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DNA Triplex Structures are Stabilized by the Incorporation of 3′-*endo* Blocked Pyrimidine Nucleosides in the Hoogsteen Strand

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Abstract—A short route to pyrimidine locked nucleosides has been developed for their incorporation in triplex forming oligonucleotides (TFO). Compared to oligonucleotides build with standard nucleosides, the modified TFOs containing 3′-*endo* blocked residues formed, with their corresponding DNA duplexes, more stable triple helix systems, an effect which might be ascribed to the 3′-*endo* pucker of the modified nucleoside residues.

At present, many advances are currently being made in the understanding of the formation and the stabilization of DNA triple helices which should help the development of the potential therapeutic applications of triple helix forming oligonucleotides (TFOs).[1] Interestingly, it has been shown that the stability of the pyrimidine motif of triple helical structure is enhanced when the TFO strand contains 2′-*O*-methyl nucleosides. In this case, the beneficial stabilizing effect has been ascribed to the 3′-*endo* pucker of these nucleosides (*N*-type conformation).[2] On the other hand, in a series of recent papers, Wengel[3] and others[4] have shown that locked nucleic acids (LNAs) incorporating 3′-*endo* blocked nucleosides form highly stabilized duplexes when hybridized with their DNA complement. Similarly, it was claimed that 2′,5′-oligonucleotides containing 2′-*endo* blocked nucleosides (*S*-type conformation) can bind with a reasonable affinity to their single-stranded DNA target.[5] These observations prompted us to investigate whether such constrained nucleosides could stabilize a pyrimidine-motif triple helix. To this aim, we developed a new and short route to these nucleoside derivatives starting from uridine (Scheme 1).

With derivative **1** in hand, readily obtained in two steps from 2′,3′-*O*-isopropylideneuridine,[6] we first tried to selectively tosylate the α-hydroxymethyl at the C-4′ position. Despite many efforts, we failed to find conditions which would provide the desired derivative in a useful yield. Finally, the di-*O*-toluenesulfonyl derivative **2** was prepared in a modest 60% yield. Treatment of **2** with *p*-methoxybenzyl chloride afforded the N-3 protected derivative **3** which, upon acidic removal of the 2′,3′-*O*-isopropylidene protection, provided quantitatively compound **4** as an immediate precursor of locked nucleosides. Indeed, the treatment of **4** in the presence of sodium hydride in dimethyl formamide (DMF) led to the formation of a 1/4 mixture of the locked nucleoside derivatives **5** and **6** in 90% combined yield,[7] and these were easily separated by flash chromatography. They were identified by ^{1}H NMR analysis and by comparison of their free nucleosides (**11** and **12**) with those previously described (Scheme 2).[3,4a]

Subsequently, compounds **5** and **6** were separately deprotected at the N-3 position by treatment with cerium ammonium nitrate in the usual manner[8] to give the 5′-*O*-toluenesulfonyl derivatives **7** and **8**, respectively. Removal of the 5′-*O*-toluenesulfonyl group could not be achieved by displacement with sodium hydroxide; however, we were pleased to find that the nucleophilic substitution took place readily using sodium benzoate in DMF at 90 °C to give the corresponding benzoates **9** and **10** in high yield (95%). Their quantitative debenzoylation was accomplished using sodium methylate in methanol to afford the desired locked nucleosides **11**[3] and **12**.[4a] For

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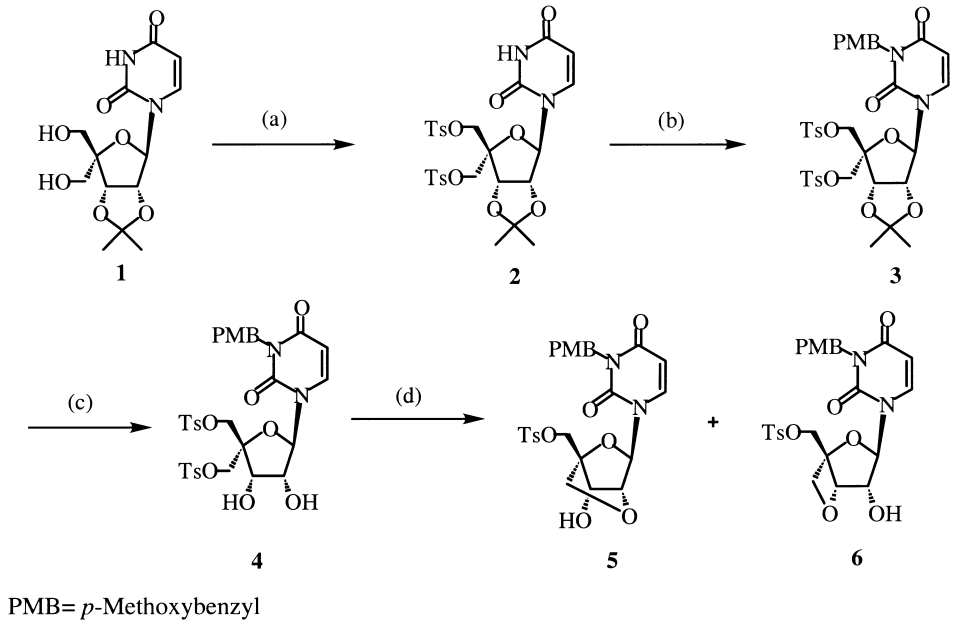
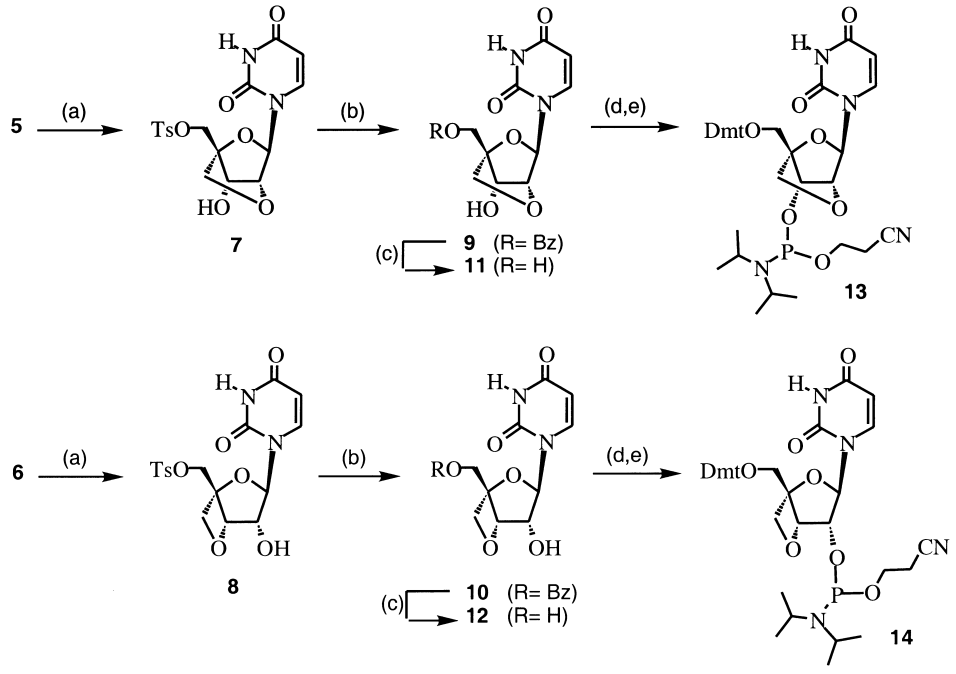


Scheme 1. Reaction conditions: (a) *p*-toluenesulfonyl chloride, DMAP, CH_2Cl_2, rt, 6 h, 60%; (b) *p*-methoxybenzyl chloride, K_2CO_3, DMF, rt, 3 h, 97%; (c) 80% acetic acid, reflux, 6 h, 100%; (d) NaH, DMF, rt, 1 h, 90% then chromatography separation (heptane:AcOEt: 1:1), **5**:**6** 1:4.



Scheme 2. Reaction conditions: (a) cerium ammonium nitrate, acetonitrile/H_2O, rt, 18 h, 95%; (b) sodium benzoate, DMF, 90 °C, 3 h, 95%; (c) NaOMe, methanol, rt, 10 min, 100%; (d) dimethoxytrityl chloride, pyridine, rt, 80%; (e) 2-cyanoethyl diisopropylchlorophosphoramidite, diisopropylethylamine, CH_2Cl_2, rt, 82%.

their final incorporation into oligonucleotides **II**–**III**, each nucleoside was first 5′-*O*-dimethoxytritylated and the resulting derivative treated with 2-cyanoethyl diisopropylchlorophosphoramidite to give the corresponding phosphoramidites **13** and **14** in good overall yields.[9]

Assays of triplex stability were made using a 29-mer double-stranded target sequence using the 16-mer probes **I**–**II**. DNA denaturation experiments were carried out to assess the thermal stability of triple helices formed by TFOs **I**–**III** in a 10 mM cacodylate buffer at pH 5.5 containing 100 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM spermine (Table 1). It was observed that the T_m value of the triplex–duplex transition ($T_m^{3\rightarrow 2}$) of **II** is 30 °C, whereas those of **III** and the unmodified TFO (**I**) are 5 and 24 °C, respectively. Consequently, one can

Table 1. The 29-mer ds DNA target was hybridized with its complementary third strand **I**–**III** (1.2 μM) in the indicated buffer

Double-stranded 29-mer DNA target

3′-CGTGAAAAA-**TTTT**CTTTTCCCCCCT-GACC-5′
5′-CCACTTTTT-**AAAA**GAAAAGGGGGGA-CTGG-3′

Complementary third strands

I 5′-TTTTCTTTTCCCCCCT-3′
II 5′-$\mathbf{L^1L^1L^1L^1}$CTTTTCCCCCCT-3′$\mathbf{L^1}$ = **11**
III 5′-$\mathbf{L^2L^2L^2L^2}$CTTTTCCCCCCT-3′$\mathbf{L^2}$ = **12**

TFO	$T_m^{3\rightarrow 2}$ (°C)[a] for triplex–duplex transition
I	24
II	30
III	5

[a] $T_m^{3\rightarrow 2}$ = melting temperature value (±1 °C).

observe that incorporation of 4′,2′-locked nucleosides in **II** provides 1.5 °C stabilization per substitution as compared to **I**.

This enhanced stability was expected since in 4′,2′-locked nucleosides the sugar is constrained in C3′-*endo* conformation.[3] In this respect, it is well known that pyrimidine-motif triple helix formation is favoured by 2′-substituents inducing C3′-*endo* conformation as observed in the 2′-methoxy or 2′-OH (RNA) series.[2] In contrast, a dramatic destabilization is observed with 4′,3′-locked nucleosides as present in **III**. This indicates that, unlike to duplex DNA hybridization,[5] such an oligonucleotide, characterized by 2′,5′-phosphodiester bonds and sugar moieties blocked in the C2′-*endo* conformation, is unable to form stable enough triple helix structures.

In conclusion, we have designed a new route to synthesize locked pyrimidine nucleosides **11** and **12** and we have shown that the substitution of 2′-deoxythymidine by the corresponding uridine derived 4′,2′-locked nucleoside residue **11** enhances the thermal stability in the case of a pyrimidine-motif triple helix. It confirms also that the correct conformational pre-organization of TFO favours the interaction between a TFO and its target duplex sequence.[10]

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References and Notes

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7. To date, we have not yet investigated the reaction conditions which would favour one cyclization pathway over the other (3′ vs 2′). An elegant solution to this problem might be adapted from a recent work (Obika, S.; Hari, Y.; Morio, K.; Imanishi, T. *Tetrahedron Lett.* **2000**, *41*, 215).
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9. Oligonucleotides syntheses were performed on an Applied Biosystem 392 synthesizer on a 1 μM scale using standard synthesis cycle. After complete deprotection, oligonucleotides were fully purified by RP18 HPLC.
10. All intermediates and final compounds were characterized by their analytical and spectral data.