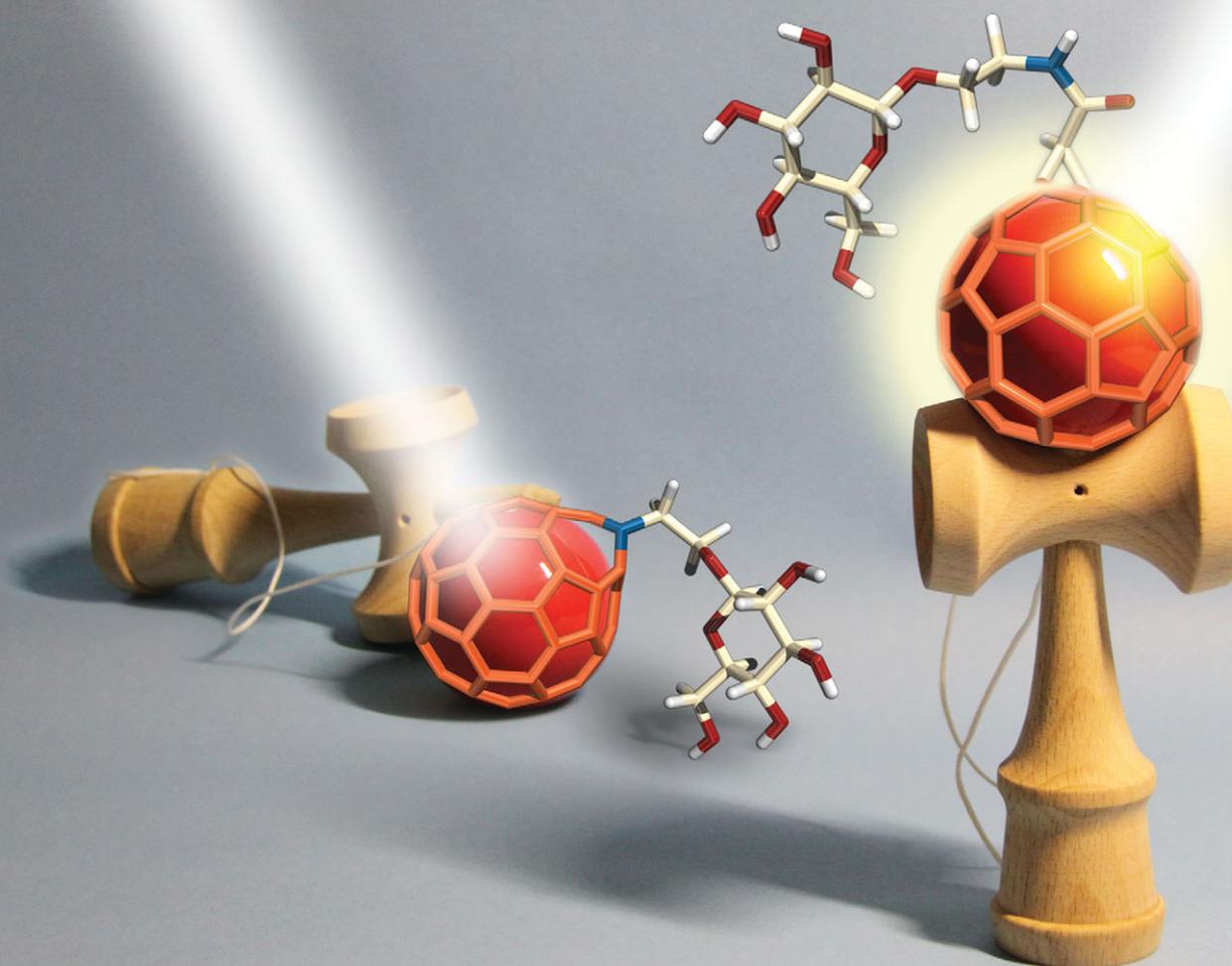


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# Efficient singlet oxygen generation from sugar pendant C<sub>60</sub> derivatives for photodynamic therapy†

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**The amidation reaction between C<sub>60</sub> with an activated ester group (1) and acetylated Glc (AcGlc) with an amino group (2) was performed to yield the target AcGlc-pendant C<sub>60</sub> compound (3). The water soluble deacetylated compound, Glc-pendant C<sub>60</sub> compound (4), exhibited high photocytotoxicity against HeLa cells due to the more efficient singlet oxygen generation as compared with that of Glc-pendant azafulleroids.**

Photodynamic action is initiated by the absorption of a photon followed by many competing radiative and nonradiative reactions, which ultimately result in the oxidation and degradation of vital biomolecules. Molecular oxygen plays a key role in the propagation of the initial molecular damage, resulting in

vascular collapse, tissue destruction, and cell death. Photodynamic therapy (PDT) has attracted much attention as a less invasive method for treating cancer, because PDT induces tumour cell necrosis and/or apoptosis by producing reactive oxygen species (ROS) through an activated photosensitizer (PS) that accumulates specifically in the tumor.<sup>1,2</sup> The potential applications of fullerenes and their derivatives have increased in recent years, particularly in the fields of biology and medicine, where they can be used as DNA photo-cleaving agents, anti-HIV protease inhibitors, antibacterial agents and PSs for PDT.<sup>3–10</sup> Although carbohydrates play essential roles in biological systems, their usage in fullerene-based PDT has yet to be fully explored. We have previously reported that sugar-pendant [60]fullerene (C<sub>60</sub>) derivatives prepared from carbohydrate linked azides exhibited the singlet oxygen producing ability in DMSO to demonstrate the carbohydrate-dependent photocytotoxicity against the HeLa cells.<sup>11,12</sup> However, the singlet oxygen yields of these D-glucose (Glc) pendant azafulleroids were significantly smaller than that of pristine C<sub>60</sub>.<sup>11</sup> It is highly desired to develop water soluble sugar-pendant C<sub>60</sub> compounds without decreasing the singlet oxygen yield upon photoirradiation.

We report herein the synthesis of a new family of Glc-pendant C<sub>60</sub> compounds, in which sugar and photosensitive units are connected *via* the cyclopropane bridged carbon-linkage to retain the conjugate properties of pristine C<sub>60</sub>, such as 2'-(2',3',4',6'-O-tetraacetyl-β-D-glucopyranosyl)ethyl 3'*H*-cyclopropa[1,9]([C<sub>60</sub>-I<sub>h</sub>])[5,6]-fullereno-3'-carboxylic amide (3) and 2'-(β-D-glucopyranosyl)ethyl 3'*H*-cyclopropa[1,9]([C<sub>60</sub>-I<sub>h</sub>])[5,6]fullereno-3'-carboxylic amide (4) (Chart 1). The photocytotoxicities against HeLa cells and photo-physical processes of 3 and 4 were compared with previously prepared Glc-pendant azafulleroids, such as 1a-aza-1a-[2'-(2',3',4',6'-O-tetraacetyl-β-D-glucopyranosyl)ethyl]-1(2)a-homo(C<sub>60</sub>-I<sub>h</sub>)[5,6]-fullerene (5) and 1a-aza-1a-[2'-(β-D-glucopyranosyl)-ethyl]-1(2)a-homo(C<sub>60</sub>-I<sub>h</sub>)[5,6]-fullerene (6) (Chart 1).<sup>11</sup>

Scheme 1 shows the synthetic procedure of 3 and 4. 3'*H*-Cyclopropa[1,9][5,6]fullereno-C<sub>60</sub>-I<sub>h</sub>-3'-carboxylic acid *N*-hydroxy-succinimide ester (1) was prepared from the corresponding C<sub>60</sub> acid derivative, 3'*H*-cyclopropa[1,9][5,6]fullereno-C<sub>60</sub>-I<sub>h</sub>-3'-carboxylic

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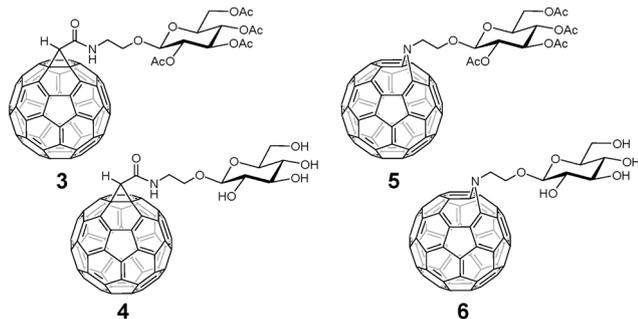
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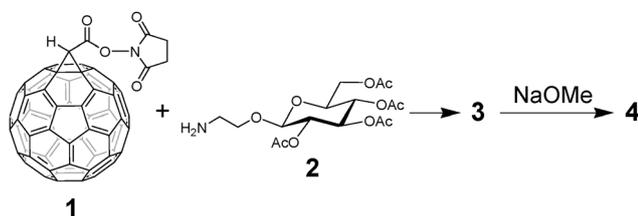
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† Electronic supplementary information (ESI) available: Experimental procedures and Fig. S1–S9, Table S1. See DOI: 10.1039/c5cc07353g





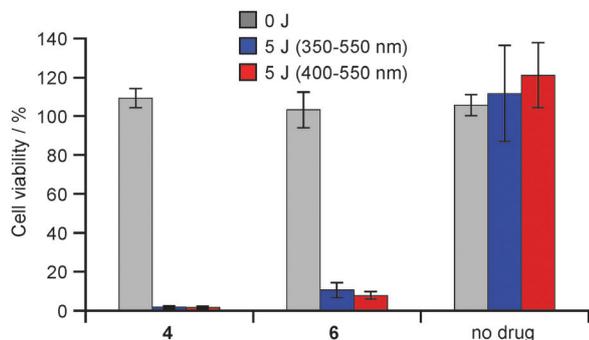
**Chart 1** Structures of Glc-pendant  $C_{60}$  compounds **3** and **4** in this study and previously prepared Glc-pendant azafulleroids **5** and **6**.



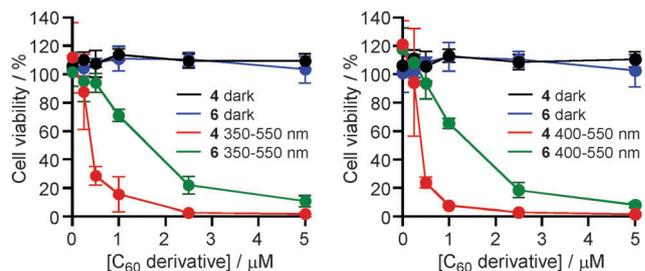
**Scheme 1** Synthesis of sugar-pendant  $C_{60}$  compounds.

acid,<sup>13</sup> and *N*-hydroxysuccinimide (NHS). 2-Aminoethyl 2,3,4,6-*O*-tetraacetyl- $\beta$ -D-glucopyranoside (**2**) was prepared by the similar procedure to that described in the literature,<sup>14</sup> in which Pd/C was used in place of the Lindlar catalyst. We performed the amidation reaction between **1** and **2** in dry  $CHCl_3$ , followed by the purification using silica gel column chromatography (eluent:  $CHCl_3/MeOH = 50/1$ ,  $R_f = 0.20$ ) to afford **3** as a brown solid. Subsequently, **3** was treated with sodium methoxide in dry THF to quantitatively afford the final target compound **4**. The detailed procedures are described in the ESI†.

The photodynamic activities were evaluated using human cervical cancer HeLa cells, to compare between two kinds of Glc-pendant  $C_{60}$  derivatives (**4** and **6**) (Fig. 1). Following incubation with the respective  $C_{60}$  derivatives, the cells were exposed to light with wavelengths between 350 and 550 nm. The results



**Fig. 1** Cytotoxicity in the dark (gray bar) and photocytotoxicity (green and red bars) of **4** and **6** in HeLa cells ( $[4$  or  $6] = 5 \mu M$ ). The cells ( $1.7 \times 10^4$  cells per well) were irradiated with a xenon lamp (total  $5 J cm^{-2}$ ) after incubation with a photosensitizer for 24 h. The cell survival was determined by the WST-8<sup>®</sup> assay. Each experiment was performed three times.



**Fig. 2** Cell viability with **4** and **6** at various concentrations under dark and photoirradiation [350–550 nm (left panel) and 400–550 nm (right panel)]. Data represent the mean values from three independent experiments. Error bars are standard deviations.

showed that no samples had dark toxicity, even at the highest concentrations used. On the other hand, the viabilities of HeLa cells were reduced depending on photoirradiation. These photodynamic activities of Glc-pendant  $C_{60}$  derivatives were drug dose-dependent and the medium inhibitory concentrations ( $IC_{50}$  values) were estimated to be *ca.*  $0.4 \mu M$  for **4** and  $1.6 \mu M$  for **6**. Furthermore, similar tendencies regarding the photodynamic activities were observed even when the light wavelengths were changed to 400–550 nm at the same light dose (Fig. 2). The  $IC_{50}$  values were estimated to be *ca.*  $0.4 \mu M$  for **4** and  $1.5 \mu M$  for **6** (Table S1 in the ESI†). These data indicate that the photodynamic activity of **4** is about 4 times higher than that of **6**.

In order to rationalize such drastic differences in the photodynamic activity, the singlet oxygen generation properties were characterized for **3** and **5**. These samples produced singlet oxygen in the oxygen-saturated  $C_6D_6$  solutions by photoirradiation, which could be quantified from the peak area due to the singlet oxygen phosphorescence at 1270 nm (Fig. S1 in the ESI†).<sup>15,16</sup> The quantum yield of singlet oxygen generation from **3** was determined to be 0.61, which is much larger than that from **5** (0.22). The larger singlet oxygen yield from **3** results from the efficient formation of the triplet excited state of the  $C_{60}$  moiety, which has the much longer lifetime as compared with that derived from **5**. This was also supported by femtosecond laser-induced transient absorption measurements of **4** and **6**. Femtosecond laser excitation of a deaerated DMSO solution of **6** at 355 nm resulted in the instant observation of a transient absorption band at 700 nm due to the triplet excited state of  $C_{60}$ , which increased at 3000 ps as shown in Fig. 3a. In contrast to the case of **4**, femtosecond laser excitation of a deaerated DMSO solution of **6** at 355 nm resulted in the formation of  $C_{60}^{\bullet-}$  as revealed by the transient absorption at 1000 nm,<sup>17</sup> together with the triplet excited state of  $C_{60}$  at 700 nm, which decayed significantly at 3000 ps as shown in Fig. 3b. Similar results were obtained for the acetylated compounds of **3** and **5** (Fig. S2 and S3 in the ESI†).

The formation of the triplet excited state of  $C_{60}$  without the formation of  $C_{60}^{\bullet-}$  of **4** was confirmed by nanosecond laser transient absorption spectra, whereas the transient absorption spectra of  $C_{60}^{\bullet-}$  were observed for **6** as shown in Fig. S4 (ESI†), where the formation of the triplet excited state of  $C_{60}$  of **3** and  $C_{60}^{\bullet-}$  of **5** are also observed. The formation of  $C_{60}^{\bullet-}$  of **5** and



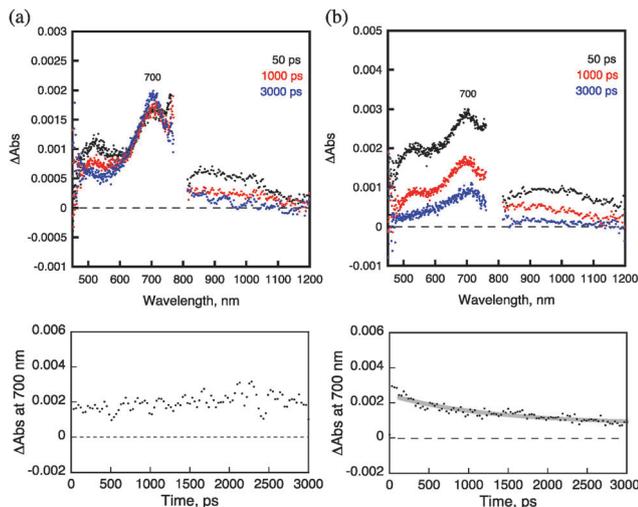
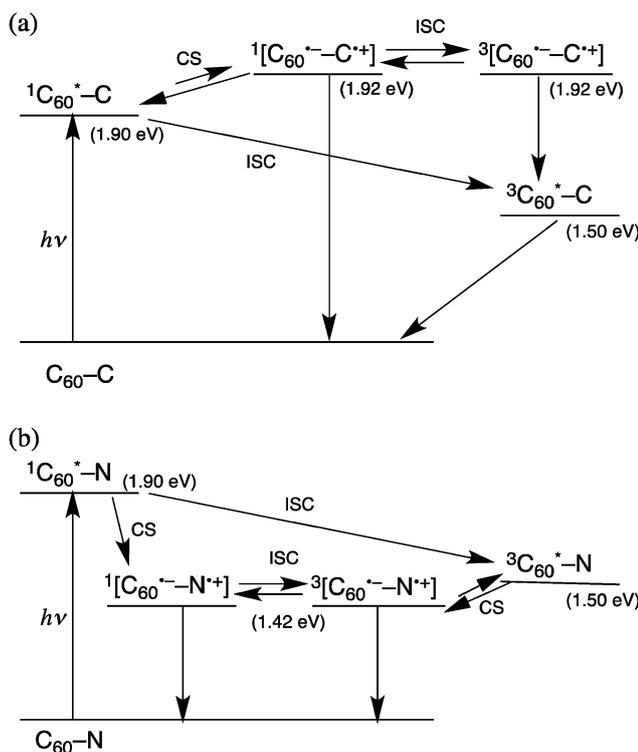


Fig. 3 Transient absorption spectra (upper panels) and time profiles (lower panels) at 700 nm of (a) **4** and (b) **6** in deaerated DMSO taken 50, 1000, and 3000 ps after laser excitation at 355 nm.

**6** was also confirmed by the EPR spectra recorded after photoirradiation at 143 K as shown in Fig. S5 (ESI<sup>†</sup>),<sup>18</sup> where only a weak EPR signal due to  $C_{60}^{\bullet-}$  was observed for **3** and **4**.

No observation of  $C_{60}^{\bullet-}$  (Fig. 3a) and the slow formation of the triplet excited state of  $C_{60}$  (Fig. 3a) indicate that no photoinduced electron transfer occurs from the Glc moiety to the singlet excited state and the triplet excited state of the  $C_{60}$  moiety. This is verified by the determination of the redox potentials of **3–6** by cyclic voltammetry and second harmonic ac voltammetry (SHACV)<sup>19</sup> as



Scheme 2 Energy diagrams of (a) **4** and (b) **6**.

shown in Fig. S6–S9 in the ESI.<sup>†</sup> The one-electron oxidation potential of the Glc moiety of **4** was determined to be 1.28 V vs. SCE by SHACV, whereas the one electron reduction potential of the  $C_{60}$  moiety of **4** was determined to be  $-0.64$  V vs. SCE by CV. Because the singlet excited state energy of the  $C_{60}$  moiety of **4** was determined to be 1.92 eV, the free energy change of electron transfer from the Glc moiety of **4** to the singlet excited state of  $C_{60}$  is evaluated to be 0.02 eV, which means that the electron transfer is slightly endergonic as shown in Scheme 2a. In such a case, electron transfer from the Glc moiety of **4** to the singlet excited state of  $C_{60}$  may be followed by faster back electron transfer to the singlet excited state of  $C_{60}$  or the triplet excited state of  $C_{60}$  without observation of  $C_{60}^{\bullet-}$ .

The occurrence of electron transfer from the Glc moiety of **6** to the singlet excited state of  $C_{60}$  (Fig. 3b) is verified by the lower oxidation potential of **6** as compared with that of **4** because of the N-linkage in **6** (Fig. S9 in the ESI<sup>†</sup>). In this case, the free energy change of electron transfer from the Glc moiety of **6** to the singlet excited state of  $C_{60}$  is evaluated to be  $-0.40$  eV, which means the electron transfer is highly exergonic (Scheme 2b). Because the energy of the charge-separated state (1.50 eV) is the same as the energy of the triplet excited state of  $C_{60}$ , the triplet excited state of  $C_{60}$  is produced *via* the intersystem crossing of the charge-separated state (Scheme 2b). However, the triplet excited state of  $C_{60}$  of **6** decays *via* the charge-separated state with the faster rate than the case of **4**.

In conclusion, the D-glucose (Glc) pendant  $C_{60}$  compound (**4**) exhibited significantly higher photocytotoxicity against HeLa cells than the corresponding azafulleroid, because of no involvement of the charge-separated state in the decay of the triplet excited state of the  $C_{60}$  moiety of **4**, which resulted in the higher yield of singlet oxygen. The present study provides a rational design of water soluble sugar-pendant  $C_{60}$  derivatives for more efficient PDT.

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