the relative STO-3G energies are at least qualitatively correct. Thus, the following discussion focuses on the STO-3G results.

First, it should be noted that the TCSCF energy of $^1A_1$, relative to the RHF energies of the other three TMM states, is anomalously low. As mentioned above and discussed in detail elsewhere,[37] this anomaly is due to the doublet instability problem. The problem is solved with an MCSCF or CI wave function, which provides electron correlation.

As observed previously by Dixon et. al.,[23] inclusion of electron correlation through use of an MCSCF wave function increases the energy difference between $^1B_1$ and $^1B_2$ from the RHF value. Our CI results confirm that this finding is not an artifact of their using a wave function with a limited number of configurations. We have previously discussed why such an increase should occur.[18,25]

The same effect is observed with the SV basis set, although the increase on going to a correlated wave function is not as great with this more flexible basis set. Our SV-CI results also provide further evidence that $^1B_1$ is lower in energy than $^1A_1$, although the energy difference is smaller than with STO-3G CI or STO-3G MCSCF.

As expected from Hammond's postulate, the energies of the transition states leading from MCP to $^1B_1$ and $^1A_1$ do, in fact, parallel the energies of these intermediates. There is also a small preference for a conrotary over a disrotary transition state connecting MCP with $^1A_1$. Planar $^1A_1$ can also close to MCP by simultaneously pseudorotating and twisting one methylene group, giving $^1B_1$ as an intermediate along this reaction path. The barrier to this process is comparable in energy to those involving the simultaneous rotation of two methylene groups and to the barrier for pseudorotation[20] to an equivalent $^1A_1$ wave function.

The energy surface that we have calculated in the region around $^1A_1$ is sufficiently flat that dynamics could play an important role in determining the partitioning of this intermediate. Our double Fourier series fit to the computed energy grid has provided a closed expression for the energy on the surface. Thus, trajectory calculations on the surface are feasible. These might provide some information regarding the molecular dynamics on the full 24-dimensional hypersurface, of which ours is but a two-dimensional projection.

Our Fourier fit to the singlet and triplet energies at the same set of geometries has enabled us to show that the lowest energy of intersection is actually above $^1B_1$. Consequently, a surface intersection below the energy of $^1B_1$ cannot be invoked to reconcile the results of Dowd's EPR study with the calculated energy difference between $^3A'_2$ and $^1B_1$. Tunnelling from one surface to another seems ruled out by Dowd's experiments.[28]

It would now seem that there is little chance of reconciling Dowd's experimental results with the results of ab initio calculations. Either the best current approximations to the solution of the Schrödinger equation for $^1B_1$ and $^3A'_2$ are inadequate to give the energy difference between these states correctly, or Dowd's experiment does not measure the energy required for crossing from the triplet to the singlet surface. It should be noted, however, that the calculations refer to isolated TMM in the gas phase, whereas Dowd's experiments were done in frozen solutions. This difference remains the last hope for bringing theory and experiment into concordance.

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**Registry No.** MCP, 6142-73-0; TMM, 13001-05-3.

# A New Method for Sequencing Fully Protected Oligonucleotides Using $^{252}$Cf-Plasma Desorption Mass Spectrometry. 1. Negative Ions of Dinucleoside Monophosphates

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**Abstract:** Using the method of californium-252 plasma desorption mass spectrometry we have established the systematics for a completely instrumental method of sequencing synthetic gene fragments which are in the intermediate stages of synthesis and therefore contain chemical protecting groups at all reactive sites. The fragmentation pattern for a variety of dinucleoside monophosphates has been identified and showed to contain ions which are diagnostic for the base sequence. The method is universally applicable to deoxy- and ribooligonucleotides containing a variety of different phosphate blocking groups.

A completely instrumental method for sequencing the chemically protected precursors of synthetic DNA and RNA has resulted from the synchronous developments in two independent fields of chemistry. The recent development of recombinant DNA techniques has stimulated the advancement of chemical procedures for synthesizing significant quantities of DNA and RNA. The synthetically assembled nucleic acids have been utilized in a multitude of novel experiments including the complete synthesis and cloning of biologically functional genes,[1] the study of the regulator regions on a gene,[2] and exploration of the structure and function of nucleic acids.[3] At the same time a new method in mass spectrometry, californium-252 plasma desorption mass spectrometry ($^{252}$Cf-PDMS),[4,5] has evolved with capabilities that

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are compatible with the analytical requirements related to the chemical synthesis of RNA and DNA. The $^{252}$Cf-PD mass spectrometer has several important features which include the abilities to investigate molecules with a wide range of masses (ions up to $m/z$ 12 637 have been detected),[6] to measure both positive and negative ion mass spectra, and to obtain mass data for very thermally-labile, polar molecules. The method also offers the advantage that the nature of the ionization method is virtually nondestructive. The application of $^{252}$Cf-PDMS to the sequence analysis of synthetic nucleic acids appeared to be a possible approach to developing a universally applicable method for the characterization of fully protected oligonucleotide intermediates which are the precursors of synthetic DNA or RNA required for recombinant studies. Although the base sequence of the phosphodiester form of DNA or RNA can be obtained by enzymatically or chemically degrading the oligonucleotide followed by chromatographic analyses of the products, information concerning the integrity of the chemical blocking groups used to direct the synthesis is necessarily lost. Knowledge of the structure of the protected precursor becomes critical in order to maintain the highest possible reaction yields during intermediate stages of the synthesis and in the rapid development and implementation of new synthetic procedures. Some information can be obtained from NMR spectra. However, the spectra tend to become exceedingly complex and the interpretation ambiguous for large protected oligonucleotides. The application of electron impact mass spectrometry has been successful in characterizing the protected nucleoside starting materials[7] but the high mass, low volatility, and thermal instability limit the method to the nucleoside or mononucleotide level. Spectra obtained by pyrolysis mass spectrometry contain low molecular weight fragments diagnostic of the protected bases and 5′ and 3′ substituents but neither the base sequence nor the molecular weight can be assessed.[8,9] The method of organic SIMS[10] (secondary ion mass spectrometry) has been successful in obtaining spectra of a variety of protected ribodinucleoside monophosphates which are almost identical with those obtained by $^{252}$Cf-PDMS. The first results obtained by $^{252}$Cf-PDMS of several chemically blocked deoxyoligonucleotides demonstrated that positive molecular ions could be detected and that the negative ion spectrum contained a simple fragmentation pattern from which the base sequence could be verified.[11] We have since investigated the application of this instrumental method to the sequence analysis of numerous protected oligonucleotides. As a result, a substantial data base has been established which as enabled us to develop a systematic analysis of this class of molecules. In this and the following papers, we report the results obtained for a variety of chemically protected ribooligonucleotides containing phosphotriester internucleotidic linkages and demonstrate how the negative ion spectrum can be used to verify the base sequence and the integrity of the blocking groups. We have also found that the negative ion spectrum can be used to characterize nucleotide impurities that might otherwise reduce reaction yields or interfere with the application of these molecules in other experiments.

The particular focus of this paper is on a set of four dinucleoside monophosphates that contain identical base sequences but which differ in the nature of the phosphate or 5′-protecting group. By comparing the $^{252}$Cf-PDMS negative ion spectra of these compounds we have been able to establish the important fragmentation pathways. In the following paper, we apply these principles in establishing a protocol for sequencing higher-order protected oligonucleotides.

## Experimental Section

**$^{252}$Cf-Plasma Desorption Mass Spectrometry ($^{252}$Cf-PDMS).** A description of the basic operational principles of the $^{252}$Cf-PD mass spectrometer has been described elsewhere.[5] The present configuration of the ion source region is also described in a recent publication.[11] Only a brief summary of the salient features of the method will be included here. The $^{252}$Cf-PDMS method is based on the interaction of nuclear fission fragments (from a $^{252}$Cf source) with molecules in a solid film, resulting in the desorption of ions from the solid film. The $m/z$ of the ions is measured by the time-of-flight (TOF) method, using standard nanosecond-resolution pulse electronics. The fission fragment flux through the samples was 2100 $s^{-1}$ $cm^{-1}$; irradiation times varied from 1 to 15 h depending on the intensity of ions in the spectrum. An acceleration voltage of 10 kV was used and the length of the field free region was 45 cm. The electronic timing resolution was 1.5 ns and the mass resolution was ~450 $M/\Delta M$ (at full width at half-maximum).

Time interval measurements were made in an event-by-event mode, using an EG&G TDC 100 time digitizer (Ortec) with a multiple stop capability (4 μs deadtime). The output of the digitizer was interfaced to a Perkin-Elmer 8/32 computer for on-line data acquisition. The same computer was used to interactively analyze each spectrum. Analysis consisted of obtaining the mass calibration for each spectrum, subtacting the background,[12] locating peaks, and determining precise peak centroids (to within 0.01 ns).

The masses of the oligonucleotide ions reported in these studies extend beyond $m/z$ 4000. A procedure has been developed for obtaining reliable mass data of high molecular weight ions. This involves a precise determination of the TOF of selected ions that appear in the low mass range of each sample and evaluation of a calibration curve for each sample under conditions where the calibration ions and sample ions were recorded simultaneously. This eliminates possible errors due to electronic drift. The negative ions selected were $H^-$, $C_2H^-$, and/or $PO_3^-$. A comparison of experimental masses determined by this technique with the calculated mass values of high molecular weight ions is included in a previous publication.[11] Unless otherwise stated, all calculated masses are reported as the averaged mass value (based upon $C$ = 12.01115). This mass closely approximates the mass of the most abundant species in the natural isotopic distribution for ions above $m/z$ 1000. The principal ion mass,[14] which is the commonly reported value in mass spectrometric studies, corresponds to the mass of a peak observed in much lower abundance for high molecular weight species. It, therefore, seems more reasonable to report the averaged mass in these studies.

**Sample Preparation.** Thin solid films of samples were prepared by the electrospray technique.[15] A solution of the sample (0.5–1 μg/μL) was prepared by dissolving the sample in high-purity acetone (Burdick and Jackson, "Distilled in Glass") and electrospraying 50–100 μL over the target backing which consisted of a 1.5 μm thick aluminized Mylar foil (Steiner Film Co.) stretched over a circular aperture with an area of 1.1 $cm^2$ and secured by a stainless steel retaining ring in the target housing. Since the $^{252}$Cf-PDMS method is nondestructive, the material could be recovered after analysis by rinsing the foil with acetone.

**Synthesis.** The protected ribonucleotides described in these studies were prepared by the general method that has been developed in our (K.K.O., N.Y.T., and M.J.N.) laboratory. This method[16] involves the use of silyl protecting groups on the 2′ position of the nucleosides and use of the phosphite coupling procedure. The sequences corresponding to the terminal units of a transfer RNA have been previously reported.[17] The synthesis of ribonucleotides bearing different phosphate protecting groups by the phosphite procedure has also been described[18] in addition to the synthesis of ribonucleosides containing the 2′-levulinyl group.[19]

## Results and Discussion

Four protected ribodinucleoside monophosphates shown in Figure 1 were selected as model compounds; all had been pre-

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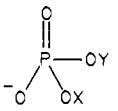
viously characterized by chromatographic methods and the purity confirmed by the $^{252}$Cf-PD positive ion spectra. Each nucleotide contains benzoylcytidine ($C^{bz}$) and adenine (A), *tert*-butyldimethylsilyl (TBDMS or Si) groups at each 2′ position, and the terminal 3′-oxygen, four different phosphate protecting groups are employed and in all but one of the samples the monomethoxytrityl group (MTr, *p*-(methoxyphenyl)diphenymethyl) is used to protect the 5′-hydroxyl (the remaining dinucleoside monophosphate bears a free 5′-hydroxyl group). By examining the $^{252}$Cf-PD negative ion spectra of these four cognates we sought to establish the identity of the negative ion fragments utilizing the characteristic mass shifts of the different protecting groups and to evaluate the effect (if any) of the various phosphate protecting groups on the mass spectra that were obtained. All are commonly encountered in different oligonucleotide syntheses.

The shorthand structure and spectrum of the dinucleoside containing the *p*-chlorophenyl phosphate protecting group are given in Figure 1A. This structure and the abbreviated symbols are presented in more detail in Figure 2. Three major fragment ions are observed in addition to the negative molecular ion. The most plausible sites for bond cleavages resulting in the formation of stable negative fragment ions appear to be along the C3′–OP, C5′–OP, and R′–OP bonds. The structures of ions produced by C3′–OP and C5′–OP cleavages and the resulting fragment ion masses are indicated in Figure 1A. Electron addition to the molecule and subsequent rupture along either of the three bonds would result in the formation of a neutral nucleoside or *p*-chlorophenyl fragment and a negatively charged nucleotide fragment (structure I) in which the charge is formally located on the oxygen atom belonging to the phosphate moiety. Heterolytic bond rupture producing an ion pair may occur to a limited extent although this process is energetically less favorable.



Ia, X = *p*-chlorophenyl; Y = $A^{Si}_{Si}$ [20]
b, X = *p*-chlorophenyl; Y = $MTr(bz)C^{Si}$
c, X = $MTr(bz)C^{Si}$; Y = $A^{Si}_{Si}$

Ion Ia is formed by cleavage along the C5′–OP bond producing ions at $m/z$ 685; ion Ib is formed by cleavage along the C3′–OP bond yielding ions at $m/z$ 923. Cleavage along the third C–O bond, the R′–OP bond, gives rise to a peak at $m/z$ 1290 designated as $(M - R')^-$ (ion Ic). Negative molecular ions are most likely formed by electron attachment and loss of a hydrogen atom or hydrogen molecule. The experimental mass is 0.75 u below the calculated mass value for $(M - H)^-$.

When the diunucleoside monophosphate is synthesized with the β-cyanoethyl functionality rather than *p*-chlorophenyl the masses of the two sequence ions (at $m/z$ 627 and 866) are shifted by 57.25 u, the mass difference between the two phosphate protecting groups (Figure 1B). Because the $m/z$ 1290 peak appears in this spectrum and the previous one it cannot contain the phosphate protecting group. This supports the proposed structure assignment, that is, $(M - R')^-$. The possibility that this peak is due to a common contaminant is unlikely because a complimentary molecular ion was not observed in the positive ion spectrum. The experimental mass of the negative molecular ion is $m/z$ 1343.48 which is within 0.48 u of the calculated average mass of $(M - H)^-$. Two additional peaks at $m/z$ 575 and 1018 are present in this spectrum which do not have analogues in spectrum 1A. From the known structure of the molecule we propose that the $m/z$ 575 peak is due to the loss of the cyanoethyl group from the 3′ sequence ion accompanied by the concomitant addition of hydrogen to give the singly charged negative ion. The broadness of the peak indicates that this species may be metastable. Corresponding loss of R′ from the 5′ sequence ion is not observed. The identity of the peak at $m/z$ 1018 has not been established. It is possible that this ion is formed by loss of both the trityl moiety and the cyanoethyl group from the parent molecule but fragments of this type were not observed in any of the cognate spectra. Furthermore no complimentary positive molecular ions, $(M + H)^+$ or $(M + Na)^+$, or detritylated sequence ions (in the negative ion spectrum) were apparent, negating the possibility that the $m/z$ 1018 species is an oligonucleotide impurity.

The third phosphate protecting group of this series is the β,-β,β-trichloroethyl (TCE) functionality. This group is employed extensively in the phosphite coupling procedure and is used as the phosphate protecting group in the majority of oligonucleotides reported in this paper. The negative ion spectrum and structure are shown in Figure 1C. This spectrum contains several peaks in addition to the four species identified in spectrum 1A. The most prominent peak is that at $m/z$ 1290 which also occurs in the two preceding spectra. This is consistent with the assignment of this peak to be $(M - R')^-$. The expected 3′ and 5′ sequence ions formed by rupture of the two C′–OP bonds are present at $m/z$ 706 and 944, respectively. The peak at $m/z$ 575 appears in both this spectrum and that of the β-cyanoethyl cognate (Figure 1B). As shown in the inset structure of both compounds, we suggest that, as in the previous spectrum, this species is formed by replacement of the phosphate protecting group by hydrogen on the 3′ sequence ion. Peaks also appear 35–36 u below the masses of both sequence ions and the negative molecular ion. These ions most probably arise by loss of HCl or chlorine from each of the three species. A peak 35 u above the parent mass is observed which we believe is due to chlorine atom addition to the molecule accompanied by electron attachment.

For unequivocably establishing the identity of the 5′ and 3′ sequence ions in this series the detritylated dinucleoside monophosphate was examined by $^{252}$Cf-PDMS. This also afforded the opportunity for investigation of the effect that the absence of this group has on the mass spectrum. This dinucleoside monophosphate was synthesized containing a methyl blocking group at the phosphate position. In Figure 1D it is observed that the 3′ sequence ion peak (at $m/z$ 589) and $(M - R')^-$ peak are shifted in mass by the expected amount due to substitution of the new R′ group. The 5′ sequence ion (at $m/z$ 589) is also shifted to lower mass but the change in mass corresponds to the combined trityl/methyl molecular weights. The peak at $m/z$ 991 has not been identified; a peak of low intensity appears ~24 u higher in mass in the positive ion spectrum suggesting that this species is a low-level impurity.

From the combined data of these four various dinucleoside monophosphates, we have clearly shown that the primary negative ion fragments above $m/z$ 500 are formed by rupture of the C3′–OP, R′–OP, and C5′–OP bonds. Other fragment ions may be observed depending on the nature of the phosphate protecting group. The identity of the phosphate blocking group does not alter the primary fragmentation pattern. Rather, relative peak intensities may change or additional fragments appear. Ions formed by loss of the R′ group appear to become more abundant (relative to the molecular ion) as the R′–OP bond attains more ionic character. In addition we observed that the presence or absence of the trityl group did not appreciably alter the relative intensities of the two sequence ions.

The mechanism of fragmentation of these molecules is of considerable interest because it forms the basis for developing $^{252}$Cf-PDMS as a method for the sequence verification of synthetic gene fragments. Previous reports of the sequence determination of dinucleoside monophosphates (naturally occurring and chemically blocked) by mass spectrometry have utilized the positive ion spectrum to provide diagnostic information.[9,21,22] In all cases fragments were formed by cleavage of the C3′–OP and C5′–OP bonds with the positive charge retained on the carbon atom (C3′ or C5′) and in some cases charge retention on O3′ or O5′ ac-

(20) The abbreviation $A^{Si}_{Si}$ refers to the adenosine residue containing TBDMS groups at the 2′ and 3′ positions. Similarly, $MTr(bz)C^{Si}$ represents the 5′ nucleoside, benzoylcytosine, which bears a TBDMS group at the 2′ position. When the functional group (such as Si) appears above the base abbreviation (e.g., A or C) it designates the 2′ position; when it appears below, it indicates the 3′ position.

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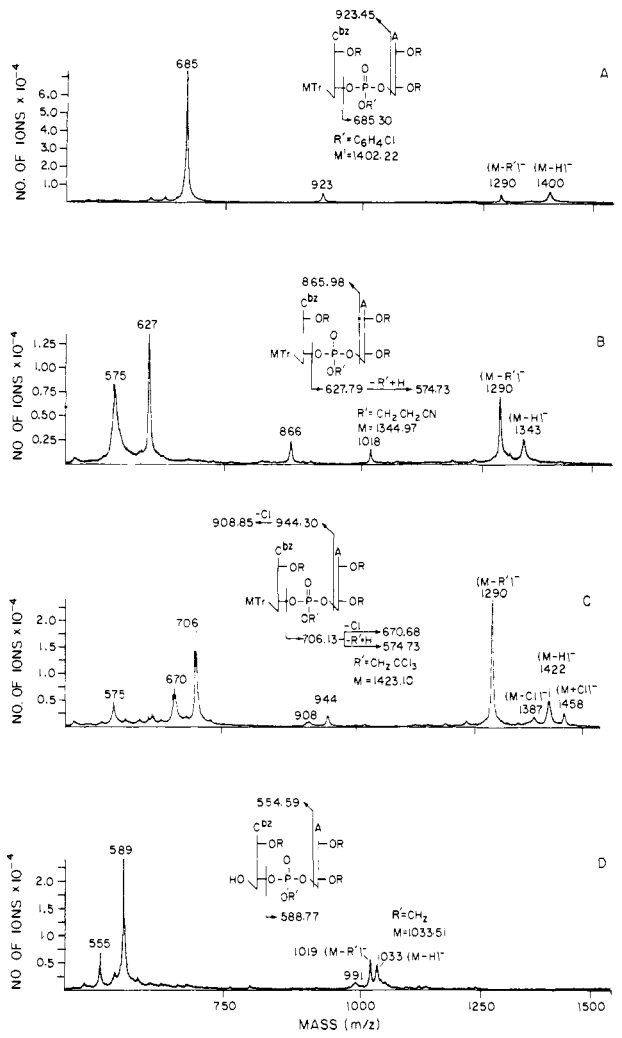


Figure 1. $^{252}$Cf PD negative ion spectra of four dinucleoside monophosphates bearing different phosphate or 5′-protecting groups. The shorthand structure and calculated average masses of the proposed fragmeant ions are shown in the inset figures. In each case data were acquired for $3.6 \times 10^2$ s and are plotted with 3 ns wide time bins. R = TBDMS (*tert*-butyldimethylsilyl), MTr = monomethoxytrityl, bz = benzoyl.

companied by the addition of two hydrogen atoms to form a positively charged nucleotide fragment. In $^{252}$Cf-PDMS, the sequence ions are only observed in the negative ion spectrum. The complimentary portion of each sequence ion, that is, the fragment bearing either a 5′- or a 3′-terminal nucleoside, formed by the rupture of the C5′–OP and C3′–OP bonds, is not typically observed. Positively charged mononucleoside fragment and several larger fragments bearing a positively charged terminal nucleoside are formed which are described in the third paper of this series but, in general, no positively charged fragments of regularly increasing size are formed. The absence of these fragments rules out the possibility that heterolytic dissociation of the C3′–OP, C5′–OP, and P–OR′ bonds occurs.

The overall conditions in $^{252}$Cf-PDMS that lead to the formation and desorption of molecular ions and to fragmentation depend on the environment around the nuclear fission track. Macfarlane first proposed that a hot spot is generated at the surface and ions are thermionically emitted from the hot spot at temperatures in excess of $10^4$ K on a picosecond time scale.[4] Although this model has been criticized in recent years, there is growing experimental and theoretical evidence that this is indeed the mechanism.[20,21] The most likely sequence of events that occurs for these molecules is that they are thermally excited while in contact with the hot spot at the surface generated by the fission fragment and have some probability for forming vibrationally-unstable states leading to fragmentation. Bond cleavage can occur during that time.

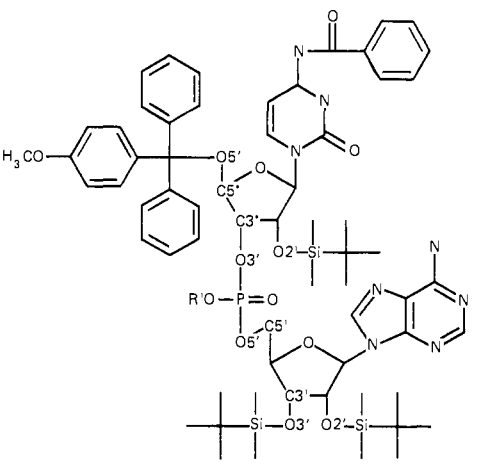

Figure 2. Structure of the dinucleoside monophosphate represented in Figure 1a. In the shorthand structure vertical lines correspond to the ribose moiety; horizontal lines to the right represent the 2′ and 3′ linkages; angled lines appearing on the left of the vertical line represent the 5′ linkage. The base linked to C1′ of each ribose sugar is indicated by the standard abbreviation at the top of the vertical line.

Recent theoretical studies indicate that the ionization of a positive or negative ion is associated with the desorption process involving a delicate interplay of the surface and adsorbate molecular states.[22,23] It is also possible that electron attachment to the adsorbate molecule in the desorption stage could be followed by dissociative resonance capture.

In negative chemical ionization mass spectrometry, one of the common ionization mechanisms leading to the formation of stable negative fragment ions is by dissociative resonance capture.[24] This process involves electron capture (via electron transfer or free electron capture) by the parent molecule followed by dissociation of the ion into a neutral fragment and a negatively charged fragment ion.

$$M^{-\cdot} \rightarrow A^- + B$$

In this scheme the $A^-$ ion would represent the negatively charged phosphate diester sequence ion (structure I) while the remaining nucleoside portion of the molecule would be lost as the neutral fragment, B. An analogous reaction involving nucleophilic attack at the carbon atom of trimethyl phosphate by anions such as $OH^-$ and $NH_2^-$ resulting in the formation of a phosphate diester anion and a small neutral molecule such as $CH_3OH$ was reported by Beauchamp, et al. utilizing the method of ion cyclotron resonance spectroscopy.[28] In this study the authors further emphasize that for phosphate triesters bearing dissimilar ester groups that phosphate diester anions characteristic of the mass of each ester group should be obtained. The results obtained by $^{252}$Cf-PDMS corroborate this observation.

**Acknowledgment.** This work was supported by grants from the National Institute of General Medical Sciences (GM26096), the National Science Foundation (CHE-79-04863), and the Robert A. Welch Foundation (A258), all awarded to R. D. Macfarlane, and the Natural Sciences and Engineering Research Council of Canada. We are indebted to Ronald Macfarlane for many fruitful discussions, and to Dennis Shelton, Randall Martin, and Michele Bailey for their technical assistance.

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