JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Article
pubs.acs.org/JACS

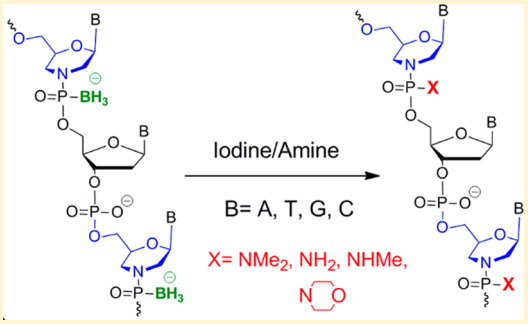
Synthesis of Phosphorodiamidate Morpholino Oligonucleotides and Their Chimeras Using Phosphoramidite Chemistry

Sibasish Paul and Marvin H. Caruthers*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309, United States

Supporting Information

ABSTRACT: Phosphorodiamidate morpholinos (PMOs) and PMO−DNA chimeras have been prepared on DNA synthesizers using phosphoramidite chemistry. This was possible by first generating boranephosphoroamidate morpholino internucleotide linkages followed by oxidative substitution with four different amines: *N,N*-dimethylamine, *N*-methylamine, ammonia, and morpholine. When compared to a natural DNA duplex, the amino modified PMO was found to have a higher melting temperature with either complementary DNA or RNA, whereas the remaining PMO analogues having morpholino, dimethylamino, or *N*-methylamino phosphorodiamidate linkages had melting temperatures that were either comparable or reduced. Additionally the *N,N*-dimethylamino PMO−DNA chimeras were found to stimulate RNaseH1 activity. Treatment of HeLa cells with fluorescently labeled PMO chimeras demonstrated that these analogues were efficiently taken up by cells in the presence of a lipid transfection reagent. Because of the simplistic synthesis procedures, various PMO analogues are now readily available and should therefore open new pathways for research into the antisense, diagnostic, and nanotechnology oligonucleotide fields.



INTRODUCTION

The ability to target genes with antisense oligonucleotides has been shown to be a useful approach for studying biological pathways and for investigating possible treatments of various diseases.[1,2] A very promising antisense oligonucleotide (PMO with *N,N*-dimethylamino, **3**, Figure 1)[3] having nonionic

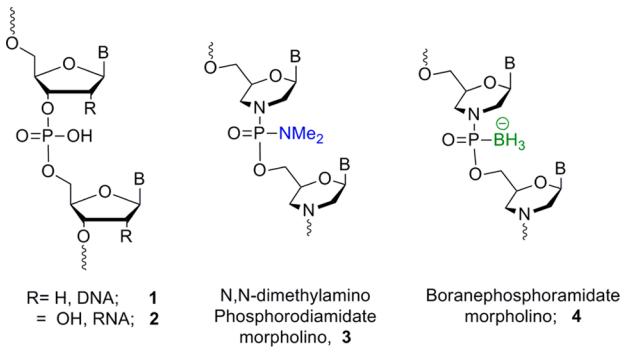


Figure 1. Chemical structures of DNA, RNA, *N,N*-dimethylamino PMOs and Boranephosphoroamidate morpholino oligonucleotides.

internucleotide linkages and morpholino replacing the deoxyribose of DNA has been investigated for almost last three decades. PMOs are known to inhibit gene expression,[4] are resistant to cellular nucleases,[5,6] and form duplexes with complementary RNA that have binding affinities comparable to natural DNA/RNA duplexes.[7] In biological studies, gene expression in embryos of Zebrafish and *Xenopus laevis* has been manipulated using PMOs.[8] The efficacy, sequence specificity, stability and low toxicity of PMOs has been verified in several publications, mostly describing work in embryos.[9] Therapeutic development of PMOs is currently underway in ongoing clinical trials for treatment of Duchenne muscular dystrophy (DMD) and against the infection and spread of the hemorrhagic filovirus Marburg.[10] Recent research has also demonstrated that PMOs may prove useful for studies in the nanotechnology[11] and surface hybridization[12] fields.

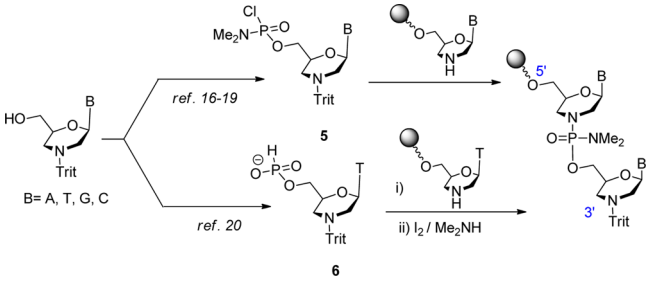
Although there are many potential applications for these promising oligonucleotide analogues, their use remains severely limited.[13] For example PMOs are uncharged and do not form complexes with cell penetrating peptides (CPP) or lipids. Therefore, simply mixing cells and a PMO with a CPP or lipid fails to produce antisense effects.[14,15] Consequently covalent conjugation of a CPP to a PMO is required for transfection.[12] Moreover morpholino nucleoside monomers, as prepared using previous chemistries, cannot be incorporated into chimeric oligonucleotides. This is because PMOs must be prepared in a 5′ to 3′ direction on a polystyrene resin[16] (Scheme 1). This requirement is not compatible with standard methods for the chemical synthesis of natural DNA/RNA or most analogues, as they are prepared in a stepwise 3′ to 5′ direction.

The most commonly used approach for preparing PMOs involves, as a first step, condensing a 5′-hydroxyl-*N*-trityl-morpholino nucleoside with *N,N*-dimethylamino-dichlorophos-

Received: September 6, 2016
Published: November 14, 2016

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

Scheme 1. Current Approaches for the Synthesis of *N,N*-Dimethylamino PMOs



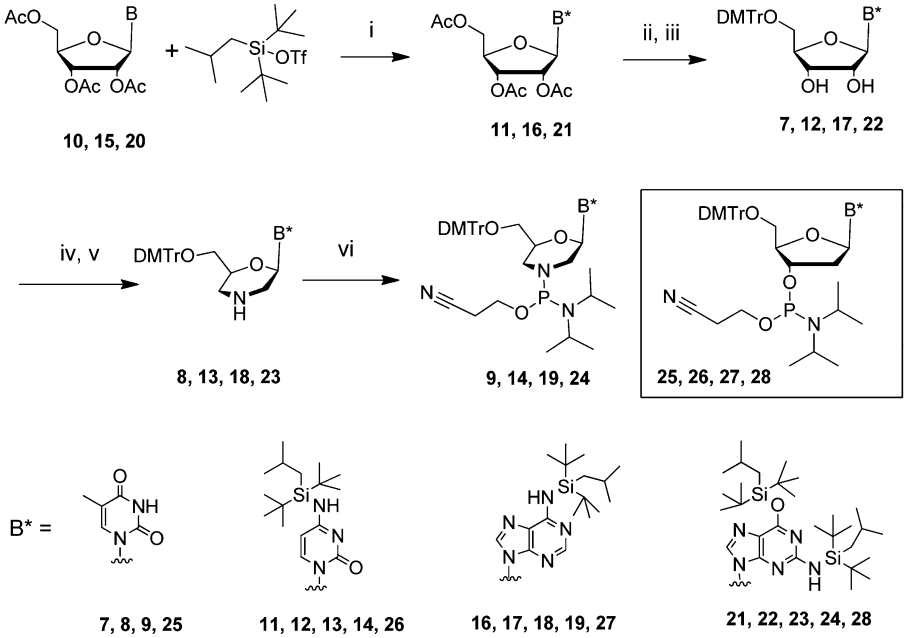
phoramidate in order to generate a *N,N*-dimethylamino-chlorophosphoramidate synthon (**5**). Coupling with base yields the dimer attached to the resin (Scheme 1). Further detritylation with an acid salt generates a product that can be elongated by repeating the cycle.[17−19] There are several recognized problems with this approach. For example condensation yields are low (the recovered yield for a dithymidine morpholino was 45%) and require long reaction times.[18] Moreover, Sinha et al. has claimed that the 5′-chlorophosphoroamidate monomers are unstable.[20] Additionally this approach requires special procedures, techniques, and materials (e.g., a swellable polystyrene resin, unique reaction vessels, and the pyridinium salt of trifluoroacetic acid as the detritylating reagent) that are not readily available to most laboratories. Recently a new method has been developed (Scheme 1) using H-phosphonate chemistry where (**6**) was used for the synthesis of polythymidine PMOs and a PMO−DNA chimera (conjugated in 3′-3′ fashion).[20] There are also problems with this approach. For example, because the phosphorus atom in H-phosphonates is electrophilic and lacks a lone pair of electrons, it is much more resistant toward oxidation under ambient conditions than most P (III) compounds. Moreover coupling yields are low (77%) toward formation of the H-phosphonatemorpholino dimer.[20]These limitations led us to explore using the phosphoramidite methodology[21] for the synthesis of various PMO derivatives.

In a recent publication, the stereoselective conversion of boranephosphonate DNA, in the presence of iodine and amines, to phosphoramidate DNA was reported.[22] Based upon these results, we outline in this manuscript a general method for synthesizing various PMO analogues. This new approach has several significant advantages. (1) The method is orthogonal and compatible with standard DNA/RNA synthesis procedures. (2) For the first time, PMOs having internucleotide linkages other than *N,N*-dimethylamino functionality can be synthesized. (3) Additionally and also for the first time, PMO−DNA chimeras can be synthesized where the internucleotide linkages are 3′ to 5′. Because these chimeras are anionic, they were found to be active with RNase H1 and were taken up by cells using a standard lipid transfecting reagent.

RESULTS

Synthon Preparation. Our initial objective was to prepare phosphordiamidite synthons (Scheme 2, compounds **9**, **14**, **19**, **24**) that could be used to generate PMO analogues (Scheme 3). Synthesis of the thymine morpholino monomer (**8**) followed literature protocols. 5′-*O*-Dimethoxytrityl ribothymidine (**7**) was treated with sodium periodate and then ammonium biborate to afford the dihydroxythymine morpholino monomer. Reduction of the hydroxyl groups was carried out (without further purification) using sodiumcyanoborohydride under mild acidic condition to yield the thymine morpholino monomer (**8**).[23,24] The thymine synthon (**9**) was then prepared in 86% yield by phosphitylation of (**8**) with 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphordiamidite and

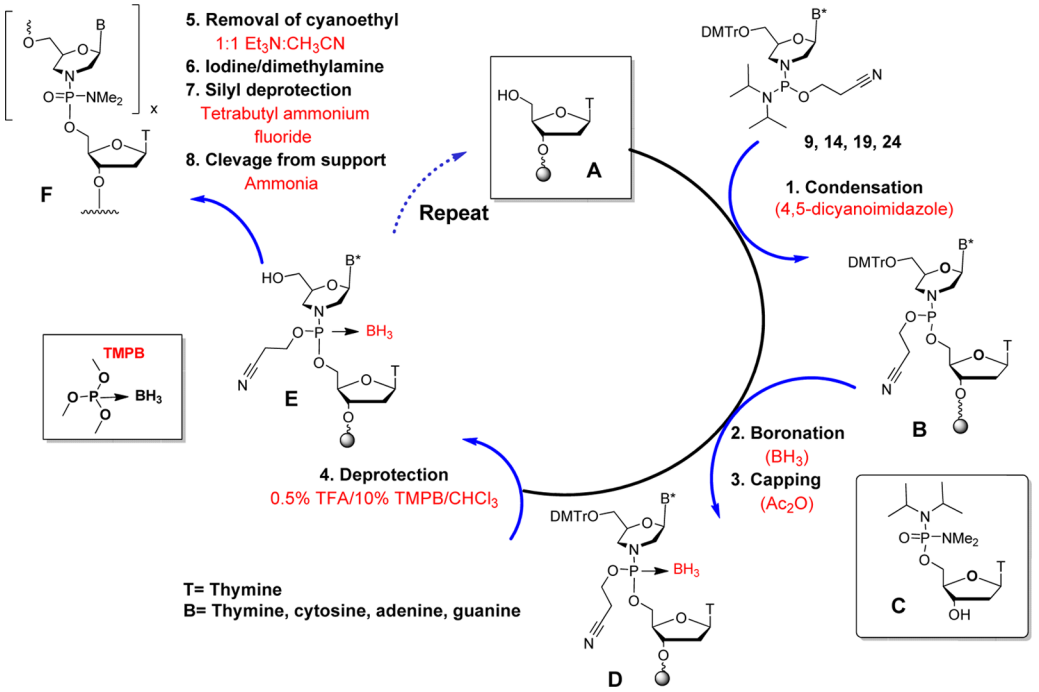
Scheme 2. Synthesis of BIBS Protected Morpholino Phosphordimidite Synthons[a]



[a](i) Lutidine (4.0 equiv)/1,4-Dioxane; (ii) 1.0 M NH_3/MeOH; (iii) DMT-Cl (1.2 equiv)/Pyridine; (iv) $NaIO_4$ (1.1 equiv)/$(NH_4)_2B_4O_7$ (1.1 equiv)/CH_3OH; (v) $NaCNBH_3$ (2.0 equiv)/AcOH (2.0 equiv)/CH_3OH; (vi) $P(OCH_2CH_2CN)(NiPr_2)_2$ (1.2 equiv)/4,5-Dicyanoimidazole (0.5 equiv)/CH_2Cl_2. DMTr: Dimethoxytrityl.

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

Scheme 3. Synthesis Cycle for the Preparation of PMOs[a]



[a]F: A PMO having only *N,N*-dimethylamino-phosphorodiamidate internucleotide linkages; "X" is a selected number of *N,N*-dimethylamino-PMO nucleotide units; Compound A can be replaced by any of the four 2′-deoxyribonucleosides or morpholino nucleosides but the cytosine, adenine, and guanine bases must be protected with the bis(*tert*-butyl)isobutylsilyl group.

4,5-dicyanoimidazole (DCI) in dichloromethane under an argon atmosphere.[23] Using a similar protocol, a thymine synthon having the *O*-methyl-*N,N*-diisopropylphosphordiamidite was prepared; however, the solid phase coupling with this synthon was found to generate a lower yield when compared to **9** (96% vs 89% respectively after the first coupling step). Synthesis of the corresponding monomers of cytosine, adenine, and guanine was initiated by first protecting the nucleoside amino groups with bis(*tert*-butyl)isobutylsilyl (BIBS).[25]The standard amide protecting groups cannot be used with cytosine, adenine, and guanine because boronation, an essential part of the synthesis cycle, would reduce these amides to the corresponding alkyl groups which are stable and cannot be removed following synthesis. In contrast, the BIBS group can readily be removed using fluoride. The reaction of 5′,3′,2′-tri-*O*-acetylcytidine (**10**) with BIBS-OTf (Tf = triflate) in the presence of 2,6-lutidine gave 76% yield of **11** after 2 h stirring at 60 °C under an argon atmosphere. However, the synthesis of **16** and **21** from **15** and **20** required much longer reaction times (3 days) with yields of 25−31% (**16**) and 74% (**21**). As outlined in Scheme 2, these silylated ribonucleosides were then treated with ammonium hydroxide to remove the acetyl protecting groups, converted to the 5′-dimethoxytrityl compounds, and then to the morpholino derivatives (**13**, **18**, and **23**), using the same chemistry as outlined for the preparation of **8**. These compounds were used to generate the phosphordiamidite synthons **14**, **19** and **24**. (See the Supporting Information and Experimental Section for details). For the synthesis of PMO−DNA chimeras, 5′-*O*-dimethoxytrityl-2′-deoxyribonucleoside-3′-phosphoramidite synthons **26**, **27** and **28** were prepared following a literature protocol[25] whereas compound **25** was obtained from commercially available sources.

Synthesis of *N,N*-Dimethylamino PMO Oligonucleotides. Once these synthons were available, our next goal was to optimize the solid-phase synthesis cycle for preparing boranephosphoroamidate morpholino derivatives and for converting these compounds to the corresponding PMOs. The synthesis cycle is outlined in Scheme 3 and Table S1 (Supporting Information). Prior to synthesis, the 5′-DMT group on the 2′-deoxyribothymidine linked to a polystyrene support was removed with 0.5% trifluoroacetic acid in chloroform containing 10% trimethylphosphiteborane (TMPB).[25] The 5′-unprotected-2′-deoxyribonucleoside (A)[26] was then allowed to react with **9**, **14**, **19** or **24** in anhydrous acetonitrile containing 4,5-dicyanoimidazole (DCI) in order to generate a dimer having a phosphoramiditediester internucleotide linkage (B). The coupling wait time was 300s. Following boronation, capping, and detritylation, repetition of this cycle generates the product ready for further conversion to the PMOs.

Post synthesis, supports were washed with acetonitrile, treated with a 1:1 mixture of triethylamine:acetonitrile for 600 s in order to remove the cyanoethyl groups from internucleotide linkages, washed with acetonitrile and dichloromethane several times to remove residual triethylamine, and dried. These polystyrene supports were next removed from columns and placed in 1.5 mL screw-cap glass reaction vials. For conversion to the *N,N*-dimethylamino PMOs, the morpholino boranephosphoroamidates were treated 2 h with a solution of 0.05 M iodine and 2.0 M dimethylamine in tetrahydrofuran. The resins were repeatedly washed with acetonitrile and oligonucleotides were next treated with a 1.0 M solution of tetrabutyl ammonium fluoride (TBAF) in THF to remove the BIBS protecting groups. ($Et_3N{\cdot}HF$ could not be used for removing these silyl groups as this reagent hydrolyzed phophorodiami-

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

Table 1. Mass Analysis (LC−MS) of PMO and PMO−DNA Chimeras (ODNs)[a]

NO	ODNs	mol wt cal	mol wt obs
ODN 1	T*T*T*T*T*T *T*T*T*t	3214.00	3214.01
ODN 2	T*T*T*T*T*T*T*T*T*T*T*T*T*T*T*T *T*T*T*t	6846.17	6848.14
ODN 3	C*C*C*T*T*T*T*T *T*T*T*t	3829.36	3828.16
ODN 4	A*A*A*T*T*T*T*T*T*T*T*t	3901.44	3902.30
ODN 5	G*G*T*T*T*T*T*T *T*T*T*t	3924.42	3925.26
ODN 6	G*A*C*T*T*T*T*T*T*T*T*t	3893.41	3894.25
ODN 7	A*T*G*T*G*C*T*G*C*T*A*t	3937.44	3938.28
ODN 8	T*A*A*C*A*C*G*A*T*A*C*G*C*G*A*t	5264.26	5264.75
ODN 9	T*T*T*T*$t_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_p$t	6430.17	6430.15
ODN 10	$t_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_p$T*T*T*T*t	6430.17	6430.15
ODN 11	$t_pt_pt_pt_pt_pt_pt_pt_p$T*T*T*T*$t_pt_pt_pt_pt_pt_pt_pt_p$t	6430.17	6430.14
ODN 12	T*T*T*T*$t_pt_pt_pt_pt_pt_pt_pt_pt_pt_pt_p$T*T*T*T*t	6534.17	6534.38
ODN 13	T*t_pt_pT*$t_pt_pt_p$T*$t_pt_pt_p$T*$t_pt_pt_p$T*$t_pt_pt_p$T*t	6482.26	6482.17
ODN 14	$t_pa_pg_pt_pt_pg_pa_pg_pt_pg_pa_pt_p$A*T*C*A*t	5343.45	5343.07
ODN 15	G*T*A*G*$t_pC_pC_pt_pg_pc_pa_pa_pt_pg_pa_p$t	5000.26	4999.99
ODN 16	T*G*A*$t_pC_pg_pC_pt_pg_pC_pa_pa_p$T*G*A*t	5052.26	5052.16

[a]Oligonucleotides containing *N,N*-dimethylamino-phosphorodiamidate (*) and phosphate (p) internucleotide linkages are shown. Morpholino and 2′-deoxyribonucleosides are shown as upper and lower case letters respectively.

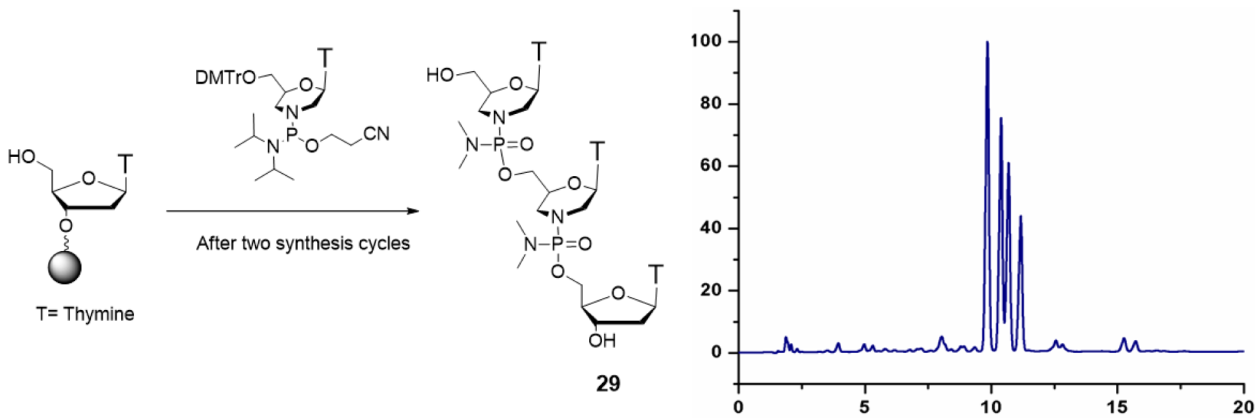


Figure 2. Synthesis of a *N,N*-Dimethylamino PMO trimer (**29**). The LC profile from the LC−MS analysis of the total reaction mixture is shown.

date linkages). When oxidative amination reactions were carried out after removal of silyl protecting groups, amine substitution at the N-4 of cytosine was observed (Supporting Information, Figure S1). This problem was overcome by performing the oxidative amination reaction prior to desilylation. Once the silyl groups were removed, oligonucleotides were cleaved from supports using 30% ammonium hydroxide, polystyrene resins were removed using 0.2 μm centrifugal filters, and solutions were passed through Illustra NAP-25 columns to remove fluoride salts. An additional purification step was performed with each PMO using an Amicon Ultra-15 3K centrifugal filter device (to remove shorter failure sequences). The oligonucleotides were then used for further characterization and other studies.

Zhang et al. reported incorporation of uridine morpholino phosphoramidite (similar to **9** except B* = uracil) into siRNA sequences by means of 5-(ethylthio)-1*H*-tetrazole (ETT) and was able to convert the resulting morpholino phosphoramidite internucleotide linkage to the corresponding phosphoramidate morpholino linkage with aqueous iodine.[23,27] However, we found that ETT (pK_a = 4.3) activated both morpholine and *N,N*-diisopropylamine in compounds **9**, **14**, **19** and **24** (Scheme 2). As a consequence we observed very low yields of the final PMO products following their protocol. This was because activation of morpholine followed by boronation and iodine/dimethylamine oxidation leads to a capped PMO oligonucleotide having a 5′-*N,N*-diisopropylamino-phosphorodiamidate (C) that cannot be further elongated (Scheme 3). In order to further investigate this problem, several less acidic activators (tetrazole, saccharin-1-methylimidazole, 4,5-dicyanoimidazole) were tested in attempts to identify one that would react with *N,N*-diisopropylamine (pK_a = 11.1) but minimally with morpholine (pK_a = 8.3).[28] Of the activators we screened by synthesizing PMOs, having a sequence corresponding to ODN 1 ((Table 1), 4,5-dicyanoimidazole (pK_a = 5.2, 0.12 M, with 300 s coupling time) proved to generate the highest yield (67%) of the desired PMO with low levels of side-products (Supporting Information, Figure S2). Since the morpholino phosphoramidite diester (B), as synthesized following the coupling step, was somewhat unstable toward the capping solution we carried out boronation in order to generate a P (IV) morpholino compound (D) prior to the capping step. After boronation, the support was washed with acetonitrile, failure sequences capped using acetic anhydride, and detritylation carried out using a solution of 10% TMBP and

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

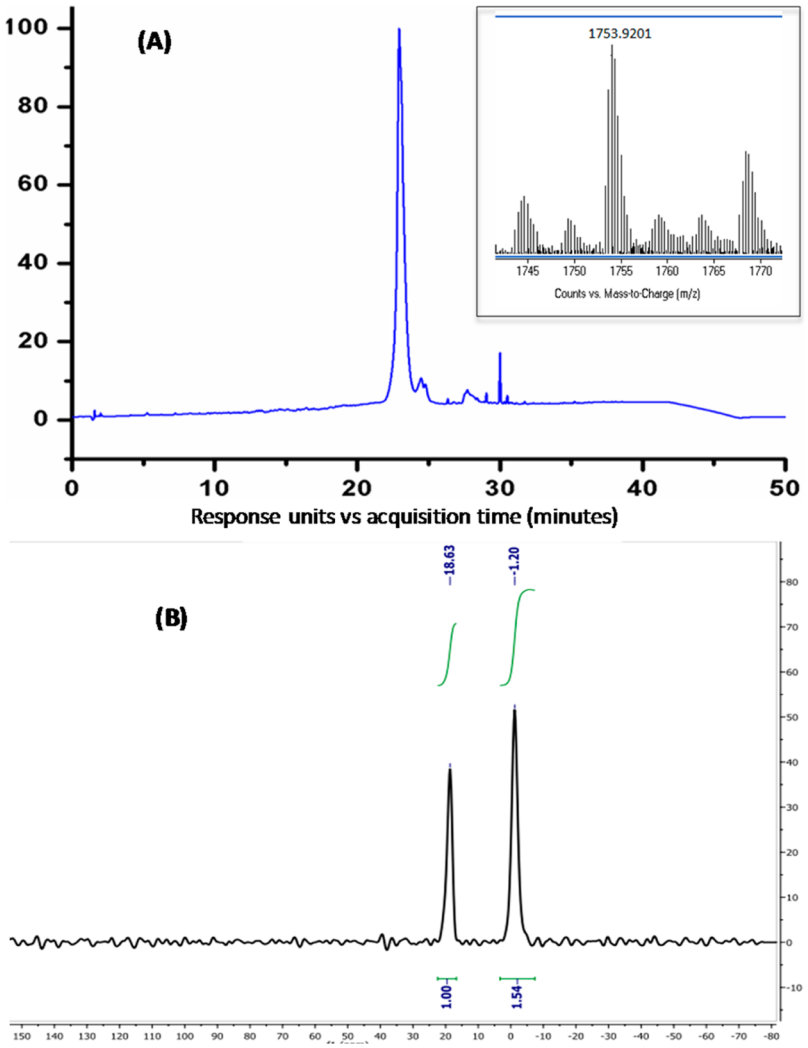


Figure 3. (A) LC−MS analysis of the total reaction mixture from the synthesis of ODN 8. The data shows the LC trace and a zoomed in view of the base peak in the mass spectra. The base peak in each case was a −3 charged species. Full mass spectra are provided in the Supporting Information. (B) ^{31}P NMR of the total reaction mixture obtained from the synthesis of ODN 16 (400 MHz, 15000 scans). The ^{31}P NMR peaks at 18.63 ppm and −1.2 ppm correspond to the *N*,*N*-dimethylamino-phosphorodiamidate and phosphodiester linkages respectfully.

0.5% TFA in chloroform (D was stable to a acidic detritylation conditions).

Using the procedure outlined in Scheme 3, initially a trimer having only thymine nucleoside bases was synthesized, converted to the *N*,*N*-dimethylamino PMO derivative with iodine/*N*,*N*-dimethylamine, removed from the support with ammonia, and analyzed by LC−MS. The four major peaks as shown in the LC profile (Figure 2) have the mass spectra expected for the four diastereomers of the product (calculated mass, 902.3, found 901.3). A 94% yield of compound **29** was calculated when the combined areas of all peaks from the crude reaction mixture were compared to the peak area for these diastereomers. In a similar experiment where a dimer was prepared, the first coupling yield was found to be 96% which is far better than other reported procedures (Supporting Information, Figure S3).[18,20]

N,*N*-Dimethylamino PMOs having oligothymidine (Table 1, ODNs 1 and 2) and all four bases (Table 1, ODNs 3−8), have been synthesized using this approach and characterized by LC−MS. Because it has been established that PMOs 12−16 in length are effective inhibitors of RNA expression, either via blockage of splicing or mRNA translation,[9] we carried out the synthesis of a 16-mer having all four bases (Table 1, ODN 8). As can be seen from the LC−MS chromatogram of the unpurified reaction mixture (Figure 3A), both the yield (10 A_{260} units isolated) and mass analysis were satisfactory.

Synthesis of PMO−DNA Chimeras. For the synthesis of PMO−DNA chimeras, 4,5-dicyanoimidazole (0.12 M and 300 s coupling time) and ETT (0.25 M and 180 s coupling time) were used respectively for the morpholino phosphordiamidite synthons (**9**, **14**, **19** or **24**) and the 5′-dimethoxytrityl-2′-deoxyribonucleoside-3′-phosphoramidites (**25**, **26**, **27** or **28**).[29] Following condensation, a morpholino phosphoramidite diester was converted to the P(IV) borane linkage and standard oxidation with 0.02 M iodine in THF/water/pyridine was used to convert a phosphite triester to the phosphate triester. These synthesis steps were repeated until the PMO−DNA chimeras of the desired sequence/length were prepared. Table S1 (Supporting Information) summarizes these synthesis steps.

Because PMO−DNA chimeras are new to the scientific community and could prove to be useful for various research projects, several were synthesized (Table 1, ODNs 9−16). Initially these chimeras, as a series of 21mer oligothymidines and containing four *N*,*N*-dimethylamino PMO linkages, were

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

prepared. In these 21mers, the morpholino diamidate linkages were placed adjacent to either the 5′ or 3′ ends, near the middle of the 21 mer, and at every third position throughout the oligomer (Table 1; ODNs, 9, 10, 11, 12 and 13). For these chimeras, substitution of borane with dimethylamine upon activation by iodine proceeded efficiently and analysis of the crude reaction mixtures by LC−MS (Supporting Information) revealed that the expected phosphorodiamidate linkages were formed in near quantitative yields. These encouraging results were followed by the synthesis of PMO−DNA chimeras containing all four nucleobases with variable locations and number of PMO linkages (Table 1: ODNs, 14, 15 and 16). LC−MS analysis and ^{31}P NMR of the reaction mixtures demonstrated that the expected PMO−DNA chimeras were obtained in high yields (Figure 3B for ODN 16; Supporting Information for ODNs 14 and 15) with an average yield being 10−20 A_{260} units (from a 0.2 μM synthesis cycle). These experiments also demonstrated that treatment with dimethylamine does not lead to measurable cleavage of the succinate linkage and loss of product during synthesis.

Solid Phase Synthesis of PMOs with Amino, *N*-Methylamino and Morpholino Linkages. From previous research,[25] it is known that the boranephosphonate linkage can be activated with iodine for displacement by a large number of nucleophiles. Therefore, in addition to testing this new synthetic route by synthesizing PMO analogues having the *N,N*-dimethylamino-phosphorodiamidate linkage, we decided to investigate other amines, which could be used in order to generate several new PMO−DNA derivatives. Initially an oligothymidine 21 mer having four boranephosphoroamidate morpholino linkages near the center of this oligomer was synthesized. The support containing this oligonucleotide was divided into three samples that were treated with *N*-methylamine, ammonia, and morpholine under iodine oxidation conditions and then purified using reverse phase column chromatography. Additionally mixed sequence PMO−DNA chimers having all four bases and amino-phosphorodiamidate internucleotide linkages were synthesized where the positions for the diamidate linkages were located at the 5′, 3′, and 5′/3′ termini of these chimeras. The mass analysis from LC−MS and the sequences of PMOs are listed in Table 2. Yields were comparable to those obtained for the *N,N*-dimethylamino PMO chimeras.

Melting Temperatures of PMO−DNA Chimeras. In order to assess the target binding ability of the modified PMO−DNA chimeras, duplex hybridization studies were performed with 2′-deoxyoligothymidines having amino, methylamino, morpholino, and dimethylamino-morpholino internucleotide linkages. PMOs 11, 17, 18, and 19 were mixed with 2′-deoxyribo or riboadenosineoligonucleotides 21 nucleotides in length (1:1 ratio) in a buffer (1.0 M NaCl, 10 mM sodium phosphate, pH 7.1) at an overall concentration of 1.0 μM of duplex. The samples were denatured at 90 °C and cooled to 15 °C. They were then heated at a rate of 1 °C/min, and A_{260} versus time was recorded. Melting temperatures were taken as the temperature of half dissociation and were obtained from the first derivative plots (Table 3).

Table 2. LC−MS Analysis of PMO−DNA Chimeras[a]

NO	ODNs	Mol wt Cal	Mol wt Obs
ODN 17	tptptptptptptptptT*T*T*Tptptptptptptptptpt	6318.21	6318.02
ODN 18	tptptptptptptptptpT♦T♦T♦Tptptptptptptptptpt	6374.32	6374.08
ODN 19	tptptptptptptptptpT●T●T●Tptptptptptptptptpt	6598.57	6598.20
ODN 20	tpapgptptpgpapgptpgpaptpA*T*C*A*t	5231.48	5230.97
ODN 21	G*T*A*G*tpCpCptpgpCpapapaptpgpapt	4888.26	4887.89
ODN 22	T*G*A*tpCpgpCptpgpCpapapT*G*A*t	4884.26	4883.96

[a]ODN 17, 20, 21 and 22: * = NH_2; ODN 18: ♦ = NHMe; ODN 19: ● = morpholine. Phosphate linkages are denoted by "p". Morpholino and 2′-deoxyribonucleosides are noted as upper and lower case letters respectively.

Table 3. T_m Results with PMO−DNA Chimeras[c]

Number	Modification	T_m [a] (°C) DNA	T_m [a] (°C) RNA	ΔT_m [b] (°C) DNA	ΔT_m [b] (°C) RNA
ODN 23	——	58.5	51.1	——	——
ODN 11	NMe_2	58.4	52.4	0.03	0.3
ODN 17	NH_2	61.4	53.4	0.8	0.6
ODN 18	NHMe	59.0	52.2	0.1	0.3
ODN 19		57.4	51.4	-0.3	0.1

[a]T_m measurements represent an average of at least three experiments. [b]ΔT_m represents (T_m ODN-T_m unmodified control)/number of modifications). [c]PMO−DNA chimeras (ODNs 11, 17, 18, and 19) and also unmodified ODN 23, $d(T)_{21}$, were mixed with complementary $d(A)_{21}$ or $r(A)_{21}$ and the melting temperatures measured (Supporting Information).

Melting temperature studies of RNA heteroduplexes with PMOs 11, 17, 18, and 19 showed that introduction of phosphorodiamidate internucleotide linkages stabilized the PMO:RNA duplex relative to the unmodified duplex. The trend in increased stabilization was amino > *N*-methylamino ~ *N,N*-dimethylamino > morpholino. When the PMO−DNA chimeras were allowed to form duplexes with a 2′-deoxyriboadenosine 21mer, the order of stabilization (when compared to the T_m of the unmodified DNA·DNA duplex) was amino > *N*-methylamino > *N,N*-dimethylamino > morpholino, where the morpholino analogue was destabilizing.

RNase H1 Activity of Chimeric PMO−RNA Heteroduplexes. *N,N*-Dimethylamino PMO−DNA chimeras were tested for their ability to stimulate RNase H1 activity. The test systems were composed of a 5′-*O*-fluorescein labeled RNA and complementary *N,N*-dimethylamino PMO−chimeras.[30] The analyzed PMOs were oligothymidine 14 mers with cap/gap sequences (ODNs 24−26, Table S2, Supporting Information) having *N,N*-dimethylamino PMO linkages on either end and 3−7 phosphodiester linkages in the center of the analogues. Controls were complementary DNA and 2′-*O*-methyl RNA (which activate and do not stimulate RNase H1 activity respectively). All these cap/gap oligonucleotide analogues were found to activate RNA hydrolysis (Figure 4).

Fluorescence Microscopy. Since uncharged PMOs cannot be delivered to cells using lipid based transfection reagents, we chose to investigate whether these PMO−DNA chimeras could be taken up by cells in the presence of Dharmafect 1, a commonly used siRNA transfection reagent. In order to test

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

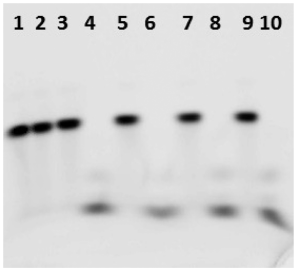


Figure 4. *E. coli* RNase H1 degradation of RNA when Catalyzed by PMO−DNA Chimeras. 5′-*O*-Fluorescein labeled $r(A)_{14}$ was allowed to form duplexes with complementary ODNs 24−26. These duplexes were then treated with *E. coli* RNase H1 for 12 h and analyzed by PAGE. Lane 1:2′-*O*-methyl RNA/RNA; Lane 2:2′-*O*-methyl RNA/RNA/RNase H1; Lane 3: DNA/RNA; Lane 4: DNA/RNA/RNase H1; Lane 5: ODN 24/RNA; Lane 6: ODN 24/RNA/RNase HI; Lane 7: ODN 25/RNA; Lane 8: ODN 25/RNA/RNase H1; Lane 9: ODN 26/RNA; Lane 10: ODN26/RNA/RNaseH1. ODN24: T*T*T*T*T*T*T*TpTpTpT*T*T*T*T*T*T; ODN25: T*T*T*T*T*TpTpTpTpTpTpT*T*T*T*T*T; ODN 26: T*T*T*TpTpTpTpTpTpTpT*T*T*T*T. Oligonucleotides containing *N*,*N*-dimethylaminophosphorodiamidate (*) and phosphate (p) linkages are shown. Morpholino and 2′-deoxynucleosidesare noted by upper and lower case letters, respectively.

transfection of these chimeras, a PMO−DNA chimera was synthesize (ODN 27, 5′-FL-T*G*T*A*apapcpcpaptpgpaptpgptpgpcptpG*C*T*A*t; see Table 1 for a description of these abbreviations) where the internal, normal nucleotides were flanked at the 5′- and 3′- ends with *N*,*N*-dimethylamino PMO nucleotides. ODN 27 also contained a fluorescein dye (FL) joined by a six-carbonlinker (Supporting Information; Figure S32) to ODN 27. HeLa cells were transfected with ODN 27 (100 nM concentration) in the presence of DharmaFECT 1 with live cells (Supporting Information) and fixed cells (Figure 5) imaged by fluorescent microscopy after 20 and 18 h incubation, respectively. Transfection was dose dependent as an increased fluorescent signal was observed when cells were incubated with 100 nM of ODN 27 (Supporting Information, Figure S33). The fluorescence appears to be distributed primarily in the nucleus although there is also evidence of cytoplasmic distribution without the common punctuated structures found for many analogues.

DISCUSSION

In this manuscript we outline a new method for synthesizing PMO and PMO−DNA chimeras. The method is very robust as oligomers containing phosphosphorodiamidate internucleotide linkages can be prepared; using phosphoramidite chemistry, in high yield on automated DNA synthesizers. The process begins by incorporating morpholino phosphoramidites into DNA, boronation, and then oxidation with iodine in the presence of various amines in order to form a diverse set of PMOs and PMO−DNA chimeras. The method appears to be quite general and should lead to the synthesis of a large number of PMO linkages. Unlike procedures that use chlorophosphoramidate synthons, the more reactive phosphordiamidite synthons were used to synthesize these analogues in high yields. Selective activation, via dicyanoimidazole, of the diisopropylamino component of the morpholino phosphordiamidite during coupling was a key step for assembling these analogues.

PMO and PMO−DNA chimeras were synthesized on a DNA synthesizer. This is because both the morpholino phosphordiamidite and 2′-deoxyribonucleoside synthons were designed for synthesis of oligonucleotides in a 3′ to 5′ direction. This is not the case with previously developed chemistries where the *N*,*N*-dimethylamino PMO synthons are designed for synthesizing these analogues in a 5′ to 3′ direction. Moreover, as this new approach builds upon using a boranephosphoramidate internucleotide linkage, the same intermediate through iodine oxidation with an appropriate amine can be used to generate a large variety of PMO chimeras. For example PMO−LNA, PMO−RNA, PMO−DNA phosphorothioate/phosphorodithioate and PMO−DNA phosphonoacetate/phosphonoformatechimeras among others can be prepared.

Relative to their potential use in various biological and biochemical applications, these PMO−DNA chimeras exhibited three possible advantages over several other analogues. For example, aminomorpholino phosphorodiamidate derivatives were found to form a more stable duplex with complementary

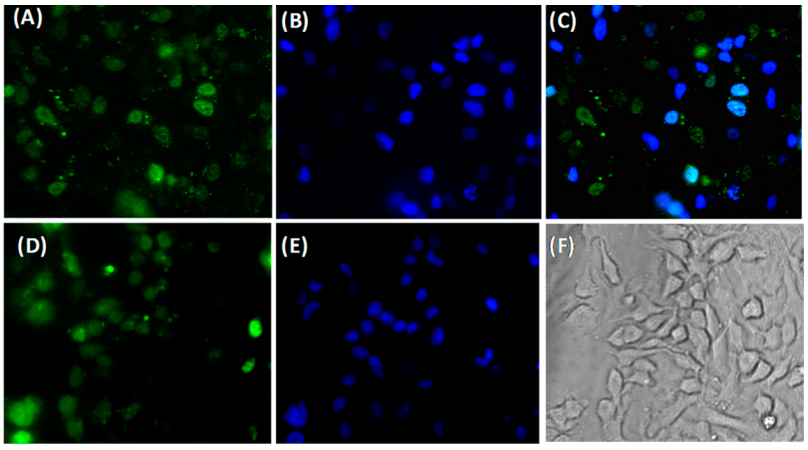


Figure 5. Visualization of ODN 27 transfection using fluorescence microscopy. ODN 27 (100 nM) in reduced serum medium (Opti-MEM) was transfected into HeLa cells using DharmaFECT 1 followed by 18 h of incubation at 37 °C. Cells were washed with PBS and fixed using buffered formalin solution. Nuclei of the cells were counterstained with DAPI (blue). (A)/(D) Image showing fluorescein localizationof ODN 27. (B)/(E) Images showing DAPI localization of nuclei in the same cells. (C). Image showing overlay of A and B. (F) Phase contrast image of the cells shown in D.

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

DNA or RNA than either unmodified DNA/RNA or the standard *N,N*-dimethylaminoPMO analogue (~1.75 times more stable per modification with RNA than the *N,N*-dimethyl analogue, see Table 3). Additionally these PMO−DNA chimeras are active with RNase H1. This new result is encouraging relative to the standard *N,N*-dimethylamino PMO analogues where the completely substituted PMO is inactive with RNase H1. As has been known for some time, several other analogues, such as methylphosphonates[31] and phosphonoacetates[32] as cap/gap oligomers are also known to activate RNase H1 activity. The observation that cap/gap *N,N*-dimethylamino PMO chimeras activate RNase H1 provides another analogue with different biochemical properties (for example the aminoamidate derivative has enhanced stability toward duplex formation) that may prove useful for various applications in biology. Thus, in a manner as observed previously with LNA/phosphorothioate oligonucleotides and due to the increased stabilization of these PMO−DNA chimeras with complementary RNA (relative to the unmodified duplexes), this kind of cap/gap analogue may decrease off-target effects because shorter single stranded antisense oligonucleotides can be used.[33]Moreover these PMO−DNA chimeras can easily be transfected into cells using regular DNA based transfecting reagents which eliminates the problems associated with delivery of PMOs by such procedures as microinjection,[34] hybridization of PMOs with DNA and delivery with ethoxylated polyethylenimine,[35] or conjugation with either peptides[36] or dendritic molecular transporters.[37]

CONCLUSIONS

This manuscript outlines a new, versatile method for synthesizing different amine substituted morpholino oligonucleotides other than the classical *N,N*-dimethylamino PMO analogue. The synthesis strategy is compatible with other current methodologies and high yielding. Moreover chimeras containing unmodified DNA/RNA and other analogues of DNA/RNA can also be prepared. Using this new chemistry, certain analogues form duplexes with complementary DNA or RNA that are more stable than natural DNA or DNA/RNA complexes. Also various PMO−DNA chimeras were shown to be active with RNase H1 and can be transfected into cells using standard lipid reagents. On the basis of these preliminary biochemical results, analogues of this type may prove quite useful for numerous applications, as they are stable toward nucleases,[38] and activate RNase H1.

EXPERIMENTAL SECTION

Chemical Synthesis. The general procedure for the synthesis of appropriately protected synthons (**9**, **14**, **19** or **24**) is as follows: 5′-*O*-DMT-N-BIBS protected morpholino nucleosides (**8**, **13**, **18** or **23**, see Supporting Information) were dried overnight in vacuum. They were dissolved in anhydrous CH_2Cl_2 followed by addition of 1.2 equiv of 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphorodiamidite under argon. After adding 0.5 equiv of 4,5-dicyanoimidazole, the reaction was allowed to proceed with stirring for 5 h under an argon atmosphere at room temperature. At this time, TLC indicated complete conversion of starting material. Reaction mixtures were diluted with CH_2Cl_2, washed first with a 5% $NaHCO_3$ solution and then brine. The organic layers were dried over Na_2SO_4, filtered and evaporated until dry. The products were purified by silica gel column chromatography. The silica gel slurry was prepared with the starting eluant mixture containing an additional 5% triethylamine. After pouring the slurry, the column was washed with two column volumes of the starting solvent mixture containing no triethylamine. Compounds **9**, **14**, **19** and **24** were purified using a gradient of 3:7 ethyl acetate:hexanes to 1:1 ethyl acetate:hexanes.

*Compound **9**: 5′-O-Dimethoxytritylmorpholinothymine-3′-O-cyanoethyl-N,N-diisopropylphosphordiamidite.* Yield: 86%. ^{31}P NMR (CD_2Cl_2) δ: 127.21, 126.08. ^{1}H NMR (CD_2Cl_2, 400 MHz) δ: 9.51 (1H, bs), 7.50−7.47 (2H, m), 7.38−7.25 (8H, m), 6.89−6.86 (4H, m), 5.78−5.75 (0.5 H, dd, J = 12.0, 4.0 Hz), 5.65−5.62 (0.5H, dd, J = 12.0, 4.0 Hz), 4.08−4.02 (1H, m), 3.99−3.86 (3H, m), 3.82 (6H, s), 3.65−3.52 (2H, m), 3.48−3.34 (1H, m), 3.31−3.27 (1H, m), 3.13−3.09 (1H, m), 2.75−2.68 (2H, m), 2.53−2.47 (2H, m), 1.96 (3H, m), 1.25−1.18 (12H, m). ^{13}C NMR (CD_2Cl_2) δ: 164.02, 163.96, 158.66, 150.13, 144.95, 135.92, 135.90, 135.79, 135.68, 135.52, 129.99, 128.07, 127.77, 126.77, 117.89, 117.74, 113.05, 110.48, 110.39, 86.05, 80.54, 80.49, 80.20, 77.36, 77.21, 64.37, 60.09, 59.84, 55.20, 49.05, 48.83, 47.58, 47.06, 46.83, 45.87, 45.78, 43.91, 43.74, 24.36, 24.29, 24.22, 24.20, 20.77, 20.33, 20.66, 12.29. ESI-MS (m/z): Calculated 744.3526, found 744.3497 $(M + H)^+$.

*Compound **14**: N^2-Di(tert-butyl)isobutylsilyl-5′-O-dimethoxytrityl-morpholinocytosine-3′-O-cyanoethyl-N,N-diisopropylphosphordiamidite.* Yield: 83%. ^{31}P NMR (CD_2Cl_2) δ: 126.32, 124.88.^{1}H NMR (CD_2Cl_2, 400 MHz) δ: 7.58−7.55 (1H, m), 7.50−7.48 (2H, m), 7.38−7.31 (6H, m), 7.28−7.24 (1H, m), 6.88−6.85 (4H, m), 5.79−5.76 (0.5H, dd, J = 12.0, 4.0 Hz), 5.63−5.59 (0.5H, dd, J = 12.0, 4.0 Hz), 4.54 (1H, bs), 4.04−3.87 (3H, m), 3.82 (6H, s), 3.74−3.71 (0.5H, m), 3.65−3.45 (3H, m), 3.30−3.24 (1.5H, m), 3.14−3.10 (1H, m), 2.80−2.75 (1H, m), 2.71−2.68 (1H, m), 2.55−2.46 (1H, m), 2.38−2.31 (1H, m), 2.11−2.07 (1H, m), 1.78 (1H, bs), 1.25−1.15 (31H, m), 1.04−1.02 (8H, m). ^{13}C NMR (CD_2Cl_2) δ:168.14, 158.61, 154.46, 144.98, 140.62, 140.42, 135.98, 135.88, 130.03, 128.07, 127.75, 126.71, 117.96, 117.75, 113.02, 96.20, 96.09, 85.97, 81.68, 81.34, 81.23, 77.05, 77.00, 64.50, 60.21, 60.09, 59.85, 55.18, 49.63, 49.41, 47.98, 47.13, 46.18, 45.91, 45.83, 43.96, 43.84, 43.77, 28.58, 26.21, 26.07, 24.78, 24.38, 24.26, 24.17, 24.12, 20.98, 20.76, 20.71, 20.62, 20.31, 20.23. ESI-MS (m/z): Calculated 927.5333, found 927.5329 $(M + H)^+$.

*Compound **19**: N^2-Di(tert-butyl)isobutylsilyl-5′-O-dimethoxytritylmorpholinoadenine-3′-O-cyanoethyl-N,N-diisopropylphosphordiamidite.* Yield: 79%. ^{31}P NMR (CD_2Cl_2) δ: 127.96, 125.36. ^{1}H NMR (CD_2Cl_2, 400 MHz): 8.38−8.37 (1H, d), 8.01−8.00 (1H, d), 7.51−7.48 (2H, m), 7.38−7.31 (6H, m), 7.28−7.24 (1H, m), 6.88−6.86 (4H, m), 5.93−5.90 (0.5H, dd, J = 12.0, 4.0 Hz), 5.82−5.79 (0.5H, dd, J = 12.0, 4.0 Hz), 5.39 (1H, bs), 4.14−4.10 (0.5H, m), 4.06−4.02 (0.5H, m), 4.0−3.90 (2H, m), 3.82 (6H, s), 3.71−3.63 (1H, m), 3.60−3.52 (2.5H, m), 3.42−3.37 (0.5H, m), 3.34−3.30 (1H, m), 3.17−3.13 (1H, m), 3.02−2.91 (1H, m), 2.78−2.75 (1H, m), 2.72−2.68 (1H, m), 2.66−2.59 (1H, m), 1.26−1.18 (31H, m), 1.10−1.08 (8H, m). ^{13}C NMR (CD_2Cl_2) δ: 158.64, 158.11, 152.55, 148.92, 144.97, 137.76, 135.92, 135.80, 130.02, 128.06, 127.77, 126.74, 121.60, 117.79, 117.71, 113.04, 86.50, 80.50, 80.44, 80.34, 80.22, 76.94, 76.81, 64.39, 60.14, 59.91, 55.18, 50.16, 49.93, 48.49, 48.44, 47.35, 47.12, 46.08, 46.00, 43.95, 43.83, 43.71, 28.62, 26.28, 26.19, 24.80, 24.35, 24.29, 24.18, 21.04, 20.79, 20.36, 20.27. ESI-MS (m/z): Calculated 951.5446, found 951.5437 $(M + H)^+$.

*Compound **24**: N^2,O^6-Bis[di(tert-butyl)isobutylsilyl]-5′-O-dimethoxytrityl-morpholinoguanine-3′-O-cyanoethyl-N,N-diisopropyl-phosphoradiamidite.* Yield: 82%. ^{31}P NMR (CD_2Cl_2) δ: 127.62, 126.99. ^{1}H NMR (CD_2Cl_2, 400 MHz): 7.79−7.76 (1H, d), 7.49−7.45 (2H, m), 7.37−7.29 (6H, m), 7.27−7.23 (1H, m), 6.87−6.84 (4H, m), 5.76−5.73 (1H, dd, J = 12.0, 4.0 Hz), 5.67−5.64 (1H, dd, J = 12.0, 4.0 Hz), 4.55 (1H, s), 4.05−3.88 (3H, m), 3.82 (6H, s), 3.67−3.54 (3H, m), 3.49−3.45 (1H, m), 3.32−3.23 (1H, m), 3.19−2.90 (2H, m), 2.71−2.68 (2H, m), 2.61−2.54 (1H, m), 2.23−2.16 (1H, m), 2.13−2.06 (1H, m), 1.27−1.15 (49H, m), 1.06−0.97 (27H, m). ^{13}C NMR (CD_2Cl_2) δ: 160.67, 159.38, 158.61, 154.33, 154.06, 144.96, 136.47, 135.90, 135.79, 130.00, 128.02, 127.76, 126.69, 117.58, 116.77, 113.03, 85.99, 80.69, 80.58, 79.55, 79.49, 76.95, 76.45, 76.36, 64.22, 60.20, 60.12, 59.88, 59.86, 55.16, 49.71, 49.47, 47.77, 47.68, 47.46, 47.26, 45.96, 45.89, 43.99, 43.88, 43.77, 43.65, 28.76, 28.07, 26.39, 26.31, 26.27, 24.87, 24.68, 24.44, 24.37, 24.27, 24.19, 21.90, 21.37, 20.83, 20.76, 20.75, 20.69, 20.35, 20.32, 20.27, 20.24. ESI-MS (m/z): Calculated 1165.7198, found 1165.7203 $(M + H)^+$.

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672

Automated PMO Synthesis. Synthesis was carried out on an ABI 394 Synthesizer. All syntheses were performed at a 0.2 μmol scale using a 5′-DMTr-2′-deoxythymidine joined to a low volume polystyrene solid support via a succinate linkage. For synthesis of morpholino oligonucleotides, a standard 0.2 μ mole synthesis cycle was used with an increased coupling time of 300 s. A wash with methanol followed the detritylation step. All the phosphordiamidites (**9**, **14**, **19** or **24**; 0.1 M) were dissolved in anhydrous CH_3CN. Detritylation was carried out using a 0.5% solution of TFA in anhydrous $CHCl_3$ containing 10% TMPB. Solutions for boronation (0.05 M BH_3-THF complex in THF) were prepared fresh prior to use. Reagents for activation (4,5-dicyanoimidazole) and capping were purchased from Glen Research. A stepwise description of the synthesis cycle is described in Table S1 (Supporting Information). Deprotection was carried out in two steps: the solid support linked boranephosphoroamidate morpholino oligonucleotides were first treated with a 1:1 solution of triethylamine in acetonitrile for 10 min followed by extensive washing with acetonitrile. The resin was then dried using a flow of argon and transferred to a glass vial. A solution of iodine (0.05 M) and dimethyl amine in THF (2.0 M) was added to the glass vial. For reactions with ammonia, iodine (0.05 M) was dissolved in a solution of ammonia (2.0 M) in isopropanol and this solution was added to the resin and stirred overnight. The glass vial was then placed on a mechanical shaker for 24 h. For the synthesis of the methylamine and morpholinodiamidate derivatives, a 2.0 M solution of each amine in THF was used.

The vials containing resins were centrifuged at 4000 rpm and the supernatants removed with a pipet. Subsequently resins were washed 4−5 times with 2 mL aliquots of acetonitrile, shaken vigorously, placed in a centrifuge at 4000 rpm for 5 min and supernatants removed. For amino modified morpholino derivatives, the ammonia solution in isopropanol was removed under vacuum. These morpholino oligonucleotides were desilylated by overnight fluoride (1.0 mL of 1.0 M solution of tetrabutylammonium fluoride in THF) treatment. The resins were then treated with 1 mL of 37% ammonium hydroxide in water for 1 h in order to remove the product from the resins and the polystyrene resin beads were removed using a 0.2 μm centrifugal filter.

The total volume of each solution was diluted to 2.5 mL by adding 0.5 mL Millipore water and the PMOs were desalted using Illustra NAP-25 Columns (GE Healthcare). Initially these columns were equilibrated with 25.0 mL of water. After allowing water to completely enter the gel beds by gravity flow, 2.5 mL of samples were loaded on to columns and the purified samples were eluted using 3.5 mL Millipore water. Second step purifications were carried out for oligonucleotides having more than 10 nucleotides by using Amicon Ultra-4 3K devices. Solutions that accumulated after Nap column purification (3.5 mL total volume) were loaded on to Amicon devices and centrifuged at 4000*g* for 30 min. Concentrated solutes were washed with 3.0 mL × 2 of Milipore water in a similar fashion. The oligonucleotides were collected and used for various experiments.

Synthesis of a 5′-Fluorescein PMO−DNA Chimera. After completion of a DMTr-ON synthesis of the boranephosphoroamidate morpholino chimera, the ODN was conjugated with 5′-Amino-Modifier C6-TFA (Glen research) using a standard DNA synthesis cycle. After carrying out the iodine oxidation reaction and desilylation of exocyclic amines, the oligonucleotide was purified using reverse phase HPLC (Buffer A: triethylammonium bicarbonate, 0.05 M; Buffer B: acetonitrile; 0−100% B in 50 min; 55 °C; 4.0 mL/min flow rate). The purified ODN was dissolved in 1 mL of a solution of 1:1 NH_4OH:CH_3NH_2 and heated at 65 °C for 3 h to remove the trifluoroacetamido group. The reaction mixture was dried and dissolved in a buffer (200 μL) containing 20 mM sodium phosphate and 0.15 M NaCl (pH 8.0) and the concentration was measured. A 20-fold molar excess of 5-(and 6-)carboxy-fluorescein succinimidyl ester (Thermo Fisher Scientific) was dissolved in DMSO and added to the ODN solution. The reaction mixture was stirred at room temperature for 1 h followed by stirring at 4 °C for a period of 3 h. The reaction mixture was diluted using 300 μL water and excess NHS-fluorescein was removed using Illustra NAP-5 Column.

***E. coli* RNase H1 Hydrolysis of RNA Heteroduplexes.** Experiments with *E. coli* RNaseH1 (Promega) were performed using the conditions described by Dellinger et al.[30] The reactions were carried out using an assay buffer of 50 mM tris-HCl (pH 8.0), 20 mM KCl, 9 mM $MgCl_2$, 1 mM β-mercaptoethanol, and 250 μg/mL bovine serum albumin. An oligodeoxynucleotide or modified oligodeoxynucleotide (200 pmol) and 5′-*O*-fluorescein labeled, complementary oligoribonucleotide were added to the assay buffer (35 μL). Following the addition of *E. coli* RNase H1 (3 units), reactions were carried out at 25 °C over 12 h. The reaction mixtures were diluted with an equal volume of 80% formamide gel loading buffer containing tracking dyes and analyzed by polyacrylamide gel electrophoresis (20%, 19:1 cross-linking, 7 M urea). All reactions were performed in triplicate. The developed gels were analyzed using a Molecular Dynamics Typhoon Phosphorimager.

Lipid Transfection As Observed by Microscope Imaging. An ODN 27 stock solution was diluted with 200 μL OptiMEM to a final concentration of 0.1 μM ODN. In a separate Eppendorf Tube, 5.0 μL DharmaFECT 1 was diluted with 200 μL OptiMEM. The 200 μL solution of ODN 27 and the DhamaFECT 1 solution were mixed, equilibrated for 20 min, and 600 μL OptiMEM was added. HeLa cells were seeded at 0.3×10^6 cells/well on a coverslip placed in a six well plate in DMEM medium containing 10% FBS and penstrep. After 24 h, medium was removed and the cells were washed twice (2.0 mL D-PBS/wash) before transfection at 80% confluency. D-PBS was removed from the HeLa cells, and 1.0 mL of the transfection mixture was added to each well. Cells were then incubated at 37 °C for 18 h and washed twice (2.0 mL D-PBS/wash). Cells were covered with 1.0 mL of 10% neutral buffered formalin for 15 min. The formalin solution was removed and the cells were covered with 3.0 mL DPBS for 10 min at RT. The coverslips were removed from the wells and mounted upside down on coverslides using Fluoromount-G with DAPI as mounting media and observed using an inverted microscope (OlympusIX 81) equipped with a Hamamatsu C4742−95 CCD and CoolSNAP ES digital camera (Photometrics).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08854.

Detailed synthetic procedures, characterization information and LC−MS spectra of synthesized oligomers (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*marvin.caruthers@colorado.edu

ORCID

Marvin H. Caruthers: 0000-0002-4931-468X

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Douglas J. Dellinger (Agilent Technologies) for providing access to an LC−MS instrument and Dr. Subhadeep Roy for meaningful discussions. We thank Joe Dragavon for fluorescent microscopy. The research was supported by the University of Colorado.

■ REFERENCES

(1) Chan, J. HP.; Lim, S.; Wong, WS. F. *Clin. Exp. Pharmacol. Physiol.* **2006**, *33*, 533−540.

(2) Khorkova, O.; Myers, A. J.; Hsiao, J.; Wahlestedt, C. *Hum. Mol. Genet.* **2014**, *23*, R54−R63.

(3) Summerton, J.; Weller, D. *Antisense Nucleic Acid Drug Dev.* **1997**, *7*, 187−195.
(4) Summerton, J. *Biochim. Biophys. Acta, Gene Struct. Expression* **1999**, *1489*, 141−158.
(5) Hudziak, R. M.; Barofsky, E.; Barofsky, D. F.; Weller, D. L.; Huang, S. B.; Weller, D. D. *Antisense Nucleic Acid Drug Dev.* **1996**, *6*, 267−272.
(6) Arora, V.; Knapp, D. C.; Reddy, M. T.; Weller, D. D.; Iversen, P. L. *J. Pharm. Sci.* **2002**, *91*, 1009−1018.
(7) Summerton, J. E. In *Morpholinos and Related Antisense Biomolecules*; Janson, C. G., During, M. J., Eds.; Kluwer Academic/Plenum Publishers: Dordrecht, the Netherlands, 2003.
(8) Ekker, S. C.; Larson, J. D. *Genesis* **2001**, *30*, 89−93.
(9) www.gene-tools.com/morpholino-publication-database.
(10) www.sarepta.com.
(11) Paul, S.; Jana, S.; Bhadra, J.; Sinha, S. *Chem. Commun.* **2013**, *49*, 11278−11280.
(12) Tercero, N.; Wang, K.; Gong, P.; Levicky, R. *J. Am. Chem. Soc.* **2009**, *131*, 4953−4961.
(13) Mangos, M. M.; Min, K. L.; Viazovkina, E.; Galarneau, A.; Elzagheid, M. I.; Parniak, M. A.; Damha, M. J. *J. Am. Chem. Soc.* **2003**, *125*, 654−661.
(14) Moulton, H. M.; Hase, M. C.; Smith, K. M.; Iversen, P. L. *Antisense Nucleic Acid Drug Dev.* **2003**, *13*, 31−43.
(15) Järver, P.; Coursindel, T.; Andaloussi, S. EL.; Godfrey, C.; Wood, M. JA.; Gait, M. *Mol. Ther.–Nucleic Acids* **2012**, *1*, e27/1−e27/17.
(16) For the morpholino derivatives, the numbering of the primary hydroxyl group should be 6′. In order to correlate with the numbering system commonly used in the nucleic acid field, we marked it as 5′ for this manuscript.
(17) Summerton, J.; Weller, D. US Patent 5217866 A 19930608, 1993.
(18) Harakawa, T.; Tsunoda, H.; Ohkubo, A.; Seio, K.; Sekine, M. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 1445−1447.
(19) Paul, S.; Pattanayak, S.; Sinha, S. *Tetrahedron Lett.* **2014**, *55*, 1072−1076.
(20) Bhadra, J.; Kundu, J.; Ghosh, K. C.; Sinha, S. *Tetrahedron Lett.* **2015**, *56*, 4565−4568.
(21) Beaucage, S. L.; Caruthers, M. H. *Tetrahedron Lett.* **1981**, *22*, 1859−1862.
(22) Paul, S.; Roy, S.; Monfregola, L.; Shang, S.; Shoemaker, R.; Caruthers, M. H. *J. Am. Chem. Soc.* **2015**, *137*, 3253−3264.
(23) Zhang, N.; Tan, C.; Cai, P.; Jiang, Y.; Zhang, P.; Zhao, Y. *Tetrahedron Lett.* **2008**, *49*, 3570−3573.
(24) Pattanayak, S.; Paul, S.; Nandi, B.; Sinha, S. *Nucleosides, Nucleotides Nucleic Acids* **2012**, *31*, 763−782.
(25) Roy, S.; Olesiak, M.; Shang, S.; Caruthers, M. H. *J. Am. Chem. Soc.* **2013**, *135*, 6234−6241.
(26) The CPG support could not be used because this support is not compatible with fluoride reagents that are required to remove N-silyl ether protecting groups from the bases.
(27) Zhang, N.; Tan, C.; Cai, P.; Zhang, P.; Zhao, Y.; Jiang, Y. *Bioorg. Med. Chem.* **2009**, *17*, 2441−2446.
(28) Perrin, D. D. In *Dissociation Constants of Organic Bases in Aqueous Solution*; Butterworths Publishers: London, 1965; pp 41, 137, 141. The values given in brackets correspond to the pK_as calculated in water and are used as formal indicators of amine basicity.
(29) The use of ETT for the synthesis of chimeras was possible because the borane/morpholino P(IV) linkage was stable towards this activator.
(30) Dellinger, D. J.; Sheehan, D. M.; Christensen, N. K.; Lindberg, J. G.; Caruthers, M. H. *J. Am. Chem. Soc.* **2003**, *125*, 940−950.
(31) Giles, R. V.; Tidd, D. M. *Nucleic Acids Res.* **1992**, *20*, 763−770.
(32) Sheehan, D. M.; Lunstad, B.; Yamada, C.; Stell, M.; Caruthers, B. G. *Nucleic Acids Res.* **2003**, *31*, 4109−4118.
(33) Obad, S.; dos Santos, C. O.; Petri, A.; Heidenblad, M.; Broom, O.; Ruse, C.; Fu, C.; Lindow, M.; Stenvang, J.; Straarup, E. M.; Hansen, H. F.; KochT; Pappin, D.; Hannon, G. J.; Kauppinen, S. *Nat. Genet.* **2011**, *43*, 371−378.
(34) Nasevicius, A.; Ekker, S. C. *Nat. Genet.* **2000**, *26*, 216−220.
(35) Morcos, P. A. *Genesis* **2001**, *30*, 94−102.
(36) Moulton, H. M.; Nelson, M. H.; Hatlevig, S. A.; Reddy, M. T.; Iversen, P. L. *Bioconjugate Chem.* **2004**, *15*, 290−299.
(37) Li, Y.-F.; Morcos, P. A. *Bioconjugate Chem.* **2008**, *19*, 1464−1470.
(38) Summerton, J. *J. Drug Discovery, Dev. Delivery* **2016**, *3*, 1019−1025.

DOI: 10.1021/jacs.6b08854
J. Am. Chem. Soc. 2016, 138, 15663−15672