Synthesis of a Benzene Metabolite Adduct, 3″-Hydroxy-1,N^2-benzetheno-2′-deoxyguanosine, and Its Site-Specific Incorporation into DNA Oligonucleotides

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p-Benzoquinone (*p*-BQ) is a stable metabolite of benzene and a number of other drugs and chemicals. 2′-Deoxyguanosine was allowed to react with *p*-BQ in aqueous solution (pH 4.5, 7.4, and 9.3). At pH 7.4 and 9.3 one major product was found in low yield; at pH 4.5 no product was detected. In nonaqueous conditions (DMF or DMSO, in the presence of K_2CO_3), an unstable intermediate with two *p*-BQ moieties was found which slowly converted to the product formed in aqueous solution. These products were isolated by silica gel, column chromatography, or reverse-phase HPLC and characterized by UV, ^{1}H NMR, FAB-MS, and electrospray MS. The major stable product of the reaction of dG with *p*-BQ is an exocyclic compound, 3″-hydroxy-1,N^2-benzetheno-2′-deoxyguanosine (*p*-BQ-dG). Incorporation of the adduct into oligonucleotides requires the protection of three hydroxyl groups (C7, 5′, 3′) and the amino group at N5. The exocyclic hydroxyl and the amino group were protected by acylation after protecting the 5′- and the 3′-hydroxyl groups of the sugar moiety by 4,4′-dimethoxytrityl and a cyanoethyl *N*,*N*-diisopropylphosphoramidite group, respectively. This is the first time the fully protected phosphoramidite of *p*-BQ-dG has been prepared and used in the synthesis of site specifically modified oligonucleotides. After deprotection with 1,3-diazabicyclo[5.4.0]undec-7-ene (DBU), in ethanol, oligomers purified by gel electrophoresis and HPLC. Enzymatic hydrolysis and analysis by HPLC confirmed the presence of *p*-BQ-dG in the oligomers. These oligomers are now under investigation for their biochemical properties.

Introduction

Humans are exposed to benzene from many environmental sources, including automobile exhaust and cigarette smoke, and in industries such as rubber manufacture, and crude oil, gasoline, and chemical manufacturing. Benzene causes acute leukemia in humans (*1*) and bone marrow toxicity (*2*). *p*-Benzoquinone (*p*-BQ)[1] is a stable metabolites of benzene (*3*−*6*), as well as of a number of drugs and chemicals (*7*−*9*). Benzene must be metabolized to exert its toxic and leukemogenic effects (*10*−*12*). The initial step in one pathway is oxidation of benzene by cytochrome P450 to form benzene oxide, which further yields phenol, and then hydroquinone which in turn is oxidized to *p*-BQ. The latter accumulates in the bone marrow (*13,14*), which is the site of the observed myelotoxic and leukemogenic effects (*12,15*).

p-BQ is one of a large family of α,β-unsaturated carbonyl compounds including acrolein, crotonaldehydes, malondialdehyde, and methyl vinyl ketone. In most cases, both mono- and diadducts are formed when these compounds are reacted with deoxyguanosine via Michael addition reaction. Shapiro *et al.* (*16*) reported the formation of more than one product resulting from the reaction of the α,β-unsaturated carbonyl compounds glyoxal and acrolein with 2′-deoxyguanosine.

Several adducts have been identified from the *in vitro* reaction of *p*-BQ with nucleosides, nucleotides, and DNA (*17*−*21*); however, the relationship between the adduct structure and biological effects has not yet been established. Both dC and dA form single exocyclic benzetheno adducts (*19,20*). Multiple adducts have been reported (*17,18*) with deoxyguanosine in *p*-BQ-treated DNA. Only one adduct has been characterized resulting from reaction of dG with *p*-BQ at neutrality (*17,18*). The total modified bases formed are less than $1/10^6$ bases in DNA from human bone marrow or HL-60 cells when treated with *p*-BQ *in vitro* (*22*). With such low levels of reaction in cells, ^{32}P-postlabeling was the only method of detection (*19*−*21*).

In order to study how *p*-BQ exerts its biological effects, it was necessary to develop syntheses of both the dA and dC adducts and their phosphoramidites. We have recently reported the large-scale synthesis of *p*-BQ-dC and *p*-BQ-dA adducts and the site-specific incorporation of their phosphoramidates into a defined deoxyoligonucleotides (*23*). In this work, we report the preparative-scale synthesis of 3″-hydroxy-1,N^2-benzetheno-2′-deoxyguanosine (*p*-BQ-dG) and its phosphoramidite and the successful site-specific incorporation into DNA oligonucleotides. These oligonucleotides are now being studied for their effect on replication, mutation, and repair *in vitro*.

Materials and Methods

Chemicals and Reagents. Caution: *p*-BQ is highly toxic and should be handled carefully. Many organic solvents used in this work should be stored and used in a well-ventilated hood. 2′-Deoxyguanosine (dG) was purchased from Sigma Chemical Co. (St. Louis, MO). *p*-BQ, DMSO, 4,4′-dimethoxytrityl chloride

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[1] Abbreviations: *p*-BQ, *p*-benzoquinone; dG, 2′-deoxyguanosine; DBU, 1,3-diazabicyclo[5.4.0]undec-7-ene; BAP, bacterial alkaline phosphatase; SVDE, snake venom phosphodiesterase; FAB, fast-atom bombardment; DMTCl, 4,4′-dimethoxytrityl chloride; DMF, *N*,*N*-dimethylformamide.

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(DMTCl), pyridine, triethylamine, 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, isobutyric anhydride, silica gel, toluene, hexane, and 1,3-diazabicyclo[5.4.0]undec-7-ene (DBU), were from Aldrich Chemical Co. (Milwaukee, WI). Methanol, methylene chloride, benzene, acetonitrile, and ethyl acetate were from J. T. Baker Inc. (Phillipsburg, NJ). Potassium carbonate, phosphorus pentoxide (P_2O_5), and thin-layer chromatography (TLC) silica plates were purchased from EM Science (Gibbston, NJ), and calcium hydride was from Fluka Chemika (Ronkonkoma, NY). Bacterial alkaline phosphatase (BAP) and snake venom phosphodiesterase (SVDE) were from Pharmacia Biotech Inc. (Alameda, CA). The PAC phosphoramidates were purchased from Pharmacia (Piscataway, NJ). All solvents were dried by distillation over CaH_2.

Proton (400 MHz) NMR spectra were recorded in DMSO-d_6 as solvent, and D_2O exchanges were carried out to assign exchangeable protons, unless otherwise indicated. NMR spectra were recorded using a Brucker AM400 spectrometer and are reported in parts per million (ppm) relative to an internal standard of tetramethylsilane. ^{31}P NMR spectra were recorded on a Bruker AM400 spectrometer, and the chemical shifts are reported relative to an external standard of phosphoric acid. Fast-atom bombardment (FAB) spectra were obtained on a VG70 SE Instrument, and glycerin or thioglycerin was used as a matrix. Electrospray mass spectra were obtained on a VG Bio-Q Instruments mass spectrometer. Loop injections of the sample were made into solvent (50:50, acetonitrile/water) flowing into the electrospray source (4 mL/min). Analytical and semipreparative HPLC were conducted using a Hewlett Packard 1050 photodiode array detector and quaternary gradient pump (solvent delivery system).

HPLC. Solvent systems included solvent A (acetonitrile), solvent B (triethylammonium acetate; 0.1 M, pH 7.0), and solvent C (potassium phosphate buffer; 0.01 M, pH 4.5). System 1: After gel electrophoresis, oligonucleotides were purified using a C18 column (150 × 3.9 mm, 5 μm, Millipore Corp.). The initial concentration of 5% solvent A, 95% solvent B was maintained for 10 min, and then solvent A was increased linearly to 29% over 20 min at a flow rate of 1 mL/min. System 2: Analysis of the enzyme digest of the oligonucleotides was performed with a supelcosil LC-18-DB column (2.5 × 0.46 cm, 5 μm, Supelco, Inc.) and 0% solvent A, 100% solvent C. Solvent A was linearly increased to 12% over 30 min, then to 40% over 15 min where it was held for 10 min at a flow rate of 1 mL/min. System 3: The progress of the reaction of *p*-BQ with dG was monitored using a Supelco C18 reverse-phase semipreparative (250 × 10 mm, 5 μm) and 0% solvent A, 100% H_2O. Solvent A was increased linearly to 30% over 30 min at a flow rate of 2 mL/min, and then to 70% over 15 min at room temperature.

Ultraviolet spectra were recorded on a Hitachi U-2000 spectrophotometer using 0.5 cm cuvettes. TLC was performed on EM5735/7 silica gel 60, F_{254} plates. Column chromatography was performed using silica gel 60 with elution under pressure.

Chemical Syntheses. Preparation of 3′′-Hydroxy-1,*N*^{2}-benzetheno-2′-deoxyguanosine (*p*-BQ-dG) (1). dG (500 mg, 1.87 mmol) was dissolved in 20 mL of dry DMSO or DMF and very fine potassium carbonate (316 mg, 2.28 mmol) was added at room temperature. After 15 min, *p*-BQ (414 mg, 3.82 mmol) was added in a single dose at room temperature and stirred for 7 h. TLC showed the formation of two products and some unreacted starting material. A further amount of a very fine K_2CO_3 (316 mg, 2.28 mmol) and *p*-BQ (207 mg, 1.41 mmol) was added and stirred for another 15 h. HPLC analysis showed only traces of starting material, one major product, and traces of an unstable intermediate. TLC on silica gel (MeOH/CH_2Cl_2; 15:85) showed the formation of a less polar product (R_f 0.30) and some very polar material in the bottom of the TLC which was attributed to decomposition. The reaction mixture was absorbed on silica gel (5−25 μm), then applied to a silica column (18 × 4 cm of silica), and eluted with 15% methanol in methylene chloride. After analysis of all fractions by TLC, the fractions containing the desired product were evaporated to dryness to yield a light brown solid material (156 mg, 28%): mp: 275 °C (the product decomposed to a black material). UV: product (**1**) (pH 6.5) λ_{max} 233, 272, 328 nm; (pH 12) λ_{max} 251, 291, 361 nm; (pH 1) λ_{max} 235, 271, 284, 329 nm. These data are similar to those reported by Jowa *et al*. (17). FAB/MS (positive ion): *m*/*z* 380 (15) $(M + Na)^+$, 358 (30) MH^+, 264 (12) $(BH + Na)^+$, 242 (100) BH_2^+. ^1H NMR (DMSO-d_6, 400 MHz): δ 9.485 (S, 1H, exch, HO-7), 8.124 (S, 1H, H-2), 8.022 (d, 1H, J = 2.3 Hz, H-6), 7.230 (d, 1H, J = 8.6 Hz, H-9), 6.866 (d, 1H, J = 2.35 Hz, H-8), 6.531 (′S, 1H, exch, HN-5), 6.257 (t, 1H, J = 6.5 Hz, H-1′), 5.329 (S, 1H, exch, HO-3′), 5.011 (S, 1H, exch, HO-5′), 4.369 (S, 1H, H-4′), 3.849 (m, 1H, H-3′), 3.518 (m, 2H, H-5′), 2.608 (m, 1H, H-2′), 2.270 (m, 1H, H-2′′).

The above reaction was repeated in DMF/K_2CO_3 and produced the same products as shown by the monitoring of the reaction by TLC and HPLC (Figure 1). In another reaction, the base, K_2CO_3, was replaced by NaOH pellets in DMSO and gave the major product and only traces of the intermediate product throughout the reaction course.

The reaction of dG with *p*-BQ was also performed in aqueous solutions, at pH 4.5, 7.4, and 9.3 using potassium phosphate buffers. HPLC analysis of these reactions after 24 h showed that the reaction at pH 7.4 and 9.3 produced the same stable product as in nonaqueous solutions (DMSO/K_2CO_3) and only traces of the unstable intermediate, but at pH 4.5 no reaction was detected.

Small-Scale Reaction of 2′-Deoxyguanosine with *p*-BQ. 2′-Deoxyguanosine (40 mg, 0.145 mmol) was dissolved in 2 mL of dry DMF, and potassium carbonate (38 mg, 0.28 mmol, 2 equiv) was added and stirred for 20 min at room temperature and then *p*-benzoquinone (18 mg, 0.168 mmol, 1.2 equiv) dissolved in 1 mL of dry DMF was added dropwise. The reaction color changed from colorless to yellow to green to dark. The reaction mixture was allowed to continue stirring at room temperature while it was monitored by HPLC at increasing times (5 min, 30 min, 1 h, 2.5 h, 3.5 h, 4.5 h, 5.5 h, 6.3 h, 12 h, 22.5 h) by using system 3. HPLC analysis showed the formation of an intermediate product which slowly converted to the stable desired product overnight (Figure 1). Analytical data for the intermediate is as follows. UV: unstable intermediate (pH 6.5) λ_{max} 238, 273, 334 nm; (pH 12) λ_{max} 250, 298, 366 nm; (pH 1) λ_{max} 240, 286, 284, 333 nm. Electrospray MS (positive ion): *m*/*z* 486 (74) $(M + Na)^+$; 464 (20) MH^+; 370 (48) $(BH + Na)^+$; 348 (100) BH_2^+. Electrospray MS (negative Ion): *m*/*z* 462 (100) $(M - H)^+$.

5′-*O*-(4,4′-Dimethoxytrityl-3′′-hydroxy-1,*N*^{2}-benzetheno-2′-deoxyguanosine (2). Compound (**1**) (50 mg, 0.14 mmol) was coevaporated from dry pyridine three times and then dissolved in 5 mL of dry pyridine and treated with dimethoxytrityl chloride (DMTCl) (142 mg, 0.42 mmol, 3 equiv) at room temperature with stirring. After 6 h, TLC on silica gel (10% MeOH + CH_2Cl_2) showed that all the starting material was consumed and a new product was formed which was less polar than the starting material. The reaction was stopped, and the pyridine was reduced to about 1 mL, then 2 mL of methylene chloride was added, and the resulting mixture was applied to a silica gel column (2 × 18 cm) equilibrated with CH_2Cl_2/MeOH/Et_3N (97:2:1), as eluant. About 200 mL of this solvent was run on the column to remove any material of low polarity, as well as any traces of pyridine. The polarity of the solvent was then increased by using CH_2Cl_2/MeOH/Et_3N (89:10:1). After analyzing all fractions by TLC, the fractions containing the product were collected and evaporated to give an off-white foamy product (72 mg, 78%): R_f (10% MeOH in CH_2Cl_2) 0.37; mp 135−137 °C. FAB/MS (positive ion): *m*/*z* 660 (42) MH^+, 242 (16) BH_2^+. ^1H NMR (DMSO-d_6, 400 MHz): δ 9.569 (S, 1H, exch, HO-7), 8.012 (S, 1H, H-2), 7.301 (d, 1H, J = 7.3 Hz, H-6), 7.178 (m, 9H, 8 aromatic H + H-9), 6.891 (d, 1H, J = 11 Hz, H-8), 6.7609 (m, 5H, aromatic H), 6.295 (t, 1H, J = 7.2 Hz, H-1′), 5.437 (d, 1H, J = 4.7 Hz, exch, HO-3′), 4.406 (m, 1H, H-4′), 3.946 (m, 1H, H-3′), 3.701 (m, 2H, H-5′), 3.649 (2s, 6H, OCH_3), 2.608 (m, 1H, H-2′), 2.270 (m, 1H, H-2′′).

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(diisopropylamino)(2-cyanoethoxy)phosphino]-3′′-hydroxy-1,*N*^{2}-benzetheno-2′-

deoxyguanosine (3). Compound **2** (125 mg, 0.19 mmol) was dried over P_2O_5 and NaOH pellets in a vacuum desiccator for 24 h and then was coevaporated with a mixture of dry CH_2Cl_2 and dry benzene, prior to reaction. To a mixture of compound **2** and dry Et_3N (1 mL to aid the solubility) in 12 mL of dry CH_2Cl_2 under N_2 was added 2′-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (53 mg, 36 mL, 0.226 mmol, 1.2 equiv) dropwise over 1 min at −72 °C and with vigorous stirring. The reaction was stirred at −72 °C for a further 20 min. The temperature was then slowly increased to room temperature and stirred for another 45 min and then analyzed by TLC (CH_2Cl_2/MeOH, 90:10) prerun in $CH_2Cl_2CH_3CO_2C_2H_5Et_3N$ (60:40:5), which showed that all the starting material was consumed and a new less polar product was formed. The reaction was stopped by adding 1 mL of dry methanol dropwise at −20 °C, and after about 3 min, all the solvent was evaporated under vacuum, without using any heat, to give a yellowish solid material. This material was dissolved in dry CH_2Cl_2 and washed twice with a 5% solution of $NaHCO_3$ and then once with a saturated solution of NaCl, dried over Na_2SO_4, filtered and evaporated under vacuum, and coevaporated twice in a mixture of dry CH_2Cl_2 and dry benzene to give a light brown gum product (113 mg, 69%). TLC analysis of this product on silica gel (CH_2Cl_2/MeOH; 90:10) gave R_f = 0.27 and ^{31}P NMR ($CDCl_3$, 400 MHz) δ 145.857 with reference to phosphoric acid as the external standard. The ^{31}P signals for the two diastereoisomers which comprise phosphoramidite **3** were unresolved.

Acylation of 5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-[(diisopropylamino)(2-cyanoethoxy)phosphino]- 3′′-hydroxy-1,*N*²-benzetheno-2′-deoxyguanosine (3). A 75 mg sample of compound **3** was coevaporated three times from dry pyridine under a very strong vacuum and then was dissolved in 5 mL of dry pyridine, and 100 mL of isobutyric anhydride was added dropwise at ambient temperature with stirring. After the mixture was stirred for 24 h, TLC on silica gel (CH_2Cl_2/MeOH; 90:10) showed that the starting material was fully consumed and gave a product of very low polarity at R_f 0.83. The reaction was stopped by evaporating all pyridine and coevaporated from dry benzene twice. The product was dissolved in 1 mL toluene and added dropwise to dry hexane at −42 °C (CH_3CN/CO_3) to precipitate. The product was centrifuged for 2 min, washed with fresh hexane at −42 °C, and again centrifuged for 2 min. The hexane was removed and the product was dissolved in dry pyridine and coevaporated three times from dry C_6H_6 to give a yellow gum product (71 mg, 81%). This product was dried over P_2O_5 and NaOH pellets in a vacuum desiccator overnight at room temperature and used to synthesize the DNA oligomers. ^{31}PNMR: ($CDCl_3$), δ 148.003, this signal for the two diastereoisomers which represent phosphoramidite **4** was unresolved.

Solid-Phase Synthesis of the Oligonucleotides. Synthesis of the oligonucleotides was done on an Applied Biosystem 394 automated DNA synthesizer on a 1 μmol scale using phosphoramidite chemistry on a controlled pore glass (CPG) support with an aminopropyl succinate linker. Phenoxyacetyl phosphoramidites (PAC) were used to synthesize the DNA oligomers. The synthesis followed standard protocol of the DNA synthesizer for phosphoramidite chemistry, except for the step in which the modified deoxynucleoside was inserted. In this step, the time of coupling was increased to 30 min, in order to maximize the coupling efficiency (96%). The oligonucleotides were recovered as the 5′-dimethoxytritylated derivatives and deprotected under nonaqueous conditions with 1,8-diazobicyclclo-[5.4.0]undec-7-ene (DBU) in ethanol (200 proof). The resin was treated with 2 mL of DBU/ethanol (10:90) under anhydrous conditions with occasional shaking. After 24 h at room temperature, the resin was removed by centrifugation, and the pellet washed with ethanol twice (0.5 mL). The combined supernatant and washings were evaporated under reduced pressure to remove all ethanol. Water was added (3 mL), and immediately the pH was adjusted to 7.0 by adding 180 μL of a solution of 50% acetic acid and stirring. This was desalted and detritylated by 2% TFA in water using two Sep-Pack Cartridges (Waters Associates) as described by the supplier. The eluted fraction from the cartridges containing the desired oligomer together with the failure sequences were concentrated and further purified by 20% polyacrylamide gel electrophoresis and HPLC. The following DNA oligomers were made.

1: 5′-CCGCTA**X**CGGGTACCGAGCTCGAAT-3′

2: 5′-**X**CGGGTACCGAGCTCGAAT-3′

X = *p*-BQ-dG

The defined oligonucleotides (0.5 A_{260} units) were dissolved in 44 mL of freshly deionized water, 0.8 mL of 1 M $MgCl_2$, and 3.5 mL of 0.5 M Tris-HCl buffer (pH 7.5) and digested with snake venom phosphodiesterase (2.5 mL) and bacterial alkaline phosphatase (4.0 mL) at 37 °C for 16 h. The digested mixture was analyzed on a C-18 reverse-phase HPLC Supelcosil column (250 × 4.5 mm) using system 2. Quantitation of the deoxynucleosides was on the basis of integration of the peak areas at 260 nm.

Results and Discussion

Synthesis of 3′′-Hydroxy-1,*N*²-benzetheno-2′-deoxyguanosine (*p*-BQ-dG, 1). In 1986, Jowa *et al.* (*17*) reported the formation and characterization of the exocyclic adduct, *p*-BQ-dG, formed from reaction of dG with *p*-BQ in aqueous solution at pH 7.2. However, the yield was low (∼1%) (*18*). The same adduct was also detected in DNA reacted with *p*-BQ, but to such a low extent that it was necessary to use a ^{14}C-labeled reagent for detection.

Our objective in this work was to synthesize oligonucleotides containing a site-specific *p*-BQ-dG adduct (**1**) using phosphoramidite chemistry and to use DNA oligomers for biochemical studies. This could be achieved only by optimizing the conditions of the reaction of *p*-BQ with dG to obtain a large amount of the product, *p*-BQ-dG, and convert it to the fully protected phosphoramidite (**4**).

The reaction of dG with *p*-BQ was performed in aqueous solutions and at different pHs in order to find the best conditions to maximize the yield of the major product. The reaction in aqueous solution at both pH 7.4 and 9.3 produced the same product as reported by Jowa (*17*), as well as traces of other products which were not studied. However, no reaction was detected at pH 4.5. The major product had the same UV spectrum as reported by Jowa, but the yield of the desired compound at both pH 7.4 and 9.3 was poor (<2%) and not suitable for our purposes. Therefore, the reaction was repeated in nonaqueous conditions, either in dry DMSO or DMF in the presence of K_2CO_3 or NaOH pellets. Under these conditions, substantial amounts of product (about 50%) were obtained, as shown by HPLC.

The reaction of dG with *p*-BQ in DMSO/K_2CO_3 first produced an unstable product which was slowly converted to the final stable product (*p*-BQ-dG). This reaction, when monitored by HPLC or TLC over a period of time, clearly showed the conversion of the intermediate which had a longer retention time (32−33 min) to the final product (Figure 1), which was eluted at 31 min. The UV spectrum of the unstable intermediate does not resemble that of the final product; the final product has a significant trough at about 250 nm and a peak at about 270 nm, whereas the intermediates have a broad shoulder from 260 to 275 nm, in addition to red-shifted maxima (Figure 2).

This intermediate showed two peaks very close to each other by HPLC (Figure 1). When either peak was collected and immediately reanalyzed under the same

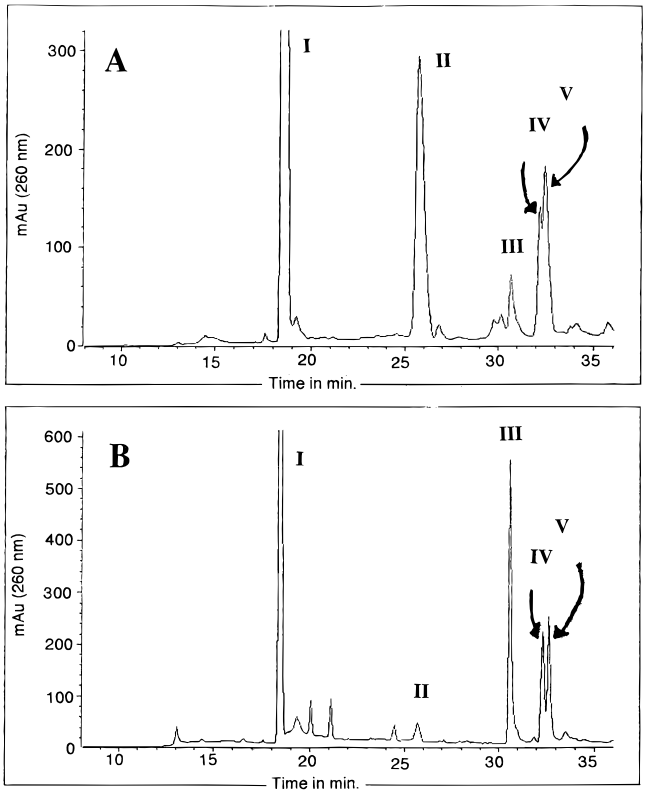


Figure 1. HPLC analysis of products of reactions of dG with *p*-BQ at 30 min and 22 h. The reaction was performed in DMF/K_2CO_3 at room temperature. (Top) 30 min reaction: peak I (~19 min) corresponds to the starting material, dG. peak II (~26 min) is *p*-BQ; peak III (~31 min) is the stable final adduct; peaks IV and V between 32 and 33 min are the two unstable intermediates. (Bottom) 22 h reaction: peak I is dG; only traces of *p*-BQ remain (peak II); the peak at ~31 min (peak III) is the stable *p*-BQ-dG product; the doublet at 32−33 min consists of two interconvertable forms of the unstable intermediates (peaks IV, V). When either of the doublets is collected and immediately reanalyzed in the same HPLC system, the same two peaks (32−33 min) result (data not shown).

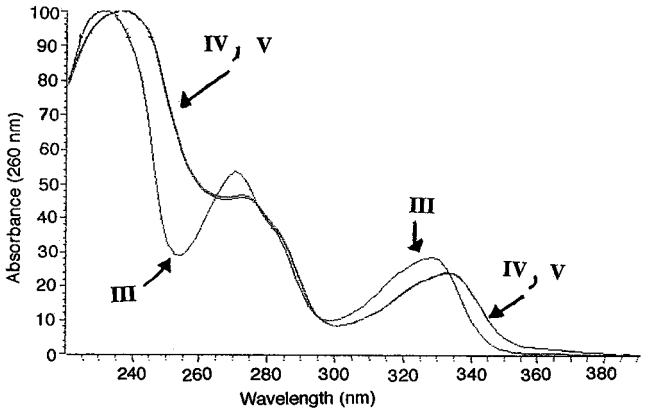


Figure 2. UV spectra of *p*-BQ-dG (peak III) and the intermediate product (peaks IV and V). The arrows indicate the spectrum of each. Note that the three maxima for *p*-BQ-dG are shifted ~5 nm.

HPLC conditions, there were again two peaks from each of the single peaks. This suggests that there is a single product in equilibrium in two forms, which are presumed to be isomers. The intermediate product was isolated by HPLC and analyzed immediately by electrospray MS both in positive and negative ion. In the positive mode, the spectrum showed a protonated molecular ion at 464 for MH^+ and a protonated base BH_2^+ at 348 (Figure 3). The negative ion electrospray showed $(M - H)^-$ at 462. The MS spectrum suggests that the molecule carries two molecules of *p*-BQ on the dG base, with removal of a molecule of water (dG + 2*p*-BQ − H_2O) = 463. A comparison of the UV spectra of the intermediate and the stable adduct gives an indication of substitution on the N-1 and N^2 of the dG by *p*-BQ, but we do not know where the second *p*-BQ reacts with dG. However we speculate that the second *p*-BQ molecule is either on the N-7 or C-8 or alternately cyclizes to form a N-7−C-8 ring as suggested by Shapiro *et al.* for acrolein reacted with dG (*16*). Due to the unstable nature of this adduct, it was not possible to use ^1H NMR to obtain further information about the structure or mechanism of the interconversion.

In order to obtain sufficient product for making the fully protected phosphoramidite of *p*-BQ-dG, the reaction was scaled up to react 500 mg of dG with *p*-BQ, which after chromatography on silica gel, yielded 28% of a light brown solid pure product **1** (Scheme 1), which was analyzed by TLC, HPLC, ^1H NMR, UV, and FAB/MS (see Materials and Methods). The ^1H NMR of the product showed, in particular, four exchangeable protons, two on the 3′- and 5′-hydroxyl groups of the sugar moiety, the third at 9.465 ppm as a singlet for the 7-hydroxyl group on the phenolic ring on the base, and the fourth one at the NH-5 of the base (at 6.531 ppm). The FAB/MS of product **1** showed an intense natriated molecule at *m/z* 380 $(M + Na)^+$ and protonated molecular ion MH^+ at *m/z* 358, an $(BH + Na)^+$ peak at *m/z* 264 and a $(BH_2)^+$ peak at *m/z* 242. These data are consistent with the addition of one molecule of *p*-BQ to the base moiety followed by dehydration to form the stable adduct (**1**). The UV spectra of this product **1** at different pHs was similar to the one reported (*17*).

Synthesis of the Fully Protected Phosphoramidite of *p*-BQ-dG(4). Compound **1** has three hydroxyl groups and an amino group. In order to incorporate this adduct in oligonucleotides, all four reactive groups must be protected. There is a hydroxyl group at position 7 and an amino group at position 5 of the exocyclic base moiety, as well as the usual two hydroxy groups (3′- and 5′-OH) on the sugar moiety. The hydroxyl group (7-OH) and the amino group (NH-5) on the base of *p*-BQ-dG would definitely interfere in the synthesis of the oligonucleotides by branching. Therefore, they must be protected with protecting group(s), which later can be easily removed in the last step of the deprotection of oligonucleotides. *p*-BQ-dG (**1**) was converted to its 5′-O-DMT product in 78% yield, which was achieved according to standard conditions (*24*,*25*). Phosphitylation of 5′-*O*-DMT-*p*-BQ-dG (**2**) with 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (*26*) yielded the phosphoramidite (**3**) (69% yield). By controlling the amount of the phosphitylating reagent and the temperature (−70 °C) only the 3′-OH was successfully reacted, with only traces of a product which was phosphitylated on both hydroxyl group (3′-OH and 7-OH). These two derivatives could be separated on the basis of polarity. Compound **3** contains a free phenolic hydroxyl group and an amino group which may lead to branching in the DNA synthesizer if not protected. Therefore, derivative **3** was acylated by using isobutyric anhydride in pyridine at room temperature (*27*). The reaction, monitored by TLC on silica gel indicated the formation of a new product which was less polar than the starting material **3**. This product **4** was purified by precipitation in dry hexane at −42 °C, which gave a yellow gum product (81%) which was coevaporated twice

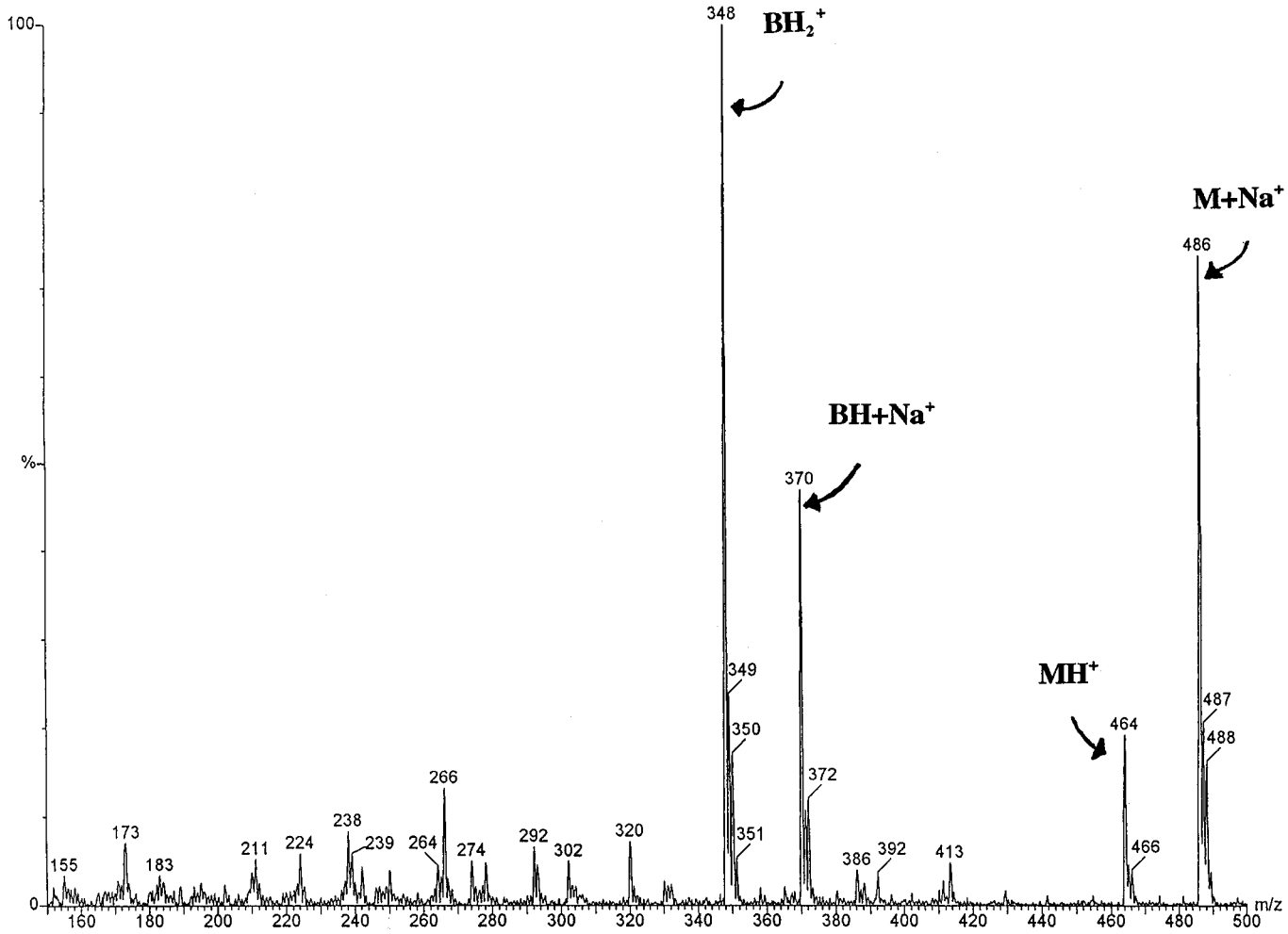
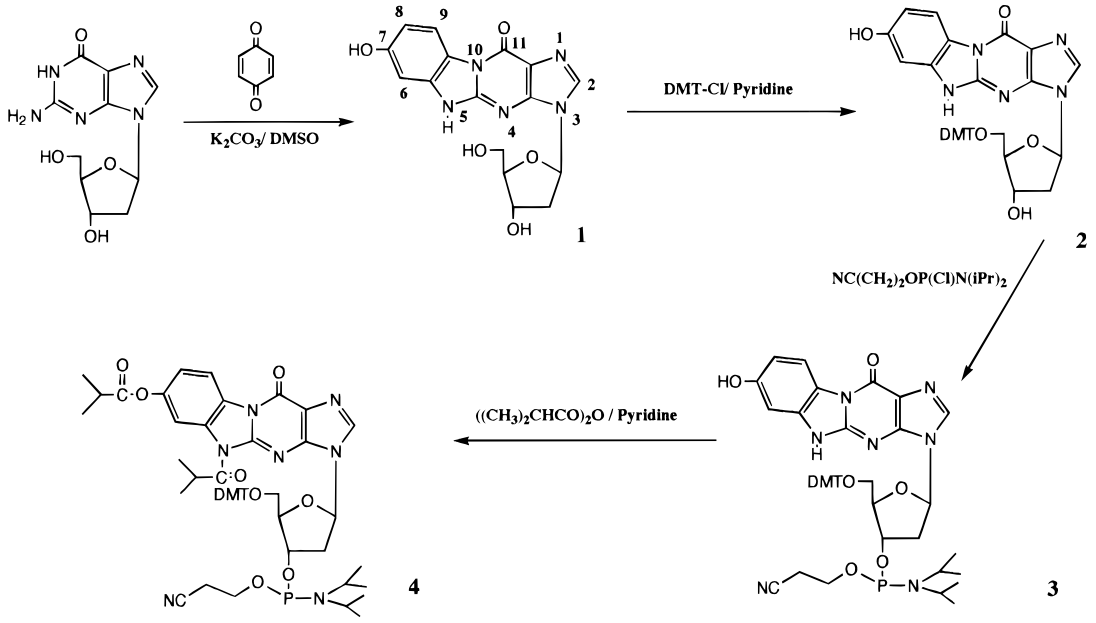


Figure 3. Electrospray mass spectrum of the unstable intermediate (combined peaks IV and V) in Figure 1.

Scheme 1. Preparation of the Phosphoramidite of *p*-BQ-dG Adduct 1



in dry benzene and kept under vacuum before being used to make the desired oligonucleotides. This fully protected compound **4** was used to synthesize two different oligonucleotides: a 19-mer and a 25-mer. The phenoxyacetyl phosphoramidites (PAC) were used to synthesize these DNA oligomers and followed the standard phosphoramidite chemistry. The coupling efficiency of *p*-BQ-dG phosphoramidite was 96%. The oligonucleotides were then deprotected under nonaqueous conditions by using 10% DBU in ethanol at room temperature for 24 h (*28*) and then purified by 20% polyacrylamide gel electrophoresis followed by HPLC. DBU was used to deprotect these oligomers instead of ammonia, since *p*-BQ-dG was not stable in 28% ammonia. In addition, the use of the

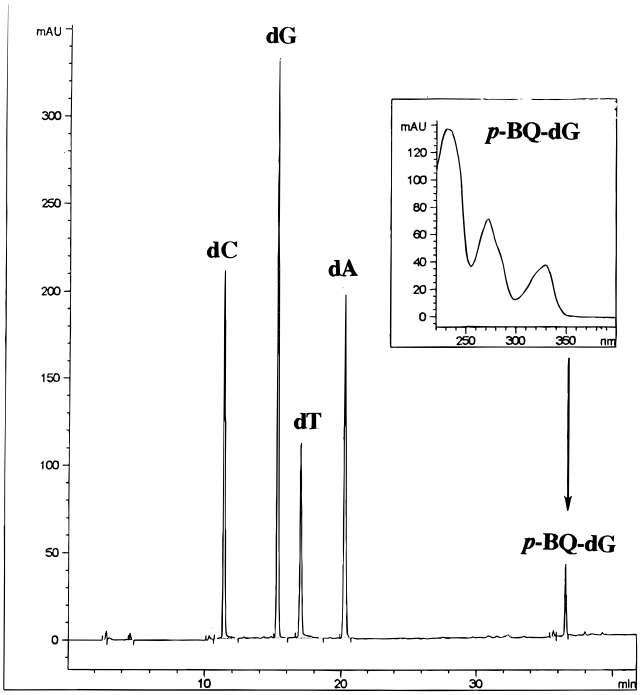


Figure 4. HPLC profile of 2′-deoxynucleosides obtained as a result of enzymatic digestion of oligonucleotide **1**, which contains *p*-BQ-dG (see Materials and Methods). HPLC was performed using system 3. The retention times of the deoxynucleosides are dC, 11.4 min; dG, 15.3 min; dT, 17.0 min; dA, 20.2 min; and *p*-BQ-dG, 36.5 min. The UV spectrum of *p*-BQ-dG is shown in the inset.

PAC protecting group allowed the deprotection of DNA oligomers at room temperature and in a shorter time (24 h) than the standard phosphoramidite, which requires several days.

The composition of the oligomers was confirmed after enzymatic digestion of the DNA oligomers to nucleosides, followed by HPLC analysis. The enzyme hydrolysis showed that the modified base survived the conditions used in the DNA synthesizer and the deprotection procedure (Figure 4).

Summary

The reaction of dG with *p*-BQ proceeded with high yield in DMF or DMSO with K_2CO_3 or NaOH pellets as the base catalyst. In contrast, in neutral or basic aqueous solution, the yield of *p*-BQ-dG was <2% and no product was detected at pH 4.5. Although only a single final stable product was obtained, a small amount of an unstable intermediate could be isolated which separated on HPLC into two interconvertable products which are apparent isomers.

This intermediate is formed under physiological conditions, as well as nonaqueous and both the final product and intermediate have significantly different UV spectra. However, electrospray MS indicates that the intermediate contains two *p*-BQ molecules. From the comparison of the two UV spectra we can conclude that one *p*-BQ forms an exocyclic ring between the 1- and N^2-positions of dG while the other *p*-BQ could be on either the N7 and/or C-8 of dG. This speculation is supported by Shapiro's finding that, in general, α,β-unsaturated carbonyl compounds can form bicyclic 1,N^2:7,8-diadducts (*16*).

The conversion of *p*-BQ-dG into the phosphoramidite was successfully accomplished in good yield with both protection and deprotection after incorporation into defined oligonucleotides. This new synthesis of a *p*-BQ-dG containing oligonucleotides will enable us to extend our biochemical studies of repair of *p*-BQ-dC and *p*-BQ-dA in defined sequences (*23*,*29*) to the only other stable *p*-BQ adducts formed under physiological conditions. Such oligonucleotides will also be used for structural studies.

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