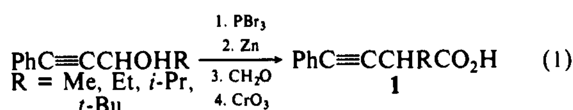
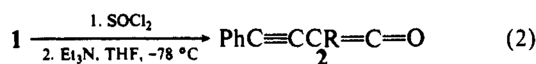
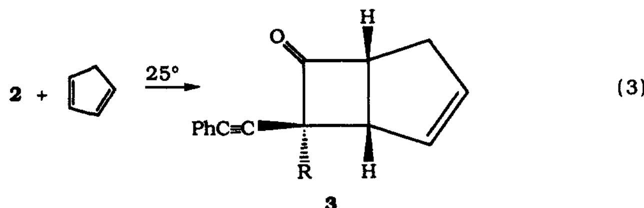
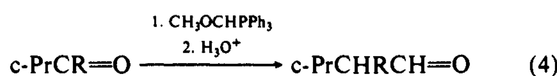
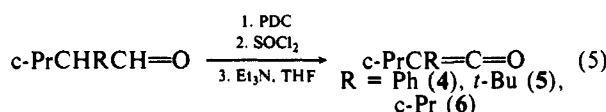




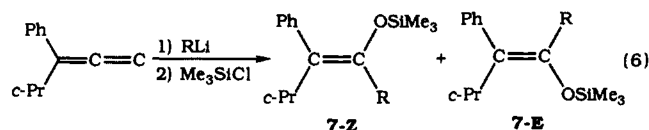


Cyclopropylketenes **4–6** prepared as shown in eqs 4 and 5 were stable enough for purification by distillation at room temperature and were characterized by their IR, NMR, UV, and mass spectra.

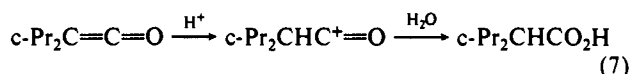




Reaction of **4** with *n*-BuLi followed by capture of the intermediate enolates with Me_3SiCl gave the stereoisomeric silyl enol ethers **7** with a 79/21 preference for formation of the *z* isomer resulting from nucleophilic attack syn to cyclopropyl, whereas the corresponding reaction of *t*-BuLi gave a 9/91 preference for attack anti to cyclopropyl (eq 6).



Results on the hydrolytic reactivity of cyclopropylketenes are summarized in Table I. The rate ratios for neutral hydrolysis $k(\text{c-Pr}_2\text{C=C=O})/k(\text{Et}_2\text{C=C=O}) = 1.2$ and $k(\text{PhC(c-Pr)=C=O})/k(\text{PhC}(i\text{-Pr})\text{=C=O}) = 500$ suggest that steric factors affect cyclopropylketene reactivity and that cyclopropyl is smaller than isopropyl. The rate ratio for acid hydrolysis $k(\text{Et}_2\text{C=C=O})/k(\text{c-Pr}_2\text{C=C=O}) = 21$ shows a rate-decelerating effect of cyclopropyl for protonation, by eq 7, as observed earlier for other alkenes.[11]



The persistent acylketene $t\text{-BuC(CO}_2\text{Et)=C=O}$[6g] reacted in H_2O to give $t\text{-BuCH(CO}_2\text{Et)CO}_2\text{H}$ with $k(\text{H}_2\text{O}) = 0.124\ \text{s}^{-1}$ and $k(\text{OH}^-) = 12.4\ \text{M}^{-1}\ \text{s}^{-1}$ in H_2O ($\mu = 0.1$, NaCl) at 25 °C. Thus this ketene is less reactive than $n\text{-BuCH=C=O}$,[2b,g] which has $k(\text{H}_2\text{O}) = 99.4\ \text{s}^{-1}$ and $k(\text{OH}^-) = 3.29 \times 10^4\ \text{M}^{-1}\ \text{s}^{-1}$.

In summary, molecular orbital calculations suggest that while conjugating substituents do not in general have a profound stabilizing effect on ketene, these species are also not prohibitively destabilized. The preparation and reactivity studies of alkynyl-, cyclopropyl-, and acylketenes are consistent with these conclusions.

Acknowledgment. Financial support by the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

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Inducible Alkylation of DNA Using an Oligonucleotide–Quinone Conjugate

Moneesh Chatterjee and Steven E. Rokita*

Department of Chemistry, State University of New York Stony Brook, New York 11794-3400

Received October 6, 1989

Messenger RNA has recently become a viable target for inhibiting the expression of a desired gene in vivo.[1] Compounds created for this purpose have drawn from the advances in site-specific modification of DNA[2,3] and the synthesis of metabolically stable oligonucleotides that can traverse cell membranes.[4] Although a large number of reactive appendages are available for related use in vitro,[3,5] only a limited set of these may be incorporated into protocols for in vivo study.[6] Naphthoquinones should neither impede the cellular uptake of appropriately modified nucleotides nor react with DNA indiscriminately; these compounds serve as the basis for our search of reactive components with a potential for in vivo application. Previously, methyl-1,4-naphthoquinone was shown to sensitize the selective oxidation of thymine.[7] We now report that related derivatives are also capable of alkylating DNA when held adjacent to a target sequence and subjected to UV irradiation.

5-Methyl-1,4-naphthoquinone[8] was condensed with 3-mercaptopropionic acid to provide a convenient method for attaching a sequence-directing oligonucleotide (Scheme I). The products of this reaction, two inseparable regioisomers (**1**),[9] were carried together throughout the following procedures. Treatment of the acid **1** with *N*-hydroxysuccinimide in the presence of 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide yielded the activated ester **2**; this was subsequently used to acylate a hexamethyleneamino linking arm that was coupled to the 5′ terminus of an oligonucleotide 15 bases in length. Preparation of the oligonucleotide plus linker relied completely on standard proce-

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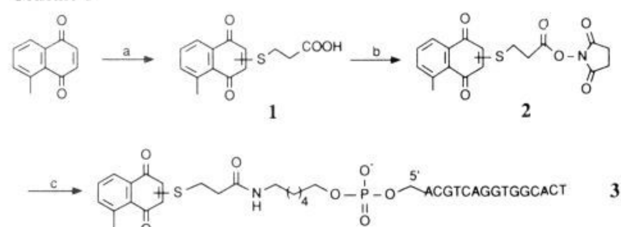
Scheme I[a]



[a] (a) 3-Mercaptopropionic acid, 70% EtOH, 4 °C; (b) *N*-hydroxysuccinamide, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide, DMF, 4 °C; (c) oligo-$(CH_2)_6NH_2$, 3-(*N*-morpholino)propanesulfonic acid, pH 7.5, 50% DMF, 4 °C.

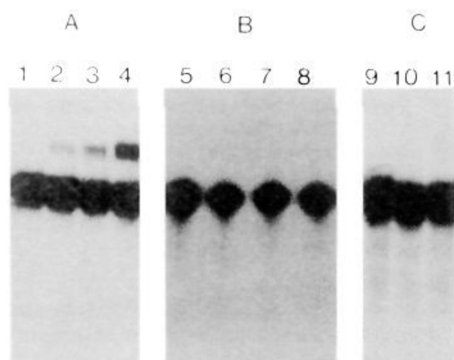


Figure 1. Autoradiograms of denaturing polyacrylamide (20%) gels used to identify the photochemical cross-link formed by duplex DNA. (A) The 20-base oligonucleotide **4** was labeled at the 5′ terminus with ^{32}P, annealed to **3**, and irradiated for 0.0, 0.5, 1.0, and 5.0 min (lanes 1, 2, 3, and 4, respectively). (B) A foreshortened derivative of **3** was annealed with labeled **4** and irradiated for 10 min (lane 5). The analogue of **3** was formed by aerobic condensation of 2-methyl-1,4-naphthoquinone and a hexamethylenethio group placed at the 5′ end of a sequence corresponding to **3**. The parent sequence of **3** was also annealed with **4** and irradiated for 10 min in the absence of **1** (lane 6) and in the presence of 100-fold (lane 7) and 1000-fold (lane 8) excesses of **1**. (C) **3** was preirradiated for 10 min, annealed with labeled **4**, and further irradiated for 0.0, 5.0, and 10 min (lanes 9, 10, and 11). In every case, the oligonucleotides (2.2 μM of each strand) were annealed in 1 mM potassium phosphate, pH 7, by slow cooling of their heat-denatured mixture (65 °C) to ambient temperature over 3–4 h. Individual samples (10 μL, 10 nCi per lane) were placed at the focal point of a 150-W Xe arc lamp and irradiated in Pyrex tubes screened by a long-pass 345-nm filter. After photolysis, samples were lyophilized and resuspended in 80% formamide for electrophoresis.

dures of solid-phase phosphoramidite chemistry. The final product, **3**, was then purified by reverse-phase chromatography. A target strand of DNA, d(AGTGCCACCTGACGTCTAAG) **4**, was prepared in parallel, and each strand was appropriately labeled with ^{32}P.[10a,b]

No oxidative strand scission was detected after the target and reactant strands were annealed, irradiated aerobically with wavelengths of greater than 345 nm, and analyzed by electrophoresis (in 7 M urea) and autoradiography (Figure 1A). Instead, a material migrating more slowly than the 20-base target sequence accumulated over the irradiation times of 0.5, 1, and 5 min. This species was evident when a ^{32}P label was appended to the 3′ or 5′ terminus of **4** or to the 3′ terminus of **3**. The only detectable product of photolysis therefore contained both complete strands of DNA in a nondissociable and likely cross-linked form. This reaction was unique to these photochemical conditions; no similar modification was apparent after treating the annealed complex with heat (90 °C, 10 min) or reducing agents. Substituting **4** with a noncomplementary sequence, d(CACGGGAACGCATG), similarly provided no detectable products after photolysis. DNA alkylation was also prevented when a shorter linking unit was used to join the site-directing oligonucleotide with an otherwise active quinone (Figure 1B). In addition, the untethered derivative **1** was unable to induce photochemical cross-linking of the two parent strands even when used in a 1000-fold excess over DNA. This result was anticipated since methylnaphthoquinone is not a bifunctional reagent like psoralen.[11] Covalent modification was detected as a cross-link in these experiments only because **1** had already been attached to one of the strands prior to irradiation.

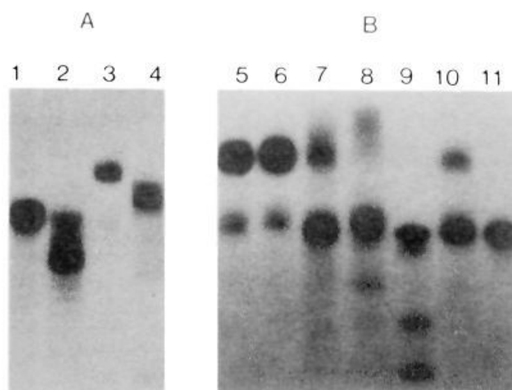


Figure 2. Chemical and enzymatic characterization of the cross-linked duplex formed by irradiation of **3** + **4**. The material under investigation was isolated by electrolution from gels that were equivalent to those used in this analysis (20% polyacrylamide, 7 M urea). (A) Annealed but unirradiated **3** + **4** was incubated for 0 (lane 1) and 5 min (lane 2) in the presence of 5 units of klenow fragment (BRL) at 37 °C. This procedure was repeated with the cross-linked species for 0 (lane 3) and 5 min (lane 4). (B) The covalently modified duplex was also treated with (lane 5) β-mercaptoethanol (10 mM, 37 °C, 30 min), (lane 6) 0.25 M piperidine formate (pH 2, 37 °C, 60 min), (lane 7) 0.1 M triethylammonium acetate (pH 11, 90 °C, 60 min), and (lane 8) 1 M piperidine (90 °C, 30 min). As a control for alkali-induced scission, **4** was modified with dimethyl sulfate and subsequently hydrolyzed with piperidine (lane 9).[13] Purified cross-link (lane 10) and annealed **3** + **4** (lane 11) were treated with 0.26 M sodium borohydride in buffer (10 mM potassium phosphate, pH 8.3, 5 mM NaCl, 50 mM thymidine) for 60 min at room temperature followed by 0.25 M piperidine formate (pH 2) for 80 min at 40 °C.[14] This mixture was lyophilized and then incubated with 1 M piperidine at 90 °C for 30 min. All samples were finally lyophilized and resuspended for electrophoresis as before.

Extensive irradiation of the hybridized complex **3** + **4** produced at most a 20% conversion to the cross-linked structure, yet the mixture of modified and unmodified material created during the initial 10 min of photolysis persisted unchanged after additional exposure to UV light (>2 h). Preirradiation of **3** prior to hybridization with **4** dramatically inhibited the formation of the product expected upon further irradiation of the duplex (Figure 1C). This suppression of activity would be consistent with a rapid and competing photodegradation of the attached 5-methyl-1,4-naphthoquinone. The formation of the cross-linked material, however, remained unaffected by the addition of mannitol (100 mM) or by the substitution of D_2O for H_2O. These results in turn suggest that diffusible oxygen intermediates such as hydroxyl radical or $^1O_2$ are not involved in the chemistry described here.

The cross-linked product of irradiation was next separated from the reaction mixture by preparative electrophoresis[10c] and subjected to enzymatic and chemical analysis. The annealed and cross-linked forms of **3** + **4** reacted equivalently with the klenow fragment of DNA polymerase.[12] The exonuclease activity of this enzyme did not appear to be inhibited during hydrolysis of the overhanging nucleotides (Figure 2A). The newly formed bond between DNA strands also proved to be quite stable under alternative exposure to excess acid or thiol (Figure 2B). The latter treatment with β-mercaptoethanol was included in an attempt to displace the

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thioether bond used to hold the quinone onto oligonucleotide **3**.

If the most nucleophilic atom (guanine N7) of DNA had been derivatized to form the cross-link, then strand scission at the site of modification should have been induced under alkaline conditions.[13] No such fragmentation was noted. Instead, covalent attachment between strands was reversed. Treatment with triethylamine or piperidine (90 °C) transformed the cross-linked duplex back to a species migrating in a manner similar to **4** (Figure 2B). In addition, a related conversion was unavoidable during the purification of the photochemical product and this decomposition is evident in lanes 5 and 6 of Figure 2B.

Finally, Becker and Wang[14] had shown that a protocol using sodium borohydride would cleave the DNA backbone at a thymine or cytosine with a saturated C5–C6 bond. A similar analysis performed on the cross-linked material described here was insufficient to cause scission at the site of modification. The surprising profile of this quinone-based photoalkylation might then suggest that an equally unusual bond reorganization occurred during irradiation. This process would likely include the formation of at least one new carbon–carbon bond and may be reminiscent of a previously characterized dimerization of naphthoquinones.[15]

Although covalent cross-linking between DNA and free methyl-1,4-naphthoquinone had not been previously detected after photolysis, such a reaction can be induced when the quinone is forced to remain in close proximity to a target sequence of DNA. This alkylation immortalizes the hybridization of a DNA probe and may be expected to serve as a basis for future protocols in nucleic acid manipulation. Whether or not other species may also form distinct products when forced to surround DNA remains to be demonstrated.

Acknowledgment. This research was supported in part by the University Exploratory Research Program of The Procter and Gamble Company, The Anna Fuller Fund, and The American Cancer Society (IN164A).

Supplementary Material Available: Preparation of oligonucleotides and the syntheses of **1–3** (2 pages). Ordering information is given on any current masthead page.

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Carbenes and the O–H Bond: Spectroscopic Evidence for Protonation of Diarylcarbenes To Give Diarylcarbenium Ions

Wolfgang Kirmse* and Jörg Kilian

Fakultät für Chemie, Ruhr-Universität Bochum
D-4630 Bochum, Federal Republic of Germany

Steen Steenken*

Max-Planck-Institut für Strahlenchemie
D-4330 Mülheim, Federal Republic of Germany

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The reaction of diarylcarbenes with O–H bonds[1] plays a central and critical role in the assignment of chemical and physical properties to specific spin states. The interpretation of absolute rates[2] suffers from mechanistic ambiguity. About 30 years ago

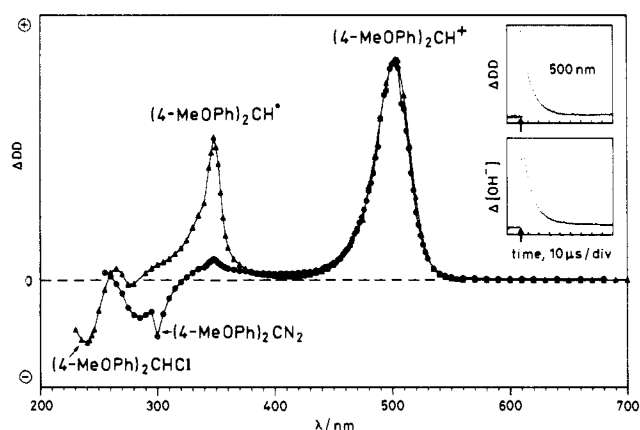
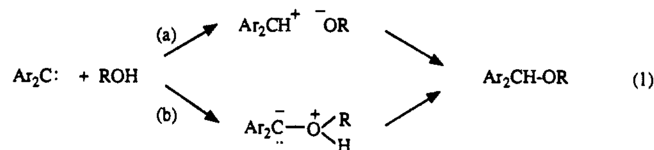


Figure 1. Changes in optical density (OD) observed on 248-nm photolysis of 0.9 mM $(4\text{-MeOC}_6H_4)_2CN_2$ in acetonitrile containing 2% of water (v/v), circles (measured at 40 ns after the pulse), and of 0.13 mM $(4\text{-MeOC}_6H_4)_2CHCl$ in acetonitrile containing 0.2 M CH_2Cl_2, triangles (recorded at 1.6 µs). The spectra were normalized to give the same OD at 500 nm. The radical $(4\text{-MeOC}_6H_4)_2CH^\bullet$ originates from H abstraction from the solvent by the triplet carbene and from photohomolysis of the chloride, respectively. Insets: Comparison of the decay of Ar_2CH^+, monitored at 500 nm (upper inset), with the decrease of conductance (lower inset), due to neutralization of HO^- by H^+ from the (rate-determining) reaction of Ar_2CH^+ with H_2O. The cation was produced by LFP of 0.12 mM $(4\text{-MeOC}_6H_4)_2CN_2$ in acetonitrile–water, 1:2 (v/v), containing 1 mM NaOH. Note: In this particular figure Ph represents C_6H_4.

we suggested, on the basis of indirect evidence, that diphenylcarbene abstracts a proton from alcohols to form the diphenylcarbenium ion,[3] eq 1, path a. On the other hand, Bethell et al., studying thermal reactions of diphenyldiazomethane with ROH and their isotope effects, favored the electrophilic attack of diphenylcarbene on the oxygen atom,[4] eq 1, path b.



Product and label distributions have revealed protonation of 7-norbornenylidenes,[5] (benzo)cycloheptatrienylidenes,[6] cyclopentenylidenes,[7] and arylbenzylcarbenes.[8] Analogous techniques are not applicable to diarylcarbenes. The reactivities of alcohols toward diphenylcarbene were found to parallel their acidity.[4,9] The kinetic range, however, was much smaller than that observed for typical nucleophilic carbenes.[10] In this communication we report the spectroscopic detection of diarylcarbenium ions, following the generation of diarylcarbenes by laser flash photolysis (LFP) in protic solvents.

Diarylcarbenium ions have recently become accessible for time-resolved measurements by heterolytic photocleavage of Ar_2CHX.[11-17] The data thus gathered are used in the present

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