Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 2453–2455

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Incorporation of 2′-Deoxy-5-(trifluoromethyl)uridine and 5-Cyano-2′-deoxyuridine into DNA

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Received 7 May 2001; accepted 28 June 2001

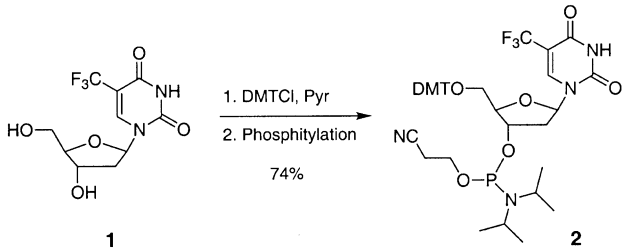
Abstract—In an attempt to synthesize DNA containing 2′-deoxy-5-(trifluoromethyl)uridine (**1**) using previously published protocols, we found that the trifluoromethyl group converted into a cyano group, resulting in DNA containing 5-cyano-2′-deoxyuridine (**3**). We show that nucleoside **1** can be incorporated into DNA using phosphoramidite **2** in combination with acetyl-protected deoxycytidine and phenoxyacetyl-protected purine phosphoramidites. Replacing thymidine in DNA with **1** caused a slight decrease in DNA duplex stability at pH 6.9. © 2001 Elsevier Science Ltd. All rights reserved.

The study of conformational changes in biopolymers as a function of conditions, mutations or interactions with other macromolecules or drugs is an important component of understanding their mechanism. Observing large-scale conformational changes requires techniques that enable measurements of long distances. Fluorescence resonance energy transfer (FRET) measurements can be used to determine distances of 35–85 Å and have, for example, been used to study ribozyme folding.[1–3] However, FRET gives a relatively low-resolution view, in part due to the flexible tethers that link the dyes to the polymer. Another technique, solution NMR spectroscopy, yields high-resolution information, but is limited to the determination of relatively short distances.

Solid-state NMR spectroscopy, however, can be used to determine long-range distances. In particular, ^{19}F–^{31}P rotational-echo double resonance (REDOR) solid-state NMR can be used to measure distances up to 16 Å.[4] This technique has been used to measure long-range distances in nucleic acids, between a phosphodiester and a fluorine atom placed on either a sugar or base moiety.[5] We are interested in determining distances longer than 16 Å by solid-state NMR and calculations suggested that using a trifluoromethyl group in place of a single fluorine atom might extend the range of distances past 25 Å. This paper describes the efficient incorporation of 2′-deoxyuridine containing a trifluoromethyl group into DNA.

The incorporation of commercially available 2′-deoxy-5-(trifluoromethyl)uridine (**1**) into DNA using phosphoramidite chemistry has been reported.[6,7] Accordingly, we converted nucleoside **1** into phosphoramidite **2** (Scheme 1) and used it in a solid-phase synthesis of a trityl-on oligonucleotide with the sequence 5′-d(CGCGAA**11**CGCG). Cleavage and deprotection of the solid support-bound DNA was accomplished using a standard deprotection protocol (concd NH_4OH at 55 °C for 16 h). The oligomer was subsequently purified by RP-HPLC and the trityl group was removed. Unexpectedly, no ^{19}F signal could be detected by ^{19}F NMR analysis of the oligomer (data not shown), indicating that the trifluoromethyl group had not survived the conditions used for the synthesis, deprotection and purification of the oligomer.

To further investigate this matter, the DNA was enzymatically digested.[8] Analysis of the digest by RP-HPLC (Fig. 1a) revealed the absence of **1** (Fig. 1b) and the appearance of a new substance with a shorter retention



Scheme 1. Synthesis of 3′-(2-cyanoethyl-*N*,*N*-diisopropyl)-2′-deoxy-5′-(4,4′-dimethoxytrityl)-5-(trifluoromethyl)uridine (**2**).

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PII: S0960-894X(01)00461-9

time. Nucleoside **1** has been observed to react with NH_4OH[9] and, in fact, treatment with concd NH_4OH at 55 °C for 16 h converted **1** to a product (>95%) which co-eluted with the new substance seen in the enzymatic digest (data not shown). High-resolution mass spectrometric and ^1H NMR analyses of the product isolated after NH_4OH treatment indicated that this substance was nucleoside **3** (Scheme 2).[10] To further verify the identity of **3**, an authentic sample was synthesized from 2′-deoxy-5-iodouridine[11] (Scheme 2) and shown to have the same physical properties (^1H NMR, ESI-MS, RP-HPLC, data not shown) as **3** synthesized from **1**.

Although perfluorinated alkyl groups are normally considered inert, the best example being Teflon®, the reactivity of the trifluoromethyl group of **1** towards nucleophiles, such as methoxyamine and bisulfite, has been documented.[12,13] It was proposed that the mechanism for the reaction starts with a nucleophilic addition in position 6 of the pyrimidine ring, resulting in elimination of a fluorine atom from the methyl group. Subsequent addition of a nucleophile, such as ammonia in our case, to the methyl group leads to the loss of the substituent in position 6. The overall result is the replacement of a fluorine atom with ammonia via an elimination–addition mechanism. After incorporation of the amino function into the methyl group, two molecules of HF are eliminated to form the cyano group. A similar mechanism has been proposed for the hydrolysis of α,α,α-trifluorocresol, where the *meta* isomer is inert while both the *ortho* and *para* isomers are readily hydrolyzed to hydroxybenzoic acid.[14]

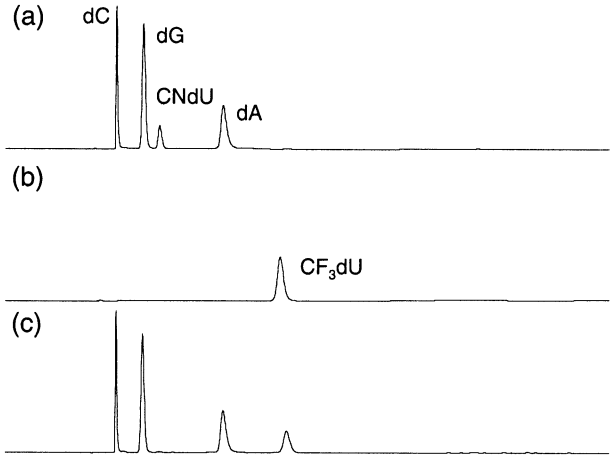
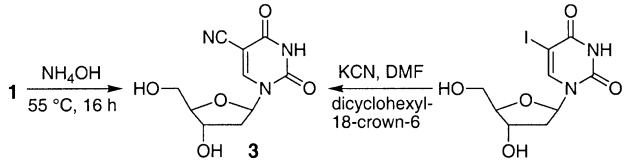


Figure 1. RP-HPLC analyses of enzymatically digested DNA containing 5-substituted 2′-deoxyuridines. Trace (a) 5′-d(CGCGAA**33**CGCG), (b) nucleoside **1**, (c) 5′-d(CGCGAA**11**CGCG).



Scheme 2. Conversion of nucleoside **1** and 2′-deoxy-5-iodouridine to 5-cyano-2′-deoxyuridine (**3**).

Because **1** converted to **3** under commonly used DNA deprotection conditions, a milder deprotection protocol was necessary. Deprotection of DNA containing **1** with concd NH_4OH at 20 °C for 12 h[9,15] yielded a substantial amount of **3** in the DNA (2:3 ratio of **1** to **3**, data not shown). Therefore, we used an alternate method for DNA synthesis, employing commercially available phosphoramidites containing phenoxyacetyl-protecting groups for the purine nucleosides and an acetyl protecting group for deoxycytidine.[16] This approach enables DNA deprotection under much milder conditions (in a methanolic solution of K_2CO_3).[17]

We first tested the stability of **1** in a methanolic solution of K_2CO_3 (50 mM) at 22 °C for 4 h. Analysis of the resulting solution by RP-HPLC showed that the nucleoside was >99% intact. However, it should be noted that prolonged incubation (37 °C, 2 weeks) resulted in quantitative conversion of **1** to a new substance. This substance was determined by ESI-MS analysis to be 2′-deoxy-5-(trimethoxymethyl)uridine, which hydrolyzed quickly in acid to form 2′-deoxy-5-(methylcarboxylate)uridine.[18]

The DNA oligomer 5′-d(CGCGAA**11**CGCG) was resynthesized using the mild-deprotecting phosphoramidites and phosphoramidite **2**, deprotected under the aforementioned conditions, purified and detritylated. ESI-MS analysis of this oligomer yielded the mass expected for a DNA oligomer containing 2′-deoxy-5-(trifluoromethyl)uridines (3754.6, calcd 3754.4). Furthermore, enzymatic digestion of the DNA and analysis of the digest by RP-HPLC (Fig. 1c) showed the presence of **1** (verified by co-injection with an authentic sample of **1**, data not shown). 2′-Deoxy-5-(methylcarboxylate)uridine was not observed in the enzymatic digests, but the mass of an oligomer containing this modification was observed at times in ESI-MS of oligomers containing **1** (data not shown). However, when the mass corresponding to a 5-methylcarboxylate-containing oligomer was observed, it was below the 1% abundance level and, therefore, we conclude that this modification was present in negligible amounts.

To determine if the incorporation of nucleoside **1** into DNA had an effect on duplex stability, we prepared four duplexes and conducted thermal denaturation experiments (Table 1). DNA duplexes **A** and **C** were unmodified whereas duplexes **B** and **D** contain one and two modifications, respectively. Contrary to a previous report,[19] the duplexes containing **1** are slightly less stable, with a melting temperature ca. three degrees lower for each modification. Duplex **D** was enzymatically digested after the thermal denaturation experiment and RP-HPLC analysis showed that nucleoside **1** did not degrade over the course of the annealing and melting experiments (data not shown). Thermal denaturation experiments of a duplex formed by the oligomer 5′-d(CGCGAA**11**CGCG) did not show a cooperative melting transition.

In conclusion, we have described an efficient method for incorporation of **1** into DNA and have verified its

Table 1. Thermodynamic data for trifluoromethylated DNA duplexes[a]

A	5′CGCAAAAATGCG 3′GCGTTTTTACGC	**B**	5′CGCAAAAA**1**GCG 3′GCGTTTTTACGC
C	5′CGCGAATTCGCG 3′GCGCTTAAGCGC	**D**	5′CGCGAA**1**TCGCG 3′GCGCT**1**AAGCGC

Duplex	T_m (°C)	$-\Delta G°_{37}$ (kcal/mol)	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ (cal/mol·K)
A	59.0 (±0.5)	4.8 (±0.1)	71.9 (±3.0)	216.5 (±9.5)
B	55.9 (±0.4)	3.3 (±0.2)	57.1 (±4.5)	173.6 (±13.8)
C	64.2 (±1.1)	5.3 (±0.7)	64.9 (±5.9)	192.3 (±17.2)
D	58.3 (±0.7)	2.9 (±0.1)	45.0 (±2.5)	135.6 (±7.6)

[a]Optical melting experiments were performed at 260 nm in 500 mM NaCl, 0.05 mM Na_2EDTA, and 5 mM sodium phosphate (pH 6.9).

intactness. Incorporation of nucleoside **1** slightly lowers DNA duplex stability at pH 6.9. We have also shown that DNA containing nucleoside **3** can be readily prepared by treating DNA containing **1** with NH_4OH. Solid-state NMR experiments on DNA containing nucleoside **1** are underway and will be reported in due course.

Acknowledgements

We thank the National Institutes of Health (GM58914 and GM56947) for financial support, Dr. G. P. Drobny and E. A. Louie for helpful discussions, Dr. M. Sadilek for help with MS analyses and members of the Sigurdsson research group for critical reading of the manuscript.

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