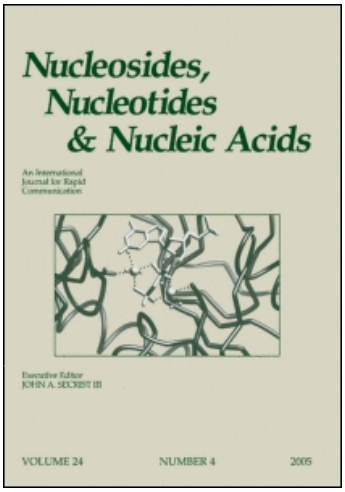

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CONCEPT, DISCOVERY AND DEVELOPMENT OF MMI LINKAGE: STORY OF A NOVEL LINKAGE FOR ANTISENSE CONSTRUCTS

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ABSTRACT: Methylene(methylimino) or MMI linkage is a novel backbone modification that has enormous potential in the oligonucleotide-based antisense therapeutics as a replacement for the natural phosphodiester linkage. This presentation synopsis covers the rationale, detailed SAR on the optimization process of this linkage vs. others, various synthetic strategies to construct MMI linkage and a brief discussion on the biological properties of the modified oligonucleotides.

INTRODUCTION

The search for synthetic backbone surrogates of natural phosphodiester linkage has become one of the most actively pursued areas of research in antisense technology.[1] Our efforts in this area began in 1989, the year Isis was founded. At that time we knew that use of unmodified DNA or RNA as antisense molecules had significant limitations.[2] One of the major problems was associated with their nucleolytic degradation. Therefore, phosphothioates were synthesized and studied as the first generation of nuclease resistant antisense oligonucleotides. It was soon realized that phosphorothioate oligonucleotides had certain limitations, such as chirality, polyanionic character, lower affinity for the target RNA compared to unmodified DNA, protein interactions, and unexplored manufacturing issues. Despite these limitations, about fifteen phosphorothioates have now made it to clinical trials with very promising drug profiles.[3]

It was mainly due to these limitations in 1989, we rationalized that the removal of both phosphorus and sulfur atoms from the backbone linkage was an improtant consideration for the design of second generation of antisense molecules. Conceptually, dephosphono-linkages that are neutral or positively charged may provide a handle to modulate the net charge of the antisense construct, thereby altering the pharmacokinetic and pharmcodynamic properties in a useful manner. What follows is a story of a novel dephosphono-linkage called methylene(methylimino) or MMI linkage. We believe that this linkage has great potential in antisense constructs.[5] The purpose of this account is to provide a pictorial summary of the key events which took place in the process of discovery

and development of the MMI linkage. Several scientists[6] have contributed to this project over the years in search of a perfect backbone surrogate resulting in twenty-five man years of work. Following is a short discussion and summary of our mostly recent results.

SAR of the backbone linkage

In order to create a large data-base with backbone modifications, we have synthesized several synthetic linkers (Figure 1), incorporated them into oligonucleotides and studied their antisense properties. Some of the general design considerations for such linkages were related to the following questions: (i) geometry, e.g. *E/Z* isomers; (ii) spatial arrangement of atoms; (iii) positioning of steric bulk; (iv) placement of charge, e.g. positive or negative; (v) conformational flexibility vs. Rigidity; (vi) hydrophobicity or hydrophilicity, and (vii) length, e.g. shorter or longer than 4-atoms. All of these design elements were evaluated by their affinity for the complement RNA, utilizing identical sequences. In brief, geometrical constraints such as *E/Z* isomers in **1** were found to be destabilizing. The spatial placement of the methyl group in **3** was found to be better than unsubstituted linkage **2**. Appropriate positioning of the steric bulk, such as the trans arrangement in **4**, was well-tolerated.[7] Correct positioning of the positive charge was also important.[8] For example, the Tm's of **5** were better than those of **6**. Use of flexible linkers, such as **7** or **8**, resulted in poor hybridization.[9] Reducing the length of the linker to 3-atom spacer (**9**) or increasing it to a 5-atom spacer (**10**) did not gain much in the affinity.[10] Incorporation of conformationally restricted linkers (**11**) via a multi-step synthesis did not provide an increase in affinity.[11] These results, taken together, clearly indicated that MMI linker **3** was the best choice among the linkers studied at Isis.

We then turned our attention to optimization of the atom arrangement within the 4-atom space of the MMI linkage (Figure 2). The 3′-C-N-O-C-4′ arrangement of MMI was shuffled to 3′-C-O-N-C-4′ (**12**) and 3′-O-N-C-C-4′ (**13**) linkers. Both isomeric linkers were found to be somewhat destabilizing compared to the parent linker **3**.[12] The role of 3′-C-C bond in MMI linkage is believed to enhance the 3′-endo sugar conformation and in turn provide preorganization of the antisense oligonucleotide to adopt an A-type conformation on hybridization to the complement RNA. Interestingly, replacemnt of 3′-C with 3′-O, as in the linkage **13**, reduced the 3′-*endo* sugar pucker resulting in lower affinity, compared to MMI linkage. Therefore, MMI linker was selected for the next round of optimization.

Selection of the best side-arm functionality (Figure 3) for MMI linkage was carried out in the following manner.[13] Four types of modifications were considered for this exercise – namely, length, steric-bulk, charge and the gauche effect. Changing of substituents on the nitrogen atom from none (i.e. H) to a 4-atom tether indicated that a methyl group was best suited in terms of affinity. Placement of an aromatic or aliphatic steric-bulk on the

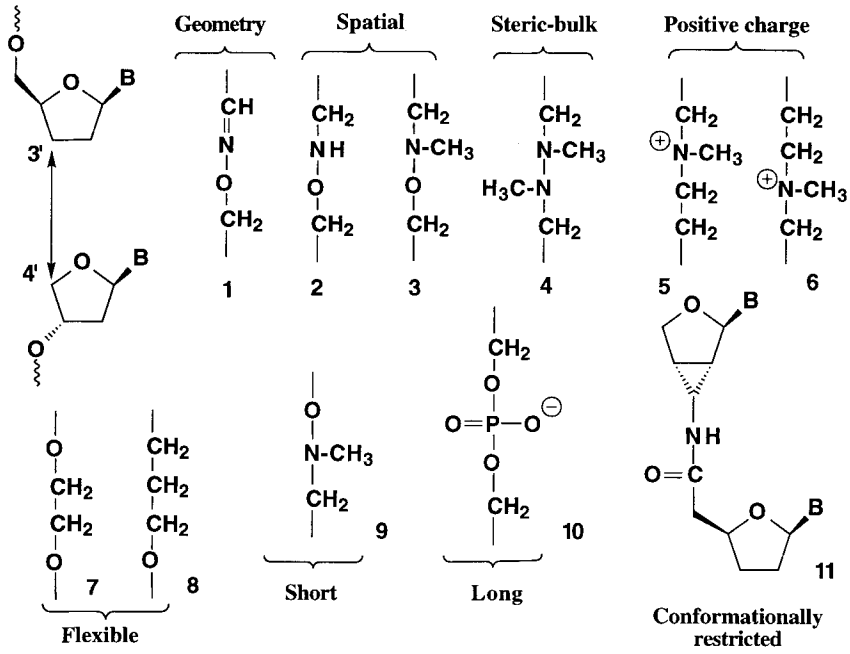


Figure 1: List of various backbone linkers studied at Isis

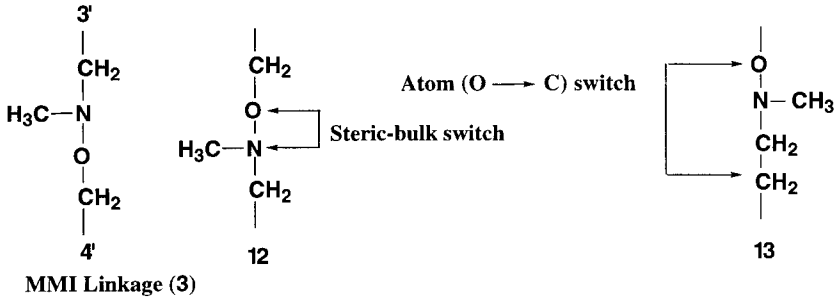


Figure 2: Atom shuffle within MMI linkage

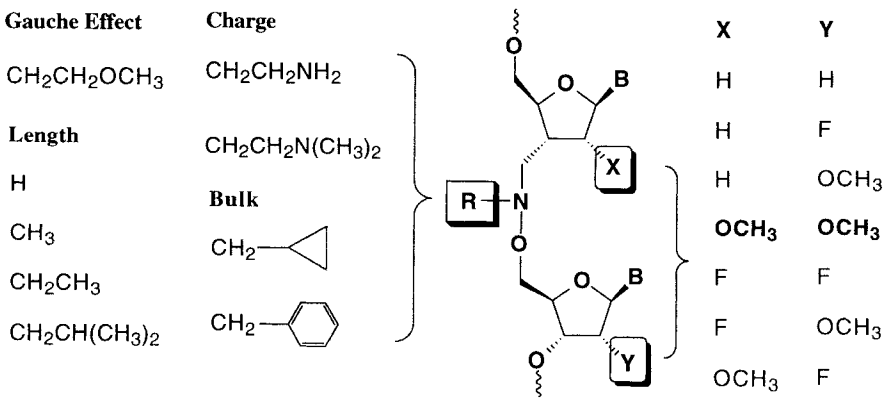


Figure 3: Optimization of the side-arm (**R**) and 2'-functionalities (**X** and **Y**)

nitrogen-atom had a negative impact on the Tm. Similarly, creation of a gauche effect at this position made no difference. Placement of a positive charge away from the backbone nitrogen provided similar affinity compared to the present linker **4**. Considering the additional synthetic efforts to attach a protonated linker off the backbone, we chose to further evaluate the MMI linker.

The enhanced stability of the hybrids of 2′-OMe and 2′-F modified oligonucleotides with RNA is well documented in the literature.[14] The increased stability is attributed to the electronegative nature of the 2′-substituent which prefers an axial orientation due to the gauche effect (with O 4′) and forces the sugar to adopt a high 3′-endo pucker. Therefore, we decided to prepare seven MMI dimers shown in Figure 3, substituted with 2′-OMe and 2′-F groups. These dimers were synthesized and their conformational properties (% of *N*-pucker) studied by proton NMR analysis. The 2′-substitution in combination with MMI backbone clearly demonstrated that "conformational preorganization" can be induced for an entropic advantage towards higher binding affinity for complement RNA. Peoc'h *et al.*[15] have summarized these results elsewhere in this volume.

Synthetic strategies

In the last seven years, several synthetic strategies were developed to prepare various MMI linked dimers. A summary is depicted in Figure 4. First and oldest approach[5] was based on an efficient coupling reaction of the 3′-C-formyl nucleoside **14** (R' = Tr, B = T) with 5′-O-amino nucleoside **15** (R = TPS, B=T) providing a stable oxime linked dimer. The latter dimer was reduced and methylated to furnish the MMI linkage. The dimer **20** was obtained in 3-steps, starting from **14** and **15**. This procedure was also applicable to the solid-support synthesis of MMI-linked oligosides.[16] A schematic representation in Figure

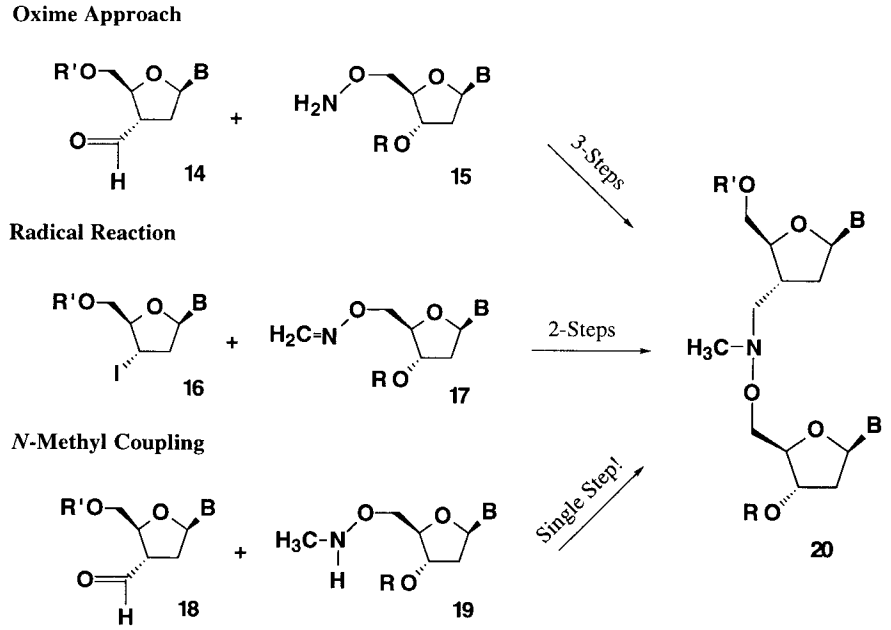


Figure 4: Strategies for the synthesis of the MMI linked dimers

5 highlights the key reactions on the support and off the support. A communication on the potential applications of this reaction was recently published. Subsequenctly, a 2-step stereoselective C-C bond formation between **16** (R = TPS, B = T) and **17** (R = TPS, B = T) was developed to provide a 3′-CH_2-NH-O-CH_2-4′ linked dimer in 80% yield.[17] The latter dimer was methylated to provide **20** in high yield. The latest synthesis of **20** was accomplished in a single-step via reductive coupling of **18** (R' = DMT, B = T, C^{BZ}, A^{BZ}) with **19** (R = TPS, B = T, A^{BZ}, C^{BZ}) in 60-80% yield. Among the three methodologies developed thus far, we believe that the N-methyl coupling procedure should be versatile and allow the prepartion of various dimers containing mixed bases. The utility of this procedure is further elaborated elsewhere in this volume by Swayze *et al.*

The SAR on various backbone and sugar modifications around the 3′-C-N-O-C-4′ motif has provided dimeric **23** as a clear winner with highest affinity and amenable synthesis. As a result, we have prepared all of the sixteen dimers (**23**, B = T, 5-MeC^{BZ}, A^{BZ}, G^{iBu}) as phosphoramidites ready for incorporation into oligonucleotides (Figure 6). A large-scale synthesis of all eight building blocks (i.e. **21** and **22** with B = T, 5-MeC^{BZ}, A^{BZ}, G^{iBu}) has been accomplished and described elsewhere by Dimock *et al.*[19] in this volume.

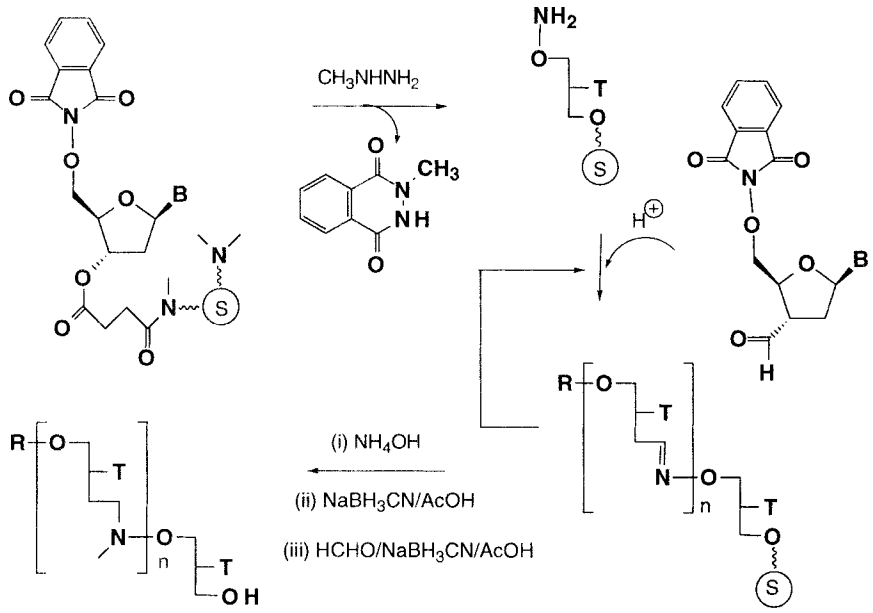


Figure 5: Synthetic scheme showing the preparation of oligosides on solid-support

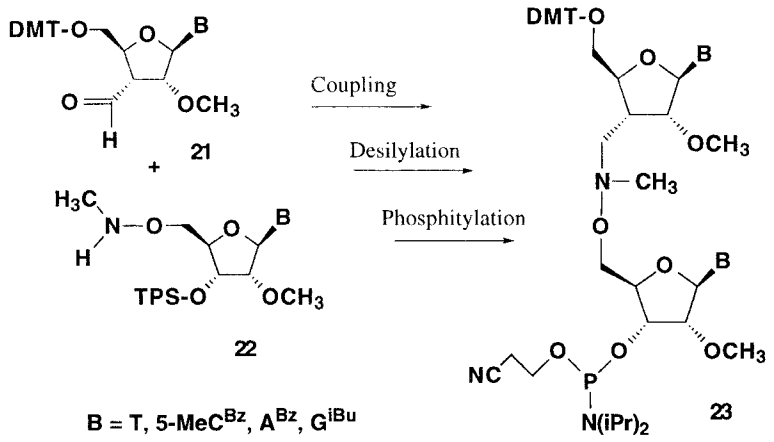


Figure 6: Synthetic scheme showing the preparation of sixteen dimers

Antisense constructs with MMI linkage

With the MMI chemistries described in the section above, three distinct classes of motifs can be assembled. Figure 7 shows a pictorial summary of these motifs. The solid-support automated synthesis provided an access to oligosides made of nucleosides connected via MMI linkages only. These molecules were neutral and exhibited poor water solubility.[16] Dimers and tetramers were somewhat soluble in water, whereas octamer and a 12-mer were found to be completely insoluble in aqueous media. Due to the lack of solubility with oligoside motif, we decided to create an alternating motif of MMI and phospho diester or phosphorothioate linkages (**2a** and **2b** respectively in Figure 7) in a uniform manner. The 5'- and 3'-ends were always capped with an MMI dimer providing the stability towards cleavage by exonucleases. Interestingly, the internal phosphodiester linkage of **2a** was not cleaved by endonucleases. One possible explanation for this stability is due to a local change in the conformation of phosphodiester linkage when flanked by MMI linkages. In view of this, use of motif **2b** may not be necessary for an antisense construct. Fully alternating motif **2** (Figure 7) does not support RNase H mediated cleavage of the RNA target. However, chimeric oligonucleotides (**3** in Figure 7) containing a gap of phosphorothioate linkages, flanked by MMI dimers or tetrameric oligoside support the cleavage of RNA by RNase H.[20] Several antisense oligonucleotides have been synthesized based on alternating MMI arrangement for RNase H independent targets and chimeric motif for RNase H dependent targets. Most of the antisense oligonucleotides containing MMI linkages have shown greater or similar biological activity (e.g. inhibition of PKC-α and H-*ras*) in cell-based assay compared to their all phosphorothioate counterparts.[21] Our preliminary animal experiments indicated that full alternating MMI-PO motif **2a** had a distinct biodistribution pattern compared to phosphorothioate oligonucleotide. At 24 hours, MMI-PO oligomer concentration in kidney was 6-fold less than the phosphorothioate oligomer and also had lower accumulation in liver compared to all thioate oligomer.

Summary

At Isis, we believe that bis 2′-OMe MMI is one of the most promising backbone modifications for incorporation into antisense oligomers. As indicated in Figure 8, this linkage provides a very high degree of nuclease resistance, as MMI backbone is not a substrate for cellular nucleases. Additionally, our results indicated that a phosphodiester linkage flanked by MMI backbone is completely protected from cleavage by endonucleases, which allows the use of dimeric phosphoramidites **23**, conveniently on a DNA synthesizer. These fully alternating oligomers demonstrated excellent water solubility, great affinity (ΔTm + 5 °C/mod. Compared to thioate oligos) and base pair specificity for complementary RNA, while reducing the overall charge by 50%. The correct conformational flexibility vs. Rigidity of MMI linkage, as well as enhanced N-pucker (due to 2′-OMe substituents) is

1. Oligosides

$(B^*B)_n$ B = T; * = MMI linkage; n = 2, 4, 6

2. Alternating MMI linkage (represented by *)

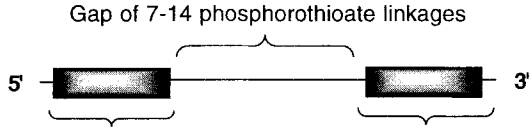
(a)

$(B^*B_{po}B^*B)_n$ B = T, 5-meC, A, G; po = phosphodiester linkage; n = 5

(b)

$(B^*B_{ps}B^*B)_n$ B = T, 5-meC, A, G; ps = phosphorothioate linkage; n = 5

3. Chimeric motif



Short segments of **2a** or **2b** containing 1-3 MMI linkages or tetrameric oligoside

Figure 7: Possible motifs for antisense constructs with MMI linkage

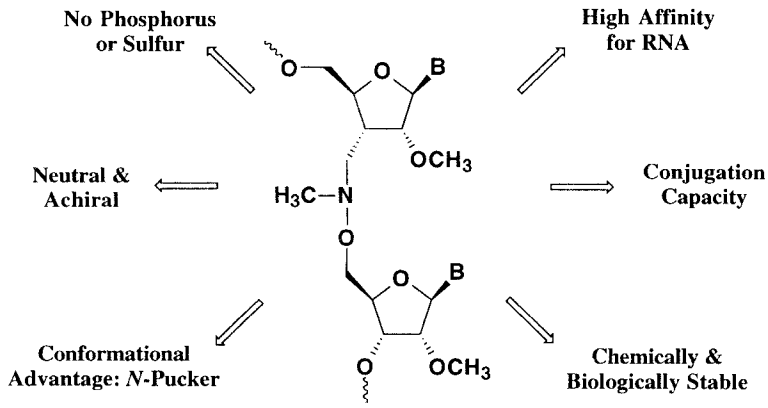


Figure 8: Various attributes of the best backbone modification from Isis

believed to play an important role in the tight binding affinities observed for RNA targets. Our amenable synthesis of various building-blocks required to prepare MMI linked dimer **23** should allow us to study the potential of this modification not only in antisense but also in other biomedical areas of research, such as triplex and ribozymes.

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