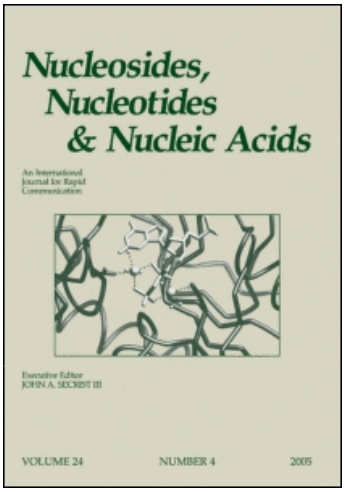

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Joeph Banoub[ab]; Stven Combden[b]; Judith Miller-Banoub[a]; George Sheppard[a]; Howard Hodder[a]
[a] Department of Fisheries and Oceans, Science Branch, Environmental Sciences Division, St. John's, NF, CANADA [b] Department of Biochemistry, Memorial University of Newfoundland, St. John's, NF, CANADA

To cite this Article Banoub, Joeph , Combden, Stven , Miller-Banoub, Judith , Sheppard, George and Hodder, Howard(1999) 'Structural Characterization of Intact Covalently Linked DNA Adducts by Electrospray Mass Spectrometry', Nucleosides, Nucleotides and Nucleic Acids, 18: 11, 2751 — 2768
To link to this Article: DOI: 10.1080/07328319908044639
URL: http://dx.doi.org/10.1080/07328319908044639

NUCLEOSIDES & NUCLEOTIDES, 18(11&12), 2751–2768 (1999)

STRUCTURAL CHARACTERIZATION OF INTACT COVALENTLY LINKED DNA ADDUCTS BY ELECTROSPRAY MASS SPECTROMETRY

Joseph Banoub,[a,b*] Steven Combden,[b] Judith Miller-Banoub,[a] George Sheppard[a] and Howard Hodder[a]

[a] *Department of Fisheries and Oceans, Science Branch, Environmental Sciences Division, St. John's, NF, A1C 5X1, CANADA*
[b] *Memorial University of Newfoundland, Department of Biochemistry, St. John's, NF, A1B 3X9, CANADA*

ABSTRACT: Electrospray mass spectrometry has aided the structural characterization of new series of intact covalently linked [DNA-(aromatic compounds)] adducts. The following DNA adducts with modified d-guanosine units were synthesized: d[CGTAC(*GBA*)], d[(CCACGTTAACGTGG)-(*AAF*)$_{n=1-4}$] and d[(GATTCGTAGCTACGAATC)-(*AAF*)$_{n=1-4}$] and were analyzed by negative electrospray mass spectrometry. These DNA adducts exhibited different series of multiply charged deprotonated molecular anions. The mass spectra so obtained were of the whole molecules, each having a number of negative charges, mostly in the range of –5 to –9. Fragmentations of the multiply charged deprotonated molecular anions formed by the [mer-14(G*AAF*)$_{n=1-4}$] were obtained by controlled collisionally activated dissociation (CAD) initiated by cone voltage fragmentation and afforded diagnostic product anions which confirmed the presence and location of the guanine nucleobase adduct [Gua-C$_8$-*AAF*] and allowed bi-directional sequencing of these DNA-carcinogen adducts. Low-energy collision activated dissocation of the precursor multiply charged anion F^{8-} at *m/z* 796.72, produced from the [mer-18(G*AAF*)$_4$+Na–H] adduct using a high collision energy, provided some characteristic fingerprint product anions.

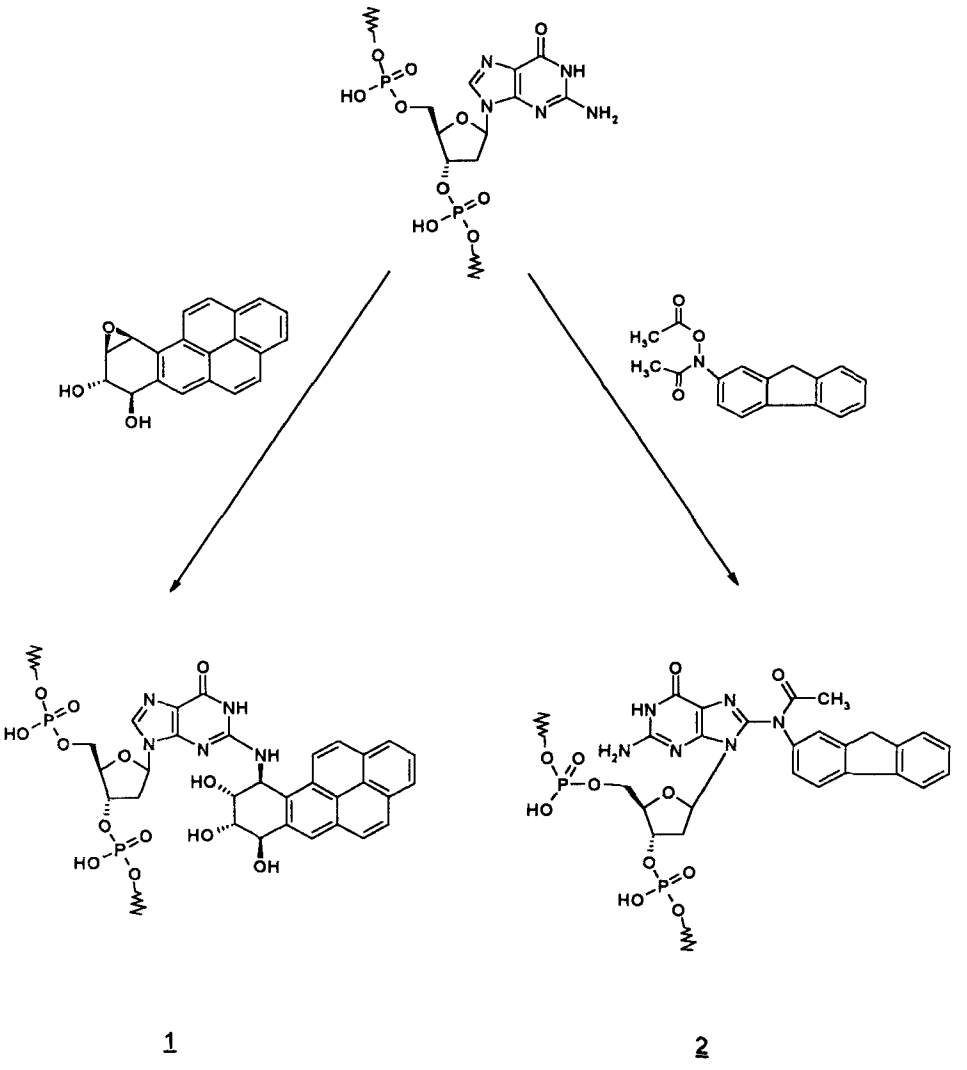
Introduction

DNA adducts are important biomarkers used to quantify exposure to mutagenic and carcinogenic substances in the environment.[1] Industrial and chemical processes, in particular combustion, produce toxic substances which, when introduced into the environment without detoxification, cause damage to DNA and, accordingly, are known to be mutagenic and/or

carcinogenic to humans. Polycyclic aromatic hydrocarbons (PAH) are a group of structurally-related chemicals which are important carcinogenic pollutants.[2,3] These compounds are metabolized into electrophilic derivatives, typically dihydrodiol-epoxides which react with DNA to elicit genetoxic, mutagenic and carcinogenic responses.[4,5] For example, the potent carcinogen benzo[*a*]pyrene is oxidatively metabolized to a diol-epoxide (BPDE) which attacks the 7-amino group of guanine bases in DNA[6] to form the DNA-carcinogen adduct **1**. Similarly, the *N*-acetoxy-2(acetylamino)fluorene (AAAF) is a known mutagenic and carcinogenic aromatic amine that reacts with DNA *in vitro* and *in vivo*[7] with a high degree of specificity for the C-8 position of guanine, forming the N(2'-deoxyguanosin-8-yl)-2-(acetylamino)fluorene DNA adduct **2** (Scheme 1). AAAF has previously been used to form DNA adduct models for the study of the nucleotide excision repair (NER) mechanism, a major pathway for the removal of bulky lesions from the genome.[8]

Since the 1960s it has been known that the mutagenic activity of many substances is linked to the degree to which they covalently bind to DNA. The analysis and measurement of carcinogen-DNA adducts has been proposed as a means of evaluation of the extent of human exposure to specific carcinogens[9] and to monitor the presence and effects of these specific carcinogens in the environment.

DNA adduct analyses are complicated by the low frequency of DNA adduction that occurs *in vivo*. In experimental animals, only about 10-100 picomoles of adduct per milligram of DNA are normally formed following treatment with tumorigenic doses of carcinogens.[10] Several techniques, including the use of radiolabelled mutagens or carcinogens[9], immunoassay,[11] fluoresence[12] and ^{32}P-post-labelling techniques,[2,13] have been developed for the analysis of small quantities of carcinogen-DNA adduct. For example, the Randerath enzymatic radiolabelling process[13] can detect 1 nucleobase-adduct in 10^8 to 10^9 normal nucleotides. Although all of the previous methods are highly sensitive, they do not allow the determination of the precise molecular structures of the detected adducts. This is a severe limitation when there is uncertainty with regard to the nature and type of carcinogen exposure. Highly sensitive, structurally informative analytical techniques are thus needed for the general characterization of DNA modifications.



1 2

Scheme (1)

Mass spectrometry has the potential to provide structural information and, as such, has played an important role in the structural elucidation of covalently modified nucleic acids.[14] Many modes of ionization have been employed including electron-impact[15], chemical ionization, field desorption[16] and thermospray ionization.[17] Although all of the previous techniques provided adequate structural information (especially when coupled with tandem mass spectrometry) they allowed only the analysis of either single nucleobase-

adducts or derivatized single nucleobase-adducts. Also, these methods suffered either from not having the sensitivity necessary for the analysis of trace levels of components derived from small amounts of DNA, or from the need to use chemical derivatization techniques to increase volatility and decrease thermal lability. Fast atom bombardment[18] (FAB) and matrix assisted laser desorption ionization-Fourier Transform mass spectrometry[19] (MALDI-FTMS) have allowed the analysis of non-derivatized adducts of nucleosides and nucleotides. Electrospray ionization (ESI) has proven to be a promising technique for the detection, identification and quantitation of such adducts. ESI is well established as a robust technique for use with combined liquid chromatography/mass spectrometry (LC/MS), which allows rapid, accurate and sensitive analysis of a wide range of analytes from low molecular weight polar compounds (less than 200 Da) to biopolymers larger than 200 kDa.[20] Besides being very sensitive for polar compounds such as nucleotides and DNA, it can also be easily interfaced with other separation techniques such as capillary electrophoresis (CE). Furthermore, MS/MS readily provides sequence information permitting rapid, accurate analysis of moderately sized oligonucleotides.[21-24] Analyses of nucleoside and nucleotide adducts have been performed using LC-ESI MS/MS[25] and CE-ESI MS/MS[26-28]. To our knowledge there are two reports on the analysis of intact, covalently linked DNA adducts using electrospray ion trap mass spectrometry (ESI-ITMS)[29,30].

In continuation of our interest in the ESI MS/MS of bioactive molecules[31-33] and as part of a program aimed at the synthesis of intact, covalently linked DNA adduct models which will be used as templates to rationalize the structural characterization of DNA modified by xenobiotics a series of standard model compounds were prepared. The self-complementary mer-6 DNA-hexamer, d(CGTACG), was reacted with trans-3,4-dihydrodiol-1,2-epoxide-anti-benz(a)anthracene. A series of covalently linked DNA adducts have been obtained by reacting, individually, self-complementary mer-14 DNA oligomer d(CCACGTTAACGTGG) and mer-18 DNA oligomer d(GATTCGTAGCTACGAATC) with *N*-acetoxy-2(acetylamino)fluorene. This rationale deals with the characterization of these novel, intact, covalently linked DNA adducts by electrospray ionization with a quadrupole-hexapole-quadrupole mass spectrometer.

Experimental

Sample preparation. DNA samples were obtained from Oswel DNA Service, Department of Chemistry, University of Southampton (Southampton, UK) and were

prepared on an Applied Biosystems ABI 380B DNA synthesizer using the phosphoramidate method[34] and purified by reversed-phase HPLC and Sephadex gel filtration.[35] The polycyclic aromatic hydrocarbons, diol epoxide and AAAF were obtained from the National Cancer Institute, Chemical Carcinogen Repository. The standard solutions used for LC/MS and LC/MS/MS were prepared with a mixture of HPLC solvent grade acetonitrile-water (CH_3CN-H_2O, 50-50) at a concentration of 50 pmol μL^{-1}. A 20 μL aliquot of sample was then introduced into the electrospray ion source by a continuous flow of (CH_3CN-H_2O, 50-50) at a flow rate of 10 $\mu L\ min^{-1}$ using a Shimadzu LC-10AD pump connected to the Rheodyne injector with a 20 μL loop.

Mass spectrometric conditions. The ESI mass spectra (negative ion mode) were recorded with a Micromass Quattro quadrupole-hexapole-quadrupole mass spectrometer equipped with a megaflow ESI source and capable of analyzing ions up to *m/z* 4000. A personal computer (Compaq, PII 266 MHz processor, running Windows NT 4, service pack 3) equipped with Micromass MASSLYNX 3.2.0 Mass Spectrometry Data System software was used for data acquisition and processing. The temperature of the ESI source was maintained at 75 ^{0}C. The operating voltage of the ESI capillary was 3.00 kV and the high voltage lens was set at 0.40 kV throughout the whole operation. ESI-MS were recorded with a cone voltage of 25 V. Component analysis of ESI-MS was performed using MASSLYNX algorithms which converts a range of multiply-charged anions into identifiable single components. ESI-MS with 'maximum entropy' (ESI-MS-MaxEnt) was performed using the Micromass MaxEnt Deconvolution (MMD) program.[36] MaxEnt automatically disentangles the *m/z* spectrum produced by the mass spectrometer and presents the data for each individual DNA and/or DNA adduct in the mixture as a single peak on a true molecular weight scale. MS/MS experiments were conducted using the same instrument. Fragment ion spectra of mass-selected were induced by collision with argon in the (r.f.-only) hexapole. The resulting fragments were analyzed by the second quadrupole. A cone voltage varying from 20 to 35 V, collision energies varying from 25 to 35 eV and a collision gas pressure in the collision cell varying from 3.5×10^{-4} to 6.5×10^{-4} mbar (1 bar = 10^5 Pa) were used in all MS/MS experiments. The collision gas pressure was increased to induce the dissociation of the sodiated adduct anions (typical settings were around 6.0×10^{-4} mbar for the $[M - 9H]^{9-}$ ions).

Covalent modification of DNA oligonucleotides. Adduct derived from the binding of *anti*-benz[*a*]anthracene-*trans*-3,4-dihydrodiol-1,2-epoxide to mer-6 DNA hexamer d(CGTACG). To a solution of 1.2 mg (6μmoles) of the mer-6 DNA hexamer d(CGTACG), **2**, (Oswel DNA Service), in 1 mL of 5 mM bis-Tris-HCl (Sigma) containing 0.01 mM EDTA (pH 7.1), *anti*-benz[*a*]anthracene-*trans*-3,4-dihydrodiol-1,2-epoxide, 556 μg (2 μmoles) in 300 μL THF was added. The mixture was maintained at 37 ^{0}C for 24h then 1 mL of water was added and the mixture extracted 3 times with diethyl ether to remove the unreacted benz[*a*]anthracene compound. Cold ethanol (0.5 mL) was added to the aqueous phase and the solution cooled to 5 ^{0}C to precipitate a mixture of unmodified d(CGTACG), **3**, and the covalently attached d[CGTAC(G*BA*)], **4**. This mixture was collected by centrifugation and used for electrospray mass spectrometric identification without further purification.

Adduct derived from the binding of AAAF to mer-14 DNA oligomer d(CCACGTTAACGTGG), 5. A stock solution was prepared by dissolving 5 mg (17.8 μmol) of AAAF in a 1 mL amber vial equipped with a septum (in the glove box) with 800 μL of degassed 100% ethanol. To a solution of 2.2 mg (0.5 μmol) of the mer-14 DNA oligomer d(CCACGTTAACGTGG), **5**, in a mixture of 1 mL of ethanol:water (1:1), 500 μL (8.9 μmol) of the stock AAAF solution was added and the reaction mixture was shaken on a flatbed shaker at 37 ^{0}C for 3 hours in a dark fume hood. The reaction mixture was dialyzed against deionized water in a dialysis bag with a molecular weight cutoff of 1000 to remove unreacted AAAF. The dialysate was then lyophilized. The obtained mixture of mer-14 covalently linked oligomer [mer-14(G*AAF*)$_n$] adducts, namely: the tetrasubstituted [mer-14(G*AAF*)$_4$] adduct d[CCAC(G*AAF*)TTAAC(G*AAF*)T(G*AAF*)(G*AAF*)], **18** and sodiated derivatives **19** to **21**; the trisubstituted [mer-14(G*AAF*)$_3$] adduct **14** and sodiated derivatives **15** to **17**; the disubstituted [mer-14(G*AAF*)$_2$] adduct **10** and sodiated derivatives **11** to **13**; the monosubstituted [mer-14(G*AAF*)] adduct **6** and sodiated derivatives **7** to **9**, was analyzed by electrospray mass spectrometry and maximum entropy processing without further purification.

Adducts derived from the binding of AAAF to the mer-18 DNA oligomer d(GATTCGTAGCTACGAATC), 22. The monosodiated mer-18 DNA oligomer d(GATTCGTAGCTACGAATC) **22** was reacted in a similar fashion to that described

above, initially for 3 hours, to afford a mixture of mer-18 covalently linked oligomer, namely: the tetrasubstituted sodiated [mer-18(G*AAF*)$_4$+Na–H] adduct d[(G*AAF*)ATTC(G*AAF*)TA(G*AAF*)CTAC(G*AAF*)AATC], **28** and di-sodiated adduct **29**; the trisubstituted sodiated [mer-18(G*AAF*)$_3$+Na–H] adduct **26** and di-sodiated adduct **27**; the disubstituted sodiated [mer-18(G*AAF*)$_2$+Na–H] adduct **24** and the di-sodiated adduct **25** and the monosubstituted sodiated [mer-18(G*AAF*)+Na–H] adduct **23** and di-sodiated adduct **24**. The obtained mixture of mer-18 [DNA-(G*AAF*)$_n$] adducts **23** to **29** were analyzed by electrospray mass spectrometry and maximum entropy processing without further purification. When the same reaction was conducted for a longer period (6 hours), a mixture of the sodiated tetrasubstituted adduct [mer-18(G*AAF*)$_4$+Na–H], **29**, and the di-sodiated adduct, **30**, was obtained.

Results and discussion

The negative ion ESI-MS of the DNA mer-6 hexamer d(CGTACG), **3** , M.Wt. 1791.3, has been described previously[37] and afforded a series of multi-charged deprotonated molecular anions corresponding to $[M-2H]^{2-}$, $[M-3H]^{3-}$, $[M-4H]^{4-}$ and $[M-5H]^{5-}$ at *m/z* 894.5, 595.8, 446.7 and 357.2, respectively. The negative ion ESI-MS of the mixture of synthetic, covalently attached *anti*-benz[*a*]anthracene dihydrodiol-epoxide deoxyguanosine d[CGTAC(G*BA*)], **4**, contained unreacted amounts of mer-6, **3**. The addition reaction was not complete since the spectrum indicates the presence of the mer-6 hexamer molecular anions, *m/z*(%) as follows: 894.5(27), 596.15(100), 446.98(59) and 357.2(31)] and the corresponding monosodiated molecular ions, *m/z*(%) as follows: 905.4(23), 603.4(34) and 452.3(14). The low abundance peaks are indicated *m/z*(%) as follows: [688.77(27), 516.44(19) and 412.74(12) originate from the adduct d[CGTAC(G*BA*)], **4** which was formed only in low yield (~30%). This spectrum was deconvoluted on the true molecular weight scale, which considerably simplifies the spectrum and facilitates the interpretation. Generally, the molecular weight determined by ESI-MS combined with MaxEnt are within 0.01% of the values calculated from the sequences. The deconvoluted spectrum of this mixture showed the presence of the non-reacted sodiated mer-6 d(CGTACG) oligomer, **3**, (*m/z* 1792.5), its sodiated adduct (*m/z* 1814) and the d[CGTAC(G*BA*)] adduct **4** (*m/z* 2070). The mass increment of 279 a.m.u. of the d[CGTAC(G*BA*)] adduct **4** corresponds to the

attachment of the 2,3,4-trihydroxy *anti*-benz[*a*]anthracene moiety which is on the 7-amino-group of the guanine base of this mer-6 hexamer. The tentative structure for adduct **4** is d[C(G*BA*)TACG] and/or d[CGTAC(G*BA*)]. This was not further pursued since the yield of this synthetic adduction reaction did not improve on varying the experimental conditions. As the synthetic adduction reaction using *anti*-benz[*a*]anthracene-3,4-dihydrodiol-1,2-diol *trans* could not be extended to other guanosine-containing DNA oligomers longer than hexamers, it was decided that the [DNA-(G*BA*)] adduct **4** was not a good model to probe the possibility of verifying the adduction sites of guanosine residues by low-energy ESI MS/MS.

Structural characterization of products obtained by reaction with AAAF was then attempted. The primary reaction that occurs is the formation of the nitrenium ion due to the loss of the acetoxy function from AAAF followed by an attack on the C-8 position of guanine. Before conducting the initial condensation adduction reaction with AAAF, the purity of the starting mer-14 DNA oligomer was assessed. The negative ion ESI-MS of the mer-14 DNA oligomer d(CCACGTTAACGTGG), **5**, was recorded with a low cone voltage (20 V) and gave a series of multi-charged deprotonated anions corresponding to $[M-5H]^{5-}$, $[M-6H]^{6-}$, $[M-7H]^{7-}$ and $[M-8H]^{8-}$ at *m/z*(%) as follows: [851.52(46), 709.51(100), 608.24(84) and 532.05(71)] assigned as the A-series. A series of monosodiated multi-charged deprotonated anions of [mer-14 + Na-H] corresponding to $[M+Na-6H]^{5-}$, $[M+Na-7H]^{6-}$, $[M+Na-8H]^{7-}$ and $[M+Na-9H]^{8-}$ at *m/z*(%) 856.3(37), 713.22(85), 64.30(61), 611.30(61) and 534.82(36) was observed and assigned as the B-series. In addition, a series of multi-charged anions of the disodiated adduct [mer-14 + 2Na-2H] was observed which corresponded to $[M+2Na-7H]^{5-}$, $[M+2Na-8H]^{6-}$, $[M+2Na-9H]^{7-}$ and $[M+2Na-10H]^{8-}$ at *m/z*(%) 860.8(21), 717.01(27), 614.48(24) and 537.5(11) and assigned as the C-series. The deconvoluted spectrum confirmed that the mer-14 DNA oligomer d(CCACGTTAACGTGG), $C_{136}H_{172}N_{53}O_{82}P_{13}$, M.Wt. 4263.8285, was indeed composed of a population of native mer-14 DNA oligomers, which has the expected molecular weight at *m/z* 4263.79, in addition to the monosodiated adduct [M+Na–H] at *m/z* 4286.01, ($C_{136}H_{171}N_{53}O_{82}P_{13}Na$, M.W.=4285.8104) and disodiated adduct [M+2Na–2H] at *m/z* 4308.00, ($C_{136}H_{170}N_{53}O_{82}P_{13}Na_2$, M.W.=4307.7923). Thus, the deconvoluted spectrum confirmed the base content and the theoretical molecular weight.

Compared to the d[CGTAC(G*BA*)] DNA adduct **4** discussed previously, the reaction of the AAAF with the mer-14 DNA oligomer d(CCACGTTAACGTGG) was completed after 3 hours and the ESI-MS of the adduction reaction product showed 16 complicated, multiply-charged DNA adduct anions. The identities of these anions are assigned in Table 1 as series A to P. They were highly charged (–8 to –5) anions corresponding to 16 different DNA adducts, originating from 4 different DNA oligomers (**6**, **10**, **14** and **18**), in addition to their monosodiated, disodiated and trisodiated species.

The ESI-MS of the adduction of the mer-14 oligonucleotide was deconvoluted and confirmed, once more, the presence of 16 different covalently linked mer-14 [DNA-$(GAAF)_n$] adducts containing the N(deoxyguanosin-8-yl)-2-*AAF* moiety, in addition to a minute amount of residual, unreacted mer-14 d(CCACGTTAACGTGG), **5**. It also indicates that the series of [mer-14(G*AAF*)$_2$] adducts **10** to **12** are in higher abundance than [mer-14(G*AAF*)$_4$], **18** to **21**. Attempts to increase the yield of the [mer-14(G*AAF*)$_4$] series by extending the reaction time or by varying the proportions of the condensation reactants did not change the ratio of the formed mer-14 DNA adducts **6** to **21**. The tentative structure of the adduct, **18**, is as follows: d[CCAC(G*AAF*)TTAAC(G*AAF*)T(G*AAF*)(G*AAF*)]. This is representative of the [mer-14(G*AAF*)$_4$] adducts **19** to **21**. Assignment of the structures for the remaining [mer-14(G*AAF*)$_{n=1-3}$] adducts **6** to **17** remains, at this stage, conjectural since the starting mer-14 DNA oligonucleotide contains 4 deoxyguanosine units and at least 15 different isomeric oligomers arising from these types of adducts can be expected, namely: the trisubstituted, disubstituted and monosubstituted [mer-14(G*AAF*)$_{n=1-3}$]. The condensation-adduction reaction with AAAF using a longer DNA oligomer was then attempted. The mer-18 DNA oligomer d(GATTCGTAGCTTAGAATC), **22**, was selected since the guanosyl moieties were located as the 5th, 10th, 13th and 18th nucleotides, in contrast to the adduction reaction conducted with the mer-14 which contained 2 penultimate and terminal -guanosyl nucleotides. The steric crowding should have caused the mer-14 to be more resistant to adduction by AAAF.

Purity of the mer-18 DNA oligomer d(GATTCGTAGCTTAGAATC), **22**, was assessed by negative ion ESI-MS and recorded with a low cone voltage (20 V). A series of multiply-charged, deprotonated anions was assigned (A series) and corresponding to $[M+Na-(n+1)H]^{n-}$ (n = 6 to 10), at *m/z*(%) as follows: [915.33(19), 784.23(46),

Table 1. Component analysis of the different series of multiply charged deprotonated molecular anions obtained from the [mer-14(G*AAF*)$_{n=1-4}$] adducts *6* to *21*.

mer-14 DNA Adducts	Compd	MaxEnt Calc. MW	Series	Deprotonated Anions, m/z (%)
[mer-14(G*AAF*)] $C_{151}H_{183}N_{54}O_{83}P_{13}$ (4485.0871)	**6**	4484.20	A	A^{7-}= 639.57 (33) A^{6-} = 746.30 (35) A^{5-} = 895.58 (5)
[mer-14(G*AAF*)+Na-H] $C_{151}H_{182}N_{65}O_{83}P_{13}Na$ (4507.0689)	7	4506.10	B	B^{8-} = 562.25 (3) B^{7-} = 642.71 (43) B^{6-} = 750.01 (56) B^{5-} = 900.22 (13)
[mer-14(G*AAF*)+2Na-2H] $C_{151}H_{181}N_{54}O_{83}P_{13}Na_2$ (4529.0507)	**8**	4528.40	C	C^{8-} = 565.05 (2) C^{7-} = 645.91 (18) C^{6-} = 753.73 (36) C^{5-} = 904.68 (15)
[mer-14(G*AAF*)+3Na-3H] $C_{151}H_{180}N_{54}O_{83}P_{13}Na_3$ (4551.0326)	**9**	4552.00	D	D^{8-} = 568.00 (1) D^{7-} = 649.28 (5) D^{6-} = 757.66 (14) D^{5-} = 909.40 (6)
[mer-14(G*AAF*)$_2$] $C_{166}H_{194}N_{55}O_{84}P_{13}$ (4706.3456)	**10**	4706.56	E	E^{8-} = 587.29 (7) E^{7-} = 671.34 (74) E^{6-} = 783.39 (64) E^{5-} = 940.27 (7)
[mer-14(G*AAF*)$_2$+Na-H] $C_{166}H_{193}N_{55}O_{84}P_{13}Na$ (4728.3274)	**11**	4727.30	F	F^{8-} = 589.91 (4) F^{7-} = 674.32 (71) F^{6-} = 786.8 (100) F^{5-} = 944.46 (18) F^{4-} = 1180.82 (2)
[mer-14(G*AAF*)$_2$+2Na-2H] $C_{166}H_{192}N_{55}O_{84}P_{13}Na_2$ (4750.3092)	**12**	4749.40	G	G^{8-} = 592.67 (2) G^{7-} = 677.48 (25) G^{6-} = 790.56 (53) G^{5-} = 948.88 (17)
[mer-14(G*AAF*)$_2$+3Na-3H] $C_{166}H_{191}N_{55}O_{84}P_{13}Na_3$ (4772.2910)	**13**	4771.40	H	H^{8-} = 595.42 (1) H^{7-} = 680.62 (7) H^{6-} = 794.23 (16) H^{5-} = 953.28 (9)

Table 1 (continued)

mer-14 DNA Adducts	Compd	MaxEnt Calc. MW	Series	Deprotonated Anions, m/z (%)
[mer-14(G*AAF*)$_3$]	**14**	4927.50	I	I^{8-} = 614.95 (10) I^{7-} = 702.94 (73) I^{6-} = 820.27 (55) I^{5-} = 984.52 (4)
$C_{181}H_{205}N_{56}O_{85}P_{13}$ (4927.6040)				
[mer-14(G*AAF*)$_3$+Na-H]	**15**	4950.30	J	J^{8-} = 617.78 (7) J^{7-} = 706.18 (56) J^{6-} = 824.05 (72) J^{5-} = 989.06 (13)
$C_{181}H_{204}N_{56}O_{85}P_{13}Na$ (4949.5859)				
[mer-14(G*AAF*)$_3$+2Na-2H]	**16**	4972.20	K	K^{8-} = 620.52 (4) K^{7-} = 709.31 (27) K^{6-} = 827.70 (36) K^{5-} = 993.44 (9)
$C_{181}H_{203}N_{56}O_{85}P_{13}Na_2$ (4971.5677)				
[mer-14(G*AAF*)$_3$+3Na-3H]	**17**	4992.60	L	L^{8-} = 623.07 (2) L^{7-} = 712.22 (10) L^{6-} = 831.10 (11) L^{5-} = 997.52 (5)
$C_{181}H_{202}N_{56}O_{85}P_{13}Na_3$ (4993.5495)				
[mer-14(G*AAF*)$_4$]	**18**	5148.80	M	M^{8-} = 642.60 (41) M^{7-} = 734.54 (32) M^{6-} = 857.10 (20)
$C_{196}H_{216}N_{57}O_{86}P_{13}$ (5148.8625)				
[mer-14(G*AAF*)$_4$+Na-H]	**19**	5169.50	N	N^{8-} = 645.18 (12) N^{7-} = 737.50 (22) N^{6-} = 860.58 (31) N^{5-} = 1032.90 (4)
$C_{196}H_{215}N_{57}O_{86}P_{13}Na$ (5170.8444)				
[mer-14(G*AAF*)$_4$+2Na-2H]	**20**	5192.80	O	O^{8-} = 648.10 (4) O^{7-} = 740.82 (7) O^{6-} = 864.46 (16)
$C_{196}H_{214}N_{57}O_{86}P_{13}Na_2$ (5192.8262)				
[mer-14(G*AAF*)$_4$+3Na-3H]	**21**	5214.20	P	P^{8-} = 650.85 (1) P^{7-} = 743.97 (2) P^{6-} = 868.13 (5)
$C_{196}H_{213}N_{57}O_{86}P_{13}Na_3$ (5214.8080)				

686.02(100), 609.62(57) and 548.45(22)]. In addition a minor series of multiply-charged, deprotonated anions was assigned (B series) and corresponded to $[M+2Na-(n+2)H]^{n-}$ (n = 6 to 10), at *m/z*(%) as follows: [918.84(19), 787.36(39), 688.74(53), 612.04(27) and 550.63(10)]. Finally, 2 small multiply-charged ions at *m/z*(%) [691.51(12) and 790.41(18)] were assigned respectively as $[M+3Na-10H]^{3-}$ and $[M+3Na-11H]^{3-}$. The deconvoluted spectrum confirmed that the mer-18 DNA oligomer d(GATTCGTAGCTTAGAATC), ($C_{175}H_{222}N_{65}O_{107}P_{17}$, M.W.=5474.6155), electrosprayed mainly as the major sodiated adduct [M+Na–H] at *m/z* 5496.61 ($C_{175}H_{221}N_{65}O_{107}P_{17}Na$, M.W. 5496.5974) and also contained traces of disodiated adduct [M+2Na–2H] at *m/z* 5518.87 ($C_{175}H_{220}N_{65}O_{107}P_{17}Na_2$, M.W. 5518.5792) and the trisodiated adduct [M+3Na–3H] at *m/z* 5540.09 ($C_{175}H_{219}N_{65}O_{107}P_{17}Na_3$, M.W. 5540.5610).

The condensation-adduction reaction of the AAAF with the mer-18, **22**, was initiated and the reaction was stopped after 3 hours. The product was lyophilized and analyzed by ESI-MS, which was recorded with a low cone voltage (20 V). Component analysis of this mixture of [mer-18(G*AAF*)$_n$] DNA adducts indicated that there were 7 series (A to G) of multiply-charged, deprotonated mer-18 DNA adduct anions. Their identities and respective molecular weights are shown in Table 2. The deconvoluted spectrum of DNA adducts **23** to **29** indicated 7 different DNA adducts and that the most abundant were obtained from the D and F series derived from [mer-18(G*AAF*)$_3$+Na–H], **26**, (*m/z* 6160.50) and [mer-18(G*AAF*)$_4$+1Na–1H], **28**, (*m/z* 6381.80), respectively. The expected structure of the [mer-18(G*AAF*)$_4$], **18**, is as follows: d[(G*AAF*)ATTC(G*AAF*)TA(G*AAF*)CTTA-(G*AAF*)AATC].

The same condensation-adduction reaction was repeated and stopped after 6 hours. The reaction mixture was analyzed by negative ion ESI-MS and gave two series (A and B) of multiply- charged deprotonated anions which correspond to the F and G series already described in Table 2. The deconvoluted spectrum indicated that the adduction reaction proceeded to completion as evidenced by the predominance of DNA adducts [mer-18(G*AAF*)$_4$+Na–H], **28**, and [mer-18(G*AAF*)$_4$+2Na–2H], **29**, at *m/z* 6381.80 and 6403.78, respectively. The major mass difference between each of the [DNA-(G*AAF*)$_n$] adducts described in this rationale is 221.3 a.m.u., which corresponds to the adduction of the C-8 position of an extra guanine unit with the AAF moiety.

The obtained synthetic, covalently linked [DNA-(G*AAF*)$_n$] adducts always formed intact multiply-charged anions, representative of the intact structure of the respective molecule.

Table 2. Component analysis of the different series of multiply charged deprotonated molecular anions obtained from the [mer-18(G*AAF*)$_{n=1-4}$] adducts *23* to *29*.

mer-18 DNA Adducts	Compd	MaxEnt Calc. MW	Series	Deprotonated Anions, m/z (%)
[mer-18(G*AAF*)+Na–H] $C_{190}H_{232}N_{66}O_{108}P_{17}Na$ (5717.8558)	**23**	5717.90	A	A^{9-} = 634.32 (5) A^{8-} = 713.73 (8) A^{7-} = 815.84 (10) A^{6-} = 951.48 (9) A^{5-} = 1142.57 (12)
[mer-18(G*AAF*)$_2$+Na–H] $C_{205}H_{243}N_{67}O_{109}P_{17}Na$ (5939.1143)	**24**	5939.20	B	B^{9-} = 658.91 (12) B^{8-} = 741.39 (31) B^{7-} = 847.45 (43) B^{6-} = 988.86 (31) B^{5v} = 1186.83 (10)
[mer-18(G*AAF*)$_2$+2Na–2H] $C_{205}H_{242}N_{67}O_{109}P_{17}Na_2$ (5961.0961)	**25**	5961.18	C	C^{9-} = 661.35 (8) C^{8-} = 744.14 (27) C^{7-} = 850.59 (30) C^{6-} = 992.52 (19) C^{5-} = 1191.23 (16)
[mer-18(G*AAF*)$_3$+Na–H] $C_{220}H_{254}N_{68}O_{110}P_{17}Na$ (6160.3728)	**26**	6160.50	D	D^{9-} = 683.49 (23) D^{8-} = 769.06 (91) D^{7-} = 879.07 (64) D^{6-} = 1025.74 (39) D^{5-} = 1231.09 (15)
[mer-18(G*AAF*)$_3$+2Na–2H] $C_{220}H_{253}N_{68}O_{110}P_{17}Na_2$ (6182.3546)	**27**	6182.48	E	E^{9-} = 685.94 (27) E^{8-} = 771.80 (40) E^{7-} = 882.21 (60) E^{6-} = 1029.41 (36) E^{5-} = 1235.49 (18)
[mer-18(G*AAF*)$_4$+Na-H] $C_{235}H_{265}N_{69}O_{111}P_{17}Na$ (6381.6313)	**28**	6381.80	F	F^{9-} = 708.08 (15) F^{8-} = 796.72 (100) F^{7-} = 910.68 (79) F^{6-} = 1062.63 (38) F^{5-} = 1275.36 (11)
[mer-18(G*AAF*)$_4$+2Na-2H] $C_{235}H_{264}N_{69}O_{111}P_{17}Na_2$ (6403.6131)	**29**	6403.78	G	G^{9-} = 710.53 (9) G^{8-} = 799.47 (26) G^{7-} = 913.82 (39) G^{6-} = 1066.29 (36) G^{5-} = 1279.75 (18)

ESI-MS showed no evidence of any fragmentation of these multiply-charged anions. This is expected since usually under appropriate ESI experimental conditions, gas phase fragmentation of the various oligonucleotide adduct anions is minimized and the anions thus formed possess low internal energy and are sufficiently stable to pass from the ion source to the detector of the mass spectrometer without dissociation. This phenomenon is characteristic for anions produced by electrospray ionization, which is a very soft ionization process. If the primary ionization process does not impart enough internal energy for spontaneous fragmentation to occur dissociation, if needed, can be induced by controlling the accelerating voltage applied to the sampling cone (focus voltage) of the electrospray source.[38,39] This procedure, known as cone voltage fragmentation, is also referred to as collisionally activated dissociation (CAD) in the atmospheric pressure/vacuum interphase.[40] In order to generate more structural information on the previous series of [mer-14(G*AAF*)$_{n=1-4}$] the ESI-MS of the mixture **6** to **21** was recorded with a higher focus voltage (50 V) to induce the fragmentation of the multiply charged anions. The genesis of the characteristic fragment ions and their relative intensities are shown in Table 3. The nomenclature adopted for the fragmentation of the series of multiply-charged anions produced by the cone voltage fragmentation of the [mer-14(G*AAF*)$_n$] adducts is the one described by McLuckey *et al* for the oligonucleotides.[41] Examination of Table 3 permitted the identification of a distinct series of very intense, diagnostic anions which corresponded to *m/z* values from simple heterolytic cleavages of the phosphate-sugar bond which causes release of the N(deoxyguanosin-8-yl)-2-*AAF* moiety or dG$_p$-C$_8$-*AAF*. The formation of the diagnostic deprotonated anion of the nucleotide adduct dG$_p$-C$_8$-*AAF* at *m/z* 567 was observed. Expulsion of the sugar-phosphate moiety from the latter afforded the high abundance anion designated as [B-H]$^-$ at *m/z* 371, which is the guanine nucleobase-adduct also known as the [Gua-C$_8$-*AAF*]. This latter can lose a molecule of ketene to afford the [B-H-CH$_2$CO]$^-$ anion at *m/z* 329. This CAD MS experiment (CV=50) also permitted a bidirectional sequencing survey of these DNA-carcinogen adducts. Initial attempts to generate by low energy activated collision[38,39,42] MS/MS from the multiply-charged oligonucleotide adduct anions obtained from the various series of either the [mer-14(G*AAF*)$_n$] or the [mer-18(G*AAF*)$_n$] adducts failed, as the majority of these DNA adduct anions were electrosprayed as sodiated adducts. It was observed that sodiated adducts, in general, are resistant to fragmentation under normal CAD MS/MS conditions using

Table 3. Characteristic fragment ions obtained from the cone voltage fragmentation of the ESI-MS of [mer-14(G*AAF*)$_{n=1-4}$] adducts **6** to **21**.

Characteristic Ion	*m/z (%)*	Characteristic Ion	*m/z (%)*
$[w_2–2(AAF)]^{1-}$	674.98 (11)	$[dA]^{1-}$	312.29 (28)
$[pdG–C_8(AAF)]^{1-}$	567.31 (16)	$[dT]^{1-}$	303.47 (41)
$[y_{8-}–(AAF)]^{5-}$	489.44 (13)	$[dC]^{1-}$	288.25 (26)
$[y_{11}–4(AAF)]^{7-}$	481.28 (17)	$[p\text{-}s]^{1-}$	195.38 (41)
$[a_{10}–(GAAF)]^{6-}$	466.32 (20)	$[(p\text{-}s)–H_2O]^{1-}$	177.15 (100)
$[a_8–(AAF)]^{6-}$	392.03 (8)	$[Gua]^{1-}$	150.31 (33)
$[Gua–C_8\text{-}AAF]^{1-}$ or $[B–H]^-$	371.66 (56)	$[Ade]^{1-}$	134.18 (71)
$[a_6–(AAF)]^{5-}$	346.39 (24)	$[Thy]^{1-}$	124.84 (53)
$[B–H–CH_2CO]^{1-}$	329.40 (80)	$[Cyt]^{1-}$	110.15 (56)

quadrupole-hexapole-quadrupole MS/MS instruments. It appears that sodiated adducts stabilize the precursor anions impeding further fragmentation into product ions. However, CAD MS/MS experiments were conducted using the F^{8-} (*m/z* 796.72) and F^{7-} (*m/z* 910.68*)* multiply-charged deprotonated anions derived from the [mer-18(G*AAF*)$_4$+Na–H], **28**. The

MS/MS spectrum of the precursor F^{8-} anion (*m/z* 796.72) measured using a high collision energy (CE=70V) afforded *inter alia* the guanine nucleobase-adduct [Gua-C_8-*AAF*]– or [B-H]– anion (*m/z* 371) and its decomposition product, the $[B\text{-}H–CH_2CO]^-$ anion, (*m/z* 329). These preliminary CAD MS/MS experiments permitted only partial sequencing of this covalently linked [mer-18(G*AAF*)$_4$+Na–H] adduct and established the presence of guanine- nucleobase adduct. It is notable that some of the obtained diagnostic anions were identical to those described for the [mer-14(G*AAF*)$_n$] adducts in Table 3. Further work on the CAD MS/MS of non-sodiated [DNA-(G*AAF*)$_n$] adducts using CE-ESI is currently in progress and will be published elsewhere.

Preliminary investigation of this novel series of DNA adducts by negative ion ESI-MS followed by deconvolution of the spectrum demonstrated that these techniques facilitated

the characterization of the proposed structures. In addition, cone voltage fragmentation and tandem mass spectra of the multiply-charged deprotonated anions obtained from the [mer-18(G*AAF*)$_n$] adducts provided fingerprints which allowed the location of the aromatic modification and the partial sequencing of these synthetic, intact, covalently attached DNA adducts to be established. Indeed, the presence of the guanine nucleobase adduct [Gua-C_8-*AAF*]$^-$ anion (*m/z* 371) and its decomposition product anion [B-H–CH_2CO] (*m/z* 329) confirms the nature and type of the aromatic adduction of the d-guanosine residue of these [DNA-(G*AAF*)$_n$] adducts .

In conclusion, ESI-MS permitted the identification of the individual synthetic DNA adducts, and augurs well as a convenient technique for the characterization of intact DNA-oligomer adducts.

Acknowledgment

Joseph H. Banoub acknowledges the financial support of the Natural Sciences and Engineering Research Council of Canada in the form of a research grant.

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Received : 4 / 11 / 99
Accepted : 8 / 22 / 99