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A NOVEL METHOD FOR PHOSPHODIESTER AND INTERNUCLEOTIDE BOND SYNTHESIS

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Abstract—The synthesis of phosphate diesters from various alcohols and nucleosides is described, using the N-methylpyridinium salt of dichlorophosphoric acid (1) as a phosphorylating agent. Internucleotide bonds are formed by stepwise addition of two suitably protected nucleosides to 1. Side products, usually formed during oligonucleotide synthesis were not observed using this new method. In addition, a nucleoside 2', 3'-cyclic phosphate was prepared by one step phosphorylation of a ribonucleoside protected at the 5'-hydroxyl. The products were isolated in relatively high yields by simple separation methods.

The use of phosphoryl chlorides (and other halides) as phosphorylating agents is a well established method for preparing phosphate esters according to the general equation:

$$(RO)_mPOCl_n + nR'OH \xrightarrow{\text{Base}} (RO)_mPO(OR')_n \quad (1)$$
$$m + n = 3$$
$$R,R' = H, \text{alkyl, aryl}$$

Thus for instance, phosphoryl chloride has been used to propare mono-, di- or triesters of phosphoric acid by adjusting the amount of alcohol reactant[2] and chlorophosphoric acid (prepared *in situ*) has been used to yield monoesters.[3]

Less known but of great potential for preparing phosphate esters is dichlorophosphoric acid. Its N-methylpyridinium salt $C_5H_5NCH_3^+ \cdot PO_2Cl_2^-$ (1) was first prepared by Smrt and Catlin from methyl dichlorophosphate and pyridine,[4] and by reacting this salt with 5'-0-dimethoxytritylideoxythymidine they obtained the monoester 5'-0-dimethoxytritylideoxythymidine-3' phosphate in 48% yield.

Symmetrical phosphodiester synthesis. The availability of a new phosphorylating agent having two reactive chlorine residues raised the possibility of synthesizing symmetrical diesters of phosphoric acid by reacting 1 with two molar equivalents of alcohol. Our studies have shown that phosphate diesters can indeed by prepared by the reaction:

$$PO_2Cl_2^- + 2ROH \xrightarrow{C_5H_5N} (RO)_2PO_2^- \quad (2)$$
$$\mathbf{2}$$
$$R = \text{alkyl, aryl or benzyl}$$

This method appears to be of general application since aliphatic, aromatic and benzylic alcohols were all smoothly converted to their phosphodiesters in good yields. Being medium-strong acids, the diesters were soluble in neutral or basic aqueous solutions and could be extracted with organic solvents from the acidified aqueous phase. This made possible their isolation as free acids or as salts, such as the cyclohexylammonium salt (Table 1).

The importance of this new, promising route arises from the limited number of other simple methods for producing phosphate diesters. Our technique has certain distinct advantages over conventional methods. The phosphorylation of two moles of an alcohol by one mole of phosphoryl chloride for example, usually leads to a mixture of mono-, di- and triesters, and the preparation of diesters by oxidation of dialkyl phosphites (obtained from phosphorous trichloride and alcohols) is limited to aliphatic alcohols and also involves many steps.

When aralkyl alcohols were phosphorylated by 1, a considerable amount of the corresponding benzyl chloride was also formed. Being non-ionic it could be easily separated from the desired product. We assume that the benzyl chloride is probably formed from the intermediate $ArCH_2OPO_2^-Cl$ and not from the diester, since in attempts to prepare benzylic monoesters of phosphoric acid according to the equation:

$$PO_2Cl_2^- + ArCH_2OH \longrightarrow ArCH_2OPO_2Cl^- \xrightarrow{H_2O} ArCH_2OPO_3H_2 \quad (3)$$

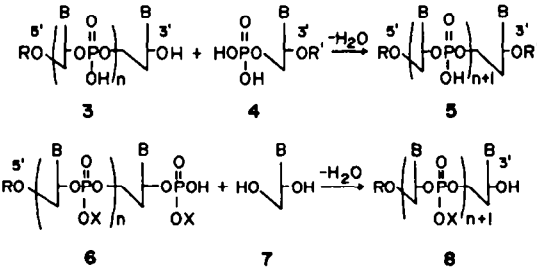
an even larger fraction of benzyl chloride by-product was obtained than with phosphodiester synthesis (Eqn 2).

Phosphomonoester synthesis. The preparation of monoesters was also investigated. When an alcohol was phosphorylated by excess of 1 the main product after aqueous workup was the monoester as already described by Smrt and Catlin.[4] However even dropwise addition of alcohol to a large excess of 1 still forms considerable amounts of a diester in addition to the monoester. This result can be explained by assuming similar reaction rates in pyridine for alcohol with $PO_2Cl_2^-$ and with $ROPO_2Cl^-$. Conducting the reaction in other solvents such as acetonitrile and using equivalent amount of base resulted in reduced yields of both mono- and diesters. It appeared that when using N-methylpyridinium dichlorophosphate to prepare monoesters, formation of diesters is inevitable and they must be separated out. However, the phosphorylation of the bulky alcohol 2,2'-dinitrobenzhydrol by an excess of 1 yielded only one phosphate-containing product, as confirmed by TLC. The product was extracted from the reaction mixture, isolated as barium salt and analysis showed it to be the salt of the monoester 2,2'-dinitrobenzhydryl phosphate. No diester was formed probably due to the difficulty of placing two bulky benzhydryl groups on the phosphate.

Mixed phosphodiester synthesis. Preparation of mixed phosphodiesters $ROPO_2(OR')^-$ is of particular interest as biologically important compounds such as nucleic acids

belong to this class of compounds. The stepwise chemical synthesis of oligonucleotides is one of the most complicated tasks in bio-organic chemistry due to two main problems: First the finding of suitable and selective protecting groups, and second, the designing of an effective method for internucleotide bond synthesis. While many protecting groups have been suggested and successfully used, not many effective methods for internucleotide bond synthesis exist. The usual methods involve condensing a suitably protected mononucleotide (4) with the 3'-terminal alcoholic function of the forming oligonucleotide chain (3) (Scheme 1).

Dicyclohexylcarbodiimide (DCCD) or aromatic sulfonyl chlorides[5,6] are usually used as dehydrating agents. These reagents however, are not selective and several undesired side reactions such as sulfonylation of hydroxyls, formation triesters and production of 5'-pyridinium nucleosides are observed. The most serious problems arise from the very nature of the condensation reaction[7] whose first step is the formation of pyrophosphate bonds from the 5'-terminal phosphate groups of 4 as well as from the internal phosphate groups of 3. These pyrophosphates are the reactive phosphorylating species, but to some extent they are also alkylating agents.[8] Therefore during the condensation and during the subsequent aqueous treatment some internucleotide bonds are degraded while some additional bonds which do not correspond to those in oligonucleotides found in nature are formed.[9]



Scheme 1. The stepwise chemical synthesis of oligodeoxyribonucleotides.
(R,R' = hydroxyl protecting groups)
B = base residues (adenine, thymine, etc.);
$n = 0, 1, 2, 3 \ldots$;
X = a phosphate protecting group.

In a newer approach to oligonucleotide synthesis (the "phosphotriester approach"), the phosphate residue are blocked by protecting groups.[10] By this approach the formation of side products is greatly reduced, and in fact, side products will only arise via the pyrophsophate of 6. This method however, has some disadvantages, such as the susceptibility of the phosphotriesters to nucleophilic attack. Actually, some triesters are partially hydrolysed even by aqueous pyridine and are completely hydrolysed under the basic conditions used to remove the acyl-type protecting groups from the 3'-OH position of the oligonucleotide (5) (this demasking is necessary after each step of condensation).

We investigated the possibility of using 1 for oligonucleotide synthesis, avoiding the use of condensing agents and hence formation of side products arising from pyrophosphates and also making the use of labile phosphate triesters unnecessary. A stepwise addition of two different alcohols to 1 would produce a mixture of all possible phosphate monoesters and diesters. If, however, one of the alcohols carries a group with a unique character, such as the hydrophobic trityl group, then all derivatives containing that group can be separated from the reaction mixture. Indeed, this principle has already been demonstrated, and trityl derivatives of nucleotides were isolated from reaction mixtures because of their relative hydrophobic nature.[5]

The mixed diester 5'-O-tritylthymidine-3' β-cyanoethyl phosphate (TrO-TpCE) was initially prepared. β-Cyanoethanol (7·2 mmol) was first added to 1 (5 mmol) to produce a mixture of the symmetrical diester and the monoester of chlorophosphoric acid. After a short time 5'-O-tritylthymidine (0·4 mmol) was added to the mixture. Analytically pure TrO-TpCE was isolated in 83% yield by simple extraction procedure and no other trityl-containing phosphate derivatives were obtained. Although the literature synthesis for this compound contains only a hint of difficulties[11] we attempted to repeat the reported work and condensed 5'-O-tritylthymidine with β-cyanoethyl phosphate in the presence of either dicyclohexylcarbodiimide or 2,4,6-triisopropylbenzenesulfonyl chloride, repeatedly obtaining a mixture of two trityl-containing phosphate derivatives.

The dinucleoside phosphate 5'-O-tritylthymidilyl (3'→5') 3'-O-acetylthymidine (TrO-TpT-OAc) was next prepared in a similar manner: by reacting 1 initially with 3'-O-acetylthymidine (T-OAc) and then with 5'-O-tritylthymidine (TrO-T) (eqn 4).

$$1 + \text{T-OAc} \rightarrow \text{Cl-}p\text{T-OAc} + p(\text{T-OAc})_2 \xrightarrow[(2)\ H_2O]{(1)\ \text{TrO-T}} \text{TrO-T}p\text{T-OAc} + p(\text{T-OAc})_2 + p\text{T-OAc}. \quad (4)$$

The phosphorylation of 5'-O-tritylthymidine was almost quantitative (>95%) as determined by TLC. Being the only trityl-containing phosphate derivative, the protected dinucleoside phosphate could be selectively extracted with organic solvent and it was isolated in 75% yield (based on the starting amount of TrO-T).

In this new approach to oligonucleotide synthesis formation of side products is avoided, apparently because the intermediates remain ionized at all reaction steps. In the "phosphotriester approach" on the other hand, the phosphate centers are neutral and subject to nucleophilic attacks. In the "phosphodiester approach", the diesters are converted at the condensation step to neutral tetraesters of pyrophosphate which are even more labile to nucleophilic attacks.

The approach suggested by us may be used to prepare higher oligonucleotides since the growing oligonucleotide chain is usually protected by a trityl group at the 5'-terminal. This enables purification of the chain by its selective retardation on a trityl-cellulose column. Dilute aqueous salt solutions will specifically elute the excess nucleoside, mononucleotide and other side products while alcohol-water mixtures will finaly elute the protected oligonucleotide chain.[12]

Methyldichlorophosphate has already been used for the stepwise synthesis of oligothymidilates on an insoluble polymeric carrier which enabled the easy separation of the desired mixed diester from excess reagents. However, the yields were rather low and a mixture of products was obtained upon cleavage from the polymeric carrier.[13]

Cyclic mononucleotides. In addition to oligonucleotides, cyclic phosphate diesters of nucleosides are of great biological interest. Adenosine-3',5' cyclic phosphate, for example, is a well known member of this group and ribonucleoside-2',3' cyclic phosphates are intermediates in the enzymatic degradation of ribonucleic acid.

Also protected ribonucleosides-2',3' cyclic phosphates are starting materials in the synthesis of oligoribonucleotides.

Cyclic mononucleotides are usually prepared by cyclization of mononucleotides using dehydrating agents such as dicyclohexylcarbodiimide.[14] We, however, investigated the possibility of phosphorylating a suitably protected ribonucleoside using N-methylpyridinium dichlorophosphate (1). Treating 5'-O-trityluridine with a 2·5-fold excess of 1 gave pyridinium 5'-O-trityluridine-2',3' cyclic phosphate in practically quantitative yield. Analytically pure product was isolated in 85% yield by the usual solvent extraction procedure. This first reported direct conversion of a ribonucleoside to a cyclic mononucleotide is very efficient and rapid.

collected, yield: 14 g (76%), m.p. 137°. (Found: C, 45·78; H, 3·70; N, 7·66; P, 8·47. Calcd. for: $C_{14}H_{13}N_2O_3P$ (368·2): C, 45·66; H, 3·56; N, 7·6; P, 8·4%). NMR: (DMSO) δ 5·4 (4H, s, J = 8 Hz) 7·5–8·3 (8H, m).

Dibenzyl phosphate and di-*p*-nitrobenzyl phosphate were similarly prepared (for result see Table 1).

Cyclohexylammonium dineopentyl phosphate. The compound was prepared from neopentyl alcohol (2 g, 22 mmol) and N-methylpyridinium dichlorophosphate (10 mmol) as above. It was extracted with chloroform-n-butanol (7:3, three 50 ml portions) and the extract was evaporated *in vacuo*. The oily residue was dissolved in anhyd ether (100 ml) and cyclohexylamine (1 ml) was added. The ppt thus formed was collected and recrystallized from methanol-ether, yield: 1·2 g (36%), m.p. 236–237°. (Found: C, 57·28; H, 11·1; N, 4·19; P, 8·86. Calcd. for: $C_{16}H_{36}NO_4P$ (337·2): C, 56·95; H, 10·75; N, 4·15; P, 9·18%). NMR:

Table 1. The preparation of Symmetrical phosphate diesters

Compound	Yield %	mp	mp Lit.	neutral equiv. found	neutral equiv. Theoretical	R_f values Solv. A	R_f values Solv.B
Dibenzyl phosphate	75	78°	78-79° [a]	284	278.1	0.93	-
Di-o-nitrobenzyl phosphate	76	137°	-	369	368.2	0.60	-
Di-p-nitrobenzyl phosphate	86	175°	175° [b]	363	368.2	0.45	-
Cyclohexylammonium dineopentyl phosphate	36	236°	-	335	337.2	-	0.06
Cyclohexylammonium dicyclohexyl phosphate	53	212°	211°[c]	357	361.27	-	0.18
Cyclohexylammonium diphenyl phosphate	75	195°	195-8°[d]	351	349.17	-	0.29

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EXPERIMENTAL

General. NMR spectra were measured at 60 Mhz on a Varian A-60 spectrometer. Molecular weights were determined by titration of the diesters or their cyclohexylammonium salts with a benzene soln of NaOMe in a nonaqueous solvent (usually DMF). Thymol blue was used as the indicator. TLC was carried out on silica gel (SiF) plates using $CHCl_3$-MeOH 7:3 (solvent A) and cellulose (CeF) plates using 2-propanol-conc ammonia-water 7:1:2 (solvent B) (Riedel de Haen, Hannover, W. Germany). P-containing compounds were detected on TLC plates by the method of Hanes and Isherwood[15] and trityl derivatives were detected by spraying with 10% perchloric acid aq and heating. Analytical pyridine (AnalaR, BDH) was dried and stored over calcium hydride. Methyl dichlorophosphate (B.P. 63°/30 mm Hg) was prepared according to Mizuma *et al.*[16] (it is also available commercially).

Di-o-nitrobenzyl phosphate. Dry pyridine (50 ml) was cooled in an ice bath. Methyl dichlorophosphate (5 ml, 50 mmol) was added dropwise over 15 min atmospheric moisture being excluded. The mixture was kept in the cold for a further 15 min. During this period a ppt of N-methylpyridinium dichlorophosphate formed. *o*-Nitrobenzyl alcohol (20 g, 125 mmol) was added and the sealed mixture was stirred overnight at room temp. It was then poured into 10% $NaHCO_3$ aq (200 ml) and the pyridine was evaporated *in vacuo*. The residue was diluted with water to 200 ml and extracted with ether (two 200 ml portions). TLC of the etheral layer on silica gel with chloroform as a solvent showed the presence of *o*-nitrobenzyl chloride together with *o*-nitrobenzyl alcohol in the etheral layer. The aqueous phase was acidified with 2N-HCl to pH 1, the precipitated oil was extracted with two 150 ml portions of chloroform-n-butanol (7:3), washed with 0·5 N HCl and water (100 ml each), dried over Na_2SO_4, filtered and concentrated *in vacuo* to 100 ml. n-Hexane (200 ml) was added and the ppt was

(CD$_3$OD) δ 0·9 (18H, s), 1·1–2·2 (11H, m), 3·5 (4H, d, J = 4Hz).

Cyclohexylammonium salts of diphenyl phosphate and dicyclohexyl phosphate were similarly prepared (for results see Table 1).

Barium 2,2'-dinitrobenzhydryl phosphate. 2,2'-Dinitrobenzhydrol (2·74 g, 10 mmol) was phosphorylated by N-methylpyridinium dichlorophosphate (30 mmol) in pyridine (30 ml) 16 hr at room temp. Water and ether (100 ml each) added to the mixture, the aqueous phase was separated and washed with ether (100 ml). Upon acidification to pH 1 (with 2N HCl) 1·4 g of crude 2,2'-dinitrobenzhydryl phosphate was precipitated. The ppt was dissolved in a mixture of water (10 ml) and pyridine (1 ml) and an aqueous barium acetate soln (1·5 g in 5 ml water) was added, the mixture being held for 1 hr at 0° to ensure complete precipitation. The product was collected and dried over P_2O_5, yield: 1·0 g, (20%). (Found: C, 31·12; H, 2·18; N, 5·54; P, 6·42. Calcd. for $C_{13}H_9N_2O_8PBa$. 1/2H_2O (498·2): C, 31·31; H, 2·02; N, 5·62; P, 6·21%).

Pyridinium,5'-O-tritylthymidine-3'β-cyanoethylphosphate (TrO-TpCE). Methyl dichlorophosphate (0·5 ml, 5 mmol) was added dropwise to anhyd pyridine (8 ml) in a drybox. After 15 min, β-cyanoethanol (0·5 ml, 7·2 mmol) was added and the mixture was stirred for 1 hr. A soln of 5'-O-tritylthymidine (227 mg 0·4 mmol) in pyridine (5 ml) was then added and the mixture was left for 16 hr. Water (20 ml) was added, the soln was washed with ether (20 ml) and then extracted with chloroform (three 30 ml portions). The chloroform soln was washed with water (20 ml), dried over Na_2SO_4, and evaporated *in vacuo* to a small volume. The product was precipitated with an excess of dry ether, yield: 230 mg (83%). The product was homogenous in TLC (R_f = 0·2, solvent A). (Found: C, 61·40; H, 5·57; N, 7·92; P, 4·38. Calcd. for $C_{37}H_{36}N_4PO_8$. 1·5 H_2O (722·57): C, 61·44; H, 5·44; H, 5·44; N, 7·75; P, 4·28%).

The dinucleotide 5'-O-*tritylthymidilyl*(3' → 5')3'-O-*acetylthymidine* (*TrO-TpT-OAc*). Methyl dichlorophosphate (0·25 ml, 2·5 mmol) was added to anhyd pyridine (12·5 ml) in a drybox. After 15 min a soln of 3'-O-acetylthymidine (855 mg, 3 mmol) in pyridine (5 ml) was added dropwise and 30 min later 5'-O-tritylthymidine (230 mg, 0·4 mmol) was added. The mixture was left for 16 hr. Water (15 ml) was then added and the mixture was washed with ether (two 20 ml portions). The ether was back extracted with water (15 ml), the combined aqueous phase was extracted with chloroform-n-butanol (5:1, three 35 ml portions) and the organic phase was washed with water (50 ml). The organic phase was dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The resulting gum was dissolved in chloroform (10 ml) containing 1 ml pyridine and was added dropwise to dry ether (100 ml). The white ppt was collected and dried. The yield of protected dinucleotide was 275 mg (75%), it was homogenous on TLC (R_f = 0·41 solvent *A*) and identical to an authentic dinucleotide prepared by other methods. The protecting groups were removed according to literature and the unprotected dinucleoside phosphate was completely hydrolysed by snake venom phosphodiesterase to thymidine and thymidine-5' phosphate which were obtained in a 1:1 ratio.

Pyridinium 5'-O-*trityluridine*-2',3' *cyclic phosphate*. 5'-O-trityluridine (2g, 4 mmol) was phosphorylated with N-methylpyridinium dichlorophosphate (10 mmol) in pyridine (20 ml) at room temp. TLC after 2 hrs showed complete disappearance of 5'-O-trityluridine and formation of a new product (R_f = 0·25, solvent *A*). Water (20 ml) was then added at 0° and the soln was washed with ether (two 30 ml portions). The aqueous phase was extracted with chloroform-n-butanol (7:3, four 25 ml portions). The organic phase was washed with 1 M NaCl (20 ml) and dried over Na_2SO_4. It was then concentrated at a reduced pressure to about 20 ml and was added dropwise to dry ether (200 ml). The white ppt was filtered, washed with a little dry ether and dried. The product obtained in 85% yield (2·2 g) was homogenous on TLC (R_f = 0·25, solvent *A*), titration with NaOMe gave neutral equivalent 610 (Theoretical = 625). (Found: N, 6·65; P, 5·01. calcd. for $C_{33}H_{28}N_3O_8P$ (625·2); N, 6·72; P, 4·95%).

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