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Bioorganic & Medicinal Chemistry Letters 18 (2008) 99–103
Bioorganic & Medicinal Chemistry Letters

Synthesis and anti-inflammation evaluation of new C_{60} fulleropyrrolidines bearing biologically active xanthine

Sheng-Tung Huang,[a,b,*] Jian-Sheng Liao,[a] Hsu-Wei Fang[b] and Chun-Mao Lin[c]

[a]*Graduate Institute of Biotechnology, National Taipei University of Technology, Taipei, Taiwan*
[b]*Department of Chemical Engineering and Biotechnology, National Taipei University of Technology, Taipei, Taiwan*
[c]*College of Medicine, Taipei Medical University, Taipei, Taiwan*

Received 24 August 2007; revised 17 October 2007; accepted 1 November 2007
Available online 5 November 2007

Abstract—We designed and prepared the new C_{60} fullerene hybrids bearing a xanthine moiety as potential double-action anti-inflammatory agents, capable of simultaneous inhibition of LPS-induced NO and TNF-α production. The 10 μM of fulleropyrrolidine-xanthine dyad **2a** and **b** were effective in suppressing LPS-induced NO production by 55.1 ± 2.1% and 58.6 ± 2.6%, respectively, but only **2b** was also effectively in suppressing LPS-induced TNF-α production by 34.0 ± 2.7%. We believed that the agents synthesized herein would hold promise for future development of a new generation of potent anti-inflammatory agents.

Research in the field of water-soluble C_{60} fullerene derivatives has significantly increased due to the broad range of biological activity that was found for these compounds.[1] However, the low water solubility of fullerenes had always been an important issue for biological use. One of the current approaches to overcome the lack of fullerenes' solubility in aqueous media is by chemical modification of the fullerenes to incorporate the polar functionalities such as carboxylate, polyethers, polyols, and dendrons, so that they acquire solubility in polar media.[2] Recently, the preparation of novel water-soluble fullerene hybrids bearing a variety of functional moieties such as peptides,[3] oligonucleotides,[4] porphyrins,[5] flavonoids,[6] have attracted much attention. Such dyad systems could amplify or alter the biochemical characteristics of their components or even produce compounds with new biological properties.

The two pro-inflammatory cytokines, nitric oxide (NO) and tumor necrosis factor α (TNF-α) have been recognized as essential components in acute and chronic inflammatory processes.[7] The production of NO and TNF-α serves to recruit other inflammatory cells, which in turn release cytokines and subsequently amplify the immune response. The cytokine responses to cell injury are a regulated process and are usually beneficial to the host, but overexpression of these cytokines can cause serious diseases including, rheumatoid arthritis, multiple sclerosis, asthma, and psoriasis.

Current therapeutic approaches to the treatment of inflammatory diseases are centered on the suppression of the NO or TNF-α production.[8] It has been reported that suppression of oxidative stress-mediated NO elevation might be beneficial in reducing the development of inflammation.[9] The intracellular second messenger, cAMP, is also a very potent immunomodulator, exerting generally suppressive effects on the function of inflammatory and immunocompetent cells.[10] Activation of the cAMP/PKA pathway inhibits the production of pro-inflammatory cytokine TNF-α.[11] The inhibitors which inhibit the activity of cyclic adenosine monophosphate-phosphodiesterase (cAMP-PDE) are helpful in the increased intracellular concentration of cAMP and subsequent suppression of the production of pro-inflammatory cytokine TNF-α.[11]

With this in mind, we desired to design a hybrid agent which simultaneously inhibited the NO and TNF-α production during inflammation. This hybrid agent would be useful for the treatment of inflammatory diseases. We envisioned that a hybrid agent, C_{60} fulleropyrrolidine-xanthine dyads **2a** and **b**, would serve this purpose (Fig. 1). The basic chemical architecture of dyads **2a** and

Keywords: Anti-inflammation; C_{60} fulleropyrrolidine; TNF-α; Nitric oxide; Xanthine.
* Corresponding author. Tel.: +886 2 2771 2171x2525; fax: +886 2 2731 7117; e-mail: ws75624@ntut.edu.tw

0960-894X/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2007.11.004

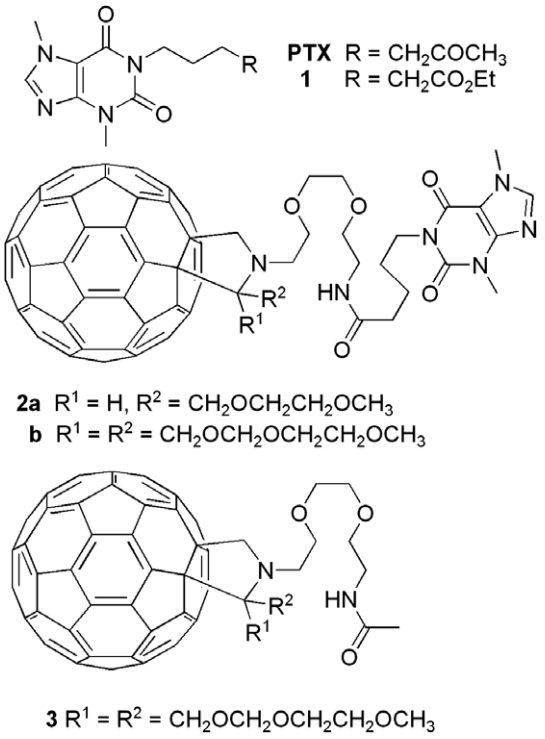


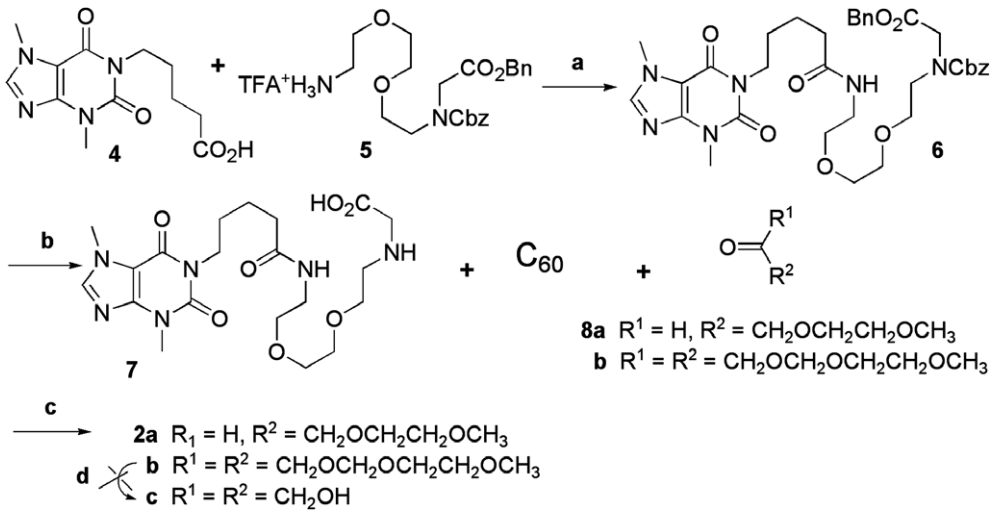
Figure 1.

b is composed of 1-pentonic acid-3,7-dimethylxanthine linked to the nitrogen of a C_{60} fulleropyrrolidine moiety through ethylene glycol chains, and an additional polar side chain was strategically positioned on the fullerene spheroid of the dyads to provide the extra hydration sites in order to enhance the solubility in the aqueous media. The peculiar structure of C_{60} fullerene which is capable of 'adding' multiple radicals per molecule serves as a 'radical sponge'.[12] It was recently shown that a number of water-soluble fullerene derivatives behaved as potent antagonists of the basal and acetylcholine-stimulated NO-mediated relaxation,[13] and the possible mechanism of these fullerene derivatives in the inhibition of NO-dependent relaxation was associated with their radical scavenging and direct NO quenching activity.[12b] Xanthine analogues, caffeine and its major metabolite, paraxanthine, suppress neutrophil and monocyte chemotaxis and also suppress production of the pro-inflammatory cytokine TNF-α from human blood.[14] Xanthine analogues, pentoxifylline (PTX), which is widely used in the treatment of cerebrovascular and peripheral vascular disease, and PTX analogue **1**[15] were known to posses anti-inflammation properties which are related to their ability to suppress the synthesis of TNF-α by macrophages through inhibition of the cAMP-PDE activity. We postulated that the fulleropyrrolidine moiety in **2** should be capable of reducing the NO released by macrophages through scavenging the excess ROS produced or by direct NO-quenching activity during inflammation. Since, Tzeng et al. had shown that water-soluble bis-malonate C_{60} derivatives did not exhibit the inhibitory effects on LPS-induced TNF-α production in microglia cells,[16] we envisaged that the xanthine moiety in **2** would act as an inhibitor of cAMP-PDE thus suppressing the production of pro-inflammatory cytokine TNF-α by macrophage. In this study, we also prepared the fulleropyrrolidine analogue **3** which lacked the xanthine moiety to evaluate its inhibitory effect on the LPS-induced NO and TNF-α production. We believed that C_{60} fulleropyrrolidine-xanthine dyads **2a** and **b** could express synergistic immunomodulatory effects during inflammation.

The synthesis of fulleropyrrolidine-xanthine dyads **2a** and **b** is outlined in Scheme 1. The synthesis began with the preparation of amide **6**. Coupling the 1-pentonic acid -3, 7-dimethylxanthine (**4**)[15] with a known amine salt **5**[17] under standard peptide coupling conditions gave the corresponding amide **6** in 77% yields. Removing two protecting groups in **6** in a palladium-catalyzed hydrogenation reaction furnished the N-alkylated glycine **7** in 91% yields. The fulleropyrrolidine moiety in **2** was constructed by the 1–3 dipolar cycloaddition reactions.[17] Condensing the N-alkylated glycine **7** with known aldehyde **8a**[17] or di-methoxyethoxymethyl (MEM) protected ketone **8b**[18] formed the corresponding azomethine ylide (not shown) which underwent the 1–3 dipolar cycloaddition reactions with C_{60} fullerene to yield the desired C_{60} fulleropyrrolidine-xanthine dyads **2a** and **b**, and the yields were 28% and 25%, respectively. The chemical structures of both dyads **2a** and **b** had been characterized by ^{1}H NMR, ^{13}C NMR, and mass.[19] Several attempts to prepare **2c** by cleaving the di-MEM protecting groups in **2b** under various conditions ($ZnBr_2$/HCl, CBr_4/IPA, HCl) were fruitless, and they gave mostly unidentifiable products. The syntheses of the C_{60} fulleropyrrolidine-xanthine dyads **2a** and **b** were completed in three steps from the known starting materials.

One of our goals in this study is to prepare the water-soluble C_{60} fullerene derivatives. Both dyads **2a** and **b** were soluble in 1% DMSO aqueous solution with a maximum concentration of 1.5×10^{-5} M, and 1.0×10^{-5} M, respectively.[20] Recently, there are several papers describing the preparation of novel fulleropyrrolidine hybrids bearing promising biological components, but their best solubility in a 10% DMSO aqueous solution was reported to be in the range of $\sim 10^{-5}$ M,[21] and this presents a challenge to the further evaluation in a mammalian cell culture model. The concentration of DMSO exceeding 1% in cell media caused instability of mammalian cells during culturing and biological evaluation. Both dyads **2a** and **b** were soluble in 1% DMSO aqueous solution, and they were suitable for evaluation with a mammalian culture model.

We utilized the murine reticulum sarcoma cell line J774A.1 with lipopolysaccharide (LPS) to simulate the type of macrophages during inflammation for biological evaluation.[22] We first analyzed the cytotoxic effects of both dyads **2a** and **b** against a J774A.1 cell culture lineage via MTT assay; and the cell viability results are shown in Supplementary data. Cytotoxicity studies after 24 h of continuous exposure to the various concentrations of dyads **2a** and **b** with or without LPS stimulation were measured with a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT



Scheme 1. Reagents and conditions: (a) EDCI, HOBT, Et_3N, DMF, rt, 16 h, 77%; (b) H_2 (60 psi), Pd/C, 18 h, 91%; (c) toluene, reflux.

as described previously.[23] The J774A.1 cells pretreated with 10 μM of either**2a** or **b** in the presence of 1 μg/ml of LPS for 24 h had a viability close to 95% with respect to the control. One interesting observation was that we observed a slight increase in the cells viability when J774A.1 incubated with 10 μM of either **2a** or **b** without LPS stimulation. Both dyads **2a** and **b** were not cytotoxic to the J774A.1 cell culture lineage.

We evaluated the influence of both dyads on the LPS-activated macrophages J774A.1 by determining the amount of NO and TNF-α released into the cell supernatants. The J774A.1 cells were pretreated with either PTX or two different concentrations of **2a** and **b** (10, and 1 μM) for 3 h, followed by stimulating them with LPS (1 μg/ml) for 24 h. The J774A.1 stimulated with LPS alone was the control. The amount of NO was determined by measuring the nitrite concentrations in the cell supernatants after 24 h treatment.[24] The concentration of TNF-α was determined by the ELISA method as described according to prior known art.[25] The results are shown in Figure 2. The macrophages J774A.1 pretreated with 10 μM of either **2a** or **b** for 24 h in the absence of LPS stimulation did not induce any NO or TNF-α production by the cells (shown in Supplementary data). All tested agents, PTX, **2a** and **b**, showed an inhibitory effect on LPS-induced NO production (Fig. 2A). The J774A.1 pretreated with 10 μM of either the PTX or the **2a** exhibited a reduction of the NO produced, and the concentrations of NO produced were 24.4 ± 0.05 μM and 16.6 ± 0.7 μM, respectively, and those were 33.9 ± 0.2% and 55.1 ± 2.1% reduction with respect to the control. In contrast, the dyad **2b** was a potent agent for reducing the NO released, and the concentrations of NO detected in the cell supernatants were 15.3 ± 1.1 μM and 19.4 ± 1.3 μM when the cells were pretreated with 10 μM and 1 μM of **2b**, respectively, and those were 58.6 ± 2.6% and 47.5 ± 2.7% reduction with respect to the control. All agents tested herein had no quenching effect on the Griess reagent at the concentrations used. On the other hand, 10 μM of either PTX or **2a** exhibited little inhibitory effect on LPS-induced TNF-α synthesis (Fig. 2B). The J774A.1 pretreated with 10 μM of either PTX or **2a** exhibited a slight reduction of the TNF-α, and the concentration of TNF-α detected in cell medium were, 28.3 ± 0.05 ng/ml and 26.7 ± 1.5 ng/ml, respectively, and those were 7.5 ± 0.6% and 12.7 ± 3.6% reduction with respect to the control. Once again, dyads **2b** exhibited an impressive effect on suppressing the TNF-α production by the LPS-induced TNF-α synthesis at either 1 or 10 μM of the pretreatments; the concentration of TNF-α detected in the cells medium were 25.8 ± 0.5 ng/ml and 20.2 ± 0.9 ng/ml, respectively, and those were 15.6 + 0.6% and 34.0 ± 2.7% reduction with respect to the control. The fulleropyrrolidine-xanthine dyad **2a** and **b** were equally effective in suppressing LPS-induced NO production. PTX and **2a** exhibited weakly inhibition effect on the LPS-induced TNF-α production, but only dyad **2b** also exhibited significant inhibition effect on LPS-induced TNF-α production by the macrophages JA774A.1.

Because dyad **2b** was the most effective agent tested herein, we were interested in evaluating the effect of dyad **2b** without the xanthine moiety on J774A.1 after the LPS stimulation. We designed and synthesized **3**, a structural analogue of **2b** which lacked the xanthine moiety. The synthesis of **3** was similar to that for dyad **2b**, and it was also completed in 3 steps from the known starting materials with overall yield of 18%.[19] We evaluated the influence of **3** on the LPS-activated J774A.1 by determining the amount of NO and TNF-α released in the cell medium, and the results are also shown in Figure 2. The 10 μM of fulleropyrrolidine **3** effectively inhibited on LPS-induced NO production by 52.1 ± 2.3%, but it had little influences on the LPS-induced TNF-α production by the macrophages. These results suggested that the C_{60} fulleropyrrolidine moiety alone was unable to inhibit the LPS-induced TNF-α production by cells in a simulated inflammation cell model.

The **2b** was more effective then PTX in inhibiting the LPS-induced NO production by the macrophage, and the chemical structure of **2b** is a combination of PTX

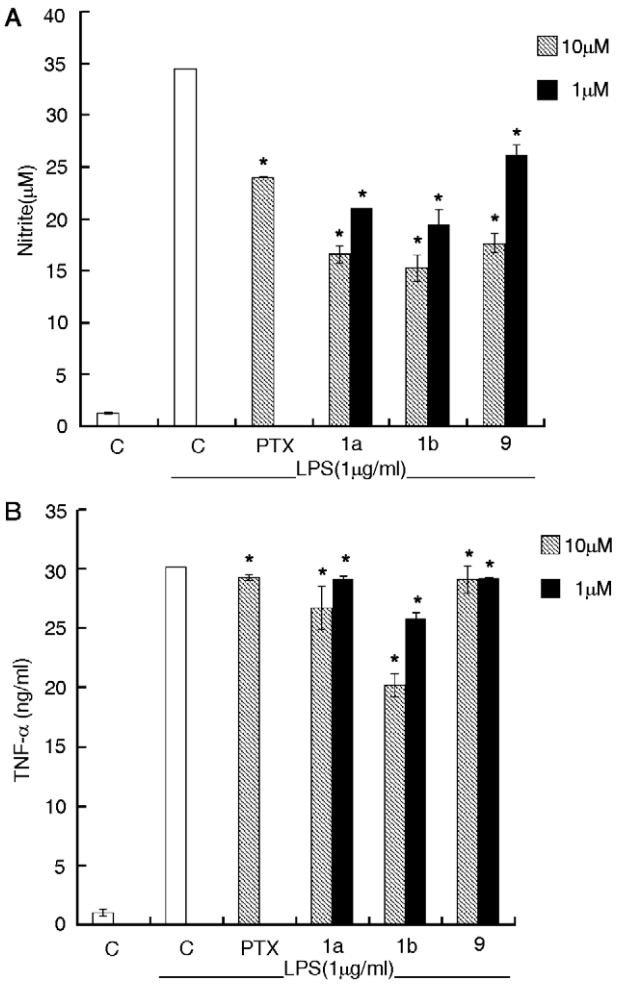


Figure 2. Inhibition of nitrite and TNF-α production by PTX, **2a**, **b**, and **3** in LPS-stimulated murin macrophage cell in J774A.1. (A) Nitrite production in J774A.1 cells were pretreated with the indicated concentration of PTX, **2a**, **b**, and **3** for 3 h. The cells were activated with LPS (1 μg/ml). Control cells were incubated with DMSO alone. Culture supernatants were collected after a 24 h activation. (B) TNF-α production in J774A.1 cells were pretreated with the indicated concentration of PTX, **2a**, **b**, and **3** for 3 h. The cells were activated with LPS (1 μg/ml). Control cells were incubated with 1% DMSO alone. Culture supernatants were collected after a 24 h activation. Error bars represent standard error of mean ($n = 3$). *Significant difference ($P < 0.01$) compared with the control treated with LPS.

and **3**, the xanthine and fulleropyrrolidine moieties in dyad **2b** could exert a synergistic inhibitory effect. The C_{60} fulleropyrrolidine moiety in **2b** could inhibit the LPS-induced NO production through scavenging the ROS produced or by direct quenching of the NO. In addition, the xanthine moiety in dyad **2b** could also play a role in suppressing the NO production by the LPS-induced macrophages. PTX possess anti-inflammatory properties which are related to cAMP-PDE inhibition. Beshay et al. had presented evidence that inhibition of cAMP-PDE could also lead to reduction of NO production in LPS-stimulated macrophages.[26] The xanthine moiety in dyad **2b** could also act by inhibiting the cAMP-PDE activities which led to the suppression of NO production by the stimulated macrophages. Further details of the molecular pharmacological studies involving dyad **2b** are underway.

In conclusion, we successfully prepared new C_{60} fulleropyrrolidines bearing biologically active xanthine, **2a** and **b**. Our preliminary biological evaluation using a cell lineage to simulate the type of macrophages present during inflammation had identified that 10 μM of dyad **2b** was the most potent agent to reduce both NO and TNF-α released into cells medium. Furthermore, integration of a xanthine onto a C_{60} fulleropyrrolidine exhibited an inhibitory effect on LPS-induced TNF-α production. The present study has demonstrated that a C_{60} fulleropyrrolidine hybrid bearing xanthine could be a potent agent to inhibit LPS-induced NO and TNF-α production. We believed that the agents synthesized herein would hold promise for future development of a new generation of potent anti-inflammatory agents.

Acknowledgment

This work was supported by Nation Science Council (NSC 94-2113-M-027-004).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.11.004.

References and notes

1. (a) Tagmatarchis, N.; Shinohara, H. *Mini-Rev. Med. Chem.* **2001**, *1*, 339; (b) Sagman, U. *Perspect. Fullerene Nanotech.* **2002**, 145; (c) Bosi, S.; Da Ros, T.; Spalluto, G.; Prato, M. *Eur. J. Med. Chem.* **2003**, *38*, 913; (d) Satoh, M.; Takayanagi, I. *J. Pharmacol. Sci.* **2006**, *100*, 513.
2. (a) Sayes, C. M.; Fortner, J. D.; Guo, W.; Lyon, D.; Boyd, A. M.; Ausman, K. D.; Tao, Y. J.; Sitharaman, B.; Wilson, L. J.; Hughes, J. B.; West, J. L.; Colvin, V. L. *Nano Lett.* **2004**, *4*, 1881; (b) Dugan, L. L.; Lovett, E.; Cuddihy, S.; Ma, B.-W.; Lin, T.-S.; Choi, D. W. *Fullerene Chem. Phys. Technol.* **2000**, 467; (c) Song, T.; Goh, S. H.; Lee, S. Y. *Macromolecules* **2002**, *35*, 4133; (d) Gutierrez-Nava, M.; Accorsi, G. p; Masson, P.; Armaroli, N.; Nierengarten, J.-F. *Chem. Eur. J.* **2004**, *10*, 5076; (e) Bosi, S.; Da Ros, T.; Spalluto, G.; Prato, M. *Eur. J. Med. Chem.* **2003**, *38*, 913; (f) Yang, J.; Barron, A. R. *Chem. Commun.* **2004**, 2884.
3. (a) Sofou, P.; Elemes, Y.; Panou-Pomonis, E.; Stavrakoudis, A.; Tsikaris, V.; Sakarellos, C.; Sakarellos-Daitsiotis, M.; Maggini, M.; Formaggio, F.; Toniolo, C. *Tetrahedron* **2004**, *60*, 2823; (b) Rouse, J. G.; Yang, J.; Barron, A. R.; Monteiro-Riviere, N. A. *Toxicol. In Vitro* **2006**, *20*, 1313; (c) Yang, J.; Alemany, L. B.; Driver, J.; Hartgerink, J. D.; Barron, A. R. *Chem. Eur. J.* **2007**, *13*, 2530.
4. Da Ros, T.; Bergamin, M.; Vazquez, E.; Spalluto, G.; Baiti, B.; Moro, S.; Boutorine, A.; Prato, M. *Eur. J. Org. Chem.* **2002**, *3*, 405.
5. (a) Imahori, H.; Fukuzumi, S. *Adv. Funct. Mater.* **2004**, *14*, 525; (b) Guldi, D. M. *Pure Appl. Chem.* **2003**, *75*, 1069; (c) Diederich, F. *Chimia* **2001**, *55*, 821.

6. (a) de la Torre, M. D. L.; Rodrigues, A. G. P.; Tome, A. C.; Silva, A. M. S.; Cavaleiro, J. A. S. *Tetrahedron* **2004**, *60*, 3581; (b) de la Torre, M. D. L.; Marcorin, G. L.; Pirri, G.; Tome, A. C.; Silva, A. M. S.; Cavaleiro, J. A. S. *Tetrahed. Lett.* **2002**, *43*, 1689; (c) de la Torre, M. D. L.; Tome, A. C.; Silva, A. M.; Cavaleiro, J. A. S. *Tetrahedron Lett.* **2002**, *43*, 4617.
7. (a) Jialal, I.; Devaraj, S.; Venugopal, S. K. *Free Radical Res.* **2002**, *36*, 1331; (b) Suryaprasad, A. G.; Prindiville, T. *Autoimmunity Rev.* **2003**, *2*, 346.
8. Kulkarni, R. G.; Achaiah, G.; Sastry, G. N. *Curr. Pharm. Des.* **2006**, *12*, 2437.
9. Komatsu, W.; Ishihara, K.; Murata, M.; Saito, H.; Shinohara, K. *Free Radical Biol. Med.* **2003**, *34*, 1006.
10. Kammer, G. M. *Immunol. Today* **1988**, *9*, 222.
11. Odingo, J. O. *Expert Opin. Ther. Patents* **2005**, *15*, 773.
12. (a) Krusic, P. J.; Wassermann, E.; Keizer, P. N.; Morton, J. R.; Preston, K. F. *Science* **1991**, *254*, 1183; (b) Yamakoshi, Y.; Umexawa, N.; Ryu, A.; Arakane, K.; Miyata, N.; Goda, Y. *J. Am. Chem. Soc.* **2003**, *125*, 12803; (c) Gharbi, N.; Pressac, M.; Hadchouel, M.; Szwarc, H.; Wilson, S. R.; Moussa, F. *Nano Lett.* **2005**, *5*, 2578.
13. (a) Satoh, M.; Matsuo, K.; Takanashi, Y.; Takayanagi, I. *Gen. Pharmacol.* **1995**, *26*, 1533; (b) Satoh, M.; Matsuo, K.; Kiriya, H.; Mashino, T.; Nagano, T.; Hirobe, M. *Eur. J. Pharmacol.* **1997**, *327*, 175; (c) Satoh, M.; Mashino, T.; Nagano, T.; Hirobe, M.; Takayanagi, I. *Fullerene Sci. Technol.* **2001**, *9*, 141.
14. Horrigan, L. A.; Kelly, J. P.; Connor, T. J. *Pharm. & Ther.* **2006**, *11*, 877.
15. Cottam, H. B.; Shih, H.; Tehrani, L. R.; Wasson, D. B.; Carson, D. A. *J. Med. Chem.* **1996**, *39*, 2.
16. Tzeng, S.-F.; Lee, J.-L.; Kuo, J.-S.; Yang, C.-S.; Murugan, P.; Tai, L. A.; Hwang, K. C. *Brain Res.* **2002**, *940*, 61.
17. Kordatos, K.; Da Ros, T.; Bosi, S.; Vázquez, E.; Bergamin, M.; Cusan, C.; Pellarini, F.; Véronique, T.; Benedetta, B.; Davide, P.; Georgakilas, V.; Giampiero, S.; Prato, M. *J. Org. Chem.* **2001**, *66*, 4915.
18. Wolleb, H.; Pfander, H. *Helv. Chim. Acta.* **1986**, *69*, 1505.
19. (a) The detail spectra descriptions for all of synthesized compounds are listed in Supplementary data. (b) The synthetic scheme of **3** is also provided in Supplementary data.
20. The UV–vis spctra of **2a**, **b** and **3** in 1% DMSO aqueous sloutions showed that the main absorption of the fullerene core was still evident, although, in analogy to other fullerene derivatives dissolved in polar solvents, the bands are broader and less structured, especially in the low-energy region (Supplementary data). Da Ros, T.; Prato, M. *J. Org. Chem.* **1996**, *61*, 9070.
21. (a) Suarez, M.; Verdcia, Y.; Illescas, B.; Martinez-Alvarez, R.; Alvarez, A.; Ochoa, E.; Seoane, C.; Kayali, N.; Martin, N. *Tetrahedron* **2003**, *59*, 9179; (b) Illescas, B.; Martinez-Grau, M. A.; Torres, M. L.; Fernandez-Gadea, J.; Martin, N. *Tetrahedron Lett.* **2002**, *43*, 4133; (c) Illescas, B. M.; Martinez-Alvarez, R.; Fernandez-Gadea, J.; Martin, N. *Tetrahedron* **2003**, *59*, 6569.
22. Ralph, P.; Prichard, J.; Cohn, M. *J. Immunol.* **1975**, *114*, 898.
23. Huang, S. T.; Tsai, H. D.; Kuo, H. S.; Yang, Y. P.; Peng, Y. C.; Lin, Y. L. *Chem. Bio. Chem.* **2004**, *5*, 797.
24. Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S. *Anal. Biochem.* **1982**, *126*, 131.
25. Cho, J. Y.; Park, J.; Yoo, E. S.; Yoshikawa, K.; Baik, K. U.; Lee, J. *Arch. Pharm. Res.* **1998**, *21*, 12.
26. Beshay, E.; Croze, F.; Prud'homme, G. *Clin. Immunol.* **2001**, *98*, 272.