Synthesis of Nucleic Acid Fragments with 3′-Deoxy-3′-*C*-Methylenephosphonate Linkages − Oxidation Of Nucleoside 3′-Deoxy-3′-*C*-Methylenephosphinate Esters

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A study on the iodine oxidation of protected thymidine 5′-(uridine 3′-deoxy-3′-*C*-methylenephosphinate) (**1**) to the corresponding thymidine 5′-(uridine 3′-deoxy-3′-*C*-methylenephosphonate) (**2**) is reported. Oxidation with 80 mM iodine in pyridine/water (98:2, v/v) required 24 h for completion. The reaction is catalyzed by pyridinium ion as well as by triethylamine (TEA). The reactions appear to occur *via* the tricoordinate form of **1**: the anion in the TEA-promoted reaction and the neutral tautomer for the acid (pyridinium ion) catalyzed reaction. Several sets of conditions are suggested for oxidation of oligo(nucleoside methylenephosphinate)s to the corresponding phosphonates. Solutions of 200 mM I_2 in pyridine/water, with either 1 M TEA or 1 M pyridine hydrochloride, gave half-lives of less than 2 and 10 min, respectively.

Introduction

A large number of modified dinucleotides and oligonucleotides have been synthesized since the 1960s.[1,2] An early modification introduced by Jones et al. is the 3′-deoxy-3′-*C*-methylenephosphonate internucleosidic linkage.[3] This modification was originally developed for enzymatic studies, but the expected properties of such analogues, for example enhanced hybridization affinity for complementary RNA and nuclease resistance, mean that they could also be of interest in the exploration of potential antisense oligonucleotides. Despite this the 3′-deoxy-3′-*C*-methylenphosphonate modification has, apart from inclusion in a trimer,[4] only recently been introduced in oligonucleotides.[5,6] The late entry of this modification into oligonucleotide analogues is at least partially due to the lack of an efficient synthesis methodology.

Alkylhydrogenphosphinates can be coupled to a hydroxyl function of a nucleoside to produce an alkylhydrogenphosphinate ester.[5−7] Subsequent oxidation gives the corresponding alkylphosphonate ester. Recent reports on the oxidation of oligonucleotide analogues with 3′-deoxy-3′-*C*-methylenephosphinate linkages show that camphorylsulfonyl-oxaziridine can be used for oxidation after prior treatment with a silylating agent such as *N*,*O*-bis(trimethylsilyl)-acetamide (BSA).[6] Alternatively, the phosphinate ester can be oxidized in a one step reaction using iodine in pyridine/water with[5] or without[6] triethylamine as promoter. Carbon tetrachloride can also be used instead of iodine.[6]

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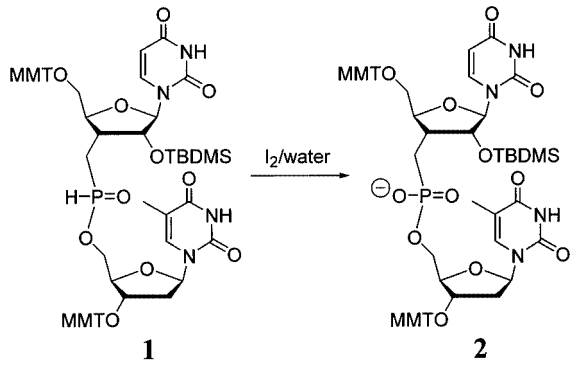
We have recently reported on several aspects of ribonucleoside 3′-deoxy-3′-*C*-methylenephosphonate synthesis, i.e. 3′-carbon extension at the nucleoside level,[8] synthesis of 3′-deoxy-3′-*C*-methylenephosphinate building blocks[9] and development of efficient condensation with the 5′-hydroxyl function of nucleosides to form internucleosidic 3′-deoxy-3′-*C*-methylenephosphinate linkages.[10] We now present our studies of the iodine-mediated oxidation of 3′-deoxy-3′-*C*-methylenephosphinate linkages to produce the desired 3′-deoxy-3′-*C*-methylenephosphonate functionalities. The influence on the rate of oxidation by various changes in reaction conditions, such as the addition of base and acid promoters, is reported.

Results and Discussion

The use of I_2 in pyridine/water is a standard oxidation method in the H-phosphonate approach to oligonucleotide synthesis.[11−16] Alkylhydrogenphosphinate esters differ in reactivity compared to H-phosphonate diesters,[7] this being one of the main issues requiring a detailed study. To investigate the oxidation, 3′-*O*-(4-methoxytrityl)thymidine 5′-(2′-*O*-*tert*-butyldimethylsilyl-5′-*O*-(4-methoxytrityl)uridine 3′-deoxy-3′-*C*-methylenephosphinate) (**1**)[10] was used as model compound. Initially, the conversion of 3′-deoxy-3′-*C*-methylenephosphinate ester **1** to 3′-deoxy-3′-*C*-methylenephosphonate **2** (Scheme 1) was carried out using 80 mM I_2 in pyridine/water (98:2, v/v) at 20 °C. In contrast to the almost instantaneous reaction of the corresponding H-phosphonate,[17] the phosphinate monoester was oxidized quite slowly. Under these conditions oxidation of **1** gave quantitative conversion into **2**, although the reaction required 24



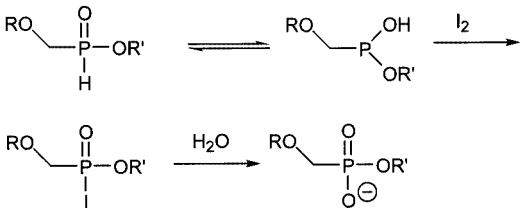
Scheme 1

hours for completion. The half-life is about 440 minutes (Table 1). Interestingly, the reaction does not obey first-order kinetics, but the rate of production of **2** displays a sigmoid behavior. This is a clear indication of autocatalysis, i.e. catalysis by a reaction product.

Since pyridine hydroiodide is formed during the oxidation, a feasible explanation for the autocatalytic behavior would be acid catalysis. This would presumably affect the tautomeric equilibrium between the tetracoordinate (H-P=O) and tricoordinate (P-OH) forms of **1**. To test this hypothesis, reactions were carried out with added pyridine hydrochloride (Table 1). Oxidation of **1** using 80 mM I_2 in pyridine/water (98:2, v/v) containing 0.5 M pyridine hydrochloride was indeed substantially faster than the reaction with no added acid, giving a half-life of 30 minutes. The reaction also displayed a good first-order dependence on the disappearance of **1** with time, in contrast to the sigmoid behavior seen earlier.

The reaction rate with 1 M pyridine hydrochloride is about twice that with 0.5 M concentration (Table 1, entries 2 and 3). The reaction rate was also found to be somewhat dependent on the amount of water present, with less water giving a higher rate (Table 1, entries 3 to 5). Oxidation of **1** in the presence of pyridinium tosylate resulted in a comparable rate to that with the corresponding hydrochloride (Table 1, entries 5 and 6) suggesting that the pyridinium ion is responsible for the catalysis, presumably acting as an acid. The reaction in presence of 1 M pyridine hydrochloride is also dependent on the concentration of iodine, with a reaction with 200 mM I_2 giving almost double the rate for 80 mM I_2. This suggests that attack of the phosphonite form of **1** on iodine is at least partially rate limiting at 1 M concentration of pyridine hydrochloride.

A pathway consisting of a tautomerisation equilibrium followed by attack of the phosphonite form of **1** on iodine seems likely. The resulting iodophosphonate (presumably short-lived since this intermediate is not observed by NMR spectroscopy) would then be rapidly hydrolyzed to the phosphonate anion (Scheme 2). It is likely that one major effect of the pyridinium ion is to accelerate (and possibly shift) the tautomerism. When pyridinium ion is present in relatively high concentration, either or both of the first two steps could be rate limiting. This may well depend on the relative concentrations of iodine and pyridinium ion.



Scheme 2

Another way to enhance the rate of the oxidation reaction could be through addition of base. Base-promoted oxidation of H-phosphonate diesters follows a mechanism where it has been established that the rate-limiting step is usually the abstraction of the P−H hydrogen. This is followed by rapid attack on iodine and substitution of the resulting iodophosphate by water.[18−20] A similar pathway could be possible for the corresponding phosphinate analogue. As we mentioned earlier, triethylamine has been reported to promote phosphinate oxidation either by carbon tetrachloride[6] or by iodine.[5] However reaction conditions and/or details about oxidation rates are not clearly stated in these reports. Hence, we decided to examine the oxidation of **1** in the presence of triethylamine and to compare the rates with different concentrations of iodine and base.

Oxidation of **1** with 0.5 M triethylamine and 80 mM I_2 in pyridine/water (98:2, v/v) at 20 °C gave a significant in-

Table 1. Oxidation of **1** by iodine in pyridine/water with and without pyridinium salt present

Entry	$[I_2]$ (mM)	Acid	[Acid] (M)	Pyridine/water	k (min^{-1})	$t_{1/2}$ (min)
1	80	–	–	98:2		438 (t_{50})[a]
2	80	Pyr·HCl	0.5	98:2	0.023 ± 0.0002	30
3	80	Pyr·HCl	1.0	98:2	0.048 ± 0.0008	14
4	80	Pyr·HCl	1.0	95:5	0.033 ± 0.001	21
5	80	Pyr·HCl	1.0	99:1	0.055 ± 0.002	13
6	80	Pyr·TsOH	1.0	99:1	0.048 ± 0.002	15
7	200	Pyr·HCl	1.0	98:2	0.083 ± 0.003	8.4

[a] Value obtained from fitting of a Boltzmann equation to the sigmoid curve (autocatalytic reaction), t_{20} and t_{80} values obtained were 211 and 665 min, respectively.

crease in reaction rate compared to the reaction without triethylamine. More than 90% conversion of **1** into phosphonate **2** was obtained in approximately 40 minutes. Further effects on the reaction rate of variations in concentration of base, iodine and/or water were examined. The base was kept at 0.5 M or 1 M, the iodine concentration was kept at 80 mM or 200 mM, and the water content in pyridine was varied between 2% and 15%. The results are summarized in Table 2.

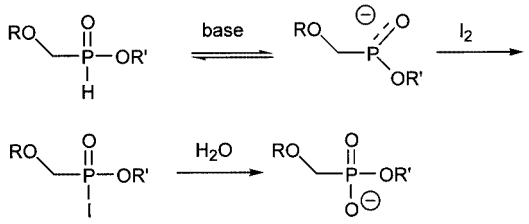
Table 2. Oxidation of **1** by iodine in pyridine/water, in the presence of triethylamine (Et_3N)

Entry	$[I_2]$ (mM)	$[Et_3N]$ (M)	Pyridine/ water	k (min^{-1})	$t_{1/2}$ (min)
1	200	0.5	98:2	–	*16*[a]
2	80	0.5	98:2	0.065 ± 0.001	11
3	80	1.0	98:2	0.13 ± 0.003	5.4
4	80	1.0	95:5	0.16 ± 0.035	4.3
5	200	1.0	95:5	*0.24*[b]	*2.9*[b]
6	200	1.0	85:15	n.e[c]	n.e[c]

[a] Observed value. The reaction did not follow first order kinetics, but deviated towards a higher order with 80% reaction obtained after 60 min. [b] Estimated from two data points, since the reaction was too fast to record more points. [c] Values could not be estimated since no starting material was detectable at the time of recording the first spectrum.

Increasing the concentration of base was found to favor the oxidation reaction, as was a high water proportion. An increase in the iodine concentration had to be accompanied by an increase in the base concentration for any increase in reaction rate to be seen. This was demonstrated by the use of 200 mM I_2 in pyridine/water (98:2, v/v), in the presence of only 0.5 M triethylamine, which resulted in a drastic decrease of the reaction rate (Table 2, entry 1); after 50 minutes 25% of the starting material was still present. The reaction also no longer followed first-order kinetics but deviated towards second-order dependence. We suggest that this is due to the depletion of triethylamine: in basic media, iodine disproportionates to iodide and iodate, accompanied by consumption of base. This disproportionation is presumably responsible for the visible partial decoloration of all investigated reactions with triethylamine present. The color faded relatively quickly but either iodine is still present in sufficient concentration, or is regenerated from iodide and iodate, to give complete oxidation in most cases.

When 80 mM iodine was used, the reaction rate was found to be proportionally dependent on triethylamine concentration (Table 2, entries 2 and 3). There is also an indication of some dependence on iodine. The reaction is likely to follow a pathway similar to that for H-phosphonate diesters,[18–20] i.e. proton abstraction to form the phosphinate anion followed by rapid attack on iodine and subsequent rapid hydrolysis of the iodophosphonate (Scheme 3). The increase in rate with increasing water content could be due to a more favorable proton abstraction as the polarity of the solvent is increased. In fact the oxidation carried out with 200 mM I_2 and 1 M triethylamine in pyridine/water (85:15, v/v) proceeded at so high a rate that only the reaction product could be detected when the first NMR spectrum was recorded (ca. 8 min).



Scheme 3

Of practical importance is that an oxidation solution (200 mM I_2 and 1 M triethylamine in pyridine/water 85:15, v/v) that was stored for 9 h at room temperature gave complete oxidation at a lower rate (0.24 min^{-1}) than for the corresponding reaction with a freshly prepared solution. However, a solution with 200 mM I_2 and 1 M pyridine hydrochloride in pyridine/water (98:2, v/v) that was stored for 8 h gave virtually identical results to that obtained with the corresponding freshly prepared solution.

Finally, we also examined oxidation via silylation of **1**, prior to treatment with iodine and water, in order to obtain a tricoordinate intermediate. Silylation was achieved by treating **1** (14 mM in pyridine) with *N*,*O*-bis(trimethylsilyl)acetamide (BSA, 23 equiv.). After about 15 minutes, 50% of **1** was converted into the corresponding tricoordinate intermediate, as judged by ^{31}P NMR spectroscopy. The silylation was complete after two hours, which is markedly slower than silylation of the corresponding H-phosphonate diester.[17,21] Addition of an iodine solution to give 80 mM I_2 in pyridine/water (98:2, v/v) resulted in subsequent oxidation of the intermediate. The formation of **2** was rapid and complete within the time required for recording the first NMR spectrum (4 min.).

Concluding Remarks

Oxidation of the alkylhydrogenphosphinate ester **1** with iodine in pyridine/water gives quantitative conversion into the alkylphosphonate ester **2**. The reaction can be promoted by triethylamine, and in this case, reaction seemingly takes place via the phosphinate anion. The rate of this reaction is dependent on concentration of base, presumably due to a rate-limiting proton abstraction and/or a higher concentration of anion. The substantially lower reaction rate than for H-phosphonate diesters is probably due to the lower acidity of the phosphinate P–H hydrogen relative to the H-phosphonate hydrogen.[22] Nevertheless, inclusion of triethylamine in the oxidation mixtures gives rapid oxidations and is a useful

tool for the conversion of methylenephosphinate esters to the corresponding methylenephosphonate functionalities.

Although an iodine solution containing triethylamine that was stored for nine hours prior to use gave complete oxidation, the rate was substantially lower than with a fresh solution. This decrease of activity on storage may be of concern when considering use on automated synthesizers. With increased base concentration, competing cleavage of the phosphinate esters could potentially become a problem. However, formation of side products was not observed in the base-promoted oxidation reactions that were carried out with the methylenephosphinate **1**. If the dinucleotide or oligonucleotide analogue were to contain other base-sensitive groups, it may be better to turn to the use of acid catalysis. For the acid-catalyzed oxidation it is possible to increase the rate by an increase in concentrations of both iodine and acid. Pyridinium salts catalyze the oxidation reaction to give a half-life of less than 10 minutes, which should be fast enough for convenient use in a final oxidation step of machine-assisted oligonucleotide synthesis. This alternative, which effectively means using a pyridine/pyridinium buffer, seems milder than the base-promoted option and may be preferred as a general method. In addition, a pyridinium chloride containing solution stored for eight hours prior to use gave an oxidation rate that was virtually identical to that obtained with a freshly prepared solution. This means that it should be relatively safe to store the solution on an automated synthesizer for some time. The method using pre-silylation also gives fast and quantitative oxidation and can be useful, but in our opinion is a less attractive option since an additional step is needed.

Experimental Section

Oxidation reactions were monitored at 20 °C using a Bruker AVANCE DRX 400 instrument. Chemical shifts are given downfield from external phosphoric acid (2% in D_2O). Pyridine (labscan p.a.) was dried over 4 Å molecular sieves. BSA and iodine were bought from Lancaster and Fluka, respectively. The reactions were carried out with R_p/S_p isomeric mixtures of **1** (in a ratio of approximately 1:1). Oxidation without a catalyst and characterization of the resulting product (**2**) are described elsewhere.[10] ^{31}P chemical shifts of the starting compounds were $\delta = 36.5-37$ ppm for one isomer and $\delta = 37.3-37.7$ ppm for the other isomer, depending on conditions. The chemical shift for the product was between $\delta = 21$ and 22 ppm in reactions with triethylamine and between $\delta = 25$ and 26 ppm in the pyridinium-catalyzed reactions.

General Procedure for the Oxidation Reactions with Acid Catalysis: Two stock solutions with double the final concentrations of all reagents were prepared prior to each acid-catalyzed oxidation reaction: one with compound **1** (40 mg, 34 μmol) in pyridine (to give a total volume of 2 mL) and the other with iodine [81 mg (0.32 mmol) or 203 mg (0.80 mmol), depending on the desired final concentration] and the pyridinium salt [either pyridine hydrochloride (231 mg, 2.0 mmol or 462 mg, 4 mmol) or pyridinium tosylate (1.0 g, 4 mmol), depending on the desired acid and final concentration] in pyridine/water (98:2, 96:4 or 90:10 v/v depending on the desired final proportion, to give a total volume of 2 mL). Oxidation reactions of **1** were performed by mixing aliquots (200 μL from each stock solution) in a 5 mm NMR tube to give a final concentration of 17 mM **1**, 80 or 200 mM I_2, 0.5 or 1.0 M pyridinium chloride/tosylate in pyridine/water (99:1, 98:2, or 95:5, v/v). Reactions were monitored by ^{31}P NMR spectroscopy at 20 °C.

General Procedure for the Oxidation Reactions with Base Catalysis: Two stock solutions with double the final concentrations of all reagents were prepared prior to each base-promoted oxidation reaction: one with compound **1** (40 mg, 34 μmol) in pyridine (to give a total volume of 2 mL) and the other with iodine [81 mg (0.32 mmol) or 203 mg (0.80 mmol), depending on the desired final concentration] and triethylamine [0.28 mL (2 mmol) or 0.56 mL (4 mmol), depending on the desired final concentration] in pyridine/water [98:2, 96:4, 90:10 or 70:30 (v/v) depending on the desired final proportion, to give a total volume of 2 mL]. Oxidation reactions of **1** were performed by mixing aliquots (200 μL from each stock solution) in a 5 mm NMR tube to give a final concentration of 17 mM **1**, 80 or 200 mM I_2, 0.5 or 1.0 M triethylamine in pyridine/water (98:2, 95:5, or 85:15, v/v). Reactions were monitored by ^{31}P NMR spectroscopy at 20 °C.

Oxidation with Pre-Silylation: Compound **1** (4 mg, 3.36 μmol) was dissolved in pyridine (240 μL) in a 5 mm NMR tube. BSA (19 μL, 77.3 μmol, 23 equiv.) was added and the reaction was carried out at 20 °C for 2 hours to give the corresponding silylated tricoordinated intermediate (^{31}P NMR spectroscopy showed two new signals with chemical shifts of $\delta = 172$ and 173 ppm). Subsequent oxidation was carried out by addition of an iodine solution (an aliquot of 160 μL was taken from a stock solution prepared by dissolving I_2 (51 mg, 200 mmol) in 1 mL pyridine/water, 95:5, v/v), to give 9 mM of the pre-silylated **1** and 80 mM I_2 in pyridine/water (98:2, v/v). The formation of **2** was complete within less than 4 minutes, as shown by ^{31}P NMR spectroscopy.

Acknowledgments

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