97. Nucleosides and Nucleotides

Part 24[1])

Investigation of Ribo- and Deoxyribonucleosides and -nucleotides by Fast-Atom-Bombardment Mass Spectrometry

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The fast-atom-bombardment mass spectra (FAB-MS) of a series of ribo- and deoxyribonucleosides and -nucleotides were examined in both positive-ion and negative-ion modes. Behaviour of cytosine analogs having unnatural bases (2(1*H*)-pyrimidone, 2(1*H*)-pyridinone, and 4-amino-2(1*H*)-pyridinone) was similar to that of compounds having naturally occurring bases. Nucleotides protected by dimethoxytrityl and *p*-chlorophenyl groups were also investigated using this technique. Use of negative-ion mode reduces interference from positive counter-ions (*e.g.*, Na^+) and allows rapid sequence determination of simple di- and trinucleotides.

Introduction. – Mass spectrometry possesses great potential as a technique for the study of biological molecules, because only small amounts of sample are required of materials which may be available only in minute quantities [2]. However, in the past, its use has been hindered by the nonvolatility of large polar molecules and by their sensitivity to the extreme conditions needed to bring them into the gas phase [3]. The advent of milder desorption ionization methods, including fast-atom bombardment has solved this problem for many types of naturally occurring molecules [4].

Fast-atom-bombardment mass spectrometry (FAB-MS) [5] is a technique, whereby a stream of fast atoms (typically Ar or Xe) impinges onto a solution of the sample to be analysed in a liquid matrix such as glycerol. Volatilization and ionization [6] of the analyte occurs, and the resulting ions, either positive or negative can be detected in the usual way. The 'sputtering' or desorption of the analyte into the gas phase is most efficient, when the fast atom beam strikes the surface of the glycerol at approximately a 70° angle of incidence [7].

The study of nucleosides and nucleotides by MS has been difficult because of the involatility and thermal instability of these compounds. However, recently, other 'soft' ionization methods [8–10] such as field desorption [11], californium-252-plasma desorption [12], secondary-ion MS [13], pulsed-laser-induced desorption [14], and atmospheric-pressure-ionization MS [15] as well as pyrolysis-electron-impact and chemical-ionization MS [16] have been applied successfully in this area. These methods have also been used to

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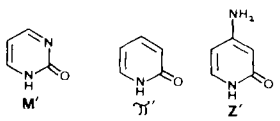
study the adducts of nucleosides with psoralens [17] and dehydroretronecine [18]. These reports indicate that FAB-MS should be a useful tool in the study of nucleosides and nucleotides, especially considering the simplicity of the technique and the ease with which existing mass spectrometers are converted to its use [19]. Not only might molecular weight be routinely determined, but, if fragmentation of oligonucleotides occurs between the sugar and phosphate residues of the backbone, sequence information might be obtained. This would provide a rapid alternative to chemical and enzymatic sequencing procedures [20]. Also, protected intermediates in the synthesis of oligonucleotides [21] might be analysed more easily [22] as well as nucleosides and nucleotides containing unnatural base residues [23].

Several publications have appeared reporting the use of FAB-MS in the study of free [24–31] and protected [32] [33] nucleosides and nucleotides. Nucleoside adducts with carcinogens [34], cyclic nucleosides [35], and chromium and cobalt complexes of nucleoside triphosphates [36] have also been studied by this technique. We, therefore, have investigated the use of FAB-MS as a tool for the characterization of nucleosides and nucleotides containing the cytosine analogues 2(1*H*)-pyrimidinone (M′), 2(1*H*)-pyridinone (π') and 4-amino-2(1*H*)-pyridinone (Z′) [37–40].



In the following discussion, the nomenclature NpN′p... will be used where N, N′, *etc.* are the usual nucleoside symbols (A, adenosine; T, ribosylthymine; U, uridine; C, cytidine; G, guanosine) and p represents a phosphate group. The nucleoside N is at the 5′-end of the oligonucleotide. In the case of protected nucleotides, ϕ represents a (*p*-chlorophenyl)phosphate group and CNEt, a cyanoethyl group.

Results and Discussion. – In preparation for the study of these unnatural nucleotides, several commercially available *(Sigma)* dinucleotides in both the ribo and deoxyribo series were studied by negative-ion FAB-MS. Quasimolecular-ion ($M - H^-$) peaks were observed in each case, as well as those corresponding to glycerol-solvated quasimolecular ions. The major mode of fragmentation is cleavage of the sugar-phosphate linkages to give nucleoside monophosphate anions. Very little, if any cleavage of the base-sugar bond was observed, in accord with the results of *Eagles et al.* [25], although *Sindona et al.* [31] reported significant fragmentation of the base. However, due to the high background below *m/z* 260, spectra were generally not recorded below this value, and thus any peaks due to free base anions would not be observed. In nearly all cases, fragmentation of the 5′-sugar residue occurred, giving peaks corresponding to the 3′-end nucleotide plus a C_3H_4O fragment. This pattern was also noted by *Crow et al.* [24]. It allows discrimination between the 5′-end and 3′-end nucleotides, and identification of the direction of the sequence. This is illustrated in *Fig. 1* for the isomeric dinucleotides ApC and CpA.

Trinucleotides were also examined by negative-ion FAB-MS. Again, quasimolecular ions and glycerol-solvated quasimolecular ions were seen. The expected mono- and dinucleotide fragments, as well as the diphosphorylated central nucleoside fragment

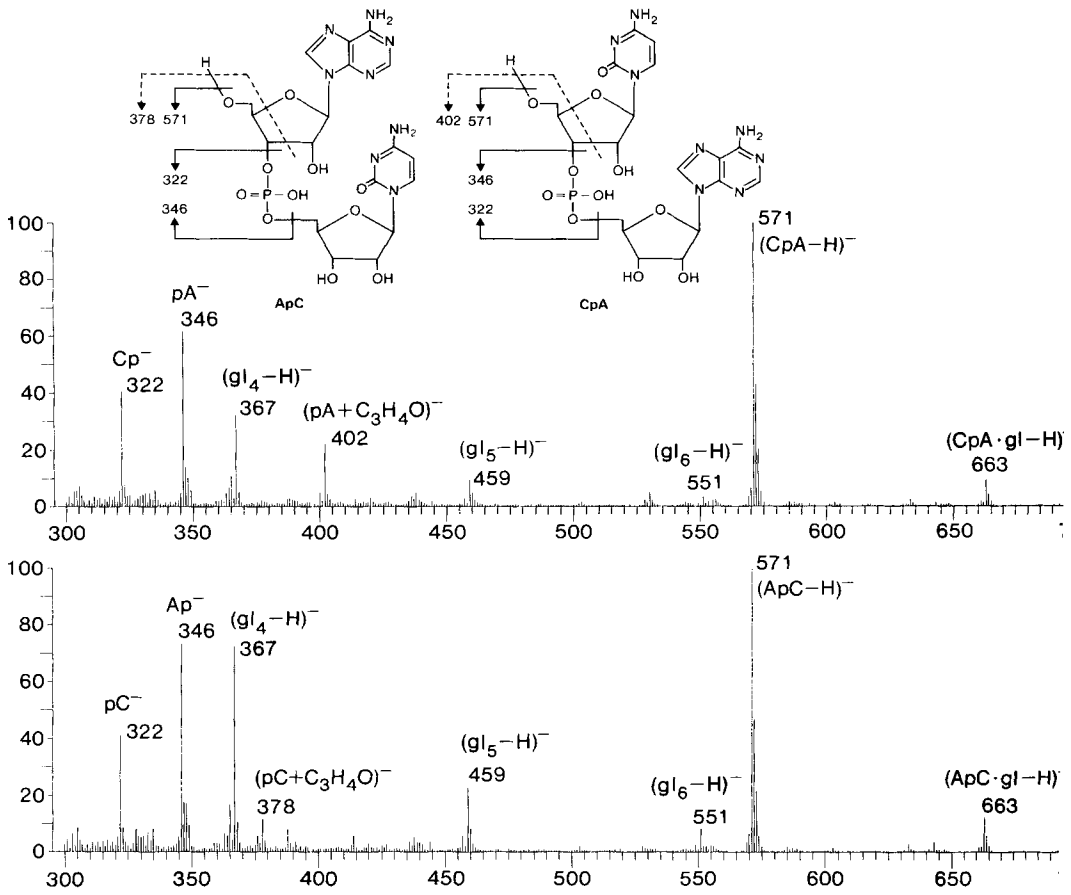


Fig. 1. *Negative-ion FAB-MS of ApC* (bottom) and *CpA* (top)

unambiguously assign the sequence. The representative spectrum of d(TpTpC) is shown in *Fig. 2*. The tetranucleotide d(TpTpTpTp) and the pentanucleotide d(TpTpTpTpTp) also give the expected negative-ion FAB-MS. However, peaks corresponding to fission of the 5′-end-sugar residue are very weak or absent in these spectra, and there is no obvious way to obtain directional information. *Grotjahn et al.* [29] reported in their study of one deca- and two octa-deoxyribonucleotides that the peaks corresponding to fragments with a terminal 5′-phosphate group were more intense than those with a terminal 3′-phosphate group. This might arise from the expected preferred cleavage of a phosphate anion from the secondary C(3′)-atom rather than from the primary C(5′)-atom of a deoxyribose moiety. However, this was the case for only three of the trinucleotides we studied, namely ApGpU, ApUpU, and ApApU. For all others, the terminal 3′-phosphate fragments gave peaks equally or more intense than those bearing a terminal 5′-phosphate group. *Panico et al.* [26] also found no correlation between peak intensities and position of terminal phosphate groups in their study of deoxyribotetranucleotides. Thus, no direct way exists of determining the direction of a small oligonucleotide sequence by negative ion FAB-MS. Selective end modification may be necessary to achieve this.

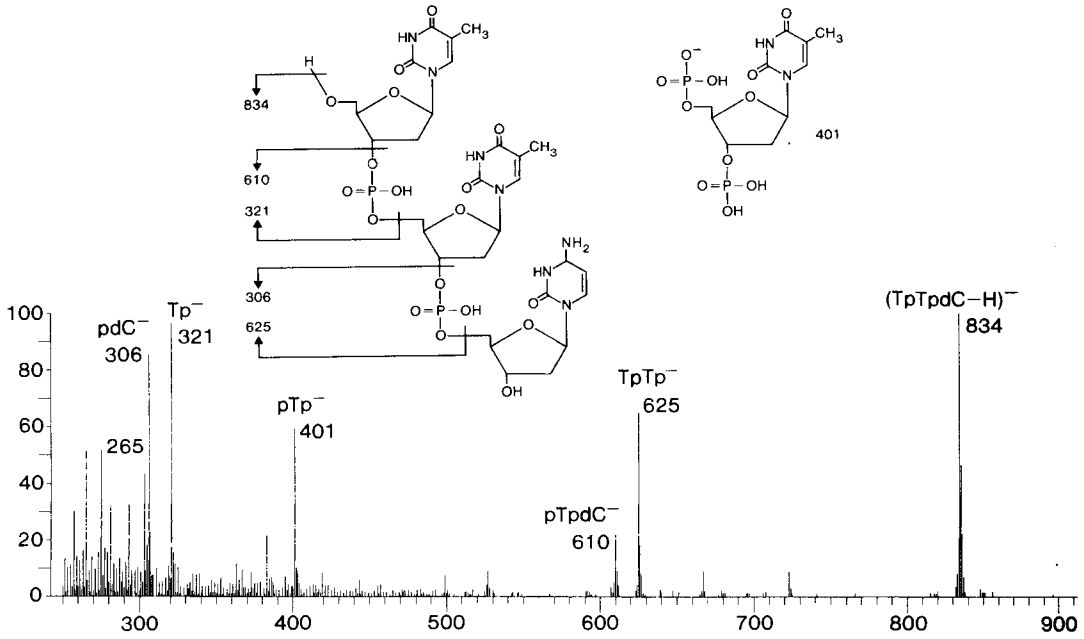


Fig. 2. *Negative-ion FAB-MS of d(TpTpC)*

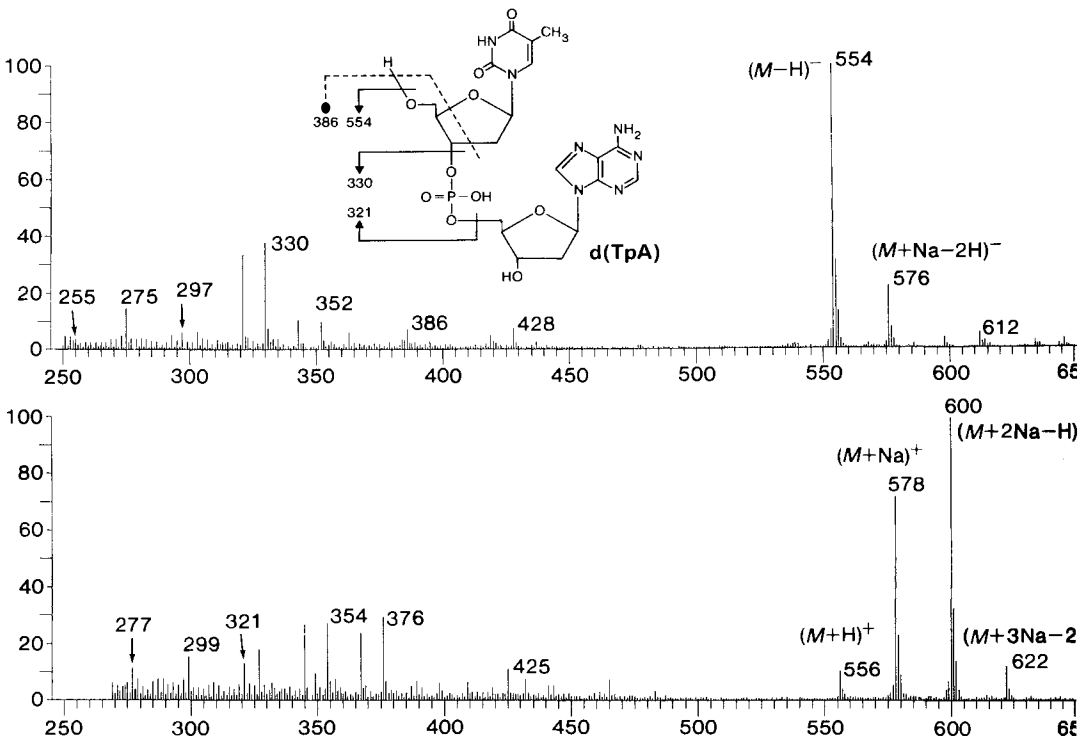


Fig. 3. *FAB-MS of d(TpA) in negatives-*(top) *and positive-*(bottom) *ion modes*

The positive-ion FAB-MS of oligonucleotides are not as useful as the negative-ion spectra. Extensive cationization by even traces of Na^+ ions occurs, and meaningful peaks are often lost in the matrix-derived background. This is illustrated for d(TpA) in *Fig. 3*. The fragment peaks at 321 $(d(Tp)^-)$ and 330 $(d(pA)^-)$ are quite intense in the negative-ion spectrum, while the expected corresponding peaks at 323 and 332 are not seen above background level in the positive-ion spectrum. Since the sequence fragments are more likely to have a negative charge, they would probably not be visible in the positive-ion spectra in any case. Also, cationization by Na^+ severely complicates the molecular-ion region in the positive-ion mode. This is illustrated further by the positive ion spectra of ApApApApA and d(TpTpTpTpTpTp) *(Fig. 4)*. Little if any MH^+ is observed while Na-containing ions are prominent. A previously reported [9] positive-ion FAB-MS of d(TpTpTpTpTpTp) showed less, but still extensive Na^+ cationization. *Aubagnac et al.* [27] also observed much Na^+ cationization and a high level of background noise in their positive-ion FAB-MS of arabinose nucleotides.

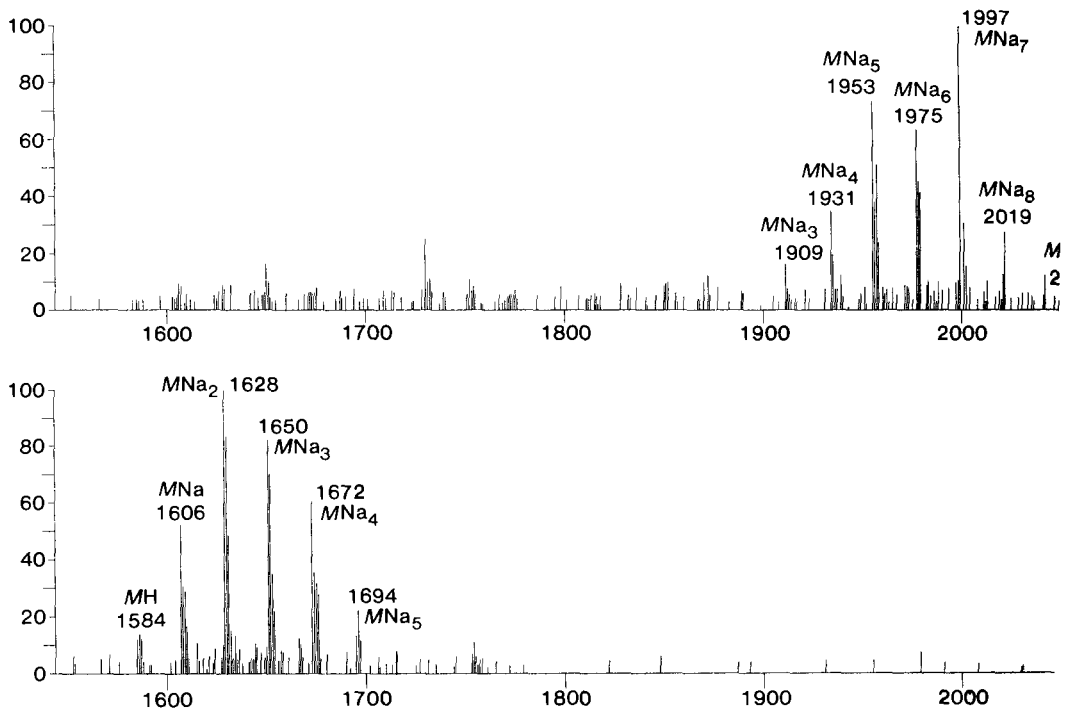


Fig. 4. *Positive-ion MS of d(TpTpTpTpTpTp)* (top) *and ApApApApA* (bottom)

Therefore, several fully protected deoxyribonucleotides [37] were studied by FAB-MS, and in all cases, little if any quasimolecular ion $(M - H^-)$ was observed. However, loss of the CNEt moiety occurred readily to give a prominent peak at 54 daltons lower mass. *Ulrich et al.* [38] observed similar behaviour in their negative-ion FAB-MS of fully protected dinucleotides. Loss of the *p*-chlorophenyl and $(MeO)_2Tr$ groups were also observed as well as cleavage of the base-sugar and phosphate-sugar bonds. The fully protected nucleotide molecule does not have a proton which can be easily lost to leave a

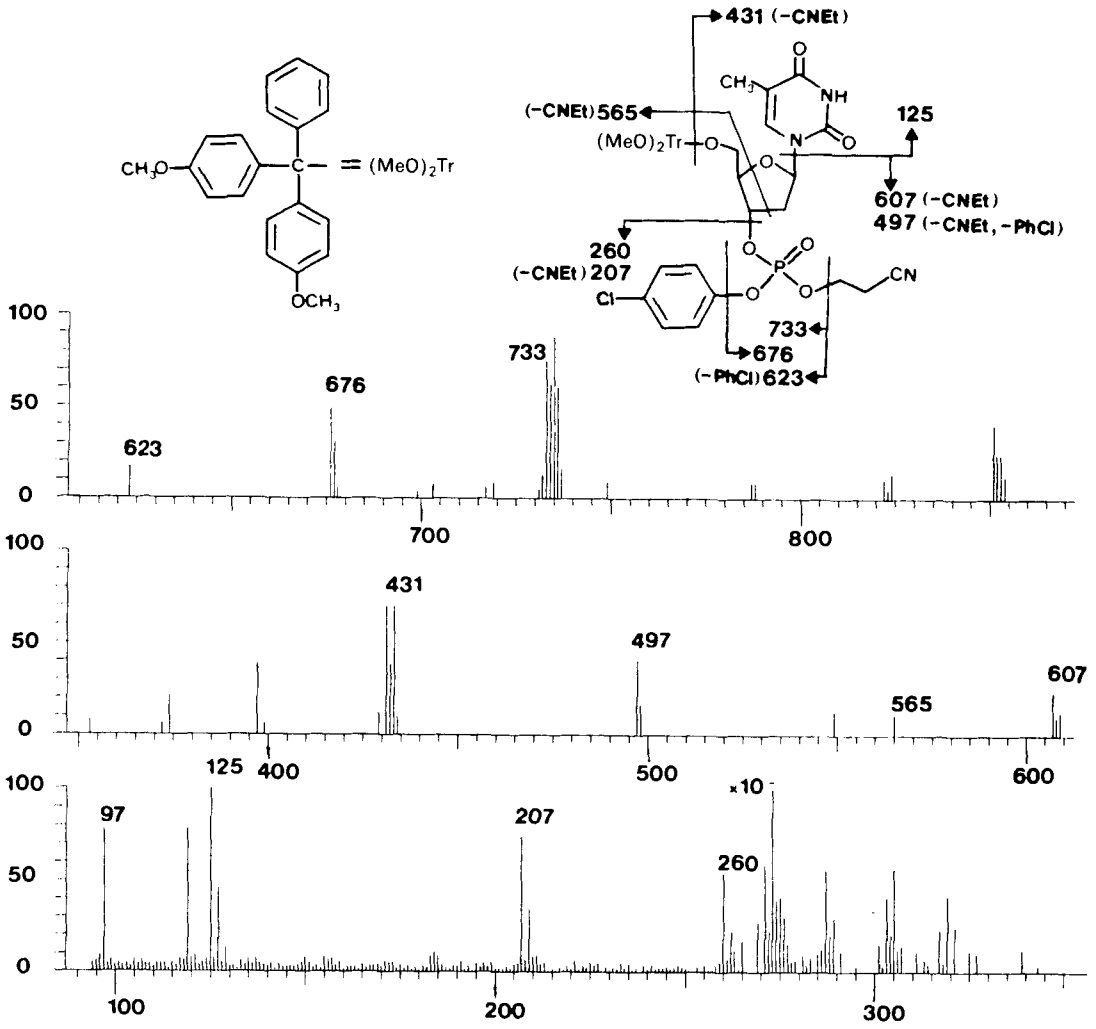


Fig. 5. *Negative-ion FAB-MS of [(MeO)$_2$Tr]T$_d\varphi$(CNEt)*

stable negatively charged fragment. As a result, loss of one of the protecting groups is necessary to obtain such a fragment. Fission of the sugar residue, as was seen in the case of the free dinucleotides, occurs, and loss of the base protecting group is observed in some cases. *Grotjahn et al.* [33] observed similar fragmentations. The representative spectrum of fully protected thymidine is shown in *Fig. 5*. The extension of this technique to unnatural nucleosides and nucleotides could now be considered.

The nucleosides dM, dπ, and dZ containing the 2(1*H*)-pyrimidone (M'), 2(1*H*)-pyridone (π'), and 4-amino-2(1*H*)-pyridone (Z') bases [38–40] gave quasimolecular-ion ($M - H^-$) peaks and showed fragmentation of the base-sugar bond. This is shown in the spectrum of dπ *(Fig. 6)*. This was also observed by *Crow et al.* [24] in their FAB-MS of natural nucleosides. In the case of dM, a high matrix-derived background made inter-

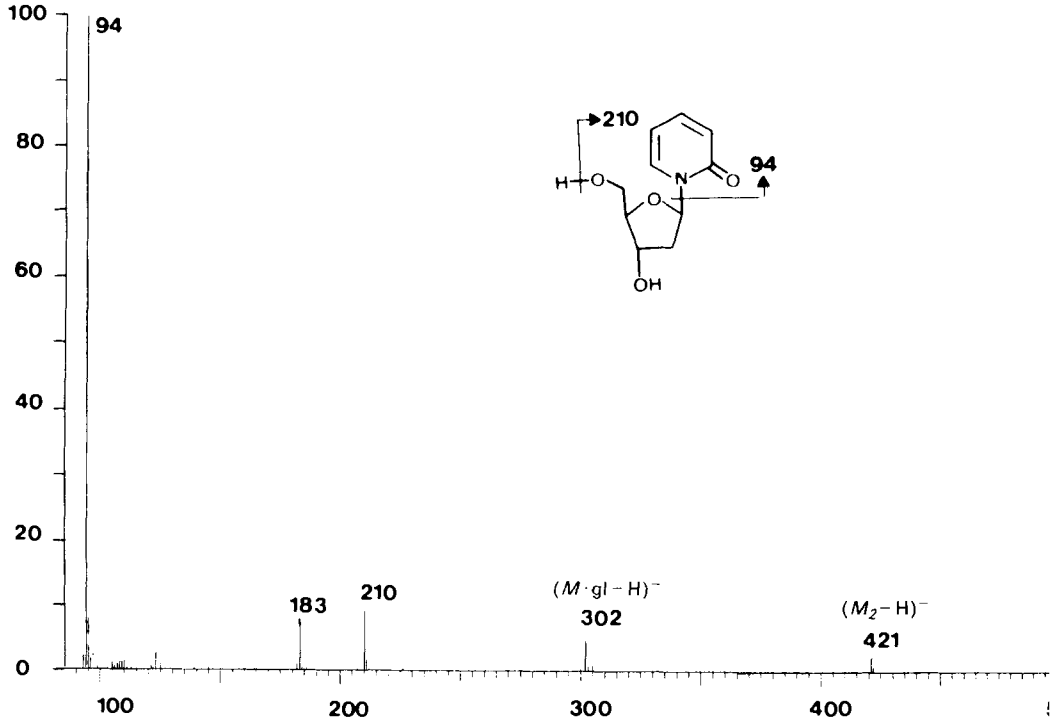


Fig. 6. *Negative-ion FAB-MS of dπ* (gl = glycerol)

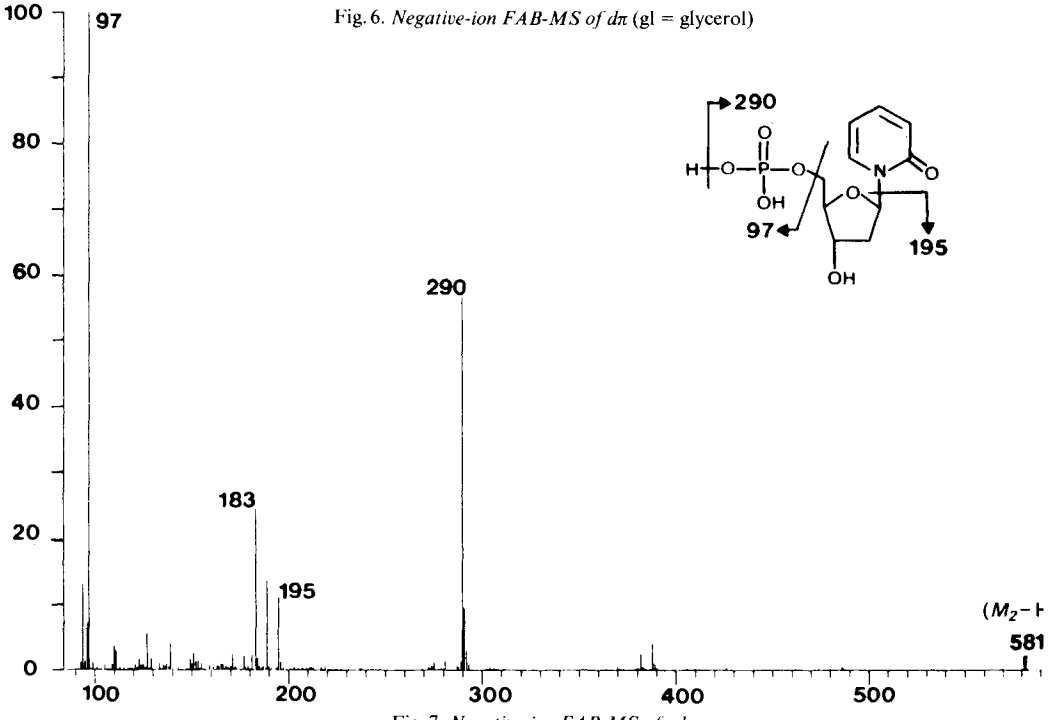


Fig. 7. *Negative-ion FAB-MS of pdπ*

pretation of the spectrum difficult. Many peaks at higher mass than the quasimolecular ion were observed, due to clustering of the quasimolecular ion with molecules of glycerol or of itself.

The 5'-mono- or 5'-triphosphates of these nucleosides were also studied. For pdπ *(Fig. 7)*, a significant quasimolecular-ion peak at 290 daltons was observed as well as fragment peaks at 195 ($(M - B)^-$) and 97 ($H_2PO_4^-$). A high degree of clustering of the quasimolecular ion with glycerol, H_3PO_4, the 195-dalton fragment, and itself occurred. The highest peak observed in the spectrum was $(M_3 - H)^-$ at 872 daltons, but this peak was of quite low intensity. For the 5'-triphosphate ppp dZ, however, little fragmentation was observed above the high background. It is possible that low solubility of this in the glycerol matrix caused a low-intensity spectrum. The Na^+-bound quasimolecular ion at 487 daltons is visible as well as glycerol-solvated quasimolecular ions at 579 and 671 daltons.

Dinucleotides containing unnatural bases appear to behave much like natural dinucleotides. For d(MpT), the quasimolecular ion at 515 daltons ($(M - H)^-$) was observed as well as the expected fragments at 291 (Mp^-), 321 (pT^-), and 377 ($(pT + C_3H_4O)^-$). The 5'-protected (MeOTr)d(MpC) was also studied *(Fig. 8)*. The expected fragmentations were seen, but it is interesting that little of the expectedly facile cleavage of the MeOTr group was observed. While this cleavage was observed by *Grotjahn et al.* [33], *Ulrich et al.* [32] did not see any loss of this group in their study.

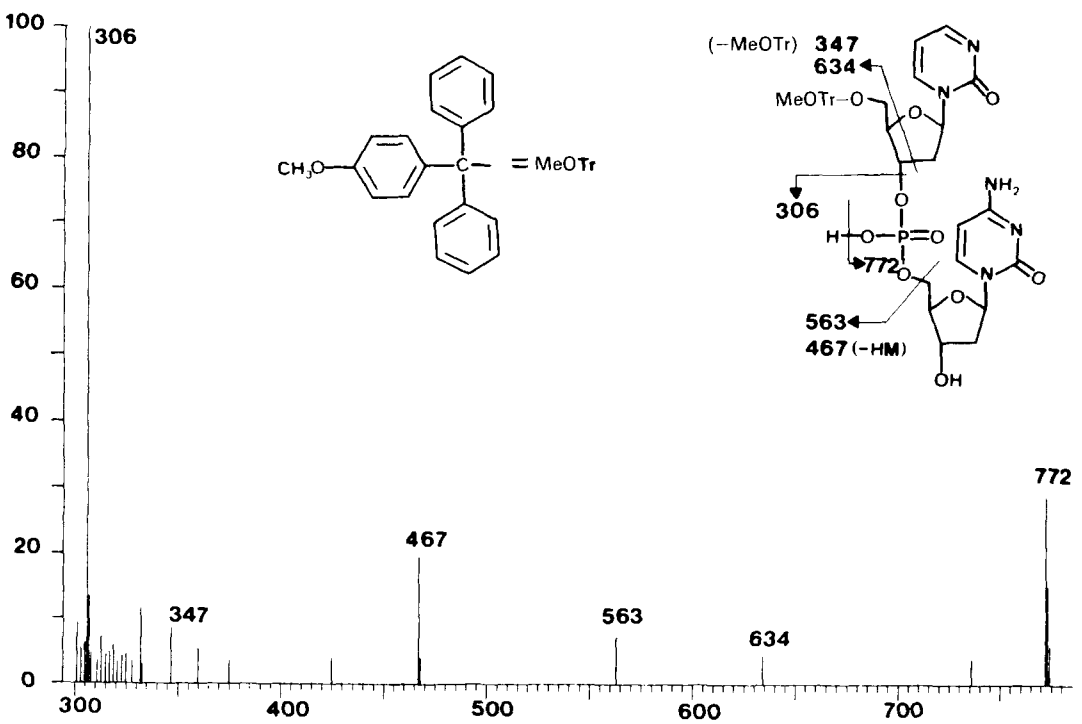


Fig. 8. *Negative-ion FAB-MS of (MeOTr)d(MpC)*

Nucleosides and nucleotides containing unnatural bases, thus, appear to behave quite similarly to the natural compounds and lend themselves well to study by FAB-MS.

Experimental. – FAB-MS were recorded on a *Kratos/AEI MS9* mass spectrometer with a mass range of 1400 daltons at an accelerating voltage of 6 kV and on a *Kratos/AEI MS50* mass spectrometer, with a mass range of 2300 daltons at an accelerating voltage of 6 kV. Both instruments were fitted with saddle-field fast-atom guns, ion sources, and copper sample probes as described in [19]. Spectra were recorded at a scan rate of 10 s/decade using a *Kratos DS55* on line-data system. Calibration of the data system for a mass range of 1300 daltons or less was carried out in the FAB mode for both positive and negative ions with perfluorotriheptyltriazine. *Fomblin* was used in the EI positive-ion mode, when mass ranges greater than 1300 daltons were required.

Commercially available free oligonucleotides (*PL Bio-chemicals, Sigma,* Amersham) were used without purification or after ion-exchange chromatography on *AG 50W-X8* (H^+ or NH_4^+ form, 50–100 mesh, *Bio-Rad*) or on *DEAE* cellulose *(Sigma)* with H_2O and 0.5M $HCOONH_4$ (pH 6.5) or 0.5M NH_3. Chromatographed samples were lyophilised under high vacuum. Protected and unnatural nucleosides and nucleotides were prepared as previously described [37–40]. Sample loading was usually about 20 µg of nucleotide dissolved in 2 µl of glycerol or sulfolane/glycerol. The saddle-field discharge was *ca.* 1 mA at 5 kV applied voltage using Xe.

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