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Communications to the Editor

Simple and Efficient Solution-Phase Synthesis of Oligonucleotides Using Extractive Work-Up

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Abstract:

A solution-phase synthesis protocol amenable to scale-up was developed for the preparation of oligonucleotides employing phosphoramidite chemistry and DMTr/iBu/Bz-protected monomers. Isolation of intermediates was accomplished by means of extractions as the only purification tool. The potential of the method is demonstrated with the synthesis of a hexameric DNA fragment in high yield and purity.

Introduction

In recent years, much effort has been devoted to the development of oligonucleotides (ONs) and their chemically modified analogues as the active compounds in antisense,[1] aptamer,[2] ribozyme,[3] microRNA,[4] RNAi,[5] and immunostimulatory CpG[6] based therapeutics. The antisense phosphorothioate ONs have thus far been the most successful with one product on the market for treatment of cytomegaloviral ritinitis in AIDS patients and several in different stages of clinical trials. The clinical success and commercialization of antisense oligonucleotides have triggered the development of efficient and cost-effective oligonucleotide synthesis protocols. Currently, all ONs in human clinical trials are produced via automated solid-supported syntheses utilizing phosphoramidite chemistry.[7] Although the solid-phase methodology produces ONs of high quality, the major limitations are the unpredictable scale-up and the use of relatively large excesses of expensive reagents. A solution-phase approach of ONs is likely to overcome these limitations, provided that time-consuming and expensive chromatographic purifications of intermediates can be avoided. In this vein, several approaches have already been explored that combine the advantages of solution- and solid-phase syntheses. For instance, the HELP protocol exploits the solubility of a PEG-based solid support, and isolation of intermediates is accomplished by precipitation and filtration.[8]

The PASS process utilizes a resin that temporarily immobilizes the growing ON chain and uses an extractive workup for removal of excess monomers.[9] Recently, the use of immobilized reagents in the solution-phase synthesis of ONs (up to hexamers) was reported that employed phosphoramidite- as well as H-phosphonate chemistry.[10] The mentioned approaches all have in common polymers (resins) that are still involved in the process and that are often combined with recurrent purification methods such as precipitation and/or chromatography.

Results and Discussion

We here report a solution-phase approach based on well-established phosphoramidite chemistry that utilizes simple extraction procedures as the only tool for the isolation of ON intermediates.[11] Our strategy for the solution-phase preparation of ONs is outlined in Scheme 1 and can be divided into two stages. First, elongation of the 5′-OH of ON **1** with a slight excess of a phosphoramidite monomer (**2a**−**e**) under the agency of a suitable activator and subsequent hydrolysis of the excess monomer gives, after oxidation and extractive workup, a mixture of fully protected oligomer **3** and monomeric phosphodiester **4a**−**e**.

The second and key step in our strategy is the removal of the DMTr group from compounds **3** and **4a**−**e** combined with the extractive removal of byproducts **6a**−**e** and **7**. To this end, novel conditions for cleavage of the DMTr group were developed that facilitated extractive removal of the

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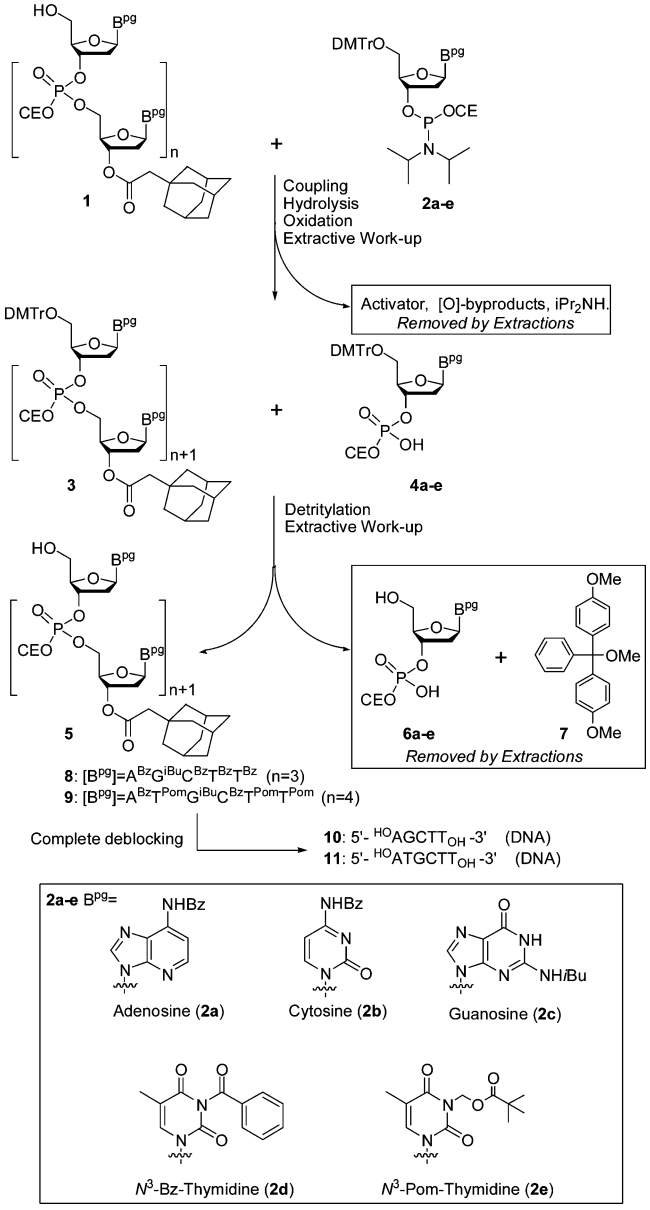
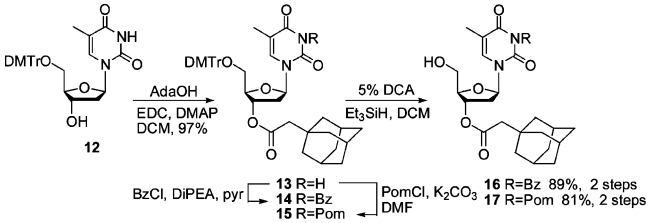
Scheme 1. **Strategy for the solution-phase synthesis of ONs**



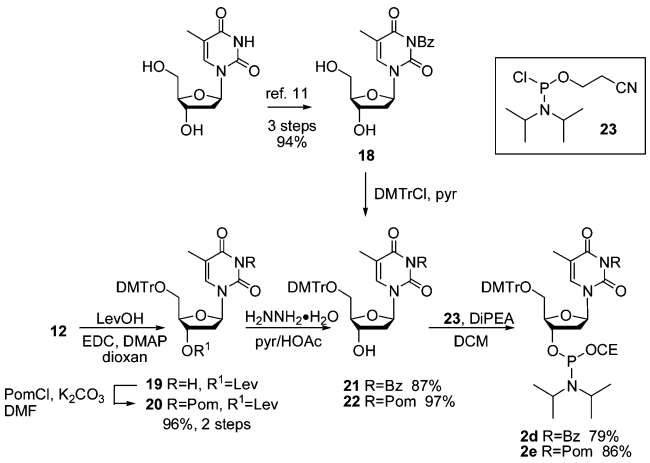
lipophilic DMTr derivative **7** from the reaction mixture. Finally, separation of the desired ON **5** and phosphodiester **6a**−**e** was accomplished by basic aqueous extraction. A crucial feature contributing to the success of our approach is the solubility of the ON in the organic phase. In the presented method, the growing ON chain is anchored in the organic phase by means of lipophilic protecting groups on the nucleobases (including N^3-protection of thymidine), the cyanoethyl moiety on the phosphates, and the adamantylacetic acid ester at the 3′ end of the ON. At first, Bz-protection of the N^3 in thymidine was chosen on the basis of an earlier observation[9] that the use of N^3-benzoylated thymidines dramatically increased the solubility of ONs.

The 3′-adamantylacetyl-N^3-benzoyl-thymidine **16** was used as the starting compound in the synthesis of ONs and was prepared as depicted in Scheme 2. Thus, esterification of dimethoxytrityl-thymidine (**12**) with adamantane acetic acid (AdaOH) under the agency of EDC/DMAP (→**13**) and subsequent reaction of the nucleobase with BzCl gave

Scheme 2. **Synthesis of N^3-protected thymidine derivatives**



Scheme 3. **Synthesis of N^3-protected thymidine phosphoramidites**



compound **14** (Scheme 2). Acid-mediated removal of the DMTr group in **14** with triethylsilane as scavenger afforded, after silica gel column chromatography, pure **16** in good yield. The requisite N^3-benzoylated thymidine phosphoramidite monomer **2d** was prepared from known N^3-benzoylthymidine **18** (Scheme 3).[12] Treatment of **18** with DMTrCl in pyridine (→**21**) followed by phosphorylation of the 3′-OH in **21** using phosphochloridite **23** gave monomer **2d** in good overall yield.

To test the feasibility of our strategy the synthesis of the pentameric sequence **10** (5′-AGCTT-3′) was undertaken and started with the condensation of nucleoside **16** and phosphoramidite **2d** (1.5 equiv) in acetonitrile (ACN) using 4,5-dicyanoimidazole (DCI) as the activator. After complete conversion of compound **16**, as judged by HPLC, water was added to hydrolyze the excess monomer. Next, a solution of 0.2 M I_2 (2 equiv relative to **2d**) in THF/pyridine was added to oxidize all phosphite- and H-phosphonate-intermediates. Dilution of the mixture with EtOAc and extraction with 1 M $Na_2S_2O_3$ (quench I_2, remove I^-), 10% $KHSO_4$ (remove pyridine, diisopropylamine), and 10% $NaHCO_3$ (remove DCI) gave, after evaporation of the solvents, a mixture of the fully protected dimer **3** ($n = 0$, Bpg = T^{Bz}) and monomeric phosphodiester **4d** (Bpg = T^{Bz}).

Detritylation of **3** and **4d** was carried out in a mixture of MeOH and ACN (6/1) containing 0.1 M HCl (1.9 equiv relative to **2d**). Reaction times varied between 5 min for short oligomers to 25 min for longer sequences.[13] After completion of the reaction (TLC) the acid was quenched with an excess

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(13) It appears that the size and composition of the oligonucleotide influences the acidity of the reaction mixture. Byproducts originating from depurination in **8** and **9** were not detected by LC−MS analysis.

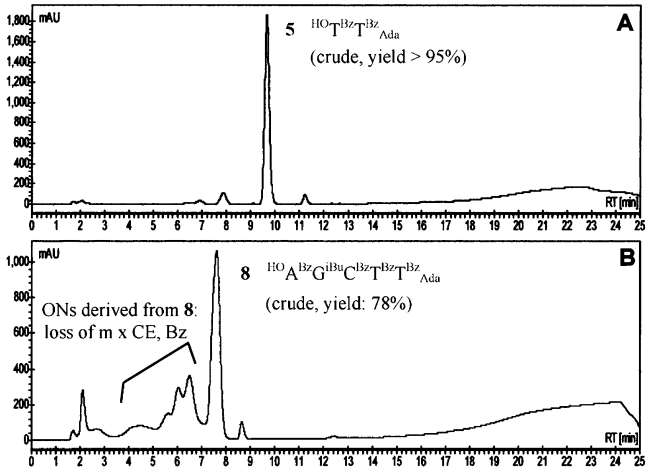

Figure 1. **HPLC traces (254 nm) of the crude products after one elongation cycle: (A) $^{HO}T^{Bz}T^{Bz}{}_{Ada}$ and four elongation cycles (B) $^{HO}A^{Bz}G^{iBu}C^{Bz}T^{Bz}T^{Bz}{}_{Ada}$ (8).**

of aqueous triethylammonium acetate (TEAA), and ACN was added to give a final mixture of ACN/MeOH/water 2/2/1 (v/v/v). Extraction of this mixture with heptane/diethylether (2/1, v/v) removed 90−95% of the DMTrOMe byproduct **7**. After concentration, the mixture was diluted with EtOAc/THF (5/2, v/v)[14] and extracted with aqueous base ($NaHCO_3$) to remove phosphodiester **6d** (Bpg = T^{Bz}). Evaporation of the solvents afforded crude **5** ($n = 0$, Bpg = T^{Bz}) in >95% yield (based on **16**)[15] and high purity (Figure 1A).

The procedure described above was repeated three times with the commercially available phosphoramidites **2a**−**c**. Analysis of the growing ON chain with HPLC after each cycle showed, apart from the formation of the desired oligonucleotide, the accumulation of several more hydrophilic byproducts. The byproducts were identified with LC−MS to be derived from the fully protected oligonucleotide and to represent different combinations of cleavage of one or more CE groups and, to a lesser extent, loss of Bz groups from the thymidine nucleobases (Figure 1B). Crude ON **8** was obtained in 78% yield (based on **16**)[15] and was subsequently treated with concentrated ammonia at 55 °C for 48 h.[16] LC−MS analysis (Figure 2) of the resulting mixture revealed the presence of a single ON (**10**) having the expected mass (found: 1478.6, calcd: 1478.3) together with minor non-ON side products originating from the protective group cleavage. This observation corroborates the above-mentioned statement that all byproducts in Figure 1B originated from **8** and that, because of the absence of shorter sequences, all reactions (i.e., coupling, oxidation, and detritylation) were essentially quantitative. We also concluded that all monomeric phosphodiesters (**6a**−**d**) could efficiently be removed by extraction and that the accumulation of internucleotidic phosphodiester groups, resulting from premature CE cleavage, did not interfere with the chemistry. However, the loss of CE groups led to a dramatic decrease

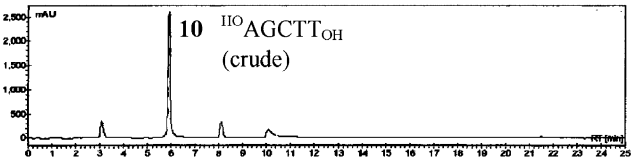

Figure 2. **HPLC trace (254 nm) of the crude, fully deprotected pentameric DNA fragment 10 ($^{HO}AGCTT_{OH}$).**

of ON solubility causing tedious extraction procedures and reduction of the overall yield.

Several modifications to the earlier described synthesis protocol were made in order to suppress premature loss of the CE- and Bz-protecting groups. First of all, the benzoyl group on thymidine was replaced with the more stable pivaloyloxymethyl (Pom) group (in **17** and **2e**). Second, the number of basic extractions could be reduced from five to three (one time after the coupling step and two times after detritylation) without compromising the removal of DCI and monomeric phosphodiesters.

Finally, in order to minimize the presence of Et_3N in the organic phase after the final basic extraction, the quenching of the HCl after detritylation was effected with an equimolar amount of TEAA and the concentration of the ACN/MeOH/water phase after extraction of DMTrOMe (**7**) was omitted.

The synthesis of the Pom-protected thymidine **17** was carried out via a route similar to that described for **16** (Scheme 2). The Pom-protected thymidine phosphoramidite monomer **2e** was prepared by levulinoylation of **12**, followed by reaction of the nucleobase with PomCl and K_2CO_3 in DMF to give the fully protected nucleoside **20** (Scheme 3). Hydrazine-mediated unmasking of the 3′-oxygen in **20** and subsequent phosphorylation gave the requisite phosphoramidite monomer **2e** in good overall yield. The benefits of these modifications were demonstrated by the preparation of the protected hexameric ON **9** ($^{HO}A^{Bz}T^{Pom}G^{iBu}C^{Bz}T^{Pom}T^{Pom}{}_{Ada}$). The synthesis started from **17** at 0.5 mmol scale, and after execution of five elongation cycles according to the modified protocol, crude **9** was obtained in 67% yield (based on **17**).[15] HPLC and LC−MS analysis of crude **9** (Figure 3A) showed the presence of **9** as the only ON accompanied with minor cleavage of the CE groups in **9**. It is of interest to note that no Pom group cleavage could be detected and that the relatively lipophilic Pom-protected phosphodiester monomers could still be efficiently removed via basic aqueous extractions. Analysis after complete deblocking (concentrated NH_3) using ion-exchange chromatography showed the presence of **11** virtually as the only product (Figure 3B). Purification (ion-exchange chromatography) and desalting (Sephadex G25) afforded the pure hexameric DNA **11** in 39% overall yield (based on **17**).[17] The identity and homogeneity of the purified hexamer **11** was confirmed by analytical ion-exchange (Figure 3C) chromatography, MALDI TOF mass spectrometry (found: 1782.37, calcd: 1782.34), and coelution of **11** with the same hexamer prepared via standard solid-phase synthesis.

(14) It is of interest to note that the EtOAc/THF organic phase posesses improved solubilizing power for ONs compared to EtOAc.

(15) Yields were estimated by comparison of the obtained and the expected amounts of material (in mg).

(16) Reaction time of 48 h was necessary for complete removal of the adamantylacetyl moiety.

(17) The yield was estimated by A_{260} absorption units using $\epsilon = 55100$ L mol^{-1} cm^{-1}.

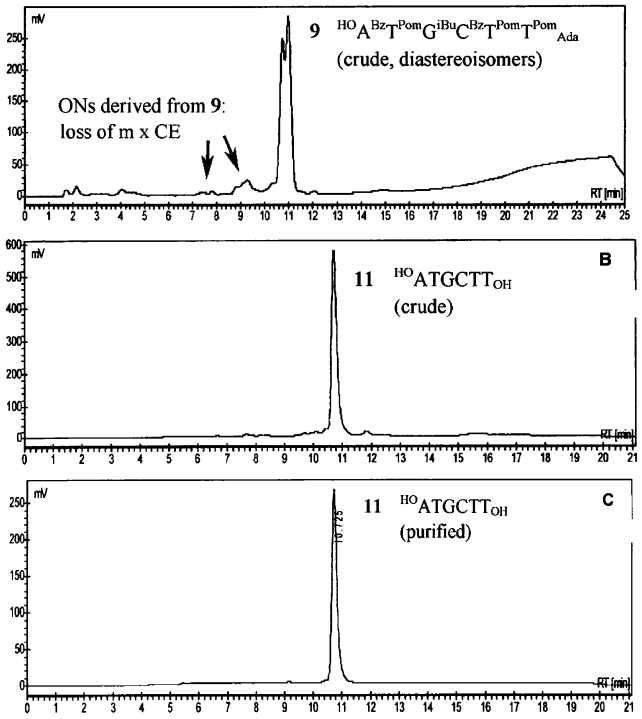

Figure 3. **(A) HPLC trace (254 nm) of fully protected ON 9. (B) Ion-exchange chromatography trace (254 nm) of crude 11. (C) Ion-exchange chromatography trace (254 nm) of purified 11.**

Conclusion

In conclusion, we developed an efficient solution-phase synthesis protocol for the preparation of DNA ONs (3′ → 5′ elongation), in which chromatographic purifications of protected intermediates are omitted. All reagents, DMTr byproducts, and excess monomeric nucleosides could be separated from the growing ON chain by simple extractions. The solubility of the ONs was assured by reducing the amount of CE-cleavage to a minimum and the use of the novel N^3-Pom-protected thymidine building blocks **17** and **2e**. The feasibility of the approach was demonstrated by the synthesis of a hexameric ON in high yield and purity. Currently, we are well underway with the implementation of a capping step as well as a sulfurization step in the presented protocols for the preparation of longer ONs and phosphorothioate ONs. The results of these efforts will be reported soon.

Experimental Section

General. All reagents were used as received. ACN (Biosolve, DNA synthesis grade) was used for the coupling reactions).

HPLCs for monitoring the progress of the coupling reactions as well as analysis of (partially) protected ONs were run on a JASCO system with detection at 214, 254, and 280 nm. A gradient of acetonitrile (solution B, 20−90%) in a mixture of 75% MeOH/water (solution A) and 0.2 M TEAA in MeOH (solution C, fixed at 10%) was used as the eluent in all cases. Analytical LC−MS was conducted on a JASCO system using an Alltima C_{18} analytical column (5 μm particle size, flow: 1.0 mL/min) or a Phenomenex Gemini C_{18} analytical column (3 μm particle size, flow: 1.0 mL/min). Absorbance was measured at 214 nm and 254 nm. Solvent system: A: 100% water, B: 100% acetonitrile, C: 1 % TFA. Gradients of B in 10% C were applied over 15 minutes (Alltima column) or over 10 minutes (Phenomenex column). Usually, for protected ONs a gradient of 50−90% B was used, while for completely deblocked ONs a gradient of 0−30% B was used. Mass spectra were recorded on a Perkin Elmer Sciex API 165 equipped with an electrospray interface (ESI). HRMS spectra were recorded on a LTQ-FT (Thermo Electron). MALDI TOF spectra were recorded on a Voyager-DE PRO mass spectrometer (PerSeptive Biosystems).

NMR spectra were measured on Bruker AC200 or AC300 spectrometers. Chemical shifts are given in ppm, relative to the signal of the internal standard tetramethylsilane. IR spectra were recorded on a Shimadzu FTIR-8300 spectrophotometer, $[\alpha]_D$ values were determined using a Propol Automatic Polarimeter, and melting points were determined using a Buchi Schmeltzpunkt Bestimmungsapparat.

5′-*O*-Dimethoxytrityl-N^3-benzoyl-thymidine-3′-*O*-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (2d). 5′-*O*-Dimethoxytrityl-N^3-benzoyl-thymidine (**21**, 13 g, 20 mmol) was coevaporated with ACN, dissolved in DCM (70 mL) and put under an argon atmosphere. DiPEA (13.6 mL, 80 mmol) was added followed by dropwise addition (dropping funnel) of a solution of chloro-2-cyanoethyl-*N,N*-diisopropylphosphoramidite (5.9 g, 25 mmol) in DCM (40 mL). When TLC analysis (EtOAc/PE 1/2) indicated complete conversion of the starting compound, the mixture was concentrated, and the residue was applied onto a silica gel column (prepared in the presence of 3% Et_3N) and eluted with a gradient of EtOAc in PE (1/2 → 1/1). Evaporation of the correct fractions yielded the title compound (13.4 g, 15.8 mmol) as a white foam (79% yield). HRMS found (calcd) for $[M + H^+]$: 849.3651 (849.3623). IR (cm^{-1}): 1747.4, 1701.1, 1654.8, 1508.2, 1247.9, 1176.5, 1029.9. $[\alpha]^{20}_D$ +20.6 (*c* 1.0, $CHCl_3$); mp 81−85 °C. ^{1}H NMR (300 MHz, $CDCl_3$, two diastereoisomers) δ 8.0 (d, J = 7.9, 2H, H arom Bz), 7.8, 7.7 (2 × s, 1H, H-6), 7.7−7.2 (m, 14H, H arom Bz, DMTr), 6.9 (m, 4H, H arom, DMTr), 6.4 (m, 1H, H-1′), 4.7 (bs, 1H, H-3′), 4.20, 4.14 (2 × s, 1H, H-4′), 3.8 (s, 6H, 2 × OMe), 3.7-3.4 (m, 4H, H-2′, H-5′), 3.3 (m, 1H, CH iPr), 2.6-2.3 (m, 5H, 2 × CH_2 CE, 1 × CH iPr), 1.4 (s, 3H, CH_3 thymidine), 1.2, 1.0 (2 × m, 12H, 4 × CH_3 iPr); ^{13}C NMR (50 MHz, $CDCl_3$ two diastereoisomers) d 168.8 (CO Bz), 162.4 (C-4), 158.3 (Cq DMTr), 148.9 (C-2), 143.9 (Cq DMTr), 135.3 (CH Bz), 134.9 (Cq DMTr), 134.6 (C-6), 131.2 (Cq Bz), 130.0, 129.7, 128.7, 127.8, 127.6, 126.8 (CH DMTr, Bz), 117.4, 117.2 (CN CE), 112.9 (CH DMTr), 110.7 (C-5), 86.5 (Co DMTr), 85.4, 84.6 (C-1′, C-4′), 73.1 (C-3′), 62.6 (C-5′), 57.9, 57.6 (CH_2 CE), 54.8 (OMe), 42.9, 42.6 (CH iPr), 39.7 (C-2′), 24.1 (CH_3 iPr), 19.8 (CH_2 CE), 11.3 (C-7); ^{31}P NMR (80.7 MHz, $CDCl_3$, two diastereoisomers) d 149.3, 148.9.

5′-*O*-Dimethoxytrityl-N^3-pivaloyloxymethyl-thymidine-3′-*O*-(2-cyanoethyl-*N,N*-diisopropyl)phosphoramidite (2e). 5′-*O*-Dimethoxytrityl-N^3-pivaloyloxymethyl-thymidine (**22**, 25.2 g, 38.3 mmol) was coevaporated with ACN, dissolved

in DCM (200 mL) and put under an argon atmosphere. DiPEA (23.2 mL, 153.2 mmol) was added, followed by slow addition (approx. 15 min.) of a solution of chloro-2-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (47.9 mmol, 10.7 mL) in DCM (200 mL) using a dropping funnel. After complete addition, the mixture was stirred for another 30 min. TLC analysis (diethylether) indicated complete conversion of the starting compound into a higher running spot. After evaporation of the solvent, the residue was applied to silica gel column (prepared with 3% Et_3N in diethylether)and eluted with diethylether. Concentration of the right fractions afforded the title phosphoramidite (28.3 g, 33.0 mmol) as a white foam in 86% yield. Upon storage, the compound turns into a glass-like substance. HRMS found (calcd) for [M + Na^+]: 881.3871 (881.3861). IR (cm^{-1}): 1662.5, 1461.9, 1249.8, 906.5. $[\alpha]^{20}_D$ +17 (*c* 1.0, $CHCl_3$); mp 70−75 °C. ^{1}H NMR (300 MHz, $CDCl_3$, two diastereoisomers) δ 7.7 (2 × s, 1H, H-6), 7.4−7.2 (m, 9H, H arom DMTr), 6.8 (m, 4H, H arom DMTr), 6.4 (m, 1H, H-1′), 6.0 (s, 2H, CH_2 Pom), 4.7 (bs, 1H, H-3′), 4.2 (2 × s, 1H, H-4′), 3.8 (s, 6H, 2 × OMe), 3.6−3.5 (m, 4H, H-2′, H-5′), 3.3 (m, 1H, CH iPr), 2.6−2.4 (m, 4H, 2 × CH_2 CE), 1.6, 1.5 (2 × s, 3H, CH_3 thymidine), 1.197 (s, 9H, tBu Pom), 1.195−1.0 (m, 12H, 4 × CH_3 iPr); ^{13}C NMR (75 MHz, $CDCl_3$ two diastereoisomers) δ 177.0 (CO Pom), 162.1 (C-4), 158.3 (Cq DMTr), 149.9 (C-2), 143.9 (Cq DMTr), 134.9 (Cq DMTr), 134.3 (C-6), 129.8, 127.8, 127.7, 127.6, 126.7 (CH DMTr), 177.3, 177.1 (CN CE), 122.9 (CH DMTr), 109.9 (C-5), 86.5 (Co DMTr), 85.3, 85.0 (C-1′, C-4′), 73.5, 73.2, 73.0, 72.8 (C-3′), 64.7 (C-5′), 62.7, 62.6 (CH_2 Pom), 58.0, 57.9, 57.8, 57.6 (CH_2 CE), 54.8 (OMe), 42.93, 42.87, 42.77, 42.70 (CH iPr), 39.7 (Co Pom), 38.4 (C-2′), 26.6 (tBu Pom), 24.2 (CH_3 iPr), 20.0, 19.9, 19.8, 19.7 (CH_2 CE), 12.0 (C-7); ^{31}P NMR (80.7 MHz, $CDCl_3$, two diastereoisomers) δ 149.4, 148.9.

General Procedure for One Elongation Cycle en Route to Hexamer 9. Compound **1** (**17** when $n = 0$, 0.5 mmol) and 1.5 equiv of phosphoramidite monomer (**2a**−**e**) were mixed and coevaporated with ACN (3×). The mixture was dissolved in ACN (3.5 mL) and put under an argon atmosphere. DCI (266 mg, 2.25 mmol) was added, and the mixture was stirred for 30 min. Samples for HPLC analysis were prepared by diluting an aliquot (10 μL) of the reaction mixture with a mixture of ACN/MeOH 1/3 (1 mL). Water (175 μL) was added, and the reaction mixture was stirred for 2 min. A solution of I_2 (0.2 M in THF/pyridine 4/1, 7.5 mL) was added, and the mixture was stirred for 5 min. The reaction mixture was transferred into a separatory funnel and diluted with EtOAc (15 mL). The mixture was subsequently extracted with a solution of 1 M $Na_2S_2O_3$ (15 mL), 10% $KHSO_4$ (2 × 15 mL), 10% $NaHCO_3$ (1 × 15 mL), and finally with a mixture of brine/water (1/1, 15 mL). The organic phase was dried ($MgSO_4$), filtrated, and concentrated to give a white foam.

A stock-solution of 0.1 M HCl was prepared by the (careful) addition of AcCl in a mixture of ACN/MeOH (1/6).

The foam containing the ON and the monomer was dissolved in the acidic stock solution (14 mL), and the progress of the detritylation was monitored by TLC (5−10% MeOH/DCM). After complete disappearance of the orange ON spot (spray with acid) 0.23 M aqueous TEAA solution (prepared by premixing 0.7 mL of the commercial 2 M TEAA solution and 5.3 mL of water) was added. The resulting mixture was transferred into a separatory funnel. The flask was rinsed with ACN (total amount 10 mL). The mixture was extracted with heptane/diethylether 2/1 (4 × 30 mL). Next, EtOAc (20 mL) and water (20 mL) were added, and the resulting layers were separated. The organic phase was washed with water (20 mL), 10% $NaHCO_3$ (2 × 20 mL), and finally with a mixture of brine/water 1/1 (20 mL). After drying ($MgSO_4$), filtration, and evaporation of the solvents the oligonucleotide was isolated as a white foam. Yields were estimated by dividing the amount of material obtained (in mg) with the expected amount (0.5 mmol × MW oligonucleotide).

General Procedure for Complete Deblocking. 5′-OH ON (50 mg) was treated with concentrated NH_3 solution (25 mL) at 55 °C for 48 h. After cooling of the reaction mixture to room temperature, the mixture was concentrated. The residue was dissolved (cloudy mixture) in water and centrifuged. The supernatant (∼6 mL) was analysed by LC−MS as well as mono-Q ion-exchange chromatography using a gradient of 1 M NaCl in 10 mM NaCl (0−50%).

Purification and Desalting of 11 . After deblocking and centrifugation as described above, the aqueous solution containing ON **11** was purified using Q-Sepharose ion-exchange chromatography utilizing a gradient of 1 M NaCl in 10 mM NaCl (0−35% over 1 CV, then 35−65% over 10 CV). After evaporation of the appropriate fractions, the residue was dissolved in water and desalted over a Sephadex G-25 column using 0.15 M NH_4HCO_3 as the eluent. After pooling, evaporation, and lyophilization, the yield was determined by measuring the absorption at 260 nm (ϵ = 55100 L mol^{-1} cm^{-1}).

3′-*O*-Adamantaneacetyl-5′-*O*-dimethoxytrityl-thymidine (13). 5′-*O*-Dimethoxytrityl-thymidine (10.9 g, 20 mmol) was coevaporated with ACN and dissolved in DCM. Molecular sieves, adamantaneacetic acid (5.8 g, 30 mmol), EDC (6.14 g, 32 mmol), and DMAP (367 mg, 3 mmol) were added, and the mixture was stirred overnight. After TLC analysis (EtOAc/PE, 1/1), which indicated completion of the reaction, the mixture was concentrated and taken up in EtOAc (700 mL). The organic phase was washed with water (250 mL), 10% $KHSO_4$ (250 mL), water (250 mL), 10% $NaHCO_3$ (250 mL), and finally with brine (250 mL). The organic layer was dried ($MgSO_4$), filtrated, and evaporated. The residue was applied onto a silica gel column (prepared in the presence of 3% Et_3N) and eluted with a gradient of EtOAc in PE (1/3 → 3/1), yielding the title compound (14.0 g, 19.4 mmol) as a white foam (yield 97%). MS found (calcd) for [M + H^+]: 721.5 (721.3). IR (cm^{-1}): 2904.6, 2846.7, 1685.7, 1249.8, 906.5. $[\alpha]^{20}_D$ +4.6 (*c* 1.0, $CHCl_3$); mp 104−108 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.6 (bs, 1H, H-3), 7.6 (s, 1H, H-6), 7.4−7.2 (m, 9H, H arom DMTr), 6.8 (d, J = 8.8, 4H, H arom DMTr), 6.4 (t, J = 7.0, 1H, H-1′), 5.5 (bs, 1H, H-3′), 4.1 (s, 1H, H-4′), 3.8 (s, 6H, 2 × OMe), 3.5

(s, 2H, H-5′), 2.4 (m, 2H, H-2′), 2.1 (s, 2H, CH_2CO Ada), 2.0 (bs, 3H, 3 × CH Ada), 1.7−1.6 (m, 12H, 6 × CH_2 Ada), 1.4 (s, 3H, CH_3 thymidine); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.0 (CO Ada), 163.9 (C-4), 158.5 (Cq DMTr), 150.6 (C-2), 144.1 (Cq DMTr), 135.2 (C-6), 135.0 (Cq DMTr), 129.9, 127.9, 127.8, 127.0, 113.1 (CH DMTr), 111.4 (C-5), 86.9 (Co DMTr), 84.2, 84.0 (C-1′, C-4′), 74.5 (C-3′), 63.5 (C-5′), 55.0 (OMe), 48.3 ($\underline{C}H_2CO$ Ada), 42.2 (CH_2 Ada), 37.8 (C-2′), 36.4 (CH_2 Ada), 32.8 (Co Ada), 28.3 (CH Ada), 11.5 (C-7).

3′-*O*-Adamantaneacetyl-5′-*O*-dimethoxytrityl-N^3-benzoyl-thymidine (14). 3′-*O*-Adamantaneacetyl-5′-*O*-dimethoxytrityl-thymidine (**15**, 23.8 g, 33 mmol) was dissolved in pyridine (200 mL); DiPEA (30.9 mL, 181 mmol) and BzCl (5.9 mL, 51 mmol) were added. When TLC analysis (PE/EtOAc, 2/1) indicated complete conversion of starting material, the solvents were removed by evaporation, and the crude mixture was taken up in EtOAc (300 mL), washed with 10% $KHSO_4$ (200 mL), water (200 mL), and 10% $NaHCO_3$ (200 mL). After drying ($MgSO_4$), filtration, and evaporation the crude material was purified by silica gel column chromatography (column prepared in the presence of 3% Et_3N). A gradient of EtOAc in PE (1/3 → 1/1) was used as the eluens giving the title compound (26.4 g, 32 mmol) in 97% yield. HRMS found (calcd) for [M + Na^+]: 847.3573 (847.3565). IR (cm^{-1}): 2904.6, 2846.7, 1747.4, 1701.1, 1658.7, 1249.8, 906.5. $[\alpha]^{20}_D$ +5.6 (*c* 1.0, $CHCl_3$); mp 97−101 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 8.0 (d, J = 7.8, 2H, H arom Bz), 7.8 (s, 1H, H-6), 7.7−7.2 (m, 12H, H arom DMTr, Bz), 6.8 (d, J = 8.5, 4H, H arom DMTr), 6.4 (t, J = 6.9, 1H, H-1′), 5.5 (s, 1H, H-3′), 4.2 (s, 1H, H-4′), 3.8 (s, 6H, 2 × OMe), 3.5 (s, 2H, H-5′), 2.5 (bs, 2H, H-2′), 2.1 (s, 2H, CH_2CO Ada), 1.9 (bs, 3H, 3 × CH Ada), 1.7−1.6 (m, 12H, 6 × CH_2 Ada), 1.4 (s, 3H, CH_3 thymidine); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.1 (CO Ada), 168.9 (CO Bz), 162.7 (C-4), 158.7 (Cq DMTr), 149.3 (C-2), 144.1 (Cq DMTr), 135.1 (CH Bz), 135.0 (Cq DMTr), 134.9 (C-6), 131.5 (Cq Bz), 130.4, 130.0, 129.0, 128.0, 127.9, 127.2 (CH arom DMTr, Bz), 113.2 (CH arom DMTr), 111.5 (C-5), 87.2 (Co DMTr), 84.5, 84.3 (C-1′, C-4′), 74.5 (C-3′), 63.5 (C-5′), 55.1 (OMe), 48.5 ($\underline{C}H_2CO$ Ada), 42.5 (CH_2 Ada), 38.1 (C-2′), 36.5 (CH_2 Ada), 32.9 (Co Ada), 28.4 (CH Ada), 11.5 (C-7).

3′-*O*-Adamantaneacetyl-5′-*O*-dimethoxytrityl-N^3-pivaloyloxymethyl-thymidine (15). 3′-*O*-Adamantaneacetyl-5′-*O*-dimethoxytrityl-thymidine (**15**, 21.7 g, 30 mmol) was dissolved in DMF (150 mL). K_2CO_3 (16.6 g, 120 mmol) and chloromethylpivalate (13.1 mL, 90 mmol) were added, and the mixture was stirred overnight. Diethylether (550 mL) and water (350 mL) were added, and the layers were separated. The aqueous layer was extracted with diethylether (200 mL). The combined organic layers were washed with water (200 mL) and brine (200 mL) and were dried ($MgSO_4$), filtrated, and evaporated. Column chromatography (preparation of the column in the presence of 3% Et_3N) using a gradient of diethylether in PE (1/1 → 1/0) gave the title compound in 89% yield (20.1 g, 26.6 mmol). HRMS found (calcd) for [M + Na^+]: 857.3976 (857.3984). IR (cm^{-1}): 2904.6, 2846.7, 1720.4, 1666.4, 1508.2, 1461.9, 1249.8. $[\alpha]^{20}_D$ +6.6 (*c* 1.0, $CHCl_3$); mp 89−92 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 7.6 (s, 1H, H-6), 7.4−7.2 (m, 9H, H arom DMTr), 6.8 (d, J = 8.5, 4H, H arom DMTr), 6.5 (t, J = 6.2, 1H, H-1′), 6.0 (s, 2H, CH_2 Pom), 5.5 (s, 1H, H-3′), 4.1 (s, 1H, H-4′), 3.8 (s, 6H, 2 × OMe), 3.5 (s, 2H, H-5′), 2.5 (bs, 2H, H-2′), 2.1 (s, 2H, CH_2CO Ada), 2.0 (bs, 3H, 3 × CH Ada), 1.7−1.6 (m, 12H, 6× CH_2 Ada), 1.4 (s, 3H, CH_3 thymidine), 1.2 (s, 9H, tBu Pom); ^{13}C NMR (75 MHz, $CDCl_3$) d 177.4 (CO Pom), 177.1 (CO Ada), 162.4 (C-4), 158.7 (Cq DMTr), 150.3 (C-2), 144.1 (Cq DMTr), 135.2 (Cq DMTr), 135.0 (C-6), 130.0, 128.0, 127.1, 113.1 (CH DMTr), 110.7 (C-5), 87.1 (Co DMTr), 85.0, 84.1 (C-1′, C-4′), 74.5 (C-3′), 65.1 (CH_2 Pom), 63.5 (C-5′), 55.1 (OMe), 48.5 ($\underline{C}H_2CO$ Ada), 42.3 (CH_2 Ada), 38.7 (Co Pom), 38.0 (C-2′), 36.5 (CH_2 Ada), 32.9 (Co Ada), 28.4 (tBu Pom), 26.9 (CH Ada), 12.1 (C-7).

3′-*O*-Adamantaneacetyl-N^3-benzoyl-thymidine (16). 3′-*O*-Adamantaneacetyl-5′-*O*-dimethoxytrityl-N^3-benzoyl-thymidine (**14**, 26.4 g, 32 mmol) was dissolved in DCM (400 mL). Triethylsilane (11 mL, 68 mmol) and dichloroacetic acid (21 mL → 5%V) were added. After completion of the reaction (45 min, TLC: PE/EtOAc 3/1), DCM (200 mL) was added, and the organic phase was extracted with 2% Na_2CO_3 (300 mL). The aqueous layer was back-extracted with DCM (200 mL), and the combined organic layers were dried ($MgSO_4$), filtrated, and concentrated. The crude material was applied onto a silica gel column and eluted with a gradient of EtOAc in PE (1/1 → 2/1) to afford the title compound (16.5 g, 29.5 mmol) in 92% yield. HRMS found (calcd) for [M + H^+]: 523.2479 (523.2439). IR (cm^{-1}): 2900.7, 1745.5, 1699.2, 1647.1, 1446.5, 1253.6, 1228.6, 1097.4. $[\alpha]^{20}_D$ −6.2 (*c* 1.0, $CHCl_3$); mp 94−96 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 7.9 (d, J = 7.2, 1H, H-6), 7.7−7.6 (m, 5H, CH Bz), 6.3 (t, J = 7.2, 1H, H-1′), 5.3 (m, 1H, H-3′), 4.1 (m, 1H, H-4′), 3.9 (bs, 2H, H-5′), 2.4 (m, 2H, H-2′), 2.3 (bs, 1H, OH), 2.1 (s, 2H, CH_2CO Ada), 1.969, 1.966 (3 × CH Ada, CH_3 thymidine), 1.7−1.6 (m, 12H, CH_2 Ada); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.3 (CO Ada), 168.8 (CO Bz), 162.8 (C-4), 149.2 (C-2), 136.2 (C-6), 135.0 (CH Bz), 131.3 (Cq Bz), 130.3, 139.0 (CH Bz), 110.9 (C-5), 85.4, 85.3 (C-1′, C-4′), 74.2 (C-3′), 62.1 (C-5′), 48.4 ($\underline{C}H_2CO$ Ada), 42.2 (CH_2 Ada), 37.5 (C-2′), 36.4 (CH_2 Ada), 32.8 (Co Ada), 28.3 (CH Ada), 12.4 (C-7).

3′-*O*-Adamantaneacetyl-N^3-pivaloyloxymethyl-thymidine (17). 3′-*O*-Adamantaneacetyl-5′-*O*-dimethoxytrityl-N^3-pivaloyloxymethyl-thymidine (**15**, 19.4 g, 25.6 mmol) was dissolved in DCM (250 mL). Triethylsilane (8.2 mL, 51.2 mmol), and dichloroacetic acid (13 mL → 5% V) were added. After 30 minutes the initial colouring disappeared, and TLC analysis (PE/diethylether 1/2) indicated complete detritylation. DCM (250 mL) was added, and the resulting mixture was extracted with 2% Na_2CO_3 (400 mL). The aqueous layer was back-extracted with DCM (150 mL), and the combined organic layers were dried ($MgSO_4$), filtrated, and concentrated. Column chromatography (diethylether/PE 2/1 → 1/0) gave the title compound (12 g, 23.3 mmol) as a

white foam (94% yield). HRMS found (calcd) for [M + H^+]: 533.2860 (533.2857). IR (cm^{-1}): 2902.7, 2848.7, 1716.5, 1652.9, 1456.2, 1132.1, 1099.3. $[\alpha]^{20}_D$ −9.0 (*c* 1.0, $CHCl_3$); mp 63−68 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.7 (s, 1H, H-6), 6.3 (t, J = 6.8, H-1′), 6.0 (s, 2H, CH_2 Pom), 5.3 (bs, 1H, H-3′), 4.1 (s, 1H, H-4′), 3.9 (s, 2H, H-5′), 2.9 (bs, 1H, OH), 2.4 (bs, 2H, H-2′), 2.1 (s, 2H, CH_2CO, Ada), 1.9 (m, 3H, 3 × CH Ada), 1.7 (m, 12H, 6 × CH_2 Ada), 1.2 (s, 9H, tBu Pom); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.0 (CO Pom), 170.7 (CO Ada), 162.2 (C-4), 149.8 (C-2), 135.1 (C-6), 109.5 (C-5), 85.4, 84.9 (C-1′, C-4′), 74.1 (C-3′), 64.4 (C-5′), 61.8 (CH_2 Pom), 47.9 ($\underline{C}H_2CO$ Ada), 41.8 (CH_2 Ada), 38.2 (Co Pom), 37.2 (C-2′), 37.2 (CH_2 Ada), 32.3 (Co Ada), 27.9 (tBu Pom), 26.3 (CH Ada), 12.5 (C-7).

5′-*O*-Dimethoxytrityl-3′-*O*-levulinoyl-thymidine (19). 5′-*O*-Dimethoxytritylthymidine (7.59 g, 14.0 mmol) was coevaporated with dioxane and dissolved in dioxane (100 mL). To this solution were added levulinic acid (2.87 mL, 28.0 mmol), EDC (5.4 g, 28 mmol), and DMAP (171 mg, 0.1 mmol). After stirring for 2.5 h TLC analysis (5% MeOH/DCM) indicated complete conversion of the starting material. The solvent was removed by evaporation in vacuo, and the residue was dissolved in DCM (100 mL). After washing of the organic phase with water, 10% $KHSO_4$, and 10% $NaHCO_3$ (3×), the organic phase was dried ($MgSO_4$), filtered, and concentrated to give a foam (**19**), which was used directly in the next reaction.

Although **19** is a known compound, no analytical data were reported. MS found (calcd) for [M + H^+]: 643.4 (643.3). IR (cm^{-1}): 2360.7, 2341.4, 1693.4, 1508.2, 1249.8. $[\alpha]^{20}_D$ +8.0 (*c* 1.0, $CHCl_3$); mp 88−92 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.7 (s, 1H, H-3), 7.6 (s, 1H, H-6), 7.4−7.2 (m, 9H, H arom DMTr), 6.9 (d, J = 8.9, 4H, H arom DMTr), 6.4 (t, J = 7.0, 1H, H-1′), 5.5 (bs, 1H, H-3′), 4.1 (s, 1H, H-4′), 3.8 (s, 6H, 2 × OMe), 3.5 (bs, 2H, H-5′), 2.8 (t, J = 6.8, 2H, CH_2 Lev), 2.6 (t, J = 6.3, 2H, CH_2 Lev), 2.5 (m, 2H, H-2′), 2.2 (s, 3H, CH_3 Lev), 1.4 (CH_3 thymidine).

5′-*O*-Dimethoxytrityl-3′-*O*-levulinoyl-N^3-pivaloyloxymethyl-thymidine (20). The foam obtained in the previous reaction (**19**, 12.1 g) was dissolved in DMF (70 mL); chloromethylpivalate (6.1 mL, 42 mmol) and K_2CO_3 (7.73 g, 56 mmol) were added, and the mixture was stirred overnight. TLC analysis (diethylether) indicated the formation of a more lipophilic product. Diethylether (250 mL) was added, and the resulting mixture was extracted with water (150 mL). The aqueous layer was back-extracted with diethylether (100 mL). The combined organic phases were concentrated in vacuo to give an oil. A silica gel column was prepared using 1% Et_3N in didiethylether. After application of the crude product onto the column, diethylether was used as the eluent. Concentration of the appropriate fractions gave 10.2 g, 13.5 mmol (96% yield) of the title compound. HRMS found (calcd) for [M + Na^+]: 779.3150 (779.3150). IR (cm^{-1}): 1716.5, 1666.4, 1508.2, 1461.9, 1249.8. $[\alpha]^{20}_D$ +7.0 (*c* 1.0, $CHCl_3$); mp 59−64 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.7 (s, 1H, H-6), 7.4−7.2 (m, 9H, H arom DMTr), 6.8 (d, J = 8.7, 4H, H arom DMTr), 6.3 (t, J = 6.6, 1H, H-1′), 5.8 (bs, 2H, CH_2 Pom), 5.4 (bs, 1H, H-3′), 4.1 (s, 1H, H-4′), 3.7 (s, 6H, 2 × OMe), 3.4 (bs, 2H, H-5′), 2.7 (t, J = 6.2, 2H, CH_2 Lev), 2.5 (t, J = 6.2, 2H, CH_2 Lev), 2.4 (m, 2H, H-2′), 2.1 (s, 3H, CH_3 Lev), 1.3 (s, 3H, CH_3 thymidine), 1.1 (9H, tBu Pom); ^{13}C NMR (75 MHz, $CDCl_3$) δ 206.6 (CO Lev), 177.8 (CO Pom), 172.5 (COO Lev), 162.8 (C-4), 159.0 (Cq DMTr), 150.6 (C-2), 144.5 (Cq DMTr), 135.5 (Cq DMTr), 134.7 (C-6), 130.4, 128.4, 127.5, 113.6 (CH DMTr), 111.1 (C-5), 87.5 (Co DMTr), 85.3, 84.3 (C-1′, C-4′), 75.8 (C-3′), 65.4 (CH_2 Pom), 64.0 (C-5′), 55.5 (OMe), 39.1 (Co Pom), 38.2, 38.0 (CH_2 Lev, C-2′), 30.0 (CH_3 Lev), 28.2 (CH_2 Lev), 27.3 (CH Pom), 12.5 (C-7).

5′-*O*-Dimethoxytrityl-N^3-benzoyl-thymidine (21). N^3-Benzoyl-thymidine[12] (9.7 g, 28 mmol) was coevaporated with and dissolved in pyridine (150 mL). Dimethoxytritylchloride (11.2 g, 33 mmol) was added, and the mixture was stirred for 45 min. TLC analysis (EtOAc/PE, 1/1) indicated complete conversion of the starting compound into a higher running product (R_f = 0.4), and the mixture was concentrated in vacuo. The residue was taken up in EtOAc (200 mL) and washed with water (100 mL), 5% $KHSO_4$ (2 × 100 mL), water (100 mL), and finally brine (100 mL). After drying ($MgSO_4$), filtration, and evaporation, the crude product was applied onto a silica gel column (prepared in the presence of 3% Et_3N) and eluted with a gradient of EtOAc in PE (1/2 → 2/1) which yielded 15.9 g, 24.5 mmol (87%) of the title compound. HRMS found (calcd) for [M + H^+]: 649.2558 (649.2544). IR (cm^{-1}): 1747.4, 1701.1, 1651.0, 1508.2, 1442.7, 1249.8, 1176.5, 1029.9. $[\alpha]^{20}_D$ +5.4 (*c* 1.0, $CHCl_3$); mp 100−105 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.9 (d, J = 7.3, 2H, H arom Bz), 7.7 (s, 1H, H-6), 7.6−7.2 (m, 12H, H arom Bz, DMTr), 6.8 (d, J = 8.5, 4H, H arom DMTr), 6.4 (t, J = 7.1, 1H, H-1′), 4.5 (bs, 1H, H-3′), 4.0 (bs, 1H, H-4′), 3.8 (s, 6H, 2 × OMe), 3.40 (dd, J = 10.6 , J = 2.5, J = 2.7, 1H, H-5′a), 3.3 (dd, J = 10.6 , J = 2.6, J = 2.7, 1H, H-5′b), 2.5 (bs, 1H, OH), 2.3 (m, 2H, H-2′), 1.4 (s, 3H, CH_3 thymidine); ^{13}C NMR (50 MHz, $CDCl_3$) δ 168.9 (CO Bz), 162.8 (C-4), 158.4 (Cq DMTr), 149.1 (C-2), 144.0 (Cq DMTr), 135.8 (C-6), 135.1 (Cq DMTr), 131.1 (Cq Bz), 130.2, 129.8, 128.9, 127.8, 126.9, 113.0 (CH DMTr, Bz), 110.8 (C-5), 86.6 (Co DMTr), 86.8, 84.8 (C-1′, C-4′), 71.8 (C-3′), 63.3 (C-5′), 55.0 (OMe), 40.8 (C-2′), 11.4 (C-7).

5′-*O*-Dimethoxytrityl-N^3-pivaloyloxymethyl-thymidine (22). 5′-*O*-Dimethoxytrityl-3-*O*-levulinoyl-N^3-pivaloyloxymethyl-thymidine (**20**, 30.2 g, 40 mmol) was dissolved in a mixture of pyridine and acetic acid (4/1, 400 mL), and hydrazine monohydrate (1.9 mL, 60 mmol) was added. TLC analysis (EtOAc) after 30 min showed the formation of a more hydrophilic compound. Acetylacetone (8.3 mL, 80 mmol) was added to quench the hydrazine, and the mixture was stirred for 5 min. The solvents were removed by evaporation, and the residue was taken up in diethylether (350 mL) and extracted with water (350 mL), 10% $KHSO_4$ (2 × 350 mL), 10% $NaHCO_3$ (2 × 350 mL), and finally brine (350 mL). After drying ($MgSO_4$) and filtration the solvents were removed by evaporation, and the crude product

was applied onto a silica gel column (prepared with 3% Et_3N in diethylether) and eluted with diethylether to give, after concentration of the appropriate fractions, 25.5 g, 38.8 mmol (97% yield) of the title compound. HRMS found (calcd) for [M + Na^+]: 681.2792 (681.2783). IR (cm^{-1}): 2904.6, 1712.7, 1654.8, 1508.2, 1461.9, 1249.8. $[\alpha]^{20}_D$ −4.0 (*c* 1.0, $CHCl_3$); mp 91−95 °C. ^{1}H NMR (300 MHz, d_4-methanol) δ 7.7 (s, 1H, H-6), 7.7−7.1 (m, 9H, CH arom DMTr), 6.8 (d, J = 8.9, 4H CH arom DMTr), 6.3 (t, J = 6.2, 1H, H-1′), 5.8 (s, 2H, CH_2 Pom), 4.5 (m, 1H, H-3′), 3.9 (bs, 1H, H-4′), 3.7 (s, 6H, 2 × OMe), 3.2 (s, 2H, H-5′), 2.3 (m, 2H, H-2′), 1.3 (s, 3H, CH_3 thymidine), 1.1 (s, 9H, 3′ CH_3 Pom); ^{13}C NMR (75 MHz, d_4-methanol) δ 178.7 (CO Pom), 164.1 (C-4), 160.0 (Cq DMTr), 151.5 (C-2), 145.9 (Cq DMTr), 136.8 (C-6), 136.6 (Cq DMTr), 131.3, 129.3, 128.9, 128.0, 114.2 (CH DMTr), 110.8 (C-5), 87.9 (Co DMTr), 87.7, 87.0 (C-1′, C-4′), 72.5 (C-3′), 66.2 (CH_2 Pom), 64.7 (C-5′), 55.7 (OMe), 41.5 (Co Pom), 39.7 (C-2′), 27.4 (tBu Pom), 12.8 (C-7).

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Supporting Information Available

Spectra for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

Abbreviations

ACN	acetonitrile
Bz	benzoyl
CNE	cyanoethyl
DMTr	dimethoxytrityl
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
DMAP	4-dimethylaminopyridine
iBu	isobutyryl
HPLC	high performance liquid chromatography
LC−MS	liquid chromatography−mass spectrometry
TEAA	triethylammonium acetate
TLC	thin layer chromatography
pg	protecting group
Pom	pivaloyloxymethyl

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