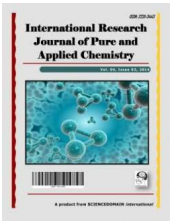

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Nucleophilic Groups Protection of Phosphoramidite for DNA Synthesis

Kabir Abdu[1*]

[1]Department of Pure and Industrial Chemistry, Bayero University Kano, Nigeria.

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Phosphoramidite are the DNA building blocks. For the DNA synthesis to proceed it is necessary to block or protect all the nucleophilic centres not involved in the reaction at each step of the synthesis. The commonly used DNA protecting groups includes; dimethoxytrityl (DMT) which is used to protect the 5'-OH, benzoyl (Bz) is used to protect the exocyclic NH_2 groups of adenine and cytosine whilst isobutyryl (Ib) group is used for guanine protection. Thymine does not need protection as it has no exocyclic NH_2 groups. The phosphate group is usually protected with the cyanoethyl group, whilst the 3'-OH is protected by virtue of the connection to the controlled pore glass (CPG) solid support. Here, the 2-amino group of the nucleoside, **1** was initially protected as its dimethylformamidine derivative (using N,N-dimethylformamide) which later decomposed into the *N*-formylamino compound which was never been used before and was found to be suitable for the protection of the exocyclic amino group for DNA synthesis. The 5'-OH group as usual was protected with dimethoxytrityl (DMT) using dimethoxytrityl chloride following which phosphitylation gave the phosphoramidite compound **4**. The chemistry involved in the conversion of the dimethylformamidine to N-formylamino compound is presented here.

**Corresponding author: E-mail: kabdu.chm@buk.edu.ng;*

Keywords: Phosphoramidite; oligodeoxyribonucleotide; synthesis; nucleophile; protection.

1. INTRODUCTION

The chemical synthesis of oligodeoxyribonucleotides (ODNs) involves relatively mild reaction conditions as the heterocyclic bases are prone to alkylation, oxidation or phosphorylation, while the phosphodiester backbone is susceptible to hydrolysis [1]. The key step in the synthesis of ODNs is the specific and sequential formation of the internucleoside 3' - 5' phosphodiester linkages [2]. In the early 1980s, Caruthers and co-workers developed a new method via phosphoramidite chemistry which was developed into an efficient and automated process of ODN synthesis on a solid support. This method is now the most efficient and chiefly used procedure for ODN synthesis with a reproducible yield of more than 98% for each step [3,4,5]. The method uses a cycle of four repeated steps. Step 1 involves the removal of dimethoxytrityl (DMT) group from the terminal nucleoside bound to the controlled pore glass (CPG) solid support [1,6]. The second step is the coupling process involving the condensation of the incoming nucleoside with the free 5' -OH group of the nucleotide attached to the CPG to form a phosphite triester intermediate. The deoxyribonucleoside-3'-phophoramidites require activation to react with the 5' –OH group of the support-bound oligonucleotide. The third step involves capping using a mixture of Ac_2O/N-methylimidazole/THF/pyridine to ease the purification of the desired synthetic ODNs. This is introduced into the reaction cycle after the coupling step to block any 5'-OH group that failed to condense with the activated phosphoramidite solution. The final step involves the oxidation of the phosphite triester intermediate using a mixture of I_2/H_2O/THF/pyridine and is completed in less than one minute [1,7,8]. The oxidation step must be performed once per cycle rather than all at the end of the synthesis, because the phosphite triester intermediates are unstable to most of the synthetic conditions, especially acid treatment.

Here, the chemistry involved in the protection of the nucleophilic centers of the nucleoside, **1** was presented. In particular the 2-exocyclic amino group (NH_2) was protected with formyl group and successfully deprotected before it was put into ODN for onward DNA synthesis.

2. EXPIREMENTAL

*4-{[(dimethylamino)methylidene]amino}-2-(2'-deoxy-β-D-erythro-pentofuranosyl)-6-oxa-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (**2**)*

This compound was prepared using a slight modification of the literature procedure [9].

Compound **1** (150 mg, 490 µmol) was dissolved in anhydrous DMF (3 mL), under an argon atmosphere. After 1 h, *N,N*-dimethylformamide dimethylacetal (0.5 mL, 3.9 mmol) was added and the solution left to stir overnight. After evaporation the residue was purified by silica gel column chromatography (0-2% MeOH / DCM (0.5% Et_3N)) which gave **2** as a white foam (100 mg, 57%).

TLC (10% MeOH / DCM); R_f = 0.47

δ_H (d_4-CD_3OD) 2.07-2.11 (2H, m, CH_2CH_2O), 2.80 (2H, t, J = 6.4 Hz, $CH_2CH_2CH_2O$), 3.00 (3H, s, N-CH_3), 3.11 (3H, s, N-CH_3), 3.45(2H, m, H2' and H2''), 3.70 (1H, m, H5'), 4.31 (1H, m, H5''), 4.39 (2H, t, J = 6.4 Hz, CH_2O), 4.93 (1H, t, J = 6.4 Hz, C4'), 5.25 (1H, d, J = 6.4 Hz, C3'), 6.05 (1H, dd, J = 3.2, 6.4 Hz, H1'), 7.15 (1H, s, H-6), 8.09 (1H, s, N=C-*H*) ppm.

δ_C (d_4-CD_3OD) 25.7 (CH_2CH_2O), 28.9 ($CH_2CH_2CH_2O$), 34.0 (N-CH_3), 39.7 (N-CH_3), 40.2 (C2'), 62.3 (C5'), 71.6 (C3'), 72.7 (CH_2O), 83.6 (C1'), 87.1 (C4'), 101.3 (C-10), 114.8 (C-6a), 118.3 (C-1), 154.4 (C-2a), 157.9 (C=N), 160.6 (C-6), 165.0 (C-4) ppm.

Mass Spec: *m/z* (ESI+) 362 $[M+H]^+$.

Acc Mass: 362.1827; calculated for $C_{17}H_{24}N_5O_4$ requires 362.1828 (deviation -0.3 ppm).

*4-[(Formyl)amino]-2-(2'-deoxy-β-D-erythro-pentofuranosyl)-6-oxa-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (**2a**)*

Compound **2** (250 mg, 692 µmol) was dissolved in 20% acetic acid (5 mL), stirred overnight at room temperature. After evaporation residue was purified by silica gel column chromatography (0-2% MeOH / DCM) which gave **2a** as a white foam (150 mg, 65%).

TLC (10% MeOH / DCM); R_f = 0.60

δ_H (d_4-CD_3OD) 2.15-2.25 (3H, m, CH_2CH_2O and *H*2'), 2.61 (1H, m, *H*2''), 2.90 (2H, t, *J* = 6.4 Hz, $CH_2CH_2CH_2O$), 3.75(2H, m, *H*5' and *H*5''), 3.94 (1H, m, *H*4'), 4.55 (3H, m, CH_2O and *H*3'), 6.70 (1H, dd, *J* = 3.2, 6.4 Hz, H1'), 7.28 (1H, s, *H*-6), 8.25 (1H, s, *NH*-CHO), 9.49 (1H, s, *CH*O) ppm.

Mass Spec: *m/z* (ESI+) 335 $[M+H]^+$.

Acc Mass: 335.1353; calculated for $C_{15}H_{19}N_4O_5$ requires 335.1355 (deviation -0.6 ppm).

*4-[(Formyl)amino]-2-(2'-deoxy-β-D-erythro-5'-O-[4,4'-dimethoxytrityl]pentofuranosyl)-6-oxa-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene (**3**)*

This compound was prepared using a slight modification of the literature procedure [9].

Compound **2a** (150 mg, 449 µmol) was dissolved in anhydrous pyridine (5 mL) under an argon atmosphere with stirring. 4,4-Dimethoxytrityl chloride (183 mg, 539 µmol) and *N*-ethyldiisopropylamine (2 mg, 15 µmol) were added. After 4 h the mixture was evaporated, redissolved in ethylacetate (20 mL) and extracted with saturated sodium acetate (10 mL), water (10 mL), brine (10 mL) and dried ($NaSO_4$). Evaporation and purification of the resulting residue by silica gel chromatography (0-2% MeOH / DCM (1% Et_3N)) gave **3** as a light yellow foam (140 mg, 50%).

TLC (5% MeOH / DCM (1% Et_3N)); R_f = 0.6, UV active, stained orange with anisaldehyde.

δ_H ($CDCl_3$) 2.17 (2H, m, CH_2CH_2O), 2.36-2.44 (2H, m, *H*2' and *H*2''), 2.71 (2H, t, *J* = 6.6 Hz, $CH_2CH_2CH_2O$), 3.33 (2H, m, *H*5' and *H*5''), 3.76 (6H, s, 2x -OCH_3, DMT), 4.12 (1H, t, *J* = 6.6 Hz, *H*4'), 4.44 (2H, t, *J* = 6.6 Hz, CH_2O), 4.51 (1H, d, *J* = 6.6 Hz, *H*3'), 6.15 (1H, dd, *J* = 3.2, 6.6 Hz, *H*1'), 6.32 (4H, m, Ar-*H*, DMT), 6.92 (1H, s, H-6), 7.20-7.91 (9H, m, Ar-*H*, DMT) 8.05 (1H, s, *NH*-CHO), 9.50 (1H, s, *CH*O) ppm.

δ_C ($CDCl_3$) 26.5 (CH_2CH_2O), 29.3 (C2'), 40.9 ($CH_2CH_2CH_2O$), 46.5 (C5'), 55.6 (2x -OCH_3), 64.5 (CH_2O), 72.8 (C3'), 73.6 (allyl-*C*, DMT), 83.5 (C1'), 86.1 (C4'), 86.9 (C-10), 113.5 (C-1), 115.5 (C-6a), (118.7, 127.3, 128.3, 128.7, 130.5, 136.1, 136.2, 145.0, 152.0, DMT), 154.0 (C-2a), 158.9 (C-6), 163.6 (*CH*O), 165.5 (C-4) ppm.

Mass Spec: *m/z* (ESI+) 637 $[M+H]^+$.

Acc Mass: 637.2654; calculated for $C_{36}H_{37}N_4O_7$ requires 637.2662 (deviation -1.3 ppm).

*4-[(Formyl)amino]-2-(2'-deoxy-β-D-erythro-5'-O-[4,4'-dimethoxytrityl]pentofuranosyl)-6-oxa-7,8,9-trihydro-2,3,5-triazabenzo[cd]azulene-3'-(2-β-cyanoethyl-N,N-diisopropyl)phosphoramidite (**4**)*

This compound was prepared using a slight modification of the literature procedure [9].

Compound **3** (110 mg, 173 µmol) was dissolved in distilled dichloromethane (2 mL, 0.1 M) under an argon atmosphere. This was followed by addition of dry diisopropylethylamine (120 µL, 694 µmol) and finally 2-cyanoethyl-*N*-diisopropylamine chlorophosphomidite (50 µL, 208 µmol) was added drowise over 15 min period. After 1 h benzyl alcohol solid support (2.5 mmol/g average) was added and the resulting suspension was stirred for 30 min. further. The reaction was then filtered to remove the solid support, the filtrate was washed with 10% $NaCO_3$ (10 ml), brine (10 mL) and dried (Na_2SO_4). The residue was evaporated and purified by silica gel column chromatography under nitrogen with 45% dichloromethane / Hexane (10% Et_3N) to give phosphoramidite **4** as a white foam (75 mg, 52%).

TLC (45% DCM / Hexane (10% Et_3N)); R_f = 0.55

^{31}P NMR δ ($CDCl_3$) +148.5 and +148.6 phosphoramidite diastereoisomers, ppm.

Mass Spec: *m/z* (ESI+) 837 $[M+H]^+$.

Acc Mass: 837.3737; calculated for $C_{45}H_{54}N_6O_8P$ requires 837.3741 (deviation -0.3 ppm).

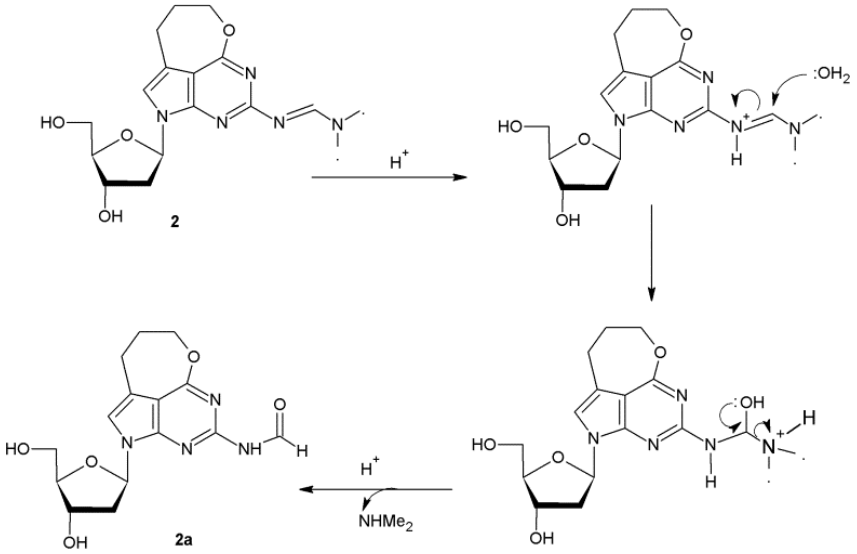
3. RESULTS AND DISCUSSION

The synthesis of the phosphoramidite, **4** from the nucleoside, **1** was carried out base on the reported literature [9]. The use of an amidine protecting group was considered. This has been used by Seela and Driller for protection of O^6-methyl-7-deaza-2'-deoxyguanosine. The synthesis of nucleoside, **1** was report in 2012 by Abdu *et al*., Protection of the 2-amino group of compound **1** as its amidine was accomplished by using *N*,*N*-dimethylformamide dimethylacetal in dry DMF [10]. Initially 8 equivalents of *N*,*N*-dimethylformamide dimethylacetal were used, the TLC analysis showed incomplete reaction after overnight stirring at room temperature. After further addition of the same amount of *N*,*N*-dimethylformamide dimethylacetal the reaction was found to complete after the second day based on TLC. However, the same reaction

carried out under the same conditions but at 50^{o}C and was found to be complete after 2 h without ring opening of the base. Following this reaction, the DMF was evaporated and silica gel column chromatography of the residue afforded a mixture of two compounds in 57% yield. The ^{1}H NMR spectrum and mass spectrum revealed the presence of a mixture of an amidine compound **2** and an *N*-formyl compound **2a** in 3:1 ratio as shown by the mass and the ^{1}H-NMR spectra. The most probable explanation for this may be the hydrolysis of the amidine due to the slight acidity of the silica gel from the column chromatography [11,12]. Also purification was performed in 5% methanol in dichloromethane. So, it was assumed that there must have been a small amount of water present in the methanol which might react with the amidine group under the acidic conditions of the silica gel. Scheme 1 shows decomposition of the amidine group under mild acid conditions to give an *N*-formyl compound.

When the reaction was repeated, triethylamine (1%) was added to the eluent during the column chromatography to neutralise the silica acidity but still the product was isolated as a mixture of both amidine and an *N*-formyl compound. It was therefore decided to convert the entire amidine compound into the formyl derivative before proceeding to the next step. The amidine compound was stirred with 20% aqueous acetic acid overnight at room temperature but based on TLC analysis, the reaction was still incomplete. It was therefore stirred over night at 50℃ to complete the decomposition. TLC analysis shows three spots, the formyl compound with the highest R_f = 0.50, followed by amidine compound with R_f = 0.47 and then the flourescent spot which is the unprotected material (starting material) at R_f = 0.40.

After converting the entire amidine compound into the formyl protected nucleoside the next step was the protection of the 5'-OH group with the dimethoxytrityl chloride (DMTrCl) to give the DMT-protected nucleoside 3. The reaction with DMTrCl is highly selective for the primary 5'-OH group over the secondary 3'-OH group [13]. The reaction requires an excess of DMTrCl and is usually completed within 1 or 2 hours using 4-dimethylaminopyridine (DMAP) as a catalyst [14]. This was carried out by dissolving the formyl compound 2a in anhydrous pyridine under dry atmosphere of argon, followed by the addition of 0.05 equivalent DMAP as a catalyst and then 1.4 equivalent triethylamine (TEA) and 1.2 equivalent of DMTrCl. The reaction was completed after 2 h, based on TLC. The TLC plates stain orange with conc. HCl vapour. Silica column chromatography was run using 2-5% MeOH/DCM (1% TEA). The 5'-DMT-deoxyribonucleoside are sensitive to acid as this could lead to the loss of the DMT group. Because of this the column chromatography was run with 1% TEA in 2-5% MeOH/DCM and the 1H NMR was run in CDCl3 passed over basic alumina silica to prevent the lost of the DMT group. Compound 3 was obtained in 50% yield.



Scheme 1. Acid catalysed decomposition of amidine to formyl

Phosphitylation of compound **3** was conveniently carried out using dry diisopropylethylamine and 2-cyanoethyl-*N,N*-diisopropylaminechlorophosphoramidite (phosphitylating reagent) in dry dichloromethane. Four equivalent of the diisopropylethylamine and 1.5 equivalent of the phosphitylating reagent were used. After 1 hour the reaction was analysed by withdrawing a small sample using a dry syringe and mixing it with water and ethyl acetate in a small tube. The ethyl acetate layer was spotted on the TLC plate and developed in 45:45:10 v/v ethyl acetate: dichloromethane: triethylamine and observed under UV light. The plate was sprayed with anisaldehyde which shows four major component of the reaction mixture on the TLC plate. The 1st spot, the least polar is the phosphoramidite **4** and it appears as a two spots very close to each other because the solvent system partially resolves the diastereomers (due to the asymmetric phosphorus atom). The 2nd spot is the deoxyribonucleoside-3'-phosphonate which may result from the hydrolysis of the phosphoramidite compound **4**, this appears to be the major component in the reaction mixture and this is assumed to be due the insufficient anhydrous reaction conditions or it may be as a result of partial hydrolysis of the phosphitylating reagent due to storage. The 3rd spot is the starting material and the 4th spot is an unknown polar material. After the TLC analysis, another equivalent of the reagents were added and after another hour the reaction was worked up and silica gel column chromatography was run in 45:45:10 v/v ethyl acetate: dichloromethane: triethylamine. ^{31}P NMR was run in $CDCl_3$ pass over basic alumina to avoid the hydrolysis of the phosphoramidite. Two peaks were observed at around +148 ppm in the ^{31}P NMR which indicates the formation of the phosphoramidite along with phosphonate at 14.1 ppm. This is presumed to be as a result of the hydrolyses of the phosphoramide due poor anhydrous reaction conditions. When the reaction was repeated under the same conditions but on a larger scale with strictly anhydrous conditions and fresh phosphitylating reagent, the reaction worked well with no phosphonate formation being observed as shown by the ^{31}P-NMR (see Fig. 1). ESI+ mass spectrum (Fig. 2) also confirm the formation of compound **4** in 52% yield.

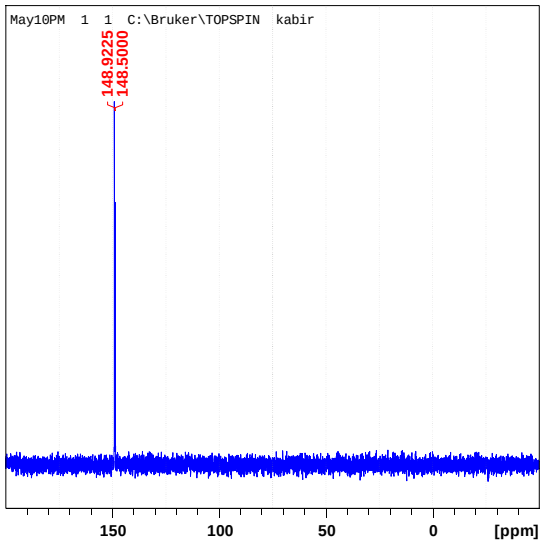


Fig. 1. ^{31}P NMR showing the two diastereomers

Deprotection of DNA is normally carried out under basic conditions and to avoid the nucleobase ring open during DNA deprotection. The deprotection process was checked prior to DNA synthesis and it was found that deprotection was completed using 10 mg of formyl compound, **2a** in 1 mL aqueous ammonia at room temperature. This was confirmed by TLC analysis which shows a single fluorescent spot of the nucleoside, **1**.

The protection of phosphoramidite **4** is therefore shown in Scheme 2.

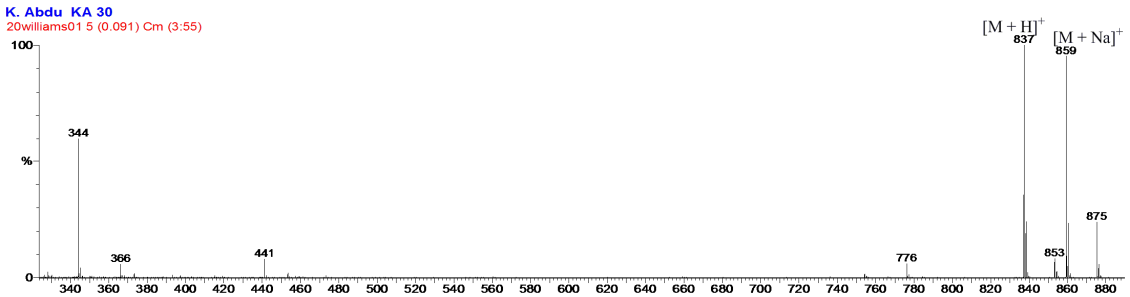
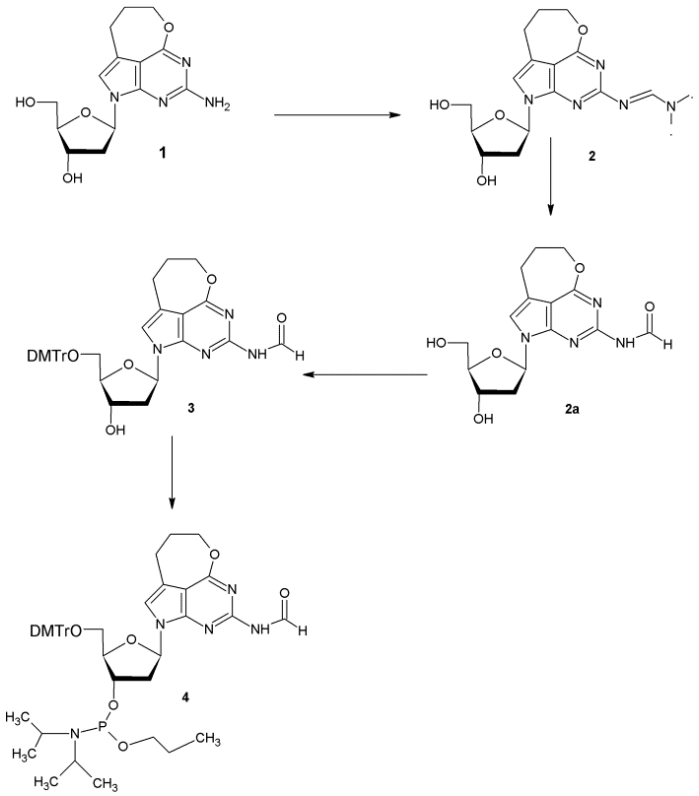


Fig. 2. Showing the ESI+ mass spectrum of the phosphoramidite 4



Scheme 2. Synthesis of phosphoramidite 4

Reagents and reaction conditions: (i) N,N-dimethylformamide dimethylacetal, DMF, 50^{o}C, 2 h, 57%; (ii) 20% AcOH, rt, overnight, 65%; (iii) DMAP, DMTCl, Et_3N,pyridine, 50%; (iv) 2-cyanoethyl-N-diisopropylamine chlorophosphoramidite, CH_2Cl_2, $^{i}Pr_2NEt$, 52%

4. CONCLUSION

The phophoramidite synthesis and the protection of its nucleophilic centres for DNA synthesis was successifully archeived. The chemistry involved in the conversion of the amidine derivative, **2** to the formyl compound, **3** which was not clear previously was now fully understood. As a result of better stability of the formyl compound it is therefore recommended for the protection of amino group in the DNA synthesis rather than the previous protecting groups used.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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