Bio-organic Applications of Mass Spectrometry. Part 6.[1] Selective Deprotection of Nucleotides by Fast Atom Bombardment Mass Spectrometry

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The fully protected nucleoside *S*-methyl *O*-2,4-dichlorophenyl phosphorothioates (**2**) and (**3**) undergo selective removal of the protecting groups when exposed, in liquid matrices, to 9.5 KeV Xe bombardment. Dimethoxytrityl and 9-phenylxanthen-9-yl cations are formed when the reactivity of the protonated species is examined, while solvolytic removal of the 2,4-dichlorophenyl protecting group afforded the nucleosides phosphodiester anions (**6**) and (**8**) as a result of matrix–substrate interaction in the energized condensed phase. The same substrates are competitively demethylated at the sulphur atom when thioglycerol is present in the solvation sphere of the reactants. Anions (**6**) and (**8**), desorbed into the gas phase by fast atom bombardment of their triethylammonium salts, displayed the same metastable ion kinetic energy spectra as those produced from nucleotides (**2**) and (**3**) by applying the same methodology.

Fast atom bombardment (FAB)[2] has been introduced as a powerful methodology in structure determination, by mass spectrometry (MS), of biologically important molecules such as peptides[3] and oligonucleotides.[4–6] The experimental conditions suitable for an FAB experiment also allow microscale monitoring of the reaction intermediates in peptide[7] and oligonucleotide chemistry.[8,9]

The success of FAB in analysing large polar molecules has overshaded to some extent the chemistry of the solvated reactants exposed to keV atom beams.[1] It has been clearly established that the matrices used in desorption ionization may act as reagents,[10] while the FWHM values[11] of fragment and molecular ions suggest that the fragment ions are not predominantly formed by unimolecular gas-phase decompositions.[11] Experimental evidence shows that halogenated nucleosides[12] and dinucleoside *O*-2-chlorophenyl phosphates[13] exchange their halogen atoms with hydrogen radicals from the matrix. Carnitine is demethylated at the ammonium moiety[1] by the nucleophilic action of glycerol, while thioglycerol, used as matrix, displaces chlorine from Ir and Rh complexes.[14] Nucleoside *S*-methyl phosphorothioates are important intermediates in the synthesis of oligodeoxyribonucleotide chains bearing terminal phosphate groups[15,16] as well as in the formation of chiral dinucleoside *S*-methyl phosphorothioates.[17,18] These thermally labile molecules are interesting substrates for FABMS since the reactivity of the polar groups, eventually involved in Brønsted-type equilibria, is reduced by the presence of the masking functions, while the population of Lewis-type equilibria could be favoured by the presence of good leaving groups.

Results and Discussion

O-2,4-Dichlorophenyl *S*-methyl phosphorochloridothioate (**1**) reacts with suitably protected thymidine and cytidine to give the building blocks (**2**) and (**3**) (Scheme 1), where the dimethoxytrityl (DMT) and 9-phenylxanthen-9-yl (pixyl, Px)[19] and 2,4-dichlorophenyl groups are masking functions easily removable by hydrolytic treatment at appropriate pH values.[17,19]

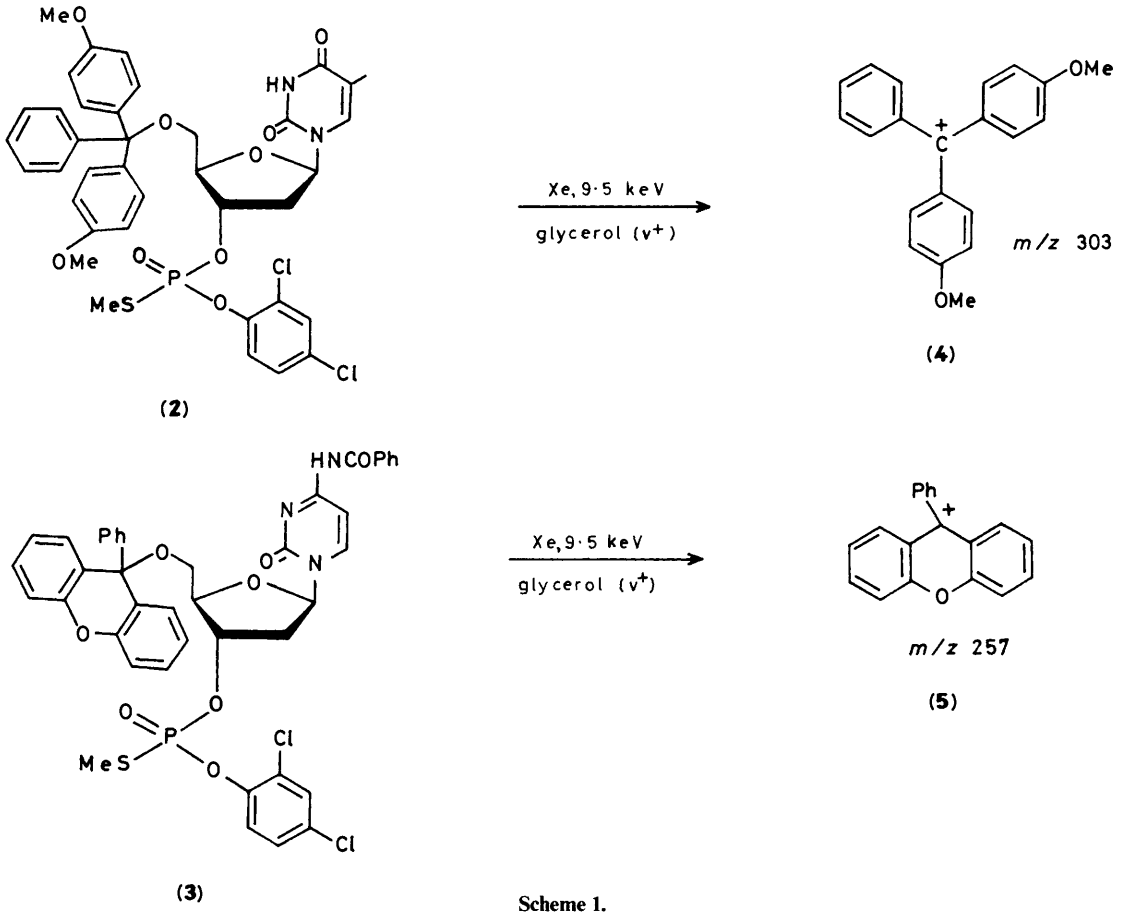
† ^{37}Cl Isotopic species are not reported in the text.

‡ G/T Values are calculated by dividing the absolute intensity of the $(M - H)^-$ peak of glycerol (m/z 91) by that of thioglycerol (m/z 107), within a given spectrum.

The positive ion spectra (v^+) of compounds (**2**) and (**3**) displayed peaks at m/z 303 and 257 respectively, which correspond to the very stable DMT (**4**) and Px (**5**) cations, while no $(M + H)^+$ or other protonated species, apart from those assignable to the matrix background, were detected. The formation of (**4**) and (**5**) can be interpreted in terms of gas-phase heterolytic cleavage of the 5-ether bonds of the very reactive protonated molecular ions produced from (**2**) and (**3**). However, since the half-lives, in acidic medium, of species similar to those here examined are in the order of 1—2 min,[19] it cannot be excluded that (**4**) and (**5**) are formed in the condensed phase as well, if the lifetimes of the protonated species initially formed on the matrix are of the same order of magnitude as the time required for the sputtering process.[1,20] From the v^+ spectra the behaviour of the conjugated acids of mononucleotides (**2**) and (**3**) both in conventional solution chemistry and under FAB conditions appears to be similar (Scheme 1).

Both the structure and the relative yield of the anions detected from the spectra of negative ions (v^-) are strongly affected by the experimental conditions. When compound (**2**), dissolved in glycerol (A), 1:1 glycerol–thioglycerol (B), and diethanolamine (C) was exposed to a 9.5 keV Xe atom beam, the relative intensity of the $(M - H)^-$ deprotonated molecular ion was almost negligible. The v^- spectra obtained in experimental conditions A and C do not show significant differences: they were characterized by the presence of abundant m/z 653 anions, which do not contain chlorine, and of m/z 351, 125, and 161 fragments, the latter being present in the spectra obtained in conditions (C) only (Figure 1).

The experiments carried out in mixture (B) gave rise to v^- spectra deeply affected by the concentration of the reactants. The most striking differences were represented by the relative intensities of the m/z 653 and 783 peaks† which were 1.3 and 3 at G/T values‡ of 1.7 and 0.85 respectively. The species at m/z 783 showed the typical pattern of dichlorinated ions and were not formed in experiments (A) and (C). The phosphodiesters (**6**) and (**7**) (Scheme 2) are the most probable nucleotides which correspond to the anionic species reported above. Their formation, in the gas phase, from the conjugated base of (**2**) at m/z 797 would require the elimination of 144 and 14 mass units which formally correspond to 2,4-dichlorobenzyne and methylene respectively. The critical energy associated with these hypothetical unimolecular processes should not be in the range of the internal energies available in gaseous molecular anions



Scheme 1.

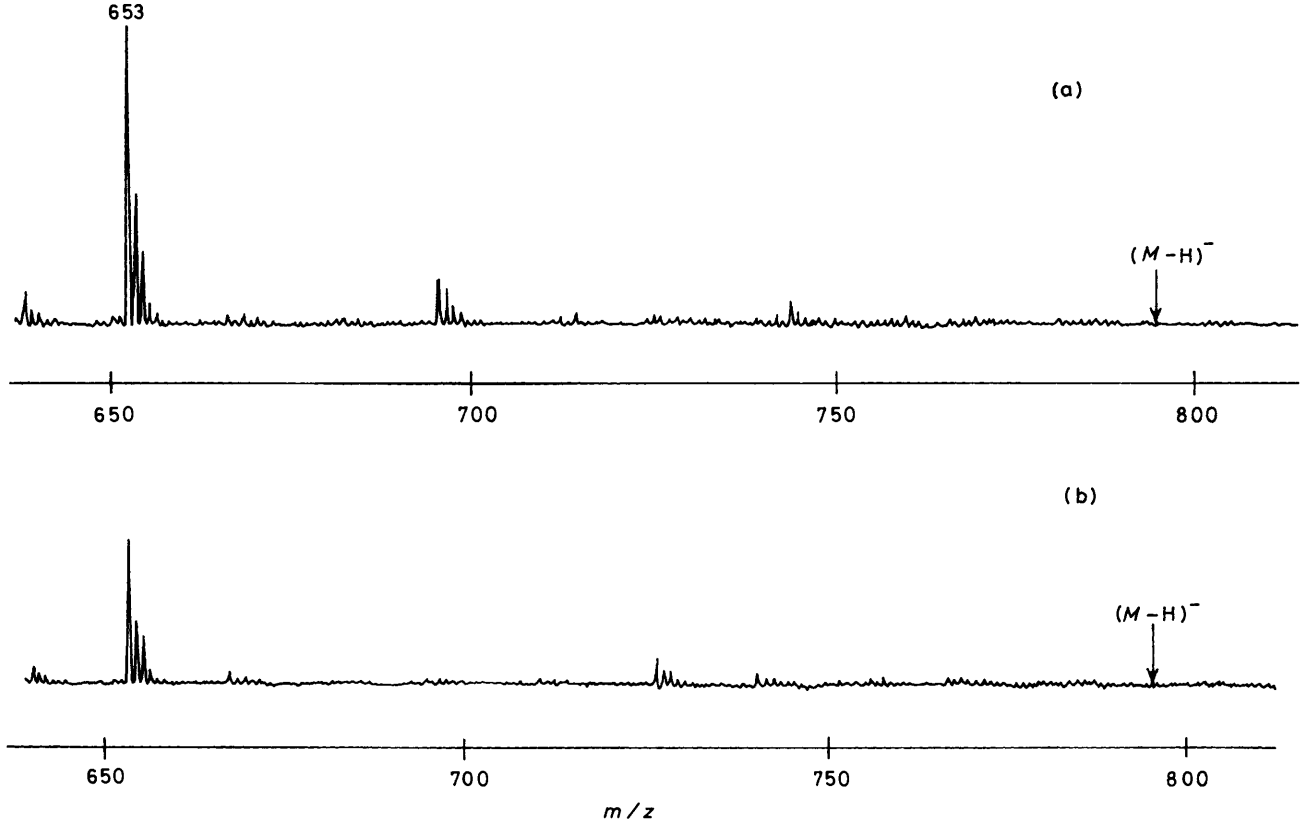
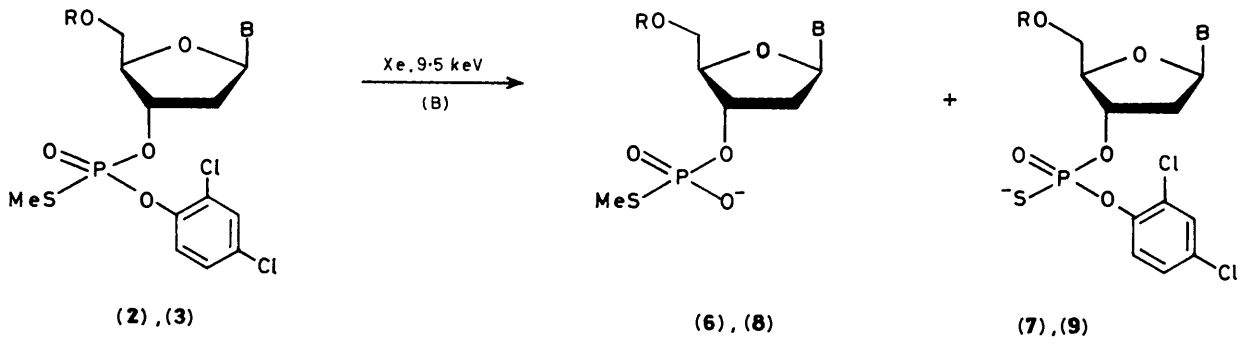


Figure 1. v^- FAB spectra of compound (**2**) (high-mass region): (a) glycerol; (b) diethanolamine



Scheme 2.

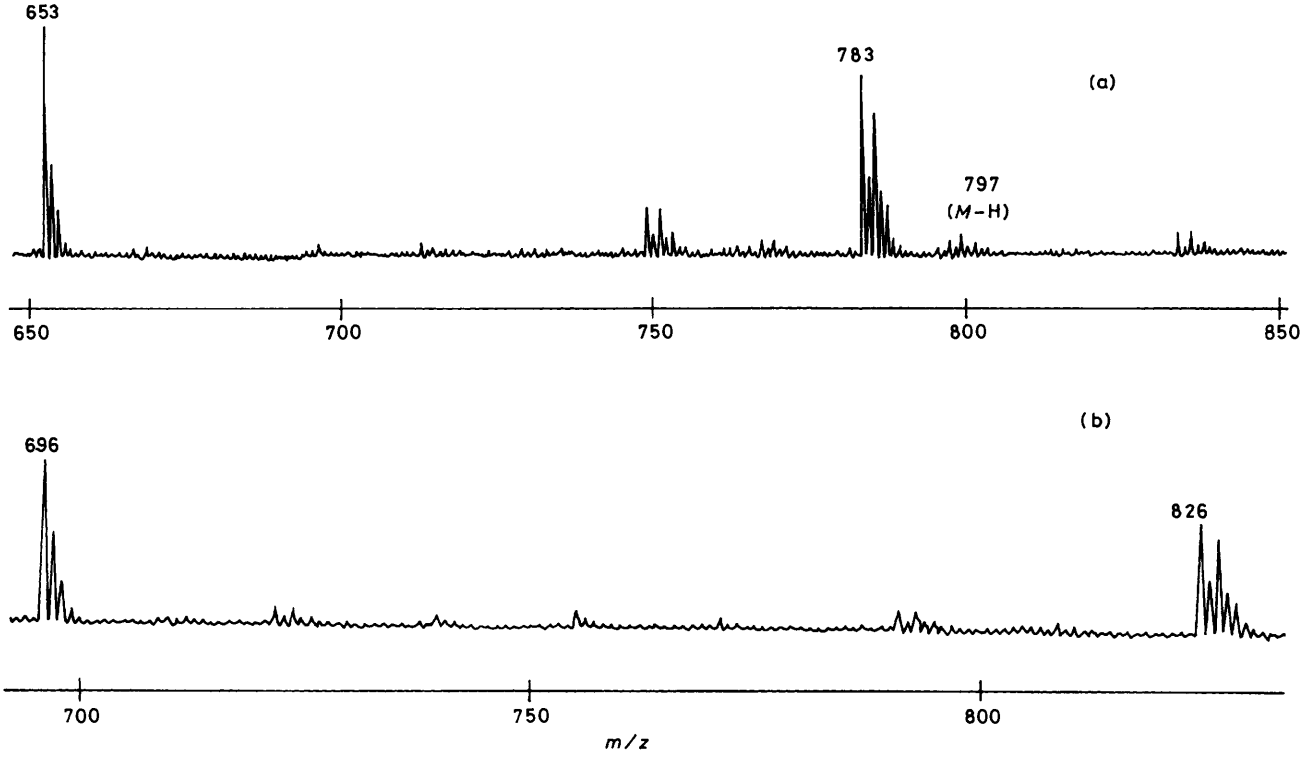


Figure 2. v⁻ FAB spectra of (a) compound (**2**) and (b) compound (**3**) from glycerol–thioglycerol (1:1), high-mass region

formed by a desorption ionization methodology such as FAB.[21] Moreover, it would be difficult to explain the matrix effect, which in some cases [(A) and (C)] suppress the formation of ions of structure (**7**) (Scheme 2). Species (**6**) and (**7**) should be formed, therefore, by bimolecular processes occurring in the condensed layers.[1,12–14]

It has been reported that demethylation of dinucleotides bearing a methylthio protecting group at the internucleotide bond can be carried out by treatment with benzenethiolate in dioxane–water at room temperature, experimental conditions which cause competitive internucleotide bond cleavage.[22] In our case the formation of (**7**) occurs only when thioglycerol is present in the matrix and its relative yield depends on the concentration of the analyte. The G/T value reported above gives an indication of the relative percentage of thioglycerol molecules 'free' and bound to the analyte. It can be assumed, therefore, that when the experiment is carried out at G/T 1.7 more thioglycerol molecules are available in the solvation sphere of the nucleotide (**2**), at the surface exposed to particle bombardment.

It can be suggested, therefore, that when Xe atom bombardment energizes the uppermost layer containing the solvated nucleotide, a nucleophilic interaction occurs between the thioglycerol and the methyl carbon of the phosphate moiety of (**2**) thus releasing the good leaving group (**7**) which is then sputtered into the gas phase. As previously mentioned, this process shows a remarkable analogy with conventional solution chemistry.[22] Similar results have been obtained when the fully protected nucleotide (**3**) was allowed to react under the same experimental conditions. In fact, species (**8**) and (**9**) at m/z 696 and 826 respectively (Scheme 2) were formed in conditions (B) while the latter was not present in experiments (A) and (C) (Figure 2).

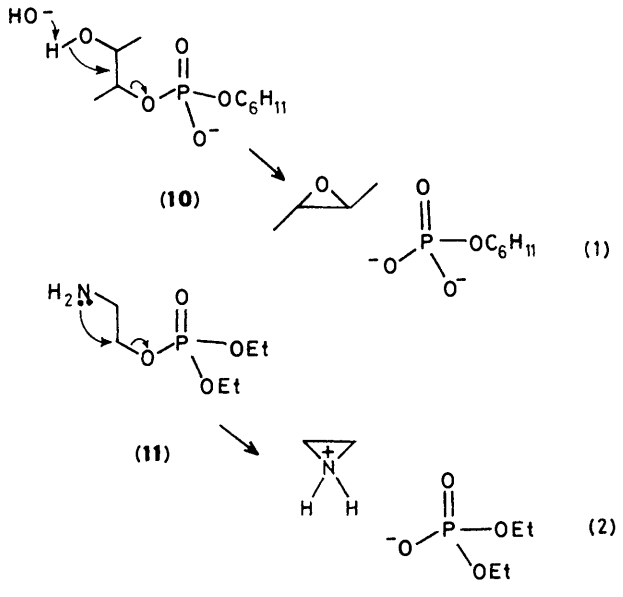
Although traces of water may be present in all the matrices employed, it seems more reasonable to consider that nucleophilic action is exhibited by the solvent itself, in agreement with

published experimental data.[1,12–14] Activated 1,2- or 1,3-diols undergo facile ring closure to three- and four-membered rings when a suitable leaving group is available on the substrate.[23–25] This process is characterized by an extremely low critical energy when the leaving group is a phosphodiester moiety[26,27] (Scheme 3). It can be assumed, therefore, that the interaction of glycerol (or other similar matrix) with the phosphotriester moiety of the reactants causes the formation of intermediates similar to (**10**) or (**11**) which decompose to (**6**) and (**8**) according to the mechanism reported in equations (1) and (2) (Scheme 3). It is worthwhile considering that compound (**11**), once formed in a thermally allowed process, undergoes spontaneous cyclization to the aziridine system.[26]

Further insights into the proposed mechanism have been obtained by using a 3-aminopropane-1,2-diol (aminoglycerol) matrix. In this case, nucleotide (**2**) gave rise to the anions (**6**) (*m/z* 653), (**7a**) (*m/z* 767), and (**12**) (*m/z* 543), whose relative intensities were 84.2, 4.4, and 11.4 respectively, *m/z* 653 being the base peak of the v^- spectrum (Scheme 4). The relative abundance of these species, even not corrected for the occurrence of consecutive processes, is in reasonable agreement with the expected chemistry of a phosphotriester compound undergoing nucleophilic substitution at the phosphate moiety. Moreover, in the aminoglycerol matrix, no species of types (**7**) and (**9**) (Scheme 2) were formed.

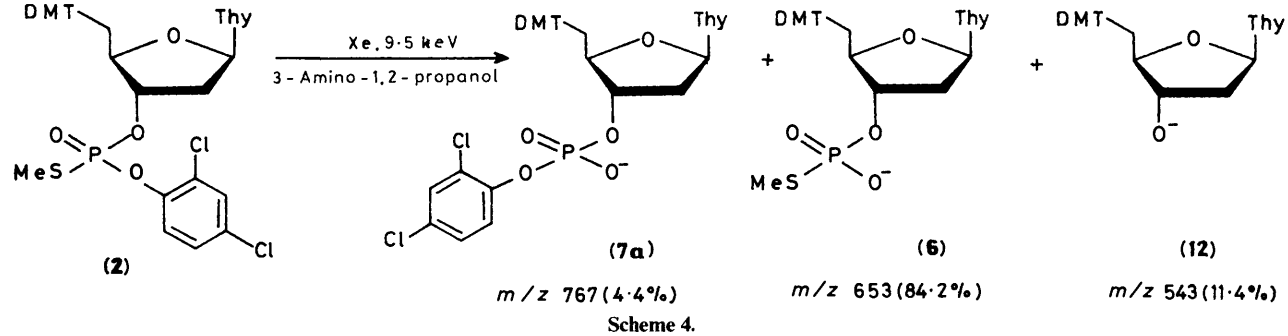
The data show that the fully protected nucleotides (**2**) and (**3**) afforded, under fast atom bombardment, the phosphodiesters (**6**) and (**8**) (Scheme 2) with a high degree of selectivity. Further characterization of these species, both as regards their structure and gas-phase reactivity, has been achieved by the MS/MS approach.[28] The metastable ion (MI) spectra, recorded according to MIKE methodology,[29] of anions (**6**) (*m/z* 653) and (**8**) (*m/z* 696) are reported in Figure 3. The lowest activation energy pathway allowed for both nucleotides is represented by the formation of *m/z* 527 and 481 daughter ions, respectively, which correspond to the elimination of the pyrimidine bases *via* a McLafferty-type rearrangement probably involving the carbonyl in position 2 of the nucleo-base and the 2′-H atom of the sugar moiety.[4,30] The other minor process in the spectra reported in Figure 3 complete the information on the gas-phase reactivity of the precursors and allow their unambiguous 'finger-printing'.

The hydrolysis of mononucleotides (**2**) and (**3**), carried out in triethylamine–water at room temperature, afforded quantitative yields of the salts (**13**) and (**14**) (Scheme 5).[17,18] The mildness and speed of this process suggest that, in the mass spectrometric experiments discussed so far, (**2**) and (**3**) produce the observed anions (Scheme 2) during sample preparation and prior to exposure of their solutions to particle bombardment. A glycerol–methanol solution of both species did not show appreciable formation of phosphodiesters when monitored over 5 h by t.l.c. ($CHCl_3$–MeOH 8:2). The same solutions, exposed to 9.5 keV Xe atom bombardment afforded the FAB spectra previously discussed; the mixture still present on the probe target after 5 min was again analysed by t.l.c., under the same conditions as before, and the nucleotides (**2**) and (**3**) were the only detectable u.v.-absorbing compounds. On the other hand, the triethylammonium salts (**13**) and (**14**) (Scheme 5), analysed by v^- FAB in a glycerol matrix, afforded quasi-molecular anions at *m/z* 653 and 696, respectively, whose MIKE spectra were completely superimposable on those obtained from the same precursors produced from (**2**) and (**3**) and reported in Figure 3. The results suggest that the observed processes occur at the liquid–gas interface under the influence of particle bombardment and that the structure of the secondary ions sputtered from the surface is determined at the moment when they are released into the gas phase.[31]



Scheme 3.

Conclusions.—A peculiar application of FABMS has been reported which takes into account some preliminary observations of the chemistry of organic molecules exposed to particle bombardment.[1,10–14] Fully protected mononucleotides show selective removal of acid-labile groups when the reactivity of protonated species was monitored (v^+ spectra), while they exhibited specific solvolysis of the phosphate moiety in basic conditions (v^- spectra). The experiments here discussed together with data in the literature throw more light on the potential of desorption ionization methodologies. In fact, nucleophilic displacement (Lewis equilibria) can predominate over proton-transfer processes (Brønsted equilibria), when low-acidity protons are available on the reactants. Bimolecular processes can be controlled by choosing a suitable matrix and,



Scheme 4.

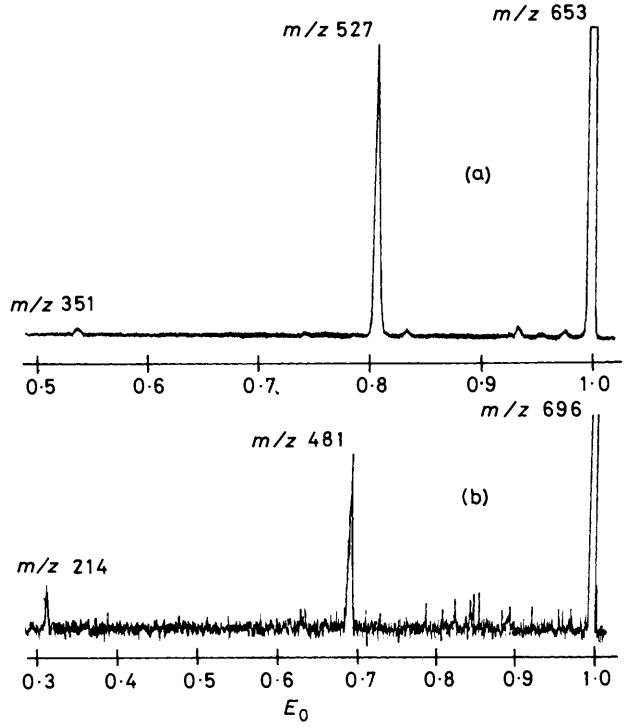
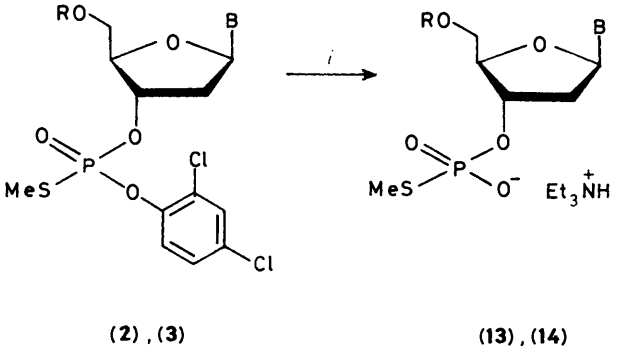


Figure 3. MIKE spectra of (a) anion (**6**) (*m/z* 653) and of (b) anion (**8**) (*m/z* 696) produced by v^- FAB of compounds (**2**) and (**3**) respectively



(**2**), (**13**), R = DMT, B = thymine

(**3**), (**14**), R = Px, B = N^4-benzoylcytosine

Scheme 5. Conditions: Et_3N–H_2O, room temperature, 5 min

at least in the case here presented, a remarkable analogy has been observed for the reaction channels populated by the same precursor both in conventional solution chemistry and under FAB conditions. In fact, the deprotection of the phosphate moiety of fully protected mononucleotides (**2**) and (**3**) can be effected with a high degree of regioselectivity.

Experimental

Glycerol, 3-aminopropane-1,2-diol, 3-mercaptopropane-1,2-diol, and diethanolamine were from Jansen. Nucleotides (**2**) and (**3**) were prepared according to literature procedures,[15] and the triethylammonium salts (**13**) and (**14**) were obtained by triethylamine–water treatment of compounds (**2**) and (**3**).[17,18] Merck silica gel pre-coated plates were used for t.l.c. and Merck Kieselgel 60 H without gypsum was used for short-column chromatography. Methanol or water solutions (2 μl) of compounds (**2**), (**3**) and (**13**), (**14**), respectively, were mixed with the appropriate matrix (2 μl) directly on the target of the FAB probe. Mass spectra were obtained on a Vacuum Generators (VG) ZAB-2F instrument operated at an accelerating potential of 8 keV by using the M-SCAN steerable FAB gun. A neutral Xe beam of 9.5 keV energy and a neutral current of *ca.* 10 μA were employed. The spectra were recorded at 1 000 resolution by scanning the magnetic field. MIKE spectra were recorded by scanning upwards the electrostatic sector potential.

Acknowledgements

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