Tetrahedron Letters No. 24, pp 2209 - 2212, 1973. Pergamon Press. Printed in Great Britain.

BLOCK SYNTHESIS OF OLIGONUCLEOTIDES BY THE PHOSPHOTRIESTER APPROACH

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(Received in UK 18 April 1973; accepted for publication 3 May 1973)

Most of the published work on the chemical synthesis of oligonucleotides[2] has involved the use of intermediates with unprotected internucleotide phosphodiester linkages. Such intermediates can usually be isolated only on a comparatively small scale and are sometimes difficult to obtain pure. Furthermore, the presence of free phosphodiester functions can lead to undesirable side-reactions during subsequent phosphorylation steps. This presumably accounts for the low yields, especially of larger oligomers, often obtained by this approach. In the hope of overcoming these problems, several groups of workers[3-7] have investigated the possibility of protecting the phosphodiester linkages in oligonucleotide synthesis. As a result of our own studies[5] in this area, we have come to believe that significant progress in the development of methods for the chemical synthesis of oligonucleotides in both the ribose and deoxyribose series will result only if the internucleotide linkages are protected. In the present communication we describe the preliminary results of our studies on the block synthesis of some oligothymidylic acids by the phosphotriester approach.

The fully-protected dinucleoside phosphate (VIa) may be regarded as the basic synthetic unit in the present work; it was prepared by allowing IIIb[8] (6.70 mmole, m.p. 145-147°, obtained from thymidine (IIIa) in 65% yield), phenyl dihydrogen phosphate (IV, 6.70 mmole) and an excess of tri-isopropyl-benzenesulphonyl chloride[9] (TPS, V, 16.1 mmole) to react together in anhydrous pyridine solution at 20° for 16 hr. and then adding IIIc (6.70 mmole, m.p. 128-130°, obtained from thymidine in 53% overall yield) and more TPS (4.1 mmole). Work-up of the products after 48 hr. and fractionation by Short Column Chromatography[10] gave VIa, which was isolated as a homogeneous (t.l.c.) colourless solid in 75% yield.

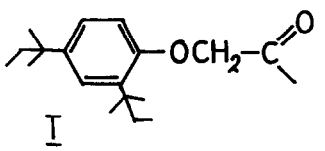
The use of Short Column Chromatography in this work should be emphasized; in our hands, this technique has proved to be far superior to conventional adsorption chromatography for the fractionation of phosphotriester intermediates and we strongly recommend its use when a high recovery of very pure material is impor-

tant. This technique may be used to fractionate quantities of up to 10-20 g. at a time. Another noteworthy practical aspect of the present approach is that phenyl and many other aryl dihydrogen phosphates are non-hygroscopic, crystalline solids which can therefore be weighed out in precisely stoicheiometric quantities.

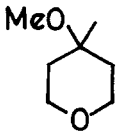
Treatment of the fully-protected dinucleoside phosphate (VIa) with dilute alkali and dilute acid at 20° gave the partially-protected dimers, VIb (>90%) and VIc (95%), respectively. The fully-protected tetramer (VII; R=I, R'=II) was then prepared from VIc (2.80 mmole), IV (2.80 mmole), TPS (6.72 + 1.7 mmole) and VIb (2.80 mmole) in the manner described above for the preparation of VIa; it was isolated as a homogeneous (t.l.c.) colourless solid in 63% yield after Short Column Chromatography. Repetition of the alkaline and acidic de-blocking processes on VII (R=I, R'=II) gave good yields of the partially-protected tetramers (VII; R=H, R'=II and R=I, R'=H, respectively). The fully-protected octamer (VIII; R=I, R'=II) was prepared from the partially-protected tetramers (0.49 mmole of each), IV (0.49 mmole) and TPS (1.15 + 0.30 mmole); it was isolated as a homogeneous colourless solid in 57% yield. Finally, a preliminary experiment, carried out on a 0.1 mmole scale, indicated that the fully-protected hexadecamer (IX; R=I, R'=II) can be prepared from stoicheiometric quantities of the partially-protected octamers (VIII; R=H, R'=II and R=I, R'=H) without a significant fall off in yield.

In order to obtain the unprotected oligonucleotides (X, XI and XII), the 5'-hydroxyl groups of the partially-protected intermediates (VIb, VII; R=H, R'=II and VIII; R=H, R'=II) were tetrahydropyranylated and the products obtained (e.g. VId) subjected first to alkaline (0.2 M alkali at 20°) and then to acidic (pH 2, 20°) hydrolysis. Examination of the hydrolysis products (paper electrophoresis and cellulose t.l.c.) revealed that the TpT (X) and $(Tp)_3T$ (XI) obtained were virtually free from contaminants. However, this was not true for the $(Tp)_7T$ (XII). The three products were purified by chromatography on DEAE-cellulose: TpT, $(Tp)_3T$ and $(Tp)_7T$ accounted for 92, 79 and 52% of the total absorbance units (267 nm) eluted from the respective columns. The purified oligomers were homogeneous (paper electrophoresis, cellulose t.l.c. and PEI-cellulose t.l.c.) and their total digestion by venom and spleen phosphodiesterases to give the expected products confirmed their structures.

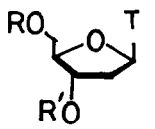
The only moderate yield of $(Tp)_7T$ (XII) obtained from the corresponding fully-protected tetrahydropyranyl derivative (VIII; R=THP, R'=II) and a preliminary examination of the structures of some of the by-products obtained indicate that phenyl is not entirely satisfactory as a protecting group for the internucleotide linkages. In effect, alkaline hydrolysis of phenyl dialkyl phosphates leads to small amounts of phenyl alkyl phosphates in addition to the expected dialkyl phosphates. Thus, when the fully-protected octamer (VIII;R=THP R'=II) is subjected to alkaline hydrolysis, the possibility exists for some



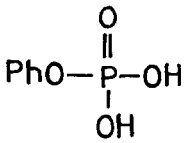
I



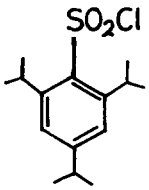
II



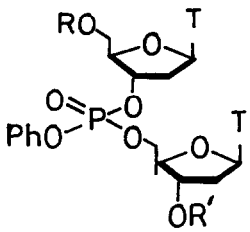
III a; R=R′=H
b; R=I, R′=H
c; R=H, R′=II



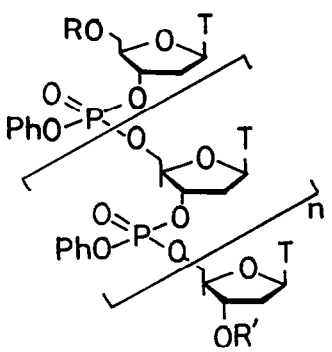
IV



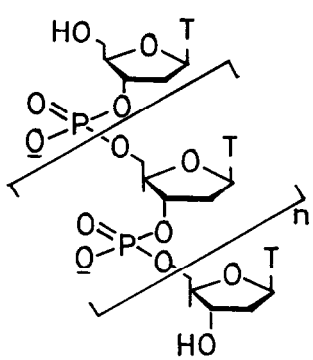
V



VI a; R=I, R′=II
b; R=H, R′=II
c; R=I, R′=H
d; R=  , R′=II



n=2, VII
n=6, VIII
n=14, IX



n=0, X
n=2, XI
n=6, XII

T=thymine-1

internucleotide cleavage to occur at each of its seven phosphotriester groups. Fortunately, recent experiments indicate that this difficulty can be virtually completely eliminated by using halogen-substituted aryl instead of simple phenyl protecting groups.

In conclusion, the present work demonstrates that comparatively large oligonucleotides can be obtained in satisfactory yields by the phosphotriester approach. The phosphorylation procedure described is effective and Short Column Chromatography is an efficient technique for the purification of phosphotriester intermediates. Following the introduction of modified protecting groups for the internucleotide linkages, this method should find general application in the synthesis of high molecular weight oligonucleotides in both the deoxyribose[11] and ribose series. Even without modification, the present block synthesis of oligothymidylic acids represents a significant advance over a corresponding synthesis by the phosphodiester approach.[12]

We thank the Science Research Council for financial support.

FOOTNOTES AND REFERENCES

1. To whom enquiries should be addressed.
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8. The 2,4-di-t-amylphenoxyacetyl protecting group (I) is both lipophilic and very sensitive to base; its lipophilic character facilitates the separation of fully-protected oligonucleotides from unchanged starting materials and its sensitivity to base permits it to be removed without concomitant hydrolysis of the phosphotriester groups.
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10. B.J. Hunt and W. Rigby, Chem. and Ind., 1868 (1967).
11. Several dinucleoside phosphates and one tetranucleoside triphosphate containing a deoxycytidine residue have also been prepared in good yields by the phosphotriester method. These experiments were carried out by Mr. P.H. van Deursen and involved intermediate o-chlorophenyl esters.
12. S.A. Narang, J.J. Michniewicz and S.K. Dheer, J. Amer. Chem. Soc., 91, 936 (1969). However, Dr. Narang is now also undertaking a block synthesis of oligothymidylic acids by the phosphotriester approach.[13] This work is being carried out in collaboration with Dr. R.H. Wightman and follows Dr. Wightman's stay of 3-4 months in the University Chemical Laboratory, Cambridge.
13. R.H. Wightman, personal communication.