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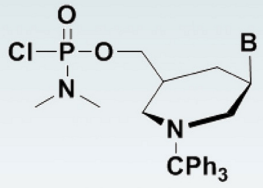
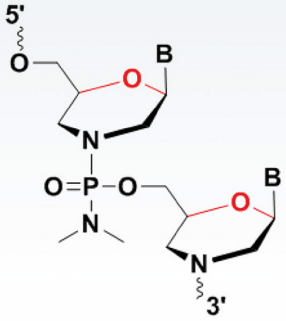
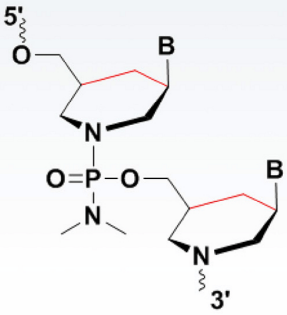


B = U, C^{Ac}, A^{Bz}, $G^{i\text{-}Bu}$

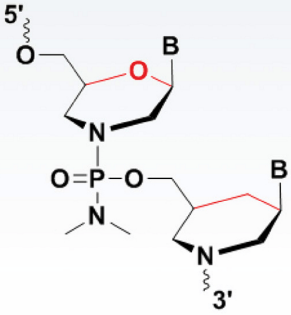
Chlorophosphoramidate
Piperidino monomer



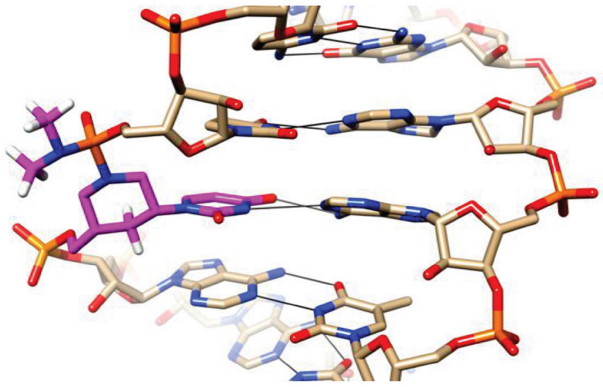
Phosphorodiamidate
Morpholino
Oligomer (PMO)



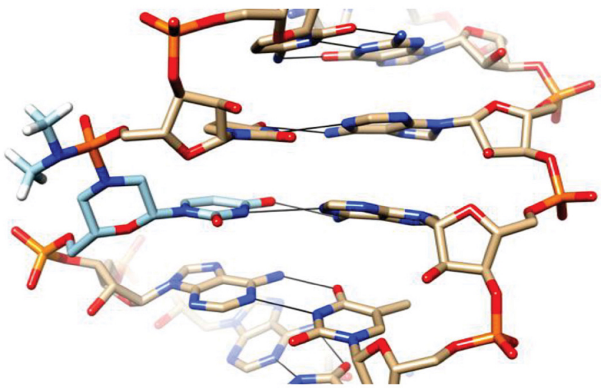
Piperidino
Phosphorodiamidate
Oligomer (PPO)



PMO-PPO
Chimera



Pip-U



PMO-U

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Synthesis and Biophysical Properties of Phosphorodiamidate Piperidino Oligomers

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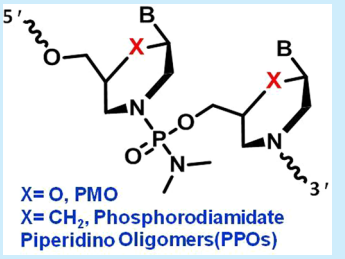
ABSTRACT: We report the synthesis of piperidino nucleoside phosphoramidates functionalized with uracil, cytosine, guanine, and adenine and their incorporation into oligomers. High-performance liquid chromatography analyses demonstrated that a phosphorodiamidate piperidino oligomer (PPO) is more lipophilic than a phosphorodiamidate morpholino oligomer (PMO) of the same tetrameric sequence. A PMO containing piperidino residues formed duplexes with both DNA and RNA, and the PPO had higher stability at endosomolytic pH and higher hydrophobicity than the PMO.



Phosphorodiamidate morpholino oligomers (PMOs), which have a neutral backbone, were developed for use in oligonucleotide-based therapeutics.[1,2] To date, four PMO-based drugs, eteplirsen, golodirsen, viltolarsen, and casimersen, have been approved by the U.S. Food and Drug Administration (FDA); these agents are used to treat patients with Duchenne muscular dystrophy.[3] PMOs have also been shown to be effective against viral and bacterial infections and cancers in cell-based and preclinical models.[4,5] In PMO, the five-membered ribose sugar unit of a natural nucleotide is replaced by a morpholino ring. Carbocyclic nucleosides, in which the furanose ring oxygen is replaced by a CH_2 group, have also been reported and have been approved as antitumor and antiviral therapies.[2,6] Carbocyclic modifications of oligonucleotides enhance resistance against nuclease degradation and stabilize the glycosidic bond against hydrolytic cleavage.

PMOs must be chemically modified to improve cellular uptake and pharmacokinetics.[7,8] Among the reported modifications, incorporation of a guanidinium linkage or guanidinium-functionalized nucleobase is notable.[9] Sinha's group demonstrated the cell-penetrating and gene-silencing properties of self-transfecting guanidinium morpholino−PMO chimeras in cell culture and zebrafish.[9d] The Hayes group reported that triazole-linked morpholino−DNA chimeras are resistant to enzymatic degradation.[10] Caruthers's group developed thiophosphoramidate morpholino oligomers and their phosphorothioate DNA chimeras.[8] Recently, Borbás's group reported alkylamine-linked, cationic morpholino analogues.[11]

Here, we report the synthesis of phosphorodiamidate piperidino oligomers (PPOs). The four piperidino nucleoside monomers, pip-A, pip-U, pip-G, and pip-C, and corresponding chlorophosphoramidate monomers were prepared (Figure 1A). These were incorporated into oligomers to evaluate the stability of their duplexes with DNA and RNA as well as their conformational properties using circular dichroism (CD) spectroscopy.

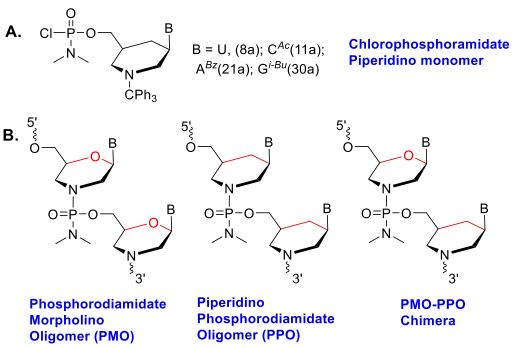


Figure 1. (A) Structure of chlorophosphoramidate piperidino monomers. (B) Structures of PMO and PPO and the PMO−PPO chimera.

The pip monomers were synthesized from commercially available cyclopentenyl-amino-methanol **1**. Car-U **2** was synthesized following the procedure previously reported for synthesis of carbocyclic RNA (Scheme 1).[2c] Silyl protection and then double bond oxidation of compound **3** with OsO_4 gave diol **4**. Diol cleavage by $NaIO_4$ and subsequent reductive cyclization with benzylamine gave *N*-benzyl pip-U monomer **5**

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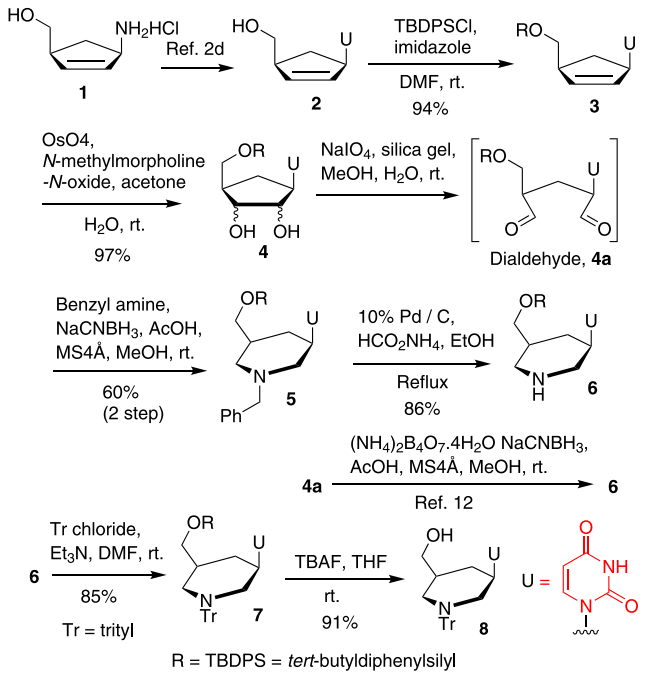
Scheme 1. Synthesis of Pip-U Monomer 8

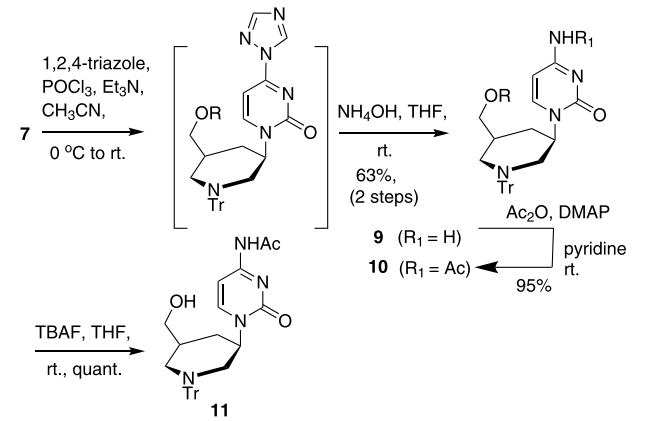


in 60% yield. Using $(NH_4)_2B_4O_7{\cdot}4H_2O$ as a nitrogen source as described previously,[12] compound **6** was obtained. After N-tritylation, compound **7** was obtained; however, in our hands, the yield was poor and an alternative route was employed. Removal of the benzyl-protecting group of compound **5** and N-tritylation afforded compound **7**; the yield was 41.5% from compound **3**. Silyl deprotection with tetra-*n*-butylammonium fluoride (TBAF) gave pip-U monomer **8**. The conformations of compound **5** and its morpholino analogue are similar, as confirmed by X-ray crystallographic data (Figure S1A of the Supporting Information).

To convert U to C, the triazole-substituted U moiety of compound **7** was converted to compound **9** by treatment with aqueous NH_4OH solution at room temperature. Acetyl protection of exocyclic amine followed by silyl deprotection of compound **10** by TBAF gave the pip-C monomer **11** in 60% yield from compound **5** (Scheme 2).

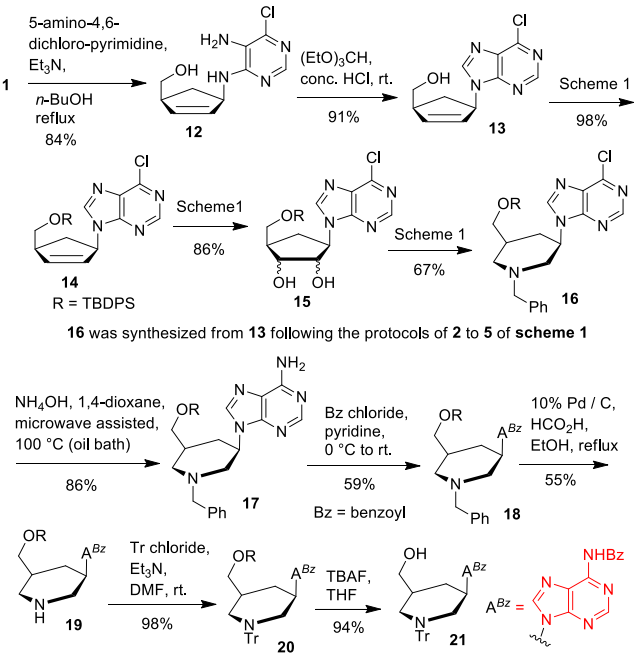
For the synthesis of pip-A monomer, compound **1** was treated with 5-amino-4,6-dichloropyrimidine, resulting in selective replacement of one chlorine to yield compound **12**.

Scheme 2. Synthesis of Pip-C Monomer 11



Compound **12** was then converted to compound **13** via the construction of an imidazole ring through a reaction with triethylorthoformate. The OH of compound **13** was protected with *tert*-butyldiphenylsilyl (TBDPS) to yield compound **14**. Dihydroxylation of the olefin afforded diol **15** as a mixture of *lyxo* and *ribo* isomers that were then converted to *N*-benzylpiperidine **16**. Subsequent treatment with NH_4OH gave compound **17** (Scheme 3). Next, the exocyclic NH_2 of

Scheme 3. Synthesis of Pip-A Monomer 21



compound **17** was protected with a benzoyl group to obtain compound **18**. For hydrogenation of compound **18**, various conditions were evaluated because the loss of the *N*-benzoyl group was observed (Table S1 of the Supporting Information). The desired product **19** was eventually obtained in 55% yield. Subsequent N-tritylation followed by silyl deprotection provided pip-A monomer **21** in 11% yield from compound **1** (Scheme 3).

For the synthesis of the pip-G monomer, we constructed the 2-amino-6-chloropurine ring through the condensation of compound **1** and 2-amino-4,6-dichloro-5-formamido-pyrimidine to obtain 2-amino-6-chloropurine carbocycle **22** (Scheme 4). Silyl protection of the OH yielded compound **23**, which was treated with the sodium salt of 3-hydroxypropionitrile, converting amino-chloropurine to the guanine ring of compound **24**. The exocyclic amine was protected with isobutyryl amide to provide compound **25**. The protocol used for synthesis of the pip-U monomer yielded the trityl-protected pip-G monomer **30** in 17% yield from compound **1**. Crystal structures of monomer intermediates (panels B−F of Figure S1 of the Supporting Information) confirmed that the desired configurations were obtained Figure S1.

We next synthesized the chlorophosphoramidate monomers necessary for the synthesis of PPOs (Table 1). Our initial attempts to synthesize the chlorophosphoramidate monomers using LiBr/1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[12] or 5-(ethylthio)-1*H*-tetrazole (ETT)[13] methods were not successful, with products obtained in less than 10% yield. We therefore performed the reaction in the presence of strong

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Scheme 4. Synthesis of Pip-G Monomer 30

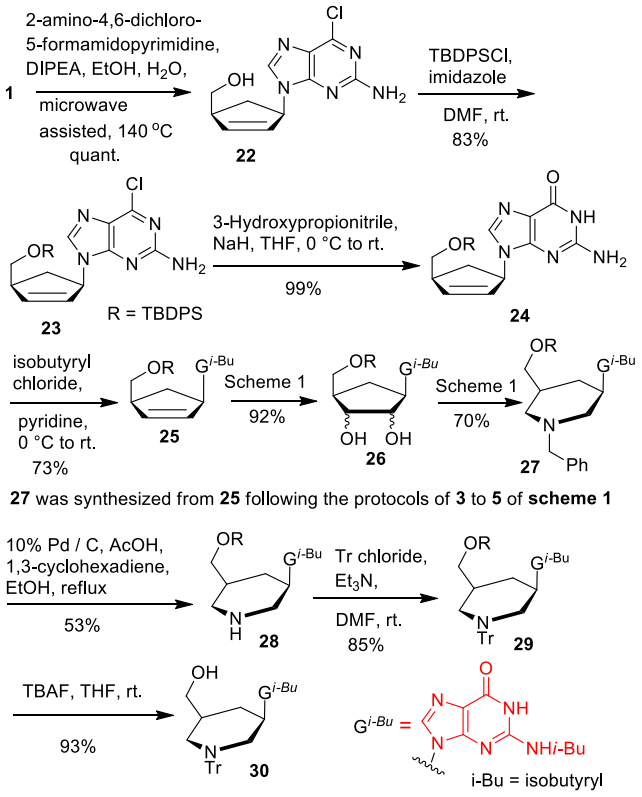
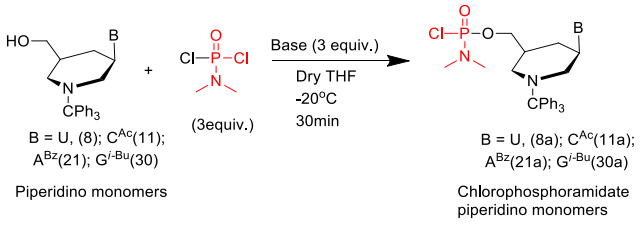


Table 1. Synthesis of Chlorophosphorodiamidate Piperidino Monomers[a]



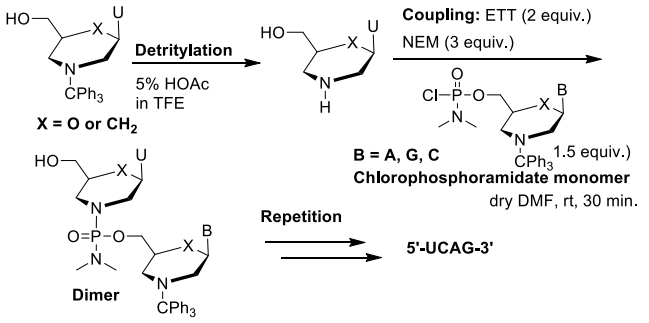
entry	monomer	base	isolated yield (%)
1	U	NaH	20
2	U	LiHMDS	22
3	U	LDA	51/20[b]
4	C^{Ac}	NaH	36
5	C^{Ac}	LDA	49
6	A^{Bz}	LDA	47
7	$G^{i\text{-}Bu}$	LDA	35

[a]Conditions: piperidino monomer (1 equiv), base (3 equiv), and *N*,*N*-dimethylphosphoramic dichloride (3 equiv) in dry tetrahydrofuran (THF) at −20 °C for 30 min. [b]The reaction was performed at −78 °C.

bases at −20 °C to prevent product decomposition. When NaH was used as a base at a temperature of −20 °C, pip-U and pip-C monomers were obtained in 20 and 36% yield, respectively. Although the reaction worked well in the presence of LiHMDS as reported earlier,[14] use of lithium diisopropylamide (LDA) resulted in better yields. At −78 °C using LDA, the reaction was very slow and the yield was approximately 20%. By comparison, at −20°C, the yield of the desired product was 51% and a side product with additional attachment of chlorophosphoramidate to C═O of U was isolated in 10% yield.

We next synthesized a PMO and PPO with the sequence 5′-UCAG-3′ in solution phase (Scheme 5 and Scheme S1 of the

Scheme 5. Solution-Phase Synthesis of Tetramer PMO and PPO



Supporting Information). In the ^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectra of the PMO and PPO tetramers, numbers, multiplicities, and distributions of peaks were similar. ^{1}H aromatic signals expected from C5 and C6 of pyrimidines and from C8 of G and C8 and C2 of A were observed, indicating that the chemical environments are similar for these protons in PMO and PPO (Figure 2, panels A and B). ^{31}P

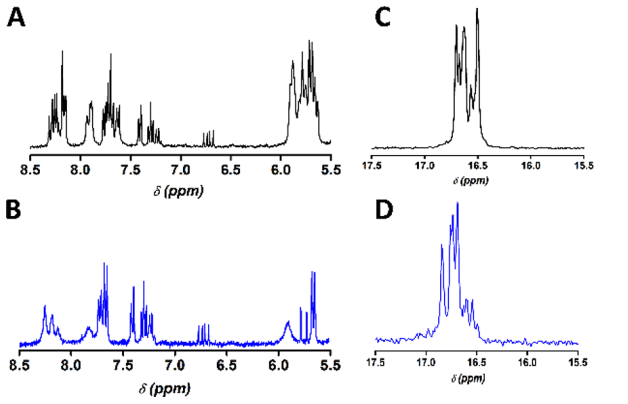


Figure 2. (A) Nucleobase ^{1}H NMR (300 MHz, CD_3OD) of PMO. (B) Nucleobase ^{1}H NMR (300 MHz, CD_3OD) of PPO. (C) ^{31}P NMR (243 MHz, DMSO-d_6) of PMO. (D) ^{31}P NMR (243 MHz DMSO-d_6) of PPO.

NMR multiplicities arising from eight diastereomeric compounds were also identical for PMO and PPO (Figure 2, panels C and D). High-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization (MALDI) analyses confirmed purities and identities (Figures S2 and S3 of the Supporting Information). Reversed-phase HPLC analyses on a C18 column indicated that the PPO tetramer was more hydrophobic than the PMO tetramer (Figure 3A) as expected because the four oxygen atoms are replaced with CH_2 groups. Thus, PPO may have enhanced cellular uptake and pharmacokinetics compared to PMO.

To evaluate the chemical stability of PPO and PMO in acidic solution, we dissolved the tetramers in formate buffer at pH 2.5 or acetate buffer at pH 5. After 12 h, reverse-phase HPLC analyses showed that the PPO was more stable than the PMO at pH 5 (Figure 3B and Figure S4 of the Supporting

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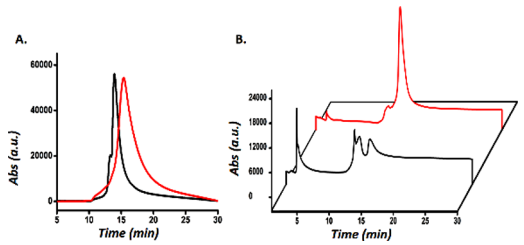

Figure 3. (A) Reverse-phase HPLC chromatograms of PMO (black) and PPO (red) tetramers to evaluate polarity. (B) Reverse-phase HPLC chromatograms of PMO (black) and PPO (red) after 12 h at pH 5.

Information), implying that the PPO will be more stable in endosomolytic compartments than the PMO. Both oligomers were degraded to unidentified fragments at pH 2.5 (Figure S5 of the Supporting Information).

We next used a combination of manual (Scheme S2 of the Supporting Information) and automated (Scheme S3 of the Supporting Information) solid-phase synthesis to synthesize oligomers as per our previous method[13] that contained both PMO and PPO residues (Table 2). The piperidino

Table 2. Sequences and Thermal Melting Temperatures (T_m) of Duplexes of PMOs and PMO−PPO Chimeras with DNA and RNA[a]

ID	Sequence (5 to 3)[b]	T_m with DNA (°C)	ΔT_m (°C)[c]	T_m with RNA (°C)	ΔT_m (°C)[c]
ON1	TTTTACTCACAT	26.0		24.0	
ON2	TTGTACTCACAT	37.0		40.4	
ON3	TTUTACTCACAT	22.1	- 3.9	28.0	+ 4.0
ON4	TTUTACTCACAT	20.7	- 5.3	28.5	+4.5
ON5	TTTTACUCACAT	12.5	- 13.5	17.5	- 6.5
ON6	TTUTACTCACAT	24.0	-2.0	29.8	+5.8
ON7	TTGTACTCACAT	32.2	-4.8	38.0	- 2.4
ON8	TTGTACTCACAT	35.0	-2.0	40.1	- 0.3
ON9	TTGTACTCACAT	32.5	-4.5	38.9	-1.5
ON10	TTGTACUCACAT	26.3	-10.7	26.7	-13.7

[a]Conditions: 40 mM phosphate buffer (pH 7), with 2 μM for each strand. T_m values reported are the averages of two independent experiments that were within ±0.5°. [b]Blue letters indicate PMO, and red letters indicate PPO. [c]The ΔT_m values are relative to T_m of the duplex formed between **ON1** or **ON2** and complementary RNA or DNA.

chlorophosphoramidate monomers were incorporated manually. The coupling efficiency for piperidino chloro−phosphoramidate monomers was an average of 95% as per the trityl assay. Yield and purity were determined using HPLC and MALDI (Table S2 and Figures S6−S14 of the Supporting Information).

Incorporation of one or two piperidinomonomers destabilized the duplex with DNA by 2−13.5 °C compared to the duplexes formed with PMO (Table 2 and Figures S15−S24 of the Supporting Information). The destabilization increased when a piperidino was incorporated in the middle (**ON5** and **ON10**). In the case of RNA, the T_m value of the duplex of the oligomer with one pip-A residue was comparable to that of PMO (**ON8**). Interestingly, chimeras with pip-U or pip-C at the 3′ or 5′ end or both termini (**ON3**, **ON4**, and **ON6**) had higher T_m values than the corresponding PMO. The pip-G-containing chimera (**ON7**) and the chimera with both pip-G and pip-A (**ON9**) had T_m values that were lower by 2.4 and 1.5 °C, respectively, than that of the PMO−RNA duplex. In the case of the oligomer with four PPO residues (**ON10**), the T_m of the duplex with RNA was 13.7 °C lower than the T_m of the duplex with PMO. We are in the process of making uniform piperidino oligomers to understand the differences noted between PMO and PPO in binding to DNA and RNA.

In the CD spectra of duplexes with DNA, there was a strong positive band at around 275 nm and a negative band at around 210 nm, which is characteristic of a B-form conformation. Even the duplex between the oligomer with four piperidino substitutions and DNA appears to have a global conformation similar to that of the PMO:DNA duplex, as shown by similarities of CD spectra (Figure S25 of the Supporting Information). In the case of duplexes with RNA, the CD spectra of duplexes formed with oligomers with one or two pip-U or pip-C residues (**ON3**, **ON4**, **ON5**, and **ON6**) were similar to that of the duplex of PMO (**ON1**) with RNA (Figure S26 of the Supporting Information). Although **ON2** differs by only substitution of PMO T by PMO G at the third position with respect to **ON1**, the CD spectrum of the duplex with RNA was significantly different than that of the duplex formed by the PMO **ON1** with RNA (Figure S27 of the Supporting Information). Duplexes between RNA and oligomers with pip-G (**ON7**, **ON8**, **ON9**, and **ON10**) and with the PMO (**ON2**) and RNA had similar CD spectra (Figure S26 of the Supporting Information). The positive bands at around 260 and 280 nm were suggestive of structural properties of a conformation in between B- and A-form helices.

Finally, to study the effect of incorporation of PMO or PPO into a RNA duplex, we modeled the structures of a RNA duplex containing a single modification of either PMO or PPO. The crystal structure of the octamer duplex [CGAA(dT)-UCG]$_2$ with a single 2′-deoxythymidine per strand served as the template [Protein Data Bank (PDB) ID 5DEK].[15] The dT in one strand was removed, and the PMO or PPO residue was manually built into the RNA backbone in UCSF Chimera,[16] using an all-equatorial conformation for the six-membered rings. Both models were energy-minimized with the program AMBER[17] using Amber-14 parameters (https://ambermd.org/). There was a virtually seamless fit of PMO and PPO monomers inside an RNA backbone (Figure 4). This analysis points out that both PMO and PPO would fit into other chimeric oligonucleotides targeting RNA using different

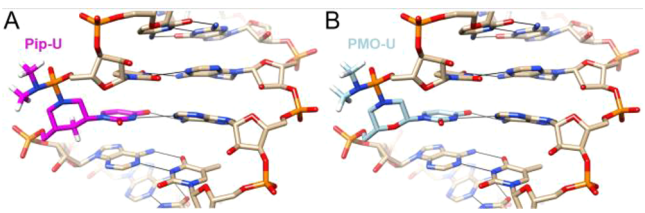

Figure 4. Models of a RNA duplex containing (A) pip-U and (B) PMO-U monomers generated with the program UCSF Chimera.

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mechanisms of action, such as RNase-H-based antisense and Argonaute-2-based RNAi therapeutics.

In summary, we have synthesized piperidino monomer building blocks and incorporated these residues using a solid-phase synthesis protocol into the PMO backbone to generate PMO−PPO chimeras. The stabilities of duplexes between DNA and PMO−PPO chimeras was lower than that of the DNA:PMO duplex. Incorporation of one or two pip-A, pip-U, or pip-C residues resulted in either comparable or higher duplex stability with RNA; however, multiple piperidino monomers destabilized the hybrid duplexes. Although stability of duplexes is one requirement for therapeutic applications of oligonucleotide-based drugs, other parameters, such as metabolic stability, cell permeation, release from the endolysosomal compartments, and target specificity, are also critical. Given the data reported here, further evaluation of the gene-silencing and splice-modulating activities of PMO−PPO chimeric oligomers in the context of both antisense and RNA interference is warranted.

■ ASSOCIATED CONTENT

Data Availability Statement

Data are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.2c04067.

Experimental procedures, compound characterization, and description of the procedures (PDF)

Accession Codes

CCDC 2202900−2202902, 2202923, and 2202926 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

†Atanu Ghosh and Masaaki Akabane-Nakata contributed equally to this work.

Notes

The authors declare no competing financial interest.

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