Use of Allylic Protecting Groups for the Synthesis of Base-Sensitive Prooligonucleotides

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The synthesis of mixed MeSATE-phosphotriester and -phosphodiester prooligonucleotides [MeSATE = 2-(acetylthio)ethyl] of various sequences is described. The strategy is based on the use of allyloxycarbonyl (AOC) protection for nucleobases and MeSATE and allyl (All) protection for internucleosidic phosphates, in combination with palladium(0) deprotection. The synthesis was achieved by the use of phosphoramidite chemistry on a photolabile solid support, enabling MALDI-TOF mass spectrometric analysis to be performed on the still anchored prooligonucleotides

Introduction

Prodrugs of oligonucleotides (prooligonucleotides)[1] have been designed with the goals of overcoming the instability of oligonucleotides towards phosphodiesterases,[2] reducing nonspecific protein interactions,[3] and enhancing their cellular uptake.[4,5] In our prooligonucleotide approach, the negative sites in some internucleoside linkages were transitorily protected with the 2-(acylthio)ethyl (SATE) group previously introduced in mononucleotide chemistry.[6,7]

In cell extracts, prooligonucleotides become fully deprotected through a carboxyesterase-mediated mechanism, to yield free oligonucleotides.[8,9] This decomposition proceeds by thioester bond breakage, releasing an unstable 2-mercaptoethyl phosphotriester that rapidly decomposes to ethylene sulfide and the corresponding ionic phosphodiester or phosphorothioate linkages. This process could also occur upon base[10] or nucleophile treatment. Prooligonucleotides therefore could not be synthesized under the common conditions of solid-support DNA synthesis, as they rapidly decompose on the ammonia[2] treatment required for the release of the oligonucleotide from succinyl solid support[11] and the deprotection of *N*-acyl-substituted nucleobases.[12] A new strategy was therefore devised for the solid-phase synthesis of these base-sensitive analogues, with the aid of a new photolabile solid support[13] and thymidine SATE phosphoramidites. This enabled prooligonucleotide models[2] to be synthesized by phosphoramidite methodology.[12]

Our attention then focused on the development of protecting groups of nucleobases for synthesis of prooligonucleotide heteropolymers containing the four common nucleobases. We have evaluated the properties of several protecting groups, such as the sulfenyl protecting groups (2-nitrophenyl)sulfenyl (NPS)[14] and tritylsulfenyl (TrS),[15] removable under mild reductive conditions and under oxidative conditions, respectively. Unfortunately, these groups are not efficient for DNA synthesis by the phosphoramidite approach.[16] In contrast, the photocleavable protecting groups (6-nitroveratryl)oxycarbonyl (NVEOC)[17] and 2,2-bis(2-nitrophenyl)ethoxycarbonyl (diNPEOC)[18] were more efficient and allowed the synthesis of several *t*BuSATE heteropolymers (pentamers and dodecamers).[19] The cleavage of the prooligonucleotides from the solid support and the removal of the protecting groups from the nucleobases were performed with the aid of UV irradiation at wavelengths greater than 300 nm. However, the longer the prooligonucleotide (the higher the number of protections), the harder the removal of all the protecting groups. Furthermore, as reaction times increased, numerous side products appeared.[19]

The allylic groups allyoxycarbonyl AOC and allyl (All)[20] were introduced and developed by Hayakawa et al.[21] for protection of nucleobases and internucleosidic linkages during the synthesis of regular oligonucleotides. Interestingly, syntheses involving the use of All/AOC protecting groups have been reported by Greenberg both for regular oligonucleotides and for oligonucleotides containing base-labile nucleosides on solid supports bearing photolabile linkers.[22,23] The orthogonality of the protecting groups and the photolabile linker allowed allylic groups to be removed before the release of oligonucleotides from the solid support. The advantage of allyl and allyloxycarbony protecting groups over the conventional cyanoethyl and acyl (benzoyl

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and isobutyryl) functions was their removal through a palladium(0)-catalyzed reaction under conditions milder than standard ammonia deprotection. Here we would like to describe the use of the allyloxycarbonyl (AOC) group for the protection of the exocyclic amines of the nucleobases, and of the allyl group for the protection of the internucleosidic linkages, in the synthesis of prooligonucleotide chimeras bearing phosphodiester and MeSATE 2-(acetylthio)ethyl phosphotriester linkages.

Results and Discussion

All-AOC Removal Conditions Suitable for Prooligonucleotide Stability

The original conditions described for the removal of allyl/AOC protecting groups from CPG-supported oligonucleotides[21] involved treatment of the compounds at 50 °C for 0.5−1 h with a THF solution of tris(dibenzylideneacetone)dipalladium(0)−chloroform complex [$Pd_2(dba)_3 \cdot CHCl_3$] as catalyst (2.5 equiv./allylic group), triphenylphosphane (25 equiv./allylic group), and a large excess of formic acid/butylamine (1:1) (250 equiv./allylic group) as a scavenger of allyl groups. The supports were then washed with THF, acetone, and, finally, an aqueous solution of sodium *N*,*N*-diethyldithiocarbamate (ddtc) at pH = 9.7 to complex and remove residual palladium.

To investigate whether this treatment was compatible with the stability of base-sensitive MeSATE prooligonucleotides, a hexathymidylate d(TpMeSATE)$_6$ (**A**) bearing six MeSATE phosphotriester linkages (Figure 1) was synthesized by using 5′-*O*-DMT thymidine 3′-*O*-MeSATE phosphoramidite (**1**) as previously described[2] on a CPG solid support with a photolabile linker bearing a 1-(*o*-nitrophenyl)-1,3-propanediol moiety between the growing oligonucleotide and the CPG.[13] This solid support releases prooligonucleotides with a 3′-phosphodiester end after UV irradiation (20 min, $\lambda > 300$ nm).

As previously described for peptides bound to resins through a photolabile linker,[24,25] the oligonucleotide could be directly analyzed by MALDI-TOF mass spectrometry while still anchored. Indeed, we found that laser UV irradiation (337 nm) was able to release the free oligonucleotides from our photolabile support and also to ionize it (Figure 2).[26] Thus, at the end of the elongation process, MALDI-TOF analysis performed on a few beads of the still anchored prooligonucleotide **A** showed one major signal (Figure 3a) corresponding to the desired prooligonucleotide (*m*/*z* calcd. 2455.1) and two other signals of lower intensity (*m*/*z* found 2355.1 and 2049.4) due to the presence of a prooligonucleotide having lost one MeSATE group (*m*/*z* calcd. 2353.0) and of a truncated prooligonucleotide d(TpMeSATE)$_5$ (*m*/*z* calcd. 2048.8).

MALDI-TOF MS of the solid-supported material after subjection to the palladium(0) mixture treatment (45 min) and its several washes showed a ladder of ions differing from one another by 102.15 Daltons, corresponding to the partial loss of 1−6 MeSATE moieties (− C_4H_6OS) from the prooligonucleotide **A** (Figure 3b). The intensity of the signal corresponding to the fully demasked phosphodiester

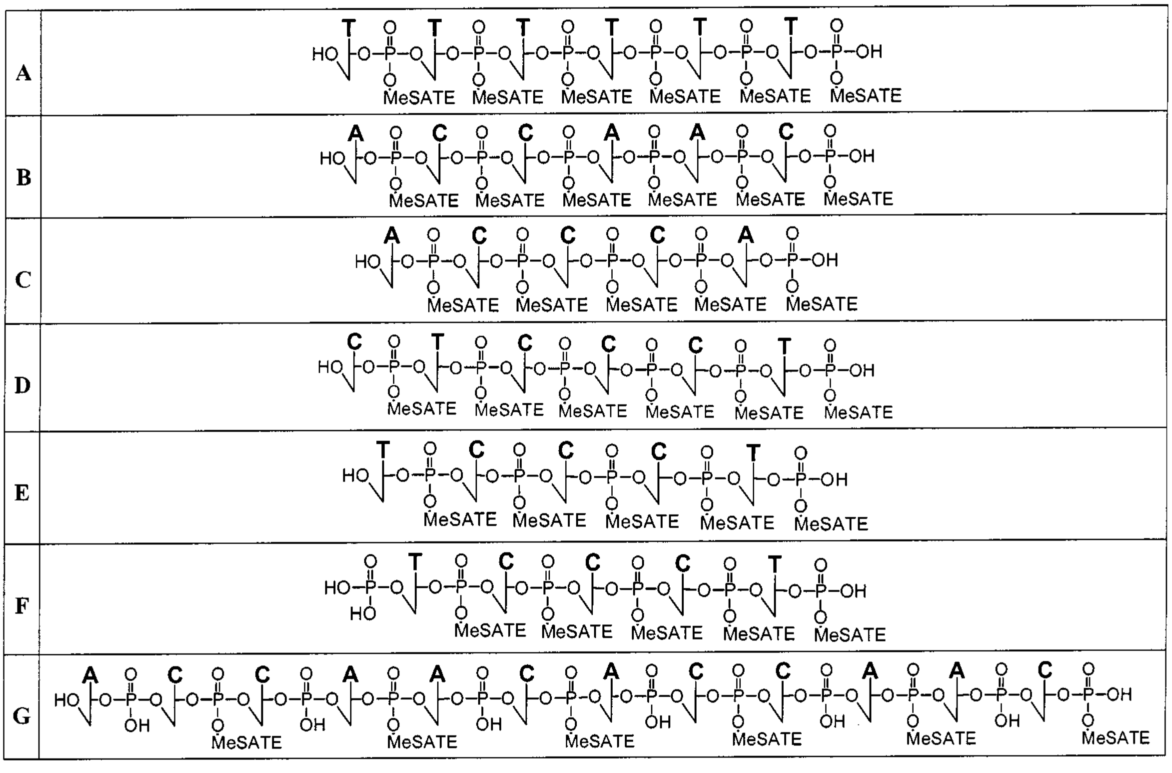

Figure 1. Schematic structure of the prooligonucleotides **A**−**G**

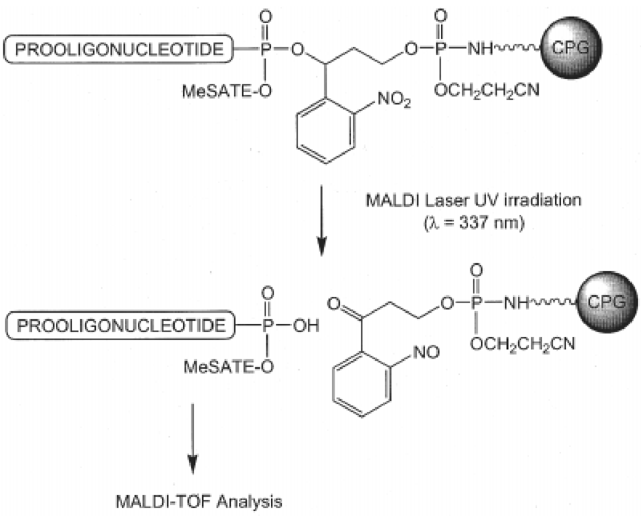

Figure 2. Release and analysis of prooligonucleotides from photolabile solid support upon laser UV irradiation during MALDI-TOF mass spectrometry analysis

oligonucleotide d(Tp)$_6$ (*m*/*z* calcd. 1842.2) was the highest in the spectrum. This result correlated well with the reverse-phase HPLC analysis of the material obtained after the release of the material from the solid support by UV irradiation, which showed extensive degradation of the prooligonucleotide A (t_R = 37.8 min) in phosphodiester d(Tp)$_6$ (t_R = 17.4 min, Figure 3b′). Thus, the Pd^0 standard treatment[21] was unsuitable for prooligonucleotide synthesis, as it induced significant loss of MeSATE groups. Similarly, MALDI-TOF analysis of a few beads of the solid-supported **A** washed by ddtc solution at pH = 9.7 without Pd treatment (Figure 3c), and also HPLC of the released material (Figure 3c′), revealed degradation of the prooligonucleotide, but to a lesser extent than that found after the complete treatment (Figure 3b and b′). To overcome the instability of MeSATE phosphotriester linkages during the wash with dttc, the solution was buffered to pH = 6.7, as it had been reported that removal of Pd traces was still efficient under these conditions.[27] Under these pH conditions, the prooligonucleotide **A** was not degraded, as shown by MALDI-TOF and HPLC analyses (data not shown).

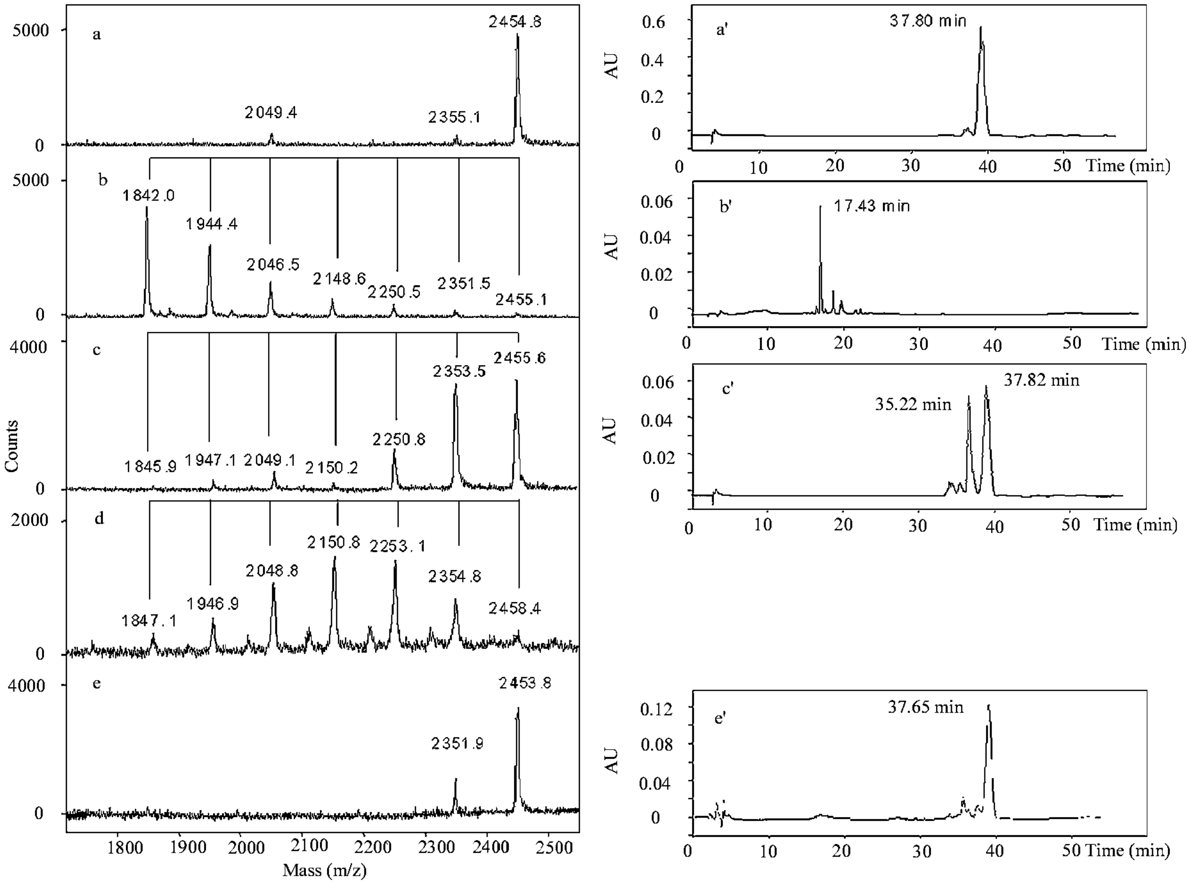

Figure 3. MALDI-TOF analysis of the still anchored prooligonucleotide **A**: a) without treatment; b) treated at 50 °C for 45 min with a THF solution of [Pd$_2$(dba)$_3$·CHCl$_3$], PPh$_3$ and formic acid/butylamine (1:1) and washed with an aqueous solution of ddtc at pH = 9.7; c) washed with ddtc at pH = 9.7 without Pd treatment; d) treated at 50 °C for 45 min with a THF solution of [Pd$_2$(dba)$_3$·CHCl$_3$], PPh$_3$ and formic acid/butylamine (1:1) and washed with an aqueous solution of ddtc at pH = 6.7; e) treated at 50 °C for 45 min with a THF solution of [pd$_2$(dba)$_3$·CHCl$_3$], PPh$_3$ and dimedone and washed with an aqueous solution of ddtc at pH = 6.7; HPLC analyses a′)−e′) of the corresponding materials obtained after the release of the prooligonucleotide **A** from the solid support

The anchored prooligonucleotide **A** was then submitted to the Pd^0 treatment followed by washes with dttc at pH = 6.7. Here, once more, MALDI-TOF mass spectrometry revealed extensive degradation of the prooligonucleotide (Figure 3d). As observed previously, this degradation generated a mass ladder of peaks that differed from one another by 1 MeSATE residue (− 102 Da). Another set of ions of lower intensity, differing from the previous set by a loss of 42 Da, were the result of thioacetyl hydrolysis of MeSATE.

The source of this instability should be nucleophilic attack on the thioester function of the MeSATE by the butylamine as allyl scavenger. To prove this, formic acid alone (150 equiv./allylic group) was used as allyl group scavenger[28] in place of formic acid/butylamine (1:1). Under these conditions, MALDI-TOF analysis of supported d(TpMeSATE)$_6$ did not show significant loss of MeSATE groups (data not shown). Further studies showed that dimedone (pK_a = 5.3)[28,29] (150 equiv./allylic group) was as efficient as formic acid (Figure 3e and e′), and this was chosen for further deprotection so as to avoid the possible depurination of oligonucleotide heteropolymers because of the presence of formic acid (pK_a = 3.75).

It is notable that the use of formic acid or dimedone as allyl group scavengers has been reported for nucleotide synthesis,[28] but never for oligonucleotide chemistry. To demonstrate the efficiency of allyl removal under these new conditions, a heteropolymer with six MeSATE phosphotriester linkages d(ACCAAC) pMeSATE **B** (Figure 1) with AOC-protected nucleobases was synthesized on an automated DNA synthesizer by phosphoramidite methodology, starting from the 3′-*O*-MeSATE phosphoramidite monomer units **2** and **3** (Figure 4). These MeSATE phosphoramidite synthons **2** and **3**, as well as the dT and dG analogs **1** and **4**, were prepared by treatment of the free 3′-hydroxy functions of the corresponding 5′-nucleosides − i.e., 5′-*O*-DMTr-4-*N*-AOC-2′-deoxycytidine, 5′-*O*-DMTr-6-*N*-AOC-2′-deoxyadenosine, 5′-*O*-DMTr-deoxythymidine, and 5′-*O*-DMTr-2-*N*-AOC-6-*O*-allyldeoxyguanosine[21,30] − with the phosphitylating agent [2-(acetylthio)ethyloxy]bis(diisopropylamino)phosphane (**5**) in the presence of diisopropylammonium tetrazolide. This method is simpler and easier than the one previously described,[2] in which the 5′-*O*-DMTr-3′-*O*-MeSATE phosphoramidite derivative of thymidine was prepared in two steps starting from 5′-*O*-DMTr thymidine, by treatment with chloro(*N*,*N*-diisopropylamino)phosphane in the presence of *N*,*N*-diisopropylamine followed by in situ reaction with 2-thioacetylethanol in the presence of tetrazole.

During the elongation process (the oxidation of phosphite triester intermediates to form MeSATE phosphotriester linkages), *tert*-butyl hydroperoxide[31,32] in anhydrous dichloromethane was preferred to the standard iodine/water treatment, so as to avoid possible loss of the SATE group through an aqueous oxidation.[32,33] Furthermore, as we had found that the capping step with acetic anhydride gave rise to side reactions (vide infra), this step was omitted for the DNA synthesis of d(ACCAAC) pMeSATE (**B**). The coupling efficiency of the *N*-AOC deoxynucleoside MeSATE phosphoramidites was determined at the end of the incorporation synthesis cycle by titration of the dimethoxytrityl cation released under acidic conditions. A coupling efficiency similar to that seen with standard *N*-acyl deoxynucleoside 2-cyanoethyl phosphoramidites was observed (> 98%). At the end of the elongation process, an aliquot of the anchored oligonucleotide was subjected to the Pd^0 treatment with dimedone as allyl scavenger, followed by washes with the ddtc solution at pH = 6.7. The solid-supported prooligonucleotide was analyzed by MALDI-TOF mass spectrometry before (Figure 5a) and after allyloxycarbonyl removal (Figure 5b). The MALDI spectrum showed complete AOC removal from the *N*-protected prooligonucleotide (*m*/*z* calcd. 2941.5) to give the expected NH$_2$-free d(ACCAAC) pMeSATE (**B**) (*m*/*z* calcd. 2437.1) without extensive loss of MeSATE group (*m*/*z* calcd.2335.0).

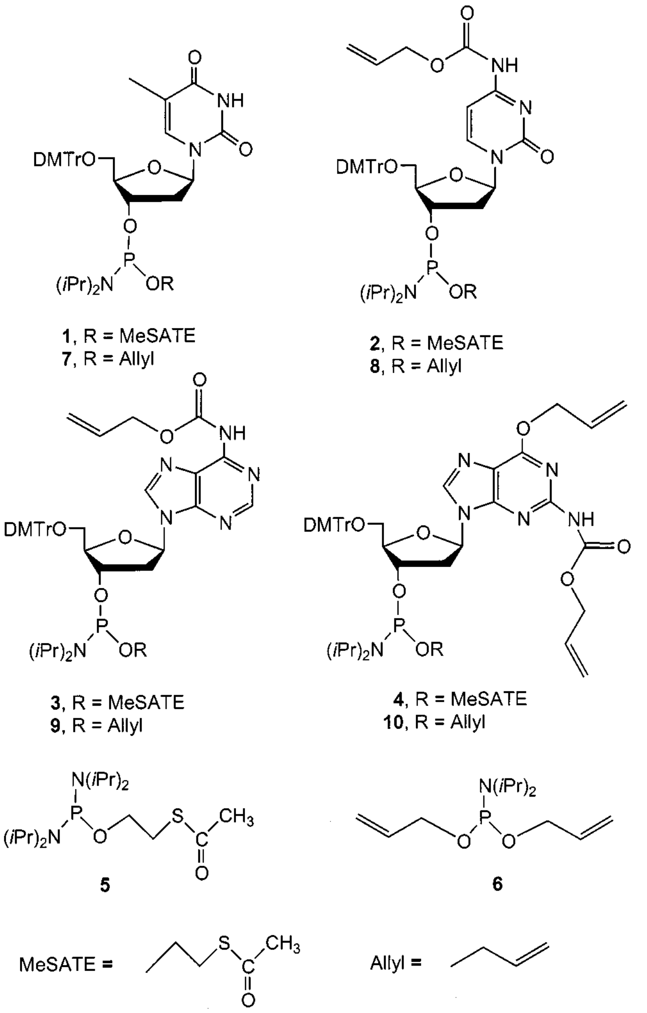

Figure 4. Phosphoramidite building blocks

A New Capping Reagent

MALDI-TOF mass spectrometric analysis of a solid-supported, fully protected prooligonucleotide d(AC$_3$A) pMeSATE (**C**), bearing 5 AOC protecting groups, revealed a mixture of the expected oligomer (*m*/*z* found 2439.4) and an adduct at *m*/*z* 2481.5 (Figure 6). This adduct was the result

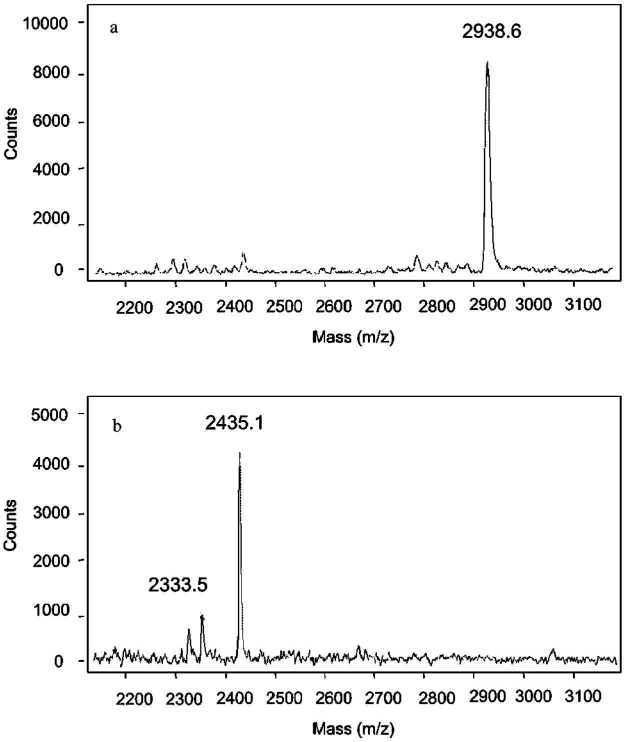

Figure 5. MALDI-TOF analysis of the solid-supported prooligonucleotide **B**: a) without treatment; b) treated at 50 °C for 45 min with a THF solution of $[Pd_2(dba)_3{\cdot}CHCl_3]$, PPh_3 and dimedone and washed with an aqueous solution of ddtc at pH = 6.7

of partial acetylation (CH_2CO, + 42) of the oligomer under the standard capping step conditions used in the oligonucleotide elongation (Ac_2O/THF/2,6-lutidine + 10% *N*-methylimidazole/THF). This undesired acetylation had previously been observed during the synthesis of prooligonucleotides with 2,2-bis(2-nitrophenyl)ethoxycarbonyl groups as nucleobase protections,[19] although this side reaction was not reported in early works on allyloxycarbonyl protection of nucleobases.[22,23]

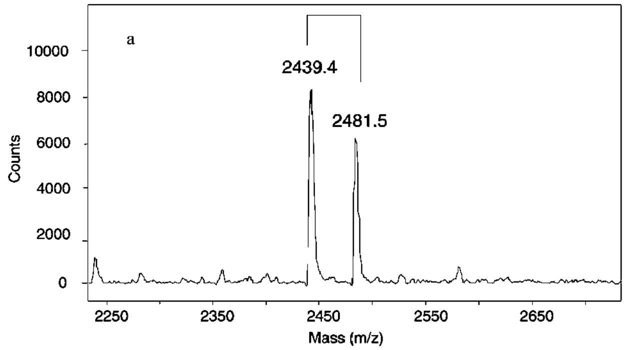

Figure 6. MALDI-TOF analysis of the still anchored prooligonucleotide **C** using Ac_2O for the capping step during oligonucleotide elongation

To circumvent this problem, the Ac_2O capping treatment was replaced with the use of diallyl *N*,*N*-diisopropylphosphoramidite (**6**) in the presence of tetrazole under standard phosphoramidite chemistry conditions. Use of this phosphoramidite reagent has been reported for the phosphorylation of hydroxy groups of nucleosides and peptides,[34] but never for capping reaction during oligonucleotide synthesis. It may be noted, however, that phosphoramidite chemistry has already been used for capping reagents.[19,35,36]

This phosphoramidite capping had another advantage, since the release of the allyl moieties under palladium treatment conditions results in the formation of hydrophilic 5′-monophosphate failure sequences, permitting their easy separation from the full length lipophilic prooligonucleotide. To demonstrate the utility of this procedure, the synthesis of d(CTC_3T) MeSATE (**D**) was performed with the use of only 4 mol-equiv. in the last 5′-dC phosphoramidite incorporation, instead of the 18 mol-equiv. required in a regular synthesis. Part of the material was then either treated or not treated with this new capping reagent **6**. After AOC deprotection and UV release of the prooligonucleotide from the solid support, RP-HPLC analysis of the crude material resulting from the synthesis without capping showed only one broad peak (t_R = 33.96 min, Figure 7a). In contrast, MALDI-TOF analysis of this peak (Figure 7a′) showed two main signals corresponding to the desired prooligonucleotide d(CTC_3T) (**D**) (*m*/*z* calcd. 2395.1) and to the shorter prooligonucleotide d(TC_3T) (**E**) (*m*/*z* calcd. 2003.7), resulting from incomplete coupling. Thus, without capping, it was not possible to distinguish the desired prooligonucleotide **D** from its failure sequence **E** by HPLC. On the other hand, RP-HPLC analysis of the synthesis with the capping step showed two peaks (Figure 7b), which were assigned by MALDI-TOF mass spectrometry (Figure 7b′ and b"). The desired full-length prooligonucleotide **D** was the compound with the higher retention time (t_R = 33.96 min). The other peak, with the lower retention time (t_R = 30.19 min), corresponded to the 5′-phosphate d(TC_3T) pMeSATE (**F**) (*m*/*z* calcd. 2083.7) contaminated with a small amount of full-length d(CTC_3T) pMeSATE (**D**) with one missing MeSATE group (− C_4H_6OS, − 102 Da) (Figure 77b").

This demonstrates that the use of diallyl *N*,*N*-diisopropylphosphoramidite (**6**) as a capping reagent for the synthesis of prooligonucleotides avoids the formation of acetylation by-products found with standard capping and yields crude materials consisting of the full-length desired oligonucleotide and hydrophilic capped species.

Synthesis of Chimeric Prooligonucleotide Heteropolymers

Replacement of all internucleosidic phosphodiesters of oligonucleotides by MeSATE phosphotriester linkages in prooligonucleotides decreases the aqueous solubility of these analogs. Fully masked dT prooligonucleotides (12mers) were thus poorly soluble in water.[2] Such excessively high hydrophobicity prompted us to synthesize mixed phosphodiester/MeSATE phosphotriester prooligonucleotides, so as to obtain compounds with sufficient lipophilicity for cell uptake and enough hydrophilicity for water solubility. The synthesis of such dT prooligonucleotide chimeras has been described previously.[37] In that work, phosphoramidite[12] and H-phosphonate[38] chemistries were

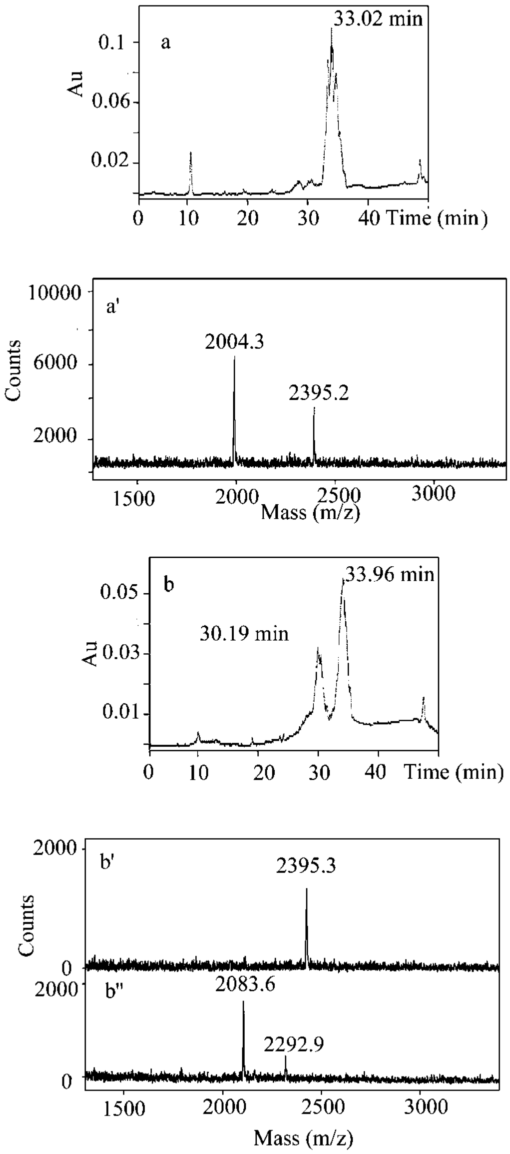

Figure 7. HPLC analyses of the crude mixtures resulting from the synthesis of the prooligonucleotide **D**: a) without capping step during the elongation process; b) with a capping step performed with diallyl *N*,*N*-diisopropylphosphoramidite (**6**); MALDI-TOF analyses of a′) the main peak (t_R = 33.02 min) resulting from the synthesis without capping step during the elongation process, b′) and b″) of the peaks (t_R = 33.96 and 30.19 min) resulting from the synthesis with diallyl *N*,*N*-diisopropylphosphoramidite capping

used to generate MeSATE phosphotriester and phosphodiester linkages, respectively. This method was subject to some constraints due to changes during the oligonucleotide elongation of specific reagents (i.e., activator, oxidant, capping agent) for each chemistry. In this work, phosphoramidite chemistry alone was used to create those chimeras. MeSATE phosphoramidites **1−4** were used to generate MeSATE phosphotriester linkages and allyl phosphoramidites[21] **7−10** were used to yield allyl phosphotriester linkages, which gave rise to phosphodiester linkages under Pd^0 conditions. To illustrate this strategy, we prepared a dodecanucleotide d(ACACCCAATTCT) (**G**), containing 6 phosphodiester internucleosidic linkages alternating with 5 MeSATE phosphotriester linkages with a 3′-MeSATE phosphodiester moiety. The efficiency of the elongation process was determined after removal of the 5′-*O*-DMTr groups, by standard 4,4′-dimethoxytrityl carbocation titration; the average incorporation yield was 98.1%. After elongation of the oligonucleotide under the conditions established above, the allylic protecting groups were removed under our new deprotection conditions. Finally, the prooligonucleotide **G** was released from the solid support by photolysis, purified by reverse-phase HPLC (Figure 8a and b), and analyzed by MALDI-TOF mass spectrometry (Figure 8b′). The resulting spectrum showed a major signal in agreement with the expected mass of **G** (*m*/*z* calcd. 4241.3). Another signal of lower intensity at *m*/*z* 4141.8 was attributed to a slight MeSATE loss (− 102 Da).

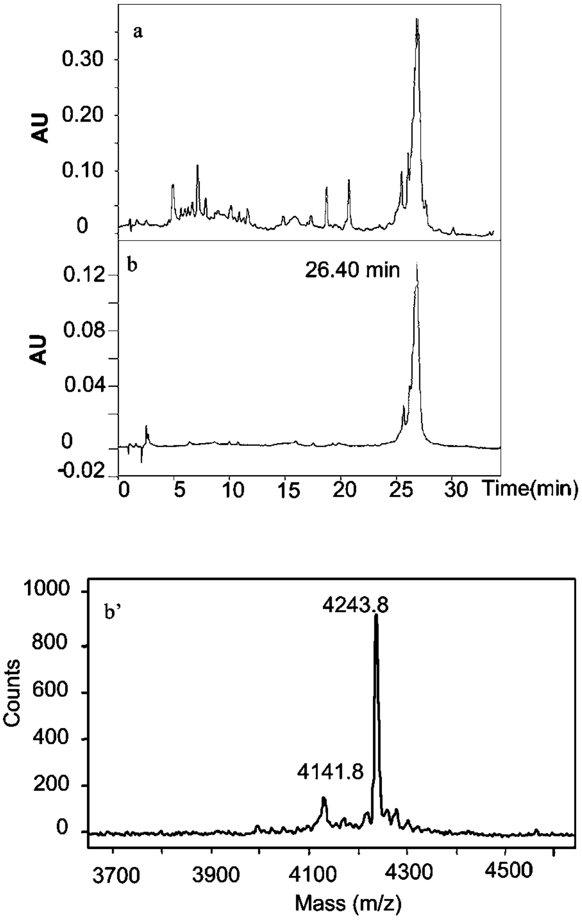

Figure 8. a) HPLC analysis of the crude mixture resulting from the synthesis of the prooligonucleotide **G**; b) HPLC analysis and b′) MALDI-TOF analysis of purified prooligonucleotide **G**

Conclusion

We have shown that use of allylic protecting groups combined with an anchored photolabile solid support allowed mixed prooligonucleotides of various sequences to be synthesized by phosphoramidite chemistry techniques. Allylic protecting groups were efficiently removed by use of dimedone as allyl scavenger, without affecting the MeSATE phosphotriester internucleosidic linkages. During the elongation process on solid support, the use of diallyl *N*,*N*-diisopropylphosphoramidite as the capping reagent allowed the desired prooligonucleotides to be easily separated from failure sequences. Furthermore, it was demonstrated that the solid-supported prooligonucleotides prepared in such a way could be directly analyzed by MALDI-TOF mass spectrometry, providing information about solid-phase chemistry in the same way that thin layer chromatography does for reactions in solution.

Experimental Section

General Remarks: All commercial chemicals were reagent grade and were used without further purification. DNA synthesis reagents, except for the oxidizer, were from Applied Biosystems Inc. (Voisins le Bretonneux, France). Anhydrous *tert*-butyl hydroperoxide (5.5 M in decane) was obtained from Fluka and was diluted with anhydrous dichloromethane (Aldrich). Triphenylphosphane, diallyl(diisopropylamino)phosphane (both under the names of “phosphines”) tris(dibenzylideneacetone)dipalladium(0)−chloroform complex, and dimedone were obtained from Aldrich. 5′-*O*-(4,4′-Dimethoxytrityl)-*N*-allyloxycarbonyl-2′-deoxynucleosides were prepared as previously described.[21,30]

[2-(Acetylthio)ethyloxy]bis(diisopropylamino)phosphane (5): Chlorobis(diisopropylamino)phosphane (8 g, 30 mmol) was added dropwise at 0 °C to a solution of 2-(acetylthio)ethanol (4.5 g, 37.5 mmol) and Et$_3$N (10.5 mL, 75 mmol) in diethyl ether (100 mL). The resulting mixture was stirred at room temperature for 4 h, diluted with an Et$_2$O/Et$_3$N mixture (9:1, v/v; 40 mL), and then filtered. The filtrate was concentrated until a viscous oil was obtained. Cyclohexane (20 mL) was added, and the crude mixture was purified by flash column chromatography [silica gel; elution with cyclohexane/triethylamine (9:1, v/v)]. The appropriate fractions were combined and the solvents were evaporated to dryness to give **5** as an oil (6 g, 57%). ^{31}P NMR (CD$_3$CN): δ = 125.68. ^{1}H NMR (CD$_3$CN): δ = 1.15 [d, 24 H, 4 CH(C*H*$_3$)$_2$], 2.31 (s, 3 H, CH$_3$), 3.09 (t, 2 H, OCH$_2$C*H*$_2$S), 3.44−3.68 [m, 6 H, OC*H*$_2$CH$_2$S, 4 (CH$_3$)$_2$C*H*N].

General Procedure for the Phosphitylation of *N*-Allyloxycarbonyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxynucleosides: Prior to use, the 5′-*O*-DMTr-*N*-AOC-2′-deoxynucleosides (2 mmol) and diisopropylammonium tetrazolide (171 mg, 1 mmol) were separately dried three times by coevaporation with anhydrous acetonitrile, and then mixed and dissolved in anhydrous dichloromethane (15 mL). A solution of [2-(acetylthio)ethyloxy]bis(diisopropylamino)phosphane (**5**) (841 mg, 2.4 mmol) in dichloromethane (5 mL) was added under argon. The resulting mixture was stirred overnight at room temperature, and then diluted with ethyl acetate (100 mL) and washed with saturated aqueous NaHCO$_3$ (50 mL) and then with brine (50 mL). The organic layer was dried (Na$_2$SO$_4$) and filtered, and the solvents were evaporated to dryness. The residues were purified by flash column chromatography (silica gel; gradient 30−100% ethyl acetate/1% Et$_3$N/cyclohexane). The appropriate fractions were combined, concentrated to dryness, dissolved in a few mL of toluene, and then precipitated in cold hexane (−78 °C). The resulting precipitate was dried under vacuum to afford the resulting phosphoramidites **2** (1.54 g, 89%), **3** (1.44 g, 81%), or **4** (1.40 g, 74%), as colorless powders.

4-*N*-Allyloxycarbonyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxycytidine 3′-*O*-{[2-(Acetylthio)ethyl] *N*,*N*-diisopropylphosphoramidite} (2): ^{31}P NMR (CD$_3$CN): δ = 148.93, 149.20. ^{1}H NMR (CD$_3$CN): δ = 0.97−1.23 [m, 12 H, 2 CH(CH$_3$)$_2$], 2.20−2.30 (m, 4 H, CH$_3$COS, H-2''), 2.41−2.59 (m, 1 H, H-2′), 2.97 (m, 2 H, OCH$_2$C*H*$_2$S), 3.36 (m, 2 H, H-5′, H-5''), 3.49−3.61 [m, 4 H, 2 C*H*(CH$_3$)$_2$, OC*H*$_2$CH$_2$S], 3.73 (s, 6 H, 2 OCH$_3$), 4.09 (m, 1 H, H-4′), 4.60 (3 H, H-3′, CH$_2$=CH−C*H*$_2$-), 5.19−5.37 (m, 2 H, C*H*$_2$=CH−CH$_2$), 5.89−6.07 (m, 1 H, CH$_2$=C*H*−CH$_2$), 6.08 (m, 1 H, H-1′), 6.81−6.85 (m, 5 H, H-5, 4 H DMTr), 7.13−7.43 (m, 9 H, DMTr), 8.15 and 8.19 (2 d, 1 H, H-6). HRMS: calcd. for C$_{44}$H$_{55}$N$_4$O$_{10}$PS 861.3298, found 861.3318. FABMS (negative mode, thioglycerol): *m*/*z* = 861 [M − H]$^-$.

6-*N*-Allyloxycarbonyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyadenosine 3′-*O*-{[2-(Acetylthio)ethyl] *N*,*N*-diisopropylphosphoramidite} (3): ^{31}P NMR (CD$_3$CN): δ = 148.86, 149.05. ^{1}H NMR (CD$_3$CN): δ = 0.98−1.24 [m, 12 H, 2 CH(CH$_3$)$_2$], 2.21 (s, 3 H, CH$_3$COS), 2.45−2.61 (m, 1 H, H-2''), 2.91−3.16 (m, 3 H, OCH$_2$C*H*$_2$S, H-2′), 3.16−3.49 (m,2 H, H-5′, H-5''), 3.41−3.67 [m, 4 H, 2 C*H*(CH$_3$)$_2$, OC*H*$_2$CH$_2$S], 3.70 (s, 6 H, 2 OCH$_3$), 4.15 (m, 1 H, H-4′), 4.45 (m, 2 H, CH$_2$=CH−C*H*$_2$), 4.51−4.90 (m, 1 H, H-3′), 5.20−5.45 (m, 2 H, C*H*$_2$=CH−CH$_2$), 5.87−6.06 (m, 1 H, CH$_2$=C*H*−CH$_2$), 6.35 (m, 1 H, H-1′), 6.73 (m, 4 H, DMTr), 7.10−7.37 (m, 9 H, DMTr), 8.20 (s, 1 H, H-2), 8.50 (s, 1 H, H-8). HRMS: calcd. for C$_{45}$H$_{54}$N$_6$O$_9$PS 885.3411, found 885.3441. FABMS (negative mode, thioglycerol): *m*/*z* = 885 [M − H]$^-$.

6-*O*-Allyl-2-*N*-allyloxycarbonyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxyguanosine 3′-*O*-{[2-(Acetylthio)ethyl] *N*,*N*-diisopropylphosphoramidite} (4): ^{31}P NMR (CD$_3$CN): δ = 148.83, 148.97. ^{1}H NMR (CD$_3$CN): δ = 0.85−1.38 [m, 12 H, 2 CH(CH$_3$)$_2$], 2.26 (s, 3 H, CH$_3$COS), 2.49−2.69 (m, 1 H, H-2''), 2.90−3.20 (3 H, OCH$_2$C*H*$_2$S, H-2′), 3.21−3.47 (m, 2 H, H-5′, H-5''), 3.49−3.72 [m, 4 H, 2 C*H*(CH$_3$)$_2$, OC*H*$_2$CH$_2$S], 3.78 (s, 6 H, 2 OCH$_3$), 4.16−4.25 (m, 1 H, H-4′), 4.68 (2 H, CH$_2$=CH−C*H*$_2$), 4.89 (m, 1 H, H-3′), 5.09 (2 H, CH$_2$=CH−C*H*$_2$), 5.25−5.55 (m, 4 H, 2 C*H*$_2$=CH−CH$_2$), 5.98−6.05 (m, 1 H, CH$_2$=C*H*−CH$_2$), 6.17−6.24 (m, 1 H, CH$_2$=C*H*−CH$_2$), 6.40 (m, 1 H, H-1′), 6.69−6.78 (m, 4 H, DMTr), 7.20−7.45 (m, 9 H, DMTr), 8.01 (s, 1 H, H-8), 8.22 (br s, 1 H, NH). HRMS: calcd. for C$_{48}$H$_{58}$N$_6$O$_{10}$PS 941.3672, found 941.3708. FABMS (negative mode, thioglycerol): *m*/*z* = 941 [M − H]$^-$.

Solid-Phase Elongation of Prooligonucleotides: The prooligonucleotides were synthesized with an ABI 394 DNA synthesizer on a 1-μmol scale, by a cycle involving phosphoramidite chemistry. An 18-fold molar excess of the nucleoside phosphoramidites **1**−**4** and **7**−**10** (0.09 M in CH$_3$CN) in the presence of tetrazole (0.45 M in CH$_3$CN) was introduced onto the column containing the photolabile CPG solid support (coupling time of 60 s).[13] A modification to the standard cycle was the replacement of the common capping step by treatment with diallyl *N*,*N*-diisopropylphosphoramidite (**6**) (0.1 M in acetonitrile) in the presence of 0.45 M tetrazole solution in acetonitrile (capping time of 3 × 2 min for the first coupling and 15 s for the others). The intermediate phosphite triesters were oxidized by treatment (60 s) with a 1.1 M solution of *tert*-butyl hydroperoxide[31,32] in toluene/dichloromethane to afford phosphotriester internucleosidic linkages. The coupling efficiency was evaluated by spectrophotometric measurement (λ = 498 nm) of the release of the 4,4′-dimethoxytrityl carbocation at the end of each incorporation cycle. Yields for couplings were > 98%.

Deprotection and Release of the Prooligonucleotides from the Solid Support: At the end of the elongation process, the columns were washed with argon-flushed THF and then disassembled. The supported prooligonucleotides (15 mg) were treated at 50 °C for 0.5−1 h with a THF solution of tris(dibenzylideneacetone)dipalladium(0)−chloroform complex [$Pd_2(dba)_3 \cdot CHCl_3$] (2.5 equiv./allylic group), triphenylphosphane (25 equiv./allylic group), and a large excess of allyl scavenger (0.6 M final concentration, 250 equiv./allylic group). The supports were then washed with THF, acetone, and finally with an aqueous solution of sodium *N*,*N*-diethyldithiocarbamate (ddtc) at pH = 6.7. The CPG-supported prooligonucleotides were suspended in a 1-cm path length quartz cell in acetonitrile/water (1:1, v/v; 1 mL) and the magnetically stirred suspensions were exposed to the light of a high-pressure Hg lamp (HPK 125, Philips), filtered with a Pyrex glass (thickness 2 mm), for 25 min at 20 °C. The glass beads were filtered off on a 0.45 µm Millex filter and washed with the same solvent mixture (2 × 0.5 mL). The prooligonucleotides were purified by reverse phase HPLC on a Waters-Millipore instrument equipped with a Model 600 E solvent delivery system, a Model U6 K injector, and an ND Model 486 absorbance detector. A reverse-phase C_{18} (5 µm) Nucleosil column (150 × 4.6 mm, Macherey−Nagel) was used with linear gradients of acetonitrile (0−90%) in 0.05 M aqueous triethylammonium acetate (pH = 7). The appropriate fractions were concentrated and redissolved in water/dioxane (1:1, v/v), and the solvents were removed. This last operation was repeated twice. The residues were then redissolved in water/dioxane and lyophilized to afford colorless powders.

MALDI-TOF MS Analysis: MALDI-TOF mass spectra were recorded with a Voyager DE mass spectrometer (Perseptive Biosystems) equipped with an N_2 laser (337 nm). MALDI conditions were: accelerating voltage 24000V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of the accelerating voltage; delay extraction time, 550 ns. For direct analysis of the CPG-supported prooligonucleotides, the solid materials (0.2 mg) were suspended in 5−10 µL of a saturated solution of the matrix 2,4,6-trihydroxyacetophenone[39] (THAP, 45 mg, ammonium citrate, 4 mg) in 500 µL of acetonitrile/water (1:1, v/v). One µL of the mixture was spotted onto the stainless steel probe plate and allowed to air-dry before analysis. For analysis of prooligonucleotides released from the solid support, 1 µL of the samples [0.1 OD_{260nm} in 100 µL water/acetonitrile (1:1, v/v)] was exchanged on DOWEX 50 W X8 resin (ammonium form) prior to the addition of the THAP matrix solution (5−10 µL). 1 µL of the resulting solution was then spotted onto the stainless steel probe plate and allowed to air-dry before analysis.

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