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Efficient Release of Base-Sensitive Oligonucleotides from Solid Supports using Fluoride Ions[‡]

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With the aim to prepare biolabile oligoribonucleotides that are partially or fully base-sensitive 2′-*O*-pivaloyloxymethyl functionalized, we investigated the cleavage of a silyl-type linker and a Q-linker anchored to a solid support under several fluoride ion conditions. Thus, an original fluoride ion treatment was perfected to release oligonucleotides bearing base-sensitive modifications from solid supports without affecting them and with high efficiency. Chimeric oligouridylates containing both 2′-OH and 2′-*O*-PivOM groups were obtained in high yield and purity. Moreover, the conditions used to cleave the linker were extended to the release of a base-sensitive DNA-oligonucleotide with 4-*N*-acetylcytosine residues; the acetyl groups were maintained.

Introduction

For several decades, chemically modified DNA or RNA oligonucleotides (ONs) have been synthesized for a broad variety of therapeutic and diagnostic applications. Usually, they are obtained on solid support by the standard phosphoramidite approach, in which ammonia treatment is required to remove base-labile protecting groups from the nucleobases and phosphates and to release the ONs from the solid support. This treatment cannot be performed on oligonucleotides bearing base-sensitive modifications, so new synthetic strategies avoiding the final ammonia treatment have been investigated. Especially, different methods using nucleoside 3′-phosphoramidites without base protection[1–3] as well as linkers cleaved by non-nucleophilic bases,[4,5] fluoride ions,[6–8] photochemical reactions,[9,10] or under mild conditions[11,12] preclude ammonia treatment in the final deprotection.

In earlier work, we studied modified homouridylates bearing base-sensitive 2′-*O*-acyloxymethyl or 2′-*O*-acylthiomethyl groups as biolabile protections, which we expect to be removed inside cells by carboxyesterases to release the functional RNA molecule.[13] These base-sensitive uridylates could not be anchored on a solid support by using the common succinyl linker, but they were obtained by using a photolabile linker orthogonally cleavable in the presence of acetalester groups.[9] The major drawback of this linker was the low recovery of the poorly soluble crude ON in a water/methanol mixture. In order to prepare oligoribonucleotides containing 2′-*O*-acyloxymethyl groups with high efficacy, we searched for an appropriate linker that could be cleaved under conditions suitable for the preservation of the base-labile pivaloyloxymethyl (PivOM) groups.

In addition, DNA oligomers containing base-labile acyl groups on the amino functions of cytosines are of interest, as it has already been reported that they exhibit a higher hybridization affinity for ssDNA relative to that of the unmodified ONs.[14] Recently, ONs with 4-*N*-acetylcytosine (C^{Ac}) and 6-*N*-acetyladenine (A^{Ac}) have been efficiently synthesized by using a silyl linker that is cleaved under fluoride ion conditions.[7] Here, the more accessible Q-linker was chosen for the synthesis of an ON bearing thymine (T) and C^{Ac}, and the conditions that allow release of this ON without affecting the acetyl groups were investigated.

The aim of this work was to optimize a fluoride ion treatment that releases 2′-*O*-PivOM oligoribonucleotides from the solid support and removes the TBDMS groups remaining in the case of chimeric ONs, leaving the 2′-*O*-acetalesters intact. In addition, the release of modified ONs with C^{Ac} was studied to extend the linker cleavage treatment to another type of base-sensitive modified ON.

Results and Discussion

For this purpose, chimeric oligouridylates (Table 1) bearing both PivOM and TBDMS 2′-*O*-protecting groups were prepared from the assembly of phosphoramidites **1** and **2** on solid supports **3** or **4** functionalized with a thymidine linked to the hydroquinone-*O*,*O*′-diacetic acid (Q-linker)[15]

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Table 1. Data for synthesized oligonucleotides.

ON[a]	5′-Sequence-3′[b]	Linker–Support[c]	AY[d]	O.D.[e]	Calculated Mass $[M - H]^-$	Observed Mass[f]	T_m [°C][g]
ON **1**	U*u*U*u*U*u*U*u*UT	Silyl linker–HCP **5**	99.0	54	4179.84	4179.06	15.5
ON **2**	U*u*U*u*U*u*U*u*UT	Q-linker–HCP **3**	99.0	55	4179.84	4180.39	15.5
ON **3**	U*u*U*u*U*u*U*u*UT	Q-linker–CPG **4**	99.1	56	4179.84	4178.58	15.5
ON **4**	UUUUU*u*UUUUUT	Q-linker–HCP **3**	98.4	55	3723.25	3723.04	17.0
ON **5**	*uuuuuuuuuu*T	Q-linker–HCP **3**	99.5	58	4864.71	4864.51	22.5
ON **6**	UUU*uuuuu*UUUT	Q-linker–CPG **4**	99.0	60	4179.84	4179.67	19.0
ON **7**	*uuu*UUUUUU*uu*T	Q-linker–CPG **4**	98.9	64	4179.84	4179.09	18.5
ON **8**	U*u*U*u*U*u*U*u*U*u*U*u*UTT	Q-linker–HCP **3**	99.0	118	7389.98	7388.50	n.d.
ON **9**	TTC^{Ac}TTC^{Ac}TTC^{Ac}TT	Q-linker–CPG **4**	99.4	48	3364.29	3364.61	n.d.

[a] ON = oligoribonucleotide. [b] *u* (italic lower case) = uridine 2′-*O*-PivOM; U = uridine. [c] CPG: controlled-pore glass; HCP: highly cross-linked polystyrene. [d] AY = average stepwise coupling yield [%]. [e] Overall material (O.D. = optical density units) measured at 260 nm UV absorption after desalting. [f] MALDI-TOF mass spectrometry in negative mode. [g] Melting temperatures were determined with the complementary RNA target $C_2A_{12}C_2$, in 10 mM sodium cacodylate, 100 mM NaCl, pH 7, at 260 nm, and 3 μM oligonucleotides concentration. n.d. = not determined. With unmodified $U_{11}T$, T_m = 16.5 °C.

anchored to highly cross-linked polystyrene (HCP) resin or controlled-pore glass (CPG) support. Moreover, for the first time, an oligoribonucleotide was synthesized from solid support **5** with a thymidine linked to the silyl linker attached to HCP (Figure 1).

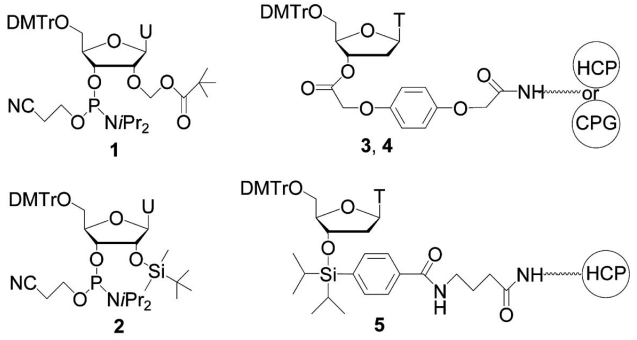

Figure 1. Phosphoramidites and solid supports for ON synthesis. 2′-*O*-PivOM **1** and 2′-*O*-TBDMS **2** uridine phosphoramidites; **3**: thymidine-Q-linker HCP support; **4**: thymidine-Q-linker CPG support;[15] **5**: thymidine-silyl-linker HCP support.[7]

2′-*O*-PivOM 3′-phosphoramidite uridine **1** was prepared as previously described.[16] To obtain thymidine-loaded HCP resin **5**, first we synthesized 2′-*O*-silyl nucleoside **6** according to the procedure reported by Kobori (Figure 2).[17] Initially, reagent **6** was introduced into aminopropyl-CPG, but the loading efficiency did not reach more than 6 μmol g^{-1}. In a second attempt, the loading reaction was performed by using amino resin **7** (25 μmol g^{-1}), which contains a 4-aminobutyric spacer arm introduced by reaction of aminomethyl-HCP resin (35.9 μmol g^{-1}) with 4-aminobutyric acid. The procedure allowed better loading for resin **5** (29 μmol g^{-1}) to be obtained. This loading method is an alternative route to a reported method[7] and similar loading efficacy was obtained.

Oligouridylates syntheses were performed on a 1 μmol scale with 3 min coupling time and 5-benzylmercaptotetrazole as activator (Table 1). Since our early work on the synthesis of 2′-*O*-acyloxymethyl uridylates,[13] the coupling yields have been improved up to 99% by use of 2-cyanoethyl (CNE) instead of 2-(trimethylsilyl)ethyl (TSE) groups as phosphate protecting groups. After chain assembly, ONs

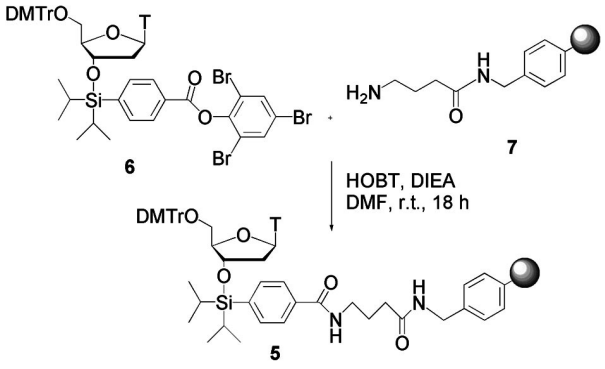

Figure 2. Loading of HCP support with thymidine bridged by a silyl linker.

1–**8** attached to solid supports **3**–**5** (Table 1) were first treated with piperidine in dry acetonitrile for 15 min to eliminate CNE from the phosphates without affecting the PivOM groups and the linkers.

Then, various fluoride ion mixtures were tested to cleave ON **2** from support **3** with the simultaneous removal of the TBDMS groups. The progress of ON **2** release was monitored by UV spectroscopy, and the material was quantified in comparison to the amount of ON **2** liberated upon the standard ammonia treatment (Figure 3). First, we applied the usual fluoride ion treatment, which was described to cleave the Q-linker.[15,18] ON **2** treated with 1 M TBAF in THF at room temperature was 80% released from HCP over the period necessary to remove the TBDMS groups (more than 15 h); moreover, this treatment led to a slight loss of the PivOM groups.

In our hands, neat $Et_3N{\cdot}3HF$ was not efficient, because this treatment liberated only 10% of ON **2** after 10 h at room temperature, whereas the TBDMS groups were removed and only 25% of ON **2** was released at 65 °C (Figure 3). In contrast, a 50 mM K_2CO_3 solution in methanol cleaved the Q-linker with high efficiency, but removed the PivOM groups within 1 min. Furthermore, the cleavage of the Q-linker was assessed with several aqueous TMEDA/HF solutions in acetonitrile. First, a 10% HF/20% TMEDA solution in acetonitrile[18] released 30% of ON **2** from HCP at room temperature and 84% of ON **2** at 65 °C

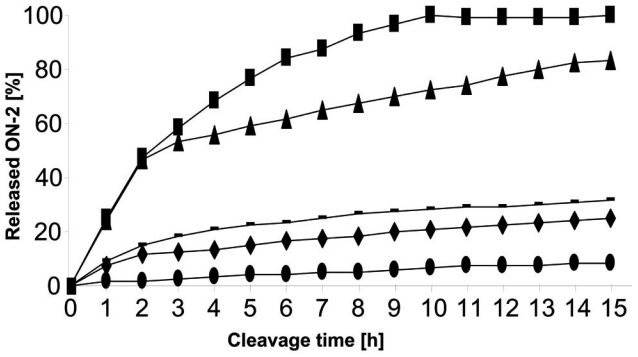

Figure 3. Q-linker cleavage kinetic curves for the release of ON **2** by using various fluoride ion solutions in comparison to ammonia treatment (100% release). Notation: HF/Et_3N (1:3) at 65 °C (■); 10% HF/20% TMEDA in CH_3CN at 65 °C (▲) or at 25 °C (–); Et_3N·3HF at 65 °C (♦) or at 25 °C (●). HF = 48 wt.-% HF in H_2O; TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylene diamine.

within 15 h. These data could be slightly improved when a 20% HF/40% TMEDA solution was used (38% ON **2** released at r.t. and 94% ON **2** at 65 °C). This last treatment at 65 °C cleaved the Q-linker efficiently but MALDI-TOF mass spectrometry and HPLC analysis of crude ON **2** revealed the presence of a minor side product in the mixture. This observation prompted us to find new optimized fluoride ion conditions for deprotection of chimeric oligomers. A solution of aqueous HF/Et_3N/CH_3CN (15:45:40) released ON **2** with 100% efficacy at 65 °C after 13 h and removed all TBDMS groups within 2 h. After desalting, MALDI-TOF MS and HPLC profiles of ON **2** showed that this treatment did not affect the PivOM groups. With the aim of shortening the time required for 100% ON release, a HF/triethylamine (1:3) mixture was used without CH_3CN, and after 10 h at 65 °C, ON **2** was completely released from solid support **3** (Figure 3). This optimized treatment was prolonged up to 15 or 24 h to evaluate RNA stability and PivOM group lability. MS and HPLC analysis attested to the integrity of chimeric ON **2**.

In the same way, the optimized mixture HF/Et_3N (1:3) successfully cleaved ON **3** anchored to the Q-linker CPG at 65 °C in 8 h. The only difference with the Q-linker HCP was the dissolution of glass beads in the fluoride mixture, which has a slight advantage, because the deprotection solution did not need stirring and the cleavage rate increased. In contrast, RNA degradation was noticed when a solution of Et_3N·3HF (0.2 M) and Et_3N (0.4 M) in THF was applied to ON **1** (for 4 h, at r.t.) to cleave the silyl linker as described in the literature for DNA oligomers synthesized on HCP.[7] These conditions were not found to be destructive for DNA but are not suitable to release RNA anchored to a solid support through a silyl liker. Therefore, HF/Et_3N (1:3) treatment was applied to ON **1**; the silyl linker anchored to HCP **5** was cleaved and the TBDMS groups were removed in only 2 h at 65 °C. Cleavage also occurred at room temperature, but with a lower rate (6 h for complete ON release) and total removal of the TBDMS groups required 11 h. In both cases, chimeric ON **1** was obtained with high purity.

To validate this new deprotection method for base-sensitive ONs, we synthesized several chimeric oligouridylates ONs **4**–**8** with a mixture of 2′-*O*-PivOM and 2′-OH groups. They were deprotected and released from HCP or CPG in 10 or 8 h upon HF/Et_3N (1:3) treatment at 65 °C, affording ONs in good yield and high purity after desalting and without further HPLC purification (Figure 4). In all cases, the TBDMS groups were totally removed within 90 min upon this treatment, as attested by MALDI-TOF analysis. Thus, these fluoride ion conditions are an alternative method for the deprotection of RNA synthesized by the standard TBDMS strategy.

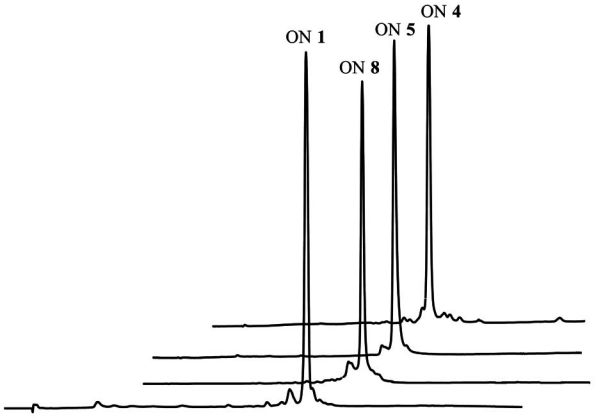

Figure 4. RP-HPLC analysis of crude chimeric oligouridylates ON **1**, **4**, **5**, and **8** after desalting.

Furthermore, we extended the application field of this fluoride treatment with another example of base-sensitive ONs. ON **9** containing T and C^{Ac} was prepared under standard DNA synthesis conditions on Q-linker-CPG, which is easily available compared to the silyl linker. After deprotection of the phosphate groups by piperidine, ON **9** in its entirety was released from CPG after 8 h in the mixture HF/Et_3N (1:3) at 65 °C. MS and HPLC analyses evidenced that this treatment left the acetyl groups intact on the cytosine residues.

Besides their use in this study, $U_{11}T$ ONs **1**–**7** were evaluated for their ability to form duplexes with complementary targets to study the effect of the number and the position of the 2′-PivOM modifications in RNA. Thus, ONs **1**–**7** were hybridized to the RNA target $C_2A_{12}C_2$, and the corresponding duplex T_m values were determined by standard UV-melting techniques (Table 1). We observed that the introduction of one 2′-*O*-PivOM ribonucleotide in oligouridylate ON **4** did not affect the duplex stability significantly (+0.5 °C). The PivOM distribution had a beneficial or a detrimental effect on the thermal stability of the RNA duplex depending on their relative position into the sequences. Five 2′-*O*-PivOM ribonucleotides incorporated alternating with 2′-OH ribonucleotides in ON **1** resulted in a slight duplex destabilization (ΔT_m = –1 °C), whereas five modifications spread out either in a central window or in the

wings of chimeric ONs **6** and **7** increased the duplex stability (ΔT_m = +2 to 2.5 °C). The stabilizing effect of these chimeric mixed RNA was less pronounced than that previously observed with fully modified 2′-*O*-PivOM ON **5** (ΔT_m = +6 °C).[13] These results demonstrate that PivOM chimeric RNA would also be potentially valuable in siRNA formation.

Conclusions

In conclusion, we found an efficient fluoride ion treatment (aq. 48% HF/Et_3N, 1:3; 65 °C, 8 to 10 h) to cleave the Q-linker. Unlike the previous existing treatments, this one ensures complete release of ONs from solid supports with a recovery similar to that obtained by ammonia treatment. Moreover, the fluoride solution cleaves a silyl linker and removes the TBDMS protecting groups with high efficacy without affecting base-labile 2′-*O*-PivOM groups in chimeric mixed oligoribonucleotides and without damaging RNA. In addition, this treatment allowed modified DNA-oligomers with acyl groups on the nucleobases to be obtained. So, this method will be practical for the release and deprotection of base-sensitive oligonucleotides. In particular, the Q-linker combined with fluoride-labile nucleobase protections orthogonal to PivOM groups would be attractive for the preparation of modified biolabile 2′-*O*-PivOM RNA.

Experimental Section

Preparation of Thymidine-Silyl-Linker HCP Support 5: *N*-γ-Fmoc-γ-aminobutyric acid (Fmoc-GABA; 193 μmol, 63 mg, 5 equiv.) was dissolved in dry DMF in the presence of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU; 189 μmol, 72 mg, 4.9 equiv.), 1-hydroxybenzotriazole (HOBT; 193 μmol, 26 mg, 5 equiv.), and diisopropylethylamine (DIEA; 385 μmol, 66 μL, 10 equiv.). This solution was withdrawn with a frit-equipped syringe containing a highly cross-linked polystyrene (HCP) resin (1 g) having a benzylamino group, and the total mixture was stirred overnight. The resin still in the reactor was then washed with dry DMF (4 × 5 mL), dichloromethane (4 × 5 mL), and ether (4 × 5 mL) and then dried under reduced pressure. To remove the Fmoc protecting group, a 20% piperidine solution in DMF was applied to the resin, which was then washed with DMF (50 mL) and dried. Compound **6** (193 μmol, 210 mg, 5 equiv.) prepared following the procedure described by Kobori[17] was dissolved in dry DMF (5 mL) in the presence of HOBT (193 μmol, 26 mg, 5 equiv.) and DIEA (385 μmol, 66 μL, 10 equiv.). This solution was added to the GABA-HCP resin in the reactor, and the mixture was stirred overnight. The solvent was removed by filtration through the frit. The residual resin was then washed with DMF (4 × 5 mL), dichloromethane (4 × 5 mL), and ether (4 × 5 mL) and then dried under reduced pressure. Subsequently, the resin still in the reactor was suspended in a mixture (1:1, 6 mL) of 10% *N*-methylimidazole in THF and Ac_2O/pyridine/THF (1:1:8) and stirred gently for 1 h. The solution was removed from the reactor, and the resin was washed with DMF (4 × 5 mL), dichloromethane (4 × 5 mL), and ether (4 × 5 mL) and then dried under reduced pressure. The amount of thymidine loaded into the HCP resin was estimated at 28.5 μmol g^{-1} by use of the DMTr cation assay.

Oligonucleotide Synthesis: Oligouridylate ONs **1**–**8** were assembled on an ABI model 381A DNA synthesizer on a 1 μmol scale by using phosphoramidite building blocks 2′-*O*-PivOM **1** prepared as described[16] and commercially available 2′-*O*-TBDMS **2**. Syntheses were carried out either on thymidine-Q-linker HCP or CPG supports **3** or **4**, or on thymidine-silyl linker HCP support **5**. 5-Benzylmercaptotetrazole (BMT) was used as the activator for coupling (180 s). After chain assembly, the cyanoethyl groups were removed by treatment with an anhydrous 10% piperidine solution in CH_3CN (3 mL) at room temperature for 15 min. The solution was removed by filtration, and the support was dried to be transferred into a screw-capped O-ring eppendorf.

Optimized Procedure for Release of the Oligonucleotides: The support containing 1 μmol of the oligonucleotide was suspended in a mixture (pH 8.3) of 48 wt.-% HF in H_2O (0.5 mL) and triethylamine (1.5 mL) at 65 °C for 10 h. After centrifugation, the supernatant was removed in a round flask, and the support was washed with water (2 × 0.5 mL). After centrifugation, the combined supernatants were concentrated under reduced pressure. The crude material diluted in water (1 mL) was loaded onto a homemade RP C_{18} column (3 cm × 1 cm) equilibrated in water. The column was first flushed with water (20 mL) to remove the salts. Then, the desalted oligonucleotide was eluted with 50% acetonitrile (10 mL) and analyzed by RP-HPLC and MALDI-TOF MS.

Melting Experiments: The T_m experiments were performed with a CARY 300 UV spectrophotometer (Varian Inc.) equipped with a Peltier temperature controller and thermal analysis software. The samples were prepared by mixing ONs **1**–**7** and the target $C_2A_{12}C_2$ together to give a final concentration of 3 μM in 10 mM sodium cacodylate, 100 mM NaCl, pH 7. A heating–cooling cycle in the 0–65 °C temperature range with a gradient of 0.5 °C min^{-1} was applied. The values of T_m were determined from the maxima of the first derivative plots of absorbance versus temperature. The T_m values from three independent experiments were accurate within ±0.5 °C (Table 1, graphs in the Supporting Information).

Supporting Information (see footnote on the first page of this article): HPLC chromatograms and MALDI-TOF MS spectra of crude ONs **1**–**8** after fluoride ion treatment; melting temperature graphs.

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