Bis(hydroxyphenylethyl)deoxyguanosine Adducts Identified by [^{32}P]-Postlabeling and Four-Sector Tandem Mass Spectrometry: Unanticipated Adducts Formed upon Treatment of DNA with Styrene 7,8-Oxide

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Calf thymus DNA was incubated with [8-^{14}C]styrene oxide in vitro, and six covalent xenobiotic–DNA adducts were detected using the [^{32}P]-postlabeling procedure. Adducts 1–3 were purified by HPLC and identified as bis-substituted-2′-deoxyguanosine 3′-phosphate derivatives using four-sector tandem mass spectrometry. These adducts represented less than 2% of the total adducts detected by [^{14}C]-radioactivity. Adducts 1–3 were also detected when styrene oxide was allowed to react with the mononucleotide, 2′-deoxyguanosine 3′-phosphate only. The elemental compositions of these adducts ($C_{26}H_{30}N_5O_9P$) were determined by measurement of their accurate masses by high-resolution mass spectrometry and revealed the unusual incorporation of 2 mol of hydroxyphenylethyl moieties. The structures of these bis(phenylethyl) adducts were established by interpretation of high-energy collision-induced dissociation (CID) mass spectra, together with UV/visible and fluorescence spectrophotometry as N^2-(2-hydroxy-1-phenylethyl)-O^6-(2-hydroxy-2-phenylethyl)-2′-deoxyguanosine 3′-phosphate (adduct 1), N^2-(2-hydroxy-1-phenylethyl)-O^6-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate (adduct 2), and N^1,N^2-bis(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate (adduct 3). The other most abundant adducts were detected only by [^{14}C]-radioactivity and represented approximately 65% of the total covalent binding. These were identified as depurinated N7-substituted guanines by tandem mass spectrometry and UV/visible spectroscopy. The combination of advanced techniques of mass spectrometry with the [^{32}P]-postlabeling assay and spectroscopic techniques is a comprehensive strategy to assure complete structural identification of all xenobiotic–DNA adducts. In this study the [^{32}P]-postlabeling assay was important in showing the presence of very hydrophobic bis-substituted adducts which were not readily detected by [^{14}C]-labeling.

Introduction

Styrene 7,8-oxide (styrene oxide)[1] is the major reactive metabolite of the industrially important compound styrene, and it is formed primarily in the liver by the P-450-catalyzed oxidation of styrene (*1*). Styrene is used widely as a starting monomer in the plastics industry, and workers are exposed to this chemical mainly via inhalation of the airborne vapor (*1*). Studies have shown styrene oxide to be mutagenic in both prokaryotic (*2*) and eukaryotic (*3*) test systems and carcinogenic in rodents (*4, 5*). In studies involving humans, increased chromosome aberration levels have been reported in workers occupationally exposed to styrene (*6–8*). In addition, preliminary studies have suggested the presence of covalent DNA adducts in the lymphocytes of workers exposed to styrene (*9, 10*).

In vitro studies have shown that styrene oxide reacts at the nucleophilic sites in nucleic acid bases (e.g., N^7, N^2, O^6 in guanine), DNA, and proteins to form covalent adducts (*11–14*). Alkylation can occur through either the C7 (α) or the C8 (β) carbon of styrene oxide, resulting in two structural isomers per reactive nucleophilic site. Using the [^{32}P]-postlabeling procedure, six covalent DNA adducts have been detected previously in calf thymus DNA incubated with styrene oxide (*10, 13, 15, 16*). In order to understand the biological significance of the covalent adducts related to styrene oxide, it is essential to establish their chemical structures. Previous work in this laboratory identified adducts 4, 5, and 6 as 5′-^{32}[P]-labeled O^6-(2-hydroxy-2-phenylethyl)-2′-deoxyguanosine 3′-phosphate, O^6-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate, and N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate, respectively (*10, 13, 15*).

Mass spectrometry (MS) has played an important role in the structural elucidation of covalently modified nucleic acids at low levels. A variety of instruments, ionization techniques, and chemical derivatives have been used (for reviews see refs 17–20). While in early studies field desorption provided the first opportunity to carry out CID experiments on underivatized DNA adducts (*18*), the advent of liquid matrix secondary ionization MS (LSIMS, or the related technique FAB) has greatly facilitated

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[1] Abbreviations: styrene oxide, styrene 7,8-oxide; 3′-dNp, 2′-deoxyribonucleotide 3′-phosphate; 3′-dGp, 2′-deoxyguanosine 3′-phosphate; 3′,5′-dGp, 2′-deoxyguanosine 3′,5′-bisphosphate; MS, mass spectrometry; LSIMS, liquid secondary ion mass spectrometry; FAB, fast atom bombardment; CID, collision-induced dissociation; t_R, retention time; TLC, thin-layer chromatography; O^6-SO-3′-dGp, O^6-(2-hydroxy-2-phenylethyl)-2′-deoxyguanosine 3′-phosphate; N^2-SO-3′-dGp, N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate.

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analogous investigations in both the positive and negative ion modes (*19, 21–26*). The combination of LSIMS and high-performance tandem MS employing electrooptical multichannel array detection (*27*) is particularly important in providing detailed structural information for covalently modified nucleosides and nucleotides available in only picomole quantities. In addition, since fragment ions in mass spectra obtained using a four-sector tandem instrument arise only from a selected ^{12}C molecular ion isotope, contributions in the spectra from other components, contaminants, and chemical noise are eliminated (except those which are isobaric).

In this study the [^{32}P]-postlabeling procedure was employed to detect covalent xenobiotic adducts present in the enzymic hydrolysate of native calf thymus DNA modified during incubation with styrene oxide. This resulted in detection of six covalent adducts, three of which were very hydrophobic, suggesting that they might be unusually modified. We focused our effort on identifying these particularily hydrophobic adducts, 1, 2, and 3, respectively. We report here their structural nature as isomeric bis-substituted guanine mononucleotides (3′-dGp) using a combination of [^{32}P]-postlabeling, mass spectrometry, and a variety of spectroscopic methods.

Materials and Methods

Experimental. (A) Chromatography. HPLC isolations were performed on a Beckman 110A HPLC system (Beckman Instruments, Palo Alto CA) using a Hewlett Packard 1040A diode array detector and a series 2, Perkin Elmer HPLC system (Perkin Elmer, Norwalk CT) equipped with an ISCO gradient programmer and Perkin Elmer LC-235 diode array detector. UV/visible spectra were obtained using a Beckman DU-7 spectrophotometer, and fluorescence spectra were recorded on a Spex Fluorolog Model 1680 spectrofluorimeter (Spex Industries Inc., Edison, NJ).

(B) Mass Spectrometry. Liquid secondary ion mass spectra (LSIMS) were recorded in the positive and negative ion modes on a Kratos MS-50S (Kratos, Manchester, U.K.) mass spectrometer equipped with a postacceleration detector. The LSIMS ion source has been described elsewhere (*28*). A Cs^+ ion primary beam of energy 10 keV was used (*29*). Spectra were recorded (300 s/decade) with a Gould ES-1000 electrostatic recorder. When the highest sensitivity was required for detection and measurement of the smallest quantities of adduct, LSIMS spectra were also recorded on a prototype Wien EXB mass spectrometer using a resistive multichannel array detector in the static mode using a primary beam of 10 keV energy, detecting ions simultaneously over a 5-min period and signal averaging by a multichannel analyzer (*30*).

Tandem MS experiments were performed on a Kratos Concept II HH (Kratos, Manchester, U.K.) four-sector instrument of EBEB geometry. The sample was ionized as above in a LSIMS ion source using a Cs^+ primary beam of energy 12 keV. Only the ^{12}C isotope peak for the MH^+ ion stable isotope cluster was selected in MS-1 and introduced into a collision cell containing helium. The collision energy was 6 keV, and the helium pressure in the cell was adjusted to obtain a MH^+ ion attenuation of 65%. The fragment ions generated in the collision cell were separated in MS-II and detected in successive 4% mass windows (2-s exposure/frame) using an electrooptically-coupled 1000-channel array detector (*27*). Samples for MS analysis were taken to near dryness under vacuum. A glycerol/thioglycerol/0.1 M HCl (1:10:trace) matrix (+ve ion mode) or glycerol matrix (−ve ion mode) was applied to the stainless steel probe tip, a small aliquot of the sample (pmol) was added, and the sample probe tip was cooled during the MS analysis (*31*). Accurate mass measurements were carried out by peak matching at an instrument resolution of 10 000.

(C) [^{32}P]-Postlabeling. DNA was incubated with styrene oxide as described below and [^{32}P]-postlabeled using the P1 nuclease-enhanced procedure (*31*). The adducts were purified on reversed-phase TLC plates which were eluted with 2.0 M ammonium formate (pH 6.2), transferred to PEI–cellulose plates, and chromatographed as previously described (*15*). Modified nucleotides in HPLC fractions were [^{32}P]-postlabeled, applied directly to PEI–cellulose plates, and chromatographed under the same conditions.

In Vitro Reactions. (A) [8-^{14}C]Styrene Oxide–DNA Adducts. Calf thymus DNA (4 aliquots, 0.5 mg each; 30 mM Tris-HCl, pH 7.5, 1 mL each) was incubated with [8-^{14}C]styrene oxide (25 μCi each, 25 mCi/mmol; Amersham, U.K.) and unlabeled styrene oxide (5 μL each, 37 °C, 24 h). Similarly, in a larger-scale unlabeled experiment, DNA (15 aliquots, 8 mg each; 30 mM Tris-HCl, pH 7.5, 10 mL each; Sigma, St. Louis, MO) was incubated with styrene oxide (30 μL each) under the same conditions. In each case, unreacted styrene oxide was removed by extraction with ethyl acetate and the DNA was further purified by repeated precipitation. The levels of total covalent binding were determined from [^{14}C]-radioactivity measurements using liquid scintillation counting (Tm analytic, Elk Grove Village, IL). The DNA from each experiment was enzymatically digested to 2′-deoxyribonucleotide 3′-phosphates (3′-dNps) in 20 mM sodium succinate and 8 mM $CaCl_2$ (pH 6) at 37 °C for 16 h with micrococcal nuclease and spleen phosphodiesterase (Worthington Biochemicals, Freehold, NJ). Fifty micrograms of each enzyme was used in a total volume of 250 μL for the [^{14}C]-labeled reaction and 250 μg in 1 mL for the unlabeled reaction. The 3′-dNps were fractionated using C-18 reversed-phase HPLC (10 × 250 mm, Alltech Associates Inc., Deerfield, IL) with a column loading of 8 mg per analysis (solvent A = 10 mM NH_4OAc, solvent B = MeOH; solvent program, t = 0 min, A = 100%, B = 0%, t = 60 min, A = 30%, B = 70%; t = 70 min, A = 0%, B = 100%; flow rate = 3 mL/min). In order to use similar column loadings in each analysis, 7.5 mg of unreacted 3′-dNps were added to the radiolabeled reaction prior to HPLC. Fractions were collected at 1-min intervals in each HPLC analysis and the radioactivity in the labeled reaction was monitored by liquid scintillation counting. The radiogram accounted for 92% of the radioactivity loaded onto the column. HPLC fractions were combined according to the peaks in the [^{14}C]-radiogram. The HPLC retention times for peaks in the [^{14}C]-radiogram were used to identify fractions containing covalently modified 3′-dNps from the unlabeled reaction. Fractions from consecutive HPLC analyses having identical retention times were pooled. Pooled fractions were further purified using C-4 reversed-phase HPLC (2.1 × 100 mm, Vydac, Hesperia, CA; solvent program, t = 0 min, A = 100%, B = 0%, t = 30 min, A = 30%, B = 70%; t = 40 min, A = 0%, B = 100%; flow rate = 0.3 mL/min). Aliquots of fractions obtained were analyzed by [^{32}P]-postlabeling. Wien MS and high-energy CID experiments in the negative ion mode were used to identify molecular weights and determine the structures of modified nucleotides, respectively.

(B) Reaction of 2′-Deoxyguanosine 3′-Phosphate with Styrene 7,8-Oxide. Twenty-five milligrams of 3′-dGP was dissolved in 3 mL of 20 mM Tris buffer (pH 7.5), and 300 μL of styrene oxide was added. The mixture was incubated overnight at 37 °C after which unreacted styrene oxide was removed by extraction with diethyl ether. The aqueous phase was diluted with water to a volume of 50 mL, and 10-mL aliquots were passed through SEP PAK C-18 cartridges (Waters Chromatography Division/Millipore Co., Milford, MA) which had been washed previously with methanol followed by water. Each cartridge was eluted with 3 mL of 50% methanol/water and the eluant was lyophilized. The total dry residue was taken up in 3 mL of water, and 1-mL aliquots were fractionated using a C-18 reversed-phase column (Econosil, 10 × 250 mm, Alltech Associates Inc., Deerfield, IL; linear gradient, 10 mM ammonium acetate/methanol (pH 5.1) 0–70% in 60 min, 70–100% in 10 min, flow rate = 3 mL/min). The fractions eluting between t_R 45 and 62 min were pooled and lyophilized. The depurinated bases, arising from N^7-

substitution, were removed by ion-exchange HPLC [Partisil PXS 10/25 SAX column, Whatman Chemical Separation Inc., Clifton NJ; linear gradient of 0.6 M KH_2PO_4 (pH 4.1) in 0.025 M KH_2PO_4 (pH 3.3), 0–70% in 30 min, flow rate = 1 mL/min]. Three fractions were collected at t_R 3.8, 12.0, and 17.1 min. The fractions at 12.0 and 17.1 min were desalted using SEP PAK cartridges. These fractions were analyzed using the [^{32}P]-postlabeling procedure, and the fraction at 17.1 min was identified as containing adducts 1–3. The adducts were purified further by reversed-phase HPLC (Alltech Associates Inc., Deerfield, IL, Econosil C-18, 4.6 × 250 mm; 10 mM ammonium acetate, pH 5.1/methanol gradient, 0–70% methanol in 60 min, 70–100% in 10 min, flow rate = 1 mL/min). Three major fractions were collected at t_R 54.9, 59.4, and 62.6 min. The [^{32}P]-postlabeling assay showed these fractions to correspond to adducts 3, 2, and 1, respectively. Adducts 1 and 2 showed UV_{max} 252, 286, UV_{min} 270 nm (pH 6 and pH 12); UV_{max} 250, 290, UV_{min} 270 nm (pH 1). Adduct 3 showed UV_{max} 256 nm (pH 6), UV_{max} 258 nm (pH 1), and UV_{max} 259 nm (pH 1). High-resolution negative ion LSIMS ($M/\Delta M$ 10 000) was used to identify the elemental compositions of adducts obtained from the reaction of 2′-deoxyguanosine 3′-phosphate with styrene oxide.

(C) Depurination of Adducts 1 and 2. Adduct 1 (t_R 62.6 min) and adduct 2 (t_R 59.4 min) from the 3′-dGp reaction were depurinated under mild acidic conditions. Each fraction (0.5 OD) was heated in 0.1 N HCl at 70 °C for 30 min. After cooling, the reaction mixture was analyzed by reversed-phase HPLC using the chromatographic conditions described above. Depurination of adduct 1 resulted in a product which eluted at t_R 44.4 min (UV_{max} 250, 275 nm). Depurination of adduct 2 resulted in a product which also eluted at t_R 44.4 min and showed UV/visible spectral characteristics identical to those obtained for the depurination product of adduct 1.

(D) Reaction of O^6-(2-Hydroxy-2-phenylethyl)-2′-deoxyguanosine 3′-Phosphate with Styrene Oxide. O^6-SO-3′-dGp (0.5 mg) (*13*) was incubated with 10 μL of styrene oxide in 0.5 mL of Tris-HCl buffer pH 7.4 at 37 °C overnight. The products were isolated by reversed-phase HPLC as above using a semi-preparative C-18 column (10 × 250 mm, Alltech Associates Inc., Deerfield, IL). Fractions collected at t_R 51 and 54 min had identical UV/visible spectral characteristics (UV_{max} 251, 288 nm; UV_{min} 270 nm at pH 6.0) and mass spectra ($[M-H]^-$ at m/z 586). The molecular weights indicated the presence of bis-substitution.

(E) Reaction of N^2-(2-Hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-Phosphate with Styrene Oxide. N^2-SO-3′-dGp (0.5 mg) (*15*) was incubated with styrene oxide, and the products were isolated using the conditions described above. Fractions collected at t_R 47, 52, and 53 min were identified as bis-substituted products by mass spectrometry ($[M-H]^-$ at m/z 586), and the fraction at t_R 57 min was identified as trisubstituted ($[M-H]^-$ at m/z 706).

Results

Calf thymus DNA was incubated with [8-^{14}C]styrene oxide and enzymatically digested to obtain 3′-dNps. [^{32}P]-Postlabeling analysis of the [^{14}C]-labeled DNA showed a characteristic autoradiogram indicating six adducts (Figure 1). The level of [^{32}P] incorporated into the adduct spots indicated the presence of 9.1 adducts per 10^5 nucleotides. The nucleotides were fractionated by HPLC, and the radioactivity levels were measured to identify fractions containing [^{14}C]-labeled covalent adducts (Figure 2). The retention times obtained for radiolabeled adducts (a–f) were used to aid HPLC isolation of adducts from a larger-scale unlabeled preparation. This was necessary since the level of modification was below the detection limits of UV/visible absorbance.

The positive ion CID mass spectrum for the major styrene oxide–DNA adduct detected by [^{14}C]-radioactivity

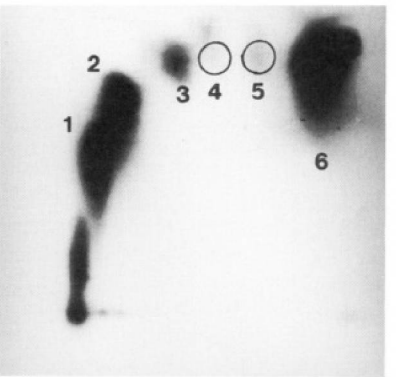

Figure 1. Autoradiogram for the [^{32}P]-postlabeled reaction products from calf thymus DNA incubated with styrene oxide in vitro.

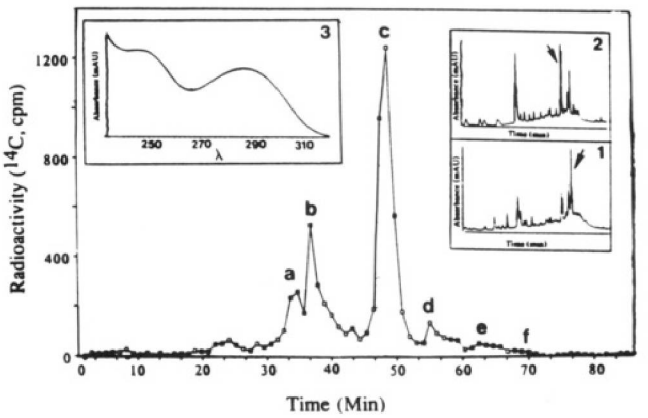

Figure 2. Reversed-phase C-18 HPLC radiogram (10 × 250 mm) of 3′-dGp covalent adducts from calf thymus DNA incubated with [8-^{14}C]styrene oxide. Inset 1 shows reversed-phase C-18 HPLC chromatogram (2.1 × 100 mm) of fraction f; arrow indicates fraction containing adduct 1. Inset 2 shows reversed-phase C-18 HPLC chromatogram (2.1 × 100 mm) of fraction e; arrow indicates fraction containing adduct 2. Inset 3 shows UV/visible spectrum for adduct 1.

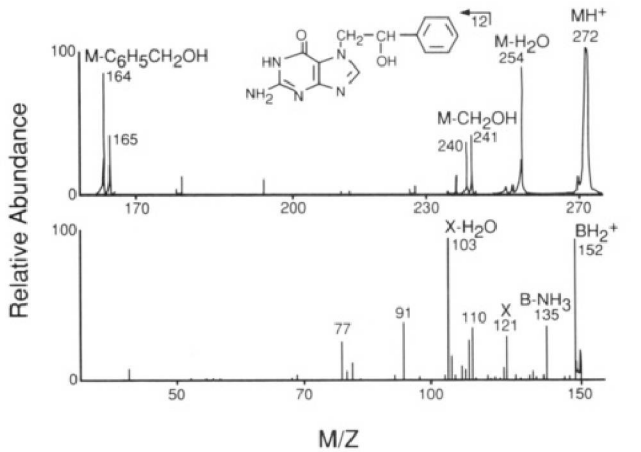

Figure 3. Positive ion high-energy CID mass spectrum for the major adduct from calf thymus DNA incubated with styrene oxide. Symbols: X, $C_6H_5C_2H_3OH$; B, base. For convenience, only one structural isomer is shown.

measurement (Figure 2, fraction c) is shown in Figure 3. Fraction c accounted for approximately 65% of the peak area in the [^{14}C]-radiogram. The measured molecular weight showed immediately that the adduct had undergone depurination (Figure 3). The MH^+ at m/z 272 corresponded to the base guanine containing one hydroxyphenylethyl moiety. The abundant ion at m/z 152 arose from loss of the hydroxyphenylethyl moiety from MH^+. Further

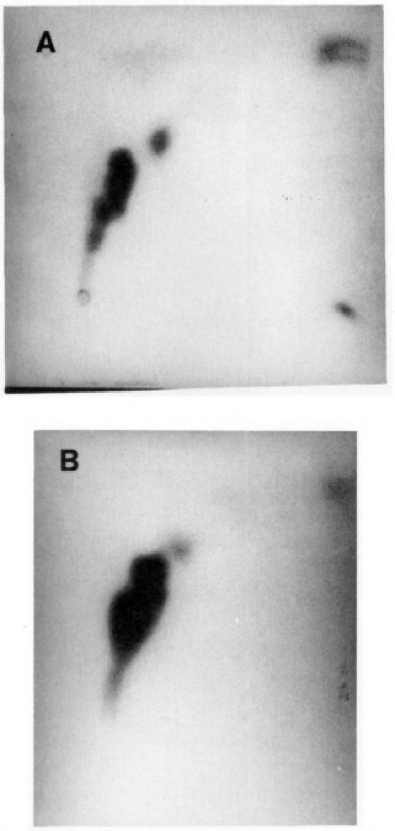

Figure 4. Autoradiogram for [^{32}P]-postlabeled adduct 2 (A) from HPLC fraction e and adduct 1 (B) from HPLC fraction f indicated in Figure 2.

interpretation of the mass spectrum indicated the presence of two chromatographically unresolved isomers, 1-hydroxy-1-phenylethyl (β-isomer) and 2-hydroxy-1-phenylethyl (α-isomer) by the observation of ions at m/z 164 and 165 corresponding to M – $C_6H_5CH_2OH$ (β-isomer) and m/z 240 and 241 corresponding to M – CH_2OH (α-isomer) (Figure 3). The four possible isomers of the N7 styrene oxide substituted guanine were synthesized (K. Pongracz, unpublished results). Chromatographic analysis of a mixture of these authentic isomers established that their elution time was identical to that observed for fraction c in Figure 2. N7-substituted adducts were shown to be the most abundant adducts of the styrene oxide–guanosine (*11*) and the styrene oxide–DNA reaction (*12*). No detectable postlabeling of fraction c was observed, which would be consistent with the fact that we were dealing with a depurination product.

In order to identify which specific HPLC fractions (a–f) contained adducts 1–3 observed in the [^{32}P]-autoradiogram (Figure 1), an aliquot of each HPLC fraction was analyzed using the [^{32}P]-postlabeling method. Two late-eluting fractions (e and f) showed autoradiograms where the retention times of the major components were characteristic of adducts 2 and 1, respectively (Figure 4, panels A and B). Fractions e and f from consecutive HPLC analyses were pooled and further purified by reversed-phase narrow-bore HPLC (Figure 2, insets 1 and 2). The narrow-bore HPLC chromatogram showed a number of peaks, and again [^{32}P]-postlabeling analysis of aliquots of these HPLC fractions was employed to identify the peaks containing adducts 1 and 2. The UV/visible spectral characteristics for adducts 1 and 2, together with radioactivity measurements, indicated that the quantities of

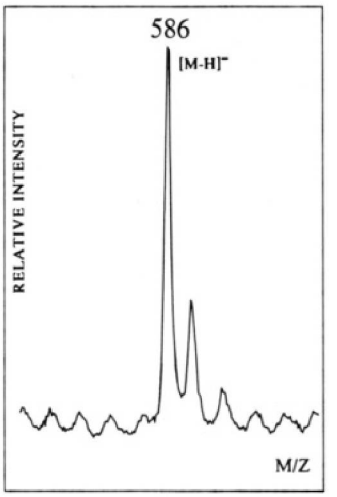

Figure 5. Negative ion LSIMS mass spectrum of adduct 1 isolated from fraction f by narrow-bore HPLC chromatography.

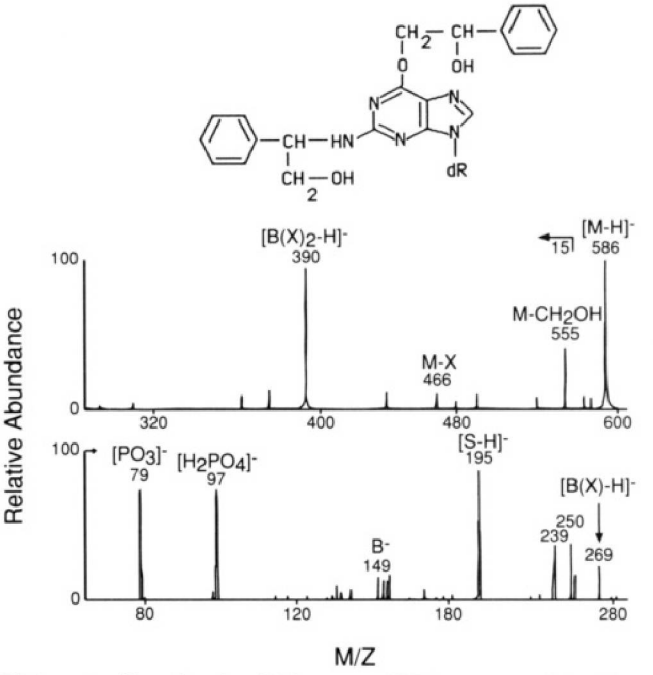

Figure 6. Negative ion high-energy CID mass spectrum for molecular ion [M – H]$^-$ m/z 586 of adduct 1 from narrow-bore HPLC purification of fraction f. Symbols: X, $C_6H_5C_2H_3OH$; B, base; S, ribonucleotide. For convenience, only one structural isomer is shown.

these adducts must be in the low picomole range (Figure 2).

The UV/visible spectra were obtained for adducts 1 and 2 using a diode array detector during narrow-bore HPLC, and the spectra were found to be identical, suggesting structural similarity of the chromophores (Figure 2, inset 3). Using the Wien instrument, negative ion LSIMS data established that the [M – H]$^-$ = m/z 586 were identical for both adducts 1 and 2 (Figure 5). This result established that adducts 1 and 2 were 3′-dGp structural isomers—each containing two hydroxyphenylethyl ($C_6H_5C_2H_3OH$) moieties. High-energy CID analyses were then employed to obtain further structural information on adducts 1 and 2. The CID mass spectrum for the ^{12}C isobar of [M – H]$^-$ at m/z 586 for adduct 1 is shown in Figure 6. Observation of the mass of the abundant B-type ion at m/z 390 indicated that both alkylations were occurring on the guanine base (*33*). Less abundant ions at m/z 269 and 149 indicated

the loss of one and two hydroxyphenylethyl groups, respectively, from m/z 390. Loss of one hydroxyphenylethyl moiety from $[M - H]^-$ resulted in the fragment m/z 466. The ions m/z 252 and 239 corresponded to loss of H_2O and CH_2OH from m/z 270, the guanine base containing one hydroxyphenylethyl group. Nucleotide CID spectra generally show the loss of H_2O and CH_2OH from $[M - H]^-$ (*21–24, 33*). In Figure 6 these losses may also have arisen from the 1-hydroxy-2-phenylethyl (β) 3′-dGp isomer, for example, loss of H_2O and CH_2OH from m/z 586 (568 and 555), m/z 390 (372 and 359), and m/z 269 (251 and 238), respectively. Loss of C_6H_5CHOH from m/z 586 and 390 in the β-isomer resulted in ions of m/z 478 and 282, respectively (Figure 6). Ions of m/z 195 (deoxyribose + $PO_4)^-$, 97 $(H_2PO_4)^-$, and 79 $(PO_3)^-$ were related to the deoxyribose phosphate moiety. Similar fragmentation was observed for adduct 2. The positive ion CID mass spectra for adducts 1 and 2 revealed analogous fragmentation, showing substitution at two different sites in 3′-dGp (data not shown).

A 3′-dGp structure containing a single hydroxyphenylethyl dimer could be ruled out from the CID spectrum shown in Figure 6. First, a characteristic fragment of m/z 163 [loss of $C_6H_5C_2H_3(OH)C_6H_4CHOH$ from m/z 390] would be expected, and this was absent. Second, ions of m/z 466 and 269 would not be expected, and these were observed (Figure 6). The CID spectrum clearly showed both modifications to be at the guanine base in 3′-dGp and not at the deoxyribose moiety. However, fragmentation across the purine ring was not observed, and therefore the two sites of substitution could not be determined from the CID experiments.

For more detailed structural characterization of the bis-substituted adducts 1 and 2, larger quantities of these adducts were prepared by incubating 3′-dGp with styrene oxide. Earlier studies from our laboratory established that 3′-dGp is the primary target for modification in the DNA–styrene oxide reaction (*16*). The reaction products were isolated using C-18 reversed-phase HPLC. The fractions collected were analyzed by high-resolution mass spectrometry and the [^{32}P]-postlabeling procedure. The high-resolution (10 000) negative ion LSIMS mass spectrum of an HPLC fraction at t_R 63 min showed $[M - H]^-$ at m/z 586.1734 (calculated 586.1703, Δ5 ppm), establishing the presence of an adduct containing two hydroxyphenylethyl moieties. The elemental composition $C_{26}H_{30}N_5O_9P$ confirmed the bis-substituted nature of this nucleotide. The negative ion CID mass spectrum for this fraction was identical to those obtained for adducts 1 and 2 above (data not shown). The positive ion CID spectrum revealed essentially analogous fragmentation (data not shown). These results showed that bis-substituted 3′-dGps are formed in reactions involving both DNA and 3′-dGp.

To obtain adducts 1 and 2 in sufficient purity for further structural analysis, additional purification steps involving ion-exchange chromatography were required. Figure 7 shows the autoradiograms for HPLC fractions at t_R 59.4 min (Figure 7A) and 62.6 min (Figure 7B) from the reaction of 3′-dGp incubated with styrene oxide. These autoradiograms were characteristic for adduct 2 (Figure 7A) and adduct 1 (Figure 7B). Adducts 1 and 2 exhibited identical UV/visible spectra, suggesting that they were isomers of the same compound. The UV/visible spectral characteristics for the bis-substituted adduct 2 under a variety of

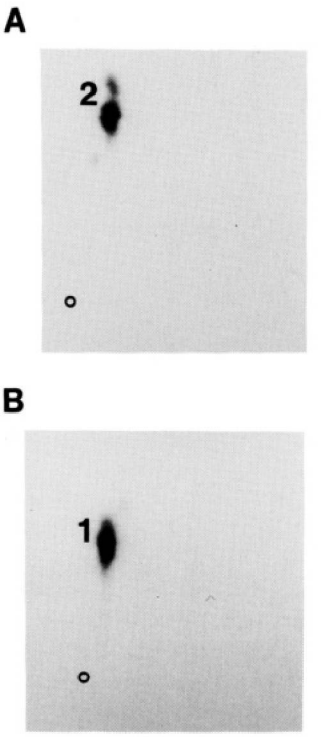

Figure 7. Autoradiogram of [^{32}P]-postlabeled adduct 1 (A) and adduct 2 (B) isolated from the reaction of 3′-dGp with styrene oxide. The film was exposed for 10 min.

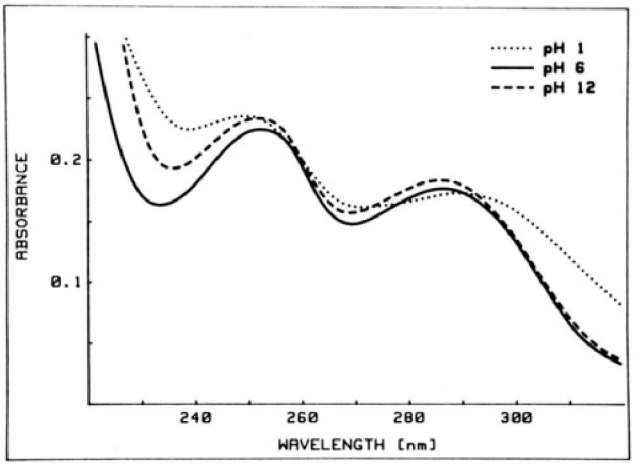

Figure 8. UV/visible absorption spectra of adduct 2 isolated from the reaction of 3′-dGp with styrene oxide.

pH conditions (Figure 8) were consistent with substitution at the N^2 and O^6 positions (*34*).

Mild hydrolysis of adducts 1 and 2 from the 3′-dGp incubation resulted in the formation of N^2-(2-hydroxy-1-phenylethyl)guanine (*15*). The fact that mild hydrolysis produced a mono-adduct suggested that the second substituent was at the O^6 position, since this is the only substitution site in guanine that is susceptible to mild hydrolysis. Fluorescence spectra of adducts 1 and 2 (Figure 9, panels A and B) showed a strong increase in fluorescence intensity under acidic conditions (excitation at 290 nm, emission peak at 390 nm). These spectra were virtually identical to that of O^6-(2-hydroxy-2-phenylethyl)-2′-deoxyguanosine (excitation at 290 nm, emission peak at 380 nm, not shown). These results were consistent with substitution at the N^2 and O^6 positions, since alkylation at the O^6 position in guanine has significantly more influence on the fluorescence properties than alkylation at the N^2 position (K. Pongracz, unpublished results).

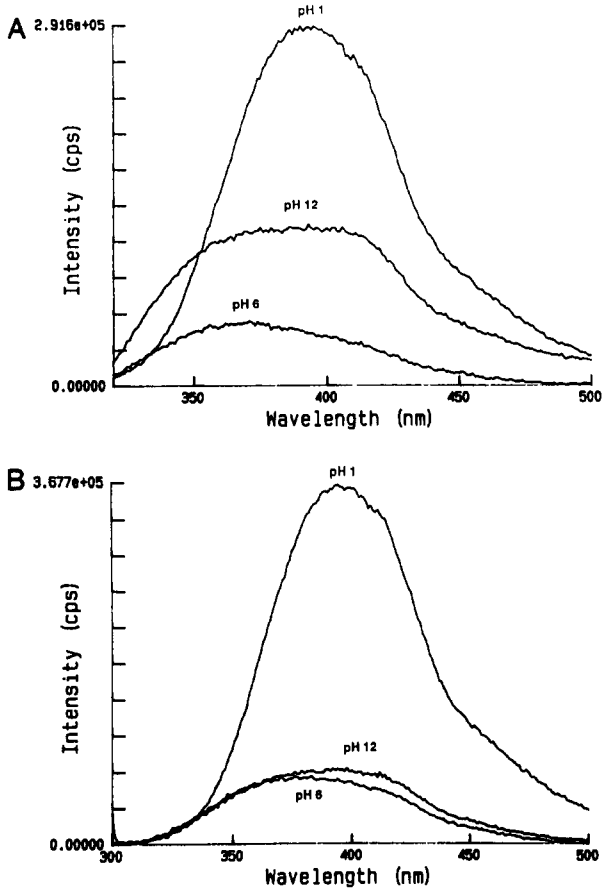

Figure 9. Fluorescence spectra of adduct 1 (A) and adduct 2 (B) isolated from the reaction of 3′-dGp with styrene oxide.

Compounds O^6-SO-3′-dGp and N^2-SO-3′-dGp were reacted with styrene oxide to form adducts with additional substitution. Bis-substituted adducts ($[M - H]^-$ at m/z 586) isolated from these reactions comigrated with adducts 1 and 2 during the [^{32}P]-postlabeling analysis.

The HPLC chromatogram for the 3′-dGp reaction products also showed a peak eluting at t_R 54.9 min. [^{32}P]-Postlabeling of this peak showed an adduct corresponding to adduct 3 (Figure 1). The negative ion LSIMS mass spectrum identified $[M - H]^-$ at m/z 586. The molecular weight and the UV/visible spectral characteristics of adduct 3 identified the structure as N^1,N^2-bis(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate (α-α) (*35*). Reaction of styrene oxide at the N^2 position of guanine produces only the α-isomer (*35*). We have found previously that reaction at the N1 position also results in only the α-isomer (K. Pongracz, unpublished results).

Discussion

The major [^{14}C]-labeled adducts from the DNA reaction with styrene oxide were identified as depurinated (α and β) N7-substituted guanine, which was consistent with previous studies (Figures 2 and 3) (*11*, *12*). Since the major adducts were depurinated, they were not detected by the [^{32}P]-postlabeling assay. This highlights the importance of using a combination of analytical techniques for the characterization of DNA adducts. [^{32}P]-Postlabeling of fractions a and b identified these structures as the two diastereoisomers of α-N^2-substituted 3′-dGp (*15*).

The hydrophobic adducts 1 and 2 from the incubation of DNA with styrene oxide were isolated and identified as N^2-(2-hydroxy-1-phenylethyl)-O^6-(2-hydroxy-2-phenylethyl)-2′-deoxyguanosine 3′-phosphate (α-β) and N^2-(2-hydroxy-1-phenylethyl)-O^6-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate (α-α). The structures were assigned as α-β (adduct 1) and α-α (adduct 2) on the basis of our experience with the HPLC and PEI chromatographic characteristics of O^6-substituted derivatives of styrene oxide (*13*). The structure of adduct 3 was determined as N^1,N^2-bis(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate (α-α). The presence of several configurational isomers is not unusual since two sites in guanine are involved and reaction can occur through both the C7 (α) and C8 (β) carbon of styrene oxide. In addition, the formation of diastereomeric products can result in further isomerism. The presence of two covalent modifications at a single DNA base (Figure 6) from the incubation of a monofunctional xenobiotic compound with DNA is without precedent to our knowledge. There is evidence for bis-substitution in reactions involving nucleosides (*36*–*39*).

The mechanisms involved in the formation of bis-substituted adducts identified in this work were not established, and further work will be required to do so. Since the overall extent of DNA modification was relatively high (1.7 adducts per 10^2 bases measured by [^{14}C]-binding), it is possible that addition of the first styrene oxide molecule activated the mono-adduct formed toward further reaction. Reaction of O^6-(2-hydroxy-2-phenylethyl)-2′-deoxyguanosine 3′-phosphate (*13*) or N^2-(2-hydroxy-1-phenylethyl)-2′-deoxyguanosine 3′-phosphate (*15*) with styrene oxide produced bis-substituted compounds which comigrated with the bis-substituted adducts from the 3′-dGp reaction with styrene oxide (data not shown). It is noteworthy that, in each case, the reaction yields for the second substitution were *higher* than the first substitution, which suggested a faster rate for the second alkylation. In addition, in the reaction of N^2-substituted 3′-dGp with styrene oxide, trisubstituted products were also detected by LSIMS. N^2-Substituted adducts were the most abundant intact adducts detected by the [^{32}P]-postlabeling assay (Figure 1) (*10*, *15*). Therefore, adducts 1 and 2 in this work presumably arose from N^2-substituted mono-adducts which underwent modification at the O^6 position. If bis-substituted adducts are formed by styrene in vivo, it is possible that DNA miscoding might occur, since the structural distortion in DNA would be significant. Further work is required to investigate the presence of covalent styrene oxide–DNA bis-substituted adducts in vivo and their effects on DNA replication.

Multichannel array detection was important to the success of low-level experiments in analyses employing both the Wien and four-sector tandem MS experiments since only picomole quantities of these adducts were available. The CID mass spectra showed that the substitutions were on the purine ring; however, fragmentation across the purine ring was not observed and hence the sites of substitution were not identified by MS. In other studies, fragmentation across the purine ring was observed for certain adducts, e.g., *N*-(deoxyguanosin-8-yl)-3-aminofluoranthene, and presumably was due to the stability of the fragment ion formed (*40*). In this study the abundances of bis-adducts were low (<2%) compared to those of the major DNA adducts, as indicated by the small size of peaks e and f in the HPLC radiogram (Figure 2). However, since the major adducts were not detected by the [^{32}P]-postlabeling assay, adducts 1 and 2 appeared

significant (ca. 20%) compared to the total adducts detected in the autoradiogram (Figure 1).

While the [^{32}P]-postlabeling method provides a highly sensitive method for the detection of DNA adducts and has been used widely to detect DNA adducts in humans (*41–43*), by itself it is grossly inadequate since it provides no structural information. The discovery and identification of the unanticipated bis-substituted covalent DNA adducts in this work highlight the importance of high-sensitivity mass spectrometric scrutiny of substances detected by [^{32}P]-postlabeling studies. Tandem MS is a particularly powerful method for use in the identification of covalent modification of DNA as well as other macromolecules. Studies using MS are independent of the type of structure under investigation; thus it is possible to detect and identify even the unanticipated modifications with equal ease using only a single physicochemical technique. Furthermore, mass spectrometric investigations reveal detailed features of molecular structure, and additional structural information a priori is not required. A combination of the [^{32}P]-postlabeling assay and high-performance tandem MS provides an ideal strategy to identify unanticipated DNA adducts which may represent minor reaction pathways, but may have unexpected, significant biological implications.

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Announcement

Fourth International Symposium on Chiral Discrimination

The Fourth International Symposium on Chiral Discrimination will be held September 19–22, 1993, at the Bonaventure Hilton Hotel in Montreal, Quebec, Canada. The purpose of the meeting is to provide a forum for the exchange of ideas, information, and knowledge concerning all aspects of the impact of chirality on biology, pharmacology, and chemistry. Invited lectures will highlight recent advances in Stereochemical issues in the agricultural and food industries; International harmonization of stereoisomeric drug regulation; Clinical implications of stereochemistry; New approaches to practical stereoselective synthesis; Chirality in ligand–biopolymer interactions; and Chirality as an investigative tool. Poster discussion sessions will be organized to give poster authors the opportunity to make five-minute oral presentations of their work and will cover the topics of Regulatory issues; Nomenclature; Stereochemical aspects of pharmacology and toxicology; Nonchromatographic approaches to the determination of stereochemical purity and structure; Chromatographic separation of stereoisomers; and Stereocontrol in organic synthesis.

For further information contact: The Chiral Secretariat, Conference Office, McGill University, 550 Sherbrooke St. West, West Tower, Suite 490, Montreal, Quebec, Canada H3A 1R9 (Phone: 514-398-3370; Fax: 514-398-4854).

Meeting Calendar

January 10–17, 1993	Second International Meeting on Molecular Mechanisms of Metal Toxicity and Carcinogenicity [*Chem. Res. Toxicol.* **5** (3), 447, 1992].
January 18–24, 1993	Free Radical Club CERLIB: "Current Problems Associated with the Role of Free Radicals in Medicine" [*Chem. Res. Toxicol.* **5** (6), 876, 1992].
June 30–July 3, 1993	EUROTOX '93 [*Chem. Res. Toxicol.* **5** (3), 447, 1992].