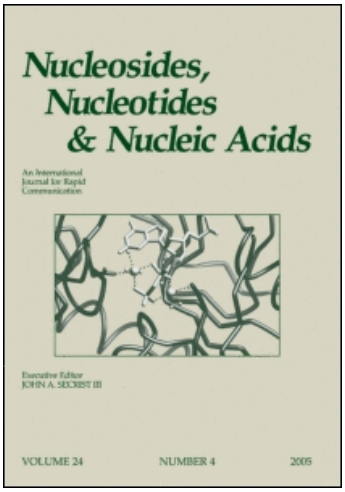

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Polymer Support Oligonucleotide Synthesis XX: Synthesis of a Henhectacosa Deoxynucleotide by Use of a Dimeric Phosphoramidite Synthon

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POLYMER SUPPORT OLIGONUCLEOTIDE SYNTHESIS XX[1]: SYNTHESIS OF A HENHECTACOSA DEOXYNUCLEOTIDE BY USE OF A DIMERIC PHOSPHORAMIDITE SYNTHON

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ABSTRACT

The dimeric phosphoramidite synthon (4) has been prepared by solution synthesis. Incorporation of (4) and the monomeric phosphoramidite (5) into a polymer supported synthesis on controlled pore glass (CPG) lead to the synthesis of a model oligodeoxynucleotide containing 101 nucleotide units.

INTRODUCTION

Chemical synthesis of long chain oligodeoxynucleotides remains still a difficult and time-consuming task although synthesis of oligomers containing 51 and 62 nucleotide units have been described in the literature[2,3].

Our present approach to oligodeoxynucleotide synthesis relies on the use of controlled pore glass (CPG) as silica based solid support[4,5]. Condensation reactions are performed following phosphoramidite chemistry[6]. The amidite synthons in use are 5'-O-(4,4'-dimethoxytrityl)-N-acyl-deoxynucleoside-3'- O^P-(2-cyanoethyl) -N,N-diisopropylphosphoramidites[1,7]. One cycle of nucleotide unit addition includes removal of 5'-O-dimethoxytrityl groups by protic acid treatment, condensation using p-nitrophenyltetrazole activated phosphoramidite units[8], capping and oxidation (Table 1).

The synthesis of long chains on a solid support requires many cycles to be performed during the assembly of

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one oligonucleotide, an approach which might lead to special problems.

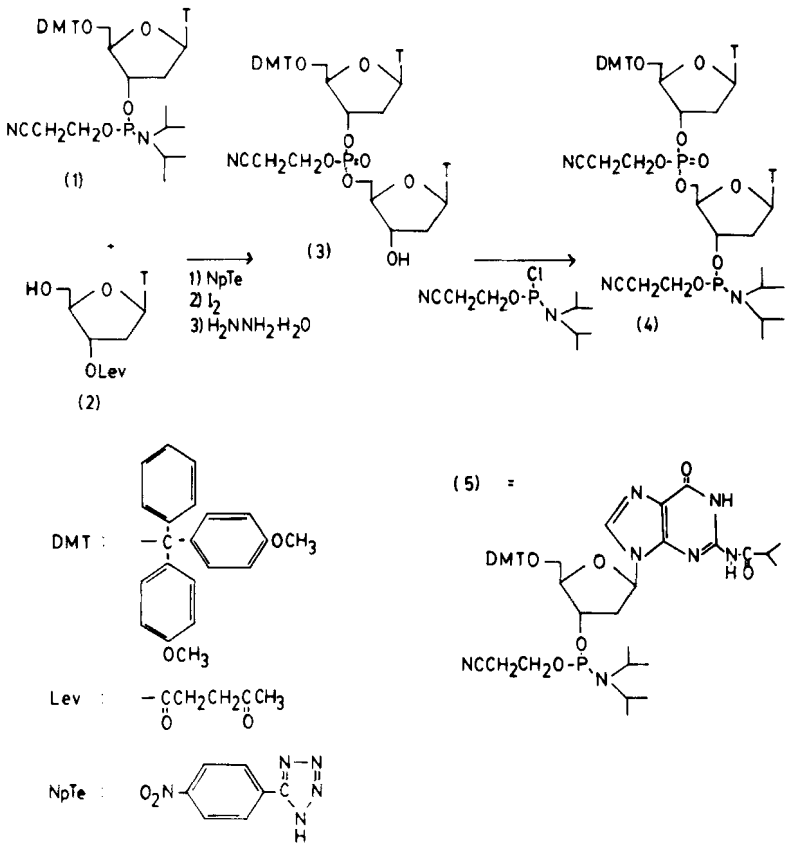
Although condensation reactions following phosphoramidite chemistry are normally high yield reactions (90-98%) accumulation of the effect of incomplete condensation may give rise to very low overall yields (100 condensations of 90% yield each would result in 0.003% overall yield, 95% each would give 0.6% overall yield). Detritylation and capping are reactions proceeding with nearly quantitative yields. However, traces of incomplete detritylation ($\lesssim 1\%$) could not be avoided in our hands (as controlled by dimethoxytrityl cation assay of detritylated material with stronger acids and prolonged reaction time). Accumulation of the effect of incomplete detritylation (and/or capping) results in contamination of the final product by failure sequences - shorter sequences bearing dimethoxytrityl groups which are not easily separable from the desired product by reversed phase HPLC. To reduce these expected problems we undertook the synthesis of a dimeric unit which would reduce the number of reaction cycles to 50%.

The superiority of the β-cyanoethyl phosphate protecting group for the synthesis of very long DNA sequences due to its efficient and highly selective removal has already been discussed [1a]. A chain scission during phosphate deprotection would also lead to DMT- containing truncated sequences which are very difficult to remove by reversed phase HPLC.

RESULTS AND DISCUSSION

In this paper the synthesis of an oligodeoxynucleotide containing 101 nucleotide units by our polymer support methodology using β-cyanoethyl phosphoramidite chemistry is described[1a,6].

The synthesis of the key intermeidate (4) is outlined in Scheme 1. The p-nitrophenyltetrazole mediated condensation reaction between (1) and (2) in the solution synthesis of the dimer (3) was complete within 2 minutes as confirmed by tlc. Experiments concerning the amount of p-nitrophenyl-tetrazole necessary to maintain high yields



Scheme 1. Synthesis of the dimeric phosphoramidite synthon (4).

in solution condensation reactions indicated the need of the presence of at minimum 1 equivalent of this activator. After oxidation by I_2 and work-up by aqueous extraction the crude mixture was subjected to delevulination. Quenching of the delevulination reaction after 6 minutes[9] was followed by stripping off 2/3 of the solvent under vacuum and direct application of the resulting mixture to a short and large diameter silica gel column. After prewash with $CHCl_3$ the product was eluted with a stepwise gradient of ethanol in $CHCl_3$ (7%, 10%, 0.5% pyridine was added to the elution medium in order to avoid dedimethoxytritylations). Pure (3) was obtained in 80% isolated yield. An important feature of the condensation reaction is the use of a very pure phosphoramidite (1). (1) and similar phosphoramidites could be purified by flash chromatography on silica gel[10]. Pur-

ified samples of (1) generally underwent nearly quantitative conversion to dinucleotides in solution at a nearly one to one stoichiometric ratio between phosphoramidite and OH-component (as confimed by tlc). (3) was phosphitylated by the usual procedure[1a,7] to give the dimeric phosphoramidite (4), which was obtained after purification by column chromatography on silica gel in 93% yield.

Another dimeric phosphite unit (of the chlorophosphite type) has recently been used in the synthesis of $d(Tp)_4T$ on a silica gel solid support[11]. During the preparation of this manuscript we became aware of a publication by Kumar and Poonian[12]. These workers described the synthesis of all 16 dimeric methylphosphoramidite synthons[13]. Machine-aided synthesis of oligodeoxynucleotides containing 16-29 base residues has been achieved with these synthons.

Chain assembly of the protected oligomer was performed by a fully automatic DNA-synthesizer[14] following the cycle outlined in Table 1.

The synthesizer allows addition of reagents and solvents by washing steps and recycling of reagents in a recycling loop. 53 reaction cycles had been performed with 22.5 mg of CPG-500 loaded with 5'-O-(4,4'-dimethoxytrityl) thymidine[5] (loading 31.2 µmole/g). The total time for the construction of the model sequence consisting of 101 nucleotide units was 48 hours. In order to facilitate characterisation of the product by Maxam-Gilbert sequence analysis six reaction cycles with the suitable protected deoxyguanosine-synthon (5)[1a,7] had been incorporated into the chain assembly:

$$d(T_{1-4}G_5T_{6-21}G_{22}T_{23-38}G_{39}T_{40-63}G_{64}T_{65-80}G_{81}T_{82-97}G_{98}T_{99-101}).$$

The 47 condensations using the TT phosphoramidite (4) required 3.0 g (2.71 mmoles) of the dimer.

Removal of the p-nitrophenyltetrazole, which is poorly soluble in acetonitrile, was best accomplished by employing acetone as washing medium after condensation. This solvent (dried over P_4O_{10} and distilled by use of a long Vigreux-column under argon) could also successfully substitute ace-

Table 1: Outline of Chain Elongation Cycle

Step	Manipulation	Reagent	Time (sec)
1	wash	detritylation reagent[1)]	450
2	wash	acetonitrile	300
3	wash	condensation reagent[2)]	90
4	recycle	-	70
5	wash	condensation reagent	60
6	recycle	-	70
7	wash	condensation reagent	60
8	recycle	-	70[5)]
9	wash	acetone	180
10	wash	capping reagent[3)]	120
11	recycle	-	90
12	wash	capping reagent	60
13	recycle	-	90
14	wash	capping reagent	60
15	recycle	-	90
16	wash	acetone	60
17	wash	oxidizing reagent[4)]	120
18	wash	acetone	210
19	wash	acetonitrile	90

1) Detritylation reagent: 3% TCA in $CH_2Cl_2/CH_3NO_2/CH_3OH$ 80/19/1 (V/V)
2) Condensation reagent: mixture of 0.1 M p-nitrophenyltetrazole in acetonitrile and 0.07 M phosphoramidite (4) or (5) in acetonitrile, 2/1 (V/V).
3) Capping reagent: mixture of a dimethylaminopyridine-solution in pyridine (300 mg in 45 ml) and acetic anhydride, 23/7 (V/V).
4) Oxidizing reagent: 0.1 M iodine solution in collidine/ THF/H_2O, 40/80/2 (V/V).
5) Chain elongation cycles 27-53 have been performed by introduction of additional steps. Cycle 27-41 steps 8a-b, Cycle 42-53 steps 8a-d:

8a	wash	condensation reagent	60
b	recycle	-	70
c	wash	condensation reagent	30
d	recycle	-	70

Thus the time for the condensation step of a cycle was increased during the chain assembly from 420 sec (cycles 1-26) to 550 sec (cycles 27-41) and 650 sec (cycles 42-53).

tonitrile as the condensation medium. The greater solubility of p-nitrophenyltetrazole in acetone (0.7 M solutions may be obtained easily) leads to shorter condensation times during monomer additions on a solid support. Condensation times of 2 minutes may be used with good yields when 0.33 M p-nitrophenyltetrazole in acetone is employed as condensation medium. Unfortunately, acetone exhibits no good solubility for the dimer (4); mixtures of acetonitrile and acetone

may be favourable to dissolve dimer blocks as well as p-nitrophenyltetrazole.

During the detritylation step the dimethoxytrityl cation is released from the support and may be collected for rough determination of the amount of dimethoxytrityl containing chains on the polymer formed during synthesis. Determination of condensation yields (by collection of detritylation washings and addition of 5% TCA in CH_2Cl_2 with ε_{DMT^+} = 71700) after 12 (0.557 µmoles $DMTr^+$), 26(0.386 µmoles), 39 (0.286 µmoles) and 52 cycles (0.214 µmoles) demonstrated tentative yields of 79, 55, 41 and 30% respectively (original loading of support 0.705 µmoles DMTr-groups).

At the end of the chain assembly the polymer was transferred to a glass reactor fitted with a frit, washed with acetone and dichloromethane and dried in vacuo. Dimethoxytrityl group assay (70% $HClO_4$/EtOH,3/2) showed 4.67 µmoles dimethoxytrityl groups per gram of polymer indicating a total yield of 15% over 53 condensation reactions (96.5% average per condensation)[15].

1.8 mg of the polymer was deprotected by incubation with 0.5 ml concentrated NH_3 (25%) for 72 hours at 60° C in an Eppendorf vial. After concentration of the supernatant, the material (12 OD) was dissolved in bidistilled water (2 ml) and filtered from some insoluble material (probably a polymer of acrylonitril) through a Sartorius membrane filter. After concentration the material was purified by reversed phase HPLC. Further purification was achieved by gel electrophoresis on a 10% polyacrylamide gel after labelling with γ-^{32}P ATP and polynucleotide kinase. Figure 1 shows an autoradiograph of a 10% polyacrylamide gel with samples of γ-^{32}P-labelled material without HPLC-separation, with HPLC-separation and after purification by gel electrophoresis. Maxam-Gilbert sequence analysis[16] of the gel purified fraction confirmed the sequence (Figure 2).

The described approach for the synthesis of long oligodeoxynucleotide chains relies on the favourable properties of our ß-cyanoethyl phosphoramidite synthons. Excellent stability of the amidite moiety under non-acidic conditions enabled us to produce very pure amidites by column chroma-

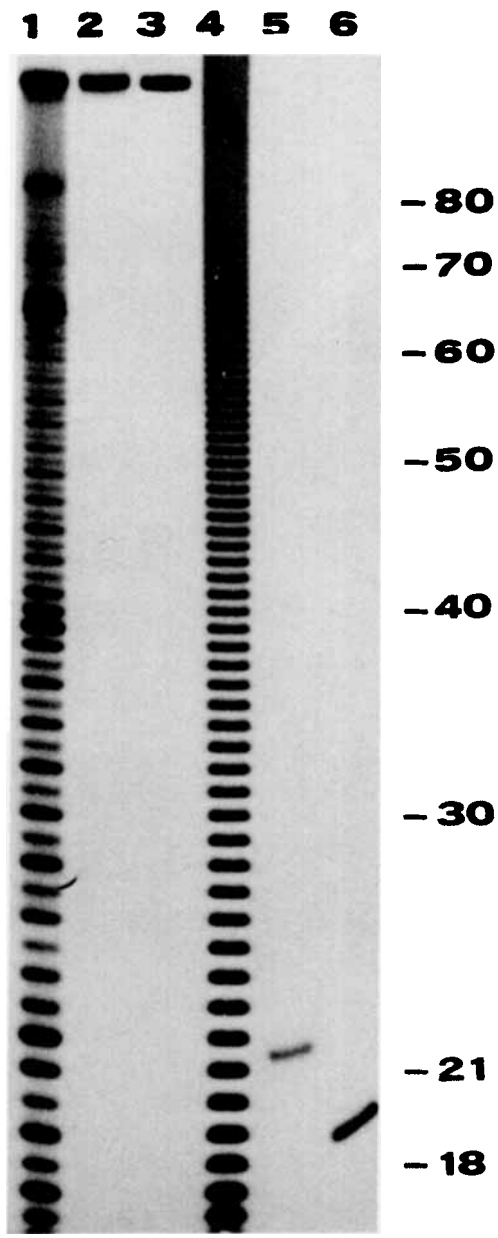


Figure 1: Electrophoresis of the 101-mer, $d(T_{(1-4)}G_5T_{(6-21)}G_{22}T_{(23-38)}G_{39}T_{(40-63)}G_{64}T_{(65-80)}G_{81}T_{(82-97)}G_{98}T_{(99-101)}$, on the 10% polyacrylamide gel after detritylation and labelling with ($-^{32}P$)ATP and T4 polynucleotide kinase. Lane 1: Material without HPLC purification. Lane 2: Material from the trityl positive peak after HPLC purification. Lane 3: Material purified by gel electrophoresis. Lane 4: Homo-oligo-dT chain length standard[17]. Lane 5 and 6: 21 and 18-mer as length standard respectively.

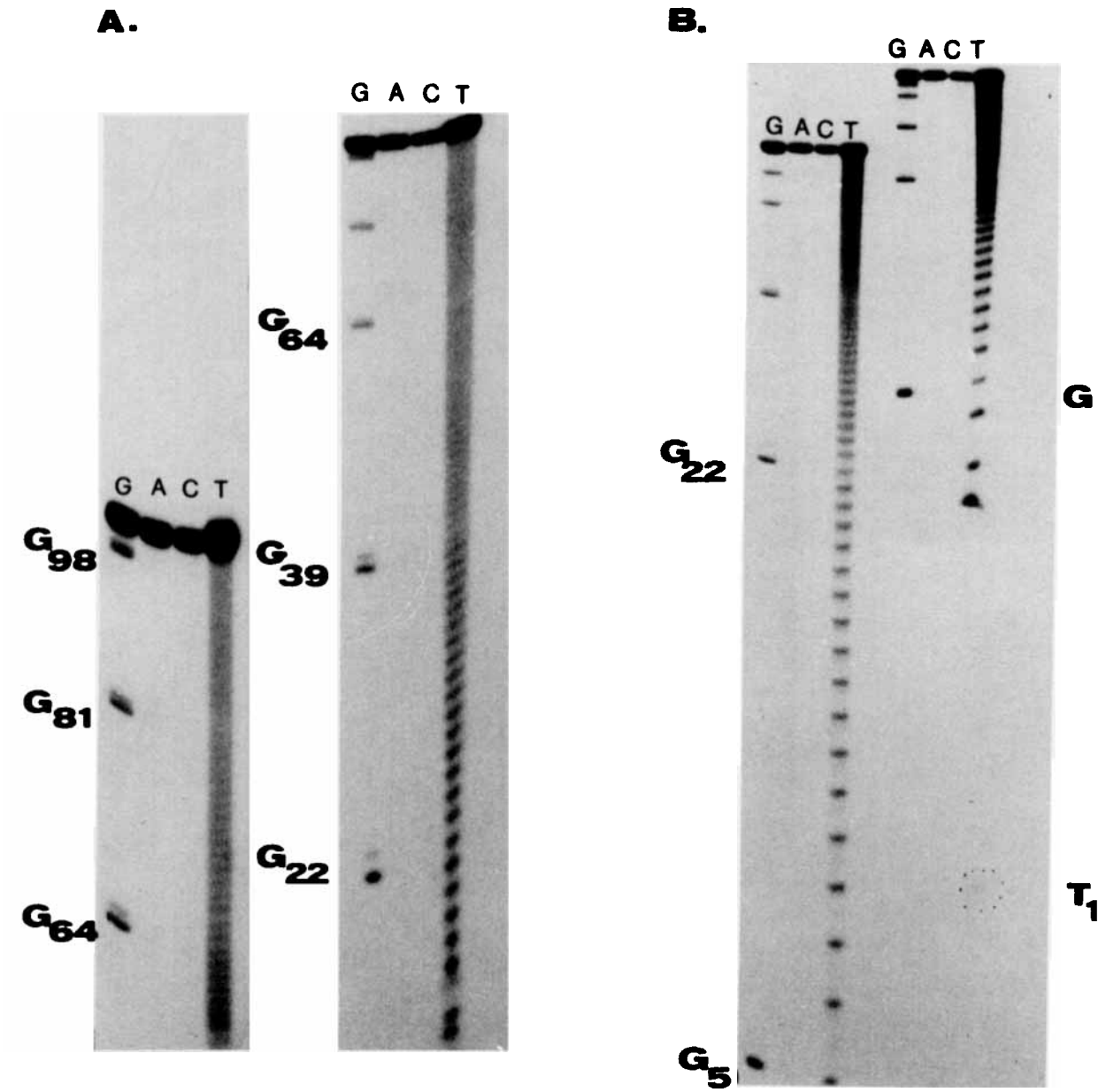


Figure 2: Sequencing of 101-mer $d(T_{(1-4)}G_5T_{(6-21)}G_{22}T_{(23-38)}G_{39}T_{(40-63)}G_{64}T_{(65-80)}G_{81}T_{(82-97)}G_{98}T_{(99-101)}$, according to Maxam-Gilbert[16].

A. Electrophoresis on 10% polyacrylamide gel. On the left side the xylene cyanol marker (XC) runs 38.5 cm, on the right side 19.5 cm.

B. Electrophoresis on 20% polyacrylamide gel. On the left side the bromophenol-blue marker (BPB) runs 28.5 cm, on the right side 9.5 cm.

tography. The pure amidites may be used with success in condensation reactions in solution at a nearly 1/1 stoichiometry with respect to OH component. Very high reactivity is exhibited in p-nitrophenyltetrazole containing solutions also at dimer level. Constant high condensation yields are ensured even in the assembyl of long oligodeoxynucleotides.

Very important for the successful synthesis of very long oligodeoxynucleotide sequences is the quantitative and regioselective removal of the phosphate protecting group after finalizing synthesis. In this respect, the ß-cyanoehtyl group apparently has very favourable properties.

Controlled pore glass serves as a highly reliable support material for long chain synthesis. The excellent properties of CPG are discussed elsewhere[4]. Another important impact in the course of this synthesis was the very high degree of reproducibility during the performance of reaction cycles by the automatic DNA-Synthesizer system used[14]. Coupling yields determined by estimation of dimethoxytrityl cation released from the polymer during detritylation steps showed a rather constant level (average 96-97% per step). The time saving properties of a machine-aided system is an additional favourable feature.

An alternative recently published concept of time saving for gene synthesis is the simultaneous synthesis of large number of medium chain length oligodeoxynucleotides on segmental solid supports[18]. In this approach the number of performed condensation reactions is drastically reduced. Nevertheless, the manual handling of the segmental support requires manwork and this does not ensure complete reproducibility during all performed reaction cycles.

Moreover, today it is a well-established experience that not oligodeoxynucleotide synthesis (chain assembly) but work-up, purification and sequencing is by far the most time-consuming part. Therefore, the most convincing strategy for the construction of long DNA duplexes is the fully automated synthesis of a few very long oligodeoxynucleotide sequences and subsequent filling in by DNA polymerase. Thus only a few sequences have to be worked up, purified and sequenced. These are operations which still have to be per-

formed manually, while the large number of identical nucleotide addition steps during chain assembly should favourably be undertaken by a highly reliable automatic DNA synthesizer.

The result of the study presented in this paper demonstrated that very long oligodeoxynucleotides can be obtained by the use of the dimeric ß-cyanoethyl phosphoramidite synthon (4). The good performance during synthesis of this model sequence suggests that oligonucleotides containing more than one hundred base residues may be obtained by application of this methodology.

EXPERIMENTAL

Commercial pyridine was distilled over tosylchloride, KOH and CaH_2. Acetonitrile was distilled over P_4O_{10}, CaH_2 and passed over basic Al_2O_3. THF was dried over Na/Benzophenone and distilled. Toluene, benzene and diisopropylethylamine were distilled from CaH_2. Ethylacetate was kept over molecular sieves (0.4 nm). Acetic anhydride was distilled over anhydrous sodiumacetate. p-Nitrophenyltetrazole[19] was crystallized from dioxane/petroleumether, 8/2. HPLC-purifications have been described previously[1,4].

Purification of phosphoamidite synthons (1) and (5) by column chromatography

The material obtained after reaction and aqueous extraction work-up[1a,7] on a 5 mmole-scale was dissolved in a small amount of ethylacetate/pyridine (19/1) and applied to a column of silica gel packed in the same solvent-mixture (100 g silica gel 60H Merck, column diameter 50 mm). Elution was performed using 1 l of the above solvent under 0.35 bar. After collection of a prefraction (220 ml) all further manipulations were performed under argon. 100 ml-fractions were analysed after evaporation by tlc (silica gel, solvent ethylacetate, diastereoisomers of (1) and (5) separate on tlc in this system). The product was usually obtained within the first 2 fractions. Pure fractions were combined and lyophilised from benzene. ^{31}P-NMR: (1): 148,5/148,3 ppm; (5): 148,3/148,1 ppm (diastereoisomers).

Preparation of 5'-O-(4,4'-dimethoxytrityl)thymidyl-3'-[O^{P}-(2-cyanoethyl)]5'-thymidine (3)

8.3 mmole phosphoamidite (1) (6.16 g) were placed in a 100 ml round bottomed flask with a side arm under argon. A mixture of 7.5 mmole 3'O-laevulinyl-thymidine (2.60 g) and 7.58 mmole p-nitrophenyltetrazole (1.44 g) was dried by co-evaporation with small amounts of a) pyridine, b) toluene and c) acetonitrile, dissolved in 60 ml acetonitrile with gentle heating and transferred to the phosphoamidite under argon. The reaction was monitored by tlc (silica gel, solvent $CHCl_3$/methanol 9/1) indicating complete reaction within 2 minutes[20]. After 30 minutes iodine solution (9 mmoles I_2 in 30 ml $CH_3CN/Py/H_2O$, 24/5/1) was added. The reaction mixture was stirred for 5 minutes and the iodine colour was quenched by addition of 0.5 ml 40% aqueous $NaHSO_3$ - solution. After evaporation of most of the acetonitrile in vacuo the material was dissolved in 250 ml $CHCl_3$. The $CHCl_3$- layer was washed twice with 150 ml 1 M $NaHCO_3$-solution. The aqueous layers were combined, saturated with NaCl and re-extracted four times with 75 ml $CHCl_3$ each (some precipitated p-nitrophenyltetrazole was filtered off). The combined $CHCl_3$-layers were dried (Na_2SO_4), filtered and evaporated in vacuo. The material was dissolved in 75 ml pyridine and 75 ml of 1 M hydrazine hydrate solution in pyridine/acetic acid (3/2) was added. After 6 minutes the reaction flask was cooled in an ice-bath and 15 ml acetylacetone were added. The reaction was concentrated in vacuo to a volume of 50 ml and then directly applied to a silica gel column (250 g silica gel 60H, Merck; column diameter 90 mm). The elution medium contained 0.5% pyridine and consisted of $CHCl_3$ (0.7 l) and $CHCl_3$/ethanole-mixtures (1.5 l 93/7; 2 l 90/10). Fractions containing pure material were pooled and 4.07 g (3) were obtained after evaporation and lyophilisation from dioxane as white powder. Some fractions containing impure material had been rechromatographed to give a second crop of 1.36 g pure material (total 80%)
^{31}P-NMR: -2,2 ppm (diastereoisomers not resolved).

Preparation of 5'O-(4,4'-dimethoxytrityl)thymidyl-3'-[O-(2-cyanoethyl)]-5'-thymidine-3'-O^{P}-[(2-cyanoethyl)]-N,N-diisopropylphosphoramidite (4)

5.40 g (3) (6.0 mmole) were phosphitylated using 24 mmole diisopropylethylamine and 12 mmole chloro-2-cyanoethoxy-N,N-diisopropylamino phosphane in 45 ml THF as previously described[1a,7]. The reaction was monitored by tlc (silica gel, solvent THF/ethylacetate, 3/1). Column chromatography was performed as described above using 130 g silica gel and THF/ethylacetate/pyridine (47.5/47.5/5) as elution medium. 5.02 g of (4) were obtained after lyophilisation from benzene (76%)[21]. ^{31}P-NMR: 158.8/148.1 ppm (1 P: P^{III}, diastereoisomeric compounds partially resolved); -2,4 ppm (1 P: P^{V}, no resolution of diastereoisomers).

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21) In a second run, starting from 3.5 mmole (3), 93% of purified (4) have been isolated.

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