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Assessment of new 2′-*O*-acetalester protecting groups for regular RNA synthesis and original 2′-modified proRNA

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ABSTRACT

New base-labile acyloxymethyl groups were evaluated to protect 2′-OH functions of ribonucleotides for regular RNA synthesis in order to shorten the deprotection procedure upon ammonia. These same acetalester groups were assessed in 2′-modified proRNA as biolabile 2′-protections removable by cell enzymes to generate parent RNA. Demasking of 2′-modified pro-uridylates was studied in cell extracts.

Since the discovery of RNA interference (RNAi)[1] as a means to silence the expression of specific genes, chemically synthesized short oligoribonucleotides (ON), such as small interfering RNA (siRNA) have attracted significant attention for therapeutic applications and biological researches.[2] RNA synthesis is more complex than DNA synthesis because of the presence of the 2′-OH requiring a permanent protecting group in the chain assembly process. The choice of this protection is crucial as it dictates the synthesis success or failure because the reactivity of phosphoramidite monomers is mainly dependent upon the nature of the group. Moreover its removal should be performed simply and efficiently without causing RNA damage.

For these last ten years, numerous 2′-*O*-protecting groups[3] were developed to overcome the drawbacks of the mostly used *tert*-butyldimethylsilyl (TBDMS) group[4] which reduces the coupling efficacy in the elongation and has a tendency for (2′→3′) migration during phosphoramidites preparation. Although all these new protection strategies allow convenient RNA chemical synthesis, some weaknesses still persist. Of special interest is our recent all-base-labile strategy with 2′-*O*-pivaloyloxymethyl (PivOM) protections which provides highly pure RNA sequences efficiently and rapidly.[5] Indeed, this improved synthetic method consists in protecting the 2′-OH with a base-labile acetalester group (PivOM) compatible with standard protections for 5′-OH (DMTr), phosphates (2-cyanoethyl), nucleobases (acyl groups). As for triisopropylsilyloxymethyl (TOM) phosphoramidites,[6] PivOM building blocks guarantee very high average coupling yields >99%, possibly due to the low hindrance of the group composed by the base-labile pivaloyl ester bound to the nucleoside through a small methylene spacer. Besides, the major benefit of this strategy using an acetalester group is the straightforward two-step all-base deprotection in 3 h at room temperature, first with dry piperidine or DBU followed by aqueous ammonia, without any concomitant RNA degradation or migration. Hence, no desalting step but a simple evaporation is required after the deprotection step affording RNA of good quality.

Parallel to the use of PivOM as a base-labile 2′-*O*-protecting group for regular RNA synthesis, we demonstrated that this acetalester protection introduced in 2′ position of uridylates was biolabile since it can be removed by cell esterases yielding to the parent RNA.[7] Within the context of using RNA in vivo, moreover the 2′-*O*-PivOM oligoribonucleotides named proRNA successfully fulfill the criteria of nuclease resistance and high affinity for complementary RNA to form a stable dsRNA. Eventually they may exhibit an improved cellular uptake as a consequence of the lipophilic character of the PivOM group. All these properties make the acetalester modification very attractive to generate bioactive RNA intracellularly.

The aim of the work reported herein is to develop new acetalester protecting groups for two main objectives: improvement of chemical method for regular RNA synthesis and design of original 2′-modified proRNA.

For ON synthesis purpose, our goal was to shorten the deprotection procedure of RNA by ammonia (less than 3 h at rt). The second objective was to modulate the biolability of acetalester groups in playing with the nature of the ester leading to the production of potential proRNA.

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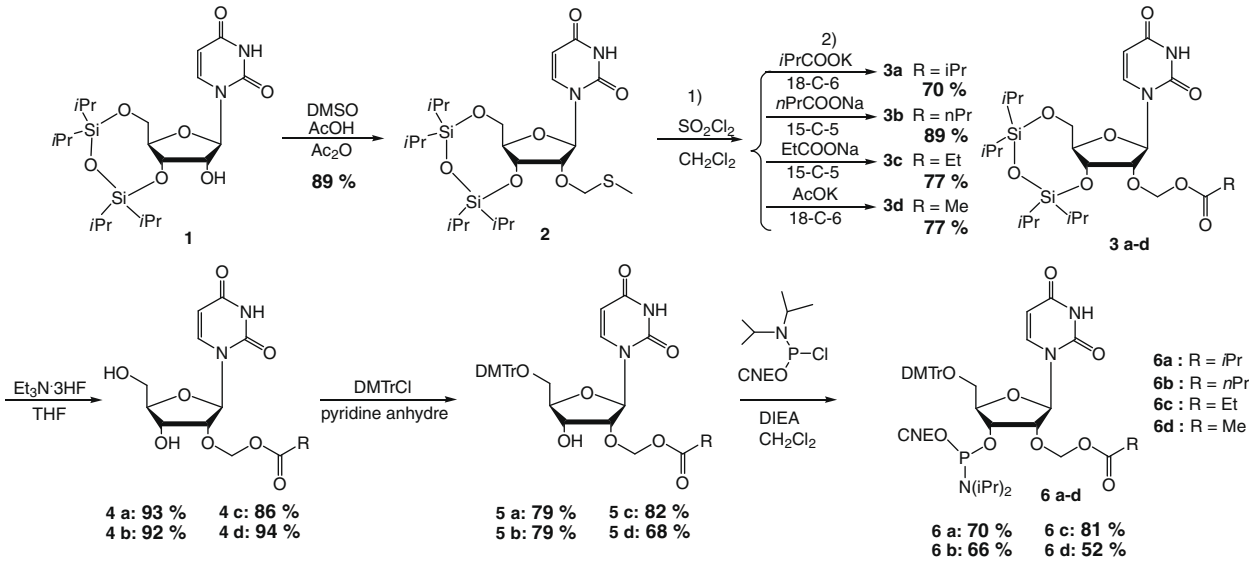

Scheme 1. Synthesis of 2′-*O*-acyloxylmethyl 3′-*O*-phosphoramidite uridine monomers.

In this study, three new acetalester protections, *iso*butyryloxymethyl (*i*BuOM), butyryloxymethyl (BuOM) and propionyloxymethyl (PrOM) groups were evaluated in comparison to the PivOM and acetyloxymethyl (AcOM) groups used in our previous study.[5,7] They were introduced in various oligo-U_n ($n = 5$, 11, 19) to study their lability towards ammonia treatment deprotection, their ability to form duplex with complementary RNA and finally their demasking mediated by cellular esterases.

The four fully protected uridine phosphoramidites **6a–d** were prepared in six steps from uridine (Scheme 1) as previously described for PivOM ribonucleoside.[7] The acetalester groups were introduced in a site-specific manner using the Markiewicz reagent ($TIPSiCl_2$) which simultaneously blocks 5′-OH and 3′-OH leaving the 2′-OH free to accept the acyloxymethyl group.[8] In this work, this route was preferred to the one consisting in the direct alkylation in 2′-position with PivOM chloride[5] because the introduction of each acetalester group was performed with good yields (70–81%) from the same key intermediate methylthiomethyl derivative **2** prepared in a large scale (93% yield). Furthermore, most of the different alkylating agents *i*BuOM-Cl, AcOM-Cl and PrOM-Cl are not commercially available whereas the corresponding potassium or sodium carboxylate salts are accessible. The three next steps leading to phosphoramidites **6a–d** were conducted in the same way than formerly reported[7] except for the use of 2-cyanoethyl (CNE) in replacement of 2-(trimethylsilyl)ethyl (TSE) group as a base-labile protection of phosphates. Furthermore, we showed that the coupling yields in oligonucleotide synthesis were highly improved to 99% with CNE-phosphoramidites. During the phosphoramidite synthons preparation, it is noteworthy that the lowest yields were observed with the acetyloxymethyl group, certainly because of the higher instability of the acetyl ester compared to the other esters (see Supplementary data).

To evaluate the new acetalester protecting groups in RNA synthesis *via* the whole base-labile strategy, phosphoramidite monomers **6a–d** and 2′-*O*-PivOM amidite monomer were assembled on a DNA synthesizer using controlled-pore glass (LCAA-CPG) linked to 5′-*O*-DMTr-dT through a 3′-*O*-succinyl linker to afford $U_{19}TT$ as models (Table 1). Syntheses were performed on a 1 μmol scale with 180 s coupling time and 5-benzylmercaptotetrazole (BMT) as activator. The average stepwise yields for **ON 2–5** were superior to 98% but slightly inferior to the yield obtained with 2′-*O*-PivOM amidite monomers (99.4%). After chain assembly, phosphates were first deprotected by 1 M DBU in CH_3CN for 1 min.[9] Then, 5 mg of each $U_{19}TT$-succinyl-CPG were treated with 28% aqueous ammonia solution and the kinetics of each acyloxymethyl group removal was monitored by MALDI-TOF mass spectrometry. The PivOM groups in **ON 1** were completely removed within 1 h 30 whereas the *i*BuOM protections in **ON 2** were cleaved after 15 min. This finding was surprising as we expected the *i*BuOM cleaved more rapidly than the PivOM but not so quickly considering the steric and inductive effects. The removal of BuOM (**ON 3**), PrOM (**ON 4**) and AcOM (**ON 5**) linear groups took less than 10 min (Table 1). 2′-positions were also deprotected upon milder conditions with ammonia/ethanol (3:1, v/v) used regularly for RNA deprotection and still BuOM, PrOM and AcOM were removed in less than

Table 1
Data for synthesized oligo-$U_{19}TT$ sequences and ammonia deprotection evaluation

ON	5′-sequence-3′		OY [a]	AY[b]	Deprotection time[c] (min)
ON 1	U_{19}dTdT	PivOM	89.2	99.4	90
ON 2	U_{19}dTdT	*i*BuOM	75.9	98.5	15
ON 3	U_{19}dTdT	BuOM	68.4	98.1	10
ON 4	U_{19}dTdT	PrOM	77.9	98.7	<5
ON 5	U_{19}dTdT	AcOM	70.7	98.3	<5

[a] OY = overall coupling yield (%).
[b] AY = average stepwise coupling yield (%).
[c] The coupling yields were calculated from cation trityl assays. ON deprotection was performed by an aqueous ammonia (28%) treatment at room temperature and was monitored by MALDI-TOF MS.

Table 2
Data for synthesized oligo-$U_{11}T$ sequences and melting temperatures (T_m, °C)

ON	5′-sequence-3′		OY[a]	AY[b]	T_m[c] (°C)
ON 6	U_{11}dT	PivOM	95.1	99.5	22.5
ON 7	U_{11}dT	*i*BuOM	84.7	98.4	14.5
ON 8	U_{11}dT	BuOM	80.8	97.9	12.0
ON 9	U_{11}dT	PrOM	88.3	98.7	12.5
ON 10	U_{11}dT	AcOM	82.8	98.1	13.5
	U_{11}dT	OH			16.5

[a] OY = overall coupling yield (%).
[b] AY = average stepwise coupling yield (%).
[c] The coupling yields were calculated from cation trityl assays. T_m values were assessed in 10 mM sodium cacodylate, 100 mM NaCl, pH 7, at 260 nm, and 3 μM oligonucleotides. RNA complementary target was $C_2A_{12}C_2$.

Table 3
Data for synthesized oligo-U_5T sequences and half-lives of demasking of 2′-*O*-acyloxymethyl **ON 11–15** in TSA cell extracts

ON	5′-sequence-3′		OY[a]	AY[b]	Half-life[c] (min)
ON 11	U_5dT	PivOM	96.9	99.2	147
ON 12	U_5dT	*i*BuOM	87.6	97.4	80
ON 13	U_5dT	BuOM	87.9	97.5	385
ON 14	U_5dT	PrOM	88.0	97.5	60
ON 15	U_5dT	AcOM	87.9	97.5	72

[a] OY = overall coupling yield (%).
[b] AY = average stepwise coupling yield (%).
[c] The coupling yields were calculated from cation trityl assays. Oligo-U_5dT demasking was performed by incubating each oligonucleotide in TSA cell extract (final concentration 47 μM) at 37 °C and was monitored by MALDI-TOF MS.

10 min. All these acyloxymethyl 2′-protections are potentially useful for RNA synthesis since ON were obtained with high purity.

In the second part of the study, these new acetalester groups were evaluated as biolabile 2′-modifications for proRNA approach concerning RNA duplex formation and demasking upon esterase hydrolysis to release the parent molecule. We first prepared $U_{11}T$ **ON 6–10** (Table 2) bearing five different acyloxymethyl groups in the same way than previously except they were assembled from a Q-linker[10]-thymidine anchored to a polystyrene support. Indeed, recently we reported an efficient fluoride ions treatment able to cleave the Q-linker without affecting the PivOM protection.[11] After chain assembly, **ON 6–10** anchored to the support were treated with 1 M DBU in CH_3CN for 1 min then the Q-linker was cleaved by a mixture of aqueous HF (48%) and triethylamine (1/3) to release ON after 8 h at 65 °C.[11] This harmless treatment for the PivOM stability was detrimental for the four other groups which were removed to some extent depending on the lability of the ester entity. The order of increasing stability was acetyl <propionyl<butyryl<*iso*butyryl. The partial loss of one or two acyloxymethyl groups for each $U_{11}T$ **ON 7–10** was evidenced by HPLC and MS analysis. Nevertheless, after HPLC purification, **ON 6–10** were isolated with suitable purity and their ability to form a duplex with complementary $C_2A_{12}C_2$ target was studied by UV melting experiments (Table 2).

All $U_{11}T$ **ON 6–10** formed a duplex with the target since a sharp transition in melting curves was observed for each duplex. Among all the 2′-*O*-modified ON, PivOM-$U_{11}T$ **6** formed the most stable duplex (T_m 22.5 °C) which was even more stable than the parent duplex (T_m 16.5 °C). In contrast, the duplex formed with the other $U_{11}T$ **7–10** were less stable than the parent duplex. **ON 8** and **ON 9** with BuOM and PrOM groups which are linear aliphatic chains were the most destabilizing with similar T_m for duplex (≈12 °C). Surprisingly, *i*BuOM group decreased the duplex stability (T_m 14.5 °C, ΔT_m −2 °C) whereas PivOM stabilized hybrids (ΔT_m +6 °C). These differences in duplex stability depending of the biolabile 2′-protection could find an application in designing pro-siRNA. Indeed, it is known that asymmetric strand incorporation into RISC, controlled by thermodynamic properties of the siRNA is fundamental to successful gene silencing in RNAi.[12] In playing with these 2′-modifications located in the two ends of the antisense and/or the sense strands, the relative stability of the two ends could be

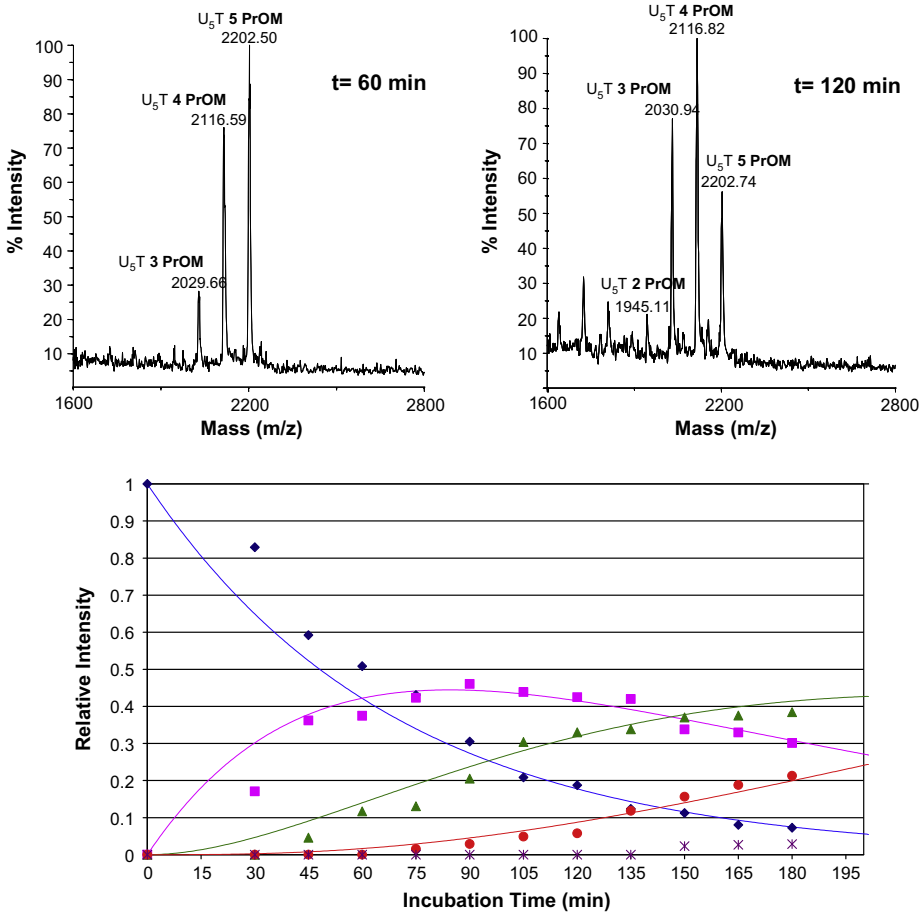

Figure 1. MALDI-TOF mass spectra of U_5T PrOM **ON 14** incubated at 37 °C in TSA cell extract for 1 h and 2 h. Demasking kinetics curves of **14**: U_5T 6 PrOM (blue), U_5T 5 PrOM (pink), U_5T 4 PrOM (green), U_5T 3 PrOM (red).

modulated and would influence the asymmetric retention of the active antisense strand into the RISC.[2]

Finally, since our proRNA strategy involves an intracellular carboxyesterase hydrolysis, we estimated the fate of 2′-*O*-acyloxymethyl U_5dT in total cell extract from TSA cells (derived from HEK293) used as mimic for the intracellular medium. Each U_5dT **ON 11–15** (Table 3) bearing different acyloxymethyl groups was synthesized similarly to **ON 6–10** and their demasking by cell enzymes was monitored by MALDI-TOF MS (Fig. 1). Each peak was assigned in the time-dependent mass spectra of each 2′-*O*-acyloxymethyl U_5T and their metabolites produced after incubation with cell extract. From each MALDI spectrum the height of each signal was measured and was converted in relative proportion, assuming that each metabolite flew in similar extent as the others.[13] The half-life ($t_{1/2}$) of each 2′-modified U_5T was determined from the kinetics (Table 3, Fig. 1).

Most acetalester groups were efficiently removed by cellular esterases with a demasking rate depending on the nature of the alkyl chain (R) in the ester moiety. Similarly to the stability data in aqueous ammonia, a significant difference was noticed between the stability of **ON 11** bearing PivOM groups (half-life 147 min) and the one of **ON 12** and **ON 14–15** bearing *i*BuOM, PrOM and AcOM, respectively (half-lives around 70 min). Surprisingly, in **ON 13**, the linear butyryl chain slowed down the demasking process (half life: 385 min).

Regarding nuclease hydrolysis of **ON 11–15** in cell extracts, the data confirmed that the acetalester groups in 2′ provided a high RNA stability towards nucleases, compared to the RNA parent degraded in one hour. Indeed the 2′-OH demasking is the major event observed in the mass spectra during the assay period.[7] In **ON 13** with a butyryl residue, the demasking process is much slower than the nuclease hydrolysis. For the other ON, the hydrolysis rate is directly related to the demasking rate since the nucleases cleave the internucleotide linkage where the 2′-OH is deprotected.

In summary, in addition to the PivOM group already studied, four new acyloxymethyl groups fulfill the requirements to protect 2′-OH in RNA synthesis efficiently. They are removed in less than 15 min upon ammonia without RNA damage. Besides, these 2′-protecting groups will be useful to design proRNA with biolabile 2′-modifications which would be demasked in cells. As the demasking rates of RNA protected with *i*BuOM, PrOM and AcOM are quite similar in cell extracts, the choice of the suitable group could be made regarding their chemical stability (AcOM<PrOM<*i*BuOM) upon the fluoride treatment during the ON release from the solid support. Thus, the *i*BuOM group could be chosen to make proRNA with biolabile protections less stable than PivOM groups.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.015.

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