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Production of reactive oxygen species induced by a new [60]fullerene derivative bearing a tetrazole unit and its possible biological applications

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ABSTRACT

The wide range of physical and chemical properties of modified fullerenes has drawn increasing attention in the past few years. As part of this research, this paper describes the preparation, characterization, and photophysical properties of a new fullerene derivative chemically modified with a tetrazole. The photophysical properties were studied by EPR radical spin-trapping technique and showed that reactive oxygen species (ROS) can be produced through UVA photosensitization. EPR spin-trapping experiments with singlet oxygen ($^1O_2$) and superoxide ($O_2^{\bullet -}$) inhibitors (β-carotene and superoxide dismutase, respectively) revealed also that: (i) the main ROS produced is $^1O_2$ and (ii) $^1O_2$ is being partially dismutated to $O_2^{\bullet -}$. The results suggest that this derivative can be used in biological applications, as for example, in topic photodynamic therapy (PDT) as a photosensitizer.

1. Introduction

Since 1990, when Krätschmer et al. [1] made C_{60} available in macroscopic quantities, the unusual properties of this fascinating allotropic carbon form and of several of its chemically modified derivatives have been intensively investigated [2–9]. Fullerenes have received considerable attention particularly due to their interesting photophysical and photochemical properties [10], which have been exploited in many biological fields [11,12]. For example, a potential biological application of fullerene derivatives is related to the easy photoexcitation of C_{60} by either UV or visible light [13]. The resulting excited-state $^1C_{60}$ is readily converted to the long-lived $^3C_{60}$ via intersystem crossing. In the presence of molecular oxygen, fullerene may decay from its triplet to the ground state, transferring its energy to O_2 and generating reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and superoxide ($O_2^{\bullet -}$), known to be a highly cytotoxic species [14]. The ability of fullerenes to catalyze the production of singlet oxygen is invaluable in the destruction of cellular targets, particularly of nucleic acids and cell membranes. Therefore, fullerenes constitute an excellent photosensitizer to be used in photodynamic therapy (PDT) of tumors or infections [15].

The interest of our research group in the synthesis of fullerenes modified with tetrazoles is due to the metabolic stability of these heterocycles and their several applications as drugs, peptide chelating agents, carboxylic surrogates and others [16,17]. The physicochemical properties of associated fullerenes and tetrazoles have been little explored and they may provide interesting and biologically active compounds. The functionalization of C_{60} with tetrazoles is unknown, existing only one report in the literature on vinylmethyltetrazole-acrylate copolymers composites with fullerene C_{60} [18]. This paper describes the synthesis and characterization of the first fullerene derivative linked with a tetrazole. EPR studies show its ability to produce reactive oxygen species.

2. Experimental

2.1. Materials

Reagents and solvents were purchased as reagent grade and used without further purification. THF was distilled over sodium benzophenone. Dry acetone was prepared after agitation with potassium carbonate for 24 h at room temperature, and then distilled. 1-Phenyl-1*H*-tetrazole-5-thiol was obtained from Sigma–Aldrich. C_{60} (99.5%) was obtained from M.E.R. Corporation. Melting points were determined on a Mettler FP80HT apparatus and are uncorrected. NMR spectra were recorded on Bruker Avance DRX-200 or DRX-400 spectrometers. Chemical shifts are

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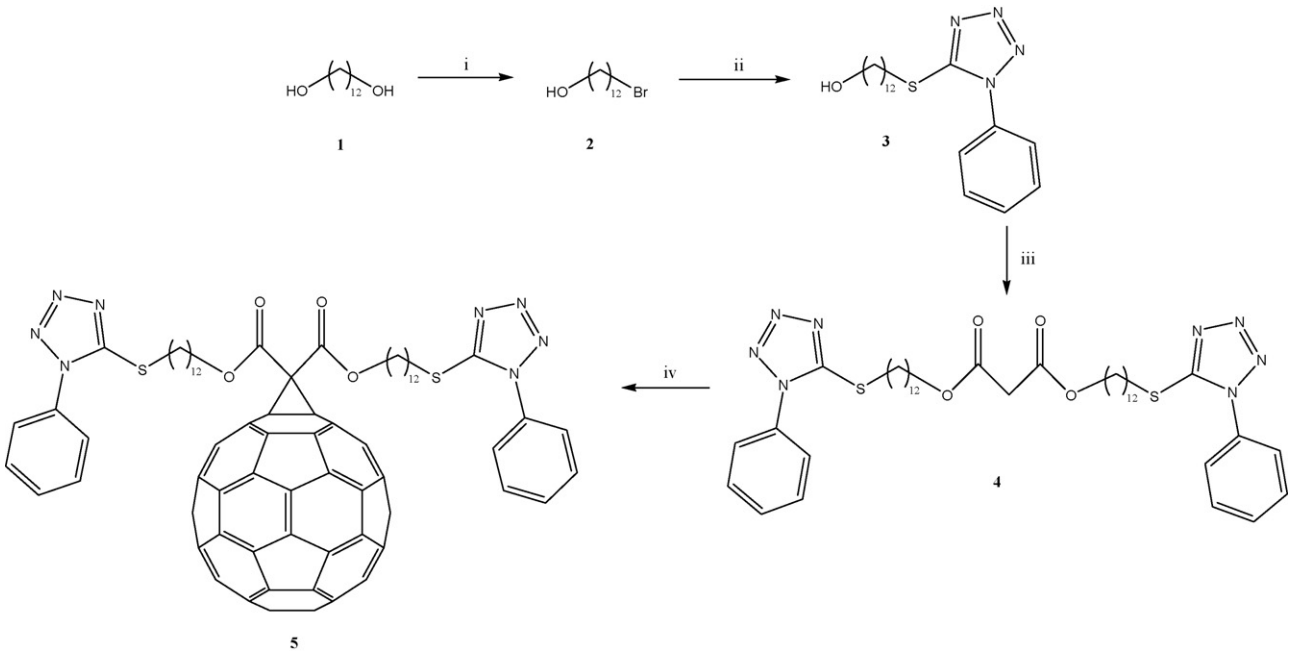

Scheme 1. Preparation of compound **5**. Reagents and conditions: (i) HBr (48%), *n*-Bu_4NBr, MW, 4 min (51%); (ii) 1-phenyl-1*H*-tetrazole-5-thiol, K_2CO_3, acetone, MW, 5 min (99%); (iii) malonyl dichloride, pyridine, THF, room temperature, 5 h (70%) and (iv) C_{60}, I_2, DBU, toluene, room temperature, 8 h (38%).

reported in δ units downfield from TMS and *J* values are given in Hz. FAB-mass spectrum (*m*/*z*, % relative intensity) was taken on a *ZA HF* instrument with 4-nitrobenzyl alcohol as matrix. Column chromatography was performed with silica gel 60, 70–230 mesh (Merck). The experiments were performed using a commercial microwave oven (Panasonic Junior Smart NNS53BH) specially modified for organic synthesis [19].

2.2. Synthesis and characterization of new fullerene derivative

The strategy employed for the preparation of the functionalized methanofullerene derivative **5** is based upon Bingel type chemistry [20,21] (Scheme 1). Malonate **4** was prepared in three steps from diol **1**. The commercially available compound **1** was converted to its bromo derivative **2** with 51% yield by a previously reported procedure [22]. Alkylation of commercial 1-phenyl-1*H*-tetrazole-5-thiol with alkyl bromide **2** in dried acetone and anhydrous K_2CO_3, under microwave irradiation [23] for 5 min produced tetrazole derivative **3** in quantitative yield after purification by silica chromatography. The product structure was clearly confirmed by ^{13}C NMR. The literature reports the ^{13}C NMR chemical shifts of C-tetrazolic of 1-phenyl-1*H*-tetrazole-5-thiol around 154 ppm [24]. Esterification of commercial malonyl dichloride with **3** in pyridine and THF at room temperature gave malonate **4** with 70% yield. The reaction of C_{60} with **4**, I_2, and DBU in toluene at room temperature afforded mono-adduct **5** with 38% yield after purification by column chromatography. The structure and purity of **5** was confirmed by ^1H and ^{13}C NMR spectroscopy and FAB-mass spectroscopy. Compounds **3**–**5** obtained in this work are unpublished.

2.2.1. 12-(1-Phenyl-1H-tetrazole-5-ylsulfanyl)dodecane-1-ol (*3*)

A mixture of 1-phenyl-1*H*-tetrazole-5-thiol (0.10 g, 0.56 mmol), anhydrous potassium carbonate (0.20 g, 1.40 mmol), 12-bromododecane-1-ol (**2**) (0.23 g, 0.84 mmol) and dried acetone (10 mL) was irradiated with microwaves for 5 min. After cooling, the solvent was removed under reduced pressure. The residue was diluted with CH_2Cl_2 and washed with water. The organic phase was dried over anhydrous Na_2SO_4 and filtered. Then the residue was concentrated and purified by column chromatography (20% EtOAc in hexane) to give product **3** in quantitative yield as white solid.

mp: 62.9–64.0 °C; ^1H NMR (200 MHz, $CDCl_3$): δ ppm 1.20–1.45 (m, 16H, CH_2), 1.45–1.60 (m, 2H, CH_2CH_2OH), 1.79 (qn, 2H, J = 7.0 Hz, CH_2CH_2S), 2.07 (s, 1H, O*H*), 3.39 (t, 2H, J = 7.0 Hz, CH_2S), 3.60 (t, 2H, J = 6.4 Hz, CH_2OH), 7.54–7.57 (m, 5H, C_6H_5). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 28.69, 28.83, 29.08, 29.14, 29.50, 29.58, 29.64, 32.84 ($10CH_2$), 33.44 (CH_2S), 62.99 (CH_2OH), 123.91, 129.86, 130.17 ($5C_6H_5$), 133.80 (C-*ipso*), 154.64 (C-tetrazole). $C_{19}H_{30}N_4OS$ (362 g mol^{-1}).

2.2.2. Bis[12-(1-phenyl-1H-tetrazole-5-ylsulfanyl)dodecyl] malonate (*4*)

Malonyl dichloride (80 μL, 0.82 mmol) diluted in anhydrous THF (2 mL) was added dropwise to an ice cold solution of alcohol **3** (0.63 g, 1.74 mmol) and anhydrous pyridine (141 μL, 1.74 mmol) in anhydrous THF (15 mL) under nitrogen atmosphere. The ice bath was removed and the reaction mixture was stirred at room temperature for 5 h. The solvent was removed under reduced pressure and the residue diluted with CH_2Cl_2 and washed with water. The organic phase was dried over anhydrous Na_2SO_4 and filtered. The crude mixture was concentrated and purified by column chromatography (15% EtOAc in hexane). Malonate **4** was obtained as a transparent oil (0.44 g, 70% yield).

^1H NMR (200 MHz, $CDCl_3$): δ ppm 1.20–1.45 (m, 32H, CH_2), 1.50–1.70 (m, 4H, CH_2CH_2O), 1.82 (qn, 4H, J = 7.4 Hz, CH_2CH_2S), 3.37 (s, 2H, $COCH_2CO$), 3.39 (t, 4H, J = 7.4 Hz, CH_2S), 4.13 (t, 4H, J = 6.8 Hz, CH_2O), 7.54–7.65 (m, 10H, C_6H_5). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 28.76, 29.14, 29.21, 29.31, 29.53, 29.61 ($20CH_2$), 33.48 ($2CH_2S$), 41.82 ($COCH_2CO$), 65.77 ($2CH_2O$), 123.95, 129.89, 130.19 ($10C_6H_5$), 133.87 (2C-*ipso*), 154.73 (2C-tetrazole), 166.82 (2C=O). $C_{41}H_{60}N_8O_4S_2$ (792 g mol^{-1}).

2.2.3. Bis[12-(1-phenyl-1H-tetrazole-5-ylsulfanyl)dodecyl] 1,2-methano[60]fullerene-61,61-dicarboxylate (*5*)

To a solution of C_{60} (0.36 g, 0.50 mmol) in toluene (400 mL) were added iodine (0.19 g, 0.75 mmol), malonate **4** (0.40 g, 0.50 mmol)

and DBU (170 μL, 1.1 mmol). The reaction mixture was stirred for 8 h at room temperature, under nitrogen atmosphere, and then filtered. The solvent was removed under reduced pressure and the residue purified by column chromatography, eluting first with toluene (to remove unreacted C_{60}). Pure mono-adduct was isolated eluting with 5% EtOAc in toluene to provide 0.27 g of **5** as a solid past black, 38% yield (55% based on recovered C_{60}).

^{1}H NMR (200 MHz, $CDCl_3$): δ ppm 1.27–1.42 (m, 32H, CH_2), 1.78–1.84 (m, 8H, CH_2), 3.30 (t, 4H, J = 7.2 Hz, CH_2S), 4.41 (t, 4H, J = 6.4 Hz, CH_2O), 7.40–7.60 (m, 10H, C_6H_5). ^{13}C NMR (50 MHz, $CDCl_3$): δ ppm 26.11, 28.71, 28.76, 28.80, 29.21, 29.31, 29.57, 29.69 (20CH_2), 33.50 (2CH_2S), 52.58 (methano bridge), 67.58 (2CH_2O), 71.79 (2C_{60}-sp^3), 123.89, 129.89, 130.17 (10C_6H_5), 133.85 (2C-*ipso*), 139.09, 141.02, 141.99, 142.27, 143.06, 143.09, 143.15, 143.95, 144.68, 144.75, 144.94, 145.27, 145.32, 145.49 (58C-fullerene), 154.58 (2C-tetrazole), 163.75 (2C=O). MS calcd for $C_{101}H_{58}N_8O_4S_2$: 1510, found (FAB$^+$) m/z: 1511 (M+H)$^+$.

2.3. Sample preparation

The samples were toluene solutions with compound **5** (1 mM) and the spin-trap PBN (α-phenyl-*N*-*tert*-butyl nitrone) with concentration of 300 mM. A control sample with PBN in the same concentration was also measured. For comparison of efficiency of the ROS produced, a solution of C_{60} (1 mM) and PBN (300 mM) was also prepared. To study the nature of the ROS produced by illumination, β-carotene or superoxide dismutase (SOD) enzyme was added to the PBN/compound **5** solution. While the former is well known as a singlet oxygen inhibitor [25,26], the latter is an enzyme known to induce a noticeable reduction in EPR intensities of superoxide spin-adducts due to the competitive reaction of SOD with $O_2^{\bullet-}$ [27]. The concentrations of β-carotene in PBN/compound **5** solution were 0.1 mM and 0.2 mM, whereas in the experiment with SOD the concentration was 2.1 mg/mL.

A fixed volume of 100 μL of the above solutions was prepared under ambient laboratory light and placed inside 2 mm i.d. EPR quartz tubes (Wilmad). *In situ* illumination was done by a UVA solid state LASER (Power Technology) with wavelength of 375 nm and 16 mW output power. The LASER beam was directed to the sample through the open top of the quartz tube placed inside the EPR cavity. The EPR measurements were made as a function of time at room temperature and with a microwave power of about 2 mW immediately after illumination.

2.4. EPR methodology

The photogeneration of reactive oxygen species assisted by the compound **5** was studied by electron paramagnetic resonance (EPR) using the spin-trapping technique. The EPR measurements were carried out with a custom-built X-band spectrometer (9.38 GHz). A commercial cylindrical resonant cavity (Bruker) was used. The microwave source was a 500 mW Klystron (Varian). Magnetic fields were produced by an electromagnet (Varian) with a fully automated current source (Heizinger). Standard lock-in detection (EG&G) and field modulation of 100 kHz were also employed. The microwave frequency was stabilized by means of an automatic frequency control and measured with precision by a frequency-meter (PTS). The magnetic field was calibrated with a diphenylpicrylhydrazyl (DPPH) standard.

To determine the absolute concentration of the photo-generated PBN spin-adducts as a function of time, their hyperfine triplet spectrum due to the ^{14}N (100% natural abundance and I = 1) was doubly integrated and the area was compared to a calibration curve using free-radical TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) solutions, with toluene as solvent, and measured under the same conditions. This calibration followed the protocol given in [28].

3. Results and discussion

3.1. EPR studies

When the solution of PBN/compound **5** was illuminated with the 375 nm LASER (16 mW), the EPR spectrum was composed of a ^{14}N hyperfine triplet due to the nitrone group of the PBN spin-adduct with $g = 2.0063 \pm 0.0001$ and $A_{hf} = 1.53 \pm 0.01$ mT. Before illumination, the signal observed for the same triplet was very weak, probably due to the preparation of the solution under laboratory light. Since we were interested in the signals generated by the LASER illumination, this weak signal was not taken into account in the analysis and was subtracted from the time-dependent LASER-induced EPR signal. In addition, the LASER-induced EPR signals of the control PBN solution, probably caused by its slow degradation under UVA light were also subtracted from the spectra of the solution PBN/compound **5**.

Under illumination and in the presence of oxygen, a photosensitizer may undergo two types of reaction mechanisms to produce ROS. The first, known as type I process, involves charge transfer from the photosensitizer to the oxygen molecule, generating either superoxide ($O_2^{\bullet-}$) or hydroxyl radicals ($^{\bullet}$OH). One typical example of type I mechanism is the production of superoxide radicals by electron transfer from photoexcited electron–hole pairs in TiO_2 nanoparticles to oxygen dissolved in TiO_2 suspension [25]. In the second energy transfer mechanism, known as type II, the photoexcitation of C_{60} fullerene and its functionalized derivatives produces singlet oxygen ($^1O_2$) by energy transfer from the lowest triplet state of $^3C_{60}$ to the oxygen molecule, whose ground state also is a triplet ($^3O_2$) [29]. Whether the ROS production mechanism is of type I or type II, or in other words, the primary ROS produced are oxygen radicals ($O_2^{\bullet-}$ or $^{\bullet}$OH) or singlet oxygen ($^1O_2$) is the main question that arises when new photosensitizers are synthesized and their photophysical and photochemical properties are investigated.

Fig. 1 shows the triplet of the spectrum of PBN spin-adducts generated in the PBN (300 mM)/compound **5** (1 mM) toluene solution,

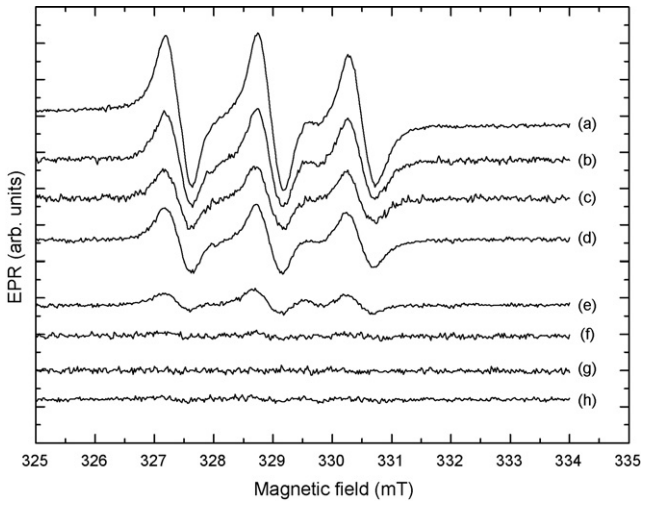

Fig. 1. EPR spectra of the photo-generated PBN spin-adducts in the presence of compound **5**: (a)–(d) after 2 h 36 min of illumination, and (e)–(h) after 150 s. The ROS inhibitors used were β-carotene (inhibitor of $^1O_2$) 0.1 mM ((b) and (f)) and 0.2 mM ((c) and (g)) as well as 2.1 mg/mL of SOD (scavenger of $O_2^{\bullet-}$) ((d) and (h)). The EPR parameters were frequency of 9.38 GHz, microwave power of 2 mW and room temperature (300 K).

for two different illumination times. Curves (a)–(d) were measured after 2 h 36 min of illumination, when all the signal intensities were saturated, i.e. did not change further in time. Curves (e)–(h) were measured in the initial stage of the illumination (150 s). Curves (a) and (e) were measured without the inhibitors (β-carotene or SOD). Curves (b) and (f) were for a solution with 0.1 mM of β-carotene, while (c) and (g) were for a solution with a concentration of β-carotene of 0.2 mM. The solution with SOD (2.1 mg/mL) yielded the spectra (d) and (h).

In Fig. 1 one can see that the maximum EPR signals were obtained without inhibitors (curves (a) and (e)), and that under experimental error and illumination time of 150 s, the triplet signal is nearly suppressed due to the presence of both β-carotene and SOD (curves (f)–(h)). For the long illumination times (curves (b)–(d)), the suppression was not 100%, being about 40% and 60% for the solutions with β-carotene (0.1 mM and 0.2 mM, respectively), and circa of 60% for the solution with SOD, measured by the absolute intensity of the central EPR line. The reduction of PBN spin-adducts with SOD indicates the production of superoxide radicals in the solution under LASER illumination. There are two possibilities for the appearance of $O_2^{\bullet -}$. The first is the direct formation by the type I mechanism that involves a charge transfer from the photosensitizer to the molecular oxygen; the second, assuming that compound **5** undergoes mechanism type II yielding singlet oxygen, as pure fullerenes, is by means of a later conversion of $^1O_2$ into $O_2^{\bullet -}$. Since the PBN spin-adducts formed, are due to $O_2^{\bullet -}$, and since the PBN is not a $^1O_2$ sensitive spin-trap, the suppression of $^1O_2$ produced by mechanism type II, in the presence of β-carotene, shows that it has being generated in high quantities and was partially converted into $O_2^{\bullet -}$ by the capture of an electron, yielding PBN spin-adducts. This is consistent with earlier observations of $^1O_2$ to $O_2^{\bullet -}$ conversions in the presence of electron donors [30]. The direct formation of $O_2^{\bullet -}$ by electron transfer (mechanism type I) cannot be ruled out, however the enhancement of the β-carotene effect by doubling its concentration, indicates that the energy transfer process (mechanism type II) followed by the formation of singlet oxygen is the dominant and primary mechanism, which is totally consistent with what is known for pure fullerene [29,31].

We can therefore conclude that for short illumination times all $O_2^{\bullet -}$ produced are being consumed by SOD or your formation inhibited by β-carotene, whereas for long illumination times, the concentrations of β-carotene and SOD may be insufficient to suppress the high concentrations of singlet oxygen and superoxide.

Fig. 2 shows the time evolution of the PBN spin-adduct absolute concentration calculated after calibration of the EPR intensities of PBN/compound **5** with TEMPOL concentration standards. The error bars represent the mean error in the estimation of the intensity. The concentration of PBN spin-adducts increases monotonically and saturates for illumination times over 2 h. The data were fitted to a single curve with equation $N = N_s[1 - \exp(-\alpha t)]$. The saturation concentration was $N_S = (3.6 \pm 0.5) \times 10^{15}$ spins/mL, with rate constant $\alpha = (4.9 \pm 0.5) \times 10^{-4}\ s^{-1}$.

From the experimental results above, one can conclude that compound **5** catalyzes the production of ROS (initially $^1O_2$, and later $O_2^{\bullet -}$ by its dismutation) under UVA illumination. The appearance of the characteristic hf triplet in the spectrum evidences that the $O_2^{\bullet -}$ produced are trapped by PBN molecules and yield PBN spin-adducts. The time evolution of the EPR spectra under illumination shows that this process is relatively slow, with a rate constant of about $\alpha = (4.9 \pm 0.5) \times 10^{-4}\ s^{-1}$. Since there is no other transient recorded in the experiment time scales, the trapping of ROS by PBN is the major photochemical reaction in compound **5** samples. The PBN spin-adduct concentration at saturation calculated after fitting the time evolution of the EPR spectra is about $(3.6 \pm 0.5) \times 10^{15}$ spins/mL for 100 μL of 1 mM compound **5** solution. Comparatively, the number of PBN spin-adducts produced in the C_{60} solution at 2 h 36 min of illumination in the same measurement conditions was about $(2.7 \pm 0.5) \times 10^{15}$ spins/mL for 100 μL (see Fig. 3). The rate constant for the formation of PBN spin-adducts in the presence of C_{60} was $\alpha = (5.4 \pm 0.5) \times 10^{-4}\ s^{-1}$. Both the concentration of PBN spin-adducts and the rate constants for compound **5** and pure C_{60} are, within experimental error similar. Since pure C_{60} has a maximum theoretical yield for the production of $^1O_2$, our results show that compound **5** is also a very efficient photosensitizer and, in addition to that, the functionalization of the C_{60} carbonic cage by the tetrazole groups in compound **5** did not change significantly its photophysical properties.

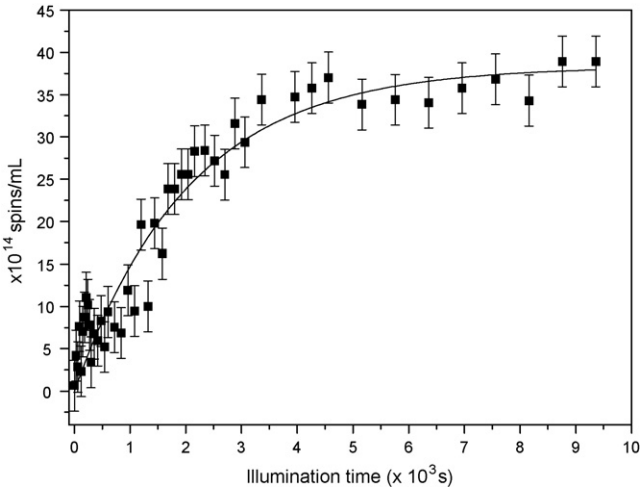

Fig. 2. Time evolution of the PBN spin-adduct concentration, formed by compound **5**, under LASER illumination. The absolute spin concentration was calculated from the EPR triplet intensities and compared with a calibration curve done with TEMPOL under the same measurement conditions (see Ref. [28] for details of the calibration).

Table 1 summarizes the EPR experiment results of compound **5** and C_{60} with PBN in toluene. The comparison of the time evolution curves of compound **5** and C_{60} shows that both present saturation behavior at long irradiation times. The adduct concentrations

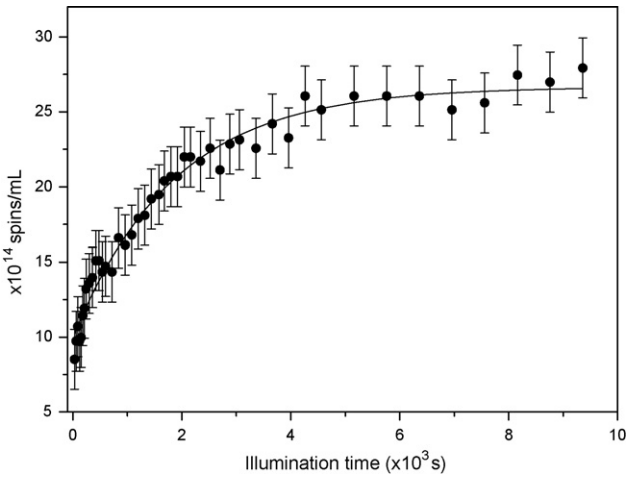

Fig. 3. Time evolution of the PBN spin-adduct concentration, formed by C_{60}, under LASER (16 mW and 375 nm) illumination. The measurements conditions were kept the same as from the experiment with the PBN/compound **5** sample (9.38 GHz, 2 mW microwave power and 300 K). The absolute spin concentration was also calculated from the EPR after calibration with TEMPOL. The full line is the best fit to a single exponential saturation curve used in Fig. 2 (see parameters in the text). The EPR hf triplet of the adduct produced by the C_{60} are the same as for compound **5** (see Table 1).

Table 1
Comparison of the experimental data for C_{60} and compound **5**

Compound	Spins produced at saturation/mL ($\pm 0.5 \times 10^{15}$)	Rate constant α in s^{-1} ($\pm 0.5 \times 10^{-4}$)	A_{hf} in mT (± 0.01)	g-Factor (± 0.0001)	Mechanism and primary ROS
Derivative **5**	3.6×10^{15}	4.9×10^{-4}	1.54	2.0060	(1) Type II/$^1O_2$ followed by conversion into $O_2^{\bullet -}$ (2) Type I mechanism ($O_2^{\bullet -}$) may not be ruled out
C_{60}	2.7×10^{15}	5.4×10^{-4}	1.54	2.0060	Type II/$^1O_2$

are also similar, with compound **5** producing, within experimental error about the same amount of adducts than pure C_{60}. While for compound **5**, the experiments with SOD demonstrate the suppression of the formation of PBN spin-adducts, indicating that the superoxide is formed by photoexcitation, it is clear that the formation of $^1O_2$ is the dominant process and that it is converted into $O_2^{\bullet -}$ that induces the formation of PBN spin-adducts. Despite the fact that for the pure C_{60} fullerene, it is well known that the quantum yield for the formation of singlet oxygen ($^1O_2$) is very high [29,31], the final amount of superoxide produced from the dismutation of singlet oxygen is similar for compound **5** and for C_{60}. This may be an evidence that this conversion is more efficient for compound **5** because of its tetrazole groups is a more efficient electron donor than the pure fullerene.

4. Conclusions

We have shown in this paper that a new fullerene modified with tetrazole may be prepared and fully characterized. We have also demonstrated by EPR spin-trapping method that this derivative presents potentially useful properties (photosensitization), i.e. superoxide radicals are generated from the photoexcited fullerene derivative under UVA light after the conversion of singlet oxygen produced by mechanism type II. The photochemical properties of the fullerene derivative are similar to pure fullerene and point to potentially promising biological applications.

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