Bioorganic & Medicinal Chemistry 24 (2016) 2108–2113

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Development of a 3′-amino linker with high conjugation activity and its application to conveniently cross-link blunt ends of a duplex

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ARTICLE INFO

Article history:
Received 16 February 2016
Revised 24 March 2016
Accepted 25 March 2016
Available online 25 March 2016

Keywords:
Amino linker
Nucleic acids
Oligonucleotides
Labeling
Cross-link
anti-microRNA

ABSTRACT

The 2-aminoethyl carbamate linker (ssH linker) exhibits high activity in modifying the 5′-termini of oligonucleotides; however, the ssH linker is not appropriate for 3′-terminal modification because it undergoes intramolecular *trans*-acylation under heat–aqueous ammonia conditions. We developed an *N*-(2-aminoethyl)carbamate linker (revH linker), in which the carbamate is oriented in the reverse direction relative to that in 2-aminoethyl carbamate. The revH linker was tolerant to heat–alkaline conditions and retained its high reactivity in conjugation with exogenous molecules. The 3′-revH linker was efficiently linked with the 5′-ssH linker at the termini of complementary double strands with a bifunctional molecule, producing a synthetic loop structure. An *anti*-microRNA oligonucleotide (AMO) was prepared from the chemical ligation of three-stranded 2′-*O*-methyl RNAs, and the AMO with two alkyl loops exhibited high inhibition activity toward miRNA function. The revH linker is not only useful for 3′-terminal modification of oligonucleotides but also expands the utility range in combination with the 5′-ssH linker.

1. Introduction

Post-synthetic modifications of oligonucleotides (ONTs) are site-specifically promoted through reactive linkers, which involve a primary amine,[1] thiol,[2] hydrazine,[3] aldehyde,[4,5] or amino-oxy moiety.[6,7] Recently, an increasing number of cycloaddition reactions have also been reported for the modification of ONTs. Copper-catalyzed azide–alkyne cycloadditions (CuAACs), known as 'click' reactions, are a very powerful tool for ONT modifications.[8,9] As metal-free cycloaddition reactions, strain-promoted azide–alkyne cycloaddition (SPAAC)[10,11] and inverse electron-demand Diels–Alder reactions have also been applied to ONT labeling.[12]

Amino linkers have been extensively used for ONT modifications because amino-modified ONTs are chemically stable and cost-effective. Modifications of ONT terminals minimize the effects on hybridization efficiency or sequence selectivity. The decision of which termini to modify depends on each application. In the synthesis of 3′-amino-modified ONTs, the first nucleotide amidite unit is coupled with an amino-linker unit that is covalently connected to a solid support such as controlled pore glass (CPG) or polystyrene resin. Primary aliphatic amino linkers with branched[13] or linear alkyl chain, such as hexyl (C6) group,[14,15] have been developed for the 3′-amino modifications. Primary amines are generally protected by alkaline labile[15–17] or photolabile groups.[18] Thus, amino-modified ONTs are cleaved from the solid support by a heat–alkaline treatment or by UV-irradiation after the ONT synthesis.

We previously developed an amino linker consisting of an 2-aminoethyl carbamate structure (ssH linker, Fig. 1b) for 5′-terminal modification. Some chemical features of a primary amine were induced by the neighboring effect of the carbamate group. The ssH-linker-modified ONTs more efficiently conjugate with exogenous molecules with active esters than do C6-ONTs.[19] In addition, the monomethoxytrityl (MMT) group protecting a primary amine can be rapidly removed, resulting in high-throughput purification of amino-modified ONTs. However, in the ssH linker, *trans*-acylation to a free primary amine occurs during the heat–aqueous ammonia treatment unless the primary amine is protected by the MMT. Thus, the ssH linker is not suitable as a 3′-terminal amino linker that cannot employ the MMT group. In this study, to develop a more suitable linker structure, we improved our carbamate-containing amino linker to be applicable to 3′-terminal modifications. The novel 3′-amino linker was stable under alkaline conditions and exhibited the same efficiency as the ssH linker in labeling with molecules of active esters. We also report here that 5′- and 3′-carbamate-containing amino linkers enabled convenient cross-linking

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http://dx.doi.org/10.1016/j.bmc.2016.03.039
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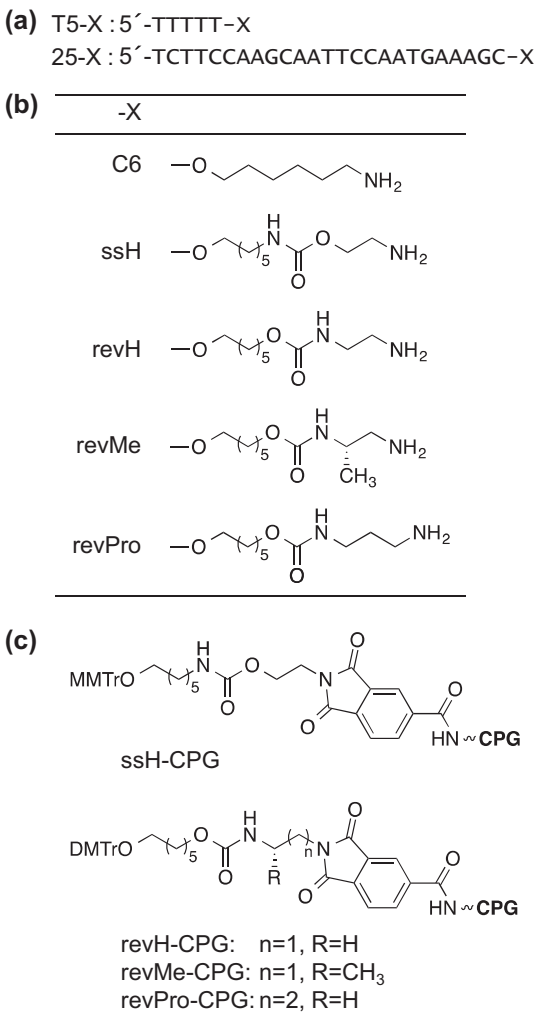

Figure 1. Sequences of 3′-amino-modified ONTs (T5-X and 25-X) (a) and the structures of 3′-terminal amino linkers (b). X indicates the 3′-amino linker (C6, ssH, revH, revMe, or revPro). (c) Structures of the 3′-amino-linker CPG units synthesized in this study.

at the terminals of complementary strands, probing the usefulness of these linkers for preparing long ONTs.

2. Results and discussion

2.1. Design and synthesis of novel 3′-amino linkers

When 5′-ssH-modified ONTs without protecting group for a primary amine are treated in heat–aqueous ammonia, a *trans*-acylation reaction occurs through the attack of a primary amino group on a carbamate carbon atom.[19] On the basis of this result, we designed an *N*-(2-aminoethyl)carbamate linkage (revH, Fig. 1b), where a 2-aminoethyl group was connected to the nitrogen atom of the carbamate moiety. We expected that the revH structure could be reformed even after the intramolecular *trans*-acylation. We also synthesized a revMe linker unit with a methyl group on an ethylenediamine chain (Fig. 1b). All primary amines of these linker units were covalently tethered with CPG using trimellitic anhydride (revH-CPG, revMe-CPG, Fig. 1c).[16,17] Although the ssH linker is originally suitable for 5′-modification, we prepared an ssH-CPG for 3′-modification to enable a comparison of the chemical stability or reactivity. The revH-CPG was observed to be advantageous in preparation because it could be synthesized in five steps from 1,6-hexanediol, whereas ssH-CPG units required seven steps for preparation from 2-aminoethanol (Schemes S1 and S2).

2.2. Stability under heat–alkaline conditions

We first synthesized penta-thymidylic acids with 3′-amino linkers (T5-X; X = C6, ssH, revH, revMe; Fig. 1a) using each amino-modified CPG. After these amino-modified ONTs were treated in AMA solution (i.e., 1:1 mixture of 40% methylamine and concentrated ammonia solution) at 65 °C for 10 min, they were purified by reversed-phase HPLC. Their stabilities in AMA and concentrated aqueous ammonia were examined (Fig. 2 and Fig. S1 in Supplementary data). All 3′-amino-ONTs were observed to be stable in the short AMA treatment (Fig. S1). On the other hand, the chromatogram of T5-ssH exhibited two peaks (P1 and P2, Fig. 2a) after incubation in concentrated aqueous ammonia at both 55 °C and 65 °C. The percentage of P2 was approximately 2.6 times greater than that of P1, and the total percentage (P1 + P2) increased with the incubation temperature (Fig. 2b). These results are consistent with the previously reported results of the aqueous ammonia treatment of 5′-ssH-modified ONTs.[19] On the basis of the previous results and the molecular-weight analyses (Table S2), P1 and P2 were identified to have 6-aminohexyl and *trans*-acylated urea structures (Fig. 2c), respectively.

In contrast to the chromatogram of T5-ssH, that of T5-revH exhibited only a low-intensity peak (P3 in Fig. 2a; 0.8% at 55 °C, 2.9% at 65 °C), whose percentage was only one-tenth the total degradation of T5-ssH. Although T5-revMe is cognate with T5-revH, its chromatogram exhibited two peaks (P4 and P5, Fig. 2a) and the total percentage was much higher than that of T5-revH (Fig. 2b). Molecular-weight analyses revealed that both P3 and P4 had 6-hydroxyhexyl structures and that P5 had the same molecular weight as the parent molecule (Table S2). These results indicate that the intramolecular *trans*-acylation reaction evidently occurred in the revMe linker (Fig. 2c) and P5 was identified to be a *trans*-acylated product. By contrast, whether the same reaction proceeded in the revH linker was unclear because the revH structure could be reformed as a consequence of its symmetrical ethylenediamine moiety, even after the intramolecular *trans*-acylation. Another possibility is that the revH linker is tolerant to the alkaline treatment. In any event, the revH linker had the alkali resistance necessary for a 3′-amino linker.

2.3. Labeling reactions with active esters

All 3′-amino-linkers were found to be stable under the AMA treatment (Fig. S1). By using the AMA deprotection, we prepared 3′-modified 25-mer (25-Y; Y = C6, ssH, revH, revMe, Fig. 1a) to investigate the labeling efficiencies with fluorescein isothiocyanate (FITC), biotin-*N*-hydroxysuccinimidyl ester (biotin-NHS), and polyethylene glycol *N*-hydroxysuccinimidyl ester (PEG-NHS). As evident in Figure 3 and 25-ssH gave several times more conjugates than 25-C6; these results are consistent with our previous results obtained for the modifications of 5′-ssH-ONTs.[19] Notably, both 25-revH and 25-revMe exhibited high labeling efficiencies comparable with that of 25-ssH. To examine the structural requirement of the *N*-(aminoalkyl)carbamate moiety, the *N*-(3-aminopropyl)carbamate structure (revPro, Fig. 1b) was prepared. The labeling reactions with the 3′-revPro-modified 25-mer (25-revPro) decreased the conjugation yields compared to those of the revH- and ssH-modified ONTs (Fig. 3). We attributed this decrease in revPro to the reduction in the neighboring effect of the carbamate group because of the insertion of 3-aminopropyl spacer, indicating that the *N*-(2-aminoethyl)carbamate structure is a more effective 3′-amino linker.

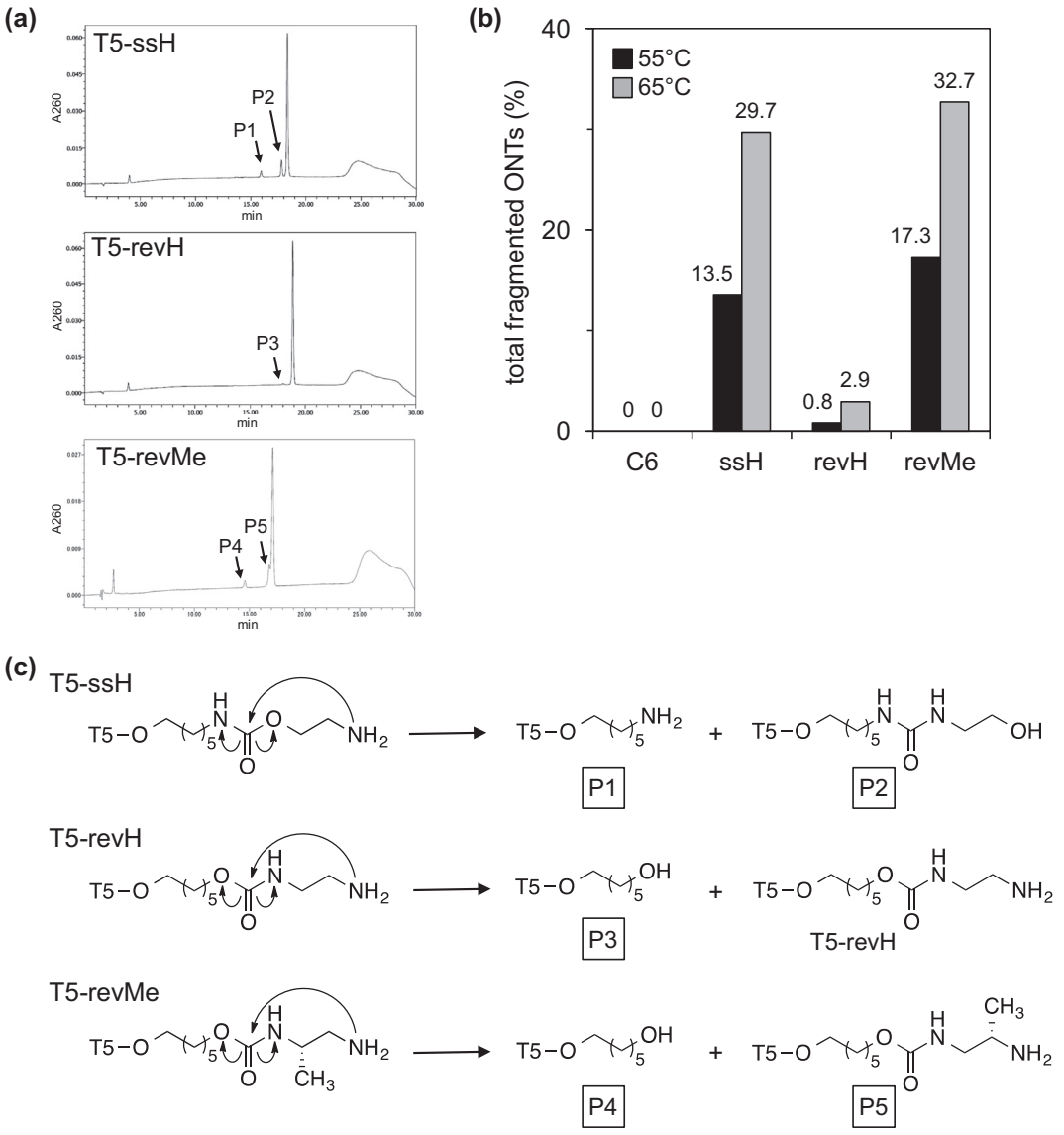

Figure 2. Stabilities of 3′-amino-modified ONTs in heat–alkaline conditions. (a) Chromatograms of HPLC analyses of T5-ssH, T5-revH and T5-revMe after treated in conc. NH_4OH at 55 °C for 16 h. (b) Percentages of ONT fragments produced by the incubation of T5-X at 55 °C (black bars) or 65 °C (gray bars) in conc. NH_4OH. (c) Schemes of the intramolecular *trans*-acylation reactions. The sum of each product is plotted in Figure 2b.

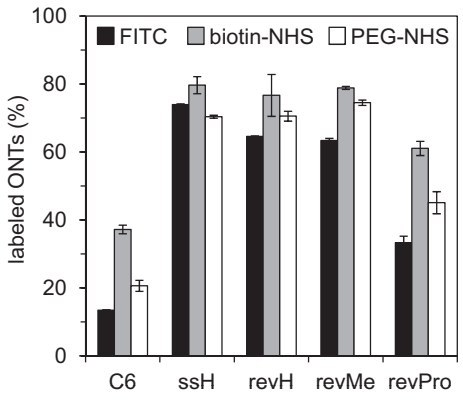

Figure 3. Conjugating reactions of 25-X ONTs with activated molecules. Percentages of ONTs labeled with FITC (black bars), biotin-NHS (gray bars), and PEG-NHS (white bars).

2.4. pK_a values of the primary amino groups

Our previous study revealed that the pK_a value of a primary amine on the ssH linker was decreased, compared with that of an aliphatic amine, C6 linker.[19] To examine the pK_a values of the *N*-(aminoalkyl)carbamate moiety, amino-linker monomer units ($revH_{OH}$ and $revPro_{OH}$, Scheme S2) were prepared. The pK_a values of the primary amines were determined on the basis of the chemical shift changes in the ^{13}C NMR spectra collected under various pH conditions (Fig. S2). The determined pK_a value of $revH_{OH}$ was 9.8. This value is substantially lower than that of $C6_{OH}$ (pK_a = 11.0) and is similar to that of ssH_{OH} (pK_a = 9.6).[19] By contrast, $revPro_{OH}$ with an *N*-(3-aminopropyl)carbamate exhibited a pK_a intermediate (pK_a = 10.4) between those of $revH_{OH}$ and C6. We think that the significant reduction in the pK_a values of $revH_{OH}$ and ssH_{OH} was derived from the electron-withdrawing effect of the carbamate group. Thus, this neighboring effect was slightly diminished in $revPro_{OH}$ containing the 3-aminopropyl spacer. In a

coupling reaction between a primary amine and an active ester, a protonated amine loses nucleophilicity. In addition, deprotonation from the amine of the intermediate is essential to complete a covalent bond formation. Hence, the reduced pK_a values of both ssH_{OH} and $revH_{OH}$ are thought to be closely related with the enhanced conjugation efficiencies of the ssH and revH linkers.

2.5. Cross-linking of amino linkers on blunt ends of duplexes

In chemical synthesis of ONTs, amount of ONTs are generally reduced as the number of nucleotides increase. In addition, it becomes difficult to purify long ONTs. To address these issues, there is a post-synthetic ligation method of short ONTs. Double strands can be cross-linked between 5′ and 3′ terminals to produce a hairpin loop ONTs. So far, the chemical ligation using aldehyde-amine coupling[20] or the CuAAC reaction have been reported.[21] Amino-modified ONTs are not only chemically stable but also are superior in terms of convenience and cost. By utilizing these advantages, we originated the cross-linking between 5′ and 3′ terminal amino linkers on a blunt end of a duplex. One of the blunt ends of a complementary 25-mer duplex was modified with an amino-linker pair of 5′-ssH/3′-revH or 5′-C6/3′-C6, followed by cross-linking with a bifunctional linker, disuccinimidyl glutarate (DSG) (Fig. 4a). As the results of the reaction, the pair of 5′-ssH/3′-revH completed the cross-linking after 5 min (98.4% yield), in contrast to 43.5% yield in the cross-linking of the 5′-C6/3′-C6 pair (Fig. 4b). Although the long alkyl-loop structure is formed after the cross-link of 5′-ssH/3′-revH, the result indicates that the ligation reaction between revH and ssH could be cost-effective and superior method in the convenient preparation of hairpin-type long ONTs.

Various types *anti*-microRNA ONTs (AMOs) have been developed to control microRNA (miRNA) function in living cells.[22] Single stranded ONTs having hairpin-stem loops at both 5′ and 3′ terminals has been reported to especially show high inhibitory activities.[23,24] To conveniently prepared these long sequence AMOs, we applied the blunt end cross-link using 5′-ssH/3′-revH linkers. We synthesized a 5′-ssH- and 3′-revH-modified 36-base 2′-*O*-methyl RNA (MeRNA) (I), which consisted of a complementary sequence for microRNA-21 (miR-21). The MeRNA-I was subjected to cross-link with 3′-revH- and 5′-ssH-modified short MeRNAs (II, III) in the presence of the DSG reagent. The cross-linked AMO (CL-AMO) was successfully obtained from the blunt end cross-links of short MeRNAs (Fig. 5a). We also prepared a single stranded MeRNA with two hairpin loops as a control AMO (HP-AMO, Fig. 5a).[22,23] To examine the inhibiting activity against miRNA, CL-AMO, HP-AMO and three-stranded AMO (I/II/III) were separately transfected into HeLa cells, and their activities were evaluated by the dual-luciferase assay. Although the three-stranded AMO exhibited no suppression of miRNA function, both HP-AMO and CL-AMO efficiently inhibited miR-21 (Fig. 5b). Because the

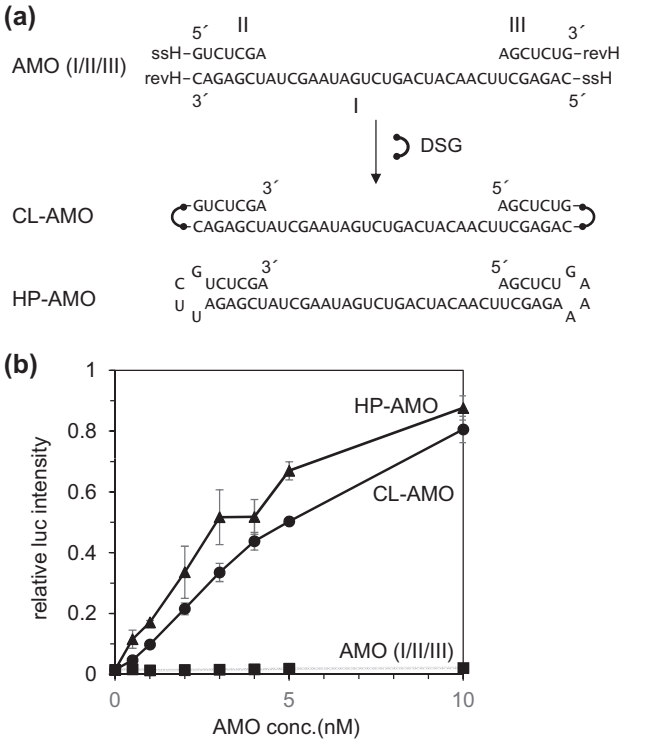

Figure 5. (a) Schematic of CL-AMO preparation and the structure of HP-AMO. (b) Plots of relative luciferase intensities (Rluc/Fluc) versus concentrations of AMOs.

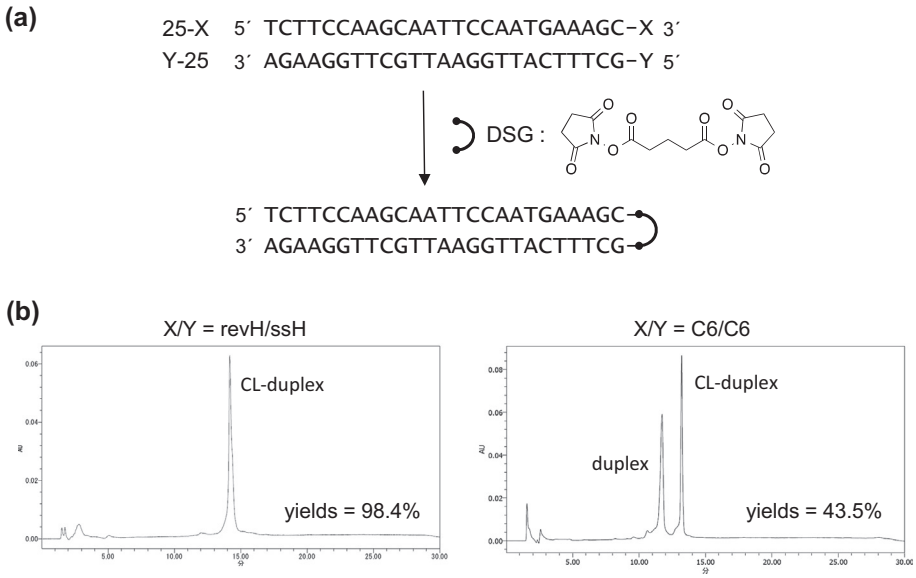

Figure 4. (a) Scheme of cross-linking reaction at a blunt end of a duplex (25-X/Y-25). (b) Analyses of the cross-linking reactions (X/Y = revH/ssH, or C6/C6).

inhibitory activity has been reported to depend on the lengths of the stems flanking a sequence complementary to miRNA,[23] these results suggest that three-stranded AMO (I/II/III) could not maintain the stem structures due to the small number of base pairs. On the other hand, it was revealed that CL-AMO could involve a stable stem-loop structure via the cross-linked linkages. It is not clear why HP-AMO exhibited the slightly higher activity than CL-AMO. Since HP-AMO involves two types of stable hairpin loops, it might be superior to CL-AMO in nuclease resistance or binding affinity against miRNA. Further experiments to reveal them are ongoing. We confirmed that the cross-links between 5′-ssH and 3′-revH are able to provide functional long ONTs conveniently.

3. Conclusion

N-(2-Aminoethyl)carbamate (revH) and 2-aminoethyl carbamate (ssH) linkers were synthesized for 3′-terminal modification of ONTs. Both the carbamate-containing linkers could exhibit efficient reactivity toward active esters compared to the aminoalkyl linker. Importantly, the revH was more stable in alkaline treatments than the ssH. This high stability was attributed to the symmetrical ethylene diamine structure in the revH. These results indicate the high potential of the revH as 3′-amino-linker.

We proved that the combination of 5′-ssH and 3′-revH linkers enable the efficient cross-links at the blunt ends of duplexes in the presence of a bifunctional reagent. An *anti*-microRNA ONT (CL-AMO) was prepared via the cross-linking of three ONT pieces, and it efficiently inhibited miRNA function compared to a noncross-linked ONT. In conclusion, the revH linker containing a carbamate linkage is very useful for various 3′-terminal modifications of ONTs and its combined use with a cognate ssH linker as a 5′-amino linker further enables versatile applications.

4. Experimental

4.1. Synthesis of 3′-amino-linker CPG units

The syntheses of ssH-, revH-, revMe-, and revPro-CPG units are presented in the Supplementary data.

4.2. Synthesis of 3′-amino-modified ONTs

All ONTs were chemically synthesized using standard phosphoramidite chemistry. 3′-Amino-modified oligoribonucleotides were synthesized using 2′-*O*-TBDMS-amidite units (Glen Research) with the trityl-off mode. Cleavage from the support and removal of the protecting groups were carried out in AMA solution at 65 °C for 10 min. The deprotection of TBDMS groups was carried out in a solution containing 3HF/TEA (75 μL), DMSO (115 μL), and TEA (60 μL) at 65 °C for 2.5 h. After the reaction solution was desalted with NAP10, 3′-modified oligoribonucleotides were purified by HPLC using a reversed-phase column.

4.3. HPLC analyses of T5-X treated with alkaline solutions

After T5-X (1.5 nmol) were incubated in various alkaline solvents (100 μL; condition 1: AMA at 65 °C for 10 min; condition 2: conc. NH_4OH at 55 °C or 65 °C for 16 h), each solvent was evaporated under reduced pressure. The residues were dissolved in water and subjected to HPLC analyses using a reversed-phase column. Figure 2a and Figure S1 show the chromatograms of the HPLC analyses before and after the alkaline treatments (AMA at 65 °C, conc. NH_4OH at 55 °C). Percentages of each peaks generated from the incubations in conc. NH_4OH at 55 °C and 65 °C are listed in Table S1. The results of molecular-weight analyses of the decomposed products (P1–P5) are listed in Table S2.

4.4. Labeling of amino-modified ONTs

The 3′-amino-modified ONT (150 pmol) was reacted with FITC (150 nmol) or biotin succinimidyl ester (15 nmol) in a solution (100 μL) containing 250 mM phosphate buffer (pH 8.0) and 10% dimethylformamide (DMF) at 25 °C. After 30 min (for biotin succinimidyl ester) or 60 min (for FITC), the reaction mixture was desalted with a cartridge column (NAP5) and the products were analyzed by HPLC equipped with a reversed-phase column and a photodiode array detector (Waters). The percentage of the product was determined by HPLC analysis.

Labeling with PEG-NHS (75 nmol) was carried out as described for the reaction with biotin-NHS but in the absence of DMF. We used branched PEG (SUNBRIGHTGL 2-400GS2, NOF Corp.).

4.5. Cross-linking reaction between amino linkers on a blunt end of a duplex

Amino-modified duplexes of complementary 25 bases (25-revH/ssH-25 or 25-C6/C6-25; 200 pmol) were dissolved in 150 mM phosphate buffer (pH 8; 200 μL). The solution was heated at 90 °C for 1 min and then cooled to room temperature. A 0.4 mM solution of disuccinimidyl glutarate (Thermo) dissolved in DMF (50 μL) was added, and the reaction solution was incubated at 27 °C. After 5 min, the reaction mixtures were analyzed as described for the analysis of labeled ONTs.

4.6. Preparation of a CL-AMO from cross-links of amino linkers

Oligo-I (1.0 nmol), oligo-II (1.2 nmol), and oligo-III (1.2 nmol) were dissolved in 156 mM phosphate buffer (200 μL), heated at 90 °C for 1 min, and then annealed to room temperature. The solution was placed on an ice bath for 5 min, followed by the addition of 2 mM DSG dissolved in DMF (5 μL). The reaction solution was incubated at 17 °C for 3 h, and CL-AMO was purified by reversed-phase HPLC. The molecular weight of CL-AMO, which was measured using liquid chromatography–mass spectrometry, was observed to be nearly identical to the calculated value: calcd 17,815.48, found 17,818.06.

4.7. Luciferase assays

A target sequence complementary to mature miR-21 was inserted into the 3′ untranslated region of the Renilla luciferase (hRluc) gene of psiCHECK-2 vector (Promega), providing the plasmid of psiCHECK-2-miR21, which contained both hRluc and firefly luciferase genes. HeLa cells were seeded at densities of 2×10^4 cells per well in 96 well plates in DMEM (200 μL) containing 10% FBS the day before transfection. After an aliquot (100 μL) was removed from each well, the cells were transfected in triplicate with DMEM solution (10 μL) containing Lipofectamine 2000 (Invitrogen; 0.3 μL), psiCHECK-2-miR21 (100 ng), and AMOs. The concentrations of the AMOs were varied from 0 to 10 nM.

Assays were performed 48 h post-transfection according to the manufacturer's instructions. Renilla luciferase/firefly luciferase (Rluc/Fluc) values are the average of triplicate wells. All values were normalized by the ratio of the plasmid signal to the control plasmid (psiCHECK-2) signal.

Acknowledgments

We thank Drs. Yu Hirano and Yasuhiro Mie (Bioproduction Research Institute, National Institute of Advanced Industrial

Science and Technology) for helpful discussions. Financial support was received, in part, from the Ministry of Education, Culture, Sports, Science and Technology, Japan (Grant-in-Aid for Scientific Research (No. 25293030)), and Akiyama Life Science Foundation.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2016.03.039.

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