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Synthesis of RNA Using 2′-*O*-DTM Protection

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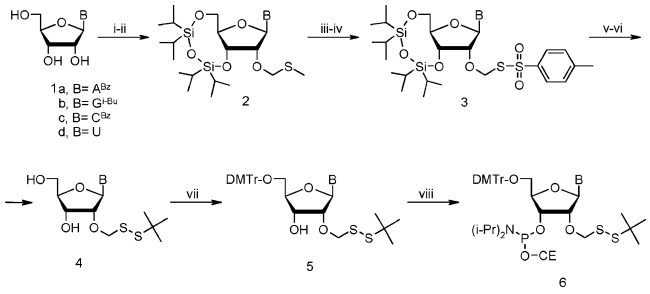
The choice of a 2′-OH protecting group is of crucial importance for chemical synthesis of RNA. It limits the range and dictates the nature of all other groups used for protection of remaining functionalities. Consequently, the origin of most shortcomings is related to the chemical characteristics of this particular protecting group.

The most widely used silyl-based protecting groups for 2′-OH are removable by fluoride anions in solvents that dissolve the 2′-*O*-modified RNA only sparingly.[1] The complete deprotection of such oligonucleotides becomes difficult, especially at the end of the process when RNA becomes even more hydrophilic.[2] Use of more active and acidic reagents ($Et_3N{\cdot}3HF$) results in the loss of 5′-*O*-DMTr groups, a function that could be of use during the oligonucleotide purification. The very acid-labile 2′-*O*-bis(2-acetoxyethoxy)methyl (2′-*O*-ACE) protection,[3] cleavable under mild and homogeneous conditions is not compatible with the standard 5′-*O*-DMTr protection. The fluoride-labile bis(trimethylsiloxy)-cyclododecyloxysilyl (DOD) protecting group, used to block the 5′-OH, cannot be used as a purification handle during RNA separation. The recently published, 2′-*O*-(2-cyanoethoxymethyl) (2′-*O*-CEM) chemistry uses also a heterogeneous condition during removal of the 2′-*O*-protecting group.[4] Further, it requires the presence of a strong organic base at this deprotection step; thus, the usefulness of this method has to be proven as it challenges the rule of RNA instability in basic media.

We have designed a new 2′-OH protecting group, *tert*-butyldithiomethyl (DTM), which is fully compatible with standard 5′-*O*-DMTr and cyanoethyl phosphate protection, completely stable during ammonia treatment, can be cleaved in a homogeneous, aqueous media under nearly neutral conditions, and is fully amenable to trityl-on-based purification. It belongs to the category of protected protecting groups—a concept that has been introduced earlier[5] and which principle has been used for several 2′-OH blocking functions. The DTM protection is introduced in a site-specific manner, avoiding separation of different isomers, and it does not migrate under any of subsequent operations. All steps involved in the synthesis of 2′-*O*-DTM nucleosides (Scheme 1) proceed with high yield and use only inexpensive reagents. Interestingly, large parts of the synthetic process can be performed as a one-pot procedure, as a number of purification steps can be omitted. In fact, during the large-scale preparation, the first flash column separation was undertaken at the level of compound **4**, as we found this to be the optimal method for scaling up and increasing the yield of the final amidite. Activation of 2′-*O*-methylthiomethyl (2′-*O*-MTM) derivative **2** upon treatment with SO_2Cl_2[6] proceeds quantitatively and can be monitored after hydrolysis of 2′-*O*-CH_2Cl to the 2′-OH nucleoside. The 2′-*O*-chloromethyl ether reacts smoothly with potassium

Scheme 1. Synthesis of 2′-*O*-DTM Protected Amidites[a]



[a] Conditions: i) TIPDS−Cl_2, 3 h; ii) DMSO, Ac_2O, AcOH, 24−48 h; iii) SO_2Cl_2, 1 h; iv) *p*-$MePhSO_2SK$, 0.5 h; v) ^{t}BuSH, 0.5 h; vi) NH_4F in MeOH, 16 h; yield of compounds **4a**−**d** are 45%, 44%, 57%, 44%, respectively (from **1a**−**d)**, vii) DMTrCl; viii) $(^i Pr)_2NP(OCE)Cl$.

p-toluenethiosulfonate, forming the reactive **3**, which is converted to the 2′-*O*-DTM derivative upon addition of *tert*-butyl mercaptan. Treatment of the 2′-*O*-MTM derivative of uridine (**2d)** with excess of SO_2Cl_2 may result in formation of a 5-chloro derivative. By performing the activation at 0 °C and using minimal amounts of base and SO_2Cl_2 we succeeded in eliminating this side product.

The tritylated compounds **5** were converted to the appropriate amidites using 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite. All amidites could be precipitated in cold hexane and were completely stable in this form during long-term storage (6 months) at −20 °C. Dissolving all amidites in acetonitrile lowers their stability particularly of the G amidite, **6b**. This degradation was found to be solvent and temperature dependent and was not influenced by addition of triethylamine. The presence of signals at ∼73 ppm in the ^{31}P NMR of partially degraded material suggests the formation of a complex mixture (S═P(V)) of derivatives, but accurate analysis of this material was hampered by its instability. An intermolecular attack of a phosphite triester on an activated disulfide usually proceeds very fast, and this process is used as a method for preparation of oligonucleotides phosphorothioates.[7,8] Although an intramolecular redox reaction would be expected to go even faster, we found that all amidite solutions were sufficiently stable within a period of 12−24 h, and this time is longer than the average turnover time for all amidites on a synthesizer. This relative high stability is caused by the strongly stabilizing electron-donating effect of the *tert*-butyl group, the steric hindrance around the disulfide bond, and the lack of a good leaving group connected to any of the sulfur atoms. No similar redox reaction has been reported for the structurally related material,[9] pointing to the limited importance of this side reaction for the performance of the building blocks. Interestingly, we observed that protection of the *O*-6 position of guanosine with diphenylcarbamoyl (DPC)[10] protecting group had a stabilizing effect on this amidite, resulting in less than 2% degradation within 24 h. Moreover, it is expected that placing an additional electron-donating group on the existing *tert*-butyl moiety

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will stabilize the dithio function even further. Such a group is also expected to drastically limit the unpleasant odor of the mercaptan, otherwise necessitating that oligonucleotide deprotection is performed in a fumehood. All amidites, when activated by 5-ethylthio-1-*H*-tetrazole (ETT), coupled within 150 s with efficiencies ranging from 98.5 to 99.8%, which is well comparable with other best-performing RNA amidites. Concentrated (0.1 M) solutions of iodine partially cleaved the S−S bond as was found in experiments using both 2′-*O*-DTM-protected nucleosides and DTM-protected RNA dimers. Surprisingly, the diluted (0.02 M) solution did not give rise to detectable DTM degradation. This peculiar behavior of dilute iodine was recently also reported by others.[11] The synthesized oligonucleotides were deprotected and cleaved from the synthesis support using aqueous concentrated ammonia at 55 °C after previously proving that such conditions are completely inert for the DTM function. The removal of the 2′-OH protecting group was performed after evaporation of ammonia and dissolution of the residual and otherwise stable material in a buffered (pH 7.6) solution containing 1,4-dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) at 55 °C. These homogeneous, aqueous, and nearly neutral conditions ensured complete removal of all 2′-OH protecting groups without any risk of internucleotide bond cleavage or isomerization. The DTM group removal consists of two steps, and at this pH the initial step of the S−S bond reduction proceeds very fast. The liberated thiohemiformacetal eliminates a molecule of thioformadehyde, yielding the free 2′-OH function. The thiohemiformacetal group was unusually stable at lower pH, compared to the stability of hemiformacetals under the same conditions, suggesting the possibility of using this function as a target for RNA labeling. It breaks down much faster at higher temperature and higher pH conditions. Thus, DTM removal from an $A_{(2'-O-DTM)}pC$ at 20 °C gave t_∞ 1200 and 120 min for reactions performed at pH 6.8 and 8.0, respectively. Increasing the temperature to 55 °C speeds this process further ($t_\infty \approx 7$ min). For deprotection of longer sequences, as the amount of reducing agent per DTM group decreases, we have used a prolonged time of 90 min at 55 °C. The acid labile 5′-*O*-DMTr group was completely stable under the above deprotection conditions. Thus tritylated oligonucleotides can be easily purified by reversed phase HPLC chromatography. Moreover, as the oligonucleotide solution neither contains any organic solvent nor any other substances deteriorating the chromatographic columns, no prior desalting step is necessary.

We prefer, however, to perform this separation using a cartridge system and a novel on-cartridge detritylation methodology,[12] allowing for parallel separation of hundreds of oligonucleotides.

The digestion of the purified products using nuclease P1 followed by alkaline phosphatase[13] resulted in the conversion to four nucleosides only, proving the absence of any unnatural phosphodiester linkages or modified bases.

The biological function of RNA synthesized by the DTM methodology was confirmed by: (i) analyzing the rate of RNase P RNA-mediated cleavage of a model RNA substrate[14] synthesized by the DTM and ACE procedures, respectively (Figure 1a, b, and c), and (ii) a fast and complete digestion of RNA/DNA hybrid upon treatment by RNase H (Figure 1d).

The 2′-*O*-DTM protection was found to be fully orthogonal to the existing silyl-based 2′-OH protecting groups. It was thus possible to perform RNA synthesis, using both types of building blocks simultaneously, and to incorporate them in any desired order and proportion. Selective cleavage of the silyl protecting groups resulted in RNA protected only by the 2′-*O*-DTM groups. For example, performing RNA synthesis using 2′-*O*-silyl-protected phosphoramidites on a 2′-*O*-DTM-protected nucleoside support results in

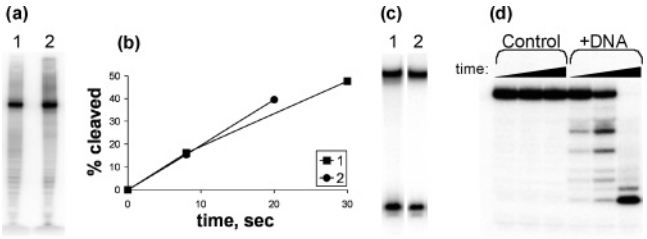

Figure 1. Identity and purity of RNA synthesized according to the DTM method. (a) Analysis of the 45-mer (5′GAUCUGAAUGGAGAGAGG GGGUUCAAAUCCCCCUCUCUCCGCCAC) model RNase P RNA substratre made by the DTM method (1) and purchased from Dharmacon (2), made by the ACE method. (b) Single-turnover measurement of the cleavage efficiency with RNase P RNA. Both RNA molecules are cleaved at the same rate. (c) Identical migration of the 5′ cleavage products. (d) RNase H digestion of a 22-mer RNA/DNA duplex (5′GAUCUGAAUGUUCAAA-UGCCAC). Control = RNA + RNase H; +DNA = RNA + complementary DNA + RNase H. The data points correspond to 40 s, 2 and 10 min, respectively.

RNA containing a 3′-terminal nucleoside bearing a single DTM function. A closely related 2′-S−S−^{n}Bu-substituted RNA derivative was recently reported to undergo spontaneous and site-specific aminoacylation with activated amino acid thioesters.[15] This interesting process is disturbed by a tendency for formation of bis-acylated products, and by the presence of the 2′-SH function in the final material. We are investigating if these drawbacks could be omitted by using the present 2′-*O*-DTM strategy.

Moreover, during incubation of 2′-*O*-DTM-protected oligonucleotides with serum we found that the DTM function is biodegradable (data not shown); hence, in in vivo experiments complete deprotection of RNA may not be necessary.

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Supporting Information Available: Experimental procedures and characterization of all new compounds; studies of amidite stabilities and kinetics of RNA deprotection and enzymatic digest. This material is available free of charge via the Internet at http://pubs.acs.org.

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