ñEffect of North bicyclo[3.1.0]hexane pseudosugars on RNA interferente. A novel class of siRNA modificationò Terrazas, M., Ocampo, S.M., Perales, J.C., Marquez, V., Eritja, R. ChemBioChem, 12(7), 1056-1065 (2011). doi: 10.1002/cbic.201000791

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# Effect of *North* bicyclo[3.1.0]hexane 2'-deoxy-pseudosugars on RNA interference. A novel class of siRNA modification

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**Keywords:** 2ô-deoxy-methanocarba (MC) nucleoside; immunostimulation; TNF-α; *Renilla* luciferase; RNA interference

**ABSTRACT**. North bicyclo methanocarba thymidine ($T^N$) nucleosides were substituted into siRNAs to investigate the effect of bicyclo[3.1.0]hexane 2ôdeoxy-pseudosugars on RNA interference activity. Here we provide evidence that these modified siRNAs are compatible with the intracellular RNAi machinery. We studied the effect of the $T^N$ modification in a screen involving residue-specific changes in an siRNA targeting Renilla luciferase and we applied the most effective pattern of modification to the knockdown of murine tumor necrosis factor (TNF-□). We also showed that incorporation of $T^N$ units into siRNA duplexes increased thermal stability, substantially enhanced serum stability and decreased innate immunostimulation. Comparative RNAi studies involving the $T^N$ substitution and locked nucleic acids (LNA) showed that the gene silencing activity of $T^N$ -modified siRNAs was comparable to that obtained with the LNA modification. An advantage of the *North* 2ôdeoxy-methanocarba modification is that it may be further explored in the future by changing the 2ôposition. The results from these studies suggest that this modification may be valuable for developing siRNAs for therapeutic applications.

## Introduction

RNA interference has become one of the most important new tools for biological research in the past decade.[1] This methodology is widely used for knocking down (i.e. regulating) expression of specific genes in cell culture and can be carried out conveniently by using synthetic 21-23 nt double stranded RNAs, which are known as short interfering RNAs or siRNAs.[2] These oligoribonucleotides are formed by a sense and an antisense (or guide) strand, which is complementary in sequence to a fragment of the target mRNA. The RNAi process is initiated with the recognition of the siRNA duplex by the RNA-induced silencing complex (RISC),[3] a protein located in the cytoplasm. After siRNA binding, RISC uses the antisense strand as a template to find the target mRNA[4] and induces endonucleolytic cleavage of the target mRNA,[5] preventing its translation into protein.

Despite the significance of RNAi both as a biological tool and as a potential therapeutic strategy, the utility of siRNAs *in vivo* faces some key hurdles including delivery, biostability,[6] and low sequence specificity that results in undesired off-target effects.[7] Moreover, cellular receptors such as toll-like receptors can bind to siRNAs and trigger innate immune responses, resulting in cytokine production.[8] To address these limitations and to further understand the mechanism of silencing, several research groups have been actively studying siRNAs with various chemical modifications,[9] specially on the sugar moiety.[10]

One widely used class of alteration that has been explored involves restriction of the conformation of the furanose system of the nucleotide building blocks of siRNAs in the *North* hemisphere of the pseudorotational cycle,[11] which is likely promising for the development of a nucleic acid analogue with high binding affinity to its RNA complement. Among this class of modification, incorporation of appropriate electronegative substituents at the 2' position of the sugar ring[12] like 2'-fluoro[10a,10b] and 2'-*O*-methyl substitutions in the ribo configuration,[10a-10c] has been found to increase stability of siRNAs to degradation by nucleases and to improve thermal stability, as measured by $T_m$ values. Moreover, Aigner *et al.* recently described siRNAs containing 2ô-azido substitutions.[10d] Interestingly, they found that this modification is well accepted by the RNAi machinery.

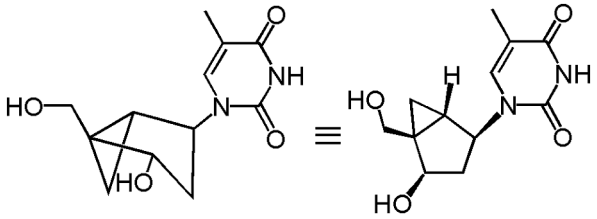
Another 2'-sugar modification that has been investigated involves addition of a methylene bridge between the 2' oxygen and the 4' carbon of the furanose ring.[13] The resulting nucleotides and oligonucleotides containing these monomers are called ñocked nucleic acidsò (LNA)[14] since the conformation of their bicyclic furanose ring is locked in the *North* hemisphere of the pseudorational cycle (3'-endo conformation),[11] thus retaining conformationally RNA-like A-type helical characteristics for effective gene silencing. Several studies have demonstrated that the LNA modification increases the thermodynamic and serum stability of siRNA duplexes to a great extent[15,10e,10i] and that single RNA to LNA exchanges at base paired positions in the antisense strand are tolerated in most of the positions that have been investigated.[10e]

Other conformationally restricted nucleoside analogues have been synthesized and incorporated into oligonucleotide strands.[16] For example, bicyclodeoxynucleosides,[16a,16b] which contain an ethylene bridge between the 3ôand the 5ôcarbons of the furanose ring, and tricyclodeoxynucleosides,[16c-g] with an additional cyclopropane ring fused to the ethylene bridge. The resulting oligonucleotides containing these units are called bc-DNA and tc-DNA, respectively. In particular, tc-DNA modifications have been found to favor an A-form double helix.[16e] In recent studies, Ittig *et al.* have found that incorporation of tc-DNA units in various positions of siRNAs can improve RNAi activity and serum stability.[16g]

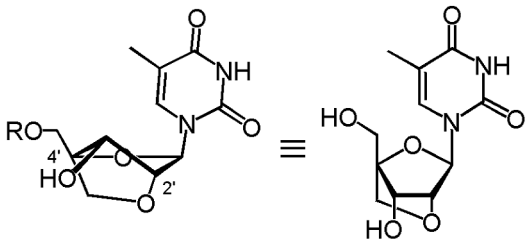
Another candidate for introducing new features into siRNAs without perturbing the overall A-form helical structure required for activity is the *North*-locked form of 2'-deoxynucleoside analogues based on a carbocyclic bicyclo[3.1.0]hexane system (2'-deoxy-methanocarba (MC) nucleosides).[17] The design and synthesis of 2'-deoxy-MC nucleosides have been summarized.[18] Moreover, it has been found that incorporation of *North* 2ô-deoxy-MC thymidine ($T^N$) units into oligodeoxynucleotide strands increases the thermal stability of the corresponding ODN/RNA heteroduplexes.[17b,18b] But despite the efficient hybridization of *North* 2'-deoxy-MC nucleoside analogues, these derivatives have never been incorporated into RNA strands and therefore, they have never been tested for their effects on the RNAi process. In this work we explore the replacement of the natural sugar ring of siRNAs by the *North* conformational restricted carbocyclic bicyclo[3.1.0]hexane system. In particular, we focus on siRNAs modified with the *North* 2'-deoxy-MC thymidine derivative ($T^N$, Figure 1). Herein we report studies which directly compare the gene silencing efficiency of $T^N$-modified siRNAs and native siRNA molecules. In

particular, we focus mainly on the modification of the guide strand, which has been reported to be more sensitive to chemical modification compared with the sense strand.[10a,19] Because of the importance of the guide strand on the RNAi process, modification of this strand can provide useful information on the RNAi mechanism and on guide siRNA-RISC interactions. However, most of the focus to date has been on modifying the sense strand, which tolerates better chemical modification.[10e] To carry out such study, we first evaluated the effect of the $T^N$ modification on the ability of siRNAs to inhibit the expression of a luciferase gene. In addition, we studied the use of this modification for inhibiting the expression of the murine tumor necrosis factor (TNF-$\alpha$) gene. This research is an ongoing project aimed to the search of anti-inflammatory siRNAs to treat Crohnôs disease. A critical issue in the design of anti-inflammatory siRNAs is the inhibition of innate immune response. In this work, we also demonstrate that RNA to $T^N$ replacement decreases immunostimulation.

Moreover, we evaluated the effect of these substitutions on the thermal and serum stability of the corresponding siRNA duplexes. We also compared the gene silencing activity of two $T^N$-modified siRNAs and their LNA thymine ($T^L$)-modified versions. Our results suggest that the RNAi activity of our $T^N$-modified siRNAs was comparable to that of their $T^L$-modified versions. These properties and the fact that the 2'-deoxy-methanocarba nucleosides have an open 2'-position allows the exploration of chemical diversity at such site to give new, *North* MC-modified siRNAs with yet unexplored therapeutic applications.




*North* methanocarba-thymidine monomer ($T^N$)




LNA-thymidine monomer ($T^L$)

Figure 1. Chemical structures of the conformationally restricted thymidine analogues used in this study

## Results and Discussion

**Design and synthesis of modified guide and sense strands targeting the *Renilla* luciferase gene**

We first designed a large number of 21-mer $T^N$-modified siRNA duplexes targeting the A-rich site 501ï 519 of *Renilla* luciferase mRNA[9d,9e,9g] (Figure 2B). We chose a luciferase model system because it allowed rapid determination of RNAi activity. This sequence allowed us to make a large number of substitutions into the guide strand by using the $T^N$ monomer (replacement of natural U by $T^N$). The *North* 2'-deoxy-MC thymidine monomer was synthesized according to the literature.[17b] This modification could be introduced into RNA without substantial alteration of standard synthesis protocols (see Materials and Methods).

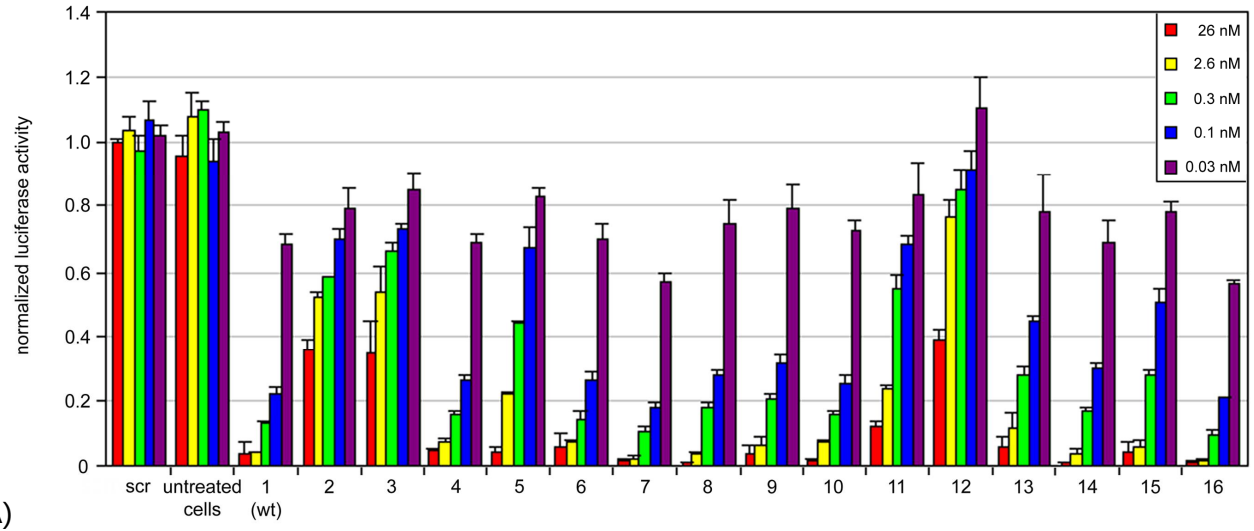
We synthesized siRNAs containing no modifications (**1**), a single $T^N$ substitution at position 1 (**2**), 2 (**4**), 10 (**5**), 11 (**6**), and 20 (**7**) of the guide strand, one modification at both ends of the guide strand (**8**), three modifications on the 5' half (**9**) and three modifications on the 3' half (**10**), six $T^N$ substitutions at spaced out positions along the guide strand (**11**) and eleven $T^N$ substitutions (**12**). We also modified the 3' dinucleotide overhangs to give siRNAs **13** (modification of the guide strand), **14** (modification of the sense strand), and **15** (modification of both strands). Moreover, we prepared siRNA **16**, which contains a single $T^N$ substitution at position 2 of the sense strand, and the 5'-phosphorylated version of siRNA **2** (**3**). Finally, we synthesized LNA-modified versions of siRNAs **7** and **11** (**17** and **18**), in order to compare their RNAi activity.

**Thermal stability of *North* methanocarba thymidine-modified siRNA duplexes**

After RNA synthesis we measured the effects of these substitutions on thermal stability of the siRNA duplexes (Figure 2B). Thermal stability is an important variable to evaluate when considering the value of chemical modifications to siRNA. For example, it may play a substantial role in the stability of the duplex to degradation by nucleases, as formation of thermodynamically stable duplexes may prevent the degradation of the constituent RNA strands by single-stranded nucleases. On the other hand, the level of siRNA duplex stability is also a critical parameter for siRNA activity.[10a] However, it has been suggested that there is a limit to how stable the duplex can be. In the work by Chiu *et al.* it was reported that an excessive thermal duplex stabilization can interfere with siRNA unwinding.[10a]

Figure 2. (A) Plot of gene-specific RNAi activity for $T^N$-modified siRNAs targeting the *Renilla* luciferase mRNA in SH-SY5Y cells. Varied amounts of siRNA were added as shown. (B) Sequences of unmodified, $T^N$-modified and $T^L$-modified siRNAs targeting the *Renilla* luciferase mRNA and $T_m$ data. Top strand depicts the sense strand in the 5ô 3ôdirection (same as the target sequence). Bottom strand depicts the antisense strand in the 3ô 5ôdirection (complementary to the target). $T^N$: *North* methanocarba thymidine monomer, $T^L$: LNA thymidine monomer. Scr: scrambled sequence. (C) Plot comparing the gene silencing activity of *North* methanocarba thymidine-modified siRNAs **7** and **11** and the LNA-modified siRNAs **17** and **18** targeting the *Renilla* luciferase mRNA in SH-SY5Y cells. Untreated cells: cells treated with plasmids alone. Bars indicate standard deviation.

A)




| siRNA | Sequence | $T_m$ (ºC) |
|---|---|---|
| **1** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCUUCUUCAGAUTT | 67.8 |
| **2** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-**T**$^{N}$UUUUCUCCUUCUUCAGAUTT | 68.6 |
| **3** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-p**T**$^{N}$UUUUCUCCUUCUUCAGAUTT | – |
| **4** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-U**T**$^{N}$UUUCUCCUUCUUCAGAUTT | 68.9 |
| **5** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCC**T**$^{N}$UCUUCAGAUTT | 69.7 |
| **6** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCU**T**$^{N}$CUUCAGAUTT | 69.1 |
| **7** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCUUCUUCAGAU**T**$^{N}$T | 67.8 |
| **8** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-U**T**$^{N}$UUUCUCCUUCUUCAGAU**T**$^{N}$T | 68.7 |
| **9** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-U**T**$^{N}$U**T**$^{N}$UC**T**$^{N}$CCUUCUUCAGAUTT | 71.4 |
| **10** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCUUCU**T**$^{N}$CAGA**T**$^{N}$**T**$^{N}$T | 69.7 |

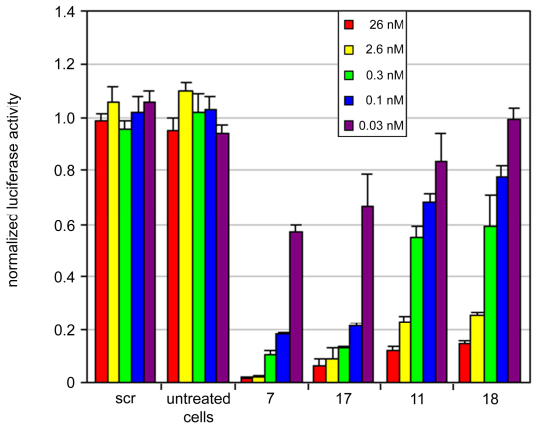
| siRNA | Sequence |
|---|---|
| **11** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-U**T**$^{N}$U**T**$^{N}$UC**T**$^{N}$CCUUCU**T**$^{N}$CAGA**T**$^{N}$**T**$^{N}$T |
| **12** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'U**T**$^{N}$**T**$^{N}$**T**$^{N}$**T**$^{N}$C**T**$^{N}$CC**T**$^{N}$**T**$^{N}$C**T**$^{N}$**T**$^{N}$CAGA**T**$^{N}$**T**$^{N}$T |
| **13** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCUUCUUCAGAU**T**$^{N}$**T**$^{N}$ |
| **14** | **T**$^{N}$**T**$^{N}$AAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCUUCUUCAGAUTT |
| **15** | **T**$^{N}$**T**$^{N}$AAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCUUCUUCAGAU**T**$^{N}$**T**$^{N}$ |
| **16** | TTAAAAAGAGGAAGAAGUC**T**$^{N}$A-5'<br>5'UUUUUCUCCUUCUUCAGAUTT |
| **17** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-UUUUUCUCCUUCUUCAGAU**T**$^{L}$T |
| **18** | TTAAAAAGAGGAAGAAGUCUA-5'<br>5'-U**T**$^{L}$U**T**$^{L}$UC**T**$^{L}$CCUUCU**T**$^{L}$CAGA**T**$^{L}$**T**$^{L}$T |
| **scr** | TTAGGAAAGAAAGAAAGCUAU-5'<br>5'-UCCUUUCUUUCUUUCGAUATT |

Compared to the unmodified RNA sequence (**1**), all substituted oligoribonucleotides containing T$^{N}$ substitutions at base-paired positions had greater duplex stability (as evaluated by higher $T_m$ values) when hybridized to their RNA complement (see data in Figure 2B). The incorporation of *North* 2'-deoxy-MC thymidine residues resulted in an increase of 0.8ï 2 ºC per modification depending on the position of the pseudonucleoside. Such stabilizing effect was found to be additive. This is in agreement with the previously reported thermodynamic stability of T$^{N}$-modified ODN/RNA heteroduplexes.[18a,18c] The rank order of duplex $T_m$ was: eleven T$^{N}$ (**12**) > six T$^{N}$ (**11**) > three T$^{N}$ on the 5' half (**9**) > three T$^{N}$ on the 3' half (**10**) ~ one T$^{N}$ at position 10 of the guide strand (**5**) > one T$^{N}$ at position 11 (**6**) > one T$^{N}$ at position 2 of the guide strand (**4** and **8**) ~ one T$^{N}$ at position 2 of the sense strand (**16**) and one T$^{N}$ at position 1 of the guide strand (**2**).

A singly modified LNA-siRNA duplex was as stable as the corresponding T$^{N}$-siRNA duplex (**17** and **7**) while an LNA-siRNA with multiple substitutions was more stable than the corresponding T$^{N}$-siRNA

duplex [$T_m$ = 79.7 ºC for siRNA **18** (containing six $T^L$ residues) versus $T_m$ = 72.8 ºC for its $T^N$-modified version (**11**)].

Figure 2. (C) Plot comparing the gene silencing activity of *North* methanocarba thymidine-modified siRNAs **7** and **11** and the LNA-modified siRNAs **17** and **18** targeting the *Renilla* luciferase mRNA in SH-SY5Y cells. Untreated cells: cells treated with plasmids alone. Bars indicate standard deviation




## CD spectra of $T^N$-modified siRNA duplexes

We determined CD spectra of selected siRNAs [siRNAs containing no modifications (**1**), one $T^N$ (**5**), two $T^N$ (**8**), three $T^N$ (**9**), six $T^N$ (**11**) and eleven $T^N$ (**12**)]. The spectra clearly indicate that the overall conformation of a typical A-form double helical geometry is retained in the $T^N$-modified siRNAs (see the Supplementary Figure S5).

## RNAi activity of $T^N$-modified siRNAs targeting *Renilla* luciferase gene

RNAi studies in SH-SY5Y cells with siRNAs containing each of the eleven $T^N$-modified guide strands (**2**-**12**), siRNAs modified at the 3' dinucleotide overhangs (**13**-**15**), siRNA **16** (containing a single $T^N$ substitution into the sense strand), as well as with the unmodified (wild type) RNA (**1**), were performed. Experiments were carried out in triplicate. The cells were first transfected with dual reporter plasmids that express *Renilla* luciferase (the target) and non-targeted firefly luciferase as an internal control. The effects of the different RNAs on luciferase expression were evaluated after dosing with 0.21-210 ng (0.03-26 nM) of double stranded RNA in the cell media, and measuring luminiscence responses after 22 h. The results, showing *Renilla* luciferase activity normalized to firefly luciferase, are represented in Figure 2A.

In a first series of experiments, we compared the positional effect of the $T^N$ modification on RNAi by making single RNA to $T^N$ exchanges at relevant base-paired positions in the guide strand (siRNAs **2**-**7**, Figure 2B). It has been suggested that the position 1 of the guide strand plays a critical role for siRNA function and is more sensitive to chemical modification. Compared to the unmodified or wild type siRNA (**1**), incorporation of one $T^N$ residue on the 5' end of the guide strand (position 1, siRNA **2**) caused a significant decrease in luciferase activity at the highest siRNA concentration (26 nM). It is known that a

5' phosphate is crucial for siRNA function; therefore, to exclude the possibility that the 5'-modified siRNA **2** was poorly recognized by cellular kinases resulting in loss activity due to the lack of a 5'-phosphate, we synthesized the 5'-phosphorylated version of siRNA **2** (**3**). However, this procedure did not recover any of the lost effect (Figure 2A). In contrast to what was observed for compound **2**, incorporation of a $T^N$ residue at position 2 of the guide strand (**4**) gave rise to a gene silencing activity comparable to that of wild type siRNA (**1**).

Next, we examined the effect of incorporating a $T^N$ residue at position 10 of the guide strand (**5**). It is known that this position plays an important role for the siRNA function too since mRNA cleavage occurs between position 10 and 11 of the guide strand counting from the 5' end. When we incorporated a $T^N$ residue at position 10 of the guide strand we observed a decrease in gene silencing activity. However, the resulting modified siRNA (**5**) retained significant activity (at the lowest siRNA concentration (0.03 nM), *Renilla* luciferase expression was ~83% for **5** and ~68% for wild type siRNA (**1**)). In contrast, RNA to $T^N$ replacement at position 11 did not disrupt RNAi activity. siRNA **6** displayed gene silencing activity comparable to that observed for wild type siRNA (**1**). Moreover, guide siRNA having one $T^N$ substitution at position 20 (**7**) displayed slightly better activity than that observed for unmodified siRNA **1**. The most significant differences were observed when very low concentrations (0.03 nM) of siRNAs were employed (significant differences were assessed by ANOVA to compare three or more groups followed by Bonferroni test (see data in Supplementary Figure S1A)). At the lowest siRNA concentration (0.03 nM), 44% ± 3 gene knockdown for **7** versus 32% ± 4 for **1** ($p < 0.05$). It is noteworthy that, compared to the 5'-modified siRNA **4** ($T^N$ at position 2), this 3'-modified siRNA (**7**) displayed slightly better activity.

Since the above described experiments suggest that, with a few exceptions, siRNAs containing a single substitution on the guide strand could effectively mediate RNAi activity to levels comparable to the wild type siRNA, it was of interest to study the effect of multiple substitutions on RNAi activity. We first incorporated one $T^N$ substitution at both ends of the guide strand (positions 2 and 20, siRNA **8**). The resulting disubstituted siRNA displayed RNAi activity similar to that observed for the wild type siRNA (**1**). On the other hand, incorporation of three $T^N$ residues on the 5' half (positions 2, 4, and 7, siRNA **9**) caused a slight loss of the interference activity. Although the resulting modified siRNA (**9**) was significantly active, it showed decreased activity as compared with the siRNA containing two $T^N$ residues (**8**). However, incorporation of three substitutions on the 3' half (positions 14, 19 and 20, siRNA **10**) recovered all the lost effect. siRNA **10** displayed gene silencing activity slightly better than that of siRNA **9** and comparable to that of the wild type siRNA (**1**) (Bonferroni test, $p < 0.05$; see the Supplementary Figure S1B) (at an siRNA concentration of 0.03 nM, *Renilla* luciferase gene silencing was 25% ± 7 for **8**, 20% ± 8 for **9**, 28% ± 3 for **10** and 32 % ± 4 for **1**). These and the above-described effects (for siRNAs **4** and **7**, containing a single $T^N$ substitution at the 5'-end and at the 3'- end, respectively) support the idea that the sequence structure within the 5' region of the antisense strand is more sensitive to chemical modification than in the 3' region.[10a] This is also consistent with previous observations that incorporation of 2'-FU, 2'-FC, dAs and dGs residues predominantly on the 5' half of a

siRNA caused a significant loss in gene silencing activity, whereas siRNAs containing 2'-FU, 2'-FC, dAs and dGs modifications predominantly on the 3' half retained a significant level of gene silencing.[10a]

Having demonstrated that three $T^N$ substitutions can be tolerated by the RNAi machinery, we studied the effect of a higher number of substitutions in the guide strand. For siRNA containing six $T^N$ substitutions at spaced-out positions along the guide strand (**11**), gene silencing activity was significantly less efficient than that observed for wild type, disubstituted and tribustituted siRNAs (**1**, **8**, **9** and **10**, respectively). However, despite this loss in activity, siRNA **11** retained significant inhibitory effect at the highest dose of 26 nM (~12% *Renilla* luciferase expression for **11** versus ~4% for **1**).

In order to ensure that the loss in activity observed for double stranded **11** was due to the presence of excessive modifications we incorporated eleven $T^N$ residues in the antisense strand (**12**). As expected, fully substituted siRNA **12** disrupted *Renilla* luciferase-specific RNAi activity. Incorporation of eleven $T^N$ substitutions within the guide strand caused loss of most of the interference activity even at the highest siRNA concentration (26 nM).

The above described results indicate that in general, incorporation of one to three nucleotides in the guide strand is well tolerated by the RNAi machinery. However, the *North* 2'-deoxy-MC modification is not tolerated when oligoribonucletides contain more than six substitutions. On the other hand, introduction of $T^N$ modifications in the 3' overhangs in the antisense (**13**), the sense (**14**) or both strands (**15**) led to similar activity (Figure 2A and Figure S1C).

In the report by Elmén *et al.* it was suggested that introduction of a single LNA modification at the 5'-terminal end (position 1) of the sense strand allows preferential incorporation of the guide strand into RISC, thereby improving gene silencing efficiency and reducing the sense strand-specific off- targeting activity.[10e] Although *North* 2'-deoxy-MC nucleosides and LNAs are different in structure, both of them possess conformationally restricted ring systems and are bridged bicyclic compounds. In order to study the effect of the modification of the sense strand with a $T^N$ residue we chose the neighboring position (position 2) (siRNA **16**, Figure 2B). Interestingly, the resulting siRNA (**16**) displayed slightly better activity than that of the unmodified siRNA **1** (Figure 2A and Supplementary Figure S1D; Bonferroni test, $p < 0.05$). At an siRNA concentration of 0.03 nM, 44% ± 1 of gene knockdown was observed for **16** versus 32% ± 4 gene knockdown for **1**. Gene silencing was comparable to that observed for the 3'-modified siRNA **7**, which contains a single $T^N$ substitution at position 20 of the guide strand.

To ensure the specificity of the above-described effects, sequence-scrambled siRNAs (as negative controls) were used. Cells co-transfected with scrambled versions of the unmodified siRNA **1** (scr) and scrambled versions of two of the more active chemically modified siRNAs (**10** and **16**) displayed *Renilla* luciferase activity similar to those of cells transfected with plasmids alone (see data in Figure 2A and in the Supplementary Figures S3 and S4). These results indicate that the siRNAs used in this study specifically induce inhibition of expression of the target gene.

Finally, we compared the RNAi activity of one of the most active and one of the less active $T^N$-modified siRNAs (**7** and **11**, respectively) versus their LNA-modified versions (siRNA **17**, which contains

a single LNA thymine substitution ($T^L$) at position 20 of the guide strand, and siRNA **18**, which contains six $T^L$ residues at spaced out position along the guide strand). The results are represented in Figure 2C. Interestingly, SH-SY5Y cells co-transfected with $T^N$-modified siRNAs **7** and **11**, and plasmids encoding *Renilla* and firefly luciferase genes showed gene silencing activity comparable to that of cells transfected with their LNA-modified versions (siRNAs **17** and **18**, respectively) and the luciferase plasmids.

**RNAi activity of $T^N$-modified siRNAs targeting murineTNF-$\alpha$**

TNF-$\alpha$ factor (a pro-inflammatory cytokine) is a major mediator of apoptosis, inflammation and immunity. Furthermore, TNF-$\alpha$ has been implicated in the pathogenesis of many human diseases; for example, Crohnôs disease, which is an inflammatory disorder. Thus, efficient knockdown of this factor could have beneficial consequences on human health. Our group has been interested in the development of anti-inflammatory siRNAs to treat Crohnôs disease. In recent studies we have developed new delivery systems which involved conjugation of lipids and peptides to the ends of an siRNA targeting murine TNF-$\alpha$ mRNA.[20] On the other hand, this work is aimed at increasing the binding affinity and nuclease stabillty of these siRNAs. For these reasons, the effect of $T^N$-modified siRNAs on the inhibition of expression of murine TNF-$\alpha$ gene were examined.

Two of the most promising modified siRNA designs, corresponding to siRNAs **7** and **16** were selected from our previous luciferase gene silencing results and used for targeting murine TNF-$\alpha$ mRNA in RAW cells (murine peritoneal macrophages) using a previously reported sequence [20] (see the sequence in Figure 3A). We synthesized unmodified siRNA **19** and $T^N$-modified siRNAs **20** and **21** targeting murine TNF-$\alpha$ (Figure 3A), which contained $T^N$ modifications at the same positions as siRNAs **7** and **16** [one $T^N$ substitution at position 20 of the guide strand (**20**) and one $T^N$ substitution at position 2 of the sense strand (**21**)]. Moreover, we synthesized two disubstituted siRNAs targeting murine TNF-$\alpha$, one of them (**22**) containing $T^N$ modifications at positions 6 and 20 of the guide strand, and the second one (**23**) containing $T^N$ modifications at positions 2 and 18 of the sense strand. Levels of TNF-$\alpha$ protein after treating the cells with siRNAs (100 nM concentration per well) were analyzed by enzyme-linked immunoabsorbent assay (ELISA) (Figure 3B). Gene silencing experiments were carried out in triplicate and included controls in which cells were treated with a non-targeting, scrambled siRNA (scr), which was selected from our luciferase siRNA assays. Disubstituted siRNAs **22** and **23** were the least potent of the set but still demonstrated gene silencing activity. On the other hand, monosubstituted siRNAs **20** and **21**, which contain the $T^N$ modification at the same positions as the most active siRNAs targeting *Renilla* luciferase, silenced the expression of murine TNF-$\alpha$, with activities comparable to the unmodified siRNA (**19**). These results suggest that the most effective patterns of modification that emerged from the *Renilla* luciferase studies (which involved one $T^N$ substitution) could be used to target

a wide variety of mRNA targets, while two $T^N$ substitutions seem to be less tolerated by this endogenous gene.

A)

| siRNA | Sequence |
|---|---|
| **19** | TTCUCCGACUCUGUAUCCGUG-5'<br>5'-GAGGCUGAGACAUAGGCACTT |
| **20** | TTCUCCGACUCUGUAUCCGUG-5'<br>5'-GAGGCUGAGACAUAGGCAC**T**$^N$T |
| **21** | TTCUCCGACUCUGUAUCCG**T**$^N$G-5'<br>5'-GAGGCUGAGACAUAGGCACTT |
| **22** | TTCUCCGACUCUGUAUCCGUG-5'<br>5'-GAGGC**T**$^N$GAGACAUAGGCAC**T**$^N$T |
| **23** | TTC**T**$^N$CCGACUCUGUAUCCG**T**$^N$G-5'<br>5'-GAGGCUGAGACAUAGGCACTT |

B)

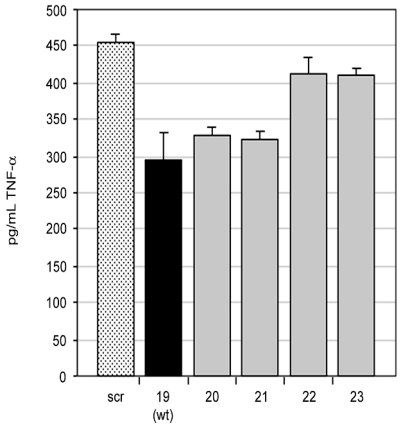



Figure 3. (A) Sequences of unmodified (**19**) and $T^N$-modified (**20**, **21**, **22** and **23**) siRNAs targeting murine TNF-α mRNA. Top strand sepicts the sense strand in the 5ô3ô direction (complementary to the target). (B) Plot showing the amount of TNF-α produced after 30 h of transfection of 100 nM unmodifed and $T^N$-modified siRNAs targeting the murine TNF-α mRNA in RAW cells (murine peritoneal macrophages). Bars indicate standard deviation

**Immunostimulation**

It is known that siRNAs can induce strong interferon-α (IFN-α) responses in human peripheral blood mononuclear cells (PBMC).[8] In order to investigate if $T^N$-modified siRNAs can reduce the undesired immunostimulatory activity in PBMCs, we treated human PBMCs with unmodified siRNA 19 (which is known to have immunostimulatory activity in human PBMCs) and with the $T^N$-modified siRNAs that had been used for targeting TNF-α in murine peritoneal macrophages (20, 21, 22 and 23) at a 10 nM siRNA concentration by using DOTAP as the delivery system. Immunostimulated PBMC produce an array of cytokines including hTNF-α, IFN-α and IL-6 after siRNA transfection, from which hTNF-α was selected and measured 24h after transfection as indicator of immunomodulation in our experiments (Figure 4). It is important to mention that murine anti-TNF-α siRNAs 19-23 do not inhibit expression of hTNF-α. Treatment of human PBMCs with siRNA 20 resulted in immunostimulation levels comparable to those obtained for unmodified siRNA 19. However, incorporation of one $T^N$ substitution at position 2 of the sense strand (siRNA 21) and two substitutions at positions 6 and 20 of the guide strand (siRNA 22) lead to immunostimulation levels significantly lower than those obtained for unmmodified siRNA (19). Production of hTNF-α (pg/mL) was 299 ± 18 for 21 and 286 ± 33 for 22, versus 390 ± 35 for 19; Bonferroni test, $p < 0.05$). Moreover, sense-disubstituted siRNA 23 lead to immunostimulation levels even lower than those obtained for siRNAs 21 and 22 (217 ± 33 pg/mL hTNF-α for 23 versus 390 ± 35 pg/mL hTNF-α for 19; $p < 0.005$). These results suggest that in siRNA $T^N$ replacement along the guide or sense strand reduce induction of hTNF-α in PBMCs and that this effect depends on the position and the number of $T^N$ substitutions.

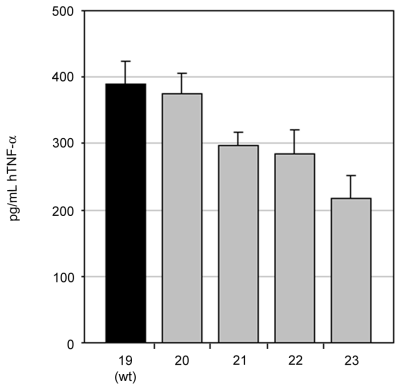



Figure 4. hTNF-α levels in human PBMC cells 24 h after treatment with unmodified and $T^N$-modified siRNAs (10 nM) transfected with DOTAP. hTNF-α levels were measured using an enzyme-linked immunoabsorvent assay (ELISA kit). hTNF-α levels in response to unmodified siRNA are shown in black and $T^N$-modified siRNAs in grey.

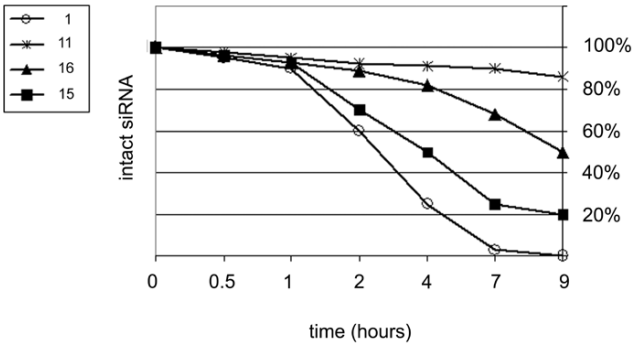
## Serum stability of *North* 2ô-deoxy-MC thymine-modified siRNAs

Since the above described experiments showed that most of the $T^N$-modified siRNAs that were tested could effectively mediate RNAi to levels comparable to wild type siRNA, and that the $T^N$ modification can be used to increase the thermal stability of siRNA duplexes, it was of interest to study the serum stability of these oligonucleotides. To carry out such studies, unmodified or modified double stranded RNAs were incubated in 50% human serum. At various time points, siRNAs were extracted, analyzed on a 14% polyacrylamide gel under denaturing conditions and visualized by staining. Here we describe the most representative examples: unmodified oligoribonucleotide **1** and siRNAs **11**, **15** and **16** (Figure 5 and Supplementary Figure S2). SiRNA **15** was designed to enhance exonuclease resistance (it contains $T^N$ modifications at both 3' ends). SiRNA **11**, which contains five substitutions at base-paired positions in the guide strand, was designed to increase the thermal stability of the duplex. Finally, siRNA **16** (one $T^N$ base-paired substitution in the sense strand) is one of the most active oligonucleotides.

Compared to unmodified siRNA **1**, the $T^N$-modified siRNAs displayed higher serum stability. As shown in Figure 5 and in Supplementary Figure S2, unmodified siRNA (**1**) was rapidly degraded, with 27%, 4% and 0% of the original siRNA remaining intact through 4, 7 and 9 h, respectively. Modification of both 3' dinucleotide ends of the siRNA (siRNA **15**) gave rise to an increase in the serum stability of the duplex, with 52%, 25% and 20% of the original siRNA remaining intact through 4, 7 and 9 h, respectively. Similar results were obtained for siRNAs containing a single $T^N$ substitutions at position 20 of the guide strand and modifications in the 3' dinucleotide overhang in either strands of the duplex (siRNAs **7**, **13** and **14**, respectively) (data not shown). On the other hand, double stranded siRNA with one $T^N$ residue at position 2 of the sense strand (**16**) demonstrated a significant increase in stability over the course of the experiment, with 50% of the original siRNA remaining intact through 9 h. Interestingly, siRNA containing six $T^N$ substitutions (**11**) displayed strongly enhanced stability, with 84% of the siRNA population remaining intact after 9 h. The original siRNA was completely degraded after 48 h in serum (see the Supplementary Figure S2). Similar results were obtained for siRNA **12**, which contains eleven $T^N$ substitutions in the guide strand (data not shown).

Thus, we can conclude that the $T^N$ modification confers a significant stabilization of siRNAs in serum.

Figure 5. Serum stability of unmodified and $T^N$-modified siRNAs. The different siRNA were incubated in 50% human serum at 37 ºC and withdrawn at indicated time points. Plot of the remaining intact siRNA (%) over incubation time.




## Conclusion

In this work we have demonstrated that the *North* 2'-deoxy-MC substitution is substantially compatible with the RNAi machinery. The gene silencing activity of the corresponding modified siRNAs depends on the position and the number of the substitutions. We have shown that incorporation of one to three substitutions into the guide strand is, in general, well tolerated in a luciferase system. In contrast to this, a greater number of substitutions are detrimental to activity, despite the enhanced serum and thermal stability of the corresponding modified siRNAs.

On the other hand, the $T^N$-modified siRNAs that were compared with the LNA thymidine modification showed similar activity (siRNAs **7** and **11** versus siRNAs **17** and **18**; see data in Figure 2C). Thus, our results suggest that the effect on RNAi activity caused by the *North* 2'-deoxy-MC modification might be similar to that observed for the LNA substitution.

Moreover, the $T^N$ modification confers resistance of siRNAs against nucleases and such stabilizing effect increases with the number of substitutions.

Finally, the *North* 2'-deoxy-MC can also be used in the design of anti-inflammatory siRNAs targeting murine TNF-$\alpha$. In this case, innate immunostimulation effects are clearly reduced by the introduction of a few units of this pseudonucleoside. This result is in agreement with the general observation that modifications at the carbohydrate moiety may reduce immunostimulation.[21]

In summary, from this work we can conclude that the effect of the *North* 2'-deoxy-MC modification on RNAi is similar to that of LNA and therefore *North* 2'-deoxy-MC nucleosides are promising candidates for developing new RNA agents for biomedical applications of siRNAs. Moreover, alteration of the 2'-position of the pseudosugar with different functional groups may offer an avenue for the development of new potentially active MC-modified siRNAs with groups that may even reach beyond the 2'-OH.

In these studies we have demonstrated for the first time that siRNAs carrying a constrained cyclopentane ring embedded in a bicyclo[3.1.0]hexane system can induce the RNAi process. These results are in agreement with previous studies reported by Hoshika *et al*.[22] which suggested that the oxygen atom of the ribose moiety was not necessary and that it could be replaced by a sulfur atom. This means that replacement of this oxygen by sulfur or a carbon might yield novel modified siRNAs with improved properties as long as the *North* sugar pucker is conserved.

## Experimental Section

**Nucleoside phosphoramidite synthesis:** The 5'-*O*-DMT protected 2'-deoxy-MC thymidine 3'-*O*-phosphoramidite was prepared as described. [23]

**CPG functionalization:** The polymer support was functionalized with *North* 2'-deoxy-MC thymidine as described for natural nucleosides. [24]

*Step I* (preparation of 5'-*O*-DMT-protected *North* 2'-deoxy-MC thymidine-3'-*O*-succinate): To a solution of 5'-*O*-DMT-protected *North* 2'-deoxy-MC thymidine[18a,24] (1 mmol) in dichloromethane (0.2M), succinic anhydride (1.3 mmol), diisopropylethylamine (1.4 mmol) and dimethylaminopyridine (0.1 equiv) were added. The resulting solution was stirred for 24 h at room temperature and then washed with 1% dihydrogen phosphate. The aqueous layer was extracted with $CH_2Cl_2$ and the organic layer was dried with $MgSO_4$ and evaporated.

*Step II*: 2,2'-Dithio-bis-(5-nitropyridine) (0.1 mmol) dissolved in 400 µL of a mixture of acetonitrile-dichloroethane (1:3) was mixed with a solution of 5'-*O*-DMT-protected *North* 2'-deoxy-MC thymidine-3'-*O*-succinate (0.1 mmol) and DMAP (0.1 mmol) in acetonitrile (500 µL). The clear solution that was obtained was added to a solution of triphenylphosphine (0.1 mmol) in acetonitrile (200 µL) at room temperature. The mixture was vortexed for few seconds and then added to a vial containing CPG (500 Å, 500 mg) and allowed to react 30 min at room temperature. Then, 500 µL of methanol were added and the support was recovered on a sintered glass funnel followed by washings with methanol (2 x 5 mL) and diethyl ether (2 x 5 mL). The support was air dried and then placed under high vacuum. The support was subjected to capping following the standard protocol.[25] The *North* 2'-deoxy-MC thymidine loading on the derivatized support was determined by the acid treatment method.[25]

**RNA synthesis and purification methods:** RNA oligonucleotides were synthesized on the 0.2 µmol scale on an Applied Biosystems 394 synthesizer using 2'-*O*-TBDMS protected phosphoramidites. Acetonitrile (synthesis grade) and the 2'-*O*-TBDMS protected phosphoramidite monomers of A, C, G and U were from commercial suppliers. Tetrazole was used as activator. The coupling time was 15 min. P (III) to P (V) oxidation was performed with a solution of 10% *tert*-butyl hydroperoxide in acetonitrile/water (96:4) with a 10 min oxidation time. Chemical phosphorylation reagent procured from

Link Technologies was used to phosphorylate the 5' end of a modified oligonucleotide. All oligonucleotides were synthesized in DMT-on mode. After the solid-phase synthesis, the solid support was transferred to a screw-cap glass vial and incubated at 55 ºC for 1 h with 1.5 mL of $NH_3$ solution (33%) and 0.5 mL of ethanol. The vial was then cooled on ice and the supernatant was transferred into a 2 mL eppendorf tube. The solid support and vial were rinsed with 50% ethanol (2 x 0.25 mL). The combined solutions were evaporated to dryness using an evaporating centrifuge. The residue that was obtained was dissolved in a total volume of 85 µL of 1M TBAF in THF and rocked at room temperature for 12 h. Then, 85 µL of 1M triethylammonium acetate and 330 µL of water were added to the solution. The oligonucleotide was desalted on a NAP-5 column using water as the eluent and evaporated to dryness. The oligonucleotide was purified by high-performance liquid chromatography (HPLC; DMT-on). The HPLC conditions were as follows: column: PRP-1 (Hamilton) 250 x 10 mm; solvent A = 5% acetonitrile in 100 mM triethylammonium acetate, pH 6.5; solvent B = 70% acetonitrile in 100 mM triethylammonium acetate pH 6.5; gradient: 0ï 30 min, from 15 to 100% in B with UV detection at 260 nm. The pure fractions were combined and evaporated to dryness. The residue that was obtained was treated with 1 mL of 80% AcOH solution and incubated at room temperature for 30 min. The deprotected oligonucleotide was desalted on a NAP-10 column using water as the eluent. All oligonucleotides were quantified by absorption at 260 nm and confirmed by MALDI mass spectrometry (see data in Supplementary Table S1).

**UV-monitored thermal denaturation studies:** Absorbance versus temperature curves of duplexes were measured at 1 µM strand concentration in 15 mM HEPES-KOH (pH 7.4), 1 mM $MgCl_2$ and 50 mM KOAc buffer. Experiments were performed in Teflon-stoppered 1 cm path lenght quartz cells on a JACSO V-650 spectrophotometer equipped with a thermoprogrammer. The samples were heated to 90 ºC, allowed to slowly cool to 25 ºC, and then warmed during the denaturation experiments at a rate of 1 ºC/min to 85 ºC, while monitoring absorbance at 260 nm. The data were analyzed by the denaturation curve processing program, MeltWin v. 3.0. Melting temperatures ($T_m$) were determined by computerfit of the first derivative of absorbance with respect to 1/$T$.

**CD measurements**

CD spectra were recorded on a Jasco J-810 spectropolarimeter equipped with a Julabo F/25HD temperature controller, using the same buffer conditions and oligonucleotide concentrations as for UV melting curves. All spectra were recorded at room temperature between 220 to 320 nm, using a 100 nm/min scan rate. The graphs were analyzed using Origin software.

**Luciferase siRNA assays:** SH-SY5Y cells were grown at 37 ºC in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and

100 µg/mL streptomycin. Cells were regularly passaged to maintain exponential growth. Twenty-four hours before transfection at 50ï 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1ï 3 x $10^5$ cells/mL) and transferred to 24-well plates (500 µL per well). Two luciferase plasmids, *Renilla* luciferase (pRL-TK) and firefly luciferase (pGL3) from Promega, were used as a reporter and control, respectively. Cotransfection of plasmids and siRNAs was carried out with Lipofectamine 2000 (Life Technologies) as described by the manufacturer for adherent cell lines; 1.0 µg pGL3-Control, 0.1 µg pRL-TK and 0.03ï 26 nM siRNA duplex formulated into liposomes were added into each well with a final volume of 600 µL. The cells were harvested 22 h after transfection and lysed using passive lysis buffer (100 µL per well) according to the instructions of the Dual-Luciferase Reporter Assay System (Promega). The luciferase activities of the samples were measured using a MicroLuma*Plus* LB 96V (Berthold Technologies) with a delay time of 2 s and an integrate time of 10 s. The following volumes were used: 20 µL of sample and 30 µL of each reagent (Luciferase Assay Reagent II and Stop and Glo Reagent). The inhibitory effects generated by siRNAs were expressed as normalized ratios between the activities of the reporter (*Renilla*) luciferase gene and the control (firefly) luciferase gene.

**TNF-α siRNA assays**: siRNA inhibition experiments with the endogeneous target murine TNF-α were performed in RAW 264.7 cells (murine peritoneal macrophages). RAW 264.7 cells were grown at 37 ºC in Dulbeccoûs modified Eagleûs medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and 10% glutamine. Cells plated a 40-60% confluence in 96-well plates were transfected using DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) at an siRNA concentration of 100 nM according to the manufacturerûs protocol. Experiments were carried out in triplicate. 20 h after transfection, the cells were stimulated with LPS (lipopolysaccharide of gram-negative bacteria) (10 ng/mL) for 10 hours and the amount of murine TNF-α produced by the cells (contained in the supernatants from cells) was analyzed by enzyme-linked immunoabsorbent assay (ELISA).

**Cell stimulation and hTNF-α detection:** PBMC cells were obtained from human blood using Ficoll density gradient separation. Enriched monocyte populations were isolated by plastic adherence. After three hours incubation at 37ºC, cells were transfected with 10 nM of siRNAs for 18 hours using DOTAP (Roche) following the manufacturerûs instructions. Culture supernatants were then collected and hTNF-Ŭ production upon immunostimulation was assessed by ELISA (Bender MedSystems). Experiments were carried out in triplicate

**Statistical analysis:** Data were analyzed by GraphPad Prism 5 program (GraphPad Software). Significant differences were assessed by ANOVA to compare three or more groups followed by Bonferroni test. In all figures, * represents *p* values <0.05.

**Stability assays in 50% human serum:** Unmodified or modified double-stranded siRNA samples (20 µM; 24 µL) were incubated in human serum (24 µL) at 37 ºC. At appropriate periods (0, 0.5, 1, 2, 4, 7 and 9 hours), 6 µL aliquots of the reaction mixture were added to 54 µL of a 1% sodium dodecyl sulphate aqueous solution and the mixtures were heated-denatured for 5 min at 90 ºC. SiRNAs were isolated by hot phenol extraction followed by ethanol precipitation. After re-suspension in 20 µL of loading buffer (90% formamide, 10% 1X TBE), the samples were run on a denaturing 14% polyacrylamide gel containing 20% formamide. RNA bands were visualized with the SYBR Green II reagent (Sigma-Aldrich) according to the manufacturer's instructions.

***Acknowledgements***

This research was supported by the European Communities (MULTIFUN consortium), the Spanish Ministry of Education (BFU2007-63287, CTQ2010-20541) and the Generalitat the Catalunya (2009/SGR/208). This work was funded in part by the Center for Cancer Research, National Cancer Institute, NIH. M. T. acknowledges the JAE-Doc 2008 contract (CSIC, Spain) and the Juan de la Cierva contract (MICINN, Spain) for financial support.

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**Supplementary Data**

# Effect of *North* bicyclo[3.1.0]hexane 2'-deoxy-pseudosugars on RNA interference. A novel class of siRNA modification

Montserrat Terrazas[1], Sandra M. Ocampo[1], José Carlos Perales[2], Victor E. Marquez[3], and Ramon Eritja[1,*]

**CONTENTS:**

**Table S1.** MALDI-TOF mass spectra of synthesized oligonucleotides.

| Oligonucleotide | Sequence | MW calcd. | MW found |
|---|---|---|---|
| ss (1) | 3'-TTAAAAAGAGGAAGAAGUCUA-5' | 6790.0 | 6787.6 |
| as (1) | 5'-UUUUUCUCCUUCUUCAGAUTT-3' | 6423.0 | 6426.4 |
| 2 | 5'-$T^N$UUUUCUCCUUCUUCAGAUTT-3' | 6429.4 | 6427.8 |
| 3 | 5'-p$T^N$UUUUCUCCUUCUUCAGAUTT-3' | 6508.0 | 6513.3 |
| 4 | 5'- U$T^N$UUUCUCCUUCUUCAGAUTT -3' | 6429.5 | 6440.1 |
| 5 | 5'- UUUUUCUCC$T^N$UCUUCAGAUTT-3' | 6429.5 | 6435.9 |
| 6 | 5'- UUUUUCUCCU$T^N$CUUCAGAUTT -3' | 6429.5 | 6437.6 |
| 7 | 5'- UUUUUCUCCUUCUUCAGAU$T^N$T -3' | 6431.3 | 6437.7 |
| 8 | 5'- U$T^N$UUUCUCCUUCUUCAGAU$T^N$T -3' | 6439.5 | 6447.3 |
| 9 | 5'- U$T^N$U$T^N$UC$T^N$CCUUCUUCAGAUTT -3' | 6445.9 | 6456.4 |
| 10 | 5'- UUUUUCUCCUUCU$T^N$CAGA$T^N$$T^N$T -3' | 6447.6 | 6454.3 |
| 11 | 5'- U$T^N$U$T^N$UC$T^N$CCUUCU$T^N$CAGA$T^N$$T^N$T -3' | 6472.4 | 6480.8 |
| 12 | 5'-U$T^N$$T^N$$T^N$$T^N$C$T^N$CC$T^N$$T^N$C$T^N$$T^N$CAGA$T^N$$T^N$T-3' | 6513.4 | 6528.5 |
| 13 | 5'- UUUUUCUCCUUCUUCAGAU$T^N$$T^N$-3' | 6441.4 | 6442.7 |
| 14 | 5'-$T^N$$T^N$AAAAAGAGGAAGAAGUCUA-3' | 6809.3 | 6807.4 |
| 16 | 3'-TTAAAAAGAGGAAGAAGUC$T^N$A-5' | 6797.5 | 6802.7 |
| 17 | 5'-UUUUUCUCCUUCUUCAGAU$T^L$T-3' | 6449.3 | 6450.1 |
| 18 | 5'- U$T^L$U$T^L$UC$T^L$CCUUCU$T^L$CAGA$T^L$$T^L$T -3' | 6435.1 (+Na) | 6451.9 |
| scr-ss (1) | 3'-TTAGGAAAGAAAGAAAGCUAU-5' | 6790 | 6796.5 |
| scr-as (1) | 5'-UCCUUUCUUUCUUUCGAUATT-3' | 6421.3 | 6425.4 |
| scr-10 | 5'-UCCUUUC$T^N$UUCUUUCGA$T^N$A$T^N$T-3' | 6447.6 | 6453.2 |
| scr-16 | 3'-TTAGGAAAGAAAGAAAGC$T^N$AU-5' | 6790.5 (+Na) | 6790.3 |
| 20 | 5'- GAGGCUGAGACAUAGGCAC$T^N$T -3' | 6782.4 | 6779.4 |
| 21 | 3'-TTCUCCGACUCUGUAUCCGU$T^N$G-5' | 6567.9 | 6573.2 |

**Figure S1.** Separate gene silencing activities for unmodified and modified siRNAs targeting the *Renilla* luciferase mRNA in SH-SY5Y cells. SH-SY5Y cells were transfected with dual reporter plasmids that express *Renilla* luciferase (the target) and nontargeted firefly luciferase as an internal nontargeted control and with siRNAs (0.03 nM per well) containing one $T^N$ substitution (**4**, **6** and **7**) (A), two and three $T^N$ substitutions (**8**, **9** and **10**) (B), two $T^N$ substitution at the 3'-dinucleotide overhangs (**13**, **14** and **15**) (C), and with the most active siRNAs (**7** and **16**) (D), and compared with the unmodified counterpart (**1**). Normalized *Renilla* luciferase activity is shown. Experiments were carried out in triplicate. Bars indicate standard deviation. A Bonferroni test was conducted to evaluate $T^N$ modifications to the unmodified control (**1**). In all figures, * indicates a significant change in *Renilla* luciferase expression from unmodified siRNA **1** ($p<0.05$).

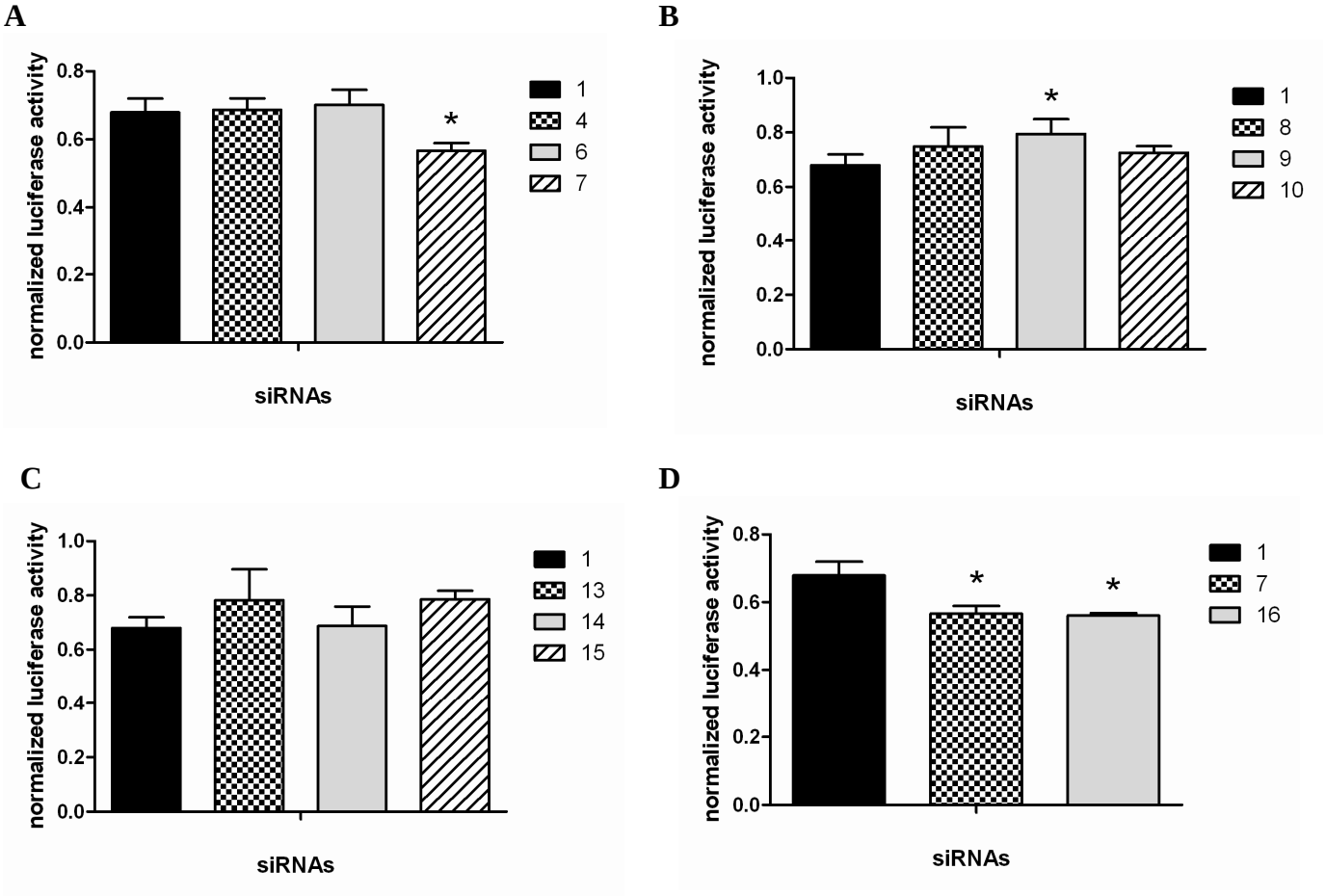

**Figure S2.** Serum stability of unmodified and $T^N$-modified siRNAs. The siRNAs were incubated in 50% human serum at 37 ºC and withdrawn at indicated time points. The RNAs were separated by polyacrylamide gel containing formamide and visualized with SYBR green II.

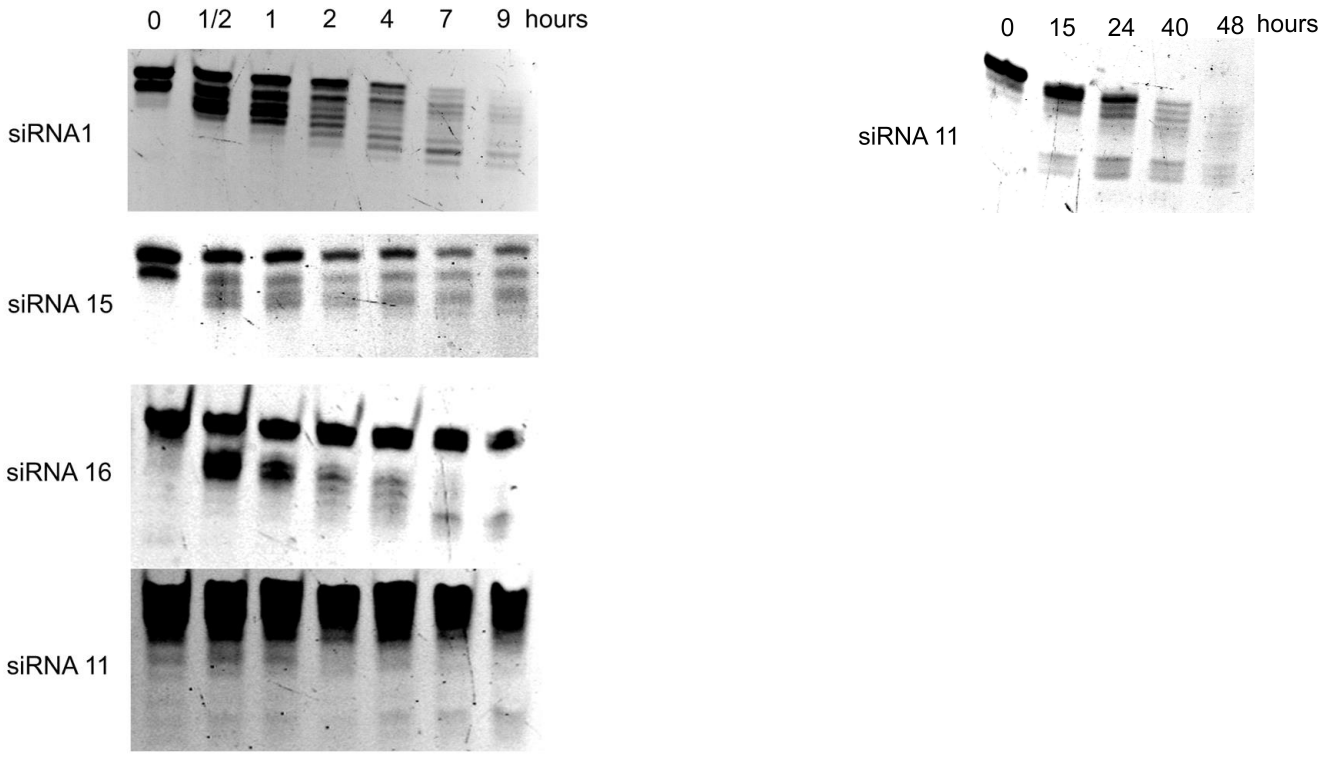

**Figure S3.** Sequences of scrambled versions of siRNAs **1**, **10** and **16** (**scr-1**, **scr-10** and **scr-16**) used in the RNA interference control experiments. Top strand depicts the sense strand in the 3'ï 5' direction (same as the target sequence). Bottom strand depicts the antisense strand in the 5'ï 3' direction (complementary to the target). $T^N$: *North* 2ô-deoxy MC thymidine monomer

**scr-1** 3'-TTAGGAAAGAAAGAAAGCUAU-5'
5'-UCCUUUCUUUCUUUCGAUATT-3'

**scr-10** 3'-TTAGGAAAGAAAGAAAGCUAU-5'
5'-UCCUUUC**T**$^N$UUCUUUCGA**T**$^N$A**T**$^N$T -3'

**scr-16** 3'-TTAGGAAAGAAAGAAAGC**T**$^N$AU-5'
5'-UCCUUUCUUUCUUUCGAUATT-3'

**Figure S4.** Plot comparing RNA interference activity of scrambled siRNAs (yellow data, **scr-1**, **scr-10** and **scr-16**) to siRNAs **1**, **10** and **16** (blue data). Suppression data are for siRNA concentrations of 0.3 nM. Untreated cells: cells transfected with plasmids alone.

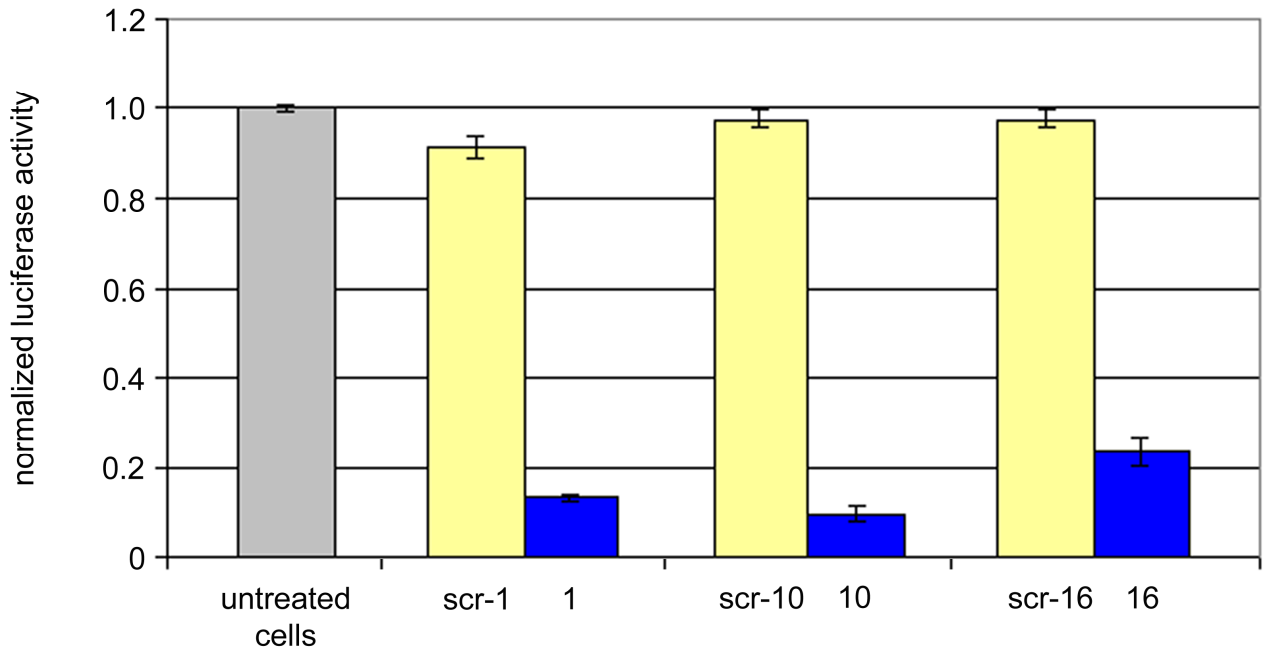

**Figure S5.** Overlay of CD spectra of siRNAs **1**, **5**, **8**, **9**, **11** and **12**. Conditions: 1 μM siRNA, 15 mM HEPES-KOH (pH 7.4), 1 mM $MgCl_2$ and 50 mM KOAc.