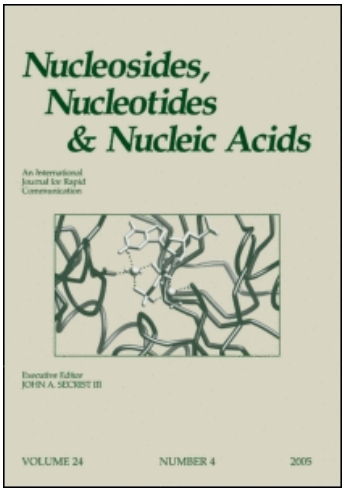

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Side Reactions in the H-Phosphonate Approach to Oligonucleotide Synthesis: A Kinetic Investigation on Bisacylphosphite Formation and 5'-O-Acylation

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Side Reactions in the H-Phosphonate Approach to Oligonucleotide Synthesis: A Kinetic Investigation on Bisacylphosphite Formation and 5′-O-Acylation

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ABSTRACT

A kinetic study on the reactions of pivaloyl chloride with a nucleoside H-phosphonate (bisacylphosphite formation), or a protected nucleoside with a free 5′-OH (5′-O-pivaloylation) has been carried out in the presence of differently substituted pyridines. The bisacylphosphite formation is a result of acylation of the mixed carboxylic-phosphonic acid anhydride to give the corresponding bispivaloylphosphite derivative. The rate of this reaction is dependent on the concentration of pyridine derivative and pivaloyl chloride. The 5′-O-pivaloylation reaction is also dependent on the concentration of pyridine derivative and pivaloyl chloride. The rate of both reactions is dependent on the basicity of the pyridine derivative, displaying a higher rate with more basic pyridines. The dependence of log k vs. pK_a of pyridine is linear but the slopes are quite different for the two reactions (0.84 for bisacylation and 0.21 for 5′-O-pivaloylation). For both reactions considerably lower rates in the presence of sterically hindered pyridines suggest the existence of nucleophilic catalysis on pivaloyl chloride in reactions with non-hindered pyridines.

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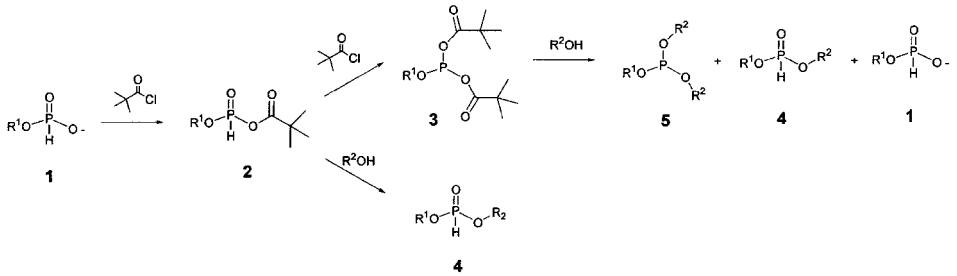
Key Words: Hydrogenphosphate; Mechanism; Kinetics; Bisacylphosphite; Acylation.

INTRODUCTION

The H-phosphonate approach to oligonucleotide synthesis[1–7] is today a relatively established method and has recently been extended to large scale synthesis of antisense oligonucleotides.[8–11] Reports on mechanistic aspects of H-phosphonate couplings have mostly concerned the pathway and/or side reactions.[12–19] The most commonly used condensing reagent for H-phosphonate couplings in oligonucleotide synthesis is pivaloyl chloride.[3–6,12] Upon prolonged treatment pivaloyl chloride can cause P-acylation of the H-phosphonate diester product.[20,21] This is in general not a problem with ribonucleoside H-phosphonates, due to a sterically more demanding 2′-substituent, and P-acylation on deoxyribonucleoside H-phosphonates can also be reduced or eliminated by use of less basic pyridine derivatives.[22]

The condensation of an H-phosphonate (**1**) with an alcohol has been reported to occur via an initially formed mixed phosphonic carboxylic anhydride[12] (**2**). It was recognised at an early stage that H-phosphonate couplings in solution were best carried out by adding the condensing agent to a solution of alcohol and H-phosphonate.[1,2,12] If an H-phosphonate (**1**) is preactivated with pivaloyl chloride before addition of the alcoholic component, the corresponding bispivaloylphosphite **3** is formed[12] (Sch. 1). The bisacylphosphite **3** gives, upon addition of alcohol, not only the desired product **4** but also substantial amounts of the phosphite triester **5** through reaction with two equivalents of the 5′-hydroxyl component (Sch. 1). In fact, addition of excess of alcohol gives no desired H-phosphonate diester **4**, but only phosphite triester **5** and H-phosphonate monoester **1**. In addition, it has been reported that bisacylphosphites react at a considerably lower rate than the mixed carboxylic phosphonic anhydrides **2** with alcohols.[18,23] As a consequence of this, substantial acylation of 5′-hydroxyls by pivaloyl chloride occurs, resulting in low coupling yields.[23]

For technical reasons, machine assisted solid support synthesis of oligonucleotides usually involves premixing of H-phosphonate and pivaloyl chloride. In order to further optimise the H-phosphonate method it would be most useful with more



Scheme 1.

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detailed knowledge on the kinetics of the preactivation and 5′-O-acylation reactions, in particular the dependence on the pyridine derivative used and how this relates to the corresponding dependence for the condensation reaction. Detailed kinetic studies have so far not been reported, neither on bisacylphosphite formation nor on 5′-O-pivaloylation. In this paper we have carried out kinetic studies of the reaction of the mixed carboxylic phosphonic anhydride with pivaloyl chloride as well as 5′-O-pivaloylation of a nucleoside in the presence of differently substituted pyridines.

RESULTS

Bisacylphosphite Formation

Reactions of 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl)uridine H-phosphonate (**6**) with pivaloyl chloride were carried out at 30 mM concentration of **6**. In order to get comparable data to previous studies on the condensation reaction most experiments were subsequently carried out in acetonitrile with 300 mM (600 mM in some cases) concentration of a pyridine derivative. The amount of pivaloyl chloride was usually 3 equiv. (90 mM) and in a few cases 5 equiv. (150 mM). Reactions were carried out in the presence of a number of substituted pyridines with pK_a values (in water) ranging from 3.2 to 6.8 (Fig. 1). The reactions were monitored by ^{31}P-NMR, and the initial activation is in almost all cases so fast that all H-phosphonate **6** is consumed and not detected in the first spectrum recorded. Only the mixed phosphono carboxylic acid anhydride **7** (2.6 ppm)[12] and bisacylphosphite **8** (134 ppm)[12] are detected. Thus the observable reaction is that from **7** to **8** (Sch. 2).

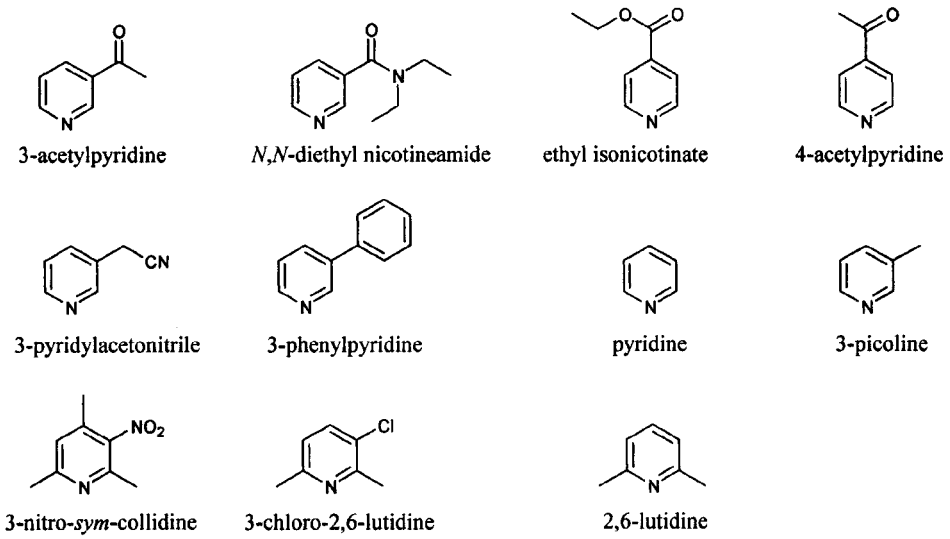

Figure 1. Pyridine derivatives used.

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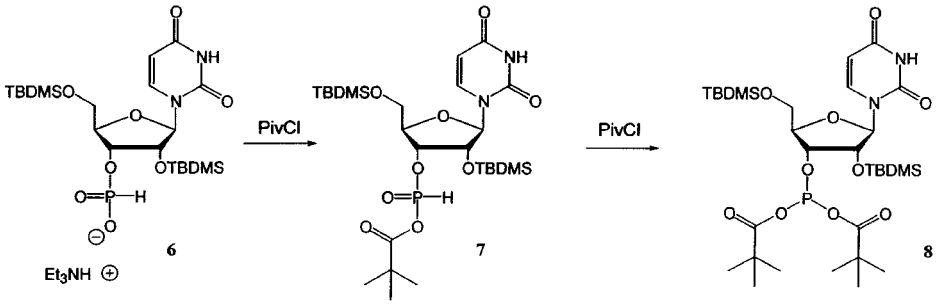

Scheme 2.

The reaction rate is as expected dependent on pivaloyl chloride (Table 1) and all reactions follow second order kinetics, first order with respect to **7** and pivaloyl chloride respectively. In principle one equivalent of pyridine is consumed in the reaction. However, reactions were followed during less than 50% conversion (typically starting at 80–90% of **7** remaining). The consumption of pyridine is then low enough to approximate the pyridine concentration as being constant. Comparing experiments with different pyridine concentrations it is clear that the rate of disappearance of **7** is dependent thereof, giving a higher rate with higher concentration of the pyridine derivative (Table 1). That dependence seems to be first order in pyridine concentration since a doubling of this gives a doubling of the second order rate constant.

The reaction rate for the bisacylphosphite formation is clearly dependent on the basicity of the pyridine derivative (Table 2). The plot of log k_{obs} vs. pK_a (in acetonitrile)[24] of pyridine derivative is linear with a slope of 0.84 (Fig. 2) as long as sterically hindered pyridines are not included. 2,6-Lutidine gives a lower rate than with the less basic pyridine or 3-picoline and no bisacylphosphite formation could be detected in overnight reactions in the presence of 3-chloro-2,6-lutidine or 3-nitro-*sym*-collidine. From the considerably lower rates of reaction with the sterically hindered pyridines it appears that nucleophilic catalysis is operating. This is most

Table 1. Observed second order rate constants for conversion of **7** (30 mM) to **8** by reaction with pivaloyl chloride in acetonitrile at 20°C, in the presence of different amounts of a pyridine derivative

		60 mM PivCl[a] $k_{obs} \times 10^3$ ($M^{-1}s^{-1}$)	120 mM PivCl[b] $k_{obs} \times 10^3$ ($M^{-1}s^{-1}$)
Pyridine	300 mM	6.5	12
	600 mM	14	
Ethyl isonicotinate	300 mM	0.047	0.081
	600 mM	0.11	

[a]2 equiv to **7** (initially 3 equiv. (90 mM) to **6**).
[b]4 equiv to **7** (initially 5 equiv. (150 mM) to **6**).

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Table 2. Observed second order rate constants for conversion of **7** (30 mM) to **8** by reaction with pivaloyl chloride (60 mM)[a] in acetonitrile at 20°C, in the presence of different pyridine derivatives (300 mM)

	pK_{awater} (pK_{aMeCN})	$k_{obs} \times 10^3$ ($M^{-1}s^{-1}$)
3-acetylpyridine	3.26 (10.21)	0.032
N,*N*-diethyl nicotineamide	3.42 (10.41)	0.052
ethyl isonicotinate	3.45 (10.44)	0.047
4-acetylpyridin	3.51 (10.52)	0.052
3-pyridyl acetonitrile	4.08 (11.22)	0.15
3-phenyl pyridine	4.80 (12.10)	1.5
pyridine	5.16 (12.55)	6.5
3-picoline	5.82 (13.36)	8.7
2,6-lutidine	6.77 (14.53)	4.5

[a]2 equiv., initially 90 mM, i.e., 3 equiv. to **6**.

likely through nucleophilic catalysis on pivaloyl chloride. It is also likely that base catalysis contributes to the reaction rate, through ionisation (or possibly general base catalysis) of **7**.

5′-O-Pivaloylation

In order to mimic the conditions of solid support synthesis, the reactions of 3′-*O*-(4-methoxytriphenyl)thymidine (**9**) with pivaloyl chloride (Sch. 3) were carried out at

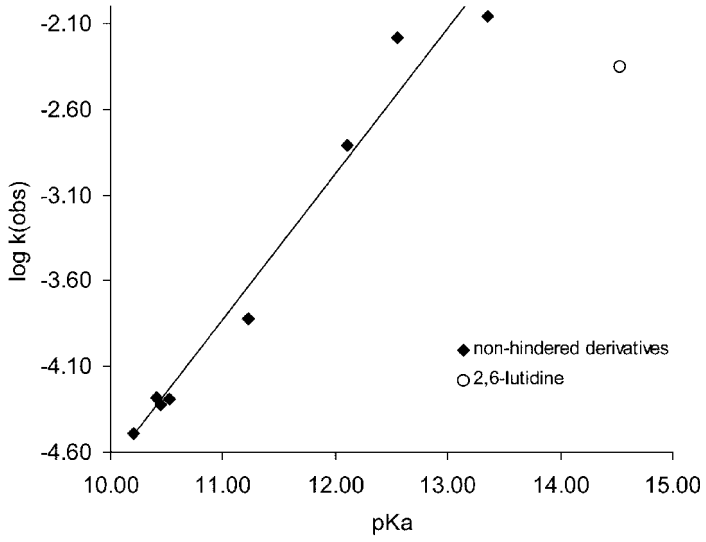

Figure 2. Plot of log k(obs) for formation of bisacylphosphite **8** from **7** by reaction with pivaloyl chloride (60 mH) *vs* pKa (MeCN) for the pyridine derivative present (300 mM).

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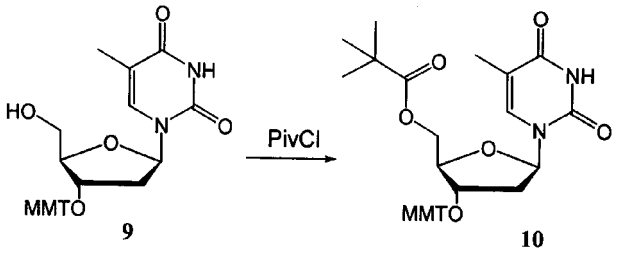

Scheme 3.

4.5 mM concentration of **9**. Most experiments were subsequently carried out at 300 mM (900 mM in some cases) concentration of the pyridine derivative used, and the pivaloyl chloride concentration was usually 90 mM, but in a few cases 150 mM. The reactions were carried out in the presence of a number of substituted pyridines with pK_a values (in water) ranging from 3.2 to 6.8 (Fig. 1). The reactions were monitored on TLC and quantifications of **9** and **10** were done with the Quantiscan programme after scanning of the acid developed plates.

All these reactions follow first order kinetics, which is not unexpected since the other reactants are in excess to **9**. Variation in the concentration of the pyridine derivative reveals a first order rate dependence (Table 3). Increase of the pivaloyl chloride concentration also shows the expected dependence. As seen in the plot of log k_{obs} vs. pK_a (in acetonitrile),[24] the rate of 5′-O-pivaloylation is also dependent on the basicity of the pyridine derivative (Fig. 3). Also in this case are the reactions using sterically hindered pyridines considerably slower than for non-hindered pyridines of similar or lower basicity, again suggesting nucleophilic catalysis on pivaloyl chloride. A major difference in the dependence of reaction rate upon basicity of pyridine is that the sensitivity to the pK_a of the pyridine is considerably higher for the bisacylation (Fig. 2) than for the O-acylation (Fig. 3).

DISCUSSIONS AND CONCLUSIONS

Both side reactions above are dependent on pivaloyl chloride and pyridine concentration and both are influenced by the basicity of the pyridine. Both reactions are

Table 3. Observed first order rate constants for conversion of **9** (4.5 mM) to **10** by reaction with excess pivaloyl chloride in acetonitrile at 20°C, in the presence of different amounts of a pyridine derivative

		90 mM PivCl $k_{obs} \times 10^5$ (s^{-1})	150 mM PivCl $k_{obs} \times 10^5$ (s^{-1})
Pyridine	300 mM	12	–
	900 mM	33	56
N,*N*-diethylnicotineamide	300 mM	4.3	–
	900 mM	12	17

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Table 4. Average first order rate constants for conversion of **9** (4.5 mM) to **10** by reaction with pivaloyl chloride (90 mM) in acetonitrile at 20°C, in the presence of different pyridine derivatives (300 mM)

	pK_{awater} (pK_{aMeCN})	$k_{obs} \times 10^5$ (s^{-1})
3-acetylpyridine[33]	3.26 (10.21)	4.0
N,*N*-diethyl nicotineamide[34]	3.42 (10.41)	4.3
ethyl isonicotinate[33]	3.45 (10.44)	4.2
4-acetylpyridin[33]	3.51 (10.52)	5.2
3-pyridyl acetonitrile[35]	4.08 (11.22)	6.7
3-phenyl pyridine[36]	4.8 (12.10)	9.2
pyridine[35]	5.16 (12.55)	12
3-picoline[37]	5.82 (13.36)	19
3-chloro-2,6-lutidine[34]	5.46 (12.92)	3.7
2,6-lutidine[37]	6.77 (14.53)	6.2

also likely to proceed through nucleophilic catalysis on pivaloyl chloride in the presence of a non-hindered pyridine. That nucleophilic catalysis can occur on pivaloyl derivatives is known from a study of imidazole catalysis on aryl pivalates.[25] The extent of the dependence of the rate of O-acylation and bisacylation on the basicity of the pyridine is, however, quite different. The slope for the log k vs. pK_a correlation of pyridine is 0.84 for the bisacylation whereas it is only 0.21 for the 5′-O-acylation.

The dependences on concentrations of reagents and on the basicity of the pyridine derivative observed in this study may have implications for the choice of

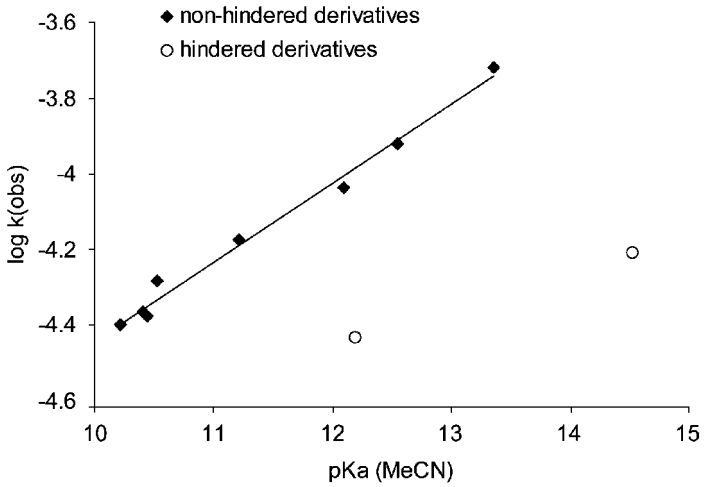

Figure 3. Plot of log k(obs) for conversion of **9** to **10** by reaction with pivaloyl cholride (90 mM) *vs* pKa (MeCN) for the pyridine derivative present (300 mM).

conditions in H-phosphonate couplings in either solid phase or solution synthesis of oligonucleotides. For pyridines with a pK_a (aq.) lower than 4.8, the condensation reaction is considerably less dependent on the basicity of the pyridine derivative[26] than the bisacylation reaction. Use of a less basic pyridine should then also cause more retardation of this side reaction than of the condensation reaction. In the previous study on the H-phosphonate condensation step we found that this reaction also was dependent on pivaloyl chloride concentration, although only for the more basic pyridines.[26] Thus, use of a less basic pyridine and a lower concentration of pivaloyl chloride should lead to a reduction of the above side reactions (as well as to less P-acylation[20–22]).

The competing 5′-O-acylation has a relatively similar dependence on the basicity of the pyridine compared to that of the condensation reaction, when less basic pyridines are used.[26] In order to obtain conditions resembling those during oligonucleotide synthesis, the O-acylation study has been carried out under pseudo first order conditions, i.e., excess of nucleoside H-phosphonate toward alcohol. Since the coupling reaction is extremely fast, the study of that reaction was performed using second order conditions, i.e., a 1:1 ratio of H-phosphonate and alcohol. A typical concentration used in oligonucleotide synthesis for the H-phosphonate is 0.03 M, and when this is in excess to alcohol (as in solid support synthesis) the second order rate constants can be converted to first order constants by multiplying with 0.03 M. Comparing the rate constants for condensation[26] obtained in this way with those of the 5′-O-pivaloylations reveals that during the time required for completion of condensation the amount of O-acylation that could be expected is approximately half a percent or less. As both these reactions have a low dependency on the basicity of the pyridine, a change of pyridine derivative does not in itself give much improvement. However, when a pyridine derivative having a pK_a (aq.) below 4.8 is used, the condensation is independent of pivaloyl chloride concentration[26] whereas the 5′-O-pivaloylation is not. Use of a less basic pyridine in combination with a lower pivaloyl chloride concentration should then suppress the competing 5′-O-acylation as well as the bisacylation reaction. Thus, provided that a lower rate of condensation is acceptable, one would expect the use of a pyridine derivative with a pK_a value below 4.8 and a lower concentration of pivaloyl chloride to be preferred for H-phosphonate couplings, in order to minimise possible side reactions.

EXPERIMENTAL

Materials and Methods

NMR spectra were recorded using a Bruker DRX 400 spectrometer. ^{31}P chemical shifts are given in ppm, using 2% H_3PO_4 in D_2O as reference. Thin layer chromatography was carried out using Merck Silica Gel 60 F_{254} pre-coated glass plates with UV light detection and/or charring with 8% sulphuric acid in ethanol. Acetonitrile (chromatography grade, Merck) was dried over 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 4 Å molecular sieves. 3-chloro-

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2,6-lutidine was synthesised from 2,5-dimethylpyrrole according to a published procedure.[27] Other pyridines were commercially available and either distilled and stored over predried 4 Å molecular sieves or recrystallised and dried under vacuum. 2′,5′-bis-*O*-(*tert*-butyldimethylsilyl) uridine 3′-hydrogenphosphonate[28] triethylammonium salt was synthesised through disilylation of uridine according to published procedure,[29] followed by phosphonylation using diphenylphosphite.[30] 3′-*O*-monomethoxytrityl thymidine[31] was synthesised essentially according to the published procedure[32] except that pivaloyl chloride was used instead of diphenylchloroacetyl chloride as temporary protection of the 5′-hydroxyl.

^{31}P NMR Studies of the Reaction of 6 (i.e., 7) with Pivaloyl Chloride (Bisacylphosphite Formation)

The reactions were performed in acetonitrile using 30 mM of H-phosphonate **6**, 300 or 600 mM (10 or 20 equiv.) pyridine and 90 or 150 mM (3 or 5 equiv. to **6**) 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 (72 μmmol) was dried twice by evaporation of added acetonitrile-pyridine 3:1 and then once with acetonitrile whereupon the residue was dried further for a few minutes in vacuo. 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 defined time intervals. The spectra were integrated and the areas of the signals for **7** (2.6 ppm) and **8** (134 ppm) were used for the calculations. The second order rate constants were obtained from the slope in plots of $1/0.03 * \ln(1-F_7/2)/(1-F_7)$ vs. time for experiments with a starting concentration of 90 mM pivaloyl chloride and of $1/0.09 * \ln(1-F_7/4)/(1-F_7)$ vs. time for experiments with a starting concentration of 150 mM pivaloyl chloride (F_7 = fraction of **7**, i.e., area of **7**/(area of **7** + area of **8**)).

TLC Studies of the Reaction of 9 with Pivaloyl Chloride

Stock solutions of the different pyridine derivatives in acetonitrile (0.3 M, 0.9 M in a few cases) were prepared, containing either 3′-*O*-(4-methoxytrityl) thymidine **9** (9 mM, solution **A**) or pivaloyl chloride (180 mM, in a few cases 300 mM, solution **B**). These solutions were tightly sealed and stored in the freezer until used.

Each reaction was carried out by addition of 0.5 mL of stock solution **B** to 0.5 mL of stock solution **A**., i.e., total concentration 4.5 mM **7**, 90 or 150 mM PivCl and 0.3 or 0.9 M pyridine in acetonitrile. Small aliquots (50 μL) were withdrawn and quenched in 2 M triethylammonium bicarbonate buffer, pH 7.5 (100 μL). Each sample was extracted with CH_2Cl_2 (100 μL) and the organic extracts analysed by TLC (toluene:ethyl acetate 1:1, R_f(**9**) ~ 0.25 and R_f(**10**) ~ 0.60). The TLC plates were developed by 8% H_2SO_4 and subsequently scanned on a CanoScan FB 320P scanner (grey scale, 150 DPI resolution). The areas of the spots from **9** and **10** were obtained using the QuantiScan software (BioSoft, Cambridge, UK). The Fraction of remain-

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ing **9** (i.e., area of **9**/area of **9** + area of **10**) was plotted vs. time to obtain the first order rate constants.

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