

High Catalytic Activity of Dendritic C₆₀ Monoadducts in Metal-Free Superoxide Dismutation**

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Water-soluble fullerenes, in particular the tris-malonyl-C₆₀ derivative **1** (so-called C₃),^[1] have been shown to exhibit strong antioxidant activity against reactive oxygen species in vitro and to protect cells and tissue from oxidative injury and cell death in vivo.^[2] The ability to destroy the toxic superoxide O₂^{•−} was suggested to be responsible for fullerene antioxidant activity,^[3] although its mechanism is still not clear. Dugan and co-workers offered evidence in support of a catalytic superoxide dismutation mechanism instead of direct radical attack on the C₆₀ moiety of **1**, thus showing that it could act as a metal-free mitochondrial manganese superoxide dismutase (MnSOD) mimetic.^[3a] They proposed the formation of a complex between C₃ and O₂^{•−}.

Herein, we present for the first time clear and unambiguous evidence for a catalytic dismutation process, the key steps of which are successive O₂^{•−} oxidation, within an outer-sphere electron-transfer process, and fullerene-derivative-mediated O₂^{•−} reduction. At the same time we are able to rationalize structure–property relationships by the systematic investigation of a series of stable, readily accessible, and nontoxic mono- and trisadducts **2–7** of C₆₀.^[2c,4] This led to the identification of new lead compounds for neuroprotective applications with significantly improved superoxide dismutation activity.

To describe the structure–reactivity relationship with respect to superoxide dismutase (SOD) activity, we first present the redox properties of **2–7**. Cyclic voltammetry measurements^[5] in DMSO have shown that in the potential range from 0 to −1 V (vs. a saturated calomel electrode, SCE) **2–6** undergo two reversible reductions, whereas **7** exhibits one reversible redox couple (Table 1, Figure 1, Figure S1 in the Supporting Information). The corresponding reduction potentials of the monoadducts **2–4** are significantly higher than those of trisadducts **5–7** and show a prominent charge

Table 1: Redox potentials, catalytic rate constants (*k*_{cat}), and IC₅₀^[a] values obtained by using direct stopped-flow measurements in DMSO (0.06 % water) and an indirect cytochrome *c* assay (*k*_{McCF})^[b] in aqueous solution (pH 7.8).

Fullerene	¹ E _{1/2} ^[c] [V]	² E _{1/2} ^[c] [V]	IC ₅₀ [μM]	<i>k</i> _{McCF} × 10 ⁶ [M ^{−1} s ^{−1}]	<i>k</i> _{cat} × 10 ⁶ [M ^{−1} s ^{−1}]	Modified NBT assay ^[d]
2	−0.248	−0.614	2.11 ± 0.02	1.3 ± 0.2	2.64 ± 0.04	+
3	−0.224	−0.647	1.86 ± 0.01	1.9 ± 0.3	4.29 ± 0.06	+
4	−0.077	−0.521	0.31 ± 0.02	8.7 ± 0.3	12.02 ± 0.22	+
5	−0.433	−0.726	2.85 ± 0.03	0.9 ± 0.2	0.26 ± 0.02	+
6	−0.436	−0.732				[e]
7	−0.585	−1.094				[e]

[a] IC₅₀ is the concentration of putative SOD mimic that induces a 50% inhibition of the reduction of cytochrome *c*. [b] McCF = McCord–Fridovich, reference [12]. [c] Calibrated by the Fc⁺/Fc couple (0.43 V vs. SCE). [d] The NBT assay was qualitatively applied; NBT = nitroblue tetrazolium. [e] A precipitate was formed.

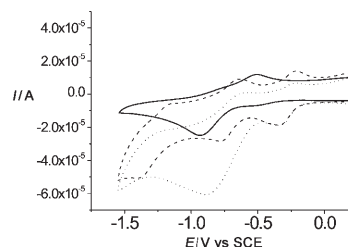


Figure 1. Cyclic voltammograms of **2** in DMSO purged with nitrogen (----) and oxygen (.....), and of pure DMSO purged with oxygen (—). Conditions: [**2**] = 0.5 × 10^{−3} M, [Bu₄NBF₄] = 0.1 M, *T* = 298 K, scan rate = 0.2 V s^{−1}.

dependence, especially for the first C₆₀/C₆₀^{•−} redox couple, with the positively charged derivative **4** being the strongest electron acceptor. The observed redox potentials are considerably higher than expected for fullerene mono- and trisadducts.^[6a] It seems that solvent effects (predominantly solvent polarity)^[6b] and the amphiphilic nature of the attached addends, which facilitate micellar organization and therefore close C₆₀–C₆₀ interaction of **2–7** in solution, are responsible for the positive shift of their redox potentials.

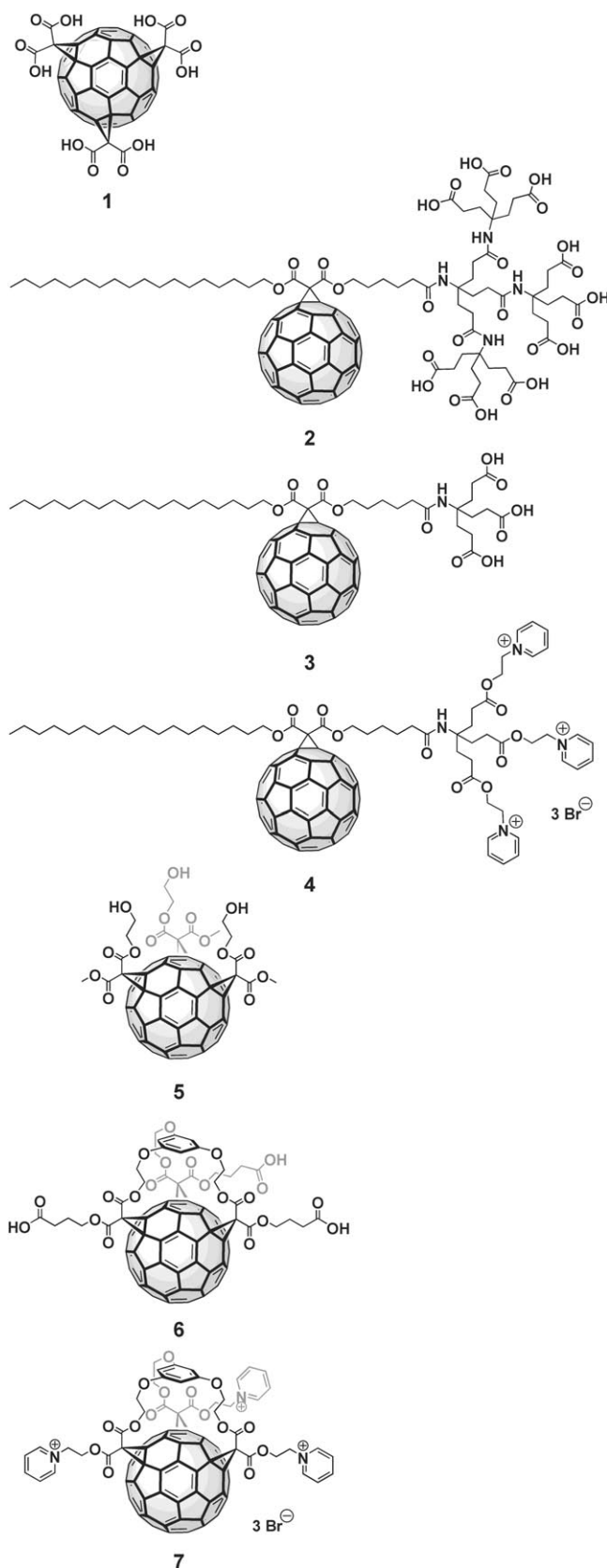
Of special importance is the fact that the first reduction potentials of **2–4** are much higher than the oxidation potential of superoxide (−0.74 V vs. SCE in DMSO). This implies that

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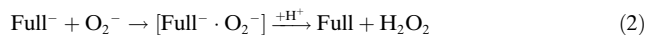


the electron transfer from $\text{O}_2^{\cdot-}$ to those fullerenes [Reaction (1)] is not only strongly driven thermodynamically in



DMSO, but also that it is possible in aqueous solution ($E^\circ(\text{O}_2/\text{O}_2^{\cdot-}) = -0.4 \text{ V vs. SCE}$ in water). Even in the case of the more difficult to reduce trisadducts **5** and **6**, this process could be energetically feasible. Note that photoinduced $\text{C}_{60}^{\cdot-}$ is able to reduce O_2 in aqueous solution, thus generating $\text{O}_2^{\cdot-}$ in an outer-sphere electron-transfer process, because its oxidation potential is more negative (-0.56 V vs. SCE) than the reduction potential of O_2 ^[7] and also more negative than the corresponding potentials of **2–6** (Table 1).

The cyclic voltammograms of **2–5** in dioxygen-saturated DMSO have revealed that in the presence of these fullerenes the reoxidation wave of superoxide (electrochemically generated in situ) disappears. The current arising from reoxidation of the fullerene anions diminishes, whereas the values of corresponding fullerene anion reoxidation potentials remain unaffected (Figure 1). This behavior clearly indicates that a reaction between electrochemically generated superoxide and the fullerene anions [Reaction (2)] takes place without



inducing chemical changes on the fullerenes. The superoxide decomposition is also observed by applying much lower (catalytic) concentrations of **2–5**, whereas **6** and **7**, independent of the applied concentrations, do not affect the superoxide reoxidation. These experiments suggest not only that **2–5** and their anions can react with $\text{O}_2^{\cdot-}$, but also that the reaction has a catalytic character.

To address this point, we further studied the reactions of **2–7** with a large excess of KO_2 in DMSO containing a controlled amount of water (0.06 %), which was in excess of the superoxide and fullerene concentrations.^[5] Time-resolved UV/Vis spectra (Figure 2) have shown that immediately after mixing of a superoxide solution with a fullerene solution,

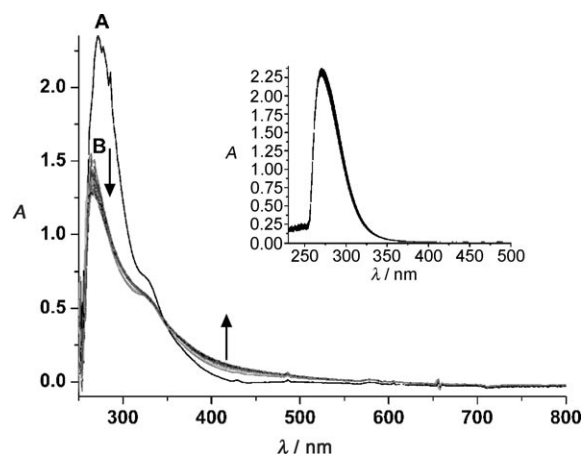


Figure 2. Time-resolved UV/Vis spectra for the reaction of **2** ($5 \times 10^{-5} \text{ M}$) with KO_2 (1 mM) in DMSO at room temperature. A) Spectrum recorded before mixing (measurements in tandem cuvette). B) First spectrum obtained after mixing (using a stopped-flow module) followed by spectra recorded at time intervals of 10 s (total observation time 30 min). Inset: control reaction without addition of the fullerene followed over 2.5 h.

rapid decomposition of $\text{O}_2^{\cdot-}$ (decrease of the absorbance in the range of 240–330 nm within the dead time of the stopped-flow instrument) is observed in the case of **2–5**.^[8] The products of superoxide disproportionation, O_2 and H_2O_2 , were qualitatively detected in all four experiments.^[5] The corresponding unattached malonate addends themselves, as well as **6** and **7**, do not induce superoxide decomposition.^[9]

The rapid process was quantified by following the corresponding absorbance decrease at 270 nm in a series of stopped-flow measurements, in which the catalytic concentration of the studied fullerenes was varied. Application of a microcuvette accessory, which reduced the dead time of the instrument to 0.4 ms, enabled the observation of the fast disappearance of the 270-nm absorption. This behavior could best be fitted as a first-order process to obtain the characteristic value of the rate constant k_{obs} (s^{-1}). A good linear correlation between k_{obs} and the fullerene concentration was observed, and the catalytic rate constants (k_{cat})^[5,10] were determined from the slopes of the corresponding plots (Figure 3, Table 1). This is the first time that the catalytic SOD activity of fullerenes has been detected by using a direct method. Interference with additional components that are always present in indirect assays can also be ruled out.

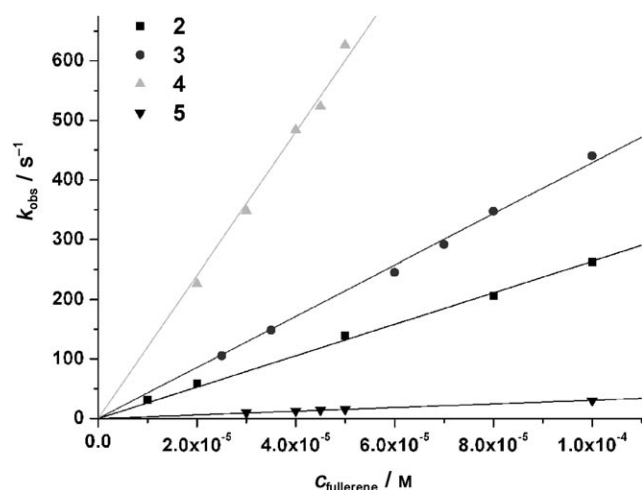


Figure 3. k_{obs} as a function of fullerene concentration for the reaction between fullerenes and saturated KO_2 in DMSO solution at room temperature.

Importantly, our results show a clear dependence of the SOD activity (k_{cat}) of the fullerenes on their reduction potentials, namely, the higher the reduction potential (ability to be reduced by $\text{O}_2^{\cdot-}$) the higher the SOD activity (Table 1). This finding suggests that the electron transfer from $\text{O}_2^{\cdot-}$ to the fullerene plays a key role in the overall catalytic dismutation of superoxide. At the same time, although **6** has almost the same reduction potential as **5**, it does not induce catalytic decomposition of $\text{O}_2^{\cdot-}$, which additionally implies that the architecture of fullerene derivatives plays an important role, too. In contrast to **5**, the capped structure of **6** most probably does not allow for a favorable attractive

interaction between $\text{O}_2^{\cdot-}$ and the functionalized region of the C_{60} cage, which could include H binding with the three OH groups of the malonate addends. Such an interaction, however, could be essential for superoxide reduction and stabilization of the resulting peroxide in the second step [Reaktion (2)] of the SOD cycle (as was observed in the case of metal-based SOD mimetics^[5,11]).

Although widely used indirect SOD assays are not very reliable in the case of enzyme mimetics (the direct stopped-flow method is a better probe for SOD activity even though it requires a nonaqueous medium),^[5,10] for comparison we also applied cytochrome *c* (cyt *c*)^[12] (Figure 4a) and modified NBT assays^[5] to investigate the SOD activity of our fullerenes

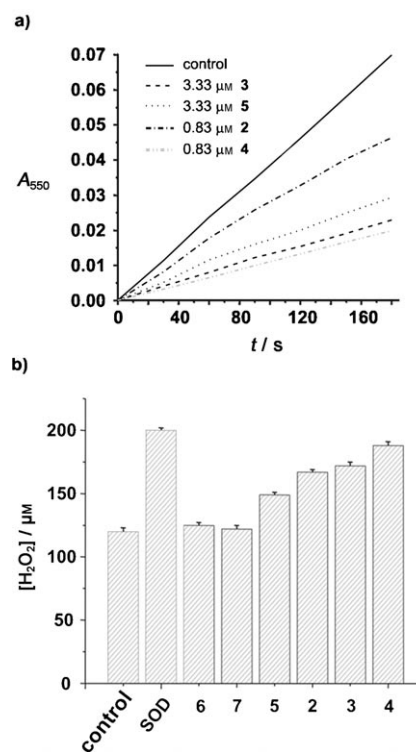


Figure 4. a) Kinetics of the reduction of ferricytochrome *c* (550 nm) without and with the fullerenes at room temperature. b) Production of H_2O_2 by SOD (25 μM) and fullerenes (25 μM) from KO_2 (500 μM ; pH 7.8; 37 °C).

in an aqueous buffer in a manner utilized in the literature. The trisadducts **6** and **7** show no SOD activity, whereas **2–5**^[13] exhibit activity with the catalytic rate constants (k_{McCF})^[5] reflecting the same trend as that determined by the direct stopped-flow measurements (Table 1). None of the fullerenes interfered with the cyt *c* assay by reoxidizing cyt *c*^{II} to cyt *c*^{III}.^[14]

A lower solubility of **2–4** in water caused the somewhat smaller k_{McCF} compared with k_{cat} determined in DMSO. Upon using an excess of superoxide over the putative SOD mimetic, we also determined the formation of H_2O_2 .^[15] The SOD-active fullerenes **2–5** yield more H_2O_2 as compared with the amount produced by spontaneous dismutation of superoxide in the aqueous buffer, in a similar fashion to the native

enzyme (Figure 4b). The amount of H_2O_2 produced correlated with the fullerene SOD activity. The capped trisadducts **6** and **7** had no effect on the formation of H_2O_2 , which again proves their lack of SOD activity.

In conclusion, we have shown by electrochemical, spectrophotometric, and submillisecond mixing UV/Vis stopped-flow measurements, as well as by indirect SOD assays and H_2O_2 detection, that **2–5** act as SOD mimetics. For the first time we have demonstrated that there is a direct correlation between the SOD activity of the water-soluble fullerenes and molecular properties such as 1) reduction potential, 2) charge, and 3) molecular structure. Monoadducts **2–4** of C_{60} have been identified as very active new lead structures. In particular, the positively charged monoadduct **4** is more active than C_3 (**1**) by one order of magnitude and approaches the performance of natural Mn- and FeSODs.^[3a] Its activity is comparable to that of the highly active metal-containing SOD mimics.^[5] The high SOD activity of **4** can be explained by its high redox potential, which facilitates its reduction by $\text{O}_2^{\cdot-}$ [Reaction (1)], and its positive charge, which through electrostatic interactions favors a closer contact between $\text{O}_2^{\cdot-}$ and the C_{60} cage assisting in the superoxide reduction [Reaction (2)].

In addition to the high activity, the monoadducts have a number of advantages over C_3 (**1**), as they are: 1) more stable, 2) considerably less toxic,^[2d] and 3) can be readily produced in large quantities. Knowing that upon irradiation solubilized C_{60} , its aggregates, and some water-soluble derivatives can generate superoxide^[7] (on which their potential application in photodynamic therapy is based), it is crucial to understand how fullerene derivatives should be designed in order to either decompose or generate $\text{O}_2^{\cdot-}$, depending on the direction of their biomedical application. In that light our results will certainly be of fundamental importance.

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- [1] I. Lamparth, A. Hirsch, *J. Chem. Soc. Chem. Commun.* **1994**, 1727–1728.
- [2] a) L. L. Dugan, E. G. Lovett, K. L. Quick, J. Lotharius, T. T. Lin, K. L. O'Malley, *Parkinsonism Relat. Disord.* **2001**, 7, 243–246; b) L. L. Dugan, D. M. Turetsky, C. Du, D. Lobner, M. Wheeler, C. R. Almlie, C. K. Shen, T. Y. Luh, *Proc. Natl. Acad. Sci. USA* **1997**, 94, 9434–9439; c) P. Witte, F. Beuerle, U. Hartnagel, R. Lebovitz, A. Savouchkina, S. Sali, D. Guldi, N. Chronakis, A. Hirsch, *Org. Biomol. Chem.* **2007**, 5, 3599–3613; d) F. Beuerle, P. Witte, U. Hartnagel, R. Lebovitz, C. Parnig, A. Hirsch, *J. Exp. Nanosci.* **2007**, 2, 147–170.
- [3] a) S. S. Ali, J. I. Hardt, K. L. Quick, J. S. Kim-Han, B. F. Erlanger, T.-T. Huang, C. J. Epstein, L. L. Dugan, *Free Radical Biol. Med.* **2004**, 37, 1191–1202; b) K. Okuda, T. Mashino, M. Hirobe, *Bioorg. Med. Chem. Lett.* **1996**, 6, 539–542.
- [4] a) F. Beuerle, N. Chronakis, A. Hirsch, *Chem. Commun.* **2005**, 3676–3678; b) for the synthesis and characterization of **4**, see the Supporting Information.
- [5] For the experimental details regarding solution preparations, instrumentation techniques, and cyt *c* and NBT assays, see: G.-F. Liu, M. Filipović, F. W. Heinemann, I. Ivanović-Burmazović, *Inorg. Chem.* **2007**, 46, 8825–8835.
- [6] a) C. Boudon, J.-P. Gisselbrecht, M. Gross, L. Isaacs, H. L. Anderson, R. Faust, F. Diederich, *Helv. Chim. Acta* **1995**, 78, 1334–1344; b) V. Krishnan, G. Moninot, D. Dubois, W. Kutner, K. M. Kadish, *J. Electroanal. Chem.* **1993**, 356, 93–107.
- [7] a) I. Nakanishi, S. Fukuzumi, T. Konishi, K. Ohkubo, M. Fujitsuka, O. Ito, N. Miyata, *J. Phys. Chem. B* **2002**, 106, 2372–2380; b) Y. Yamakoshi, N. Umezawa, A. Ryu, K. Arakane, N. Miyata, Y. Goda, T. Masumizu, T. Nagano, *J. Am. Chem. Soc.* **2003**, 125, 12803–12809.
- [8] Less prominent spectral changes at wavelengths higher than 350 nm, in the case of **2** and **3**, were found to be caused by KOH which is inevitably present in KO_2 . By using an electrochemically generated superoxide solution these spectral changes were not observed.
- [9] At a much longer timescale the pyridinium groups of two cationic dendrimers and **7** react stoichiometrically with KO_2 , which results in a product with strong absorbance at 273 nm (Figure S2). The same product is formed upon reaction with KOH.
- [10] a) D. P. Riley, W. J. Rivers, R. H. Weiss, *Anal. Biochem.* **1991**, 196, 344–349; b) R. H. Weiss, A. G. Flickinger, W. J. Rivers, M. M. Hardy, K. W. Aston, U. S. Ryanll, D. P. Riley, *J. Biol. Chem.* **1993**, 268, 23049.
- [11] D. T. Sawyer, J. S. Valentine, *Acc. Chem. Res.* **1981**, 14, 393–400.
- [12] J. M. McCord, I. Fridovich, *J. Biol. Chem.* **1969**, 244, 6049–6055.
- [13] **5** has quite low, but still detectable, SOD activity.
- [14] The time-resolved UV/Vis spectra for the reaction between electrochemically reduced cyt *c* and the fullerenes under an argon atmosphere were monitored over 30 min and 12 h. No change in the redox state of cyt *c*^{II} was observed. However, in the entire spectral range a slow shift of the cyt *c* spectrum was observed in the case of **5** and **6**, which suggests that these fullerenes induce precipitation of cyt *c* from the solution (Figure S3). This finding is attributed to the somewhat higher value of apparent k_{cat} for **5** in comparison to the value obtained in the stopped-flow experiment, by causing an additional decrease of the absorbance at 550 nm (Figure S3).^[5,11]
- [15] Experimental details are given in the Supporting Information.