Site-Specific Incorporation of the Alkaline Labile, Oxidative Stress Product (5*R*)-5,6-Dihydro-5-hydroxythymidine in an Oligonucleotide

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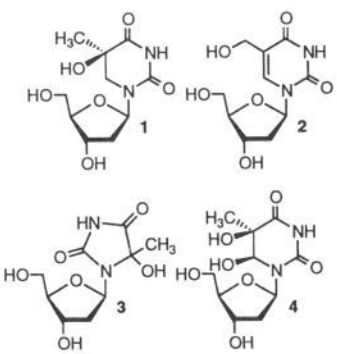
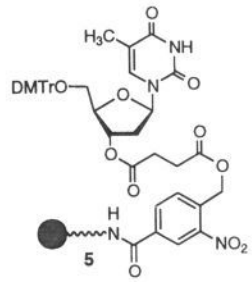
The nucleobases within nucleic acids are alkylated by electrophiles such as oxygenated benzo[*a*]pyrenes and are oxidized by activated oxygen species.[1,2] The damage resulting from such chemical reactions can be mutagenic and/or cytotoxic. In order to understand the origin of mutagenicity, it is important to correlate changes induced by mutated nucleosides on the function and structure of nucleic acids. Determination of the effects that mutated nucleosides impart on the function of polymerase and repair enzymes *in vitro* and *in vivo*, as well as spectroscopic characterization of structural alterations induced by these molecules, is made possible by their site-specific incorporation in oligonucleotides.[1,3-7] In some instances, mutations are introduced in the oligonucleotide postsynthetically.[1,5] This strategy is limited by the structural variation of the biopolymers containing the mutated nucleoside that can be prepared, as well as the types of mutations that one can introduce. Incorporation of the mutated monomer as its nucleotide triphosphate or phosphoramidite provides the most versatile synthesis of modified oligonucleotides.[4] However, site-specific incorporation of some mutated nucleosides in biopolymers during solid-phase synthesis protocols is prohibited by their lability to the alkaline conditions typically used to deprotect chemically synthesized nucleic acids.[5] We wish to report the first chemical synthesis of an oligonucleotide containing alkaline labile (5*R*)-5,6-dihydro-5-hydroxythymidine (**1**), in which Pd^0 labile protecting groups are utilized for carrying out the synthesis on a photolabile solid-phase synthesis support.



(5*R*)-5,6-Dihydro-5-hydroxythymidine (**1**) is one of several mutated forms of thymidine that are believed to be mutagenic and/or cytotoxic. These mutated nucleosides are formed as a result of the interaction between reactive oxygen species and pyrimidine nucleosides.[2a] Oligonucleotides containing 5-(hydroxymethyl)-2′-deoxyuridine (**2**) and 5-hydroxy-5-methylhydantoin (**3**) were synthesized using conventional protecting groups, because the modified nucleosides withstood deprotection with concentrated ammonium hydroxide.[4a-c] Site-specific incorporation of a mixture of stereoisomers of alkali labile thymidine glycol (**4**) was achieved postsynthetically via osmylation of an oligonucleotide containing a single thymidine.[5] To our knowledge, there is no chemical reaction that enables one to transform a nucleoside into **1** postsynthetically. Furthermore, **1** decomposes when exposed to concentrated ammonium hydroxide. Hence, the integrity of **1** cannot be maintained when it is incorporated into chemically synthesized oligonucleotides that require alkaline deprotection conditions.

In order to insure that oligonucleotides containing **1** are sufficiently pure for use as mechanistic probes *in vitro* and *in vivo*, we sought conditions that obviate the need for harsh base treatment during biopolymer synthesis. With this requirement in mind, allyloxy protecting groups were chosen in order to take advantage of their lability to Pd^0 at pH 5.5.[8] We also elected to utilize a solid-phase synthesis support from which oligonucleotides are cleaved under nonalkaline conditions. Oligonucleotides bound to solid-phase supports via disulfide linkages are cleaved under mild reductive conditions. However, the oligonucleotides released by this method contain 3′-termini that are not compatible with subsequent enzymatic manipulation unless they are subjected to further alkaline hydrolysis.[9] We recently reported on a family of photolabile orthogonal linkers that release oligonucleotides containing 3′-hydroxyl groups.[10] *O*-Nitrobenzyl support **5** releases oligonucleotides under conditions that result in less than 2% thymine·thymine dimer formation. The resulting 3′-succinato moiety undergoes hydrolysis under the mildly basic conditions (pH 8.3) encountered during denaturing polyacrylamide gel electrophoresis, yielding oligonucleotides containing 3′-termini that are suitable for enzymatic reactions.



Phosphoramidite **10** was synthesized using methodologies previously described for the synthesis of **1** and other (allyloxy)-carbonyl phosphoramidites (Scheme 1).[8,11] The stereochemistry at C5 is established during the dihydroxylation of 3′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)thymidine by OsO_4. Thymidine and hydroxy-protected derivatives are predisposed to favor formation of the respective 5*R*,6*S* stereoisomer, such as **6**, which was isolated as a single diastereomer in 87% yield.[11] Following esterification of **6** (78%), the protected thymidine C5-hydrate (**7**) was isolated in 68% yield via photoinduced SET reduction.[11,12] Bis-allyloxy nucleoside **8** was obtained via acylation of the dianion of **7** by diallyl pyrocarbonate. A number of deprotonation methods were investigated, but *t*-BuMgCl in THF was found to be the most effective. In order to prevent cleavage of the allyloxy carbonate protecting groups, desilylation was carried out using buffered TBAF (0.5 M AcOH, 0.5 M TBAF).[13]

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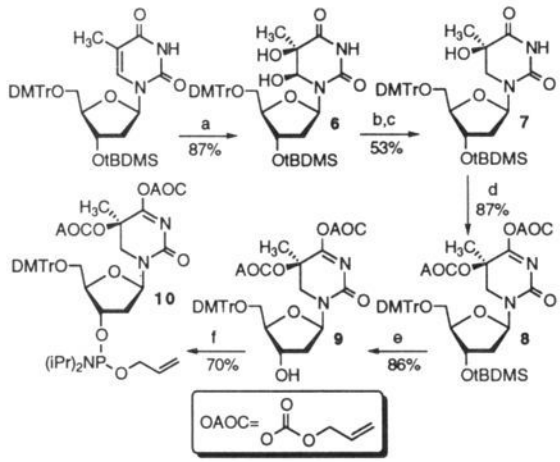
Scheme 1



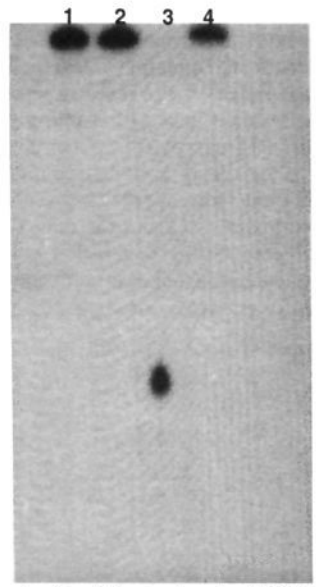
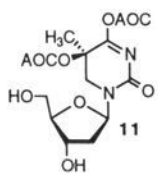


Figure 1. Polyacrylamide gel electrophoresis of 5′-^{32}P-labeled **12** and **13**. Lanes 1 and 3, **12**. Lanes 2 and 4, **13**. Lanes 1 and 2, no alkali treatment. Lanes 3 and 4, treatment with 1 M NaOH, 6 h, 55 °C.

Phosphitylation of **9** was carried out in the final step using literature procedures.[14]

The stability of **1** to conditions typically employed for the deprotection of allyloxy protecting groups was examined prior to synthesizing an octadecamer containing (5*R*)-5,6-dihydro-5-hydroxythymidine. Treatment of **11** with $Pd_2(dba)_3CHCl_3$ and PPh_3 in 0.5 M $BuNH_2/HCO_2H$ at 55 °C for 1 h resulted in complete conversion of **11** to **1**, as analyzed by TLC and reverse-phase HPLC.[8] Neither thymidine nor any other nucleoside product was detected when **11** was subjected to these reaction conditions.



5′-d(CAT A1G CAT GTA GCT GAT)
12

5′-d(CAT ATG CAT GTA GCT GAT)
13

An octadecamer (**12**) containing a single molecule of **1** was synthesized via automated synthesis using previously reported cycles.[8,10a] The only restriction imposed upon the design of the oligonucleotide's sequence was the avoidance of consecutive thymidines, in order to guard against small amounts of photodimer formation. The crude oligonucleotide was subjected to Pd^0 deprotection, followed by band-pass photolysis (λ_{max} = 400 nm) for 3 h in order to cleave the oligonucleotide from the controlled pore glass support. Following the prescribed workup, the oligonucleotide was purified by denaturing polyacrylamide gel electrophoresis (20% polyacrylamide) and desalted using a reverse-phase purification cartridge.[10a]

Purified **12** and an octadecamer (**13**) that differs from **12** solely by having a thymidine substituted for **1** were labeled with ^{32}P at their 5′-termini and shown to comigrate on a 20% denaturing polyacrylamide gel (Figure 1). Treatment of the labeled oligonucleotides with 1 M NaOH does not affect **13** but results in complete cleavage of **12** to yield a labeled fragment that moves faster than the Bromophenol Blue dye. The mobility of the fragment is consistent with the presence of an alkali labile site at the position where **1** is located in **12**.[15] Further evidence for the presence of **1** in the oligonucleotide was elicited by acidic hydrolysis of **12** and subsequent silylation of the nucleobases released.[16,17] The mixture of silylated nucleobases was analyzed by GC/MS using selected ion monitoring (supplementary material).[17] The peak corresponding to trisilylthymine C5-hydrate coeluted with material produced from hydrolysis of independently synthesized **1**.[11a]

The methodology discussed above will enable the investigation of the effects of alkali labile mutated nucleosides (e.g., **1**) on the structure and biological function of the nucleic acids in which they are present. Furthermore, this methodology demonstrates that photolabile orthogonal solid-phase synthesis supports can be used to incorporate alkali labile nucleosides in oligonucleotides which contain 3′-termini suitable for enzymatic manipulation.[10]

Acknowledgment. Financial support of this research from the National Institutes of Health (GM-46534) is appreciated. The authors are grateful to Professor Robert M. Williams for helpful discussions.

Supplementary Material Available: Experimental procedures, spectral characterization for all new compounds, and GC/MS SIM trace of hydrolyzed **12** (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(16) Compositional analysis of oligonucleotides is often carried out via enzymatic digestion, followed by HPLC analysis of the liberated nucleosides. However, **1** does not absorb strongly above 214 nm, which hindered its detection via the UV detectors available to us. See: (a) Crain, P. F. *Methods Enzymol.* **1990**, *193*, 782. (b) Eadie, J. S.; McBride, L. J.; Efcavitch, J. W.; Hoff, L. B.; Cathcart, R. *Anal. Biochem.* **1987**, *165*, 442.

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