Photoinduced Biochemical Activity of Fullerene Carboxylic Acid

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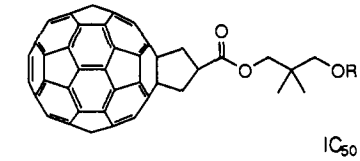
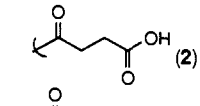
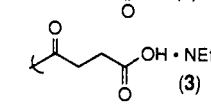
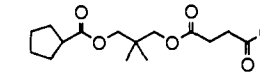
Recent intensive studies on the chemistry of buckminsterfullerene have revealed the diverse reactivity of this molecule,[1,2] and some of these transformations have provided access to functionalized fullerenes with useful applications.[3,4] The wealth of such chemical information notwithstanding, there has been a conspicuous lack of data on the biological activity of C_{60},[5] perhaps because of its insolubility in aqueous solution. C_{60}, which has an intense color, may be expected to display rich photochemistry in both the UV and visible light regions.[6] Indeed, light irradiation effectively excites the molecule to the triplet state,[7] which, in turn, can convert molecular oxygen to singlet oxygen with a quantum yield of nearly unity.[8] Here we report the preparation of water-miscible fullerene carboxylic acid **2** and its biological activity–cytotoxicity and G-selective DNA cleaving ability. What is truly remarkable is that the biological activity of C_{60} was observed only under irradiation with visible light and not in the dark, suggesting that fullerenes may serve as useful photosensitive biochemical probes.

We have previously shown that the cycloaddition reaction between C_{60} and a trimethylenemethane biradical species[9] produces fullerene alcohol **1** in ca. 30% yield.[4] Although this alcohol is much more soluble in organic solvents than C_{60} itself, it is still only sparingly soluble in polar solvents. 4-(Dimethylamino)pyridine-catalyzed esterification of **1** with succinic anhydride in a toluene/methylene chloride mixture (0.2 mL/mg) gave the fullerene carboxylic acid **2** in 62% yield (Table I). This compound is a brown solid, and its UV–visible spectrum (supplementary material) is similar to the UV–visible spectra of C_{60} and the starting alcohol **1**.[4] Unlike the parent C_{60}, the acid **2** and its triethylamine salt **3** are soluble in polar solvents and form emulsions[10] in aqueous dimethyl sulfoxide, and thus are amenable to biological investigation.

Table I. Cytotoxicity against the HeLa S3 Cell Line[a]



R	IC_{50} (μM) dark	light
H (1)	>100	>100
 (2)	>100	6.3
 (3)	>100	6.6
 (4)	>100	>100
mitomycin C	0.06	0.06

[a] The *in vitro* cytotoxicity against HeLa S3 cells was evaluated by the study of the inhibition of growth according to the reported method (ref 9). To examine the effect of light, incubation was carried out with or without light irradiation (with two 6-W fluorescent lights at a 3–5-cm distance, total twice, each for 1 h in every 24 h during a 72-h period of incubation at 37 °C).

The biological activity of these fullerene derivatives was investigated first in a whole cell system. The *in vitro* cytotoxicity against the HeLa S3 cell line was evaluated by the study of the inhibition of growth rate (Table I).[11] When compound **2** was incubated at 37 °C for 72 h in the dark, no measurable activity was observed. However, when this experiment was repeated with 6-W fluorescent light irradiation (total twice, every 24 h for 1 h each time), distinctive inhibition of the inhibition by this simple compound approaches 1% of that of the potent cytotoxic agent mitomycin C.[12] The triethylamine salt **3** showed a parallel response to light. Alcohol **1**, on the other hand, did not show any activity either in the dark or under irradiation, probably because of its insolubility in aqueous media. A reference compound lacking the fullerene moiety (**4**) was also totally inactive. These results clearly indicate that photoactivated C_{60} strongly interacts with living cells either directly or indirectly through some chemical mediator.

Encouraged by the results with the whole cell system, we next examined a more specific target, DNA. Supercoiled pBR322 DNA was incubated with **3** in the dark and under visible light irradiation. As in the cytotoxicity studies, **3** was photosensitive, showing the DNA-cleaving activity only under photoirradiation and not in the dark. Thus, incubation under light irradiation converted the covalently closed supercoiled (form I) DNA into nicked circular (form II) and linear duplex (form III) DNAs (Figure 1, lane 4), whereas incubation without irradiation caused no cleavage (lane 3). The photoinduced action of **3** was more pronounced in D_2O (results not shown), in which singlet oxygen has a longer lifetime.[13] Under the same experimental conditions,

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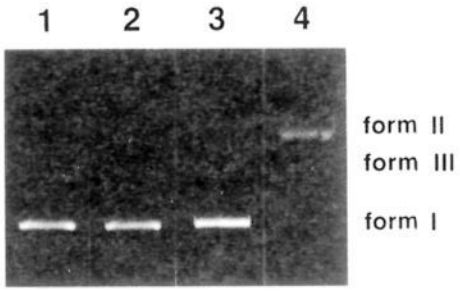

Figure 1. Agarose gel electrophoretic patterns of DNA nicking by compound **3**. Lanes 3 and 4 show DNA cleavage by **3** (100 μM) in the absence or presence of visible irradiation, respectively. Lane 1 shows intact DNA, and lane 2 presents DNA irradiated in the absence of **3**. The reaction samples contained 100 μM **3**, 0.4 μg of pBR322 plasmid DNA, 20 mM Tris-HCl buffer (pH 7.5), and 20% THF. After irradiation at a distance of 10 cm by a commercial 300-W photoreflector lamp (Toshiba, Tokyo; color temperature of 3150 K) at 20 °C. Electrophoresis was performed by using 1% agarose gel containing ethidium bromide (0.5 $\mu g/mL$).

carboxylic acid **2** showed lower DNA-cutting ability. The alcohol **1** and reference compound **4** were found to be unable to cleave DNA.

Next, the site specificity of DNA cleavage by **3** was examined with the 3′-end-^{32}P-labeled 182-base-pair DNA fragment. Of special interest is that compound **3** induced cleavage at guanine bases[14] with considerable selectivity (Figure 2, lane 4). Base treatment after incubation led to more extensive cleavage (results not shown). The above observations suggest that the single-strand breaks were generated by singlet oxygen[15] which could be produced by interaction of the photoexcited C_{60} group with molecular oxygen.[8]

The foregoing experiments establish that fullerene carboxylic acid **2** and its amine salt **3** exhibit distinct biochemical activity under the influence of low-energy light.[16] In view of the sequence specificity of the DNA cleavage as well as the activity observed for two vastly different probes, singlet oxygen is the most likely agent with the observed photoinduced biochemical activity. Further studies are obviously needed,[17] however, to substantiate this attractive hypothesis. In summary, we have found, for the first time, that even low-energy visible light is sufficient to induce biological activity in fullerene derivatives. Among the numerous implications of the present findings, the most exciting prospect includes the use of fullerene derivatives[18] for photodynamic therapy.

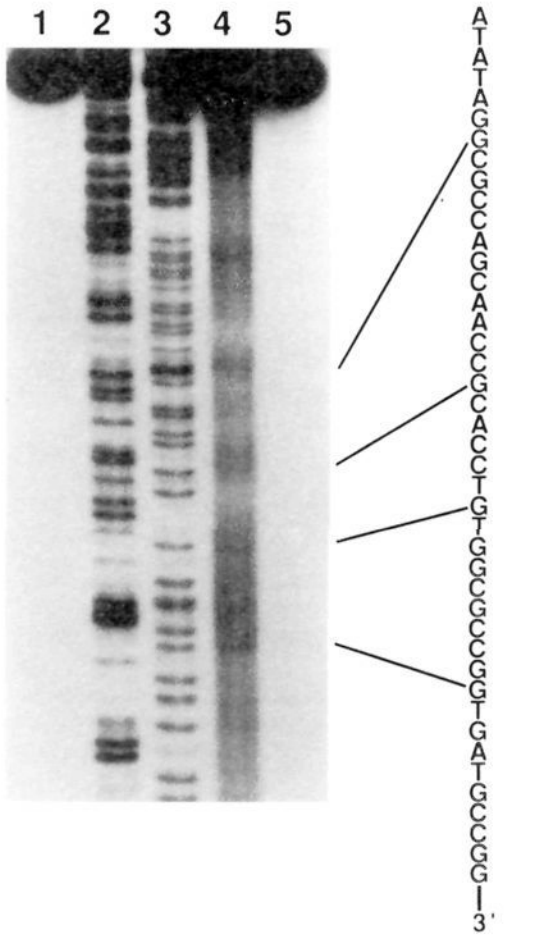

Figure 2. Strand scission of the 3′-end-^{32}P-labeled DNA sequence by compound **3**. Lane 4 shows the DNA-cleavage pattern by **3** (100 μM) under light irradiation at 37 °C for 1 h. Lane 1 presents intact DNA, and lane 5 shows DNA irradiated in the absence of **3**. Lanes 2 and 3 indicate the Maxam–Gilbert sequencing reactions for C + T and G + A, respectively. The reaction samples contained the 3′-labeled 182-bp (*Bam*HI–*Sph*I) pBR322 DNA fragment, 100 μM **3**, 20 mM Tris-HCl buffer (pH 7.5), and 20% THF. Nucleotide sequence cleavage was initiated by irradiation with visible light at a distance of 10 cm at 37 °C for 1 h. Electrophoresis was performed on 10% polyacrylamide/7 M urea slab gel.

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Supplementary Material Available: Experimental procedure for the assay of cytotoxicity and preparation and spectral data for compounds **2** and **4** (4 pages). Ordering information is given an any current masthead page.

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(16) We also made preliminary examinations of inhibitory activity against various enzymes to investigate the interaction between fullerene and protein. The fullerene carboxylic acid **2** was found to show considerable activity against cysteine proteinases (*m*-calpain IC_{50} = 3.6 μM; papain IC_{50} = 43 μM) and serine proteinases (trypsin IC_{50} = 5.6 μM, plasmin IC_{50} = 3.2 μM; thrombin 24% inhibition at 10 μM) and to show considerable selectivity: **2** was totally or almost inactive against cathepsin D, acyl-CoA cholesterol acyl transferase, diacylglycerol acyltransferase, HIV-reverse transcriptase, and the whole system of sterol biosynthesis. Since the experimental protocols necessitate that light be used, all experiments were conducted under light irradiation. Preliminary kinetic studies on calpain suggested a rather complex kinetic behavior. For other aspects of the enzyme inhibition issue, see: Ando, R.; Morinaka, Y.; Tokuyama, H.; Isaka, M.; Nakamura, E. *J. Am. Chem. Soc.* **1993**, *115*, 1174.

(17) We have recently confirmed that irradiation of the carboxylic acid **2** under aerobic conditions in aqueous DMSO does generate a preparatively useful amount of singlet oxygen. Details will be published in due course.

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