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Synthesis of DNA oligonucleotides containing 5-(mercaptomethyl)-2′-deoxyuridine moieties

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Abstract—Recently thiolated oligonucleotides have attracted significant interest due to their ability to efficiently undergo stable bond formation with gold nanoparticles and surfaces to form DNA conjugates. In this respect we became interested in the synthesis of oligonucleotides that bear short thioalkyl functions located at the nucleobase. Here we present a strategy for the synthesis of DNA oligonucleotides that bear 5-(mercaptomethyl)-2′-deoxyuridine moieties. The building blocks were synthesized in a straightforward manner from thymidine. Only moderate changes of standard protocols for automated DNA synthesis are required for the generation of modified oligonucleotides containing the thiolated building blocks.

1. Introduction

Efficient chemical modification of DNA and RNA oligonucleotides is the basis for numerous subsequent studies like conjugation of reporter molecules, investigation of biological systems or immobilization on surfaces.[1,2] Among the numerous functionalities conjugated to DNA, thiol moieties are of particular interest, for example, for conjugation of reporter groups, immobilization of DNA oligonucleotides on gold surfaces or nanoparticles.[1–7] In most current studies thioalkyl functions are coupled to the 5′- or 3′-end of the oligonucleotides, respectively, employing thioalkylated phosphoramidites or solid supports.[8,9] Recently, oligonucleotides that were thioalkylated at the 3′-terminus were employed in studies dedicated to directly measure electrical transport through single DNA molecules.[10,11] To fulfill these tasks, respective oligonucleotide duplexes were equipped with thioalkyl functions and employed to form double stranded DNA (dsDNA) bridges between two gold contacts. In this respect we became interested in the synthesis of oligonucleotides that bear short thioalkyl functions in close proximity to the π-system of the nucleobase to facilitate electrical transport through dsDNA. Thus, our first aim was the synthesis of DNA oligonucleotides that bear 5-(mercaptomethyl)-2′-deoxyuridine moieties (Fig. 1). We intended to develop a strategy that allows positioning of the thiolated building blocks at the 3′- or 5′-terminal nucleotide or at internal locations to ensure maximum flexibility.

Recently, a route for the synthesis of oligonucleotides containing 5-(mercaptomethyl)-2′-deoxyuridine moieties was developed.[12] The reported approach harbors several disadvantages like the usage of organomercury species and comparatively expensive starting materials. Here we present an improved and concise strategy for the synthesis of oligonucleotides that bear 5-(mercaptomethyl)-2′-deoxyuridine moieties in principle at any desired position. The herein depicted approach relies on the selective activation of the 5-methyl group of thymidines through bromination and the subsequent nucleophilic replacement of bromide by thioacetate.

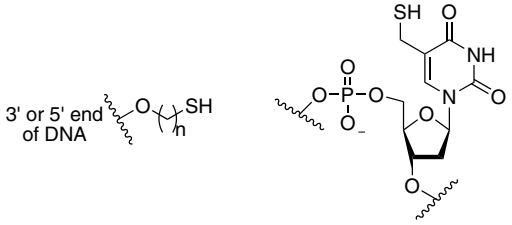

Figure 1. Thioalkyl functions conjugated to the 5′- or 3′-end of DNA (left), 5-(mercaptomethyl)-2′-deoxyuridine moieties incorporated into DNA oligonucleotides.

Keywords: DNA; Thiol functions; Conjugation; Oligonucleotide; Nucleoside; Phosphoramidite; Gold.
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2. Results and discussion

2.1. Nucleoside synthesis

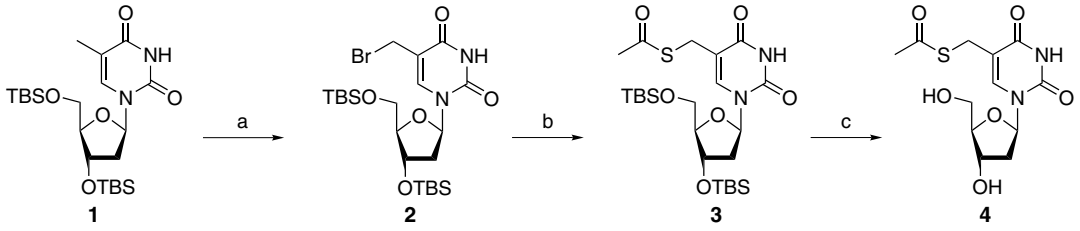
Our synthesis of suitable building blocks for the synthesis of modified DNA oligonucleotides started with the 3′,5′-bis-*O*-(*tert*-butyl dimethylsilyl) protected thymidine derivative **1**[13] that was easily accessible from thymidine in high yields (Scheme 1).

Radical bromination in the benzylic position was achieved with *N*-bromo succinimide (NBS) and 2,2′-azo-bis-isobutyronitrile (AIBN) at elevated temperature. The resulting bromide **2** turned out to be rather labile and was thus used crude for further transformation after filtration of the reaction mixture. Substitution of bromide with potassium thioacetate afforded the protected 5-(mercaptomethyl)-2′-deoxyuridine analog **3**. Selective 3′,5′-bis-O-desilylation was achieved smoothly to yield **4** under acidic conditions employing a 4:1 mixture of acetic acid (75%) and THF. Noteworthy, attempts to synthesize **4** directly from thymidine without any protection of the nucleoside failed as well as cleavage of the silyl ethers of **3** employing tetrabutyl ammonium fluoride (TBAF).

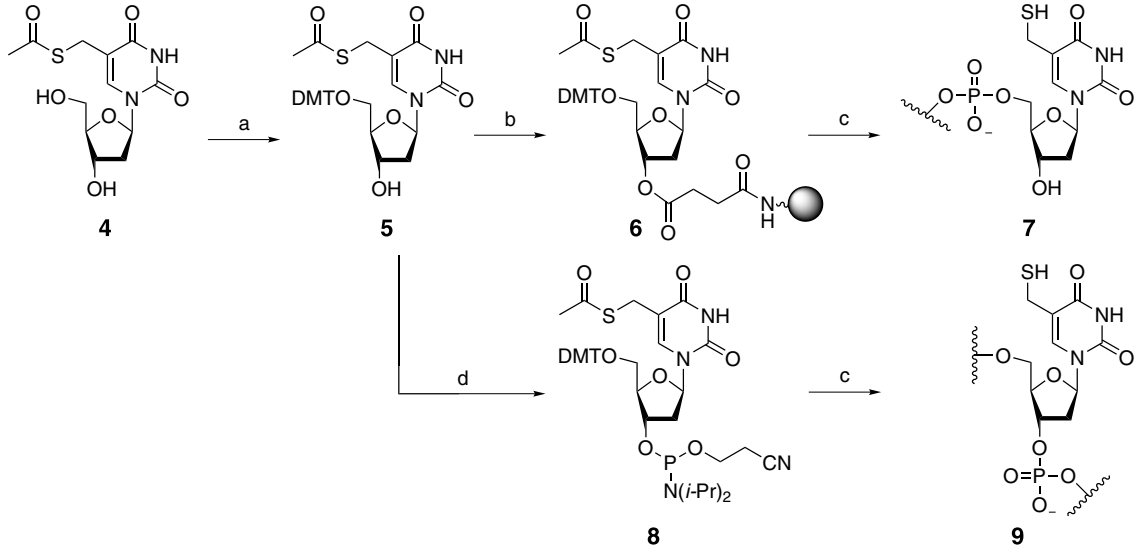
2.2. Synthesis of modified oligonucleotides

Next modified nucleoside **4** was converted into building blocks suited for automated DNA synthesis (Scheme 2).

Thus, **4** was 5′-O-protected with 4,4′-dimethoxytrityl (DMT) employing standard conditions to yield **5** in very good yields. In order to obtain oligonucleotide strands **7** that bear modified thymidine residues at the 3′-terminal position, **5** was coupled to solid support to afford **6**. Employing solid support **6** oligonucleotides **7** were synthesized using standard β-cyanoethyl phosphoramidites and coupling conditions. However, the deprotection protocol had to be adjusted: After completion of the synthesis, solid supports were first treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and afterwards with concentrated ammonia in the presence of 1,4-dithiothreitol (DTT). In order to synthesize oligonucleotides that bear the 5-thiolated thymidine moiety at the 5′-terminal position or at an internal position, phosphoramidite **8** was synthesized by phosphitylation of **5** in good yields. Employing **8** oligonucleotides **9** were synthesized that contain the modified building block at the 5′-terminal position or within the DNA strand. Table 1 lists the synthesized oligonucleotides and provides the analytical



Scheme 1. Synthesis of 5-(mercaptomethyl)-2′-deoxyuridine. Reagents and conditions: (a) NBS, AIBN, CCl_4, reflux, 2.5 h; (b) potassium thioacetate, DMF, 1.5 h, (39%, over two steps); (c) AcOH, THF, 24 h (73%).



Scheme 2. Synthesis of DNA oligonucleotides containing 5-(mercaptomethyl)-2′-deoxyuridines. Reagents and conditions: (a) DMT-Cl, DMAP, pyridine, 0 °C, 24 h, (90%); (b) EDC, DMAP, succinylated LCAA-CPG, pyridine; then 4-nitrophenol; then piperidine, then acetic anhydride/pyridine/THF (cap A) and 1-methylimidazole/THF (cap B); (c) i—oligonucleotide synthesis, ii—DBU, then 33% NH_4OH, 0.2 M DTT; (d) $[(^iPr_2N)(NCCH_2CH_2O)P]Cl$, NEt_3, CH_2Cl_2, rt, 4 h, (66%).

Table 1. Synthesized DNA oligonucleotides

	Sequence	Calcd mass $[M-H]^-$	Found mass $[M-H]^-$
S1	5′-TTT TTT TTT TTT*-3′	3619.4	3619.6
S2	5′-CGT TGG TCC TGA AGG AGG AT*-3′	6244.1	6243.7
S3	5′-CGT TGG TCC T*GA AGG AGG AT-3′	6244.1	6246.8
S4	5′-T*CG TTG GTC CTG AAG GAG GAT-3′	6548.3	6551.1

T*, 5-(mercaptomethyl)-2′-deoxyuridine.

data obtained by electrospray ionization mass spectrometer (ESI-MS).

3. Conclusions

Acetylated 5-(mercaptomethyl)-2′-deoxyuridine **4** was synthesized in good yields in a straightforward synthesis from thymidine. Compound **4** was subsequently converted into suitable building blocks for the automated synthesis of DNA oligonucleotides that contain the modified residues at desired positions. Through only moderate changes of standard protocols for automated DNA synthesis the generation of modified oligonucleotides containing the thiolated building blocks was feasible. Currently, we are investigating the properties of the synthesized oligonucleotides for immobilization on gold surfaces.

4. Experimental

4.1. General

All temperatures quoted are uncorrected. All reagents are commercially available and used without further purification. Solvents are purchased over molecular sieves (Fluka) and used directly without further purification unless otherwise noted. All reactions were conducted under rigorous exclusion of air and moisture. Elemental analysis was carried out by the microanalysis facility of the University of Konstanz. NMR spectra were recorded on Bruker AC 250 Cryospec (^{1}H: 250 MHz), Jeol JNA-LA-400 (^{1}H: 400 MHz, ^{13}C: 100 MHz), and Bruker DRX 600 (^{1}H: 600 MHz, ^{13}C: 150 MHz). Chemical shifts are given in parts per million (δ) relative to the residual solvent signal. MALDI-TOF mass spectra were recorded on a Kompact MALDI II mass spectrometer (Kratos Analytical) in positive, linear mode with delayed extraction MALDI source and a nitrogen laser (337 nm). ESI-IT mass spectra were recorded on a Bruker Daltonics esquire 3000+ in positive or negative mode with a flow rate of 3 μl/min. DNA oligonucleotides were synthesized on an Applied-Biosystems 392 DNA/RNA-synthesizer employing a standard phosphoramidite strategy. Flash chromatography: Merck silica gel G60 (230–400 mesh). Thin-layer chromatography: Merck precoated plates (silica gel 60 F_{254}). Reversed-phase HPLC was performed on a prominence-line HPLC (Shimadzu) with a Nucleosil-100-5-(250/4)-C18-column from Macherey-Nagel and a binary gradient system (TEAA-buffer (0.1 M), acetonitrile).

4.1.1. 5-Bromomethyl-3′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-2′-deoxyuridine (2). To a solution of **1**[13] (1.00 g, 2.13 mmol) in dry CCl_4 (20 ml) under argon atmosphere NBS (567 mg, 3.18 mmol) and AIBN (35 mg, 0.21 mmol) were added and heated at reflux for 2.5 h. The brownish reaction mixture was then filtered through a sintered glass frit (D4) to remove the succinimide and evaporated to dryness. Compound **2** was used immediately without further purification.

4.1.2. *S*-Acetyl-3′,5′-bis-*O*-(*tert*-butyldimethylsilyl)-5-(mercaptomethyl)-2′-deoxyuridine (3). To a solution of crude **2** in dry DMF (20 ml) under argon atmosphere potassium thioacetate (1.00 g, 8.75 mmol) was added and heated to 75 °C for 1.5 h. The reaction mixture was diluted with CH_2Cl_2 (100 ml) and washed with water (3× 50 ml). The organic phase was dried with magnesium sulfate and evaporated to dryness. The product **3** was purified by column chromatography (petrol ether–ethyl acetate, 3:1) to a yield of 444 mg (39%, over two steps). ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.08 (s, 6H, $Si(CH_3)_2$), 0.12 (s, 6H, $Si(CH_3)_2$), 0.89 (s, 9H, $SiC(CH_3)_3$), 0.92 (s, 9H, $SiC(CH_3)_3$), 1.92–2.02 (m, 1H, H2′A), 2.22–2.23 (m, 1H, H2′B), 2.30 (s, 3H, $(CO)CH_3$), 3.75 (s, 2H, $CH_2$5), 3.79–3.84 (m, 2H, H5′A and H5′B), 3.92–3.98 (m, 1H, H4′), 4.41–4.46 (m, 1H, H3′), 6.28 (dd, 1H, J = 6.4 Hz and 6.4 Hz, H1′), 7,75 (s, 1H, H6), 9.70 (br s, 1H, NH); MS (MALDI-TOF, DHB): Calcd for $C_{24}H_{44}N_2O_6SSi_2$ $[M+Na]^+$: 567.24, $C_{24}H_{44}N_2O_6SSi_2$ $[M+K]^+$: 583.22. Found: 567.1, 583.1.

4.1.3. *S*-Acetyl-5-(mercaptomethyl)-2′-deoxyuridine (4). Compound **3** (440 mg, 0.81 mmol) was deprotected in a mixture (20 ml) of 75% acetic acid and THF (4:1). The reaction was monitored by TLC (CH_2Cl_2–methanol, 95:5). After complete conversion (24 h), the reaction mixture was evaporated to dryness and purified by column chromatography (CH_2Cl_2–methanol, 95:5) to yield 187 mg (73%) of **4**. ^{1}H NMR (250 MHz, MeOH-d_4) δ 2.07–2.23 (m, 2H, H2′A and H2′B), 2.35 (s, 3H, $(CO)CH_3$), 3.71–3.86 (m, 4H, H5′A, H5′B and SCH_2) 3.93 (dt, 1H, J = 3.7 Hz and 4.3 Hz, H4′), 4.41 (dt, 1H, J = 2.7 Hz and 4.3 Hz, H3′), 6.22 (dd, 1H, J = 6.7 Hz and 6.7 Hz, H1′), 8.00 (s, 1H, H6); Calcd for $C_{12}H_{16}N_2O_6S$: C, 45.56; H, 5.10; N, 8.86. Found: C, 45.33; H, 5.19; N, 8.87; MS (ESI-MS, MeOH): Calcd for $C_{12}H_{16}N_2O_6S$ $[M+H]^+$: 316.07, $C_{12}H_{16}N_2O_6S$ $[M+Na]^+$: 338.07. Found: 317.1, 338.1.

4.1.4. *S*-Acetyl-5′-*O*-(dimethoxytrityl)-5-(mercaptomethyl)-2′-deoxyuridine (5). A solution of **4** (180 mg, 0.57 mmol) in dry pyridine (10 ml) under argon atmosphere was cooled to 0 °C, dimethoxytritylchloride (212 mg, 0.63 mmol) and DMAP (7 mg, 0.06 mmol) were added. The reaction was kept at 0 °C for 24 h and then quenched with 1 ml methanol. The solution

was evaporated to dryness, dissolved in CH_2Cl_2 (10 ml), and washed with saturated $NaHCO_3$ solution. The organic phase was dried with magnesium sulfate, evaporated to dryness, and purified by column chromatography (1% Et_3N in ethyl acetate) to yield 317 mg (90%) of **5**. ^1H NMR (400 MHz, acetone-d_6): δ 2.06–2.09 (m, 2H, H2′A and H2′B), 2.20 (s, 3H, (CO)CH_3), 3.29–3.55 (m, 4H, H5′A, H5′B and SCH_2), 3.79 (s, 6H, OCH_3), 4.06 (dt, 1H, J = 4.0 Hz and 4.4 Hz, H4′), 4.51 (dt, 1H, J = 4.0 Hz and 6.0 Hz, H3′), 6.29 (dd, 1H, J = 6.6 Hz and 6.6 Hz, H1′), 6.89 (d, 4H, J = 9.0 Hz, Ar), 7.23 (t, 1H, J = 7.3 Hz, Ar), 7.32 (dd, 2H, J = 7.3 Hz and 7.5 Hz, Ar), 7.38 (d, 2H, J = 9.0 Hz, Ar), 7.39 (d, 2H, J = 9.0 Hz, Ar), 7.51 (d, 2H, J = 8.5 Hz, Ar), 7.77 (s, 1H, H6), 10.25 (br s, 1H, NH); ^{13}C NMR (100 MHz, acetone-d_6): δ 42.0 (C2′), 56.5 (OCH_3), 65.8 (C5′), 73.0 (C3′), 86.6 (C1′), 88.0 (C4′), 88.3 (CAr_3), 111.9 (C5), 115.0 (Ar), 127.2 (Ar), 128.7 (Ar), 129.8 (Ar), 130.1 (Ar), 130.9 (Ar), 132,1 (Ar), 132.1 (Ar), 137.8 (C6), 147.1 (C2) 160.8 (C4), 196.8 (CO); MS (ESI-MS, acetone): Calcd for $C_{33}H_{34}N_2O_8S$ $[M+H]^+$: 619.20, $C_{33}H_{34}N_2O_8S$ $[M+Na]^+$: 641.20, $C_{33}H_{34}N_2O_8S$ $[M+K]^+$: 657.18. Found: 619.1, 641.1, 657.1.

4.1.5. *S*-Acetyl-4′-*O*-(2-cyanoethoxy)(diisopropylamino)phosphino-5′-*O*-(dimethoxytrityl)-5-(mercaptomethyl)-2′-deoxyuridine (6). To a solution of **5** (100 mg, 0.162 mmol) in dry CH_2Cl_2 (5 ml) under argon atmosphere triethylamine (82 mg, 0.81 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite (77 mg, 0.32 mmol) were added. The reaction was monitored by TLC (petrol ether–ethyl acetate, 1:2). After complete conversion (4 h), the reaction mixture was evaporated to dryness and purified by column chromatography (1% Et_3N in petrol ether–ethyl acetate, 1:2) to yield 88 mg (66%) of **6**. ^1H NMR (600 MHz, acetone-d_6): δ 1.18 (d, 6H, J = 7.5 Hz, NCH(CH_3)), 1.20 (d, 6H, J = 7.5 Hz, NCH(CH_3)), 2.20 (s, 3H, (CO)CH_3), 2.36–2.48 (m, 2H, H2′A and H2′B), 2.64 (t, 2H, J = 5.9 Hz, CH_2CN), 3.44–3.48 (m, 2H, H5′A and H5′B), 3.62–3.68 (m, 2H, CH_25), 3.70–3.76 (m, 2H, N(CH)), 3.80 (s, 6H, OCH_3), 4.17–4.22 (m, 1H, H4′), 4.66–4.73 (m, 1H, H3′), 6.29 (dd, 1H, J = 6.6 Hz and 6.6 Hz, H1′), 6.91 (d, 4H, J = 9.0 Hz, Ar), 7.25 (t, 1H, J = 7.3 Hz, Ar), 7.34 (dd, 2H, J = 7.7 Hz and 7.7 Hz, Ar), 7.41 (d, 4H, J = 8.9 Hz, Ar), 7.53 (d, 2H, J = 7.3 Hz, Ar), 7.80 (s, 1H, H6), 10.16 (br s, 1H, NH); ^{13}C NMR (150 MHz, acetone-d_6): δ 20.6 (CH_2CN), 24.4 ($CH(CH_3)_2$), 26.5 (5-CH_2), 30.1 ((CO)CH_3), 40.0 (C2′), 43.9 (NCH), 44.0 (NCH), 55.0 (OCH_3), 64.1 (C5′), 74.0 (C3′), 85.7 (C1′), 86.1 (C4′), 87.3 (CAr_3), 110.9 (C5), 114.0 (Ar), 118.8 (CN), 127.6 (Ar), 128.7 (Ar), 129.1 (Ar), 131.1 (Ar), 136.6 (C6), 138.9 (Ar), 145.9 (Ar), 150.8 (C2), 159.7 (Ar), 162.9 (C4), 195.5 (CO); MS (ESI-MS, acetone): Calcd for $C_{42}H_{51}N_4O_9PS$ $[M+Na]^+$: 841.31, $C_{42}H_{51}N_4O_9PS$ $[M+K]^+$: 857.28. Found: 842.0, 857.9.

4.2. Synthesis of modified oligonucleotides

For the synthesis of oligonucleotides **S1** and **S2** solid-phase support **6** was used, in all other cases the modified phosphoramidite **8** (0.1 M in acetonitrile). The synthesis of oligonucleotides was carried out on a DNA-synthesizer on 0.2 μmol scale applying commercially available 2-cyanoethylphosphoramidites. A standard method for 2-cyanoethylphosphoramidites was used, with the exception that the coupling time from the modified nucleotides was extended to 10 min. Yields for modified oligonucleotides are comparable to those obtained for unmodified oligonucleotides. The synthesized oligonucleotide sequences are listed in Table 1. Following deprotection strategy was used: The solid-phase support was treated with 1 ml of 10% DBU in acetonitrile for 30 min followed by washing with 5 ml acetonitrile. The deprotection with ammonium hydroxide (33%) occurred in the presence of excess DTT (0.2 M) at 56 °C for 16 h. This deprotection procedure leads to the desired product and a minor product with a mass 120 Da higher, presumably the DTT-adduct, which can be easily separated by HPLC. The oligonucleotides were purified by RP-HPLC with a binary gradient system (A: 0.1 M TEAA-buffer and B: acetonitrile) and a gradient of 5–20% B over 25 min. Integrities of all modified oligonucleotides were confirmed by ESI MS.

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