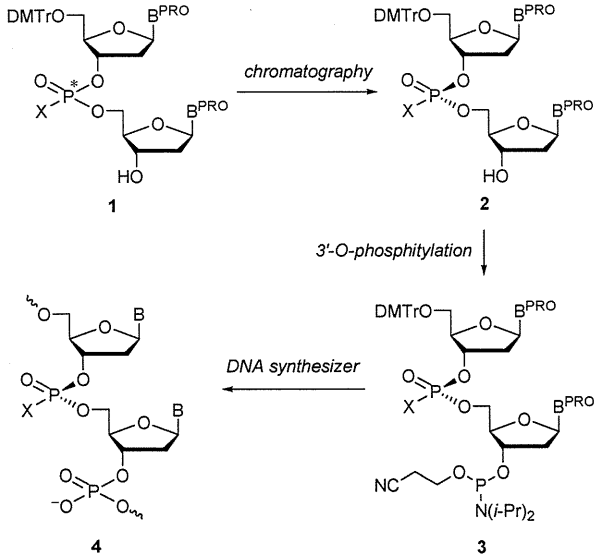
exponentially. In other words, an oligonucleotide analog with n chiral phosphorus atoms would have 2^n different *P*-diastereomers. For example, an oligodeoxyribonucleoside phosphorothioate (PS-ODN) 10mer, which contains nine chiral phosphorus atoms, is obtained as a mixture of 2^9 (= 512) *P*-diastereomers by a nonstereocontrolled synthesis. The individual diastereomers are very similar to each other with respect to the retention time and thus practically inseparable. Therefore, in cases of oligonucleotide analogs with predominantly chiral phosphorus atoms, only short oligomers (generally up to 5mers) can be separated into their *P*-diastereomers even with high-performance liquid chromatography (HPLC).[38,39]

3. Use of diastereopure dimer building blocks

To obtain diastereopure *P*-chiral dinucleoside phosphate analogs by chromatography, fully deprotected 2mers are separated using reversed-phase HPLC in most cases, but those bearing protecting groups can be separated into diastereomers by either normal- or reversed-phase chromatography. When protected 2mers in which only the 3′-OH group is free are available in a diastereopure form, they can be derivatized into the corresponding 3′-phosphoramidite dimer building blocks, which are applicable to solid-phase syntheses of oligonucleotide analogs by the conventional phosphoramidite method (Scheme 1).[40,41] This method can produce oligonucleotides containing multiple stereodefined chiral phosphate analogs, which are difficult to obtain by chromatographic separation. Another advantage of this method is that a stereocontrolled synthetic method is unnecessary, although stereocontrolled reactions can also be used to synthesize the dimer building blocks. There are also several disadvantages: chromatographic separation is not always assured. Up to 32 types of dimer building blocks may be necessary to synthesize the desired oligonucleotides (four types of nucleosides for the 3′- and 5′-nucleosides and two *P*-diastereomers). The coupling efficiency of the dimer building blocks is generally lower than that of the regular monomer units. Oligonucleotides containing consecutive *P*-chiral nucleotides are not available.



Scheme 1 Synthesis of ODNs containing stereodefined *P*-chiral phosphate analogs **4** using diastereopure dimer building blocks **3**. B^{PRO} = protected nucleobase.

4. Enzymatic synthesis

Enzymatic synthesis has also been used since as early as the 1960s to obtain *P*-chiral oligonucleotide analogs.[16,18,24,25,42–48] Four types of *P*-chiral nucleotide analogs, including nucleoside 5′-phosphorothioates,[42–44] phosphoroselenoates,[24,25] methylphosphonates[45,46] and boranophosphates,[47,48] have been enzymatically incorporated into oligonucleotides. The enzymatic synthesis uses an enzyme, such as *E. coli* DNA polymerase I or T7 RNA polymerase, ribo- or 2′-deoxyribonucleoside 5′-triphosphates (rNTPs, dNTPs), and template and primer oligonucleotides. The enzyme extends the primer from the 5′- to the 3′-end by incorporating nucleotides in a template-dependent manner. To incorporate *P*-chiral nucleotide analogs into oligonucleotides, α-*P*-modified NTPs (Fig. 3) are used in place of natural NTPs. For example, oligodeoxyribonucleotides in which all of the thymidine 5′-phosphate residues are replaced by their phosphorothioate counterparts are synthesized by using α-*P*-thiothymidine 5′-triphosphate (TTPαS) in place of natural TTP in conjunction with the other three types of natural dNTPs. Fully modified PS-ODNs are synthesized by using four types of dNTPαSs. Unlike the approach employing chromatographic separation, enzymatic synthesis can provide oligonucleotides containing multiple *P*-chiral phosphate analogs as well as fully modified *P*-chiral oligonucleotides. The products are always diastereopure. Because stereocontrolled chemical syntheses of *P*-chiral oligonucleotide analogs still have some limitations as described below, particularly in the synthesis of long oligomers, an enzymatic synthesis may still be the method of choice to obtain stereoregulated oligonucleoside phosphorothioates, phosphoroselenoates, methylphosphonates, and boranophosphates longer than 10–30mers. However, the enzymatic synthesis has its own limitations: (1) the enzymes only use the (*S*p)-isomers of NTPαSs, (*R*p)-isomers of α-*P*-borano-NTPs and α-*P*-methyl-dNTPs as substrates. As a result, only (*R*p)-phosphorothioate, (*S*p)-boranophosphate, and (*S*p)-methylphosphonate linkages can be incorporated into oligonucleotides. Surprisingly, both (*R*p)- and (*S*p)-α-*P*-seleno-TTPs (TTPαSes) are used as substrates by the Klenow fragment of *E. coli* DNA polymerase I, and both (*R*p)- and (*S*p)-phosphoroselenoate linkages can be incorporated into oligonucleotides using this system.[24] (It has been reported that the recognition of rATPαSe by T7 RNA polymerase was stereospecific, and only one isomer of rATPαSe was incorporated into oligoribonucleotides.[25]) (2) Modification sites cannot be freely selected

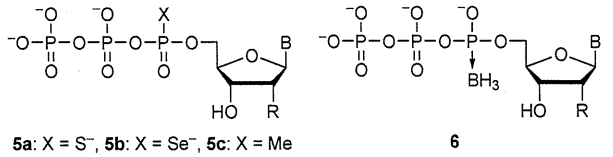


Fig. 3 α-*P*-Modified nucleoside 5′-triphosphates for incorporation of nucleoside phosphorothioate (**5a**), phosphoroselenoate (**5b**), methylphosphonate (**5c**) and boranophosphate (**6**).

because altered residues are universally incorporated, for example, the use of α-*P*-modified TTP results in complete replacement of thymidine 5′-phosphates throughout the oligomer. (3) Because of the substrate recognition specificity of the enzymes, only the above four types of *P*-chiral nucleotides can be incorporated at the current time. Base and sugar modifications are also limited. (4) The product is covalently linked to the primer. (5) The method is not suitable for large-scale syntheses. (6) Undesired byproducts, such as oversized transcripts, are concomitantly produced.[47,48]

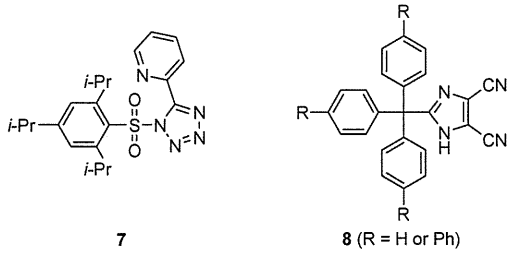
5. Stereocontrolled chemical synthesis of *P*-chiral oligonucleotide analogs

As described above, *P*-chiral oligonucleotide analogs which can be prepared by the aforementioned methods are limited. For this reason, the stereocontrolled chemical synthesis of *P*-chiral oligonucleotide analogs has been extensively studied over the past few decades, and a variety of stereoselective and stereospecific reactions have been developed. This review classifies these studies into four categories as outlined below.

5.1. Stereoselective synthesis of *P*-chiral oligonucleotide analogs by utilizing the chirality of ribose

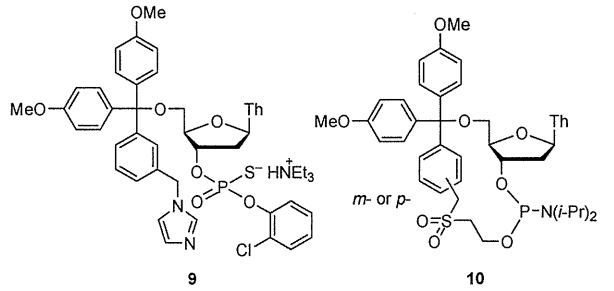
The chirality of ribose and deoxyribose moieties affects the stereoselectivity of the synthesis of *P*-chiral internucleotidic phosphate analogs. For instance, it has been reported that the *Rp* to *Sp* ratios of dinucleoside phosphorothioates are not exactly 50 : 50, but range from *Rp* : *Sp* = 63 : 37 to 43 : 57[38] even when they are synthesized with a "nonstereoselective" conventional phosphoramidite method.[49] This is due to the chirality of deoxyribose. Although this "stereoselectivity" is far from sufficient to synthesize stereoregulated *P*-chiral oligonucleotide analogs, a number of studies designed to enhance this selectivity have been reported to date. Since several reviews[16–18] have covered this topic, here we highlight only representative examples.

Ohtsuka *et al.* developed a 2,4,6-triisopropylbenzenesulfonyl tetrazolide derivative (Fig. 4, **7**) incorporating a 5-(2-pyridyl)tetrazole in place of the typical 1*H*-tetrazole as a nucleophilic catalyst. This condensing agent was used to synthesize dinucleoside phosphate derivatives *via* the phosphotriester method and only the (*Sp*)-isomers were obtained with complete stereoselectivity.[50] The 2-pyridyl group may stabilize the transition states yielding the (*Sp*)-diastereomers. Engels *et al.* designed new 2-trityl-4,5-dicyanoimidazole derivatives (**8**) and



7 **8** (R = H or Ph)

Fig. 4 New condensing agent **7** and azoles **8** used for stereoselective synthesis of *P*-chiral dinucleoside phosphate analogs.

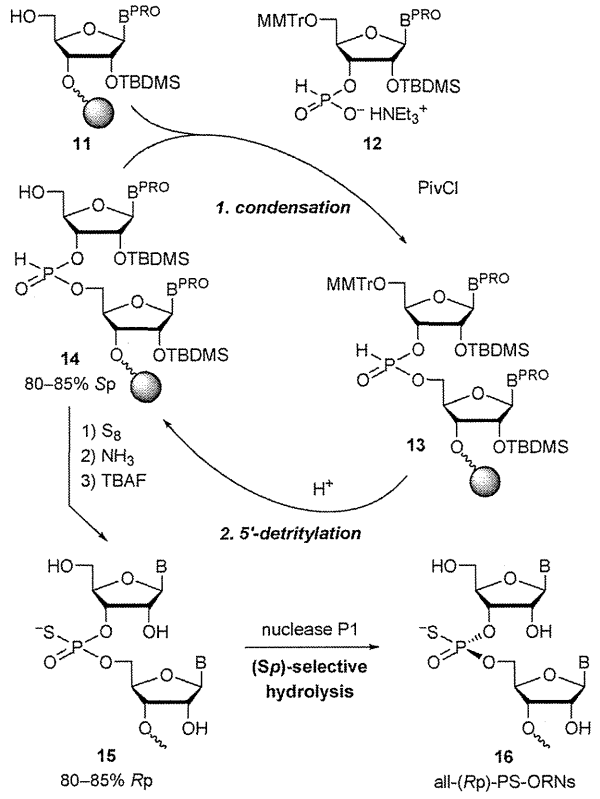


9 **10**

Fig. 5 Monomer units having new trityl group (**9**, **10**) used for stereoselective synthesis of dinucleoside phosphorothioates.

used these to activate 2′-deoxyribonucleoside 3′-methylphosphonamidite monomers. The desired dinucleoside methylphosphonates were obtained stereoselectively (from *Rp* : *Sp* = 89 : 11 to 76 : 24) probably resulting from dynamic kinetic resolution of the methylphosphonazolide intermediates.[51] Sekine *et al.* developed new trityl groups that have an imidazolyl group (Fig. 5, **9**)[52] or a linker to the phosphorus atom (**10**).[53] Compound **9** was used in the phosphotriester method to give the corresponding dithymidine phosphorothioate derivative stereoselectively (*Rp* : *Sp* = 18 : 82). The authors confirmed that the imidazolyl moiety was involved in the diastereoselection process (probably *via* an intramolecular nucleophilic attack on the phosphorus atom) by replacing the imidazolyl–dimethoxytrityl group with a traditional dimethoxytrityl group, resulting in an almost complete loss of stereoselectivity. Compound **10** was used to synthesize dithymidine phosphorothioate *via* the phosphoramidite method. The linker was readily removed from the product by treatment with ammonia. The stereoselectivity of the product varied from 50 : 50 to 86 : 14 depending on the structure of the linker and the activator used. The monomer having a standard dimethoxytrityl group showed little stereoselectivity. The authors attributed the stereoselectivity to the dynamic kinetic resolution of the phosphorazolide intermediate. Steric hindrance around the phosphorus atom of **10** probably hampers one of the two reaction paths.

It has been reported that the stereoselectivity of the synthesis of oligonucleoside *H*-phosphonates can be significantly enhanced by the existence of a bulky substituent at the 2′-position.[54,55] This enhancement of stereoselectivity enables a stereoselective synthesis of *P*-chiral oligoribonucleotide analogs, although the same strategy is not applicable to 2′-deoxyribonucleotide analogs. For example, Strömberg *et al.* developed a method to synthesize diastereopure all-(*Rp*)-oligoribonucleoside phosphorothioates (PS-ORNs) *via* the *H*-phosphonate method (Scheme 2).[55] The condensation of 2′-*O*-(*tert*-butyldimethylsilyl)-5′-*O*-(4-monomethoxytrityl)-ribonucleoside 3′-*H*-phosphonate monomers **12** with the 5′-OH of a nucleoside on a solid support **11** and the subsequent 5′-detritylation step give protected oligoribonucleoside *H*-phosphonate **14** in a stereoselective manner (80%–85% *Sp*). Because the sulfurization of *H*-phosphonate diesters proceeds with retention of configuration (according to the Cahn–Ingold–Prelog rules, P–S$^-$ has the highest priority among the four substituents around the phosphorus atom, while P–H has the lowest priority), crude PS-ORN obtained from **14** is enriched with (*Rp*)-PS-linkages (**15**). Finally, crude **15** is treated with nuclease P1, which exclusively



Scheme 2 Synthesis of all-(*R*p)-PS-ORNs by the *H*-phosphonate method.

hydrolyzes (*S*p)-PS-diester linkages, to digest all the oligomers containing one or more (*S*p)-PS-linkages so that the desired all-(*R*p)-PS-ORN **16** is easily purified by HPLC.

Thus, the chirality of ribose sometimes produces a highly asymmetric environment. However, it is difficult to further enhance the stereoselectivity shown in these examples because the methodology offers little flexibility in designing chiral sources. More importantly, only one of the two *P*-isomers is available through these methods because of the homochirality of ribose.

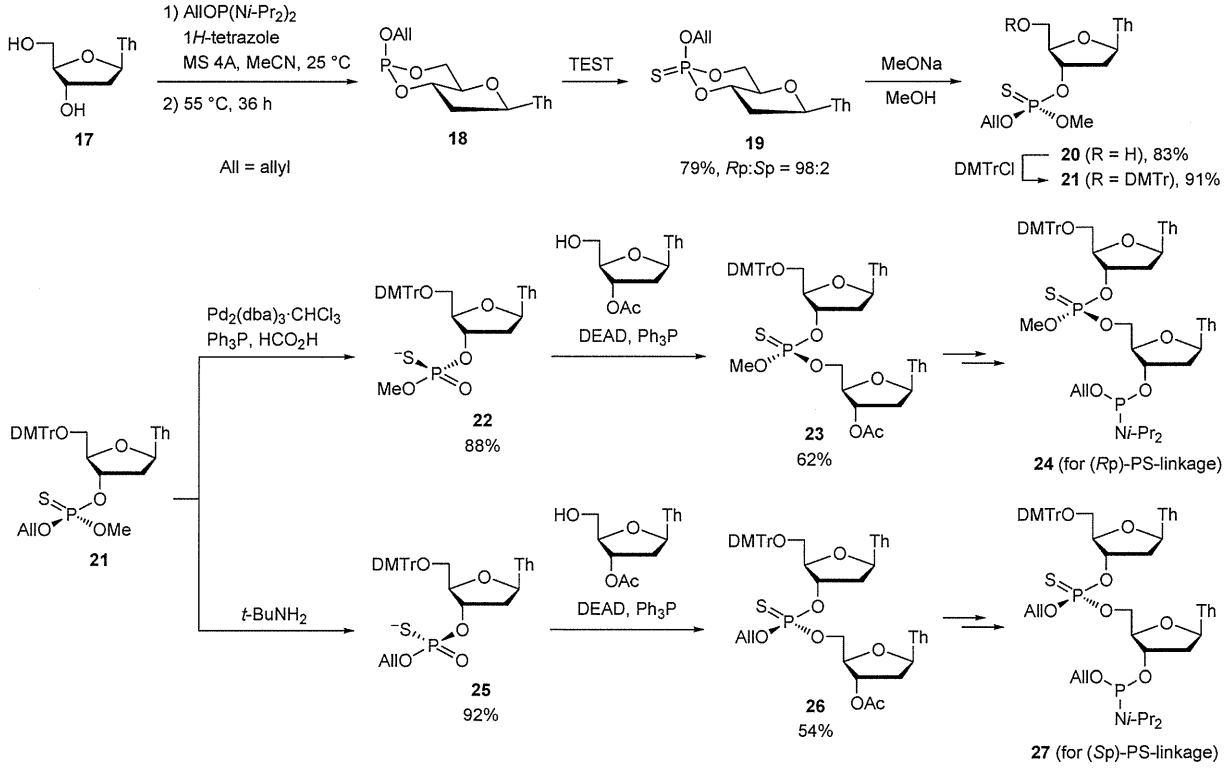
Recently, Hayakawa *et al.* reported a method to solve these problems, in which they successfully transferred the chirality of 2-deoxy-D-ribose to both (*R*p)- and (*S*p)-phosphorothioate internucleotide linkages (Scheme 3).[56] First, they synthesized thymidine 3′,5′-cyclic phosphite derivative **18** from thymidine **17** stereoselectively. The more thermodynamically stable isomer having the allyloxy group at the axial position was preferentially generated upon heating the reaction mixture. Subsequent sulfurization with bis[3-(triethoxysilyl)propyl] tetrasulfide (TEST) afforded the 3′,5′-cyclic phosphorothioate derivative **19** (*R*p : *S*p = 98 : 2). Treatment of **19** with MeONa preferentially cleaved the P–O-5′ bond to give the thymidine 3′-phosphorothioate triester derivative **20**. A small amount of the 5′-phosphorothioate counterpart was also generated because of P–O-3′ bond cleavage (*ca.* 10%), which was removed by chromatography. 5′-*O*-Dimethoxytritylation of **20** gave **21**. This compound functioned as a common intermediate to synthesize both (*R*p)- and (*S*p)-PS-internucleotidic linkages owing to the orthogonality of the allyl and methyl protecting groups. Thus, the allyl and methyl groups were selectively removed by using Pd(0) and *t*-BuNH_2, respectively, in a stereoretentive manner. Subsequent Mitsunobu reactions with 3′-*O*-acetyl-thymidine afforded the dithymidine phosphorothioate triesters **23** and **26**, which were converted into the corresponding 3′-phosphoramidite dimer building blocks **24** and **27** and used to incorporate (*R*p)- and (*S*p)-PS-linkages into ODNs, respectively. This method is advantageous compared to the other methods described in this section in that both (*R*p)- and (*S*p)-PS-linkages can be synthesized by using 2-deoxy-D-ribose as the sole chiral source. However, because the P–S^- groups of **22** and **25** also work as a nucleophile in the Mitsunobu reaction, the yields of the desired products **23** and **26** are relatively low, and thus it is currently not practical to use this reaction for the chain elongation of PS-ODNs. Therefore, this method has the same limitations as those of the other methods using dimer building blocks.

5.2. Use of chiral activators

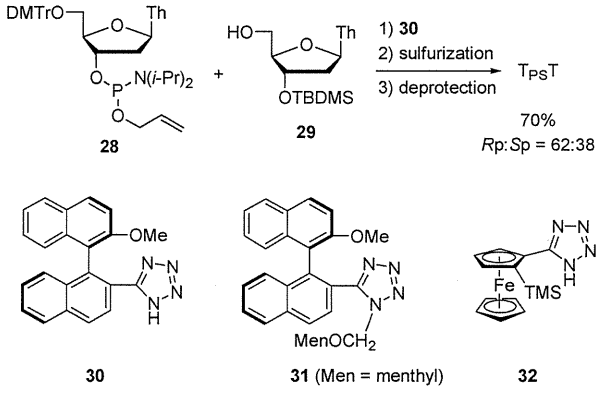
In order to develop a more efficient and versatile method that can equally incorporate both (*R*p)- and (*S*p)-*P*-chiral internucleotidic phosphate analogs at any position of oligonucleotides, stereoselective or stereospecific syntheses using chiral sources other than the ribose moieties have been studied. The general strategy described in this section uses nucleophilic acids with a chiral moiety to activate diastereomeric mixtures of monomers having a P^{III} atom. From the viewpoint of synthetic organic chemistry, this may be one of the best strategies if accomplished because diastereomixtures of monomer units can be easily prepared and the chiral components are not covalently linked to the substrates. Hence, additional steps for their introduction and removal are unnecessary. However, there have been only a few reports using this strategy, and all of them resulted in the synthesis of dinucleoside phosphate analogs with low to modest diastereoselectivity.[57,58] For example, Hayakawa *et al.* synthesized tetrazole derivatives having a chiral moiety (Scheme 4, **30–32**) and applied them to the synthesis of dithymidine phosphorothioate, but the level of diastereoselectivity was not sufficient.[57] Schell and Engels also synthesized a camphor-derived tetrazole derivative and used it to synthesize dinucleoside methylphosphonates, but the diastereomer ratio (dr) of the product only reached 64 : 36.[58] Thus, it is still difficult to induce high stereoselectivity with this strategy. It should be noted that this difficulty is not limited to the case of nucleotide analogs. In general, a highly stereoselective synthesis of *P*-chiral phosphite triesters has not yet been achieved without using a chiral auxiliary that is covalently bonded to the starting materials.[59] It is also worth noting that the effects of the chirality of ribose on stereoselectivity further complicate the design process in the case of nucleotide analogs.

5.3. Diastereomeric separation of monomer units and their application to the synthesis of stereoregulated *P*-chiral oligonucleotide analogs

As described in the previous section, there are continuing difficulties related to the development of an efficient method to synthesize stereoregulated *P*-chiral oligonucleotide analogs



Scheme 3 Synthesis of (*R*p)- and (*S*p)-phosphorothioate dimer building blocks (**24**, **27**) *via* common intermediate **21**.

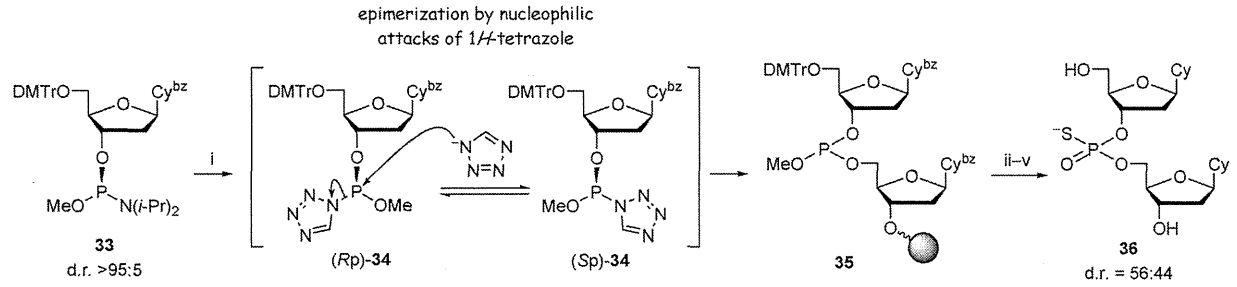


Scheme 4 Synthesis of dithymidine phosphorothioate with optically pure tetrazole derivatives **30–32** as activators.

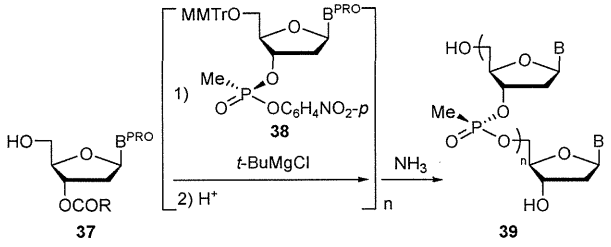
with a stereoselective reaction. In contrast, some reliable methods using stereospecific reactions have been developed in which diastereopure monomers are prepared, and individual isomers are synthetically incorporated into an oligonucleotide containing the corresponding *P*-chiral internucleotidic phosphate analogs without loss of diastereopurity. The diastereopure monomers are prepared by either chromatographic separation of diastereomixtures or stereoselective synthesis. This section highlights examples of the former approach.

As in the case of *P*-chiral dinucleoside phosphate analogs and their oligomers, nucleoside analogs bearing a chiral phosphorus atom have (*R*p)- and (*S*p)-diastereomers, and they can theoretically be separated by chromatography. In general, diastereomers have similar chromatographic retention times, and their separation requires a relatively long elution time. Therefore, it is necessary to use monomers with sufficient stability on stationary phase materials (typically silica gel). For this reason, monomers having a P^V atom have been employed in most cases as described below. Conventional nucleoside 3′-phosphoramidite monomers with a P^{III} atom can also be separated into *P*-diastereomers. However, it has been reported that the synthesis of a dinucleoside phosphorothioate using a separated phosphoramidite *P*-diastereomer in the presence of 1*H*-tetrazole was accompanied by nearly complete loss of *P*-diastereopurity.[60] This was attributed to repetitive attacks of 1*H*-tetrazole at the chiral phosphorus atom (Scheme 5).

In contrast, several types of P^V monomers that undergo a base-promoted stereospecific reaction have been developed. For example, nucleoside 3′-methylphosphonate derivatives having a *p*-nitrophenoxy group (Scheme 6, **38**) have been introduced by Stec *et al.* as monomer units for stereocontrolled synthesis of oligodeoxyribonucleoside methylphosphonates (PMe-ODNs) **39**.[19,61,62] Monomers **38** are synthesized nonstereoselectively and separated into (*R*p)- and (*S*p)-diastereomers by silica gel column chromatography. A *p*-nitrophenoxy group is displaced by a nucleoside that has been activated by *t*-BuMgCl to yield a methylphosphonate diester linkage stereospecifically. This method was used to synthesize all-(*R*p)- and (*S*p)-PMe-ODN 2–5mers in a solution phase. This method, which employs a *p*-nitrophenoxy group and *t*-BuMgCl,[63] was also used to synthesize short PS-ODN[64] and ORN[65] oligomers. Later, the same research group developed new monomers having a SMe[66] or SeMe[67] group instead of the *p*-nitrophenoxy group to synthesize PMe-ODN 5mers in a solution phase. The improved reactivity of these monomers



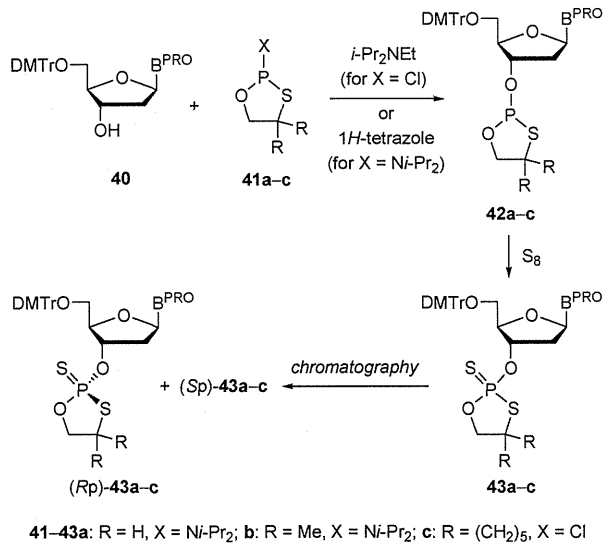
Scheme 5 Solid-phase synthesis of d[C_{PS}C] using diastereopure nucleoside 3′-phosphoramidite monomer and 1*H*-tetrazole. Reagents: (i) support-bound N^4-benzoyl-2′-deoxycytidine, 1*H*-tetrazole; (ii) S_8, 2,6-lutidine; (iii) 3% $CHCl_2CO_2H$ in CH_2Cl_2; (iv) PhSH, Et_3N; (v) NH_3 aq.



Scheme 6 Stereospecific synthesis of oligodeoxyribonucleoside methylphosphonates **39** using *t*-BuMgCl.

enabled the use of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) with LiCl in place of *t*-BuMgCl to promote the reaction. This reaction is completely stereospecific, but the application of this method is still limited to solution-phase syntheses, and only the synthesis of 2mers has been achieved on a solid support.[68] Further investigation of solid-phase synthesis is necessary to synthesize longer oligomers.

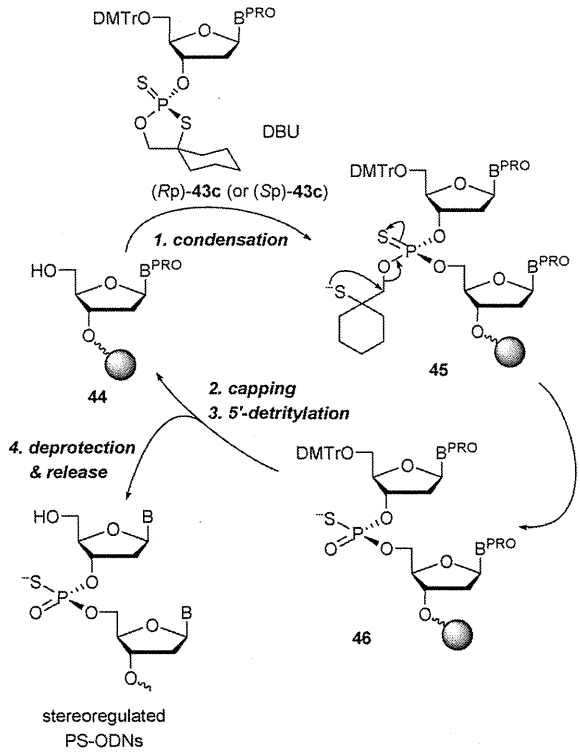
Stec *et al.* have also developed a method to synthesize *P*-chiral oligonucleotide analogs by using a DBU-promoted stereospecific reaction of nucleoside 3′-*O*-oxathiaphospholane monomers. (For more detailed reviews on the oxathiaphospholane method, see ref. 17, 69 and 70 by Stec *et al.*) Scheme 7 shows the synthesis of oxathiaphospholane monomers **43** used



Scheme 7 Synthesis of 2′-deoxyribonucleoside 3′-*O*-oxathiaphospholane monomers **43**.

for the preparation of PS-ODNs from appropriately protected nucleosides **40** and 2-chloro- or 2-diisopropylamino-1,3,2-oxathiaphospholane derivatives **41a–c**. Nonstereoselectively synthesized monomers **43a–c** were separated into (*R*p)- and (*S*p)-isomers by silica gel column chromatography. The 4,4-pentamethylene group of the oxathiaphospholane ring of **43c** was introduced to enhance the asymmetry of the chiral phosphorus atom, resulting in easier chromatographic separation of diastereomers.[71] In fact, *ca.* 70% recovery of diastereopure (*R*p)- and (*S*p)-**43c** was achieved by a single passage through a silica gel column, which was a significant improvement compared to the separation of **43a,b**.[72–74]

Chain elongation of a PS-ODN is carried out by the nucleophilic substitution reaction of diastereopure oxathiaphospholane monomers **43a–c** with the 5′-OH of a support-bound nucleoside or an oligonucleotide **44** in the presence of a strong base (DBU, Scheme 8). The cleavage of the P–S bond by nucleophilic substitution and the subsequent elimination of an



Scheme 8 Synthetic cycle for stereoregulated PS-ODNs by the oxathiaphospholane method.

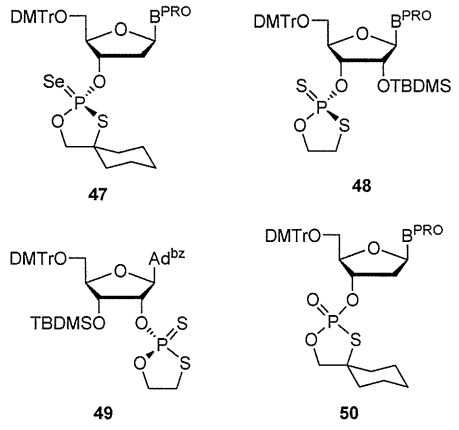


Fig. 6 Nucleoside oxathiaphospholane monomers for stereospecific introduction of 2′-deoxyribonucleoside phosphoroselenoate (**47**), ribonucleoside phosphorothioate (**48**), adenosine (2′–5′) phosphorothioate (**49**) and 2′-deoxyribonucleoside phosphate (**50**).

episulfide yielded the extended PS-ODN **46** stereospecifically. It has been confirmed that the reaction proceeds with retention of configuration. The chain elongation cycle consists of nucleophilic substitution, capping of unreacted 5′-OH by Ac_2O, and 5′-detritylation by dichloroacetic acid in CH_2Cl_2. The efficiency per cycle was typically 92%–96%. Deprotection and cleavage from the solid support can be carried out by standard treatment with ammonia. Synthesis of up to 28mer PS-ODNs was achieved.[71]

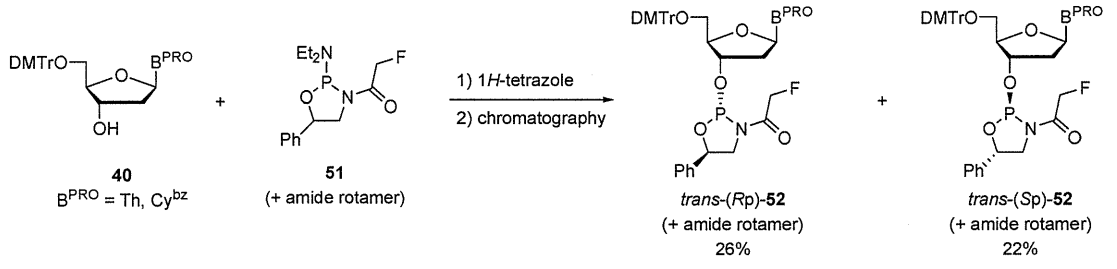
The oxathiaphospholane method is also applied to the synthesis of other *P*-chiral oligonucleotide analogs. The monomers used for these syntheses are shown in Fig. 6. These monomers can be used for stereospecific chain elongation of the corresponding *P*-chiral oligonucleotides through the same synthetic cycle. Stereoregulated oligodeoxyribonucleoside phosphoroselenoates (PSe-ODNs),[75] PS-ORNs,[76] and oligoadenosine (2′–5′) phosphorothioates[77] are synthesized by using **47**, **48**, and **49**, respectively. Monomer **50** has been developed to incorporate achiral phosphate diester linkages into PS-ODNs[71] so that stereoregulated chimeric PS/PO-ODNs can be synthesized by the same cycle. This is advantageous because the oxathiaphospholane method is not compatible with either the phosphoramidite or the *H*-phosphonate method generally used for incorporating these achiral phosphate monomers. The incompatibility is due to the sensitivity of phosphorothioate diester linkages of PS-ODN intermediates (*e.g.* **46** in Scheme 8) toward the oxidizing reagent (I_2–H_2O) used in these two methods.[71]

Thus, the oxathiaphospholane method can produce relatively long stereoregular *P*-chiral oligonucleotide analogs, particularly, PS-ODNs. The resultant oligomers have been used in many studies to identify the effects of the configuration of the phosphorus atoms on their physicochemical and biological properties.[71–84] However, the method has the following disadvantages: (1) chromatographic separation of diastereomixtures of monomers (*ca.* 1 : 1) into individual diastereopure monomers is required. (2) The efficiency of the DBU-promoted condensation is relatively low. As a result, in particular, the synthesis of PS-ORNs by using monomers **48** bearing a bulky 2′-*O*-TBDMS group is limited to 2mers.[76] (3) The method is not compatible with the most widely used phosphoramidite method because the two methods use completely different types of monomers and reactions. The sensitivity of the phosphorothioate diester linkages toward oxidizing reagents is another factor contributing to the incompatibility, as described above.

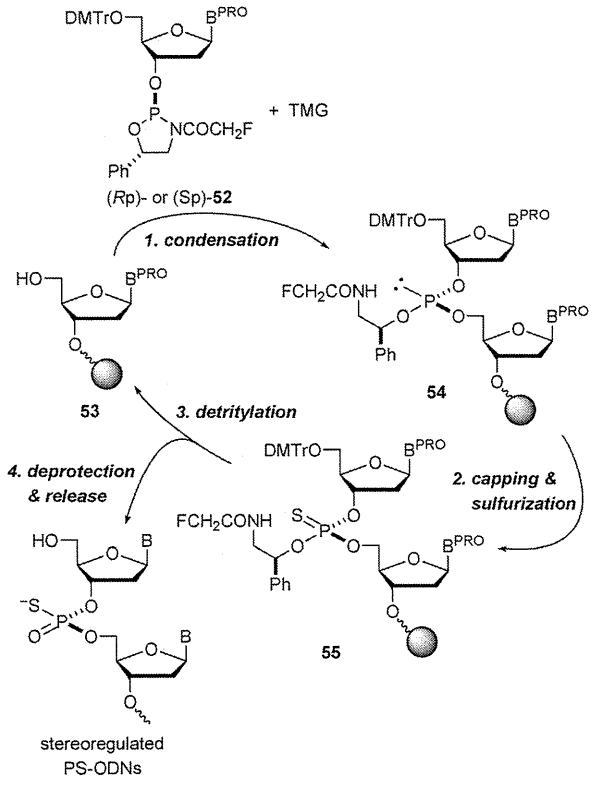
On the other hand, Beaucage *et al.* have reported the synthesis of stereoregulated PS-ODNs using 2′-deoxyribonucleoside 3′-*O*-(3-*N*-acyl-1,3,2-oxazaphospholidine) derivatives **52** as monomers, which bear a chiral P^{III} atom.[85] Monomers **52** were synthesized as shown in Scheme 9 from the appropriately protected nucleosides **40** and the 2-(diethylamino)-3-*N*-acyloxazaphospholidine derivative **51**, which was prepared from (±)-2-amino-1-phenylethanol. Diastereopure *trans*-(*Rp*)- and *trans*-(*Sp*)-**52**, in which the nucleoside moiety and the phenyl group are oriented on opposite sides of the oxazaphospholidine ring, were isolated from the resultant diastereomixtures by silica gel column chromatography. The isolated yields were relatively low because monomers **52** were unstable on silica gel and partially decomposed during purification.

Monomers **52** undergo a stereospecific condensation with the 5′-OH of a support-bound nucleoside or an oligonucleotide **53** in the presence of a strong base 1,1,3,3-tetramethylguanidine. The synthetic cycle for stereoregulated PS-ODNs using this stereospecific condensation is shown in Scheme 10. The resulting phosphite intermediate **54** is sulfurized with 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Beaucage reagent)[86] after capping the unreacted 5′-OH by Ac_2O to give the phosphorothioate triester intermediate **55**. The average coupling yield per cycle estimated by the $DMTr^+$ assay was *ca.* 98%. Deprotection of the phosphorothioate linkages can be carried out together with base deprotection and cleavage from the solid support by treatment with ammonia without affecting diastereopurity. All-(*Rp*)-$[T_{PS}]_{11}T$ and (*Rp*,*Sp*,*Rp*)-$d[C_{PS}C_{PS}C_{PS}C]$ were synthesized using this cycle.

It is worth noting that monomers **52** can be used to synthesize PS/PO-chimeric oligonucleotides by switching the sulfurization/oxidation (with *t*-BuOOH) steps. This is advantageous compared to the oxathiaphospholane method,



Scheme 9 Synthesis of diastereopure 2′-deoxyribonucleoside 3′-*O*-(3-*N*-acyl-1,3,2-oxazaphospholidine) monomers **52**.



Scheme 10 Synthetic cycle for stereoregulated PS-ODNs using *N*-acyl-oxazaphospholidine monomers **52**.

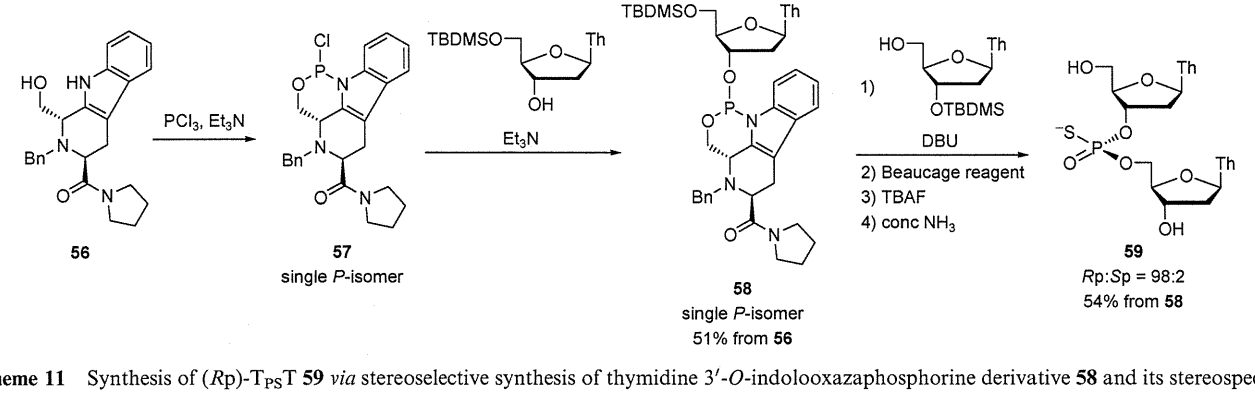
which requires switching monomer sets to synthesize PS/PO-chimeric oligonucleotides, as described above. This method may also be compatible with the phosphoramidite method because the intermediates do not have phosphorothioate diesters, which are sensitive to oxidizing agents, although two sets of monomers and activators would be necessary.

5.4. Stereoselective synthesis of monomer units and their application to the synthesis of stereoregulated *P*-chiral oligonucleotide analogs

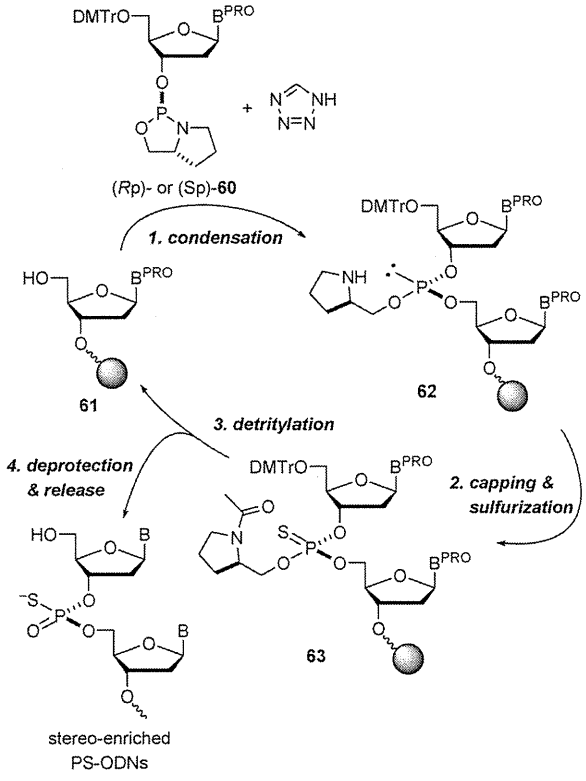
The methods using a base-promoted condensation of diastereopure monomers give the desired *P*-chiral oligonucleotides with complete stereospecificity as described in the previous section. However, the separation of monomers from diastereomixtures (*ca.* 1 : 1) by chromatography is troublesome. The reactive P^{III} monomers suffer from partial decomposition during the chromatographic resolution, whereas the efficiency of condensation reactions of the more stable P^{V} monomers is relatively low, although they can be separated without decomposition. To overcome this problem, various studies targeting the development of monomers that can be stereoselectively synthesized have been conducted.

Just *et al.* have reported several types of 2′-deoxyribonucleoside 3′-*O*-indolooxazaphosphorine monomers that can be stereoselectively synthesized using chiral auxiliaries and applied them to the synthesis of PS-ODN 2mers.[87,88] For example, they synthesized an optically pure amino alcohol (Scheme 11, **56**) from N_b-(benzyloxycarbonyl)-tryptophan and used it as a chiral auxiliary in the synthesis of a thymidine 3′-*O*-indolooxazaphosphorine derivative **58**.[88] Only one of the two possible *P*-isomers of **58** was obtained stereoselectively. (Configuration of the phosphorus atom was not assigned.) Compound **58** was then allowed to react with 3′-*O*-TBDMS-thymidine in the presence of DBU. The reaction was almost completely stereospecific, and after subsequent sulfurization and deprotection, (*R*p)-dithymidine phosphorothioate (**59**) (*R*p : *S*p = 98 : 2) was obtained. Although this monomer and the other indolooxazaphosphorine derivatives have not been applied to the synthesis of oligonucleotides longer than 2mers, these studies have clearly demonstrated that such monomers, which can be stereoselectively synthesized by using chiral auxiliaries, can be employed for the synthesis of internucleotidic chiral phosphate analogs *via* a base-promoted stereospecific reaction.

On the other hand, Agrawal *et al.* have reported the stereoselective synthesis of nucleoside 3′-*O*-oxazaphospholidine derivatives and their applications as monomer units for oligonucleoside phosphorothioates (Scheme 12).[89–92] Monomers **60** were synthesized by the reaction of the corresponding protected nucleosides bearing a 3′-OH with the prolinol-derived 2-chloro-1,3,2-oxazaphospholidine.[90,92] Only the *trans* isomers were obtained stereoselectively from both L- and D-prolinols. Unlike the *N*-acyl-oxazaphospholidine monomers **52** shown in Scheme 10 and the indolooxazaphosphorine monomer **58** shown in Scheme 11, the condensation reactions of the oxazaphospholidine monomers **60**, which were activated by 1*H*-tetrazole like the widely used phosphoramidite monomers, were not stereospecific. When eight dinucleoside



Scheme 11 Synthesis of (*R*p)-$T_{PS}T$ **59** *via* stereoselective synthesis of thymidine 3′-*O*-indolooxazaphosphorine derivative **58** and its stereospecific condensation promoted by DBU.



Scheme 12 Synthetic cycle for stereo-enriched PS-ODNs using prolinol-derived oxazaphospholidine monomers **60**.

phosphorothioates (*Rp*)- and (*Sp*)-d[N_{PS}T] (N = A, T, G, C) were synthesized by using this cycle, it was observed that diastereoselectivity varied from *Rp* : *Sp* = 10 : 90 to 14 : 86 for (*Sp*)-d[N_{PS}T] and from *Rp* : *Sp* = 93 : 7 to 91 : 9 for (*Rp*)-d[N_{PS}T].[90,92] The partial loss of diastereopurity can be attributed to the repetitive nucleophilic attacks of 1*H*-tetrazole at the chiral phosphorus atom of **60** leading to its epimerization, similar to the case of the phosphoramidite monomer (Scheme 5). The subsequent condensation reactions *via* both of the resulting two *P*-isomers of **60** and/or its tetrazolide active intermediate give rise to the diastereomixtures of the products. Although stereoselectivity is not very high, the method can produce relatively long stereoenriched PS-ODN oligomers on an automated DNA synthesizer by using the same protocol as that of the conventional phosphoramidite method. All-(*Rp*)- and (*Sp*)-PS-ODN 18–20mers as well as those containing both (*Rp*)- and (*Sp*)-PS-linkages were synthesized in *ca.* 98% average yield per cycle (estimated by the $DMTr^+$ assay).[92]

The stereoselectivity of this method is dependent on the structure of the monomers, activators, and reaction conditions. For example, Hayakawa *et al.* have reported that the stereoselectivity of the reactions between **60** and a nucleoside in a solution phase varied from *Rp* : *Sp* = 86 : 14 to >99 : 1 with D-prolinol-derived monomers and from *Rp* : *Sp* = 29 : 71 to 1 : 99 with the L-prolinol-derived counterparts depending on the activator.[93] Best results were obtained by using *N*-phenylimidazolium triflate (PhIMT)[94] or benzimidazolium triflate (BIT).[95] (Stereoselectivity was slightly lower on a solid support (up to 94 : 6).) Agrawal *et al.* have reported that the ephedrine-derived monomer **64** (Fig. 7) gave T_{PS}T with little stereoselectivity

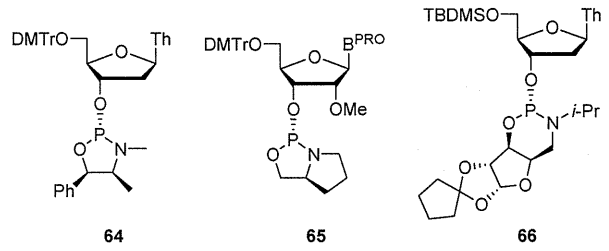


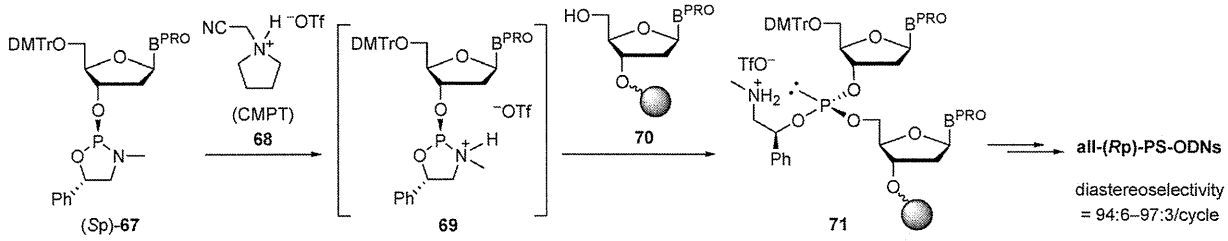
Fig. 7 Nucleoside 3′-*O*-oxazaphospholidine (**64**, **65**) and 3′-*O*-oxazaphosphorinane (**66**) monomers for stereoselective synthesis of dinucleoside phosphorothioates.

(*Rp* : *Sp* = 40 : 60) under conditions similar to those used for **60**.[89] The L-prolinol-derived 2′-OMe-ribonucleoside 3′-*O*-oxazaphospholidine monomers **65** gave (*Sp*)-2′-OMe-diribonucleoside phosphorothioates with diastereoselectivity ranging from *Rp* : *Sp* = 2 : 98 to 4 : 96, whereas the D-prolinol-derived counterpart gave (*Rp*)-2mers with significantly lower stereoselectivity (from *Rp* : *Sp* = 76 : 24 to 63 : 37).[91] This indicates that the biased effects of the ribonucleoside chirality on stereoselectivity can be an important factor in the synthesis of *P*-chiral oligonucleotides with a stereoselective reaction. Just *et al.* synthesized a series of thymidine 3′-*O*-oxazaphosphorinane derivatives (*e.g.* **66**) with complete stereoselectivity from 2-chloro-oxazaphosphorinanes and used them to synthesize T_{PS}T under various reaction conditions.[96,97] They found that T_{PS}T was obtained with excellent diastereoselectivity at low temperatures (*Rp* : *Sp* = 1 : 40 at 0 °C; 1 : 68 at −15 °C, 2-bromo-4,5-dicyanoimidazole, $CHCl_3$), while a significantly lower diastereoselectivity was observed at rt (*Rp* : *Sp* = 1 : 6, 2-bromo-4,5-dicyanoimidazole, MeCN).[97]

Thus, the diastereomeric resolution of monomers by chromatography can be circumvented by using monomers that can be stereoselectively synthesized. Among these, the monomers that can be activated by weak acids[89–93,96,97] are particularly attractive because they are fully compatible with the phosphoramidite method, and the synthesis of oligomers can be performed with a commercial DNA synthesizer using this method. However, the loss of diastereopurity of the monomers during the acid-promoted condensations is problematic. As shown above, it is difficult to develop a stereospecific azole-promoted condensation reaction applicable to both ribonucleotide and 2′-deoxyribonucleotide analogs. Although diastereoselectivity can be improved by lowering the reaction temperature,[96,97] this approach is not suitable for syntheses on an automated synthesizer.

Considering this background, our group initiated a project to develop a method consisting of a stereoselective synthesis of monomers, which could be used to generate stereoregulated *P*-chiral oligonucleotide analogs under stereospecific, acid-promoted reaction conditions.[98–103] Ideally, the method should be fully compatible with the phosphoramidite method and applicable to a solid-phase synthesis on an automated DNA synthesizer. Such a method would greatly facilitate the synthesis of stereoregulated *P*-chiral oligonucleotides and encourage their use.

It is reasonable to predict that the condensation of diastereopure nucleoside 3′-*O*-oxazaphospholidine derivatives would proceed stereospecifically if they are activated only by



Scheme 13 Stereocontrolled synthesis of PS-ODNs using less nucleophilic activator **68**.

N-protonation of the oxazaphospholidine ring without using a nucleophilic activator. Although it was shown that weaker nucleophilic activators were not effective with phosphoramidite derivatives,[104,105] it was found that less nucleophilic *N*-(cyanomethyl)dialkylammonium salts efficiently activated nucleoside 3′-*O*-oxazaphospholidine monomers.[98,99] Stereoregulated PS-ODNs were synthesized by using one of these activators, *N*-(cyanomethyl)pyrrolidinium triflate (CMPT, **68**), on a solid support (Scheme 13).[99] 2′-Deoxyribonucleoside 3′-*O*-oxazaphospholidine monomers (**67**) were generated from the corresponding 2-chlorooxazaphospholidine derivative and protected nucleosides having a 3′-OH with dr of 93 : 7–96 : 4 (*trans* : *cis*) and the *trans* isomers were isolated by silica gel column chromatography in 62%–75% yields. It was expected that CMPT **68** would activate the P–N bond of monomers **67** only by *N*-protonation, and their subsequent condensation with the 5′-OH of nucleosides or oligonucleotides **70** would proceed without loss of diastereopurity. However, the HPLC analysis of the resultant PS-ODNs showed that the average diastereoselectivity per cycle was *ca.* 94 : 6–97 : 3, although the major products were generated with inversion of configuration, as expected.

Because it was found that the partial loss of diastereopurity was due to epimerization of the monomers **67** by the acidity of **68**,[99] the configurational stability of oxazaphospholidine monomers was investigated.[101] Four types of nucleoside 3′-*O*-oxazaphospholidine monomers, shown in Fig. 8 (**60**, **67**, **72**, and **73**), were synthesized with dr ranging from 95 : 5 to >99 : 1 (*trans* : *cis*) and treated with 2 equiv. of CMPT **68** in CH_3CN–CD_3CN (4 : 1, v/v) at rt. After 4 h, ^{31}P NMR analysis showed that the *trans* : *cis* ratio of monomer **67** changed from 95 : 5 to 58 : 42. In sharp contrast, the other bicyclic oxazaphospholidine derivatives showed significantly improved stability to epimerization. In particular, monomer **72** showed only negligible inversion. In addition, the *trans* isomer of **72** was exclusively generated from the corresponding 2-chlorooxazaphospholidine and the protected nucleoside. Therefore, diastereomeric resolution by chromatography was not necessary.

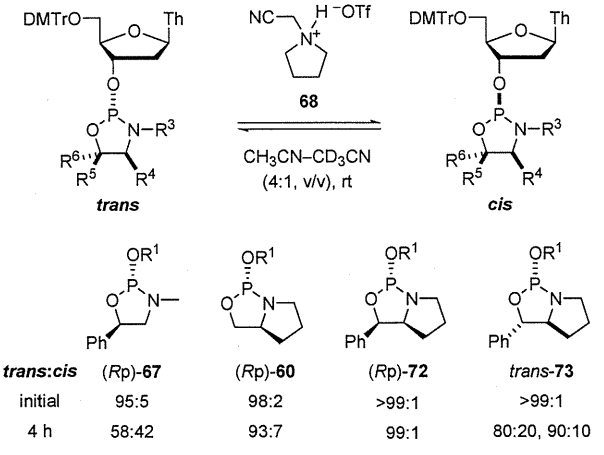
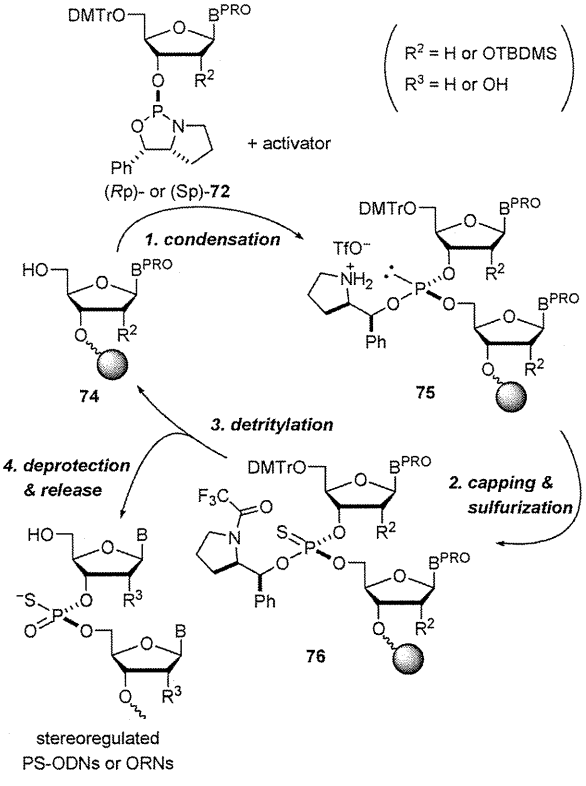


Fig. 8 Epimerization of *trans*-nucleoside 3′-*O*-oxazaphospholidine monomers **67**, **60**, **72** and **73** in the presence of CMPT **68**. R^1 = 5′-*O*-DMTr-thymidin-3′-yl. *trans*-**73** is a mixture of (2*S*,4*R*,5*R*)- and (2*R*,4*S*,5*S*)-isomers. dr of (2*S*,4*R*,5*R*)-**73** and (2*R*,4*S*,5*S*)-**73** were 80 : 20 and 90 : 10, respectively, after treatment with **68**.

Monomer **72** was then applied to the synthesis of stereoregulated PS-ODNs on a DNA synthesizer.[101] The eight types of monomers **72** ((*R*p)- and (*S*p)-isomers for each of the four nucleobases) were synthesized in 44%–58% yields. The synthetic cycle (Scheme 14) is very similar to that shown in Scheme 12 as well as to that of the conventional phosphoramidite method except for the reagents. Monomers **72** and CMPT **68** are used for the condensation step. *N*-Trifluoroacetylimidazole (CF_3COIm) and *N*,*N*′-dimethylthiuram disulfide (DTD)[106]

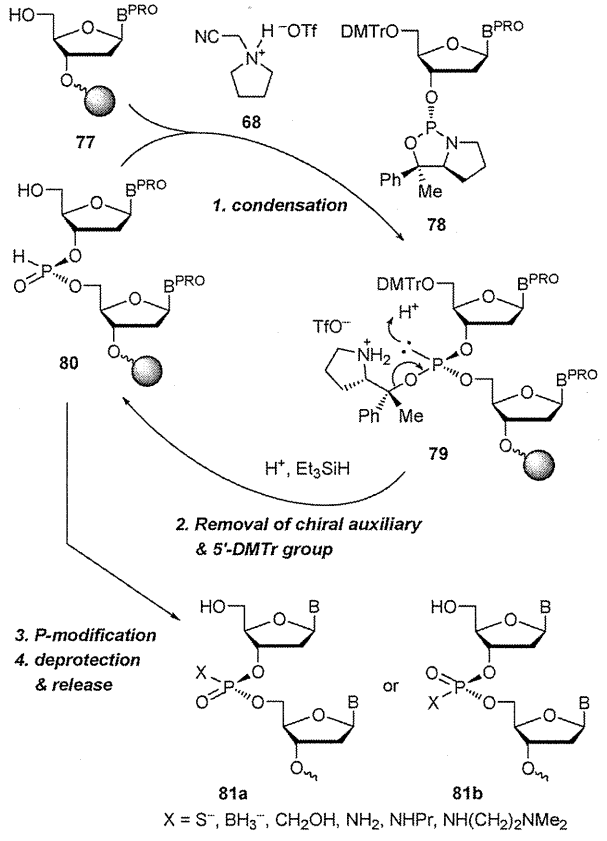


Scheme 14 Synthetic cycle for stereoregulated PS-ODNs and ORNs by the oxazaphospholidine method.

are used for the capping and sulfurization steps. Trifluoroacetylation was used for capping instead of acetylation because the *N*-acetylated pyrrolidinylmethyl moiety on the PS-linkages was too stable for complete deprotection by ammonia. The average coupling yields and diastereoselectivity for the synthesis of 8–12mers were 95%–99% and $\geq$ 99%, respectively. Thus, stereospecific condensation reactions were achieved by the use of the configurationally stable bicyclic oxazaphospholidine monomers **72**.

Next, the oxazaphospholidine method was applied to the synthesis of stereoregulated PS-ORNs.[102] The *trans* isomers of **72** bearing a 2′-*O*-TBDMS group were stereoselectively synthesized (*trans* : *cis* >99 : 1, 41%–75% isolated yields) and applied to the same synthetic cycle. However, it was found that the average coupling yields for the synthesis of 4mers were significantly lower (67%–94%) than those observed with the 2′-deoxyribonucleoside monomers. The lower reactivity was attributed to steric hindrance by the 2′-*O*-TBDMS group. To our surprise, this problem was overcome by using highly nucleophilic azole activators. The use of PhIMT or BIT greatly improved the efficiency of the condensation reactions, and all-(*R*p)- and (*S*p)-[U_{PS}]$_9$U were synthesized in 97%–99% average coupling yields ($DMTr^+$ assay). The diastereoselectivity of the couplings was comparable or nearly comparable to that obtained by using CMPT (96 : 4 to >99 : 1). The mechanism of the reaction promoted by these azoles is not clear. It may proceed *via* *N*-protonation of the oxazaphospholidine ring as in the case of CMPT, or the dynamic kinetic resolution of the diastereomixture of azolide intermediates may occur. However, the success could be attributed to the configurational stability of the bicyclic oxazaphospholidine monomers **72** because condensation of the monocyclic oxazaphospholidine monomer **67** resulted in low diastereoselectivity when nucleophilic 1*H*-tetrazole was used as an activator.[98]

It has also been demonstrated that the oxazaphospholidine method can be used to synthesize other *P*-chiral oligonucleotide analogs. As shown in Scheme 15, the synthesis uses the bicyclic oxazaphospholidine monomers **78** bearing an additional methyl group at the 5-position of the oxazaphospholidine ring in place of the monomers **72**.[103] The *trans* isomers of **78** have been stereoselectively synthesized (dr > 99 : 1) and isolated in 43%–83% yields. The condensation reactions of **78** promoted by CMPT **68** give diastereopure phosphite intermediates **79**. Treatment of **79** with an acidic solution causes an E1 reaction, generating the 5′-detritylated *H*-phosphonate diester intermediates **80** and a tertiary carbocation stabilized by the phenyl group. The acidic solution contains Et_3SiH as a carbocation scavenger. Thus, this two-step cycle produces oligodeoxyribonucleoside *H*-phosphonates stereospecifically. To the best of our knowledge, this is the first example of a stereocontrolled synthesis of *H*-phosphonate diesters. Because *H*-phosphonate diesters can be converted into a variety of *P*-modified phosphate analogs,[26] this method can greatly expand the availability of stereoregulated *P*-chiral oligonucleotides. In fact, dinucleoside boranophosphates, hydroxymethylphosphonates, and several types of phosphoramidates were synthesized with dr ranging from 98 : 2 to >99 : 1. Oligodeoxyribonucleoside boranophosphates and phosphoramidate 4mers were also synthesized in addition to PS-ODN



Scheme 15 Synthetic cycle for stereoregulated *P*-chiral oligonucleotide analogs **81** *via* oligonucleoside *H*-phosphonates **80** by the oxazaphospholidine method.

10mers, although the conversion of the *H*-phosphonates into boranophosphates and phosphoramidates was less efficient compared to the case of PS-ODNs.[103]

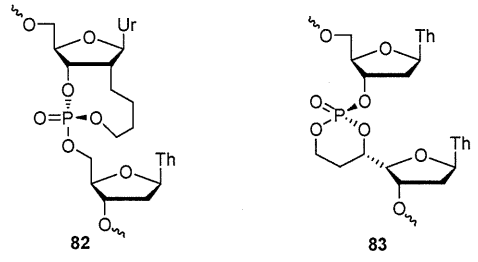
Thus, the oxazaphospholidine method that uses configurationally stable bicyclic oxazaphospholidine monomers and CMPT (or azoles) can produce various *P*-chiral oligonucleotide analogs in a stereospecific manner. Because this method is compatible with the phosphoramidite method, it is expected to be applicable to the stereocontrolled synthesis of chimeric *P*-chiral oligonucleotide analogs as well as oligonucleotides with a fully-modified backbone by varying oxazaphospholidine/phosphoramidite monomers, CMPT/azole activators, and/or *P*-modification/oxidation steps. However, the method still has some problems: (1) because the oxazaphospholidine monomers are more sensitive to acids than the corresponding phosphoramidites, their isolation yields by chromatography are modest, although diastereomeric resolution is not necessary. (2) The efficiency of the condensation reactions needs improvement for the synthesis of long oligomers. In the case of ORN derivatives, the combinational use of CMPT and a less-bulky protecting group for 2′-OH than the conventional TBDMS group can be studied. (3) The efficiency of conversion of *H*-phosphonate diester linkages into *P*-modified phosphate analogs, such as boranophosphates, on a solid support must be improved. (4) Protocols for the synthesis of oligonucleotides with a chimeric backbone must be studied. A study is currently underway to address these problems.

6. Applications of stereoregulated *P*-chiral oligonucleotide analogs

The *P*-chiral oligonucleotide analogs have been primarily used for therapeutic applications and for studies of enzymatic reactions. For the latter purposes, those having a single *P*-modification at a specific site have been used in most cases. Because these oligonucleotides are available by HPLC separation or by using dimer building blocks, many studies have employed them to elucidate the interactions of specific pro-*R*p or *S*p oxygens with metal ions or amino acid residues in enzymatic reactions.[10,21] However, many internucleotidic phosphates are involved in the recognition of an oligonucleotide by an enzyme,[21,107] and thus, many oligonucleotides with a modified phosphate at specific sites are required to study such a recognition process in detail. Because it is laborious to prepare many oligonucleotides by HPLC separation or by using dimer building blocks, the development of a method that can readily produce such oligonucleotide analogs in a stereocontrolled manner is required.

In contrast, oligonucleotides with multiple modifications or a fully-modified backbone are used for therapeutic applications because such modifications are necessary to enhance their stability to nucleases. Many studies on their physicochemical and biological properties, such as the stability of duplexes with complementary oligonucleotides, cellular uptake, stability to nucleases, nonspecific interactions with proteins, and the ability to suppress gene expression, have been performed using oligonucleotides prepared by stereocontrolled chemical syntheses, HPLC separation, the dimer building block method, or enzymatic synthesis, and these studies have shown that their properties were affected by the configuration of the phosphorus atoms. For example, in the case of PS-ODN, (*R*p)-PS-linkages are favored for duplex formation with complementary ORN strands,[73] while (*S*p)-PS-linkages are chosen to enhance the stability to nucleases.[78] Therefore, PS-ODNs in which both (*R*p)- and (*S*p)-PS-linkages are properly arranged would be better drug candidates than those synthesized in a nonstereocontrolled manner. However, more research is required prior to any clinical trials. For this purpose, it is necessary to expand the availability of stereoregulated *P*-chiral oligonucleotide analogs—particularly, the most widely used PS-ODNs—by developing more efficient methods for their stereocontrolled synthesis. Moreover, new methods may lead to the identification of novel types of *P*-modifications suitable for therapeutic applications. Currently, only a few types of *P*-chiral oligonucleotide analogs, such as PS-ODNs, are used for therapeutic studies, partly because the introduction of a large substituent into an internucleotidic phosphate of an oligonucleotide in a nonstereocontrolled manner significantly destabilizes its duplex with a complementary oligonucleotide.[108,109] Because the destabilization is reduced when the substituent is oriented outward from the duplex, the variety of *P*-chiral oligonucleotide analogs potentially suitable for therapeutic applications may increase.

Stereoregulated *P*-chiral oligonucleotides have rarely been used beyond the two objectives highlighted above. But the limited studies carried out to date have demonstrated their potential. For example, oligonucleotides having a stereodefined PS-linkage can be used to synthesize their conjugates with other functional molecules, such as reporter molecules, through S-alkylation.[10,110,111] Stereodefined phosphoramidate linkages (PN-linkages) have also been used to make such conjugates.[112,113] Duplex formation of these conjugates with complementary oligonucleotides is not severely hampered by using cross links through stereodefined (*S*p)-PS- or (*R*p)-PN-linkages because the substituents are oriented outward,[110] although it has also been reported that the introduction of an intercalator through an (*R*p)-PS-linkage stabilized the duplex.[111,113] A stereocontrolled synthesis is also advantageous because a more precise orientation of these functional molecules around duplexes is possible. Analyses of these diastereopure conjugates are also easier than their stereochemically-randomized counterparts. Moreover, it has been reported that duplex structures can be globally transformed by changing the absolute configuration of the phosphorus atoms. For instance, Endo and Majima have reported that double-stranded ODNs consisting of two self-complement ODN strands, which are cross linked to each other through stereodefined PN-linkages, form either double helices or hairpins depending on the absolute configuration of the PN-linkages.[114] They have also reported stereodependent behaviors of various DNA nanostructures consisting of oligonucleotides cross linked to each other through stereodefined PN-linkages.[115–117] In addition, significant stabilization of an ORN hairpin structure has been reported by introducing an (*R*p)-PS-linkage at a specific site in its loop region.[118] Such stabilization of oligonucleotide secondary structures has also been achieved with some conformationally restricted nucleotide analogs[119–123] in which one of the non-bridging oxygen atoms of a phosphodiester is covalently linked to the ribose (*e.g.*, **82**,[121] **83**[123] in Fig. 9). It has also been reported that left-handed Z-form duplexes can be stabilized by using ODNs with a specific arrangement of (*R*p)- and (*S*p)-PS-linkages.[82] Such stereodependent behaviors of *P*-chiral oligonucleotides and stabilization of specific oligonucleotide structures by using stereodefined *P*-modifications may be useful for DNA nanotechnology.[6–9] However, in many cases, the oligonucleotide analogs for such studies have been prepared by HPLC separation or the dimer building block method because the methods for stereocontrolled synthesis of *P*-chiral oligonucleotides, such as the oxathiaphospholane and oxazaphospholidine methods, are still too complicated for a typical end-user. The development of a more user-friendly method to synthesize stereoregulated *P*-chiral oligonucleotides or commercialization of the monomers or the oligonucleotides themselves should encourage their use and promote studies of DNA nanotechnology as well as those for the biological and therapeutic applications described above.



82 83

Fig. 9 Conformationally restricted *P*-chiral nucleotide analogs.

7. Conclusion

In conclusion, stereoregulated *P*-chiral oligonucleotide analogs have been used for biological and therapeutic studies. In addition, they are potentially useful for the synthesis of oligonucleotide conjugates and also for DNA nanotechnology. In order to promote studies on these applications, the development of a user-friendly method to produce these oligonucleotide analogs is required such that nonspecialists can perform the synthesis, preferably on an automated synthesizer. We expect that this goal is achievable either by further studies on the aforementioned methods or by the development of a novel, more efficient approach, such as a catalytic asymmetric synthesis of *P*-chiral phosphate analogs. New developments from other areas of chemistry, such as those from ongoing research on organophosphorus compounds or catalytic asymmetric synthesis, may be important to achieve this challenging goal.

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