DOI: 10.1002/ejoc.200500547

Solution-Phase Synthesis of Phosphorothioate Oligonucleotides Using a Solid-Supported Acyl Chloride with *H*-Phosphonate Chemistry

Ilaria Adamo,[a] Cécile Dueymes,[a] Andreas Schönberger,[a,b] Aude-Emmanuelle Navarro,[a] Albert Meyer,[a] Meinolf Lange,[b] Jean-Louis Imbach,[a] Fritz Link,[b] François Morvan,*[a] and Jean-Jacques Vasseur[a]

Keywords: Oligonucleotides / *H*-Phosphonate / Solution-phase synthesis / Solid-supported reagents / Nucleosides

The synthesis of oligonucleotides from dimers to a hexamer by a *H*-phosphonate approach in solution using solid-supported acyl chloride is reported herein. This strategy avoids the use of chromatography. The work-ups have been simplified and involve filtration, aqueous extraction and precipitation. The synthesis was scaled up to 1 mmol for the dimers and 340 μmol for the hexamer.

Introduction

Oligonucleotides and their phosphorothioate analogues are mainly synthesized with DNA synthesizers on a solid support. In this way, batches of kilograms of oligonucleotides can be obtained. Nevertheless, when antisense compounds reach the market, hundreds of kilograms or perhaps even tonnes of them will be needed and all at a relatively low price.[1] If an increase in the number of machines could increase the quantity of oligonucleotides produced, the global cost would remain the same. An alternative to solid-phase synthesis is solution-phase synthesis. Along this line, some solution-phase syntheses have already been described, such as the HELP,[2] which uses a soluble PEG solid support, or the PASS[3] in which the growing oligonucleotide reacts with a phosphoramidite derivative temporarily immobilized on a solid support. In both of these processes, the precipitation or the removal of the excess phosphoramidite by aqueous extraction avoids the use of costly and time-consuming chromatography to obtain the pure oligonucleotide. Furthermore, solution-phase synthesis using *H*-phosphonate chemistry has been reported[4] but this protocol still requires the use of chromatographic separation after each coupling step. To best accomplish the goal of an efficient large-scale synthesis, it is clear that chromatographic purification of the intermediates must be avoided.

The tremendous need to decrease the cost of oligonucleotide synthesis has prompted us to develop a new process that could be scaled up. Our goal was to develop a solution-phase process that does not require chromatography after each step. To accomplish this, we envisioned the use of solid-supported reagents that would be removed after reaction by filtration. Both phosphoramidite[5] and *H*-phosphonate chemistries were investigated. We report herein the synthesis of fully protected building blocks and a hexamer by a convergent strategy using *H*-phosphonate chemistry with a solid-supported activator. This process requires only filtration, extraction or precipitation as work-up.

Results and Discussion

According to the *H*-phosphonate methodology, the synthesis of oligonucleotides on a solid support requires two recurrent steps: coupling and detritylation (since the oxidation is performed at the end of the elongation). Coupling occurs between a nucleoside 5′-*O*-Dmtr-3′-*H*-phosphonate monoester, activated with an acyl chloride, and the 5′-hydroxy group of a nucleoside or an oligonucleotide immobilized on a solid support to form a *H*-phosphonate diester linkage. This linkage is stable in the acidic conditions required for detritylation and the acid is then removed by washing the solid support. In solution, the moiety at the 3′-position is replaced by a nucleoside protected at the 3′-position by a levulinyl group. Theoretically, oxidation of the *H*-phosphonate linkage formed could be delayed and further detritylation and coupling steps applied. However, since the *H*-phosphonate diester linkage is relatively sensitive to alkaline conditions,[6] the quenching of the acidity should be well controlled. In addition, it has been reported that the *H*-phosphonate diester could be cleaved by intramolecular transesterification of the resulting 5′-hydroxy function on the phosphorus atom leading to hydrolysis of

[a] ERT "Oligonucléotides: Méthodologie, Valorisation", UMR 5625 CNRS-UM2 Université Montpellier II,
34095 Montpellier, France
Fax: +33-467-042-029
E-mail: morvan@univ-montp2.fr
[b] Girindus AG,
33790 Halle-Kuensebeck, Germany

the linkage.[7] To avoid such degradation, the *H*-phosphonate diester linkage was oxidized after the coupling reaction to a more stable phosphorothioate triester linkage.[4]

Solid-Supported Coupling Reagents: Preparation of Acyl Chloride Resins and Loading

In *H*-phosphonate chemistry, the standard activator used in the coupling reaction is a sterically hindered acyl chloride, such as pivaloyl chloride[8,9] or adamantoyl chloride[10] (Figure 1), which reduces the extent of 5′-*O*-acylation and overactivation.[11] Alternatively, the coupling reactions can be performed using carbodiimide,[12] phosphorochloridate[13] or sulfonyl chloride[13] derivatives. First, we wanted a commercially available solid-supported reagent that bears one of these functionalities. Polymer-bound sulfonyl chloride (Aldrich) and chlorocarbonyl-polystyrene (FLUKA), both commercial reagents, were selected (Table 1). Secondly, we wished to use a solid-supported reagent that could be recycled. For this purpose we prepared polymer-bound acyl chlorides starting from the corresponding polymer-bound carboxylic acids. One was the Dowex MWC-1 polyacrylic acid and two were benzoic acids on polystyrene resin (from Novabiochem and Rapp polymers). The acyl chloride resins were prepared from the corresponding solid-supported acid by using thionyl chloride with a catalytic amount of DMF in dichloromethane or toluene under reflux for 3–12 h (Scheme 1 and Table 2).

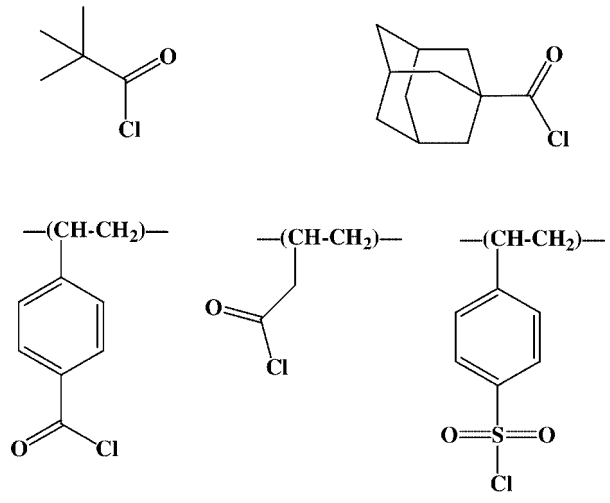
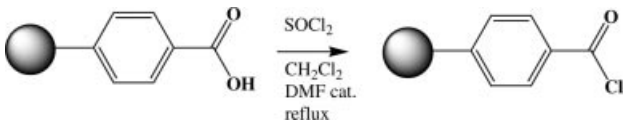


Figure 1. Soluble and solid-supported acyl and sulfonyl chlorides.

Table 1. Commercial coupling reagents: acyl and sulfonyl chlorides.

	Commercial specificities	Cl^- content [mmol/g]
Chlorocarbonyl-polystyrene; PS acid chloride, FLUKA 12973	≈ 2.1 mmol/g	1.2
Polymer-bound sulfonyl chloride, Aldrich 51,622-8	2.5–3.0 mmol/g; 70–90 mesh	1.71



Scheme 1. Preparation of the acyl chloride resins.

The amount of chlorine in the resins was determined by elemental analysis. We noticed that the chlorine content of the commercial chlorocarbonyl-polystyrene from FLUKA was only 1.2 mmol/g instead of the 2.1 mmol/g given. The chlorine content was around 3.6 mmol/g in the acyl chloride resin derived from the Dowex polyacrylic and between 1.8 and 2.2 mmol/g in the two polystyrene-derived resins that we prepared.

Evaluation of Polymer-Bound Coupling Agents

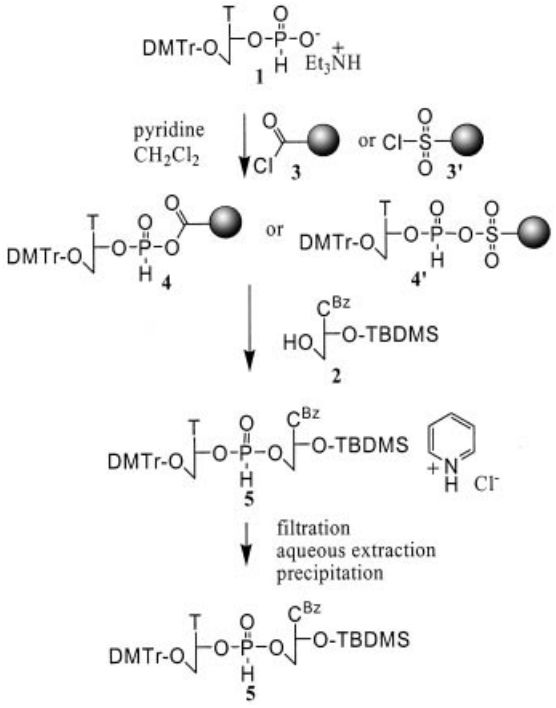
The efficiency of each solid-supported activator was established based on the following criteria: 1) its efficiency in forming an *H*-phosphonate diester (yield), 2) the number of molecular equivalents used, 3) the rate of reaction, 4) its ability to trap excess *H*-phosphonate monoester, **5**) cost and 6) recycling potential.

The coupling reaction was performed at a 33 μmol scale with 10–30 molar equivalents of resin **3** or **3′** with respect to the 3′-*H*-phosphonate monoester nucleoside (3′-HP nucleoside) (Scheme 2). The resin was allowed to swell in dichloromethane/pyridine (1:1, v/v) and then the two monomers, 5′-*O*-Dmtr-d**T**-3′-HP (**1**) (1.2 equiv.) and 5′-OH-d$\mathbf{C}^{Bz}$-3′-*O*-TBDMS (**2**) (1 equiv.), were added. The resulting heterogeneous mixture was shaken and the reaction monitored by ^{31}P NMR spectroscopy and HPLC. The coupling reaction proceeds through a catch-and-release

Table 2. Conversion of carboxylic resins into acyl chloride resins.

Commercial carboxy resin	Properties	Acyl chloride resin, Cl^- content	Treatment
Macroporous polyacrylic carboxy DOWEX MWC-1 FLUKA 44551	6.84 mmol/g of COOH 20–50 mesh	3.41 mmol/g	toluene, DMF cat., 4 equiv. $SOCl_2$ reflux 12 h
PS carboxy, carboxypolystyrene VHL NOVABIOCHEM 01-64-0150	2.10 mmol/g 100–200 mesh	1.82 mmol/g	CH_2Cl_2, DMF cat., 4 equiv. $SOCl_2$ reflux 3 h
PS carboxy, polystyrene-COOH Rapp Polymers H110008	1.96 mmol/g 100–200 mesh	2.15 mmol/g	CH_2Cl_2, DMF cat., 4 equiv. $SOCl_2$ reflux 4 h

mechanism (Scheme 2). First, a mixed anhydride **4** or **4′** is formed between the *H*-phosphate monoester nucleoside and the resin (acyl or sulfonyl chloride) after which the free hydroxy of the other unit attacks the phosphorus atom to release the dimer with an *H*-phosphonate diester linkage **5**. Thus, it is expected that excess of the 3′-HP nucleoside is trapped on the resin and only the resulting *H*-phosphonate diester derivative and pyridinium chloride remain in solution after the consumption of the 5′-hydroxy derivative. Theoretically, the *H*-phosphonate diester derivative is isolated by filtration and the salt is removed by simple aqueous extraction. Note that the resin is also a dehydrating agent.



Scheme 2. Synthesis of the dimer *H*-phosphonate diester through the *H*-phosphonate SOLIGO process.

Using the sulfonyl chloride resin, we observed, by ^{31}P NMR spectroscopy, only the dimer *H*-phosphonate diester (9.7 and 10.5 ppm) after 5 or 6 h, indicating that excess of the 3′-HP nucleoside is trapped on the resin. However, monitoring by HPLC showed only a 51% extent of coupling with 20 equiv. of coupling reagent after 6 h and a 61% extent of coupling with 30 equiv. of coupling reagent after 5 h. In both experiments, some of the monomers remained in solution.

With 20 equiv. of the commercial acyl chloride resin (FLUKA, 12973), HPLC showed a yield of about 70% after 4 h with both monomers still in solution. With the polyacrylic DOWEX MWC-1 acyl chloride resin, only 24% of dimer was observed after 140 h. Fortunately, under the same conditions, using the acyl chloride resin prepared from the Novabiochem polystyrene resin, we observed almost quantitative dimer formation after 3 h. After filtration, only the dinucleoside *H*-phosphonate diester was observed by ^{31}P NMR spectroscopy (9.3 and 9.9 ppm), indicating that excess of the 3′-HP nucleoside had been caught by the resin. The HPLC-measured purity of the dinucleoside *H*-phosphonate diester at 260 nm was higher than 90%. The dimer was characterized by MALDI-TOF MS ($C_{53}H_{63}N_5O_{13}PSi$ positive mode: calcd.: 1037.17; found 1037.02). With only 10 equiv. of resin, the reaction was monitored by HPLC and the yield of dimer formation was 59% after 1 h, 66% after 2 h and 88% after 4 h.

Similarly, using the acyl chloride resin prepared from the Rapp Polymer polystyrene resin, we observed almost quantitative dimer formation after 2 h. After filtration, only *H*-phosphonate diester was observed by ^{31}P NMR spectroscopy (8.4 and 8.9 ppm), indicating that excess of the *H*-phosphonate monoester had been caught by the resin. The HPLC-measured purity at 260 nm was higher than 90%. With a smaller amount of resin (10 equiv.), the coupling reaction performed on the 125 μmol scale was complete within 2 h with a purity of 98%. However, with only 5 equiv. of resin, the reaction was still not complete after 5 h (74%).

In conclusion, the two resins prepared from the Novabiochem and Rapp acid-supported polystyrene resins gave the best results with a high amount of coupling. The coupling reaction performed with the Rapp polymer resin was faster, required fewer equivalents of resin, trapped excess monomer and was cheaper. Hence, this resin was selected for further investigation.

The *H*-phosphonate method is somewhat less popular than the phosphoramidite one. This is partly because several side-reactions occur during the coupling step.[11,14] Indeed, the acyl chloride can react with the 5′-OH oligonucleotide giving rise to a 5′-*O*-acylated oligonucleotide or could overactivate the 3′-HP nucleoside leading to phosphorus bis-acylation. To better understand the various aspects of these possible side-reactions with solid-supported reagents, we studied the kinetics of the reactions of the acyl chloride resin with the 5′-OH and 3′-HP nucleosides and the overactivation reaction. Furthermore, we wanted to confirm that, as in the solution-phase methodology,[14] the resulting *H*-phosphonate diester dinucleoside did not react with the acyl chloride resin.

Kinetics of 5′-OH Trapping: *O*-Acylation

The rate of the reaction between a 5′-OH nucleoside and the acyl chloride resin was evaluated under the same conditions as those used for the coupling reaction. To this end, 5′-OH-thymidine-3′-*O*-Lev was mixed with the Rapp acyl chloride resin (5 equiv.) in the presence of 3′,5′-di-*O*-acetylthymidine as an internal standard in pyridine/CH_2Cl_2 (1:1, v/v). The reaction was monitored by HPLC by withdrawing an aliquot every 5 min. The disappearance of the 5′-OH nucleoside was calculated from the area of its peak relative to that of the diacetylthymidine. The kinetic study showed that 26% of the nucleoside was acylated after 5 min, 65% after 15 min and that acylation was complete within 90 min. Note that in a standard coupling reaction, the 3′-HP nucleoside competes with the 5′-OH nucleoside.

Kinetics of *H*-Phosphonate Trapping on the Rapp Acyl Chloride Resin

The rate of reaction of a 3′-HP nucleoside with the acyl chloride resin was evaluated under the conditions used above for the *O*-acylation experiments. 5′-*O*-Dmtr-thymidine-3′-HP was mixed with the acyl chloride resin (5 equiv.) in the presence of 3′,5′-di-*O*-acetylthymidine as an internal standard in pyridine/CH_2Cl_2 (1:1, v/v). The reaction was monitored by HPLC every 5 min. The HPLC profile showed that 88% of the 3′-HP-thymidine reacted with the acyl chloride resin within 5 min and that the reaction was complete within 10 min.

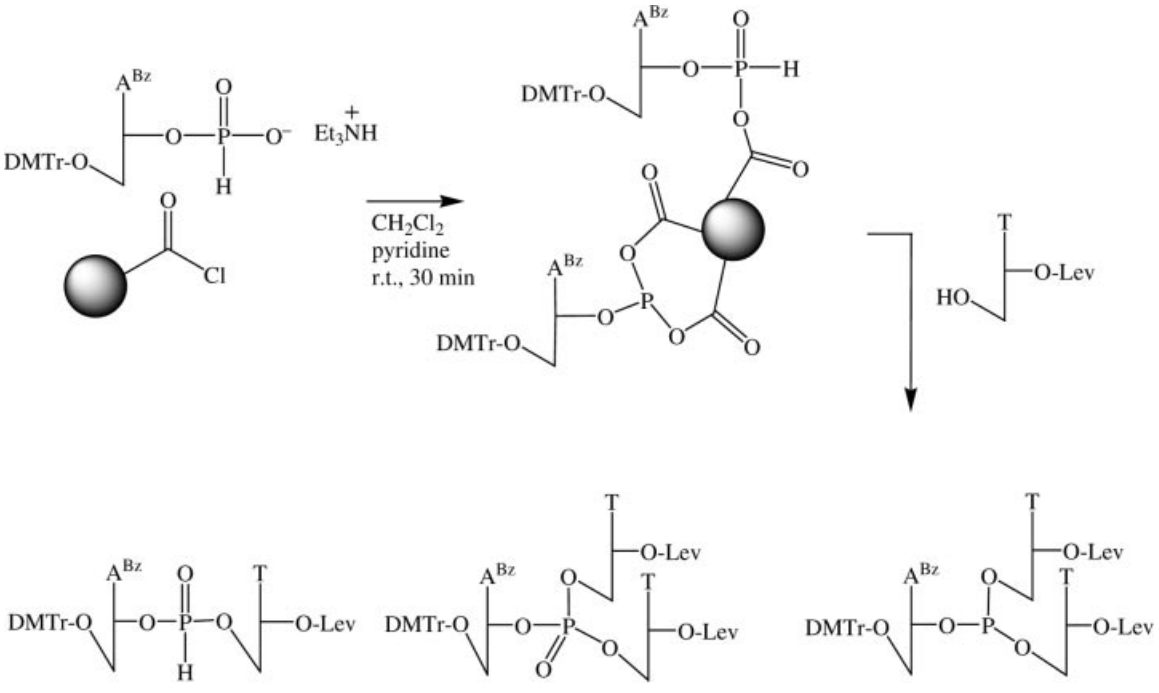
Just as in solution phase, the 3′-HP nucleoside reacts more rapidly with a solid-supported acyl chloride than the 5′-OH nucleoside.

Overactivation of the 3′-HP Nucleoside with the Acyl Chloride Resin

Overactivation is a known side-reaction in *H*-phosphonate chemistry that results from the reaction of a second molecule of acyl chloride with the mixed phosphonocarboxylic acid anhydride.[11] The use of supported acyl chlorides such as those described here, in which steric hindrance is due to the polystyrene resin, could help prevent this overactivation side-reaction. To evaluate it, Dmtr-d$\mathbf{A}^{Bz}$-3′-HP was mixed with the acyl chloride resin for 30 min and then 3′-*O*-levulinylthymidine was added. HPLC, ^{31}P NMR spectroscopy and MALDI-TOF MS showed that three compounds were formed (Scheme 3): the expected *H*-phosphonate diester dimer (AT) as a minor compound (21.3% of the area at 260 nm, HPLC retention times: 20.32 and 20.54 min; δ = 9.0 and 10.0 ppm; *m*/*z* = 1043.70, calcd. 1045.03), the phosphotriester derivative bearing two thymidine residues (45.7% of the area at 260 nm, HPLC retention time: 20.05 min; δ = 141.3 ppm; *m*/*z* = 1365.0, calcd. 1367.35) and its phosphite triester precursor (11.8% of the area at 260 nm, HPLC retention time: 21.15 min; δ = –1.0 ppm; *m*/*z* = 1380.7, calcd. 1383.35). Partial oxidation of the phosphite triester precursor could be explained by the possibility that some anhydride was present on the resin and acted as an oxidizer. Note that the ε value for the dimer is lower than that of the trimer hence the percentage of dimer is higher than that calculated from the area of its peak. Therefore, despite the steric hindrance of the resin, the overactivation side-reaction was not prevented. So, just as for solid-phase synthesis using pivaloyl chloride, the order in which the reactants are added is important for the efficiency of *H*-phosphonate diester formation and premixing of the 3′-HP nucleoside and the acyl chloride resin must be avoided. Fortunately, when both monomers were introduced together no overactivation was detected.

Synthesis of Dimers with the Rapp Acyl Chloride Resin

Among the 16 possible dimers, 10 of them (Table 3) were synthesized using 1.2 equiv. of the 3′-HP monomer in anhydrous CH_2Cl_2/pyridine (1:1, v/v) on a scale between 125 and 1000 μmol with respect to the 5′-OH nucleoside. The number of molar equivalents of acyl chloride used varied between 3.0 and 7.7 equiv. with respect to the *H*-phosphonate nucleoside (Table 3). We noticed that the number of equivalents of resin could be reduced when the scale of the synthesis was increased. Indeed, on the 125 μmol scale,



Scheme 3. Overactivation of H-phosphonate monoester by acyl chloride resin.

Table 3. Dimers synthesized by activation with the Rapp acyl chloride resin using 1.2 equiv. of Dmtr nucleoside 3′-*H*-phosphonate in solution in CH_2Cl_2/pyridine (1:1, v/v).

Entry	Dimer sequence	5′-OH nucleoside [μmol]	[mg]	3′-*H*-Phosphonate [mg]	Resin [equiv.]	Time	Yield [%]	HPLC purity [%] at 260 nm	*m/z* (MALDI-TOF)	^{31}P NMR [ppm]
1	AT	125	42.5	123.4	7.7	30 min	98	88.5	1043.02	9.08, 10.02
		438	149.0	412.0	3.5	1 h	100	100	1044.96	8.4, 9.8
									1045.03 calcd.	
2	AC	125	53.7	123.4	5.5	1 h	89	94	1133.75	9.5, 10.0
		125	53.7	123.4	5.5	1 h 30	85	>94	1133.51	8.8, 9.5
									1134.13 calcd.	
3	CT	125	42.5	120.0	5.5	1 h	90	>91	1020.46	8.4, 9.8
		417	142.0	399.8	3.5	1 h	100	100	1020.21	8.7, 10.2
									1020.01 calcd.	
4	CA	435	197.3	400.0	3.5	1 h	95	95	1132.14	8.9, 9.9
									1133.13 calcd.	
5	CG	417	181.0	400.0	3.5	4 h + 15 min	80	>92	1115.29	8.8, 12.4
									1116.11 calcd.	
6	GT	125	42.5	120.7	5.5	1 h 30	70	97	1025.72	9.0, 10.2
									1027.02 calcd.	
7	GC	125	53.7	120.7	5.5	1 h 30	72	100	1116.43	9.2, 9.8
		500	215.0	483.0	3.5	1 h	87	92	1117.77	9.2, 10.1
									1116.11 calcd.	
8	GA	125	56.6	120.7	5.5	1 h	69	>90	1138.33	8.9, 9.6
		417	189.0	402.0	3.5	1 h	83	85	1140.30	6.6, 6.7
									1140.14 calcd.	
9	TT	417	142.0	354.8	3.5	1 h	100	100	928.75	8.6, 10.0
		125	106.4	51.7	5	1 h 30	99	100	929.91 calcd.	8.8, 10.4
		417	142.0	354.8	3.5	1 h	98	99	Neg. mode	8.6, 10.0
10	TG	1000	435.4	850.0	3	2 h 15 + 15 min	89	96	1026.61	8.5, 12.7
									1027.02 calcd.	

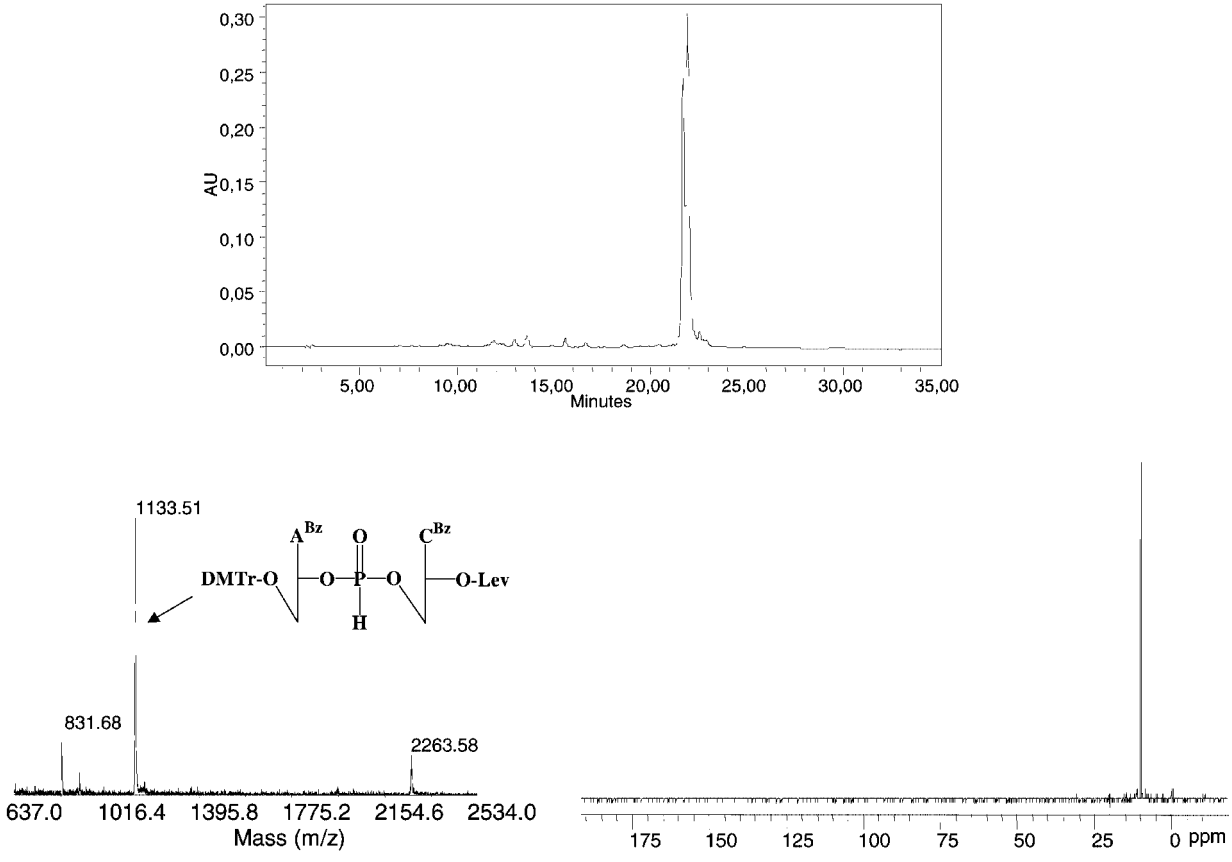


Figure 2. HPLC profile, MALDI-TOF MS and ^{31}P NMR spectra of dimer Dmtr-d**A**^{Bz}-HP-d**C**^{Bz}-3′-Lev after precipitation.

5.5 equiv. of acyl chloride were necessary to drive the reaction to completion whereas above the 400 μmol scale, 3.5 equiv. were sufficient. This observation can be explained by the more anhydrous conditions of larger-scale syntheses. We hypothesized that the residual water content was lower for larger-scale syntheses and thus less acyl chloride was necessary to obtain a complete reaction. Most of the reactions were complete within a time of between 1 h and 1 h 30 (HPLC monitoring). After completion, the resin was filtered off and the pyridine evaporated. The pyridinium salt was then removed by aqueous extraction. The organic layer was evaporated and the dimers were precipitated from diethyl ether as white powders. Compounds were analyzed by HPLC and characterized by MALDI-TOF MS and ^{31}P NMR spectroscopy. Figure 2 displays the results of HPLC, MALDI-TOF MS and ^{31}P NMR analyses of the dimer Dmtr-d**A**^{Bz}-HP-d**C**^{Bz}-3′-Lev after precipitation.

Note that in two cases (entries 5 and 10), there was insufficient resin to completely scavenge the excess 3′-HP nucleoside. However, after complete dimer formation, this excess was successfully trapped in 15 min by adding extra (3 equiv.) acyl chloride resin.

Thus, this protocol has allowed us to prepare, at up to 1 mmol scale, fully protected dimers with an *H*-phosphonate diester linkage in both high yield and high purity, yet without the need for any chromatography. Furthermore, the used resin was recycled up to five times with no decrease in its efficacy observed and with a chlorine content of between 2.1 and 2.3 mmol per gram of resin.

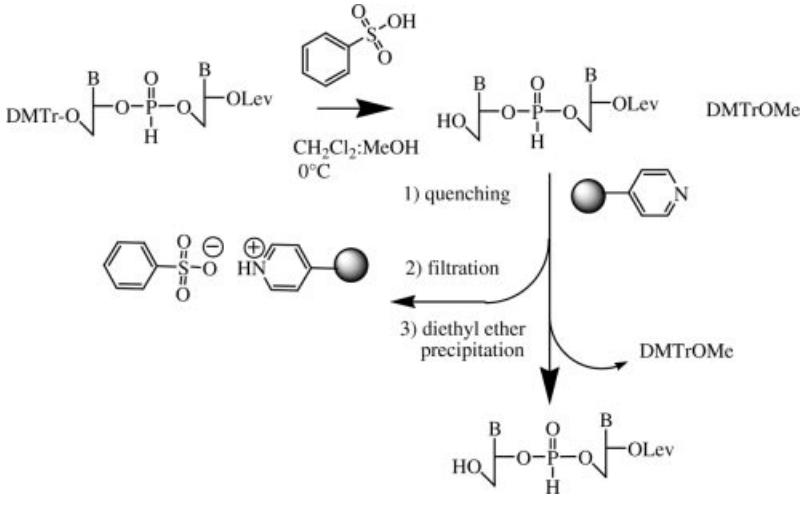
Detritylation

To further elongate the dimers, the Dmtr group needs to be removed next. Detritylation was achieved using a solution of 2% benzenesulfonic acid (BSA) in CH_2Cl_2/MeOH (7:3, v/v) at 0 °C for 15–30 min (Table 4). In the first attempt, the reaction was quenched by the addition of

Table 4. Detritylation of dimer using a 2% BSA solution in CH_2Cl_2/MeOH (7:3, v/v).

Sequence	Scale [μmol]	Scale [mg]	BSA [mL]	Quenching	Time [min]	Yield [%]	Yield [mg]	*m/z* (positive mode)
AT	100	105	5	$NaHCO_3$	15	70	52	742.14 742.66 calcd.
AC	106	120	5	$NaHCO_3$	15	65	66	830.58
	106	120	5	$NaHCO_3$	15	88	73.2	830.75 831.76 calcd.
TT	183	115	5	PVP	15	93.5	73	NA[a]
	410	382	10	PVP	30	86	223	
CT	594	426	10	PVP	30	98	330	717.71 718.63 calcd.
GC	88.7	98.9	5	PVP	30	94	68	812.08 813.74 calcd.
CA	158	180	5	PVP	20	83	109	831.91 831.76 calcd.
AT	438	458	10	PVP	20	98	322	742.16 742.66 calcd.
TG	420	430	10	PVP	20	100	320	725.24
	450	460	10	PVP	15	93	303	725.15 724.64 calcd.

[a] Thymine is not protonated.



Scheme 4. Schematic protocol for detritylation with benzensulfonic acid and quenching with PVP resin.

1 equiv. (with respect to BSA) of aqueous $NaHCO_3$ and then the mixture was washed with water. The trityl group was removed from the mixture by precipitation with diethyl ether. The resulting free 5′-OH dimers were obtained in a moderate yield (65–88%). We assumed that some cleavage of the internucleosidic linkage occurred during the quenching since it is known that *H*-phosphonate diesters are sensitive to the basic conditions of the work-up.[6] Thus, to prevent partial cleavage, we quenched the reaction further by adding poly(4-vinylpyridine) resin (PVP resin, 5.5 equiv. of resin per equiv. of acid) (Scheme 4). After filtration and evaporation, the dimer was precipitated from diethyl ether to remove the trityl derivative. The yields were higher (83–100%) than those obtained with $NaHCO_3$ quenching and aqueous work-up and the purities were generally greater than 92%. Quenching of the reaction with a resin had another advantage in that 5′-OH-$\mathbf{T}_{HP}$**T**-3′-Lev is soluble in water, which made it compulsory to avoid aqueous treatment. Note that some degradation of the dimer was observed when the more basic resin Amberlyst A21 (polystyrene-bound dimethylammonium) was used.

Synthesis of Short Oligonucleotides: Trimer to Pentamer

The solid-phase synthetic approach was also used to synthesize short oligonucleotides without oxidizing the *H*-phosphonate diester linkages. Several trimers were made in this way, at scales ranging from 70–420 μmol. Using 4–5 equiv. of acyl chloride resin, the coupling time was generally longer than that required to synthesize the dimers (3–5 h instead of 1 h). To increase the reaction rate, 8–12 equiv. of acyl chloride resin were used which enabled the coupling reaction to be finished within 1 h. Trimers were obtained in yields of between 55 and 91% and with purities of between 67 and 99% (Table 5).

Therefore, by following the sequence of 1) coupling, 2) filtration, 3) aqueous washing, 4) precipitation (optional), 5) detritylation, 6) PVP quenching, 7) filtration and 8) precipitation, short oligonucleotides have been synthesized. As an example, we report the synthesis of the pentamer Dmtr-**TG**$^{i\mathrm{Bu}}$**C**$^{\mathrm{Bz}}$**A**$^{\mathrm{Bz}}$**T**-Lev. (Table 6). The dimer Dmtr-**A**$^{\mathrm{Bz}}$**T**-Lev *H*-phosphonate diester was prepared on a 438 μmol scale (149 mg) from **T**-3′-Lev and 1.15 equiv. of Dmtr-**A**$^{\mathrm{Bz}}$-3′-*H*-phosphonate monoester with 3.5 equiv. of acyl chloride resin in 1 h in 100% yield after precipitation (98% purity; MS: *m*/*z* = 1044.96; ^{31}P NMR: δ = 8.41 and 9.77 ppm). The dimer was detritylated using 2% BSA in CH_2Cl_2/MeOH (8 mL, 7:3, v/v) at 0 °C for 20 min. The reaction was quenched with poly(4-vinylpyridine) resin (800 mg) for 5 min and then filtered. After evaporation, the residue was precipitated from diethyl ether (322 mg, 98%, 430 μmol; MS: found 742.16; calcd. 742.66: ^{31}P NMR: δ = 8.54 and 9.73 ppm).

The resulting dimer reacted with Dmtr-**C**$^{\mathrm{Bz}}$-3′-HP activated with 3.5 equiv. of acyl chloride resin to yield the *H*-phosphonate diester trimer Dmtr-**C**$^{\mathrm{Bz}}$**A**$^{\mathrm{Bz}}$**T**-Lev in 3 h (556 mg, 91% yield, 90% purity; MS: *m*/*z* = 1420.0). Then the trimer was detritylated and the reaction was quenched (as described previously) to give the 5′-OH-**C**$^{\mathrm{Bz}}$**A**$^{\mathrm{Bz}}$**T**-Lev trimer (432 mg, 99% yield, 386 μmol; MS: found 1118.3; calcd. 1118.95). A further coupling with Dmtr-**G**$^{i\mathrm{Bu}}$-3′-HP activated with 4.5 equiv. of resin gave the *H*-phosphonate diester tetramer Dmtr-**G**$^{i\mathrm{Bu}}$**C**$^{\mathrm{Bz}}$**A**$^{\mathrm{Bz}}$**T**-Lev in 3 h (520 mg, 75% yield, 96% purity; MS: found 1803.45; calcd. 1804.63) with little trace of any remaining trimer (MS: *m*/*z* = 1119). After detritylation (solubility was poor), the tetramer reacted with Dmtr-**T**-3′-HP (1.5 equiv.) activated with 8 equiv. of acyl chloride resin to yield the *H*-phosphonate diester pentamer Dmtr-**TG**$^{i\mathrm{Bu}}$**C**$^{\mathrm{Bz}}$**A**$^{\mathrm{Bz}}$**T**-Lev in 5 h (394 mg, 60% yield, 188 μmol, 92% purity).

The rate of the reaction as well as the yields both decreased with the length of the oligomer although the amount of acyl chloride resin was increased to increase the rate of the reaction. Note that even when yields were lower, the HPLC purity of the oligomer remained high.

Unfortunately, after detritylation of the pentamer some impurities corresponding to shorter sequences were observed. We assumed that a side-reaction occurred involving

Table 5. Fully protected trimers synthesized by activation with 4–11.7 equiv. of the Rapp acyl chloride resin using 1.2 equiv. of 5′-Dmtr 3′-HP nucleoside in solution in CH_2Cl_2/pyridine (1:1, v/v).

Sequence	Scale [μmol] 5′-OH	Resin [equiv.]	Conc. HP [mM]	Time	Yield [%]	HPLC purity [%] at 260 nm	*m*/*z* (MALDI-TOF)
AAT	70	7.7	24	3 h	75	91	1445.60 1446.36 calcd.
TAC	79.5	11.7	13	1 h	65	67	1419.86
	88.2	8	22	1 h	82	82	1421.06 1422.33 calcd.
GTT	116	8	22	1 h	55	93	1312.50
	355	4	46	3 h	70	67	1312.26 1315.21 calcd.
GCT	410	4	63	3 h 30	detritylated	90	1402.52 1404.31 calcd.
GCA	131	5	30	1 h	50	74	1516.14 1517.43 calcd.
CAT	430	4.5	65	3 h	91	99	1420.23 1421.33 calcd.
CTG	430	4.5+0.5	58	4 h	89	99	1403.95 1404.31 calcd.

Table 6. Synthesis of pentamer Dmtr-**TG**$^{i\text{Bu}}$**C**$^{\text{Bz}}$**A**$^{\text{Bz}}$**T**-Lev H-phosphonate.

Oligonucleotide	Starting material		Resin/	Time	Isolated product	HPLC	*m/z*
	H-phosphonate	5′-OH-oligo	*H*-phos		yield	purity [%]	
DMTr-**A**$^{\text{Bz}}$**T**-Lev	412 mg, 500 μmol, 1.15 equiv.	149 mg, 438 μmol, 1 equiv.	830 mg, 3.5 equiv.	1 h	458 mg, 100%	100	1045.03 calcd. 1044.96 exp.
HO-**A**$^{\text{Bz}}$**T**-Lev		458 mg, 438 μmol		20 min	322 mg, 98%	100	742.66 calcd. 742.16 exp.
DMTr-**C**$^{\text{Bz}}$**A**$^{\text{Bz}}$**T**-Lev	413 mg, 516 μmol, 1.2 equiv.	322 mg, 430 μmol, 1 equiv.	860 mg, 3.5 equiv.	3 h	556 mg, 91%	100	1422.33 calcd. 1420.23 exp.
HO-**C**$^{\text{Bz}}$**A**$^{\text{Bz}}$**T**-Lev		556 mg, 391 μmol		20 min	432 mg, 99%	79	1118.95 calcd. 1118.30 exp.
DMTr-**G**$^{i\text{Bu}}$**C**$^{\text{Bz}}$**A**$^{\text{Bz}}$**T**-Lev	373 mg, 463 μmol, 1.2 equiv.	432 mg, 386 μmol, 1 equiv.	990 mg, 4.5 equiv.	3 h	520 mg, 75%	96	1804.63 calcd. 1803.45 exp.
HO-**G**$^{i\text{Bu}}$**C**$^{\text{Bz}}$**A**$^{\text{Bz}}$**T**-Lev		520 mg, 288 μmol		20 min	450 mg, 100%	45	1502.26 calcd. 1501.17
DMTr-**TG**$^{i\text{Bu}}$**C**$^{\text{Bz}}$**A**$^{\text{Bz}}$**T**-Lev	277 mg, 390 μmol, 1.3 equiv.	450 mg, 300 μmol, 1 equiv.	1500 mg, 8 equiv.	5 h	394 mg, 60%	92	2092.83 calcd. 2091.85 exp.

the transesterification of a free 5′-hydroxy group on the *H*-phosphonate diester linkage, as reported by Wada et al.[7] One way to avoid this side-reaction is to oxidize the *H*-phosphonate linkage to a stable phosphotriester linkage.

Oxidation Protocol

Oxidizing Reagents

In early 1990, Van Boom and co-workers reported that the oxidation of a dinucleoside *H*-phosphodiester in solution with *N*-(phenylthio)- and *N*-(benzylthio)succinimide yielded a phosphorothioate dimer after deprotection.[15] This idea was investigated further by using, specifically, *N*-(2-cyanoethylthio)phthalimide or *N*-(2-cyanoethylthio)succinimide (Figure 3) for the elaboration of building blocks for the synthesis of phosphorothioates in solution using phosphotriester chemistry[16] and for the synthesis of phosphorothioate oligonucleotides in solution using *H*-phosphonate chemistry.[4]

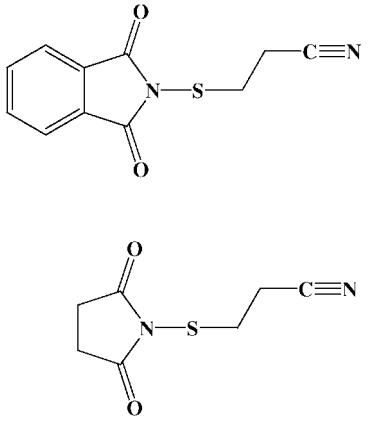


Figure 3. Reagents used for the oxidation of the *H*-phosphonate diester linkage.

We evaluated the use of both oxidizing reagents, the *N*-(2-cyanoethylthio)phthalimide and the *N*-(2-cyanoethylthio)succinimide, in the oxidation of our *H*-phosphonate diester dimers. Starting with the isolated dimer, oxidation reactions were performed with 3 equiv. of *N*-(2-cyanoethylthio)phthalimide in the presence of 0.5 equiv. of *N*-methylmorpholine (NMM) as an activator and the reactions were complete within 1 h. The oxidation reactions performed with *N*-(2-cyanoethylthio)succinimide were more efficient as these reactions were complete within 30 min and required only 1.8 equiv. of reagent and no NMM. Note that the *H*-phosphonate diester trimers were also sulfurized to give products of excellent purity.

Furthermore, we observed that the oxidation reactions could be performed directly on the crude *H*-phosphonate diesters, that is, without removing the acyl chloride resin.

It has been demonstrated in the synthesis of phosphorothioate dinucleosides in solution that coupling and sulfurization can be achieved selectively, though both reagents (i.e. bis-arylphosphorochloridate and *N*-(2-cyanoethylthio)succinimide) were added together to a 3′-HP nucleoside and a 5′-OH nucleoside.[4] Indeed, the *H*-phosphonate diester, obtained in situ, reacts with the sulfurizing reagent approximately an order of magnitude faster than the *H*-phosphonate monoester, hence the monoester is not oxidized. An attempt to do the same with our solid-supported coupling reagent was not successful. Indeed, when the two reagents [i.e. the acyl chloride resin and *N*-(2-cyanoethylthio)succinimide] were added simultaneously to a mixture of 3′-HP and 5′-OH nucleosides in pyridine/dichloromethane, we observed the formation of a byproduct. The reaction was monitored by HPLC and MALDI-TOF MS, and these analyses showed that not only the expected sulfurized dimer was formed, but that a side-sulfurization of the *H*-phosphonate monoester had also occurred. This result could be explained by the fact that the coupling reaction using solid-supported acyl chloride proceeded much more slowly than the coupling reaction in solution with pivaloyl chloride. As a consequence, the *H*-phosphonate diester dimer was formed more slowly and a competitive sulfurization between the *H*-phosphonate mono- and diester had time to occur. Therefore, in all subsequent syntheses of oligonucleotides, the coupling and the sulfurization steps were performed successively.

Synthesis of Hexamer Dmtr-C^{Bz}pScneA^{Bz}pScneG^{iBu}pScneC^{Bz}pScneG^{iBu}pScneT-Lev with Thiolatecyanoethyl Phosphotriester Linkages

The main advantage of the synthesis in solution is that it can proceed by a convergent approach (Scheme 5). Thus, risk of failure is minimized and the overall yield is improved. To synthesize a hexamer, we first prepared the two trimers Dmtr-**C**^{Bz}pScne**A**^{Bz}pScne**G**^{iBu}-3′-HP and 5′-OH-**C**^{Bz}pScne**G**^{iBu}pScne**T**-Lev which were then coupled together. This necessitated the previous conversion of one trimer to its 3′-*H*-phosphonate monoester derivative.

Synthesis of Trimer 5′-OH-C^{Bz}pScneG^{iBu}pScneT-Lev

A Dmtr-**G**^{iBu}pScne**T**-Lev dimer was synthesized (on a 0.66 mmol scale) as described above using 4 equiv. of supported acyl chloride. After coupling, oxidation was achieved by the direct addition of *N*-(2-cyanoethylthio)succinimide. The resin was filtered off and the pyridinium hydrochloride was removed by aqueous extraction. The dimer was isolated by precipitation from diethyl ether (79% yield, 92% HPLC purity). The Dmtr group was removed using BSA and the mixture was quenched with $NaHCO_3$ instead of PVP resin owing to the better stability of the phosphorothiolate triester linkage under basic conditions. After extraction and evaporation the 5′-OH dimer was precipitated (92%). Following the same protocol, Dmtr-**C**^{Bz}-3′-HP was coupled with this dimer and sulfurized. After detritylation and precipitation, the 5′-OH trimer was obtained in a 52% overall yield over the six steps (89.7% average yield) and with a 95% HPLC purity at 260 nm (Figure 4).

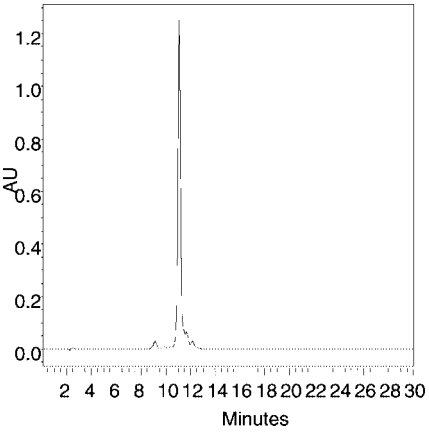
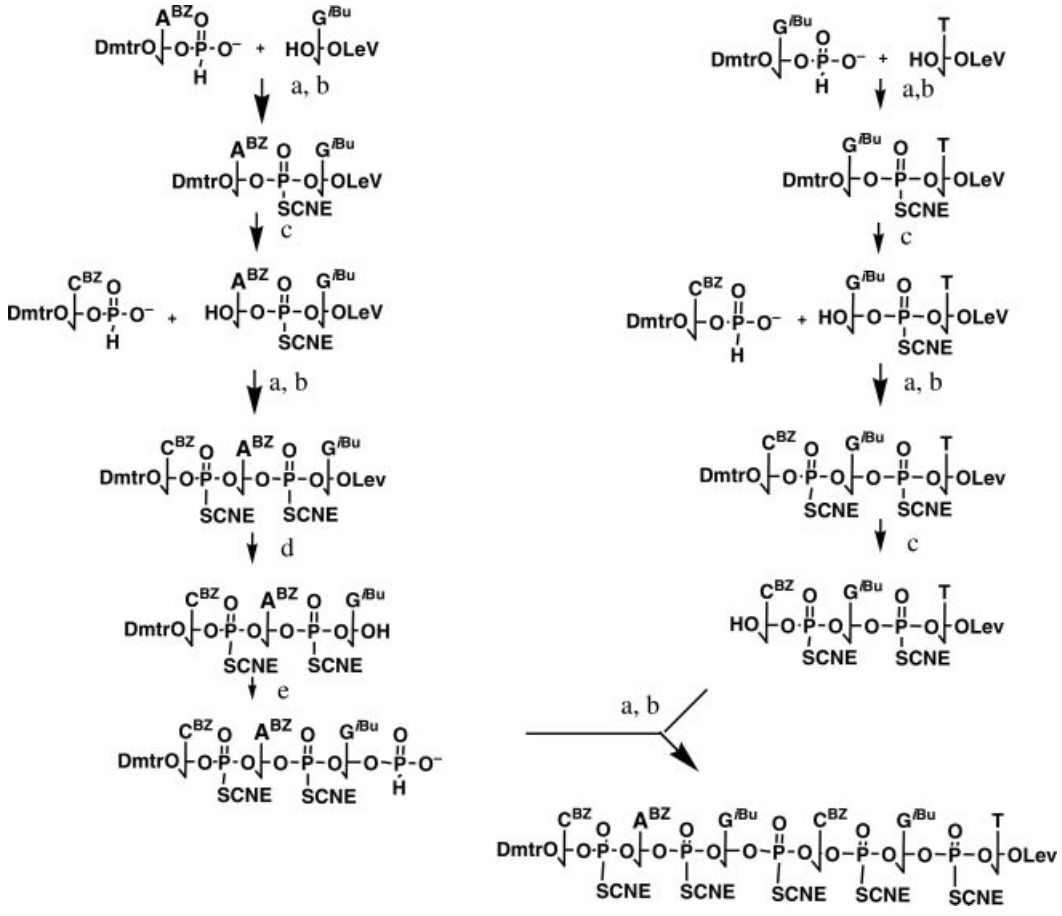


Figure 4. HPLC profile of 5′-OH-**C**^{Bz}pScne**G**^{iBu}pScne**T**-Lev after precipitation.



Scheme 5. Convergent synthesis of fully protected hexamer CAGCGT. Reagents and consitions: a. acyl chloride resin, CH_2Cl_2, pyridine; b. succ-Scne; c. 2% BSA, CH_2Cl_2/MeOH; d. hydrazine resin; e. salicylchlorophosphite, CH_2Cl_2, pyridine.

Synthesis of Trimer Dmtr-C^{Bz}pScneA^{Bz}pScneG^{iBu}-3′-HP

Initially, the Dmtr-**C**^{Bz}pScne**A**^{Bz}pScne**G**^{iBu}-3′-Lev trimer was synthesized, starting from 1 mmol of 5′-OH-**G**^{iBu}-Lev, following the same procedure as that outlined above. The levulinyl group was removed in 2 h by using a solid-supported hydrazine (Amberlyst A15 in hydrazine form, 15 equiv.) to trap the levulinyl group on the resin. After filtration and precipitation, the 3′-OH trimer was obtained in a 54.8% overall yield over the six steps (90.5% average yield) and with 79% purity (Figure 5).

The next key step was phosphitylation. Several protocols for this reaction have been described in the literature[17–21] and two of them have given good results. Since, from the outset, our highest priority was to develop a process that did not involve chromatography, we tried a phosphitylation

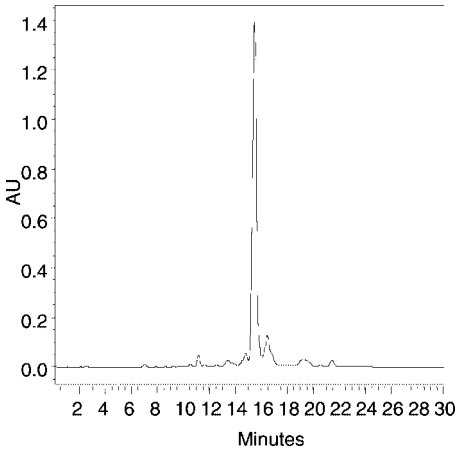


Figure 5. HPLC profile of trimer Dmtr-**C**^{Bz}pScne**A**^{Bz}pScne**G**^{iBu}-3′-OH.

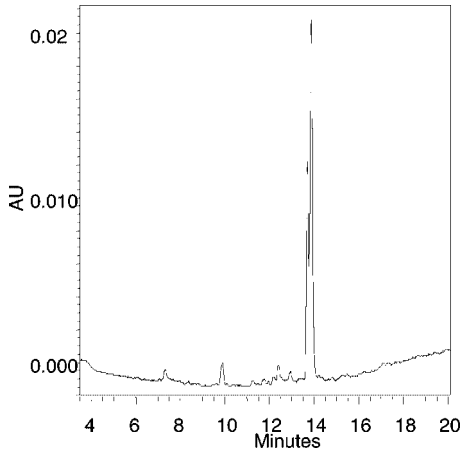


Figure 6. HPLC profile of trimer Dmtr**C**^{Bz}pScne**A**^{Bz}pScne**G**^{iBu}-3′-HP.

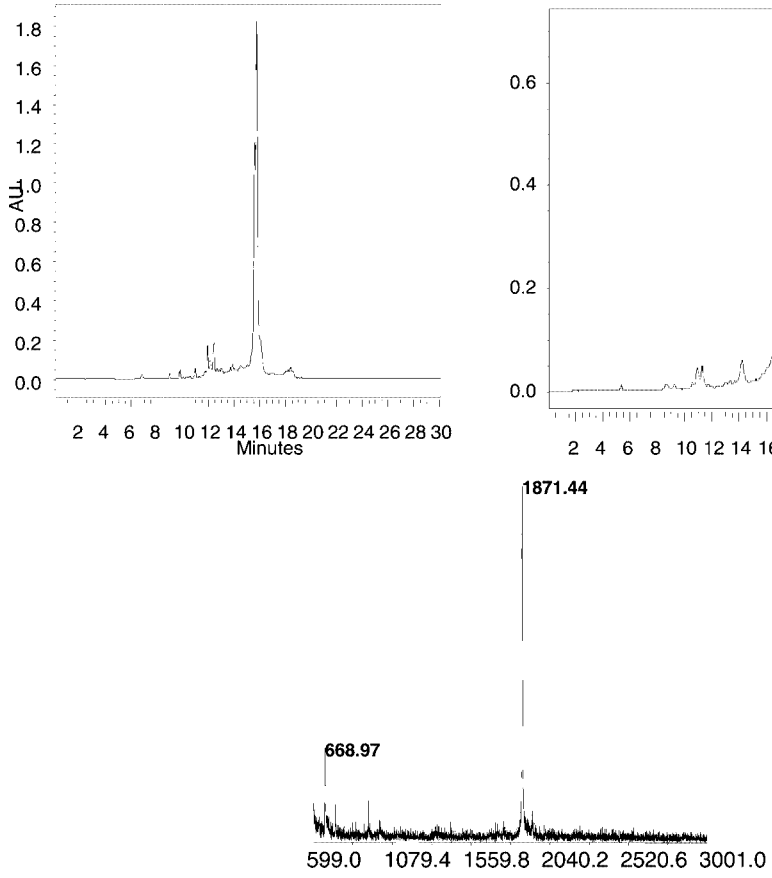


Figure 7. HPLC profiles of fully protected (left) and deprotected (right) hexamer CAGCGT and MALDI-TOF MS of deprotected hexamer.

protocol using 4-methylphenyl *H*-phosphonate[21] and the same solid-supported acyl chloride as used in the coupling reaction in pyridine/CH_2Cl_2 (1:1, v/v). The reaction was usually complete in 2–3 h. The main advantage of this approach is that the excess activator is easily removed by filtration and did not need to be quenched by washing with different buffers. The mixture was treated with water and a sulfonic acid resin (in triethylammonium form) and the 3′-HP trimer was obtained in an 83–95% yield and with 83–85% HPLC purity. However, this phosphitylating reagent is not commercially available and releases foul-smelling fumes of cresol. Therefore, we used the salicylchlorophosphite reagent[17] which does not need to be activated and is commercially available.

Hence, the trimer was phosphitylated using salicylchlorophosphite in pyridine/CH_2Cl_2 (1:1, v/v) at 0 °C for 3 h 30. After hydrolysis of the excess chlorophosphite, the mixture was quenched by the addition of a sulfonic acid resin (in triethyammonium form). After filtration, the expected trimer was precipitated (83% yield, 90% purity) (Figure 6).

Both trimers, dissolved in pyridine/CH_2Cl_2 (1:1, v/v), were coupled by the addition of solid-supported acyl chloride (10 equiv.) for 3 h 30 and then the *H*-phosphonate linkage was oxidized with *N*-(2-cyanoethylthio)succinimide. After work-up, the hexamer was precipitated (49% yield, 89% HPLC purity) (Figure 7).

Unblocking of Hexamer Phosphorothioate

Since the hexamer contains phosphorothioate linkages, direct treatment with ammonia could not be performed as partial desulfurization of the phosphorus atoms would occur. It was therefore necessary to first remove the cyanoethyl group using DBU in dry acetonitrile. A portion of the fully protected hexamer in dry acetonitrile solution was treated with DBU (in the presence of chlorotrimethylsilane as a scavenger of water) for 30 min. After evaporation of the solvent, concentrated ammonia and mercaptoethanol were added and the resulting mixture was heated at 55 °C overnight. The solvent was removed under vacuum and the residue was treated with 80% aqueous acetic acid for 1 h. Acetic acid was removed by coevaporation with water and ethanol. The resulting residue was precipitated from cold ethanol. The solid was dissolved in water and the amount of hexamer was determined from its absorbance at 260 nm (330 $OD^{260\ nm}$, 5.3 μmol). The hexamer gave a broad HPLC peak owing to the presence of 2^5 diastereoisomers and was characterized by MALDI-TOF MS (found 1871.44; calcd. 1871.56) (Figure 7).

Conclusions

We have developed a strategy for synthesizing oligonucleotides in solution using solid-supported reagents for the coupling, delevulinylation and phosphitylation steps. The synthesis of the dimers was scaled up to 1 mmol with high purity without chromatography. The polystyrene acyl chloride resin was recycled up to five times without any quantifiable decrease in its efficacy. Short oligonucleotide *H*-phosphonate diesters were synthesized, but were found to be unstable after detritylation. A hexamer exhibiting a thiolatecyanoethyl phosphotriester linkage was synthesized on the 340 μmol scale without chromatography and with a 90% HPLC purity at 260 nm.

Experimental Section

General Remarks: All commercial chemicals were reagent grade and were used without further purification, except where otherwise stated. *H*-Phosphonate monoester nucleosides were synthesized according to literature procedures.[19]

HPLC Analysis and MS Characterization: High-performance liquid chromatography (HPLC) analyses were performed with a Waters-Millipore instrument equipped with a Model 600E solvent delivery system, a reodyne injector and a Model 996 PDA detector. A reversed-phase C18 (5 μm) Nucleosil column (150 × 4.6 mm, Supelco) with acetonitrile (linear gradient of 10–72%) in 0.05 M aqueous triethylammonium acetate (pH 7) as eluent at a flow rate of 1 mL/min for 13 min.

MALDI-TOF mass spectra were recorded with a Voyager DE mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) equipped with an N2 laser. Prooligo samples (1 μL, 0.1 $OD^{260\ nm}$ in 100 μL water/acetonitrile, 50:50, v/v) were exchanged on an ammonium DOWEX 50W X8 resin prior to the addition of an acetonitrile/water (10 μL, 1:1, v/v) solution saturated with 2,4,6-trihydroxyacetophenone (THAP) and ammonium citrate (1 μL, pH 9.4, 100 mM in water). This mixture was placed on a plate and dried at ambient temperature and pressure.

Preparation of Polystyrene Acyl Chloride Resin: Polystyrene carboxy resin (6 g, ≈12 mmol) from Rapp-Polymers (load: 1.96 mmol/g) was suspended in anhydrous CH_2Cl_2 (80 mL) in a round-bottomed flask to which DMF (300 μL) and $SOCl_2$ (3.8 mL, 48 mmol) were added. The mixture was stirred under reflux for 3 h and then the resin was filtered under argon and vacuum and washed twice with CH_2Cl_2 and diethyl ether. The resin was dried under vacuum for 4 h. Chlorine content, determined by elemental analysis, was in the range of 2.1–2.3 mmol/g.

General Protocol for Coupling: The 5′-Dmtr-3′-HP (0.60 mmol) and 5′-OH-3′-Lev nucleosides (0.50 mmol) were dissolved in CH_2Cl_2:pyridine (5 mL, 1:1, v/v) and added to the polystyrene-acyl chloride resin (1.0 g, 3.5 equiv.) suspended in CH_2Cl_2/pyridine (5 mL, 1:1, v/v). The mixture was shaken and the reaction monitored by HPLC. After completion (1–2 h), the resin was filtered off and washed with CH_2Cl_2. The organic layer was washed with water, dried with Na_2SO_4 and removed by evaporation under reduced pressure. The residue was dissolved in toluene and precipitated from diethyl ether.

General Protocol for Detritylation of *H*-Phosphonate Diester Derivatives: 10% BSA (2.0 mL) in CH_2Cl_2/MeOH (7:3, v/v) was added to a 5′-Dmt-3′-Lev *H*-phosphonate diester dinucleoside (0.438 mmol) in CH_2Cl_2/MeOH (8.0 mL, 7:3, v/v) cooled to 0 °C. The reaction was monitored by TLC. After 20–60 min, the solution was diluted with CH_2Cl_2 (40 mL) and water (1 mL). Then, poly(4-vinylpyridine) (FLUKA 81391) (800 mg) was added and the mixture was shaken for 5 min. The resin was filtered off and pyridine (1 mL) added to the filtrate. The organic layer was evaporated under reduced pressure and the residue dissolved in toluene and precipitated from diethyl ether.

Synthesis of 5′-OH-dA^{Bz}-dG^{iBu}-3′-*O*-Lev Thiolatecyanoethyl Phosphotriester: The monomer 5′-*O*-DMTr-dA^{Bz}-3′-HP (987 mg, 1.2 mmol, 1.2 equiv.) was coevaporated twice with anhydrous pyridine (10 mL). 5′-OH-d**G**^{iBu}-3′-*O*-Lev (435 mg; 1 mmol) was added and two more coevaporations with anhydrous pyridine (10 mL) were performed. The resulting foam was dissolved in CH_2Cl_2/pyridine (7 mL, 1:1, v/v) and added to a round-bottomed flask containing polystyrene-supported acyl chloride (2.1 g, 4.75 mmol) swollen in CH_2Cl_2/pyridine (5 mL, 1:1, v/v). The reaction was monitored by reversed-phase HPLC. After shaking for 2 h 30 at room temperature, *N*-(2-cyanoethylthio)succinimide (442 mg, 2.4 mmol) was added. After stirring for 30 min, the resin was filtered off and washed with CH_2Cl_2 (50 mL). The organic layer was washed with deionized water (2 × 50 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The crude dimer was dissolved in cold (0 °C) CH_2Cl_2/MeOH (14 mL, 7:3, v/v) and a solution of 10% benzenesulfonic acid in CH_2Cl_2/MeOH (3.7 mL, 7:3, v/v) was added. The reaction was stirred for 15 min at 0 °C (TLC monitoring: CH_2Cl_2/MeOH 95:5, v/v). Then CH_2Cl_2 (50 mL), distilled water (1 mL) and a saturated solution of $NaHCO_3$ (50 mL) were successively added to the solution. After phase separation, the organic layer was washed with distilled water (3 × 50 mL), dried with Na_2SO_4, and concentrated under vacuum. The residue was precipitated in diethyl ether (100 mL) to give 760 mg (0.824 mmol, 83%) of the desired product (92% HPLC purity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 922.89; found 921.62.

Synthesis of 5′-*O*-DMTr-dC^{Bz}-dA^{Bz}-dG^{iBu}-3′-OH Thiolatecyanoethyl Phosphotriester: The monomer 5′-*O*-DMTr-d**C**^{Bz}-3′-HP (799 mg, 0.99 mmol) was coevaporated twice with anhydrous pyridine (10 mL). 5′-OH-d**A**^{Bz}-d**G**^{iBu}-3′-*O*-Lev (760 mg, 0.82 mmol) was added and two more coevaporations with anhydrous pyridine (10 mL) were performed. The resulting foam was dissolved in CH_2Cl_2/pyridine (5 mL, 1:1, v/v) and added to a round-bottomed flask containing polystyrene-supported acyl chloride (950 mg, 3.96 mmol) swollen in CH_2Cl_2/pyridine (3 mL, 1:1, v/v). The reaction was monitored by reversed-phase HPLC. After shaking for 3 h at room temperature, *N*-(2-cyanoethylthio)succinimide (304 mg, 1.65 mmol) was added. After stirring for 30 min, the resin was filtered off and washed with CH_2Cl_2 (50 mL). The organic layer was washed with deionized water (2 × 50 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The residue was dissolved in pyridine (16 mL) and acetic acid (4 mL). Then Amberlyst A15 NH_3^+-NH_2 (3.1 g, 12.36 mmol) was added. The mixture was shaken at room temperature for 2 h [TLC monitoring: CH_2Cl_2/MeOH (9:1, v/v)] and the resin was filtered off and washed with CH_2Cl_2 (100 mL). A cold (4 °C) saturated solution of $NaHCO_3$ (55 mL) was added. After phase separation, the organic layer was washed with distilled water (2 × 55 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The residue was precipitated in cooled diethyl ether (400 mL) to obtain 860 mg (0.54 mmol, 66%) of the desired product (79% HPLC purity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 1588.59; found 1587.27.

Synthesis of 5′-*O*-DMTr-dC^{Bz}-dA^{Bz}-dG^{iBu}-3′-HP Thiolatecyanoethyl Phosphotriester: The trimer 5′-*O*-DMTr-d**C**^{Bz}-d**A**^{Bz}-d**G**^{iBu}-3′-OH (860 mg, 0.54 mmol) was coevaporated with pyridine (2 × 6 mL) and dissolved in CH_2Cl_2/pyridine (10 mL, 1:1, v/v). Salicylchlorophosphite (274 mg, 1.35 mmol) in CH_2Cl_2/pyridine (10 mL, 1:1, v/v) was added at 0 °C and the reaction mixture was stirred for 15 min at 0 °C and then at room temperature for 3 h. Then water (150 μL) and sulfonyl resin as the triethylammonium form (4.2 g, 13.5 mmol) were added and the mixture was shaken for 1 h. The resin was filtered and washed with CH_2Cl_2 (50 mL). The organic layer was washed with water (2 × 160 mL), dried with Na_2SO_4 and evaporated under reduced pressure to obtain 600 mg (0.34 mmol, 63%) of an oil of the desired phosphorylated trimer (90% HPLC purity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 1652.56; found 1652.69.

Synthesis of 5′-OH-dG^{iBu}-dT-3′-*O*-Lev Thiolatecyanoethyl Phosphotriester: The monomer 5′-*O*-DMTr-d**G**^{iBu}-3′-HP (640 mg, 0.8 mmol, 1.2 equiv.) was coevaporated twice with anhydrous pyridine (10 mL). 5′-OH-d**T**-3′-*O*-Lev (226 mg, 0.66 mmol) was added and two more coevaporations with anhydrous pyridine (10 mL) were performed. The resulting foam was dissolved in CH_2Cl_2/pyridine (5 mL, 1:1, v/v) and added to a round-bottomed flask containing the polystyrene-supported acyl chloride (1.4 g, 3.12 mmol) swollen in CH_2Cl_2/pyridine (3 mL, 1:1, v/v). After shaking for 3 h at room temperature, *N*-(2-cyanoethylthio)succinimide (260 mg, 1.4 mmol) was added. After stirring for 30 min, the resin was filtered off and washed with CH_2Cl_2 (50 mL). The organic layer was washed with deionized water (2 × 50 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The residue was precipitated in diethyl ether (70 mL) to give 580 mg (0.52 mmol, 79%) of the desired product (92% HPLC purity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 1112.14; found 1111.49.

The dimer (80 mg, 0.52 mmol) was dissolved in cold CH_2Cl_2/MeOH (9 mL, 7:3, v/v) and 10% benzenesulfonic acid in CH_2Cl_2/MeOH (2.4 mL, 7:3, v/v) was added at 0 °C. The reaction was stirred for 15 min at 0 °C [TLC monitoring: CH_2Cl_2/MeOH (9:1, v/v)] and then CH_2Cl_2 (50 mL), distilled water (1 mL) and a saturated solution of $NaHCO_3$ (50 mL) were successively added. After phase separation, the organic layer was washed with distilled water (3 × 50 mL), dried with Na_2SO_4 and concentrated under vacuum. The residue was precipitated in diethyl ether (200 mL) to give 386 mg (0.48 mmol, 92%) of the desired product (95% HPLC purity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 809.77; found 808.93.

Synthesis of 5′-OH-dC^{Bz}-dG^{iBu}-dT-3′-*O*-Lev Thiolatecyanoethyl Phosphotriester: The monomer 5′-*O*-DMTr-d**C**^{Bz}-3′-HP (455 mg, 0.57 mmol, 1.2 equiv.) was coevaporated twice with anhydrous pyridine (10 mL). 5′-OH-d**G**^{iBu}-d**T**-3′-*O*-Lev (386 mg, 0.47 mmol) was added and two more coevaporations with anhydrous pyridine (10 mL) were performed. The resulting foam was dissolved in CH_2Cl_2/pyridine (5 mL, 1:1, v/v) and added to a round-bottomed flask containing polystyrene-supported acyl chloride (950 mg, 3.96 mmol) swollen in CH_2Cl_2/pyridine (3 mL, 1:1, v/v). After shaking for 3 h at room temperature, *N*-(2-cyanoethylthio)succinimide (173 mg, 0.94 mmol) was added. After stirring for 30 min, the resin was filtered off and washed with CH_2Cl_2 (50 mL). The organic layer was washed with deionized water (2 × 50 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The residue was precipitated in diethyl ether (50 mL) to give 650 mg (0.41 mmol, 88%) of the desired product (85% HPLC purity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 1574.57; found 1571.00.

The trimer (650 mg, 0.41 mmol) was dissolved in CH_2Cl_2/MeOH (7 mL, 7:3, v/v) and 10% benzenesulfonic acid in CH_2Cl_2/MeOH (1.9 mL, 7:3, v/v) was added at 0 °C. The reaction was stirred for 30 min at 0 °C [TLC monitoring: CH_2Cl_2/MeOH (9:1, v/v)] and then CH_2Cl_2 (50 mL), distilled water (1 mL) and a saturated solution of $NaHCO_3$ (50 mL) were successively added. After phase separation, the organic layer was washed with distilled water (3 × 50 mL), dried with Na_2SO_4 and concentrated under vacuum. The residue was precipitated in diethyl ether (200 mL) to give 440 mg (0.35 mmol, 84%) of the desired product (92% HPLC pu-

rity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 1272.19; found 1269.96.

Synthesis of the Hexamer 5′-*O*-DMTr-dC^{Bz}-dA^{Bz}-dG^{iBu}-dC^{Bz}-dG^{iBu}-dT-3′-*O*-Lev Thiolatecyanoethyl Phosphotriester: The trimer 5′-*O*-DMTr-d**C**^{Bz}-d**A**^{Bz}-d**G**^{iBu}-3′-HP (600 mg, 0.34 mmol) was coevaporated twice with anhydrous pyridine (5 mL) and 5′-OH-d**C**^{Bz}-d**G**^{iBu}-d**T**-3′-*O*-Lev (440 mg, 0.34 mmol) was added and coevaporated twice more with anhydrous pyridine (5 mL). The resulting foam was dissolved in CH_2Cl_2/pyridine (5 mL, 1:1, v/v) and added to a round-bottomed flask containing polystyrene-supported acyl chloride (5 g, 3.4 mmol) swollen in CH_2Cl_2/pyridine (4 mL, 1:1, v/v). After shaking for 3 h 30 at room temperature, *N*-(2-cyanoethylthio)succinimide (125 mg, 0.68 mmol) was added. After stirring for 30 min, the resin was filtered off and washed with CH_2Cl_2 (50 mL). The organic layer was washed with distilled water (2 × 50 mL), dried with Na_2SO_4 and concentrated under reduced pressure. The residue was precipitated in cooled diethyl ether (300 mL) to obtain 500 mg (0.17 mmol, 49%) of the desired product (89% HPLC purity at 260 nm). MALDI-TOF MS (positive mode, THAP): calcd. 2991.87; found 2988.95.

Unblocking of the Hexamer: Fully protected hexamer (50 mg, 17 μmol) was first dried by coevaporating twice with dry pyridine. Then, after dissolving in dry acetonitrile (1 mL), chlorotrimethylsilane (25 μL) and DBU (114 μL) were added. After 30 min at room temperature, the solvents were evaporated and concentrated ammonia (1 mL) and mercaptoethanol (0.1 mL) were added. The mixture was heated at 55 °C overnight. The solvent was removed under vacuum and the residue was treated with 80% aqueous acetic acid (400 μL) for 1 h. The acetic acid was removed by coevaporation with water and ethanol. The resulting residue was precipitated from cold ethanol (–10 °C). The solid was dissolved in water and the amount of hexamer was determined from its absorbance at 260 nm (330 $OD^{260\ nm}$, 5.3 μmol, 32%).

Acknowledgments

This work was supported by the Ministère de l′Education Nationale, de la Recherche et de la Technologie, France, by Girindus AG, Germany, and the CNRS. We thank Natalie L. Smith from Girindus America Inc. for careful reading of this manuscript.

[1] C. B. Reese, *Tetrahedron* **2002**, *58*, 8893–8920.
[2] G. M. Bonora, C. L. Scremin, F. P. Colonna, A. Garbesi, *Nucleic Acids Res.* **1990**, *18*, 3155–3159.
[3] C. Mihaichuk, T. B. Hurley, K. E. Vagle, R. S. Smith, J. A. Yegge, G. M. Pratt, C. J. Tompkins, D. P. Sebesta, W. A. Pieken, *Org. Process Res. Dev.* **2000**, *4*, 214–224.
[4] C. B. Reese, H. B. Yan, *J. Chem. Soc., Perkin Trans. 1* **2002**, 2619–2633.
[5] C. Dueymes, A. Schönberger, I. Adamo, A. E. Navarro, A. Meyer, M. Lange, J. L. Imbach, F. Link, F. Morvan, J. J. Vasseur, *Org. Lett.* **2005**, *7*, 3485–3488.
[6] F. H. Westheimer, S. Huang, F. Covitz, *J. Am. Chem. Soc.* **1988**, *110*, 181–185.
[7] T. Wada, F. Honda, Y. Sato, M. Sekine, *Tetrahedron Lett.* **1999**, *40*, 915–918.
[8] P. J. Garegg, I. Lindh, T. Regberg, J. Stawinski, R. Stromberg, C. Henrichson, *Tetrahedron Lett.* **1986**, *27*, 4051–4054.
[9] B. C. Froehler, *Tetrahedron Lett.* **1986**, *27*, 5575–5578.
[10] A. Andrus, J. W. Efcavitch, L. J. McBride, B. Giusti, *Tetrahedron Lett.* **1988**, *29*, 861–864.
[11] T. Regberg, J. Stawinski, R. Stromberg, *Nucleosides Nucleotides* **1988**, *7*, 23–35.
[12] R. Zain, J. Stawinski, *J. Org. Chem.* **1996**, *61*, 6617–6622.
[13] P. J. Garegg, J. Stawinski, R. Stromberg, *J. Org. Chem.* **1987**, *52*, 284–287.
[14] J. Garegg, T. Regberg, J. Stawinski, R. Strömberg, *Nucleosides Nucleotides* **1987**, *6*, 655–662.
[15] C. E. Dreef, C. M. Dreef-Tromp, G. A. van der Marel, J. H. van Boom, *Synlett* **1990**, 481–483.
[16] X. H. Liu, C. B. Reese, *J. Chem. Soc., Perkin Trans. 1* **1995**, 1685–1694.
[17] J. E. Marugg, M. Tromp, E. Kuyl-Yeheskiely, G. A. van der Marel, J. H. van Boom, *Tetrahedron Lett.* **1986**, *27*, 2661–2664.
[18] B. C. Froehler, P. Ng, M. D. Matteucci, *Nucleic Acids Res.* **1986**, *14*, 5399–5407.
[19] J. Stawinski, M. Thelin, *Nucleosides Nucleotides* **1990**, *9*, 129–135.
[20] J. Jankowska, M. Sobkowski, J. Stawinski, A. Kraszewski, *Tetrahedron Lett.* **1994**, *35*, 3355–3358.
[21] V. Ozola, C. B. Reese, Q. Song, *Tetrahedron Lett.* **1996**, *37*, 8621–8624.

Received: July 20, 2005
Published Online: October 31, 2005

