Universal linker phosphoramidite

Andrey Yagodkin and Alex Azhayev*

Department of Pharmaceutical Chemistry, University of Kuopio, FIN-70211 Kuopio, Finland and Metkinen Chemistry, Microkatu 1, Microtower, Osa R, FIN-70211 Kuopio, Finland
E-mail: Andrey.Yagodkin@uku.fi, Alex.Azhayev@uku.fi

This paper is dedicated to Professor Harri Lönnberg on the occasion of his 60th birthday

Abstract
Universal Linker phosphoramidite was synthesized and employed for conversion of conventional nucleoside bound solid phase into the universal support. The latter was successfully tested in preparation of the 20mer oligonucleotide.

Keywords: Oligonucleotide synthesis, Universal Solid support, Universal Linker phosphoramidite

Introduction

Standard oligonucleotide synthesis uses a solid support that contains the first nucleoside covalently bound to the solid phase by a linker that is hydrolyzed during the cleavage step following solid-phase assembly. This support-bound nucleoside becomes the 3'-terminal residue of the final oligonucleotide after the cleavage and deprotection steps. Clearly, this approach requires the use of at least four solid supports for general DNA synthesis along with four additional supports for RNA synthesis. Various solid supports containing unusual nucleosides for specific applications are also required.

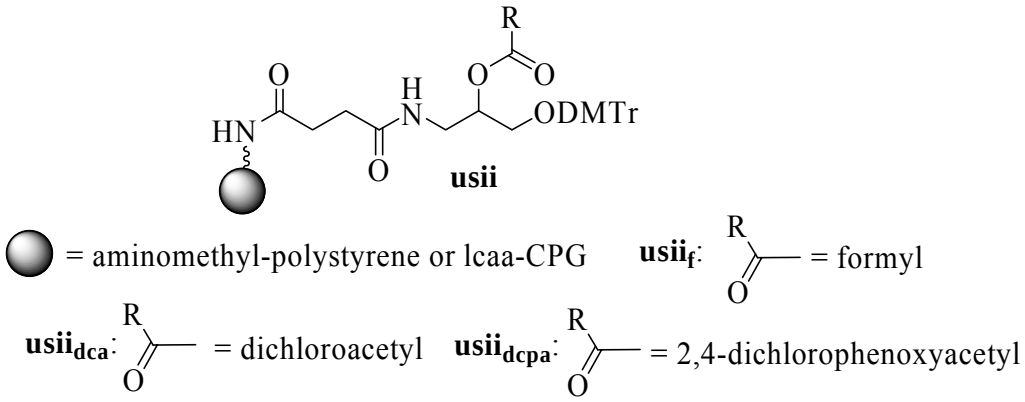
A universal support has the intended 3'-nucleoside added in the first cycle, generating an undesired phosphate linkage between this nucleoside and the universal support. The approach requires that this phosphate linkage be removed during the cleavage and/or deprotection steps. However, the universal support strategy offers the following clear advantages: eliminates the possibility of errors in parallel synthesis applications where up to 384 wells may contain different supports; eliminates the need for at least four supports for DNA synthesis and four for RNA synthesis.

A universal linker phosphoramidite that may be employed for conversion of conventional nucleoside bound solid phase into the universal support directly on the oligonucleotide

synthesizer will be of certain value in research laboratories, performing syntheses of modified oligonucleotides, incorporating modified or unusual nucleosides at the 3'-terminus.

Results and Discussion

Earlier we reported the Universal solid support for oligonucleotide synthesis **usii** (Scheme 1).[1-3]



Scheme 1

The **usii** support was thoroughly compared to a number of commercially available Universal solid phases and was demonstrated to be superior in terms of mildest conditions and shortest time of target oligonucleotide cleavage from the solid phase.[4] The main advantage of the **usii** support consists of the fact that processes of cleavage and 3'-dephosphorylation appear to be, in essence the same extremely fast reaction that releases only the desired product – 3'-dephosphorylated oligonucleotide.

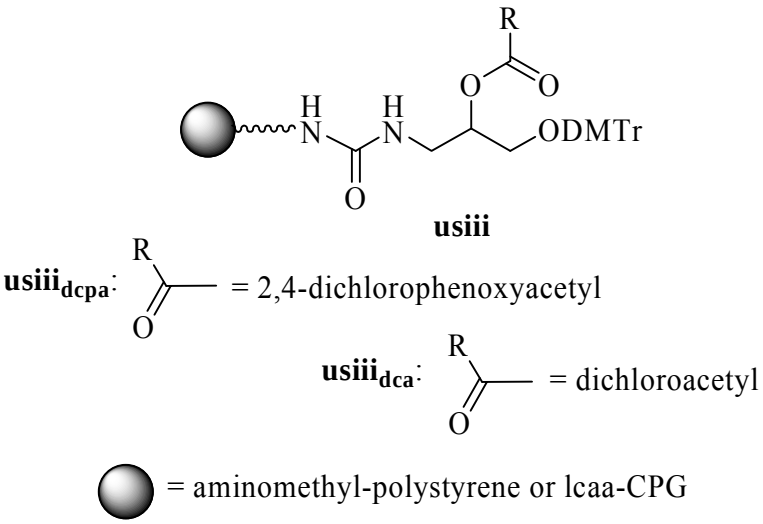
In our previous studies[1,2] we have found that basically three acyl groups, protecting the 2-hydroxyl function of 3-acylamido-1,2-propanediol tether may be nearly completely cleaved with ammonia *prior* to phosphate deprotection (β-elimination of the cyanoethyl group). These groups were: formyl-, dichloroacetyl and 2,4-dichlorophenoxyacetyl, listed in accordance with increasing stability towards ammonolisis.

Yields of oligonucleotides, synthesized on versions of the **usii**, bearing the formyl (**usii$_f$**) and dichloroacetyl (**usii$_{dca}$**) were either equal or even exceeded yields of oligomers, prepared using conventional nucleoside bound solid supports. The **usii$_f$** version failed to meet the criteria of prolonged storage – this support proved to be not stable enough as a potential commercial product. The **usii$_{dca}$** version appeared satisfactory, allowing synthesizing oligonucleotides with yields equal to oligomers generated on nucleoside bound supports and having a reasonable shelf life – 1 year at +4 ºC. The versions of the **usii**, bearing the 2,4-dichlorophenoxyacetyl group (**usii$_{dcpa}$**) proved to be the most stable – more than 1 year at r.t. However, yields of

oligonucleotides assembled on the **usii$_{dcpa}$** and cleaved as described earlier[1,2,3] were always about 15-25 % lower than yields of the same oligomers, generated on either nucleoside bound supports or the **usii$_{dca}$**.

The main disadvantage of the **usii** is that its preparation takes 5 steps of modification of solid phase – construction of linker and capping steps. Evidently, this lengthy and laborious method does not allow to precisely loading the solid phase with the Universal Linker. Moreover, this fact makes synthesis of the **usii** support on the industrial scale questionable.

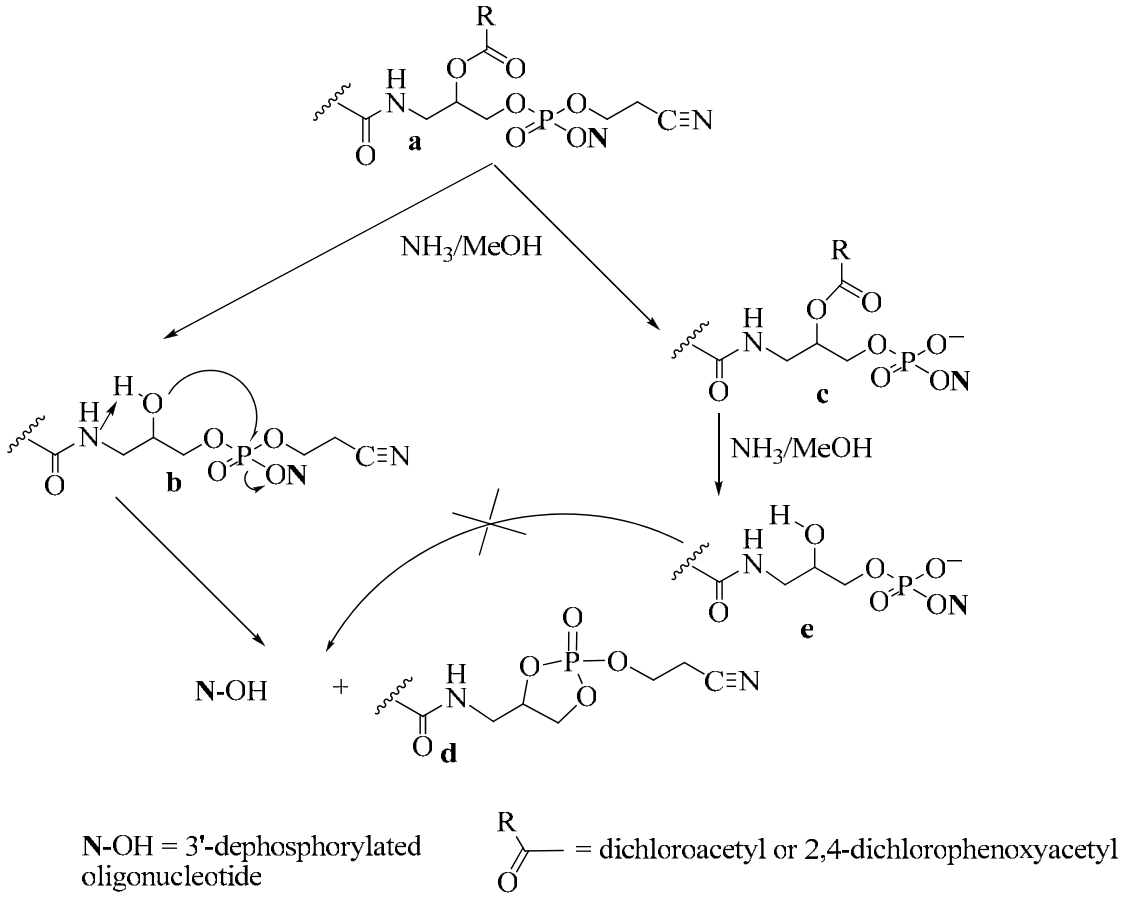
Recently we developed a new simple and very efficient method of carbomoylation of amines.[6] This reaction was successfully employed to develop a straightforward, considerably simplified method to prepare the universal solid supports for oligonucleotide synthesis, **usiii**[6] as alternative to the lengthy and laborious construction of linker by consecutive reactions on the solid phase (preparation of the **usii**).[1-3] Scheme 2 shows structures of the **usiii** supports, where the urea function bridges the solid phase and the universal linker.



Scheme 2

As in the case with the **usii,** we have tested two variants of **usiii –** the one bearing the dichloroacetyl group (**usiii$_{dca}$**) and another, bearing the 2,4-dichlorophenoxyacetyl group (**usiii$_{dcpa}$**). Both supports proved to be as stable upon storage as their **usii** counterparts. The yields of oligonucleotides, synthesized on the **usiii$_{dca}$** normally exceeded yields of oligomers, prepared using conventional nucleoside bound supports by about 5%. However, yields of oligonucleotides assembled on the **usiii$_{dcpa}$** and cleaved as described earlier[1-4] were again as with **usii$_{dcpa}$** about 15-25 % lower than yields of the same oligomers, generated on either nucleoside bound supports or the **usiii$_{dca}$**.

We believe that mechanistically the cleavage/dephosphorylation reaction proceeds as shown in Scheme 3. The fastest reaction upon treatment with NH_3/MeOH cleaves the acyl group of **a** and gives rise to intermediate **b.** Amide group of either succinyl linker (**usii**) or urea linker (**usiii**), bridging the solid phase and the 3-amino-1,2-propanediol tether assists an instant attack of the 2-hydroxyl group on the phosphorous atom (**b**) *prior* to phosphate deprotection (β-elimination of cyanoethyl function that gives rise to undesired phosphodiester **c**). This attack leads to the elimination of the 3'-dephosphorylated target ologonucleotide **N**-OH along with the formation of the cyclic phosphotriester **d**. The competing reaction, if any leads to the formation of a solid phase bound oligonucleotide **c** with the phosphodiester group at the 3'-terminus. This intermediate ultimately converts into a stable phosphodiester **e** linked to the solid phase. We assume that in the case of **usiii$_{dca}$**, bearing dichloroacetyl group a reaction, leading to intermediate **b** appears much faster than that leading to **c**. Therefore, practically no stable polymer bound phosphodiester **c** is being formed in this case and this fact may explain high yields of oligonucleotides **N**-OH, when dichloroacetyl group is used for protection of the 2-hydroxyl function of 3-acylamido-1,2-propanediol tether.

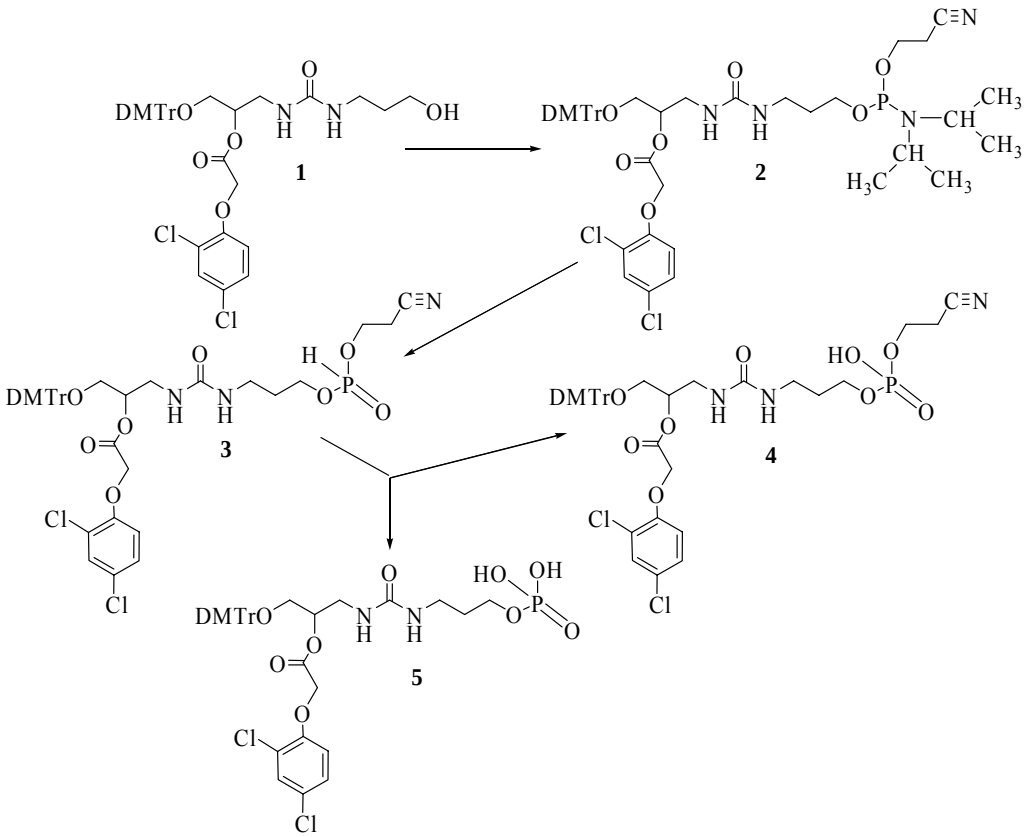


Scheme 3

On the other hand, in the case of **usiii$_{dcpa}$** reaction rates, leading to intermediates **b** and **c** may appear comparable due to the higher stability of 2,4-dichlorophenoxyacetyl group towards ammonolisis. This phenomena brings about formation of certain fraction of stable polymer bound phosphodiester **c** and hence the somewhat decreased yields of desired oligonucleotides **N**-OH (15-25 % lower than yields of the same oligomers, generated on either nucleoside bound supports or the **usiii$_{dca}$**).

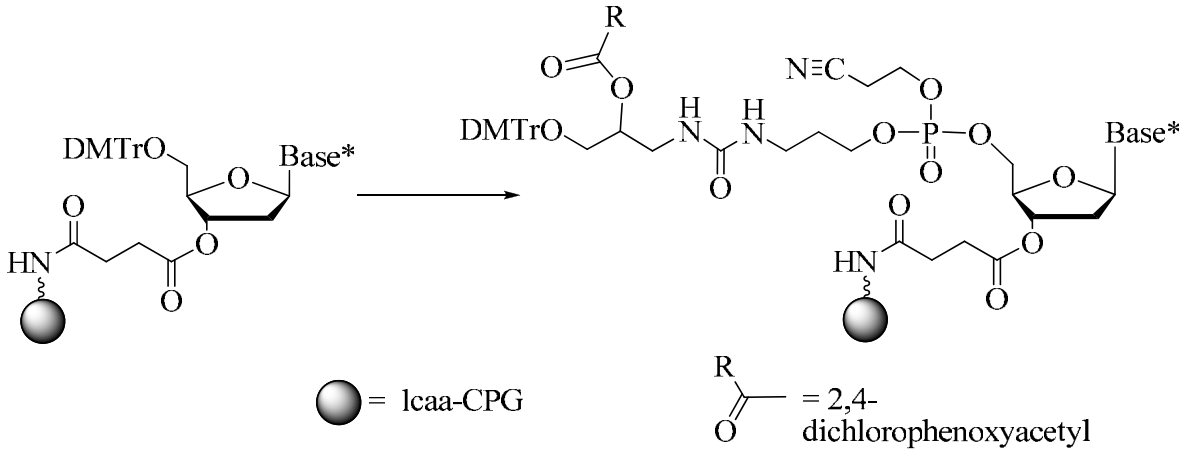
Obviously, mechanistic considerations discussed above look reasonable. However, no experimental evidence seems feasible due to the fact that in case of **usiii$_{dcpa}$**, the formation of diester **c** and ultimately polymer bound tethered oligonucleotide **e** appear difficult to support by conventional physico-chemical methods.

Our new carbomoylation procedure proved applicable for the preparation of various types of urea derivatives.[5] Among other compounds we have synthesized urea **1**[6] (Scheme 4). This compound bears a free hydroxyl that allows for one step transformation[7] into the universal linker phosphoramidite **2** in about 80% yield (Scheme 4). The structure of phosphoramidite **2** was supported by NMR spectroscopy. The further characterization was achieved by hydrolyzing **2** into H-phosphonate **3** and subsequent oxidation of **3** into a mixture of phosphodiester **4** and phosphomonoester **5** (Scheme 4 and Experimental Section).



Scheme 4

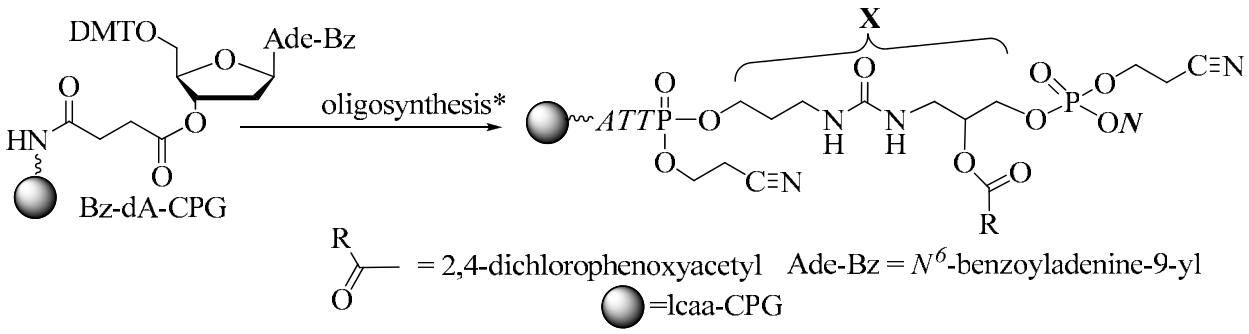
Compound **2** may be employed to convert any kind of conventional nucleoside bound solid support for oligonucleotide synthesis into the universal phase, employing standard steps on a DNA synthesizer (Scheme 5).



Base* = thymin-1-yl or N^6-benzoyladenine-9-yl

Scheme 5

We performed the following experiment: the protected modified oligonucleotide 5'-*ATACCGATTAAGCGAAGTTT***X***TTA*-3'(protected nucleoside units are given in uppercase italics) was assembled on a commercial solid support (Bz-dA-CPG), employing DMTr-off mode. No changes of standard synthesizer protocol were made to introduce the unit **X**, employing phosphoramidite **2** (Scheme 6). After the assembly, the solid phase bound protected oligomer was treated with 3.5 M ammonia in methanol for 40 min. at r.t. (methanolic ammonia treatment converts unit **X** into the de-acylated unit **Y**, Scheme 7). Methanolic solution was removed from the solid phase, diluted (3 times) with concentrated aqueous ammonia and heated at 55 ºC for additional 8 h. The sample was evaporated to dryness and the resulting oligonucleotide material was dissolved in water and analyzed, using IE HPLC (Figure 1).



*addition of two T-units, one **X**-unit, followed by conventinal oligosynthesis steps
Prortected nucleoside units are given in uppercase italics; protected target oligonucleotide is given in uppercase bold italics (***N***).

Scheme 6

The peak with τ_R 21.5 min corresponds to the 20mer deprotected oligonucleotide 5'-ATACCGATTAAGCGAAGTTT-3', **N**-OH (deprotected nucleoside units are given in uppercase plain), as was proved by co-injection with the standard 20mer (data not shown). The structure of oligonucleotide with τ_R 22.5 min was tentatively assigned as the 24mer 5'-ATACCGATTAAGCGAAGTTT**Y**TTA-3', **f**.

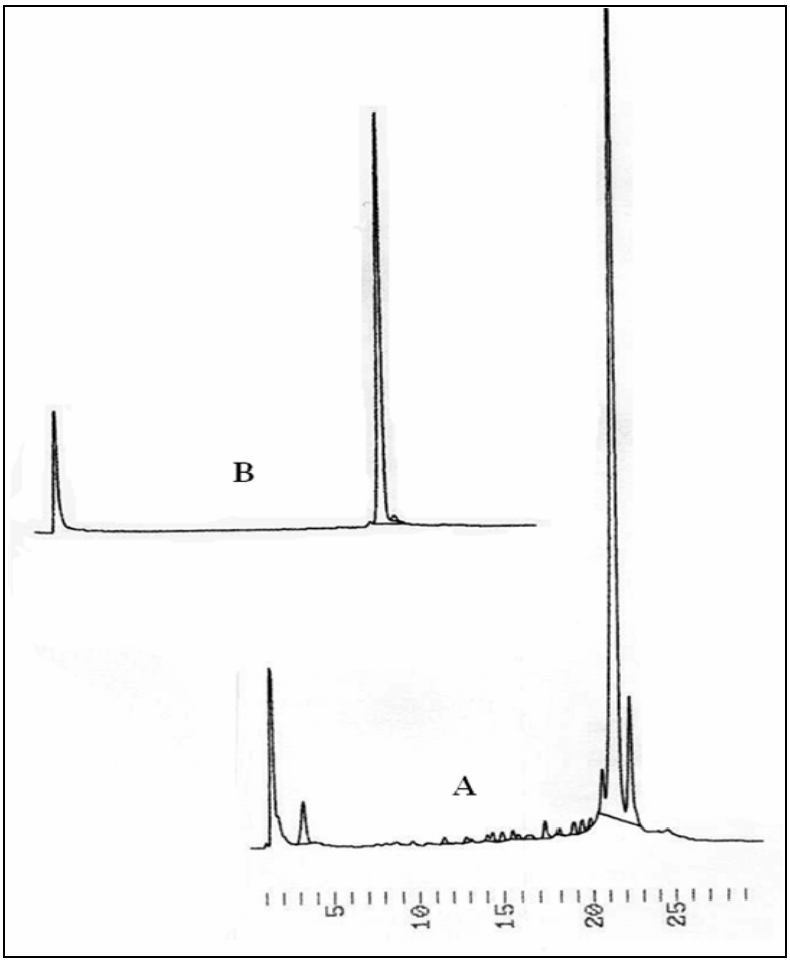


Figure 1. **A** - IE HPLC trace of reaction mixture, resulting from cleavage/deprotection of CPG bound protected 5'-*ATACCGATTAAGCGAAGTTT***X***TTA*-3'. The major peak, eluting at 21.5 min corresponds to the deprotected 20mer oligonucleotide 5'-ATACCGATTAAGCGAAGTTT-3', **N**-OH. The minor peak, eluting at 22.5 min corresponds to 24mer ATACCGATTAAGCGAAGTTT**Y**TTA-3', **f**. **B** - standard 20mer oligonucleotide 5'-ATACCGATTAAGCGAAGTTT-3', **N**-OH, retention time (τ_R) 21.5 min.

In order to precisely assign structure of - **Y**TTA-3' tether we have assembled two shorter oligonucleotides – protected 5'-TTTTTT**Y**TTA-3', and 5'-TTTTTT**Y**TTT-3', on the corresponding dA-bound and dT-bound supports and employing the Universal Phosphoramidite **2.** After the assembly, the solid phase bound oligomers were treated with 3.5 M ammonia in

methanol for 40 min. at r.t. Concentrated aqueous ammonia (2 volumes) was then added to each mixture and the resulting suspensions were left for additional 24 h at r.t. Solid material was removed, both solutions were evaporated to dryness and the resulting oligonucleotide material was dissolved in water and analyzed, using IE HPLC (data not shown; IE HPLC traces were similar to that in Figure 1). Two major eluting peaks were collected in each case and their sequences were established. In both cases, the earlier eluting peak corresponded to the **oligo-T_6** as was proved by co-injection with the standard **oligo-T_6** (data not shown). The later eluting peaks from both mixtures (5'-TTTTTT**Y**TTA-3', and 5'-TTTTTT**Y**TTT-3') were collected and characterized with ESI MS. Found molecular masses, being 2940.0 (calcd. 2938.8) and 2931.0 (calcd. 2929.8), univocally supported their sequences to be 5'-TTTTTT**Y**TTA-3' and 5'-TTTTTT**Y**TTT-3' correspondingly.

The syntheses of **oligo-T_6** and 20mer **N**-OH, employing Universal Phosphoramidite **2** appeared to be reasonably good, when the final cleavage/deprotection was performed with 3.5 M ammonia in methanol within maximum 40 min. at r.t and subsequent immediate removal of the methanolic solution from the solid phase. In that case, additional treatment of methanolic solution with concentrated aqueous ammonia (55ºC for additional 8h) facilitated only the cleavage of nucleic bases' protecting groups and the ratio of target 20mer **N**-OH to the 24mer **f** varied in several similar experiments from about 10:1 to 12:1.

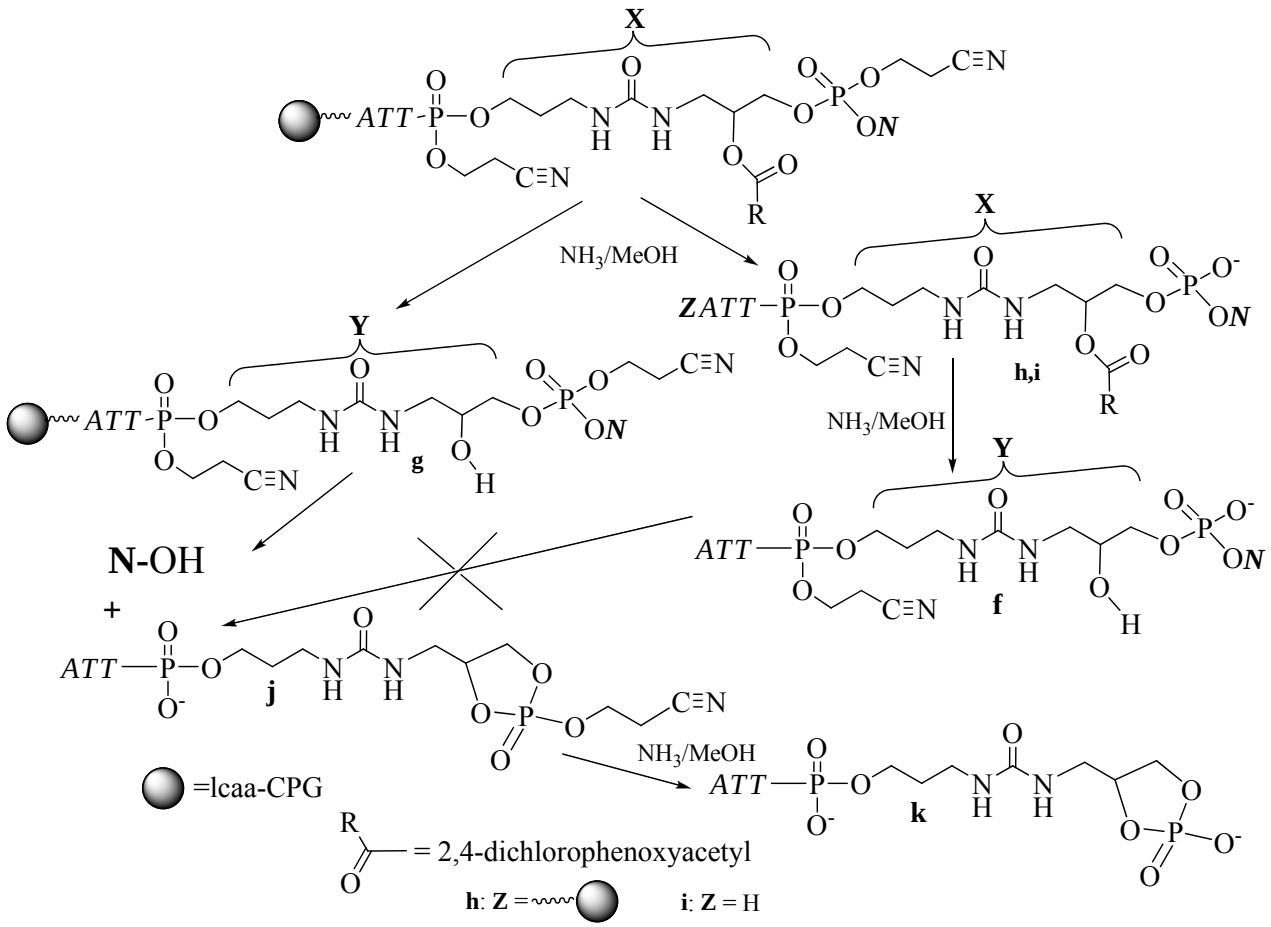
We believe that the 40 min. treatment of solid support bound protected oligonucleotide with ammonia in methanol at r.t. leads to the release of three products (**N**-OH, **k** and **f**, Scheme 7). The fastest reaction – removal of 2,4-dichloroacetyl group proceeds completely and instantly leads to the release of the desired 20mer oligonucleotide **N**-OH as a major component in accordance with mechanism, shown in Scheme 7 (formation of triester **g** leads to **N**-OH plus cyclic triester **j**, followed by conversion of **j** into cyclic diester **k**) . Two competing slower reactions - β-elimination of cyanoethyl function and ammonolisis of the succinate linker (bridging the controlled pore glass and the nucleoside unit) bring about the release of two minor phosphodiester products – **h** and **I**, both subsequently giving rise to tethered oligonucleotide **f.**

However, when the assembled solid support bound protected oligonucleotide was subjected to a treatment with ammonia in methanol followed by aqueous ammonia (24 h at r.t) without the removal of the solid phase, all three competing reactions appear complete. For example, in case with the synthesis of standard 20mer **N**-OH, this procedure leads to a lower content of desired oligonucleotide along with substantial amount of 24mer **f** in a ratio of about 7:3.

Conclusions

Phosphoramidite **2** helps to clarify why the yields of oligonucleotides assembled on the **usii$_{dcpa}$** or **usiii$_{dcpa}$** (bearing 2,4-dichlorophenoxyacetyl group) are giving rise to oligonucleotides with yields about 15-25% lower than yields of the same oligomers, generated on either nucleoside bound supports or universal supports **usii$_{dca}$** or **usiii$_{dca}$** (bearing dichloroacetyl group). In

addition, universal linker phosphoramidite **2** may be successfully employed for conversion of conventional nucleoside bound solid phase into the universal support.



Prortected nucleoside units are given in uppercase italics; deprotected nucleoside units are given in uppercase plain; protected target oligonucleotide is given in uppercase bold italics (***N***); **N**-OH = 3'-dephosphorylated target oligonucleotide

Scheme 7

Experimental Section

Column flash chromatography was run using C-601 pump module, C-610 pump controller and borosilicate glass columns (Büchi). Lichroprep RP-18 (40-63 μm) (Merck) was used for column flash chromatography. A gradient of acetonitrile in water (from 50% to 100%, 25 ml/min) was used for purification of compound **2**. IE HPLC of oligonucleotides was performed employing a Merck-Hitachi gradient chromatograph on a DNAPac PA-100 (4.0 x 250mm, Dionex) column, using a gradient elution of sodium perchlorate (from 0 to 0.1M) in (0.1M sodium acetate in 20% acetonitrile) over 30 min.; flow rate 1 ml/min (system A). RP HPLC was performed was

performed employing a Waters chromatograph on a xBridge C18, 5 μm (4.6 x 150mm, Waters) column, using a gradient elution of acetonitrile (from70 to 95%) in 0.1M triethylammonium acetate, pH 8.5 over 20 min.; flow rate 1 ml/min (system B).
IR spectra were obtained with Thermo Nicolet Nexus 470 FT-IR spectrometer. NMR spectra were taken with Avance 500 spectrometer (Bruker) in CD_3CN. Elemental analysis was performed using Thermo Quest CE Instruments EA 1110 analyser. Electrospray Ionization Mass spectra (ESI MS) were obtained with Finnigan LTQ MS spectrometer in negative mode. Deconvolution of raw files obtained was performed with BioworksBrowser Rev 3.1 SR1 program. Mongo Oligo Mass Calculator v2.06 and ChemDraw Ultra 8.0 programs were used for calculation of theoretical molecular masses. Oligonucleotide syntheses were performed on an ASM-800 synthesizer (Biosset) on a 0.1 μmolar scale, employing a universal solid support **usiii** of Metkinen Chemistry (for synthesis of control 20mer oligonucleotide **N**-OH) or a nucleoside bound supports of Glen Research (for testing of universal phosphoramidite **2**) and using standard protocol for DNA synthesis. Monomer concentrations were about 0.1M in all cases. After completion of assembly, oligonucleotides were cleaved from the solid phase with 3.5 N ammonia in methanol (7 N ammonia in methanol of Aldrich was diluted two times with dry cold methanol) and deprotected as described above.

Universal Linker phosphoramidite (2). Compound **2** was prepared from urea **1**,[6] 2-cyanoethyl tetraisopropylphosphorodiamidite (Rhodia) and sublimed 1*H*-tetrazole (Glen Research), following the standard protocol.[7] The phosphoramidite **2** (6 g) was isolated using RP flash chromatography in 78% yield as a 96% pure (RP HPLC in system B, τ_R 14.3 min) colorless glass. IR (KBr) ν_{max} 3372, br, s; 2966, s; 2933, s; 2873, m; 2838, m; 2259, m; 1761, s; 1643, s; 1608, s; 1571, s; 1509, s; 1480, s; 1364, s; 1301, s; 1251, s; 1200, s; 1084, s; 1035, s; 978, s. ^{31}P-NMR: δ, ppm 147.72, s. ^{1}H-NMR: δ, ppm 7.48-6.86, m, 16H, arom.; 5.15, m, 1H, -CHOAcyl; 5.05, m, 2H, 2 x -NH; 4.80, s, 1 H, H_a of -CH_2OAryl; 4.79, s, 1 H, H_b of -CH_2OAryl; 3.80, m, 2H, -OCH_2CH_2CN; 3.78, s, 6H, 2 x -OCH_3; 3.71-3.12, m, 10H, -CH_2ODMTr, 2 x -CH_2 (3-aminopropanol), 2 x -N$CH(CH_3)_2$, -NHCH_2CHOAcyl,; 2.65, dt, 2H, *J*=1.4 Hz, *J*=6.0 Hz, -OCH_2CH_2CN; 1.67, m, 2H, -$CH_2CH_2CH_2$ (3-aminopropanol); 1.19, d, 6H, *J*=6.8 Hz, $(CH_3)_a$ of -$CH(CH_3)_2$; 1.17, d, 6H, *J*=6.8 Hz, $(CH_3)_b$ of -$CH(CH_3)_2$. ^{13}C-NMR: δ, ppm 167.92, s, -COO-; 158.78, s, 2 x C-4 of 4-methoxyphenyl; 158.15, s, -$CO(NH-)_2$; 152.68, s, C-1 of 2,4-dichlorophenyl; 145.04, s, C-1of phenyl; 135.92 and 135.90, 2s, 2 x C-1 of 4-methoxyphenyl; 130.04, d, 4 x C-2 of 4-methoxyphenyl; 129.89, d, C-3 of 2,4-dichlorophenyl; 128.07 and 127.98, 2d, 2 x C-2 of phenyl; 127.95, d, C-5 of 2,4-dichlorophenyl; 126.98, d, C-4 of phenyl; 126.13, d, C-4 of 2,4-dichlorophenyl; 123.25, s, C-2 of 2,4-dichlorophenyl; 117.38, s, CN; 115.07, d, C-5 of 2,4-dichlorophenyl; 113.20, d, 4 x C-3 of 4-methoxyphenyl; 86.09, s, C-(4-methoxyphenyl)$_2$Phenyl; 73.97, d, -OCH_2CH(O-)CH_2N-; 66.01, t, -OCH_2CH(O-)CH_2N-; 62.84, 7, -OCH_2CO-; 61.31, 61.17, 2t, –NH$CH_2CH_2CH_2$O-; 58.50, 58.35, 2t, -OCH_2CH_2CN; 55.03, q, 2 x -OCH_3; 42.97, 42.88, 2d, 2 x –N$CH(CH_3)_2$; 40.18, t, -NHCH_2CH(O-)CH_2O-; 37.07, t, -NH$CH_2CH_2CH_2$O-; 31.81, 31.75, 2t, NH$CH_2CH_2CH_2$O-; 24.11, 24.08, 24.05, 24.02, 4q, 4 x –

$CH\underline{C}H_3$; 20.20 and 20.14, t and t, $-CH_2\underline{C}H_2CN$. Anal. Calc. for $C_{45}H_{55}Cl_2N_4O_9P$: C 60.20, H 6.17, N 6.24; found: C 60.02, H 6.29, N 6.51.
Phosphoramidite **2** was hydrolyzed with water in the presence of tetrazole to give H-phosphonate **3**, ESI MS: 813.0 (calcd. 813.2). H-phosphonate **3** was oxidized with peroxide to give mixture of phosphodiester **4**, ESI MS: 829.3 (calcd. 829.2) and phosphomonoester **5**, ESI MS: 776.3 (calcd. 776.2).

Acknowledgements

We thank Dr. Hugh Mackie for inspiring this work. Financial Support from Glen Research Corporation is gratefully acknowledged.

References

1. Azhayev, A.; Antopolsky, M. *Tetrahedron* **2001**, *57*, 4977.
2. Azhayev, A.; Antopolsky, M. U.S. Patent 6 770 754, 2004 and Eur. Patent 1404695, 2005.
3. Antopolsky, M.; Roivainen, J.; Randolph, J.; Mackie, H.; Azhayev, A. In *Innovations and Perspectives in Solid Phase Synthesis & Combinatorial Libraries*; Epton, R., Ed., Mayflower Worldwide, 2002, p 51.
4. Azhayev, A. V.; Antopolsky, M. L.; Tennilä, T. M. L.; Mackie, H.; Randolph, J.B. *Genetic Engineering News* **2005,** *25*, 54.
5. Yagodkin, A.; Azhayev, A. U.S. Patent Appl. 60/854,721, 2007 and Int. Pat. Appl. WO 2008/049972, 2008.
6. ^{1}H-NMR: δ ppm 7.50-6.82, m, 16H, arom.; 5.13, m, 1H, $\underline{CH}$-O-Acyl; 5.07, m, 2H, 2 x NH; 4.78, dd, $\underline{CH_2}$OAryl; 3.76, s, 6H, 2 x OCH_3; 3.45-3.12, m, 9H, $\underline{CH_2}$ODMTr, 2 x CH_2 (3-aminopropanol), OH, -NH-$\underline{CH_2}$-CH-OAcyl,; 1.51, m, 2H, $CH_2\underline{CH_2}CH_2$ (3-aminopropanol).
7. Nielsen, J.; Dahl, O. *Nucleic Acids Res.***1987**, *15*, 3626.