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Tetrahedron Letters
Tetrahedron Letters 48 (2007) 469–472

A base-stable dithiomethyl linker for solid-phase synthesis of oligonucleotides

Andrey Semenyuk[a] and Marek Kwiatkowski[a,b,*]

[a]*Department of Genetics and Pathology, Uppsala University, 75185 Uppsala, Sweden*
[b]*Quiatech AB, 75108 Uppsala, Sweden*

Received 14 September 2006; revised 1 November 2006; accepted 8 November 2006
Available online 4 December 2006

Abstract—A novel linkage, useful for the synthesis of oligonucleotides is described. The linking function is compatible with all conditions used for oligonucleotide synthesis, orthogonal to all other protecting groups, but regenerates 3′-OH rapidly upon mild reduction under aqueous conditions. This method is employed in the removal of depurinated fragments during the synthesis of oligonucleotides.

Oligonucleotides conjugated to other oligonucleotides, proteins, labels, haptens or separation tags by means of a linker that can be cleaved with liberation of the native oligonucleotide, have potential to find application in all fields of molecular biology. Solid-phase based methods for chemical synthesis are the most attractive routes for the synthesis of such conjugates. This requires, however, an access to a linkage that can withstand all reaction conditions for oligonucleotide synthesis and deprotection, being at the same time cleavable under mild and preferably aqueous conditions. Preferentially, the cleavage of the conjugate should liberate 3′-OH of the oligonucleotide, since it is often needed as a starting point for template-based DNA extension. These highly demanding conditions are not easy to realize. It is thus not surprising that amongst all existing linkages only photolabile[1] and silyl-based linkers[2] tend to approach these demands. However, photolabile functions often demand a long deprotection time, and are inadequate for application in sites which are nonaccessible by light. The cleavage of silyl-based functions proceeds under nonaqueous conditions, thus it is cumbersome and incompatible with most biological systems.

Recently, we reported the use of *tert*-butyldithiomethyl as a 2′-OH protecting group for solid-phase RNA synthesis.[3] Herein, a strategy for the conjugation of oligonucleotides via the 3′-*O*-dithiomethyl linkage is described and applied for the removal of depurinated oligonucleotides during the synthesis and purification of synthetic DNA.

Introduction of the dithiomethyl group onto a nucleoside was achieved via 5′-*O*-MMTr-3′-*O*-(methylthiomethyl)thymidine **1** obtained according to the published procedure[4] from 5′-*O*-MMTr-thymidine. This synthon, upon activation, reacted with 6-mercapto-1-hexanol, 3-mercapto-3-methyl-1-butanol[5] or 6-mercapto-6-methyl-1-heptanol (prepared according to the modified procedure)[6] to produce several nucleoside derivatives (**2**, **4**, **5**, and **6**) (Scheme 1). These nucleosides were converted to respective phosphoramidites (**3**, **7**, **8**, and **9**) bearing the dithiomethyl group. The stability of both nucleosides and nucleotide amidites was examined. It was found that tertiary alkyl substituents had pronounced stabilizing effects on the disulfide bond toward aqueous ammonia and iodine compared to the less stable primary analogues. Additionally, the stability of the disulfide bond in the obtained phosphoramidites was dependent on the presence of a bulky, tertiary carbon atom, flanking the dithio function. Thus, nucleoside **2** was not completely stable in 0.02 M iodine oxidation solution and phosphoramidite **3** had limited stability when dissolved in acetonitrile.

It was found that phosphoramidite **7** was prone to degradation. After isolation, derivative **7** underwent decomposition within few hours at room temperature in dry acetonitrile. HPLC studies showed the formation of intermediate **10**, which was further converted to **11**

* Corresponding author. Tel.: +46 18 4714809; fax: +46 18 47 14808; e-mail: marek.kwiatkowski@genpat.uu.se

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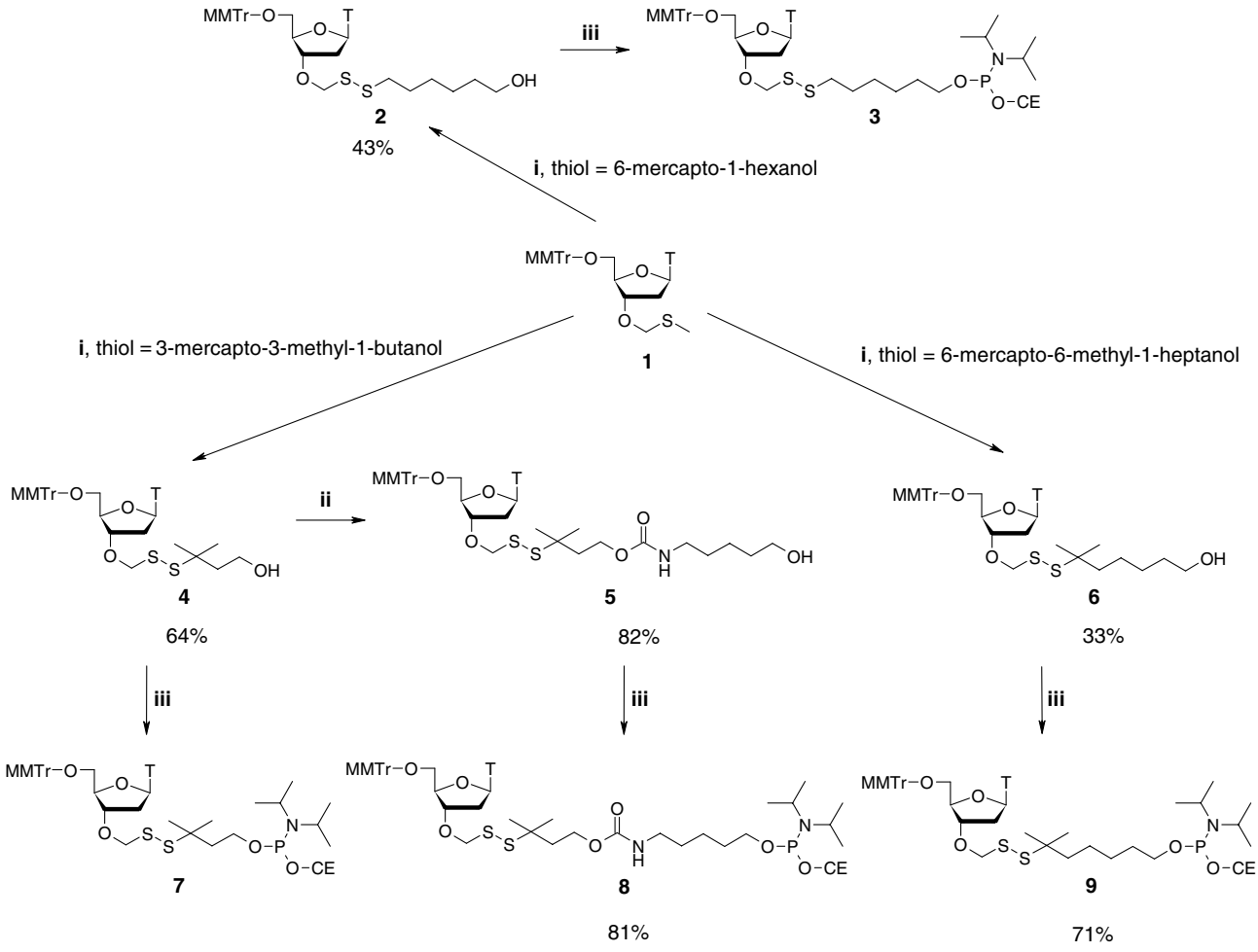
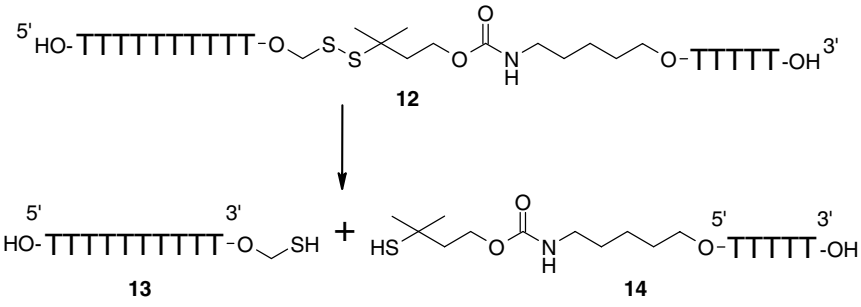
doi:10.1016/j.tetlet.2006.11.045

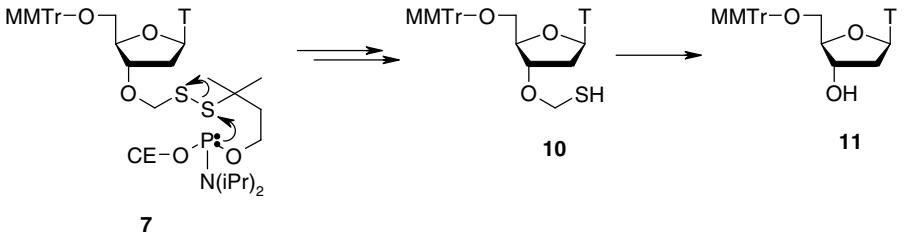


Scheme 1. Synthesis of phosphoramidites used in the present study: (i) a. SO_2Cl_2 (1.1 equiv), TEA (1.2 equiv) in 1,2-dichloroethane; b. *p*-MeC_6H_4SK (3.0 equiv) in DMF; c. thiol (5.0 equiv), DIEA (5.0 equiv); (ii) a. CDI (2.0 equiv) in Py; b. 5-aminopentanol (5.0 equiv); (iii) $(i\text{Pr})_2$NP(Cl)OCH_2CH_2CN (1.5 equiv), TEA (4.5 equiv) in CH_2Cl_2.

after the addition of pH 8.0 aqueous buffer. A possible explanation of such rapid conversion of **7** could be made by assuming neighboring attack of the trivalent phosphorus atom on the disulfide bond, favored by a six-membered ring intramolecular conformation (Scheme 2). This hypothesis was supported by the observation that phosphoramidites **8** and **9** were much more stable in acetonitrile at room temperature.

The destabilizing effect of the hydroxyl group located in close proximity to the disulfide bond was also observed, similar to the described effect of the neighboring amino group on the stability of the disulfide bond.[7,8] Thus, compound **4** degraded completely after 14 h incubation in 32% aq NH_3–EtOH (1:1) at 55 °C. In contrast, derivative **5** obtained from **4**, and nucleoside **6** did not show any sign of disulfide bond cleavage under identical conditions. The fast decomposition of **4** cannot be explained only by the electronegativity of the hydroxyl group destabilizing the dithio bond, since the presence of the carbamido group in compound **5** did not result in a similar rate of decomposition. The stability of oligonucleotides bearing dithiomethyl linkers toward ammonia was verified by the synthesis of oligonucleotide **12** using the standard conditions for DNA synthesis and deprotection.





Scheme 2. Putative mechanism of amidite **7** decomposition.

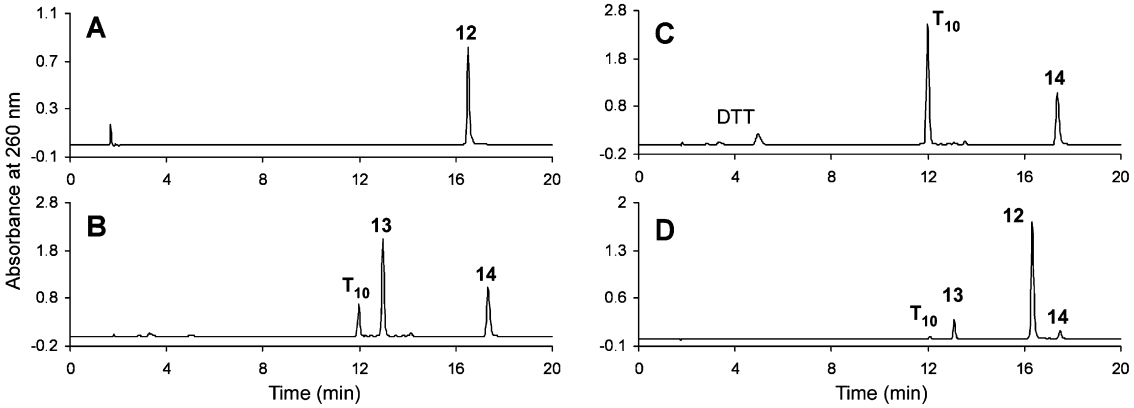


Figure 1. Deprotection of **12** studied by RP HPLC: (A) crude oligonucleotide **12**; (B) 0.2 mM **12** incubated with 50 mM DTT, 5 min, 20 °C, pH 10; (C) 0.2 mM **12** incubated with 50 mM DTT, 120 min, 20 °C, pH 10; (D) 0.2 mM **12** incubated with 2 mM DTT, 30 min, 20 °C, pH 8.5.

Cleavage of the dithiomethyl linker in **12** with 1,4-dithiothreitol (DTT) proceeded through the formation of intermediate **13** (Fig. 1), yielding finally oligonucleotide T_{10} and 5′-modified T_5 fragment **14**. The rate of linker cleavage was dependent both on DTT concentration and on the pH of reaction. The above example illustrates the general possibility for the synthesis of oligonucleotide conjugates linked by cleavable units. The pentathymidilic acid (T_5) present in oligonucleotide **12** can be easily substituted by any existing functional or labeling group to form the desired conjugate. When necessary these functions can be cleaved releasing free oligonucleotide with intact 3′-OH.

We realized early that the present dithiomethyl linker could be used as an alternative to the disiloxyl linker employed during oligonucleotide synthesis in order to remove failure fragments arising from the cleavage of abasic sites.[2] The latter linkage has some disadvantages as it demands an extra drying step since the cleavage of the siloxyl bond has to be maintained under anhydrous conditions. Additionally, cleavage of the disiloxyl group, linked to the polystyrene, is not very efficient, limiting this chemistry only to controlled pore glass (CPG) supports only. In contrast, the dithiomethyl linker can be easily applied for oligonucleotide synthesis on a polystyrene support. It can be cleaved fast in aqueous media, withstanding the harsh conditions of deprotection in ammonia, but the shorter fragments formed due to the cleavage of abasic sites, under these basic conditions, can be easily washed away from the support-bound product.

To demonstrate the applicability of this novel linker, the oligonucleotide 5′-d$(AG)_{30}$T, was synthesized on an amino modified polystyrene-grafted poly(tetrafluoro-

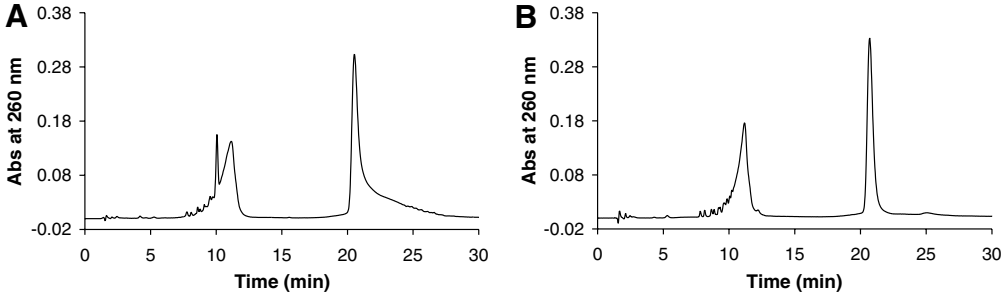


Figure 2. RP HPLC analysis of 5′-d$(AG)_{30}$T released from the polystyrene support: (A) oligonucleotide was attached to the support via a succinyl linker; (B) oligonucleotide was attached via a dithiomethyl linker and released after the ammonia deprotection step according to the described procedure.

ethylene) support,[9] further functionalized to obtain hydroxyalkyl functions according to the described procedure.[2] The support with oligonucleotide was treated with aqueous ammonia at 55 °C overnight (16 h) to ensure a complete cleavage of all apurinic sites and aglycone protecting groups, then washed with aqueous ammonia, and treated again with aqueous ammonia containing 50 mM DTT at 55 °C for 10 min to cleave the oligonucleotide from the support. Alternatively, the dithiomethyl linker cleavage can be performed at an ambient temperature within 1 h. The collected ammonia fraction containing the crude oligonucleotide was analyzed by HPLC and compared to the crude oligonucleotide of the same sequence synthesized on the same support, but derivatized with succinyl linker (Fig. 2). The oligonucleotides were purified using a cartridge-based separation methodology,[10] which in conjunction with the present method produced a good quality purine-rich material.

Thus, it has been shown that the dithiomethyl linker construct can be used as a linker between a solid support and the oligonucleotide synthesized. We have demonstrated the expected base stability of the novel linker and the mild conditions for its cleavage. It was proven that oligonucleotides made according to this procedure have purities similar to oligonucleotides, which were made on disiloxyl support and purified on RP cartridges. The present methodology represents an improvement over the existing one, making purification more user friendly by eliminating a time consuming and laborious step.

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