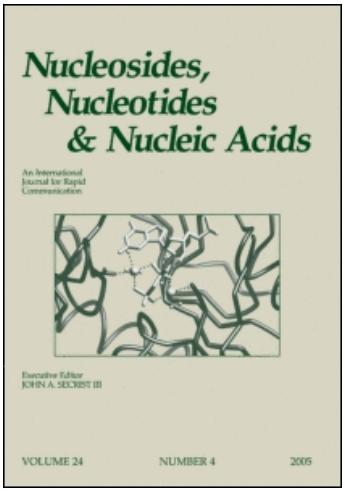

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Stability of Phosphorothioate Oligonucleotides in Aqueous Ammonia in Presence of Stainless Steel

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Stability of Phosphorothioate Oligonucleotides in Aqueous Ammonia in Presence of Stainless Steel

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Abstract: Oligodeoxyribonucleotide phosphorothioates undergo desulfurization in presence of metal in aqueous ammonia at elevated temperatures. The extent of sulfur loss and chain cleavage have been investigated.

Antisense oligonucleotides as modulators of gene expression represent an exciting new drug technology.[1-3] Oligodeoxyribonucleotide phosphorothioates are now among the most intensively investigated nuclease-resistant antisense analogs, as evidenced by a number of ongoing clinical trials against several disease targets.[4-6] Structurally, these differ from natural oligonucleotides by the replacement of one of two nonbridging oxygen atoms by a sulfur atom at each internucleotide linkage. Among factors in the successful development of these complex molecules to support broad clinical trials have been advances made in automation, analysis and purification. The large scale synthesis[7-9] of oligonucleotide phosphorothioates is presently carried out by initial formation of the internucleotide phosphite linkage using solid-phase phosphoramidite chemistry[10,11] followed by sulfurization. Efficient synthesis of 20-mer oligophosphorothioates has been achieved on 80-100 mmole scale (800 - 1000 g solid support) with only 1.5-fold excess of amidite synthons.[12] However, as the scale of synthesis increases to meet future market demands, issues related to fast and efficient synthesis, automation, scalability and product purification are also being investigated. One issue has been the protocol for final product deprotection. Since deprotection involves large quantities of saturated aqueous ammonium hydroxide, one might consider use of stainless steel reactors to withstand resulting vapor pressure at 55°C. A recent report,[13] however, discusses the instability of dimer phosphorothioates in aqueous ammonia in the presence of metal ions. As this is potentially an important issue for

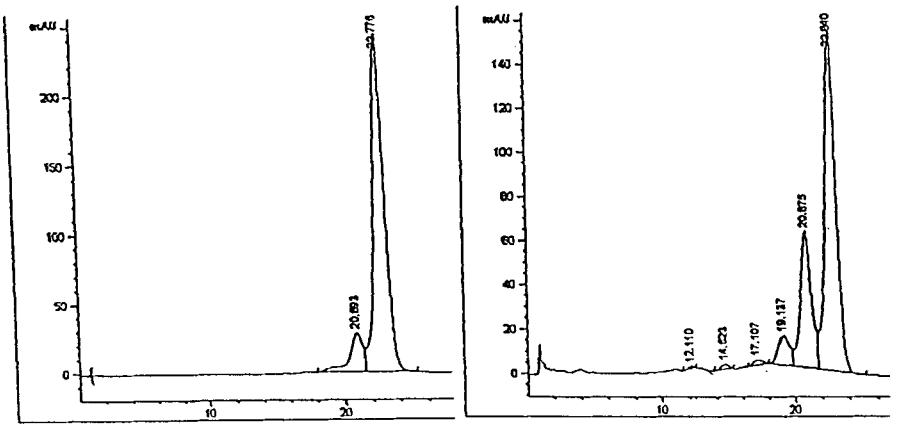

Fig. 1: Anion exchange HPLC of the analyzed sample at time 0 h and 34 h.

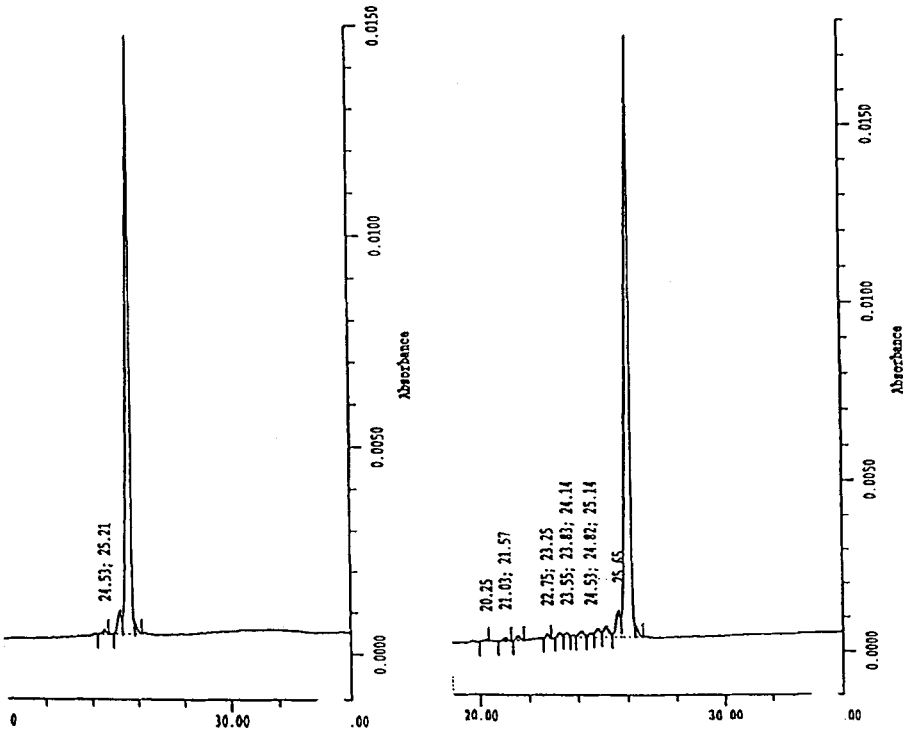

Fig. 2: CGE of the analyzed sample at time 0 h and 34 h.

phosphorothioate oligonucleotide synthesis, we describe herein our findings regarding deprotection of a 20-mer oligodeoxyribonucleotide phosphorothioate with aqueous ammonia during process development studies.

In a typical experiment, 10 mg of a purified 20-mer oligophosphorothioate bulk drug substance (ISIS 5132/CGP 69846A) [ds(TCCCGCCTGTGACATGCATT)][14] were dissolved in aqueous ammonia (33%, 10 ml) and divided into six equal batches. Samples were taken up in bottles containing stainless steel shavings (type 316L), sealed, and held in an oven at 55°C. Samples were removed at time intervals of 2, 4, 6, 10, and 34 h, cooled, concentrated and analyzed by anion exchange chromatography (Fig. 1) and capillary gel electrophoresis (Fig. 2). The amount of partially desulfurized material present was found to increase with contact time (Table 1).

In another recent literature report concerning deprotection of phosphorodithioates,[16] low levels of base-catalyzed desulfurization were again reported under standard deprotecting conditions in a glass reactor, but desulfurization extent appeared to vary depending on the ammonia lot used. In view of this observation, we repeated the above experiment using ammonia from five different lots. We observed consistent levels (± 5%) of desulfurization and chain cleavage across lots (data not shown).

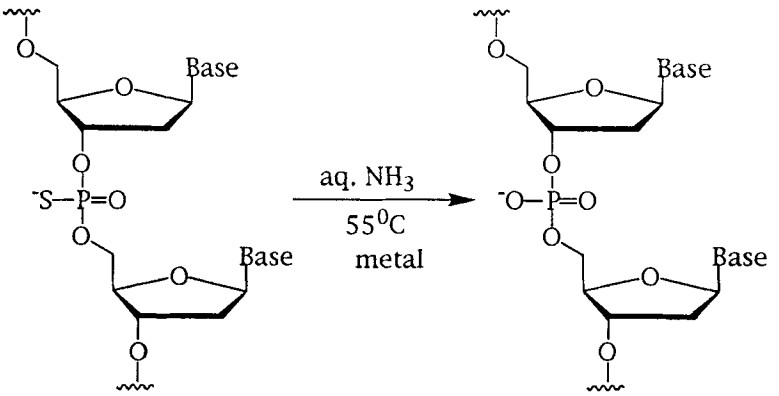

Table 1: Stability of oligophosphorothioates in ammonia in presence of stainless steel.

Contact Time (h)	% P=S based on SAX	% Full length based on CGE[15]
0	87.85	94.17
2	79.04	93.64
4	76.28	93.42
6	73.75	89.79
10	68.68	88.50
34	65.10	86.60

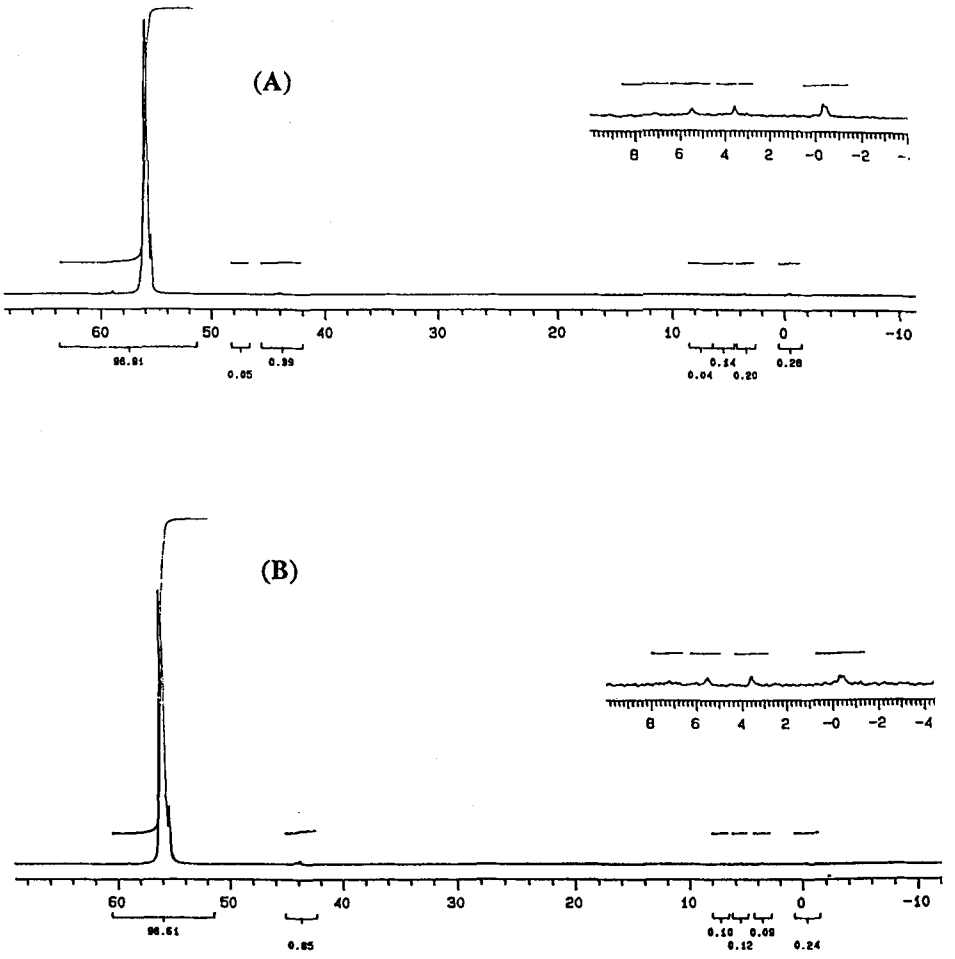

Fig. 3: ^{31}P NMR of the sample incubated in glass reactor (A) and stainless steel (B)

Finally, in order to find whether stainless steel containers could be used for base-catalyzed deprotection, the following experiment was performed: The 20-mer oligonucleotide phosphorothioate was synthesized on a very large scale (80 mmole; loading 85 μmole/g) using polystyrene primer support on Pharmacia OligoProcess synthesizer. At the end of the synthesis, two 10 grams lots of the support were taken separately and incubated for 12 h with 200 ml of 30% aqueous ammonium hydroxide in a 2-litre glass container and an electropolished stainless steel (316L) container repectively. The solutions were filtered, solid support washed with ethanol and the combined mixture concentrated and analyzed by ^{31}P NMR (Fig. 3) and anion exchange HPLC. No significant difference was observed between the two experiments.

In summary, from the above results, it appears that surface integrity of the stainless steel reactor may be more important to get consistently good results than lot-to-lot ammonia variations.

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