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RESEARCH ARTICLE

Building siRNAs with cubes: synthesis and evaluation of cubane-modified siRNAs

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Abstract: Cubane molecules hold great potential for medicinal chemistry applications due to its inherent stability and low toxicity. In this manuscript, we report the synthesis of a cubane derivative phosphoramidite for the incorporation of cubane into small interfering RNAs (siRNAs). Synthetic siRNAs rely on chemical modifications to improve their pharmacokinetic profiles. However, they are still able to mediate sequence-specific gene silencing via the endogenous RNA interference pathway. We designed a library of siRNAs bearing the cubane at different positions within the sense and antisense strands. All siRNAs showed excellent gene-silencing activity, with IC_{50} values ranging from 45.4 to 305 pM. Incorporating the cubane modification in both the sense and antisense strand led to viable duplexes with good biological activity. To the best of our knowledge, this is the first report of siRNAs bearing a cubane derivative within the backbone.

Introduction

Short-interfering RNAs (siRNAs) belong to a class of double-stranded RNA molecules that can induce gene silencing via the RNA interference (RNAi) pathway.[1] The siRNA is first incorporated into an RNA-induced silencing complex (RISC) as a duplex, but one of the strands, called the passenger strand must be removed for proper RNAi activity. The guide strand, which is complementary to the target mRNA, remains bound to the active complex and directs mRNA cleavage to achieve sequence-specific gene silencing.[2] Recently, these biomolecules have gained widespread interest due to the US FDA-approval of three siRNA-based formulations for the treatment of rare genetic conditions.[3] Despite this progress, there are many limitations associated with the inherent structure of siRNAs, so they must be chemically modified to improve their pharmacokinetic profiles. Several chemical modifications, such as 2'-fluoro and 2'-*O*-methyl have been reported and extensively used for both *in vitro* and clinical applications.[4-6] While these modifications have been instrumental in opening the field up to new investments, the future and pace of siRNA development will depend on how quickly novel chemical modifications can be synthesized, tested, and applied within biological systems.

A common strategy involves replacing the phosphate backbone with phosphorothioate or boranophosphate to increase nuclease resistance.[7] However, these modifications do not reduce the anionic charge of the RNA molecule. This has led to the development of neutral backbone replacements such as amides,[8] triazoles,[9] neutral phosphate triesters,[10] and aromatic scaffolds.[11] While this is an important limitation to overcome, it bears mentioning that this work is focused on the compatibility of the cubane moiety in the RISC complex, not necessarily improving the pharmacokinetic properties of the siRNA at this stage. With this in mind, we present the following results on the compatibility of cubane modifications within the RNAi pathway.

Cubane is a saturated hydrocarbon system, with a three-dimensional cube-like arrangement. This synthetically challenging molecule has been of great interest in theoretical organic chemistry and, more recently, has become a promising molecule for medicinal chemistry applications.[12] Cubane can act as a bioisostere for benzene, but it has the added benefit of being less toxic and more biologically stable.[13] As a result, there is increasing interest in the synthesis of novel, cubane-bearing small molecules that could maintain the same functionality as the previous generation of benzene-based therapies. One such case is the use of a cubane derivative in a nucleic acid aptamer for selective recognition of malaria biomarkers.[14] Cubane molecules also share a similar reactivity profile to benzene, in that they undergo substitution reactions despite them not being aromatic themselves.[15] Therefore, they can be further modified with any number of substituents depending on the desired application.

In this study, we employed a reported synthetic approach to prepare a cubane ethoxy derivative which was then used as the starting material to synthesize a novel cubane derivative phosphoramidite for oligonucleotide synthesis. We designed a small library of siRNAs bearing a single cubane derivative at different positions within the sense or antisense strand (Figure 1). We then investigated the gene-silencing activity of duplexes modified at either the sense strand, the antisense strand, or both. All duplexes adopted the appropriate A-form helical conformation required for incorporation into the RISC. Most siRNAs induced potent gene silencing activity and offered some enhanced target specificity compared to unmodified wildtype siRNA.

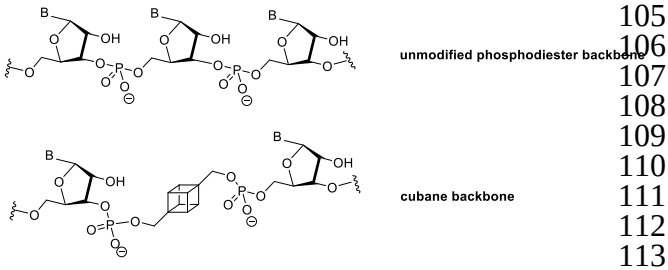
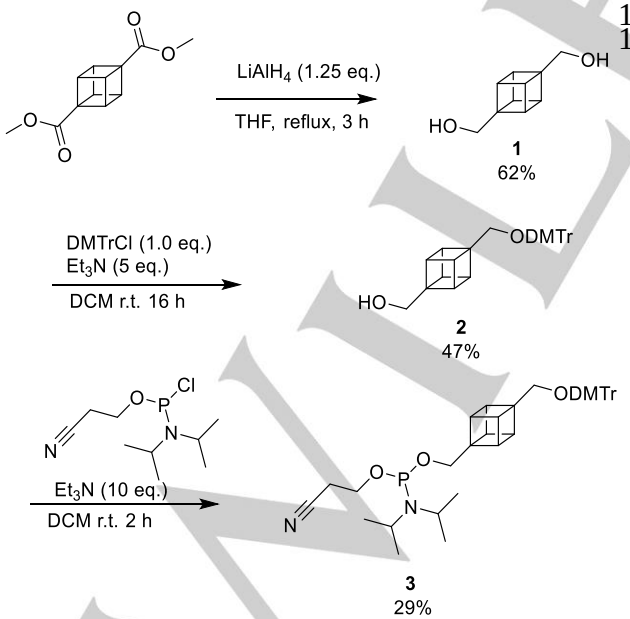


Figure 1. Structure of RNA with native phosphate backbone (top) and the cubane modification (bottom).

Results and Discussion

To synthesize siRNAs incorporating the 1,4-ethoxy cubane derivative, we followed a standard cubane synthesis procedure developed by the Tsanaktsidis lab.[15,16] This was followed by the reduction of the acetylated diol, monoprotection using dimethoxytrityl chloride (DMT-Cl), and phosphitylation of the DMT-protected monoalcohol for incorporation into siRNAs (Scheme 1). We focused on developing a symmetrical 1,4 ethoxy cubane derivative that could be used as a backbone replacement for benzene. Starting with the acetylated cubane, reduction of the esters with lithium aluminum hydride in tetrahydrofuran (THF) yielded the 1,4-ethoxy cubane derivative **1** (62%). Next, diol **1** was treated with DMT-Cl in dichloromethane (DCM) to give the singly protected monoalcohol **2** (47%), which was then reacted with 2-cyanoethyl diisopropylchlorophosphoramidite in the presence of triethylamine in DCM. The resulting phosphoramidite **3** represents our solid-phase synthesis building block, which was employed to prepare a library of siRNAs. To the best of our knowledge, this is the first report of an siRNA bearing a cubane replacement within its backbone.



Scheme 1. Synthesis of cubane DMT-protected phosphoramidite.

After standard oligonucleotide synthesis, cleavage and deprotection, RNA strands were purified by high-performance liquid chromatography (HPLC) (Supplementary Figure S-1) and characterized by quadrupole time-of-flight mass spectroscopy (qTOF-MS). siRNA synthetic yields were >99% based on the colour of the DMT cation that was removed at each step, using standard synthetic procedures (See supplement S1 general section for synthetic parameters). The cubane modification was placed at several locations of interest. Modifications at the 3' end are generally well tolerated and were included to investigate whether the cubane modification dramatically impacts siRNA activity. Modification of the seed region (nucleotides 2 to 8 from the 5' end) can dramatically reduce activity of the associated strand as it interrupts primary target recognition. Thus, the cubane was only placed within the seed region of the sense strand. Central modifications may prevent maturation of the RISC and/or reduce the ability for the mature RISC to cleave target mRNA strands. The impact of the cubane within the central region was therefore explored on both the antisense and sense strand. The resulting sequences are summarized in Table 1 and mass spectroscopy data can be found in the Supplementary Table S-1. Sense and antisense strands were annealed prior to biophysical and biological assays. We prepared twelve siRNA duplexes, with siRNAs **1**-**4** containing the cubane modification within the sense strand, siRNAs **5** and **6** containing the cubane modification within the antisense strand and siRNAs **7**-**12** being modified on both strands. To confirm that the resulting duplexes adopted the active A-form helical conformation recognized by the RISC, circular dichroism (CD) spectroscopy was performed (See Figure S2 in supplement S1). All siRNAs, including those that were modified on both the sense and antisense strand (siRNAs **7**-**12**) formed viable duplexes and adopted the desired conformation (Supplementary Figure S-2). Since the potency of siRNAs can be modulated by their thermodynamic properties, we also evaluated the thermal stability of each duplex. The melting temperature (T_m) data is summarized in Table 1.

Table 1. Sequences, melting temperatures and IC_{50} values of anti-luciferase cubane siRNAs

siRNA	Sequence	T_m (°C)	Δ T_m (°C)	IC_{50} ± SE (pM)
wt	5' CUU ACG CUG AGU ACU UCG ATT 3' 3' TTG AAU GCG ACU CAU GAA GCU 5'	76	-	5.9 ± 2.3
1	5' CUU ACG CUG AGU ACU UCG ATX 3' 3' TTG AAU GCG ACU CAU GAA GCU 5'	72	-4	83.9 ± 46.7
2	5' CUU ACG XUG AGU ACU UCG ATT 3' 3' TTG AAU GCG ACU CAU GAA GCU 5'	72	-4	45.4 ± 11.7
3	5'CUU ACG CUG AXU ACU UCG ATT -3' 3' TTG AAU GCG ACU CAU GAA GCU 5'	71	-5	108.8 ± 11.7
4	5' CUU ACG CUG AGU ACU UCG AX 3' 3' TTG AAU GCG ACU CAU GAA GCU 5'	63	-13	152.5 ± 46.3
5	5' CUU ACG CUG AGU ACU UCG ATT 3' 3' TTG AAU GCG XCU CAU GAA GCU 5'	65	-11	305.5 ± 6.5
6	5' CUU ACG CUG AGU ACU UCG ATT 3' 3' XG AAU GCG ACU CAU GAA GCU 5'	72	-4	101.9 ± 97.9
7	5' CUU ACG CUG AGU ACU UCG ATX 3' 3' TTG AAU GCG XCU CAU GAA GCU 5'	69	-7	754.3 ± 10.2
8	5' CUU ACG CUG AGU ACU UCG ATX 3' 3' XG AAU GCG ACU CAU GAA GCU 5'	63	-13	816.0 ± 20.5
9	5' CUU ACG XUG AGU ACU UCG ATT 3' 3' TTG AAU GCG XCU CAU GAA GCU 5'	65	-11	1259 ± 410.8
10	5' CUU ACG XUG AGU ACU UCG ATT 3' 3' XG AAU GCG ACU CAU GAA GCU 5'	61	-15	557.7 ± 68.8

11	5' CUU ACG CUG AXU ACU UCG ATT 3' 3' TTG AAU GCG XCU CAU GAA GCU 5'	52	-24	573.1 ± 110.6
12	5' CUU ACG CUG AXU ACU UCG ATT 3' 3' XG AAU GCG ACU CAU GAA GCU 5'	61	-15	436.7 ± 22.2

The top strand corresponds to the sense strand. The bottom strand corresponds to the antisense strand. X corresponds to the cubane moiety. The antisense strand is phosphorylated at the 5' end.

All siRNAs were designed to target firefly luciferase, so we used the standard Dual-Luciferase Reporter Gene Assay (Promega) to assess their gene-silencing activity. We used two expression plasmids coding for either firefly luciferase (target) or *Renilla* luciferase (internal control). HeLa cells were transfected with both plasmids as well as the appropriate siRNA using the transfection reagent Lipofectamine 2000™. Cells were lysed 24 hours post-transfection after which the Dual-Luciferase Reporter Gene Assay was performed following the manufacturer's procedure. We first investigated the biological activity of the siRNAs modified on either the sense or antisense strand (siRNAs **1**-**6**). The results of this assay are illustrated in Figure 2 and IC_{50} values for each siRNA are summarized in Table 1. The corresponding dose-response curves are found in Supplementary Figure S-3.

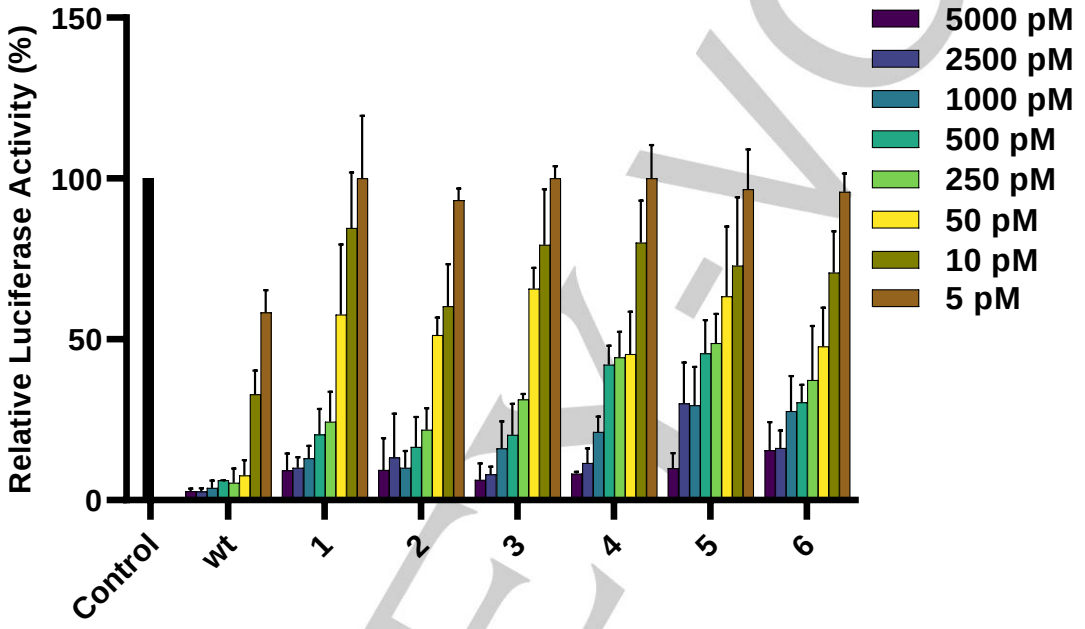


Figure 2. Relative normalized expression of firefly luciferase in HeLa cells after 24 hours treatment with unmodified (wt) siRNA or siRNAs bearing a single cubane modification within either the sense or antisense strand. Firefly luciferase expression was normalized to *Renilla* luciferase. Error bars indicate standard deviation of at least two independent biological replicates.

The resulting gene-silencing effect was position-dependent, with siRNAs modified within the sense strand showing higher knockdown than those modified within the antisense strand. We found that siRNA **1** (replacing a single dT in the sense strand 3' overhang) and siRNA **2** (modified at the sense strand position 2) showed the highest activity with IC_{50} values of 83.90 and 45.4 pM, respectively. These results are comparable to other reported neutral backbone replacements in the literature.[16b] We also investigated the effect of replacing both dT nucleotides in the sense strand 3' overhang (siRNA **4**) but found that this reduced siRNA activity by nearly 2 fold, with an IC_{50} of 152.5 pM, compared to the single dT replacement (siRNA 1). A similar effect was observed when we modified position 11 from the sense strand 5' end (siRNA **3**; IC_{50}: 108.8 pM). On the other hand, we found the cubane modification was better tolerated in antisense strand when placed at the 3' end (siRNA **6**; IC_{50}: 101.9 pM) as opposed to internally (siRNA 5; IC_{50}: 305.5 pM). Taken together, these results indicate that the cubane modification is suitable for incorporation into siRNAs, in either the sense or antisense strand.

Given the success of these cubane-modified siRNAs, we decided to investigate the effect of combining the modified strands used in siRNA duplexes **1-6** to yield a library of six additional siRNA duplexes modified within both the sense and antisense strands (siRNAs **7-12**). While less effective than their singly modified counterparts, these doubly modified duplexes still retain good biological activity with IC_{50} values ranging from 436.7 to 1259 pM (Figure 3). Out of this small library, siRNA **12**, which is modified at the antisense strand 3' end and at position 11 from the sense strand 5' end, displayed the highest gene-silencing efficacy (IC_{50}: 436.7 pM). Similarly, siRNA **10**, which is modified at the antisense strand 3' end and at position 7 from the sense strand 5' end, displayed good activity with an IC_{50} of 557.7 pM. On the other hand, modifying the 3' end of the sense strand and position 12 from the antisense strand 5' end (siRNA **7**) led to a slight decrease in activity (IC_{50}: 754.3 pM). Modifying both strands at their respective 3' ends (siRNA **8**) did not improve gene-silencing activity (IC_{50}: 816.0 pM). Notably, modifying position 11 from the sense strand 5' end and position 12 from the antisense strand 5' end led to significant thermal destabilization (ΔT_m: -24 °C) yet this siRNA displayed effective gene-silencing activity (IC_{50}: 573.1 pM).

On the other hand, modifying position 7 from the sense strand 5' end and position and position 12 from the antisense strand 5' end led to the lowest reported potency (IC_{50}: 1259 pM) even though this duplex was only destabilized by -11°C (siRNA **9**). Previous reports suggest that thermal destabilization can induce stronger gene-silencing activity, where the secondary pathway without strand cleavage is activated. This increase in activity is compared to a non-cleavable spacer in the same location, where destabilization can assist with unwinding to improve silencing activity.[17]

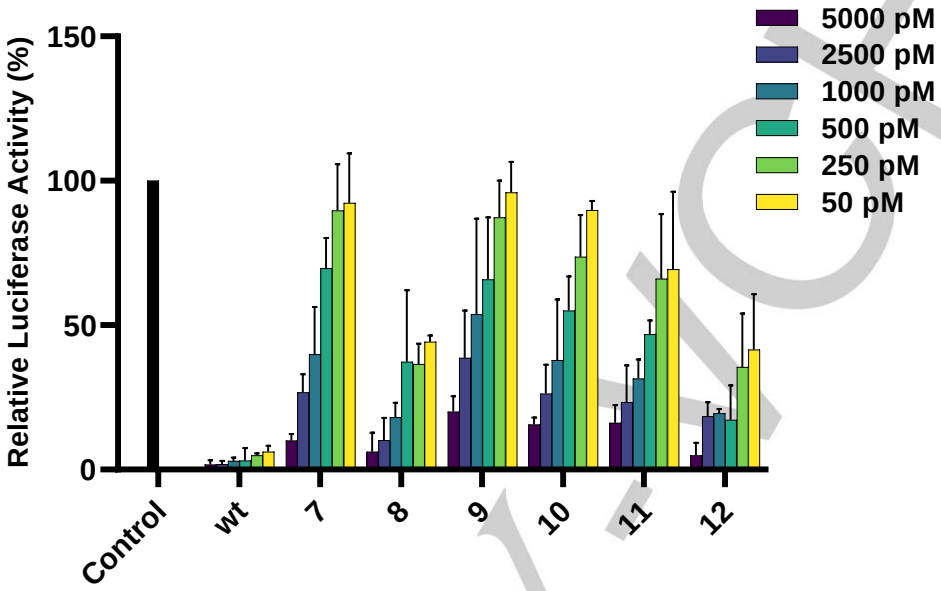


Figure 3. Relative normalized expression of firefly luciferase in HeLa cells after 24 hours treatment with siRNAs bearing a single cubane modification within both the sense and antisense strands. Firefly luciferase expression was normalized to *Renilla* luciferase. Error bars indicate standard deviation of at least two independent biological replicates.

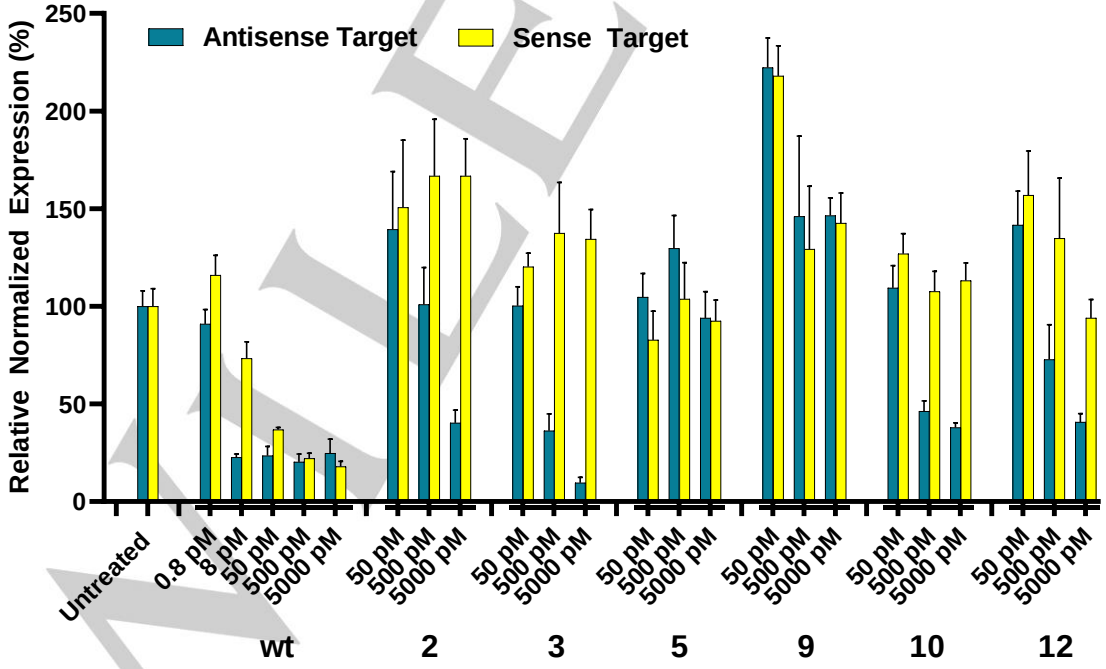


Figure 4. Relative normalized expression of firefly luciferase mRNA by the activity of either the antisense target (black) or the sense targe (gray) of unmodified (wt) or cubane-modified siRNAs . Singly modified siRNAs (2, 3 and 5) bear the cubane modification at a single position within either the sense or antisense strand whereas doubly modified siRNAs (9, 10 and 12) bear the cubane modification at a single position within both the sense and the antisense strand.

The selection of the guide strand during RISC maturation is a crucial step for RNAi function and is dependent on factors such as the siRNA's 5' sequence identity, thermodynamic stability, and chemical modifications.[18] Ideally, the antisense strand, which is complementary to the target mRNA, should be selected for and become the guide strand. To further investigate the effect of our cubane modification, we employed an RT-qPCR assay to measure mRNA cleavage due to the incorporation of either the antisense or sense strand within the RISC.[19] As is observed in Fig 4, wild-type siRNA exhibits significant gene-silencing for both antisense and sense strand targets. In order to improve and examine the effect of cubane placement on siRNA off-target effects, we selected three singly modified and three doubly modified cubane siRNAs and tested them at 50, 500 and 5000 pM. As seen in Figure 4, siRNAs **2** and **3**, which were modified internally within the sense strand, retained excellent targeted antisense gene-silencing activity, but no off-target sense strand mediated gene-silencing. On the other hand, no significant cleavage was observed for siRNA **5**, which was modified internally within the antisense strand. In addition, and unsurprisingly, siRNA **9**, which is modified internally within both the sense and antisense strand, led to no target cleavage. When the cubane modification was placed at the 3' end of the antisense strand (siRNAs **10** and **12**), significant antisense activity is observed despite the presence of an internally-modified sense strand in these duplexes. Taken together, siRNAs that have the cubane modification positioned internally within the sense strand (without a cubane modification placed internally on the complementary antisense strand), exhibit the best strand selection, and desired antisense targeted gene-silencing (siRNAs **2**, **3**, **10** and **12**).

Conclusion

In conclusion, we report the synthesis of a novel cubane derivative phosphoramidite used to prepare a small library of siRNAs modified at the sense strand, antisense strand, or both strands. We then assessed the biophysical properties and biological activity of these siRNAs. All siRNAs adopted the desired A-form helical conformation and were able to induce gene silencing. In addition, placing the cubane moiety within the central region of the sense strand led to desirable increases in targeted strand selection, thus reducing off-target effects. To the best of our knowledge, this is the first report of an siRNA bearing a cubane modification within its backbone. Cubane is of great interest for medicinal chemistry applications as it can act as a bioisostere for benzene while being less toxic and more biologically stable. Future directions include incorporating other cubane derivatives into the siRNA sense and antisense strands for improving the structure-activity relationship of siRNAs.

Acknowledgements

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Keywords: cubane • siRNA • gene-silencing• strand selection • phosphoramidite

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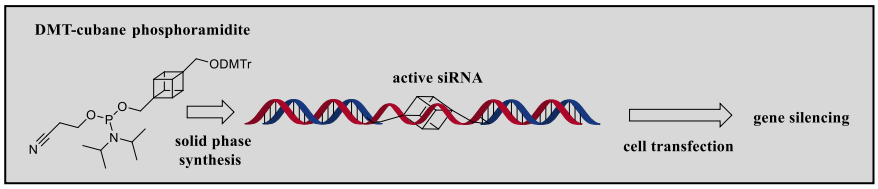

siRNA lego: cubane-functionalized siRNAs lead to potent RNAi-mediated gene-silencing activity, and improved strand selection.

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