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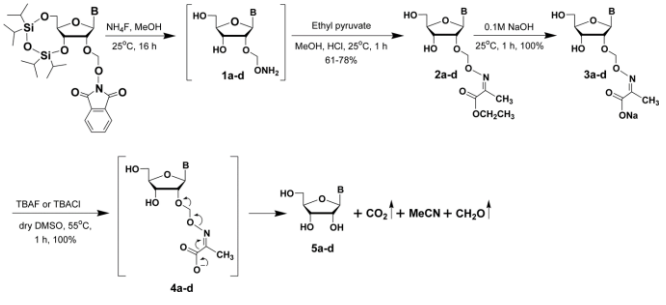
Protection of the 2'-Hydroxy Function of Ribonucleosides as an Iminooxymethyl Propanoate and its 2'-*O*-Deprotection via an Intramolecular Decarboxylative Elimination Process

Jacek Cieślak, Andrzej Grajkowski, Cristina Ausín, and Serge L. Beaucage*[a]

Abstract: The design and implementation of 2'-hydroxy protecting groups for ribonucleosides is still a daunting challenge to overcome when assembling RNA sequences for therapeutic applications. The reaction of 2'-*O*-aminooxymethylribonucleosides with ethyl pyruvate results in the formation of 2'-*O*-iminooxymethyl propanoic acid ethyl esters. The deprotection of this type of 2'-*O*-protecting groups is demonstrated through saponification of the esters to 2'-*O*-imino-oxymethyl propanoate salts, which when needed, decarboxylate quantitatively at 55 °C in the presence of tetra-*n*-butylammonium fluoride or chloride in dimethyl sulfoxide to produce all four native ribonucleosides.

On the basis of recent developments in RNA interference to silence the expression of specific genes, the availability of rapid and efficient methods for the chemical synthesis of RNA sequences in sufficient quantity and purity for pharmaceutical applications has become an urgent issue. A formidable challenge in the chemical synthesis of oligoribonucleotides is designing a suitable 2'-hydroxy protecting group for ribonucleosides. Indeed, the protecting group should optimally: (i) be easy to introduce; (ii) remain completely stable throughout the full assembly of the RNA sequence and particularly under the conditions used for nucleobase and phosphate deprotection, and release of the sequence from the solid support; (iii) be totally removable under conditions that do not compromise the structural integrity of the RNA sequence.[1,2a-c] The 2'-hydroxy protecting group should preferably be structurally flexible and sterically small enough to permit rapid phosphoramidite coupling kinetics and high coupling efficiencies. In this regard, a number of acetal, acetalester and orthoester protecting groups have been investigated with the objective of imparting ribonucleoside phosphoramidites with coupling kinetics and efficiencies matching those of deoxyribonucleoside phosphoramidites. These protecting groups include the: 2-nitrobenzyloxymethyl,[3] 4-nitrobenzyloxymethyl,[4] bis(2-acetoxyethoxy)methyl,[5] triisopropylsilyloxymethyl,[6] 2-cyanoethoxymethyl,[7a,b] 2-*tert*-butyldithiomethyl,[8] 2-(4-tolylsulfonyl)ethoxymethyl,[9a,b] levulinyloxymethyl,[10a,b] pivaloyloxymethyl,[11a,b] 4-(*N*-dichloroacetyl-*N*-methyl)aminobenzyloxymethyl [12a,b] and the 2-cyano-2,2-dimethylethanimine-*N*-oxymethyl[13] groups. The use of 2'-*O*-aminooxymethylribonucleosides[13,14] in the synthesis of the latter 2'-hydroxy protecting group has proven to be a straightforward, efficient and cost-effective approach to the chemical synthesis of high quality RNA sequences in the context of RNA interference applications. However, the fluoride-assisted deprotection rates of the 2-cyano-2,2-dimethylethanimine-*N*-oxymethyl group, in addition to those of any fluoride-sensitive 2'-hydroxy protecting groups, decrease with increasing RNA chain length. Electrostatic repulsion between fluoride ions and the negatively charged phosphodiester functions of RNA sequences is likely to increase proportionally with the number of phosphodiesters.[13] With the objective of mitigating this drawback, we report herein the application of an iminooxymethyl group for 2'-hydroxy protection of ribonucleosides and demonstrate its unprecedented cleavage through an innovative, entropically-driven, intramolecular decarboxylative process.

As shown in Scheme 1, the usefulness of 2'-*O*-aminooxy-methylribonucleosides (**1a-d**) in the development of novel 2'-hydroxy protective groups is further demonstrated by mixing the ribonucleoside **1a**[13,14] with ethyl pyruvate in the presence of drops of concd. HCl in MeOH. After 1 h at 25 °C, the 2'-*O*-iminooxymethyl propanoic acid ethyl ester derivative **2a** was isolated in a post-silica gel purification yield of 78%. Saponification of **2a** upon treatment with 0.1 M NaOH produced the 2'-*O*-iminooxymethyl propanoate salt **3a** in a quantitative yield. Inspired by reports on the decarboxylation of α-keto acids,[15a,b] we hypothesized that α-ketoxime acid derivatives could perhaps be amenable to decarboxylation under mild conditions. Indeed, heating **3a** in the presence of



Scheme 1. Implementation of a 2'-*O*-iminooxymethyl propanoate protecting group for ribonucleosides and its removal through a decarboxylative elimination reaction. Abbreviations: B, uracil-yl (a), cytosin-1-yl (b), adenin-9-yl (c) or guanin-9-yl (d); TBAF, tetra-*n*-butylammonium fluoride; TBACl, tetra-*n*-butylammonium chloride.

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Supporting information for this article is given via a link at the end of the document.

tetra-*n*-butylammonium fluoride (TBAF) or chloride (TBACl) in dry DMSO (Scheme 1) resulted in a clean decarboxylation of **4a** with the concomitant formation of MeCN and release of formaldehyde to provide uridine (**5a**) in quantitative yield based on RP-HPLC analysis of the reaction presented in Figure 1.

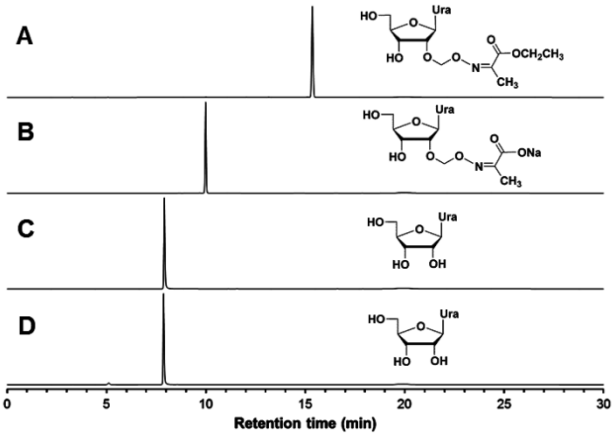


Figure 1. RP-HPLC profiles demonstrating the sequential conversion of the ribonucleoside **2a** → **3a/4a** → **5a**. (A) silica gel purified 2'-*O*-protected uridine. (B) de-esterified 2'-*O*-protected uridine. (C) 2'-*O*-deprotected uridine. (D) commercial sample of uridine. Chromatographic conditions are described in the Materials and Methods section of the Supporting Information.

Although other salts including tetra-*n*-butylammonium acetate (TBAOAc) or tetra-*n*-butylammonium cyanide (TBACN) could be used in various solvents (e.g., MeCN, MeOH or H_2O) for this purpose, the kinetics of the decarboxylation reaction were somewhat slower. The mechanistic features of this novel 2'-*O*-decarboxylative elimination reaction has been scrutinized by ^{13}C-NMR spectroscopy. Figure 2A displays the ^{13}C-NMR spectrum of de-esterified 2'-*O*-protected uridine (**3a**). Decarboxylation of **3a/4a** has been carried out using ammonium fluoride, instead of TBAF, in order to prevent interference from the ^{13}C-NMR signals associated with the tetra-*n*-butylammonium cation that would otherwise obscure the diagnostic ^{13}C-signal shifts resulting from the decarboxylative elimination of MeCN. As shown in Figure 2B, the signal (11.93 ppm) corresponding to the methyl group of **3a** in Figure 2A, has now shifted to 1.63 ppm; this signal corresponds to the primary carbon of MeCN. Consistent with this assessment is the presence of a new signal at ~118 ppm corresponding to the quaternary carbon of MeCN. The formation of acetonitrile is further demonstrated by spiking the NMR sample with commercial MeCN. Figure 2C reveals a significant increase in the intensity of both ^{13}C signals corresponding to MeCN, thereby convincingly supporting the proposed mechanistic pathway taking place during the cleavage of the iminooxymethyl propanoate group protecting the 2'-hydroxyl of uridine. With the aim of assessing the generality of our proposed approach to the protection of ribonucleosides as 2'-*O*-iminooxymethyl propanoic acid ethyl esters, the 2'-*O*-amino-oxymethylribonucleosides **1b-d**[13,14] have also been converted to **2b-d** under conditions similar to those employed for

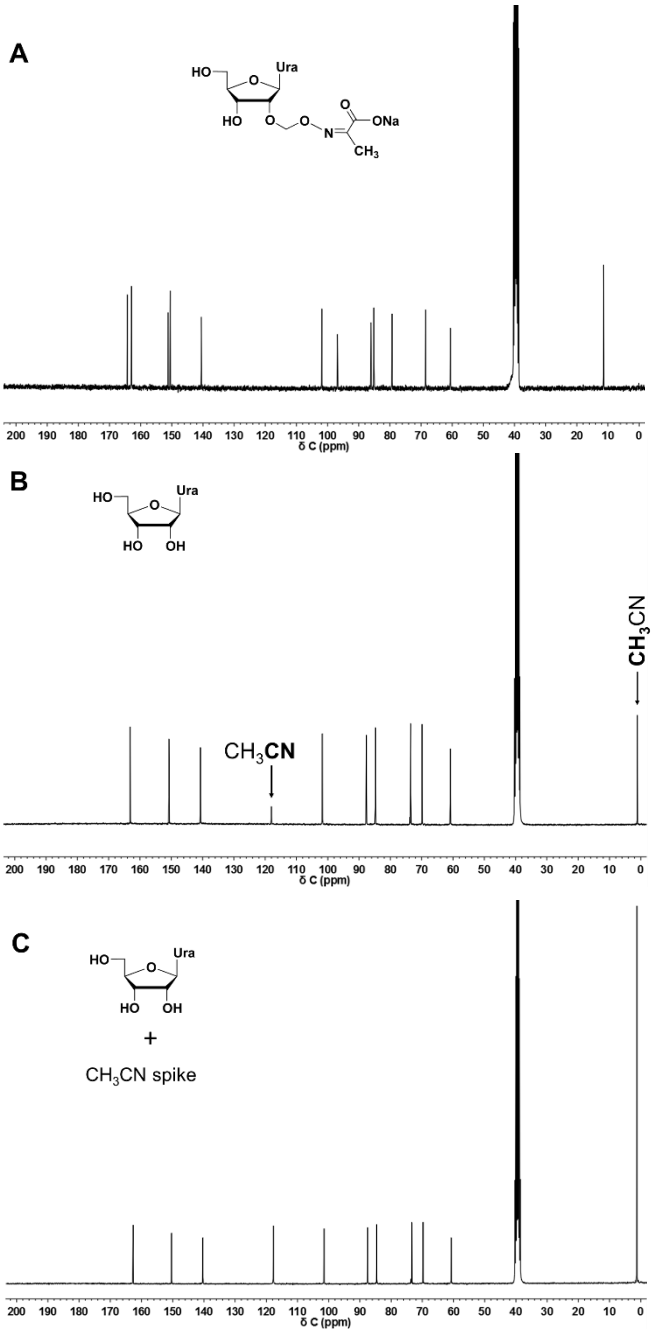
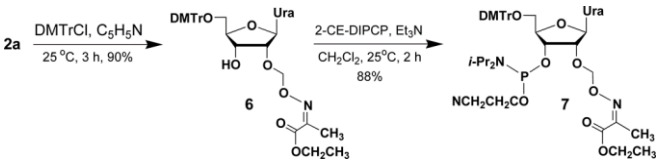


Figure 2. ^{13}C-NMR analysis of the decarboxylative elimination of acetonitrile from **3a/4a** in [D_6]DMSO. (A) Spectrum of de-esterified 2'-*O*-protected uridine **3a**. (B) Spectrum of fully 2'-*O*-deprotected **3a/4a**. (C) Spectrum of fully 2'-*O*-deprotected **3a/4a** that is spiked with commercial acetonitrile.

the preparation of **2a** (see experimental details in the Supporting Information). Treatment of **2b-d** with 0.1 M NaOH at 25 °C for 1 h provides after neutralization with AcOH, the 2'-*O*-iminooxymethyl propanoate salt **3b-d** in yields similar to that obtained for **3a**. TBAF or TBACl is added to a solution of **3b-d** in dry DMSO; the solution is then heated at 55 °C to induce decarboxylation of **3b-d** over a period of 1 h. Similar to the

decarboxylation of **3a** under those conditions, the concurrent formation of MeCN and formaldehyde occurred and resulted in the production of cytidine, adenosine and guanosine in quantitative yields based on RP-HPLC analysis of the decarboxylative elimination reactions displayed in Figures S1, S2 and S3.

Our findings show that an iminooxymethyl propanoic acid ethyl ester can protect the 2'-hydroxyl of all four ribonucleosides and be quantitatively removed when needed. In the context of solid-phase RNA synthesis, it is critically important to assess the adequacy of the 2'-*O*-iminooxymethyl propanoate protection/de-protection process for this purpose. Thus, the solid-phase synthesis of a chimeric polyuridylic acid $(Up)_{20}dT$ served as a preliminary and simplistic model for such an assessment. First, the 5'-*O*-protection of the ribonucleoside **2a** upon reaction with 4,4'-dimethoxytrityl chloride in dry pyridine produced **6** (Scheme 2) in a yield of 90% after silica gel purification. The reaction of **6** with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite in the presence of triethylamine gives the ribonucleoside phosphoramidite **7** in a post-purification yield of 88%.



Scheme 2. Synthesis of the ribonucleoside phosphoramidite **7**. Experimental details are provided in the Supporting Information. Abbreviations: DMTrCl, 4,4'-dimethoxytrityl chloride; Ura, uracil-1-yl; 2-CE-DIPCP, 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite.

The coupling kinetics and efficiency of the ribonucleoside phosphoramidite **7** were evaluated through the solid-phase synthesis of $(Up)_{20}dT$. An identical RNA control sequence was also prepared using a commercial uridine 2'-*O*-(*tert*-butyldimethylsilyl) phosphoramidite[16] with the purpose of comparing the quality of the RNA sequences produced from each phosphoramidite monomer. Upon completion of the solid-phase RNA syntheses, the RNA sequence constructed from the use of **7** was subjected to treatment with 0.5 M tetra-*n*-butylammonium hydroxide for 3 h at 25 °C to concurrently de-esterify the 2'-*O*-protecting groups, remove the 2-cyanoethyl phosphate protective groups and release the RNA sequence from the solid support. The basic nucleic acid solution was then neutralized by adding a four-molar equivalent of glacial acetic acid and evaporated to dryness. Without isolation, the crude RNA sequence was dissolved in a 0.5 M solution of TBAF or TBACl in dry DMSO and heated at 55 °C for 3 h to induce decarboxylation of the 2'-*O*-iminooxypropanoate groups. The control RNA sequence was fully deprotected and released from the support under published conditions.[16,17a,b] Analytical samples of the unpurified and desalted RNA sequences were analysed by RP-HPLC and polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (Figures 3 and 4, respectively). Moreover, enzymatic hydrolysis of the crude de-esterified 2'-*O*-protected $(Up)_{20}dT$ (Figure 3A) catalyzed by snake venom phosphodiesterase and alkaline phosphatase cleanly led to the production of **3a** and thymidine (Figure 5A), whereas the enzymatic hydrolysis of crude de-esterified 2'-*O*-protected $(Up)_{20}dT$ revealed, after decarboxylation, only the presence of uridine and thymidine (Figure 5B), thereby indicating complete cleavage of the 2'-*O*-protective groups from the chimeric RNA sequence.

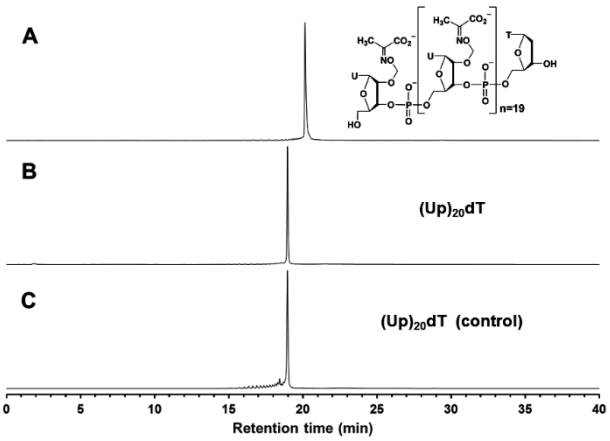


Figure 3. RP-HPLC analysis of unpurified and desalted chimeric RNA sequences. (A) Chromatogram of de-esterified 2'-*O*-protected $(Up)_{20}dT$. (B) Chromatogram of fully 2'-*O*-deprotected $(Up)_{20}dT$. (C) Chromatogram of fully 2'-*O*-deprotected $(Up)_{20}dT$ control sequence. Abbreviations: U, uracil-1-yl; T, thymin-1-yl. Chromatographic conditions are defined in the Materials and Methods section of the Supporting Information.

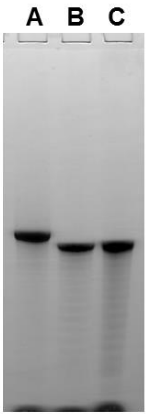


Figure 4. PAGE analysis of unpurified and desalted chimeric RNA sequences. Lane A: De-esterified 2'-*O*-protected $(Up)_{20}dT$. Lane B: Fully 2'-*O*-deprotected $(Up)_{20}dT$. Lane C Fully 2'-*O*-deprotected $(Up)_{20}dT$ control sequence. Bromophenol blue is used as a marker and appears as a large band at the bottom of the gel. Electrophoretic conditions are reported in the Materials and Methods section of the Supporting Information.

Although the iminooxymethyl propanoic acid ethyl ester function has been demonstrated to be generally applicable to the 2'-*O*-

protection/deprotection all the four canonical ribonucleosides, the solid-phase synthesis of RNA sequences carrying all four 2'-*O*-protected ribonucleoside is beyond the scope of this communication and will be dealt with in the near future.

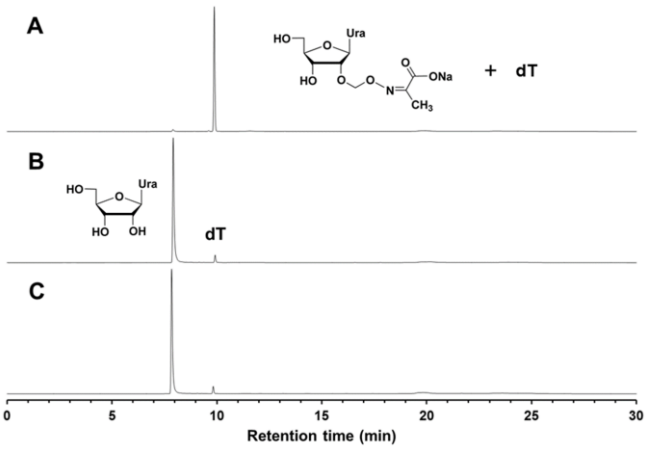


Figure 5. RP-HPLC analysis of SVP/BAP hydrolysates. (A) Chromatogram of unpurified, de-esterified and desalted 2'-*O*-protected $(Up)_{20}dT$ digest. (B) Chromatogram of unpurified and desalted 2'-*O*-deprotected $(Up)_{20}dT$ digest. (C) Chromatogram of unpurified 2'-*O*-deprotected $(Up)_{20}dT$ control sequence digest. Abbreviations: SVP, snake venom phosphodiesterase; BAP, bacterial alkaline phosphatase; Ura, uracil-1-yl; T, thymin-1-yl. Experimental details and chromatographic conditions are provided in the Materials and Methods section of the Supporting Information.

In summary, the application of 2'-*O*-iminooxymethyl propanoic acid ethyl esters, as a novel class of protected protecting groups,[18] for the 2'-hydroxy protection of ribonucleosides is unprecedented in that the removal of these protecting groups led to positive entropic changes through the release of carbon dioxide, acetonitrile, and formaldehyde. This entropically-driven process ensures a quantitative intramolecular cleavage of the 2'-*O*-protective group under mild and neutral conditions. Given the notorious complexity of protecting and deprotecting the 2'-hydroxy function of ribonucleosides, our findings should be of interest to the community of organic and medicinal chemists dedicated to the manufacturing of 2'-*O*-protected ribonucleosides that will ultimately be used in the production of RNA sequences for therapeutic and/or synthetic biology applications.

Experimental Section

Materials and Methods, full experimental procedures and characterization data including ^{1}H, ^{13}C, ^{31}P NMR and mass spectra of all new compounds are provided in the Supporting information of this article.

Acknowledgements

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Keywords: nucleosides • 2'-hydroxy protection • protected protecting group • 2'-*O*-iminooxymethyl propanoate • decarboxylative elimination reaction

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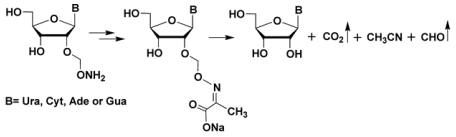
Layout 2:

COMMUNICATION

2'-*O*-Aminooxymethylribonucleosides react with ethyl pyruvate to provide, after saponification, 2'-*O*-iminooxymethyl propanoate derivatives. Deprotection of the 2'-hydroxy protecting group to native ribonucleosides is demonstrated through of a tetraalkylammonium salt-mediated decarboxylative elimination reaction.



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Key topic: Decarboxylative Elimination Reaction