HYBRIDIZATION OF OLIGODEOXYNUCLEOTIDE WITH REDOX COENZYME MODEL; SYNTHESIS AND PROPERTIES OF THYMIDINE DECAMERS COVALENTLY LINKED TO 5-DEAZAFLAVIN

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Modified thymidine decamers covalently linked to 5-deazaflavin derivatives through aminoalkyl spacer arm in the form of phosphoramidate bond, were prepared and characterized. Chemical and physical properties of the hybrid molecules are discussed.

KEYWORDS DNA hybrid; 5-deazaflavin; antisense molecule; synthesis; fluorescence

5-Deazaflavin cofactor, such as Factor 420, is an essential coenzyme and is involved in the reduction of carbon dioxide to methane in biological systems.[1] More interestingly, 5-deazaflavin catalyzes the splitting of thymine dimer as a photoreactivating coenzyme,[2] and some related chemical studies have been reported.[3] We have reported a model system in which a reduced form of 5-deazaflavin derivative contributes to reductive repair of oxidatively damaged nucleic acid related compounds.[4]

In connection with chemical studies on coenzyme model and functionalized antisense molecules, we describe here the synthesis and properties of the thymidine decamers modified with 5-deazaflavin derivatives at the specific site of the phosphorous backbone in oligodeoxynucleotides. In addition to redox reactivity, 5-deazaflavin has the inherent characters of planarity of the aromatic rings, strong visible and ultraviolet absorptions, and characteristic fluorescence. These functions might play an important role as an intercalator, a non-radioactive reporter as well as a functionalized antisense molecule[5] with specific endonuclease-like activity[6] based on an activation of molecular oxygen by a reduced form of 5-deazaflavin, if oligodeoxynucleotide is tagged with 5-deazaflavin.

In order to incorporate the 5-deazaflavin molecule into DNA-oligomer, the synthesis of 5-deazaflavin derivatives bearing primary aminoalkyl side-chains from the N(3) or C(8) position, **1** or **2**, [7,10] was performed as shown in Chart 1.[7] For the substituent of the N(10) position, primary alkyl group rather than aromatic ring was adopted to avoid an orthogonal structural feature and keep the molecule planar[8] as well as to improve solubility toward organic solvents, especially carbon tetrachloride employed for oxidative coupling (*vide infra*). Thus, 3-methyl-6-octylaminouracil was condensed with 2,6-difluorobenzaldehyde to yield 5-deazaflavin skeleton,[9] **3**, whose fluoride was substituted by N-1-trifluoroacetylhexanediamine[11] in DMF. Deprotection and subsequent spontaneous air oxidation gave **1**. On the other hand, the 5-deazaflavin derivative (**2**) was furnished by successive reaction of **4** with 1,6-dibromohexane, sodium azide and catalytic hydrogenation.

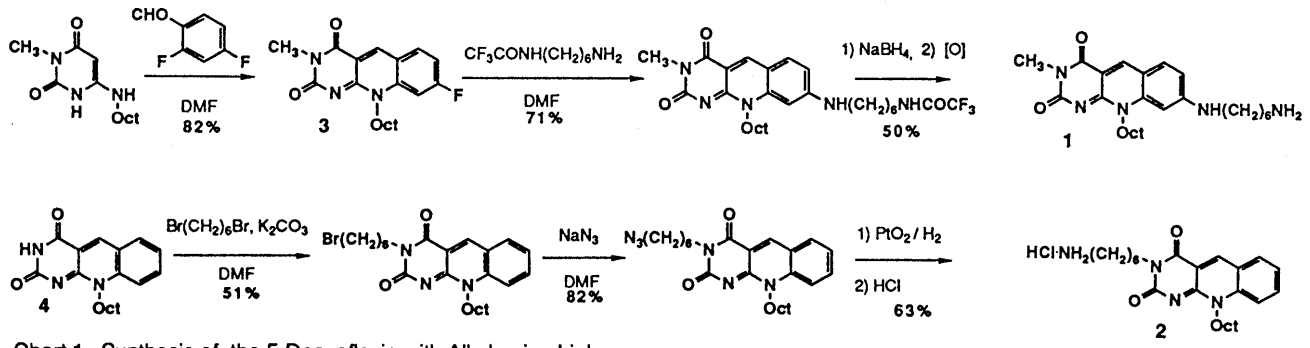
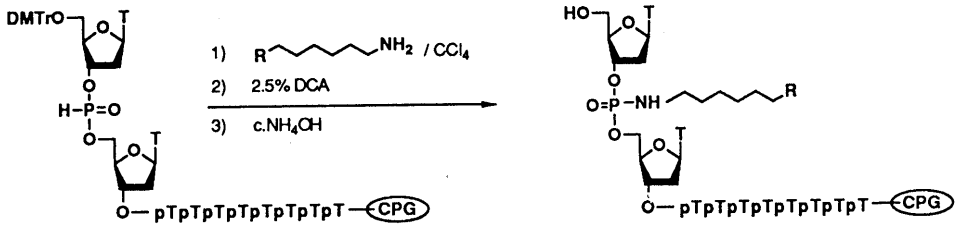
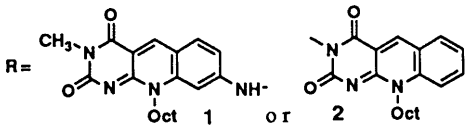


Chart 1. Synthesis of the 5-Deazaflavin with Alkylamino Linker

The connection of 5-deazaflavin derivatives to the backbone of DNA oligomer is based on oxidation of the H-phosphonate linkage with the primary aminoalkyl group in the presence of carbon tetrachloride resulting in a phosphoramidate bond.[11,12] Using the standard syringe works[13] for polymer support DNA synthesis, the oxidative coupling with excess **1** or **2** was carried out in CCl_4/pyr./CH_3CN (1/1/1) for 7-12 min in the presence of small amounts of triethylamine to yield the modified dimer after detritylation and detachment from the support. In the case of **1**, coupling occurred exclusively at the primary amine due to the poorer nucleophilic nitrogen at the C(8) position.[14] The successful formation of the modified dithymidylate prompted synthetic work on a longer oligomer modified at the specific site of sequence. Three modified decathymidylates, **5-7**, were prepared and purified by reverse-phase HPLC; two of them have the modified internucleotide linkage at the 5'-end in the strand, and the remainder has it at the central phosphorous backbone (Chart 2).





$^{5'}$Tp*(1)TpTpTpTpTpTpTpT$^{3'}$ **5**
$^{5'}$Tp*(2)TpTpTpTpTpTpTpT$^{3'}$ **6**
$^{5'}$TpTpTpTpTp*(1)TpTpTpTpT$^{3'}$ **7**

p: natural phosphodiester bond
p*: modified phosphodiester bond

Chart 2

In the UV spectra, these conjugate molecules have absorption maxima at 436nm as well as those at 260nm. Since one of the internucleotide linkages is neutral in the modified compound, the relative mobility of strands **5-7** in 20% PAGE analysis was smaller (83%) than that of the natural type of thymidine decamer (T_{10}), and no significant difference of mobility within the modified strands was observed. When exposed to ultraviolet light of wavelength 254nm, the bands of the modified single strand fluoresced an extremely light yellow color. As expected, the phosphoramidate internucleotide linkage is resistant to phosphodiesterase; therefore, enzymatic digestion[15] by sequential treatment with phosphodiesterase I and alkaline phosphatase of strands **5** and **6** resulted in formation of thymidine and the modified dithymidylate in a consistent ratio (Fig. 1).

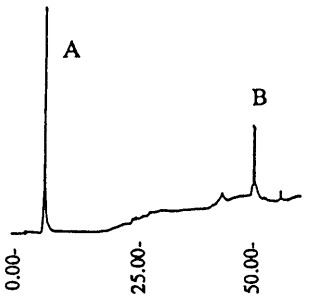
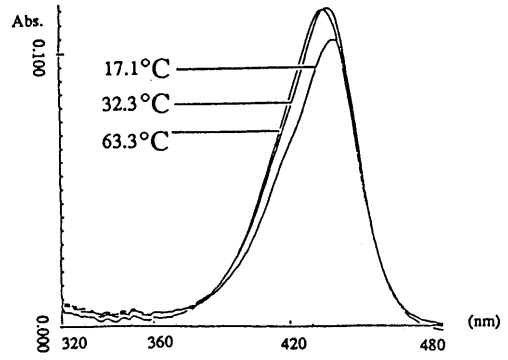


Fig. 1. HPLC Profile[a] of the Reaction Products in Enzymatic Degradation of the Strand **5**

a) Conditions: CAPCELL PAK C_{18} AG-120 (SHISEIDO Co. Ltd.) column, 0.1M TEAA buffer (pH 7.0), 1% / min, CH_3CN linear gradient starting at 5% CH_3CN, 1ml / min flow rate.
A: thymidine, B: modified dimer.

Fig. 2. Absorption Spectra of **5** with poly dA (1:1 Mole Equivalents) at Different Temperatures in 0.01M Tris-HCl Buffer (pH 7.0) under the Concentration of 0.1M NaCl



The melting curve of the duplex with poly dA was taken, and from it the melting temperature was determined in order to assess the effects of introduction of 5-deazaflavin molecule on the thermal stability of the double helical structure; these results are tabulated (Table I). As seen in Table I,

introduction of the 5-deazaflavin derivative at the central position of the decamer **7** causes lowering of the temperature, probably due to steric distortion of the duplex, whereas duplexes of both **5** and **6** bearing the modified amidate linkage at the 5'-end in the strand exhibit higher Tm values as compared with that of the natural type of T_{10}. The stability might come from an intercalative contribution[16] by the 5-deazaflavin moiety. In fact, with measurement of the ultraviolet spectra of the duplex with **5** at different temperatures, both the red shift and hypochromicity were observed in the absorption region around 430 nm, attributable to 5-deazaflavin moiety, at a temperature below Tm (Fig. 2). Additionally, in the fluorescence spectrum of the duplex with **5** excited at 375nm, the temperature-independent wavelength of which is attributable to the 5-deazaflavin, a quenching phenomenon of fluorescence was observed in some degree. These results suggest that 5-deazaflavin moiety contributes to stability of the duplex by an intercalative effect.[16]

Table I. Melting Temperature (Tm) of the Duplexes of **5** - **7** with Poly dA (1:1 Mole Equivalents) in 10 mM Tris-HCl Buffer (pH 7.0)

Strand	Tm (°C) in 0.1M NaCl	Tm (°C) in 1.0M NaCl
T_{10} (native)	24	38
5	31	41
6	26	46
7	17	29

In conclusion, the present study on hybridization of the 5-deazaflavin derivatives with deoxynucleotide oligomer might give useful information about the design of a new type of fluorescence probe[17] and a thermally stable functionalized antisense molecule.

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