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PAPER

Reactive intermediates in the H-phosphonate synthesis of oligonucleotides†‡

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The formation of H-phosphonate diesters is an important step in the synthesis of oligonucleotides. Using diphenylchlorophosphate as the activator for the coupling step is often accompanied by side reactions as a result of self 'capping' and other reactions of the reactive intermediate. In the absence of base, the activation of ethyl H-phosphonate with diphenylchlorophosphate probably occurs through the intermediate formation of bis diethyl pyro-di-H-phosphonate rather than the expected diphenyl ethyl pyro-H-phosphonate. Pyridine acts as a nucleophilic catalyst converting diphenylchlorophosphate to its pyridinium adduct. Several side and unwanted reactions are quantified so that conditions to minimise these can be identified.

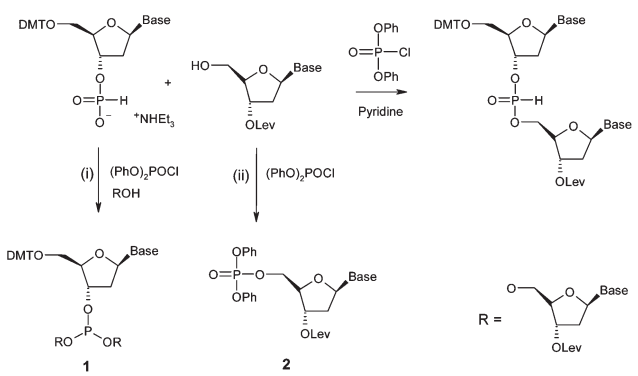
Introduction

Synthetic oligonucleotides are used as substrates for polymerase chain reactions, DNA sequencing and mutagenesis experiments and modified ones are of interest as potential antisense drugs, the first of which, vitravene, was for the treatment of cytomegalovirus-induced retinitis in AIDS patients.[1] Since the discovery of RNA interference (RNAi) as a means to silence the expression of specific genes, small interfering RNA (siRNA) oligonucleotides have been recognized as powerful tools for targeting and inhibiting therapeutically important mRNAs.[2] This discovery has created a high demand for synthetic oligonucleotides as potential therapeutics and has spurred a renaissance in the development of rapid, efficient methods for solid-phase RNA synthesis.[3] There are many challenges to the large scale manufacture of synthetic oligonucleotides and the current cost of a large scale manufactured drug is about £1000 per gram and is dependent on the structure, length, sequence and nature of side chain modifications.

With the exception of the phosphoramidite methodology,[4] the synthesis of oligonucleotides is usually from monomeric units using phosphorylating agents such as phosphites and phosphonates with a dehydrating agent.[5] Coupling reactions can be carried out on a solid supported resin with the 3′-hydroxyl of the first nucleoside base in the sequence bound directly to an insoluble support such as glass beads, silica, polystyrene resin derivative or swellable polymers.[6]

In general, coupling reactions involving phosphorus(III) are much more reactive than those with phosphorus(V), both towards water and 5′ nucleosidic hydroxyl groups. Currently, most oligonucleotides are synthesised using phosphate tri- or di-esters, phosphoramidites or H-phosphonates. Ribonucleoside H-phosphonates[7–9] are a simple and efficient way of producing diester linkages and dinucleoside H-phosphonates with very high yields (Scheme 1).[10] The coupling step requires a dehydrating agent or activator,[11] such as an acyl chloride or diphenylchlorophosphate, which is then often accompanied by side reactions as a result of self 'capping' and other reactions of the reactive intermediate.[12] H-phosphonate nucleosides are relatively stable compounds even in the presence of water, however as only the activator reacts with any water present during coupling, the production of oligonucleotides using H-phosphonate chemistry is becoming an attractive option.

H-Phosphonates exist in two tautomeric forms – the three coordinate phosphite and the usually more stable four coordinate H-phosphonate (Scheme 2), the fraction of phosphite species in



1 2

Scheme 1

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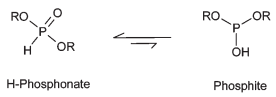


Scheme 2

ethyl H-phosphonate in aqueous solution is estimated to be below 10^{-9} at most pHs, although above pH 13 it is the dominant form of diethyl H-phosphonate, the pK_a of which is 12.7.[13] Proton abstraction of H-phosphonates to produce the phosphite anion has been studied by the oxidation of H-phosphonates and by H–D exchange.[14] However, despite much qualitative work on the H-phosphonate coupling step there is little quantitative information, especially about the competing processes:[15] the over-activation of the H-phosphonates leading to unreactive phosphite triesters[16,17] and the phosphorylation of the 5′-hydroxyl of the nucleoside to be coupled.[17] Herein, we provide quantitative mechanistic information on the reaction rates of the competing processes using diphenylchlorophosphate as the activator, with and without base, to establish the active phosphonylating agent and investigate the variables which determine whether the desired coupling or side reactions dominate.

Results and discussion

The major loss in yield in the coupling of 3′ protected nucleosides and H-phosphonate nucleosides using diphenylchlorophosphate (DPCP) as the activator to form the H-phosphonate diester is due to: (i) 'over-reaction' of the presumed intermediate or product diester with the activator, leading to a phosphite triester (**1**)[18] and (ii) 5′-phosphorylation of the 3′ protected nucleoside by DPCP to give (**2**) (Scheme 1). The proportions of these unwanted and desired products are determined by the relative rates of the reactions and so we investigated the kinetics and mechanisms of the reactions involved.

The formation of the unwanted phosphite triesters (**1**) (Scheme 1) occurs particularly under basic conditions.[12,19] The initial reaction in the coupling of H-phosphonates promoted by acid chlorides is thought to be activation of the H-phosphonate monoester by acylation to form a 'H-phosphonate anhydride' intermediate,[20,21] which subsequently reacts with the 5′-hydroxyl of the 3′ protected nucleoside to form the desired H-phosphonate diester (Scheme 1). However, either this diester product or the intermediate could react further with the activator to form an 'over-activated' species which subsequently reacts with the 3′ protected nucleoside to produce phosphite triester by-products (**1**).[16,17] Our initial assumption was that similar reactions could occur using DPCP as the activator by initial phosphorylation rather than acylation. Thus the coupling of tetrahydrofurfuryl alcohol with ethyl H-phosphonate using DPCP as the activator would give initially diphenyl ethyl pyro-H-phosphonate (**3**) as the reactive intermediate, which could then react either with the alcohol to give the desired diester (**4**) or further with DPCP to give an 'over-phosphorylated' intermediate (**5**) which could react with the alcohol to give phosphite triesters (**6**) (Scheme 3).

However, in the absence of alcohol, ethyl H-phosphonate triethylammonium salt in $CDCl_3$ reacts with 1 equivalent of diphenylchlorophosphate (DPCP) to give the novel symmetrical bis diethyl pyro-di-H-phosphonate (**7**) and diphenylphosphate (DPP) and a small amount of tetraphenylpyrophosphate (TPPP) which is more slowly formed. The ^{1}H decoupled ^{31}P NMR analysis showed no peaks attributable to diphenyl ethyl pyro-H-phosphonate (**3**), although it is presumably formed as an intermediate which then reacts with ethyl H-phosphonate to produce bis diethyl pyro-di-H-phosphonate (**7**) and DPP. TPPP is formed through the reaction of DPP with DPCP (Scheme 3).

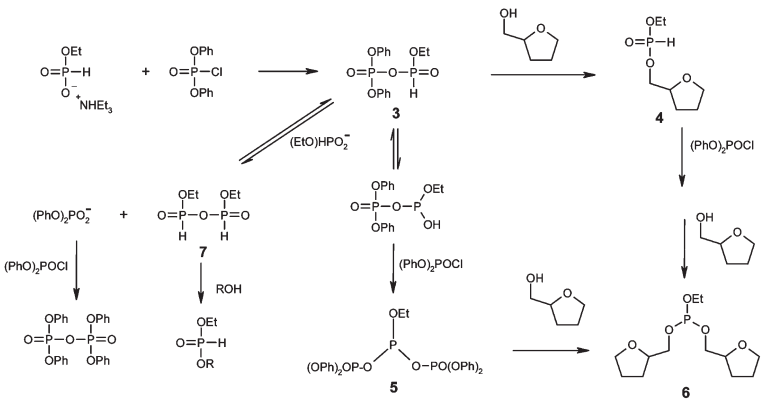
The question then arises as to whether any of these products are the reactive phosphonylating agents in the coupling reaction, particularly the relative activities of (**7**) and (**3**) towards alcohols. Addition of excess tetrahydrofurfuryl alcohol to the product mixture described above produced tetrahydrofurfuryl ethyl H-phosphonate diester (**4**) and an equal molar increase in the amount of ethyl H-phosphonate present (Scheme 4). This is indicative of the novel H-phosphonate (**7**) being the active phosphonylating agent.

During these reactions, none of the unwanted phosphorylation of the hydroxyl group occurred and no phosphite triesters (**6**) were formed. The rates of formation of the various species were determined from ^{1}H decoupled ^{31}P NMR time scans of a 1 : 1 mixture of DPCP and triethylammonium ethyl H-phosphonate in $CDCl_3$ at 25 °C with triphenylphosphate as an internal standard. After 100 min, the reaction mixture gives approximately 85% bis diethyl pyro-di-H-phosphonate (**7**) and DPP and the second order rate constant for formation of (**7**) is 6.8×10^{-3} M^{-1} s^{-1}. During the reaction, the ^{1}H decoupled ^{31}P NMR chemical shift for ethyl H-phosphonate anion increases from 4.5 ppm to 6.7 ppm, indicating a change from the anionic salt to its protonated form. Presumably, the DPP formed is a stronger acid than ethyl H-phosphonic acid in $CDCl_3$, and so DPP exists as its anion and ethyl H-phosphonate is present as its undissociated acid, and so its rate of reaction with DPCP decreases.

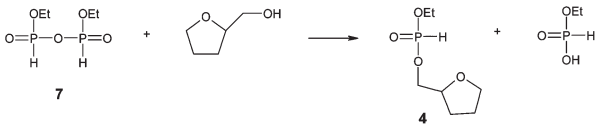
The suspected reactive intermediate, bis diethyl pyro-di-H-phosphonate (**7**) was prepared from ethyl H-phosphonic acid in benzene using dicyclohexylcarbodiimide as the coupling agent.[22]

The ^{1}H decoupled ^{31}P NMR spectrum of the diastereoisomeric (**7**) shows two singlets due to the racemic mixture of the two enantiomers and the meso form. The equilibrium between (**7**) and DPP to diphenyl ethyl pyro-H-phosphonate (**3**) and ethyl H-phosphonic acid (Scheme 5) depends markedly on whether the free acids or their salts are involved. With the former, aliquots of DPP added to (**7**) in $CDCl_3$ produced increasing amounts of (**3**) and ethyl H-phosphonate, from which an equilibrium constant $K = 0.37$ was obtained. The reaction is reversible as confirmed by adding ethyl H-phosphonic acid to the mixture which regenerated bis diethyl pyro-di-H-phosphonate (**7**). The equilibrium constants for the free acids in d_3-acetonitrile and d_6-benzene both give $K = 0.15$, showing little dependence on solvent and all show bis diethyl pyro-di-H-phosphonate (**7**) as the more stable derivative. With salts, the overall equilibrium is shifted even more towards bis diethyl pyro-di-H-phosphonate (**7**) and DPP triethylammonium salt, with no (**3**) detectable, as expected from our earlier results.

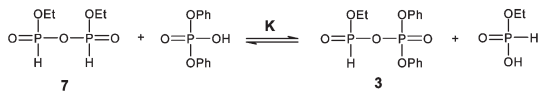
These observations suggest that the activation of ethyl H-phosphonate with DPCP probably occurs through the intermediate formation of bis diethyl pyro-di-H-phosphonate (**7**) rather than diphenyl ethyl pyro-H-phosphonate (**3**). The rate



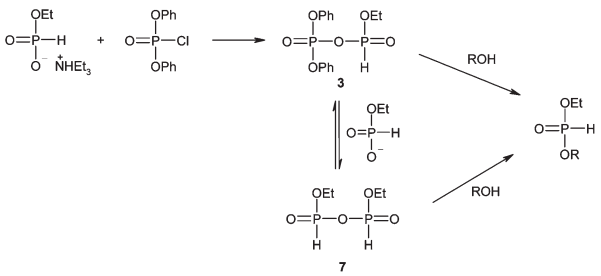
Scheme 3



Scheme 4



Scheme 5



Scheme 6

of ethanolysis of (**7**) in $CDCl_3$ at 25 °C is too fast to follow, but that of iso-propanol, although complete within two minutes, could be followed using ^{1}H- decoupled ^{31}P NMR time scans, with triphenylphosphate as an internal standard and using only 1 scan per spectrum acquisition rather than the normal 16, to give an approximate second order rate constant of 7.8×10^{-2} M^{-1} s^{-1}. In summary, the activation of ethyl H-phosphonate by DPCP in the absence of base proceeds through the intermediate formation of diphenyl ethyl pyro-H-phosphonate (**3**) which is in equilibrium with the thermodynamically more stable symmetrical bis diethyl pyro-di-H-phosphonate (**7**) (Scheme 6). Furthermore, no over-activation arises in the coupling of DPCP with ethyl H-phosphonate in the absence of base to give either tri-ester formation (**6**) or phosphorylation of the alcohol by DPCP.

Scheme 7

Scheme 8

Reactions in the presence of base

The large scale production of H-phosphonate diesters using DPCP as an activator is usually carried out in the presence of base but does produce unwanted reactions including the phosphorylation of the 5′-hydroxyl of the nucleoside to be coupled (Scheme 1). The rate of phosphorylation of hydroxyl groups by DPCP was initially investigated using benzyl alcohol as a model by monitoring ^{1}H decoupled ^{31}P NMR spectra. Although there is no reaction between benzyl alcohol and DPCP (1 : 1 ratio) in d_3-acetonitrile, the addition of 1 equivalent of pyridine to the solution resulted in total conversion of DPCP to benzyldiphenylphosphate. If traces of water were present small amounts of diphenylphosphate (DPP), from hydrolysis of DPCP, and tetraphenylpyrophosphate (TPPP), from the reaction of DPCP with DPP, were formed.

The phosphorylation of tetrahydrofurfuryl alcohol by DPCP in DMF (Scheme 7) with pyridine produces pyridinium hydrochloride and so the rate of reaction was measured by conductance. However, there is a non-linear dependence of the latter on the concentration of pyridinium hydrochloride. The data could not be fitted to the Ostwald or Kohlrausch equations but a quadratic conductance model based on two equilibria (Scheme 8) and, using $[Py]_{tot} = [Py] + [PyH^+] + [PyHCl]_{ip}$, where ip represents the non-conducting ion-pair, eqn (1) can be derived.

The concentration of pyridinium hydrochloride $[PyH^+]$ is related to the conductance Λ of the solution through ε, the specific conductivity of PyH^+ ions.

$$[PyH^+] = \frac{(-(1+\sqrt{K_a}) + \sqrt{((1+\sqrt{K_a})^2 + 4[Py]_t/K_{diss}))K_{diss}}}{2} \quad (1)$$

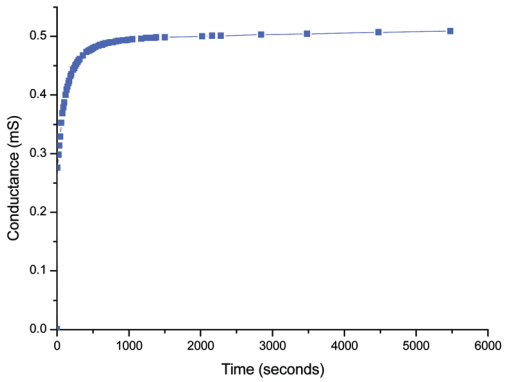


Fig. 1 The change in conductance with time for the alcoholysis of DPCP (0.05 M) with tetrahydrofurfuryl alcohol (1.4×10^{-3} M) and pyridine (0.1 M) in DMF at 25 °C.

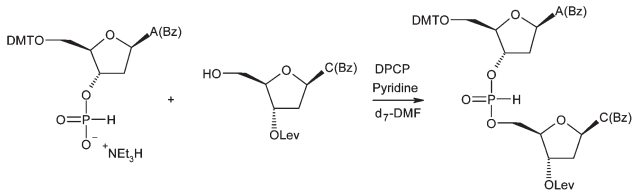
A calibration graph was determined by adding hydrogen chloride in DMF to 0.1 M pyridine in DMF. With excess pyridine K_a can be neglected and the pyridine concentration taken as constant. The concentration of conducting ions is then approximated by eqn (2) using $\lambda_o = 7.54 \times 10^{-7}$ S, $K_{diss} = 6.96 \times 10^{-4}$ M and $\varepsilon = 0.077$ S M^{-1}. Using a pK_a of pyridinium hydrochloride in DMF of 3.0,[23] eqn (2)

$$[PyH^+] = \frac{(-K_{diss} + \sqrt{(K_{diss}^2 - 4[HCl]_t K_{diss})})}{2} \quad (2)$$

gives an excellent fit to the calibration curve for pyridinium hydrochloride. A similar calibration for hydrogen chloride in DMF was undertaken, so that the rate of phosphonylation could be measured in the absence of base, using $\varepsilon = 4.0 \times 10^{-4}$ S M^{-1} and a pK_a = 3.40 for hydrogen chloride in DMF.[24] The rate of alcoholysis of DPCP (0.05 M) by tetrahydrofurfuryl alcohol with pyridine in DMF at 25 °C was followed by conductance (Fig. 1) and the second order rate constant found to be 2.4×10^{-3} M^{-1} s^{-1} compared with that for water = 1.66×10^{-2} M^{-1} s^{-1}.

Alcoholysis of DPCP is slower than hydrolysis and so, for example, the reaction of equimolar quantities of water, benzyl alcohol and DPCP gave a ratio of 7 : 1 diphenyl phosphate to phosphate triester. Although DMF is often used for H-phosphonate coupling reactions, its unsuitability was also confirmed by another side reaction of DPCP with DMF to give a reactive Vilsmeier intermediate, chloroiminium ion, leading to formylation of nucleoside residues.

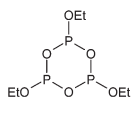
Although the coupling of ethyl H-phosphonate triethylammonium salt with ethanol to form diethyl H-phosphonate using DPCP activation occurs in d_7-DMF and $CDCl_3$ without base, there is a faster reaction with added pyridine. Using 0.14 M pyridine the yield of diethyl H-phosphonate from ethyl H-phosphonate (0.1 M) and ethanol (0.15 M) in d_7-DMF at 25 °C also depends on the amount of DPCP and increases linearly from 10 to 70% as the DPCP increases from 0.02 to 0.14 M. However, with increasing molar equivalents of DPCP above 0.9 DPCP, the initial product diphenyl phosphate is increasingly converted to tetraphenylpyrophosphate (TPPP) presumably through its slower reaction with DPCP, compared with ethyl H-phosphonate (Scheme 3). The yield of diethyl H-phosphonate increases with



Scheme 9

increasing molar equivalents of pyridine which also gives a decreasing concentration of TPPP. The optimum coupling ratios found for diethyl H-phosphonate synthesis were used for the coupling of DMT-dA-Bz-H-phosphonate triethylammonium salt and HO-dC-Bz-OLev in d_7-DMF at 25 °C using DPCP and pyridine (Scheme 9) gave a very fast reaction in which the only phosphorus component observed in the ^{1}H decoupled ^{31}P NMR spectrum is the product DMT-dA-Bz-dC-Bz-OLev formed in 99% yield.

Catalysis by pyridine could be due either to nucleophilic catalysis through the formation of an active phosphoryl pyridinium adduct from the reaction of pyridine with DPCP or to general base catalysis of hydroxyl group attack on the active phosphonylating agent. Triethylamine is unlikely to act as a nucleophilic catalyst because of steric hindrance but the addition of the base to the reaction of ethyl H-phosphonate triethylammonium salt and DPCP in $CDCl_3$ increases the yield of bis diethyl pyro-di-H-phosphonate (**7**). However, with excess base (**7**) is unstable and is converted to a phosphite species. The reaction of ethyl H-phosphonate triethylammonium salt with ten equivalents of DPCP in $CDCl_3$ containing 22% v/v of triethylamine gives, after two minutes of reaction, a phosphite species thought to be 2,4,6-triethoxy-1,3,5-trioxa-2,4,6-triphosphinane (tris-ethylmetaphosphite) (**8**).[25] This probably explains the poor yield obtained when using pre-activation[26] which encourages metaphosphite formation and any phosphite by-products formed from the alcoholysis of the metaphosphite. The hydrolysis of tris-ethyl metaphosphite (**8**) gives only ethyl H-phosphonate, whilst alcoholysis gives ethyl H-phosphonate, the H-phosphonate diester (**4**) and a phosphite triester (**6**), indicating that the alcohol hydroxyl group preferentially reacts at phosphorus(III) rather than phosphorus(V). Reaction at phosphorus(V) would produce only H-phosphonate diester, with no phosphite triesters. Metaphosphite formation could occur during oligonucleotide synthesis on solid supports using high loadings on porous resins.[26]



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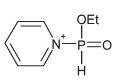

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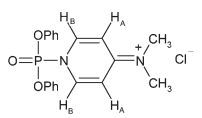

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Using one equivalent each of DPCP, ethyl H-phosphonate triethylammonium salt and triethylamine in $CDCl_3$ there is no ethyl H-phosphonate anion remaining after 2 min but large quantities of DPP which subsequently reacts with DPCP to form TPPP, which in turn reacts further and its concentration decreases with time (Fig. 2).

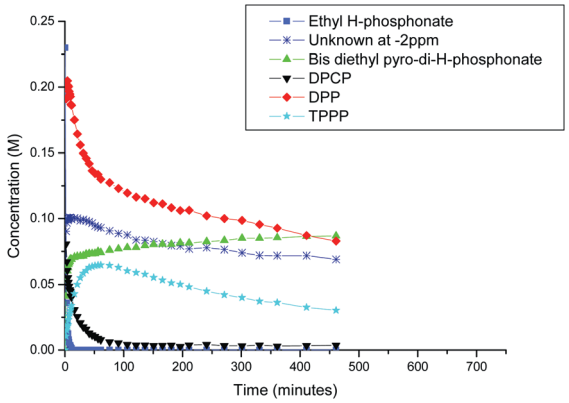


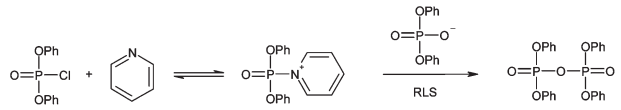
Fig. 2 Concentration–time plots for the reaction of DPCP (0.24 M) and ethyl H-phosphonate triethylammonium salt (0.23 M) with triethylamine (0.23 M) and triphenylphosphate (0.14 M) as internal standard in $CDCl_3$ measured by ^1H decoupled ^{31}P NMR at 298 K.

DPCP reacted completely within 100 min but there is no metaphosphite produced. Before the first spectrum was obtained, bis-diethyl pyro-di-H-phosphonate is formed and an unknown compound with a ^1H decoupled ^{31}P NMR chemical shift of −2 ppm is produced the concentration of which then slowly reduces. In addition to base facilitating formation of active phosphonylating agents, it could also have a catalytic role in the coupling step and so the reactions of the individual components with base were investigated. Both DPCP and TPPP show no detectable reaction with pyridine or triethylamine in $CDCl_3$, but bis-diethyl pyro-di-H-phosphonate (**7**) and diphenyl ethyl pyro-H-phosphonate (**3**) with pyridine both form the unknown compound with a ^1H decoupled ^{31}P chemical shift of −2 ppm and a small amount of tris-ethyl metaphosphite (**8**). The compound with a ^1H decoupled ^{31}P chemical shift of −2 ppm (Fig. 2) could be the pyridinium adduct of ethyl H-phosphonate (**9**) formed directly from the reaction of bis-diethyl pyro-di-H-phosphonate with pyridine,[27] but mass spectral analysis to trap the intermediate proved inconclusive. Although the reaction of pyridine with bis-diethyl pyro-di-H-phosphonate gives the compound with a ^1H decoupled ^{31}P chemical shift of −2 ppm but no ethyl H-phosphonate anion, with diphenyl ethyl pyro-H-phosphonate the former is formed with an equivalent amount of DPP. Although H-phosphonate–pyridinium adducts are reported in the literature the evidence is far from conclusive.[27]

Pyridine does catalyse the reaction of diphenyl phosphate and DPCP to give TPPP and the corresponding second order rate constant shows a first order dependence on the concentration of pyridine and the overall rate law is given by eqn (3).

$$\text{Rate of TPPP formation} = k_3[\text{DPCP}][\text{DPP}^-][\text{Pyridine}] + k_{2\text{uncat}}[\text{DPCP}][\text{DPP}^-] \quad (3)$$

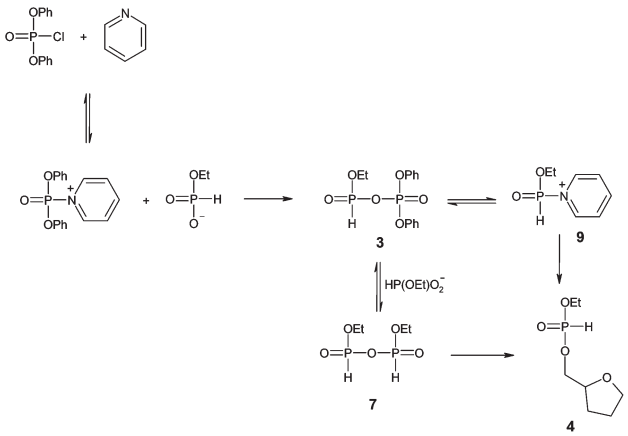
The third order rate constant, k_3, is 2×10^{-2} M^{-2} s^{-1} and the second order uncatalysed rate constant, $k_{2\text{uncat}}$, is 8.9×10^{-3} M^{-1} s^{-1}. The overall third order term is compatible with nucleophilic catalysis (Scheme 10) with a pre-equilibrium formation of the pyridinium adduct which then reacts with DPP in the rate limiting step. In support of this proposal, the ^1H decoupled ^{31}P NMR of an equimolar mixture of DMAP and



Scheme 10

Table 1 The third order rate constants for the reaction of DPCP with DPP to give TPPP in $CDCl_3$ at 25 °C, the pK_a of the bases are in water

Base	pK_a	k_3/M^{-2} s^{-1}
4-Cyanopyridine	1.12	2.00×10^{-4}
Pyridine	5.25	2.04×10^{-2}
2,6-Lutidine	6.65	2.00×10^{-3}
4-Methoxypyridine	6.9	8.91×10^{-2}
Triethylamine	10.75	3.80×10^{-3}



Scheme 11

DPCP gives a single signal of chemical shift of −12.8 ppm assumed to be the DMAP–DPCP adduct (**10**).[28] The hydrogens *ortho* to the pyridine nitrogen shift from 6.47 to 7.36 upon phosphorylation, compared with protonation which causes a shift to 6.91.

The third order rate constants for the formation of TPPP using a variety of substituted pyridines and triethylamine are given in Table 1. The third order rate constants increase with the basicity of the substituted pyridine and, excluding the sterically hindered lutidine, generate a linear Bronsted plot of log k_3 against the pK_a of the base (in water) giving an apparent Bronsted β_{nuc} of 0.46. With increasing basicity of the substituted pyridine, the equilibrium constant for formation of the adduct is expected to increase but the rate of the second step would decrease so the observed value is compatible with rate limiting displacement of pyridine by attack of DPP on the adduct.

Conclusions

The coupling reaction of ethyl H-phosphonate and tetrahydrofurfuryl alcohol promoted by DPCP and pyridine in $CDCl_3$ is given in Scheme 11. DPCP reacts initially with pyridine to form a reactive intermediate (**9**) that is attacked by ethyl H-phosphonate to

generate diphenyl ethyl pyro-H-phosphonate (**3**) which in turn reacts either with pyridine to form another reactive intermediate or with ethyl H-phosphonate to give bis diethyl pyro-di-H-phosphonate (**7**). It is these two activated H-phosphonate derivatives that then react with the alcohol to form the desired diester product (**4**). However, although added base does increase the overall rate of reaction it also increases the amount of unwanted side products.

Experimental

Diphenylalkylphosphates

The alcohol (46.4 mmol), pyridine (5.55 g, 70.2 mmol) and DCM (50 ml) were cooled to ~0 °C over a nitrogen atmosphere. Diphenylchlorophosphate (15.04 g, 55.5 mmol) was added over 5 min using a syringe and septa. The reaction was stirred for 5 min before quenching and washing with water (2 × 50 ml). The DCM phase was dried over magnesium sulphate and evaporated under reduced pressure. To remove pyrophosphates the residue was dissolved in DCM (50 ml), washed with acid (50 ml at pH 2), then base (50 ml sat. sodium bicarbonate solution) before a final water wash (50 ml). The organic phase was dried with magnesium sulphate and evaporated to dryness.

Diphenylbenzylphosphate. (10.41 g) White solid. GC-MS analysis: 5.50 min (M + 1, 341). ^{1}H decoupled ^{31}P NMR δ (CD_3CN) −10.9 (s, 1P); ^{1}H NMR δ (CD_3CN) 7.0–7.5 (m, 15H), 5.26 (d, J_3 = 8.3 Hz, 2H).

Diphenylisopropylphosphate. (12.1 g) White crystals. GC-MS analysis: (DCM) (M + 1, 293); ^{1}H decoupled ^{31}P NMR: δ (CD_3CN) −11.8 (s, 1P); ^{1}H NMR δ (CD_3CN) 7.0–7.5 (m, 10H), 4.90 (m, 1H, J_3 = 6.3 Hz), 1.36 (dd, 6H, J_3 = 6.14 Hz, J_4 = 0.88 Hz).

Diphenylethylphosphate. (13.16 g) White crystals. GC-MS analysis: 4.41 min (M + 1, 279); ^{1}H decoupled ^{31}P NMR δ (CD_3CN) −11.0 (s, 1P); ^{31}P coupled NMR δ (CD_3CN) −11.0 (t, J_3 = 8.4 Hz); ^{1}H NMR: δ (CD_3CN) 7.0–7.5 (m, 10H), 4.35 (m, 2H, J_3 = 7.02 Hz, J_4 = 1.32 Hz), 1.36 (dt, 3H, J_3 = 7.02Hz, J_4 = 1.32 Hz).

Ammonium ethyl H-phosphonate

To a concentrated ammonia solution (675 ml, 13.87 mol) was slowly added diethyl H-phosphonate (129 ml, 1 mol) with stirring. The flask was sealed and stirred at room temperature for 24 hours, before evaporation to dryness. The resulting thick clear solution was suspended in acetonitrile (500 ml) upon which a crystalline white solid formed. The solid was filtered and dried by desiccation overnight to yield solid ammonium ethyl H-phosphonate (120.6 g, yield 96.8%). ^{1}H decoupled ^{31}P NMR δ (D_2O) 6.77 (s, 1P,) ^{1}H decoupled ^{31}P NMR δ (D_2O + HCl) 7.27 (s, 1P). ^{31}P coupled NMR δ (D_2O) 8.69 and 4.85 (dt, P–H 621.04 Hz, J_3 = 8.47 Hz); ^{1}H NMR δ (D_2O) 7.64 (s, 0.5H), 5.53(s, 0.5H), 3.78 (q, 2H), 1.13 (t, 3H).

Triethylammonium ethyl H-phosphonate

Ammonium ethyl H-phosphonate (12.96 g, 0.1 mol) was dissolved in acetonitrile (300 ml) and triethylamine (75 ml, 0.54 mol) added. The reaction was stirred for 24 h under nitrogen. The mixture was then evaporated under reduced pressure, acetonitrile (100 ml) was added and evaporated. Triethylamine was removed under vacuum 1 mmHg for 24 h. ^{1}H decoupled ^{31}P NMR δ ($CDCl_3$) 4.56 (s, 1P); ^{1}H NMR δ ($CDCl_3$) 7.6 (s, 0.5H), 6.05 (s, 0.5H), 3.92 (q, 2H), 3.1 (t, 6H), 1.34 (t, 9H), 1.28 (t, 3H).

Tetrabutylammonium ethyl H-phosphonate

Ammonium ethyl H-phosphonate (2.02 g, 15.9 mmol) was dissolved in acetonitrile (50 ml). Tetrabutylammonium chloride (4.49 g, 16 mmol) was added and the reaction left stirring overnight. DCM (20 ml) and water (20 ml) was added and stirred for 24 h. The DCM phase was then separated and evaporated under reduced pressure. ^{1}H decoupled ^{31}P NMR δ (CD_3CN) 1.61 (s, 1P); ^{1}H NMR δ (CD_3CN) 7.6 (s, 0.5H), 5.6 (s, 0.5H), 3.7 (q, 2H), 3.1 (t, 8H), 1.6 (m, 8H), 1.3 (m, 8H), 1.2 (t, 3H), 0.9 (t, 12H).

Ethyl H-phosphonic acid

Hydrogen chloride in ether (2 M, 100 ml, 0.2 mol) was added to ammonium ethyl H-phosphonate (15 g, 0.12 mol) in DCM (100 ml) and left stirring overnight under a nitrogen atmosphere. After filtering and evaporation under reduced pressure, trace amounts of ethyl H-phosphonate ammonium salt were removed with piperidine on a resin (12.5 g, 94.7%). MS (+ve ion, CD_3CN): 111.2; ^{1}H decoupled ^{31}P NMR: δ ($CDCl_3$) 8.02 (s, 1P); ^{1}H NMR: δ ($CDCl_3$) 12.40 (s, 1H), 7.66 (s, 1H), 4.12 (q, 2H), 1.36 (t, 3H).

Bis diethyl pyro-di-H-phosphonate[29]

Ethyl H-phosphonate free acid (2.01 g, 18.2 mmol) in benzene (5 ml) was placed in oven dried glassware and a nitrogen flushed filtration apparatus. Dicyclohexylcarbodiimide (2.23 g, 10.7 mmol) in benzene (10 ml) was added over 30 min then stirred for 1 h before filtering under nitrogen. The filter cake was washed with benzene (3 ml) and the product stored in the fridge as the benzene solution for later use. For NMR analysis bis diethyl pyro-di-H-phosphonate solution (0.5 ml) was evaporated under reduced pressure in a Schlenk flask. ^{1}H decoupled ^{31}P NMR: δ ($CDCl_3$) −3.1 (d, 2P); MS (+ve ion, CD_3CN): (M + 1, 203). All pyrophosphonates were manipulated in a sealed nitrogen glove box.

DMT-dA-Bz-H-phosphonate triethylammonium salt

1,2,4-Triazole (12.3 g, 0.175 mol), triethylamine (24.6 g, 0.242 mol) and tetrahydrofuran (317 ml) were cooled to below −10 °C under a nitrogen atmosphere. Phosphorus trichloride (5.3 ml, 30.8 mmol) in THF (10 ml) was added over 30 min with a THF wash (10 ml). The reaction was stirred for 30 min,

DMT-dA-Bz-OH (10 g, 15.2 mmol) in THF (263 ml) was then added over 3 h, again maintaining the temperature at −10 °C. The reaction was then left stirring until HPLC analysis showed less than 1% starting material. The reaction mixture was warmed to room temperature and water (80 ml) and triethylamine (80 ml) were added. A quench of DCM (500 ml), water (750 ml) and acetic acid (1 ml) adjusted to pH 7 using triethylamine was then prepared. The reaction mixture was then quenched into the DCM–water maintaining the pH at 6–8 using acetic acid with a final pH of 7. The aqueous/organic layers were separated and the water phase washed with DCM (200 ml). After combination the DCM layer was dried over magnesium sulphate before concentrating under vacuum. The off-white solid (9.3 g) was purified by dissolving in a minimum of DCM and loaded onto a packed A60 silica column (150 g) and eluting with DCM–methanol–triethylamine mixtures and analysing by HPLC. Evaporation of solvent gave a white solid with water content 0.24%. ^{1}H decoupled ^{31}P NMR: δ 3.70 ppm (s, 1P) MS (acetonitrile, +ve): M + 1, 722, (acetonitrile, −ve): M − 1, 720.

Alcoholysis of DPCP

DPCP (199.2 mg, 0.7 mmol) was added to a solution of benzyl alcohol (95.9 mg, 0.9 mmol) in CD_3CN (1.5 ml) and analysed by NMR. Pyridine (70.3 mg, 0.9 mmol) was added and the sample re-analysed by NMR.

DPCP and pyridine

DPCP (202.0 mg, 0.7 mmol) was added to a solution of pyridine (74.9 mg, 0.9 mmol) in CD_3CN (1.5 ml) and analysed by ^{1}H decoupled ^{31}P NMR.

TPPP and benzyl alcohol

Benzyl alcohol (88.2 mg, 0.8 mmol) was added to a solution of TPPP (0.34 g, 0.7 mmol) in CD_3CN (1.5 ml) and analysed by NMR, then repeated with pyridine (63.5 mg, 0.8 mmol).

Pyrophosphoric acid and benzyl alcohol

Benzyl alcohol (87.2 mg, 1.1 mmol) was added to pyrophosphoric acid (0.19 g, 1.1 mmol) in CD_3CN (1.5 ml) and analysed by NMR; then repeated with pyridine (87.2 mg, 1.1 mmol).

Alcoholysis of DPCP

DPCP (81.4 mg, 0.3 mmol) was added to benzyl alcohol (43.7 mg, 0.4 mmol) in d_7-DMF (0.75 ml) and analysed by NMR; then repeated with pyridine (31.7 mg, 0.4 mmol).

Conductance studies

Glassware was oven dried and the conductance cell purged with nitrogen prior to use. The conductance was calibrated using KCl (0.745 g) in water (1 l) giving a conductance of 1413 mS. For the alcoholysis of DPCP : DMF (100 ml) was added to the flask and the initial conductance value noted, DPCP (2.7 g, 10 mmol) was added to the cell and the datalog started. After one hour pyridine (0.79 g, 10 mmol) was added, after a further hour tetrahydrofurfuryl alcohol (10.3 mg, 0.1 mmol); after two hours a further amount of tetrahydrofurfuryl alcohol was added (20.6 mg, 0.2 mmol). After a total of five hours the datalog was stopped and the data retrieved. For the hydrolysis of DPCP: DMF (100 ml) containing water (1.3%w/w, 0.78 M) was added to the flask and the initial conductance value noted. DPCP (2.7 g, 10 mmol) was added to the cell and the datalog started. After one hour an additional aliquot of DPCP (2.7 g, 10 mmol) was added to the cell. After a further hour of data collection the datalog was stopped and results retrieved. For competitive alcoholysis and hydrolysis of DPCP: DPCP (92.7 mg, 0.34 mmol) was added to a mixture of benzyl alcohol (40.5 mg, 0.37 mmol) and water (6.7 mg, 0.37 mmol) in d_3-acetonitrile (0.75 ml) and analysed by ^{1}H decoupled ^{31}P NMR: δ (d_3-acetonitrile) −4.7 ppm (s, 0.609P, DPCP), −9.9 ppm (s, 0.322P, DPP), −10.9 ppm (s, 0.046P, benzyldiphenylphosphate), −24.4 ppm (s, 0.024P, TPPP).

DPCP activation

DPCP (65.1 mg, 0.24 mmol, 0.32 M) was added to a solution of ethyl H-phosphonate triethylammonium salt (52.6 mg, 0.25 mmol, 0.33 M) in $CDCl_3$ (0.75 ml) and monitored by ^{1}H decoupled ^{31}P NMR: δ ($CDCl_3$) 4.4 ppm (s, 0.94P, ethyl H-phosphonate), −3.05 ppm (d, 0.48P, bis diethyl pyro-di-H-phosphonate), −4.7 ppm (s, 1P, DPCP), −11.6 ppm (s, 0.29P, DPP), −25.0 ppm (s, 0.06P, TPPP). ^{1}H NMR: δ ($CDCl_3$) 8.0 ppm (s, 0.5H, P–**H** bis diethyl pyro-di-H-phosphonate), 7.6 ppm (s, 0.5H, P–**H** ethyl H-phosphonate), 6.9–7.4 ppm (m, aromatic protons DPCP, DPP and TPPP), 6.1 ppm (s, 0.5H, P–**H** bis diethyl pyro-di-H-phosphonate), 5.9 ppm (s, 0.5H, P–**H** ethyl H-phosphonate), 4.3 ppm (m, 4H, $OC\mathbf{H}_2CH_3$ bis diethyl pyro-di-H-phosphonate), 4.0 ppm (m, 2H, $OC\mathbf{H}_2CH_3$ ethyl H-phosphonate), 3.0 ppm (q, 6H, $HN^+(C\mathbf{H}_2CH_3)_3$), 1.4 ppm (m, 6H, $OCH_2C\mathbf{H}_3$ from bis diethyl pyro-di-H-phosphonate), 1.3 ppm (t, 9H, $HN^+(CH_2C\mathbf{H}_3)_3$).

Ethyl H-phosphonate triethylammonium salt (31.7 mg, 0.15 mmol, 0.2 M) was then added to a solution of DPCP (51.6 mg, 0.19 mmol, 0.25 M) in $CDCl_3$ (0.75 ml) and monitored by ^{1}H decoupled ^{31}P NMR.

DPCP (51.6 mg, 0.19 mmol, 0.25 M) was added to a solution of ethyl H-phosphonate triethylammonium salt (38.0 mg, 0.18 mmol, 0.24 M) and triphenylphosphate (31.3 mg, 0.1 mmol, 0.13 M) in $CDCl_3$ (0.75 ml), left for two hours and analysed by ^{1}H decoupled ^{31}P NMR. After analysis tetrahydrofurfuryl alcohol (19.6 mg, 0.19 mmol, 0.25 M) was added and the sample re-analysed by ^{1}H decoupled ^{31}P NMR. Pyridine (15.1 mg, 0.19 mmol, 0.25 M) was added and the mixture again analysed by ^{1}H decoupled ^{31}P NMR: δ ($CDCl_3$) 6.8 ppm (s, 0.84P, ethyl H-phosphonate), −3.0 ppm (d, 1.59P, bis diethyl pyro-di-H-phosphonate), −4.7 ppm (s, 1.06P, DPCP), −11.6 ppm (s, 0.90P, DPP), −17.1 ppm (s, 1.0P, triphenylphosphate), −25.0 ppm (s, 0.37P, TPPP). ^{1}H decoupled ^{31}P NMR: δ ($CDCl_3$ + tetrahydrofurfuryl alcohol) 9.2 ppm (s, 0.40P, tetrahydrofurfuryl ethyl H-phosphonate diester), 8.6 ppm (s, 0.47P, tetrahydrofurfuryl ethyl H-phosphonate diester), 7.0 ppm (s, 1.50P,

ethyl H-phosphonate), −4.7 ppm (s, 1.01P, DPCP), −11.6 ppm (s, 0.97P, DPP), −17.1 ppm (s, 1.0P, triphenylphosphate), −25.0 ppm (s, 0.38P, TPPP). ^{31}P coupled NMR: δ ($CDCl_3$ + tetrahydrofurfuryl alcohol) 11.3 and 6.9 ppm (dq, P–H coupling 706.6 Hz, J^3 = 9.36 Hz, tetrahydrofurfuryl ethyl H-phosphonate diester), 10.8 and 6.5 ppm (dq, P–H coupling 704.3 Hz, J^3 = 9.14 Hz, tetrahydrofurfuryl ethyl H-phosphonate diester), 9.1 and 5.0 ppm (dt, P–H coupling 686.5 Hz, ethyl H-phosphonate). ^{1}H NMR: δ ($CDCl_3$ + tetrahydrofurfuryl alcohol) 7.73 and 5.95 ppm (d, P–H coupling 706.61 Hz, tetrahydrofurfuryl ethyl H-phosphonate diester), 7.69 and 5.93 ppm (d, P–H coupling 704.3 Hz, tetrahydrofurfuryl ethyl H-phosphonate diester), 7.60 and 5.89 ppm (d, P–H coupling 686.8 Hz, ethyl H-phosphonate). ^{1}H decoupled ^{31}P NMR: δ ($CDCl_3$ + tetrahydrofurfuryl alcohol + pyridine) 9.2 ppm (s, 0.53P, tetrahydrofurfuryl ethyl H-phosphonate diester), 8.6 ppm (s, 0.67P, tetrahydrofurfuryl ethyl H-phosphonate diester), 7.0 ppm (s, 1.0P, ethyl H-phosphonate), −4.7 ppm (s, 0.81P, DPCP), −11.6 ppm (s, 1.18P, DPP), −17.1 ppm (s, 1.0P, triphenylphosphate), −25.0 ppm (s, 0.35P, TPPP). MS (+ve, acetonitrile): (M + 1, 195, tetrahydrofurfuryl ethyl H-phosphonate diester).

^{1}H decoupled ^{31}P NMR timescan kinetics

DPCP (51.6 mg, 0.19 mmol, 0.25 M) was added to a solution of triethylammonium ethyl H-phosphonate (38.0 mg, 0.18 mmol, 0.24 M) and triphenylphosphate (31.3 mg, 0.1 mmol, 0.13 M) in $CDCl_3$ or d_3-acetonitrile (0.75 ml). The sample was then analysed continuously by ^{1}H decoupled ^{31}P NMR. All individual NMR spectra were 16 scans per spectra.

Equilibrium studies

DPP (156.7 mg, 0.62 mmol) was dissolved in the chosen solvent (0.4 ml) and aliquots (50 µl) were added to a solution of bis diethyl pyro-di-H-phosphonate containing triphenylphosphate (17.6 mg, 0.05 mmol) in the same solvent (0.6 ml) under a nitrogen atmosphere. The sample was then analysed by ^{1}H decoupled ^{31}P NMR and ^{31}P coupled–^{31}P coupled NMR COSY. The position of DMAP–DPCP adduct formation was determined from DPCP (78.0 mg, 0.29 mmol) in $CDCl_3$ (0.75 ml) analysed by both ^{1}H and ^{1}H decoupled ^{31}P NMR. DMAP (55.mg, 0.45 mmol) in $CDCl_3$ (0.75 ml) analysed by ^{1}H NMR. DPCP (0.16 g, 0.57 mmol) was added to a solution of DMAP (62.5 mg, 0.51 mmol) in $CDCl_3$ (0.75 ml) and analysed by both ^{1}H and ^{1}H decoupled ^{31}P NMR. DMAP hydrochloride (80.9 mg, 0.51 mmol) in $CDCl_3$ (0.75 ml) analysed by ^{1}H NMR: δ (DPCP in $CDCl_3$) 7.32 ppm (m, phenyl protons DPCP) ^{1}H decoupled ^{31}P NMR: δ (DPCP in $CDCl_3$) −4.6 ppm (s, 1P, DPCP), −24.9 ppm (s, 0.02P, TPPP); ^{1}H NMR: δ (DMAP in $CDCl_3$) 8.22 ppm (d, 2H, $\mathbf{H}_A$ DMAP), 6.47 ppm (d, 2H, $\mathbf{H}_B$ DMAP), 2.97 ppm (s, 6H, $N(C\mathbf{H}_3)_2$ DMAP). ^{1}H NMR: δ (DPCP and DMAP in $CDCl_3$) 8.23 ppm (broad s, 2H, $\mathbf{H}_A$ DMAP-DPCP), 7.36 ppm (broad s, 2H, $\mathbf{H}_B$ DMAP-DPCP), 6.89 ppm (m), 3.09 ppm (s, 6H, $N^+(C\mathbf{H}_3)_2$ DMAP-DPCP); ^{1}H decoupled ^{31}P NMR: δ (DPCP and DMAP in $CDCl_3$) −5.0 ppm (s, 3.09, DPCP), −13.1 ppm (s, 11.53, DMAP-DPCP), −25.1 ppm (s, 1.0, TPPP). ^{1}H NMR: δ (DMAP-HCl in $CDCl_3$) 14.63 ppm (broad s, 1H, DMAP-$\mathbf{H}_C$Cl), 8.15 ppm (s, 2H, $\mathbf{H}_A$ DMAP-HCl), 6.91 ppm (s, 2H, $\mathbf{H}_B$ DMAP-HCl), 3.31 ppm (s, 6H, $N^+(C\mathbf{H}_3)_2$ DMAP-HCl).

Abbreviations

DPCP	diphenylchlorophosphate
DPP	diphenylphosphate
TPPP	tetraphenylpyrophosphate

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References

1 The Vitravene Study Group, *Am. J. Opthalmol.*, 2002, **133**, 467.
2 S. L. Beaucage, *Curr. Opin. Drug Discovery Dev.*, 2008, **11**, 203.
3 J. Tian, K. Ma and I. Saaem, *Mol. BioSyst.*, 2009, **5**, 714.
4 M. H. Caruthers, *Science*, 1985, **230**, 281.
5 G. Zon, *New J. Chem.*, 2010, **34**, 795.
6 R. B. Merrifield, *Angew. Chem., Int. Ed. Engl.*, 1985, **24**, 799.
7 R. H. Hall, A. Todd and R. F. Webb, *J. Chem. Soc.*, 1957, 3291.
8 B. C. Froehler and M. D. Matteucci, *Tetrahedron Lett.*, 1986, **27**, 469.
9 B. C. Froehler, P. G. Ng and M. D. Matteucci, *Nucleic Acids Res.*, 1986, **14**, 5399.
10 E. de Vroom, C. E. Dreef, H. van den Elst, G. A. van der Marel and J. H. van Boom, *Recl. Trav. Chim. Pays-Bas*, 1988, **107**, 592.
11 H. G. Khorana, *Science*, 1979, **203**, 614.
12 P.J. Garegg, T. Regberg, J. Stawinski and R. Stromberg, *Nucleosides, Nucleotides Nucleic Acids*, 1987, **6**, 655.
13 P. J. Guthrie, *Can. J. Chem.*, 1979, **57**, 236.
14 Z. Luz and B. Silver, *J. Am. Chem. Soc.*, 1961, **83**, 4518.
15 J. Stawinski and A. Kraszewski, *Acc. Chem. Res.*, 2002, **35**, 952.
16 R. Stromberg, *Nucleosides, Nucleotides Nucleic Acids*, 2003, **22**, 1.
17 Y. V. Dankov, N. V. Batchikova, N. V. Scaptsova, E. S. Besidsky and A. V. Azhayev, *Bioorg. Khim.*, 1988, **14**, 615.
18 P. J. Garegg, T. Regberg, J. Stawinski and R. Stromberg, *Nucleosides, Nucleotides Nucleic Acids*, 1987, **6**, 283.
19 P. J. Garegg, I. Lindh, T. Regberg, J. Stawinski, R. Stromberg and C. Henrichson, *Tetrahedron Lett.*, 1986, **27**, 4051.
20 B. C. Froehler and M. D. Mattuecci, *Nucleosides Nucleotides*, 1987, **6**, 287.
21 P. J. Garegg, T. Regberg, J. Stawinski and R. Stromberg, *Chemica Scripta*, 1986, **26**, 59.
22 A. Zwierzak and A. Koziara, *Tetrahedron*, 1967, **23**, 2243.
23 M. Breant and G. Demanga-Guerin, *Bull. Soc. Chim. Fr.*, 1969, 2935.
24 R. L. Beniot and C. Buisson, *Electrochim. Acta*, 1973, **18**, 105.
25 P. J. Garegg, J. Stawinski and R. Stromberg, *J. Org. Chem.*, 1987, **52**, 284.
26 J. H. Atherton, Avecia Biotechnology, personal communication.
27 I. Y. Dubey, V. A. Efimov, T. V. Lyapina and D. M. Fedoryak, *Bioorg. Khim.*, 1992, **18**, 911; S. Sigurdsson and R. Stromberg, *J. Chem. Soc., Perkin Trans. 2*, 2002, 1682.
28 V. F. Zarytova, E. M. Ivanova, D. G. Knorre, A. V. Lebedev, A. I. Rezvukhin and E. V. Yarmolinskaya, *Dokl. Akad. Nauk. SSSR.*, 1979, **248**, 1124.
29 A. Zwierzak and A. Koziara, *Tetrahedron*, 1967, **23**, 2243.