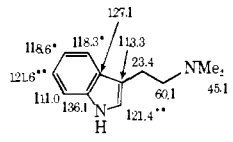
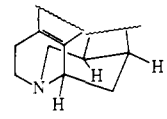


i

(37) D. W. Cochran, Ph.D. Dissertation, Indiana University, 1971.
(38) Three investigations on the carbon shift assignments of a limited number of Rauwolfia alkaloids were published[39-41] some time after completion of the 1971 study.[37]
(39) R. H. Levin, J.-Y. Lallemand, and J. D. Roberts, *J. Org. Chem.*, **38**, 1983 (1973).
(40) T. Kametani, T. Higa, M. Ithara, K. Fukumoto, M. Kajiwara, Y. Fujimoto, and G. Tanaka, *J. Heterocycl. Chem.*, **12**, 259 (1975).
(41) G. van Binst and C. Tourwe, *Heterocycles*, **1**, 257 (1973).
(42) G. W. Gribble, R. B. Nelson, G. C. Levy, and G. L. Nelson, *J. Chem. Soc., Chem. Commun.*, 703 (1972); 148 (1973).
(43) J. B. Stothers, "Carbon-13 NMR Spectroscopy", Academic Press, New York, N.Y., 1972.
(44) E. L. Eliel, W. F. Bailey, L. D. Kopp, R. L. Willer, D. M. Grant, R. Bertrand, K. A. Christensen, D. K. Dalling, M. W. Duch, E. Wenkert, F. M. Schell, and D. W. Cochran, *J. Am. Chem. Soc.*, **97**, 322 (1975).
(45) E. Wenkert, B. L. Buckwalter, I. R. Burfitt, M. J. Gasić, H. E. Gottlieb, E. W. Hagaman, F. M. Schell, and P. M. Wovkulich, in "Topics in Carbon-13 NMR Spectroscopy", Vol. II, G. C. Levy, Ed., Wiley-Interscience, New York, N.Y., 1976.
(46) The ^{13}C NMR spectrum of a sixth substance, reserpiline (10,11-dimethoxyakuammigine), was inspected also. Its nonaromatic carbons exhibit shifts identical with those of like carbon centers of akuammigine (**34**), while its extra methoxy shifts are 56.0 and 56.4 ppm and the shifts of its indole carbons 2, 7, 8, 9, 10, 11, 12, and 13 are 131.9, 107.2, 120.2, 100.4, 146.3, 144.7, 95.2, and 130.1 ppm, respectively.
(47) This is based on conformation **48** always representing the allo configuration. There is, however, the remote possibility of conformationally unfavorable substitution of ring E leading to the alternate conformation ii. The low δ(C-3) value of raunitidine (**51**) probably is the consequence of a low concentration of conformer ii for this alkaloid in deuteriochloroform solution.



ii

(48) In yohimboid compounds possessing a 16-carbomethoxy group the α-ketomethine signal appears in the 50–57-ppm range. While the C(3) signal of pseudo and epiallo systems lies within this region, no confusion arises in the ^{13}C NMR analysis, since the absence of a 60 ± 1 ppm methine signal negates a normal or allo configurational interpretation.
(49) While the ring E unsubstituted bases may exhibit an additional upfield methylene signal, the C(6) signal is recognized easily by virtue of its unique coupling characteristics (benzylic $^{R}J_{CH} \neq$ alicyclic $^{R}J_{CH}$).[45]
(50) Cf. D. K. Dalling and D. M. Grant, *J. Am. Chem. Soc.*, **94**, 5318 (1972).
(51) Cf. F. Näf, B. L. Buckwalter, I. R. Burfitt, A. A. Nagel, and E. Wenkert, *Helv. Chim. Acta*, **58**, 1567 (1975).
(52) The reciprocity of the γ effect is difficult to assess in the case of **42a** in view of its spectrum having been run in Me_2SO-d_6 solution, wherein the shift of the aminocarbon, C(2), is affected strongly by the polar solvent.
(53) D. K. Dalling and D. M. Grant, *J. Am. Chem. Soc.*, **96**, 1827 (1974).
(54) E. Wenkert and B. Wickberg, *J. Am. Chem. Soc.*, **87**, 1580 (1965).
(55) NOTE ADDED IN PROOF. A recent ^{13}C NMR analysis of **45** and its derivatives [G. W. Gribble, R. B. Nelson, J. L. Johnson, and G. C. Levy, *J. Org. Chem.*, **40**, 3720 (1975)] necessitated the revision of the C(9) and C(10) shifts of ring A unsubstituted, tetrahydrocarboline-based alkaloids and derivatives, initially proposed by R. G. Parker and J. D. Roberts, *J. Org. Chem.*, **35**, 996 (1970). The analysis contains an incorrect assignment of the aminomethylene shifts of the H(3)–H(15) cis isomer of 15-*t*-Bu-**45**. The interchange of the δ values for C(5) and C(21) removes the apparent anomaly of the C(5) shift.

Synthesis of Thymidine Oligonucleotides by Phosphite Triester Intermediates[1]

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Contribution from the Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received November 12, 1975*

Abstract: A synthetic procedure for building phosphotriester derivatives of oligothymidylates is described which involves four chemical steps for addition of each nucleotide unit: (1) reaction of $ROPCl_2$ with 5′-*O*-phenoxyacetylthymidine in tetrahydrofuran at −78° (5 min); (2) reaction of the resulting phosphoromonochloridite with 3′-*O*-mono-*p*-methoxytritylthymidine or a related oligonucleotide phosphotriester which possesses a 5′-OH group, also in tetrahydrofuran at −78° (10–20 minutes); (3) oxidation of the resulting phosphite with iodine and water (a few minutes at −10 to 0°); and (4) hydrolytic cleavage of the phenoxyacetic ester at the terminal 5′ position by ammonium hydroxide in aqueous dioxane (10 min). Best results were achieved with R = Cl_3CCH_2–; yields at the di, tri, tetra, and penta stage of synthesis were 95, 69, 75, and 69%, respectively. The corresponding phosphodiesters, dTpT, dTpTpTpT, and dTpTpTpTpT were obtained from the triesters by reaction with sodium–naphthalene in hexamethylphosphoric triamide (94, 69–71, and 59% yield, respectively). *o*-$ClC_6H_4OPCl_2$ was also employed as the phosphorylating agent. This reagent was satisfactory for the synthesis of triesters related to dithymidine phosphate, but did not prove suitable for synthesis of longer chains.

In spite of extensive experimental work and numerous improvements in methodology,[2] the stepwise synthesis of arbitrarily defined oligonucleotides remains a difficult and time consuming operation. Ideally, one would hope to automate repetitive step syntheses of the type required in preparing oligonucleotides. To date, however, the yields in condensation reactions conducted on insoluble supports, which offer the best opportunity for automation, have not been sufficiently high to afford a practical synthetic procedure.[3]

In pursuing this problem we have explored new approaches to generating internucleotide links. We report in this paper a novel coupling procedure that involves intermediate phosphite links. Although the potential and limitations of the method have not yet been fully investigated, the short reaction times and the relatively good yields are distinctive features of considerable promise.

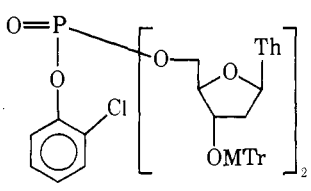
The basis for this approach was the observation that phosphorochloridites, such as $(C_2H_5O)_2PCl$ and $C_6H_5OPCl_2$, react very rapidly at the 3′-OH of nucleosides in pyridine, even at very low temperatures.[4] By contrast, the reactions of the analogous chloridates $(RO)_2POCl$ and $ROP(O)Cl_2$ require several hours at room temperature.[5,6] The latter condensations can be accelerated by *N*-alkylimidazoles;[7] however, such reactions are complicated by the fact that the base ring (e.g., the thymine or *N*-benzoyladenine moiety) may be attacked as well as the sugar hydroxy groups.[8] Two subsequent findings of importance were: (1) dinucleoside monophosphite triesters can be oxidized essentially quantitatively to the corresponding

phosphates by iodine and water;[9] and (2) condensations of phosphorochloridites with nucleosides are cleaner when conducted in tetrahydrofuran in the presence of small amounts of pyridine or 2,6-lutidine than in pyridine as a solvent.[10] Best results have been achieved in tetrahydrofuran at −78°. A preliminary report of the phosphite procedure has been published.[11]

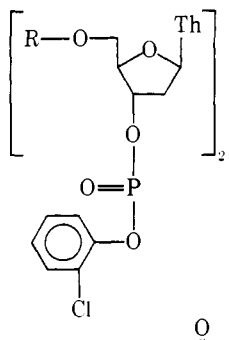
***o*-Chlorophenyl Phosphotriesters.** To develop this chemistry into a useful synthetic procedure, it was necessary to establish which protecting groups are compatible with the highly reactive phosphorochloridite reagents. For this purpose, samples of deoxyribonucleosides protected at the 3′-O and 5′-O and at the amino group (in dA, dG, and dC) were exposed to excess phenyl phosphorodichloridite in pyridine for 1 h at room temperature. The reaction products were examined by thin layer chromatography on silica gel plates. It was found that *O*-trityl, mono-*p*-methoxytrityl, acetyl, phenoxyacetyl, benzoyl, and β-benzoylpropionyl derivatives are stable. Esters with benzoylformyl and 2,4-dinitrobenzenesulfenyl groups are unstable. Of special interest was the observation that, in spite of the unusually high reactivity of the dichloridite reagent toward hydroxyl groups, no evidence for attack at the thymine ring or *N*-benzoyl protected adenine, guanine, and cytosine rings was found.

Phenoxyacetyl was selected to protect the 5′-O of nucleosides used in stepwise addition, since this group can be readily cleaved from oxygen under mild alkaline conditions.[12] Mono-*p*-methoxytrityl was chosen to protect the 3′-O of the nucleoside destined to be the 3′ terminus of the oligonucleotide. This group is stable to alkali and, in addition, provides a convenient marker for following reaction products on thin layer chromatography.[13] Initially, *o*-chlorophenyl[14] was used to protect P–O; the triester derivatives can be converted to phosphodiesters relatively satisfactorily under alkaline conditions more severe than those employed in hydrolyzing phenoxyacetic esters.

The feasibility of the "phosphite" approach for preparation of dinucleoside monophosphates was examined by the synthesis of the 3′–3′ and 5′–5′ derivatives, compound **1** and **2a.** Thus,



1

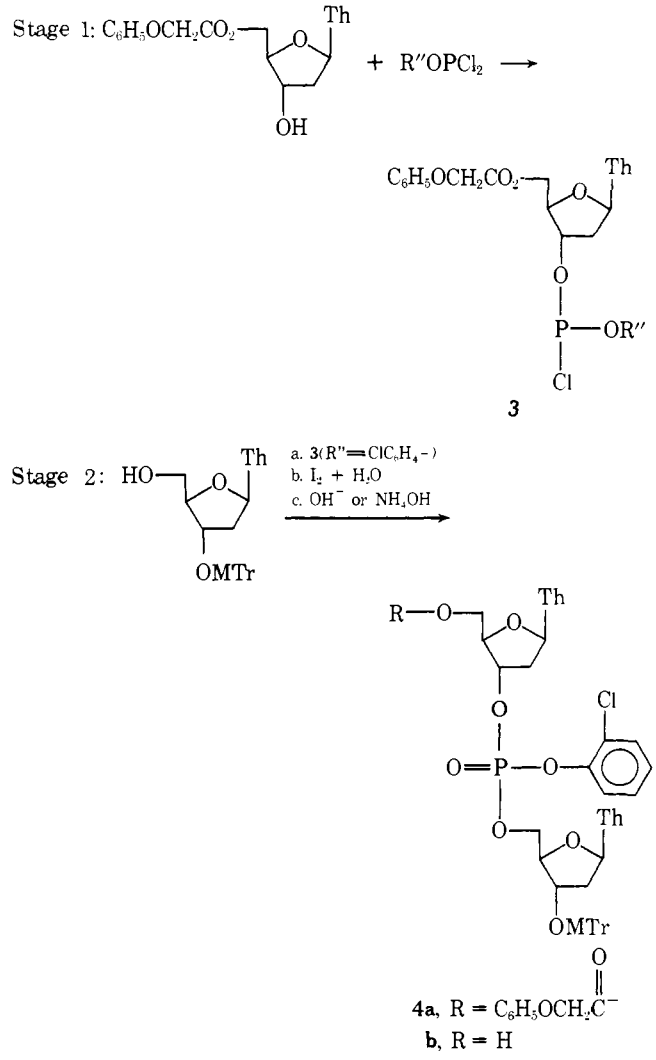


2a, R = $C_6H_5OCH_2C(=O)-$
b, R = H

reaction (30 min) of 3′-*O*-mono-*p*-methoxytritylthymidine with 0.55 mol equiv of *o*-chlorophenyl phosphorodichloridite and pyridine in tetrahydrofuran at −78°, followed by oxidation with iodine and water at ~−10°, afforded **1** in 76% yield. Compound **2a** was similarly isolated in 66% yield from a reaction of 5′-*O*-phenoxyacetylthymidine. These triesters yielded the dithymidine phosphates (5′–5′ and 3′–3′ isomers, respectively) on deblocking with alkali and acid. It may be noted that these experiments show that both the 3′ and the 5′ hydroxyl groups of the nucleoside react efficiently with both the dichloridite ($ArOPCl_2$) and the intermediate monochloridite (ArO(RO)PCl) reagents.

The scheme developed for synthesis of the unsymmetrical

Scheme I



3′–5′ isomers is outlined in Scheme I. Although the reactions are readily carried out in a single flask without transfer of the reaction mixture, it is convenient to consider the sequence in two stages: (1) formation of the activated phosphorylnucleoside **3**, and (2) chain extension from the 5′-OH of an oligonucleotide or nucleoside derivative.

A general problem in preparing unsymmetrical compounds by condensation reactions with symmetrical reagents (e.g., $ROPCl_2$) is that mixtures containing symmetrical as well as unsymmetrical derivatives are usually produced. Thus, if excess nucleoside is present in stage 1, some of the 3′–3′ isomer will form: if excess phosphorodichloridite is used, it will carry over into stage 2 and afford a 5′–5′ isomer. This problem was handled by employing excess 5′-phenoxyacetylthymidine in stage 1 to insure complete consumption of the dichloridite. The mixture containing **3** was then treated with 3′-*O*-mono-*p*-methoxytritylthymidine, the limiting reagent in stage 2, to give the desired unsymmetrical dinucleoside phosphate derivate. In the general scheme, an oligonucleotide is constructed by stepwise additions at the 5′ terminus of the growing oligonucleotide derivative. The procedure, therefore, conserves the reactant with the 5′-OH (the most valuable reagent) and employs an excess of the mononucleoside component. At the end of each full cycle one must separate the 3′–3′ isomer formed in stage 1 from the desired compound produced in stage 2.

Appropriate mole ratios for 5′-phenoxyacetylthymidine/$R''OPCl_2$/3′-*O*-mono-*p*-methoxytritylthymidine were found to be 1:0.9:0.3–0.5. The evidence (see below) shows that at a

nucleoside/dichloridite ratio of 1:0.9 all of the dichloridite is converted within 10 min at −78° to intermediate **3** and inactive derivatives, consisting primarily of the 3′–3′ isomer.[15] The ratio of 1:0.3–0.5 for phenoxyacetylthymidine/methoxytritylthymidine was selected to ensure an excess of active intermediate **3** even when traces of moisture are present in the reaction vessel. With this ratio all of the 5′-OH component (3′-*O*-mono-*p*-methoxytritylthymidine in Scheme I) is consumed in the reaction.

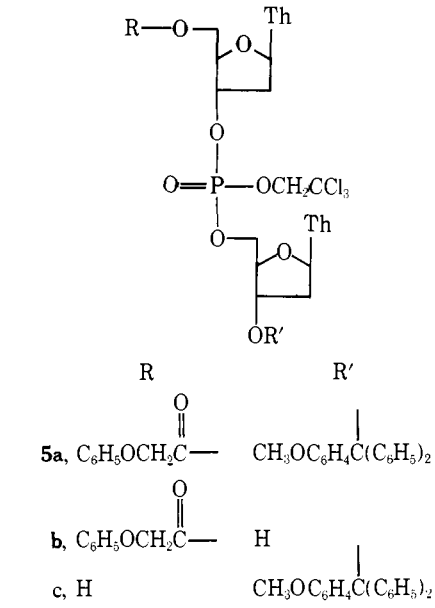
The manipulations for this synthetic sequence are simple and the reaction times are short. For the preparation of **4b**, 5′-*O*-phenoxyacetylthymidine was added to a mixture of *o*-chlorophenyl phosphorodichloridite and 2,6-lutidine in tetrahydrofuran at −78°. This was followed after 10 min by addition of 3′-*O*-mono-*p*-methoxytritylthymidine and, after an additional 20 min at −78° and rapid warming to −10°, by addition of iodine and water. The oxidation proceeded about as rapidly as the reagent was added, as indicated by disappearance of the iodine color. In the absence of water, no decoloration occurred. To facilitate chromatographic separation, the phenoxyacetic esters were cleaved by treatment with ammonium hydroxide in an equal volume of dioxane (20 min at room temperature). Under these conditions hydrolysis of the *o*-chlorophenyl phosphotriester is negligible. Chromatography on silica afforded the partially protected dinucleoside phosphate **4b** in 65% yield, based on methoxytritylthymidine. Compounds **4b, 2b,** and **1** can be readily separated and distinguished on silica slides. The absence of detectable amounts of **1** in the reaction products confirms the absence of any significant amounts of *o*-chlorophenyl phosphorodichloridite in the phosphorylating reagent used in stage 2.

When the synthetic sequence (stages 1 and 2 and the iodine oxidation) was carried out in the same manner but at 0°, more products were formed (as indicated by appearance of six distinct spots on thin layer chromatography) and the yield of **4b** was somewhat lower (57% isolated). The results of a reaction carried out at 25° were even less favorable.

Attempts to extend the sequence to prepare a trinucleoside diphosphate derivative from **4b** were only partially successful. At best only low yields (~23%) of the protected trithymidine product could be realized, and numerous side products were observed. The most plausible explanation for the low yields is that the phosphotriester link is unstable toward the phosphorochloridite reagent. This view is strengthened by the observation that a reaction occurs (indicated by appearance of new spots on thin layer chromatography) when **2a** is treated with *o*-chlorophenyl phosphorodichloridite. Since the phenoxyacetic ester, methoxytrityl ether, and thymine moieties are stable under the conditions employed (see previous discussion) the vulnerable link in **2a** is most likely the *o*-chlorophenyl phosphotriester. Attention was therefore turned to other groups for protecting the phosphoryl function. Of the available groups, 2,2,2-trichloroethyl appeared especially attractive. Phosphorotriester derivatives of oligonucleotides are known to be relatively stable,[6] and the requisite reagent, 2,2,2-trichloroethyl phosphorodichloridite, can be readily prepared from 2,2,2-trichloroethanol and phosphorus trichloride.[21]

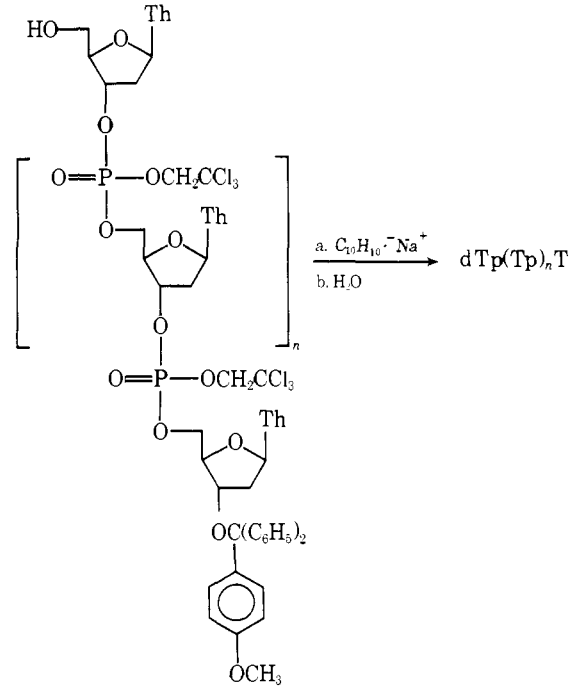
2,2,2-Trichloroethyl Phosphotriesters. The condensations outlined in Scheme I were repeated with 2,2,2-trichloroethyl phosphorodichloridite ($Cl_3CCH_2OPCl_2$) in place of *o*-chlorophenyl phosphorodichloridite. Since preliminary experiments indicated that the reaction time could be further reduced, the time for the initial condensation (stage 1) was shortened to 5 min. On oxidation with iodine and water, the desired dinucleoside phosphate triester derivative (**5a**) was isolated (silica gel chromatography) in 82% yield.

Cleavage of the methoxytrityl ether with aqueous acetic acid afforded the corresponding 3′-OH compound (**5b**). Similarly, treatment of **5a** with ammonium hydroxide yielded the 5′-OH



member of the series (**5c**). Compound **5c** was best prepared by repeating the synthesis and hydrolyzing the crude reaction mixture containing **5a** prior to purification on silica gel, since **5c** is easier to purify than **5a.** In this manner, **5c** was isolated in 95% yield based on the methoxytritylthymidine used in the reaction.

The synthetic sequence, involving condensation with the phosphorochloridite, oxidation, and cleavage with ammonium hydroxide, was then repeated twice, starting with compound **5c** in place of 3′-*O*-mono-*p*-methoxytritylthymidine. The first cycle yielded the trinucleotide derivative **6** (69%), and the



6, $n = 1$
7, $n = 2$
8, $n = 3$

second cycle gave the tetranucleotide derivative **7** (75%). In contrast to the *o*-chlorophenyl esters, therefore, the trichloroethyl phosphotriesters are suitable for extended syntheses by the phosphite procedure. For a single reaction cycle the overall time needed to carry out the reactions was less than 1 h, and the time for workup of the mixture and isolation of the product by thick layer chromatography was 5–9 h.

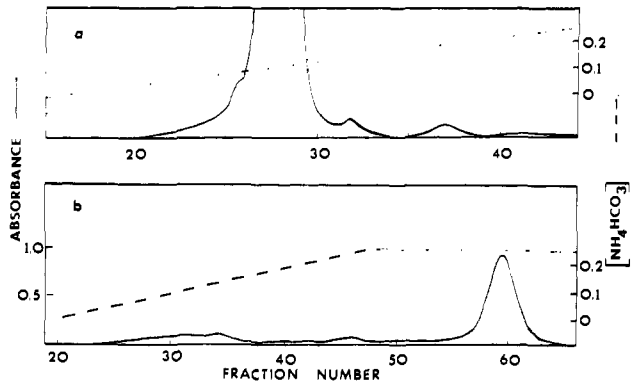


Figure 1. (a) Chromatography on DEAE Sephadex A-25 (bicarbonate form; 1 × 17 cm) of products from the reaction of **5c** (2.65 mg, 2.79 μmol) with sodium naphthalene. Elution was carried out with a linear gradient made from 100 ml of 0.01 M ammonium bicarbonate (mixing vessel) and 100 ml of 0.25 M ammonium bicarbonate (reservoir). Fractions (7.5 ml) were collected every 18 min. (b) Chromatography on DEAE Sephadex A-25 (bicarbonate form; 1 × 19 cm) of products from the reaction of **7** (2.50 mg, 1.37 μmol) with sodium naphthalene. Elution was carried out successively with ammonium bicarbonate solutions at 0.01, 0.01–0.25 (a linear gradient), and 0.25 M. Fractions (7 ml) were collected every 11 min.

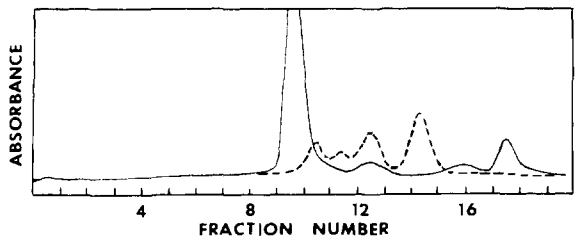


Figure 2. (—) Chromatography on a Bio-bead SX-1 column (8 × 98 cm) of reaction mixture from preparation of **8**. Tetrahydrofuran was the eluting solvent and the volume of each fraction was 3 ml. (- - -) Similar chromatography of a calibration mixture consisting of 3′-*O*-monomethoxytritylthymidine and trichloroethyl triester derivatives of dTpT, $dT(pT)_2$, and $dT(pT)_3$. The nucleoside is in fractions 14 and 15. The elution volumes of the oligomers decrease with increasing molecular weight. These separations were made by S. A. Jacobs.

Several procedures have been reported for removing the trichloroethyl group from nucleoside phosphotriesters.[6] Yields of dinucleoside monophosphates ranged from 50 to 73% for reductive deblocking with zinc or zinc-copper alloy;[6] however, the procedures have proven unsatisfactory for deprotecting some of the higher oligonucleotide derivatives.[16] A more efficient and reproducible method for converting the triesters to the diesters was therefore desirable.

In view of the facile conversion of *O*-methoxytritylthymidine to thymidine by sodium naphthalene in hexamethylphosphoric triamide,[17] we investigated the reaction of sodium naphthalene with trichloroethyl ester **5c**. The reaction proceeded to completion rapidly (<5 min) and efficiently, with elimination of both the trichloroethyl group from phosphorus and the mono-*p*-methoxytrityl group from oxygen. Following quenching with water and purification of the product on a DEAE-Sephadex column, thymidylyl[3′-5′]thymidine (dTpT) was obtained in 94% yield based on triester **5c**. The elution pattern is shown in Figure 1a. The tetranucleoside triphosphate, dTpTpTpT, was similarly obtained from **7** in 69–71% yield (see Figure 1b for elution pattern).

An interesting property of the trichloroethyl ester of thymidylyl[3′-3′]thymidine, which is produced on alkaline hydrolysis of the protected triester (**3**) formed as a by-product in stage 1 of the synthetic sequence (analogous to **2b**), is that this compound can be extracted from chloroform solutions of methoxytrityl ethers, such as **5c**, with water–ethanol (9:1, v/v).

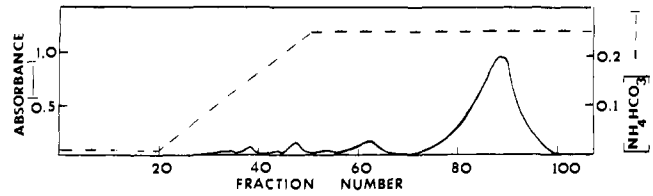


Figure 3. Chromatography on DEAE Sephadex A-25 (bicarbonate form, 1 × 22 cm) of products from the reaction of **8** (101 OD_{26} units) with sodium naphthalene. The same gradient used in Figure 1b was employed, but 6.5-ml fractions were collected.

Since the 3′–3′ isomer is the major nucleotidic side product in the reaction series, an appreciable degree of purification of the desired 3′–5′ phosphotriester can therefore be achieved simply by employing several aqueous extractions from chloroform. As a test of the efficiency of this purification procedure, we carried through a stepwise synthesis of the tetranucleotide derivative (**7**) from 3′-*O*-mono-*p*-methoxytritylthymidine. In this sequence the separations on silica gel plates were omitted at the di- and trinucleotide stage, the only purification being the aqueous extractions. At the tetramer stage the phosphotriesters were purified by thick layer chromatography on silica plates. Tetramer **7**, which separated cleanly from the other components, was recovered in 34% overall yield based on the methoxytritylthymidine. This procedure is considerably more rapid than that involving chromatography at each stage, and the final product was of good quality; however, the overall yield was somewhat lower than realized in the sequence involving purification at each step (49% overall).

As a further check on the synthetic procedure, a small scale reaction was carried out to extend the chain of the tetramer produced in the last experiment by one nucleotide unit. Repetition of the condensation, oxidation, and hydrolysis sequence afforded a product which, by analysis by gel filtration, corresponded to a pentamer with small amounts of dimer (3′–3′ isomer from the stage 1 reaction). The elution pattern is shown in Figure 2. Purification by silica gel chromatography afforded the pentamer product (**8**, 69%), which was then deprotected by reaction with sodium naphthalene. dTpTpTpTpT was recovered in 59% yield (based on **8**) by chromatography on DEAE Sephadex. The elution profile (Figure 3) indicates that some degradation of the pentamer chain occurred in the deprotection step.

Work is in progress to adapt the phosphite procedure to the synthesis of oligonucleotides containing all four common nucleotides and to explore the possibilities for building oligonucleotide chains on polymer supports by the phosphite procedure.

Experimental Section

The apparatus and general methodology for electrophoretic and chromatographic separations were the same as previously described.[18] R_m^{dpT} refers to electrophoretic mobility on paper relative to thymidine 5′-phosphate at pH 7.2 (phosphate buffer). R_f (solvent A) refers to mobility on paper chromatography (3MM paper) with *i*-C_3H_7OH–NH_4OH–H_2O (7:1:2), and R_f (THF) and R_f (EtOAc) express data for thin layer chromatography on silica gel (Quantum Industries Silica Gel Plates Q5F, 5 cm × 20 cm × 250 μm) with tetrahydrofuran and ethyl acetate, respectively. Preparative chromatography was carried out on 1000 μm Quantum Industries Plates. Elemental analyses were performed by Micro Tech Laboratories of Skokie, Ill.

A critical feature of the syntheses is the dryness of the solvents and reagents. Tetrahydrofuran was dried by refluxing over lithium aluminum hydride and distilling onto Linde 4A molecular sieves. 2,6-Di-*tert*-butyl-4-methylphenol (0.025%) was added as an antioxidant. Pyridine and 2,6-lutidine were distilled successively from toluenesulfonyl chloride and calcium hydride and stored over Linde 4A molecular sieves. Hexamethylphosphoric triamide[19] was treated with two batches of Linde 13X molecular sieves, distilled under vacuum,

and stored over Linde 13X molecular sieves. Nucleosides were dried by dissolving in anhydrous pyridine and distilling off the pyridine three times.

Phosphorylations were carried out in a 15-cm test tube closed with a rubber septum. Solutions were added by piercing the septum with a hypodermic syringe. Another syringe filled with calcium sulfate was used to equilize the pressure inside and outside the reaction vessel.

o-Chlorophenyl phosphorodichloridite[20] and 2,2,2-trichloroethyl phosphorodichloridite[21] were prepared from phosphorus trichloride and the appropriate phenol or alcohol by reported procedures. 5′-*O*-Phenoxyacetylthymidine (58%; mp 135–136.5 °C) was prepared by reaction of equivalent amounts of phenoxyacetyl chloride and thymidine in pyridine at 0° for 30 min, analogous to 5′-*O*-isobutyloxycarbonylthymidine.[22]

The nucleotide phosphotriester derivatives were obtained as white powders by precipitating from organic solvents or concentrating the solvents. As in the case of triesters prepared from phosphoryl compounds, stereoisomers would be expected, although no clear separation of stereoisomers on the silica slides was observed. Melting ranges are reported for transitions from the solid to a glassy melt. In general, softening occurred prior to melting and the melting range depended somewhat on the rate of heating. Melting points are therefore of less diagnostic value for these derivatives than for crystalline compounds (compare ref 25).

***o*-Chlorophenyl Ester of 3′-*O*-Mono-*p*-methoxytrityl[5′–5′]-3′-*O*-mono-*p*-methoxytritylthymidine (1).** To a solution of 3′-*O*-mono-*p*-methoxytritylthymidine (100 mg, 0.19 mmol) in 1 ml of tetrahydrofuran at −78° was added pyridine (18 μl, 0.21 mmol) and *o*-chlorophenyl phosphorodichloridite (17 μl, 0.11 mmol). A heavy white precipitate formed during the addition. The mixture was stirred for 30 min at −78° and then allowed to warm to about −10°, whereupon a solution containing iodine (27 mg, 0.11 mmol) in 2:1 tetrahydrofuran–water (2 ml) was added. The mixture was stirred as it warmed to room temperature. Solvent was removed in vacuo, and the residual gum was taken up in 2 ml of 10% 1-butanol in chloroform and washed with freshly prepared aqueous sodium bisulfite (5%). The organic layer was washed with water (three 1-ml portions) and concentrated. Chromatography of the residue on silica gel with 2:1 ethyl ether–ethyl acetate afforded 79 mg (76%) of compound **1** (isolated from the solvent by precipitation with hexane); mp 141 °C with softening at 136 °C; R_f (EtOAc) 0.24; R_f (THF) 0.62; R_f (ether) 0.07; uv (CH_3OH) λ_{max} 232 (sh, ε 33 000), 265 nm (ε 20 100), and λ_{min} 251 nm (ε 17 200).

Anal. Calcd for $C_{66}H_{62}ClN_4O_{14}P$: C, 65.97; H, 5.20; N, 4.66. Found: C, 65.82; H, 5.19; N, 4.57.

For further characterization this substance (5 mg) was treated with 80% acetic acid (1 ml) for 10 min at 100° and, after cooling and removal of the acetic acid, with 1 ml of 0.05 M sodium hydroxide in 1:1 dioxane–water (3 h). Paper chromatography in solvent A and paper electrophoresis yielded a single nucleotidic spot, which corresponded to the dinucleoside monophosphate [R_f (solvent A) 0.38; R_m^{dpT} 0.32]. Hydrolysis of 15 OD_{267} units with snake venom phosphodiesterase[23] afforded dT and dpT in a 1:1 ratio. As expected, the thymidylyl-[5′–5′]thymidine was not degraded by spleen phosphodiesterase.

***o*-Chlorophenyl Ester of 5′-*O*-Phenoxyacetylthymidylyl[3′–3′]-5′-*O*-phenoxyacetylthymidine (2a).** This substance was obtained in 66% yield by essentially the same procedure used in preparing **1** except that 5′-*O*-phenoxyacetylthymidine (100 mg, 0.27 mmol) and 2,6-lutidine (34 μl, 0.29 mmol) were used in place of monomethoxytritylthymidine and pyridine. The quantity of the dichloridite and iodine was 0.15 mmol each. Compound **2a** melted at 91–94 °C; λ_{max} (CH_3OH) 265 nm (ε 21 500) and λ_{min} 234 nm (ε 5030).

Anal. Calcd for $C_{42}H_{42}ClN_4O_{16}P\cdot H_2O$: C, 53.48; H, 4.70; N, 5.94. Found: C, 53.62; H, 4.44; N, 6.03.

Treatment (5 min) of a dioxane solution of **2a** with an equal volume of 9 M ammonium hydroxide gave a single nucleotidic product (**2b**) on thin layer chromatography (see Table I for properties). Deblocking **2a** by treatment with 0.10 M sodium hydroxide in 1:1 dioxane–water for 3 h, as in the characterization of **1**, gave a single nucleotidic product which corresponded to dTpT on paper chromatography in solvent A (R_f 0.42) and on paper electrophoresis at pH 7.2 (R_m^{dpT} 0.37).

***o*-Chlorophenyl Ester of Thymidylyl[3′–5′]-3′-*O*-mono-*p*-methoxytritylthymidine (4b).** 5′-*O*-Phenoxyacetylthymidine (100 mg, 0.27 mmol) in 1 ml of tetrahydrofuran was added over a 4 min period to *o*-chlorophenyl phosphorodichloridite (37 μl, 0.24 mmol) and 2,6-lutidine (111 μl, 0.96 mmol) in 0.5 ml of tetrahydrofuran at −78°. After 6 min of stirring, 3′-*O*-mono-*p*-methoxytritylthymidine (68 mg, 0.13 mmol) in tetrahydrofuran (0.5 ml) was added, the mixture was stirred at −78° for 20 min, and the vessel was removed from the cooling bath. When the temperature reached ~−10°, a solution of iodine (61 mg, 0.24 mmol) and 2,6-lutidine (55 μl, 0.48 mmol) in 2:1 tetrahydrofuran–water (2 ml) was added. The resulting solution was stirred for 5 min, then the solvent was removed in vacuo and the residue was taken up in 10% 1-butanol in chloroform (2 ml). After treatment with freshly prepared aqueous sodium bisulfite (5%) to reduce the excess iodine, and separation and concentration of the organic layer, a mixture of dioxane (25 ml) and 9 M ammonium hydroxide (25 ml) was added to hydrolyze the phenoxyacetic ester. The resulting solution was stirred for 20 min, concentrated, and partitioned between water and a mixture of chloroform/1-butanol (9:1, v/v). The organic layer was dried over sodium sulfate. Analytical thin layer chromatography on silica with EtOAc–THF (2:1, v/v), followed by observation with ultraviolet light and spraying with aqueous perchloric acid, showed the following spots: R_f 0.26 (very strong, yellow after acid spray) (**4b**); R_f 0.07 (moderate under uv, no coloration after acid spray) (**2b**); R_f 0–0.02 (moderate, some yellowing after acid spray, unidentified); R_f 0.44 and 0.55 (extremely weak; pale yellow after acid spray, unidentified by-products, possibly halonucleosides bearing the methoxytrityl group). No trace of **1** (R_f 0.38) was found. Likewise, the absence of a spot in the region of 0.34, where compound **4a** moves, showed removal of phenoxyacetyl from **4a** was complete. Chromatographic data are summarized in Table I. Preparative thick layer chromatography was carried out with THF–1,2-dichloroethane (1:3 v/v). Compound **4b** was recovered by extraction from the silica with THF–EtOH (2:1, v/v) followed by precipitation with hexane: weight 80 mg (65%); mp 130–133 °C; λ_{max} (CH_3OH) 232 (sh, ε 18 600), 265 nm (ε 20 800), and λ_{min} 246 nm (ε 14 600).

Anal. Calcd for $C_{46}H_{46}ClN_4O_{13}P\cdot\frac{1}{2}H_2O$: C, 58.88; H, 5.04; N, 5.97. Found: C, 58.94; H, 5.07; N, 6.18.

For further characterization, 5 mg of compound **4b** was converted to dTpT by (i) treatment with 80% aqueous acetic acid at 100° for 10 min, followed by removal of the acetic acid under reduced pressure and removal of methoxytritanol by extraction with hexane, and (ii) treatment with 1 ml of 0.1 M sodium hydroxide for 3 h. Neutralization with Dowex 50 resin (pyridinium form) afforded the pyridinium salt of dTpT as the only nucleotidic product: R_m^{dTp} 0.40. It was completely degraded to dT and dpT by snake venom phosphodiesterase and to dT and dTp by spleen phosphodiesterase.

Experiments on Hydrolysis of Phenoxyacetic Esters. Several tests were carried out to establish suitable conditions for selectively removing the phenoxyacetyl group in the presence of the *o*-chlorophenyl group. The course of the reactions was followed by spotting aliquots on silica slides and developing with ETOAc–THF (2:1) or THF.

Ammonium hydroxide (4.5 M) in dioxane–water (1:1, v/v) proved satisfactory for these thymidine derivatives. The *O*-phenoxyacetyl group was completely removed from **2a** within 5 min at room temperature to give **2b** (see Table I for properties). Under these conditions no reaction of compound **2b** was observed.

Table I. R_f Values for Thin Layer Chromatography[a]

Compound	Solvent		
	EtOAc	EtOAc:THF[b]	THF
2b	0.02	0.07	0.20
Thymidine	0.05	0.11	0.32
4b	0.07	0.15	0.55
5′-*O*-Phenoxyacetylthymidine	0.16	0.32	0.52
2a	0.18	0.31	0.64
1	0.24	0.38	0.62
4a	0.25	0.34	0.62
3′-*O*-Mono-*p*-methoxytritylthymidine	0.39	0.48	0.65

[a] These values are for solutions of individual compounds run separately; in crude reaction mixtures the values may be somewhat different. Charged phosphoryl derivatives did not move from the origin with these solvents. [b] 2:1.

Sodium hydroxide (0.1 M) in dioxane–water (1:1) at 0° was also satisfactory. Phenoxyacetic esters were completely hydrolyzed within 5 min. Under the same conditions, no cleavage of *o*-chlorophenyl ester **4b** was observed over a period of 30 min, but traces of hydrolyzed material could be found at the origin of the silica plates when the time of reaction was extended to 50 min.

Saturated ammonia in methanol led to cleavage of the phenoxyacetic ester, but over long time periods the *o*-chlorophenyl triester group was also attacked. Thus, a reaction of **4a**[24] with a saturated solution of ammonia in methanol for 5 h at 0° afforded only a small amount of **4b** (15% yield). The major product (65%) was a solid, mp 132–134 °C, R_f (2:1 EtOAc–THF) 0.21, which on the basis of the analytical data appears to be the methyl ester of thymidylyl[3′–5′]-3′-*O*-mono-*p*-methoxytritylthymidine.

Anal. Calcd for $C_{41}H_{45}N_4O_{13}P\cdot H_2O$: C, 57.88; H, 5.57; N, 6.58. Found: C, 58.00; H, 5.30; N, 6.36.

Similarly, thin layer chromatographic data showed that triester **4b** was extensively converted to this same material when stirred in saturated ammonia in methanol for 2 h at room temperature. After 12 h the reaction was complete, and the new product was the only material observable on the chromatogram.

2,2,2-Trichloroethyl Ester of 5′-*O*-Phenoxyacetylthymidylyl[3′–5′]-3′-*O*-monomethoxytritylthymidine (5a). 5′-*O*-Phenoxyacetylthymidine (150 mg, 0.40 mmol) was added over a 2-min period to a stirred solution of 2,2,2-trichloroethyl phosphorodichloridite (53 μl, 0.36 mmol) and 2,6-lutidine (170 μl, 1.44 mmol) in THF (0.5 ml) in a test tube cooled by dry ice–isopropyl alcohol. A white precipitate formed immediately. The tube which had contained the nucleoside was rinsed with 0.5 ml of THF and this rinse was added to the reaction vessel. After an additional 3 min of stirring, 3′-*O*-mono-*p*-methoxytritylthymidine (102 mg, 0.20 mmol) in THF (0.5 ml) was added. Again, the test tube containing the nucleoside was rinsed with 0.5 ml of dry THF and this rinse was added to the reaction vessel. The mixture was stirred for a total of 15 min at −78° and then removed from the bath. When the temperature reached about −10° (approximately 2 min) a solution of iodine (92 mg, 0.36 mmol) and 2,6-lutidine (84 μl, 0.72 mmol) in 3 ml of THF–H_2O (2:1 v/v) was added. After 5 min the solvent was removed in vacuo and the resulting gum was taken up in 2 ml of 10% 1-butanol in chloroform. Water (2 ml) and a few drops of sodium bisulfite were added to reduce excess iodine. The chloroform layer was separated and the aqueous layer was washed with two 2-ml portions of chloroform. The combined chloroform layers were then concentrated and the resulting material was purified on silica gel preparative plates using chloroform–ether–ethanol (20:20:1 v/v/v) as developing solvent (multiple developments were necessary). Compound **5a** (176 mg, 82% yield) melted at 115–119 °C; R_f (THF) 0.47; R_f (2:1 EtOAc–THF) 0.35; λ_{max} (CH_3OH) 232 (sh, ε 19 200), 264 nm (ε 21 200), and λ_{min} 244 nm (ε 15 000).

Anal. Calcd for $C_{50}H_{50}Cl_3N_4O_{15}P$: C, 55.39; H, 4.65; N, 5.17. Found: C, 55.22; H, 4.59; N, 5.05.

2,2,2-Trichloroethyl Ester of 5′-*O*-Phenoxyacetylthymidylyl[3′–5′]thymidine (5b). Compound **5a** (145 mg, 0.134 mmol) was dissolved in 1 ml of 80% aqueous acetic acid and heated on a steam bath for 10 min. The acetic acid was removed in vacuo and the resulting gum was purified by chromatography on silica plates with ether–chloroform–ethanol (1:1:1) to give 98 mg (89%) of **5b**: mp 103–106 °C; R_f (THF) 0.23; R_f (2:1 EtOAc–THF) 0.07; λ_{max} (CH_3OH) 264 nm (ε 19 000) and λ_{min} (ε 5220).

Anal. Calcd for $C_{30}H_{34}N_4Cl_3O_{14}P\cdot\frac{1}{2}H_2O$: C, 43.89; H, 4.30; N, 6.82. Found: C, 43.85; H, 4.09; N, 7.04.

Trichloroethyl Ester of Thymidylyl[3′–5′]-3′-*O*-mono-*p*-methoxytritylthymidine (5c). The reaction conditions used to prepare **5a** were repeated with 68 mg (0.13 mmol) of 3′-*O*-mono-*p*-methoxytritylthymidine. Quantities of other reactants were scaled accordingly. The workup differed in that the crude product from the chloroform extractions (i.e., just before purification by chromatography) was treated with 25 ml of concentrated ammonium hydroxide for 10 min. The solution was concentrated rapidly (under vacuum) and the residue was taken up in a small amount of pyridine.

Analytical thin layer chromatography of an aliquot with EtOAc–THF (2:1) showed three spots under ultraviolet light: R_f 0.22 (very strong, compound **5c**); R_f 0.04 (weak, residual 3′–3′ isomer which had not been removed by extraction with water), and R_f 0.0 (small tight spot of unidentified organic matter which failed to move on silica). On spraying with aqueous perchloric acid a very strong yellow spot developed at R_f 0.22 and a pale yellow spot appeared at R_f 0.39 (unidentified trityl positive material which was present in too low a concentration to observe under ultraviolet light, prior to spraying). Some yellowing at the origin also was observed.

Separation of the reaction products by preparative thick-layer chromatography with EtOAc–THF (2:1, v/v) gave 120 mg (95%) of the title compound: mp 106–109 °C, R_f (solvent A) 0.85; R_f (THF) 0.37; λ_{max} 232 (sh, ε 18 200), 265 nm (ε 20 300); λ_{min} 246 nm (ε 14 200).

Anal. Calcd for $C_{42}C_{44}Cl_3N_4O_{13}P$: C, 53.09; H, 4.67; N, 5.90. Found: C, 52.98; H, 4.72; N, 5.87.

Tris(trichloroethyl) Ester of Thymidylyl[3′–5′]thymidylyl[3′–5′]-thymidylyl[3′–5′]-3′-*O*-mono-*p*-methoxytritylthymidine (7) (First Sequence). This compound was prepared from **5c** by two reaction cycles utilizing **3** (R″ = Cl_3CCH_2–). The first cycle was carried out by addition of **5c** (50 mg, 0.053 mmol) in tetrahydrofuran (0.6 ml) to **3** prepared from 5′-*O*-phenoxyacetylthymidine (50 mg, 0.13 mmol) and trichloroethyl phosphorodichloridite (17.5 μl, 0.118 mmol) with 2,6-lutidine (55 μl, 0.47 mmol) in tetrahydrofuran (0.9 ml). The procedure was the same employed for the preparation of **5c.** The trinucleotide derivative (**6**) was isolated by chromatography on preparative silica plates with chloroform–ether–ethanol (3:3:1): weight, 50.7 mg (69%); R_f (THF) 0.11. For the next cycle, 45 mg of **6** in tetrahydrofuran (0.6 ml) was added to **3** prepared from phenoxyacetylthymidine (30 mg, 0.08 mmol), trichloroethyl phosphorodichloridite (11 μl, 0.072 mmol), and 2,6-lutidine (33 μl, 0.29 mmol) in tetrahydrofuran (0.9 ml). Temperature and conditions were the same as previously employed for **6,** except that 20 min was allowed for the condensation at −78° (rather than 10 min) and water saturated with sodium chloride rather than pure water was used in the extraction steps in isolating the product. Compound **7** was purified by chromatography on silica with chloroform–ether–ethanol (3:3:1): weight 43 mg (75%); mp 148–152 °C; R_f (solvent A) 0.73; R_f (THF) 0.08; R_f ($CHCl_3$–C_2H_5OH–$C_2H_5OC_2H_5$ 1:1:1) 0.14; λ_{max} (CH_3OH) 264 nm (ε 38 000) and λ_{min} 242 (ε 22 800).

Anal. Calcd for $C_{66}H_{72}Cl_9N_8O_{27}P_3$: C, 43.52; H, 3.98; N, 6.15. Found: C, 43.11; H, 3.95; N, 6.11.

Second Sequence: No Isolation of Intermediates. To test the need for purification of the intermediates, the synthesis of **7** was repeated, starting with 5′-*O*-phenoxyacetylthymidine (100 mg) and 3′-*O*-mono-*p*-methoxytritylthymidine (68.4 mg). In these cases, purification by chromatography on silica gel (a slow step in the sequence) was omitted. The 3′–3′ isomer of the thymidylylthymidine ester was removed at each stage by extracting a chloroform solution of the reaction products (after removal of the phenoxyacetyl group) four times with 20-ml portions of water–ethanol (9:1, v/v). Following evaporation of the chloroform, the residual gum was dried (by addition and evaporation in vacuo of pyridine three times) and dissolved in tetrahydrofuran for the next reaction cycle. At the tetranucleotide stage the product was purified by chromatography on silica gel with ether–chloroform–ethanol (3:3:1, v/v). The overall yield of **7** from 3′-*O*-monomethoxytritylthymidine was 34% (82 mg): mp 146–151 °C; identical in chromatographic properties with the substance prepared in the first sequence.

Deblocking the Trichloroethyl Esters. Sodium (23 mg, 1 mmol) was stirred with 4 ml of hexamethylphosphoric triamide (HMPT)[19] until a deep blue color developed. Naphthalene (150 mg, 1.2 mmol) was added and the solution was stirred for 5 min after the green color characteristic of the naphthalene radical ion was obtained. A solution of the nucleotide material (2.5–3 mg) in 0.1 ml of pyridine was then added, and after 5 min the reagent was quenched with addition of a few drops of water. The solution was diluted to 50 ml with water and, after adjustment of the pH to 8, the HMPA and naphthalene were removed by extraction with chloroform (four extractions with 50-ml portions). The aqueous layer was diluted to 50 ml with water, adjusted to pH 8.5 with ammonium hydroxide, and applied to a DEAE Sephadex column equilibrated with 0.01 M aqueous ammonium bicarbonate. After washing the column with water (30 ml) and 0.01 M ammonium bicarbonate (50 ml), the product was eluted with ammonium bicarbonate (0.01–0.25 M). Elution profiles for dTpT and dTpTpTpT (from **5c** and **7**) are shown in Figure 1. Fractions 27–30 (Figure 1) on pooling afforded 51.1 OD_{264} units of dTpT. The yield based on an extinction coefficient of 19 400 for dTpT and on the amount of starting triester (2.79 μmol) is 94%. Based on the total material eluted from the column which exhibited the thymidine ultraviolet spectrum (53.6 OD_{264} units), the yield is 95%. Fractions 53–66 (Figure 2) similarly yielded 35.4 OD_{266} units of dTpTpTpT

Table II. Enzymatic Hydrolysis of Nucleotide Products from Sodium Naphthalene Reductions

Compd	OD_{267} units of sample	Nucleoside:Nucleotide ratio		
		From snake venom enzyme	From spleen enzyme	Calcd
dTpT	10	1.06	0.9	1
dTpTpT-pT	14	3.1	3.5[a]	3
dTpTpT-pTpT	17	3.9	4.0	4

[a] In this experiment 93% of the substrate was hydrolyzed and 7% remained undegraded. In all other cases the initial nucleotide was hydrolyzed completely and only thymidine and thymidine phosphate were found.

(71% based on an extinction coefficient of 36 400 for this tetramer and on starting triester, 1.37 μmol). Another reaction gave a 69% yield of dTpTpTpT.

dTpTpTpTpT. Chain extension of compound **7** (from the second synthetic sequence; 70 mg, 0.038 mmol) was accomplished by reaction with **3** prepared from 5′-*O*-phenoxyacetylthymidine (54 mg, 0.143 mmol) by the same procedure used for constructing **7.** The resulting products were analyzed (after extraction of the 3′-3′ dinucleotide isomer derivative with four portions of 10% ethanol in water and before purification on silica gel) by gel filtration on a Bio-bead column. The elution pattern indicated good conversion to the protected pentamer (Figure 2). Purification of the main portion obtained from the extractions by chromatography on silica gel plates using ether–chloroform–ethanol (1:1:2 v/v/v, four developments) afforded 1235 OD_{267} units (69% based on an extinction coefficient of 46 900) of purified pentamer: λ_{max} 267 nm and λ_{min} 243 nm; R_f (THF) 0.05; R_f($CHCl_3$–C_2H_5OH–$C_2H_5OC_2H_5$ 1:1:1) 0.06. Deblocking by use of sodium naphthalene in HMPA as described above, using 101 OD_{267} units of the protected pentamer, yielded 57.5 OD_{267} units (59%) of dTpTpTpTpT; R_m^{dpT} 0.81; R_f (solvent A) 0.012, R_f (solvent F) 0.30. The elution pattern for fractionation of the reaction products is given in Figure 3.

Enzymatic Degradation. The nucleotide products were hydrolyzed by use of snake venom phosphodiesterase and by spleen phosphodiesterase by conventional procedures.[23] The products were characterized by paper electrophoresis and chromatography (solvent A). For quantitative analysis, they were eluted from the paper (solvent A) and determined by the absorbance at 267 nm. The results are given in Table II.

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Synthesis of the Witchweed Seed Germination Stimulant (+)-Strigol[1-3]

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Abstract: A synthesis of (±)-strigol (**24**) and (±)-4′-epistrigol (**25**) has been carried out by formylation of (±)-1α,4α-dihydroxy-7,7-dimethyl-4,5,6,7-tetrahydroindan-2α-acetic acid γ-lactone (**21**) followed by alkylation of the resulting hydroxymethylene lactone (**2**) with the bromobutenolide **4.** Similarly prepared were (±)-4-epistrigol (**25**) and (±)-4,4′-diepistrigol (**26**). Resolution of (±)-**21** was accomplished by its conversion into the diastereomeric 3β-acetoxyandrost-5-ene-17β-carboxylate derivatives. Using the same reaction sequence as for the racemic series, (+)-**21** was converted into (+)-strigol and (+)-4′-epistrigol while (−)-**21** yielded (−)-strigol and (−)-4′-epistrigol. Routes to **21** from both α-cyclocitral (**11a**) and β-cyclocitral (**11b**) via the common intermediate methyl 3-oxo-2,6,6-trimethylcyclohex-1-ene-1-carboxylate (**9b**) are described.

Witchweed (*Striga lutea* Lour.) is an angiospermous root parasite that attacks corn, sorghum, sugarcane, rice, and more than 60 other important crop plants and weeds of the grass family.[4] *Striga* species occur commonly in the Eastern Hemisphere and inflict serious crop damage in many parts of the world.[4,5] The discovery of witchweed in the United States in 1956,[6] the first report of this pest in the Western Hemisphere, has been a matter of concern.

The germination of the seeds of witchweed, which may lie dormant for 15–20 years, is induced by a stimulant released by the roots of the host plant.[5] This stimulant is also released by certain other plant species which are not parasitized by witchweed.[7-9] Cook et al.[10] isolated this germination stimulant, strigol, from the root exudates of cotton (*Gossypium hirsutum* L.) and determined its structure and relative configuration as depicted in **1**[11] largely by spectroscopic and x-ray crystallo-