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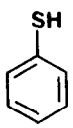
2-MERCAPTOBENZOTHIAZOLE-- AN IMPROVED REAGENT FOR THE REMOVAL OF METHYL PHOSPHATE PROTECTING GROUPS FROM OLIGODEOXYNUCLEOTIDE PHOSPHOTRIESTERS.

Alex Andrus [1a] and Serge L. Beaucage[1b]
Beckman Instruments Inc., 1050 Page Mill Road, Palo Alto, CA 94303.

An equimolar (1.5M) solution of 2-mercaptobenzothiazole and N,N-diisopropylethylamine in 1-methyl-2-pyrrolidinone efficiently removed the methyl phosphate protecting groups from deoxynucleotide phosphotriesters and yielded synthetic oligomers of high genetic integrity. This odorless reagent can therefore substitute for hazardous thiophenol usually used for this purpose.

Recently, several laboratories have reported the use of the methyl group as a phosphate protecting group during solid-phase synthesis of oligodeoxynucleotides,[2] oligoribonucleotides[3] and α-anomeric oligodeoxynucleotides.[4] An inherent disadvantage in using this phosphate protecting group is the requirement for noxious triethylammonium thiophenate[5] to effect the SN2 displacement of the methyl groups upon completion of the synthesis.

To alleviate this problem, we wish to report that commercially available mercaptans, such as 2-mercaptobenzothiazole (**2**), 2-mercaptobenzoxazole (**3**) and 2-mercaptopyridine (**4**), can substitute thiophenol (**1**) for the removal of methyl phosphate protecting group without exhibiting its foul-smelling characteristic.[6]



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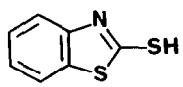

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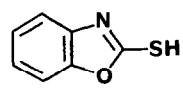

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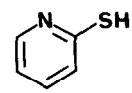

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To evaluate the effectiveness of these mercaptans relative to thiophenol, the dealkylation of trimethyl phosphate was carried out under conditions where solvents, bases, reagent concentrations and reaction temperatures were varied. The rates at which trimethyl phosphate was converted into phosphoric acid dimethyl ester were monitored by ^{31}P NMR and used to define the optimal conditions for the demethylation reaction.[7]

The effect of the solvent on the rate of demethylation was examined by reacting trimethylphosphate (85mM) in solvent such as *N,N*-dimethylformamide (DMF), *N,N*-dimethylacetamide (DMA), 1,1,3,3-tetramethylurea (TMU), 1-methyl-2-pyrrolidinone (NMP), dioxan or tetrahydrofuran (THF), with equimolar (2.0M) thiophenol and triethylamine at ambient temperature in a NMR tube. Under these conditions, the fastest rate of demethylation was observed with NMP as a solvent. 50% of trimethyl phosphate was converted into phosphoric acid dimethyl ester within 5 min.

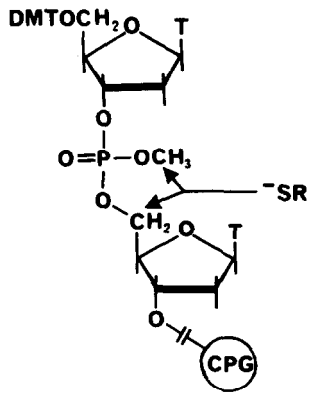
Using NMP as solvent, **2**, **3** and **4** were then evaluated in the dealkylation of trimethylphosphate under the conditions delineated above. According to ^{31}P NMR data, **2** produced 50% demethylation within 50 min whereas **3** and **4** required 150 min respectively. This experiment suggests that 2-mercaptobenzothiazole, despite its moderate demethylation kinetics, offers an odorless alternative to

thiophenol in the removal of methyl phosphate protecting groups.

To improve the dealkylation kinetics of **2**, the effect of the strength of the base used and its concentration was investigated. 2,6- lutidine, triethylamine (Et_3N) and *N,N*-diisopropylethylamine (iPr_2NEt) were each tested at various concentrations (0.5M-2.0M) in the demethylation of trimethylphosphate (85mM) by **2** (2.0M) in NMP at ambient temperature. As anticipated, the strength of the base and its concentration were rate limiting[8] and thus, reflected the effective concentration of benzothiazolyl mercaptide anion generated. Optimally, an equimolar (1.5M) solution of iPr_2NEt and **2** in NMP[9] produced 50% demethylation of trimethylphosphate within 25 min at 22°C. Heating an identical reaction mixture at 55°C generated the same extent of demethylation within 5 min. This rate of dealkylation is comparable to the one previously obtained with an equimolar (2.0M) solution of thiophenol and triethylamine in NMP at 22°C. The efficacy of the 2-mercaptobenzothiazole formulation was then evaluated in the deprotection of deoxynucleotide methylphosphotriesters.

A critical requirement in the removal of methyl phosphate protecting group from oligonucleotide phosphotriesters by mercaptide anions is the stability of the internucleotidic linkage under the deprotection conditions. Several years ago, Reese and his coworkers[10] reported significant internucleotidic bond cleavage caused by the reaction of *p*-methylphenylthiolate ion with deoxynucleotides harboring aryl phosphate protecting groups. To our knowledge, such a study has never been achieved with oligonucleotides carrying methyl phosphate protecting groups. Consequently, the dinucleotide phosphotriester **5** immobilized on controlled-pore glass (CPG) was treated with an equimolar (1.5M) solution of **2** and iPr_2NEt in NMP at ambient temperature for 24 hr. The solid phase was then thoroughly washed with NMP and the dimer was detritylated and released from the support according to standard procedures.[11] HPLC analysis of the reaction mixture indicated only the presence of the dinucleoside monophosphate d(TpT) and traces amounts of unreacted 2'-deoxythymidine (2%). There was no evidence of 5'-S-benzothiazolyl-2'-deoxythymidine that could have been produced from the nucleophilic attack of the benzothiazolyl mercaptide ion at the 5'-carbon of the internucleotidic linkage. Except for the presence of unreacted 2'-deoxythymidine, the HPLC profile is superimposable to the HPLC profile obtained from a sample of d(TpT) (purchased from Sigma Chemicals), which, has only been treated with concentrated ammonium hydroxyde as a positive control experiment. Substituting **2** by **1** did not produce detectable amounts of internucleotidic bond cleavage under optimal dealkylation conditions. These experiments suggest that the nucleophilic attack by the thiophenate or benzothiazolyl mercaptide ion at the methyl phosphate protecting group is rapid relative to the attack at the 5'-carbon of the internucleotidic link[12] and that upon removal of the methyl group, the anionic character of the phosphodiester may prohibit further nucleophilic attack at the 5'-carbon of the internucleotidic bond, presumably because of significant electrostatic repulsion towards incoming thiolate anions.[13]

When applied to the deprotection of methyl phosphate protecting groups from lengthy oligomers, the 2-mercaptobenzothiazole formulation appeared as efficient as the thiophenol solution (Compare Lanes 1 and 2 with Lanes 3, 4, 5 and 6 in Figure 1). Because of earlier reports[14,15] indicating that deoxynucleotide methylphosphotriesters may induce methylation of thymine residues at N-3, the extent of thymine methylation that may have occurred during solid-phase synthesis and phosphate deprotection of a polydeoxythymidylic acid methyl ester (65-mer), was evaluated.



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DMT: di-p-anisylphenylmethyl
R: benzothiazolyl
T: 1-thyminyl

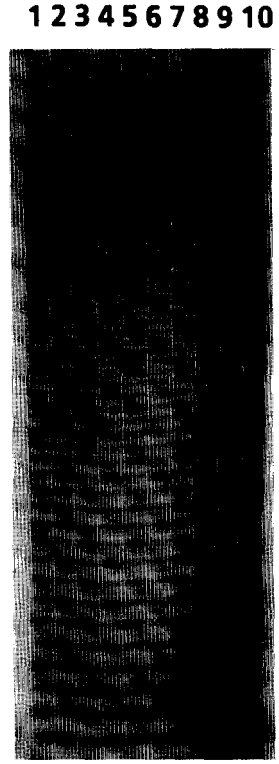

Fig. 1. Autoradiogram of crude 5'-radiolabeled oligomers obtained after deprotection of the methyl phosphate protecting groups under various conditions.[16]

Lane 1: poly-T (65-mer); 2.0M thiophenol-Et_3N in dioxan 90 min; 22°C.
Lane 2: As in lane 1; 24 hr; 22°C.
Lane 3: poly-T (65-mer); 1.5M **2**-iPr_2NEt in NMP; 90 min; 22°C.
Lane 4: As in Lane 3; 24 hr; 22°C.
Lane 5: As in lane 3; 2 hr; 55°C.
Lane 6: poly-T (65-mer); 2.0M **2**-Et_3N in NMP; 24 hr; 22°C.
Lane 7: CED-poly-T deprotected with conc. NH_4OH.
Lane 8: Heteropolymer (63-mer) deprotected with 1.5M **2**-iPr_2NEt in NMP; 1 hr; 55°C.
Lane 9: As in lane 8; 24 hr; 22°C.
Lane 10: CED-Heteropolymer deprotected with concentrated NH_4OH.

The crude 65-mer (poly-dT, Lane 5 of Fig.1) was digested by snake venom phosphodiesterase and bacterial alkaline phosphatase according to published procedures.[14,17] HPLC analysis of the digest revealed the presence of negligible amounts of 3-N-methyl-2'-deoxythymidine (0.2% of the integrated area corresponding to 2'-deoxythymidine).[18]

We have demonstrated that an equimolar (1.5M) solution of 2-mercaptobenzothiazole and *N,N*-diisopropylethylamine in NMP efficiently removed methyl phosphate protecting groups from oligodeoxynucleotide phosphotriesters without detectable internucleotidic bond cleavage and negligible amounts of methylated thymine residues. Because of the odorless nature of the reagent and its rapid demethylation kinetics, it seems reasonable to believe that the reagent may also be useful in the general area of synthetic organic chemistry wherever harmful thiolates may be required.

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References and notes.

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(b) Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305.

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6. **2**, **3** and **4** were purchased from Aldrich and were used without further purification.

7. The ^{31}P NMR spectra were recorded on a Varian 200 NMR spectrometer operating at 81 MHz under proton-decoupling mode.

8. For instance, an equimolar (1.0M) solution of **2** and triethylamine in NMP demethylates trimethylphosphate (85mM) in essentially the same rate as the one obtained with a 2.0M solution of **2** and triethylamine (1.0M) in the same solvent. 50% demethylation was observed within 150min and 140 min respectively at 22°C. Additionally, an equimolar (1.5M) solution of **2** and iPr_2NEt in NMP demethylate trimethylphosphate at 50% within 25 min whereas 95 min will be required to an equimolar (1.5M) solution of **2** and triethylamine to achieve the same results.

9. Typically, **2** (2.5g, 15 mmol) was dissolved with minimum amount of NMP in a graduated flask. iPr_2NEt (2.6 mL, 15 mmol) was then added to the solution followed by NMP to a volume of 10 mL.

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12. Our views are supported by the dealkylation of the mixed phosphotriester MeO(EtO)P(O)OPh by phenylthiotrimethylsilane in the presence of catalytic amounts of phenylthiolate ions. At 70°C the methyl group was cleaved at least 360 times faster than the ethyl group which resembles the 5'-carbon of an internucleotidic bond. (See Takeuchi, Y., Demachi, Y., Yoshii, E. *Tetrahedron Lett.* **1979**, 1231-1232)

13. This notion is consistent with the fact reported herein and by others (see Savignac, P., Lavielle, G. *Bull. Soc. Chim. France*, **1978**, 1506-1508) that thiophenate and thiolate ions only induced the monodealkylation of trimethylphosphate.

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16. The oligomers were prepared using methyl and cyanoethyl deoxynucleoside phosphoramidites on a Beckman System One Plus DNA Synthesizer. A fraction of the fully deprotected oligomers were radiolabeled at the 5'-end with γ-^{32}P ATP and T4- polynucleotide kinase before electrophoresis on a 15% 7M urea-polyacrylamide gel.

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18. McBride *et al.*[2] reported similar amounts of 3-N-methyl-2'-deoxythymidine when thiophenol was used for the removal of methyl phosphate protecting groups from oligodeoxynucleotide phosphotriesters (72-mer and 150-mer).

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