Pergamon

Tetrahedron Vol. 50, No. 24, pp. 7203-7218, 1994

0040-4020/94 $7.00+0.00

0040-4020(94)E0341-P

Novel Solid Supports for the Preparation of 3'-Derivatized Oligonucleotides: Introduction of 3′-Alkylphosphate Tether Groups Bearing Amino, Carboxy, Carboxamido, and Mercapto Functionalities

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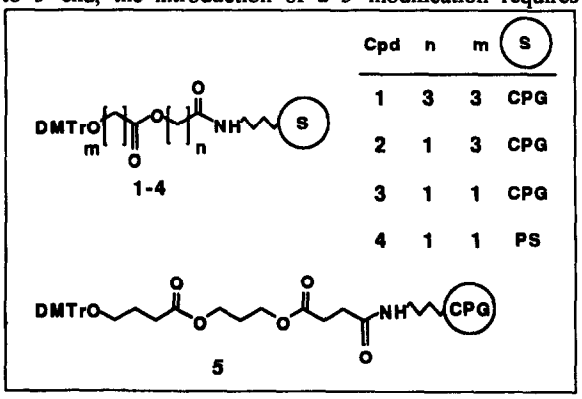
Abstract: Syntheses of 5 non-nucleosidic solid supports (1-5) that enable preparation of oligonucleotides bearing a carboxy,- amino,- carboxamido,- or mercaptoalkyl spacer arm at their 3′-terminus are described. They all contain an ester bond of moderate susceptibility toward nucleophiles. Upon the completion of oligonucleotide chain assembly, this bond may be cleaved by a variety of nucleophiles. These release the oligonucleotide from the support and simultaneously introduce the desired functionality. Differences in the reactivity between the supports prepared are discussed.

INTRODUCTION

Oligonucleotides that bear a functional group tethered to their 3′-terminus have recently received increasing interest for two main reasons. On one hand, attachment of luminescent, intercalating or chemically reactive groups to these functions affords oligonucleotide conjugates that may be used as tools in molecular biology.[1] On the other hand, 3′-modified oligonucleotides are often rather resistant towards the action of nucleases, and hence they have found usage as antisense inhibitors.[2] Since the solid phase synthesis of oligonucleotides usually proceeds from the 3′- to 5′-end, the introduction of a 3′-modification requires preparation of a modified solid support.[1] Some methods have been reported[3-5] that enable synthesis of various 3′-functionalized oligonucleotides on a single polymer support. They either include the preparation of oligonucleotide 3'-phosphates[3] or 3'-thiophosphates,[4] followed by post-synthetic esterification[3] or alkylation[4] of the deprotected and isolated material, or they are based on a machine assisted synthesis of peptide - oligonucleotide conjugates that allow the



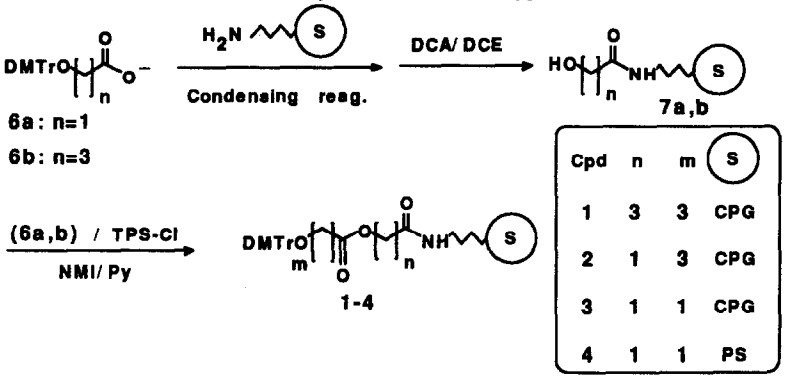
Cpd	n	m	S
1	3	3	CPG
2	1	3	CPG
3	1	1	CPG
4	1	1	PS

introduction of several amino groups at the 3′-terminus of the DNA sequence.[5] Some additional methods for the synthesis of 3'-tethered oligonucleotides are available, but the general applicability of them is restricted by the fact that the structure of the solid support employed strictly determines the nature of the functional group and the length of the spacer arm.[1, 6-10]

Recently we have reported on the preparation of solid supports containing either a 3'-derivatized thymidine unit[11] or a non-nucleosidic spacer[12] that enable a versatile functionalization of the 3′-terminus. The key feature of the structure of these supports is an ester bond of moderate reactivity. The functional groups are introduced by cleavage of this bond with appropriate nucleophilic reagents. Supports of this kind have to meet the contrary requirements of stability and reactivity. The reactivity of the ester bond should be high enough to warrant the release from the solid support without competitive side reactions at the base moieties, but it should still remain intact during the oligonucleotide chain assembly steps. In order to find a support that optimally fulfils these requirements, 5 homologous solid supports, having a general structure of **1-5**, were prepared. To provide a generally applicable structure, they were tested in oligonucleotide synthesis, and their applicability for the preparation of 3'-functionalized oligonucleotides was compared.

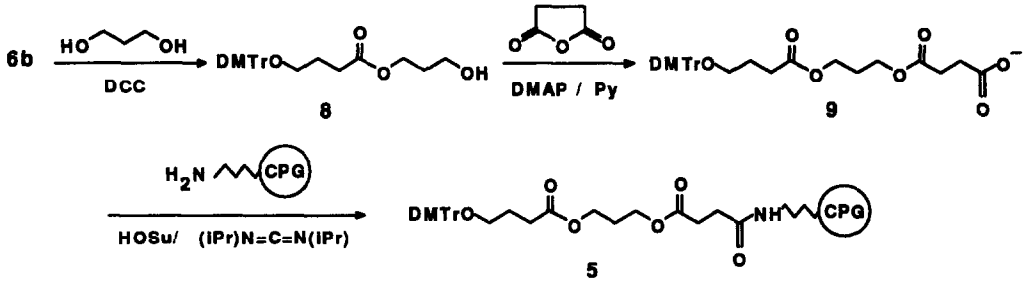
RESULTS AND DISCUSSION

Synthesis of the solid supports (**1-5**). - Five different solid supports were prepared, each containing an ester bond, the susceptibility of which to nucleophiles was varied. Four of them (**1-4**) were obtained by successive acylations of aminoalkylated particles, as depicted in *Scheme 1*. Accordingly, 4,4′-dimethoxytritylated glycolic acid (**6a**) or 4-hydroxybutyric acid (**6b**)[12] was initially immobilized on the long chain alkylamine controlled pore glass (CPG) with the aid of *N,N′*-diisopropylcarbodiimide and *N*-hydroxysuccinimide. Capping of the unreacted amino functions and subsequent detritylation then gave ω-hydroxyalkanoyl derivatized supports, **7a,b**, which were further acylated to **1-3** with the mixture of **6a** (or **6b**) and 2,4,6-triisopropylbenzenesulfonyl chloride in pyridine, using *N*-methylimidazole as catalyst. The latter mixed-anhydride approach was selected, since the carbodiimide-assisted methods have been reported[13] to be ineffective in attachment of carboxylic acids to the solid supports bearing hydroxyl groups. The aminomethylated polystyrene beads were derivatized to **4** in a similar manner, but now the mixed-anhydride method was applied to both acylations.



Scheme 1. Condensing reag.- *N,N′*-diisopropylcarbodiimide and *N*-hydroxysuccinimide in pyridine (synthesis of **1-3**) or triisopropylbenzenesulfonyl chloride (TPS-Cl) and *N*-methylimidazole (NMI) in pyridine (synthesis of **4**); DCA - dichloroacetic acid. DCE - dichloroethane. DMTr - 4,4′-dimethoxytriyl; CPG - controlled pore glass; PS - polystyrene.

The fifth solid support, **5**, was prepared as depicted in *Scheme 2*: **6b** was esterified with an excess of 1,3-propanediol in the presence of DCC, the product, **8**, was acylated with succinic anhydride to give **9**, and this was bound by the carbodiimide method to the long chain alkylamine controlled pore glass.



Scheme 2. DCC - *N,N'*-dicyclohexylcarbodiimide; DMAP - *N,N*-dimethylaminopyridine; HOSu - *N*-hydroxysuccinimide.

Synthesis of the 3'-derivatized oligonucleotides on solid supports **1-5**. - In order to investigate the usefulness of the supports **1-5** in preparation of 3'-modified oligonucleotides, the sequence d(GCCGTGGAGTCGTT) bearing various functionalities at its 3'-terminus was synthesized (*Schemes 3-7*). The protected oligonucleotides, **10-14**, were assembled on an Applied Biosystems 392 DNA Synthesizer. Phosphoramidite chemistry and recommended protocols were used in all cases (0.04-1.0 μmol scale). No differences in coupling efficiency (>98 % as determined from trityl responses) were detected between the modified supports and commercial T-derivatized columns, consistent with the stability of spacer arms under the conditions of oligonucleotide synthesis. The assembled chains were cleaved from the solid supports with various nucleophiles, as described below in detail. Oligonucleotides prepared were isolated by ion exchange HPLC, purified on reversed phase column and finally desalted by gel filtration. Their HPLC retention times are listed in Table 1. It has been shown previously that oligonucleotides tethered with a single functional group could be easily separated by ion exchange chromatography.[13] Our preliminary reports[11,12] confirmed that oligonucleotides of the same sequence but with different functional groups at 3'-end elute from the ion exchange column in the order amino<unmodified 3'-end<carboxamido<carboxy showing a remarkable difference in retention times. This difference in the mobility was used for the preliminary characterization of the structure of the 3'-modified oligonucleotides prepared. The final characterization was performed by enzymatic digestion, as described below.

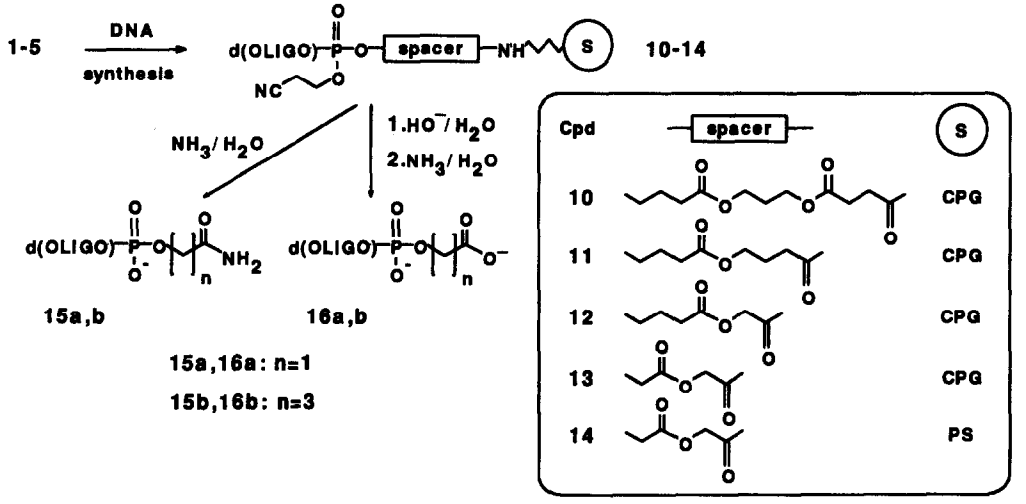
Table 1. HPLC retention times (in minutes) of the oligonucleotides prepared.

Oligonucleotide[a]	**15a**	**16a**	**17a**	**18a**	**19a**	**20a**		**22a**	**23a**
Ion exchange[b]	22.4	25.1	20.7	20.7	20.7	20.9		22.4	-
Reversed phase[b]	25.6	24.8	26.0	26.0	26.3	26.9		26.9	30.7
Oligonucleotide	**15b**	**16b**	**17b**	**18b**	**19b**	**20b**	**21**	**22b**	**23b**
Ion exchange[b]	22.3	24.0	20.7	20.7	20.4	20.9	21.7	22.6	-
Reversed phase[b]	26.1	25.1	26.1	26.0	26.4	27.5	27.3	27.3	31.0

a. For structures, consult *Schemes 3-7*; b. for conditions, see experimental section.

Introduction of functional groups to 3′-terminus.

Cleavage with aqueous ammonia. - When the oligonucleotides assembled on the modified solid supports **1-5** were released with aqueous ammonia, ω-carboxamidoalkyl esters of oligonucleotide 3′-monophosphate (**15a,b**) were obtained (*Scheme 3*). Tables 1 and 3 record their HPLC retention times and the results of the enzymatic digestion, which clearly verify the assumed structures. Ion exchange HPLC profile of crude **15b** obtained from **10** is shown on Fig 1.



Scheme 3.

Cleavage with hydroxide ion. - ω-Carboxyalkyl esters of oligonucleotide 3′-monophosphates (**16a,b**) were prepared by treating **10-14** with 0.1 M aqueous sodium hydroxide or 0.5 M aqueous 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; *Scheme 3*). This cleaves the β-cyanoethyl phosphate protections and creates a carboxylate group at the 3′-terminus. The partially deprotected oligomers were further treated with concentrated aqueous ammonia to remove all remaining base protecting groups. The HPLC retention times and the results of enzymatic digestions are collected in Tables 1 and 3. Ion exchange HPLC profile of crude **16b** obtained from **11** is shown on Fig. 2.

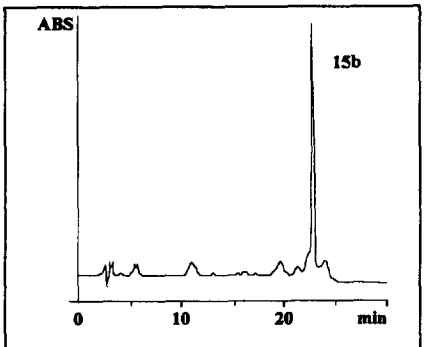


Fig. 1. Ion exchange HPLC profile of crude **15b**.

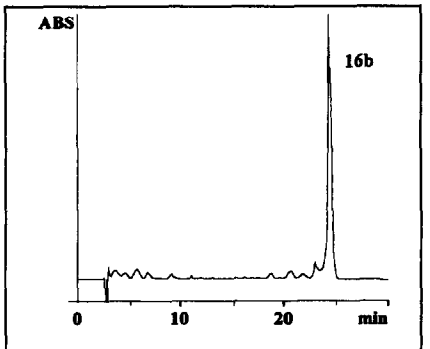
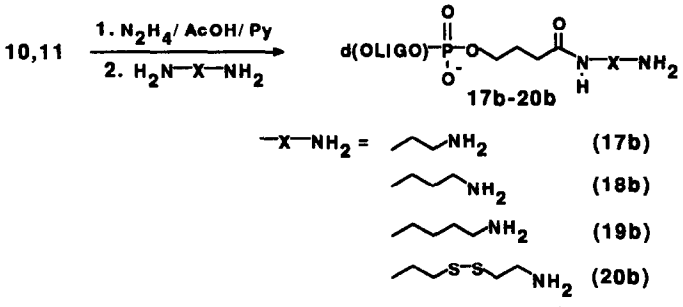


Fig. 2. Ion exchange HPLC profile of crude **16b**.

Cleavage with α,ω-alkanediamine and cystamine. - Introduction of amino tails to the 3'-terminus may be achieved by treating the oligonucleotide attached to the solid support (**1-5**) with α,ω-alkanediamines. However, the action of primary alkylamines on the protected oligonucleotides also leads to undesired transamination of the N^3-benzoylated cytosine residues. This side reaction has previously been avoided by selective debenzoylation of cytosine and adenine residues with hydrazinium acetate.[14] Unfortunately, of the modified supports prepared in the present study, only **1** and **5** stand this treatment, as evidenced by the fact that after hydrazinium acetate deblocking the carboxamido functionalized oligonucleotides (**15b**) could be obtained by ammonia treatment. By contrast, hydrazinium acetate quantitatively cleaved oligonucleotides from the other solid supports (**2-4**). Accordingly, with these supports other approaches had to be used to suppress the transamination.

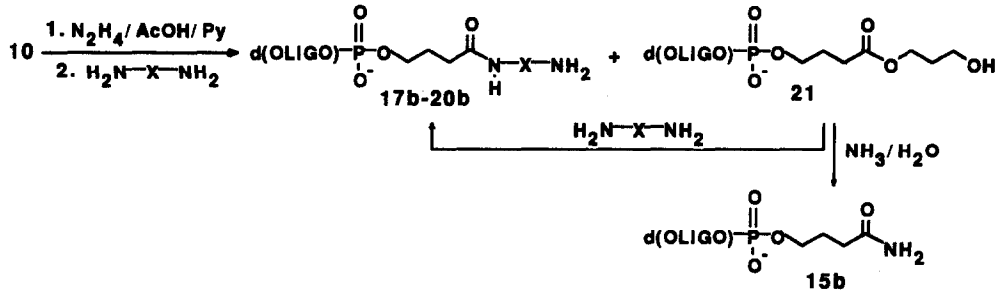
Since supports **1,5** were observed to be stable towards hydrazinium acetate, the oligonucleotides bound to them (**10**, **11**) were first deblocked with this reagent, and then treated with 50 % (v/v) solutions of α,ω-alkanediamines or cystamine in 2-propanol or pyridine (*Scheme 4*). Under these conditions the ester bond of the solid support was aminolyzed, releasing the oligonucleotide to solution and introducing the appropriate aminoalkyl tail to the 3'-end. Simultaneously, the internucleosidic phosphate groups and guanine residues also became deblocked, and hence the 3'-functionalized oligonucleotides (**17b-20b**) were obtained in a completely deprotected form. Simple precipitation of the oligonucleotides with dioxane removed the excess of diamines. Alternatively, the crude reaction mixture was passed through a short Dowex 50Wx8 column in pyridinium form. The results of the characterization of **17b-20b** are given in Tables 1 and 3.



Scheme 4.

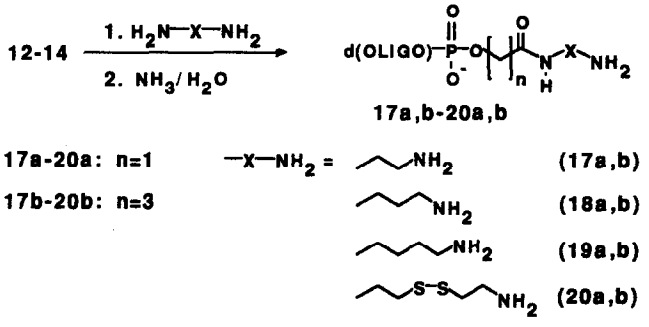
Although the 3'-aminoalkylated oligonucleotides, **17b-20b**, may be prepared on supports **1** and **5**, the procedure described above suffers from some drawbacks. While the cleavage from the solid support was relatively rapid with 1,2-ethanediamine and 1,3-propanediamine (8 h at room temperature), a prolonged treatment was needed when 1,4-butanediamine or cystamine was used as cleaving agent (30 h and 3 d at 55 °C, respectively). Addition of DBU to the reaction mixture (0.5 M) accelerated the cleavage in 2-propanol by a factor of 2, but still the reaction was undesirably slow. When 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) was used instead of DBU, the cleavage from solid support was markedly accelerated, but the transamination of cytosine residues started to compete. Another complication is that cleavage from support **5** was accompanied by formation of a side product that on the basis of the following observations was assigned as **21** (*Scheme 5*).

(i) Cleavage with all the diamines tested gave the same side product. (ii) The ion exchange HPLC retention time of this product was longer than those of the desired products (**17b-20b**) and shorter than that of **16b** (Table 1). (iii) Prolonged treatment of the isolated side product with α,ω-alkanediamines converted it to the desired product, and treatment with aqueous ammonia gave **15b**.



Scheme 5. **17b-19b:** X = -$(CH_2)_n$-; **20b:** -$(CH_2)_2SS(CH_2)_2$-; **17b:** n = 2; **18b:** n = 3; **19b:** n = 4;.

For these reasons the attention was focused on supports **2-4**. As already mentioned, protected oligonucleotides assembled on these supports cannot be debenzoylated by treatment with hydrazinium acetate. Cleavage of protected oligonucleotides with 1,4-butanediamine (50 % v/v in *i*PrOH), in turn, resulted in complicated mixtures of the 3′-functionalized oligonucleotides (**19a,b**) and their mono-, bis-, and tris-transaminated derivatives. The proportion of the desired product was less than 30 %. However, cleavage from the solid support **3** with a 5 % solution (v/v) of 1,4-butanediamine in either 2-propanol or water gave a rather satisfactory result (*Scheme 6*). With both solutions, the oligonucleotide was released within 18 h. Simultaneously the phosphate functions were completely deblocked, whereas the isobutyryl protections remained practically intact. After conventional ammonolysis, the reaction mixture in 2-propanol contained the desired oligonucleotide as the major component and mono-transaminated oligonucleotides as by-products (4.5:1). Surprisingly, no transamination took place in the aqueous solution. The desired oligonucleotide was, however, contaminated (8:1) with oligonucleotide 3'-phosphoglycolate **16a**, the hydrolysis product of the ester bond.



Scheme 6.

In order to minimize the amount of side products, the reaction conditions were optimized with respect to the α,ω-alkanediamine concentration and reaction time. The following HPLC analyses were carried out. (i) After the aminolysis and removal of the excess of diamine, the products soluble in water were chromatographed to monitor the completeness of the base deprotection. (ii) This solution and the solid

support were ammonolyzed. The amount of oligonucleotides transaminated, hydrolyzed to the 3′-carboxyalkyl derivatives (**16a,b**), or ammonolyzed to 3′-carboxamidoalkyl derivatives (**15a,b**) was determined and compared to that of the desired aminoalkylated product (**17a,b-20a,b**). The proportion of **15a,b** is equal to the amount of the oligonucleotide remained bonded to the solid phase during the aminolysis with α,ω-alkanediamine. The results obtained are listed in Table 2. The most relevant observations may be summarized as follows. (i) α,ω-Alkanediamines do not deprotect, at concentrations less than 50 % (v/v), all the base residues even in 18 h. An additional treatment with aqueous ammonia is thus necessary, quite independently of which diamine is employed. (ii) The benzoylated cytosine moieties are not markedly transaminated, when the aminolysis with diamines is carried out in aqueous solution instead of organic solvent. (iii) Higher concentrations of α,ω-alkanediamines and/or longer reaction times are needed to cleave the oligonucleotides from support **2** than from supports **3,4**. This difference in susceptibility to aminolysis is expected on the basis of the carbonyl group substitution.[15] Moreover, hydrolysis to **16a,b** competes from 1.5 to 3 times more efficiently with aminolysis from **2** than from **3,4**.

In summary, the solid supports **3** and **4** (based on long chain alkylamine controlled pore glass and aminomethyl polystyrene, respectively) give the highest yields of 3′-aminoalkyl derivatized oligonucleotides, and they also allow the introduction of the amino tails under mildest conditions. Ion exchange HPLC profiles of crude **18a** and **20a** are shown as illustrative examples on Fig. 3. The only marked side product, *viz.* the 3′-carboxyalkyl oligonucleotide, **16a**, is easy to separate by ion exchange chromatography from the desired aminoalkyl product. With these supports, the aminolysis with diamines is a rather fast reaction in aqueous solutions, being usually completed within 10 h. Furthermore, the reaction time is not crucial: the yield of **17a-20a** did not appreciably decrease on a prolonged treatment (24 h). About 90 % of the oligonucleotide material may be released in 2.5 h. If such short cleavage times are used, one should, however, carefully separate the solid and solution phase from each other before the subsequent ammonolysis. Otherwise the desired oligonucleotide will be contaminated by the corresponding carboxamido derivative (**15a**).

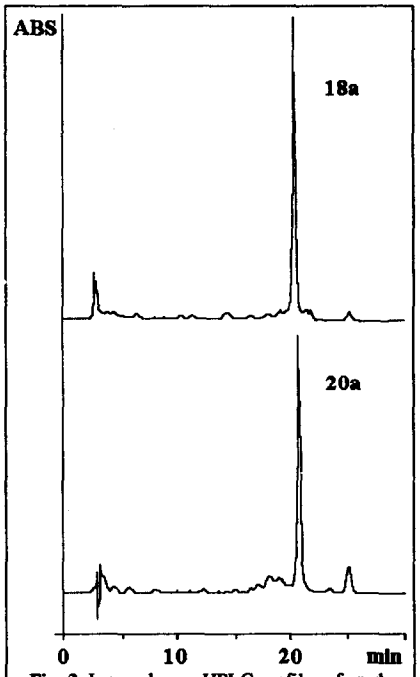
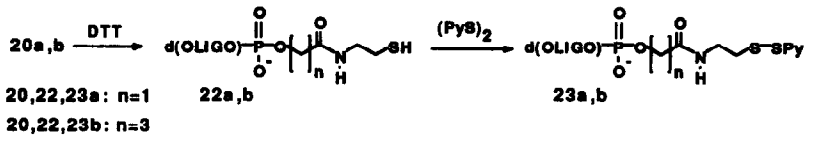


Fig. 3. Ion exchange HPLC profiles of crude **18a** and **20a** prepared on **3** and **4**, respectively.

Release of the masked thiol function. - The method described above allows a simple attachment of a cystamine arm, containing a masked mercapto group, to the 3′-end of oligonucleotide, as demonstrated by the preparation of oligonucleotides **20a,b**. The mercapto function was subsequently released by reducing the disulfide bond with 1,4-dithio-**D,L**-threitol (*Scheme 7*). The 3′-mercaptoalkyl oligonucleotides (**22a,b**) obtained were isolated by reversed phase HPLC, and the presence of the mercapto group was shown by quantitative conversion of **22a,b** to their 2-pyridyldithio derivatives (**23a,b**) under the conditions described previously.[7a]



Scheme 7. DTT - 1,4-dithio-D,L-threitol; $(PyS)_2$ - 2,2'-dipyridyl disulfide.

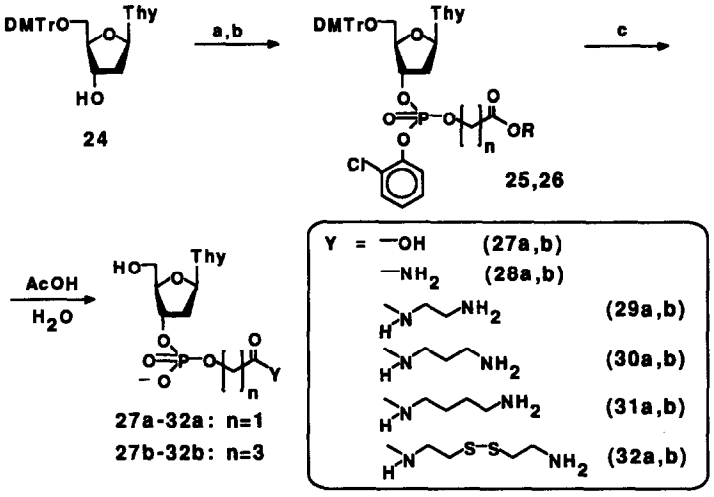
Table 2. Deprotection procedures to obtain the 3'-modified oligonucleotides **15a,b-20a,b**.

Solid Support	Protected oligo	Product	Deprotection procedure	Product distribution, %[a]				
				target oligo	trans amination	15[b]	16	others
5	10	15b	conc. $NH_3 \cdot H_2O$; 7 h at 55 °C	97	-	97	3	-
1	11	15b		98	-	98	2	-
2	12	15b		94	-	94	6	-
3	13	15a		95	-	95	5	-
4	14	15a		95	-	95	5	-
5	10	16b	(i) 0.1 M aq. NaOH; 4 h at r.t;	98	-	2	98	-
1	11	16b	(ii) conc. $NH_3 \cdot H_2O$; 7 h at	95	-	5	95	-
2	12	16b	55 °C.	100	-	-	100	-
3	13	16a		100	-	-	100	-
4	14	16a		100	-	-	100	-
5	10	17b	(i) hydrazine acetate[c]; (ii) 1,2-	87	10	-	3	1(21)
5	10	18b	ethanediamine or 1,3-propane-	87	13	-	<1	<1(21)
1	11	17b	diamine in *i*PrOH (50 %, v/v);	78	21	1	-	-
1	11	18b	8 h at r.t.	78	16	2	2	-
5	10	19b	(i) hydrazine acetate[c]; (ii) 0.5 M	72	23	1	3	-
1	11	19b	DBU in 1,3-butanediamine / *i*PrOH (50 %, v/v); 16 h at r.t	88	12	-	<1	-
5	10	20b	(i) hydrazine acetate[c]; (ii) 0.5 M DBU	74	7	-	3	19(21)
1	11	20b	in cystamine/Py (50 %, v/v);36 h at r.t.	67	14	8	11	-
2	12	17b	(i) 20 % aq. α,ω-alkanediamine;	81	4	-	15	-
2	12	18b	18 h at r.t; (ii) conc. $NH_3 \cdot H_2O$;	85	3	-	12	-
2	12	19b	7 h at 55 °C	83	2	-	15	-
2	12	20b	(i) 30 % aq. cystamine 18 h at r.t. (ii) conc. $NH_3 \cdot H_2O$; 7 h at 55 °C	85	2	-	13	-
3	13	17a	(i) 10 % aq. α,ω-alkanediamine;	89	<1	2	9	-
3	13	18a	10 h at r.t; (ii) conc. $NH_3 \cdot H_2O$;	91	<1	1	8	-
3	13	19a	7 h at 55 °C	90	<1	2	8	-
4	14	17a		89	<1	2	9	-
4	14	18a		93	<1	3	4	-
4	14	19a		89	<1	3	8	-
3	13	20a	(i) 20 % aq. cystamine; 10 h at r.t;	88	-	5	7	-
4	14	20a	(ii) conc. $NH_3 \cdot H_2O$; 7 h at 55 °C 90	90	-	2	8	-

a. peaks resulting from the capped oligos have not been included in calculation; b. equal to the amount of oligonucleotide material remaining on solid support after treatment with nucleophile; c. for the conditions, see experimental section.

ymatic digestion of the oligonucleotides prepared. - The structures of the modified oligonucleotides prepared were verified by enzymatic digestion. Accordingly, **15a,b-20a,b** were digested by phosphodiesterase I in the presence of alkaline phosphatase, and the product distribution was analyzed by reversed phase HPLC (Table 3 and Fig. 4). Along with the natural deoxynucleosides, the enzymatic digestions gave in each case an additional product, the identity of which was verified by HPLC comparison with the authentic samples obtained as described below. The ratios of the digestion products were found to be in agreement with the predicted values. By contrast, digestion with phosphodiesterase II and alkaline phosphatase resulted in hydrolysis of the modified phosphodiester bond at 3′-terminus, and only nucleosides were detected as products.

Modified thymidine 3'-alkylphosphates were synthesized according to *Scheme 8*: 5′-*O*-(4,4′-dimethoxytrityl)thymidine (**24**) was phosphorylated with 2-chlorophenylphosphoro-bis-(triazolide) and converted to phosphorotriesters with ethyl 4-hydroxybutyrate or methyl glycolate. The products, **25** and **26**, were isolated by silica gel chromatography and treated with appropriate nucleophiles in water. After subsequent detritylation, the desired products **27a,b-32a,b** were purified by preparative reversed phase HPLC. Their structure was confirmed by ^{1}H and ^{31}P NMR spectroscopy (Tables 4 and 5).



Scheme 8. **a.** 2-chlorophenylphosphoro-bis-(triazolide); **b.** methyl glycolate or ethyl 4-hydroxybutyrate **c.** appropriate nucleophile in water. **25**: R = Me; n=1; **26**: R = Et; n=3.

Concluding remarks. Among the solid supports prepared, the *O*-[*O*-(4,4'-dimethoxytrityl)glycolyl] glycolyl derivatized long chain alkylamine controlled pore glass **3** or aminomethyl polystyrene **4** give the best yields (*ca* 90 %) of oligonucleotides bearing 3′-functional group under mildest conditions, and can be used in routine preparation of 3'-tethered oligonucleotides. If necessary, longer spacer arm may be introduced by using support **2**. However, the conditions needed to introduce the 3′-end modifications are slightly more drastic. The usage of solid supports **1** and **5** is limited.

Table 3. Product distribution after the digestion of the 3′-modified oligonucleotides (**15a,b-20a,b**) by phosphodiesterase I and alkaline phosphatase.[a]

Oligo	dG	dC	T	T*	dA
15a	5.89	2.87	3.01	0.96 (**28a**)	1
16a	5.93	2.69	3.10	0.96 (**27a**)	1
17a	6.01	2.98	3.02	0.98 (**29a**)	1
18a	5.97	2.97	3.03	0.96 (**30a**)	1
19a	6.02	3.17	3.02	0.92 (**31a**)	1
20a	5.89	3.00	2.99	0.99 (**32a**)	1
15b	5.84	2.84	3.02	1.09 (**28b**)	1
16b	6.16	2.85	3.02	1.11 (**27b**)	1
17b	6.20	2.96	3.02	0.90 (**29b**)	1
18b	6.09	3.00	3.03	1.05 (**30b**)	1
19b	6.09	2.87	3.05	0.99 (**31b**)	1
20b	6.03	3.01	3.03	0.95 (**32b**)	1

a. Relative ratios, compared to 2′-deoxyadenosine (=1). Theoretical ratio dG:dC:T:T*:dA = 6:3:3:1:1, where T* is **27a,b-32a,b**.

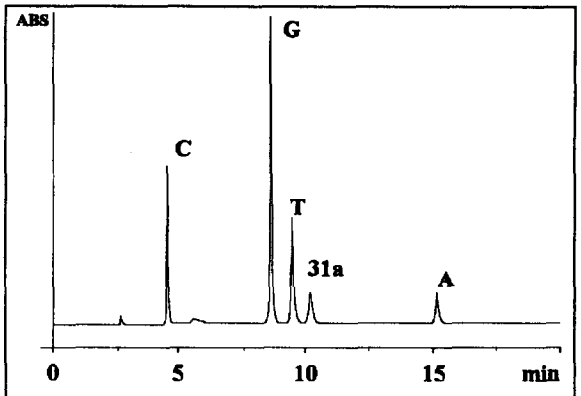


Fig. 4. HPLC profile after enzymatic digestion of **19a**.

EXPERIMENTAL

General. Thymidine and long chain alkylamine controlled pore glass were purchased from Sigma, and reagents for machine assisted oligonucleotide synthesis from ABI (Applied Biosystems; Foster City, CA). Cystamine dihydrochloride (Aldrich) was converted to free base form[16] and used without purification. Aminomethyl polystyrene (1000 Å; 23 μmol of NH_2 groups per gram) was a generous gift of ABI. Adsorption column chromatography was performed on colums packed with Kieselgel 60 (Merck). Preparative TLC was conducted on Kieselgel 60 F_{254} plates (Merck). Phosphodiesterase I (from *Crotalus adamanteus*) and phosphodiesterase II (from bowine spleen) were purchased from USB and calf thymus alkaline phosphatase from Boehringer Mannheim. NMR spectra were recorded on Jeol GX-400 spectrometer operating at 399.8 and 161.9 MHz for ^1H and ^{31}P, respectively. The signal of HDO was used as internal (^1H) and H_3PO_4 as external (^{31}P) reference. UV spectra were recorded on Perkin Elmer Lamda-2 spectrophotometer.

HPLC techniques. HPLC analyses were carried out on Merck - Hitachi instrument consisted of L-6200A Gradient Pump, L-4000 UV Detector, and D-2500 Chromato - Integrator. The crude reaction mixtures containing the synthesized and deprotected oligonucleotides were analyzed by ion exchange chromatography (column: Synchropak AX-300, 4.6 x 250 mm, 6.5 μm, SynChrom; buffer A: 0.05 M KH_2PO_4 in 50 % aq. formamide, pH 5.60; buffer B: A + 0.6 M $(NH_4)_2SO_4$; flow rate: 1 mL min^{-1}; a linear gradient from 10 to 80 % B in 30 min). The oligonucleotides were then preparatively isolated under the same conditions, purified by reversed phase chromatography (column: Nucleosil 300-5C18, 4.0 x 250 mm, Macherey-Nagel; buffer A: 0.05 M aq. NH_4OAc; buffer B: 0.05 M NH_4OAc in 50 % aq. MeCN (v/v); flow rate: 1 mL min^{-1}; a linear gradient from A to 40 % B in 45 min), and finally desalted by gel filtration (column: TSKgel G2000SW 7.5 x 300 mm, Toso Haas; water; flow rate: 0.5 mL min^{-1}). Thymidine 3′-phosphodiesters **27a,b-32a,b** were purified by

semipreparative reversed phase chromatography (column: Reliasil 300A 9.0 x 300, 5 μm, Column Engineering; flow rate: 3 mL min^{-1}); isocratic elution of water containing 1-3 % MeCN). The product distributions after enzymatic digestions of oligonucleotides were determined by reversed phase chromatography (column: Hypersil C-18; 4.6 x 240 mm, 6 μm; Merck; an isocratic elution of 0.05 M NH_4OAc containing 1-4 % MeCN; flow rate: 1 mL min^{-1})

Synthesis of the solid supports **1 - 3**. - Long chain alkylamine controlled pore glass (1.0 g) was treated with the mixture of 10 % Et_3N in 80 % aqueos ethanol, washed with acetonitrile and dried. **6a** (0.50 mmol, 0.23 g) or **6b** (0.50 mmol, 0.25 g) as pyridinium salt,[12] *N,N'*-diisopropylcarbodiimide (1.0 mmol, 157 μL), and *N*-hydroxysuccinimide (0.5 mmol, 58 mg) were added to the suspension of solid support in dry pyridine (5 mL), and the mixture was shaken overnight at room temperature. The suspension was filtered, washed with pyridine, kept in the mixture of Ac_2O:pyridine:*N*-methylimidazole (1:5:1, v/v) for 10 min, and finally washed with ether. The material obtained was washed with dichloroethane containing 5 % of dichloroacetic acid, with dichloromethane and finally dried *in vacuo* to give **7a,b**. 2,4,6-Triisopropylbenzenesulfonyl chloride (0.9 mmol, 273 mg, predissolved in 500 μL of pyridine), **6a** or **6b** (1.0 mmol, 0.49 g) and *N*-methylimidazole (72 μL, 0.9 mmol) were added to the suspension of **7a,b** (0.5 g) in dry pyridine (2.5 mL). The mixture was shaken overnight at room temperature and filtered. After capping of the unreacted hydroxyl functions of **7a,b**, the solid supports **1-3** were washed and dried. Loadings of the spacer arms according to DMTr-cation assay[17] for **1-3** were 20, 21 and 15 μmols per gram, respectively.

Synthesis of the solid support **4**. - Aminomethylated polystyrene beads (0.5 g) were suspended in dry pyridine (2 mL). 2,4,6-Triisopropylbenzenesulfonyl chloride (0.45 mmol, 137 mg, predissolved in 250 μL of pyridine), **6a** (0.5 mmol) and *N*-methylimidazole (36 μL, 0.45 mmol) were added. The mixture was shaken overnight at room temperature and filtered. After capping and detritylation, as described above for **7a,b**, another coupling was performed under the same conditions, followed by capping and final washing. The product, **4**, contained 5 μmols of DMTr-groups per gram.

3-Hydroxypropyl 4-(4,4'-dimethoxytrityloxy)butyrate **8**. - Compound **6b** (2.50 g, 5.0 mmol; as pyridinium salt), dry 1,3-propanediol (25 mmol, 1.81 mL) and DMAP (0.25 mmol, 30 mg) were dissolved in dichloromethane (25 mL), and DCC (5.5 mmol, 1.13 g; predissolved in 2 mL of CH_2Cl_2) was added. After 4 h at ambient temperature the *N,N'*-dicyclohexylurea formed was filtered. The filtrate was diluted with methylenechloride (50 mL), washed with aq. $NaHCO_3$ (2 x 50 mL), dried over Na_2SO_4, and concentrated. The resulting oil was purified on silica gel column, eluting with dichloromethane containing 2 % of MeOH and 0.5 % of pyridine (v/v). Pure fractions were pooled and concentrated to give **8** (1.86 g, 80 %) as a pale yellow oil. ^1H NMR ($CDCl_3$): 7.42 - 6.81 (13H, DMTr); 4.21 (2H, t, $OCOCH_2$); 3.79 (6H, s, 2 x OCH_3); 3.62 (2H, m, CH_2OH); 3.09 (2H, t, OCH_2); 2.45 (2H, t, CH_2COO); 1.92 (2H, p, CH_2); 1.82 (2H, p, CH_2); 1.66 (1H, br, *OH*). Found: C, 72.01; H, 6.89 %. Calcd. for $C_{28}H_{32}O_6$: C, 72.38; H, 6.95 %.

Triethylammonium 3-[4-(4,4'-dimethoxytrityloxy)butyryloxy]propyl succinate **9**. - Compound **8** (0.50 mmol, 0.266 g) was dried by coevaporation with dry pyridine (3x25 mL), and dissolved in 5 mL of the same solvent. Succinic anhydride (1 mmol, 200 mg) and DMAP (0.025 mmol, 3 mg) were added and the mixture was stirred

overnight at room temperature. The reaction was quenched with ice and concentrated. The residue was dissolved in ethyl acetate (25 mL) and washed twice with aq. $NaHCO_3$ (25 mL). The organic layer was dried (Na_2SO_4) and concentrated. Purification on preparative TLC plate (CH_2Cl_2 /EtOH /Et_3N, 94:5:1, v/v) gave **9** (0.46 g, 91 %) as a pale yellow oil. ^1H NMR ($CDCl_3$): 7.26 - 6.83 (13H, DMTr); 4.16, 4.12 (4H, 2x t, 2 x CH_2OCO); 3.09 (2H, t, OCH_2); 2.66 (4H, m, 2 x CH_2COO); 2.42 (2H, t, CH_2COO^-); 1.92 (4H, m, 2 x CH_2). The spectrum exhibited also signals of the triethylammonium group. For elemental analysis a part of the oil obtained was dissolved in CH_2Cl_2 and washed several times with sat. $NaHCO_3$. The organic phase was dried over Na_2SO_4, concentrated and kept overnight *in vacuo* to give **9** as sodium salt. Found: C, 65.26; H, 5.78 %. Calcd. for $C_{32}H_{35}NaO_9$: C, 65.50; H, 6.02 %.

Synthesis of the solid support **5**. - Compound **9** was bound to long chain alkylamine controlled pore glass by the carbodiimide method described for **7a,b**. DMTr-cation assay showed a loading of 21 μmol of DMTr-groups per gram.

Synthesis of protected oligodeoxyribonucleotides. - The oligonucleotides **10-14** were assembled on an Applied Biosystems 392 DNA Synthesizer using the solid supports **1-5** (0.04 -1.0 μmol scale). The corresponding natural sequence was synthesized by using a commercial thymidine derivatized column. Phosphoramidite chemistry and recommended protocols (DMTr-Off synthesis) were used in all cases.

Preparation of 3'-carboxamido derivatized oligonucleotides (**15a,b**). - The protected oligonucleotides **10-14** were kept in concentrated aqueous ammonia (2 mL) for 7 h at 55 °C. The resulting solutions were evaporated to dryness. The crude products were dissolved in water (1 mL) and purified by HPLC.

Preparation of 3'-carboxy derivatized oligonucleotides (**16a,b**). - The protected oligonucleotides **10-14** were treated with aq. NaOH (0.1 M; 0.5 mL) or aq. DBU (0.5 M; 0.5 mL) for 4 h. The solution was diluted with concentrated ammonia (2 mL), kept for 7 h at 55 °C, and concentrated to one half. The residue was neutralized with acetic acid and evaporated to dryness. Purification of **16a,b** was performed as described above.

Introduction of amino function at the 3'-terminus of oligonucleotides assembled on the solid supports **1,5**. - The oligonucleotides attached to the solid supports (**10** and **11**) were treated with 0.5 M hydrazine in pyridine:acetic acid (4:1, v/v) for 24 h,[14] washed with MeOH (5 mL) and acetonitrile (5 mL), and finally dried *in vacuo*. The debenzoylated material was transferred to 1.5 mL test tube and a solution of α,ω-alkanediamine (50 % in *i*-PrOH) or cystamine (50 % in pyridine) containing 0.5 M DBU was added (for reaction times and temperatures, consult Table 2). After appropriate time period, the mixture was diluted with aqueous pyridine (30 %; 15 mL), passed through Dowex-50x8 resin (pyridinium form; 2 mL) to remove the excess of diamine, and concentrated *in vacuo*. After coevaporation with water (2x3 mL), the crude product was dissolved in the same solvent, and purified by HPLC.

Introduction of amino function at the 3'-terminus of oligonucleotides assembled on the solid supports **2-4**.

Optimization experiments. - Part of the oligonucleotide attached to the solid supports (*cca.* 0.02 μmol of **12** or

13) was placed into 1.5 mL test tube. A solution of appropriate α,ω-alkanediamine or cystamine (50 μL; 5, 10, 20, 50 % in water or 5, 50 % in *i*-PrOH) was added, and the mixture was incubated a fixed time period (2.5, 5, 10, 18 h) at room temperature. Dioxane (1.3 mL) was added and the resulting suspension was shaken vigorously and centrifuged for 1 min. The supernatant was discarded, and the precipitate was washed with fresh dioxane (2x1.3 mL). After removal of the organic solvent, water (0.5 mL) was added to the residue, and the test tube was shaken and centrifuged. The progress of deprotection was monitored by taking a probe of the supernatant (25 μL) and injecting it into an ion exchange column. Main material was dried in a vacuum centrifuge. The cleavage of oligonucleotide material from the solid support was completed by adding aqueous ammonia (1 mL) to the residue and keeping the mixture at room temperature for 2 h. The liquid phase was collected in a screw-cap vial, kept at 55 °C for 7 h, evaporated, and analyzed by ion exchange chromatography. Peaks of oligonucleotides **15,16** and the appropriate target oligo **17-20** were integrated. The total yield of cleavage during diamine treatment was calculated from the ratio A(**16**+T) / A(**15**+**16**+T), where A = integration area of each peak, T - corresponding target oligonucleotide **17a,b-20a,b**. The optimized reaction conditions together with the yields of 3′-aminoalkylated oligonucleotides (**17a,b-20a,b**) and side products (**15a,b** and **16a,b**) are presented in Table 2.

Optimized procedures to introduce amino function at 3'-terminus of oligonucleotides assembled on the supports **2-4**.

A. Conventional method. - Protected oligonucleotide **12-13** was placed into 1.5 mL test tube. A solution of α,ω-alkanediamine or cystamine (150 μL; for concentrations, see Table 1) was added, and the mixture was gently shaken for 10 h (standard procedure) or for 2.5 h (fast cleavage) at room temperature. Dioxane (1.3 mL) was added, the resulting suspension was shaken vigorously and centrifuged for 1 min. The supernatant was discarded, and the precipitate was washed with fresh dioxane (2x1.3 mL). After removal of dioxane the test tube was filled with conc. aqueous ammonia, shaken, and centrifuged for 1 min. The supernatant was collected in a screw-cap vial, kept at 55° C for 7 h, evaporated, and chromatographed.

B. Two syringe method. - The synthetic column containing one of oligonucleotides **12-14** attached to the solid supports **2-4** was attached to two 1 mL syringes, one of those loaded with aqueous α,ω-alkanediamine or cystamine (0.5 mL; for concentrations, see Table 2). The solution was flushed through the column for several times, and the reaction mixture was left for 10 h (standard procedure) or for 2.5 h (fast cleavage) at room temperature. The resulting mixture was withdrawn into one of the syringes and transferred into screw-cap vial. The support remaining in the column was washed with conc. aqueous ammonia (2 mL), and the washings were collected to the same vial. The solution was kept at 55 °C for 7 h and evaporated. Low-boiling α,ω-alkanediamines were removed by evaporation followed by coevaporation with water (4x3 mL). Removal of cystamine by filtering through Dowex 50x8 resin is described above for the solid supports **1,5**. The target oligonucleotides were isolated by ion exchange chromatography.

3′-(ω-alkoxycarbonylalkyl, 2-chlorophenyl) esters of 5′-O-(4,4′-dimethoxytrityl)thymidine 3′-monophosphate (**25, 26**). - The compounds **25,26** were prepared by a modification of the reported method.[18] Dry triethylamine (2.55 mL 18.4 mmol) and freshly distilled 2-chlorophenyl phosphorodichloridate (1.51 mL, 9.18 mmol) were added to the suspension of dry triazole (1.27 g, 18.4 mmol) in acetonitrile (30 mL). The mixture was stirred at room temperature for 40 min and filtered on predried 5′-*O*-(4,4′-dimethoxytrityl)thymidine **24**[19] (2.50 g, 4.56

Table 4. ^{1}H NMR chemical shifts (in D_2O) of compounds **27a,b-32a,b**.

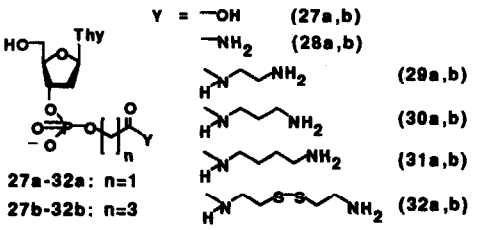
Compound	H-6	H-1′	H-2′ / H-2″	H-3′	H-4′	H-5′ / H-5″	$POCH_2$	5-Me	others
27a	7.46	6.10	2.20 / 2.34	4.63	3.99	3.63 / 3.59	4.30	1.68	
28a	7.48	6.14	2.25 / 2.36	4.65	4.02	3.65 / 3.61	4.19	1.71	
29a	7.46	6.09	2.23 / 2.35	4.64	4.01	3.66 / 3.60	4.23	1.69	3.43 ($CONHCH_2$); 3.02 (CH_2NH_2)
30a	7.46	6.12	2.23 / 2.34	4.65	4.01	3.65 / 3.59	4.19	1.70	3.20 ($CONHCH_2$); 2.85 (CH_2NH_2); 1.74 (CH_2)
31a	7.47	6.11	2.23 / 2.34	4.63	4.01	3.63 / 3.59	4.19	1.70	3.14 ($CONHCH_2$); 2.84 (CH_2NH_2); 1.52 and 1.46 (2xCH_2)
32a	7.49	6.14	2.26 / 2.37	4.64	4.02	3.67 / 3.62	4.21	1.73	3.46 ($CONHCH_2$); 3.19 (CH_2S); 2.83 (CH_2NH_2); 2.76 (SCH_2)
27b[a]	7.65	6.31	2.40 / 2.52	4.75	4.17	3.84 / 3.77	3.90	1.88	2.48 (CH_2COO^-); 1.92 (CH_2)
28b	7.51	6.16	2.23 / 2.37	4.70	4.04	3.68 / 3.63	3.76	1.74	2.24 (CH_2CONH_2); 1.78 (CH_2)
29b	7.51	6.16	2.26 / 2.27	4.65	4.04	3.70 / 3.63	3.76	1.74	3.02 ($CONHCH_2$); 2.83 (CH_2NH_2); 2.16 (CH_2CONH); 1.78 (CH_2)
30b	7.48	6.13	2.21 / 2.32	4.58	4.07	3.66 / 3.60	3.71	1.71	3.01 ($CONHCH_2$); 2.84 (CH_2NH_2); 2.15 (CH_2CONH); 1.75 and 1.50 (2xCH_2)
31b	7.47	6.12	2.20 / 2.33	4.59	4.00	3.65 / 3.60	3.71	1.70	3.03 ($CONHCH_2$); 2.82 (CH_2NH_2); 2.18 (CH_2CONH); 1.74; 1.50; 1.42; (3 x CH_2)
32b	7.52	6.16	2.26 / 2.37	4.60	4.05	3.70 / 3.63	3.75	1.75	3.38 ($CONHCH_2$); 3.22 (CH_2S); 2.84 (CH_2NH_2); 2.73 (SCH_2) 2.25 (CH_2CONH); 1.78 (CH_2)

a. as tetramethylguanidinium salt

Table 5. ^{1}H,^{1}H coupling constants,[a] ^{31}P NMR chemical shifts[a,b] and HPLC retention times[c] of **27a,b-32a,b**.

Compound	J(1′,2′)	J(1′,2″)	J(2′,2″)	J(2′,3′)	J(2″,3′)	J(4′,5′)	J(4′,5″)	J(5′,5″)	δ (^{31}P)	t_R (min)
27a	6.8	6,8	14.1	6.9	2.4	3.4	4.4	12.7	-1.01	2.8
28a	7.3	6.8	14.2	7.3	3.4	3.4	4.9	12.2	-1.98	4.5
29a	6.8	6.4	14.7	6.8	2.9	3.4	4.9	12.2	-1.30	5.9
30a	7.3	6.3	14.2	6.8	2.9	3.4	4.9	12.2	-1.33	6.8
31a	7.8	6.3	14.2	7.3	2.9	3.4	4.9	12.2	-1.93	7.8
32a	6.4	6.8	14.2	7.3	2.9	3.4	4.9	12.2	-2.10	25.4
27b[c]	7.3	6.7	14.4	7.6	3.1	3.4	4.9	12.5	0.23	3.0
28b	7.3	6.8	14.3	d	2.7	3.4	4.9	12.7	-3.71	5.6
29b	7.6	6.4	14.3	d	3.1	3.4	4.9	12.5	-3.65	7.7
30b	7.0	6.4	14.0	d	3.1	3.4	4.6	12.5	-0.31	8.7
31b	7.0	6.4	14.3	7.6	3.4	3.4	4.9	12.5	-0.23	10.9
32b	6.8	6.8	14.2	d	3.4	3.4	4.4	12.2	-3.64	35.2

a. in D_2O; b. external reference H_3PO_4; c. isocratic elution of 0.05 M NH_4OAc + 4 % MeCN, for other detales, see experimental section; d. Could not be measured due to overlapping; e. as tetramethylguanidinium salt.



mmol). After 10 min, dry methyl glycolate (in the case of **25**; 1.42 mL, 18.4 mmol) or ethyl 4-hydroxybutyrate[20] (in the case of **26**; 2.34 mL, 18.4 mmol) were added. The mixture was stirred for 4 h and quenched by addition of ice. The mixture was concentrated, dissolved in methylene chloride (50 mL) and extracted twice with an equal volume of sat. $NaHCO_3$. The organic layer was dried over Na_2SO_4, and concentrated. Purification on silica gel column, eluting with dichloromethane containing 2 % of MeOH and 0.1% of pyridine (v/v) gave **25** or **26** as white foam. ^{31}P NMR ($CDCl_3$, external ref. H_3PO_4 at 0.00 ppm) **25**: -8.25 ppm; **26**: -7.60 ppm.

Thymidine 3'-(ω-carboxyalkyl)phosphates (**27a,b**). - *Method A.* Compound **25** (0.24 mmol) was dissolved in 0.1 M aq. NaOH containing 5 % pyridine (20 mL) and kept overnight at room temperature. The mixture was concentrated and the residue was dissolved in 80 % aq. acetic acid (20 mL) and stirred at room temperature for 1 h. The mixture evaporated *in vacuo*, suspended in water (50 mL) and extracted with diethyl ether (5 x 50 mL). The crude product was purified on a semipreparative RP-column to give **27a** as a sodium salt. *Method B.* The solution of *syn*-2-nitrobenzaldoxime (225 mg, 1.36 mmol) and 1,1,3,3-tetramethylguanidine (160 µL, 1.36 mmol) in dioxane:water (3 mL; 1:1, v/v) was added to **26** (0.2 g, 0.24 mmol). The mixture was kept for 20 h at room temperature and evaporated. Detritylation and work up were performed as above. Purification by HPLC as described above gave **27b** as tetramethylguanidinium salt.

Thymidine 3'-(ω-carboxamidoalkyl)phosphates (**28a,b**). - **25** or **26** (0.25 mmol) was dissolved in aqueous ammonia containing 5 % pyridine (25 mL), and stirred overnight at ambient temperature. The mixture was concentrated *in vacuo*. Detritylation, work up and purification were performed as described above.

*Thymidine 3'-[*N*-(ω-aminoalkyl)-ω-carboxamidoalkyl]phosphates* (**29a,b-31a,b**) and *thymidine 3'-[*N*-(6-amino-3,4-dithiahexyl)-ω-carboxamidoalkyl]phosphates* (**32a,b**). - **25** or **26** (0.25 mmol) was dissolved in 2 M aq. α,ω-alkanediamine (*i.e.* 1,2-ethanediamine, 1,3-propanediamine or 1,4-butanediamine) or cystamine (10 mL) and stirred overnight at ambient temperature (in the case of cystamine 48 h at 55 °C). The reaction mixture was concentrated, neutralized with aq. KH_2PO_4 and extracted with methylenechloride. The organic phase was evaporated and dissolved in 80 % aq. acetic acid (25 mL). After 1 h the mixture was concentrated, suspended in water (50 mL), and extracted with ether (5 x 50 mL). The final purification was performed by HPLC as described above. Compounds **27a,b-32a,b** exhibited identical UV spectra with λ_{max} (H_2O) at 267 nm. Their ^1H and ^{31}P NMR chemical shifts, $^1H,^1H$ coupling constants and HPLC retention times are listed in Tables 3 and 4.

Enzymatic digestion of the modified oligonucleotides **15a,b-20a,b**. - Each oligonucleotide (0.5 - 1.0 OD) was treated with phosphodiesterase I (from *Crotalus adamanteus*) and alkaline phosphatase overnight at 37 °C (0.05 M Tris·HCl, 0.10 M NaCl, 0.014 M Mg^{2+}; pH = 8.0). The products were analyzed by reversed-phase HPLC. The product distribution was determined by comparing the integration areas of each product to the areas of authentic samples of known concentrations. The extinction coefficients of thymidine 3'-phosphodiesters, **27a,b-32a,b**, were assumed to be the same as that of thymidine.

Acknowledgments. Financial support from the Research Council for Natural Sciences, the Academy of

Finland, is gratefully acknowledged. Authors wish to thank Dr. A. Andrus, Applied Biosystems, Inc. for providing aminomethylated polystyrene.

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(*Received in UK* 25 *February* 1994; *accepted* 15 *April* 1994)