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RESEARCH ARTICLE

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Synthesis of fulleropyrrolidine dicarboxylic acid and its scavenging activity to superoxide radicals ($O_2^{\bullet-}$)

Abstract Photochemical reaction of C60 with iminodiacetic ester ($NH(CH_2COOR)_2$, R=Me, Et, *t*-Bu) produced fulleropyrrolidine derivatives **2** in a 55%–36% yield (based on consumed C60). The reaction activity correlated with the steric hindrance of R groups. The order of the reaction rates decreased from Me to *t*-Bu (R=Me＞Et＞*t*-Bu). Fulleropyrrolidine derivative **2** (R=Me) were hydrolyzed with NaH and methanol in toluene, and then acidified with HCl to result in the corresponding fulleropyrrolidine dicarboxylic acid **3** in a 65% yield (relative to fulleropyrrolidine derivative **2**). C60 derivatives **2** and **3** were structurally characterized by ^{1}H NMR, ^{13}C NMR, IR, and elementary analysis, MS. A chemiluminescence technique was applied to study the effects of their scavenging superoxide radicals ($O_2^{\bullet-}$) generated by pyrogallol autoxidation. The results show fulleropyrrolidine dicarboxylic acid **3** had scavenging activity and the efficiencies were dependent on their concentrations. At the concentration of 3.0×10^{-4} mol·L^{-1}, fulleropyrrolidine dicarboxylic acid **3** showed a radical scavenging efficiency of approximately 70%. Finally, the influence of structure factor on the scavenging activity was discussed. The results show that the monoadduct of C60, owing to keeping almost intact double bands in C60 moiety, had obvious scavenging activity for superoxide radicals ($O_2^{\bullet-}$).

Keywords photochemical reaction, fulleropyrrolidine derivative, chemiluminescence, superoxide radicals ($O_2^{\bullet-}$)

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The investigation of the modern free radicals biology and medical sciences has suggested that the reactive oxygen species (ROS) in biological system were associated with the pathogenesis of a number of diseases, and it is believed to be an especially prominent contributor initiating these diseases [1]. Among various active oxygen species, superoxide radical ($O_2^{\bullet-}$) is especially the important kind, which could produce other active oxygen species [2]. Hence, it is of very important significance to look for antioxidants, which can efficiently eliminate superoxide radical ($O_2^{\bullet-}$). C60 and its derivatives, owing to its electronic structure as an electron-poor polyolefine，readily react with various organic radicals, and then exhibit excellent free radical scavenger properties for the absorption of active oxygen radicals. Many biomedical experiments demonstrated that C60 and its derivatives have an excellent efficiency in eliminating active oxygen radicals generated in a biological system, which result in their biological activity, including neuroprotective properties [3], antiapoptotic activity [4], and radical scavenging activity [5].

Many reports have been devoted to the interesting subject of the elimination of C60 and its derivatives for active oxygen radicals ($O_2^{\bullet-}$, ·OH) in a chemical system. Chiang and Zhu have studied scavenging effects of fullerenol for superoxide radical ($O_2^{\bullet-}$) and hydroxyl radicals (·OH) using chemiluminescence, ESR spectroscopy, and spin-trapping technique, respectively [6, 7]. Cheng and Okuda also described the scavenging activity of malonic acid C60 for superoxide radical ($O_2^{\bullet-}$) and hydroxyl radicals (·OH) [8, 9]. Recently, Sheng reported that *β*-alanine C60 adducts have an excellent efficiency in eliminating superoxide radicals [10]. All these investigations suggest that C60 derivatives possess a scavenging activity for active oxygen radicals ($O_2^{\bullet-}$, ·OH) with excellent efficiency that is equal to some natural antioxidants. Here we report the synthesis of the fulleropyrrolidine carboxylic acid **3** by a photochemical reaction between C60 and iminodiacetic ester ($NH(CH_2COOR)_2$) and hydrolysis reaction. We also wish to examine the scavenging ability of the fulleropyrrolidine derivative for superoxide

radical ($O_2^{\bullet-}$).

1 Experimental

1.1 Reagents and instruments

IR spectra were obtained from Spectrum One Perkin Elmer spectrometer in pellets with KBr. Chemiluminescence were detected with RF-546 spectrometer. ^1H and ^{13}C NMR spectra were recorded on Varian INOVA 600NMR instrument in $CDCl_3$ or D_2O (600 and 150 MHz, respectively). Elemental analyses were performed using an MOD1106 instrument.

C_{60} of purity 99% was prepared at the Institute of Fullerenes of Wu Han University. Luminol (BR) was purchased from Luo-Na Chemical Engineering Company (Nan Jing, China). Pyrogallol was from Ke-Long Chemical Reagent Factory (Cheng Du, China). Iminodiacetic acid (AR) was from Guang-Fu Institute of Fine Chemical Engineering (Tian Jin, China). All other chemicals (NaH, $SOCl_2$, NaOH, methanol, and toluene) were of reagent grade, and used on AR or CR.

1.2 Preparation of iminodiacetic ester $NH(CH_2COOR)_2$

Iminodiacetic ester $NH(CH_2COOR)_2$ were prepared according to the procedure given in Ref. [11]. Dimethyl iminodiacetate was obtained as a colorless liquid (77%). b.p. ~127°C /4.399×10^3 Pa (Ref. [11]: 126°C /4.399×10^3 Pa).

1. 3 Preparation of fulleropyrrolidine Derivatives **2**

Fulleropyrrolidine derivatives **2** were synthesized according to reported procedure [12]. The synthetic routes are presented in Scheme 1.

The crude products were purified using chromatography on silica gel using toluene as eluent to produce unconverted C60 (1st fraction) and then derivatives **2** (2nd fraction) as a brown solid (55%). ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 3.91 (*S*, 3.54H, CH_3), 5.53 (*S*, 1H, *trans*-methine proton), 6.00 (*S*, 0.19H, *cis*-methine proton);^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm):170.20(COO), 153.2–135.50 (fullerene sp^2 carbon), 77.23 (fullerene sp^3 carbon), 73.80 (HC-N), 53.01 (OMe); FT-IR (KBr), ν/ cm^{-1}:3288, 2970, 1755, 1450, 1435, 1272, 1170, 1085, 850, 760, 580; Anal. calcd for $C_{66}H_9NO_4$: C 90.10, H 1.02, N 1.59; found C 90.22, H 0.92, N 1.52.

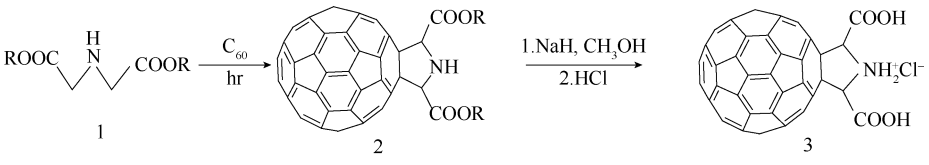
1.4 Preparation of fulleropyrrolidine Dicarboxylic Acid **3**

Fulleropyrrolidine dicarboxylic acid **3** were synthesized as described by Lamparth and Hirsch [14]. Briefly, fulleropyrrolidine derivative **2** (R=Me) (100 mg) was dissolved in 120 mL of dry toluene. A 60-fold molar excess of NaH (165 mg) was added to the solution above. The reaction mixture was stirred at 70°C under N_2 for 12 h. After the heating source was removed, methanol (3 mL), followed by 2 mol/L HCl (80 mL), was added dropwise. The resulting brown precipitate was collected by centrifugation. The brown powder was washed with 2 mol/L HCl, H_2O, acetone, then toluene and finally dried under a vacuum at 70°C for 12 h. (65%). ^1H NMR (600 MHz, D_2O) δ (ppm): 5.70 (*S*, 1*H*, *trans* -methine proton), 6.16 (*S*, 0.18*H*, *cis*-methine proton); ^{13}C NMR (150 MHz, D_2O) δ(ppm):171.03(COO), 154.10–135.60 (fullerene sp^2 carbon), 77.56 (fullerene sp^3 carbon), 73.94 (HC-N); FT-IR (KBr), σ/cm^{-1}: 3252, 2965, 2848, 1750, 1625, 1460, 1425, 1252, 1187, 1105, 850, 740, 550; Anal. calcd for $C_{64}H_6O_4NCl$: C 86.54; H 0.68; N 1.58 ; found C 86.22, H 0.71, N 1.62.

1.5 Generation and determination of superoxide radicals ($O_2^{\bullet-}$)

The ability of fulleropyrrolidine dicarboxylic acid **3** in scavenging superoxide radical ($O_2^{\bullet-}$) was determined according to a modification of the literature procedure [13] and is briefly outlined below. In the chemiluminescenct glass tube, 0.1 mL of solution of fulleropyrrolidine dicarboxylic acid **3** and 0.8 mL of luminal (0.3 mmol/L) were added. After mixing, the photo emission level of this solution was measured as the dark average of background intensity. 0.1 mL of pyrogallol (0.625 mmol/L) was then added and the chemiluminescenct emission from the resulting mixture was measured. The area of the chemiluminescenct curve recorded within 200 seconds indicated the relative quantity of superoxide radical ($O_2^{\bullet-}$) in the system of pyrogallol-luminol. In the control group, 0.1 mL of fulleropyrrolidine dicarboxylic acid **3** were replaced by the second distilled water.

Fulleropyrrolidine dicarboxylic acid **3** was dissolved in a buffer solution (0.05 mol/L Na_2CO_3-$NaHCO_3$) and prepared as a series of solutions with different concentrations. Luminol was prepared as 0.3 mmol/L stock solution in 0.05 mol/L Na_2CO_3-$NaHCO_3$ buffer solution. Pyrogallol was prepared as 0.625 mmol/L stock solution in 1 mmol/L HCl solution.



Scheme 1 R=Me, Et, t-Bu

2 Results and discussion

2.1 Photochemical reactions of C60 with iminodiacetic ester and hydrolysis of fulleropyrrolidine derivatives **2**

Gan et al. [12] have reported photochemical reactions between C60 and glycine ester (NH_2CH_2COOR, R=Me, Et, CH_2Ph), and described that the steric effect of R groups has an influence on the photochemical reaction rate, the order of which matches their steric hindrance increasing from methyl to benzyl. Photochemical reactions between C60 and iminodiacetic ester ($NH(CH_2COOR)_2$) were also studied, and it was found that the reaction of iminodiacetic ester $NH(CH_2COOR)_2$ is faster and the yield is higher. In our experiment, iminodiacetic ester $NH(CH_2COOR)_2$ was selected to react with C60. The effect of different R groups in iminodiacetic ester ($NH(CH_2COOR)_2$) on the reaction rate was investigated. The results showed that photochemical reactions of iminodiacetic ester ($NH(CH_2COOR)_2$) have the same regulation as in the case of the glycine esters, and the yields of photochemical reactions were 55% (50 min) for the methyl ester, 47% (70 min) for the ethyl ester and 36% (160 min) for the *t*-butyl ester, respectively.

For C60 derivatives containing ester groups, transforming their ester groups into corresponding carboxyl groups through hydrolyzation to increase their polarity is a useful approach in solubilizing these derivatives in water. Hirsch et al. [14] had succeed in hydrolyzing malonic ester C60 derivatives ($C_{60}(C(COOEt)_2)_3$) into malonic acid C60 derivatives ($C_{60}(C(COOH)_2)_3$) using NaH and methanol. Through Hirsch's method, fulleropyrrolidine derivatives **2** was converted into the corresponding fulleropyrrolidine dicarboxylic acid **3** in the presence of NaH and methanol. The carboxylic acid **3** is very soluble in dimethyl sulfoxide, and moderately soluble in basic aqueous solution.

2.2 Scavenging activity of fulleropyrrolidine derivative **3** against active oxygen radicals

Under basic conditions, pyrogallol was rapidly auto-oxidized to yield a series of intermediate products, and superoxide radicals ($O_2^{\bullet}$) was simultaneously released. Luminol as a chemiluminescenct probe was able to enhance the detectability of superoxide radicals ($O_2^{\bullet}$). In this procedure, luminol reacted with superoxide radicals ($O_2^{\bullet}$), and was converted to its analogous form in an electronically excited state, which relaxed back to its ground state resulting in chemiluminescence. C60 derivative in the chemical systems, owing to its antoxidant activity of scavenging superoxide radicals ($O_2^{\bullet}$), could restrain the chemiluminescence. The eliminating efficiency of C60 derivative on superoxide radicals ($O_2^{\bullet}$) could be determined from the equation below:

$$S=(I_0-I_1)/I_1\times 100\%$$

Where I_0 and I_1 are chemiluminescent integral values before and after fulleropyrrolidine dicarboxylic acid **3** was added, respectively.

Scavenging efficiency on superoxide radicals ($O_2^{\bullet}$), obtained in the presence of a variable concentration of fulleropyrrolidine dicarboxylic acid **3** is depicted in Fig. 1. These data display that fulleropyrrolidine dicarboxylic acid **3** had the ability of eliminating superoxide radicals ($O_2^{\bullet}$) generated in the system of pyrogallol–luminol. The relative quantity of superoxide radical ($O_2^{\bullet}$) in the system decreased with increasing concentration of fulleropyrrolidine dicarboxylic acid **3** fom 0.05 to 0.30 mmol L^{-1}. At the highest concentration of 0.30 mmol L^{-1}, its eliminating efficiency reached 70%.

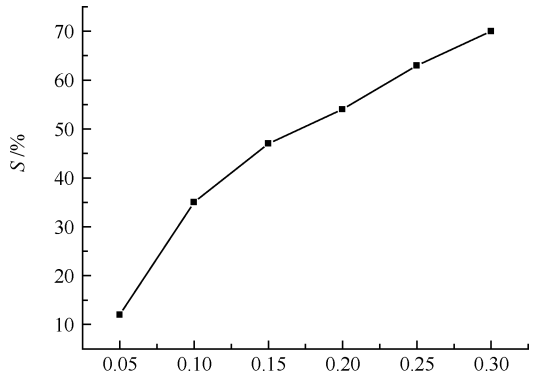


Fig. 1 The dependence of scavenging efficiency on fulleropyrrolidine dicarboxylic acid concentration

So far as fulleropyrrolidine dicarboxylic acid 3 was concerned, the side carboxyl groups had no scavenging activity against radicals. Hence, the scavenging action of fulleropyrrolidine dicarboxylic acid **3** could be due to the addition of radicals to the double bonds of C60 moiety. Obviously, monoadducts of C60 should possess better scavenging activity against radicals, because, their conjugated bonds existed in the C60 ball is almost kept intact and the steric hindrance of addends existed in the C60 ball to attack superoxide radical ($O_2^{\bullet}$) was the least. On the other hand, monoadducts of C60, compared to polyadducts of C60, have worse solubility in water, and more easily aggregates in polar solvent. These are adverse factors in eliminating active oxygen radicals.

In summary, our experiments have studied the scavenging activity of fulleropyrrolidine derivative **3** against active oxygen radicals. The results provided basic information and data for further investigation of its antioxidant activity in the cell and sub-cell level. In view of favorable biological compatibility of amino acids, through various methods of C60 functionalizations established in the fields of fullerene chemistry, the introduction of amino acids into C60 ball to synthesize C60 derivatives with biological compatibility and water solubility could be expected. This may be a worthy approach in the application of C60 in biological and medical fields.

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