Functionalization of C_{60} with diphosphonate groups: a route to bone-vectored fullerenes

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Received (in Cambridge, UK) 8th January 2002, Accepted 4th April 2002
First published as an Advance Article on the web 23rd April 2002

A facile synthesis for derivatizing fullerenes with diphosphonate groups based on the Bingel reaction has been explored. Five different bisadduct isomers of C_{60} with tetraethyl methylenediphosphonate, $CH_2(PO_3Et_2)_2$, have been synthesized, separated and characterized by MALDI-TOF mass spectrometry and $^{31}P\{^1H\}$ and ^{13}C NMR spectroscopy. Hydrolysis of the $C_{60}[C(PO_3Et_2)_2]_2$ isomers generates water-soluble diphosphonic acids, $C_{60}[C(PO_3H_2)_2]_2$ for future *in vitro* and *in vivo* studies in which mineralized bone tissue will be selectively targeted.

Introduction

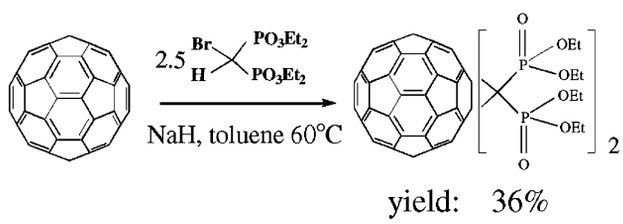
Since the discovery of a method in 1990 for the efficient production of macroscopic quantities of fullerenes, potential applications for these fascinating materials have been explored intensively. One of the most promising areas now appears to be in the field of medicine. To date, several C_{60} derivatives have been shown to possess a variety of potential medical applications ranging from photodynamic therapy to the treatment of HIV and various neurological diseases.[1] In addition, recent advances in the production of radioactive ($^{166}Ho@C_{82}$)[1b,2–4] and paramagnetic ($Gd@C_{82}$)[1b,5] endohedral metallofullerenes demonstrate the possibility of using such materials as radiotracers, radiopharmaceuticals and magnetic resonance imaging contrast agents. The continued development of such applications will, in general, require functionalization of fullerenes with tissue-targeting moieties to help ensure the delivery of any drug to a specific site in the body.

The long-term goal of this work has been to synthesize water-soluble, tissue-vectored derivatives of C_{60} which might be considered as a model system for the design and study of tissue-selective fullerenes, in general. Hydrophilic diphosphonate groups are known to possess high affinity for the bone mineral hydroxyapatite (HAP).[6] Thus, functionalization of C_{60} with diphosphonate groups should lead to bone-vectored, water-soluble fullerene derivatives. This concept has already been demonstrated, in principal, by a water-soluble C_{60} derivative having one diphosphonate and 16 hydroxy groups.[1b,d] *In vitro* HAP crystal growth inhibition studies with this molecule demonstrated high affinity and specificity for bone tissue, but the molecule is difficult to synthesize (multi-step low-yield procedure) and thus impractical for further drug development.

A more convenient, one step method for derivatizing fullerenes with diphosphonate groups is based on the Bingel reaction[7,8] which involves cyclopropanation of a 6,6 double bond in C_{60} with a methylenediphosphonate group. Addition of only one hydrophilic group to the fullerene cage is, in general, insufficient to confer water solubility of the adduct, but bis- and higher adducts have been found to be reasonably soluble in water.[9] Multiple Bingel additions usually lead to the formation of mixtures of regioisomers.[10] Thus, in this study we have focused on the synthesis and isolation of the individual isomers of the bis(methylenediphosphonate) derivatives of C_{60}.

Results and discussion

The $C_{60}[C(PO_3Et_2)_2]_2$ was obtained according to Scheme 1. The



Scheme 1 Synthesis of $C_{60}[C(PO_3Et_2)_2]_2$.

high yield of the bisadducts was achieved using a 2.4–2.6 excess of tetraethyl bromomethylenediphosphonate. Flash chromatography on silica gel ($CHCl_3$ eluent) was used for the preliminary separation. After removal of the monoadduct as a purple–red colored band, a second dark-amber fraction containing mainly the bisadduct isomers was collected. Further separation was achieved by HPLC on a preparative "Econosil Silica" column. A toluene–methanol mixture (30 : 1) was found to be the best eluent for the separation of the different $C_{60}[C(PO_3Et_2)_2]_2$ isomers.

The HPLC trace of the mixture of the $C_{60}[C(PO_3Et_2)_2]_2$ isomers displays five major, well-separated peaks. MALDI-TOF MS data of all five fractions are almost identical, containing two peaks corresponding to the molecular ion, $[M]^+$ (m/z = 1292.1) and $[M + Na]^+$ (m/z = 1315.1), confirming the formation of the $C_{60}[C(PO_3Et_2)_2]_2$ bisadducts.

In some cases, the structures of the different $C_{60}[C(PO_3Et_2)_2]_2$ isomers can be identified by their $^{31}P\{^1H\}$ and ^{13}C NMR spectra, but, in general, additional comparison of the relative polarities of the isomers with the order of elution is necessary. For this reason, the dipole moments of all eight possible geometrical isomers of $C_{60}[C(PO_3Et_2)_2]_2$ were calculated by the PM3 semi-empirical method using Hyperchem-6 software. The results are listed in Table 1.

Among the eight possible structural isomers of $C_{60}[C(PO_3Et_2)_2]_2$ (Fig. 1) there is only one containing three different types of phosphorus atoms (*e*), as well as only one isomer in which all the phosphorus atoms are equivalent (*trans-1*). In addition, the *trans-1* isomer should have a characteristic ^{13}C NMR spectrum, which should differ considerably

DOI: 10.1039/b200145d

Table 1 Calculated dipole moments of the $C_{60}[C(PO_3Et_2)_2]_2$ isomers

Isomer	Symmetry	Dipole moment/D
cis-1	C_S	3.9
cis-2	C_S	3.1
cis-3	C_2	2.6
e	C_S	2.4
trans-4	C_S	2.1
trans-3	C_2	2.0
trans-2	C_2	1.2
trans-1	D_{2h}	0.0

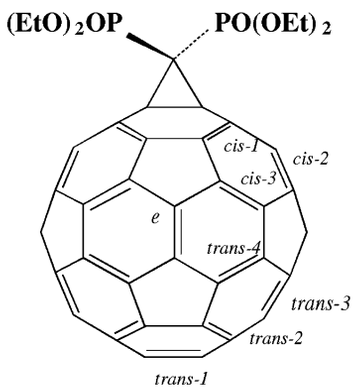


Fig. 1 Positional notation for the $C_{60}[C(PO_3Et_2)_2]_2$ bisadducts.

from the spectra of the other isomers. The D_{2h} symmetry of the substituted fullerene cage for the *trans-1* isomer produces only nine different types of carbon atoms (eight in the sp^2 region), compared to 28–30 different types of sp^2 carbon atoms for the other isomers. $^{31}P\{^1H\}$ NMR data for all five of the isolated isomers are presented in Fig. 2. The spectrum of fraction five

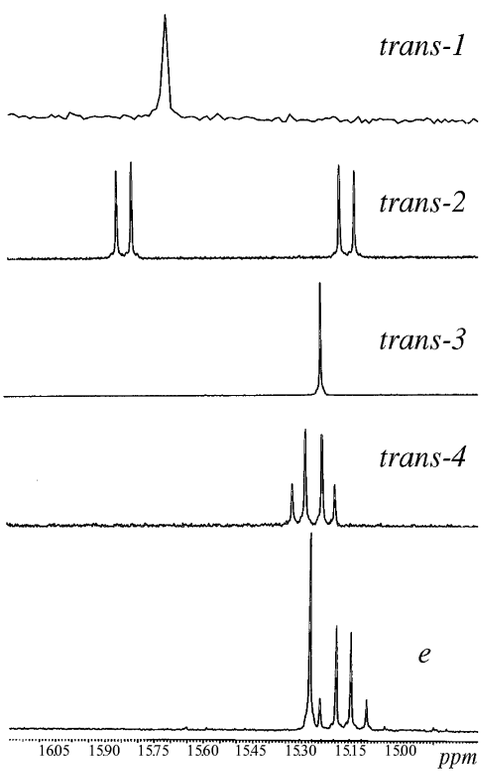


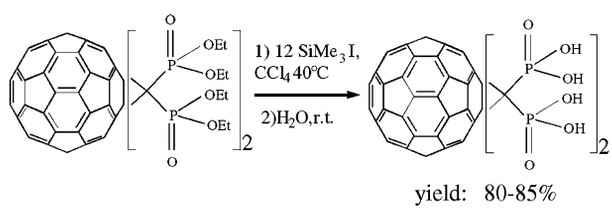
Fig. 2 The $^{31}P\{^1H\}$ NMR spectra of the isomers of $C_{60}[C(PO_3Et_2)_2]_2$, 202.4 MHz.

(Fig. 2) reveals three different types of phosphorus atoms in the ratio 2 : 1 : 1. This is the expected case of the *e*-isomer. The ^{13}C spectrum of fraction five is also in good agreement with the C_S symmetry of the *e*-isomer (Table 2). Because the last peak on the HPLC profile obtained from the normal phase column is for the *e*-$C_{60}[C(PO_3Et_2)_2]_2$, any of the *cis* isomers (which are more polar than *e*) is not expected to be among the isolated compounds.

Surprisingly, two different isomers, showing only one resonance in the $^{31}P\{^1H\}$ NMR spectrum were found (Fig. 2). As might be expected from the elution number, fraction one turned out to be *trans-1* isomer, as clearly confirmed by its carbon NMR which contains exactly eight lines in the sp^2 region. The ^{13}C NMR spectrum of fraction three exhibits 26 signals, one of which is three times more intense than the others, corresponding to the C_2 structure of the *trans-3* isomer with 28 different types of carbon atoms of equal intensities in the sp^2 region. The existence of only one signal in the $^{31}P\{^1H\}$ NMR spectrum of fraction three, instead of two doublets, as would be expected for the two non-equivalent phosphorus atoms of the diphosphonate group in the C_2 *trans-3* adduct, may be explained if it is assumed that the difference in chemical shifts of the two different phosphorus atoms is negligibly small compared to the coupling constant ($J/\Delta\nu \gg 1$) (an extreme AB case). This leads to a merging of the inner components of the two doublets and the disappearance of their outer components, resulting in only one signal. This explanation is indirectly confirmed by the non-equal intensities of the doublet components (AB case) in the $^{31}P\{^1H\}$ NMR spectra of three other isomers of $C_{60}[C(PO_3Et_2)_2]_2$ (Fig. 2).

Relative polarity-based assignment of the fractions 2 and 4 as the *trans-2* and *trans-4* isomers of $C_{60}[C(PO_3Et_2)_2]_2$ becomes evident from Table 2, in which the experimentally obtained $^{31}P\{^1H\}$ and ^{13}C NMR data are compared to expectations derived from symmetry considerations alone.

Finally, the three major isomers, *trans-2*-, *trans-3*-, and *e*-$C_{60}[C(PO_3Et_2)_2]_2$, were hydrolyzed to obtain the corresponding diphosphonic acids, as shown in Scheme 2. Completion of



Scheme 2 Synthesis of $C_{60}[C(PO_3H_2)_2]_2$.

the reactions was established by the absence of an ethyl group signal in ^1H NMR spectra of the resulting products. This mild hydrolytic method produced relatively high yields, typically in the 80–85% range. Formation of the diphosphonic acids was confirmed by the MALDI-TOF mass spectra, which contained peaks for the molecular ion, $[M]^+$ (m/z = 1068.1), and for the $[M + Na]^+$ (m/z = 1091.1) species.

The $^{31}P\{^1H\}$ NMR spectra of *trans-2*- and *e*-$C_{60}[C(PO_3H_2)_2]_2$ revealed exactly the same features as the spectra of the corresponding esters, but with a substantial upfield shift (~2 ppm) of the signals. In contrast, the $^{31}P\{^1H\}$ NMR spectrum of *trans-3*-$C_{60}[C(PO_3H_2)_2]_2$ is remarkably different from the spectrum of the parent ester. Apparently, the substitution of an ethyl group by a proton creates a difference in chemical shifts of the two non-equivalent phosphorus atoms of the *trans-3* structure that is sufficient to resolve their signals at 202.4 MHz NMR. Thus, instead of one signal, as for the ester compound, the $^{31}P\{^1H\}$ NMR spectrum of *trans-3*-$C_{60}[C(PO_3H_2)_2]_2$ displays two doublets with unequal intensities of components, as expected for the *trans-3* structure. This observation further confirms the correct assignment of the structure for the parent ester.

The diphosphonic acids obtained show moderate solubility in polar organic solvents (DMF, DMSO) and also in water. Converting to the sodium or potassium salts leads to an

Table 2 ^{13}C and ^{31}P NMR characterization of the $C_{60}[C(PO_3Et_2)_2]_2$ isomers

Fraction number (ret. time/min)	Relative yield (%)	Isomer, assigned	No. of C in C_{60}		No. of sp^2 C in C_{60}		No. of P	
			Theor.	Exp.	Theor.	Exp.	Theor.	Exp.
1 (26.8)	1.5	*trans-1*	9	9	8 (2–1; 6–2) [a]	8 (2–1; 6–2)	1	1
2 (31.2)	23.5	*trans-2*	30	30	28 (28–1)	28 (28–1)	2	2
3 (39.9)	32.0	*trans-3*	30	28	28 (28–1)	26 (25–1; 1–3)	2	1
4 (45.3)	10.5	*trans-4*	32	31	30 (4–1; 26–2)	29 (4–1; 24–2; 1–4)	2	2
5 (53.5)	32.5	*e*	32	29	29 (2–1; 27–2)	26 (2–1; 21–2; 3–4)	3	3

No.—number of signals in the spectrum. [a] *a* (*b–c*; ...) = total number of lines (number of lines–intensity; ...).

increase in water solubility. For example, the solubility of *trans-3*-$C_{60}[C(PO_3Na_2)_2]_2$ in water at room temperature is approximately 2 mg ml^{-1}, which is sufficient for *in vitro* hydroxyapatite crystal growth inhibition studies or *in vivo* testing of its effectiveness as a drug for osteoporosis and other bone disorders.

Some fullerene derivatives have been shown to form aggregates in water solutions.[11] This can affect important properties such as the biodistribution in animals. To investigate the possibility of aggregation for the present diphosphonic acid derivatives, the UV–Vis absorption spectrum of *trans-3*-$C_{60}[C(PO_3Na_2)_2]_2$ was examined over a range of concentrations from 4 to 18 μM at the two characteristic wavelengths (226.0 and 423.3 nm). No shift in position of these peak maxima with concentration was observed, thus suggesting the absence of aggregates. Furthermore, dynamic light scattering measurements on a 0.5 mM solution of $C_{60}[C(PO_3Na_2)_2]_2$ showed almost the same scattering intensity as for a filtered distilled water blank, again indicating the absence of any significant aggregation in solution.

Cell culture and animal studies using these diphosphonic acids and their sodium salts are currently in progress and will be reported subsequently.

Experimental

General

C_{60} (>99.5%) was purchased from MER Corp., Tucson, Arizona, and used without further purification. All solvents were reagent grade. Toluene was dried over sodium in the presence of dibenzophenone, CCl_4 was dried over P_2O_5. Tetraethyl bromomethylenediphosphonate was prepared according to the method of McKenna[12] from tetraethyl methylenediphosphonate, yield 76%. All reactions were performed under dry argon atmosphere.

NMR spectra were recorded on Bruker Avance 500 and Bruker Avance 400 instruments. $Si(CH_3)_4$ and 85% H_3PO_4 were used as chemical shift references for ^{13}C and $^{31}P\{^1H\}$ NMR respectively. All δ values are reported in ppm. Mass spectra were recorded on a Bruker MALDI-TOF MS. A saturated solution of sulfur in CS_2 was used as the matrix. For the dynamic light scattering experiment, a Coulter N4 Plus submicron particle sizer was used. Data were taken at a 90° detecting angle. UV–Vis absorption spectra were recorded on the Cary-4E UV–Vis spectrophotometer in the 200–900 nm range.

Syntheses

$C_{60}[C(PO_3Et_2)_2]_2$. A solution of C_{60} (360 mg, 0.5 mmol) and tetraethyl bromomethylenediphosphonate (460 mg, 1.25 mmol) in dry toluene was prepared and heated to 60 °C. A suspension of NaH (36 mg, 1.5 mmol) in toluene was then added dropwise over two hours with stirring (Scheme 1). Addition of sodium hydride caused a distinct change in color of the reaction mixture from purple to reddish-brown. After stirring for an additional hour at 60 °C, the reaction mixture was cooled, filtered and reduced to dryness under vacuum.

$C_{60}[C(PO_3H_2)_2]_2$. In a typical synthesis (Scheme 2), the diphosphonic ester (30 mg, 0.023 mmol) was treated with a 1.5 fold excess of trimethylsilyl iodide (56 mg, 0.28 mmol) in anhydrous CCl_4 at 40–50 °C for 35–40 minutes. Transformation to the diphosphonic acid was then achieved by treatment of the reaction mixture with excess of H_2O for 15–30 minutes at room temperature.

Selected spectroscopic data

***trans-1*-$C_{60}[C(PO_3Et_2)_2]_2$.** $^{31}P\{^1H\}$ NMR ($CDCl_3$, 202.4 MHz) δ = 15.79; ^{13}C NMR ($CDCl_3$, 125.7 MHz): δ = 16.73, 32.37 (t, J = 154.0 Hz), 64.05, 67.08, 140.76, 142.47, 142.81, 143.90 (t, J = 5.2 Hz), 143.99, 145.02, 145.14, 145.08.

***trans-2*-$C_{60}[C(PO_3Et_2)_2]_2$.** $^{31}P\{^1H\}$ NMR ($CDCl_3$, 202.4 MHz) AB quartet: δ_1 = 15.15, δ_2 = 15.89 (J = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 125.7 MHz): δ = 16.60†, 16.65†, 16.70†, 16.74†, 16.80†, 36.73 (t, J = 154.0 Hz), 63.89 (t, J = 7.1 Hz), 64.04 (t, J = 6.3 Hz), 67.53, 68.16, 139.64, 140.65, 140.87, 140.97, 141.47, 141.75, 141.90, 142.09, 142.35, 143.24 (t, J = 5.6 Hz), 143.40, 143.77 (t, J = 5.6 Hz), 143.81, 144.06, 144.26, 144.50, 144.72, 144.90 (t, J = 5.6 Hz), 145.09, 145.29, 145.36, 145.73, 145.76, 145.94, 146.47, 146.69, 147.00, 148.62 (t, J = 5.6 Hz).

***trans-3*-$C_{60}[C(PO_3Et_2)_2]_2$.** $^{31}P\{^1H\}$ NMR ($CDCl_3$, 202.4 MHz) δ = 15.22; ^{13}C NMR ($CDCl_3$, 125.7 MHz): δ = 16.53 (t, J = 2.9 Hz), 16.60 (t, J = 2.9 Hz), 16.62 (t, J = 2.9 Hz) 16.72 (t, J = 2.9 Hz), 39.11 (t, J = 153.8 Hz), 63.74 (t, J = 3.2 Hz), 63.85 (t, J = 2.9 Hz), 63.89 (t, J = 2.9 Hz), 63.97 (t, J = 3.2 Hz), 67.75 (t, J = 4.1 Hz), 68.19 (t, J = 4.1 Hz), 139.04, 140.76, 141.83, 141.95, 141.96, 142.29, 142.37, 142.89, 142.93, 142.10, 143.56, 144.43, 144.58, 144.72, 145.29, 145.67, 146.10 (t, J = 5.2 Hz), 146.43 (t, J = 5.2 Hz), 146.57, 146.62, 146.68 (t, J = 5.2 Hz), 146.69, 146.78, 147.02, 147.28 (t, J = 5.2 Hz), 147.30.

***trans-4*-$C_{60}[C(PO_3Et_2)_2]_2$.** $^{31}P\{^1H\}$ NMR ($CDCl_3$, 202.4 MHz) AB quartet: δ_1 = 15.23, δ_2 = 15.32 (J = 9.1 Hz); ^{13}C NMR ($CDCl_3$, 125.7 MHz): δ = 16.51†, 16.56†, 16.58†, 16.60†, 16.62†, 16.67†, 16.71†, 37.36 (t, J = 154.0 Hz), 63.73†, 63.77†, 63.83†, 63.92†, 63.97†, 67.79 (t, J = 4.1 Hz), 68.02 (t, J = 4.1 Hz), 137.49, 139.81, 140.48, 140.94, 141.24, 141.78, 142.09, 142.17 (t, J = 5.2 Hz), 142.19, 142.30, 142.41, 143.12, 143.73 (t, J = 5.2 Hz), 143.98, 144.16, 144.33, 144.86 (t, J = 5.2 Hz), 145.15, 145.29, 145.44, 145.52, 145.62, 145.70, 145.75 (J = 5.2 Hz), 146.16, 146.30, 146.78, 147.39, 148.52.

***e*-$C_{60}[C(PO_3Et_2)_2]_2$.** $^{31}P\{^1H\}$ NMR ($CDCl_3$, 202.4 MHz) singlet: δ = 15.28, AB quartet: δ_1 = 15.14, δ_2 = 15.22 (J = 9.8 Hz); ^{13}C NMR ($CDCl_3$, 125.7 MHz): δ = 16.41†, 16.46†, 16.50†,

† Due to overlapping of the different peaks, their multiplicity could not be determined.

16.52†, 16.56†, 16.59†, 39.00 (t, J = 154.0 Hz), 40.92 (t, J = 154.0 Hz), 63.67†, 63.69†, 63.71†, 63.75†, 63.80†, 63.84†, 66.43 (t, J = 4.1 Hz), 67.94 (t, J = 4.1 Hz), 68.01 (t, J = 4.1 Hz), 136.81, 140.70, 141.24, 142.45, 142.64, 142.79, 142.84 (t, J = 5.2 Hz), 142.89, 143.82, 144.15 (t, J = 5.2 Hz), 144.21, 144.33, 144.52, 144.77, 144.93, 145.18 (t, J = 5.2 Hz), 145.22, 145.44, 145.67, 145.68, 146.39, 146.45, 146.53, 146.70, 147.30, 148.46 (t, J = 5.2 Hz).

***trans-2*-$C_{60}[C(PO_3H_2)_2]_2$.** $^{31}P\{^1H\}$ NMR (DMF–D_2O, 161.9 MHz) AB quartet: δ_1 = 12.84, δ_2 = 13.40 (J = 21.8 Hz).

***trans-3*-$C_{60}[C(PO_3H_2)_2]_2$.** $^{31}P\{^1H\}$ NMR (DMF–D_2O, 161.9 MHz) AB quartet: δ_1 = 13.19, δ_2 = 13.04 (J = 18.4 Hz).

***e*-$C_{60}[C(PO_3H_2)_2]_2$.** $^{31}P\{^1H\}$ NMR (DMF–D_2O, 161.9 MHz) singlet: δ = 12.96, AB quartet: δ_1 = 12.21, δ_2 = 12.75 (J = 21.5 Hz).

***trans-3*-$C_{60}[C(PO_3Na_2)_2]_2$.** UV–Vis absorption spectrum (H_2O, 298 K): max. at 226.0 nm, 423.2 nm; shoulders around 300 and 440 nm.

Acknowledgements

This work was supported by The Robert A. Welch Foundation (C-0627) and C Sixty Inc., Toronto, Canada. The authors also wish to thank Dr Lawrence B. Alemany at Rice University for useful discussions.

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