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SOLID-PHASE STEREOSELECTIVE SYNTHESIS OF 2'-*O*-METHYL-OLIGORIBONUCLEOSIDE PHOSPHOROTHIOATES USING NUCLEOSIDE BICYCLIC OXAZAPHOSPHOLIDINES

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Abstract: The use of 2'-OMe-ribonucleoside bicyclic oxazaphospholidines derived from (*R*)- or (*S*)-2-pyrrolidinemethanol has enabled the stereoselective synthesis of (R_p)-, and (S_p)-2'-*O*-methyloligoribonucleoside phosphorothioates. Interestingly, higher stereoselectivity (96-98%) was observed in the synthesis of (S_p)-2'-*O*-methyl-oligoribonucleoside phosphorothioates compared to that in the case of (S_p)-oligodeoxyribonucleoside phosphorothioates (90%).

Introduction

The concept of using antisense oligonucleotides for the inhibition of gene expression has been explored in a number of *in vitro*, and *in vivo* systems with an intent to develop them into potential therapeutic agents.[1] Phosphorothioate oligonucleotides (PS oligos), in which one of the non-bridging oxygens in a phosphodiester linkage is replaced by sulfur, have been extensively studied as a promising class of antisense agents. The PS oligos have a number of desirable characteristics: (a) they are isosteric and isoelectronic with phosphoric diester oligonucleotides and are relatively easier to synthesize, (b) they form stable duplexes with complementary RNA, (c) they are resistant to nucleolytic enzymes, and (d) they are capable of activating cellular RNase H that selectively cleaves the RNA chain hybridized to the PS oligo.

The replacement of the oxygen in a phosphodiester linkage by sulfur introduces chirality at the phosphorous center. Consequently, the PS oligos obtained by routine synthesis are a mixture of 2^n diastereomers where n = number of internucleotidic linkages in the oligonucleotide. To date, most studies that are related to the biological activity, pharmacokinetics, and toxicity of PS oligos have employed PS oligos as diastereomeric mixtures. Recently however, it has been shown that the nuclease stability of PS oligos, their affinity towards RNA target, and their ability to activate RNase H are influenced by the *P*-chirality.[2] Thus, for example, the 3'-exonuclease present in human plasma was reported to be R_p-specific preferentially cleaving the internucleotidic phosphorothioate linkages with the R_p configuration, while sparing those with the S_p configuration. Furthermore, the DNA-RNA duplex containing the PS oligo of "all R_p" configuration was shown to be more susceptible to RNase H degradation compared to the S_p counterpart.[2,3] These observations are consistent with the known stereodiscriminatory power of the nucleolytic enzymes.[4]

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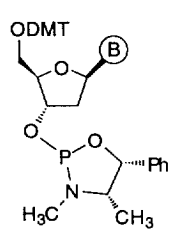
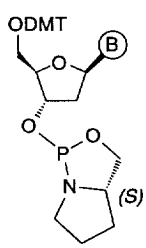
Clearly, additional studies, using stereodefined oligonucleoside phosphorothioates, are warranted to gain further insights on the impact of *P*-stereoselectivity on antisense activity, both in *in vitro,* and in *in vivo* systems. For this purpose, a practical synthetic approach is required that would provide access to stereopure R_p or S_p phosphorothioate oligonucleotides. Recently, two promising methods have been reported. In the first report, Stec and coworkers used nucleoside oxathiaphospholane building blocks to prepare short stereodefined phosphorothioates.[3] Wang and Just showed that nucleoside indoloxazaphosphorine synthon can be used to prepare stereopure dinucleoside phosphorothioates.[5] We have recently shown that nucleoside bicyclic oxazaphospholidines are novel synthons for the solid-phase stereoselective synthesis of dinucleoside phosphorothioates.[6] We have continued the development of this synthon for the stereoselective synthesis of phosphorothioates, and report here our model studies on the synthesis of 2'-*O*-methylribonucleoside phosphorothioates.

Design of the oxazaphospholidine synthon

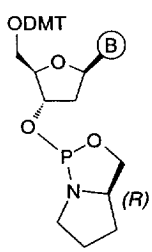
Previously, we used the nucleoside oxazaphospholidines **1**, derived from 1*R*, 2*S*-ephedrine,[7] as a novel sterically encumbered synthon for the solid-phase stereoselective synthesis of oligonucleotides. However, in model studies, the use of either R_p or S_p nucleoside oxazaphospholidines **1** did not give the expected stereoselectivity in the coupling reaction, but instead the formation of $[R_p,S_p]$ diastereomeric mixture of phosphorothioates was observed.[7] Presumably, the use of tetrazole as activator during the coupling step, resulted in the epimerization of the phosphorus center of **1** *via* pseudo rotation. We therefore rationalized that the rate of *P*-epimerization in the oxazaphospholidines can be slowed down by increasing the energy barrier for pseudo rotation. This reasoning led to the design of the more conformationally restricted bicyclic oxazaphospholidines **2a-b** derived from the corresponding (*R*)- or (*S*)-2-pyrrolidine methanol.



1



2a



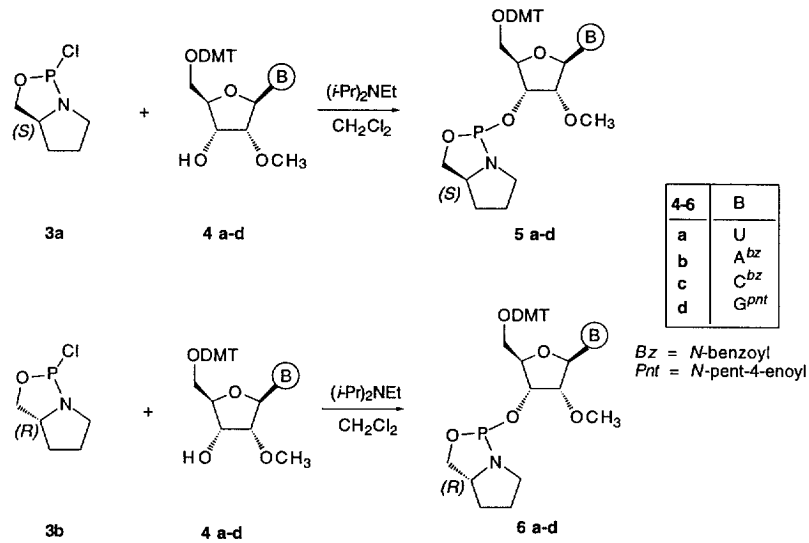
2b

Using these new deoxynucleoside synthons **2a** and **2b**, we were able to achieve stereoselectivity of 90:10, in the solid-phase synthesis of TpsT dimers.[6] We have now prepared the corresponding 2'-OMe-ribonucleoside

oxazaphospholidines, and evaluated their potential in the stereoselective synthesis of 2'-OMe-ribonucleoside phosphorothioates.

Results

The requisite chlorophosphite **3** derived from (*R*)- or (*S*)-2-pyrrolidine methanol, obtained as previously described,[6] was reacted with the *N*-benzoyl, and *N*-pent-4-enoyl (*PNT*)-protected nucleosides[8] **4a-d** (Scheme 1) in the presence of diisopropylethylamine at -78 °C. This reaction sequence, in each case, gave the corresponding bicyclic oxazaphospholidine **5a-d** and **6a-d** in greater than 90% yield. Importantly, the examination of the crude product that was obtained following the workup of the reaction revealed the presence of a single diastereoisomer as ascertained by ^{31}P NMR (Figure 1).[9,10]



Scheme 1. Preparation of the synthons **5** and **6** by the reaction of the chlorophosphite **3a** or **3b** with the 2'-OMe-ribonucleosides **4a-d**.

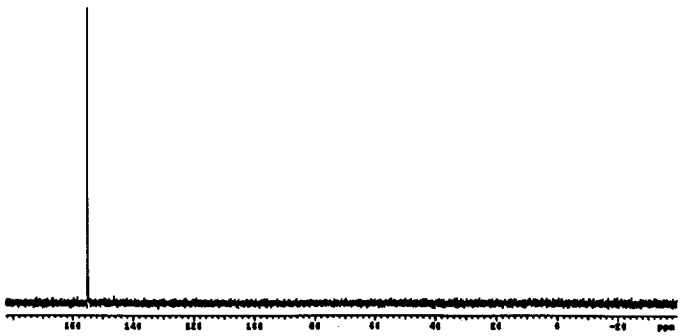
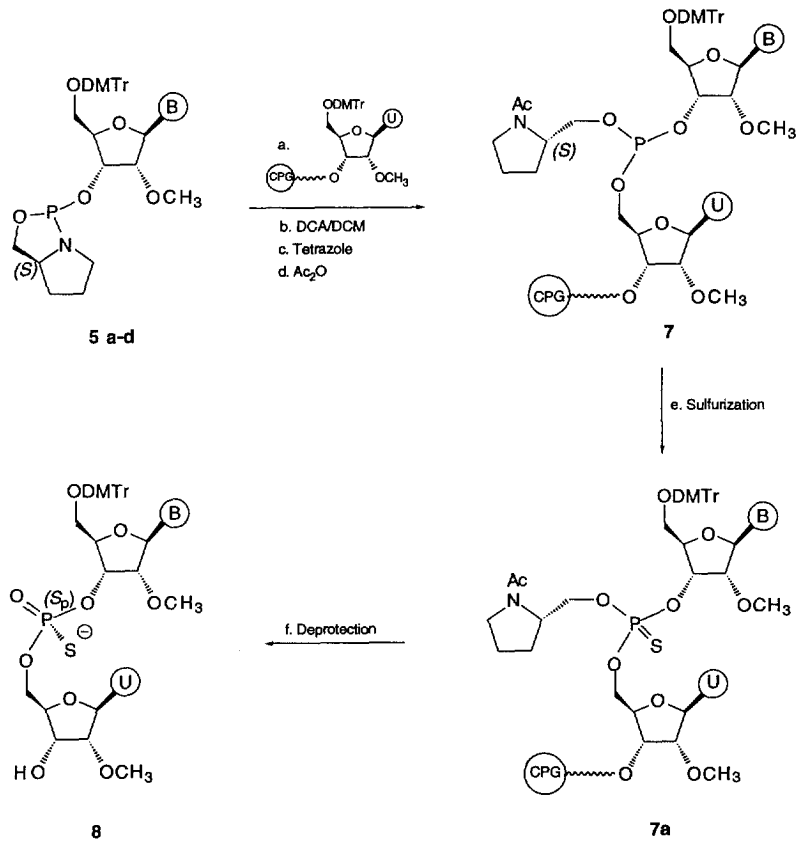


Figure 1. ^{31}P NMR spectrum of 2'-*O*-methylribonucleoside oxazaphospholidine **5a**.

Next, the crude oxazaphospholidines **5** and **6** were used in solid-phase oligonucleotide synthesis on a 1 μmol scale (Scheme 2).[11] In a typical experiment, the CPG-bound 2'-*O*-methyl-uridine was coupled with either nucleoside oxazaphospholidine **5** or **6** using 1*H*-tetrazole as activator. Then, following capping, the dinucleoside phosphite was oxidatively sulfurized with 3*H*-1,2-benzodithiole-3-one-1,1-dioxide[12] to obtain the CPG-bound dinucleoside thiophosphotriester **7a**. The coupling efficiency was found to be greater than 98%.[13]



Scheme 2. Solid-phase synthesis of 2'-OMe-ribonucleoside phosphorothioates; sulfurization was done with 3*H*-benzodithiole-3-one-1,1-dioxide;[12] deprotection was done with 28% NH_4OH at 55 °C for 12 h.

Following a final capping, washing, and removal of the DMTr group, the CPG-bound dimer was heated with aqueous ammonium hydroxide (28%, 65 °C, 12 h). Examination of the crude products by reversed-phase HPLC,[14] and ^{31}P NMR revealed the predominant formation of a single diastereoisomer. The R_p and S_p configurations of the phosphorothioates were assigned as previously described.[6] Table 1 shows the diastereoisomeric composition of the dinucleotides as determined by HPLC. Representative HPLC, and ^{31}P-NMR profiles of dimer **8** are shown in Figure 2.

Table 1.
Stereoselective synthesis of 2'-*O*-methyl-ribonucleoside phosphorothioate dimers

Using oxazaphospholidines 5 a-d[@]				Using oxazaphospholidines 6 a-d[@]			
Dimer	R_p	:	S_p	Dimer	R_p	:	S_p
8a	3	:	97	**8a**	73	:	27
8b	3	:	97	**8b**	76	:	24
8c	4	:	96	**8c**	75	:	25
8d	2	:	98	**8d**	63	:	37

[@] For structures, refer to Schemes 1 and 2

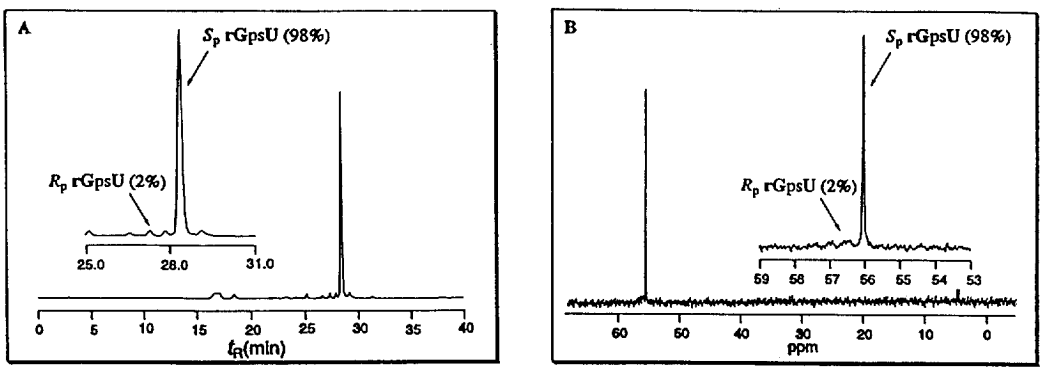


Figure 2. (**A**) HPLC profile of the 2'-*O*-methylribonucleotide dimer **rGpsU** prepared using **5d**; (**B**) ^{31}P NMR spectrum of the dimer **rGpsU**

Discussion

Starting from (*S*)-(+)-2-pyrrolidinemethanol, a sequence of reactions *via* the synthons **5a-d** (Scheme 2) led to (S_p)-2'-*O*-methyl ribodinucleoside phosphorothioate, and that with (*R*)-(-)-2-pyrrolidinemethanol *via* the synthons **6a-d** gave (R_p)-2'-*O*-methyl-ribodinucleoside phosphorothioate. Interestingly, we obtained higher stereoselectivity during the preparation of (S_p)-2'-*O*-methyl-ribodinucleoside phosphorothioates compared to (R_p)-2'-*O*-methyl ribodinucleoside phosphorothioates (Table 1). Furthermore, under identical synthesis conditions, higher stereoselectivity was achieved during the synthesis of (S_p)-2'-*O*-methyl-ribonucleoside phosphorothioates compared to (S_p)-deoxyribonucleoside phosphorothioates.[6] The mechanistic basis for such differences is not known at this time.

In summary, stereo-enriched 2'-*O*-methyl ribodinucleoside phosphorothioates have been synthesized by using the novel oxazaphospholidines **5** and **6**. Additional studies are ongoing to understand the factors which govern the stereoselectivity, and optimization of conditions to improve the stereoselectivity. Results from these studies will be reported in a future publication.

Experimental Procedure

In a typical experiment, 2'-*O*-methylribonucleoside **4a** (2.14 g, 3.82 mmol) was dissolved in dry dichloromethane (DCM) (40 mL), diisopropylethylamine (1 mL) was added, and the solution was cooled to -78 °C under argon. The chlorophosphite **3** (0.62 g, 3.75 mmol),[6] dissolved in dry DCM (20 mL) and pre-cooled to -78 °C, was then added under positive argon pressure, and the reaction mixture gradually allowed to warm to room temperature. The reaction mixture was stirred overnight, and washed with cold 5% sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate, and the solvent removed *in vacuo*. The residue was dried overnight *in vacuo*, to give **5a** (2.42 g); white solid; yield 93%.

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9. The absolute configurations of **5**, and **6** have not been assigned as yet.

10. The ^{31}P NMR was recorded on a Varian 600 MHz NMR Spectrometer; **5a**: δ 152.56 *ppm*, **6a**: δ 154.30 *ppm*.

11. The synthesis was done on a PerSeptive Expedite Nucleic Acid Synthesizer using a slightly modified 1 μM scale synthesis program.

12. Iyer, R. P.; Regan, J. B.; Egan, W.; Beaucage, S. L. *J. Am. Chem. Soc.* **1990**, *112*, 1253.

13. As ascertained by trityl yields.

14. HPLC was done on a Waters 600E instrument using 8NV C-18 4μ Radial Pak cartridge column, gradient (100% A to 60% B over 60 minutes) of buffer A (0.1 M ammonium acetate) and buffer B (80/20, v/v, acetonitrile/ammonium acetate), at a flow rate of 1.0 ml/min.