# Synthesis and Transfection Capability of Multi-Functionalized Fullerene Polyamine

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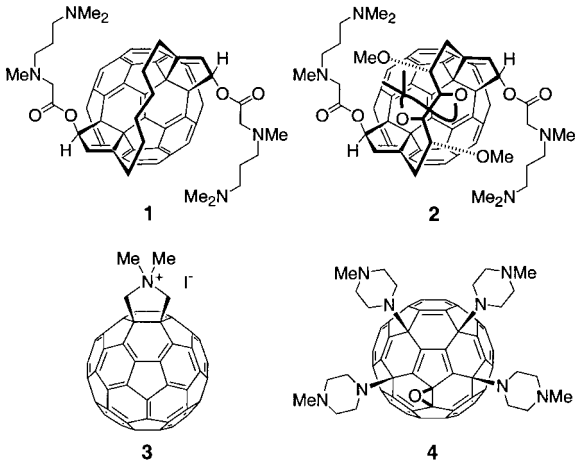
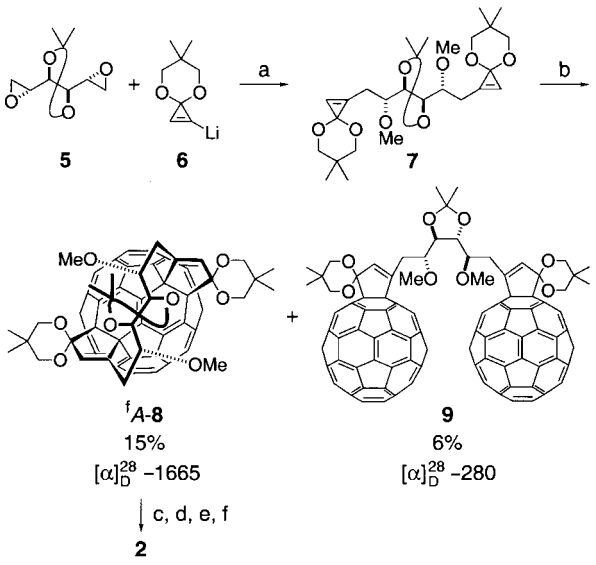
A new fullerene transfection reagent bearing multiple-functional groups has been synthesized by diastereoselective double cycloaddition reaction. The highly oxygenated reagent transfers extra-cellular DNA into mammalian cells with the efficiency comparable to that of a nor-analogue.

Interaction of organofullerenes and DNA has attracted considerable interest of chemists since the report that a water-soluble fullerene cleaves DNA under irradiation with visible light.[1,2] Recently an entirely new possibility of fullerene biology was pointed out—transfection of mammalian cells.[3] Thus, the fullerene tetramine **1** was found to be able to bind to DNA and to deliver the bound DNA into mammalian cells with efficiency comparable to conventional lipid-based transfection agent. This prototype reagent **1** embodies a very simple design principle possessing two sets of trimethylene diamine side chains, which are separated by 1.2 nm from each other so that they may have maximum electrostatic interaction with the two phosphate side chains of double strand DNA. To further develop the fullerene-mediated transfection technology, we felt it necessary to study the installation of functional sites, on which additional functional groups such as base-recognition groups may be attached. To this end, we investigated the synthesis of **2**, which is a highly oxygenated analogue of **1**, and its transfection ability to examine whether or not such heavy substitution on the hexamethylene moiety of **1** affects the biological activity. The transfection capability of **2** was found to be of the same order as **1**, suggesting that installation of elaborate functionality on the hexamethylene tether is tolerable. Two other DNA-binding fullerenes **3** and **4** did not show any transfection ability, which demonstrates that the electrostatic interaction with DNA is not the only structural requirement for the fullerene-transfection reagents, but suitable spatial arrangement of the functional groups is crucial.




We started the synthesis of **2** with the known diepoxide **5**[4] (prepared from D-mannitol), which was allowed to react with the lithiated cyclopropenone acetal **6**[5] to obtain the bis-cyclopropenone acetal **7**. Diepoxide **5** was added to a THF solution of **6** at –40 °C and the mixture was stirred for 20 h. To the reaction mixture was added 10 equiv of MeI and the reaction mixture was warmed to room temperature. Double addition of **6** and double methylation of the resulting alkoxide took place in one pot to give the desired biscyclopropenone acetal **7** in 45% yield after silica gel column chromatography.




**Scheme 1.** (a) HMPA, THF, −40 °C, 20 h, then MeI, rt, 2 h, 46%; (b) $C_{60}$, degassed 1,2-$Cl_2C_6H_4$, 150 °C, 7 d; (c) $H_2SO_4$, $H_2O$, THF, PhCl, 50 °C, 21 h, 64%; (d) DIBAL-H, PhCl, –25 °C, 14 min, 82%; (e) $BrCH_2COBr$, pyridine, DMAP, PhCl, rt, 6 h, 73%; (f) $HNMe(CH_2)_3NMe_2$, degassed PhCl, dark, rt, 5 h, 23%.

In the next stage, **7** was heated with [60]fullerene ($C_{60}$) to obtain regioselectively the double cycloaddition product **8**. In the previous report, we used a nor-analogue of **7** that lacks the oxygen substituents, and carried out the reaction at a relatively low concentration (1.5 mmol·$L^{-1}$) to obtain a racemic single regioisomer in 41% isolated yield.[6] Under the same reaction conditions, the desired double cycloadduct **8** did not form at all, and 85% of unreacted $C_{60}$ was recovered even after 11 d. Such low reactivity of **7** is likely due to both steric and electronic effects of the substituents in **7**. The reaction was therefore carried out for 7 d at a higher concentration (14 mmol·$L^{-1}$ in $C_{60}$) in a sealed glass tube which was previously treated with *N*,*O*-bis(trimethylsilyl)acetamide (Scheme 1). Purification with silica gel column chromatography afforded the desired double cycloadduct $^fA$-**8**[7] in 15% yield. No other diastereomeric double adduct was detected in the product

mixture. FABMS spectrum was consistent with the 1:1 adduct. Unreacted $C_{60}$ was recovered in 42% yield and 1:2 double cycloadduct **9** was also obtained in 6% yield. $^{f}A$-**8** was subsequently converted to tetramine **2** in 4 steps (Scheme 1) by the synthetic route reported for tetramine **1**.[3a]

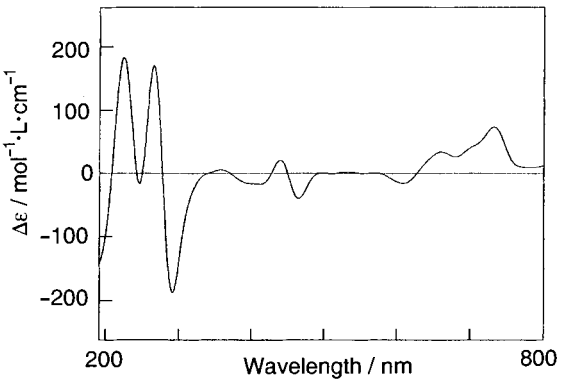



**Figure 1.** CD spectrum of $^{f}A$-**6** (7.0 μmol·L$^{-1}$ in cyclohexane).

Stereochemical assignment of $^{f}A$-**8** needed considerable care. $^{1}$H NMR and $^{13}$C NMR indicated that the double cycloadduct is a single isomer of $C_2$-symmetry. Very large optical rotation of the product ($[\alpha]_D$ –1665°, $c$ = 0.01, $CHCl_3$, 28 °C) is characteristic to non-racemic fullerenes produced by chiral double cycloaddition.[6a] The optical rotation of 1:2 adduct **9** was much smaller ($[\alpha]_D$ –280°, $c$ = 0.01, $CHCl_3$, 28 °C). In spite of previous efforts[8] to determine the absolute configuration of such double cycloaddition products, no conclusive method of structural assignment has been obtained. In an attempt to assign the stereochemistry of our double cycloadduct **8**, we performed molecular mechanics analysis[6b,9] to find that $^{f}A$ diastereomer is slightly more stable than the $^{f}C$ diastereomer (by 0.41 kcal/mol). The CD spectrum of **8** showed strong positive Cotton effect in the region of 210–230 nm (Figure 1). Such positive Cotton effect was also observed for a simpler analogue of **8**[6] as well as for a fullerene bismalonate, which was proposed to have $^{f}A$ configuration deduced from theoretical analysis of CD spectra.[8]

DNA-binding ability of tetramine **2** was then examined with competitive binding assay using ethidium bromide.[10] In order to investigate the structure–activity relationship in the transfection ability, two other fullerenes **3**[11] and **4**[12] were examined as reference. The compound **3** showed moderate binding ability ($C_{50}$ = 2.8 μmol·L$^{-1}$), and **2** ($C_{50}$ = 1.6 μmol·L$^{-1}$) and **4** ($C_{50}$ = 1.2 μmol·L$^{-1}$) showed comparable binding ability as our previous transfection reagent **1** (1.9 μmol·L$^{-1}$).[3] All compounds (**1**–**4**) strongly bind to DNA. In addition, the side chain substitution in two-handed fullerene did not interfere the binding of the molecule with DNA.

With this knowledge in hand, we carried out transfection experiments with the DNA binding fullerene under the optimized conditions (reagent/base pair = 13).[3] Each DNA binding fullerene (**1** and **2**) was mixed with plasmid DNA (pGreen LANTERN-1) that contains a reporter gene of green fluorescent protein (GFP). After 30 min, the resulting fullerene-DNA complex was added to cultured NIH 3T3 cells. After 2-day incubation, GFP expression in the transfected cells was observed with fluorescence microscope to find transfection efficiency of **2** to be $3.8 \times 10^{-4}$, which is comparable to the value obtained for **1** under the same conditions ($2.1 \times 10^{-4}$). On the other hand, experiments with the other reference compounds **3** and **4** did not show any transfection capability.

In summary, we reported the second example of gene delivery with functionalized fullerene, and found that installation of polar substituents does not hinder the binding of the fullerene **2** to DNA. This observation combined with the propensity of fullerene to form stable aggregates in water[13] suggest that, in the fullerene–DNA condensate obtained from **1** or **2**, the molecule is located in such a way that the hydrophilic oxygenated moiety points toward the interior of the groove, while the unmodified fullerene core points outward so that it can maintain hydrophobic interaction with other fullerene molecule. With such a working model, one may expect that installation of a base-selective functionality on the oxygenated side chain will create a base-selective DNA binding agent. This intriguing possibility will be the subject of further studies.

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Dedicated to Prof. Hideki Sakurai on the occasion of his 70th birthday.

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