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SYNTHESIS OF 5'-PHOSPHORYLATED DNA FRAGMENTS

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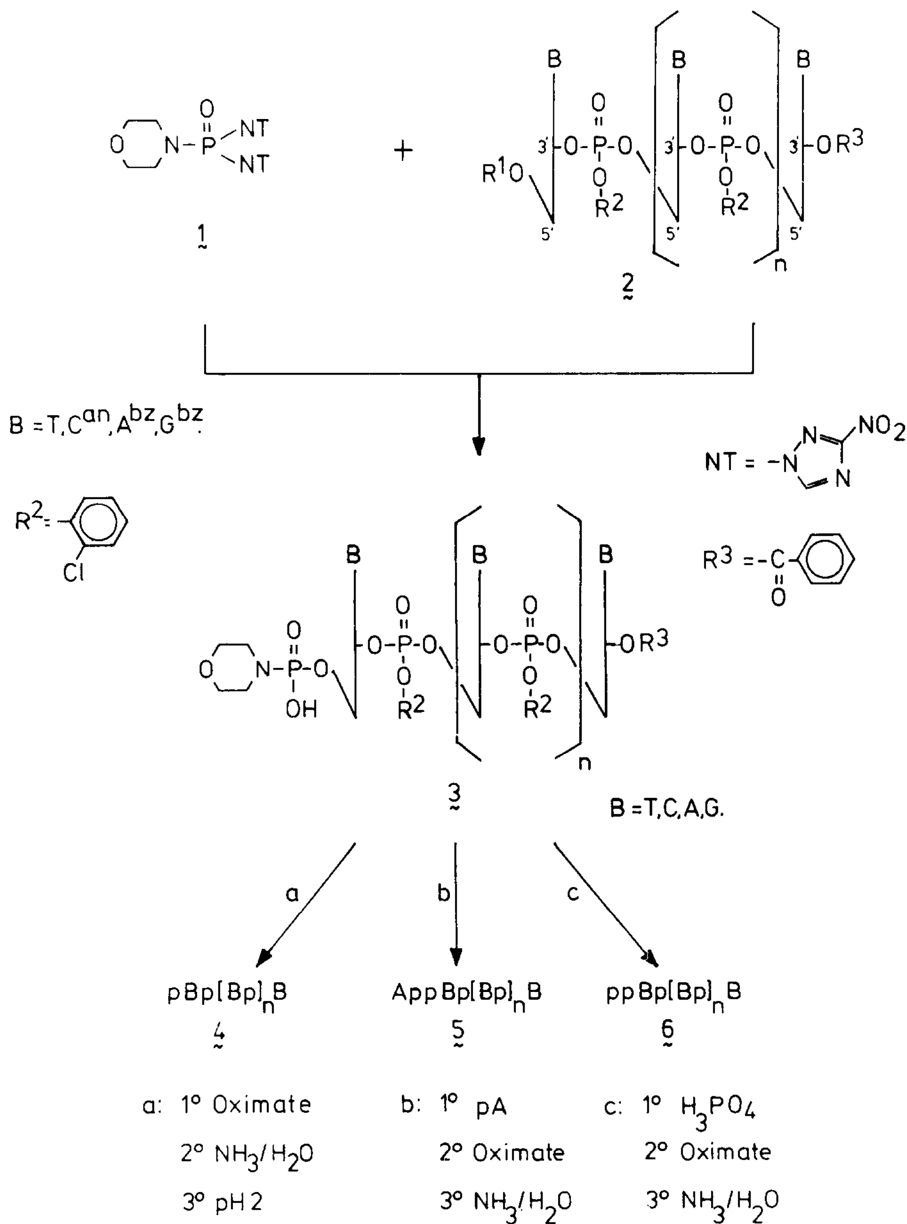
Summary: Morpholino phosphorobis-3-nitro-1,2,4-triazolidate (1) has been applied to the phosphorylation of partially protected (5'-OH free) DNA intermediates (2). Using this procedure, the following types of compounds were synthesized: $pBp(Bp)_nB$; $AppBp(Bp)_nB$ and $ppBp(Bp)_nB$.

The synthesis of DNA fragments (i.e. 2; R^1=R^2=R^3=H; B=T,C,A or G) with a defined sequence and length via phosphotriester intermediates is a well established technique[1]. In a strategy based on the phosphotriester approach to the synthesis of DNA fragments, we may discern two distinct stages. The first one involves the synthesis of a fully-protected intermediate (i.e. compound 2 in which R^1, R^2 and R^3 present protective groups). The last step consists of a complete removal of the protective groups from the fully-protected intermediate to afford a DNA fragment (i.e. 2) containing naturally occurring 3'-5'-phosphodiester linkages (R^2=H) as well as free hydroxyl (R^1=R^3=H) and exocyclic amino functions (B=C,A or G). Although different routes to the synthesis of fully protected intermediates have been developed, they all have in common the feasibility to remove the protective group R^1 at the 5'-end selectively in the presence of the other groups. This property of group R^1 is an essential part in a phosphotriester approach to the synthesis of DNA fragments: it allows elongation of a nucleic acid chain in the 5'-direction. However, the unique property of the protective group at the 5'-end is lost during the deblocking of all protecting groups.

We now wish to report that the partially protected derivatives 2(R^1=H), which were obtained after selective removal of protective group R^1, can be phosphorylated with the morpholino phosphoro derivative 1 to give valuable 5'-phosphorylated DNA fragments (i.e. 4, 5 and 6).

We chose for the phosphorylation of the primary hydroxy group of DNA fragments 2 (R^1=H, R^2=2-ClC_6H_4, R^3=Benzoyl) the bifunctional phosphorylating agent morpholino phosphorodichloridate[2]. This reagent is easily accessible by reacting together phosphoroxytrichloride (0.9 mol) with morpholine (0.15 mol) in dry toluene (250 ml), and is a well defined liquid (b.p. 84-85°/0.3 mm Hg). The nature of the morpholino group not only allows the synthesis of mono--phosphates but also of di- and tri-phosphates. Further, the reactivity of the phosphorylating agent can be modified[3] by substituting the chlorine atoms by 1,2,4-triazole or derivatives thereof. However, an obvious disadvantage inherent to the use of this phosphorylating agent is that acidic conditions, which may lead to depurination[4], are necessary for the removal of the morpholino group. We found that the half-time of removal of the morpholino group from a 5'-phosphate function of a dimer[5] (i.e. 3; B=T, n=0, R^2=R^3=H), using aqueous HCl (0.01 N, pH 2), was 6 min. On the other hand, depurination of adenine or N^6-benzoyladenine from the partially protected dimer[1e] TA (i.e. 2; n=0, R^1=R^2=R^3=H) or dimer TA^{Bz} (i.e. 2; n=0, R^1=R^2=H, R^3=Bz), under the same acidic conditions, proceeded with half-times of hydrolysis of 90 and



and 8 hr[6], respectively. The above data clearly indicate that the removal of the morpholino function can be performed with a neglible amount of depurination, if performed on DNA fragments containing purine bases having free exocyclic amino functions (i.e. 3; $R^2=R^3=H$, B=A or G).

Having established the conditions which guarantee a safe removal of the morpholino group, we turned our attention to the phosphorylation of DNA fragments (e.g. 2) having solely a free 5'-OH group with the unmodified reagent morpholino phosphorodichloridate. The DNA fragments

2 (R^1=H, R^2=2-ClC_6H_4, R^3=Bz), which were used in this study, were obtained by treating the fully protected derivatives 2 (R^1=Levulinyl, R^2=2-ClC_6H_4, R^3=Bz) with hydrazine hydrate[1e]. Phosphorylation of a solution of DNA fragments 2 (R^1=H) in pyridine with an excess of morpholino phosphorodichloridate gave, after complete deblocking according to procedure a (see later) of products 3 thus obtained, a low yield of the required compounds 4. Especially very low yields of 4 were obtained when we started from DNA fragments containing d-guanosine nucleosides. However, smooth and quantitative phosphorylation of DNA fragments 2 occurred, and satisfactory yields[7] (60-70%) of compounds 4 were obtained, by using reagent 1 which was obtained by replacing the two chlorine atoms of morpholino phosphorodichloridate by 3-nitro-1,2,4-triazole[8]. The synthesis of the DNA fragment 4 (pTAGGAT) will be demonstrated.

To a solution of morpholino phosphorodichloridate (2.5 mmol) in dry dioxan (10 ml) was added 3-nitro-1,2,4-triazole[9] (5 mmol) and triethylamine (0.7 ml). After 5 hr, the triethylamine-HCl salt was filtered off, and the filtrate containing 1 was added to hexamer 2 (R^1=H, R^2=2-ClC_6H_4, R^3=Bz, sequence $TA^{Bz}G^{Bz}G^{Bz}A^{Bz}T$; 0.05 mmol). After 24 hr, when TLC analysis showed the reaction to be complete (R_f 0.5 → 0.0; $CHCl_3$/MeOH; 92:8, v/v), the reaction mixture was diluted with $CHCl_3$ and washed with aq. triethylammonium bicarbonate (4x2.0 M TEAB). The organic layer was dried and concentrated to give 3 as an oil. The compound thus obtained was deblocked as follows (procedure a). The 2-chlorophenyl groups were removed by treating 3 with syn-4-nitrobenzaldoximate according to the procedure of Reese[1f,10]. After 48 h, aq. ammonia was added to the reaction mixture which was kept at 50° for 48 h. The solution was concentrated to a small volume (5 ml) and acidified with 0.1 N HCl until pH 2. After 1 h, the aq. solution was extracted (4x) with ether and chloroform, neutralized with aq. ammonia and finally concentrated to a small volume (1 ml). HPLC analysis[1e] of the crude reaction mixture showed the presence of mainly one product having a longer retention time than the hexamer TAGGAT (deblocked starting product). Purification of the product on Sephadex G50, followed by freeze-drying, afforded pTAGGAT (4) as a fluffy solid. HPLC analysis revealed the presence of one product which was completely digested with venom phosphodiesterase into the expected d-nucleotides. Treatment of pTAGGAT with alkaline phosphatase afforded, as expected, one product which was identical (HPLC analysis) with hexamer TAGGAT. Further, ^{31}P-NMR spectroscopy showed the presence of five phosphodiester linkages between -1.049 and -1.351 ppm and one phosphomomoester at 0.858 ppm. In the same way, we prepared the following 5'-phosphorylated DNA fragments 4 : $pAp(Ap)_6A$, $pTp(Tp)_2T$, $pTp(Tp)_5A$, $pTp(Tp)_5G$ and $pTp(Tp)_6T$.

The intermediate morpholino derivative 3 could also be converted into a DNA fragment bearing a 5'-diphosphate function (i.e. 6). Thus, treatment of 3 (n=2, B=T, R^2=2-ClC_6H_4, R^3=Bz: 0.03 mmol) with the tri-n-butylammonium salt of phosphoric acid (0.5 mmol) in dry DMF (2 ml) for 24 h at 50° gave, after deblocking (procedure c) and finally acid treatment (1 h at pH 2), crude 6 (N=2, B=T, R^2=R^3=H). Analysis of crude 6 by HPLC revealed the presence of 4 (pTTTT) and 6 (ppTTTT) in nearly equal amounts. Purification of the crude mixture on Sephadex-A25 anion-exchange chromatography afforded homogeneous ppTTTT (6) in a yield of 40%. The identity of the compound was confirmed by ^{31}P-NMR data and the finding that it was completely digested by venom phosphodiesterase to give solely the d-nucleotide pT.

Finally, we also demonstrated that intermediate 3 could be used to synthesize DNA fragments 5. This type of compounds, which is formed by treating DNA fragments 4 with the enzyme T_4-DNA

ligase in the presence of ATP[11]), can serve as donor in the enzymatic ligation with acceptor DNA fragments. Thus fragment 3 (n=2, B=T, R^2=2-ClC_6H_4, R^3=Bz; 0.025 mmol) was treated with the pyridinium salt of adenosine-5'-phosphate (pA; 100 mg) in dry DMF (5 ml) for 24 h at 50°. Deblocking of the reaction product (procedure b) followed by short acid treatment (0.1 N HCl, pH 2, 1 h) gave, after work up, crude product. Analysis of the crude reaction product by HPLC showed the presence of the required product 5 (AppTTTT) and 4 (pTTTT) in the ratio 1:1. Purification of the crude product by Sephadex-A25 or Sephadex-G25 column chromatography afforded homogeneous 5 (AppTTTT) in a yield of 40%. The identity of the product was inter alia corroborated by enzymatic digestion. Thus treatment of the product with phosphodiesterase (venom) gave solely pA and pT in the ratio 1:4.

An alternative route to the synthesis of 5'-phosphorylated fragments 4, 5 and 6 would have been the phosphorylation of compounds 2 (R^1=H) with suitably protected monofunctional phosphorylating agents (see phosphorylating agents mentioned in references 5, 12 and 13). However, in applying these reagents we found that the monitoring (TLC-analysis) of the phosphorylation step {e.g., conversion of 2 (R^1=H; sequence TAGGAT) into 3} was impossible: no difference in R_f-value between 2 and 3 was observed. Furthermore,HPLC-analysis of the products obtained after complete deblocking still showed the presence of starting material.

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6. The identity of the depurinated product was established by ^{1}H-NMR. The progress of the hydrolysis was monitored by HPLC.
7. Yields were determined as described in reference 1e.
8. Substitution of the chlorine atoms by 1,2,4-triazole gave a phosphorylating agent which reacted very slowly with 2 (R^1=H).
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