200. Synthesis of Oligodeoxynucleotides by the Phosphite-Triester Method Using Dimer Units and Different Phosphorous-Protecting Groups

by **Willi Bannwarth**

F. Hoffmann-La Roche & Co. Ltd., Central Research Units, CH-4002 Basel

(22.VII.85)

A simple synthesis of phosphoamidite dinucleotides with two different phosphorous-protecting groups and their use for the synthesis of 2'-oligodeoxynucleotides on a polymer support is reported.

Introduction. – For the economical synthesis of oligodeoxynucleotides, the method of choice is the solid-phase chemistry using either the triester or the phosphite-triester approach [1]. The advantage of the triester method is the possibility of using dimers, trimers, or even higher units as building blocks in the preparation of oligonucleotides, thus decreasing the coupling steps for a given sequence and thereby enhancing the speed and effectiveness of synthesis and simplifying the purification of the final product. The phosphite-triester method has so far been limited to monomer additions, but extension to the coupling of larger units would be desirable.

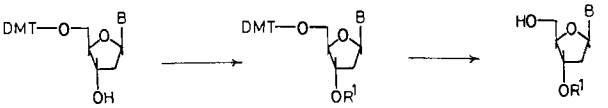
Lately, *Kumar* and *Poonian* [2] described two ways for the synthesis of dimer units which were used with high efficiency in the preparation of several oligodeoxynucleotides by the phosphite-triester strategy. The route for the synthesis of these methyl (*N,N*-dialkyl)phosphoamidite dinucleotides **3** *(Scheme 1)* is relatively complicated. It involves the preparation of the 3'-protected compounds **1**. After condensation and oxidation to compound **2**, the protecting group R^1 has to be cleaved off before a phosphinylation leads to the desired compound of type **3**.

We would like to report a simpler method for the preparation of dimer units and their successful use in the synthesis of oligodeoxynucleotides by the phosphite-triester strategy on a polymer support. These dimer units are compounds of the general structure **6** *(Scheme 2)*. The P-atoms in these molecules are present in two oxidation levels, *i.e.* P(III) and P(V) and each carries a different protecting group. The phosphate part of the molecule carries the *o*-chlorophenyl group mainly used in the triester strategy, whereas the phosphoamidite part carries the methyl group mainly used in the phosphite-triester method.

Compounds of type **4** are easily prepared in high yield by standard procedures from the protected nucleosides [3] *(Scheme 2)* or purchased from a supplier. For being used as dimers in the triester strategy, they are transformed to the triesters **5** according to [4] which, after removal of the cyanoethyl group [5] can be used for chain elongations on polymer support (I).

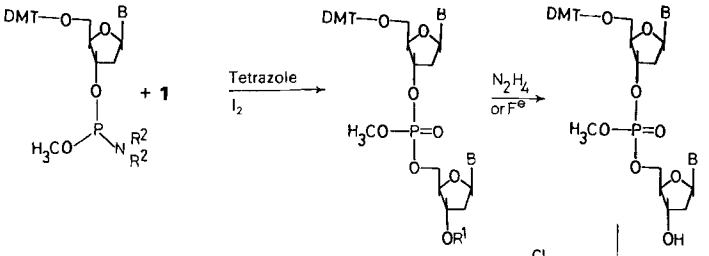
On the other hand, we have shown that **4** can be transformed by phosphinylation into dimers **6** (II) usable for phosphite-triester chemistry by activation with an acid-like

Scheme 1



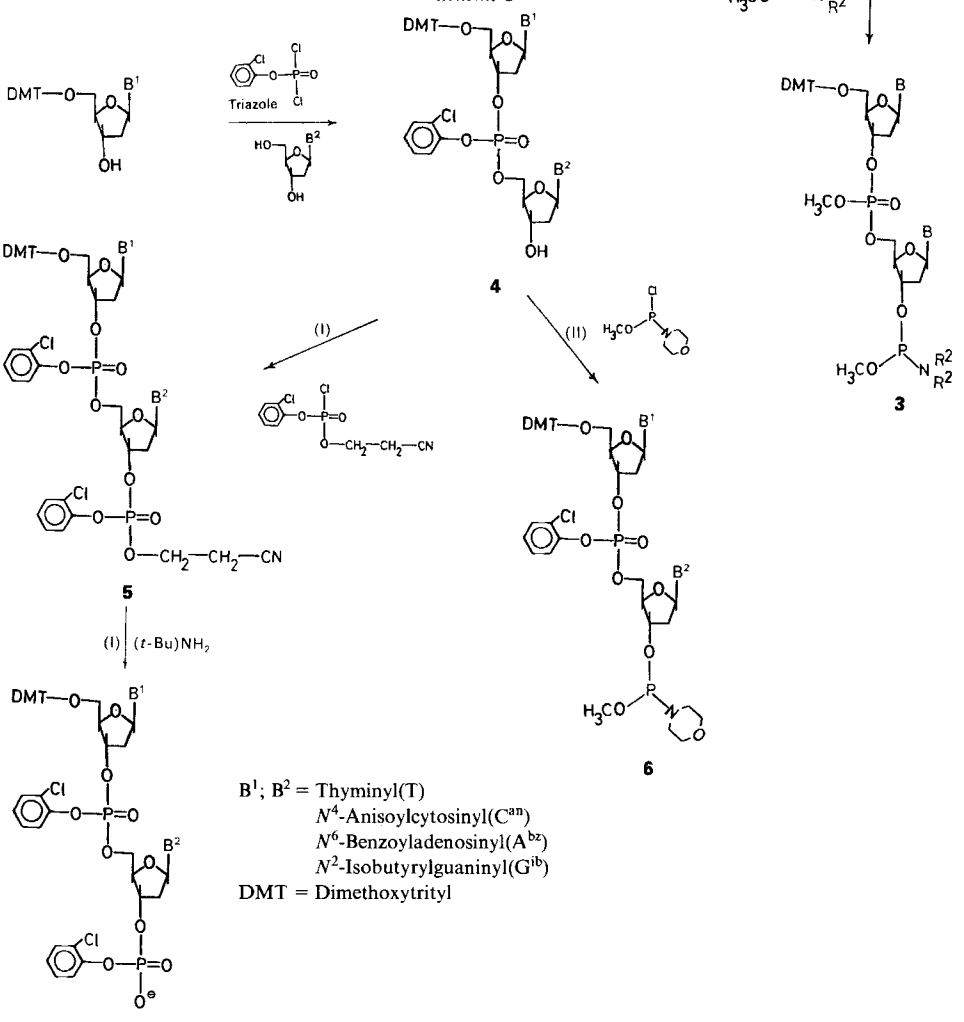
1

DMT = Dimethoxytrityl
B = Base
R^1 = Levulinyl- or (*tert*-butyl)dimethylsilyl
R^2 = CH_3 or $CH(CH_3)_2$



2

Scheme 2



3

4

5

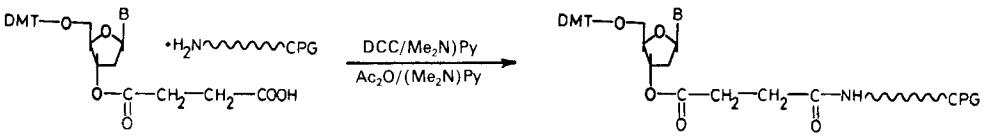
6

B^1; B^2 = Thyminyl(T)
N^4-Anisoylcytosinyl(C^{an})
N^6-Benzoyladenosinyl(A^{bz})
N^2-Isobutyrylguaninyl(G^{ib})
DMT = Dimethoxytrityl

tetrazole. In this strategy, there is no need for a 3'-protecting group as in the previously reported synthesis [2].

Synthesis of Oligodeoxynucleotides. – 1. *Support.* As support we used controlled pore glass (CPG) attached to a long-chain alkylamine as manufactured by *Pierce*. The corresponding 3'-succinylated nucleoside was linked to the alkylamine chain of the support directly by coupling with dicyclohexylcarbodimide (DCC; *Scheme 3*) [6]. Unreacted amino functions were capped by acylation with Ac_2O/pyridine. After 24 h, the loading was in a range of 10–30 μmol/g.

Scheme 3



B = Thyminyl (T)
N^4-Anisoylcytosinyl (C^{an})
N^6-Benzoyladenosinyl (A^{bz})
N^2-Isobutyrylguaninyl (G^{ib})

DMT = Dimethoxytrityl
DCC = Dicyclohexylcarbodiimide
$(Me_2N)Py$ = 4-(dimethylamino)pyridine

2. *Synthesis of Oligonucleotides on Controlled Pore Glass.* In order to test the suitability of the prepared dimer components **6**, we have synthesized the following sequences **7**, sometimes in combination with monomers (*e.g.* **7c** and **7e**): $^{5'}$TC TC TC TC $T_{3'}$ (**7a**), $^{5'}$GA GA GA GA $G_{3'}$ (**7b**), $^{5'}$CT A GG TA $C_{3'}$ (**7c**), $^{5'}$TT TT TA AA AA $A_{3'}$ (**7d**), and $^{5'}$AG CT GG TA C $C_{3'}$ (**7e**).

The syntheses were carried out in the simple glass-frit system originally developed by *Caruthers* [1]. We started with *ca.* 2.5 μmol of functionalized support and used a 20fold excess of dimer or monomer units. For the coupling step, the elongation units were dissolved in 0.75 ml of a standard solution of tetrazole in anh. MeCN containing 1 g of tetrazole/30 ml of MeCN. The complete cycle for the addition of one building-block unit is outlined in *Table 1*.

Table 1. *Cycle for the Addition of One Building Block*

Step	Reagent or solvent	Amount	Time
1	$ZnBr_2$	3 × 2 ml	3 × 2 min
2	MeOH	2 ml	30 sec
3	CH_2Cl_2/i-PrOH 85:15 *(v/v)*	2 ml	wash
4	MeCN	6 × 2 ml	wash
5	Condensation mixture	0.8 ml	5 min
6	Capping (Ac_2O/$(Me_2N)Py$)	3 ml	2 min
7	THF	2 ml	wash
8	THF/lutidine/H_2O 2:1:2 *(v/v)*	2 ml	2 min
9	I_2 in THF/lutidine/H_2O	2 ml	2.5 min
10	THF	2 ml	wash
11	MeOH	2 ml	wash
12	CH_2Cl_2/i-PrOH (85:15)	2 ml	wash

The cleavage of the DMT group was performed with $ZnBr_2$ in CH_2Cl_2/i-PrOH [7] 85:15, thus avoiding any possible depurination or cleavage of the protecting group of the exocyclic amino function of A. This step was followed by a MeOH wash to destroy any possible Zn complexes.

The capping step was performed with a freshly prepared mixture of a 6.5% solution *(w/v)* of 4-(dimethylamino)pyridine ($(Me_2N)Py$) in THF (2 parts) and an Ac_2O/lutidine solution 1:1 *(v/v)* (1 part). This step can be omitted if the coupling yield is reasonably high and the sequence is not too long. *Step 8* was introduced in order to avoid branching, but was omitted in later syntheses because this step seems to be of no importance since no $n + 1$ product could be seen during syntheses.

The oxidation step was performed with a 0.2M solution of I_2 in THF/lutidine/H_2O 2:1:2 after each coupling, since a single oxidation step after completion of the desired sequence leads to the formation of much more side products.

3. *Cleavage of the Protecting Groups.* The protecting groups at the P-atoms have to be cleaved off first in order to prevent isomerisation during the basic conditions used for the cleavage of the base-protecting groups. The Me group is cleaved off first by the nucleophilic attack of thiophenol [8]. Treatment with (*Z*)-*o*-nitrobenzaldoximate cleaves off the *o*-chlorophenyl group [9] and releases the oligonucleotide from the support. The base-protecting groups are then removed by the action of NH_3 and finally the DMT group by 80% AcOH. The whole procedure is summarized in *Table 2.* After deprotection, the crude mixtures were purified on a *DEAE-Sephadex* column using a gradient of $Et_3NH^{\oplus}$ $HCO_3^{\ominus}$.

Table 2. *Cleavage of the Protecting Groups*

Step	Reagent	Step	Reagent
1	thiophenol/Et_3N/dioxane 1:2:2 *(v/v)*	*3*	conc. NH_3 (min. 25%)
2	(*Z*)-*o*-nitrobenzaldoximate/tetramethylguanidine	*4*	AcOH (80%)

Experimental Part

Solvents and Reagents. Pyridine: refluxed and distilled from TsCl, refluxed over CaH_2: distilled and kept over molecular sieve (4 Å) in tightly closed bottles. $CHCl_3$: *p.a.*, filtered through basic alumina (act. I; 10 g/14 ml). MeCN: refluxed and distilled from P_2O_5, refluxed over CaH_2, and distilled just prior to use. Et(i-Pr)$_2$N: distilled over a *Vigreux* column (15 cm) and stored over molecular sieve. DCC, $(Me_2N)Py$; Ac_2O, (*t*-Bu)NH_2, and triazole, all *puriss. (Fluka)*, were used without further purification. Tetrazole *(Fluka)* was sublimed. *o*-Chlorophenylphosphorodichloridate and protected nucleotides were prepared by standard procedures [1]. Chloro(methoxy)morpholinophosphine was prepared starting from methoxy(dichloro)phosphine *(Aldrich)* [1]. The monomer units used for oligonucleotide synthesis were prepared by a modified procedure according to [1]. The dimer components of general structure **4** which were used for the preparation of the building blocks were synthesized by the standard procedure and purified by short-column chromatography [1].

Functionalization of the Support. The 3'-succinylated nucleosides needed for the functionalization were prepared according to [1]. The *Pierce* glass support material did not need any further treatment. It could be used directly for the functionalization with the corresponding 3'-succinylated nucleoside [6]. A suspension of 3 g of support in 50 ml of abs. pyridine and 0.9 ml of Et_3N was evaporated. After addition of 2.5 mmol of 3'-succinylated nucleoside, the mixture was again taken up in 50 ml of pyridine and evaporated once more. Finally, it was taken up in 50 ml of pyridine and 22 mmol (4.5 g) of DCC, and 0.5 mmol (61.2 mg) of $(Me_2N)Py$ were added. The suspension was reacted with occasional shaking *(Vortex)*. After 24 h, the mixture was filtered through a glass-sinter funnel and washed with pyridine and Et_2O and dried *in vacuo*. Then, it was suspended in 30 ml of pyridine and 3 ml of Ac_2O and reacted for 2 h. It was filtered again through a glass-sinter funnel, and the functionalized support was washed with pyridine and Et_2O and finally dried *in vacuo*. The functionalization ranged from *ca.* 10 to 32 μmol of nucleoside/g support.

Dimer Phosphoamidites **6**. A mixture of 1 mmol **4** in 6 ml of dry $CHCl_3$ was kept under Ar. With ice cooling and stirring, 2.0 ml of $Et(i\text{-}Pr)_2N$ were added followed by 6 mmol of chloro(methoxy)morpholinophosphine (2 min). After complete addition, stirring was continued for another 30 min. The mixture was taken up in 70 ml of AcOEt

Table 3. *^{31}P-NMR Data of the Synthesized Dimer Units*

B^1	B^2	δ_P [ppm][a])							
		P(III)				P(V)			
A^{bz}	T	146.3	145.3	145.2		–7.0	–7.2		
A^{bz}	A^{bz}	146.17	146.09	145.64	145.5	–7.0	–7.4		
A^{bz}	G^{ib}	146.0	145.6	145.4		–7.5	–7.7		
A^{bz}	C^{an}	146.3	146.2	145.2	145.1	–6.6	–6.9		
T	A^{bz}	146.2	146.1	145.6	145.5	–6.9	–7.3		
T	T	146.29	145.30	145.21		–7.0	–7.1		
T	G^{ib}	146.1	146.0	145.7	145.3	–7.4	–7.5	–7.7	–7.8
T	C^{an}	146.3	146.2	145.1	145.0	–6.8	–6.9		
C^{an}	A^{bz}	146.2	146.1	145.5	145.4	–7.1	–7.2		
C^{an}	C^{an}	146.3	145.2	145.0		–6.7	–7.0		
C^{an}	G^{ib}	146.3	146.0	145.7	145.5	–7.5	–7.6		
C^{an}	T	146.4	145.3	145.2		–6.8	–6.8	–7.1	
G^{ib}	A^{bz}	145.9	145.8	145.7	145.6	–6.7	–6.9	–7.1	–7.3
G^{ib}	T	146.3	146.2	145.6		–6.3	–6.4	–7.0	–7.2
G^{ib}	G^{ib}	146.0	145.7			–7.9	–8.0		
G^{ib}	C^{an}	146.8	146.3	146.1	145.6	–6.9	–7.3	–7.4	

[a]) Relative to the standard H_3PO_4.

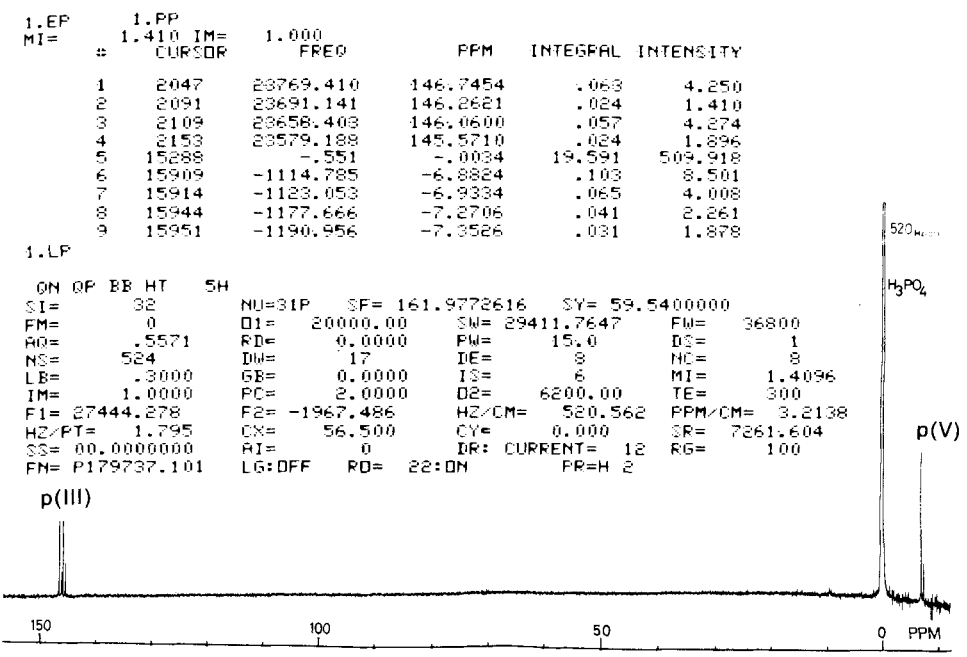


Fig. 1. *^{31}P-NMR spectrum of the completely protected dimer unit d(GC)*. The signals of the P(III) moiety as well as for the P(V) moiety show the appearance of diastereoisomeric forms of the molecule due to the asymmetric properties of the P-centers.

and extracted 6× with half-sat. and 2× with sat. NaCl soln. The org. layer was dried with Na_2SO_4 and evaporated. The residue was purified by short-column chromatography over 20 g of silica *H* with CH_2Cl_2/AcOEt/Et_3N (45:45:10 *(v/v)*). The fractions containing the pure product (TLC) were evaporated, taken up in toluol/AcOEt, and the product was precipitated with pentane, filtered, and dried. The yield ranged from 50 to 60%. The compounds were characterized by ^{1}H-NMR and ^{31}P-NMR *(Table 3)*. See also *Fig. 1.*

Syntheses of the Oligodeoxynucleotides. The syntheses were performed as mentioned above using the simple frit-system developed by *Caruthers* [1]. All solvents and reagents were added by *Pasteur* pipettes and removed by suction. For the coupling steps, the sintered-glass funnel was closed by a ruhher septum and kept under Ar, and the coupling soln. containing the corresponding monomer or dimer unit and tetrazole as activating agent in abs. MeCN was added with a syringe.

Deprotection and Purification. After completion of the synthesis, the support was washed with THF and Et_2O, dried, and treated with a mixture of thiophenol/dioxane/Et_3N 1:2:2 *(v/v)* for 2 h. Then, the mixture was filtered by suction into a soln. of I_2 in EtOH or 2.5% aq. NaOCl (*Javel* water). The support was washed with dioxane, MeOH, THF, and Et_2O and dried. Then 87.5 mg of (*Z*)-*o*-nitrobenzaldoximate and 67.7 µl of tetramethylguanidine in 1.5

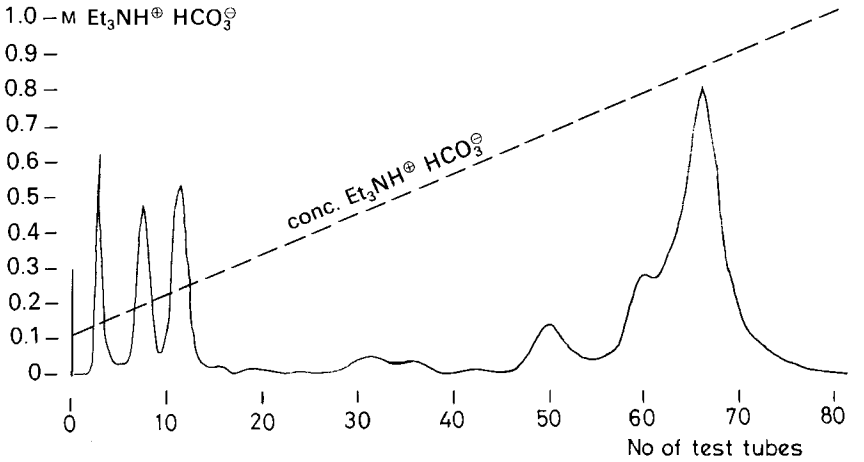


Fig. 2. *Purification of d(GAGAGAGAG) on DEAE-Sephadex.* The main peak containing the product was evaporated, and part of the material was purified further by reversed-phase HPLC or PAGE.

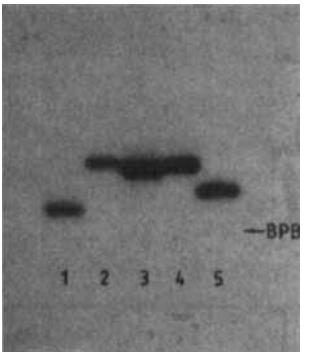


Fig. 3. *Autoradiograph of the purified oligomers after labelling with ^{32}P by T4-polynucleotide kinase.* The sequences of the oligomers were confirmed by the wandering-spot method of *Tu* and *Wu* [10].
1: d(CTAGGTAC); *2:* d(AGCTGGTACC); *3:* d(GAGAGAGAG); *4:* d(TTTTTAAAAA); *5:* d(TCTCTCTCT).

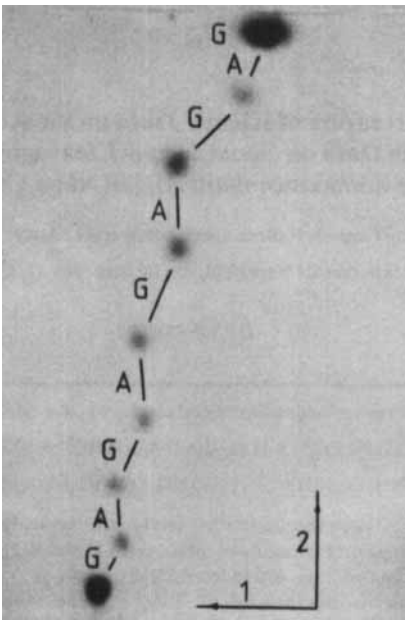


Fig. 4. *Sequence of the oligonucleotide d(GAGAGAGAG)*

ml of dioxan/H_2O 1:1 were added and reacted for 20 h. After filtration, the support was washed with 8 ml of pyridine/H_2O, and the filtrate was evaporated. The residue was taken up in 5 ml of NH_3 (conc.: min. 25%) and kept over the week-end in a tightly closed small flask. After evaporation, the residue was taken up in 4 ml of AcOH (80%) and reacted for 1 h, evaporated, taken up in H_2O and extracted several times with Et_2O. The H_2O layer was evaporated, the residue taken up in a little 0.1M $Et_3NH^{\oplus}$ $HCO_3^{\ominus}$, applied to a *DEAE-Sephadex* column and eluted with a linear gradient of $Et_3NH^{\oplus}$ $HCO_3^{\ominus}$ (500 ml of 0.1M $Et_3NH^{\oplus}$ $HCO_3^{\ominus}$/500 ml of 1.4M Et_3NHCO_3; see *Fig. 2*). The main peak containing the product was evaporated several times after addition of a little EtOH and part of it was then further purified by polyacrylamide-gel electrophoresis (PAGE) or reversed-phase HPLC. For the autoradiograph and an example of sequence analysis, see *Fig. 3* and *4*.

I would like to thank *B. Heierli, E. Küng,* and *M. Ott* for technical assistance, Dr. *D. Gillessen* for helping in the preparation, and Ms. *M. Pfendler* for typing the manuscript. Furthermore, I would like to thank our department of spectroscopy for the ^{31}P-NMR spectra.

REFERENCES

[1] 'Chemical and Enzymatic Synthesis of Gene Fragments', Eds. H. G. Gassen and A. Lang, Verlag Chemie, Weinheim, 1982.
[2] G. Kumar, M. S. Poonian, *J. Org. Chem.* **1984,** *49,* 4905.
[3] S. S. Jones, B. Rayner, C. B. Reese, A. Ubasawa, M. Ubasawa, *Tetrahedron* **1980,** *36,* 3975.
[4] S. de Bernardini, F. Waldmeier, Ch. Tamm, *Helv. Chim. Acta* **1981,** *64,* 2142.
[5] H. M. Hsiung, *Tetrahedron Lett.* **1981,** *23,* 5119.
[6] F. Chow, T. Kempe, G. Palm, *Nucleic Acids Res.* **1981,** *9,* 2807.
[7] R. Kierzek, H. Ito, R. Bhatt, K. Itakura, *Tetrahedron Lett.* **1982,** *22,* 3761.
[8] E. E. Van Tamelen, S. V. Daub, *J. Am. Chem. Soc.* **1977,** *99,* 3526.
[9] C. B. Reese, R. C. Titmas, L. Yau, *Tetrahedron Lett.* **1978,** *19,* 2727.
[10] C.-P. D. Tu, R. Wu, *Meth. Enzymol.* **1980,** *65,* 621.