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The 4-oxopentyl group as a labile phosphate/thiophosphate protecting group for synthetic oligodeoxyribonucleotides†

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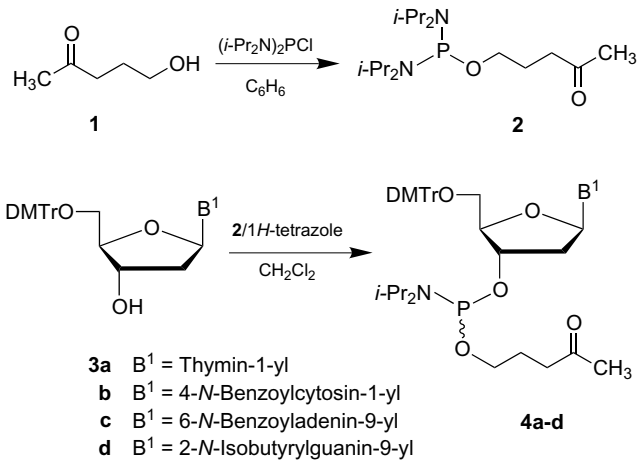
Abstract—An efficient and economical method for the solid-phase synthesis of oligodeoxyribonucleotides and their phosphorothioate analogues is described. The method entails the use of the 4-oxopentyl group for phosphate/thiophosphate protection. Post-synthesis removal of the protecting group is easily and rapidly achieved under mild conditions at ambient temperature using either pressurized gaseous amines or concentrated ammonium hydroxide. © 2001 Elsevier Science Ltd. All rights reserved.

The recent manufacturing of an anti-viral oligodeoxyribonucleotide phosphorothioate on a kilogram scale[1] underscores the potential therapeutic value of synthetic oligonucleotides against infectious diseases and various types of cancer in humans. This remarkable achievement strongly challenges the current methods used to synthesize oligonucleotides. Of critical importance, the synthetic methods must not lead to chemical modification of the therapeutic oligonucleotides that would adversely affect potency, and must also be economical to ensure a reasonable cost per dose of these drugs.

During the course of our search for cost-effective phosphate/thiophosphate protecting groups, several possibilities attracted our attention. For example, we found the 2-(*N*-formyl-*N*-methyl)aminoethyl group to be adequate for this purpose and performed the synthesis of oligodeoxyribonucleotides, such as dT_{18} and $d(AG)_{10}$, using this group for phosphate protection.[2] The 2-(*N*-formyl-*N*-methyl)aminoethyl group is easily cleaved from these oligonucleotides in an aqueous buffer (pH ~7) via a unique thermolytic cyclodeesterification process.[2,3] Other cost-effective phosphate/thiophosphate protecting groups such as the 2-(*N*-acetyl-*N*-methyl)aminoethyl, the 4-oxopentyl, or the 3-[(*N*-*tert*-butyl)carboxamido]-1-propyl also exhibit similar thermolytic deprotection properties.[2] Moreover, the 4-oxopentyl group can be cleaved completely from oligonucleotides upon exposure to pressurized ammonia/methylamine gas[4] or concentrated ammonium hydroxide. Given the versatility with which oligonucleotides carrying 4-oxopentyl groups for phosphate/thiophosphate protection can be deprotected, we now report the syntheses of deoxyribonucleoside phosphoramidites **4a**–**d**, and the use of these compounds in the solid-phase preparation of an oligodeoxyribonucleotide (20-mer) and its phosphorothioate analogue.

The synthesis of **4a**–**d** is illustrated in Scheme 1 and begins with the preparation of the phosphinylating



Scheme 1. Synthesis of the deoxyribonucleoside phosphoramidites **4a**–**d**.

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† This paper is dedicated to the memory of Dr. Krystyna Lesiak.

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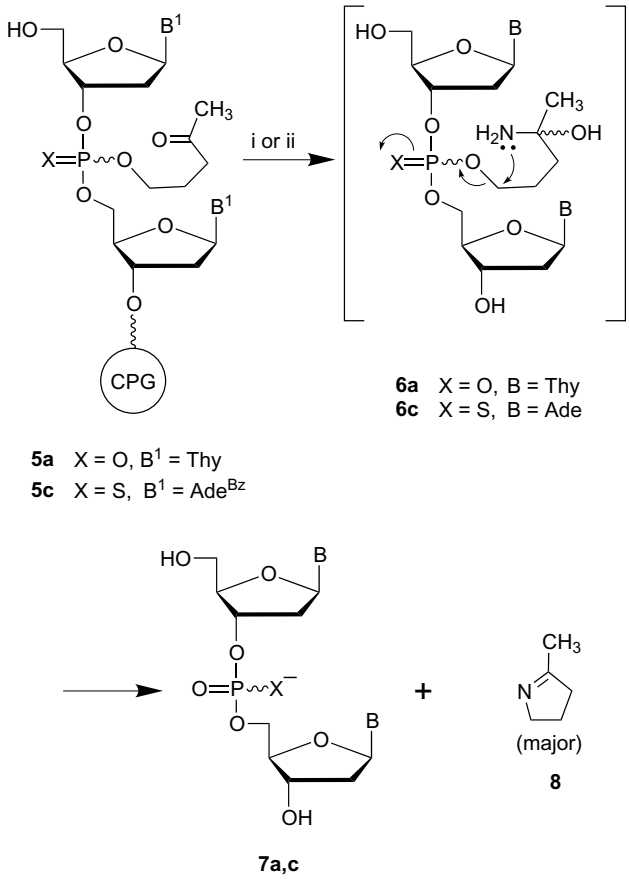
reagent **2**. Typically, a solution of anhydrous diisopropylamine (19.6 mL, 140 mmol) is added dropwise under an argon atmosphere, over a period of 30 min, to a cold solution (5°C) of PCl_3 (1.75 mL, 20 mmol) in dry benzene (100 mL).[5] The cold suspension is then allowed to stir at ambient temperature and the formation of bis-(*N*,*N*-diisopropylamino)chlorophosphine is monitored by ^{31}P NMR spectroscopy. Upon completion of the reaction (2 days), 5-hydroxy-2-pentanone (**1**)[6] (2.43 mL, 24 mmol) is added by syringe to the stirred suspension. ^{31}P NMR analysis of the reaction mixture indicates that bis-(*N*,*N*-diisopropylamino)chlorophosphine (δ_P 135.9 ppm in C_6D_6) is converted to the corresponding phosphordiamidite **2** (δ_P 123.5 ppm in C_6D_6) within 2 h at 25°C. The suspension is filtered and the filtrate is evaporated under reduced pressure to afford a yellow oil which is used without further purification in the preparation of the phosphoramidites **4a**–**d** (Scheme 1).

Crude phosphordiamidite **2** (980 μL, ~3 mmol) is then added by syringe under an inert atmosphere to a stirred solution of the deoxyribonucleoside **3a**–**d**[7] (2 mmol) in dry CH_2Cl_2 (10 mL). Sublimed 1*H*-tetrazole[7] (120 mg, 1.6 mmol) is added portionwise under a positive pressure of argon to the solution. The reaction is monitored by TLC until **4a**–**d** is no longer produced. Triethylamine (1 mL) is added to the solution, which is then evaporated under reduced pressure to give a gum. The material is purified by silica gel chromatography to afford pure **4a**–**d** in yields ranging from 68 to 80%.[8]

The dinucleoside phosphotriesters **5a**,**c** are then prepared under standard conditions by mixing **4a**,**c** (0.2 M in MeCN), 1*H*-tetrazole (0.45 M in MeCN), and either 5′-unprotected thymidine or 5′-unprotected N^6-benzoyl-2′-deoxyadenosine (0.2 μmol) covalently attached to long chain alkylamine controlled pore glass (LCAA-CPG). The resulting dinucleoside phosphite triester intermediates are oxidized or sulfurized by treatment with 0.02 M aqueous iodine or 0.05 M 3*H*-1,2-benzodithiol-3-one 1,1-dioxide[9] in MeCN, respectively. Release of the dinucleoside phosphotriesters from LCAA-CPG is effected by pressurized gaseous amines, or concentrated NH_4OH at 25°C. Analysis of the crude dinucleotides by reversed-phase (RP) HPLC[10] reveals, to our surprise, that the 4-oxopentyl phosphate protecting group is completely removed from **5a** within 15 min, 90 min, or 4 h when using methylamine gas (~2.5 bar), ammonia gas (~10 bar), or concentrated NH_4OH,[11] respectively. A proposed mechanism for the conversion of **5a**,**c** to **7a**,**c** is shown in Scheme 2.

The reaction of ammonia with the ketone function of the phosphate/thiophosphate protecting group in **5a**,**c** most likely leads to an hemiaminal intermediate **6a**,**c**.[12] This intermediate may then undergo rapid cyclodeesterification to produce **7a**,**c** with the concomitant formation of 2-methyl-1-pyrroline (**8**) as the major deprotection side-product.[13] This deprotection pathway is consistent with the removal of 4-aminobutyl or 4-*N*-methylaminobutyl phosphate/thiophosphate protecting groups reported earlier under similar conditions.[14]

Given that the 4-oxopentyl phosphate/thiophosphate protecting group is easily removed from **5a**,**c** under mild conditions, the deoxyribonucleoside phosphoramidites **4a**–**d** are then applied to the solid-phase synthesis of an oligonucleotide (20-mer)[15] and its fully phosphorothioated analogue[9a,15] to further demonstrate the utility and convenience of this protecting group. Upon completion of the synthesis, the oligonucleotide is released from the CPG support and fully deprotected by treatment with either pressurized ammonia gas (~10 bar, 25°C, 12 h) or concentrated NH_4OH (55°C, 10 h). The crude oligonucleotide is analyzed 'DMTr-on' and 'DMTr-off' by RP-HPLC[10] to estimate the overall yield of the synthetic oligonucleotide. Purity is determined by RP-HPLC comparison with an oligonucleotide identical in sequence that was synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites. Fig. 1A shows that the overall yield of the 'DMTr-on' 20-mer deprotected under gas-phase conditions is 93%, based upon areas of all relevant integrated RP-HPLC peaks, thereby indicating an average yield of greater than 99% for each coupling step. The RP-HPLC profiles of the 'DMTr-off' 20-mer deprotected by contact with pressurized ammonia gas (Fig. 1B) and concentrated NH_4OH (Fig. 1C) show that the purity of the



Scheme 2. Proposed mechanism for the phosphate/thiophosphate deprotection of **5a**,**c**. *Reagents and conditions*: (i) ammonia gas (~10 bar, 25°C); (ii) conc. NH_4OH, 25°C.

oligonucleotide synthesized from **4a–d** is equivalent to, if not better than, that of the same 20-mer prepared from 2-cyanoethyl deoxyribonucleoside phosphoramidites (Fig. 1D).

In order to assess whether deprotection of the 4-oxopentyl phosphate protecting group (under the conditions described above) results in nucleobase modification, the crude 20-mer is subjected to enzymatic hydrolysis catalyzed by snake venom phosphodiesterase and bacterial alkaline phosphatase.[16] RP-HPLC analysis of the enzymatic digest shows that the crude 20-mer is completely hydrolyzed to the corresponding nucleosides without detectable modification of the nucleobases (see Fig. 2).[17]

The fully phosphorothioated 20-mer oligonucleotide analogue (synthesized from **4a–d**) is also completely deprotected by pressurized ammonia gas and is obtained in yields comparable to that of the unmodified 20-mer as judged by RP-HPLC analysis of the crude oligonucleotide product (data not shown). ^{31}P NMR analysis of the crude phosphorothioated 20-mer is pre-

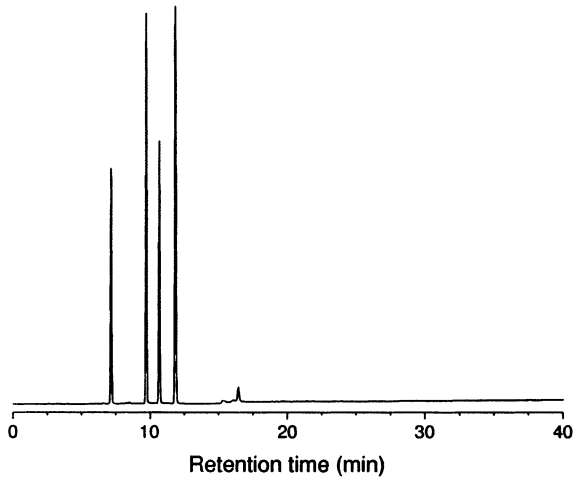


Figure 2. RP-HPLC analysis of the crude 20-mer d(ATCCGTAGCTAAGGTCATGC) synthesized from **4a–d**, deprotected by NH_3 (~10 bar, 12 h, 25°C), and digested by snake venom phosphodiesterase and bacterial alkaline phosphatase (12 h, 37°C). Identities of the RP-HPLC peaks from left to right are as follows: dC, dG, dT, dA and benzamide (~17 min) when compared to authentic commercial samples.

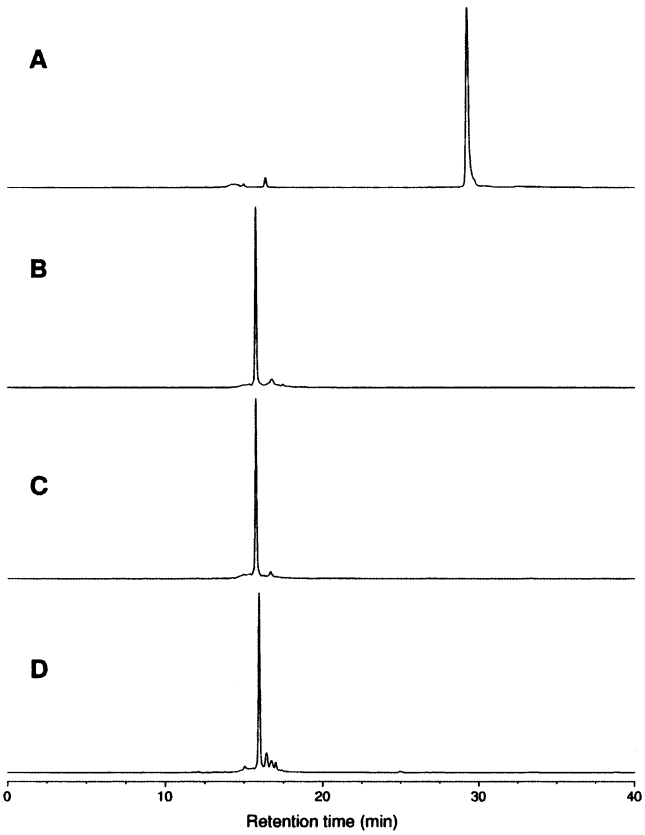


Figure 1. RP-HPLC profiles of a crude 20-mer synthesized from **4a–d**. A: 5′-DMTr-d(ATCCGTAGCTAAGGTCATGC) deprotected by NH_3 (~10 bar, 12 h, 25°C). B and C: d(ATCCGTAGCTAAGGTCATGC) deprotected by NH_3 (~10 bar, 25°C, 12 h) and by concentrated NH_4OH (55°C, 10 h), respectively. D: d(ATCCGTAGCTAAGGTCATGC) synthesized from 2-cyanoethyl deoxyribonucleoside phosphoramidites and deprotected by concentrated NH_4OH (55°C, 10 h).

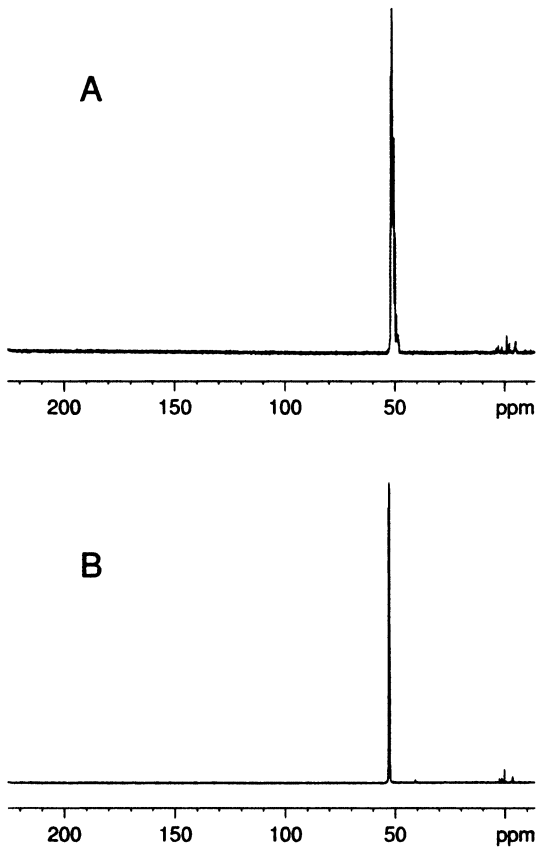


Figure 3. 121 MHz ^{31}P NMR spectra of crude 20-mer d($A_{PS}T_{PS}C_{PS}C_{PS}G_{PS}T_{PS}A_{PS}G_{PS}C_{PS}T_{PS}A_{PS}A_{PS}G_{PS}G_{PS}T_{PS}C_{PS}$-$A_{PS}T_{PS}G_{PS}C$) in aqueous solvents. A: 20-mer synthesized from commercial 2-cyanoethyl deoxyribonucleoside phosphoramidites and deprotected by concentrated NH_4OH (55°, 10 h). B: 20-mer prepared from **4a–d** and deprotected by NH_3 (~10 bar, 12 h, 25°C).

sented in Fig. 3, and demonstrates that the unwanted production of desulfurized material ($\delta_P \sim 0$ ppm) is minimal (<2%)[18] whether the 4-oxopentyl group or the 2-cyanoethyl group is used for thiophosphate protection during synthesis.

Thus, the data presented herein support the use of the 4-oxopentyl group for phosphate/thiophosphate protection in the synthesis of *alkylation-free* oligodeoxyribonucleotides. The deoxyribonucleoside phosphoramidites **4a–d** are easily prepared and are coupled in high efficiency in the stepwise solid-phase synthesis of oligodeoxyribonucleotides. As a phosphate protecting group, the 4-oxopentyl group is more versatile than the 2-cyanoethyl group in that it is completely removed from oligonucleotides under neutral conditions (<1 h at 90°C in an aqueous buffer at pH 7.0). Such deprotection conditions should be ideal for oligonucleotides carrying nucleobases and/or reporter groups that are base-sensitive. In regard to thiophosphate protection, the 4-oxopentyl group is as versatile as the 2-cyanoethyl group as they are both rapidly removed when treated with pressurized gaseous amines (NH_3 or CH_3NH_2) or concentrated ammonium hydroxide. Considering the simple and economical syntheses of **4a–d**, these phosphoramidites are suitable for the large-scale preparation of therapeutic oligonucleotides that is necessary for clinical studies. It is also likely that the 4-oxopentyl group will be useful for phosphate/thiophosphate protection in the synthesis of oligoribonucleotides and their phosphorothioated analogues.

Acknowledgements

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4. Boal, J. H.; Wilk, A.; Harindranath, N.; Max, E. E.; Kempe, T.; Beaucage, S. L. *Nucleic Acids Res.* **1996**, *24*, 3115–3117.
5. Diisopropylamine and benzene (Aldrich) are dried by refluxing over CaH_2, distilled, and transferred under anhydrous conditions to amber glass bottles containing 4 Å molecular sieves.
6. Purchased from Aldrich and used as received.
7. The deoxyribonucleosides **3a–d** and sublimed 1*H*-Tetrazole are obtained from Chem-Impex International and Aldrich, respectively. These are kept overnight over P_2O_5 in a dessicator under high vacuum prior to use.
8. Compound **4a**: 80%; ^{31}P NMR (121 MHz, C_6D_6): δ 148.38, 149.04 ppm; FAB-HRMS: calcd for $(C_{42}H_{54}N_3O_9P+Na)^+$ 798.3496, found 798.3541. Compound **4b**: 78%; ^{31}P NMR (121 MHz, C_6D_6): δ 148.87, 149.16 ppm; FAB-HRMS: calcd for $(C_{48}H_{57}N_4O_9P+Na)^+$ 887.3761, found 887.3682. Compound **4c**: 72%; ^{31}P NMR (121 MHz, C_6D_6): δ 148.61, 148.85 ppm; FAB-HRMS: calcd for $(C_{49}H_{57}N_6O_8P+Na)^+$ 911.3873, found 911.3881. Compound **4d**: 68%; ^{31}P NMR (121 MHz, C_6D_6): δ 148.03, 148.503 ppm; FAB-HRMS: calcd for $(C_{46}H_{59}N_6O_9P+Na)^+$ 893.3980, found 893.3965.
9. Purchased from Glen Research or prepared according to: (a) Iyer, R. P.; Phillips, L. R.; Egan, W.; Regan, J. B.; Beaucage, S. L. *J. Org. Chem.* **1990**, *55*, 4693–4699. (b) Regan, J. B.; Phillips, L. R.; Beaucage, S. L. *Org. Prep. Proc. Int.* **1992**, *24*, 488–492.
10. RP-HPLC analyses are performed using a 5 μm Supelcosil LC-18S column (4.6 mm×25 cm) and a linear gradient of 1% MeCN/min starting from 0.1 M triethylammonium acetate (pH 7.0) pumped at a flow rate of 1 mL/min.
11. Comparatively, the removal of an ethyl phosphate protecting group from DNA oligonucleotides is reported to proceed to the extent of only ~10% when exposed to concentrated NH_4OH for at least 36 h at 25°C; see: Koziolkiewicz, M.; Wilk, A. In *Methods in Molecular Biology*; Agrawal, S., Ed. Protocols for Oligonucleotides and Analogs; Humana Press: Totowa, 1993; Vol. 20, pp. 207–224.
12. March, J. *Advanced Organic Chemistry—Reactions, Mechanism, and Structure*, 4th ed.; John Wiley & Sons: New York, 1992; p. 897.
13. ^{13}C NMR analysis of a model experiment aimed at inducing cyclodeesterification of *O*-(4-oxopentyl)-*O*,*O*-diethyl phosphate (see Ref. 19) to *O*,*O*-diethyl phosphate, when left standing in concentrated NH_4OH, reveals the presence of five major signals exhibiting chemical shifts (175.0, 60.7, 38.5, 22.6 and 19.3 ppm) identical to those of an authentic sample of **8** (Aldrich) in concentrated NH_4OH. These findings strongly support the deprotection mechanism proposed in Scheme 2.
14. (a) Wilk, A.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1997**, *62*, 6712–6713; (b) Wilk, A.; Grajkowski, A.; Phillips, L. R.; Beaucage, S. L. *J. Org. Chem.* **1999**, *64*, 7515–7522; (c) 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.
15. Phosphoramidites **4a–d** are kept overnight over P_2O_5 in a dessicator under high vacuum prior to being dissolved in dry MeCN to a concentration of 0.2 M. The wait time required for the coupling step and the oxidation reaction is 150 s and 60 s, respectively.
16. Enzymatic digestion is performed as described in: Scremin, C. L.; Zhou, L.; Srinivasachar, K.; Beaucage, S. L. *J. Org. Chem.* **1994**, *59*, 1963–1966.

17. Incubation of thymidine, N^4-benzoyl-2′-deoxycytidine, N^6-benzoyl-2′-deoxyadenosine, and N^2-isobutyryl-2′-deoxyguanosine with **8** under condition identical to those used to evaluate nucleobase alkylation by acrylonitrile (see Ref. 14b) was performed. No nucleobase modification caused by **8** in a setting similar to that of large-scale oligonucleotide deprotection was detected by RP-HPLC.
18. The extent of desulfurization is more pronounced when 4-oxopentyl thiophosphate protecting groups are thermolytically removed under neutral conditions (see Ref. 2). The use of pressurized gaseous amines is therefore strongly recommended for the cleavage of 4-oxopentyl groups from phosphorothioated oligonucleotides.
19. *O*-(4-Oxopentyl)-*O*,*O*-diethyl phosphate is synthesized as reported for the preparation of *O*,*O*-diethyl-*O*-[4-[*N*-methyl-*N*-(2,2,2-trifluoroacetyl)amino]butyl] phosphate (see Ref. 14b) and is purified by silica-gel chromatography prior to use.