Tetrahedron Letters 50 (2009) 2545–2547

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Synthesis of hairpin siRNA using 18β-glycyrrhetinic acid derivative as a loop motif

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ARTICLE INFO

Article history:
Received 24 February 2009
Accepted 10 March 2009
Available online 13 March 2009

Keywords:
18β-Glycyrrhetinic acid
Hairpin ODN
shRNA
RNA interference

ABSTRACT

We synthesized a new phosphoramidite building block from 18β-glycyrrhetinic acid. This compound was introduced in the middle of the palindromic oligonucleotides and they formed hairpin structure. This 18β-glycyrrhetinic acid derivative excellently performed its role as hairpin loop. These hairpin ODNs had higher T_m values than the natural one and formed stable hairpin structure without any structural distortion. We found that stability of hairpin ODNs was changed according to the length of hairpin stem and modified hairpin ODNs, which had longer than seven base pair stem, were more stable than natural one based on T_m values. The shRNAs bearing similar modified loop were also synthesized. These modified shRNAs could suppress their target gene expression without losing their RNAi efficiency.

Nucleic acid has been an attractive material in this century because of their highly specific recognition between nucleobases.[1] Since the solid phase synthesis based on phosphoramidite chemistry by automatic synthesizer has been established, it has been possible for easy modification of oligonucleotide using fuctional building blocks.[2]

RNA interference (RNAi) is known as the most effective gene knock-down process since A. Fire and C. Mello reported.[3] In RNAi, double-stranded RNA known as small interfering RNA (siRNA) is used to suppress the expression of targeted genes.[4] The short hairpin RNA (shRNA) also has ability to silence a target gene through the similar RNAi mechanism.[4c] The purpose of shRNA was reducing unspecific side effect and generating the therapeutic siRNAs.[5] To improve their stability, cellular uptake properties, and target specificity, modification of these therapeutic RNAs has been widely explored.[6] Although various modified siRNAs have been reported, modified hairpin siRNA (shRNA) is rare.

Hairpin structure is the basic secondary structure in nucleic acid world. Because of their high stability, intramolecular hairpin structure with various loop units has been reported. Introducing foreign loop molecules, intramolecular structures showed better properties such as structural stability[7], nuclease resistance[8], electron transfer[9], structural reversibility[10], and therapeutic ability[11].

We previously reported the hairpin oligonucleotide bearing cholane-3,24-diol moiety as a loop.[12] In a similar manner, we inserted non-nucleosidic molecule at the loop position and synthesized hairpin oligonucleotide. The loop molecule was derived from 18β-gycyrrhetinic acid, which is one of the components of extracts of licorice and reported as the compound having anti-inflammatory, anti-viral, anti-allergic, and anti-tumor activities.[13] The molecule has similar size with cholane-3,24-diol but more rigid structure, so it could act as a stable loop in the hairpin structure.

We synthesized the phosphoramidite from 18β-glycyrrhetinic acid as described in Scheme 1. 18β-glycyrrhetinic acid was reduced by lithium aluminum hydride for 4 h to give **1** in 95% yield.[14] After hydrogenolysis of two diastereomers of compound **1**, compound **2** was obtained by filtration quantitatively. Then, primary alcohol was protected by the treatment of 4,4′-dimethoxytrityl chloride and catalytic amount of dimethylaminopyridine in pyridine at 50 °C for 2 h. The total yield of two steps was 47%. The phosphoramidite **4** was obtained from compound **3**, reacting with chloro-(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphine in 4-methylmorpholine and CH_2Cl_2 for 5 min. All compounds were characterized by ^1H, ^{13}C, ^{31}P NMR, and mass spectrometry.

The phosphoramidite **4** was firstly introduced in the middle of the oligodeoxyribonucleotide (ODN) to determine its property as a hairpin loop. We synthesized four hairpin ODNs by oligonucleotide synthesizer. The modifier **4** could easily couple with natural sequences under the normal conditions and the synthesized oligonucleotides were characterized by MALDI-MS.[15]

Table 1 shows the T_m values of hairpin ODNs compared with those of natural one. Compound **4** and natural loop unit, $\mathbf{C_4}$, were inserted in the middle of the palindromic DNA sequences. The T_m value of **A4T** was higher than that of $\mathbf{AC_4T}$. It means that **A4T** had more stable structure than $\mathbf{AC_4T}$. In case of $\mathbf{Y_1XY_1}$, when the stem length was just six base parings, T_m of $\mathbf{Y_14Y_1}$ was slightly lower than the natural one, $\mathbf{Y_1C_4Y_1}$. However, $\mathbf{Y_24Y_2}$ containing seven base pair stem formed stable hairpin by introducing the compound **4** in the loop position, accompanied with much higher ΔT_m value

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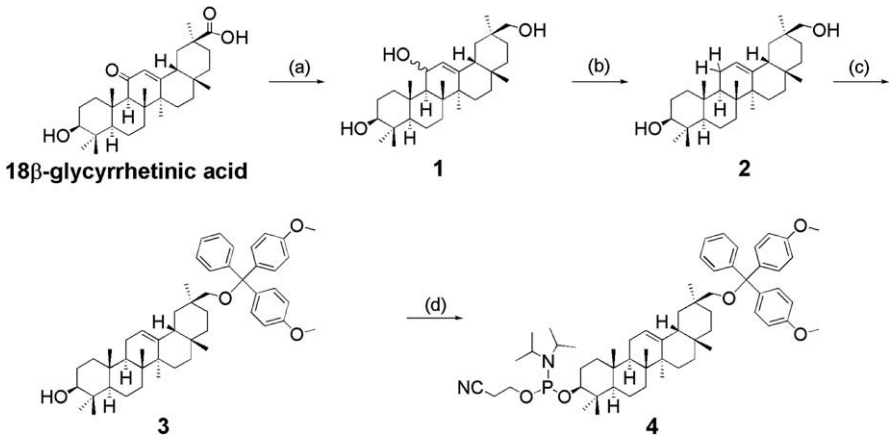
doi:10.1016/j.tetlet.2009.03.049



Scheme 1. Reagents and conditions: (a) $LiAlH_4$, THF, 60 °C, 4 h, 95%; (b) Pt/C(10 wt %), H_2 (1 atm), $CHCl_3$/THF(1/1), rt, 2 h; (c) 4,4′-dimethoxytrityl chloride, dimethylaminopyridine, pyridine, 45–50 °C, 2 h, 47% (2 steps); (d) 4-methylmorpholine, chloro-(2-cyanoethoxy)-*N*,*N*-diisopropylaminophosphine, CH_2Cl_2, rt, 5 min, 81%.

Table 1
Sequence of ODNs and their T_m values

Name	Sequence (5′–3′)	T_m (°C)	
		X = **4**	X = **C_4**
AXT	d(A_{12} X T_{12})	73	68
Y_1XY_1	d(AACGTT X AACGTT)	63	66
Y_2XY_2	d(CAA CGT T X A ACG TTG)	75	—
Y_3XY_3	d(CC AAC GTT X AA CGT TGG)	80	—

T_m values were determined by measuring changes in absorbance at 260 (cuvette, 1 cm path length) as a function of temperature in Tris–HCl buffer (10 mM, pH 7.2) containing NaCl (100 mM) and $MgCl_2$ (20 mM). The temperature was raised at a rate of 1.0 °C/min. Total ODN concentration is 3 μM.

between **$Y_1$4Y_1**, and **$Y_2$4Y_2** than between **$Y_2$4Y_2**, and **$Y_3$4Y_3**. In comparison with our previous data of another stable loop modifier (T_m values were 72, 76, and 82 °C for **Y_1XY_1**, **Y_2XY_2**, and **Y_3XY_3** respectively.),[12] **$Y_2$4Y_2** has minimum stem length to form highly stable hairpin structure.

Modification of the loop position could not disturb their whole structures as illustrated in Figure 1. These are CD spectra of **A4T** and **AC_4T**. These two ODNs had nearly similar patterns of curves, representing positive bands at 218 and 283–284 nm and a negative band at 248–249 nm. These CD spectra were almost identical to that of known B-form duplex structures.

By using this hairpin loop mimic compound **4**, we synthesized shRNAs as shown in Table 2. We incorporated compound **4** and

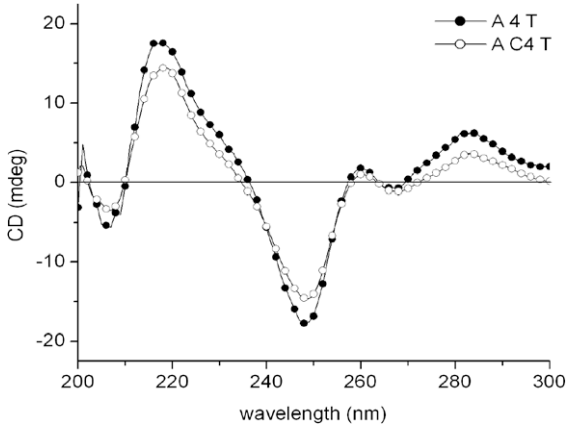


Figure 1. CD spectra of A4T and AC_4T. The curves were obtained at 10 °C and the condition was as shown in Table 1.

Table 2
Sequence of synthesized shRNAs

Name	Sequence
	(sense (5'-3')-linker- / antisense (3'-5')-linker-) loop
L-C4	5' ACCCUGAAGUUCAUCUGCACCCCGG C 3' ACUGGGACUUCAAGUAGACGUGGGGCC C C
L-4	5' ACCCUGAAGUUCAUCUGCACCCCGG 3' ACUGGGACUUCAAGUAGACGUGGGGCC 4
S-C4	5' ACCCUGAAGUUCAUCUGCACC C 3' ACUGGGACUUCAAGUAGACGUGG C C
S-4	5' ACCCUGAAGUUCAUCUGCACC 3' ACUGGGACUUCAAGUAGACGUGG 4

natural hairpin loop unit, **C_4**, between sense and antisense strand. Our synthesized shRNAs are classified into two groups, **L**-group and **S**-group. **L**-group has longer linker than **S**-group between loop and siRNA sequences. The shRNAs were synthesized by oligonucleotide synthesizer and purified by HPLC.

As a target of all shRNAs, EGFP gene was used to determine their interfering efficiency by measuring fluorescence of remained GFP. The EGFP suppression assay using synthesized shRNAs was done in collaboration with Dr. Dong-Ho Kim (Beckman Research Institute of the City of Hope). The experimental protocols were exactly similar to their previous methods except for using NIH3T3 cell line here.[16] If the anti-EGFP shRNAs worked through RNAi mechanism, reduced fluorescence would be detected because GFP was downregulated.

After transfection with Lipofectamine 2000 (invitrogen), all synthetic RNAs showed highly reduced fluorescence signal comparing with control treatment without shRNA (Fig. 2). It suggests that modified shRNAs have potent activity as a gene silencer and changing loop position with foreign molecule does not suppress their

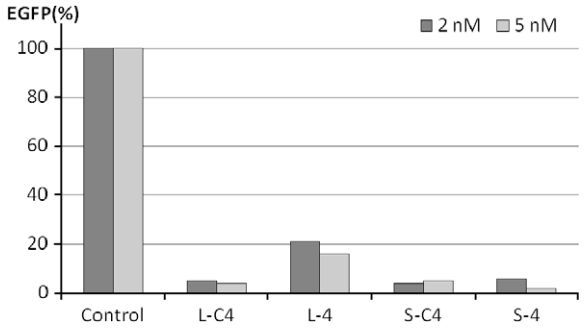


Figure 2. RNA interference efficacy test: all shRNAs were annealed in sodium phosphate buffer (20 mM, pH 6.8) containing NaCl (100 mM).

RNAi activity. **S-4** had higher RNAi efficacy than **L-4**, but this linker-dependent phenomenon was not applicable to natural one.

In conclusion, we synthesized a new building block which was introduced into the oligonucleotide to function as a hairpin loop. The hairpin oligonucleotides with this rigid molecule formed stable hairpin systems and more than seven base-paired stems were needed to have higher T_m value than natural one. We could observe that the novel loop modifier did not distort original duplex structure. The shRNAs bearing derivative of 18β-glycyrrhetinic acid could interfere with their target gene expression without affecting their RNAi efficiency, by carefully designing the linker sequences between the loop and siRNA part. It indicates that we can modify the loop position of shRNA with not only nucleotide but also with any possible molecule such as fluorophores, membrane permeable molecules, and receptor binding molecules.

Acknowledgments

We thank Dr. Dong-Ho Kim for his contribution on the EGFP suppression assay in Beckman Research Institute of the City of Hope in the USA. This work has been supported by KOSEF (Gene Therapy R&D and KNRRC programs).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.03.049.

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