Polyamine-assisted rapid and clean cleavage of oligonucleotides from cis-diol bearing universal support

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ABSTRACT

Polyamine-assisted deprotection conditions have been developed for the rapid and clean cleavage of oligonucleotide chains from a cis-diol group bearing universal polymer support, making it compatible with modern oligonucleotide synthesis via all types of phosphoramidite synthons, including base labile protecting group bearing synthons as well. The synthesized oligonucleotides were found to be comparable with the corresponding standard oligomers with respect to their retention time on HPLC, mass on MALDI-TOF and biological activity in PCR amplification.

INTRODUCTION

The last decade has seen tremendous improvements in the existing protocols for the synthesis of oligonucleotides (1,2). Basically, the solid phase methodology for their synthesis involves three steps: functionalization of polymer supports, sequential assembly of nucleotides and deprotection of oligomer chains from the support and simultaneous removal of protecting groups from internucleotide phosphates and exocyclic amino groups of nucleic bases. The last two steps have already been perfected, whereas the first step still requires functionalization of a number of polymer supports. The first nucleoside is attached to a polymer support via a base labile linkage, namely, an oxalyl (3), succinyl (4) or hydroquinone-\(O,\ O\)-diacetyl (5) linker. Therefore, four polymer supports are required for the synthesis of oligodeoxyribonucleotides. The availability of various types of phosphoramidite synthons carrying base labile and conventional protecting groups for nucleic bases (1) and 2'-hydroxyl function in ribonucleosides has further necessitated the need to have a large number of pre-derivatized polymer supports. Some research groups have tried to address this problem by evolving the concept of a universal polymer support. Gough et al. (6,7) were the first to propose a universal support comprised of a linker, 3-anisoyl-2'-O-benzoyluridine-5'-\(O\)-succinyl, attached to controlled pore glass (CPG). In another modified version, they reported the preparation of a universal support by using an adapter, 2'(3')-,O-benzoyluridine-5'-O-cyanoethyl-\(N,\ N\)-diisopropylphosphoramidite, coupled to a standard T-support (8). The functionalized support was subsequently used for the synthesis of oligonucleotides. The time taken for the cleavage of oligomers from these supports was considerably long, making these supports non-compatible with modern oligonucleotide synthesis, particularly with base labile synthons. Moreover, these supports employ nucleosidic material, which does not get incorporated into oligonucleotide chains and hence goes to waste. Since then, some non-nucleoside-based universal supports (9–14) have also been proposed, but the cleavage of oligomers from these is either very time consuming or tedious, which restricts their commercial exploitation for DNA and RNA synthesis employing synthons carrying conventional and base labile protecting groups for nucleic bases.

Recently, Komiyama and Yoshinari (15) have reported the use of diamines for hydrolysis of RNA. Encouraged by this finding, Azhayev (16) has proposed the incorporation of ethylenediamine residue in a cis-diol-based universal support to enhance the elimination of a terminal phosphodiester linkage. He has proposed a set of deprotection conditions for the cleavage of oligonucleotides from the universal support. More recently, Earnshaw and Gait (17) have reported the cleavage of hairpin and hammerhead ribozymes by a polyamine, spermine, in the presence of Mg\(^{2+}\). Since the cleavage of oligonucleotides from a cis-diol bearing universal support resembles base-catalyzed hydrolysis of RNA, we therefore propose to study the cleavage of oligonucleotides from 1,4-anhydroerythritol (cis-diol) bearing universal support using amines and oligoamines in the presence of a metal ion (18). In addition, in the present investigation, we have also carried out a detailed study of the effect of metal ions, namely, Ni\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Li\(^{+}\), and temperature on deprotection of oligonucleotides from the universal support.

MATERIALS AND METHODS

Long-chain alkylamine-controlled pore glass (LCAA-CPG), 4-dimethylaminopyridine and spermine were procured from...
Sigma Chemical Co. (St Louis, MO). Tris (2-aminoethyl) amine and 1,4-anhydroerythritol were obtained from Aldrich Chemical Co. (USA). All other chemicals, reagents and solvents were purchased from local suppliers and purified prior to their use.

Thin-layer chromatography was performed on silica gel 60F-254 plates (Merck, Darmstadt, Germany) and compounds were detected under short-wavelength UV light.

Oligonucleotide synthesis was carried out at 0.2 mol/g scale using two types of nucleoside phosphoramidites, carrying (dA bz, dAc, dGac) and labile monomers, namely, d(A), d(C), d(T), d(G). DMTr-dC-phosphoramidite was employed in the present study. Oligonucleotides, respectively.

Preparation of cis-diol group bearing universal polymer support I

Universal polymer support I was prepared according to the procedure reported previously (12). The loading on the functionalized polymer support was obtained in the range of ~40 μmol/g of support.

Oligonucleotide synthesis

A number of oligonucleotides, namely, d(TTT), d(CTC TCT CTC T), d(TTT TTT TTT TTT TTT), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TGA CT), d(AAT CAA GGT ATG AAG ATC G), d(CCG), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TCT C), d(TTT TTT TT), d(TTT TTT TTT TTT TTT), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TGA CT), d(AAT CAA GGT ATG AAG ATC G), d(CCG), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TCT C), d(TTT TTT TT), d(TTT TTT TTT TTT TTT), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TGA CT), d(AAT CAA GGT ATG AAG ATC G), d(CCG), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TCT C), d(TTT TTT TT), d(TTT TTT TTT TTT TTT), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TGA CT), d(AAT CAA GGT ATG AAG ATC G), d(CCG), d(CAT GAA TGT TGA TA), d(CTG CTA TCA ACA CAC TCT C),

Table 1. Time needed for complete cleavage of oligomers from universal support I under various conditions

<table>
<thead>
<tr>
<th>Oligo-T3</th>
<th>LiCl (0.5 M)</th>
<th>LiCl (1.0 M)</th>
<th>LiCl (1.5 M)</th>
<th>ZnCl2 (0.2 M)</th>
<th>ZnCl2 (0.5 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature = 60°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH4OH</td>
<td>3.0 h</td>
<td>1.0 h</td>
<td>50 min</td>
<td>6.0 h</td>
<td>3.5 h</td>
</tr>
<tr>
<td>NH4OH + MeNH₂ (1:1)</td>
<td>1.0 h</td>
<td>0.5 min</td>
<td>30 min</td>
<td>–</td>
<td>3.5 h</td>
</tr>
<tr>
<td>NH4OH + EDA (1 M)</td>
<td>1.5 h</td>
<td>0.5 min</td>
<td>30 min</td>
<td>–</td>
<td>3.5 h</td>
</tr>
<tr>
<td>NH4OH + Spm (0.5 M)</td>
<td>2.0 h</td>
<td>0.1 h</td>
<td>30 min</td>
<td>–</td>
<td>2.0 h</td>
</tr>
<tr>
<td>NH4OH + Spm (1 M)</td>
<td>1.5 h</td>
<td>0.1 h</td>
<td>20 min</td>
<td>–</td>
<td>1.0 h</td>
</tr>
<tr>
<td>NH4OH + PVA (2.6 μmol)</td>
<td>2.5 h</td>
<td>1.0 h</td>
<td>30 min</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>NH4OH + TAEA (1 M)</td>
<td>2.5 h</td>
<td>1.0 h</td>
<td>40 min</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Temperature = 70°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH4OH + Spm (1 M)</td>
<td>40 min</td>
<td>10 min</td>
<td>10 min</td>
<td>–</td>
<td>1.0 h</td>
</tr>
<tr>
<td>Temperature = 80°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH4OH + Spm (1 M)</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>–</td>
<td>1.0 h</td>
</tr>
</tbody>
</table>

EDA, ethylenediamine; Spm, spermine; PVA, polyvinylamine; TAEA, tris (2-aminoethyl) amine; ND, not detected.

to avoid modification at the stage of deprotection with oligoamines. The corresponding oligonucleotides for comparison purposes were synthesized on the standard polymer supports. The coupling efficiencies in both cases were found to be comparable. In the case of the phosphorothioate oligomer, oxidation was performed with tetraethylthiuram disulfide (TETD) (0.5 M) in acetonitrile instead of iodine solution in the synthesis of unmodified oligomers.

Deprotection and purification of oligonucleotides

Cleavage of oligonucleotide chains from the support and removal of protecting groups from the exocyclic amino groups of the nucleic bases and internucleotidic phosphates were achieved following one of the conditions given in Table 1. In one of the deprotection experiments, the support-bound oligo-T3 was taken in a deprotection vial and treated with aqueous ammonium hydroxide (32%) containing lithium chloride (0.5 M). The suspension was kept at 60°C for 3 h followed by cooling and concentration in a speed vac. The residue was re-dissolved in water (~200 μl), applied on to a reverse-phase silica gel column. The elution was effected with 30% acetonitrile in water and the solution was concentrated in a speed vac. The residue was dissolved in ammonium acetate buffer and subjected to RP-HPLC analysis. The oligomers prepared on standard supports were deprotected using a standard protocol (aqueous ammonium hydroxide, 16 h, 60°C). For oligomers prepared on universal support I with the conventional type of protecting groups, a longer exposure (usually ~8 h) was given.

Effect of metal ion on the cleavage of oligomers from universal polymer support I

Support-bound oligo-T3 was divided into small portions and treated with ammonium hydroxide (32%) containing lithium chloride (0.5 M) at 60°C. The vials were removed at regular time intervals (10–30 min) and after the usual work-up, the oligomers were analyzed on RP-HPLC and further characterized by MALDI-TOF. Similarly, deprotection of oligomers from the support was repeated with lithium chloride (1.0 and 1.5 M), zinc chloride (0.2, 0.5 and 1.0 M), copper chloride (0.5 M), cobalt chloride (0.5 M) and nickel chloride (0.5 M) in aqueous ammonium hydroxide.
Effect of oligoamines on the cleavage of oligomers from support I

As above described, the support-bound oligo-T3 was divided into small portions in deprotection vials and treated with aqueous ammonium hydroxide containing ethylenediamine (1 M) and zinc chloride (0.5 M) at 60°C. Vials were removed at regular time intervals (10–30 min). After the usual work-up, the deprotected oligomers were analyzed on RP-HPLC and characterized by MALDI-TOF. Similarly, deprotection time was obtained with other amines, namely, methylamine, spermine, polyvinylamine and tris (2-aminoethyl) amine, and metal ions, namely, zinc chloride (0.5 M) and lithium chloride (0.5, 1.0 and 1.5 M) in aqueous ammonium hydroxide.

Effect of temperature on the cleavage of oligomers from support I

Support-bound oligo-T3 was divided into small portions and treated with ammonium hydroxide (32%) containing spermine (1 M) and lithium chloride (0.5 M). The vials were kept at three different temperatures (60, 70 and 80°C). After regular intervals (10 min), vials were removed, cooled in an ice-bath and concentrated in vacuo. Then, to record the extent of cleavage, the oligomers were analyzed on RP-HPLC. Similarly, deprotection kinetics were studied with other amines and metal ions in different concentrations. Further characterization was done on MALDI-TOF.

RESULTS AND DISCUSSION

The present investigation has been performed to design a set of rapid deprotection conditions for the cleavage of oligonucleotides from cis-diol group bearing universal polymer supports. Recent studies (15) on the cleavage of RNA have provided enough information either for designing an effective universal polymer support or to develop rapid deprotection conditions for the cleavage of oligonucleotides from the support. Exploiting this information, Azhayev (16) has reported the preparation of a new universal polymer support making ethylenediamine an integral part of the support and discussed the cleavage of oligonucleotides from the universal support under different conditions. However, the multistep preparation of the support and falling yields at each step, makes this support unattractive for routine synthesis of oligonucleotides. But these studies clearly demonstrated the role of ethylenediamine as an effective agent for the cleavage of oligomers from cis-diol universal supports. Encouraged by these studies, including the one reported by Earnshaw and Gait (17) on polyamine-assisted RNA hydrolysis, we have now designed a set of rapid deprotection conditions for the cleavage of oligonucleotides from the universal support, making it truly compatible for oligonucleotide synthesis using all types of phosphoramidite synthons, including the base labile types.

The preparation of universal support I (Scheme 1) starting from 1,4-anhydroerythritol has already been reported (12).

![Scheme 1. Structure of universal polymer support I and synthesis of oligomers.](image1)

**Figure 1.** RP-HPLC profile of crude d(TTT) deprotected with aqueous ammonium hydroxide (32%, 4 h, 60°C). HPLC conditions: column, Lichrosphere RP-18; gradient, 0–25% B in 25 min; buffer A, 0.1 M ammonium acetate and solvent B, acetonitrile; Auf, 0.01. Inset: MALDI-TOF spectrum, m/z: 849.8 (oligo-T3) and 1016.1 (oligo-T3-universal linker).
The loading of universal linker, based on released dimethoxytrityl cation, was found to be in the range of ~40 μmol/g of LCAA-CPG. A number of oligonucleotides, namely, d(TTT), d(TTT TTT T), d(CTC TCT CTC T), d(TTT TTT TTT TTT TTT), d(CAT GAA TGT TGA TA), d(CGT CTA CAC TGA CT), d(AAT CAA GGT ATG AAG ATC G), d(CCG CCC TCC ACT ACT ACT), d(AGG AAC CTC TGC ACG GCC) (forward primer), d(CAG AAA ATC CCT CCC CCC TA) (reverse primer) and d(TpTpTpTpTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTps) were synthesized on the support at 0.2 μmol scale following the manufacturer’s standard protocol (20). The same oligomers were also assembled on the standard polymer supports for comparison purposes. Oligo-T3 was selected as a model sequence for carrying out kinetic studies on deprotection of oligonucleotides from the universal support I, as its modified analog (oligo-T3 with universal linker) could easily be identified on a reverse-phase column (21).

Cleavage of oligo-T3 from universal polymer support I

The support-bound oligo-T3 was first treated with aqueous ammonium hydroxide (4 h, 60°C). The extent of cleavage of oligomer from the universal support was investigated on RP-HPLC and MALDI-TOF (Fig. 1).

Effect of metal ions on cleavage of oligomer from universal support I. It can be deduced from the above study as well as our earlier observations (11,12,14) that aqueous ammonium hydroxide alone is not sufficient for the complete cleavage of oligomers from support I. Therefore, the role of metal ions in conjunction with aqueous ammonium hydroxide was investigated. For this purpose, the support-bound oligo-T3 was divided into vials and treated with aqueous ammonium hydroxide containing zinc chloride (0.2 M) at 60°C. The vials were removed at definite time intervals and after the usual post-deprotection work-up, the deprotected oligomers were analyzed on RP-HPLC. The optimum time was calculated after analysing these aliquots. Since it was observed that zinc ions facilitated the release of oligomer chains from the universal support, we therefore decided to use higher concentrations of zinc ions (0.5, 1.0 and 1.5 M). Up to 0.5 M zinc chloride the reaction proceeded very well, but at higher concentrations (1.0 and 1.5 M) the formation of a viscous semi-solid during post-deprotection work-up hampered the analysis of cleaved oligomers. In addition, the role of other transition metal ions, namely, Cu²⁺ (0.5 M), Ni²⁺ (0.5 M) and Co²⁺ (0.5 M) (Fig. 2A), was investigated, but no significant difference in deprotection time was noticed. Therefore, the studies with higher concentrations of these metal ions were not pursued.

Figure 2. Bar diagrams showing (A) effect of metal ions on cleavage of oligomers from support I using aqueous ammonium hydroxide at 60°C; (B) effect of amines on cleavage of oligomers from support I using aqueous ammonium hydroxide containing metal ions at 60°C; (C) effect of temperature on cleavage of oligomers from support I using aqueous ammonium hydroxide containing spermine (1.0 M) in the presence of metal ions. Time (min) represents complete deprotection time.

Also, as mentioned in earlier reports, the effect of lithium ions was re-investigated and we found that it was more effective than the transition metal ions. Therefore, the deprotection studies were carried out at three different concentrations (0.5, 1.0 and 1.5 M) and the optimum time, in each case, was calculated as discussed above.
Effect of amines on the cleavage of oligomers from support I. In order to investigate the role of amines, similar experiments, as described above, were carried out using amines, namely, methylamine (1:1, v/v, with aqueous NH$_4$OH), spermine, ethylenediamine, tris (2-aminoethyl) amine and polyvinylamine (Fig. 2B), at different concentrations. The optimum time, in each case, was calculated by kinetic studies. The minimum time required for complete cleavage of the oligomer chain from the universal support was obtained with aqueous ammonium hydroxide containing spermine (1.0 M) and lithium chloride (1.5 M) at 60°C for 20 min. Deprotection kinetics could not be pursued with polyvinylamine and tris (2-aminoethyl) amine in conjunction with zinc chloride (0.5 M), as these resulted in the formation of semi-solid complex materials during post-deprotection work-up. The results are summarized in Table 1.

Effect of temperature on the cleavage of oligomers from support I. Similarly, in an attempt to study the effect of temperature, the kinetics were carried out at three different temperatures (60, 70 and 80°C) (Fig. 2C). Since the minimum time required at 60°C for complete deprotection of oligomers from the support was obtained with a spermine and lithium chloride mixture, as discussed above, the same composition was kept at 70 and 80°C. The complete cleavage of oligomers from the universal support occurred in 10 min in both cases, using spermine (1.0 M) and lithium chloride (1.0 M) at 70°C and spermine (1.0 M) and lithium chloride (0.5 M) at 80°C. However, the deprotection kinetics could not be performed below a 10 min time interval, as spermine takes a few minutes for complete dissolution.

The deprotected oligomers were analyzed on RP-HPLC and further characterized by MALDI-TOF and compared with the corresponding oligomers with respect to their retention time. Figure 3A shows the elution profile of d(CAT GAA TGT TGA TA) synthesized on support I and deprotected with aqueous ammonium hydroxide containing spermine (1 M) and lithium chloride (1.5 M) at 65°C for 1 h, whereas its co-injection with standard oligomer is shown in Figure 3B. The single peak obtained on RP-HPLC confirms the complete cleavage of universal linker from the oligomer. The inset of the figure shows the MALDI-TOF spectrum of the cleaved oligomer from the universal support (expected 4301.8, found 4302.9).

Figure 3. RP-HPLC profiles of d(CAT GAA TGT TGA TA) (A) treatment with aqueous NH$_4$OH containing spermine (1 M) and lithium chloride (1.5 M) at 65°C for 1 h, and (B) co-injection with d(CAT GAA TGT TGA TA) synthesized on standard dA-support and deprotected under standard conditions. HPLC conditions: column, Lichrosphere RP-18; gradient, 0–50% B in 25 min; buffer A, 0.1 M ammonium acetate and solvent B, acetonitrile; Auf, 0.01. Inset: MALDI-TOF spectrum, m/z: expected 4301.8, found 4302.9.

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Figure 4. RP-HPLC profile of d(AAT CAA GGT ATG AAG ATC G) treated with aqueous NH$_4$OH containing ethylenediamine (1 M) and lithium chloride (1 M) at 65°C for 1 h. HPLC conditions: as given in Figure 3. Inset: MALDI-TOF spectrum, $m/z$: expected 5884.8, found 5883.1.

Figure 5. RP-HPLC profiles of d(TTT) (A) treatment with aqueous NH$_4$OH containing spermine (1 M) and lithium chloride (0.5 M) at 80°C for 10 min, and (B) co-injection with standard d(TTT) synthesized on standard dT-support. HPLC conditions: as given in Figure 1. Inset: MALDI-TOF spectrum, $m/z$: expected 850.6, found 849.5.
Furthermore, the presence of free 3’-hydroxyl group in deprotected oligomers was established by performing a PCR using d(AGG AAC CTC TGC ACG GCC) (forward primer) and d(CAG AAA ATC CCT CCC CCC TA) (reverse primer), respectively. The amplified products were used for the detection of an intron 2 conversion polymorphism in the CyP11B2 gene. The results are shown in Figure 6.

**Effect of amines/oligoamines on nucleic bases during deprotection**

Cleavage of oligonucleotides from cis-diol-based universal supports and simultaneous removal of protecting groups from nucleic bases and internucleotidic phosphates is very fast under the proposed amine/oligoamine-assisted deprotection. Then, it became essential to ascertain that no modification of nucleic bases had taken place under these conditions. It has already been reported (22–25) that thymine, guanosine and adenosine do not undergo any modification under amine-assisted deprotection conditions. N-protected 2’-deoxyctydine was found to be the most vulnerable to modification under these conditions and the extent of modification depends on the type of protection employed for exocyclic amino function of 2’-deoxyctydine. It has been proposed (22,24) that this problem can be circumvented by employing base-labile protecting groups. N-Isobutyl (ibu), N-isoproxyacetyl (ipa), N-tert-butylphenoxyacetyl (t-bpa) and N-acetyl (ac), in increasing order, increase the electropositive character of carbonyl carbon which facilitates the nucleophilic attack at this site compared to C-4 of the pyrimidine ring. In the case of such protecting groups, the formation of the alkyl derivatives during the deprotection with amines/oligoamines can be suppressed or substantially decreased. The N-benzoyl-dC was found to be the most vulnerable and N-acetyl-dC the least (in fact no modification of dC was noticed under the proposed deprotection conditions). In order to further establish the above facts, HPLC and MALDI techniques were used to analyze the deprotected oligonucleotides. Oligomer d(CAT GAA TGT TGA TA) was deprotected in aqueous ammonium hydroxide containing spermine (1 M) and lithium chloride (1.5 M). In order to demonstrate the utility of the proposed deprotection conditions in modified oligonucleotides, an oligomer d(TpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsTpsT) synthesized on support 1 and deprotected with aqueous NH₄OH containing ethylenediamine (1 M) and lithium chloride (1.5 M) at 60°C for 30 min. HPLC conditions: column, Lichrosphere RP-8; gradient, 0–25% B in 25 min; buffer A, 0.1 M ammonium acetate and solvent B, acetonitrile; Auf, 0.01.

The elution profile is shown in Figure 7.

**CONCLUSION**

A set of new conditions have been reported for the first time, which makes the cis-diol group bearing universal support I fully compatible with modern oligonucleotide synthesis, using a wide variety of phosphoramidites synthons, including base labile ones. The use of spermine (1.0 M) in conjunction with Li⁺ ions (0.5 M) in aqueous ammonia (32%) at 80°C cleaves the oligomers from the universal support in just 10 min, making the universal support fully compatible with oligonucleotide synthesis using base labile synthons. The use of polyamines in the deprotection step, however, requires the use of a non-conventional cytidine derivative, but this does not impose any limitation, as acetylcytidine phosphoramidite is commercially available and used in routine oligomer synthesis.
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