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Synthesis and Incorporation into DNA of a Chemically Stable, Functional Abasic Site Analogue

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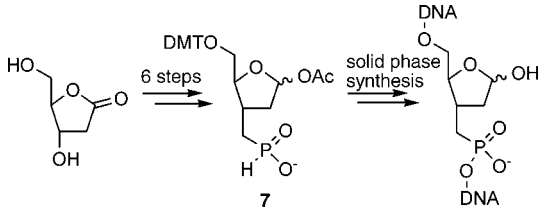
ABSTRACT



The abasic site building block 7 for DNA synthesis, containing a methylenephosphinic acid group at C3′, was prepared in six steps and was incorporated into DNA via a combination of H-phosphonate and phosphoramidite chemistry. Corresponding oligodeoxynucleotides were shown to be chemically stable under basic conditions and fully functional at the respective hemiacetal center.

Abasic sites are DNA lesions that either occur spontaneously by hydrolysis of purine bases or enzymatically as intermediates in the base excision pathway of the DNA repair machinery.[1] Due to the missing coding information, such sites are highly mutagenic and are thus a threat to the integrity of the genome. Chemically, abasic sites contain a hemiacetal function at the anomeric center and are thus intrinsically unstable leading to DNA strand scission at the 3′- and 5′-site of the abasic unit via β- and δ-elimination (Figure 1).

Due to the low chemical stability, most of the investigations on the biological, biophysical, and structural impact of abasic sites in the past have been performed on analogues in which the hemiacetal function was reduced to a cyclic ether (THF analogue) or to open-chain analogues (propanediol analogues). Both of these variants are not optimal as the chemical characteristics of the hemiacetal function that crucially determine its reactivity and that may also influence its structural and biophysical properties is lost.

A conceptually simple way to preserve the hemiacetal function and to maintain chemical stability of the DNA backbone exists in the conversion of the 3′-oxygen of an abasic site into a methylene unit, resulting in a phosphonate

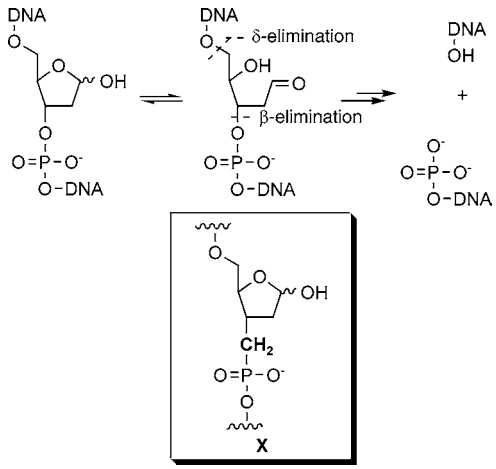


Figure 1. β,δ-Elimination pathways leading to DNA strand cleavage at abasic sites, as well as the chemical structure of the 3′-methylene analogue **X**.

(1) (a) Lhomme, J.; Constant, J. F.; Demeunynck, M. *Biopolymers* **1999**, *52*, 65. (b) Schärer, O. D. *Angew. Chem., Int. Ed.* **2003**, *42*, 2946.

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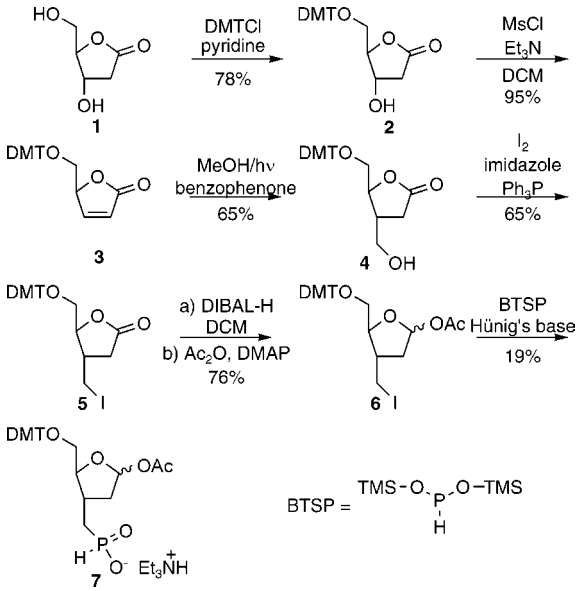
function that is isostructural and isoelectrostatic to a natural backbone phosphodiester function. This modification is expected to block the β-elimination reaction at abasic sites, thus inhibiting strand scission.

3′-Deoxy-3′-hydroxymethylene nucleosides and corresponding phosphonates were synthesized before. Their syntheses typically started from the natural nucleosides using different C-1 synthons.[2] DNA oligonucleotides containing phosphonate backbone linkages were also prepared before in the context of antisense research, and it was shown that they slightly enhance duplex stability,[3] indicating that the mutation of a 3′-oxygen into a methylene group does not interfere with double helix formation.

Here, we report on the successful synthesis of oligodeoxynucleotides containing the 3′-methylene abasic site analogue **X** (Figure 1) and the characterization of its functional properties and its chemical stability.

Scheme 1. Synthesis of Building Block **7**



We planned to use the H-phosphinate **7** (Scheme 1) as a building block for **X**, reasoning that it could be directly used in the automated synthesis of oligodeoxynucleotides and that it would be compatible with standard phosphoramidite chemistry. For the elaboration of the C−C bond between the methylene unit and the deoxyribose ring we intended to utilize the photoinduced radical conjugate addition of methanol, previously developed by Mann.[4] As a protecting group for the anomeric center we chose an acetyl group which would liberate the hemiacetal function concomitantly during deprotection and detachment from the solid support of the oligonucleotide. The synthesis of building block **7** is outlined in Scheme 1.

While the synthesis up to iodide **6** proceeded smoothly and in good yields, the following Arbusov reaction proved to be difficult. Reaction of **6** with in situ prepared bis-trimethylsilylphosphonite (BTSP) led to only ca. 20% of the desired building block **7** after aqueous workup, even after considerable efforts of optimization. A major side reaction observed was the (known) reduction by BTSP to the corresponding 3′-methyl derivative.[5] Changing the leaving group to a tosylate inhibited the reduction pathway but surprisingly did not improve the yield. Attempts to use the more active triflate leaving group failed due to the instability of the corresponding compound. To test whether a TMS-based Lewis acid originating from the TMS phosphinate intermediate of **7** was responsible for the low yield, we also subjected lactone **5** to BTSP treatment. But again the corresponding Arbusov product could only be isolated in ca. 25% yield, indicating that Lewis acid-catalyzed elimination of the anomeric acetate group is not a significant side reaction responsible for the low yields of **7**. Despite the difficulties in the last synthetic step, **7** could be produced in sufficient quantities for the following synthetic and biophysical studies.

In a series of model experiments using 3′-*O*-TBDMS-protected thymidine and **7** we first tested a variety of carbodiimide-, uronium-, and phosphonium-based coupling reagents and were surprised to find that, contrary to the reported high coupling efficiencies in the ribo series, building block **7** reacted not at all under standard coupling conditions (30 min, rt, 20-fold molar excess). It thus seems that a missing electronegative substituent at the remote 2′-position decelerates the condensation reaction for a yet unknown reason. A way out was finally found by adding a nucleophilic catalyst to the coupling mixture as was recently reported for the synthesis of boranophosphates.[6] We found that the combination of BOP-Cl and 3-nitro-1,2,4-triazole was perfectly suited for coupling the H-phosphinate intermediate (Figure 2), leading to complete conversion within 30 min. The related NEP-Cl was under these conditions less active and needed 24 h for complete conversion.

Having optimized the coupling conditions in solution, we next approached the solid-phase oligonucleotide synthesis. Within the amidite protocol, a separate coupling and oxidation step for **7** had to be accommodated. Coupling was effected with BOP-Cl and 3-nitro-1,2,4-triazole as activator while oxidation was performed using iodine (200 mM) under basic (1 M NEt_3) conditions for 5 min.[7] The detailed protocol is described in the Supporting Information. With this protocol, we prepared the two oligonucleotides **8** and **9** (Table 1). Coupling efficiencies for the incorporation of **7** as determined from the trityl assay were typically >95%. Oligonucleotides were detached from solid support and deprotected under standard conditions (NH_3 concd, 55 °C,

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(3) An, H.; Wang, T.; Maier, M. A.; Manoharan, M.; Ross, B. S.; Dan Cook, P. *J. Org. Chem.* **2001**, *66*, 2789.

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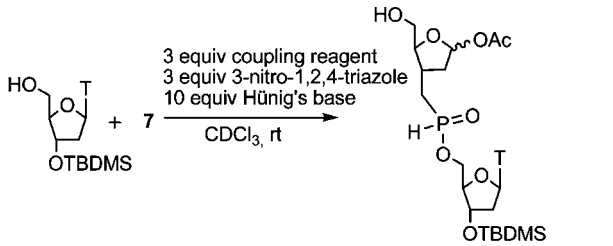


coupling reagent	30 min	3 h	24 h
NEP-Cl	<2%	67%	100%
BOP-Cl	100%	100%	100%

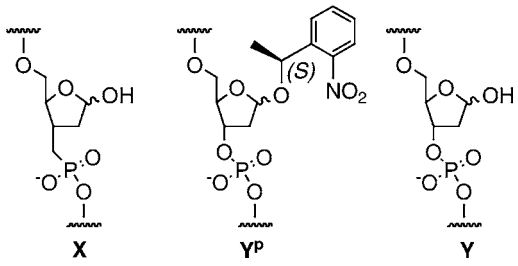
Figure 2. Coupling yields for H-phosphinate **7** as judged from integration of ^{31}P NMR peaks after the indicated reaction time. NEP-Cl = 2-chloro-5,5′-dimethyl-1,3,2-dioxaphosphorinane-2-oxide; BOP-Cl = bis(2-oxo-3-oxazolidinyl)phosphinic chloride.

16 h) and purified by standard RP-HPLC. Isolated yields after purification were in the range of 20−30%, and ESI-mass spectrometric analysis confirmed the expected masses (Table 1). From these results we conclude that the condensation chemistry for the introduction of **7** is orthogonal to the phosphoramidite chemistry of oligonucleotide synthesis despite the fact that there is no protecting group on the newly formed phosphonate function.

For comparison, we also prepared oligonucleotides **10** and **11** having a natural protected or unprotected abasic site **Y** from a phosphoramidite building block containing a photocleavable *S*-1-(2-nitrophenyl)ethyl (1NPE) group at the anomeric center.

The next task was to prove the presence of the hemiacetal function in oligonucleotides. For this we recorded a ^{1}H NMR spectrum of **8**, of which the aromatic and anomeric proton section is depicted in Figure 3.

Table 1. Sequence and Analytical Data of Oligonucleotides Containing the Abasic Site Analogue X or the Natural Abasic Site Y



X **Y^{p}** **Y**

	sequence	ESI$^-$-MS calcd	ESI$^-$-MS found	yield[a] OD^{260}	yield[a] %
8	d(TTT**X**TTT)	1957.3	1956.7	17.3	27.0
9	d(GTAC**X**ATCG)	2603.7	2602.9	23.9	23.1
10	d(GTAC**Y^{p}**ATCG)	2754.9	2754.1	27.8	26.8
11	d(GTAC**Y**ATCG)				

[a] All oligonucleotides were synthesized on a 1.3 μmol scale. Yields are calculated after RP-HPLC purification.

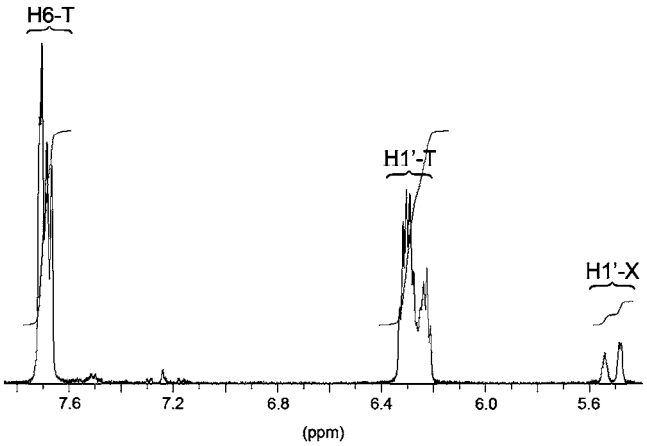


Figure 3. Section of the ^{1}H NMR spectrum of **8** (D_2O, ≈ 0.65 mM, 500 MHz).

Besides the typical resonances of the aromatic H6 and the anomeric H1′ of the thymidine residues there appear two signals at around 5.5 ppm that can be assigned to the H1′ of the two anomeric forms of the corresponding abasic unit **X**. No signal at around 10 ppm could be detected ruling out a significant contribution of the aldehyde form to the equilibrium mixture.

To prove the functionality of the abasic site, we incubated oligonucleotide **8** with varying amounts of dansylhydrazine (DNSH), a reagent typically used for labeling aldehydes and ketones,[8] and analyzed the progress of hydrazone formation by polyacrylamide gel electrophoresis (PAGE). As can be seen from Figure 4, a new (fluorescent) band with lower

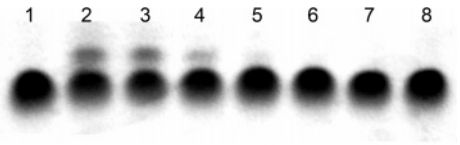


Figure 4. UV shadow of a denaturating 20% PAGE of mixtures of **8** with varying amounts of dansylhydrazine (DNSH). Each lane contains 0.4 OD^{260} (13.2 μg, 6.75 nmol) of **8**. Lane 1: control without DNSH. Lanes 2−8: 10, 5, 2, 1, 0.5, 0.25, 0.1 equiv of DNSH.

mobility than the parent oligonucleotide **8** occurred at high DNSH excess in a concentration dependent manner. This band is ascribed to the hydrazone of oligonucleotide **8** and fully proves the functionality of the abasic site analogue **X**.

Last but not least, we determined the base stability of the abasic site analogue **X**. For this oligonucleotide **9** and for comparison also **10** and **11**, containing a protected and unprotected natural abasic site were radiolabeled at their 5′-end with T4 polynucleotide kinase and [γ-^{32}P]ATP. Oligo-

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nucleotides were then incubated in 0.2 M NaOH at 65 °C for 30 min and the reaction products analyzed by PAGE.

As can be seen from Figure 5, no degradation of oligonucleotide **9**, containing the abasic site analogue **X**, was observed under these conditions, while oligonucleotide **11**, having a natural abasic site, underwent complete β- and δ-elimination leading to a shorter labeled fragment with higher mobility. This experiment clearly demonstrates the stability of **X** toward basic conditions.

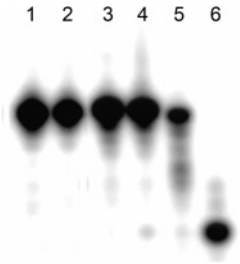

Figure 5. Denaturing 20% PAGE of radiolabeled **9** and control oligonucleotides **10** and **11**. Lanes 1, 3, and 5: oligonucleotides **9**, **10**, and **11**, without base treatment. Lane 2, 4, and 6: oligonucleotides **9**, **10**, and **11** after treatment with 0.2 M NaOH at 65 °C for 30 min.

In conclusion, we have shown that phosphinate **7** is a useful building block for the abasic site analogue **X**. The underlying coupling and oxidation chemistry was found to be orthogonal to the phosphoramidite chemistry despite the missing protecting group at the phosphonate function. Further improvement in the synthesis of **7** has to be achieved in future work in order to make the use of this building block more practical.

We also showed that **X**-containing oligonucleotides can be deprotected via standard methods and that **X** occurs in aqueous solution as expected predominantly in the two hemiacetal forms. A hydrazine dye labeling test and NaOH treatment clearly demonstrated the functionality and the base stability of oligonucleotides containing **X**. Thus, this phosphonate unit constitutes the first chemically stable and functional abasic site analogue.

Potential applications of this unit can be envisaged in experiments directed to determine the effect of the hemiacetal function on duplex structure and stability as well as on DNA polymerase dependent template primer extension reactions. Furthermore, this unit could be of interest in the context of reactivity based screening or inhibition of enzymes involved in the DNA base excision repair pathway.

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Supporting Information Available: Procedures and analytical data for the synthesis of compounds **2**−**7** as well as oligonucleotides **8** and **9**. Detailed procedures for the experiments summarized in Figures 4 and 5 as well as ^{1}H and ^{13}C NMR spectra for compounds **2**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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