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A Base-Labile Group for 2′-OH Protection of Ribonucleosides: A Major Challenge for RNA Synthesis

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A great interest in the chemical synthesis of RNA has grown up since the advent of RNA interference (RNAi)[1] with the crucial need of a large number of short RNA molecules for biological research and therapeutic applications.[2] Compared to DNA synthesis, RNA production is more complex. In well-established DNA synthesis, all the nucleophilic functions are protected with base-labile protecting groups that are removed at the end of the elongation process with a base treatment. Beside lower coupling yields in chain assembly compared to DNA synthesis, the main difficulty of RNA chemistry results from the instability of RNA in basic media. It is generally admitted that the 2′-OH protection must not be base-labile to avoid the 2′-OH nucleophilic attack on the phosphorus atom of the internucleoside linkages resulting in 3′–5′ to 2′–5′ isomerisation or 3′–5′ cleavage of the linkages under deprotection conditions.[3] With regard to the use of acyl (acetyl or benzoyl) protection for 2′-OH reported in an early work, this resulted in very poor yields.[4] The *tert*-butyldimethylsilyl (TBDMS) group is certainly the most commonly utilized group for 2′-OH protection.[5] Several protecting groups[6,7] have been proposed in place of TBDMS, such as triisopropylsilyloxymethyl (TOM),[8] bis(2-acetoxyethyloxy)methyl (ACE),[9] *tert*-butyldithiomethyl (DTM),[10] 1-(2-cyanoethoxy)ethyl (CEE),[11] 2-cyanoethoxymethyl (CEM),[12,13] 2-(4-toluylsulfonyl)ethoxymethyl (TEM),[14] and 2-cyanoethyl[15]; most of them, like TBDMS, are removed by fluoride ions. This deprotection is a major hurdle because it requires a desalting step by precipitation or cartridge purification leading to additional time-consuming workup procedures to obtain pure oligoribonucleotides.

In our search for a radically improved synthetic method to obtain RNA efficiently, rapidly, and in high purity, we now report a new RNA synthesis strategy based on protecting the 2′-OH with a base-labile pivaloyloxymethyl (PivOM) group compatible with standard protection for 5′-OH (DMTr), phosphates (2-cyanoethyl), and nucleobases (acyl groups). The main advantage of this RNA strategy with an acetal ester group is a straightforward two-step all-base deprotection in a short period of time (3 h total) at room temperature. It consists of 1) the selective removal of the phosphate protecting group by β-elimination induced by a non-nucleophilic strong organic base (1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU) or piperidine), and 2) the simultaneous liberation of the nucleobases, the 2′-OH, and the rupture of the succinyl or the Q-linker by an ammonia treatment without migration or chain rupture. This approach, which makes exclusive use of base-labile protecting groups, is challenging because of the well-known RNA instability in basic media.[3] To illustrate it, we report on the use of 2′-*O*-PivOM phosphoramidites to synthesize RNA oligomers up to 21 nucleotides in length and we demonstrate that they were obtained in high yield and high purity without chain rupture or migration.

First, our investigations began with the synthesis of the four 3′-phosphoramidite ribonucleosides (Scheme 1). Previously we described the site-specific introduction of the PivOM protecting group in which the Markiewicz reagent ($TIPSiCl_2$)[16] simultaneously blocks 5′-OH and 3′-OH and leaves the 2′-OH free to accept the PivOM. This method avoids separation of different isomers, but it requires six steps for the whole uridine phosphoramidite synthesis.[17] Although all these steps proceed in high yields, they are time consuming and, moreover, $TIPSiCl_2$ is an expensive reagent. Alternatively we developed a convenient method with only

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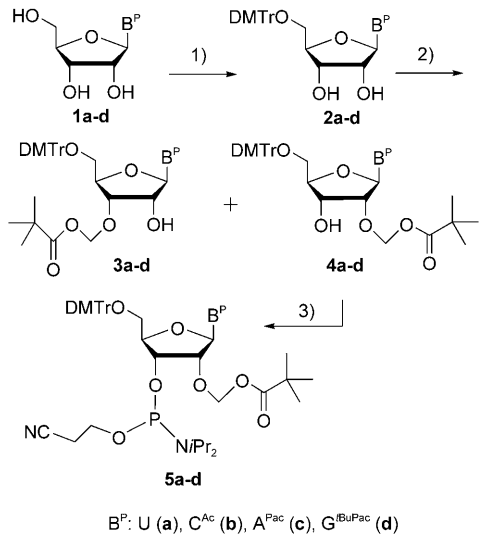

Scheme 1. Synthesis of 2′-O-PivOM protected phosphoramidites **5a–d**. Reagents and conditions: 1) DMTrCl, pyridine, RT; 2) Bu_2SnO, Bu_4NBr, **1a–d**, 2,2-dichloroethane, $ClCH_2OCOC(CH_3)_3$, microwave, 75 °C, 2.5 h (**4a** 36 %, **4b** 38 %, **4c** 34 %, **4d** 49 % from **2a–d**); 3) (iPr_2N)PCl(OCH_2CH_2CN), iPr_2NEt, CH_2Cl_2, RT, 3 h (**5a–d** 75-82 % from **4a–d**). DMTrCl : 4,4′-dimethoxytrityl chloride, B^P: base-protected, Ac: acetyl, Pac: phenoxyacetyl, *t*BuPac: *tert*-butylphenoxyacetyl.

four steps affording amidites **5a–d** with the same overall yield. The first step consists in the protection of the exocyclic amines of nucleobases with fast labile groups, such as acetyl (Ac) for cytosine,[18] phenoxyacetyl (Pac) for adenine,[19] and *tert*-butyl phenoxyacetyl (*t*BuPac) for guanine.[20] Starting from uridine **1a** or base-protected nucleosides **1b–d**, the 5′-OH group was blocked with DMTr. Then the 2′-OH group was derivatized with PivOM via a 2′,3′-*O*-dibutylstannylidene intermediate, which was treated with the cheap commercial alkylating agent pivaloyloxymethyl chloride to give a mixture of the 3′-*O*-PivOM **(3a–d)** and 2′-*O*-PivOM **(4a–d)** derivatives. This reaction must be conducted under microwave irradiation at 75 °C. The desired 2′-*O*-PivOM compounds **4a–d** were obtained in 34–49 % yield from **2a–d** after silica gel chromatography. For all ribonucleosides, the fast eluting 2′-isomers were isolated with higher yield than the undesired 3′-isomers, and for guanosine the 2′- to 3′-isomer ratio was the highest, which explains a better yield (49 %). The tritylated 2′-*O*-PivOM compounds **4a–d** were converted to the corresponding amidites **5a–d** with 75–82 % yield by using 2-cyanoethyl *N,N*-diisopropylchloro-phosphoramidite. All amidites were lyophilized and were completely stable during long-term storage at −20 °C.

With phosphoramidite monomers **5a–d** in hand, various oligoribonucleotides (Table 1) were prepared on an automated DNA synthesizer by using commercially available controlled-pore glass (LCAA-CPG) linked to 5′-*O*-DMTr-dT or 5′-*O*-DMTr-dC through a 3′-*O*-succinyl linker or Q-linker. We first synthesized U_{12}dC (**ON-1**; ON = oligoribonucleotide) as a model RNA oligonucleotide to test the efficacy of PivOM as 2′-OH protection. Syntheses were performed on a 1 μmol scale with 180 s coupling time and 5-benzylmercaptotetrazole (BMT) as activator. The average stepwise yield was 99.7 %, which is comparable with DNA or other efficient RNA amidites. Upon completion of chain assembly, **ON-1** anchored to CPG solid support was first treated with DBU in dry THF to remove phosphate protecting groups (2-cyanoethyl) over a period of 45 min, after having proved that such conditions are completely inert to PivOM groups in monomers. Then, simultaneous cleavage from the solid support and 2′-OH deprotection with concentrated ammonia at room temperature for 3 h led to a very pure U_{12}dC without any cleavage products. In the first step of this process, DBU was used to deprotect the internucleoside linkages, because phosphodiesters are less prone to nucleophilic attack than phosphotriesters. On the other hand, **ON-2** was treated with piperidine, instead of DBU, in acetonitrile for 15 min to eliminate cyanoethyl groups with similar efficacy. After ammonia treatment, RP-HPLC profile of the crude U_{19}TT (Figure 1, top) reveals efficient elongation and deprotection without chain rupture. The proposed mechanism for base-mediated hydrolysis of the acetal ester PivOM group consists of cleavage of the ester function by ammonia with formation of a formaldehyde hemiacetal. This intermediate is stable enough to ensure protection of oligoribonucleotides in aqueous ammonia and upon evaporation, when pH decreases, the hemiacetal undergoes fragmentation to the 2′-OH ribonucleoside and formaldehyde (see scheme in the Supporting Information).

This promising data prompted us to synthesize heteropolymers **(ON-3–5)** under the same conditions, except for the capping step in which phenoxyacetic anhydride was used instead of acetic anhydride to prevent replacement of *t*BuPac group by Ac group of guanine residues.[19]

ON-3 was initially deprotected similarly to U_{19}TT. However, formation of adducts was evidenced by MALDI-TOF MS analysis (peaks at +41). These by-products resulted from a side reaction of guanines as nucleophiles (at N1 and NH_2 in position 2, see Supporting Information) with formaldehyde generated by liberation of PivOM in the presence of ammonia.[21] We noted that guanosine adducts were unstable and disappeared after several hours in water or in triethyl-

Table 1. Data for synthesized oligoribonucleotides.

	5′-sequence-3′	CT[a]	OY[b]	AY[c]	Crude material[d]
ON-1	U_{12}dC	180	96.5	99.7	n.d.[e]
ON-2	U_{19}TT	180	94.2	99.7	140
ON-3	CCC GUA GCU GTT	180	91.1	99.1	86
ON-4	UGG AUC CUC GAU GGU AAC GdCT	180	82.1	99.0	130
ON-5	CGU UAC CAU CGA GGA UCC AdAT	180	83.8	99.1	125

[a] CT = coupling time [s] in automated synthesis cycle. [b] OY = overall coupling yield [%]. [c] AY = average stepwise coupling yield [%]. [d] Overall crude material O.D units measured at 260 nm UV absorption. [e] n.d. = not determined.

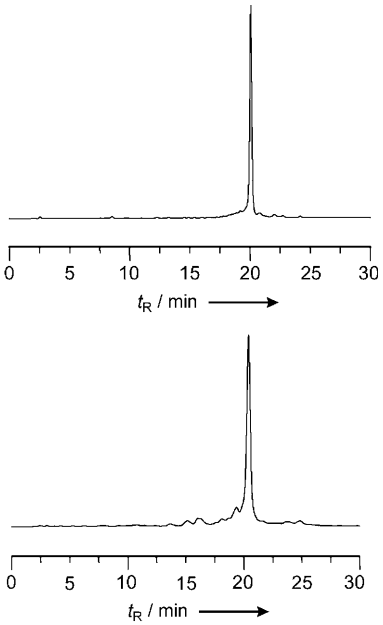

Figure 1. RP-HPLC analysis of crude $U_{19}TT$ (top) and crude **ON-4** (bottom).

ammonium acetate buffer. However, to avoid the formation of adducts that occurred during ammonia concentration under reduced pressure, isopropylamine was added just before evaporation, affording the desired **ON-3**. No chain cleavage was detected.

Then, the fully deprotected 21-mers **ON-4** and **ON-5** were obtained following the optimized protocol: first piperidine in acetonitrile for 15 min, secondly concentrated ammonia at room temperature for 3 h and finally addition of isopropylamine just before evaporation. The HPLC profile of unpurified **ON-4** (Figure 1, bottom) illustrates the efficiency of the chain assembly and of the deprotection. For comparison, **ON-4** was prepared according standard conditions with TBDMS chemistry. In our hands, the average stepwise coupling yield was 96.8% providing **ON-4** with a lower purity than that obtained with our new methodology.

The digestion of the purified **ON-4** and **ON-5** by nuclease P1 and alkaline phosphatase gave the four natural ribonucleosides indicating the absence of any nucleobase modification or unnatural internucleotide linkages. Hybridization of 21-mer **ON-4** with its complementary **ON-5** resulted in duplex formation with a single cooperative transition (T_m = 79 °C).

The activity of siRNA duplex **4**/**5** was evaluated in an RNAi assay that targets the Ret/PTC1 junction oncogene involved in papillary thyroid carcinoma (Figure 2). The siRNA duplex **4**/**5** obtained with the PivOM method had a similar gene silencing activity (60% inhibition) to a purchased siRNA duplex (**siRNA AS**) with the same sequence (40% inhibition). This result confirms the integrity and purity of the synthesized RNA by the PivOM method.

In conclusion, in the need for more robust RNA routine-synthesis strategies to prepare siRNA, we developed a novel

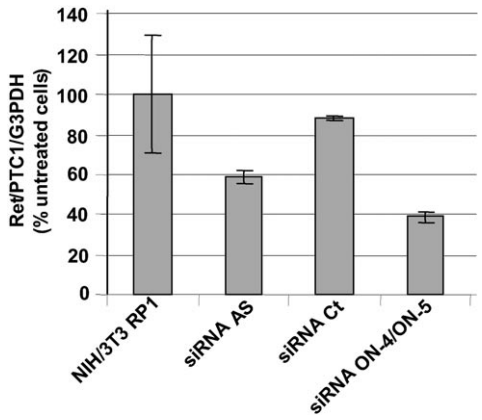

Figure 2. Analysis after RT-PCR quantification of Ret/PTC1 expression inhibition by cytofectine transfected siRNA in vitro. siRNA AS: duplex purchased, same sequences than **ON-4**/**ON-5**; siRNA Ct: control siRNA purchased.

methodology that disrupts the dogma "RNA synthesis is incompatible with a whole base labile strategy".[3] The key feature is the 2′-*O*-pivaloyloxymethyl protecting group of the nucleotide building blocks that guarantees very high average coupling yields >99% by using benzylmercaptotetrazole as activator. As for TOM phosphoramidites, these excellent yields are possibly the consequence of the low hindrance of the PivOM group, such that the base-labile pivaloyl ester is bound to the nucleoside through a small formaldehyde spacer. Furthermore PivOM is easy to introduce, stable during each synthetic step, and easily removed under basic conditions. In our strategy, RNA was protected with exclusively base-labile groups, which were completely removed in less than 3 h at room temperature in a straightforward all-base two-step procedure, first with piperidine in acetonitrile followed by aqueous ammonia without concomitant degradation of the RNA. A further strength of the PivOM method is that it readily provides highly pure RNA without any additional desalting step. The PivOM strategy is a powerful and efficient method for siRNA synthesis.

Experimental Section

After chain assembly of oligoribonucleotides on an ABI model 381 A DNA synthesizer, the solid support still in the column was dried by blowing argon through it and was first treated with 10% anhydrous piperidine in CH_3CN at room temperature for 15 min to eliminate cyanoethyl groups from phosphates. Then the piperidine solution was removed from the column and the solid support was washed with CH_3CN (3×2 mL). Secondly, a 28% aqueous ammonia solution was applied to the column in three batches (1.5 mL, 1 mL, 0.5 mL) for 30 min each. The three ammoniacal eluates were collected in a screw-capped glass vial and were left at room temperature for a further 1.5 h to completely deprotect nucleobases and 2′-hydroxyl groups. The fully deprotected oligonucleotide was transferred to a 50 mL round-bottomed flask and isopropylamine (15% of total volume: 0.45 mL) was added to the solution before evaporation to dryness.

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