RNAs Containing Carbocyclic Ribonucleotides

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ABSTRACT: Toward the goal of evaluation of carbocyclic ribonucleoside-containing oligonucleotide therapeutics, we developed convenient, scalable syntheses of all four carbocyclic ribonucleotide phosphoramidites and the uridine solid-support building block. Crystallographic analysis confirmed configuration and stereochemistry of these building blocks. Duplexes with carbocyclic RNA (car-RNA) modifications in one strand were less thermodynamically stable than duplexes with unmodified RNA. However, circular dichroism spectroscopy indicated that global conformations of the duplexes containing car-RNAs were similar to those in the unmodified duplexes.
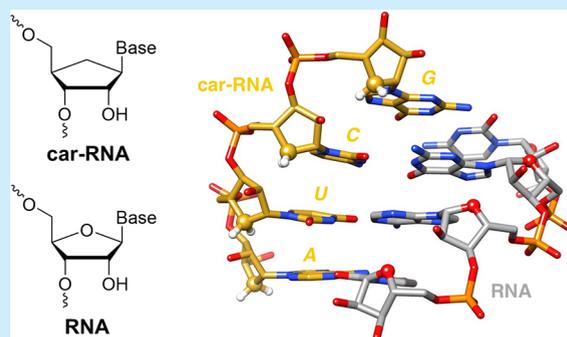
The pentofuranose ring 4′-oxygen and 2′-OH form critical intramolecular interactions within RNA strands and interactions with proteins through stereoelectronic effects and hydrogen bonding. For example, in therapeutics based on RNA interference (RNAi), antisense, splice modulation, mRNA vaccines,[1,2] gene editing,[3] and aptamers, these groups affect an eventual biological effect. The secondary and tertiary structures of RNA such as pseudoknots, loops, bulges, hairpins, and kissing complexes are also governed by the interactions arising from these two functional groups.[4] We are interested in how loss of the 4′-oxygen impacts RNA-based therapeutics and RNA structures.

Nucleosides that lack the 4′-oxygen have been characterized.[5] Compared to natural nucleosides, the carbocyclic 4′-CH$_2$ nucleosides have enhanced flexibility and lipophilicity and increased metabolic stability toward phosphorylases and hydrolases, which cleave the glycosidic bond of natural nucleosides. Additionally, the loss of the furanose oxygen results in an altered conformation of the pseudosugar ring due to the loss of the anomeric effect and gauche interactions between the 4′-oxygen and 2′- and 3′-OH groups.[5–9] In solution, the loss of the gauche and anomeric effects results in a relatively low-energy barrier between the pseudorotational forms of the cyclopentane ring relative to conventional nucleosides.[10]

Our laboratory has been interested in studying noncanonical nucleic acid modifications,[1] and we recently reported the synthesis of small interfering RNAs (siRNAs) containing 2′-fluorinated *northern*-methanocarbacyclic (2′-F-NMC) nucleotides (Figure 1A).[11,12] The 2′-F-NMC scaffold preorganizes the carbocyclic sugar into a pseudo-boat C2′-*exo* (north) conformation.[13] 2′-F-NMC was generally well tolerated in both the guide and passenger strands of siRNAs with some
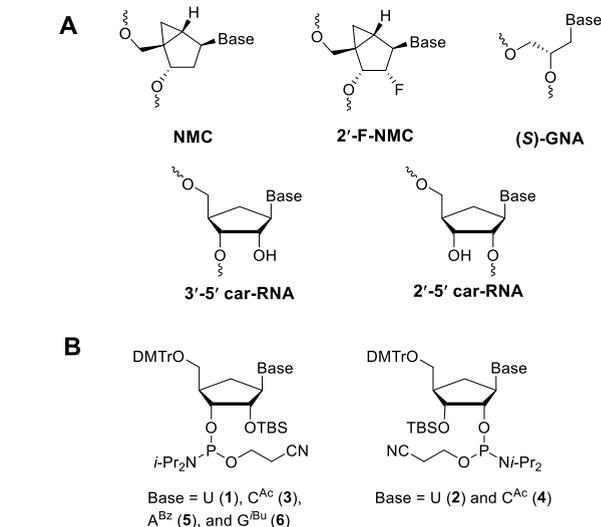

Figure 1. (A) Noncanonical nucleic acid modifications devoid of 4′-oxygen described in this work, NMC, 2′-F-NMC, (*S*)-GNA, and car-RNA residues. (B) Car-RNA 3′- and 2′-phosphoramidites **1**−**6**.
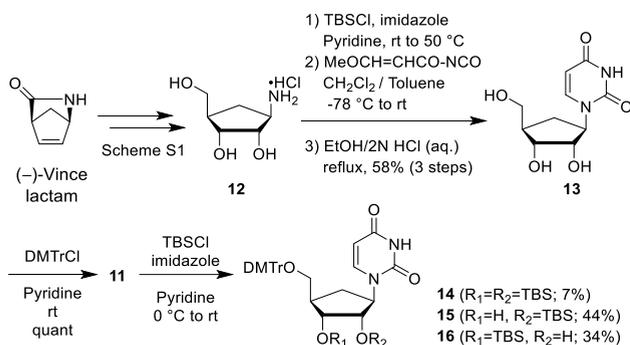
exceptions, such as positions 1 and 2 on the guide strand.[1,12] This prompted us to evaluate the carbocyclic nucleotides, the parent compounds of the NMC scaffold. We have also studied
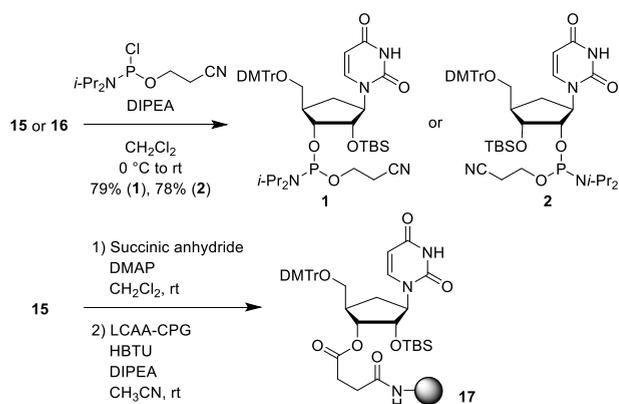
Scheme 1. Synthesis of 5′-DMTr-Protected *lyxo* and *ribo* Isomers 10 and 11
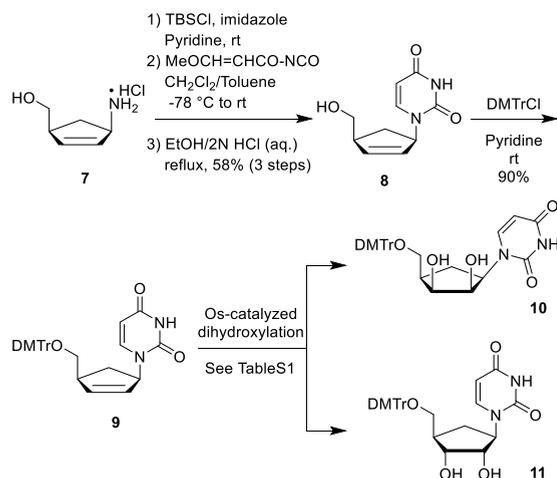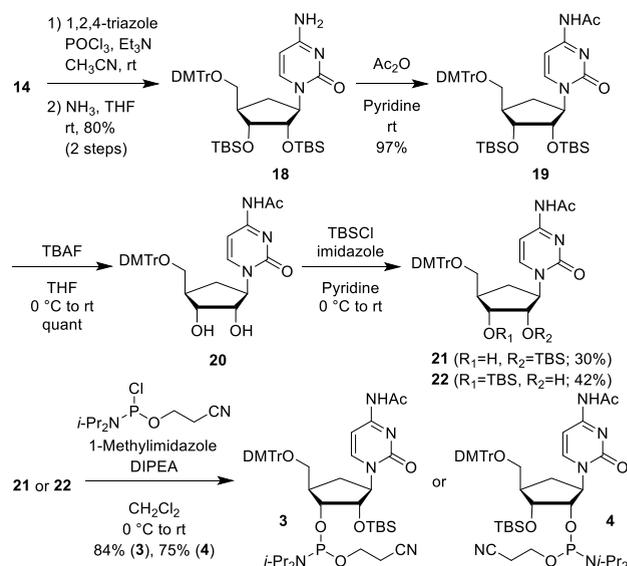

Scheme 2. Synthesis of 2′-O-TBS and/or 3′-O-TBS Carbocyclic Uridine Intermediates 14, 15, and 16 from (−)-Vince Lactam
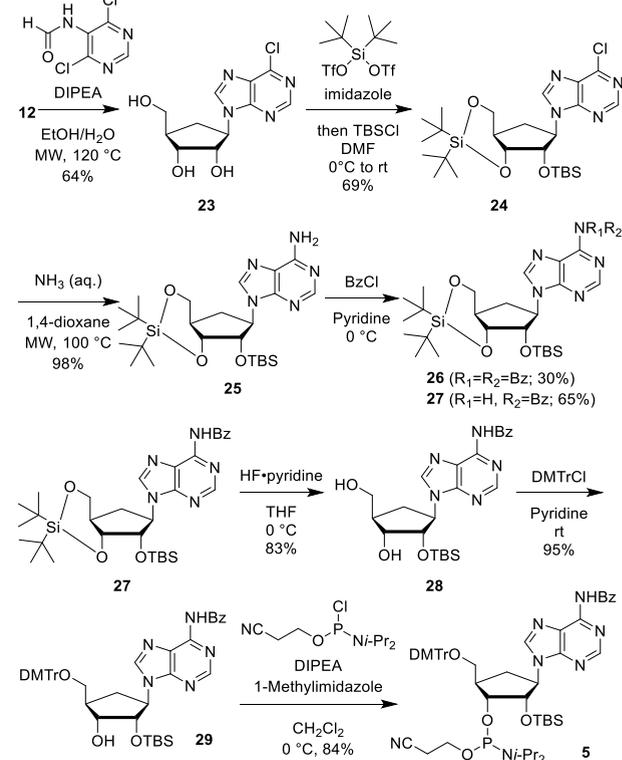

Scheme 3. Synthesis of Carbocyclic Uridine Phosphoramidites 1 and 2 and Solid Support 17

Scheme 4. Synthesis of Carbocyclic Cytidine Phosphoramidites 3 and 4

Scheme 5. Synthesis of Carbocyclic Adenosine 3′-Phosphoramidite 5

how modifications with low thermal binding affinity such as (S)-glycol nucleic acid ((S)-GNA), which is also devoid of the 4′-oxygen, mitigates off-target effects of siRNAs.[14]

To investigate the therapeutic potential of the car-RNA modification, car-RNA analogues bearing all four RNA nucleobases are required (Figure 1B). Here, we report the synthesis of six phosphoramidite building blocks of car-RNA from a common starting material in a convergent approach. We also report the synthesis of oligonucleotides containing

these building blocks and their thermodynamic binding affinities with DNA, RNA, and with car-RNA.

Syntheses of carbocyclic derivatives have been reported.[15−17] Fourrey et al. synthesized carbocyclic adenosine (aristeromycin) and cytidine 3′-phosphoramidites and incorporated them into a hammerhead ribozyme domain.[18] Their approach used Pd-catalyzed substitution of an allylic acetyl ester, with cytosine and adenine bases. This direct coupling is tedious, and a regioisomer can be produced by attack of N7 of the purine base. Another approach involves an

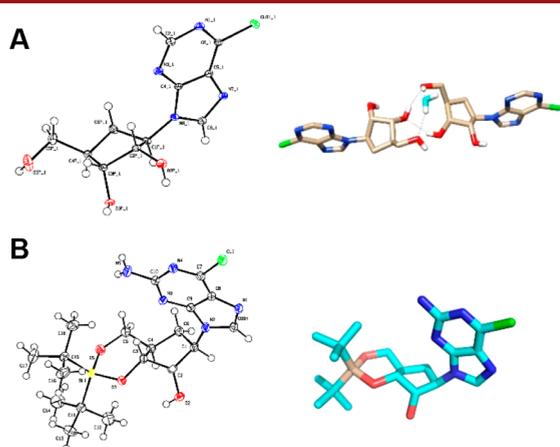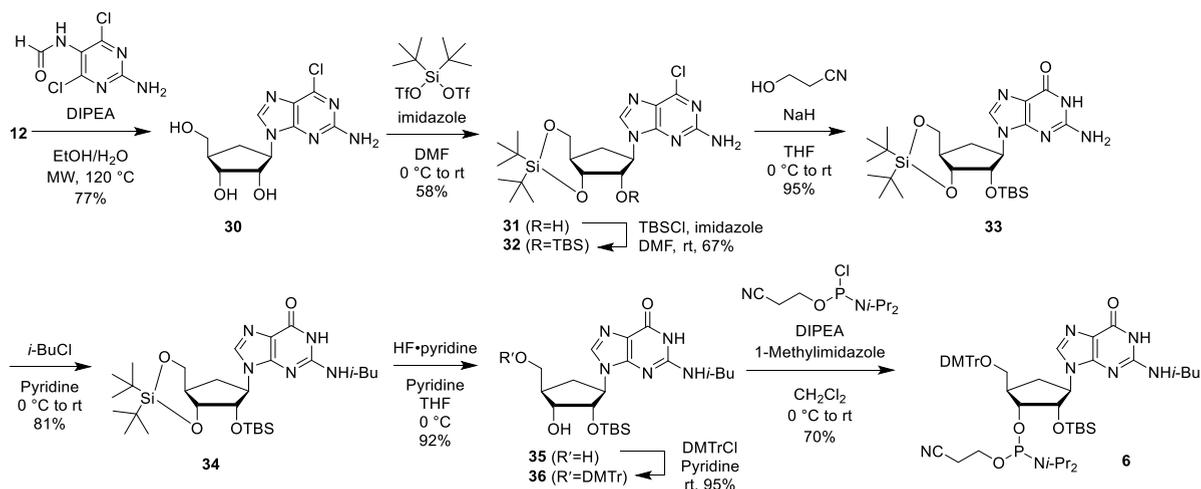## Scheme 6. Synthesis of Carbocyclic Guanosine 3′-Phosphoramidite 6

Figure 2. X-ray crystal structures of car-RNA building blocks. ORTEP style drawings with thermal ellipsoids (left) and the contents of the asymmetric unit (right) for (A) compound **23** and (B) compound **31**. Atoms are colored red, blue, white, and green for oxygen, nitrogen, hydrogen, and chlorine, respectively For compound **23**, carbon is in tan; for compound **31**, carbon is in cyan. The ellipsoid contour % probability levels are 50%.

aminocyclopenta(e)ne, synthesized from (±)-2-azabicyclo[2.2.1]hept-5-en-3-one, also known as the Vince lactam.[19] The racemic lactam can readily be converted to the optically pure intermediate, which has the same configuration as the carbocyclic nucleosides, through enzymatic resolution,[20] and this intermediate can serve as the precursor for various carbocyclic nucleosides.[21]

As depicted in Scheme 1, the synthesis of car-RNA uridine started from aminocyclopentene **7**.[19,22] The olefin **7** was coupled with 3-methoxyacryloyl isocyanate, prepared in situ from 3-methoxyacrylic acid, and cyclized under acidic conditions to afford uridine derivative **8** as described.[23,24] The olefin **8** was reacted with 4,4′-dimethoxytriphenylmethyl (DMTr) chloride to give the protected compound **9**. To introduce 2′- and 3′-OH groups to the olefin, we attempted osmium-catalyzed dihydroxylation as previously described (Table S1).[25,26] However, the dihydroxylation conditions, including those of AD-mix α and β, yielded *lyxo* and *ribo* isomers **10** and **11** as a mixture, and the desired compound **11** was not preferentially obtained.[27]

To overcome the difficulty of separation of **10** and **11**, we synthesized **11** from aminotriol **12**, prepared from (−)-Vince lactam (Scheme 2 and Scheme S1). **12** was treated with TBSCl to protect the hydroxyl groups and to improve the solubility in organic solvent. The resulting silyl-protected compound was then coupled with 3-methoxyacryloyl isocyanate and cyclized under acidic conditions to yield uridine nucleoside **13**. Selective DMTr protection of the 5′-OH group of **13** gave diol **11**. The 2′- or/and 3′-OH groups of **11** were protected with TBSCl to obtain intermediates **14**, **15**, and **16** (Scheme 2). Phosphitylation of **15** and **16** produced the phosphoramidites **1** and **2**, respectively (Scheme 3). **15** was reacted with succinic anhydride in the presence of DMAP, followed by coupling to CPG support functionalized with long chain amino alkyl to obtain solid support **17**.

To synthesize the cytidine analogue of car-RNA, protected uridine **14** was converted into the corresponding cytidine **18** by reacting with 1,2,4-triazole in the presence of Et₃N and POCl₃, followed by treatment with aqueous NH₃ (Scheme 4). The exocyclic amine of **18** was acetylated by acetic anhydride, and the TBS groups of the obtained N-acetyl cytidine **19** were removed using tetra-n-butylammonium fluoride (TBAF) to yield 2′,3′-diol **20**. The 2′- or 3′-OH groups of **20** were reprotected with TBSCl to yield monosilyl compounds **21** and **22**. Phosphitylation of **21** and **22** afforded the phosphoramidites **3** and **4**, respectively.

To construct the car-RNA purine monomers on amine **12**, we utilized a microwave-assisted, one-pot procedure to obtain 6-chloro- or 2-amino-6-chloropurines as previously reported.[11] As shown in Scheme 5, the microwave-assisted reaction of **12** with 4,6-dichloro-5-formamidopyrimidine gave carbocyclic 6-chloropurine nucleoside **23**, which was treated with di-*tert*-butylsilyl bis(trifluoromethanesulfonate) to protect 5′- and 3′-OH groups, followed by protection of the 2′-OH group with TBSCl.[23] Ammonolysis of the obtained compound **24** using a microwave reactor produced adenine-functionalized **25**. The exocyclic amino group was protected with benzoyl chloride to furnish the dibenzoyl compound **26** and the monobenzoyl compound **27**. Selective deprotection of the di-*tert*-butylsilyl group of **27** was achieved with HF-pyridine at 0 °C,[23] resulting in 2′-O-TBS nucleoside **28**. Subsequent dimethoxytritylation of the 5′-OH followed by phosphitylation of **29** yielded the adenosine 3′-phosphoramidite **5**.

Table 1. UV Melting Temperatures of Duplexes with a Car-RNA-Modified Strand

duplex	sequence	T_m^a (°C)	ΔT_m^b (°C)
1	5′-r(UACAG**U**CUAUGU) 3′-r(AUGUCAGAUACA)	46.3 ± 0.0	−5.0
2	5′-r(UACAGU**C**UAUGU) 3′-r(AUGUCAGAUACA)	46.3 ± 0.0	−5.0
3	5′-r(UACAGUCUAUGU) 3′-r(AUGUC**A**GAUACA)	47.4 ± 0.1	−3.9
4	5′-r(UACAGUCUAUGU) 3′-r(AUGUCA**G**AUACA)	46.4 ± 0.0	−4.9
5	5′-r(**UACAGUCUAUGU**) 3′-r(AUGUCAGAUACA)	32.7 ± 0.3	−18.6
6	5′-r(UACAGUCUAUGU) 3′-r(**AUGUCAGAUACA**)	32.2 ± 0.4	−19.1
7	5′-r(**UACAGUCUAUGU**) 3′-r(**AUGUCAGAUACA**)	nd	nd
8	5′-r(UACAG**U**CUAUGU) 3′-d(ATGTCAGATACA)	32.6 ± 0.3	−6.8
9	5′-r(UACAGU**C**UAUGU) 3′-d(ATGTCAGATACA)	31.2 ± 0.5	−8.3
10	5′-d(TACAGTCTATGT) 3′-r(AUGUC**A**GAUACA)	36.5 ± 0.3	−4.4
11	5′-d(TACAGTCTATGT) 3′-r(AUGUCA**G**AUACA)	34.6 ± 0.3	−6.3
12	5′-r(**UACAGUCUAUGU**) 3′-d(ATGTCAGATACA)	nd	nd
13	5′-d(TACAGTCTATGT) 3′-r(**AUGUCAGAUACA**)	nd	nd

$^a T_m$ (mean ± standard deviation) values were obtained in PBS (pH 7.4) using 2.0 μM concentrations of each strand and are averages of two independent experiments. Bold red letters indicate sites of car-RNA modification. $^b \Delta T_m$ is the difference in melting temperature between the duplex with the modified strand and the unmodified reference duplex: 5′-r(UACAGUCUAUGU)-3′:3′-r-(AUGUCAGAUACA)-5′ (T_m = 51.3 °C) for duplexes 1−7, 5′-r(UACAGUCUAUGU)-3′:3′-d(ATGTCAGATACA)-5′ (T_m = 39.4 °C) for duplexes 8, 9, and 12, and 5′-d(TACAGTCTATGT)-3′:3′-r(AUGUCAGAUACA)-5′ (T_m = 40.9 °C) for duplexes 10, 11, and 13; nd = melting transition not detectable.

Synthesis of the guanosine 3′-phosphoramidite **6** was accomplished, as shown in Scheme 6. The microwave-assisted reaction of the amine **12** with 2-amino-4,6-dichloro-5-formamidopyrimidine afforded carbocyclic 2-amino-6-chloropurine **30**, a guanosine precursor. The hydroxyl groups of **30** were protected by treatment with di-*tert*-butylsilyl bis-(trifluoromethanesulfonate), yielding **31**, and subsequently with TBSCl to obtain silyl-protected compound **32**. The precursor **32** was converted to guanosine nucleoside **33** by reacting with 3-hydroxypropionitrile in the presence of NaH.

Protection of the exocyclic amine using iso-butyryl chloride (*i*-BuCl) yielded fully protected nucleoside **34**. The di-*tert*-butylsilyl group of **34** was selectively removed using HF-pyridine to obtain 2′-O-TBS nucleoside **35** in 92% yield. Dimethoxytritylation of diol **35** gave 5′-O-DMTr intermediate **36**, which was converted to the corresponding 3′-phosphoramidite **6**.

To confirm the stereochemistry of these building blocks, an X-ray crystallographic analysis of the intermediates was performed. Analyses of **23** and **31** revealed expected chemistries and configurations. The crystallographic asymmetric unit of **23** contains two independent molecules that dimerize via their 2′- and 3′-hydroxyl groups (Figure 2A). Both nucleosides adopt the 1′-*exo* pucker. In the structure of **31**, the single nucleoside per asymmetric unit adopts the 3′-*endo* pucker forced by the simultaneous protection of 5′- and 3′-OH groups (Figure 2B). The parent compound **30** without the 5′ and 3′ protecting groups may adopt a different sugar conformation.

To evaluate the effect of the carbocyclic modification on thermodynamic stabilities of RNA/RNA and RNA/DNA duplexes, the building blocks **1**, **3**, **5**, and **6** were incorporated into oligoribonucleotides via standard solid-phase synthesis. The synthesized oligonucleotides were annealed with complementary RNA or DNA in PBS, and the melting temperatures (T_m) were compared with those of the corresponding unmodified duplexes. A single car-RNA nucleotide at the center of an RNA homoduplex resulted in a decrease of 4 to 5 °C in the melting temperature compared to that in the unmodified duplex (Table 1, duplexes 1−4). Multiple incorporations of car-RNA nucleotides resulted in decreases of 18 to 19 °C in the melting temperatures of the duplexes with complementary unmodified RNA. No melting transition was detected when the strand fully modified with car-RNA was mixed with the complementary RNA (duplex 7). The modified RNA strands containing a single car-RNA nucleotide also destabilized the heteroduplex with DNA to extents similar to or higher than those of the destabilization of the RNA homoduplex (duplexes 8−11). No duplex binding was observed from the fully car-RNA strand either with the complementary DNA or RNA strand (duplexes 12 and 13). Car-RNAs bind more stably to RNA than to DNA. Fully modified car-RNAs can form duplexes with native RNA but not with a complementary car-RNA.

The global conformations of modified RNA/RNA and RNA/DNA duplexes containing single or multiple car-RNA nucleotides were evaluated using circular dichroism (CD) spectroscopy. In CD spectra of modified RNA/RNA duplexes 1−6 and the unmodified duplex, there was a strong positive band at around 260 nm and a negative band at around 210 nm, which is characteristic of an A-form duplex (Figure S1A). There was no CD signal from the sample of duplex 7, suggesting that a duplex was not formed, consistent with the lack of a melting transition. The CD spectra of modified RNA/DNA duplexes 8−11 also suggest formation of A-form duplexes (Figure S1B). Spectra of duplexes 12 and 13 were more typical of a B-like helix. These data indicate that although a single car-RNA residue does not significantly distort the global geometry of RNA/RNA or RNA/DNA duplexes, multiple incorporations can significantly affect the duplex formation. These interesting findings and the study of corresponding (2′−5′) RNAs deserve follow-up, which will be enabled by the synthetic methods described here.

In summary, we present efficient routes for the synthesis of car-RNA 2′- and 3′-phosphoramidites and a uridine solid support. The configurations of the intermediates were confirmed by X-ray crystallographic analysis. The car-RNA residues were incorporated into oligonucleotides using standard solid-phase synthesis. A single car-RNA thermally destabilized duplexes with both complementary RNA and DNA without altering the global structures of the duplexes. The car-(3′−5′)-RNA and car-(2′−5′)-RNA building blocks will allow us to incorporate these modifications into RNA-based therapeutics to explore their effects on metabolic stability, cellular uptake, and pharmacological properties. The placement of single thermally destabilizing modification mitigates off-target effects due to binding of the siRNA guide strand to nontargeted unrelated mRNAs.[14] Thermally destabilizing modifications may also facilitate allele-specific targeting and product release from mRNA-antisense duplexes after mRNA cleavage. Finally, car-RNAs and their related analogues (e.g., 2′-OMe) can be evaluated in RNA-based therapeutics. The car-RNA modification should also prove useful in studies of the relationship between RNA structure and function as substitution with this residue will shed light on the importance of the 4′-oxygen to secondary and tertiary structures of RNA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.1c03936.

Experimental, compound characterization, and assays (PDF)

Accession Codes

CCDC 2082820 and 2082823 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Author Contributions

The manuscript was written through contributions of all authors.

Notes

The authors declare no competing financial interest.

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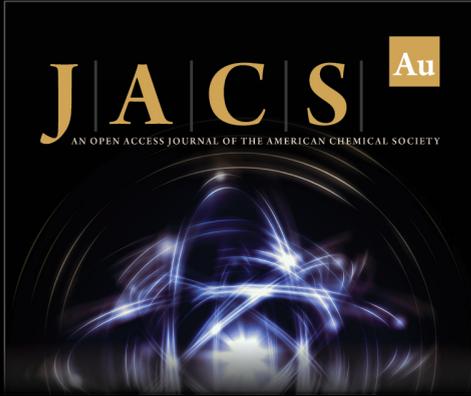
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