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A Direct, Efficient Method for the Preparation of siRNAs Containing Ribo-like *North* Bicyclo[3.1.0]hexane Pseudosugars

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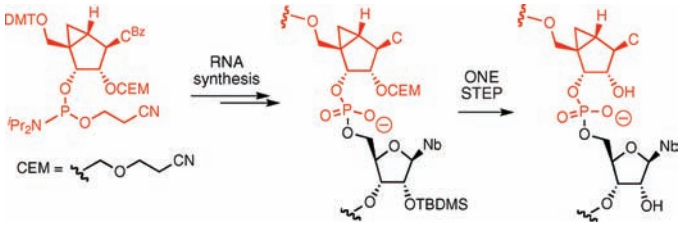
ABSTRACT



An efficient method for the preparation of siRNAs modified with ribo-like *North* bicyclo[3.1.0]hexane pseudosugars is described. The combined use of 2′-*O*-(2-cyanoethoxymethyl) (CEM) and 2′-*O*-TBDMS protection was successfully employed for RNA synthesis with the added advantage that both groups were efficiently removed in a single step. The resulting *North* ribo-methanocarba-modified siRNAs are compatible with the intracellular RNAi machinery and can mediate specific degradation of target mRNA.

Nucleotide analogues that exhibit the *North*-type sugar puckering[1] and possess the overall A-RNA-type conformation have attracted much interest in the field of RNA interference (RNAi) therapy.[2] RNAi is a sequence-specific RNA silencing mechanism[3] triggered by double-stranded RNA or short interfering RNA molecules (siRNA), which are formed by a sense and a guide strand.[4] Within the cells, an RNA-induced silencing complex (RISC)[5] unwinds the siRNA duplex and uses the guide strand as a template to find the complementary target mRNA,[6] an event that induces the endonucleolytic cleavage of the mRNA[7] and prevents its translation into protein. For efficient gene silencing to take place, the guide siRNA:mRNA duplex must adopt an A-type helical structure.[8] To fulfill these requirements and improve target-binding affinity, several

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North-locked nucleotide building blocks have been designed, synthesized, and incorporated into siRNAs.[9] Examples are 2′-*O*-alkylated RNAs,[8b,10] 2′-fluoro-RNAs,[8b,10a,11] and Locked Nucleic Acids (LNA), the latter containing a methylene bridge between the 2′ oxygen and the 4′ carbon.[12] In particular, LNAs have been widely explored, and it is well-known that LNA-modified siRNAs efficiently induce the RNAi process and increase the thermodynamic and serum stability of RNA duplexes to a great extent.[13]

Another candidate for introducing new features into siRNAs without perturbing the A-type helical structure they require for activity is the *North*-locked form of nucleotide analogues based on a carbocyclic bicyclo-[3.1.0]hexane system [methanocarba (MC) nucleosides]. Preliminary studies on the effect of *North* 2′-deoxy-MC nucleosides on the RNAi process have shown that these pseudonucleosides are accepted by the RNAi machinery.[14] However, the effect of a hydroxyl group at the 2′ position of any of the modified sugars, or the carbocyclic pseudosugar, on the RNAi process has never been investigated. The design and synthesis of *North* ribo-MC nucleosides (Figure 1) has been reported.[15] Nevertheless, these derivatives have never been incorporated into RNA strands.

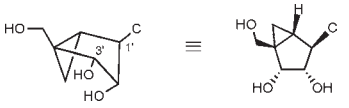
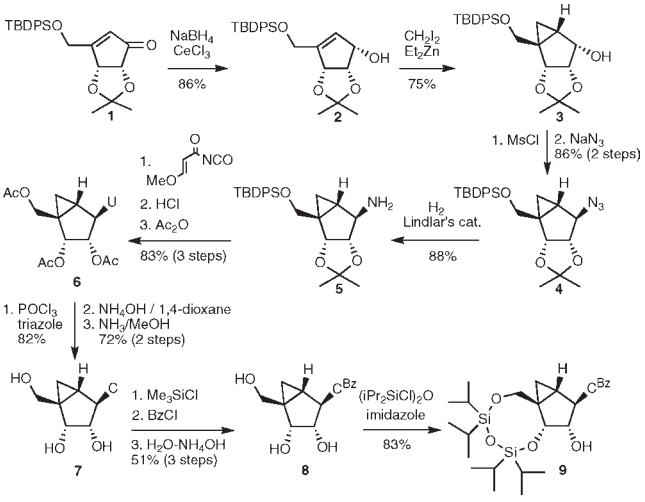


Figure 1. *North* ribo-methanocarba cytidine monomer (C^N).

Herein we describe a synthetic strategy for the preparation of conveniently 2′-*O*-protected phosphoramidite of *North* ribo-MC cytidine (C^N) (Figure 1). We also describe its incorporation into mixed 2′-*O*-protected RNA strands and the removal of the 2′-*O*-protecting groups of the RNAs in a single step. This approach permits an easy access to the preparation of *North* ribo-MC cytidine-modified siRNAs with potential therapeutic applications.

An important challenge in the preparation of RNA strands modified with *North* ribo-MC is the protection of the 2′-OH group of the pseudoribose. Preliminary studies on the reactivity of the 2′-OH group of *North* ribo-MC cytidine carried out in our group have shown that the 3′-OH is much more reactive than the 2′-OH group (data not shown). Thus, in contrast to what has been reported for a great number of nucleoside derivatives,[16] the direct protection of the 2′-OH group of 5′-*O*-protected ribo-MC cytidine derivatives with a free 3′-hydroxyl group becomes a serious problem. The attempted strategy was to employ a 3′,5′-*O*-disiloxan diprotected intermediate with a free 2′-OH group (**9**, Scheme 2). Our first try involved the protection of the free 2′-OH in **9** with a benzoyl group. However, this route was abandoned due to the rapid migration of the 2′-*O*-benzoyl from the 2′-OH to the 3′-OH after deprotection.

Scheme 1. Synthesis of Intermediate **9**



A wide variety of 2′-*O*-protecting groups have been developed for RNA synthesis.[16,17] For example, fluoride-labile protecting groups such as *tert*-butyldimethylsilyl (TBDMS),[17a] [(triisopropylsilyl)oxy]methyl (TOM),[17b] and 2-cyanoethoxymethyl (CEM)[17c] groups, or the photolabile [(2-nitrobenzyl)oxy]methyl group[17d] among others.[16] TBDMS and TOM are popular 2′-OH protecting groups whose phosphoramidites are commercially available. We decided to use the cheaper 2′-*O*-TBDMS-protected

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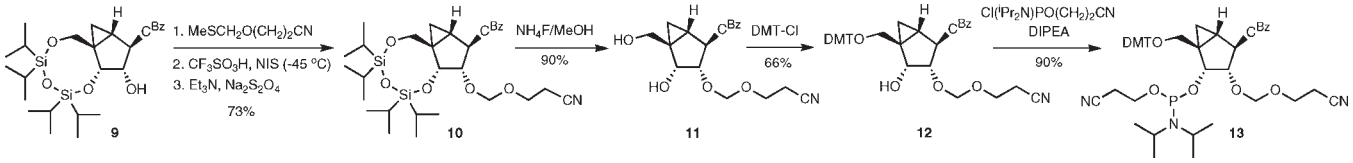
Scheme 2. Preparation of 2′-*O*-CEM-protected Ribo-MC Cytidine Building Block



phosphoramidites of natural ribonucleosides for RNA synthesis. However, for the protection of the 2′-OH group of the 3′,5′-*O*-disiloxan protected MC nucleoside **9**, the fluoride-labile TBDMS and TOM groups had to be avoided since the disiloxane protecting group is also removed by a fluoride reagent like TBAF. The CEM group is sensitive toward fluoride anion too, but only in aprotic solvents. In this regard, the group of Wada et al. have found a way to prevent the removal of the CEM group during the removal of the disiloxan group of 3′,5′-*O*-disiloxan-2′-*O*-CEM-protected nucleosides by treatment with TBAF in the presence of AcOH.[18]

As for RNA synthesis, removal of the CEM group from fully 2′-*O*-CEM-protected RNA strands has been successfully achieved under aprotic conditions.[19] In our search for a synthetic method that would allow ready cleavage of the 2′-*O*-protecting group of the MC nucleoside and the 2′-*O*-TBDMS protecting group of natural nucleosides from the final RNA product in only one step, we decided to focus on the CEM group.

The synthesis of intermediate **9** began by preparing cyclopropane **3** from cyclopentenone **1**[20] according to a previously reported procedure (Scheme 1),[21] which involves regio- and stereoselective Luche reduction followed by hydroxyl-directed Simmons−Smith cyclopropanation. Subsequently, the MC uridine derivative **6** was prepared from alcohol **3** following conventional published methods:[22,15a] (i) conversion of alcohol **3** into amine **5** via formation of the azido intermediate **4**,[22] and (ii) construction of the uracil ring by reacting amine **5** with 3-methoxyacryloyl isocyanate.[15a] Acetylation of the resulting uridine derivative gave **6**, which was converted into the unprotected cytidine derivative **7** by treatment with $POCl_3$, triazole, and ammonia.[23] Protection of the free 4-amino group as the *N*-benzoyl amide **8** was followed by treatment with 1,3-dichlorotetraisopropyl-disiloxane to give 3′,5′-*O*-disiloxan diprotected derivative **9**. As described in Scheme 2, compound **9** was treated with 2-cyanoethyl methylthiomethyl ether to give the desired 2′-*O*-CEM-3′,5′-*O*-disiloxan-protected derivative **10** in 73% yield. Following the fluoride-catalyzed removal of the disiloxan protecting group the desired 2′-*O*-CEM protected diol **11** was obtained in 90% yield. The 5′ hydroxyl group was then protected by a 4,4′-dimethoxytrityl group (DMT), and the resulting 5′-*O*-DMT-2′-*O*-CEM-protected nucleoside **12** was converted into the desired phosphoramidite (**13**).

With compound **13** in hand we proceeded to incorporate this nucleotide building block (C^N) into different positions in place of the natural ribocytidine along a 21-mer RNA guide strand that targets the *Renilla* luciferase mRNA by using a DNA/RNA synthesizer. The solid-phase synthesis was carried out on a 0.2 μmol scale with a coupling time of 10 min. For C^N-modified RNAs, P(III) to P(V) oxidation was performed with a solution of 10% *tert*-butyl hydroperoxide in acetonitrile/water (96:4). Commercially available succinyl polystyrene functionalized with 5′-*O*-DMT-2′-deoxythymidine was used as the solid support and tetrazole as the activator. Phosphoramidites of 2′-*O*-TBDMS protected natural ribonucleosides (A, U, G, and C) were obtained from commercial sources. We prepared RNA guide strands containing no substitutions (**14**, wt), one C^N substitution at position 8 (**15**), and two C^N substitutions spaced-out along the guide strand at positions 8 and 15 (**16**). We also prepared the corresponding unmodified sense strand (Table 1).

Table 1. Sequences of RNAs and T_m Data

entry	sequence	T_m[a]
1	3′-TTAAAAAGAGGAAGAAGUCUA (sense)	
2	5′-UUUUUCUCCUUCUUCAGAUTT (guide, **14**, **wt**)	67.6
3	5′-UUUUUCU**C^N**CUUCUUCAGAUTT (guide, **15**)	65.9
4	5′-UUUUUCU**C^N**CUUCUU**C^N**AGAUTT (guide, **16**)	64.5

[a] T_m's were measured in 15 mM HEPES-KOH (pH 7.4), 1 mM $MgCl_2$, and 50 mM KOAc.

After RNA synthesis, the DMT-on polystyrene solid support was treated with a mixture of 33% ammonia solution and ethanol [3:1 (v/v)] for 24 h at 35 °C[17c] for the cleavage from the polystyrene solid support and the removal of the phosphate-protecting and base-protecting

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groups. In the next step, the 2′-*O*-TBDMS protecting group of unmodified guide (**14**) and sense strands was completely removed by treatment with 1 M TBAF/THF for 12 h.

For the removal of 2′-*O*-TBDMS and 2′-*O*-CEM protecting groups of RNAs **15** and **16**, 10% *n*-propylamine and 1% of bis(2-mercaptoethyl) ether in 1 M TBAF/THF was used instead of 1 M TBAF/THF to prevent formation of CEM adducts,[24] a side reaction reported by Ohgi et al.[19] These authors report complete removal of 2′-*O*-CEM protecting groups of fully 2′-*O*-CEM protected RNA strands after 6 h at rt. Under these conditions, we obtained mixtures of 2′-*O*-protected and 2′-*O*-deprotected RNAs (as evaluated by HPLC). We increased the reaction time (to 12, 15, and 24 h), and after the mixtures were shaken at room temperature for 24 h, HPLC analysis of the crude oligo showed a single main peak corresponding to the fully deprotected product and only small amounts of shorter products (Figure 2A). Fully 2′-*O*-deprotected RNAs (**14**, **15**, **16**, and sense) were purified by reversed-phase semipreparative HPLC in the DMT-on mode. Finally, the DMT-on products were treated with 80% AcOH solution to remove the DMT group and the deprotected products were desalted on NAP columns. The desired products were obtained in good overall yields (**15**, 58%, 22 OD; **16**, 35%, 13 OD).

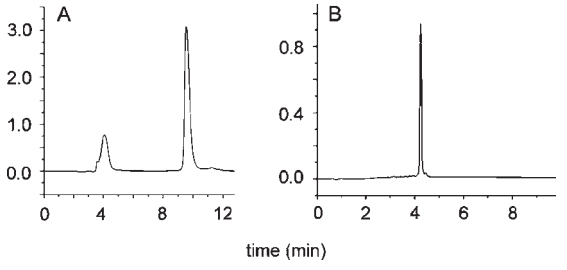

Figure 2. HPLC analysis of RNA **15**: (A) unpurified fully deprotected RNA **15**; (B) purified fully deprotected RNA **15**.

RNA strands **15** and **16** were treated with nuclease P1 and then alkaline phosphatase, and the digestion products were analyzed by HPLC. No modified ribonucleosides (2′-*O*-CEM protected ribo-MC cytidine or 2′-*O*-TBDMS protected U, A, C, and G) were observed, showing that complete removal of the CEM and TBDMS protecting groups and base-protecting groups was achieved.

Finally, to study the effect of the ribo *North* MC cytidine substitution on the RNA interference process, we carried out separate RNAi studies in SH-SY5Y cells with siRNA duplexes containing each of the modified guide strands (**15** and **16**), as well as with the unmodified (wt) guide RNA (**14**). Experiments were carried out in triplicate. The cells were first transfected with dual reporter plasmids that express *Renilla* luciferase (the target) and nontargeted firefly as an internal control. The effects of the different siRNAs on luciferase expression were evaluated using 26−0.03 nM siRNA concentrations in the cell media and measuring luciferase responses after 22 h. The results show that the RNAi machinery is not impaired by the *North* MC riboC modification (Figure S27). The best results were obtained for siRNA containing one substitution in the guide strand (**15**), which showed activity comparable to that of wt siRNA (**14**). To confirm the specificity of the observed effects, sequence-scrambled siRNAs were used (Table S1). As expected, scrambled sequences gave no *Renilla* inhibition (Figure S27). Thermal denaturation studies suggested that the C^N modification caused a slight destabilization of the siRNA duplex ($\Delta T = -1.6$ °C per substitution; Table 1). However, such small destabilization did not significantly affect RNAi activity.

In conclusion, in this work we have reported a strategy for (i) the preparation of *North* ribo-MC nucleosides conveniently protected at the 2′-OH position, (ii) their incorporation into mixed 2′-*O*-protected RNA strands, and (iii) the removal of the 2′-*O*-protecting groups of the RNA in only one step. To the best of our knowledge, this is the first time that the synthesis and one-step deprotection of 2′-*O*-TBDMS/2′-*O*-CEM oligoribonucleotides have been described.

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Supporting Information Available. Procedures, characterizations, copies of NMR spectra. This material is available free of charge via the Internet at: http://pubs.acs.org.