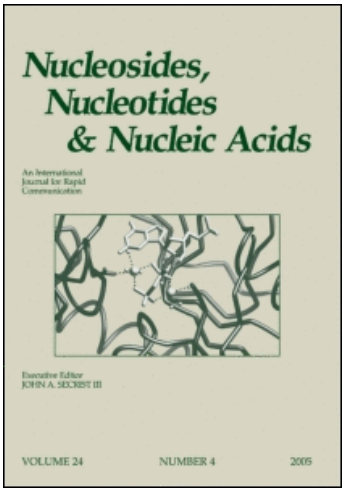

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APPLICATION OF 2-AMINO-6-VINYLPURINE AS AN EFFICIENT AGENT FOR CONJUGATION OF OLIGONUCLEOTIDES

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□ *Attempts have been made to conjugate a variety of molecules with oligonucleotides to achieve useful functions. In this study, we have established a new efficient method for post-synthetic conjugation of oligonucleotides with the use of the 2-amino-6-vinylpurine nucleoside. Amino nucleophiles form the corresponding conjugates under acidic conditions, whereas thiol nucleophiles reacted efficiently under alkaline conditions. Thus, glutathione and HS-Cys-(Arg)$_8$ without protecting groups were efficiently conjugated to the 2-amino-6-vinylpurine–bearing ODN under alkaline conditions. The use of 2-amino-6-vinylpurine as an agent for conjugation is advantageous in that it is stable during the reaction and may be applied to conjugation of ODNs with multiple functional molecules.*

Keywords Oligodeoxynucleotide; Conjugation; 2-Amino-6-vinylpurine; Antisense

INTRODUCTION

Molecules that can target DNA or RNA with high efficiency and specificity are of great interest because of potential applications to new methods for diagnosis[1–6] and therapy,[7–12] which may detect or interfere in gene expression in a sequence-specific manner.[13–17] The ODNs (oligodeoxynucleotides) attaching a radio-ligand have shown potential ability in imaging of the *m*RNA level in vivo.[1,3,18–20] The ODNs conjugating fluorophores[21–25] or stable free radicals (spin-label)[26–28] have been used in the study of the hybridization behavior of duplex or triplexes. ODN conjugates of enzyme have

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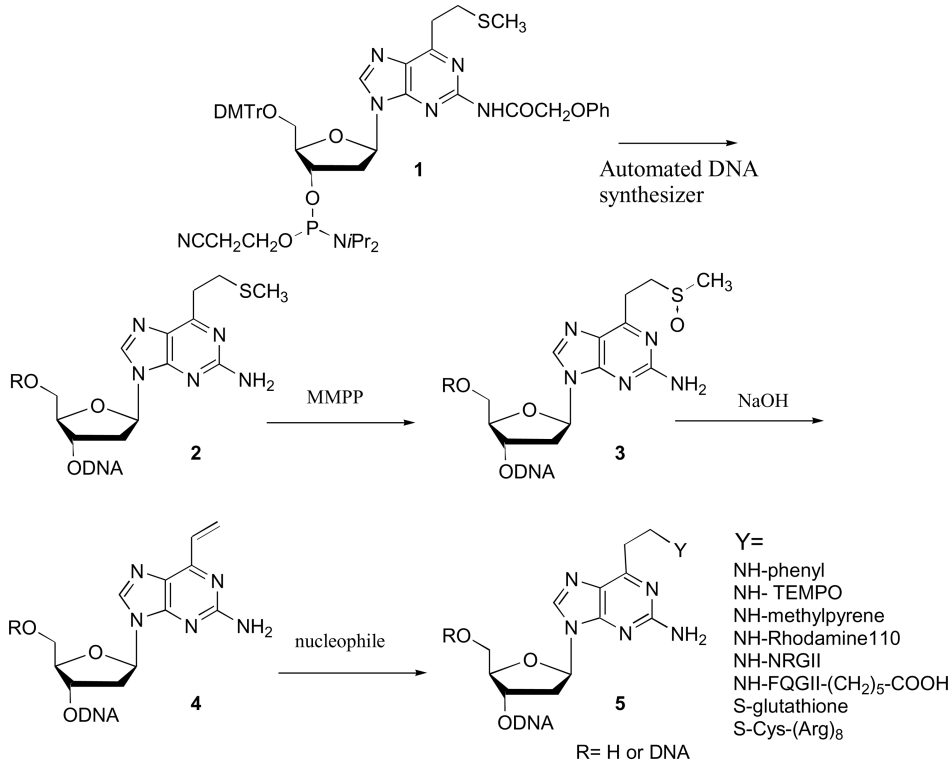
been synthesized to provide sequence-specificity to the enzyme.[29–31] Attaching some additional functional molecules to the ODNs have been shown to be useful for tolerance against hydrolytic enzymes, improvement of cell permeability, decrease of toxicity, enhancement of specific binding, and so on.[17,32,33] For example, ODNs conjugated with peptides or polymers have exhibited improved delivery and cell permeability in vivo.[17,34–37] Thus, conjugation of ODNs with a wide variety of functional molecules would facilitate their application for diagnosis as well as therapy. In most cases, conjugation has been carried out either at the 5′ or 3′ end of an ODN using an amino or a thiol group. In order to conjugate peptide at the 3′ amino group of an ODN some coupling reagents such as EDC or DCC were used.[38] Utilization of such coupling reagents may lead to formation of by products, which are sometimes troublesome in HPLC purification. In a different approach, the ODN having a pyridine sulfenyl group at the 5′ end was successfully coupled to the thiol group of the peptide.[39] This method was not successfully applied to conjugate arginine-rich peptide to ODN in the solution phase.[40] Also, the disulfide bond is in equilibrium with the corresponding thiols and is not stable in the living system. Another major challenge is the conjugation of ligands in an interior region of the ODN other than a terminal end, and some methods have been developed based on solid-phase procedures.[41–44] However, the existing methods need either high concentration of nucleophiles or high reaction temperature. Therefore, development of a more convenient and versatile method of ODN conjugation is highly desired. Here, we describe in detail an efficient method for post-synthetic conjugation of ODNs with the use of the 2-amino-6-vinylpurine derivative as the precursor for conjugation.

We have previously demonstrated 2-amino-6-vinylpurine as an efficient cross-linking agent with high selectivity towards cytidine.[45–47] Alkylation of 2-amino-6-vinylpurine has been shown to be auto-generated within a duplex from its phenylsulfide or phenylsulfoxide precursors.[48,49] Efficient reactivity of 2-amino-6-vinylpurine skeleton has been successfully applied to triplex-triggered cross-linking.[50,51] More recently, we have found that the cross-links of the triplexes caused point mutations in a site-specific manner.[52] During our study of 2-amino-6-vinylpurine derivatives as cross-linking agents, we have observed that the reaction with amino nucleophiles needs acidic conditions, whereas thiol nucleophiles efficiently form adducts under neutral to alkaline conditions.[45–47] Although the nucleophiles are needed in large excess for conjugation, it is advantageous that 2-amino-6-vinylpurine is stable enough over longer reaction period. Thus, we planned to apply the 2-amino-6-vinylpurine nucleoside analog to conjugation of oligonucleotides with a variety of amino- and thiol-nucleophiles. Here, we describe a new versatile method for conjugation of oligodeoxynucleotides with the use of 2-amino-6-vinylpurine to construct ODN-conjugates with a variety of functional molecules for radio-, spin-, fluorescence-labels and peptide ligands.

RESULTS AND DISCUSSION

Synthesis of DNA Incorporating 2-Amino-6-vinylpurine Analog

In this study, a series of nucleophiles were subjected to the conjugation with 2-amino-6-vinylpurine (**4**)-bearing ODNs and the strategy for the conjugation is shown in Figure 1. The phosphoramidite precursor of the methyl sulfide derivative of 2-amino-6-vinyl purine was synthesized from 2′-deoxyguanosine following the standard protocol described in our previous report[48] and was incorporated into the oligonucleotides by an automated DNA synthesizer according to the standard phosphoramidite chemistry. The ODNs incorporating the 2-amino-6-vinylpurine analog either at the interior



ODN1: 5'-**X**AG GAC AGG TTC AGT GGA TC-3'
ODN2: 5'-CTT T**X**T TCT CCT TTC T-3'
ODN3: 5'-**X**AT GCC CAT ACT GTT GAG-fluorescien 3'
X indicates the position of 2-amino-6-vinylpurine analog.
MMPP=magnesium monoperpthalate, TEMPO=2,2,6,6-Tetramethyl-1-piperinyloxy
NRGII=Asn-Arg-Gly-Ile-Ile, FQGII=Phe-Gln-Gly-Ile-Ile

FIGURE 1 Conjugation strategy with amino and thiol nucleophiles using 2-amino-6-vinylpurine, and oligodeoxynucleotides used in this study. ODN1: antisense to D2 dopamine receptor,[7] ODN2: sequence used in the cross-linking study,[45–47] ODN3: antisense to luciferase.[53]

position or at the 5′ end (ODN **1-3**) were obtained in good yields (40–60% after isolation).

Conjugation

Prior to the conjugation reaction, the methyl sulfide derivative of 2-amino-6-vinylpurine of the ODN was converted to the vinyl form **4** by treatment with MMPP (magnesium monoperoxyphthalate) followed by NaOH in aqueous solution (Figure 1).[45–47] Then, the nucleophiles were added to the reaction mixture and incubated at room temperature. The progress of the conjugation reactions was monitored by RP-HPLC, and the peak corresponding to the conjugate products was isolated to confirm the structure by MALDI-TOF MS measurements. Figure 2 illustrates an example of the HPLC profiles for the conjugation of **4** (ODN1 sequence) with aniline, indicating that the reaction took place efficiently. Other conjugations with amino nucleophiles such as 1-pyrenemethylamine and the peptide NRGII proceeded similarly, although a large excess amount is needed to afford the product in over 90% conversion yield. In contrast, the conjugation with 4-amino TEMPO (4-amino-2,2,6,6-tetramethyl-1-piperidinyloxy, free radical), the pentapeptide FQGII-$(CH_2)_5$-COOH, and Rhodamine110 needed a longer reaction period, probably due to the steric hindrance and less nucleophilicity of the amino group (Table 1). It was observed in the case of the conjugation with amino nucleophile that the conjugation reaction proceeded efficiently only under acidic conditions (pH $\sim$ 5). Computational calculations in the previous study have suggested that protonation at 1N of 2-amino-6-vinylpurine lowers activation energy for the reaction with amino nucleophiles.[46] The cross-linking within the duplex between the 2-amino-6-vinylpurine and the amino group of cytosine proceeded faster under acidic conditions than that under neutral conditions.[45] Due to the proximity effect, the cross-linking

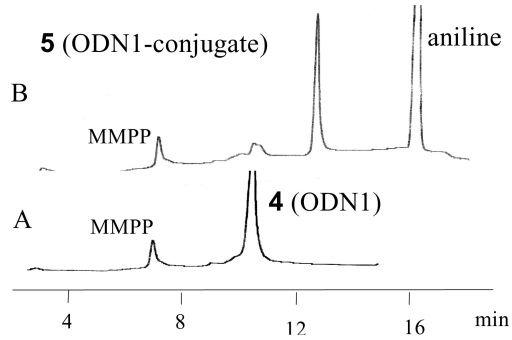


FIGURE 2 The HPLC change by conjugation of **4** (ODN1) with aniline. A) ODN1: **4** after the treatment of **3** with MMPP followed by NaOH. B) The reaction mixture after 3 h. HPLC conditions: A: 0.1M TEAA, B: CH_3CN, 10–30%/20 min, 30–100%/30 min, flow rate: 1 mL/min, UV: 254 nm, column: Nacalai tesque, COSMOSIL 5C 18-AR-II (ODS) (4.5 × 250 mm).

TABLE 1 Results of Conjugation of ODN **4** Incorporating 2-Amino-6-vinylpurine with a Variety of Substrates

Product ODN	ODN (position)[a]	Conjugated group (Y=)	Nucleophile	Reaction time (h)	pH	Yield (%)[d]	MS, found (calcd)
2	ODN1 (End)	Aniline[b]	NH_2	3	5	93	6323.45 (6320.23)
3	ODN2 (Int)	4-Amino TEMPO[c]	NH_2	12	5	64	4934.90 (4932.45)
4	ODN2 (Int)	1-Pyeremethylamine[b]	NH_2	3	5	89	4995.97 (4995.76)
5	ODN2 (Int)	Rhodamine110[c]	NH_2	12	5	21	5096.90 (5091.06)
6	ODN2 (Int)	NRGII[b]	NH_2	3	5	91	5330.69 (5332.06)
7	ODN2 (Int)	FQGII-$(CH_2)_5$-COOH[b]	NH_2	12	5	60	5457.98 (5451.06)
8	ODN3 (End)	Glutathione[b]	SH	3	10	80	5829.80 (5828.56)
9	ODN3 (End)	Cys-$(Arg)_8^b$	SH	5	10	92	7430.69 (7431.03)

[a] Int.: internal conjugate, End: conjugate at 5′ end.
[b] 20–50 equivalent of nucleophile was used.
[c] More than 100 equivalent of nucleophile was used, FQGII (Phe-Gln-Gly-Ile-Ile); NRGII (Asn-Arg-Gly-Ile-Ile).
[d] HPLC conversion yield from **4** to the corresponding conjugate.

reaction proceeded smoothly in equimolar concentrations of the vinyl ODN and the target ODN.[47] In this study we found that excess amount of nucleophiles (20–100 equivalents) was needed for successful conjugation.

In contrast to amino nucleophiles, thiol nucleophiles were found to produce conjugates under neutral to alkaline conditions.[48,49] It should be also noted that the 2-amino-6-vinylpurine unit incorporated in ODN is stable under alkaline conditions at about pH 10. From the fact that amino nucleophiles do not show significant reactivity to the 2-amino-6-vinylpurine unit under alkaline conditions, we next investigated conjugation with non-protected peptides bearing a thiol nucleophile. The reaction of **4** (ODN3 without 3′ amino linker) with glutathione proceeded rapidly at pH 10 to form the corresponding conjugate in high yield (Table 1). Enzymatic hydrolysate was analyzed and compared with an authentic nucleoside adduct with glutathione, confirming that the reaction took place at cystein of glutathione.

Conjugation with a cationic peptide such as the HIV Tat fragment or oligoarginine has attracted much attention because of enhancement of cell permeability of large molecules such as proteins and plasmids;[54–56] hence, we became interested in conjugation of the oligoarginine Cys-$(Arg)_8$ to the ODN with use of the 2-amino-6-vinylpurine unit. Thus, we investigated conjugation of the ODN labeled with fluorescein at the 3′ end with non-protected Cys-$(Arg)_8$, and the HPLC change is summarized in Figure 3.

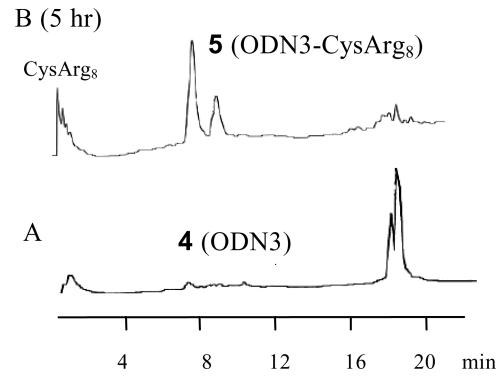


FIGURE 3 Conjugation of oligoarginine with **4** (fluorescein-ODN3). A) **4** (fluorescein-ODN3). Two peaks correspond to isomers of fluorescein. B) the reaction mixture after 5 h. HPLC conditions: solvent A: 25 mM phosphate buff. 10% CH_3CN, pH 7, B: 25 mM phosphate buff. 10% CH_3CN, 1 M NaCl, pH 7, 25–50%/30 min, 50–100%/40 min flow rate 1.0 mL/min, UV 254 nm, column: Shodex IEC DEAE-420 (4.6 × 35 mm).

At first, the ODN bearing the methylsulfide precursor of 2-amino-6-vinylpurine at the 5′ end together with the amino group at the 3′ end was synthesized. Fluorescein was conjugated at the 3′ amino group using its carboxyfluorescein succinimidyl ester (FAMSE) as described in the Experimental section. The methylsulfide derivative of the fluorescein-ODN conjugate was converted into the vinyl derivative by the treatment of MMPP followed by alkaline conditions to give **4**(ODN3) containing the 2-amino-6-vinylpurine unit at the 5′ end and fluorescein at the 3′ end (Figure 3, A). Then, an aqueous solution of Cys-$(Arg)_8$ was added into the reaction mixture and incubated at room temperature. It was shown by HPLC analysis that the reaction almost completed after 5 h in 92% conversion yield (Figure 3, B). The two new peaks in the HPLC chart produced the same MS spectra by MALDI-TOF MS measurements (Table1), showing that these two peaks correspond to the isomers of the fluorescein. This successful conjugation also indicates that the fluorescence label is compatible with MMPP or NaOH treatment for activation of the methylsulfide protecting group of the 2-amino-6-vinylpurine unit.

EXPERIMENTAL

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. The amidite precursors of 2′-deoxynucleotides were purchased from Glen Research or Proligo Biochemi GmbH. CPG columns were purchased from PE Biosystems or Proligo Biochemi GmbH. ODN synthesis was carried out by the use of an automated DNA synthesizer (ABI, 394 DNA/RNA synthesizer) following the standard phosphoramidite chemistry. The other reagents used in DNA synthesis, were purchased from Applied Biosystems Incorporation (ABI).

Acetonitrile and dichloromethane were purified by distillation from CaH_2. DMF (dimethylforamide) was distilled from molecular sieves 4 A. All other chemicals and solvents used in this study were purchased either from Nacalai tesque, Aldrich, Wako Pure Chemical Industry, Kanto, or TCI.

Ultraviolet-visible (UV-vis) absorption spectra were recorded on a JASCO V-550 UV-VIS spectrophotometer. Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectra were determined with a Voyager Elite Perseptive Biosystems mass spectrometer. Matrix used for preparing MALDI-TOF mass samples was 3-hydroxy-2-picolinic acid and diammonium hydrogen citrate in acetonitrile and water.

DNA and DNA-ligand conjugates were analyzed and purified by high-performance liquid chromatography (HPLC) using a Shimadzu apparatus that consisted of a UV-VIS detector (Shimadzu, SPD-10A), a liquid chromatogragh (Shimadzu, LC-10AD), and a degasser (Shimadzu, DGU-12A). The HPLC chromatogram was recorded with a Shimadzu C-R6A Chromatopac.

All centrifugations were carried out with a high-speed thermoelectric cooling microcentrifuge (TOMY, ECO-Fuge, EF-13000) at 13,000 rpm at 4°C. All freeze-drying operations were carried out with the help of LABCNCO FREEZONE or EYELA freeze dryer FD-1000. The samples were pre-frozen in glass vials or nash flasks either in liquid nitrogen or in a freezer at −40°C.

General Procedure for Conjugation of ODN with Amino Nucleophiles

Prior to conjugation, the oligonucleotides were converted into the vinyl form (**4**). Three equivalents of MMPP (in 24 mM carbonate buffer, pH 10) were added to a solution of oligonucleotides (**4**). After mixing, the reaction mixture was incubated at room temperature and the reaction progress was analyzed by RP-HPLC (Nacalai Tesque Cosmosil Packed Column, C18-ARII, 4.6 × 250 mm). After 1 h, an excess amount of aqueous NaOH solution (0.20 mM final conc.) was added to the reaction mixture and incubated at room temperature. RP-HPLC analysis of the reaction mixture showed that the reaction completed within 1 h. The pH of the reaction mixture was then adjusted to ∼5 with acetic acid. The amino nucleophiles (listed in Table 1) were added to the reaction mixture, mixed well, and incubated at room temperature. The progress of the reaction was analyzed by RP-HPLC (HPLC condition described in Figure 2). The conjugates were purified by RP-HPLC and the structures were confirmed by MALDI-TOF mass measurements. Conjugates **2–7** (Table 1) were synthesized following this procedure.

Conjugation of 4 (ODN3 Excluded 3′-Amino Linker) with Glutathione. Vinyl form **4** of the ODN3 (without 3′-linker) was generated by the treatment of the methyl sulfide derivative with 3 equivalents of MMPP followed by the

treatment of NaOH similarly described above. Then 20 equivalents of glutathione (aqueous) was added and kept at room temperature. The reaction progress was analyzed by RP-HPLC (HPLC condition given in Figure 2). After purification the structure was confirmed by MALDI-TOF measurement (conjugate **8** in Table 1).

Conjugation of Fluorescein-Labeled ODN with Oligoarginine. Fluorescein was conjugated with the ODN at the 3′ amino group using the 5(6)-fluoresceinsuccinimidyl ester following the manufacturer's instruction (Molecular probe). Briefly, 4 μL (16 nmol) of amino-modified ODN was added in 75 μL sodium tetraborate buffer (0.1 M, pH 8.5) and subsequently added 15 μL of (30 mM dissolved in DMSO) FAM.SE. After removal of an excess unbound fluorescein reagent by ethanol precipitation, the conjugate was purified by RP-HPLC. After determination of the structure by MALDI-TOF measurement, the conjugate was used in the next conjugation reaction with oligoarginine (Cys-R8). Prior to conjugation with oligoarginine, the ODN-fluorescein (8.30 nmol) in 50 μL H_2O was treated with MMPP (6.0 mM in standard carbonate buffer, pH 10, 7.00 μL, 42.00 nmol) at room temperature. After 1 h, NaOH (4.0 M, 7.0 μL, 28 mmol) was added to the reaction mixture and incubated at room temperature. After 1 h, 40.0 μL aqueous solution of Cys-R8 (15 mM, 581 nmol) was added to the reaction mixture, mixed and incubated at room temperature. The progress of the reaction was analyzed by HPLC using an ion exchange column (Figure 3). After 5 h, the peak of the vinyl ODN **4** disappeared producing two new peaks at faster retention time. The new peaks were isolated by HPLC using the ion exchange column (Figure 3). The isolate was lyophilized and the structure was confirmed by MALDI-TOF mass measurement (calculated 7431.03, found 7430.69). 92% conversion yield of oligoarginine conjugate was obtained (conjugate **9** in Table1).

CONCLUSION

We have successfully established a new efficient method for conjugation of oligonucleotides with use of the 2-amino-6-vinylpurine unit. It should be noted that a non-protected form of the polycationic oligo-arginine was conjugated to the ODN in high yield. As the 2-amino-6-vinylpurine nucleoside analog may be incorporated into any site of the ODN and its conjugate ODN is stable, a variety of ODN conjugates can be synthesized with amino- and thiol-nucleophiles. The conjugation methods so far described are not sufficiently general because in each method a single kind of molecule or nucleophile was conjugated.[38–44] Moreover, these methods followed either a solid-phase synthesis in the protected form of DNA or peptides or used some coupling agents such as EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

hydrochloride) or DCC (dicyclohexylcarbodiimide).[38–41] In comparison to these methods, our conjugation method is simple and more advantageous as a variety of functional molecules can be conjugated using a single agent. In addition, this new method is advantageous in that the methylsulfide precursor of 2-amino-6-vinylpurine is very stable until it is activated by oxidation following alkaline treatment, and that the active species of the 2-amino-6-vinylpurine unit is stable under alkaline conditions. Thus, the 2-amino-6-vinylpurine unit may be applied to conjugation of ODNs with multiple functional molecules. Application of this method is now ongoing to multiple conjugation of ODN with polymers, signal peptides, or proteins.

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