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Stereochemistry of internucleotide bond formation by the *H*-phosphonate method. Part 6: Optimization of the reaction conditions towards highest stereoselectivity

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ABSTRACT

The condensations of ribonucleoside 3′-*H*-phosphonates with simple alcohols and nucleosides are known to be stereoselective reactions that favour formation of $D_P(S_P)$-diastereomers of the produced *H*-phosphonate diesters without engaging any chiral auxiliaries. We have investigated various reaction conditions in order to attain the highest stereoselectivity for the condensation. With an optimal choice of solvents, reagent concentrations, temperature and the condensing agent used, the diastereomeric excess of the $D_P(S_P)$-isomers of dinucleoside *H*-phosphonates was enhanced from the initial 50–70% to ca. 85%.

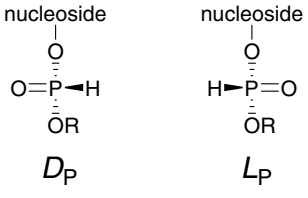
1. Introduction

P-Modified oligonucleotide analogues (e.g., phosphorothioates) with a defined configuration at the chiral phosphorous centre are valuable tools in the investigations of interactions between nucleic acids and other biomolecules. This has stirred the interest of several research groups to design methods for the stereocontrolled formation of P-chiral internucleotide linkages.[1–5] To achieve the desired stereoselectivity, in most of the known methods, the phosphorous atom is incorporated into a relatively rigid five- or six-membered ring system. The necessity of the preparation of unique synthons dedicated to a particular synthetic scheme seems to be the major inconvenience of such approaches. Also, due to synthetic limitations, most of the methods for the stereocontrolled synthesis of nucleotide analogues are focused on only one type of a phosphate moiety modification, usually phosphorothioates and, to a lesser extent, methylphosphonates or boranophosphates. Only recently, Wada et al. reported preliminary data on a stereocontrolled synthesis of oligodeoxynucleotide *H*-phosphonates by an oxazaphospholidine approach[6] that, by taking advantage of the flexibility of the *H*-phosphonate chemistry, can provide various nucleotide analogues from one common precursor.

Another important issue concerns the efficiency and stereochemistry of P-chiral oligonucleotide synthesis in the deoxy versus ribo series. At present, most of the published methods deal with P-chiral oligodeoxynucleotide analogues, but the growing interest in RNAi and its application for therapeutic purposes[7,8] have made the stereocontrolled synthesis of P-chiral oligoribonucleotides a particularly attractive goal. Wada et al. showed that the oxazaphospholidine approach developed for oligodeoxynucleotides could also be applied to oligoribonucleotides,[9] but the oxazaphospholidine derivatives optimized for the highest stereoselectivity in the deoxy series required significant changes to be effective in the ribo series. It may be anticipated that also in other approaches to stereocontrolled synthesis of oligonucleotide analogues, switching from DNA to RNA chemistry might not be straightforward or even possible.

In this context, the stereoselective oligoribonucleotide synthesis via the *H*-phosphonate approach might appear as an attractive alternative, as the condensation of ribonucleoside *H*-phosphonates with nucleosides (or alcohols) produces D_P-diastereomers[†] of the ribonucleoside *H*-phosphonate diesters in de (diastereomeric excess) of 50–70% (Fig. 1).[14–17] In this approach, commercially available *H*-phosphonate synthons can be used, which make the method particularly attractive since nucleoside *H*-phosphonates are known to be superior substrates for oligonucleotide synthesis in the ribo series.[15,18,19]

[†] For the compounds presented herein, the D_P-descriptor refers to a structure in which the P–H bond is directed to the right in the Fischer projection, while for the L_P one, to the left. The full D_P/L_P notation is described in Refs. 10–13



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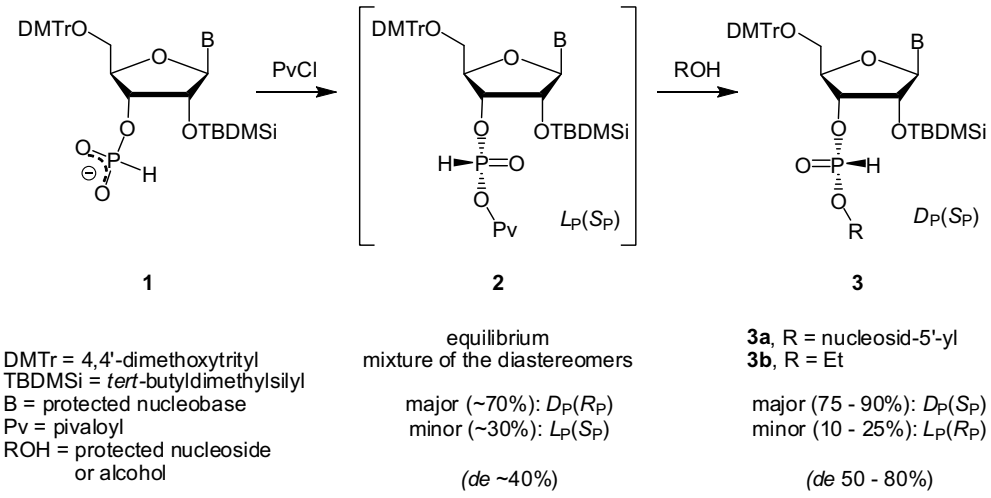

Figure 1. Stereoselective formation of the $D_P(S_P)$-diastereomer of *H*-phosphonate diesters of type **3**.

In the paper by Almer et al.,[15] several helpful hints for tuning the stereoselectivity of the *H*-phosphonate internucleotide bond formation were given. Recently, we have resumed the evaluation of this method in a more systematic way to gain a better understanding of the underlying mechanism,[16,17,20–24] and to achieve further improvement of stereoselectivity.

2. Results

2.1. Effects of amines on the stereoselectivity

The influence of tertiary amines and pyridine derivatives on the stereoselectivity of the model reaction (Fig. 1, B = Ura, R = Et) was discussed in detail in the previous parts of this series.[23,24] The most meaningful results for uridine derivatives are shown in Figure 2 (left panel) along with the results of analogous reactions for cytidine *H*-phosphonate **1** (B = Cyt^{Bz}), which is known to react with a significantly lower stereoselectivity than the other nucleoside *H*-phosphonates.[15–17]

Good stereoselectivity, together with quantitative yields of the reactions, pointed to 2,6-lutidine as the amine of choice.[23] However, the excellent stereoselectivity observed in the presence of TEA (de 75% for B = Ura, and 61% for B = Cyt^{Bz}, Fig. 2) prompted us to carry out similar condensations using 2′,3′-*O*-dibenzoyl uridine (1.2 equiv) as a hydroxylic component instead of ethanol (Fig. 1, R = uridin-5′-yl). As expected, under such reaction conditions *H*-phosphonate diester **3a** (B = Ura) was formed with high stereoselectivity (de 84–88%); however, the yields of *H*-phosphonate diester did not exceed 71%. For the other substrates of type **1** (B = Ade^{Bz}, Cyt^{Bz} and Gua^{ibu}) the stereoselectivities in the presence of TEA were also very high (de 84%, 74% and 83%, respectively), but the yields were again low (ca. 65%). For this reason, we left the investigations of the use of tertiary amines in the condensation of *H*-phosphonates as a subject of separate study.[24]

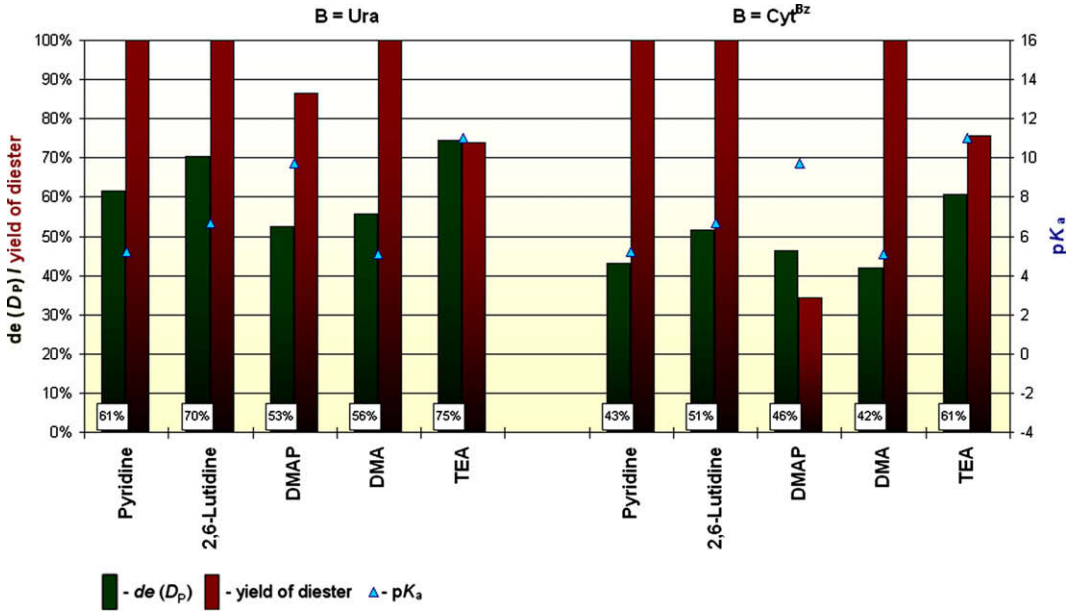

Figure 2. Diastereomeric excess (de) of the $D_P(S_P)$-diastereomers of the *H*-phosphonate diesters **3b** (Fig. 1, B = Ura and B = Cyt^{Bz}) formed in the presence of several amines. The reaction conditions: 0.05 mmol of **1** (B = Ura) + EtOH (3 equiv) + amine (3 equiv) + PvCl (1.5 equiv) in DCM (0.5 mL). Green bars: de of D_P diastereomer; red bars: the total yield of the diester **3b** (sum of diastereomers); triangles: pK_a of amines.[25,26] Abbreviations: DMA, *N*,*N*-dimethylaniline; DMAP, *N*,*N*-dimethylaminopyridine; TEA, triethylamine.

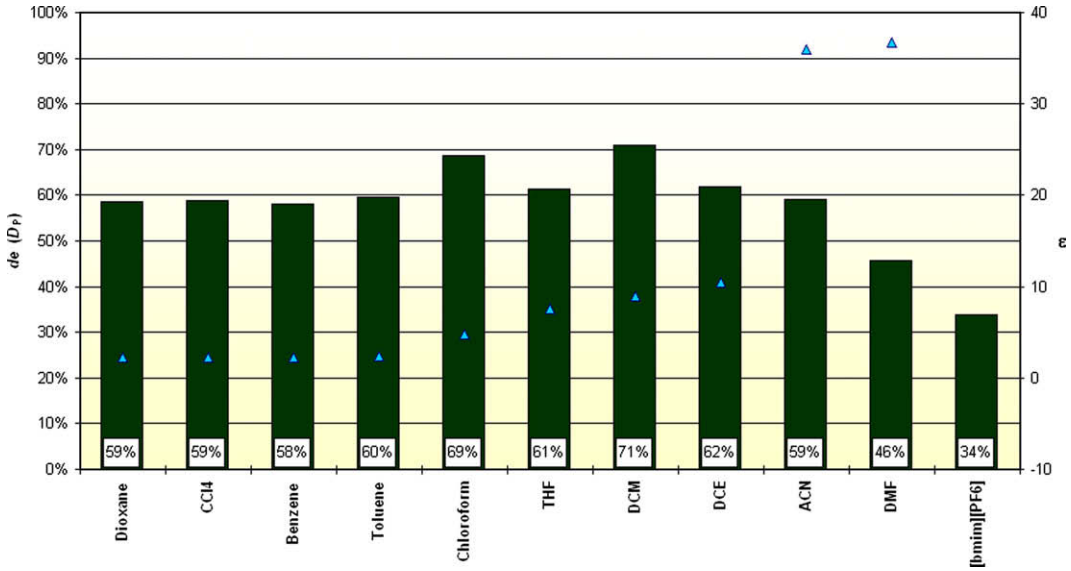

Figure 3. Diastereomeric excess (bars) of the $D_P(S_P)$ diastereomer of *H*-phosphonate diester **3b** formed in solvents of various relative permittivities (triangles).[26] The reaction conditions: 0.05 mmol of **1** (B = Ura) + EtOH (3 equiv) + 2,6-lutidine (3 equiv) + PvCl (1.5 equiv) in a given solvent (0.5 mL). For the ionic liquid, [bmim][PF_6], the value of permittivity is not available. Abbreviations: ACN, acetonitrile; DCE, 1,2-dichloroethane; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; THF, tetrahydrofuran; [bmim][PF_6], 1-*n*-butyl-3-methylimidazolium hexafluorophosphate.

2.2. Effect of solvents on the stereoselectivity

Several solvents were analyzed with respect to the stereoselectivity of the condensations of ribonucleoside 3′-*H*-phosphonates. In Figure 3, the solvents were arranged according to their relative permittivity. The best results, in terms of stereoselectivity (de 71%), were found for a moderately polar DCM.

2.3. Effect of base concentration on the stereoselectivity

In order to estimate the optimal concentration of the base in the reaction mixture, four ribonucleoside 3′-*H*-phosphonates **1** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu}‡ and Ura; 0.0125 mmol) were reacted with ethanol (6.8 equiv) in the presence of PvCl (2.5 equiv) in DCM–2,6-lutidine mixtures (total volume 0.5 mL). Lowering the concentration of *H*-phosphonate **1** to 25 mM (instead of standard 100 mM) helped diminish any possible disturbance due to the protonation of the amine by acidic species released during the condensation. As shown in Figure 4, for B = Ade^{Bz}, Cyt^{Bz} and Ura, the highest stereoselectivity was obtained when the concentration of 2,6-lutidine was ca. 10%, while for B = Gua^{ibu}, ca. 3%. The advantage of lower content of an amine in the case of guanosine *H*-phosphonate was in line with the earlier findings of Almer et al.[15]

2.4. Effects of condensing agents (C.A.'s) on the stereoselectivity

The results of investigations on the influence of the kind of a C.A. on the stereoselectivity are summarized in Figure 5. Acyl chlorides,§ triphosgene and DCC were able to secure fast condensations (completion before the first ^{31}P NMR spectrum was recorded, i.e., in less than 1 min) under standard conditions used in this study. For the other C.A.'s, the reactions were complete after several minutes or hours. No traces of by-products due to bis-activation, P-acylation or oxidation were noticed in the ^{31}P NMR spectra in either case.

The use of phenoxyacetyl chloride (PAC-Cl) caused pronounced detritylation (probably due to a relatively high acidity of the released phenoxyacetic acid, pK_a 3.17), and this experiment could be successfully repeated with 5 equiv of lutidine.

H-Phosphonate diesters **3b** were formed efficiently over the course of the transesterification of *p*-nitrophenyl–ribonucleoside *H*-phosphonates.[22] The stereoselectivity in this reaction was found to be very low (de ~ 10%); however, when the aryl *H*-phosphonate diester was reacted with 2′,3′-O-protected uridine instead of EtOH (yielding *H*-phosphonate diester **3a**), the stereoselectivity increased significantly (de 72%), that is, to the level only slightly lower than that of the reaction promoted by PvCl (cf. Fig. 11).¶ Nevertheless, the regular esterification using PvCl as a C.A. was still more stereoselective and experimentally simpler.

In contrast to the so far presented formation of *H*-phosphonate diesters via activation of the *H*-phosphonate monoester, the condensations under Mitsunobu conditions, that is, via activation of a hydroxylic component with TPP/DIAD tandem,[29] appeared to be a non-stereoselective reaction (de 2%).

2.5. Effects of chiral bases and chiral C.A. on the stereoselectivity

Chiral amines [(−)-sparteine and (*S*)-(−)-nicotine] or chiral C.A. [(−)-camphanoyl chloride] were used in the reactions of *H*-phosphonate **1** with EtOH (under standard conditions) in order to investigate the influence of this chiral components on the stereoselectivity. The obtained de values 70% and 65%, respectively, for the chiral amines,[23] and 67% for the chiral C.A. were very similar to the analogous reactions without chiral auxiliaries (de 70%, vide supra). In the experiment with (±)-nicotine the stereochemical outcome of the condensation (de 64%) was found to be almost identical to that of enantiopure (*S*)-(−)-nicotine. In a supplementary reaction in neat (*S*)-(−)-nicotine the stereoselectivity dropped significantly (de 53%), analogously as it was observed for the reaction in neat pyridine (de 42%) versus the reaction in a DCM–pyridine mixture (de 62%).[17]

‡ In these studies, we did not see any significant differences in stereoselectivity between reactions of phenylacetyl[15] and isobutyryl N-protected guanosine *H*-phosphonate **1** (B = Gua^{PAC} and B = Gua^{ibu}, respectively).

§ With an exception of 2,4,6-trimethyl- and 2,4,6-triisopropyl-benzoyl chlorides that appeared to be poor activators of nucleoside *H*-phosphonates.

¶ The phenomenon of diverse stereoselectivity of aryl *H*-phosphonate transesterification was consistent with the DYKAT mechanism and is discussed in more detail in Ref. 22.

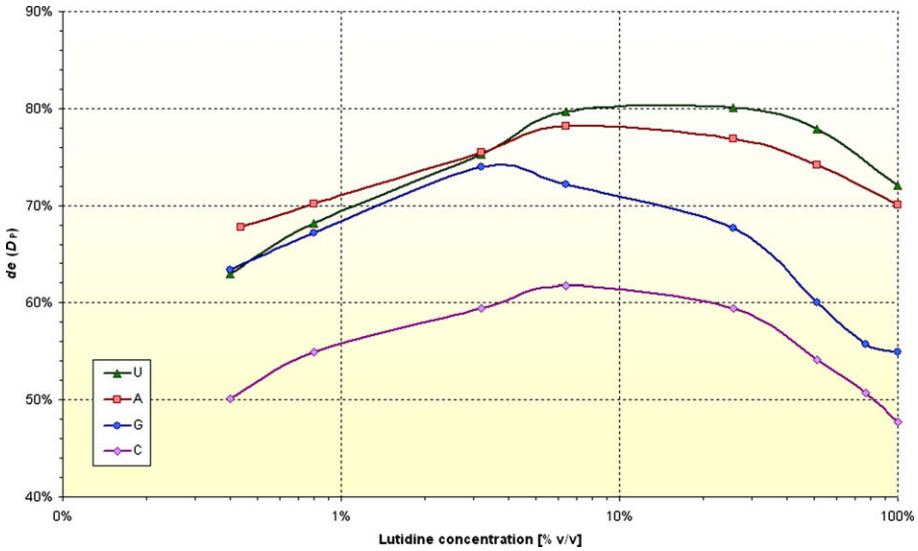

Figure 4. Diastereomeric excess of the $D_P(S_P)$-diastereomer of *H*-phosphonate diester **3b** formed in the presence of 2,6-lutidine in various concentrations. Reaction conditions: 0.0125 mmol of **1** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu} and Ura) + EtOH (6.8 equiv) + 2,6-lutidine (0.3–100% v/v) in DCM (up to 0.5 mL) + PvCl (2.5 equiv).

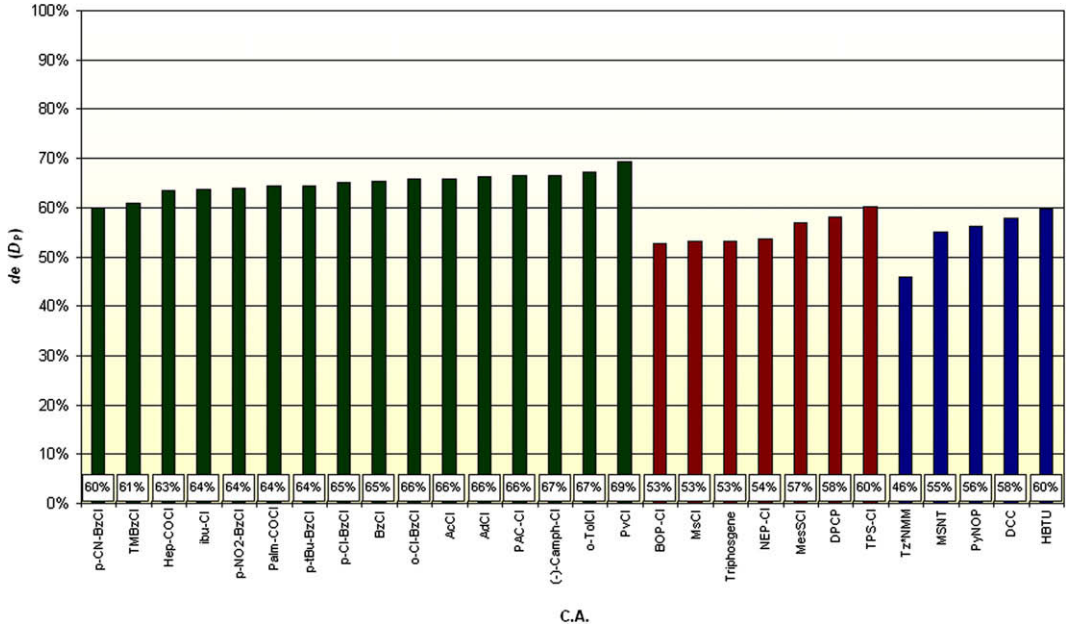

Figure 5. Diastereomeric excess of the $D_P(S_P)$-diastereomer of *H*-phosphonate diester **3b** formed in the presence of various condensing agents. Reaction conditions: 0.05 mmol of **1** (B = Ura) + EtOH (3 equiv) + 2,6-lutidine (3 equiv) + C.A. (3 equiv) in DCM (0.5 mL). Green bars: acyl chlorides; red bars: other chlorides; blue bars: other C.A.'s. Abbreviations: *p*-CN-BzCl, *p*-cyanobenzoyl chloride; TMBzCl, 3,4,5-trimethoxybenzoyl chloride; Hep-COCl, heptanoyl chloride; ibu-Cl, isobutyryl chloride; *p*-NO_2-BzCl, *p*-cyanobenzoyl chloride; Palm-COCl, palmitynoyl chloride; *p*-tBu-BzCl, 4-*tert*-butylbenzoyl chloride; *p*-Cl-BzCl, *p*-chlorobenzoyl chloride; *o*-Cl-BzCl, *o*-chlorobenzoyl chloride; AdCl, adamantanecarbonyl chloride; PAC-Cl, phenoxyacetyl chloride; (−)-Camph-Cl, (−)-camphanoyl chloride; *o*-TolCl, *o*-toluoyl chloride; NEP-Cl, neopentylene chlorophosphate; DPCP, diphenyl chlorophosphate; PyNOP, 6-nitrobenzotriazol-1-y1-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; Tz*NMM, *N*-methyl-*N*-[4,6-dimethoxy-1,3,5-triazin-2-yl)-morpholinium chloride][28] (without additional base). DCC required an acid catalyst[27] added to the reaction mixture in the form of lutidinium hydrochloride (0.1 equiv).

Another coupling reagent system, 2-chloro-4,6-dimethoxy-1,3,5-triazine/(−)-sparteine, which is known to promote an enantioselective peptide bond formation,[30] appeared to be unreactive towards the investigated *H*-phosphonate monoesters.

Other attempts to increase the stereoselectivity of ribonucleoside 3′-*H*-phosphonate condensations by introducing stereospecific interactions, via magnesium ions or dimethylaluminium ligand,[31] which were hypothesized to be capable of establishing conformational constrains in the mixed anhydride **2**, were unsuccessful. The condensations performed in the presence of anhydrous magnesium acetate displayed decreased stereoselectivity (de < 60%), while dimethylaluminium chloride caused a substantial decomposition of *H*-phosphonate **1**.

2.6. Effect of temperature on the stereoselectivity

The influence of temperature on the chemical shifts and the ratio of the diastereomers of the mixed anhydrides **2** that are

Table 1
Chemical shifts of ^{31}P NMR signals of diastereomers of the mixed anhydride **2** and diastereomeric excess of **2**-D_P(R_P) at various temperatures in toluene and in acetonitrile

Temperature (°C)	δ_P [**2**-D_P(R_P)]	δ_P [**2**-L_P(S_P)]	$\Delta\delta_P$	de (%) (D_P)
In toluene				
0	1.55	2.31	−0.76	32
10	1.47	2.11	−0.64	34
20	1.43	1.95	−0.52	31
30	1.37	1.74	−0.37	28
40	1.27	1.43	−0.16	22
50	1.19	1.15	0.04	30
60	1.12	0.94	0.18	38
70	1.06	0.86	0.20	26
In acetonitrile				
20	2.53	2.72	−0.19	25
35	2.47	2.63	−0.16	25
50	2.42	2.55	−0.13	25
65	2.36	2.44	−0.08	26

Reaction conditions: 0.05 mmol of **1** (B = Ura) + 2,6-lutidine (3.6% v/v; 3 equiv) + PvCl (2 equiv) in appropriate solvent (0.5 mL).

summarized in Table 1, show continuous changes in the chemical shifts of the ^{31}P NMR signals of both diastereomers with the constant ratio of their integrals.[‖]

Temperature dependence of the condensation was investigated for three solvents: DCM, from −78 °C to 40 °C (bp); ACN, from −46 °C (mp) to 80 °C (bp); and toluene, from −78 °C to 80 °C (Fig. 6). The highest stereoselectivity for all solvents investigated was observed at ca. 20 °C.

2.7. Effect of the substrate concentrations on the stereoselectivity

In the experiments described in this section, various concentrations and ratios of *H*-phosphonate monoester **1** (B = Ura) and EtOH were used for the condensations.

In the ^{31}P NMR spectra of the mixed anhydride intermediate **2** (B = Ura), a downfield shift of the signals of both diastereomers as a function of the increasing concentration of the reaction mixture was observed along with a decreasing separation of the resonances (Fig. 7). No signal broadening was observed and the ratio of the diastereomers of **2** remained unchanged in these experiments (D_P:L_P = 70:30 in the investigated range of 10–200 mM concentrations of *H*-phosphonate **2**).

During the condensations of ribonucleoside *H*-phosphonate with EtOH (Table 2), a positive effect of a low excess of both the hydroxylic (Fig. 8) and *H*-phosphonic (Fig. 9) components on the stereoselectivity in the condensation of ribonucleoside *H*-phosphonates was observed. Also the decreased concentration in both reactants yielded an enhanced stereoselectivity for the condensations (Fig. 10).

2.8. Condensation of H-phosphonates 1 with 2′,3′-*O*-dibenzoyluridine

The optimized reaction conditions were validated for the internucleotide bond formation in which ribonucleoside 3′-*H*-phosphonate monoesters of type **1** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu} and Ura) were condensed with 2′,3′-*O*-dibenzoyl-uridine bearing a free 5′ hydroxyl group. The results are shown in Figure 11 in comparison with those obtained under non-optimized conditions.

For three *H*-phosphonate monoesters of type **1** (B = Ade^{Bz}, Gua^{ibu} or Ura), the diastereoselectivity could be enhanced from de 50–60% (the initial reaction conditions; Fig. 11, set A) to ca. 70% when the amount of pyridine was reduced (Fig. 11, set B), and to ca. 80% when pyridine was replaced with 2,6-lutidine (Fig. 11, set C). A further increase in de to ca. 85% was achieved when fourfold dilution of the reactants ([**1**] = 25 mM) was used along with 7% (v/v) concentration of 2,6-lutidine in DCM (Fig. 11, set D).

For cytidine *H*-phosphonate **1** (B = Cyt^{Bz}), the optimization of the reaction conditions allowed us to increase de to ca. 75%, which was a significant improvement in comparison to the starting value of ca. 20%. Using an amidine protection[15,32] of the cytosine *exo*-amino function [e.g., 4-*N*-(dimethylamino)methylene or 4-*N*-(*N*-methylpyrrolidin-2-ylidene)], it was possible to enhance the stereoselectivity to the level of other *H*-phosphonates (de ca. 82%). Such derivatives are not commercially available, but can be readily prepared[32,33] by the reaction of cytidine with an appropriate *N*,*N*-dialkylformamide dimethylacetal.[††]

3. Discussion

The reaction of ribonucleoside *H*-phosphonates of type **1** with hydroxylic components (suitably protected nucleosides or alcohols) promoted by pivaloyl chloride yields *H*-phosphonate diesters of type **3** with the predominant D_P(S_P) configuration at the phosphorous centre (Fig. 1).[14–17] It was established that the most plausible mechanism responsible for the observed stereochemistry was Dynamic Kinetic Asymmetric Transformation (DYKAT) (Fig. 12).[22] According to this scenario, the diastereomers of the mixed anhydride **2** exist in a rapid equilibrium, in which the minor diastereomer of **2** (ca. 30%) was identified as having L_P(S_P)-configuration at the phosphorous centre. This minor isomer is much more reactive towards hydroxylic compounds (nucleosides or alcohols) than its major counterpart ($k_{D_p} \gg k_{L_p}$), yielding the D_P(S_P) *H*-phosphonate diesters of type **3** as the prevailing products. The depletion in concentration of **2**-L_P(S_P) during the esterification is rapidly replenished by a fast equilibration of both diastereomers of **2**, catalyzed probably by pivalate or chloride anions present in the reaction mixtures.[‡‡]

It was also found that the stereoselectivity of the condensation significantly depends on the kind and excess of the alcohol used, and on the method of activation of *H*-phosphonate monoester **1**.[22] We assumed that variations of these factors affected the rates of P-epimerization of the diastereomers of the mixed anhydride **2** and their subsequent esterification. This suggested that the relationship between the reactions involved in the DYKAT mechanism, which is responsible for the investigated stereoselectivity, might be susceptible to changes in the reaction conditions, such as temperature, concentration of the reactants, the type of a solvent and a

[‖] The minor variations in the ratio of diastereomers of the mixed anhydride **2** could be attributed to experimental errors. Compound **2** was unstable at higher temperatures and its content in the reaction mixture continuously decreased during the NMR experiment, down to only ca. 10% at 70 °C. Moreover, the ^{31}P NMR signals of the simultaneously formed *H*-phosphonate monoester **1** overlapped partly with the signals of **2**, making precise integration difficult.

[††] The (dimethylamino)methylene protection of cytosine is very labile and was easily cleaved during the purification process. Thus, *H*-phosphonate **1** (B = Cyt^{dmm}) was prepared from **1** (B = Cyt^{Bz}) by deprotection of the cytosine moiety with pyridine–water–ammonia solution, followed by re-protection with *N*,*N*-dimethylformamide dimethylacetal.

[‡‡] For the reactions performed in the presence of nucleophilic catalysts, an equilibrium of diastereomers of P-[nucleophile]$^+$ phosphoammonium adducts was found to take over the role of the **2**-D_P ⇌ **2**-L_P equilibrium shown in Figure 12. This did not change the general idea of the mechanism and may be neglected in general considerations. The effect of acid, base and nucleophilic catalysts on the ribonucleoside 3′-*H*-phosphonate condensation with alcohols was discussed in detail in a separate article.[23] Among the bases investigated, 2,6-lutidine was found to be the amine of choice (quantitative yield, de ~ 70%) and it was used in further experiments.

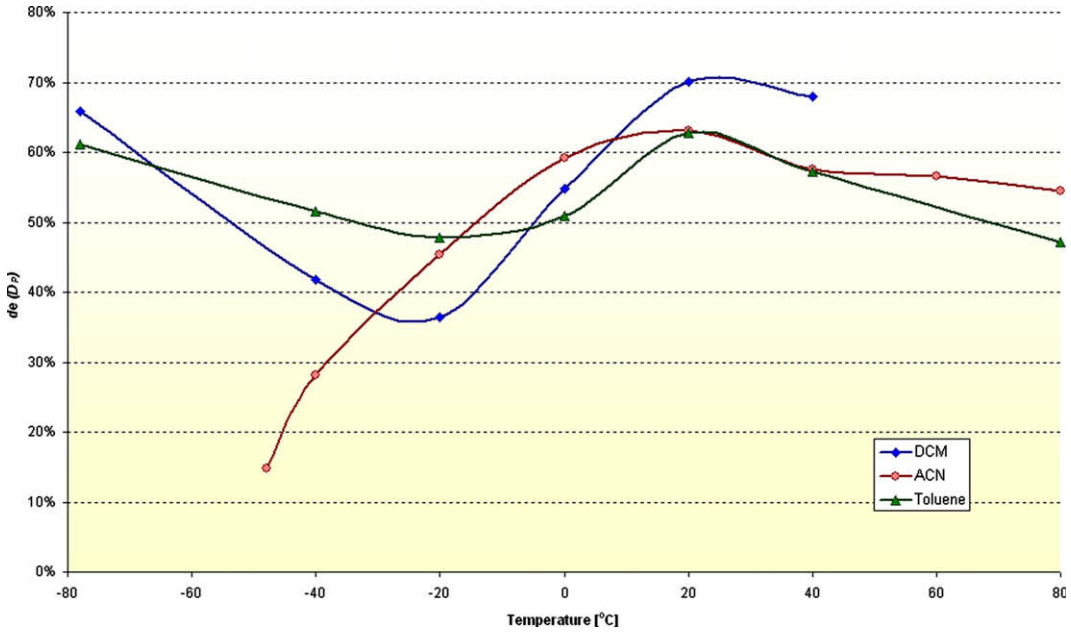

Figure 6. Diastereomeric excess of the $D_P(S_P)$-diastereomer of *H*-phosphonate diester **3b** formed at various temperatures. Reaction conditions: 0.05 mmol of **1** (B = Ura) + EtOH (3 equiv) + 2,6-lutidine (3.6% v/v; 3 equiv) + PvCl (1.5 equiv) in DCM (0.5 mL).

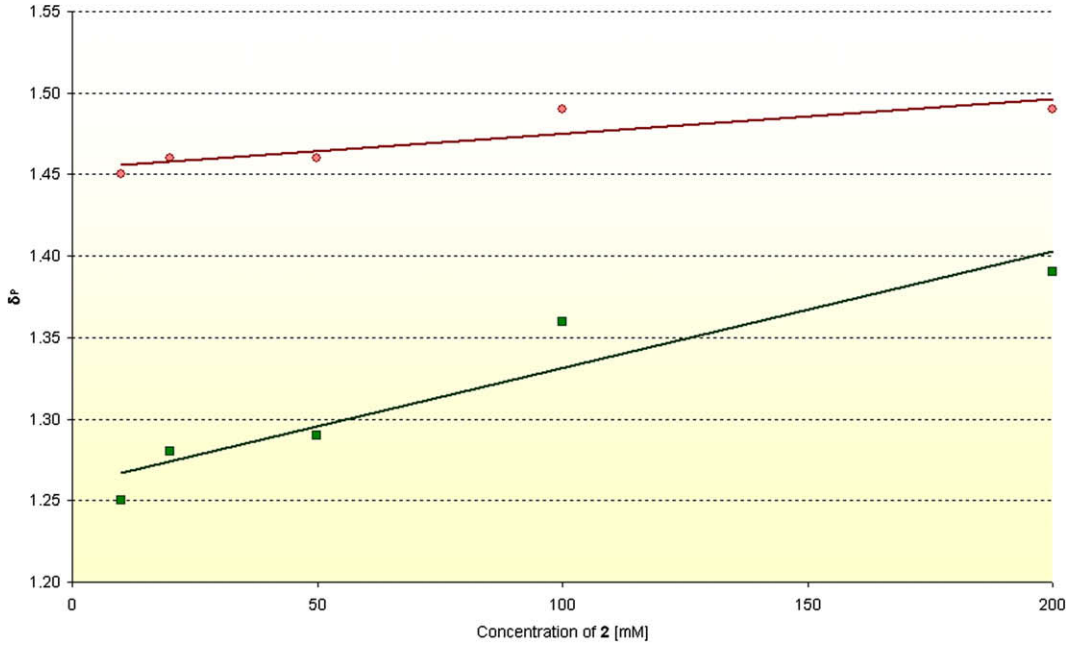

Figure 7. ^{31}P Chemical shifts of the diastereomers of the mixed anhydride **2** (B = Ura) [○—L_P (minor); ■—D_P (major)] in DCM as a function of concentration of **2** ($D_P + L_P$).

Table 2
Compilation of reaction conditions used for studies on the effects of the substrates concentrations

Entry	*H*-Phosphonate **1** (mM)	EtOH equiv (mM)	Best stereoselectivity
1 (Fig. 8)	25	1.2–150	<3 equiv EtOH
2 (Fig. 9)	10–200	(1700)	[**1**] = 10 mM
3 (Fig. 10)	6.25–200	5.0	[**1**] < 25 mM

base used, the nature of an acid and the type of a condensing agent. These variables were investigated in this study.

3.1. Effect of solvents on the stereoselectivity

The kind of a solvent may affect stereoselectivity of the condensation of ribonucleoside *H*-phosphonates in several ways. Firstly, since esterification of the mixed anhydride **2** is believed to be of $S_N2(P)$ type and involves uncharged species, the increase in polarity of a solvent should enhance the rate of this reaction, while the reaction with the pivalate anion (P-epimerization) might be expected to be slowed down. Furthermore, it was plausible that diverse solvation of ribonucleoside 3′-*H*-phosphonate

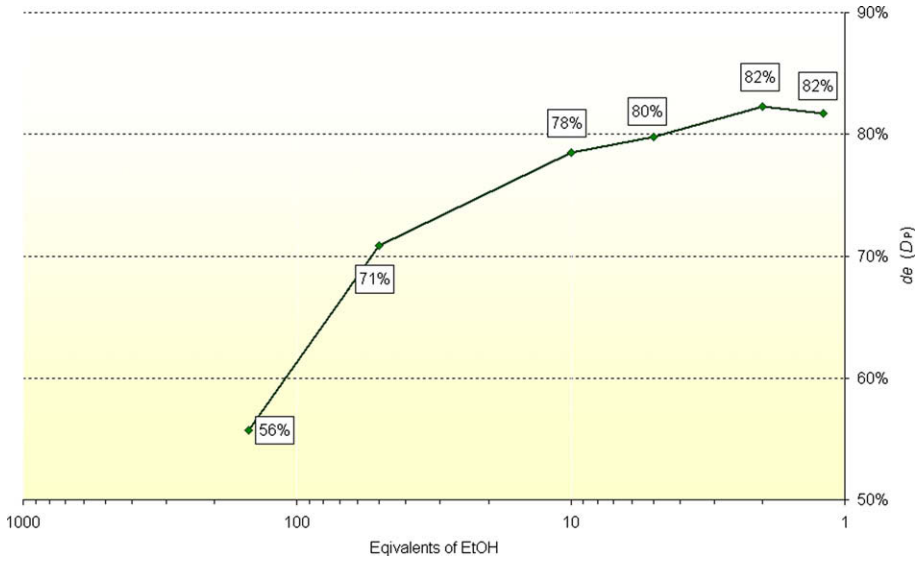

Figure 8. Diastereomeric excess of the $D_P(S_P)$-diastereomer of *H*-phosphonate diester **3b** formed in the presence of different excesses of EtOH. Reaction conditions: 50 μmol of **1** (B = Ura) + EtOH (1.2–150 equiv) + 2,6-lutidine (20 equiv) + PvCl (2 equiv) in DCM (up to total volume of 2.0 mL).

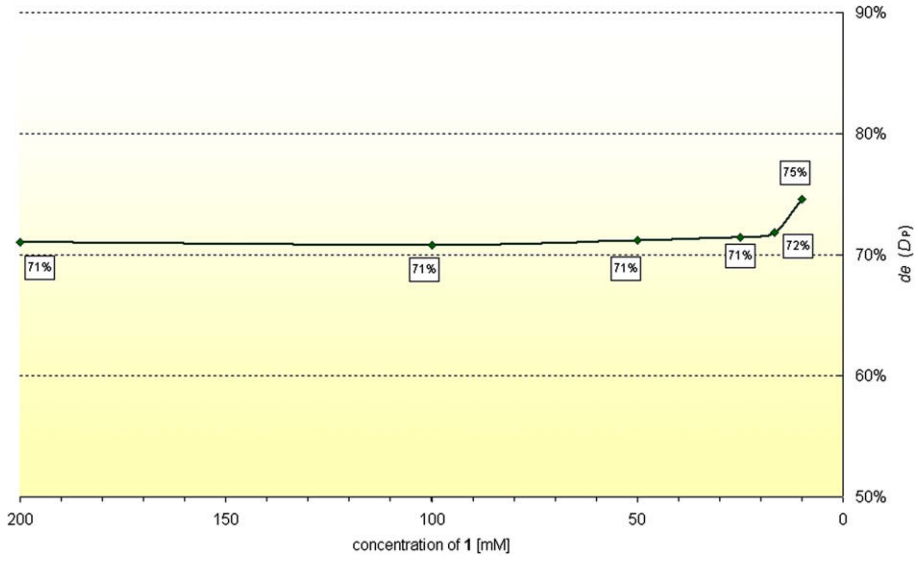

Figure 9. Diastereomeric excess of the $D_P(S_P)$-diastereomer of *H*-phosphonate diester **3b** formed in various concentrations of *H*-phosphonate **1**. Reaction conditions: 5–100 μmol of **1** (B = Ura) + EtOH (50 μL, 850 μmol) + 2,6-lutidine (100 μL, 850 μmol) + PvCl (3 equiv with respect of **1**) in DCM (350 μL). A higher excess of 2,6-lutidine and EtOH was used in order to maintain pseudo-first order kinetic conditions for all reactions investigated.

molecules in various solvents might create conformational changes in the ribose ring, altering in that way the spatial environment of the activated *H*-phosphonate function. Additionally, the solvent may affect a hypothetical P–H···$O^{2'}$ electrostatic interaction that can be considered as an underlying source of different reactivity of the P-epimers of the mixed anhydride **2**. In order to evaluate the possible influence of different solvents on stereoselectivity, de was plotted against principal properties (PPs)[34] or against the relative permittivity of the solvents used. Neither of the PPs parameters showed regularity with respect to the stereoselectivity achieved. However, when the solvents were arranged according to their relative permittivity, a maximum stereoselectivity was found for a moderately polar DCM (de 71%), while less or more polar solvents gave inferior results (Fig. 3). These changes in stereochemistry indicated that possibly neither of the solvent effects mentioned above played a dominant role during the condensations, and the reactive species formed during the course of reactions were probably involved in complex interactions with the solvents at different stages of the *H*-phosphonate diester formation.

3.2. Effect of base concentration on the stereoselectivity

According to Almer et al., the replacement of neat pyridine with mixtures of ACN–pyridine had a profound favourable effect only on stereoselectivity of the condensation of a guanosine derivative **1** (B = Gua^{PAC}), while for the other ribonucleoside *H*-phosphonates the effect was rather small or none at all.[15] However, when DCM–pyridine (2.4% v/v) was used as a reaction medium, an improved stereoselectivity was observed for almost all 24 investigated reactions of *H*-phosphonates **1** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu} and Ura) with four nucleosides and ethanol.[17]

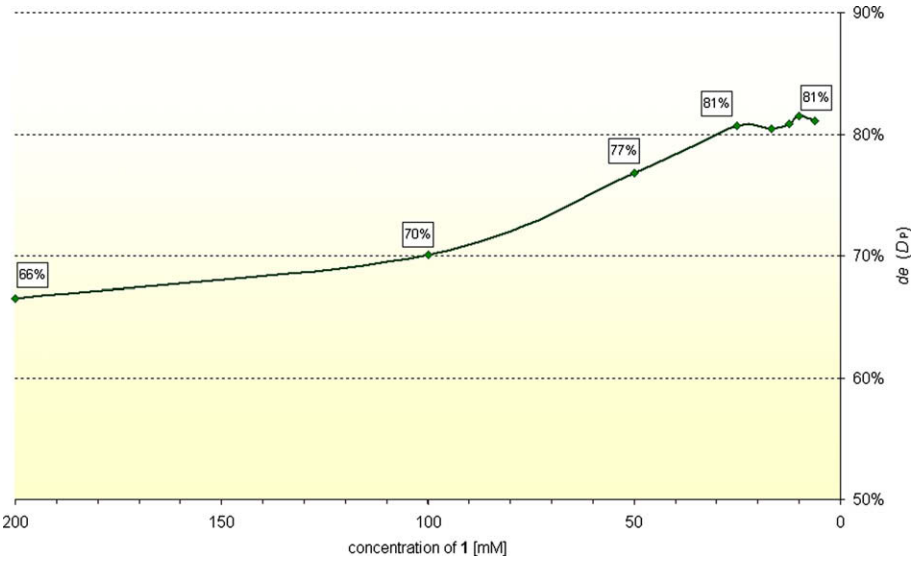

Figure 10. Diastereomeric excess of the $D_P(S_P)$ diastereomer of diester **3b** formed in various dilutions of reagents. Reaction conditions: 50 μmol of **1** (B = Ura) + EtOH (5 equiv) + 2,6-lutidine (3.6% v/v) + PvCl (2 equiv) in DCM (up to total volume of 0.5–5.0 mL).

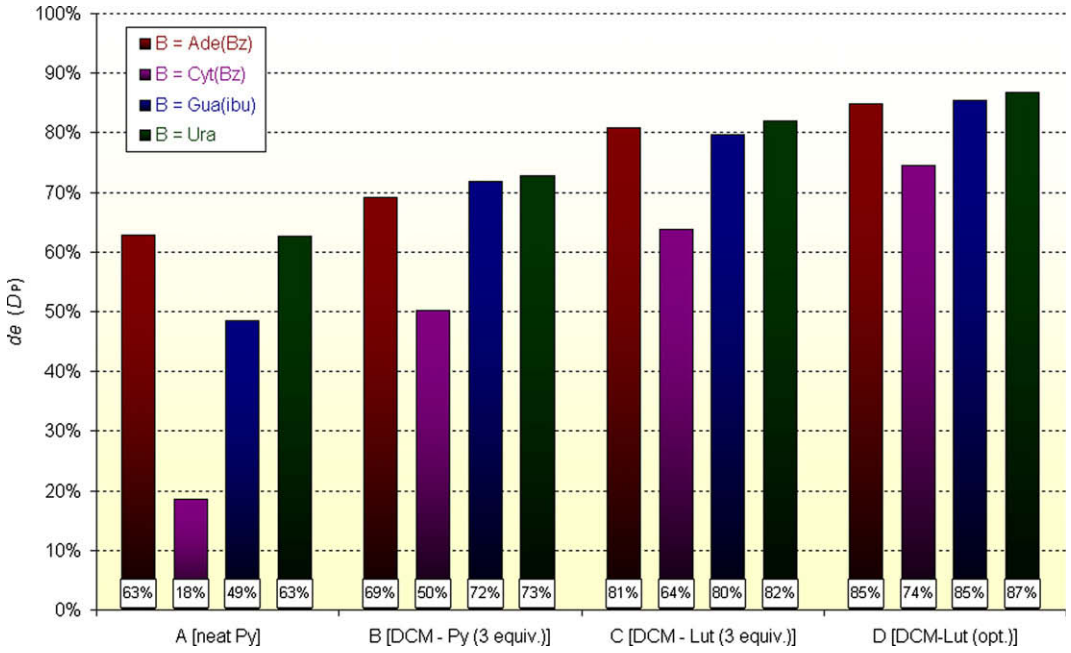

Figure 11. Diastereomeric excess of the $D_P(S_P)$-diastereomer of dinucleoside *H*-phosphonate **3a** under various reaction conditions: 50 μmol of **1** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu} or Ura) + 2′,3′-di-*O*-benzoyluridine (1.2 equiv) + PvCl (2.5 equiv) in (A) neat pyridine (0.5 mL); (B) DCM (0.5 mL) containing pyridine (3 equiv); (C) DCM (0.5 mL) containing 2,6-lutidine (3 equiv); (D) DCM (2.0 mL) containing 2,6-lutidine (7% v/v; 24 equiv).

In this study, we tried to optimize the contents of 2,6-lutidine in the reaction mixtures to maximize the stereoselectivity. According to the results shown in Figure 4, for adenosine, cytidine and uridine *H*-phosphonates **1** (B = Ade^{Bz}, Cyt^{Bz} and Ura), the optimal concentration of 2,6-lutidine was ca. 7–12% (v/v), and for guanosine derivative (B = Gua^{ibu}), ca. 2–4% (v/v). These variations in optimal base concentration may be attributed to a different reactivity of various nucleoside *H*-phosphonates, and thus, different DYKAT parameters. Although the above concentrations of 2,6-lutidine gave the highest stereoselectivity for individual nucleoside *H*-phosphonates, for practical purposes, it may be more convenient to use a concentration of ca. 7% (v/v) of this base in DCM for all four *H*-phosphonate monoesters **1**. At this concentration of 2,6-lutidine, the stereoselectivity of the condensations was close to its highest value in all the cases.

3.3. Effect of condensing agents (C.A.'s) on the stereoselectivity

Pivaloyl chloride, which is the most commonly used C.A. for *H*-phosphonate diester formation,[35] was used as a primary C.A. in our previous and current studies. However, several other C.A.'s were also reported to be effective in *H*-phosphonate condensations.[27,35–41] Since for various C.A.'s, different mechanisms might be involved or the rates of the DYKAT reactions could be altered, the kind of C.A. may affect the stereochemical outcome of reactions.

Thus, several C.A.'s, including various acyl chlorides and chlorophosphates, as well as representatives of other classes of activating reagents, such as arylsulfonyl derivatives, onium salts and diimides, were tested with respect to their ability to promote stereoselective condensation of ribonucleoside 3′-*H*-phosphonate monoesters (Fig. 5). The best results were obtained for acyl chlorides, with PvCl being slightly better over the others; this C.A. was therefore used in further studies. The 1.2 molar excess of PvCl (relatively to *H*-phosphonate monoester **1**) was found to be sufficient for effective condensation of **1** with ethanol. However, in order to avoid the detrimental effects of adventitious water, using 2–3 equiv of PvCl is advisable. These variations in the excess of PvCl were found to have no effect on the stereoselectivity of the reactions investigated (data not shown).

3.4. Effects of chiral bases and chiral C.A. on the stereoselectivity

According to the DYKAT mechanism of asymmetric induction assumed for the investigated reactions, an application of chiral axillaries should have little or no effect on the stereoselectivity of the condensations. Indeed, the stereochemistry of reactions performed in the presence of (−)-sparteine and (*S*)-(−)-nicotine did not differ significantly from those in which pyridine or (±)-nicotine was used as bases. Also, a chiral condensing agent, (−)-camphanoyl chloride, gave similar stereochemical output as did achiral acyl chlorides (Fig. 5). Thus, it was concluded that the chiral properties of the reaction medium and the sense of chirality of the P-leaving group did not alter the k_{D_P}/k_{L_P} ratio (Fig. 12) in the ribonucleoside *H*-phosphonate condensations.

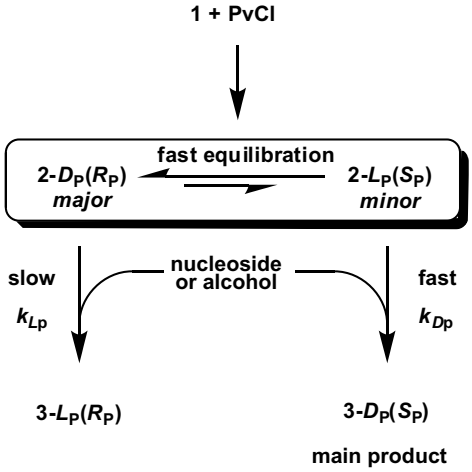

Figure 12. The DYKAT mechanism for the stereoselective formation of the $D_P(S_P)$-diastereomer of *H*-phosphonate diester **3**.

3.5. Effect of temperature on the stereoselectivity

Since the temperature dependence of the equilibrium (**2**-$D_P \rightleftharpoons$ **2**-L_P) and that of the esterification of diastereomers of the mixed anhydride **2** might be different, the stereoselectivity might vary with temperature. We previously found[17] that the chemical shifts of the ^{31}P NMR signals of the individual diastereomers of the mixed anhydride **2** in toluene were changed to a different extent with temperature, and above 50 °C the positions of the signals were inverted. The signals, however, remained sharp in the whole temperature range investigated and their ratio underwent only minor variations that could be attributed to experimental errors (Table 1). Thus, it was concluded that the observed changes in the chemical shifts did not reflect changes in the equilibrium rates.

The influence of temperature on the stereoselectivity of the condensations was determined for DCM, ACN and toluene, and the results are summarized in Figure 6. For all solvents investigated, the maximum of stereoselectivity was observed at ca. 20 °C. Additionally, in DCM and toluene, a minimum stereoselectivity was observed around −20 °C, while for ACN, the stereoselectivity decreased steadily until the freezing point of the system was reached. At the moment, we cannot provide a mechanistic explanation for these results. Based on the collected data, the optimal temperature of 20 °C was used in the further condensation reactions.

3.6. Effect of the substrates ratios on the stereoselectivity

According to the DYKAT mechanism (Fig. 12), it might be expected that by decreasing the rates of esterification of the mixed anhydride **2** and maintaining a high rate of **2**-$D_P \rightleftharpoons$ **2**-L_P equilibrium, the stereoselectivity of the condensation could be improved. This, in principle, could be achieved by decreasing the excess of an alcohol used for the reaction, and indeed, it was found that the stereoselectivity was increasing gradually with a decreasing concentration of ethanol, reaching a plateau of de ∼ 82% at 1.2–3 equiv of EtOH (Fig. 8). These results were consistent with the previously observed decrease or reversal in stereoselectivity, when the esterification rate was significantly enhanced by using a large excess of a reactive alcohol (methanol).[22]

3.7. Effect of concentration of the substrates on the stereoselectivity

In the section above, the positive effect of a low excess of the hydroxylic component on stereoselectivity in the condensation of ribonucleoside *H*-phosphonates was found. The influence of a concentration of *H*-phosphonate monoesters **1** on the stereoselectivity was less obvious, so at first we investigated the effect of concentration of **1** on a diastereomeric composition of the intermediate mixed anhydride **2** formed in situ. In the ^{31}P NMR spectra, only minor changes in the chemical shifts of the signals from **2** (B = Ura) were observed, and the ratio of its diastereomers remained constant (Fig. 7). Similarly, as for the variable temperature experiments (vide supra), these changes could not be attributed to the averaging of the signals due to ligand exchange, as no broadening of the ^{31}P resonances was observed. However, a larger separation of the ^{31}P NMR signals of the diastereomers of **2** in diluted solutions might suggest larger structural differences between the diastereomers under such reaction conditions.

In next experiments, *H*-phosphonate monoester **1** in various concentrations (10–200 mM) was condensed with EtOH (1.7 M). A moderate increase in stereoselectivity (de ∼ 71%→∼75%) was observed only at the highest dilution of **1** (10 mM; Fig. 9). However, when the concentrations of **1** and EtOH were changed simultaneously, a distinct increase in stereoselectivity with a decreasing concentration of the substrates (**1** + 5 equiv of EtOH) was found (from de ∼ 70% at 100 mM of **1** to de ∼ 81% at 25 mM of **1**; Fig. 10). Decreasing the concentration further had no effect on the stereochemistry of the reaction, while completion of the condensation became difficult. Therefore, the optimal concentration of *H*-phosphonate **1** was established as 25 mM.

Summarizing these model experiments, the reaction conditions under which the highest yield and stereoselectivity of the reaction of *H*-phosphonate monoester **1** with ethanol could be achieved were found to be 25 mM concentration of *H*-phosphonate **1** in DCM, 1.2 equiv of an alcohol, 2,6-lutidine (ca. 7% v/v), PvCl as a

condensing agent (2–3 equiv) and at ambient reaction temperature.

When the above conditions were applied for the condensation of *H*-phosphonates **1** with a nucleoside (2′,3′-*O*-dibenzoyl uridine), a practically quantitative yield and an excellent stereoselectivity were obtained (^{31}P NMR). For 3′-*H*-phosphonates derived from various nucleosides, diastereomeric excess of 82–87% for B = Ade^{Bz}, Cyt^{pya}, Gua^{ibu} or Ura, and of ca. 75% for B = Cyt^{Bz} was achieved (Fig. 11). This was a considerable improvement in comparison to the de of ca. 50–60% (B = Ade^{Bz}, Gua^{ibu} or Ura) and 20% for B = Cyt^{Bz} when the reactions were performed in pyridine.

An application of the developed reaction conditions to the solid phase stereoselective synthesis of oligoribonucleotide *H*-phosphonates is currently in progress in our laboratory.

4. Conclusions

We have investigated the reaction conditions for the condensations of ribonucleoside 3′-*H*-phosphonates with alcohols and nucleosides in order to attain the highest stereoselectivity. In this respect, the most important factors were found to be the type of a base, concentration of the reactants and the ratio of ribonucleoside *H*-phosphonate and the hydroxylic component. The stereoselectivity also appeared to be sensitive to changes in temperature of the reaction and to polar properties of the medium, while the influence of chirality of the solvents, bases and condensing agents was insignificant. Acyl chlorides, especially pivaloyl chloride, were the condensing agents of choice.

The application of the developed conditions to the condensations of commercially available ribonucleoside *H*-phosphonates bearing a standard set of protecting groups, in most cases yielded a diastereomeric excess of ca. 85% in favour of the $D_P(S_P)$-diastereomer of the formed dinucleoside *H*-phosphonates.

The optimized reaction conditions were consistent with the assumed DYKAT scheme for the asymmetric induction, but the mechanistic role of several factors remains unclear and requires further investigations. This study also indicated that by tuning the steric and electronic properties of the protecting groups in the *H*-phosphonate monoesters **1**, further increase in the stereoselectivity might be possible, and this is a subject of current study in our laboratory.

5. Experimental

5.1. General

Chemicals, instruments and procedures were similar to those reported earlier.[22–24] The yields and diastereomeric excess were calculated with accuracy of ±1.5 percentage points (an average of three ^{31}P NMR measurements).

1-*n*-Butyl-3-methylimidazolium hexafluorophosphate ([bmim][PF_6]) was rendered anhydrous by triple co-evaporation with dry acetonitrile. 5,5-Dimethyl-2-oxo-2-chloro-1,3,2-dioxaphosphorinane (NEP-Cl)[42] and 6-nitrobenzotriazol-1-y1-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyNOP)[43] were obtained according to the published methods. Racemization of (*S*)-(−)-nicotine was performed according to Ref. 44.

Nucleoside *H*-phosphonates **1** (B = Ade^{Bz}, Cyt^{Bz}, Gua^{ibu} and Ura)[45] and (B = Cyt^{pya})[32,45] were obtained according to the published methods. Immediately prior to a reaction, all nucleosidic derivatives in appropriate amounts were rendered anhydrous by dissolving in DCM (0.5 mL/0.05 mmol) followed by addition of toluene (3 mL/0.05 mmol) and evaporation of these solvents under reduced pressure (pyridine was avoided in order not to introduce traces of a nucleophilic catalyst to the reaction mixture). After drying under vacuum (15 min, 0.5 Torr), the flask was filled with air dried by passing through Sicapent (Merck).

5.2. Standard procedure for condensation of *H*-phosphonates of type 1 with ethanol

Nucleoside *H*-phosphonate **1** (0.05 mmol) was dissolved in 0.5 mL of DCM, and 2,6-lutidine (3 equiv) and EtOH (3 equiv)[§§] were added, followed by PvCl (1.5 equiv). ^{31}P NMR spectra were recorded immediately after mixing the reactants. For details and variations, see in the text.

5.3. General procedure for condensation of *H*-phosphonates of type 1 with 2′,3′-*O*-dibenzoyl-uridine

Nucleoside *H*-phosphonate **1** (0.05 mmol) and 2′,3′-*O*-dibenzoyl-uridine (1.2 equiv, 28 mg) were mixed together, rendered anhydrous (vide supra) and dissolved in appropriate solvent mixture (as described in the text). To this, PvCl (2.5 equiv) was added and after ca. 10 min of vigorous stirring ^{31}P NMR spectra were recorded.

5.4. Variable temperature ^{31}P NMR studies on the *H*-phosphonic–pivalic mixed anhydride 2

Nucleoside *H*-phosphonate **1** (0.05 mmol) was dissolved in 0.5 mL of DCM containing 3 equiv of 2,6-lutidine. PvCl (2 equiv) was added and the mixture was transferred to the NMR tube. The temperature of the sample was set to the lowest investigated value (0 or 20 °C) and increased gradually, recording the spectra at the desired temperatures.

5.5. Condensation of *H*-phosphonates of type 1 with ethanol under the Mitsunobu conditions

To a solution of 0.15 mmol of diethoxytriphenylphosphorane (prepared in situ according to the procedure of Camp and Jenkins[46]) in DCM (250 μL) a solution of 0.05 mmol of *H*-phosphonate 1 (B = Ura) in DCM (250 μL) was added under nitrogen atmosphere at rt and after ca. 10 min a ^{31}P NMR spectrum was recorded.

5.6. 5′-*O*-Dimethoxytrityl-4-*N*-[*N*-(dimethylamino)methylene]-2′-*O*-*tert*-butyldimethylsilylcytidine 3′-*H*-phosphonate triethylammonium salt, 1 (B = Cyt^{dmm})

H-Phosphonate **1** (B = Cyt^{Bz}, 2.79 g, 3 mmol) was dissolved in pyridine (3 mL) and 25% aqueous ammonia (10 mL) was added. The progress of the debenzoylation was monitored with TLC. After completion of the reaction (5 h), the solvents were evaporated under reduced pressure and the residue obtained was dissolved in DCM and applied to a short silica gel column using 5% (v/v) MeOH in DCM followed by 5% (v/v) MeOH + 2% (v/v) TEA in DCM to elute by-products, and 10% (v/v) MeOH + 3% (v/v) TEA in DCM to elute pure **1** (B = Cyt^{NH_2}). Yield: 2.18 g (88%). R_f 0.29 (DCM–MeOH–TEA, 85:10:5); ^{31}P NMR (DCM): δ = 1.67 (dd, $^1J_{PH}$ = 613.6 Hz, $^3J_{PH}$ = 9.6 Hz); ^{1}H NMR (in $CDCl_3$) δ = 7.92 (d, ^3J = 7.4 Hz, 1H, 6-H), 7.13–7.39 (m, 9H, aromatic protons), 6.88 (d, ^1J = 621 Hz, 1H, P–H), 6.78 (d, ^3J = 8.6 Hz, 4H, aromatic protons), 5.93 (d, ^3J = 3.2 Hz, 1H, 1′-H), 5.40 (d, ^3J = 7.6 Hz, 1H, 5-H), 4.67 (m, 1H, 3′-H), 4.38 (m, 1H, 2′-H), 4.35 (m, 1H, 4′-H), 3.72 (s, 6H, CH_3O), 3.52 (dd, ^1J = 10.8 Hz, ^3J = 1.5 Hz, 1H, 5′-H), (dd, ^1J = 10.8 Hz, ^3J = 2.6 Hz, 1H, 5″-H), 2.95 (q, ^3J = 7.3 Hz, 6H, CH_3CH_2N), 1.23 (t, ^3J = 7.3 Hz, 9H, CH_3CH_2N), 0.89 (s, 9H, CH_3C), 0.11 (2 × s, 6H, CH_3Si).

[§§] Using EtOH instead of a nucleosidic substrate circumvented possible solubility problems, while the stereochemical outcome was expected to be very similar to the condensations with nucleosides.[17]

Compound **1** (B = Cyt^{NH_2}, 1.65 g, 2 mmol) was dissolved in DMF (15 mL) after which *N*,*N*-dimethylformamide dimethylacetal (1.5 mL) was added.[32,33] After 4 h, the reaction was complete (TLC analysis) and the reaction mixture was evaporated to dryness under reduced pressure. Silica gel column chromatography using a stepwise gradient of MeOH (1–5%) in DCM–TEA (99:1, v/v) as an eluent afforded the title compound contaminated with ca. 15% of **1** (B = Cyt^{NH_2}) due to on-column decomposition. Yield: 1.45 g (82%). R_f 0.45 (DCM–MeOH–TEA, 85:10:5); ^{31}P NMR (DCM): $\delta = 1.76$ (dd, $^1J_{PH} = 614.5$ Hz, $^3J_{PH} = 9.7$ Hz); ^1H NMR (in $CDCl_3$) $\delta = 8.80$ (s, ^1H, N=*CH*–N<), $\delta = 8.02$ (d, $^3J = 7.2$ Hz, 1H, 6-H), 7.16–7.42 (m, 9H, aromatic protons), 6.87 (d, $^1J = 621$ Hz, 1H, P–H), 6.81 (d, $^3J = 8.1$ Hz, 4H, aromatic protons), 6.02 (d, $^3J = 3.6$ Hz, 1H, 1′-H), 5.67 (d, $^3J = 7.5$ Hz, 1H, 5-H), 4.68 (m, 1H, 3′-H), 4.44 (t, 1H, $^3J = 3.9$ Hz, 2′-H), 4.38 (m, 1H, 4′-H), 3.75 (s, 6H, CH_3O), 3.50 (m, 2H, 5′-H, 5″-H), 3.09 (2 × s, 6H, NMe_2), 2.98 (q, $^3J = 7.3$ Hz, 6H, CH_3CH_2N), 1.25 (t, $^3J = 7.3$ Hz, 9H, CH_3CH_2N), 0.88 (s, 9H, CH_3C), 0.15 and 0.13 (2 × s, 6H, CH_3Si).

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