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First Evaluation of Acyloxymethyl or Acylthiomethyl Groups as Biolabile 2′-*O*-Protections of RNA†

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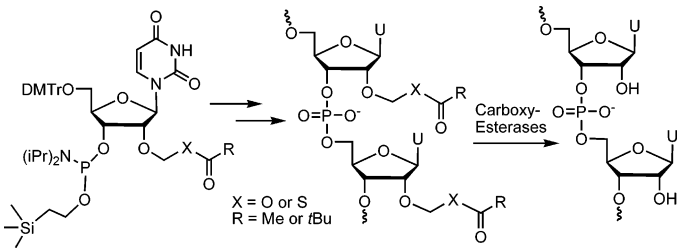
ABSTRACT



Short oligo-U sequences containing 2′-*O*-acyloxymethyl or acylthiomethyl groups as biolabile 2′-*O*-protections of RNA have been synthesized. These modified homouridylates are deprotected upon cellular esterase activation to release the parent RNA. They exhibit exceptional resistance to nuclease degradation, and the evaluation of their pairing properties shows that the 2′-acyloxymethyl groups do not prevent the duplex dsRNA formation. These biolabile 2′-modifications overcome the first hurdle to turn oligoribonucleotides into canditates for RNA interference drugs.

RNA and more precisely double-stranded RNA (dsRNA) are powerful tools for the suppression of gene expression in a variety of organisms and for gene therapy. Indeed, this agent is the central player in RNA interference (RNAi) to target and destroy specific cellular or viral RNAs.[1−3] Moreover dsRNA is responsible for stimulating immune response through nucleic acid interactions with pattern recognition receptors.[4] The critical role of dsRNA in these different biological processes triggered an impressive interest in the production of synthetic dsRNA. However, unmodified RNA is rapidly degraded in cells and has poor cell permeation. Chemists have a major role to play in synthesizing modified RNA to enhance their stability, cellular delivery, and biodistribution and consequently to turn RNA into a practically viable drug.

Among the numerous chemical modifications known to improve oligonucleotides (ON) properties (affinity, nuclease resistance, membrane permeability), some of them were introduced in synthetic siRNA but only a few are well tolerated for effective gene silencing.[5,6] A crucial need in a very accessible and nondetrimental modification arose. Our idea was to synthesize RNA protected by biolabile groups in the 2′-position, which should increase the nuclease stability of RNA, and the cell uptake should be enhanced in playing around the lipophilic character of the 2′-protections. For this purpose, we chose acetalester groups which should be cleaved by carboxyesterases to liberate the parent RNA into the cells (Figure 1). Moreover, acetal protecting groups have

† This paper is dedicated to Professor J.-L. Imbach on the occasion of his 70th birthday.

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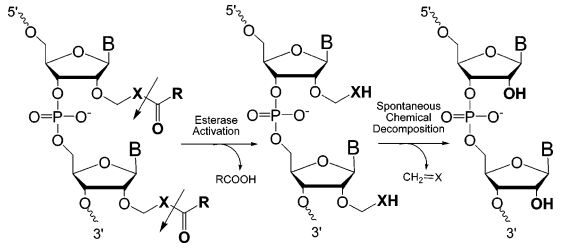


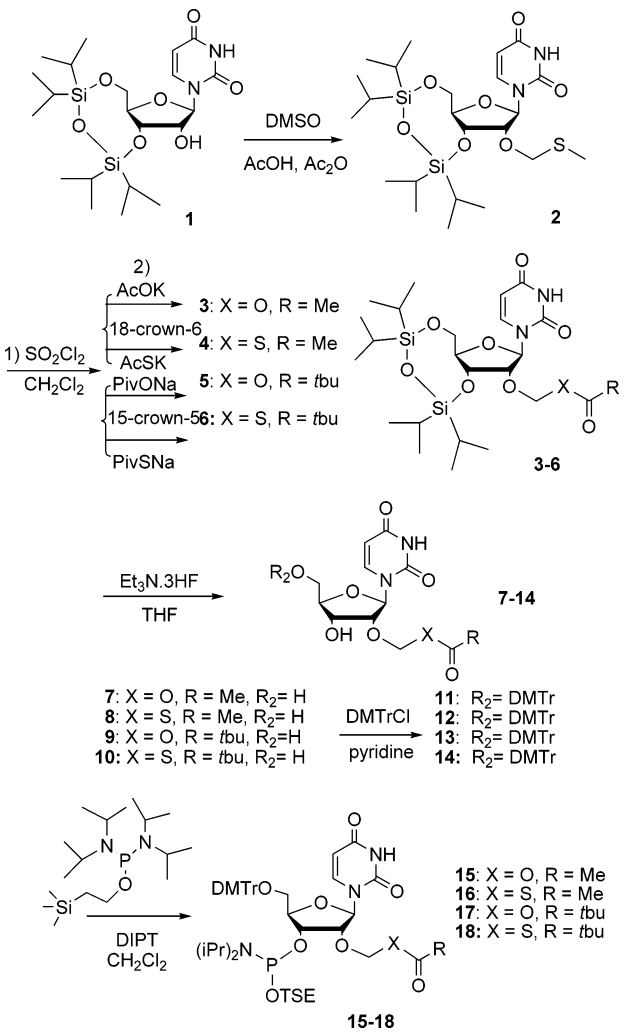
Figure 1. 2′-*O*-Protected RNA with biolabile acetalester groups release parent RNA upon carboxyesterase hydrolysis

several advantages over a more readily accessible and possible protection: the acetyl group. As far as the protection of the 2′-OH functions in RNA synthesis is concerned, acetal groups cannot migrate from the 2′-position to the 3′-position of the ribonucleoside and give a mixture of 2′- and 3′-isomers. Furthermore, acetyl prodrugs are very labile in protic solvents whereas acyloxymethyl prodrugs are more stable chemically and enzymatically.[7,8]

This Letter reports the initial synthesis of short oligo-U sequences containing 2′-*O*-acyloxymethyl or acylthiomethyl groups where acyl refers to acetyl or pivaloyl groups, the evaluation of their stability in the presence of esterases, and their pairing properties. RNA containing 2′-*O*-pivaloyloxymethyluridines (2′-*O*-PivOM-U) or pivaloylthiomethyluridines (2′-*O*-PivSM-U) should be more lipophilic than the corresponding 2′-*O*-acetyl counterparts (2′-*O*-AcOM-U or 2′-*O*-AcSM-U), and thus may have better cellular permeation properties.

The four fully protected uridine phosphoramidites **15**−**18** were synthesized in 6 steps from uridine (Scheme 1). The selective 2′-*O*-protection was achieved from 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine **1**, which was converted to its methylthiomethyl ether derivative **2**. This key intermediate was then cleaved with sulfuryl chloride to give the chloromethyl ether.[9] Reaction of this chloromethyl ether with the potassium salt of acetate or thioacetate in the presence of 18-crown-6 gave the desired 2′-*O*-AcOM **3** and 2′-*O*-AcSM uridine **4**, respectively, with a good yield (91%). Similarly, when the sodium salt of pivaloate or thiopivaloate was reacted with the chloromethyl ether in the presence of 15-crown-5, the 2′-*O*-PivOM and 2′-*O*-PivSM uridines **5** and **6** were obtained with 69% and 98% yield, respectively. Among the reagents commonly used to remove silyl groups,[10] only $Et_3N{\cdot}3HF$ was convenient to cleave the disiloxane group while keeping intact the acetalester functions in the different 2′-*O*-acetalester monomers **7**−**10**. Then the dimethoxytrityl group was typically introduced in the 5′-position of the four different synthons **11**−**14** and their corresponding phosphoramidites **15**−**18** were prepared.

Scheme 1. Synthesis of 3′-Phosphoramidites of 2′-*O*-Acetalester Protected Uridine



The most common 2-cyanoethyl (CNE) phosphate protecting group could not be used in the case of these RNA analogues, because the acetalester functions are sensitive to basic conditions required to remove it. Thus, the fluor-labile 2-(trimethylsilyl)ethyl (TSE) group was chosen as the phosphate protection.[11] In the same way, the succinyl linker that usually anchored the oligonucleotide to the solid support has been replaced with a photolabile cleavable linker.[12]

To rapidly evaluate the acyloxy- or acylthiomethyl groups as biolabile 2′-*O*-protections of RNA, we prepared homouridine hexamers (U_6) **19**−**22** and dodecamers (U_{12}) **23**−**26**, which avoid nucleobase protection (Table 1). Syntheses were carried out on a 1 μmol scale with 5-(benzylthio)-1*H*-tetrazole (BTT) as activator for the phosphoramidite coupling reaction and coupling times were extended to obtain the newly formed phosphite triester with yields >93%. It is noteworthy that the internucleotide TSE groups were removed by treatment with the solution of iodine during the

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Table 1. MALDI-TOF-MS Analysis of Oligouridylates with 2′-*O*-Acetalester Protections and Their Effect on Duplex Stability with the Complementary RNA Target $C_2A_{12}C_2$[a]

		MALDI-TOF-MS[b]			
no.	sequence	calcd	found	T_m[c] (°C)	T_m[d] (°C)
19	AcOM-U_6	2286.42	2287.64		
20	AcSM-U_6	2382.81	2382.45		
21	PivOM-U_6	2538.91	2542.22		
22	PivSM-U_6	2635.30	2635.73		
23	AcOM-U_{12}	4554.82	4554.70	12.7	25.4
24	AcSM-U_{12}	4747.60	4746.69	nd	nd
25	PivOM-U_{12}	5059.80	5057.60	22.0	40.5
26	PivSM-U_{12}	5252.57	5250.08	nd	<12
27	OH−U_{12}	3691.06	3690.18	16	29.4

[a] AcOM = acetyloxymethyl, AcSM = acetylthiomethyl, PivOM = pivaloyloxymethyl, PivSM = pivaloylthiomethyl. [b] Negative mode. [c] T_m values were assessed in 10 mM sodium cacodylate, 100 mM NaCl, pH 7, at 260 nm, and 3 μM oligonucleotides. [d] T_m values were assessed in 10 mM sodium cacodylate, 1 M NaCl, pH 7, at 260 nm, and 2 μM oligonucleotides. nd = no T_m value could be determined for duplex melting.

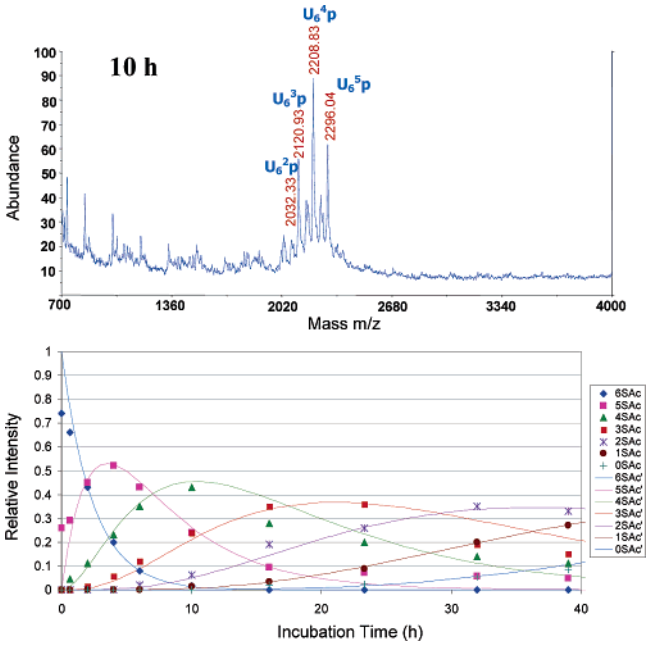

Figure 2. MALDI-TOF mass spectrum of AcSM-U6 **20** incubated at 37 °C in the presence of pig liver esterase (10 U/mL) for 10 h. In U_6^np, where n = number of 2′-*O*-protecting groups and p = 3′-phosphate. Demasling kinetics curves of the hexauridylate **20**.

normal oxidation protocol. Indeed, MALDI-TOF mass spectrometry of the U_6 or U_{12} still covalently linked to solid support CPG through the photolabile linker revealed the absence of the TSE groups in the uridylates whereas no fluoride ion treatment was applied.[13] This finding was not previously reported by Sekine and Wada since they applied a treatment with 1 M Bu_4NF in THF to deprotect the oligonucleotides bearing the TSE groups.[11] Furthermore, some years ago it was found that the *o*-methylbenzyl protection as a phosphate protecting group was quantitatively removed during the aqueous iodine oxidation step and the absence of phosphate protecting groups did not inhibit the subsequent elongation of the ON.[14] In our case, the early phosphate deprotection was not too harmful to the success of the synthesis since an average stepwise yield of 93% was obtained. Finally, the four hexauridylates **19**−**22** and the four dodecauridylates **23**−**26** were released from the solid support by UV irradiation.[15] The crude mixtures were analyzed and purified by C_{18} reverse-phase HPLC. These oligouridylates were characterized by MALDI-TOF MS (Table 1).

First, the demasking kinetics of the 2′-modified U_6 were studied in the presence of the pig liver esterase (PLE) and monitored by MALDI-TOF MS (Figure 2). It was easy to assign each peak in the time-dependent mass spectra of each 2′-*O*-protected U_6 and their metabolites produced after incubation with PLE. From each MALDI spectrum the height of each signal was measured and it was converted in relative proportion, assuming that each metabolite flew in a similar extent as the others.[16] The half-lives ($t_{1/2}$) of each 2′-modified U_6 were determined from the kinetics and a stability order as a function of the 2′-protecting group was established (Table 2). Hexuridylates bearing acetyl groups AcOM **19**

Table 2. Half-Lives of Demasking of 2′-*O*-Acyl(oxy or thio)methyl Hexauridylates **19**−**22**[a]

no.	sequence	PLE	CEM cell extracts
19	AcOM-U_6	13 h	55 min
20	AcSM-U_6	3.9 h	30 min
21	PivOM-U_6	64 h	7.7 h
22	PivSM-U_6	34.7 h	25.7 h

[a] PLE = pig liver esterase.

($t_{1/2}$ 13 h) and AcSM **20** ($t_{1/2}$ 3.9 h) were deprotected faster than U_6 bearing pivaloyl groups PivOM **21** ($t_{1/2}$ 64 h) and PivSM **22** ($t_{1/2}$ 34.7 h). Moreover, thioacetalesters (AcSM and PivSM) were removed earlier than acetalester groups (AcOM and PivOM) in view of the same acyl groups. This first study shows that the protecting groups were efficiently removed by an esterase.

Next, we checked if these protections enhanced the nuclease resistance of the four U_6 models **19**−**22** in the presence of an endonuclease (Nuclease S1) and 3′- and 5′-exonucleases (respectively snake venom phosphodiesterase and calf spleen phosphodiesterase). Furthermore, the stabili-

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ties of the modified U_6 were evaluated in human serum. In all these media, the four U_6 **19**−**22** exhibited a high resistance against enzymatic phosphodiester hydrolysis compared to the parent hexuridylate degraded within 30 min. The stabilities of **19** and **21** with acetalesters were greater than those of **20** and **22** with thioacetalesters considering the same ester group: acetyl or pivaloyl. Furthermore, the two U_6 bearing pivaloyl esters **21** and **22** were more stable against nuclease hydrolysis than the two U_6 with acetyl esters **19** and **20**. In all these media, the less stable RNA was the hexauridylate **20** bearing acetylthioester groups. So the 2′-modifications enhance the lifetime of the RNA by protection against nuclease hydrolysis.

Finally, since our strategy involves an intracellular carboxyesterase activation, we studied the fate of the modified U_6 in total cell extract from CEM-SS cells used as mimic for the intracellular medium. Their metabolization was monitored by MALDI-TOF MS. The data confirmed that the acetalester groups in 2′ provide a relative RNA stability against nuclease hydrolysis inside the cells compared to the parent RNA, completely degraded in 30 min. Moreover, this study showed that the 2′-*O*-protecting groups were efficiently demasked by cellular carboxyesterases (Table 2). We found that the pivaloyl groups slowed the demasking process compared to the acetyl groups. In conclusion of these stability studies, we established that the acetylthiomethyl group was not a good candidate for biolabile 2′-protection of RNA because of its low stability in human serum. Indeed, a relative stability in extracellular medium associated with a rapid intracellular demasking are key factors for compounds designed to selectively release inside the cells the parent RNA through enzymatic activation.[17]

The next step was to examine the ability of these RNA analogues to efficiently hybridize to their complementary RNA sequence keeping in mind that dsRNA are the active molecules in several biological phenomena, especially in the RNAi mechanism. The four dodecauridylates **23**−**26** were hybridized to RNA target $C_2A_{12}C_2$ and the corresponding T_m values for the melting of the duplex were determined by standard UV/melting-curve techniques (Table 1). The melting curves of U_{12} **24** and **26** bearing thioacetalesters (AcSM and PivSM) showed that no duplex transition could be detected in 0.1 M NaCl (entry 1) and PivSM-U_{12} **26** only formed a duplex in 1 M NaCl (entry 2) with a very low stability (T_m < 12 °C). In contrast, in the case of U_{12} with acetalester **23** and **25**, the stability of the duplex with RNA target was much higher and PivOM-U_{12} **25** formed a more stable duplex (T_m 22 °C at 0.1 M NaCl) than the parent duplex (T_m 16 °C at 0.1 M NaCl). Although the T_m value (12.7 °C) of the duplex AcOM **23**-U_{12}/RNA target was lower than the T_m of the parent duplex, the melting curve indicates a duplex formation that was enhanced when the salt ionic strength was 1 M NaCl (entry 2). Among the four 2′-*O*-protected U_{12} tested, only the uridylates bearing the acetyloxymethyl or pivaloyloxymethyl groups were able to form a stable duplex with $C_2A_{12}C_2$ RNA.

To be effective for gene silencing, the RNA duplex must retain conformationally RNA-like A-type helical characteristics. We try to explain the differences in duplex stability between the different RNA analogues studying the sugar puckering modes of the new modified ribonucleosides in D_2O by ^1H NMR. The percentage of the C3′-*endo* form (% N) was determined for each ribonucleoside and compared with this of the uridine (% N 52).[18] It was found that the % N values of the 2′-*O*-acetyloxymethyl (% N 53) and 2′-*O*-acetylthiomethyl (% N 53) uridine were similar to that of uridine but in the 2′-*O*-pivaloyloxymethyl (% N 47) or pivaloylthiomethyl (% N 40) uridine, the C3′-*endo* form was decreased to a degree of 5% and 12% respectively. Sugar puckering modes of the ribonucleosides are not sufficient to explain the thermal stability of the modified duplexes since PivOM-U_{12} **25** formed the more stable duplex and AcSM-U_{12} **24** the less stable one. As the substitution of the 2′-*O*-acyloxymethyl for 2′-acylthiomethyl groups had an adverse effect on the binding affinity, the steric hindrance of the sulfur atom larger than the oxygen atom may play an essential role in the destabilization of the RNA duplex.

In conclusion, we have synthesized novel 2′-*O*-modified oligouridylates with four different biolabile 2′-protections. Within the context of using RNAs in vivo, the 2′-*O*-acyl-(Ac or Piv)oxymethyl uridylates successfully fulfill the criteria of nuclease resistance, demasking upon esterase activation in intracellular medium and good affinity for RNA target to form a stable dsRNA. A potentiel advantage of our biolabile protecting groups approach is that the 2′-modifications are removed into the cells to release the functional RNA molecule. These properties make this acetalester modification a promising candidate for further evaluation for RNAi and such efforts are in progress since we are currently preparing the 2′-protected ribonucleotides containing the nucleobases A, C, and G for RNA synthesis.

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Supporting Information Available: Experimental procedures, spectral data for compounds, synthesis of oligonucleotides, enzymatic studies, and hybridization experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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