2,2,2-Tribromoethyl 2-Chloro-4-*t*-butylphenyl Phosphorochloridate: A Convenient Phosphorylating Agent for the Synthesis of DNA-Fragments by the Phosphotriester Approach

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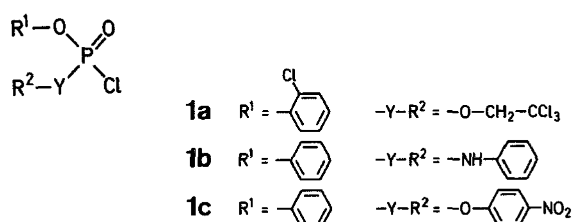
The method of choice for the synthesis of oligonucleotides with a defined sequence and length appears, at present, to be the phosphotriester approach[1,2]. The crucial step in a strategy based on a phosphotriester approach is the introduction of an intermediate 3′–5′ internucleotide phosphotriester linkage (e.g. **9**). To achieve this aim, bifunctional phosphorylating agents, e.g. aryl or alkyl dihydrogen phosphates[1–5], have repeatedly been used. However, a serious drawback of these bifunctional reagents is the concomittant formation of unnatural 3′–3′ and 5′–5′ internucleotide phosphotriester linkages[6,7]. To avoid the formation of these unwanted products, several monofunctional phosphorylating agents (**1a, b, c**)[8,9,10] have been used for the introduction of 3′–5′ internucleotide phosphotriester linkages.

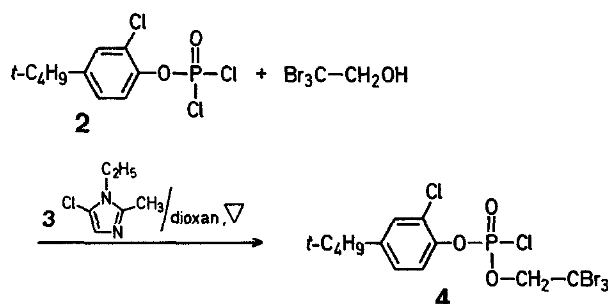


A reagent of this type suitable for the introduction of internucleotide linkages should have the following properties: (a) it should be an efficient phosphorylating agent; (b) the nature of one protective group at phosphorus should be such that it can be selectively removed in the presence of the other; (c) it should be easily accessible and should preferentially be crystalline. We now wish to report that 2,2,2-tribromoethyl 2-chloro-4-*t*-butylphenyl phosphorochloridate (**4**) possesses the desired properties.

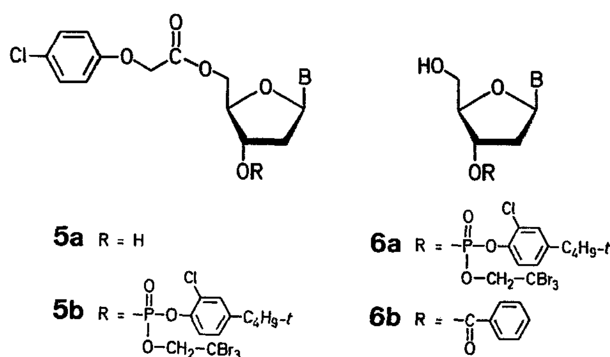
The utility of the new reagent **4** is demonstrated by its use in the synthesis of the fully protected oligodeoxyribonucleotide dTATCAAGTTG. Reagent **4** shows three distinct advantages over the previously reported 2,2,2-trichloroethyl 2-chlorophenyl phosphorochloridate[8] (**1a**):

- the selective removal of the 2,2,2-tribromoethyl group from the phosphotriester intermediates (e.g. **7**) by reduction with zinc/2,4,6-triisopropylbenzenesulfonic acid (TPSOH)/pyridine[8,11] is even more easily accomplished[12] than the removal of the 2,2,2-trichloroethyl group;
- the 4-*t*-butyl group at the aryl ring of our reagent increases the lipophilicity of the phosphotriester intermediates[14] (e.g. **9**); this property not only enhances the solubility of triesters of oligonucleotides in organic solvents (especially in chloroform) but also simplifies their purification by short-column chromatography[15]; it should be noted that the *t*-butyl group does not hinder the final cleavage of the *O*-aryl group by fluoride ion[16];
- reagent **4** is an easily accessible, well defined, and stable crystalline compound.

2,2,2-Tribromoethyl 2-chloro-4-*t*-butylphenyl phosphorochloridate (**4**) is prepared from 4-*t*-butyl-2-chlorophenol by a two-step sequence: reaction of the phenol with phosphoryl chloride in the presence of catalytic amounts of 5-chloro-1-ethyl-2-methylimidazole (**3**) at 150° to give 2-chloro-4-*t*-butylphenyl phosphorodichloridate[17] (**2**; 94%), and reaction of **2** with tribromoethanol in boiling dioxan in the presence of 5-chloro-1-ethyl-2-methylimidazole (**3**)[10,18].



Phosphorylation of protected deoxyribonucleosides[7,19] (**5a**) having a free 3′-hydroxy function with **4** gives the fully protected 3′-phosphotriester intermediates **5b** in good yields (Table). Compounds **5b** are the starting material for the synthesis of two building blocks (**6a** and **7**), which are required for a systematic synthesis of oligodeoxyribonucleotides. Thus, building blocks **6a** are obtained by treatment of **5b** with potassium carbonate in anhydrous methanol/dioxan[11]. Protection of the 5′-hydroxy function of **6a** with the levulinyl group[20,21], which can be selectively removed with hydrazine hydrate in pyridine/acetic acid[11,20,21], gives the building blocks[22] **7**.



For the synthesis of the fully protected oligodeoxyribonucleotide dTATCAAGTTG we prepared five fully protected dimers, dTA, dTC, dAA, dGT and dTG. The fully protected dinucleoside diphosphate dAA (**9a**, $B=A^{Bz}$, $B'=A^{Bz}$) was synthesized as follows: The 2,2,2-tribromoethyl group of the fully protected phosphotriester intermediate **7** ($B=A^{Bz}$) was removed with zinc dust/TPSOH in anhydrous pyridine[8,11] to give the phosphodiester intermediate **8** ($B=A^{Bz}$). Condensation of the latter (**8**, $B=A^{Bz}$) with the partially protected phosphotriester intermediate **6a** ($B=A^{Bz}$) in anhydrous pyridine in the presence of 1-(2,4,6-triisopropylbenzenesulfonyl)-4-nitroimidazole (TPSNI)[11] as condensing agent gave the fully protected dinucleoside diphosphate dAA (**9a**, $B=A^{Bz}$, $B'=A^{Bz}$) in 80% yield. The fully protected dinucleoside diphosphates dTA, dTC, and dGT were similarly obtained in good yields. According to this procedure, the 3′-terminal fully protected dinucleoside monophosphate dTG (**9b**, B=T, $B'=G^{Bz}$) was synthesized by condensation of **8** (B=T) with **6b** ($B=G^{Bz}$)[22].

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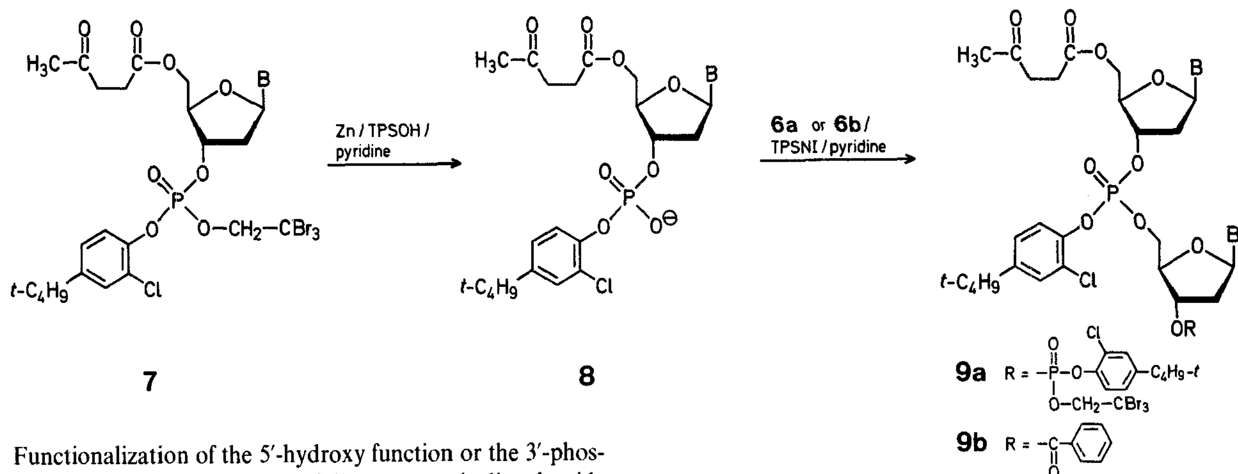

Functionalization of the 5'-hydroxy function or the 3'-phosphotriester function of the fully protected dinucleoside diphosphates **9a** by treatment with hydrazine hydrate in pyridine/acetic acid or zinc dust/TPSOH in anhydrous pyridine affords building blocks with a free 5'-hydroxy function or a 3'-phosphodiester function, respectively. Using this strategy, the fully protected tetramers dTATC and dGTTG were synthesized by condensing the appropriately functionalized dimers.

The fully protected hexamer dAAGTTG was obtained starting from the partially protected dimer dAA (3'-phosphodiester) and the tetramer dGTTG (free 5'-OH). Finally, the fully-protected decamer dTATCAAGTTG was obtained by condensation of the functionalized tetramer dTATC (3'-phosphodiester) and hexamer dAAGTTG (free 5'-OH). It is worth mentioning that the yield of the decamer in the final coupling step did not decrease significantly as compared to those for the dimers, tetramers, and hexamer. After unblocking of the aryl protective groups with fluoride ion[16] and removal of the remaining base-labile protective groups with aqueous ammonia, the product was purified by Sephadex G50 column chromatography. The isolated pure (by H.P.L.C.) product underwent complete digestion in the presence of snake venom phosphodiesterase, yielding pT, pA, pC, pG, and T in a ratio of 3.3:2.9:0.9:1.9:1.0, as analyzed by H.P.L.C.

2-Chloro-4-*t*-butylphenyl Phosphorodichloridate (2)[17]:

A mixture of commercially available (Aldrich) 2-chloro-4-*t*-butylphenol (92.3 g, 0.5 mol), freshly distilled phosphoryl chloride (137 ml, 1.5 mol), and 5-chloro-1-ethyl-2-methylimidazole[10,18] (**3**; 0.5 g, 3.45 mmol) is heated at reflux temperature (oil-bath, 150°). After 3 h, when I.R. spectroscopy reveals that no more starting material is present, the products are allowed to cool to room temperature and the excess phosphoryl chloride is removed by distillation at water aspirator pressure. The residual product is fractionally distilled in vacuo to give **2** as a colorless liquid; yield: 142 g (94 %); b.p. 116°/0.4 torr. The product solidifies upon standing at 4°; m.p. 45°.

^{1}H-N.M.R. (CCl_4): δ = 1.32 (s, 9H, *t*-butyl); 7.14–7.54 ppm (m, 3H_{arom}).

2,2,2-Tribromoethyl 2-Chloro-4-*t*-butylphenyl Phosphorochloridate (4):

A mixture of 2-chloro-4-*t*-butylphenyl phosphorodichloridate (**2**; 36.2 g, 0.12 mol), 2,2,2-tribromoethanol (28.3 g, 0.1 mol), and 5-chloro-1-ethyl-2-methylimidazole (**3**; 1 ml) in anhydrous dioxan (125 ml) is heated at reflux temperature (oil bath, 150°). After 4 h, when I.R. spectroscopy shows that no more 2,2,2-tribromoethanol is present, the mixture is allowed to cool to room temperature and dioxan is removed on a rotatory evaporator under reduced pressure. Traces of dioxan in the remaining syrup are removed by coevaporation with anhydrous petroleum ether (40–60°, 2 × 150 ml). The residue is dissolved in hot anhydrous petroleum ether (40–60°, 250 ml) and the solution is carefully decanted from some residual oil. This oil is washed with hot anhydrous petroleum ether (40–60°, 2 × 75 ml) and the washings decanted. Crystallization of **4** occurs at 4°. The crystals are isolated by filtration, washed with a small volume of anhydrous petroleum ether (40–60°, 50 ml), and dried in vacuo over phosphorus pentoxide. A second portion of the pure product is obtained from the concentrated mother liquor; yield: 38.5 g (70 %, based on 2,2,2-tribromoethanol); m.p. 85–87°.

$C_{12}H_{14}Br_3Cl_2O_3P$ (547.8)	calc.	C 26.31	H 2.58
	found	26.50	2.74

^{1}H-N.M.R. ($CDCl_3$): δ = 1.32 (s, 9H, *t*-butyl); 5.00 (d, 2H, —CH_2—, J = 6.7 Hz); 7.20–7.52 ppm (m, 3H_{arom}).

^{13}C{^{1}H}-N.M.R. ($CDCl_3$): δ = 31.1 (s, —CH_3, *t*-butyl); 33.4 (d, —CBr_3); 34.7 (s, C_{quart}, *t*-butyl); 80.5 (d, —CH_2—); 121.1 (d, C-4$_{arom}$); 125.0 (d, C-6$_{arom}$); 125.1 (d, C-5$_{arom}$); 128.0 (d, C-3$_{arom}$); 143.1 (d, C-2$_{arom}$); 151.0 ppm (d, C-1$_{arom}$).

Phosphorylation of Protected Deoxyribonucleosides (5a)[7,19] with Reagent 4:

A solution of 2,2,2-tribromoethyl-2-chloro-4-*t*-butylphenyl phosphorochloridate (**4**; 4.1 g, 7.5 mmol) in anhydrous pyridine (5 ml) is added dropwise, over a period of 30 min, to a cooled (ice/water bath) solution of **5a** (B = T; 5 mmol)[7] in anhydrous pyridine (5 ml) under exclusion of moisture. After the addition is complete, the cooling bath is removed and the reaction allowed to proceed at room temperature. After ~2.5 h, pyridine is removed by evaporation under reduced pressure and the residue partitioned between chloroform (150 ml) and 5 % aqueous sodium hydrogen carbonate (75 ml). The organic layer is washed with water (50 ml), dried with magnesium sulfate, concentrated, and triturated with petroleum ether (40–60°, 2 × 100 ml). The solid is redissolved in chloroform and purified by short-column chromatography[15] on silica gel (Merck Kieselgel H, Typ 60; 100 g) in chloroform/methanol (98:2, v/v). Elution of the column with the same solvent mixture and concentration of the appropriate fractions gives a foam. A solution of the latter in chloroform (5 ml) is added dropwise, with stirring, to petroleum ether (40–60°, 150 ml). The precipitated product (**5b**, B = T) is isolated by filtration and dried in vacuo over phosphorus pentoxide at 20°.

Phosphorylation of **5a** (B = A^{Bz})[19] with **4** is carried out similarly. Phosphorylation of **5a** (B = C^{An})[19] and **5a** (B = G^{Bz})[19] with **4** is carried out in anhydrous acetonitrile in the presence of 1-methylimidazole. Relevant data are given in the Table.

Preparation of Fully Protected Dinucleoside Diphosphates (9a); General Procedure:

5'-*O*-Levulinylnucleoside 3'-*O*-(2,2,2-tribromoethyl 2-chloro-4-*t*-butylphenyl) phosphate (**7**; 1.1 mmol) and TPSOH (0.11 mmol) are dissolved in anhydrous pyridine (5.5 ml), and activated zinc dust[11] (~10 mmol) is added. The suspension stirred. After ~5 sec,

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the temperature of the mixture increases sharply and reaches a maximum after ~30 sec. After 1 min, the mixture is filtered to remove excess zinc. The filtrate is diluted with chloroform (200 ml) and washed with 1 molar triethylammonium hydrogen carbonate (TEAB; pH 7.5, 16.5 ml) and 0.1 molar TEAB (16.5 ml), respectively. The organic layer is filtered and concentrated to an oil. This oil is transferred to a small flask containing the 5'-hydroxynucleoside 3'-*O*-(2,2,2-tribromoethyl 2-chloro-4-*t*-butylphenyl) phosphate (**6a**; 1.0 mmol). The mixture is dried by repeated coevaporation with anhydrous pyridine (3 x 10 ml). TPSNI[11] (1.2 mmol) is added to the resulting viscous oil and the reaction allowed to proceed, under exclusion of moisture, at 20°. After ~24 h, the reaction mixture is partitioned between chloroform (100 ml) and 5% aqueous sodium hydrogen carbonate (50 ml). The organic layer is washed with water (50 ml), dried with magnesium sulfate, and concentrated to an oil. Trituration with petroleum ether (40–60°, 2 x 100 ml) affords a solid which is redissolved in chloroform and purified by short-column chromatography[15] on silica gel (Merck Kieselgel H, Typ 60; 30–50 g) in chloroform/methanol (97.5–96.5:2.5–3.5, v/v). Elution of the column with the same solvent mixture and precipitation of the appropriate concentrated fractions from petroleum ether (40–60°) as described above, affords the fully protected dinucleoside diphosphates **9a** as colourless solids in good to high yields.

Table. Phosphorylation of Deoxynucleosides (**5a**)[7,19] with 2,2,2-Tribromoethyl 2-Chloro-4-*t*-butylphenyl Phosphorochloridate (**4**)

B	**5a**/**4** [mmol]	Reaction conditions[a]	Reaction time[b] [h]	Yield of **5b**[c] [%]
T	5/7.5	(A)	2.5	90
A^{Bz}	5/7.5	(A)	2.5	80
C^{An}	5/10	(B)	5.5	75
G^{Bz}	5/10	(B)	4.5	70

[a] (A): A solution of **4** in anhydrous pyridine (5 ml) was added dropwise to a solution of **5a** in anhydrous pyridine (5 ml) at 0°.
(B): A solution of **4** in anhydrous acetonitrile (15 ml) was added dropwise to a stirred suspension of **5a** in anhydrous acetonitrile (45 ml) containing 1-methylimidazole (1.6 ml, 20 mmol) at 0°.

[b] Reaction times were established by T.L.C. on silica gel (TLC-Ready Plastic Sheets F1500 LS 254 Silica Gel, Schleicher and Schüll) in chloroform/methanol (92:8, v/v).

[c] Yields are based on the weight of precipitated products after purification by column chromatography. Satisfactory analytical data have been obtained for all four compounds: C, ±0.14; H, ±0.09; N, ±0.02.

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[22] The synthesis of these compounds will be published elsewhere.

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