Synthesis, structure and biological activity of nitroxide malonate methanofullerenes

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Two nitroxide methanofullerenes was synthesized for the first time, and their structures and biological activities studied. It was shown by X-ray single crystal analysis that the methanofullerene with two nitroxide groups forms a 1 : 2 inclusion complex with chloroform and has a nearly tetrahedral (diamond-like) arrangement of fullerene–fullerene interactions in the crystal. For the first time, it has been found that malonate nitroxide methanofullerene in combination with the known anticancer drug cyclophosphamide (CPA) shows high antitumor activity against leukemia P-388.

Introduction

Functionally substituted fullerene derivatives are of interest since it is possible to use them to obtain both biologically active substances and new materials for nanotechnology. The presence of the fullerene core allows the design of molecules containing units designed to give particular properties.

Numerous investigations in recent years have revealed that fullerenes exhibit various types of biological activity.[1–11] Fullerenes can be used for the photodynamic therapy of tumors,[1–5] can exhibit antiviral activity against human immunodeficiency virus,[6,7] and a fullerene–paclitaxel complex has substantial antitumor activity.[8] It has been shown that fullerenes possess a high degree of penetration through cellular membranes, and localize preferentially in the mitochondria.[7,9] It has been proposed that fullerenes can be used as drug delivery agents, in particular, as a vector for anticancer drug delivery directly into the tumor. Water-soluble fullerene derivatives have been found to exhibit considerable antioxidant activity.[10,11]

There have been several studies into the synthesis and study of nitroxide fullerene derivatives, mainly fulleropyrrolidines,[12–14] but it is interesting to note that there has been practically no work into the synthesis of nitroxide methanofullerenes.[15] Methanofullerenes are, from the point of view of synthesis, one of the most developed classes of organic fullerene derivatives.[16–18] The fullerene core in methanofullerenes usually contains 1–4 nitroxide groups, but can contain more. Methanofullerenes with such a high content of nitroxide radicals are possible high-spin organomagnetics. We have previously shown that pulsed photoexcitation of a new fullerene-linked bis-nitroxide gave a compound with a well-resolved transient EPR spectrum, assigned to an excited quintet spin state generated by spin-coupling of the nitroxides and the fullerene excited triplet.[19,20] The synthesis, structure and biological activity of nitroxide methanofullerenes **2** and **4** has not been previously described. In this paper, the synthesis, structure and biological activities of methanofullerenes containing one or two nitroxide fragments are reported.

Results and discussion

Compounds **2** and **4** were synthesized by a Bingel–Hirsch reaction[21] by the reaction of fullerene and the corresponding nitroxide ethers of malonic acids **1** and **3** (obtained from TEMPOL and the chloroanhydrides of these acids according to a literature procedure[22]). These reactions proceed in the presence of tetrabromomethane (CBr_4) and 1,8-diazabicyclo-[5.4.0]-undec-7-ene (DBU) to give the nitroxide methanofullerenes **2** and **4** (Scheme 1).

Compounds **2** and **4** were isolated by column chromatography on silica gel and identified by UV, IR, ^{13}C NMR, ESR methods and by single-crystal X-ray diffraction analysis. The compositions of all compounds were confirmed by MALDI-TOF mass spectrometry. The mass spectra of compounds **2** and **4** contain molecular ion peaks at m/z 1005.08 (calculated 1004.99) and 1131.21 (calculated 1131.18), respectively. The purity of compounds **2** and **4** was checked by HPLC data (Fig. 1).

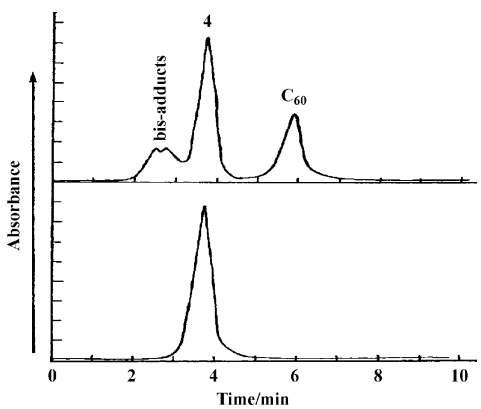

Fig. 1 HPLC data for the reaction mixture (top) and pure compound **4** (bottom). Chromatographic conditions: C_{18} reversed-phase column (250 × 4.6 mm, Partisil-5 ODS-3), toluene–MeCN (1 : 1, v/v) as the eluent, UV detection at $\lambda = 328$ nm.

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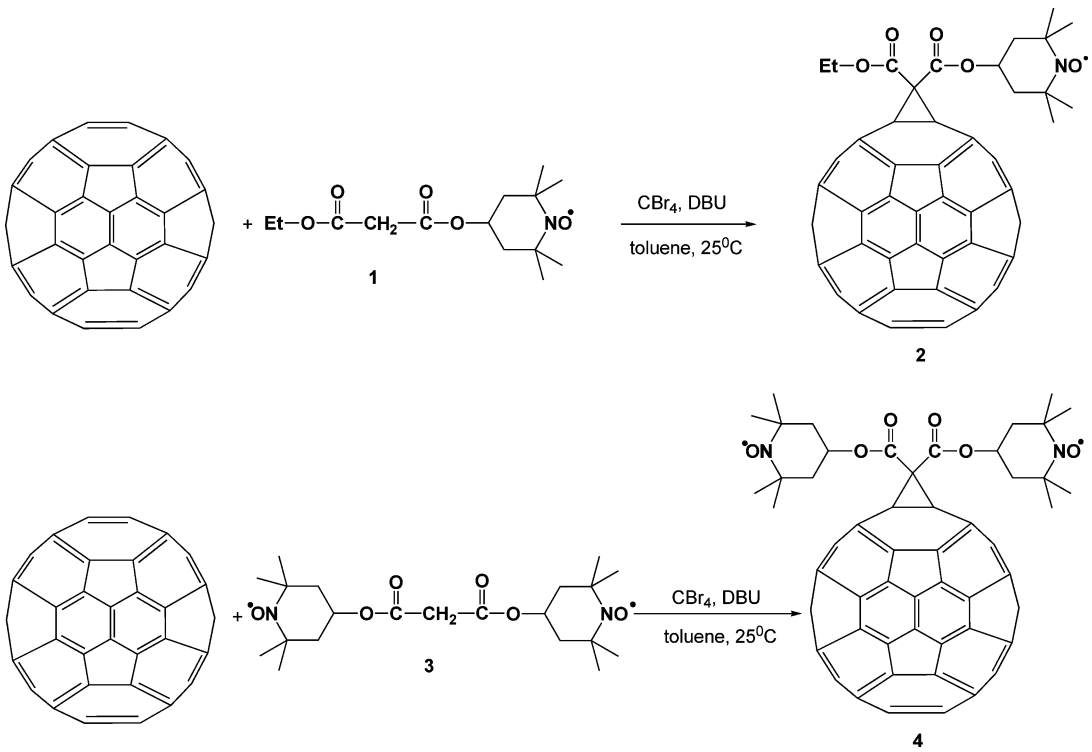


Scheme 1 Synthesis of compounds **2** and **4**.

The UV spectra of compounds **2** and **4** have characteristic absorption bands at 256, 327, 429, 493 and 696 nm. The absorption band at 429 nm indicates the formation of [6,6]-closed monoadducts. The IR spectra of these compounds have absorption bands at 527 and 1428 cm^{-1} (characteristic for vibrations of the fullerene cage), at 1745 cm^{-1} (C=O groups), and a series of other bands corresponding to stretching vibrations of the attached fragments.

The ^{1}H NMR spectra of **2** and **4** are not informative, as signals of all protons are paramagnetically broadened owing to the presence of nitroxide radicals. In the ^{13}C NMR spectra of these compounds the signals of sp^2 carbon atoms of the fullerene cage are observed in the 136–144 ppm region. The signals of the sp^3-hybridized carbon atoms of the fullerene cage are observed at δ 70.42 (**2**) and 70.80 (**4**). The weak broadened signals due to the C(61) atom of the methano fragment appear at 55.64 (**2**) and 54.96 ppm (**4**).

The presence of the nitroxide fragments in the synthesized compounds **2**, **3** and **4** was also confirmed by ESR spectroscopy (Fig. 2). The initial TEMPOL and nitroxide methanofullerenes **2**, **3** and **4** in the solid state, taken in equimolar amounts, are characterized by singlets with integrated intensities close to theoretical ratio 1 : 1 : 2 : 2, and widths 0.93, 1.30 1.40 1.20, mT, respectively. The intermolecular magnetic exchange interactions are strong for the more magnetically concentrated TEMPOL. Compounds **2** and **4** in solid state do not exhibit their biradical nature at room temperature. Magnetic intramolecular exchange with $J \sim a_N$ is observed in toluene solutions of compound **4** (Fig. 3).

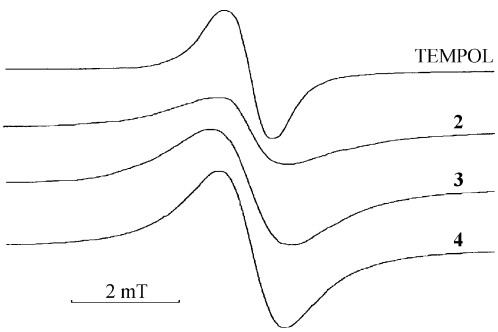


Fig. 2 EPR spectra of TEMPOL and compounds **2**, **3** and **4** in the solid state at 295 K.

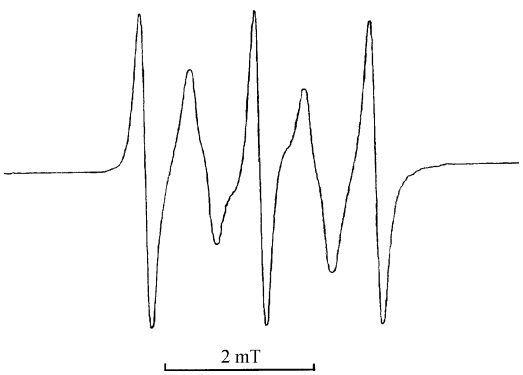


Fig. 3 EPR spectrum of compound **4** in toluene solution ($c = 1 \times 10^{-4}$ M) at 295 K.

Crystal structure of 4

Single crystals of **4** were obtained by the evaporation of a chloroform solution. The single-crystal X-ray diffraction pattern showed that it forms a 1 : 2 inclusion complex with chloroform (Fig. 4). In the crystal, the $C_{81}H_{34}N_2O_6$ molecule is situated on a two-fold axis, which is aligned with the center of gravity of the

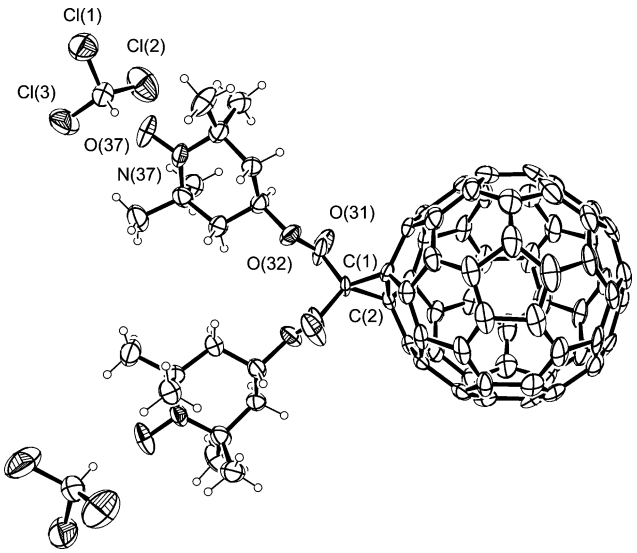


Fig. 4 ORTEP view of the molecular structure of compound **4**. Displacement ellipsoids are drawn at the 30% probability level. H atoms are represented by circles of arbitrary size. Selected bond lengths and angles are: N(37)–O(37) 1.276(7), N(37)–C(36) 1.486(10), N(37)–C(38) 1.498(11), C(1)–C(2) 1.513(9), O(32)–C(32) 1.218(9), C(1)–C(32) 1.481(9), O(33)–C(32) 1.266(9), O(33)–C(34) 1.467(8) Å; C(2)–C(1)–C(2′) 62.5(6)°, C(32)–C(1)–C(2) 120.2(5)°.

fullerene fragment and the C(1) position of the methano fragment; the nitroxide groups are directed away from the fullerene fragment.

The methanofullerene molecules are linked by two chloroform molecules by C–H $\cdots$ O intermolecular interactions between H(44) of the solvate molecule and O(37) of the nitroxide group (H(44) $\cdots$ O(37) = 2.22 Å,; C(44)–H(44) $\cdots$ O(37) = 160°).

The supramolecular structure of compound **4** in the crystal is apparently determined mainly by the fullerene–fullerene interactions. In spite of the presence of large substituents in the methanofullerene and solvate molecules in the crystal of **4**, which hinders such interactions, the fullerene fragments are closely packed with two slightly different fullerene environments, each fullerene molecule having four neighbors.

Interactions between the aromatic ring systems of methanofullerene molecules result in the formation of continuous zigzag chains along the crystallographic *b* axis (fig. 5). These chains are located in the *bc* plane of the unit cell, with a distance between the fullerene centroids of 10.14 Å, and an angle between the centroids of the three fullerene fragments of 116.2°. This distance is close to that of pure C_{60} crystals (9.94 Å).[23a] The fullerene molecules in zigzag chains with five- and six-membered rings are assembled facing each other. The distance between the centers of the nearest aromatic 5- and 6-rings of the fullerene fragments is 4.00 Å, and the dihedral angle is 37.7°; the shortest carbon–carbon distance, C(7) $\cdots$ C(8)′ and C(8) $\cdots$ C(7)′, is 3.30 Å (symmetry operation $1-x, -y, 2-z$).

Two other neighboring fullerene fragments are arranged along the diagonal to the *ac* plane with a centroid-to-centroid distance of 10.08 Å and an angle between centroids of 138.5°. Fullerene–fullerene interactions also form continuous zigzag chains in the *ac* plane. The distance between the centers of interacting 5- and 6-membered aromatic rings of the fullerene fragments is 4.15 Å, and the dihedral angle is 38.0°; the shortest carbon–

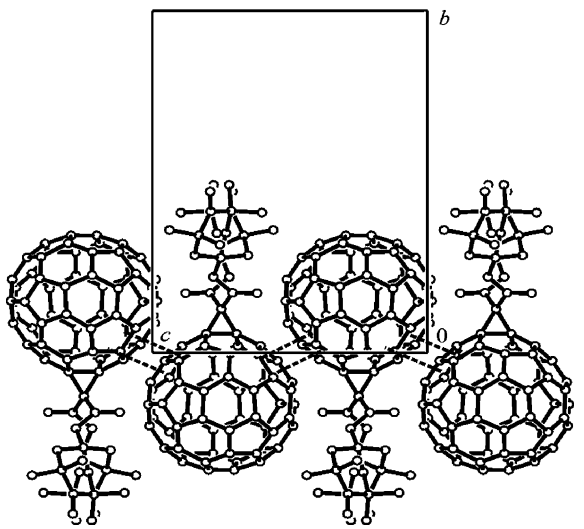


Fig. 5 Projection on the *bc* plane showing the zigzag fullerene arrangement in crystalline **4**. H atoms are omitted for clarity. Dashed lines indicate the fullerene–fullerene interactions.

carbon distance, C(23) $\cdots$ C(23)″, is 3.10 Å (symmetry operation $\frac{3}{2}-x, -\frac{1}{2}-y, 2-z$). It has recently been shown[23b] that in C_{60} intercalation complexes the zigzag arrangement of fullerenes encourages fullerene–fullerene interactions more strongly than in their linear counterparts.

In general, the indicator for fullerene–fullerene interactions is any centroid-to-centroid distance being close to 10.0 Å (vdW diameter of C_{60} is 10.18 Å). From this point of view, the methanofullerene molecules have a nearly tetrahedral environment in the solid state (Fig. 6), with the angles between fullerene centroids of 100.3, 100.3, 101.2, 101.2, 116.2 and 138.5° resulting in a diamond-like supramolecular structure.

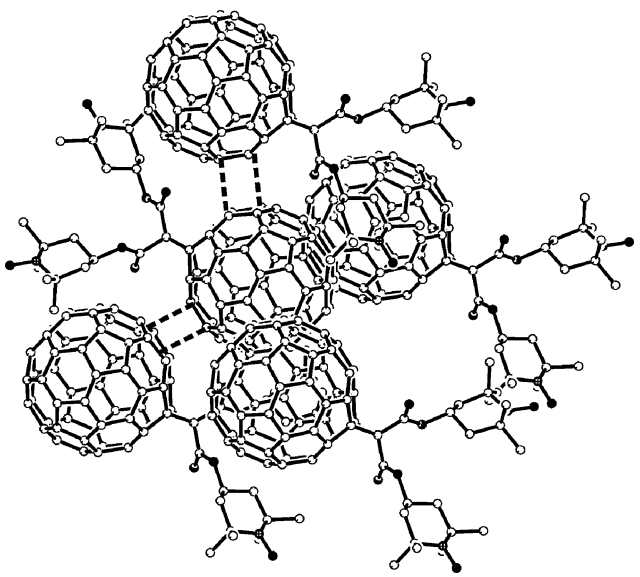


Fig. 6 Diamond-like arrangement of the methanofullerene molecules in the crystal of **4**. Dashed lines indicate the fullerene–fullerene interactions. H atoms are omitted.

The solvate molecules are distributed in the cavities of the supramolecular structure in such a manner that close contacts

between the chloroform molecules (including Cl···Cl contacts) are not detected. Such mutual arrangement of the molecules in crystalline **4** leads to a high packing coefficient (68.8%).

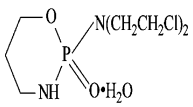
Biological activity of compounds 3 and 4

We have previously shown that functionalized fullerene derivatives are more reactive towards free organic radicals than free C_{60}.[24,25] In this regard, nitroxide methanofullerenes are of special interest, as the fullerene cage and the nitroxide fragment can be used both as radical traps, and spin-labels.[26–28]

In modern tumor chemotherapy, anticancer drugs are often used at high doses, which induces severe side effects. Therefore, the search for methods of increasing tumor chemotherapeutic sensitivity and protecting normal tissues is of great importance. Nitroxide radicals are of prime interest for the protection of normal cells and tissues from the toxic side effects of cytostatics,[29,30] since, being powerful antioxidants, they can normalize the level of cytochrome P-450 in the liver and protect the cells against toxic products.[31]

It is predicted that compounds containing fullerene and nitroxide radical moieties could be used as modifiers of biological reactions (MBR) for anticancer chemotherapy.[31,32] Based on this literature data, we have studied the anticancer activity of the nitroxide malonate **3** and nitroxide methanofullerene **4**.

An experimental tumor model, leukemia P-388, was used for the evaluation of the therapeutic activity of compounds **3** and **4**. These compounds were injected in combination with the anticancer drug cyclophosphamide (CPA).



CPA

Leukemia P-388 (10^6 cells) was transplanted intraperitoneally into male BDF_1 mice. The number of recovered mice (alive after 60 days) and the increase of the average life span (ILS%) of treated mice compared to a control group were used as criteria of therapeutic efficacy. Compound **3** was dissolved in 10% ethyl alcohol, and compound **4** was dissolved in Tween-80 (10%). Compounds **3** and **4** were injected subcutaneously into the mice on days 1–7 after tumor transplantation, and a solution of CPA in water was injected subcutaneously into the mice on days 1 and 6 after tumor transplantation. The mice were observed for 60 days; the results are shown in Fig. 7 and Table 1.

No tumor-bearing mice survived after the injection of cyclophosphamide, **3**, or **4** individually. However, treatment of mice with cyclophosphamide in combination with **3** or **4** resulted in the survival of 20% and 70% animals, respectively. The surviving animals had no symptoms of leukemia. It should be noted that the use of fullerene **4** produced a greater therapeutic benefit than **3**. Similar effects were obtained when ILS% was used as a criterion of treatment efficiency (Table 1).

Conclusion

Two nitroxide methanofullerenes, demonstrating the properties of bis- and poly-centric radical traps in a living body, were synthesized for the first time. The presence of the fullerene cage and nitroxide radicals in one of the molecules allowed their protective action on leukemia P-388-bearing animals to be revealed, when applied in combination with the anticancer drug cyclophosphamide. The results obtained suggest that nitroxide fullerene derivatives are promising modifiers of biological reaction for tumor chemotherapy. These new fullerene derivatives are also candidates for pharmacokinetic studyies *in vivo* by means of ESR spectroscopy.

Table 1 Combined activity against leukemia P-388by cyclophosphamide (CPA) and complexes CPA + **3** and CPA + **4**

Drug	Single dose/mg kg^{-1}	ILS (%)
Control	—	0
CPA	30	219
3	50	0
4	200	0
CPA + **3**	30 + 50	252
CPA + **4**	30 + 200	325

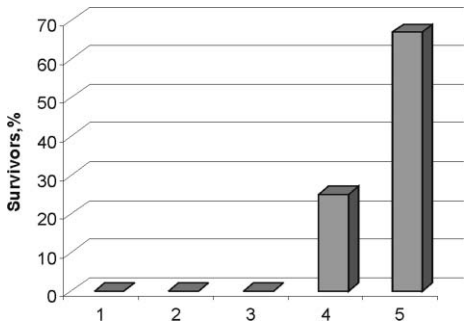


Fig. 7 Increase of sensitivity of leukemia P-388 to treatment with cyclophosphamide and **3** or **4**. Column 1: 30 mg kg^{-1} of CPA. Column 2: 50 mg kg^{-1} of **3**. Column 3: 200 mg kg^{-1} of **4**. Column 4: CPA + **3** (the same doses). Column 5: CPA + **4** (the same doses). Each group comprised 10 animals.

Experimental

Analysis by HPLC was carried out on a Gilson chromatograph equipped with a UV detector (C_{18} reversed-phase column, Partisil-5 ODS-3; toluene–MeCN 1 : 1 (v/v) as the eluent). Organic solvents were dried and distilled before use. C_{60} of 99.9% purity (produced by the G. A. Razuvaev Institute of Organometallic Chemistry of the Russian Academy of Sciences, Nizhny Novgorod) was used. The starting nitroxide compounds **1** and **3** were prepared according to literature procedures.[22] All chemical operations were carried out under dry argon.

UV spectra were recorded on a Specord M-40 spectrophotometer in CH_2Cl_2. IR spectra were measured on a Bruker Vector 22 Fourier-transform spectrometer (KBr pellets). ^1H and ^{13}C NMR spectra were recorded on Bruker MSL-400 (400.00 MHz for ^1H and 100.6 MHz for ^{13}C) and Bruker Avance-600 (100.57 MHz for ^{13}C and 600.00 MHz for ^1H) spectrometers in $CDCl_3$ at 30 °C. The ESR spectra were recorded using an SE/X-2544 (Radiopan) ESR spectrometer. Mass spectra were obtained on MALDI-TOF instrument (Dynamo Thermo-BioANALYSIS) using trihydroxyanthracene as the matrix.

61-*O*-Methyl-*O*-(2′,2′,6′,6′-tetramethyl-4′-oxypiperidinyloxyl)ethoxycarbonyl-61-methano-[60]-fullerene (2)

DBU (0.137 g, 0.9 mmol) was added to a mixture of C_{60} (0.216 g, 0.3 mmol), CBr_4 (0.149 g, 0.45 mmol) and **1** (0.128 g, 0.45 mmol) in 200 ml toluene under argon at room temperature. The reaction mixture stirred for 5 h at temperature 25° C. The mixture was then washed with water (3 × 30 ml) and purified by column chromatography on SiO_2 (silica gel Merk 60). Unreacted C_{60} (0.034 g) was obtained after elution by toluene–hexane (1 : 3). Compound **2** was isolated by elution with toluene–CH_3CN (40 : 1). Yield: 0.114 g (37.9% based on consumed C_{60}). MALDI-TOF MS, found: m/z 1005.08 [M^+], 959.98 [$M^+ - 3CH_3$]; calculated: 1004.99; UV-Vis (CH_2Cl_2), λ_{max}/nm: 258.0, 325.0, 430.0, 494.0, 696; FTIR (KBr), ν/cm^{-1}: 526, 675, 709, 743, 837, 972, 1004, 1059, 1179, 1219, 1363, 1425, 1461, 1636, 1740, 2851, 2922, 2971; ^{13}C NMR (C_6D_6 + $CDCl_3$): 13.01, 20.18, 55.64, 70.42, 95.32, 136.90, 138.48, 140.45, 141.07, 141.42, 142.28, 142.35, 142.47, 143.14, 143.84, 143.95, 144.09, 144.16, 144.33, 144.48, 144.61, 162.02 (C=O).

61-Bis-(*O*-2′,2′,6′,6′-tetramethyl-4′-oxypiperidinyloxyl)carbonyl-61-methano-[60]-fullerene (4)

Compound **4** was obtained from fullerene C_{60} (0.216 g, 0.3 mmol), bis-*O*-(2,2,6,6-tetramethylpiperidinyl-4-oxyl)malonate **3** (0.185 g, 0.45 mmol), CBr_4 (0.149 g, 0.45 mmol) and DBU (0.137 g, 0.9 mmol) in toluene (200 ml) using the conditions described above. Yield: 0.096 g (34% based on consumed C_{60}). MALDI-TOF MS, found: m/z 1131.21; calculated: 1131.18; UV-Vis (CH_2Cl_2), λ_{max}/nm: 256.0, 327.0, 429.9, 493.0, 699; FTIR (KBr), ν/cm^{-1}: 527, 577, 672, 707, 740, 813, 837, 961, 1006, 1058, 1176, 1230, 1267, 1362, 1428, 1462, 1636, 1745, 2854, 2924, 2963; ^{13}C NMR ($CDCl_3$): 54.96, 63.24, 70.80, (sp^3 carbons), 95.71, 135.81, 137.18, 138.35, 138.44, 140.33, 140.40, 141.23, 141.56, 142.32, 142.40, 143.24, 144.05, 144.27, 144.37, 144.48, 144.65, 162.69 (C=O).

Crystal data for 4. This was collected on a CAD 4 diffractometer (graphite-monochromated CuKα radiation, $\lambda = 1.54184$ Å),[33] and the structure solved by direct methods using SIR.[34] Full-matrix least-squares refinement on F^2 was performed using SHELXL-97[35] with anisotropic displacements for non-H atoms. All hydrogen atoms were placed in idealized positions and refined using the riding model. Illustrations were generated using Platon.[36] $C_{81}H_{34}N_2O_6{\cdot}2(CHCl_3)$, M 1369.84 g mol^{-1}, black prism of dimensions 0.24 × 0.20 × 0.18 mm^3, monoclinic, space group $C2/c$, $a = 25.403(9)$, $b = 17.845(4)$, $c = 17.218(4)$ Å, $\beta = 132.09(2)°$, $V = 5792(3)$ Å^3, $T = 293$ K, $Z = 4$, μ(CuKα) 32.52 cm^{-1}, $d_{calc} = 1.57(1)$ g cm^{-3}. 9464 reflections measured, 5631 unique ($R_{int} = 0.221$), 984 observed with $F_{obs} > 4\sigma(F_{obs})$, 442 parameters. The final $R1(F^2)$ was 0.0579 ($>2\sigma I$) and $wR2$(all data) = 0.1923. CCDC reference number 630090. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b617892h.

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