ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2021, 57, 6808

Received 30th April 2021,
Accepted 15th June 2021

DOI: 10.1039/d1cc02328d

rsc.li/chemcomm

Synthesis of a novel cyclopropyl phosphonate nucleotide as a phosphate mimic†

Erich F. Altenhofer, *[a] Michael J. Lawler,[a] Pankaj Kumar,[a] Leo A. Joyce,[a] Matthew Fowler-Watters,[a] Tao Pei[a] and Zhen Li[b]

The inherent *in vivo* instability of oligonucleotides presents one of many challenges in the development of RNAi-based therapeutics. Chemical modification to the 5′-terminus serves as an existing paradigm which can make phosphorylated antisense strands less prone to degradation by endogenous enzymes. It has been recently shown that installation of 5′-cyclopropyl phosphonate on the terminus of an oligonucleotide results in greater knockdown of a targeted protein when compared to its unmodified phosphate derivative. In this paper we report the synthesis of a 5′-modified uridine.

RNAi-based therapeutics have received increasing attention in the last few decades, especially as drug candidates for diseases that are difficult to access with small molecules. Chemical modification of oligonucleotides is an effective strategy to increase selectivity, *in vivo* stability, and accumulation in tissue.[1] To direct the therapeutic to a cell of interest, small molecules serving as targeting ligands can be conjugated to a single strand RNA (ss-RNA). For example, *N*-acetylgalactosamine (GalNAc or NAG) has been shown to be highly efficient in delivering exogenous RNA to hepatocyte cells.[2] Additionally, chemical modifications to nucleotides can greatly impact the stability of ss-RNA. Specifically, the use of 2′-fluoro and 2′-methoxy modified nucleotides has been shown to enhance stability of antisense (guide) strands *in vivo*.[3] This work primarily focuses on the chemical modification of the oligonucleotide at the 5′-terminus as a means to increase the metabolic stability.

Once an exogenously administered double-stranded RNAi trigger (*e.g.*, an siRNA or dsRNA) is internalized in the cell type of interest, a cascade of processes must occur before gene expression can be silenced. The RNAi trigger loads onto the RNA-induced silencing complex (RISC) where the duplex is unwound, and the guide strand binds to exonuclease Argonaute-2 (Ago2) protein *via* 5′-phosphate interaction.[4] The RISC complex hybridizes with a complementary, targeted messenger RNA (mRNA) and exonuclease Ago2 cleaves the targeted mRNA. Before this can successfully happen a 5′-phosphorylation of the externally delivered RNAi trigger must occur to enable RISC loading. Phosphorylation of the 5′-alcohol occurs through kinase Clp1.[5] The *in vivo* stability of the newly formed 5′-phosphate plays a key role in the propagation of the RNAi pathway.

Upon phosphorylation, the guide strand is prone to enzymatic degradation. The labile P–O bond at the 5′-terminus of the guide strand (Fig. 1, **1a**) is prone to cleavage by phosphatases, leading to degradation before entering the RNAi pathway.[6] Additionally, exonucleases cleave monomeric units of the oligonucleotide which also reduces efficacy of the therapeutic. Chemical modification of the terminal phosphate serves as an existing approach to prevent these two degradation pathways.[7]

A cyclopropyl phosphonate moiety in place of the 5′-terminal phosphate addresses this shortcoming by removing the necessity for intracellular phosphorylation of the guide strand and increasing the metabolic stability of the strand (Fig. 1, **1b**). In a head-to-head study, the 5′-cyclopropyl phosphonate was

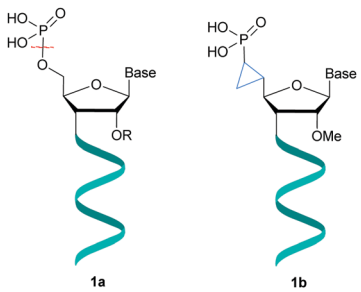
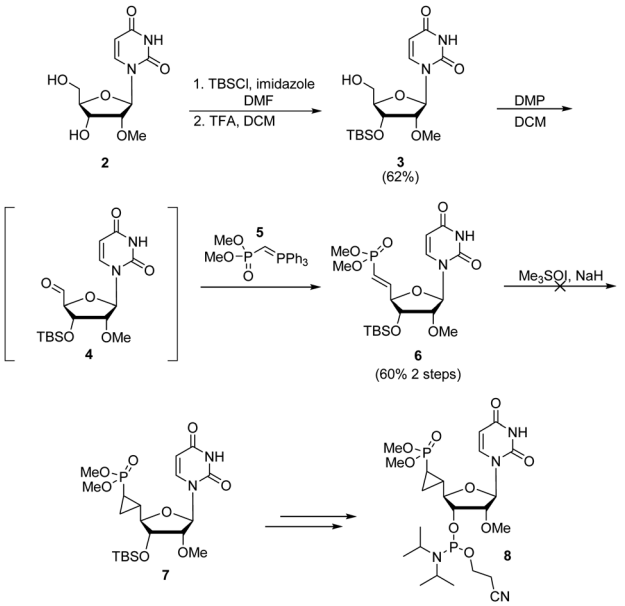


Fig. 1 Chemical modification of the 5′-terminus of ss-RNA.

[a] *Arrowhead Pharmaceuticals, Inc., 502 S Rosa Rd, Madison, WI 53705, USA. E-mail: ealtenhofer@arrowheadpharma.com*
[b] *5871 Oberlin Drive, San Diego, CA 92121, USA*

† Electronic supplementary information (ESI) available: Detailed experimental procedures and compound characterization data. CCDC 2048176. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/d1cc02328d



Scheme 1 Original route to access 5′-modified uridine **8**.

observed to achieve a greater knockdown of a targeted protein than its 5′-phosphate derivative.[8] Herein, we describe the synthesis of a 5′-modified uridine which was achieved through a novel cyclopropanation reaction.

Synthesis of oligonucleotides is typically carried out on solid phase, coupling a 3′-phosphoramidite in solution with a resin bound 5′-OH nucleoside. Using this traditional phosphoramidite chemistry to incorporate a 5′-cyclopropyl phosphonate onto the terminus of the antisense strand would require synthesis of **8** containing a phosphoramidite moiety at the 3′-position of the ribose (Scheme 1). Commercially available 2′-OMe uridine **2** was converted to 3′-OTBS **3** through a bis-TBS protection, followed by selective TBS mono-deprotection at the 5′-position.[9] Oxidation of resulting 5′-alcohol with Dess–Martin periodinane (DMP) resulted in clean conversion to aldehyde **4**. Running the oxidation at 0 °C with an equimolar amount of DMP was key to completely consuming **3**, while avoiding over-oxidation of the aldehyde to a carboxylic acid.

Unfortunately, the aldehyde was found to decompose during aqueous workup and could not be isolated. The crude solution of aldehyde was then treated with ylide **5**, affording the *E*-vinylphosphonate **6** exclusively in 60% yield over two steps. This sequence is an improvement over Horner–Wadsworth–Emmons chemistry that gives an inseparable 9 : 1 *E*/*Z* mixture of the vinylphosphonate. During the scale up synthesis of dimethyl hydroxymethylphosphonate, an intermediate to compound **5**, a runaway exotherm was observed. The original procedure heated a neat mixture of reagents until the reaction initiated, causing an uncontrollable exotherm.[10] This necessitated modification of the process to improve safety parameters. To that end, methanol was added as a solvent to aid in heat distribution and eliminate hot spots. Furthermore, a dose-controlled addition of dimethylphosphite at 50 °C prevented reagent accumulation, providing a more controlled process (see ESI†).

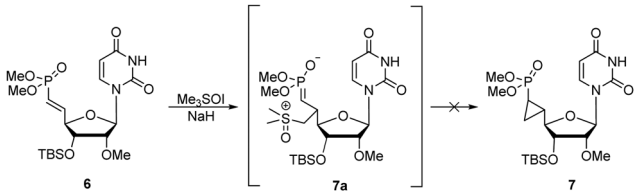
With compound **6** in hand, the key step to this route relied upon a cyclopropanation of the olefin adjacent to the phosphonate. Although the cyclopropanation of diethyl vinylphosphonate is known,[11] it was unclear whether chemoselectivity issues would arise from the neighboring nucleobase. Side reactions such as *N*-methylation or cyclopropanation of the vinyl group on uracil would be problematic.

After initial screening experiments failed, it became clear that the cyclopropanation of a 5′-vinylphosphonate was not trivial. Simmons–Smith chemistry[12] and other carbene reagents were either unreactive with vinylphosphonate **6** or resulted in substrate decomposition. The most promising methodology involved Corey–Chaykovsky conditions[13] using sulfoxonium salts which were observed to give clean conversion of **6** to a more polar product, though unfortunately not the desired cyclopropyl derivative **7**. This methodology was investigated further since it was the only set of conditions that gave clean conversion of compound **6**. Upon closer examination of the crude reaction mixture by ^{1}H NMR, the vinylphosphonate protons of **6** were no longer present (see ESI†), indicating that reaction of the α,β-unsaturated system had indeed occurred. The reaction was found to be highly chemoselective, as all uracil protons were still intact.

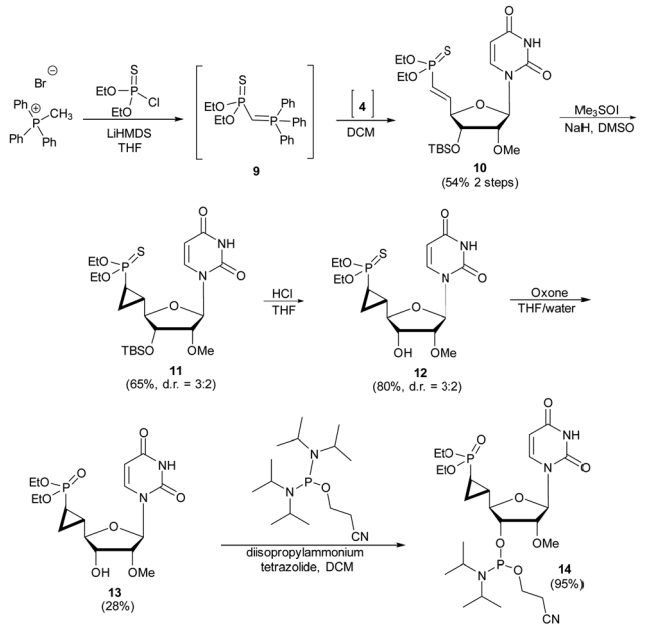
Mass spectrometry (MS) data were inconclusive, however, as the base peak observed was [M] + 78 rather than the expected [M] + 1. It was unclear whether this was a result of a DMSO solvent adduct of the desired product **7**, or the molecular ion for a different higher molecular weight species. The ^{1}H NMR spectrum showed an absence of signals around 1 ppm where cyclopropyl protons would be expected to resonate. Given these data, it was hypothesized that the cyclopropanation stalled at intermediate **7a** (Scheme 2). This intermediate was found to be highly stable and could not be converted to the desired product even at elevated temperatures. Attempts to isolate and characterize the intermediate were unsuccessful as it could not be extracted out of the DMSO reaction solvent.

The electron deficient character of the olefin that made 1,4-addition proceed very rapidly unfortunately also seemed to stabilize intermediate **7a** and prevent conversion to desired product **7**. It was hypothesized that replacing the oxygen atom with less electronegative sulfur would further delocalize the electron density across the olefin and allow displacement of the dimethylsulfoxonium adduct to give the desired cyclopropane product. We therefore envisioned the synthesis of vinyl thiophosphonate derivative **10** (Scheme 3).

Direct conversion of vinylphosphonate **6** to the corresponding vinyl thiophosphonate **10** would appear to be a



Scheme 2 Formation of locked 1,4-adduct.



Scheme 3 Revised route to access cyclopropyl phosphoramidite **14**.

reasonable solution. However, using Lawesson's reagent on large scale was not desirable. Having a well-defined Wittig olefination procedure optimized for aldehyde **4**, a route which used a thiophosphonate analogue of ylide **5** was explored. Due to limited commercial availability of *O*,*O*′-dimethyl chlorothiophosphate, the ethyl thioester analogue was used to make compound **9**. To our knowledge, this is the first reported synthesis and application of this unknown ylide, although a similar phenyl derivative[14] has been reported. The stabilized ylide was found to exist as an amorphous solid which complicated any attempts at purification. Nevertheless, the ylide was found to be stable for several months at 5 °C and the crude amorphous solids were used to carry out the next step.

To circumvent the previously mentioned instability of aldehyde **4**, it was generated *in situ* and reacted with the corresponding ylide, leading to clean conversion to *E*-vinyl thiophosphonate **10**. Similar to our previous findings, ^{1}H NMR did not show evidence of *Z*-isomer of vinyl thiophosphonate **10**. Subjecting *E*-vinyl thiophosphonate **10** to Corey–Chaykovsky conditions led to clean conversion to the desired cyclopropyl thiophosphonate **11** in 65% yield with a 3 : 2 diastereomeric ratio. Previously mentioned side reactions such as *N*-methylation and cyclopropanation on uracil were not detected during analysis of crude ^{1}H NMR spectra. After a search of current literature, this appears to be the only documented cyclopropanation of a vinylphosphonate using a sulphur ylide. Efforts to increase the diastereoselectivity, using different trialkylsulfoxonium reagents were explored, but in all cases, the 1,4-addition did not occur.

Deprotection of the 3′OTBS group with hydrochloric acid resulted in clean conversion of **11** to corresponding alcohol **12**. Oxidation of thiophosphonate **12** was first explored with Oxone, as it is a relatively safe oxidant and its application spans a wide range of functional groups.[15] Conversion of **12** to phosphonate

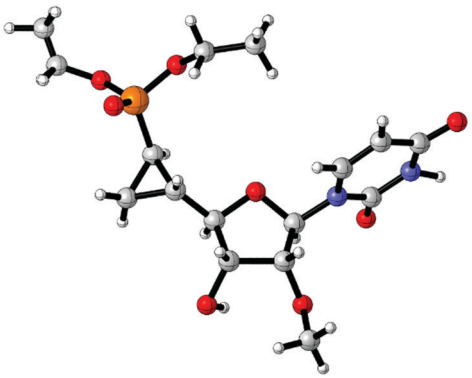


Fig. 2 X-Ray structure of compound **13** (atoms: carbon is gray, hydrogen is white, oxygen is red, nitrogen is blue, and phosphorus is orange).

13 was observed in under four hours, which to the best of our knowledge, constitutes a novel use of Oxone.

After workup, compound **13** was found to be crystalline, providing an opportunity to further purify the crude material without using silica. The penultimate compound was screened for solubility in a variety of solvents and appeared to be insoluble in ethyl acetate. Analysis of the undissolved solids by ^{31}P NMR showed enrichment of the major diastereomer. After some development, the major diastereomer could be enriched with an ethyl acetate trituration giving diastereomeric purities greater than 90%. To further improve the diastereomeric purity, using a 4 : 6 mixture of ethanol and heptane, the solids could be recrystallized allowing for isolation of **13** as a single diastereomer. The absolute stereochemistry around the cyclopropyl group was then confirmed by X-ray crystallography (Fig. 2).

To complete the synthesis, only the major diastereomer was carried through to the final step. Using optimized phosphitylation conditions, the penultimate alcohol **13** underwent clean conversion to phosphoramidite **14** as a 1 : 1 mixture of diastereomers resulting from installation of the new phosphorus stereocenter.

In conclusion, chemical modification of the 5′-terminus of oligonucleotides constitutes a promising methodology to impart increased stability and promote enhanced accumulation in tissues. The present work improves upon existing synthetic procedures towards the synthesis of 5′-vinylphosphonate precursors, allowing access to these substrates. Most importantly, a novel cyclopropanation reaction on a vinyl thiophosphonate has been described. Cyclopropanation has been accomplished by tuning the electronics of the phosphonate, which nicely illustrates the power of fundamental chemical knowledge in solving synthetic challenges.

Conflicts of interest

Arrowhead Pharmaceuticals, Inc. has filed a patent application covering the process: U.S. patent application no. PCT/US2017/036108.

Notes and references

1 (*a*) R. Haraszti, L. Roux, A. H. Coles, A. A. Turanov, J. F. Alterman, D. Echeverria, B. M. D. C. Godinho, N. Aronin and A. Khvorova, *Nucleic Acids Res.*, 2017, **45**, 7581; (*b*) G. F. Deleavey and M. J. Damha, *Chem. Biol.*, 2012, **19**, 937.
2 A. D. Springer and S. F. Dowdy, *Nucleic Acid Ther.*, 2018, **28**, 109.
3 C. R. Allerson, N. Sioufi, R. Jarres, T. P. Prakash, N. Naik, A. Berdeja, L. Wanders, R. H. Griffey, E. E. Swayze and B. Bhat, *J. Med. Chem.*, 2005, **48**, 901.
4 (*a*) S. M. Hammond, E. Bernstein, D. Beach and G. J. Hannon, *Nature*, 2000, **404**, 293; (*b*) A. Khvorova, A. Reynolds and S. D. Jayasena, *Cell*, 2003, **115**, 209; (*c*) A. L. Jackson, S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet and P. S. Linsley, *Nat. Biotechnol.*, 2003, **21**, 635.
5 M. Manoharan, *Curr. Opin. Chem. Biol.*, 2004, **8**, 570.
6 A. Khvorova and J. K. Watts, *Nat. Biotechnol.*, 2017, **35**, 238.
7 (*a*) R. Parmar, J. L. S. Willoughby, J. Liu, D. J. Foster, B. Brigham, C. S. Theile, K. Charisse, A. Akinc, E. Guidry, Y. Pei, W. Strapps, M. Cancilla, M. G. Stanton, K. G. Rajeev, L. Sepp-Lorenzino, M. Manoharan, R. Meyers, M. A. Maier and V. Jadhav, *ChemBioChem*, 2016, **17**, 985; (*b*) W. F. Lima, T. P. Prakash, H. M. Murray, G. A. Kinberger, W. Li, A. E. Chappell, C. S. Li, S. F. Murray, H. Gaus, P. P. Seth, E. E. Swayze and S. T. Crooke, *Cell*, 2012, **150**, 883; (*c*) W. Chang, Y. Pei, E. N. Guidry, D. Zewge, C. A. Parish, E. C. Sherer, J. DiMuzio, H. Zhang, V. J. South, W. R. Strapps, L. Sepp-Lorenzino, S. L. Colletti and M. G. Stanton, *Bioorg. Med. Chem. Lett.*, 2016, **26**, 4513; (*d*) T. P. Prakash, W. F. Lima, H. M. Murray, W. Li, G. A. Kinberger, A. E. Chappell, H. Gaus, P. P. Seth, B. Bhat, S. T. Crooke and E. E. Swayze, *Nucleic Acids Res.*, 2015, **43**, 2993.
8 Z. Li, T. Pei and M. Lawler, *PCT International Publication*, n.d., WO2017/214112A1, 2017.
9 A. V. Kel'in, I. Zlatev, J. Harp, M. Jayarman, A. Bisbe, J. O'Shea, N. Taneja, R. M. Monoharan, S. Khan, K. Charisse, M. A. Maier, M. Egli, K. G. Rajeev and M. Monoharan, *J. Org. Chem.*, 2016, **81**, 2261.
10 (*a*) J. Gajewiak, Y. Xu, S. A. Lee, T. G. Kutateladze and G. D. Prestwich, *Org. Lett.*, 2006, **8**, 2811; (*b*) D. P. Phillion and S. S. Andrew, *Tetrahedron Lett.*, 1986, **27**, 1477.
11 L. Tokes and G. Jones, *Org. Mass Spectrom.*, 1975, **10**, 241.
12 H. E. Simmons and R. D. Smith, *J. Am. Chem. Soc.*, 1958, **80**, 5323.
13 E. J. Corey and M. Chaykovsky, *J. Am. Chem. Soc.*, 1965, **87**, 1353.
14 H. Run-Qiu, D. She-Shiang and Y. Shi-Xian, *Acta Chim. Sin.*, 1989, **7**, 64.
15 A. V. Narsaiah, *Synlett*, 2002, 1178.