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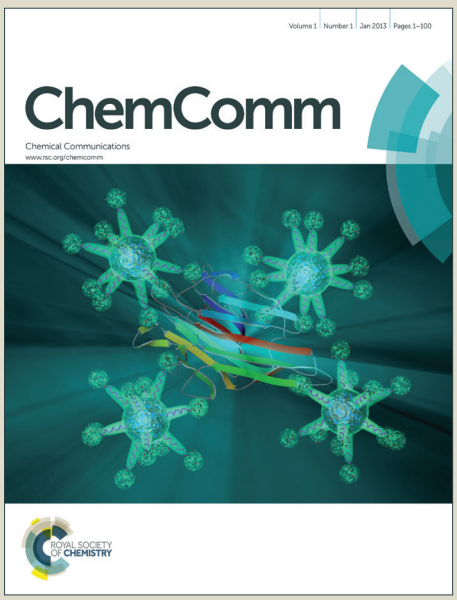

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ARTICLE TYPE

A New Strategy for Site-Specific Alkylation of DNA using Oligonucleotides Containing an Abasic site and Alkylating Probes

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Selective chemical reactions with DNA, such as its labelling, are very useful in many applications. In this paper, we discuss a new strategy for the selective alkylation of DNA using an oligonucleotide containing an abasic site and alkylating probes. We designed three probes consisting of 2-AVP as a reactive moiety and three kinds of binding moiety with high affinity to duplex DNA. Among these probes, Hoechst-AVP probe exhibited high selectivity and efficient reactivity to thymine bases at the site opposite an abasic site in DNA. Our method is potentially useful for inducing site-directed reactions aimed at inhibiting polymerase reactions.

Alkylating agents react with DNA bases in the generation of a variety of covalent adducts and can cause cancer by inducing mutagenic modifications of the constitutive nucleic acid bases in DNA.[1] However, certain alkylating agents are commonly used as chemotherapeutic drugs in cancer treatment.[2] In light of the double-edged properties of alkylating agents, methods for the site-specific alkylation of DNA with sequence selectivity offer new potential strategies for the development of selective chemotherapeutic drugs. In addition, selective chemical modification of DNA and RNA can significantly impact their respective functions. Reports exist on the development of small probes for selective reactions with DNA[3,4] or RNA.[5,6] Aryl nitrogen mustard conjugated to various functional groups has demonstrated selective alkylation with DNA[7-11] and RNA.[12] Rhodium-catalyzed carbene transfer was achieved in the selective alkylation of cytosine and adenine in the unpaired DNA or RNA sequences.[13] The combination of catalytic RNA and electrophilic probes was developed as a tool for selective, irreversible RNA modification.[14]

Here, we propose a strategy for the site-specific alkylation of DNA using oligonucleotides containing an abasic (AP) site and the alkylating probes. We previously reported that a 2'-OMe oligonucleotide containing 2-amino-6-vinylpurine (**1**: AVP) efficiently forms a covalent linkage with the complementary sequence of mRNA at the uridine residue across from the AVP.[15,16] The efficient reactivity observed with AVP was attributed to the proximity effect enabled by the formation of hydrogen bonds with the target uridine. Based on these results, we anticipated that 2-AVP derivatives might also form hydrogen bonds with target nucleobases located in a hydrophobic pocket, forcing the reactive moiety into close proximity and inducing selective alkylation. As a hydrophobic pocket, an AP site in duplex DNA has been configured in our study, because it has been reported that a variety of ligands can recognize the nucleobase opposite the AP site through hydrogen bonding even in the aqueous media.[17-22]

In this study, we have designed the new probes for reactions involving an AP site in duplex DNA. These probes consist of 2-AVP as a reactive moiety and penta-arginine (**2**: $(Arg)_5$), acridine (**3**), or Hoechst (**4**) as a binding moiety with high affinity to duplex DNA (Fig.1.).

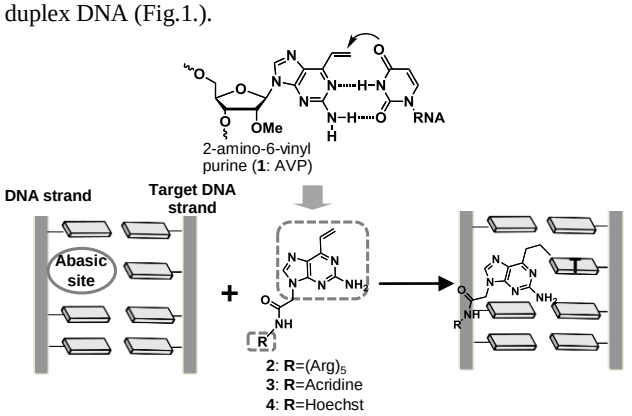
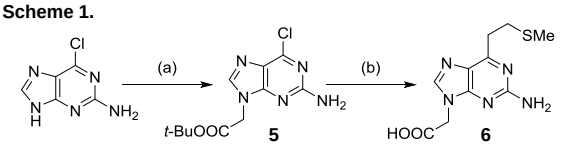


Fig. 1 Schematic representation of the strategy for site-specific alkylation to DNA

Penta-arginine binds to DNA with high affinity by a non-specific, electrostatic interaction. The acridine moiety binds to the duplex DNA as an intercalator.[23-24] Hoechst is a representative minor grove binder with high affinity and high specificity to sequences rich in adenine and thymine.[25-26] These binding moieties are postulated to be capable of promoting access of 2-AVP to the AP site. The 2-AVP derivatives are expected to form hydrogen bonds and to react selectively with the target base opposite the AP site.

Scheme 1.



(a) $BrCH_2CO_2t$-Bu, *t*-BuOK, DMSO, 75 % (b) 1) CH_2=$CHSnBu_3$,$(Ph_3P)_2Pd_2Cl_2$, dioxane 2) MeSNa, CH_3CN, 54 % for 2 steps 3) TFA, 98 %

The reactive base portion (**6**) was synthesized as shown in Scheme 1. The N9 position of chloropurine was alkylated with *t*-

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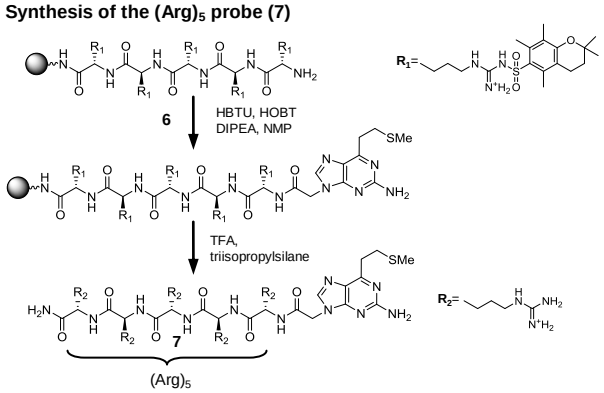
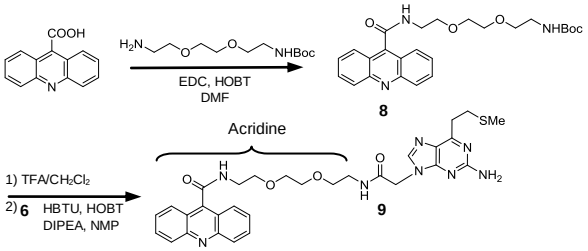
butyl bromoacetate to yield the *t*-butyl ester (**5**) in 75% yield. The cross-coupling reaction of (**5**) with nBu$_3$SnCHCH$_2$ gave the purine derivative, which was, without purification, reacted with methanethiol to give the thiol protected purine derivative (54% for 2 steps). The removal of *t*-butyl group was performed with TFA to afford **6**.

Scheme 2.

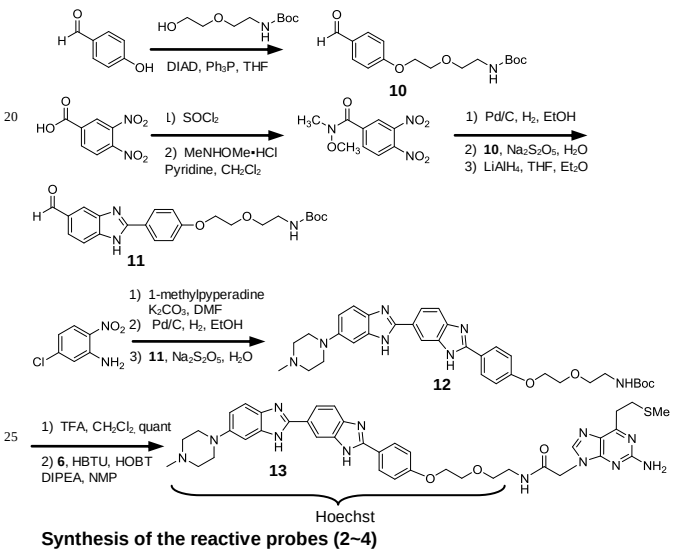
Synthesis of the (Arg)$_5$ probe (7)



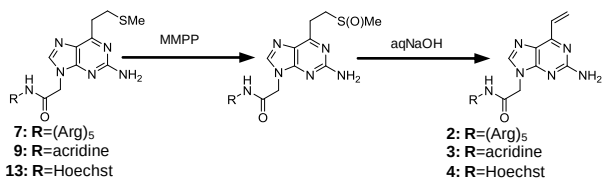
Synthesis of the acridine probe (9)



Synthesis of the Hoechst probe (13)



Synthesis of the reactive probes (2~4)



Each probe was synthesized as shown in Scheme 2. The 2-AVP probe conjugated to penta-arginine was prepared by (Fmoc)-based solid-phase synthesis. The protected penta-arginine on the resin was coupled with the 2-AVP derivative (**6**) at the N-terminal position. Subsequently, the stable precursor of the 2-AVP probe conjugated with the penta-arginine (**7**) was cleaved from the resin by treatment with TFA and triisopropylsilane. The acridine derivative (**8**) was obtained from 9-acridinecarboxylic acid and coupled with **6** to produce the stable precursor of the 2-AVP probe conjugated to the acridine derivative (**9**). The Hoechst derivative (**12**) was synthesized through a modification of a method described in the literature[27,28] and conjugated with **6** to afford the stable precursor of the 2-AVP probe (**13**). The sulphide-protected precursors of the 2-AVP probes (**7**, **9, 13**) were converted to the sulphoxides by oxidation with magnesium monoperphtalate (MMPP), followed by elimination under alkaline conditions to yield the AVP probes (**2**, **3**, **4**). The reactivities of synthesized probes were evaluated using duplex DNA containing an AP site and fully-matched DNA. In our experiments, tetrahydrofuranyl residue (dSpacer) was used as an AP site. The duplexes were formed with 5' fluorescein labelled DNA**2**, which was complementary to DNA**1**, containing thymine (T), cytosine (C), adenine (A) and guanine (G) bases opposite the AP site. Each probe (**2**, **3**, **4**) was added to both duplex DNA and single-stranded DNA. After 24 h, each reaction mixture was analysed by gel electrophoresis with 20% denaturing gel (Fig. 2A).

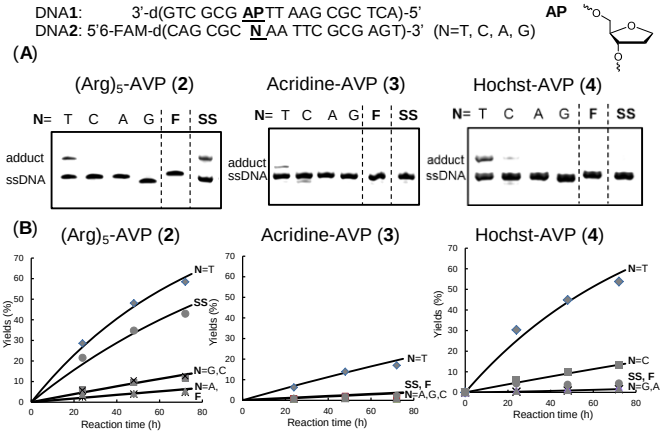


Fig. 2 Evaluations of the reactivities of the probes by the denaturing PAGE
The reaction was performed with 5 uM target DNA and 100 uM (Arg)$_5$-AVP (**2**), 100 uM Acridine-AVP (**3**), or 25 uM Hoechst-AVP (**4**) in 100 mM NaCl, 50 mM MES pH 7 at 37 ^{o}C. (**A**) Gel electrophoresis analysis performed on the products of the alkylation reaction after 24 h, **SS**: single strand (DNA**2**, **N**=T) **F**: full match (DNA**1**, **AP**=A, DNA**2**, **N**=T) (**B**) The time course for alkylation reactions.

The higher-molecular-weight band detected in reactions between the probes (**2**, **3**, **4**) and duplex DNA contained thymine opposite an AP site. The adduct was purified from each higher molecular-weight band and measured by MALDI TOF mass spectra. Each isolated species was shown to have a molecular weight consistent with a probe-DNA**1** (**N**=T) adduct (supporting information). No adducts were observed in reactions between the probes (**2**, **3**, **4**) and fully matched DNA duplexes. The yields of the alkylated products (Y-axis) were determined by quantification of the fluorescent bands in comparison to the bands corresponding to non-reacted target DNA. Fig. 2B shows the time-dependent changes observed in the alkylation yields with the three probes. The (Arg)$_5$-AVP (**2**) probe exhibited efficient reactivity with the duplex DNA containing thymine opposite the AP site, but similar reactivity to single-stranded DNA. These results indicated that the (Arg)$_5$-AVP (**2**) may also bind to single-stranded DNA through electrostatic interactions, arranging itself in sufficiently

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close proximity to react the target base. The acridine-AVP (**3**) and Hoechst-AVP (**4**) probes did not produce adducts with single-stranded DNA. These results suggested that acridine and Hoechst do not interact with single-stranded DNA sufficiently to enable selective reactivity with duplex DNA containing an AP site. The reactivity of **4** to duplex DNA containing a thymine opposite the AP site was higher than that of **3**. This more efficient reactivity might be due to a higher binding affinity between duplex DNA and Hoechst-AVP (**4**) than shown by acridine-AVP (**3**).

The alkylation of duplex DNA containing an AP site using Hoechst-AVP (**4**) was monitored by HPLC (Fig. S7). After the newly observed peak was purified, the isolated species was confirmed by MALDI TOF mass spectrometry to be DNA alkylated with Hoechst-AVP (**4**). We tried enzymatic hydrolysis of the purified alkylated DNA to determine the structure; however, we could not isolate the hydrolyzed product derived from the alkylated nucleoside. The potential alkylated sites with Hoechst-AVP (**4**) are O4, O2 and N3 of thymine (Fig. 3)

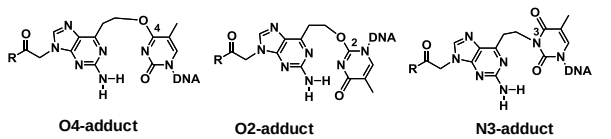

Fig.3. Proposed structures for the adduct to thymine with Hoechst-AVP (**4**)

For the considerations on the adduct structure, we examined the stability of the purified adduct. This adduct was highly stable under acidic (pH 5.0) or basic conditions (pH 9.0) for 24 hr, and heating conditions (for 10 min, 90 °C) (Fig. S8A~D). In addition, treatment of the adduct with 20 % acetic acid (pH 1.9) at 50 ºC for 30 min did not induced the remarkable degradation product (Fig. S8E). Under these conditions, O2-alkylthymidine is cleaved of the glycosyl bond so rapidly and O4-alkylthymidine is induced dealkylation.[29] N3-alkyl derivative is stable under the strong acidic conditions.[30] Based on these reports and our results, we propose that the major adduct structure might be the N3-adduct.

Next, primer extension reaction assays were used to confirm that the alkylation took place at the thymine opposite the AP site. Either purified DNA alkylated by Hoechst-AVP (**4**) or non-modified DNA was annealed to the fluorescein labelled primer, and each mixture was subjected to the primer extension reaction using Klenow DNA polymerase. The DNA fragments extended by nucleotide incorporation were visualized by fluorescence through sequencing polyacrylamide gel (PAGE) analysis. Fig. 4A shows the denatured PAGE bands obtained through the elongation of the fluorescein-labelled 9 mer primer annealed with DNA alkylated by Hoechst-AVP (**4**) or non-modified DNA. Two base elongations were observed using unmodified DNA as a template with TTP to produce 11 mer DNA (lane 2). Complete elongation to the full-length product was detected in the presence of four dNTP in the reference experiment (lane 6). In contrast, when using alkylated DNA as a template, 11 mer DNA was produced in the presence of dTTP (lane 8) and four dNTP (lane 12). In Fig. 4B, single nucleotide incorporations were examined using the fluorescein-labelled 11mer primer. When un-modified DNA was used as a template, elongation by one or two bases was found in the presence of dATP (Lane 4) and full length elongation in the presence of four dNTP (Lane 6) was observed. No elongation was observed with any dNTP using the alkylated DNA as a template (lane 8~12). These results suggested that the alkylation by Hoechst-AVP (**4**) took place at the thymine opposite the AP site, stopping the primer extension at that site.

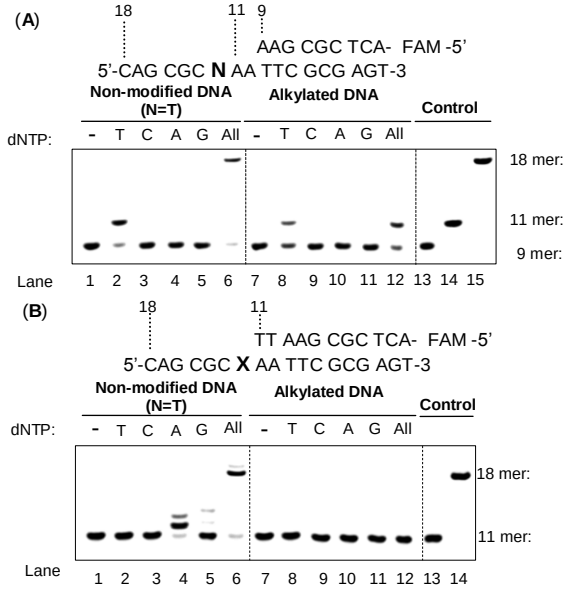

Fig.4. Gel analysis of primer extension assay using alkylated DNA by Hoechst-AVP or non-modified DNA as the template. The template DNA (**X**=T or T-HoechstAVP) was annealed with the primer (5' FAM labeled 9 mer DNA (**A**) or 11 mer DNA (**B**). The primer extension reactions were catalyzed by Klenow fragment in the presence of dTTP (Lane 2 and 8), dCTP (Lane 3 and 9), dATP (Lane 4 and 10), dGTP (Lane 5 and 11) and four dNTP (Lane 6 and 12). Lane 13~15: 5'FAM labeled DNA as a size marker.

Next, we investigated the influence of the distance between the AP site and the Hoechst binding site (AATT) in the target DNA on Hoechst-AVP (**4**) reactivity.

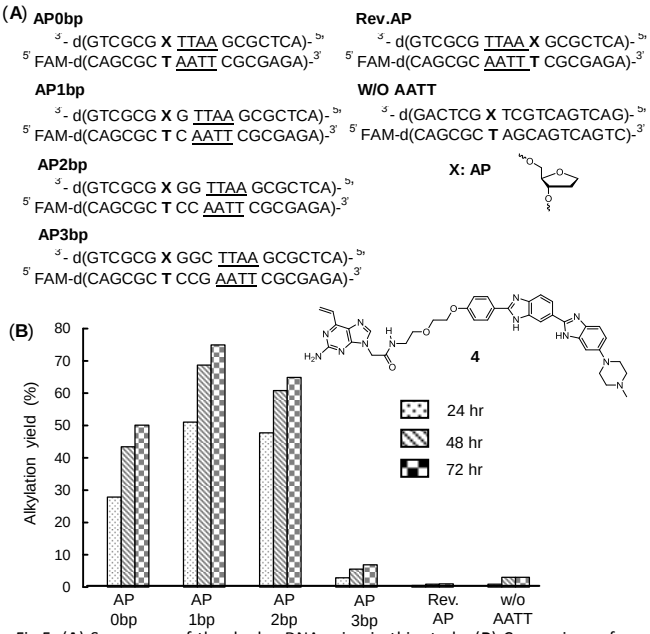

Fig.5. (**A**) Sequences of the duplex DNA using in this study. (**B**) Comparison of the alkylation yields with **16** to target duplex DNA contained the different distances between AATT and an AP site.

Fig. 5A shows the sequences of duplexes in this experiment. The reactivity of **4** with duplex DNA decreased in order of AP1bp≥AP2bp>AP0bp>>AP3bp, showing a dependence on the distance between AATT site and the AP site. The duplex DNA

without an AATT site (w/o AATT) gave only a modest yield of the alkylated product.

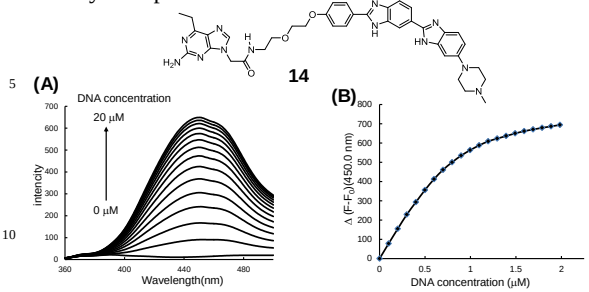

Fig.6. (**A**) Changes in fluorescence spectra of **14** (0.1μM) in 50 mM MES (pH 7.0), 100 mM NaCl with increasing duplex DNA (AP0bp) concentration. (**B**) Changes in fluorescence intensity at 450 nm are plotted versus the DNA concentration.

Interestingly, the alkylated product was nearly absent in the duplex DNA containing the Hoechst binding site (AATT) at the 3' side of the target base in the fluorescein-labelled strand (Rev.AP). For the non-reactive Hoechst-AVP derivative (**14**) that was prepared by a route described in the supporting information, the binding constants to duplex DNA targets were determined by fluorescence titrations. Fig. 6 shows the change in the fluorescence spectra of **14** with increasing concentrations of duplex AP-site-containing DNA. The binding constants were obtained by analysis of the titration curve (Table 1).

Table 1 Binding constants of **14** to duplex DNA

DNA	Ks $10^8 M^{-1}$	DNA	Ks $10^8 M^{-1}$
AP0bp	3.0	AP3bp	3.0
AP1bp	2.3	Rev.AP	3.3
AP2bp	2.1	w/oAATT	weak

Values were obtained by a non-linear least squares data analysis.

The non-reactive Hoechst-AVP derivative (**14**) exhibited the similar binding constants for duplex DNA, AP0~3bp and Rev.AP, indicating that the binding affinity of **14** did not depend on the location of the Hoechst binding site (AATT) in the target DNA. Taken together, the efficient reactivity of the Hoechst-AVP derivative (**4**) required appropriate positioning of the AVP moiety for interaction with the thymine opposite the AP site.

Conclusions

In conclusion, we have developed a strategy for the selective alkylation of DNA using single-stranded DNA containing an abasic site and an alkylating probe. The Hoechst-AVP probe exhibited high selectivity and efficient reactivity to thymine bases at the site opposite an abasic site in DNA. In addition, the primer extension reaction with polymerase was stopped at the alkylated site by the Hoechst-AVP probe. These results suggested that Hoechst-AVP probe may exert inhibitory effects on DNA replication. Our studies provide the proof-of concept that AVP derivatives conjugated with DNA binding molecules might form hydrogen bonds with target nucleobases located in a hydrophobic pocket and induce the selective alkylation. Based on the concept, the investigations of the probes for inducing selective alkylation in other hydrophobic pockets are now in progress.

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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