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PHOSPHORYLATING AGENT FOR THE SYNTHESIS OF OLIGONUCLEOTIDE WITH ALIPHATIC AMINO GROUP AT 5' END

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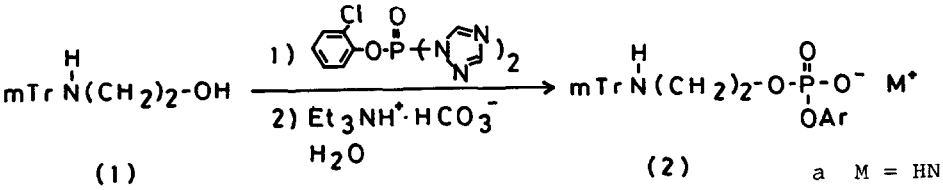
Summary
A lipophilic phosphorylating agent was prepared and used for the synthesis of pentadeoxyribonucleotide with aminoethyl group at 5' end on a polymer support by the phosphotriester method.

Chemically synthesized oligodeoxyribonucleotide with defined sequence is widely used as the primer for DNA sequence determination procedure[1]. Such oligonucleotide which has hydroxyl group at 5' end is first labeled by means of [γ-^{32}P]ATP and polynucleotide kinase. However, since this method requires the use of radioisotopes, other alternative means of detection procedure were investigated. Recently the fluorescent compounds such as fluorescein, tetramethylrhodamine, Texas Red and 7-nitrobenzo-2-oxa-1-diazole were applied for this purpose and successfully used[2]. This strategy is very attractive since the primer is non-radioactive and safe. As the fluorescent compounds were attached to the primary amino group of oligonucleotide, the synthesis of oligonucleotide with primary amino group at 5' end was necessary. Smith and co-workers[2a] prepared 5'-amino-5'-deoxythymidine and converted it to its phosphoramidite derivative. However this procedure needs the four types of 5'-amino-5'-deoxynucleotides. Coull and co-workers[3] and Agrawal and co-workers[4] prepared phosphitylating reagent for the synthesis of oligonucleotide with 5'-aminoethyl group. However, the amino-protecting groups they used were base labile. Accordingly, after the treatment with the basic condition, all the protecting groups used were removed and thus the oligonucleotide has become unprotected. On the other hand, it is known that the oligonucleotide which has lipophilic dimethoxytrityl group was easily separated by the reversed phase C-18 column[5].

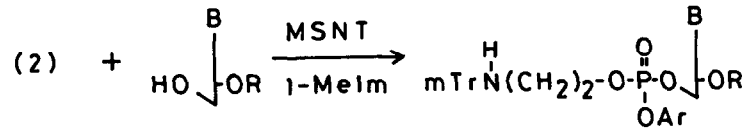
In this paper, we wish to report a lipophilic phosphorylating agent for the synthesis of oligonucleotide with aminoethyl group at 5' end. N-Monomethoxytritylaminoethanol (1)[6] was treated with o-chlorophenylphosphorditriazole (1.5 equiv) in CH_2Cl_2 at 0°C for 30 min followed by the hydrolysis with 50 mM triethylammonium bicarbonate (TEAB, pH 7.5) to give (2a). After extraction with CH_2Cl_2, the triethylammonium salt of 2a was converted to cyclohexylammonium salt by adding cyclohexylamine (1 equiv). The

cyclohexylammonium salt of (2b) was crystallized after evaporation and 87 % total yield was obtained[7]. Though compound 2b had poor solubility in dry pyridine or CH_2Cl_2 which was the solvent used for the condensation reaction, the mixture of 2b (2 equiv) and 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT) (3 equiv) was gradually clear. However the reaction of 2b with



(1) (2)

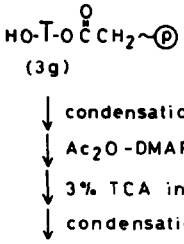
a M = $HN(C_2H_5)_3$
b M = $H_3NC_6H_{11}$
c M = HNC_5H_5



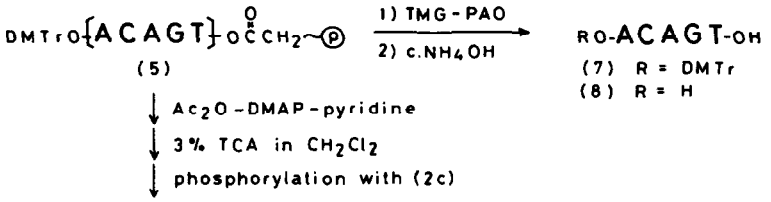
(3) (4)

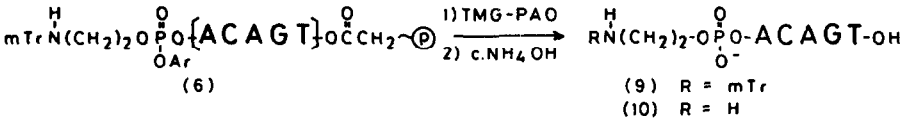
	B	R
a	T	COC_6H_5
b	T	P(O)(OAr)(OCE)
c	T	P(O)(OAr)O^- ($\overset{+}{H}N(C_2H_5)_3$)
d	bzC	P(O)(OAr)(OCE)
e	bzA	P(O)(OAr)(OCE)
f	ibG	P(O)(OAr)(OCE)
g	T	$CO(CH_2)_2CO$–Ⓟ

Ar = o-chlorophenyl
CE = β-cyanoethyl



DMAP = 4-dimethylaminopyridine
TCA = trichloroacetic acid





3'-O-benzoylthymidine (3a) was very slow. For the increase of the solubility of 2b in anhydrous organic solvent, 2b was converted to pyridinium salt by using the pyridinium Dowex 50W x 2. Compound (2c) was very soluble in dry pyridine but the condensation reaction between 2c and 3a by MSNT was still slow. Even after 2 hr, starting material 3a still remained and several side products were seen on the silica gel TLC plate. It is known that 1-methylimidazole accelerates the condensation reaction[8]. Therefore, 2c (2 equiv) and 3a were condensed with MSNT (3 equiv) in the presence of 1-methylimidazole (6 equiv). The reaction was completed after 30 min at rt and (4a) was obtained in a yield of 91 % after the isolation by the silica gel column. With the same procedure, the phosphorylation of 3'-O-phosphorylated compound (3b) by 2c gave (4b) in a yield of 88 %. The treatment of 4b with a trietylamine-pyridine-H_2O (1:3:1, v/v) mixture for 10 min at rt gave (4c) which can be used for the last condensation step during the oligonucleotide synthesis on a polymer support. Other nucleotides (4d, e, f) were also prepared in 87, 88 and 81 % yields, respectively, by the same procedure.[9]

It is considered that the phosphorylating agent 2c can be used for the phosphorylation of 5'-hydroxyl group of oligonucleotide on a polymer support. A thymidine bound to a polystyrene support (3g) was treated with 2c (6 equiv) by MSNT (18 equiv) and 1-methylimidazole (36 equiv) for 1 hr at rt. From the calculation of released MTr group by treatment with 3 % trichloroacetic acid in CH_2Cl_2, the coupling yield was estimated to be 94 %. Next, the preparation of pentanucleotide with aminoethyl group at 5' end on a polymer support was demonstrated. According to our procedure[5], the thymidine resin 3g (6μmol) was first treated with protected dimer [AG] (2.7 equiv)[5,10] by MSNT (14 equiv) for 40 min at rt to give trimer. Then after the acetylation of unreacted hydroxyl group of 3g and acid treatment to remove DMTr group, the protected dimer [AC][5,10] was condensed by MSNT to give protected pentamer (5). One half of the resin 5 was treated with 0.5 M N^1,N^1,N^3,N^3-tetramethylguanidium syn-pyridine-2-aldoximate (TMG-PAO) overnight at rt and conc. NH_4OH at 55 C for 6 hr. The other half of the resin 5 was treated with 2c (8 equiv) by MSNT (24 equiv) and 1-methylimidazole (48 equiv) for 1 hr at rt after the acetylation and the acid treatment. The resin (6) was subjected to the deblocking procedure as above. The partially deblocked pentamers (7) and(9) were isolated by the reversed phase C-18 silica gel column which was performed by the linear gradient of CH_3CN (10→35 %) in 50 mM triethylammonium acetate (TEAA, pH 7). Compounds 7 and 9 were eluted slowly with 24 % CH_3CN and the yields of 7 and 9 were 117 and 102 A_{259} units, respectively. Then the treatment of 7 with aq 80 % AcOH at rt for 30 min gave the pentamer (8) with hydroxyl group at 5' end. From the compound 9, the pentamer (10) with aminoethyl group at 5' end was obtained after treatment with 80 % AcOH at rt for 30 min. The structures of 8 and 10 were confirmed enzymatically. Both 8 and 10 were hydrolyzed to 2d-pA, d-pG, d-pC and pT by venom phosphodiesterase

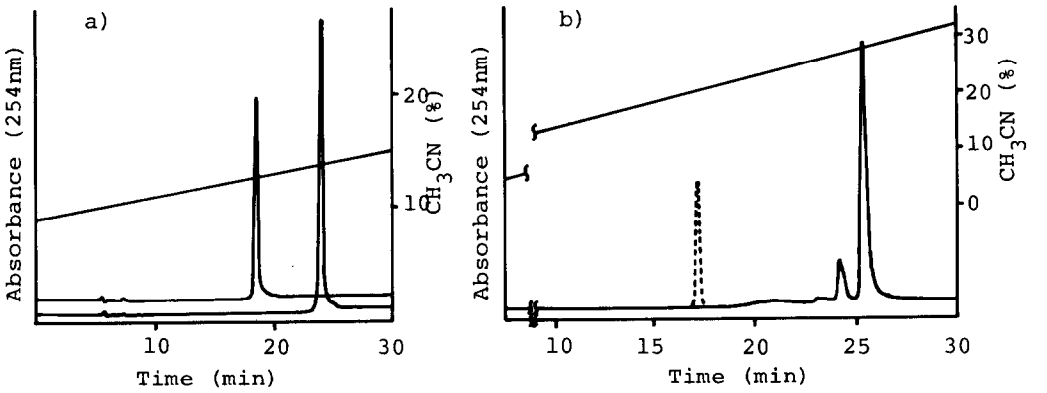


Fig 1. a) HPLC analysis of d-ACAGT (lower line) and d-$NH_2(CH_2)_2$OpACAGT (upper line) on the reversed phase C-18. b) HPLC analysis of d-(FITC)$NH(CH_2)_2$OpACAGT on the reversed phase C-18. The dotted line indicates the plase where d-$NH_2(CH_2)_2$OpACAGT is eluted.

and 10 was hydrolyzed to d-$NH_2CH_2CH_2$OpA, d-pA, d-pG, d-pC and pT by nuclease P1. At the last step, the 5'-terminal amino group of compound 10 was reacted with fluorescein isothiocyanate (FITC)[4] to give d-FITC-pACAGT (Fig. 1b, retention time 25.4 min).

In conclusion, the compound 2 is a useful phosphorylating agent for the synthesis of oligonucleotide with an aminoethyl group at 5' end by the phosphotriester method.

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6. N-Monomethoxytritylaminoethanol was prepared in 71 % yield by the reaction of ethanolamine (1.5 equiv) with monomethoxytrityl chloride in the presence of diisopropylethylamine (1.5 equiv) at rt for 1 hr.
7. Anal. Calcd. for $C_{34}H_{40}N_2O_5PCl \cdot 1/2H_2O$: C, 64.60; H, 6.54; N, 4.43. Found: C, 64.58; H, 6.43; N, 4.74.
8. a) V. A. Efimov, S. V. Reverdatto and O. G. Chakhmakhcheva, Tetrahedron Lett., 23, 961 (1982). b) R. L. Letsinger, E. P. Groody and T. Tanaka, J. Am. Chem. Soc., 104, 6805 (1982).
9. Satisfactory elemental analyses were obtained for compounds (4a,b,d,e,f)
10. 5'-Hydroxyl, heterocyclic amino and phosphate groups were protected by dimethoxytrityl, benzoyl or isobutyryl and o-chlorophenyl groups, respectively.

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