JOC Article

Thermolytic Properties of 3-(2-Pyridyl)-1-propyl and 2-[*N*-Methyl-*N*-(2-pyridyl)]aminoethyl Phosphate/Thiophosphate Protecting Groups in Solid-Phase Synthesis of Oligodeoxyribonucleotides

Jacek Cieślak† and Serge L. Beaucage*

Division of Therapeutic Proteins, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892

beaucage@cber.fda.gov

Received October 2, 2003

Thermolytic groups may serve as alternatives to the conventional 2-cyanoethyl group for phosphate/thiophosphate protection in solid-phase oligonucleotide synthesis to prevent DNA alkylation by acrylonitrile generated under the basic conditions used for oligonucleotide deprotection. Additionally, thermolytic groups are attractive in the context of engineering a "heat-driven" process for the synthesis of oligonucleotides on diagnostic microarrays. In these regards, the potential application of pyridine derivatives as thermolytic phosphate/thiophosphate protecting groups has been investigated. Specifically, 2-pyridinepropanol and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethanol were incorporated into deoxyribonucleoside phosphoramidites **7a**−**d** and **9**, which were found as efficient as 2-cyanoethyl deoxyribonucleoside phosphoramidites in solid-phase oligonucleotide synthesis. Whereas the removal of 3-(2-pyridyl)-1-propyl phosphate/thiophosphate protecting groups from oligonucleotides is effected within 30 min upon heating at 55 °C in concentrated NH_4OH or in an aqueous buffer at pH 7.0, cleavage of 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl groups occurs spontaneously when their phosphate or phosphorothioate esters are formed during oligonucleotide synthesis. The deprotection of these groups follows a cyclodeesterification process generating the bicyclic salts **13** and **14** as side products. These salts do not alkylate or otherwise modify any DNA nucleobases and do not desulfurize a phosphorothioate diester model under conditions mimicking large-scale oligonucleotide deprotection.

Introduction

In recent years we have been investigating cyclic *N*-acylphosphoramidites in the solid-phase synthesis of DNA oligonucleotides and their phosphorothioate analogues.[1] This method led to the discovery of several thermolabile phosphate/thiophosphate protecting groups.[1,2] Interestingly, the thermolytic cleavage of these protecting groups proceeds through an intramolecular cyclodeesterification mechanism reminiscent to that of the base-assisted deprotection of 4-[*N*-(2,2,2-trifluoroacetyl)amino]butyl and 4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl phosphate protecting groups described earlier by us.[3] Such a deprotection mechanism departs from that of the 2-cyanoethyl phosphate/thiophosphate protecting group,[4] which occurs under basic conditions via elimination of acrylonitrile, a potent carcinogen known to alkylate the nucleobases of nucleosides and nucleic acids.[3a] By comparison, deprotection of the thermolabile groups investigated so far for phosphate/thiophosphate protection has not generated mutagenic side products.[2,3] The use of these phosphate/thiophosphate protecting groups has therefore been recommended for large-scale syntheses of alkylation-free therapeutic oligonucleotides.

Although the discovery of thermolytic phosphate/thiophosphate protecting groups may be valuable in large-scale preparations of oligonucleotide drugs, it may also lead to the development of thermolabile 5′-/3′-hydroxyl protecting groups in the synthesis of oligonucleotides on diagnostic microarrays. In this context, given that ketoalkyl and amidoalkyl phosphate/thiophosphate protecting groups (shown as **1** and **2**) exhibited striking thermolytic deprotection properties,[2] we rationalized that

* To whom correspondence should be addressed. Tel: (301) 827-5162. Fax: (301) 480-3256.

† On leave from the Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan, Poland.

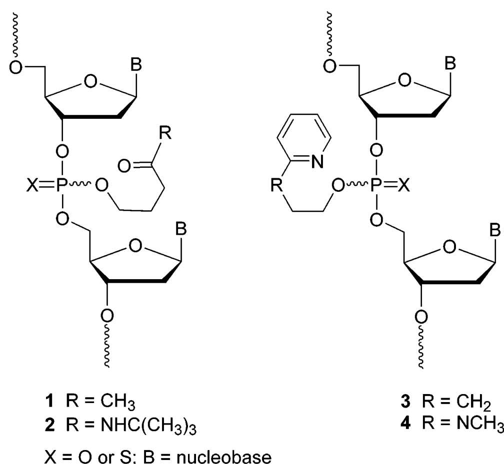
(1) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Am. Chem. Soc.* **2000**, *122*, 2149−2156.

(2) (a) Grajkowski, A.; Wilk, A.; Chmielewski, M. K.; Phillips, L. R.; Beaucage, S. L. *Org. Lett.* **2001**, *3*, 1287−1290. (b) Wilk, A.; Chmielewski, M. K.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *Tetrahedron Lett.* **2001**, *42*, 5635−5639. (c) Wilk, A.; Chmielewski, M. K.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Org. Chem.* **2002**, *67*, 6430−6438. (d) Wilk, A., Chmielewski, M. K.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A., Eds.; John Wiley & Sons: New York, 2002; pp 3.9.1−3.9.15.

(3) (a) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Org. Chem.* **1999**, *64*, 7515−7522. (b) Wilk, A.; Grajkowski, A.; Chmielewski, M. K.; Phillips, L. R.; Beaucage, S. L. In *Current Protocols in Nucleic Acid Chemistry*; Beaucage, S. L., Bergstrom, D. E., Glick, G. D., Jones, R. A. Eds.; John Wiley & Sons: New York, 2001; pp 2.7.1−2.7.12. (c) Wilk, A.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1997**, *62*, 6712−6713.

(4) Tener, G. M. *J. Am. Chem. Soc.* **1961**, *83*, 159−168.

10.1021/jo0354490 This article not subject to U.S. Copyright. Published 2003 American Chemical Society
Published on Web 12/03/2003



2-pyridinylalkyl and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoalkyl phosphate/thiophosphate protecting groups (shown as **3** and **4**) may significantly accelerate cyclodeesterification deprotection reactions, considering the inherent nucleophilicity of pyridine derivatives.

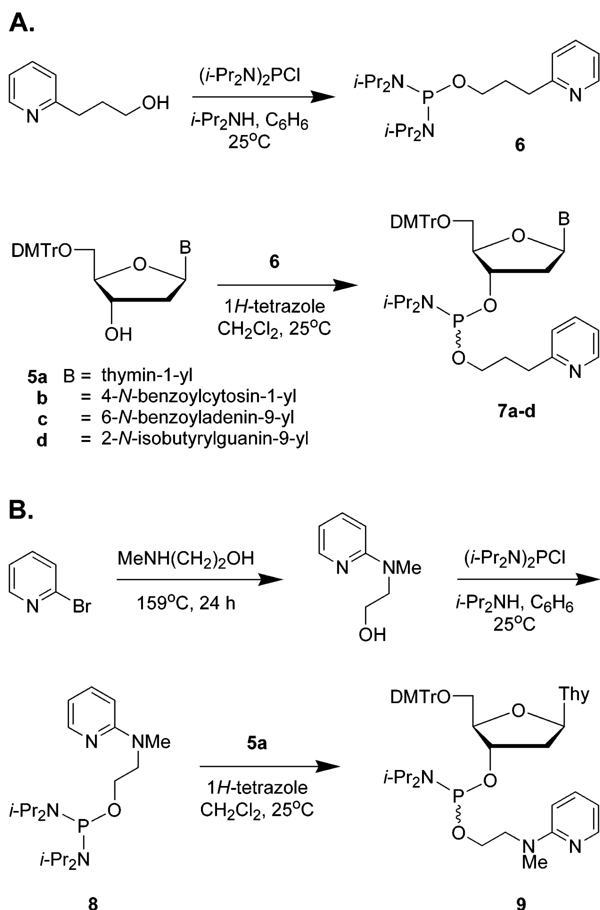


FIGURE 1. Synthesis of deoxyribonucleoside phosphoramidites **7a–d** and **9**. Keys: DMTr, 4,4-dimethoxytrityl; Thy, thymin-1-yl.

We set out to investigate this rationale through the preparation of deoxyribonucleoside phosphoramidites **7a–d** (Figure 1A) and **9** (Figure 1B), and incorporation of these monomers into oligonucleotides.

Results and Discussion

Synthesis of phosphoramidites **7a–d** is achieved by condensing each of the commercially available deoxyribonucleosides **5a–d** with phosphorodiamidite **6** in the presence of 1*H*-tetrazole[5] in anhydrous dichloromethane (Figure 1A). The phosphorodiamidite **6** is obtained from the reaction of 2-pyridinepropanol with bis(*N*,*N*-diisopropylamino)chlorophosphine generated in situ upon mixing phosphorus trichloride with an excess *N*,*N*-diisopropylamine in dry benzene.[2] The preparation of phosphoramidite **9** is accomplished through the condensation of **5a** with phosphorodiamidite **8** (Figure 1B) in a manner similar to that described for the synthesis of phosphoramidites **7a–d**. As for **6**, phosphorodiamidite **8** is generated from bis(*N*,*N*-diisopropylamino)chlorophosphine and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethanol, which is obtained by heating 2-bromopyridine and 2-methylaminoethanol as reported in the literature.[6] The crude deoxyribonucleoside phosphoramidites **7a–d** and **9** are purified by silica gel chromatography and are isolated in yields ranging from 70% to 80%. Identity of these phosphoramidites is confirmed by ^{31}P NMR spectroscopy and high-resolution mass spectrometry. The phosphoramidites **7a** and **9** are then used in the solid-phase synthesis of dinucleotides to independently assess the thermolytic deprotection kinetics of both 3-(2-pyridyl)-1-propyl and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl phosphate protecting groups through reversed-phase high performance liquid chromatography (RP-HPLC) analyses.

Thermolytic Properties of the 3-(2-Pyridyl)-1-propyl and 2-[*N*-Methyl-*N*-(2-pyridyl)]aminoethyl Groups as Phosphate/Thiophosphate Protecting Groups for DNA Oligonucleotides. Treatment of the solid-phase-linked dinucleoside phosphotriester **10** (Figure 2) with pressurized methylamine gas for 3 min at 25 °C, followed by washing of the support with 0.1 M triethylammonium acetate (TEAA, pH 7.0)/MeCN (3:2 v/v), afforded a mixture of dinucleotides composed of phosphotriester **11** (40%) and phosphodiester TpT (**12**, 60%) on the basis of RP-HPLC analysis of the eluates. A longer exposure of **10** to pressurized methylamine gas (30 min, 25 °C) did not change the ratio of **11** and **12**. Complete removal of the 3-(2-pyridyl)-1-propyl group from **11** is accomplished within 30 min ($t_{1/2}$ ~225 s) or 5 min ($t_{1/2}$ ~40 s) upon heating the eluates at 55 or 90 °C,[7] respectively. By comparison, treatment of **10** with concentrated NH_4OH for 30 min at 25 °C followed by heating the ammoniacal solution for an additional 30 min at 55 °C also led to complete cleavage of the 3-(2-pyridyl)-1-propyl group from **11**, as **12** was the only nucleotidic species detected by RP-HPLC analysis of the deprotection reaction.

In the absence of NH_4OH, the rates at which the 3-(2-pyridyl)-1-propyl group is removed from **11** in aqueous buffers are pH-dependent. Specifically, when a citric acid buffer (pH 4.0) is used instead of 0.1 M TEAA (pH 7.0)/MeCN (3:2 v/v), removal of the 3-(2-pyridyl)-1-propyl phosphate protecting group is 99% complete only after heating for 60 min at 55 °C. The slower deprotection rates, relative to those determined at pH 7.0 (100%, 30 min, 55 °C), are likely due to the decreased nucleophi-

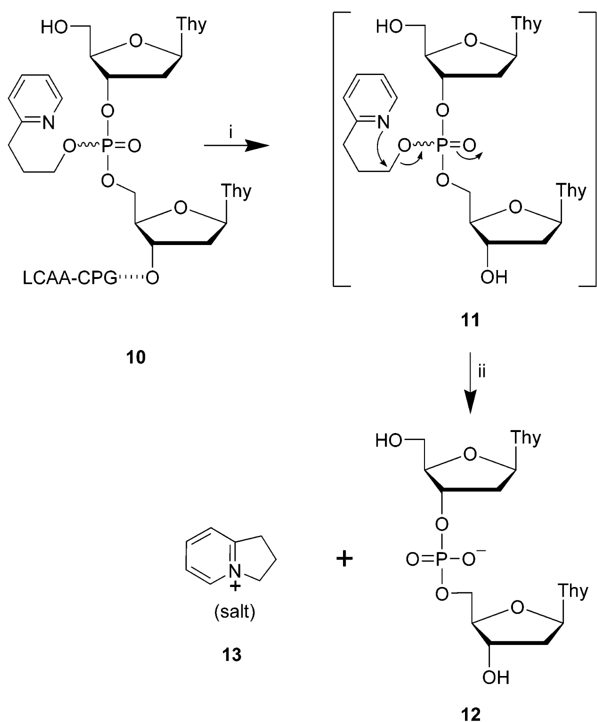


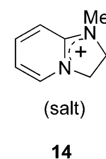
FIGURE 2. Thermolytic cleavage of the 3-(2-pyridyl)-1-propyl phosphate protecting group from a dinucleotide. Conditions: (i) $MeNH_2$ gas (2.5 bar), 3 min, 25 °C or concentrated NH_4OH, 30 min, 25 °C; (ii) 0.1 M TEAA (pH 7.0)/MeCN (3:2 v/v), 30 min, 55 °C or concentrated NH_4OH, 30 min, 55 °C. Keys: Thy, thymin-1-yl; LCAA-CPG, succinyl long chain alkylamine controlled-pore glass.

licity of the partially protonated pyridyl group at pH 4.0 inhibiting the cyclodeesterification reaction (Figure 2).

Replacement of the 3-(2-pyridyl)-1-propyl group with the 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl group for phosphate protection in **10** and exposure of the support-bound dinucleoside phosphotriester to pressurized methylamine gas for 3 min result in the quantitative formation of **12** according to RP-HPLC analysis of the deprotection reaction. This result is consistent with the increased nucleophilicity of the pyridyl group caused by the electron-donating 2-dialkylamino function, which accelerates the cyclodeesterification reaction leading to the formation of **12**. Because it has been demonstrated that exposure of **10** to pressurized methylamine for either 3 or 30 min has no significant effect on the production of **12** (vide supra), it would appear that the cleavage of both 3-(2-pyridyl)-1-propyl and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl phosphate protecting groups begins when the phosphite triester is oxidized to the corresponding phosphate triester during solid-phase oligonucleotide synthesis. Particularly noteworthy is the complete removal of the 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl phosphate protecting group during the oxidation reaction (vide infra). Thermal deprotection of the 3-(2-pyridyl)-1-propyl and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl groups from dinucleoside phosphorothioate triesters under conditions identical to those used for the parent dinucleoside phosphotriesters (Figure 2) proceeds, as expected, with similar kinetics (data not shown). No other thermolytic phosphate/thiophosphate protecting groups reported earlier by us exhibited such a rapid deprotection kinetics.

Synthesis and Characterization of Cyclodeesterification Products. On the basis of our previous experience with cyclodeesterification of thermolytic phosphate/thiophosphate protecting groups,[1,2] formation of the bicyclic pyridinium salt **13** is expected from the thermal deprotection of **10** and **11**. Indeed, RP-HPLC analysis of the deprotection reaction revealed, aside from **12** (t_R = 14.7 min), a new fast-eluting compound (t_R = 3.4 min).[8] This compound was collected and characterized by high-resolution mass spectrometry. The accurate mass found for the fast-eluting material is consistent with that expected for **13** (see Experimental Section). To further corroborate the identity of **13**, its synthesis is achieved by heating a solution of 2-pyridinepropanol and trifluoroacetic anhydride (TFAA) in MeCN (1:5:5 v/v/v). RP-HPLC analysis of the reaction product showed a single peak exhibiting a retention time (3.4 min) identical to that recorded for **13** under the same chromatographic conditions. ^{1}H and ^{13}C NMR spectra of the reaction product are in agreement with the structure proposed for **13** (see Experimental Section).

When the 3-(2-pyridyl)-1-propyl phosphate protecting group in **10** is replaced with the 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl group, the cyclodeesterification reaction product **14** is immediately formed and washed off the support. Short exposure of the support-linked dinucleotide to pressurized methylamine gas leaves **12** as the only species detectable by RP-HPLC.



Thus, a strategy different than that used for isolating **13** had to be devised to capture and identify **14**. Specifically, condensation of the phosphoramidite **9** with a slight excess of 3′-*O*-acetylthymidine and 1*H*-tetrazole in anhydrous MeCN afforded the dinucleoside phosphite triester product in essentially quantitative yield according to ^{31}P NMR analysis of the reaction mixture, which displayed two characteristic signals at ~144 ppm. Addition of solid 3*H*-1,2-benzodithiol-3-one 1,1-dioxide to the solution rapidly converted the phosphite triester to the parent dinucleoside phosphorothioate diester characterized by two ^{31}P NMR signals at ~60 ppm. These signals confirmed the rapid formation of **14** given the absence of ^{31}P NMR signals corresponding to the dinucleoside phosphorothioate triester expected at ~70 ppm. RP-HPLC analysis of the solution validated the presence of **14** as a peak displaying a retention time (4.9 min) comparable to that of **13** (t_R = 3.4 min) under identical chromatographic conditions. The fast-eluting material was collected and characterized by high-resolution mass spectrometry. The results of accurate mass determination are in accordance with the mass expected for **14** (see Experimental Section). Identity of the bicyclic salt is further established through its chemical synthesis by heating a solution of 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethanol and TFAA in MeCN, as described above for the preparation of **13**. The reaction product shows as a single RP-HPLC peak (t_R = 4.9 min) identical to that of **14** under the same chromatographic conditions. ^{1}H and ^{13}C NMR spectra of

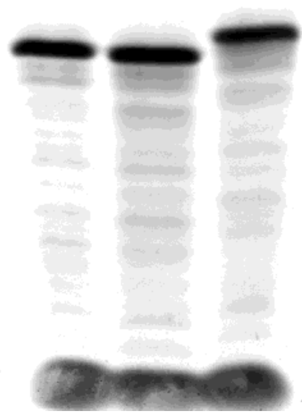


FIGURE 3. Polyacrylamide gel electrophoresis analysis of d(ATCCGTAGCTAAGGTCATGC) and its phosphorothioated analogue under denaturing conditions (7 M urea, 1X TBE buffer, pH 8.3). Left lane: crude oligomer synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites and deprotected by treatment with concentrated NH_4OH for 10 h at 55 °C. Middle lane: crude oligomer synthesized from 1-week-old solutions of **7a**−**d** and deprotected under conditions identical to those used for the 20-mer shown in the left lane. Right lane: crude phosphorothioated oligomer synthesized from fresh solutions of **7a**−**d** and deprotected under conditions identical to those used for the 20-mer shown in the left lane. Unmodified oligonucleotides are visualized as blue bands and fully phosphorothioated oligonucleotides as purple bands, upon staining the gel with Stains-all. Bromophenol blue is used as a marker and shows as a large band in each lane at the bottom of the gel.

the reaction product agree with the structure proposed for **14** (see Experimental Section) and support an operative cyclodeesterification processs for the removal of 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl phosphate/thiophosphate protecting groups.

Synthesis and Characterization of Oligodeoxyribonucleotides. Automated solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) and that of its fully phosphorothioated analogue[9] is performed using fresh and 1-week-old solutions of **7a**−**d** for the purpose of comparing coupling efficiency and stability of the phosphoramidites in solution with that of commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites.

The solid-phase-linked oligonucleotides are released from the support by treatment with concentrated NH_4OH, and the eluates are heated for 10 h at 55 °C to complete nucleobase and phosphate/thiophosphate deprotection. The crude oligonucleotides are compared with each other using RP-HPLC and polyacrylamide gel electrophoresis (PAGE) techniques. Figure 3 shows that synthesis of DNA oligonucleotides using phosphoramidites **7a**−**d** is as efficient as that achieved with commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites when evaluating the relative intensity of bands corresponding to shorter than full-length sequences on the gel. RP-HPLC chromatograms of the crude oligonucleotides are consistent with PAGE data (see Supporting Information).

Given the structural similarities of phosphoramidites **9** and **7a**−**d** in regard to phosphorus protection, preparation of the remaining three 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl deoxyribonucleoside phosphoramidites to assess their suitability for solid-phase oligonucleotide synthesis seems unnecessary. Instead, the site-specific incorporation of **9** depicted as T* into the DNA oligonucleotide sequence d(AT*T*CGT*AGCT*AAGGT*CAT*GC) and into that of its phosphorothioated analogue should be sufficient to demonstrate the comparability of these oligonucleotides with those synthesized using **7a**−**d** or 2-cyanoethyl deoxyribonucleoside phosphoramidites in terms of ease of synthesis and deprotection, purity, and yields. Such a comparability study is validated on the basis of RP-HPLC and PAGE data (shown as Supporting Information). In addition, native DNA oligonucleotides prepared via **7a**−**d**, **9**, and 2-cyanoethyl deoxyribonucleoside phosphoramidites were exposed to bacterial alkaline phosphatase and snake venom phosphodiesterase to assess any nucleobase modifications that might have occurred through the use of these phosphoramidites. RP-HPLC analysis of the enzymatic digests shows no detectable nucleobase modification and indicates that the use of **7a**−**d** or **9** (data shown as Supporting Information) produces oligonucleotides of quality comparable to those prepared using 2-cyanoethyl deoxyribonucleoside phosphoramidites.

We have earlier reported that the concentration of acrylonitrile that is generated from base-assisted β-elimination of 2-cyanoethyl phosphate/thiophosphate groups, under conditions mimicking large-scale oligonucleotide deprotection (>50 mmol), converted thymidine to N^3-(2-cyanoethyl)thymidine to the extent of 11%.[3a] Because large-scale preparations of therapeutic oligonucleotides are required for clinical studies, it becomes imperative to investigate whether oligonucleotides synthesized via **7a**−**d** or **9** would undergo nucleobase alkylation under deprotection conditions comparable to those used for large-scale syntheses of therapeutic oligonucleotides.

Thus, thymidine, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, N^4-benzoyl-2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, and N^2-isobutyryl-2′-deoxyguanosine are mixed with the 2,2,2-trifluoroacetate salt of **13** or that of **14** in NH_4OH in concentrations representative to those existing in large-scale (>50 mmol) oligonucleotide deprotection and heated for 10 h at 55 °C.[10] The simulation reaction involving either **13** or **14** is then analyzed by RP-HPLC to assess the extent of nucleobase modification. No nucleobase alkylation products or other nucleoside modifications are detected under these conditions, thus indicating that the use of phosphoramidites **7a**−**d** and/or **9** and its congeners is recommended over 2-cyanoethyl deoxyribonucleoside phosphoramidites for the large-scale preparation of *alkylation-free* therapeutic oligonucleotides.

Because small-scale (0.2 μmol) solid-phase synthesis of phosphorothioated oligonucleotides via **7a**−**d** or **9** proceeds without significant desulfurization during either chain assembly or oligonucleotide deprotection, as judged by ^{31}P NMR analysis of fully deprotected phosphorothioated oligonucleotides (see Supporting Information), it still

(5) (a) Lee, H.-J.; Moon, S.-H. *Chem. Lett.* **1984**, 1229−1232. (b) Barone, A. D.; Tang, J.-Y.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 4051−4061.

(6) Weiner, N.; Kaye, I. A. *J. Org. Chem.* **1949**, *14*, 9353−9372.

remains to demonstrate whether **13** or **14** can induce desulfurization of phosphorothioated oligonucleotides when produced in concentrations similar to those prevailing under large-scale oligonucleotide deprotection conditions. To confirm or reject this possibility, the potassium salt of *O,O*-diethyl thiophosphate is used as a model and is mixed with the 2,2,2-trifluoroacetate salt of **13** or that of **14** under conditions simulating large-scale oligonucleotide deprotection (see Experimental Section). ^{31}P NMR analysis of the reactions did not reveal any conversion of *O,O*-diethyl thiophosphate (δ_P ~55 ppm) to *O,O*-diethyl phosphate (δ_P ~2 ppm) caused by either **13** or **14** (see Supporting Information). It is therefore likely that **13** or **14** will not induce desulfurization of thioated oligonucleotides produced on a large-scale process.

Conclusion

We have demonstrated that the 3-(2-pyridyl)-1-propyl and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl groups are appealing phosphate/thiophosphate protecting groups for solid-phase DNA oligonucleotide synthesis. These protecting groups are cleaved from DNA oligonucleotides under the mildest conditions ever applied to thermolytic phosphate/thiophosphate protecting groups, whether in the presence or absence of concentrated NH_4OH. Like all thermolytic phosphate/thiophosphate protecting groups we have studied so far, removal of the 3-(2-pyridyl)-1-propyl and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl groups from oligonucleotides follows a cyclodeesterification pathway with the concomitant formation of bicyclic salt side products, which are innocuous to both DNA nucleobases and phosphorothioate diester groups. It should be pointed out that exposure of these functional groups to the bicyclic salts is minimal during oligonucleotide deprotection. Indeed, cleavage of the 3-(2-pyridyl)-1-propyl or 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl phosphate/thiophosphate protecting group is ~60% or 100% complete, respectively, after each oxidative conversion of an internucleosidic phosphite triester linkage to its phosphate/thiophosphate counterpart throughout oligonucleotide assembly. Consequently, the bicyclic salts **13** and **14** are washed off the solid support as they are generated, thereby minimizing potential nucleobase modification and desulfurization of phosphorothioate diesters. This feature would certainly be valuable in the large-scale preparation of therapeutic DNA oligonucleotides as it would preserve genetic fidelity and maintain the inherent ability of thioated oligonucleotides to resist ubiquitous nucleases. It is also very likely that the 3-(2-pyridyl)-1-propyl or 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl phosphate/thiophosphate protecting group may find applications in the solid-phase synthesis of oligoribonucleotides, since these oligomers are currently in high demand considering the biomedical significance of short interfering double-stranded RNAs[11] in silencing gene expression. It is worth restating that the use of thermolytic groups for phosphate and hydroxyl protection in oligonucleotide synthesis on planar glass surfaces is very attractive because it would decrease exposure of both oligonucleotides and glass surface to the harsh chemical reagents that are currently employed in conventional solid-phase oligonucleotide synthesis. The 3-(2-pyridyl)-1-propyl and 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethyl groups for phosphate/thiophospate protection thus add to the repertoire of protecting groups exhibiting thermolytic properties.

(7) Comparatively, thermal deprotection of the 3-(*N*-*tert*-butylcarboxamido)-1-propyl group from dinucleoside phosphotriesters is significantly slower ($t_{1/2}$ ~100 s, see ref 2c) under similar conditions (pH 7.0, 90 °C).

Experimental Section

***O*-[3-(2-Pyridyl)-1-propyl] *N,N,N′,N′*-Tetraisopropylphosphorodiamidite (6).** 2-Pyridinepropanol (3.1 mL, 24 mmol) is added by syringe to a stirred solution of bis(*N,N*-diisopropylamino)chlorophosphine[2c,d] (20 mmol) in dry benzene (100 mL) at 25 °C. ^{31}P NMR analysis of the reaction mixture indicates that bis(*N,N*-diisopropylamino)chlorophosphine (δ_P 135.5 ppm) is converted to the corresponding phosphorodiamidite (δ_P 123.3 ppm) within 2 h. The suspension is filtered, and the filtrate is evaporated under reduced pressure to afford an oil, which is used without further purification in the preparation of **7a**−**d**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.51 (ddd, J = 1.0, 1.8, 4.9 Hz, 1H), 7.56 (ddd, J = 1.8, 7.6, 7.8 Hz, 1H), 7.15 (m, 1H), 7.07 (ddd, J = 1.0, 4.9, 7.6 Hz, 1H), 3.60 (dt, $^3J_{PH}$ = 7.3 Hz, J = 6.3 Hz, 2H), 3.54 (sept, J = 6.8 Hz, 2H), 3.50 (sept, J = 6.8 Hz, 2H), 2.89 (m, 2H), 2.02 (m, 2H), 1.16 (d, J = 6.8 Hz, 12H), 1.15 (d, J = 6.8 Hz, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 23.7, 23.8, 24.6, 24.7, 31.6 (d, $^3J_{PC}$ = 9.5 Hz), 35.1, 44.2, 44.3, 63.6 (d, $^2J_{PC}$ = 21.2 Hz), 120.9, 122.8, 136.1, 149.3, 162.1. ^{31}P NMR (121 MHz, $CDCl_3$): δ 124.6.

***O*-[2-[*N*-Methyl-*N*-(2-pyridyl)]aminoethyl] *N,N,N′,N′*-Tetraisopropylphosphorodiamidite (8).** This phosphinylating agent is prepared under conditions identical to those described for the preparation of **6**. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.12 (ddd, J = 0.9, 2.0, 4.9 Hz, 1H), 7.39 (ddd, J = 2.0, 7.1, 8.8 Hz, 1H), 6.49 (m, 2H), 3.73 (m, 4H), 3.51 (sept, J = 6.8 Hz, 2H), 3.48 (sept, J = 6.8 Hz, 2H), 3.1 (s, 3H), 1.14 (d, J = 6.8 Hz, 12H), 1.12 (d, J = 6.8 Hz, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 23.77, 23.83, 24.4, 24.5, 37.2, 44.2, 44.4, 51.4 (d, $^3J_{PC}$ = 9.6 Hz), 61.9 (d, $^2J_{PC}$ = 20.3 Hz), 105.6, 111.2, 128.2, 136.8, 147.8, 158.5. ^{31}P NMR (121 MHz, $CDCl_3$): δ 123.8.

General Procedure for Preparation of Deoxyribonucleoside Phosphoramidites 7a−**d.** Crude **6** (700 mg, ~2 mmol) is added by syringe under an inert atmosphere to a stirred solution of **5a**−**d** (2.2 mmol) in dry CH_2Cl_2 (10 mL). Sublimed 1*H*-tetrazole (112 mg, 1.6 mmol) is then added, portionwise, under a positive pressure of argon to the solution. The reaction is monitored by ^{31}P NMR spectroscopy until **6** is all reacted (<2 h). Triethylamine (1 mL) is added to the solution, which is then immediately evaporated under reduced pressure. The material left is purified by silica gel chromatography using benzene/triethylamine (9:1 v/v) as the eluent. Fractions containing the product are pooled together and rotoevaporated under reduced pressure to a white foam. A solution of the foamy material in dry benzene is frozen and lyophilized under high vacuum to afford pure **7a**−**d** as white powders in yields ranging from 70% to 80%.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N,N*-diisopropylamino)-[3-(2-pyridyl)-1-propyloxy]phosphinyl-2′-deoxythymidine (7a). ^{31}P NMR (121 MHz, $CDCl_3$): δ, 147.1, 147.6. FAB−HRMS: calcd for $C_{45}H_{55}N_4O_8P$ $(M + Cs)^+$ 943.2819, found 943.2812.

***N*[4]-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*N,N*-diisopropylamino)-[3-(2-pyridyl)-1-propyloxy]phosphinyl-2′-deoxycytidine (7b).** ^{31}P NMR (121 MHz, $CDCl_3$): δ, 147.4, 147.7. FAB−HRMS: calcd for $C_{51}H_{58}N_5O_8P$ $(M + Cs)^+$ 1032.3075, found 1032.3077.

***N*[6]-Benzoyl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*N,N*-diisopropylamino)-[3-(2-pyridyl)-1-propyloxy]phosphinyl-2′-deoxyadenosine (7c).** ^{31}P NMR (121 MHz, $CDCl_3$): δ, 147.3, 147.6. FAB−HRMS: calcd for $C_{52}H_{58}N_7O_7P$ $(M + Cs)^+$ 1056.3176, found 1056.3190.

N^2-Isobutyryl-5′-*O*-(4,4′-dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)-[3-(2-pyridyl)-1-propyloxy]phosphinyl-2′-deoxyguanosine (7d). ^{31}P NMR (121 MHz, $CDCl_3$): δ, 147.3, 147.5. FAB−HRMS: calcd for $C_{49}H_{60}N_7O_8P$ $(M + Cs)^+$ 1038.3329, found 1038.3295.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*N*,*N*-diisopropylamino)-[2-[*N*-methyl-*N*-(2-pyridyl)]aminoethoxy]phosphinyl-2′-deoxythymidine (9). This compound is prepared from **5a** and **8**, purified, and isolated in a manner identical to that described for the preparation of **7a**−**d**. ^{31}P NMR (121 MHz, $CDCl_3$): δ, 152.0, 152.2. FAB−HRMS: calcd for $C_{45}H_{55}N_5O_8P$ $(M - H)^+$ 824.3820, found 824.3788.

Preparation and Characterization of Oligonucleotides. It is important to dissolve each of the purified deoxyribonucleoside phosphoramidites **7a**−**d** in dry benzene and lyophilize each frozen solution to eliminate residual triethylamine that is carried through the phosphoramidite purification and postpurification processes. Automated solid-phase synthesis of d(ATCCGTAGCTAAGGTCATGC) and that of its phosphorothioated analogue is performed on a 0.2-μmol scale using phosphoramidites **7a**−**d** as 0.2 M solutions in dry MeCN. The preparation of d(AT*T*CGT*AGCT*AAGGT*CAT*GC) and that of its phosphorothioated analogue is also achieved under similar conditions via 2-cyanoethyl deoxyribonucleoside phosphoramidites and **9** (T*) as 0.1 M solutions in dry MeCN. All reagents needed for the preparation of oligonucleotides were purchased and used as recommended by the instrument's manufacturer. The sulfurization reaction that is indicated for the preparation of phosphorothioated oligodeoxyribonucleosides is performed employing 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN as recommended in the literature.[12] The crude oligomers and their phosphorothioated analogues are released from the CPG support by treatment with concentrated NH_4OH for 30 min at 25 °C. Each of the oligonucleotide solutions is then heated for 10 h at 55 °C to ensure complete nucleobase and phosphate/thiophosphate deprotection. The crude oligonucleotides are analyzed "DMTr-ON" by RP-HPLC to estimate purity and compare synthesis yields with that of control oligonucleotides synthesized from 2-cyanoethyl deoxyribonucleoside phosphoramidites. RP-HPLC chromatograms are shown in Supporting Information.

Fully deprotected oligomers (0.25 OD_{260} unit, each) are further characterized by electrophoresis on 20% polyacrylamide-7 M urea gels (40 cm × 20 cm × 0.75 mm), which were prepared using electrophoresis purity reagents. Gels are stained by soaking in a solution of Stains-all as described elsewhere.[3a] Photographs of such gels are shown in Figure 3 and in Supporting Information.

Phosphorothioated oligonucleotides prepared through the use of phosphoramidites **7a**−**d** and **9** were analyzed for desulfurization by ^{31}P NMR spectroscopy in concentrated NH_4OH. Data are shown in Supporting Information.

Enzymatic digestion of crude d(ATCCGTAGCTAAGGTCATGC) and d(AT*T*CGT*AGCT*AAGGT*CAT*GC) is performed with snake venom phosphodiesterase (*Crotalus adamanteus*) and bacterial alkaline phosphatase according to a published procedure[13] to assess nucleobase modifications that might have occurred during oligonucleotide synthesis and deprotection. Prior to enzymatic digestion, crude d(ATCCGTAGCTAAGGTCATGC) (~25 OD_{260} units) in ddH_2O (250 μL) is applied to the top of a prepacked PD-10 Sephadex G-25M column equilibrated in ddH_2O to eliminate the bicyclic salt **13**. The crude oligonucleotide is eluted with ddH_2O, and 1 mL fractions are collected. Fractions containing the oligonucleotide are identified by UV at 260 nm and pooled together. One OD_{260} unit of the desalted oligonucleotide is evaporated to dryness under reduced pressure and then subjected to enzymatic digestion. Oligonucleotide d(AT*T*CGT*AGCT*AAGGT*CAT*GC) does not require desalting prior to enzymatic digestion. Aliquots of either digests are analyzed by reversed-phase HPLC under the conditions reported in ref 8. RP-HPLC profiles of the digests are shown in Supporting Information.

Isolation and Characterization of 2,3-Dihydro-1*H*-indolizinium Salt 13. This compound is isolated from the thermal deprotection of dinucleotide **11** via RP-HPLC. The dinucleoside phosphotriester **10** is first prepared manually by condensing phosphoramidite **7a** with thymidine, covalently attached through a 3′-*O*-succinyl linker to LCAA-CPG, and a 0.45 M solution of 1*H*-tetrazole in MeCN. Following standard iodine oxidation and cleavage of the 5′-dimethoxytrityl group, **10** is treated with concentrated NH_4OH for 30 min at 25 °C, affording **11**. Heating the ammoniacal solution for 30 min at 55 °C results in complete removal of the phosphate protecting group to produce dinucleotide **12** and some bicyclic salt **13**. RP-HPLC analysis of the deprotection reaction shows a fast-eluting compound (t_R = 3.4 min) under chromatographic conditions identical to those used for the characterization of oligonucleotides. The fast-eluting compound is collected, and the eluate is evaporated to dryness under reduced pressure. The residue, generated from multiple RP-HPLC runs, is characterized by high-resolution mass spectrometry. The mass spectral data agree with the accurate mass expected for **13**. EI−HRMS: calcd for $C_8H_{10}N_1$ (M^+) 120.0811, found 120.0813. To further confirm its structure, **13** is synthesized by mixing a solution of 2-pyridinepropanol (100 μL) in MeCN (0.5 mL) with TFAA (0.5 mL). The solution is heated in a tightly closed vial overnight at 55 °C. Evaporation of excess TFAA gives an oil. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.02 (d, J = 6.0 Hz, 1H), 8.47 (dt, J = 1.2, 8.1 Hz, 1H), 8.08 (d, J = 8.1 Hz, 1H), 7.94 (m, 1H), 4.82 (t, J = 7.7 Hz, 2H), 3.47 (t, J = 7.7 Hz, 2H), 2.39 (qt, J = 7.7 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 21.0, 31.8, 58.6, 116.7 (q, $^1J_{CF}$ = 299 Hz), 124.4, 125.2, 141.2, 144.8, 157.8 (q, $^2J_{CF}$ = 32.3 Hz), 158.5. EI−HRMS: calcd for $C_8H_{10}N_1$ (M^+) 120.0811, found 120.0813. RP-HPLC analysis of the oil shows a peak having a retention time identical to that obtained for **13** isolated from the deprotection of **11**.

Isolation and Characterization of 1-Methyl-2,3-dihydroimidazo[1,2-*a*]pyridinium salt 14. In a 5-mm NMR tube are added **9** (16.5 mg, 0.020 mmol), 3′-*O*-acetylthymidine (6.2 mg, 0.022 mmol), 1*H*-tetrazole (2 mg, 0.028 mmol), and dry MeCN (0.5 mL). ^{31}P NMR analysis of the reaction indicates immediate formation of the corresponding dinucleoside phosphite triester (δ_P ~144 ppm). To the reaction mixture is added 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (6.0 mg, 0.03 mmol), which converted the phosphite triester to a dinucleoside phosphorothioate diester (δ_P ~60 ppm) with the concomitant release of **14**. RP-HPLC analysis of the solution reveals the presence of a fast-eluting compound having a retention time of 4.9 min under the chromatographic conditions used to isolate **13**. The fast-eluting compound is collected, and the material accumulated from multiple RP-HPLC runs is characterized by high-resolution mass spectrometry. The mass spectral data are consistent with the accurate mass expected for **14**. EI−HRMS: calcd for $C_8H_{11}N_2$ (M^+) 135.0922, found

(8) RP-HPLC analyses were performed using a 5-μm Supelcosil LC-18S column (25 cm × 4.6 mm) under the following conditions: starting from 0.1 M triethylammonium acetate pH 7.0, a linear gradient of 1% MeCN/min is pumped at a flow rate of 1 mL/min for 40 min and then held isocratic for 20 min.

(9) Solid-phase synthesis of the fully phosphorothioated 20-mer requires replacing the standard iodine solution with a solution of 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide in MeCN and performing the capping reaction after the sulfurization step (see ref 12).

(10) Since **13** and **14** are ~60% and 100% eliminated, respectively, during each oxidation/sulfurization step of the oligonucleotide assembly, the concentrations of **13** or **14** in the simulation experiments are that of a worst-case scenario.

(11) Novina, C. D.; Murray, M. F.; Dykxhoorn, D. M.; Beresford, P. J.; Riess, J.; Lee, S.-K.; Collman, R. G.; Lieberman, J.; Shankar, P.; Sharp, P. A. *Nat. Med. (N. Y.)* **2002**, *8*, 681−686.

(12) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693−4699. See also: Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proc. Int.* **1992**, *24*, 488−492.

(13) Scremin, C. L.; Zhou, L.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1994**, *59*, 1963−1966.

135.0928. To further establish its identity, **14** is synthesized by mixing a solution of 2-[*N*-methyl-*N*-(2-pyridyl)]aminoethanol (100 μL) in acetonitrile (0.5 mL) with TFAA (0.5 mL). Heating the solution overnight at 55 °C in a tightly closed vial, followed by evaporation of excess TFAA, produces an oil, which is characterized by NMR spectroscopy. ^{1}H NMR (300 MHz, DMDO-d_6): δ 8.23 (d, J = 6.5 Hz, 1H), 7.99 (ddd, J = 1.7, 7.3, 8.9 Hz, 1H), 7.2 (d, J = 8.9 Hz, 1H), 6.90 (ddd, J = 0.8, 6.5, 7.3 Hz, 1H), 4.64 (t, J = 9.9 Hz, 2H), 3.90 (t, J = 9.9 Hz, 2H), 3.07 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 32.4, 49.6, 50.0, 107.5, 113.5, 115.6 (q, $^{1}J_{CF}$ = 289 Hz), 137.3, 145.0, 154.8, 160.4 (q, $^{2}J_{CF}$ = 37.9 Hz). RP-HPLC analysis of the oil shows a peak having a retention time identical to that of **14** isolated after sulfurization of the dinucleoside phosphite triester that was synthesized from **9** and 3′-*O*-acetylthymidine.

Procedure for Determining the DNA-Modifying Properties of 13 and 14. To thymidine, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, N^4-benzoyl-2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, and N^2-isobutyryl-2′-deoxyguanosine (0.5 mmol each) in a 7-mL screw-capped glass vial are added **13** or **14** (4 mmol), concentrated NH_4OH (1.1 mL), and MeCN (3 mL). The suspension solubilizes within 10 min upon heating at 55 °C. The solution is then kept at 55 °C for a total time of 10 h. A control reaction is performed under identical conditions in the absence of **13** or **14** for comparison purposes. Aliquots of the reaction mixtures are analyzed by RP-HPLC under the conditions delineated in ref 8. No nucleobase modifications are detected as only thymidine, 2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine, benzamide, **13** or **14**, and their individual trace amount impurities are present relative to that observed from the analysis of the control reaction.

Procedure for Determining the Phosphorothioate Desulfurization Properties of 13 or 14. A solution of 0.5 M potassium *O,O*-diethyl thiophosphate and 0.75 M **13** or **14** in concentrated NH_4OH is heated for 10 h at 55 °C. Analysis of the reaction mixture by ^{31}P NMR spectroscopy does not reveal any conversion of *O,O*-diethyl thiophosphate (δ_P ~55 ppm) to *O,O*-diethyl phosphate (δ_P ~2 ppm) (Data shown in Supporting Information).

Acknowledgment. This research is supported in part by an appointment to the Postgraduate Research Participation Program at the Center for Biologics Evaluation and Research administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration. We thank Andrzej Wilk for performing a preliminary experiment in this project.

Supporting Information Available: Materials and methods; ^{1}H, ^{13}C, and ^{31}P NMR spectra of crude **6** and **8**; ^{31}P NMR spectra of **7a**−**d** and **9**; ^{1}H and ^{13}C NMR spectra of **13** and **14** as 2,2,2-trifluoroacetate salts; RP-HPLC chromatograms of crude 5′-DMTr-d(ATCCGTAGCTAAGGTCATGC), 5′-DMTr-d(AT*T*CGT*AGCT*AAGGT*CAT*GC), and that of their fully phosphorothioated analogues synthesized using either **7a**−**d** or **9** as indicated; RP-HPLC chromatograms of the snake venom phosphodiesterase and bacterial alkaline phosphatase digestion of crude d(ATCCGTAGCTAAGGTCATGC) and d(AT*T*CGT*AGCT*AAGGT*CAT*GC); ^{31}P NMR spectra of crude phosphorothioated d(ATCCGTAGCTAAGGTCATGC) and d(AT*T*CGT*AGCT*AAGGT*CAT*GC); photograph of a stained 20% polyacrylamide-7 M urea gel showing the electrophoretic profiles of crude d(AT*T*CGT*AGCT*AAGGT*-CAT*GC) and that of its phosphorothioated analogue, both synthesized from **9** and standard 2-cyanoethyl deoxyribonucleoside phosphoramidites; ^{31}P NMR spectra of the reaction of *O,O*-diethyl thiophosphate with either **13** or **14** under conditions simulating those of large-scale oligonucleotide deprotections. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0354490