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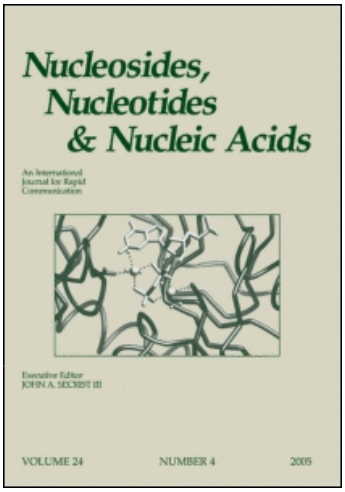

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
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Nucleoside H-Phosphonates. V. The Mechanism of Hydrogenphosphonate Diester Formation Using Acyl Chlorides as Coupling Agents in Oligonucleotide Synthesis by the Hydrogenphosphonate Approach

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To cite this Article Garegg, Per J. , Regberg, Tor , Stawinski, Jacek and Strömberg, Roger(1987) 'Nucleoside H-Phosphonates. V. The Mechanism of Hydrogenphosphonate Diester Formation Using Acyl Chlorides as Coupling Agents in Oligonucleotide Synthesis by the Hydrogenphosphonate Approach', Nucleosides, Nucleotides and Nucleic Acids, 6: 3, 655 — 662
To link to this Article: DOI: 10.1080/07328318708069994
URL: http://dx.doi.org/10.1080/07328318708069994

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NUCLEOSIDES & NUCLEOTIDES, 6(3), 655-662 (1987)

NUCLEOSIDE H-PHOSPHONATES. V. THE MECHANISM OF HYDROGENPHOSPHONATE DIESTER FORMATION USING ACYL CHLORIDES AS COUPLING AGENTS IN OLIGONUCLEOTIDE SYNTHESIS BY THE HYDROGENPHOSPHONATE APPROACH

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Abstract

The H-phosphono-acyl mixed anhydrides of type III were found to be the main intermediates during H-phosphonate diester formation using acyl chlorides as coupling agents in the reaction of hydrogenphosphonate monoesters with hydroxylic components.

Recently we have reported on the use of nucleoside hydrogenphosphonates as new intermediates in oligonucleotide synthesis[1,2]. It was found that H-phosphonate diesters are formed rapidly and in high yield when nucleoside 3'- H-phosphonates are activated in the presence of a nucleoside with a free 5'-OH function ("regular" coupling), but the yield decreased significantly when H-phosphonate monoesters were mixed with the coupling agent (diester chlorophosphate[1,2], aryl sulfonic acid derivatives[1,2] or pivaloyl chloride[2,3]) before the addition of the hydroxylic component (coupling with preactivation).

These rather unexpected findings prompted us to gain deeper insight into these reactions and thus we decided to investigate the activation process and the coupling reaction using ^{31}P NMR spectroscopy. Since pivaloyl chloride (PV-Cl) proved to be a most suitable coupling agent, both for solution[1] and solid phase[2] synthesis of oligonucleotides, we chose it for these studies.[4]

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RESULTS AND DISCUSSION

In the three regular coupling reactions investigated, Ia + 3'-O-benzoylthymidine (HO-T-OBz), Ia + ethanol, and Ib + ethanol, in the presence of pivaloyl chloride (PV-Cl, 2.5 equiv.) in pyridine, no reactive intermediates could be detected by ^{31}P NMR spectroscopy, and the first spectrum recorded (after ca. 2 min) showed quantitative conversion of the starting materials into H-phosphonate diesters IIa-c.

Completely different ^{31}P NMR spectra emerged when the above reactions were carried out with preactivation (ca. 30 sec) of I using an acyl chloride, before the addition of the hydroxylic component. In all cases the reaction mixtures then consisted of three compounds with the ^{31}P NMR chemical shifts at ca. 139, 132 and 122 ppm and practically no H-phosphonate diesters II were detected.

The chemical shifts and the absence of $^{1}J_{PH}$ coupling constants in undecoupled spectra indicated the presence of trivalent species. The signals at 122 and 132 ppm disappeared immediately upon addition of water and that at 139 ppm after ca. 5 min, producing signals at ca. 8 ppm (H-phosphonate diesters) and 1.5 ppm (H-phosphonate monoesters).

To identify these intermediates, the activation of I has been carried out without a hydroxylic component. By adding 2.5 equiv. of PV-Cl to Ia or Ib in pyridine, intermediates with chemical shifts 122.3 ppm (doublet, $^{3}J_{PH}$ 9.7 Hz) and 122.6 ppm (triplet, $^{3}J_{PH}$ 7.0 Hz) respectively, were observed. When both Ia and Ib were activated together, the ^{31}P NMR spectrum showed only those two intermediates. Both were converted into the respective starting materials upon addition of water. These findings indicate that the reactive species generated from I and PV-Cl contain one phosphorus atom and one residue of nucleoside or one ethyl group. The only structure which is consistent with this, is the bis-acylphosphite V.[5]

Further support for this structure came from the reaction of V with ethanol. According to our expectations, the addition of 1 equiv. of ethanol to Va resulted in two singlets at 132.0 and 131.6 ppm (diastereoisomers of diester acyl phosphite VIb), a singlet at 138.0 ppm (phosphite triester VIIb) and the starting material, bis-acylphosphite Va, at 122.3 ppm. A spectrum without ^{1}H-heteronuclear decoupling confirmed the above assignments. Thus the two singlets at 132.0 and 131.6 ppm appeared as two overlapping quartets ($^{3}J_{PH}$ 7.9 Hz and $^{3}J_{PH}$ 9.3 Hz) and

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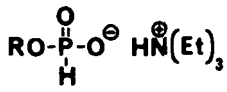
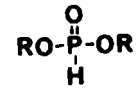
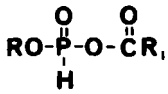


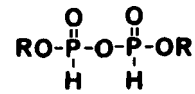
Ia R=5'-O-dimethoxytritylthymidine-3'-yl(DMT-T-)
Ib R=ethyl



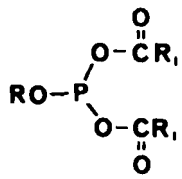
IIa R=DMT-T-, R_1=-T-OBz
IIb R=DMT-T-, R_1=ethyl
IIc R=R_1=ethyl



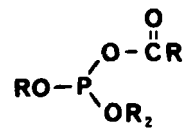
IIIa R=DMT-T-, R_1=t-butyl
IIIb R=ethyl , R_1=t-butyl



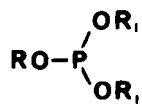
IVa R=DMT-T-
IVb R=ethyl



Va R=DMT-T-, R_1=t-butyl
Vb R=ethyl , R_1=t-butyl



VIa R=DMT-T-, R_1=t-butyl, R_2=-T-OBz
VIb R=DMT-T-, R_1=t-butyl, R_2=ethyl
VIc R=R_2=ethyl, R_1=t-butyl



VIIa R=DMT-T-, R_1=-T-OBz
VIIb R=DMT-T-, R_1=ethyl
VIIc R=R_1=ethyl

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Table 1. ^{31}P NMR data (ppm, relative to 2% H_3PO_4)

Ia	1.5	IVa	-2.3	VIc	131.9
Ib	1.3	IVb	-4.0	VIIa	139.1
IIa	8.1; 9.6	Va	122.3	VIIb	138.0
IIb	7.5*	Vb	122.6	VIIc	138.8
IIc	7.3	VIa	132.2*		
IIIa	1.1	VIb	132.0; 131.6		
IIIb	1.2				

*Diastereoisomers not resolved

the singlet at 138.0 ppm, as a sextet ($^3J_{PH}$ 7.9 Hz). Addition of another equivalent of ethanol caused immediate disappearance of the signals from Va and VIb, and only the signal from phosphite triester VIIb at 138.0 ppm was present in the ^{31}P NMR spectrum.

Upon addition of water, the latter compound was converted into a mixture of the H-phosphonate diesters IIb and IIc, apparently as a result of unspecific hydrolysis.

The same pathway was found to be true also for the reactions of nucleoside bis-acylphosphite Va and ethyl bis-acylphosphite Vb with other hydroxylic components (for chemical shifts, see the Table).

Formation of diester acyl phosphites VI and phosphite triesters VII, may theoretically also arise as a consequence of activation of H-phosphonate diesters II by PV-Cl. This, however, has to be excluded in light of complete resistance of H-phosphonate diesters towards further activation, even with an excess (10 equiv.) of PV-Cl. Thus, the reaction pathway which can lead to VI and VII, must have bis-acylphosphite V as an intermediate.

The fact that none of the compounds of type V, VI or VII were detected during the coupling reaction without preactivation, strongly indicates that V can not be considered as an intermediate during the regular coupling reaction in solution.

Searching for reactive intermediates, which can be involved in the coupling reaction when activation is carried out in the presence of a hydroxylic component, we investigated the reaction of I with limited amounts of acyl chloride.

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With 0.5 equiv. PV-Cl one would expect formation of the H-phosphono-acyl anhydride of type III, which should react with I forming the H-pyrophosphonate IV. With 1.5 equiv. of coupling agent, the ^{31}P NMR spectrum should be similar or, if the formation of III is faster than the subsequent reaction with I, the mixed anhydride III should be the main reactive intermediate.

Indeed, we found, that addition of 1.5 equiv. of PV-Cl to Ib in pyridine mainly caused the formation of IIIb (1.2 ppm, $^{1}J_{PH}$ 732 Hz, $^{3}J_{PH}$ 9.8 Hz)[7] and small amounts (less then 10%) of H-pyrophosphonate IVb (-4.0 ppm, $^{1}J_{PH}$ 780 Hz)[8]. However, when more H-phosphonate Ib was added to such a mixture, we did not observe the expected reaction Ib + IIIb ⟶ IVb. Since it is rather unlikely that Ib is not nucleophilic enough to react with the mixed anhydride IIIb, these findings indicate, that there is an equilibrium IIIb ⇌ IVb, which is to the left. The reaction of Ib with 0.5 equiv. of PV-Cl, as expected, also failed to produced substantial amounts of H-pyrophosphonate IVb, and the reaction mixture consisted of Ib (80%) and IIIb (20%).

The ^{31}P NMR spectra of the analogous reaction of nucleoside H-phosphonate Ia with 1.5 equiv. of PV-Cl showed similar types of intermediates, but because of significant broadening of peaks, detailed ^{31}P NMR analysis could not be done.

Thus, we continued with ethyl H-phosphonate as a model compound to clarify the activation process further. Since, in the activation of H-phosphonate monoesters by PV-Cl, nucleophilic or base catalysis may be involved, we investigated the role of pyridine in the above reaction. When Ib was allowed to react with 1.5 equiv. of PV-Cl in acetonitrile, the ^{31}P NMR spectrum showed formation of mixed anhydride IIIb and H-pyrophosphonate IVb (ratio ca 1 : 1), which upon addition of ethanol (1.5 equiv.) were converted into H-phosphonate diester IIc and H-phosphonate monoester Ib (ratio ca 2 : 1). When, instead of ethanol, more PV-Cl was added (3 equiv.), the only change observed in the ^{31}P NMR spectrum was the amost complete conversion of H-pyrophosphonate IVb into the mixed anhydride IIIb. However, addition of 4 equiv. of pyridine, resulted in appearance of the signal at 122.6 ppm (bis-acylphosphite Vb).

These experiments demonstrated that formation of the mixed anhydride IIIb and H-pyrophosphonate IVb, the conversion of IVb ⟶ IIIb, as well as the subseqent reaction of these intermediates with added

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ethanol, do not require pyridine. However, pyridine, or another base[9], is necessary for the further activation of IIIb by PV-Cl, which results in the formation of bis-acylphosphite Vb.

To sum up, we conclude on the basis of the above experiments, that the mixed anhydrides of type III are the main reactive species involved in H-phosphonate diester formation using acyl chlorides as activators. Since H-pyrophosphonates IV also seem to be reactive species, they can contribute to the formation of H-phosphonate diesters, but probably to a lesser extent, because of the unfavourable equilibrium III $\rightleftharpoons$ IV, which is to the left.

The important synthetic implication of these studies is, that preactivation of H-phosphonate I should be avoided since it causes the alternative reaction pathway *via* intermediate V to become the predominant one. Another possibility to eliminate this undesired reaction pathway during condensation, is to carry out the coupling in acetonitrile with limited amounts of pyridine, since the latter promote the conversion of mixed anhydride III into bis-acylphosphite V.

During the regular coupling reaction, the pathway *via* intermediate V is suppressed even in pyridine since the reaction of III with the hydroxylic component is faster than conversion of III into V. However, if for any reason, the coupling reaction is slowed down (e. g. coupling with nucleoside bound to solid support), this alternative reaction pathway may start to compete with the desired one, proceeding *via* intermediates III (or *via* III and IV). Synthesis of the hexamer $d(Ap)_5A$ on solid support has shown, that the coupling reaction was very fast[2] and only the desired product was formed. However, when the same synthesis was carried out with preactivation of nucleoside H-phosphonate with PV-Cl, polyacryamide gel electrophoresis (PAGE) revealed presence of the desired hexamer together with shorter oligonucleotides[2]. Interestingly, the difference between the two syntheses was smaller than one could expect from the synthesis of dimers (with *versus* without preactivation) in solution. This probably reflects the differences in reaction conditions during solid phase and solution synthesis of oligonucleotides, and also indicates, that the extent of side reactions can be different in the two types of synthesis.

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EXPERIMENTAL PART

Reactions were carried out in NMR tubes and spectra were recorded on a Varian Associates XL-100 FT (40.48 MHz) spectrometer. Chemical shifts are reported relative to 2% H_3PO_4 in D_2O (inner tube). The value of chemical shifts for the intermediates produced *in situ*, in some cases varied (± 1 ppm), depending on the reaction conditions.

Pyridine was refluxed and distilled over P_2O_5, then refluxed and distilled over CaH_2 and stored over 3Å molecular sieves. The same procedure was used for the preparation of anhydrous acetonitrile.

5'-O-dimethoxytritylthymidine 3'- hydrogenphosphonate[1] and ethyl hydrogenphosphonate[6] (both as triethylammonium salts) were prepared according to published procedures. Phosphite triesters VIIa,b, as references for the ^{31}P NMR analysis, were prepared in the reaction of 5'-O-dimethoxytritylthymidine with PCl_3 (1.2 equiv.) in pyridine, followed by addition of 3'-O-benzoylthymidine or ethanol.

Pivaloyl chloride, diethyl hydrogenphosphonate and triethyl phosphite were commercial grade (Aldrich).

General procedure for the "regular" coupling reactions (synthesis of II).

H-phosphonate I (0.2 mmoles) and a hydroxylic component (0.22 mmoles), after concentration from pyridine solution, were dissolved in the same solvent (3 ml) and pivaloyl chloride (0.5 mmoles) was added. The ^{31}P NMR spectra were recorded after mixing the reagents.

General procedure for "preactivation".

H-phosphonate Ia (0.2 mmoles), concentrated from a pyridine solution, was dissolved in pyridine (3 ml), or in the case of Ib, in pyridine or in acetonitrile, and pivaloyl chloride (2.5 equiv. or as stated in the text) was added. To identify the intermediates formed during the activation process and to investigate their chemical reactivity, hydroxylic components or water were added as specified in the text. The ^{31}P NMR spectra were recorded after each step.

ACKNOWLEDGEMENTS

We are indebted to Prof. Bengt Lindberg for his interest, to the Swedish National Board for Technical Development and The Swedish Natural Science Research Council for financial support.

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3. B. C. Froehler and M. D. Matteucci, Tetrahedron Lett., 27, 469 (1986).
4. Studies on the activation of H-phosphonate monoesters by aryl sulfonyl derivatives and by chlorophosphates are in progress and will be published eleswhere.
5. With other acyl chlorides, the same type of intermediates were formed. E. g. with anisolyl chloride, the intermediate of type V (R=ethyl, R_1= pCH_3O-Ph-) and VI (R=R_2=ethyl, R_1= pCH_3O-Ph-) were observed at 123.9 ppm (triplet, $^3J_{PH}$ 7.6 Hz) and at 132.4 (quintet, $^3J_{PH}$ 7.8 Hz) respectively.
6. P. R. Hammond, J. Chem. Soc., 252, (1962).
7. Compound IIIb was produced as a single intermediate in the reaction of Ib with 2 equiv. of PV-Cl and pyridine in acetonitrile. Addition of ethanol (1 equiv.) to such a reaction mixture, produced immediately and almost quantitatively, as judged from ^{31}P NMR spectra, H-phosphonate diester IIc.
8. Compound IVb was produced as a single intermediate in the reaction of Ib with 1 equiv. of benzenesulfonyl chloride (or diphenylchlorophosphate) and 2 equiv. of pyridine, in acetonitrile. Addition of ethanol (1 equiv.) to this reaction mixture resulted in the formation of H-phosphonate diester IIc and H-phosphonate monoester Ib in the ratio of ca. 1 : 1.
9. In this reaction, pyridine seems to act as a base, since the same results were obtained when pyridine was replaced by triethylamine.

Received July 8, 1986.