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Synthesis of Oligonucleotides Containing 2'-Azido- and 2'-Amino-2'-deoxyuridine Using Phosphotriester Chemistry[1]

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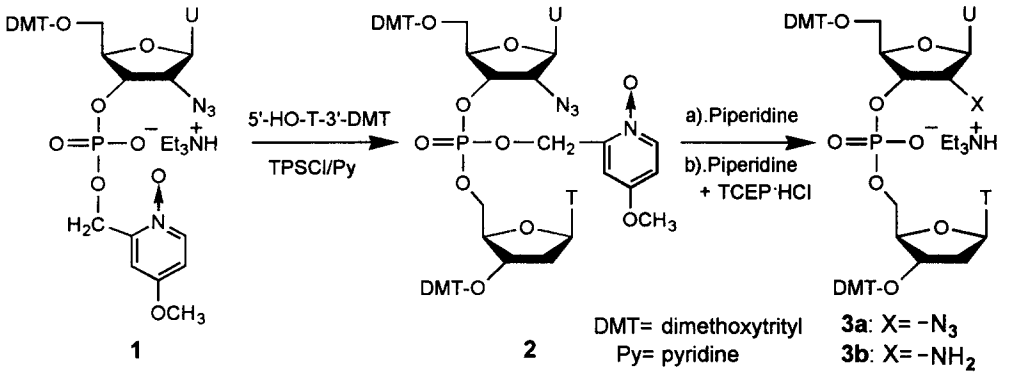
Abstract: *The phosphotriester method of oligonucleotide synthesis is shown to be useful for assembly of oligomers containing multiple internal 2'-azido substituents. Subsequent reduction of the azido group(s) with trialkylphosphines provides 2'-amino substituted oligonucleotides.*

Chemical synthesis of modified oligonucleotides as potential therapeutic agents has attracted great interest over the last decade. Modifications have been introduced at various points throughout the oligonucleotide molecule. Among the analogues with modified sugar moieties, 2'-O-alkyl, 2'-fluoro and 2'-amino substitutions have been described.[2] Some of these exhibit both increased stability against enzymatic degradation and improved ability to bind RNA target.[2] Despite the fact that 2'-azido-2'-deoxy ribonucleosides have been known for some time, to our knowledge there has only been one report of a synthetic oligonucleotide containing a 2'-azido group. This report described a thymidine oligomer, containing a single azido group at the 2'-position of the 3'-terminal T residue.[3] Because we believe that oligonucleotide analogues bearing 2'-azido substituted ribose moieties might possess some valuable characteristics, an effort to develop general methods for their synthesis was undertaken. Attempts to prepare 5'-dimethoxytrityl-2'-azido-2'-deoxyuridine-3'-cyanoethyl phosphoramidite failed as this compound was not stable and began to decompose immediately upon formation. This was not surprising as it is well known that phosphites are able to reduce the azido group. And this behavior excluded the possibility of using phosphoramidite methods for the synthesis of 2'-azido oligonucleotides. The phosphotriester method remained a possible viable approach.

From the many published variations of phosphotriester-based oligonucleotide synthesis, we chose to explore the highly efficient method based on O-nucleophilic intramolecular catalysis.[4] Nucleotide component **1** bearing the catalytic 1-oxido-4-methoxy-2-picolyl phosphate protecting group was synthesized from 5'-DMT-2'-N_3-2'-dU[5] in a manner analogous to the preparation of 2'-unmodified deoxynucleotide monomers.[4,6] The suitability of the catalytic approach was proven by coupling of **1** (2 eq) with 5'-HO-T-3'-DMT[7] (1 eq) in the presence of triisopropylbenzenesulfonyl chloride (TPSCl, 4 eq) in a dry $Py/CH_2Cl_2/CH_3CN$ [8] mixture (1/3/9, v/v/v, 0.04-0.05 M in **1**) at room temperature. Under these conditions the coupling reaction reached

completion in less than 2 min giving the desired diastereomeric phosphotriester dimer (**2**) in high yield, 85% after chromatographic isolation on silica gel.



Next, we checked the stability of the 2'-azido moiety towards the deblocking and capping reagents which are commonly used in solid-phase synthesis. Thus, 5'-DMT-2'-N_3-2'-dU was treated with a 1:1 mixture of Ac_2O/THF (1/9, v/v) and methylimidazole/Py/THF (1/1/8, v/v/v) for 3 hr at room temperature. The only two products were the corresponding 3'-acetylated and 3',N^3-bisacetylated (<2% by TLC) derivatives. The major product, 5'-DMT-2'-N_3-2'-dU-3'-OAc, was purified and further treated with a 3% solution of dichloroacetic acid in CH_2Cl_2 for 3 hr at room temperature to give exclusively the 5'-hydroxyl derivative, 5'-HO-2'-N_3-2'-dU-3'-OAc. Thus, the azido group was proven stable to standard capping and detritylation conditions.

As an example of the synthesis of 2'-azido modified oligonucleotides, the automated solid-phase synthesis of 10-mer 5'-HO-[U(2'-N_3)p]$_5$(Tp)$_4$T-OH-3' containing 5 consecutive 2'-azido-2'-deoxyuridines was accomplished. In this regard, a 0.056 M solution of monomer **1** as the triethylammonium salt in 5% collidine in CH_3CN/CH_2Cl_2 (3/2, v/v) [8] was mixed with an equal volume of 0.2 M TPSCl in 5% collidine in CH_3CN. This activated nucleotide mixture was then immediately applied to a 5'-detritylated-3'-support-bound thymidine pentamer previously prepared by standard cyanoethyl phosphoramidite chemistry. The synthesis cycle, comprised of coupling, capping and detritylation was then repeated four more times to generate the support-bound decamer. A coupling time of 4 min provided coupling yields of 95-98% per cycle based on dimethoxytrityl cation release. Final deprotection was achieved by treatment with piperidine for 12 hr at room temperature to remove the 1-oxido-4-methoxy-2-picolyl and β-cyanoethyl phosphate protecting groups, followed by methylamine/ammonia treatment for 1 hr at room temperature to cleave the succinate linkage to the support. The crude deprotected 5'-HO-[U(2'-N_3)p]$_5$(Tp)$_4$T-OH-3', 53% purity by capillary electrophoresis (CE), was purified by denaturating 20% polyacrylamide gel electrophoresis and its integrity and structure were confirmed by a variety of analytical methods. Thus, both RP-HPLC and CE analyses showed one dominant peak accounting for more than 90% of the absorbance at 260 nm. Delayed extraction

matrix-assisted laser desorption-ionization time of flight (DE-MALDI-TOF) MS analysis [9] gave a 135.0 mass difference between the azido-modified 10-mer and a standard 5'-HO-(Tp)$_9$T-OH-3', in agreement with the calculated mass difference of 134.9 Da. Enzymatic digestion and base composition analysis revealed two nucleoside components in 1:1 ratio having HPLC retention times identical to authentic thymidine and 2'-azido-2'-deoxyuridine. Finally, the structure was further confirmed by DE-MALDI-TOF MS assisted exonuclease sequencing (Fig. 1).[9]

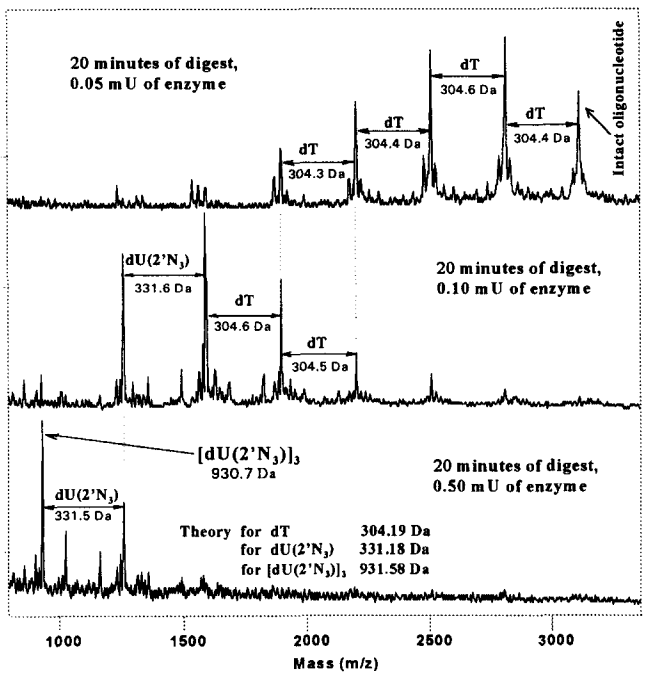


Figure 1. 3 '–> 5' sequence by Snake Venom Phosphodiesterase. The signals from monomers and dimer can not be identified due to high noise below 800 Da caused by MALDI matrix and fragmentation patterns.

Although the phosphotriester method is generally acceptable for the synthesis of short 2'-azido oligonucleotides as demonstrated here, we have encountered difficulties during the synthesis of modified oligos containing longer stretches of 2'-azido-2'-deoxyuridine. The reasons for this we believe are attributable to aggregation of the growing 2'-azido chain and/or a preactivation problem during mixing of nucleotide component with coupling agent within the lines of the synthesizer.[10] Efforts to improve solid phase synthesis protocols both chemically and mechanically are now in progress.

Apart from their potential value as antisense drugs, we also reasoned that 2'-azido-modified oligos might prove useful for the synthesis of 2'-amino oligonucleotides as the 2'-azido group might be reduced at the end of the oligomer synthesis. Thus, 5'-DMT-2'-azido-2'-deoxyuridine was readily transformed to the corresponding 2'-amino derivative in a matter of minutes by treatment with triphenylphosphine (TPP, 1.5 eq) in Py/CH_3CN (1/4, v/v) in the presence of H_2O (10-20 eq) as a proton source. Unfortunately, a similar attempt to reduce dimer **3a**[11], which was prepared from **2** by treatment with piperidine, proceeded very slowly reaching completion only after 2 weeks! This result was rationalized by the fact that the 2'-azido group in the context of dimer **3a** is spatially less accessible for reaction with the bulky TPP. Use of the more water soluble and less bulky tris(2-carboxyethyl)-phosphine hydrochloride (TCEP·HCl)[12], however, reduced dimer **3a** to dimer **3b**[13] in less than 10 min at room temperature in

Py/H_2O/CH_3CN (2/1/4, v/v/v, 0.01 M **3a**, 4 fold excess of TCEP·HCl). These results are now being extended to the synthesis of longer molecules containing multiple internal amino substituents.

References and notes

1. The results of this work were presented at the *Nucleic Acids Symposium*: 6-11 August, **1995**, Noordwijkerhout, the Netherlands.
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5. 5'-DMT-2'-N_3-2'-dU was synthesized by standard dimethoxytrytilation of 2'-azido-2'-deoxyuridine. For the synthesis of 2'-N_3-2'-dU see: Verheyden, J.P.H., Wagner, D., Moffatt, J.G. *J. Org. Chem.* **1971**, *36*, 250-254.
6. Compound **1** was prepared with 55% overall yield from 5'-DMT-2'-N_3-2'-dU; ESI MS (negative mode): 787.21 (M^-), calc. 787.21; ^{1}H-NMR (200 MHz, DMSO-d6): δ 11.43 (d, 1H, NH (U)), 8.14 (d, 1H, H-6 (Py)), 7.66 (d, 1H, H-6 (U)), 7.41-7.11 (m, 9H, H-Ar (DMT)), 7.08 (d, 1H, H-3 (Py)), 6.83 (d, 4H, H-Ar (DMT)), 5.80 (d, 1H, H-1'), 5.28 (d, 1H, H-5 (U)), 4.91-4.62 (m, 3H, H-3' + OCH_2-2 (Py)), 4.34 (t, 1H, H-2'), 4.15 (br. s, 1H, H-4'), 3.79 (s, 3H, OCH_3 (Py)), 3.70 (s, 6H, OCH_3 (DMT)), 3.30 (m, 2H, CH_2-5'), 3.02 (q, CH_2 (Et_3NH)), 1.13 (t, CH_3 (Et_3NH)).
7. 5'-HO-T-3'-DMT was purchased from Glen Research.
8. Because nucleotide **1** is poorly soluble in neat CH_3CN addition of CH_2Cl_2 was necessary to obtain a homogeneous solution.
9. Smirnov, I.P.; Roskey, M.T.; Juhasz, P.; Takach, E.J.; Martin, S.A.; Haff, L.A. Submitted to Anal. Biochem, 1996.
10. Efimov, V.A.; Chakhmakhcheva, O.G.; Dubey, I.Y.; Polushin, N.N. *Nucl. Acids Res. Symp.Ser.* **1987**, *18*, 213-216.
11. Spectral data for **3a**: MS ESI (positive mode): 1222.24 ($M^- + 2Na^+$), calc. M^- =1176.38; ^{1}H-NMR (200 MHz, DMSO-d6): δ 11.47 (br. s, 1H, NH (U)), 11.19 (s, 1H, NH (T)), 7.82 (s, 1H, H-6 (T)), 7.58 (d, 1H, H-6 (U)), 7.45-7.11 (m, 18H, H-Ar (DMT)), 6.83 (d, 8H, H-Ar (DMT)), 6.24 (dd, 1H, H-1' (T)), 5.83 (d, 1H, H-1' (U)), 5.31 (d, 1H, H-5 (U)), 4.64 (q, 1H, H-3' (U)), 4.22 (d, 1H, H-3' (T)), 4.12 (t, 1H, H-2' (U)), 4.04 (br. s, 1H, H-4' (U)), 3.70 (s, 12H, OCH_3 (DMT)), 3.70-3.00 (m, 5H, H-4' and CH_2-5' (T) + CH_2-5' (U)), 3.00 (q, CH_2 (Et_3NH)), 1.88-1.44 (m, 2H, H-2',2'' (T)), 1.68 (s, 3H, CH_3 (T)), 1.11 (t, CH_3 (Et_3NH)); ^{31}P-NMR (200 Mhz, DMSO-d6): δ -1.53 (d).
12. TCEP·HCl is available from Pierce Chemical, Rockford, IL USA.
13. Spectral data for **3b**: MS ESI (positive mode): 1196.41 ($M^- + 2Na^+$), 1174.43 ($M^- + H^+ + Na^+$), calc. M^- =1150.38; ^{1}H-NMR (200 MHz, DMSO-d6): δ 11.45 (br. s, 1H, NH (U)), 11.20 (br. s, 1H, NH (T)), 7.69 (s, 1H, H-6 (T)), 7.59 (d, 1H, H-6 (U)), 7.43-7.08 (m, 18H, H-Ar (DMT)), 6.84 (dd, 8H, H-Ar (DMT)), 6.23 (dd, 1H, H-1' (T)), 5.90 (d, 1H, H-1' (U)), 5.50 (d, 1H, H-5 (U)), 4.59 (q, 1H, H-3' (U)), 4.19 (d, 1H, H-3' (T)), 4.04 (br. s, 1H, H-4' (U)), 3.79 (t, 1H, H-2' (U)), 3.70 (d, 12H, OCH_3 (DMT)), 3.70-2.95 (m, 5H, H-4' and CH_2-5' (T) + CH_2-5' (U)), 3.00 (q, CH_2 (Et_3NH)), 1.88-1.44 (m, 2H, H-2',2'' (T)), 1.68 (s, 3H, CH_3 (T)), 1.14 (t, CH_3 (Et_3NH)); ^{31}P-NMR (200 MHz, DMSO-d6): δ 0.20 (s). These data are identical to that from a separate sample prepared by coupling of 5'-DMT-2'-NHAc-2'-dU cyanoethyl phosphoramidite with 5'-HO-T-3'-DMT followed by oxidation and ammonia deprotection.

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