

A New Multi-Charged C₆₀ Derivative: Synthesis and Biological Properties

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A new water-soluble multi-charged monoadduct fullerene [60]pyrrolidine derivative with three ethylene glycol chains and three ammonium groups has been synthesized by means of two alternative synthetic pathways. Increasing the concentration of this C₆₀ derivative did not show a significant modification of concentration of superoxide anion radical O₂^{•-},

generated by the xanthine/xanthine oxidase system. Moreover, this C₆₀ derivative was ineffective for neuroprotection in quantitative assessment of a neuronal injury considered to occur via radicals.

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Introduction

The unusual spheroidal shape, the exclusive presence of carbon atoms in their structures and the big π -electron cloud make fullerenes good candidates for novel approaches to chemotherapy. Unmodified fullerenes, although relatively soluble in some apolar solvents, are virtually insoluble in biologically relevant media.^[1] Thus, the attachment of hydrophilic groups to C₆₀ is necessary to render the fullerene soluble in aqueous solutions and hence more amenable to biological experiments.^[2] So far, fullerene derivatives have shown interesting properties in different fields such as apoptosis, neuroprotection, anti-HIV therapy, antibacterial and DNA-photocleavage activities.^[3–6] In particular, neuroprotection activity seems to be related to the fullerene's ability to take up many radicals on the carbon cage (up to 34 methyl radicals on a single C₆₀ molecule, as demonstrated by Krusic^[7]).

The formation of reactive oxygen species (ROS), including superoxide radical anion (O₂^{•-}), or ROO[•] and [•]OH radicals, is considered to be one of the processes involved in neuronal injury. One mechanism of production of these ROS is the hyperstimulation of *N*-methyl-D-aspartate (NMDA) glutamate receptors.^[8–10] The overproduction of

these species during oxidative stress is capable of inducing biological damage.^[11] The antioxidant properties of C₆₀ could therefore be used to prevent free radical stress and all the related diseases.

In this field, several C₆₀ derivatives have been investigated as neuroprotecting agents.^[12,13] In particular, two C₆₀-tris(malonic acid) derivatives C₆₀[C(COOH)₂]₃, formed as two different isomers, called C₃ (**1**) and D₃ (**2**), depending on the addition pattern of the carbon cage, have proved to be the most promising compounds for preventing neuronal damage (Figure 1).^[14,15]

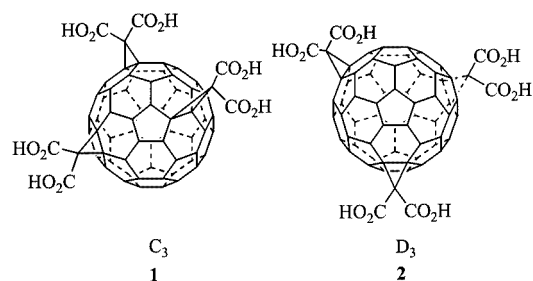


Figure 1. Isomeric C₆₀-tris(malonic acid) derivatives **1** (C₃) and **2** (D₃)

The presence of six carboxylic functions improves the solubility in biological media and avoids the aggregation problems commonly encountered in polar solvents. As a result of its better penetration into lipids, compound **1** has been studied in more detail than other isomers and it has been demonstrated to be a good radical scavenger against superoxide anions and hydroxyl radicals at micromolar concentrations. In vivo studies have also shown that **1** is effective in neuroprotection by chronic administration.^[15]

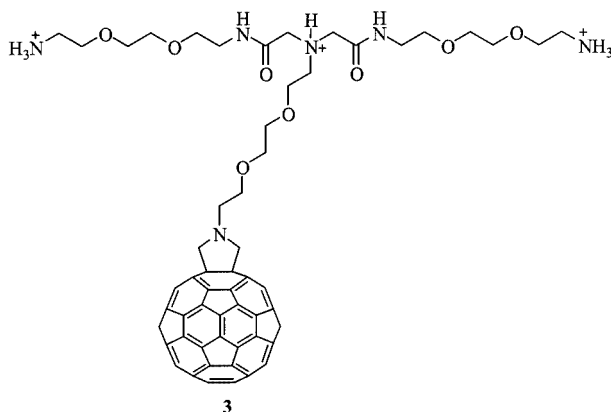
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Starting from these observations, we have designed a new water-soluble fullerene derivative **3**, with three ethylene glycol chains and the same number of ammonium groups for increasing water solubility, as a potential neuroprotecting agent.



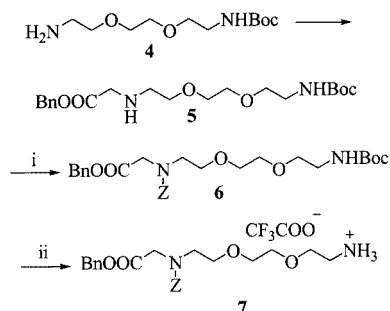
The rational design of this compound was aimed at obtaining a mono-functionalized water-soluble C₆₀ derivative with increased potential free radical loading capacity, since a higher number of addends on the fullerene moiety should decrease its radical scavenging properties.^[7]

Results and Discussion

Synthesis

Two different synthetic approaches have been utilized to reach the goal, as depicted below, in which a solubilizing appendage was introduced on a suitably functionalized fulleropyrrolidine or was already present in the amino acid used in the cycloaddition step.

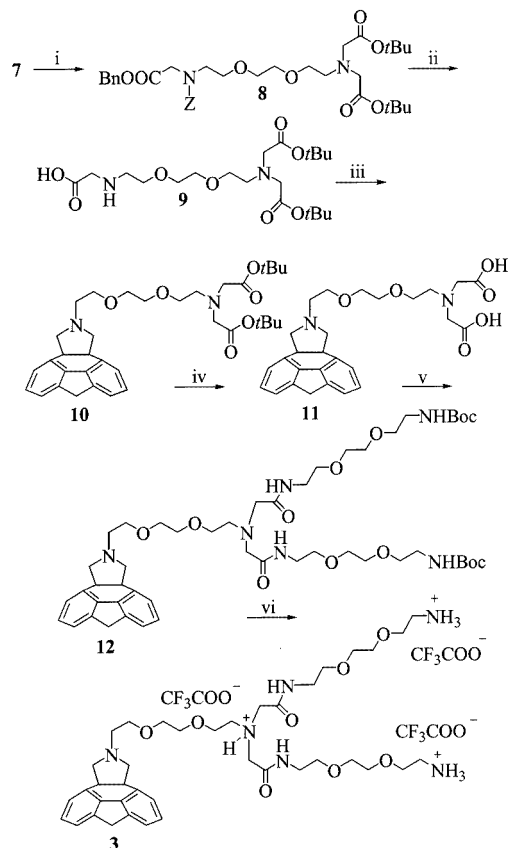
In both cases, the synthetic pathway started from the monoprotected 2,2'-(ethylenedioxy)diethylamine (**4**),^[16] which was allowed to react with benzyl bromoacetate to give amino ester **5**.^[17] Protection of the amino group with benzyl chloroformate led to **6**, which was deprotected with trifluoroacetic acid to yield the corresponding salt **7** (Scheme 1).



Scheme 1. i: ZCl, DCM; ii: TFA, DCM

Compounds **4** and **7** were the common key-products utilized in the two different synthetic pathways.

In synthetic route 1 (Scheme 2), **7** was allowed to react with an excess of *tert*-butyl bromoacetate to obtain **8**, which, after simultaneous deprotection of amino and carboxylic functions under catalytic hydrogenation conditions, gave amino acid **9**. The cycloaddition step was performed in the presence of C₆₀, paraformaldehyde and **9** in refluxing toluene (Scheme 2).^[18–20]

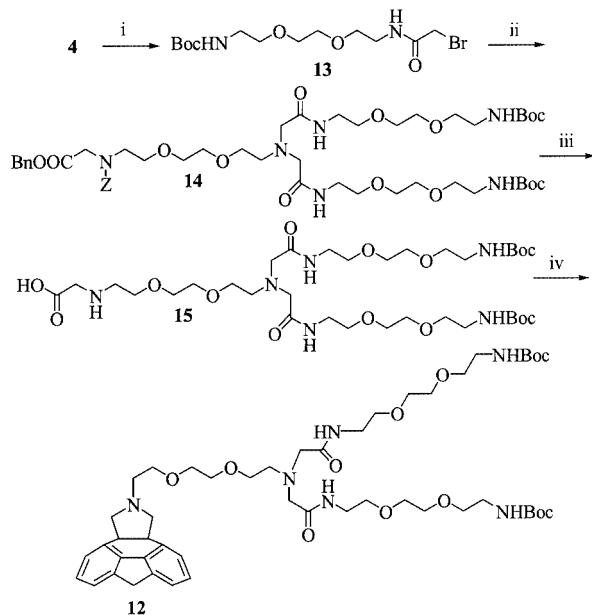


Scheme 2. i: K₂CO₃, *tert*-butyl bromoacetate, THF; ii: H₂, Pd/C, methanol; iii: C₆₀, paraformaldehyde, toluene, Δ; iv: TFA, CH₂Cl₂; v: EDC·HCl, HOBt, **4**, dioxane; vi: TFA, CH₂Cl₂

Fulleropyrrolidine **10** was obtained in 20% yield, while subsequent deprotection with trifluoroacetic acid afforded the bis(acid) **11**. The latter was coupled with **4** in the presence of EDC·HCl and HOBt to give **12**, which, after deprotection, afforded the desired final compound **3**. However, purification of derivative **12** was very difficult and the yield very low (5%).

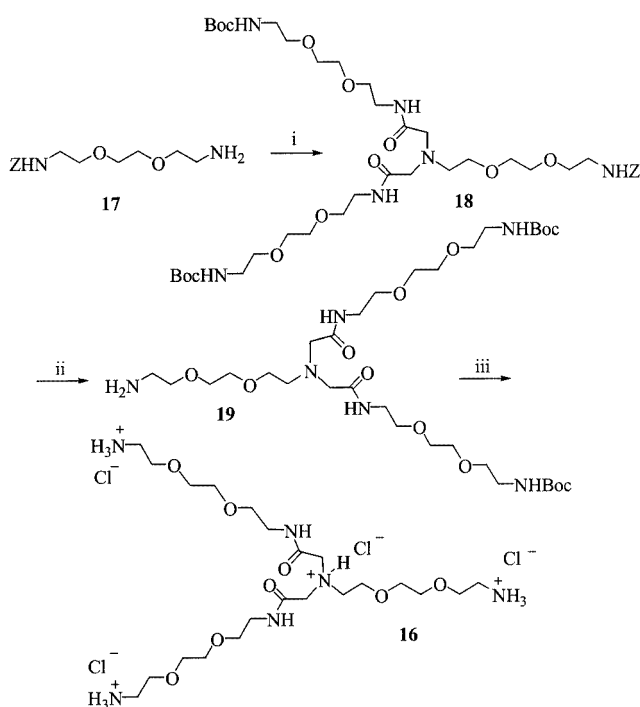
To circumvent this problem, we focused our attention on synthetic pathway 2, in which the final ramification (amino acid **15**) was performed before the cycloaddition. Pathway 2 starts with the reaction of key-product **4** with bromoacetyl bromide in the presence of TEA, leading to compound **13** (Scheme 3). This compound was allowed to react with **7**, giving amino ester **14**, which was treated with hydrogen in the presence of Pd/C (10%), leading to amino acid **15**. The cycloaddition step was performed in the presence of C₆₀, paraformaldehyde and **15** in refluxing toluene affording the

desired product **12** with higher yields (14%) and avoiding the purification problems found in synthetic pathway 1. Deprotection of **12** with trifluoroacetic acid afforded the desired compound **3** in quantitative yield.



Scheme 3. i: Bromoacetyl bromide, TEA, CH_2Cl_2 ; ii: **7**, TEA, CH_2Cl_2 ; iii: H_2 , Pd/C, methanol; iv: C_{60} , paraformaldehyde, toluene, Δ

To better evaluate the effect of fullerene derivative **3** as a neuroprotecting agent, the branched chain without C_{60} (**16**) was synthesized for use as a control in the biological assays (Scheme 4).



Scheme 4. i: **13**, TEA, CH_2Cl_2 ; ii: H_2 , Pd/C, methanol; iii: HCl, methanol

Derivative **13** was used as alkylating agent with *N*-(benzyloxycarbonyl)-2,2'-bis(ethylenedioxydiethylene)-diamine (**17**)^[21] to obtain **18**, which gave **19** after hydrogenolysis. Total deprotection of **19** by HCl/methanol afforded the desired triamino compound **16** as its hydrochloride salt.

Reactivity Towards Radicals: Chemical Assays for Superoxide Anion

As easily observed by UV/Vis spectrophotometry,^[22] solutions of **3** show aggregation at concentration values of approximately 10^{-5} M not only in water but also in mixtures of water/methanol (75:25) and water/DMF (75:25) where the solubility reaches values of 0.34 and 0.46 mM, respectively. This was deduced from the deviation from the Lambert–Beer law. In fact, the optical density changes non-linearly with the fullerene concentration increase, leading to saturation above 10^{-5} M.

The reactivity of the fullerene derivative **3** towards $\text{O}_2^{\cdot-}$, thought to be one of the oxy radicals involved in neuronal degeneration,^[8–10] was studied by spectrophotometric assays employing the xanthine/xanthine oxidase system for the generation of superoxide radicals and ferricytochrome c reduction for the measurement of superoxide concentration. Conversion of xanthine to uric acid by xanthine oxidase in the presence of oxygen is accompanied by the production of $\text{O}_2^{\cdot-}$. The capture of this species in situ in a buffer solution is possible using ferricytochrome c (Fe^{3+}) resulting in the formation of ferrocyanochrome c (Fe^{2+}) which absorbs optically at 550 nm.^[23] The absorbance change (increase in absorbance $\Delta\epsilon_{(550)} = 2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) due to the production of ferrocyanochrome by an electron-transfer reaction between superoxide ions and ferricytochrome c is a measure of the reaction. In all experiments the optical density increased rapidly during the first 10 min and then leveled off between 20 and 30 min. The change in absorbance at 550 nm during the first 5 min of the reaction was used to calculate the reduction of the ferricytochrome c and was found to be linear within the 95% confidence limit. The presence of **3** did not cause a significant modification of the concentration of $\text{O}_2^{\cdot-}$, within the experimental errors observed for the determination of the reduced cytochrome c (Table 1).

Table 1. Kinetics of cytochrome c reduction by superoxide anion ($\text{O}_2^{\cdot-}$); all values are the average of three experiments

[Compound 3] [μM]	μM reduced cytochrome c min^{-1}
0.0	505 ± 50
2.26	410 ± 41
5.64	405 ± 40
11.3	489 ± 48

Biological Evaluation

We analyzed the fullerene derivative **3** in a model of neuroprotection in cerebral cortical cells. In fact, for evaluating the protection against glutamate-induced neuronal death, compound **3** and its analogue **16** were tested in cortical cell culture treated with 100 μ M glutamate. As shown in Figure 2, 24 h after exposure to glutamate a metabolic impairment was observed, as indicated by the decrease ($53 \pm 4\%$, $n = 25$, of the control cells) in the cell viability based on the MTT assay.

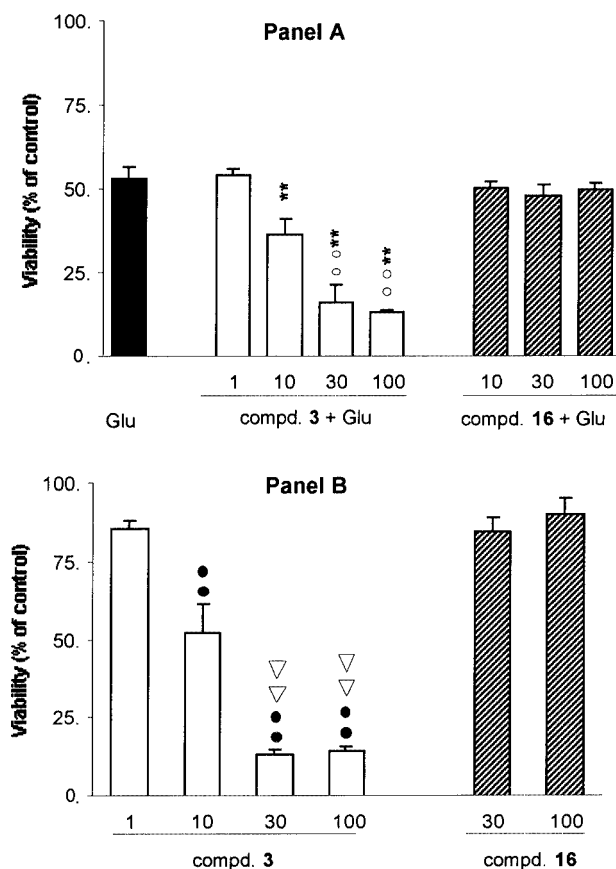


Figure 2. Cell viability assessed with MTT assay 24 h after 100 μ M exposure (10 min) in cortical cell culture; effect of **3** and **16** in the presence (panel A) and in the absence (panel B) of glutamate; the results are expressed as a percentage of control cells not exposed to glutamate; the values represent the mean $\pm$ SEM; significantly different from Glu and **3** (1 μ M) + Glu: ** $P < 0.01$; significantly different from **3** (10 μ M) + Glu: $\circ\circ$ $P < 0.01$ (panel A); significantly different from **3** (1 μ M): $\bullet\bullet$ $P < 0.01$; significantly different from **3** (10 μ M): $\nabla\nabla$ $P < 0.01$ (panel B); according to one-way ANOVA with Newman-Keuls test for multiple comparisons

Pretreatment with **3** (1–100 μ M) surprisingly enhanced the glutamate-induced cell death, while **16** (10–100 μ M) did not effect the injury induced by the excitatory amino acid (Figure 2, panel A). For better analyzing these results, the effect of both derivatives (**3**, **16**) alone on cell viability was tested (Figure 2, panel B). It is clearly evident that while compound **3** (1, 10, 30, 100 μ M) behaves as a toxic agent since it induces a concentration-dependent decrease of the cell viability, derivative **16** at 30 and 100 μ M concentration

is ineffective. A possible explanation of this undesirable toxicity can be ascribed to the lipophilic character of **3**, which, coupled with its hydrophilic part, confers surfactant properties favoring its interaction with cell membranes with their possible disruption and subsequent cell death.

Conclusion

A new water-soluble multi-charged monoadduct fullerene-[60]pyrrolidine derivative with three ethylene glycol chains and three ammonium groups has been synthesized by means of two alternative synthetic pathways. In spectrophotometric assays using the reduction of ferricytochrome c, this new C₆₀ derivative did not show a significant reaction with O₂^{•−}, the oxy radical considered to be one of the species involved in neuronal degeneration.^[8–10] In a neuroprotection model, in cerebral cortical cell death induced by glutamate, the compound was not only found to be ineffective, but also showed a significant concentration-dependent toxicity. Nevertheless, the results presented here give useful information about the chemical requirements necessary for a C₆₀ derivative to have neuroprotective activity. This seems to be correlated more with the number of substitutions than with their nature. An increased number of polar groups on C₆₀ probably prevents its interaction with the cell membrane, and consequently its disruption. Further work in this field should therefore focus on multiple addition products to guarantee a better solubility and a lower degree of aggregation than monoadducts.

Experimental Section

Synthesis

General Remarks: ¹H and ¹³C NMR spectra were recorded at 200 MHz and 50 MHz, respectively, in CDCl₃, unless otherwise noted. Chemical shifts are given in parts per million (δ) relative to tetramethylsilane. To record ES-MS spectra, the compounds were dissolved in THF/methanol (4:1), unless otherwise noted. FT-IR spectra were recorded using NaCl cells (oils) or KBr powder (DRIFT system). Yields are reported as absolute values without taking into account C₆₀ recovery (30–40% of the initial fullerene was routinely recovered). C₆₀ was purchased from Bucky-USA (99.5%); all other reagents and solvents were used as purchased from Fluka, Aldrich, J. T. Baker and Cambridge Isotope Laboratories. Silica gel NM Kieselgel 60 (70–230 mesh ASTM) was obtained from Macherey–Nagel and Merck. The syntheses of **4**,^[16] **5**^[17] and **17**^[21] were performed as described in the literature.

Synthesis of 6: ZCl (7.5 mL, 44.8 mmol) in 40 mL of CH₂Cl₂ was added to a solution of **5** (8.1 g, 20.3 mmol) in dichloromethane (60 mL). The mixture was stirred over a period of 2 h at room temp. and then washed with basic, acidic and neutral water. The organic phase was dried with CaCl₂ and the solvents were removed by evaporation, giving the pure product **6** as a colorless oil (10.8 g, 20.3 mmol, yield 99%). FT-IR: $\tilde{\nu}$ = 3367, 2936, 1715, 1707, 1508, 1460, 1365, 1245, 1178, 999, 914, 741 cm^{−1}. ¹H NMR: δ = 1.41 (s, 9 H), 3.24 (m, 2 H), 3.39–3.49 (m, 6 H), 3.52–3.64 (m, 4 H), 4.14 and 4.19 (s, 2 H), 4.98 (br. s, 1 H), 5.06 and 5.07 (s, 2 H), 5.15 (s,

2 H), 7.39–7.24 (m, 10 H) ppm. ^{13}C NMR: δ = 28.3, 40.2, 47.8, 48.4, 50.1, 66.6, 67.3, 67.4, 70.0, 70.1, 70.2, 79.0, 127.6, 127.7, 127.8, 127.9, 128.1, 128.2, 128.2, 128.3, 128.4, 135.3, 136.3, 155.7, 169.6 ppm. ES-MS: m/z = 531 $[\text{MH}^+]$.

Synthesis of 7: A solution of **6** (12.5 g, 23.6 mmol) in CH_2Cl_2 (60 mL) and TFA (60 mL) was stirred at room temp. for 24 h then the solvent and the acid were eliminated by evaporation and the pure product **7** was isolated as a colorless oil (12.9 g, 23.6 mmol, yield 99%). FT-IR: $\tilde{\nu}$ = 2945, 1710, 1690, 1463, 1360, 1197, 997, 803, 702, 601, 445 cm^{-1} . ^1H NMR: δ = 3.03 (m, 2 H), 3.42–3.61 (m, 10 H), 4.06 (br. s, 2 H), 5.11 (m, 4 H), 7.38–7.13 (m, 10 H), 7.58 (br. s, 3 H) ppm. ^{13}C NMR: δ = 39.7, 48.1, 48.6, 49.9, 50.5, 66.6, 67.1, 67.7, 69.4, 70.1, 70.2, 127.7, 128.1, 128.2, 128.5, 128.5, 128.6, 129.1, 135.3, 136.3, 156.4, 169.8 ppm. EI-MS: m/z = 431 (20) $[\text{M}^+]$, 91 (100).

Synthesis of 8: A solution of **7** (3.0 g, 5.5 mmol), K_2CO_3 (1.9 g, 13.8 mmol) and *tert*-butyl bromoacetate (2.0 mL, 13.8 mmol) in THF (50 mL) was stirred at room temp. over a period of 48 h. The solvent was then removed by evaporation and the crude material was purified by chromatography (eluent: CH_2Cl_2 /ethyl acetate, 9:1) to give **8** as a colorless oil (2.2 g, 3.4 mmol, yield 61%). FT-IR: $\tilde{\nu}$ = 2931, 1720, 1710, 1460, 1145, 744, 701 cm^{-1} . ^1H NMR: δ = 1.41 (s, 18 H), 2.89 (t, J = 5.9 Hz, 2 H), 3.44 (s, 4 H), 3.45 (m, 4 H), 3.54 (m, 6 H), 4.13 and 4.17 (s, 2 H), 5.06 and 5.07 (s, 2 H), 5.14 (s, 2 H), 7.27 (m, 5 H), 7.32 (m, 5 H) ppm. ^{13}C NMR: δ = 28.2, 50.3, 53.3, 56.4, 56.4, 56.7, 66.7, 67.4, 70.2, 70.4, 70.4, 70.5, 80.8, 127.7, 127.9, 128.2, 128.4, 128.5, 135.5, 136.4, 155.9, 156.2, 169.7, 170.7 ppm. EI-MS: m/z = 658 (5) $[\text{M}^+]$, 601 (3), 557 (50), 528 (10), 501 (100), 393 (30), 91 (100).

Synthesis of 9: A solution of **8** (1.0 g, 1.5 mmol) in methanol (12 mL) was stirred under hydrogen pressure with 10% Pd/C (0.05 g) as catalyst. After 24 h, the crude material was purified by filtration through Celite and the solvent evaporated to yield **9** as a colorless oil (0.6 g, 1.5 mmol, yield 99%). FT-IR: $\tilde{\nu}$ = 3440, 2926, 1727, 1646, 1368, 1150, 847 cm^{-1} . ^1H NMR (D_2O): δ = 1.28 (s, 18 H), 2.74 (t, J = 5.4 Hz, 2 H), 3.11 (t, J = 5.1 Hz, 2 H), 3.31 (s, 4 H), 3.45 (m, 2 H), 3.51 (m, 6 H), 3.62 (t, J = 4.0 Hz, 2 H) ppm. ^{13}C NMR (D_2O): δ = 26.9, 46.4, 48.6, 52.5, 56.0, 65.0, 68.2, 69.0, 69.1, 69.2, 82.9, 171.6 ppm. EI-MS: m/z = 434 (10) $[\text{M}^+]$, 333 (30), 116 (60), 70 (100).

Synthesis of 10: A mixture of C_{60} (502.0 mg, 0.7 mmol), paraformaldehyde (104.5 mg, 3.5 mmol) and **9** (502.0 mg, 1.2 mmol) in toluene (500 mL) was heated at reflux for 2 h. After cooling the solution to room temp., the product was purified on a chromatography column (eluent: toluene, then toluene/ethyl acetate, 9:1), and then the pure compound **10** was precipitated by addition of methanol to a CH_2Cl_2 solution giving a brown solid (160.5 mg, 0.2 mmol, yield 20%). DRIFT: $\tilde{\nu}$ = 2908, 2805, 1735, 1461, 1360, 1146, 759, 518, 441 cm^{-1} . ^1H NMR: δ = 1.45 (s, 18 H), 2.96 (t, J = 5.8 Hz, 2 H), 3.35 (t, J = 5.5 Hz, 2 H), 3.49 (s, 4 H), 3.69 (t, J = 6.1 Hz, 2 H), 3.79 (m, 4 H), 4.03 (t, J = 5.5 Hz, 2 H), 4.49 (s, 4 H) ppm. ^{13}C NMR: δ = 28.2, 53.4, 54.2, 56.7, 58.4, 70.4, 70.5, 70.5, 70.6, 70.8, 80.8, 136.1, 140.0, 141.8, 142.0, 142.1, 142.5, 143.0, 144.4, 145.2, 145.3, 145.6, 145.9, 146.0, 146.1, 147.2, 155.0, 170.7 ppm. ES-MS: m/z = 1123 $[\text{MH}^+]$. UV/Vis (CH_2Cl_2): λ_{max} = 325, 430 nm. $\text{C}_{80}\text{H}_{38}\text{N}_2\text{O}_6$ (1122): calcd. C 85.55, H 3.41, N 2.49; found C 85.50, H 3.32, N 2.48.

Synthesis of 11: Trifluoroacetic acid (5 mL) was added to a solution of **10** (40.0 mg, 35.6 μmol) in CH_2Cl_2 (5 mL) and the reaction mixture was stirred for 24 h at room temp. The solvent was evaporated and the salt was washed with toluene and dried under vacuum,

yielding **11** as a brown powder (35.0 mg, 34.6 μmol , yield 97%). FT-IR: $\tilde{\nu}$ = 3568, 2929, 1641, 1119, 520 cm^{-1} . ^1H NMR (DMSO): δ = 2.96 (m, 2 H), 3.46 (s, 4 H), 3.37–3.54 (m, 2 H), 3.62 (t, J = 4.5 Hz, 2 H), 3.76 (m, 4 H), 4.03 (t, J = 6.0 Hz, 2 H), 4.59 (s, 4 H) ppm. ^{13}C NMR (DMSO): δ = 53.7, 54.3, 59.2, 69.3, 69.9, 70.5, 70.6, 136.1, 140.0, 141.8, 141.9, 142.1, 142.5, 143.0, 144.4, 145.1, 145.3, 145.9, 146.0, 146.1, 147.2, 155.0, 176.0 ppm. ES-MS: m/z = 1112 $[\text{MH}^+]$. UV/Vis (CH_2Cl_2): λ_{max} = 306, 430, 461 (sh), 691 nm. $\text{C}_{72}\text{H}_{22}\text{N}_2\text{O}_6$ (1011): calcd. C 85.54, H 2.19, N 2.77; found C 83.60, H 2.23, N 2.65.

Synthesis of 12: A solution containing **11** (15.0 mg, 14.8 μmol), EDC-HCl (7.1 mg, 37.0 μmol), HOBT (3.5 mg, 37.0 μmol) and **4** (9.2 mg, 37.0 μmol) in dioxane (10 mL) was stirred at 50 °C for 1 h then the mixture was cooled to room temp. and the product was purified by chromatography (eluent: toluene/methanol, 4:1). Compound **12** was precipitated by addition of diethyl ether to a CH_2Cl_2 solution yielding a brown powder (1.1 mg, 0.7 μmol , yield 5%). ES-MS: m/z = 1472 $[\text{MH}^+]$.

Synthesis of 13: Bromoacetyl bromide (1.2 mL, 13.9 mmol) was added dropwise to a cold solution of **4** (3.4 g, 13.9 mmol) and TEA (2.2 mL, 15.9 mmol) in CH_2Cl_2 (20 mL). After 1 h, the mixture was washed with brine and the collected organic phases were dried with CaCl_2 . The product was purified by chromatography (eluent: petroleum ether/ethyl acetate, 7:3, then ethyl acetate) and crystallized from diethyl ether yielding **13** as a white solid (4.5 g, 12.0 mmol, yield 86%). M.p. 57–58 °C. DRIFT: $\tilde{\nu}$ = 3296, 3077, 2875, 1711, 1641, 1539, 1250, 1100, 657 cm^{-1} . ^1H NMR: δ = 1.43 (s, 9 H), 3.26 (m, 2 H), 3.51 (m, 6 H), 3.58 (m, 4 H), 3.84 (s, 2 H), 4.98 (br. s, 1 H), 6.93 (br. s, 1 H) ppm. ^{13}C NMR: δ = 28.1, 39.8, 40.0, 69.0, 69.8, 69.9, 78.8, 155.6, 165.6 ppm. ES-MS: m/z = 370 $[\text{MH}^+]$. $\text{C}_{13}\text{H}_{25}\text{BrN}_2\text{O}_5$ (369.3): calcd. C 42.29, H 6.82, N 7.59; found C 42.00, H 6.72, N 7.51.

Synthesis of 14: Compound **13** (1.9 g, 5.2 mmol) was added in seven portions (one per day) to a solution of **7** (200.0 mg, 0.4 mmol) and 151.2 μL (1.1 mmol) of TEA in CH_2Cl_2 (50 mL). After one week, the mixture was purified by chromatography (eluent: ethyl acetate, then ethyl acetate/2-propanol, 4:1), yielding **14** as a colorless oil (172.0 g, 0.2 mmol, 46% yield). DRIFT: $\tilde{\nu}$ = 3398, 2923, 1702, 1687, 1454, 1284, 1132, 843 cm^{-1} . ^1H NMR: δ = 1.41 (s, 18 H), 2.35 (br. s, 2 H), 2.75 (t, J = 5.1 Hz, 2 H), 3.26 (m, 4 H), 3.54 (s, 4 H), 3.36–3.48 (m, 30 H), 3.55 (s, 4 H), 4.10 and 4.16 (s, 2 H), 5.05 and 5.07 (s, 2 H), 5.14 (s, 2 H), 7.27 (m, 5 H), 7.33 (m, 5 H) ppm. ^{13}C NMR: δ = 28.1, 38.3, 39.9, 47.5, 48.1, 49.7, 49.8, 53.2, 58.8, 66.3, 66.4, 67.0, 67.1, 69.0, 69.3, 69.7, 78.6, 127.2, 127.4, 127.5, 127.6, 127.8, 128.0, 128.1, 128.1, 134.9, 135.0, 135.8, 135.9, 155.4, 155.5, 155.7, 169.2, 170.4 ppm. ES-MS: m/z = 1006 $[\text{MH}^+]$, 1029 $[\text{M} + \text{Na}^+]$.

Synthesis of 15: A solution of **14** (1.1 g, 1.1 mmol) in methanol (20 mL) was stirred under hydrogen pressure, with 10% Pd/C (0.02 g) as catalyst. After 24 h, the crude material was purified by filtration through Celite and the solvent evaporated, giving the pure product **15** as a colorless oil (0.9 g, 1.1 mmol, yield 99%). FT-IR: $\tilde{\nu}$ = 3335, 2870, 1700, 1530, 1365, 1250, 1115, 730 cm^{-1} . ^1H NMR (D_2O): δ = 1.24 (s, 18 H), 2.63 (t, J = 4.9 Hz, 4 H), 3.06 (t, J = 5.1 Hz, 4 H), 3.14 (s, 4 H), 3.25 (br. t, J = 4.9 Hz, 2 H), 3.31–3.42 (m, 20 H), 3.48 (s, 4 H), 3.89 (m, 8 H) ppm. ^{13}C NMR: δ = 28.2, 38.8, 40.0, 58.5, 58.5, 58.6, 65.8, 69.2, 69.8, 70.0, 78.8, 155.7, 169.3, 170.5 ppm. ES-MS: m/z = 783 $[\text{MH}^+]$.

Synthesis of 12: A mixture of C_{60} (52.0 mg, 72.0 μmol), paraformaldehyde (10.8 mg, 0.3 mmol) and **15** (283.0 mg, 0.4 mmol) in toluene (50 mL) was heated at reflux for 1 h. After cooling the solution to

room temp., the product was purified by chromatography (eluent: toluene, then toluene/methanol, 97:3) and then compound **12** (14.0 mg, 9.5 μ mol, yield 14%) was precipitated as a brown solid by addition of diethyl ether to a CH₂Cl₂ solution. DRIFT: $\tilde{\nu}$ = 3424, 2918, 1703, 1680, 1456, 1371, 1092, 452 cm⁻¹. ¹H NMR: δ = 1.43 (s, 18 H), 2.82 (t, J = 4.8 Hz, 2 H), 3.24–3.39 (m, 12 H), 3.46–3.57 (m, 16 H), 3.61 (s, 4 H), 3.78 (m, 4 H), 4.05 (t, J = 5.7 Hz, 2 H), 4.50 (s, 4 H), 5.21 (br. t, J = 5.7 Hz, 2 H), 7.54 (br. s, 2 H) ppm. ¹³C NMR: δ = 28.4, 39.0, 40.2, 40.4, 54.1, 55.1, 59.4, 68.4, 69.8, 70.2, 70.2, 70.7, 79.2, 141.8, 140.1, 136.7, 142.0, 142.1, 142.5, 143.0, 144.4, 145.2, 145.3, 145.5, 145.9, 146.0, 146.1, 147.2, 154.8, 155.9, 170.7 ppm. ES-MS: m/z = 1472 [MH⁺]. UV/Vis (CH₂Cl₂): λ_{max} = 314, 457 (sh), 544 (sh), 702 nm. C₉₄H₆₆N₆O₁₂ (1472): calcd. C 76.72, H 4.52, N 5.71; found C 74.90, H 4.56, N 5.46.

Synthesis of 3: Trifluoroacetic acid (2 mL) was added to a solution of **12** (14.0 mg, 9.5 μ mol) in CH₂Cl₂ (2 mL), and the reaction mixture was stirred for 3 h at room temp. The solvent was then evaporated and the salt washed with toluene and dried under vacuum, yielding **3** as a brown powder (14.3 mg, 9.5 μ mol, yield 99%). DRIFT: $\tilde{\nu}$ = 3505, 2921, 1682, 1432, 1129, 832, 520, 414 cm⁻¹. ¹H NMR (CD₃OD): δ = 2.86 (br. s, 2 H), 3.12 (br. t, 4 H), 3.20 (m, 4 H), 3.34–3.37 (m, 8 H), 3.51–3.71 (m, 20 H), 3.85 (m, 2 H), 4.06 (br. s, 2 H), 4.61 (br. s, 2 H) ppm. ¹³C NMR (CD₃OD): δ = 38.3, 38.8, 42.7, 57.8, 63.0, 66.0, 67.9, 68.7, 69.4, 69.5, 70.2, 135.8, 139.5, 141.3, 141.6, 142.0, 142.6, 144.0, 144.6, 144.8, 145.0, 145.6, 146.7, 154.4 ppm. ES-MS: m/z = 1272 [M⁺], 635 [M/2]⁺. UV/Vis (H₂O): λ_{max} = 217, 271, 337 nm. C₉₂H₅₄F₁₂N₆O₁₆ (1727): calcd. C 63.97, H 3.15, N 4.86; found C 63.70, H 3.27, N 4.96.

Synthesis of 18: Compound **13** (2.7 g, 7.2 mmol) was added in seven portions (one per day) to a solution of **17** (100.0 mg, 0.3 mmol) and TEA (151 μ L, 1.1 mmol) in CH₂Cl₂ (50 mL). After one week, the mixture was purified by chromatography (eluent: ethyl acetate, then ethyl acetate/2-propanol, 24:1), yielding **18** as a colorless oil (101.0 mg, 0.1 mmol, yield 34%). DRIFT: $\tilde{\nu}$ = 3333, 2869, 1713, 1531, 1455, 1390, 1366, 1246, 1115, 1045, 863, 779, 738, 699 cm⁻¹. ¹H NMR: δ = 1.40 (s, 18 H), 2.74 (t, J = 11.8 Hz, 2 H), 3.12–3.66 (m, 38 H), 5.06 (s, 2 H), 5.19 (br. s, 2 H), 5.89 (br. s, 1 H), 7.27–7.35 (m, 5 H), 7.55 (br. s, 2 H) ppm. ¹³C NMR: δ = 28.5, 39.1, 40.4, 40.9, 55.1, 59.5, 66.7, 69.1, 69.9, 70.2, 79.3, 128.2, 128.2, 128.5, 136.6, 156.0, 156.6, 170.9 ppm. ES-MS: m/z = 449 [(M + K)/2]⁺, 858 [M]⁺, 880 [M + Na]⁺, 898 [M + K]⁺.

Synthesis of 19: A solution of **18** (0.1 g, 0.1 mmol) in methanol (10 mL) was stirred under hydrogen pressure, with 10% Pd/C (0.05 g) as catalyst. After 24 h, the crude material was purified by filtration through Celite, the solvent was evaporated to give **19** as a colorless oil (84.0 mg, 0.1 mmol, yield 99%). FT-IR: $\tilde{\nu}$ = 3329, 2871, 1703, 1529, 1360, 1274, 1113, 733 cm⁻¹. ¹H NMR: δ = 1.22 (s, 18 H), 2.64–2.76 (t, J = 10.5 Hz, 2 H), 2.95–3.10 (m, 6 H), 3.18–3.30 (m, 8 H), 3.32–3.60 (m, 24 H) ppm. ¹³C NMR: δ = 27.2, 38.3, 38.6, 39.1, 57.8, 65.9, 67.8, 68.3, 68.9, 69.1, 80.5, 157.6, 172.0 ppm. ES-MS: m/z = 724 [M]⁺, 747 [M + Na]⁺.

Synthesis of 16: HCl gas was bubbled through a cold solution of **19** (80.0 mg, 0.1 mmol) in methanol (15 mL) for 15 min, then the solvent was removed by evaporation affording the pure product **16** as a colorless oil (95.3 mg, 0.1 mmol, yield 99%). FT-IR: $\tilde{\nu}$ = 3370, 2034, 1678, 1281, 1113, 1024 cm⁻¹. ¹H NMR: δ = 2.98–3.08 (br. m, 6 H), 3.24–3.35 (br. m, 6 H), 3.43–3.69 (m, 24 H), 3.87–3.95 (br. s, 4 H) ppm. ¹³C NMR: δ = 38.6, 55.9, 65.9, 68.1, 69.0, 69.9, 165.8 ppm. EI-MS: m/z = 439 (25), 482 (100), 525(75) [M]⁺, 561 (25) [M + Cl]⁺, 597 (25) [M + 2 Cl]⁺.

Biology

General: Cytochrome c, xanthine and xanthine oxidase (Sigma) were all used as received. All solvents used were of spectroscopic grade and were used without further purification.

Spectrophotometric Assay for Superoxide Anion (O₂^{•-}): Superoxide radicals were generated by employing the xanthine/xanthine oxidase/cytochrome c system. The reaction was initiated by the addition of xanthine oxidase (7.5×10^{-3} units) to the incubation mixture and the reaction was followed in terms of the reduction of cytochrome c and the corresponding increase in the absorbance at 550 nm. The reduction of ferricytochrome c into ferrocyclochrome c was determined using the molar absorption coefficients of 9 mmol⁻¹ cm⁻¹ and 27.7 mmol⁻¹ cm⁻¹ for the oxidized and reduced forms, respectively. Cytochrome c was fully oxidized as demonstrated by the absence of the absorption band at 550 nm. All assays were performed in triplicate at 25 °C. The incubation mixture consisted of 10 μ M cytochrome c, 0.05 mM xanthine and 0.1 mM EDTA in a buffer solution of KH₂PO₄/K₂HPO₄ (50 mM, pH = 7.4), along with the indicated concentration of fullerene derivative. A total volume of 3 mL was used for all experiments.

Evaluation of Neuroprotection in Cortical Cell Culture: Cerebral cortical cells were prepared from 1 d old Sprague–Dawley.^[24] After resuspension in the plating medium, the cells were counted and then plated on poly-L-lysine-coated (5 μ g/mL) 24-well plates (NUNC) at a density of 5×10^5 cells/plate. The culture medium consisted of Eagle's Basal Medium supplemented with inactivated fetal calf serum, 25 mM KCl, 2 mM glutamine and 100 μ g/mL gentamycin. Cultures were grown at 37 °C in a humidified atmosphere, 5% CO₂/95% air. Cytosine arabinoside (10 μ M) was added within 24 h of plating to prevent glial cell replication. The cultures were used in experiments after 8 d in vitro. The culture medium was removed and 100 μ M glutamate was added in 1 mL of Mg²⁺-free Krebs–Ringer bicarbonate buffer at 37 °C in 5% CO₂/95% air. After 10 min, glutamate was quickly removed. The cells were returned to the incubator in their culture medium for 24 h, when injury was assessed. To determine the degree of neuroprotection, the tested compounds (**3**, **16**) were added to the cultures 20 min before glutamate and maintained in contact with the cells during and 30 min after glutamate exposure. At the end of the treatment with the tested compounds, the cells were washed three times and then returned to their culture medium. Quantitative assessment of neuronal injury was obtained by a colorimetric assay for cell survival using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as previously described by Mosmann.^[25] In live cells mitochondrial enzymes have the capacity to transform MTT tetrazolium salt into MTT formazone. Briefly, MTT tetrazolium salt was added to the cultures and incubated for 4 h at 37 °C. The precipitated dye was dissolved in 2-propanol with 1 M HCl and quantified colorimetrically (absorbance at 570 nm). The results were expressed as the percentage of vital neurons compared with the cells not exposed to glutamate.

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