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Syntheses of water-soluble [60]fullerene derivatives and their enhancing effect on neurite outgrowth in NGF-treated PC12 cells

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ABSTRACT

Water-soluble [60]fullerene (C_{60}) derivatives were synthesized to examine their bioactivities. PC12 cells were used as a model of nerve cells and the bioactivities of synthesized C_{60} derivatives together with some reported ones were tested. Among the compounds tested, C_{60}/(γ-CyD)$_2$, C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**), and C_{60}/PVP were sufficiently soluble in water and showed an enhancing effect on the neurite outgrowth of NGF-treated PC12 cells.

Since the discovery of [60]fullerene (C_{60}) by Kroto et al. in 1985,[1] extensive studies aimed at utilizing this molecule in different scientific fields have been carried out because of its unique physical and chemical properties (antioxidant, radical scavenger, photosensitizer, etc.). As regards the biological activities of fullerenes, antiviral,[2] antitumor,[3] antimicrobial,[4] and antioxidant activites,[5] including inhibition of HIV protease,[6] HIV reverse transcriptase,[7] and DNA damage,[8] have been reported. One drawback, however, is that the extremely low solubility of fullerenes in water hinders their application in biological systems. Various trials to make fullerenes water-soluble have been carried out. The tris-malonic acid derivative of C_{60} (carboxyfullerene),[9] polyhydroxylated C_{60} (fullerol), C_{60} wrapped with poly(vinylpyrrolidone) (C_{60}/PVP),[10] and C_{60} bicapped with γ-cyclodextrine (γ-CyD) [C_{60}/(γ-CyD)$_2$][11] have been demonstrated as water-soluble C_{60} derivatives. Using C_{60}/PVP, we previously demonstrated its effect on promoting mouse embryonic limb bud cell differentiation[12] and its DNA cleaving activity.[13]

In this study, we synthesized other water-soluble C_{60} derivatives and examined their bioactivities as well as those of previously reported water-soluble C_{60} derivatives on nerve cells. As regards the bioactivity of fullerenes on nerve cells, carboxyfullerene is reported as a neuroprotective agent.[14]

In this Letter, we describe that water-soluble C_{60} derivatives are able to enhance neurite outgrowth in nerve growth factor (NGF)-treated PC12 cells that were used as a model of nerve cells.

The syntheses of water-soluble C_{60} derivatives **1**–**4** are shown in Scheme 1. The condensation reaction of compound **6**[15] with β-D-glucopyranosylamine 2,3,4,6-tetraacetate[16] yielded C_{60} derivative **1**. Hydrolysis of compound **1** gave C_{60} derivative **2**. Activated ester **7**[17] was prepared by the condensation of compound **6** with *N*-hydroxysuccinimide (NHS). The reactions of compound **7** with mono(6-amino-6-deoxy)-γ-cyclodextrin (NH_2-γ-CyD)[18] gave C_{60} derivative **3** in 78% yield. Activated ester **9** of compound **8**[19] was prepared following the procedure for compound **7** and it was allowed to react with NH_2-γ-CyD to give C_{60} derivative **4** in 80% yield. The synthesis of water-soluble C_{60} derivative **5** is shown in Scheme 2. C_{60} derivative **10**[20] was prepared by use of the Bingel reaction where 1,3-bis[2-(1,1-dimethylethoxy)-2-oxoethyl]propanedioate and C_{60} were allowed to react with I_2 and DBU in dry toluene. Removal of the *t*-butyl groups of compound **10** gave compound **11**. The condensation reaction of compound **11** with NHS gave activated ester **12**. The reaction of compound **12** with NH_2-γ-CyD gave a mixture of C_{60}-bis(γ-CyD) (**5**) and C_{60}-mono(γ-CyD) (**5′**), the existence of which was confirmed by MALDI mass spectroscopy. The C_{60}-bis(γ-CyD)/C_{60}-mono(γ-CyD) ratio was estimated to be almost 1/1 on the basis of MALDI signal intensities. Without further separation, C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) was used in the biological assay. For the synthesis of C_{60}-bis(γ-CyD) and C_{60}-mono(γ-CyD), activated ester **12**

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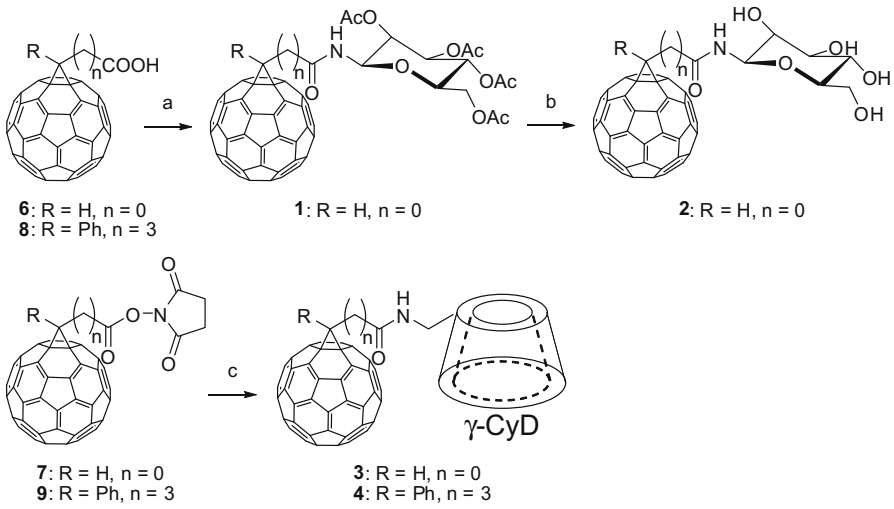
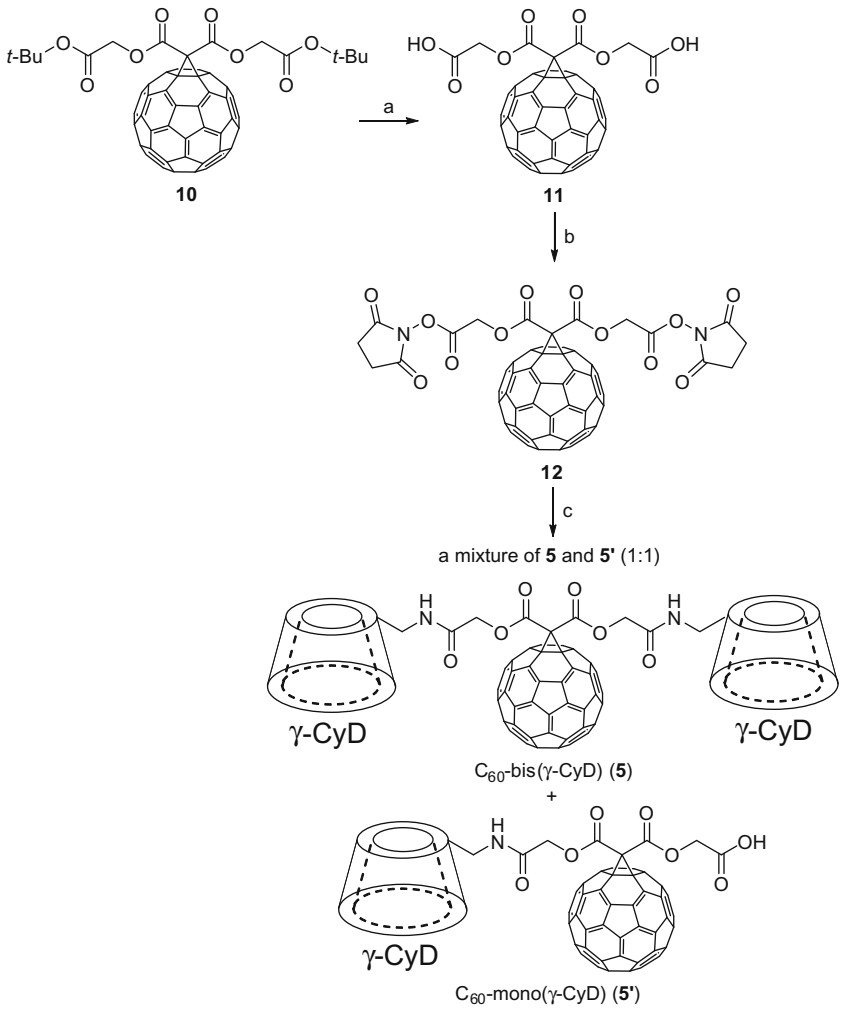


Scheme 1. Syntheses of C_{60} derivatives **1**–**4**. Reagents, conditions, and yields: (a) β-D-glucopyranosylamine 2,3,4,6-tetraacetate, EDCI, HOBt, DMF, 27–45%; (b) K_2CO_3, MeOH/$CHCl_3$, 76%; and (c) NH_2-γ-CyD, DMF, 78–80%.



Scheme 2. Synthesis of C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**). Reagents, conditions, and yields: (a) *p*-TsOH, toluene, reflux, 96%; (b)NHS, EDCI, DMF; and (c) NH_2-γ-CyD, DMF, 19% from **11**.

was used instead of compound **11** to introduce the NH_2-γ-CyD moiety. By using the activated ester, purification of C_{60}-bis(γ-CyD) and C_{60}-mono(γ-CyD) was facilitated because the separation of unreacted compound **11** was extremely difficult. Details of the synthetic procedures and the characterization of new compounds **1**–**4**, **11**, and **12** are available in Supplementary data.

C_{60}/(γ-CyD)$_2$ is reported to form a complex in which two γ-CyDs are capping the C_{60} molecule.[11] The ^{1}H NMR spectrum of C_{60}/(γ-CyD)$_2$ showed that the signals of the γ-CyD unit shifted slightly up- or downfield compared to those of γ-CyD itself. The UV spectrum of C_{60}/(γ-CyD)$_2$ in aqueous solution showed a sharp absorption peak of C_{60} appearing at approximately 330 nm.[21] In contrast, in the ^{1}H NMR spectrum of C_{60}-bis(γ-CyD), the same chemical shifts of the γ-CyD unit as those of γ-CyD itself were noted. Further, the UV spectrum of C_{60}-bis(γ-CyD) in aqueous solution showed a broad peak indicating that C_{60}-bis(γ-CyD) forms aggregates in aqueous solution. From these results, together with the results of a computer-based model study (data not shown), the γ-CyD moieties of C_{60}-bis(γ-CyD) may not form complex structures similar to those of C_{60}/(γ-CyD)$_2$.

The antioxidant activity of compounds **1**–**4** and C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) was examined using ESR spectrometry. ·OH was generated by use of the H_2O_2–Cu^{2+} system in the absence or presence of C_{60} derivatives, and the generated ·OH was trapped by 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). Then, the ESR spectra of ·OH-trapped DMPO (DMPO-OH) were measured. All C_{60} derivatives examined reduced the signal intensity of DMPO-OH, indicating that these derivatives have antioxidative activity (Supplementary data).

The solubility in water of the fullerenes synthesized in this study was compared with typical water-soluble fullerenes, C_{60}/(γ-CyD)$_2$, C_{60}/PVP, and fullerol, which were prepared according to reported procedures or purchased. The most soluble fullerene was C_{60}/(γ-CyD)$_2$ (more than 100 μM), followed by C_{60}/PVP, C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**), compound **3**, and fullerol (less than 1 μM). As the solubility in water of the other compounds synthesized in this study was much lower than 1 μM, only C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) and compound **3** were used together with C_{60}/(γ-CyD)$_2$, C_{60}/PVP, and fullerol for the biological assay using PC12 cells.

Rat pheochromocytoma PC12 cells were used as a model of nerve cells. Treatment of PC12 cells with NGF resulted in the induction of differentiation and the formation of neurites. First, we examined whether or not C_{60} derivatives have similar activity to NGF. Experiments were performed using the most water-soluble C_{60}, C_{60}/(γ-CyD)$_2$. However, no effects as seen with NGF were observed for C_{60}/(γ-CyD)$_2$ up to the examined concentration of 100 μM. Next, the effect of C_{60} derivatives on neurite outgrowth in NGF-treated PC12 cells was examined. The experimental protocol is as follows: PC12 cells were seeded and cultured for 2 days in the medium with serum, and then the medium was changed to a fresh one without serum. NGF (50 ng/mL) and appropriate concentrations of fullerenes were added. After incubating for 3 days, the number of neurites was counted and the length of the outgrowth was measured. Figure 1 shows PC12 cells that were treated with NGF plus 0–50 μM C_{60}/(γ-CyD)$_2$. NGF-treated PC12 cells formed neurites and both neurite number and length of outgrowth increased in a dose-dependent manner. The length of outgrowth, which was expressed as the number of times the cell body diameter, and the ratio of neurite cells to total cells are shown in Figure 2. The total number of neurites increased in a dose-dependent

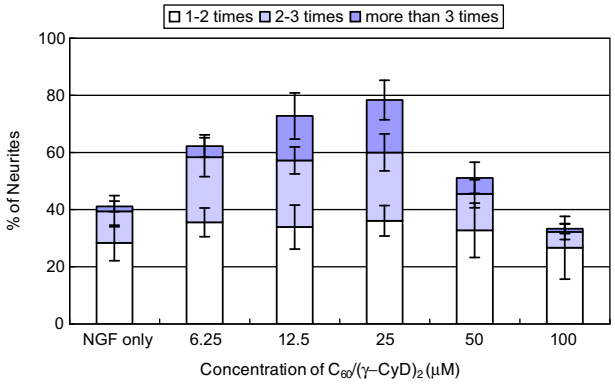


Figure 2. Ratio of neurites to total number of cells when treated with C_{60}/(γ-CyD)$_2$. The length of neurite outgrowth was expressed as the number of times the cell body diameter. Data are expressed as means ± S.D.

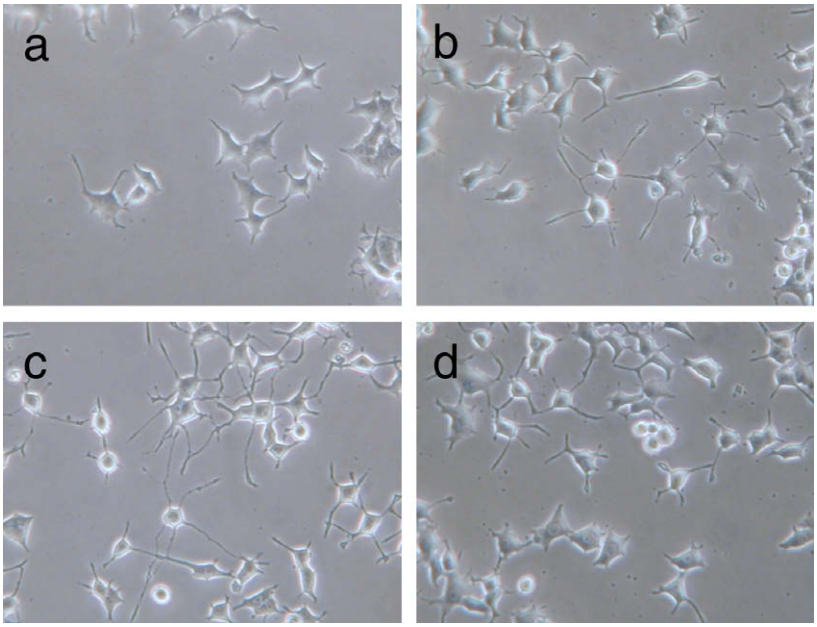


Figure 1. Neurite outgrowth enhancing effect of C_{60}/(γ-CyD)$_2$. NGF 50 ng/mL plus C_{60}/(γ-CyD)$_2$: (a) 0 μM, (b) 12.5 μM, (c) 25 μM, and (d) 50 μM.

manner, reaching a maximum at 25 μM. At higher concentrations of 50 and 100 μM, a decrease in the total number of neurites was observed. The reason is not clear at present, although we speculate that some toxic effects of the compounds may have caused the decrease. Free γ-CyD is present in C_{60}/(γ-CyD)$_2$ and its presumed maximum concentrations are 0.32–5.1 mM relative to the concentrations of C_{60}/(γ-CyD)$_2$ of 6.25–100 μM. Then, the effect of γ-CyD itself on neurite outgrowth was examined, but no enhancing effect of γ-CyD was observed (Supplementary data). A similar experiment was performed with C_{60}/PVP. The ratio of C_{60} to PVP was approximately 1–17.6, and the concentrations of C_{60}/PVP (12.5 μM/2.2 mM, 25 μM/4.4 mM, and 50 μM/8.9 mM) were examined. While PVP itself (2.2, 4.4, and 8.9 mM) had no effect, C_{60}/PVP showed neurite outgrowth enhancing effect (Supplementary data).

C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) was dissolved in 1% DMSO and its activity was examined. The concentration of C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) was determined based on the average molecular weight of the mixture. The results in Figure 3 indicate that the ratio of neurites to the total number of cells increased in a dose-dependent manner to reach a maximum at 25 μM. This concentration is the same as that of C_{60}/(γ-CyD)$_2$. At 50 μM, the ratio of neurites to the total number of cells decreased, as seen for C_{60}/(γ-CyD)$_2$. At 100 μM, precipitates appeared. In the case of compound **3** and fullerol, precipitation was noted at 5 μM and 1 μM, respectively, and neither of them showed any enhancing effects probably because of their low concentration in the medium. C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) was the most water-soluble among the compounds synthesized and its solubility was sufficient for use in biological assays. However, the water solubility of C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) was lower than that of C_{60}/(γ-CyD)$_2$. Nevertheless, compounds **5** and **5′** have the advantage of being not a mixture of γ-CyD, such as C_{60}/(γ-CyD)$_2$. In addition, further chemical modification of compounds **5** and **5′** is possible. Therefore, compounds **5** and **5′** could be used to study the real effects of C_{60}.

The effects of pretreatment with C_{60}/(γ-CyD)$_2$ and C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**), which showed the most potent neurite outgrowth enhancing effect, prior to NGF treatment on neurite outgrowth were examined. PC12 cells were seeded and cultured for 1 day in the medium with serum, and then 25 μM C_{60}/(γ-CyD)$_2$ or C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) was added and the cells were incubated for another 2 days. The medium was changed to a fresh one without serum and only NGF (50 ng/mL) was added. After incubating for 3 days, the number of neurites was counted and the length of outgrowth was measured. The results are shown in Figure 4. Pre-incubation had a greater neurite outgrowth enhancing effect than co-incubation for both C_{60} derivatives.

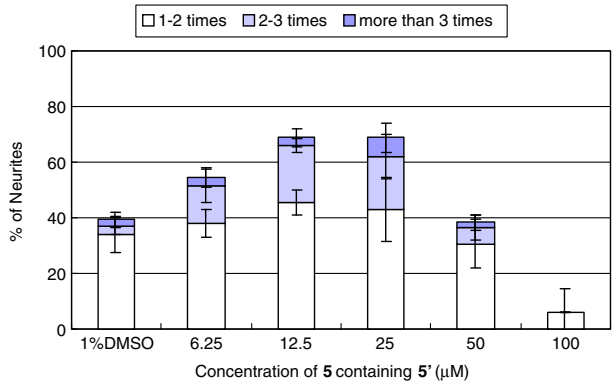


Figure 3. Ratio of neurites to total number of cells when treated with C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**). The length of neurite outgrowth was expressed as the number of times the cell body diameter. Data are expressed as means ± S.D.

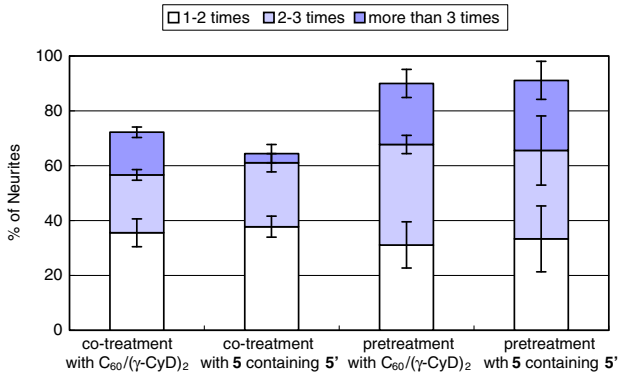


Figure 4. Effect of pretreatment of C_{60}/(γ-CyD)$_2$ or C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) on neurite outgrowth. Ratio of neurites to total number of cells when pretreated for 2 days with 25 μM C_{60}/(γ-CyD)$_2$ or C_{60}-bis(γ-CyD) (**5**) containing C_{60}-mono(γ-CyD) (**5′**) before NGF (50 ng/mL) treatment. The length of neurite outgrowth was expressed as the number of times the cell body diameter. Data are expressed as means ± S.D.

The mechanisms of neurite outgrowth enhancement by C_{60} derivatives are obscure. Ibi et al. reported that reactive oxygen species were produced by NADPH oxidase when PC12 cells underwent differentiation into neurites by NGF treatment.[22] Reactive oxygen species inhibit neurite growth. As C_{60} is a scavenger of reactive oxygen species, it is possible that the C_{60} derivatives scavenged neurodegenerative reactive oxygen species and as a result, neurite outgrowth was enhanced. Pretreatment with C_{60} derivatives had a greater neurite outgrowth enhancing effect than co-treatment. However, it is not clear if the C_{60} derivatives remained on the cell membrane even after the cells were washed or if the pretreatment with C_{60} derivatives resulted in switching on some receptor of cell membrane leading to show the activity. Further study is in progress.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.01.142.

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