Preparation, identification and analysis of stereoisomeric *anti*-benzo[*a*]pyrene diol epoxide–deoxyguanosine adducts using phenyl liquid chromatography with diode array, fluorescence and tandem mass spectrometry detection

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Abstract

Benzo[*a*]pyrene, a common environmental pollutant, can be metabolized into reactive *anti*-benzo[*a*]pyrene diol epoxide (*anti*-BPDE), which predominantly binds to deoxyguanine in DNA and forms four stereoisomeric adducts. To characterize the stereochemistry of these adduct isomers, preparation of single adducted deoxyguanosine (dG) is required for efficient enantiomeric analysis. Here, we demonstrate an improved method for preparation, identification, and analysis of four BPDE-adducted dGs, including (+)-*trans*-, (−)-*trans*-, (+)-*cis*-, and (−)-*cis*-*anti*-BPDE-*N*²-dG. These stereoisomerically adducted nucleosides were first synthesized by a direct reaction of (±)-*anti*-BPDE with dG, followed by optimized solid-phase extraction (SPE) and HPLC purification. The reaction of (±)-*anti*-BPDE and dG displayed a yield as high as 45%. The developed preparation method does not require any enzymatic digestion. Based on highly efficient separation achieved by optimization of stationary phase and mobile phase, LC-UV-MS/MS and LC-diode array detection (DAD)-fluorescence detection (FL) methods were established for characterization and analysis of the four stereoisomeric *anti*-BPDE-dGs. The established LC-DAD-FL method may provide characterization and analysis of four stereoisomeric *anti*-BPDE-dGs and two interfering *anti*-BPDE tetrols by taking advantage of their distinct fluorescence quenching.

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1. Introduction

Benzo[*a*]pyrene (B[*a*]P) is one of those carcinogenic polycyclic aromatic hydrocarbons (PAHs), which are widely present in polluted environment. B[*a*]P can be stereoselectively metabolized *in vivo* to a reactive metabolite, (+)-*anti*-benzo[*a*]pyrene diol epoxide (*anti*-BPDE), which may predominantly bind to deoxyguanosine (dG) in DNA and preferentially form BPDE-DNA adducts at lung cancer mutational hotspots in tumor suppressor gene *p53* [1]. A number of animal and human cell culture experiments have shown that (±)-*anti*-BPDE was more mutagenic than its stereoisomeric (±)-*syn*-BPDE [2–4]. Each enantiomer of racemic *anti*-BPDE can react at the C10 position

of deoxyguanosine in DNA and form *trans* and *cis* stereoisomeric BPDE-*N*²-dG adducts (Fig. 1). Striking differences in the biological activities of these four stereoisomeric *anti*-BPDE-*N*²-dG adducts have been reported [5–9]. It is important to qualitatively and quantitatively evaluate stereoisomeric *anti*-BPDE-*N*²-dG adducts in DNA to understand the mutagenic and carcinogenic effects of B[*a*]P.

A number of methods have been developed for characterization and/or detection of BPDE-DNA adducts, including capillary electrophoresis (CE)-laser-induced fluorescence (LIF) and CE-LIF immunoassays [10–13], accelerator mass spectrometry [14], liquid chromatography or capillary electrophoresis coupled to mass spectrometry [15–21], and ³²P-postlabelling [20,22] and spectroscopic analysis [23,24]. Among these methods, CE-LIF immunoassays display advantages of measuring trace levels of DNA adducts without digestion of DNA [10,11]. However, no method is available to characterize the stereochemistry of DNA

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Fig. 1. Structures of *anti*-BPDE enantiomers (top) and *anti*-BPDE-*N*²-dG stereoisomers (bottom).

adducts without the need of DNA digestion. In fact, enzymatic digestion of damaged DNA into single adducted nucleosides and subsequent stereoselective separation is required to identify and characterize the stereochemistry of various adduct isomers [2,18]. A large amount of pure single BPDE-adducted nucleosides, which are not commercially available, are also required to be prepared in individual laboratories to characterize the stereochemistry of BPDE-DNA adducts generated *in vitro* or *in vivo*. Previously, single adducted nucleosides were obtained by reaction of *anti*-BPDE with DNA, followed by enzymatic digestion, liquid–liquid or solid-phase extraction, and HPLC purification [19,20,23]. In these procedures, multiple enzymatic digestions involved are time-consuming (~24–72 h), which may cause excess hydrolysis of the BPDE-nucleosides into BPDE tetrols. Another disadvantage is the low yield of *anti*-BPDE-dG in DNA except (+)-*trans*-*anti*-BPDE-dG [24]. To produce enough *anti*-BPDE-dG standards, a large amount of highly carcinogenic *anti*-BPDE is often required (1–10 mg) [19,20,23]. An alternative is to use the reaction of dGMP and *anti*-BPDE, which only requires one-step enzymatic digestion for preparation of *anti*-BPDE-dGs [25]. However, only two optically active *anti*-BPDE-dGMPs were observed in our work (unpublished data). Here, we developed a simple preparation method of four optically active *anti*-BPDE-dGs by a direct reaction of deoxyguanosine and racemic *anti*-BPDE, followed by optimized SPE and HPLC purification. The stereochemistry of prepared *anti*-BPDE-dG was identified and characterized by HPLC–MS/MS, UV spectroscopy, and circular dichroism (CD).

Recently, we have reported that nonchiral aromatic (phenyl) stationary phases display better stereoisomeric resolution of four *anti*-BPDE-dG adducts in short oligonucleotides over nonaromatic C18 stationary phases [26]. In this work, a baseline

separation of four *anti*-BPDE-dGs and two related tetrols was developed. This is achieved by using a silicone polymer coating phenyl column and optimizing mobile phase. The established HPLC separation not only fits to MS/MS detection but also fits to more sensitive fluorescence detection, in which two BPDE tetrols interfere seriously with the separation of the four optically-active BPDE-dG adduct isomers. The established LC separation method was also used for the preparation and purification of synthesized BPDE-dG isomers. They may be applied to identify and to detect the optically active adducts in short oligonucleotides and genomic DNA after enzymatic digestion. We have also found that each stereoisomer of the four *anti*-BPDE-dGs has unique fluorescence quenching observed with the established HPLC-DAD-FL method. The fluorescence quenching property of these stereoisomers may be potentially used for studying the stereochemistry of the BPDE-dG adducts.

2. Materials and methods

2.1. Chemicals and reagents

Caution: BPDE is carcinogenic and must be handled with caution.

(±)-*anti*-BPDE was a kind gift from Dr. X. Chris Le at the University of Alberta. Deoxyguanosine (dG), tetrahydrofuran, and triethylamine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were of HPLC grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Ultrapure water was prepared by a Milli-Q water system (Millipore, Bedford, MA, USA). Other chemicals were used of analytical or HPLC grade.

2.2. Apparatus

Two sets of HPLC system were used in this work. The first is a Hitachi L-2000 series liquid chromatography system (Hitachi, Tokyo, Japan). It consisted of a L-2130 low-pressure gradient pump, a L-2200 autosampler with an injection volume from 0 to 100 μ L, and two detectors, which are a L-2455 (1024 bits) diode array detector and a L-2485 fluorescence detector. A Hitachi EZChrom Elite chromatography data system was used for the operation and data acquisition. This system was used for HPLC purification and HPLC-DAD-FL analysis.

The other HPLC system was used for HPLC-Q (quadrupole)/TOF(time-of-flight)-MS/MS analysis. It consisted of an Alliance 2695 HPLC (Waters, Manchester, UK) and a Waters Micromass Quattro Micro mass spectrometer, which was equipped with an electrospray ionization (ESI) source. Data acquisition was conducted by MassLynx 4.0 software (Waters, Milford, MA, USA).

2.3. Preparation of BPDE- N^2 -dG stereoisomeric adducts

Four *anti*-BPDE- N^2 -dG stereoisomers were synthesized by a direct reaction of deoxyguanosine with ($\pm$)-*anti*-BPDE. In brief, 0.1 mg ($\pm$)-*anti*-BPDE dissolved in 100 μ L of freshly prepared tetrahydrofuran/triethylamine (19:1, v/v) was mixed with 1.2 mL deoxyguanosine solution (4 mg/mL in 50 mM Tris-HCl buffer, pH 7.5). The reaction solution was divided into two equal aliquots. Both aliquots were incubated in dark for 24 h but at 25 and 37 $^{\circ}$ C, respectively. The aliquots were then applied to the Cleanert C18 SPE cartridges (100 mg, Agela Technologies, Newark, DE, USA), which were pre-conditioned with 2 mL methanol and then with 2 mL deionized water. The cartridges were rinsed with 1 mL deionized water twice, and then sequentially washed with 1 mL 10, 30, and 50% methanol aqueous solution. Finally, the cartridges were eluted by 1 mL methanol twice.

The collected SPE fractions were analyzed by HPLC-DAD-FL. A Capcell Pak Ph UG column (250 mm $\times$ 4.6 mm I.D., 5 μ m particle size) from Shiseido (Tokyo, Japan) was employed in this experiment. Two solvents (solvents A and B) were prepared as mobile phase for gradient and isocratic elution. The solvent A consisted of methanol/10 mM acetate buffer containing 0.14% triethylamine (pH 7.0) (6/94, v/v), and the solvent B consisted of methanol/10 mM acetate buffer containing 0.14% triethylamine (pH 7.0) (80/20, v/v). An isocratic elution with the mobile phase consisted of 45.9% solvent B and 54.1% solvent A was used at the first 10 min, then a linear gradient elution from 45.9% to 73% solvent B was used for the next 20 min, followed by a 10 min isocratic elution of 73% solvent B. The flow-rate was maintained at 0.75 mL/min.

The fractions of *anti*-BPDE- N^2 -dGs were further purified by HPLC with an isocratic elution. The mobile phase consisted of acetonitrile/10 mM acetate buffer containing 0.14% triethylamine (pH 7.0) (18.5/81.5, v/v). The flow-rate was kept at 0.75 mL/min.

2.4. Optimization of HPLC separation of BPDE- N^2 -dG

To achieve optimum separation of the four *anti*-BPDE- N^2 -dGs, silicone polymer coating phenyl column that displays well separation of BPDE-DNA adducts was chosen, and two sets of mobile phases, methanol/water/acetate-triethylamine buffer (set 1) and acetonitrile/water/acetate-triethylamine buffer (set 2), were tested. With both mobile phases, 10 mM acetate buffer with 0.14% triethylamine (pH 7.0) was included to inhibit peak tailing. For set 1, the volume percentage of methanol was varied from 52 to 36%. For set 2, the volume percentage of acetonitrile was varied from 30 to 18%. Isocratic elution of each tested mobile phase was used to optimize the type and the composition of mobile phase. The solutes were simultaneously monitored by a DAD system and a fluorescence detector. It was found that the optimum mobile phase consisted of 18.5% acetonitrile, 10 mM acetate buffer (pH 7.0) with 0.14% triethylamine.

2.5. HPLC/ESI-Q-TOF-MS/MS analysis of four BPDE- N^2 -dGs

HPLC-MS analysis of the four *anti*-BPDE- N^2 -dGs was conducted under optimum chromatographic conditions. The flow-rate was kept at 0.75 mL/min for HPLC separation. The effluent from the HPLC column was split into a stainless capillary at 0.2 mL/min for Q-TOF-MS analysis. The mass spectrometer was operated in either positive ionization mode or negative ionization mode. The desolvation temperature and source temperature were set at 200 and 80 $^{\circ}$ C, respectively. The cone voltage was 20 V, and the capillary voltage was maintained at 3.5 kV for positive mode and 2.5 kV for negative mode. Nitrogen was chosen as a nebulizer gas at a flow-rate of 10 L/min.

For collision-activated dissociation (CAD) MS/MS fragmentation analysis, positive ionization mode was chosen. Argon was used as the collision gas to fragment the parent ions of the protonated adducts at m/z of 570. The collision energy was 20 V, and the gas pressure was kept at 0.3 MPa.

2.6. Circular dichroism (CD) spectra of anti-BPDE- N^2 -dGs

The BPDE- N^2 -dG adducts of high purity (purified as described above) were individually dissolved in 33% methanol aqueous solution. Their CD spectra were measured on a Jasco Model J-810 spectropolarimeter. The measurement was carried out at 4 $^{\circ}$ C.

3. Results and discussion

3.1. Synthesis of anti-BPDE- N^2 -dGs

In the synthesis of *anti*-BPDE- N^2 -dGs, 100-times (molar ratio) excess dG over *anti*-BPDE was used to make full use of carcinogenic *anti*-BPDE. Racemic *anti*-BPDE of about 37.8–45.8% could react with dG and form four *anti*-BPDE- N^2 -dGs with a varied percentage (2.2–50.5%) (Tables 1 and 2). Reaction temperature may affect the total yield but not the

Table 1

Percentages of the four BPDE-*N*²-dG stereoisomers in the total BPDE-*N*²-dG adducts from reaction of *anti*-BPDE with deoxyguanosine at 25 and 37 °C

	Peak 2 (–)- <i>trans</i>	Peak 3 (+)- <i>cis</i>	Peak 4 (+)- <i>trans</i>	Peak 5 (–)- <i>cis</i>	Total percentage
25 °C	18.6 ± 0.15	2.5 ± 0.14	50.5 ± 0.02	28.5 ± 0.04	100
37 °C	19.2 ± 0.13	2.2 ± 0.10	49.4 ± 0.04	29.2 ± 0.03	100

The yield was calculated using peak area percentage by UV absorbance at 343 nm. The measurement was conducted in triplicate (means ± SD).

Table 2

Percentages of the total BPDE-*N*²-dG adducts and the two BPDE tetrols in total BPDE products from reaction of *anti*-BPDE with deoxyguanosine at 25 and 37 °C

	BPDE-dG adducts	BPDE tetrol (peak 1)	BPDE tetrol (peak 6)	Total percentage
25 °C	45.8 ± 0.51	46.4 ± 0.23	7.8 ± 0.30	100
37 °C	37.8 ± 0.43	52.6 ± 0.46	9.6 ± 0.27	100

The yield was calculated using peak area percentage by UV absorbance at 343 nm. The measurement was conducted in triplicate (means ± SD).

relative ratios of the adduct isomers. It was found that the yield of BPDE-*N*²-dG adducts at 25 °C (45.8%) is higher than that at 37 °C (37.8%). Meanwhile, there is no significant difference in the ratio of four adducts between 25 and 37 °C. The yield of *cis*-(+)-*anti*-BPDE-*N*²-dG is the lowest (~2.2–2.5% of total adducts), which is 20 times lower than that of *trans*-(+)-*anti*-BPDE-*N*²-dG (~50% of total adducts). The low yield of the *cis*-(+)-*anti*-BPDE-*N*²-dG was consistent with its chemical instability [27]. By the established synthesis method, enzymatic digestion process is no longer required, and carcinogenic *anti*-BPDE (~0.1 mg) is 10–100 fold less consumed than reported methods [19,20,23].

3.2. SPE fractionation

A SPE fractionation procedure was developed to remove excess dG and minor byproducts in the reacted solution of *anti*-BPDE and dG. Fig. 2 illustrates SPE removal of unreacted dG and byproducts and extraction of *anti*-BPDE-dG adducts. It is evident that unreacted dG can be removed after washing by 2 mL water and 1 mL 10% methanol/water (v/v) (see Fig. 2A). Some unknown fluorescent byproducts could be removed by 1 mL 30% methanol/water (Fig. 2B). During these washing steps, no *anti*-BPDE-dG adducts and *anti*-BPDE tetrols were lost. However, they could be co-eluted by 1 mL 50% methanol/water and

Fig. 2. HPLC-DAD-FL analysis of each SPE fraction obtained from the reaction of deoxyguanosine (dG) with (±) *anti*-BPDE. UV absorbance was monitored at 252 nm (A and C) and fluorescence response (B and D) was monitored at Ex 343 nm, Em 400 nm. (a) Sample loading, (b) first water washing, (c) second water washing, (d) 10% methanol washing, (e) 30% methanol washing, (f) 50% methanol elution, (g) 100% methanol elution, and (h) second 100% methanol elution. The peaks of 1 and 6 are BPDE tetrols, and the peaks labeled with asterisk are unassigned *anti*-BPDE-*N*²-dGs.

1 mL 100% methanol (Fig. 2C and D). The *anti*-BPDE tetrols are the byproducts resulting from hydrolysis of *anti*-BPDE and *anti*-BPDE-dG adducts [28].

3.3. Optimization of HPLC separation and purification of *anti*-BPDE-dGs

The fractions of *anti*-BPDE-dGs and *anti*-BPDE tetrols collected from SPE fractionation needed further HPLC purification to prepare *anti*-BPDE-dG stereoisomers. To achieve the optimum separation and purification efficiency, HPLC stationary phase and mobile phase were first optimized. Our previous work demonstrated that the silicone polymer coating phenyl stationary phase could provide a unique capacity of separating stereoisomeric *anti*-BPDE-DNA adducts in short oligonucleotides, and exhibited a better resolution over C18 stationary phases coated or uncoated by the same silicone polymer [26]. Therefore, HPLC separation was conducted on the silicone polymer coating phenyl column. The next step was the optimization of mobile phase. Fig. 3 shows the separation of the four *anti*-BPDE-dGs under the optimum composition of two mobile phases, acetonitrile/water and methanol/water. The four *anti*-BPDE-dGs (peaks 2–5) and two *anti*-BPDE tetrols (peaks 1 and 6) were baseline resolved in 80 min when acetonitrile/water (18.5%) was used as mobile phase (Fig. 2A). The adducts were eluted out in the order of *trans*-(–)-*anti*-BPDE-*N*²-dG (59.8 min), *cis*-(+)-*anti*-BPDE-*N*²-dG (63.1 min), *trans*-(+)-*anti*-BPDE-*N*²-dG (69.8 min) and *cis*-(–)-*anti*-BPDE-*N*²-dG (72.9 min). The identification and characterization of these adduct isomers are described later in Section 3.4. In contrast, the four *anti*-BPDE-*N*²-dG adducts (labeled with asterisk) and the two *anti*-BPDE tetrols (peaks 1 and 6) were just partially separated in 80 min using the optimum composition of methanol/water mobile phase (38% methanol) (Fig. 3B). The results suggest that acetonitrile/water is a better mobile phase than methanol/water.

Fig. 4 shows the effects of the composition of two mobile phases (acetonitrile/water and methanol/water) on the separation of the four *anti*-BPDE-dGs. It was found that the retention of the BPDE-*N*²-dGs and BPDE tetrols is more sensitive to the concentration of acetonitrile than that of methanol. For example, the separation of the four *anti*-BPDE-dGs and two tetrols is completed in 85 min under optimum separation for acetonitrile/water (18.5% acetonitrile) (Fig. 4A). However, these adduct peaks could not be eluted in 100 min when the concentration of acetonitrile slightly decreased down to 18% in the mobile phase, and could be eluted in 40 min when the concentration of acetonitrile increased up to 20% in the mobile phase. It was also observed that the two *anti*-BPDE tetrols could seriously interfere with the separation of the four BPDE-*N*²-dGs unless the appropriate concentrations of acetonitrile and methanol were used (Fig. 4). It is difficult to remove the two tetrols by SPE or liquid–liquid extraction without the loss of the adducted nucleosides. The first *anti*-BPDE tetrol (peak 1) was overlapped with the *anti*-BPDE-dGs and interfered with their separation when the concentration of acetonitrile was in the range of 23–20%. The second *anti*-BPDE tetrol (peak 6) tended to be overlapped with the *anti*-BPDE-dGs that have stronger retention if lower concentration of acetonitrile ($\leq 18\%$) was used. The four *anti*-BPDE-dGs could be well separated between the peaks of two *anti*-BPDE tetrols only when 18.5% acetonitrile was used. Similar interference by the two tetrols was also observed in methanol/water mobile phase, however, the major interference resulted from the second *anti*-BPDE tetrols (peak 6) (Fig. 4B). Nevertheless, it is interesting that a baseline separation of the four stereoisomeric *anti*-BPDE-dGs and the two *anti*-BPDE tetrols can be achieved using nonchiral column.

The optimized HPLC separation was applied to the purification of the *anti*-BPDE-dG fractions collected from SPE fractionation. Under the optimized chromatographic conditions,

Fig. 3. HPLC separation of two BPDE tetrols and four *anti*-BPDE-*N*²-dGs using two optimum mobile phases, 18.5% acetonitrile concentration (A) and 38% methanol concentration (B). The peaks of 1 and 6 represent two BPDE tetrols, and peaks of 2–5 are corresponding to (–)-*trans*-, (+)-*cis*-, (+)-*trans*-, and (–)-*cis*-*anti*-BPDE-*N*²-dG. The peaks labeled with asterisk are unassigned *anti*-BPDE-*N*²-dG stereoisomers when methanol/water was used as mobile phase.

Fig. 4. HPLC chromatograms of *anti*-BPDE- N^2 -dGs and BPDE tetrols obtained by using two mobile phases of acetonitrile/water (A) and methanol/water (B), showing the effects of the type and composition of the mobile phase on the separation and the optimization of the mobile phase. The HPLC separation under 30% acetonitrile (a), 26% acetonitrile (b), 23% acetonitrile (c), 20% acetonitrile (d), 18.5% acetonitrile (e), and 18% acetonitrile (f) were illustrated for optimization of acetonitrile/water mobile phase. The separation under 48% methanol (a), 46% methanol (b), 44% methanol (c), 42% methanol (d), 38% methanol (e), and 36% methanol (f) were investigated to optimize the methanol/water mobile phase. The peaks of 1 and 6 are two BPDE tetrols, and the peaks labeled with asterisk are unassigned *anti*-BPDE- N^2 -dGs.

the four *anti*-BPDE-dG adducts can be simultaneously purified using an analytical-scale column.

3.4. Characterization and identification of *anti*-BPDE-dGs

The structure and stereochemistry of the four *anti*-BPDE-dGs were identified and characterized by a combination of HPLC–MS/MS, UV spectroscopy, and CD spectroscopy.

These adducts were first subjected to HPLC–Q–TOF–MS analysis for structure confirmation. Analysis of the three *anti*-BPDE- N^2 -dG adducts ((*−*)-*trans*, (*+*)-*trans*, and (*−*)-*cis*) indicated a protonated molecular ion $[(M+H)^+]$ at m/z 570 in the positive operation mode (data not shown), and a deprotonated molecular ion $[(M-H)^-]$ at m/z 568 in the negative operation mode (Fig. 5). However, we did not observe the peak of protonated molecular ion (m/z 570) for the (*+*)-*cis* adduct. It was found that peak of (*+*)-*cis* adduct appeared in HPLC–MS analysis if selected primary ion at m/z 454 was monitored. All the other three adducts also displayed this fragment ion (at m/z 454). The results suggest that the *anti*-BPDE-dG can be fragmented

Fig. 5. HPLC-ESI-Q-TOF-MS analysis of stereoisomers of BPDE- N^2 -dG adducts by monitoring at m/z 568 corresponding to $[(M-H)^-]$ (upper panel) and UV absorbance at 252 nm (lower panel). The peaks of 1 and 6 are two BPDE tetrols, and the peaks of 2–5 are (*−*)-*trans*-, (*+*)-*cis*-, (*+*)-*trans*-, and (*−*)-*cis*-*anti*-BPDE- N^2 -dG.

in the initial electrospray ionization process. Disappearance of intact molecular ion of (+)-*cis* adduct (m/z 570) and observation of fragment ion (m/z 454) in primary ionization source together indicates that the (+)-*cis*-*anti*-BPDE-dG is unstable. The observation is consistent with previous work [17].

Product ion spectra of the *anti*-BPDE-dGs at the selected precursor ion of m/z 570 obtained from LC-ESI-Q-TOF-MS analysis were shown in Fig. 6. The *anti*-BPDE- N^2 -dG of (–)-*trans*, (+)-*trans*, and (–)-*cis* displayed the formation of fragments at m/z 454, 303, 285 and 257 (Fig. 6). The fragment ion at m/z 454 is the protonated *anti*-BPDE-guanine with a neutral loss of deoxyribose (Fig. 7). The presence of this fragment ion (m/z 454) suggested that the reaction of *anti*-BPDE with dG occurred between *anti*-BPDE and the base of the deoxyguanosine but not between *anti*-BPDE and the deoxyribose of dG. The fragment ion at m/z 303 corresponding to a loss of dG from protonated parent ion $[(M+H)^+]$, which further dehydrated ($-H_2O$) into the fragment ion at m/z 285. Loss of the CO group from m/z 285 gave rise to m/z 257 (Fig. 7). It is surprising that LC-Q-TOF-MS/MS analysis of the (+)-*cis*-*anti*-BPDE- N^2 -dG at m/z 570 can give a fragment ion peak at m/z 257 but no fragments at m/z 454, 303 and 285 (Fig. 5A). As described above, the positive ion of the (+)-*cis*-*anti*-BPDE- N^2 -dG (m/z 570) cannot be observed in primary Q-TOF-MS analysis. So it is interesting that its product ion at m/z 257 could be detected in tandem MS/MS analysis of precursor ion at m/z 570. The results may suggest that MS/MS analysis has better sensitivity for detection of adducted nucleosides. Therefore, it is reasonable that all the four adducts could be detected at m/z 257 when using MS/MS analysis (Fig. 6B).

UV and CD spectra were investigated to validate assigned stereochemistry of each adduct. These adducts were classi-

fied into two groups by absorbance at 320–350 nm, which is the characteristic range for the aromatic ring moiety of *anti*-BPDE (Fig. 8). The adducts of assigned (+)-*cis* and (–)-*cis* exhibited a characteristic absorbance maximum at 346 nm, and the adducts of (–)-*trans* and (+)-*trans* exhibited a maximum absorbance at shorter wavelength (344/345 nm). Since *anti*-BPDE-mononucleosides in the *cis* configuration show a stronger red shift than those in *trans* configuration [23], these recorded UV spectra are in agreement with their *cis* or *trans* configurations. Two BPDE tetrols resulting from hydrolysis of *anti*-BPDE showed the maximum absorbance at a shortest wavelength of 343 nm. The covalent binding of BPDE to dG shifted the absorbance toward longer wavelength. The red shift (1–2 nm) was also observed for the absorbance at 250–280 nm (Fig. 8). The two BPDE tetrols display a maximum absorbance at 277 nm, and the *anti*-BPDE- N^2 -dGs display a maximum absorbance at 278 or 279 nm. The relative intensity of UV maximum absorbance at 277–279 nm to that at 343–346 nm may be useful to indicate whether *anti*-BPDE- N^2 -dG or BPDE tetrols. The two BPDE tetrols have a higher intensity of UV absorbance at 343 nm than that at 277 nm. On the contrary, the UV absorbance intensities of four BPDE- N^2 -dGs at 278 or 279 nm are higher than that at 344–346 nm. This effect may be useful to differentiate the BPDE- N^2 -dG adducts from BPDE tetrols and to identify the different stereochemistry of the *anti*-BPDE- N^2 -dGs.

Fig. 9 shows CD spectra of three purified *anti*-BPDE- N^2 -dGs ((–)-*trans*, (+)-*trans* and (–)-*cis*). Stereoisomeric adducts ((+)-*trans* vs. (–)-*trans*; (+)-*cis* vs. (–)-*trans*) exhibited similar CD spectra that were opposite in sign. (+)-*trans*- and (–)-*cis*-*anti*-BPDE- N^2 -dGs having a 10*S* absolute configuration both exhibit a positive signal for their most intense CD band. (–)-*trans*-*anti*-

Fig. 6. (A) Product ion spectrum of the four BPDE- N^2 -dGs from LC/ESI-MS/MS analysis. (B) HPLC chromatogram of the four BPDE- N^2 -dGs by ESI-MS/MS monitoring selected fragment of (m/z 570 $\rightarrow$ 257) (upper panel), and UV absorbance at 252 nm (lower panel).

Fig. 7. The major fragments derived from CAD-MS/MS analysis of the protonated *anti*-BPDE- N^2 -dG molecules $[(M+H)^+]$ (m/z 570).

Fig. 8. Ultraviolet spectra of the two BPDE tetrols and the four *anti*-BPDE- N^2 -dGs. The spectra were extracted from HPLC-DAD-FL analysis shown in Fig. 2A. The peak number (1–6) has same meaning as that in Fig. 2A.

Fig. 9. Measured circular dichroism spectra of three purified *anti*-BPDE- N^2 -dG stereoisomers.

Table 3

The ratios of fluorescence (excitation 343 nm, emission 400 nm)/UV 343 nm absorbance of the four *anti*-BPDE- N^2 -dGs and two BPDE tetrols were listed

Signal intensity	(-)- <i>trans</i>	(+) - <i>cis</i>	(+) - <i>trans</i>	(-) - <i>cis</i>	BPDE tetrol	
					Peak 1	Peak 6
FL/UV (343 nm)	32.1 ± 4.4	50.0 ± 3.4	20.4 ± 1.9	12.7 ± 1.9	1440 ± 96	1510 ± 70
Relative fluorescence quenching	0.395	0.254	0.621	1.0 ^a	0.0088	0.0084

The measurement was conducted in triplicate (means ± SD).

^a The strongest fluorescence quenching is defined as 1.0.

BPDE- N^2 -dG having a 10R absolute configuration exhibits a negative signal for its most intense CD band. The measured CD spectra of the purified (-)-*trans*-, (+)-*trans*- and (-)-*cis*-*anti*-BPDE- N^2 -dGs are consistent with previous work [19,25], further validating the assigned chirality and stereochemistry. The purified amount of (+)-*cis*-*anti*-BPDE- N^2 -dG is not enough to measure their CD spectrum due to its low yield (Table 1, less than 3% in the total adducts). However, its assigned stereochemistry can be supported and validated from its instability, low yield, UV spectrum, and HPLC–MS/MS data as described above.

3.5. Effect of stereochemistry of *anti*-BPDE-dG on fluorescence quenching

The covalent binding of BPDE to the exocyclic amino groups of guanine may cause fluorescence quenching of BPDE [29]. Here we demonstrate that the fluorescence quenching of *anti*-BPDE- N^2 -dGs is correlated well with their stereochemistry. By taking advantage of HPLC–DAD–FL, we estimated the ratios of fluorescence/UV absorbance for the four stereoisomeric BPDE- N^2 -dGs as well as two BPDE tetrols. Therefore, the fluorescence quenching can be evaluated from the ratio of emitted fluorescence to the absorbance at excited wavelength (343 nm). The larger ratio indicates higher fluorescence quantum yield and weaker fluorescence quenching, and smaller ratio indicates lower fluorescence quantum yield and stronger fluorescence quenching. The results were shown in Table 3. The BPDE tetrols that do not conjugate with dG show highest ratio of fluorescence/absorbance (about 1440–1510). In contrast, *anti*-BPDE- N^2 -dGs only show the ratios of 12.7–50.0, suggesting that the strong fluorescence quenching has taken place once *anti*-BPDE is bound to dG. It is interesting that

the four *anti*-BPDE- N^2 -dGs have significantly different signal ratios (fluorescence/absorbance at 343 nm). The ratio of fluorescence/absorbance of the four *anti*-BPDE- N^2 -dGs increases in the order of (-)-*cis*-, (+)-*trans*-, (-)-*trans*- and (+)-*cis*-*anti*-BPDE- N^2 -dG, suggesting the least fluorescence quenching of (+)-*cis*-*anti*-BPDE- N^2 -dG and the strongest quenching of (-)-*cis*-*anti*-BPDE- N^2 -dG. The least quenching of (+)-*cis*-*anti*-BPDE- N^2 -dG is consistent with its weak bond of C10 at BPDE with N^2 of guanine (chemical instability). The ratio of fluorescence/absorbance of the four *anti*-BPDE- N^2 -dGs is in the essence of the reflection of their stereochemistry. Therefore, this parameter may be useful to identify the stereochemistry of the *anti*-BPDE-dGs in combination of HPLC separation.

4. Conclusions

In this work, a simple and systematic method for preparation, identification, and analysis of the four *anti*-BPDE-dG isomers was established. It is also found that each of the four *anti*-BPDE-dG isomers has unique relative fluorescence quenching. In combination of the established method and our findings, it is promising to identify and to analyze the stereochemistry of optically active BPDE–DNA adducts in short oligonucleotides and DNA without the need of large-scale preparation of *anti*-BPDE-dG isomers.

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