Synthesis and DNA binding affinity of irregular sequence oligonucleotides with triazole internucleotide linkages.

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Abstract

A novel class of backbone-modified oligonucleotide analogs has emerged since the discovery of Cu^{I}-catalyzed [3+2] azide-alkyne cycloaddition. These are oligonucleotide analogs with 1,4-substituted 1,2,3-triazoles in internucleotide linkages. Of all such analogs known to date, only the triazole-linked deoxythymidine decamer has been reported to show enhanced binding affinity to complementary DNA. Importantly, it is a fully modified $(dT)_{10}$ analog. Irregular oligonucleotides bearing the same backbone modification have not been described so far. With a goal of investigating sequence and regularity dependence of the effect of this modification on duplex stability, we have designed sequentially heterogenous modified oligonucleotides, which can be prepared using a modified dinuleoside block. In this paper we report on the synthesis of the dithymidine phosphoramidite block with the triazole linker, its utilization in oligonucleotide synthesis and hybridization data of thus obtained oligonucleotide analogs. The effect of single and multiple modifications on stability of irregular sequence duplexes is assessed and compared with published data for the oligo(T)/oligo(A) duplex. We also compare the effect of the linker concerned with that of a shorter triazole linker.

Keywords: click chemistry; oligonucleotide analogs; hybridization

Introduction

The discovery of copper(I)-catalysis of Huisgen 1,3-dipolar cycloaddition by groups of Sharpless and Meldal in 2002 had a marked impact on the field of artificial nucleosides and oligonucleotides (ONs). A number of 1,2,3-triazole-containing nucleoside analogs exhibiting antibacterial or antiviral activity have been recently obtained using Cu^{I}-catalyzed [3+2] azide-alkyne cycloaddition (CuAAC) as the key reaction.[1] What is more, a novel class of backbone-modified ONs has emerged in the last decade. In the search for potent posttranscriptional gene-silencing agents, several teams have designed and synthesized ONs with 1,4-substituted 1,2,3-triazoles in internucleoside linkages.[2] Of all triazole-linked ONs known to date, only the deoxythymidine decamer analog reported by Isobe *et al*. exhibits enhanced binding affinity to complementary DNA.[3] This $(dT)_{10}$ analog is a fully modified ON, which can be prepared from a thymidine derivative by repeating functionalization and CuAAC ligation steps. Melting temperature (Tm) of the corresponding duplex is about 40°C higher than that of the isosequential wild-type duplex. The authors conjecture that this is due to the structural complementarity and the absence of repulsion between neutral triazole and anionic natural phosphodiester linkages. We believe it would be of interest to clarify whether this remarkable enhancement in duplex stability is sequence-dependent. Considering that synthesis of fully modified triazole-linked ONs with random sequences would be elaborate and lengthy (it requires preparation of four different monomers and probably reoptimization of oligomerization conditions), we employed a different strategy, based on the use of a modified dinucleoside block.

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In this paper, we report on the synthesis of the dithymidine phosphoramidite block with a triazole linker, its incorporation in ONs and hybridization data of modified ONs. The effect of single and multiple modifications on stability of irregular sequence duplexes is assessed and compared with published data for the oligo(T)/oligo(A) duplex. We also compare the effect of the linker concerned with that of a shorter triazole linker, reported in our previous work.[4]

Experimental

ONs were synthesized on an Applied Biosystems 3400 DNA synthesizer (USA) using standard phosphoramidite protocols and purified using preparative scale reverse-phase HPLC on a 250 mm x 4.0 mm^2 Hypersil C18 column with detection at 260 nm. Chromatography of dimethoxytrytil-protected ONs was performed using 10-50% gradient of CH_3CN in 0.05 M TEAA. Detritylated oligonucleotides were further purified in 0-25% gradient of CH_3CN in TEAA buffer.

MALDI TOF mass spectra were acquired on a Bruker Microflex mass spectrometer (Bruker, Germany) in a linear mode (+20 kV). Each spectrum was accumulated using 200 laser shots (N_2 gas laser, 337 nm). The solution of 35g/ml of 3-hydroxypicolinic acid with dibasic ammonium citrate was used as a matrix.

Melting curves of the duplexes were recorded on a Shimadzu UV 160-A spectrophotometer (Japan) using a thermostated cell in 20 mM sodium phosphate buffer, 100 mM NaCl, 01 mM EDTA, pH 7.0, concentration of each duplex being $2.5 \cdot 10^{-6}$M. Samples were denatured at 95°C for 5 min and slowly cooled to 20°C prior to measurements. Duplex absorbance at 260 nm was measured as a function of temperature. It was registered every 0.5°C from 20 to 70°C. Thermodynamic parameters of duplex formation were obtained by performing nonlinear regression analysis using DataFit version 9.0.059 (Oakdale Engineering, USA). The calculation method taking into account temperature dependence of UV absorbance of duplexes and single strands was applied.

Circular dichroism (CD) spectra were obtained on a Jasco J-715 spectropolarimeter at 20°C using samples annealed in the same buffer and under the same conditions as for the thermal denaturation studies. The CD values ($\Delta\varepsilon$) are given per moles of nucleotides.

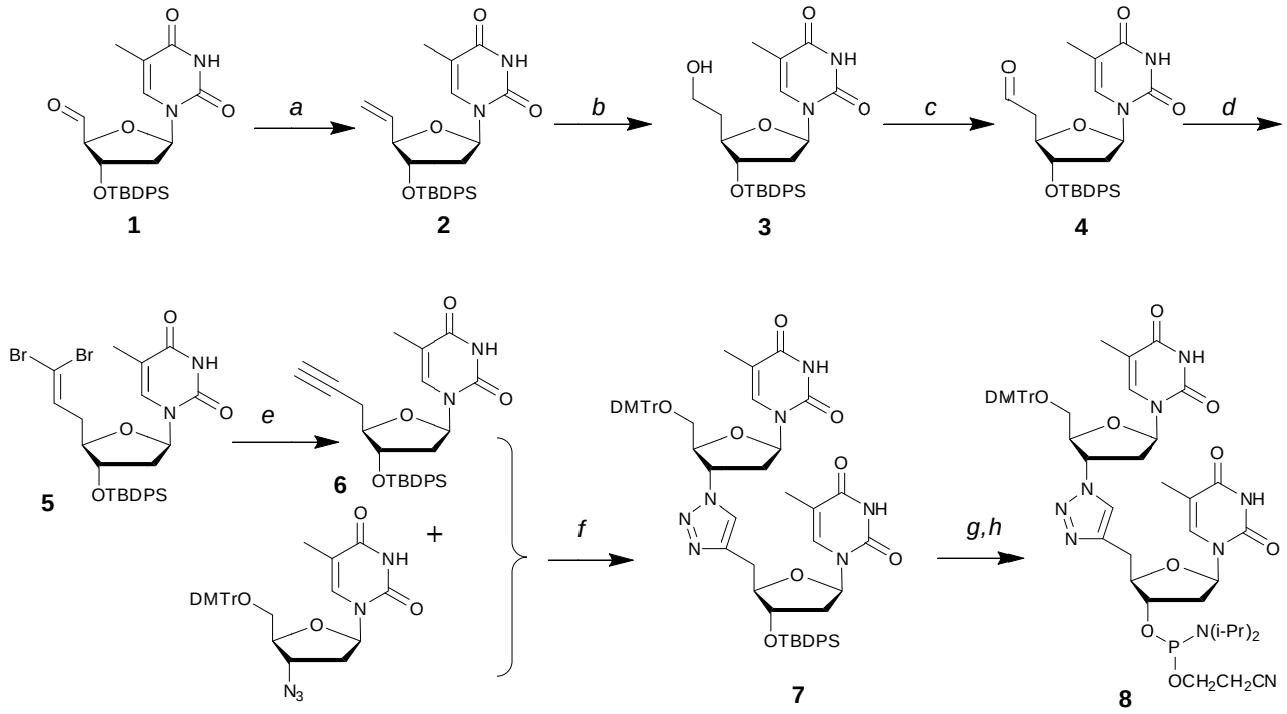
Conformational analysis of the modified duplex fragment was performed by molecular mechanics calculations using the HyperChem 8.0.9 Amber force field (HyperCube, Inc., USA).

Results and discussion

We commenced the synthesis of the target triazole-linked dinucleotide block with the preparation of acetylenic nucleoside component **6** from aldehyde **1** (Scheme). The latter was readily accessed from 3'-*O*-(*tert*-butyldiphenylsilyl)thymidine[5] by oxidation with *o*-iodoxybensoic acid (IBX) using a reported method.[4] Wittig-type condensation of aldehyde **1** with the ylide derived from methyltriphenylphosphonium bromide in the presence of BuLi in abs. THF (18 h, 20°C) afforded terminal olefin **2** in 84% yield. Nucleoside:BuLi ratio of 1:1 is crucial for high yield of nucleoside **2**, since the lack of BuLi leads to formation of a polar by-product (presumably the phosphonium salt similar to the one reported by Matsuda *et al.*[6]), and in the presence of the excess of BuLi beta-elimination occurs. Compound **2** was subjected to hydroboration with 9-borabicyclo[3.3.1]nonane (9-BBN-H) in abs. THF (0.5 h at 0°C, then 20 h at 20°C) and subsequent oxidative treatment with $NaBO_3 \cdot 4H_2O$ (30 h, 20°C) to give compound **3** in 86% yield. Sodium perborate was chosen as an oxidizing agent since the use of the conventional reagent, H_2O_2 in alkaline solution, has been reported to result in side reactions in the case of a similar nucleoside.[7] Oxidation of alcohol **3** with IBX gave carboxylic acid. However, when Dess-Martin periodinane was used, aldehide **4** was obtained in 89% yield. All attempts to convert aldehyde **4** to terminal alkyne **6** via Ohira-Bestmann reaction were unsuccessful. Neigther one-pot procedure with *in situ* generation of dimethyl-1-diazo-2-oxopropylphosphonate (Ohira-Bestmann reagent) from tosyl azide and dimethyl-2-oxopropylphosphonate,[8] nor more elaborate one with intermediate isolation of Ohira-Bestmann reagent afforded alkyne **6**. Therefore, formyl to ethynyl group transformation was realized

in 2 steps, via intermediate dibromoalkene. Aldehyde **4** was reacted with dibromomethyltriphenylphosphonium bromide (prepared by Wolkoff's method[9]) in the presence of Zn in abs. dioxane under reflux (3h) to give dibromomethylene derivative **5** in 80% yield. Treatment of compound **5** with BuLi in abs. THF (0.5 h, -70°C) afforded acetylenic nucleoside **6** in 77% yield.

Alkyne **6** was coupled with 3'-azido-3'-deoxy-5'-*O*-dimethoxytritylthymidine (Scheme 3) in the presence of $CuSO_4 \cdot 5H_2O$ and sodium ascorbate (2h, 20°C). CuAAC was carried out in a two-phase solvent system (H_2O/CH_2Cl_2)[10], which has proven to be favorable for click coupling of poorly water-soluble compounds. Dinucleoside **7** was obtained in a yield of 80%. The silyl protection was removed with 0.5 M TBAF in THF (20°C, 2 h). Treatment of the dinucleoside bearing free hydroxyl with 2-cyanoethyl-*N*,*N*,*N*',*N*'-tetraisopropylphosphoramidite in the presence of 1*H*-tetrazole and pyridine in abs. CH_2Cl_2 (2h, 20°C) gave target phosphoramidite **8** in 72% yield.



Scheme. Synthesis of the phosphoramidite dinucleoside block with a triazole internucleoside linkage. Reagents and conditions: (a) $PPh_3CH_3^+Br^-$, BuLi, THF; (b) *i* - 9-BBN-H, THF, *ii* – $NaBO_3 \cdot 4H_2O$, THF-H_2O-MeOH (5:2:3); (c) Dess-Martin periodinane, CH_2Cl_2; (d) $PPh_3CHBr_2^+Br^-$, Zn, dioxane, reflux; (e) n-BuLi, THF, -70°C, (f) $CuSO_4$, sodium ascorbate, CH_2Cl_2/H_2O; (g) TBAF, THF; (h) $NCCH_2CH_2OP(NPr^i_2)_2$, 1*H*-tetrazole, pyridine, CH_2Cl_2.

All nucleosides and dinucleosides were characterized by ^1H and ^{13}C NMR spectroscopy as well as ESI HR mass spectrometry. Dinucleoside structures were confirmed by COSY NMR spectroscopy (data is not presented).

Dinucleoside block **9** was utilized directly for solid-phase synthesis of modified ONs using standard phosphoramidite protocols. Coupling time was increased to 15 minutes for the modified phosphoramidite. No decrease in coupling efficiency was observed (98-99% step-wise coupling yields for both modified and unmodified amidites). Along with the modified ONs, a wild-type complement and wild-type isosequential ONs were synthesized. All the ONs were purified by reverse-phase HPLC and characterized by MALDI mass spectrometry. Modified ONs and their wild-type counterpart were hybridized to ssDNA. Thermal stability of the duplexes was examined by monitoring the change in hyperchromicity at 260 nm (Figure, A). Sequences and melting temperatures of the duplexes are shown in Table.

Table. Modified oligonucleotides and melting temperatures of their duplexes with a wild-type DNA complement (duplex concentration $2.5 \cdot 10^{-6}$).

Code	Sequence of a modified strand (5'→3')*	T_m, °C ± 0.5, (ΔT_m, °C**)
1	TTAACTTCTTCACATTC	50.3
2	**X**AACTTCTT**C**ACATTC	50.4 (+0.1)
3	TTAACTTCTT**C**ACA**X**C	47.2 (-3.1)
4	TTAACTTC**XC**ACATTC	41.9 (-8.4)
5	**X**AACTTC**XC**ACA**X**C	37.7 (-12.6)

* X –triazole-linked dithymidine fragment.

** T_m difference between modified and natural duplexes

As evident from Table, the influence of 5'-terminal modification on ON hybridization is insignificant. 3'-Terminal modification destabilizes the duplex to a certain extent (ΔT_m = -3.1°C), and middle-strand modification leads to a drastic loss in duplex stability (ΔT_m = -8.4°C).

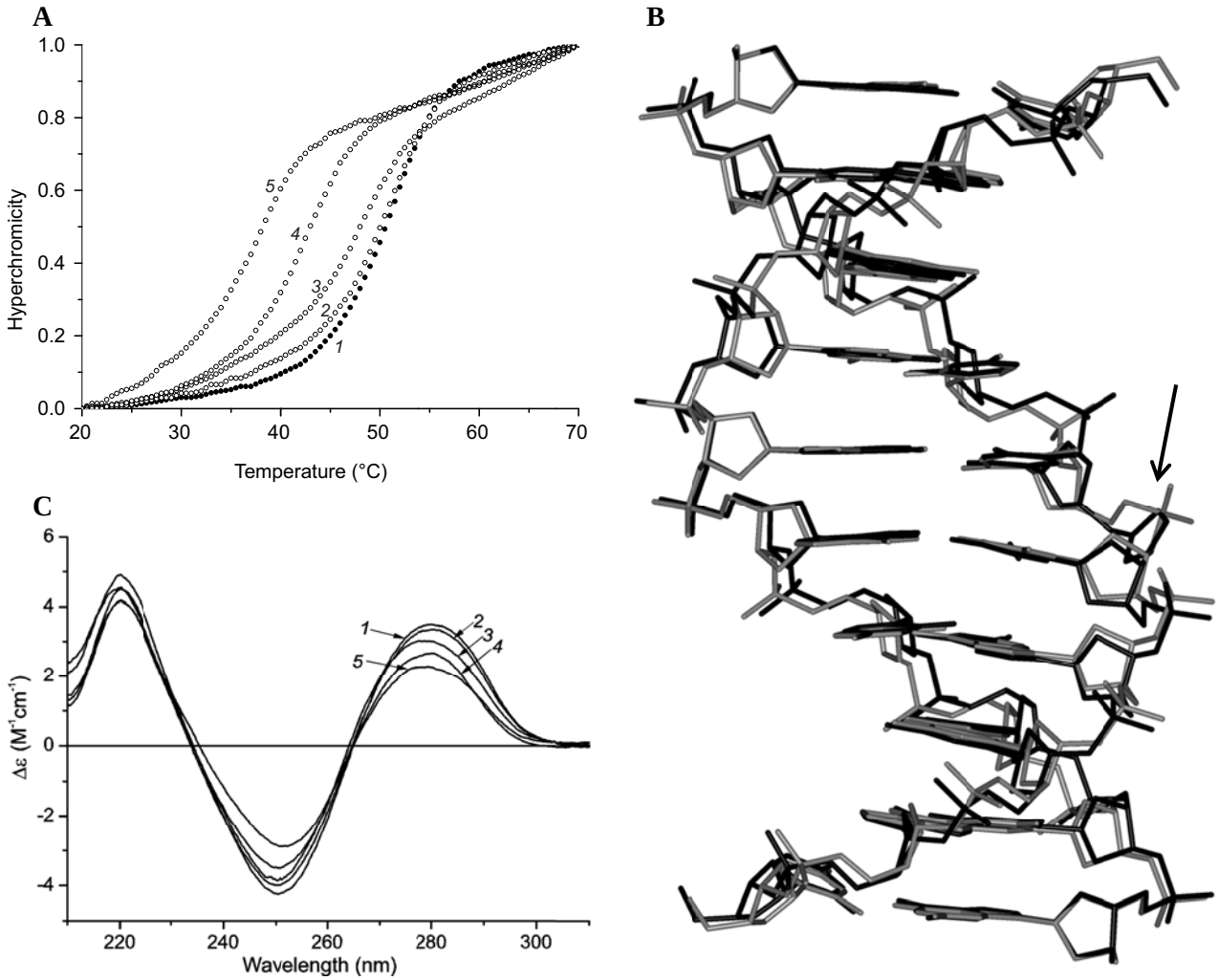


Figure. Geometry and hybridization properties of modified oligonucleotides. (**A**) Melting curves of the wild-type and modified duplexes. Legends represent duplex code numbers (see Table). (**B**) Superimposition of optimized conformations of a wild-type duplex fragment (grey strands) and a modified one (black strands). The arrow points to the triazole internucleotide linker. Modified strand sequence: 5'-CATGTtriazoleTCATG-3'. (**C**) CD spectra of the wild-type and modified duplexes. Legends represent duplex code numbers (Table).

To understand what changes in ON geometry underlie this destabilization, we performed conformational analysis of a modified backbone fragment in a duplex. The results imply that duplex distortion may arise from the lesser twist of the triazole linker in comparison with the phosphordiester one (Figure, B). Overall duplex conformation is, however, rather close to classical B-helix. CD spectra of modified ONs hybridized to complementary ssDNA confirm that the former adopt B-form geometry upon duplexation (Figure, C). Average ΔTm per modification is -4.0 °C (calculated as a sum of all ΔTm values divided by the total number of modifications).

Our results are consistent with that obtained earlier for mixed sequence ON analogs with shorter triazole linkers.[4]. Interestingly, they do not accord with hybridization data reported by Isobe *et al*. for the similarly modified regular ON, i.e. the $(dT)_{10}$ analog.[3] We suggest that distinct geometry of oligo(T)/oligo(A) DNA fragments[11] may be somewhat responsible for the outstanding stability of the regular triazole-modified decamer duplex.

Conclusions

To sum up, we have reported on the synthesis of the dinucleoside phosphoramidite block with the triazole internucleoside linkage. Surprisingly, incorporation of this block in sequentially heterogenous ONs was found to be disadvantageous for their hybridization ability, while the fully modified oligo(dT) analog is known to bind tightly with a complementary DNA fragment. We conclude that the effect of this modification is highly dependent on ON primary structure. The phosphoramidite block we have described is a useful intermediate for further synthesis and investigation of triazole-functionalized ONs.

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