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**Comparison of collision-induced dissociation and electron-induced dissociation of singly
charged mononucleotides**

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Abstract

The dissociation of singly charged mononucleotides by electron induced dissociation (EID) and sustained off resonance irradiation/collision induced dissociation (SORI-CID) was investigated. The experiments have been carried out on deprotonated mononucleotides monomers. In general the same product ions are observed in the EID and SORI-CID spectra but some additional product ions have been obtained with EID. It was shown by MS³ experiments that all these additional product ions have been produced through consecutive dissociations of the [M-2H]⁻ radical anion. In addition, H/D exchange and sequential MS³ experiments demonstrated that the *m/z* 247 fragment ion produced in EID of deprotonated 2'-deoxyguanosine 5'-monophosphate corresponds to the association of the phosphate group and the nucleobase. They were most likely linked by a non-covalent bond that was initially present in the precursor ion.

Introduction

The decomposition of oligonucleotide even electron ions has been successfully carried out using several activation methods such as CID [1-3] and IRMPD [4]. The obtained activation spectra present similar fragment ions because they are based on ergodic processes involving vibrational excitation [5, 6]. Under such activation conditions, mostly charge driven processes take place from even-electron precursor ions. Consequently, the orientation of the fragmentations strongly depends on the proton affinity of each functional group in the charged molecular species [7]. A few years ago, activation methods involving radical driven pathways have been developed such as ECD (electron-capture dissociation) [8-10]. In ECD, multiply charged positive ions can capture one or several electrons. Such process is exothermic (5-7 eV) [11] due to the charge state decrease and generates a radical cation. The distonic charge reduced species consecutively yields covalent bond cleavages (similar or not to CID) with enhanced dissociation rate constants. ECD as well as ETD [12] is widely used for characterization of macromolecules. However, this method is rarely applied for DNA, because they are generally analyzed by mass spectrometry in the negative ion mode. More recently electron-photo-detachment (EPD) [13, 14] and electron-detachment-dissociation (EDD) [15-17] have been used for multiply deprotonated DNA. The electron detachment (electron stripping) processes require electron kinetic energy higher than 20 eV. EDD was successfully applied for multi-deprotonated deoxyoligonucleotides providing complementary fragment ions compared to those formed with vibrational excitation methods [18, 19]. For EDD, the electron irradiation duration (*i.e.*, several seconds) is significantly longer than for ECD (typically below 100 ms) [16, 20, 21]. In particular, it was shown that electron-induced dissociation (EID) occurred concomitantly with purely EDD processes [19, 21]. As EDD, EID is performed with relatively high-kinetic-energy electrons but can be used with

negatively or positively charged singly charged species [22-26]. O'Hair *et al*, have compared CID and EID of singly protonated aromatic amino acid [27]. They observed that EID yielded quite different and complementary fragmentations compared to those provided by low-energy CID. Zubarev *et al* [28] investigated activation of peptide ions by electron ionization dissociation. Compared to CID, they observed some different fragment ions due to distonic structures.

In our previous work [21], sequential EDD/SORI-CID MS³ and double resonance-EDD (DR-EDD) experiments allowed to clarify the origin of the observed fragment ions produced by either EDD or EID [21]. These results showed in particular that EDD processes are very random along the sequence, whereas EID processes yield mostly nucleobase dependent cleavages.

In the present work we are interested in the comparison of the dissociation of deprotonated 2'-deoxynucleotide-5'-monophosphate by EID and CID. The dissociation of 2'-deoxynucleotide-5'-monophosphate was already investigated by CID [29-31] and more recently, by in vacuum photodissociation [32-34] and APPI [35]. Habibi-Goudarzi and McLuckey, investigated the low-energy CID of 2'-deoxynucleotide-3' and -5'-monophosphate [29]. They demonstrated that the base loss is significantly favored by the presence of a phosphate group at the 3' position. On the other hand, both the nucleobase acidity and basicity seem to play an important role. EID results in more decomposition pathways than SORI-CID. In particular, the EID spectra display some odd-electron fragment ions not detected in the corresponding SORI-CID spectra. Our principal aim was to obtain a better mechanistic understanding on the EID processes. Sequential MS³ experiments, double resonance and H/D exchanges were also performed to clarify some mechanisms. By this way, our results suggested that fragment ions remained as ion-dipole complexes, and their

formation involve the cleavage of covalent bonds although non covalent bond is maintained intact.

Experimental

Sample preparation

All mononucleotides were purchased from Sigma-Aldrich Chemicals (St Quentin Fallavier, France) and used without further purification. Sample solutions in water/methanol 80/20 have been prepared using deionized water (18.2 M Ω) and HPLC grade methanol. The final concentration of the mononucleotide solutions was 20 μ M.

Mass spectrometry

All experiments were conducted on an actively shielded 7 T hybrid quadrupole Fourier transform ion cyclotron resonance (hQh-FT/ICR) mass spectrometer (ApexQe, Bruker Daltonics, Billerica, USA). The 2'-deoxynucleotide-5'-monophosphate solutions were infused in an Apollo II electrospray ion source at a flow rate of 135 μ L h⁻¹ with the assistance of N₂ nebulizing gas. Ionization was performed in the negative mode with an ESI high voltage of 3500 V. Voltage applied in the desolvation zone were; capillary exit -300 V; skimmer -15 V; and skimmer II -8 V. For EID, the ECD lens was set to 20 V and the ECD bias was set to 13 V. Sustained off-resonance irradiation collision-induced dissociations (SORI-CID) were performed using Argon as collision gas introduced using a pulse valve. The ions were excited using excitation amplitude of 3.4 V_{p-p} with a frequency offset of 600 Hz applied for 250 ms. A pumping delay of 2 s was used before the excitation/detection step. All mass spectra and activation spectra were acquired with XMASS (version 6.1, Bruker Daltonics) in broadband mode from m/z 50 to m/z 500. The image signal was amplified and

digitized using 512 K data point resulting in the recording of a 0.47 s time domain which was transformed into the corresponding frequency domain by Fourier transform (one zero fill and Sinbell apodization). All ESI mass spectra were internally calibrated from unambiguous signals. Reported m/z ions were compared to the theoretical m/z and ions with an error higher than 5 ppm were not considered. IRMPD was performed with a 25 W CO₂ laser (Synrad, Mukilteo, WA, USA) operating at 10.6 μm . Photon irradiation energy was set to 50 % of full laser power. The irradiation time was varied from 50 to 80 ms.

Because of the relatively low efficiency of the EID process, the relative abundance values were determined by excluding the abundance of the precursor ion.

Results and discussion

SORI-CID and EID spectra of 2'-deoxynucleotide-5'-monophosphate

Table 1 (Figure 1s) displayed the relative intensities of product ions produced from deprotonated 2'-deoxynucleotide-5'-monophosphates in SORI-CID and EID modes. The SORI-CID and EID spectra present similar fragment ions. In all cases, the main fragment ions are $[(\text{M}-\text{H})-\text{BH}]^-$ produced through the loss of nucleobase. The complementary fragment ions (the deprotonated nucleobases (B^-)) are observed in the SORI-CID spectra of $[\text{dpC}-\text{H}]^-$ (m/z 306), $[\text{dpU}-\text{H}]^-$ (m/z 307), $[\text{dpA}-\text{H}]^-$ (m/z 330). The formation mechanism of these fragment ions has been studied in detail by Rodger et al [36] who proposed that both the $[(\text{M}-\text{H})-\text{BH}]^-$ and B^- complementary ions are considered to be produced by an intramolecular E2-type mechanism from the deprotonated structure. Actually, this process is a *syn*-elimination, promoted by the negative charge, in which the deprotonated 5'-phosphate site attacks the hydrogen atom at 2' β C (i.e. the β face of the deoxyribose ring) since this reaction is sterically

more favorable. It should be noted that, the E₂ elimination requires that the 2'C-hydrogen and the leaving group are antiperiplanar [7], which is sterically not possible with the studied system. This could explain the higher energy threshold observed for base released from 2'-deoxynucleotide-5'-monophosphate than that observed from the 2'-deoxynucleotide-3'-monophosphate [7]. An alternative mechanism can be proposed by considering that the dissociation of the deprotonated 2'-deoxynucleotide-5'-monophosphate occurs from a population of anions with zwitterion (ZW) structures (*i.e.*, two negative charges and one positive charge) rather than a canonic form. During the desolvation, in the charged aggregate, the proton can be transferred to the nucleobase. Such a protonation should not occur with thymine and uracil, because of their respective low basicity [37]. Consequently, the competitive formation of U⁻ and [(dpU-H)-UH]⁻ should not be observed. But both these complementary ion species are detected at *m/z* 111 at *m/z* 195, respectively (Table 2). Therefore, the mechanism involving ZW as an intermediate form must be ruled out. So, to observe deprotonated uracil (and competitively, its neutral form), an assistance mechanism is required. By considering a stepwise process, it can be assumed that a proton transfer from the 2_βC site to the deprotonated phosphate group takes place to assist the deprotonated base release. From this process (Figure 2), formation of an anion-dipole ([[(dpU-H)-B], B⁻) complex occurs with an unsaturation at the C₂-C₁ position. During the second step, an intra ion-dipole proton transfer takes place from the phosphate group to the deprotonated uracil. Thus, the gas phase acidity of the nucleobase must play a role in the evolution of the ([[(M-H)-BH]⁻]+[(M-H)-BH-H₂O]⁻)/[B⁻] ratio. From our results with SORI-CID this ion abundance ratios evolves as: dpG>dpA>dpU>dpC. This evolution suggests comparing only purines (G and A bases) and pyrimidines (U and C bases). Indeed, it seems necessary to separate these two classes of 2'-deoxynucleotide-5'-monophosphates, for such comparison. The role played

by the acidity is not the only one because the excitation energy effect influences strongly this ratio as shown in the work of Kebarle [30].

A similar trend is observed in EID mode. However, the consecutive losses of [BH+H₂O] are reinforced whereas the B⁻ ion decrease partially for the purine bases and disappears for pyrimidine bases. Note that the consecutive losses reflected higher internal energy on the deprotonated 2'-deoxynucleotide-5'-monophosphate. Nevertheless, from Kebarle's study, it should be expected enhanced B⁻ anion abundances with higher internal energy which is not observed from our results, since the B⁻ abundance decreased (or disappeared) [30].

The loss of H₂O from the [(dpX-H)-XH]⁻ (X=C, U, A or G) fragment ions was observed from the deprotonated 2'-deoxynucleotide-5'-monophosphate. McLuckey et al [7] proposed that this arises from the hydroxyl loss at 3'_αC and the proton at 4'_αC. Minor contributions are observed for H₂PO₄⁻ (*m/z* 97), the formation of this ion is explained by an E2 type mechanism in which the phosphate alkoxide attacks the 4'_αC-H of the sugar while the phosphate ester linkage oxygen becomes the anion leaving group. The abundance of H₂PO₄⁻ ion in EID spectra is generally greater than the one in SORI-CID spectra. This trend cannot be explained only by considering the acidity and the activation mode (SORI-CID vs EID). Most likely, it also depends on the initial conformation of the precursor ion. Interestingly the fragment ion [M-H-H₃PO₄]⁻ was not detected while the H₂PO₄⁻ ion is detected. This indicates that the acidity of H₃PO₄ is much higher than that of the [M-H₃PO₄] neutral, and therefore that the lifetime of the [H₂PO₄, (M-H₃PO₄)]⁻ ion dipole complex is too short to allow proton exchange. The loss of 43 u from the precursor ions corresponds to CONH *via* a retro Diels-Alder like mechanism [7]. The 2'-deoxyguanosine-5'-monophosphate presenting particular behavior, it will be discussed latter.

Compared to the SORI-CID, the EID spectra of the deprotonated 2'-deoxynucleotide-5'-monophosphates displayed more information. The H₂O loss is also detected with larger abundance in the EID spectra. Under EID conditions, additional fragment ions such as sugar cross-ring cleavages are significantly more abundant than those detected in the SORI-CID spectra [38]. These fragment ions are very useful for determining the deoxyribose substituent location. These cleavages are mainly observed using keV range collisions as well as from multiply charged oligonucleotide by ECD and EDD [39-41]. Beside the loss of nucleobase and the formation of the H₂PO₄⁻ fragment ion, the EID spectra displayed in several cases radical fragment ions. The formation of these radical ions will be discussed in detail with the deprotonated 2'-deoxyguanosine-5'-monophosphate (*vide infra*).

SORI-CID and EID spectra of 2'-deoxyguanosine-5'-monophosphate

The SORI-CID spectrum of deprotonated 2'-deoxyguanosine-5'-monophosphate (m/z 346) is reported in Figure 3a. The main fragment ions were detected at m/z 195 corresponding to the loss of the nucleobase (GH) and deprotonated guanine (G⁻, m/z 150). The EID spectrum of deprotonated 2'-deoxyguanosine-5'-monophosphate (Figure 3b) displays the m/z 195 ion as main fragment ion. Its consecutive water loss yielding m/z 177 is produced in a similar abundance in the other EID spectra. The m/z 166 and m/z 139 fragment ions were identified as odd electron species that were produced from the rupture of the ribose ring (^{1,4}A and ^{0,3}A). Interestingly additional ions are detected at m/z 247 and m/z 149 which were not observed in CID and on other nucleotide EID spectra. The latter is attributed to [G-H]^{-•} radical anion (as confirmed by its elemental composition C₅H₃N₃O^{-•} from the accurate mass measurements). The non-radical deprotonated base expected at m/z 150 (C₅H₄N₃O⁻) is detected with very low abundance. The m/z 149 ion, C₅H₃N₃O^{-•}, odd-electron anion corresponds to dehydrogenated guanine. Its formation could formally involve the release of one H[•] from the m/z 150

deprotonated guanine. In fact, the formation of such odd-electron $[B-H]^{\bullet}$ species has been already considered for explaining some fragment ions generated through particular pathways under EID conditions [27]. The following equation may explain the origin of such charged radical species using fast electron bombarding (*i.e.*, electron kinetic energy of about 20 eV) which yields very likely vertical electronic excitation resulting into vibrational energy in the excited electronic level rather than vibrational energy in the fundamental electronic level:



This process can be considered rather than a stepwise pathway formally proposed in the literature for positive ion $[M+H]^+$ which is described by an electronic excitation yielding electron stripping followed by capture of the produced slow electron [27]. In fact, both pathways are similar formally since they assumed, an electronic excited state; the electron can migrate to different sites.

In the EID spectra of the deprotonated 2'-deoxyguanosine-5'-monophosphate (Figure 3b), the dehydrogenated nucleobase radical anion $[G-H]^{\bullet-}$ is produced as the product ion base peak. This means that its formation is favored and this oxidized species is stable toward further dissociations. Different structures could be proposed (Figure 4) by considering formation of distonic forms (*e.g.*, the *a* and *a_{di}* series calculations are in progress) [42].

Note that the guanine is the nucleic base characterized by the highest electron affinity [43] and the previous observation of the *m/z* 149 ion suggests also by analogy that the $C_5H_3N_5O$ neutral must be characterized by a higher EA value than that of the other nucleic bases [44].

The second additional peak at *m/z* 247 with the $C_5H_6N_5O_5P^{\bullet-}$ elemental composition, is intriguing because the formation of this radical anion involves, formally the loss of the ribose residue radical (*i.e.*, $C_5H_5O_3^{\bullet}$). The existence of this ion implies that both the phosphate and

guanine moieties are maintained together. Two hypotheses can be considered to rationalize the formation of the m/z 247 ion:

(i) either the even-electron precursor ion in its excited electronic state isomerizes into one particularly stable ion-dipole complex allowing the oxidized ribose residue radical $C_5H_5O_3^{\bullet}$ release. This could take place by a stepwise process, which could be initiated by the homolytic cleavage of the C-C linkage between the sugar moiety and the nucleic base.

(ii) or according to a similar molecular isomerization pathway, the complex which, instead of yielding an ion-dipole complex after the sugar residue release, results into a covalent system in which the nucleic base and the phosphate are bound.

Independently of the previous assumption, the produced m/z 247 ion must be an odd-electron species in a distonic form. This will be discussed later (*vide infra*).

H/D exchange

H/D exchange experiments were conducted using CH_3OD solution (Figure 5). The precursor ion at m/z 346 was shifted to m/z 351 consistent with 5 H/D exchanges. The m/z 247 fragment ion was shifted to m/z 252 indicating the presence of 5 deuterons. The oxidized guanine [B-H] $^{\bullet}$ radical anion was detected at m/z 151 (the guanine radical anion without H/D exchange was detected at m/z 149). This indicates that two labile deuterium remained on this radical ion. This is consistent with the structures proposed in Figure 4.

Sequential MS³ experiments

Figure 6 displayed the sequential MS³ experiments from the deprotonated 2'-deoxyguanosine-5'-monophosphate, (EID (m/z 346)/IRMPD (m/z 247)). The EID/IRMPD spectrum displayed only the m/z 149 fragment ion corresponding to the radical nucleobase, demonstrating that the radical nucleobase was produced consecutively from the ion-complex species (m/z 247). Double resonance (DR-IRMPD) experiments with variable excitation energy on the m/z 247 ion was carried out (data not reported), but the relative abundance of the m/z 149 ion remained unchanged [45]. This indicates that the ion-dipole complex is dissociated consecutively with a too high rate constant to observe the DR effect. In this case, the decomposition can be faster than the time of ejection. Bowers and co-workers [43] have proposed that the conformation of 2'-deoxyguanosine-5'-monophosphate was in a *syn* conformation in which the phosphate group is linked to the nucleobase (guanine) by a intramolecular non covalent bond. Our experiments demonstrated that this non covalent bond was preserved during EID of [dpG-H]⁻ (*e.g.* m/z 247 fragment ion (Figure 7)); this behavior presents some analogy to ECD that can allow the cleavage of covalent bond without affecting labile groups (*e.g.* phosphate group) and preserved non-covalent bonds [46, 47].

Sequential MS³ experiments on [dpG-2H]⁻

Interestingly, the H[•] loss from the precursor ion was produced in significant amount; such radical release was also reported recently [48]. This H[•] loss was proposed to be the initial step for the formation of fragment ions through radical driven pathways [27, 48]. To demonstrate this suggestion, sequential MS³ experiments have been carried out. In this case, the double resonance experiment cannot be concomitantly applied with EID, because the [M-2H]⁻ ion (m/z 345) that should be ejected is too close to the [M-H]⁻ precursor ion (m/z 346). The Figure 8a displayed the isolation spectrum of [dpG-2H]⁻, this showed an effective isolation to

perform the sequential MS³ experiment. In the EID/IRMPD MS³ spectrum (Figure 8b), the *m/z* 247 ion was detected as well as the guanine radical ion (*m/z* 149). Interestingly, the [M-GH-2H]^{•-} ion (*m/z* 194) was observed. This contrasts with the EID spectrum in which both the [M-GH-2H]^{•-} and [M-GH-H]⁻ ions (*m/z* 194 and *m/z* 195) were detected. The fragment ion at *m/z* 166 (^{1,4}A) in this sequential MS³ spectrum, identified as a radical fragment ion (C₄H₇O₅P^{•-}), is also detected. So this experiment confirmed that the fragment ions characteristic of the EID mode are produced through the consecutive dissociation of the unexpected [M-2H]^{•-} ion. In the H/D exchange experiment (Figure 5) the *m/z* 247 ion was shifted to *m/z* 252, indicating the introduction of 5 deuterons; this means that the H[•] loss from the precursor ion during EID activation is not one of the exchangeable protons.

Conclusion

The EID spectra displayed more fragment ions than the SORI-CID spectra but all fragment ions observed in the SORI-CID spectra are also detected in the EID spectra. In SORI-CID, the fragmentations from vibrationally activated species yielded mainly charge driven fragmentations, while in EID, radical driven decomposition pathways took place. The principal difference between SORI-CID and EID is the formation of odd-electron fragment ions produced only in EID. Interestingly, cross ring cleavages were detected in the EID spectra which are useful for ribose substituent location. These product ions are in particular very important for location of the substituent in the ribose. Sequential MS³ experiment (EID/IRMPD) demonstrated the stepwise process via formation of a stable ion-dipole complex, consistent with the initial precursor ion gas phase conformation, likely due to the formation of intra molecular salt bridge (calculations are in progress). This demonstrated that non covalent bonds can be preserved in EID. Sequential MS³ (EID/IRMPD) spectrum of

[dpG-2H]⁺ evidenced that all the fragments specific of the EID mode were produced consecutively to the loss of H⁺ from the [dpG-H]⁺ precursor ion. The EID mode appears to be a useful tool to investigate molecular structure and non-covalent interactions.

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Figure Captions

Figure 1: Structure of a) 2'-deoxycytidine-5'-monophosphate, b) 2'-deoxyuridine-5'-monophosphate, c) 2'-deoxyadenosine-5'-monophosphate, d) 2'-deoxyguanosine-5'-monophosphate.

Figure 2:a) Mechanism of 2'-deoxynucleotide-5'-monophosphate fragmentation [36], b) Nomenclature for cross-ring cleavages [38].

Figure 3: Activation spectra of deprotonated 2'-deoxyguanosine-5'-monophosphate (m/z 346) recorded in the a) SORI-CID and b) EID modes.

Figure 4: Different structures proposed for radical guanine anions [42]

Figure 5: EID spectrum of $[\text{dpG}_{\text{d6-D}}]^-$ (m/z 351).

Figure 6: MS^3 (EID/IRMPD) experiment of m/z 247 for deprotonated 2'-deoxyguanosine-5'-monophosphate.

Figure 7: Proposed structure for m/z 247 ion-dipole complex.

Figure 8: a) Isolation spectrum of $[\text{dpG-2H}]^-$ (m/z 345) after EID activation of m/z 346, b) MS^3 spectrum (EID/IRMPD) of $[\text{dpG-2H}]^-$ (m/z 345).

Table 1. Relative abundance of fragment ions produced in the SORI-CID and EID spectra of deprotonated 2'-deoxynucleotide-5'-monophosphate (radical fragment ions are in bold).

	dpC		dpU		dpA		dpG	
	m/z	CID(EID)	m/z	CID(EID)	m/z	CID(EID)	m/z	CID(EID)
[M-H-43] ⁻	263	9 (0)						
[(M-H)-BH] ⁻	195	64 (39)	195	79 (43)	195	54 (43)	195	58 (31)
[(M-2H)-BH] ⁻	194							0(3)
[(M-H)-BH-H ₂ O] ⁻	177	18 (29)	177	15 (25)	177	21 (41)	177	6 (1)
^{1,4} A ⁻			166	0 (8)			166	0 (5)
^{2,4} A ⁻			136	0 (7)				
B ⁻	110	5 (0)	111	7 (0)	134	24 (16)	149	32 (5)
[B-H] ⁻								0(3)
H ₂ PO ₄ ⁻	97	3 (32)	97	0 (17)	97	1 (0)	0 (0)	0 (0)
[M-H- C ₅ H ₇ O ₂] ⁻								0(16)
R ^(a)		16		13.5		3 (5)		2 (2.5)

^(a)R=([(M-H)-BH]⁻+[(M-H)-BH-H₂O]⁻)/[B⁻]

Table 2: Elemental composition of fragment ions observed in the SORI-CID and EID spectra of 2'-deoxynucleotide-5'-monophosphate.

Exact mass (u)	Elemental composition	Error (ppm)	mononucleotides	Comment
96.9696	H ₂ O ₄ P ⁻	0	All mononucleotides	Phosphate group
110.0360	C ₄ H ₄ N ₃ O ⁻	5	dpU	Uridine
111.0200	C ₄ H ₃ N ₂ O ₂ ⁻	1.7	dpC	cytosine
134.0472	C ₅ H ₄ N ₅ ⁻	1.5	dpA	adenosine
135.9931	C ₃ H ₅ O ₄ P ⁻	0.7	dpU	Cleavage ribose (^{2,4} A)
138.9802	C ₂ H ₄ O ₅ P ⁻	3	dpC, dpG	Cleavage ribose (^{0,3} A)
149.0343	C ₅ H ₃ N ₅ O ⁻	1.4	dpG	Radical guanosine
150.0421	C ₅ H ₄ N ₅ O ⁻	1.6	dpG	Guanosine
150.9802	C ₃ H ₄ O ₅ P ⁻	7	dpU, dpC, dpA	unknown
166.0037	C ₄ H ₇ O ₅ P ⁻	2.5	dpC, dpU, dpG	ribosecleavage(^{1,4} A)
176.9958	C ₅ H ₆ O ₅ P ⁻	0	All mononucleotides	Nucleobase and H ₂ O loss
195.0064	C ₅ H ₈ O ₆ P ⁻	0	All mononucleotides	Nucleobaseloss
247.0112	C ₅ H ₆ N ₅ O ₅ P ⁻	2	dpG	Phosphate group + guanosine

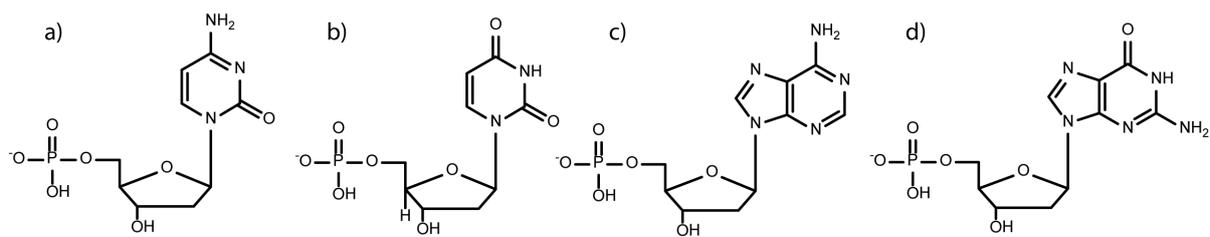


Figure 1

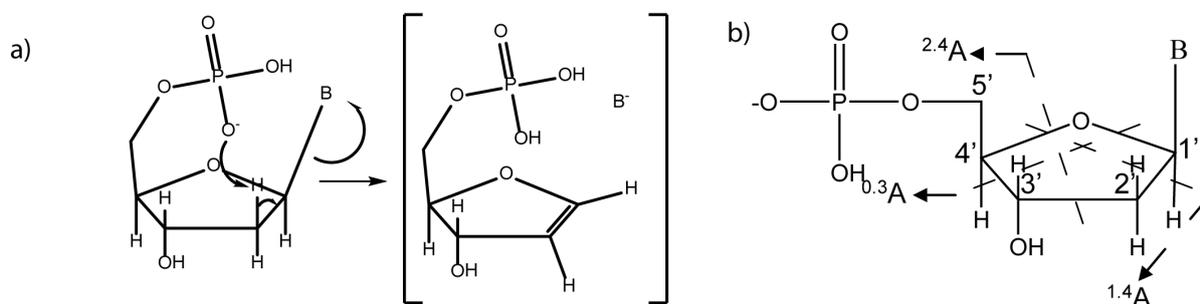


Figure 2

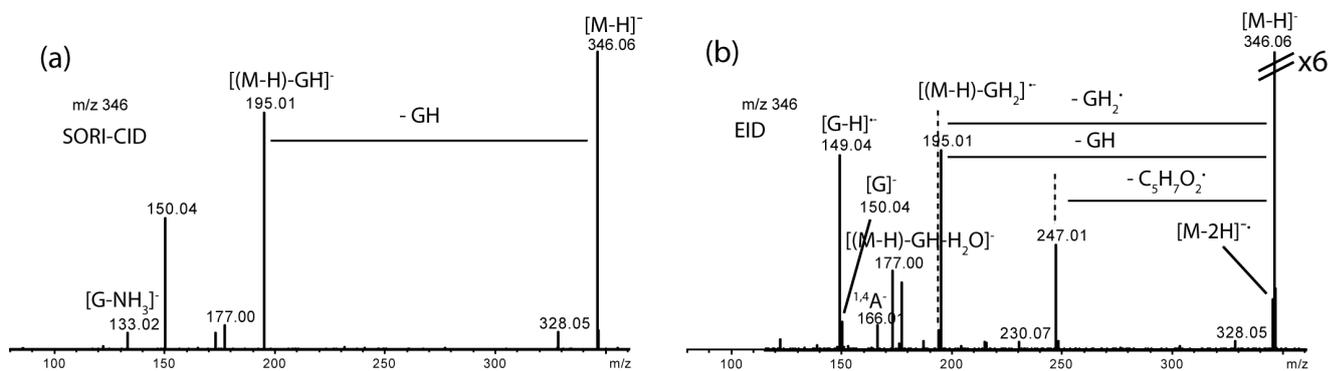


Figure 3

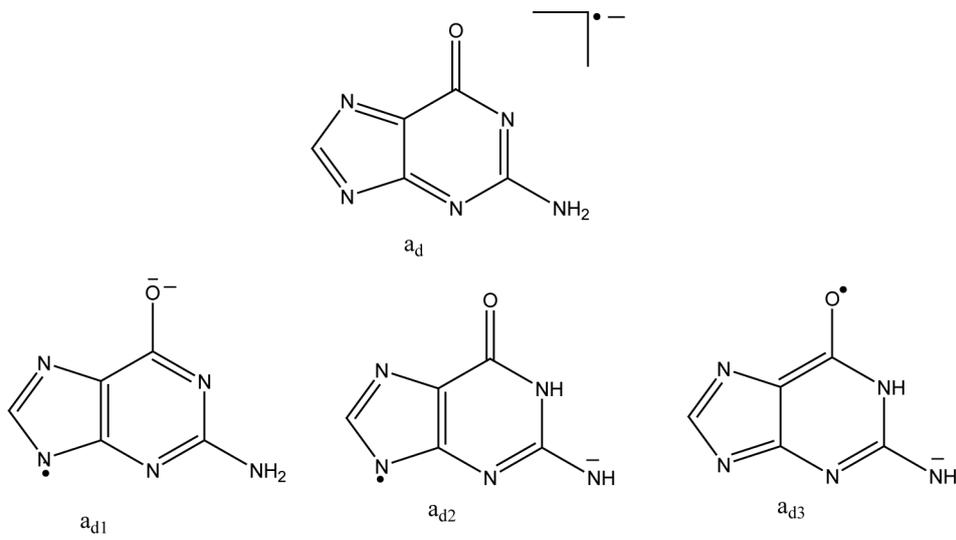


Figure 4

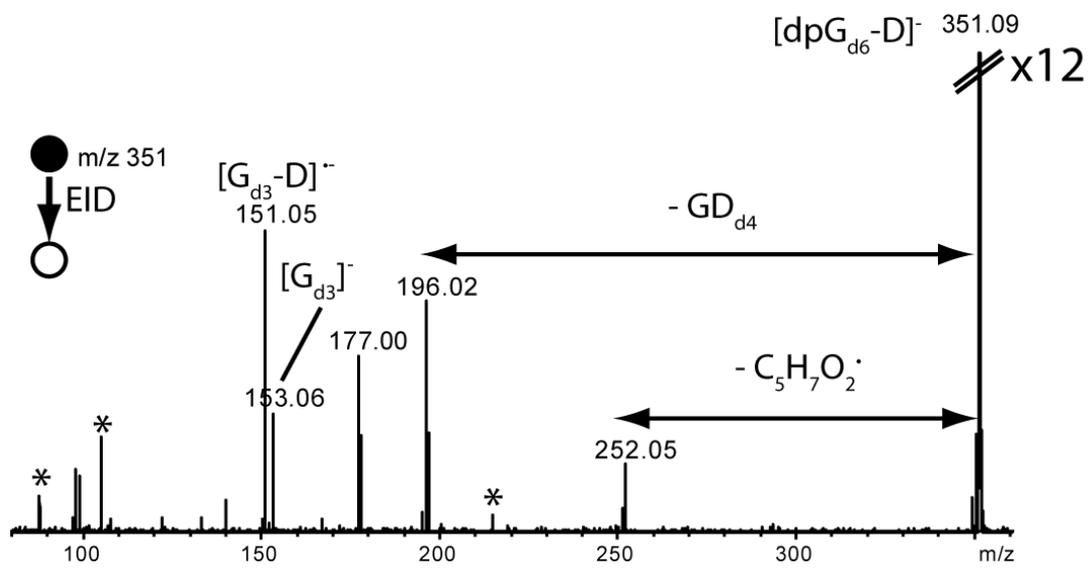


Figure 5

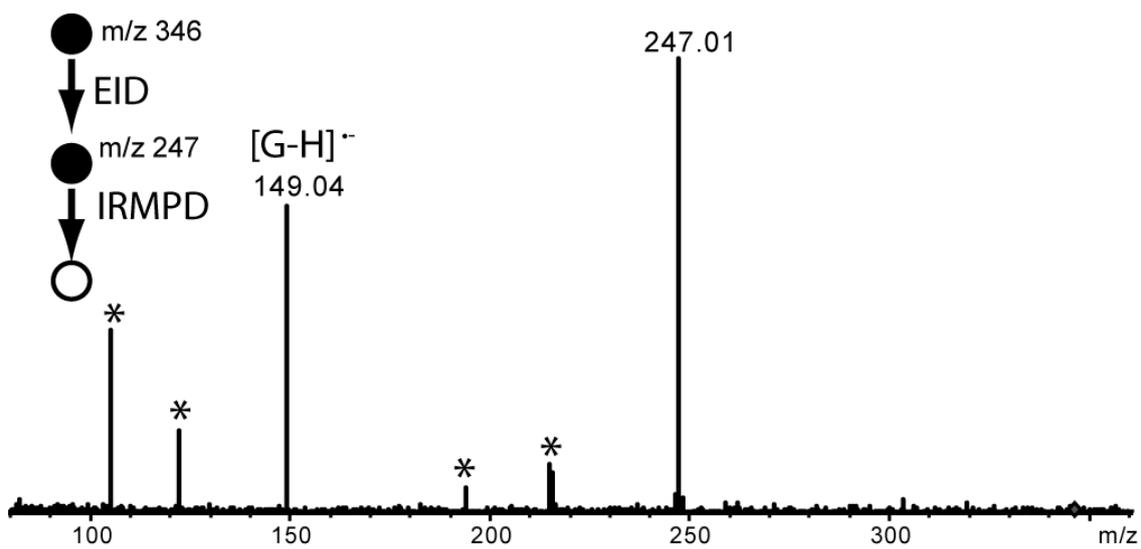


Figure 6

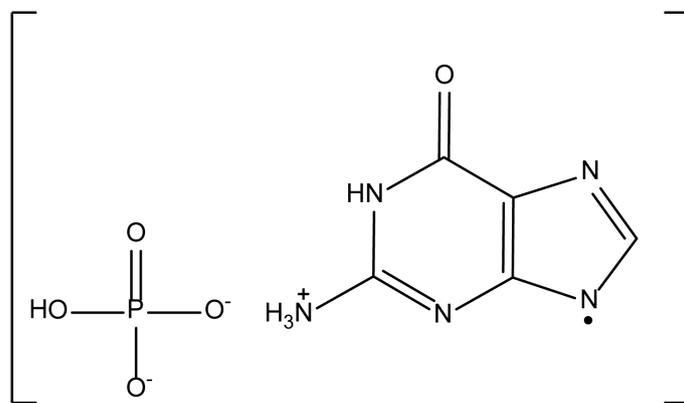


Figure 7

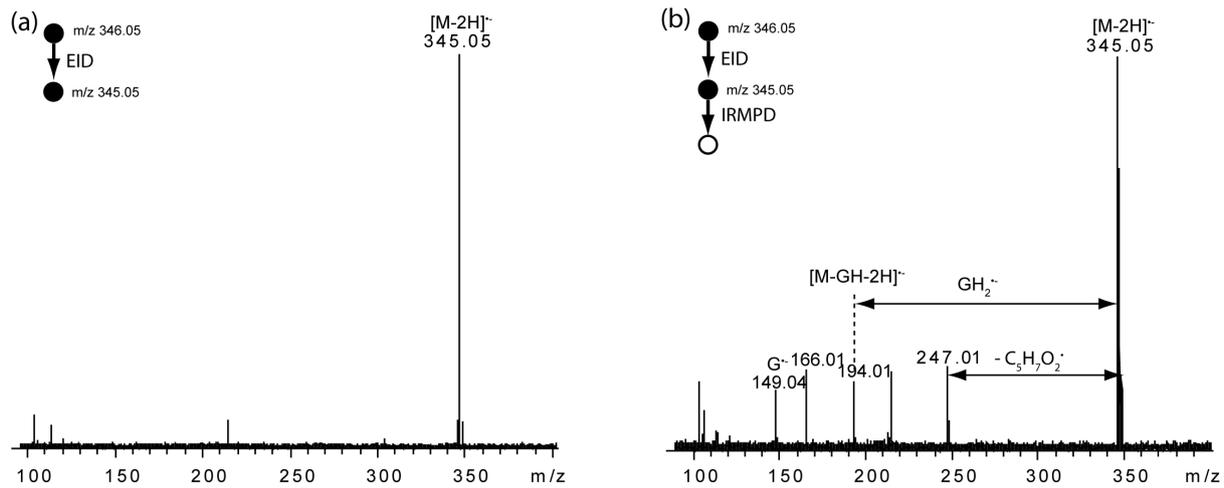


Figure 8