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CHEMICAL 5'-PHOSPHORYLATION OF OLIGONUCLEOTIDES VALUABLE IN AUTOMATED DNA SYNTHESIS

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Abstract: A new method has been developed for the chemical 5'-phosphorylation of oligonucleotides starting from easily available phosphorous-III compounds using phosphate protecting groups which can be cleaved off via ß-elimination. The hydrophobic 2-(p-nitrophenyl)-ethyl group, at the phosphodiester level can serve as a purification handle in reversed phase HPLC. Moreover, the procedure has been adapted to automated DNA synthesis.

The synthesis of oligonucleotides by current methods usually yields after deprotection a product with a free 5'-hydroxyl group. However, most biological processes require oligonucleotides containing a 5'-terminal monophosphate function. Although there exist a number of chemical procedures for the synthesis of oligonucleotide-5'-phosphates on the basis of phosphotriester chemistry[1-4], the phosphorylation of oligonucleotides by enzymatic means with polynucleotide kinase and ATP is still the most widely used. We present here a simple chemical method involving a new type of monovalent phosphorylating agent based on phosphoramidite chemistry[5]. The new procedure offers great advantages in automated DNA synthesis[6].

The phosporylating agents 1a - 1d are relatively stable compounds which can be stored at -20°C for several months without decomposition. The reaction of 1 equivalent diisopropylaminophosphoramidite 2 with 2 equivalents 2-(p-nitrophenyl)-ethanol or of 2-cyanoethanol in the presence of DIPEA (diisopropylethylamine) results in the symmetric phosphorylating agent 1c or 1d, whereas the asymmetric phosphorylating agents 1a or 1b are obtained by the reaction of equimolar amounts of diisopropylaminomethoxychlorophosphine 3 ($R^1 = CH_3$)[5] with 2-(p-nitrophenyl)-ethanol or 2-cyanoethanol respectively. Fully protected oligonucleotides, but bearing a free 5'-hydroxy function (type 4), can be converted to the 5'-phosphotriesters 5 by the reaction with agent 1 and tetrazole and subsequent oxidation with iodine/water. We have synthesized the 5'-phosphorylated Nco I linker d(pCCATGG) by means of a DNA synthesizer (Applied Biosystems Inc.) under standard conditions, except that in the phosphorylation

cycle the time for coupling and oxidation was prolonged. Deprotection of the oligonucleotides by successive treatment with thiophenolate and ammonia yields oligonucleotide-5'-phosphodiesters of type 6 provided that phosphorylation has been carried out with reagent 1a or 1c, and oligonucleotide-5'-phosphates 7 in the case of reagents 1b or 1d. However, use of the 2-(p-nitrophenyl)-ethyl group[7] proved to be advantageous in that phosphodiesters of type 6 are relatively hydrophobic and therefore easily separated from nonphosphorylated compounds by reversed phase HPLC (Fig. 1). The p-nitrophenylethyl group can be eliminated quantitatively by DBU, TBD, MTB or DMETP[8] (0.5 M solution of the base in pyridine or acetonitrile with 1 - 5 % water) to yield the desired monophosphate 7. The chemically phosphorylated d(pCCATGG) is identical by HPLC and polyacrylamide gel electrophoresis with the enzymatically phosphorylated product. Biological suitability of the chemically phosphorylated product was checked by polymerisation of the duplex linker with T4-DNA ligase (Fig. 2, lane 2) and subsequent digestion by the restriction endonuclease Hae III yielding again the original monomeric linker.

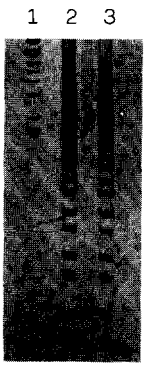
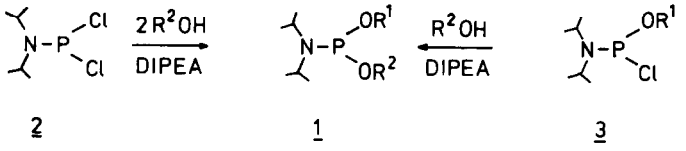


Fig. 2: 12 % polyacrylamide gel (native) of Ligation mixtures from pCCATGG with T4-DNA-Ligase
lane 1: Ø X 174 digested with Hinf I
lane 2: pCCATGG chemically phosphoryl. and ligated
lane 3: pCCATGG enzymatically phosphoryl. and ligated

It is obvious that the method presented for phosphorylation is not restricted to 5'-hydroxy groups of oligonucleotides and to the protecting groups exemplified, but is of general value. Furthermore, by carrying out the oxidation step with sulphur, S-thiophosphates are available.

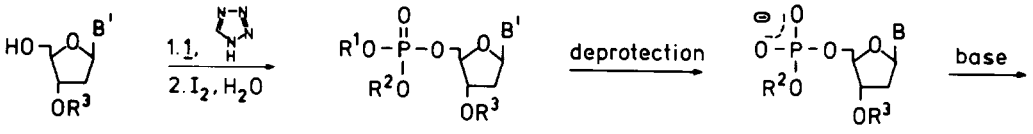
Preparation of symmetrical phosphoramidite 1c and 1d:
A solution of 0.1 mol alcohol in 40 ml anhydrous tetrahydrofuran and 26 ml DIPEA was cooled to 0°C. To this solution, 0.05 mol diisopropylaminophosphodichloridite were added with vigorours stirring within 15 min. After stirring for further 30 min at room temperature the solution was separated from the amine hydrochloride. The filtrate was diluted with 1 l acid-free ethyl acetate and the organic layer was extracted three times with 150 ml phosphate buffer pH7, dried over sodium sulfate, and concentrated in vacuo. The crude product was obtained in almost quantitative yield and was purified by short column chromatography in the presence of triethylamine (Tab. 1).



2 1 3

1a: $R^1 = CH_3$, $R^2 = CH_2CH_2$-C$_6$H$_4$-NO$_2$ 1c: $R^1 = R^2 = CH_2CH_2$-C$_6$H$_4$-NO$_2$

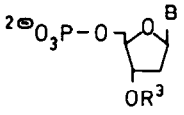
1b: $R^1 = CH_3$, $R^2 = CH_2CH_2$-CN 1d: $R^1 = R^2 = CH_2CH_2$-CN 3: $R^1 = CH_3$



4 5 6

$R^2 = CH_2CH_2$-C$_6$H$_4$-NO$_2$, R^3 = oligonucleotide chain

B = A, G, C, T ; B' = A^{bz}, G^{ib}, C^{bz}, T



7

base = DBU, TBD, MTBD, DMETP

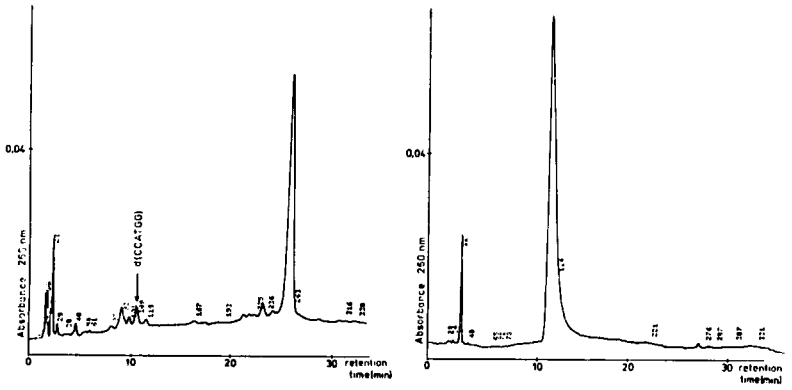


Fig. 1:
reversed phase HPLC on C8-Zorbax column (Dupont); linear gradient of 12% A to 42% A in B (A = acetonitrile : B = 1:1 (v:v); B = 0.1 M Triethylammoniumacetate pH 7

a) crude phosphorylated oligonucleotide 6 (R_2 = CH_2CH_2-C_6H_4-NO_2)

b) pCCATGG after isolation of main peak from b) and treatment with DBU

The preparation of unsymmetric phosphoramidite (1a, 1b) was carried out as above by reaction of 0.11 mol alcohol in 200 ml dichloromethane and 80 ml DIPEA with 20 ml diisopropylaminomethoxychlorophosphine.

Table 1: Data of the phosphorylating reagents

compound	^{31}P-NMR H_3PO_4 as standard	purity[a] crude	purity[a] purified[b]
1a	- 149 ppm	86 %	95 %
1b	- 150 ppm	94 %	-
1c	- 149 ppm	-	96 %
1d	- 150 ppm	83 %	93 %

a) based on total phosphorous (^{31}P NMR)
b) purified by short column chromatography

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