Pergamon

Tetrahedron Letters, Vol. 37, No. 19, pp. 3231-3234, 1996

0040-4039/96 $15.00 + 0.00

S0040-4039(96)00537-0

Synthesis of Functionally Modified Oligonucleotides from Methoxyoxalamido Precursors[1]

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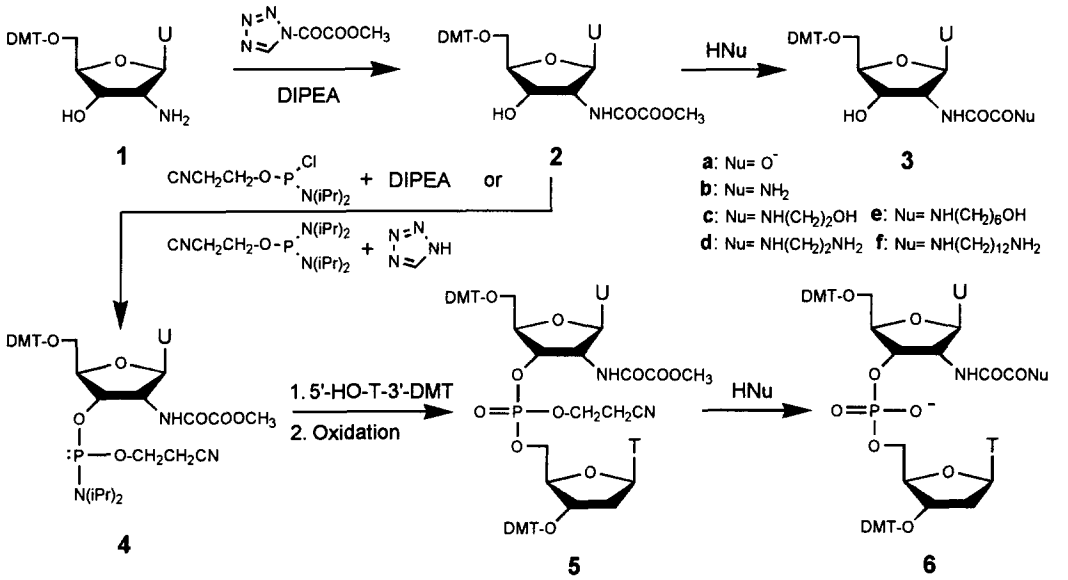
Abstract: *2'-Methoxyoxalamido-2'-deoxyuridine was incorporated into an oligonucleotide molecule and shown to be a useful precursor for the post synthetic introduction of various functional additives.* Copyright © 1996 Elsevier Science Ltd

Oligonucleotides bearing various functionalities have become commonplace tools in molecular biology and diagnostics.[2] One of the most efficient routes to the synthesis of functionally modified oligonucleotides (FMOs) is the introduction of a precursor which at the end of solid phase synthesis can react with a desirable linker or modifier.[3] This strategy enables one to synthesize a wide variety of FMOs from a single parent oligonucleotide.

The methoxyoxalamidoalkyl moiety (alkyl-NHCOCOOCH$_3$), if designed as part of an oligonucleotide molecule, might be a convenient site for the introduction of various functional additives. The ester carbonyl carbon of the methoxyoxalyl residue is highly electrophilic due to electron withdrawing effects of the adjacent methoxy and carbonyl groups. Indeed, rapid and quantitative reactions are observed with strong nucleophiles such as primary amines, ammonia or hydroxyl anion. Thus, a desirable additive, if it contains an aliphatic primary amino group, might easily derivatize an oligonucleotide precursor at a methoxyoxalyl site to afford the corresponding conjugate via a stable alkylamidooxalamidoalkyl bridge (alkyl-NHCOCONH-alkyl)[4]. The stability of the alkoxyoxalamidoalkyl moiety towards the reagents used in solid-phase phosphoramidite synthesis has been previously demonstrated by Alul et al.[5] who used the oxalyl group to anchor nucleosides to amino-derivatized CPG.

From the many possible placements of the methoxyoxalamido moiety within an oligonucleotide, the approach of introducing methoxyoxalyl modification at the 2'-position of 2'-amino-2'-deoxyuridine was chosen. Phosphoramidite **4** was envisioned to be easily prepared from 5'-dimethoxytrityl-2'-amino-2'-deoxyuridine (**1**)[6] in two steps for introduction at any point in the oligonucleotide chain. Furthermore, 2'-modification is believed to alter the hybridization properties of an oligonucleotide to a lesser extent than modification at a nucleobase or along the phosphate backbone.



The 2'-amino group in **1** was selectively methoxyoxalylated with methyl oxalyl tetrazolide prepared *in situ* (1.2 eq, 0.45 M CH_3CN solution)[7] in CH_2Cl_2 (0.1M in **1**) in the presence of diisopropylethylamine (DIPEA, 2eq) at room temperature. After the reaction was complete (15 min as monitored by TLC), the reaction mixture was extracted with 1 M TEAB and the desired 2'-methoxyoxalamido derivative **2** was isolated by chromatography on silica gel in 70 % yield.[8] Phosphitylation of **2** was accomplished conventionally[9] except that the aqueous workup was omitted and the reaction mixture in CH_2Cl_2 was directly loaded onto a silica gel column. Elution with a pyridine/hexane/ethyl acetate mixture (0.5/10/89.5, v/v/v) afforded pure phosphoramidite **4** in 80% yield.[10] Purified **4** (2 eq) was coupled with 5'-HO-T-3'-O-DMT[11] (1 eq) in CH_3CN (0.15-0.20 M in **4**) in the presence of tetrazole (5 eq) as a catalyst. Under these conditions the coupling reaction reached completion in less than 5 min providing, after oxidation with iodine,[12] the desired diastereomeric phosphotriester dimer **5** in 80% isolated yield. The diastereomers were further separated by preparative TLC and characterized.[13]

The ability of the 2'-methoxyoxalamido group to undergo rapid transformation upon treatment with different nucleophiles was first investigated at the nucleoside level. Compound **2** (0.1 M CH_2Cl_2 solution) was treated with excess (50-100 eq) LiOH (1 M aqueous-methanolic solution), methanolic ammonia (saturated at 0° C), ethanolamine (EA), ethylenediamine (EDA), 6-amino-1-hexanol (AH, 1 M CH_2Cl_2 solution) and 1,12-diaminododecane (DAD, 0.5 M pyridine solution) to produce compounds **3a-f**. As expected, efficient derivatization occurred with all nucleophiles. Nucleosides **3a-e** were formed nearly instantaneously, <1 min, and quantitatively.[14] In the case of the bulky DAD group the reaction time was slightly longer, ~3 min, and, apart from the expected nucleoside **3f**, about 5% of a disubstituted product was also formed.

Similar experiments were performed with phosphotriester dimer **5**. In this case, however, the reaction time was increased to 30 min to ensure complete removal of β-cyanoethyl phosphate protecting group. Again, quantitative formation of phosphodiesters **6a-e** was observed. Treatment with DAD resulted in a small amount, < 5% by TLC, of disubstituted product along with the dimer **6f**[15].

The novel convergent strategy was next established at the oligonucleotide level. A 10-mer consisting of nine thymidines and one 2'-methoxyoxalamido-2'-deoxyuridine at the 5'-end was synthesized by automated solid-phase β-cyanoethyl phosphoramidite chemistry (3x1μmol scale) on an Expedite DNA Synthesizer. Coupling time for phosphoramidite **4** (0.1 M CH_3CN solution) was 33 min, which provided a coupling yield of 75-80% based on dimethoxytrityl cation release. The reason for the rather poor yield is most likely attributable to steric hindrance by the rigid 2'-methoxyoxalylamido residue. Detritylated support-bound decamer was treated with DAD (0.5 M pyridine solution, 500 μl) for 3 hrs at room temperature. Aqueous ammonia (500 μl) was subsequently added and the reaction mixture was kept for another hour to ensure complete cleavage of the succinate linkage and removal of β-cyanoethyl phosphate protecting groups. Finally, the reaction mixture was desalted over Sephadex G25 to give 198 A260 units of crude material consisting primarily of the desired derivatized 10-mer, 5'-HO-U[2'-NHCOCONH$(CH_2)_{12}NH_2$](Tp)$_8$T-OH-3', and the unreacted 9-mer 5'-HO-(Tp)$_8$T-OH-3'. A total of 60 ODUs (30%) of the DAD-oligonucleotide conjugate was isolated by denaturating 20% PAAG electrophoresis and the structure of the conjugate was confirmed by a variety of analytical methods.

Enzymatic digestion and base composition analysis revealed two nucleoside components in approximately 9:1 ratio having HPLC retention times identical to authentic thymidine and 2'-NHCOCONH$(CH_2)_{12}NH_2$-2'-dU. Delayed extraction MALDI-TOF MS analysis[16] yielded a 255.5 mass difference between the DAD-modified 10-mer and a standard 5'-HO-(Tp)$_9$T-OH-3', in agreement with the calculated mass difference of 255.2 Da. Finally, the structure was confirmed by ^{1}H-NMR analysis (Fig.1).

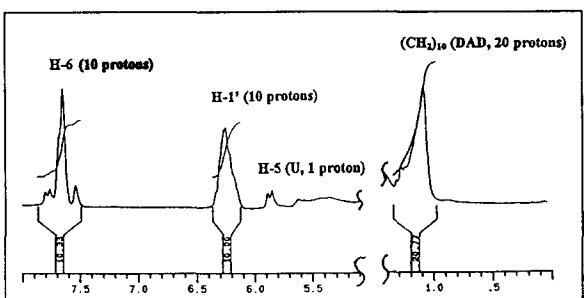


Figure 1. The 200 MHz ^{1}H-NMR spectrum of the 10-mer **5'-HO-U[2'-NHCOCONH$(CH_2)_{12}NH_2$](Tp)$_8$T-OH-3'** confirming incorporation of the DAD residue.

In conclusion, the utility of 2'-methoxyoxalamido modification for the post synthetic introduction of various functional additives into an oligonucleotide molecule has been demonstrated. Efforts to improve solid-phase coupling of phosphoramidite **4** are now in progress. Alternative routes to the synthesis of FMOs through 2'-methoxyoxalamido oligonucleotides generated *in situ* from 2'-amino precursors will be reported elsewhere.

Acknowledgments: The author is indebted to Dr. J.Coull and Dr. G.Vella for editorial assistance. I am also very grateful to Dr. I.Smirnov for MALDI MS analysis and to Dr. A.Verentchikov for ESI MS analysis.

References and notes

1. The results of this work were presented at the *Nucleic Acids Symposium*: 6-11 August, **1995**, Noordwijkerhout, the Netherlands.
2. Goodchild, J. Conjugates of Oligonucleotides and Modified Oligonucleotides: A Review of Their Synthesis and Properties. In *Perspectives in Bioconjugate Chemistry*; Meares, C.F. Ed.; ACS, Washington, DC, **1993**; pp. 77-99, and references cited therein.
3. Ferentz, A.E.; Verdine, G.L. *Nucleosides & Nucleotides.* **1992**, *11*, 1749-1763.
4. Phosphodiester **6f** was stable in 0.5 M methanolic KOH for at least 6 hrs at room temperature and in saturated aqueous ammonia for at least 16 hr at 55° C.
5. Alul, R.H.; Singman, C.N.; Zhang, G.; Letsinger, R.L. *Nucl. Acids Res.* **1991**, *19*, 1527-1532.
6. For the synthesis of 5'-DMT-2'-NH_2-2'-dU see: Polushin, N.N.; Smirnov I.P.; Verentchikov A.N.; Coull, J.M. *Tetrahedron Lett.* **1996**, in press, and references cited therein.
7. To a solution of tetrazole in anhydrous CH_3CN (0.45 M, 1.2 eq, Activator Solution, DNA Synthesis Reagent, PerSeptive Biosystems) were consecutively added with stirring methyl oxalyl chloride (1 eq, Aldrich) and 2,4,6-collidine (1 eq, Fluka). After 30 min the reaction mixture was centrifuged and the supernatant was used for the methoxyoxalylation reaction.
8. Spectral data for **2**: MS ESI (positive mode): 654.26 ($M + Na^+$), 1285.45 ($2M + Na^+$), calc. M= 631.22; ^{1}H-NMR (200 MHz, DMSO-d6): δ 11.32 (s, 1H, NH (U)), 8.54 (d, 1H, 2'-NH), 7.65 (d, 1H, H-6 (U)), 7.45-7.15 (m, 9H, H-Ar (DMT)), 6.87 (d, 4H, H-Ar (DMT)), 5.97 (d, 1H, H-1'), 5.86 (d, 1H, 3'-OH), 5.46 (d, 1H, H-5 (U)), 4.57 (dd, 1H, H-2'), 4.22 (dd, 1H, H-3'), 4.04 (dd, 1H, H-4'), 3.78 (s, 3H, $COCOOCH_3$), 3.71 (s, 6H, OCH_3 (DMT)), 3.36-3.12 (m, 2H, CH_2-5').
9. Polushin, N.N.; Chen, B-c.; Anderson, L.W.; Cohen, J.S. *J. Org. Chem.* **1993**, *58*, 4606-4613.
10. MS ESI data for **4** (positive mode): 933.59 ($M + Et_3NH^+$), 832.48 ($M + H^+$), 854.42 ($M + Na^+$), calc. M= 831.32.
11. 5'-HO-T-3'-DMT was purchased from Glen Research.
12. Oxidizer Solution, DNA Synthesis Reagent, PerSeptive Biosystems.
13. Selected spectral data for **5**: MS ESI (positive mode): upper isomer: 1313.52 ($M + Na^+$), lower isomer: 1313.51 ($M + Na^+$), calc. M= 1290.42; ^{1}H-NMR (200 MHz, DMSO-d6): upper isomer: δ 11.41 (s, 1H, NH (U)), 11.24 (s, 1H, NH (T)), 9.31 (d, 1H, 2'-NH (U)), 6.87 (t, 4H, H-Ar (DMT)), 5.37 (d, 1H, H-5 (U)), 3.71 (s, 12H, OCH_3 (DMT)), 3.68 (s, 3H, $COCOOCH_3$), lower isomer: δ 11.44 (s, 1H, NH (U)), 11.26 (s, 1H, NH (T)), 9.35 (d, 1H, 2'-NH (U)), 6.86 (d, 4H, H-Ar (DMT)), 5.43 (d, 1H, H-5 (U)), 3.75 (s, 3H, $COCOOCH_3$), 3.68 (m, 12H, OCH_3 (DMT)); ^{31}P-NMR (200 MHz, DMSO-d6): upper isomer: δ −2.38, lower isomer: δ −1.58.
14. MS ESI data (positive mode) for **3**: **a**: 662.19 ($M^- + 2Na^+$), 719.35 ($M^- + Et_3NH^+ + H^+$), 820.42 ($M^- + 2Et_3NH^+$), calc. M^- = 616.19; **b**: 639.21 ($M + Na^+$), 718.33 ($M + Et_3NH^+$), calc. M= 616.22; **c**: 683.29 ($M + Na^+$), calc. M= 660.24; **f**: 800.37 ($M + H^+$), calc. M= 799.42.
15. Selected spectral data for **6f**: MS ESI (positive mode): 1428.68 ($M^- + H^+ + Na^+$), 1406.63 ($M^- + 2H^+$), calc. M^- =1404.58; ^{1}H-NMR (200 MHz, DMSO-d6): δ 7.67 (s, 1H, H-6 (T)), 7.60 (d, 1H, H-6 (U)), 7.45-7.11 (m, 18H, H-Ar (DMT)), 6.84 (t, 8H, H-Ar (DMT)), 6.24 (dd, 1H, H-1' (T)), 5.94 (d, 1H, H-1' (U)), 5.35 (d, 1H, H-5 (U)), 3.70 (d, >6H, OCH_3 (DMT)), 3.67 (s, >6H, OCH_3 (DMT)), 1.70 (s, >3H, CH_3 (T)), 1.20 (br. s, 20H, $(CH_2)_{10}$ (DAD)).
16. Smirnov, I.P.; Roskey, M.T.; Juhasz, P.; Takach, E.J.; Martin, S.A.; Haff, L.A. Submitted to Anal. Biochem, **1996**.

(*Received in USA* 15 *February* 1996; *revised* 6 *March* 1996; *accepted* 13 *March* 1996)