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Bioconjugation of Oligodeoxynucleotides Carrying 1,4-Dicarbonyl Groups via Reductive Amination with Lysine Residues

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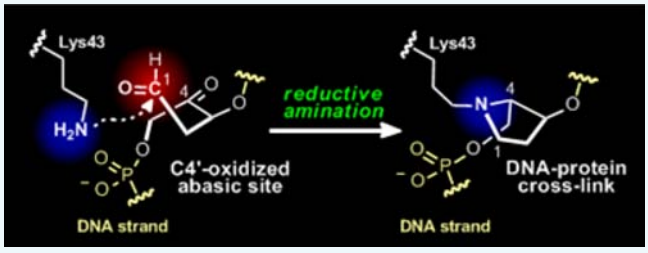
Supporting Information

ABSTRACT: We evaluated the efficacy of bioconjugation of oligodeoxynucleotides (ODNs) containing 1,4-dicarbonyl groups, a C4′-oxidized abasic site (**OAS**), and a newly designed 2′-methoxy analogue, via reductive amination with lysine residues. Dicarbonyls, aldehyde and ketone at C1- and C4-positions of deoxyribose in the ring-opened form of **OAS** allowed efficient reaction with amines. Kinetic studies indicated that reductive amination of **OAS**-containing ODNs with a proximal amine on the complementary strand proceeded 10 times faster than the corresponding reaction of an ODN containing an abasic site with C1-aldehyde. Efficient reductive amination between the DNA-binding domain of *Escherichia coli* DnaA protein and ODNs carrying **OAS** in the DnaA-binding sequence proceeded at the lysine residue in proximity to the phosphate group at the 5′-position of the **OAS**, in contrast to unsuccessful conjugation with abasic site ODNs, even though they have similar aldehydes. Theoretical calculation indicated that the C1-aldehyde of **OAS** was more accessible to the target lysine than that of the abasic site. These results demonstrate the potential utility of cross-linking strategies that use dicarbonyl-containing ODNs for the study of protein−nucleic acid interactions. Conjugation with a lysine-containing peptide that lacked specific affinity for ODN was also successful, further highlighting the advantages of 1,4-dicarbonyls.



INTRODUCTION

Bioconjugation of oligodeoxynucleotides (ODNs) with biomolecules that interact with DNA provides information about the interactions, structures, and functions of the biomolecules in question.[1,2] Bioconjugation of ODN has also been used to introduce useful functions into ODN (e.g., therapeutic nucleic acids).[3,4] Nucleic acids containing carbonyl groups have been used extensively for conjugate formation and labeling applications.[5−9] The reactivity of the carbonyl groups and their stability in aqueous environments are advantageous for the modification of biomolecules at naturally available amino groups. An ODN containing an abasic site is an example of such a reactive species; the predominant ring-closed form exists in equilibrium with the ring-opened form (**1**) bearing an aldehyde group at the C1-position of deoxyribose (C1-aldehyde). It reacts with an amine-containing molecule to form a Schiff base, and the following reduction affords conjugate **2** (Scheme 1).[10,11] Its reductive amination with biomolecules that interact with nucleic acids, such as complementary DNA strands, enzymes, and proteins, yields cross-linked products at the amino groups in proximity to the aldehyde.[12,13] The C4′-oxidized abasic site (**OAS**) is one of the oxidatively damaged DNA lesions induced by antitumor bleomycins.[14] **OAS** also exists as an equilibrating mixture of the predominant ring-closed hemiacetal and ring-opened form with C1-aldehyde and C4-ketone (**3**) (Scheme1).[15] We found that **OAS** reacted efficiently with a primary amine under mild conditions to yield lactam **4** and ODN fragments; this did not require additional reagents.[16−18] The ability of the 1,4-dicarbonyl system in **3** to form kinetically and thermodynamically favored five-membered-ring aminal **5**[19] results in facile lactam formation. We envisioned that bioconjugation of an ODN containing an **OAS** with lysine residues in close proximity to the C1-aldehyde and/or the C-4 ketone in the ODN−protein complex might be effective. In addition, a cooperative reaction of two carbonyl groups in the DNA structure might effect bioconjugation via reductive amination, but this would not occur in an ODN containing an abasic site, which carries only one aldehyde group. An ODN containing an **OAS**, which lacks one nucleobase, can still form a duplex and interact with proteins. Thus, it functions as a DNA analogue, and the resulting cross-linked proteins retain DNA, rather than releasing the DNA during lactam formation. This strategy therefore provides significant information regarding nucleic acid−protein interactions. Previously, we synthesized ODN **8**, which carries an **OAS** in the sequence of a specific binding site

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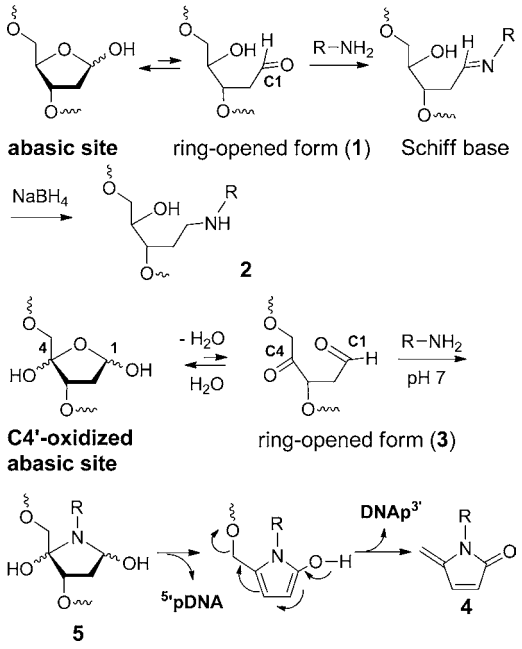
Scheme 1



(**6**:**14**) of the DnaA protein, a key factor involved in the initiation of chromosomal replication in *Escherichia coli* (Figure 1).[20] DnaA protein binds to specific sites with the consensus

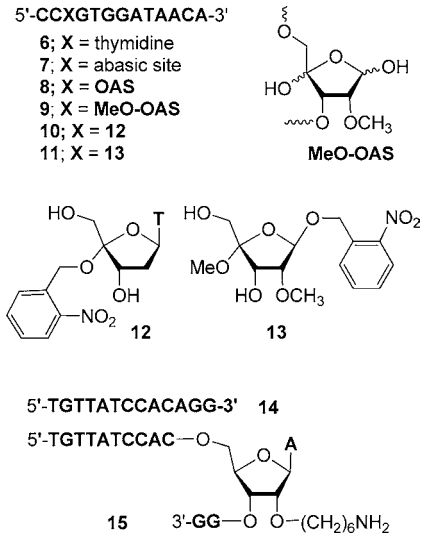


Figure 1. ODNs containing DnaA binding site.

sequence TTATNCACA in the chromosomal replication origin.[21] We first evaluated the cooperative effect of dicarbonyl groups by comparing the rates and yields of reductive amination of ODNs **8** and **7** with a proximal amine on the complementary strand of the DNA-binding sequence (**15**). The versatility of ODNs carrying **OAS** in bioconjugation was studied by lysine modification of the DNA-binding domain (domain IV) of *Escherichia coli* DnaA with **8**:**14**.

It has been reported that β-eliminations of ODNs that contain **OAS** take place under basic conditions and in the presence of amine; this holds even under near neutral conditions and at faster rates than similar reactions involving ODNs with abasic sites.[22,23] In spite of the possible advantages of amine modification, one concern is that the 1,4-dicarbonyl system in **3** could undergo strand cleavage at the β-position of the aldehyde (β-elimination) during the bioconjugation; this would generate ODN fragments that are unsuitable for conjugation. Leumann reported that the strand cleavage of abasic RNA sites and their 2′-*O*-methylated analogues under alkaline conditions was slower than that observed with abasic DNA.[24] We designed an ODN **9** containing a 2′-methoxy analog of **OAS** (**MeO-OAS**), as a stable and amine-reactive ODN for bioconjugation (Figure 1). The reactivity of **9** was elucidated and compared with the reactivities of ODNs containing an **OAS** and an abasic site. Finally, we investigated ODN−peptide conjugate formation by reductive amination using ODNs carrying **MeO-OAS** and lysine-containing peptides that lack a specific affinity for the ODN. The practical applications of such bioconjugates are also discussed.

RESULTS AND DISCUSSION

Synthesis of ODN Containing MeO-OAS. Previously, we reported the preparation of ODN **8** containing **OAS** by the photoirradiation of caged precursor ODNs which contained **12**[17] and 2-nitrobenzyloxy-4′-methoxy-2′-deoxy-D-ribofuranoside.[18] In analogy, caged sugar **13**, 2-nitrobenzyloxy-2′-*O*-methyl-4′-methoxy-D-ribofuranoside, was synthesized for preparation of **MeO-OAS** by oxidation of 4,5-unsaturated sugar **21** with *m*-CPBA followed by the reaction with MeOH (Figure 1 and Scheme 2). *o*-Nitrobenzyl-β-D-ribofuranoside **16**[25] was converted into 3′,5′-TIPDS ether **17** for the following 2′-*O*-methylation. Treatment of **17** with MeI in the presence of Ag_2O afforded **18**. Silyl groups of **18** were removed and the resulting **19** was converted to iodide **20**, which was further converted into 4,5-unsaturated sugar **21**. Treatment of **21** with *m*-CPBA in methanol yielded **13** and its C-4 epimer (***epi*-13**) in 61% yield as a 2:3 inseparable mixture. This mixture was subjected to dimethoxytritylation (DMTr), furnishing DMTr-protected **22** and its epimer (***epi*-22**) in 32% and 49% isolated yields, respectively, the stereochemistry of both compounds was confirmed by their NOESY spectra. Compound **22** was transformed into phosphoramidite **23** in 80% yield, which was subsequently incorporated into a 13-mer ODN by automated DNA synthesizer in the DMTr-on mode. Finally, ODN **11** was obtained after removal of the terminal DMTr group by treatment of the obtained ODN with 5% acetic acid (MALDI-TOF MS data for **11** (m/z): calculated, 4045; found, 4044).

ODN **9**, containing **MeO-OAS**, was next generated by photoirradiation of precursor ODN **11** at 365 nm in phosphate buffer (10 mM, pH 7). The reaction progress followed by HPLC showed efficient conversion of **11** to **9** after 2 h irradiation (Figure 2), which was supported by MALDI-TOF MS analysis of the photolyzed sample (m/z: calculated, 3896; found, 3898). The yield of **9** (ca. 84%) was estimated on the basis of a comparison of its peak area with that of an internal standard (**IS**; 5′-d(TTTTT)-3′) at 25 min.

As expected, **9** containing **MeO-OAS** was more stable than the corresponding **OAS**-containing **8** (Supporting Information). After incubation of a 20 μM solution in phosphate buffer (10 mM, pH 7) at 37 °C for 2 d, **9** appeared essentially unchanged. In contrast, 60% of **8** remained intact under the same conditions and the 5′-phosphorylated 10-mer **25** (Scheme 3) was formed by β-elimination. Heating **8** at 90 °C for 1 h led to its complete degradation, whereas 77% of **9** remained under the same conditions.

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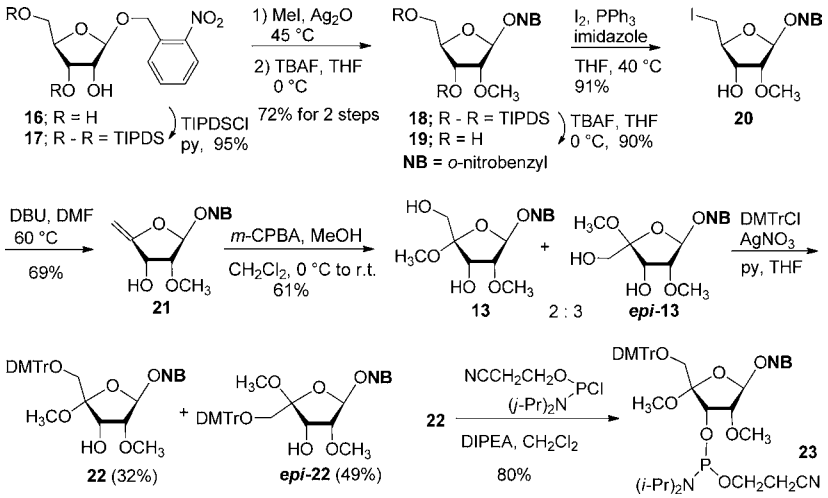
Scheme 2. Synthesis of Caged Sugar 13 and Phosphoramidite 23



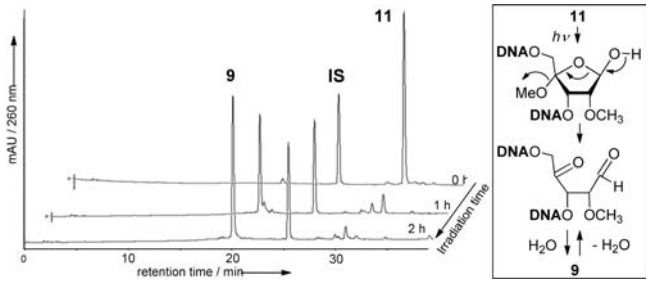
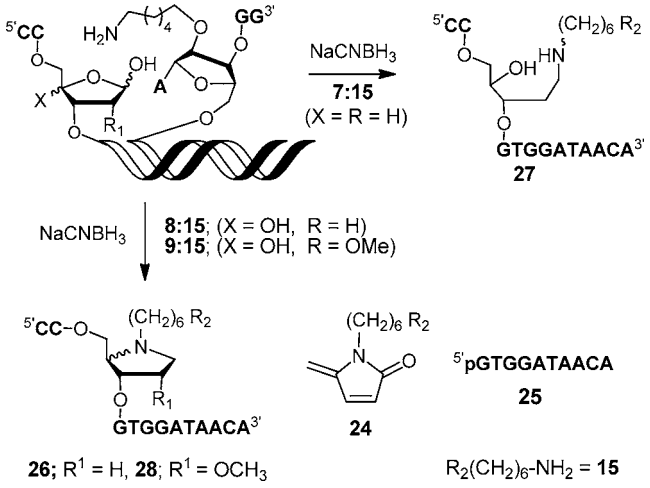


Figure 2. HPLC analysis of the photoreaction of **11** at different time intervals. ODNs **11** and **9** were eluted at retention times of 31 and 20 min, respectively. 5′-d(TTTTT)-3′ was added as an internal standard (**IS**) (retention time of 25 min).

Scheme 3. Reactions of Duplexes 7:15, 8:15, and 9:15



Reactions of 7, 8, and 9 with a Proximal Amine on Complementary Strand 15. The reactivities of the dicarbonyl-carrying ODNs **8** and **9** with an amine were evaluated in the context of duplexes containing **15**,[9,26] in which the aliphatic amino group of **15** could approach dicarbonyls (Scheme 3).

First, the duplex with **OAS**, **8**:**15**, was prepared by photoirradiation of precursor duplex **10**:**15** in phosphate buffer (10 mM, pH 7) containing 100 mM NaCl. HPLC analysis of the photolyzed sample indicated the formation of **8** as a major product. During photolysis, lactam formation (**24**) along with liberation of ODN fragment **25** shown in Scheme 1 had already proceeded (**24** at 32 min; 19%, **25** at 16 min; 25%, respectively; Figure 3a, 0 h).[27] Then, $NaCNBH_3$ (1000 equiv) was added to the photolyzed sample and the mixture was incubated at 25

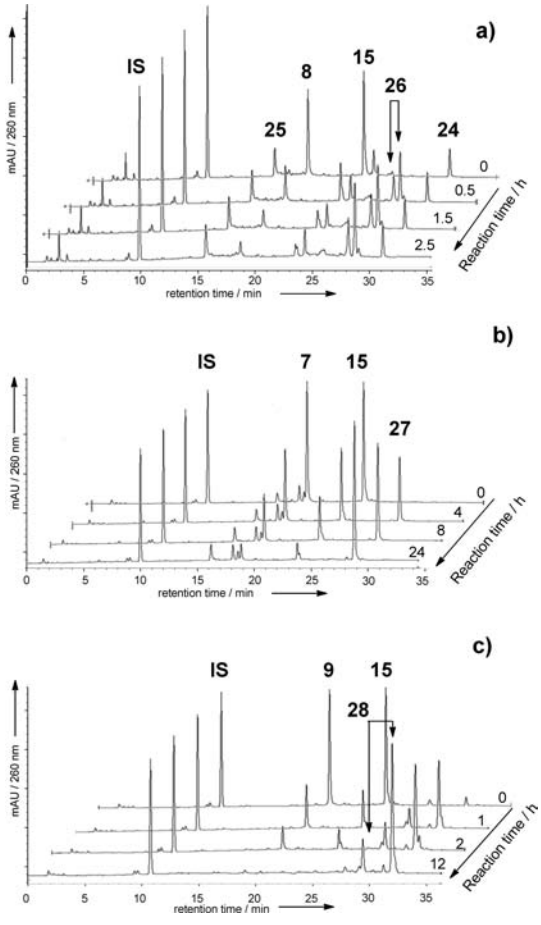


Figure 3. HPLC analysis of reductive amination of duplexes and rate constants and half-lives of cross-link formation: (a) **8**:**15** (k = 3.8 × 10 M^{-1} s^{-1} and $t_{1/2}$ = 43 min), (b) **7**:**15** (k = 3.6 M^{-1} s^{-1} and $t_{1/2}$ = 7.6 h), and (c) **9**:**15** (k = 5.3 × 10 M^{-1} s^{-1} and $t_{1/2}$ = 32 min).

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°C.[28] HPLC analysis of the reaction mixture revealed a time-dependent increase in new peaks at retention times of 28 and 29 min with a concomitant decrease in the starting duplex (Figure 3a, 0 to 2.5 h). Attempted separation of these products by preparative HPLC for was unsuccessful. MALDI-TOF MS analysis of the mixture of new peaks showing a major peak at m/z 7880 indicated a cross-link formation within the duplex. The possibility of cross-link formation at nucleobases of the complementary strand was excluded by the fact that a similar reaction using duplex between **8** and **14**, which lacks the aliphatic amine, did not proceed. Consequently, formation of diastereomer cross-links **26** at an aliphatic amine was supported (MW: 7882, Scheme 3). After 24 h, most of **8** disappeared and the combined yield of the diastereomers **26**, based on comparison with their peak areas with those of **8** and **15** at 0 h, was 73% (sum of 29% and 44%), whereas the amount of **24** did not increase during the reaction. Studies of the reaction kinetics showed that cross-link formation followed second-order kinetics with a rate constant and half-life of $k = 3.8 \times 10$ M^{-1} s^{-1} and $t_{1/2}$ = 43 min, respectively (Supporting Information).

The amine reactivity of ODN **7** containing the abasic site[18,29] was next examined. HPLC analysis of the reaction of duplex **7**:**15** showed the cross-link formation of **27** (Figure 3b, 0 to 24 h; Scheme 3), which, on isolation, was supported by MALDI-TOF MS (m/z: calculated, 7990; found, 7990). After 72 h, **7** and **15** were mostly consumed and the yield of **27** was essentially quantitative. Cross-link formation also followed second-order kinetics with $k = 3.6$ M^{-1} s^{-1} and $t_{1/2}$ = 7.6 h (Supporting Information). Notably, the reaction with **OAS**-containing **8**:**15** proceeded 10 times faster than with **7**:**15**, which contains an abasic site. Although the resulting cross-linked products were a mixture of diastereomers, the contribution of the dicarbonyl groups to the rapid reductive amination of **8**:**15** was evident.

The reaction of **MeO-OAS** containing **9**:**15** was similarly analyzed by HPLC. A time-dependent increase in new peaks at retention times of 29 and 32 min with a concomitant decrease in the starting duplex was observed (Figure 3c, 0 to 12 h). Again, the reaction of a duplex between **9** and **14**, lacking an aliphatic amine, did not yield cross-links. The new peaks were isolated, and the results of MALDI-TOF MS were consistent with diastereomers of **28** (Scheme 3, m/z: calculated, 7911; found, 7912 (both major and minor isomers)). After 12 h, **9** and **15** were mostly consumed and the combined yields of the minor and major isomers of **28** were essentially quantitative (ca. 1:4). Cross-link formation was as rapid as that observed with **OAS**-containing **8**:**15** ($k = 5.3 \times 10$ M^{-1} s^{-1} and $t_{1/2}$ = 32 min, Supporting Information). Thus, the reductive amination with the **MeO-OAS** duplex **9**:**15** proceeded more rapidly than that of the abasic site-containing duplex, and displayed more selectivity than that observed with the **OAS** duplex. Recently, we reported the reaction of an ODN containing a 2,2-difluoro analogue of C4′-OAS.[9] In this case, cross-link formation proceeded efficiently without the addition of reagents. However, the reaction rate was unfavorably slow compared to reductive amination of **OAS** duplexes. The faster reaction times associated with **OAS** molecules compared to the difluoro congener thus appeared preferable for biomolecules, and therefore we studied conjugation of a DNA-interacting protein with **OASs 8** and **9**.

Reactions of Domain IV of DnaA Protein with OAS, MeO-OAS, or an Abasic Site in Its Binding Sequence. The versatility of ODNs containing abasic sites, **OAS**, and **MeO-OAS** in reductive amination was examined by cross-link formation with a DNA interacting protein, DNA binding domain (domain IV) of DnaA protein. A truncated DnaA protein bearing only domain IV specifically binds to a DNA-binding sequence with an affinity comparable to the full-length DnaA.[30] The X-ray crystal structure of a complex of domain IV with a 13-mer duplex **6**:**14**, derived from the DnaA-binding site in the replication origin, suggested that Lys43 (corresponding to Lys415 of DnaA) is located near the phosphate group between C_2 and T_3 in **6** (Figure 4a).[31] Thus, reductive amination of domain IV for cross-linking at Lys43 was conducted using duplexes **7**:**14**, **8**:**14**, and **9**:**14**, which contain an abasic site, **OAS**, and **MeO-OAS** at the position corresponding to T_3 in **6**, respectively.

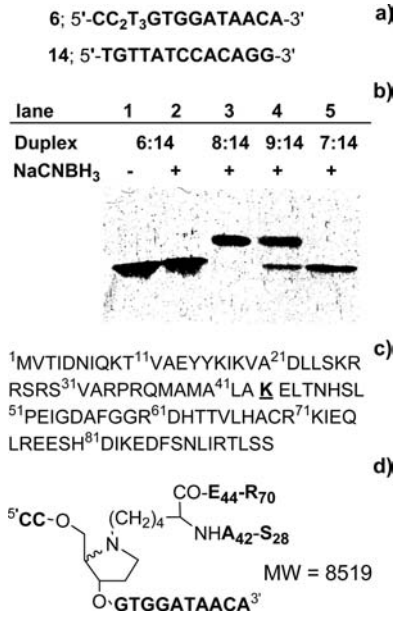

Figure 4. (a) 13-Mer duplex **6**:**14** derived from DnaA-binding site, (b) SDS-15% PAGE analysis of reaction between domain IV with duplexes, (c) amino acid sequence of domain IV, and (d) proposed structure of the digestion product of the cross-linking.

Binding affinities of **7**:**14**, **8**:**14**, and **9**:**14** for domain IV, at a concentration of domain IV that yielded 50% binding of 1 μM duplex, ranged 1.1−1.3 μM, and were comparable to that of unmodified **6**:**14** (1.0 μM). Next, domain IV (1.7 μM in reaction buffer) was incubated with duplexes (1.8 equiv) in the presence of $NaBCNH_3$ (1000 equiv) overnight. Reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Figure 4b). Reaction of domain IV and **8**:**14** with **OAS** led to the formation of a new, slow-migrating band that was attributable to cross-linking (lane 3, Figure 4b). Similar cross-linking was seen between domain IV and duplex **9**:**14** with **MeO-OAS**, albeit with slightly lower efficiency (lane 4, Figure 4b). In contrast, a similar reaction with **7**:**14**, which contains an abasic site, did not lead to cross-link formation, and only a band corresponding to intact domain IV was observed (lane 5, Figure 4b). These results were confirmed by HPLC analysis of the reaction mixture. The reaction mixture of **8**:**14** with **OAS** showed a peak at 15 min detected at 215 and 260 nm (Figure S39a, Supporting Information). That peak was ascribed to cross-linked ODN−protein. However, the corresponding

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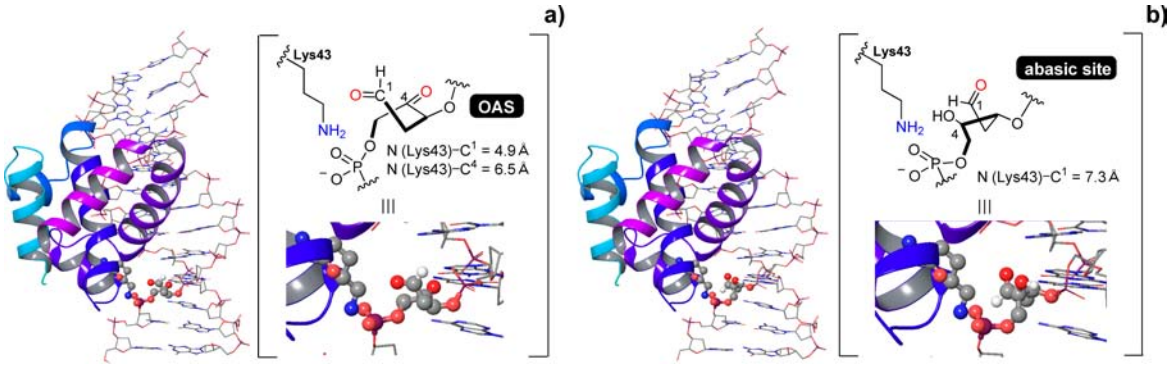

Figure 5. Modeled complexes of domain IV with 13-mer duplexes containing a ring-opened abasic analogue (OAS (a) and abasic site (b)).

260 nm peak at 15 min from the reaction of **7**:**14** with abasic site was not detected. This indicated that no cross-link was present, since cross-linked ODN–protein should exhibit a peak at 260 nm. For analysis of the cross-linking position, the cross-linked molecules of domain IV and **8** were successively digested with V8 protease and trypsin; under these conditions, proteins are first cleaved at the carboxyl group of glutamic acid residues, and then at lysines and arginines (Figure 4c). The digested sample was analyzed by HPLC, upon which an ODN–peptide conjugate was eluted and shown to form a single major peak. MALDI-TOF MS of the obtained ODN–peptide conjugate showed a peak at 8523, which suggested that conjugates included residues 28–70 of domain IV, since this is the only fragment with a molecular weight within an acceptable error (0.1%) for the observed peak[32] (MW = 8519, Figure 4d, Supporting Information). In the obtained peptide fragment, the target Lys43 and an additional four arginine residues are nucleophilic. Since reductive amination of **OAS** in **8** with arginine (200 equiv of α-dansyl-L-arginine[33] was used) did not provide any products, the possibility of cross-linking at arginine can be excluded. These results suggest that duplexes containing dicarbonyls are capable of inducing reductive amination of domain IV at site-specific lysine residues. As already mentioned, the crystal structure shows that Lys43 is located near the phosphate group between C_2 and T_3. The efficient cross-linking suggests that the Lys43 is mobile within the complex and/or **OAS** molecules can reach Lys43 through conformational flexibility. Previously, structural studies of DNA duplexes containing abasic site and **OAS** in the same sequence showed that **OAS** exists as a ring-closed hemiacetal, and adopts a predominantly intrahelical conformation; the abasic site in this context exists mainly as a ring-closed form, and adopts a partial extrahelical conformation.[15] Without detailed conformational studies of the abasic site and **OAS** in duplexes used in this study, it is not possible to determine whether such conformational differences were determinants of the cross-link efficiency of **7**:**14** and **8**:**14**. We had inferred that the C4-ketone of **OAS** was closer to Lys43 than the C1-aldehyde, and the first reaction was that between the amine and the ketone, followed by subsequent reaction of the C1-aldehyde to yield cross-linked protein with high efficiency. On the other hand, the aldehyde group appears to be restricted from approaching Lys43 in the complex with abasic site ODN. Computational models of the complex consisting of domain IV and **8**:**14** and **7**:**14** in the ring-opened form were constructed and visualized by MacroModel.[34] The theoretical model indicated that the C1-aldehyde of **OAS** was closer to Lys43 than the C4-ketone [distance between the nitrogen atom of Lys43 (N(Lys43)) and the carbon atom of the aldehyde (C1); 4.9 Å, that between N(Lys43) and the C4-carbon of the ketone; 6.5 Å, Figure 5a]. A similar calculation related to a complex between domain IV and **7**:**14**, which carries an abasic site, indicated the distance between N(Lys43) and the C1-carbon atom of the aldehyde is 7.3 Å. Those conformational differences might be determinants of reaction efficiency. Although cross-linking with **MeO-OAS** duplex **9**:**14** was less efficient than with the **OAS** duplex, we note that proteins that interact with DNA can be modified with ODNs carrying artificial RNA-like **MeO-OAS**. ODNs with dialdehyde groups introduced at a single defined position in an oligonucleotide strand have previously been reported, and are able to undergo cross-linking to enzymes.[35,36] In those reports, 1,5-dialdehyde groups derived from a ribose were attached to the 2′-hydroxyl group of a nucleoside, or 1,6-dialdehyde groups were in an acyclic sugar; this retains the functions of the nucleoside and affinity for proteins. ODNs carrying **OAS** and **MeO-OAS** at a defined site also retain sufficient DNA structure in their ring-closed forms. Together, our results suggested that reductive amination using ODNs carrying **OAS** and **MeO-OAS** might work with other proteins including those that interact with DNA via lysine–phosphate interaction. This would be valuable for the study of protein–nucleic acid interactions, since lysine–phosphate interaction is important for many proteins, and these new ODNs might react with proteins that were inert with ODNs that contain abasic sites. **OAS** ODNs participate in fast and efficient DNA interstrand cross-link formations,[37] in contrast to abasic site ODNs.[11] One of the two types of cross-links is proposed to form by condensation of two carbonyl groups with the exocyclic amine of the nucleobase.[38] **OAS** might also react efficiently with lysine amino groups which react with abasic sites; this encourages us to pursue further studies of protein modification using such ODNs.

Reaction of ODNs Containing an Abasic Site, or MeO-OAS, with Lysine-Containing Peptides. Chemical conjugation of oligonucleotides with signal peptides for increased cellular uptake and delivery to cellular target sites is a promissing approach for development of therapeutic nucleic acids that modulate gene expression.[4] The reductive amination between ODNs containing 1,4-dicarbonyls and lysine-containing peptides can provide such conjugates. Although reactions can be less efficient compared to reductive aminations within a duplex or those between a protein and an ODN in complex, and the efficacy of the reaction was evaluated for downstream applications. Efficient peptide labeling on a solid support via reductive amination of a reactive aldehyde site in the peptide proceeded with 1 equiv of a fluorescent aromatic amine and $NaCNBH_3$.[39] For reactions in solution phase, an excess amount

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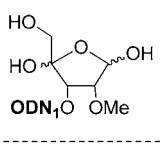
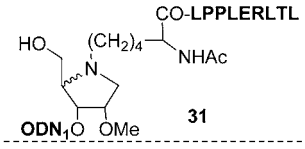
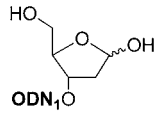
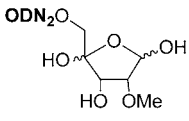
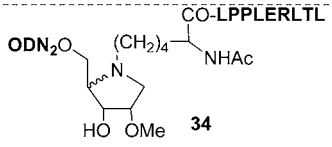
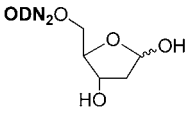
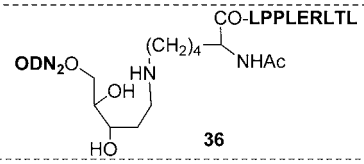
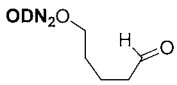
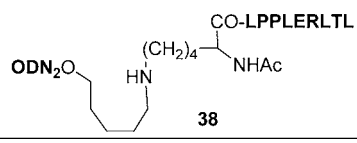
Table 1. Reductive Amination of ODN with 30; AcNHKLPPLERLTL[a]

Entry	ODN		pH	product	yield (%)
1	**29**		6		56
2	**29**		7		72
3	**29**		8	**31**	73
4	**32**		6		n.d.[b]
5	**32**		7		n.d.[b]
6	**32**		8		n.d.[b]
7	**33**		6		87
8	**33**		7		93
9	**33**		8	**34**	92
10	**35**		6		9
11	**35**		7		11
12	**35**		8	**36**	7
13	**37**		6		20
14	**37**		7		32
15	**37**		8	**38**	43

[a]ODN (20 μM) and **30** (400 μM) were incubated at 37 °C for 24 h. [b]Products were not detected. **ODN$_1$** = p**CAGTTAGGGTTAG**$^{3'}$, **ODN$_2$** = $^{5'}$**CAGTTAGGGTTAG**p.

of peptide is generally used to increase the yields of conjugates (10- to 50-fold peptide in the reductive amination with aldehyde-containing DNAs).[7,8]

The reductive amination of **29** containing **MeO-OAS** at the 5′-terminus of the complementary sequence of the RNA template of human telomerase[40,41] was performed. Photoirradiating the corresponding precursor containing **13** yielded **29** in essentially quantitative yield (Supporting Information). A reaction was then performed with 20 equiv of **30** (400 μM), which contains peptide derived from a nuclear export signal peptide sequence of HIV-1 rev[41,42] with Nα-Ac-Lys at the *N*-terminus, in phosphate buffer. The reaction at pH 7 and 8 (37 °C, 24 h) produced a higher yield of conjugate than reactions conducted at pH 6 (entries 1−3, Table 1, HPLC analysis in Supporting Information). The possibility of reaction of **30** at arginine was excluded since the reaction of **29** with α-dansyl-L-arginine[33] (1000 equiv) did not provide any products under similar conditions. The structure of conjugate **31** (MW: 5443) was consistent with its MALDI-TOF MS spectrum: the conjugate exhibited an m/z of 5444. Although observed as a single peak in HPLC analysis, conjugate **31** could be a mixture of diastereomers. With 10 equiv of **30** (200 μM), the yield of **31** was slightly lower (53% at pH 8) than when using 20 equiv. Surprisingly, the reaction of **32** (which contains an abasic site) with **30** was very poor under these conditions. It was also unexpected that the reaction of **33** (which contains **MeO-OAS** at the 3′-terminus) yielded the conjugate **34** (a mixture of diastereomers observed as 2 peaks), at greater yields than those for **31** under similar conditions. With 10 equiv of **30** (200 μM), the yield of **34** was 81% at pH 8. We infer that the position of **MeO-OAS** influences the position of equilibrium, thereby allowing **MeO-OAS** to open into a ketoaldehyde structure and react more easily at the 3′-terminus than the 5′-terminus. Reductive amination of **35** (which carries an abasic site) with **30** was also inefficient (entries 10−12, Table 1). In **35**, the shift in equilibrium to the ring-closed form may be greater than in **33**, which contains **MeO-OAS**. Such a "rate-limiting" ring-opening process may explain the significant difference between the reactivities of **33** and **35**. The reactions of **33** and **35** require a ring-opening process prior to reductive amination; this made it difficult to compare only the efficiency of reductive amination. Therefore, we used **37**, since it is a one carbonyl substrate which does not require ring-opening for the reaction. Similarly, it would be ideal to use 1,4-dicarbonyls, which, in contrast to **33**, exist exclusively in a ring-opened form. However, such 1,4-dicarbonyl compunds are not available since they are readily cylized to a ring-closed form. Conjugate formation of **37** yielded more conjugate (**38**) than a similar reaction that produced **36** but less than the reaction that produced **34** (entries 13−15, Table 1). This indicates that the dicarbonyl groups of **33** effected reductive amination at the ε-amino group of lysine, presumably via a 5-membered-ring aminal intermediate, such as **5** in lactam formation (Scheme 1). The formation of conjugates still required excess peptide, but conjugation proceeded efficiently with ODN carrying **MeO-OAS** at the 3′-terminus. Further studies will examine the biological properties and activities of obtained conjugates in order to determine the functional importance of the conjugation method (5-membered-ring amine as in **31** and **34** or aliphatic chain amine as in **36** and **38**) and the position of conjugation (3′- or 5′-terminus).

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Bioconjugate Chem. 2015, 26, 1830−1838

CONCLUSION

We investigated the reductive amination of ODNs containing a C4′-oxidized abasic site and a newly designed 2′-methoxy-substituted analogue. The 1,4-dicarbonyl systems underwent rapid reductive amination, as demonstrated by a 10-fold acceleration of cross-link formation between ODNs containing **OAS** and **MeO-OAS** and the proximal amines on complementary strands, in contrast to the similar reaction using an ODN containing an abasic site. Efficient cross-link formation between **OAS** or **MeO-OAS** and the DNA binding domain of DnaA indicated that this cross-linking strategy can be useful for identification of proteins that interact with DNA. The bioconjugation of ODN containing **MeO-OAS** with a lysine-containing peptide proceeded with high yield especially at the 3′-terminus of ODNs. Efficient conjugation through the cooperative reaction of two carbonyl groups has been demonstrated using three types of substrates.

EXPERIMENTAL PROCEDURES

Generation of ODN 9 by Photoirradiation of 11. A solution of **11** (100 μL, 20 μM, containing 5′-d(TTTTT)-3′ (30 μM) as an internal standard (**IS**)) in phosphate buffer (10 mM, pH 7, containing 100 mM NaCl) was irradiated in a microtube with a UV lamp (λ_{max} = 365 nm, intensity: 2.5 mW cm^{-2} @ 364 nm) and 10 μL aliquots were analyzed at different time intervals by HPLC using a X-bridge C18 column (4.6 mm I.D. × 150 mm). Solvent A: CH_3CN; Solvent B: 0.1 mM triethylammonium acetate (pH 7). Gradient: initial, 96% B, linear to 14% A at 40 min. Detection was carried out at 260 nm. The yield of **9** was determined by comparison of its peak area with that of **IS** based on their extinction coefficients (10^5 M^{-1} cm^{-1}); **11**: 1.25, **9**: 1.22, **IS**: 0.39. ODNs **29** and **33** were synthesized similarly.

Reductive Amination of Duplexes. ODNs **10** or **11** (15 μM considering efficiency of decaging) in 10 mM phosphate buffer (pH 7) containing 100 mM NaCl and 5′-d(ACC)-3′ (50 μM, ε_{260} = 27000) as an internal standard was hybridized with **15** (10 μM) by heating at 85 °C for 1 min and allowed to cool to room temperature slowly. Obtained duplex was photolyzed similarly to single strand and the resulting duplex was incubated at 25 °C and 10 μL aliquots were analyzed at different time intervals by HPLC using a X-bridge C18 column (4.6 mm I.D. × 150 mm). Solvent A: CH_3CN; Solvent B: 0.1 mM triethylammonium acetate (pH 7). Gradient: initial, 4% A, linear to 14% A at 40 min. Detection was carried out at 260 nm. The yields of **26**, **27**, **28**, **24**, and **25** were determined by comparison of peak areas with that of IS based on their extinction coefficients (10^5 M^{-1} cm^{-1}); **26**, **27**, **28**: 2.46, **24**: 1.24, **25**: 1.09.

Reductive Amination of Domain IV. A mixture of duplex 13-mer (3 μM) and domain IV (1.7 μM) in 30.6 μL reaction buffer (20 mM Hepes-KOH (pH 7.6), 5 mM $Mg(OAc)_2$, 1 mM EDTA, 11.6% (v/v) glycerol, 0.2% Triton X-100, 15% 0.9 mM ATP) was incubated for 20 min on ice and $NaBCNH_3$ (1000 equiv) was added and the mixture was incubated at 25 °C overnight. The reaction mixtures were analyzed by SDS-polyacrylamide (15%) gel electrophoresis. Proteins were visualized by CBB staining.

V8 Protease/Tpck-Trypsin Digestion of Isolated Cross-Link of Domain IV with 8. Cross-link prepared from domain IV (702 pmol) was isolated by HPLC (the peak eluted at 15 min, protein R column (4.6 mm I.D. × 150 mm; solvent A: CH_3CN containing 0.05% TFA; solvent B: H_2O containing 0.05% TFA. gradient: initial, 20% A, linear to 80% A at 30 min; detection at 215 and 260 nm). After neutralization, the eluent was concentrated under reduced pressure and 6 M urea solution (20 μL) was added to the residue. After incubation at 37 °C for 1 h, 121 μL Milli-Q water, ammonium bicarbonate solution (1 M, 7.5 μL), and V8 protease (400 μg/mL solution, 1.5 μL) were added and the mixture was incubated at 37 °C 2 d. Tpck-trypsin (200 μg/mL solution, 4 μL) was added and the mixture was incubated at 37 °C for 36 h. The mixture was separated by HPLC (X-bridge column (4.6 mm I.D. × 150 mm; solvent A: CH_3CN; solvent B: 0.1 mM triethylammonium acetate (pH 7), gradient: 5% A to 20% A in 30 min, then to 80% in 30 min; detection at 215 and 260 nm, detection by fluorescence; excitation at 286 nm, emission at 304 nm). The peak at 46 min was collected and was concentrated to 2 μL. After desalting, sample was mixed with 1 μL of 5% 3-hydrocypicolinic acid in H_2O/CH_3CN containing 10% diammonium hydrogen citrate, and was analyzed by MALDI-TOF MS.

Reductive Amination of Lysine Containing Peptides. A mixture of ODN (20 μM) and peptide (200 or 400 μM) was incubated in phosphate buffer (50 mM) at 37 °C for 24 h. Aliquots (10 μL) were analyzed at different time intervals by HPLC using a X-bridge C18 column (4.6 mm I.D. × 150 mm). Solvent A: CH_3CN; Solvent B: 0.1 mM triethylammonium acetate (pH 7). Gradient: 5% A to 20% A at 40 min, then to 50% A at 60 min. Detection was carried out at 260 nm. The yields of conjugates were determined by comparison of peak areas with that of IS (5′-d(TTTTT)-3′) based on their extinction coefficients (10^5 M^{-1} cm^{-1}); 1.31. For reaction of 37, reaction mixture was analyzed using a COSMOSIL 5-C18-AR-II column (4.6 mm I.D. × 250 mm). Solvent A: CH_3CN; Solvent B: 20 mM sodium phosphate/MeOH (V/V = 95:5, pH 7). Gradient: 100% B to 80% B at 40 min.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00361.

Experimental procedures for compounds **13**, ***epi*-13**, **17**−**23**, **S1**−**S3** for synthesis of **37**, studies on stability of **8** and **9**, UV-melting curve measurements of **9**:**14**, determination of binding affinity of duplexes to domain IV, and theoretical calculation of complexes of domain IV with duplexes ^1H and ^{13}C NMR spectra of the compounds **17**−**21**, a mixture of **13** and ***epi*-13**, **22**, epimer of **22**, **23**, **S1**−**S3** for synthesis of **37**, $^1H-^1H$ NOESY spectra of **22**, epimer of **22**, COSY spectrum of **S1** for synthesis of **37**, MALDI-TOF MS of **11**, **9**, **24**, **25**, **26** (mixture), **7**, **27**, **28** (major), **28** (minor), digestion product from cross-link of domain IV with **8**, precursor of **29**, **31**, precursors of **32** and **33**, and **34** and its isomer, precursor of **35**, **36**, **S4**, **37**, **38**, HPLC analyses of incubation of **8** and **9** at **37** and 90 °C, plots to determine reaction rates of reductive amination of duplexes **8**:**15**, **7**:**15**, and **9**:**15**, HPLC analysis of the reaction mixtures of domain IV with **8**:**14** and **7**:**14** and digestion of cross-link by V8 protease and trypsin, HPLC analyses of photogeneration of **29**, **32**, **33**, and **35**, reductive amination of **29**, **32**, **33**, **35**, and **37** (PDF)

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Notes

The authors declare no competing financial interest.

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