA cleavage activity greatly depends on the generation of Reactive Oxygen Species (ROS) including singlet oxygen (\( ^1\text{O}_2 \)), superoxide radical anion (\( \text{O}_2^- \)) and hydroxyl radical (\( \cdot\text{OH} \)) which are proposed to be the definite active species [31-34]. The \( ^3\text{C}_{60} \) triplet form of fullerene formed on photoirradiation will generate \( ^1\text{O}_2 \) by transferring its energy to molecular oxygen. This \( ^3\text{C}_{60} \) can also be easily reduced to \( \text{C}_{60}^- \) anion in the presence of a reductant such as NADH and this reduced \( \text{C}_{60}^- \) may produce \( \text{O}_2^- \) or \( \cdot\text{OH} \) in the presence of molecular oxygen by transferring its electron [30-33]. All these ROS can collectively cause significant DNA cleavage but the presence of s-triazine and the positive charge on the fullereopyrrolidine improves the selectivity of cleavage.

We presume that the positive charge on fullereopyrrolidine ring in cationic fullerene s-triazine conjugates participates in molecular interaction with negatively charged phosphate backbone of DNA and thereby initiating the DNA scissoring process. The substituted s-triazine is known to be a minor groove binder due to its efficient interaction with AT-rich sequences of DNA and the morpholine attached to triazine moiety can stack inside the minor groove and establish \( n\)-interaction with nitrogenous bases of DNA [23]. Hence due to all these interactions we can say that the synthesized compounds act as minor groove binders and cause DNA cleavage.

For a complete understanding of the interaction of molecules 7a-f with the DNA coil and the mechanism of cleaving, molecular docking simulations were carried out. The complete discussion of the docking studies is covered in chapter 7.
4.6 Conclusion

We report here a novel cationic fullerene-s-triazine conjugates with supercoiled DNA cleaving ability under photoirradiation in the presence of NADH. Compounds 7a, 7d, 7e and 7f cleaves DNA more efficiently than the substituted triazine analogues 5a-f. Results indicates that s-triazine and positive charge near fulleropyrrolidine ring preferentially interacts with negatively charged phosphate of DNA minor groove whereas conjugated fullerene interacts hydrophobically. We anticipate that compounds under photoirradiation may generate reactive molecule species facilitating DNA cleavage. This work opens up interesting prospects in the field of DNA cleavage by this novel class of fullerene-s-triazine derivatives.
FIGURES

Figure 5. Histogram results of the particle size distribution obtained from the DLS measurements of the solution of three representative compounds (a) 7d (b) 7e and (c) 7f compounds with concentration of (1 × 10⁻⁵ M) used for DNA cleavage test.

Figure 6. TEM images of a representative compound 7f. Scale bars corresponds to 20 nm (a) and 2 nm (b), respectively.
Figure 7. Photocleavage of pBR322 supercoiled DNA by cationic fullerene-s-triazine conjugates 7a-f analyzed by agarose gel electrophoresis (concentration of compounds 7a-f = 1×10^{-5} M and [NADH] = 1×10^{-3} M).

Figure 8. Photocleavage of pBR322 supercoiled DNA by substituted-s-triazine analogs 5a-f analyzed by agarose gel electrophoresis. Compounds concentration= (1.5×10^{-3} mM), [NADH] = (1×10^{-3} M)
Chapter 4

References


