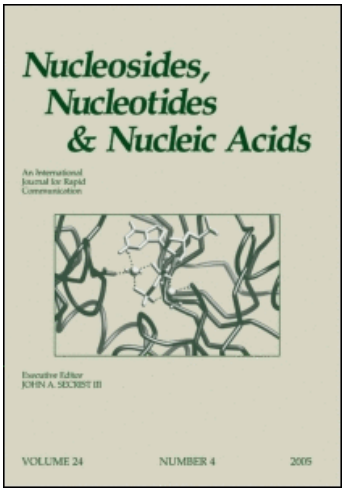

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Base Modification and the Phosphoramidite Approach

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BASE MODIFICATION AND THE PHOSPHORAMIDITE APPROACH

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Abstract. Oligodeoxynucleotides 18-150 bases in length were synthesized with both O-(2-cyanoethyl)- and O-(methyl)-phosphoramidites. After enzymatic degradation of the purified products, base modification and composition were evaluated by HPLC. Additionally, synthesis of 5'-d[GCGCGCTT] with O-(methyl) phosphorus protection generated 3-methylthymidine when thiophenoxide was omitted from the deprotection protocol.

Use of nucleoside phosphoramidites[1] on controlled pore glass polymer supports (CPG)[2] allows synthesis of oligodeoxynucleotides (oligomers) greater than 100 bases in length.[3] Recent reports suggest that during lengthy syntheses, chemical modifications of deoxyadenosine (A),[4] deoxyguanosine (G)[5] and thymidine (T)[6] become significant. Some of these events are reported to depend upon the phosphorus protecting group employed in the phosphoramidite approach.[7] We have investigated the extent of oligonucleotide damage that occurs under a defined scenario of purified reagents, automated synthesis, deprotection, and purification.

Each of four oligomers, 18, 34, 72, and 150 in length (TABLE 1) were synthesized once by (methyl)- and once by (2-cyanoethyl)- N,N-diisopropylphosphoramidites using standard protocol.[8] Following purification (TABLE 1), products were degraded with snake venom phosphodiesterase and alkaline phosphatase to nucleosidic components.[6] Digestions were monitored for 48 h by autoradiography (not shown) and were complete in 4-8 h. Following ethanol precipitation, nucleosides were analyzed by RP-HPLC (FIG. 1) with UV_{254nm} detection. Deoxyinosine (I), present at 1-2% in most digests, was determined to be a product of adenosine deaminase activity in snake venom phosphodiesterase. There-

TABLE 1. Comparison of Polydeoxynucleotide Syntheses Using (Methyl)- and (2-Cyanoethyl)-phosphoramidites. (a) Synthesized with methyl protection; (b) Synthesized with 2-cyanoethyl protection; (c) Based on relative peak areas normalized to a chromatogram of a standard solution of A, G, C, T (25 mole % of each); (d) Total yield in O.D. (260nm) units following polyacrylamide gel electrophoresis and desalting with Sep-Pak (Waters Assoc.).

Length	Theoretical Composition	Empirical Composition[c]	Isolated[d] Yield
18[a]	$A_5\ G_6\ C_4\ T_1$	$A_{4.89}\ G_{6.01}\ C_{4.06}\ T_{1.00}$	4.4
18[b]	$A_5\ G_6\ C_4\ T_1$	$A_{4.90}\ G_{6.09}\ C_{4.03}\ T_{0.97}$	4.9
34[a]	$A_8\ G_{11}\ C_8\ T_7$	$A_{7.90}\ G_{1.11}\ C_{8.03}\ T_{6.91}$	4.8
34[b]	$A_8\ G_{11}\ C_8\ T_7$	$A_{7.89}\ G_{1.11}\ C_{8.00}\ T_{6.96}$	3.5
72[a]	$A_{15}\ G_{19}\ C_{20}\ T_{18}$	$A_{14.8}\ G_{18.6}\ C_{20.0}\ T_{18.6}$	3.3
72[b]	$A_{15}\ G_{19}\ C_{20}\ T_{18}$	$A_{15.0}\ G_{18.9}\ C_{19.7}\ T_{18.4}$	3.9
150[a]	$A_{24}\ G_{41}\ C_{39}\ T_{46}$	$A_{22.6}\ G_{41.9}\ C_{39.0}\ T_{46.5}$	0.9
150[b]	$A_{24}\ G_{41}\ C_{39}\ T_{46}$	$A_{21.1}\ G_{41.9}\ C_{39.4}\ T_{47.6}$	0.3

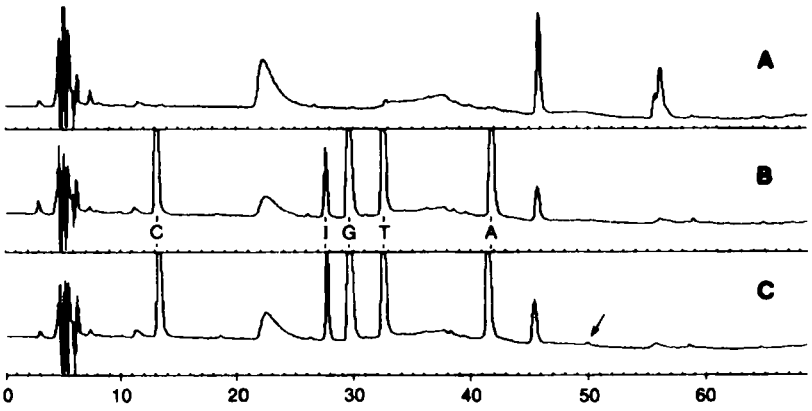

FIG. 1. Chromatograms of Enzymatic Digests of Purified Polydeoxynucleotides 150 Bases in Length Synthesized by the Phosphoramidite Approach. (a) Control digest (no oligomer); (b) 2-cyanoethylphosphoramidites were used; (c) Methylphosphoramidites were used. The arrow indicates an impurity (0.15% abundance relative to thymidine) which comigrates (50 min.) with a 3-methylthymidine standard.

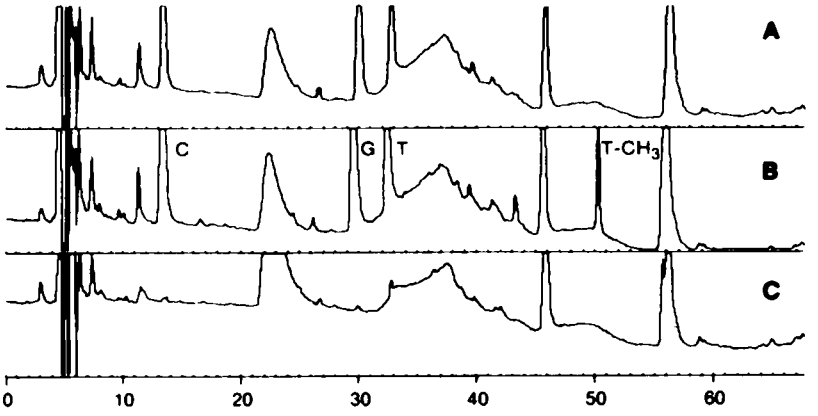

FIG.2 Chromatograms of Enzymatic Digests of 5'-d[GCGCGCTT] Synthesized With and Without Thiophenoxide. The protected oligomer was divided in half, and deprotected with (Panel A) and without (Panel B) thiophenoxide. Panel C was a digestion control (no oligomer). The peak labeled T-CH_3, 10% in abundance, comigrates with a 3-methylthymidine standard.

fore, the relative abundance of (A) was determined by adding the (A) and (I) peak areas. Empirical compositions (TABLE 1) for methyl and 2-cyanoethyl syntheses were equivalent within experimental error, and both generally agreed well with theoretical. At the 150-base level, however, the (A) composition was 5-11% below theoretical in both syntheses. The fate of (A) was not determined here since no modified nucleosidic species (0.1% by UV_{254nm}) could be detected in any of the eight syntheses listed in TABLE 1. One explanation for this deficiency is that apurinic sites exist in the purified product.

Low levels of modification products (0.01-0.1%) were detected when chromatographic sample sizes were increased five fold (0.2 - 0.3 $O.D._{260nm}$). These impurities were in similar abundance regardless of synthesis length. For example, the most intense side product observed during this study, 3-methylthymidine, was present in 0.23 and 0.15% (relative to thymidine) for the 72-mer and 150-mer, respectively, when methyl phosphoramidites were used with the standard deprotection protocol. However, deletion of thiophenoxide treatment in the deprotection protocol yielded 10% 3-methylthymidine (FIG. 2) in the synthesis of 5'-d[GCGCGCTT].

CONCLUSION

Isolated yields from 2-cyanoethyl and methyl syntheses were similar at all lengths (18-150 bases). Following purification and enzymatic degradation, RP-HPLC (UV_{254nm}) of these syntheses failed to reveal any modified nucleoside in excess of 0.1% of total nucleoside integrated area. These low levels of base modification are supported by the close agreement of empirical base compositions with theoretical values. The quantities of modified nucleoside material did not increase with increasing chain length. For example, the 150-mer and 72-mer synthesized with (methyl)-phosphoramidites contained similar levels (1-2 ppt relative to thymidine) of 3-methylthymidine. Lower levels of base modification seen here relative to other reports suggest that certain deviations from standard protocol[8] enhance base modification. For example, methylation of thymidine takes place during NH_3(aq) deprotection after incomplete thiophenoxide treatment.

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8. All oligomers were synthesized at 0.2 umol scale on an Applied Biosystems 381A DNA Synthesizer with the same set of reagents all commercially available from Applied Biosystems (ABI) as of 9/1/86, with the exception that the 150-mers were synthesized on a "wide pore" CPG support (1000A°, 18 umol/g deoxycytidine). Cycle-2 (ABI User Bulletin No. 6, Revised 7/1/86) and Cycle-1 (ABI User Bulletin No. 1, 11/1/85), were used for "2-cyanoethyl" and "methyl" syntheses, respectively. Deprotections were performed manually: 1) Thiophenoxide solution (0.5 h, 25°C, methyl syntheses only) followed by conc NH_3 (aq) (53°C, 10-16h).
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