Detection of phosphodiester adducts formed by the reaction of benzo[a]pyrene diol epoxide with 2'-deoxynucleotides using collision-induced dissociation electrospray ionization tandem mass spectrometry

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ABSTRACT

In this study, we investigated the products formed following the reaction of benzo[a]pyrene-7,8-dihydridiol-9,10-epoxide (B[a]PDE) with 2'-deoxynucleoside 3'-monophosphates. The B[a]PDE plus 2'-deoxynucleotide reaction mixtures were purified using solid phase extraction (SPE) and subjected to HPLC with fluorescence detection. Fractions corresponding to reaction product peaks were collected and desalted using SPE prior to analysis for the presence of molecular ions corresponding to \( m/z \) 648, 632, 608 and 623 \([\text{M-H}]^-\) consistent with B[a]PDE adducted (either on the base or phosphate group) 2'-deoxynucleotides of guanine, adenine, cytosine and thymine, respectively, using LC-ESI-MS/MS collision-induced dissociation (CID). Reaction products were identified having CID product ion spectra containing product ions at \( m/z \) 452, 436 and 412 \([\text{B[a]Ptriol+base}-\text{H}]^-\), resulting from cleavage of the glycosidic bond between the 2'-deoxyribose and base, corresponding to B[a]PDE adducts of guanine, adenine and cytosine, respectively. Further reaction products were identified having unique CID product ion spectra characteristic of B[a]PDE adduct formation with the phosphate group of the 2'-deoxynucleotide. The presence of product ions at \( m/z \) 399 and 497 were observed for all four 2'-deoxynucleotides, corresponding to \([\text{B[a]Ptriol+phosphate}-\text{H}]^-\) and \([\text{2'-deoxyribose+phosphate+B[a]Ptriol}-\text{H}]^-\), respectively. In conclusion, this investigation provides the first direct evidence for the formation of phosphodiester adducts by B[a]PDE following reaction with 2'-deoxynucleotides.

INTRODUCTION

A potential site in DNA for the interaction of genotoxic species is the phosphodiester linkages between the 2'-deoxynucleosides, which constitute the sugar-phosphate backbone of DNA, resulting in esterification of the phosphate group and formation of phosphotriester adducts. The properties of phosphotriester adducts have been extensively studied using simple alkylating agents as model compounds showing that they represent long lived biomarkers of exposure (1–3). In contrast the potential of polycyclic aromatic hydrocarbons (PAHs) to form phosphodiester adducts with 2'-deoxynucleotides as well as phosphotriester adducts in DNA has not been clearly ascertained (4).

Studies involving human fibroblast cells and rodents have shown that alkyl phosphotriester adducts are more stable to DNA repair when compared to base adducts (5–7). For alkylating agents it has been shown that the relative abundance of phosphotriester DNA adducts formed depends on the chemical nature of the genotoxic species (1,3,8). The biological consequences of alkyl phosphotriester adducts is not fully understood, though they are chemically stable under physiological conditions and may potentially alter the binding/function of proteins such as DNA repair or replication enzymes (1,9). To date, the compounds investigated for the formation of phosphodiester or phosphotriester adducts include alkylating agents, such as dialkylsulphates, alkyl methanesulphonates and N-nitroso compounds cyanoethylene oxide, cyclophosphamide and phenyl glycidyl ether (10–16).

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PAHs of which benzo[a]pyrene (B[a]P) is a well-studied example, represent an important class of compounds found as ubiquitous environmental pollutants or in certain occupational settings (17,18). PAHs are produced by the incomplete combustion of fossil fuels and the chargrilling of food as well as being present in automobile exhaust and cigarette smoke (19). PAHs have been shown to be carcinogenic in animals and potentially carcinogenic to humans (20–22). Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (B[a]PDE) is the reactive species formed by the cytochrome P450 mediated metabolism of B[a]P (23,24). The reaction of B[a]PDE via a B[a]Ptriol carbocation intermediate with purine and pyrimidine bases present in DNA has been well characterized, the predominant product formed is by reaction with the exocyclic amino group of guanine and to a lesser extent with the exocyclic amino groups of adenine and cytosine (25–27). The base adducts formed by B[a]PDE exist as diastereoisomers following cis or trans addition at C-10 of the hydrocarbon and studies show that the (+)-anti-B[a]PDE isomer with the 7R,8S,9S,10R configuration has greatest carcinogenic activity in vivo (28). B[a]PDE produces concentration-dependent strand breaks in DNA in vitro with the fragmentation of the DNA being attributable to the formation of a phosphotriester adduct rather than a base adduct. A mechanism for DNA strand scission has been proposed that involves the C-9 hydroxyl group of B[a]PDE attacking the phosphodiester group and the formation of a cyclic triester intermediate as shown in Scheme 1 (29).

The technique of collision-induced dissociation (CID) tandem mass spectrometry provides structural information, which is important since both the base and phosphodiester adducted 2'-deoxynucleotides have the same molecular mass. This approach has been used to characterize the phosphodiester adducts formed by phenyl glycidal ether and ethylating agents (11,16). We investigated the formation of phosphodiester adducts resulting from the reaction of B[a]PDE with 2'-deoxynucleotides using negative electrospray ionization tandem mass spectrometry CID.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

(±)-Anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (B[a]PDE) was purchased from the NCI Chemical Carcinogen Repository, Midwest Research Institute.
(Kansas City, Missouri, USA). The 2'-deoxynucleoside 3'-monophosphates and TRIS base were purchased from Sigma (Poole, Dorset, UK). All other reagents (analytical grade), HPLC (fluorescence) grade methanol, were purchased from Fisher Scientific (Loughborough, UK). HPLC grade water, 18.2 MΩ output quality was obtained from Maxima purification equipment (Elga, High Wycombe, UK).

Caution B[a]PDE is a mutagen and carcinogen. Protective clothing should be worn and appropriate safety procedures followed when working with the compound.

Reaction of 2'-deoxynucleotides with B[a]PDE

The four 2'-deoxynucleosides 3'-monophosphates (1 mg dissolved in 750 μl of 0.1 M TRIS base, pH 7.0) were each incubated with B[a]PDE (250 μg; 1 μg/μl dissolved in methanol) for 18 h at 37°C. Unreacted starting material containing 2'-deoxynucleosides 3'-monophosphates was removed by subjecting the reaction mixtures to solid phase extraction using Oasis HLB columns (1 cc, 30 mg, Waters Ltd, Elstree, UK) connected to a vacuum manifold (Phenomenex, Macclesfield, UK) maintained at a vacuum of 5 mmHg. The columns were initially conditioned with 1.0 ml of methanol followed by 1.0 ml of HPLC grade water. The reaction mixtures were then loaded onto the columns and washed with 1.0 ml of 5:95 methanol/water (v/v). The reaction products were eluted from the columns with 2.0 ml of methanol. Samples were dried to completeness using a centrifugal vacuum evaporator (Speedvac, Savant, Farmingdale, NY, USA) and resuspended in 400 μl of 10:90 acetonitrile/HPLC grade water (v/v). The purified HPLC fractions were then loaded onto the columns and washed with 1.0 ml of 5:95 methanol/water (v/v). The reaction products were eluted from the columns with 2.0 ml of methanol. Samples were dried to completeness using a centrifugal vacuum evaporator (Speedvac, Savant, Farmingdale, NY, USA) and resuspended in 400 μl of 10:90 acetonitrile/HPLC grade water (v/v). The purified fractions were then subjected to solid phase extraction using Oasis HLB columns (1 cc, 30 mg) connected to a vacuum manifold (Phenomenex, Macclesfield, UK) maintained at a vacuum of 5 mmHg. The columns were initially conditioned with 1.0 ml of methanol followed by 1.0 ml of HPLC grade water. The reaction mixtures were then loaded onto the columns and washed with 1.0 ml of 5:95 methanol/water (v/v). The reaction products were eluted from the columns with 2.0 ml of methanol. Samples were dried to completeness using a centrifugal vacuum evaporator and re-dissolved in 400 μl of methanol/HPLC grade water (20:80, v/v).

Mass spectrometric analysis

The LC-MS/MS consisted of a Waters Alliance 2695 separations module with a 100 μl injection loop connected to a Micromass Quattro Ultima Pt. (Micromass, Waters Ltd, Manchester, UK) tandem quadrupole mass spectrometer with an electrospray interface. The temperature of the electrospray source was maintained at 110°C and the desolvation temperature at 350°C. Nitrogen gas was used as the desolvation gas (6501/h) and the cone gas was set to zero. The capillary voltage was set at 3.00 kV. The cone and RF1 lens voltages were 42 and 25 V, respectively.

The mass spectrometer was tuned by using a dGp standard solution (10 pmol/μl) dissolved in methanol/HPLC grade water (45:65, v/v) introduced by continuous infusion at a flow rate of 10 μl/min with a Harvard model 22 syringe pump (Harvard Apparatus Ltd, Edenbridge, UK). Initial analysis of the reaction products was performed using continuous infusion and full scan negative ESI-MS over the m/z range from 60 to 800, following a 1:10 dilution with methanol/HPLC grade water (45:65, v/v) of each of the purified reaction products.

A 20 μl undiluted aliquot of each purified reaction product was injected onto a HyPurity C18 (3 μm, 150 × 2.1 mm) column (Thermo Electron Corporation, Runcorn, UK) connected to a Uniguard HyPurity C18 (3 μm, 10 × 2.1 mm) guard cartridge attached to KrudKatcher (Phenomenex) disposable pre-column (5 μm) filter. The column was eluted isocratically with solvent A, methanol/HPLC grade water (45:65, v/v) at a flow rate of 120 μl/min for 45 min. It was then washed with solvent B, methanol at a flow rate of 200 μl/min for 10 min and then equilibrated to starting conditions with solvent A at a flow rate of 120 μl/min for 15 min. The collision gas was argon (indicated cell pressure 3.0–3.5 × 10⁻³ mbar) and the collision energy set at 21 eV. The dwell time was set to 200 ms and the resolution was one m/z unit at peak base. The samples were analysed in negative electrospray ionization (ESI) mode MS/MS CID for the deprotonated molecular ion [M–H]⁻ for each B[a]PDE adducted 2'-deoxynucleotide: 2'-deoxyguanosine 3'-monophosphate (dGp) [C₃₀H₂₈N₅O₉P⁻]⁻ m/z 648.15; 2'-deoxyadenosine 3'-monophosphate (dAp) [C₃₀H₂₈N₅O₉P⁻]⁻ m/z 632.16; 2'-deoxyadenosine 3'-monophosphate (dAp) [C₃₀H₂₈N₅O₉P⁻]⁻ m/z 608.14 and thymidine 3'-monophosphate (Tp) [C₃₀H₂₈N₅O₁₄P⁻]⁻ m/z 623.14. The mass spectral data was acquired in continuum mode and processed using MassLynx version 4.0 (Micromass, Waters Ltd).

RESULTS

HPLC-fluorescence analysis of the B[a]PDE plus 2'-deoxynucleotide reaction products

The reaction mixtures for the four different 2'-deoxynucleotides and B[a]PDE were initially subjected to solid phase extraction to remove any unreacted 2'-deoxynucleoside 3'-monophosphates, followed by separation using HPLC with fluorescence detection.
The typical HPLC-fluorescence chromatogram of a control reaction mixture containing only [B[a]PDE and 0.1 M TRIS base pH 7.0 buffer incubated at 37°C for 18 h and subjected to solid phase extraction is shown in Figure 1. The typical HPLC-fluorescence chromatograms for 2'-deoxynucleotides plus [B[a]PDE reaction mixtures are shown in Figures 2A, 3A, 4A and 5A. Fractions corresponding to the peaks that eluted before retention time 35 min, since these were unique to the reaction mixtures and not present in the control reaction mixture, were collected, pooled and evaporated to dryness and then subjected to a further purification by solid phase extraction (to remove any salts) prior to analysis by continuous infusion full scan negative ESI-MS to determine the presence of the deprotonated molecular [M-H]⁻ ion for each adducted 2'-deoxynucleotides. Each fraction was then further characterized using LC-ESI-MS/MS CID.

### Reaction of 2'-deoxynucleotides with B[a]PDE—phophodiester adducts

For each 2'-deoxynucleotide, the phosphodiester adducts eluted before the corresponding base adducts following analysis by HPLC-fluorescence and then by LC-ESI-MS/MS CID. Typically, the LC-ESI-MS/MS retention times ranged from 7 to 12 min using the microbore C18 column (data not shown). The LC-ESI-MS/MS CID product ion spectra for each adducted 2'-deoxynucleotide are shown in Figures 2B, 3B, 4B and 5B. Two distinct product ions at m/z 399 and 497 were observed corresponding to the [(B[a]Ptriol + phosphate)−H]⁻ and [(2'-deoxyribose + phosphate + B[a]Ptriol)−H]⁻, respectively, resulting from the fragmentation of the precursor molecular ion of each of the four adducted 2'-deoxynucleotides. The ion at m/z 381 corresponds to the loss of H2O from the ion at m/z 399. These results indicated that the B[a]PDE modification was present on the phosphate moiety of the 2'-deoxynucleotide resulting in the formation of a phosphodiester adduct. Further product ions were observed corresponding to m/z 79 [PO3]⁻, m/z 97 [H2PO4]⁻, m/z 195 [(2'-deoxyribose + phosphate)−H]⁻ that were common to the spectra for all four adducted 2'-deoxynucleotides. The spectra also contained product ions that correspond to the unadducted 2'-deoxynucleotide 3'-monophosphate and base and at m/z 346 [dGp−H]⁻, m/z 150 [guanine−H]⁻, m/z 330 [dAp−H]⁻; m/z 134 [adenine−H]⁻, 306 [dCp−H]⁻; m/z 110 [cytosine−H]⁻ and m/z 321 [Tp−H]⁻; m/z 125 [thymine−H]⁻. The fragmentation pathway of phosphodiester adducted 2'-deoxynucleotides is shown in Scheme 2. It was noted that there was a complete absence of products ions corresponding to the adducted bases in the spectra.

### Reaction of 2'-deoxynucleotides with B[a]PDE—base adducts

For each 2'-deoxynucleotide, the base adducts eluted after the phosphodiester adducts following analysis by HPLC-fluorescence and then by LC-ESI-MS/MS CID. Typically, the LC-ESI-MS/MS retention times ranged from 13 to 28 min using the microbore C18 column (data not shown). The LC-ESI-MS/MS CID product ion spectra for each adducted 2'-deoxynucleotide are shown in Figures 2C, 3C and 4C showing adduct formation with the base. Product ions at m/z 452 and 550 were observed resulting from the fragmentation of the B[a]PDE adducted dGp precursor molecular ion at m/z 648 [M−H]⁻ (Figure 2C), corresponding to [(B[a]Ptriol + guanine)−H]⁻ following cleavage of the glycosidic bond and [(B[a]Ptriol + 2'-deoxyguanosine)−H]⁻ following loss of one H2O molecule plus the phosphate group, respectively. The ions at m/z 434 and 416 correspond to the loss of one and two H2O molecules from the ion at m/z 452, respectively. Similarly, the ions at m/z 532 and 514 correspond to the loss of one and two H2O molecules from the ion at m/z 550, respectively. These results imply the presence of a B[a]PDE modification on the guanine base of dGp. A product ion at m/z 436 was observed resulting from the fragmentation of the B[a]PDE adducted dAp precursor molecular ion at m/z 632 [M−H]⁻ (Figure 3C) corresponding to [(B[a]Ptriol + adenine)−H]⁻ following cleavage of the glycosidic bond. The ions at m/z 418 and 400 correspond to the loss of one and two H2O molecules from the ion at m/z 436, respectively. These results imply the presence of a B[a]PDE modification on the adenine base of dAp. A product ion at m/z 412 was observed resulting from the fragmentation of the B[a]PDE adducted dCp precursor molecular ion at m/z 608 [M−H]⁻ (Figure 4C) corresponding to [(B[a]Ptriol + cytosine)−H]⁻ following cleavage of the glycosidic bond. The ions at m/z 394 and 376 correspond to the loss of one and two H2O molecules from the ion at m/z 412, respectively. These results imply the presence of a B[a]PDE modification on the cytosine base of dCp. The fragmentation pathway of base adducted 2'-deoxynucleotides is shown in Scheme 3. It was noted that there was a complete absence of products ions characteristic of an

![Figure 1. HPLC-fluorescence chromatogram of a reaction mixture containing (±)-anti-B[a]PDE (250μg) and 0.1 M TRIS base pH 7.0 buffer incubated at 37°C for 18 h and subjected to solid phase extraction. The analysis was performed using gradient elution with 0.05 M potassium phosphate buffer, pH 7.2 (solvent A) and acetonitrile (solvent B) at a flow of 1 ml/min (s = refer to Figures 2A, 4A and 5A).](https://academic.oup.com/nar/article-abstract/35/15/5014/1076447)
Figure 2. HPLC-fluorescence chromatogram of the reaction of B[a]PDE (250 μg) with dGp (1 mg) following gradient elution with 0.05 M potassium phosphate buffer, pH 7.2 (solvent A) and acetonitrile (solvent B) at a flow of 1 ml/min (A). Typical negative ESI LC-MS/MS CID product ion spectra for fractions 1 and 2 corresponding to phosphodiester adducts (B) and fraction 3 corresponding to base adduct (C). Both spectra were obtained from the molecular ion [M−H]− at m/z 648 following isocratic elution with methanol/HPLC grade water (45:65, v/v) at a flow rate of 120 μl/min and collision energy of 21 eV (⋆ = peaks present in control reaction mixture (Figure 1)).
Figure 3. HPLC-fluorescence chromatogram of the reaction of B[a]PDE (250 μg) with dAp (1 mg) following gradient elution with 0.05 M potassium phosphate buffer, pH 7.2 (solvent A) and acetonitrile (solvent B) at a flow of 1 ml/min (A). Typical negative ESI LC-MS/MS CID product ion spectra for fractions 1, 2 and 3 corresponding to phosphodiester adducts (B) fraction 4 corresponding to base adduct (C). Both spectra were obtained from the molecular ion [M–H]⁻ at m/z 632 following isocratic elution with methanol/HPLC grade water (45:65, v/v) at a flow rate of 120 μl/min and collision energy of 21 eV.
Figure 4. HPLC-fluorescence chromatogram of the reaction of B[a]PDE (250 μg) with dCp (1 mg) following gradient elution with 0.05 M potassium phosphate buffer, pH 7.2 (solvent A) and acetonitrile (solvent B) at a flow of 1 ml/min (A). Typical negative ESI LC-MS/MS CID product ion spectra for fractions 1, 2 and 3 corresponding to phosphodiester adducts (B) fraction 4 corresponding to base adduct. (C) Both spectra were obtained from the molecular ion [M–H]$^-$ at m/z 608 following isocratic elution with methanol/HPLC grade water (45:65, v/v) at a flow rate of 120 μl/min and collision energy of 21 eV [* = peaks present in control reaction mixture (Figure 1)].
adducted phosphate group. All three spectra for the base adducted 2′-deoxynucleoside 3′-monophosphates contained a product ion at \( m/z \) 283 corresponding to B[a]Pdiol.

Confirmation that the structural identity of the base adducts was due to the reaction of B[a]PDE with the exocyclic –NH2 group was ascertained following analysis by positive ESI. The LC-ESI-MS/MS CID product ion spectra for each base adducted 2′-deoxynucleotide are shown in Figure 6. The spectrum for each base adducted 2′-deoxynucleotide contained common product ions. The ion at \( m/z \) 303 corresponds to B[a]Ptiol. The ion at \( m/z \) 285 corresponds to B[a]Pdiol following loss of H2O from the ion at \( m/z \) 303. The ion observed at \( m/z \) 257 corresponds to the loss of CO from the ion at \( m/z \) 285. The spectra also contained product ions that correspond to the unadducted 2′-deoxynucleoside 3′-monophosphate and base at \( m/z \) 348 [dGp + H2]+, \( m/z \) 152 [guanine + H2]+, \( m/z \) 332 [dAp + H2]+, \( m/z \) 136 [adenine + H2]+, and \( m/z \) 308 [dCp + H2]+, \( m/z \) 112 [cytosine + H2]+. Product ions were observed corresponding to the adducted base following cleavage of the glycosidic bond at \( m/z \) 454 [(B[a]Ptiol + guanine) + H2]+, at \( m/z \) 438 [(B[a]Ptiol + adenine) + H2]+, and at \( m/z \) 414 [(B[a]Ptiol + cytosine + H2]+. No product ions resulting from the neutral loss of 17 u corresponding to NH3 were observed, thus confirming that B[a]PDE adduct formation for each 2′-deoxynucleotides was by reaction with the
exocyclic –NH₂ group at position N² for guanine, N⁶ for adenine and N⁴ for cytosine.

**DISCUSSION**

The results of this investigation provide the first direct evidence for the formation of phosphodiester adducts with 2'-deoxynucleotides that are normally present in DNA by the reactive PAH epoxide metabolite, B[a]PDE, thus providing supporting evidence to the supposition that B[a]PDE can react with the sugar-phosphate backbone of DNA resulting in the formation of phosphotriester adducts. Koreeda *et al.* were the first to provide indirect evidence that B[a]PDE may form phosphotriester adducts as well as base adducts following their experiments involving the reaction of tritium labelled B[a]PDE with polyguanylic acid (4). Chan and Raddo hypothesized that the formation of phosphotriesters from the reaction of B[a]PDE with DNA was chemically feasible using initial model experiments investigating the formation of phosphotriesters following the reaction of cyclohexene oxides with dibenzyl or diethyl phosphates resulting in the formation of phosphotriester adducts in a regio- and stereo-specific manner as determined by NMR spectroscopy (31). We investigated the reaction of B[a]PDE with 2'-deoxynucleotides, which resulted in the formation of a number of additional early eluting product peaks when compared to a control reaction mixture containing only the B[a]PDE and buffer following HPLC-fluorescence analysis, which were further characterized using LC-ESI-MS/MS CID. The peaks present in the control reaction mixture (Figure 1) represent breakdown products such as B[a]Ptetraols following hydrolysis of the B[a]PDE. The direct HPLC-fluorescence analysis of (±)-anti-B[a]PDE following hydrolysis in aqueous methanol resulted in the detection of two major peaks (corresponding to the peaks labelled with an asterisk in Figure 1) and two later eluting minor peaks (data not shown). We hypothesize that the other later eluting peaks observed may be impurities derived from the interaction of B[a]PDE with the TRIS base reaction buffer. Previous studies have shown that hydrolysis of B[a]PDE in aqueous solutions at neutral pH results in the formation of two B[a]Ptetraol products with *trans* and *cis* configurations (32). It was noted that each HPLC fraction analysed contained several product peaks, the reason

**Scheme 2.** Negative ESI-MS/MS CID fragmentation pathway for B[a]PDE 2'-deoxynucleotide phosphodiester adducts.
being that each peak corresponded to the different stereoisomers of adducts resulting from the cis or trans addition at C-10 of the (±)-anti-B[a]PDE (33). The interaction of the (+)- or (−)-anti-enantiomers of B[a]PDE with either 2'-deoxyguanosine or 2'-deoxyadenosine will result in the formation of two pairs of stereoisomer adducts, namely, (+)-anti-trans-, (+)-anti-cis-, (−)-anti-trans- and (−)-anti-cis- following reaction with the exocyclic amino group of the base (34). Furthermore, the phosphate of the phosphodiester group is chiral with adduct formation resulting in two configurations, Rp and Sp (35). Using LC-ESI-MS/MS CID reaction products were differentiated having a unique CID product ion spectra characteristic of a phosphodiester adduct. The presence of product ions at m/z 399 and 497 was observed for all four 2'-deoxynucleotides, corresponding to [(B[a]Ptriol + phosphate)−H]− and [(2'-deoxyribose + phosphate + B[a]Ptriol)−H]−, respectively. There was an absence of product ions in the spectra corresponding to B[a]PDE adducted bases. For the three B[a]PDE and 2'-deoxynucleotide reaction mixtures the phosphodiester adducts eluted before the corresponding base adducts following HPLC-fluorescence analysis. Canella et al. noted the presence of uncharacterized products in their investigation of the synthesis of base adducts by the reaction of B[a]PDE with 2'-deoxynucleotides. Two early eluting products were detected following HPLC-UV analysis of a B[a]PDE plus 2'-deoxyadenosine 5'-monophosphate reaction mixture that were resistant to hydrolysis by alkaline phosphatase unlike the base adducts (36). The identity of these products was not elucidated but it was concluded that their structure contained a nucleotide component that required the presence of 2'-deoxy nucleotides in the B[a]PDE reaction mixture for their formation (36). It is feasible to assume that these unidentified products were phosphodiester adducts.

Further reaction products were identified having CID product ion spectra characteristic of adduct formation with the bases of the 2'-deoxynucleotides resulting from cleavage of the glycosidic bond, with prominent product ions at m/z 452, 436 and 412 [(B[a]Ptriol + base)−H]− corresponding to B[a]PDE adducts of guanine, adenine and cytosine, respectively. Consistent with previous reports in the literature B[a]PDE adduct formation with the base was confined to dGp, dAp and dCp (27,37,38).

Confirmation that the reaction with exocyclic −NH2 group of each base had occurred was obtained following positive
ESI LC-MS/MS analysis of the reaction products. The CID product ion spectra revealed that no product ions resulting from the neutral loss of 17 u corresponding to NH₃, were observed. Thus, confirming that B[a]PDE adduct formation for each 2'-deoxynucleotide was by reaction with the exocyclic –NH₂ group at position N² for guanine, N⁶ for adenine and N⁴ for cytosine. For example, the CID product ion spectra of N-7 alkyl guanine adducts.

Figure 6. Typical positive ESI LC-MS/MS CID product ion spectra for the base adducted 2'-deoxynucleotides. The spectra were obtained from the molecular ions [M+H]⁺ at m/z 650, B[a]PDE plus dGp (fraction 3, Figure 2A) (A), m/z 634 B[a]PDE plus dAp (fraction 4 (×2 concentrated), Figure 3A) (B) and m/z 610 B[a]PDE plus dCp (fraction 4 (×2 concentrated), Figure 4A) (C) following isocratic elution with 0.1% acetic acid/methanol (50:50, v/v) at a flow rate of 120 µl/min. The MS conditions used are as described in the Experimental Procedures section except for the following; capillary voltage, 3.20 kV and collision energy, 16 eV.
such as those derived from ethylating agents contain a product ion corresponding to the neutral loss of NH$_3$ from the alkylated [M + H]$^+$ ion (39,40). No base adducts of Tp were detected which is again consistent with previous findings. Due to later elution of the phosphodiester adducts of Tp following HPLC-fluorescence analysis (Figure 5A) compared to the other three 2'-deoxynucleotides, any base adducts formed may have eluted in the region of the hydrolyzed break down products of B[a]PDE. Studies in vitro have shown that the stereochemistry of B[a]PDE influences the extent of formation of the different base adducts following the reaction with DNA. The (+)-anti-B[a]PDE isomer with the 7R,8S,9S,10R configuration has the highest reactivity resulting in 95% guanine and 5% adenine of total adducts being formed (24,41). The results described in the present study do not allow for the estimation of the extent B[a]PDE adduct formation with the phosphate group relative to the base since the HPLC-fluorescence detector response may be significantly different for the phosphodiester adducts compared to the base adducts.

The formation of B[a]PDE phosphoryl triester adducts in DNA has the potential for producing more profound adverse biological consequences when compared to the stable alkyl phosphoesters adducts formed by simple alkylating agents. This is due to the presence of an oxygen atom in the β position with respect to the ester group that can lead to the alteration in the integrity of the DNA via mechanism of internal oxygen nucleophile displacement at the phosphate atom, resulting in strand scission (29,42). The confirmation of the formation of B[a]PDE phosphodiester adducts should allow for the investigation of methodology for the detection of B[a]PDE phosphodiester adducts in DNA. The first methods developed to detect alkyl phosphodiester adducts in DNA relied on 32P postlabelling techniques (8,11,44,45). In the last few years, liquid chromatography coupled to mass spectrometry has been increasingly used for not only for the characterization of DNA adducts but also their detection (46). Recently, LC-MS/MS methods have been described for the detection of alkyl phosphodiester adducts in DNA relying on selective enzymatic digestion (47–50). The transalkylation approach involves the alkyl group of the phosphodiester adduct in di-2'-deoxynucleoside monophosphates, formed following enzymatic digestion of DNA, undergoing nucleophilic displacement by cob(II)alamin. The resulting alkyl-cob(II)alamin product can then be determined by LC-MS/MS (47, 48). The enzymatic digestion relies on the presence of a phosphodiester adduct in DNA generating di-2'-deoxynucleoside monophosphate adduct triesters, since the internucleotide bonds adjacent to a completely esterified phosphate group are resistant to enzymatic cleavage by nucleases (48,51). The method described by Haglund et al. uses enzymatic digestion with nuclease P1 in combination with 5'-phosphodiesterase and alkaline phosphatase for the direct detection of di-2'-deoxynucleoside monophosphate ethyl triesters by online column switching LC-MS/MS (48). However, optimization of the enzymatic digestion may be required for the detection of B[a]PDE phosphodiester adducts, since it is found for bulky DNA adducts such as those derived from B[a]PDE, that di-2'-deoxynucleoside monophosphates are generated by the presence of base adducts, which are particularly resistant to enzymatic digestion (52,53). These B[a]PDE di-2'-deoxynucleoside monophosphate adducts have been observed to occur at a 50 times lower level than the main base adduct formed at the exocyclic –NH$_2$ group of guanine in DNA (54).

In conclusion, the formation of phosphodiester adducts following the reaction of B[a]PDE with 2'-deoxynucleotides has been confirmed, having been identified with distinct CID product ion spectra when compared to base adducted 2'-deoxynucleotides.

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