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PHOSPHORUS–OXYGEN BOND CLEAVAGES DURING TRIMETHYLSILYL-DERIVATIZATION OF CONDENSED POLYPHOSPHATES AND RELATED NUCLEOTIDES

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SUMMARY

The importance of unavoidable phosphorus–oxygen bond cleavage reactions during trimethylsilyl (TMS) derivatization of condensed polyphosphates is demonstrated. Pyrophosphate linkages are cleaved to produce significant amounts of the per-TMS-orthophosphate derivative whereas triphosphate linkages are cleaved to produce mixtures of per-TMS-ortho- and per-TMS-pyrophosphates. Present evidence is sufficient to show that all these processes occur primarily in the reagent solution during the derivatization procedures and not after injection into the gas–liquid chromatographic (GLC) column. A general mechanism to explain these complex reactions is discussed. It is concluded that these cleavage processes are sufficient to preclude trimethylsilylation as a useful approach to achieve quantitative GLC analysis of condensed polyphosphates and related nucleotides.

INTRODUCTION

Trimethylsilylation has found widespread usage for the rapid and quantitative preparation of thermally stable, volatile derivatives of compounds unsuited for direct analysis by gas–liquid chromatography (GLC). A wide range of organic compounds are amenable to trimethylsilyl (TMS) derivatization and subsequent GLC analysis[1]. Even certain inorganic anions such as borate, phosphite, and phosphate have been rendered volatile by TMS derivatization[2–9].

The general convenience and wide-range applicability of this class of derivatizing agents suggested they might be useful in the GLC analysis of some nucleotides, and indeed several papers demonstrating qualitative separations of TMS-derivatized nucleoside monophosphates have appeared in the literature[10–19]. However, it is noteworthy that no report of the GLC elution of TMS-derivatized nucleoside di- and triphosphates or of any other condensed organic phosphates has been published. To

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explain why is the subject of this paper, the first of several reports on our researches into a number of special problems associated with the TMS-derivatization and GLC analysis of inorganic and organic phosphates.

We have found that the major difficulty in the GLC analysis of nucleoside di- and triphosphates stems from unavoidable cleavages of the phosphorus–oxygen linkages during TMS-derivatization of these compounds[20]. These cleavage processes are confirmed by the model compound experiments reported in this paper using pure inorganic polyphosphates as analogs for the nucleoside polyphosphates. Our evidence establishes that these cleavages occur primarily in the reagent solution during the derivatization procedures and not after injection of this solution into the GLC column.

EXPERIMENTAL

Materials

The principal chemicals and other materials used in this study are listed as follows indicating chemical formula or name, special treatments, common acronyms used in this paper and supplier: $Na_4P_2O_7 \cdot 10H_2O$ and pyridine (redistilled reagent grade, stored over activated molecular sieve 13×) (Fisher Scientific, Pittsburgh, Pa., U.S.A.); $Na_5P_3O_{10}$ (Alfa Division, Ventron, Danvers, Mass., U.S.A.); hexamethyldisilizane (HMDS), trimethylchlorosilane (TMCS) and bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Pierce, Rockford, Ill., U.S.A.); 0.3-ml reaction vials (Regis, Morton Grove, Ill. U.S.A.); all GLC stationary phases and solid supports (Applied Science Labs., State College, Pa., U.S.A.).

Preparation of model compounds and TMS derivatization procedures

Ammonium salts of ortho-, pyro-, and triphosphoric acids were prepared by conversion of the respective sodium salts to their free acids via ion-exchange column chromatography using a 10-fold excess of Dowex 50W-X8 resin (protonic form), followed by neutralization in methanol with 1 *N* ammonium hydroxide. Precipitation of the ammonium salts was achieved by dilution with acetone. The gently air-dried salts were stored in a desiccator over indicating silica gel.

A typical TMS derivatization was performed by weighing out about 5.0 mg of each phosphate substance into a reaction vial and adding a known amount of phenanthrene as an internal standard. This sample was then TMS-derivatized by adding either solution system I (composed of 0.1 ml HMDS, 0.05 ml TMCS, and 0.2 ml of pyridine solvent) or solution system II (composed of 0.1 ml BSTFA, 0.01 ml TMCS and 0.2 ml of pyridine) and sealing with a serum cap. The tightly sealed reaction vials were then heated at 60° ± 2° on a hot plate fitted with an asbestos-insulated soft steel block drilled to accept the vials. The block temperature was regulated with a Variac connected to the hot plate, and monitored visually with a laboratory thermometer positioned in an oil-filled well in the same steel block.

GLC analysis

Aliquots of 1 or 2 μl were withdrawn directly from the sample vials with a 10-μl Series 701 Hamilton syringe and injected into a Hewlett-Packard model 5750 gas chromatograph equipped with dual column and dual flame ionization detectors.

The phosphate samples were run on a 4 ft. × 0.25 in. O.D. silanized glass column packed with 5% OV-17 on 80–100 mesh, acid-washed, dimethyldichlorosilane-treated Chromosorb W. A reference column of similar dimensions and packing but coated with 1% OV-101 was found ideal for the methylene unit measurements of product peaks reported herein. All GLC coated packings were prepared by the evaporative method[21]. Column temperatures were programmed from 72° (with a 2-min isothermal hold) to 180° at 20°/min using a helium carrier gas flow regulated at 25 ml/min. Injection block and detector chamber temperatures were each maintained at 210°.

RESULTS AND DISCUSSION

For convenience in the following discussion the abbreviated notations p-TMS-Pi, p-TMS-PPi, and p-TMS-PPPi refer to the per-trimethylsilylated derivatives of inorganic ortho-, pyro-, and triphosphate anions, respectively. When more detailed chemical structural formulae are required, the symbol TMS is substituted to represent the trimethylsilyl functional group, $(CH_3)_3Si$.

We find that phosphorus–oxygen bonds in both inorganic pyrophosphate (PPi) and triphosphate (PPPi) anions are cleaved in the reagent solution during TMS derivatization. Both substances produce GLC peaks identical to p-TMS-Pi and p-TMS-PPi, but no peak corresponding to p-TMS-PPPi is observed most probably because the phosphorus–oxygen linkages in the triphosphate anion are cleaved too rapidly and completely. No other single product peaks are recovered from the derivatization and cleavage reactions of these two polyphosphates.

Pyrophosphate is a simple and useful model for examining the phosphorus–oxygen cleavage process because the relative yields of both the parent derivative, p-TMS-PPi, and one of the cleavage products, p-TMS-Pi, are observed together by GLC as shown in Fig. 1. By measuring all peak areas relative to the internal standard, phenanthrene, we were able to monitor changes in each product yield during a typical 60° TMS derivatization procedure after a sample of ammonium pyrophosphate was exposed to the system I reagent combination for varying lengths of time as illustrated in Fig. 2. The slight amount of p-TMS-Pi observed initially is believed to be the result of a small unavoidable Pi contamination in the sample. The gradual decrease of p-TMS-PPi, after reaching its maximum yield at about 40-min reaction time, and the concomitant slow increase of p-TMS-Pi, indicates that phosphorus–oxygen bond cleavage is a gradual, long-term process.

Our data show considerably more scatter in measuring the yields of p-TMS-PPi than p-TMS-Pi. We believe this variation in reproducibility is real and stems from new phenomena taking place in the GLC inlet system after sample injection and is not to be confused with the unavoidable phosphorus–oxygen cleavage process occurring slowly in the derivatizing solution prior to injection. It is clear that any decomposition in the GLC apparatus would not produce the gradual shifts in p-TMS-PPi and p-TMS-Pi yields observed in Fig. 2. Rather, such decompositions would be expected to produce a more constant ratio of these two species. Furthermore, to produce a p-TMS-derivatized reaction product observable by GLC (such as p-TMS-Pi) solely from the decomposition of p-TMS-PPi after its injection, requires at least two consecutive and complex chemical processes: initial phosphorus–oxygen bond cleavage and subsequent p-TMS-derivatization of the fragments. It is difficult

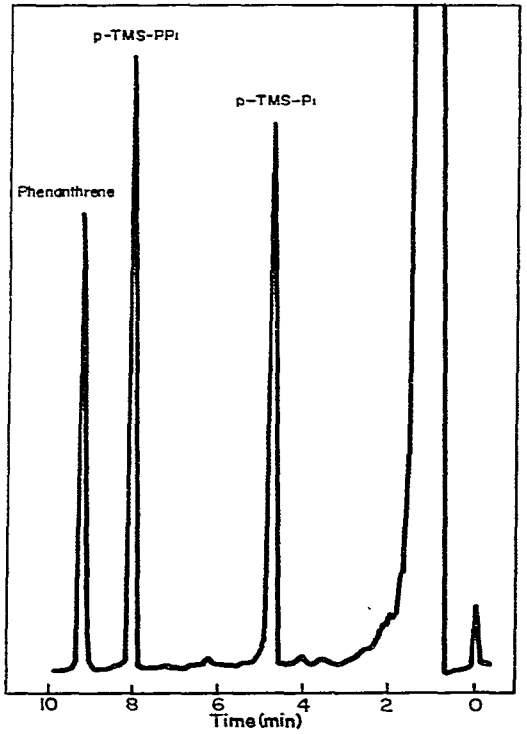


Fig. 1. Typical GLC trace following p-TMS derivatization of pyrophosphate.

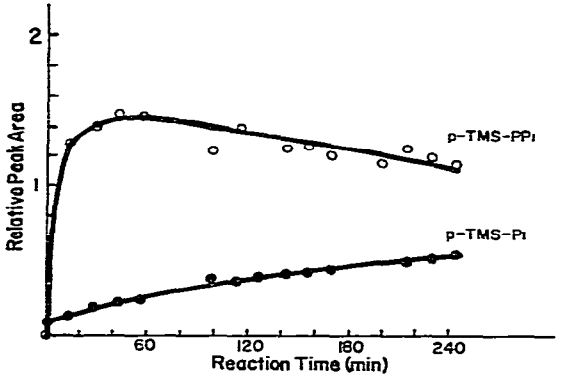


Fig. 2. Product yield *vs.* reaction time study for pyrophosphate in system I. Reagent at 60°.

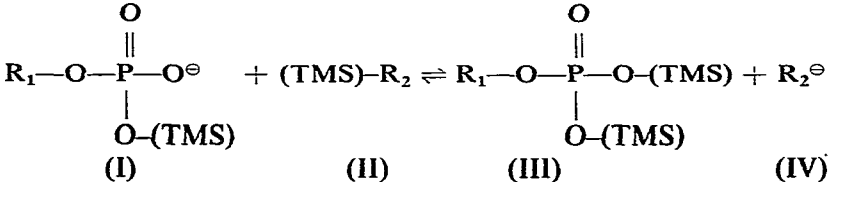
to imagine such a multi-step mechanism being completed in the gas phase during GLC analysis under conditions where the volatile excess TMS-reagents are being rapidly separated from the substrate as well as rapidly diluted by mixing with the carrier gas. Hence, we conclude that the required consecutive reactions are probable only in the reagent solution during TMS derivatization and that these reactions are

responsible for the time dependence revealed in Fig. 2. This conclusion is reinforced also by results obtained on derivatizing PPi with the system II reagent combination. In this case the yield of p-TMS-PPi rises more rapidly to a maximum after a reaction time of only 20 min at 60° before it slowly declines at a rate similar to that observed with system I in Fig. 2. This more rapid initial rise is consistent with the greater TMS donor ability previously reported for system II (ref. 1).

Cleavage of the phosphorus–oxygen bonds in pyro- and triphosphate was noted previously by Harvey and Horning[9]. They suggested that their p-TMS-Pi product was formed by "slow decomposition" on the GLC column. However, they presented no results to substantiate this suggestion or data similar to our Fig. 2. In addition, we believe the GLC peak shapes displayed in ref. 9 and in this paper further support our claim that the cleavage products are predominantly formed prior to GLC injection because chromatographic peaks derived by thermal decomposition on the GLC column would be expected to appear quite unsymmetrical and to tail considerably more than the relatively sharp and symmetric peaks observed in our Fig. 1 or in Fig. 2 of ref. 9. Indeed, Schep and De Clerk[22], investigating the decomposition of methylpolyphosphate to trimethylphosphate in the GLC inlet, observed a pronounced tailing of their pyrolysis product trimethylphosphate peak as compared to the peak obtained when an authentic sample of trimethylphosphate was injected for routine GLC analysis. In fact, these workers were able to estimate the rate constant for the GLC pyrolysis of methylpolyphosphate from these differences in peak shape.

We measured methylene unit values of our observed product peaks for p-TMS-Pi and p-TMS-PPi and found them to be 13.02 ± 0.05 and 17.34 ± 0.03, respectively. These data define the GLC retention behavior of these substances. The low standard deviations cited for these measurements indicate good reproducibility of the instrumental conditions. Our methylene unit values are consistent with but slightly higher than those reported previously[3,9]. These small differences probably arise from variations in the temperature programs used in each study, since GLC retentions are well known to be quite temperature-dependent[23].

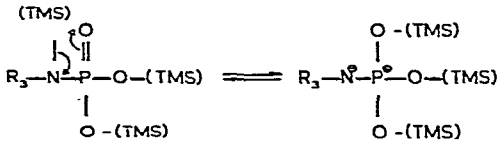
Although the exact mechanism by which both p-TMS derivatization and phosphorus–oxygen bond cleavage takes place is not fully established, the main features can be understood in terms of the following six prototype equilibrium reactions (written in a generalized notation to be explained) which can account for most of the data now known from model systems such as the ortho-, pyro- and triphosphate anions used in this study. Since polar or ionic compounds are involved in these TMS derivatizations, it is necessary to employ some polar, aprotic, and non-interfering solvent. We found pyridine (Pyr) to be the best choice for maximizing the formation of p-TMS-derivatives while minimizing undesirable cleavage reactions. Unfortunately, we found that significant phosphorus–oxygen bond cleavage is always unavoidable in polyphosphate systems.



(1)

$$\underset{\text{(V)}}{\text{II}} + n\,\text{Pyr} \rightleftharpoons \text{IV} + \underset{\text{(VI)}}{(\text{TMS})^{\oplus}(\text{Pyr})_n} \tag{2}$$

$$\text{III} + \text{IV} \rightleftharpoons \underset{\text{(VIIa)}}{R_2\text{—P(=O)(O–(TMS))—O–(TMS)}} + \underset{\text{(VIII)}}{R_1\text{–O}^{\ominus}} \tag{3}$$



(VIIa) (VII b) (4)

$$\text{VIII} + \text{II} \rightleftharpoons \underset{\text{(IX)}}{R_1\text{—O–(TMS)}} + \text{IV} \tag{5}$$

$$\text{VIII} + \text{VI} \rightleftharpoons \text{IX} + \text{V} \tag{6}$$

The first principal action of either TMS reagent system described in this work is p-TMS derivatization of each phosphate anion. This is a multi-step process which seeks to combine every available anionic and nucleophilic oxygen site with an electrophilic TMS group. Most available evidence suggests that each TMS derivatization is best described mechanistically as a bimolecular, nucleophilic substitution on silicon, or a S_N2 (Si) reaction. The last step in the substitution sequence of any specific phosphate substrate is the step which adds the last TMS group and forms each p-TMS product. This is illustrated by generalized reaction 1, where R_2 stands for the NH(TMS) group when using the HMDS reagent, or is the $CF_3CON(TMS)$ group when using BSTFA; and R_1 stands for the TMS group on the Pi substrate, but represents the $(TMS)_2PO_3$ and $(TMS)_3P_2O_6$ groups for the PPi and PPPi substrates, respectively. Hence, product III symbolizes the ultimate p-TMS-derivative obtainable from the penultimate intermediate I; and IV symbolizes the specific anion displaced when using either of our TMS reagent systems, with the understanding that one such anion is produced each time a TMS group is transferred to a phosphate oxygen.

Anion IV is also formed along with the solvated electrophile VI via the slow, solvent-assisted reaction 2. This process is analogous to the first ionization step in a unimolecular, nucleophilic substitution, or S_N1 mechanism, whereby the bulky TMS group tends to ionize to relieve steric strain and is further stabilized in polar solvents by solvation and by hyperconjugation effects which minimize electrostatic forces between the product ions.

The second principal action of TMS reagents, characterized experimentally and discussed here for the first time, is their unavoidable ability to induce phosphorus–oxygen bond cleavage. We postulate that such cleavage is the result of bimolecular,

nucleophilic substitution on phosphorus, $S_N2(P)$, as displayed in general reaction 3 for which there are other precedents in phosphorus chemistry[24]. Product VIIa lacks the fourth oxygen atom on one phosphorus and instead contains a phosphorus–nitrogen bond. VIIa can also be rearranged intramolecularly to the more ionic ylid isomer, VIIb, as shown in more detail in reaction 4, where R_3 is simply H when using the HMDS reagent or is the CF_3CO group when using BSTFA. This rearrangement process is favored in the latter system because of the greater inductive effect of the CF_3CO group. Both isomers confer considerable polarity and consequent non-volatility on these molecules. Furthermore, we believe such structures are sensitive to thermal cracking which leads inevitably to the accumulation of a gray polymeric residue in the GLC inlet chamber[20].

Finally, to complete our generalized mechanism, the anion VIII displaced via reaction 3 is TMS derivatized by competing reactions 5 or 6. Either process converts VIII to the volatile p-TMS product IX having one less phosphorus atom than its precursor, reactant III, assuming as we do from our evidence that the terminal phosphate group is the one preferentially cleaved-off by reaction 3. As long as product IX contains a terminal phosphate, it can in turn play the role of a new species III and be recycled beginning with reaction 3 to undergo further cleavage. However, we note that the probability for reaction 3 diminishes with structure in the order: p-TMS-PPPi $\gg$ p-TMS-PPi $\gg$ p-TMS-Pi.

It is worth noting that although it is theoretically possible for any p-TMS-monophosphate to undergo cleavage analogous to reaction 3, the tendency to do so is negligibly small in the case of p-TMS-Pi. However, this is not so for all organic monophosphates, including the nucleoside monophosphates. Also there is no doubt that nucleoside di- and triphosphates are far more unstable toward phosphorus–oxygen bond cleavage and the thermal cracking reactions than the inorganic model compounds used in this study[20]. Hence, we conclude that reaction 3 processes are both sufficiently unavoidable and indeterminate enough to preclude trimethylsilylation as a useful approach to achieve a general method for the quantitative GLC analysis of condensed polyphosphates and related nucleotides.

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