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# Synthesis and RNA Binding Selectivity of Oligonucleotides Modified with Five-Atom Thioacetamido Nucleic Acid Backbone Structures

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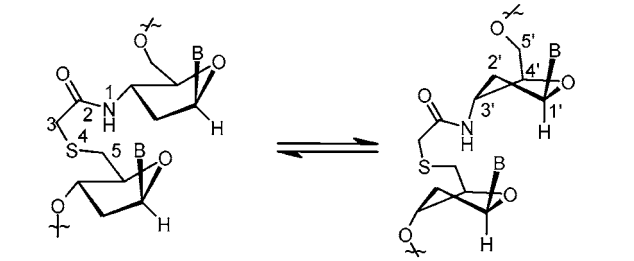
## ABSTRACT




**Convenient chemical synthesis and incorporation of dithymidine and thymidine–cytidine dimer blocks connected with a five-atom amide linker N3′–CO–$CH_2$–S–$CH_2$ into oligonucleotides (ONs) are reported. The UV–$T_m$ experiments for binding affinities of these mixed backbone ONs with complementary DNA and RNA sequences revealed important results such as significantly higher RNA-binding selectivity as compared with complementary DNA. NMR studies of the dimer blocks suggested a marginal increase in the N-type sugar conformations over that of the native DNA.**

The last two decades have witnessed an upsurge in the synthesis of several modified nucleic acid derivatives. The intentions have been to synthesize therapeutically suitable and commercially viable nucleic acid analogues.[1] The more recent developments such as splice correcting and exon skipping strategies require highly robust nucleic acid analogues[2] that are stable under physiological conditions as single strands as well as in the form of duplexes with complementary RNA sequences. The enzymatic stability[3] of peptide nucleic acids (PNAs) gains importance for such applications.[2] The uncharged PNAs (Figure 1) are poorly soluble in water and bind to DNA in either parallel or antiparallel orientation.[4] Also, PNAs require assistance in the form of covalent conjugation with cell-penetrating peptides (cpp)[5] or additional positive charges on PNA[6,7] to cross the cell membranes for their activity. The replacement of a few or several internucleoside phosphate linkages by the robust amide bond as in PNA could be an alternate solution so that the advantages of chirality and 3′−5′

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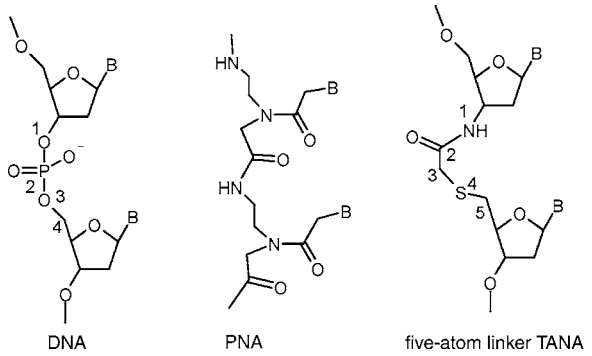



**Figure 1.** Structures of DNA, PNA, and TANA.

directionality of the sugar are maintained in the analogue along with the enzymatic stability of the amide bond. The retention of some negatively charged phosphate groups would be advantageous for water solubility. Cell-penetration experiments then could be performed using cationic lipids,[8] complexation, or conjugation with cpp.[8] Several four- and five-atom amide-linked oligonucleotide analogues and those containing thioether linkages are known in the literature.[1,9] The five-atom amide linkers were introduced to compensate the shorter amide bond in the dinucleoside as compared with the four-atom phosphate linkers to maintain the internucleoside distance complementarity.[9] The strategy was found to be successful as some of the five-atom-linked dimers when substituted in oligonucleotide (ON) sequences lead to significantly higher RNA affinities compared with that of the native DNA. It has been shown by CD spectroscopy and crystal structure that the longer amide backbones do not disrupt the duplex geometries.[10] The application of these ONs with superior binding properties could be stymied by the multistep synthetic procedures to arrive at the desired monomers. Our continuing efforts toward finding probable optimum oligonucleotide mimics resulted in a new five-atom thioacetamido nucleic acid (TANA, Figure 1) backbone.[11] The convenient synthetic methodology to convert pyrimidine nucleosides to a sugar−amino acid monomer unit by using thiol functionality in ethyl mercaptoacetate as a requisite nucleophile was reported in our previous communication. The homooligomeric pyrimidine ONs were found to bind to complementary RNA sequences significantly better than their DNA counterparts, and the binding efficiency was found to be as good as PNA itself. However, introduction of these amino acid nucleoside derivatives in PNA sequences was found to be detrimental for RNA/DNA binding. In this communication, we present the synthesis and incorporation of thymidine and thymidine−cytidine dimer blocks (**tst** and **cst**) connected with a five-atom amide linker N3′−CO−$CH_2$−S−$CH_2$ (TANA) (Figure 2) into oligonucleotide oligomers. The assessment of the compatibility of the TANA dimer blocks in a sugar−phosphate backbone was studied by UV−$T_m$ measurements of the resulting mixed-backbone ON complexes with DNA and RNA.

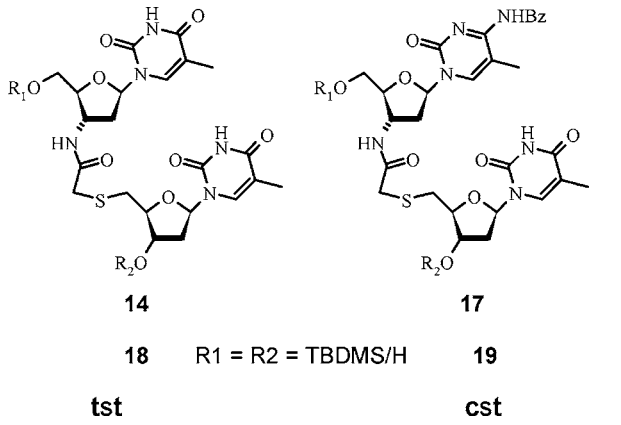
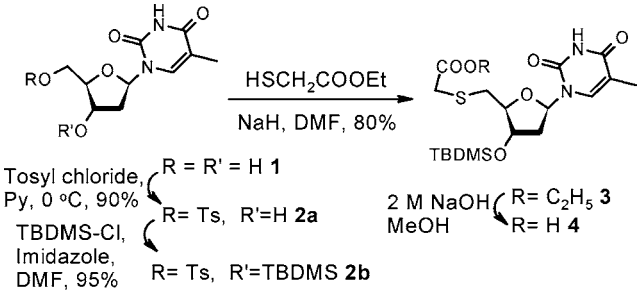



**Figure 2.** **tst** and **cst** building blocks.

Thymidine was selectively tosylated at the 5′ position, and the 3′-OH was then protected as a 3′-*O*-TBDMS group. Treatment of 3′-O-protected 5′-*O*-tosyl-thymidine with ethyl mercaptoacetate followed by ester hydrolysis gave the acid synthon **4** (Scheme 1). The amine synthon of thymidine was synthesized from 3′-azidothymidine **5** by protection of the free hydroxy group as 5′-*O*-DMTr in **6** and reduction of azide to amine to get **7**. The 5′-*O*-DMTr, 3′-azidothymidine **6** was converted[12] to a cytidine derivative by known procedures via C4-triazolide (**8**) followed by amination (**9**) and benzoylation (**10**), and reduction of the azide gave the desired

**Scheme 1.** Synthesis of a Thymidine Monomer Unit

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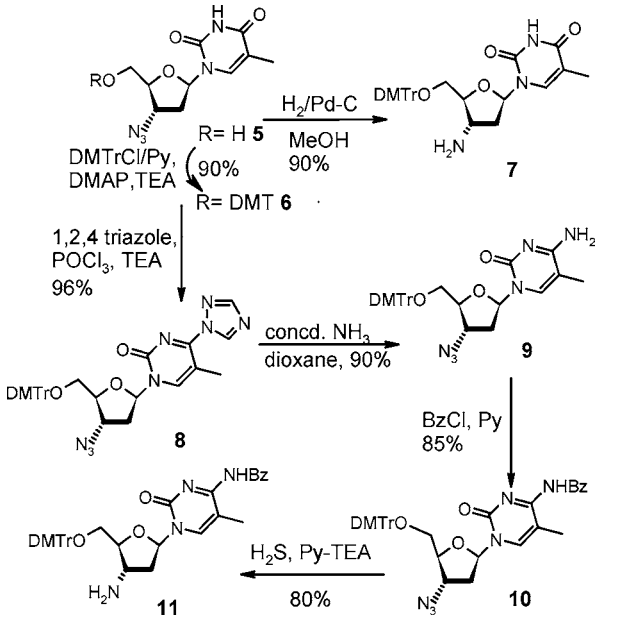
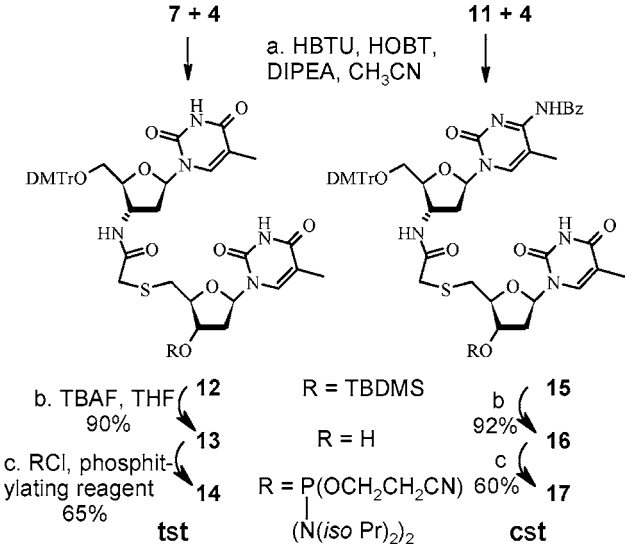
**Scheme 2.** Synthesis of a Cytidine Monomer Unit




cytidine derivative **11** (Scheme 2). The synthesis of the dimer building blocks **tst 14** and **cst 17** was then achieved by using peptide-coupling chemistry from the monomer units, deprotection, and phosphitylation (Scheme 3). All the new

**Scheme 3.** Synthesis of **tst** and **cst** Dimer Blocks




compounds were characterized by $^1$H NMR, $^{13}$C NMR, and mass spectral analysis. The structures of the dimer blocks **18** and **19** (Figure 2) were extensively studied by using 2D NOESY, COSY, and TOCSY $^1$H spectral analysis. The sugar ring conformations in the dinucleosides were elucidated using an empirical method of analysis of the vicinal coupling constants $^3J_{1'2'}$ and $^3J_{1'2''}$.[13] The population of the 2′-*endo* sugar conformations (% S) was calculated from the equation used in this analysis.[20]

A series of chimeric ONs (Tables 1 and 2) containing one to four **tst** and **cst** blocks were synthesized by automated solid-phase synthesis using the phosphoramidite approach and an Applied Biosystems 3900 DNA Synthesizer. After cleavage from the support, the oligomers were purified by

**Table 1.** Modified ON−Pyrimidine Sequences and UV−$T_m$ of Complexes with Complementary DNA and RNA[a]

| | ON sequences mass (calcd/obsd) | ON:DNA $T_m$ (°C) | ON:RNA $T_m$ (°C) |
|---|---|---|---|
| 1 | 5′ CGTTTTTTTTGC **33** | **33:34** | **33:35** |
| | | 40 | 32.0 (−8.0)[b] |
| 2 | 5′ CG TT**tst**TTT TGC **20** | **20:34** | **20:35** |
| | (3606.5/3606.6) | 23.7 | 32.3 (+8.6) |
| 3 | 5′ CGTT **tst** TT **tst** GC **21** | **21:34** | **21:35** |
| | (3596.6/5597.0) | nd | 50.0 |
| 4 | 5′ CG **tst tst tst tst** GC **22** | **22:34** | **22:35** |
| | (3576.8/3576.6) | nd | 47.81 |
| 5 | 5′ TCT CTT TCT T **39** | **39:40** | **39:41** |
| | | 23.6 | 25.4 (+0.8) |
| 6 | 5′ TCT C **tst** TCT T **23** | **23:40** | **23:41** |
| | (2933.1/2933.1) | nd | 29.6 |
| 7 | 5′ TCT C **tst** TC **tst 24** | **24:40** | **24:41** |
| | (2923.2/2924.0) | nd | 33.8 |
| 8 | 5′ T**cst** CTT TCTT **29** | **29:40** | **29:41** |
| | (2948.2/2948.1) | 19.5 | 26.2 |
| 9 | 5′ T **cst** CTT T **cst** T **30** | **30:40** | **30:41** |
| | (2938.3/2938.7) | nd | 33.8 |
| 10 | 5′ T**cst cst** T T **cst** T **31** | **31:40** | **31:41** |
| | (2928.4/2928.7) | nd | 39.7 |

[a] DNA (**34**, **40**) and RNA (**35**, **41**) sequences **34** 5′ GCAAAAAAAACG 3′, **40** 5′AAG AAA GAG A3′, **35** r (5′ GCAAAAAAAACG 3′), and **41** r (5′ AAG AAA GAG A 3′). [b] Figures in parentheses indicate the difference in $T_m$ between complexes with RNA and DNA.

**Table 2.** Modified ON−Purine−Pyrimidine Sequences and UV−$T_m$ of Complexes with Complementary DNA and RNA[a]

| | ON sequence (mass cald/obsd) | ON:DNA $T_m$ (°C) | ON:RNA $T_m$ (°C) |
|---|---|---|---|
| 1 | 5′ CCT CTT ACC TCA GTT ACA | **36:37** | **36:38** |
| | **36** | 54.6 | 54.7 (+0.1) |
| 2 | 5′ CCT C **tst** ACC TCA G TT ACA | **26:37** | **26:38** |
| | **26** (5366.7/5366.8) | 39.6 | 47.5 (+7.9) |
| 3 | 5′ CCT C **tst** ACC TCA G **tst** ACA | **27:37** | **27:38** |
| | **27** (5356.9/5356.9) | 43.5 | 52.8 (+9.3) |
| 4 | 5′ TCA CTA GAT G | **42:43** | **42:44** |
| | **42** | 24.3 | 24.7 (0.4) |
| 5 | 5′ TCA **cst** A GAT G 3′ | **32:43** | **32:44** |
| | **32** (3036.2/3037.0) | 16.3 | 29.6 (+13) |

[a] DNA (**37**, **43**) and RNA (**38**, **44**) sequences **37** 5′ TGT AAC TGA GGT AAG AGG 3′, **43** 5′CAT CTA GAG A3′, **38** r (5′ UGU AAC UGA GGU AAG AGG 3′), and **44** r (5′CAU CUA GAG A3′). [b]Figures in parentheses indicate the difference in $T_m$ between complexes with RNA and DNA.

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gel filtration and reverse-phase HPLC. The purity of the oligomers was checked by reverse-phase HPLC analysis on a C18 column and was characterized by mass spectrometry. These ONs were tested for their binding affinity to complementary DNA and RNA sequences in thermal denaturation−UV measurement experiments and the data are summarized in Tables 1 and 2. The unmodified sequence $GCT_8CG$ **33** was found to form complexes with both cDNA and RNA with a higher melting temperature for the ON:DNA complex over ON:RNA ($\Delta T_m = -8$).[14] Introduction of a single thioacetamido-linked **tst** dimer unit in **20** reversed this selectivity, and the ON:RNA duplex, **20:35**, was more stable ($\Delta T_m = +8.6$) than its complex with cDNA (**20:34**). A cumulative effect was observed in stabilizing the ON:RNA complex, **21:35**, and at the same time, the complex with DNA (**21:34**) was destabilized, when two units of modified **tst** dimer were incorporated in the ON. Similarly, the ON sequence **22** with alternating phosphate−TANA linkers exhibited binding only with RNA **35** and no observable melting transition with cDNA **34**. The unmodified mixed pyrimidine ON **39** formed complexes with either cDNA or RNA and exhibited almost equal binding strength. The preferential binding to RNA was consistently observed when one or two phoshate linkers were replaced by TANA by incorporation of either one or two modified (**tst** or **cst**) dimer units, almost independent of their position in the ON. Inclusion of two or more modified units in ONs leads to the significant stabilization of their complexes with complementary RNA, whereas complexes with the DNA counterpart did not show a detectable transition (Table 1, entries 3, 4, 7, 9, and 10). To verify the usefulness of these modified units in the mixed purine−pyrimidine sequence context, we synthesized two different unmodified sequences **36** and **42** (Table 2). The 18mer sequence **36** was modified by introduction of one **tst** unit in **26** and two **tst** units in **27**. The unmodified 18mer **36** recognized both cDNA **37** and RNA **38** with equal affinity ($\Delta T_m = +0.1$). One **tst** unit caused destabilization of complexes with both DNA (**36:37**) and RNA (**36:37**), but to a much lesser extent with RNA than with DNA. The discrimination between RNA and DNA recognition was observed with a $\Delta T_m$ of about 8 °C. Introduction of two **tst** units in **27** increased this stabilization of ON:DNA/RNA (**27:37**/**27:38**) complexes and RNA vs DNA discrimination to 9.3 °C. In a 10mer ON **32**, a single **cst** unit caused stabilization of the ON:RNA complex (**32:44**) compared to the control of the unmodified complex (**42:44**) and destabilized the ON:cDNA complex (**32:43**, Table 2, entries 4 and 5). The binding was found to be sequence specific as a single mismatch in the target RNA highly destabilized the modified DNA:RNA complexes.[20] Thus, the modified units consistently destabilized the complex formation of the modified ONs with cDNA and stabilized the ON:RNA complexes.

A single modified unit of LNA with a locked N-type sugar conformation in an ON is known to effectively stabilize duplex structures with both DNA and RNA.[15] The electronegativity of the 3′-substituents in 3′-*N*-phoramidates was shown to set the sugars in a preferred N-type conformation but, unlike LNA, to show preferential binding to RNA over DNA.[14,16] Several other examples in the literature such as 2′−5′ DNA[17]/RNA[18] prefer to bind to RNA over DNA, and it is not therefore entirely certain which factors differentiate the DNA vs RNA selectivity.[1d] Native DNA and RNA prefer to be in S- or N-type sugar conformations giving rise to either B- or A-form structures in equilibrium. In this particular case, however, the $^1$H NMR studies point out conformational equilibration in either the 3′-amino or 5′-thioacetamido sugars to be similar to the native 3′−5′ phosphate linked DNA.[20] The structural similarity between unmodified and modified RNA:DNA complexes was also evident by CD studies.[20] The RNA selectivity of binding seems to be arising from the extended backbone linker that is probably inherently folded to be competent to bind to RNA over DNA as was found with the reported five-atom-linked ON analogues.[10,19] The **tst** and **cst** dimer blocks were found to be compatible in the DNA backbone to selectively stabilize the ON:RNA complexes. Further work to exploit their utility is currently in progress in our laboratory. The preferential sequence-independent RNA binding ability of these evolved modified ONs will find applications in current antisense research.

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**Supporting Information Available:** Experimental and spectral data of the compounds in Schemes 1−3. $^1$H NMR of dinucleosides. Mass and $^{31}$P spectra of **14** and **17**. Mass spectra and CD and UV−$T_m$ plots of the synthetic ONs with complementary DNA/RNA/mismatched RNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) Alternatively, in duplexes, the extra distance between sugars might be compensated by compact N-type sugar conformations that favor RNA binding (we thank an annonymous referee for this useful suggestion).
(20) See Supporting Information.