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Structural characterization of carcinogen-modified oligodeoxynucleotide adducts using matrix-assisted laser desorption/ionization mass spectrometry

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The aim of this study was to determine the chemical structure of *in vitro* 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP)-modified oligodeoxynucleotides (ODNs) by exonuclease digestion and matrix-assisted laser desorption/ionization mass spectrometry. A single-stranded 11-mer ODN, 5′-d(CCATCGCTACC), was reacted with *N*-acetoxy-PhIP, resulting in the formation of one major and eight minor PhIP–ODN adducts. A 10 min treatment of the major and one minor PhIP–ODN adduct with a 3′-exonuclease, bovine intestinal mucosa phosphodiesterase (BIMP), and a 5′-exonuclease, bovine spleen phosphodiesterase, results in inhibition of the primary exonuclease activity at deoxyguanosine (dG) producing 5′-d(CCATCG(PhIP)) and 5′-d(G(PhIP)CTACC) product ions, respectively. Post-source decay (PSD) of these enzymatic end products identifies dG as the sole modification site in two 11-mer ODN–PhIP adducts. PSD of the minor PhIP–ODN adduct digestion end product, 5′-d(CCATCG(PhIP)), also reveals that the PhIP adducted guanine moiety is in an oxidized form. Prolonged treatment of the PhIP–ODN adducts at 37 °C with BIMP induces a non-specific, or endonuclease, enzymatic activity culminating in the formation of deoxyguanosine 5′-monophosphate-PhIP (5′-dGMP–PhIP). The PSD fragmentation pattern of the 5′-dGMP–PhIP $[M + H]^+$ ion of the major adduct confirms PhIP binds to the C-8 position of dG. For the minor adduct, PSD results suggest that PhIP binds to the C-8 position of an oxidized guanine, supporting the hypothesis that this adduct arises from oxidative degradation, resulting in a spirobisguanidino structure. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: MALDI; mass spectrometry; PSD; PhIP; DNA; adducts; exonuclease digestion

INTRODUCTION

Many chemical carcinogens bind covalently to DNA, either directly or after metabolic activation, resulting in the formation of DNA adducts. The biological effects of DNA adducts are believed to be influenced by the chemical structure of the adduct and DNA sequence context.[1,2] Significant effort has been directed towards the detection and quantitation of DNA adducts using methods such as ^{32}P-postlabeling,[3,4] immunoassays,[5] fluorescence spectroscopy[6] and accelerator mass spectrometry (AMS).[7] These techniques of DNA adduct detection do not, however, provide any direct information on sequence specificity or the covalent nature of adduct formation. Furthermore, although ^{32}P-postlabeling and AMS can be used for adduct identification and quantification, characterization is based on chromatographic comparison with adduct standards, which are often not available. For example, in the ^{32}P-postlabeling assay, an adduct containing DNA is isolated and digested to 3′-monophosphate deoxynucleosides. The DNA adducts are labeled with ^{32}P at the 5′-terminus, separated chromatographically and detected by radioactivity counting. Digestion into nucleosides eliminates information relating to the adduct sequence location, and detection by radioactivity counting provides no structural information.

In contrast to the techniques mentioned above, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) mass spectrometry (MS) are becoming viable techniques for determining both sequence specificity and chemical structure of DNA modifications. Tandem mass spectrometry (MS/MS) of MALDI or ESI generated ions is one MS approach employed to identify DNA modification sites. MS/MS has been used by several researchers to identify modification sites in short, *in vitro* adducted oligodeoxynucleotides (ODNs). For example, Stemmler *et al*.[8] used MALDI Fourier transform (FT) MS/MS to sequence and identify the location of a benzo[*a*]pyrene adduct in single-stranded 4-mer and 6-mer ODNs. However, when the same MS/MS methods

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were utilized with benzo[*a*]pyrene-modified 11-mer ODNs, significant fragmentation inhibited complete sequence determination. In a similar manner, Glover *et al*.[9] identified the full sequence and benzoquinone adduction site in a synthesized 7-mer. Using liquid chromatography (LC)/ESI-MS/MS, Ni *et al*.[10] identified and quantified the guanine specific adduction of benzo[*a*]pyrene in an 11-mer containing three dG residues. By this method they confirmed three unique isomeric benzo[*a*]pyrene–ODN adduct structures with each containing one modified dG; however, in each unique structure, full sequence information was unattainable. Thus, because MS/MS induces considerable non-sequence-specific fragmentation, sequence determination and positional mapping of modified ODNs has been limited to short, single-stranded oligomers.

An alternative method to MS/MS for determining the sequence and modification site in larger modified ODNs is ladder sequencing by time-dependent exonuclease digestion. In this method, a 3′- and/or 5′-exonuclease sequentially removes single nucleotides from the 3′- or 5′-terminus, creating a mixture of ODNs that can be rapidly detected by MALDI-MS[11–19] or ESI-MS.[20–22] Bentzley *et al*.[12] and Smirnov *et al*.[13] demonstrated the applicability of sequencing 24- and 50-mer ODNs by exonuclease digestions and MALDI-MS analysis. Pieles *et al*.[11] were the first to demonstrate the utility of this methodology for sequencing modified ODNs. They subjected a modified 12-mer ODN containing a 2′-*O*-methyladenosine at position 5 to a 5′ -and a 3′-exonuclease and observed complete inhibition of the 5′-exonuclease at the modified nucleoside. However, the 3′-exonuclease was not blocked at the modified nucleoside and allowed sequencing of the entire ODN. More recently, Bourdat *et al*.[15], Tretyakova *et al*.[16] and Zhang and co-workers[17,18] utilized exonuclease digestion and MALDI-MS to determine modification sites in single and tandem base damaged ODNs. Although the types of damage varied, from abasic sites[18] and photomodified thymines[17] to oxidized guanine,[15] each revealed a change in the rate of enzymatic hydrolysis as the exonucleases approached the site of damage. In all of these cases, the 5′-exonuclease was completely inhibited by the modification. Zhang *et al*.[19] utilized MALDI post-source decay (PSD) MS analysis of exonuclease digestion product ions to distinguish isomeric species resulting from the 3′- and 5′- exonuclease digestion of photomodified ODNs where the nucleotide sequence was identical on either side of the modified site.

In this paper, we report methods utilizing MALDI time-of-flight (TOF) MS in combination with exonuclease digestion and PSD to identify the location and chemical structure of DNA adducts formed by the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in a defined sequence ODN. PhIP is a heterocyclic amine formed during the cooking of protein-rich foods such as beef, chicken and fish.[23] Formation of PhIP–DNA adducts has been implicated in the development of colon, breast and prostate cancers in humans.[24–26] Several PhIP–DNA adducts formed *in vivo* have been detected using ^{32}P-postlabeling methods; however, *N*-(deoxyguanosin-8-yl)PhIP (C8-dG-PhIP) is the only PhIP–DNA adduct unequivocally identified to date.[27,28] In a previous paper, we reported the synthesis and isolation of multiple PhIP–ODN adducts formed by reaction of the metabolite *N*-acetoxy-PhIP with an 11-mer, 5′-d(CCATCGCTACC).[29] This particular sequence was chosen because it has been used by others for the production of a variety of carcinogen-modified ODNs in order to determine adduct solution structures. The conformation adopted by DNA adducts is dependent on the sequence context, therefore producing the PhIP adducts in the same sequence allowed us to directly compare the structure adopted by PhIP adducts with adducts formed by other carcinogens.[30] Here, we have developed MALDI-TOF-MS methods that utilize exonuclease digestions to determine the sequence location of the PhIP modified base. In addition, PSD experiments were performed directly on ions resulting from digestion (i.e. without prior LC separations) not only to identify the covalent binding site, but also to determine the chemical structure of individual PhIP adducts. The methods were first developed on the major adduct, believed to be the C8-dG–PhIP adduct, and then carried out on one of the less abundant, minor PhIP–ODN adducts.

EXPERIMENTAL

Instrumentation

MALDI mass spectra were acquired on a PerSeptive Biosystems Voyager DE-STR TOF mass spectrometer equipped with a nitrogen laser (337 nm). Mass spectra of the exonuclease digests of the unmodified ODN, 5′-d(CCATCGCTACC), the primary and one minor PhIP–ODN adducts were obtained in positive ion and reflectron mode with an acceleration voltage of 25 kV, a grid voltage of 64.8% and a delay time of 175 ns. Negative ion mass spectra were also acquired; however, all data presented here are positive ion data. We are in agreement with Koomen *et al*.[31] in that the ion abundance and mass resolution were superior in the positive ion mode with this particular sample preparation (see below). PSD focusing methods with a single-stage reflection were utilized to generate tandem mass spectra of several enzymatic digestion product ions without prior LC separations. In order to generate significant fragment ion abundance, PSD spectra were acquired at a greater laser fluence than the mass spectra of the exonuclease digests. All mass spectra contain an average of 100 laser shots.

MALDI mass spectra of time-dependent exonuclease digestions were externally calibrated using monoisotopic masses of ACTH 1–17 (m/z 2093.0867), angiotensin I (m/z 1296.6853) and ACTH 7–38 (m/z 3657.9294), resulting in mass measurement accuracies of better than ±0.1 Da (50 ppm). Calibration of PSD spectra was performed with the Data Explorer (v. 5.01) software package provided by PerSeptive Biosystems. The mass measurement accuracy of the fragment ions produced by PSD focusing was ±0.3 Da. Fragment ions were assigned based on the nomenclature for ODN phosphodiester backbone cleavages proposed by McLuckey *et al*.[32]

Sample preparation

The 11-mer ODN 5′-d(CCATCGCTACC) and PhIP–ODN adducts were synthesized at Lawrence Livermore National

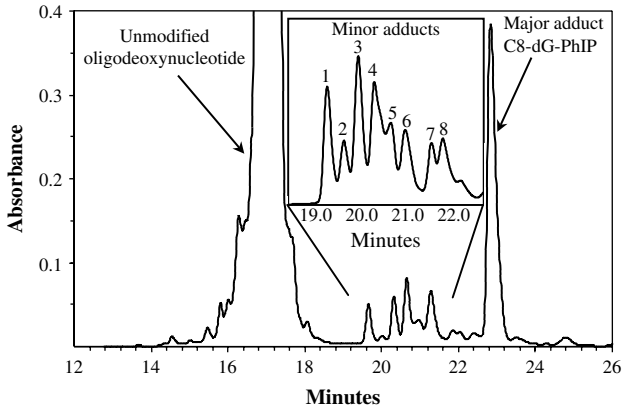


Figure 1. Liquid chromatogram of the nine, structurally unique, 5′-d(CCATCGCTACC)–PhIP adducts. Separations were performed using a gradient mobile phase of 20 mM sodium phosphate (pH 7.0)–methanol on an analytical Hypersil ODS column (250 × 4.6 mm i.d.) (Keystone Scientific, Bellefonte, PA, USA).

Laboratory using methods described previously.[29] Briefly, *N*-acetoxy-PhIP was reacted in pH 7.0 buffer with an equimolar concentration of the 11-mer ODN, at 37 °C for 1 h. Semi-preparative LC separation of the reaction mixture revealed one major PhIP–ODN adduct peak and eight minor, more polar adduct peaks, as shown in Fig. 1. It is important to note that in total, ~8% percent of the unmodified ODN reacted with *N*-acetoxy-PhIP. The yield of the major product was ~4.0% and the combined yield of the remaining eight minor adduct peaks was estimated as 3.5–4.0%. LC fractions containing the unmodified ODN, the major adduct peak and one minor product, designated adduct peak 1, were collected and repurified by analytical LC for MS analysis. Prior to enzyme digestions and MS analysis, the ODN and PhIP–ODN adducts were desalted by drop dialysis on a 0.0025 mm Micropore filter (Millipore) for 45 min, then cation-exchange beads in the NH_4^+ form were used to remove residual Na^+ and K^+ ions. Dowex 50WX8-200 ion-exchange beads (Aldrich, Milwaukee, WI, USA) were received in the H^+ form and converted to the NH_4^+ moiety according to published methods.[33]

The matrix preparation was similar to that of Koomen *et al.*,[31] who used a fast evaporation-overlayer sample preparation. We prepared a base matrix layer by depositing 3 µl of 40 mg ml^{-1} 2,4,6-trihydroxyacetophenone (2,4,6-THAP) dissolved in methanol on the MALDI plate. Fast evaporation of the methanol deposited an opaque matrix crystal bed on which 0.5 µl of each sample (see below) was applied.

Bovine intestinal mucosa phosphodiesterase (BIMP), a 3′ → 5′ exonuclease (Sigma, St. Louis, MO, USA) and bovine spleen phosphodiesterase (BSP), a 5′ → 3′ exonuclease (Worthington Biochemical), were used to digest the unmodified ODN and two PhIP–ODN adducts. These enzymes liberate 5′- or 3′-monodeoxynucleotides, respectively, via hydrolysis of phosphodiester bonds. Aliquots (2 µl, 0.4 mM) of the ODN and major PhIP–ODN adduct were each added to 1 µl of BIMP (0.016 units μl^{-1}) and 12 µl of 18 mΩ purified water, resulting in a final sample concentration of 50µM. At time intervals of 1, 3, 5, 10, 30 and 60 min, a 1 µl aliquot was removed and added to 2µl of 100 mM ammonium citrate, 2 µl of water and 1 µl of formic acid to quench BIMP activity. A 1 µl volume of this mixture was added to 4 µl of water and 2 µl of the matrix, 2,4,6-THAP (8 mg ml^{-1}). The same procedure was carried out for BSP digestion of the unmodified ODN and the major PhIP–ODN adduct, except that 2 µl of BSP (0.01 units μl^{-1}) and 11 µl of water were added to each enzyme incubation. The initial concentration of the minor PhIP–DNA adduct, peak 1, was 60 µM, therefore 3 µl of this adduct were added to the digestion mixture containing 1 µl of BIMP and 10 µl of water. The sample was then processed in an identical manner to the other digests. All incubations were carried out at room temperature, unless noted otherwise, to prevent the digestion from proceeding too quickly.

RESULTS AND DISCUSSION

Determination of the PhIP modification site in the major adduct

Administration of PhIP to rodents induces a spectrum of mutations, predominantly single base substitutions and single base deletions.[34,35] A similar profile has been observed in cultured cells treated with PhIP.[36] Metabolically activated PhIP forms a variety of DNA adducts as detected by ^{32}P-postlabeling;[37,38] however, these types of studies provide no information on which adduct might be responsible for which mutations. In order to understand how adduct structure influences biological outcome, site-specific adducts are required, containing single, pure DNA adducts in a defined sequence ODN. As part of an effort to understand the mechanisms through which PhIP causes mutations, we have demonstrated that the metabolite *N*-acetoxy-PhIP forms at least nine products when reacted with 5′-d(CCATCGCTACC) and have determined the structure and conformation of the major product, the C8-dG–PhIP–11mer adduct by 2-D NMR spectroscopy.[29,30] Furthermore, we have proposed structures for three previously unidentified adducts formed in this reaction, based on accurate mass measurements using MALDI-TOF-MS and UV spectroscopy. However, even after large-scale synthesis, these three minor adducts, and the other, as yet, uncharacterized products are not generated in sufficient quantities to permit absolute structural assignment by NMR. Therefore, in this paper we describe the development of MS-based methods to obtain definitive structural information on the PhIP–ODN adducts isolated in our studies.

The procedures, which utilize MALDI-TOF-MS, exonuclease digestion and PSD, have been developed using the major C8-dG–PhIP–ODN adduct, and one of the minor adducts (peak 1). In future studies we intend to apply these techniques to the identification of the remaining PhIP–ODN adducts. Figure 2 shows the MALDI mass spectra of a BSP digestion of the unmodified ODN, 5′-d(CCATCGCTACC)-3′. The digest serves as a control with which the rate of exonuclease digestion of the PhIP–ODN adducts can be compared. A 1 min digest reveals that the first six nucleotides have been cleaved from the 5′ terminus, as evidenced by the appearance of singly charged ions corresponding to the sequential loss of individual nucleotides. After 10 min, digestion has reached the nucleotide at position 8 and loss of nucleotides

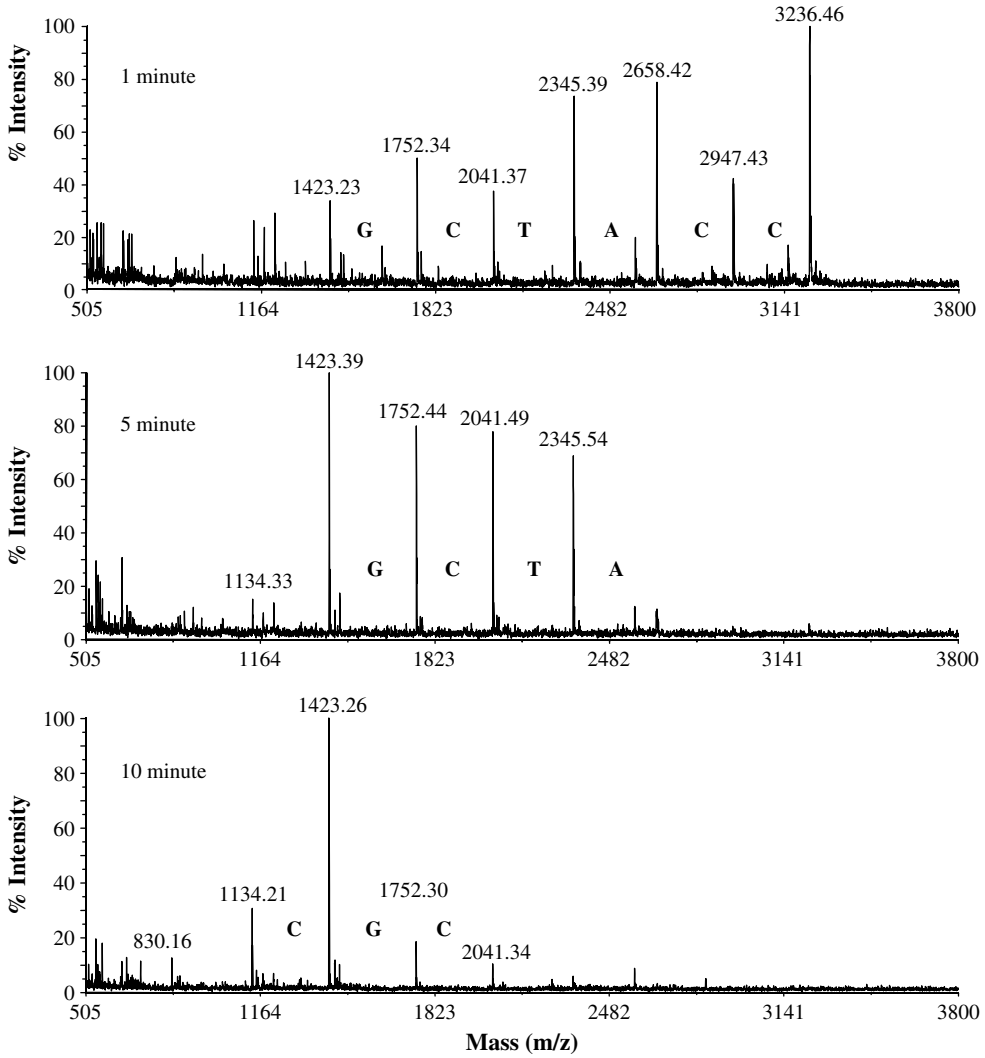


Figure 2. Positive ion MALDI-TOF mass spectra of the BSP digestion end products of 5′-d(CCATCGCTACC) at 1, 5 and 10 min digestion times.

at positions 5–8 from the 5′-end are observed. Similar results were obtained with a BIMP digestion of the unmodified 11-mer ODN.

To demonstrate the value of this methodology in identifying sites of PhIP modification, the major PhIP–ODN adduct was subjected to BSP digestion. Figure 3 shows the MALDI mass spectra of 1, 5, 10 and 60 min BSP digests of the major PhIP–ODN adduct isolated from the *N*-acetoxy-PhIP–ODN reaction. After a 1 min digestion, cleavage of only the first three nucleotides from the 5′-end is observed. After 5 min, nucleotides at positions 1–4 are observed, and a further 5 min digestion results in the removal of only one more nucleotide, the fifth base from the 5′-end, deoxycytosine (dC). Comparison of the MALDI mass spectra of 1, 5 and 10 min digests of the major PhIP–ODN adduct and the unmodified ODN reveals a decrease in the rate of digestion for the adducted species. Furthermore, the mass spectrum of the 60 min BSP digest reveals that not only is the digestion rate restricted, but also completely blocked at deoxyguanosine (dG), as represented by the digest product ion at m/z 1974.52, 5′-d(GCTACC)PhIP $[M + H]^+$ ions. Digest inhibition at dG suggests dG is the site of PhIP adduction and is consistent with results reported by others of exonuclease inhibition at modified nucleotides.[15–18] However, it is possible that PhIP does not bind dG, but rather, structural effects of the PhIP–ODN adduct could inhibit enzymatic degradation beyond dG.

To verify the hypothesis that PhIP is covalently adducted to dG, PSD was performed on the exonuclease digestion product 5′-d(GCTACC)PhIP $[M + H]^+$ ions, and Fig. 4 shows the resulting MALDI PSD spectrum. Two fragment ions are observed that contain both guanine and PhIP, the ion at m/z 374.1, $(\text{guanine–PhIP})^+$, and the ion at m/z 1556.5, $(\text{a5-CH})^+$. This clearly identifies guanine as an adduction site. Knowing that guanine binds PhIP, the other fragment ions can be assigned based on familiar backbone cleavages, as labeled in the PSD spectrum, confirming guanine as the sole site of PhIP adduction in the major adduct.

To understand fully the covalent nature of the major PhIP–ODN adduct, we compared the 3′ → 5′ digest using BIMP exonuclease (shown in Fig. 5), with the 5′ → 3′ BSP digestion. After 1 min the first four bases from the 3′-end are revealed and by 5 min the first five bases can be sequenced. Similarly to the BSP digestion, at 10 min the rate

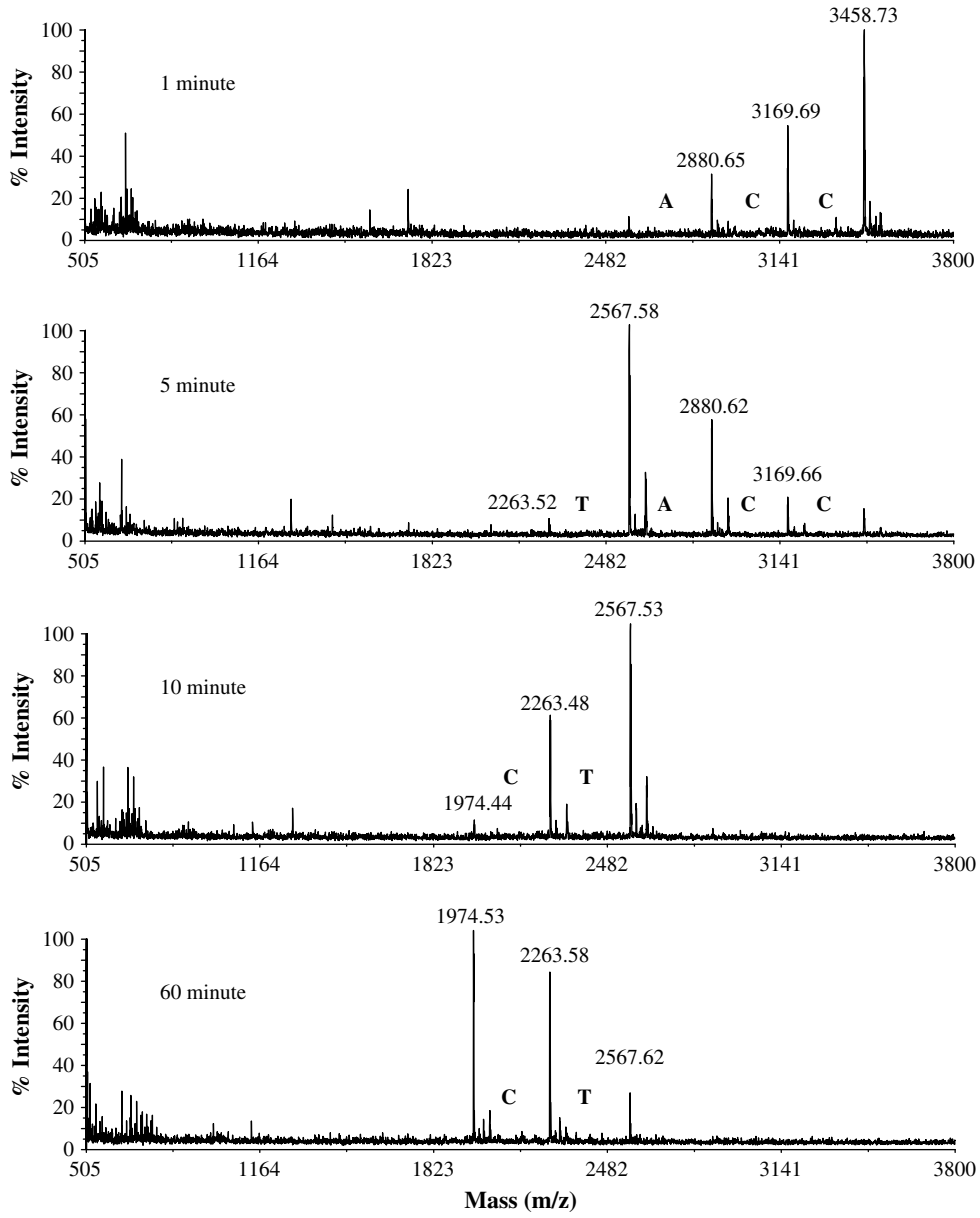


Figure 3. Positive ion MALDI-TOF mass spectra of the BSP digestion end products of the major 5′-d(CCATCGCTACC)–PhIP adduct at 1, 5, 10 and 60 min digestion times.

of nucleolysis has been restricted at dG and still only five bases from the 3′-end can be identified. Interestingly, in the mass spectrum of the 5 and 10 min digests, unique ions, labeled with an asterisk, are observed. In each case, this ion corresponds to a loss of 209.08 Da from the previous sequence ion and is identified as dC, the 5′-nucleoside, which is indicative of endonuclease activity in the BIMP enzyme. Residual endonuclease activity of snake venom phosphodiesterase (SVP), another 3′ → 5′ exonuclease, has previously been observed when the 3′ → 5′ activity was quenched at an abasic site[18] or at an 8-oxo-7,8-dihydro-2′-dG in modified ODNs.[15] However, in each of these cases, SVP was used in excess and digestion times were of the order of 60 min. It is important to note that BIMP endonuclease activity was also observed in the time-dependent digestion of the unmodified 11-mer ODN (data not shown), where it was first evident in the loss of the 5′-dC from the digestion product 5′-d(CCATCG). This indicates that BIMP does not require inhibition by DNA modification, an excess of enzyme, or long digestion times in order to exhibit endonuclease activity. This is in accordance with the supplier's indication that there is some non-specific activity in the 5′ → 3′ direction with this enzyme that results in minimal amounts of loss of the 5′-end nucleoside.

With the aim of obtaining information on the covalent structure of guanine–PhIP, the BIMP digestion product at m/z 1974.52 was also subjected to PSD analysis. The precursor ion in Fig. 6 bears the guanine–PhIP moiety at the 3′-end of the sequence, 5′-d(CCATCG(PhIP)), in contrast to the structural isomer resulting from BSP digestion where the guanine–PhIP moiety resides at the 5′-end, 5′-d(G(PhIP)CTACC). The PSD spectrum in Fig. 6 reveals vastly different fragmentation pathways compared with its isomer (Fig. 4) with the exception of the characteristic

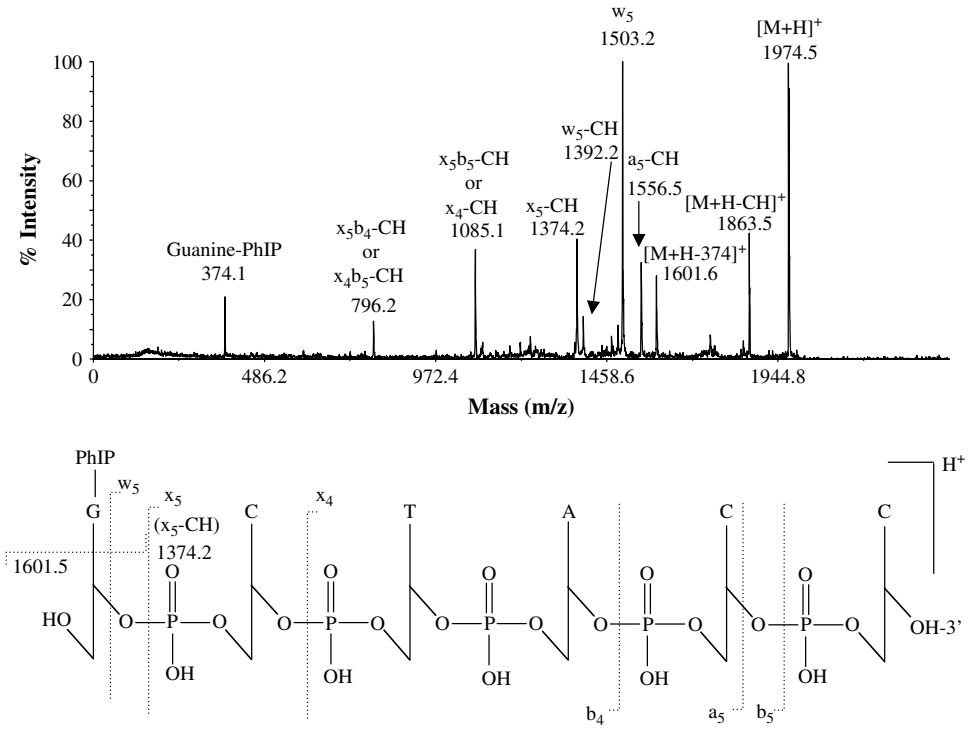


Figure 4. PSD spectrum of the BSP digestion end product containing 5′-d(GCTACC)–PhIP from the major PhIP–ODN adduct.

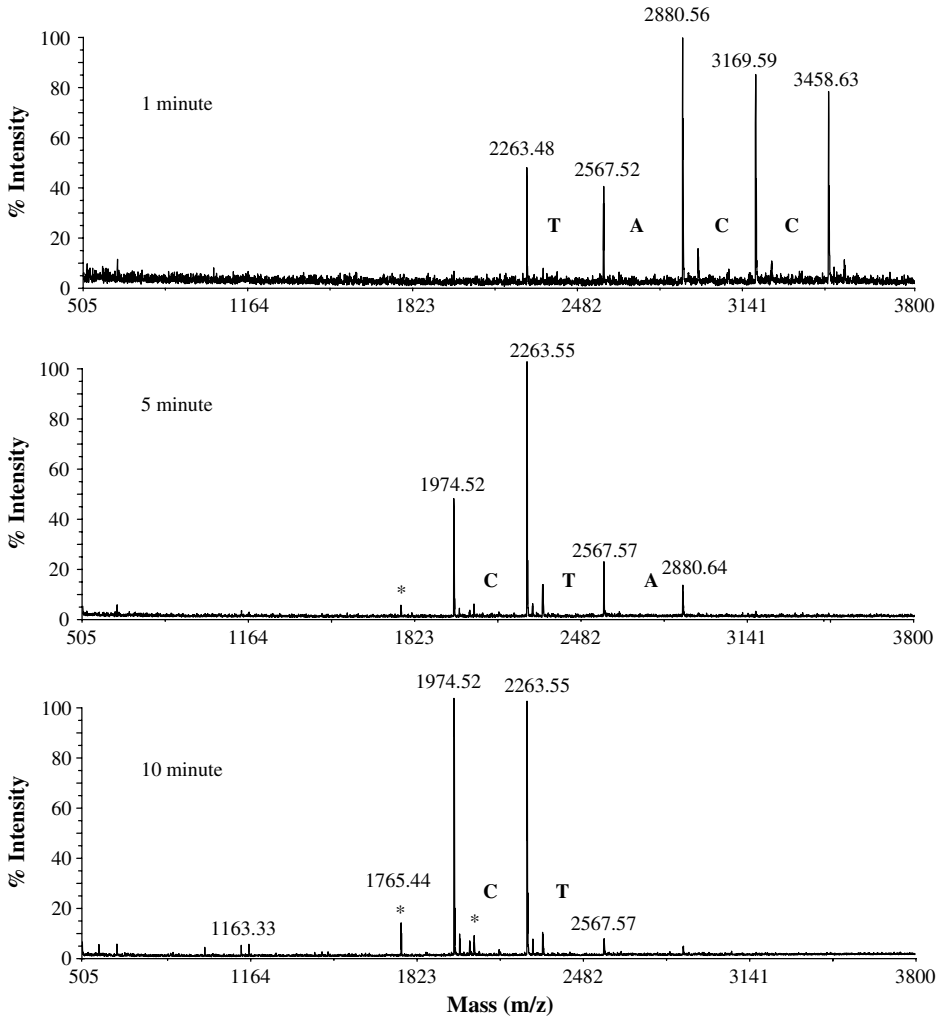


Figure 5. Positive ion MALDI-TOF mass spectra of the BIMP digestion end products of the major 5′-d(CCATCGCTACC)–PhIP adduct at 1, 5 and 10 min digestion times.

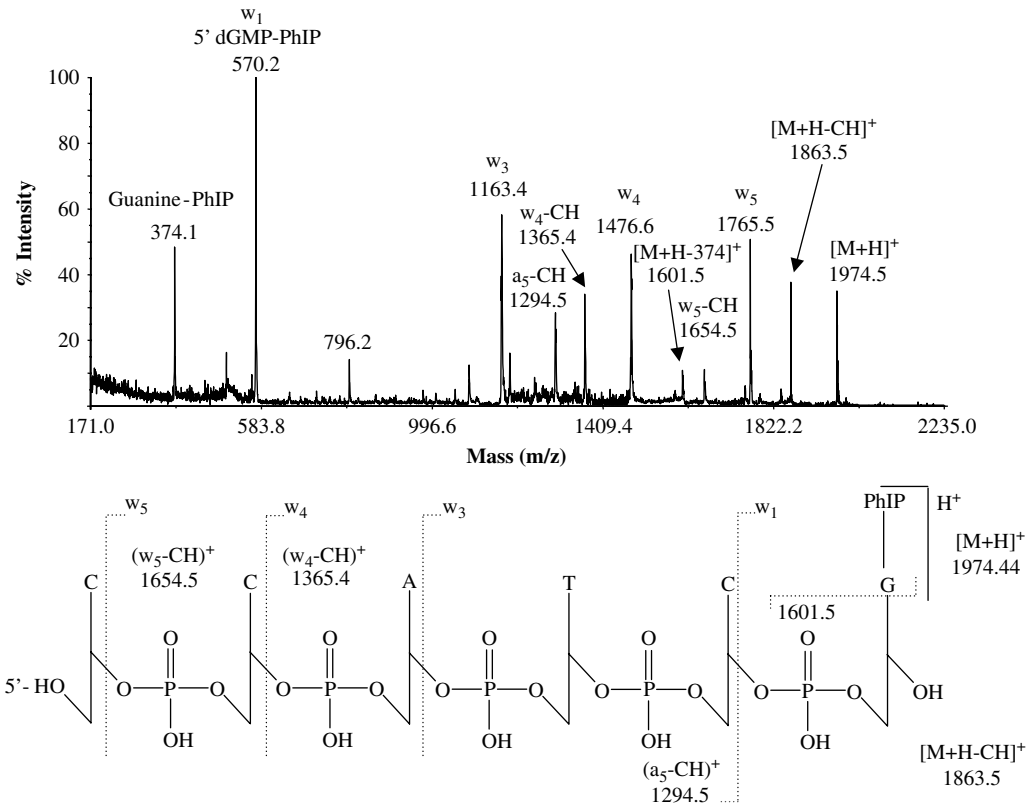


Figure 6. PSD spectrum of the BIMP digestion end product containing 5′-d(CCATCG)–PhIP from the major PhIP–ODN adduct, the structural isomer of 5′-d(GCTACC)–PhIP.

(guanine–PhIP)$^+$ cation at m/z 374.1, identifying guanine as the primary site of adduction. The PSD spectrum contains nearly a complete series of w_n-type fragment ions with the w1 ion, deoxyguanosine 5′-monophosphate-PhIP (5′-dGMP–PhIP) being the base peak at m/z 570.2. The 5′-dGMP–PhIP fragment ion was not observed in the PSD spectrum of 5′-d(G(PhIP)CTACC), where guanine is linked to cytosine through a 3′-rather than a 5′-phosphodiester functionality. The diverse dissociation pathways of the structural isomers suggests that guanine–PhIP interacts differently with a 5′-phosphodiester versus a 3′-phosphodiester backbone resulting from different gas-phase structures. The fact remains however, that the PSD spectrum in Fig. 6 does not reveal any additional information about the covalent structure of the guanine–PhIP covalent bond for this particular adduct.

Ideally, one would like to isolate the guanine–PhIP fragment ion at m/z 374.1 or the 5′-dGMP–PhIP fragment ion at m/z 570.2 to obtain information about the precise binding site of PhIP to guanine; however, in the two-stage MALDI-TOF experiment this is not possible. In order to produce 5′-dGMP–PhIP $[M + H]^+$ ions in a single-stage MALDI-TOF experiment, we took advantage of the non-specific, or endonuclease, activity of BIMP. Although the primary activity in the $3' \rightarrow 5'$ direction is quenched at dG where PhIP binds, the endonuclease activity from the $5' \rightarrow 3'$ end was exploited by performing a 60 min digest at 37 °C with the same PhIP–ODN adduct and BIMP concentrations as described earlier. The elevated temperature accelerates enzymatic degradation in both the $5' \rightarrow 3'$ and $3' \rightarrow 5'$ direction. Figure 7 shows the mass spectrum of the 60 min PhIP–ODN adduct enzymatic incubation. The base peak in the mass spectrum is m/z 570.1, corresponding to 5′-dGMP–PhIP $[M + H]^+$ ions, confirming that the PhIP–ODN adduct has been digested from the 3′-end to the dG–PhIP adduct and subsequently from the 5′-end to the dG–PhIP adduct site.

The 5′-dGMP–PhIP $[M + H]^+$ ions could then be isolated and dissociated in the two-stage MALDI-TOF experiment to identify the covalent nature of the guanine–PhIP interaction. Based on our accurate mass assignments of four of the PhIP–ODN adducts[29] and results published by Rindgen *et al.*,[39] two isomeric dG–PhIP adducts have been proposed. One contains PhIP bound to the C-8 position of guanine and the other at the N^2 position (Scheme 1). Figure 8 contains the PSD spectrum of 5′-dGMP–PhIP $[M + H]^+$ ions. (It is

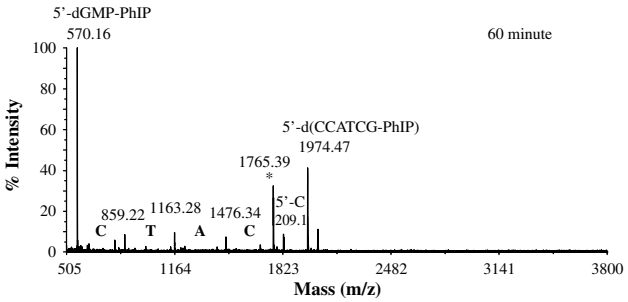
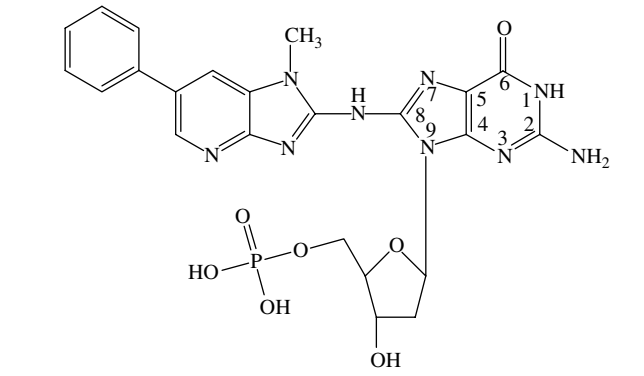
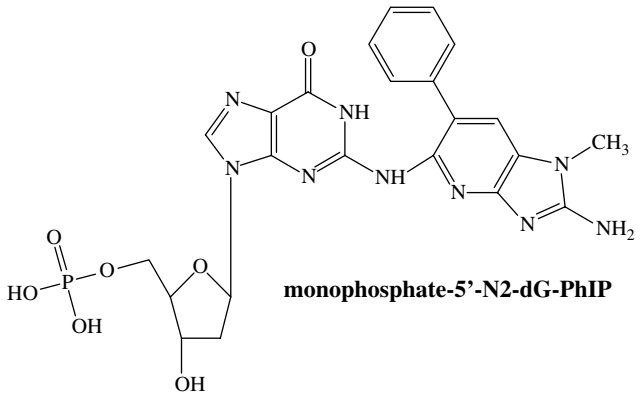
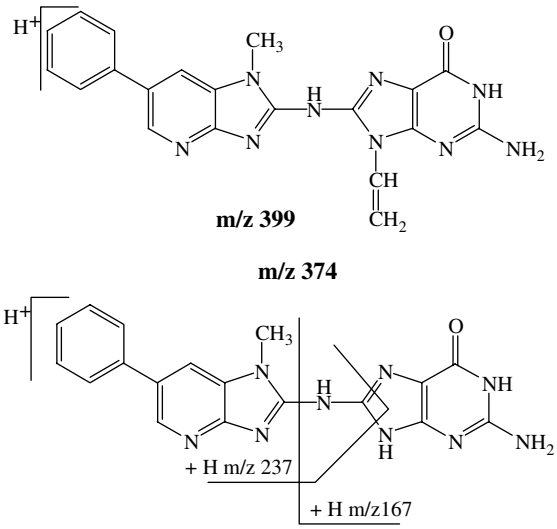


Figure 7. Positive ion MALDI-TOF mass spectra of the BIMP digestion end products of the major 5′-d(CCATCGCTACC)–PhIP adduct at a 60 min digestion time and carried out at 37 °C.





Scheme 1. Structure of the C8-dG–PhIP and N2-dG–PhIP adducts.



Scheme 2. Proposed structures for the fragment ions observed in the dissociation of 5′-dGMP–PhIP $[M + H]^+$ ions resulting from BIMP digestion of the major PhIP–ODN adduct.

important to note that in the negative ion mode, there was not sufficient production of 5′-dGMP–PhIP $[M - H]^-$ ions to allow for PSD analysis). Scheme 2 shows proposed structures of the fragment ions observed in Fig. 8. The most abundant fragment ion at m/z 374.2 arises through cleavage of the glycosidic bond of guanine with the charge remaining on guanine–PhIP and is also observed in the PSD spectrum of 5′-d(G(PhIP)CTACC) and 5′-d(CCATCG(PhIP)) digest product ions. The fragment ion at m/z 167.1 corresponds to cleavage of the C—N bond in PhIP, with the free amine remaining bound to the C-8 position of guanine. Formation of this fragment ion would not be possible from 5′-monophosphate-N^2-dG–PhIP $[M + H]^+$ ions because the amine on PhIP is not bound to the C-8 of guanine in this isomer. The ion at m/z 237.1 is tentatively assigned as the product from simultaneous cleavage of the C-8—N-7 and the C-8—N-9 bonds of guanine and is consistent with a fragment ion identified by Rindgen *et al*.[39] from collision-induced dissociation in an ion trap mass spectrometer of a C8-dG–PhIP nucleotide adduct. This fragment ion is also not plausible from an N^2-dG–PhIP covalent bond. Thus, in agreement with the NMR structural data, the dissociation pathways of the MALDI identify the major PhIP–DNA adduct as containing PhIP covalently linked to the C-8 position of dG.

Determination of the PhIP modification site in one minor adduct

The minor PhIP–ODN adduct, peak 1 (Fig. 1), is 16 Da greater in mass than the C8-dG–PhIP–ODN adduct. The measured mass of the minor adduct $[M + H]^+$ ions is 3474.6719 Da, which is a 1.2 ppm mass measurement error from the

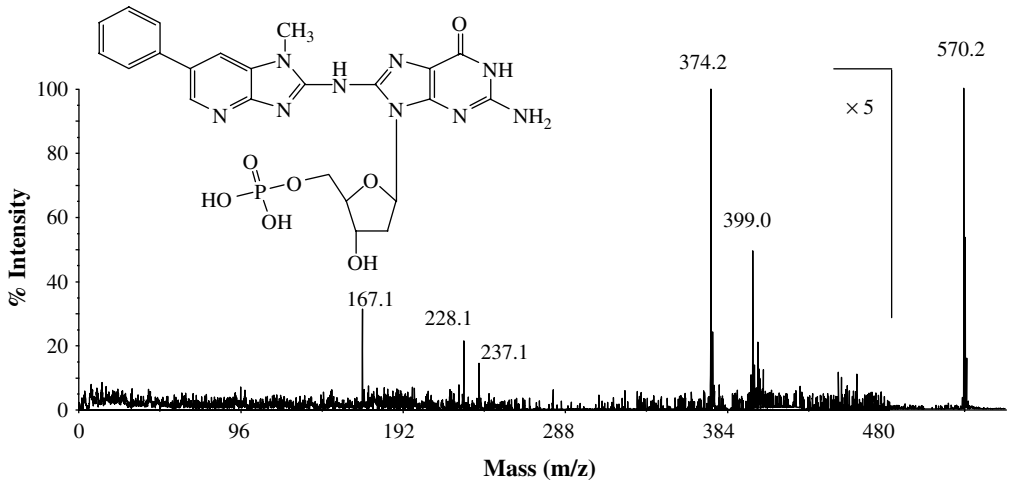
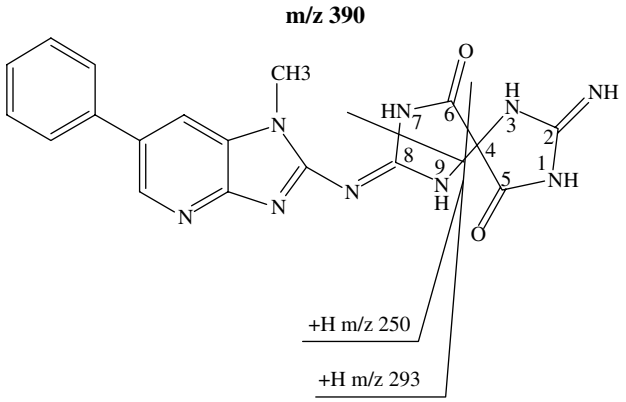


Figure 8. PSD spectrum of the BIMP digestion end product containing 5′-dGMP–PhIP from the major PhIP–ODN adduct.



Scheme 3. Proposed structures for the fragment ions observed in the dissociation of 5′-dGMP–PhIP $[M + H]^+$ ions resulting from BIMP digestion of the minor PhIP–ODN adduct.

proposed structure of a spirobisguanidino–PhIP adduct (Scheme 3),[29] which has an exact mass of 3474.6761 Da. This adduct is thought to be formed through oxidative degradation of the C8-dG–PhIP adduct similar to that proposed by Shibutani *et al.*[40] for C8-dG–2-aminofluorene adducts.

The first experiment performed on the minor adduct was a 3′ → 5′ BIMP digest (Fig. 9). After 1 min, the first four nucleotides from the 3′-end are identified. The rate of digestion again slows as the enzyme reaches dG and after 10 min we again observe complete enzymatic inhibition at dG with the ion at m/z 1990.42 being consistent with 5′-d(CCATCG(PhIP)) $[M + H]^+$ ions, except 16 Da larger. Also observed at 10 min is the ion at m/z 1781.34, 5′-d(CATCG(PhIP)), resulting from BIMP endonuclease activity. Recognition of this BIMP enzymatic behavior identifies dG as the primary PhIP binding site in this minor adduct. The increase in mass by 16 Da of the digestion end products also demonstrates the oxidized moiety is contained in the digestion end product 5′-d(CATCG(PhIP)).

To identify the location of oxidation and PhIP adduction, PSD was performed on the 5′-d(CCATCG(PhIP)) $[M + H]^+$ ions and Fig. 10 shows the resulting PSD spectrum. The dissociation of this ion is analogous to the BIMP digestion end products of the C8-dG–PhIP–ODN adduct (Fig. 6) except each fragment ion observed is 16 Da greater in mass than the same sequence fragment ions observed in Fig. 6. Importantly, the $(G–PhIP)^+$ ion at m/z 390.1 designates that indeed PhIP is bound to guanine and either guanine or PhIP has been oxidized.

The next step is to determine the covalent structure of the oxidized guanine–PhIP moiety. To accomplish this, again BIMP digestion was carried out at 37 °C for 60 min to allow the endonuclease activity of BIMP to occur, digesting

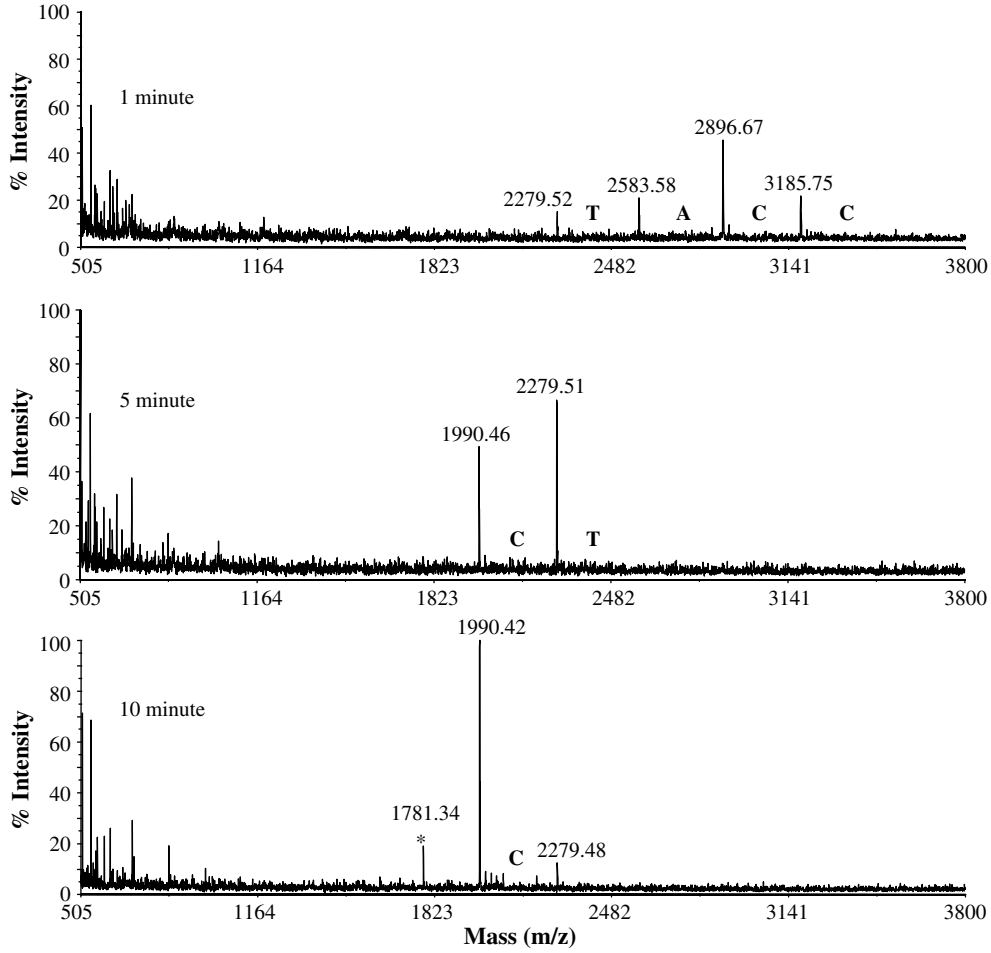


Figure 9. Positive ion MALDI-TOF mass spectra of the BIMP digestion end products of the minor 5′-d(CCATCGCTACC)–PhIP adduct (peak 1 in Figure 1A) at 1, 5 and 10 min digestion times.

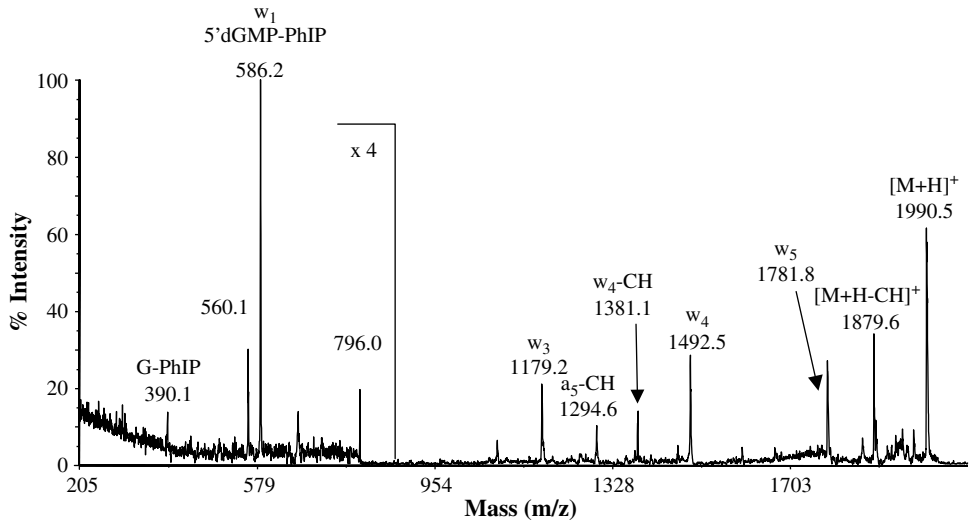


Figure 10. PSD spectrum of the BIMP digestion end product containing 5′-d(CCATCG–PhIP) from the minor PhIP–ODN adduct.

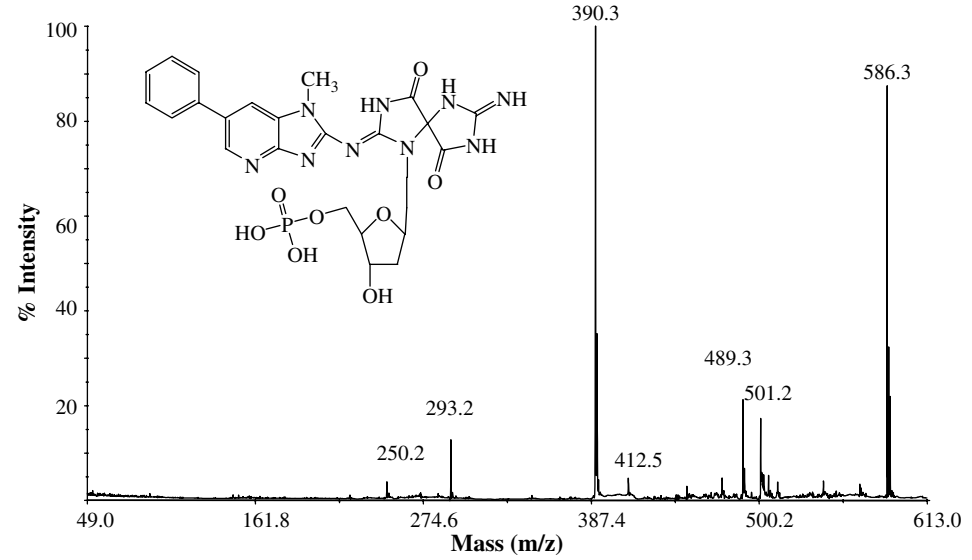


Figure 11. PSD spectrum of the BIMP digestion end product containing 5′-dGMP–PhIP from the minor PhIP–ODN adduct.

the PhIP–ODN to the 5′dGMP–PhIP $[M + H]^+$ ion at m/z 586.2 for PSD analysis (Fig. 11). The primary fragment ion observed is the $(dG–PhIP)^+$ ion at m/z 390.2 with several lower abundance ions apparent at m/z 293.2 and 250.1. Their proposed structures are shown in Scheme 3. The proposed structure of the ion at m/z 293.2 is consistent with the dissociation mechanisms of spiroiminodihydantoin as suggested by Luo *et al*.[41] They used ^{18}O labeling and ESI-MS/MS methods to dissociate spiroiminodihydantoin, which is a major oxidation product of 8-oxoguanine. The structure of spiroiminodihydantoin is similar to that of the spirobisguanidino–PhIP adduct proposed, with the exception that instead of PhIP bound to the C-8 position, this carbon position is oxidized. Luo *et al*. reported a fragment ion resulting from simultaneous dissociation of the N-3—C-4 and C-4—C-5 bonds with the charge remaining on the C-5—N-1—C-2—N-3 fragment. We suggest a similar fragmentation pathway for the spirobisguanidino–PhIP adduct ion where dissociation of the C-4—C-6 and C-4—N-9 bond is observed with the charge remaining on the PhIP-containing fragment.

CONCLUSIONS

Nine, structurally unique, PhIP–ODN adducts were synthesized and isolated. Of the nine adducts, only one major adduct structure could be unambiguously identified by NMR even after large-scale synthesis. This paper demonstrates the applicability and sensitivity of MALDI-TOF-MS, exonuclease digestion and PSD for identifying sequence location of modification sites in DNA and also the covalent nature of the modification of structurally unknown ODN adducts. The primary activity of the $3' \rightarrow 5'$ exonuclease, BIMP, and the $5' \rightarrow 3'$ exonuclease, BSP, is quenched at dG in the PhIP-adducted defined sequence ODN, 5′-d(CCATCGCTACC), indicating that PhIP binds to dG in both adducts investigated. PSD of the digestion end products, 5′-d(CCATCG(PhIP)) and 5′-d(G(PhIP)CTACC), confirms that dG is the primary

binding site in both the major adduct and minor adduct analyzed. PSD of the minor adduct also reveals that PhIP not only binds to guanine, but also the guanine–PhIP moiety (m/z 390.1) is oxidized in comparison with the same fragment ion of the major adduct (m/z 374.1). Exonuclease digestion and PSD of the digestion end products define the ODN sequence and identify the PhIP adduction site. Non-specific activity of BIMP further digested the PhIP–ODN adducts to 5′-dGMP–PhIP at which point PSD was again utilized to determine the covalent structure of guanine–PhIP. In both the major and minor adducts, PhIP binds to guanine through the C-8 position. In the minor adduct, however, guanine is oxidized at position 5 and a rearrangement of guanine to a spirobisguanidino structure is hypothesized.

The MALDI-TOF-MS methods incorporating mass measurements of the intact PhIP–ODN adducts, exonuclease digestion and PSD described here are carried out on a defined sequence ODN containing a single dG in which PhIP binds. By performing these experiments on ODNs with multiple dGs, one could begin to estimate the importance and specificity of nucleotide sequence on the reactivity of *N*-acetoxy-PhIP to dG. Certainly, in ODNs containing multiple dGs or even dsODNs, this combination of techniques would be essential in determining PhIP binding site(s). Mass measurements of PhIP-modified ODNs containing multiple dGs would determine the number of PhIP molecules adducted to the ODN. Here, we demonstrated that the primary activity of both the 3′ → 5′ and 5′ → 3′ exonucleases are completely inhibited at the PhIP modification site. Utilizing both enzymes to digest an ODN with two or more PhIP adducts could easily determine two binding sites, and sequencing the digest product ions by PSD (or MS/MS in an o-MALDI-Q-TOF mass spectrometer) could locate additional PhIP–dG adduct sites.

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