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Novel amino linkers enabling efficient labeling and convenient purification of amino-modified oligonucleotides

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Abstract—We developed new amino linker reagents for an oligonucleotide (ONT) terminus. These reagents consist of an aminoethyl carbamate main linkage and a side-chain residue, which was a naphthylmethoxymethyl, methoxymethyl, or methyl group or a hydrogen atom. The primary amine was protected with a monomethoxytrityl (MMT) group. The chemical properties of ONTs containing these amino-modifications were investigated. The MMT group of these amino-modifications could be quite rapidly removed from the amine under very mild acidic conditions, which are not strong enough for the deprotection of a conventional aliphatic amine. This significant feature enabled the amino-modified ONTs to be conveniently purified with a reverse phase column. Furthermore, the amino-modifications efficiently reacted to active esters, as compared with other amino-modifications. We also found that the pK_a values of the amino-modifications were lower than that of the aliphatic amine. All of the experimental results showed that these chemical properties are closely related to their structures. We report here the chemical properties and the availability of the new amino linker reagents.

1. Introduction

DNA and RNA detection techniques have been remarkably advanced by including physicochemical techniques. Oligonucleotide array and beads-based systems are representative technologies, which utilize chemically synthesized oligonucleotides (ONTs). There are in situ synthesis and deposition methods for the preparation of oligonucleotide arrays. In the in situ synthetic method, ONT probes are directly synthesized on a solid surface in combination with the standard amidite chemistry,[1,2] photolithography[3,4] or electrochemical techniques.[5] In the deposition method, synthesized ONT probes are spotted on the array surface by an ink-jet[6] or thin needles,[7,8] and then become immobilized on the surface by forming a covalent bond. ONT probes are also chemically immobilized on micro beads by immersing the beads in a reaction solution. The probe immobilization requires functional groups, such as an aliphatic amine or a sulfhydryl group at the ONT terminus, and the modified ONT probes are used after purification. This purification process is not carried out for in situ synthetic method.

A sulfhydryl group is frequently used for ONT-immobilization on a gold surface.[9–11] However, a sulfhydryl group requires thiol-protection, to prevent intermolecular dimerization, and this protecting group should remain attached to the sulfhydryl group until just before the ONTs are used. In addition, the sulfhydryl modification is expensive. Although there are other terminal modifications such as hydrazide,[12] anthraquinone,[13] and acrylamide,[14] these modifications yield side products under the standard deprotecting conditions for synthesized ONTs. On the other hand, an aliphatic amine is cost-effective and is very easy to handle in comparison with other modifications. From this point of view,

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amino-modification is frequently used for the preparation of diverse ONT probes.[15,16]

An amino hexyl amidite reagent is a typical aliphatic amino-modification,[17,18] and the amino group is introduced to the 5′-end of the ONT at the final step of the chemical synthesis. There are mainly two kinds of commercially available reagents, which differ in the protecting groups for the primary amine. One is a monomethoxytrityl (MMT) group, and the other bears a trifluoroacetyl group (TFA) as the amino-protection. The MMT group assists in the purification of the amino-modified ONTs by reverse phase column chromatography, due to its strong hydrophobicity; however, the removal of the MMT requires stringent acid treatment. The stringent acidic treatment is unfavorable for deoxyribonucleotides, because it leads to the generation of apurinic sites. Furthermore, the long deprotection time is disadvantageous in terms of the high throughput preparation of diverse ONT probes. On the other hand, the TFA group is easily removed during the standard alkaline deprotection step, but it is difficult to purify the amino-modified ONTs from impurities, such as defective ONTs, because there is little difference in the hydrophobicity between the amino-modified ONTs and the defective ONTs, which fail to combine with the amino linker reagent. Therefore, some problems still exist in the current procedure to achieve the high throughput preparation of an amino-modified ONT library. In addition, the reaction efficiency of the amino-modification should be increased in the labeling reaction in aqueous solution.

We previously constructed new types of amino linker reagents with aromatic residues.[19,20] The introduction of the aromatic residue resulted in an increase of the labeled products in the coupling reactions with active esters, due to the enhanced hydrophobic interaction between the aromatic residue and the target molecule. However, these amino linker reagents require many synthetic steps, and the high throughput purification of the ONTs was insufficient.

In our further development of amino linker reagents, we found that a simple aminoethyl carbamate structure can improve the reaction efficiencies and the purification process, in comparison with the conventional amino-modification. This amino modification is very useful for not only labeling of ONTs but also preparation of diverse probes for gene expression analysis. We report here the properties and the application of this amino-modification for the ONT-terminus.

2. Results and discussion

2.1. Synthesis of amino linker reagents containing an aminoethyl carbamate linkage

We previously reported amino linker reagents, which were connected to an aromatic residue by various alkyl linkers.[19,20] The amino-modified ONTs were efficiently labeled with a fluorophore in aqueous solution. Among those amino linker reagents, one amino linker reagent, ssN, which consists of a naphthylmethoxymethyl group at an aminoethyl carbamate linkage displayed the most efficient reactivity. To examine the high reactivity of the ssN-modification, we synthesized three amino linker amidite units, with methoxymethyl (ssMeO), methyl (ssMe), and hydrogen (ssH) in place of the naphthylmethoxymethyl group of the ssN (Fig. 1). All of these amino linker reagents (ssR; R = N, MeO, Me, H) had an aminoethyl carbamate structure in the main chain. In the first report describing the ssN-modification, the primary amine of the reagent was protected by a trifluoroacetyl group (TFA). Since the TFA was removed by the standard alkaline treatment, the hydrophilic amino group was exposed. The hydrophobicity of only the naphthalene residue was insufficient to retain the amino-modified ONTs within a reverse phase column. Thus, there were still some difficulties in purifying the ssN-modified ONTs with a reverse phase column. To achieve easier purification by using a reverse phase column, all of the primary amines of the ssR-amidite units were prepared with monomethoxytrityl (MMT) protection, which is highly hydrophobic. However, the removal of an MMT group from a primary amine requires a stringent acid treatment, and the MMT group thus has a great disadvantage in terms of high throughput and convenient purification of diverse amino-modified ONTs. Therefore, various deprotecting conditions for the MMT group were first examined.

2.2. MMT-deprotection reaction

We synthesized amino-modified ONTs of 25-bases, which had the MMT group at the 5′-terminus (MMT-X-25; Fig. 2a). The commercially available C5 reagent, which bears an aminoethoxyethyl structure, and a conventional aliphatic amino reagent (Con) were used for the preparation of control ONTs (Fig. 2b). After aqueous ammonia treatment of these ONTs, we checked

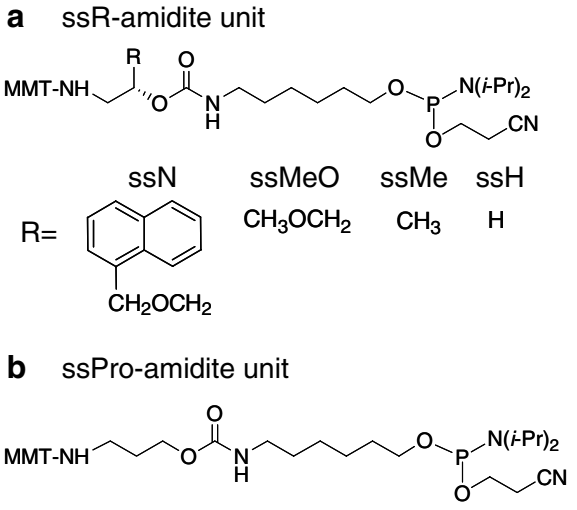

Figure 1. Structures of amino linker amidite reagents. (a) ssR-amidite units. (b) ssPro-amidite unit. R indicates a naphthylmethoxymethyl (N), methoxymethyl (MeO), or methyl (Me) group or a hydrogen atom (H).

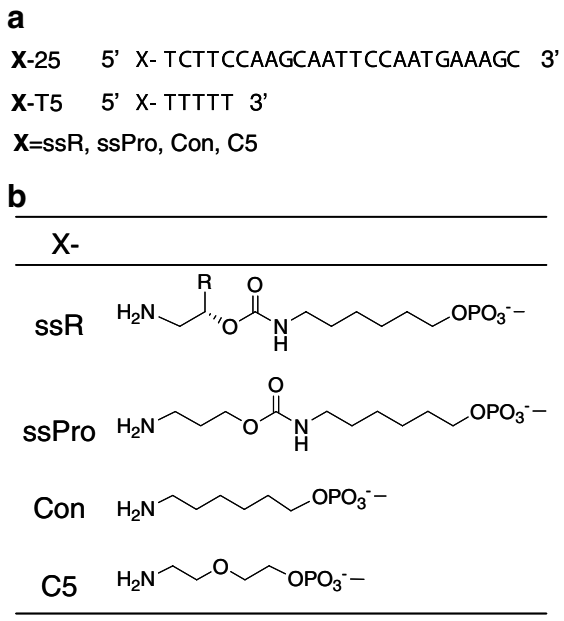

Figure 2. Amino-modified ONTs (X-25 and X-T5). (a) Sequences of X-25 and X-T5. X indicates ssR, ssPro, Con or C5. (b) 5′-Terminal structures of amino-modified ONTs. Con and C5 were used as control modifications.

the acid lability of the MMT group of each amino-modification. First, MMT-X-25 was treated with an 80% acetic acid solution for 20 min, and the ratios of 'trityl off' ONTs were calculated from the HPLC analysis (Table 1). Interestingly, the MMT groups were completely deprotected from the MMT-ssR-25 under these conditions, whereas MMT-Con-25 gave only 20% 'trityl off' products. The same reactions were then performed under milder conditions (1% acetic acid). The MMT groups of the ssR were still removed very fast, within 10 min, under the mild acidic conditions (Table 1), whereas MMT-Con-25 gave only a small amount of 'trityl off' ONTs. The observed rate constants in the 1% acetic acid solution were obtained (Table 1). Although ssH showed a slightly slower deprotection rate than the other ssR, the rate of ssH was still much faster than that of Con-modification. The deprotection rate of the C5-modification was an intermediate value between Con and ssH. The fast MMT-deprotection of the ssR-modification was also observed in other ONTs with different sequences (data not shown).

To investigate the structure–function relationship more precisely, an amino linker reagent with an aminopropyl carbamate linkage (ssPro) was synthesized (Figs. 1b and 2a), and the MMT deprotection rate was measured. ssPro showed a much slower deprotection rate than ssH, even though the structural difference is only one methylene between these molecules. The results obtained from the ssR, ssPro, and C5-modifications indicated that the aminoethyl carbamate linkage is an adequate structure for the fast removal of the MMT group from the primary amine under mild acidic conditions.

2.3. Cartridge column purification

Trityl groups can facilitate the purification of trityl-protected ('Tr on') ONTs with the use of a reverse phase column, because the trityl groups have high affinity to the reverse phase resin. Especially, the dimethoxytrityl (DMT) group, which is a protecting group for a primary alcohol of the 5′-terminus, enables ONTs to be conveniently purified, because the DMT can be rapidly removed within the resin, by passing an acidic solution through the column. On the other hand, the MMT group protecting an amine cannot be deprotected completely within the column resin, even by adding an acidic solution.

Since it was found that the MMT deprotection rate of the ssR was very fast under mild acidic conditions, we investigated whether the ssR-modified ONTs would be rapidly purified by using a cartridge column filled with reverse resin. ssH, which showed the slowest deprotection rate among the ssR-modifications, was tested in the cartridge purification. After ssH- or Con-modified ONTs (MMT-ssH-25, MMT-Con-25) were subjected to the standard alkaline treatment, each ONT was loaded on the column resin. After the resin was washed, 3% aqueous trifluoroacetic acid was passed through the column to promote the MMT-deprotection. The total exposure times to the acidic solution for the ssH- and Con-modified ONTs were 10 or 60 min. After neutralization of the column resin, the amino-modified ONTs were eluted. HPLC analysis of each eluted fraction indicated that ssH-25 could be purified by the 10-min acid treatment but Con-25 still retained the MMT group even after the longer acid treatment (Supplementary data). The 10-min treatment of Con-25 yielded almost all of the ONT in the 'Tr on' form. We also found that other ssH-modified ONTs of 40 bases could be purified with a cartridge column (Supplementary data). These results indicate that the ssR-modification enables the rapid and high quality purification of the amino-modified ONTs with the use of a cartridge column, and this feature provides a great advantage for the high throughput purification of diverse ONT probes.

Table 1. Analysis of MMT-deprotection reactions by treatment with aqueous acetic acid (AcOH)

	Con	ssN	ssMeO	ssMe	ssH	C5	ssPro
80% AcOH (20 min)[a]	20.7	100	100	100	100	91.5	82.5
1% AcOH (pH 2.7; 10 min)[a]	0	97.3	100	97.6	88.5	45.0	32.1
1% AcOH (pH 2.7; 20 min)[a]	4.5	98.7	100	100	98.4	79.4	46.5
k_{obs} (min^{-1})[b]	N.D.	0.37	1.3	0.49	0.25	0.067	0.033

N.D., not detected.

[a] Percentages of 'Tr off' amino-modified ONTs (X-25) after 10 or 20-min reaction.

[b] Observed rate constants were calculated from the percentages of the products, which were generated from 1% acetic acid treatment.

2.4. Labeling reactions with active esters

Amino-modified ONTs are frequently used as biological probes by conjugation with various reporter groups, such as fluorophores or biotin. Therefore, we investigated the labeling efficiency of the ssR-modification, using succinimidyl (NHS) esters (Cy3-NHS and biotin-NHS) and isothiocyanate group (FITC). These reactions were carried out under standard labeling conditions using bicarbonate (pH 9) or phosphate (pH 8) buffers.

First, the labeling reactions to Cy3-NHS and biotin-NHS were examined. The ssR-modified ONTs (ssN-25, ssMeO-25, ssMe-25, and ssH-25) were labeled less efficiently than Con-25 in the bicarbonate buffer (Fig. 3a and b; white bars). On the other hand, the same coupling reactions using the phosphate buffer resulted in a drastic increase in the product for the ssR (Fig. 3a and b; black bars). Especially, the ssN- and ssMeO-modifications gave approximately 6-fold higher amounts of the labeled products by this buffer change. C5 and ssPro also yielded slightly increased labeled products in the phosphate buffer. In contrast to the ssR-modifications, Con-25 did not exhibit any difference in the labeling efficiency between both reaction buffers.

In contrast to the NHS ester, FITC reacted with the ssR-modified ONTs efficiently in both the bicarbonate and phosphate buffers (Fig. 3c). The labeling efficiency of the ssR-modifications remarkably depended on the side-chain size. On the other hand, the Con-modification decreased the reaction products in the phosphate buffer. Since the pK_a value of the aliphatic amine (Con) is about 11 (the pK_a is described in Section 2.5), it is generally thought that the decrease in the labeled product is derived from the increase in the protonated amino form, by changing the buffer's pH from pH 9 to 8. Although the reactions of the ssN, ssMeO, and ssMe-modifications were not influenced by this buffer change, the labeled products of ssH were slightly decreased. The C5- and ssPro-modifications showed similar reactivities to Con-modification.

The results of the labeling reactions revealed that the reaction property of the ssR was different from that of the standard aliphatic amine, and an apparent structure–function relationship was observed from the reactions of ssR, ssPro, and C5. Among all of the reactions, the ssR-NHS ester reactions performed in the carbonate buffer were quite characteristic. To further examine the effect of the buffer composition on the ssR reactivity, the labeling reactions of ssN-25, ssH-25, and Con-25 to biotin-NHS were carried out with various concentrations of the three reaction buffers [bicarbonate (pH 8, 9), phosphate (pH 8) and borate (pH 8)]. These reactions revealed that the ssN- and ssH-modified ONTs reacted to the NHS ester more efficiently than the Con-

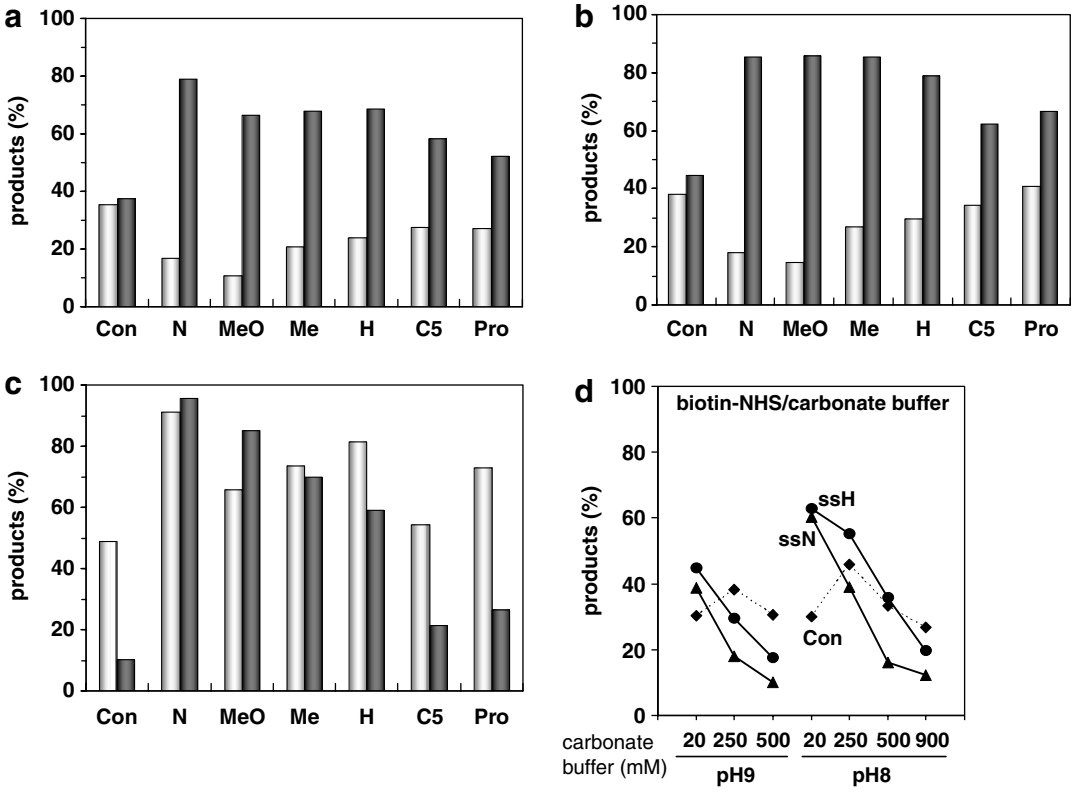

Figure 3. Labeling reactions of X-25 (X = Con, ssN, ssMeO, ssMe, ssH, C5, ssPro) with reporter groups. Each ONT was labeled with Cy3-NHS (a) biotin-NHS (b) or FITC (c). The reactions were carried out in 250 mM carbonate buffer (pH 9; white bars) or 250 mM sodium phosphate buffer (pH 8; black bars) for 30 min. Con-25 (solid diamonds), ssH-25 (solid circles) and ssN-25 (solid triangles) were labeled with biotin-NHS in the presence of various concentrations of the carbonate buffer (pH 8 and 9) (d). Percentages of the products are plotted versus the buffer concentrations.

modified ONT in either the phosphate or the borate buffer, and the percentages of the products were the same at all concentrations (Supplementary data). On the other hand, a decrease in the product was observed as the concentration of the bicarbonate buffer increased (Fig. 3d). It is unclear why the ssR reaction to the NHS ester was inhibited at high bicarbonate ion concentrations. There might be some interactions between the aminoethyl carbamate structure and the bicarbonate ions. However, the results show that the labeling reaction proceeds efficiently at the low concentrations (about 20 mM) of the bicarbonate ions.

2.5. pK_a measurements

The pK_a is an important factor to characterize the amino group, and thus the pK_a value of each amino-modification was examined. In order to determine the pK_a, each cognate alcohol unit of ssN, ssH, C5, and Con was prepared. ^{13}C NMR analyses of these monomer units (ssN_{OH}, ssH_{OH}, $C5_{OH}$, Con_{OH}) were measured in various pH buffers, and each pK_a value was determined from the chemical shift changes[21,22] (Supplementary data). The pK_a values of ssN_{OH}, ssH_{OH}, $C5_{OH}$ and Con_{OH} were found to be 8.9, 9.6, 10.1, and 11.0, respectively. The ssN_{OH} and ssH_{OH} units containing the aminoethyl carbamate linkage, showed lower pK_a values than the aliphatic amine, and the pK_a values of ssN_{OH} and ssH_{OH} are probably derived from the electron withdrawing effect of the carbamate linkage. This effect might be also responsible for the rapid MMT removal and the efficient labeling reaction. The difference in the pK_a values between ssN_{OH} and ssH_{OH} is notable. The pK_a difference is probably related to their tertiary structures induced by the side chains at the aminoethyl carbamate linkage.

2.6. Computational simulations

The ssR-modification exhibited unique chemical properties, which are expressed by the presence of the aminoethyl carbamate structure. In addition, the chemical properties of the ssR series depended on the side-chain residues. Especially, ssN showed the highest reactivity among the ssR series, and therefore, we performed a computational simulation of the ssN-monomer (ssN_{OH}) to elucidate the structural effect based on the main and side chains. MacroModel version 9.0 was used for the computational simulation,[23] and most stable structure of ssN_{OH} is shown in Figure 4. The modeling of ssN_{OH} indicates that the primary amine and the naphthylmethoxymethyl residue are aligned with an *anti*-conformation, and that the primary amine is close to the O4 oxygen atom of the carbamate linkage. This conformation allows a hydrogen bond between the amino proton and the oxygen atom. If the primary amine forms an intramolecular hydrogen bond with the O4 atom, then the nucleophilicity of the amine (N1) might be increased, leading to reduction in the pK_a, which is consistent with the results of the pK_a measurement.

The stability of the *anti*-conformation is thought to depend on the bulkiness of the side-chain residue on the C3 atom of ssR. Actually, the MMT-deprotection rate and the labeling efficiency were also related to the R residues. Although we could not detect this intramolecular hydrogen bond by the NMR measurement, this structural effect induced by the side chain is probably related to the chemical properties of the ssR-modification. ssH, C5, and ssPro have also the potential to form the conformation of five- or six-membered rings by forming the intramolecular hydrogen bonds; however, these modifications have no side-chain residues, and the conformations might not be stabilized unlike ssN. Therefore, the reactivities of these modifications would mainly associate with the electron withdrawing inductive effect of the main chain structures as discussed in the pK_a measurement rather than the hydrogen bond factor.

2.7. Stability of ssR-modified ONTs

If the ssN adopts the *anti*-conformation, as predicted in the computational simulation, then *trans*-acylation may occur at the primary amine. Therefore, to examine the stability of the ssR-modified ONTs, penta thymidylic acids containing ssR- or Con-modifications (ssR-T5 and Con-T5; Fig. 2) were subjected to the standard aqueous ammonia treatment without MMT-protection, and the reactions were analyzed by reverse phase HPLC.

Aqueous ammonia treatment of ssN-T5 provided new two peaks (P1 and P2), in addition to the starting material (ssN-T5) (Fig. 5a). Both products were purified by HPLC, and their molecular weights were measured. P1 was identified as Con-T5 from its molecular weight and the retention time of the HPLC analysis. Although the molecular weight of P2 was the same as that of ssN-T5, the retention time of P2 was different from that of ssN-T5. Alkaline treatment of ssR-T5 also yielded the same decomposition pattern as ssN-T5 (Supplementary data), but the product yields were different from each other (Fig. 5b) and depended on the R residue on the carbamate linkage. ssH-T5 provided the least amount of the products among the ssR series, and ssPro-T5 gave much less product than ssH-T5, while no side products were found with the same treatment of the Con-modification. These reactions required heating conditions and did not proceed under the labeling conditions [at 40 °C in bicarbonate buffer (pH 9)]. Furthermore, the P1 and P2 products were not detected after the alkaline treatment of MMT-protected ONTs. This result shows that

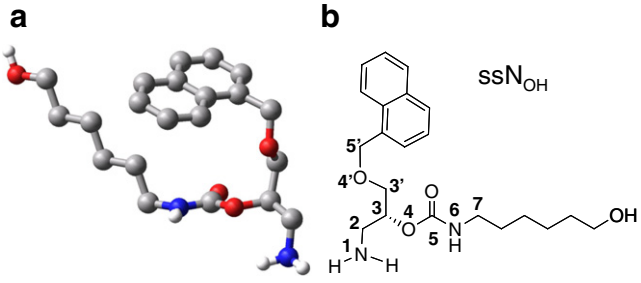

Figure 4. Computational analysis of the ssN-monomer (ssN_{OH}). The most stable *anti*-conformation structure (a) and its schematic drawing (b).

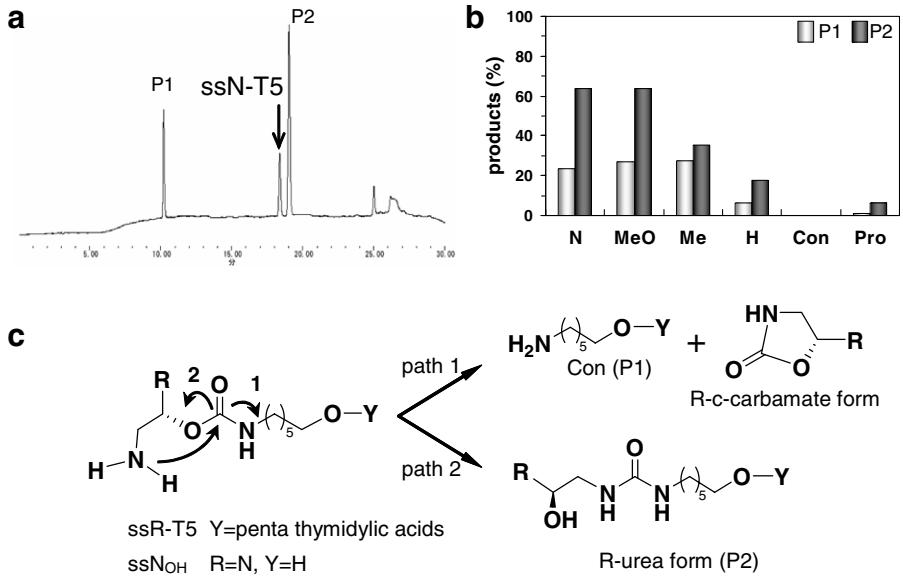

Figure 5. Stability of free ssR-modifications under heated alkaline conditions. (a) HPLC analysis of ssN-T5 without trityl-protection after aqueous ammonia treatment at 60 °C for 16 hr. (b) Products (P1 and P2) of each ONT after alkaline treatment at 60 °C for 16 hr. White and black bars indicate percentages of P1 and P2. (c) Scheme of the intramolecular reaction of ssR-modified ONT and ssN-monomer (ssN$_{OH}$). Y indicates ONT or a hydrogen atom. Aliphatic amine and cyclic-carbamate (c-carbamate) forms were generated by path 1, and the urea-form was generated by path 2.

the presence of a free primary amine of the ssR is associated with the decomposition reaction.

To investigate this reaction mechanism, the ssN-monomer unit (ssN$_{OH}$, Fig. 5; R = N, Y = a hydrogen atom) was also subjected to the alkaline treatment. As expected, two main products with UV-absorbance were obtained, and their structures were determined to be cyclic carbamate (c-carbamate; yield 11%) and urea-ssN$_{OH}$ forms (yield 52%), as shown in Figure 5. These products were generated from two reaction routes, which are triggered by the attack of the primary amine. From this result, it is thought that P1 and P2, generated from the alkaline treatment of ssR-T5, were Con-T5 and urea-R-T5 (Fig. 5c; Y = penta thymidylic acid), and this identification is consistent with the results of the molecular weight and the HPLC analyses. P1 and P2 of each ssR-T5 were subjected to labeling reactions with FITC, which revealed that only P1 was labeled with FITC. This result is consistent with the identification that P1 is Con-T5 and P2 is urea-R-T5 lacking the primary amine. The efficiency of the intramolecular *trans*-acylation depended on the R residue. This result might be derived from the *anti*-conformational effect, as shown in Figure 4, which places the primary amine proximal to the carboxyl group.

2.8. Application to microarray

It was proven that ssR modification enables high throughput purification and increases reactivity to active esters. Then, to investigate their capability as microarray probes, ssH-modified ONTs were subjected to a microarray analysis. The RNA expression of cell lines (K562 and HepG2) was analyzed using microarrays with immobilized ssH-modified ONTs. All of the ONTs consisted of poly(dT)$_{10}$-linkers and 30-base capture sequences, which were designed to be complementary to the *anti*-sense RNAs (aRNAs) of selected endogenous mRNAs. These ONTs were chemically synthesized and purified with a reverse phase column, according to our purification protocol. All of the ONTs were highly purified with high quality and then were immobilized on a plastic slide. The aRNAs were generated from total RNA preparations of K562 and HepG2 cells, followed by labeling with Cy3 and Cy5, respectively.

The relative RNA expression levels of these cell lines were obtained from microarray analyses (Fig. 6). A quantitative PCR (QPCR) experiment was also performed using the same RNAs. The expression ratios of genes obtained using microarray were compared to those obtained using QPCR. For a few genes, namely

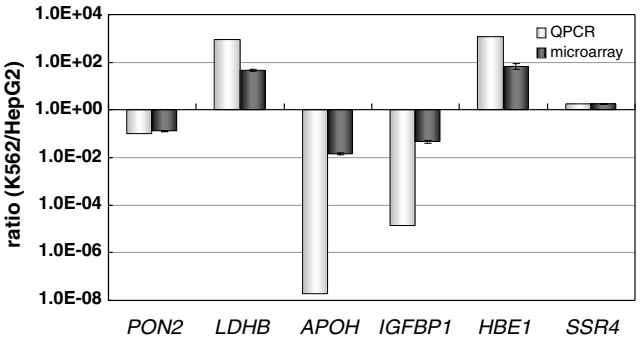

Figure 6. Microarray analysis using ssH-modified ONTs as probes. The gene expression of two cell lines was examined by the microarray and quantitative PCR (QPCR). Concordance in the profiling of gene expression is shown.

APOH and *IGFBP1*, the ratios of QPCR were larger than those of microarray. This is partly due to the difference in calculation methods. Overall, the direction of the expression ratios of all genes was concordant to each other for both systems. This result demonstrates that ssH-modified ONTs are applicable to microarray analyses.

3. Conclusions

We have developed novel amino linker reagents (ssR), which have an aminoethyl carbamate structure. The aminoethyl carbamate structure increased both the MMT-deprotection rate and reactivity to active esters, as compared to a standard aliphatic amine. The fast deprotection of the MMT group from the aminoethyl carbamate linkage facilitated the rapid and convenient purification of the amino-modified ONTs with the reverse phase cartridge column. The pK_a value of the aminoethyl carbamate structure became lower than that of the aliphatic amine. These significant properties were derived from the aminoethyl carbamate structure and the substituent at the linkage. Computational simulation of the ssN-monomer, which contains a naphthylmethoxymethyl group on the main chain, revealed the stable *anti*-conformation between the primary amine and the naphthylmethoxymethyl residue, leading to an intramolecular hydrogen bond between the primary amine and the oxygen atom of the carbamate structure. Due to the *anti*-conformation, intramolecular *trans*-acylation occurred at the free aminoethyl carbamate structure under heated alkaline conditions, and this intramolecular reaction depended on the substituent size.

The terminal modification containing an aminoethyl carbamate linkage was superior to the aliphatic amino-modification, in terms of the reactivity and the purification. Especially, ssH lacking an asymmetric atom was the most stable and cost-effective modification. It was proven that ssH-modified ONTs could function as microarray probes. We have revealed here the unique chemical properties of the amino-modification containing an aminoethyl carbamate linkage. The simple aminoethyl carbamate structure is a useful modification for the functionalization of ONT termini.

4. Experimental

4.1. Synthesis of phosphoramidite units

The syntheses of the ssN, ssMeO, ssMe, ssH, and ssPro phosphoramidite units are presented in the Supplementary data.

4.2. Synthesis and deprotection of oligonucleotides

All oligonucleotides (ONTs) were chemically synthesized by using standard phosphoramidite chemistry. The amidite units of the canonical bases and the control amino linker reagents (5′-amino-modifier C6-MMT, 5′-amino-modifier C5) were purchased from Glen Research. All amino linker units were coupled for 600 s at the final step of the ONT synthesis. The coupling efficiencies of ssR and Con were calculated by measuring the 478 nm absorbance of the monomethoxytrityl group, which was removed from the termini. Cleavage from the CPG support and deprotection of the ONTs were carried out by incubating the CPG in concentrated ammonium hydroxide (2 mL) at 55 °C for 16 h.

4.3. Cartridge column purification of 5′-MMT-oligonucleotides

A reverse phase cartridge column (YMC, C18, 500 mg) was washed with acetonitrile (6 mL) and was equilibrated with 0.2 M TEAA (6 mL). After the deprotection of amino-modified oligonucleotides (MMT-ssR-25, MMT-Con-25, MMT-ssR-T5, and MMT-Con-T5), a concentrated ammonium hydroxide solution was mixed with the same volume of 0.2 M TEAA, and then, the solution was loaded on the cartridge column. The column was washed with 0.2 M TEAA (4.2 mL), 10% acetonitrile-0.2 M TEAA (4 mL), and distilled water (3 mL). To remove the MMT group from the primary amine, 3% aqueous trifluoroacetic acid (TFA; 5 mL) was slowly passed through the column over 10-min period (60 min for the Con-modified ONT). The column was washed with distilled water (3 mL), 0.1 M TEAA (3 mL), and distilled water (3 mL) to neutralize and desalt the column resin. Finally, the amino-modified ONTs were eluted from the column with 20% aqueous acetonitrile (3 mL). The oligonucleotides were further analyzed by HPLC, using reverse phase column chromatography.

4.4. Analysis of MMT-deprotection from the synthesized ONTs

After the standard deprotection step of the amino-modified ONTs (MMT-Con-25, MMT-ssR-25, MMT-ssPro-25, MMT-C5-25; 0.2 μmol), an aliquot (10 μL) was taken from the concentrated ammonium hydroxide solution (2 mL), and was immediately transferred into distilled water (5 μL). The solution was evaporated under reduced pressure. Aqueous acetic or trifluoroacetic acid solutions of various concentrations (25 μL) were added to the amino-modified ONTs to deblock the trityl group. Aliquots (5 μL each) were taken from the reaction solution at time intervals and were neutralized with a dilute aqueous ammonia solution. The percentages of 'trityl off' products were determined by an HPLC analysis, using a reverse phase column. These HPLC charts are presented in the Supplementary data. The observed rate constants of ssN, ssMeO, ssMe, and ssH were calculated from the reactions using a 1% aqueous acetic acid solution.

4.5. Coupling reaction with reporter groups

The amino-modified ONTs (1 nmol) were reacted with an active ester group (Cy5-succinimidyl ester, biotin-succinimidyl ester, fluorescein isothiocyanate (FITC)) in a solution (100 μL) containing 250 mM sodium carbonate buffer (Na_2CO_3–$NaHCO_3$, pH 9) and 10%

dimethylformamide. The buffer composition was changed to 250 mM sodium phosphate (pH 8) or 100 mM sodium tetraborate (pH 8), and labeling reactions were also carried out. The concentrations of the succinimidyl ester groups (Cy3 and biotin) were 0.3 mM, and the FITC labeling reactions were carried out at 5 mM. Aliquots were taken from the reaction solutions after 30 and 60 min, and were desalted with a cartridge column (NAP5, Amersham Pharmacia). The products were analyzed by HPLC, using a reverse phase column and a photodiode array detector (Waters). The percentages of the products at each time point were determined from the HPLC analysis.

To study the effect of the carbonate ion on the coupling reaction, 10 μM of the amino-modified ONT (Con-25, ssN-25) was reacted with 0.3 mM biotin-NHS in the presence of 250 mM sodium phosphate buffer (pH 8), containing 0, 50, 100, 250 or 500 mM sodium carbonate buffer (pH 8). The reactions were analyzed by the same method as that used for the coupling reaction described above.

4.6. Computational simulation and pK_a determination

See the Supplementary data.

4.7. Alkaline treatment of amino-modified ONTs

After the amino-modified ONTs (Con-T5, ssR-T5, ssPro-T5; 50 nmol) were purified on the reverse phase column, they were dissolved in concentrated aqueous ammonia (3.3 mL), and heated at 60 °C. After 16 h, the ammonia was removed under reduced pressure, and then the residue was dissolved in distilled water (1100 μL). The products (P1 and P2) were purified by HPLC, and the molecular weights were determined by MALDI-TOF/MS measurements (Supplementary data). P1 and P2 were mixed with Con-T5 and the starting material (ssR-T5, Con-T5 or ssPro-T5), respectively, and the HPLC analysis using the reverse phase column was carried out to compare the retention times.

4.8. Microarray analyses of mRNAs collected from cell lines

ssH-modified ONTs were designed and synthesized to detect *anti*-sense RNAs (aRNAs) of *PON2*, *LDHB*, *APOH*, *IGFBPI*, *HBE1*, *GAPDH*, and *SSR4* (Supplementary data). All ssH-modified ONTs were purified with cartridge columns, according to our purification protocol.

Microarrays were fabricated using a Prime Surface kit^R (BS-11607 Sumitomo Bakelite Co., Ltd.). The ssH-modified ONTs were dissolved in the spotting buffer to a concentration of 10 μM, and then each ONT was deposited in triplicate on the plastic plate with a spotting machine (MARKS-I, Hitachi Software Engineering, Co., Ltd.). Cy3 or Cy5 labeled *anti*-sense RNA (aRNA) was prepared from HepG2 and K562 cells, and then these aRNAs were subjected to microarray analyses (Supplementary data). QPCR was also carried out, according to the standard method (Supplementary data).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.10.011.

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