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SOLID-SUPPORTED SYNTHESIS, DEPROTECTION AND ENZYMATIC PURIFICATION OF OLIGODEOXYRIBONUCLEOTIDES

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A simple solid-phase scheme for deprotection and enzymatic purification of oligodeoxyribonucleotides synthesized by a phosphoramidite method using hydrazine-sensitive protection is presented.

The synthesis of DNA by automated instruments has become an integral technology in the molecular biology laboratory. However, the purification of synthetic oligomers remains a "hands-on" time-consuming procedure. Here we report our investigations of a simple solid-supported deprotection and purification scheme for oligomers produced by a modified phosphoramidite synthetic method. Since the process described is conducted while the crude product remains bound to the same support used for its synthesis, it should prove possible to fully automate this method on existing synthesis machines. Like HPLC purification of 5'-dimethoxytrityl oligonucleotides, the purification method described takes advantage of the chemical difference between the desired product and truncated failure sequences.

Subsequent to synthesis and deprotection, purification is achieved by solid-supported enzymatic degradation of failure sequences. Since the exonuclease spleen phosphodiesterase requires a free 5'-hydroxyl for recognition and cleavage of oligonucleotides (1), we reasoned that it should be possible to specifically hydrolyze only failure sequences on the support in the presence of the 5'-protected target sequence. Provided that both the detritylation and capping steps are quantitative during the synthesis, the process should yield pure target product.

In order to conduct a solid-supported enzymatic purification, it is necessary to remove the exocyclic nitrogen and phosphate protecting groups and capping groups from the oligonucleotide without cleavage of the anchor to the support. With a 3'-nucleoside succinate linkage and typical protection and capping strategies, complete solid-supported deprotection is not possible. Therefore an alternate blocking and capping protection scheme was developed that is based on hydrazine-sensitive bonds.

Letsinger et al. (2) demonstrated that a mixture of hydrazine hydrate, pyridine and acetic acid (HPAA) efficiently hydrolyzes the amide of N^6-benzoyldeoxyadenosine and N^4-benzoyldeoxycytidine without concurrent hydrolysis of 3'- and 5'- benzoyl esters. Even after a 50 h exposure no side products of the bases were noted. Our results indicate that

a 3'-nucleoside succinate ester link to control pore glass (CPG) is also completely stable to the hydrazine reagent. Although the amide linkage of N^6-isobutyryldeoxyguanosine is stable in HPAA (2), we have shown that N^6-(N',N'-dibutylformamidine)deoxyguanosine is easily hydrolyzed. 5'-Acetyl esters generated with acetic anhydride are HPAA stable precluding the use of this capping method. However, we have found that levulinic anhydride and 4-dimethylaminopyridine is an efficient capping reagent and consistent with the observations of van Boom and Burgers (3), 5'-levulinyl esters are hydrolyzed rapidly and quantitatively in HPAA.

Either methyl or β-cyanoethyl phosphorous protection can be used since solid-supported oligomers can be deprotected with either thiophenol in triethylamine/dioxane (4) or tributylamine in pyridine (5), respectively, without hydrolysis of the succinate linkage.

Thus, complete solid-phase deprotection was made possible by altering the automated solid-supported phosphoramidite method previously described (6) in only two ways. First, N^6-isobutyryldeoxyguanosine was replaced by N^6-(N',N'-dibutylformamidine)deoxyguanosine. Second, levulinic anhydride was used instead of acetic anhydride in the capping step.

5'-Protection of the target sequence during the spleen phosphodiesterase hydrolysis of failures was initially attempted with the 5'-dimethoxytrityl group that remained on the target after synthesis and solid-supported deprotection. Unfortunately, under the conditions necessary for optimal enzymatic activity (pH 6), the 5'-dimethoxytrityl ether is hydrolyzed. Therefore, we replaced this acid labile blocking group with an acid and hydrazine stable 5'-benzoyl function prior to deprotection.

The post synthesis solid-phase deprotection and purification process employed is outlined in Table I. After detritylation of the crude material, the product is benzoylated with benzoic anhydride, treated with thiophenolate (for methylphosphoramidites) and then HPAA. After washing, the support is mixed with spleen phosphodiesterase in pH 6 phosphate buffer. Subsequently, the purified target oligonucleotide is removed from the support with ammonium hydroxide at room temperature and debenzoylated at 60°C. Finally, the product is dried by evaporation and resuspended in water.

Table I. Deprotection and Enzymatic purification protocol.

1. Detritylate the completed synthesis and wash with CH_2Cl_2.
2. Benzoylate 10 m with 2 M benzoic anhydride in 6.5% 4-dimethylaminopyridine (W/V) in 2,6-lutidine/THF (1:10 V/V), then wash with CH_3CN.
3. Demethylate phosphates for 1 h with thiophenol/triethylamine/dioxane (1:1:2 V/V) and wash with methanol.
4. Deprotect exocyclic nitrogen and 5'-O capping groups for 18 h with 0.5 M hydrazine hydrate in pyridine/glacial acetic acid (4:1 V/V), then wash with methanol and 0.1 M sodium phosphate, pH 6.0.
5. Digest for 18 h with 1 unit (per mg of support) of spleen phosphodiesterase in 0.1 M sodium phosphate pH 6.0, then wash with 0.1 M sodium phosphate, pH 6.0 and water.
6. Remove the fragment from the support with a 2 h treatment with NH_4OH.

7. Transfer and seal the supernatant in a glass vial for a 4 h debenzoylation at 60°C.
8. Dry in a Speed-Vac and resuspend in water.

In order to test the method, sequences were synthesized on a "homemade" automated synthesizer (the Gene-O-Matic) using long chain alkylamine CPG (500A) with a limiting concentration of deoxyguanosine phosphoramidite to purposely introduce truncated failures.

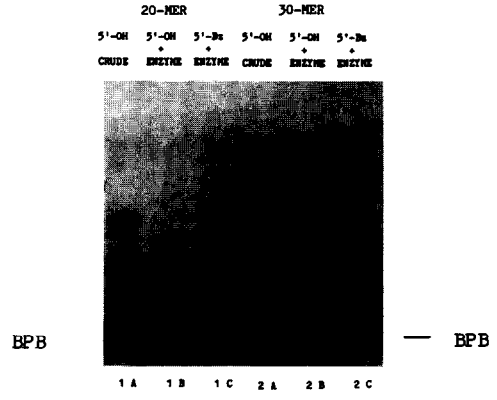

Figure I presents the polyacrylamide gel analysis of crude, fully degraded and purified samples of two different fragments (fragment 1, 5'-AGTTGGCAGTACAGCCTAGC-3'; fragment 2, 5'-TGTCAGTTCCGATGAGCTTTGCTCCAGCAGACC-3'). Visualization was performed by U.V. shadowing. The A lane in each panel shows the crude deprotected material prior to enzymatic treatment. In the B lanes, a control enzymatic hydrolysis was conducted on the crude sample that had not been 5'-benzoylated after detritylation; therefore, the target sequence as well as the failures should be fully degraded. The final enzymatically purified oligomers obtained from the crude 5'-benzoylated material are pictured in lanes C. As can be seen, failure sequences greater than approximately ten nucleotides are thoroughly degraded under the conditions employed. Also, comparison of the B and C lanes suggests that 5'-benzoylation fully protects the product from spleen phosphodiesterase cleavage.

We believe that the inability of the phosphodiesterase to hydrolyze fragments smaller than ten bases is due to hindered access of the enzyme into the pores of the CPG support since neither additional aliquots of enzyme nor longer hydrolysis times decrease their concentration. Although for most applications of synthetic oligomers these very short contaminating fragments would not present a problem, their presence makes determination of the concentration of the target product by U.V. spectral analysis impossible. Investigations of alternate support materials with longer linker arms and increased pore sizes are in progress to improve enzyme access. It may also be possible to remove these short failures using a rapid reverse-phase cartridge procedure (7).

Several alternate synthesis-deprotection schemes in combination with the enzymatic purification are possible including the use of ammonium hydroxide stable linkages of the oligomer to the support (8). This would permit the use of more typical blocking and capping reagents. Alternatively it is possible to employ the enzymatic purification step

on deprotected sequences (save a 5'-hydroxyl protection of the target fragment) in solution (data not shown) but this process is somewhat less attractive due to the difficulties in automation.

In conclusion, the use of N',N'-dibutylformamidine instead of isobutyryl to protect the N^6 of deoxyguanosine and levulinic anhydride as opposed to acetic anhydride for capping permits the complete solid phase deprotection of oligodeoxyribonucleotides synthesized by a phosphoramidite method on a succinate linkage CPG support. This can be achieved due to the use of hydrazine-sensitive protecting functions. After 5'-benzoylation the solid-supported target oligomer can be substantially purified by treatment with spleen phosphodiesterase. Since the new deprotection and purification method involves only reagent addition and washing steps, automation should be easily achieved.

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