Tricyclic 2′-Deoxycytidine Analogs: Syntheses and Incorporation into Oligodeoxynucleotides Which Have Enhanced Binding to Complementary RNA

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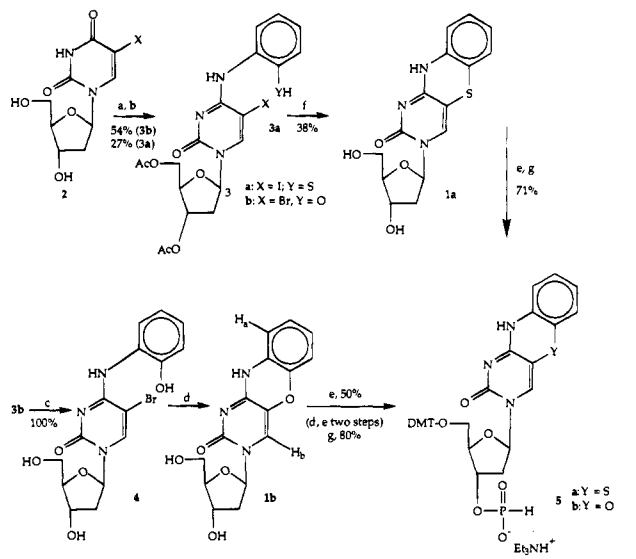
Stacking interactions between the planar heterocycles of nucleic acids are largely responsible for the stability of DNA and RNA duplexes.[1] Maximizing stacking interactions through chemical modification provides a means of creating duplex helices of greater stability. Such helix-stabilizing analogs are of interest in the area of sequence-specific regulation of gene expression by targeting mRNA (antisense approach).[2]

Pyrimidine analogs with extended aromatic and hydrophobic faces have previously been shown to stabilize DNA/DNA and DNA/RNA helices. A bicyclic pyridopyrimidine deoxynucleoside incorporated into a self-complementary dodecadeoxyribonucleotide was shown to hybridize specifically to a guanine residue and to enhance hybrid stability.[3] 5-Propynylpyrimidine deoxynucleosides within oligodeoxynucleotides (ODNs) have been shown to enhance duplex stability,[4] and this stability has resulted in enhanced antisense RNA potency in cell culture.[5] 5-Heteroaryluridines have recently been shown to possess similar helix-stabilizing properties relative to the 5-propynyl modification.[6] Molecular modeling of the previously mentioned analogs suggests that enhanced binding could be due to modest extended stacking interactions.[6,7] These stacking interactions could be further increased by constructing tricyclic pyrimidine analogs.[8] This report focuses on the synthesis of two related tricyclic pyrimidine nucleoside analogs, phenothiazine **1a** and phenoxazine **1b** (Scheme 1),[9] their incorporation into ODNs, and characterization of helix formation with complementary RNA.

Phenothiazine **1a** and phenoxazine **1b** derivatives were synthesized by similar synthetic approaches (Scheme 1). The sulfur analog **1a** was derived from 5-iodo-2′-deoxyuridine **2**. Acetylation of the hydroxyls followed by activation of the O^4 position with mesitylsulfonyl chloride and base produced the reactive O^4 sulfonate ester.[10] This was reacted with excess 2-aminothiophenol in the presence of DBU to yield **3a**. Cyclization and deacetylation to **1a** was effected using potassium *tert*-butoxide in refluxing EtOH. The analogous route to **1b** failed at the cyclization stage, with the major product being that of simple deiodination.

The oxygen analog **1b** was synthesized from 5-bromo-2′-deoxyuridine (**2**). Acetylation, mesitylsulfonylation, and displacement with 2-aminophenol furnished intermediate **3b**. Cyclization was effected after acetyl deprotection by the use of the unusual system of excess potassium fluoride in refluxing EtOH.[11,13]

Scheme 1



[a] Conditions: (a) Ac_2O in Py, RT; (b) 2-mesitylsulfonyl chloride, TEA, then 2-aminophenol or 2-aminothiophenol, DBU, RT; (c) NH_3 in CH_3OH, RT; (d) 10 equiv of KF, ethanol, reflux; (e) 4,4′-dimethoxytrityl chloride, Py, RT; (f) *t*-BuOK in ethanol, reflux; (g) 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, pyridine, CH_2Cl_2, 0 °C.

Sequential dimethoxytritylation followed by phosphitylation[14] produced the corresponding H-phosphonates **5a,b**. Compounds **5a,b** were incorporated into 15-mer ODNs by solid phase DNA synthesis chemistry using a H-phosphonate protocol.[15] Both monomers were incorporated without heterocyclic protection, and the integrity of the ODNs was confirmed by nuclease and phosphatase digestion followed by HPLC analysis of resulting deoxynucleosides.[16] All ODNs showed the expected ratio of mononucleosides.[17]

The hybridization properties of ODNs containing the tricyclic deoxynucleosides **1a,b** were determined in thermal denaturation experiments (T_m). Tricyclic heterocycles were shown to specifically hybridize with a complementary guanosine. This was determined by hybridizing the ODNs independently with two RNA targets, one where the tricycle would pair with guanosine **6**, and the other with adenosine **7** (Table I).[18] The 5-methyl-2′-deoxycytidine control **8** is specific for the guanosine target **6**, with the T_m difference being 16.5 °C between the guanosine

(1) (a) DeVoe, H.; Tinoco, I., Jr. *J. Mol. Biol.* **1962**, *4*, 500. (b) Petersheim, M.; Turner, D. H. *Biochemistry* **1983**, *22*, 256. (c) Saenger, W. *Principles of Nucleic Acid Structure;* Springer-Verlag: New York, 1984; pp 132–133.

(2) (a) Uhlmann, E.; Peymen, A. *Chem. Rev.* **1990**, *90*, 543. (b) Milligan, J. F.; Matteucci, M. D.; Martin, J. C. *J. Med. Chem.* **1993**, *36*, 1923.

(3) Inoue, H.; Imura, A.; Ohtsuka, E. *Nucleic Acids Res.* **1985**, *13*, 7119.

(4) Froehler, B. C.; Wadwani, S.; Terhorst, T. J.; Gerrard, S. R. *Tetrahedron Lett.* **1992**, *33*, 5307.

(5) Wagner, R. W.; Matteucci, M. D.; Lewis, J. G.; Gutierrez, A. J.; Moulds, C.; Froehler, B. C. *Science* **1993**, *260*, 1510.

(6) Gutierrez, A. J.; Terhorst, T. J.; Matteucci, M. D.; Froehler, B. C. *J. Am. Chem. Soc.* **1994**, *116*, 5540.

(7) Colocci, N.; Dervan, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 785.

(8) Modeling studies performed using Biograf software from Molecular Simulations, Inc.

(9) The IUPAC names for **1a** and **1b** are 3-β-D-2′-deoxyribofuranosyl-3,5-diaza-4-oxophenothiazine and 3-β-D-2′-deoxyribofuranosyl-3,5-diaza-4-oxophenoxazine, respectively.

(10) (a) Gaffney, B. L.; Jones, R. A. *Tetrahedron Lett.* **1982**, *23*, 2253. (b) Bischofberger, N. *Tetrahedron Lett.* **1987**, *28*, 2821. (c) Bischofberger, N.; Matteucci, M. D. *J. Am. Chem. Soc.* **1989**, *111*, 3041.

(11) These conditions were identified after extensive experimentation. A key observation was the need to remove the acetyl protecting groups prior to cyclization. Free 5′-hydroxyl groups have previously been shown to be important for substitutions at the 5 position of uridines.[12] Presumably, a transient Michael addition of the hydroxyl to the 6 position at the 5,6 double bond then labilizes the 5 position toward substitution.

(12) Santi, D. V.; Brewer, C. F. *J. Am. Chem. Soc.* **1968**, *90*, 6326.

(13) The structural assignment of **1b** (Scheme 1) was supported by a NOE experiment in DMSO-d_6. The NH proton gives a NOE with H_a but not H_b (Scheme 1). If the positions of the N and O had reversed during cyclization from **4**, the NH proton of the resulting product would be expected to give NOEs with both H_a and H_b. The structures are further supported by the fact that **1a,b** behave as cytidine analogs when incorporated into ODNs and specifically base pair with a complementary guanosine.

(14) Marugg, J. E.; Tromp, M.; Kuyl-Yehskiely, E.; van der Marel, G. A.; van Boom, J. H. *Tetrahedron Lett.* **1986**, 27, 2661.

(15) (a) Froehler, B. C.; Ng, P. G.; Matteucci, M. D. *Nucleic Acids Res.* **1986**, *14*, 5399. (b) Froehler, B. C. In *Protocols for Oligonucleotides and Analogs: Synthesis and Properties;* Agrawal, S., Ed.; Humana: Totowa, NJ, 1993; pp 63–80.

(16) Eadie, J. S.; McBride, L. J.; Efcavitch, J. W.; Huff, L. B.; Cathcart, R. *Anal. Biochem.* **1987**, *165*, 442.

(17) Data shown in the supplementary material.

(18) The T_m curves for Table 1 are shown in the supplementary material.

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Table 1. T_m Study on Tricyclic 2′-Deoxyriboside Incorporations

6 RNA target: 3′-AGAGGGAGAGAAAAA[a]

ODN		Z	ODN sequence	T_m (°C)[b]	T_m/substn (°C)[c]
8	control		5′-TCTCCCTCTCTTTTT	69.0	
9	1	**1a** (X = S)	5′-TCTCZCTCTCTTTTT	70.0	+1.0
10	1	**1b** (X = O)	5′-TCTCZCTCTCTTTTT	71.0	+2.0
11	3 apart	**1a**	5′-TZTCCCTZTZTTTTT	76.0	+2.3
12	3 apart	**1b**	5′-TZTCCCTZTZTTTTT	75.5	+2.2
13	3 together	**1a**	5′-TCTZZZTCTCTTTTT	81.0	+4.0
14	3 together	**1b**	5′-TCTZZZTCTCTTTTT	84.0	+5.0

[a] Sequence of mismatched adenosine target **7** was 3′-AGAGAGA-GAGAAAAA. [b] T_m values were assessed in 140 mM KCl/5 mM Na_2HPO_4/1 mM $MgCl_2$, pH = 7.2, at 260 nm, and the final concentrations of all ODNs and the RNA were approximately 2 μM. C = 5-methyl-2′-deoxycytidine. T_m values are ±0.5 °C. [c] Melting temperature change per substitution relative to 5-methyl-2′-deoxycytidine containing ODN.

(G) match **6** and the adenosine (A) mismatch **7** (data not shown). The phenothiazine **1a** and phenoxazine **1b** tricycles in ODN **9** and **10** behave as cytidine analogs with ΔT_m values of 14.5 °C and 12 °C, respectively, between the G and A targets (data not shown).[19] Both show higher thermal stability of the resulting duplex for the guanosine target relative to 5-methyl-2′-deoxycytidine.

Molecular modeling suggested that the tricyclic heterocycles would show the greatest duplex stability effect when a tricyclic ring could stack with the nearest neighbor tricycle. To test this, the guanosine RNA target **6** (Table 1) was hybridized to the ODN containing three tricyclic cytidine analogs either clustered together or separated by at least one thymidine. The results are shown in Table 1, with both the tricycle analogs giving similar results. Both the phenothiazine (ODN **11**) and phenoxazine (ODN **12**) result in stabilization of the duplexes to thermal denaturation when incorporated in three separated positions (ΔT_m per substitution of ~2 °C relative to 5-methyl-2′-deoxycytidine in both cases). When the tricycles are clustered together to permit tricyclic−tricyclic base stacking, the T_m goes up dramatically, with the ΔT_m relative to 5-methyl-2′-deoxycytidine being 5 °C per substitution in the case of phenoxazine moiety (ODN **14**).[20]

The enhanced thermal stability of the ODN/RNA duplexes observed when three tricyclic heterocycles are positioned together is consistent with the maximizing of $\pi-\pi$ overlap between adjacent aromatic faces. The model shown in Figure 1A provides a structural rationale for this additional overlap. The two adjacent phenoxazine rings modeled onto a canonical A helix possess substantial overlap between the second ring of one tricycle and the third ring of the adjacent tricycle. Such overlap is not possible in analogs of less than three rings. The corresponding cytidine−cytidine overlap shown in Figure 1B is virtually nonexistent.[21]

The exact nature of aromatic−aromatic overlap between the heterocycles of DNA and RNA helices is more complicated than a simple hydrophobic effect.[1c] Early surveys of stacking patterns of nucleic acids in crystal structures point to the

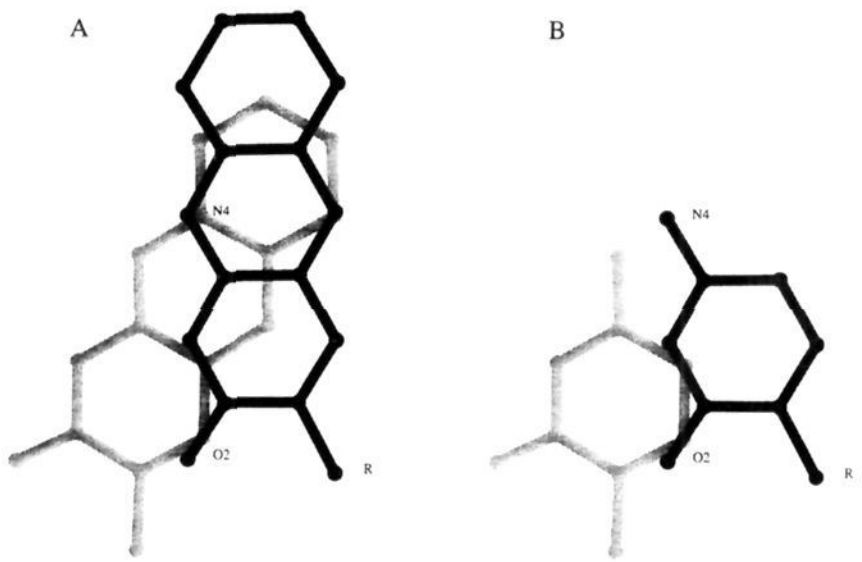

Figure 1. (A) Phenoxazine−phenoxazine overlap of adjacent heterocycles in a canonical A-form duplex. Complementary guanines are not shown. O^2 and N^4 labels denote normal pyrimidine numbering of black structures; R denotes ribose connection. Viewing perspective is sighting down the axis of the helix in a 5′ to 3′ direction, with the black image being above the gray image. (B) Cytosine−cytosine overlap of adjacent bases in a canonical A-form duplex. Labeling and viewing perspective are as in (A).

importance of dipole-induced dipole interactions.[22] A recent study[23] has shown a lack of interaction between two naphthalene groups connected by a small tether, while with the same tether two adenines or one adenine and one naphthalene interact. These results are also consistent with the induced dipoles between neighboring rings being important. The thermal stability of tricycle−tricycle stacking interactions could be due to such an induced dipole effect.[24]

The synthesis of additional polycyclic analogs bearing different charge distributions coupled with binding and structural studies will allow for greater insight into what are the important parameters for stacking interactions. The simple syntheses of the phenothiazine and the phenoxazine deoxynucleosides are the first examples of tricyclic pyrimidine analogs capable of complementary base pairing.[25] These analogs, which are capable of enhanced binding, are of interest for the regulation of gene expression by sequence-specific helix formation with targeted RNA *in vivo*.

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Supplementary Material Available: Experimental details and characterization data are reported for the compounds described herein; nuclease and phosphatase digestion analysis data and thermal denaturation curves are provided (10 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(19) The reason for the somewhat lower specificity of the tricyclic recognition of guanosine relative to 5-methylcytosine is unknown. Potentially, the tautomeric equilibrium could be shifted slightly from the cytosine-like form to the uracil-like form relative to 5-methylcytosine. Alternatively, enhanced stacking forces in the case of the tricycles could stabilize a mismatch relative to 5-methylcytosine.

(20) Formal proof that the clustering of tricycles always results in enhanced thermal stability of duplexes will require the study of more sequences and contexts.

(21) The same modeling exercise performed on a canonical B helix scaffold shows similar results with substantial overlap in the phenoxazine−phenoxazine context and the lack of overlap in the cytidine−cytidine context. A more complete description of overlap awaits a structural determination by high-field NMR or X-ray crystallography.

(22) Bugg, C. E.; Thomas, J. M.; Sundaralingam, M.; Rao, S. T. *Biopolymers* **1971**, *10*, 175.

(23) Newcomb, L. F.; Gellman, S. H. *J. Am. Chem. Soc.* **1994**, *116*, 4993.

(24) Alternately, as noted by a reviewer, London dispersion forces, dipole−dipole interactions, or simple hydrophobic effects could be contributing to the stability.[1c]

(25) Polycyclic pyrimidine analogs which can bridge a helix[26] or intercalate have been reported.[10c]

(26) Leonard, N. J.; Devadas, B. *J. Am. Chem. Soc.* **1987**, *109*, 623.