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A Simple Preparation of 5'-O-Dimethoxytrityl Deoxyribonucleoside 3'-O-Phosphorbisdiethylamidites as Useful Intermediates in the Synthesis of Oligodeoxyribonucleotides and Their Phosphorodiethylamidate Analogs on a Solid Support

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ABSTRACT: 5'-O-Dimethoxytrityl deoxyribonucleoside 3'-O-phosphorbisdiethylamidites were prepared by the selective phosphitylation of 5'-O-dimethoxytrityl deoxyribonucleosides with tris(diethylamino)phosphine in the presence of amine and tetrazole as catalyst. The bisamidites activated by p-nitrophenyltetrazole can be coupled efficiently with 5'-hydroxyl group of nucleoside on a solid support. Oligodeoxyribonucleotides were obtained in high yields. The phosphorodiethylamidate linkage was obtained by non-aqueous oxidation using tert-butyl hydroperoxide of the phosphordiethylamidite formed after the coupling.

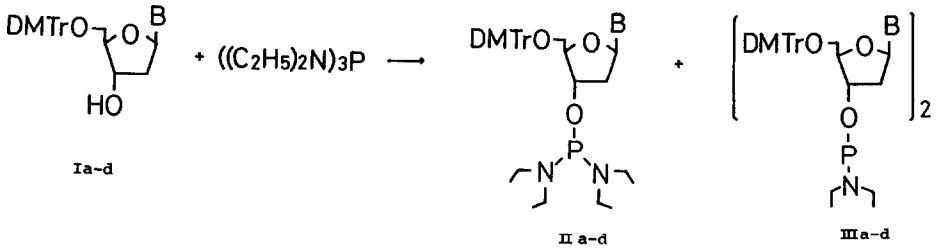
Recent developments of the phosphite approach based on phosphoramidite chemistry[1)] enable us to synthesize biochemically useful DNA fragments in a reasonable time without tedious efforts. On the other hand, hydrogen phosphonate chemistry[2)] has proven to be useful for generating a variety of modified internucleotide linkages such as phosphorothioate[3)] and phosphoroamidates.[3,4)] Recent reports have shown that the alternative method utilizing deoxyribonucleoside 3'-O-phosphorbismorpholidite[5)] and diisopropylamidite[6)] as new class of intermediates can be adapted to the synthesis of oligodeoxyribonucleotides[5)] and their phosphotriester[5)] and phosphorodithioate analogs.[6)]

We describe here the simple preparation of this type of intermediates, which can be successfully achieved by the selective reaction of the protected deoxyribonucleoside with tris(dialkylamino)phosphine as a phosphitylating reagent. Utilities of these intermediates are demonstrated in the solid-phase synthesis of oligodeoxyribonucleotides and their phosphoroamidate analogs.

RESULTS AND DISCUSSIONS

We have expected that the selective phosphitylation of 5'-O-dimethoxytrityl deoxyribonucleoside with tris(dialkylamino)phosphines under appropriate conditions could be adapted to the simple preparation of deoxyribonucleoside 3'-O-phosphorbisdialkylamidite in situ. For this purpose, tris(diethylamino)phosphine and tris(morpholino)phosphine were prepared by the reaction of PCl_3 with appropriate amines. It was found that the former reagent is particularly stable under the normal laboratory conditions.

The phosphitylations of 5'-O-dimethoxytrityl thymidine (Ia) with tris(diethylamino)phosphine (equiv) were carried out in the presence of various amounts of tetrazole as a catalyst. In these phosphitylations, the high selectivity to give the bisamidite(IIa) could not be achieved and a



Ia-d IIa-d IIIa-d

DMTr=dimethoxytrityl

B= a) thymine b) N-benzoyl adenine c) N-benzoyl cytosine d) N-isobutyryl guanine

Table 1. Effect of amines on the selective preparation of 5'-O-dimethoxytrityl thymidine 3'-O-phosphorbisdiethylamidite.

Catalyst	Yield of IIa(%)	Yield of IIIa(%)
tetrazole	71.5	25.9
tetrazole-diethylamine	97.9	1.0
tetrazole-diisopropylamine	98.5	0.5
tetrazole-morpholine	65.7	1.22
tetrazole-pyridine	96.2	3.8
tetrazole-triethylamine	93.4	2.3

The reaction of 5'-O-dimethoxytrityl thymidine Ia with tris(diethylamino)phosphine(equiv) was carried out at room temperature for 15 min in the presence of tetrazole and amine(equivs to the phosphine). The mixture was treated with excess tetrazole in CH_3CN/H_2O(9:1, v/v) and then the products were analyzed by h.p.l.c.

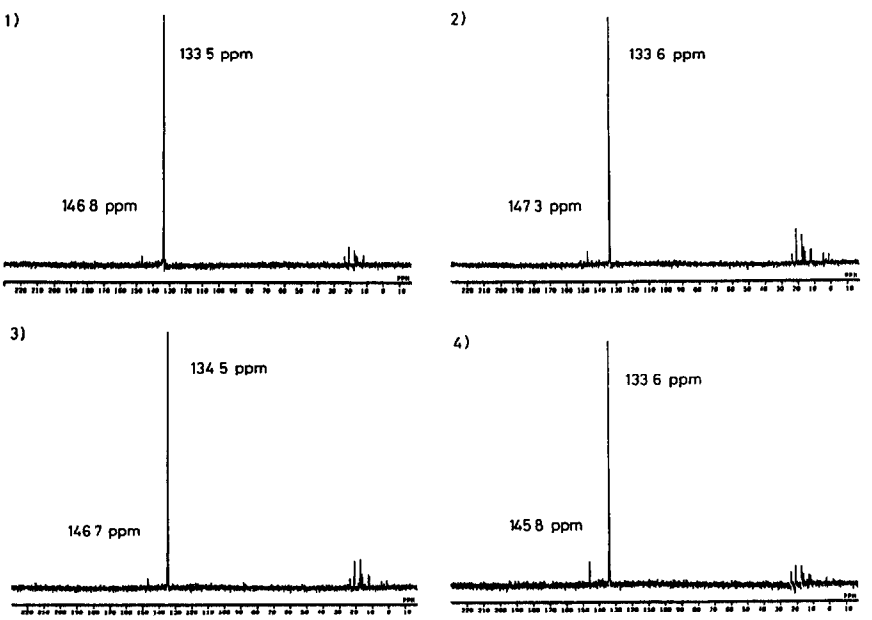
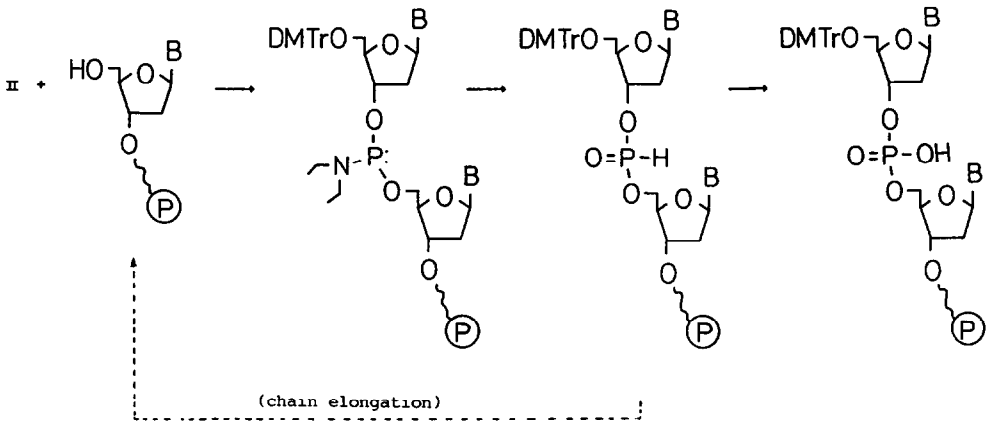


Figure 1. ^{31}P nmr spectra of 5'-O-dimethoxytrityl deoxyribonucleoside 3'-O-phosphorbis-diethylamidites; 1) IIa 2) IIb 3) IIc 4) IId.



considerable amount of the unwanted monoamidite(IIIa) was formed. On the contrary, the selectivity was significantly improved by addition of amines to this system as shown in Table I. Diethylamine and diisopropylamine were found to be most satisfactory. ^{31}P nmr spectra of deoxyribonucleoside phosphorbisdiethylamidites(IIa-d) synthesized using tetrazole(equiv) and diethylamine(equiv) as catalyst are shown in Figure 1. In all cases, the main peaks(133.5 ppm, 133.6 ppm, 134.5 ppm, 133.6 ppm) due to IIa-d, respectively, were observed together with minor peaks from the hydrolysis products. There remained no trace amount of the phosphitylating reagent and only a little amount of IIIa-d(appeared at 146.8 ppm, 147.3 ppm, 146.7 ppm and 145.8 ppm, respectively). These results clearly indicate that the bisamidites (IIa-d) thus obtained can be used without purification for the solid-phase synthesis of oligodeoxyribonucleotides. We have done the same experiments by using tris(morpholino)phosphine and found that this phosphitylating reagent is difficult to be handled because of its hygroscopic character.

Utility of all four bisamidites(IIa-d) is demonstrated in the synthesis of dCCTAGCTAGG on the solid support. Preliminary experiments showed that the amine used in the phosphitylation should be removed and p-nitrophenyltetrazole is useful for the efficient coupling of IIa-d. The synthetic cycle contains a coupling reaction of 0.1 M IIa-d and 0.1 M p-nitrophenyltetrazole in dry acetonitrile(3 min), a hydrolysis reaction using 0.5 M tetrazole in acetonitrile and water(4:1, v/v)(1 min), and dimethoxytrityl deprotection using 3 % dichloroacetic acid in dichloromethane(1.5 min). With this procedure, avarage coupling yield was estimated to be 97%

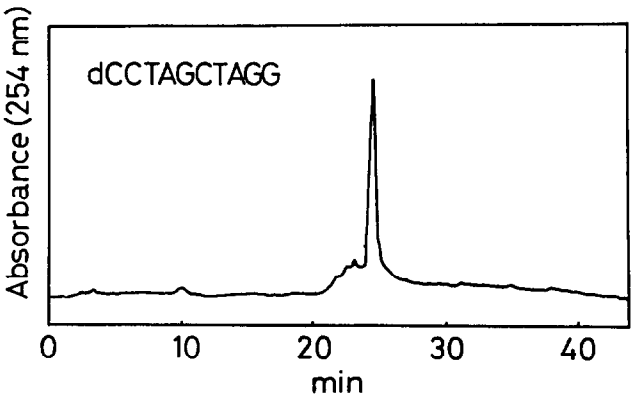
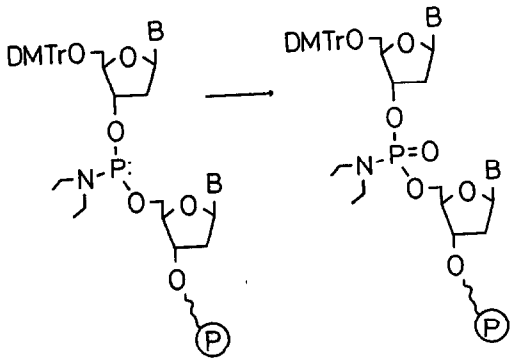


Figure 2. H.p.l.c.(Cosmosil $5C_{18}$-300) analysis of crude reaction mixture from preparation of dCCTAGCTAGG.



based on dimethoxytrityl cation test.[1] After the end of cycle, the loaded support was treated with iodine and water, followed by conc. ammonium hydroxide in an usual manner. The product was analyzed by h.p.l.c. which is shown in Figure 2. The chromatographic profile shows that all reactions in the synthetic cycle proceed efficiently. The product is identical on h.p.l.c. and silica gel t.l.c. to the authentic oligomer synthesized by β-cyanoethylphosphoramidite chemistry[7] and completely degraded by snake venom phosphodiesterase.

Another possibilities of our approach involve the synthesis of oligodeoxyribonucleotides possessing modified backbone. We have synthesized thymidine tetramer containing two phosphodiesters and one phosphorodiethylamidate bond at the 5'-side($^{TpTpTpT}_{\ulcorner N \urcorner}$). The phosphodiester linkage was generated by using β-cyanoethyphosphoramidite chemistry.[7] The phosphoroamidate was obtained by modification of the synthetic cycle described above. After the coupling of IIa, the non-aqueous oxidation using tert-butylhydroperoxide(5 min)[8] was carried out instead of the hydrolysis. In this synthesis, the each coupling yield exceeded 99 %. The product was released from the support by treatment with conc. ammonium hydroxide. Figure 3a shows the h.p.l.c. of the tetramer. Two major peaks were appeared at the different position from that of $(Tp)_3T$ containing all diester bonds[9] shown in Figure 3b. Both products separated by h.p.l.c. were completely digested by snake venom phosphodiesterase to give the same products of pT and dithimidine linked by the phosphorodiethylamidate in a molar ratio of 2:1. These results indicate that the non-aqueous oxidation of the amidite proceed smoothly giving two diastereoisomers due to the chiral phosphorus.

The further study to evaluate the potential of our approach in connection with the synthesis of oligonucleotides having modified backbone is now in progress.

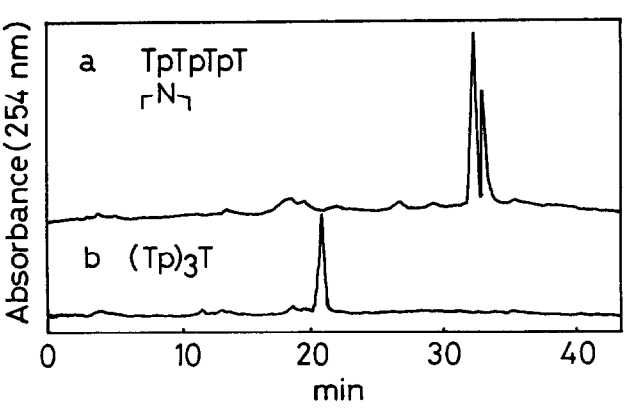


Figure 3. H.p.l.c.(μ-Bondapak C_{18}) analysis of a) $^{TpTpTpT}_{\ulcorner N \urcorner}$ and b) $(Tp)_3T$.

EXPERIMENTAL

High-performance liquid chromatography(h.p.l.c.) was carried out on a Waters 600 E instrument using Cosmosil 5C_{18}-300 column(0.46x15 cm); a linear gradient of CH_3CN(1%/min) starting at 3% in 0.1 M triethylammonium acetate(pH 7.0) was used at a flow rate of 1.0 ml/min or μ-Bondapak C_{18} column(0.46x25 cm)(Waters); 0.5%/min CH_3CN linear gradient starting at 5% CH_3CN in the same buffer was used at a flow rate of 1.5 ml/min. The chromatographic data were analized using a Waters M730 Data Module. Silica gel t.l.c. was carried out on Kiesel gel 60 F254 plate using n-propylalcohol/water/conc. ammonium hydroxide(55:10:35, v/v). UV-Vis spectra were obtained on a Hitachi 100-60 spectrophotometer. ^{31}P nmr spectra(161.7 MHz) were recorded on a JEOL GX-400 instrument. ^{31}P nmr chemical shifts(ppm) were reported relative to external 85% H_3PO_4.

All solvents used here(except for ether) were dried by refluxing over CaH_2, distilled, and stored over Molecular Sieves or CaH_2. Ether was refluxed in the presence of $LiAlH_4$ and distilled. 5'-O-Dimethoxytrityl thymidine, 5'-O-dimethoxytrityl N-benzoyl deoxyadenosine, and 5'-O-dimethoxytrityl N-benzoyl deoxycytidine were synthesized according to the literature.[10] p-Nitrophenyltetrazole was prepared by the method described in the literature.[11] Nucleoside loaded CPG supports were obtained from Biosearch, Inc. All other chemicals were commercially obtained.

Preparation of tris(diethyl)aminophosphine

To a solution of diethylamine(95 ml, 0.9 mol) in dry ether(200 ml), PCl_3(12.7 ml, 0.14 mol) in 100 ml of dry ether was added slowly under N_2 atmosphere at 0°C. The reaction was carried out for 2 h. After removal of the diethylamine hydrochloride, the solvent was evaporated. The remaining material was purified by distillation under the reduced pressure to give the pure phosphine(14.1 g, 61.7%). B.p.=57-58°C at 3 mmHg, d^{20}=0.906, δ(^{31}P nmr)=116.3 ppm(from 85% H_3PO_4). Elementary analysis for $C_{12}H_{30}N_3P$: Found(Calcd)%; C=58.03(58.27), H=12.49 (12.22), N=16.91(16.99), P=12.46(12.52).

Preparation of tris(morpholino)phosphine

PCl_3(1.83 ml, 0.02 mol) was added dropwise to trimethylsilylmorpholine[12] (17 ml, 0.15 mol) at -5°C. The volatile materials were removed under the reduced pressure. The remaining solid was washed well with dry ether. The product was purified by recrystallization from CH_2Cl_2 to give the pure phosphine (5.0 g, 86.7%). M.p.=160-165°C, δ(^{31}P nmr)=114.6 ppm(from 85% H_3PO_4).

Preparation of 5'-O-dimethoxytrityl deoxyribonucleoside 3'-O-phosphorbisdiethylamidite

All reactions were carried out at room temperature. To a solution of 5'-O-dimethoxytrityl deoxyribonucleoside(0.1 mmol) in 0.5 ml of dry CH_2Cl_2, an equivalent of amine(diethylamine, diisopropylamine, morpholine, pyridine, triethylamine) and a solution(0.27 ml) of tris(diethylamino)phosphine(0.1 mmol) in CH_2Cl_2(1:9, v/v) were added. To the mixture, 0.2 ml(0.1 mmol) of 0.5 M tetrazole solution in CH_3CN was added.

From the solution obtained using diethylamine, all volatile materials were carefully removed under the reduced pressure. The remaining solid was dissolved in dry CH_3CN(0.5 ml). This stock solution was used for the solid-phase synthesis.

In order to estimate of the selectivity of the reaction, the reaction mixture was further treated with 0.5 ml of 0.5 M tetrazole in CH_3CN and water(9:1, v/v). The products were analyzed by h.p.l.c. The results are shown in Table 1. It is noteworthy that this procedure is useful for the convenient synthesis of deoxyribonucleoside 3'-O-phosphonates.

Synthetic cycle for the solid-phase synthesis of oligodeoxyribonucleotides

The solid-phase synthesis was carried out on the CPG support by using the syringe technique.[13] The chain elongation cycle on the solid support is as follows; 1) wash with CH_2Cl_2(x3), 2) detritylation with 3 % dichloroacetic acid in CH_2Cl_2(1.5 min), 3) wash with dry CH_3CN(x5), 4) 0.2 M bisamidite in CH_3CN / 0.2 M p-nitrophenyltetrazole in CH_3CN(1:1, v/v)(3 min), 6) hydrolysis with 0.5 M tetrazole in CH_3CN/H_2O(4:1, v/v)(1 min), 7) wash with CH_3CN(x3). After the end of cycle, the iodine oxidation(0.2 M I_2 in THF/2,6-lutidine/H_2O, 2:2:1, v/v)(15 min) was carried out. The removal of the oligomer from the support was done by treatment with ammonium hydroxide(55°C, 12h).

Synthesis of thymidine tetramer containing phosphorodiethylamidate linkage

Trithymidine diphosphate β-cyanoethyl ester was synthesized on the CPG support by using β-cyanoethylphosphoramidite chemistry.[7] IIa prepared in the similar way described above was coupled with the 5'-hydroxy group of the thymidine trimer. To obtain the phosphorodiehtylamidate linkage, the oxidation using 1.0 M tert-butyl hydroperoxide in toluene(5 min) was carried out at step 6 instead of the hydrolysis in the above synthetic cycle. The product was released from the support by using the similar way described above.

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