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Deprotection of methylphosphonate oligonucleotides using a novel one-pot procedure

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ABSTRACT

Deprotection of methylphosphonate oligonucleotides with ethylenediamine was evaluated in a model system. Methylphosphonate sequences of the form 5'-TTTNNTTT, where N was either N^4-bz-dC, N^4-ibu-dC, N^2-ibu-O^6-DPC-dG, N^2-ibu-dG, N^6-bz-dA, or T, were used to determine the extent of modifications that occur during deprotection. Up to 15% of N^4-bz-dC was found to transaminate at the C^4 position when treated with ethylenediamine. A similar displacement reaction with ethylenediamine was observed at the O^6 position of N^2-ibu-O^6-DPC-dG, and to a much lesser extent of N^2-ibu-dG. Side reactions were not observed when oligonucleotides containing N^4-ibu-dC, N^6-bz-dA, or T were treated with ethylenediamine. A novel method of deprotecting methylphosphonate oligonucleotides was developed from these studies. The method incorporates a brief treatment with dilute ammonia for 30 minutes followed by addition of ethylenediamine for 6 hours at room temperature to complete deprotection in a one-pot format. The solution is then diluted and neutralized to stop the reaction and prepare the crude product for chromatographic purification. This method was used to successfully deprotect a series of oligonucleotides at the 1, 100, and 150 μmole scales. These deprotection results were compared to a commonly used two-step method and found to be superior in yield of product by as much as 250%.

INTRODUCTION

Oligonucleotides that contain modified phosphate backbones have shown great potential as therapeutic agents.[1–3] Neutral methylphosphonate oligonucleotides satisfy the necessary criteria of nuclease resistance and cell membrane permeability.[4,5] Methylphosphonate oligonucleotides have been used to successfully inhibit cellular activity in several systems [6–10] and to exhibit single base mismatch discrimination in at least one system.[11]

Although the methylphosphonamidite synthesis reagents are commercially available, the preparation of methylphosphonate oligonucleotides is more difficult than the preparation of phosphodiester oligonucleotides. The published procedures for deprotecting methylphosphonate oligonucleotides require a considerable amount of experience.

Concentrated ammmonium hydroxide traditionally used to deprotect phosphodiester oligonucleotides rapidly degrades the methylphosphonate backbone. To avoid this, the more gentle deprotecting reagent, ethylenediamine (EDA) 1/1 in 95% ethanol (EtOH), has been employed.[12,13]. One draw-back to this strategy is that EDA readily transaminates the N^4 amine of the commonly used N^4 benzoylated deoxycytidine (N^4-bz-dC)[13]. This side reaction was reduced by pre-treatment of the oligonucleotides with either an overnight reaction with hydrazine hydrate[14,15] or by treatment with concentrated ammonium hydroxide for two hours at room temperature[9,16]. These pre-treatments removed most of the N^4 benzoyl protecting group prior to treatment with EDA. Following the pre-treatment, deprotection of the oligonucleotide is then completed by treatment with a 1/1 mixture of EDA and 95% EtOH at room temperature for four to six hours. In our hands, neither pre-treatment method was found to be completely satisfactory: the hydrazine hydrate method yielded a variable amount of transaminated side-products; and the method using ammonium hydroxide gave us low yields upon scale-up. Furthermore, the half-life of the cleavage of the benzoyl group from dC with ammonium hydroxide at room temperature has previously been shown to be two hours[17], which suggests that some bz-dC will still be left to transaminate.

We have investigated a number of deprotection schemes with the goal of simplifying the production of methylphosphonate oligonucleotides while minimizing side-products. Toward that end, we evaluated the replacement of N^4-bz-dC with the more base-labile isobutyryl protected dC (N^4-ibu-dC)[17]. We also investigated the degradation of methlyphosphosphonate backbones by concentrated ammonium hydroxide. Currently there is widespread use of 2 hour room temperature pre-treatments with concentrated ammonium hydroxide as described above, but no reports of the effects of such a mild treatment. We were concerned that our low yields were caused by a level of degradation that was greater than previously thought.[18]

This report describes a simple, one-pot procedure that utilizes a short pre-incubation with a mild ammonia solution, followed by treatment with EDA. The reaction is terminated by dilution and neutralization of the sample, yielding a solution that can be directly applied to reverse-phase HPLC columns for purification.

RESULTS

Backbone cleavage

As mentioned above, lability of the methylphosphonate backbone to concentrated NH_4OH was the primary reason for the switch to EDA as the main deprotecting reagent for these compounds. Because of the low yields and low quality crude mixtures we

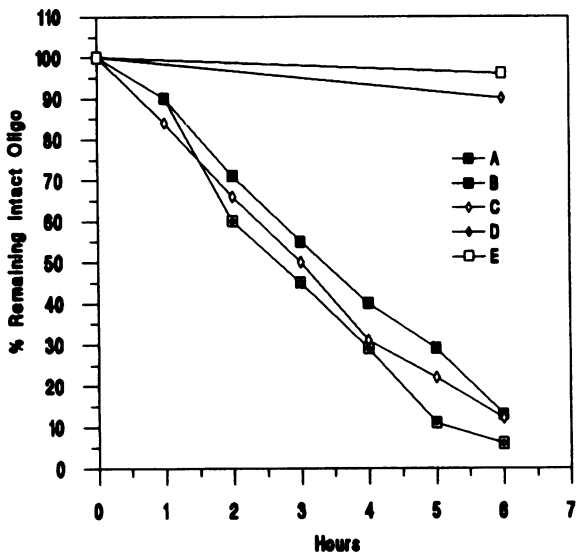

Figure 1. Rate of backbone digestion of methylphosphonate oligonucleotides by EDA and conc. NH_4OH at room temperature. A—Digestion of GpTC-TTC-CTG-CCC-CAT-TGC with conc. NH_4OH at RT; B—digestion of $(AG)_8$ as in A; C—digestion of $(CT)_8$ as in A; D—digestion of GpTC-TTC-CTG-CCC-CAT-TGC with EDA/ACN/EtOH/H_2O (50:23.75:23.7:2.5) at RT; E—digestion of CAT-TGC as in D. (Only analyzed 6 hour time points for EDA rxns.) Analyses performed on RP-HPLC using Whatman RAC II analytical column, 0–30% ACN over 30 minutes in 0.05 M TEAA, pH 7, flow at 1 ml/min. Underlined nucleosides have methylphosphonate linkages.

had obtained using the method utilizing a two hour incubation with conc. NH_4OH followed by EDA [9,16], we investigated the rate of backbone degradation by conc. NH_4OH at room temperature. We included digestions by EDA/acetonitrile (ACN)/EtOH/H_2O (50:23.75:23.75:2.5) at room temperature for comparison. The room temperature digests were performed on several oligonucleotides and followed by reverse-phase HPLC. The results (Figure 1) indicated that the average rate of product loss using conc. NH_4OH was about 15% per hour. Thus, a two hour incubation in conc. NH_4OH at room temperature, which is common practice, results in 30% loss of product. The loss of product due to degradation by EDA was only approximately 1% per hour

Model study of base modifications

Transamination of dC by EDA is greatly enhanced by the presence of a benzoyl protecting group.[13] The need to remove this group prior to treatment with EDA led to the two-step deprotection schemes described above. [9,12–16] In order to reduce the amount of transamination that occurs we replaced the benzoyl protected dC (bz-dC) with the more labile isobutyryl protected dC (ibu-dC). Model methylphosphonate oligonucleotides with the sequence dTTTCCTTT were synthesized with either bz-dC or ibu-dC and deprotected with an EDA reagent mixture consisting of EDA/ACN/EtOH/H_2O (50:23.75:23.75:2.5) for 6 hours at room temperature. Figure 2 shows chromatograms on a polar-stationary phase column of the resulting product mixtures. It can be seen (Figure 2a) that the oligonucleotide prepared with bz-dC had additional side-products which were more polar in nature based on their HPLC mobilities and which amounted to about 36% of the integrated area. The oligomer prepared with ibu-dC (Figure 2b) was cleanly deprotected with the EDA reagent mixture.

The model sequence dTTTGGTTT, synthesized with N^2-ibu-dG, was prepared and subjected to the same EDA reagent treament described above. This oligonucleotide was also modified (~20%) to a more polar product. We subsequently found that a short, 10 minute treatment with conc. ammonium hydroxide prior to treatment with EDA was sufficient to revert the modified guanosine back to natural state and yield clean product. (Data not shown.)

The oligonucleotides dTTTAATTT, synthesized with benzoyl protected adenosine, and dTTTTTTTT were also included in the study and were both found to be cleanly deprotected with the EDA reagent mixture.

One pot procedure for the deprotection of methylphosphonate oligonucleotides

Based on the model studies described above, our approach to deprotection was to pre-incubate the support-bound methylphosphonate oligonucleotide with a mildly basic solution to revert the dG modifications mentioned above, to which the EDA could be added directly to complete deprotection. Initially, the ACN/EtOH/water (47.5:47.5:5) co-solvent used for the EDA reactions described above was saturated with ammonia in an ice bath. More recently, we have found that a solution consisting of ACN/EtOH/conc. NH_4OH (45:45:10) to be just as effective and much easier to prepare. Data using both reagents are presented here, including a direct comparison to demonstrate equivalency.

Deprotections were carried out by treating the support-bound oligonucleotide with the mild ammonia-bearing co-solvent for one half hour at room temperature. An equal volume of EDA was then added to the reactions and mixed for 6 additional hours.

The suggested work-up from this point included taking the EDA reaction mixture to dryness[12–14]. This had the undesirable effect of concentrating the EDA with the oligonucleotide which had the potential for cleaving the backbone. We found that stable solutions can be achieved by decanting the deprotection solution from the support, diluting the solution to 10% organic content with water, and neutralizing the solution with either acetic acid or HCl. This neutralized solution is then ready for purification by reverse-phase HPLC. We found that methylphosphonate oligonucleotides are stable in this solution for at least two weeks at room temperature. Another concern was solubility. Some concentrated methylphosphonate oligonucleotides are not fully soluble in 10% organic, neutral solutions. In cases when cloudiness or precipitation was experienced, the percent of organic in the solution was increased with acetonitrile, or the sample further diluted with 10% acetonitrile/water. Though the described procedure worked well for a vast majority of compounds prepared in this manner, occasionally particularly insoluble oligonucleotides were encountered.

Deoxycytidine and deoxyguanosine model studies

The conditions described above using the 8% ammonia solution were initially tried on the dTTTCCTTT and dTTTGGTTT model systems prepared with ibu-dC and ibu-dG, respectively. The results compare favorably to those obtained with the commonly used two-step procedure using conc. NH_4OH for two hours followed by EDA (hereafter referred to as the two step method), yielding clean material with no polar modifications (data not shown). The experiments were repeated using the solution consisting of ACN/EtOH/conc. NH_4OH (45:45:10) with the same results.

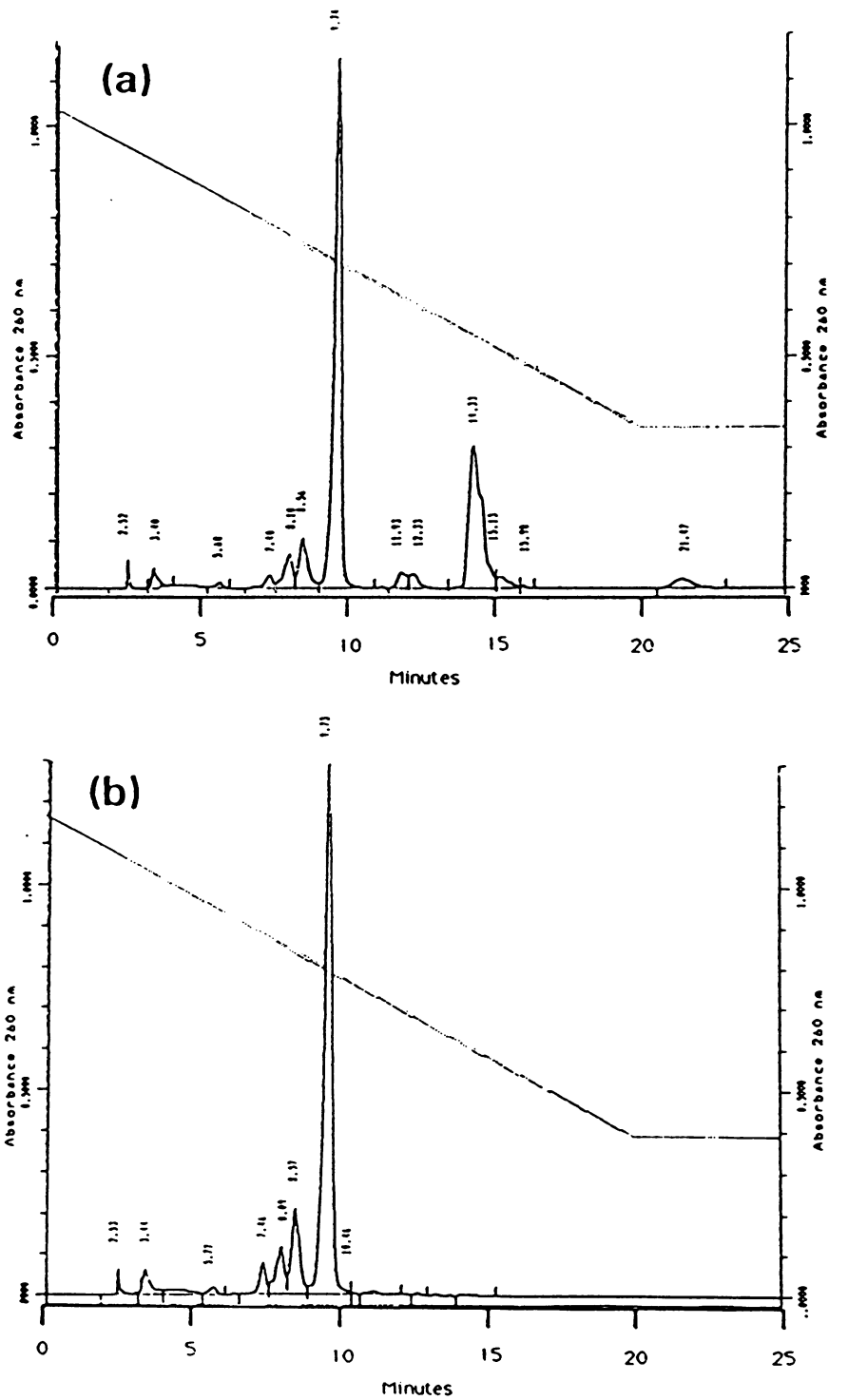

Figure 2. Polar-stationary phase analyses of deprotections of model sequence dTTTCCTTT. a) N^4-bz protected dC, deprotected with EDA reagent mixture. b) N^4-ibu protected dC, deprotected with EDA reagent mixture. Analyses were performed on a PolyLC polyhydroxylethyl A column. Buffer A—0.05 M TEAA, pH 7.0–7.2, buffer B—acetonitrile. Gradient 70–30% B over 20 min. Flow—1 ml/min. Observed at 260 nm.

Deprotection of oligonucleotides using the improved procedure

A series of methylphosphonate oligonucleotides (**1-5**) were synthesized on a 15 μmole scale (Table 1). An aliquot of each (1 μmole) was subjected to either the two step method, or the new one-pot procedure described above. The oligonucleotides had all-methylphosphonate backbones, were 15 to 18 bases in length, and contained mixed sequences. The crude product mixures were analyzed by reverse-phase HPLC (Whatman RAC II ODS-3 column) and the integrated peak area was used to estimate the relative purity of each mixture. Table 2 lists the product yields obtained based on these HPLC analyses. Figure 3

shows reverse-phase HPLC chromatograms of oligonucleotide (**3**) by both the two step method and the one-pot procedure. The improved procedure yielded more product in every case, and double the product in several cases. This increased yield was probably because of the improved solubility of the oligos in the EDA deprotection mixture as compared to concentrated ammonium hydroxide.

To further demonstrate the effectiveness of our improved procedure, deprotections were attempted on a larger scale. Two all-methylphosphonate oligonucleotides (**6**) and (**7**) (Table 1), 15 bases in length and of mixed base composition, were prepared on 300 μmole and 200 μmole scales, respectively. The syntheses were equally divided and deprotected by the two step and one-pot procedures described above. The reagents were proportionately scaled up to afford concentrations of 10 theoretical milligrams of product per ml of final deprotection solution. The results are shown in table 3. (In order to increase yield even further we have tried to use more deprotection reagent to a final concentration of theoretical 0.1 milligrams/ml with no appreciable increase in yield. Data not shown.)

Table 1. Oligonucleotide Sequences.

Oligonucleotide	Sequence
1	5′-TCT-CAG-GGC-AAT-GTT-TTT
2	5′-CCT-CCG-GCA-CAG-ACA-AGG
3	5′-ATT-GGT-CAA-ACT-CAG-GCA
4	5′-CAC-ACA-CAT-GAA-CCA-CAC
5	5′-CCA-CGA-AAG-GCA-TGA-CCG
6	5′-TTC-TGC-CAT-GGC-TGC
7	5′-GTC-AGC-CAT-CTT-GCG

The oligonucleotides contained all methylphosphonate linkages, as indicated by the underlining.

After the 6 hour treatment with the one-pot EDA reagent mixture, samples were diluted to a final concentration of 10% organic (includes the deprotecting reagents and any subsequent washes with acetonitrile/water) and neutralized with HCl (6 N in 10% acetonitrile/water). We found that the diluted, neutralized solution can be loaded directly onto a high capacity column such as the Hamilton PRP-1 polymeric reversed -phase column using a small Eldex B-100 pump[19]. After the compound is loaded onto the column it can then be reattached to the HPLC pumps and the compound chromatographed as normal. This greatly simplifies the final purification step. An in-line filter unit is also used to prevent particulate material from clogging the column frit.

The portion of the oligonucleotides deprotected by the two-step procedure was dried down after EDA treatment using a rotory evaporator followed by several co-evaporations with ethanol. The oligonucleotides were then reconstituted to 0.5 mg/ml in 10% ACN/water in preparation for HPLC purification.

Products from the large scale deprotections were isolated using reverse-phase HPLC. The column used was a preparative PRP-1 column from Hamilton and the loading was done using the method involving the Eldex B-100 pump. Purified yields for the improved procedure were higher in both cases, and more than double with oligonucleotide (**7**) (Table 3). Comparisons of the isolated products showed that the product peaks were identical by reverse-phase and polar stationary phase chromatography.

The atomic mass of (**6**) deprotected by either the two-step method or the one-pot procedure was found to be 4506.6 and 4507.1 atomic mass units (AU), respectively, by electrospray mass spectrometry positive ion mode. (The theoretical mass for

Table 2. Results of comparisons of two-step method and one-pot deprotection yields for oligonucleotides (1) through (5); small (1 μmole) scale.

Oligo	Deprotection	Crude Yield (OD Units A_{260})	% Int. Area of Prod. (RP-HPLC)	Prod. Yield, Calculated (OD Units A_{260})
1	Two-Step	62.1	35.0	21.7
	One-Pot	76.8	52.8	40.6 (187%)
2	Two-Step	44.7	48.8	21.8
	One-Pot	99.3	55.5	55.1 (253%)
3	Two-Step	62.1	36.2	22.5
	One-Pot	120.6	46.2	55.7 (248%)
4	Two-Step	57.0	31.2	17.8
	One-Pot	99.6	39.5	39.3 (221%)
5	Two-Step	72.3	46.7	33.8
	One-Pot	103.8	51.1	53.0 (157%)

Two-step deprotection refers to the use of conc. ammonium hydroxide for two hours at room temperature, decanting from the beads, drying the solution, then treating with EDA/ACN/EtOH/H_2O (50/23.75/23.75/2.5) for 6 hours at room temperature. The last column (product yield) contains in parentheses the percent increase in yield of the one-pot method over the two-step method.

Table 3. Results of comparisons of two-step method and one-pot deprotection yields for oligonucleotides (6) and (7); large scale.

Oligo	Deprotection	Scale (μmole)	Crude OD Units A_{260}	% Int. Area	OD Units A_{260} Prod. Isolated
6	Two-Step	150	9195	47.5	2720
	One-Pot	150	11732	55.4	3519 (129%)
7	Two-Step	100	2800	64.0	885
	One-Pot	100	7331	69.8	2996 (226%)

The last column (product yield) contains in parentheses the percent increase in yield of the one-pot method over the two-step method.

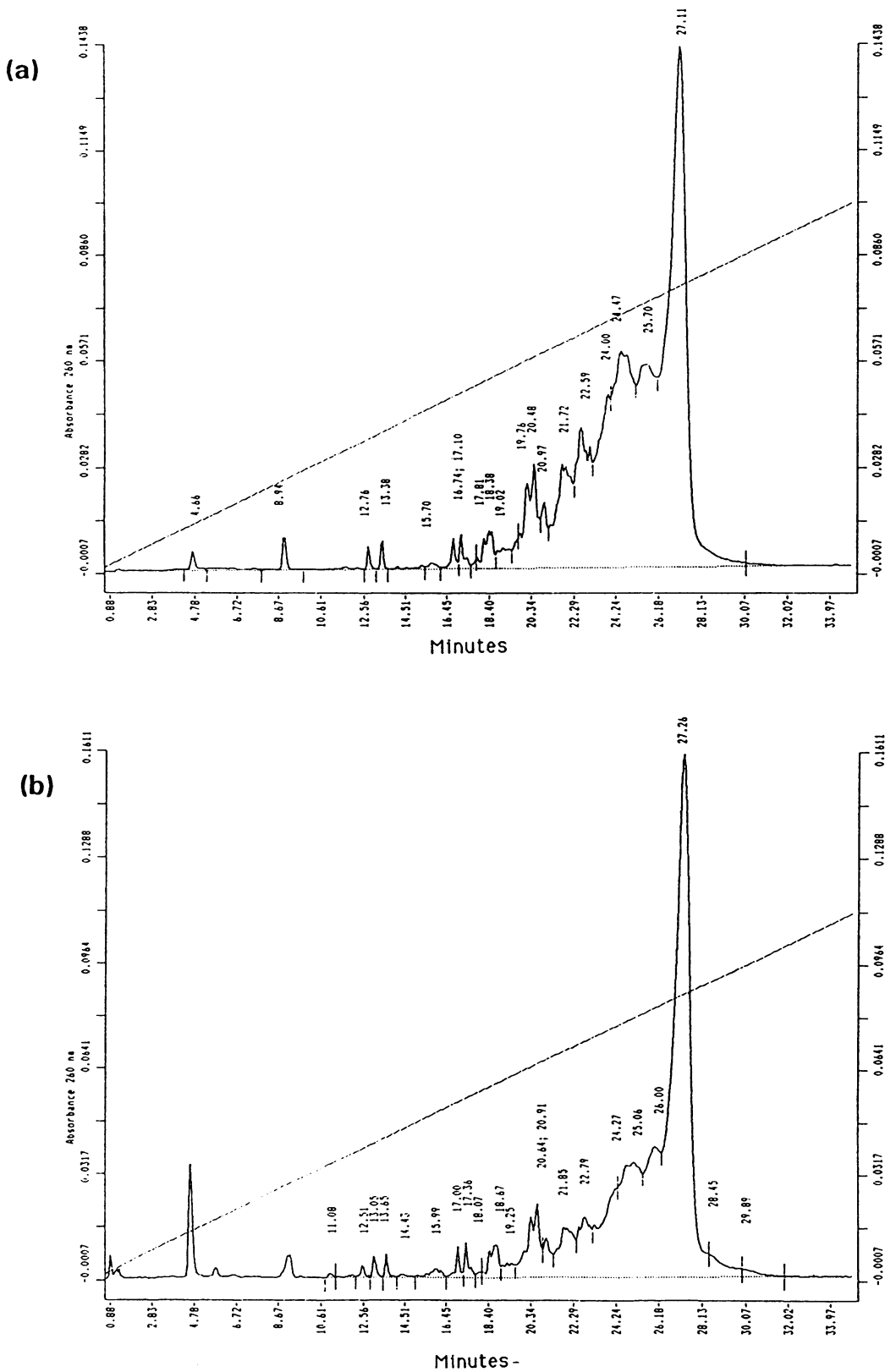

Figure 3. Representative reverse-phase analyses of the comparative 1 μmole deprotections of oligonucleotide (3) by the two-step (method b) and one-pot methods. a) Oligo (**3**) deprotected by *method b*. Integrated area of product—36.2%. b) Oligo (**3**) deprotected by the one-pot method. Integrated area of product—46.2%. Analyses were performed on a Whatman RAC II C-18 analytical column. Buffer A—0.05 M TEA, pH 7.0–7.2; buffer B—50% acetonitrile in A. Gradient—0–60% B over 30 min. Flow—1.5 ml/min. Observed at 260 nm.

(**6**) is 4507.4 AU.) The mass of oligonucleotide (**7**) deprotected by the two-step method was found to be 4516.0 AU, compared to the one-pot value of 4515.4 AU. (Theoretical mass for (**7**) is 4516.4 AU).

DISCUSSION

The clean deprotection of ibu-dC with EDA without significant transamination and the ease of reversion of dG adducts to native dG using a very dilute ammonium solution that does not cause significant backbone degradation enabled the development of the improved deprotection scheme. We were quite pleased that such a minor change in base protection allowed us to make a major improvement on the deprotection methodology. In the course of this work we also investigated the use of a dG base that was protected at the O-6 position, N^2-isobutyryl-O^6-diphenylcarbamoyl-2′-deoxyguanosine (N^2-ibu-O^6-DPC-dG) in the hope that it would block any modifications that occur during synthesis. On the contrary, the base was modified to about twice that of ibu-dG (data not shown). We did not attempt other protecting groups such as dimethylformamidine or phenoxyacetyl in light of the success we were acheiving with the singly protected dG.

The rapid degradation by conc. NH_4OH at room temperature was very surprising. The literature leads one to believe that a 2 hour treatment with conc. NH_4OH should not cause undue degradation.[18] However, our finding of 30% product degradation in two hours makes use of conc. NH_4OH prohibitive even for those short periods, if product yield is of any importance. Solutions of NH_4OH in pyridine were previously reported, but are inefficient.[15]

Polar-stationary phase HPLC analysis of the model oligonucleotides was a useful tool in following the effects of deprotection chemistry. The column used has an aspartamide phase bonded to silica gel. The transaminated side-products are more polar than the desired product and elute with longer retention times when a decreasing gradient of acetonitrile in aqueous 0.05 M triethylammonium acetate (TEAA) buffer was used. We found that the one-pot deprotection scheme gave crude products that were essentially free of these polar side-products using the model systems.

The deprotection of oligonucleotides in side by side comparisons demonstrated the advantages of the improved deprotection method. The comparative small scale deprotections (oligonucleotides **1-5**) originated from single 15 μmole syntheses from which aliquots of support were removed for each study to avoid variables due to different syntheses. In each case, the one-pot procedure yielded a larger amount of crude material with greater purity based on HPLC profiles than the two-step method. The amount of crude product obtained with the one-pot procedure was significantly better than the amounts obtained using the two-step method in every instance because of the increased solubility of the oligonucleotides in the EDA mixture compared to their solubility in concentrated ammonium hydroxide The percentage of desired product in the crude mixtures of oligonucleotides deprotected with the one-pot procedure were also higher than with the two step method as evidenced by HPLC because of the milder, less basic reagent mixture used.

On a 100 μmole scale (a single 200 μmole scale synthesis divided into two aliquots), 885 OD Units of oligonucleotide (**7**) was purified from a mixture deprotected using the two step method, whereas 2996 OD Units of similar quality product was obtained from the portion deprotected by the one-pot method. With the 150 μmole scale (aliquoted from a single 300 μmole scale synthesis), (**6**), the numbers were somewhat closer, but the one-pot method still yielded a significant amount more of the oligonucleotide; 3519 OD Units, compared to the 2720 OD Units obtained with the two step method. As stated earlier, the large scale deprotections were aliquoted from single 200 and 300 μmole scale syntheses to ensure comparability.

The isolated products from the two types of deprotection of both (**6**) and (**7**) were found to be indistinguishable by HPLC and mass-spectrometry. In all cases, the purified products did not contain the more polar late running peaks that we have observed using the polar-stationary phase HPLC chromatography system. These peaks are associated with side-products.

The use of an ACN/EtOH/conc. NH_4OH (45:45:10) mixture as the pre-incubation reagent prior to the addition of the EDA has proven to be as efficient as the 8% ammonia in ACN/EtOH/water (47.5:47.5:5) solution. We are now evaluating the former reagent in large scale deprotections. This solution is recommended because of the simplicity of preparation as well as the lower concentration of base.

The deprotected oligomer may be recovered from the EDA reaction mixture by evaporation of the organic reagents and solvents under vacuum. This is time consuming, however, and can lead to variable amounts of backbone hydrolysis. We prefer simple dilution and neutralization as described above. This takes only 5 minutes and leaves the sample ready for HPLC purification.

The oligonucleotides were purified using a Hamilton Prep PRP-1 preparative reverse-phase column and an HPLC system with preparative pump heads. A linear gradient of acetonitrile in 0.05 M TEAA, 20−60% over 40 minutes was used to elute the product. This method gave products of sufficient purity for most applications. However, we have observed that reverse-phase purifications are not always capable of resolving the full length product from the failure sequences. The use of the polar-stationary phase column as an analytical tool has been helpful in providing a cross-check of purity. Unfortunately, attempts to use this column in a preparative mode have failed because of the inherent insolubilty of many methylphosphonate oligonucleotides in the solvent system (65% acetonitrile) required for efficient loading onto this column.

The purified oligonucleotides were analyzed by HPLC and mass-spectrometry. Unfortunately, enzymatic methods for determining base sequence and/or base composition are not applicable to methylphosphonate oligonucleotides because they are not recognized as substrates. This, in fact, is one of their advantages as therapeutic agents. Hybridization analyses of methylphosphonate oligonucleotides are very sensitive to internal mismatches and to a lesser extent terminal mismatches, but we are not certain at this time of the effect of minor modifications. Miller *et al.* [13] have described a method whereby the purine and pyrimidine bases can be distinguished in sequence, but the results have been difficult to reproduce. Electron spray ion mass-spectrometry, however, has proven to be an excellent tool for the analyses of methylphosphonate oligonucleotides.

In summary, we have used model oligonucleotides to investigate deprotection using EDA. Through this model system we have been able to develop a convenient one-pot deprotection protocol. This method substantially increases the yield of material and minimizes the extent of base modifications.

METHODS AND MATERIALS

Materials

All of the methylphosphonamidite synthesis monomers were obtained from JBL Scientific, Inc. (San Luis Obispo, CA). The synthesis support containing N^4-isobutyryl deoxycytidine and the two large scale oligonucleotides, (**6**) and (**7**), still attached to the synthesis support were likewise obtained from JBL.

The other support bound nucleosides were obtained from Milligen/Biosearch (Novato, CA). The activating reagent (0.45 M tetrazole in acetonitrile) was obtained from Glen Research Corporation (Sterling, VA).

All solvents were obtained from Fisher Scientific, and all solid reagents from Aldrich Chemical Co., Inc.

General techniques/instruments

Absorbance readings were obtained in 1/1 acetonitrile/water solutions on a Miltron Roy Array 3000 diode-array spectrophotometer. The absorbance at 260 nm was converted to OD Units (A_{260}×volume of sample in ml)).

Analytical HPLC was done on Beckman System Gold 126 pump systems with a Model 168 diode-array detector. Preparative HPLC was done on a Beckman System Gold 126 pump with preparative heads with a Model 166 detector.

Analytical reverse-phase HPLC was done using a Whatman RAC II C-18 column, 4.6 mm×100 mm. The solvents used were: A—0.05 M triethylammonium acetate (TEAA) pH 7.0−7.2; and B—50% acetonitrile in 0.05 M TEAA, pH 7.0−7.2. The gradient system used was 0−60% B over 30 minutes (1% acetonitrile increase per minute). The flow rate was 1.5 ml/minute.

Reverse-phase purifications were done on a preparative (2.5×30.5 cm) PRP-1 column from Hamilton Co. (Las Vegas, NV). Samples were diluted to 10% organic (acetonitrile), then loaded onto the column using an Eldex Model B-100-S pump. The column was reattached to the HPLC and the sample eluted as described below. The flow rate was 13 ml/min.

Analytical polar-stationary chromatography was done using a Polyhydroxylethyl A column, 5 micron, 4.6 mm×200 mm, obtained from PolyLC (Columbia, MD). Buffer A was 0.05 M triethylammonium acetate (TEAA), pH 7.0−7.2, and buffer B was acetonitrile. The gradient was 70−30% B over 20 min. at a flow of 1.0 ml/min. An analytical sample, typically 0.2 OD Units, was dissolved in 100 μl of 65% acetonitrile/water. The sample sometimes required sonication and mixing to go into solution.

All analytical chromatograms were monitored at 260 nm; preparative runs were monitored at 290 nm.

Mass-spectometry was performed on a Fisons Trio2000 ESM mass-spectrometer using electrospray in the positive ion mode.

Preparation of oligonucleotides

Oligonucleotides (**1**−**5**) and the $dT_3N_2T_3$ (N = dA, dC, dG or T) model sequences described in the text were prepared on a 15 μmole scale using a Biosearch 8750 DNA synthesizer as described elsewhere.[19] Average coupling efficiencies ranged from 94 to 96%. The compounds were all detritylated as a part of the synthesis routine.

The large scale syntheses (**6**−**7**) were prepared for us by JBL Scientific, Inc. of San Louis Obisbo, CA. The oligonucleotides were delivered attached to the solid support with the dimethoxytrityl protecting group removed.

Deprotection procedures

EDA. EDA only deprotections were accomplished by adding 1 ml of EtOH/ACN/water (47.5:47.5:5) to the support bound oligonucleotide (5 mg), followed by 1 ml of EDA. The reaction was mixed for 6 hours at room temperature and then dried under vacuum. The residue was co-evaporated with ethanol 3 times to remove excess EDA, then reconstituted in 1 ml of 1/1 ACN/water for analysis.

Two step (small scale). The two-step deprotections were carried out similar to that previously described [9,15]. Support-bound oligonucleotide (**1**−**5**) (23 mg; ~1 μmole) was treated with 1 ml of conc. NH_4OH for two hours at room temperature. The ammonium hydroxide solution was then removed from the beads and the beads rinsed twice with 1 ml of 1/1 ACN/water each. The combined supernatant and rinsings was evaporated to dryness. The residue was then treated with 1 ml of EtOH/ACN/water (47.5:47.5:5), followed by 1 ml of EDA. After 6 hours at room temperature, the reaction mixture was handled as described for method a.

Two step (large scale). The large scale two-step deprotections on oligonucleotides (**6**) and (**7**) were accomplished as follows:

The synthesis support was placed in a screw cap vessel and 20 ml of conc. ammonium hydroxide was added. The vessel was sealed and mixed for two hours at room temperature. The ammonium hydroxide solution was then decanted, and the beads rinsed twice with 10 ml of 1/1 ACN/water. The ammonium solution and rinsings were combined and dried using a rotory evaporator under vacuum. To the residue was added 5 ml of, followed by 5 ml of EDA. This was mixed for 6 hours at room temperature. The reaction was then evaporated to dryness using a rotary evaporator under vacuum at room temperature. The residue was co-evaporated three times with ethanol. The sample was reconstituted in 1/1 ACN/water for analysis and purification. Table 3 lists the results from the large scale deprotections.

One-pot deprotections (small scale). The small scale ($dT_3N_2T_3$ model sequences and oligonucleotides (**1**−**5**)) one-pot deprotections were carried out as follows:

The support, 5 mg in the case of the $dT_3N_2T_3$ model sequences and 1 μmole (~23 mg) of oligonucleotides (**1**−**5**), was treated for 30 minutes at room temperature with 1 ml of the EtOH/ACN/water (47.5/47.5/5) solution containing 8% ammonia (w/w; which was prepared by bubbling ammonia gas through a solution of ACN/EtOH/water (47.5:47.5:5) at 0°C for one hour), or the mixture containing EtOH/ACN/ammonium hydroxide (45/45/10). After 30 minutes an equal volume of EDA was added to the reaction mixture. After 6 hours at room temperature, the solution was decanted from the beads and the beads washed twice with 1 ml each of 1/1 ACN/water, which was combined with the reaction mixture. The solution was then diluted to a volume of 30 ml with water and neutralized (pH 7) with 6 N HCl containing 10% ACN.

One-pot deprotections (large scale). Large scale deprotections were done in the same manner described for the small scale deprotections except that all volumes were scaled accordingly. The 100 μmole scale synthesis (**7**) was deprotected with 12.5 ml of each reagent, washed twice with 12.5 ml 1/1 ACN/water, and diluted to 375 ml with water. The 150 μmole scale synthesis (**6**)

required 20 ml of each reagent and wash and was diluted with water to 600 ml total volume.

Tables 2 and 3 summarize the crude yields obtained from deprotection of these oligonucleotides.

Purification of oligonucleotides (6) and (7)

Purifications of the oligonucleotides were done on the preparative Hamilton PRP-1 column. Solvents used were: A—0.05 M triethylammonium acetate (TEAA), pH 7.0–7.2; and B—50% acetonitrile in 0.05 M TEAA, pH 7.0–7.2. The gradient was 20–60% B over 40 minutes (0.5% acetonitrile increase per minute). The flow rate was 13 ml/minute.

We found it convenient to load the dilute samples resulting from our deprotection stratagy onto a reverse-phase HPLC column using a small high pressure pump such as the Eldex Model B-100-S[17]. (We do not recommend using HPLC pumps for this purpose because of the possibility of sample precipitation.) If the sample is in 10% ACN or less, it will adsorb to the top of the column.

Purifications for the large scale oligonucleotides (**6** and **7**) were done in the same manner. The purification of (**6**) deprotected by the one-pot method will be used as an example.

The crude sample of (**6**) (1200 ml; 11,858 OD Units; approximately 400 mg), which was diluted to 10% organic content after deprotection, was pumped directly onto the column using an Eldex pump. The column was reattached to the HPLC and the gradient run.

Fractions were collected every 0.5 minutes and the absorbance at 260 nm was measured for each fraction around the product peak. These fractions were then analyzed by reverse-phase HPLC using the Whatman RAC II C-18 column as described in the General Techniques/Instruments section. Fractions of the desired purity were pooled, evaporated to dryness, and reconstituted in 5 ml 1/1 ACN/water for final quantification. Yield: 3519 OD; approximately 117 mg; 30% recovery. Analysis by ES mass-spectrometry: determined mass—4507.1; calculated mass—4507.4.

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ABBREVIATIONS

dA, 2′-deoxyadenosine; dC, 2′-deoxycytidine; dG, 2′-deoxyguanosine; T, thymidine; N^4-bz-dC, N^4-benzoyl-2′-deoxycytidine; N^4-ibu-dC, N^4-isobutyryl-2′-deoxycytidine; N^2-ibu-dG, N^2-isobutyryl-2′-deoxyguanosine; N^2-ibu-O^6-DPC-dG, N^2-isobutyryl-O^6-diphenylcarbamoyl-2′-deoxyguanosine; N^6-bz-dA, N^6-benzoyl-2′-deoxyadenosine; ACN, acetonitrile; AU, atomic units; bz, benzoyl; conc., concentrated; DMAP, dimethylaminopyridine; DPC, diphenylcarbamoyl; EtOH, ethanol; EDA, ethylenediamine; HPLC, high performance liqiud chromatography; ibu, isobutyryl; NMI, N-methylimidazole; OD, optical density; TEAA, triethylammonium acetate; UV, ultraviolet

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