The H-phosphonate approach to oligonucleotide synthesis. An investigation on the mechanism of the coupling step

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A kinetic study on the pivaloyl chloride promoted H-phosphonate condensation step in the presence of differently substituted pyridines has been carried out. The kinetics of the system follow second order kinetics with a 1 : 1 ratio of nucleoside 3′-H-phosphonate and nucleoside component (with free 5-OH). Irrespective of the pyridine derivative used, the reaction rate is dependent on the concentration of this as well as of the hydroxy component and H-phosphonate. A reactive intermediate is indirectly identified from the kinetic evidence. This intermediate is suggested to be a pyridinium adduct formed by attack of the pyridine derivative on the initially formed mixed phosphonic carboxylic anhydride. Considerably lower rates of condensation in the presence of sterically hindered pyridines further support the existence of nucleophilic catalysis in the reactions with non-hindered pyridines. In addition, the rate of reaction in the presence of pyridines with pK_a values above ~4.8 is enhanced by an increase in pivaloyl chloride concentration. The main reason for this enhancement is most likely the fact that pivaloyl chloride removes pivalate ion, which retards the reaction by influencing the equilibrium between the mixed anhydride and the pyridinium intermediate. Although the observed rate constants are composed of several constants, their temperature dependence gives some indication of the nature of the transition-state of the rate-limiting step. Entropies of activation are estimated to be slightly positive, suggesting a transition state arising from attack of the hydroxy component on the pyridinium intermediate but involving a fair degree of bond breakage to the leaving group, *i.e.* the pyridine.

Introduction

The development of methods for the synthesis of oligonucleotides has been most important for progress in molecular biology. The most commonly used method for the key step, *i.e.* the formation of the internucleoside phosphate linkages, is the phosphoramidite approach.[1–3] Since the mid-eighties the H-phosphonate approach[4–9] has emerged as a viable alternative and, as suggested from the work of Reese *et al.*,[10–13] may be the method of choice for large-scale production of antisense oligonucleotides for therapeutic use.

There have been several mechanistic investigations on the coupling step of the amidite approach,[14–18] but little has been carried out on H-phosphonate couplings. The brief reports published so far have mainly been less detailed investigations, where the pathway and/or side-reactions of the condensation step have been in focus.[19–26] The most commonly used condensing reagent for H-phosphonate couplings in oligonucleotide synthesis is pivaloyl chloride.[6–9] The condensation in the presence of pivaloyl chloride has been reported to occur *via* the initially formed mixed phosphonic carboxylic anhydride.[19,20] This initial activation takes place also in the absence of base, but the subsequent reaction with alcohol is then severely retarded.[19] It has been suggested that pyridine acts not only as base but also as a nucleophilic catalyst,[19,25,26] for which there is some experimental support.[25,26]

No detailed kinetic studies have so far been reported. For further optimisation of the H-phosphonate method it would be most useful to have a more detailed knowledge of the mechanisms involved. In this paper we have carried out a detailed kinetic study of the pivaloyl chloride promoted H-phosphonate condensation step in the presence of differently substituted pyridines.

Results and discussion

The reaction investigated is that of 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine 3′-H-phosphonate (**1**) with 3′-*O*-(4-methoxytrityl)thymidine (**2**) in the presence of pivaloyl chloride, resulting in the dinucleoside H-phosphonate **3**, which in the making of a phosphate linkage would be oxidised to the corresponding phosphodiester **4** (Scheme 1). All reactions were monitored by ^{31}P-NMR and were carried out with 30 mM concentration of **1**, generally using 3 equivalents of pivaloyl chloride and equimolar amounts of **1** and **2**, except when **2** was varied. An excess of one of a number of substituted pyridines was used, their pK_a values (in water) ranging from 3.2 to 6.8 (Fig. 1). Reactions using equimolar amounts of **1** and **2** in neat pyridine or acetonitrile–pyridine (3 : 1) are virtually complete upon recording of the first spectrum. Due to this, the study was carried out using acetonitrile as solvent and the excess of pyridine was reduced to 300 or 600 mM (10 or 20 equivalents), enabling a reasonable number of data points to be collected. There is some variation in the rate constants, probably partially dependent on some variation in moisture content. Therefore, experiments were repeated numerous times in order to get more statistically certain values. For reasons clarified later (see below) the variation was most noticeable for the faster reactions.

The initial activation is in almost all cases so fast that all H-phosphonate **1** is consumed and not detected in the first spectrum recorded. Only the mixed phosphono-carboxylic anhydride **5** (2.6 ppm in ^{31}P-NMR)[19] and product **3** (8.0 and 9.5 ppm in ^{31}P-NMR) are detected. Thus the observable reaction is that from **5** to **3** (Scheme 2), and at our starting point there is the mixed anhydride **5**, along with alcohol **2**, pivaloyl chloride (less one reacted equivalent) and pyridine. As the reaction proceeds we would form the product, **3**, and pyridinium pivalate. The

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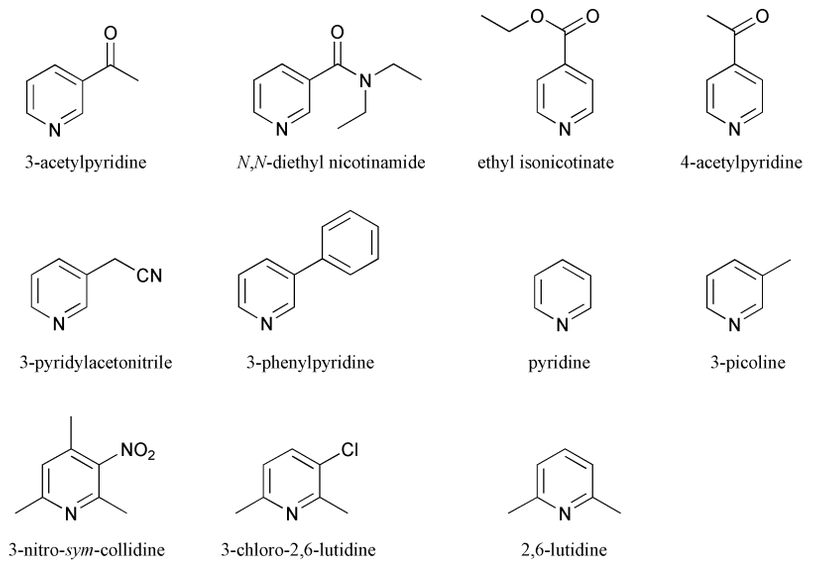
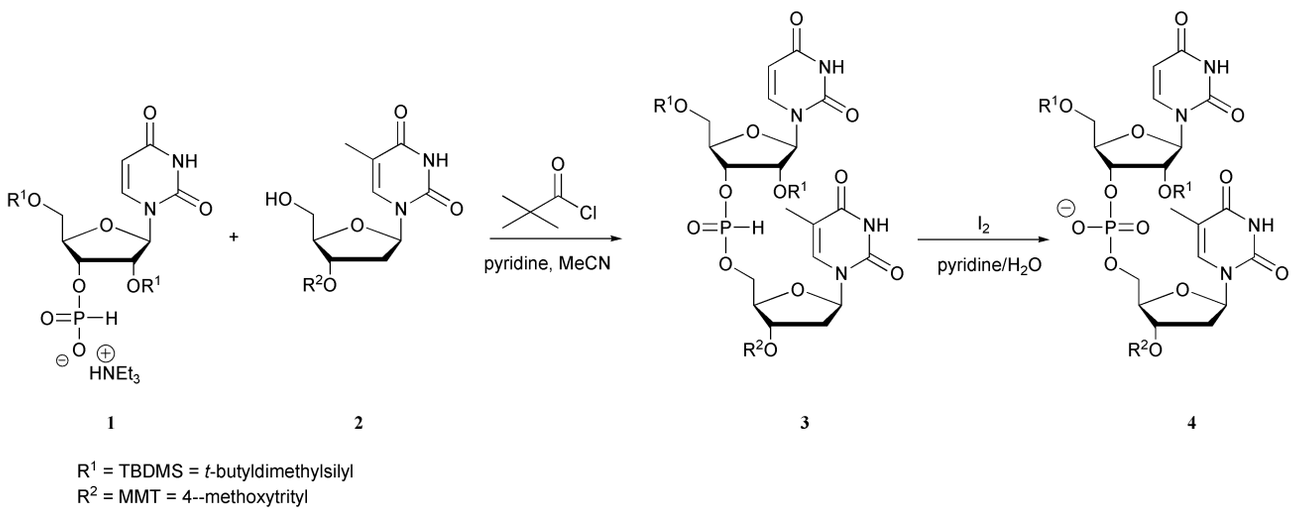
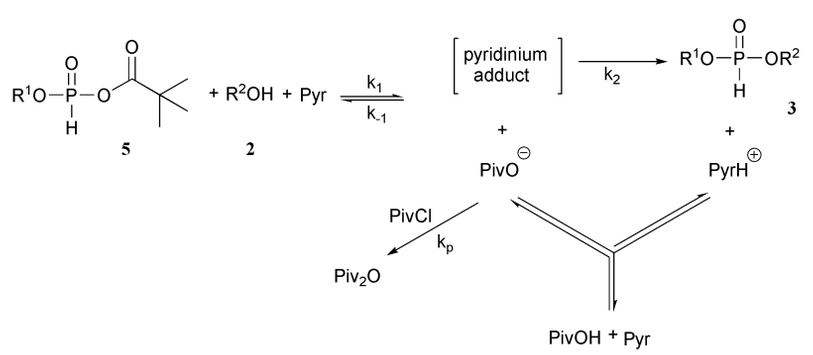


Fig. 1 Pyridine derivatives used in the present study.



R^1 = TBDMS = *t*-butyldimethylsilyl
R^2 = MMT = 4--methoxytrityl

Scheme 1 The pivaloyl chloride promoted coupling of 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine 3′-hydrogenphosphonate with 3′-*O*-(4-methoxytrityl)thymidine.



R^1 = 2',5'-bis-*O*-(TBDMS) uridine-3'-yl; R^2OH = **2**
Piv = Pivaloyl; Pyr = pyridine derivative

Scheme 2 A proposed reaction scheme for H-phosphonate condensation aided by pivaloyl chloride in the presence of non-hindered pyridine derivatives.

acid–base equilibrium of this salt in acetonitrile is, however, almost exclusively shifted to pyridine and pivalic acid[27] and the pyridine concentration may thus be considered constant throughout the reaction.

As previously mentioned, the rate of disappearance of **5** is clearly dependent on the concentration of pyridine, giving a higher rate with increased concentration (Table 1). This dependence seems to be first order in pyridine, for which a doubling of the concentration gives nearly a doubling of the rate. This appears to cover the whole pK_a range studied since a similar behaviour is exhibited when using 3-phenylpyridine or ethyl isonicotinate instead of pyridine.

Some other general influences on the rate of condensation could immediately be observed. The rate of reaction is also dependent on the concentration of alcohol **2**, giving higher rates with increased concentration. If an intermediate is present between the mixed anhydride **5** and product **3**, the generated pivalate could retard the reaction by shifting the equilibrium

Table 1 Observed second order rate constants for couplings of **1** with **2** (both at a concentration of 0.03 M) in acetonitrile at 20 °C, in the presence of different amounts of pyridine derivatives and 3 equiv. of PivCl

	Equiv. pyridine derivative	$k_{obs}/M^{-1}\ s^{-1}$
Pyridine	10	1.17 ± 0.10
	20	1.82 ± 0.18
Ethyl isonicotinate	10	0.27 ± 0.020
	20	0.41 ± 0.011
3-Phenylpyridine	10	0.42 ± 0.027
	20	0.93 ± 0.009

towards the mixed anhydride, *i.e.* the common ion effect. Pivalate ions will be largely protonated by the pyridinium ion formed after the reaction of alcohol **2** with the intermediate (the difference in pK_a value between acetic acid and the pyridinium ion is about 10 units in acetonitrile, pyH^+ being the more acidic[27]). We could, however, not exclude that even a very low concentration of pivalate could retard the reaction substantially. Therefore condensations were carried out with two equivalents of pivalic acid added, and indeed the reaction rate was reduced (approximately halved) compared to the reactions without added pivalic acid. This was observed both in the presence of pyridine and ethyl isonicotinate. It may be concluded that at least one intermediate, although at non-detectable concentrations, is present in the reaction.

The most obvious intermediate would be a pyridinium adduct formed from nucleophilic attack on **5**, *i.e.* nucleophilic catalysis, which has been suggested earlier.[19,26] If we assume a pathway (Scheme 2) *via* such an intermediate (without firmly suggesting structure or coordination number) it must be present in low concentration and we can make a standard steady state approximation for that intermediate. This leads to the rate expression for the disappearance of **5** shown in eqn. (1).

$$-d[\mathbf{5}]/dt = k_1' k_2[\mathbf{5}][\mathbf{2}]/(k_{-1}[PivO^-] + k_2[\mathbf{2}]) \quad (1)$$

where $k_1' = k_1$[pyridine derivative] and $PivO^-$ = pivalate ion.

If [**2**] = [**5**], k_2[**2**] < $k_{-1}[PivO^-]$ and $[PivO^-] \approx$ constant

$$\Rightarrow -d[\mathbf{5}]/dt = k_{obs}[\mathbf{5}]^2 \quad (2)$$

where $k_{obs} = k_1' k_2/k_{-1}[PivO^-]$.

However, plotting the data from reactions with a 1 : 1 ratio of **1** (*i.e.* **5**) and **2** reveals that simple second order kinetics (1/concentration *vs.* time) is followed quite well (Fig. 2). We suggest that, at least with a 1 : 1 ratio of **1** (*i.e.* **5**) and **2**, the term k_2[**2**] is negligible compared to $k_{-1}[PivO^-]$. In the case of a typical common ion effect retardation of rate with time, due to build up of the common ion (*i.e.* pivalate), would be expected. This would have been seen as a deviation from linearity in plots of 1/concentration *vs.* time, despite the fact that the reactions are usually half over by the time the first data point is recorded. If there is some retardation, it is not clearly detectable and a lot less pronounced than would be expected with a simple common ion effect. As the equilibrium between mixed anhydride and pyridinium intermediate is unfavorable, and pivalic acid has a pK_a value considerably lower than pyridinium ion, the pivalate ion concentration will be largely determined by its acid–base equilibrium, and largely buffered. Since it is difficult to completely eliminate water from the reaction mixture there will also always be a background of pivalic acid/pivalate present. This would make any changes in pivalic acid/pivalate concentration even less visible, especially since in most cases it is only possible to follow the later stages of the reaction. Thus, it seems realistic to approximate the pivalate concentration to be constant, which leads to the rate expression in eqn. (2). The above are probably the reasons why the reactions follow simple second order kinetics well, despite the fact that added pivalic acid retards the rate.

The situation is, however, further complicated. When a higher initial concentration of alcohol is used, a considerably poorer linearity is observed, *i.e.*, there is not a straightforward linear relationship upon increase in the initial alcohol concentration. With a 1 : 1 ratio of **5** and **2**, the term k_2[**2**] seems negligible compared to $k_{-1}[PivO^-]$. However, this is probably a borderline case and at higher alcohol concentrations this is no longer true. If at moderately higher alcohol concentrations, the term k_2[**2**] is no longer negligible, then when using an excess of alcohol one would reach the situation where instead $k_{-1}[PivO^-]$ would become negligible. From eqn. (1) it is clear that if using excess alcohol (**2**) the reaction would in fact become independent of the alcohol concentration. To test this hypothesis we carried out reactions with 10 or 20 equivalents of the alcohol **2** in the presence of three different pyridine derivatives. These reactions followed first order kinetics with respect to **5** and did indeed display an independence of concentration of **2** (Table 2).

The observed rate constants (k_{obs}) obtained at a 1 : 1 ratio of **5** and **2** will represent a composite of different constants (and concentrations). However, the k_{obs} values are informative on how the rate is affected by various factors. The observed rate constant is comprised of not only k_2 and the initial equilibrium from mixed anhydride **5** to pyridinium intermediate, but also of the acid–base equilibrium between pivalate ion and pivalic acid (Table 3). When varying the basicity of the pyridine one could expect several different and opposing effects. Probably these counteracting effects are the reasons why the dependence of the observed rate constants on the pK_a value (in acetonitrile)[28] of the pyridine derivative is quite low (Fig. 3) up to a pK_a value (aq.) of 4.8. However, for pyridines of higher basicity there is a considerably higher dependence of k_{obs} on the pK_a value of the

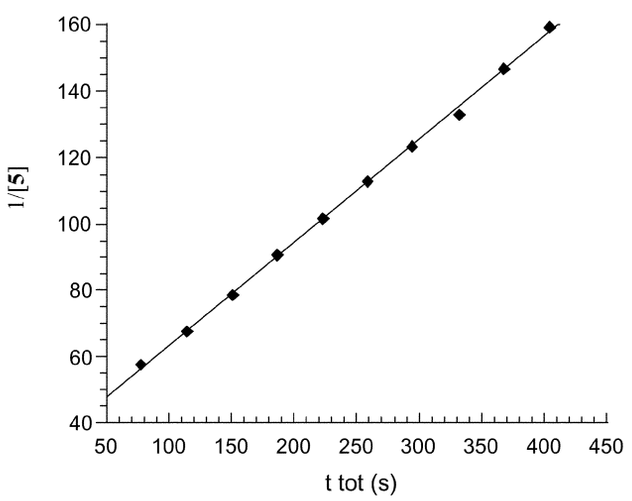


Fig. 2 A plot of the reaction using a 1 : 1 ratio of **1** and **2**, 3 equiv. PivCl and 10 equiv. ethyl isonicotinate.

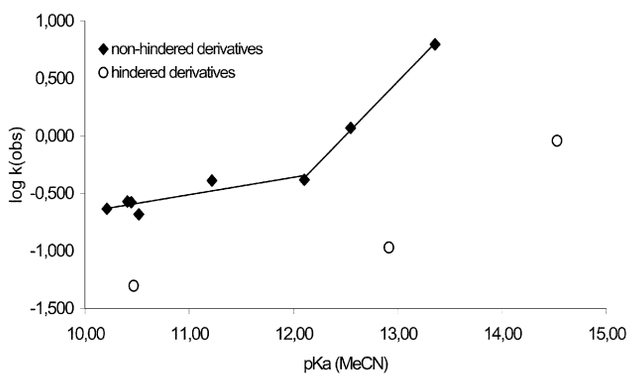


Fig. 3 Plot of log k_{obs} for the condensation reaction *vs.* pK_a (in MeCN) of the various pyridine derivatives.

Table 2 Observed first order rate constants for couplings of **1** (0.03 M) with excess of **2**, in acetonitrile using 7.5 equiv. PivCl and different pyridine derivatives (300 mM)

Ratio of alcohol (**2**) : phosphonate (**1**)	k_{obs} pyridine[a]	k_{obs} ethyl isonicotinate[b]	k_{obs} 3-phenylpyridine[a]
10 : 1	0.014 ± 0.00062	0.0033 ± 0.00009	0.0086 ± 0.00033
20 : 1	0.012 ± 0.00027	0.0038 ± 0.00012	0.0081 ± 0.00017

[a] Reactions carried out at 5 °C. [b] Reactions carried out at 10 °C.

Table 3 Observed second order rate constants for couplings of **1** with **2** (both at a concentration of 0.03 M) in acetonitrile at 20 °C, in the presence of different pyridines (300 mM) and 3 equiv. of pivaloyl chloride

	$pK_{a\ water}$ ($pK_{a\ MeCN}$)	k_{obs} ($K'k_2$/[PivO$^-$])/M^{-1} s^{-1}
3-Acetylpyridine[29]	3.26 (10.21)	0.23 ± 0.036
N,*N*-Diethylnicotinamide[30]	3.42 (10.41)	0.27 ± 0.005
Ethyl isonicotinate[29]	3.45 (10.44)	0.27 ± 0.020
4-Acetylpyridine[29]	3.51 (10.52)	0.21 ± 0.014
3-Pyridylacetonitrile[31]	4.08 (11.22)	0.41 ± 0.027
3-Phenylpyridine[32]	4.8 (12.10)	0.42 ± 0.015
Pyridine[31]	5.16 (12.55)	1.17 ± 0.10
3-Picoline[33]	5.82 (13.36)	6.27 ± 0.065
3-Nitro-*sym*-collidine[30]	3.47 (10.47)	0.05 ± 0.012
3-Chloro-2,6-lutidine[30]	4.87 (12.19)	0.11 ± 0.011
2,6-Lutidine[33]	6.77 (14.53)	0.91 ± 0.13

Table 4 Observed second order rate constants for couplings of **1** with **2** (both at a concentration of 0.03 M) in acetonitrile at 20 °C, in the presence of different pyridines (300 mM) and various equivalents of PivCl

	Equiv. PivCl			
k_{obs} ($K'k_2$/[PivO$^-$])	3	5	7.5	15
Pyridine	1.17 ± 0.10	1.79 ± 0.59	3.7 ± 0.23	10 ± 0.79
Ethyl isonicotinate	0.27 ± 0.02	0.28 ± 0.013	—	—
3-Phenylpyridine	0.42 ± 0.015	0.61 ± 0.12	—	—

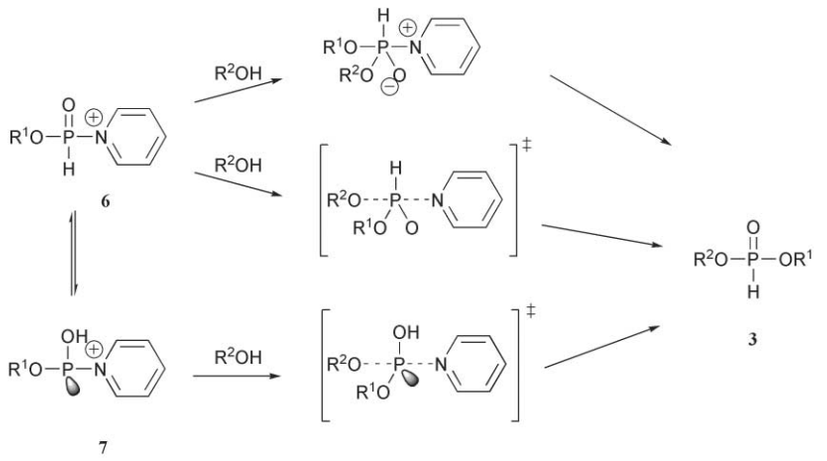
pyridine derivative. The situation is complicated further by the observation that different pyridines give different rate dependences on pivaloyl chloride. In the simple case where alcohol attacks **5**, or a subsequent pyridinium intermediate, it is to be expected that the rate of disappearance of **5** would be independent of pivaloyl chloride. This is also the case for pyridines with a pK_a value (aq.) below 4.8, but for 3-phenylpyridine and pyridine itself the rate is clearly dependent on pivaloyl chloride concentration (Table 4).

It is conceivable that in the presence of a stronger base/nucleophilic catalyst excess pivaloyl chloride reacts with pivalate giving pivaloyl anhydride, which is known to be unreactive in coupling.[34] To investigate this, experiments were performed where pivaloyl chloride was reacted with pivalic acid in the presence of ethyl isonicotinate, 3-phenylpyridine or pyridine. With ethyl isonicotinate only barely detectable amounts of the pivalic acid were consumed during the time needed for complete condensation. For 3-phenylpyridine the reaction was clearly visible but somewhat slower than condensations using the same base. In pyridine, the reaction was even faster with a rate virtually on a par with that for the condensation reaction. These observations provide a feasible explanation for the dependence of the rate of condensation on the pivaloyl chloride concentration. In the presence of a pyridine derivative with a pK_a value higher than about 4.8, the pivalate ion concentration is not only restricted by the acid–base equilibrium to pivalic acid, but also through reaction with pivaloyl chloride at a rate of the same order as the rate of its formation. Raising the concentration of pivaloyl chloride will lead to an increase of the reaction rate by giving a lower pivalate ion concentration, *i.e.*, through less retardation from the "common ion" concentration, thereby shifting the equilibrium towards the pyridinium intermediate. As the rate of the reaction between pivalate and pivaloyl chloride, in pyridine, is of the same magnitude as the overall condensation reaction, the kinetics of the overall condensation reaction under these conditions would still follow the same order and within error with a seemingly constant, but lower, pivalate concentration.

From the above experiments it is clear that the reaction is somewhat complicated, but a number of approximations make it possible to obtain rate constants that are useful in sorting out the mechanism of condensation. In particular they give information on the dependence of rate on the nature of different pyridine derivatives. The upwards deviation in the plot of log k_{obs} *vs.* pK_a of pyridine derivative (Fig. 3) is most likely not due to a change in the mechanism of the condensation reaction. Instead this is probably due to the fact that in the presence of the more basic pyridines the pivalate ion reacts sufficiently fast with excess pivaloyl chloride to affect its concentration and thus diminish the "common ion" retardation effect. The reaction between pivalate and pivaloyl chloride clearly has a much higher dependence on the pK_a value of the pyridine derivative than the condensation reaction has.

The dependence of the rate of condensation on the pK_a value of the pyridine derivative is more clearly represented by the reactions of the less basic pyridines. This dependence is quite modest (the slope for the left half of the plot in Fig. 3 is 0.15), which is not unexpected since the rate constants are dependent on several counteracting effects. The rate of formation of pyridinium intermediate should increase when increasing the basicity of the pyridine. On the other hand the reactivity of the pyridinium intermediate decreases and the higher basicity also gives an increase in pivalate ion concentration, which retards the formation of intermediate.

It has been suggested previously that pyridine acts as a nucleophilic catalyst in H-phosphonate condensations.[5,26] There is also experimental support in favour of this, *i.e.* that a considerably lower rate of condensation is observed in the



R^1 = 2',5'-bis-*O*-(TBDMS) uridine-3'-yl;
R^2OH = **2**

Scheme 3 Plausible transition states/intermediate in the rate limiting step.

presence of *N*,*N*-dimethylaniline than in the presence of pyridine.[26] However, the solvent dependences of the basicities of *N*,*N*-dimethylaniline and pyridine are not completely identical. In several aprotic solvents, as opposed to in water, pyridine is more basic than *N*,*N*-dimethylaniline (from 0.8 to 1.2 pK_a units),[27] which in the case of base catalysis also would mean a lower rate. Furthermore, in view of our present results, the higher rate with pyridine compared to with *N*,*N*-dimethylaniline could also be due to nucleophilic catalysis on pivaloyl chloride in the trapping of pivalate. Although mostly convinced by the mentioned study of Efimov *et al.*,[26] we thought it valuable to solidify the evidence by carrying out experiments with sterically hindered pyridines and therefore condensations were carried out in the presence of 2,6-lutidine, 3-chloro-2,6-lutidine and 3-nitro-*sym*-collidine.

As can be seen in Table 3 the rate of condensation with 2,6-lutidine is lower than for pyridine even though the pK_a value is substantially higher. With only this in mind one could perhaps not rule out that this could depend on less efficient trapping of pivalate, which is responsible for additional rate enhancement with pyridine. The other less basic, sterically hindered pyridines, however, give a substantially lower rate than the non-hindered pyridines with similar pK_a values, resulting in a separate correlation of basicity to k_{obs} for the hindered pyridine derivatives (Fig. 3). This correlation probably reflects the dependence on pK_a value of the base, in base catalysis for the reaction of alcohol with the mixed anhydride **5**. Most importantly, though, is that we can consider it confirmed that non-hindered pyridine derivatives exert nucleophilic catalysis in the reaction of **5** to **3**. Moreover, this appears to be the case over the whole range of pK_a values investigated here. This brings us to the nature of the intermediate. The experiments support a pyridinium intermediate, but the species reacting with alcohol in a subsequent step may not necessarily be tetracoordinated, *e.g.* **6**, since it will be in equilibrium with the tautomeric tricoordinated form, *e.g.* **7** (Scheme 3). That this tautomerism readily occurs under the conditions is evidenced from the observation that without alcohol present a bisacyl phosphite is rapidly formed.[19]

An interesting mechanism would be *via* the tricoordinated form **7** since this is essentially similar to the intermediate suggested to be formed in pyridinium catalysed phosphoroamidite coupling,[18] the only difference being that one phosphorus ligand is here an OH instead of an O-alkyl. For H-phosphonate diesters the tetracoordinated tautomer predominates, indicating this is thermodynamically more stable than the phosphite tautomer. However, a lone pair on phosphorus could well stabilise the positive charge of a pyridinium intermediate, thus stabilising the tricoordinated pyridinium tautomer. In order to get some idea about this possibility we carried out quantum mechanical calculations on both intermediates. Hartree–Fock calculations using the MIDI! basis set have been shown to give surprisingly accurate geometries (MP2-like) of phosphorus acid derivatives as well as subsequent single point energies with the EDF1 functional.[35] Using this combination we carried out calculations on compounds similar to **6** and **7** but with the nucleoside moiety replaced by an isopropyl group. The calculations gave only a 0.6 kcal difference in energy between the tetracoordinated and tricoordinated tautomers. Although the calculations were performed without inclusion of solvation they give an indication that the reaction path can involve either or both pyridinium intermediates. At this stage we can, however, not be certain as to which intermediate the alcohol **2** predominantly or exclusively reacts with in formation of the H-phosphonate diester **3**. Further calculations including transition states may aid in this elucidation and are in progress.

Although the observed rate constants are dependent on several steps, some indications of the nature of the transition state of the rate-limiting step may be obtained from their temperature dependence. Hence we carried out condensations at different temperatures with 3 equivalents of pivaloyl chloride in the presence of pyridine, 3-phenylpyridine or ethyl isonicotinate. The resulting Arrhenius plots are shown in Fig. 4. The ΔS values obtained from these are for pyridine 11.6 (± 1.7) J K^{-1} mol^{-1}, for 3-phenylpyridine 10.3 (± 1) J K^{-1} mol^{-1} and for ethyl isonicotinate 9.6 (± 0.3) J K^{-1} mol^{-1}. Apart from the rate constants for attack of alcohol on a pyridinium intermediate, the observed rate constants also involve two equilibria. Since the

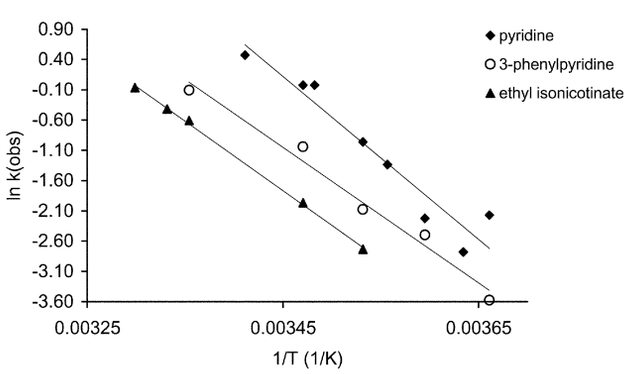


Fig. 4 Arrhenius plots for ethyl isonicotinate, 3-phenylpyridine and pyridine.

number of reactants is equal on both sides of both equilibria, we should be able to approximate the change in entropy for these equilibria to be relatively modest. As the overall ΔS values obtained from the intercepts of the Arrhenius plots are quite small, we can assume that the entropy of activation, $\Delta S^{\neq}$, is also quite modest and slightly positive.

The $\Delta S^{\neq}$ values suggest that the reaction from pyridinium intermediate **6** or **7** to diester **3** does not involve a transition state arising from a rate-limiting strongly associative mechanism. Although one would usually expect a more positive activation entropy for a completely dissociative mechanism, this cannot be excluded on the basis of the entropy value alone. A completely dissociative mechanism can nevertheless be excluded since this is inconsistent with the observed rate dependence on alcohol concentration. Thus, initial rate-limiting formation of a metaphosphite species from **6** or **7** is unlikely leaving as options initial attack of alcohol on either of the pyridinium intermediate tautomers. This could be *via* an intermediate or a transition state that is tetra- or pentacoordinated, depending on which tautomer is being considered (Scheme 3). If an intermediate with a reasonable lifetime is formed and its formation would be rate limiting, a more clearly negative $\Delta S^{\neq}$ would be expected. If on the other hand the breakdown would be rate limiting, one would expect a more positive $\Delta S^{\neq}$. Although there is some degree of uncertainty in the entropies of activation, we would suggest that it is more likely that the reaction is concerted, and that the transition state involves a fair degree of bond breakage to the leaving group, *i.e.* the pyridine. It has also been argued convincingly that nucleophilic displacement of a good leaving group from a phosphate is either concerted, or involves an intermediate in a very shallow well at the top of a barrier.[36] By similarity this would not be unexpected in the case of the more reactive H-phosphonate derivatives.

Experimental

Materials and methods

NMR spectra were recorded using a Bruker DRX 400 MHz spectrometer, or in some cases a JEOL GSX-270 FT spectrometer. Chemical shifts are given in ppm, using 2% H_3PO_4 in D_2O as reference. Acetonitrile (chromatography grade, Merck) was dried over predried 3 Å molecular sieves. Pivaloyl chloride (Acros) was distilled at reduced pressure and stored at −20 °C in sealed flasks. Anhydrous pyridine (Labscan) was stored over predried 4 Å molecular sieves. 3-Chloro-2,6-lutidine was synthesised according to a published procedure[37] from 2,5-dimethylpyrrole. Other pyridines were commercially available and either distilled and stored over predried 4 Å molecular sieves or recrystallised and dried under vacuum, except for 3-nitro-*sym*-collidine (Maybridge), which was used without further purification. 2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)uridine 3′-hydrogenphosphonate triethylammonium salt[38] was synthesised through disilylation of uridine according to a published procedure,[39] followed by phosphonylation using diphenyl phosphite.[40] 3′-*O*-(Methoxytrityl)thymidine[41] was synthesised according to a published procedure,[42] except that pivaloyl chloride was used instead of diphenylchloroacetyl chloride as temporary protection of the 5′-hydroxy.

^{31}P NMR studies of the coupling reaction

The couplings were studied using H-phosphonate and alcohol in a ratio of 1 : 1 (in a few cases 1 : 10 or 1 : 20), 10 equivalents (in a few cases 20 equiv.) pyridine and 3 equivalents (in some cases 5, 7.5 or 15 equiv.) of PivCl, in a total volume of 2.4 ml. An inner tube containing D_2O or 2% phosphoric acid in D_2O was used for locking and as reference.

H-phosphonate and alcohol (72 μmol of each, or 72 μmol H-phosphonate and the correspondingly greater amount of alcohol for the 1 : 10 and 1 : 20 ratios respectively) were weighed and dried by evaporation of added MeCN–pyridine 3 : 1 (twice) and MeCN, whereafter it was kept for ten minutes under vacuum. The samples were dissolved in the appropriate amount of acetonitrile and, along with the desired pyridine (0.72 or 1.44 mmol), transferred to an NMR tube. At the start of each experiment PivCl (0.216 mmol or 0.36 mmol) was added and spectra were recorded at various times. The spectra were integrated and the areas of the signals from **5** (2.6 ppm) and **3** (8 and 9.5 ppm) used for kinetic calculations. The second order rate constants for the reactions were obtained as the slope in standard plots of $1/0.03*F(\mathbf{5})_t$ *vs.* time ($F(\mathbf{5})$ = area of **5**/area of **5** + area of **3**). The first order rate constants (in experiments with excess of **2**) were obtained as the slope in plots of ln $F(\mathbf{5})$ *vs* time.

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