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

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A R T I C L E   I N F O
Article history:
Received 10 November 2009
Revised 21 January 2010
Accepted 25 January 2010
Available online 2 February 2010

Keywords:
[60]Fullerene
C 60
Water-soluble
Neurite outgrowth
PC12 cell

A B S T R A C T
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 mono(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.

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-o-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...
Scheme 1. Syntheses of C60 derivatives 1–4. Reagents, conditions, and yields: (a) β-D-glucopyranosylamine 2,3,4,6-tetraacetate, EDCI, HOBt, DMF, 27–45%; (b) K2CO3, MeOH/CHCl3, 76%; and (c) NH2-γ-CyD, DMF, 78–80%.

Scheme 2. Synthesis of C60-bis(γ-CyD) (5) containing C60-mono(γ-CyD) (5'). Reagents, conditions, and yields: (a) p-TsOH, toluene, reflux, 96%; (b) NHS, EDCI, DMF; and (c) NH2-γ-CyD, DMF, 19% from 11.
was used instead of compound 11 to introduce the NH2-γ-CyD moiety. By using the activated ester, purification of C60-bis(γ-CyD) and C60-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.

C60(γ-CyD)2 is reported to form a complex in which two γ-CyDs are capping the C60 molecule.11 The 1H NMR spectrum of C60(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 C60(γ-CyD)2 in aqueous solution showed a sharp absorption peak of C60 appearing at approximately 330 nm.21 In contrast, in the 1H NMR spectrum of C60-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 C60-bis(γ-CyD) in aqueous solution showed a broad peak indicating that C60-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 C60-bis(γ-CyD) may not form complex structures similar to those of C60(γ-CyD)2.

The antioxidant activity of compounds 1–4 and C60-bis(γ-CyD) (5) containing C60-mono(γ-CyD) (5’) was examined using ESR spectrometry. OH was generated by use of the H2O2−Cu2+ system in the absence or presence of C60 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 C60 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, C60(γ-CyD)2, C60/PVP, and fullerol, which were prepared according to reported procedures or purchased. The most soluble fullerene was C60(γ-CyD)2 (more than 100 μM), followed by C60/PVP, C60-bis(γ-CyD) (5) containing C60-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 C60-bis(γ-CyD) (5) containing C60-mono(γ-CyD) (5’) and compound 3 were used together with C60(γ-CyD)2, C60/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 C60 derivatives have similar activity to NGF. Experiments were performed using the most water-soluble C60, C60(γ-CyD)2. However, no effects as seen with NGF were observed for C60(γ-CyD)2 up to the examined concentration of 100 μM. Next, the effect of C60 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 C60(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 1. Neurite outgrowth enhancing effect of C60(γ-CyD)2. NGF 50 ng/mL plus C60(γ-CyD)2: (a) 0 μM, (b) 12.5 μM, (c) 25 μM, and (d) 50 μM.](image-url)
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 C60/(γ-CyD)2 and its presumed maximum concentrations are 0.32–5.1 mM relative to the concentration of C60/(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 C60/PVP. The ratio of C60 to PVP was approximately 1–17.6, and the concentrations of C60/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, C60/PVP showed neurite outgrowth enhancing effect (Supplementary data).

C60-bis(γ-CyD) (5) containing C60-mono(γ-CyD) (5′) was dissolved in 1% DMSO and its activity was examined. The concentration of C60-bis(γ-CyD) (5) containing C60-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 C60/(γ-CyD)2. At 50 μM, the ratio of neurites to the total number of cells decreased, as seen for C60/(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. C60-bis(γ-CyD) (5) containing C60-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 C60-bis(γ-CyD) (5) containing C60-mono(γ-CyD) (5′) was lower than that of C60/(γ-CyD)2. Nevertheless, compounds 5 and 5′ have the advantage of being not a mixture of γ-CyD, such as C60/(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 C60.

The effects of pretreatment with C60/(γ-CyD)2 and C60-bis(γ-CyD) (5) containing C60-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 C60/(γ-CyD)2 or C60-bis(γ-CyD) (5) containing C60-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 C60 derivatives.

The mechanisms of neurite outgrowth enhancement by C60 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. Reactive oxygen species inhibit neurite growth. As C60 is a scavenger of reactive oxygen species, it is possible that the C60 derivatives scavenged neurodegenerative reactive oxygen species and as a result, neurite outgrowth was enhanced. Pretreatment with C60 derivatives had a greater neurite outgrowth enhancing effect than co-treatment. However, it is not clear if the C60 derivatives remained on the cell membrane even after the cells were washed or if the pretreatment with C60 derivatives resulted in switching on some receptor of cell membrane leading to show the activity. Further study is in progress.

Acknowledgment

This work was supported in part by MEXT-HAITEKU (2008).

Supplementary data

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

References and notes


Figure 3. Ratio of neurites to total number of cells when treated with C60-bis-(γ-CyD) (5) containing C60-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 C60/(γ-CyD)2 or C60-bis(γ-CyD) (5) containing C60-mono(γ-CyD) (5′) on neurite outgrowth. Ratio of neurites to total number of cells when pretreated for 2 days with 25 μM C60/(γ-CyD)2 or C60-bis(γ-CyD) (5) containing C60-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.