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Stereocontrolled Synthesis Of Nucleoside-Methylphosphonate-Methyl Ester Diastereomers From Mixed Anhydride

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Abstract: *Nucleoside-3'-methylphosphonate was converted to a diastereomeic mixture of di-n-butylphosphiothioic anhydride and the stereoisomers were separated. Activation of each isomer with silver nitrate and coupling with methanol resulted in the formation of a methyl ester with inversion of configuration.* Copyright © 1996 Elsevier Science Ltd

Recent evidence demonstrates that enrichment of the Rp-isomer of methylphosphonate internucleotide linkages of oligonucleotides (Rp-MPO's) results in enhancement of duplex stability and antisense activity.[1-5] Therefor, stereospecific synthesis of Rp-MPO's is crucial for continuing development of these compounds into therapeutic agents. In this report, we introduce a novel concept for P(V) stereospecific synthesis using a relatively stable chirally pure analog of nucleoside-3'-methylphosphonic-di-n-butylphosphinothioic anhydride. In general, pure isomers of MPO's are obtained by either separation of the diastereomers after the synthesis or by stereocontrolled strategies.[6,7]

In a non-stereocontrolled chemical synthesis, the dinucleotide is prepared routinely as a diastereomeeric mixture using commercially available phosphonamidites. The diastereomers are separated and coupled again to make a tetramer.[6] A chirally pure homo-T octamer has been made by this laborious procedure but preparation of longer oligomers is not been practical.

Stereocontrolled synthesis of Rp-MPO's, on the other hand, could be applicable for the synthesis of chirally pure oligomers of any length and sequence. Methods for the diastereoselective synthesis of MPO,s by P(III) and P(V) chemistries are reported where one stereoisomer is formed as a predominant product. For example, using methyldichlorophosphine [P(III)] and collodine at low temperature results in the formation of a predominantly Rp-methylphosphonate internucleoside linkage.[7] Diastereoselective synthesis of MP dinucleotides using P(V) chemistry has been observed by Wang et al[8] with ribo-2',5' favoring Sp formation and ribo-3',5' favoring Rp formation. Enzymatic polymerization of nucleoside-5'-α-methylphosphonyl-β,γ-diphosphate is an attractive approach for stereoselective synthesis. Formation of the Sp isomer has been reported by Higuchi and co-workers with di-thymidine.[9] More recently, Victorova and co-workers[10] found similar activity by AMV reverse transcriptase which incorporates up to eight nucleotides. There is no report, however, for the formation of Rp linkages via an enzymatic route.

Because of the stability of tetra-coordinated methylphosphonate, P(V) chemistry has been the focus of most recent investigations for the synthesis of chirally pure MPO.s. In general, purified stereoisomer of tetra-coordinated nucleoside-3'-phosphonate are coupled with the 5'-OH of the second nucleoside. The reaction must be driven by pre-activation of either 5'-hydroxy (with negligible epimerization) or 3'-phosphorous which could lead to considerable epimerization.[11-13]

We have designed a novel tetra-coordinated mixed anhydride of a nucleoside which can be readily activated under mild conditions and coupled with an alcohol without epimerization as demonstrated in Figure 1.

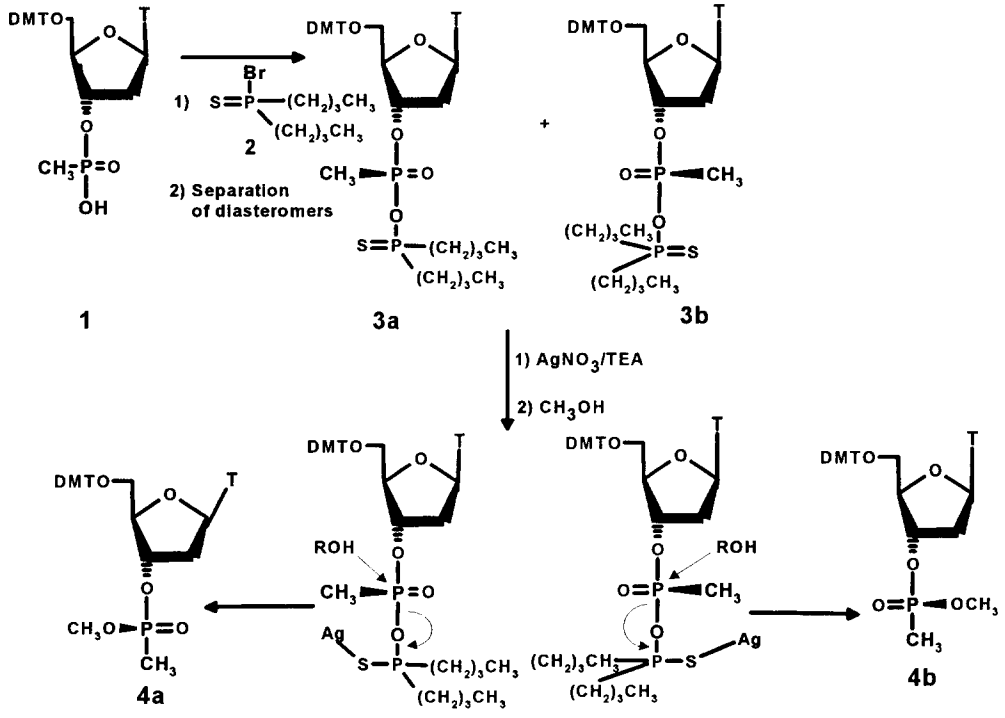

Figure 1

Mixed anhydride condensation of nucleotides was first introduced by Furrasawa et al[14] for the synthesis of nucleoside-5'-diphosphates and nucleoside-3',5'-cyclic phosphates. Later, Vaghefi et al[15] utilized this concept for the synthesis of nucleoside-diphosphate sugar analogs. Formation of a relatively stable mixed anhydride intermediate is the first goal toward this approach. Furrasawa suggested that the stability of these compounds is due to an intermolecular hydrogen bonding between the OH of one phosphorus and the sulfur of the other. In this report, however, the OH has been replaced with -CH_3 which eliminates the possibility of such an interaction. Nevertheless, the mixed anhydride is stable under neutral to slightly basic conditions which allows us to isolate the diasteriomers by column chromatography.

The reaction of mixed anhydrides with weak nucleophiles such as methanol proceeds by activation of the phosphinothioyl group. In this experiment, silver ion was used as the activator reagent[16], which forms complex with sulfur and makes phosphorus more electrophilic. Methanol was used as a model nucleophile to couple with purified slow and fast isomers of the mixed anhydride. The reaction resulted in only one isomer in each case with reversed mobility of the products on a silica gel plate. The faster running **3a** has an Rf value of about 0.9 and the product (**4a**) runs at Rf of 0.32. The Rf value for the slower running compound **3b** is 0.75 and the product of this compound **4b** has an Rf value of 0.42.

The above synthetic procedure provides a simple example of stereospecific synthesis of the methylphosphonodiester compounds with P(V) chemistry by activation of the phosphorous moiety. Further exploration of this chemistry is underway in our laboratory which could lead to the stereospecific synthesis and characterization of methylphosphonate oligonucleotides.

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16. *5'-Dimethoxytritylthymidine-3'-methylphosphonate:(1).* 5'-Dimethoxytritylthymidine (DMT-T) (5 g, 10 mmole) was dried by twice co-evaporation with anhydrous pyridine and dissolved in 250 ml of anhydrous pyridine and cooled to -10°C. To this solution, methylphosphonic dichloride (6.0 g, 45 mmole) in anhydrous pyridine (60 ml) was added slowly while stirring at -10°C to -13°C. The reaction was stirred for 2 hours (95% complete by TLC). The reaction was quenched by dropwise addition of 1.5 ml of water. The reaction mixture was dried down on rotary evaporator. The residue was dissolved in 50 ml

dichloromethane and washed with 2x50 ml water. The organic layer was separated and dried ($MgSO_4$) to give 5 g of crude product which was purified by column chromatography..

5'-Dimethoxytritylthymidine-3'-methylphosphonic-di-n-butylphosphiothioic anhydride:(3). Compound 1 (3.49 g, 10 mmole) was dried by twice co-evaporation with anhydrous pyridine and dissolved in anhydrous pyridine in a septum-sealed round bottom flask under argon. Two equivalents of freshly prepared di-n-butylphosphinothioyl bromide (2)[13] (3.0 g, 12 mmole) was added via syringe and stirred for 30 min. The progress of the reaction was monitored by TLC which indicated formation of 1:1 mixture of the two diasteriomers of the anhydride in almost quantitative yield. The Rf values of stereo-isomers of the mixed anhydride are different on silica gel plate which was pretreated with triethylamine in order to neutralize the silica gel. The solvent was removed from the reaction mixture by evaporation and co-evaporation with toluene and the residue was dissolved in 10 ml dichloromethane for column purification. A 2.5x40 cm column was packed with 230-400 mesh silica gel in dichloromethane with 0.5% triethylamine (to neutralize silica hence avoid detritylation) and washed with DCM. The product was loaded and eluted with 200 ml portions of dichloromethane/acetone mixture with concentrations of 10:1, 7:1, 5:1 and 2:1. The faster running stereoisomer (**3a**) was eluted first which was followed by mixture of two isomers and finally the slower running isomer (**3b**). The products were dried down to yield 540 mg of pure Rp and 480 mg of pure Sp isomers. The Products were analyzed by Mass spectra and NMR. 31P NMR of Rp isomer shows two doublets at 24.7 ppm (P1) and 104.7 ppm (P2) with P-P coupling constant of 25 MHz. The Sp isomer also shows two doublet at 24.3 ppm (P1) and 104.3 ppm

5'-Dimethoxytritylthymidine-3'-methylphosphonic acid methyl ester (4). In a 10 ml round bottom flask, 20 mg of fast isomer, **3a**, was dried by co-evaporation with anhydrous pyridine (2x2 ml). The residue was dissolved in 2 ml of pyridine and adder to a mixture of 20 mg of silver nitrate and 30 μl of diisopropylethylamine in 2 ml of methanol under an argon balloon at room temperature. The progress of the reaction was monitored by TLC on silica gel plate with 5% methanol in dichloromethane. Under this condition the Rf for compound **3a** is about 0.9 and the product (**4a**) runs at Rf of 0.32. This procedure was repeated for the slow isomer, **3b**. The TLC analysis of this isomer reaction shows an Rf of 0.75 for the starting pyrophosphate and 0.42 for the product **4b**. Both products were purified on a silica gel preparative plate using dichloromethane acetone (5:4) as solvent. The yield of **4a** was 12 mg (75%) and **4b** was 9 mg (56%). The ^{31}P-NMR of the product shows a singlet at 32.28 for **4a** and 32.34 for **4b**.

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