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Solid support synthesis of ester linked hydrophobic conjugates of oligonucleotides

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Abstract

Novel non-nucleosidic phosphoramidite building block **2** was employed for multiple modification of oligonucleotides with hydrophobic octyl groups. Hydrophobic sites are attached *via* potentially biodegradable ester bonds that are demonstrated to withstand the conditions of DNA deprotection. The chimeric oligonucleotides are capable of forming triple helix complexes that are stabilized by forming a hydrophobic clamp consisting of terminal octyl groups. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: carboxylic acids and derivatives; nucleic acid analogues; phosphoramidites

INTRODUCTION

Antisense oligonucleotides are a novel class of potential therapeutics [1]. Major obstacles that prevent wider application of these oligonucleotides are their limited oral bioavailability and permeability through cell membranes. Modification of oligonucleotides with hydrophobic groups has been considered as one possible method to improve their pharmacokinetics. It has been demonstrated previously that even a single incorporation of a hydrophobic tether greatly enhances the cellular uptake [2,3] and antisense activity [4].

Oligonucleotides attached to lipophilic groups may become adhered to cell membranes and could therefore become unavailable for binding to mRNA [3]. This problem may be addressed by attachment of hydrophobic moieties *via* a biodegradable linker, an approach that is well known as a prodrug strategy for the delivery of therapeutic molecules [5]. Previously, several potentially cleavable linkers have been used to tether a hydrophobic conjugate group to a synthetic oligonucleotide. A tetrathymidylate sequence with unmodified phosphodiester bonds may in principle be cleaved by intracellular endonucleases considerably faster than the phosphorothioate backbone of a first generation antisense oligonucleotide [6,7]. More recently, a triglycyl spacer, the target for intracellular peptidases, has been used to conjugate cholesterol to the 3'-terminus of triple helix forming oligonucleotides [8]. A biodegradable

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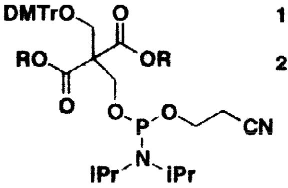
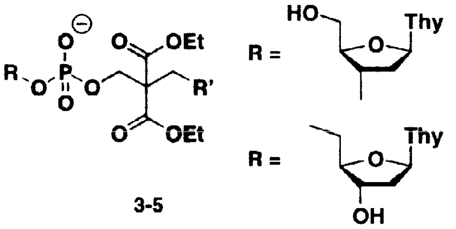


1: R = Et;

2: R = n-C_8H_{17}



3-5

3: R' = H

4: R' = $-OPO_2^{\ominus}$

5: R' = $-OPO_2^{\ominus}$

disulfide bridge has also been employed to attach an alkyl chain [9], a cholesteryl [10] or a phospholipid [11] group to the base moiety [9] or the 5'-terminus [10,11] of an oligonucleotide.

An ester bond may be hydrolyzed by intracellular esterases [12]. Attachment of a lipophilic linker through an ester bond is hence an attractive approach to obtaining prodrug forms of oligonucleotides. This approach has previously been employed for derivatization of oligonucleotides at the 5'-terminus with 1,2-di-*O*-miristoylglycerolphosphoric acid [13], dexamethasone [14], or palmitoyl [15] residues, or at internucleosidic phosphates with an *S*-pivaloylthioethyl group [16]. However, the generally known sensitivity of esters towards nucleophilic agents severely limits the applicability of this approach. Under conditions of oligonucleotide deprotection, esters are usually cleaved more rapidly than base protecting groups. The cleavage of the succinyl linker, the one most widely used in oligonucleotide synthesis, upon base deprotection serves as a well-known example [17]. Similarly, several solid supports and phosphoramidite reagents that we have previously introduced for terminal derivatization of oligonucleotides use the reactivity of the ester function towards nucleophiles [9,10]. In fact, the majority of methods reported for the preparation of ester conjugates of oligonucleotides involve performing the conjugation step postsynthetically in solution and thus lack the main advantages of solid phase synthesis [11,13,14]. Thus far, only oligothymidylate ester conjugates where the base residue required no protection have been assembled successfully using entirely solid phase chemistry [15,16]. For instance, the cleavage of 5'-palmitoyl decathymidylate from CPG with ethanolamine or aqueous ammonium hydroxide gave the desired conjugate in 68 and 15% yield, respectively [15]. Alternatively, use of a photolabile linker enabled preparation of phosphotriester oligonucleotide analogs uniformly modified with pivaloylthioethyl group [16].

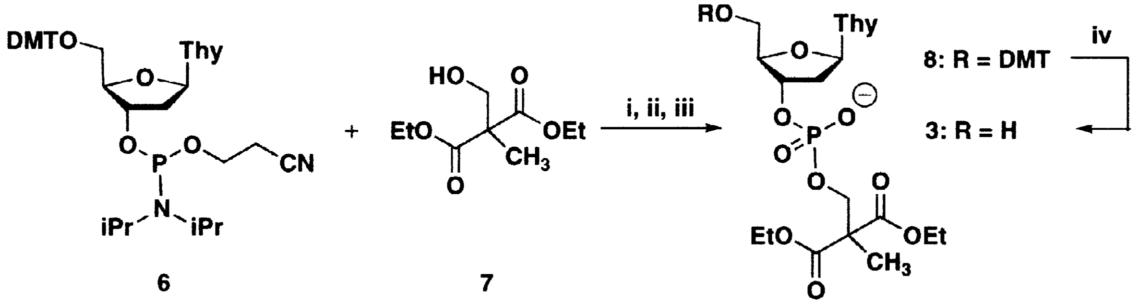
We now report on a novel phosphoramidite reagent **2** in which hydrophobic octyl groups are attached *via* an ester bond. We demonstrate that the use of **2** in DNA synthesis allows efficient multiple modification of oligonucleotides with lipophilic pendants.

RESULTS AND DISCUSSION

We previously reported several applications of novel phosphoramidite reagents derived from diamides of 2,2-bis(hydroxymethyl)malonic acid [18]. A structurally similar building block **1** has been designed for chemical phosphorylation of oligonucleotides [19]. Somewhat unexpectedly, neither of the two ester functions that the phosphoramidite **1** brought into an oligonucleotide reacted with ammonia, methylamine, or 1,3-propanediamine [19]. One tentative explanation was that the phosphodiester oligonucleotide backbone stabilized the proximate ester functions. To reveal this effect in more detail, three thymidine phosphates, **3-5**, were synthesized. The reactivity of these model compounds under conditions most frequently used for deprotection of synthetic oligonucleotides suggested that flanking the dialkylmalonate moiety with electron-rich phosphate groups severely affects the reactivity of ester functions towards nucleophiles. This observation was demonstrated to be of practical interest. A phosphoramidite reagent **2**, was synthesized and employed in the preparation of hydrophobic oligodeoxynucleotide conjugates which proved to be stable towards conditions of DNA deprotection. Finally, hybridization properties of these oligonucleotide conjugates were investigated.

Synthesis of model compounds.

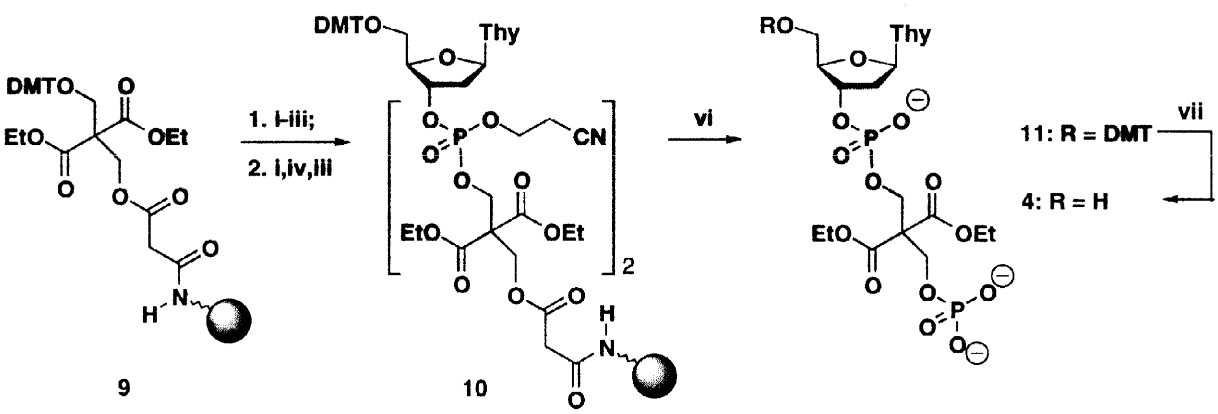
Three model thymidine phosphates, **3-5**, in which either one (**3**) or two phosphate residues (**4** and **5**) flank the 2,2-disubstituted diethyl malonate fragment, were synthesized.



Scheme 1. **i:** 1*H*-tetrazole/MeCN; **ii:** I_2/Py/H_2O/THF; **iii:** *t*-BuNH_2/MeCN; **iv**: 80% aq AcOH.

To obtain **3**, thymidine phosphoramidite **6** was coupled to diethyl 2-hydroxymethyl-2-methylmalonate **7** [20] in the presence of 1*H*-tetrazole as depicted in Scheme 1. The resulting phosphite triester intermediate was oxidized with iodine and the 2-cyanoethyl protecting group was cleaved with *t*-butylamine [21] to give **8**, which was isolated by column chromatography on silica gel and subsequently detritylated with 80% aq AcOH. The product was purified by semipreparative RP HPLC and desalted on the same column to afford **3** in 69% overall yield.

Compounds **4** and **5** were assembled on an ABI 392 DNA Synthesizer on 40 μmol scale. Accordingly, to obtain **4**, two elongation cycles were carried out. In the first cycle, the solid support **9** [22] was detritylated with 2% trifluoroacetic acid in CH_2Cl_2, and the solid support was washed with MeCN (Scheme 2). The free hydroxy group was reacted with phosphoramidite reagent **1** in the presence of 1*H*-tetrazole and the excess reagents were removed by washing. Standard iodine oxidation was followed by a MeCN wash to complete



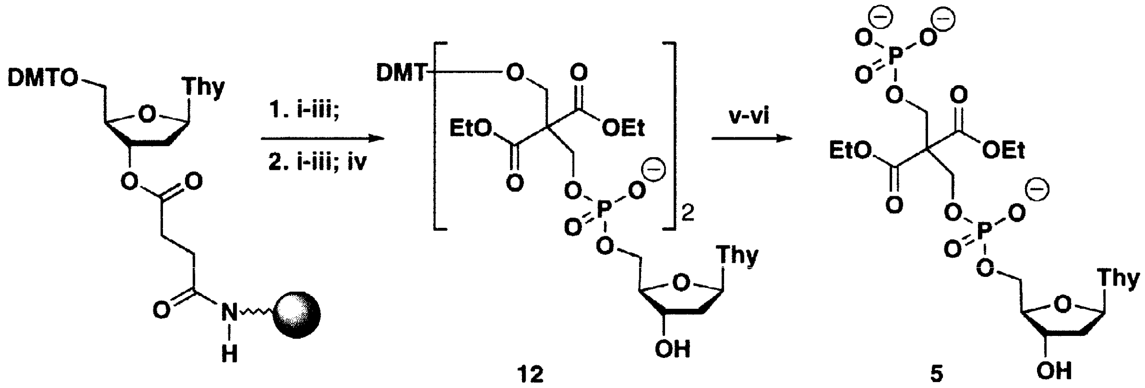
Scheme 2. **i**: CF_3COOH/CH_2Cl_2; **ii**: **1**/1*H*-tetrazole/MeCN; **iii**: I_2/H_2O/Py/THF; **iv** **6**/1*H*-tetrazole/MeCN; **vi**: conc. aq NH_3-H_2O/ 30 min/ r.t.; **vii**: 80% aq AcOH/20 min.

the cycle. The second cycle was carried out analogously using the phosphoramidite **6** in place of **1**. Support-bound **10** was treated with concentrated ammonium hydroxide. This effected release from the solid support. Simultaneously, the 3'-terminal non-nucleosidic moiety was degraded to release the 3'-phosphate group [22] thus giving rise to compound **11**. Finally, **11** was detritylated, and **4** was isolated in 75% yield by HPLC.

Compound **5** was synthesized analogously (Scheme 3). Starting from commercial CPG-thymidine, two successive coupling cycles using phosphoramidite **1** were carried out. The support-bound material was treated with concentrated aqueous ammonium hydroxide to give an intermediate **12**. This was detritylated, and the 5'-terminal non-nucleosidic moiety was removed with ammonia as reported previously [19]. The final product, **5**, was isolated by HPLC in 79% yield.

Stability of model nucleoside phosphates 3-5 towards the DNA deprotection conditions.

To examine the influence of adjacent phosphate residues on the reactivity of ester functions in more detail, model nucleoside phosphates **3**-**5** were subjected to treatment with a variety of bases. With possible practical application of the results in mind, conditions that are



Scheme 3. **i**: CF_3COOH/CH_2Cl_2; **ii**: **1**/1*H*-tetrazole/MeCN; **iii**: I_2/H_2O/Py/THF; **iv**: conc. aq NH_3-H_2O/ 2 h/ r.t.; **v**: 80% aq AcOH/ 20 min; **vi**: conc. aq NH_3-H_2O/ 0.5 h/ r.t.

most widely applied to the deprotection of synthetic oligonucleotides were chosen: a) conc. ammonium hydroxide at room and elevated temperatures; b) mixture of conc. ammonium hydroxide and 40% $MeNH_2$ (1:1) [23]; c) 1,2-ethanediamine (50% in EtOH) [24]; and d) 0.05 M K_2CO_3 in MeOH [25]. The data obtained is presented in Table 1 in terms of the half-lives of the disappearance of **3-5** and the percentage of their conversion under conditions required by a given protecting strategy.

Table 1.

Stability of **3-5** Towards the DNA Deprotection Conditions.

Reagent	Conditions	Appropriate Protection Strategy[a]	Half life $\tau_{1/2}$, h			Extent of conversion, %		
			3	**4**	**5**	**3**	**4**	**5**
conc. NH_3-H_2O	22 °C	-	3.5	66	75	-	-	-
	22 °C/1.5 h	AMB [25], *i*-PrOAc [26], PhOAc [27]	-	-	-	26	<2	<2
	50°C/2 h	a) A^{dmf}; C^{dmf}; G^{dmf} [28]	-	-	-	nd	6	6.5
	65°C/1 h	b) A^{bz}; C^{bz}; G^{dmf} [29]	-	-	-	nd	4	5
NH_4OAc/NH_3-H_2O (1:10)	50°C/1 h	A^{dmf}; C^{dmf}; G^{dmf} [28,30]	-	-	-	nd	<2	<2
Conc. NH_3-H_2O - 40% $MeNH_2$ (1:1)	22 °C	-	nd	6.9	7.5	-	-	-
	22 °C/1.5 h	A^{bz}; C^{Ac}; G^{ib} [23]	-	-	-	nd	14	13
50% 1,2-diaminoethane in EtOH	22 °C	-	15.5	78	119	-	-	-
	22 °C /7 h	A^{bz}; C^{bz}; G^{ib} [24]	-	-	-	27	6	4
0.05 M K_2CO_3 in MeOH	22 °C	-	4.9	90	81	-	-	-
	22 °C /6 h	AMB [25]	-	-	-	57	4.5	5.0

a. Abbreviations used for base protection groups: *i*-PrOAc, (isopropyloxy)acetyl; PhOAc, phenoxyacetyl; AMB, (2-acetoxy)benzoyl; dmf, *N,N*-dimethylformamidino; bz, benzoyl; ib, 2-methylpropionyl.

The results show that compounds **4** and **5** are dramatically less reactive under basic conditions than **3**. Depending on the conditions employed, **3** reacts from 5 to 20 times as fast as diphosphates **4** and **5**. This difference in reactivity is more pronounced in conc. ammonium hydroxide and 0.05 M K_2CO_3/MeOH (*ca.* 20 times) than in 50% ethanolic 1,2-diaminoethane (*ca.* 5 to 7 times). The increased stability of ester bonds in **4** and **5** may be attributed to the presence of an additional phosphodiester group that flanks the non-nucleosidic moiety. At the same time, location of the non-nucleosidic moiety (attachment at 5'- *vs.* 3'-hydroxy group of thymidine) does not significantly influence the reactivity of the ester groups.

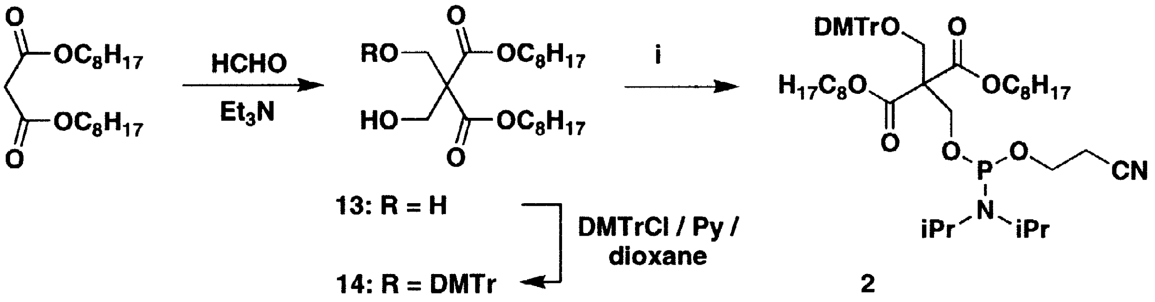
In another set of experiments, **3-5** were treated under conditions recommended for removal of protecting groups most widely used in DNA synthesis. The protection strategies, the corresponding deprotection conditions, and the observed extent of conversion of **3-5** are presented in Table 1. These results demonstrate that **3** is not sufficiently stable under conditions of DNA deprotection. In contrast, diphosphates **4** and **5** are stable in concentrated ammonium hydroxide at 22 to 50 °C for at least 1.5 h. They are moderately stable towards even more drastic deprotection conditions listed in Table 1, whereas treatment with ammonium hydroxide – methylamine [23] may not be recommended.

From the results obtained, one may conclude that the ester bond is not necessarily labile under the conditions of DNA deprotection. In particular, analogs of the phosphoramidite building blocks **1** can be used safely for the preparation of multiply labeled oligonucleotides. A tentative explanation of the above results is that the negative charge of proximal phosphate groups protects the carbonyl carbon from nucleophilic attack.

Synthesis of ester linked hydrophobic oligonucleotide conjugates on solid support

We demonstrated above that the proximate phosphate residues stabilize the carboxylic ester functions to the extent that they can withstand the conditions of DNA deprotection. This observation opened new possibilities for application of reagents analogous to **1** in oligonucleotide synthesis. The ethyl groups in **1** were substituted by pendants of practical interest and biological value, hydrophobic octyl groups. The utility of hydrophobic phosphoramidite, **2**, was rigorously tested to reveal its compatibility with the conditions of oligonucleotide synthesis and deprotection.

Synthesis of phosphoramidite building block 2. The hydrophobic dioctyl phosphoramidite **2** was prepared as described previously for **1** (Scheme 4) [19]. Accordingly, dioctyl malonate was *bis*hydroxymethylated with formaldehyde in the presence of triethylamine to give **13** [20]. Selective mono-*O*-protection with 4,4'-dimethoxytrityl chloride (0.8 equiv.) led to **14**, which was converted into phosphoramidite **2** with 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite in the presence of 1H-tetrazole. The building block **2** was purified by column chromatography on silica gel and characterized by ^{1}H, ^{13}C, and ^{31}P NMR, and combustion analysis. Phosphoramidite **2** was stable at –20 °C for at least 6 months.



Scheme 4. **i:** 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite / 1*H*-tetrazole / MeCN.

Preparation of hydrophobic oligonucleotides. The utility of building block **2** in DNA synthesis was demonstrated by preparation of oligonucleotide conjugates **16-22** (Table 2) where **Y** stands for a hydrophobic moiety derived from **2** (Scheme 5). The following observations appear relevant.

Detritylation of the non-nucleosidic moiety. The hydrophobic moiety **Y** could not be conveniently detritylated by standard reagents (3% DCA or 2% TCA in CH_2Cl_2). Therefore, synthetic columns were disconnected from the instrument, and the moiety **Y** was detritylated with trifluoroacetic acid (TFA, 2% in methylene chloride). Quantitative detritylation, achieved in 40s, was followed by washing with methylene chloride and MeCN. Despite the potential risk of acid catalyzed depurination, no chain scission with any of the oligonucleotides **16**, **17**, and **22** that required this treatment, was detected by HPLC. In contrast to the above, detritylation under aqueous conditions proceeded as for unmodified oligonucleotides. Hence treatment of the ammonia deprotected oligonucleotides with 80% aqueous acetic acid afforded removal of the DMT groups in 15 min.

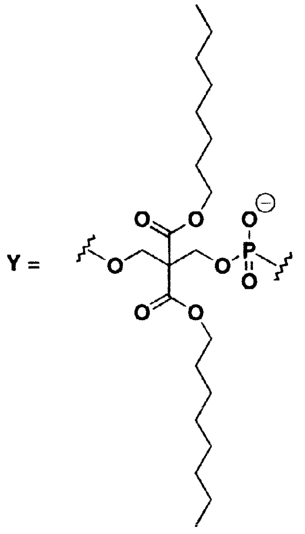
Condensation step. In contrast to previously reported structurally related building blocks [18,19], phosphoramidite **2** exhibited relatively slow coupling. Under standard conditions (0.1M **2** in MeCN, 1H-tetrazole as activator), acceptable coupling yields (>98%) were obtained only when a longer coupling time (600s) was employed.

Terminal protection of pseudo-5'- and 3'- hydroxy groups. Derivatives of *bis*(hydroxymethyl)malonic acid are known to decompose under basic conditions if they possess at least one free hydroxy group [18,19]. Protection of the terminal hydroxy groups of the non-nucleosidic moieties **Y** was therefore required during deprotection with ammonium hydroxide. At the 5'-terminus, this was readily achieved by subjecting 5'-DMT-protected oligonucleotides to the final ammonolysis. Alternatively, the 5'-terminal non-nucleosidic

Table 2.
Hydrophobic Oligonucleotide Conjugates.

Compd.	Oligonucleotide sequence	Retention time, min	
		C4^a	C18^a
DMTr-**15**	5'-DMTrGAACATCATGGTCGT-3'	-	19.4
16	5'-p**Y**GAACATCATGGTCGT-3'	-	26.4
17	5'-p**Y**$_2$GAACATCATGGTCGT-3'	-	36.2
18	5'-ACT$_{11}$CACACT$_{11}$CA**Y**OH-3'	16.4	22.5
19	5'-H**Y**ACT$_{11}$CACACT$_{11}$CA**Y**OH-3'	21.6	28.0
20	5'-ACT$_{11}$CACACT$_{11}$CA**Y**$_2$OH-3'	26.6	32.1
21	5'-H**Y**ACT$_{11}$CACACT$_{11}$CA**Y**$_2$OH-3'	27.6	33.4
22	5'-H**Y**$_2$ACT$_{11}$CACACT$_{11}$CA**Y**$_2$OH-3'	31.8	37.5
DMTr-**23**	5'-DMTr-ACT$_{11}$CACACT$_{11}$CA-3'	10.8	16.9

a. for conditions, see experimental section.



Scheme 5. Structure of non-nucleosidic moiety **Y**.

moiety could be permanently protected by phosphorylation with the aid of **1**. This was done for oligonucleotides **16 and 17** by the previously reported method [19].

Temporary protection of oligonucleotides at the 3'-terminus with a base-resistant group is not trivial. Hence, the pseudo 3'-hydroxy group of oligonucleotides **18-22** had to be protected permanently. This has been previously achieved by 3'-phosphorylation [18] with the aid of *bis*(2-hydroxyethyl)sulfone derivatized solid supports. The latter, however, require prolonged treatment with ammonium hydroxide at elevated temperature to effect complete deblocking of the 5'-phosphate [31]. To perform the ammonolysis under milder conditions, a solid support **9** was employed as a carrier of the 3'-phosphorylated oligonucleotide [22]. With the use of **9**, the deprotection of the 3'-phosphate group was completed simultaneously with the release of the oligonucleotide from the solid support (20 min at room temperature).

Base protection strategy and final deprotection. Oligonucleotides **16** and **17** were first synthesized by exploiting phosphoramidites protected at the base moiety with the 2-(acetoxymethyl)benzoyl (AMB) group [25]. For comparison, each of the oligonucleotides was assembled, deprotected, and analyzed in parallel with the unmodified oligonucleotide **15**. On deprotection with concentrated ammonium hydroxide for 2 h at room temperature, **16** and **17** were obtained in 90 to 100% isolated yield with respect to the oligonucleotide **15**.

Since the octyl ester in **2** should be more stable towards basic treatment than its ethyl counterpart in **1**, the compatibility of phosphoramidite **2** with other protection strategies was studied. The isolated oligonucleotides were treated with concentrated ammonium hydroxide for 3 days at room temperature and for 1h at 65°C. HPLC analysis of the reaction mixtures revealed no substantial conversion of **16** and **17** (less than 5%). Longer treatment at elevated temperature (7 h at 55°C) resulted in a more extensive loss of the octyl groups (10 to 15% for **16** and 20 to 25% for **17**). Accordingly, in comparison with model compounds **4** and **5** the non-nucleosidic moiety **Y** is notably more stable. This makes phosphoramidite **2** compatible with all strategies of DNA synthesis that use labile protecting groups at the base moieties. Compatibility with the standard protecting scheme (A^{bz}, C^{bz}, and G^{ib}) is limited but may be considered as an option if either longer deprotection time (3 d/RT) or 30% reduction in yield of final product is acceptable. In the present work, we chose to use the standard protection strategy and deprotect oligonucleotides with ammonium hydroxide for 2 d at room temperature. HPLC profiles of crude deprotection mixtures for **16** and **17** are presented in Figure 1.

In striking contrast, treatment of **16** and **17** with 0.05 M K_2CO_3 in anhydrous MeOH for 4 h led to severe hydrolysis of the non-nucleosidic units **Y** (more than 75%).

Isolation of hydrophobic oligonucleotides **16-22**. To the best of our knowledge, only oligonucleotides with a maximum number of four hydrophobic groups have been synthesized [32]. The moderate hydrophobicity of these compounds allowed their isolation on anion exchange [33], C8 [6,34], or, more often, C18 HPLC columns [35]. In this work, oligonucleotides **16**-**22** demonstrated a rather high hydrophobicity that may make their analysis and isolation difficult and lead to considerable loss of material. The following observations are of crucial importance for successful isolation. a) Analysis of the DMT-Off oligonucleotides on C18 RP column required a descending gradient of salt along with ascending concentration of organic solvent (0.05 M NH_4OAc to 60% aqueous MeCN). However, preparative isolation of oligonucleotides **19**, **21**, and **22** that bear hydrophobic groups at both termini was unsuccessful. None of the 5'-DMT protected oligonucleotides could be eluted with 95% aq MeCN, dioxane, or THF. b) A less hydrophobic C4 column enables reproducible purification of **16**-**22** (DMT-Off). For analytical separations, buffer B may contain 0.05 M NH_4OAc. DMT-On oligonucleotides could be chromatographed only on analytical scale. Attempts to scale up the separation led to irreproducible results.

Under optimal conditions, oligonucleotide conjugates **16**-**22** are eluted as single sharp peaks. HPLC profiles obtained from **16**-**22** were reproducible over a wide range of the separation scales (0.2 to 60 AU). Retention times of the conjugates **16**-**22** are listed in Table 2. Typical HPLC profiles of **16** and **17** are presented in Figure 1.

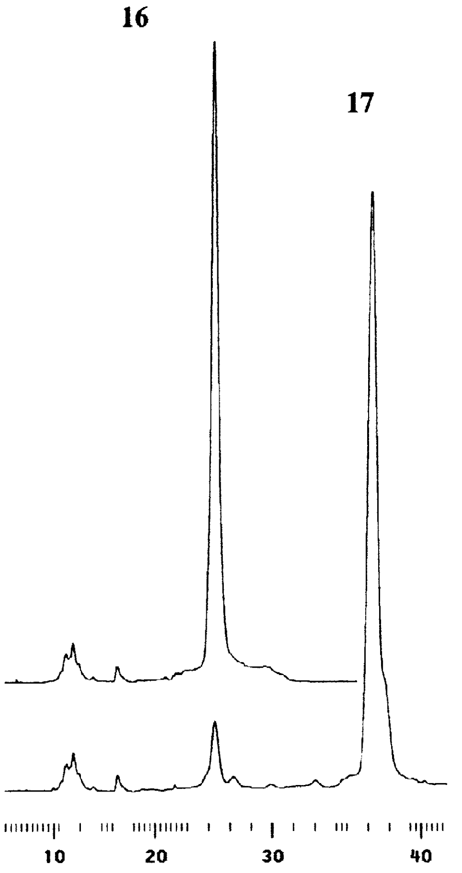


Figure 1. HPLC profiles of **16** and **17** (crude reaction mixtures).

Hybridization properties of hydrophobic oligonucleotides.

The influence of hydrophobic pendants **Y** on the stability of double and triple helical complexes formed by modified oligonucleotides **16**-**22** was evaluated by the thermal denaturation method. The conjugates **16** and **17** were hybridized to a complementary DNA template, 5'-d(cag tct ACG ACC ATG ATG TTC gtt cag)-3', **24**, having six overhanging nucleotides at the 3'-end. The overhang in the template strand was included to clarify whether the pendants cause steric hindrance and therefore result in duplex destabilization. The observed melting temperatures are presented in Table 3. It is seen that the stability of the duplexes formed by hydrophobic oligonucleotides was either essentially unaffected (**16/24**) or even increased (**17/24**) in comparison to that of the parent oligonucleotide (**15/24**).

It has been shown previously [36] that attachment of cholesterol to both termini of a triple helix forming oligonucleotide stabilizes the complex by hydrophobic interaction. In order to reveal the minimal extent of hydrophobic octyl modification that is required for a similar stabilization, the melting temperatures of oligonucleotides **18**-**22** prehybridized to deoxyundecaadenylate were measured (Table 3 and Scheme 6). The T_m values obtained were compared to those of parent complex **23**/d(A_{11}) and the circular oligonucleotide **25**/d(A_{11}) reported previously [37]. As seen from Table 3, the octyl group at the 3'-terminus of oligonucleotide **18** slightly destabilizes triplex formation. In contrast, when alkyl groups are tethered to both 3'- and 5'-termini the triple helix is stabilized. The magnitude of this effect correlates with the number of octyl groups. The T_m of the most hydrophobic complex, **22**/d(A_{11}), falls between the two extremes, *i.e.* complexes of linear and circular oligonucleotides, **23** and **25**. This suggests that, upon the hybridization event, **22** may form a pseudo-circular structure that is stabilized by a hydrophobic clamp consisting of eight octyl groups.

CONCLUSIONS

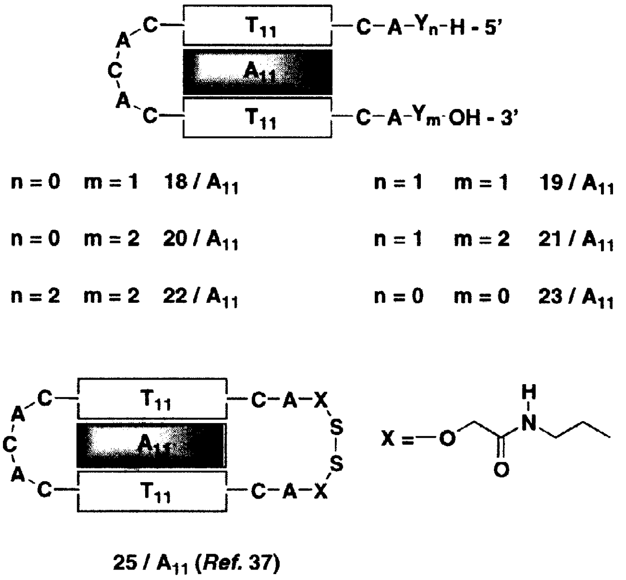
The easily available phosphoramidite building block **2** allows convenient preparation of synthetic oligonucleotides tethered with hydrophobic octyl groups. Since **2** does not terminate the chain assembly, the hydrophobicity of the conjugate may be tuned to a desired extent. Upon incorporation, **2** creates an achiral, non-nucleosidic moiety **Y**. Terminal modification of oligonucleotides with these moieties stabilizes double helical complexes. Placement of at least one hydrophobic unit **Y** at both 5'- and 3'-termini of a triple helix forming oligonucleotide

Table 3.

Melting Temperatures of Complexes Formed by Oligonucleotides **15**-**23** and **25**.[a]

Complex	T_m, °C	ΔT_m, °C	ΔT_m, °C
15/24	58.4	-	-
16/24	57.4	-1.0[b]	-
17/24	63.9	+1.5[b]	-
18/d(A_{11})	46.6	-1.0[c]	-10.6[d]
19/d(A_{11})	49.7	+2.1[c]	-7.5[d]
20/d(A_{11})	46.4	-1.2[c]	-10.8[d]
21/d(A_{11})	50.5	+2.9[c]	-6.7[d]
22/d(A_{11})	52.0	+4.4[c]	-5.2[d]
23/d(A_{11})	47.6 (*Ref.* 37)	-	-9.6[d]
25/d(A_{11})	57.2 (*Ref.* 37)	+9.6[c]	-

a. For conditions, see experimental section.
b. As compared to **15/24**.
c. As compared to **23**/d(A_{11}).
d. As compared to **25**/d(A_{11}).



Scheme 6. Structures of triple helical complexes formed by d(A_{11}) and oligonucleotides **18**-**23** and **25**.

leads to stabilization of triple helical complexes.

EXPERIMENTAL

General. Dioctyl malonate was prepared by the reported method [20]. Reagents for oligonucleotide synthesis were purchased from Glen Research. Adsorption column chromatography was performed on silica gel 60 (Merck). NMR spectra were recorded on a JEOL GX-400 spectrometer operating at 399.8 and 161.9 MHz for ^{1}H and ^{31}P, respectively. $CDCl_3$ was used as a solvent, with either tetramethylsilane (TMS) as an internal (^{1}H) or H_3PO_4 as an external (^{31}P) standard. The thermal denaturation curves were recorded as described previously at 260 nm in a buffer containing 20 mM Tris-HCl and 0.1 M NaCl (pH 7.0) [37].

Oligonucleotide synthesis. The protected oligonucleotides were assembled on an Applied Biosystems 392 DNA Synthesizer on 0.2 and 1.0 μmol scales using either commercial solid supports or **9**, and phosphoramidite chemistry. Phosphoramidite **2** was used as 0.1M solution in anhydrous MeCN, with 600 s coupling time.

Detritylation of non-nucleosidic units (solid support **9** and moiety **Y**) was carried out manually. The synthetic column was detached from the instrument, and a solution of TFA in methylene chloride (2%, 3 mL) was passed through the column for 40 s followed by CH_2Cl_2 (5 mL) and finally, MeCN (5 mL). The column was then replaced on the instrument and chain assembly was continued.

The capping subroutine was excluded from the synthetic protocol throughout the synthesis when the solid support **9** was employed (oligonucleotides **18-22**).

The support-bound material was deprotected with concentrated aqueous NH_3 (48 h at room temperature). The oligonucleotides bearing a 5'-terminal moiety **Y** were subjected to ammonolysis in DMT-On mode. Upon evaporation of aqueous ammonia *in vacuo*, the 5'-DMT group was cleaved with 80% aqueous AcOH for 20 min at room temperature. The hydrophobic oligonucleotides **16-22** were isolated by RP HPLC, desalted, and finally characterized by MALDI/TOF mass spectrometry using 3-hydroxypicolinic acid as a matrix [38] to give the expected masses (±1.2 amu).

HPLC Techniques. The oligonucleotides were analyzed and isolated by reverse phase chromatography on a Nucleosil C4 column (300-5 4×250 mm) or a Hypersil ODS column (5 μm, 4×250 mm). 0.05 M NH_4OAc was used as a buffer A, and 65% aqueous MeCN as a buffer B at a flow rate 1.0 mL min^{-1}. Linear gradients from 0 to 60 and 0 to 100% B in 40 min were employed for C4 and C18 columns, respectively. Retention times are presented in Table 2. Desalting was performed by gel filtration on a TSKgel column (G2000SW 7.5×300 mm, Toso Haas) eluting with water at a flow rate 0.5 mL min^{-1}.

Triethylammonium 2,2-bis(ethoxycarbonyl)propyl 5'-O-(4,4'dimethoxytrityl)thymidin-3'-yl phosphate **7**.

A solution of **6** (745 mg, 1.0 mmol) and **7** [12] (306 mg, 1.5 mmol) in anhydrous MeCN (2.0 mL) was treated with 0.45 M 1*H*-tetrazole in MeCN (2.2 mL, 1.0 mmol). The reaction was stirred for 2 h and found to be completed by TLC. Anhydrous *t*-butylamine (5.0 mL) was added, and the mixture was stirred for 3 days at room temperature. The solvent was evaporated *in vacuo*, the residue was dissolved in CH_2Cl_2 (100 mL), washed with $NaHCO_3$ (3 × 25 mL), and dried over Na_2SO_4. The solvent was evaporated, and the product was isolated on a silica gel column eluting with a step gradient from 0 to 15% MeOH in CH_2Cl_2/Et_3N (98:2) to give **8** (748 mg, 82%) as a colorless foam. High resolution electron spray MS (HR ESMS): 810.2752 (Found); 810.2765 (Calculated for $C_{40}H_{47}N_2O_{14}P$). A NMR sample (15 mg) was converted in pyridinium salt by treatment with Dowex 50W×8 (PyH^+), evaporated, and dried *in vacuo*. ^1H NMR ($CDCl_3$): 12.50 (1H, s, NH), 7.63 (1H, s, H-6), 7.41-7.35 (7H, m, aromatic), 7.30-7.20 (7H, m, aromatic), 6.85-6.80 (4H, m, aromatic), 6.48 (1H, dd, J^3 = 9.1, 5.2 Hz, H-1'), 5.01 (1H, m, H-3'), 4.35 (1H, br.s, H-4'), 4.24 (1H, dd, J^2_{HH} = 10.0 Hz, J^3_{HP} = 4.4 Hz, $C\mathit{H}^AH^B$-O-P), 4.21 (1H, dd, J^2_{HH} = 10.0 Hz, J^3_{HP} = 4.6 Hz, $CH^A\mathit{H}^B$-O-P), 4.18-4.05 (4H, m, 2 × CH_2OOC), 3.78 (6H, s, 2 × CH_3O), 3.47 (1H, dd, J^2 = 10.4 Hz, J^3 = 2.2 Hz, H-5'), 3.43 (1H, dd, J^2 = 10.4 Hz, J^3 = 2.1 Hz, H-5"), 2.66 and 2.38 (2 × 1H, m, J^2 = 13.2 Hz, H-2',2"), 1.47 (3H, s, 5-CH_3), 1.30 (6H, t, J^3 = 7.3 Hz, 2 × $C\mathit{H}_3CH_2OOC$), 1.27 (3H, s, C-CH_3). ^{13}C NMR ($CDCl_3$): 170.2 (COO); 163.8 (C4); 158.6 (C2); 136.0 (C6); 150.4, 144.3, 135.4, 135.3, 130.2, 130.1, 128.3, 127.9, 127.0, 113.2 (Arom.); 111.1 (C5); 86.9 (Ar_3C); 85.4 (C1'); 84.6 (C4'); 76.5 (C3'); 67.8 (P-O-CH_2); 64.1 (C5'); 61.3 ($MeCH_2O$); 55.2 (CH_3O); 54.9 (Cq); 39.5 (C2'); 18.0 (CH_3-Cq); 13.9 (CH_3CH_2); 11.4 (C5-CH_3). ^{31}P NMR ($CDCl_3$): -1.70.

Ammonium 2,2-bis(ethoxycarbonyl)propyl thymidine-3'-yl phosphate **3**. Compound **8** (50 mg, 55 μmol) was shaken with 80% aq AcOH (10 mL) for 1 h, and the solution was evaporated to dryness. The residue was dissolved in water (5 mL), extracted with CH_2Cl_2 (3 × 2 mL), evaporated, and re-dissolved in water (5 mL). The product was isolated by reverse phase HPLC on a LiCrospher® column (100 RP-18 5 μm, 10 × 250 mm) using 0.05 M NH_4OAc as buffer A, 60% aq MeCN as buffer B, and a linear gradient from 5 to 50% B in 25 min. Collected fractions were evaporated, dissolved in water (5 mL), and desalted by injecting on the same column, then washing with 0.1 M NH_4OAc (12 min), water (12 min) and eluting the product with 60% aq. MeCN at a flow rate 3 mL min^{-1}. Evaporation *in vacuo* gave **3** (NH_4 salt; 24.3 mg, 84%). ^{31}P NMR (DMSO-d_6): -6.12. HR ESMS: 508.1450 (Found); 508.1458 (Calculated for $C_{19}H_{29}N_2O_{12}P$).

Thymidylyl-(3'→1-O) 2,2-bis(ethoxycarbonyl)-1,3-propanediol 3-O-phosphate, triammonium salt **4**. Compound **10** was assembled on an ABI 392 DNA Synthesizer starting from the solid support **9** [22] (40 μmol) and using phosphoramidite **1** [19] for the first coupling and commercial T phosphoramidite **6** for the second one (Scheme 2). A user-defined elongation protocol was employed. The detritylation was done with continuous flow of 2% TFA in CH_2Cl_2 (25 s) and was followed by washing cycle, 2 × (MeCN wash, 1 min; reverse flush, 30 s). The coupling step consisted of delivering a mixture of a phosphoramidite reagent (0.1 M in MeCN) and 1*H*-tetrazole (0.45 M in MeCN) to the column for 40 s and waiting for 180 s, and was followed by washing cycle. Oxidation was done with commercial I_2/Py/THF/H_2O reagent (120 s delivery, 30 s waiting). Finally, the washing cycle completed the elongation protocol. The product was released from the solid support with conc. ammonium hydroxide for 30 min, and the solution was evaporated. The residue was treated with 80% aq AcOH (10 mL) for 20 min and evaporated to dryness. The resulting material was dissolved in water (5 mL), extracted with CH_2Cl_2 (3 × 2 mL), evaporated, and re-dissolved in water (5 mL). The isolation and desalting was performed as described for **3** to give **4** (19.7 mg, 75%). ^{1}H NMR (DMSO-d_6) δ 11.32 (1H, s, NH), 7.81 (1H, s, H-6), 6.16 (1H, dd, J^3 = 7.8, 6.3 Hz, H-1'), 4.76 (1H, m, H-3'), 4.25-4.15 (4H, m, 2 × CH_2-O-P), 4.15-4.05 (4H, m, 2 × CH_2OOC), 3.97 (1H, m, H-4'), 3.63 and 3.54 (2 × 1H, 2 × dd, J^2 = 9.5 Hz, $J^3 \approx$ 1 Hz, H-5',5"), 2.23 and 2.11 (2 × 1H, m, H-2',2"), 1.77 (3H, s, 5-CH_3), 1.16 and 1.14 (2 × 3H, 2 × t, J^3 = 7.1 Hz, 2 × $C\mathit{H}_3CH_2OOC$). ^{31}P NMR (DMSO-d_6) δ -1.2 (1P, $ROPO_3^{2-}$), -3.2 [1P, phosphodiester]. HR ESMS: 604.1059 (Found); 604.1071 (Calculated for $C_{19}H_{30}N_2O_{16}P_2$).

Thymidylyl-(5'→1-O) 2,2-bis(ethoxycarbonyl)-1,3-propanediol 3-O-phosphate, triammonium salt **5**. A solid support-bound material was prepared starting from commercial thymidine CPG (40 μmol). Phosphoramidite **1** was used to carry out two elongation cycles as described above for **10** (Scheme 3). Compound **12** was released from the solid support with conc. aqueous ammonia (2 h; RT). The solution was evaporated, and the residue was treated with 80% aq AcOH (10 mL) for 20 min and evaporated to dryness. The resulting material was dissolved in 5% aq ammonium hydroxide (10 mL), which effected the release of 5'-phosphate group. The solution was extracted with CH_2Cl_2 (3 × 2 mL), evaporated, and re-dissolved in water (5 mL). The isolation and desalting was performed as described for **3** to give **5** (20.7 mg, 79%). ^{1}H NMR (DMSO-d_6): δ 11.25 (1H, s, NH), 7.78 (1H, s, H-6), 6.21 (1H, dd, J^3 = 7.4, 6.5 Hz, H-1'), 4.32 (1H, m, H-3'), 4.22-4.10 (4H, m, 2 × CH_2-O-P), 4.17-4.02 (4H, m, 2 × CH_2OOC), 3.88 (1H, br.s, H-4'), 3.81 (2H, br.s, H-5',5"), 2.10 and 2.02 (2 × 1H, m, H-2',2"), 1.81 (3H, s, 5-CH_3), 1.14 (6H, t, J^3 = 7.1 Hz, 2 × $C\mathit{H}_3CH_2OOC$. ^{31}P NMR (DMSO-d_6) δ -1.9 (1P, $ROPO_3^{2-}$), -2.9 [1P, $RO(R'O)PO_2^-$]. HR ESMS: 604.1060 (Found); 604.1071 (Calculated for $C_{19}H_{30}N_2O_{16}P_2$).

Experiments on stability of **3**, **4**, *and* **5** *towards various deprotection conditions.* Aliquots of **3**, **4**, or **5** (20 to 40 AU/mL; 50 μL) in water, MeOH or EtOH were diluted to 500 μL with appropriate deprotection mixture to give the following final concentrations: 25% ammonium hydroxide; NH_4OAc/25% ammonium hydroxide (1:10); 25% ammonium hydroxide - 40%

$MeNH_2$ (1:1); 50% 1,2-diaminoethane in EtOH; 0.05 M K_2CO_3 in MeOH. The reaction mixtures were kept at temperatures specified in Table 1. Samples (50 µL) were taken, briefly evaporated in high vacuum, re-dissolved in water, and analyzed by HPLC. Analysis was carried out on a Nucleosil column (300-5 C18; 4.6×250 mm) using 0.05 M NH_4OAc as buffer A, 60% aq MeCN as buffer B, and a linear gradient from 0 to 60% B in 20 min at a flow rate 1 mL min^{-1}. The reactions were followed for 4 to 5 half-lives. The results are presented in Table 1.

Dioctyl 2,2-bis(hydroxymethyl)malonate (**13**). Compound **13** was synthesized by a modification of the reported method [20]. Triethylamine (1.0 M in THF; 0.10 mL, 0.1 mmol) was added to a stirred solution of dioctyl malonate (1645 mg, 5.0 mmol) in 25% aq. formaldehyde (1.8 mL, 15 mmol) and pyridine (6 mL), and the mixture was stirred for 6 h at room temperature. The reaction mixture was diluted with water (100 mL), and the product was extracted with benzene (3 × 50 mL). Organic extracts were dried over Na_2SO_4 and evaporated. The product was isolated by a silica gel column chromatography eluting with a gradient from CH_2Cl_2 to CH_2Cl_2/ethyl acetate (7:3) to give **13** (1410 mg 72.6%): n^D_{20} 1.4553; ^1H NMR ($CDCl_3$) δ 4.15 (4H, t, J^3=6.8, 2 × CH_2OOC), 4.09 (4H, d, J^3=6.3, 2 × $C\mathit{H}_2OH$), 3.44 (2H, br. t, J^3=6.3, 2 × OH), 1.63 (4H, m, 2×J^3=6.8, 2 × $C\mathit{H}_2CH_2O$), 1.31-1.27 (20H, m, 2 × $(CH_2)_5$), 0.88 (6H, t, J^3=6.8, 2 × CH_3); ^{13}C NMR ($CDCl_3$) δ 169.5 (C=O), 66.0, 31.8, 29.2, 28.5, 26.0, 22.7, 14.1 (C-1, C-6, C-4,5, C-2, C-3, C-7, CH_3), 63.1 (CH_2OH), 61.2 (C). Found, %: C 64.89, H 10.45 ($C_{21}H_{40}O_6$). Calculated, %: C 64.92, H 10.38.

Dioctyl 2-[[(4,4'-dimethoxytrityl)oxy]methyl]-2-(hydroxymethyl)malonate (**14**). Solution of 4,4'-dimethoxytrityl chloride (1085 mg, 3.2 mmol) in dry dioxane (20 mL) was added dropwise to dioctyl 2,2-*bis*(hydroxymethyl)malonate, **13**, (1555 mg, 4.0 mmol) and dry pyridine (0.64 mL, 8.0 mmol) in dioxane (10 mL). The reaction was left overnight at room temperature. Liquid phase was separated from viscous precipitate, diluted with benzene (100 mL), washed with $NaHCO_3$ (5% in water; 10 mL), saturated aqueous NaCl (2×50 mL), and dried over Na_2SO_4. The solvent was removed *in vacuo*, and the residue was separated on a silica gel column eluting with a gradient from benzene to benzene/ethyl acetate (9:1) to give **14** (1700 mg, 77% on DMTrCl) as a colorless oil. ^1H NMR ($CDCl_3$) δ 7.42-7.35, (2H, m, aromatic), 7.33-7.25 (7H, m, aromatic), 6.87-6.80 (4H, m, aromatic), 4.18-4.12 (6H, m, $C\mathit{H}_2OH$ and 2 × CH_2OOC), 3.78 (6H, s, 2 × CH_3O), 3.63 (2H, s, CH_2ODMT), 2.05 (1H, t, J^3 = 6.9 Hz, OH), 1.59 (4H, m, 2 × J^3 = 6.9 Hz, 2 × $C\mathit{H}_2CH_2OOC$), 1.26 (20H, m, 10 × CH_2), 0.87 (6H, t, J^3 = 6.9 Hz, 2 × CH_3); ^{13}C NMR ($CDCl_3$) δ 169.3 (C=O), 158.5, 135.5, 130.0, 113.2 (C-4, C-1, C-2,6, C-3,5 in An), 144.4, 128.1, 127.9, 126.9 (C-1, C-2,6, C-3,5, C-4 in Ph), 86.3 (Ar_3C-), 65.7, 31.8, 29.2, 28.4, 25.8, 22.6, 14.1 (C-1, C-6, C-4,5, C-2, C-3, C-7, CH_3 in Oct), 63.6 ($DMTOCH_2$), 61.9 (C), 60.6 (CH_2OH), 55.2 (CH_3O). Found, %: C 73.12, H 8.43 ($C_{42}H_{58}O_8$). Calculated, %: C 73.01, H 8.46.

2-Cyanoethyl 3- [(4,4'-dimethoxytrityl) oxy]-2,2-bis (n – octyloxycarbonyl) propyl (N,N-diisopropylamino) phosphoramidite (**2**). Dry 1*H*-tetrazole (0.46 M in MeCN; 2.38 mmol; 5.3 mL) was added to a solution of **14** (1645 mg, 2.38 mmol) predried by coevaporation with MeCN (3×20 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (860 mg; 2.85 mmol) in dry MeCN (5 mL). The reaction was left at ambient temperature and found to be completed in 30 min (TLC, benzene- ethyl acetate; 6:1). The reaction was stopped by adding 5% aqueous $NaHCO_3$ (10 mL). The viscous product was extracted with benzene (2×20 mL), washed with saturated aqueous NaCl, and dried over Na_2SO_4. The solvent was removed under reduced pressure; the residue was separated on a silica gel column, eluting with a gradient from hexane/TEA (95:5) to hexane/benzene/TEA (60:35:5) to give **2** (1793 mg, 84.5%) as a colorless oil. ^1H NMR ($CDCl_3$) δ 7.40-7.35, (2H, m, aromatic), 7.30-7.17 (7H, m, aromatic), 6.83-6.78 (4H, m, aromatic), 4.29 (1H, dd, J^2 = 10.0 Hz, J^3_{HP} = 6.6 Hz, $C\mathit{H}^AH^BOP$), 4.17-3.97 (5H, m, $CH^A\mathit{H}^BOP$ and 2 × CH_2OOC), 3.78 (6H, s, 2 × CH_3O), 3.71 and 3.63 (2 × 1H, 2 × d, J^2 = 8.7 Hz, CH_2ODMT), 3.65-3.43 (4H, m, $CH_2C\mathit{H}_2OP$, 2 × N-CH), 2.43 (2H, td, J^3 = 6.5 Hz, J^4_{HP} = 2.7 Hz, CH_2CN), 1.55 (4H, m, 2 × $C\mathit{H}_2CH_2OOC$), 1.29-1.22 (20H, m, 10 × CH_2), 1.15 (6H, d, J^3 = 6.8 Hz, 2 × N-C- CH_3), 1.10 (6H, d, J^3 = 6.8 Hz, 2 × N-C- CH_3), 0.88 (6H, t, J^3 = 7.0 Hz, 2 × CH_3); ^{13}C NMR ($CDCl_3$) δ 168.4 (C=O), 158.4, 135.7, 130.2, 112.9 (C-4, C-1, C-2,6, C-3,5 in An), 144.6, 128.3, 127.7, 126.7 (C-1, C-2,6, C-3,5, C-4 in Ph), 117.6 (CN), 85.9 (Ar_3C-), 65.5, 31.8, 29.2, 28.4, 25.8, 22.6, 14.0 (C-1, C-6, C-4,5, C-2, C-3, C-7, CH_3 in Oct), 61.6, 60.8, 59.8, 59.3 (C-$\mathit{C}H_2OP$, $DMTOCH_2$, C, $CH_2\mathit{C}H_2OP$), 55.1 (CH_3O), 43.1 (CHN), 24.5 ($\mathit{C}H_3CH$), 20.2 ($\mathit{C}H_2CN$); ^{31}P NMR ($CDCl_3$) δ 148.07. Found, %: C 68.77, H 8.49, N 3.18 ($C_{51}H_{75}N_2O_9P$). Calculated, %: C 68.74, H 8.48, N 3.14.

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