Participation of the Nucleobases in the Regioselective Backbone Fragmentation of Nucleic Acids. A Molecular Dynamics and Tandem Mass Spectrometric Investigation on a Model Dinucleoside Phosphotriester

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The anions (**I–III**) obtained from *O*-methyl 5′-*O*-(5′-deoxythymidine) 3′-*O*-(2′,3′-dideoxyuridine) phosphate by the competitive removal of the 3-*N*-H protons of the nucleobases and of the methyl group from the phosphotriester bond, assume in the gas phase stable conformations as a function of their charge site. The mass-analyzed ion kinetic energy (MIKE) spectra of **I** and **III** show that the regioselective backbone cleavage of the internucleotidic linkage is controlled by the 2′-H proton transfer to the nucleobase within the 5′-end nucleoside. Similar pathways are taken by species **II** when the nucleobase is eliminated as neutral from the 5′-end nucleoside. (J Am Soc Mass Spectrom 1997, 8, 1257–1261) © 1997 American Society for Mass Spectrometry

The combined use of molecular dynamics (MD) [1], for the designing of appropriate dinucleotide models, and of tandem mass spectrometry (MS/MS) [2, 3], for the investigation of the chemistry of their singly charged anions sputtered by fast atom bombardment (FAB) [4], provides evidence on the mechanism for the competitive dissociations of the phosphate bonds from the 3′ and 5′ termini of a nucleic acid backbone.

Electrospray ionization [5] permits accurate molecular weight (M_r) determination [6] in the 40-kDa range, attomole sensitivity [7], and sequence information [8], which give mass spectrometry a fundamental role in nucleic acid chemistry [9–11], especially when modified molecules are investigated [12].

A mass spectrum of a natural nucleotide or oligonucleotide displays peaks due to competitive glycosyl and internucleotidic bond breakage, regardless the complexity of the investigated species and the ionization method used. Both processes usually require a prototropic shift and cannot be considered as simple bond cleavage reactions.

The gas-phase chemistry of the $[M–H]^-$ ions of dinucleotide models lacking both 5′ and 3′ hydroxyl groups should allow the evaluation of the fragmentation mechanism of the phosphate bond in the absence of fast exchangeable protons. The model mimics to some extent the "chemical environment" experienced by a given dinucleotide moiety within a DNA strand, in this case, in fact, no "acidic protons" are available at the 5′- and 3′-ends.

The sequential fragmentation of the polyanion backbone of gaseous DNA strands seems to be unaffected by the charge status of the oligomer [8], whereas it seems that the formation of an "apurinic" site by elimination of a nucleobase can induce, by analogy with chemical degradation methods [13], the fragmentation of the phosphate bond from the 3′-end [14]. The base elimination process has been widely documented for single-charged nucleosides [15–17] and dinucleotides [18–20], and many mechanisms have been suggested for a similar reaction path taken by longer multiple charged oligomers [8, 14].

The observation that the base-driven sequencing of single stranded DNA can occur remotely from the charge site [8], has prompted the investigation of the gas-phase chemistry of appropriate dinucleotide models. The dinucleotide anions **I–III**, lacking free hydroxyl groups at both 5′- and 3′-ends, facilitates the evaluation of the role of a localized charge in the heterolysis of the phosphotriester group. The difference of 14 Da between the two nucleobases allows an easy distinction between

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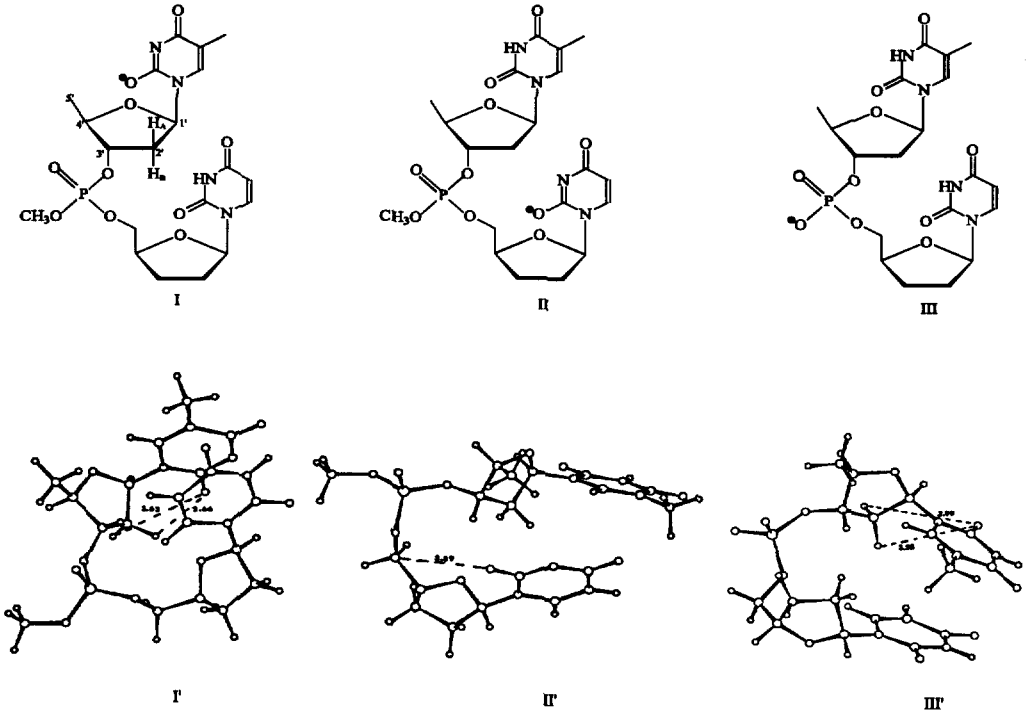


Figure 1. Anions **I–III** are obtained by fast atom bombardment from *O*-methyl 5′-*O*-(5′-deoxythymidine) 3′-*O*-(2′,3′-dideoxyuridine) phosphate in glycerol matrix. The gas-phase conformations **I′–III′** are simulated with the DISCOVER program from standard fragment geometries of **I–III**. **I′** and **III′** are obtained by energy minimization; **II′** corresponds to the most stable conformation of anion **II** when the uracil moiety is fixed in its *endo* position.

fragments originating from similar breakdown processes, i.e., glycosyl and/or internucleotidic bond dissociations. Moreover, the similarity in their electronic [21, 22] and geometric [23] parameters and their nearly identical pK_as [24] ensure that the ionization from both 5′-end and 3′-end nucleobases causes similar energy deposition into the resulting ions. The species **I–III** can be produced from the corresponding *O* methyl 3′-*O*-(5′-deoxythymidine) 5′-*O*-(2′,3′-dideoxyuridine) phosphate (**1**) by FAB-induced ionization in glycerol matrix. **I** and **II** are formed by a competitive proton abstraction from the acidic sites of the molecule (i.e., the 3-NH functions of both thymine and uracil residues [24]). The anion **III** is delivered into the gas phase after a well documented transmethylation process occurring in the selvedge [25–27].

The synthesis of **1** was accomplished by the phosphoroamidite approach [28], from the appropriate commercially available modified nucleosides. Energy minimization and molecular dynamics were performed on **I–III** with the DISCOVER program, as an application within Biosyn's graphical molecular modeling interface, Insight II [29]. The systems were generated in their first configuration by using standard fragment geometries, whereas the CVFF force Field [1] has been employed in all simulations. The atoms, in MD simulations, follow a trajectory according to Newton's laws of motion, where the mobility is determined by the temperature (or kinetic energy) of the system.

The most stable conformations **I′** and **III′** achieved by **I** and **III**, at the temperature of 300 K (Figure 1), show that, regardless the localization of the charge, the distances between the 2-O and 2′-H_A atoms are 2.66 and 3.53 Å and those between 2-O and 2′-H_B are 3.62 and 3.99 Å, respectively. These ground state values are, at least for the species **I′**, well within the 3.10–4.00 range of a C–H–O hydrogen bond [30] and suggest that a 2′-H_A can be abstracted by the nucleobase through a favorable six-membered cyclic transition state. When the MD calculations were performed at higher temperatures (up to 600 K) both the distances between the interacting atoms and the potential energy of the conformer did not change significantly. Therefore the conformation **I′** is located in a potential well in the diagram of the total strain energy versus conformational changes of the ion.

The conformation **II′** was obtained by freezing the rotation along the glycosyl bond of the 3′-end nucleoside and fixing the uracil base in its *endo* position. The observed distance between the 2-O of uracil and the 5′-C carbon of the 3′-end residue is 3.89 Å. When no restrictions are imposed, a totally different conformer is obtained where the 2-O/5′-C distance becomes 6.39 Å and the uracil base is in its more stable *exo* conformation. The calculated difference in the total strain energy content of the *endo* versus *exo* conformers is approximately +0.45 eV.

The chemistry of **I–III** was investigated by MS/MS, detecting the product ions formed from each single

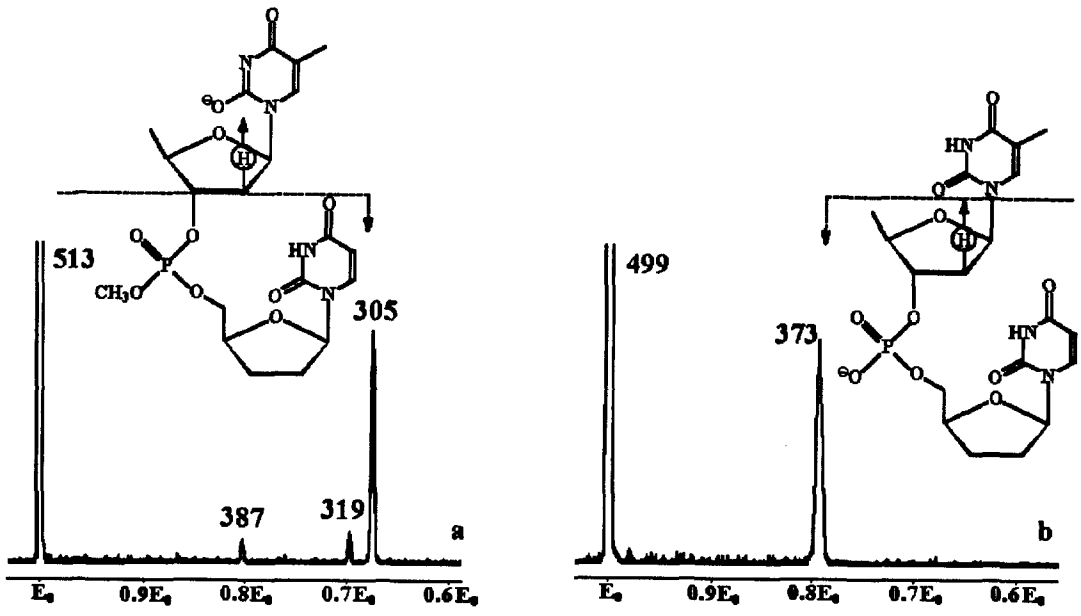


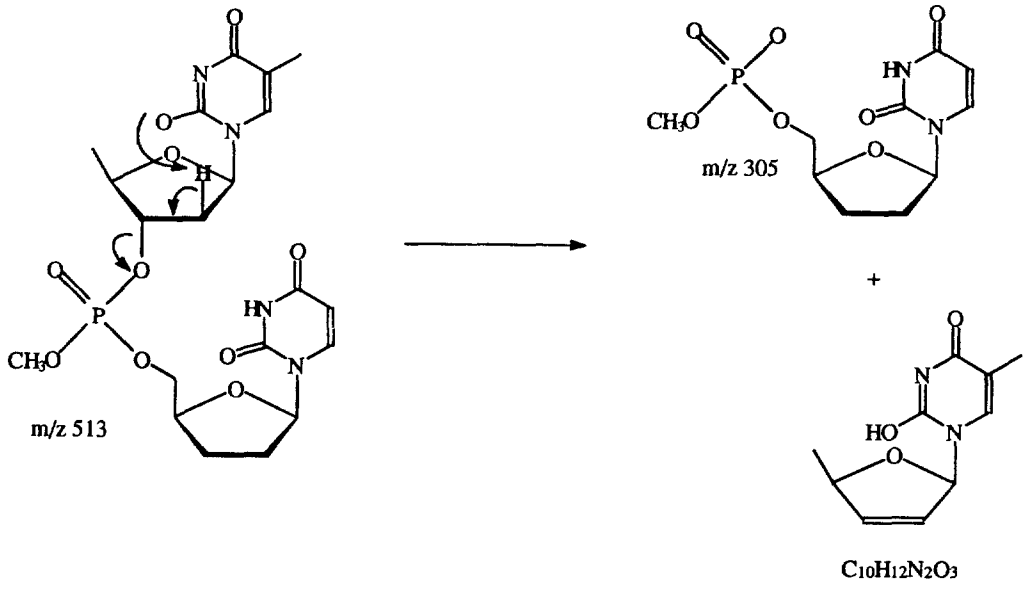
Figure 2. MIKE spectra of the $[M-H]^-$ (**A**) and $[M-CH_3]^-$ (**B**) ions obtained on a (VG-Micromass) ZAB-2F spectrometer from 2 μL glycerol solutions of *O*-methyl 5′-*O*-(5′-deoxythymidine) 3′-*O*-(2′,3′-dideoxyuridine) phosphate.

precursor in a collision chamber ($P \cong 10^{-7}$ torr) preceding the electrostatic sector of a *B-E* type mass spectrometer [3] (MIKE spectra, Figure 2). **I** and **II** are isobaric ions at m/z 513 that cannot be distinguished by the methodology but both can be formed in the ionization process. Their product ions, however, can be selectively detected at different mass-to-charge values, as a consequence of the differentiation of the nucleobases. The product ions of the m/z 513 precursor are mainly formed by a regioselective fragmentation of the phosphotriester bonds. The dissociation of the internucleotidic linkage from the 3′-end causes the formation O-methyl 5′-(2′,3′-dideoxyuridine) monophosphate (m/z 305, 86%; scheme), whereas the less competitive fragmentation path produces O-methyl 3′-(5′-deoxythymidine) monophosphate (m/z 319, 9%). The side process leading to the formation of the m/z 387 ion (5%) by releasing thymine as a neutral provides additional data for the understanding of the reaction paths involving the nucleobase.

On the basis of the MD calculations previously reported it is reasonable to suggest that the sequencing of the phosphotriester bond from the 3′-end is driven by a β-elimination process that involves the 2-O basic site of the 5′-end nucleobase and the 2′-H_A proton, with concomitant release of the good leaving group, here a phosphodiester anion. The most important aspect of the proposed mechanism is represented by the breakage of the phosphate link, which for these model systems does not require any proton transfer from the sugar to the leaving phosphate moiety [31]. The formation of the m/z 387 can occur from the precursor ion at m/z 513 in its reacting configuration **II′**, through a charge remote fragmentation which involves the 2′-H_A proton and leads to the elimination of the thymine residue with 5% relative abundance. The same process accounts for the formation of the species at m/z 373 from the lowest excited parent (**III**) at m/z 499 (Figure 2B). The 2-O/2′H_A distance (3.55 Å, Figure 1) in its most stable conformer **III′**, calculated at 300 K does not change significantly when the temperature is raised up to 600 K. However, by analogy with the MD calculations performed on ion **II**, different higher energy conformers can be obtained by fixing the distance of the two interacting atoms at given values. The simulation shows that when the 2′-H_A and 2-O atoms are fixed at 1.85 Å only, which represents the minimum value before atom repulsion, the 2′-H_B/2-O distance becomes 3.22 Å and the total strain energy of the conformer is 1.96 eV higher than that of **III′**. This values suggest that the reacting configuration for the elimination of the 5′-end nucleobase from the anion **III**, according to the proposed mechanism, can be easily achieved. The 1,2-elimination requiring the 2′-H_A transfer to the adjacent nucleobase, already proposed in the early application of tandem mass spectrometry to dinucleotide anions [32], closely resembles the formation of enol-ethers from acetals in the condensed phase [33, 34]. The data discussed so far show that the sequencing of the model dinucleotide can be described by two charge-driven competing processes (scheme, eqs 1 and 2)

$$[\text{dinucleotide-H}]^- \rightarrow {}^-O_2(CH_3O)PO\text{-}(2',3'\text{-dideoxyuridine}) \quad (1)$$

$$[\text{dinucleotide-H}]^- \rightarrow (5'\text{-deoxythymidine})\text{-}OP(CH_3O)O_2^- \quad (2)$$



Scheme I

The transfer of the 2′-H_A proton to the 5′-end nucleobase (eq 1) is a low activation energy process that favors the heterolysis of the phosphate bond at its 3′-end. The competing process (eq 2) involves the isomeric species **II**, in the reacting configuration **II′**, where the fragmentation reaction occurs at the 5′-end and may be driven by an intramolecular nucleophilic displacement reaction. The preferential formation of the 5′-monophosphate fragment cannot be due to differences in the pK_as of the nucleobases [24] or to different stabilization of the anions through the formation of intramolecular hydrogen bonds that might occur if the 5′-end nucleoside were a natural thymidine residue.

We ascribe the observed regiochemistry to differences in the critical energies associated with the two competing processes. Assuming that the most stable conformer of ion **II** has the ionized base in its *exo* position, the breakage of the internucleotidic bond requires, according to the proposed mechanism, a conformational change along the reaction coordinate that keeps the uracil base in its *endo* position. The difference in the total energy strain of the two rotamers (~0.45 eV) can be consider the minimum energy to attain the transition state configuration for the formation of the 5′-end fragment. The evaluation of the lowest activated fragmentation processes of the parent species at *m/z* 513 (MIKE spectra, Figure 2) ensures that the range of internal energies of the reactants falls within 0.1 to 1.0 eV above the threshold for their unimolecular dissociations [35]. In this experimental conditions it is possible to discriminate between two competing processes of similar critical energy.

From the theoretical and experimental data presented here we conclude that the nucleobase at the 5′-end of a given sequence can be involved in the fragmentation of the internucleotidic bond from its 3′-end and that the elimination of the base can simply occur through the facile transfer of the *syn* 2′-H_A proton. Finally, when $(M–H)^-$ anions are formed from longer oligomeric strands by MALDI [36], protonated nucleobases may occur within a "zwitterionic" structure [37] of this multiply charged gaseous species. The preferential release of nucleobases with the higher proton affinity values [21, 22] may occur through a similar reaction path, as shown for the depurination of protonated nucleosides [16].

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