Investigation of activation of phosphate groups in mono- and oligonucleotides with mesitoyl chloride


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ABSTRACT

It has been demonstrated with the use of $^{31}$P NMR pulsed spectroscopy that the reaction of mesitoyl chloride (MsCOCl) both with terminal and internucleotide phosphate groups pA, d(MeOTr)TpT and dpTpT (Ac) proceeds in a quantitative fashion within less than 2 min at 0°C with the respective mixed anhydrides being thereby formed. The anhydrides of phosphomonoesters are resistant, unlike those of phosphodiesters which may be readily split by water, alcohol or amine without the internucleotide bonds being broken.

Treatment of poly(U) with an excess of MsCOCl leads to rapid cyclization followed by formation of phosphotriesters. A comparatively easy hydrolysis leads to partial cleavage and isomerization of internucleotide bonds. A similar treatment of UpC showed that about 20% of the internucleotide bonds are cleaved, the remaining UpC being a mixture of approximately equal amounts of 3'-5' - and 2'-5' -isomers.

INTRODUCTION

It was previously shown that mixed anhydrides of mono- and oligonucleotides with mesitioic acid are active phosphorylating agents for various nucleophiles (1). The anhydrides were found to be sufficiently stable in aqueous solution to be used as affinity labelling reagents (2). They also proved to be useful as reactive intermediates for preparing some other mono- and oligonucleotide derivatives with a modified end phosphate group (3).

The wide application of the anhydrides made it feasible to study the key reaction, namely interaction of mesitoyl chloride (MsCOCl) with oligonucleotides. Of special interest was the reaction of MsCOCl with internucleotide phosphates.

In recent years pulsed $^{31}$P NMR spectroscopy was demonstrated to be a highly effective method for studying conversions of phosphate groups in the course of oligonucleotide synthesis (4), formation of some oligonucleotide derivatives (5) and other reactions.

The present paper deals with a $^{31}$P NMR spectroscopy investigation of the reaction.
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of MsCOCl with mono- and oligonucleotides and subsequent reactions of the mixed anhydrides formed with nucleophilic reagents. It was found that MsCOCl acylates internucleotide phosphate groups as well as the end phosphate groups both in deoxyribo- and ribonucleotides.

MATERIALS AND METHODS

d(MeOTr) TpT and dp-TpT(Ac) were prepared according to (6). UpC kindly provided by N. S. Sidorova (Leningrad Institute of High Molecular Weight Compounds, the USSR Academy of Science. MsCOCl was prepared as described in (7). The reactions of mono- and oligodeoxynucleotides with MsCOCl were performed as described previously (3) in 10 mm tubes (1.5 ml of the reaction mixture). Paper chromatography was performed by the ascending technique on FN-1 (Filtrak, DDR) in solvent system ethanol-1 M ammonium acetate (7:3 v/v), pH 7.5. In this system the Rf values of nBuNHpA and CH3OpA were 0.70 and 0.68, respectively. Pyridine with water content less than 0.5% stored above molecular sieves 4A was used as a solvent.

The 31P NMR spectra were taken with a Bruker HX-90 pulse spectrometer operating at 36.43 MHz. Fourier transform was performed using a Bruker B-NC 12-FFT computer after 100-500 accumulations. The pulse width was 15μsec and the time between pulses was 0.7-0.8 sec. D2O was used as an external standard for stabilization of the resonance conditions. The majority of the spectra were recorded with heteronuclear spin-spin decoupling 31P-1H. The chemical shifts are reported in ppm related to external 85% H3PO4 and are judged to be 0 ppm accurate. The initial concentration of 31P atoms was 0.07-0.15 M in all experiments. Poly(U) from Calbiochem Co was converted into the cetavlon salt with the help of a 4-fold excess of cetyltrimethylammonium bromide. After the preparation had been dried over P2O5, 121 mg of cetavlon salt of poly(U) was dissolved in 1.5 ml of anhydrous pyridine and 3.6 ml of MsCOCl were added. 30 Min later 0.5 ml of water was added to the reaction mixture. 2 Hours later the solution was evaporated and the precipitate was washed 3 times with ether. After that the precipitate was dissolved in 1 ml 1.5 M NaCl and precipitated with a 3-fold volume of ethanol which was adjusted to pH 5 by 0.02 M CH3COOK. This was carried out three times and the Na salt of poly(U) was precipitated from water by a 3-fold excess of ethanol.

12 ou of the isolated polymer of uridylic acid were hydrolysed by RNase (E.C. 2.7.16) for 20 hours at 37° as described in (8). The hydrolysate was analyzed by microcolumn chromatography as described by Tomlinson-Tener in a MSTP-1 device as described.
UpC treated with MsCOCl as described in (3) was isolated by t.l.c. on Silufol sheets in system iPrOH-NH$_3$-H$_2$O (7:1:2 v/v). The $R_f$ value for UpC was 0.4. RNase hydrolysis and analysis of the hydrolysate was carried out as in the case of poly(U).

RESULTS AND DISCUSSION

Fig. 1a shows a $^{31}$P NMR spectrum of the reaction mixture obtained by treatment at 30° of pA in pyridine solution with 5 eqv of MsCOCl recorded 1 hour after addition of anhydride. No signal of the starting pA ($\delta = 0.72$ ppm) is seen. Two main signals are singlets at 8 ppm and 16.6 ppm (intensities ratio 3:1) are present in the spectrum. Addition of water results 2 min later in complete disappearance of the latter signal.

From the previous chemical data it is well known that the above procedure leads to mixed anhydride MsCOpA as a single product of the nucleotide conversion. Therefore the signal at 8 ppm may be identified as corresponding to MsCOpA. The upfield shift of the signal as compared to pA is typical of the formation of a phosphoanhydride bond (10).

The signal at 16.6 ppm due to additional upfield shift may be identified as corresponding to some compound with two anhydride bonds at P atom. This may be either I or II:

\[
\begin{align*}
&\text{MsCO-O} \quad \text{MsCO-O-P-O-AdO} \\
&\text{MsCO-O-} \quad \text{O} \\
&\text{O} \\
&\text{O} \\
&\text{MsCO-O-P-O-AdO} \\
&\text{O}
\end{align*}
\]

(I) \hspace{1cm} (II)

To discriminate between these two possibilities n-BuNH$_2$ or CH$_3$OH were added to the reaction mixture. In both cases signal at 16.6 ppm disappeared immediately and no new signal appeared. This means that the compound with $\delta = 16.6$ ppm converted completely to MsCOpA. This may only be in case I. Compound II should react with n-BuNH$_2$ and CH$_3$OH with formation of n-butylamide and methyl ester derivative of pA or asymmetric pyrophosphate which had the specific signals in the NMR spectra (11).

The data presented demonstrate that the nucleophiles attack I at the carbonyl group of mesitoic acid with the anion of the stronger acid being eliminated.

The reaction of pA with MsCOCl is extremely fast. At 0° 2 min after addition of 5 eqv of MsCOCl to a pyridine solution of pA, the $^{31}$P NMR spectrum of the reaction
mixture is mostly represented by a signal with $\delta = 7.7$ ppm (>98% of intensity). The signal with $\delta = 16.6$ ppm is very small (Fig. 1b).

Incubation of MsCOmA in pyridine solution with either n-BuNH$_2$ or CH$_3$OH results in a lower intensity of the signal of MsCOmA at 7-8 ppm and in the appearance of signals at - 7.1 ppm and -0.6 ppm, respectively. The former is typical of a phosphoamide derivative (5), the latter - of a phosphodiester (10). In accordance with the $^{31}$P NMR data, the presence of either n-BuNHpA or CH$_3$OpA was demonstrated in the respective reaction mixtures by paper chromatography and microcolumn chromatography on DEAE-cellulose. As seen in Fig. 1c, 60% of MsCOmA at 30° are converted to n-BuNHpA within 70 min. The reaction of MsCOmA with CH$_3$OH proceeds significantly slower: 45% of MsCOmA are converted within 24 hours (Fig. 1d.) The data are in agreement with the previously found dependence of the reaction rate of MsCOmA on the nucleophilicity of the reagent.

![Fig. 1](image)

To study the interaction of MsCOCl with internucleotide phosphate, 3 eqv of MsCOCl were added to pyridine solution of d(MeOTr)TpT at 30°; 2 min later in the $^{31}$P NMR spectrum of the reaction mixture there is no signal of the starting dinucleoside phosphate ($\delta = 1.0$ ppm) and the spectrum represents two close singlets at 9.0 and 9.3 ppm (Fig. 2a). The upfield shift is typical of anhydride bond formation (10); therefore the signals may be assigned to two diastereomers (12) of compound III:

$$\text{MeOTr-T-O-P-O-T}$$

$$\text{CoMs}$$

(III)
Addition of water results in immediate (2 min) disappearance of both signals with regeneration of the starting signal of dinucleoside phosphate ($\delta = 1.05$ ppm, Fig. 2b). The same changes occur after addition to the solution of III of n-BuNH$_2$ or C$_2$H$_5$OH.

![Fig. 2.](image)

The regeneration of dinucleoside phosphate is accomplished within less than 2 min with BuNH$_2$ and within 30 min with ethanol.

Similarly to the reaction with I, nucleophiles attack the carbonyl group of mesitoic acid of III.

Addition of a six-fold excess of MsCOCl to pyridine solution of dpTpT (Ac) at 0° results in 2 min in complete disappearance in the $^3$P NMR spectrum of the signals of the end ($\delta = -0.12$ ppm) and internucleotide ($\delta = 2.3$ ppm) phosphate groups (Fig. 3a, b). Instead, two new signals of equal intensities with $\delta = 7.9$ and 9.4 ppm appear, thus indicating immediate conversion of both end and internucleotide phosphate to mixed anhydrides with mesitoic acid. Due to significant broadening of the signal

![Fig. 3.](image)
with the centre at 9.4 ppm two signals for diastereomers of compound IV could not be resolved.

Subsequent addition of water at 0° results in rather rapid hydrolysis of the anhydride bond with internucleotide phosphate. In 25 min the $^{31}$P NMR spectrum is represented by three signals 1.5, 8.4 and 9.4 ppm with an intensity ratio of 33:50:17. (Fig. 3c). The signals may be assigned to internucleotide phosphate and mixed anhydrides of the end and internal phosphates, respectively. The $\delta$ values differ from those in absolute pyridine, probably due to association of the charged phosphate groups with water. The mixed anhydride with the end phosphate group remains unchanged in these conditions. Therefore, the reaction scheme may be visualized as follows:

$$
\text{dpTpT(Ac)} \stackrel{\text{MsCOCl}}{\rightarrow} \text{dMsCOpTpT(Ac)} \stackrel{\text{H}_{2}\text{O}}{\rightarrow} \text{dMsCOpTpT(Ac)}
$$

The final $^{31}$P NMR spectrum of the reaction mixture (30 min after addition of water) had three signals at -18.5, 0.6 and 0.8 ppm, the integral intensities ratio being 1:2:2 (Fig. 4c).
The signal at $\delta = -18.5$ ppm which is retained on a long-term treatment with water may be assigned to the end cyclodiesteric group in compounds of type VIII formed as a result of hydrolysis of compound VII. (see scheme below)

The signals at 0.6 and 0.8 ppm are in the range typical of internucleotide phosphate groups. These groups have to appear due to hydrolysis of one of the P-O-C bonds of the cyclotriester VII. Two signals are likely to belong to 3'-5' (IX) and 2'-5' (X) isomers. The isomerisation of phosphodiester bonds was proved by pancreatic RNase hydrolysis of poly(U) pretreated with MsCOCl and water. Microcolumn chromatography of the digest on DEAE-cellulose in 7 M urea demonstrates the presence of non-hydrolysed oligonucleotides of various lengths (Fig. 5a)

Therefore the scheme of the reactions proceeding on subsequent treatment of poly(U) with MsCOCl and water may be represented as follows
In agreement with this scheme are the data obtained with natural ribodinucleoside phosphate, UpC. The oligonucleotide was treated by a 100-fold excess of MsCOCl in anhydrous pyridine (20 min, 20°C). After treatment with water 18% Up was found in the reaction mixture by thin layer chromatography. This means about 20% hydrolysis of internucleotide bond. RNAse digest of the remaining UpC consist of cytidine, uridine-3'-phosphate and non-hydrolysable UpC (Fig. 5b). The 1:1 ratio of Up and UpC means that the phosphodiester bonds are isomerised by 50%.

The data obtained demonstrate that acylation of internucleotide phosphate groups takes place by treatment with MsCOCl. Further conversions of the mixed anhydrides formed by sterically hindered carboxylic acid and phosphodiester group depend in the nature of oligo(poly)nucleotide. In deoxyribooligonucleotide derivatives acylphosphate is stable in absolute pyridine and is easily split by nucleophilic agents, the nucleophile attacking exclusively the carbons of a carbonyl C atom. With oligo (poly)ribonucleotides acylphosphate group (VI) is unstable and converts immediately to cyclophosphotriester group (VIII) with the acyl residues being split off. Further reaction with water results in the splitting of internucleotide bond (about 20%) and isomerisation of the remaining internucleotide bonds.

Fig. 5. Chromatographic analysis of ribonuclease hydrolyzate of (a) poly(U) and (b) UpC treated successively with MsCOCl and water in a 1x60 mm micro-column packed with cellulose DEAE. Elution was performed in a linear NaCl gradient in 7 M urea at pH 7.5.
REFERENCES