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A CHEMICAL 5'-PHOSPHORYLATION OF OLIGODEOXYRIBONUCLEOTIDES THAT CAN BE MONITORED BY TRITYL CATION RELEASE

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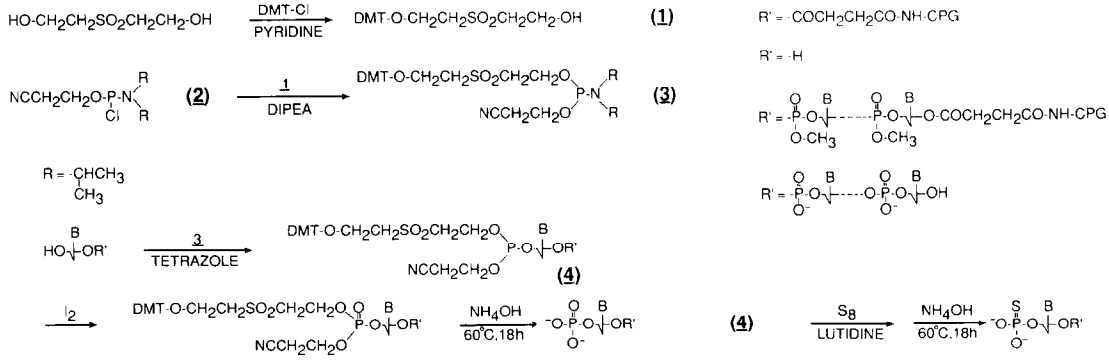
ABSTRACT: A new phosphoramidite-derived reagent, (2-cyanoethoxy)-2-(2'-O-4,4'-dimethoxytrityloxyethylsulfonyl)ethoxy-N,N-diisopropylaminophosphine, for the 5'-phosphorylation of oligodeoxyribonucleotides has been developed. Phosphorylation efficiency can be determined by the release of 4,4'-dimethoxytrityl cation in acid.

5'-Phosphorylation of synthetic oligodeoxyribonucleotides which are synthesized as the 5'-hydroxyl form is often necessary for molecular biological applications. Usually this is conducted by treating purified oligomers with ATP and T_4 polynucleotide kinase. Alternatively, chemical methods have been developed for phosphorylation of fully protected fragments (1,2), some of which can be used on automated DNA synthesizers that employ solid-supported phosphoramidite chemistry (2).

One drawback with most chemical phosphorylation reagents reported to date is that there is no convenient way to monitor the coupling reaction. Whether or not the 5'-phosphorylated fragment was actually synthesized is usually determined after purification by an electrophoretic mobility shift or by a change in the HPLC retention time of the 5'-phosphate versus the 5'-hydroxyl species. This requires an additional work-up and purification of the unphosphorylated oligomer. Also, the difference in the mobility or retention time between the two forms is minimal for polynucleotides larger than 20 bases.

We chose to design a phosphorylation reagent that could be monitored by the release of the orange 4,4'-dimethoxytrityl cation after acid deprotection the same way coupling of nucleotides during DNA synthesis is traditionally determined. Also, we were interested in



Scheme I

making the reagent fully compatible with phosphoramidite chemistry (3). These requirements suggested the use of a set of NH_4OH sensitive blocking groups where one carries a dimethoxytrityl ether for a dialkylaminophosphine.

Since removal of protecting functions from phosphate in the diester form typically requires more rigorous conditions than internucleotide triester deprotection, the choice of suitable blocking groups for phosphorylating agents has been an important consideration. Halogenated aromatic and simple alkyl phosphate diesters are particularly resistant to hydrolysis (4). It is for this reason that protecting groups that can be ω-eliminated such as 2-cyanoethyl and p-nitrophenylethyl have proven useful (2), the former being particularly attractive since it can be fully eliminated from a phosphate diester in NH_4OH. It would however be difficult to derive a trityl ether-containing protection scheme for phosphorus based on either of these functions.

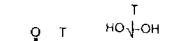
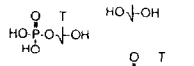
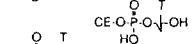
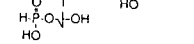
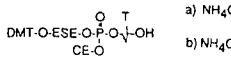
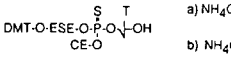
Another potential protecting function that could be tailored to our needs appeared to be the alk(ar)ylsulfonylethyl group reported by Tesser et al (5,6). As for the 2-cyanoethyl function, complete elimination of both alkyl- and arylsulfonylethyl groups from phosphate diesters is possible in NH_4OH solution.

Thus, we designed and prepared (2-cyanoethoxy)-2-(2'-4,4'-dimethoxytrityloxy ethylsulfonyl)ethoxy-N,N-diisopropylaminophosphine(3) and tested it as a phosphorylation reagent (Scheme I).

Commercially available sulfonyldiethanol (65% w/v in H_2O) was dried by repeated coevaporation with dry acetonitrile to give a viscous oil which crystallized on standing. To solid sulfonyldiethanol (10.6 g, 68.6 mmole) in pyridine (150 ml) was added 4,4'-dimethoxytrityl chloride (DMT-Cl; 16.95 g, 50 mmole) and the mixture was left stirring in the dark for 18h. The reaction solution was then concentrated in vacuo. The residue dissolved in ethyl acetate (500 ml) was extracted with 5% aq. $NaHCO_3$ and 80% saturated aq. NaCl and the organic phase was dried over anhydrous Na_2SO_4. After removal of solvents the product was purified by silica gel column chromatography to give 10.0 g of pure 1 (TLC, silica in CH_2Cl_2; R_f=0.015). Chloro-N,N-diisopropylamino-2-cyanoethoxy-phosphine 2 (4.6 mmole) was added rapidly under argon to a stirred solution of 1 (4.6 mmole) and N,N-diisopropylethylamine (DIPEA; 4.6 mmole) in methylene chloride (10 ml) at 0 C. The solution was allowed to warm to room temperature, diluted with ethyl acetate (50 ml) and washed with 80% saturated aq. NaCl (2 X 20 ml). The organic phase was dried with anhydrous Na_2SO_4 and concentrated by rotary evaporation. The oily product was dissolved in acetonitrile and then aliquoted into 1.5 ml septum-sealed Wheaton vials each containing 100 μmole of 3. The solvent was removed by evacuation and the product was stored under argon at -20 C (7). This crude product was used without further purification.

Reagent 3 was activated with 0.75 ml of 0.5 M 1H-tetrazole in dry acetonitrile and added to a detritylated thymidine CPG support. After standard oxidation with aqueous iodine and thorough washing, the support was treated with 2 ml of 5% w/v DCA in CH_2Cl_2 for 90 sec. The bright orange solution obtained was compared by visible absorption spectroscopy to the material collected from deprotection of the original thymidine support suggesting a coupling efficiency of 96%. The polymer was exposed to 2 ml of NH_4OH for 2h at 20 C and the supernatant was heated at 60 C for an additional 5h. TLC, HPLC and ^{31}P-NMR studies indicated quantitative synthesis of thymidine 5'-monophosphate without

TABLE I.

Compound	TLC(R_f) Sol I[a]	TLC(R_f) Sol II[a]	HPLC[b] (R_t;min)	^{31}P-NMR[c] (ppm)
	0.90	0.77	1.66	-
	0.59	0.14	0.67	4.01
	0.69	0.29	1.84	+0.7(pyridinium salt)[d]
	0.70	0.30	1.10	-6.0,-5.9(NH_4^+-salt)[d]
 a) NH_4OH, 20°C	0.59,0.69[f]	0.33[f],0.29,0.14	1.84,0.67	N.D.
b) NH_4OH, 60°C	0.59	0.14	0.67	4.02[g]
 a) NH_4OH, 20°C	N.D.	N.D.	4.10,4.50	N.D.
b) NH_4OH, 60°C	0.70[e]	0.22[e]	0.70	-43.25

CE - $NCCH_2CH_2$- ESE - $CH_2CH_2SO_2CH_2CH_2$-

a) Sol I and Sol II are 8:2 and 9:1 (v/v) isopropanol: 0.1 M Triethylammonium acetate pH 7.5, respectively; b) C18 reverse phase; 2.5 to 10% CH_3CN in 0.05 M $NH_4H_2PO_4$, pH 4.0; c) relative to 1 M H_3PO_4 in D_2O; measured in 50% D_2O and 50% 1 M Tris-HCl-0.05M EDTA, pH 8.8; (10) d) in D_2O, relative to H_3PO_4; e) becomes bright yellow when sprayed with 10 mM DTNB in 1 M Tris, pH 8.0; f) DMT positive as evidenced by 10% H_2SO_4 spray; g) an identical NMR spectrum was obtained if detritylation was performed prior to NH_4OH treatment.

concomitant production of phosphonate or partially protected nucleotide (Table I).

When deprotection of 5'-phosphorylated thymidine was conducted with NH_4OH at 20 C only, a mixture of both 2-(2'-hydroxyethylsulfonyl)ethyl- and 2-cyanoethyl-5'-phosphate thymidine was noted. If 5'-phosphitylated thymidine was deprotected with 60 C NH_4OH without prior oxidation, a mixture of both 5'-phosphonate and nucleoside was obtained (8).

By substituting sulfur for iodine in the oxidation step (9), the 5'-phosphitylated thymidine was quantitatively converted to the 5'-phosphorothioate derivative (Table I) as evidenced by ^{31}P-NMR (10). Since the sulfur in phosphorothioates is a reasonably good nucleophile, maleimido- and bromoacetyl-containing reagents and disulfide exchange can be used to modify oligonucleotides specifically at this function (11). Solid-supported and non-radioactively labeled oligonucleotide probes have been produced in this manner.

The palindromic BamHI linker sequence GGATCCGGATCC was synthesized on an automated instrument (the Geno-O-Matic) using a solid-supported phosphoramidite chemistry (12). One-half of the support was phosphorylated with reagent 3, detritylated to check the coupling efficiency and fully deprotected. The product was then purified by polyacrylamide gel electrophoresis. The second half of the material was deprotected and purified as the 5'-hydroxyl form which was then 5'-phosphorylated with T_4 polynucleotide kinase and ATP. The PAGE analyses of T_4 DNA ligase reactions using the chemically and enzymatically phosphorylated fragments are presented in Figure 1. Both sequences are near fully phosphorylated as evidenced by the lack of starting material after ligation.

The use of a bifunctional protecting group such as sulfonyldiethanol reported here permits the introduction of a monitoring moiety that can be removed without concomitant phosphorus deprotection. Thus it is possible to repeat the reaction if monitoring reveals a low coupling. Although compound 1 is not ideal since detritylation results in the unmasking of a new hydroxyl group, the side-product obtained with an additional phosphorylation is easily removed from the oligonucleotide upon work-up. We are investigating other similar bifunctional protecting groups for chemical phosphorylation

and other synthetic processes that would allow monitoring yet remain masked.

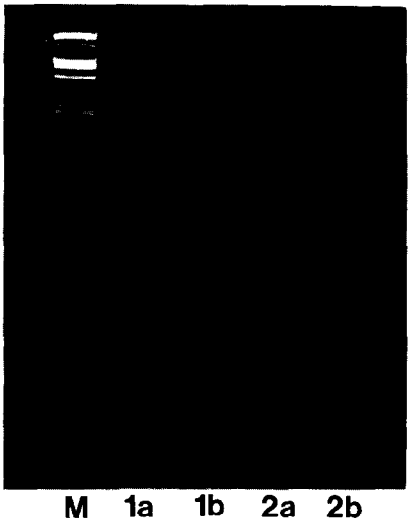


Figure 1. Comparison of 18h ligation reactions for enzymatically (1b) and chemically (2b) phosphorylated GGATCCGGATCC. (a) lanes = no ligation, M = marker DNA.

In conclusion, (2-cyanoethoxy)-2-(2'-O-4,4'-dimethoxytrityloxyethylsulfonyl)ethoxy-N,N-diisopropylaminophosphine (3) has been shown to be useful for the incorporation of a 5'-phosphate or 5'-phosphorothioate into oligodeoxyribonucleotides. Coupling efficiency can be monitored by trityl cation release. The reagent can be used on automated DNA synthesis instruments employing phosphoramidite coupling methods without changing current synthesis, deprotection or work-up procedures.

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