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SYNTHESIS OF FUNCTIONALIZED DNA FRAGMENTS SUITABLE FOR REVERSIBLE ATTACHMENT TO ACTIVATED CELLULOSE†

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Abstract—A system consisting of donor (1), template (2) and immobilized acceptor molecules (**58**, **59**, **64–67**) which may be used for an enzymatic extension (T4 DNA ligase) of immobilized DNA fragments is described. The synthesis of the donor (1), template (2) and the functionalized acceptor molecules (**3–8**) was performed via phosphotriester intermediates. The functionalized acceptor molecules used in this study contained: base labile bonds (i.e. compounds **3–6**), an acid labile bond (i.e. compound **7**) or a RNase labile bond (i.e. compound **8**). The functionalized acceptor molecules (**3**,**4**) could be immobilized to cellulose activated with 2, 4, 6-trichloro-s-triazine and the other molecules (**5–8**) to 2-amino-4, 6-dichloro-s-triazine to give the immobilized DNA complexes **58**, **59** and **64–67**, respectively, in high yield. The immobilized DNA fragments could be released quantitatively from the solid support by: base treatment (i.e. **58**, **59**, **64** and **65** gave DNA-fragments **60**, **61**, **68** and **69** respectively), acid treatment (i.e. **66** gave DNA-fragment **70**) or RNase digestion (i.e. **67** gave deoxythymidine).

It is well recognized now that nucleic acids covalently linked to a solid support (e.g. cellulose or agarose) find an ever increasing use in molecular biology. For instance, these polymeric nucleic acid complexes have been applied successfully in isolation and purification of enzymes or nucleic acids,[1] and to examine the interaction between nucleic acids[2–4] or enzymes.[5–8]

However, up to now, no approach to the synthesis of immobilized nucleic acids which entails the following combined elements has been proposed: (i) synthesis, via phosphotriester intermediates, of nucleic acids which are functionalized at the 5′-position with base or acid labile bonds (temporary linkage, e.g. compounds **3–6** and **7**, respectively, in Fig. 2) or having a stable bond (persistent linkage) but containing a specific site which may be cleaved selectively by an enzyme (e.g. **8** in Fig. 2); (ii) linking of these nucleic acids to preactivated solid particles; (iii) enzymatic extension of the immobilized nucleic acids with other nucleic acid fragments designed for this purpose.

This approach has two advantages; firstly, well defined and immobilized nucleic acids may be obtained and, secondly, selective cleavage of the temporary linkage with solid particles may afford nucleic acids with a length which is not attainable by using existing methods devised for the chemical synthesis of nucleic acids.

In this paper we wish to report on the immobilization via temporary and persistent linkages of synthetically prepared DNA fragments (e.g. **3–8** in Fig. 2) on preactivated cellulose.

The results of the studies concerning the enzymatic extension of the immobilized DNA fragments using T4 DNA ligase will be published elsewhere.

RESULTS AND DISCUSSION

Enzymatic joining of chemically prepared oligonucleotides has been proven to be very successful for the synthesis of relatively large DNA fragments.[9–12] The enzymatic reaction involves a T4 DNA ligase [5,13,14] catalysed formation of a 3′–5′ phosphodiester bond between an oligonucleotide with a terminal 3′-OH (acceptor) and an oligomer with a terminal 5′-phosphate (donor). This enzymatic joining requires that the two segments are held in juxtaposition by a complementary strand (template) by Watson–Crick base-pairs. It has also been reported[15–17] that DNA ligase of phage T4 joins fully base-paired termini of bihelical DNA (blunt end joining).

The model system we set up to pursue the possibility of synthesizing immobilized DNA fragments by T4 DNA ligase catalysis is outlined in Fig. 1. The 5′-phosphate of the decamer of deoxythymidine was destined to function as the donor molecule (1). In order to prevent polymerisation of the donor the 3′-OH was protected with the acid labile methoxytetrahydropyranyl (MTHP) group.[18] As acceptor molecules we chose oligothymidylic acids of different length and with three different functionalizing groups at the 5′-position (e.g. **3–8** in Fig. 2) which were immobilized on activated cellulose. For the template we constructed, because of the fact that the donor and acceptor molecules only consisted of deoxythymidine units, an oligomer of deoxyadenosine.

In order to obtain immobilized acceptor molecules which, after enzymatic extension with the donor, could be removed quantitatively from the solid support to give the free DNA fragments, we prepared three different functionalized thymidylic acids (Fig. 2): (a) one set of molecules which contain a 5′-O-β-alanine ester (**3–6**; n = 5, 8, 11, 14); (b) a thymidine nonamer with an acid

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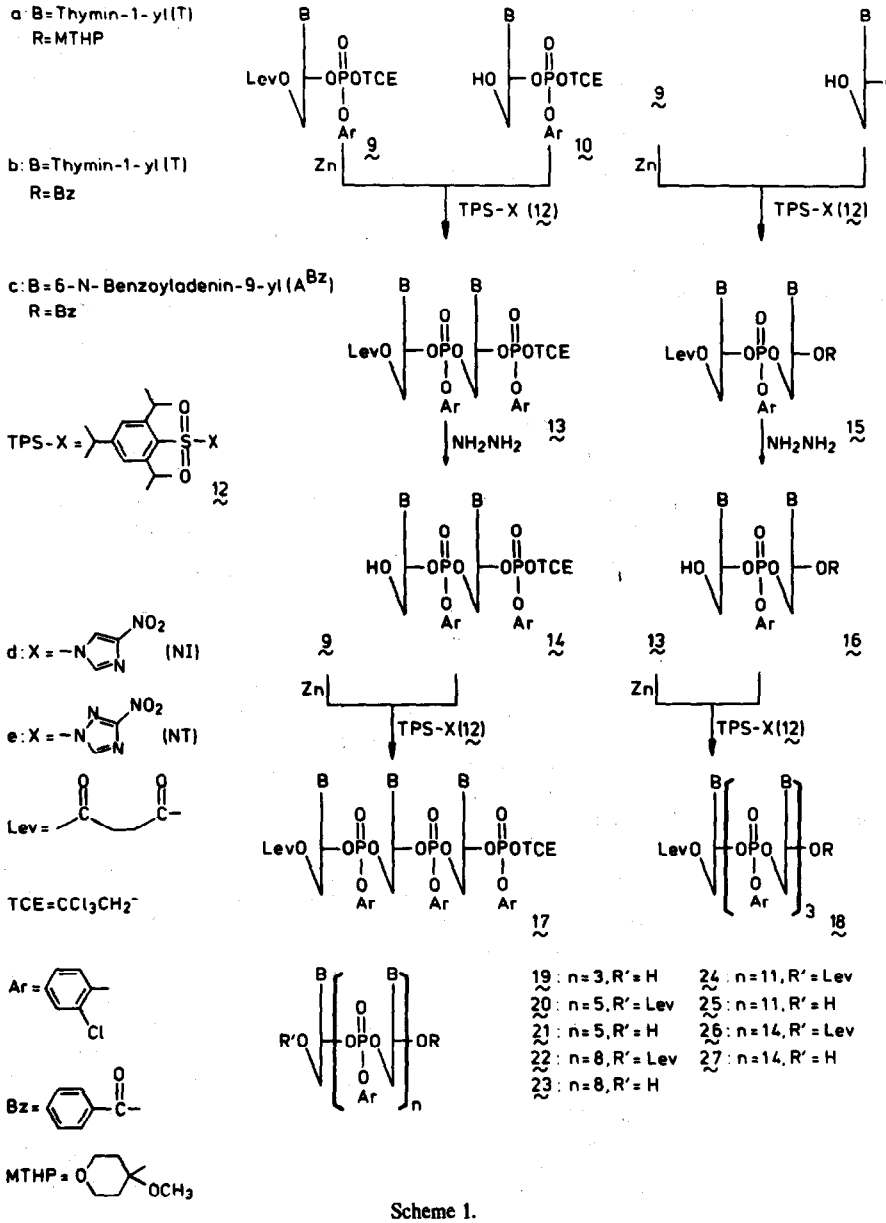
labile 5'-phosphoro 4-aminobenzylamidate and finally a 5'-amino-5'-deoxy-tridecanucleotide (**8**) consisting of twelve deoxythymidine units and one uridine which creates by the presence of a 2'-hydroxyl group a RNase labile bond.

The strategy we followed to prepare the required molecules (i.e. **1–8** in Fig. 1 and 2) implied the following: (a) synthesis of the protected oligodeoxynucleotides **13–27** (Scheme 1) which serve as building blocks for the preparation of donor (**1**), template (**2**) and acceptor molecules (**3–8**); (b) preparation of a fully protected 5'-phosphate of a decanucleotide (**30**) which after partial deblocking afforded the donor **1** (Scheme 2); (c) deblocking of the fully protected dodecamer **24c** (see Scheme 1) to give the template **2**; (d) synthesis of the fully protected oligonucleotides **31–34** which gave after deblocking the base labile acceptor molecules **3–6** (Scheme 3); (e) preparation of the fully protected nonanucleotide **43** which, after deblocking, afforded the acid labile acceptor (**7**) (Scheme 4); (f) synthesis of the fully protected tridecamer **54** which, after deblocking, gave the RNase labile acceptor **8** (Scheme 5); (g) immobilisation of the functionalized acceptor molecules **3–8** with 2, 4, 6-trichloro-s-triazine activated cellulose (**57**, Scheme 6) or 2-amino-4, 6-dichloro-s-triazine activated cellulose (**63**, Scheme 7).

Synthesis of the protected oligonucleotides **13–27** (Scheme 1). Three different types of protected oligonucleotides were required as building blocks in the synthesis of oligonucleotides **1–8**: (i) four partially protected oligothymidylic acids with a 3'-terminal methoxytetrahydropyranyl group (**21a**, **23a**, **25a** and **27a**) which served as precursors for the donor **1** and the base labile acceptor molecules **3–6**; (ii) a partially protected hexamer of deoxythymidine with a 3'-terminal benzoyl group (**21b**) which functioned as a building block for the synthesis of



Scheme 1.

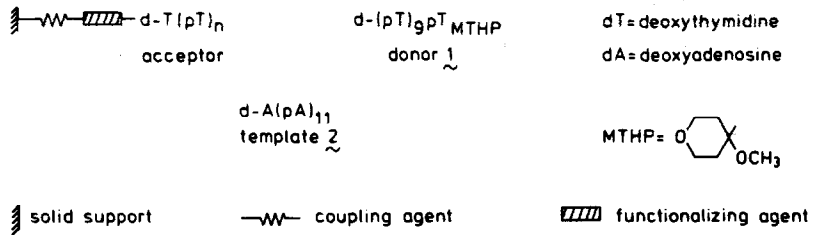


Fig. 1.

the acid labile acceptor **7**; (iii) a fully protected dodecamer of deoxyadenosine (**24c**) destined as precursor for the template **2**.

The synthesis of oligonucleotides **13–27** as outlined in Scheme 1 was accomplished according to a modified phosphotriester approach.[19,20] In this strategy the oligonucleotides **13–27** were constructed starting from the three monomeric units[20] **9**, **10** and **11**. The 3'-terminal unit **11** was protected at the 3'-OH with the acid labile methoxytetrahydropyranyl group in case of **11a**[21] or the base labile benzoyl group in case of **11b** and **11c**[20].

In the condensation reactions two activating agents were used, 2, 4, 6-triisopropylbenzenesulphonyl-4-nitroimidazole[22] (TPS-NI, **12d**) or 2, 4, 6-triisopropylbenzenesulphonyl-3-nitro-1, 2, 4-triazole[20] (TPS-NT, **12e**).

Thus, monomer **9** (B = T) was treated with zinc and 2, 4, 6-triisopropylbenzenesulphonic acid in pyridine[19,22] to remove the 2, 2, 2-trichloroethyl group. The resulting phosphodiester was condensed with the 3'-terminal unit **11b** using TPS-NT (**12e**) as the activating agent. Work-up of the reaction mixture after 1 hr, and purification of the crude product by short column chromatography[23] afforded dimer **15b** in 97% yield. Dimer **13** (B = T) was prepared analogously as described for the synthesis of the dimer **15b** in 88% yield.

Block condensation of the oligonucleotides mentioned in Scheme 1 was performed according to a general four-step cycle which comprised of: (i) removal of the levulinyl group from dinucleotide **15b** by hydrazine-treatment[24] to afford the 5'-hydroxy component **16b**; (ii) reductive cleavage of the 2, 2, 2-trichloroethyl group from dimer **13** (B = T) by Zn-treatment to produce a 3'-phosphodiester; (iii) condensation of the phosphodiester obtained from dimer **13** (B = T) with the 5-hydroxy-component **16b** using TPS-NT (**12e**) as activating agent to give the fully protected tetramer **18b**; (iv) purification of the condensation product (**18b**) by short column chromatography.[23] Tetranucleotide **18b** was isolated in 96% yield.

The tetranucleotides **18a** and **18c** were prepared according to the same procedure. One four-step cycle, starting from the two dimers **13** (B = T, or A^{Bz}) and the three tetramers **18** (**a–c**), afforded the hexamers **20** (**a–c**). Hydrazine-treatment of the hexanucleotides **20a** and **20b** gave two of the required products: **21a** and **21b**.

The trinucleotides **17a** and **17c** were prepared by one four-step cycle starting from the corresponding monomers **9** (**a**, **c**) and dimers **13** (**a**, **c**). The required oligonucleotides **23a**, **25a** and **27a** could now be obtained by elongation of hexamer **21a** at the 5'-end with trimer **17a** in respectively one, two and three four-step cycles followed by a final hydrazine treatment.

The only oligonucleotide which remained to be prepared was the fully protected template precursor d-$A(pA)_{11}$ (**24c** in Scheme 1). This molecule could easily be obtained starting from hexamer **20c** and trimer **17c** in two successive four-step cycles. Data relevant to the synthesis of oligonucleotides **21a**, **b**, **23a**, **25a**, **27a** and **24c** are summarized in Table 1.

It is interesting to note (see Table 1) that the yield of the intermediates (**17c**, **18c**, **20c**, **22c** and **24c**) necessary for the synthesis of the template d-$A(pA)_{11}$ decreased with growing chain length. This phenomenon can be attributed to the loss of nucleotide material during purification of the crude condensation products by short column chromatography on silica gel. For instance, only a relatively small quantity of crude dodecamer **24c**, applied on a column of Kieselgel H, could be isolated. The recovery of the required product could not be raised by addition of water or excessive methanol to the eluting solvent. However, we are confident that application of gel permeation chromatography on Sephadex LH60[25] for the purification of relatively long oligonucleotides will solve this problem.

Preparation of the donor d-$(pT)_9pT_{MTHP}$ (**1**, Scheme 2). The crucial step in the synthesis of the fully protected donor **1** is the introduction of a 5'-terminal phosphate. To attain our goal we adopted a method, published by Smrt,[26] which implies the use of the monofunctional phosphorylating agent dianilido phosphorochloridate[27] (**28**, Scheme 2). However, phosphorochloridate **28** was not reactive enough to introduce directly a 5'-terminal phosphate on a relatively large oligonucleotide (e.g. **23a**). For this reason we followed the route outlined in Scheme 2.

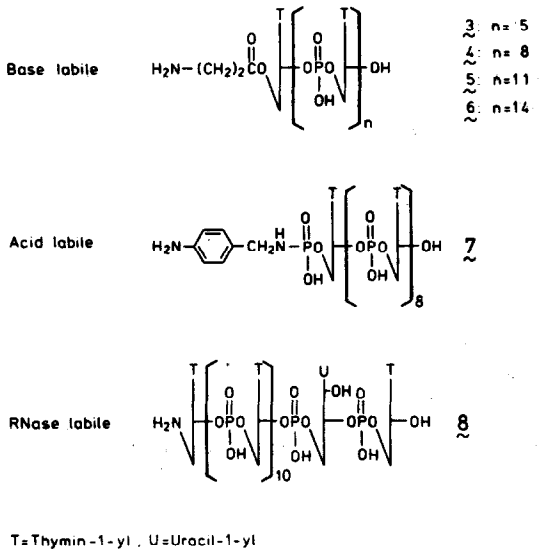
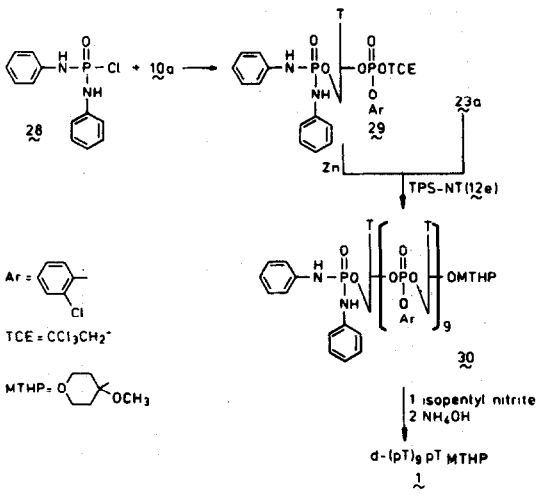


Fig. 2. Functionalized acceptor molecules.

Table 1. Relevant data on the synthesis of oligonucleotides in Scheme 1.

3'-Phosph. component		5'-Hydroxy component		Activating agent		Reaction time	Product			Delevulination product		
No.	mmole	No.	mmole	No.	mmole	(hours)	No.	yield[a]	Rf[b]	No.	yield	Rf[b]
9(B=T)	4.50	10(B=T)	4.10	12e	5.00	1	13(B=T)	88%	0.47	14(B=T)	93%	0.34
9(B=T)	1.40	11a	1.30	12d	1.50	20	15a	92%	0.44	16a	95%	0.27
9(B=T)	0.88	14a	0.80	12e	1.00	1.5	17a	84%	0.38			
13(B=T)	1.10	16a	1.00	12d	1.20	20	18a	92%	0.29	19a	98%	0.17
13(B=T)	0.47	19a	0.36	12d	0.52	30	20a	72%	0.16	21a	90%	0.12
17a	0.45	21a	0.30	12d	0.50	48	22a	69%	0.13	23a	98%	0.08
17a	0.06	23a	0.04	12e	0.07	2	24a	83%	0.10	25a	94%	0.06
17a	0.03	25a	0.018	12e	0.04	3	26a	89%	0.06	27a	94%	0.04
9(B=T)	3.30	11b	3.00	12e	3.6	1	15b	97%	0.46	16b	90%	0.30
13(B=T)	1.15	16b	1.05	12e	1.3	1.5	18b	96%	0.31	19b	97%	0.19
13(B=T)	1.10	19b	0.95	12e	1.3	1.5	20b	89%	0.20	21b	95%	0.14
9c	6.40	10c	6.00	12e	7.0	1	13c	97%	0.54	14c	90%	0.47
9c	1.45	11c	1.30	12e	1.6	1	15c	91%	0.52	16c	95%	0.46
9c	1.50	14c	1.30	12e	1.65	1.5	17c	76%	0.45			
13c	1.25	16c	1.03	12e	1.4	1	18c	74%	0.40	19c	91%	0.33
13c	0.75	19c	0.65	12e	0.9	2.5	20c	60%	0.30	21c	92%	0.26
17c	0.375	21c	0.25	12e	0.45	3	22c	60%	0.21	23c	89%	0.18
17c	0.09	23c	0.055	12e	0.1	3	24c	29%	0.15			

[a]Based on 5'-hydroxy component.
[b]System A.



Scheme 2.

Thus, phosphorylation of mononucleotide **10a** with dianilido phosphorochloridate **28** in pyridine gave deoxythymidine-5'-phosphorodianilidate 3'(2, 2, 2-trichloroethyl 2-chlorophenyl) phosphate (**29**) in 83% yield. Cleavage of the 2, 2, 2-trichloroethyl group from **29** by Zn-treatment and condensation of the resulting phosphodiester with nonanucleotide **23a** using TPS-NT (**12e**) as activating agent afforded the fully protected decamer **30** in 86% yield.

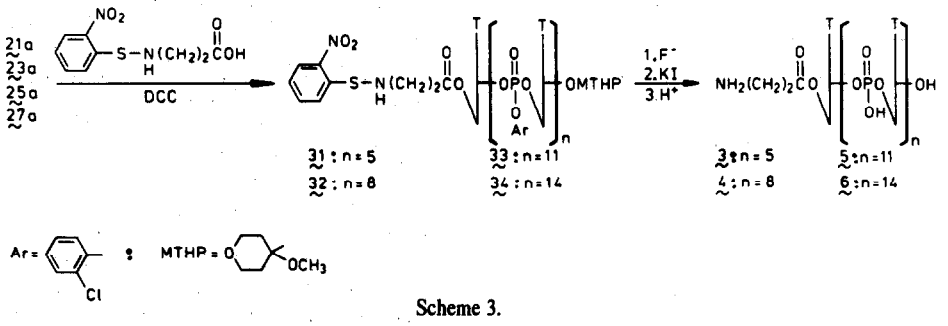
To obtain donor molecule **1** all protecting groups, except the 3'-terminal methoxytetrahydropyranyl group had to be cleaved from the fully protected decamer **30**. In the first step the terminal 5'-phosphate was deblocked by treating **30** with isopentyl nitrite in pyridine-acetic acid (1:1 v/v)[28] for 24 h. The second step consisted of the removal of the 2-chlorophenyl groups with 25% aqueous ammonia, followed by purification of the crude decamer on Sephadex G50.

The yield of the deblocking and purification process was established spectrophotometrically and the value thus obtained (570 OD_{max}) was converted into μmoles using the extinction coefficient ($\epsilon_{max} = 86{,}000$) published by Cassani and Bollum.[29] This procedure to establish the yield of a deprotected oligonucleotide was followed throughout this study.

Donor molecule **1** was isolated in 66% yield and the purity and identity of the decanucleotide was established by hplc analysis (system B). The presence of a terminal 5'-phosphate was demonstrated by the susceptibility of **1** to alkaline phosphatase. Hplc analysis (system C) of the venom phosphodiesterase digestion of **1** afforded pdT and pdT_{MTHP} in the correct ratio.

Preparation of the template d-A(pA)$_{11}$ (**2**, in Fig. 1). To acquire the template **2** fully protected d-A(pA)$_{11}$ (**24c** in Scheme 1) had to be deblocked. As reported previously[20] dodecamer **24c** was treated with 0.05 M tetrabutylammonium fluoride[30] (TBAF) in tetrahydrofuran-pyridine-water (8:1:1 v/v) to remove the 2-chlorophenyl groups. The remaining protecting groups, benzoyl and levulinyl, were cleaved with aqueous ammonia (25%, w/w). The crude oligonucleotide **2** thus obtained was purified on Sephadex G50 and isolated in 62% yield. The identity and purity of dodecamer **2** was confirmed by hplc analysis (system B) and by digestion with venom phosphodiesterase and spleen phosphodiesterase.

Preparation of the base labile acceptor molecules **3–6** (Scheme 3). In order to obtain oligonucleotides, which could be immobilized on cellulose via a base labile linkage, we esterified four partially protected oligothymidylic acids of different length (**21a**, **23a**, **25a** and **27a**)



Scheme 3.

with β-alanine (see Scheme 3). The amino function of β-alanine was protected with the 2-nitrophenylsulphenyl group[31] which has the advantage that it can be cleaved under very mild conditions[32]: potassium iodide in methanol-acetic acid (9:1, v/v) for 5 min.

Thus, treatment of oligonucleotides **21a**, **23a**, **25a** and **27a** with N-2-nitrophenylsulphenyl-β-alanine,[33] dicyclohexylcarbodiimide and N-dimethylaminopyridine[34] in dioxan gave the fully protected functionalized oligomers **31–34** which were isolated, after short column chromatography, in good yields (Table 2).

Deblocking of oligonucleotides **31–34** was performed in three stages. Firstly, the internucleotide phosphotriesters in oligomers **31–34** were hydrolysed, without affecting the 5'-β-alanine ester, by fluoride ion treatment using 0.05 M TBAF in tetrahydrofuran-pyridine-water (8:1:1, v/v).

Both remaining protecting groups (methoxytetrahydropyranyl and 2-nitrophenylsulphenyl) are acid labile. However, we decided, because of the relative stability of the 2-nitrophenylsulphenyl group, to cleave the latter with potassium iodide in methanol-acetic acid (9:1, v/v). A mild acid treatment (0.01 N HCl for 2 hr), to remove the methoxytetrahydropyranyl group, and purification of the completely deblocked product on Sephadex G50 afforded the base labile acceptor molecules **3–6** in good yield (Table 2).

It is worthwhile mentioning that the 5'-(2-nitrophenylsulphenyl)-β-alanine ester was much more stable towards base ($t_{1/2}$ in 12.5% aqueous ammonia 2 hr) than the unprotected 5'-β-alanine ester which was hydrolysed within a few hours at pH 8.0.

The purity of the base labile acceptor molecules **3–6** was verified by hplc analysis (system B, Table 2). Treatment of oligomers **3–6** with 25% aqueous ammonia afforded quantitatively the corresponding oligothymidylic acids d-T(pT)$_n$ (n = 5, 8, 11, 14) as was established by hplc analysis (system B, Table 2).

Preparation of the acid labile acceptor molecule **7** (Scheme 4). In nonanucleotide **7** (Fig. 2) the phosphoramidate bond is susceptible to acid. The rate of acidic hydrolysis of nucleoside phosphoramidates has been reported[35,36] to be dependent on the N-substitution. Because of the pronounced acid lability of the glycosidic bond in deoxynucleosides,[37] especially in case of deoxyadenosine and deoxyguanosine, we chose a system which contained the relatively labile phosphorobenzylamidate[38] function. The amino group at the para position of the phosphorobenzylamidate function allows immobilisation of the nonamer **7** on activated cellulose.

The other phosphate function of the 5'-phosphoro-4-aminobenzylamidate in the fully protected acid labile acceptor **43** (Scheme 4) was protected with a 2, 4-dichlorophenyl group. The choice of a 2, 4-dichlorophenyl was based upon the observation that the removal of a 2-chlorophenyl group from a nucleoside aryl phosphoramidate by fluoride ion was extremely slow. However, we found that the 2, 4-dichlorophenyl group could be removed fast and selectively from a fully protected oligonucleotide 5-phosphoro-4-aminobenzylamidate (e.g. **43**) by fluoride ion treatment.

The preparation of the acid labile acceptor molecule **7** can be divided into three stages (Scheme 4): (i) synthesis of the properly protected nucleoside 5'-aryl phosphoroamidate **39**; (ii) preparation of the fully protected nonamer **43**; (iii) deblocking of **43** to afford the acid labile acceptor **7**.

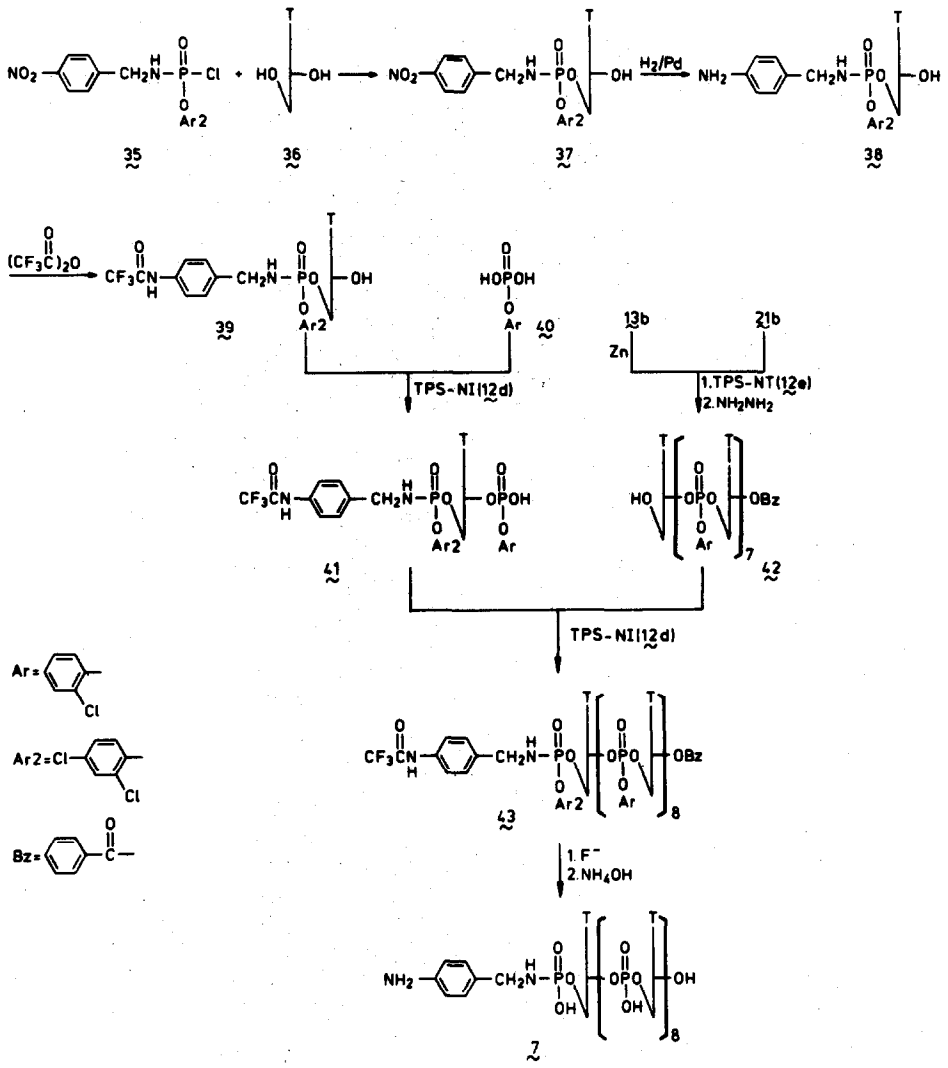
Introduction of a precursor of the 5'-phosphoro-4-aminobenzylamidate was accomplished using the monofunctional phosphorylating agent 2, 4-dichlorophenyl phosphoro-4-nitrobenzylamidochloridate (**35**) which was obtained as a pure solid from equimolar amounts of 2,-4-dichlorophenyl phosphorodichloridate, 4-nitrobenzylamine[39] and triethylamine.

Thus, treatment of deoxythymidine (**36**) with phosphorochloridate **35** in pyridine afforded deoxythymidine-

Table 2. Preparation of 5'-O-β-alanine acceptor molecules 3, 4, 5 and 6

Synthesis of the fully protected oligomers					Deprotection			Ammonolysis	
5'-Hydoxy oligomer		Product							
No.	mmole	No.	yield	Rf[a]	No.	yield	Rt(min)[b]	Products	Rt(min)[b]
21a	0.10	31	65%	0.18	3	82%	7.5	d-T(pT)$_5$	12.4
23a	0.05	32	61%	0.11	4	69%	11.0	d-T(pT)$_8$	15.1
25a	0.01	33	78%	0.08	5	50%	14.4	d-T(pT)$_{11}$	18.0
27a	0.015	34	62%	0.05	6	62%	17.6	d-T(pT)$_{14}$	20.7

[a]System A.
[b]System B.



Scheme 4.

5′ 2, 4-dichlorophenyl phosphoro-4-nitrobenzylamidate (**37**) in 79% yield. The nitro function in mononucleotide **37** was converted quantitatively into an amino group by reduction with hydrogen and Pd–C to give monomer **38**. The free amino function thus obtained was protected with the trifluoroacetyl group to avoid side reactions in the following phosphorylation step. This amino protecting group could easily be introduced by treatment of monomer **38** with trifluoroacetic anhydride to afford mononucleotide **39** in 75% yield.

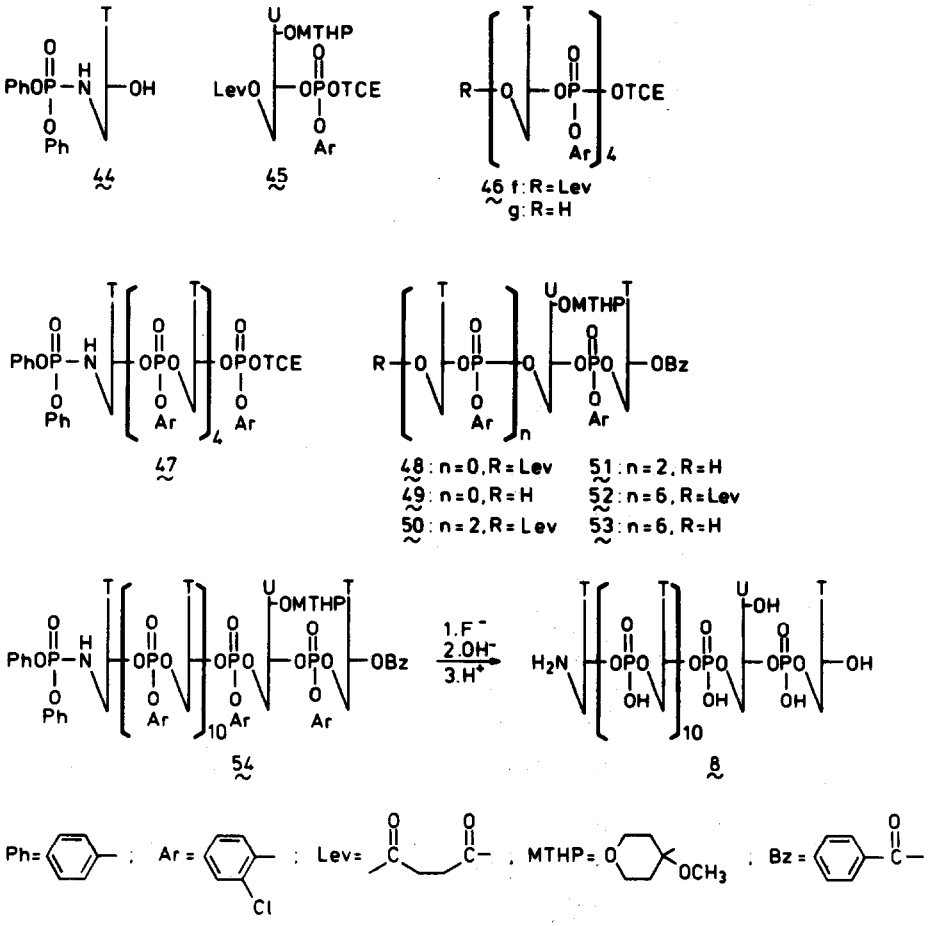
Octanucleotide **42** was obtained after Zn-treatment of dimer **13b** (Scheme 1) and condensation of the resulting phosphodiester with hexanucleotide **21b** (Scheme 1) using TPS-NT (**12e**) as the activating agent. Hydrazinolysis of the fully protected octamer gave the required compound in an overall yield of 83%.

Mononucleotide **39** was phosphorylated at the 3′-hydroxyl with 2-chlorophenyl phosphate[40] (**40**) in the presence of TPS-NI (**12d**). After 16 hr, the mixture was diluted with chloroform and the solution was extracted with aqueous triethylammonium bicarbonate (TEAB, pH 7.5) to remove excess 2-chlorophenyl phosphate (**40**). The organic layer containing **41** was dried by repeated coevaporation with anhydrous pyridine and the nucleotide thus obtained was used directly in the last condensation step. Condensation of phosphodiester **41** with octamer **42** using TPS-NI (**12d**) as the activating agent afforded the fully protected nonanucleotide **43** in 49% yield.

Deblocking of nonamer **43** was accomplished by treatment with TBAF to remove the aryl groups, followed by 25% aqueous ammonia to cleave the trifluoroacetyl and the benzoyl group.

The acid labile acceptor molecule **7** was purified on Sephadex G50 and isolated in 72% yield. The nonamer **7** was resistant to spleen phosphodiesterase, but was completely digested by venom phosphodiesterase. Hplc analysis (system C) of the digestion products revealed the presence of deoxythymidine 5′-phosphoro-4-aminobenzylamidate and pdT in the correct ratio.

Preparation of the RNase labile acceptor molecule **8**. (Scheme 5). The synthesis of the fully protected tridecamer **54** (Scheme 5) was performed according to the same strategy as described for oligonucleotides **13–27** in Scheme 1, starting from four building units: (i) N-5′-deoxy-deoxythymidine-5′-diphenyl phosphoramidate[41,42]



Scheme 5.

(44); (ii) 5'-O-levulinyl-2'-O-methoxytetrahydropranyluridine 3'-O-2, 2, 2-trichloroethyl 2-chlorophenyl phosphate[24] (45); (iii) 3'-O-benzoyldeoxythymidine[20] (11b, Scheme 1); (iv) fully protected dinucleotide 13 (Scheme 1).

Starting from dinucleotide 13 tetramer 46 was prepared by the condensation of Zn-treated 13 with hydrazine-treated 13 using TPS-NT (12e) as activating agent.

Ribonucleotide 45, after treatment with Zn, was condensed with 3'-O-benzoyldeoxythymidine (11b) in the presence of TPS-NT (12e) to afford dimer 48 in 76% yield. The dinucleotide 48 was elongated in the 5'-direction with dimer 13 in one four-step cycle to give tetranucleotide 50 in 91% yield. According to the same procedure octamer 52 was prepared starting from tetranucleotides 50 and 46f.

The first step in the synthesis of the fully protected pentamer 47, starting from mononucleotide 44 and tetramer 46g (R = H), consists of the introduction of an aryl phosphodiester function at the 3'-position of monomer 44. However, we found that phosphorylation of 44 with the monofunctional phosphorylating agent 2, 2, 2-trichloroethyl 2-chlorophenyl phosphorochloridate[22] gave the required product in very low yield. The latter may be ascribed to side reactions at the phosphoramidate function in nucleotide 44. For this reason monomer 44 was phosphorylated with the bifunctional agent 40 (Scheme 4) in the presence of TPS-NI (12d) after which excess aryl phosphate (40) was removed by TEAB extraction. The resulting phosphodiester was condensed with tetramer 46g using TPS-NI (12d) as the activating agent to afford pentanucleotide 47.

The final four-step cycle, starting from pentamer 47 and octamer 52, using TPS-NT (12e) as the activating agent, gave tridecanucleotide 54. The fully protected tridecamer 54 was purified by gel permeation chromatography on Sephadex LH60[25] and isolated in 93% yield. Data relevant to the synthesis of tridecamer 54 are listed in Table 3.

In order to obtain the RNase-labile acceptor 8 the fully protected tridecanucleotide 54 had to be deblocked. The crucial step in the deprotection of oligomer 54 was the removal of one of the phenyl groups from the 5'-phosphoramidate function. We found that fluoride treatment of this diaryl phosphoramidate was extremely slow. On the other hand, alkaline hydrolysis (0.1 N NaOH) of nucleoside aryl phosphoramidates was reported[36] to proceed readily. However, alkaline hydrolysis, instead of a fluoride ion promoted hydrolysis, of the internucleotide phosphotriesters in tridecamer 54 would lead to an unacceptable high percentage of internucleotide cleavage[20,24,43,44] products and, too, promote neighbouring group participation[45] of the 3'-hydroxyl which is deprotected during the alkaline treatment. Fortunately, a deblocking procedure consisting of a short fluoride ion treatment (30 min) followed by alkaline hydrolysis (0.1 N NaOH, 36 hr) proved to be an acceptable alternative for the removal of the 2-chlorophenyl

Table 3. Data on the synthesis of the fully protected tridecanucleotide **54** in Scheme 5

3'-Phosph. component		5'-Hydroxy component		Activating agent		Reaction time	Condensation product			Delevulination product		
No.	mmole	No.	mmole	No.	mmole	(hours)	No.	yield[a]	Rf[b]	No.	yield	Rf[b]
13(B=T)	2.2	14(B=T)	2.3	12e	2.75	1.5	46f	81%	0.32	46g	93%	0.22
44	1.3	46g	1.0	12d	1.4	45	47	38%	0.25			
45	2.2	11b	2.0	12e	2.4	1.5	48	76%	0.50	49	88%	0.30
13(B=T)	1.15	49	1.05	12e	1.3	1.5	50	91%	0.31	51	92%	0.21
46f	0.8	51	0.6	12e	0.88	2	52	90%	0.17	53	96%	0.13
47	0.225	53	0.15	12e	0.24	2	54	93%	0.09			

[a]Based on 5'-hydroxy component.
[b]System A.

groups and one phenyl group from the fully protected tridecamer **54**.

Cleavage of the remaining 5'-phenyl phosphoramidate and 2'-O-methoxytetrahydropyranyl groups with acid (0.01 N HCl) afforded the RNase-labile acceptor **8**, which was isolated, after purification on Sephadex G50, in 54% yield.

The purity of the tridecamer **8** was established by hplc analysis (system B) and digestion with venom phosphodiesterase which gave 5'-amino-5'-deoxy-deoxythymidine,[42,46] pdT and pU. Hplc analysis (system B) of the RNase digest of **8** showed the presence of solely 5'-NH_2-dT $(pdT)_{10}$pUp and dT.

Immobilisation of the functionalized acceptor molecules on activated cellulose (Schemes 6 and 7). Several methods have been developed to immobilize nucleic acids on polysaccharide matrices.[1,47,48] Gilham introduced the covalent attachment of oligonucleotides to cellulose by condensation of the terminal 5'- or 3'-phosphate of an oligonucleotide with a hydroxyl function of the sugar matrix using dicyclohexylcarbodiimide[49,50] or a water-soluble carbodiimide.[51] A disadvantage of this method is the occurrence of side reactions at nucleoside bases and with internucleotide phosphodiesters.[52]

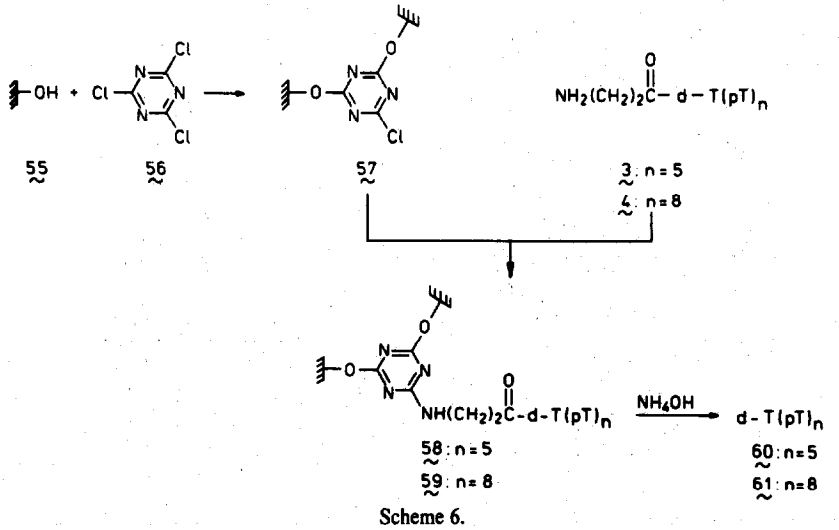
Another general approach[53,54] implies the use of reactive functions, especially amino groups, naturally present in nucleic acids to accomplish coupling with cyanogen bromide (CNBr) activated polysaccharides. However, the presence of multiple reactive functions in one molecule entails crosslinking with the solid support, making the ligand less accessible for interaction with molecules in solutions. The same disadvantage adheres to the immobilisation of nucleic acids via bisoxirane activated polysaccharides.[55]

Recently we reported[56] on the coupling of 5'-NH_2 terminated oligo- and poly-deoxythymidylates with CNBr- activated cellulose. In this way, oligo- and polynucleotides were immobilized in high yield and exclusively via the 5'-end. However, a problem which remained to be solved was the leakage of nucleotide material from the solid support, especially in basic medium and at elevated temperatures, a phenomenon inherent to CNBr-activation.[57]

Polysaccharides can also be activated with 2, 4, 6-trichloro-s-triazine, as described by Kay and Crook for the immobilisation of enzymes on cellulose.[58] During the activation reaction, which was performed in basic medium, two chlorine atoms are replaced by hydroxyls of the matrix with alkaline hydrolysis as a competing reaction. The third and less reactive chlorine atom can be replaced by an amino group of the compound to be incorporated.

We now wish to report on the immobilisation of 5'-NH_2-terminated oligonucleotides (e.g. compounds **3**–**7** in Fig. 2) on 2, 4, 6-trichloro-s-triazine activated cellulose.

The efficiency of the coupling and the effect of the pH on this reaction were investigated by measuring the immobilisation of 5'-NH_2-dT$(pdT)_5$[56] (125 nmoles) in potassium phosphate buffers (0.1 ml) containing 2, 4, 6-trichloro-s-triazine activated cellulose (**57**, 5 mg, Scheme 6). The extent and rate of the coupling process was monitored by hplc (system B) using d-T$(pT)_2$ as



Scheme 6.

Table 4. Coupling of oligonucleotides with 2, 4, 6-trichloro-s-triazine activated cellulose 57

Reactants[a]				Oligonucleotides linked		Oligonucleotides recovered	
Oligonucleotides		Activ.cell.(57)	pH				
Compound	nmoles	mg		nmoles	yield	No.	yield
NH_2-dT(pdT)$_5$	125	5	7.0	69	55%		
NH_2-dT(pdT)$_5$	125	5	8.0	103	82%		
NH_2-dT(pdT)$_5$	125	5	11.0	111	89%		
3	125	5	7.0	60	48%	60	91%
4	200	10	7.0	60	30%	61	90%

[a]The data refer to reaction mixtures (0.1 ml) containing the indicated amounts of reactants. (See text for further details).

internal standard. The reactions proceeded fast in the first hour, levelled off subsequently, and reached a maximum after 24 hr. The results which are listed in Table 4 show a high incorporation yield when the coupling was carried out in basic medium, and a somewhat lower value in a neutral buffer.

The linkage between oligonucleotide and solid support was stable in both acidic and basic medium: no leakage of oligonucleotide material could be detected after 120 hr at 20° when the polymer was suspended in buffer with pH ranging from 3 to 11.

Acceptor molecules 3 and 4, containing the base labile ester function, could also easily be immobilized on 2, 4, 6-trichloro-s-triazine activated cellulose (57, Scheme 6) at pH 7.0. The incorporation yield was again measured by hplc analysis (system B) of the supernatant containing d-T(pT)$_2$ as internal standard (Fig. 3). Data relevant to the coupling reactions are listed in Table 4.

The alanine ester linkage between cellulose and oligo-(dT) in the polymers **58** and **59** remained unaffected in acidic and neutral buffers. At pH 8.0 the polymeric compounds **58** and **59** were still reasonably stable: after 120 h at 20°, only 24% of the oligonucleotide was cleaved from the polysaccharide matrix. However, when the polymers **58** and **59** were suspended in 25% aqueous ammonia for 16 h, oligonucleotides d-T(pT)$_5$ (**60**) and d-T(pT)$_8$ (**61**) were recovered in 90% and 91% yield respectively, as established by hplc analysis using d-T(pT)$_2$ as internal standard (see Fig. 3). These findings, together with the observation that solely pure oligomers **60** and **61** were released from polymers **58** and **59** respectively, indicate that by this method oligonucleotides may be immobilized in high yield to cellulose, that the functionalized oligonucleotides **3** and **4** are linked specifically and with stable bonds to activated cellulose (**57**), and, finally, that the immobilized nucleic acids may be released quantitatively by base treatment.

However, cross-linking of the matrix during the activation step may influence the accessibility of the immobilized molecules and, further, the fast hydrolysis of the first and second chlorine atom in 2, 4, 6-trichloro-s-triazine may prevent higher coupling yields. Recently, Finlay *et al.*[59] have evaded this problem, in the immobilisation of proteins on 2, 4, 6-trichloro-s-triazine activated Sepharose, by stepwise replacement of the chlorine atoms in anhydrous organic medium.

We chose another alternative by using 2-amino-4, 6-dichloro-s-triazine (**62**, Scheme 7) as the activating agent: an approach developed by Kay and Lilly[60] to immobilize chymotrypsin. Replacement of one chlorine atom in 2, 4, 6-trichloro-s-triazine by an amino group decreases the susceptibility towards nucleophilic attack of the remaining Cl atoms which react now at convenient rates, and also prevents cross-linking.

Optimum reaction conditions for the activation of cellulose with 2-amino-4, 6-dichloro-s-triazine and subsequent incorporation of oligonucleotides were corroborated by measuring the immobilisation of 5'-NH_2-dT(pdT)$_8$[56] under various conditions. The highest incorporation yields were obtained when cellulose was activated according to the following procedure. Dry cellulose (100 mg) was suspended in 0.5 N NaOH (0.6 ml) and after 1 hr 2-amino-4, 6-dichloro-s-triazine (**62** in Scheme 7, 50 mg) in acetone–water (1:1, v/v) was added. The mixture was shaken for 10 min, the activated cellulose (**63**) was filtered off, washed and used immediately for coupling of oligonucleotides.

To determine the effect of the pH on the efficiency of the immobilisation process, 5'-NH_2-dT(pdT)$_8$ (167 mmole) was coupled with 2-amino-4, 6-dichloro-s-triazine activated cellulose (**63**, 10 mg) in 0.1 M potassium phosphate buffers (0.1 ml) of different pH. The results, as given in Fig. 4, show that the incorporation, after 24 hr at

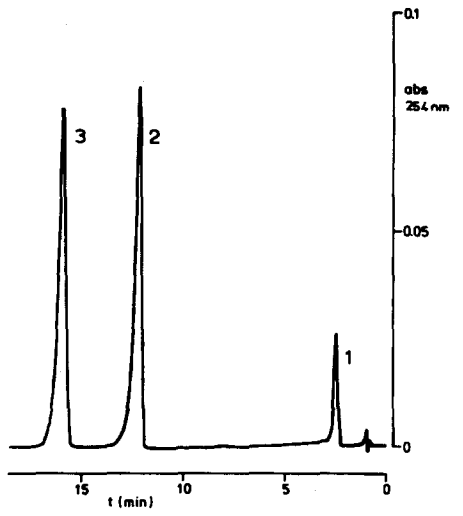
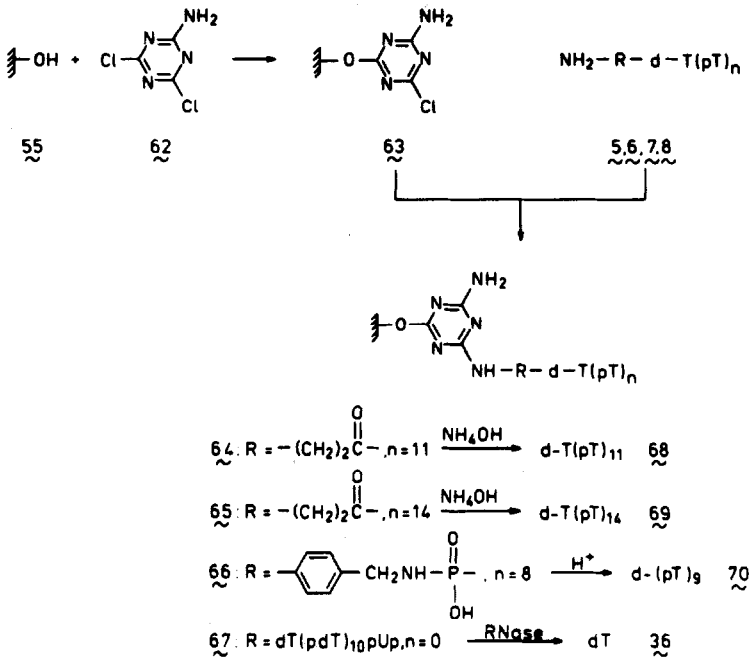


Fig. 3. The coupling of a functionalized oligonucleotide (e.g. 4, peak 2) with activated cellulose was measured with respect to an internal standard (d-T(pT)$_2$, peak 1) by hplc analysis (system B) of the supernatant. The quantity of an oligonucleotide (e.g. 61, peak 3) released from a solid support (e.g. 59) was determined according to the same procedure. See text for details.



Scheme 7.

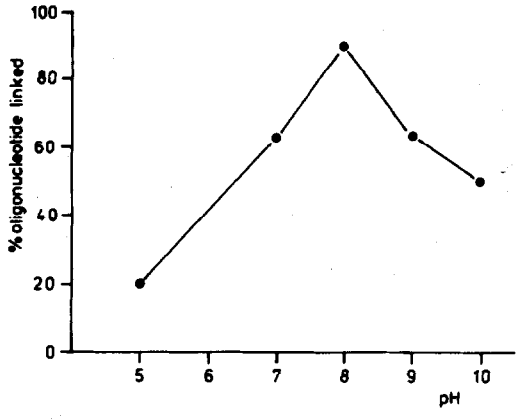


Fig. 4. Effect of the pH on the immobilisation of NH_2-dT(pdT)$_8$ on 2-amino-4, 6-dichloro-s-triazine activated cellulose (**63**). See text for details.

20°, had an optimum at pH 8.0; at this stage 90% of the nonamer was immobilized. A three times higher concentration of 5'-NH_2-dT(pdT)$_8$, under the same conditions, afforded a maximum incorporation of 6.9% (w/w) of the nonamer on the solid support.

Immobilisation of the functionalized acceptor molecules **5**–**8** (Scheme 7) occurred at pH 6.0 in case of alanine derivatives **5** and **6**, and at pH 8.0 in case of nonamer **7** and tridecamer **8**. Data relevant to the incorporation reactions are given in Table 5.

Cleavage of oligomers **68** and **69** was achieved by treating the corresponding polymers **64** and **65** with 25% aqueous ammonia for 16 hr. Both oligonucleotides (**68** and **69**) were recovered in a pure state and in high yield (Table 5).

A mild acid treatment (0.01 N HCl) of polymer **66** for 3 hr ($t_{1/2}$ = 26 min) afforded d-(pT)$_9$ (**70**) in 97% yield.

When polymer **67** was incubated with Pancreatic RNase in 0.1 M Tris-CHl buffer (pH 8.0) at 37°, deoxy-

Table 5. Coupling of oligonucleotides with 2-amino-4, 6-dichloro-s-triazine activated cellulose **63**

Reactants[a]			Oligonucleotides linked		Oligonucleotides recovered	
Oligonucleotides		Activ.cell.(63)				
Compound	nmoles	mg	nmoles	yield	No.	yield
NH_2-dT(pdT)$_8$	1670	100	1500	90%		
NH_2-dT(pdT)$_8$	5000	100	2300	46%		
5	1000	100	240	24%	68	91%
6	1000	100	280	28%	69	95%
7	1000	100	680	68%	70	97%
8	1000	100	750	75%		
8	1670	100	1080	65%	36	88%

[a]The data refer to reaction mixtures (1 ml) containing the indicated amounts of reactants (see text for further details).

thymidine was isolated as the sole nucleotide material in 88% yield.

CONCLUSION

In this paper we demonstrated that functionalized oligodeoxynucleotides of defined length (e.g. **3–8**) may be attached reversibly and in high yield to activated cellulose. From the observation that pure oligonucleotides **60**, **61**, **68–70** and deoxythymidine could be recovered from the solid support we may conclude that the DNA fragments were linked exclusively via the functionalized 5'-end to the matrix. Furthermore it is evident that both immobilization and subsequent cleavage from the solid support are devoid of side reactions at the nucleic acid material.

The bond between ligand and 2, 4, 6-trichloro-s-triazine or 2-amino-4, 6-dichloro-s-triazine activated cellulose was stable in acidic and basic medium (pH 3–11) as was established via polymers containing immobilized NH_2-dT(pdT)$_n$ (n = 5 or 8).

EXPERIMENTAL

General methods and materials. UV absorption spectra were measured with a Cary C14 recording spectrophotometer. ^{1}H NMR spectra were measured at 100 MHz with a Jeol JNMPS 100 spectrometer; chemical shifts are given in ppm (δ) relative to TMS.

Schleicher & Schüll DC Fertigfolien F 1500 LS 254 were used for tlc in solvent system A ($CHCl_3$–MeOH, 92:8, v/v). The high-performance liquid chromatography system used in this study has been described elsewhere.[61] High-performance anion-exchange chromatography was performed with the strong anion-exchange resin Permaphase AAX (DuPont, U.S.A.) dry-packed into a stainless-steel column (1 m × 2.1 mm). Elution was effected, starting with buffer A (0.005 M KH_2PO_4, pH 4.5) and applying 4% buffer B (0.05 M KH_2PO_4, 0.5 M KCl, pH 4.5) per min (system B). A flow rate of 1 ml/min at a pressure of 70 kp/cm^2 at 20° was standard. In system C high-performance anion-exchange chromatography was performed on a column (25 cm × 4.6 mm) packed with Partisil PXS 10/25 SAX (Whatman). The column was eluted with a mixture of buffer A and buffer B (95:5, v/v) at a flow of 1 ml/min and a pressure of 45 kp/cm.^{2} Retention times (Rt) in systems B and C were measured relative to the injection peak. Short column chromatography was performed on Merck Kieselgel H.

All solvents were dried as described previously.[20] Nucleosides were purchased from Waldhof (GFR). Microcrystalline cellulose (Merck) was mercerized[62] before use. 2, 4, 6-Trichloro-s-triazine was purchased from Aldrich.

General procedure for the preparation of the fully protected oligonucleotides **13–27**

The synthesis of the oligonucleotides **13–27** (Scheme 1) was performed according to the same procedure as previously described.[20] All fully protected oligonucleotides were purified by short column chromatography: the crude oligomers were dissolved in $CHCl_3$–MeOH (98–93:2–7, v/v) and applied on to a column of Kieselgel H (*ca.* 20 g per g of crude oligonucleotide) suspended in the same solvent mixture. If the oligonucleotide to be purified was 10 or more units long, 0.25% water was added to the mobile phase. The appropriate fractions were concentrated to a small volume and the pure oligonucleotides were precipitated from pet. ether (40–60°), filtered off and dried *in vacuo* (P_2O_5). Data relevant to the synthesis are summarized in Table 1.

Deblocking of the fully protected oligonucleotide **24c**

Oligonucleotide **24c** (64 mg, 0.01 mmole) was dissolved in tetrahydrofuran (7 ml) and 0.25 M tetrabutylammonium fluoride (TBAF, 4 equiv. per phosphotriester moiety) in pyridine–water (1:1, v/v, 1.75 ml) was added. After 2 hr at 20°, Dowex 50 W cation–exchange resin (4.4 g, 100–200 mesh, ammonium form) was added. The resin was removed by filtration and washed with water (2 × 5 ml). The combined filtrates were concentrated and 25% aqueous ammonia (20 ml) was added. After 40 hr at 20°, the soln was concentrated to a small volume which was analysed by hplc (system B). Crude **2** was purified on two columns (1 m × 3 cm^2) in series connection of Sephadex G50 suspended in 0.05 M triethylammonium bicarbonate (TEAB, pH 7.5). Elution occurred with the same buffer at a flow of 14 ml per hr. The chromatographic process was monitored by UV (254 nm) and the fractions (3 ml) containing pure **2**, as established by hplc analysis (system B), were collected and lyophilized. The dodecamer **2** was brought into the ammonium form by passing it through a column (8 cm × 2 cm^2) of Dowex 50W cation-exchange resin (100–200 mesh, ammonium form). The resulting aqueous solution was rely-ophilized. Yield 744 OD (ϵ_{max} = 120,000).[29] 6.2μ mole (62%). R_t 19.7 min (system B).

Synthesis of the fully protected d-thymidine derivative **29**

Phosphorochloridate **28**[27] (0.29 g, 1.1 mmole) was added to a solution of **10a** (0.56 g, 1 mmole) in anhydrous pyridine (5 ml). Tlc (system A), after 4 hr at 20°, showed the reaction to be complete. The mixture was concentrated to an oil which was dissolved in $CHCl_3$ (100 ml) and washed with 5% $NaCHO_3$ aq (50 ml) and water (50 ml). The organic layer was dried ($MgSO_4$), concentrated to a small volume and triturated with pet. ether (40–60°, 150 ml). A soln of the ppt in $CHCl_3$–MeOH (95:5, v/v) was applied on to a column (9 cm × 5 cm^2) of Kieselgel H (15 g) suspended in the same solvent.

Elution of the column with the same solvent mixture afforded pure **29**, which was precipitated from pet, ether (40–60°), R_f 0.37 (system A.) calc. for $C_{30}H_{30}Cl_4N_4O_9P_2$ (794.35): C, 45.36; H, 3.81; N, 7.05; P, 7.80.) (Found: C, 45.47; H, 3.93; N, 7.07; P, 7.78. UV (95% EtOH) λ_{max} 266 (ϵ 10,200) λ_{min} 247 nm (ϵ 6200). NMR ($CDCl_3$) 6.18 ($H_{1'}$, t, J 7Hz); 5.39 ($H_{3'}$, m); 4.64 (CH_2, d, J 7Hz); 1.72 (CH_3, s).

Synthesis of the fully protected decamer **30**

Reductive cleavage of the 2, 2, 2-trichloroethyl group from **29** (143 mg, 0.18 mmole) by treatment of **29** with activated Zn and 2, 4, 6-triisopropylbenzenesulphonic acid (TPS-OH) in pyridine and condensation of the resulting phosphodiester with **23a** (339 mg, 0.09 mmole) using TPS-NT (**12e**, 76 mg, 0.2 mmole) as activating agent, was performed as described previously. The reaction time at 20° was 1 hr. Crude **30** was purified on a column (3 cm × 7 cm^2) of Kieselgel H (9g) suspended in $CHCl_3$–MeOH-water (92.75:7:0.25). Elution of the column with the same solvent mixture afforded pure **30**. Yield 340 mg (86%), R_f 0.04 (system A).

Deblocking of the fully protected decamer **30**

Freshly distilled isopentyl nitrite (0.33 ml, 3 mmole) was added to a soln of **30** (44 mg, 0.01 mmole) in pyridine-AcOH (1:1, v/v, 2 ml). After 24 hr at 20°, the mixture was poured into stirred ether (100 ml), the ppt was filtered off, washed with ether (2 × 20 ml) and dissolved in dioxan (2 ml). Immediately 25% aqueous ammonia (8 ml) was added and the soln was left for 24 hr at 20°. The mixture was concentrated to a small volume and crude **30** was purified on Sephadex G50 as described for compound **2**, yield 570 OD (ϵ_{max} = 86,000),[29] 6.6 mmole (66%), R_t 17.7 min (system B). Oligonucleotide **1** was resistant to spleen phosphodiesterase. Treatment of **1** with alkaline phosphatase gave quantitative conversion into 3'-0-methoxytetrahydropyranyl-d-T(pT)$_9$. R_t 16.1 min (system B). Digestion of **1** with venom phosphodiesterase afforded 3'-0-methoxytetrahydropyranyl-deoxythymidine 5'-phosphate (R_t 7.3 min, system C) and deoxythymidine 5'-phosphate (R_t 5.1 min, system C).

Preparation of the fully protected oligonucleotides **31–34**

(*a*) *Hexamer* **31**. Dicyclohexylcarbodiimide (DCC, 20.6 mg, 0.1 mmole) was added to a magnetically stirred soln of **21a** (243 mg, 0.1 mmole), N-2-nitrophenylsulphenyl-β-alanine (24.2 mg, 0.1 mmole) and 4-dimethylaminopyridine (10 mg, 0.08 mmole) in anhyd dioxan (3 ml). After 1 hr and 2 hr, the same quantities (0.1 mmole) of DCC and N-nitrophenylsulphenyl-

β-alanine were added. Tlc ($CHCl_3$–MeOH 85:15, v/v) after 3 hr at 20° showed that the reaction was complete. The mixture was filtered, the filtrate was diluted with $CHCl_3$ (20 ml) and washed with 5% $NaHCO_3$ aq (2 × 20 ml) and water (20 ml). The organic layer was concentrated under reduced pressure to a small volume (3 ml) and triturated with pet. ether (40–60°, 75 ml). A soln of the ppt in $CHCl_3$–MeOH (95.5:4.5, v/v) was applied on to a column (3 cm × 7 cm^2) of Kieselgel H (9 g) suspended in the same solvent. Pure **31** was eluted from the column with $CHCl_3$–MeOH (95:5, v/v) and, after evaporation of the appropriate fractions, precipitated from pet. ether (40–60°, 100 ml).

(*b*) *Nonanucleotide* **32** was prepared starting from **23a** (189 mg, 0.05 mmole), N-2-nitrophenylsulphenyl-β-alanine (36 mg, 0.15 mmole), 4-dimethylaminopyridine (6 mg 0.05 mmole) and DCC (31 mg, 0.15 mmole) in anhyd dioxan (2 ml) according to the same procedure as described for **31**. Purification of **32** was performed on a column (3 cm × 7 cm^2) of Kieselgel H (9 g) suspended in $CHCl_3$–MeOH (93:7, v/v).

(*c*) *Dodecamer* **33** was prepared starting from **25a** (50 mg, 0.01 mmole), N-2-nitrophenylsulphenyl-β-alanine (15 mg, 0.06 mmole), 4-dimethylaminopyridine (2.4 mg, 0.02 mmole) and DCC (12.6 mg, 0.06 mmole) in anhyd dioxan (1.5 ml) in the same way as described for **31**. Crude **33** was purified on a column (2.5 cm × 3 cm^2) of Kieselgel H (2.5 g) suspended in $CHCl_3$–MeOH–water (92.75:7:0.25, v/v).

(*d*) *Pentadecamer* **34** was prepared, starting from **27a** (94 mg, 0.015 mmole) and purified in the same way as described for **33**. Yields and analytical data pertaining to the synthesis of oligomers **31–34** are given in Table 2.

Deblocking of the fully protected oligonucleotides **31–34**

Hexamer **31** (40 mg, 0.015 mmole) was dissolved in THF (4.8 ml) and 0.25 M TBAF in pyridine–water (1:1, v/v, 1.2 ml) was added. After 2 hr at 20°, the reaction was quenched by the addition of Dowex 50 W cation-exchange resin (3.0 g, 100–200 mesh, ammonium form). The resin was filtered off and washed with water (2 × 5 ml). The combined filtrates were concentrated to dryness and coevaporated with dioxan (2 × 10 ml). The residue was dissolved in methanol–acetic acid (9:1, v/v, 0.3 ml) and KI (10 mg, 0.06 mmole) was added. After 5 min at 20°, the mixture was partitioned between aqueous 0.01 N HCl (3 ml) and ether (3 ml). The aqueous layer was washed with ether (3 × 3 ml), the ethereal layers were extracted with 0.01 N HCl (2 ml) and the combined aqueous layers were adjusted to pH 2.0 with 0.1 N HCl.

After 2 hr, the pH was adjusted to 5.0 by the addition of 0.5 M aqueous ammonia and the soln was concentrated to a small volume (1 ml). Crude **3** was purified on two columns (1 m × 3 cm^2) in series connections of Sephadex G25, swollen in 0.005 M ammonium formate (pH 4.0). The columns were eluted with the same buffer at a flow of 14 ml/hr. The fractions (3 ml) of the main peak were analysed by hplc (system B) and those containing pure **3** were lyophilized, yield 640 OD (ϵ_{max} = 51,900),[29] 12.3 μmole (82%). According to the same procedure as described for **31** the 2-chlorophenyl groups were removed from the oligomers **32**, **33** and **34** using 4 equiv. TBAF per phosphotriester in THF-pyridine-water (8:1:1, v/v). The removal of the nitrophenylsulphenyl group and the methoxytetrahydropyranyl group was performed in exactly the same way as described for **31**. Oligomers **4**, **5** and **6** were purified on two columns (1 m × 3 cm^2) in series connection of Sephadex G50 according to the same procedure as described for **3**, yields and retention times (system B) of oligomers **3–6** are given in Table 2. Treatment of the oligonucleotides **3–6** (1 mg) with 25% aqueous ammonia (1 ml) for 2 hr afforded the corresponding oligomers d-T(pT)n (n = 5, 8, 11 and 14) (Table 2) which were completely digested by venom phosphodiesterase and spleen phosphodiesterase to give the expected products in the correct ratio.

2, 4-*Dichlorophenyl phosphoro-4-nitrobenzylamidochloridate* **(35)**

A soln of 4-nitrobenzylamine (1.52 g, 10 mmole) and triethylamine (1.53 ml, 11 mmole) in anhyd dioxan (3.3 ml) was added dropwise, over a period of 15 min, to a stirred soln of 2, 4-dichlorophenyl phosphorodichloridate (2. g, 10 mmole) in anhyd-dioxan (3 ml). After stirring for another hr, the mixture was filtered and the filtrate was concentrated to an oil which was dissolved in hot anhyd chlorobenzene (*ca.* 100°, 50 ml). Pure **35** solidified upon standing at 0°, yield 2.4 g (61%), m.p. 80–83°. NMR ($CDCl_3$) 8.18 (C_6H_4, d, J 7Hz); 7.55 (C_6H_4, d, J 7Hz); 7.30 (C_6H_3, m); 5.58 (NH, dt, J_{PH} 15 Hz, J_{HH} 7.5 Hz); 4.47 (CH_2, dd, J_{PH} 14 Hz, J_{HH} 7.5 Hz).

Deoxythymidine-5′ 2, 4-dichlorophenyl phosphoro-4-nitrobenzylamidate **(37)**

A soln of **35** (1.78 g, 4.5 mmole) in anhyd pyridine (9 ml) was added dropwise, over a period of 1 hr, to a stirred soln of **36** (0.73 g, 3 mmole) in anhyd pyridine (12 ml). After stirring for another hour, the mixture was concentrated to an oil. A soln of the oil in $CHCl_3$-dioxan (2:1, v/v, 100 ml) was washed with 5% $NaHCO_3$ aq (50 ml) and water (50 ml). The organic layer was dried ($MgSO_4$), concentrated to a small volume and triturated with pet. ether (40–60°, 2 × 150 ml). The ppt was dissolved in $CHCl_3$–MeOH (95:5, v/v) and applied on to a column (11 cm × 9 cm^2) of Kieselgel H (40 g) suspended in the same solvent. Elution of the column with $CHCl_3$–MeOH (94:6, v/v) and evaporation of the appropriate fractions afforded pure **37** as a glass, yield 1.43 g (79%), R_f 0.16 (system A). NMR ($(CD_3)_2SO$) 8.14 (C_6H_4, d, J 8 Hz); 7.60 (C_6H_4, d, J 8 Hz); 7.50 (C_6H_3, m); 6.18 ($H_{1'}$, t, J 7 Hz).

Deoxythymidine-5′ 2, 4-dichlorophenyl phosphoro-4-aminobenzylamidate **(38)**

Hydrogen was bubbled through a soln of **37** (0.6 g, 1 mmole) in absolute alcohol (100 ml) containing Pd-C (10%, 0.2 g). After 30 min at 20°, tlc (system A) showed that the reaction was complete. The mixture was filtered over hyflo-super-cel and concentrated to afford **38** as a glass. Yield 0.565 g (99%), R_f 0.10 (system A). NMR ($(CD_3)_2SO$) 6.86 (C_6H_4, d, J 8 Hz); 6.52 (C_6H_4, d, J 8 Hz); 6.18 ($H_{1'}$, and NH).

Deoxythymidine-5′ 2, 4-dichlorophenyl phosphoro-4-trifluoroacetylaminobenzylamidate **(39)**

Trifluoroacetic anhydride (0.14 ml, 1 mmole) was added to a soln of **38** (286 mg, 0.5 mmole) in anhyd pyridine (5 ml). Tlc (system A), after 5 min at 20°, showed that the reaction was complete. The mixture was concentrated to an oil which was dissolved in $CHCl_3$–MeOH (4:1, v/v, 100 ml) and washed: 5% $NaHCO_3$ aq (30 ml) and water (30 ml). The organic layer was dried ($MgSO_4$), evaporated down to a small volume and triturated with pet. ether (40–60°, 150 ml). A soln of the ppt in $CHCl_3$–MeOH (94:6, v/v, 3 ml) was applied on to a column (3 cm × 7 cm^2) of Kieselgel H (9 g) suspended in the same solvent. Elution with the same solvent mixture afforded pure **39** which was precipitated from pet. ether (40–60°, 150 ml), yield 250 mg (75%), R_f 0.13 (system A.). (Found: C, 45.13; H, 3.75; N, 8.31; P, 4.93). (Calc. for: $C_{25}H_{24}Cl_2F_3N_4O_8P$ (667.36): C, 44.99; H, 3.63; N, 8.40; P, 4.64) UV (95% EtOH) λ_{max} 257 (ε 19,100)λ_{min} 234 nm (ε 11,800). NMR ($(CD_3)_2SO$) 7.55 (C_6H_4, d, J 8 Hz); 7.26 (C_6H_4, d, J 8 Hz); 6.34 (PNH, m); 6.17 ($H_{1'}$, t, J 7Hz).

Preparation of octanucleotide **42**

The synthesis of octamer **42** was performed analogous to a procedure described previously[20]. Thus, dimer **13b** (0.86 g, 0.8 mmole) was treated with zinc and triisopropylbenzenesulphonic acid in pyridine to remove the 2, 2, 2-trichloroethyl group and the resulting phosphodiester was condensed with **21b** (1.45 g, 0.6 mmole) using TPS-NT (**12e**, 0.34 g, 0.88 mmole) as activating agent. Purification of the fully protected octamer by short column chromatography, followed by removal of the levulinyl group by hydrazine treatment, afforded **42**. Yield 1.6 g (83% based on **21b**), R_f 0.13 (system A).

Preparation of the fully protected nonanucleotide **43**

To a soln of **39** (90 mg, 0.135 mmole) and 2-chlorophenyl phosphate[40] (31 mg, 0.15 mmole) in anhyd pyridine (3 ml) was added TPS-NI (**12d**, 133 mg, 0.35 mmole). After 16 hr at 20°, the mixture was diluted with $CHCl_3$ (50 ml) and washed with 1 M TEAB (2 ml) and 0.1 M TEAB (2 ml). The organic layer was concentrated to an oil which was transferred to a smaller flask containing octamer **42** (98 mg, 0.03 mmole). The mixture was

dried by repeated coevaporation with anhyd pyridine (3 × 10 ml). TPS-NI (**12d**, 38 mg, 0.10 mmole) was added and after 20 hr at 20°, tlc (MeOH–$CHCl_3$ 9:1, v/v) showed the reaction to be complete. The mixture was concentrated to an oil which was diluted with $CHCl_3$–dioxan (2:1, v/v, 100 ml) and washed with 5% $NaHCO_3$aq (25 ml) and water (25 ml). The organic layer was concentrated to a small volume and crude fully protected nonamer **43** was precipitated from pet. ether (40–60°, 100 ml). A soln of **43** in $CHCl_3$–MeOH-water (92.75:7:0.25, v/v, 3 ml) was brought on to a column (4 cm × 3 cm^2) of Kieselgel H (4 g) suspended in the same solvent. Elution of the column with the same solvent mixture afforded pure **43** which was precipitated from pet. ether (40–60°, 100 ml), yield 60 mg (49% based on **42**), R_f 0.15 (system A).

Deblocking of the fully protected nonanucleotide **43**

Nonamer **43** (49 mg, 0.012 mmole) was deblocked and purified in the same way as described for **24c**, yield 667 OD (ϵ_{max} 77,500),[29] 8.6 μmole (72%), R_t 15.0 min (system B). Nonamer **7** was resistant to spleen phosphodiesterase, but was completely digested by venom phosphodiesterase to give d-thymidine-5′ phosphoro-4-aminobenzylamidate (R_t 1.2 min, system C) and d-thymidine 5′-phosphate (R_t 5.0 min, system C).

Preparation of the fully protected tridecanucleotide **54**

The synthesis of **46** and **48–54** (Scheme 5) was performed according to the same procedure as described for oligonucleotides **13–27** in Scheme 1. The tridecamer **54** was not purified on silicagel but on a column (1 m × 7 cm^2) of Sephadex LH60[25] using THF–MeOH (19:1, v/v) as the mobile phase. The preparation of **47** was analogous to the synthesis of **43**: monomer **44** (0.62 g, 1.3 mmole) was phosphorylated with the bifunctional agent **40** (0.29 g, 1.4 mmole) using TPS-NI (**12d**, 1.10 g, 2.9 mmole) as activating agent. Condensation of the resulting phosphodiester with **46g** (1.81 g, 1.0 mmole) and TPS-NI (**12d**, 0.53 g, 1.4 mmole) as activating agent gave **47** which was purified by short column chromatography. Data relevant to the synthesis of oligomers **46–54** are given in Table 3.

Deblocking of the fully protected tridecanucleotide **54**

To a soln of **54** (283 mg, 0.05 mmole) in THF (38.4 ml) was added 0.25 M TBAF in pyridine–water (1:1, v/v, 9.6 ml). After 30 min at 20°, Dowex 50 W cation-exchange resin (24 g, 100–200 mesh, ammonium form) was added to the mixture. The resin was filtered off and washed with water (2 × 5 ml). The combined filtrates were concentrated to dryness, dissolved in 0.1 N NaOH (70 ml) and stored for 36 hr at 20°. Dowex 50 W cation-exchange resin (10 g, 100–200 mesh, ammonium form) was added and after filtration the soln was concentrated to a small volume (2 ml) which was diluted with 0.01 N HCl (15 ml) and adjusted to pH 2.0 with 0.1 N HCl. After 16 hr at 20°, the soln was neutralized with 0.5 M aqueous ammonia and concentrated to a small volume. Purification of **8** on Sephadex G50 was analogous to the purification of **2**, yield 3041 OD (ϵ_{max} 111,800)[29] 0.0272 mmole (54%), R_t 15.8 min (system B). RNase treatment of **8** afforded 5′-amino-5′-deoxy-dT(pdT)$_{10}$pUp (R_t 16.7 min, system B) and dT. Tridecamer **8** was resistant to spleen phosphodiesterase but was completely digested by venom phosphodiesterase affording 5′-amino-5′-deoxy-dT,[46] pdT and pU.

Activation of cellulose with 2, 4, 6-trichloro-s-triazine

Cellulose (100 mg) was treated with 1 N NaOH (1 ml), after 2 hr the excess base was removed by filtration and the moist cellulose was transferred to a soln of 2, 4, 6-trichloro-s-triazine (**56**, 100 mg) in acetone (2.5 ml). Water (2.5 ml) was added and the mixture was shaken for 30 sec at 20°. The reaction was quenched by the addition of 10% aqueous AcOH (2.5 ml). The activated cellulose (**57**, Scheme 6) was filtered off and washed with acetone–water (1:1, v/v, 3 × 5 ml), water (2 × 5 ml) and aqueous 0.1 M KH_2PO_4 (pH 7.0, 2 × 5 ml). The moist activated cellulose (**57**) was used immediately for coupling reactions.

Coupling of d-oligonucleotides with 2, 4, 6-trichloro-s-triazine activated cellulose (**57**)

A quantity, corresponding to 10 mg dry weight, of activated cellulose (**57**) was added to a 0.1 M potassium phosphate buffer (for pH Table 4) containing the oligonucleotide and 0.1 μmole d-T(pT)$_2$. To determine the amount of oligonucleotide, immobilized on the solid support, 2 μl of the supernatant was analysed by hplc (system B). After 24 hr at 20° 1 M NH_4Claq(50 μl) was added to the mixture which was left for another hour. The polymer was filtered off and washed with buffer (0.1 M KH_2PO_4, 1 M NaCl, pH 5.0, 3 × 1 ml), water (3 × 1 ml), acetone–water (respectively 1:3, 1:1, 3:1, v/v) and acetone. The polymer was dried and stored *in vacuo* at 0°. Data relevant to the coupling reactions are given in Table 4.

Removal of the oligonucleotides **60** *and* **61** *from cellulose*

Cellulose-oligo-dT (**58** or **59**, 5 mg) was treated with 25% aqueous ammonia (0.25 ml) for 16 hr at 20°, after which 10 mM d-T(pT)$_2$ (10 μl) was added. The supernatant was removed and concentrated to a small volume (pH 7). The quantity of oligonucleotide, recovered from cellulose was determined by hplc analysis (system B) of the neutral solution. Other relevant data are summarized in Table 4.

Activation of cellulose with 2-amino-4, 6-dichloro-s-triazine

Cellulose (100 mg) was treated with 0.5 N NaOH (0.6 ml). After 1 hr a soln of 2-amino-4, 6-dichloro-s-triazine[60] (**62**, 50 mg) in acetone–water (5:3, v/v, 2, 4 ml) was added. The mixture was shaken for 10 min at 20°, the activated cellulose (**63**, Scheme 7) was filtered off and washed with actone–water (1:1, v/v, 3 × 5 ml), water (2 × 5 ml) and the buffer, to be used in the coupling reaction (Table 5, 2 × 5 ml). The activated cellulose (**63**) was used immediately for the coupling reaction.

Coupling of d-oligonucleotides with 2-amino-4, 6-dichloro-s-triazine activated cellulose (**63**)

The coupling was performed at pH 8.0 in the same way as described for **57**. The immobilization of the alanine derivatives **5** and **6** was performed at pH 6.0. After immobilization of **7**, **66** was washed with a basic buffer (0.05 M $NaCHO_3$, 1 M NaCl, pH 9.0). Data relevant to the coupling reactions are given in Table 5.

Removal of oligonucleotides **68–70** *and d-thymidine* (**36**) *from cellulose*

Oligomers **68** and **69** were removed analogous to **60** and **61**. Nonanucleotide **70** was removed by treatment of **66** (2 mg) with 0.01 N HCl (100 μl) for 4 hr. After addition of 10 mM d-T(pT)$_2$ (4 μl) the supernatant was isolated, neutralized and analysed by hplc (system B). The immobilized tridecamer **67** (10 mg) was suspended in 0.1 M Tris-HCl (pH 8.0, 0.1 ml), containing 0.5 mg Pancreatic Ribonuclease (Merck). After 16 hr at 37°, tlc ($CHCl_3$–MeOH, 3:1, v/v) of the supernatant showed deoxythymidine as the sole UV absorbing product. Data relevant to the removal of **68–70** and **36** from cellulose are given in Table 5.

Enzymatic digestion of the deblocked oligonucleotides

(a) *Venom phosphodiesterase.* A soln of the oligonucleotide (2 OD) in a buffer (0.05 ml) containing 25 mM Tris-HCl (pH 9.0), 5 mM $MgCl_2$ and 2 μg snake venom phosphodiesterase (Crotalus terr. terr., Boehringer) was incubated at 37° for 3 hr.

(b) *Spleen phosphodiesterase.* A soln of the oligonucleotide (2 OD) in a buffer (0.05 ml) containing 0.1 M NH_4OAc (pH 5.7) and 4 μg spleen phosphodiesterase (Boehringer) was incubated at 37° for 3 hr.

(c) *RNase.* The oligonucleotide (2 OD) was incubated with 10 μg Pancreatic Ribonuclease (Merck) in 0.2 M Tris-HCl buffer (pH 8.0, 0.05 ml) at 37° for 3 hr.

(d) *Alkaline phosphatase.* A soln of the oligonucleotide (2 OD) in a buffer (0.05 ml) containing 0.2 M Tris-HCl (pH 8.0) and 0.5 U alkaline phosphatase (Sigma) was incubated at 37° for 3 hr.

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