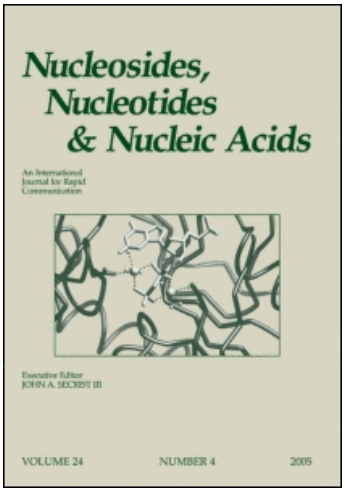

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ANALYSIS OF MODIFIED DEOXYNUCLEOSIDES BY ELECTROSPRAY IONIZATION MASS SPECTROMETRY.

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ABSTRACT

Electrospray mass spectra for selected modified deoxynucleosides and deoxynucleoside monophosphates have been determined. Protonated molecular ions are abundant in the positive ion spectrum, while $(M-H)^-$ appears in the negative ion spectrum. However, fragment ion intensities are usually low in both spectra. Conditions which promote collision-induced dissociation within the electrospray source facilitate fragment ion formation, and the intensity of BH_2^+ and S^+ (positive ion spectrum) and $(M-BH)^-$ and B^- (negative ion spectrum) are enhanced by increasing the skimmer cone voltage. MH^+ was detected with as little as 3 pmol of deoxynucleoside, and the protonated molecular ion intensity is linear with respect to analyte concentration over two orders of magnitude.

Electrospray ionization (ESI) mass spectrometry[1,2] is a sensitive technique for the analysis of nonvolatile compounds. Developed by Dole[3] and established by Fenn and coworkers[4,5] as an indispensable, new ion source for mass spectrometry, ESI is a soft ionization technique that forms predominantly molecular ions, and few fragment ions appear in the mass spectrum. It has been used extensively for the analysis of large biomolecules such as peptides,[6-12] oligonucleotides,[1,6,13] and oligosaccharides,[14-16] and spectra of these substances contain multiply charged ions formed by either addition of protons to the molecule (positive ion spectrum) or loss of protons (negative ion spectrum). Ions which are adducts with Na^+ and K^+ are also common when small amounts of these substances contaminate the sample. Factors affecting the distribution of charged states are not well understood, but

currently are under study.[17,18] Numerous multiply-charged molecular ions may be found in a single spectrum; hence, the precision of molecular weight determinations is excellent because of the large number of independent measurements.[19] Sensitivity for large biomolecules is good (pmol) and usually exceeds that found in FAB-MS.

Application of ESI to small, non-volatile molecules has been less extensive.[20] Electrospray has been used successfully for the analysis of esters of boron acids,[21] zwitterionic acylcarnitines,[22] and pyrimidine cyclobutane dimers.[23] Preliminary ESI results have also been reported for acetylaminofluorene adducts of 2′-deoxyguanosine[24] and alkylated nucleobases and deoxynucleosides.[25] Nucleoside adducts are formed in reactions of specific chemicals and free radicals with genetic material and are often excised from DNA by repair enzymes. Thus, sensitive analytical methods for the identification and quantitation of these substances may be important for the identification of disease states, monitoring drug efficacy, and investigating chemical carcinogenesis, mutagenesis, and the aging process. In the past, various mass spectrometric techniques have been used for analysis. Among those, gas chromatography coupled with mass spectrometry[26] has been the method of choice at the subnanogram level because of the high degree of selectivity and sensitivity. However, this procedure requires chemical conversion of enzymatically liberated nucleosides to volatile substances, and losses incurred during this procedure may be significant.

We have employed ESI mass spectrometry for the analysis of modified deoxynucleosides and nucleoside monophosphates, and conditions for collisional activation within the ESI source have been established. In addition, flow injection has been used to develop parameters for low-level detection and to establish the linearity of response for low concentrations of modified deoxynucleosides.

EXPERIMENTAL

Materials: 3,N^4-Ethano-2′-deoxycytidine **1**, 3,N^4-etheno-2′-deoxycytidine **2**, N^2,3-ethenoguanosine **3**, 5-hydroxymethyl-2′-deoxyuridine **4**, 8-oxo-2′-deoxynebularine **5**, 8-oxo-2′-deoxyadenosine **6**, 8-oxo-2′-deoxyinosine **7**, 8-oxo-2′-deoxyguanosine **8**, and 8-oxo-2′-deoxyxanthosine **9** were kindly provided by Dr. Francis Johnson (SUNY-Stony Brook, Stony Brook, NY). Chemical structures of the modified deoxy-

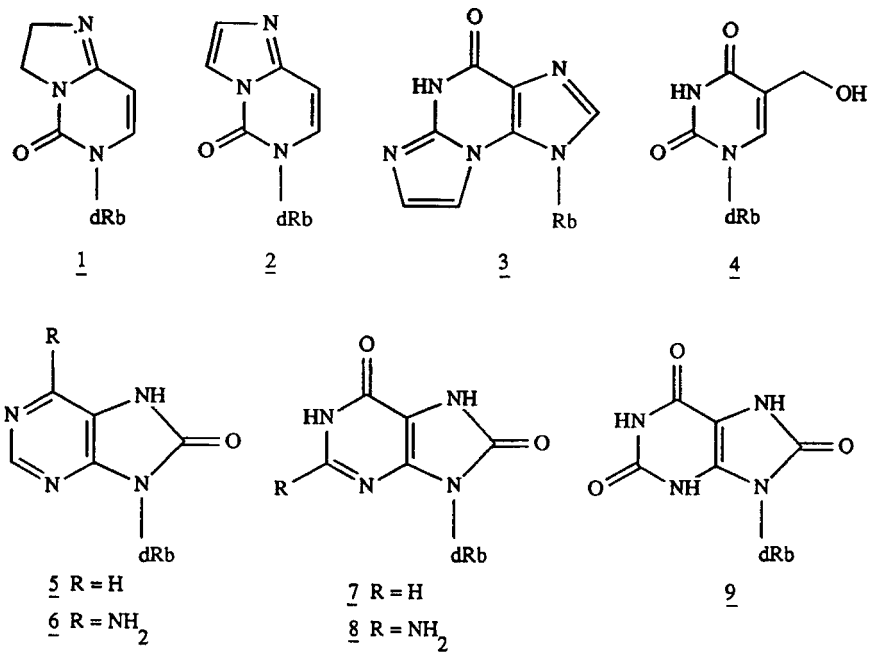

Figure 1. Chemical structures of modified deoxynucleosides.

nucleosides are shown in Figure 1. 2′-Deoxyadenosine **10**, 2′-deoxycytidine **11**, 2′-deoxyguanosine **12**, 2′-deoxyuridine **13**, thymidine **14**, adenosine 5′-monophosphate **15**, cytidine 5′-monophosphate **16**, 2′-deoxyadenosine 3′-monophosphate **17**, 2′-deoxyadenosine 5′-monophosphate **18**, 2′-deoxycytidine 5′-monophosphate **19**, 2′-deoxyguanosine 5′-monophosphate **20**, thymidine 3′-monophosphate **21**, and thymidine 5′-monophosphate **22** were obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile, ammonia and formic acid were from Fisher Scientific (Fairlawn, NJ).

Sample Preparation: Deoxynucleosides were dissolved in acetonitrile:water (50:50) containing 0.5% formic acid at a concentration of 15 pmol/μL for positive ion experiments. For negative ion electrospray deoxynucleosides and deoxynucleoside monophosphates were dissolved in either acetonitrile:water (50:50) or methanol:water (90:10) containing 1% ammonia in place of formic acid at a concentration of 150 pmol/μL. An aliquot (10 μL) of each sample was injected into a continuous flow of the same solvent entering the electrospray source.

Instrumentation: Mass spectra were acquired on a TRIO-2000 quadrupole mass spectrometer (Fisons Instruments, Danvers, MA) equipped with an electrospray ion source. Samples were introduced by flow injection at 6 μL/min using a syringe pump (Harvard Apparatus Inc., South Natick, MA) connected to a Reodyne injector with a 10 μL sample loop. The injector is connected to the stainless steel ion source capillary by 1 m of fused silica (75 μm ID). A capillary potential of 3-4 kV was applied to produce the spray. Nitrogen drying gas was maintained at 40 psi for positive ion electrospray and 20 psi for negative ion electrospray. Lens potentials were adjusted for maximum ion current. 2′-Deoxyadenosine and a deoxynucleotide trimer (5′-CTA-3′) were used to tune the instrument in the positive and negative ion modes, respectively. The instrument was scanned in the multichannel analyzer/acquisition mode (MCB) over the mass range (*m/z*) 100 to 600 with a 10 sec scan time. The mass range was calibrated in the positive ion mode with polyethylene glycol. For spectra acquired under conditions of enhanced collision-induced dissociation in the source, the skimmer cone voltage was increased from 20-30 V to 60-70 V. All mass spectra comprise an average of 10-12 scans with background substraction.

For determining the linearity of the MH^+ intensity versus analyte concentration, the instrument was tuned in the positive ion mode by injecting standards and adjusting source parameters to obtain a stable spray. Parameters were then left undisturbed throughout the experiment. Nine sets of samples (8-oxo-2′-deoxyadenosine) were prepared in concentrations ranging from 0.5-50 pmol/μL, each with an internal standard (2′-deoxyadenosine) concentration of 5 pmol/μL. A 10 μL aliquot was injected, and eleven scans were accumulated in the MCB mode of data acquisition over a narrow mass range (*m/z* 550-575) with a 5 sec scan time. Spectra were smoothed and plotted, and the peak height of ions at *m/z* 252 and 268 was used to plot a standard curve.

RESULTS AND DISCUSSION

Nine modified nucleosides (Figure 1) were analyzed by positive and negative electrospray ionization, and spectra were acquired on a single quadrupole mass spectrometer. Five nucleosides and eight nucleoside monophosphates (see Experi-

Table I. Positive ion relative intensities for electrospray mass spectra of modified deoxynucleosides.

Compound	MH^+	BH_2^+	S^+	Compound	MH^+	BH_2^+	S^+
1	254 (100)	138 (51)	117 (1)	8	284 (100)	168 (3)	
1[a]	254 (8)	138 (100)		8[a]	284 (27)	168 (100)	117 (11)
2	252 (100)	136 (7)		9	285 (100)	169 (28)	117 (18)
2[a]	252 (100)	136 (100)	117 (1)	9[a]	285 (100)	169 (100)	117 (98)
3	308 (100)	176 (50)		10	252 (100)	136 (4)	
3[a]	308 (8)	176 (100)		10[a]	252 (100)	136 (100)	117 (3)
4[b]	259 (100)	143 (37)	117 (2)	11[b]	228 (100)	112 (96)	
4[a,b]	259 (100)	143 (51)	117 (20)				
5	253 (100)	137 (17)		12	268 (100)	152 (59)	
5[a]	253 (15)	137 (100)	117 (1)	12[a]	268 (11)	152 (100)	117 (3)
6	268 (100)	152 (15)		13[b]	229 (100)	113 (21)	117 (36)
6[a]	268 (5)	152 (100)					
7	269 (100)			14	243 (100)	127 (49)	117 (3)
7[a]	269 (100)	153 (44)	117 (8)	14[a]	243 (33)	127 (100)	117 (21)

[a] Spectrum generated under conditions of enhanced collision induced dissociation.
[b] Protonated dimer also present in spectrum: 4 m/z 517 (38); 4[a] m/z 517 (12); 11 m/z 455 (6); 13 m/z 457 (19).

mental) were also analyzed for comparison purposes. Relative intensities of all ions observed in the analysis of compounds 1-14 are listed in Tables I and II for positive and negative ESI, respectively. Data were obtained for eight nucleoside monophosphates only in the negative ionization mode and are found in Table III.

Figure 2 shows the positive and negative ion ESI spectrum of 8-oxo-2′-deoxyadenosine (6). The principal ions observed in the positive ion spectrum are a protonated molecular ion MH^+, the base peak, and an intense fragment ion BH_2^+. This fragment ion, which forms as a result of protonation of the base and glycosidic bond cleavage with transfer of a hydrogen atom from the sugar, is also observed in deoxynucleoside mass spectra generated by thermospray[27] and fast atom bombardment.[28] A single ion $(M-H)^-$ is observed in the negative ion mass spectrum.

Table II. Negative ion relative intensities for the electrospray mass spectra of modified deoxynucleosides.

Compound	$(M-H)^-$	B^-	Compound	$(M-H)^-$	B^-
1	252 (100)		8	282 (100)	
2	250 (57)	134 (100)	9	283 (100)	
3	306 (100)		10	250 (100)	134 (37)
4	257 (100)	141 (22)	11	226 (100)	110 (35)
5	251 (100)		12	266 (100)	
6	266 (100)		13	227 (100)	111 (9)
7	267 (100)		14	241 (100)	
7[a]	267 (100)	177 (14)			

[a] Spectrum generated under conditions of enhanced collision-induced dissociation.

Table III. Negative ion intensities for the electrospray mass spectra of nucleoside monophosphates.

Compound	$(M-H)^-$	$(M-BH)^-$	B^-	Compound	$(M-H)^-$	$(M-BH)^-$	B^-
15	346 (100)			19	306 (100)		
15[a]	346 (100)	212 (5)		19[a]	306 (100)	195 (10)	111 (3)
16	322 (100)			20	346 (100)		
16[a]	322 (100)			20[a]	346 (100)	212 (4)	150 (9)
17	330 (100)			21	321 (100)		
17[a]	330 (100)	195 (68)		21[a]	321 (26)	195 (100)	126 (21)
18	330 (100)			22	321 (100)		
18[a]	330 (100)	195 (9)	134 (1)	22[a]	321 (100)	195 (26)	126 (11)

[a] Spectrum generated under conditions of enhanced collision-induced dissociation.

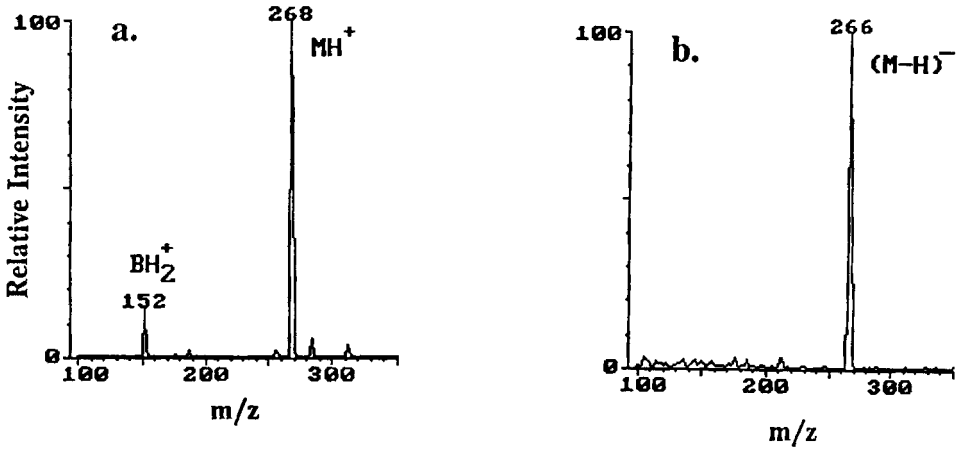

Figure 2. Electrospray mass spectra for 8-oxo-2′-deoxyadenosine; (**a**). positive ion spectrum, (**b**). negative ion spectrum.

Presumably, $(M-H)^-$ is formed by the abstraction of a proton from the molecule by a base (ammonia). A base fragment ion, B^-, analogous to BH_2^+, is not present.

The spectra of other deoxynucleoside adducts are found in Tables I and II. In general, the positive ion spectra contain a strong MH^+, and the BH_2^+ fragment is usually present. An ion S^+, representing the deoxyribose moiety, is not found consistently in the spectra, and no ions consisting of the base moiety and portions of the deoxyribose are present. In the case of 5-hydroxymethyl-2′-deoxyuridine (HMDU), 4, an intense protonated dimer $(2M+H)^+$ is present with MH^+ and BH_2^+ ions. The $(2M+H)^+$ is also observed in normal deoxynucleosides such as 2′-deoxyuridine and 2′-deoxycytidine. The $(M-H)^-$ dominates the negative ion spectrum except for 3,N^4-etheno-2′-deoxycytidine 2 and 5-hydroxymethyl-2′-deoxyuridine 4, which contain B^- ions as well; however, B^- is not observed routinely in negative ion spectra. In general, the sensitivity in the negative ion mode is less than that of the positive ion by a factor of 5-10. Both positive and negative ion spectra are free of adducts ions such as $(M+Na)^+$, $(M+Na-2H)^-$, or $(M+Cl)^-$. However, an equimolar addition of sodium acetate to the analyte suppressed the intensity of MH^+ and BH_2^+ in the positive ion spectrum and gave an intense ion corresponding to $(M+Na)^+$ (spectrum not shown).

Negative ion ESI spectra of eight nucleoside monophosphates were also recorded, including adenosine 5′-monophosphate **15**, cytidine 5′-monophosphate **16**, 2′-deoxyadenosine 3′-monophosphate **17**, 2′-deoxyadenosine 5′-monophosphate **18**, 2′-deoxycytidine 5′-monophosphate **19**, 2′-deoxyguanosine 5′-monophosphate **20**, thymidine 3′-monophosphate **21**, and thymidine 5′-monophosphate **22**. These compounds have intense $(M-H)^-$ under normal ionization conditions. Little fragmentation occurs, and $(M-BH)^-$ and B^- ions are not detected. However, the latter ions are detected easily under conditions of enhanced collision-induced dissociation. Data for these compounds are found in Table III.

Fragment ion intensities in ESI spectra can be enhanced by collision-induced dissociation in the ESI source by increasing ion energies in the high pressure region of the source to promote energetic collisions between ions and neutral molecules.[29,30] This effect was observed with modified nucleosides on the TRIO-2000 instrument by increasing the skimmer cone voltage over a range of 20-140 V. Nucleoside spectra obtained at high skimmer cone voltages contain a higher degree of fragmentation (Table I). In most spectra the deoxyribose ion (S^+) is detected only at these higher energies, and the relative intensities of MH^+ and BH_2^+ are reversed when compared to low energy spectra. Similarly, in the spectra of deoxynucleoside monophosphates, $(M-BH)^-$ and B^- are found only at elevated skimmer cone voltages (Table III). Figure 3 shows the relative intensities of MH^+ and BH_2^+ as a function of skimmer cone voltage for 2′-deoxyinosine. It is evident that MH^+ is most abundant at low cone voltages and maximizes at approximately 30 V. As the voltage is increased, MH^+ intensity decreases while that of the BH_2^+ fragment increases and passes through a broad

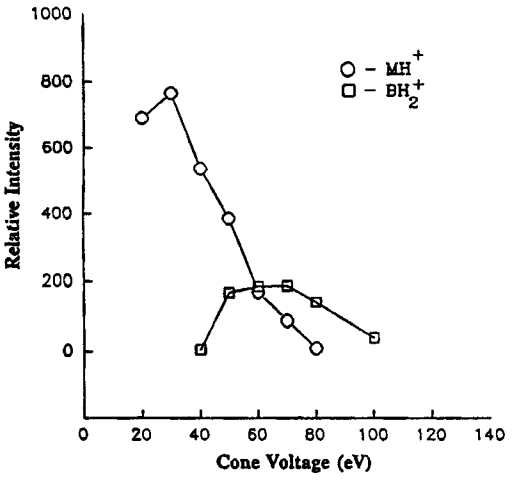

Figure 3. Effect of skimmer cone voltage on ion intensity in the ESI mass spectrum of 8-oxo-2′-deoxyinosine.

maximum at 60-70 V. Similar behavior was exhibited by the other nucleosides as well.

Recently, there has been considerable interest in the quantitation of modified nucleosides and nucleobases in damaged DNA[31-33]. Quantitative mass spectrometric methods not only can provide highly selective detection of modified nucleosides, but do so with extremely good sensitivity. ESI may provide a sensitive means of quantitation of intact nucleosides without chemical derivatization. Since the ESI spectrum of modified nucleosides affords mainly MH^+ with little or no fragmentation at low skimmer cone voltages, the technique might be used to considerable advantage because a high percentage of the total ion current exists in a single ion, namely MH^+. In view of this we have evaluated the linearity of the MH^+ ion signal as a function of analyte concentration for the ESI source using flow injection of nucleoside adducts. MH^+ from 8-oxo-2′-deoxyadenosine was selected to be monitored, while MH^+ from 2′-deoxyadenosine was used as the internal standard. Nine samples were prepared in the concentration range of 0.5-50 pmol/μL, each with an internal standard concentration of 5 pmol/μL. Approximately 4-5 μL of each sample was consumed during data acquisition, and a nine point calibration curve was constructed using the MH^+ ion intensity ratio of sample and internal standard versus the sample concentration (Figure 4). The curve is linear over the full range with a regression coefficient of 0.998. The lower limit of detection is less than 3 pmol of sample.

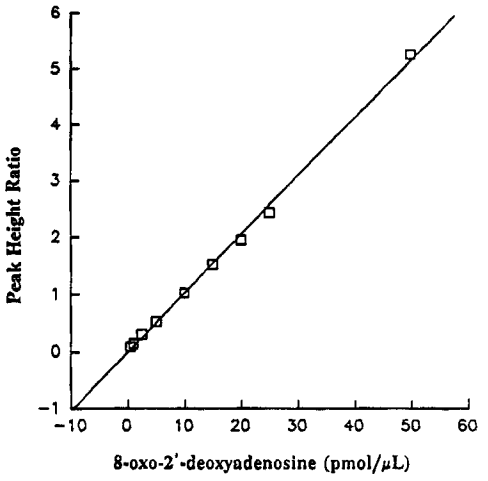

Figure 4. Measured MH^+ intensity for 8-oxo-2′-deoxyadenosine.

ESI mass spectra of modified nucleosides and nucleotides have been acquired from samples in the picomole range. These results show that basic structural information can be extracted from the data, including, for example, the nature of the

base and the sugar, and simple modifications of either or both moieties. In addition, collision-induced dissociation within the ESI source augments molecular ion fragmentation, reducing the need for MS/MS studies. The ESI source possesses excellent detection limits and displays a superb linear relationship between MH^+ intensity for modified deoxynucleosides and analyte concentration, suggesting that ESI may be effective for the quantitation of modified deoxynucleosides.

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