Pergamon

*Tetrahedron Letters*, Vol. 35, No. 36, pp. 6741-6744, 1994
Elsevier Science Ltd
Printed in Great Britain
0040-4039/94 $7.00+0.00

0040-4039(94)01409-4

# Solid Phase Synthesis of Phosphorothioate Oligonucleotides Using Benzyltriethylammonium Tetrathiomolybdate as a Rapid Sulfur Transfer Reagent

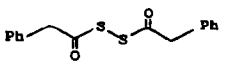
**M. Vaman Rao* and K. Macfarlane**
Cruachem Limited, Todd Campus,
West of Scotland Science Park, Glasgow G20 0UA, U.K.

**Abstract:** Benzyltriethylammonium tetrathiomolybdate (8), an easily prepared compound has been found to be a rapid, efficient and reliable sulfur-transfer reagent in the automated synthesis of phosphorothioate oligonucleotides via the phosphoramidite approach.

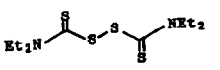
Phosphorothioate analogues are regarded as the first generation of antisense oligonucleotide analogues which have been successfully tested *in vitro* and *in vivo* as inhibitors of gene expression.[1-5] Phosphorothioate oligonucleotides are isoelectronic analogues of natural nucleotides in which one of the non-bridging internucleotide oxygen atoms is replaced by a sulfur-atom. The solid phase synthesis of phosphorothioate oligonucleotides has been achieved using the H-phosphonate approach[6], where only one sulfur transfer step is required after assembling the desired sequence to convert all the internucleotide linkages to phosphorothioates, or the phosphoramidite approach[7], where a stepwise sulfurization instead of iodine oxidation step in an otherwise standard synthetic cycle is required. The phosphoramidite chemistry, due to its superior coupling efficiency and its suitability to synthesize designed mixtures of phosphorothioate and phosphodiester internucleotide linkages in an oligonucleotide, appears to be the method of choice[8-13].

Although both approaches require the use of a sulfur-transfer reagent, it is of crucial importance that an efficient sulfur-transfer reagent is used for the synthesis of phosphorothioate oligonucleotides via the phosphoramidite approach. Elemental sulfur(9), which was initially used for this purpose, was not efficient due to its poor solubility properties and its slow sulfurization reaction. In order to alleviate these problems, recently more suitable sulfur-transfer reagents were recommended, viz., di-(phenylacetyl)-disulfide[10](1), tetraethylthiuram disulfide[11](2), 3H-1,2-benzodithiole-3-one1,1-dioxide[8](3), dibenzoyltetrasulfide[12](4) and bis(O,O-diisopropoxy phosphinothioyl) disulfide[(13)](5), for the synthesis of phosphorothioate oligonucleotides via the phosphoramidite approach. Of these, only 3H-1,2-benzodithiole-3-one 1,1-dioxide[8](3) appears to transfer sulfur very rapidly, however its synthesis and stability are not particularly suitable for large-scale use. In addition, the co-product formed during its sulfurization of internucleotide phosphite linkages can potentially transfer oxygen and thus can result in undesired phosphodiester linkages under certain conditions[8].

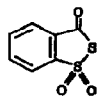
In this communication we wish to report another sulfur-transfer reagent, benzyltriethyl-ammonium tetrathiomolybdate [BTTM][14](8), which has been found to be particularly efficient. Furthermore, BTTM (8) is readily prepared (Scheme) in 80% yield in a one step reaction[15] from commercially available ammonium tetrathiomolybdate(6) and benzyltriethylammonium chloride(7). BTTM (8) is isolated as a stable, deep red coloured crystalline solid which is readily soluble in acetonitrile[11].



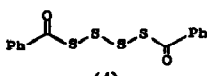
(1)



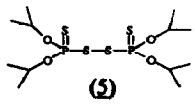
(2)



(3)



(4)



(5)

Scheme:

$$[NH_4]_2\,MoS_4 \;+\; PhCH_2N(CH_3CH_2)_3Cl \xrightarrow{H_2O}$$

(6) (7)

$$[PhCH_2N\,(CH_3CH_2)_3]_2MoS_4$$

(8)

| Concentration of Sulfur-transfer Reagent | Solvent(s) | Duration of Solution (Days) | Delivery Time (Seconds) | Reaction Time (Seconds) | % of Tp(s)T |
|---|---|---|---|---|---|
| 0.2M of (8) | $CH_3CN$ | 1 | 15 | 60 | 99.47 |
| 0.1M of (8) | $CH_3CN$ | 1 | 15 | 60 | 99.40 |
| 0.05M of (8) | $CH_3CN$-$H_2O$ (99:1) | 1 | 15 | 60 | 96.50 |
| 0.05M of (8) | $CH_3CN$-$H_2O$ (96:4) | 1 | 15 | 60 | 99.01 |
| 0.075M of (8) | $CH_3CN$-$H_2O$ (96:4) | 1 | 15 | 60 | 99.17 |
| 0 1M of (8) | $CH_3CN$-$H_2O$ (96:4) | 1 | 15 | 60 | 99.95 |
| 0.1M of (8) | $CH_3CN$-$H_2O$ (96:4) | 7 | 15 | 60 | 99.28 |
| 0.5M of (2) | $CH_3CN$ | 1 | 15 | 900 | 98.86 |
| 0.5M of (2) | $CH_3CN$ | 7 | 15 | 900 | 98.30 |
| 0.2M of (3) | $CH_3CN$ | 1 | 15 | 30 | 98.76 |
| 0.2M of (3) | $CH_3CN$ | 7 | 15 | 30 | 97.30 |

Table: Sulfurization of 5'-O-DMT-dinucleoside (3'-5')-O-β-Cyanoethyl phosphite, bound via 3'-terminal succinate to LCAA-CPG

In order to evaluate the efficacy of BTTM (8) for the synthesis of phosphorothioate oligonucleotides and also to optimise the sulfurizing conditions, dinucleoside phosphorothioates d(Tp(s)T) were synthesized on 1.0μmol scale using different concentrations of (8) in acetonitrile for 60 seconds to effect sulfurization instead of iodine oxidation step, but before the capping step, in an otherwise standard synthetic cycle[16]. After deprotection, the unpurified dimers were analysed by HPLC and found to contain greater than 99% of d(Tp(s)T). In addition, the efficacy of BTTM (8) as a sulfur-transfer reagent was compared with that of (2) and (3) under appropriate conditions, on day 1 and 7, in the solid phase synthesis of dinucleoside phosphorothioates d(Tp[s]T), (see table).

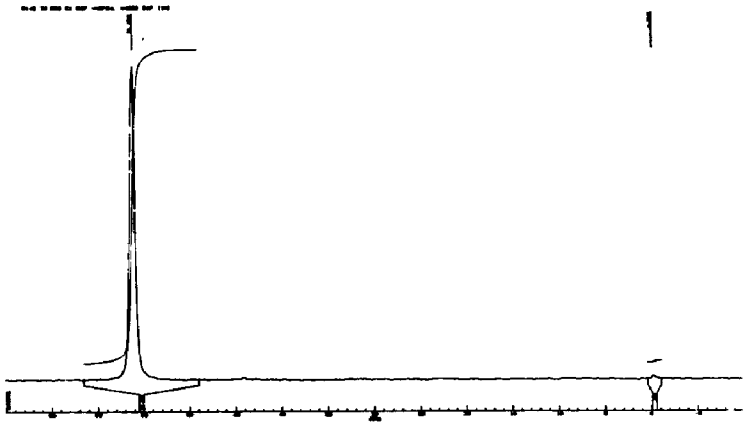


Figure 1: $^{31}$P-NMR Spectra (101.3 MHz, $D_2O$) of unpurified phosphorothioate 28-mer.

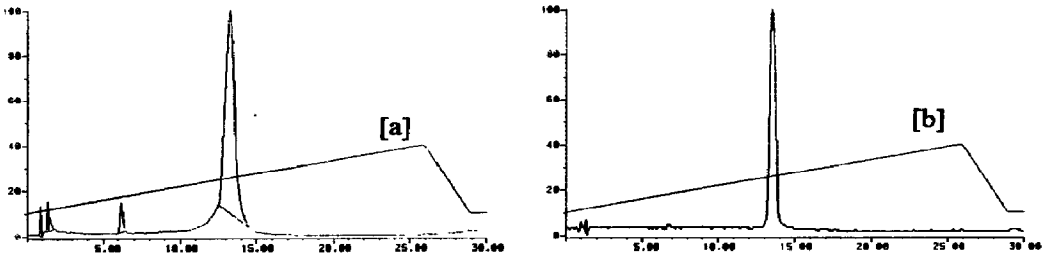



Figure 2. LC profiles (on an ODS, 5μ column, eluted with 0.1M triethylammonium acetate-acetonitrile) of phosphorothioate 28-mer: [a] unpurified and [b] purified.

To further evaluate the usefulness of BTTM (8) as an efficient sulfurizing agent, an oligodeoxyribonucleoside phosphorothioate S-d[TCG TCG CTG TCT CCG CTT CTT CCT GCC A], 28-mer, complementary to the mRNA of the HIV-1 *rev* gene was synthesized on 1.0μmol scale using a 0.1M solution of BTTM in acetonitrile-water (96:4) for 60 seconds to effect sulfurization in each synthetic cycle[16].The unpurified 28-mer was analysed by $^{31}$P-NMR spectroscopy after ammonolytic release from the solid support and deacylation; Figure 1. The ratio of the integrals of the signals at δca 56.2 (assigned to phosphorothioate phosphorus) and at δca - 0.3 (assigned to phosphate phosphorus) is estimated to be 99.46:0.54. Figure 2a and 2b shows the reverse phase liquid chromatograms of unpurified and purified phosphorothioate 28-mer, respectively. Finally oligodeoxyribonucleotide treated with a 0.1M solution of BTTM (8) in acetonitrile at r.t. for 24 hours did not show any detectable modification of the nucleosidic bases[17].

In summary, it has been demonstrated that BTTM (8), due to its ease of preparation, solubility in desirable solvents, its highly efficient and rapid sulfurization reaction, is useful for the solid phase synthesis of phosphorothioate oligonucleotides via the phosphoramidite approach.

**Acknowledgements:** The authors thank Professor S. Chandrasekaran for useful discussions and Mr. Ian Wilkie for his encouragement.

## References and Footnotes


1 Oligodeoxynucleotides - Antisense Inhibitors of Gene Expression; Cohen, J.S. Ed.; Macmillan, London, **1989**.

2 Mirabelli, C.K., Bennet, C.F., Anderson, K. and Crooke, S.J. Anti-Cancer Drug Design, **1991**,6, 647.

3 Agrawal, S. in 'Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS', Ed. Wickstrom, E. Wiley-Liss, New York, **1992**, 143-158.

4. Simons, M., Edelman, E.R., Dekeyser, J.L., Langer, R. and Rosenberg, R.D. Nature **1992**, 359, 67.

5 Ratajczak, M.Z., Kant, J.A., Luger, S.M., Hijiya, N., Zhang, J., Zon, G. and Gewirtz, A.M. Proc. Natl. Acad. Sci. USA **1992**, 89, 11823-11827.

6. Froehler, B.C. Tetrahedron Lett. **1986**, 27, 5575-5578.

7. **(a)** Stec, W.J., Zon, G., Egan, W. and Stec, B. J. Am. Chem. Soc. **1984**, 106 6077-6079; **(b)** Stein, C.A., Subasinghe, C., Shinozuka, K. and Cohen, J.S. Nucleic Acids Res. **1988**, 16, 3209-3222.

8 **(a)** Iyer, R.P., Egan, W., Ryan, J.B. and Beaucage, S.L. J. Am. Chem. Soc. **1990**, 112, 1253-1254; **(b)** Iyer, R.P., Phillips, L.R., Egan, W., Regan, J.B. and Beaucage, S.L. J. Org. Chem. **1990**, 55, 4693-4699.

9. Zon, G. and Stec, W.J. Phosphorothioate oligonucleotides - In oligonucleotides and their analogues: A practical approach. Eckstein, F. Ed., IRL Press: Oxford, **1991**, 87-108.

10. Kamer, P.C.J., Roelen, H.C.P.F., Van der Elst, H., Vander Marel, G.A. and Van Boom, J.H. Tetrahedron Lett. **1989**, 30, 6757-6760.

11. Vu, H. and Hirschbein, B.L. Tetrahedron Lett. **1991**, 32, 3005-3008.

12. Rao, M.V., Reese, C.B. and Zhengyun, Z. Tetrahedron Lett. **1992**, 33, 4839-4842.

13 Stec, W.J., Uznanski, B., Wilk, A., Hirschbein, B.L., Fearon, K.L. and Bergot, B.J. Tetrahedron Lett. **1993**, 34, 5317-5320.

14. Ramesha, A.R. and Chandrasekaran, S. Synth. Comm. **1992**, 3277-3284.

15 Benzyltriethylammonium Tetrathiomolybdate[14] (8) was prepared by the following procedure: To a pre-filtered (through a 0.45µm nylon filter) solution of ammonium tetrathiomolybdate (260g, 1.0mol) in HPLC-grade water (2.5L), was addded a pre-filtered solution of benzyltriethylammonium chloride (466g, 2.0mol) in HPLC-grade water (500ml), over 1 hour and was stirred in dark at r.t. for 10 hours. The deep red fine crystalline solid precipitate was filtered under nitrogen atmosphere, washed with HPLC-grade water ( 2 x 750ml), then with 2-propanol (2 x 750ml) and finally with diethyl ether (2 x 750ml). The solid product was freeze-dried for 20 hours to give (8) [yield, 486g (80%); m.p.126-128°C; (decomp); UV/Vis. λ-max: 476-478 (14,600), 322-326 (22,000), 270-274 (24,700) nm; IR(KBr): 470 $cm^{-1}$ (Mo=S)].

16. **(a)** Solid-phase syntheses were performed on 1.0µmol scale using Cruachems' PS250 DNA/RNA synthesizer, β-cyanoethyl deoxyribonucleoside phosphoramidites and reagents. A solution of BTTM(8) [see table] was placed on the synthesizer at the oxidation port and a 60 seconds reaction time (with a 15 second delivery time) was used to effect complete sulfurization, soon after the coupling reaction but before the capping step. BTTM solution, 0.1M in acetonitrile: water (96:4) was used within 1 week and we have not experieinced any clogging problems. **(b)** Rao, M.V. GB-Patent Application No: GB. 921659.9, **1992**.

17. **(a)** Deoxyribonucleoside (N-benzoyl adenosine and cytidine, N-isobutyryl guanosine and thymidine) functionalized CPG were separately treated with a 0.1M solution of (8) in acetonitrile at r.t. for 24 hours. LC analysis of the nucleoside released from the respective supports, after standard deprotection, indicated no nucleosidic base modification. **(b)** An oligodeoxyribonucleoside d[TCG TCG CTG TCT CCG CTT CTT CCT GCC A] carrying exclusively P (O) linkages was synthesized. The fully protected oligomer covalently attached to the solid support was incubated at r.t. with a 0.1M solution of (8) in acetonitrile for 24hours. An identical experiment in which (8) was omitted was also performed. After deprotection, the samples were subjected to enzymatic digestion with snake venon phosphodiesterase and alkaline phosphotase. LC analysis of the hydrolysate indicated no nucleosidic base modification.


(*Received in UK* 28 *March* 1994; *revised* 15 *July* 1994; *accepted* 22 *July* 1994)