The mechanism of the phosphoramidite synthesis of polynucleotides†

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The mechanism of the coupling step in polynucleotide synthesis using 5′-4,4′-dimethoxytritylthymidine-3′-β-cyanoethyl-*N,N*-diisopropylphosphoramidite as the phosphitylating agent and catalysed by the salt of saccharin and *N*-methylimidazole in acetonitrile has been studied by ^{31}P NMR. Pre- and post-equilibria between the activator salt and released diisopropylamine have been examined by ^{1}H NMR and ITC, which show that the salt between saccharin and diisopropylamine will be present in acetonitrile. Activation of the phosphoramidite by the salt of saccharin and *N*-methylimidazole involves nucleophilic catalysis and the formation of a reactive saccharin adduct bonded through its carbonyl oxygen to phosphorus. The rate constants for the reaction of the 4-methoxyphenol with 5′-4,4′-dimethoxytritylthymidine-3′-β-cyanoethyl-*N,N*-diisopropylphosphoramidite in the presence of saccharin–*N*-methylimidazole salt show a non-linear dependence on phenol concentration, becoming independent at high phenol concentrations, compatible with a change in rate limiting step from the alcoholysis step to the activation step.

Introduction

There is an increasing need to synthesise large quantities of high purity oligonucleotides for use in nucleic acid research and for the manufacture of anti-sense oligonucleotides.[1–3] While there have been numerous studies centred around finding new synthetic routes and on improving existing methods to increase yield and product purity, detailed kinetic and mechanistic studies on the individual steps involved in these syntheses are largely lacking.

Arguably the most important step in oligonucleotide synthesis is the coupling of the nucleotide units. One such method that has shown great efficiency is the phosphoramidite route and is, today, the most widely used. The phosphoramidite method utilises solid phase methodologies, where the growing oligonucleotide is covalently anchored to a solid matrix within a column and reagents are washed down for reaction.[3–8] The nucleotide to be coupled to the growing oligomer is often presented as a phosphoramidite containing a diisopropylamine leaving group and cyanoethyl and 4,4′-dimethoxytrityl (DMTr) as P–O and 5′ hydroxy protecting groups, respectively (Scheme 1).

The phosphoramidite coupling reaction (often incorrectly referred to as phosphorylation rather than phosphitylation) is the nucleophilic substitution of the amine moiety of the nucleosidic phosphoramidite by the 5′ hydroxy function of the solid support bound nucleoside. The reaction must be performed in the presence of a suitable acid/base activator as the reactants are otherwise inert (Scheme 1).

Scheme 1

There have been many studies involving screening of numerous activators to measure their relative efficiencies, examples include: 1H-tetrazole;[9,10] 2,4-dinitrophenol;[11] 2-bromo-4,5-dicyanoimidazole;[12] various carboxylic acids;[13] 4,5-dicyanoimidazole;[14] 5-phenyltetrazole;[15] arylsulfonyl-tetrazoles;[16] which are used for activation in phosphoramidite methodologies and benzylthiotetrazole and ethylthiotetrazole[17] for H-phosphonate routes. One of the most widely used activators is 1H-tetrazole, first used by Caruthers *et al.*[9,10] Studies by Dahl *et al.*,[18] and later by Nurminen *et al.*,[19–22] of the phosphitylation reaction suggested that 1H-tetrazole has a dual role. Firstly, the activator acts as an acid to protonate the nitrogen of the amine leaving group. Secondly, it acts as a nucleophile to displace isopropylamine from the protonated amidite to form a highly reactive tetrazolide intermediate. The tetrazolide intermediate then undergoes nucleophilic attack by the nucleosidic alcohol to produce the phosphite product, one equivalent of amine and tetrazole. The difference in pK_a between the departing amine and tetrazole means that the final acid base products react to generate a salt (Scheme 2).

Although it has been suggested that protonation of the phosphoramidite occurs on phosphorus,[18,21,23,24] it seems likely that for a reaction to occur, nitrogen protonation is required.[21,25,26] Evidence supporting this has been reported by Korkin and Tvetkov[27,28] who

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Scheme 2

showed, by molecular modelling of $H_2P\text{-}NH_2$, that protonation on nitrogen lengthens and weakens the phosphorus nitrogen bond, whereas phosphorus protonation shortens and strengthens the phosphorus nitrogen bond. Nurminen[21,22] has claimed that phosphorus protonation could be achieved with strong acids, although subsequent nucleophilic substitution of the amine was much slower than with the corresponding nitrogen protonated species.

Efficient activation requires an acid to protonate the phosphoramidite and a base to act as both a good nucleophile, to facilitate rapid conversion to the activated intermediate, and as a good leaving group to enable formation of the phosphite triester. HX type activators, such as 1H-tetrazole, do not meet these requirements of strong acid and good nucleophile; as a strong acid is likely to generate a weakly nucleophilic conjugate base whereas a strong nucleophile is likely to be derived from a weak acid.[5,29,30] There have been a number of studies undertaken to find alternative activators to these HX types with salts of strong acids and nucleophilic bases showing great potential as effective promoters of phosphitylation. Examples of these activators include: salts of benzimidazole with trifluoroacetic acid, tetrafluoroboric acid, hexafluorophosphoric acid and trifluoromethansulfonic acid;[31,32] imidazolium triflate;[33] *N*-methylimidazolium triflate;[34] salts of 4-dimethylaminopyridine with 5-(*o*-nitrophenyl)tetrazole and 5-(*p*-nitrophenyl) tetrazole;[35] pyridinium trifluoroacetate;[36] *N*-methylimidazolium triflate and trifluoroacetate; *N*-methylbenzoimidazolium triflate; and *N*-phenylimidazolium triflate.[29]

Herein we report mechanistic studies on the phosphitylation of nucleosidic species in acetonitrile using saccharin and *N*-methylimidazole as an activator, which is currently used as part of the manufacture of oligonucleotides on an industrial scale.

Results and discussion

Pre- and post-equilibria

Initially it was important to establish details of the equilibria between saccharin and *N*-methylimidazole, and also that with the amine generated during phosphitylation–diisopropylamine. The phosphoramidites used in these studies all contained diisopropylamine due to its preferred use in oligonucleotide manufacture.

^1H NMR studies performed in deuterated acetonitrile of mixtures of saccharin (SH) and *N*-methylimidazole (NM) indicate full proton transfer from saccharin to *N*-methylimidazole. In the presence of diisopropylamine (DIA), full proton transfer from both saccharin and the protonated *N*-methylimidazle to diisopropylamine occurs (Table 1). Thus, similar to the order in water, the increase in basicity in acetonitrile is in the order of: saccharin anion < *N*-methylimidazole < diisopropylamine. Isothermal titration calorimetery was used to calculate protonation equilibrium constants between saccharin and *N*-methylimidazole, and between saccharin and diisopropylamine. These also indicate the same order of basicity as determined by ^1H NMR with equilibrium constants of 1.27×10^4 (SH + NM) and 5.59×10^5 (SH + DIA) respectively. In terms of salt formation between all three components, that between saccharin anion and diisopropylaminium will be predominant in acetonitrile by a factor of 44 (Scheme 3).

Scheme 3

Although these equilibria are expected on the basis of the pK_a's in water, this order of basicity is contrary to that indicated by pK_a values in acetonitrile reported in the literature, which puts the

Table 1 ^1H chemical shifts of *N*-methylimidazole (NMI), saccharin and diisopropylamine (DIA) in deuterated acetonitrile

Sample composition	δ NMI H^2 (ppm)	δ NMI H^4 (ppm)	δ NMI H^5 (ppm)	δ NMI CH_3 (ppm)	δ DIA CH (ppm)	δ DIA CH_3 (ppm)
NMI	7.39	6.95	6.90	3.63	—	—
NMI + saccharin	8.43	7.39	7.27	3.81	—	—
DIA	—	—	—	—	2.84	0.95
DIA + saccharin	—	—	—	—	3.45	1.30
NMI + saccharin + DIA	7.50	6.98	6.93	3.64	3.48	1.31

Table 2 pK_a values of aliphatic ammonium ions in water and acetonitrile.[38]

Acid	pK_a in water	pK_a in acetonitrile
Diisopropylammonium	11.05[37]	16.5[22]
Di-*n*-butylammonium	11.25	18.31
Diethylammonium	10.98	18.75
Tri-*n*-butylammonium	10.89	18.09
Triethylammonium	10.65	18.46
Tri-*n*-propylammonium	10.65	18.1
Diisobutylammonium	10.50	17.88

acidity of the conjugate acid of *N*-methylimidazole (pK_a 17.1)[22] above that of diisopropylamine (pK_a 16.5).[22] An inspection of the pK_a values (Table 2) for a range of aliphatic ammonium ions show a good correlation between those in water and in acetonitrile. The reported pK_a of diisopropylammonium ion in acetonitrile of 16.5 is almost two units lower than the average for ammonium ions and lies well outside the pK_a range. It therefore seems likely that the reported pK_a value of 16.5 is incorrect and its pK_a should be approximately 18.5. This would place the relative basicity of saccharin, *N*-methylimidazole and diisopropylamine as similar to that determined by our experiments.

As the released diisopropylamine is the most basic component of the phosphitylation reaction mixture, a salt will be formed between saccharin and the displaced diisopropylamine, which will contribute to the driving force for the reaction. As well as salt formation, there will be a large thermodynamic driving force due to the positive enthalpy change accompanying the formation of a strong phosphorus to oxygen bond in the phosphite triester product *versus* the phosphorus nitrogen bond in the phosphoramidite reactants. This is illustrated by P–O and P–N bond dissociation energies of 360 and 230 kJ mol^{-1}, respectively.[39,40]

Phosphoramidite activation

If the phosphitylation of alcohols occurs by activation of the phosphoramidite with saccharin–*N*-methylimidazole salt *via* nucleophilic catalysis and a two step mechanism (Scheme 2), then there are three possibilities for the activating nucleophile. After initial protonation of the diisopropylamine group, substitution of the protonated amine could occur by either *N*-methylimidazole to form a charged intermediate or by the saccharin anion, through either its carbonyl oxygen or nitrogen atom, to give two possible neutral intermediates (Scheme 4).

To distinguish between these possibilities, studies were conducted of the reaction of the phosphoramidite, saccharin and *N*-methylimidazole in the absence of an alcohol. Addition of increasing molar equivalents of saccharin–*N*-methylimidazole salt to 5′-4,4′-dimethoxytritylthymidine-3′-β-cyanoethyl-*N,N*-diisopropylphosphoramidite in acetonitrile gave ^{31}P NMR spectra that showed a decrease in the signal due to the starting amidite, and an increasing proportion of a relatively broad signal centred at δ 134 ppm, which is assigned to the activated species. There is also the presence of two small additional signals, of equal peak area, at δ 7 ppm due to the formation of diastereoisomers of the H-phosphonate species produced by reaction of traces of water in the system with the activated species. There is also a set of three small signals at approximately δ 125 ppm, which are due to the formation of a P–O–P anhydride type species arising from reaction of the H-phosphonate/phosphite and the activated species.[22] Addition of excess *N*-methylimidazole and/or diisopropylamine prior to or after initial activation showed an increase in the concentration of reactants, demonstrating a true equilibrium between the species.

Saccharin is a strong enough acid to cause detritylation of the 5′ protecting group of the phosphoramidite and the presence of *N*-methylimidazole as a base also helps to reduce the acidity of the medium to prevent unwanted detritylation. To study activation with saccharin in the absence of *N*-methylimidazole, a simple phosphoramidite (dimethyl *N,N*-diisopropylphosphoramidite) was used. Addition of increasing molar equivalents of saccharin to the phosphoramidite in both acetonitrile and chloroform showed, from ^{31}P NMR spectra, an increase in the activated species at the expense of starting amidite.

The same signal for the activated species was observed upon the addition of *N*-methylimidazole–saccharin to the amidite in chloroform, indicating that the activated species must be a saccharin adduct. In acetonitrile, there was a small shift in the position of the signal for the activated species, which is probably due to a medium effect rather than to the formation of a different adduct.

To identify the position of the ^{31}P NMR chemical shift of P–O–C bonds, acetic acid was used instead of saccharin–*N*-methylimidazole to activate the thymidine phosphoramidite in acetonitrile. Addition of increasing molar equivalents of acetic acid showed an increase in the proportion of a pair of signals at δ 131.7 and 131.1 ppm due to the two diastereoisomers formed upon substitution of diisopropylamine with acetic acid. The chemical

P-N -methylimidazole

P-O-Saccharin

P-N-Saccharin

Scheme 4

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Table 3 Summary of the ^{31}P NMR chemical shifts of phosphorus III species in deuterated acetonitrile

(MeO)$_2$P–N(i-Pr)$_2$	Thymidine(CEO)P–N(i-Pr)$_2$	(EtO)$_2$P–N-methylimidazolium	(EtO)$_2$P–N-dimethylaminopyridinium
δ 149 ppm	δ 149 ppm	δ 150-162 ppm[a]	δ 142 ppm
Thymidine(CEO)P–O–C(O)CH$_3$	Thymidine(CEO)P–O-saccharin	(MeO)$_2$P–O-saccharin	(EtO)$_2$P–OEt
δ 132 ppm	δ 134 ppm	δ 138 ppm	δ 139 ppm

[a] Varies with NMI concentration.

shift of 132 ppm can be compared with the δ 134/139 ppm observed with saccharin and saccharin–*N*-methylimidazole, compatible with P–O bond formation. To confirm these observations and to determine the ^{31}P NMR chemical shift of P–N–C bonds, the reaction between diethylchlorophosphite and *N*-methylimidazole was studied. Addition of *N*-methylimidazole to the diethylchlorophosphite in acetonitrile showed formation of a broad ^{31}P NMR signal, which showed a large up-field movement in chemical shift on increasing the concentration of *N*-methylimidazole from δ 162 ppm with one molar equivalent to δ 151 ppm with five molar equivalents. The shape of the signal became broader and less symmetrical on addition of excess *N*-methylimidazole. This is thought to be due to association of *N*-methylimidazole molecules to the charged activated species reducing the amount of positive charge on the phosphorus compatible with the observed change in chemical shift. Reducing the temperature of the five molar equivalent sample showed a further up-field shift in the signal and a sharpening in its shape. Addition of dimethylaminopyridine to diethylchlorophosphite in acetonitrile gave an activated species with a ^{31}P NMR signal at δ 142 ppm, corresponding to an adduct with a more delocalised charge. Table 3 shows a summary of the ^{31}P NMR chemical shifts in acetonitrile of the relevant adducts with the chemical shifts of P–N species being between δ 142–162 ppm whereas the P–O phosphite ester species resonate further up-field at δ 132–139 ppm. The chemical shifts of the P–saccharin adduct resonate at approximately δ 135 ppm, compatible with bonding through the saccharin's carbonyl oxygen atom.

Further evidence for the active intermediate being the P–O saccharin adduct was obtained from FT-IR. The spectrum of saccharin in acetonitrile shows the presence of a signal at 1744 cm^{-1} assigned to the stretching frequency of the carbonyl group, which is lost on addition of one molar equivalent of *N*-methylimidazole, indicative of proton transfer and enolate anion formation. Addition of either one molar equivalent of dimethylphosphoramidite or one molar equivalent of both dimethylphosphoramidite and *N*-methylimidazole to saccharin in acetonitrile also results in the loss of the carbonyl signal, compatible with formation of a P–O rather than a P–N adduct, which would retain a carbonyl stretch (Scheme 4).

In summary, phosphoramidite activation with saccharin–*N*-methylimidazole salt results in the formation of an activated intermediate that is an adduct of saccharin with bonding to phosphorus being through the carbonyl oxygen atom of saccharin. This is compatible with studies performed by Sekine *et al.*, who, in a not too dissimilar reaction, demonstrated the reaction of activated phosphonates with an oxygen nucleophile in the presence of nitrogen nucleophiles.[17]

A function of *N*-methylimidazole in the reaction is to reduce the acidity of the reaction mixture so as to prevent unwanted side reactions, such as de-purination and premature detritylation. The saccharin–*N*-methylimidazole salt is an effective activator of the phosphoramidites and activation occurs rapidly and stoichiometrically. The saccharin anion is more nucleophilic towards the phosphoramidite than the *N*-methylimidazole.

Alcoholysis

The alcoholysis of thymidine phosphoramidite in acetonitrile requires only one molar equivalent of saccharin–*N*-methylimidazole salt and one molar equivalent of alcohol to give quantitative formation of the phosphite triester product. The alcoholysis reaction could proceed by either direct displacement of the protonated amine or by initial nucleophilic attack of the activator and subsequent displacement of saccharin by the incoming alcohol (Scheme 5).

Scheme 5

The rate of the alcoholysis reaction of the thymidine phosphoramidite with the 5′ hydroxyl group of a nucleoside is very rapid but can be reduced by using weakly nucleophilic alcohol or phenol so that kinetics can be monitored by rapid ^{31}P NMR techniques (see ESI†). The change in concentration with time for the formation of phosphite triester from the reaction of thymidine phosphoramidite with one molar equivalent of saccharin–*N*-methylimidazole shows second order kinetics for a range of substituted phenols and alcohols at varying initial concentrations. However, the calculated rate constants show a non-linear dependence upon the concentration

Table 4 Observed second order rate constants for reaction of thymidine phosphoramidite with 4-methoxyphenol catalysed by one molar equivalent of saccharin–*N*-methylimidazole salt in acetonitrile

4-Methoxyphenol concentration (M)	$k_{obs}/M^{-1}\ s^{-1}$
0.05	0.230
0.10	0.285
0.15	0.307
0.20	0.330
0.30	0.403
0.40	0.417
0.50	0.400
0.75	0.410

of 4-methoxyphenol (Table 4). At low phenol concentrations, the rate of alcoholysis is dependent on the 4-methoxyphenol concentration, but above 0.3 M 4-methoxyphenol, the rate of phosphite triester formation becomes independent of the phenol concentration. This change in dependence on concentration is indicative of a change in rate limiting step and is compatible with the presence of an intermediate on the reaction pathway.

If phosphoramidite (PA) activation involves nucleophilic catalysis by saccharin to form a P–saccharin intermediate by displacement of the diisopropylamine (DIA) so that alcoholysis proceeds by a two step mechanism, there are two elementary reactions involved in formation of the phosphite trieseter (POR) product (Scheme 6). The rate of phosphite triester formation is given by eqn (1), but at low concentration of alcohol, where k_{-1}[NMI][DIA] > k_2[ROH], this simplifies to eqn (2) and the rate is dependent on alcohol concentration and the rate limiting step is k_2, the rate of breakdown of the intermediate.

$$\frac{d[POR]}{dt} = \frac{k_1 k_2 [PA][ROH][Sac^{-}NMIH^{+}]}{k_{-1}[NMI][DIA] + k_2[ROH]} \quad (1)$$

$$\frac{d[POR]}{dt} = \frac{k_1 k_2 [PA][ROH][Sac^{-}NMIH^{+}]}{k_{-1}[NMI][DIA]} \quad (2)$$

$$PA + Sac^{-} + NMIH^{+} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PSac + DIAH + NMI$$

$$PSac + HDIA + HOR \xrightarrow{k_2} POR + Sac^{-} + DIAH_2^{+}$$

Scheme 6

Conversely, at high concentration of alcohol, where k_2[ROH] > k_{-1}[NMI][DIA], the rate of formation of the intermediate, k_1 is rate limiting and the reaction is independent of alcohol concentration, eqn (3).

$$\frac{d[POR]}{dt} = k_1[PA][Sac^{-}NMIH^{+}] \quad (3)$$

There is little dependence on the nature of the phenol or alcohol on the rate of phosphite triester formation (Table 5) as expected if the rate limiting step is formation of the intermediate.

In conclusion, phosphoramidite activation with saccharin–*N*-methylimidazole salt results in the formation of an activated phosphite ester intermediate that is an adduct of saccharin with bonding to phosphorus being through the carbonyl oxygen atom of saccharin. In the presence of alcohols, this intermediate reacts in a fast step to form the product, so that formation of the intermediate is rate-limiting.

Table 5 Observed second order rate constants for reaction of thymidine phosphoramidite with various phenols and alcohols catalysed by one molar equivalent of saccharin–*N*-methylimidazole salt

Alcohol	$k_{obs}/M^{-1}\ s^{-1}$
4-Cyanophenol	0.237
4-Chlorophenol	0.227
Phenol	0.227
4-Methylphenol	0.218
4-Methoxyphenol	0.228
Propan-2-ol	0.238
t-Butanol	0.185

Experimental

^{1}H, ^{13}C, ^{31}P and ^{15}N NMR spectra were recorded on a Bruker 400 MHz or Bruker 500 MHz spectrometer. Coupling constants *J* are quoted in Hz.

Isothermal titration calorimetry was performed on a MicroCal VP-ITC microcalorimeter. For determination of the saccharin–diisopropylamine equilibrium constants, a solution of saccharin (0.25 mM) in acetonitrile was placed in the cell block and the titrating syringe was loaded with diisopropylamine (7.3 mM) in acetonitrile. Titre volumes of 4 μl were used in this experiment with a 150 second delay between additions. For the determination of the saccharin–*N*-methylimidazole equilibrium constants, saccharin (1 mM) in acetonitrile was placed in the cell block and *N*-methylimidazole (14.6 mM) in acetonitrile was loaded into the titration syringe. In this case, titre volumes of 2 μl were titrated with a 150 second delay between injections.

Infra-red experiments were performed on a Thermo Nicolet 380 FT-IR. All reagents were pre-prepared in deuterated acetonitrile and stored over 4 Å molecular sieves under dry argon for at least 24 hours before use. In a typical experiment, saccharin and dimethyl *N,N*-diisopropylphosphoramidite were mixed together in acetonitrile under a dry argon atmosphere and the resulting mixture injected into a 1 mm quartz cell, which had been flushed with dry acetonitrile and sealed with rubber septa; the spectrum was then recorded.

The kinetic measurements were carried out using rapid ^{31}P NMR techniques on a Bruker 400 spectrophotometer. All reagents were pre-prepared in deuterated acetonitrile and stored over 4 Å molecular sieves under dry argon for at least 24 hours prior to use. In a typical kinetic run, two identical samples of saccharin–*N*-methylimidazole salt and 4-methoxyphenol in deuterated acetonitrile were prepared in sealed/argon flushed NMR tubes. To one of these samples, the NMR shimming and deuterium locking protocols were performed after addition of thymidine phosphoramidite. Thymidine phosphoramidite was then added to the other sample, which was placed in the NMR magnet as quickly as possible and the experiment was started. ^{31}P NMR spectra were recorded successively at intervals of 23 seconds. This time base was chosen to gain the maximum number of data points over the kinetic run, but to also give adequate signal resolution. Rate constants were obtained by fitting concentration *versus* time data to either first or second order exponential decays

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using Scientist software. Errors in rate constants are typically below ±5%, data with errors above this were discarded.

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