Synthesis of Triazole-Linked Oligonucleotides with High Affinity to DNA Complements and an Analysis of Their Compatibility with Biosystems

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ABSTRACT: New oligonucleotide analogues with triazole internucleotide linkages were synthesized, and their hybridization properties were studied. The analogues demonstrated DNA binding affinities similar to those of unmodified oligonucleotides. The modification was shown to protect the oligonucleotides from nuclease hydrolysis. The modified oligonucleotides were tested as PCR primers. Modifications remote from the 3′-terminus were tolerated by polymerases. Our results suggest that these new oligonucleotide analogues are among the most promising triazole DNA mimics characterized to date.

INTRODUCTION

Oligonucleotide (ON) analogues with triazole internucleotide linkages have shown great promise in the field of antisense technology and as DNA hybridization probes.1−4 These analogues can be obtained by the iterative click ligation of azide- and alkyn-bearing nucleoside derivatives5,6 or by automated phosphoramidite synthesis with modified dinucleoside blocks.7 Although a significant number of triazole-linked ONs have been described to date,5−9 few have demonstrated hybridization properties that meet the criteria for duplex stability.8 Isobe et al. reported a triazole modification that is particularly favorable for duplex stabilization.9 Unfortunately, this stabilization was later shown to be sequence-dependent.10 Nevertheless, triazole-linked ONs remain an intensively studied class of DNA mimics, primarily due to their potential for biocompatibility.11,12 Unlike most internucleotide modifications, some triazole modifications can be tolerated by polymerases,13,14 which makes triazole-linked ONs attractive as candidate diagnostic tools and therapeutic agents. Several promising types of triazole internucleotide modifications are shown in Scheme 1.

This paper introduces a new triazole internucleotide modification. The triazole linkage described herein is an isomer of a previously reported one (Scheme 1C).6,13 A series of ON analogues bearing the triazole modification were obtained by automated solid-phase synthesis with a modified dinucleoside phosphoramidite block. The properties of the triazole-linked ONs are described.

RESULTS AND DISCUSSION

The triazole-linked dithymidine block used for incorporation into ONs was obtained by click-ligation of two nucleoside components (Scheme 2). The azido component was synthesized from 5′-O-benzoylthymidine 1. An O-azidomethyl group was introduced into the 3′-position of 1 in two steps using a modified version of a method reported by Lonnberg and co-workers.15 The first step was methyliodmethylation of the 3′-hydroxide group, which could be performed either by a modified Pummerer rearrangement15 or by treatment with DMSO and benzoyl peroxide.16,17 Both approaches are rather efficient and afford 3′-O-methylthiomyethyl deoxynucleoside derivatives in comparable yields.15,16,18 We used the modified Pummerer rearrangement. Nucleoside 1 was treated with a mixture of dimethyl sulfoxide and acetic anhydride in the presence of acetic acid. The 3′-O-methylthiomyethyl derivative 2 was obtained in 80% yield.

Nucleoside 2 was reacted with molecular bromine followed by lithium azide in dry CH3CN to produce the 3′-O-azidomethyl derivative 3 in 83% yield. The treatment of 3 with a K2CO3 suspension in dry methanol resulted in the removal of the benzoyl protecting group. A dimethoxytrityl protecting group was introduced by treatment with dimethoxytrityl chloride in dry pyridine. The resulting azide 5 was coupled with alkyne 619 in the presence of CuSO4·SH2O and...
sodium ascorbate to give dinucleoside 7 with a yield of 88%. The silyl protection was removed with 0.5 M TBAF in THF. The treatment of dinucleoside 8, bearing a free hydroxyl, with 2-cyanoethyl-\(N,N,N',N'\)′-tetraisopropylphosphoramidite in the presence of 1H-tetrazole and pyridine in dry CH\(_2\)Cl\(_2\) afforded the target phosphoramidite 9 in 74% yield.

Dinucleoside block 9 was used directly for the solid-phase synthesis of modified ONs using standard phosphoramidite protocols. The coupling time was increased to 15 min for the modified phosphoramidite. No decrease in coupling efficiency was observed (98−99% stepwise coupling yields for both modified and unmodified amidites).

ONs bearing triazole fragments at the 5′-terminus, near the 3′-terminus, and/or in the middle of the chain were obtained and hybridized to complementary ssDNAs. The CD spectra of the duplexes (Figure 1A) suggest that the modification did not cause any significant changes in duplex geometry.

The impact of the modification on duplex stability was evaluated by UV-melting experiments (Figure 1B). The results of the UV-melting experiments are summarized in Table 1. As is evident from these data, the modification tends to destabilize duplexes slightly. However, the destabilization is minor (average \(\Delta T_m\) per linkage −1.4 °C) compared to those of previously described isosequential duplexes bearing other...
triazole modifications (average ΔT_m = −5.3 °C for the triazole modification A (Scheme 1)) and −4.0 °C for the modification B (Scheme 1). Thus, the new ON analogues are among the most promising triazole-bearing DNA mimics in terms of hybridization properties.

Because the new modification has a relatively insignificant impact on duplex geometry, we hypothesized that it might be tolerated by ON-recognizing enzymes. Oligonucleotides with a triazole linkage isomeric with that described here ("isotriazole"); C in Scheme 1) are known to be recognized by DNA and RNA polymerases.13,14 Plasmid constructs with such linkages have proven functional in E. coli.19 Although the "isotriazole" modification appeared to reduce duplex stability (ΔT_m of approximately −8 °C per modification) and caused local duplex distortion,12 it was tolerated in both primers and templates by Taq polymerase.13 To investigate the compatibility of the new triazole linkage with the thermostable polymerases Taq and Pfu, we synthesized a series of PCR primers with modifications at various positions. PCR with the Taq polymerase was quite efficient with all but the 3′-modified primers, while the Pfu polymerase only tolerated modifications near the 5′-terminus (Figure 2A). The sensitivity of Taq to primer modification was evaluated more rigorously by qPCR (Figure 2B). Modifications at up to eight nucleotides from the 3′-end resulted in a substantial decrease in the product accumulation rate. Thus, the new triazole-linked ONs are generally usable as PCR primers, but only some polymerases can tolerate the modifications, and the dependence of PCR efficiency on the modification position should be taken into account. Modified PCR primers may be useful for SNP-based allele detection should they demonstrate an enhanced sensitivity to mismatches. To assess the hybridization specificity of the new triazole-linked ONs, we synthesized a set of ON4 complements with mismatches opposite to the modified fragment of ON4. Melting temperatures of mismatched duplexes were obtained and compared with that of a fully matched duplex. Sequences of the mismatched duplexes and T_m data are given in the Supporting Information. Generally, the triazole modification appears to impart slightly increased sensitivity to mismatches opposing the nucleoside which precedes the triazole fragment.

An important aspect of compatibility with biosystems is stability in biological liquids. Non-native internucleotide fragments are well-known to confer increased stability to fully modified ONs. We tested the effect of partial triazole modification on the rate of ON hydrolysis by DNase I. The triple modification of the 17-mer caused an approximately 1.5- to 2-fold increase in ON half-life. The previously reported triazole modification10 (B in Scheme 1) had a similar effect on the rate of enzymatic hydrolysis (see the Supporting Information). It can be concluded that even the partial triazole modification of internucleotide linkages may substantially improve ON biostability.

■ CONCLUSION

A dinucleotide with a new triazole internucleotide modification was synthesized and incorporated into ONs. The ONs bearing triazole fragments demonstrated high hybridization affinity to DNA complements and increased resistance to nuclease digestion. ONs with 5′-modified termini were functional as PCR primers. Our results suggest that these new ON analogues are adequate mimics of native DNA with enhanced biostability and may be attractive tools for diagnostics or the investigation of DNA structure.

■ EXPERIMENTAL SECTION

All reagents were commercially available unless otherwise mentioned and used without further purification.1-(2,5,6-Trideoxy-3-O-(tert-butyldiphenylsilyl)-β-D-erythro-hex-5-ynofuranosyl)-5-methyluracil (6) was synthesized as previously described.19 All solvents were purchased from Khimmed (Russia). Dichloromethane was distilled from phosphorus pentoxide, pyridine was distilled from calcium hydride, and THF was distilled from lithium alumohydride prior to use. Column chromatography (CC) was performed on silica gel Kieselgel 60 (0.040−0.063 mm, Merck, Germany). TLC was performed on silica gel Kieselgel 60 F254 precoated plates (Merck) with detection by UV using the following solvent systems (compositions expressed as v/v): ethanol/methylene chloride 1/49 (A), 1/20 (B). 1H NMR and 13C NMR spectra were recorded on a Bruker AMXIII-400 NMR spectrometer (Germany). Chemical shifts are given in parts per million (ppm). The coupling constants (J) are given in Hz. Abbreviations used: dmT, 5′-deoxy-5′-methylenethymidine moiety; aT, 3′-O-aminomethylthymidine moiety. Spectral assignments were completed by COSY DQF and [1H-13C]HSQC experiments (see the Supporting Information). The NMR data were processed and presented using MestReNova version 7.0.3 (Mestrelab Research SL, Spain).

Table 1. Sequences, MALDI-TOF MS Data, and Melting Temperatures of the Duplexes Formed by Modified ONs and Their Unmodified Complement

<table>
<thead>
<tr>
<th>code</th>
<th>sequence of a modified strand (5′→3′)</th>
<th>m/z, found (calcd for [M + H]+)</th>
<th>T_m ±0.5 °C (ΔT_m °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ON1</td>
<td>TTAACTCTTCTACCATTTC</td>
<td>5072.2 (5071.3)</td>
<td>50.3</td>
</tr>
<tr>
<td>ON2</td>
<td>XAACTCTTCTACCATTTC</td>
<td>5059.3 (5058.4)</td>
<td>50.0 (−0.3)</td>
</tr>
<tr>
<td>ON3</td>
<td>TTAACTCTTCCACCX</td>
<td>5091.0 (5084.4)</td>
<td>48.2 (−2.1)</td>
</tr>
<tr>
<td>ON4</td>
<td>TTAACTCXXACACTTC</td>
<td>5058.5 (5058.4)</td>
<td>49.1 (−1.2)</td>
</tr>
<tr>
<td>ON5</td>
<td>XAACTCXXACACX</td>
<td>5032.3 (5032.5)</td>
<td>45.4 (−4.9)</td>
</tr>
</tbody>
</table>

“Duplex concentration S × 10^-6 M. %X denotes the triazole-linked dithymidine fragment. ΔT_m is the difference between modified and natural duplexes.

Figure 2. PCR with modified primers. (A) Relative sensitivity of the Taq (left) and Pfu (right) polymerases to primer modification. The first four lanes of the upper gels are the products of PCR with unmodified primers. The numbers represent the positions of the modifications (number of nucleotides from the 3′-terminus). For primer sequences, see the Supporting Information. (B) Accumulation kinetics curves obtained during qPCR with Taq polymerase.
Mass Spectra. 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 (N₂ gas, laser, 337 nm). A solution of 35 g/mL of 3-hydroxypropionic acid with dibasic ammonium citrate was used as a matrix. ESI HR mass spectra were acquired on a Bruker microTOF II spectrometer (Germaoy (FD/ESI) in positive ion mode (capillary voltage 4 kV, sample plate offset –500 V, interface capillary temperature 180 °C, nitrogen dry gas, 4.0 L/min, scanning range from m/z 50 to 3000 Da, scanning frequency 1 scan/s). Internal calibration was done using Electrospray Calibration Solution (Fluka).

Oligonucleotides. 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 mm² Hypersil C18 plate with 20 μm particles (Deltix Technology, Russia) and plates were dried, and recrystallized from a mixture of ethyl acetate and hexane.

UV Melting and CD. Melting curves of the duplexes were recorded on a Shimadzu UV 160-A spectrophotometer (Japan) using a thermostatted cell in 20 mM sodium phosphate buffer, 0.1 mM EDTA, and pH 7.0, with the concentration of each duplex being 2.5 x 10⁻⁶ M. Samples were denatured at 95 °C for 5 min and slowly cooled to 20 °C prior to measurements. The duplex absorbance at 260 nm was monitored 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). A calculation method taking into account the 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 in using samples annealed in the same buffer under the same conditions as for the thermal denaturation studies. The CD values (Δε) are given per mole of nucleotides.

Nuclease Hydrolysis. ON (0.35 mmol) was dissolved in 15 μL of a reaction buffer (100 mM Tris-HCl (pH 7.5), 25 mM MgCl₂, and 1 mM CaCl₂). A 5 μL aliquot was taken as a control, and 1 unit of DNaseI (Fermentas, US) in 1 μL of storage buffer was added to the remaining solution. The mixture was incubated at 37 °C for 15 min, an aliquot was removed and stored in a freezer at –20 °C until the end of the reaction, and the mixture was incubated at 37 °C for another 15 min. Once all three samples were collected, 1 μL of formamide was added to each sample. The samples were heated to 95 °C and then cooled on ice and loaded onto a denaturing 20% polyacrylamide gel for electrophoresis. The gel was run in TBE buffer (90 mM Tris-Borate (pH 8.0), and 20 mM EDTA).
evaporated under vacuum. The residue was dissolved in 50 mL of chloroform, washed with water (20 mL) and saturated NaHCO3 (20 mL), dried over Na2SO4, and concentrated. The residue was purified by CC in 3% ethanol in CH2Cl2 to give nucleoside 7 (1.6 g, 92% yield) as a hard foam: Rf = 0.48 (solvent system A).

1H NMR (400 MHz, DMSO): δ 11.32 (s, 1 H, 3-H), 11.28 (s, 1 H, 3-H), 8.39 (s, 1 H, 6'-H, dmT), 7.66 (d, J = 10.0 Hz, 1 H, 6-H, dmT), 6.34 (1 H, d, J = 0.8 Hz, 6-H, t), 7.38–6.84 (m, 13 H, ArH DMTr), 6.14 (t, J = 2.9 Hz, 6-H, dmT), 5.86 (d, J = 11.0 Hz, 1H, 3'-OCH3, t), 5.84 (d, J = 11.0 Hz, 1H, 3'-OCH3, t), 5.57 (d, J = 4.5 Hz, 1H, 3-CH2, dmT), 4.93 (d, J = 2.9 Hz, 1H, 4'-H, dmT), 4.61–4.56 (m, 1 H, 3'-H, dmT), 4.47–4.41 (m, 1 H, 3'-H, dmT), 3.98 (td, J = 3.0 Hz, 1H, 5-CH2, dmT), 3.20 (dd, J = 3.9 Hz, J = 10.9 Hz, 1H, 5-H, dmT), 2.74–2.73 (m, 1 H, 2'-H, dmT), 2.35–2.21 (m, 3 H, 2'-CH2 t, and 2'-H, dmT). 13C NMR (101 MHz, DMSO) δ 163.52 (4-C, at), 158.14 (p, C, PhOMe), 150.25 (2-C, 144.55 (C, DMTr), 135.54 (6-CH), 135.26 and 135.17 (C, PhOMe), 129.63 (o-C, PhOMe), 127.82 (o-C, Ph), 127.58 (m-C, Ph), 126.75 (p-C, Ph), 113.19 (m-C, PhMe), 109.62 (5-C, 85.95 (C, DMTr), 83.71 (1'-CH), 82.71 (4'-CH), 81.03 (OCH3N), 78.00 (3'-CH), 63.34 (5'-CH2), 54.98 and 54.79 (CH3O DMTr), 36.59 (2'-CH2), 11.64 (5'-CH). HR MS ESI: m/z 622.2271, calc for [C36H39N5NaO12P + Na]2+ m/z 622.2272.

1-(5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl-O-methyl)-4-(3'-O-(tert-butylidiphenylsilyl)-5'-deoxy-5'-yl)-1H,1,2,3-triazole (7). To a stirred solution of compound 5 (0.54 g, 4.9 mmol) and 3'-O-(tert-butylidiphenylsilyl)-5'-deoxy-5'-ethynylthymidine (0.4 g, 0.8 mmol) in dichloromethane (5 mL) and water (5 mL) were added CuSO4•5H2O (30 mg, 0.13 mmol) and sodium ascorbate (130 mg, 0.7 mmol). The resulting solution was stirred for 3 h at room temperature. The reaction mixture was diluted with dichloromethane (25 mL) and brine (25 mL). The organic layer was separated, washed with water (20 mL) and saturated NaHCO3, dried over Na2SO4, and concentrated. The residue was purified by CC in 1% ethanol in CH2Cl2 to give nucleoside 7 as a hard foam (0.77 g, 88% yield): Rf = 0.74 (solvent system B).

1H NMR (400 MHz, DMSO): δ 11.37 (s, 1 H, 3-H, dmT), 11.26 (s, 1 H, 3-H), 8.16 (s, 1 H, 6'-H, dmT), 7.57 (δ, J = 1.3 Hz, 1 H, 6-H, dmT), 7.49 (1 H, d, J = 11.0 Hz, 6-H, t), 7.50–6.83 (m, 23 H, ArH DMTr and TDBPS), 6.42 (t, J = 2.9 Hz, 7.5 Hz, 1 H, 1'-H, dmT), 6.24 (dd, J = 6.5 Hz, 1.7 Hz, 1 H, 1'-H, at), 5.84 (d, J = 11.0 Hz, 1H, 5-a-OCH3, t), 5.78 (d, J = 11.0 Hz, 1H, 3'-b-OCH3, t), 5.07 (d, J = 2.7 Hz, 1 H, 4'-H, dmT), 4.61–4.52 (m, 2 H, 2'-H dmT and 3'-H, dmT), 3.93 (dd, J = 3.9 Hz, J = 3.3 Hz, 3.3 Hz, 1H, 4'-H, at), 3.72 (s, 6 H, CH3O DMTr), 3.15 (dd, J = 3.3 Hz, 1H, 3'-CH2, dmT), 3.01 (s, 9 H, CH3, tBu). 13C NMR (101 MHz, DMSO): δ 161.36 (4-C, dmT and at), 158.1 (p, C, PhOMe DMTr), 150.4 and 150.2 (2-C, dmT and at), 145.6 (5'-C, dmT), 144.5 (C, Ph DMTr), 138.5 (6-CH, at and dmT), 135.1 (p-C, PhOMe DMTr), 135.1 and 135.0 (o-C, PhPh3), 132.6 and 132.4 (C, PhPh3), 130.0 and 129.9 (p-C, PhPh3), 129.6 (o-C, PhOMe DMTr), 127.64 (m-C, PhOMe), 127.75 (o-C, PhDMTr), 127.5 (m-C, PhDMTr), 126.7 (p-C, Ph DMTr), 124.0 (6'-CH, dmT), 113.2 (m-C, PhDMTr), 110.9 (5-C, dmT), 109.7 (5-C, at), 86.1 (C, DMTr), 84.8 (1'-CH, dmT), 83.6 (1'-CH, t), 82.8 (4'-CH2, t), 79.9 (4'-CH, dmT), 78.7 (3'-CH, t), 76.9 (3'-CH, dmT), 76.6 (OCH3N), 63.5 (5'-CH2, at), 55.0 (CH2O DMTr), 38.9 (2'-CH2, dmT), 36.7 (2'-CH2, t), 26.6 (CH3 tBu), 18.5 (C, t-Bu), 12.0 (5'-CH2, at), 11.5 (5'-CH, at). HR MS ESI: m/z 1096.4252, calc for [C89H82N8O22P7Na2]2+/m/z 1096.4247.

1-(5'-O-(4,4'-Dimethoxytrityl)thymidin-3'-yl-O-methyl)-4-(5'-deoxy-5'-yl)-1H,1,2,3-triazole (8). To a stirred solution of nucleoside 7 (0.077 g, 0.7 mmol) in dry THF (1.4 mL) was added a 1 M solution of tetrabutylammonium fluoride in dry THF (1.4 mL). The mixture was stirred for 2 h at room temperature, diluted with saturated NaHCO3 (50 mL), and extracted with CHCl3 (3 × 40 mL). The combined organic layers were washed with water, dried over Na2SO4, filtered, and concentrated. The residue was purified by CC in 6% ethanol in CH2Cl2 + 0.1% TEA to give nucleoside 8 as a hard foam (0.58 g, 99% yield): Rf = 0.43 (solvent system B).
ASSOCIATED CONTENT

Supporting Information

Tables and figures giving sequences of PCR primers and MALDI-TOF MS data, an electrophoretic analysis of DNase I cleavage of native and triazole-modified ONs, sequences, MALDI-TOF MS data, Tm values, and UV-melting profiles of duplexes with mismatches, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank V. A. Karpov (Institute for Physical-Chemical Medicine) for ON synthesis. This work was supported by the Russian Foundation for Basic Research [11-04-00131-a].

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