2-(Phenylthio)ethyl as a Novel Two-Stage Base Protecting Group for Thymidine Analogues

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Abstract: 2-(Phenylthio)ethyl is here proposed for temporary masking the thymine residue in the synthesis of sugar modified thymidine derivatives. This protection has been devised as a 'two-stage' system. The 2-(phenylthio)ethyl residue can be easily and regiospecifically inserted at the N3-position of the pyrimidine by a Mitsunobu reaction with 2-(phenylthio)ethanol and is perfectly stable also to strongly basic conditions. This allowed us to selectively achieve O-alkylation of the ribose moieties in satisfactory yields, avoiding undesired base alkylations. After oxidation of the thioether to sulfone, the thymine protecting group can be totally removed, by a β-elimination mechanism, upon the same basic treatment required for the final deprotection and detachment of oligonucleotides from the support in solid-phase synthesis protocols.

Key words: protecting groups, Mitsunobu reactions, thymidine, oligonucleotides, solid-phase synthesis

Crucial issue in a convergent, multistep synthesis is a careful choice of the protecting groups to transiently mask potentially interfering functions. Since Khorana's precious pioneering work, several protocols have been developed and optimized in the last decades for the sequential addition of nucleotides to give an oligonucleotide sequence.[1] The most efficient methodologies for the oligonucleotide synthesis, i.e. the phosphoramidite[2] and the H-phosphonate[3] chemistry, routinely used in fully automated solid-phase synthetic processes, are both based on a unique protection strategy of the nucleotide monomers.

In order to obtain the regioselective formation of 3′→5′-phosphodiester linkages in the oligonucleotide chain, orthogonal protections are required for the 5′-OH groups, typically transiently blocked as acid-labile 5′-*O*-(4,4′-dimethoxytriphenylmethyl)ethers (DMT-ethers), and for the exocyclic amino groups of purines and pyrimidines. These nucleophiles are normally rendered innocuous by conversion into amides, then hydrolyzed at the end of the synthesis by the final basic treatment. No explicit protection is usually adopted for the thymine base, where the potential nucleophilic centers, i.e. the N3- and O4-positions, under normal conditions, interfere to a very marginal extent with the phosphodiester bond formation, either using phosphoramidite or H-phosphonate reactive intermediates. A survey of the literature revealed that only a few examples of thymine protections have been described. The most commonly used protecting group is the phenyl,[4] introduced in the thymidine O4-position by reaction of phenol with the activated 4-triazolyl intermediate. Mild basic conditions (typically an overnight oximate ions treatment) ensure their complete removal. To the best of our knowledge, there is only one example reported in the literature of alternative protection for thymine residues in oligonucleotide synthesis, involving N-acylation of the heterocycle with *p*-anisoyl chloride.[5] More stringent is the choice of the protecting groups for thymine and uracil nucleosides, in case a selective manipulation of the ribose functions is desired to obtain sugar-modified analogues. Benzyl, *p*-methoxybenzyl and benzyloxymethyl groups have been preferentially adopted to this purpose.[6]

In the course of our studies[7] on modified oligonucleotides, and particularly aiming at preparing the $^{5'}$TGGGAG$^{3'}$ sequence carrying the 3,4-dibenzyloxybenzyl (3,4-DBB) group at its 5′-end (Figure 1), discovered by Hotoda and his group to be endowed with a potent anti-HIV-1 activity,[8] we encountered serious synthetic problems in the alkylation of the thymidine monomer to be inserted in Hotoda's hexamer as the 5′-terminal nucleoside.

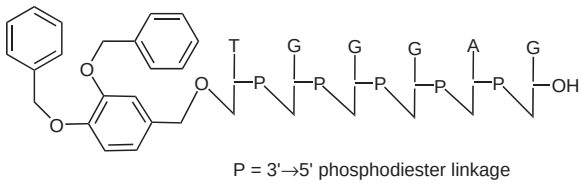

Figure 1

In the procedures described by Hotoda and co-workers,[9] the formation of the 5′-DBB-ether was achieved by reaction of DBB-bromide with 3′-*O*-TBDMS-thymidine in THF in the presence of NaH. The target molecule was finally obtained, even if in modest yields (23%) and, after TBDMS removal, phosphitylated to give the corresponding 3′-phosphoramidite derivative, used as building block in the automated oligonucleotide synthesis. When we repeated this procedure, with the sole difference of using the commercially available DBB-chloride in lieu of the bromide adopted by Hotoda, we always observed exclusively the formation of the N-alkylation product over the desired 5′-O-alkylation one, with the N3-linked DBB adduct obtained also in high yields. Several conditions were tested,

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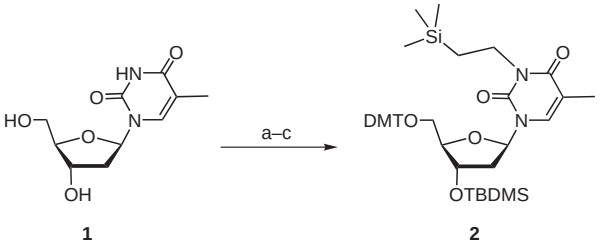
varying the solvent, temperature, activation and excess of the reagents, but in all cases we could isolate from the reaction mixtures only the N-alkylated thymidine or, when further forcing the reaction conditions, N3,O-bis-alkylated derivatives. In fact, an additional side reaction was observed in the DBB-ether formation starting from 3′-*O*-TBDMS-thymidine: the concomitant formation, in almost 1:1 ratio, of both the N3,O-5′- and the N3,O-3′-bis-alkylated derivative, as a result of the hydride-mediated migration of the TBDMS group from the 3′- to the 5′-position, which was also reported by Kobe et al.[10]

In order to get a deeper insight into the structure–activity relationships of this G-rich, quadruplex-forming hexamer and related analogues, the large scale preparation of these oligonucleotides was a prerequisite. In this frame, protection of the thymine base of the nucleoside was the necessary next step. However, all base-labile protecting groups for thymine seemed not appropriate in our case, nor in all those cases where specific transformations of the ribose moieties requiring quite strong basic media are desired. Therefore we have undertaken a more detailed study aimed at regiospecifically protecting thymidine on the heterocycle with a group satisfying the following criteria: i) facile, high-yielding and highly reproducible installation; ii) elevated stability to a wide range of different conditions, including strongly basic conditions, so allowing to differently manipulate the sugar functions; iii) high compatibility with the standard oligonucleotide synthesis conditions; iv) facile and total removal, in conditions not affecting the integrity of the 5′-DBB-ether function. The latter requirement led us to discard benzyl-type protecting groups, for their incomplete orthogonality with respect to the DBB group. Candidate protecting groups could therefore be 'safety catch' or 'two-stage' systems,[11] designed to be very stable in their native structure, but, after a simple and quantitative conversion into an 'activated' form, easily removable under mild conditions when required.

Our first attempts were carried out using as starting material 5′-*O*-DMT-3′-*O*-TBDMS-thymidine, as in Hotoda's synthetic scheme. We tested the 2-(trimethylsilyl)ethyl group as the protecting group for the thymine base, previously reported in the literature as a suitable protection for carboxyl and phosphate moieties,[12] which could be cleaved upon the same fluoride treatment required for TBDMS removal (Scheme 1). In our experiments, TBDMS proved to be unsuitable to protect the 3′-OH function, for its above-mentioned tendency to migrate to the 5′-OH under basic conditions. In addition, the 2-(trimethylsilyl)ethyl group in **2**[13,14] was found to be not completely cleaved even after repeated treatments with fluoride ions and was therefore finally discarded.

We then moved to a different protection strategy. Our previous results on the solid-phase immobilization of pyrimidine nucleosides through the base showed that regioselective N3-alkylation could be easily achieved under Mitsunobu conditions, allowing high incorporation yields of the nucleoside onto the solid matrix.[15] In order to ensure a facile and complete detachment of the nucleotide



1 2

Scheme 1 *Reagents and conditions*: a) DMTCl, DMAP, pyridine, 5 h, r.t. (97%); b) TBDMSCl, imidazole, DMF, 12 h, r.t. (95%); c) $(CH_3)_3SiCH_2CH_2OH$, PBu_3-ADDP in benzene, 18 h, 0 °C to r.t. (65%).

from the support, a suitable bifunctional linker was designed, bearing a 2-mercapto-ethanol moiety. After nucleoside incorporation and oligonucleotide chain assembly, the cleavage from this support involved two steps: oxidation with 3-chloroperbenzoic acid (MCPBA) of the sulfur atom to sulfone, followed by a standard aqueous ammonia treatment (Figure 2). We reckoned that a similar system could be profitably introduced as a new 'two-stage' protecting group for thymine base in nucleoside and oligonucleotide analogues synthesis.

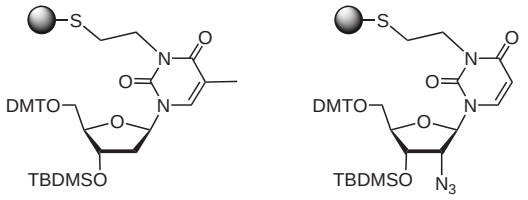
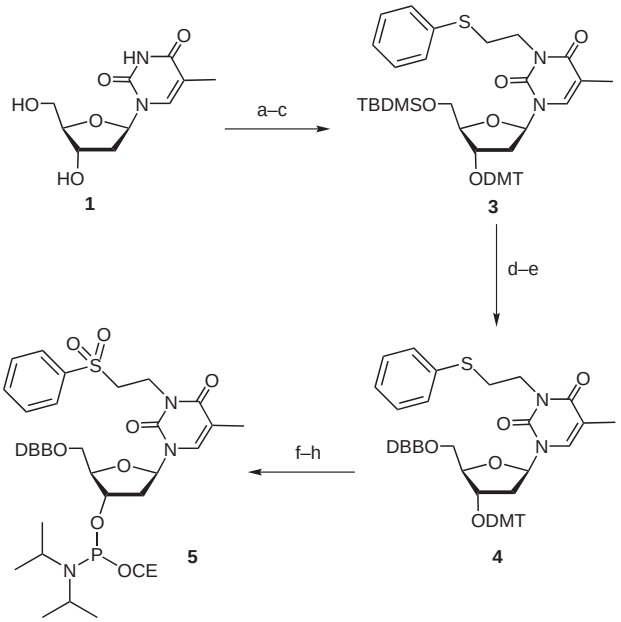


Figure 2

We therefore synthesized 5′-*O*-TBDMS-3′-*O*-DMT-thymidine as the starting material and reacted it with 2-(phenylthio)ethanol under standard Mitsunobu conditions. The 2-(phenylthio)ethyl group had been previously tested only for protection of phosphodiester functions in oligonucleotide synthesis through the phosphotriester chemistry.[16] To the best of our knowledge, its use for pyrimidine protection is unprecedented. The desired N3-alkylated nucleoside **3**[17,18] (Scheme 2) was obtained in 90% yields. Subsequent standard triethylammonium fluoride treatment furnished the 5′-OH derivative in almost quantitative yields, which was then reacted with DBBCl and NaH with cat. NaI, to give the desired 5′-DBB adduct **4** in 70% yields. In no case were side reactions observed and the unreacted nucleoside could be recovered intact after chromatography and recycled. Next, the thioether function was quantitatively oxidized with MCPBA to sulfone. This reaction, which in principle can be conveniently carried out also in a successive step, at the level of the already assembled oligonucleotide, was here realized on the monomer in solution in order to more directly control the 'two-stage' removal process: the efficiency of the crucial 'activation' of the protecting group and, then, of its base-induced scission from the heterocycle. Successively, addition of 3% TFA in CH_2Cl_2 cleanly liberated the 3′-OH

moiety, not affecting the 5′-DBB-ether function. The obtained nucleoside could then be phosphitylated by a standard reaction with 2-cyanoethyl, *N*,*N*-diisopropylchlorophosphoramidite and DIPEA in anhydrous CH_2Cl_2, to give **5**.[19] Starting from 5′-*O*-TBDMS-3′-*O*-DMT-thymidine, 3′-phosphoramidite building block **5** was prepared in five, easy synthetic steps and 48% overall yields (46% starting from thymidine).



Scheme 2 *Reagents and conditions*: a) TBDMSCl, imidazole, DMF, 5 h, r.t. (quant.); b) DMTCl, DMAP, pyridine, 12 h, r.t. (95%); c) $PhSCH_2CH_2OH$, PBu_3-ADDP in benzene, 18 h, 0 °C to r.t. (90%); d) 1 M TBAF in THF, 30 min, r.t. (98%); e) NaH, DBBCl, NaI, DMF, 18 h, r.t. (70%); f) MCPBA, CH_2Cl_2, 1 h, r.t. (98%); g) 3% TFA in CH_2Cl_2, 30 min, r.t. (94%); h) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, TEA, CH_2Cl_2, 30 min, r.t. (85%).

Assembly of the $^{5'DBB}$TGGGAG$^{3'}$ oligomer could then be achieved on an automated DNA synthesizer by exploiting standard phosphoramidite chemistry protocols. In all cases, DMT tests, monitoring the efficiency of the oligodeoxyribonucleotide (ODN) chain elongation, indicated yields not inferior to 98% for each coupling cycle. The last coupling was carried out using DBB-thymidine phosphoramidite **5** as the building block, in a standard DMT-on protocol.

Once the ODN chain assembly was complete, detachment from the solid support and full deprotection of the 5′-conjugated oligomer, including thymine protecting group removal, were achieved by treatment with 0.1 M NaOH.[20] After HPLC purification and desalting by gel filtration chromatography, the synthesized ODN was characterized by mass analysis.[21]

In conclusion, highly selective alkylation of the ribose moiety of thymidine could be finally achieved by introducing a new, two-stage protecting group on thymine, namely the 2-(phenylthio)ethyl group, which prevented otherwise predominant N-alkylation of the base. This protecting group is stable to a large variety of conditions, including strongly basic media. Only after oxidation of the thioether to sulfone, the system can fragment via a β-elimination mechanism, induced by 0.1 M NaOH treatment, basic conditions required also for the final deprotection and detachment of oligonucleotides from the solid support. The utility of this new protection was demonstrated through a more efficient synthesis of 5′-DBB-thymidine, which, after standard phosphitylation, was then used for the solid phase synthesis of anti-HIV-1 active Hotoda's hexamer. Application of this two-stage protecting group can offer easy and large scale synthetic access to a variety of ribose-modified thymidine derivatives and be profitably extended also to uridine nucleosides, allowing a wide set of O-alkylated analogues, including benzyl ether derivatives. These modified nucleosides can be of interest per se, for their biological activity, or as suitable building blocks for the synthesis of a number of sugar-modified oligonucleotides, attractive tools as potential antisense and/or antigene agents.

Acknowledgment

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References and Notes

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(13) **Selected Data for Compound 2.**
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.61–6.82 (13 H, complex signals, arom. protons), 6.38 (1 H, dd, H-1′, *J* = 6.2, 6.8 Hz), 4.49 (1 H, m, H-3′), 4.01–3.96 (3 H, overlapped signals, CH_2N and H-4′), 3.79 (6 H, s, OCH_3), 3.45 (1 H, dd, H-5′$_a$, *J* = 4.0, 12.0 Hz), 3.25 (1 H, dd, H-5′$_b$, *J* = 4.0, 12.0 Hz), 2.33–2.17 (2 H, complex signal, H_2-2′), 1.54 (3 H, s, CH_3 T), 0.97 (2 H, m, CH_2Si), 0.83 {9 H, s, $[(CH_3)_3C]Si$ of TBDMS group}, 0.07 [9 H, s, $(CH_3)_3Si$], –0.02 and –0.04 [3 H each, s, $Si(CH_3)_2$ of TBDMS group]. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 163.22 (C-4), 150.57 (C-2), 135.40 (C-6), 158.61, 144.28, 133.19, 129.95, 126.98, 113.15 (arom. carbons), 110.18 (C-5), 86.81 (quat. C of DMT group), 86.56 (C-1′), 85.35 (C-4′), 71.97 (C-3′), 62.82 (C-5′), 55.14 (OCH_3), 41.53 (C-2′), 37.96 [$(CH_3)_3SiCH_2CH_2N$], 25.59 {[$(CH_3)_3C$]Si of TBDMS group}, 17.81 [$(CH_3)_3C$ of TBDMS group], 15.87 [$(CH_3)_3SiCH_2CH_2N$], 12.54 (CH_3 T), –1.89 [$(CH_3)_3SiCH_2CH_2N$], –4.98 [$Si(CH_3)_2$ of TBDMS group]. MS: *m/z* calcd for $C_{42}H_{58}N_2O_7Si_2$: 758.38. ESI-MS found: *m/z* = 759.61 [M + H^+]; *m/z* = 781.53 [M + Na^+]; *m/z* = 797.50 [M + K^+].

(14) **Typical Procedure for the Insertion of 2-(Trimethylsilyl)ethyl Group: Synthesis of 2**
The amount of 1.0 g (1.52 mmol) of 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-3′-*O*-(*tert*-butyldimethylsilyl)thymidine was dissolved in 10 mL of benzene at 0 °C and treated with 160 μL (1.14 mmol) of 2-(trimethylsilyl)ethanol and 470 μL (1.90 mmol) of tributylphosphine. After 10 min the reaction mixture was taken to r.t. and treated with 480 mg (1.90 mmol) of 1,1′-(azodicarbonyl)dipiperidine (ADDP). After 18 h, the crude was taken to dryness, redissolved in 100 mL of EtOAc and washed twice with H_2O. The organic layer was concentrated under reduced pressure and purified by silica gel chromatography [eluent system: 2% MeOH in $CHCl_3$–pyridine (1:0.05)], affording 745 mg (0.98 mmol, 65%) of pure **2**.

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(17) **Selected Data for Compound 3.**
^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.47–6.85 (18 H, complex signals, arom. protons), 6.48 (1 H, dd, H-1′, *J* = 5.6, 9.6 Hz), 4.27 (1 H, d, H-3′, $J_{3',2'b}$ = 12.0 Hz), 4.22 (2 H, m, CH_2N), 4.01 (1 H, br s, H-4′), 3.82 (6 H, s, OCH_3), 3.68 (1 H, dd, H-5′$_a$, *J* = 4.0, 12.0 Hz), 3.29 (1 H, dd, H-5′$_b$, *J* = 4.0, 12.0 Hz), 3.19 (2 H, t, CH_2S, *J* = 8.0, 8.0 Hz), 1.87 (3 H, s, CH_3 T), 1.76 (1 H, dd, H-2′$_a$, *J* = 4.0, 12.0 Hz), 1.57 (1 H, complex signal, H-2′$_b$), 0.83 {9 H, s, $[(CH_3)_3C]Si$}, –0.02 and –0.08 [3 H each, s, $Si(CH_3)_2$]. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 162.83 (C-4), 150.37 (C-2), 135.86 (C-6), 158.29, 150.37, 144.70, 135.95, 135.28, 133.27, 129.87, 129.75, 128.51, 127.99, 127.86, 127.51, 126.63, 125.35, 112.83 (arom. carbons), 109.62 (C-5), 86.81 (C of DMT group), 86.16 (C-1′), 85.12 (C-4′), 74.55 (C-3′), 63.12 (C-5′), 54.80 (OCH_3), 40.59 (CH_2N), 39.55 (C-2′), 29.22 (CH_2S), 25.34 {[$(CH_3)_3C$]Si}, 17.77 [$(CH_3)_3C$], 12.68 (CH_3 T), –5.86 and –6.18 [$Si(CH_3)_2$]. MS: *m/z* calcd for $C_{45}H_{54}N_2O_7SSi$: 794.34. ESI-MS (positive ions) found: *m/z* 833.75 [M + K^+].

(18) **Typical Procedure for the Insertion of 2-(Phenylthio)-ethyl Group: Synthesis of 3**
The amount of 900 mg (1.37 mmol) of 5′-*O*-*tert*-butyldimethylsilyl-3′-O-(4,4′-dimethoxytriphenylmethyl)thymidine was dissolved in 10 mL of benzene at 0 °C and treated with 38 μL (1.02 mmol) of 2-(phenylthio)ethanol and 422 μL (1.71 mmol) of tributylphosphine. After 10 min the reaction mixture was taken to r.t., treated with 431 mg (1.71 mmol) of ADDP and left at r.t. for 18 h. The crude was then taken to dryness, redissolved in 100 mL of EtOAc and washed twice with H_2O. The organic layer was concentrated under reduced pressure and purified by silica gel chromatography [eluent system: 2% acetone in $CHCl_3$–pyridine (1:0.05)], affording 980 mg (1.23 mmol, 90%) of pure **3**.

(19) **Selected Data for Compound 5.**
^{31}P NMR (161.98 MHz, $CDCl_3$): 149.3. MS: calcd for $C_{48}H_{57}N_4O_{10}PS$: 912.35. ESI-MS (positive ions) found: *m/z* = 935.89 [M + Na^+]; *m/z* = 952.90 [M + K^+].

(20) Removal of the 2-(phenylsulfonyl)ethyl group by aq NH_3 at 55 °C after an overnight treatment was only partial, requiring longer reaction times to go to completion (ca. 72 h). On the other hand, a 0.1 M NaOH solution ensured complete thymine deprotection after an overnight treatment at 50 °C, i.e. conditions usually adopted also for oligonucleotide deprotection and detachment from the solid support.

(21) Data for $^{5'DBB}$**TGGGAG**$^{3'OH}$
MS: *m/z* calcd for $C_{81}H_{92}N_{27}O_{36}P_5$: 2173.49; MALDI-TOF (negative ions) found: 2171.76 [M – H]$^-$.