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Synthesis and Evaluation of Oligodeoxynucleotides Containing 4'-*C*-Substituted Thymidines

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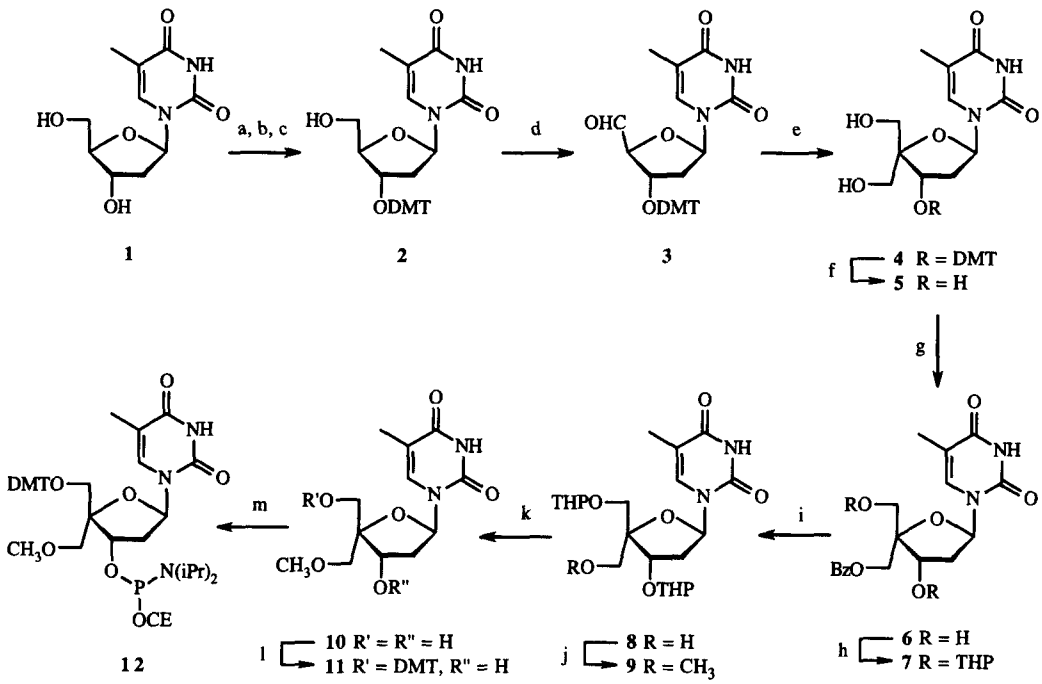
Abstract: 4'-*C*-Hydroxymethylthymidine was converted to 4'-*C*-methoxymethylthymidine and 4'-*C*-aminomethylthymidine, which were incorporated into oligodeoxynucleotides by phosphoramidite chemistry. The modified oligonucleotides exhibit excellent hybridization and significant improvement in stability to snake venom phosphodiesterase. Copyright © 1996 Elsevier Science Ltd

Antisense oligonucleotides as potential human therapeutics have been explored for more than a decade.[1-3] In order to improve the binding affinity, enzyme stability, cell-uptake, and other pharmacokinetic properties, a variety of oligonucleotide analogs have been synthesized and evaluated recently.[4-6] Oligonucleotides containing sugar-modified nucleosides have also received considerable attention.[6] 2'-*O*-Alkyl oligonucleotides have shown superior hybridization to RNA and certain enzyme stability.[7-9] Very recently, oligonucleotides containing 3'-*C*-hydroxymethylthymidine,[10] 5'-*C*-branched nucleosides,[11-12] and 4'-*C*-substituted nucleosides[13-14] have also been reported. Most of these sugar-modified oligonucleotides have satisfied hybridization and enhanced enzyme stability compared to unmodified oligonucleotides. In search for oligonucleotide analogs possessing enhanced hybridization and enzyme stability as well as ability to induce RNase H activity, we have independently explored a variety of sugar modifications. In this communication we will describe synthesis and evaluation of oligodeoxynucleotides containing 4'-*C*-aminomethyl- and 4'-*C*-methoxymethylthymidine.

Synthesis of 4'-*C*-methoxymethyl- and 4'-*C*-aminomethylthymidine derivatives is shown in Scheme 1 and 2. For introducing functional groups at C4' position of thymidine, a known nucleoside, 4'-*C*-hydroxymethyl-thymidine[15-16] was used as a key intermediate, which was prepared by a similar, but improved procedure (Scheme 1, a-f). Instead of t-butyldimethylsilyl (TBDMS), 4,4'-dimethoxytrityl (DMT) was used to protect the 3'-hydroxyl of thymidine since DMT group, unlike TBDMS, is stable to the basic condition used for condensation of **3** and formaldehyde and the following Cannizzaro reaction. Thymidine was converted, in six steps, to **5** in 35% overall yield. **5** was selectively benzoylated at the 4'-hydroxymethyl to give **6** in 50-67% yield. The structure of **6** was assigned by a NOE ^{1}H NMR experiment.[17] By-products including mono-, di- and tribenzoylated products were hydrolyzed to regenerate **5**. Both 3'- and 5'-hydroxyl groups of **6** were protected with tetrahydropyranyl and the resulting **7** was hydrolyzed to give **8** in 94% overall yield. Methylation of **8** with methyl iodide yielded the 4'-methoxymethyl derivative **9**,[18] which was deprotected to give **10** in 72% overall yield. **10** was treated with DMT-Cl to give **11** in 82% yield and treatment of **11** with 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite gave the phosphoramidite **12** in 79% yield.

Scheme 1.

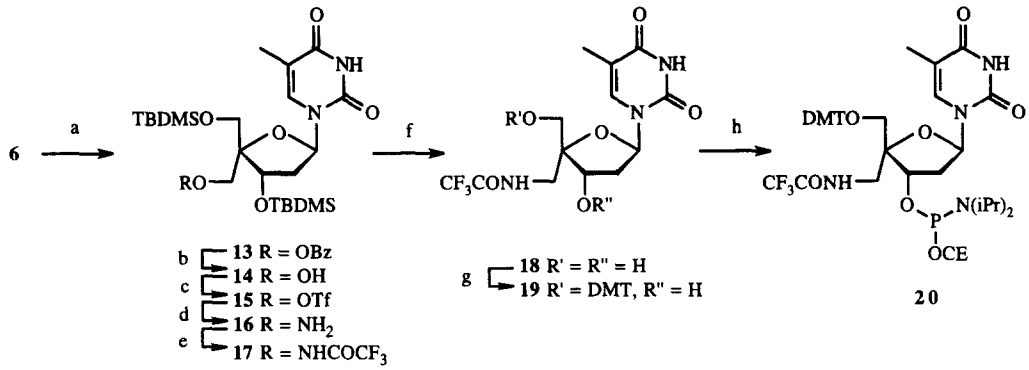


a) TBDMS-Cl (1.15 eq.), pyridine, r.t., 20 h; b) DMT-Cl, pyridine, 50 °C, 20 h; c) TBAF in THF; d) CF_3COOH, pyridine, DCC, DMSO, r.t., overnight; e) CH_2O, NaOH, dioxane, H_2O, r.t., overnight; f) 80% AcOH, 45 °C, 2 h; g) BzCl, pyridine, 0 °C, 2 h; h) dihydropyran, TsOH, CH_2Cl_2, r.t., 2 h; i) NaOH, H_2O, dioxane; 4 h; j) CH_3I (5.0 eq.), NaH (3.0 eq.), THF, 0 °C, 1.5 h; k) AcOH, THF, H_2O, 45 °C, 3 h; l) DMT-Cl, pyridine, 20 °C, overnight; m) $NCCH_2CH_2OP(Cl)N(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, 20 °C, 30 min. (r.t. = room temperature)

Reaction of **6** with TBDMS-Cl gave **13** in quantitative yield and the benzoyl group of **13** was removed by treatment with methylmagnesium bromide to afford **14** in 72% yield. Reaction of **14** with trifluoromethanesulfonic anhydride gave the triflate **15**, which was treated with ammonia in dioxane to give **16** in 63% overall yield. Reaction of **16** with S-ethyl thiotrifluoroacetate gave **17** in 88% yield. Treatment of **17** with TBAF gave **18** in 94% yield and treatment of **18** with DMT-Cl gave **19** in 82% yield. **19** was converted to **20** by 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite in 90% yield.

By using the phosphoramidites **12** and **20**, 4'-*C*-methoxymethylthymidine and 4'-*C*-aminomethyl-thymidine were incorporated into oligodeoxynucleotides on an ABI 394 Synthesizer. The coupling time for the modified phosphoramidites and the unmodified phosphoramidites adjacent to the modified was raised up to five minutes and excellent coupling yields (97-99%) were achieved. The synthesized sequences (Table 1) were worked up according to the standard procedure for unmodified oligonucleotides. The crude oligonucleotides were purified by a C8 reverse phase column with 0.1 M TEAA buffer (containing 5% acetonitrile, pH 7.5) on a Waters preparative HPLC. The purified oligonucleotides were obtained in 60-80 ODs on 1.0 μmol scale. The purified oligonucleotides were characterized by electrospray mass spectrometry.

Scheme 2.



a) TBDMS-Cl, imidazole, pyridine, r.t., 3 days; b) CH_3MgBr, THF, 0 °C, 3 h; c) Tf_2O, pyridine, CH_2Cl_2, 0 °C, 30 min; d) NH_3, dioxane, 45 °C, 4 days; e) $CF_3C(O)SEt$, pyridine, dioxane, r.t., overnight; f) TBAF in THF, neutralized to pH 7.5 with AcOH, r.t., overnight; g) DMT-Cl, pyridine, r.t., 5 h; h) $NCCH_2CH_2OP(Cl)N(iPr)_2$, $EtN(iPr)_2$, CH_2Cl_2, 20 °C, 40 min.

Hybridization properties of these 4'-*C*-substituted oligonucleotides were studied by thermodynamic melting (Tm) experiments.[19] The experiments were conducted on a Varian UV spectrometer equipped with an electronic temperature controller and a Cary hybridization software. As shown by the results in Table 1, most of these modified sequences exhibit similar or better hybridization to both complementary DNA and RNA as compared with the unmodified sequence. Moreover, these modified oligonucleotides seem to have better binding affinity to DNA than those containing 4'-*C*-hydroxymethylthymidine (about one degree drop per modification),[14] and those containing 5'(*S*)-*C*-aminomethyl- and 5'(*S*)-*C*-methoxymethylthymidine (about one degree drop per modification).[12]

Table 1. Sequences synthesized, hybridization and enzyme stability data

	Sequence	Tm °C DNA	ΔTm °C/Mod.	Tm °C RNA	ΔTm °C/Mod.	$t_{1/2}$* min.
1.	5'-ATCTCTCCGCTTCCTTTC-3'	58.3		64.4		5
2.	5'-ATCTCTCCGCTTCCTTXC-3'	59.4	+1.1	64.8	+0.4	
3.	5'-ATCTCTCCGCTTCCTXXC-3'	59.0	+0.4	65.3	+0.5	122
4.	5'-ATCTCTCCGCTTCCXTXC-3'	59.1	+0.4	65.6	+0.6	60
5.	5'-AXCTCTCCGCTTCCTTTC-3'	59.9	+1.6	64.5	+0.1	
6.	5'-ATCTCXCCGCTXCCTTTC-3'	58.9	+0.3	63.3	-0.6	
7.	5'-ATCTCTCCGCTTCCTTYC-3'	60.1	+1.8	64.5	+0.1	
8.	5'-ATCTCTCCGCTTCCTYYC-3'	61.2	+1.4	64.1	-0.2	46
9.	5'-ATCTCTCCGCTTCCYTYC-3'	61.1	+1.4	64.2	-0.1	>180
10.	5'-AYCTCTCCGCTTCCTTTC-3'	59.0	+0.7	64.9	+0.5	
11.	5'-ATCTCYCCGCTYCCTTTC-3'	57.2	-0.6	63.6	-0.4	
12.	5'-CTTCCTGTCTGATGGCTTC-3'					9
13.	5'-CXXCCXGXCXGAXGGCXXC-3'					>>180
14.	5'-CYYCCYGYCYGAYGGCYYC-3'					>>180

X = 4'-*C*-methoxymethylthymidine, Y = 4'-*C*-aminomethylthymidine. The samples for Tm measurements contain 2 μM of modified oligos and 2.0 μM of either complementary DNA or RNA in a buffer (10 mM sodium phosphate, 0.1 mM EDTA, and 0.1 M sodium chloride, pH = 7.0). * The half-lives of the 4'-*C*-substituted oligonuceotides were calculated from UV absorbance curves of oligonucleotide samples during degradation by SV phosphodiesterase at 25 °C.

Since 3'-exonucleases play a predominant role in cellular DNA degradation, the stability of oligonucleotides to snake venom phosphodiesterase was investigated by using a similar procedure as described by Wengel and collaborators.[20-21] Oligonucleotides (0.75 OD) were incubated with snake venom phosphodiesterase (1.2 units) in 1.5 mL of buffer (0.1 M Tris·HCl, pH 7.5; 0.1 M NaCl; 14 mM $MgCl_2$) in a cuvette on a Varian UV spectrometer at 25 °C, and the increase of absorbance at 260 nm during the degradation versus time was recorded. From these absorbance curves (not shown), the half-lives of oligonucleotides were calculated. At these conditions, the unmodified oligonucleotides (Sequence 1 and 12) were almost completely digested within 20 minutes and had half-lives of 5 and 9 minutes, respectively. In contrast, oligonucleotides containing two 4'-*C*-substituted thymidines at the 3'-end region had half-lives of 46 to more than 180 min. Incorporation of eight 4'-*C*-substituted thymidines into a 19 mer oligonucleotide (Sequence 13 and 14) almost completely prevents the degradation by SV phosphodiesterase (<5% degradation in 3 h).

In summary, 4'-*C*-methoxymethylthymidine and 4'-*C*-aminomethylthymidne have been synthesized and incorporated into oligodeoxynucleotides. Oligonucleotides containing these 4'-*C*-substituted thymidines hybridize with both complementary DNA and RNA with virtually identical or better binding affinity compared to the unmodified and have significant improvement in stability against SV phosphodiesterase, which indicates that these modified oligonucleotides are potentially useful in oligonucleotide therapeutics and diagnostics.

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REFERENCES AND NOTES.

1. Crooke, S. T.; Lebleu, B. *Antisense Research and Applications*; CRC Press: Boca Raton, 1993.
2. Lisziewicz, J.; Sun, D.; Metelev, V.; Zamecnik, P.; Gallo, R. C.; Agrawal, S. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 3860.
3. Stein, C. A.; Cheng, Y. -C. *Science* **1993**, *261*, 1004.
4. Uhlmann, E.; Peyman, A. *Chemical Reviews* **1990**, *90*, 543-584.
5. Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1993**, 49, 6123-94.
6. Sanhvi, Y. S.; Cook, P. D. *Carbohydrate Modifications in Antisense Research*; *ACS Symposium Series, No. 580*; American Chemical Society: Washington, D.C., 1994.
7. Inoue, H.; Hayase, Y.; Imura, A.; Iwai, S.; Miuta, K.; Ohtsuka, E. *Nucleic Acids Res.* **1987**, *15*, 6131.
8. Lesnik, E. A.; Guinosso, C. J.; Kawasaki, A. M.; Sasmor, H.; Zounes, M.; Cummins, L. L.; Ecker D. J.; Cook, P. D.; Freier, S. M. *Biochemistry* **1993**, *32*, 7832.
9. Sproat, B. S.; Lamond, A. I.; Beijer, B.; Neuner, P.; Ryder, U. *Nucleic Acids Res.* **1989**, *17*, 3373.
10. Jorgensen, P. N.; Stein, P. C.; Wengel, J. *J. Am. Chem. Soc.* **1994**, *116*, 2231.
11. Saha, A. K.; Caulfield, T. J.; Hobbs, C.; Upson, D. A.; Waychunas, C.; Yawman, A. M. *J. Org. Chem.* **1995**, *60*, 788.
12. Wang, G.; Middleton, P. J. *Tetrahedron Lett.* **1996**, 37, 2739.
13. Maag, H.; Schmidt, B.; Rose S. J. *Tetrahedron Lett.* **1994**, *35*, 6449.
14. Fensholdt, J.; Thrane, H.; Wengel, J. *Tetrahedron Lett.* **1995**, *36*, 2535.
15. Jones, G. H.; Taniguchi, M.; Tegg, D.; Moffatt, J. G. *J. Org. Chem.* **1979**, *44*, 1309.
16. O-Yang, C.; Wu, H. Y.; Fraser-Smith, E. B.; Walker, K. A. M. *Tetrahedron Lett.* **1992**, *33*, 37.
17. Saturation at 3.79-3.86 ppm (H5') gave 4.5% enhancement for H3', which is consistent with the results from another laboratory for 4'-benzoyloxymethyl-3'-(t-butyldimethylsilyl)thymidine (Reference 14).
18. Methylation of **8** yielded **9** as the only product, and no thymine-methylated product was observed at these conditions. However, the reaction yielded appreciable amount of the dimethylated product when it was run for more than four hours at 0 °C. The structure of **9** (diastereomers) was confirmed by ^{1}H NMR (δ 9.93, NH of thymine) and MS. FABMS m/z 455 (MH^+), 371 (-THP), 287 (-2THP), 127 (thymine).
19. Puglisi, J. D.; Tinoco, I. Jr. *Methods Enzymol.* **1989**, *180*, 304.
20. Svendsen, M. L.; Wengel, J.; Dahl, O.; Kirpekar, F.; Roepstorff, P. *Tetrahedron* **1993**, *49*, 11341.
21. We simply followed the procedure in Reference 20 except that the pH of the buffer was 7.5 (higher nuclease activity at pH 7.5) and amount of oligonucleotides was 0.75 OD.

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