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THE USE OF GASEOUS AMMONIA FOR THE DEPROTECTION AND CLEAVAGE STEPS DURING THE SOLID-PHASE SYNTHESIS OF OLIGONUCLEOTIDES, AND ANALOGS

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Abstract. The use of NH_3 gas under pressure offers an efficient alternative to hot aqueous ammonium hydroxide for the deprotection, and cleavage steps during the large-scale synthesis of oligonucleotides, and their analogs prepared using *N*-pent-4-enoyl (*PNT*) nucleoside phosphoramidites and *H*-phosphonates.

The automated solid-phase synthesis of oligonucleotides using phosphoramidite chemistry[1] is widely employed in the synthesis of oligonucleotides for various applications in diagnostics,[2a-b] and in the area of oligonucleotide-based therapeutics.[2b-c] The consequent high demand for synthetic oligonucleotides of superior quality (as a measure of "full length" oligomer), with batch to batch reproducibility, has rejuvenated the development of new protecting groups, coupling chemistries, supports and support-anchoring groups,[3a] as well as, improvements in deprotection strategies, processing, and purification protocols.[3b]

In the context of oligonucleotide-based therapeutics, we have found that the synthesis of mixed backbone oligonucleotides (MBOs) containing ionic and non-ionic linkages pose a special synthetic challenge. For example, during the synthesis of MBOs having internucleotidic primary phosphoramidate (PO-NH_2) and phosphorothioate (PS) linkages, in conjunction with *PNT* nucleosides, we recently reported that DMF saturated with ammonia gas (65 °C, 12 to 16 h) was the only suitable medium for the deprotection, and cleavage of the oligonucleotide from the support, that would ensure the integrity of the labile PO-NH_2 linkages.[4] In seeking an efficient alternative, we considered the use of ammonia gas under pressure. We envisioned that the resulting "gas-solid-phase methodology" will be applicable as a convenient method for the deprotection of oligonucleotides and their analogs as well, especially during their large-scale manufacture. Recently, Beaucage and coworkers[5] employed gas-phase deprotection (stainless steel pressure vessel, 100 psi, rt) for the small-scale, rapid synthesis of phosphoric diester (PO) oligonucleotides using *tert*-butylphenoxyacetyl (*t*-PAC)-protected nucleoside phosphoramidite synthons. We report here our results on the synthesis of PO, PS, and PO-NH_2 oligonucleotide analogs prepared using *PNT* nucleoside synthons, and adopting gas-phase deprotection strategy for deprotection and cleavage.

In order to validate the gas-phase deprotection using *PNT* monomers, we initially prepared PO, and PS oligonucleotides. For our pilot studies, we employed a glass pressure vessel, although its use limited the operating pressure range, that could be used for the deprotection and cleavage.[6a] The choice of the glass vessel was dictated by several criteria: (a) to minimize direct contact with metallic surfaces, and the resultant potential contamination of oligonucleotides with trace metals, (b) to avoid the known propensity of surface-bound trace metals to induce desulfurization of the phosphorothioate, as well as, dithioate linkages,[6b] and (c) to avoid the potential metal-mediated side reactions of other modified oligonucleotides.

For our study, we used a modified Parr hydrogenation apparatus[7] (shaker assembly). In this assembly, the intervening brass-lined tank (being incompatible with NH_3 gas) was removed, and the glass vessel (rated to a pressure of 120 psig) was attached to the ammonia gas tank through polypropylene tubings and stainless steel fittings, both of which are known to be compatible with ammonia gas (Figure 1). Initially,

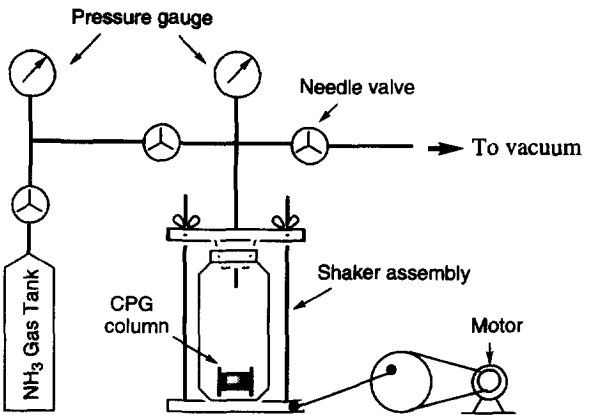

Figure 1. Pressure assembly used for gas-phase deprotection of oligonucleotides

such an assembly was used to assess the kinetics of deprotection, and cleavage of dC^{PNT}, dA^{PNT}, and dG^{PNT} nucleosides anchored to CPG supports via succinyl linkage. Thus, following the exposure of the CPG-bound nucleoside (1 μmol, packed into DNA synthesis columns) to NH_3 gas (40 to 60 psi, rt), the CPG was flushed with water to dissolve the cleaved nucleoside, which was then analyzed by reversed-phase HPLC. A time-course study conducted at a pressure of 60 psi, revealed that both quantitative cleavage, and simultaneous deprotection of dC^{PNT}, dA^{PNT}, and dG^{PNT} was achieved within 4 h. Next, a series of PO, and PS dinucleosides were synthesized on CPG supports on a 1 μM scale, using phosphoramidite chemistry, and exposed to NH_3 gas (60 psi, rt, 4 to 6 h), and the crude product evaluated by reversed-phase HPLC.[8a] In each case, complete deprotection, and quantitative cleavage was observed. Under the same conditions, dC^{Bz}, dA^{Bz}, and dG^{iBu} nucleosides were only partially deprotected (data not shown). These preliminary results suggested that gaseous NH_3 deprotection could be conveniently employed for the synthesis of PO, and PS oligonucleotides using *PNT* nucleosides, the advantage being a much simplified workup protocol which followed the deprotection.

Next, a series of 10- to 20-mer PO oligonucleotides (sequences **1-5**) were prepared on a 1 μmol scale.[9a] The deprotection and cleavage was accomplished using NH_3 gas (60 psi, rt, 10 h). The oligonucleotides were purified and representative sequences subjected to base-composition analysis, which revealed that the nucleosides were present in the expected ratio (Figure 2, typical study).[9b]

To extend the utility of the gas-phase methodology, a series of PS oligonucleotides were synthesized ("DMT-on") on 1 to 10 μmol scale. Following the gas-phase deprotection using NH_3 gas (60 psi, rt, 10 h),[6a,6c] the CPG was taken up in water to recover the crude oligonucleotides for further purification by reversed-phase HPLC. The products were evaluated by ^{31}P NMR which showed the expected peak at δ 56 ppm corresponding to the phosphorothioate diester, the PO content in the oligonucleotide being < 0.2% in each case (Figure 3, typical result).

To assess the other benefits of the gas-phase method, we carried out a comparative study of the gas-phase methodology vis a vis the aqueous NH_4OH protocol. For this purpose, we prepared a number of PS oligonucleotides (sequences **1 - 5**) on 10 to 15 μmol scale using *PNT* nucleoside phosphoramidites, as well as,

5'- GGA ACC GGT T (1)
5'- GCA GGT CAG T (2)
5'-ATG CGT GCA ATA GCC TT (3)
5'-GCG TGC CTC CTC ACT GGC (4)
5'-GCA GGT CAT TTC GAC AGC AT (5)

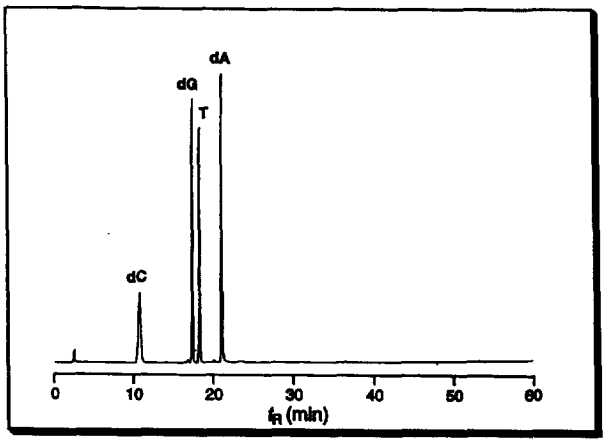

Figure 2. Base composition analysis of the oligonucleotide **3** prepared using the gas-phase protocol.

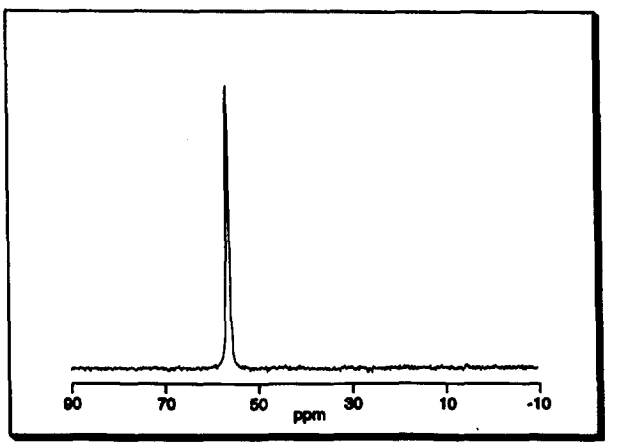

Figure 3. ^{31}P NMR spectrum of oligonucleotide **4** (all PS analog) prepared using the gas-phase protocol.

dC^{Bz}, dA^{Bz}, and dG^{iBu} amidites. Following DMT-on synthesis, the support-bound oligonucleotides were exposed to either: (a) NH_3 gas (60 psi, rt, 10 h) (for synthesis using *PNT* amidites) or, (b) aqueous NH_4OH (28%, 55 °C, 12 to 14 h). In addition to simplified work-up, the sample processing time prior to HPLC,

following deprotection in the gas-phase method was less than 5 min, compared to that of the aqueous NH_4OH method which was ca. 2 h. Both analytical and preparative reversed-phase HPLC[8b] of the resulting material revealed that the ratio of the area under the DMT-on peak to that of the DMT-off peak (representing truncated sequences) was ca. 5 to 10% higher with the gas-phase methodology compared to the aqueous NH_4OH method (Figure 4, typical result). Perhaps, the mildness of the deprotection protocol, as well as, the simplified recovery procedure[10a-b] coupled with the other favorable attributes of the *PNT* group[9b] contributed to

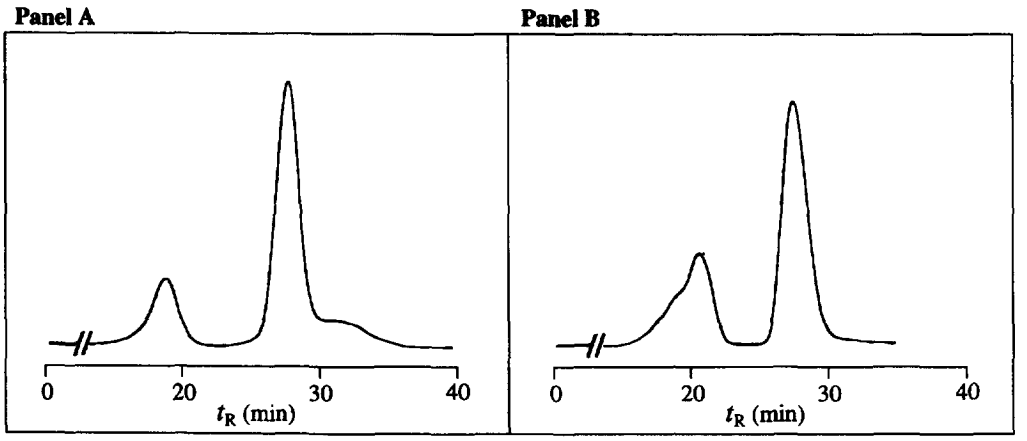

Figure 4. Comparative preparative HPLC profiles of the oligonucleotide **4** (all PS analog) prepared using the gas-phase protocol in conjunction with *PNT* amidites (**Panel A**), and that prepared with commercial amidites employing aqueous ammonia protocol (**Panel B**)

more of the DMT-on product.[10a-b] Additionally, capillary gel electrophoretic (CGE) analysis of the crude oligonucleotide revealed that its N - 1 content was lower in the case of the gas-phase method compared to the aqueous protocol (Figure 5).[11,12]

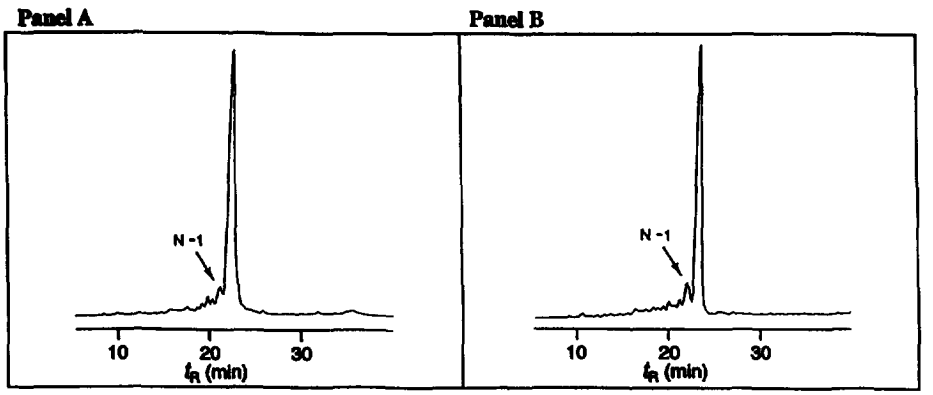

Figure 5. Comparative CE profiles of *crude* **4**: **Panel A**, that prepared using gas-phase protocol (*PNT* amidites); **Panel B** that prepared using aqueous ammonia protocol (commercial amidites).

From a process technology perspective, our studies reveal that during the large scale manufacture of oligonucleotides, the gas-phase method might be advantageous because the laborious steps of pH adjustments, and concentration of large volumes of aqueous NH_4OH, prior to chromatography, that is inherent in the aqueous protocol, could be bypassed.

The gas-phase methodology was also investigated for the synthesis of oligonucleoside PO-NH_2 analogs, using *PNT*-protected nucleoside *H*-phosphonates.[4a] Thus, following their synthesis, the CPG-bound dinucleoside *H*-phosphonates were treated with CCl_4/NH_3/dioxane (30 min) and the resulting support-bound dinucleoside phosphoramidates were exposed to NH_3 gas (60 psi, 6 h). Isolation, and analysis of the crude product revealed that complete deprotection and cleavage had been achieved while essentially preserving the structural integrity of the PO-NH_2 linkage (Figure 6, Panel A). A similar method was employed in the

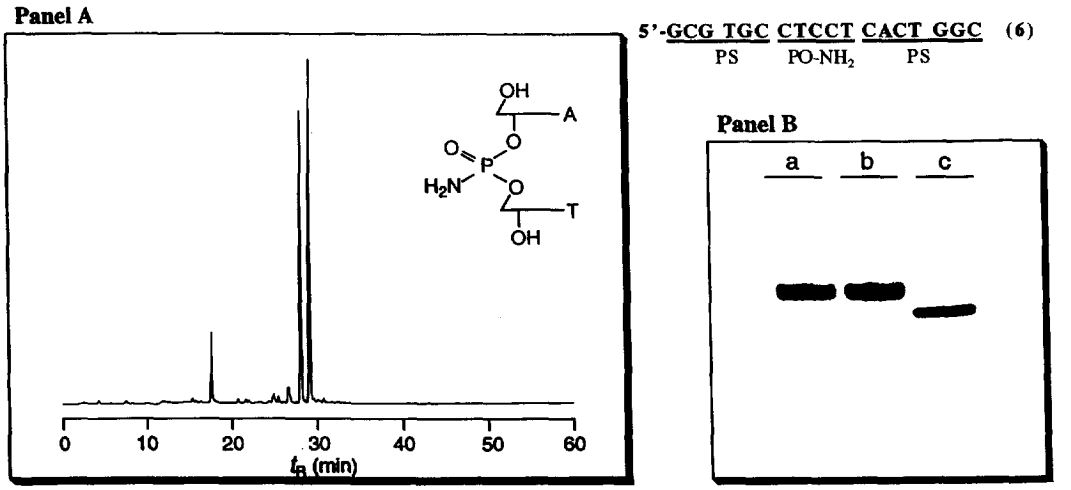

Figure 6. **Panel A.** Reversed-HPLC profile of crude 5'GT(PO-NH_2) prepared using gas-phase method; **Panel B.** PAGE of MBO **6**; lane a, **6** prepared using DMF/NH_3 procedure; lane b, **6** prepared using NH_3 gas under pressure; lane c, the corresponding "all PS analog" of **6** (standard).

synthesis of MBOs containing PS and PO-NH_2 linkages. Figure 6 (Panel B) reveals the polyacrylamide gel electrophoresis (PAGE) of the MBOs. Thus, we found that the gas-phase methodology in conjunction with *PNT* nucleosides provided rapid, and convenient access to these novel analogs as compared to the earlier protocol.[4a]

Overall, our initial studies reveal significant advantages in employing gas-phase deprotection in the synthesis of oligonucleotides, analogs, and MBOs in conjunction with *PNT*-nucleoside monomers. This methodology will be especially suitable for large-scale work. Accordingly, we are currently developing "on-line gas-phase deprotection" strategy by designing reactors of suitable material of construction that can withstand higher pressure, and which would facilitate better mass transfer between the gas- and solid-phases. Consequent significant reduction in the time of deprotection, and cleavage is anticipated when using *PNT* nucleoside synthons. The results of these studies will be reported elsewhere.

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6. (a) It is expected that by operating under pressures higher than that used in the present study, significant reduction in the time of deprotection, and cleavage can be achieved. Work is ongoing in this regard; (b) Kodra, J. T.; Kehler, J.; Dahl, O. *Nucl. Acids Res.* **1995**, *23*, 3349; (c) Following deprotection, the reaction vessel was depressurized in a well-ventilated hood.
7. The Parr apparatus was purchased from Ace Glass Inc., Vineland, New Jersey.
8. For conditions of analytical, and preparative reversed-phase HPLC analysis, see: (a) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 2471; (b) Iyer, R. P.; Yu, D.; Agrawal, S. *Bioorg. Chem.* **1995**, *23*, 1.
9. (a) DNA synthesis was performed on an EXPEDITE synthesizer (PerSeptive Biosystems); (b) For details see: Iyer, R. P.; Yu, D.; Habus, I.; Ho, N.-H.; Johnson, S.; Devlin, T.; Jiang, Z.; Zhou, W.; Xie, J.; Agrawal, S. *Tetrahedron* **1997**, *53*, 2731.
10. (a) It is reported that some detritylation of the DMT-on full-length oligonucleotide might occur during the processing step that follows aqueous ammonia deprotection; the resulting DMT-off material being bereft of the "hydrophobic handle" might co-elute with the failure sequences, and not recovered. Therefore, certain protocols recommend the addition of triethylamine to the solution of the oligonucleotide in ammonium hydroxide, prior to concentration, to minimize this premature detritylation, see: (i) Warren, W. J.; Vella, G. In *Protocols for Oligonucleotide Conjugates*, Agrawal, S., Ed.; Humana: Totowa, NJ., 1994, pp 233-264; (ii) Applied Biosystems User Bulletin, No. 50, August 1988; (b) This ratio was ca. 3 to 5% higher when such a comparative study was done using *PNT* nucleoside monomers in each case.
11. Several factors may contribute to the reduced N - 1 content; For a discussion, see reference 9b.
12. CGE analysis was done on a Beckman P/ace 2200 instrument operating at 14.1 Kv; before CGE, the samples were desalted using a SEP-PAK cartridge.

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