Headline Articles

Biological Activity of Water-Soluble Fullerenes. Structural Dependence of DNA Cleavage, Cytotoxicity, and Enzyme Inhibitory Activities Including HIV-Protease Inhibition

Eiichi Nakamura,* Hidetoshi Tokuyama,† Shigeru Yamago,†† Takashi Shiraki,††† and Yukio Sugiura*,†††

Department of Chemistry, School of Science, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

†Faculty of Pharmaceutical Sciences, the University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113

††Department of Synthetic Chemistry and Biological Chemistry, Kyoto University, Sakyo-ku, Kyoto 606-01

†††Institute for Chemical Research, Kyoto University, Uji, Kyoto 611

(Received March 11, 1996)

Two different classes of water-soluble fullerene derivatives, detergent-type and sphere-type, were synthesized. The derivatives were evaluated for their biological activities including cytotoxicity, DNA cleavage, and inhibition of HIV-protease and other enzymes. Both classes of compounds display generally similar behavior except for their cytotoxicity spectra against several cell lines. The fullerene derivatives bearing *N*-methylpyrrole were found to be photo-inactive with respect to DNA cleaving activity and cytotoxicity. A study on the kinetics for the inhibition of HIV-protease with detergent type derivative revealed that the compound is a potent fullerene-based HIV protease inhibitor, inhibiting the enzyme activity in a reversible and competitive manner with a K_i value of 0.32 μM.

Buckminsterfullerenes and their organofunctionalized derivatives have recently become a topic of interest in biochemistry and medicinal chemistry.[1] Recent investigations of biological activity have revealed that fullerene derivatives exhibit several types of biological activities both in vitro and in vivo.[2—8] For instance, we reported that the fullerene carboxylic acid **1** displays inhibitory activity toward various enzymes, cytotoxicity against tumor cells, and DNA cleaving activity under irradiation with visible light.[2] Further, the fullerene-oligonucleotide conjugate **2** bearing complementary nucleic acid sequence cleaves DNA site-specifically through duplex or triplex formation.[3] In addition, we recently published the results of the pharmacokinetic studies using ^{14}C-labeled **1** and the lack of acute toxicity of a water-miscible fullerene derivative.[4] Kenyon and Wudl studied enzyme inhibition and reported that the methanofullerene derivative **3** bearing two carboxylic acids possesses inhibitory activity against human immuno deficiency virus (HIV) protease (Chart 1).[5] It was reported that a fullerene peptide also inhibits HIV protease.[6] Schinazi, Hill, and Wudl found that compound **3** also possesses anti-HIV transcription activity.[7] On the other hand, Miyata et al. reported recently a novel promoting action of C_{60} on the chondrogenesis.[8]

In most of these studies, C_{60} has been solubilized in water by attachment of a carboxylic acid residue(s) connected to C_{60} with an organic linker moiety.[9] This class of compounds possess amphiphilic character similar to detergent (detergent-type).[10] Other known water-soluble derivatives are those having a number of polar groups distributed (randomly) on the fullerene sphere (sphere-type). Such polar groups include hydroxyl (fullerol)[11] and amino groups.[12] These compounds are highly soluble in aqueous media, but no information on their biological behavior has thus far been reported.

With the aim of further designing fullerene-based biologically active agents, we felt it necessary to obtain basic information on structure-activity relationships, which has so far been lacking. We describe herein in full the results of the studies on DNA cleavage, cytotoxicity, and enzyme inhibitory activity for several compounds belonging to the two classes of water-soluble fullerene derivatives. The range of compounds we studied includes detergent-types such as **1**,[2] **4**,[4] **5**, **6**, and C_{60} and C_{70} based sphere-types such as **7**, **8**, and **9** (Chart 2). We found that both classes of compounds generally show similar behavior, yet sometimes behave in different manners. They exhibit considerable inhibitory activity against HIV-protease.

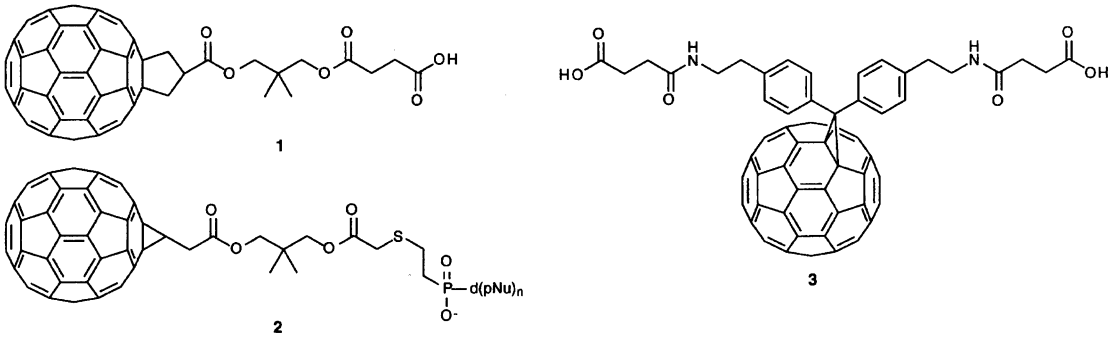


Chart 1.

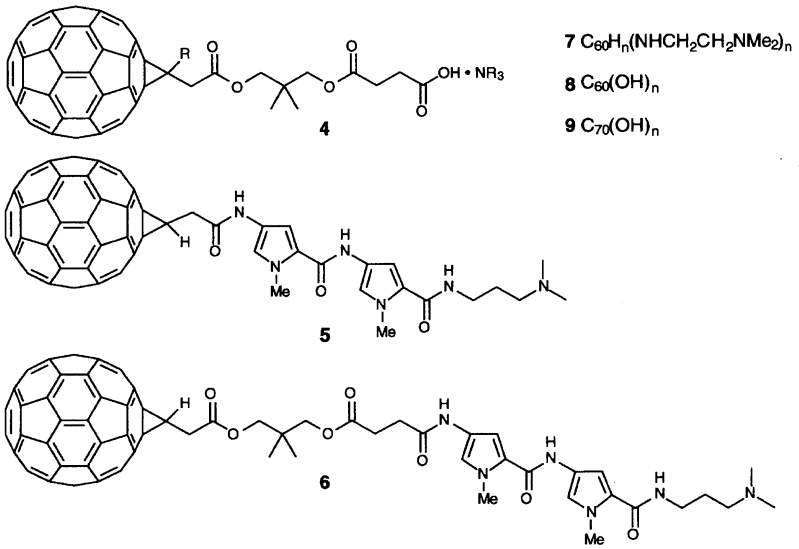


Chart 2.

Results and Discussion

DNA Cleaving Activity. We have previously reported that the carboxylic acid **1** cleaves double strand DNA with moderate efficiency (at ca. 100 μM concentration, 1 M=1 mol dm^{-3}) upon irradiation with a visible light source.[2] The cleavage took place predominantly at guanine base, which is known to be most susceptible among nucleotides based on oxidative cleavage,[13] proceeded much more efficiently in D_2O than in H_2O (Fig. 1), and was inhibited by the presence of a singlet oxygen quencher. We have shown by chemical experiments that photolysis of **1** in solution containing molecular oxygen does indeed generate singlet oxygen.[14] These results were taken as evidence of singlet oxygen mediated DNA cleavage, whereas they do not exclude the possibility of direct oxidation of DNA with excited (hence highly oxidative) fullerene core.

With these results on the detergent-type compound in hand, we examined the DNA cleaving activity of the sphere-type compound **7**. It has been reported that addition of primary amine to C_{60} affords multiple amine adducts,[12] and we synthesized the C_{60} amine multi-adduct **7** by the reaction of C_{60} and excess *N,N*-dimethylethylenediamine (Eq. 1).[14] Elemental analysis of the material suggested that about six amine residues are attached on C_{60}. Upon examination of the DNA cleaving activity with supercoiled pBR322, compound **7** cleanly cut supercoiled DNA (form I) into nicked circular one (form II) under irradiation with visible light (Fig. 2). The activity depended linearly on the concentration of **7** (Fig. 3). The relative potency of DNA cleavage was slightly higher for **7** than for the detergent type derivative **1**, possibly due to the higher water solubility of the former. Interestingly, **7** displayed weak DNA cleavage even under total darkness (Fig. 3), which stands in contrast to compound **1** which showed no activity in the dark. As with **1**, however, the light-induced DNA-cleavage with **7** took place selectively at guanine bases (Fig. 4).

$$C_{60} \xrightarrow[\text{toluene, r.t.}]{\text{excess}} C_{60}H_n(NHCH_2CH_2NMe_2)_n \quad \mathbf{7} \qquad (1)$$

In many of our DNA cleaving experiments, we observed low recovery of DNA due to fullerene-induced aggregation of DNA, suggesting that the fullerene are bound to the DNA chain (randomly) and are cleaving DNA by an oxidative mechanism. Thus, we have envisioned that introduction of an appropriate DNA binding moiety would effect sequence selective binding of C_{60} to DNA, and hence site selective photocleavage of DNA. We focused on the use of an analog of the natural product antibiotic netropsin[15] as a DNA binder. Netropsin contains two *N*-methylpyr-

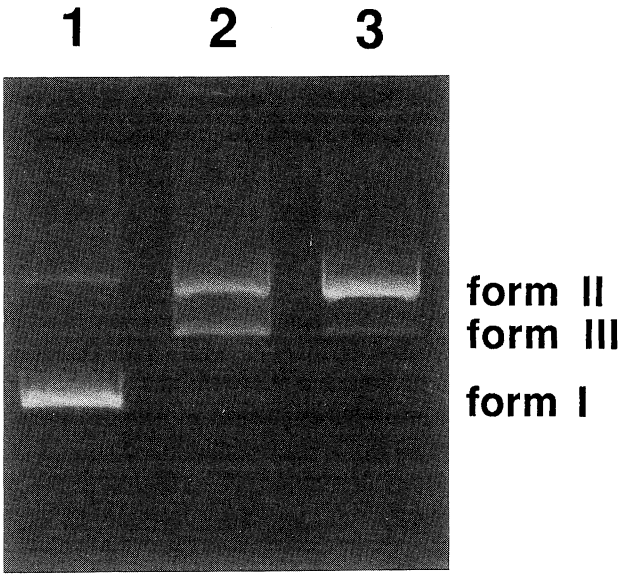


Fig. 1. Agarose gel electrophoretic patterns of DNA-nicking by compound **1**. Lane 1 shows intact DNA. Lanes 2 and 3 show DNA cleavage by **1** (100 μM) in D_2O or H_2O, respectively. The reaction sample contained 100 μM of compound **1**, 0.4 μg of pBR322 plasmid DNA, 20 mM Tris-HCl buffer (pH 7.5), and 20% THF. After irradiation at a distance of 10 cm by a commercial 300-W photo-reflector lamp (Toshiba, Tokyo; color temperature of 3150 K) at 20 °C, electrophoresis was performed by using 1% agarose gel containing ethidium bromide (0.5 μg mL^{-1}).

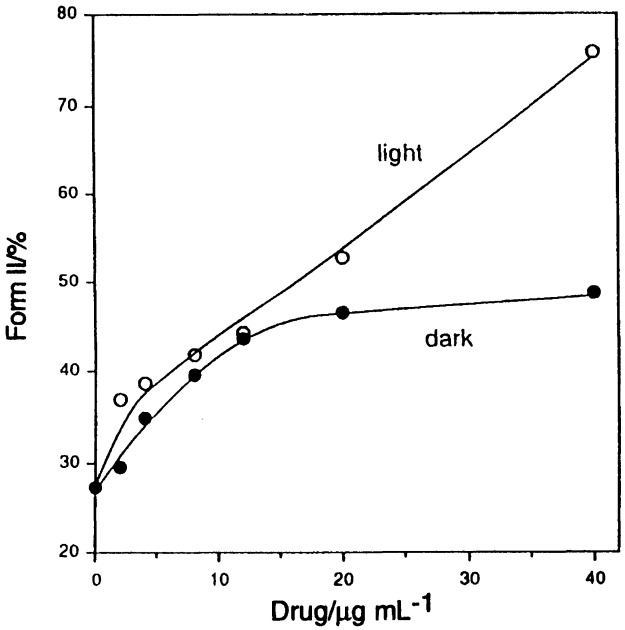


Fig. 3. Concentration dependence of the DNA cleavage by **7**. Supercoiled pBR322 DNA (20 μg mL^{-1}) was incubated for 30 min at 37 °C with (○) or without (●) irradiation of visible light in the presence of **7** at various concentrations.

role carboxamides which bind to a minor groove of double helical DNA, exhibiting a strong preference for A+T rich regions (Chart 3).[16] We therefore designed and synthesized the fullerene-netropsin conjugate **5**, as shown in Scheme 1.[17]

Hydrolysis of the methanofullerene ester **10**[18] gave the carboxylic acid **11**. Although this compound was insoluble in a variety of organic solvents, extended heating in excess oxalyl chloride converted it to the corresponding acid chloride **12**, which is soluble in various nonpolar solvents.[9c] The acid chloride **12** was coupled with the netropsin analog **13**, prepared from *N*-methylpyrrole according to the reported method,[19] leading to the conjugate **5** as a brown solid in 42% yield from **10** after column chromatography on alumina (eluent: Et_3N/CS_2, 5/95 to $Et_3N/MeOH/CS_2$ 1/5/50, v/v). The compound, which was relatively unstable in air, is soluble in aqueous media such as DMSO/water and could hence be used for the DNA cleavage studies.

To our disappointment, the fullerene-netropsin conjugate **5** displayed no DNA cleavage ability under irradiation with visible light. In light of the high electron affinity of triplet C_{60}[20,21] and the high electron donating ability of *N*-methylpyrrole moiety, intramolecular quenching[22] was suspected. Considering that a longer linker may retard intramolecular quenching,[23] we then investigated compound **6** bearing a ca.

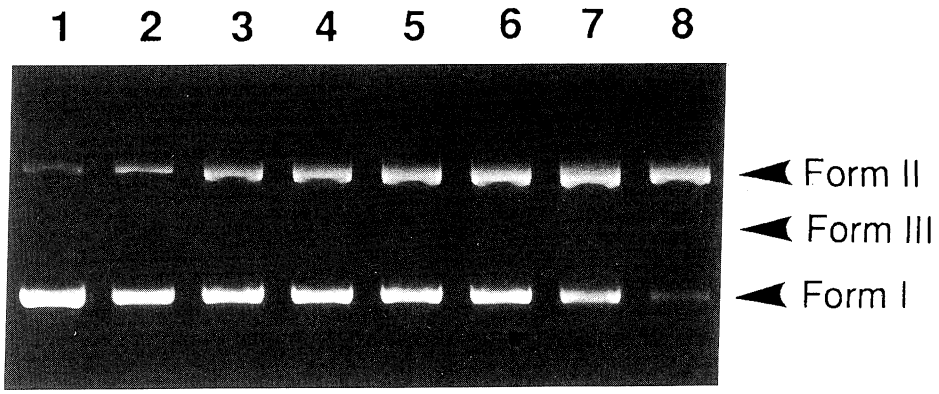


Fig. 2. DNA cleavage by **7**. Supercoiled pBR322 DNA (20 μg mL^{-1}) was irradiated with visible light for 30 min at 37 °C in the presence of **7**. The concentrations of **7** were 0 (lane 2), 2 (lane 3), 4 (lane 4), 8 (lane 5), 12 (lane 6), 20 (lane 7), and 40 μg mL^{-1} (lane 8). Lane 1 shows intact DNA alone. The DNA samples were precipitated in ethanol to remove **7** and separated on a 1% (w/v) agarose gel stained with ethidium bromide. The position of the supercoiled (form I), nicked circular (form II), and linear (form III) plasmid molecules are indicated.

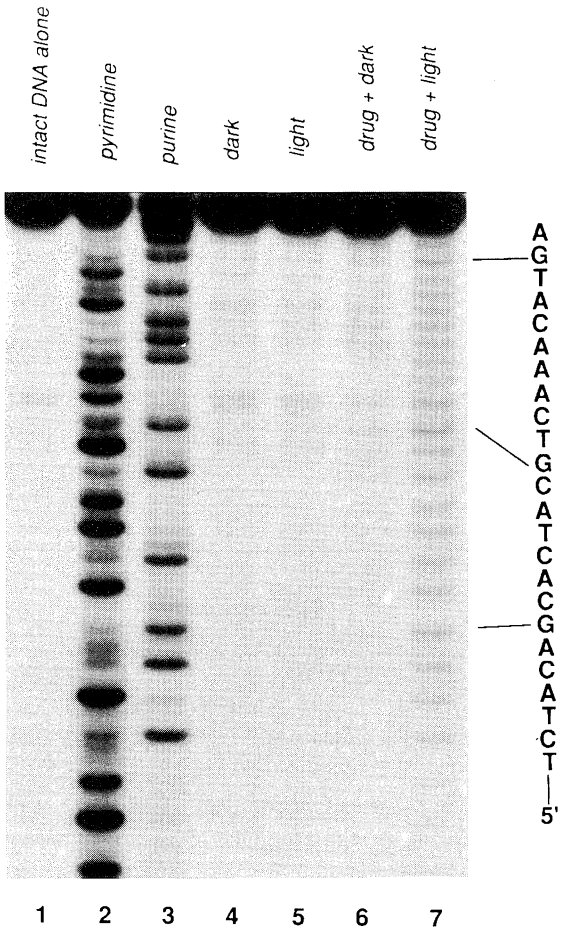
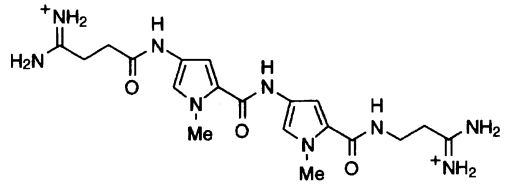


Fig. 4. Strand scission of 5′-^{32}P end labeled DNA sequence by compound **7**. The 5′-labeled oligomer duplex was irradiated with visible light for 30 min at 37 °C in the presence (lane 7) or absence (lane 5) of **7**. Lanes 4 and 6 indicate no visible light treatment. Lane 1 shows intact DNA alone. Lanes 2 and 3 are the Maxam–Gilbert pyrimidine and purine sequencing ladders.

18 Å linker (in its extended form, as opposed to ca. 8 Å in **5**) (Chart 4). However, **6** did not cause photocleavage of DNA, either. The lack of DNA cleavage by these compounds were corroborated by their lack of ability to generate singlet oxygen in test tube chemical experiments.[14] Molecular modeling

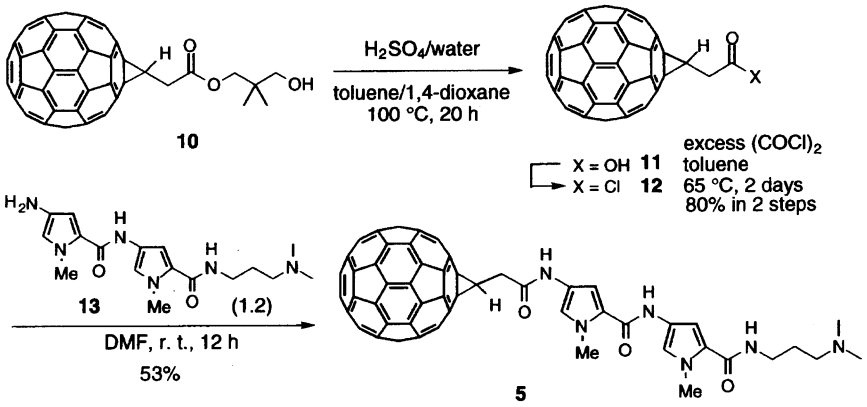


Netropsin

Chart 3.

of **6** suggested that the linker moiety interconverts between its folded conformation and its extended conformation as shown below with equal facility, and therefore that such a flexible linker may not retard intramolecular triplet quenching.[24] The goal of selective binding of organofullerene to DNA sequences has recently been achieved by the use of a C_{60}-oligonucleotide conjugate **2**.[3] Being much weaker electron-donors than pyrroles, the nucleotide bases apparently did not act as a triplet quencher in this case. On the positive side of the matter, the above results suggested that the introduction of an electron donor nearby C_{60} could be exploited for killing the photoactivities of fullerenes when such activities are undesirable.

Cytotoxicity against Tumor Cells. The fullerene carboxylic acid **1** has previously been shown to exhibit cytotoxicity against HeLa S3 cell line upon irradiation of weak visible light. No inhibition ($IC_{50} > 100$ μg mL^{-1}) was observed in total darkness. The photosensitivity was so high that all manipulation of the control experiments had to be conducted in the dark. In the present studies, we compared the in vitro cytotoxicity for the detergent-type and sphere-type compounds by the study of inhibition of growth rate[25] (Table 1). Results for the series of detergent type derivatives **1a** and **4a—c** indicate that the level of the photo cytotoxicity does not depend too much on the minor structural difference of the organic residue on the fullerene sphere. The fullerene-netropsin hybrid **5** was exceptional in that it showed light-independent cytotoxicity of considerable strength. The light-independence is consistent with the inability of **5** to form singlet oxygen, and suggests that the cytotoxicity is caused by the bis-pyrrole moiety. This conjecture was fully supported by the control for the bis-pyrrole compound **14** lacking C_{60}, which also showed a similar level of photo-independent cy-



Scheme 1.

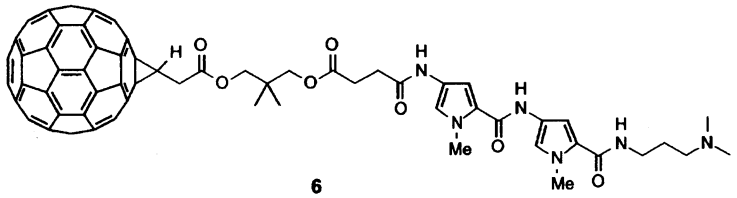


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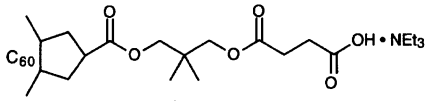
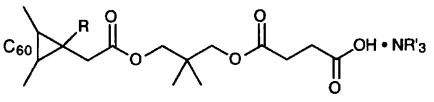
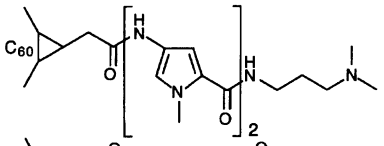
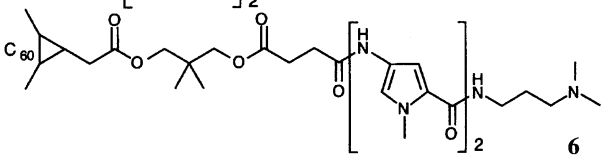
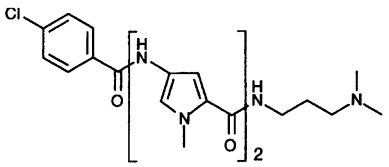
Chart 4.

Table 1. Cytotoxicity of Fullerene Derivatives (HeLa S3 cells; IC_{50} [μg mL^{-1}])[a)]

Fullerene derivatives		Dark	Light
	**1a**	>100	7.1
	**4a** R=H R′=Et	>100	2.7
		66.2	1.6
	4b R=H R′=CH_2CH_2OH	>100	2.0
	4c R=Ph R′Et	>100	4.8
	**5**	9.2	10.0
		>100	5.9
		56.3[b)]	5.3[b)]
	6		
	**14**	31.6	N.D.[c)]
$C_{60}(OH)_n$	**8**	>100	4.3
$C_{60}H_n(NHCH_2CH_2NMe_2)_n$	**7**	40.8	3.8

a) To examine the effect of light, incubation was carried out with or without irradiation of light with two 6-W fluorescent lights at a 3—5 cm distance, twice, each for 1 h in every 24 h during a 72 h period of incubation at 37 °C. b) The compound was treated with aq HCl. c) Not determined.

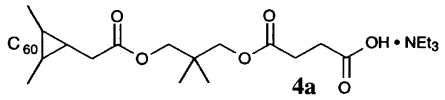
totoxicity (Table 1).

Sphere-type compounds were examined next. Photo-activation effect has clearly been seen for polyhydroxylated C_{60} (fullerol) **8** prepared by the method of Saigo.[11b)] Visible light irradiation enhanced the cytotoxicity from >100 μg mL^{-1} to 4.3 μg mL^{-1}, the level of enhancement being of the same level as that observed for **1a**. The amine adduct **7** showed weak activity in the dark, which was markedly enhanced by visible light irradiation, showing similarity to the light sensitivity of this compound in the DNA cleavage experiments (vide supra).

Cytotoxicity spectrum was briefly examined for several other cell lines, NIH/3T3 (closer to normal cells), SW-480 (*colon adenocarcinoma, human*) and HCT-116 (*colon carcinoma, human*). The results summarized in Table 2 indicates that there is considerable difference in the cytotoxicity between the detergent-type and sphere-type. Thus, compound **4a** showed a broad spectrum of cytotoxicity and possesses the same level activity against all the cell lines including NIH/3T3. Interestingly, the compound **8** was inactive against SW-480 and HCT-116 both in the dark and upon irradiation of light, and this difference may be related to the different solubility of **8** (freely soluble in aqueous medium) and **4a** (amphiphilic).

The action mechanisms for the observed photo-induced cell killing process still remain unclear. The high lipophilicity ($\log P_{ow}$=4.5)[26)] and the strong affinity of **1a** with the tissue as revealed by our recent pharmacokinetic studies[4)] may suggest that the drug taken inside of the cell acts as a photo-cytotoxic agent. More recent experiments suggested, however, that this may not be the case. Namely, studies on the cells prepared by preincubation with **1a** followed by aqueous washing to remove unbound **1a** indicated no photo-induced cyototoxicity of **1a**, strongly suggesting that cell-

Table 2. Cytotoxicity Spectrum (IC_{50}; μg mL^{-1})[a]

Compound	Cell line	HeLa S3		NIH/3T3		HCT-116		SW-480	
	Condition	Dark	Light	Dark	Light	Dark	Light	Dark	Light
 **4a**		71.5	1.64	>100	4.9	>100	2.07	>100	0.64
$C_{60}(OH)_n$ **8**		>100	4.32	N.D.[b]	N.D.[b]	>100	>100	>100	>100

a) To examine the effect of light, incubation was carried out with or without irradiation of light with two 6-W fluorescent lights at a 3—5 cm distance, twice, each for 1 h in every 24 h during a 72 h period of incubation at 37 °C. b) Not determined.

bound **1a** is photo-inactive.[27] Thus the cytotoxicy shown in Tables 1 and 2 may be due to fullerenes in the medium.

Enzyme Inhibition Activity. In addition to photochemical behavior, fullerene shows unique characters including hydrophobicity, electophilicity, and high reduction potential. We envisioned that the fullerenes may exhibit enzyme inhibitory activity by mechanisms related to these properties, and set out to investigate the inhibitory activity of detergent-type fullerene derivatives against various kinds of enzymes (Table 3). Both detergent-type compounds **1a**[2] and **4a**[4] exhibited distinct inhibitory activity against proteases including cysteine proteases (*m*-calpain, papain, and cathepsin) and serine proteases (trypsin, plasmin, and thrombin), whereas both compounds did not show high activity against sterol biosynthesis and HIV transcriptase. The small skeletal change from **1a** to **4a** slightly increased the activity.

In order to obtain an insight into the inhibition mechanism, we chose *m*-calpain and determined the activity of enzyme after incubation with compound **1a**. The enzyme activity of *m*-calpain after incubation for 15 and 30 min with excess **1a** was nearly the same, 50 and 49%, respectively. Further elongation of incubation did not decrease the enzyme activity. Although this observation suggests reversible inhibition, the Lineweaver–Burk plot did not afford a conclusive support.

In light of Kenyon's pioneering studies on **3**,[5] we also investigated inhibitory activity of two classes of water-soluble fullerene derivatives, detergent-type and sphere-type against HIV-protease by a published inhibition assay system,[28] in which the reference compound **15**[29] possesses IC_{50} of 0.38 nM (Chart 5). The detergent type derivative **4a** and **5** displayed activity at IC_{50} of 0.24 μM and 0.59 μM, respectively. Spherical derivatives also possessed inhibitory activity. Indeed, fullerol **9** prepared from C_{70} showed the same level of activity, IC_{50} of 0.46 μM. C_{60} fullerol **8**, on the other

Table 3. Enzyme Inhibitory Activity of Fullerene Carboxylic Acids.[a]

Enzyme	IC_{50} (μM) or inhibition (%) at [(μM)] **1a**	**4a**
Cystein Protease		
m-calpain	3.6	2.5
papain	43	9.0
cathepsin B	10.5	6.7
Serine Protease		
cathepsin G	N.D.[f]	n.d.[g]
thrombin	24% [10]	n.d.
trypsin	5.6	n.d.
palsmin	3.2	n.d.
Aspartic Protease		
cathepsin D	15% [50]	n.d.
Others		
ACAT[b]	17% [33]	n.d.
DGAT[c]	0% [33]	n.d.
Sterol Biosynthesis[d]	5% [33]	n.d.
HIV[e] reverse transcriptase	0% (10)	n.d.

a) Experiments were carried out under ambient light. b) ACAT: Acyl–CoA cholesterol acyl transferace. c) DGAT; diacylgycelol acyl transferase. d) Sterol biosynthesis: whole system of biosynthesis of sterol from acetic acid. e) HIV: human immuno deficiency virus. f) Not detected. g) Not determined.

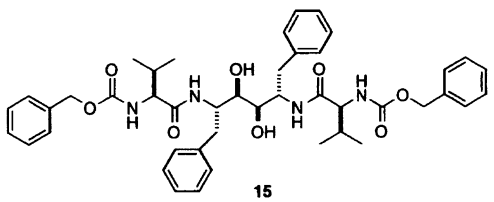


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Chart 5.

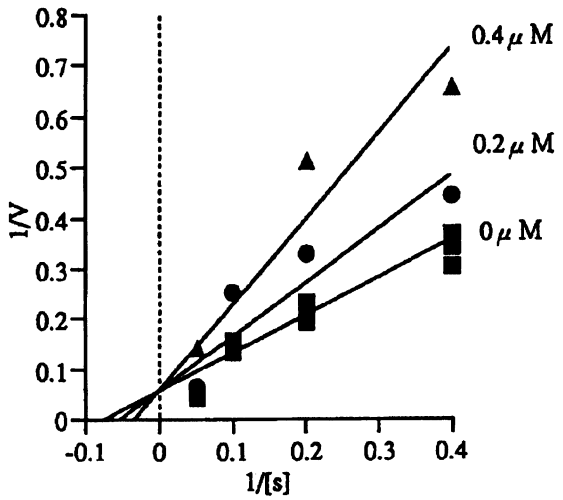

Fig. 5. Lineweaver–Burk plot of inhibition of HIV-protease by the methanofullerene derivative **4**. Concentrations of **4** are 0 (■), 0.2 μM (●), and 0.4 μM (▲).

hand, displayed less potent activity, IC_{50} of 8.3 μM.

We found that the mono-substituted methanofullerene derivative **4a** also displays activity, which is more potent than the disubstituted methanofullerene derivative **3**. A kinetic study indicated typical reversible competitive inhibition (Fig. 5) and the K_i value of 0.32 μM found for **4a** is one order of magnitude higher than that reported for the diaryl methanofullerene derivative **3**. The result of these kinetics is consistent with Kenyon's rationale for the HIV inhibitory action of **3** and suggests that the compound **4a** becomes hydrophobically bound inside the active site of the enzyme.[5)]

Conclusion

We have found, for the first time, that the spherical fullerol and the amine adduct exhibited photo-induced DNA cleaving activity and cytotoxicity that are comparable with those observed for detergent-type derivatives. However, fullerol may sometimes show a different cytotoxicity spectrum from that of the detergent-type derivative, which is likely due to the large differences of the molecular shape and solubility. The presence of an internal electron donor turns off the photo-induced DNA cleaving activity and cytotoxicity. This may provide important information for designing photo-inactive fullerene-based biologically active compounds. Some recent observations[27)] suggest that the observed photo-induced cytotoxicity is likely due to fullerene derivatives existing in the medium (not inside of the cells). Finally, we have demonstrated that the fullerene derivatives have a considerable potential as enzyme inhibitors including HIV-protease inhibitor.

Experimental

General. All reactions dealing with air- and moisture sensitive compounds were undertaken in a dry reaction vessel under nitrogen. Toluene, *o*-dichlorobenzene, triethylamine, *N,N*-dimethylformamide, and dimethyl sulfoxide were distilled from calcium hydride under nitrogen and stored over molecular sieves. Methanol was distilled from magnesium methoxide and stored over molecular sieves. Infrared spectra were recorded on a JASCO IR-800; absorptions are reported in cm^{-1}. ^1H NMR spectra were taken at 400 MHz or 500 MHz using a JEOL EX-400 or JEOL GSX-500 instrument, respectively, and are reported in parts per million from internal tetramethylsilane. Routine chromatography on silica gel was performed on Kiesel gel 60 (Merck). Gel permeation chromatography was performed on a Japan Analytical Industry LC-908 machine equipped with JAIGEL-1H (20×600 mm) and -2H (20×600 mm) GPC columns. High pressure liquid chromatography was performed on a Shimadzu LC10A machine equipped with YMC-Pack ODS-A (250 mm × 10 mm I. D.).

Materials. Plasmid pBR322 DNA was isolated from *Escherichia coli* C600. T4 polynucleotide kinase and [γ-^{32}P]ATP were purchased from New England Biolabs and DuPont, respectively. Stock drug solutions were made by dissolving weighed amounts of the drug in water. Distilled water was purified through a Sybron Nanopure II system. Oligonucleotides were synthesized on an Applied Biosystems 391 synthesizer. The oligomers were then deprotected with aqueous ammonia at 55 °C for 10 h, and purified by reversed phase HPLC. The 200 pmol of oligonucleotide was labeled at the 5′ terminal using T4 polynucleotide kinase, and then purified on a 15% (w/v) denaturing polyacrylamide gel. All other chemicals used were commercial reagent grade.

Assay for DNA-Cleaving Efficiency of Fullerene Derivative (7). A standard reaction sample contained 20 μg mL^{-1} pBR322 plasmid DNA and 40 μg mL^{-1} of **7** with 0.1 M Tris-HCl (pH 8.0). The sample was irradiated at a distance of 12 cm by a 300-W photoreflector lamp (National, Osaka; color temperature of 3200 K) for 30 min at 37 °C. After ethanol precipitation, the DNA pellets were dissolved in 20 μL of the loading buffer containing 0.05% (w/v) bromophenol blue and 10% (v/v) glycerol. Electrophoresis was performed by using a 1% (w/v) agarose gel containing ethidium bromide (0.5 mg/mL) in TBE buffer (89 mM Tris-borate, 2.5 mM Na_2EDTA, pH 8.0). The gel was photographed with Polaroid 665 film. The resulting forms of the plasmid were quantitated by measuring the intensities of the DNA bands on the negative film.

Nucleotide Sequence Analysis. A reaction sample contained 40 mg mL^{-1} of **7** with 1 μM oligonucleotide duplex and a trace (<0.1 μM) of the end-labeled oligonucleotide in a total volume of 20 μL buffered to pH 8.0 with 0.1 M Tris-HCl and 0.2 M NaCl. Prior to addition of **7**, the sample was heated at 90 °C for 5 min, slowly cooled to room temperature, and then allowed to reanneal at 4 °C for at least 60 min. The sample was then irradiated at a distance of 12 cm by a 300-W photoreflector lamp (National, Osaka; color temperature of 3200 K) for 30 min at 37 °C. After ethanol precipitation, the sample was dissolved in 5 μL of the loading buffer (90% (v/v) formamide containing 7 M urea). Electrophoresis was performed at 2000 V in TBE buffer (89 mM Tris-borate, 2.5 mM EDTA, pH 8.0). DNA sequencing was carried out by the Maxam–Gilbert method.

Cytotoxicity. The in vitro cytotoxicity against HeLa S3 cells was evaluated by the study of inhibition of growth rate. In order to examine the effect of light, incubation was carried out with or without irradiation of light for 1 h each day during the 3-day period of incubation. The fullerene was dissolved in a small amount of DMSO, and these solutions were further diluted with DMEM medium to afford solutions suitable for the fullerene concentration range studied. Each solution was placed into a 96-well microplate (100 μL/well). HeLa S3 cells were suspended in DMEM medium containing 10% of fetal calf serum at a concentration of 1×10^5 cells/mL, and 100 μL of the solution were added to 96-well plate. After 3 days incubation at 37 °C, XTT (2,3-bis[2-methoxy-4-nitrosulfophenyl]-2*H*-tetrazolium-5-carboxanilide inner salt) solution was added to each well and the plate was incubated at 37 °C for 4 h. The mitochondrial dehydrogenase of viable cells reduces XTT to a blue formazan product that can be measured spectrometrically. The IC_{50} values, defined as the concentration required to reduce the growth rate to 50% of the control, were determined by plotting the fullerenes concentration versus the cells' viability.

Assay for Inhibitory Activity of HIV-Protease. Inhibitory activity against HIV-protease was determined with HIV-protease assay kit (Nova biochem). The substrate was dissolved in DMSO (10 mM), followed by dilution with 0.1 M MES buffer (pH 6.5) to obtain 1 μM solution. To a 2 mL solution of the substrate in a cuvette was added HIV-protease (0.5 μL, 0.65 μg) at 37 °C. Fluorescence was monitored continuously at an excitation wavelength of 325 nm and an emission wavelength of 420 nm. After stirring 5 min, 1 μL of fullerene derivative in DMSO or water was added to the mixture. Activity was calculated based on decrease of the rate of cleavage of chromogenic substrate after the addition of fullerene derivative. The K_i value for inhibitor binding was estimated by Lineweaver–Burk plot for experiments at fixed concentration of substrate and inhibitor

Propanofullerene-C_{60} Carboxylic Acid Derivative (1). Compound **1** was prepared from the corresponding alcohol and succinic anhydride.[2] IR (KBr) 3000—3700, 2923, 2854, 2363, 2346, 1735, 1715, 1383, 1170, 753, 575, 528 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ=1.13 (s, 6 H, CH_3), 2.74 (br s, 4 H, $CH_2CH_2CO_2H$), 3.85—3.94 (m, 4 H), 4.09 (s, 2 H, CO_2CH_2), 4.18 (s, 2 H, CO_2CH_2), 4.22—4.29 (m, 1 H, CHCO); ^{13}C NMR (125 MHz, $CDCl_3$, measured for triethylamine salt of the titled compound due to low solubility of the free carboxylic acid) δ=8.61 (CH_3, Et_3N), 21.91 (2 CH_3), 29.68 (CH_2), 29.78 (CH_2), 35.04 (C), 42.40 (CH), 45.12 (CH_2, Et_3N), 46.63 (2 CH_2), 69.18 (CH_2), 69.84 (C_{60}, 2 C), 69.96 (CH_2), 135.51 (C_{60}, 2 C), 135.62 (C_{60}, 2 C), 140.14 (C_{60}, 2 C), 140.24 (C_{60}, 2 C), 141.86 (C_{60}, 2 C), 141.94 (C_{60}, 2 C), 142.05 (C_{60}, 4 C), 142.16 (C_{60}, 2 C), 142.35 (C_{60}, 2 C), 142.59 (C_{60}, 2 C), 142.69 (C_{60}, 2 C), 143.14 (C_{60}, 1 C), 143.18 (C_{60}, 1 C), 144.53 (C_{60}, 2 C), 144.62 (C_{60}, 2 C), 145.25 (C_{60}, 2 C), 145.30 (C_{60}, 2 C), 145.34 (C_{60}, 2 C), 145.39 (C_{60}, 2 C), 145.41 (C_{60}, 2 C), 145.62 (C_{60}, 1 C), 145.85 (C_{60}, 1 C), 145.97 (C_{60}, 2 C), 146.11 (C_{60}, 2 C), 146.15 (C_{60}, 2 C), 146.25 (C_{60}, 2 C), 146.32 (C_{60}, 2 C), 147.41 (C_{60}, 2 C), 156.21 (C_{60}, 2 C), 156.25 (C_{60}, 2 C), 172.93 (2 C, two C=O). Found: C, 79.26; H, 1.63%. Calcd for $C_{73}H_{20}O_6 \cdot 1.1CHCl_3$: C, 79.16; H, 1.89%.

Methanofullerene-C_{60} Carboxylic Acid Derivative (4). Compound **4** was prepared as described for **1**. IR ($CHCl_3$) 3045, 2840, 2830, 1735, 1600, 1180, 1060, 1000, 670 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ=1.06 (s, 6 H, CH_3), 2.72 (br s, 4 H, $CH_2CH_2CO_2H$), 3.62 (d, J=7.3 Hz, 2 H, $CHCH_2$), 4.04 (s, 2 H, CO_2CH_2), 4.14 (s, 2 H, CO_2CH_2), 4.42 (t, J=7.3 Hz, 1 H, $CHCH_2$); ^{13}C NMR (100 MHz, $CDCl_3$) δ=21.92 (2 CH_3), 28.59 (CH_2), 28.99 (CH_2), 31.77 (CH_2), 33.33 (CH), 34.88 (CCH_3), 69.30 (CH_2), 69.92 (CH_2), 75.19 (C_{60}, 2 C), 136.35 (C_{60}, 2 C), 137.95 (C_{60}, 2 C), 141.05 (C_{60}, 2 C), 141.06 (C_{60}, 2 C), 142.11 (C_{60}, 2 C), 142.15 (C_{60}, 2 C), 142.36 (C_{60}, 2 C), 142.63 (C_{60}, 4 C), 142.98 (C_{60}, 2 C), 143.01 (C_{60}, 2 C), 143.06 (C_{60}, 2 C), 143.62 (C_{60}, 2 C), 143.70 (C_{60}, 2 C), 144.25 (C_{60}, 2 C), 144.35 (C_{60}, 2 C), 144.37 (C_{60}, 2 C), 144.57 (C_{60}, 1 C), 144.67 (C_{60}, 2 C), 144.78 (C_{60}, 3 C), 145.06 (C_{60}, 2 C), 145.16 (C_{60}, 8 C), 145.21 (C_{60}, 2 C), 145.73 (C_{60}, 2 C), 146.96 (C_{60}, 2 C), 149.55 (C_{60}, 2 C), 170.78 (C=O), 171.86 (C=O), 178.02 (C=O).

Methanofullerene-C_{60} Netropsin Hybrid Compound (5). Compound **13** was prepared from the corresponding terminus nitro derivative. To a methanol (0.50 mL) solution of the nitro compound (16.0 mg, 0.046 mmol) was added Pd/C (5 mg) and the resulting suspension was stirred under hydrogen (1 atm) at room temperature for 6 h. The catalyst was removed by filtration through celite and the filtrate was concentrated to afford the aminopyrrole **13**, which was used for the subsequent condensation reaction without purification. The aminopyrrole **13** was dissolved in 2 mL of DMF and the solution was concentrated to ca. 1 mL in order to remove any remaining small amounts of methanol. To the DMF solution was added a toluene solution of fullerene acid chloride via cannula over 5 min at 0 °C. The acid chloride **12** was previously prepared from the carboxylic acid **11** by treatment of excess $(COCl)_2$. After stirring overnight, the black precipitate which formed was collected by filtration (36.0 mg). Purification of the crude product was carried out by column chromatography on basic Alumina (Merck Aluminum oxide 60 active basic (activity stage I)) with 5% Et_3N in CS_2, and 2% Et_3N in EtOH/CS_2 (1/10) as eluent. The crude product (13.0 mg) was subjected to purification to obtain the titled product (7.3 mg, 47 %); IR (KBr) 3100—3600, 3200 (shoulder), 2900, 1647, 1578, 1535, 1464, 1435, 1404, 1261, 775, 526 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ=1.54 (q, J=6.5 Hz, 2 H), 2.10 (s, 6 H, $N(CH_3)_2$), 2.20 (m, 2 H), 3.09 (m, 2 H), 3.63 (d, J=7.4 Hz, 2 H), 3.70 (s, 3 H), 3.78 (s, 3 H), 4.70 (t, J=7.4 Hz, 1 H, $CHCH_2$), 6.73 (br s, 1 H), 6.92 (br s, 1 H), 7.10 (br s, 1 H), 7.22 (br s, 1 H), 8.00 (br t, J=5.6 Hz, 1 H, $NHCH_2$), 9.82 (s, 1 H, NH), 10.28 (s, 1 H, NH); MS (FAB) [M + H] 1121, [C_{60}] 720. Due to poor solubility of the compound in various solvents (CS_2, DMF, DMSO, methanol, water, toluene, 1,2-dichlorobenzene, THF, and various mixture of these solvents), we could not obtain satisfactory ^{13}C NMR data.

Fullerene-Netropsin Conjugate with Longer Linker (6). ^1H NMR (400 MHz, DMSO-d_6) δ=1.06 (s, 6 H, two CH_3), 1.70 (m, 2 H), 2.13 (s, 6 H, $N(CH_3)_2$), 3.21 (m, 6 H), 3.66 (d, J=7.4 Hz, 2 H), 3.82 (s, 3 H, NCH_3), 3.86 (s, 3 H, NCH_3), 4.00 (s, 2 H), 4,12 (s, 2 H), 4.71 (t, J=7.4 Hz, 1 H), 6.87 (s, 1 H), 6.95 (s, 1 H), 7.20 (br s, 2 H), 8.10 (m, 1 H), 9.87 (s, 1 H), 9.96 (s, 1 H).

Preparation of the C_{60}/*N,N*-Dimethylethylenediamine Adduct (7). The experiment was carried out as described briefly in the literature.[12] To a toluene (30 mL) solution of C_{60} (30 mg) was added 2 mL of *N,N*-dimethylethylenediamine and the mixture was stirred at 25 °C for 3 d. The solvent was removed in vacuo to afford a brown solid (ca. 60 mg). Purification was achieved by precipitation from $CHCl_3$ solution of the crude product by addition of hexane. Results of elemental analysis of this material (Found: C, 67.70%; H, 5.03%; N, 13.54%) were difficult to reconcile with a rational formula, but the ratios of H/C and H/N, indicated that the average number of *N,N*-dimethylethylenediamine added was six: IR (CCl_4) 3300, 2950, 2850, 2800, 2760, 1460, 1120, 1040, 650, 540 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ=1.9—2.4 (br, 6 H, CH_3), 2.4—3.0 (br, 4 H, CH_2), 3.2—3.7 (br, 1 H, NH).

Reference Compound (14). ^1H NMR (400 MHz, DMSO-d_6) δ=1.63 (q, J=6.8 Hz, 2 H, $CH_2CH_2N(CH_3)_2$), 2.19 (s, 6 H, $N(CH_3)_2$), 2.30 (t, J=6.8 Hz, 2 H, $CH_2N(CH_3)_2$), 3.19 (dd, J=5.9, 6.8 Hz, 2 H, $NHCH_2$), 6.83 (d, J=1.5 Hz, 1 H), 7.08 (d, J=1.5 Hz, 1 H), 7.20 (d, J=1.5 Hz, 1 H), 7.34 (d, J=1.5 Hz, 1 H), 7.49 (d, J=8.3 Hz, 1 H), 7.60 (d, J=8.8 Hz, 1 H), 7.92 (br d, J=8.3 Hz, 1 H), 7.97 (br d, J=8.8 Hz, 1 H), 8.10 (t, J=5.9 Hz, 1 H, $NHCH_2$), 9.96 (br s, NH), 10.44 (br s, NH).

This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture. We thank Dr. Ando for the helpful discussions on the cytotoxicity experiments and assay of enzyme inhibition, and Drs. M. Isaka and S. Ojima for assay of inhibition and kinetic study for HIV protease. Provision of C_{60} and C_{70} samples to E. N. by Hoechst AG is gratefully acknowledged. H. T. was a recipient of JSPS pre-doctoral fellowship (1992—1994).

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