BIOORGANIC CHEMISTRY **21**, 127–155 (1993)

REVIEW

Stereocontrolled Synthesis of P-Chiral Analogues of Oligonucleotides

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Received January 4, 1993

INTRODUCTION

Use of synthetic antisense oligodeoxyribonucleotides or oligoribonucleotides for specific gene inhibition is a novel approach to therapy in cases where a disease originates from expression of genes of known sequence (*1–7*). A genetic target, polydeoxyribonucleic acid (DNA) or polyribonucleic acid (RNA), may be approached in a highly sequence-specific fashion by hybridization of a complementary (antisense) DNA, RNA, or oligonucleotide sequence, according to Watson–Crick's rule. Malignancies and viral infections, including AIDS, are prominent examples of disease for which the antisense gene inhibition approach is being actively pursued (*5, 6*). The recent growing interest in the use of synthetic oligonucleotides in fundamental research, as tools in cell biology, illustrates another rapidly expanding field of their applications (*8*).

Several requirements for antisense agents for use in the modulation of gene expression were defined, among which the most important are: *selectivity*, *stability*, and *transportability* (*1*). Unmodified oligonucleotides are readily available and have low toxicity; however, they do not satisfy most of the requirements for antisense agents, and hence oligonucleotide analogues are often used. Essentially, all constituents of the oligonucleotide chain: bases, sugar, and phosphate residues, can be modified. Most frequently applied modifications of phosphate residues are phosphorothioates (X = S), methylphosphonates (X = CH_3), phosphoramidates (X = NR_2), and alkylphosphotriesters (X = OR) (*3, 8*) (Fig. 1).

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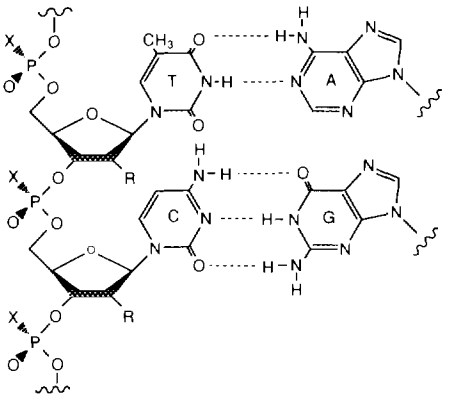

FIG. 1. Structure of P-chiral, P-tactic oligonucleotide analogues and their mode of interaction as antisense agents with complementary nucleic acid sequence, according to Watson–Crick's rule.

By virtue of phosphorus chirality and nonstereoselectivity of standard methods of oligonucleotide synthesis, phosphorus modifications are usually produced as a random mixture of 2^n diastereoisomers (n = number of P-chiral internucleotide functions). It is likely that only some of the diastereoisomers will have desirable physicochemical characteristics facilitating the most favorable biological effects (*1*, *3*, *4*).

The influence of absolute configuration at phosphorus of P-chiral antisense oligonucleotide analogues on antisense agent *selectivity* (effect on heteroduplex melting temperature, sequence specificity, and possible side effects), and *stability* (resistance against digestion by cellular nucleases) is well-recognized for methylphosphonates, phosphotriesters, and phosphorothioates (*1*, *3*, *9*, *10*).

Recent work on molecular modeling of P-stereoregular (P-homochiral, P-tactic) methylphosphonate and phosphorothioate DNA analogues suggests a strong influence of phosphorus stereochemistry upon the hydration pattern (*11*, *12*) and electrostatic potential surface (*13*, *14*) of P-modified oligonucleotides. Together with the recent discovery of putative oligonucleotide receptor (*15*, *16*), this may achieve stereodependable *transportability* of P-chiral oligonucleotides through cellular membranes. Besides the concern of P-chiral oligonucleotides stereocontrolled synthesis for their present and future practical applications, stereocontrolled methods of synthesis of any new molecule containing several chiral centers provide a perennial challenge to organic chemists. The present review summarizes achievements to date in this field.

STEREOCHEMICAL CONSIDERATIONS

Isotopic or elemental replacement of any of two nonbridging oxygens attached to the internucleotide phosphorus atom by X (Fig. 1) creates, by virtue of

asymmetry, new center(s) of chirality and results in formation of oligonucleotide diastereoisomers. The chirality at phosphorus is designated as R_P and S_P, according to the Cahn–Ingold–Prelog rules, and means opposite (mirror) space orientation of the ligands of a phosphorus atom. It should be pointed out that, because of the priority rules, for different substituents there may be the same spatial orientation, as for instance in methylphosphonates and phosphorothioates, corresponding to different configurational descriptors according to the *R*/*S* nomenclature (Fig. 1).

If P-chiral oligonucleotide is a component of double-stranded structure, the substituent X that has been introduced can point, in principle, toward the interior of the DNA double helix or away from it and into the surrounding area. This difference is essential for helix features. It has been proposed that the influence of the modification at the phosphorus on single- or double-stranded oligonucleotide physicochemical properties should depend upon (a) the electronic nature of *X*, (b) its steric "bulk," and (c) absolute stereochemistry at phosphorus (*9*). For duplexes, at least one more factor can be added, namely: (d) the differences in hydration of the analogue/phosphodiester backbone of the oligomer in duplex compared to that in the single-stranded forms (*17*).

Elimination of negative charge from the phosphate center should increase the duplex stability, as measured by its melting temperature (T_M) due to reduction in charge repulsion between the nonionic backbone of the oligomer and the negatively charged sugar–phosphate backbone of the polynucleotide. Furthermore, since nucleic acids are polyelectrolytes whose anionic phosphate groups strongly affect their structure and interactions, the deletion of phosphate charges causes charge density change, which in turn may alter nucleic acid conformation, interactions with counterions and other molecules, and hydration patterns.

The steric effect of the substituent X should depend strongly on its bulkiness so that the destabilizing influence should be more pronounced with the increase of substituent X size (*18*). However, this is not always the case, and the role of steric effects in the thermal stability of complementary oligonucleotide complexes has been occasionally questioned (*19*, *20*).

According to available data on nucleic acid hydration, phosphate anionic oxygen atoms are the most hydrated centers in DNA (~50% of the average number of contacts made by the water molecule with nucleic acid) (*21*, *22*); moreover, DNA conformation is affected by economics in the hydration of phosphate groups (*23*). Water molecules surrounding a nucleic acid single- or double-stranded structure are able to bridge hydration sites of the same residue, of adjacent residues on the same strand, or of distant residues on the two strands, forming hydration shells (*24*). Water bridges between phosphate anionic oxygen atoms are typical of each conformation form. The participation of pro-*S* and pro-*R* anionic oxygens in water bridges is different due to varying distances between two adjacent pro-*S* and two pro-*R* atoms.Therefore, it is reasonable to assume that replacing one nonbridging oxygen at the internucleotide phosphorus atom by substituting X may change the oligonucleotide pattern of hydration. If such modifications were introduced stereospecifically, one could further suppose that hydration of P-stereoregular oligonucleotide analogues would differ; for example, all-R_P and all-S_P oligomers. This may also influence physicochemical and biological properties of P-modified

oligonucleotides and complexes which they form with complementary nucleic acid.

It is not yet possible to describe the exact features of perturbations due to internucleotide linkage modification. The literature suggests that, although the duplex stabilization or destabilization results from the balance between many factors, the balance itself can be shifted into a desired direction by means of a carefully designed sequence of absolute configuration of modified P-chiral internucleotide functions. From our own and other's observations, the hypothesis emerges that the influence of factors mentioned above is modulated by the desired P-tacticity of P-chiral oligonucleotide analogues which results from the predetermined sense of chirality at phosphorus atoms. This probably also holds true as long as the nucleic acid electrostatic potential surface and hydration pattern are considered.

METHODS OF SYNTHESIS

The methods of synthesis of the oligonucleotides bearing P-chiral internucleotide linkage with defined sense of chirality can be divided into three general categories:

1. Approaches based on separation of a mixture of P-diastereoisomeric oligonucleotides obtained by means of nonstereocontrolled methods.
2. Methods utilizing block condensation, usually of dimers with a predetermined sense of chirality at modified internucleotide phosphate obtained through separation of diastereoisomeric mixture.
3. Stereoselective or stereospecific methods employing stereocontrolled methods of P-chiral internucleotide bond formation or modification.

The effectiveness of diastereoisomeric oligonucleotide separation depends on the number (n) of modifications within the oligonuclotide chain; in practice, this is limited to $n = 4$. The lack of success in separation of diastereoisomers of oligonucleotide congeners calls for the choice of another strategy and introduction to the protocol of oligonucleotide synthesis reagents consisting of oligonucleotide building blocks with a defined sense of chirality at phosphorus. These are obtained by chromatographic separation of a mixture of diastereoisomeric oligonucleotides obtained by nonstereocontrolled methods. The block approach leads to a sugar–phosphate backbone with a predetermined sense of chirality at modified phosphates under such condition that these modified groups are separated by at least one "regular" internucleotide phosphate function in an alternating mode. Perhaps the most difficult problem is posed by the requirement of introduction of stereo-defined modification at neighboring (adjacent) positions. However, this kind of P-tactic oligonucleotides expresses the most enhanced effect of modification on its physicochemical properties.

This review focuses on stereocontrolled formation of P-chiral internucleotide linkages with predetermined sense of chirality at the phosphorus atom. The methods based on separation of mixture of diastereoisomeric oligonucleotides obtained by means of nonstereospecific methods were summarized recently by Zon (*25*), and for DNA triesters by Koziolkiewicz and Wilk (*26*) and will not be discussed

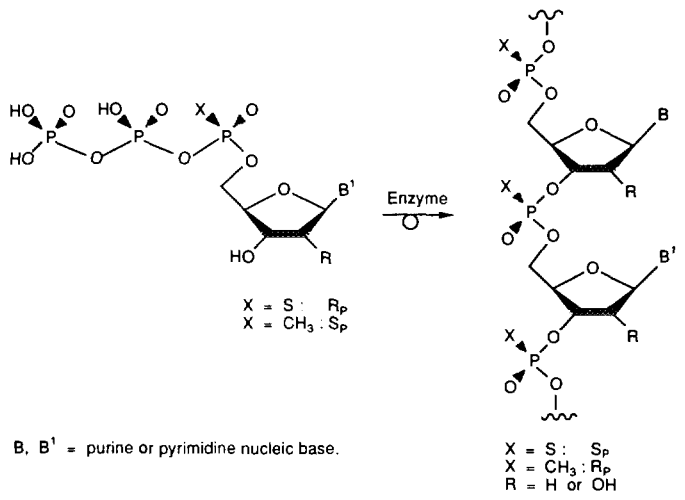

FIG. 2. Enzymatic synthesis of phosphorothioate and methylphosphonate nucleic acid analogues.

herein. The methods based on block condensation of diastereoisomerically pure oligonucleotide blocks will be omitted since it does not involve the stereocontrolled formation or modification of internucleotide bonds. The stereocontrolled methods leading to P-chiral oligonucleotides due to replacement of ^{16}O nonbridging atoms by ^{17}O or ^{18}O isotopes will also not be discussed since this kind of modification does not seem to influence the physicochemical and biological properties of oligonucleotides.

Since the term *stereospecific* is sometimes used in the literature when in effect *stereoselective* is meant, the following definitions are used in this review. The reaction in which one stereoisomer is formed predominantly at the expense of other stereoisomers is called stereoselective synthesis. In a stereospecific reaction, a given isomer leads to one product while another stereoisomer leads to the opposite product. A consequence of these definitions is that, if the reaction is carried out on a compound which has no stereoisomers, it cannot be stereospecific but at most it is stereoselective; also all stereospecific reactions are necessarily stereoselective while the converse is not true (*27*).

Enzymatic Methods

Enzymatic synthesis of P-chiral DNA and RNA described so far is limited to phosphorothioate and methylphosphonate analogues. Polyribonucleotide-containing phosphorothioate linkages were obtained as early as 1967 by Eckstein and associates using DNA-dependent RNA polymerase from *Escherichia coli* (*28*). DNA-dependent RNA polymerase is a complex enzyme whose essential function is to transcribe the base sequence in a segment of DNA into a complementary base sequence of a messenger RNA molecule. Nucleoside triphosphates are the substrates that serve as the nucleotide units in RNA (Fig. 2). In the polymerization

of triphosphates, the enzyme requires a DNA segment that serves as a template for the base sequence in the newly synthesized RNA. In the original procedure, uridine 5′-*O*-(1-thiotriphosphate), adenosine 5′-*O*-triphosphate, and poly d(AT) as a template were used. As a result, an alternating copolymer [$A_p(S)U_pA_p(S)U_p$] was obtained, in which every other phosphate was replaced by a phosphorothioate group.

Using the same approach and uridine 5′-*O*-(1-thiotriphosphate) and adenosine 5′-*O*-(1-thiotriphosphate), polyribonucleotide containing an all-phosphorothioate backbone was also synthesized (*29*). In both cases, nucleoside 5′-*O*-(1-thiotriphosphates) as a mixture of two diastereoisomers were used.

The stereochemical course of polymerization catalyzed by *E. coli* polymerase was determined by Eckstein using the 1-thio analog of adenosine 5′-*O*-triphosphate as a substrate (*30, 31*). The S_p epimer was found to serve as an enzyme substrate and was further used with uridine 5′-*O*-triphosphate and a DNA template of poly d(AT). The resulting RNA polymer was the complementary alternating copolymer of adenosine and uridine linked by alternating 3′,5′-phosphodiester and 3′,5′-thiophosphodiester bonds.

The configuration at the 3′,5′-thiophosphodiester linkage resulting from the inversion at the 1-phosphorus in the S_p epimer of nucleoside 5′-*O*-(1-thiotriphosphate) was established by enzymatic assay to be R_p (*30*). Several other polymerases proved useful in the synthesis of the phosphorothiate backbone bearing polyribo- and polydeoxyribonucleotides are shown in Table 1. An interesting example of enzyme application to the synthesis of P-tactic thiophosphate oligonucleotide analogues is preparation of 2′,5′-phosphorothioate oligoadenylates described by Suhadolnik and associates (*44, 45*). The 2′,5′-oligoadenylate [$2',5'\text{-}_{ppp}(A_p)_nA$] system is widely accepted as being involved in the antiviral mechanism of interferon and may also be involved in the regulation of cell growth and differentiation. However, 2′,5′-oligoadenylates are rapidly hydrolyzed by 2′,5′-phosphodiesterase. Therefore, a modified 2′,5′-oligoadenylate e.g., phosphorothioates which would still exhibit the biological activity and yet be resistant to enzymatic hydrolysis, would be useful as biochemical probes. This was achieved using 2′,5′-synthetase from L929 cell extracts or rabbit reticulocytes (*45*) and S_p isomer of adenosine 5′-O-(1-thiotriphosphate); the R_p isomer was not a substrate for the enzyme. Trimers and tetramers (n = 1 or 2, see above) were obtained with ~20% yield. The R_p absolute configuration at phosphorus of 2′,5′ internucleotide phosphorothioate functions assignment was accomplished by enzymatic analysis and comparison with P-stereodefined trimers and tetramers obtained by chemical methods (*45*). The rigorous stereochemical analysis led the authors to the conclusion that the reaction catalyzed by 2′,5′-synthetase proceeds with inversion of the configuration on the phosphorus atom of adenosine-5′-triphosphate. Therefore, it appears that all polymerases for which the stereochemical course was determined catalyze the polymerization reaction with inversion of configuration at phosphorus, resulting in P-tactic nucleic acid analog with all phosphorothioate internucleotide groups of the R_p configuration. This phenomenon may reflect an important feature of evolution of a machinery responsible for the genetic information flow.

It took 24 years following Eckstein's pioneering work on enzyme catalyzed

TABLE 1

Stereochemical Course of Enzyme-Catalyzed Formation of Internucleotide Phosphorothioate and Methylphosphonate Linkage

Enzyme	Stereochemical course	Reference(s)
Enzyme-catalyzed formation of internucleotide phosphorothioate linkage		
Polymerases		
E. coli DNA-dependent RNA polymerase	Inversion	*31, 32*
DNA-dependent DNA polymerases:		
Escherichia coli	Inversion	*33, 34*
Phage T4	Inversion	*35, 36*
Phage T7	Inversion	*37*
Micrococcus luteus	Inversion	*38*
Reverse transcriptase	Inversion	*39*
Other		
Polynucleotide phosphorylase	Inversion (elongation)	*40, 41*
tRNA nukleotidyl transferase	Inversion	*42*
RNA ligase	Inversion (ligation)	*43*
2′,5′-oligoadenylate synthetase	Inversion	*38–40, 44, 45*
Enzymes-catalyzed formation of internucleotide methylphosphonate linkage		
Polymerases		
DNA-dependent DNA polymerases:		
Escherichia coli, Klenow fragment	Stereospecific (?)	*49*
Rat liver, polymerase β	Stereospecific (?)	*49*
HIV-1 reverse transcriptase	Stereospecific (?)	*49*
AMV reverse transcriptase	Stereospecific (?)	*49*
Other		
Calf thymus terminal deoxynucleotidyltransferase	Stereospecific	*46, 49*

polymerization of 1-thio analogue of nucleoside 5′-*O*-triphosphates to prove that some enzymes can catalyze formation of the interneucleotide methylphosphonate bond utilizing nucleoside 5′-*O*-(1-methyltriphosphonates) as a substrate (*46*). Attempts at enzymatic synthesis of oligonucleotide analogues bearing internucleotide phosphoroamide (*47*) or phosphorotriester (*48*) bonds have so far been unsuccessful. The first enzymatic synthesis of oligonucleotide containing methylphosphonate internucleotide linkage was described by Kaji and coworkers in 1990 (*46*), who showed that enzyme, terminal deoxynucleotidyltransferase from calf thymus can elongate primer oligonucleotide by one or two nucleotide units when thymidine 5′-*O*-(1-methyltriphosphonate) is used as an active monomer. The polymerization reaction is most probably stereospecific since formation of only S_P internucleotide methylphosphonate linkage was detected. However, the stereochemistry of coupling reaction is not yet clear as the 1-methylphosphonate analogue of thymidine

5′-*O*-triphosphate was obtained by a nonstereospecific method, and its diastereoisomeric purity was not rigorously determined although the product resulted in only a single peak as characterized by anion-exchange chromatography and RP-HPLC. Unfortunately, no ^{31}P- NMR spectra were provided for this compound.

Recently, it was reported by Kukhanova and associates (*49*) that rat liver DNA polymerase β, human immunodeficiency virus (HIV-1), and avian myeloblastosis virus (AMV) reverse transcriptases, and *E. coli* polymerase I Klenow fragment, are also capable to different extents of catalyzing formation of internucleotide methylphosphonate linkage. The most efficient is AMV reverse transcriptase incorporating seven to eight nucleoside methylphosphonate units into oligonucleotide primer. The lowest efficacy is exhibited by *E. coli* DNA polymerase I Klenow fragment, probably due to proofreading 3′-5′ exonuclease activity. If this is the case, the *E. coli* DNA polymerase I is the first enzyme known to cleave internucleotide methylphosphonate linkage. The stereochemistry of polymerization reaction was not established (*49*). The thymidine 5′-*O*-(1-methyltriphosphonate) used was a mixture of R_P and S_P isomers as shown by means of the ^{31}P-NMR technique. The absolute stereochemistry of the internucleotide methylphosphonate function of the resultant modified oligonucleotides was also not determined.

Despite the above-mentioned lack of data, taking into account that internucleotide methylphosphonate linkage formation catalyzed by terminal deoxynucleotidyltransferase is stereospecific (*46*) and that enzymatic polymerization of nucleoside S_P 5′-*O*-(1-thiotriphosphate) in all cases known to date is stereospecific as well (Table 1), it seems reasonable to assume that enzymatic polymerization of nucleoside 5′-*O*-(1-methyltriphosphonate) proceeds stereospecifically.

It is also worth noting that nucleoside S_P 5′-*O*-(1-thiotriphosphate) polymerazing enzymes yield the polynucleotide products with R_P internucleotide phosphorothioate functions, and that nucleoside 5′-*O*-(1-methyltriphosphate) incorporation into oligonucleotide chain catalyzed by terminal deoxynucleotidetransferase proceeds with formation of S_P internucleotide methylphosphonate linkage. In spite of different configurational descriptors, the spatial orientation of sulphur and methyl group replacing phosphate nonbridging oxygen in both cases is the same. This in turn may support the thesis that substrate stereopreference of enzymes catalyzed polymerization process is the same for thiophosphate and methylphosphonate poly(oligo)nucleotide analogues as well as stereochemistry, and that negative charge density in nucleoside 5′-*O*-(1-thiotriphosphate) and its polymers is higher on the oxygen than on the sulfur atom, which is sometimes questioned (*50*). Stereospecific, enzymatic syntheses of phosphorothioate DNA and RNA analogues using appropriate templates, polymerases, and nucleoside 5′-*O*-(thiotriphosphates) proved a valuable tool in molecular biology; however, to date it has only yielded the R_P configuration. Enzymatic syntheses of P-stereoregular methylphosphonate oligonucleotide analogues are at the initial stage of development and need improvement and further studies on stereochemistry of polymerization reaction. While a hypothetical polymerase with opposite diastereoselectivity, or a hypothetical postenzymatic inversion scheme, could provide access to the opposite configuration, a general stereochemically controlled method will most likely be an entirely chemical process.

Chemical Methods

A variety of chemical methods are applied for the synthesis of P-chiral oligonucleotide analogues with predetermined sense of chirality at the phosphorus atom. All of them are based on previous achievements of phosphoroorganic chemistry resulting from the studies on synthesis and transformations of less complex phosphorus compounds. It should be emphasized that methods elaborated for small molecules often fail to work in the case of more intricate structures such as mono- and oligonucleotides, or require significant modification. Two qualitatively different approaches are commonly used. One involves the stereoselective or stereospecific *de novo* internucleotide linkage formation, and another utilizes stereoselective or stereospecific modification of an already formed P-chiral internucleotide bond. They will be discussed in that order in following sections.

Stereoselective Methods

The first stereoselective formation of internucleotide linkage was reported by Ikehara, Ohtsuka, and coworkers in 1981 (*51*). The authors described the synthesis of phosphate aryl esters using the new condensing reagent, arenesulfonyl 5-(pyridin-2-yl)tetrazole. The reaction was performed in pyridine. The molar ratio of nucleoside component/diester nucleotide component/condensing reagent was 1.25/1.0/2.5. Purine and pyrimidine nucleoside derivatives were used as nucleoside and nucleotide components. Formation of only one (faster on TLC) diastereoisomer was observed. The amount of the other isomer was estimated to be <5% (*52*, *53*). The absolute configuration at the phosphorus atom of the major dinucleotide product is S_P as established by the ^{1}H-NMR and CD techniques. The authors hypothesized that stereoselective reaction occurred at the stage of the mixed anhydride formation. According to the proposed mechanism, it is assumed that the pro-S oxygen atom undergoes sulfonation selectively, since pro-*R* oxygen interacts in the transition state with the nitrogen of pyridine moiety of 5-(pyridin-2-yl)tetrazolyl residue of the condensing agent. Mixed anhydride formation is followed in turn by two successive substitutions at phosphorus, first by the 5-(pyridin-2-yl) tetrazole molecule, released from the condensing agent at the sulfonation step, and next by the 5′-hydroxyl group of the nucleoside component (Fig. 3). As shown recently by Niewiarowski *et al.*, if diastereoisomerically pure 5′-*O*-monomethoxytritylthymidine 3′-*O*-methylthiophosphonate was used in the condensation reaction in the presence of triisopropylbenzenesulfonylchloride or triisopropylbenzenesulfonyl-3-nitro-1,2,4-triazolide as condensing agents, desulfuration as well as mononucleotide P-epimerization leading to diastereoisomeric mixture of dinucleoside methylphosphonates takes place (*54*). This is in agreement with the widely accepted mechanism for the phosphotriester condensation reaction, assuming that mixed anhydride is formed in the first step of the activation process (*55*). An exchange of the sulfonic acid ligand, in an equilibrium mode, with other nucleophiles present in the reaction mixture (e.g., another mononucleotide or solvent molecule such as pyridine, sulfonic acid, or tetrazole released from the activating agent, etc.), may be responsible for epimerization at the phosphorus atom (*56*, *57*). The presence of pyrdin-2-yl substituent in the condensing agent

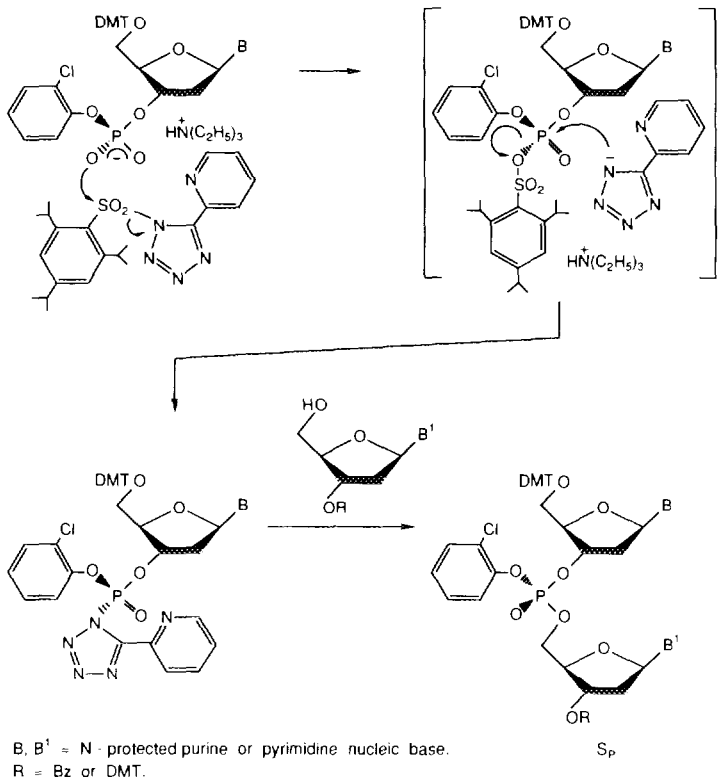

FIG. 3. Stereoselective synthesis of S_P di[(2′-*O*-deoxyribonucleoside) *O*-arylphosphate] using 1-[2,4,6-triisopropylbenzene-5-(pyridin-2-yl)tetrazole] as activating reagent.

seems, therefore, essential for stereoselectivity of the coupling reaction, since neither (1-mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazolide nor 1-arenesulfonyl-5-phenyltetrazolide prompts the stereoselective synthesis (*53*). The presence of aryloxy-type phosphate protecting group may also play a role. The importance of unique intramolecular interactions is further emphasized by the fact that no stereoselective synthesis of oligonucleotides longer than dimers has been reported so far.

An application of mesitilenesulfonyl-5-(pyridin-2-yl)tetrazole to the stereoselective synthesis of dinucleoside phosphorothioates was described by Cosstick and Williams (*58*) (Fig. 4). They also found that a new phosphodiester bond is formed selectively with pro-*S* oxygen atom as described by Ikehara *et al.* (*51*). The degree of stereoselectivity is, however, less pronounced than in the synthesis of dinucleoside phosphate aryl esters. The reaction between nucleotide monomer N^4-benzoyl-5′-*O*-dimethoxytrityl-2′-*O*-deoxycytidine-3′-*O*-[*S*-(2-cyanoethyl)]phosphorothioate and nucleoside 3′-*O*-acetylthymidine was performed in a mixture of methylene chloride and pyridine (20/1, v/v). The molar ratio of nucleoside component/diester

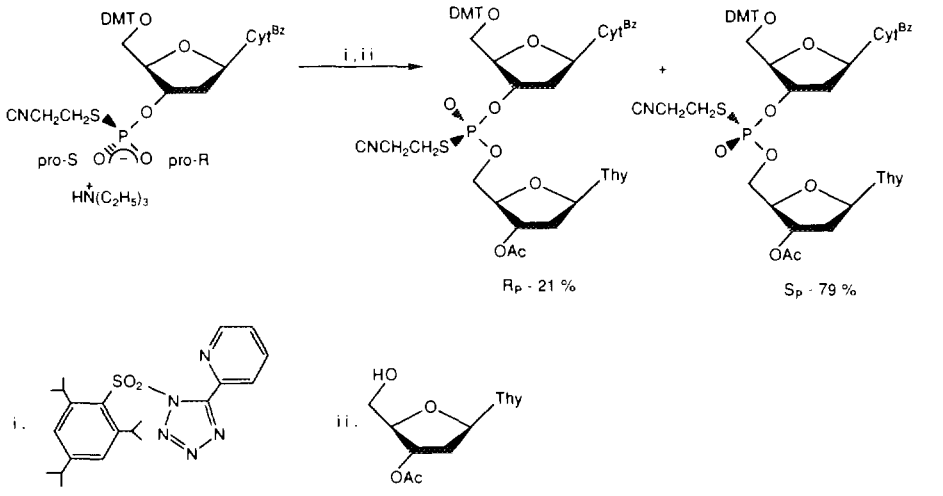

FIG. 4. Stereoselective synthesis of N,O-protected 2′-*O*-deoxyribocytidylyl(3′,5′)thymidine S-(2-cyanoethyl)phosphorothioate using 1-[2,4,6-triisopropylbenzene-5-(pyridine-2-yl)tetrazole] as activating reagent.

nucleotide component/condensing reagent was 1.0/1.25/2.5. The ratio of S_P/R_P diastereoisomer obtained was 79/21. When the reaction was performed in pyridine only, the amount of R_P isomer increased slightly to 73/27. The absolute configuration at the phosphorus atom was established by enzymatic assay and RP-HPLC retention time criteria. The stereoselective methods of P-chiral oligonucleotide synthesis discussed above deal with the formation of common 3′-5′ internucleotide linkage.

There are few examples of stereocontrolled synthesis of oligonucleotides bearing P-chiral 2′–5′ linkage. The enzymatic method was discussed above. An efficient, stereoselective chemical synthesis of 2′,5′-oligo-(S_P)-thioadenylates was described recently by Battistini *et al.* using H-phosphonate chemistry (*59*). Three cases differing in combination of sugar residue hydroxyl protecting groups were studied. In all cases, marked prevalence of the S_P isomer was observed, but to a different extent. In the first example, coupling between 5′-*O*-dimethoxytrityl-3′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyladenosine 2′-*O*-hydrogenphosphonate and 2′,3′-*O*,*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-N^6-benzoyladenosine, followed by sulfurization of intermediate dinucleoside H-phosphonate, led to mixture of S_P and R_P isomers in a ratio 7/3. Similar condensation between 5′-*O*-dimethoxytrityl-3′-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyladenosine 2′-*O*-hydrogenphosphonate and 2′,3′-*O*,*O*-bis(*t*-butyldimethylsilyl)-N^6-benzoyladenosine gave higher enrichment of diastereoisomeric mixture with S_P isomer. The ratio of S_P/R_P diastereoisomer was 8/2. The influence of the structure of the reacting nucleoside and nucleotide components upon the degree of asymmetric induction is illustrated by the fact that the reaction between 3′,5′-*O*,*O*-(1,1,3,3-tetraisopropyl-1,3-disiloxanediyl)-N^6-benzoyladenosine 2′-*O*-hydrogenphosphonate, and 2′,3′-*O*,*O*-(1,1,3,3-tetraiso-

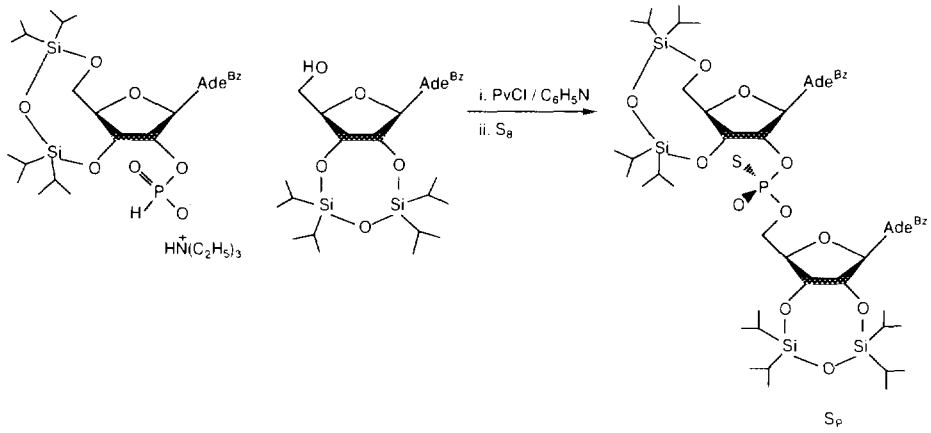

FIG. 5. Stereoselective synthesis of N,O-protected S_P adenosinylyl(2',5')adenosine phosphorothioate.

propyl-1,3-disiloxanediyl)-N^6-benzoyladenosine, leads exclusively to diastereoisomer S_P of suitable 2',5'-dinucleotide. In all cases, the molar ratio of nucleoside/nucleotide component/condensing reagent (pivaloyl chloride) was 1/1/2.5 (Fig. 5).

The condensation reaction used pyridine as the reaction medium. An elongation reaction leading from dimer to trimer also occurs with substantial stereoselectivity. This author has found that condensation of 5'-*O*-dimethoxytrityl-3'-*O*-(*t*-butyldimethylsilyl)-N^6-benzoyladenosine 2'-*O*-hydrogenphosphonate and 3'-*O*-(*t*-butyldimethylsilyl) adenosinylyl(2',5')2',3'-*O*,*O*-bis(*t*-butyldimethylsilyl)adenosine S_P-phosphorothioate, followed by oxidation with sulfur, provides the S_PS_P/R_PS_P mixture of trimers in a ratio of 8/2. The absolute configurations at the P-chiral centers of internucleotide phosphorothioate functions were established by enzymatic assay.

The phosphorothioate internucleotide linkage formation by the present methodology involves two steps: the coupling reaction leading to suitable H-phosphonates, and their subsequent oxidation with sulfur. It has been recently proved by Seela and coworkers (*60*) that sulfurization of the diastereoisomerically pure dinucleoside H-phosphonates is a stereoselective process (see above). This finding led the authors to the conclusion that asymmetric induction takes place at the level of the H-phosphonate diester formation. The demonstrated stereoselectivity of elongation step and utilization of the standard H-phosphonate chemistry make it possible to apply this methodology to the synthesis of the stereoenriched 2',5'-oligonucleotide phosphorothiate analogues on the solid support.

Recently, Stawinski *et al.* demonstrated that formation of ribonucleosidylyl(3',5') ribonucleoside internucleotide linkage by the H-phosphonate method is also stereoselective in some cases; however, no stereoselectivity in the deoxyribo series was observed (*61*). The authors showed that condensation of 5'-*O*-monomethoxytrityl-2'-*O*-(*t*-butyldimethylsilyl)uridine 3'-*O*-hydrogenphosphonate, or 3'-*O*-hydrogenthiophosphonate and 2',3'-*O*,*O*-dibenzoyluridine, in the presence of condensing agent proceeds with predominant formation of dinucleotide resonating at lower field in ^{31}P-NMR for both types of the nucleotide components. The

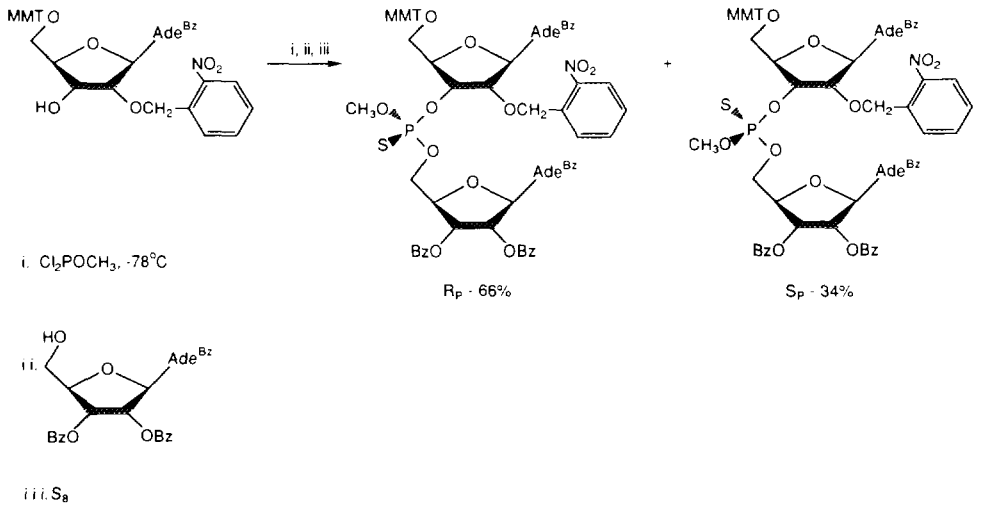

FIG. 6. Stereoselective synthesis of N,O-protected adenosinylyl(3',5')adenosine *O*-methylphosphorothioate using tricoordinated phosphorus chemistry.

ratio of lower to higher field resonating diastereoisomer was 6/1. The molar ratio of nucleoside/nucleotide/condensing agent was 2.2/1.0/4.0. Reaction was performed in pyridine as reaction medium.

Contrary to the methods based on the chemistry of tetracoordinated phosphorus, there are only a few examples of the stereocontrolled synthesis of P-chiral oligonucleotides utilizing the tricoordinated phosphorus compounds. Reactions at trivalent phosphorus atom are complicated due to pseudorotations within a trigonal–bipyramidal transition state resulting from the attack of free nucleophiles. Furthermore, P-chiral derivatives of tricoordinated phosphorus easily undergo P-epimerization due to low configurational stability (*62*). Bencovic and Marlier found that reaction of 5'-*O*-monomethoxytrityl-2'-*O*-(2-nitrobenzyl)-N^6-benzoyladenosine with methoxydichlorophosphite, followed by 2',3'-*O*,*O*-dibenzoyl-N^6-benzoyladenosine and sulfur, leads preferentially to the R_P isomer of dinucleoside phosphorothioate (*63*). The molar ratio of 3'-hydroxyl nucleoside/5'-hydroxyl nucleoside/phosphitylating agent was 1/2.2/1.5. The reaction was performed in the mixture pyridine/THF 1/7.5 at −78°C. The ratio of R_P and S_P diastereoisomers was 2/1. The absolute configuration of the internucleotide phosphrothioate function was established enzymatically (Fig. 6).

Engels and Loschner worked out a similar methodology for diastereoselective synthesis of dinucleoside methylphosphonates (*64*). They found that reaction of suitably protected 3'-hydroxyl nucleoside with methyldichlorophosphine, in the presence of collidine, followed by the 5'-hydroxyl nucleoside and oxidation step, provided a mixture of diastereoisomeric dinucleotides substantially enriched with R_P isomer. The molar ratio of 3'-hydroxyl nucleoside/methyldichlorophosphine was 1.05/1.00. The reaction was performed in THF at +4°C for the first step and −80°C for the coupling reaction (Fig. 7).

Besides purine, pyrimidine nucleosides were tested. The R_P/S_P diastereoiosomer

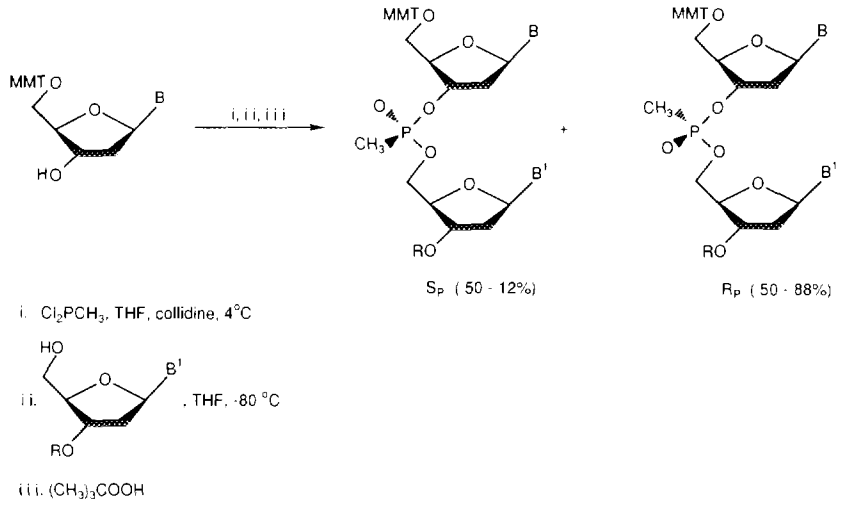

FIG. 7. Stereoselective synthesis of di[2′-*O*-deoxyribonucleoside) methylphosphonate] using tricoordinated phosphorus chemistry.

ratio varied from 8/1 to 1/1. The diasteroisomeric excess depends on the temperature and the nucleoside used. The induction is highest at low temperature (− 80°C) and with small bases (T,C). The diastereoselectivity is less with purine bases (A,G) even at low temperature. 2′-Deoxyguanosine is worst, showing only a small induction in all cases. The absolute configuration at the phosphorus atom of the internucleotide methylphosphonate group was established by means of 2D NMR using the NOE-derived ROESY technique (*65*), and in some cases by comparison with authentic samples. The preliminary mechanistic studies by ^{31}P-NMR suggest that the observed asymmetric induction arises in the second step, when the more stable dinucleoside methylphosphinic diesters are formed. The intermediate 5′-protected 3′-*O*-methylphosphonic chloride exists, even at − 80°C, as a 1/1 mixture of diastereoisomers. The final oxidation with *t*-butyl peroxide proceeds with retention of configuration and does not change the absolute stereochemistry. It was shown only recently, however, that proper modification of the phosphonylating agent may cause a substantial asymmetric induction also in the first step of condensation reaction. Thus, when bis(proline derivative)methylphosphondiamidite was reacted with 5′-*O*-tritylthymidine, in the presence of acid catalyst, induction was observed up to 5/1, R_P/S_P or S_P/R_P, respectively, subject to configuration of chiral auxiliary (*66*) (Fig. 8).

The degree of asymmetric induction in the 5′-*O*-tritylthymidine 3′-*O*-(proline derivative)methylphosphonamidite formation step depends also on temperature, solvent, and activating agent used [1,2,4-triazole, 3-nitro-1.2.4-triazole, tetrazole, 5-(4-nitrophenyl)tetrazole]. Unfortunately, the asymmetric induction gained in the first step was to a large extent lost in the reaction with nucleoside component, 3′-*O*-(*t*-butyldimethylsilyl)thymidine, leading to internucleotide bond formation;