# Oligonucleotide Mimics for Antisense Therapeutics: Solution Phase and Automated Solid-Support Synthesis of MMI Linked Oligomers

François Morvan, Yogesh S. Sanghvi,* Michel Perbost, Jean-Jacques Vasseur, and Laurent Bellon

*Medicinal Chemistry Department, Isis Pharmaceuticals 2292 Faraday Avenue, Carlsbad, California 92008*

*Received October 6, 1995*

Antisense oligonucleotides (AOs) have shown great promise as agents for inhibiting gene expression.[1] In principle, AOs interfere in a sequence-specific manner with processes such as translation of mRNA into protein. In recent years, significant advances have been made in chemical modifications of AOs that can enhance both their stability and their potency.[2] One of the main focal points of the research has been the complete replacement of natural phosphodiester (P=O) backbone with synthetic linkages.[3] Among the various surrogates of the P=O backbone studied in our group, we have selected *m*ethylene (*m*ethyl*i*mino) (MMI) as a linkage of choice for advanced studies and for incorporation into AOs.[4]

The MMI linkage is achiral and neutral, readily incorporated into AOs, and stable under physiological conditions (Figure 1). AOs containing MMI linkages hybridize to the complementary RNA with high affinity and base-pair specificity. NMR and modeling studies have indicated that the 3′-$CH_2$ group of the MMI linkage shifted the sugar conformation to a desired 3′-*endo* pucker, thus helping the AOs to preorganize into a preferred A-geometry for duplex formation.[5] Biological studies showed that incorporation of MMI linkages into a phosphorothioate (PS) AOs substantially improved the pharmacological properties of the parent oligomer.[6] Our prior incorporation of the MMI linkage into AOs has been achieved by a nucleosidic phophoramidite dimer, creating alternate P=O or P=S/MMI linkages. This procedure, therefore, does not enable the synthesis of oligonucleosides[7a] that are uniformly modified with MMI linkages.

This communication reveals a flexible synthetic strategy for constructing AOs containing the MMI backbone in any desirable configuration with the P=O and/or P=S backbone. We have accomplished the synthesis of essential nucleosidic building blocks (**1**−**8**), thus enabling us to construct chimeric[7b] AOs as potential drugs. The solution phase (SP) methodology described herein is simple to manipulate. The couplings are efficient, and the process is transferable to solid-support (SS) synthesis, which can be further automated. As a demonstration, chimeric oligomers have been assembled on SS utilizing a standard DNA synthesizer. To prepare an oligonucleoside connected via MMI linkages only, four essential nucleosidic units (**1, 2, 5, 6**) were synthesized.

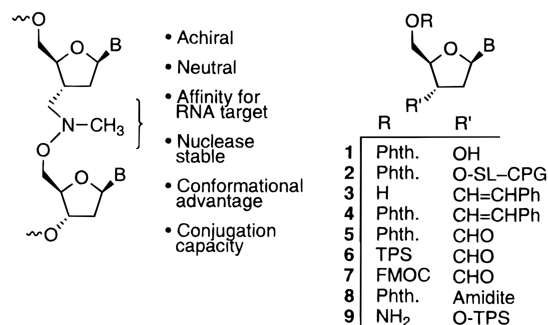



**Figure 1.** Structure and attributes of the MMI linkage.

2′-Deoxy-5′-*O*-phthalimidonucleosides[8] **1a**−**d** served as a precursor for the 3′-terminal unit. The nucleosides **1a**−**d** were successfully anchored onto the SS (CPG) via a succinyl linker[9] in good yield (∼35−40 μmol/g). To avoid the side reaction of incoming 3′-CHO nucleosides (**5** or **6**) with unprotected $NH_2$ groups left on the CPG, methylation ($HCHO/NaBH_3CN/AcOH$) of the SS provided fully protected CPG units **2a**−**d**. Alternatively, CPG units **2a**−**d** can be prepared from the commercial CPG loaded with 5′-*O*-DMT deoxynucleosides in three steps. For example, CPG anchored with 5′-*O*-DMT thymidine was treated with acid to remove the DMT group, followed by a Mitsunobu reaction,[10] and capping off the CPG $NH_2$ groups via methylation provided **2a** (30 μmol/g). The bifunctional units **4a**−**d** were prepared from 3′-*C*-styrene nucleosides[11] **3a**−**d**. Mitsunobu reaction[12] of **3a**−**d** provided the 5′-*O*-phthalimido-3′-*C*-styrene nucleosides **4a**−**d** in excellent yields. One-pot oxidative cleavage ($OsO_4/NaIO_4$) of **4a**−**d** gave **5a**−**d**, generating the 3′-CHO functionality. Syntheses of the 5′-terminal units **6a**,**c**,**d** have been published.[11] Preparation of 2′-deoxycytosine derivative **6b** was accomplished via triazolation and amination procedures.[13] Nucleoside **7** was prepared from **3a** via 5′-*O*-FMOC protection[14] and oxidative cleavage of the 3′-*C*-styrene group. Phosphitylation[14] of **1a** furnished **8** in 70% yield.

SP synthesis of $T_4$ was accomplished in the following manner. Coupling[4] of **9** with **5a** gave an oxime dimer (**12**, R = Phth, R′ = TPS, $n$ = 1, B = T), which on hydrazinolysis ($H_3CNHNH_2$) furnished 5′-*O*-$NH_2$-oxime dimer **14** (R = $NH_2$, R′ = TPS, $n$ = 1, B = T). Another round of coupling of **5a** with **14**, followed by hydrazinolysis, provided an oxime trimer (**14**, R = $NH_2$, R′ = TPS, $n$ = 2, B = T), which coupled with **6a** to give an oxime tetramer **15** ($n$ = 3, B = T). Reduction of **15** gave **16** (R = R′ = TPS, R″ = H, $n$ = 3), which on methylation followed by TBAF treatment gave MMI tetramer **17** ($n$ = 3, B = T) in 79% overall yield. Coupling of 3′-CHO nucleosides with the 5′-*O*-$NH_2$ nucleosides was quick and almost quantitative, thus allowing the manual synthesis of **17** in <8 h. Tetramers $T_3C$ and $T_2CT$ were assembled in a similar manner in high yields utilizing appropriate building blocks. The general

(1) Selected books and reviews published in 1995: (a) *Therapeutic Applications of Oligonucleotides*; Crooke, S. T., Ed.; R. G. Landes Co.:, Austin, TX, 1995. (b) *Carbohydrate Modifications in Antisense Research*; Sanghvi, Y. S., Cook, P. D., Eds.; ACS Symposium Series 580; American Chemical Society: Washington, DC, 1995. (c) Crooke, S. T. *Hematol. Pathol.* **1995**, *9*, 59. (d) Crooke, S. T. In *Burger's Medicinal Chemistry and Drug Discovery*, Vol 1; Wolff, M. E., Ed.; John Wiley: New York, 1995: p 863. (e) Mesmaeker, A. D.; Häner, R.; Martin, P.; Moser, H. E.; *Acc. Chem. Res.* **1995**, *28*, 366.

(2) Sanghvi, Y. S.; Cook, P. D. In ref 1b; Chapter 1, p 1. Mesmaeker, A. D.; Altman, K.-H.; Waldner, A.; Wendeborn, S. *Curr. Opin. Struct. Biol.* **1995**, *5*, 343.

(3) Sanghvi, Y. S.; Cook, P. D. In *Nucleosides and Nucleotides as Antitumor and Antiviral Agents*; Chu, C. K., Baker, D. C., Eds.; Plenum Press: New York, 1993; p 311.

(4) Vasseur, J.-J.; Debart, F.; Sanghvi, Y. S.; Cook, P. D. *J. Am. Chem. Soc.* **1992**, *114,* 4006.

(5) (a) Mohan, V.; Griffey, R. H.; Davis, D. R. *Tetrahedron* **1995**, *51*, 8655. (b) In addition, substitution of the O3′ by a $CH_2$ group reduces the ring gauche effects and may enhance conformational stability. See: Roughton, A. L.; Portmann, S.; Benner, S. A.; Egli, M. *J. Am. Chem. Soc.* **1995**, *117,* 7249.

(6) Sanghvi, Y. S.; Bellon, L.; Morvan, F.; Hoshiko, T.; Swayze, E.; Cummins, L.; Freier, S.; Dean, N.; Monia, B.; Griffey, R.; Cook, P. D. *Nucleosides Nucleotides* **1995**, *14*, 1087.

(7) (a) We refer to modified oligonucleotides that lack the phosphorus atom in the backbone linkage as oligonucleosides. (b) Chimeric AOs are oligomers that contain more than one type of modifications to create a *gap* for RNase H activity.

(8) Perbost, M.; Hoshiko, T.; Morvan, F.; Swayze, E.; Griffey, R. H.; Sanghvi, Y.S. *J. Org. Chem.* **1995**, *60*, 5150.

(9) Pon, R. T. In *Protocols for Oligonucleotides and Analogs*; Agraval, S., Ed.; Humana Press: Totowa, NJ, 1993; p 465.

(10) (a) Hughes, D. L. In *Organic Reactions*; Paquette, L. A., Ed.; John Wiley: New York, 1992; Vol. 42, p 335. (b) Rano, T.A.; Chapman, K. T. *Tetrahedron Lett.* **1995**, *36,* 3789.

(11) (a) Sanghvi, Y. S.; Ross, B.; Bharadwaj, R.; Vasseur, J.-J. *Tetrahedron Lett.* **1994**, *35*, 4697. (b) Sanghvi, Y. S.; Bharadwaj, R.; Debart, F.; Mesmaeker, A. D. *Synthesis* **1994**, 1163.

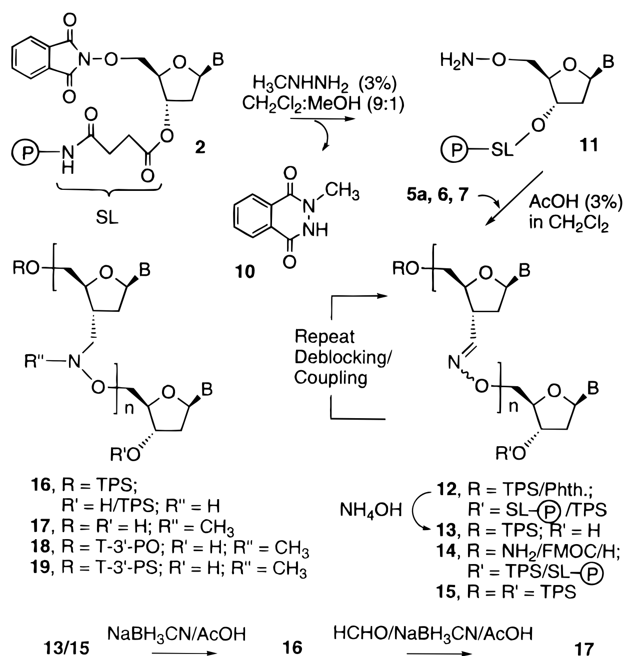
(12) (a) Mitsunobu, O. *Synthesis* **1981**, 1. (b) Debart, F.; Vasseur, J.-J.; Sanghvi, Y. S.; Cook, P. D. *Tetrahedron Lett.* **1992**, *33*, 2645.

(13) (a) Divakar, R. J.; Reese, C. B. *J. Chem. Soc., Perkin Trans. 1* **1982**, 1171. (b) Perbost, M.; Sanghvi, Y. S. *J. Chem. Soc., Perkin Trans. 1* **1994**, 2051.

(14) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223.

0002-7863/96/1518-0255$12.00/0 © 1996 American Chemical Society

**Scheme 1**[a]




[a] Abbreviations: in **1**−**6**, B = thymine (**a**); *N*-4-benzoyl-5-methylcytosine (**b**) adenine (**c**); *N*-2-isobutyrylguanine (**d**); Phth = *N*-phthalimido group; TPS = *tert*-butyldiphenylsilyl group; FMOC = 9-fluorenylmethoxycarbonyl group; amidite = $\beta$-cyanoethoxy-*N*-diisopropylaminophosphityl group; Ⓟ = controlled pore glass (CPG); T-3′-PO = 3′-phosphorylthymidine; T-3′-PS = 3′-thiophosphoryl thymidine; SL = succinyl linker.

SP strategy was extended toward the synthesis of $T_8$ (**17**, $n$ = 7) and $T_{12}$ (**17**, $n$ = 11) on SS in the manner described below. Thymidine-derivatized CPG **2a** was packed into a 1 $\mu$mol column and connected to an automated DNA synthesizer (Scheme 1). Hydrazinolysis of **2a** resulted in the formation of 1,2-dihydro-4-hydroxy-2-methyl-1-oxophthalazine (**10**) instantaneously. Attempts to estimate the release of **10** utilizing the standard UV technique was found to be inefficient (low $\epsilon_{max}$). We now report[15] an efficient and highly sensitive method of detection of **10** using the emission spectra, based on the luminescence property of the phthalazines. Treatment of **2a** with a solution of $H_3CNHNH_2$ (3%, $CH_2Cl_2$−MeOH, 9:1 v/v) for 2 min plus a 5 min wait step generated 5′-*O*-$NH_2$ derivative **11** (B = T) quantitatively. In the next step, coupling of **5a** with **11** was found to be most efficient when a solution of **5a** (0.1 M, $CH_2Cl_2$−AcOH, 97:3 v/v) was passed through the column for 15 s with a 10 min wait step. Unreacted **5a** was fully recovered on evaporation of solvent and recycled for subsequent reactions. Subsequent iterations of deprotection and coupling steps resulted in the formation of oxime oligomer **12** (R = TPS, R′ = O-SL−CPG, B = T, $n$ = 7, 11). A wash cycle ($CH_2Cl_2$) was essential between each step. An average coupling efficiency of 97−99% was obtained for the two oligonucleosides (**12**, B = T, $n$ = 7, 11) prepared.[16] Treatment of the CPG with ammonia released the free oxime oligomer **13** ($n$ = 7, 11), which was then reduced/methylated and desilylated to furnish crude **17** ($n$ = 7, 11). HPLC purification of the crude products gave analytically pure oligonucleosides. Purified **17** ($n$ = 7, 11) exhibited poor water solubility, which was not altogether unexpected, as other completely neutral oligonucleosides were also found to be sparingly soluble.[17]

**Table 1.** MMI Analogs of Isis 3521 as Inhibitors of PKC-α Protein Expression[18]

| Isis No. | sequence,[a] 5′ → 3′ | $T_m$[b] | $IC_{50}$[c] |
|---|---|---|---|
| 3521 | $G_ST_ST_S$ $C_ST_SC_S$ $G_SC_ST_S$ $G_SG_ST_S$ $G_SA_SG_S$ $T_ST_ST_S$ $C_SA$ | 52.1 | 100 |
| 9500 | $G_ST_ST_S$ $C_ST_SC_S$ $G_SC_ST_S$ $G_SG_ST_S$ $G_SA_SG_S$ T*T*T*C | 51.5 | 100 |
| 10403 | T*T*C′*T $C_SG_SC_S$ $T_SG_SG_S$ $T_SG_SA_S$ $G_ST_ST_S$ $T_S$ $C_SA$ | 52.4 | 125 |
| 10404 | T*T*C′*T $C_SG_SC_S$ $T_SG_SG_S$ $T_SG_SA_S$ $G_S$ T*T*T*C | 52.5[d] | 100[d] |

[a] An asterisk indicates MMI linkage; S, phosphorothioate linkage; C′, 5-methylcytosine. [b] $T_m$ values are in °C measured with RNA complements. [c] See ref 18 for experimental details. [d] $T_m$ of parent 18-mer PS oligomer was 51.3 °C, with an $IC_{50}$ of 120 nM.

The water solubility of oligonucleosides can be restored by incorporating nucleoside **7**, which provides a negative charge at the 5′-end of the molecule. For example, oxime dimer **14** ($n$ = 1, R = $NH_2$, R′ = O-SL-CPG) was prepared and coupled with **7** to provide a trimer (**14**, R = FMOC, R′ = O-SL-CPG, B = T, $n$ = 2). Deprotection of the latter trimer with piperidine in $CH_3CN$ gave **14** (B = T, $n$ = 2, R = H, R′ = O-SL-CPG), which on standard phosphoramidite coupling[14] with T gave a tetramer, which on cleavage from the CPG, followed by reduction/methylation, furnished **18** ($n$ = 2) with a negative charge. Tetramer **18** ($n$ = 2), with a P=S linkage, was also prepared in an analogous way. Three chimeric AOs were synthesized as analogs of Isis 3521, a PS oligomer which inhibits PKC-α protein expression at ∼100 nM concentration.[18] Manipulation of the chemistries described above in an appropriate manner[19] allowed us to synthesize Isis 9500 (3′-capped), 10403 (5′-capped), and 10404 (3′- and 5′-capped) as the first examples of chimeric AOs containing multiple MMI linkages in a row. The 5′ → 3′ unidirectional uninterrupted conjugation of MMI and DNA pieces has proven very useful due to the fact that the entire synthesis was performed on a single instrument by simply replacing the reagent bottles. The novel chimeric AOs were found to hybridize to their complementary RNA with better affinity and specificity compared to the unmodified PS oligomer (Table 1) and were found to inhibit the PKC-α expression. Tetramer **17** ($n$ = 3) was found to be completely resistant to cleavage by purified exo- (SVPD) and endo- (S1) nucleases.[20] Therefore, capping of an AOs at the 3′- and 5′-ends with MMI blocks should provide significant resistance to degradation by nucleases.

In conclusion, this work helps define chemical strategies for SP and SS synthesis of MMI-type oligonucleosides which may lead to a new class of chimeric AOs with improved pharmacological properties. The methodology also allows the MMI portion to be further elongated with additional PO or PS linkages while the oligomer is still attached to the SS, thus indicating the compatibility of MMI and phosphoramidite chemistry. These advances in the R-*O*-$NH_2$ and R-CHO coupling have also shown applications in combinatorial chemistry.[21] An appropriate mix of such studies should eventually lead to the discovery of novel drugs.

**Acknowledgment.** We thank P. Dan Cook for his interest, support, and encouragement throughout this project. We are also grateful to Elena Lesnik for the $T_m$ measurements, Lendell Cummins for nuclease studies, Nicholas Dean for PKC-α data, Patrick Wheeler for NMR studies, and Ramesh Bharadwaj for preparing the starting materials.

**Supporting Information Available:** Details of the experimental procedures, synthesis, and characterization data for nucleosides, oligonucleosides, and oligonucleotides (14 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

JA9533959

(15) Cook, P. D.; Sanghvi, Y. S.; Morvan, F. PCT Int. Appl. *WO 95/18136*, July 6, 1995.

(16) In addition to the pyrimidine oligonuclesides, we have also synthesized several purine-containing dimers (A*T, T*A, G*A, G*T) according to the SS methodology.

(17) For a sulfonate backbone, see: Huang, J.; McElroy, E. B.; Widlanski, T. S. *J. Org. Chem.* **1994**, *59*, 3520. For a silyl backbone, see: Cormier, J. F.; Ogilvie, K. K. *Nucleic Acids Res.* **1988**, *16*, 4583. For an extensive list, see ref 2.

(18) Dean, N. M.; McKay, R.; Condon, T. P.; Bennett, C. F. *J. Biol. Chem.* **1994**, *269*, 16416.

(19) White, M. A. In *Solid Supports Catalysts in Organic Synthesis*; Smith, K., Ed.; Horwood: Chichester, U.K., 1992; p 225.

(20) For experimental conditions, see: Cummins, L.; Owens, S. R.; Risen, L. M.; Lesnik, E. A.; Freier, S. M.; McGee, D.; Guinosso, C. J.; Cook, P. D. *Nucleic Acids Res.* **1995**, *23*, 2019.

(21) Cook, P. D.; Sanghvi, Y. S.; Kung, P.-P. PCT Int. Appl. *WO 95/18623*, July 13, 1995.