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Sequence-Specific Modification of Guanosine in DNA by a C_{60}-Linked Deoxyoligonucleotide: Evidence for a Non-Singlet Oxygen Mechanism

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Abstract: A C_{60}-linked deoxyoligonucleotide (C_{60}-DON-1) was prepared from bromoacetate **3**. This C_{60}-oligonucleotide conjugate was hybridized to a complementary single-stranded DNA. This system reacted with light and oxygen to damage only guanosines in the single-stranded region which are closest to C_{60}. The damage did not involve $^1O_2$ as the active species but rather resulted from a single electron-transfer mechanism between guanosine and $^3C_{60}$, as shown by comparison experiments with eosin-attached DON-1 and by the use of singlet oxygen quenchers. Copyright © 1996 Elsevier Science Ltd

Buckminsterfullerene (C_{60}) exhibits important photophysical properties (Fig. 1).[1] Upon light excitation, the triplet state is produced with near unit efficiency, and can form singlet oxygen ($^1O_2$) in high yield by energy transfer.[1a] The triplet species can also oxidize electron-rich substrates.[1b] Until recently, the study of the interaction of C_{60} derivatives with biomolecules such as nucleic acids and proteins had been hampered by their insolubility in water.[2,3] We have reported the preparation of water-soluble derivatives of C_{60} by the attachment of a polar functional group to the framework of C_{60}.[4a] The photophysical and chemical properties of these dihydrofullerenes (DHF) are very similar to those of C_{60} and the mechanism of the site-selective modification of single-stranded regions in DNA by a suitably linked C_{60}-DNA conjugate (C_{60}-DON-1) is investigated in this paper.

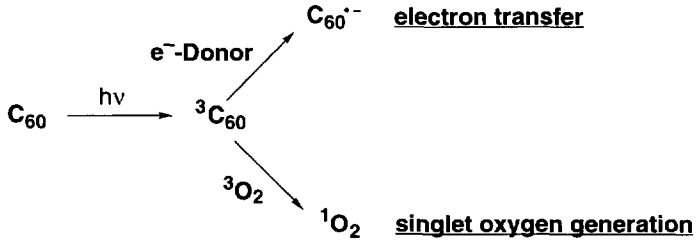
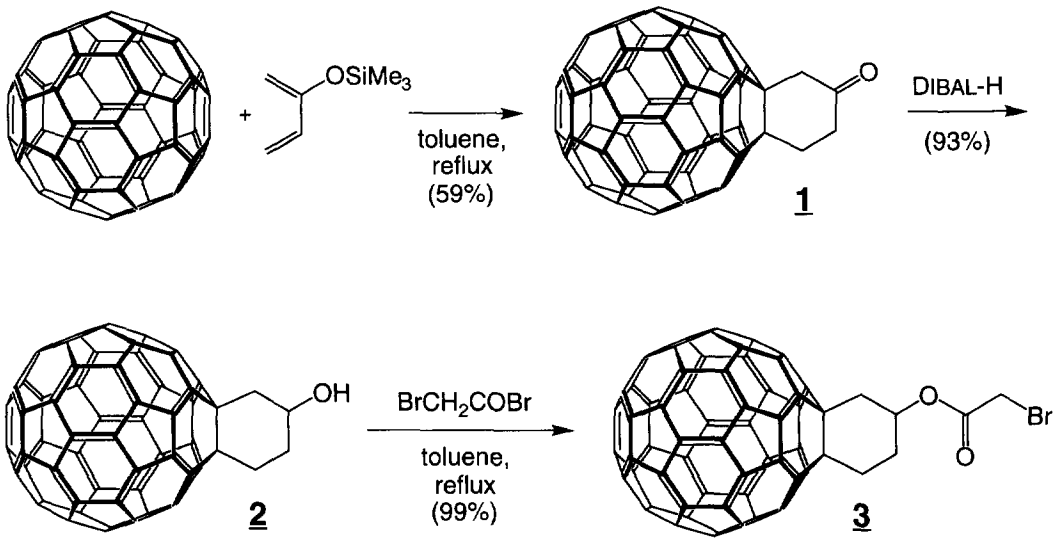


Figure 1. Photochemical pathways of C_{60} and 1,2-dihydrofullerenes upon light excitation.

Results and Discussion

A simple functional handle on C_{60} was needed to initiate the study of the biological properties of C_{60} derivatives. The Diels-Alder reaction of 2-trimethylsilyloxy-1,3-butadiene with C_{60} provided straightforward access to a functional derivative of C_{60} (Scheme 1).[4] The cyclohexanone derivative **1** was formed in good yield and its stability was further enhanced over that of other diene adducts by shutting down the *retro*-Diels-Alder pathway through hydrolysis of the intermediate silyl enol ether functionality. We also found that we could prevent the vexing destruction of **1**, which occurred with most hydride reducing reagents ($LiAlH_4$, $NaBH_4$, $NaBH_3CN$, etc.), by using DIBAL-H in toluene. In this fashion, the racemic cyclohexanol **2** was obtained in excellent yield and its versatility in further derivatizations was demonstrated by esterification with bromoacetyl bromide to give the activated functional ester **3** in quantitative yield.

Scheme 1.



It was also important to demonstrate that the photophysical properties of C_{60} are retained in derivatives of the 1,2-dihydrofullerene type, in which one of the original double bonds of C_{60} has been saturated. We found that all 1,2-dihydrofullerene derivatives prepared by us so far, including **1-3**, have practically identical UV-vis absorption spectra, as represented by compound **3** (Fig. 2). An interesting absorption band appears in the visible region centered at 705 nm, which is shifted far to the red from the lowest energy absorption for C_{60} (620 nm). Although this absorption is weak, it is situated in the ideal range for photodynamic applications since near-IR light penetrates tissue more efficiently than shorter wavelength light. Upon light excitation, the triplet state of alcohol **2** was produced

with near unit efficiency.[5] Singlet oxygen ($^1O_2$) was produced with a quantum yield of 84% at 532 nm, a value only 10% lower than that for C_{60}. In addition, C_{60} and alcohol **2** are remarkably stable towards singlet oxygen, which is a clear advantage in their potential application to photodynamic therapy. The triplet energy of **2** was estimated to be 33-34 kcal/mol, a value close to that of tetraphenylporphyrine. The triplet of **2** could oxidize electron-rich substrates such as *N,N*-dimethylaniline with a rate constant of 2.5 x 10^8 M^{-1} s^{-1}. The electron transfer mechanism from donor groups to C_{60} is an important aspect of the likely mechanism of DNA modification reported below.

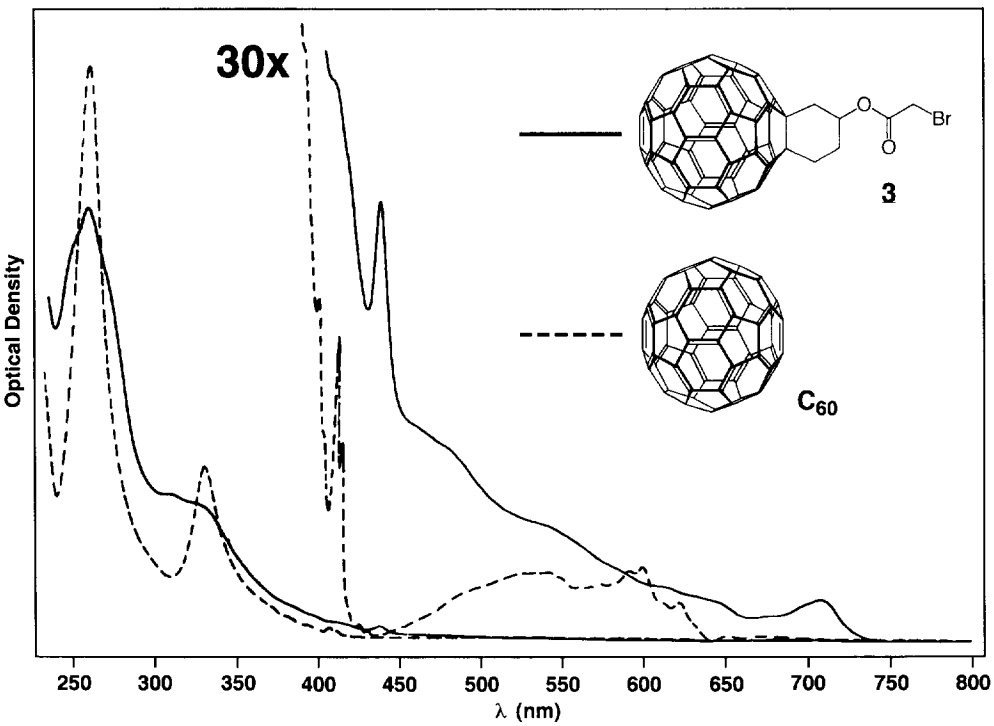


Figure 2. UV-vis absorption spectra of C_{60} and dihydrofullerene **3** in CH_2Cl_2.

The photophysical properties of alcohol **2** made it ideally suited for its use as a targeting system in the site-specific modification of DNA. It was shown independently that a water-soluble derivative of C_{60} damages DNA at its guanosine sites upon visible light irradiation.[2a] Such action is the expected reactivity of singlet oxygen with DNA in solution.[6,7] Singlet oxygen modifies guanosines by either [4+2] or [2+2] cycloadditions to the five-membered imidazole ring of the purine base.[6] This modification greatly enhances the alkaline hydrolysis rate of the phosphate diester bond in DNA. We wanted to extend this effect to a system which would lend itself to site-specific modification by placing the sensitizer in close proximity to the DNA fragment (Fig. 3).

A dihydrofullerene-linked deoxyoligonucleotide (DHF-DON-1) was prepared and hybridized with a single-stranded DNA containing a complementary sequence (Fig. 4, lower). This reaction system was setup to position C_{60} in proximity to both single and double-stranded guanosines and to permit comparison of the reactivity of guanosines in these two environments. In the cleavage experiments described below, we found that only guanosines in single-stranded regions were damaged, but in contrast to suggestions in a recent similar experiment,[2b] the modification does not involve $^1O_2$. Initial evidence suggests that DNA damage is caused by single electron-transfer reaction between guanosine and $^3C_{60}$.

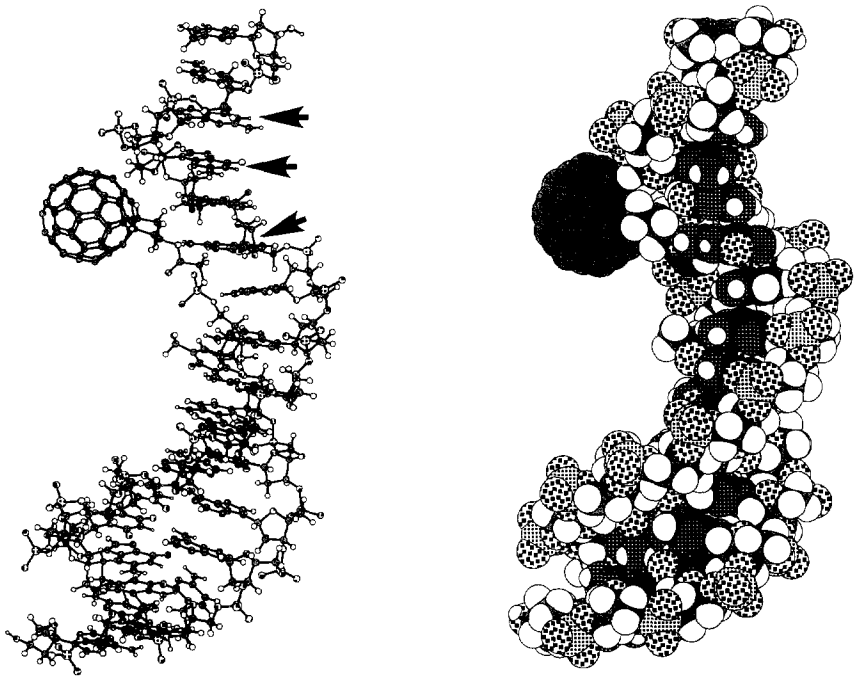


Figure 3. Ball-and-stick and space-filling representations of a portion of the C_{60}-oligonucleotide conjugate used in this study. The three guanosine moieties on the hybridized fragment which are most strongly modified by C_{60} are indicated by arrows.

DHF-DON-1 was prepared by thiophosphorylation of DON-1 with ^{35}S-gamma-ATP and polynucleotide kinase[8] followed by alkylation with bromoacetate **3** (Scheme 2). This reaction can be conveniently assayed if ^{35}S-gamma-ATP is used, since the DONs and their dihydrofullerene-alkylated products are radioactive. The alkylated DON-1 migrates more slowly on a conventional sequencing gel than the unmodified DON-1 and can be purified by elution.

In order to compare the reactivity of the bound C_{60} to that of a known $^1O_2$ generator previously used in nucleic acid chemistry,[7] eosin was tethered to DON-1 by coupling ethylenediamine to the 5'-phosphorylated-DON-1 using 1-ethyl-3(3-dimethylaminopropyl)-

carbodiimide, then reacting the free amine terminus with eosin 5-isothiocyanate to give Eosin-DON-1 (Scheme 2). The 285 nt single stranded DNA used to assay the reactivity of both DON-1 derivatives was prepared by denaturing a double-stranded EcoR1-Nhe restriction fragment isolated from a pGEM-4 vector. The sequence complementary to DON-1 is 65 nts from the ^{32}P-labeled 3'-terminus. After annealing the two modified DON-1s, photolysis was

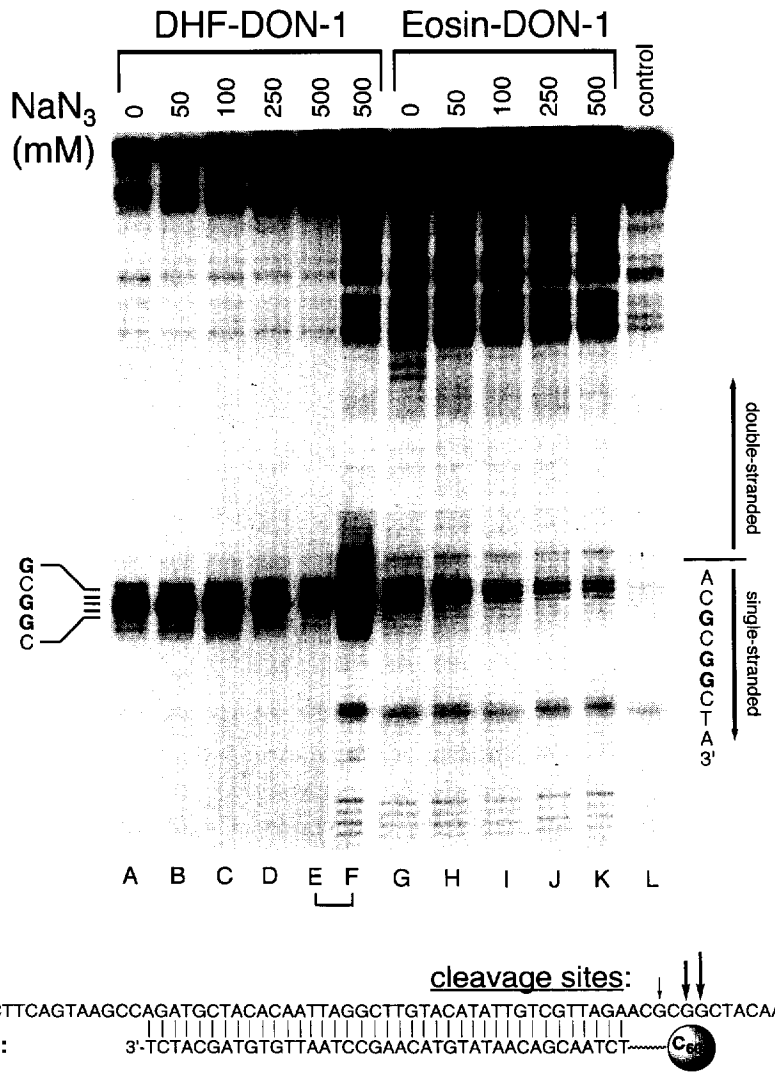
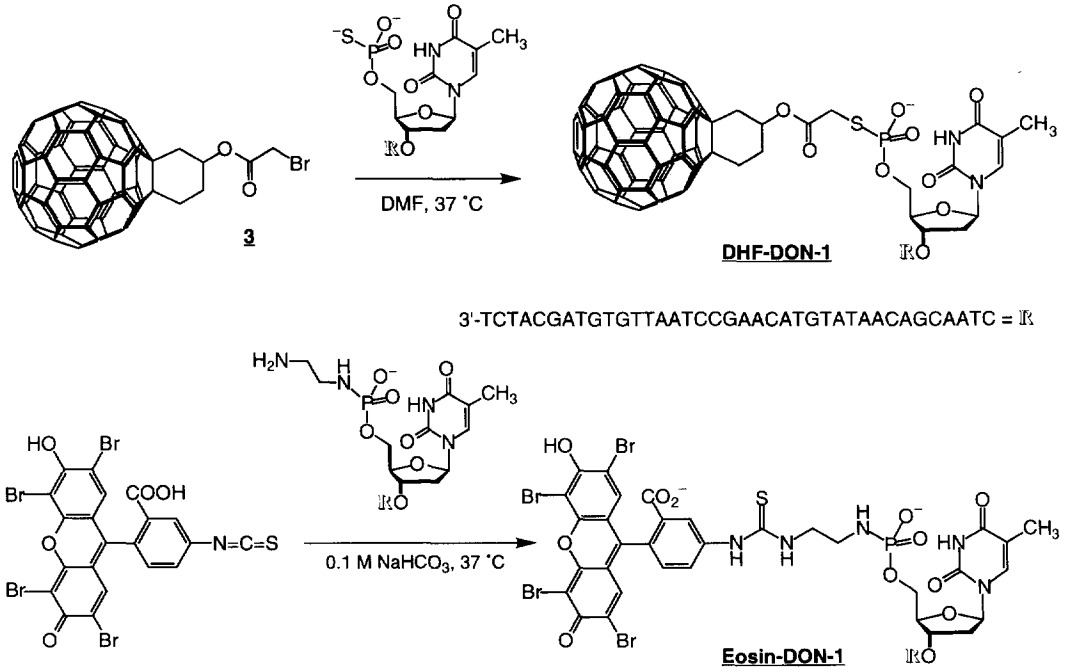


Figure 4. Comparison of the effect of the singlet oxygen quencher NaN_3 (concentrations shown on gel) on the modification of hybridized deoxyoligonucleotide promoted by DHF-DON-1 and Eosin-DON-1. Lanes **A-F** are targeted strand scission using DHF-DON-1 whereas lanes **G-K** are scissions with Eosin-DON-1. Lane **F** is the same run as lane **E**, but the film exposure time is the same as for lanes **G-K**. Lane **L** is irradiated DNA in absence of DHF-DON-1 or Eosin-DON-1.

carried out above 300 nm at 25 °C for two hours under air. The products were analyzed on a sequencing gel following reaction of the DNA with piperidine (Fig. 4). Piperidine treatment was necessary to allow visualization of alkali-labile sites caused by modification of the guanosines.

Scheme 2.



Cleavage at three guanosines in a single-stranded region near the sensitizing "warhead" of the DON-1 was observed with both derivatives. However, the reactivity observed with C_{60} was remarkably different from that observed with eosin in several respects. Although both derivatives caused preferential modification of single stranded guanosines, the reactivity of DHF-DON-1 was much greater than that of Eosin-DON-1 (lanes **F** and **K**, Fig. 4). However, photolysis in D_2O, which enhances $^1O_2$ reactions, had no apparent effect on the reaction with DHF-DON-1 but markedly increased the yield of modified guanosines with the eosin adduct (Fig. 5). In addition to its insensitivity to D_2O, the C_{60}-dependent reaction was not inhibited by $^1O_2$ quenchers such as sodium azide and DABCO at concentrations as high as 100 mM, in sharp contrast to the reaction with the eosin derivative (Fig. 4).[9] If the reaction were occurring with $^1O_2$, 50% inhibition would be expected at ~0.02 M azide. These experiments strongly suggest that the light-dependent DHF reaction with single-stranded guanosines is not mediated through $^1O_2$.

With Eosin-DON-1, the three single-stranded guanosines nearest to the 5'-terminus of the DON-1 showed equivalent reactivity (Fig. 4). In contrast, these residues showed very

different reactivity with the DHF-DON-1: the guanosine three positions downstream (G-223) from the terminus of the DON-1 was least reactive, G-225 was more strongly modified, and G-226 was the most extensively reacted. The difference in reactivity and the absence of influence of $^1O_2$ quenchers is evidence against the intermediacy of a diffusible species like $^1O_2$ in the C_{60}-mediated reaction, especially when compared to the eosin reactivity. Eosin differs from C_{60} in not being electron deficient, which should preclude Type I electron transfer reactions with this sensitizer. Guanines in double stranded regions, besides being more remote from the C_{60} framework, should be sterically inhibited from reacting with triplet dihydrofullerene; they would also be expected to be less accessible to $^1O_2$ attack because of the steric shielding from the stacked base pairs.

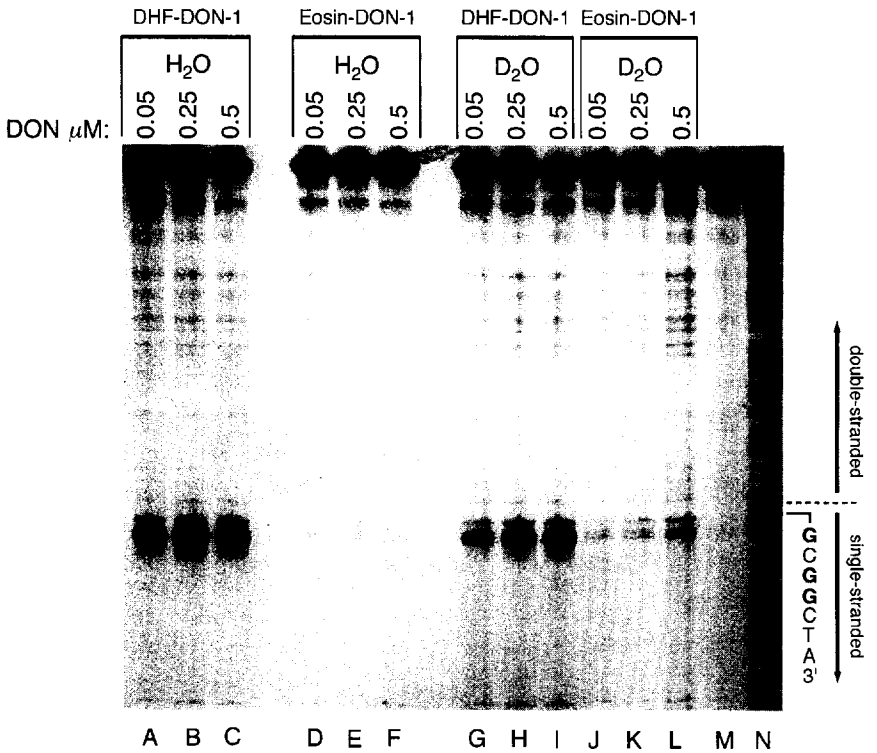


Figure 5. Comparison of the effect of H_2O and D_2O on the modification of hybridized deoxyoligonucleotide promoted by DHF-DON-1 and Eosin-DON-1. Concentrations of reactants are as indicated on the Figure. Lane **M** is irradiated DNA in absence of DHF-DON-1 or Eosin-DON-1. The dashed line indicates the adenosine complementary to the thymidine bearing C_{60}. Bold G's are the major sites of reaction.

The cause of sequence dependence of the C_{60}-mediated reaction is most likely direct electron transfer from guanosines to photoexcited dihydrofullerene. As noted above, the dihydrofullerene **2** is a particularly strong oxidant in its triplet state. Electron transfer

reactions between amines and $^3C_{60}$ proceed with second order rate constants that range from 5.2 x 10^9 M^{-1}·sec^{-1} for N,N,N',N'-tetramethyl-*p*-phenylenediamine to 7 x 10^4 M^{-1}·sec^{-1} for pyridine;[1b] the dihydrofullerene **2** reacts at only slightly lower equivalent rates.[5] One-electron oxidation of guanine has been shown to give the radical cation followed by depurination after further reaction with H_2O.[10]

Although dihydrofullerenes produce singlet oxygen efficiently, oxygen and electron-donors compete for the triplet (Fig. 1), as has been shown with other electron-poor sensitizers.[11] Because of the attachment of C_{60} in close proximity to the DNA strand, the high *local* concentration of the electron-rich guanine base must inhibit singlet oxygen formation. Dihydrofullerene photoreactions can also result in covalent adducts. Photolysis of *N,N*-diethylpropynylamine in presence of C_{60} gives a [2+2] cycloadduct in greater than 50% yield, presumably *via* electron transfer.[12] Similarly, the photochemical reaction of C_{60} with ketene silyl acetals produces α-substituted carboxylic esters[13] and irradiation of ethyl azidoformate in benzene gives [2+4] and [2+6] photoadducts of *N*-ethoxycarbonylazepine.[14] A photochemical reaction between C_{60} and guanosine could lead to crosslinks between DON-1 and the target DNA, but no evidence for such adducts formed *via* recombination of the intermediate radical species could be obtained.

Conclusion

This investigation demonstrates that the light-induced, site-selective modification of single-stranded regions in DNA by a C_{60} derivative does not proceed *via* singlet oxygen as the active species. The mechanism most likely involves single electron transfer followed by further hydrolytic degradation of the affected guanosines. The excellent selectivity of C_{60}-induced guanosine cleavage and its high activity compared to other sensitizers should make it a reagent of choice in DNA cleavage studies. We are also interested in further probing the mechanistic aspects of this reaction with simple model systems, for example using a C_{60}-linked protected guanosine.[6]

Experimental Section

1,2-(4'-Oxocyclohexano)buckminsterfullerene (1). A solution of 236.7 mg (1.67 mmol) of freshly redistilled 2-(trimethylsilyloxy)-1,3-butadiene in 20 mL of dry toluene was added over 1 h *via* a syringe pump to a refluxing solution of 1.00 g (1.39 mmol) of C_{60} in 350 mL of dry toluene under argon. After complete addition, reflux was continued for 24 h and the reaction mixture was cooled to 25 °C. The solvent was evaporated and the crude product was redissolved in the minimum amount of CS_2. This solution was loaded on top of a column packed with silica gel (flash) in hexanes and elution with hexanes/CS_2 1:1 afforded unconsumed C_{60} (127 mg, 13%). Further elution with toluene gave 562 mg (51%; 59% based on recovered C_{60}) of **1** as a shiny black crystalline material; ^1H NMR (500 MHz, CS_2/$CDCl_3$ 2 : 1,

–60 °C) δ (ppm) 3.54 (ddd, J = 20.0, 3.6, 2.5 Hz, 1H), 3.62 (ddd, J = 14.1, 4.2, 2.5 Hz, 1H), 3.77 (ddd, J = 20.0, 14.1, 4.2 Hz, 1H), 4.06 (d, J = 14.5 Hz, 1H), 4.24 (td, J = 14.1, 3.6 Hz, 1H), 4.89 (d, J = 14.5 Hz, 1H); ^{13}C NMR (90.6 MHz, CS_2/$CDCl_3$ 2 : 1) δ (ppm) 37.54, 39.67, 51.87, 62.42, 62.92, 134.96, 135.37, 140.27, 140.27, 141.60, 141.67, 141.75, 141.92, 141.98, 142.06, 142.55 (2 C's), 143.12, 144.55, 144.57, 145.04, 145.40, 145.42, 145.47, 145.52, 145.64, 146.21, 146.23, 146.42 (2 C's), 147.63, 155.05, 155.19, 207.53; UV/vis (CH_2Cl_2) λ_{max} (nm) 256 (ε 153,400), 263 (160,400), 269 (137,500), 310 (33,800), 322 sh (32,200), 404 sh (4,290), 433 (3,270), 472 sh (1,500), 526 sh (910), 701 (430); FT-IR (KBr) ν (cm^{-1}) 2940 (w), 1723 (s), 1459 (m), 1425 (m), 1420 (m), 1188 (m), 1184 (m), 728 (vs); MS (FAB) m/z (rel. intensity) 791 (42, M^+ + 1), 790 (58, M^+), 720 (100, C_{60}^+); HRMS: Calcd for $C_{64}H_7O$ (M^+ + 1): 791.0497; Found: 791.0529.

1,2-(4'-Hydroxycyclohexano)buckminsterfullerene (2). To a solution of 350 mg (0.443 mmol) of 1,2-(4'-oxocyclohexano)buckminsterfullerene (**1**) in 150 mL of dry toluene was added dropwise 1.1 mL (1.1 mmol) of a 1.0 M solution of diisobutylaluminum hydride in hexane at 25 °C. The reaction mixture was stirred further for 5 h at 25 °C and then treated with 40 mL of saturated NH_4Cl for 3 h. The organic layer was separated and the aqueous layer was extracted with toluene (2 x 50 mL). The combined organic phases were dried over Na_2SO_4 followed by evaporation of the solvent. Flash chromatography on silica gel with toluene afforded 326 mg (93%) of alcohol **2** as a shiny black crystalline solid; ^{1}H NMR (500 MHz, CS_2/$CDCl_3$ (2 : 1) δ (ppm) 1.99 (br s, 1H, OH), 2.68 (dddd, J = 15.4, 5.6, 4.2, 4.0 Hz, 1H), 3.26 (ddd, J = 13.6, 5.6, 4.0 Hz, 1H), 3.35 (dddd, J = 15.4, 11.7, 9.4, 4.0 Hz, 1H), 3.55 (dd, J = 13.4, 5.3 Hz, 1H), 3.68 (dd, J = 13.4, 9.1 Hz, 1H), 3.78 (ddd, J = 13.6, 11.7, 4.2 Hz, 1H), 5.21 (dddd, J = 9.4, 9.1, 5.3, 4.0Hz, 1H); ^{13}C NMR (125.7 MHz, CS_2/acetone-d_6 1 : 1) δ (ppm) 33.54, 35.94, 45.03, 63.18, 64.40, 66.87, 135.44, 135.72, 135.94, 136.32, 140.70, 140.74, 140.79, 142.05, 142.08, 142.13, 142.15, 142.47, 142.52, 142.55, 142.67, 143.00, 143.03, 143.06, 143.70, 145.14, 145.22, 145.28, 145.83, 145.85, 145.89, 145.92, 146.01, 146.16, 146.35, 146.44, 146.70, 146.87, 148.09, 157.35, 157.61, 157.65, 158.64; UV/vis (CH_2Cl_2) λ_{max} (nm) 249 sh (ε 87,600), 256 (93,400), 304 sh (32,200), 322 sh (30,100), 406 sh (3,800), 436 (2,980), 706 (240); FT-IR (KBr) ν (cm^{-1}) 3380 (br), 2928 (m), 2862 (w), 1511 (w), 1463 (w), 1427 (m), 1215 (m), 1184 (m), 1100 (w), 1069 (m), 1033 (m), 1009 (m), 960 (w), 767 (s); MS (FAB) m/z (rel. intensity) 792 (65, M^+), 720 (100, C_{60}^+); HRMS: Calcd for $C_{64}H_8O$ (M^+): 792.0575; Found: 792.0530. Anal. Calcd for $C_{64}H_8O \cdot 1/2C_6H_{12} \cdot 1/2H_2O$ (843.87): C, 95.36; H, 1.79; Found: C, 95.49; H. 1.73.

1,2-(4'-Bromoacetyloxycyclohexano)buckminsterfullerene (3): Alcohol **2** (110.9 mg, 0.14 mmol) and bromoacetyl bromide (1.41 g, 7.0 mmol, 0.6 mL) in 80 mL of toluene were heated under reflux for 30 min. Evaporation and flash chromatography on silica gel with toluene afforded 128 mg (quant.) of bromoacetate **3** as black crystals; ^{1}H NMR (500 MHz, CS_2/$CDCl_3$ 1:4) δ 6.19 (dddd, J = 9.6, 8.8, 5.5, 3.7 Hz, 1H), 3.98 (s, 2H), 3.81 (dd, J = 13.8, 8.8 Hz, 1H), 3.79 (ddd, J = 13.8, 10.3, 3.9 Hz, 1H), 3.73 (dd, J = 13.8, 5.5 Hz, 1H), 3.46 (dddd, J = 14.4, 10.3, 9.6, 3.9 Hz, 1H), 3.37 (ddd, J = 13.8, 6.8, 3.9 Hz, 1H), 2.82 (dddd, J = 14.4, 6.8, 3.9, 3.7 Hz, 1H); ^{13}C NMR (90.6 MHz, CS_2/$CDCl_3$ 1:4) δ 25.8, 29.1, 34.7, 40.1, 61.5, 63.3, 71.8, 135.0, 135.2, 135.3, 135.4, 140.1,

140.2, 140.3, 141.5, 141.6, 141.8, 141.86, 141.92, 142.0, 142.47, 142.52, 143.1, 144.5, 144.59, 144.64, 145.0, 145.2, 145.4, 145.5, 145.69, 145.75, 146.2, 146.35, 146.38, 147.6, 155.5, 155.7, 155.9, 156.6, 166.5; UV/vis (CH_2Cl_2) λ_{max} (nm) 246 sh (ε 101,700), 254 (107,100), 302 sh (38,100), 320 sh (35,200), 403 sh (5,480), 432 (4,040), 703 (408); FT-IR (KBr) ν (cm^{-1}) 2924.6 (s), 2851.2 (s), 1728.4 (s), 1421.7 (m), 1273.2 (s), 1103.4 (m), 756.2 (m), 526.6 (s); MS (MALDI) *m/z* (rel. intensity) 912 (18, M^-), 774 (20, M^- - $BrCH_2CO_2H$), 720 (100, C_{60}^-); HRMS: Calcd for $C_{66}H_9{}^{79}BrO_2$ (M^+): 911.9786; Found 911.9545.

DHF-DON-1: Bromoacetate **3** (200 µl of 0.6 mM solution in *amine-free* DMF) was reacted with thiophosphorylated DON-1 for two hours at 37 °C in DMF and overnight at 25 °C. After dilution with an equal volume of water, the solution was extracted with $CHCl_3$ to remove DMF and unreacted **3**. The product was purified by gel electrophoresis using a 15% denaturing acrylamide gel.

Eosin-DON-1: The ethylene diamine adduct was synthesized[15] using a ^{32}P-5-DON-1 and purified on a 15% denaturing polyacrylamide gel. The ethylenediamine derivative was reacted with 10-fold excess eosin 5-isothiocyanate in 0.1 M $NaHCO_3$ buffer (pH 9) at 25 °C for 1 h. Excess reagent was removed by passing the reaction mixture twice through G-50 spin columns. Measurement of the Eosin-DON-1 concentration in the eluent by radioactivity and visible absorption at 520 nm indicated that the reaction was stoichiometric.

Hybridization and Cleavage: The double stranded substrate was generated by digesting pGEM-4 plasmid with EcoR1, end filling with alpha ^{32}P-αATP and Klenow fragment, and finally digesting with Nhe I. The resulting fragment was then isolated by a 8% non-denaturing acrylamide gel. The restriction fragment (0.1 pmole) and the DHF-DON-1 or Eosin-DON-1 (5 pmoles) were concentrated to dryness and then re-suspended in 50 mM Tris/50 mM NaCl/H_2O *or* 50 mM Tris/50 mM NaCl/D_2O). Total volume was 20 µl. Calf thymus DNA (2 µg) was added as a carrier. This mixture was denatured at 90 °C for 5 min and reannealed at 25 °C for 1 h. Photolysis was carried out under air in Pyrex tubes (25 °C, 2 h, 300-W xenon lamp filtered with a water-cooled Pyrex cell). Samples were treated with 10% piperidine solution at 90 °C for 1/2 h to reveal the modification sites. The products were then analyzed on a 8% denaturing polyacrylamide gel.

Acknowledgments

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