Recent Advances in the Stereocontrolled Synthesis of Antisense Phosphorothioates

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Abstract: Antisense technology has advanced substantially in the past few decades and now is a well-established therapeutic approach in medicinal chemistry, and it may prove to be a valuable tool in the treatment of a wide range of diseases. Phosphorothioate oligonucleotides are among the most important and promising antisense agents. However, the key drawback lies in their polydiastereomerism, which manifests itself in the different chemical and biological properties of the diastereomeric species. Methodologies towards the stereocontrolled synthesis of antisense phosphorothioate oligonucleotides have been well investigated in recent years. In this review, the progress in this field is summarized.

Keywords: Antisense strategy, phosphorothioate oligonucleotides, phosphoramidites, stereocontrolled synthesis.

INTRODUCTION

Antisense Strategy

The drug discovery is a lengthy process, often involving the optimization of lead compounds which are derived from a painstaking process of synthesis and screening. A large number of compounds need to be prepared in order to find something worth developing. Frequently, the active compound is directed against proteins such as enzymes or receptors, the structures and modes of action of which are usually very complicated. Bindings to non-target proteins can also lead to the undesired side effects.

Oligonucleotide-based drugs [1], on the other hand, intervene earlier in the disease process – at messenger RNA (mRNA) level, by interrupting the process by which disease causing proteins are produced. Therefore, this approach is considered to be a route to rational drug design. The predictable way of interaction represents one of the most powerful systems for molecular recognition designed by nature. Moreover, since protein synthesis is dictated by gene, the antisense approach should be widely applicable to various diseases.

The potential of oligonucleotides to act as antisense agents that inhibit viral replication in cell culture was discovered by Zamecnik and Stephenson in 1978 [2]. Since then antisense technology has been investigated for therapeutic purposes and developed as a powerful tool for target validation. With the first antisense drug – Vitravene™ in the market, and a number of others in clinical trials, antisense technology is well-posed to revolutionize pharmaceutical industry and make real "sense" in rational drug design and discovery [3].

Antisense strategy is theoretically very simple and elegant, but several hurdles must be overcome for successful clinical applications. The major concerns with antisense oligonucleotides are their *in vivo* stability, efficiency of cellular uptake, binding affinity and toxicity. Since naturally occurring oligonucleotides are not suitable drug candidates due to their inability to reach the interior of the cell and lack of stability towards extra- and intracellular enzymes, various chemical modifications have emerged [4-6]. Phosphorothioate oligonucleotides (PS-Oligos) have stood out as the most promising and advanced antisense agents.

Phosphorothioates and their Chirality

In the structures of PS-Oligos, one of the oxygen atoms in the phosphate bridge is replaced by a sulfur atom [7, 8]. Thus, PS-Oligos possess polydisatereomeric phosphorus centers (Scheme **1**).

PS-Oligos have increased *in vitro* nuclease stability and better cellular uptake as compared with the naturally occurring phosphates [9-11]. One of the most important advantages of PS-Oligos is their ability to elicit the activity of RNase H [12]. This enzyme, found predominantly in the cell nucleus, cleaves the RNA strand of a RNA-DNA duplex. The PS-Oligos thus can be thought of as a catalyst for mRNA cleavage. All the advantages mentioned above combine to make PS-Oligos extremely interesting for use in antisense technology. The major disadvantages of PS-Oligos are their bindings to certain proteins [13] and reduced binding affinity [14] towards complementary RNA molecules as compared to unmodified oligonucleotides.

Currently, almost all PS-Oligos are prepared by non-stereocontrolled synthetic methods – a large number of diastereomers are generated due to the chiral centers at the internucleotide phosphorus bridges. Recent *in vitro* studies showed that the properties of PS-Oligos, such as binding affinity, stability to nucleases and RNase H activity were affected by the configurations at the phosphorus atoms [15]. In a recent report, inhibition of human immunodeficiency virus type 1 (HIV-1) replication by PS-Oligos was studied. Whereas all-S_P-phosphorothioates showed better inhibition than all-R_P-oligomers, stereochemically random PS-Oligos could not give satisfactory inhibition [16].

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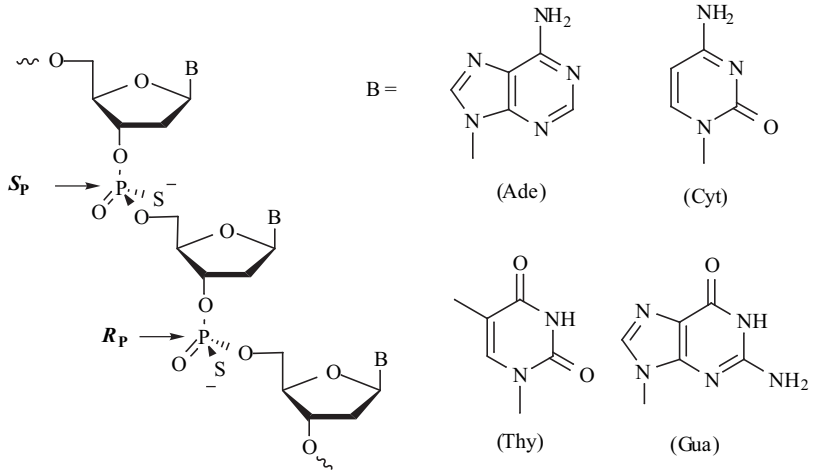


Scheme 1. Stereochemistry of PS-Oligos.

Practical methods for the preparation of PS-Oligos with well-defined stereochemistry at each internucleotide phosphorus linkage would be highly desirable. In recent years, methodologies towards the stereocontrolled synthesis of antisense PS-Oligos have been well studied. This review summarizes the latest developments in this field.

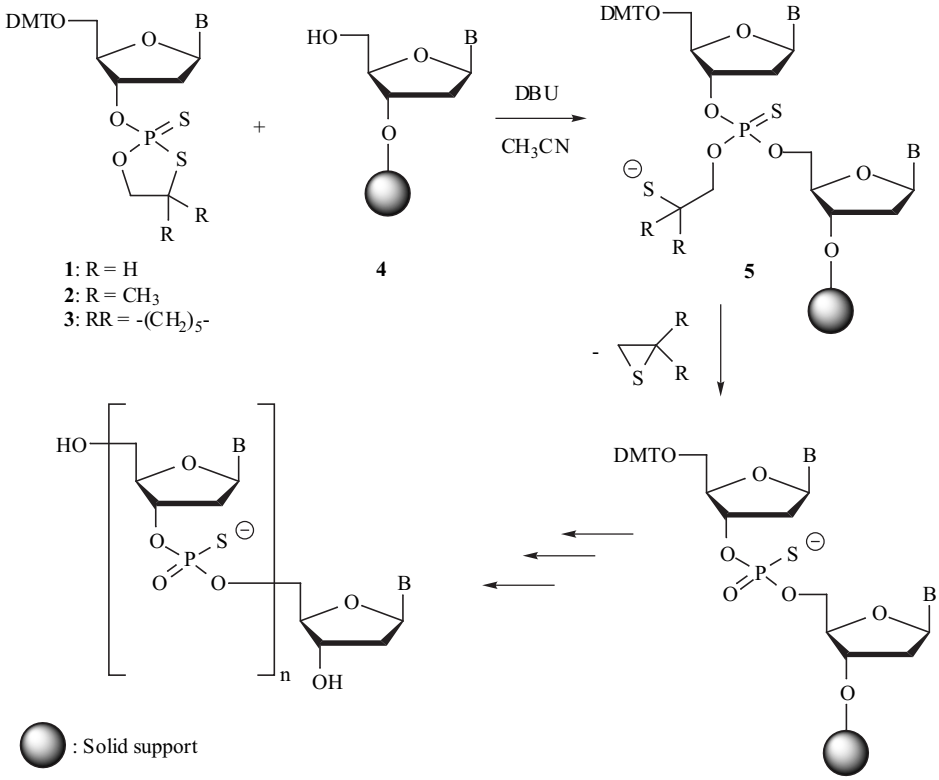
RECENT ADVANCES

The Oxathiaphospholane Approach

The oxathiaphospholane approach was first developed by Stec and co-workers in the early 1990s [17], and had been refined by the same group in recent years [18, 19].

In the oxathiaphospholane method, the starting oxathiaphospholanes 1, 2 or 3 were prepared as a pair of diastereomers, which were then separated by careful silica gel column chromatography. Each isomer was reacted with nucleoside 4 which was bound to the solid support at the 3'-position. Intermediates 5 were formed accordingly. Subsequently spontaneous loss of episulfide led to the formation of the internucleotide phosphorothioate function in over 95% yield and over 98% diastereomeric excess (de). After repetition of the coupling cycles, stereochemically well-defined PS-Oligos could be prepared (Scheme **2**).

The oxathiaphospholane approach is the only method currently available to allow for the stereocontrolled synthesis



Scheme 2. Oxathiaphospholane approach.

of PS-Oligos with a 16-28 base length, which is required for the applications in antisense strategy. The applications and various aspects of oxathiaphospholane approach have been extensively reviewed by Stec and co-workers in a number of excellent reviews [20-22].

However, this ingenious method suffers from some drawbacks. A serious problem is the difficulty associated with the separation of diastereomerically pure phospholane starting materials. Such column chromatographic separation on silica gel was difficult and tedious [17, 18]. Even though Stec *et al.* were able to achieve relatively easy separation of monomers in their latest improved system [19], it does not seem ideal and practical that such column separation can be adapted to the large scale, routine preparation of PS-Oligos. In addition, the condensation step required a large excess of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and precursors. The combination of the above made this method not applicable to large scale preparations in a practical sense.

The Cyclic Phosphoramidite Approaches

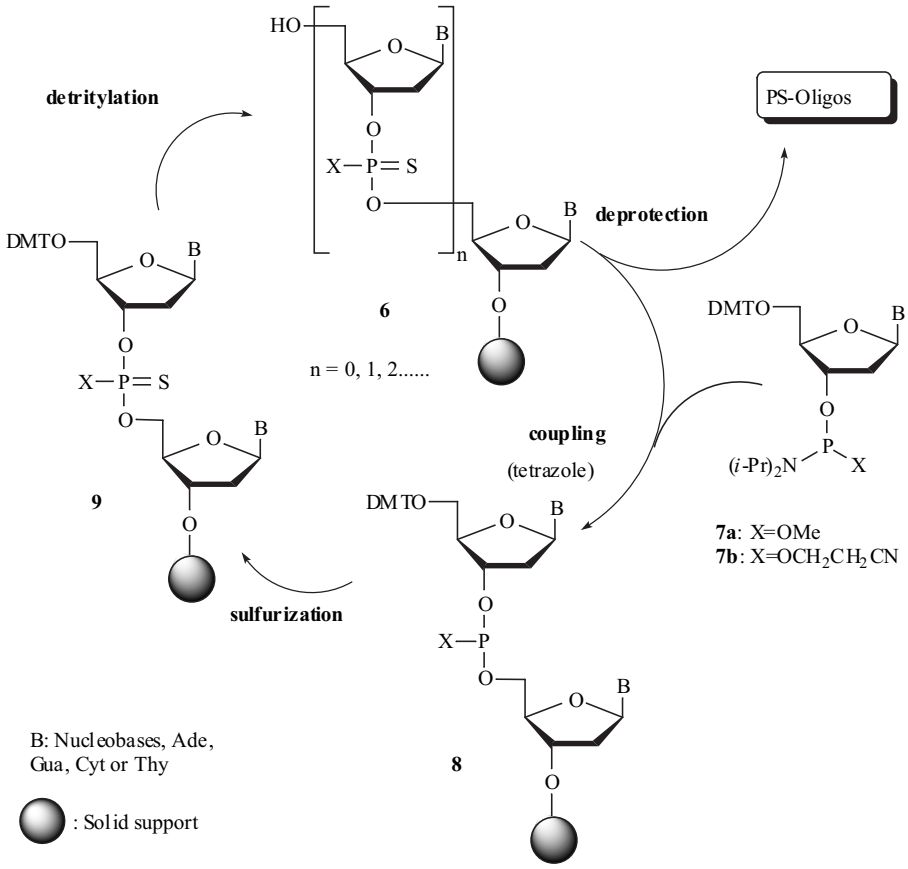
Synthesis of PS-Oligos by Phosphoramidite Approaches

Whereas the oxathiaphospholane method focuses on the nucleophilic displacements at the tetracoordinate phosphorus species, manipulations of the tricoordinate phosphorus species leading to PS-Oligos are more common approaches. Ever since its discovery more than twenty years ago [23], phosphoramidite approach has been an exceptionally versatile method for the preparation of various DNA analogues, including phosphorothioates [24] (Scheme **3**).

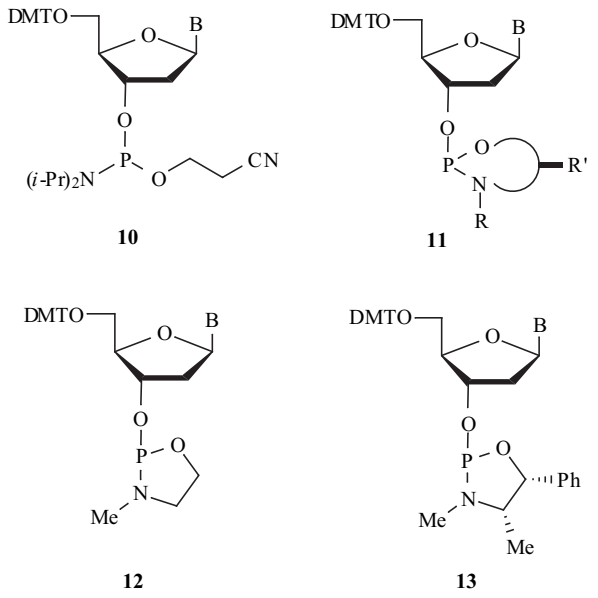
Since phosphoramidite method is so well-established, the adaptation of which for the stereocontrolled synthesis of phosphorothioates would be ideal. In Stec's earlier attempt [25], HPLC-separated phosphoramidite **7a** (B=Cyt^{Bz}) underwent a tetrazole-catalyzed coupling reaction with solid support-bound nucleoside to give fully–epimerized products. In view of this early result, two key issues need to be well addressed in order to make the phosphoramidite approach a practical solution for the stereocontrolled synthesis of PS-Oligos. The first one is to access chirally pure phosphoramidites without tedious separation of diastereomers, and the second issue is to transform the above diastereomerically pure phosphoramidites into phosphite triesters in a stereospecific manner. Many recent reports are aimed to address these problems, which we shall discuss in detail in the following sections.

The Bicyclic Oxazaphospholidine as Synthons

Iyer *et al.* used the phosphoramidite synthons of type **11** into which chiral auxiliaries were incorporated to replace the most common nucleoside β-cyanoethyl phosphoramidite **10** in the synthesis of phosphorothioates. Synthons **12** and **13** were the earlier ones employed in their synthesis [26, 27]. However, even phosphoramidite **13** was obtained as a single diastereomer, the subsequent solid phase synthesis employing **13** did not generate phosphorothioate dimer with any meaningful stereocontrol (Scheme **4**).

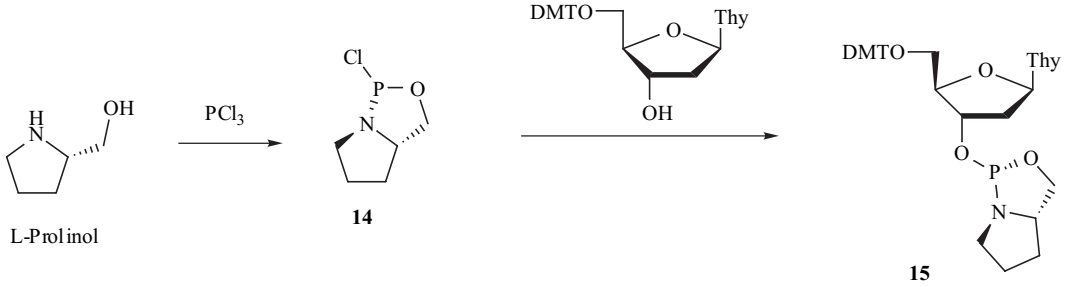


Scheme 3. Solid phase synthesis of PS-Oligos by phosphoramidite approach.



Scheme 4. Oxazaphospholidine synthons.

In a later improvement, Agrawal and coworkers employed a bicyclic oxazaphospholidine **15** for the solid phase synthesis of phosphorothioates [28]. The bicyclic structure was designed to provide more conformational restriction to limit the pseudo rotation which was believed to result in the *P*-epimerization during the coupling reaction of phosphoramidite **13**.



Scheme 5. The bicyclic oxazaphospholidine synthon.

The bicyclic oxazaphospholidine **15** was easily derived from L-prolinol. The intermediate *P*-chloro-oxazaphospholidine **14** was obtained as a single stereoisomer, which was then further converted to bicyclic oxazaphospholidine **15** as a single diastereomer, as revealed by ^{31}P NMR (Scheme **5**). Chirally pure **15** was subjected to the routine solid phase synthesis of dimeric phosphorothioates. After tetrazole-mediated coupling reaction with controlled pore glass (CPG)-bound nucleoside and sulfurization, the thymidine-thymidine dinculeotide phosphorothioates ($T_{PS}T$) was obtained in a greater than 90% diastereoselectivity upon cleavage from the solid support. Recently, this methodology was extended to the preparation of stereo-enriched phosphorothioate oligonucleotides [15] and 2'-*O*-methyl PS-Oligos [29].

The Cyclic N-Acylphosphoramidite Monomer

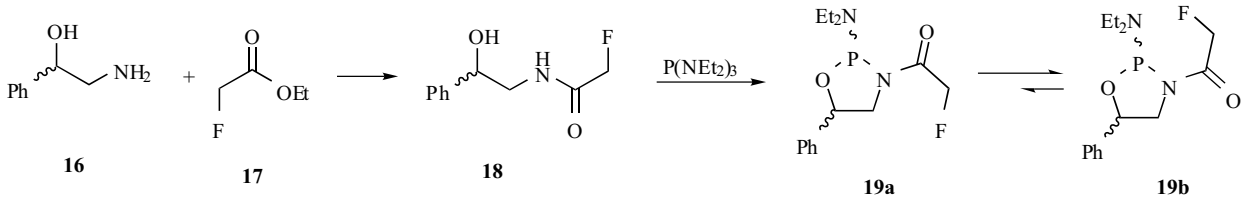
Beaucage and co-workers recently disclosed an interesting method in which the cyclic *N*-acylphosphoramidite monomers (**21**) were employed for the synthesis of PS-Oligos [30].

Racemic 2-amino-1-phenylethanol **16** was selectively *N*-acylated by ethyl fluoroacetate **17** to give **18**. The subsequent treatment with hexaethylphosphorus triamide afforded the cyclic *N*-acylphosphoramidites **19**, existing as a mixture of diastereomeric rotamers **19a** and **19b** (Scheme **6**).

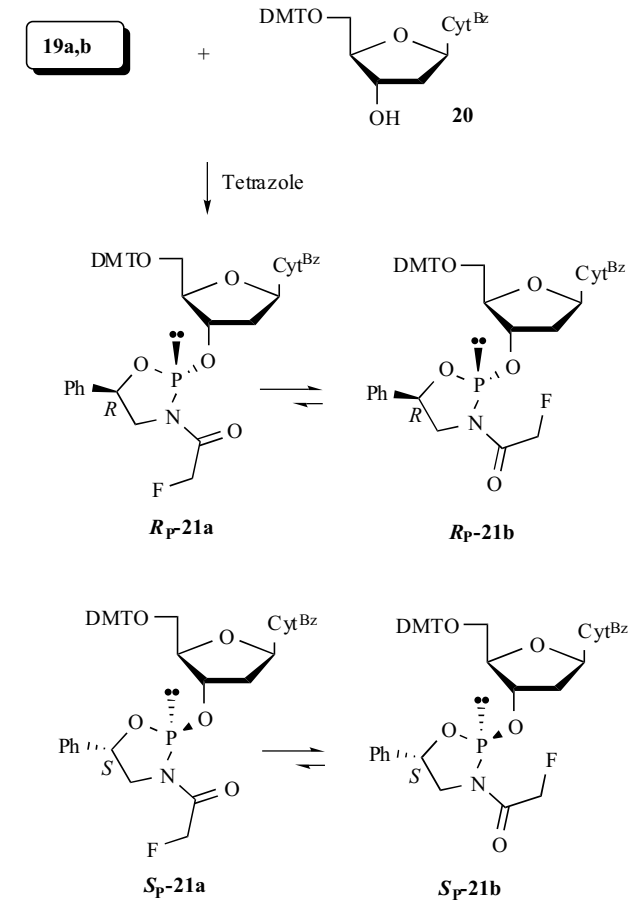
Reaction of **19** with N^4-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxycytidine **20** in the presence of 1*H*-tetrazole gave chirally pure $\boldsymbol{R_P}$**-21** and $\boldsymbol{S_P}$**-21** after silica gel chromatographic purification (Scheme **7**).

Phosphoramidites $\boldsymbol{R_P}$**-21** or $\boldsymbol{S_P}$**-21** were reacted with N^2-isobutyryl-3'-*O*-acetyl-2'-deoxyguanosine **22** and *N*,*N*,*N'*,*N'*-tetramethylguanidine (TMG) in CD_3CN, the dinucleotide phosphite triester **23** was formed in a near quantitative yield with total *P*-stereospecificity. Sulfurization of **23** generated phosphorothioate **24** with complete stereospecificity. In the end, **24** was subjected to the standard basic treatment, the complete deprotection yielded *P*-stereodefined phosphorothioate dimer **25** (Scheme **8**).

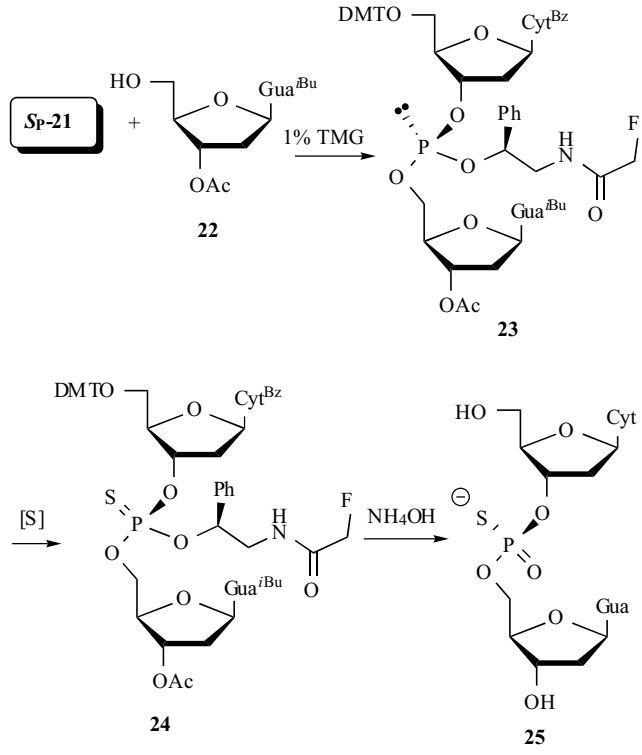
The 2'-deoxycytidine cyclic *N*-acylphosphoramidite derivatives $\boldsymbol{R_P}$**-21** or $\boldsymbol{S_P}$**-21** were also applied successfully to the solid phase synthesis of [R_P, R_P]- and [S_P, S_P]-trideoxycytidilyl diphosphorothioate d($C_{PS}C_{PS}C$) and [R_P, S_P, R_P]-tetradeoxycytidilyl triphosphorothioate d($C_{PS}C_{PS}C_{PS}C$).



Scheme 6. The cyclic N-acylphosphoramidites.



Scheme 7. The nucleoside N-acyl phosphoramidites.



TMG: *N,N,N',N'*-tetramethylguanidine;
[S]: Sulfurization

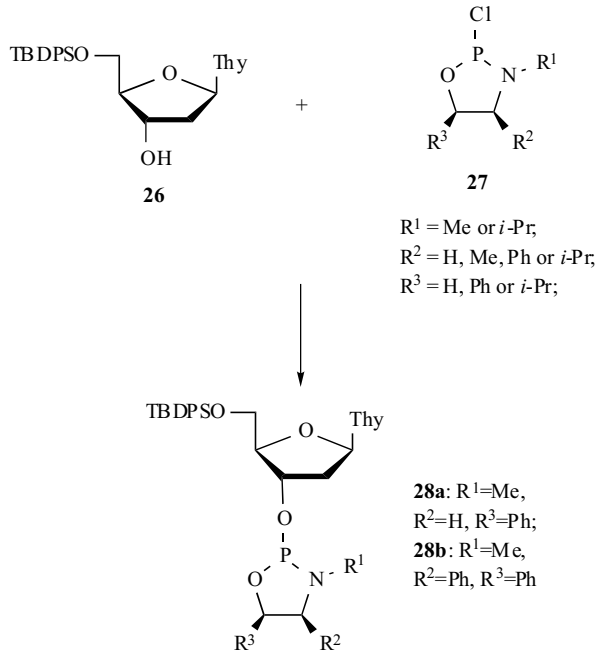
Scheme 8. The N-acyl phosphoramidite method.

The Novel Ammonium Salts as Activators in the Oxazaphospholidine Approach

An elegant approach using less-nucleophilic dialkyl(cyanomethyl)ammonium salts as activators for the synthesis of PS-Oligos was recently developed by Wada and co-workers [31-34].

Whereas the condensations promoted by strong bases such as DBU and TMG proceeded with full stereospecificity in Stec and Beaucage' methods [20, 30], the weak acid-activated coupling reactions between phosphoramidite monomers and nucleosides are rather non-stereospecific. The nonstereospecificity can be attributed to the nucleophilicity of activators, the repetitive attacks of which at the phosphorus center result in epimerization. Thus, Wada et al developed a new class of less-nucleophilic activators to circumvent the problem of epimerization.

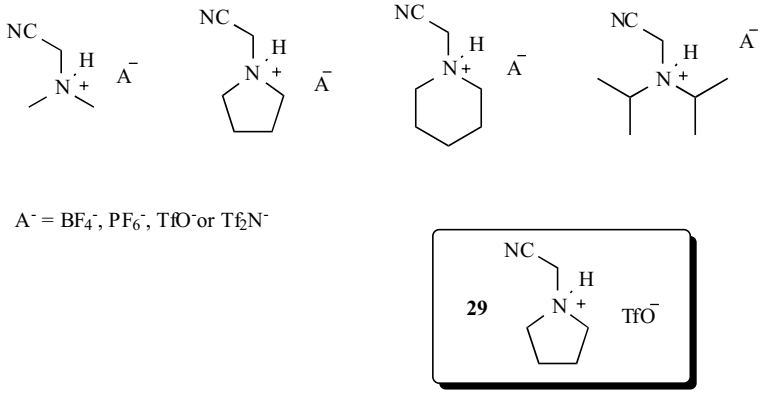
The oxazaphospholidine monomer units **28** were easily prepared (Scheme **9**). 2-Chlorooxazaphospholidine derivatives **27** were reacted with 5'-*O*-(*tert*-butyldiphenylsilyl)thymidine **26** under various conditions to give the corresponding oxazaphospholidine monomers **28**, two of which, **28a** and **28b** were obtained in excellent diastereoselectivity under kinetic and thermodynamic conditions, respectively. Upon simple silica gel chromatographic separation, diastereomerically pure monomers **28** were obtained.



Scheme 9. Oxazaphospholidine monomers.

The structures of the new class of activators are summarized (Scheme **10**). Such activators comprise two parts – ammonium ions with similar acidity to 1*H*-tetrazole to provide proton-donating ability and the less nucleophilic counter ions to alleviate the potential attacks at the phosphorus center. Based on the ease of preparation and handling, **29** was one of the best activators.

(S_P)-Dithymidine phosphorothioate was synthesized (Scheme 11). The condensation of monomer **28a** with **30** in the presence of activator **29** went to completion within 5



Scheme 10. Dialkyl(cyanomethyl)ammonium salt activators.

minutes to afford phosphite triester **31** with complete stereocontrol. The secondary amino function in **31** needed to be capped by acylation to avoid side reactions during the subsequent sulfurization reaction. The acylated chiral auxiliary in **32** could be removed by treatment with DBU. The final removal of silyl protective groups afforded the fully deprotected dimer **33** with more than 99% diastereomeric excess.

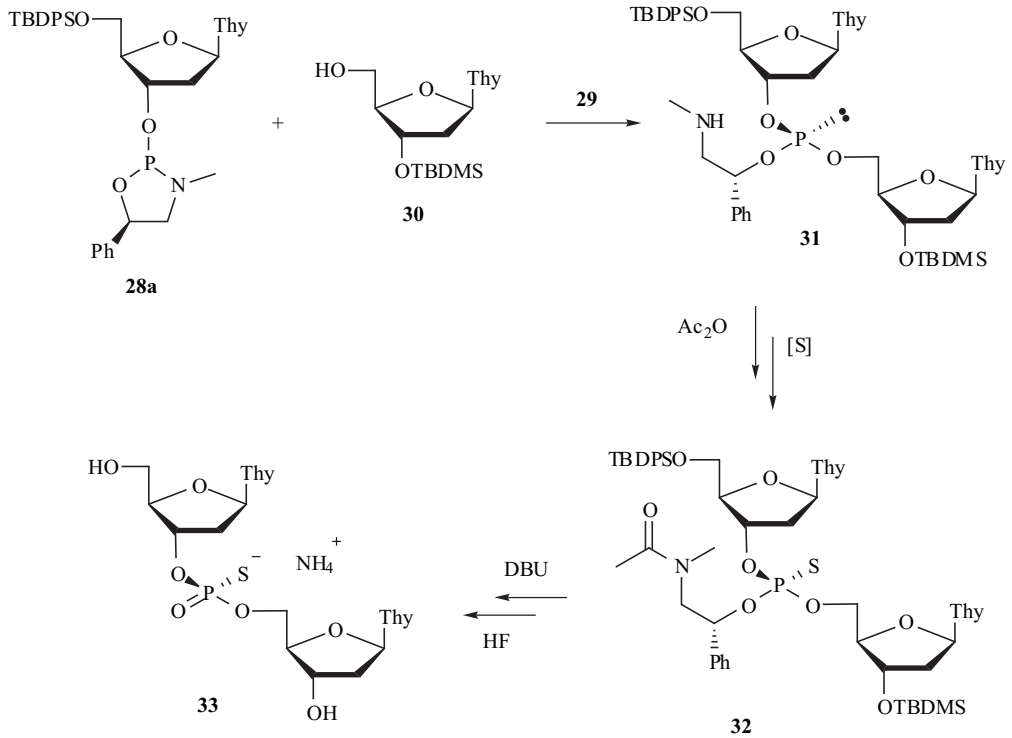
Solid phase synthesis of stereodefined PS-Oligos was carried out. Under optimized conditions, the fully *P*-stereoregulated *all*-(R_P)-[T_{PS}]$_3$T, *all*-(S_P)-[T_{PS}]$_3$T, *all*-(R_P)-d[$C_{PS}A_{PS}G_{PS}$]T and *all*-(R_P)-[T_{PS}]$_9$T had all been successfully prepared.

The Cyclic Phosphoramidites with a Six-membered Ring Structure

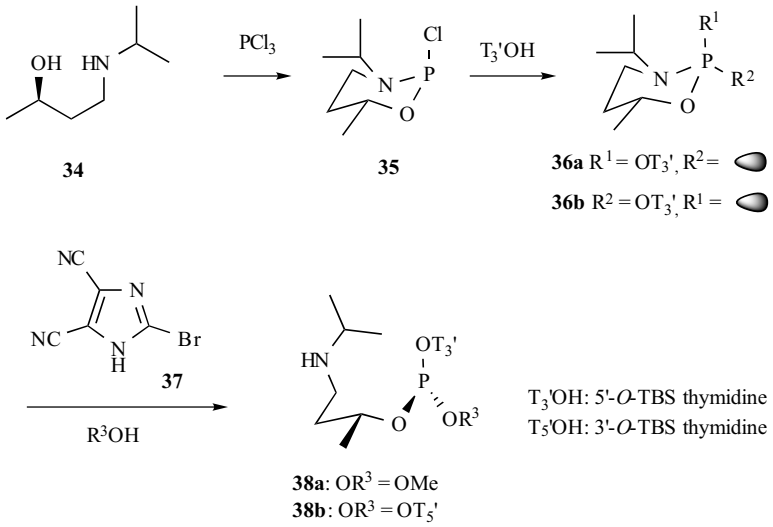
Just and co-workers intensively investigated the diastereocontrolled synthesis of phosphorothioates in the past few years. Their first key effort was to prepare easily obtainable diastereomerically pure cyclic phosphoramidites.

The first chirally pure phosphoramidite with a six-membered ring structure was prepared by Xin and Just (Scheme 12) [35]. Chiral γ-amino alcohol **34** was reacted with phosphorus trichloride to give diastereomerically pure **35** with a single ^{31}P NMR resonance. The subsequent reaction with 5'-*O*-*tert*-butyldimethylsilyl thymidine (T_3'OH) afforded cyclic phosphoramidites **36a** and **36b** in a ratio of 3:1 at room temperature, which could be improved to 20:1 upon heating in $CDCl_3$. Passing through a short column provided diastereomerically pure **36a**.

Phosphoramidite **36a** was apparently the thermodynamically more stable conformer. The configuration of **36** was assigned based on the carbon-phosphorus coupling constants, analogous to the relevant studies in the literature [36, 37]. The preferred axial orientation of OT_3' in **36a** is likely due to the anomeric effect - the favorable interaction



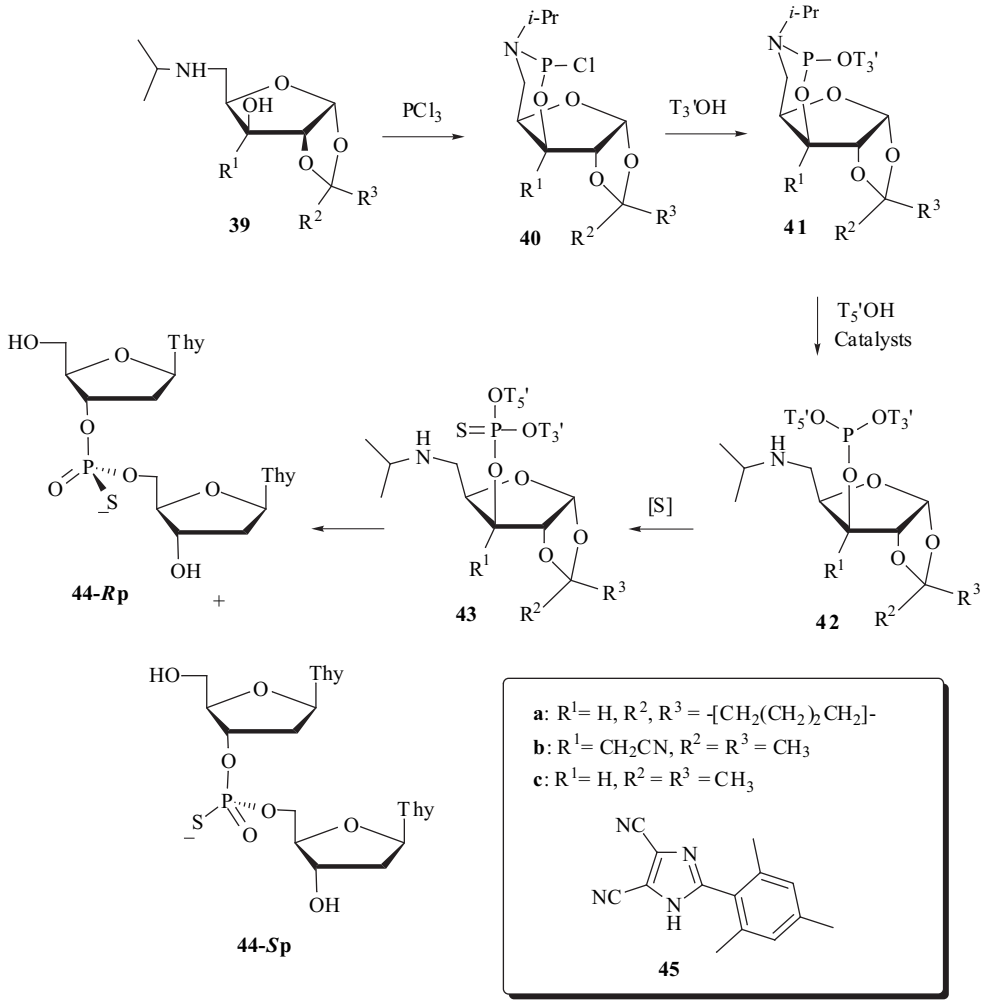
Scheme 11. Synthesis of (S_p)-dithymidine phosphorothioates.



Scheme 12. Phosphoramidite with six-membered ring.

between the nitrogen lone pair and the anti-bonding orbital of P-OT_3' σ bond.

Studies on the phosphite triester formation were carried out by reacting phosphoramidite **36a** and methanol in the presence of various azole activators. While tetrazole gave fully-epimerized products, an excellent ratio of 50:1 for the resulting phosphite triesters was obtained when 2-bromo-4,5-dicyanoimidazole **37** was tested. Unfortunately, the selectivity dropped to 3:1 when silylated thymidine was used as a nucleophile.



Scheme 13. The xylose-derived oxazaphosphorinane approach.

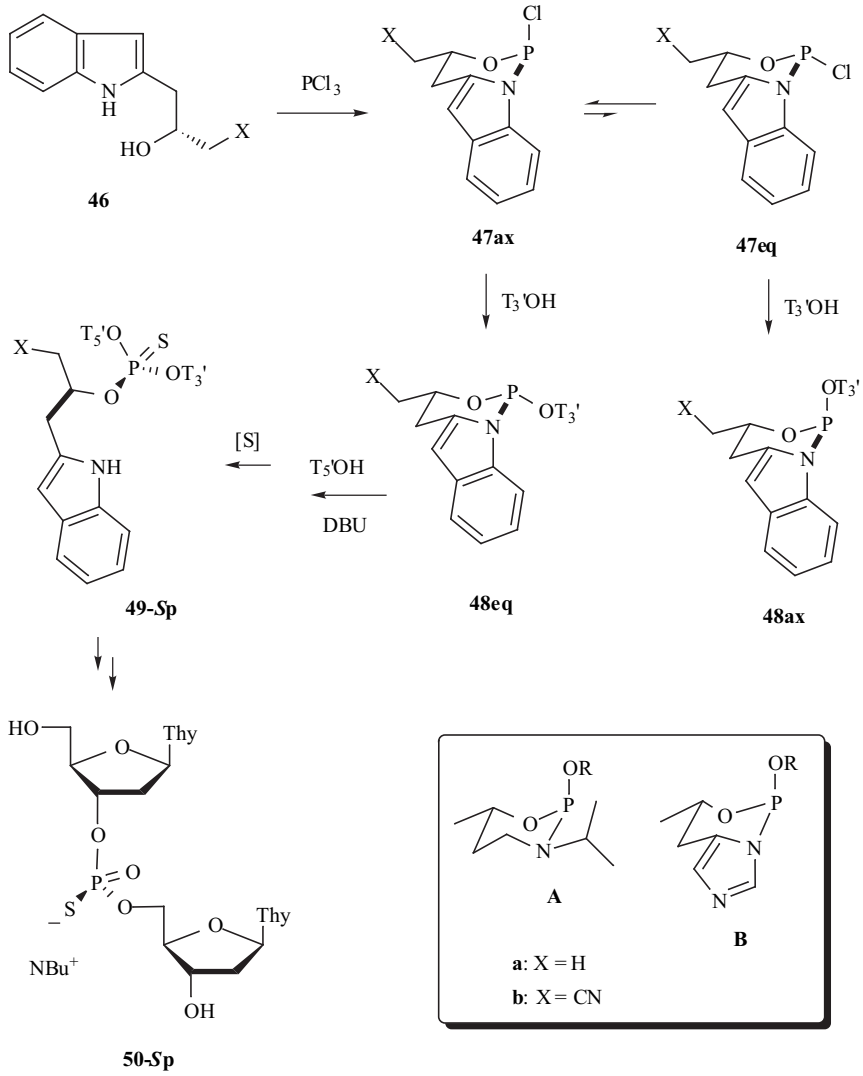
The Xylose-derived Oxazaphosphorinanes

The studies by Xin *et al.* demonstrated that the cyclic phosphoramidite possessing a six-membered ring structure could be prepared as one diastereomer without cumbersome silica gel chromatographic separation. However, the chiral auxiliary could not be removed in the end of synthesis. In addition, the stereoselectivity of phosphite triester forming reactions needed to be greatly improved. The xylose-derived oxazaphosphorinane methods were aimed to solve the above problems.

Jin *et al.* used xylose-based chiral auxiliary of type **39** for the synthesis of dinucleotide phosphorothioates [38, 39]. Xylose was chosen as a chiral structural scaffold since it is readily available in either chiral form, and it contains a masked aldehyde function β to the hydroxyl group, thus may permit a base or acid-catalyzed elimination of chiral auxiliaries in the end of synthetic sequences.

Xylose-based chiral auxiliaries were used for the synthesis of phosphorothioates (Scheme **13**). The chiral auxiliaries **39** were prepared from D-xylose. When **39a** was reacted with phosphorus trichloride, a single diastereomer of phosphorochloridite **40a** was formed as evidenced by ^{31}P NMR. When **40a** was further reacted with T_3'OH, two isomers of **41a** were formed, the ratio of which was time and temperature dependent. Equilibration of the two diastereomeric isomers at 50 °C gave **41a** as a thermodynamically more stable isomer. Passing through a quick column afforded pure **41a** as a single diastereomer. Following the coupling reaction between **41a** and T_5'OH in the presence of 2-bromo-4,5-dicyanoimidazole, and *in-situ* sulfurization with Beaucage's reagent [40], phosphorothioates **43a** were formed with a 68:1 diastereomeric ratio. After hydrolysis with 70% trifluoroacetic acid (TFA) at room temperature, fully deprotected dithymidine phosphorothioates **44** were obtained with 98% de, in favor of **44-S_P**. In a parallel run in which L-xylose was used, **44-R_P** was obtained as the major diastereoisomer.

In their later efforts, Lu *et al.* made uses of chiral auxiliaries of type **39b** and **45** as a novel activator for the diastereoselective synthesis of phosphorothioates [41, 42]. Indeed, installation of the β-cyanoethyl group allowed easy removal of chiral auxiliary upon rapid treatment with concentrated ammonia at room temperature. When the sterically more hindered activator **45** was used, the



Scheme 14. The indolo-oxazaposphorines.

diasteroselectivities were improved for the coupling reactions of phosphoramidites derived from **39b**, but remained about the same for the ones derived from **39c**.

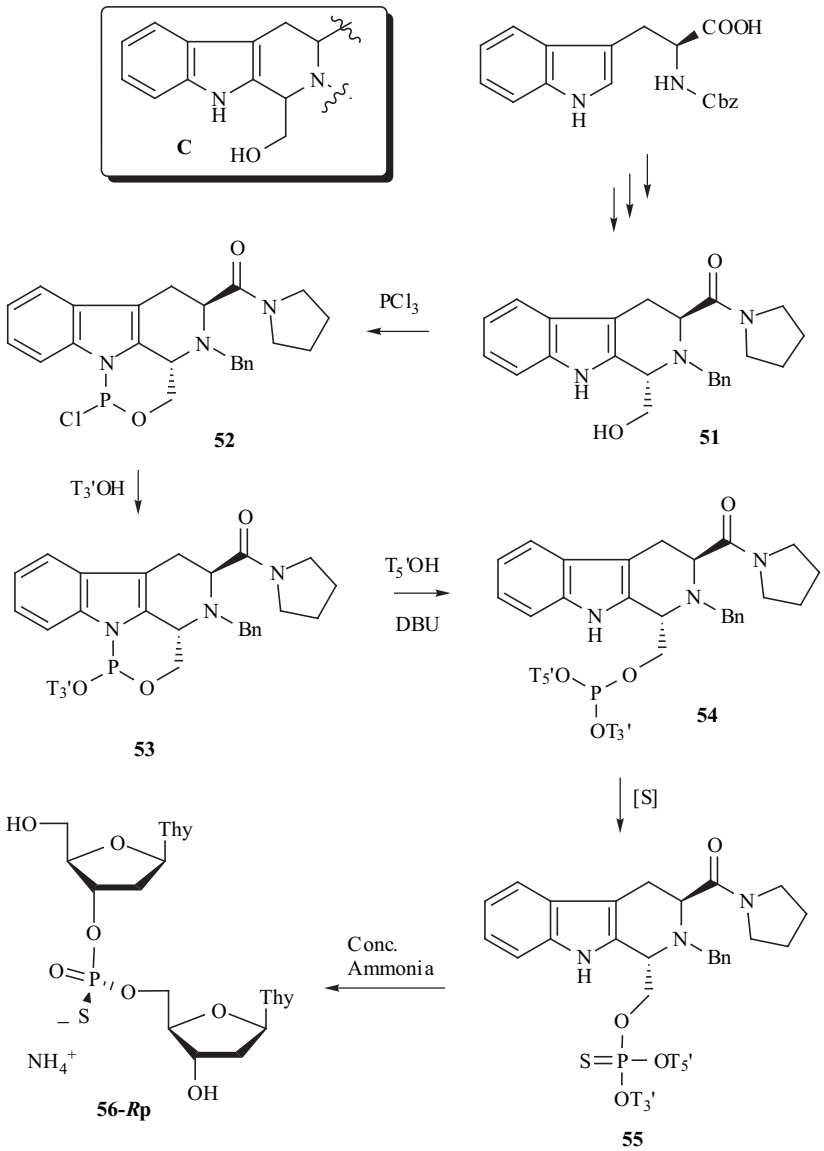
The structural assignments of phosphoramidites **41** were made based on carbon-phosphorus coupling constants according to the literature [43, 44]. After examinations of the stereochemistry of the final phosphorothioates **44**, it was proposed that the coupling reactions of **41a** and **41c** proceeded through a single protonation mechanism. In such coupling reactions, the role of the activators was to solely serve as proton sources. On the other hand, the double inversion mechanism operated in coupling reactions of **41b**. The residue of the activator displaced the protonated amino moiety in the phosphoramidites to form imidazolide intermediates, which were further displaced by nucleobases to generate phosphite triesters. The proposed two mechanistic pathways were consistent with the stereochemical outcomes of the coupling reactions in which the different activators **37** or **45** were employed.

The xylose-derived oxazaphosphorinane approaches do not constitute practical methods, but they showed that the phosphoramidite methods can be advantageously modified toward a diastereocontrolled synthesis of *P*-chiral DNA analogues, including phosphorothioates.

The Oxazaphosphorines Incorporating Heterocycles

Even though the bulky acidic imidazoles (e.g. **37**) offer excellent stereoselectivity in the coupling reactions of phosphoramidites, they are not practical in the routine solid phase synthesis of PS-Oligos. For instance, the required temperatures to achieve excellent diastereoselectivity were too low, and the strong acidity of the activator would result in depurination. Alternatively, Just and co-workers utilized the oxazaphosphorines incorporating heterocycles as precursors for the synthesis of phosphorothioates.

Marsault *et al.* initially attempted to incorporate imidazole into a ring system to eliminate the uses of acidic activators [45]. Novel bicyclic imidazo-oxazaphosphorine was prepared, and the imidazole moiety could be displaced by different alcohols in a diastereospecific manner. Unfortunately, such imidazo-oxazaphosphorine intermediate (**B** in Scheme **14**) was too unstable to be used routinely.



Scheme 15. The indolo-oxazaposphorines derived from tryptophan.

Wang *et al.* then investigated indole moiety as a leaving group for the synthesis of phosphorothioates (Scheme **14**) [46-48]. In search of a more stable and viable leaving group instead of imidazole in the design of chiral auxiliary, a comparison of the pKa values of imidazole (pKa 14.10) and indole (pKa 16.97) suggested that the latter might be a suitable leaving group. Following positive model studies, chiral auxiliary **46a** was prepared. When **46a** was treated with phosphorus trichloride, phosphorochloridites **47** were obtained as a mixture of isomers, in which probably **47ax** predominated. The subsequent *in-situ* reactions with T_3'OH at room temperature gave phosphoramidites **48** as a pair of diastereomers in a ratio of 7 to 1. Efforts were made to equilibrate **48** to a single diastereomer. In a stark contrast to the related systems of type **A** or **B** in which a thermodynamically more stable phosphoramidite with an axially oriented OR group was readily obtainable upon heating, **48ax** and **48eq** could not be equilibrated to a single isomer. The ratio of two isomers remained unchanged upon heating, and the two diastereomers **48a** and **48eq** could not be separated by silica gel chromatography. Yet, interesting enough, when a mixture of isomers of **48** were subjected to the DBU-promoted coupling reactions with T_5'OH, two isomers showed markedly different reactivities. The major diastereomer **48eq** reacted much faster than the minor isomer **48ax**. When the reaction of **48eq** was completed, **48ax** almost did not react. After filtering off DBU with a short column and treatment with Beaucage's reagent, phosphorothioate **49-S_P** was obtained with a 97% de. However, the chiral auxiliary could not be removed by treatment with concentrated ammonia. In a later refinement [47] in which chiral auxiliary **46b** was employed, reactions were carried out similarly with about the same diastereoselectivity. Chiral auxiliary bearing a β-cyano group could be easily released by standard protocols in the end of synthesis. The cyano indolo-oxazaphosphorine method was applied to the solid phase synthesis of PS-Oligos. Unfortunately, the application was unsuccessful since the use of a large excess of DBU during the solid phase synthesis led to the formation of alkylphosphonates *via* a β-elimination caused intramolecular rearrangement [49].

The potential of indolo-oxazaphosphorine for the synthesis of PS-Oligo was adequately demonstrated, although its application to the routine solid phase synthesis was unsuccessful. Lu *et al.* envisaged that a more rigid structure of type **C** would be more efficient for the formation of cyclic phosphoramidite. In addition, the elimination of troublesome β-cyano group, in combination with the readily availability of such chiral auxiliaries from natural amino acids made such approach more attractive and practical (Scheme **15**) [50].

Chiral auxiliary **51** was easily prepared from L-tryptophan. When **51** was treated with phosphorus trichloride, phosphorochloridite **52** existing as a single diastereomeric form was observed after equilibration at 50 °C. When the intermediate **52** was further reacted with T_3'OH, phosphoramidite **53** was found to be a single isomer at room temperature, no equilibration was necessary. Passing through a column on silica gel afforded pure **53** as a single isomer. DBU-promoted coupling reaction with T_5'OH transformed **53** in a stereospecific manner to diastereomerically pure phosphite triester **54**. After obtaining phosphorothioates **55**, standard treatment with concentrated ammonia at 55 °C allowed for the removal of chiral auxiliary and dithymidine phosphorothioate **56-R_P** was obtained with 95% de. This methodology was applied on solid phase for validation, both isomers of dithymidine phosphorothioates were satisfactorily prepared.

Other Methods

Recently, Stawinski and co-workers reported an interesting approach in which intramolecular nucleophile catalysis was utilized [51]. The key idea was to use a condensing agent promoted reaction of nucleosides with phosphorothioate diesters bearing a nucleophile catalytic group (Scheme **16**).

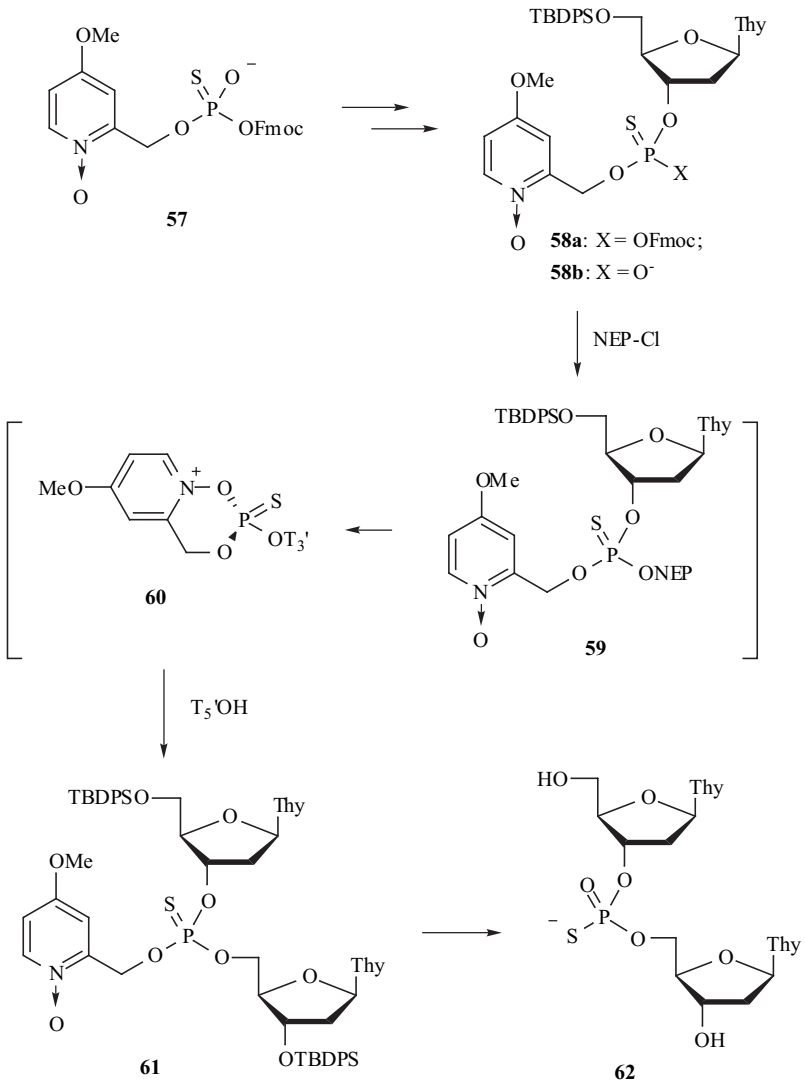
Compound **57** was a specially designed reagent. Thiophosphorylation of T_3'OH with **57** provided **58a** as a mixture of diastereomers, which were separated by silica gel chromatography. The 9-fluorenylmethoxycarbonyl (Fmoc) protective group in **58a** was removed to afford diastereomerically pure **58b**, which was thiophospholated with T_5'OH in the presence of condensing agent 2-chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane (NEP-Cl) to give **61** with complete stereocontrol. The mechanistic proposition was that the phosphorothioate diesters were activated by reactions with condensing agent NEP-Cl, the intramolecular reaction then converted **59** to **60**, the second nucleopilic displacement by T_5'OH led to double-inverted **61** stereosepecifically. After complete deprotections, *P*-chiral phosphorothioates were obtained. Although this method is still far from practical for the stereocontrolled synthesis of PS-Oligos, it represents a conceptually novel approach to the stereospecific synthesis of phosphorothioates.

SUMMARY AND OUTLOOK

Stereocontrolled synthesis of phosphorothioates has advanced enormously in the past years. Now, there are a wide range of methods available which allow for the stereocontrolled preparation of phosphorothioates with various degrees of success. With current advanced methods, it is not difficult to prepare PS-Oligos of certain length (*vide supra*) with well-defined stereochemistry at each phosphorus linkage.

Nevertheless, the true challenge lies in that if phosphorothioates are to be used as potential therapeutics, a practical synthetic methodology which permits the large scale preparation of stereocontrolled PS-Oligos at low cost is desperately needed. The synthetic PS-Oligos need to be 16-28 base long, according to the requirements of antisence technology [52].

Among the current synthetic methods for the preparation of stereodefined antisense PS-Oligos, only the oxathiaphospholane method [17-19], the cyclic *N*-acylphosphoramidite approach [30] and the novel ammonium salts activated oxazaphospholidine method [32] can partially meet aforementioned synthetic challenges. The drawbacks of Stec's oxathiaphospholane method have been discussed earlier in this review. Beaucage's cyclic *N*-acylphosphoramidite approach is interesting. The method has so far allowed for the synthesis of dodeca-thymidine



Scheme 16. The intramolecular nucleophile catalysis.

phosphorothioates with total stereocontrol at each phosphorus center, and further progress is well expected. However, one big hurdle of this approach is the difficult silica gel chromatographic separation of *N*-acylphosphoramidite monomers. The isolated yields of phosphoramidites were low, likely due to their modest stability to silica gel. Another serious drawback is the necessity of using very strong base (TMG), this can be particularly troublesome when a large amount of bases have to be used for the solid phase reactions. Wada's elegant method was very promising. Nonetheless, when the method was adapted to the solid phase synthesis, acidic activators induced epimerization of the monomer units was problematic, large quantities of monomers were necessary to overcome this problem. It is not clear whether this method will be suitable for the preparation of therapeutically useful PS-Oligos with the desired length.

The key merit of the approaches using cyclic phosphoramidites with a six-membered ring structure is the ease of obtaining diastereomerically pure monomers, a filtration-like silica gel chromatography normally is enough to provide chirally pure phosphoramidites. However, up to now, such methods have not been adapted to the solid phase synthesis of PS-Oligos. To make such approaches practical, chiral starting materials need to be readily available, and preparations of chiral auxiliaries must be simple and straightforward. Moreover, the coupling steps of phosphoramides should be efficient and stereospecific.

The intensive investigations over the past decade into the stereocontrolled synthesis of phosphorothioates have greatly advanced this largely-unexplored field. Such adventures have led to many exciting discoveries, which will surely open up new avenues in the synthesis of PS-Oligos, and pave the way to the ultimate conquest of this long-standing challenge. A practical answer to the stereocontrolled synthesis of PS-Oligos will likely to advance antisense-based therapeutics to a higher level and may greatly contribute to functional genomics.

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ABBREVIATIONS

Ade	=	Adenine
Cyt	=	Cytosine
Cyt^{Bz}	=	N^4-benzoyl-cytosine
CPG	=	Controlled pore glass
DBU	=	1,8-Diazabicyclo[5.4.0]undec-7-ene
de	=	Diastereomeric excess
DMT	=	4,4'-Dimethoxytrityl
Fmoc	=	9-Fluorenylmethoxycarbonyl
Gua	=	Guanine
HIV-1	=	Human immunodeficiency virus type 1
mRNA	=	Messenger ribonucleic acid
NEP-Cl	=	2-Chloro-5,5-dimethyl-2-oxo-1,3,2-dioxaphosphinane
PS-Oligos	=	Phosphorothioate oligonucleodies
[S]	=	Sulfurization
Thy	=	Thymine
TBDMS	=	*tert*-Butyldimethylsilyl
TBDPS	=	*tert*-Butyldiphenylsilyl
TFA	=	Trifluoroacetic acid
TMG	=	*N*,*N*,*N'*,*N'*-Tetramethylguanidine
$T_{PS}T$	=	Thymidine-thymidine dinculeotide phosphorothioates
T_3'OH	=	5'-*O-tert*-Butyldimethylsilyl thymidine
T_5'OH	=	3'-*O-tert*-Butyldimethylsilyl thymidine

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