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Synthesis of Phosphorodiamidate Morpholino Oligonucleotides and Their Chimeras Using Phosphoramidite Chemistry

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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 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 on-going 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 nanotechnology¹¹ and surface hybridization¹² 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 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 resin¹⁶ (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.

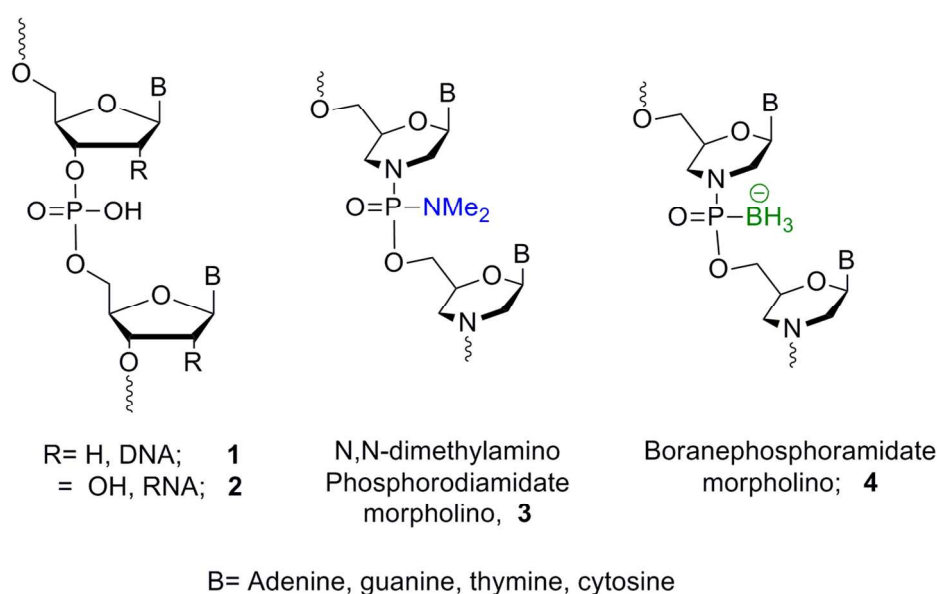
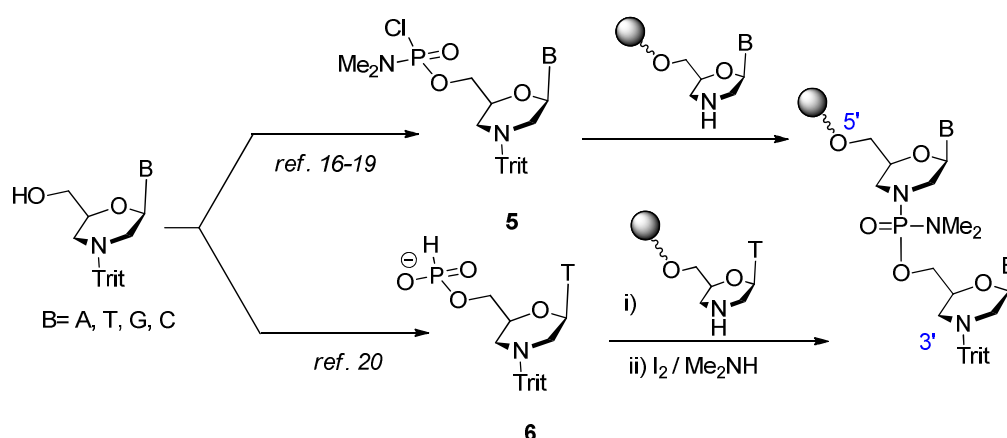


Figure 1. Chemical structures of DNA, RNA, N,N-dimethylamino PMOs and Boranephosphoroamidate morpholino oligonucleotides.

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-dichlorophosphoramidate 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.^{17,18,19} 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 towards oxidation under ambient conditions than most P (III) compounds. Moreover coupling yields are low (77%) towards formation of the H-phosphonatemorpholino dimer.²⁰ These limitations led us to explore using the phosphoramidite methodology²¹ for the synthesis of various PMO derivatives.



Scheme 1. Current approaches for the synthesis of N,N-dimethylamino PMOs.

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 baborate 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 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

10, 15, 20 + **Si-OTf** $\xrightarrow{\text{i}}$ **11, 16, 21** $\xrightarrow{\text{ii, iii}}$ **7, 12, 17, 22**

8, 13, 18, 23 $\xrightarrow{\text{iv, v}}$ **9, 14, 19, 24** $\xrightarrow{\text{vi}}$ **25, 26, 27, 28**

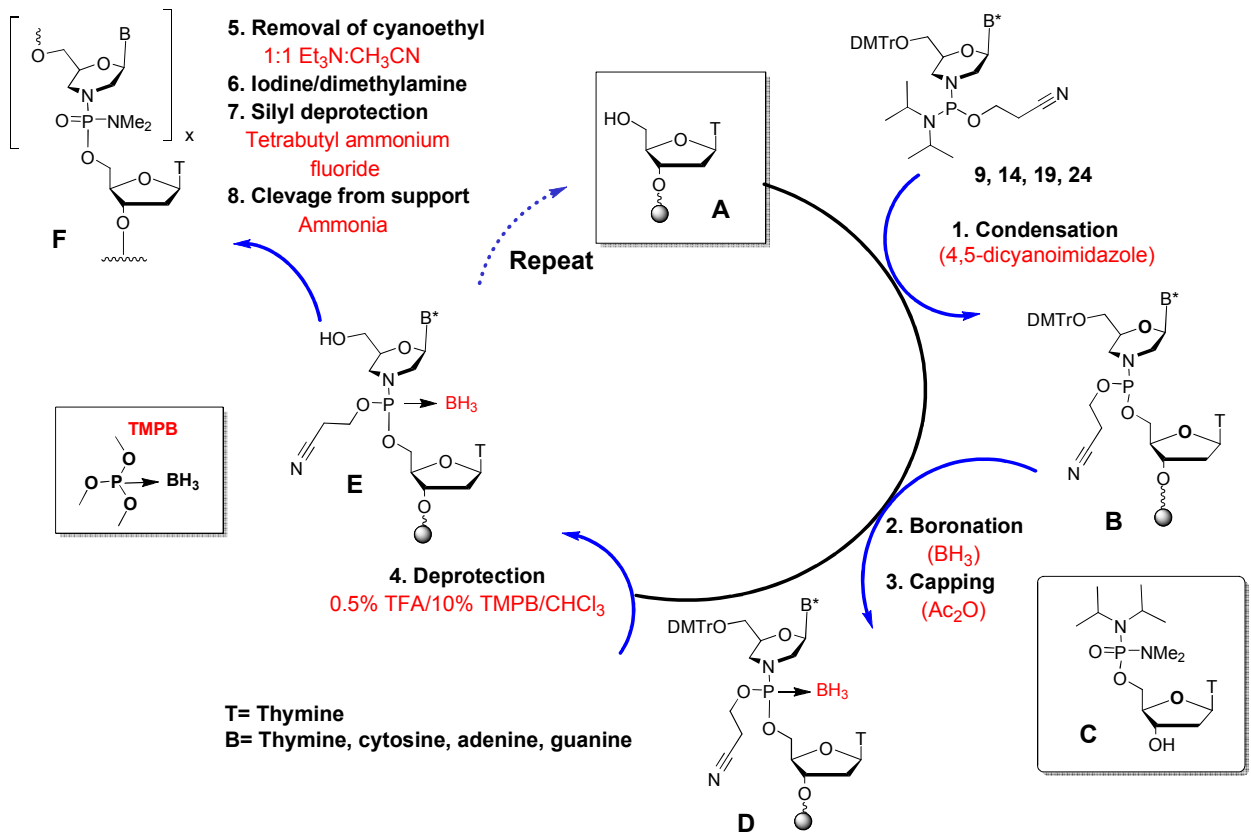
B* =

7, 8, 9, 25 **11, 12, 13, 14, 26** **16, 17, 18, 19, 27** **21, 22, 23, 24, 28**

Synthesis of N,N-Dimethylamino PMO Oligonucleotides.

4

phosphoramidite 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.



Scheme 3. Synthesis cycle for the preparation of PMOs. **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.

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 two hours 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. (Et₃N·HF could not be used for removing these silyl groups as this reagent hydrolyzed phosphorodiamidate 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)-1H-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 towards 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 0.5% TFA in chloroform (**D** was stable to a acidic detritylation conditions).

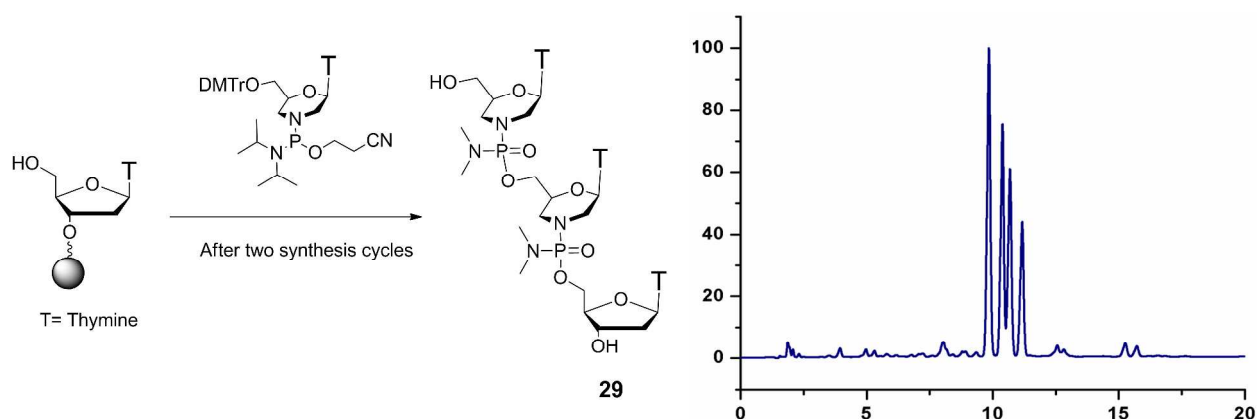


Figure 2. Synthesis of a N,N-Dimethylamino PMO trimer (**29**). The LC profile from the LCMS analysis of the total reaction mixture is shown.

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 LCMS. 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 LCMS. 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,²⁹ we carried out the synthesis of a 16-mer having all four bases (Table 1, ODN 8). As can be seen from the LCMS chromatogram of the unpurified reaction mixture (Figure 3A), both the yield (10 A₂₆₀ 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 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 LCMS (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). LCMS analysis and ³¹P 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 A₂₆₀ 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.

NO	ODNs	Mol wt	
		Cal	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	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 _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t	6430.17	6430.15
ODN 10	t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p T*T*T*T*t	6430.17	6430.15
ODN 11	t _p t _p t _p t _p t _p t _p t _p T*T*T*T*t _p t _p t _p t _p t _p t _p t _p t _p t	6430.17	6430.14
ODN 12	T*T*T*T*t _p t _p t _p t _p t _p t _p t _p t _p t _p t _p T*T*T*T*t	6534.17	6534.38
ODN 13	T*t _p t _p T*t _p t _p t _p T*t _p t _p t _p T*t _p t _p t _p T*t _p t _p t _p T*t	6482.26	6482.17
ODN 14	t _p a _p g _p t _p t _p g _p a _p g _p t _p g _p a _p t _p A*T*C*A*t	5343.45	5343.07
ODN 15	G*T*A*G*t _p C _p C _p t _p g _p C _p a _p a _p t _p g _p a _p t	5000.26	4999.99
ODN 16	T*G*A*t _p C _p g _p C _p t _p g _p C _p a _p a _p T*G*A*t	5052.26	5052.16

Table 1. Mass analysis (LCMS) of PMO and PMO-DNA chimeras (ODNs). 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.

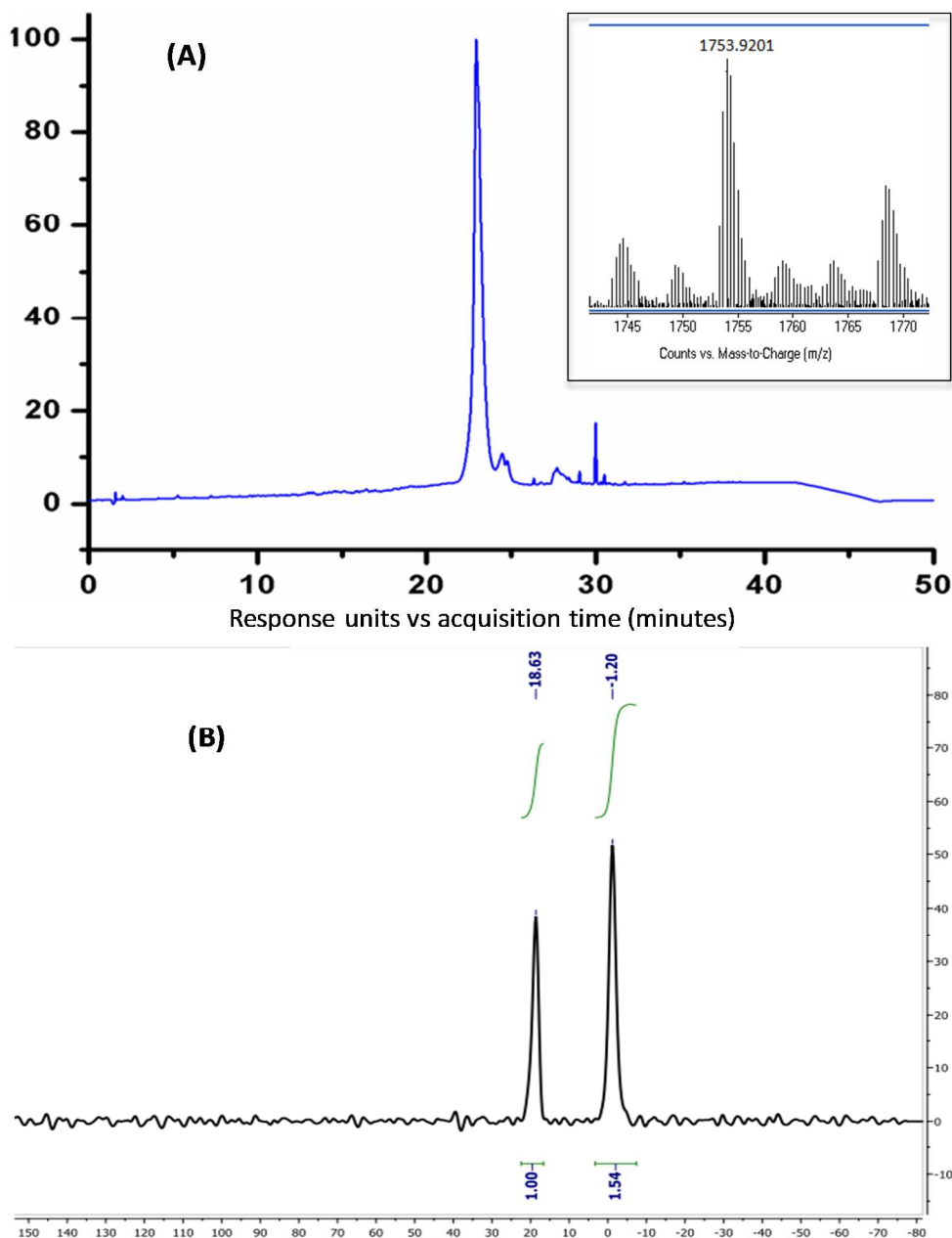


Figure 3. (A) LCMS analysis of the total reaction mixture from the synthesis of ODN 8. The data shows the LC trace and a zoomed in view of the base peak in the mass spectra. The base peak in each case was a -3 charged species. Full mass spectra are provided in the supplementary section. (B) ^{31}P NMR 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 respectively.

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 chimeras 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 LCMS and the sequences of PMOs are listed in Table 2. Yields were comparable to those obtained for the N,N-dimethylamino PMO chimeras.

NO	ODNs	Mol wt	
		Cal	Obs
ODN 17	t _p t _p t _p t _p t _p t _p t _p T*T*T*T*t _p t _p t _p t _p t _p t _p t	6318.21	6318.02
ODN 18	t _p t _p t _p t _p t _p t _p t _p T♦T♦T♦T♦t _p t _p t _p t _p t _p t _p t	6374.32	6374.08
ODN 19	t _p t _p t _p t _p t _p t _p t _p T•T•T•T•t _p t _p t _p t _p t _p t _p t	6598.57	6598.20
ODN 20	t _p a _p g _p t _p t _p g _p a _p g _p t _p g _p a _p t _p A*T*C*A*t	5231.48	5230.97
ODN 21	G*T*A*G*t _p C _p C _p t _p g _p C _p a _p a _p t _p g _p a _p t	4888.26	4887.89
ODN 22	T*G*A*t _p C _p g _p C _p t _p g _p C _p a _p a _p T*G*A*t	4884.26	4883.96

Table 2. LCMS Analysis of PMO-DNA Chimeras. ODN 17, 20, 21and 22: * = NH₂; 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.

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 1.0μM 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 A₂₆₀ versus time was recorded. Melting temperatures were taken as the temperature of half dissociation and were obtained from the first derivative plots (Table 3).

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.

Fluorescence Microscopy

12

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 analogs.

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 boranephosphoroamidate 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-DNAphosphorothioate/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 phosphordiamidate derivatives were found to form a more stable duplex with complementary 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 analogs where the completely substituted PMO is inactive with RNase H1. As has been known for some time, several other analogs, such as methylphosphonates³¹ and phosphonoacetates³² 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 analog 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 peptides³⁶ 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 analogs of DNA/RNA can also be prepared. Using this new chemistry, certain analogs 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 CH₂Cl₂ followed by addition of 1.2 equivalents of 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite under argon. After adding 0.5 equivalent 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 CH₂Cl₂, washed first with a 5% NaHCO₃ solution and then brine. The organic layers were dried over Na₂SO₄, filtered and evaporated to dryness. 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 ethylacetate:hexanes to 1:1 ethylacetate:hexanes.

Compound 9: 5'-O-Dimethoxytritylmorpholinothymine-3'-O-cyanoethyl-N,N-diisopropylphosphordiamidite: Yield: 86%. ³¹P NMR (CD₂Cl₂) δ: 127.21, 126.08. ¹H NMR (CD₂Cl₂, 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). ¹³C NMR (CD₂Cl₂) 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%. ³¹P NMR (CD₂Cl₂) δ: 126.32, 124.88. ¹H NMR (CD₂Cl₂, 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). ¹³C NMR (CD₂Cl₂) 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%. ³¹P NMR (CD₂Cl₂) δ: 127.96, 125.36. ¹H NMR (CD₂Cl₂, 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). ¹³C NMR (CD₂Cl₂) 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*-diisopropylphosphoradiamidite: Yield: 82%. ³¹P NMR (CD₂Cl₂) δ: 127.62, 126.99. ¹H NMR (CD₂Cl₂, 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). ¹³C NMR (CD₂Cl₂) 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)⁺.

Automated PMO Synthesis. Synthesis was carried out on an ABI 394 Synthesizer. All syntheses were performed at a 0.2 μ mol 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 CH₃CN. Detritylation was carried out using a 0.5% solution of TFA in anhydrous CHCl₃ containing 10% TMPB. Solutions for boronation (0.05 M BH₃-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 minutes 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 (0.05M) 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 hrs. 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 pipette. Subsequently resins were washed 4-5 times with 2 mL aliquots of acetonitrile, shaken vigorously, placed in a centrifuge at 4000 rpm for 5 minutes 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 μ m 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 4000xg for 30 minutes. Concentrated solutes were washed with 3.0 mL X 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 mins; 55 °C; 4.0 mL/min flow rate). The purified ODN was dissolved in 1 mL of a solution of 1:1 NH₄OH: CH₃NH₂ and heated at 65 °C for three hours 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, 9mM MgCl₂, 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 200µL 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 hours, 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 hours and washed twice (2.0 mL D-PBS/wash). Cells were covered with 1.0 mL of 10% neutral buffered formalin for 15 mins. 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 Flouromount-G with DAPI as mounting media and observed using an inverted microscope (Olympus IX 81) equipped with a Hamamatsu C4742-95 CCD and CoolSNAP ES digital camera (Photometrics).

ASSOCIATED CONTENT

Supporting Information

Detailed synthetic procedures, characterization information and LCMS spectra of synthesized oligomers are provided. This information is provided free of charge via the Internet at <http://pubs.acs.org>.

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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 Drug Dev.* **1997**, *7*, 187-195.
4. Summerton, J. *Biochim. Biophys. Acta.* **1999**, *1489*, 141-158.
5. Hudziak, R. M.; Barofsky, E.; Barofsky, D. F.; Weller, D. L.; Huang, S. B.; Weller, D. D. *Antisense Nucleic 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 P.O. Box 17, 3300 AA 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 Drug Dev.* **2003**, *13*, 31-43.
15. Järver, P.; Coursindel, T.; Andaloussi, S. EL.; Godfrey, C.; Wood, M. JA.; Gait, M. *Molecular Therapy 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. *Nucleo. Nucleot. Nucl. 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, Halsbury House, 35, Chancery Lane, 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 Research* **1992**, 20, 763–770.
32. Sheehan, D. M.; Lunstad, B.; Yamada, C., M.; Stell, B. G.; Caruthers, M. H. *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.; Koch, T.; 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 Discov. Develop. And Deliv.* **2016**, 3, 1019–1025.

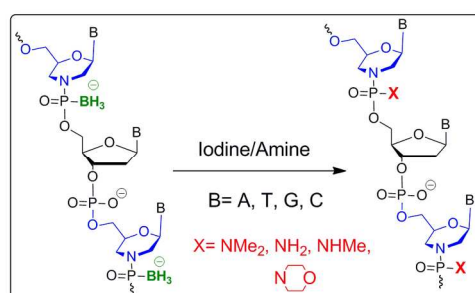
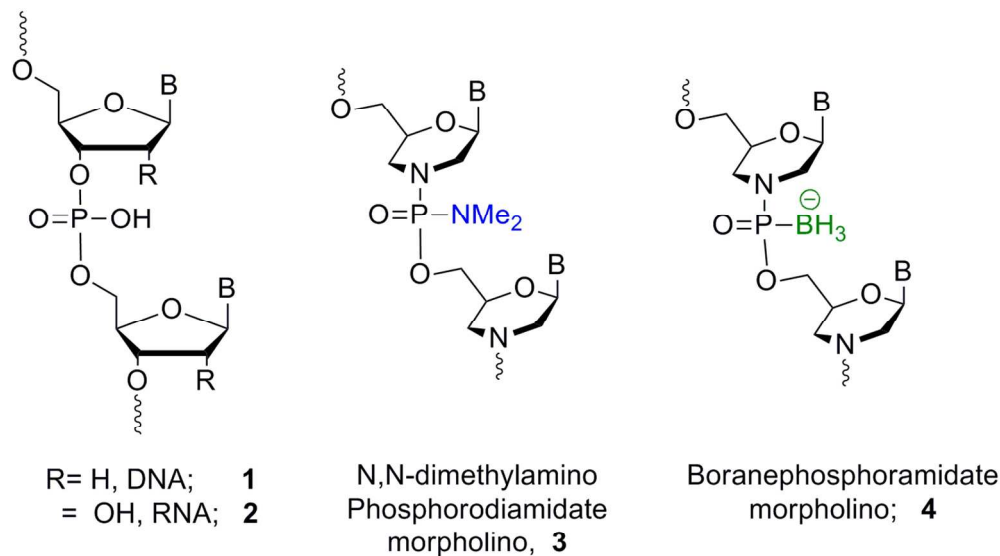


Table of Contents Figure



B= Adenine, guanine, thymine, cytosine

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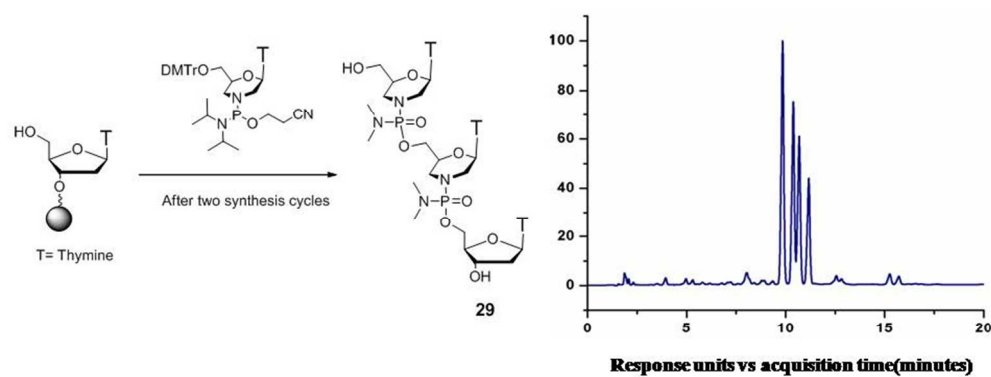


Figure 2. Synthesis of a N,N-Dimethylamino PMO trimer (29). The LC profile from the LCMS analysis of the total reaction mixture is shown.

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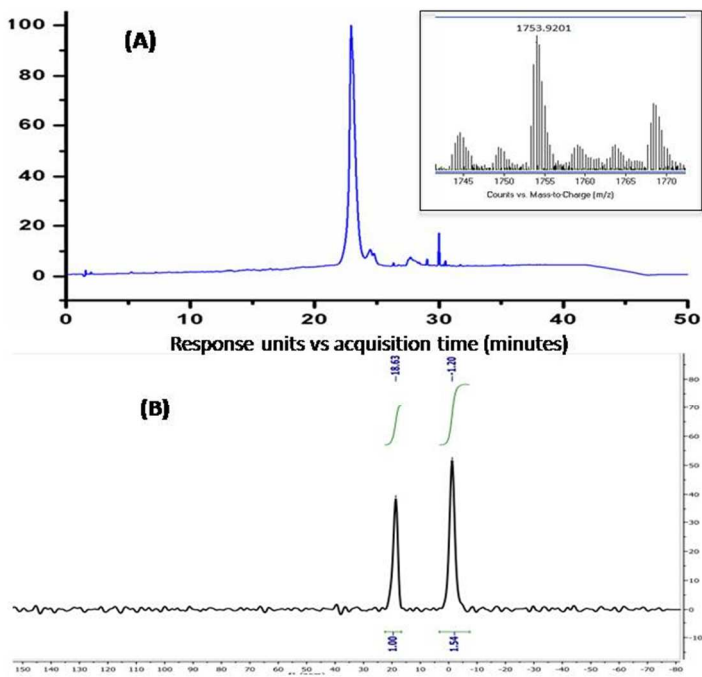


Figure 3. (A) LCMS analysis of the total reaction mixture from the synthesis of ODN 8. The data shows the LC trace and a zoomed in view of the base peak in the mass spectra. The base peak in each case was a -3 charged species. Full mass spectra are provided in the supplementary section. (B) ³¹P NMR of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

254x190mm (96 x 96 DPI)

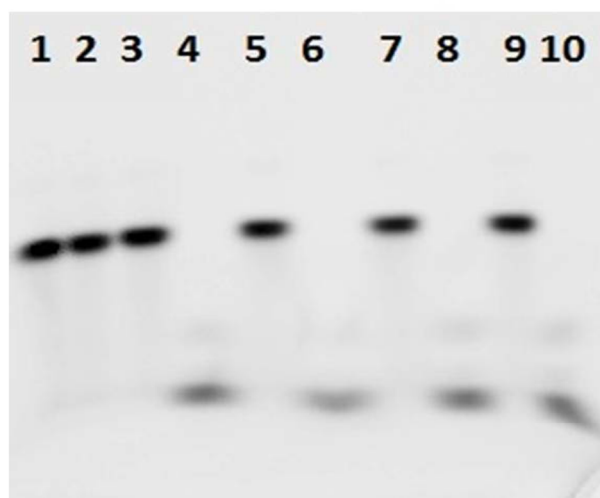


Figure 4. *E. coli* RNase H1 degradation of RNA when Catalyzed by PMO-DNA Chimeras. 5'-O-Fluorescein labeled dr(A)₁₄ was allowed to form duplexes with complementary ODNs 24-26. These duplexes were then treated with *E. coli* RNase H1 for 12 h and analyzed by PAGE. Lane 1: 2'-O-methyl RNA/RNA; Lane 2: 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 H1; Lane 7: ODN 25/RNA; Lane 8: ODN 25/RNA/RNase H1; Lane 9: ODN 26/RNA; Lane 10: ODN 26/RNA/RNase H1.

254x190mm (96 x 96 DPI)

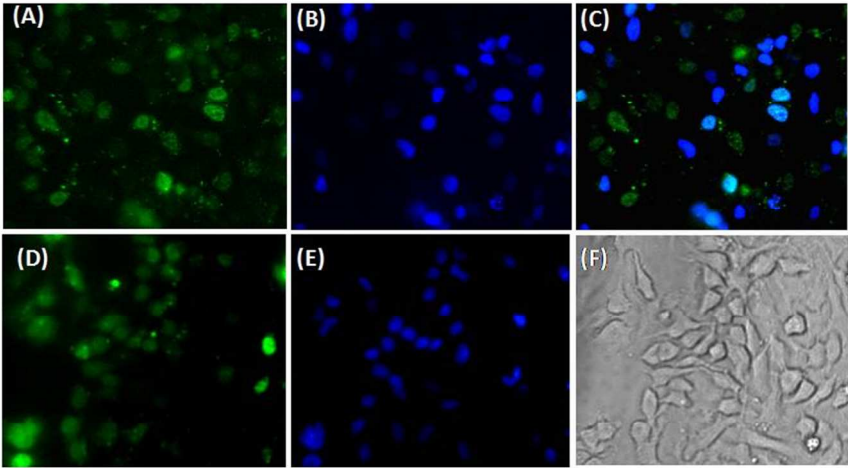
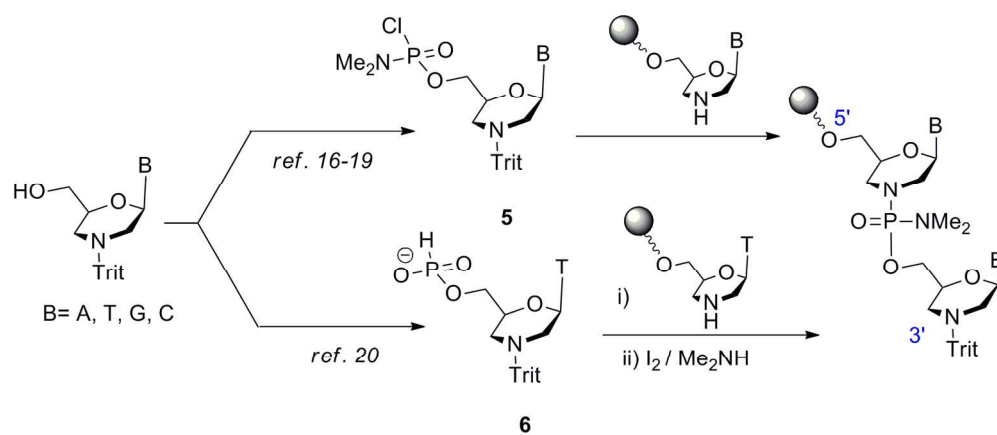


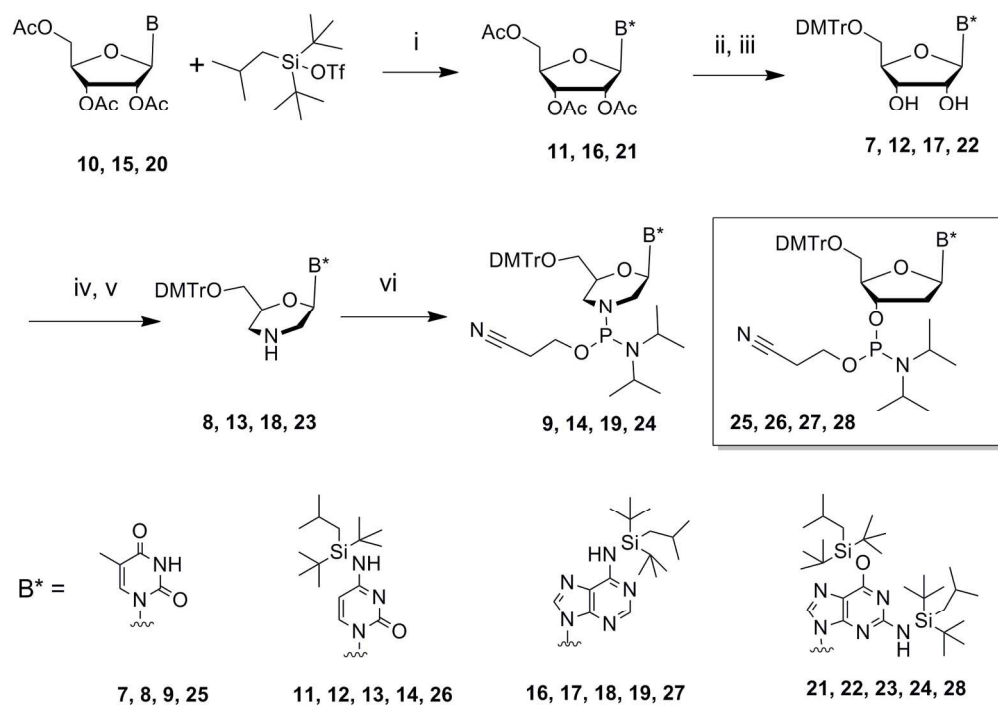
Figure 5. Visualization of ODN 27 transfection using fluorescence microscopy. ODN 27 (100 nM) in reduced serum medium (Opti-MEM) was transfected into HeLa cells using Dharmafect 1 followed by 18 h of incubation at 37 °C. Cells were washed with PBS and fixed using buffered formalin solution. Nuclei of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

254x190mm (96 x 96 DPI)



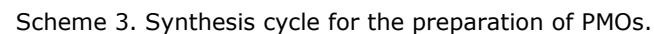
Scheme 1. Current approaches for the synthesis of N,N-Dimethylamino PMOs.

159x69mm (300 x 300 DPI)



Scheme 2. Synthesis of BIBS protected Morpholino Phosphordimidite synthons. (i) Lutidine (4.0 eq.)/1,4-Dioxane; (ii) 1.0 M NH_3/MeOH ; (iii) DMT-Cl (1.2 eq.)/Pyridine; (iv) NaIO_4 (1.1 eq.)/ $(\text{NH}_4)_2\text{B}_4\text{O}_7$ (1.1 eq.)/ MeOH ; (v) NaCNBH_3 (2.0 eq.)/ AcOH (2.0 eq.)/ MeOH ; (vi) $\text{P}(\text{OCH}_2\text{CH}_2\text{CN})(\text{N}i\text{Pr}_2)_2$ (1.2 eq.)/4,5-Dicyanoimidazole (0.5 eq.)/ CH_2Cl_2 .

170x119mm (300 x 300 DPI)



208x138mm (300 x 300 DPI)

Reactions	Wash/reagents/solvents	Time(s)
Detritylation	10% TMPB + 0.5% TFA in CHCl ₃	Flow 45 s
Wash	MeOH	Flow 20 s, wait 10 s, flow 10 s.
Condensation	0.1 M phosphoradimidite 9 , 14 , 19 or 24 (in CH ₃ CN) + Activator (0.12 M dicyanoimidazole in CH ₃ CN) Or 0.1 M phosphoramidite 25 , 26 , 27 or 28 in CH ₃ CN + Activator (0.25 M ethylthio tetrazole in CH ₃ CN)	Wait 300 s Wait 180 s
Boronation Or Oxidation	0.05 M BH ₃ -THF in THF 0.02 M iodine in THF/water/pyridine	Flow 15 s, wait 45 s Flow 8 s, wait 15 s
Wash	Anhydrous CH ₃ CN	Flow 10s
Capping	Cap A (THF/Pyridine/Ac ₂ O) + Cap B (16% 1-methylimidazole in THF)	Flow 10 s, wait 5 s
Wash	Anhydrous CH ₃ CN	Flow 10 s
Wash	Anhydrous CH ₂ Cl ₂	Flow 25 s

Table 1. Synthesis cycle for preparing PMOs and PMO-DNA chimeras.

254x190mm (96 x 96 DPI)

Table 2. Mass analysis (LCMS) of PMO and PMO-DNA chimeras (ODNs). 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.

ACS Paragon Plus Environment

NO	ODNs	Mol wt	
		Cal	Obs
ODN 17	t _p t _p t _p t _p t _p t _p T*T*T*t _p t _p t _p t _p t _p t	6318.21	6318.02
ODN 18	t _p t _p t _p t _p t _p t _p T♦T♦T♦t _p t _p t _p t _p t _p t	6374.32	6374.08
ODN 19	t _p t _p t _p t _p t _p t _p T•T•T•t _p t _p t _p t _p t _p t	6598.57	6598.20
ODN 20	t _p a _p g _p t _p t _p g _p a _p g _p t _p g _p a _p t _p A*T*C*A*t	5231.48	5230.97
ODN 21	G*T*A*G*t _p c _p c _p t _p g _p c _p a _p a _p t _p g _p a _p t	4888.26	4887.89
ODN 22	T*G*A*t _p c _p g _p c _p t _p g _p c _p a _p a _p T*G*A*t	4884.26	4883.96

Table 3. LCMS Analysis of PMO-DNA Chimeras. ODN 17, 20, 21and 22:*= NH2; 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.

254x190mm (96 x 96 DPI)


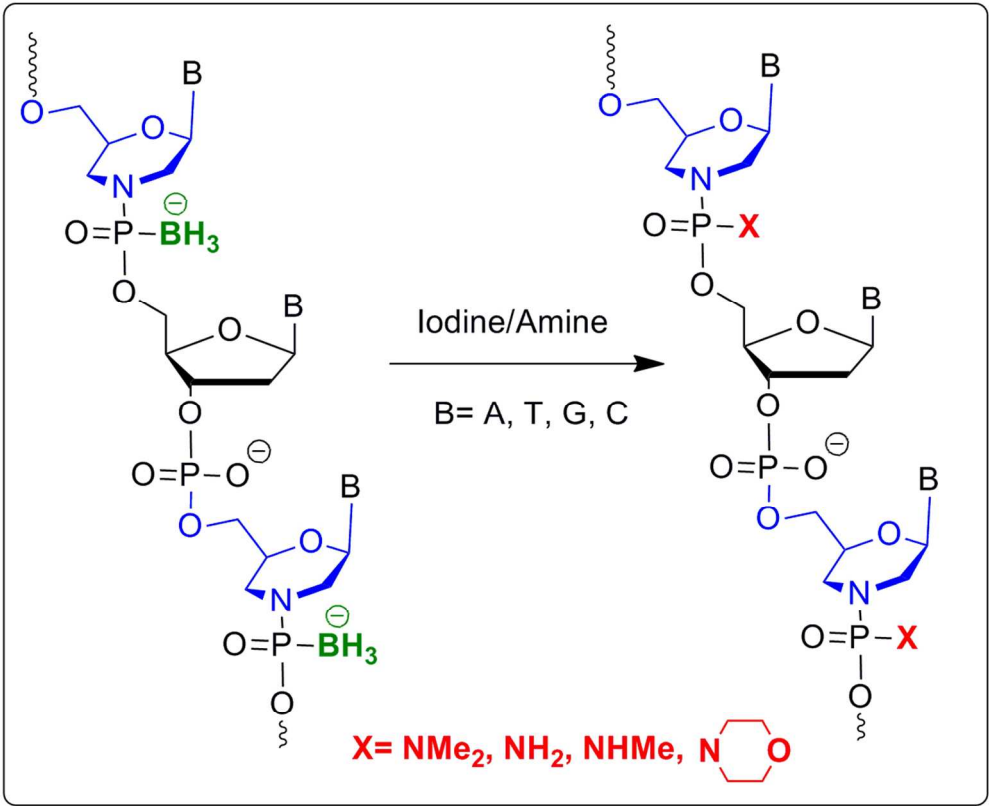
Number	Modification	T _m ^a (°C)		ΔT _m ^b (°C)	
		DNA	RNA	DNA	RNA
ODN 23	—	58.47	51.13	—	—
ODN 11	NMe ₂	58.37	52.42	0.025	0.32
ODN 17	NH ₂	61.37	53.37	0.75	0.56
ODN 18	NHMe	59.03	52.17	0.14	0.26
ODN 19		57.37	51.37	-0.27	0.06

Table 4. T_m Results with PMO-DNA Chimeras. 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 (Supplementary Section). aT_m measurements represent an average of at least three experiments. bΔT_m represents (T_m ODN-T_m unmodified control)/number of modifications).

254x190mm (96 x 96 DPI)



107x87mm (300 x 300 DPI)