

Cleavage of oligodeoxyribonucleotides from controlled-pore glass supports and their rapid deprotection by gaseous amines

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ABSTRACT

A novel method for the deprotection of oligodeoxyribonucleotides has been developed. Gaseous amines such as ammonia or methylamine were employed under pressure to achieve mild and rapid deprotection conditions. For example, oligodeoxyribonucleotides having a (*tert*-butyl)phenoxyacetyl group for the protection of the exocyclic amino function of cytosine, adenine and guanine were released from controlled-pore glass supports and fully deprotected by ammonia or methylamine under gas phase conditions, at room temperature, within 35 or 2 min respectively.

The automation of oligonucleotide synthesis via the phosphoramidite approach (1,2) has had a tremendous impact on the progress of biomedical sciences, and led to the creation of a world-wide industry dedicated to the production of oligonucleotides (3). The high demand for synthetic oligonucleotides has provided the impetus to improve the chemistry involved in post-synthesis oligonucleotide processing. In particular, rapid removal of oligonucleotide protecting groups has attracted considerable attention and has necessitated the development of novel base-labile blocking groups for nucleobases (4–8). Concentrated solutions of ammonia in water, methanol or ethanol have generally been used for the cleavage of these protecting groups (2,4–8). More recently, an aqueous solution of methylamine and ammonium hydroxide has been employed for the deprotection of those oligonucleotides having the exocyclic amino function of cytosine protected exclusively with an acetyl group (9). In this context, the use of ammonia or methylamine under gas phase conditions for the release of nucleobase protecting groups has not, to our knowledge, ever been reported. Herein, we describe a convenient method which uses these gaseous amines under pressure to generate rapid deprotection of oligodeoxyribonucleotides at room temperature.

For our preferred experiments, oligodeoxyribonucleotides were generally synthesized on controlled-pore glass (CPG) supports (0.2 or 1 μ mol scale) by the use of commercial phosphoramidite monomers having the 4-(*tert*-butyl)phenoxy-

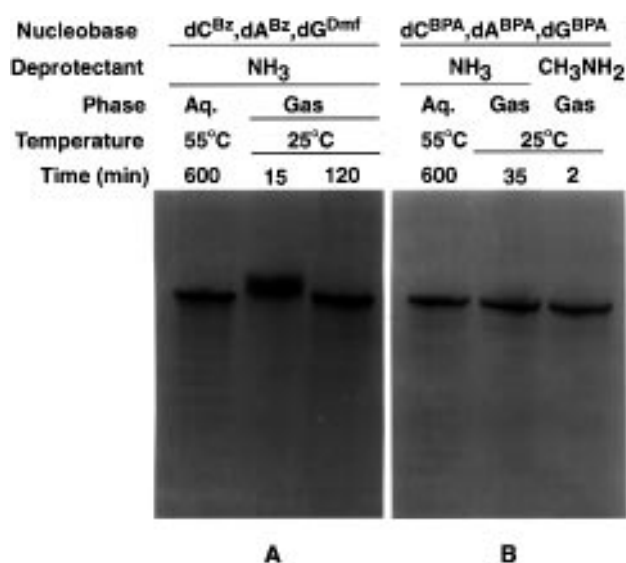


Figure 1. Completeness of the deprotection of oligodeoxyribonucleotides having selected nucleobase protecting groups by amines under gas phase conditions. Electrophoretic analysis of crude d(ATCAGTGAGGCACCTATCTCAGCGAT) on a 20% polyacrylamide-7 M urea gel at pH 8.3 (1× TBE buffer). Bands were visualized upon staining the gel with Stains-all. (A) Comparison of oligomer deprotection (Table 1, entry 2) performed under gas phase conditions (middle and right lanes) against that effected by concentrated ammonium hydroxide for an extended period of time at elevated temperature (left lane). (B) Same as (A) but for oligomers having nucleobases protected according to entry 3 of Table 1.

acetyl (BPA) group (6,10) for the protection of the exocyclic amino function of cytosine, adenine and guanine (Expedite™, PerSeptive Biosystems). Upon completion of the syntheses, the dry CPG columns were placed in a stainless steel pressure container (Barrskogen, Inc.) for deprotection. The vessel was evacuated briefly and then pressurized with either ammonia (~10 bar at 25°C, Matheson®) or methylamine (~2.5 bar at 25°C, Matheson®) (11). After 35 min (NH₃) or 2 min (CH₃NH₂) the gas cylinder was closed off, and the deprotection vessel was

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Table 1. Deprotection of oligodeoxyribonucleotides carrying various nucleobase protecting groups by ammonia or methylamine under aqueous or gas phase conditions

Entry	Nucleobase Protecting Group ^a			Time for Oligodeoxyribonucleotide Post-synthesis Processing (Release from CPG and Deprotection) in Minutes						
	dC	dA	dG	Concentrated Ammonium Hydroxide			Ammonia Gas (ca. 10 bar at 25°C)		Methylamine Gas (ca. 2.5 bar at 25°C)	
				Release Step from CPG at 25°C	Deprotection Step at 25°C	Total Processing Time ^b	Release and Deprotection Steps	Total Processing Time ^c	Release and Deprotection Steps	Total Processing Time ^c
1	Bz	Bz	iBu	60	2100 ^{d,e}	2180	~420 ^f	~421 ^f	-	-
2	Bz	Bz	Dmf	60	420 ^g	500	120	121	-	-
3	BPA	BPA	BPA	h	120 ⁱ	140	35	36	2	3
4	BPA	BPA	IPA	-	-	-	20	21	-	-

^aAbbreviations: Bz, benzoyl; iBu, isobutyl; Dmf, *N,N*-dimethylformamido; BPA, 4-(*tert*-butyl)phenoxyacetyl; IPA, isopropoxyacetyl (5).

^bIncludes a minimum of 20 min for evaporation of ammoniacal solutions and reconstitution in 300 µl of water.

^cOne minute is usually required to elute an oligonucleotide from a column with 300 µl of water.

^dSee also ref. 14.

^e600 min at 55°C is an alternative deprotection step.

^fEstimated from the half-time of the deprotection reaction which was determined by HPLC analysis of nucleolytic hydrolysates at various time points.

^gSixty minutes at 65°C is an alternative deprotection step (8).

^hDeprotection was performed in the presence of the solid support (6).

ⁱFifteen minutes at 55°C is an alternative deprotection step (6).

depressurized in a well-ventilated fume hood. The fully deprotected oligonucleotides were isolated by flushing ~300 µl of water back and forth through each column using two syringes.

The efficiency of oligonucleotide release from CPG under gas phase conditions was measured by UV spectroscopy. A complete cleavage of oligonucleotides from CPG was achieved within 15 min with ammonia or 2 min with methylamine. In addition, the completeness of oligonucleotide deprotection under these conditions was compared with that achieved with concentrated ammonium hydroxide for an extended period of time (10 h) at 55°C, and evaluated by polyacrylamide gel electrophoresis (see Fig. 1). For example, when a benzoyl group was used for the protection of the exocyclic amino function of cytosines and adenines, and *N,N*-dimethylformamido (8,12,13) for that of guanines (see Table 1, entry 2), treatment of oligodeoxyribonucleotides carrying these nucleobases with ammonia under gas phase conditions for 15 min at 25°C led to an incomplete deprotection (see Fig. 1A, middle lane). A partial deprotection affects the mass-charge ratio of a given oligonucleotide and decreases its electrophoretic mobility. It required at least 120 min under these conditions to effect complete removal of the nucleobase protecting groups (Fig. 1A, compare the right lane with the left lane). However, when BPA served as a nucleobase protecting group (see Table 1, entry 3), oligodeoxyribonucleotides were completely deprotected within 35 min with ammonia or within 2 min with methylamine (see Fig. 1B, compare the middle or right lane respectively with the left lane). A rapid deprotection (~7 h) of oligodeoxyribonucleotides having standard nucleobase protecting groups was also accomplished by gaseous ammonia when compared to that effected by concentrated ammonium hydroxide which required 36 h for completion at the same temperature (25°C) (see Table 1, entry 1).

It is important to note that oligodeoxyribonucleotides with BPA nucleobase protecting groups were, upon full deprotection by

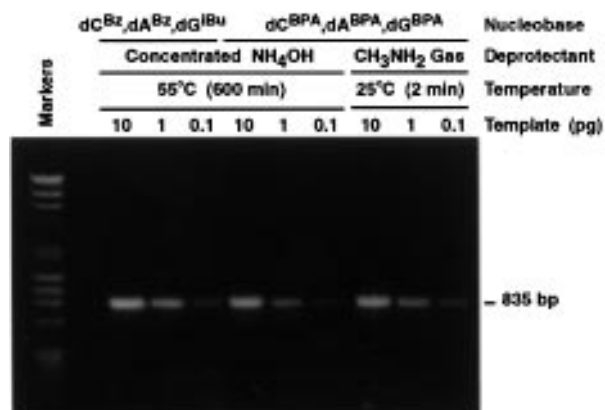


Figure 2. Electrophoretic analysis of an amplification experiment performed with a 5'-primer [d(CATTCGGTGTGCGCCCTTATCCC)] and a 3'-primer [d(ATCAGTGAGGCACCTATCTCAGCGAT)] for ampicillin resistance gene. These primers were synthesized from deoxyribonucleoside phosphoramidites carrying nucleobase protecting groups as shown, and deprotected under standard conditions as well as under gas phase conditions. Deprotected primers were desalted on NAP-25 columns and used in PCR reactions to amplify a 835 bp fragment. Amplifications were performed in a 25 µl volume using *Taq* polymerase XL (Perkin-Elmer) and different amounts of plasmid DNA as template. An amplification cycle consisted of 30 s at 94°C, 1 min at 64°C and 6 min at 72°C. The products of 32 cycles of amplification were electrophoresed on a 0.8% agarose gel. The gel was stained with ethidium bromide, and the bands were visualized on a UV transilluminator. The DNA size markers consist of a mixture of λ -HindIII and ϕ X174 *Hae*III digests.

ammonia or methylamine under gas phase conditions, chemically indistinguishable from those deprotected under standard conditions. Complete hydrolysis of these oligonucleotides by snake venom phosphodiesterase and alkaline phosphatase did not show detectable nucleobase modification according to HPLC analysis

of the digests (data not shown). Furthermore, these oligodeoxyribonucleotides were equally effective as primers for PCR experiments as those deprotected under standard conditions (see Fig. 2).

The gas phase method described herein allows rapid and simultaneous deprotection of a large number of oligodeoxyribonucleotides at ambient temperature. The number of oligonucleotides or CPG columns that can be deprotected is limited only by the size of the pressure vessel used. This deprotection procedure eliminates the hazards inherent to the tedious handling and heating of aqueous amine solutions in glass vials, and most importantly, the time-consuming evaporation of these solutions.

Thus, deoxyribonucleoside phosphoramidites having the BPA group for the protection of the exocyclic amino function of cytosine, adenine and guanine are recommended for solid-phase oligonucleotide synthesis given the speed and efficiency with which the resulting oligonucleotides are released from CPG and deprotected by ammonia or methylamine under gas phase conditions. Furthermore, the stability of these commercial phosphoramidites in acetonitrile is such that they can be used reliably for >1 week after solubilization (data not shown).

Although the gas phase deprotection method described herein has been applied to oligodeoxyribonucleotides covalently bound to CPG, it seems reasonable to speculate that this deprotection procedure can also be applied to other types of polynucleotides. These may include oligoribonucleotides and oligonucleotide analogues that are synthesized on CPG or other polymeric supports. For example, oligodeoxyribonucleoside phosphorothioates have been deprotected by ammonia under gas phase conditions without apparent adverse effect according to

³¹P-NMR spectroscopy (data not shown). Thus, it is likely that the gas phase deprotection methodology would be particularly attractive to academic and commercial oligonucleotide synthesis facilities because it can substantially accelerate the production of synthetic oligonucleotides.

REFERENCES

- 1 Beaucage, S.L. and Caruthers, M.H. (1981) *Tetrahedron Lett.*, **22**, 1859–1862.
- 2 Beaucage, S.L. and Iyer, R.P. (1992) *Tetrahedron*, **48**, 2223–2311.
- 3 Pon, R.T., Buck, G.A., Niece, R.L., Robertson, M., Smith, A.J. and Spicer, E. (1994) *Biotechniques*, **17**, 526–534.
- 4 Schulhof, J.C., Molko, D. and Teoule, R. (1987) *Nucleic Acids Res.*, **15**, 397–416.
- 5 Uznanski, B., Grajkowski, A. and Wilk, A. (1989) *Nucleic Acids Res.*, **17**, 4863–4871.
- 6 Sinha, N.D., Davis, P., Usman, N., Pérez, J., Hodge, R., Kremesky, J. and Casale, R. (1993) *Biochimie*, **75**, 13–23.
- 7 Kuijpers, W.H.A., Huskens, J. and van Boeckel, C.A.A. (1990) *Tetrahedron Lett.*, **31**, 6729–6732.
- 8 Vu, H., McCollum, C., Jacobson, K., Theisen, P., Vinayak, R., Spiess, E. and Andrus, A. (1990) *Tetrahedron Lett.*, **31**, 7269–7272.
- 9 Reddy, M.P., Hanna, N.B. and Farooqui, F. (1994) *Tetrahedron Lett.*, **35**, 4311–4314.
- 10 Köster, H., Kulikowski, K., Liese, T., Heikens, W. and Kohli, V. (1981) *Tetrahedron*, **37**, 363–369.
- 11 The deprotection of oligonucleotides under gas phase conditions is a process that has been claimed under *US Patent* 5,514,789.
- 12 Zemlicka, J. (1963) *Collect. Czech. Chem. Commun.*, **28**, 1060–1062.
- 13 McBride, L.J., Kierzek, R., Beaucage, S.L. and Caruthers, M.H. (1986) *J. Am. Chem. Soc.*, **108**, 2040–2048.
- 14 Koziolkiewicz, M. and Wilk, A. (1993) In Agrawal, S. (ed.), *Methods in Molecular Biology: Protocols for Oligonucleotides and Analogs*. Humana Press, Totowa, Vol. 20, pp. 207–224.