Pergamon

Tetrahedron 57 (2001) 7331–7335

TETRAHEDRON

Organophosphorus chemistry of fullerene: synthesis and biological effects of organophosphorus compounds of C_{60}

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Received 23 December 2000; revised 30 May 2001; accepted 20 June 2001

Abstract—Synthesis of novel methanophosphonate and methanophosphonic acid C_{60} derivatives was achieved, and their cytotoxicity was studied. In particular, a bis-methanophosphonate C_{60} derivative displayed photo-induced cytotoxicity on HeLa cells with IC_{50} value of 1.30 μM.

1. Introduction

Organophosphorus chemistry is an important branch of hetero-organic chemistry, and organophosphorus compounds are widely applied in medicine or as pesticides, extraction agents in the metallurgy industry, and additives for the petroleum industry. For example, some organophosphonic acids may act as insecticides and sterilizing agents;[1] some diphosphonic acids may display favorable ostaxis[2] and anti-inflammatory and pain-easing activities.[3] Since the bulk preparation of C_{60} in 1990, many different kinds of functional groups have been covalently linked to the parent C_{60}.[4] The preparation and properties of fullerene derivatives is being intensively studied,[5–7] while some of these compounds were found to exhibit versatile biological effects.[8] However, the synthesis and biological applications of C_{60}-organophosphorus compounds have received less attention. So far, only a few papers have been published concerning the synthesis of fullerene derivatives bearing phosphorus substituents.[9–11] In a recent communication,[11] we reported the synthesis of the first diphosphonate derivative of C_{60}, tetraethyl methano[60]fullerenediphosphonate **1**. In this paper, the chemistry and biological effects of several organophosphorus compounds of fullerene are investigated, including the preparation of phosphonate derivatives of C_{60} by an improved Bingel reaction, and of water-soluble phosphonic acid derivatives of C_{60}, and the photo-induced toxic effects of the organophosphorus compounds on HeLa cells (Scheme 1).

2. Results

Diethyl ethoxycarbonylmethano[60]fullerenephosphonate **2** was prepared by a method similar to that for the synthesis of tetraethyl methano[60]fullerenediphosphonate **1** but using a different base.[11] Triethyl phosphonoacetate was allowed to react with C_{60} in the presence of I_2 and DBU in toluene at room temperature, from which, after purification by chromatography, compound **2** was isolated as a single product. A similar procedure using diethyl cyanomethylene-phosphonate and DBU as a base was employed for the preparation of the diethyl cyanomethano[60]fullerenephosphonate **3**.

The mechanism of the reaction of C_{60} with various methylenephosphonates can be compared to that of the Bingel reaction.[12] The reaction of I_2 with the phosphonate in situ produces iodo-methylenephosphonate which then generates α-iodocarbanions in the presence of base. The products are formed by the addition of the stabilized α-iodocarbanions to C_{60}, followed by an intramolecular displacement of iodide by the anionic center generated on the fullerene core.

The methanofullerene structures with C_{2V} symmetry for compound **1** and C_s symmetry for compound **2** and **3** were assigned on the basis of FT-IR, MALDI-TOF-MS, ^{1}H-, ^{31}P-, ^{13}C-NMR, and UV–Vis spectra. In the UV–Vis spectra of these three compounds, two strong peaks are observed at 282 and 329 nm which are due to the characteristic absorption of C_{60}; at 427 nm, a medium intensity peak appears which proves that they are 6,6-closed products. The ^{13}C NMR spectrum of **1** exhibits 23 signals, 19 of which are in the sp^2 hybridized fullerene region (140.3–146.1 ppm), while the similar fullerene region of **2** and **3** shows 26 and 28 signals respectively, some of which are overlapping. A comparison of the ^{31}P NMR resonance (ppm) of **1** (15.57), **2**

Keywords: fullerene; organophosphorus compounds; photo-induced; cytotoxicity.

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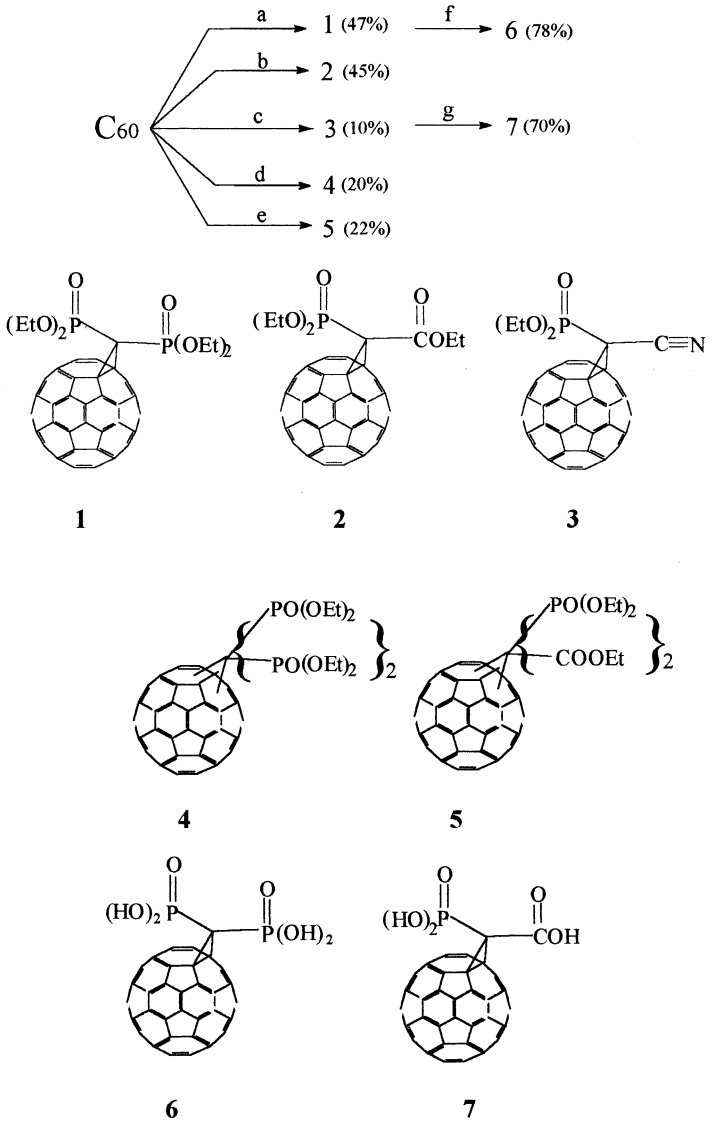
0040–4020/01/$ - see front matter
PII: S0040-4020(01)00670-6



Scheme 1. Synthesis of compounds **1–7** (a) I_2 (1 equiv.), $CH_2(PO(OEt)_2)_2$ (1 equiv.), NaH (20 equiv.), toluene, 40°C, 9 h; (b) I_2 (1 equiv.), $CH_2(PO(OEt)_2$-(COOEt) (1 equiv.), DBU (3 equiv.), toluene, rt, 4 h; (c) I_2 (1 equiv.), $NCCH_2(PO(OEt)_2$ (1 equiv.), DBU (3 equiv.), toluene, rt, 4 h; (d) I_2 (6 equiv.), $CH_2(PO(OEt)_2)_2$ (6 equiv.), NaH (120 equiv.), toluene, rt, 5 h; (e) I_2 (3 equiv.), $CH_2(PO(OEt)_2(COOEt)$ (3 equiv.), DBU (10 equiv.), toluene, rt, 5 h; (f) HCl, reflux, 3d; (g) HCl, reflux, 3d.

(13.15) and **3** (10.27) may demonstrate the shielding effect of the substituted groups.

Compared with C_{60}, the polarities of its monoadducts **1**, **2** and **3** increase remarkably and their solubilities also improve greatly. They are easily solubilized in solvents such as toluene, $CHCl_3$ and DMF. In $CHCl_3$ the solubility is more than 60 mg ml^{-1}. However, they cannot be solubilized in highly polar solvents with strong polarities such as alcohols and DMSO. From the reaction of C_{60} with excess methylenephosphonates (1:3–1:6) and the purification of the products by chromatography, two bis-adducts of phosphonate derivatives of C_{60} **4** and **5** were obtained. The solubilities of compounds **4** and **5** are very good, since they can be easily dissolved into quite different solvents, including toluene, chloroform, acetone, ethanol and DMSO. It is worth noting that, due to the difficult separation only by column chromatography, neither of **4** and **5** is a pure compound, but may be a mixture of different regioisomers of their bis-adducts.

The corresponding acid derivatives from compounds **1**, **2** and **3** were expected to be obtained by hydrolysis. However, only semi-dealkylated product was obtained when compound **1** was hydrolyzed by the mildest method of dealkylation with bromotrimethylsilane,[13] probably due to the steric hindrance of fullerene core and the two close phosphonate groups. Alternatively, the phosphonic acid derivatives **6** and **7** could be readily formed after reaction of compounds **1** and **3** at reflux in concentrated aqueous HCl under an Ar atmosphere for three days. Compounds **6** and **7** were completely insoluble in aqueous acids and moderately soluble in neutral water. Interestingly, these compounds were not soluble in aqueous NaOH or $NaCO_3$, but were soluble in an aqueous solution containing a small amount of triethylamine (1%). These results show that the sodium

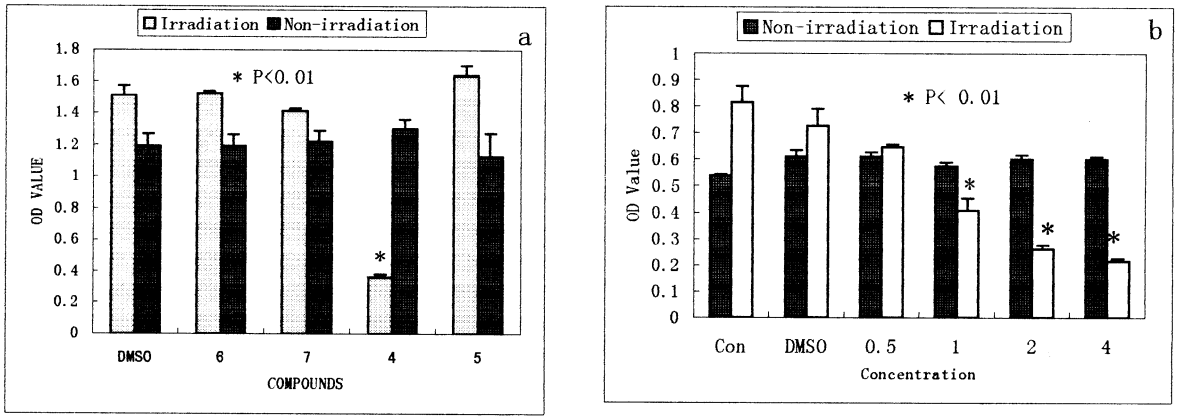


Figure 1. The cytotoxicity of compounds **4**, **5**, **6** and **7** at the concentration of 4 μM (a) and of compound **4** at various concentrations (0–4 μM) (b) in 0.1% DMSO aqueous solutions against HeLa cells.

salts of compounds **6** and **7** are not soluble in aqueous solution while their triethylammonium salts are. Compounds **6** and **7** are very soluble in DMSO as are the bis-adducts **4** and **5**.

Due to their organophosphorus function groups, compounds **4**, **5**, **6** and **7** are expected not only to display the properties of fullerene itself, but also to show some unique biological effects of the side groups. Since DMSO is easily soluble in water and does little damage to cells at low concentration, the DMSO aqueous solutions of compounds **4**, **5**, **6** and **7** have prospective applications in the field of biomedicine. The effects of the DMSO aqueous solutions of these four compounds at low concentration (4 μM) on the growth of HeLa cells in vitro were investigated by the MTT test.[14] Some significant results were obtained. Under non-irradiation, all of them had no effects on the cells; under irradiation, bisadduct **4** showed cytotoxicity, while the monoadducts **6**, **7** and the bisadduct **5** had no effect (Fig. 1a). The cytotoxicity of compound **4** at different concentrations is shown in Fig. 1b. As compared with the control (Con), 0.1% DMSO itself had no cytotoxicity on cells under both conditions, neither compound **4** at the concentrations of 0–4 μM had under non-irradiation. However, compound **4**, even at low concentration (1.0 μM), could inhibit the cell growth under irradiation ($p<0.01$, compared with the treatment of 0.1% DMSO). Therefore, the photo-induced cytotoxicity of compound **4** was dose-dependent. Furthermore, IC_{50} value of compound **4** was calculated as 1.30 μM from Fig. 1b. Observed under converted optical microscope, the shape of the cells with the compound exposed to light became round, indicating the cells were damaged severely.

3. Discussion

The results we present for the first time about the effects of the organophosphorus derivatives of C_{60} on HeLa cells are very meaningful. Many researchers have previously concluded that the photo-induced cytotoxicity of the fullerenes and their derivatives was mainly due to the reactive oxygen species (ROS) produced by them.[15–18] According to the studies of Hamano and his coworkers,[19] the ROS producing efficiency of fullerene derivatives is independent of the kinds of addends, but decreases with an increase of the addend number. So, only analyzing from the photosensitive efficiency of fullerene derivatives, the photo-induced cytotoxicity of the monoadducts **6** and **7** should be stronger than the bisadduct **4**, and that of **5** should be near to that of **4**. However, our experimental results are obviously different. The activity of **4** was found much higher than compounds **5**, **6**, and **7** (Fig. 1). Therefore it is suggested that due to the complexity of the biological system, besides of the involvement of the ROS produced by the fullerene core, the structures and the properties of the addition groups may also play an important role in the cytotoxicity of the fullerene derivatives, especially at low concentrations. The mechanism of the photo-induced cytotoxicity of these organophosphorus compounds is now under investigation in our lab.

4. Conclusion

In conclusion, several specific organophosphorus derivatives of fullerene were synthesized and their cytotoxicity tested. Among them compound **4** was toxic towards HeLa cells when photo-induced, which implies its potential application in the photodynamic therapy.

5. Exprimental

5.1. General

The IR spectra were recorded using a MAGNA FT/IR-560 (Nicolet Company, KBr pellet). The ^{1}H-, ^{31}P- and ^{13}C NMR were recorded on a DMX-300 NMR (Bruker Company) and the chemical shifts, δ, are referred to TMS (^{1}H), H_3PO_4 (^{31}P) and $CDCl_3$ (77.0 ppm, ^{13}C), respectively. MALDI-TOF-MS were measured on a EIFLEX-3 MS (Bruker Company) with α-cyano-4-hydroxyl-cinamic acid (CCA) as matrix. UV–Vis spectra were detected by a Ultraspec 2000 UV–Visible spectrophotometer (Amersham Pharmacia). For MTT assay, OD values were examined by a Model 550 microplate reader (Bio-Rad).

5.1.1. Synthesis of tetraethyl methano[60]fullerene-diphosphonate 1. Tetraethyl methano[60]fullerene-diphosphonate **1** was prepared according to the method described previously.[11]

5.1.2. Synthesis of diethyl ethoxycarbonylmethano[60]-fullerenephosphonate 2. To a stirred dry toluene solution (200 ml) containing C_{60} (250 mg, 0.35 mmol), I_2 (91 mg, 0.35 mmol) and DBU (150 μl, 1.0 mmol) was added triethyl phosphonoacetate (70 μl, 0.35 mmol). After stirring at room temperature under Ar for 4 h, the mixture was submitted to column chromatography (SiO_2). Residual C_{60} was eluted with toluene and the reaction product with 2:1 toluene/$CHCl_3$. After evaporation, the obtained solid product was washed with methanol and dried in vacuum at 60°C for 20 h to give a black solid: 150 mg, (45%). 2: FT-IR ν (cm^{-1}) (KBr):3438, 2980, 2922, 2843, 2239, 1629, 1435, 1277, 1046, 1028, 852, 742, 527. MALDI-TOF-MS (α-cyano-4-hydroxyl-cinamic acid (CCA) as matrix) *m/z* 965 (100) $[M+Na]^+$, 942 (78) $[M]^+$, 733 (10) $[M-PO(OEt)_2\cdot COOEt+1]^+$, 720 (28%) $[C_{60}]^+$. ^1H NMR (300 MHz, $CDCl_3$):δ 4.55 (q, J=7.0 Hz, 6H), 1.56 (t, J=7.0 Hz, 6H),1.47 (t, J=7.0 Hz, 3H).^{31}P NMR (300 MHz, $CDCl_3$): δ13.15. ^{13}C NMR (300 MHz, $CDCl_3$): δ 164 1, 147.6, 147.5, 145.3, 145.3, 145.1, 145.1, 145.0, 144.9, 144.8, 144.8, 144.7, 144.6, 144.6, 144.6, 144.0, 143.8, 143.2, 143.1, 143.1, 142.7, 142.2, 142.1, 142.0, 141.9, 140.8, 136.9, 70.4, 64.6, 63.4, 29.7, 16.6, 14.3. UV–Vis (toluene) λ_{max} (nm) (ϵ): 282 (126840), 331 (40680), 428 (2080).

5.1.3. Synthesis of diethyl cyanomethano[60]fullerene-phosphonate 3. Using diethyl cyanomethylenephosphonate as starting material, by a similar prepared method to above but with 1:1 toluene/$CHCl_3$ as eluant the final products were separated. The products divided two parts: the first fraction as a brown solid was the pure monoadduct **3** (10%) and the second was a mixture of bisadducts to pentaadducts. **3**: FT-IR ν (cm^{-1}) (KBr):3438, 2980, 2922, 2843, 2239, 1629, 1435, 1277, 1046, 1028, 852, 742,527. MALDI-TOF-MS (CCA as matrix) *m/z* 895 (96) $[M]^+$, 870 (35) $[M-CN+1]^+$, 758 (74) $[M-PO(OEt)_2]^+$, 720 (100%) $[C_{60}]^+$. ^1H NMR (300 MHz, $CDCl_3$): δ 4.63 (q, J=7.0 Hz, 4H), 1.65 (t, J=7.0 Hz, 6H). ^{31}P NMR (300 MHz, $CDCl_3$): δ 10.27. ^{13}C NMR (300 MHz, $CDCl_3$): δ 146.5, 146.5, 146.4, 146.3, 146.2, 145.9, 145.2, 145.9, 145.8, 145.7, 145.6, 145.5, 145.4, 145.4, 145.3, 145.0, 144.9, 144.3, 144.1, 144.0, 144.0, 143.9, 143.2, 143.1, 143.1, 142.6, 142.2, 142.0, 139.7, 114.5, 69.0, 66.4, 30.9, 17.6. UV–Vis (toluene) λ_{max} (nm) (ϵ): 283 (46750), 331 (14500), 428 (2400).

5.1.4. Synthesis of bisadduct of tetraethyl methano[60]-fullerenediphosphonate 4. To a stirred dry toluene solution (500 ml) containing C_{60} (500 mg, 0.7 mmol), I_2 (728 mg, 4.2 mmol) and NaH (3 g) was added tetraethyl methylene-diphosphonate (696 μl, 4.2 mmol). After stirring at room temperature under Ar for 5 h, the mixture was filtered off and the filtrate was submitted to column chromatography (SiO_2). The monoadduct was eluted with $CHCl_3$ and the bisadduct **4** with ethyl acetate. After evaporation, the solid product was dried in vacuum at 60°C for 20 h to give a black solid and weighed 160 mg (20%). **4**: FT-IR ν (cm^{-1}) (KBr): 2921, 2873, 1260, 1067, 1028, 527. MALDI-TOF-MS (CCA as matrix) *m/z* 1314 (96) $[M+Na-1]^+$, 1292 (100%) $[M]^+$. ^1H NMR (300 MHz, $CDCl_3$):δ 4.46 (p, J=7.0 Hz, 16H), 1.41 (t, J=7.0 Hz, 24H). ^{31}P NMR (300 MHz, $CDCl_3$): δ 16.52. ^{13}C NMR (300 MHz, $CDCl_3$): δ 147.3, 147.0, 146.7, 146.6, 145.6, 145.2, 144.7, 144.5, 144.4, 143.6, 143.0, 142.9, 142.3, 141.9, 141.8, 140.7, 139.0, 127.8, 63.9, 29.7, 16.6.

5.1.5. Synthesis of bisadduct of diethyl ethoxycarbonyl-methano[60]fullerenephosphonate 5. To a stirred dry toluene solution (200 ml) containing C_{60} (250 mg, 0.35 mmol), I_2 (285 mg, 1.05 mmol) and DBU (450 μl, 3.0 mmol) was added triethyl phosphonoacetate (210 μl, 1.05 mmol). After stirring at room temperature under an Ar for 5 h, the mixture was submitted to column chromatography (SiO_2). The minor monoadduct and major bisadduct **5** were eluted in this order with 2:1 toluene/$CHCl_3$. After evaporation, the product was dried in vacuum at 60°C for 20 h to give a black solid and weighed 90 mg (22%). **5**: FT-IR ν (cm^{-1}) (KBr): 2931, 1737, 1279, 1236, 1057, 1019, 530. MALDI-TOF-MS (CCA as matrix) *m/z* 1186 (100) $[M+Na]^+$, 1163 (73%) $[M]^+$. ^1H NMR (300 MHz, $CDCl_3$):δ 4.50 (p, J=7.0 Hz, 12H), 1.55 (t, J=7.0 Hz, 12H), 1.43 (t, J=7.0 Hz, 6H). ^{31}P NMR (300 MHz, $CDCl_3$): δ 12.64. ^{13}C NMR (300 MHz, $CDCl_3$): δ 164.0, 146.5, 146.4, 146.3, 146.2, 145.3, 144.9, 144.5, 144.2, 143.9, 143.7, 143.6, 143.2, 141.6, 64.3, 63.1, 29.5, 16.4, 14.2.

5.1.6. Synthesis of methano[60]fullerenediphosphonic acid 6. A solution of 20 mg of **1** in concentrated aqueous HCl (30 ml) was allowed to reflux for three days. The resulting mixture was centrifuged. The precipitate was washed four times with distilled water and dissolved in water containing triethylamine (1%). After centrifugation to remove insoluble side products, and the aqueous phase was acidified with concentrated aqueous HCl. The resulting precipitate was centrifuged, washed with water and finally dried in vacuum to give a brown powder **6** (14 mg, 78%): FT-IR ν (cm^{-1}) (KBr): 2921, 2873, 1260, 1067, 1028, 527. MALDI-TOF-MS (CCA as matrix, negative ion) *m/z* 893 (100%) $[M-1]^-$. ^{31}P NMR (300 MHz, ^{6}d-DMSO): δ 8.43. UV–Vis (H_2O/Et_3N, 1%) λ_{max} (nm) (ϵ): 220 (54600), 263 (48750), 329 (1850).

5.1.7. Synthesis of methano[60]fullerenediphosphonic acid 7. Compound **7** as a brown powder in 70% yield was prepared by a similar method to **6** starting from compound **3**. **7**: FT-IR ν (cm^{-1}) (KBr): 1227, 1181, 1103, 1093, 586, 574, 563, 527. MALDI-TOF-MS (CCA as matrix, negative ion) *m/z* 856 (100%) $[M-1]^-$. ^{31}P NMR (300 MHz, ^{6}d-DMSO): δ 7.21. UV–Vis (max/nm, H_2O/Et_3N): 220 (67950), 265 (57931), 330 (2200).

5.2. Cytotoxicity determination

The stock solutions of compound **4**, **5**, **6** and **7** were prepared in DMSO (4 mM). Sterile solutions for cell experiments were obtained by filtering these solutions through 0.2 μM-pored membranes.

Human cervix uteri tumor-derived HeLa Cells were cultured in an atmosphere with 5% CO_2 and at 37°C maintained in a NAPCO CO_2 incubator and in RMPI 1640 medium containing 15% heat-inactivated fetal calf serum and 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cytotoxicity determination of C_{60} derivatives was carried out with 24-well plastic culture plates. The cell

growth in culture medium without (Con) or with 0.1% of DMSO was also detected. Four repeats were undertaken for each sample.

When cells have spread well on the bottom of culture plates, C_{60} derivatives were added into the cultures. Then the cultures were irradiated with a 300 W halogen lamp setting apart from 30 cm distance over the cultures. To avoid the heat-damage to cells, the heat produced by the lamp was removed by a piece of heat shield glass. The irradiation was carried out three times, every 24 h for 0.5 h. After the third irradiation has been finished, the viable cell number was determined by the MTT assay immediately.[14] The optical density value (OD value) which represented the cell number was measured with a microplate reader in a dual wavelength way. The obtained data were statistically analyzed.

Acknowledgements

We thank the National Natural Science foundation of China for providing the financial aid (No. 59702009). We are also very grateful to Professor Jack Cousseau of Université d'Angers, France, who kindly gave great help in the improvement of this manuscript.

References

1. Kakimoto, N.; Yoshvara, T.; Akiba, M. *J. Organomet. Chem.* **1990**, *385*, 247–252.
2. Tan, W. C. *J. Chin. Nucl. Med.* **1987**, *7*, 60–64.
3. Isormura, Y.; Sakamoto, S.; Abe, T. Jpn. Kokai Tokyo Kobo JP63295595; *Chem. Abstr.* 111, 174388h.
4. Taylor, R.; Walto, D. R. M. *Nature* **1993**, *363*, 685–693.
5. Diederich, F.; Thilgen, C. *Science* **1996**, *271*, 317–323.
6. Prato, M. *J. Mater. Chem.* **1997**, *7*, 1097–1109.
7. Echegoyen, L.; Echegoyen, L. E. *Acc. Chem. Res.* **1998**, *31*, 593–601.
8. Jensen, A. W.; Wilson, S. R.; Schuster, D. I. *Bioorg. Med. Chem.* **1996**, *4*, 767–169.
9. Yamago, S.; Yanagawa, M.; Mukai, H.; Nakamura, E. *Tetrahedron* **1996**, *52*, 5091–5098.
10. Chuang, S. C.; Lee, D. D.; Santhosh, K. C.; Cheng, C. H. *J. Org. Chem.* **1999**, *64*, 8868–8872.
11. Cheng, F. Y.; Yang, X. L.; Zhu, H. S. *Tetrahedron Lett.* **2000**, *41*, 3947–3950.
12. Bingel, C. *Chem. Ber.* **1993**, *126*, 1957–1959.
13. McKenna, C. E.; Higa, M. T.; Cheung, N. H.; McKenna, M. C. *Tetrahedron Lett.* **1977**, *2*, 155–158.
14. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–60.
15. Nakajima, N.; Nishi, C.; Li, F. M.; Ikada, Y. *Fullerene Sci. Technol.* **1996**, *4*, 1–19.
16. Cheng, F. Y.; Yang, X. L.; Zhu, H. S. *Fullerene Sci. Technol.* **2000**, *8*, 113–124.
17. Satoh, M.; Matsuo, K.; Kiriya, H.; Mashino, T.; Nagano, T.; Hirobe, M.; Takayanagi, I. *Eur. J. Pharmacol.* **1997**, *327*, 175–181.
18. Richmond, R. C.; Gibson, Y. *Proc. Electrochem. Soc.* **1995**, *10*, 684–695.
19. Hamano, T.; Okuda, K.; Mashino, T.; Hirobe, M. *Chem. Commun.* **1997**, 21–22.