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SYNTHESIS AND PROPERTIES OF OLIGOTHYMIDYLATE CONTAINING SULFUR-MODIFIED THYMIDINE: EFFECT OF THIATION OF PYRIMIDINE RING ON THE THERMOSTABILITY AND CONFORMATION OF THE DUPLEX

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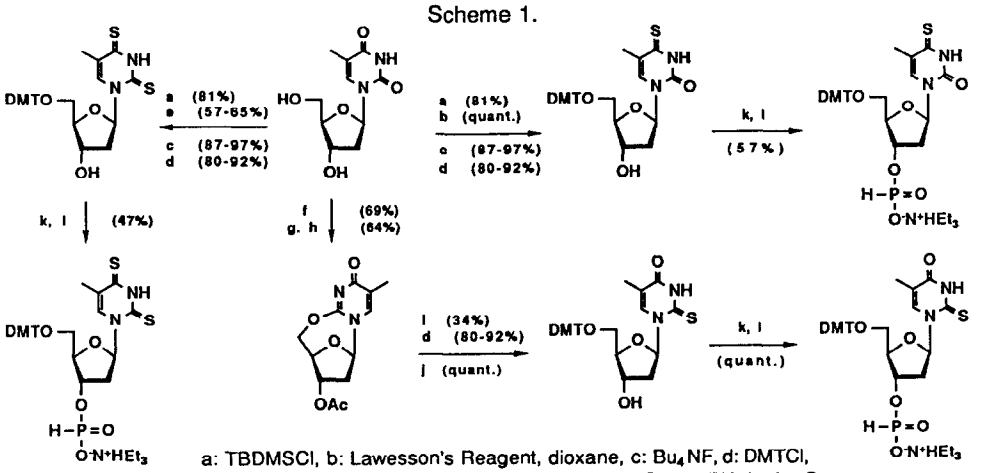
Abstract : Three kinds of thiothymidine derivatives were synthesized and successfully incorporated into oligothymidylates by using H-phosphonate chemistry at the desired site of the sequences. Among the strands prepared, the alternate strands containing 2-thiothymidine exhibited significant stability of double helical structures with their complementary strands.

Certain class of nucleic acids, especially ^{t}RNAs, contain minor nucleosides in small amount. Particularly interesting is extremely thermophilic archaebacteria that grows in the temperature up to 105°,[1] and whose ^{t}RNAs have several modified nucleosides.[2] Among those modifications, 2-thiation of ribothymidine in TΨC loop has been anticipated to be largely responsible for the thermostability of the ^{t}RNAs in their higher-order structure.[3] Thermal stability is an important factor for design of efficient antisense molecules[4] and it might be anticipated that some sulfur-modified nucleic acids provide a useful information about protein nucleic acid interaction.[5] In the course of our intensive study on functionalized antisense molecules, we have synthesized oligodeoxythymidylates containing 2-thio-, 4-thio- and 2,4-dithiothymidine using H-phosphonate chemistry and investigated their physical and chemical properties, such as duplex stability and reactivity toward nuclease.

Scheme 1.

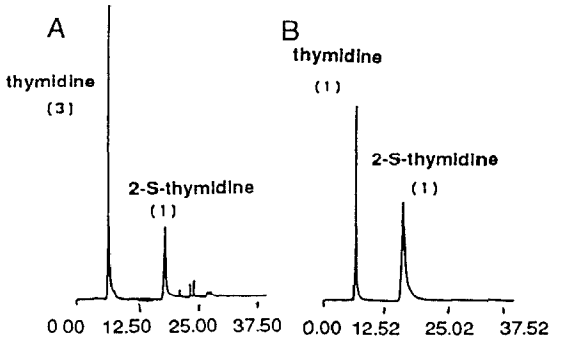


a: TBDMSCl, b: Lawesson's Reagent, dioxane, c: Bu_4NF, d: DMTCl, e: Lawesson's Reagent, toluene, f: *p*-TsCl, -20°C, g: DBU, h: Ac_2O, i: H_2S, j: NH_4OH, k: imidazole, PCl_3, l: TEAB buffer (pH 8.5)

Although syntheses of thiothymidines have been reported,[5,8] the modified procedures were undertaken for their preparations and shown in Scheme 1. Thus, a published method for the synthesis of 2-thiouridine via 2,5'-anhydro derivative,[5,6] originally reported by Todd et al.,[7] was successfully applied to the synthesis of 2-thiothymidine,[8] which was transformed into 5'-DMT derivative.. The 4-thiothymidine derivative was furnished by the treatment of 3',5'-di-O-silylated thymidine with Lawessons' reagent in dioxane[5] or with P_2S_5 in pyridine. Changing the solvent to toluene in this reaction with excess Lawessons' reagent, 2,4-dithiothymidine was afforded as a sole product in good yield. Similarly, those modified nucleosides were converted to the corresponding 5'-DMT derivatives.[9]

The H-phosphonate approach[10] is favorable for incorporation of the labile modified nucleosides into oligodeoxynucleotides as compared with the phosphoramidite method.[11] Because the number of the oxidation step can be minimized and undesired side reactions with phosphitylating agents or tetrazole activated phosphoramidite is avoidable. Consequently, the sulfur-modified nucleosides were transformed into their H-phosphonate units by a standard procedure.[10] After confirmation of incorporation of these bases into nucleotides sequences by effective formation of the modified *T-T dimer, several different type of oligothymidylates analogues were synthesized using a standard syringe work[12] for polymer support DNA synthesis (Table 1). In this synthesis the crude products bearing DMT protective group at the 5' end were first purified by HPLC, and then its detritylated products were repurified in the same manner.

Fig. 1. HPLC[a] profile of the crude reaction mixture in enzymatic digestion of the modified octamers, 2 and 3.



a) Conditions: Cosmosil $5C_{18}$ column, starting from 5% CH_3CN, 1ml/min flow rate, isocratic for 5min, then linear gradient 1%/min CH_3CN, detected at 254nm; A; digestion of the octamer **2**, B; that of octamer **3**. The products ratios are represented in parentheses.

For characterization of the synthesized strands, 20% PAGE analysis and enzymatic digestion were carried out. No significant difference in the mobility was observed in PAGE within the same size of strands due to isoelectric nature of the sulfur, and it turned out that conventional nuclease, such as snake venom phosphodiesterase, can not discriminate the modified base from the native one (Fig. 1). To assess the effects those sulfur-modified bases on duplex stability, the melting temperature of each modified strand with its complimentary strand, poly dA or dA_{15}, was determined and compared them to those of unmodified oligomers.[13] These results and melting curves are shown in Table 1 and Fig. 2, respectively. As can be seen from the Table, the oligomer modified at the 4-position, the strand **4**, shows appreciable helix destabilization and one at both the 2- and 4-position, the strand **5**, has no clear sigmoid melting curve. On the other hand, the alternate strand, **3** and **7**, considerably contribute to stability of the helical structure.

Fig.2. Absorbance profile at pH 7.0 (10mM Tris-HCl buffer)

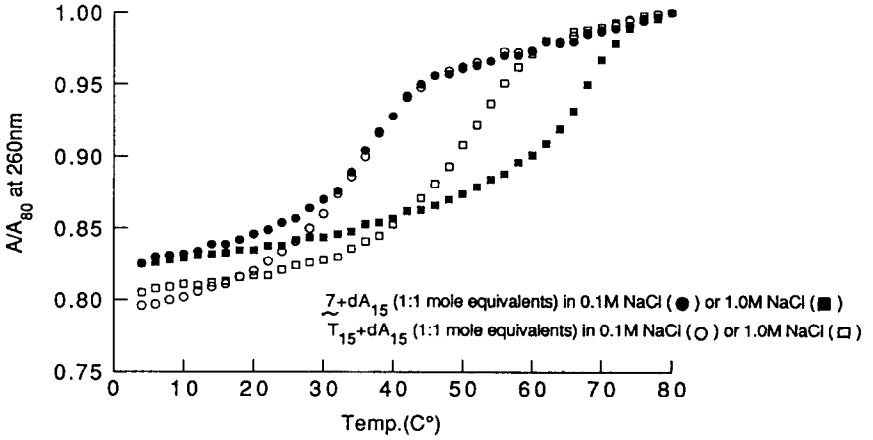


Table 1. Melting temperature of the duplexes.[a)]

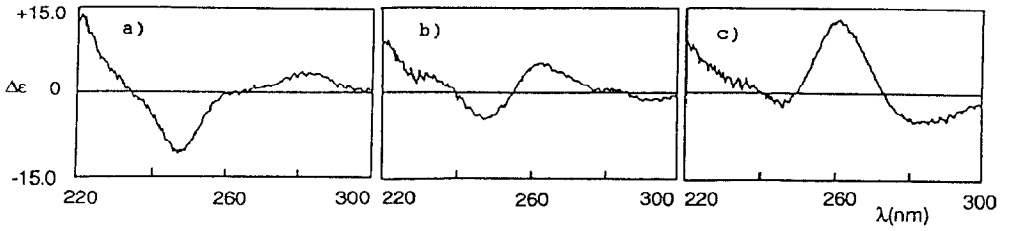
T strand[b)]		A strand	conc.(NaCl)	Tm value
T_8	1	poly dA	1.0 M	36 °C
$5'Tp^{2S}Tp(Tp)_4{}^{2S}TpT^{3'}$	2	poly dA	1.0 M	33 °C
$^{2S}TpTp^{2S}TpTp^{2S}TpTp^{2S}TpT$	3	poly dA	1.0 M	39 °C
$Tp^{4S}Tp(Tp)_4{}^{4S}TpT$	4	poly dA	1.0 M	20 °C
$Tp^{dS}Tp(Tp)_4{}^{dS}TpT$	5	poly dA	1.0 M	Not Clear
T_{15}	6	dA_{15}	0.1 M	35 °C
		dA_{15}	1.0 M	50 °C
$Tp(^{2S}TpTp)_6{}^{2S}TpT$	7	dA_{15}	0.1 M	38 °C
		dA_{15}	1.0 M	66 °C

a) T strand + dA strand (1:1 mole equivalents) in 10mM Tris-HCl buffer (pH 7.0).

b) p: natural phosphodiester link, ^{2S}T: 2-thiotnymidine, ^{4S}T: 4-thiothymidine, ^{dS}T: 2,4-dithiothymidine

Finally, circular dichroism spectra of the oligomers containing the modified base with their complimentary strands were measured at 278K in order to explain the stability of the duplex and some of them are presented in Fig. 3. As expected, the parent T8-dA10 double helix shows a typical B-DNA spectrum.[14] Contrary to this, a large peak at 265 nm has appeared concomitantly with reduction of the peak at 250 nm in the duplex of the strand **3** and dA10. This implies that the later duplex forms an A-DNA structure and therefore that of the strand **2** exists as an intermediate or transition state between A and B structure, which causes lowering the melting temperature. It is well documented that the furanose ring has a C3'-endo-pucker in A-DNA[15] contrary to C2'-endo in B-DNA.[16] In fact, Miyazawa and co-workers reported that the preferred conformation of 2-thioribothymidine is rigid C3'-endo form and such a rigidity possibly contributes to the thermostability.[8,17]

Fig. 3. CD spectra of the duplexes.



a) $T_8(1)$ + dA_{10}, b) 2 + dA_{10}, c) 3 + dA_{10} CD spectra were taken in 10mM Tris-HCl buffer, 1.0M NaCl pH 7.0 at a temperature of 278K.

From another point of view, the stability might be attributed to inherent nature of sulfur, large polarizability, lowering the level of LUMO and elevation of HOMO, which causes the higher stacking energy.[18] The sulfur at the 2-position stacks over N_1 of the adjacent nucleotide[14] and this particular position (in minor groove) does not play an important role in the hydrogen bonding of Watson-Crick base pairing. On the other hand, introduction of sulfur at the 4-position loses such a direct S-N interaction and brings about a destabilization due to the weakened hydrogen bonding and probably to steric bulkiness of sulfur atom in major groove.

In conclusion, the present study reveals successful introduction of the sulfur-modified thymidine nucleosides into synthetic oligonucleotide at the desired site of sequence by using H-phosphonate chemistry, and that number, position in the sequence as well as identity of the modified base are closely related to the thermal stability and conformation of the duplex.

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