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Solution-Phase Synthesis of Short Oligo-2′-deoxyribonucleotides by Using Clustered Nucleosides as a Soluble Support

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5′-*O*-(4,4′-Dimethoxytrityl)thymidine was attached to a pentaerythrityl-derived core, and the resulting tetravalent nucleoside cluster and the next dendritic generations served as a soluble support for the synthesis of short oligo-2′-deoxyribonucleotides in solution. Couplings using a small excess (1.5 equiv. per 5′-OH group) of the standard phosphoramidite building blocks proved efficient, and the products could be purified by quantitative precipitation from methanol. Ammonolysis released nearly homogeneous oligonucleotides (CCT, GCT, ACT, and AGCCT) in high yields.

Introduction

Oligonucleotides are currently prepared by phosphoramidite chemistry on a solid support both in the laboratory[1,2] and on a large scale.[3] Although this will likely remain the case for the near future, there seems to be space for the development of a solution-phase synthesis for specific purposes. Short oligonucleotides are sometimes needed in research laboratories in a scale of hundreds of milligrams for DNA-based material chemistry and for spectroscopic and physical organic studies on structure, modification and interaction with protein fragments or small molecular entities, including metal complexes. Convenient solution-phase methodology would undoubtedly be useful for the preparation of such oligonucleotides in-house without any special equipment. In fact, numerous strategies for the elongation of oligonucleotides on soluble supports have been developed. Phosphoramidite,[4] *H*-phosphonate,[5] and phosphotriester[6,7] chemistries have been used for the solution-phase synthesis of oligonucleotides and their monothioate analogues[8–10] on a soluble polyethylene glycol (PEG) support.[11] Once the coupling is performed, the PEG-supported oligonucleotides may be isolated by precipitation in diethyl ether. More recently, oligonucleotides have been attached to an imidazolium tetrafluoroborate,[12] to an adamantyl tag,[13] and to acetylated and methylated β-cyclodextrin[14] for the same purpose, facilitating purification of oligonucleotides by precipitation, extractive workup, and flash chromatography, respectively.

We now report a novel soluble-support-based strategy with properties that are highly useful for the synthesis of short oligonucleotides. The growing oligonucleotides, which are tetrahedrally branched from a pentaerythritol-derived core, were found to precipitate virtually quantitatively from methanol; a feature that could be utilized as a purification step in the liquid-phase synthesis. Two precipitations are needed in each coupling cycle when using commercially available phosphoramidite building blocks: one after the oxidation step and the second after removal of the 5′-*O*-(4,4′-dimethoxytrityl) protecting group. The atom economy is good, because the support is small and still bears four oligonucleotides. The efficiency of coupling is as high as for previously described liquid-phase methods.[4–14] Advantageously, the small size and radial-symmetric structure of the support allows easy MS, NMR and HPLC analysis of the products (and side-products). Structurally related branched oligonucleotides have recently been described by the group of Richert,[15,16] but, instead of synthetic applications, prefabricated oligonucleotide segments (dimers and trimers) were attached to a tetravalent core structure (5–10 μmol scale) for nanochemical purposes.

Results and Discussion

Synthesis of Tetravalent Nucleoside Cluster 9

Pentaerythrityl-derived tetraazide **5** meets the requirements for a good branching unit (Scheme 1); it is a compact and radial-symmetric structure, its synthesis is straightforward, and clustering of the first nucleoside on this branching unit may be efficiently carried out. Additionally, the residue of the core structure (**17** see Scheme 2 below) may be isolated from the deprotected oligonucleotides by simple filtration, because it precipitates in aqueous ammonia. Tetrakis{[4-(chloromethyl)phenoxy]methyl}methane

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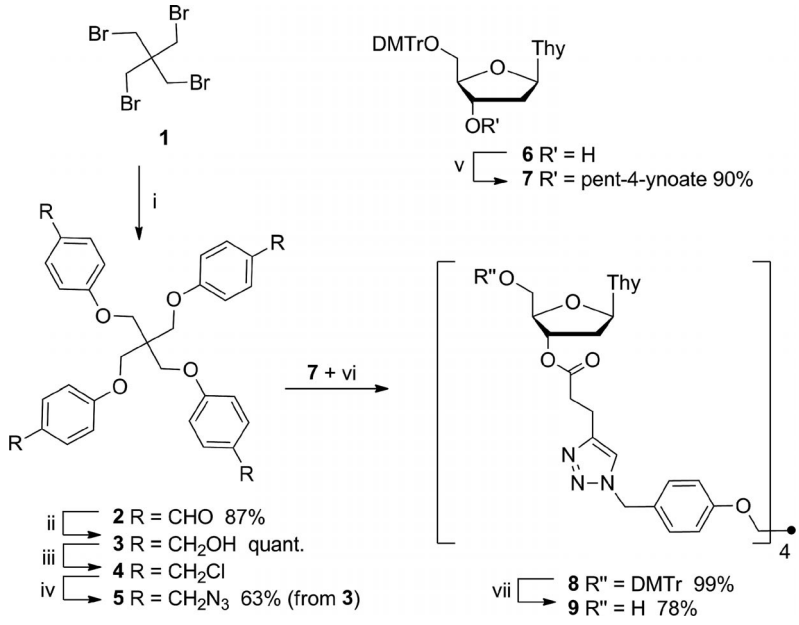


Scheme 1. Reagents and conditions: (i) 4-hydroxybenzaldehyde, KOH, TBAI, DMF, 120 °C; (ii) $NaBH_4$, MeOH at room temperature; (iii) $SOCl_2$, dioxane, 70 °C; (iv) NaN_3, DMF; (v) pent-4-ynoic anhydride, DMAP, pyridine; (vi) 5′-*O*-DMTr-3′-*O*-(pent-4-ynoyl)thymidine, $CuSO_4$, sodium ascorbate, H_2O, dioxane, 40 °C; (vii) HCl (13 mmol L^{-1}) in MeOH/CH_2Cl_2 (1:1 v/v) at room temperature.

(**4**) was prepared from pentaerytrityl tetrabromide (**1**) according to a reported procedure[17] (with some modifications), and the benzylic chlorides were then replaced by azides to give **5** in 55% overall yield. 5′-*O*-DMTr-thymidine (**6**) was acylated to give pent-4-ynoic anhydride (**7**)[18] and attached to **5** by using the click reaction.[19] It is worth noting that a relatively small excess of the alkyne-derived nucleoside **7** (1.25 equiv. per azide group) could be used, and the remaining excess could be recovered. Thus, wastage of the first nucleoside was modest, with the overall yield of **8** from the commercially available nucleoside **6** being 98%. The DMTr groups of cluster **8** were removed by a carefully treatment with HCl (13 mmol L^{-1}) in a mixture of MeOH/CH_2Cl_2 (1:1 v/v; see details for the detritylation below), which gave **9** in 78% yield.

The Phosphoramidite Coupling

For each coupling, 6.0 equiv. of a nucleoside phosphoramidite [2-cyanoethyl ^{DMTr}T, $^{DMTr}dC^{Bz}$, $^{DMTr}dG^{iBu}$ or $^{DMTr}dA^{Bz}$ *N*,*N*-diisopropylphosphoramidite, 1.5 equiv. per 5′-OH group, 0.13 mol L^{-1} solution in *N*,*N*-dimethylformamide/acetonitrile (1:1 v/v)] activated by 6.0 equiv. of 4,5-dicyanoimidazole (DCI) was used to give the desired phosphite triester (2 h at room temperature under N_2). Completion of the reactions were verified by RP HPLC analysis (Figure 1, plots i–iii). Incomplete couplings, if present, were observed as sets of un-, mono-, di- and trisubstituted starting materials. The major byproducts were, as expected, hydrolyzed phosphoramidites (i.e., *H*-phosphonates such as **19**), but traces of oxidized phosphoramidites (i.e., phosphoramidates such as **20**) and moderate amounts of unreacted phosphoramidites (such as **18**) could also be detected in the crude product mixtures. The desired products were obtained quantitatively.

The Oxidation

Phosphoramidite coupling results in the formation of P^{III} phosphite triesters, which should be subsequently oxidized to stable P^{V} phosphotriesters. When conducted on a solid phase, the oxidation may be carried out by simply using an excess of oxidant (usually aqueous iodine[1,2]), which can then be removed by extensive washings; however, this extra step complicates the synthesis in solution. The oxidant reagents should be quenched and/or removed at the latest prior to the next coupling (cf. **20** in Figure 1). In spite of the promising results obtained by using alternative oxidants (e.g., iodobenzene diacetate,[20] tetrabutylammonium periodate,[21] *N*-oxides, peroxides,[21,22] and a mixture of carbon tetrachloride, *N*-methylmorpholine and water[23,24]) conventional aqueous iodine was applied. Once the phosphoramidite coupling was completed, an aqueous solution of iodine (0.2 mol L^{-1} I_2 in pyridine/H_2O/THF, 2:4:8 v/v/v, added until the dark color remained; see conversion of **10** into **11** in Scheme 2 and Figure 1, plots i–ii) was added to the crude mixture, and the oxidation was quenched by the addition of trimethyl phosphite (1.0 mol L^{-1} in DMF, added until the dark color disappeared). Precipitation in methanol removed all traces of reagents and nucleoside derivatives (cf. **18**–**22** in Figure 1) and gave the desired phosphotriesters, which were then subjected to the detritylation step.

The Detritylation

In the standard solid-phase synthesis of oligonucleotides, deprotection of the DMTr group is carried out by an expo-

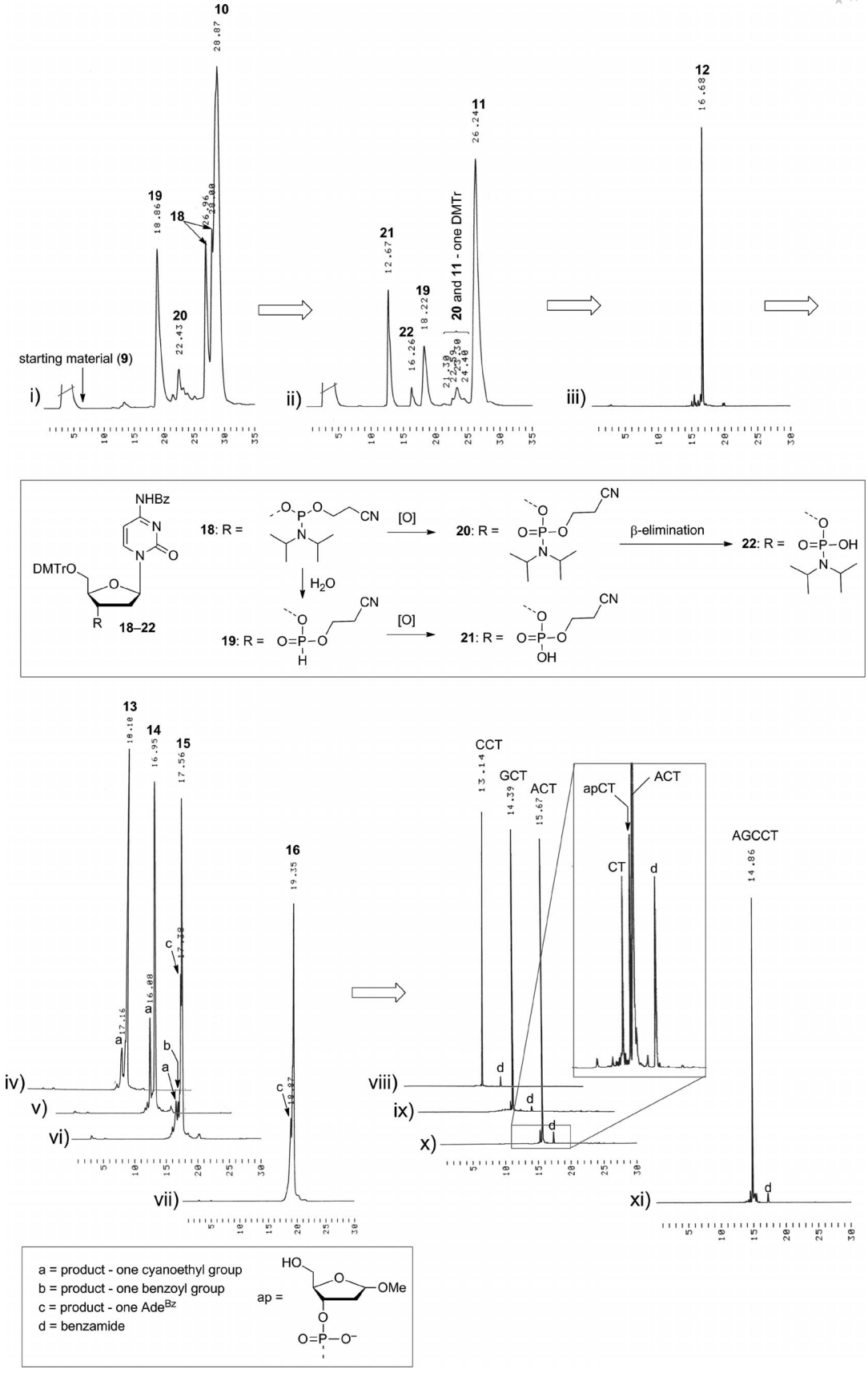
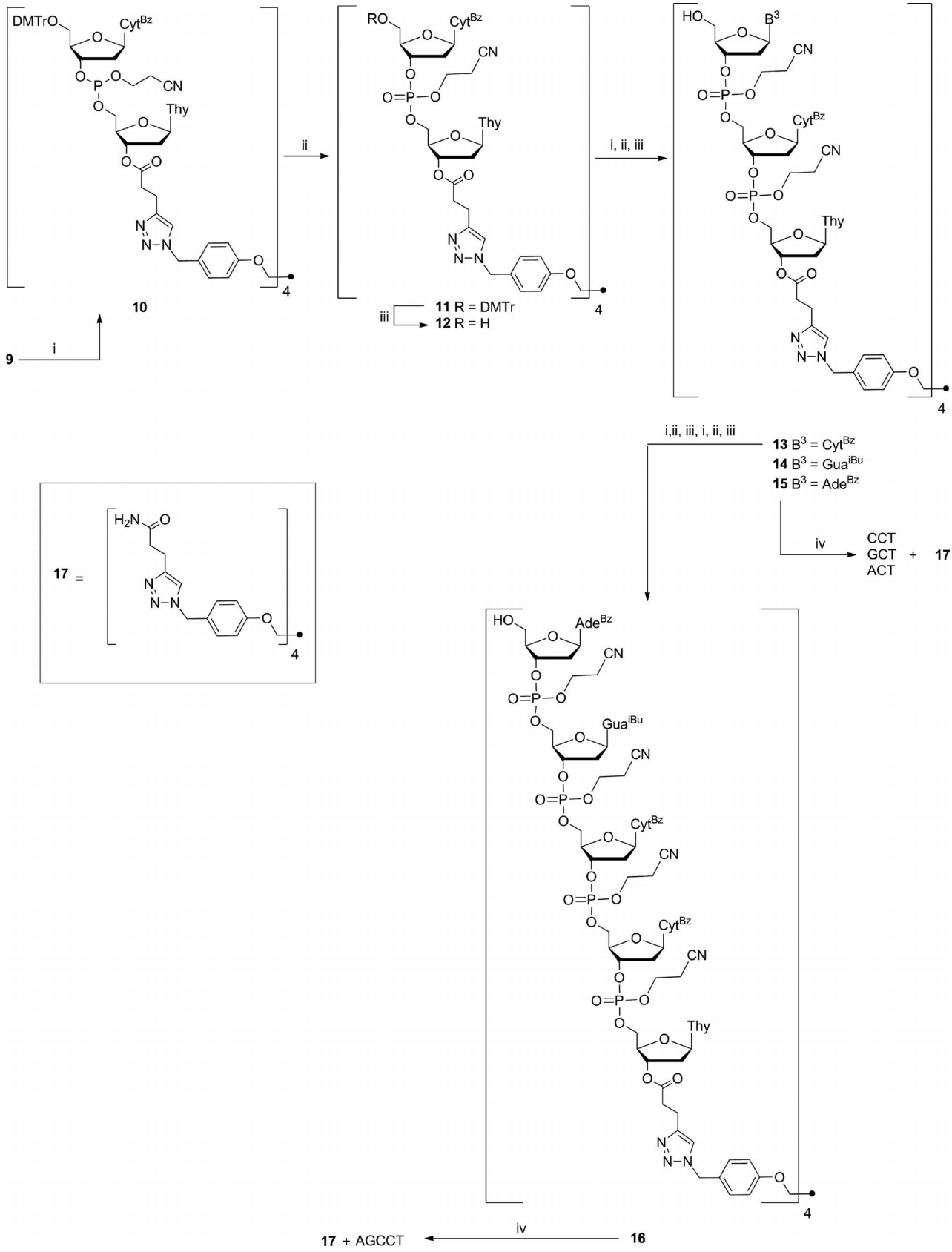


Figure 1. RP HPLC chromatograms of the crude product mixtures (for the reaction conditions and structures of **10**–**16**, see Scheme 2). Details of RP HPLC conditions A (plots i–iii), B (plots iv–vii), and C (plots vii–xi) are provided in the Exp. Sect. The identities of all species in the RP HPLC profiles were verified by MS (ESI) analysis.



Scheme 2. Reagents and conditions: (i) Phosphoramidite building block (6.0 equiv.), DCI (6 equiv.), DMF, acetonitrile, under N_2; (ii) I_2 (1.3 equiv.), pyridine, H_2O, THF [the reaction was quenched by the addition of $(MeO)_3P$ and the product was precipitated in MeOH]. (iii) HCl (13 mmol L^{-1}) in $MeOH/CH_2Cl_2$ (1:1 v/v) (the reaction mixture was neutralized with pyridine and the product precipitated in MeOH). (iv) aq. NH_3 (33%).

sure to a short treatment of tri- or dichloroacetic acid in dichloromethane.[1,2] The acidic medium is then rapidly removed by extensive washings, which minimizes the time for undesired *N*-glycosyl cleavage.[25] The reversibility of the reaction makes the situation more challenging in solution. The reactive DMTr cation needs a scavenger, and the acid treatment should additionally be neutralized either by addition of a base or by a basic extractive workup. The applicability of three volatile acids, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP),[26] aqueous acetic acid, and dilute HCl (in MeOH/CH_2Cl_2, 1:1 v/v) were initially evaluated for the detritylation. The progress of the reactions were roughly monitored by TLC and, after precipitation, the product distribution was quantified by RP HPLC. Although HFIP (neat) can be used for the detritylation of monomeric nucleosides,[26] the treatment proved ineffective for the nucleoside clusters (especially **8**). Additionally, partial premature cleavage of the benzoyl group of Ade (**15**, Bz of Cyt to a lesser extent) was observed. A similar trend was observed with 90% aqueous acetic acid. The reaction rate was sufficient at 50 °C, but a remarkable acid-catalyzed cleavage of the Bz group of Ade was observed. Carefully adjusted acidic conditions by using HCl (13 mmol L^{-1} HCl in MeOH/CH_2Cl_2, 1:1 v/v, for 15 min) proved most promising, although traces of benzoyl removal and depurination of Ade could be observed (see Figure 1, b and c). After completion, the reaction mixture (ca. 15 min reaction time) was neutralized by addition of pyridine and concentrated to an oil, but not to dryness, because pyridinium hydrochloride in concentrated solutions catalyzed the reverse reaction. Precipitation of the products finally removed the reactive DMTr traces.

Synthesis of Oligonucleotides

The synthesis cycle included phosphoramidite coupling, oxidation, detritylation, and two precipitations described above. The steps were repeated to elongate three branched trimers and one pentamer (**13**–**16** in Scheme 2) on a 27 μmol (**13**–**15**) and a 35 μmol scale (**16**) (0.11 and 0.14 mmol of oligonucleotides). RP HPLC chromatograms of the crude product mixtures (**13**–**16**) are shown in Figure 1, plots iv–vii. In addition to traces of depurination of Ade and debenzoylation (Figure 1, b and c, discussed above for the detritylation), a remarkable premature cleavage of the cyanoethyl groups was observed (Figure 1, a). Although the exposed exocyclic amino groups may result in undesired phosphoramidites, the latter side reaction is quite harmless for potential further chain elongation.[27] The identities of **13**–**16** were verified by MS (ESI) analysis, and one of the clustered trimers (**15**) was additionally characterized by NMR spectroscopy (^1H, ^{13}C, ^{31}P, COSY and HSQC). Aliquots (20 mg) of **13**–**16** were treated with concentrated aqueous ammonia, the resulting white precipitate was removed by filtration, and the filtrates were analyzed by RP HPLC. Traces of incomplete couplings and depurination of Ade may be seen upon more detailed inspection (Figure 1, plot x) of the chromatograms, but nearly homogeneous products (GCT, AGT, CCT, AGCCT) could be obtained (still containing released benzamide, d in Figure 1, plot xi). According to UV absorbance, overall yields (from **9**) for the trimers were 67–73% and 43% for the pentamer. The white precipitate obtained upon ammonolysis was also characterized, and was found to be the expected tetrakis[(4-{[4-(3-amino-3-oxopropyl)-1*H*-1,2,3-triazol-1-yl]methyl}phenoxy)methyl]methane (**17** in Scheme 2).

Conclusions

The application of clustered nucleosides as a soluble support for the synthesis of short oligo-2′-deoxynucleotides in solution has been described. Couplings using a small excess (1.5 equiv. of a nucleoside phosphoramidite per 5′-OH group) of the standard phosphoramidite building blocks proved efficient, and purification of the products could be carried out by simple precipitation (over 90% of the products usually gained) in methanol. Ammonolysis gave nearly homogeneous nucleotide trimers in ca. 70% yield and a pentamer in 43% yield. It is noteworthy that the couplings were nearly quantitative (according to RP HPLC profiles in Figure 1, plots viii–xi), and the yields indicate that the overall isolation of the products was comparable to those obtained by other liquid-phase methods.[4–14] Problems related to standard protecting groups (premature cleavage of cyanoethyl and Bz protections and susceptibility of dA for depurination upon DMTr removal) arose, but the principle of the strategy with more compatible protecting groups[14] and/or with an alternative coupling chemistry may be very useful for the production of short oligonucleotides on a scale of hundreds of milligrams without the need for any special equipment.

Experimental Section

General Remarks: NMR spectra were recorded with a 500 MHz spectrometer (Bruker Avance). Chemical shifts are given in ppm and referenced to the solvent signals.[28] RP HPLC conditions: (A) gradient elution from 50% acetonitrile in 0.1 mol L^{-1} $Et_3NH^+AcO^-$ to 100% acetonitrile in 25 min, then continued with acetonitrile; (B) gradient elution from 25% acetonitrile in 0.1 mol L^{-1} $Et_3NH^+AcO^-$ to 100% acetonitrile in 25 min, then continued with acetonitrile; (C) gradient elution from 2.5% acetonitrile in 0.1 mol L^{-1} $Et_3NH^+AcO^-$ to 50% acetonitrile in 0.1 mol L^{-1} $Et_3NH^+AcO^-$ in 25 min, then continued with 50% acetonitrile in 0.1 mol L^{-1} $Et_3NH^+AcO^-$. An analytical C-18 RP column (250 × 4.6 mm, 5 μm, flow rate 1.0 mL min^{-1}, λ = 260 nm) was used.

Tetrakis{[4-(hydroxymethyl)phenoxy]methyl}methane (3): Alcohol **3** was prepared according to the literature,[17] with the exception that pentaerythrityl tetrabromide (**1**) was used as a starting material instead of the corresponding tetratosylate. Pentaerythrityl tetrabromide (**1**; 5.0 g, 13 mmol), 4-hydroxybenzaldehyde (7.9 g, 65 mmol), KOH (3.6 g, 65 mmol) and tetrabutylammonium iodide (TBAI; 40 mg, 0.11 mmol) were dissolved in *N*,*N*-dimethylformamide (40 mL), and the mixture was stirred at 120 °C overnight, cooled

to room temperature and poured into water. The crude product was extracted with dichloromethane, washed with saturated $NaHCO_3$, dried with Na_2SO_4, and the solvents were evaporated to dryness. The residue was purified by silica gel chromatography (MeOH/CH_2Cl_2, 5:95 v/v) to yield **2** (6.0 g, 87%) as a white foam. Aldehyde **2** (6.0 g, 11 mmol) was dissolved in methanol (140 mL), and $NaBH_4$ (5.0 g, 130 mmol) was slowly added at 0 °C. The mixture was warmed and stirred at room temperature overnight. The reaction was quenched by the addition of water, and the mixture was concentrated to a viscous oil. The oil was dissolved in ethyl acetate and washed with water and brine. The organic layer was dried with Na_2SO_4, filtered, and the solvents were evaporated to dryness to give **3** (6.0 g, quant.) as white solid flakes (according to NMR analysis, further purification was not required). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.24 (d, J = 8.5 Hz, 8 H), 6.90 (d, J = 8.6 Hz, 8 H), 4.53 (s, 8 H), 4.35 (s, 8 H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 158.0, 133.4, 128.1, 114.2, 66.3, 63.6, 44.6 ppm. ESI (MS): m/z = 583.3 $[M + Na]^+$.

Tetrakis{[4-(azidomethyl)phenoxy]methyl}methane (5): Alcohol **3** (2.3 g, 4.1 mmol) was dissolved in dioxane (60 mL), and thionyl chloride (2.4 mL, 31 mmol) was added. The mixture was heated to reflux with stirring under nitrogen overnight, then the solvents were evaporated to dryness. The resulting oil (**4**; 2.9 g) and NaN_3 (3.2 g, 49 mmol) were dissolved in *N*,*N*-dimethylformamide (40 mL), and the mixture was stirred at room temperature overnight, poured into water, and the crude product was extracted with diethyl ether. The product fractions were combined, dried with Na_2SO_4, filtered, and the solvents were evaporated to dryness. The residue was purified by silica gel chromatography (CH_2Cl_2/hexane, 1:1 v/v) to give **5** (1.7 g, 63%) as a colorless oil that crystallized spontaneously upon further storage. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.26 (d, J = 8.5 Hz, 8 H), 6.97 (d, J = 8.5 Hz, 8 H), 4.40 (s, 8 H), 4.28 (s, 8 H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 158.8, 129.8, 127.9, 115.0, 66.6, 54.3, 44.8 ppm. HRMS (ESI): calcd. for $C_{33}H_{32}ClN_{12}O_4$ $[M + Cl]^-$ 695.2358; found 695.2375.

5′-*O*-(4,4-Dimethoxytrityl)-3′-*O*-(pent-4-ynoyl)thymidine (7): The synthesis of **7** has previously been reported by using pent-4-ynoyl fluoride as a starting material.[18] An anhydride method was, however, applied in the present study. Pent-4-ynoic acid (3.4 g, 34 mmol) was dissolved in dioxane (40 mL) and dicyclohexylcarbodiimide (3.6 g, 17 mmol) was added. The mixture was stirred at room temperature for 2 h, filtered, and concentrated to a viscous oil. The resulting pent-4-ynoic anhydride was dissolved in a small amount of pyridine and added to a mixture of 5′-*O*-(4,4-dimethoxytrityl)thymidine (**6**; 6.4 g, 12 mmol) in pyridine (70 mL). A catalytic amount of DMAP was added, and the mixture was stirred at room temperature overnight. The reaction mixture was concentrated to a smaller volume, dissolved in ethyl acetate, washed with saturated $NaHCO_3$, dried with Na_2SO_4, and the solvents were evaporated to dryness. The residue was purified by silica gel chromatography (EtOAc/petroleum ether/Et_3N, 70:30:1 v/v/v) to give **8** (7.3 g, 99%) as a white foam. The authenticity of the product was verified by ^{1}H, ^{13}C NMR and HRMS (ESI) analysis, and the data was compared to the reported values.

Tetrakis({4-[(4-{3-[5′-*O*-(4,4-dimethoxytrityl)thymidin-3′-*O*-yl]-3-oxoprop-1-yl}-1*H*-1,2,3-triazol-1-yl)methyl]phenoxy}methyl)methane (8): Compounds **5** (1.4 g, 2.1 mmol) and **7** (7.4 g, 12 mmol) were dissolved in dioxane (25 mL). Aqueous solutions of $CuSO_4$ (0.050 mol L^{-1}, 2.3 mL, 0.12 mmol) and sodium ascorbate (0.10 mol L^{-1}, 0.60 mL, 0.60 mmol) were added, and the mixture was stirred at 40 °C for 48 h. The reaction mixture was then concentrated to a smaller volume, diluted with water, and the product was extracted with ethyl acetate. The ethyl acetate layers were combined, washed with saturated $NaHCO_3$ and brine, dried with Na_2SO_4, and the solvents were evaporated to dryness. The residue was purified by silica gel chromatography (MeOH/Et_3N/CH_2Cl_2, 5:1:95 v/v/v) to give **8** (6.7 g, quant.) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.61 (s, 4 H), 7.40 (m, 8 H), 7.32–7.23 (m, 32 H), 7.18 (m, 8 H), 6.88–6.84 (m, 24 H), 6.39 (dd, J = 7.3, 6.7 Hz, 1 H), 5.47 (br., 4 H), 5.39 (s, 8 H), 4.30 (s, 8 H), 4.12 (br., 4 H), 3.79 (s, 24 H), 3.50–3.44 (m, 8 H), 3.00 (m, 8 H), 2.75 (m, 8 H), 2.43 (m, 8 H), 1.39 (s, 12 H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.1, 164.0, 158.9, 158.8, 150.8, 146.3, 144.2, 135.4, 135.3, 135.2, 130.12, 130.09, 129.6, 128.14, 128.05, 127.5, 127.2, 120.9, 115.1, 113.3, 111.7, 87.2, 84.3, 83.9, 75.5, 66.0, 63.7, 55.3, 53.5, 53.5, 44.7, 37.9, 33.5, 20.9, 11.7 ppm. MS (ESI): m/z = 1601.7 $[M + 2 Na]^{2+}/2$.

Tetrakis{[4-({4-[3-(thymidin-3′-*O*-yl)-3-oxoprop-1-yl]-1-*H*-1,2,3-triazol-1-yl}methyl)phenoxy]methyl}methane (9): Compound **8** (1.2 g, 0.38 mmol) was dissolved in 50 mL of 10 mmol L^{-1} HCl in MeOH/CH_2Cl_2 (1:1 v/v). The mixture was stirred at room temperature for 1 h, neutralized by the addition of pyridine, and the solvents were evaporated to dryness. Completion of the reaction was verified by RP HPLC, and the residue was purified by silica gel chromatography (MeOH/CH_2Cl_2, 1:9 v/v) to give **12** (0.58 g, 78%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.75 (s, 4 H), 7.44 (s, 4 H), 7.14 (d, J = 8.6 Hz, 8 H), 6.84 (d, J = 8.6 Hz, 8 H), 6.19 (dd, J = 7.3, 7.1 Hz, 4 H), 5.36 (s, 8 H), 5.26 (br., 4 H), 4.25 (s, 8 H), 3.95 (br., 4 H), 3.75 (s, 8 H), 2.93 (dd, J = 7.3, 7.2 Hz, 8 H), 2.68 (dd, J = 7.4, 7.2 Hz, 8 H), 2.23 (m, 8 H), 1.82 (s, 12 H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 176.2, 168.8, 162.9, 154.9, 150.2, 140.4, 133.5, 131.4, 125.6, 119.0, 114.9, 89.1, 88.8, 79.3, 70.3, 65.7, 57.3, 48.7, 41.4, 37.3, 24.5, 15.8 ppm. MS (ESI): m/z = 973.4 $[M – 2 H]^{2-}/2$.

Synthesis of Branched Oligonucleotides 12–16: The dT cluster **9** (0.30 g, 0.15 mmol) and 2-cyanoethyl ^{DMTr}dC^{Bz} *N*,*N*-diisopropylphosphoramidite (**18**; 0.77 mg, 0.92 mmol, 6.0 equiv.) were dissolved in anhydrous DMF (3.7 mL) and a solution of DCI (0.25 mol L^{-1} in acetonitrile, 3.7 mL, 6.0 equiv.) was added. The completed coupling (2 h at room temperature under N_2) was verified by RP HPLC (Figure 1, plot i), and then an aqueous solution of iodine (0.2 mol L^{-1} I_2 in H_2O/pyridine/THF, 2:4:8 v/v/v, titrated until the dark color remained, ca. 1.3 equiv. per mol **18**) was added. After 5 min, a solution of $P(OMe)_3$ (1.0 mol L^{-1} in DMF; titrated until the dark color disappeared, ca. 0.3 equiv. per mol **18**) was added (complete oxidation was verified by RP HPLC analysis, Figure 1, plot ii), and the reaction mixture was added to cold methanol (150 mL). The precipitate was isolated, dissolved in MeOH/CH_2Cl_2 (1:1 v/v, 100 mL), and HCl in MeOH/CH_2Cl_2 (0.125 mol L^{-1}, 10 mL) was added. Complete detritylation was verified by TLC analysis (ca. 15 min reaction time), then the mixture was neutralized by addition of pyridine and concentrated to an oil. The oil was dissolved in a mixture of MeOH/CH_2Cl_2 (1:1 v/v, 2.0 mL) and added to cold methanol (150 mL). The precipitate was isolated and dried under vacuum to give **12** (0.57 g, quant.) as a white powder.

Table 1. MS (ESI) data of the branched oligonucleotides.

Compound	Calcd. monoisotopic mass	Found mass
12	1865.6 $[(M – 2 H)/2]^{2-}$	1866.2 $[(M – 2 H)/2]^{2-}$
13	1838.2 $[(M – 3 H)/3]^{3-}$	1838.2 $[(M – 3 H)/3]^{3-}$
14	1846.1 $[(M – 3 H)/3]^{3-}$	1846.2 $[(M – 3 H)/3]^{3-}$
15	1870.2 $[(M – 3 H)/3]^{3-}$	1870.2 $[(M – 3 H)/3]^{3-}$
16	2300.6 $[(M – 4 H)/4]^{4-}$	2300.6 $[(M – 4 H)/4]^{4-}$

The purity of **12** is illustrated in Figure 1, plot iii. Three 0.10 g batches of **12** (27 μmol) were mixed with 2-cyanoethyl $^{DMTr}C^{Bz}$, $^{DMTr}G^{iBu}$ and $^{DMTr}A^{Bz}$ *N*,*N*-diisopropylphosphoramidite (160 μmol) in DMF (0.64 mL) and DCI (0.25 mol L^{-1} solution in acetonitrile, 0.64 mL, 6.0 equiv.) was added to each batch. Once the phosphoramidite couplings were complete (2 h at room temperature under N_2, verified by RP HPLC analysis), the mixtures were oxidized as described above by using iodine solution (0.2 mol L^{-1}), and the remaining iodine was quenched by the addition of a solution of trimethyl phosphite (1.0 mol L^{-1} in DMF), then the mixtures were precipitated in cold methanol (30 mL). The precipitates were dissolved in MeOH/CH_2Cl_2 (1:1 v/v, 20 mL), and HCl in MeOH/CH_2Cl_2 (0.125 mol L^{-1}, 2 mL) was added. After the detritylation reactions (monitored by TLC, ca. 15 min reaction time), the mixtures were neutralized by the addition of pyridine, concentrated to oils, dissolved in MeOH/CH_2Cl_2 (1:1 v/v, 0.5 mL) and precipitated in cold methanol (30 mL). The precipitates were isolated and dried under vacuum to give **13**–**15** (ca. 0.13 g) as white powders (88–89%, approximated as homogeneous products). The branched pentamer **16** was synthesized on a 35 μmol scale (by using 0.070 g of **9**) under the conditions described above (the first two couplings with Cyt repeated) to give **16** (0.22 g, 67%, approximated as homogeneous product) as a white powder. RP HPLC chromatograms of the branched oligonucleotides **13**–**16** are shown in Figure 1, plots iv–vii. The authenticity of the products was verified by ESI (MS) (Table 1). Compound **15** was additionally characterized by NMR spectroscopic analysis.

Ammonolysis of Branched Oligonucleotides 13–16: A sample of **13**–**16** (20 mg) was treated with concentrated aqueous ammonia (55 °C, overnight), and the obtained white precipitate was removed by filtration. The filtrate was concentrated to dryness, dissolved in water, and analyzed by RP HPLC and UV spectroscopy. The purity of the desired oligonucleotides is illustrated in Figure 1, plots viii–xi. According to UV spectroscopic analysis, the trimers (CCT, GCT and ACT) were obtained in 67–75% yields and the pentamer (AGCCT) in 43% yield (overall from **9**). The filtered white precipitate obtained in the ammonolysis was the released branching unit **17**. ^{1}H NMR [500 MHz, [D$_6$]DMSO]: δ = 7.74 (s, 4 H), 7.20 (d, J = 8.2 Hz, 8 H), 6.93 (d, J = 8.2 Hz, 8 H), 5.41 (s, 8 H), 4.23 (s, 8 H), 2.78 (m, 8 H), 2.37 (m, 8 H) ppm. ^{13}C NMR [125 MHz, [D$_6$]DMSO]: δ = 173.7, 158.7, 146.9, 129.9, 129.0, 122.1, 115.2, 66.5, 52.6, 44.7, 34.9, 21.5 ppm. MS (ESI): m/z = 1049.5 [M + H]$^+$.

Supporting Information (see footnote on the first page of this article): ^{1}H and ^{13}C NMR spectra of **5**, **8**, **9**, **15** (supported by HSQC) and **17** (supported by HMBC) and ^{31}P NMR spectrum of **15**.

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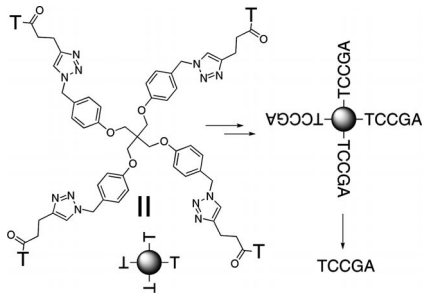
Oligonucleotide Synthesis

A liquid-phase method for the synthesis of short oligonucleotides is described that is based on the efficient precipitation of tetrahedrally branched oligonucleotides in methanol.



Keywords: Synthetic methods / Template synthesis / Oligonucleotides / Solution-phase synthesis

