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2′-DEOXYRIBONUCLEOSIDE 3′-ARYL PHOSPHORANILIDATES

KEY INTERMEDIATES IN THE STEREOSPECIFIC SYNTHESIS OF 2′-DEOXYRIBONUCLEOSIDE CYCLIC 3′,5′-PHOSPHOROTHIOATES AND DINUCLEOSIDE(3′→5′)-PHOSPHOROTHIOATES†

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Abstract—Phosphorylation of the 5′-O-monomethoxytrityl-2′-deoxyribonucleosides by means of aryl phosphoranilidochloridates gives the diastereoisomers of 5′-O-monomethoxytrityl-2′-deoxyribonucleoside 3′-aryl phosphoranilidates Their separation can be performed by means of chromatographic techniques They can be further converted to the 2′-deoxyribonucleoside cyclic 3′-5′ phosphoranilidates, which are intermediates in the stereospecific synthesis of 2′-deoxyribonucleoside cyclic (3′-5′)phosphorothioates of known absolute configuration at phosphorus

The stereospecific synthesis of nucleoside P-chiral phosphorothioates and [^{18}O]-phosphates is the continuing goal in our research program. The availability of such compounds of known absolute configuration at P facilitates investigations of the stereochemical course of enzymatic reactions involving cleavage of P–O bond in nucleotides[1] as well as the studies on the "mapping" of active sites of enzymes responsible for phosphoryl and nucleotidyl transfer.[2] In this paper we wish to describe the synthesis of deoxyribonucleoside 3′-aryl phosphoranilidates (**1**), their transformations leading into P-achiral deoxyribonucleoside phosphoranilidates (**2**) and aryl phosphates (**3**), or separation of **1** into diastereoisomers and further stereospecific synthesis of deoxyribonucleoside 3′-aryl phosphorothioates (**4**). The synthetic potential of the phosphoranilidate intermediates (**1**) is further emphasized in their simple transformation into deoxyribonucleoside cyclic 3′,5′-phosphoranilidates (**5**) which can be easily converted to diastereoisomeric nucleoside cyclic 3′,5′-phosphorothioates (**6**) and [^{18}O]-phosphates (**7**). Independently, the compounds (**2**) can be used in the synthesis of dideoxyribonucleoside (3′→5′)-phosphoranilidates (**15**) which after separation into pure diastereoisomers may be stereospecifically converted into dideoxyribonucleoside(3′→5′)-phosphorothioates (**9**). These results were reported briefly in several preliminary communications [3–9]

RESULTS

The synthesis of key-intermediates

2′-deoxyribonucleoside 3′-aryl phosphoranilidates. With few exceptions,[10–14] the syntheses of diastereoisomerically pure nucleoside phosphorothioates[15–18] have been based on the stereoselective enzymatic degradation of mixtures of diastereoisomers Our original strategy for the stereospecific chemical synthesis of nucleoside phosphorothioates relies upon the use of chiral (*but racemic*) phosphorylating agents, aryl phosphoranilidochloridates (**10**)[4–8] in the synthesis of 2′-deoxyribonucleoside 3′-aryl phosphoranilidates (**1**) which, by virtue of chirality of 2′-deoxyribose moiety and dissymmetry at P consist of diastereoisomeric mixtures, which are easily separable by chromatography. From several aryl phosphoranilidochloridates (**10**), we have found the 2-chlorophenyl derivative (**10γ**) to be the most convenient phosphorylating agent

Besides its good phosphorylating properties towards 5′-protected 2′-deoxyribonucleoside derivatives (**12**), **10γ** is an easily available and stable compound, and the corresponding 5′-O-monomethoxytrityl-2-′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphoranilidates (**11**) may readily be separated into their constituent diastereoisomers. It has to be emphasized that the diastereoisomeric homogeneity of 2-chlorophenyl esters (**11**) can be easily monitored by means of ^{31}P-NMR spectroscopy as the phosphorus resonance signals of pairs of diastereoisomers differ. Phosphorylation of 5′-O-monomethoxytrityl-2′-deoxyribonucleosides (**12**) may be performed in pyridine solution using a 50% molar excess of **10γ** In the case of **12** (B=Thy), and **12** (B=Ade) the yields of **11** were satisfactory (Table 2, method A). However, when the same conditions were used for **12** (B=Gua) and **12** (B=Cyt) the yields of compounds **11** (B=Gua) and **11** (B=Cyt) were very low (6 and 16%, respectively)

Such poor results were avoided when corresponding 5′-O-monomethoxytrityl-2′-deoxyribonucleosides were treated with 50% molar excess of **10γ** in acetonitrile solution in the presence of 6-fold molar excesses of 1,2,4-triazole and triethylamine (Table 2, method B). The progress of the reaction was monitored by ^{31}P-NMR spectroscopy. The optimal reaction time was estimated to 20 hr. The nearly equi-

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†Dedicated to Prof F Cramer on the occasion of his 60th birthday

Table 1 The yields, ^{31}P-NMR and chromatographic characteristics of 5′-O-monomethoxytritylthymidine 3′-aryl phosphoranilidates (**11**, B=Thy)

Compounds **11**	Ar	Yield [a] (%)	^{31}P-NMR δ (ppm) [b]	Δδ (ppm)	R_f [c]	ΔR_f
11α	C_6H_5-	80	-2 55 -2 49	0.06	0.20 0.27	0 07
11β	4-ClC_6H_4-	100	-2.83 -2.72	0 11	0 24 0 33	0.09
11γ	2-ClC_6H_4-	87	-3.03 -2.75	0 28	0.20 0.31	0.11
11δ	2,4-Cl_2-C_6H_3-	87	-2 81 -2.59	0.22	0 24 0 38	0 14
11	4-NO_2-C_6H_4-	90	-3.17 -3 37	0 20	0 24 0.33	0 09

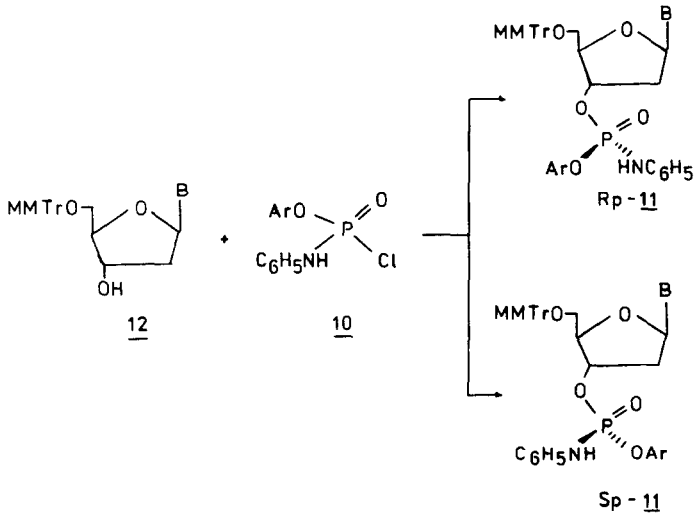
[a] Yield was assayed by means of ^{31}P-NMR
[b] Parameters taken from recording the spectra of reaction mixture
[c] Silica gel, developing system chloroform-acetone (10 3)

Table 2 The yields and physico-chemical characteristics of 5′-O-monomethoxytrityl-2′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphoranilidates (**11**)

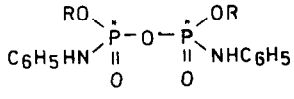
Compounds **11**	Yield [a] (%)	TLC R_f	UV (96% C_2H_5OH) (nm) λ_{max}	UV (96% C_2H_5OH) (nm) λ_{min}	[α] ($CHCl_3$) λ_{589}	[α] ($CHCl_3$) λ_{439}	^{31}P-NMR (C_5H_5N) δ (ppm)	MS (m/z)
Sp-**11γ** (B=Thy)	78[b]	0 74(S_1) 0 34(S_5)	268.7	251.1	+12 1	+24 1 (c 2 1)	-2.25 (c 2 1)	780 $(M+1)^+$
Rp-**11γ** (B=Thy)		0.60(S_1) 0 26(S_5)	270 5	251.0	+21.7	+45.9 (c 1 9)	-2.75 (c 1 91	780 $(M+1)^+$
Sp-**11γ** (B=Ade)	67[b]	0.39(S_1) 0.46(S_4)	263.5	24/ 0	-7 3	-17.4 (c 1 3)	-2.50 (c 1 3)	788 $M^{+\cdot}$
Rp-**11γ** (B=Ade)		0 33(S_1) 0.41(S_4)	263 5	247 0	+14 7	+32 1 (c 0.7)	-2.76 (c 0 7)	788 $M^{+\cdot}$
Sp-**11γ** (B=Gua)	51[c]	0.34(S_1) 0.81(S_2)	shoulders	267.5, 250 0, 227.5	+57 8	+136.6 (c 1.0)	-2 73 (c 1 0)	804±2 [d]
Rp-**11** (B=Gua)		0.29(S_1) 0.72(S_2)	shoulders	267.5, 250.0, 227 5	-8.2	-23.2 (c 3 1)	-3 18 (c 3.1)	804±2 [d]
Sp-**11ε** (B=Cyt)	55[c]	0.69(S_1) 0 47(S_4)	275 5	258.3	+33.8	+88 3 (c 1.1)	-2.60 (c 1 1)	763 $(M-1)^+$
Rp-**11ε** (B=Cyt)		0.62(S_1) 0 38(S_4)	275 5	258.3	+38 9	+95.0 (c 1 3)	-2.82 (c 1.3)	763 $(M-1)^+$

[a] Yield of diastereoisomeric mixture after isolation from reaction mixture by means of short column chromatography
[b] Phosphorylation according to method A
[c] Phosphorylation according to method B
[d] **11γ** (B=Gua), mol weight 805 28

molar ratio of diastereoisomers was observed in crude mixtures with only one exception, in the case of thymidine the ratio of the less polar to the more polar (as indicated by TLC) diastereoisomers (**11γ**) was 1 6. ^{31}P-NMR spectroscopy indicated that the phosphorus atom in the predominant diastereoisomer resonated at lower field The separation of individual diastereoisomers (**11**) was achieved by means of short-column silica gel chromatography. The structure of the diastereoisomeric species was ascertained by means of FD–MS technique (Table 2) It should be noticed that during the preparation of **11**, a side-product **19a** and, in the case of **12** (B=Ade) and **12** (B=Gua), corresponding diphosphorylated species, **11γ** {B=[N^6-(2-chlorophenyl)phosphoranilido-]2′-deoxyadenin-9-yl, *m/z* 1053}, and **11γ** {B=[O^6-(2-chlorophenyl)-phosphoranilido-]2′-deoxyguanin-9-yl, *m/z* 1069}, were isolated and identified by means of FD–MS



Scheme 1.



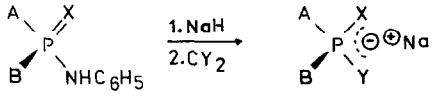
19a, R = 2-chlorophenyl, $\delta_{31p} = -14.1$ ppm and -14.3 ppm

19b, R = 5′-monomethoxytritylthymidyl-1-yl, $\delta_{31p} = -8.80$ ppm, -9.04 ppm and -9.22 ppm

The synthesis of 5′-O-monomethoxytrityl-2′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphorothioates (**13**) *and their 5′-unprotected derivatives* (**4**)

In our earlier work, we have described the simple conversion of dialkyl phosphoranilidates into dialkyl phosphates, dialkyl phosphorothioates and dialkyl phosphoroselenoates. We have also proved the full stereospecificity of this conversion and its stereoretentive nature.[19,20] This reaction is crucial for our amidodiester approach to the synthesis of nucleoside P-chiral phosphorothioates As in our model studies, we have found that treatment of each of diastereomer of **11**, in pyridine or dioxan solution, with sodium hydride followed by carbon disulphide gives diastereoisomers (**13**) in good yields. In the cases, **11γ** (B=Gua) and **11γ** (B=Cyt) dimethylformamide (DMF) was found to be better reaction medium due to the poor solubility of these substrates in pyridine or dioxan. Unfortunately, although the rate of conversion of **11γ** (B=Gua, Cyt) to **13** (B=Gua, Cyt) in DMF was markedly enhanced, as compared with that observed in pyridine or dioxan, the desired products were accompanied by larger amounts of unidentified side-products

Compounds **13**, in the form of pyridinium salts, were isolated from reaction mixtures and purified by means of preparative TLC on silica gel using



$A \neq B$, $X \neq Y$ $X = O, S, Se$ $Y = {}^{16}O, {}^{18}O, S, Se$

acetonitrile–water (9:1) as the eluting solvent. Table 3 contains the physicochemical characterization of individual phosphorothioates **13**.

For removal of 5′-O-monomethoxytrityl protective group compounds **13** were treated with 80% acetic acid and products **4** were purified by chromatography on DEAE-cellulose (triethylammonium bicarbonate as eluting buffer) and lyophilized Their yields and physical characteristics are collected in Table 4 The reaction sequence leading to **4** is shown in Scheme 2.

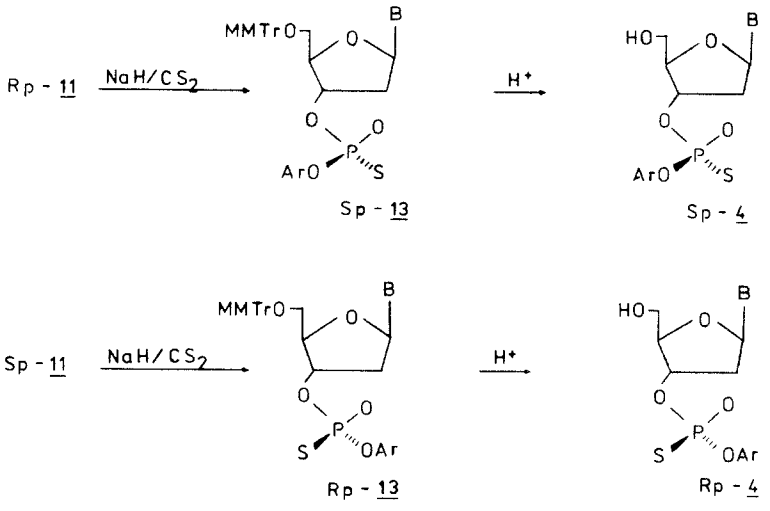
It has to be emphasized that treatment of **11γ** (B=Thy) with NaH/CO_2 gave sodium 5′-O-monomethoxytritylthymidine 3′-O-aryl phosphate (**3**) in 95% yield.

The synthesis of 2′-deoxyribonucleoside cyclic 3′,5′-phosphoranilidates (**5**) *and -phosphorothioates* (**6**)

As indicated in the Scheme 3, the conversion of the key intermediates (compounds **11**) into 2′-deoxyribonucleoside cyclic 3′,5′-phosphorothioates (**6**) had required the deprotection of the 5′-OH functions The latter were further involved in the process of intermolecular nucleophilic attack on the P atoms leading to 2′-deoxyribonucleoside cyclic 3′,5′-phosphoranilidates (**5**).

For this reason diastereoisomers of **11** were individually exposed to the action of 2% solution of toluene-*p*-sulphonic acid in chloroform–methanol (7.3 v/v) for 10 min [21] The desired 2′-deoxyribonucleoside 3′-(2-chlorophenyl phosphoranilidates (**1**) were obtained in good to excellent yields. Their characteristics are given in Table 5.

Treatment of **1** with 10-molar excess of t-BuOK in dimethylacetamide (DMAc) solution at room temperature led to the corresponding cyclic phosphoranilidates **5** If an unseparated mixture of diastereoisomers of **1** was used, the diastereoisomeric mixture of cyclic phosphoranilidates **5** was obtained However, short-column chromatagraphy gave a satisfactory separation of the diastereoisomers **5** obtained in gram-scale experiments. The reaction of a pure diastereoisomer of **1** with t-BuOK in DMAc solution showed that intramolecular substitution at P is stereospecific and gives a pure diastereoisomeric



Scheme 2

Table 3 Diastereoisomeric 5′-O-monomethoxytrityl-2′-deoxyribonucleoside 3′-(2-chlorophenyl)phosphorothioates (**13**)

Compounds 13	Yield [%] [a]	TLC R_f	UV (96% C_2H_5OH) [nm] λ_{max}	UV (96% C_2H_5OH) [nm] λ_{min}	$[\alpha]_D^{20}$ $^{\lambda}589$	$[\alpha]_D^{20}$ $^{\lambda}435$	^{31}P-NMR (C_5H_5N) [ppm]
Rp-13 (B=Thy)	80	0.43 (S_3) 0.76 (S_6)	267.0	250.0	+15.7 [b] (c 0.6)	+43.3 [b] (c 0.6)	51.37
Sp-13 (B=Thy)	81	0.43 (S_3) 0.80 (S_6)	267.0	250.0	+39.1 [b] (c 0.9)	+85.8 [b] (c 0.9)	52.02
Rp-13 (B=Ade)	60	0.40 (S_3) 0.73 (S_6)	262.3	247.0	+6.4 [c] (c 1.4)	-	51.47
Sp-13 (B=Ade)	40	0.40 (S_3) 0.79 (S_6)	262.3	248.0	+14.6 [c] (c 0.7)	-	51.67
Rp-13 (B=Gua)	57	0.72 (S_6)	shoulders 270.5, 250.0		-	-3.3 [d] (c 1.2)	52.05 [b]
Sp-13 (B=Gua)	38	0.75 (S_6)	shoulders 270.5, 250.0		+20.5 [d] (c 1.0)	+46.3 [d] (c 1.0)	52.78 [b]
Rp-13 (B=Cyt)	54	0.53 (S_7) 0.90 (S_6)	276.1	258.8	+75.8 [b] (c 0.6)	+137.5 [b] (c 0.6)	51.81
Sp-13 (B=Cyt)	50	0.56 (S_7) 0.83 (S_6)	274.9	260.0	+56.4 [b] (c 0.6)	+136.5 [b] (c 0.6)	52.37

[a] Yield of isolated product, [b] in $CHCl_3$, [c] in methanol, [d] in dimethylformamide

cyclic phosphoranilidate (**5**). From our earlier studies on model 4-methyl-2-oxo-2-phenylamino-1,3,2-dioxaphosphorinanes[22] we could assign an axially orientated N-phenyl function to that diastereoisomer of **5**, which absorbed at higher field in its ^{31}P-NMR spectrum (assuming that dioxaphosphorinanyl part of **5** exists in chair-like conformation). Assignment of the spatial orientation of anilino substituent in **5** is equivalent to assignment of the absolute configuration at phosphorus and was crucial for the stereochemical correlation, explained in detail in the discussion section. 2′-Deoxyribonucleoside cyclic 3′,5′-phosphoranilidates (**5**) were characterized by

Their physico-chemical characteristics are presented in Table 6.

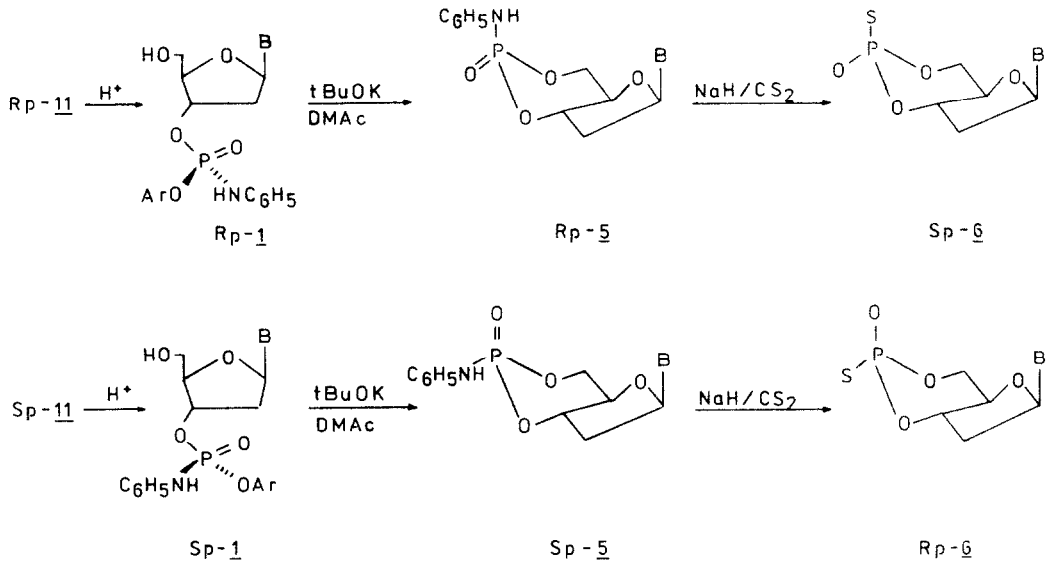
When each purified diastereoisomer of **5** was treated with sodium hydride, followed by carbon disulphide in DMF solution, it was converted to corresponding cyclic phosphorothioate (**6**). It should be mentioned that other functional groups in **5**, e.g. *exo*-amino functions in **1e–h**, were not protected. The yields and physico-chemical parameters of 2′-deoxyribonucleoside cyclic 3′,5′-phosphorothioates (**6**) are collected in Table 7.

The conversion **5**→**6** is also fully stereospecific as has been proved by many of ^{31}P-NMR [illegible] the [illegible]ed

Table 4 Diastereoisomeric ammonium 2'-deoxyribonucleoside 3'-(2-chlorophenyl) phosphorothioates (**4**)

Compounds 4	Yield [%] [a]	TLC R_f	Paper chromatography R_f	Electrophoretic mobilities		UV (96% C_5H_5OH) [nm]		Σ_{max} [c] $\times 10^3$	$\lvert\alpha\rvert$ (H_2O)		^{31}P-NMR(H_2O) δ [ppm]
				E_{cAMP}	E_PA	λ_{max}	λ_{min}		λ_{589}	λ_{435}	
Rp-4a (B=Thy)	85 [b]	0.69(S_6)	0.74 (S_6)	1.05	0.54	268.7	238.0	10.6 [d]	+2.1 (c 2.2)	+9.5 (c 2.2)	51.67
Sp-4b (B=Thy)	95 [b]	0.70(S_6)	0.78 (S_6)	1.08	0.55	269.9	238.0	10.8 [d]	+11.6 (c 1.9)	+30.5 (c 1 9)	51.87
Rp-4c (B=Ade)	66	0.64(S_6) 0.70(S_8)	0.67 (S_6)	0.61	0.35	263.5	235.9	16.7	-17.0 (c 1.6)	-	51.67
Sp-4d (B=Ade)	63	0.64(S_6) 0.73(S_8)	0.72 (S_6)	0.65	0.37	262.3	236.3	14.6	-7.4 (c 1.4)	-	51.97
Rp-4e (B=Gua)	82	0.63(S_6) 0.62(S_8)	0.49 (S_6)	0.67	0.45	255.4	233.4	9.4	-14.5 (c 1.1)	-32.5 (c 1.1)	51.33
Sp-4f (B=Gua)	80	0.63(S_6) 0.62(S_8)	0.53 (S_6)	0.73	0.49	255.4	233.8	9.5	-9.0 (c 0.8)	-21.5 (c 0.8)	51.73
Rp-4g (B=Cyt)	60	0.71(S_6) 0.54(S_8)	0.76 (S_6)	1.05	0.61	274 3	253.3	9.9	+21.9 (c 0.7)	+60.3 (c 0.7)	51.67
Sp-4h (B=Cyt)	75	0.71(S_6) 0.54(S_8)	0.70 (S_6)	1.07	0.62	274.3	253.3	9.5	+37.7 (c 1.0)	+91.7 (c 1 0)	52.07

a/ Yield of the products after purification by means of ion-exchange chromatography
b/ *Yield of precipitated product (see experimantal)*
c/ Measurement in dist. H_2O alkalized with NEt_3
d/ In H_2O



Scheme 3

Table 5 Diastereoisomeric 2′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphoranilidates (**1**)

Compounds **1**	Yield [%] a/	TLC R_f	UV (96% C_2H_5OH) [nm] λ_{max}	UV (96% C_2H_5OH) [nm] λ_{min}	$[\alpha]_D^{20}$ (CH_3OH) 589	$[\alpha]_D^{20}$ (CH_3OH) 435	^{31}P-NMR (C_5H_5N) ppm	MS [m/z, $[H]^+$]
Sp-**1a** (B=Thy)	85	0.20 (S_1)	268.7	244 0	-2 1 (c 2 0)	+7.5 (c 2 0)	-2 5	>07
Rp-**1b** (B=Thy)	80	0.22 (S_1)	268.7	244 0	+7 4 (c 0 5)	+28 9 (c 0 5)	-2 66	507
Sp-**1c** (B=Ade)	88	0.22 (S_1) 0.17 (S_4)	262 3	242.0	-18 0 (c 1 1)	-41 1 (c 1.1)	-2 46	516
Rp-**1d** (B=Ade)	91	0.22 (S_1) 0.17 (S_4)	262.3	242.0	-6 3 (c 1 1)	-13 6 (c 1 1)	-2 56	516
Sp-**1e** (B=Gua)	96	0 08 (S_1) 0.43 (S_2)	255.4	242 5	-13 0 b/ (c 2.1)	-30 7 b/ (c 2 1)	-2 78	748 [M+3TMS $^+$
Rp-**1f** (B=Gua)	98	0.08 (S_1) 0 43 (S_2)	256.5	242 5	+8 7 b/ (c 1.7)	+18.4 b/ (c 1 7)	-2 84	748 [M+3TMS $^+$
Sp-**1g** (B=Cyt)	70	0.06 (S_1) 0.38 (S_2)	274.3	252.7	+17.9 c/ (c 1 2)	+51 7 c/ (c 1 2)	-2.60	-
Rp-**1h** (B=Cyt)	60	0.06 (S_1) 0.38 (S_2)	274.3	252 7	+28 1 c/ (c 0.5)	+79 2 c/ (c 0 5)	-2 75	-

a/ Yield of precipitated product, b/ in DMF, c/ in acetone

with retention of configuration[19,20] we were able to assign absolute configurations to all the cyclic phosphorothioates (**6**) obtained

The synthesis of diastereoisomers of thymidine-3′ thymidine-5′ phosphorothioate (**9**)

Treatment of **11** with 0 125 N NaOH–dioxan (3 1) solution causes the cleavage of P–OAr bond and 5′-O-monomethoxytrityl-2′-deoxynucleoside phosphoranilidates (**14**) are prepared in quantitative yield[3] Condensation of **14** (B=Thy) with 2,4,6-triisopropylbenzenesulphonyl chloride (TPS–Cl)[23] gave **15** or **15′** in the yield 5 and 6%, respectively

Interestingly, attempts of condensation of **14** (B=Thy) with 3′-O-acetylthymidine carried out in the presence of TPS- tetrazole/triethylamine[24] were even less successful and, contrary to our expectations, the yield of **15** was less than 5%. The predominant product of both reactions (yield 75–80%) was identified, by means of ^{31}P-NMR and hydrolytic degradation, as P^1,P^2-di-(phenylamino)-P^1,P^2-di-[3′-(5′-monomethoxytritylthymidyl-1-yl)]-pyrophosphor-

mix-

ture **15** can be prepared according to the method described by Myshenina *et al.*[25] 5′-O-Monomethoxytritylthymidylyl(3′→5′)-3′-O-monomethoxytritylthymidine readily reacts with aniline in the presence of triphenylphosphine and carbon tetrachloride in pyridine to give **15** in 42% yield (not optimized) Isolation of **15** has been performed by means of preparative TLC on silica gel plates F_{254}(Merck) in solvent system $CHCl_3$–96%C_2H_5OH (10:0.6). Compounds **15** (**15a, b** and **15a′, b′**) were identified by their conversion by means of NaH/CO_2 to 3′,5′-diprotected thymidylyl-(3′→5′)-thymidine, which was further hydrolysed [after removal of 5′-monomethoxytrityl group(80% AcOH) and 3′-acetyl group (NH_4OH), or

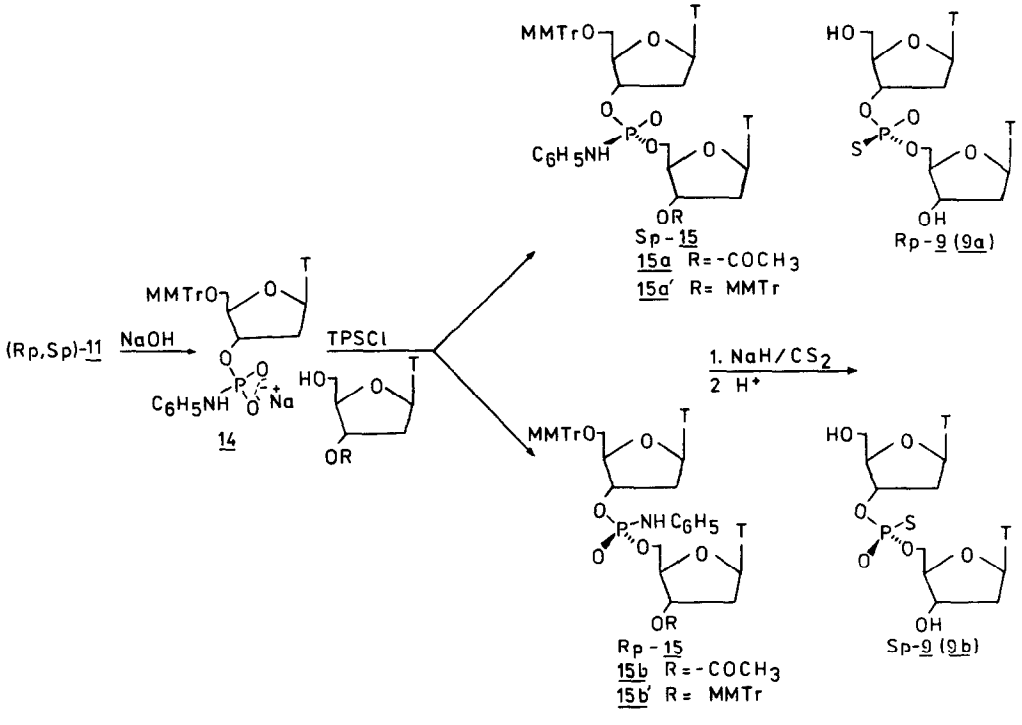
Table 6 Diastereoisomeric 2′-deoxyribonucleoside cyclic 3′-5′-phosphoranilidates (**5**)

Compounds 5	Yield [a] [%]	TLC R_f	UV (96% C_2H_5OH) [nm] λ_{max}	UV (96% C_2H_5OH) [nm] λ_{min}	[α] (CH_3OH) λ_{589}	[α] (CH_3OH) λ_{435}	^{31}P-NMR (C_5H_5N) δ [ppm]	MS [m/z], $[M]^{+\cdot}$
Sp-5a (B=Thy)	84	0.56[b]	268.2	244.0	+3.0 (c 0.6)	+11.5 (c 0.6)	0.64	379
Rp-5b (B=Thy)	90	0.45[b]	268.7	244.0	-79.4 (c 0.3)	-167.7 (c 0.3)	-3.56	379
Sp-5c (B=Ade)	53	0.15(S_1) 0.69(S_6)	258.8	240.5	-105.0 (c 2.2)	-219.8 (c 2.2)	0.72	388
Rp-5d (B=Ade)	55	0.12(S_1) 0.69(S_6)	260.6	242.5	-129.2 (c 2.7)	-282.0 (c 2.7)	-3.83	388
Sp-5e (B=Gua)	35	0.36(S_2) 0.67(S_6)	256.5	240.0	-71.4[c] (c 0.8)	-	0.89[c]	404
Rp-5f (B=Gua)	47	0.23(S_2) 0.67(S_6)	256.5	242.5	-91.9[c] (c 0.4)	-196.0[c] (c 0.4)	-4.43	404
Sp-5g (B=Cyt)	40	0.31(S_2) 0.49(S_7)	272.4	254.4	-31.4 (c 1.0)	-	0.52	364
Rp-5h (B=Cyt)	45	0.27(S_2) 0.49(S_7)	271.1	252.2	-17.4 (c 0.7)	-	-3.91	364

[a] Yield od cyclisation of individual diastereomeric species, Sp- and Rp-5

[b] Developing system chloroform-methanol (85:15)

[c] Measurement in dimethylformamid



Scheme 4.

Table 7 Diastereoisomeric ammonium 2′-deoxyribonucleoside cyclic 3′,5′-phosphorothioates (**6**)

Compounds 6	Yield [%] [a]	TLC R_f	Paper chromatography R_f (S_6)	UV (96% C_2H_5OH) [nm]		$[\alpha]^{\circ}$ (H_2O)		^{31}P-NMR (H_2O) δ [ppm]	MS [m/z]
				λ_{max}	λ_{min}	λ_{589}	λ_{435}		
Rp-6a (B=Thy)	53	0 54 (S_6)	0 56	267 0	235.5	-42 5 (c 0,5)	-81 1 (c 0.5)	54.73	320 [M-NH_3]$^{+\cdot}$ [b]
Sp-6b (B=Thy)	56	0 51 (S_6)	0.57	267.0	235 5	-14,8 (c 0,6)	-24.6 (c 0.6)	52 11	320 [M-NH_3]$^{+\cdot}$ [b]
Rp-6c (B=Ade)	26	0,62 (S_6)	0 52	260 0	227 5	-71,8 (c 0 6)	-145.7 (c 0 6)	54 47	473 [M+2TMS]$^{+}$
Sp-6d (B=Ade)	69	0 63 (S_6)	0 49	262.3	230.7	-37 5 (c 0 9)	-80 1 (c 0 9)	52.94	473 [M+2TMS]$^{+\cdot}$
Rp-6e (B=Gua)	38	0 52 (S_6) 0 50 (S_8)	0 25	255.4	222 5	-71 3 (c 0 8)	-145.6 (c 0 8)	54 47	561 [M+3TMS]$^{+}$
Sp-6f (B=Gua)	76	0 52 (S_6) 0 50 (S_8)	0.25	256 0	225 0	-54 0 (c 0 5)	-112.0 (c 0 5)	52 78	561 [M+3TMS]$^{+}$
Rp-6g (B=Cyt)	44	0,58 (S_6) 0 48 (S_8)	0 49	272 4	258 8	-3 9 (c 0 8)	+16.1 (c 0 8)	54 54	449 [M+2TMS]$^{+}$
Sp-6h (B=Cyt)	62	0 58 (S_6) 0 48 (S_8)	0 45	273 6	260 6	+21 7 (c 0 8)	+66.5 (c 0.8)	52.70	449 [M+2TMS]$^{+}$

[a] Yields calculated for the isolated products. [b] Mass spectra of ammonium salt of 6

both MMTr groups with 80% AcOH (**15a′, b′**)] by means of spleen phosphodiesterase (E C. 3.1.4.18)[26] to thymidine and thymidine 3′-monophosphate.

However, it was of interest to separate **15** into diastereoisomeric species and this has been achieved in both cases **15a, b** and **15a′, b′** by means of preparative TLC [$CHCl_3$–96% C_2H_5OH (100:6)]. It is worthwhile to mention that in the case when both 5′- and 3′-OH functions of thymidine-3′ thymidine-5′ phosphoranilidate are protected with monomethoxytrityl groups (**15a′, b′**), its resolution into diastereoisomers, **15a′** (high R_f), $\delta_{31P} = 2.3$ ppm ($CHCl_3$), **15′** (low R_f), $\delta_{31P} = 1.8$ ppm ($CHCl_3$), is much more efficient due to the greater difference in the chromatographic mobilities [developing system $CHCl_3$–96% EtOH (100:6)][27] of the latter. Individual diastereoisomers of **15** undergo facile stereospecific conversion, on treatment with NaH/CS_2 in DMF solution, followed by deprotection of OH groups, to thymidine-3′ thymidine-5′ phosphorothioate (**9**); thus, from **15a′** (high R_f) compound **9a**, $\delta_{31P} = 55.6$ ppm (H_2O), and from **15b′** (low R_f) compound **9b**, $\delta_{31P} = 55.1$ ppm (H_2O), were obtained. The absolute configuration at P in both diastereoisomers of **9** was assigned enzymatically and has been described in detail only recently.[9,28] The resistance of **9b** (prepared from **15b′**) towards hydrolysis in the presence of snake venom phosphodiesterase (E.C 3.1 4.1.)[29,30] is indicative of the S_P configuration of this dithymidine phosphorothioate, while facile hydrolysis of **9a** (obtained from higher R_f **15a′**) under identical conditions indicates the R_P configuration of diastereoisomer **9** absorbing in ^{31}P-NMR spectrum at 55.6 ppm.[31]

*Substitution of oxygen-*18 *for sulphur in dinucleoside phosphorothioates*

In the light of growing interest in bio-phosphates chiral at P by virtue of the presence of different stable isotopes of O[1,32–36] we have used diastereoisomers of **16** as substrates for the synthesis of thymidine-3′ thymidine-5′[^{18}O, ^{16}O]-phosphates. The method of stereoinversion recently reported by Frey *et al* [37] has been applied. Thus, **15a′** (the high R_f diastereoisomer of **15**) was converted by the NaH/CS_2 procedure into 5′-O-monomethoxytritylthymidine-3′ 3′-O-monomethoxytritylthymidine-5′ phosphorothioate(**16a′**), $\delta_{31P} = 52.3$ ppm ($CHCl_3$), in 85% yield. This compound was treated with lutidine, [^{18}O]-H_2O and cyanogen bromide in pyridine solution. After 30 min the reaction was quenched by addition of cysteine. The monomethoxytrityl groups were removed by means of a solution of 2% toluene-*p*-sulphonic acid in $CHCl_3$–MeOH (7·3) **17a**, purified by chromatography on DEAE-Sephadex A-25, was obtained after lyophilisation in 42% yield, similarly **16b′**, $\delta_{31P} = 54.3$ ppm ($CHCl_3$), prepared from **15b′**, was converted to **17b** in 32% yield. Although Frey's procedure is claimed to be highly stereospecific and gives the product with inverted configuration, the confirmation of the stereochemistry of conversion **16**→**17** is now in progress and will be published elsewhere.[38]

Cyclisation of thymidine 3′-(4-*nitrophenyl*) *phosphorothioate* (**4**, B=Thy)

Access to the diastereoisomers of **4** prompted us to attempt to elucidate the stereochemistry of the Borden–Smith cyclisation which is a general method for the preparation of nucleoside cyclic 3′-5′-phosphates.[40] Phosphorylation of **12** (B=Thy) with 4-nitrophenyl phosphoranilidochloridate[8] gave **11ϵ** (B=Thy, Ar=4–NO_2–C_6H_4–) as a mixture of two diastereoisomers absorbing [^{31}P-NMR] at − 3.37 and − 3 17 ppm ($CDCl_3$). Their separation was performed on silica gel using $CHCl_3$–acetone (10:3) as the developing system. **11ϵ** (B=Thy, Ar=4–$NO_2C_6H_4$–) of higher mobility ($\delta_{31P} = -3$ 37 ppm) after deprotection of 5′-OH function and treatment with t-BuOK in DMF solution gave **5a** (B=Thy), $\delta_{31P} = +0.64$ ppm Similarly **11ϵ** (B=Thy, Ar=4–$NO_2C_6H_4$–), of lower mobility $\delta_{31P} = -3.17$ ppm, was converted into **5b** (B=Thy), $\delta_{31P} = -3.56$ ppm. Since the cyclisation **11**→**5** proceeds with inversion of configuration,[14] a stereochemical correlation between diastereoisomers **11** and **5** was achieved. With that in mind, we have converted each diastereoisomer **11ϵ** (B=Thy, Ar=4–$NO_2C_6H_4$–) by means of NaH/CS_2 into 5′-O-monomethoxytritylthymidine 3′-(4-nitrophenyl)-phosphorothioates· thus, from **11ϵ** of higher mobility (B=Thy, Ar=4–$NO_2C_6H_4$–) compound **13k** (B=Thy, Ar=4–$NO_2C_6H_4$–), $\delta_{31P} = 53.2$ ppm, and from **11ϵ** of lower mobility (B=Thy, Ar=4–$NO_2C_6H_4$–) diastereoisomer **13l** (B=Thy, Ar=4–$NO_2C_6H_5$–), $\delta_{31P} = 51.0$ ppm, were obtained. Compound **13k**, after removal of the monomethoxytrityl group gave **4k** (B=Thy, Ar=4–$NO_2C_6H_4$–), $\delta_{31P} = 50.68$ ppm, which was converted upon treatment with t-BuOK into **6a** (B=Thy), $\delta_{31P} = 54$ 7 ppm Similarly, **13l** was converted into **4l** (B=Thy, Ar=4–$NO_2C_6H_4$–), $\delta_{31P} = 50.76$ ppm and transformed further into **6b** (B=Thy), $\delta_{31P} = 52.1$ ppm. The complete stereospecificity of conversion **4**→**6** was established by means of ^{31}P-NMR. These experiments constitute of the first demonstration of the total stereospecificity of the Borden–Smith cyclisation. Its stereochemical mode is described in the Discussion

DISCUSSION

Since the pioneering work of Usher and Eckstein in the early 1970s, demonstrating the application of diastereoisomers of uridine cyclic 2′,3′-phosphorothioate for elucidation of the mode of action of ribonuclease,[41] several new examples employing the potential of stereochemical methods in studies on enzyme–substrate interactions were demonstrated.[1] The second milestone marking the progress in understanding the molecular basis of the mechanisms of phosphoryl and nucleotidyl transfer enzymes has been laid by Knowles and independently by Lowe, who first employed the chiral [^{16}O,^{17}O,^{18}O]-phosphoryl group and created the basis for determination of stereochemistry of isotopically labelled phosphates.[42,43]

Having in mind that the early work employing the concept of phosphorothioate chirality was based on the phenomenon of chiral recognition of the substrate(s) by the active centre(s) of enzyme,[44] which by nature of enzymatic reactions, limited the scale of experiments, we decided to design a chemical method which would enable investigators to synthesize the quantities of nucleoside P-chiral phosphorothioates without the necessity of employing of enzymes, and

to determine their absolute configurations by physico-chemical methods The simplest way leading to nucleoside P-chiral phosphorothioates consisted of phosphorylation of nucleosides by means of chiral, but racemic, bifunctional phosphorylating agents, separation of the nucleotides into diastereoisomeric components and stereospecific, selective replacement of one of the substituents at P by S

In the course of independent studies on the specific transformations of phosphoramidates we have reinvestigated the Staudinger Horner–Wittig reaction,[19] which in the early 1960s has been demonstrated by Wadsworth and Emmons as the simple way to the synthesis of organic molecules bearing a C=N bond.[45] To our knowledge neither Wadsworth and Emmons nor their followers, were interested in the fate of the phosphate moiety We have demonstrated that chiral dialkyl phosphoramidates, derivatives of primary amines, can be successfully used for the synthesis of chiral dialkyl phosphorothioates [19] Of essential importance is the nearly complete stereospecificity and stereoretentive nature of this transformation. These findings prompted us to synthesize and use aryl phosphoranilidochloridates (**10**) as convenient phosphorylating agents for use in nucleotide chemistry [3,4,6,8,46] These compounds can be easily prepared from aryl phosphorodichloridates and aniline They are relatively stable, crystalline compounds which are very reactive phosphorylating agents with respect to primary and secondary alcohols They react with chiral molecules to give, due to the chirality (at P) of the phosphoramidate moiety, diastereoisomeric mixtures which can be separated into individual diastereoisomers We have found that 5′-protected nucleoside 3′-aryl phosphoranilidates (**11**) can be easily separated on silica gel. In addition to the TLC assay, the diastereoisomeric purity can be determined by ^{31}P-NMR spectroscopy As the model compound for phosphorylation studies we chose 5′-O-monomethoxytritylthymidine (**12**, B=Thy) Amongst a number of phosphorylating agents we have found 2-chlorophenyl phosphoranilidochloridate (**10γ**) to be the most convenient.

The overall combination of protective groups should be considered Dialkyl-(2-chlorophenyl) phosphoramidates are relatively stable under acidic conditions which do not affect the P–N bond or, as in the case of **11**, the monomethoxytrityl group protecting 5′-OH function of thymidine The monomethoxytrityl group can be removed under mild acidic conditions, which do not affect the P–N bond of phosphoranilidates The chemoselective and stereospecific transformations of the anilino function attached to the phosphate moiety are discussed below

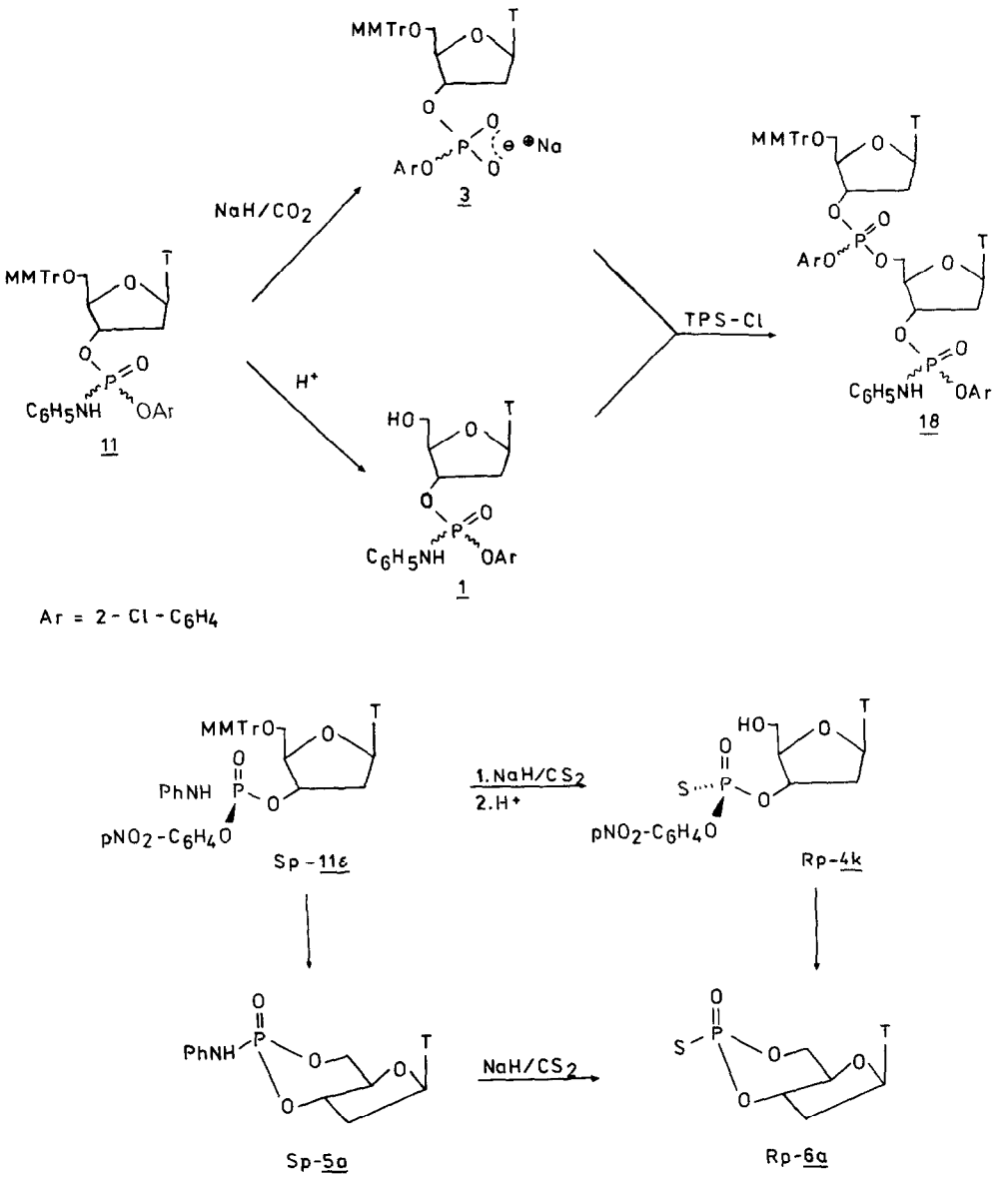
5′-O-Monomethoxytritylthymidine 3′-(2-chlorophenyl) phosphoranilidate (**11γ**) was prepared in high yield, its separation into diastereoisomers was easy due to the difference in chromatographic mobilities of diastereoisomers (ΔR_f) The NMR assay of diastereoisomeric purity was also simple and reliable due to the large difference in chemical shifts between diastereomeric species (Table 1) Additionally, the chemical reactivity of the 2-chlorophenyl protecting group was appropriate to the further transformation

the assessment of the activity of several enzymes, we recommend and have used, 4-nitrophenyl phosphoranilidochloridate (**10α**, Ar=4-$NO_2C_6H_4$-)[8] as an alternative phosphorylating agent Although successful phosphorylation of 5′-O-monomethoxytritylthymidine (**12**, B=Thy)[3] and 5′-O-monomethoxytrityl-2′-deoxyadenosine (**12**, B=Ade)[6] was performed in pyridine solution medium using a 50% molar excess of phosphorylating agent over a period of 18 hr, a fourfold excess of phosphorylating agent and the presence of tetrazole and triethylamine in acetonitrile solution were necessary for the efficient phosphorylation of 2′-deoxycytidine and 2′-deoxyguanosine derivatives (**12**, B=Cyt, B=Gua), respectively (Table 2, method B) Nucleosides unprotected on their *exo*-amino functions were used in these cases. The compounds (**11**) which were isolated following short column chromatography, can be further separated into individual diastereoisomers using a higher proportion of silica gel The need to separate the diastereoisomeric species results from the purposes for which they are required If P-achiral 5′-O-monomethoxytrityl-2′-deoxyribonucleoside 3′-aryl phosphates (**3**) are required for the further synthesis of oligonucleotides, the separation of **11** into diastereoisomers is not necessary Treatment of **11** with isoamyl nitrite in acetic anhydride/pyridine buffer brings about the cleavage of P–N bond very effectively [48] Alternatively, this transformation can be achieved by means of $NaHCO_2$, under conditions elaborated in this laboratory (Scheme 5) [4,19]

Compounds **3** are valuable intermediates for activation with 2,4,6-triisopropylbenzenesulphonyl chloride[23] (tetrazolide)[24] and condensation with e g thymidine 3′-(2-chlorophenyl) phosphoranilidate (which may also be obtained from **11** following removal of monomethoxytrityl group by means of 80% AcOH) gives compound **18** (Scheme 5) This phosphoramidate approach to the oligonucleotide synthesis which has been demonstrated in the preparation of tetrathymidylic acid, is beyond the scope of this publication [49]

Another possibility offered by **11** is the removal, by treatment with 0 125 N NaOH, of its aryl protective group Another P-prochiral compound, e g 5′-O-monomethoxytritylthymidine 3′-phosphoranilidate (**14**) is obtained. Its condensation with 3′-O-acetylthymidine or 3′-O-monomethoxytritylthymidine gives 5′-O-monomethoxytritylthymidine-3′ 3′-O-acetylthymidine-5′ phosphoranilidate (**15a, b**)[7] or its 3′-O-monomethoxytrityl analog **15a′, b′** (Scheme 4) Compounds **15**, like **11**, can be separated into diastereoisomeric species **15a** and **15b** and **15a′** and **15b′**, respectively The synthetic value of **15** lies in the presence of anilino functionality, which allows its stereospecific conversion to a dinucleoside phosphorothioate (**16**) This conversion emphasizes the potential of nucleoside phosphoranilidates and will be discussed below in detail

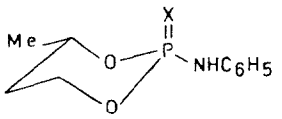
In addition to the use of compounds **11** in oligonucleotide synthesis, we have considered their application in the stereospecific preparation of nucleoside cyclic 3′,5′-phosphates and -phosphorothioates Although the role of cyclic 2′-deoxyribonucleotides is not so well recognized as that of their ribonucleotide



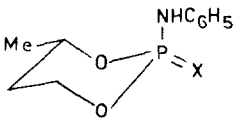
Scheme 5

no. 7 has been reported[50] in the literature. This means that enzymes responsible for the conversion of dATP to cdAMP and for the hydrolysis of the latter compound to 5′-dAMP, must exist in nature. Apparently, the synthesis of 2′-deoxyribonucleoside cyclic 3′,5′-phosphorothioates opens the possibility of stereochemical studies on the mode of action of this class of enzymes. Additionally, the lack of a convenient method for stereospecific synthesis of diastereoisomers of adenosine cyclic 3′,5′-phosphorothioate has given further impetus to studies relating to the use of **11** for preparation of diastereoisomers of **6**. The selective removal of the monomethoxytrityl group from **11** gives a 2′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphoranilidate (**1**). Intramolecular cyclization was possible by the presence of a good leaving group attached to P. Indeed, we have demonstrated that a 10-fold molar excess of t-BuOK in anhydrous dimethylacetamide converted **1** to a diastereoisomeric mixture of the two 2′-deoxyribonucleoside cyclic 3′,5′-phosphoranilidates (**5**) The latter can be separated into the individual diastereoisomers.[5,6] As in the case of **15**, the presence of the anilino function attached to P created the attractive possibility for the conversion of **5** to **6**. Moreover, the experience gained from our studies on the model 2-oxo-(thioxo-, selenoxo-)-(2-N-phenylamino-4-methyl-1,3,2-dioxaphosphorinanes[22] enabled us to asign the spatial orientation of the anilino group with respect to the rest of the molecule of **5**; we established earlier the empirical rule that an isomer with an equatorial disposition of the anilino group with respect to 1,3,2-dioxaphosphorinanyl ring system absorbs (^{31}P-NMR spectrum) at lower field than one with an axial disposition of the anilino group [22]

Assuming that P-containing 6-membered ring



X = O $\delta_{^{31}P}$ = 0 92 ppm
X = S $\delta_{^{31}P}$ = 63 89 ppm
X = Se $\delta_{^{31}P}$ = 63 43 ppm



$\delta_{^{31}P}$ = −3 95 ppm
$\delta_{^{31}P}$ = 59 5 ppm
$\delta_{^{31}P}$ = 60 31 ppm

trans-fused with the 2′-deoxyribose moiety exists in a chair-like conformation, we predicted for diastereoisomers **5** absorbing (^{31}P-NMR spectrum) at higher field, the R_P-configuration and for those absorbing at lower field, the S_P-configuration This assignment, crucial to our further configuration assignments, was proved independently 2′-Deoxyadenosine cyclic 3′,5′-phosphate was converted under conditions developed in this laboratory[31] to the diastereoisomeric mixture of 2′-deoxyadenosine cyclic (3′→5′)[^{15}N]-phosphoranilidates (**5**) by means of $Ph_3P/CCl_4/[^{15}N]C_6H_5NH_2$ This mixture was separated into its two constituent diastereoisomers, and ^{31}P-NMR spectra were recorded. It appeared that an isomer absorbing at lower field (high R_f) possesses an absolute value of spin-spin coupling constant between ^{15}N and ^{31}P nuclei larger than that absorbing at higher field This measurement again confirmed, according to our empirical rule[40] for coupling between P and several I = 1/2 nuclei in the 1,3,2-dioxaphosphorinane ring system "*for* ^{1}H, ^{13}C, ^{15}N, ^{19}F *and* ^{77}Se *the coupling to the axially positioned magnetically active nucleus is smaller than that to the equatorially positioned nuclei*",

$$|^{1}J_{PX}|_{ax} < |^{1}J_{PX}|_{eq}$$

X=^{1}H, ^{13}C, ^{15}N, ^{77}Se (I = 1/2)

the correctness of our configurational assignments In the meantime an X-ray structure analysis of the low R_f diastereoisomer **5d** (B=Ade, $\delta_{^{31}P}$ − 3 38 ppm) was carried out The results obtained have fully confirmed both the assignment of absolute configuration and the former assumption that the dioxaphosphorinanyl ring constituting a part of the molecule (**5**) exists in chair conformation, as correct [53] Having this case fully solved we have separated other deoxyribonucleoside 3′-(2-chlorophenyl) phosphoranilidates (**11**) into diastereoisomeric species and, after removal of 5-O-monomethoxytrityl group, individual diastereoisomers (**1**, Table 5) were cyclized by means of t-BuOK/DMAc First, what has to be emphasized, is the complete stereospecificity of the cyclisation process The products of transformation **1**→**5** were not contaminated by their diastereoisomers, which could be easily detected by TLC and ^{31}P-NMR spectroscopy This fact seems to be of special importance regarding the mechanism of intramolecular cyclisation Since the process was fully stereospecific, the conclusion about an S_N2-type mechanism of cyclisation process was reasonable We concluded that cyclisation proceeds with inversion of configuration at phosphorus atom of **1** This allowed us to assign the absolute configuration at P in all the compounds (**1** and **11**) which were converted to **5** (Scheme 3) The absolute configurations of these last compounds were assigned by means of our criteria Thus, it has been established that each diastereoisomer of **11**, which, after deprotection of its 5′-OH function is converted to an R_P-cyclic anilidate **5**, has R_P-absolute configuration, and *vice versa*, each S_P-cyclic anilidate results from an S_P-**11** substrate (Table 6) This regressive type of stereochemical analysis let us, on the basis of the formerly established stereoretentive nature of the PN → PX conversion (Fig. 1), to assign the absolute configuration to the 2′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphorothioates (**13**) resulting from individual compounds of structure **11** (Table 3) as well as of **4** (Table 4) It should be noticed that our assignments are consistent with these described by Gerlt *et al*, who applied our procedure to the synthesis of thymidine 3′- and 5′-(4-nitrophenyl) phosphoranilidates and by means of an enzymatic assay,[39,40] had assigned the absolute configurations of thymidine 5′-(4-nitrophenyl) phosphorothioates [14]

Since the procedure known as the Borden-Smith reaction leading to nucleoside cyclic 3′,5′-phosphates, involves the intramolecular cyclisation of nucleoside 5′-diaryl phosphates,[40] it was of interest to elucidate the stereochemistry of conversion of **4** to **6** Individual diastereoisomers of **4** (B=Thy, Ar=4-NO_2-C_6H_4-) were cyclised under conditions originally proposed by Borden and Smith. It appears that the Borden-Smith procedure is also fully stereospecific thus **4** (B=Thy, Ar=4-$NO_2C_6H_4$-), $\delta_{^{31}P}$ = 50 68 ppm (H_2O) gives **6**, $\delta_{^{31}P}$ = 54 7 ppm (C_5H_5N), and **4**, $\delta_{^{31}P}$ = 50.76 ppm, gives **6**, $\delta_{^{31}P}$ = 52 1 ppm

The stereochemical mode of this reaction has been further established due to the stereoretentive conversion of each diastereoisomer of **5** into the corresponding diastereoisomer of **6** (Scheme 3) We have proved that the low R_f R_P-**5** diastereoisomer is converted to the low R_f S_P-**6** one (B=Thy) and the high R_f S_P-**5** diastereoisomer into the high R_f R_P-**6** phosphorothioate. The chemical shift order of these two compounds is also indicative of the correctness of assignment of the absolute configuration at P in both diastereoisomers of **6**. The correlation of the absolute configuration of **4**, which gives **6** after cyclisation, is indicative for the inversion of configuration under conditions of Borden-Smith cyclisation In addition to the elucidation of the stereochemical course of the Borden-Smith reaction, this is an example of one of only a few known nucleophilic substitution reactions at phosphorus in dialkyl phosphorothioate chemistry.

Since the stereochemical correlation between the products **11(1)**, **5**, **6** and **13(4)** has been established and the physico-chemical parameters of all diastereoisomeric pairs of compounds have been measured, the question arises as to what extent parameters like chromatographic mobilities and chemical shifts in ^{31}P-NMR spectra can be applied for assignment of the absolute configuration within a given pair of diastereoisomeric species. An inspection of data included in Table 2 clearly demonstrates that 5′-O-monomethoxytrityl-2′-deoxyribonucleoside-3′-(2-chlorophenyl) phosphoranilidates can be recognized by means of the chromatographic mobilities and their chemical shifts (^{31}P-NMR spectroscopy) For the four pairs of compound, R_P-**11**γ is always less mobile on

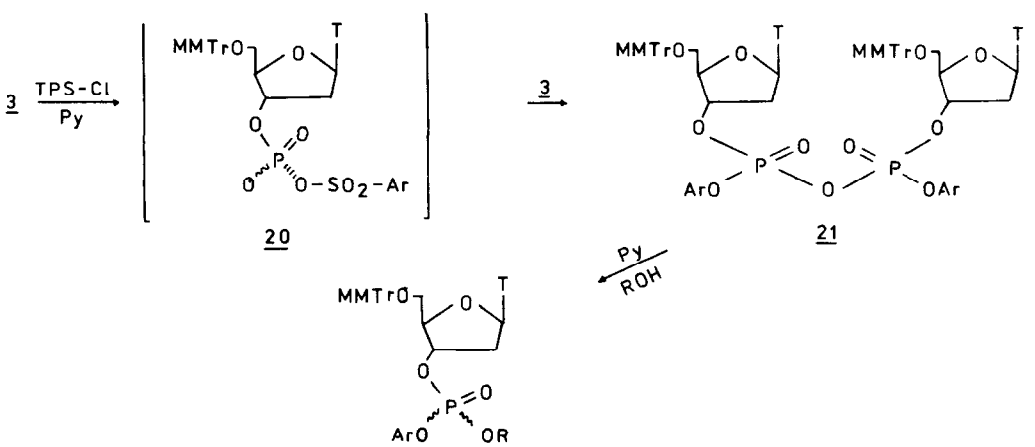
silica gel than its S_P-counterpart and R_P-**11γ** absorbs (^{31}P-NMR) at higher field than S_P-**11γ**. Unfortunately the chemical shift criterion does not hold within the whole range of aryl derivatives. For example, in the case of **11ε** (Ar=4–$NO_2C_6H_4$–) R_P-**11ε** is less mobile, but it absorbs (^{31}P-NMR) at lower field than its S_P-**11** isomer. This simple comparison shows the danger of using the ^{31}P-NMR chemical shift as an exclusive criterion, and chromatographic mobility is recommended as being indicative of absolute configuration in the family of 2′-deoxyribonucleoside aryl phosphoranilidates. However, it is interesting to analyse the data in Table 5 In the case where the 5-OH function is unprotected, the differences between the mobilities of diastereoisomers are negligible, and the only criterion which allows one to distinguish between diastereoisomers appears to be the ^{31}P-NMR chemical shift parameter Compounds **1** of S_P absolute configuration absorb at lower field than their diastereoisomers, R_P-**1**. This correlation is also true for the 5′-OH protected derivatives of **1**, the compounds **11** (Table 2). Similar inspection of Tables 3 and 4, shows that diastereoisomeric 5′-O-monomethoxytrityldeoxyribonucleoside 3′-(2-chlorophenyl) phosphorothioates (**13**), as well as detritylated **4**, show only a slight tendency for the R_P-isomers to absorb at a higher field than the S_P-diastereoisomers. This difference is of the order of 0.2–0.5 ppm. However, if both diastereoisomers are available, this criterion can be sufficient for the assignment of absolute configuration. Again this criterion does not hold for other aryl esters, for example the two diastereoisomers of thymidine 3′-(4-nitrophenyl) phosphorothioate (**4k, l**) have very similar ^{31}P-NMR chemical shifts.

The above consideration was of interest with respect to the necessity of assignment of the absolute configuration at P within the pairs of **15**, **16** and **17**. The separated diastereoisomers of **15** have the following characteristics: **15a**, R=acetyl-, high R_f, $\delta_{31P} = 2\,69$ ppm (C_5H_5N); **15a′**, R=MMTr-, high R_f, $\delta_{31P} = 2.3$ ppm ($CHCl_3$); **15b**, R=acetyl-, low R_f, $\delta_{31P} = 2.32$ ppm (C_5H_5N), **15b′**, R=MMTr-, low R_f, $\delta_{31P} = 1.8$ ppm ($CHCl_3$).

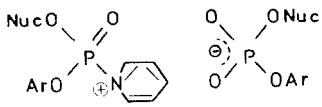
If the relationship characteristic for **11** (Ar=2–ClC_6H_4-) were obeyed by dinucleoside phosphoranilidate (**15**), compound **15a**, high R_f, should have an S_P-configuration and the resulting diprotected dithymidyl(3′-5′)phosphorothioate (**16a**) should have an R_P configuration.

Indeed, the results of experiments of enzymatic digestion of **9a**, indicate that this compound has an R_P configuration. As **9a** was obtained from **15a′** (via **16a′**) and this reaction is known to proceed with retention of configuration, compounds **15a**, **15a′**, **16a** and **16a′** all have an S_P configuration. Similarly, the resistance of **9b** towards hydrolysis in the presence of snake venom phosphodiesterase is evidence for its S_P configuration, and on the basis of arguments presented above, compounds **15b**, **15b′**, **16b** and **16b′** possess an R_P configuration. Comparison of the chromatographic mobilities of the pairs of compounds (**15** and **11**) clearly shows, that both S_P-**11γ** high R_f (B=Thy) and S_P-**15a′** possess faster migrating ability and absorb (^{31}P-NMR) at lower field than their diastereoisomeric counterparts (R_P-**11γ** and low R_f R_P-**15b′**), respectively We realize, however, that in the case of **11ε** (B=Thy) this correlation does not hold and we cannot propose or recommend the use of the criterion of chemical shift in ^{31}P-NMR spectra for the assignments of absolute configuration at P within pairs of dinucleoside phosphorothioates or their precursors, dinucleoside phosphoranilidates. Concerning the characteristics of products described in the paper, the wide use of FD–MS should be emphasized. Application of this technique was crucial for the unambiguous demonstration of the diastereoisomeric character of pairs of compounds (**11**, **1**, **5** and **6**).

The compounds described in this paper were usually obtained in satisfactory yields. Exceptionally, the conversion of **14** to **15**, by means of TIPS–chloride proceeded in low yield. However, an even poorer yield was obtained when condensation was performed by means of TIPS–Cl in the presence of tetrazole. The pyrophosphate (**19b**) was obtained as the main product. It seems to us that this case calls for special comment. According to the data reported by Knorre *et al*[54] the reaction of a nucleoside aryl phosphate with TIPS–Cl in pyridine solution in the presence of a nucleoside derivate involves the formation of an intermediate mixed anhydride (**20**), which then undergoes a fast reaction with unreacted nucleoside aryl phosphate to give a P′,P″-dinucleoside P′,P″-diaryl-pyrophosphate (**21**). This intermediate



Scheme 6

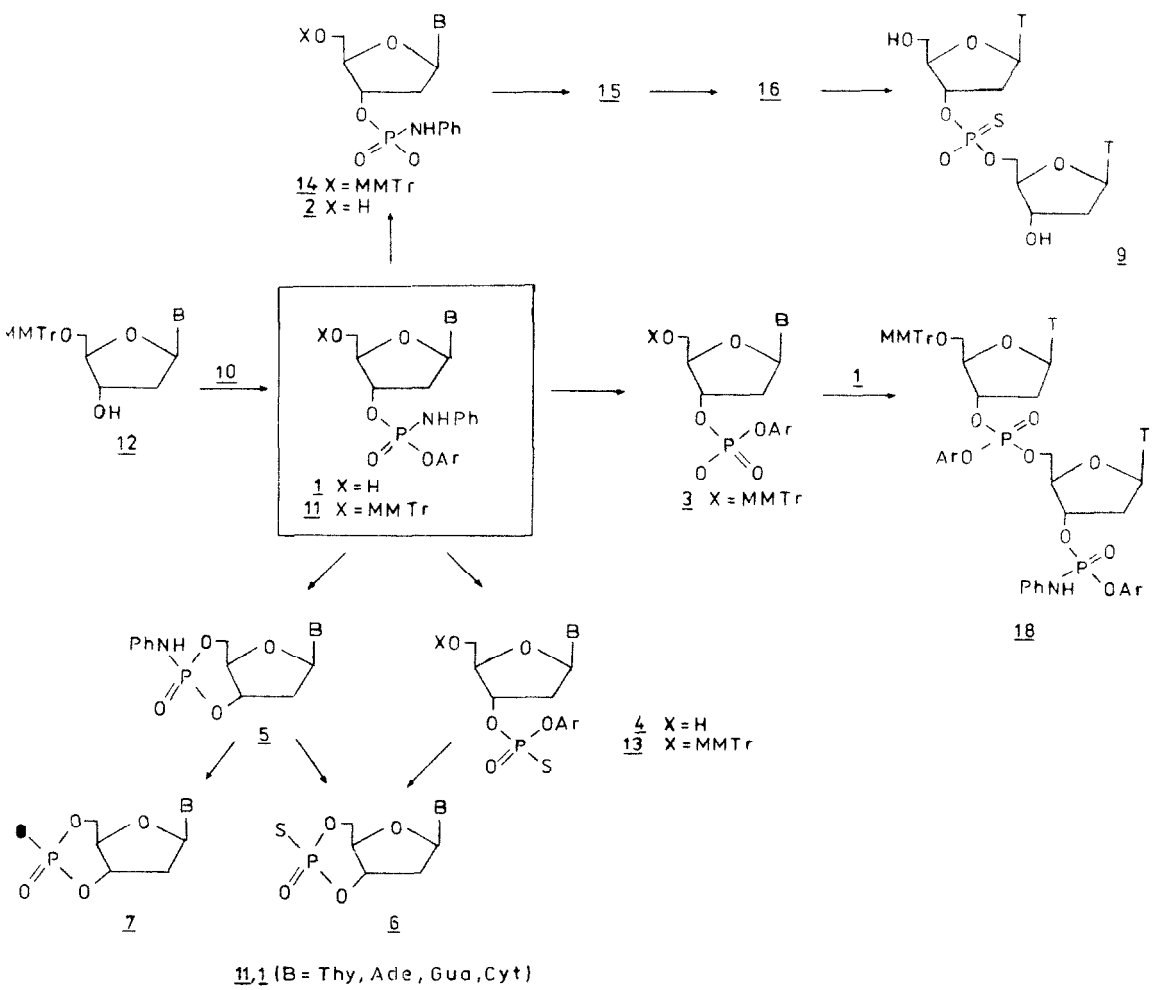


22

has been detected by the Russian workers by means of ^{31}P-NMR spectroscopy It is supposed that pyrophosphate (**21**) undergoes reaction with pyridine or another heterocyclic base present in the reaction mixture to give an ion-pair **22**, which reacts further with the nucleoside derivative present in the reaction mixture, leading to desired dinucleoside aryl phosphate In the light of data published by other authors[55] the role of pyrophosphate (**21**) as the crucial intermediate may be questioned. However, our data support the reaction scheme proposed by Knorre and Zarytova [54] Our finding that the reaction of 5′-O-monomethoxytritylthymidine phosphoramidate (**14**) with 3′-O-monomethoxytritylthymidine in the presence of TIPS–Cl in pyridine gives pyrophosphate (**19b**) as a main product supports the conclusion that pyrophosphate is really the main intermediate in the reaction under discussion The presence of an anilino group at each phosphorus atom of pyrophosphate (**19**) increases the electron density at phosphorus and thereby makes it more resistant to nucleophilic attack. However, the mode of formation of **19** remains obscure and studies on the behaviour of mixed phosphoric-sulphonic anhydrides are desirable [56]

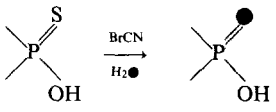
CONCLUSIONS

The application of chiral phosphorylating reagents, phosphoranilidochloridates had opened up a new way to the synthesis of S- and isotopically [^{18}O]labelled, diastereoisomeric nucleotides Easy separation of diastereoisomeric dialkyl phosphoranilidates into individual isomers gives the key intermediates required in the preparation of dialkyl phosphorothioates[51 57] and dialkyl[^{18}O]phosphates [58 59] In the meantime, Gerlt has used our methodology in the synthesis of a number of [^{17}O, ^{18}O]deoxyribonucleoside phosphates and has used the latter successfully in the elucidation of the mode of action of such enzymes like, e g adenylate cyclase[60] and spleen phosphodiesterase [61] Ikehara has



Scheme 7

used 4-chlorophenyl phosphoranilidochloridate in the synthesis of oligonucleotides[62] but has not taken advantage of the chirality of this reagent. Our results, concerning the synthesis of ribonucleoside phosphorothioates, and described so far in communications, are not discussed in this paper. However, it should be mentioned that our phosphoranilidate approach when applied to ribonucleosides allowed us to solve the problem of stereospecific synthesis of cAMPS[51] and [^{18}O]cAMP[59] diastereoisomers. This has opened the way to studies on the mechanism of action of such enzymes as a cyclic phosphodiesterase[36,63] and adenylate cyclase.[57,64] A recently developed aspect of phosphorothioate chemistry lies in the possibility of stereospecific conversion of mono and dialkyl phosphorothioates into [^{17}O]- and [^{18}O]-labelled phosphates



● = 17_0, 18_0

Our method employing [^{18}O]DMSO[65] is not as efficient as these described recently by Frey,[37] Eckstein[39] and Lowe,[66] who have used cyanogen bromide, NBS and Br_2, respectively, for activation of phosphorothioate S atoms. All of them have observed the inversion of configuration at P In the light of these findings our stereospecific synthesis of dialkyl phosphorothioates, together with all assignments of absolute configuration, have gained a new dimension

EXPERIMENTAL

^{31}P-NMR spectra were recorded with a Jeol FX60 spectrometer operating at 24.3 MHz using solns as indicated, with 85% H_3PO_4 as external standard Positive chemical shift values are assigned for compounds absorbing at lower field than H_3PO_4 UV spectra were recorded with Specord UV–VIS Spectrometer (Carl–Zeiss–Jena) Mass Spectra were obtained by means of LKB-2091 (EI) and Varian-Mat 7 (FD–MS) spectrometers Electrophoretic mobilities were assigned with Camag HVE instrument using Whatman no 1 paper at pH 7 5 (0 05 M phosphate buffer) TLC and PTLC was performed on Silica gel plates (E. Merck) and Cellulose F_{254} plates (Serva) Column chromatography was performed on Silica gel 200–300 mesh (Serva) The following developing solvent systems were applied

S_1 $CHCl_3$–MeOH (9 1)
S_2 $CHCl_3$–MeOH (8 2)
S_3 $CHCl_3$–MeOH (3 1)
S_4 $CHCl_3$–iPrOH (8 2)
S_5 $CHCl_3$–$(Me_2)CO$ (10 3)
S_6 iPrOH–NH_3–H_2O (7.1:2)
S_7 MeCN–H_2O (9 1)
S_8 nBuOH–AcOH–H_2O (5.2 3)
S_9 $CHCl_3$–96%EtOH (100.6)
S_{10} $CHCl_3$–MeOH (5 5)
S_{11} $CHCl_3$–Me_2CO (10 1)
S_{12} $CHCl_3$–Me_2CO (10 4)
S_{13} $CHCl_3$–Me_2CO (10 5)
S_{14} $CHCl_3$–MeOH (7 3)

Products were eluted from silica gel with $CHCl_3$–MeOH (1 1), if not stated otherwise

Solvents were of commercial grade and were dried and distilled before use Pyridine dried over KOH was refluxed with $KMnO_4$, distilled, dried over CaH_2, and redistilled Fraction collected at 114–116° was stored over granulated CaH_2 NaH was used as 50% dispersion in mineral oil. All evaporations under reduced pressure were performed at bath temp not exceeding 40°. Crystalline and ppts were dried in dessicator over P_2O_5 at room temp under 10^{-2} mm Hg Snake venom phosphodiesterase from Crotalus terr (1 mg/ml suspension in glycerine) and phosphodiesterase from calf spleen (2 mg/ml suspension in glycerine) were obtained from Boehringer Mannheim GmbH (W. Germany) Aryl phosphoranilidochloridates (**10**) were prepared according to the method described by Zieliński and Leśnikowski [3] The following compounds were obtained

10α Ar=C_6H_5–, yield 73%,
m p. 136–137°, δ_{31P} (acetone) = 1.53 ppm;
10β, Ar=4–ClC_6H_4–, yield 81%,
m p 152–153°, δ_{31P} = 1 61 ppm,
10γ Ar=2–ClC_6H_4–, yield 75%,
m p 94–96°, δ_{31P} = 1 77 ppm;
10δ Ar=2,4–$Cl_2C_6H_3$–, yield 68%,
m p 121–123°, δ_{31P} = 2 01 ppm,
10ε Ar=4–NO_2–C_6H_4–, yield 83%,
m.p. 126–128°, δ_{31P} = 1.61 ppm

Compound **12** (B=Thy) was obtained according to Schaller *et al*[67] yield 85%, λ_{max} 269.3 nm (96% EtOH), λ_{min} 250 nm, $R_f(S_1)$0 45, $R_f(S_2)$0 76 Other 5′-MMTrdN (N=Ade, Cyt, Gua) were obtained by modified procedure described by Żemlicka *et al.*[68] **12** (B=Ade), yield 77%, λ_{max} 263 5 nm, λ_{min} 247 nm, $R_f(S_1)$0 21, $R_f(S_2)$0 77; **12** (B=Cyt), yield 80%, λ_{max} 276 1 nm, λ_{min} 260 6 nm, $R_f(S_1)$0 26, $R_f(S_2)$0 61, **12** (B=Gua), yield 74%, λ_{max} 237 6 nm, λ_{min} 225 5 nm, $R_f(S_1)$0 15, $R_f(S_2)$0.45, 3′-0-acetylthymidine and 3′-0-monomethoxytritylthymidine were obtained according to Verheyden *et al*[69] and Davies *et al*[70] respectively

Phosphorylation of 5′-O-monomethoxytritylthymidine (**12**, B=Thy) *by means of* **10**

The soln of **12** (B=Thy, 10 mmol) in pyridine (10 ml) was evaporated to dryness. This operation was repeated twice and the residue was dissolved in pyridine (100 ml) Into this soln corresponding **10** (15 mmol) was added and the mixture was left at room temp for 18 hr without access of moisture Then water (150 ml) was added and product was extracted with $CHCl_3$ (4 × 50 ml) The organic layer was washed with phosphate buffer (pH 7 5, 3 × 50 ml) and dried over $MgSO_4$ Solvents were evaporated and corresponding **11α, β, γ, δ, ε** were purified by chromatography The yields and spectral characteristics are presented in Table 1

5′-O-*Monomethoxytrityl-2′-deoxyribonucleoside* 3′-O-(2-*chlorophenyl*) *phosphoranilidates* (**11**)

Method A. Phosphorylation of **12** (B=Thy) and **12** (B=Ade) by means of **10γ** was performed according to procedure described above Diastereoisomeric mixtures of **11γ** (B=Thy) and **11γ** (B=Ade) were isolated by means of short column chromatography on silica gel using the ratio of crude products to silica gel 1 30 (*ca* 300 g of SiO_2 washed with cyclohexane followed by $CHCl_3$). Products were eluted with following solvent systems **11γ** (B=Thy)-S_{11} or S_1, **11γ** (B=Ade)-S_{13} followed by S_1 or $CHCl_3$–i-PrOH (10 1) Separation of diastereoisomers was performed by means of short column chromatography using the ratio product-SiO_2 (1 100 w/w) and the same eluting solvent systems The efficiency of separation was monitored on TLC plates and by ^{31}P-NMR spectroscopy Fractions containing the desired homogeneous products were pooled together, solvents were evaporated and residues were dissolved in benzene, and these solns were dropped into n-hexane. The ppts were filtered off, washed with n-pentane and dried under reduced pressure. The data are collected in Table 2

Method B Into the soln of **10γ** (4.5 g, 15 mmol) and 1,2,4-triazole (4 1 g, 60 mmol) in MeCN (100 ml) Et_3N (6 1 g, 60 mmol) was added, followed by the corresponding **12** (B=Cyt, Gua, 10 mmol) and mixture was gently heated until it became homogenous Then it was left at room temp

for 20 hr Further work-up was identical to that described for method A **11γ** (B=Gua)-S_{13} followed by $CHCl_3$–MeOH (97 3), **11γ** (B=Cyt) $CHCl_3$–i-PrOH (95 5) followed by $CHCl_3$–i-PrOH (9 1) Separation of diastereoisomers was performed, as described under method A Compounds **11γ** (high R_f) and **11γ** (low R_f) after separation and evaporation of solvents, were dissolved in $CHCl_3$ and these solns were dropped into n-hexane for precipitation of solid products **11** The data are presented in Table 2

5′-O-Monomethoxytrityl-2′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphorothioates (**13**)

Each of diastereoisomers of **11γ** (1 mmol) was dissolved in DMF (10 ml) and NaH (2 mmol, 0 1 g) was added This mixture was stirred for 15 min at room temp and then was treated with CS_2 (4 ml) The reaction was controlled by means of TLC (S_2) Usually after 30 min a substrate disappeared The mixture was cooled on CO_2–EtOH bath (− 79°) and an excess of pyridinium form of Dowex 50W × 8 was added (pH 7–6 5) The ion exchange resin was filtered off and washed with pyridine Combined solns were evaporated Oily residues were evaporated with pyridine (3 × 5 ml), dissolved in pyridine (5 ml) and dropped into n-hexane The ppt was dried under reduced pressure and purified on PTLC (S_7) For the elution of products the solvent system $CHCl_3$–MeOH (1 1) containing 0 2% NEt_3 was used After solvent evaporation, the residue was dissolved in pyridine and dropped into n-hexane The ppt was washed with n-pentane and dried in desiccator Data for pyridinium salts of **13** are collected in Table 3

2′-Deoxyribonucleoside 3′-(2-chlorophenyl) phosphorothioates (**4**, B=Thy, Ade, Gua, Cyt)

The corresponding diastereoisomer of **13** (0 2 mmol) was dissolved in 80% AcOH (5 ml) at a temp not exceeding 40° and the reaction progress was monitored by means of TLC (S_7) After *ca* 40 min n-BuOH was added (5 ml) and the solvents were evaporated to dryness The residue was dissolved in pyridine (*ca* 5 ml) and this soln was dropped into diisopropyl ether The ppt was dissolved in H_2O and product was isolated on DEAE-Sephadex A-25 (HCO_3^- form, LKB 25 × 600 column) using linear gradient of TEAB (0 05–0 08 M), 60 ml/hr Fractions containing the desired product were pooled together, concentrated under reduced pressure and lyophilized The yields, and results of chromatographic and spectral control, are collected in Table 4

2′-Deoxyribonucleoside 3′-(2-chlorophenyl) phosphoranilidates (**1**, B=Thy, Ade, Gua, Cyt)

Into the soln of 2% of toluene-*p*-sulphonic acid monohydrate in solvent S_{14} (20 ml) the corresponding diastereoisomer of **11γ** (1 g) was added and the mixture was stirred at room temp for 10 min The removal of the 5′-O-monomethoxytrityl group was followed by means of TLC (S_1) The mixtures were washed with 5% $NaHCO_3$ (3 × 15 ml) The combined aqueous layers were extracted with $CHCl_3$ (4 × 15 ml) and combined organic fractions were dried over $MgSO_4$ Solvents were evaporated and the residues were dissolved in pyridine (5 ml) Pyridine was evaporated and the residues were coevaporated with toluene (3 × 5 ml) Products **1** were dissolved in acetone (B=Ade, Cyt), acetone–benzene (B=Thy) or $CHCl_3$ (B=Gua), and these solns were dropped into n-hexane The ppt was washed with n-pentane and dried under reduced pressure Further purification was achieved by means of column chromatography using solvent system as indicated in Table 5

5′-O-Monomethoxytritylthymidine 3′-phosphoranilidate (**14**)

Compound **11γ** (B=Thy, 4 3 g, 5.5 mmol) was dissolved in the mixture of dioxan (55 ml) and 0 125 N NaOH (165 ml) The reaction progress was followed by means of TLC (S_5) After *ca* 20 hr into the mixture Dowex 50W × 8 (pyridinium form) was added The resin was filtered off and washed with pyridine (50 ml) The filtrates were combined and evaporated to *ca* one-half of their original volume The residue was extracted with diethyl ether (3 × 30 ml) and water fraction was concentrated The oily residue was co-exaporated with pyridine (3 × 5 ml) and toluene (3 × 5 ml), and dissolved in $CHCl_3$ and dropped into n-hexane The ppt was centrifuged and purified by means of short column chromatography (Silica gel 70–230 mesh, 300 g) using $CHCl_3$–MeOH containing 0 2% NEt_3 as eluting systems 10 2, 10 3 and 10 4, respectively The yield of pyridinium salt of **14** was 85% TLC R_f (MeCN–H_2O 9 1) 0 28, R_f (S_6) 0 58, UV λ_{max} 270 5 nm, λ_{min} 253 8 nm (96% C_2H_5OH), $\delta_{^{31}P}$ − 2 34 ppm ($CHCl_3$)

5′-O-Monomethoxytritylthymidine 3′-(2-chlorophenyl) phosphate (**3**)

Compound **11γ** (B=Thy, 0 78 g, 1 mmol) was dissolved in pyridine (10 ml) and evaporated to dryness The residue was again dissolved in pyridine (10 ml) and into this soln NaH (0 24 g) was added The mixture was stirred for 15 min at room temp and treated with the stream of CO_2 dried over P_2O_5 The progress of the reaction was followed by TLC (S_1) The isolation of the product was analogous to that described for **14** Precipitated **3**, centrifuged from n-hexane, can be used for oligonucleotide synthesis without further purification For analytical purpose it was chromatographed on preparative TLC (S_7) Yield of pyridinium salt of **3** was 95% TLC R_f (S_6) 0 78, UV λ_{max} 269 3 nm, λ_{min} 250 0 nm (96% EtOH), $\delta_{^{31}P}$ − 5 96 ppm (C_5H_5N)

Conversion of 2′-deoxyribonucleoside 3′-(2-chlorophenyl) phosphoranilidates (**1**) *into 2′-deoxyribonucleoside cyclic 3′,5′-phosphoranilidates* (**5**)

Into the soln of corresponding **1** (1 5 mmol) in N,N-dimethylacetamide (15 ml) freshly prepared t-BuOK (15 mmol, obtained from the reaction between 15 mmol of K metal with 10 ml of t-BuOH, followed by removal of the excess of t-BuOH under reduced pressure, bath temp 60) was added, and the mixture was left for 20 hr at room temp with the exclusion of moisture After cooling to − 70° an excess of Dowex 50W X8 (pyridinium form) was added until pH 7 0 The resin was filtered off and washed with pyridine (3 × 10 ml) In the case of **1e** and **1f**, the resin was washed additionally with DMF (2 × 10 ml) Combined filtrates were evaporated and coevaporated with pyridine (3 × 5 ml) After evaporation of most of the pyridine its traces were removed by coevaporation with toluene (3 × 5 ml) Products **5** were isolated by means of preparative TLC **5a, 5b, 5c, 5d**-developing system S_1, **5e, 5f**-$CHCl_3$–MeOH (8 2) containing 1% of NEt_3, **5g, 5h**-S_7 Products were eluted from Silica gel by means of $CHCl_3$–MeOH–DMF (1 1 1) Removal of solvents left residues which after dissolution in pyridine were dropped into diisopropyl ether The ppt was centrifuged, washed with n-pentane and dried under reduced pressure If, as a starting material, the mixture of diastereoisomers (**1**) were used, the products containing both diastereoisomers of corresponding **5** were separated by means of short-column chromatography (250 g of silica gel 200–300 mesh for 1 g of **5**) Eluting systems **5a, b**-$CHCl_3$–MeOH (95 5), **5c, d**-S_9 followed by $CHCl_3$–96% EtOH (85 15), **5e, f**-$CHCl_3$–MeOH (95 5) and (S_{14}), **5g, h**-$CHCl_3$–i-PrOH (70 30) and (60 40) Yields and physico-chemical data relating to **5** are presented in Table 6

Conversion of 2′-deoxyribonucleoside cyclic 3′,5′-phosphoranilidates (**5**) *into 2′-deoxyribonucleoside cyclic 3′,5′-phosphorothioates* (**6**)

The soln of corresponding **5** (0 25 mmol) in DMF (3 ml) was treated with NaH (0 025 g of 50% NaH in mineral oil) and after stirring at room temp for 15 min CS_2 (1 ml) was added into the mixture Reaction progress was controlled by means of TLC (S_7) After *ca* 30 min mixture was cooled to − 70 and pyridinium form of Dowex 50W X8 was added

(pH 7–6 5) Further work-up was analogous to that described for **5** Precipitates **6** were purified on DEAE-Sephadex A-25 (HCO_3^-), LKB 25 × 600 column, using linear gradient of TEAB (pH 7–6.8), 60–100 ml/hr **6a, 6b, 6c, 6d**-0.05–0 8 M, **6e, 6f**-0 05–1 5 M, **6g, 6h**-0 05–1 0 M Fractions containing the desired products were pooled together and concentrated Syrupy oils were co-evaporated three times with EtOH and concentrated under reduced pressure Yields and physico-chemical data relating to the pyridinium salts of **6** are presented in Table 7

Cyclisation of thymidine 3′-(4-nitrophenyl) phosphorothioates (**4k, 4l**)

Diastereoisomeric mixture of **11ε** (Table 1) was separated into S_P-**11ε** and R_P-**11ε** on Silica gel (S_{11} and S_5) S_P-**11ε**, R_f 0 33 (S_5), δ_{31P} − 3 37 ppm ($CDCl_3$), *m/z* 789 ($M^+ - 1$), R_P-**11ε**, R_f 0 24 (S_5), δ_{31P} − 3 17 ppm ($CDCl_3$), *m/z* 789 ($M^+ - 1$) Each diastereoisomer was transformed to **13k** and **13l** in a manner analogous to conversion **11**→**13** (*vide supra*) Thus, from S_P-**11ε** (high R_f) compound **13k**, δ_{31P} 53.2 ppm, and from R_P-**11ε** (low R_f) compound **13l**, δ_{31P} 51 0 ppm, were obtained, respectively Removal of monomethoxytrityl group (procedure as described above for **1**) from each diastereoisomer left **4** (B=Thy, Ar=4–NO_2–C_6H_4–) Thus, from S_P-**11ε** diastereoisomer R_P-**4k** was obtained, R_f 0 69 (cellulose F, S_8), δ_{31P} 50 68 ppm (C_5H_5N). R_P-**11ε** gave S_P-**41**, R_f 0 71, δ_{31P} 50 76 Final purification of each diastereoisomer **4k** and **4l** was performed on Whatman 3MM paper (S_8) S_P-**41** (0 5 mmol) under treatment with t-BuOK, under conditions described for preparation of **5**, gave S_P-**6b**, δ_{31P} 52 1 ppm (H_2O), in 70% yield Analogously, from R_P-**4k** compound R_P-**6a**, δ_{31P} 54 7 ppm (H_2O), was obtained in 76% yield

Condensation of 5′-O-monomethoxytritylthymidine 3′-phosphoranilidate (**14**) *with 3′-O-acetylthymidine*

Into the soln of **14** (B=Thy, pyridinium salt, 2 5 mmol) and 3′-0-acetylthymidine (3 8 mmol) in pyridine (10 ml) 2,4,6-triisopropylbenzenesulphonic chloride (7 5 mmol) was added and solvent was evaporated under reduced pressure. Syrupy residue was left at room temp for 40 hr † After cooling to − 70° the mixture of pyridine and water (1 1, 50 ml) was added This solution was extracted with $CHCl_3$ (3 × 30 ml) and the organic phase was washed with phosphate butter (4 × 50 ml) and then dried over $MgSO_4$ The soln was evaporated, the residue dissolved in pyridine and dropped in a mixture of n-hexane and diethyl ether (1.1) The ppt was filtered off, washed with n-pentane and dried under reduced pressure Isolation of the diastereoisomeric mixture (**15a, b**) was achieved by means of preparative TLC (S_{12}) The product was eluted from silica gel with $CHCl_3$–MeOH (1 1) and, after evaporation of the solvent, the product **15a, b** was dissolved in pyridine and this soln was dropped into diethyl ether-n-pentane (1.1) The yield was 0 12 g (5%) Separation of diastereoisomers was performed on TLC plates (S_9) giving **15a** (high R_f), δ_{31P} 2.69 ppm (C_5H_5N) and **15b** (low R_f), δ_{31P} 2 32 ppm Condensation of **14** with 3′-0-monomethoxytritylthymidine was performed as described above The yield of **15a′, b′** was 6%.

†^{31}P-NMR spectrum of this mixture (in C_5H_5N) contained the signals at 2 3 and 2 7 ppm (*ca* 10%) and unsymmetrical triplet of signals at − 8 80, − 9 04 and − 9 22 ppm (*ca* 80%) High-field signals are due to the presence of **19** (2-chiral P-centres) Its structure was proved in the following way a sample of reaction mixture characterized by means of ^{31}P-NMR spectrum containing high-field triplet of signals (*ca* 80%), dissolved in H_2O, was heated at 40° for 5 hr The ^{31}P-NMR spectrum has shown the disappearance of high-field triplet of signals due to hydrolysis of **19** and, besides the unchanged signals of **15** at 2 3 and 2 7 ppm, the new intensive signal at − 1 40 ppm, corresponding to **14**, have appeared

15a′ R_f (S_9) 0 32, δ_{31P} 2.3 ppm ($CHCl_3$), **15b′** R_f (S_9) 0 28, δ_{31P} 1 8 ppm ($CHCl_3$).

Diastereoisomeric mixture **15a, b** was treated with C_5H_5N/AcOH/i-$C_5H_{11}ONO$,[62] then with 9 M NH_4OH, followed by 80% AcOH The product was identical with genuine sample of T_PT. Its hydrolysis in the presence of spleen phosphodiesterase (EC 3 1 4 18) in 0 05 M NaOAc buffer gave thymidine and thymidine 3′-phosphate

Thymidine-3′ thymidine-5′ phosphorothioates (**9**)

The soln of each diastereoisomer of **15a′** and **15b′** (30 mg) in DMF (10 ml) was treated with NaH/CS_2 analogously as for transformation **11**→**13**. Thus, from **15a′** (high R_f) **16a′** was obtained in 85% yield, δ_{31P} 52 3 ppm ($CHCl_3$) Its treatment with 80% AcOH gave **9a**, yield, 70% Similarly, from **15b′** (low R_f) compound **16b′** was obtained in 76% yield, δ_{31P} 54 3 ppm ($CHCl_3$), which was further transformed into **9b** in 72% yield Both **9a** and **9b** were purified on DEAE Sephadex A-25 column using a linear gradient of TEAB buffer (0 05–1 0 M), then with Dowex (H^+) and neutralisation with 0 1 N NaOH solution Chemical shifts are as follows: **9a**—δ_{31P} 55.6 ppm (H_2O), **9b**—δ_{31P} 55 1 ppm (H_2O) HPLC of **9** on Spherisorb S5 ODS column [eluent CH_3OH–H_2O (15 85)] has shown the homogeneity of each diastereoisomer and shorter retention time for **9a**

Thymidine-3′ thymidine-5′ [^{18}O]phosphates (**17**)

The separated diastereoisomers of **16–16a′** and **16b′** (each of 41 μmol) were dissolved in the mixture of pyridine (2 ml), lutidine (45 μmol) and $H_2{}^{18}O$ (100 μl), and a soln of cyanogen bromide (120 μmol) in THF (0 5 ml) was added The reaction was terminated after 30 min by addition of cysteine (120 μmol) [37] The mixtures were evaporated to dryness and the crude products were dissolved in a soln of 2% toluene-*p*-sulphonic acid in solvent system S_{13} (20 ml) The deprotection of 5′- and 3′-OH groups was completed after 40 min (TLC assay-S_7) Then the mixtures were extracted with H_2O (3 × 10 ml), water fractions were collected and crude **17** were separated on DEAE-Sephadex A-25 column eluted with linear gradient of TEAB buffer (0 05–1 0 M), and lyophilised Compounds **17a** and **17b**, prepared from **16a′** and **16b′**, respectively, were obtained in 42% (**17a**) and 32% (**17b**) yields Their chromatographic mobilities were the same as T_PT-standard [cellulose plates (S_8 and S_9)]

Assignment of the absolute configuration of **9**

The solns of **9a** or **9b** (65 μmol of Na salts) in 100 mmol Tris–AcOH, 20 mmol $MgCl_2$ buffer pH 8 0 (500 μl) were treated with phosphodiesterase from snake venom (E C 3 1 4 1, 0 2 mg) for 15 hr at 37° The products of the digestion were analysed by TLC (Silica gel plates, solvent system MeCN– 100 ml Tris–AcOH, pH 8 0, 10 1), HPLC and ^{31}P-NMR spectroscopy by comparison with standards of 5′-TMPS and thymidine Under these conditions, compound **9a** was digested to the extent of 50%. ^{31}P-NMR examination showed the decreasing of the intensity of the signal at 55 56 ppm (corresponding to substrate **9a**) and appearance of signals at 43 07 ppm (thymidine 5′-phosphorothioate) and others at 4 32, 3 72 and 2 62 ppm (unidentified products) Under these conditions, **9b** was not digested and its ^{31}P-NMR spectrum did not show of appearance of any new signals According to reports of Eckstein[29] and Benkovic[30] compound **9a** has the R_P absolute configuration

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