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Stereochemistry of internucleotide bond formation by the *H*-phosphonate method. 7. Stereoselective formation of ribonucleoside (R_P)- and (S_P)-3′-*H*-phosphonothioate monoesters

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ABSTRACT

Ribonucleoside 3′-*H*-phosphonothioate monoesters exist in the form of (R_P)- and (S_P)-diastereomers. In order to obtain them in good yields and in high stereochemical purity, stereoselective strategies for their preparation were investigated. For the synthesis of the (R_P)-isomer, a stereoselective sulfhydrolysis of an activated nucleoside *H*-phosphonate was developed, while the monoesters with an (S_P)-configuration were prepared by asymmetric transformation of diastereomeric mixtures of nucleoside 3′-*H*-phosphonothioates using either a condensation with 9-fluorenemethanol, followed by β-elimination, or via pivaloylation-hydrolysis reaction sequence. A tentative assignment of the absolute configurations of the obtained diastereomers of 3′-*H*-phosphonothioate esters was carried out via a stereochemical correlation analysis.

1. Introduction

Ribonucleoside 3′-*H*-phosphonates react with nucleosides and other alcohols with high diastereoselectivity towards (S_P)-diastereomers (de >80%), which are substrates for stereospecific transformations to various P-chiral derivatives, for example (R_P)-phosphorothioate diesters.[1,2] This strategy has been successfully applied in the preparation of all-(R_P)-oligo(ribonucleotide phosphorothioate)s using a combination of chemical synthesis and post-synthetic enzymatic processing.[1] More recently, it was found that a plausible source of asymmetric induction in these reactions is the dynamic kinetic asymmetric transformation (DYKAT).[3] Stereocontrolled preparation of ribonucleoside phosphorothioate diesters with an opposite [i.e., (S_P)] sense of P-chirality may be achieved by using ribonucleoside 3′-*H*-phosphonothioate monoesters as starting materials, since their condensations with alcohols and nucleosides are stereoselective, while the main diastereomers formed, (S_P)-*H*-phosphonothioate diesters, could be oxidized stereospecifically to (S_P)-phosphorothioates.[4]

In contrast to nucleoside *H*-phosphonate monoesters, the corresponding *H*-phosphonothioates are chiral at the phosphorus centre and may be separated into individual P-epimers. Access to isolated (R_P)- and (S_P)-diastereomers of ribonucleoside *H*-phosphonothioates might be helpful in mechanistic and synthetic studies. According to the DYKAT mechanism (cf. Fig. 2), during the formation of *H*-phosphonothioate diesters from diastereomerically pure precursors, their P-epimerisation might be expected to occur at the level of the *H*-phosphonothionic—carboxylic mixed anhydride, and from the degree of stereospecificity, approximate rates of formation and collapse of reactive intermediates can be inferred. This would be helpful in mechanistic studies in the early stages of *H*-phosphonate diester formation, in order to obtain a better understanding of the underlying chemistry or to evaluate differences in the reactivity of the chalcogen atoms in (S_P)- and (R_P)-nucleoside *H*-phosphonothioates towards electrophiles.

In addition to the applications in basic studies, the availability of separate (R_P)- and (S_P)-diastereomers of nucleoside *H*-phosphonothioates could also be useful in the stereospecific synthesis of stereochemically pure hypermodified nucleoside derivatives, for example, dinucleoside[5,6] or alkyl nucleoside[7] phosphoramidothioates, currently only available present only as pools of diastereomers.

Herein we report our synthetic and mechanistic studies on the preparation of both P-diastereomers of ribonucleoside 3′-*H*-phosphonothioate monoesters, and the determination of their absolute configurations.

2. Results and discussion

In the deoxyribo series, the diastereomers of (5′-*O*-DMTr)thymidine 9-fluorenemethyl 3′-*H*-phosphonothioate[8,9] can be separated chromatographically as FAST [R_f 0.42,[‡] (S_P)] and SLOW [R_f 0.35,

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[‡] *n*-Hexane–dichloromethane–ethyl acetate 5:5:1.

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(R_P)] isomers, and subsequently converted via β-elimination to the corresponding nucleoside *H*-phosphonothioate monoesters[§] in a stereospecific manner.[10] Unfortunately, and somewhat unexpectedly, the corresponding diastereomers of 9-fluorenemethyl 5′-*O*-(DMTr)-2′-*O*-(TBDMSi)uridine 3′-*H*-phosphonothioate **8b** (Fig. 2) were chromatographically indistinguishable irrespective of the stationary and mobile phase used. We assumed that a possible reason for this could be the high lipophilicity of the compounds, due to the presence in three highly hydrophobic groups, namely dimethoxytrityl (DMTr), TBDMSi, and 9-fluorenemethyl units.

Indeed, in contrast to the deoxy series, the negatively charged diastereomers of (5′-*O*-DMTr-2′-*O*-TBDMSi)-uridine 3′-*H*-phosphonothioate monoester **5** had different TLC mobilities and could be resolved into individual species by silica-gel column chromatography using 0–30% gradient of acetonitrile in 95:5 toluene–TEA. Although this allowed a significant enrichment of each of the diastereomers (de 40–50%), the mixed fractions still contained substantial amounts of both diastereomers (ca. 50% of the recovered compound).

In order to improve the efficiency of this procedure, we took advantage of the stereoselective formation of each of the diastereomers of *H*-phosphonothioate **5** under different experimental conditions, and achieved a significant increase in the content of the desired isomer in the reaction mixture which simplified its chromatographic separation.

2.1. Synthesis of (R_P)-uridine 3′-*H*-phosphonothioate (5-FAST)

In previous papers on the preparation of nucleoside 3′-*H*-phosphonothioates it was noted that one of the diastereomers of uridine *H*-phosphonothioate was formed in significant excess during sulfhydrolysis of an activated uridine *H*-phosphonate; however, little attention was paid to stereochemistry of the reaction.[11–14] Two kinds of reactive intermediates were exploited for the syntheses, either aryl nucleoside *H*-phosphonate of type **3a** or nucleoside *H*-phosphonic–pivalic mixed anhydride **3b**. These were sulfhydrolysed using hexamethylenedisilathiane (HMDST), which was either a commercial product or was generated in situ in the reaction of H_2S with TMS-Cl. Herein we found that in all cases the main diastereomer formed was the one migrating faster on silica-gel (**5**-FAST, Fig. 1) and concluded that sulfhydrolysis of reactive *H*-phosphonate **3** may proceed via two pathways. In Path A the activated *H*-phosphonate **3** is presumably attacked by TMS_2S (HMDST) or TMS-SH, followed by rapid S→O silyl migration and formation of O-silylated compound **4**.[¶] In the alternative Path B, *H*-phosphonate **3** is silylated initially to a tervalent species of type **6** which is sulfhydrolysed to the silyl ester **4**. Path A is about twice as fast as Path B. Typically, ca. 30–50% of sulfurization of *H*-phosphonate **3** proceeded via the tervalent intermediate.

The stereochemical outcome of Path B was assessed by sulfhydrolysis (H_2S in dioxane)[16] of phosphite **6** (prepared in situ by silylation of active *H*-phosphonate **3** with *N*,*O*-bis(trimethylsilyl) acetamide (BSA) or TMS-Cl).[16] It was found that the distribution of diastereomers of *H*-phosphonothioate **4** formed did not depend on the diastereomeric ratio of the intermediate **6**, and that in the sulfhydrolysis of the phenyl derivative **6a**, the **4**-R_P/S_P ratio was ca. 2:1, while for the pivaloyl derivative **6b**, it was ca. 3:2. The underlying mechanism of the observed stereochemistry is currently under investigation.

Since the sulfhydrolysis of phosphites of type **6** (Path B) was found to be ca. 2 orders of magnitude slower than that of Path A, the **4**-R_P/S_P ratio during the initial stages of the process presumably reflected the stereochemistry of Path A. Accordingly, it was found that upon sulfhydrolysis of the derivatives **3a** and **3b**, the **4**-R_P/S_P ratio was ca. 3:1 when HMDST was used as a silylating/sulfhydrolytic agent and ca. 2:1, for the TMS-Cl/H_2S mixture. Sulfhydrolysis of **3a** and **3b** probably owes its stereoselectivity to the DYKAT mechanism,[3] and thus, the configuration of the main diastereomers of *H*-phosphonothioates **4** and **5** was tentatively assigned as (R_P).

Concluding the above experiments, in order to achieve highest stereoselectivity, HMDST should be used rather than the TMS-Cl/H_2S mixture. Both pathways (A and B, Fig. 1) yielded the (R_P)-diastereomer of *H*-phosphonothioate **4** as the main isomer, however, the Path B was less stereoselective [for R = Pv (pivolyl or timethyl acetyl)] and slower than the Path A and should be avoided during the sulfhydrolysis of *H*-phosphonates of type **3**.

Having established the above, two approaches were developed for the stereoselective synthesis of the FAST diastereomer of uridine *H*-phosphonothioate **5**. In the first one, 2′,5′-di-*O*-protected nucleoside **1** was reacted with diphenyl *H*-phosphonate in pyridine[17] to form uridine phenyl *H*-phosphonate **3a**, which was sulfhydrolysed[13] in situ with HMDST (2.5 equiv).[‖] Diester **3a** was consumed completely within ca. 1 h (^{31}P NMR) to yield the silylated *H*-phosphonothioate **4** (ca. 84%) and phenyl trimethylsilyl uridine phosphite **6a** (ca. 14%). To this mixture additional 2 equiv of HMDTS were added to prevent the formation of the *H*-phosphonodithioate derivative[11,13,14] and the reaction mixture was left overnight to complete the sulfhydrolysis of phosphite **6a**. After quenching with water, ^{31}P NMR showed the formation of uridine *H*-phosphonothioate monoester **5** in ca. 90% yield and a **5**-FAST/**5**-SLOW ratio of ca. 3:1.

In the second method for the preparation of **5**-FAST, uridine *H*-phosphonate **2** was converted into *H*-phosphonic–pivalic mixed anhydride **3b** in the reaction with pivaloyl chloride in acetonitrile containing 6 equiv of pyridine.[††] The subsequent reaction with HMDST (3 equiv) yielded the desired silylated *H*-phosphonothioate **4** accompanied by ca. 10% of the tervalent intermediate **6b**. Sulfhydrolysis of the latter compound was accelerated by the addition of 2,6-lutidinium hydrochloride[11] and the whole process was completed within 30 min (^{31}P NMR yield: 94%; **4**-FAST/**4**-SLOW 3:1). After work-up, the main product **5**-FAST was isolated chromatographically with de >80% and in a yield of ca. 60% (the remaining material was eluted as a diastereomeric mixture; total yield 86%).

2.2. Synthesis of (S_P)-uridine 3′-*H*-phosphonothioate (5-SLOW)

In contrast to the uridine **5**-FAST diastereomer, there was no method available for the stereocontrolled formation of its SLOW counterpart as a major product. During these studies however, we found two straightforward routes for asymmetric transformations favouring the formation of this diastereomer (Fig. 2).

In the first method, uridine *H*-phosphonothioate **5** (R_P/S_P ca. 1:1) was reacted with 9-fluorenemethanol using pivaloyl chloride as a condensing agent (the reagent of choice for stereoselective condensations of ribonucleoside 3′-*H*-phosphonates[18]). The reaction was performed in acetonitrile containing 3 equiv of pyridine. Under these mild conditions the side reactions, which were observed previously,[19] were eliminated and the *H*-phosphonothioate diester **8b** was formed almost quantitatively, as a ca. 9:1 mixture of

[§] R_f 0.15 for both diastereomers; toluene–MeCN–TEA 3:2:1.

[¶] The ^{31}P chemical shift observed for **4** (ca. 57 ppm) was located in the region of *H*-phosphonothioates with a double P=S bond, while for a O=P–S-TMS isomer, a δ_P of ca. 15 ppm would be expected.[15]

[‖] It is essential to use HMDTS from a freshly opened bottle. Upon multiple opening and storage for several weeks (even at −25 °C), HMDTS undergoes significant decomposition and must be used in uncontrolled excess to prevent *H*-phosphonodithioate formation.

[††] The use of a polar solvent in sulfhydrolysis was critical for the yield of reaction. The best results were obtained for acetonitrile, in methylene chloride some by-products were formed, while in toluene nucleoside trimethylsilyl *H*-phosphonate and nucleoside *H*-dithiophosphonate were the main products of the process.

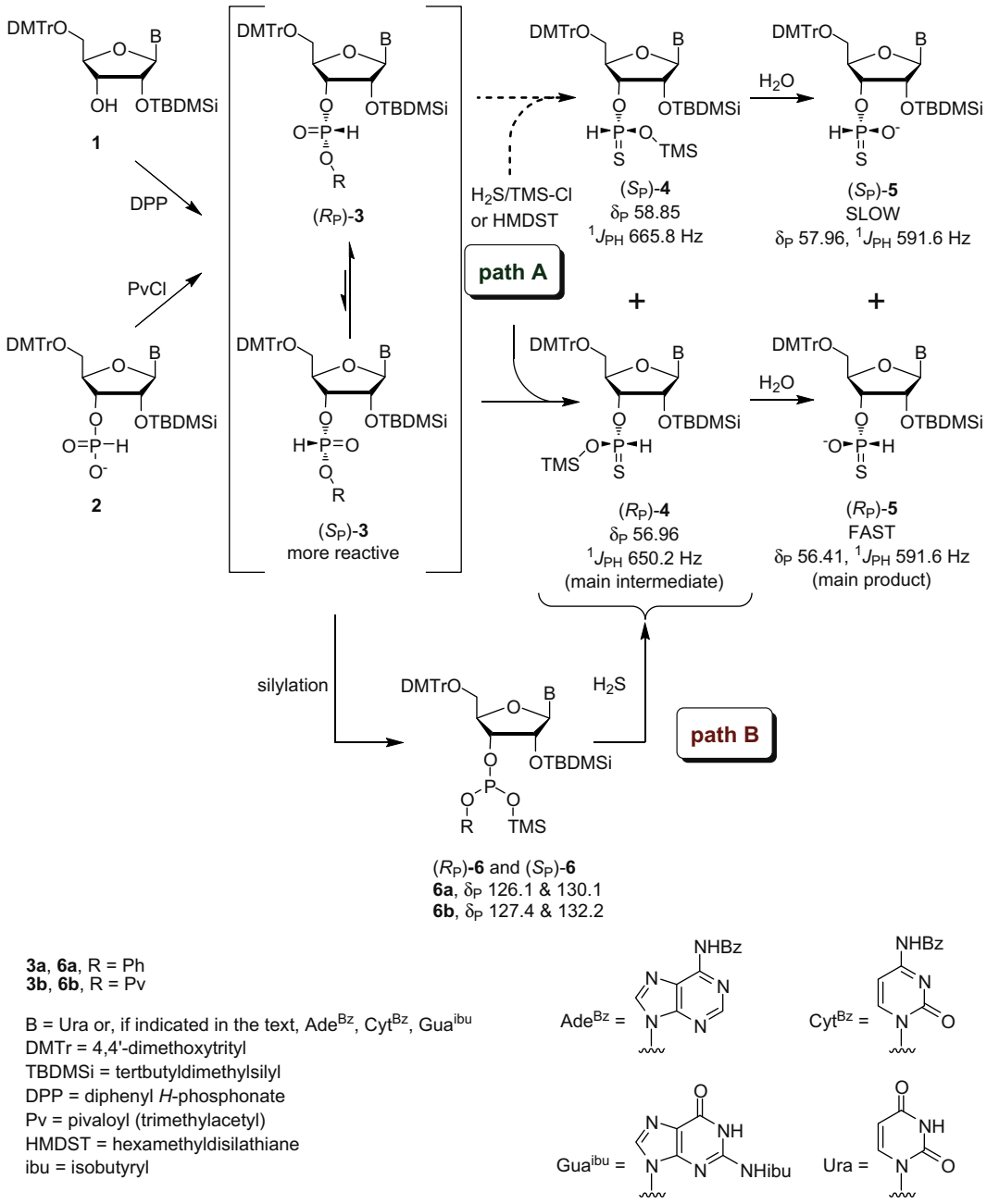
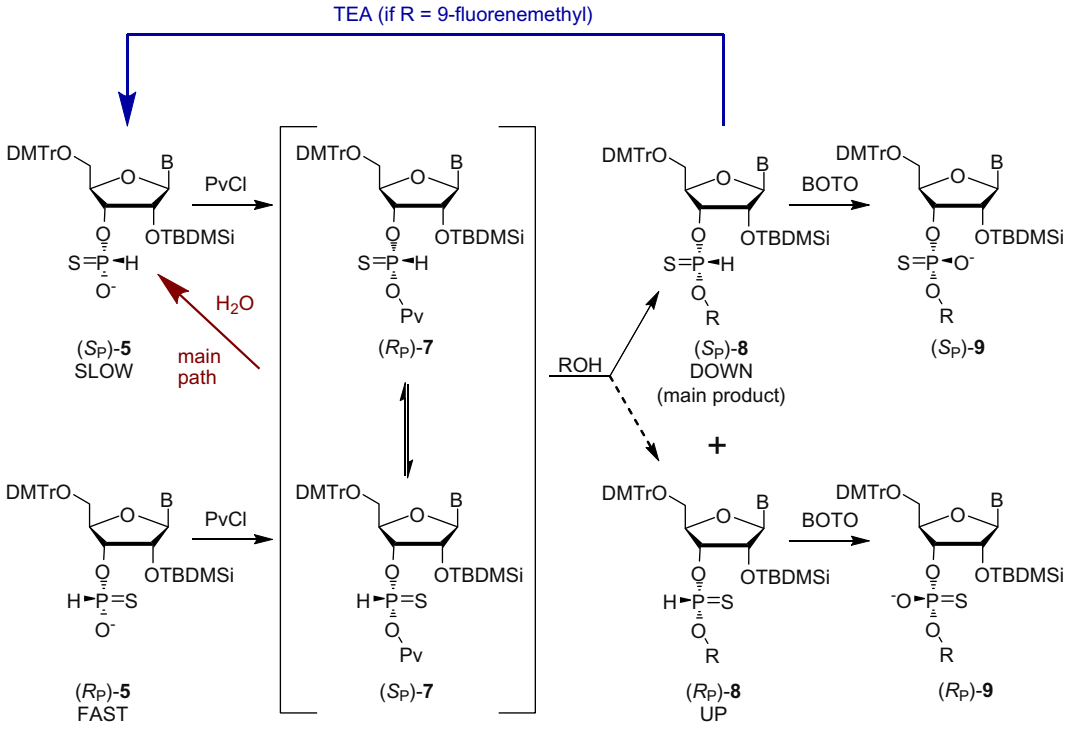


Figure 1. The assumed mechanism of stereoselective formation of uridine *H*-phosphonothioate (R_P)-**5** in the course of sulfhydrolysis of intermediate uridine phenyl *H*-phosphonate **3b**. The ^{31}P NMR data are given for uridine derivatives in the crude reaction mixtures.

diastereomers [de 83%; δ_P 72.24 ppm (the main, downfield resonating diastereomer, **8b**-DOWN) and 71.17 ppm (**8b**-UP), respectively]. The ratio of the diastereomers of *H*-phosphonothioate diesters **8** did not depend on diastereomeric composition of the starting *H*-phosphonothioate monoester **5**, confirming that the reaction was stereoselective rather than stereospecific. Upon the addition of TEA (20% v/v) a rapid β-elimination[10] of the fluorenemethyl moiety occurred, producing *H*-phosphonothioate **5** (yield 96%, ^{31}P NMR) in an unaltered 9:1 ratio [de 82%; δ_P 55.05 (**5**-SLOW) and 53.43 (**5**-FAST)]. This method is effective and requires only a brief pre-drying of the starting *H*-phosphonothioate monoesters and maintaining anhydrous conditions of the reaction.

The second approach to generate uridine derivatives **5**-SLOW as a major product was based on the observation that hydrolysis of the mixed anhydride **7** was a stereoselective reaction affording **5**-SLOW as a dominant product. Thus, *H*-phosphonothioate **5** in MeCN containing 3 equiv of pyridine was treated with PvCl (1.2 equiv) to generate the mixed anhydride **7**, which was then hydrolyzed back to monoester **5** by the addition of water to the reaction mixture. During the reaction no overactivation[19] of *H*-phosphonothioates occurred while a predominant formation of the SLOW diastereomer was observed (ca. 65:35, irrespective of a diastereomeric composition of the starting compound). These results seemed promising and prompted us to search for reaction conditions to gain higher stereoselectivity and yield.

To this end, the acylation-hydrolysis procedure was carried out in the presence of amines of different basicity and nucleophilicity (Fig. 3). The mixed anhydride **7** was either pre-formed in situ



B = Ura or, if indicated in the text, Ade^{Bz}, Cyt^{Bz}, Gua^{ibu}
Pv = pivaloyl
R = Et (**8a, 9a**), 9-fluorenemethyl (**8b, 9b**), nucleoside-5'-yl (**8c, 9c**)

Figure 2. Stereochemistry of reactions of nucleoside *H*-phosphonothioate derivatives.

and subsequently hydrolysed ('preactivation' procedure; Fig. 3, lines 3 and 4),[‡‡] or PvCl was added to a solution of substrate **5** in aqueous acetonitrile ('aqueous activation' procedure; lines 1, 2 and 5). In all cases, with an increasing amount of PvCl added, SLOW:FAST ratio of **5** tended towards an equilibrium value of ca. 75:25 (lines 1, 3 and 5). Also some desulfurization occurred and its extent was proportional to the quantity of PvCl used (lines 2 and 4).

Preactivation of uridine *H*-phosphonothioate **5** with PvCl apparently favoured the predominant formation of one diastereomer of the mixed anhydride **7**[§§] which upon hydrolysis afforded **5**-SLOW, and thus the amount of the condensing agent required for reaching the endpoint of asymmetric transformation was lower (ca. 2 equiv, Fig. 3A, line 3) than in the 'aqueous activation' procedures. The procedure, unfortunately, had two main drawbacks: the relatively high degree of desulfurization (line 4) and a significant detritylation, particularly in the reaction mixtures containing pyridine as a base (ca. 30% for 4 equiv of PvCl and 10 equiv of pyridine; ^{31}P NMR and TLC). For these reasons, this approach was not pursued further.

For the 'aqueous activation' procedure, in which the condensing agent was added to the aqueous MeCN solution of **5,** a larger excess of PvCl was necessary for the completion of the process when highly enriched **5**-FAST (de 84%) was used as the starting material. When **5**-SLOW (de 84%) was used as a substrate, the same endpoint of de 30% was reached after the addition of 5–6 equiv of PvCl (Fig. 3A, line 5).

This procedure was particularly efficient in the presence of pyridine (line 1), for which desulfurization was also at the lowest level (line 2). With more basic pyridine derivatives, higher levels of desulfurization were observed, while less basic derivatives were less efficient in the asymmetric transformation (data not shown). The non-nucleophilic tertiary amines appeared to be unsuitable for this purposes due to the desulfurization of **5** and inefficient formation of **5**-SLOW.[¶¶] On the other hand, in the presence of *N*-methyllimidazole (NMI) both desulfurization and interconversion of diastereomers proceeded reluctantly, indicating that this strong nucleophilic catalyst favoured the hydrolysis of PvCl over the acylation of *H*-phosphonothioate monoester **5**.

When diphenyl chlorophosphate (DPCP; a reagent of choice for standard nucleoside 3′-*H*-phosphonothioate condensations[19]) was used as the activating agent under similar reaction conditions (MeCN/pyridine/H_2O), only a marginal conversion of **5**-FAST to **5**-SLOW was observed due to the rapid hydrolysis of DPCP. Diethylchlorophosphate (DECP), which is less susceptible to hydrolysis, was able to promote the asymmetric transformation of *H*-phosphonothioate **5** nearly as efficiently as PvCl and without any desulfurization. The optimal pyridine content for this reaction was established to be ca. 20%. These prevented detritylation of **4** and kept hydrolysis of DECP at a low level.

Since this reaction was clean and the by-products formed could be easily monitored (^{31}P NMR) and removed during the work-up

[‡‡] Generation of the mixed anhydride **7** was carried out in the presence of catalytic amounts (0.5 equiv) of weakly basic *N*,*N*-dimethylaniline (DMA). In the presence of stronger bases, for example, TEA,, no signals of the mixed anhydride 7 could be detected in the 31P NMR spectra due to its rapid P-acylation (not observed in the oxo series under similar conditions).[19]

[§§] The mixed anhydride **2** was always formed in ca. 3:1 ratio of diastereomers (^{31}P NMR), irrespective of the diastereomeric composition of the starting monoester, presumably due to their rapid equilibration under the reaction conditions.

[¶¶] The 'aqueous activation' procedure in the presence of DMA can be exploited for a rapid and efficient desulfurization of *H*-phosphonothioate monoesters, an alternative to commonly used oxidative desulfurization procedures (see the Experimental part).

and silica-gel chromatography, it may be recommended as a second best method for the preparation of uridine *H*-phosphonothioate monoester **5**-SLOW. Thus, when *H*-phosphonothioate **5** (FAST:SLOW ≈ 2:1) was dissolved in 1% (v/v) aqueous acetonitrile containing pyridine (20% v/v), and treated with DECP (6 equiv), the diastereomeric ratio of *H*-phosphonothioate monoester **5** recovered changed to FAST:SLOW ≈ 1:4. A typical work-up of such reaction mixture and chromatographic separation afforded **5**-SLOW in de 84%. It is noteworthy that the process was very rapid and did not require prior preparation of monoester **5**. However, the diastereomeric excess of **5**-SLOW was lower than that obtained by the 9-fluorenemethyl method.

Several mechanistic interpretations of the observed stereoselectivity during the 'aqueous activation' of uridine *H*-phosphonothioate **5** are possible. In the simplest scenario, the major diastereomer of **5** (**5**-SLOW) was formed from the major diastereomer of the mixed anhydride **7** and the reaction followed a *dynamic thermodynamic resolution* (DYTR)[20,21] type of asymmetric induction. The reaction required several molar equivalents of PvCl to reach the endpoint, apparently due to the competitive reactions of the condensing agent with water or pivalic acid (formed during hydrolysis of PvCl and/or **7**).[22] Another possible mechanism would involve the formation, P-epimerisation, and hydrolysis of the mixed anhydride **7** (cf. Fig. 1), according to a *cyclic* de-*racemization* type of asymmetric induction.[21] Some other mechanisms seem to be less probable (e.g., the diverse reactivity of diastereomers of *H*-phosphonothioate **5** towards PvCl). While the available data cannot delineate which mechanism is actually operating, similar ratios of diastereomers of the intermediate mixed anhydride **7** (74:26) and the monoester **5** formed (77:23) point to the DYTR mechanism. However, when a more sterically demanding dimethoxytrityl group was present in the 2′-*O* position instead of the TBDMSi group, a SLOW/FAST ratio of ca. 9:1 could be achieved via the same procedure.[||||] This result cannot be explained in terms of the DYTR and points to the cyclic de-racemization process in this case.

The above methods can also be applied to achieve a comparable diastereomeric enrichment of other fully protected ribonucleoside *H*-phosphonothioate monoesters **5** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu}; Figs. 1 and 2). The ratios of diastereomers obtained and their chromatographic and ^{31}P NMR data are collected in Table 1. The lowest stereoselectivity in the asymmetric transformations was observed for the cytidine derivative, similarly as it was found in the oxo series.[1,18,23] Diastereomers of all the *H*-phosphonothioate monoesters of type **5** had comparable differences in mobilities on silica-gel (δR_f ca. 0.12) and could be separated chromatographically.

2.3. A tentative assignment of the configuration of the diastereomers of nucleoside 3′-*H*-phosphonothioate 5

In the first step of the correlation analysis, the configuration of the alkyl uridine *H*-phosphonothioate diesters of type **8** was assigned, and this subsequently used for the determination of the configuration of uridine *H*-phosphonothioate monoester of type **5**. The discussion below refers to uridine derivatives but it also holds for the other ribonucleoside *H*-phosphonothioates.

The diastereoselectivity of formation of diribonucleoside *H*-phosphonothioates **8c** (Fig. 2) is known and the (S_P)-diastereomer was identified as the main product.[4] Thus, the same (S_P)-configuration was tentatively assigned to the main diastereomers of alkyl uridine 3′-*H*-phosphonothioate diesters (**8**-DOWN) (note; a similar correlation was found for alkyl ribonucleoside 3′-*H*-phosphonates[2,3]).

[||||] Stereoelectronic effects that possibly govern the stereoselective transformations of nucleoside 3′-*H*-phosphonates and their analogues, are a subject of separate studies.

The assignment of the configuration of diastereomers of uridine *H*-phosphonothioate monoester **5** was based on the stereospecific β-elimination of the 9-fluorenemethyl group from 9-fluorenemethyl uridine *H*-phosphonothioate diester **8b**. As it was already shown (vide supra), diester **8b** formed in a S_P/R_P ratio of ca. 9:1 [Fig. 4, reaction **(a)**]. Treatment of such mixture with TEA [reaction (**b**)] afforded uridine *H*-phosphonothioate **5** in the same ratio of 9:1, with predominance of the **5**-SLOW diastereomer. The full stereospecificity of this reaction was proved by using as the starting material the diester **8b** in other diastereomeric ratios (55:45 and 7:3), which were accurately preserved in the reactions, proving that **5**-SLOW derived from (S_P)-**8b**. Since the β-elimination of the 9-fluorenemethyl group is a stereoretentive process, the configuration of **5**-SLOW was assigned as (S_P), while that of **5**-FAST, as (R_P). The same procedure was applied to *H*-phosphonothioates of other three ribonucleosides of type **5** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu}) and the same correlation was found in all cases.

Since the above correlation analysis for *H*-phosphonothioate monoester **5** relied on the correct assignment of configuration of diastereomers of 9-fluorenemethyl uridine *H*-phosphonothioate diester **8b**, we verified it in an independent procedure (Fig. 4, non-shaded area). To this end, the diester **8b** as previously obtained in the ratio of **8**-DOWN:**8**-UP ≈ 9:1 [reaction **(a)**] was oxidized in situ stereospecifically with 3*H*-2,1-benzoxathiol-3-one 1-oxide (BOTO)[4] yielding two diastereomers of 9-fluorenemethyl uridine 3′-phosphorothioate **9b**, also in a 9:1 ratio [reaction **(c)**].

In a separate experiment, the same phosphorothioate diester **9b** was prepared by the sulfurization of 9-fluorenemethyl uridine *H*-phosphonate **10b** [reactions (**d**) and **(e)**] and its configuration was established as described previously.[1,2,24] The (S_P)-product obtained in this way had identical ^{31}P chemical shits and coupling constants as the major diastereomer of **9b** in the analyzed reaction mixture, while the opposite (R_P)-diastereomer, was the minor one. Since oxidation with BOTO proceeds with retention of configuration,[4] diastereomer **8b**-DOWN could be assigned as (S_P), and **8b**-UP, as (R_P), which is in agreement with the initial correlation. Since the same procedure repeated for ethyl nucleoside diesters **8a**–**10a** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu}, Ura) gave the same results (e.g., for B = Ura, see Fig. 5), the formation of the (S_P)-diastereomer as the main product in the condensations of nucleoside 3′-*H*-phosphonothioates may be considered as a general rule.

3. Conclusion

The *H*-phosphonothioate esters of protected thymidine and uridine differ substantially in their ability to be isolated as individual diastereomers: in the deoxy series only alkyl nucleoside diesters are separable by silica-gel column chromatography, while in the ribo series, only the monoesters. In order to enhance the yields of diastereomers of protected nucleoside 3′-*H*-phosphonothioate with a desired configuration at the phosphorus atom, four stereoselective synthetic procedures were developed.

The 'FAST' diastereomers, showing higher chromatographic mobility on silica-gel, were synthesised via sulfhydrolysis of activated derivatives of nucleoside 3′-*H*-phosphonates with diastereomeric excess (de) 35–50%. The 'SLOW' diastereomers were prepared using (i) stereoselective formation of 9-fluorenemethyl nucleoside 3′-*H*-phosphonothioate (de 60–80%) followed by stereospecific removal of the 9-fluorenemethyl group, or (ii) asymmetric transformation of nucleoside 3′-*H*-phosphonothioate in the course of an activation—hydrolysis one pot process (de 35–50%). Using stereochemical correlation analysis of ^{31}P NMR spectra, the absolute configuration of 'FAST' and 'SLOW' diastereomers of protected ribonucleoside 3′-*H*-phosphonothioates and of 'DOWN' and 'UP'

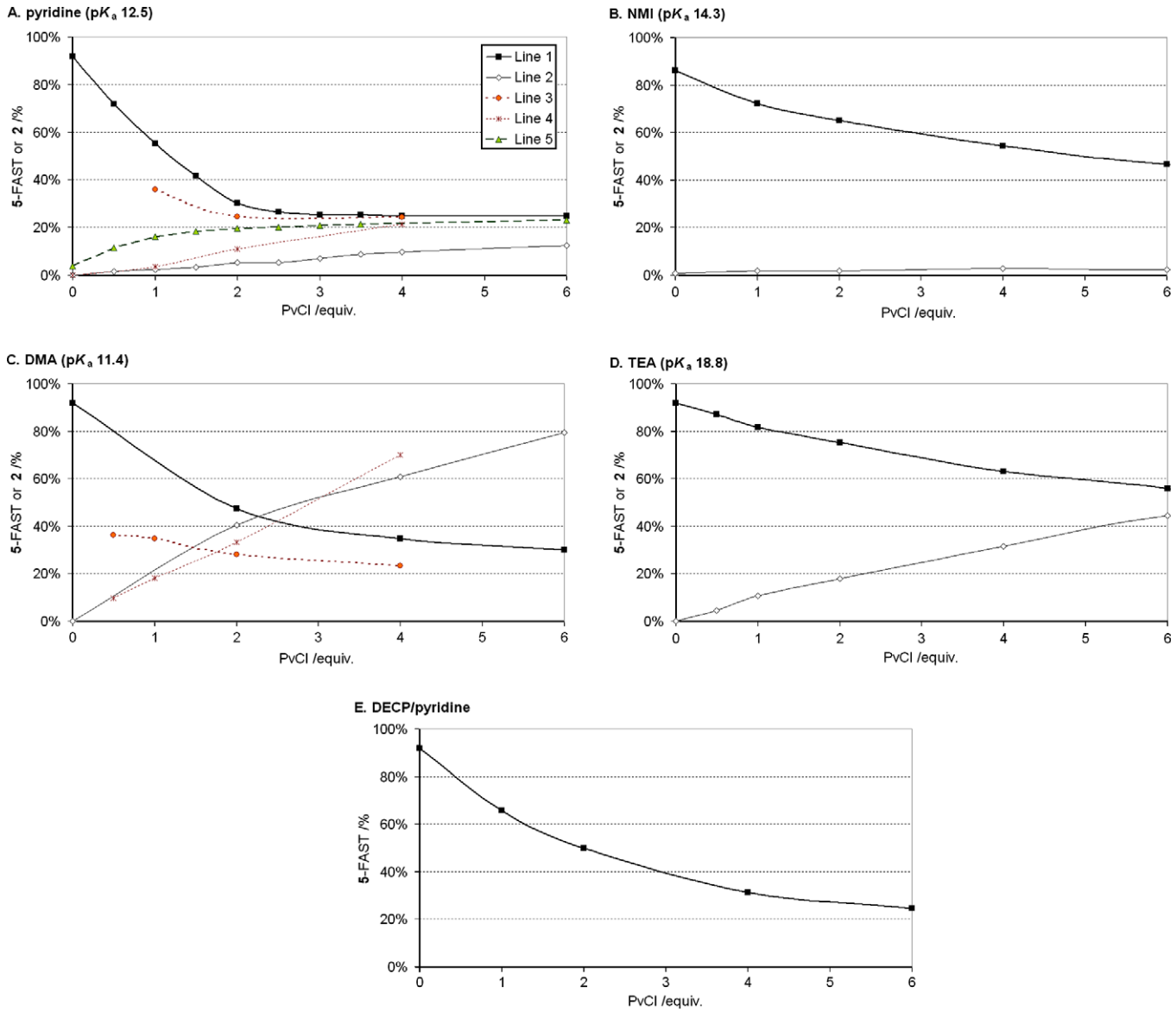


Figure 3. Charts A–D: Diastereomeric composition of uridine 3′-*H*-phosphonothioate monoester **5** during acylation–hydrolysis procedure in the presence of various amines (in parentheses, pK_a values in MeCN). The legend in chart A applies to all charts: Lines 1 and 5, de of **5**-FAST during a stepwise addition of PvCl to a solution of **5** (**5**-FAST, de 84% or **5**-SLOW, de 84%, respectively) in 90:6:4 (v/v/v) MeCN–amine–H_2O; Line 2, % of desulfurization of **5** during this process; Line 3, de of **5**-FAST during hydrolysis of the mixed anhydride **7** formed in situ using a given amount of PvCl; Line 4, % of desulfurization of **5** during this process. Chart E: The same process promoted by diethyl chlorophosphate (DECP) in the presence of pyridine.

Table 1
^{31}P NMR data, R_f values, and ratios of diastereomers for nucleoside *H*-phosphonothioates **5** obtained under various conditions

Nucleoside[a]	Diastereomer	δ_P in DCM/ppm ($^1J_{PH}$, $^3J_{PH}$/Hz)	R_f[b]	Ratio of diastereomers of **5** (FAST/SLOW)[c]		
				4b + HMDST	1. **5** + FMOH + PvCl 2. +TEA	**5** + DECP in MeCN–H_2O
A	FAST	54.25 (576.9; 12.8)	0.54	3:1	1:9	1:3
	SLOW	56.16 (587.0; 12.8)	0.37			
C	FAST	54.27 (576.0; 12.8)[d]	0.59	2:1	1:4	1:2
	SLOW	54.35 (577.9; 12.8)[d]	0.44			
G	FAST	54.91 (580.6; 15.6)	0.36	3:1	1:7	1:3
	SLOW	55.65 (588.4; 14.2)	0.25			
U	FAST	53.80 (576.0; 12.8)	0.42	3:1	1:9	1:4
	SLOW	55.62 (582.4; 13.7)	0.32			

For reactions details, see Experimental. FMOH = (9*H*-fluoren-9-yl)methanol. Pv = pivaloyl (trimethacetyl).
[a] Fully protected nucleosides (5′-O-DMTr, 2′-O-TBDMSi; B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu}, Ura).
[b] Toluene–MeCN–TEA 3:2:1.
[c] Integration of ^{31}P NMR signals.
[d] The signals overlapped in DCM and the ratio of diastereomers was established in toluene (FAST: δ_P 55.18, $^1J_{PH}$ 578.3 Hz, $^3J_{PH}$ 13.3 Hz; SLOW: δ_P 54.88, $^1J_{PH}$ 577.4 Hz, $^3J_{PH}$ 12.4 Hz).

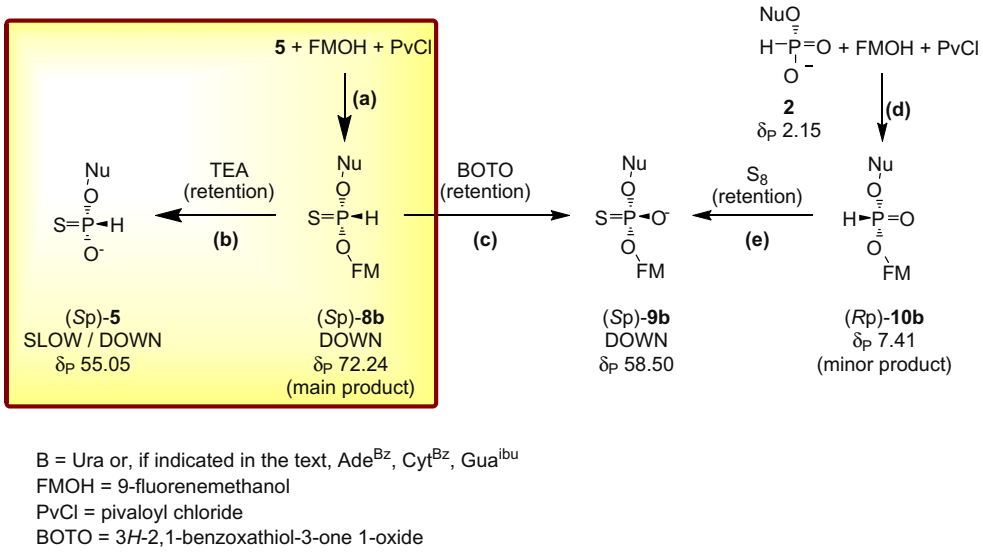


Figure 4. Stereochemical correlation analysis for assignment of configuration of nucleoside *H*-phosphonothioates **5** and **8b**. The chemical shifts given are of uridine derivatives in DCM.

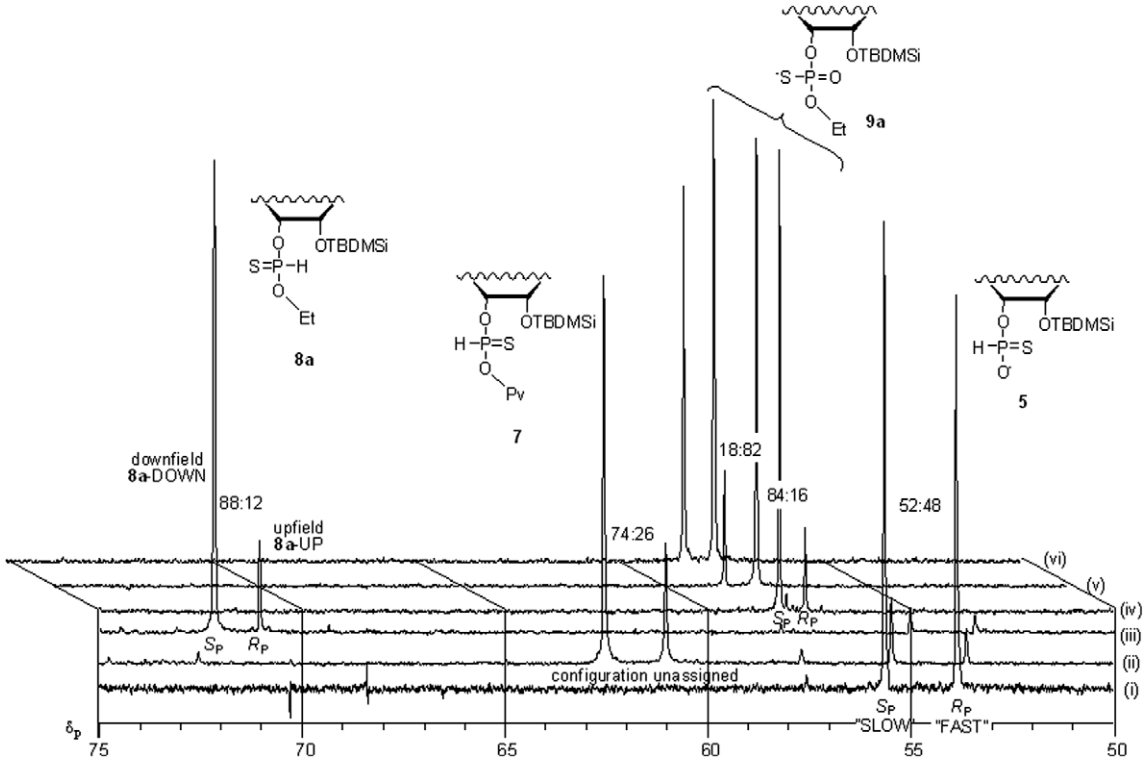


Figure 5. ^{31}P NMR spectra of the reaction mixtures during esterification of uridine 3′-*H*-phosphonothioate **5** with EtOH: (i) starting monoester **5** in DCM; (ii) reaction of **5** with PvCl; (iii) reaction of **5** with EtOH and PvCl; (iv) mixture (iii) after addition of BOTO; (v) phosphorothioate **9a** obtained by sulfurization of ethyl uridine *H*-phosphonate; (vi) mixtures (iv) and (v) combined.

diastereomers of alkyl ribonucleoside 3′-*H*-phosphonothioates was tentatively assigned as R_P/S_P, respectively.

4. Experimental

^{31}P NMR spectra were recorded at 121 MHz on a Varian Unity BB VT spectrometer. ^{1}H and ^{13}C NMR were recorded at 400 and 100 MHz, respectively, on a Bruker Avance II 400 MHz spectrometer. Commercial (Sigma–Aldrich, Alfa Aesar, Merck, POCh-Poland) reagents and were used as purchased unless otherwise noted. For column chromatography, Silica Gel 60 (70–230 mesh) was used. Anhydrous solvents (stored over molecular sieves 4 Å) contained below 20 ppm of water (Karl Fischer coulometric titration, Metrohm 684 KF). Ethanol was distilled over magnesium. Pivaloyl chloride was distilled and used within one month. 1 M triethylammonium bicarbonate (TEAB) buffer was prepared by

bubbling CO_2 through aqueous TEA at 0 °C until pH 7.0 was reached. All reactions were monitored using ^{31}P NMR spectroscopy.

4.1. Typical procedures for the preparation of diastereomers of 2′-*O*-(*t*-butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)nucleoside -3′-yl *H*-phosphonothioate 5

4.1.1. Isomer(R_P)-5 (FAST; B = Ura). Method A

The compound was prepared according to slightly modified published procedures.[13,14] 2′-*O*-(*t*-Butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)uridine **1** (1.32 g, 2 mmol) was rendered anhydrous by evaporation of added pyridine (20 mL) under reduced pressure. The residue was dissolved in 20 mL of the same solvent and diphenyl phosphite (1.26 mL, 4 mmol) was added, while stirring vigorously. After ca. 1 h, TLC showed complete phosphitylation and HMDST (1.06 mL, 5 mmol) was added slowly (ca. 15 s) with stirring. After ca. 1 h the sulfhydrolysis of diester **3a** was complete (^{31}P NMR) and the reaction mixture was left overnight with another portion of HMDST (0.85 mL, 4 mmol). The reaction was quenched with water, after which the solvent and volatile products were removed in a rotary evaporator and the residue was worked up (3 × DCM/1 M TEAB buffer) and concentrated.

4.1.2. Isomer (R_P)-5 (FAST; B = Ura). Method B

This method is based on a published procedure.[11] 2′-*O*-(*t*-Butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)uridine *H*-phosphonate **2** (1.65 g, 2 mmol) was rendered anhydrous by the evaporation of added toluene (20 mL) under reduced pressure. The residue was dissolved in 20 mL of acetonitrile and 1 mL of pyridine (12 mmol), and PvCl (0.76 mL, 6 mmol) was added. After 5 min HMDST (1.27 mL, 6 mmol) was added rapidly, while stirring vigorously, followed by 2,6-lutidinium hydrochloride (0.86 g, 6 mmol) and the vigorous stirring of the suspension was continued until sulfhydrolysis was complete (ca. 30 min, ^{31}P NMR). The reaction was quenched with water, after which the solvent and volatile products were removed in a rotary evaporator and the residue was worked up (DCM/1 M TEAB buffer) and concentrated.

In both methods crude **5** contained ca. 3:1 ratio of R_P:S_P diastereomers (^{31}P NMR). A silica-gel column chromatography using 10–30% gradient of MeCN in toluene containing 5% of TEA followed by lyophilization from benzene yielded (Method B) 760 mg of (R_P)-**5** (de 84%, yield 57%), 440 mg of a mixture of diastereomers (ca. 1:1), and 50 mg of (S_P)-**5** (de 80%). Total yield 79% (Method A) or 86% (Method B).

^1H NMR ($CDCl_3$) δ_H 0.17 (3H, s, Si–CH_3); 0.18 (3H, s, Si–CH_3); 0.88 (9H, s, Si–*t*Bu); 1.27 (9H, t, *J* 7.5 Hz, NCH_2CH_3); 3.04 (6H, q, NCH_2CH_3); 3.49 (1H, dd, $J_{5',5''}$ 11.1 Hz, $J_{4',5'}$ 2.3 Hz, H5′); 3.55 (1H, dd, $J_{4',5''}$ 2.1 Hz, H5″); 3.75 (6H, s, 2 × OCH_3); 4.33 (1H, br d, $J_{3',4'}$ 6.5 Hz, H4′); 4.42 (1H, dd, $J_{1',2'}$ 2.9 Hz, $J_{3',2'}$ 4.5 Hz, H2′); 5.05 (^1H, ddd, $J_{P,3'}$ 13.7 Hz, H3′); 5.16 (1H, d, $J_{H6,H5}$ 8.2 Hz, H5); 5.90 (^1H, d, H1′); 6.80–7.42 (13.5H, m, Ar + 1/2 P–H); 8.03 (1H, d, H6); 8.68 (0.5H, 1/2 d, 1/2 P–H); 9.48 (1H, br s, NH); 12.19 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −5.35 (Si–CH_3); −4.91 (Si–CH_3); 8.07 (NCH_2CH_3); 17.65 (Si–$C(CH_3)_3$); 25.28 (Si–$C(CH_3)_3$); 45.01 (NCH_2CH_3); 54.71 (OCH_3); 60.97 (C5′); 71.46 (d, $J_{P,C3'}$ 4.7 Hz, C3′); 75.18 (d, $J_{P,C2'}$ 3.5 Hz, C2′); 80.97 (d, $J_{P,C4'}$ 5.2 Hz, C4′); 89.20 (C1′); 101.33 (C5); 139.78 (C6); 149.82 (C2); 162.88 (C4); ^{31}P NMR (DCM) δ_P 53.87 (dd, $^1J_{H,P}$ 576.0 Hz, $^3J_{H,P}$ 13.7 Hz).

4.1.2.1. (R_P)-5. (FAST; B = Ade^{Bz}). ^1H NMR ($CDCl_3$) δ_H −0.08 (3H, s, Si–CH_3); 0.08 (3H, s, Si–CH_3); 0.78 (9H, s, Si–*t*Bu); 1.27 (9H, t, *J* 7.2 Hz, NCH_2CH_3); 3.03 (6H, q, NCH_2CH_3); 3.43 (1H, dd, $J_{5',5''}$ 10.8, $J_{4',5'}$ 3.6 Hz, H5′); 3.54 (1H, dd, $J_{4',5''}$ 2.6 Hz, H5″); 3.74 (6H, s, 2 × OCH_3); 4.50 (1H, br t, $J_{3',4'}$ 3.2 Hz, H4′); 5.02 (1H, br t, $J_{1',2'}$ 5.2 Hz, $J_{2',3'}$ 4.4 Hz, H2′); 5.12 (^1H, dt, $J_{P,3'}$ 12.8 Hz H3′); 6.19 (1H, d, H1′); 6.78–7.45 (13.5H, m, Tr + 1/2 P–H); 7.49 (2H, t, *J* ~7.6 Hz, *m*-Bz); 7.57 (1H, t, *J* ~7.6 Hz, *p*-Bz); 8.00 (2H, d, *J* 7.6 Hz, *o*-Bz); 8.26 (1H, s, H2); 8.69 (1H, s, H8); 8.75 (0.5H, 1/2 d, 1/2 P–H); 9.15 (1H, br s, NH); 12.17 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −5.12 (Si–CH_3); −4.52 (Si–CH_3); 8.47 (NCH_2CH_3); 17.94 (Si–$C(CH_3)_3$); 25.60 (Si–$C(CH_3)_3$); 45.44 (NCH_2CH_3); 55.12 (OCH_3); 62.87 (C5′); 73.57 (d, $J_{P,C3'}$ 5.5 Hz, C3′); 75.02 (d, $J_{P,C2'}$ 4.4 Hz, C2′); 82.98 (d, $J_{P,C4'}$ 4.4 Hz, C4′); 86.54, 88.59 (C1′, C5); 141.65 (C2); 144.46 (C6); 158.40 (C4); 164.50 (C(O)Ph); ^{31}P NMR (DCM) δ_P 54.25 (dd, $^1J_{H,P}$ 576.9 Hz, $^3J_{H,P}$ 12.8 Hz).

4.1.2.2. (R_P)-5. (FAST; B = Cyt^{Bz}). ^1H NMR ($CDCl_3$) δ_H 0.20 (3H, s, Si–CH_3); 0.25 (3H, s, Si–CH_3); 0.91 (9H, s, Si–*t*Bu); 1.23 (9H, t, *J* 7.4 Hz, NCH_2CH_3); 3.00 (6H, q, NCH_2CH_3); 3.51 (1H, dd, $J_{5',5''}$ 11.2, $J_{4',5'}$ 2.5 Hz, H5′); 3.66 (1H, br d, H5″); 3.79 (6H, s, 2 × OCH_3); 4.41 (1H, br t, $J_{3',4'}$ 8.5 Hz, H4′); 4.45 (1H, br d, $J_{2',3'}$ 4.0 Hz, H2′); 5.05 (^1H, ddd, $J_{P,3'}$ 13.7 Hz H3′); 5.88 (1H, br s, H1′); 6.84–7.42 (14.5H, m, Tr + H5 + 1/2 P–H); 7.46 (2H, t, *J* ~7.8 Hz, *m*-Bz); 7.58 (1H, t, *J* ~7.5 Hz, *p*-Bz); 7.87 (2H, d, *J* 7.5 Hz, *o*-Bz); 8.59 (1H, d, $J_{5,6}$ 7.5 Hz, H6); 8.62 (0.5H, 1/2 d, 1/2 P–H); 12.03 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −4.74 (Si–CH_3); −4.67 (Si–CH_3); 8.42 (NCH_2CH_3); 18.04 (Si–$C(CH_3)_3$); 25.78 (Si–$C(CH_3)_3$); 45.40 (NCH_2CH_3); 55.09 (OCH_3); 60.58 (C5′); 73.04 (d, $J_{P,C3'}$ 4.0 Hz, C3′); 75.56 (d, $J_{P,C2'}$ 4.0 Hz, C2′); 80.59 (d, $J_{P,C4'}$ 6.7 Hz, C4′); 91.60 (C1′); 96.2 (br, C5); 143.86 (C2); 144.9 (br, C6); 158.50 (C4); 161.94 (C(O)Ph); ^{31}P NMR (DCM) δ_P 54.43 (dd, $^1J_{H,P}$ 576.0 Hz, $^3J_{H,P}$ 12.8 Hz); ^{31}P NMR (toluene) δ_P 55.18 (dd, $^1J_{H,P}$ 578.3 Hz, $^3J_{H,P}$ 13.3 Hz).

4.1.2.3. (R_P)-5. (FAST; B = Gua^{ibu}). ^1H NMR ($CDCl_3$) δ_H −0.03 (3H, s, Si–CH_3); 0.11 (3H, s, Si–CH_3); 0.80 (9H, s, Si–*t*Bu); 0.98 and 1.07 (2 × 3H, 2 × d, *J* 6.6 Hz, 2 × $CHCH_3$) 1.25 (9H, t, *J* 7.5 Hz, NCH_2CH_3); 2.31 (1H, septet, *J* 7.7 Hz $CHCH_3$), 3.04 (6H, q, NCH_2CH_3); 3.12 (1H, dd, $J_{5',5''}$ 11.2 Hz, $J_{4',5'}$ 2.5 Hz, H5′); 3.49 (1H, br d, H5″); 3.71 and 3.72 (2 × 3H, 2 × s, 2 × OCH_3); 4.29 (^1H, br d, $J_{3',4'}$ 5.5 Hz, H4′); 5.06 (^1H, dd, $J_{1',2'}$ 3.8 Hz, $J_{3',2'}$ 4.6 Hz, H2′); 5.56 (1H, ddd, $J_{P,3'}$ 15.3 Hz, H3′); 5.79 (1H, d, H1′); 6.70–7.38 (13.5H, m, Ar + 1/2 P–H); 7.79 (1H, d, H8); 8.63 (0.5H, 1/2 d, 1/2 P–H); 11.67 (1H, br s, NH–ibu); 11.99 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −5.09 and −4.31 (2 × Si–CH_3); 8.51 (NCH_2CH_3); 17.95 (Si–$C(CH_3)_3$); 18.63 and 18.73 (C(O)$CH(CH_3)_2$); 25.59 (Si–$C(CH_3)_3$); 35.89 (C(O)$CH(CH_3)_2$); 45.59 (NCH_2CH_3); 55.09 (OCH_3); 61.97 (C5′); 72.40 (d, $J_{P,C3'}$ 3.6 Hz, C3′); 74.60 (d, $J_{P,C2'}$ 4.0 Hz, C2′); 81.83 (d, $J_{P,C4'}$ 4.3 Hz, C4′); 89.51 (C1′); 121.73 (C5); 147.31 and 147.96 (C2 and C4); 155.66 (C6); 179.09 [NHC(O)]; ^{31}P NMR (DCM) δ_P 54.58 (dd, $^1J_{H,P}$ 577.9 Hz, $^3J_{H,P}$ 15.6 Hz).

4.1.3. Isomer (S_P)-5 (SLOW; B = Ura). Method A

A mixture of uridine *H*-phosphonothioate **5** (750 mg, 0.89 mmol) and (9*H*-fluoren-9-yl)methanol (FMOH) (345 mg, 1.76 mmol) was rendered anhydrous by evaporation of added toluene (10 mL) under reduced pressure. The residue was dissolved in 10 mL of DCM containing pyridine (240 μL, 3.0 mmol) and PvCl (166 μL, 1.32 mmol) was added. After *ca.* 5 min 2 mL of TEA was added and the reaction was monitored with ^{31}P NMR or TLC until the signal of diester **8b** disappeared (ca 45 min). The crude reaction mixture contained ca. 10% of (S_P)-**5** and 90% of (R_P)-**5** (^{31}P NMR). After typical work-up and rapid column chromatography using 20–30% gradient of acetonitrile in toluene containing 5% of TEA, followed by lyophilization from benzene, 40 mg of mixture of diastereomers and 660 mg of (R_P)-**5** (de 96%) were obtained. Total recovery of **5**, 93%.

4.1.4. Isomer (S_P)-5 (SLOW; B = Ura). Method B

Crude **5** (842 mg, 1.0 mmol, R_P:S_P = 55:45) was dissolved in 10 mL of MeCN containing pyridine (20%, v/v) and water (1.2% v/v), and to this diethylchlorophosphate (DECP, 0.96 mL, 6 mmol) was

added slowly (ca. 15 s) with vigorous stirring. This yielded **5** as a diastereomeric mixture containing ca. 20% of (R_P)-**5** and 80% of (S_P)-**5** (^{31}P NMR). After work-up (1 × DCM/TEAB) the product was isolated as described for the (R_P)-isomer, yielding 90 mg of (R_P)-**5** (de 79%), 280 mg of mixture of diastereomers, and 290 mg of (S_P)-**5** (de 84%) after lyophilization from benzene. Total yield 78%.

^{1}H NMR ($CDCl_3$) δ_H 0.08 (3H, s, Si–CH_3); 0.14 (3H, s, Si–CH_3); 0.87 (9H, s, Si–*t*Bu); 1.30 (9H, t, *J* 7.5 Hz, NCH_2CH_3); 3.05 (6H, q, NCH_2CH_3); 3.37 (1H, dd, $J_{5',5''}$ 10.8, $J_{4',5'}$ 2.0 Hz, H5′); 3.65 (1H, dd, $J_{4',5''}$ 1.9 Hz, H5″); 3.77 (6H, s, 2 × OCH_3); 4.40 (1H, br d, $J_{3',4'}$ 2.2 Hz, H4′); 4.48 (^{1}H, dd, $J_{1',2'}$ 5.8 Hz, $J_{3',2'}$ 4.7 Hz, H2′); 5.16 (^{1}H, ddd, $J_{P,3'}$ 13.7 Hz, H3′); 5.21 (1H, d, $J_{H6,H5}$ 8.2 Hz, H5); 6.06 (1H, d, H1′); 6.81–7.42 (13.5H, m, Ar + 1/2 P–H); 7.82 (1H, d, H6); 8.89 (0.5H, 1/2 d, 1/2 P–H); 9.25 (1H, br s, NH); 12.09 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −4.97 (Si–CH_3); −4.70 (Si–CH_3); 8.51 (NCH_2CH_3); 17.93 (Si–*C*(CH_3)$_3$); 25.58 (Si–C(*C*H$_3$)$_3$); 45.54 (NCH_2CH_3); 55.17 (OCH_3); 62.70 (C5′); 74.66 (d, $J_{P,C3'}$ 5.0 Hz, C3′); 75.12 (d, $J_{P,C2'}$ 3.7 Hz, C2′); 83.99 (d, $J_{P,C4'}$ 4.3 Hz, C4′); 87.83 (C1′); 102.22 (C5); 140.24 (C6); 150.49 (C2); 163.08 (C4); ^{31}P NMR (DCM) δ_P 55.66 (dd, $^1J_{H,P}$ 582.4 Hz, $^3J_{H,P}$ 13.7 Hz).

4.1.4.1. (S_P)-5. (SLOW; B = Ade^{Bz}). ^{1}H NMR ($CDCl_3$) δ_H −0.26 (3H, s, Si–CH_3); 0.05 (3H, s, Si–CH_3); 0.75 (9H, s, Si–*t*Bu); 1.32 (9H, t, *J* 7.3 Hz, NCH_2CH_3); 3.09 (6H, q, NCH_2CH_3); 3.47 (1H, dd, $J_{5',5''}$ 10.3, $J_{4',5'}$ 2.6 Hz, H5′); 3.54 (1H, dd, $J_{4',5''}$ 3.3 Hz, H5″); 3.77 (6H, s, 2 × OCH_3); 4.54 (1H, br s, H4′); 5.05 (1H, br t, $J_{2',3'}$ 4.7 Hz, H2′); 5.30 (^{1}H, br dd, $J_{3',4'}$ 1.1 Hz, $J_{P,3'}$ 13.8 Hz H3′); 6.24 (1H, d, $J_{1',2'}$ 6.8 Hz, H1′); 6.81–7.48 (13.5H, m, Tr + 1/2 P–H); 7.51 (2H, t, *J* ~7.4 Hz, *m*-Bz); 7.59 (1H, t, *J* ~7.4 Hz, *p*-Bz); 8.03 (2H, d, *J* 7.4 Hz, *o*-Bz); 8.22 (1H, s, H2); 8.71 (1H, s, H8); 8.93 (0.5H, 1/2 d, 1/2 P–H); 9.20 (1H, br s, NH); 12.06 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −5.37 (Si–CH_3); −4.71 (Si–CH_3); 8.51 (NCH_2CH_3); 17.75 (Si–*C*(CH_3)$_3$); 25.47 (Si–C(*C*H$_3$)$_3$); 45.49 (NCH_2CH_3); 55.14 (OCH_3); 63.26 (C5′); 75.40 (m, C2′, C3′); 84.96 (d, $J_{P,C4'}$ 4.0 Hz, C4′); 86.74, 87.51 (C1′, C5); 141.50 (C2); 144.56 (C6); 158.43 (C4); 164.52 (C(O)Ph); ^{31}P NMR (DCM) δ_P 56.79 (dd, $^1J_{H,P}$ 585.2 Hz, $^3J_{H,P}$ 13.7 Hz).

4.1.4.2. (S_P)-5. (SLOW; B = Cyt^{Bz}). ^{1}H NMR ($CDCl_3$) δ_H 0.15 (3H, s, Si–CH_3); 0.16 (3H, s, Si–CH_3); 0.90 (9H, s, Si–*t*Bu); 1.29 (9H, t, *J* 7.3 Hz, NCH_2CH_3); 3.04 (6H, q, NCH_2CH_3); 3.54 (1H, br d, $J_{5',5''}$ 11.0, H5′); 3.65 (1H, dd, $J_{4',5''}$ 2.7 Hz, H5″); 3.80 (6H, s, 2 × OCH_3); 4.47 (2H, m, H2′, H4′); 5.05 (^{1}H, dt, $J_{P,3'}$ 12.5 Hz, $J_{2',3'}$ ~5.2 Hz, $J_{4',3'}$ ~5.2 Hz H3′); 6.05 (1H, d, $J_{1',2'}$ 3.4 Hz, H1′); 6.84–7.42 (14.5H, m, Tr + H5 + 1/2 P–H); 7.49 (2H, t, *J* ~7.8 Hz, *m*-Bz); 7.58 (1H, t, *J* ~7.9 Hz, *p*-Bz); 7.90 (2H, d, *J* 6.3 Hz, *o*-Bz); 8.36 (1H, d, $J_{5,6}$ 6.5 Hz, H6); 8.82 (0.5H, 1/2 d, 1/2 P–H); 12.13 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −4.91 (Si–CH_3); −4.73 (Si–CH_3); 8.51 (NCH_2CH_3); 18.03 (Si–*C*(CH_3)$_3$); 25.78 (Si–C(*C*H$_3$)$_3$); 45.50 (NCH_2CH_3); 55.19 (OCH_3); 61.85 (C5′); 73.03 (br d, $J_{P,C3'}$ 4.4 Hz, C3′); 75.67 (br d, $J_{P,C2'}$ 2.6 Hz, C2′); 82.61 (br d, $J_{P,C4'}$ 3.7 Hz, C4'); 90.22 (C1′); 96.4 (br, C5); 144.18 (C2); 144.9 (br, C6); 158.57 (C4); 161.96 (br C(O)Ph); ^{31}P NMR (DCM) δ_P 54.43 (dd, $^1J_{H,P}$ 576.0 Hz, $^3J_{H,P}$ 12.8 Hz); ^{31}P NMR (toluene) δ_P 54.88 (dd, $^1J_{H,P}$ 577.4 Hz, $^3J_{H,P}$ 12.4 Hz).

4.1.4.3. (S_P)-5. (SLOW; B = Gua^{ibu}). ^{1}H NMR ($CDCl_3$) δ_H −0.16 (3H, s, Si–CH_3); 0.06 (3H, s, Si–CH_3); 0.77 (9H, s, Si–*t*Bu); 0.84 and 0.97 (2 × 3H, 2 × d, *J* 6.8 Hz, 2 × CHC*H*$_3$) 1.27 (9H, t, *J* 7.4 Hz, NCH_2CH_3); 1.95 (^{1}H, septet, *J* 6.6 Hz *C*HCH_3), 3.05 (6H, q, NCH_2CH_3); 3.31 (1H, dd, $J_{5',5''}$ 10.2 Hz, $J_{4',5'}$ 3.5 Hz, H5′); 3.45 (1H, dd, $J_{4',5'}$ 1.9 Hz, H5″); 3.76 (6H, s, OCH_3); 4.36 (^{1}H, br d, $J_{3',4'}$ 1.8 Hz, H4′); 5.04 (1H, t, $J_{1',2'}$ 4.0 Hz, $J_{3',2'}$ 7.1 Hz, H2′); 5.50 (1H, ddd, $J_{P,3'}$ 13.6 Hz, H3′); 5.81 (^{1}H, d, H1′); 6.70–7.50 (13.5H, m, Ar + 1/2 P–H); 7.78 (1H, d, H8); 8.63 (0.5H, 1/2 d, 1/2 P-H); 11.76 (1H, br s, NH–ibu); 11.99 (1H, br s, NH); ^{13}C NMR ($CDCl_3$; DMTr resonances not listed) δ_C −5.26 and −4.61 (2 × Si–CH_3); 8.55 (NCH_2CH_3); 17.97 (Si–*C*(CH_3)$_3$); 18.90 and 18.97 (C(O)CH(*C*H$_3$)$_2$); 25.60 (Si–C(*C*H$_3$)$_3$); 36.16 (C(O)CH(CH_3)$_2$); 45.68 (NCH_2CH_3); 55.18 (OCH_3); 61.76 (C5′); 72.54 (d, $J_{P,C3'}$ 4.8 Hz, C3′); 74.93 (d, $J_{P,C2'}$ 3.8 Hz, C2′); 83.69 (d, $J_{P,C4'}$ 3.7 Hz, C4′); 90.72 (C1′); 122.04 (C5); 147.41 and 148.12 (C2 and C4); 155.42 (C6); 179.09 [NHC(O)]; ^{31}P NMR (DCM) δ_P 54.93 (dd, $^1J_{H,P}$ 590.7 Hz, $^3J_{H,P}$ 14,66 Hz).

4.2. Typical procedures for desulfurization of *H*-phosphonothioate 5

Pivaloyl chloride (250 μL, 4 equiv) was added to a solution of uridine *H*-phosphonothioate **5** (421 mg, 0.5 mmol) in MeCN (10 mL) containing dimethylaniline (DMA, 600 μL) and water (100 μL, 11 equiv). After 5 min DMA (500 μL), water (40 μL), and PvCl (250 μL) were added successively two times in 5 min intervals. The ^{31}P NMR spectrum showed 65%, 88%, and >99% of desulfurization of **5** after addition of each portion of PvCl. Triethylamine (1 mL) was added to prevent detritylation and the solvents were evaporated under reduced pressure. The residue was dissolved in DCM and extracted with TEAB buffer. Silica gel chromatography (initial isocratic 3% MeOH in DCM, followed by 3→5% gradient of MeOH in DCM containing 2% TEA) and lyophilization from benzene afforded 2′-*O*-(*t*-butyldimethylsilyl)-5′-*O*-(dimethoxytrityl)uridin-3′-yl *H*-phosphonate as a white amorphous solid (345 mg, 83%). Analytical data were consistent with the literature data.[25,26]

4.3. Determination of the configuration of diastereomers of *H*-phosphonothioate 8

3*H*-2,1-Benzoxathiol-3-one 1-oxide (BOTO) was prepared in situ by treating 3*H*-l,2-benzodithiol-3-one 1,l-dioxide (Beaucage reagent, 0.15 mmol/100 μL of MeCN) with TEA (10 μL).[27] The precipitated sulfur was centrifuged and the supernatant was added to an MeCN solution of alkyl nucleoside *H*-phosphonothioates **8a** or **8b** (0.05 mmol/400 μL) obtained in situ as described above (Method A). The diastereomeric composition of the resulting diesters **9a** or **9b** was analyzed by ^{31}P NMR spectroscopy and their configurations were identified by spiking the reaction mixtures with a solution of **9a** or **9b**, respectively, prepared as a S_P/R_P 1:4 diastereomeric mixture as described previously.[2]

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