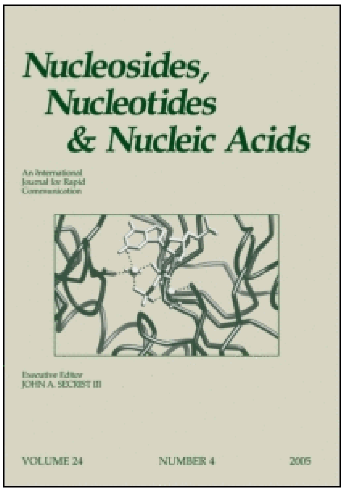

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2-Methyl-5-*tert*-butylthiophenol—An Odorless Deprotecting Reagent Useful in Synthesis of Oligonucleotides and Their Analogs

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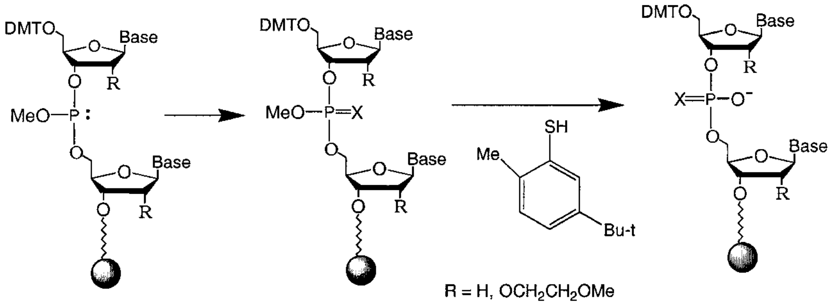
ABSTRACT

A solution of 2-methyl-5-*tert*-butylthiophenol and triethylamine in acetonitrile efficiently removed the methyl protecting groups from phosphate and phosphorothioate triesters and yielded oligonucleotides of high quality. This inexpensive odorless liquid is not toxic and is a suitable replacement for hazardous thiophenol and other reagents often used for this purpose.



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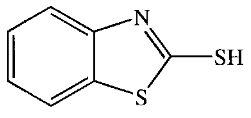
Key Words: Oligonucleotides; Phosphorothioates; Deprotection; Odorless reagent; Phosphoramidite.

Antisense oligonucleotides as modulators of gene expression represent an exciting new drug technology.[1–7] Phosphorothioate oligonucleotides are among the most intensively investigated nuclease-resistant antisense analogs, as evidenced by a number of ongoing clinical trials and one FDA approved drug. With the first antisense drug (Vitravene$^{\text{TM}}$) being approved by the US Food and Drug Administration and potentially several systemic drugs to reach market in next several years, the development of economical and environmentally safe methods for synthesis of high quality oligonucleotides has become a major focus of our research. Currently, synthesis of phosphorothioate oligodeoxyribonucleotides of 20-mer in length are routinely performed on scales up to 300–600 mmole in a cyclic manner on automated solid-phase synthesizers using β-cyanoethyl protected phosphoramidite monomers. However, this commonly used protecting group has some undesirable properties. The by-product formed during deprotection of oligonucleotide viz., acrylonitrile adds to N3 of thymine to form an irreversible adduct.[8] In addition, the manufacturing scale up of high quality phosphitylating reagent (β-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphorodiamidite) to make phosphoramidites of high grade is an issue faced by many vendors. For example, the starting alcohol viz., 3-hydroxypropionitrile is contaminated with other reactive alcohols such as methanol or ethylene glycol and also react to form corresponding amidites that get incorporated into oligonucleotide.[9] In addition, the phosphitylating reagent during distillation undergoes Arbuzov rearrangement to form undesired products. Thus, there is an urgent need to eliminate the above problems or identify an alternative protecting group.

Besides β-cyanoethyl, the most extensively investigated protecting group that does not possess the above limitations is the *O*-methyl group0.[10–15] Several laboratories have reported use of the methyl group as a phosphate protecting group during solid-phase synthesis of oligodeoxynucleotides,[16–24] oligoribonucleotides,[25–27] and α-anomeric oligodeoxyribonucleotides.[28] This group can be selectively removed by a mixture of thiophenol and triethylamine.[29] However, the reagent has an obnoxious smell and has led virtually every researcher to switch to β-cyanoethyl group. The higher reactivity of methyl compared to β-cyanoethyl phosphoramidites and a relatively low boiling point (75–78 °C/0.5 mm Hg) of corresponding phosphitylating reagent (methyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite) makes it highly desirable to have as an alternative to current β-cyanoethyl protection. To alleviate this problem other reagents such as 2-mercaptobenzothiazole **1** and disodium 2-carbamoyl-2-cyanoethylene-1, 1-dithiolate **2** have also been reported.[30,31] We report here that commercially available 2-methyl-5-*tert*-butylthiophenol **3** meets all of our



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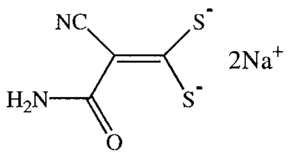

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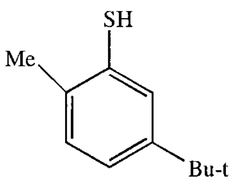

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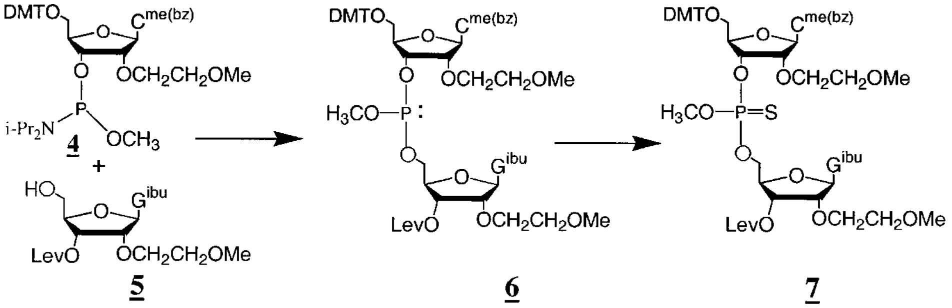
requirements and the reagent is odorless, inexpensive, non-toxic and removes methyl groups from phosphate and phosphorothioate triesters fast and clean.

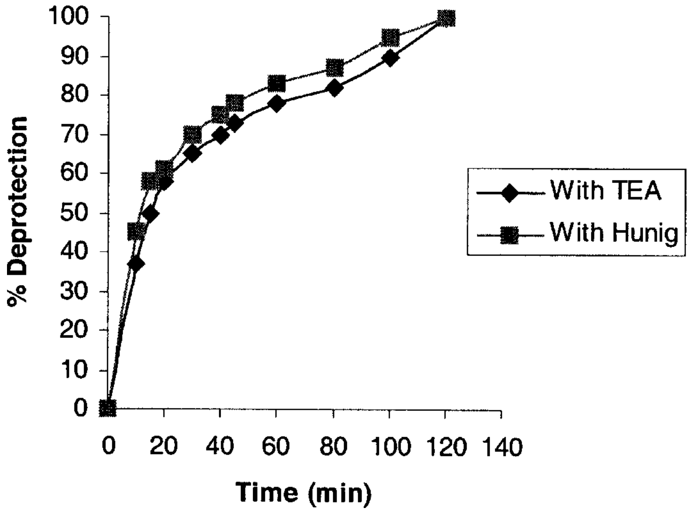
2-methyl-5-*tert*-butylthiophenol is commercially available from Clariant LSM as a colorless, odorless liquid.[32] It is miscible in organic solvents such as acetonitrile, dioxane, DMF, toluene and the solutions are stable at least for two weeks when stored under proper conditions. To investigate the deprotection reaction, a fully-protected 2′-*O*-methoxyethyl substituted phosphorothioate triester dimer 7 was synthesized in solution by coupling 5′-*O*-dimethoxytrityl-2′-*O*-methoxyethyl-N^4-benzoyl-5-methylcytidine-3′-*O*-methyl *N*, *N*′-diisopropylphosphoramidite 4 with 3′-*O*-levulinyl-2′-*O*-methoxyethyl-N^2-isobutyrylguanosine 5 in anhydrous acetonitrile in presence of 1*H*-tetrazole as activator (Sch. 1). The phosphite triester intermediate 6 was sulfurized using diethyldithiocarbonate disulfide (DDD)[33] (82% combined coupling and sulfurization yield based on 5). Sulfurization could also be efficiently performed using phenylacetyl disulfide (PADS).[34,35]

The dealkylation reactions were monitored by ^{31}P NMR spectroscopy on a Varian 200 spectrometer at 28°C in 5 mm tubes. A CD_3CN solution of 7 (0.4 M, 0.2 mL) was mixed with a CH_3CN solution of 2-methyl-5-*tert*-butylthiophenol 3 and triethylamine (1:1 mol/mol; 0.4 M, 0.2 mL) at zero time, and ^{1}H-decoupled ^{31}P NMR spectra were obtained at different time intervals. Complete deprotection of dimer was observed in 2 h. Effect of base on rate of demethylation was examined by repeating above experiment in *N*, *N*-diisopropylethylamine under identical conditions. No significant increase in the rate of deprotection was observed (Sch. 2).

Influence of solvent on rate of demethylation was also investigated. Acetonitrile was found to be the best among a variety of solvents (DMF, 1,4-dioxane, 1,2-dimethoxythane, 1-methyl-2-pyrrolidinone) evaluated. A critical requirement for removal of methyl protecting group from oligonucleotide phosphate and phosphorothioate triesters by thiolate anion is stability of the internucleotidic linkage under deprotecting conditions. The dinucleotide phosphate triester 9 (X = O) and phosphorothioate triester 9 (X = S) (Sch. 3) immobilized on Pharmacia Primer support were treated with an equimolar (1.0M) solution of 3 and triethylamine in acetonitrile at ambient temperature for 2 h. The solid support was then thoroughly washed with acetonitrile and the dimer cleaved from the support according to standard procedures. HPLC analysis of the reaction mixture indicated only the presence of product along with traces of unreacted monomer. There was no evidence of 5′-S-(2-methyl-



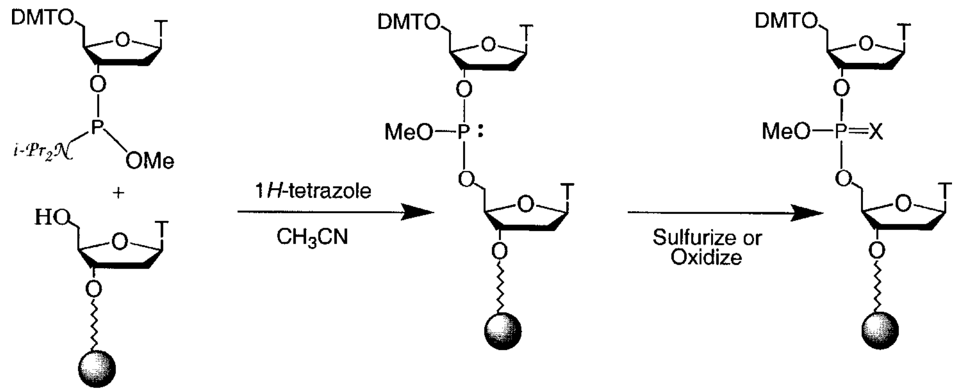
Scheme 1. Preparation of methyl protected dimer.



Scheme 2. Rate of deprotection with different bases.

5-*tert*-butyl)-thiophenylthymidine that could have been produced from nucleophilic attack of thiolate anion at 5′-carbon of the internucleotidic linkage. In addition, the HPLC profile of the dimer was superimposable with that obtained using β-cyanoethyl as protecting group. This indicates that deprotection of the methyl group by 2-methyl-5-*tert*-butylthiophenol is clean and fast relative to internucleotidic cleavage. Further attack after formation of the phosphate/phosphorothioate diester is prohibited, presumably due to significant electrostatic repulsion toward the incoming thiolate anion.

Having established the effectiveness of 3 as a fast and clean deprotecting reagent for dimers, applicability was extended to solid-supported synthesis of oligonucleotides using methoxy phosphoramidites. A homothymidine 20-mer DNA was



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Scheme 3. Synthesis of methyl protected dimers on solid support.

Table 1. Synthesis of different oligonucleotides for investigation.

Oligonucleotide	HPLC Retention time[a] (min)	Mass	
		Calculated	Found
T_{20}	17.678	6021.9	6021.2
5′-[$^{me}C^{me}UG$]-AGTCTGTTT-[$^{me}U^{me}C^{me}CA^{me}U^{me}U^{me}C^{me}U$]	21.076	6910.9	6910.8

[a] Analysis and purification of oligonucleotides by reversed phase high performance liquid chromatography (RP-HPLC) was performed on a Phenomenex Luna C_{18} column (250 × 4.6 mm, 5 μ size) using a Waters HPLC system (600E System Controller, 996 Photodiode Array Detector, 717 Autosampler). For analysis an acetonitrile (B)/0.1 M triethylammonium acetate (A) (pH 7.0) (flow rate = 1 mL/min) gradient was used: 0% to 40% B from 0 to 25 min, 40% B from 25 to 30 min, 100% B from 30 to 39 min, 100% A from 39 min to 45 min. The DMT-on fraction was collected and was evaporated in vacuum, redissolved in 50 μL water and DMT group removed as follows: An aliquot (50–100 OD) was transferred into an Eppendorff tube (1.5 mL), and 0.01 M sodium acetate (pH 3, 175 μL) was added. After 30 to 60 min at room temperature, sodium acetate (3 M, 10 μL) was added, centrifuged to remove trityl and to the supernatent was added cold ethanol (1.2 mL). The mixture was vortexed and cooled in dry ice for 20 min. The precipitate was spun down with a centrifuge, the supernatant was discarded and the precipitate was rinsed with ethanol and dried under vacuum.

synthesized on an ABI DNA/RNA synthesizer using controlled-pore glass support (loading 47 μmole/g). Detritylation was effected using 3% trichloroacetic acid in dichloromethane and oxidation was performed using iodine/water under standard conditions. The crude oligonucleotide was demethylated on support by treatment with a solution of 3:triethylamine:acetonitrile (1:1:3; v/v/v; 5 ml/g of support) at ambient temperature for 2 h. The partially deprotected oligonucleotide was cleaved from the support using concentrated aqueous ammonium hydroxide under standard conditions. Purification by reversed phase HPLC followed by LC-MS analysis indicated purity comparable to that obtained using β-cyanoethyl phosphoramidite (Table 1).

The scope of this methodology was further extended to synthesis of 2′-O-alky-oligoribonucleotides. A chimeric 20-mer oligonucleotide containing 2′-O-methoxyethyl wings [5′-($^{me}C^{me}UG$)-AGTCTGTTT-($^{me}U^{me}C^{me}CA^{me}U^{me}U^{me}C^{me}U$)] was synthesized on an ABI DNA/RNA synthesizer using controlled-pore glass support (loading 74 μmole/g). Similar oligomerization and deprotection conditions were followed as above for synthesis of homothymidine 20-mer. Purification by reversed phase HPLC followed by LC-MS analysis indicated purity comparable to those obtained using β-cyanoethyl phosphoramidite (Table 1).

In conclusion, we have demonstrated that 3 is an excellent alternative reagent for deprotection of methyl phosphotriester groups. Because of the odorless and inexpensive nature of this reagent and its rapid deprotection kinetics, it could be applicable to synthesis of RNA and other oligomeric analogs as well as in general synthetic organic chemistry where odiferous thiols have been employed.

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REFERENCES

1. Crooke, S.T. *Antisense Therapeutics in Biotechnology & Genetic Engineering Reviews*; Intercept Ltd: Hampshire, U.K. 1998; Vol. 15, 121–157.
2. Crooke, S.T. *Basic Principles of Antisense Therapeutics in Handbook of Experimental Pharmacology: Antisense Research & Application*; Crooke, S.T., Ed.; Springer-Verlag: Berlin, Germany, 1998.
3. Crooke, S.T. An overview of progress in antisense therapeutics. Antisense & Nucleic Acid Drug Dev **1998**, *8*, 115–122.
4. Crooke, S.T. *Burger's Medicinal Chemistry and Drug Discovery*, John Wiley: New York, 1995, and references cited therein.
5. Monia, B.P.; Johnston, J.F; Muller, M.; Geiger, T.H.; Fabbro, D. Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-raf kinase. Nature Med. **1996**, *2*, 668–675.
6. Knorre, G.K.; Vlassov, V.V.; Zarytova, V.F.; Lebedev, A.V.; Federova, O.S. *Design and Targeted Reactions of Oligonucleotide Derivatives*; Knorre, G.K., Vlassov, V.V., Zarytova, V.F., Lebedev, A.V., Federova, O.S., Eds.; CRC Press: Boca Raton, 1994.
7. Crooke, S.T.; Lebleu, B. *Antisense Research and Application*; CRC Press: Boca Raton, 1993: .
8. Capaldi, D.C.; Gaus, H.J.; Krotz, A.H.; Arnold, J.; Carty, R.L.; Moore, M.N.; Scozzari, A.N.; Lowery, K.; Cole, D.L.; Ravikumar, V.T. Synthesis of high quality antisense drugs: Addition of acrylonitrile to phosphorothioate oligonucleotides – Adduct characterization and avoidance. Org. Process Res. & Dev. (in press).
9. Unpublished work.
10. Beaucage, S.L.; Caruthers, M.H. Deoxynucleoside phosphoramidites – a new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Lett. **1981**, *22*, 1859–1862.
11. McBride, L.J.; Caruthers, M.H. An investigation of several deoxynucleoside phosphoramidites useful for synthesis of synthesizing deoxyoligonucleotides. Tetrahedron Lett. **1983**, *24*, 245–248.
12. Barone, A.D.; Tang, J-Y.; Caruthers, M.H. In situ activation of bis-dialkylaminophosphines – a new method for synthesizing deoxyoligonucleotides on polymer supports. Nucleic Acids Res. **1984**, *12*, 4051–4061.
13. Lee, H-J.; Moon, S-H. Bis-(N,N-dialkylamino)-alkoxyphosphines as a new class of phosphite coupling agent for the synthesis of oligonucleotides. Chem. Lett. **1984**, 1229–1232.
14. Marugg, J.E.; Burik, A.; Tromp, M.; van der Marel, G.A.; van Boom, J.H. A new and versatile approach to the preparation of valuable deoxynucleoside 3′-phosphite intermediates. Tetrahedron Lett. **1986**, *27*, 2271–2274.

15. Moore, M.F.; Beaucage, S.L. Conceptual basis of the selective activation of bis(dialkylamino)methoxyphosphines by weak acids and its application toward the preparation of deoxynucleoside phosphoramidites in situ. J. Org. Chem. **1985**, *50*, 2019–2025.
16. Neuner, P.; Cortese, R.; Monaci, P. Codon-based mutagenesis using dimer-phosphoramidites. Nucleic Acid Res. **1998**, *26*, 1223–1227.
17. Chan, Y-L.; Lin, A.; Paz, V.; Wool, I.G. The primary structure of rat ribosomal protein S8. Nucleic Acid Res. **1987**, *15*, 9451–9459.
18. Seela, F.; Kehne, A.; Herdering, W.; Kretschmer, U. Phosphoramidites of [oxygen-18] chiral (Rp)- and (Sp)-configurated dimer-blocks and their use in automated synthesis. Nucleosides Nucleotides **1987**, *6*, 451–456.
19. Haralambidis, J.; Duncan, L.; Tregear, G.W. The solid phase synthesis of oligonucleotides containing a 3′-peptide moiety. Tetrahedron Lett. **1987**, *28*, 5199–5202.
20. McBride, L.J.; Eadie, J.S.; Efcavitch, J.W.; Andrus, W.A. Base modification and the phosphoramidite approach. Nucleosides Nucleotides **1987**, *6*, 297–300.
21. Bannwarth, W. Synthesis of oligodeoxynucleotides by the phosphite-triester method using dimmer units and different phosphorous-protecting groups. Helv. Chim. Acta. **1985**, *68*, 1907–1913.
22. Kumar, G.; Poonian, M.S. Improvments in oligodeoxyribonucleotide synthesis: Methyl N,N-dialkylphosphoramidite dimmer units for solid support phosphite methodology. J. Org. Chem. **1984**, *49*, 4905–4912.
23. Dorman, M.A.; Noble, S.A.; McBride, L.J.; Caruthers, M.H. Synthesis of oligodeoxynucleotides and oligodeoxynucleotide analogs using phosphoramidite intermediates. Tetrahedron **1984**, *40*, 95–102.
24. Smith, D.J.H.; Ogilvie, K.K.; Gillen, M.F. The methyl group as phosphate protecting group in nucleotide synthesis. Tetrahedron Lett. **1980**, *21*, 861–864.
25. Scaringe, S.A.; Wincott, F.E.; Caruthers, M.H. Novel RNA synthesis method using 5′-*O*-silyl-2′-*O*-orthoester protecting groups. J. Am. Chem. Soc. **1998**, *120*, 11,820–11,821.
26. Usman, N.; Ogilvie, K.K.; Jiang, M-Y.; Cedergren, R.J. The automated chemical synthesis of long oligoribonucleotides using 2′-O-silylated ribonucleoside 3′-O-phosphoramidites on a controlled-pore glass support: synthesis of a 43-nucleotide sequence similar to the 3′-helf molecule of an Escherichia coli formylmethionine tRNA. J. Am. Chem. Soc. **1987**, *109*, 7845–7854.
27. Damha, M.J.; Ogilvie, K.K. Protocols for oligonucleotides and Analogs. In *Methods in Molecular Biology*; Agrawal, S., Eds.; Humana Press: Totowa, NJ 1993, Vol. 20, 81–114.
28. Morvan, F.; Rayner, B.; Leonetti, J.P.; Imbach, J.L. Nucleic Acids Res. **1988**, *16*, 833–847.
29. Daub, G.W.; van Tamelen, E.E. Synthesis of oligoribonucleotides based on the facile cleavage of methyl phosphotriester intermediates. J. Am. Chem. Soc. **1977**, *99*, 3526–3528.
30. Andrus, A.; Beaucage, S.L. 2-Mercaptobenzothazole – an improved reagent for the removal of methyl phosphate protecting groups from oligodeoxynucleotide phosphotriesters. Tetrahedron Lett. **1988**, *29*, 5479–5482.

31. Dahl, B.H.; Bjergarde, K.; Henriksen, L.; Dahl, O. A highly reactive, odourless substitute for thiophenol/triethylamine as a deprotection reagent in the synthesis of oligonucleotides and their analogues. Acta Chem. Scand. **1990**, *44*, 639–641.
32. The price of the reagent is ca $65/Kg and can be used without further purification.
33. Eleuteri, A.; Cheruvallath, Z.S.; Capaldi, D.C.; Cole, D.L.; Ravikumar, V.T. Synthesis of dimer phosphoramidite synthons for oligodeoxyribonucleotide phosphorothioates using diethyldithiocarbonate disulfide as an efficient sulfurizing reagent. Nucleosides Nucleotides **1999**, *18*, 1803–1807.
34. Cheruvallath, Z.S.; Wheeler, P.D.; Cole, D.L.; Ravikumar, V.T. Use of phenylacetyl disulfide (PADS) in the synthesis of oligodeoxyribonucleotide phosphorothioates. Nucleosides Nucleotides **1999**, *18*, 485–492.
35. Cheruvallath, Z.S.; Carty, R.L.; Moore, M.N.; Capaldi, D.C.; Krotz, A.H.; Wheeler, P.D.; Turney, B.J.; Craig, S.R.; Gaus, H.J.; Scozzari, A.N.; Cole, D.L.; Ravikumar, V.T. Synthesis of antisense oligonucleotides: Replacement of 3H-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent) with phenylacetyl disulfide (PADS) as efficient sulfurization reagent: From bench to bulk manufacture of active pharmaceutical ingredient. Org. Process Res. Dev. **2000**, *4*, 199–204.

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