123. Nucleosides and Nucleotides. Part 2: Synthesis of Both Anomers of 1-(5′-O-Phosphoryl-2′-deoxy-D-ribofuranosyl)-2(1*H*)-pyridone [1][1])

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Summary. The two anomeric 1-(2′-deoxy-D-ribofuranosyl)-2(1*H*)-pyridones **6** and **7** were synthesized from 2-pyridone and 3,5-di-(O-*p*-toluoyl)-2-deoxy-D-ribofuranosyl chloride (**2**) via the di-O-*p*-toluoyl derivatives **3** and **4** using the mercuric halide procedure. Phosphorylation of the nucleosides **6** and **7** by bis-(2,2,2-trichloroethyl)-chlorophosphate gave the phosphate esters **8** and **9** together with some 2-(bis-[2,2,2-trichloroethyl]-phosphoryloxy)-pyridine **10**, which proved to be very labile. Structure and configuration of compounds **6** to **9** were established by spectral methods, the configurations being derived from the chemical shifts of the sugar protons and the splitting patterns of the anomeric protons ('triplet-quartet rule'). The specific rotations of **3**, **4**, **6**, **7**, **8** and **9** show that the three pairs of anomers represent exceptions to *Hudson*'s rule of isorotation.

Reductive removal of the trichloroethyl groups in **8** and **9** with zinc proceeds stepwise, yielding the phosphoro-diesters **13** and **14** and the two desired anomeric 5′-nucleotides **15** and **16**. These latter were purified and characterised as the ammonium salts.

Enzymatic cleavage by the 5′-nucleotidase of *Crotalus adamanteus* venom took place only in the 'natural' β-series. The 'unnatural' α-anomers were resistent to the enzyme.

The structure of **10** was established by spectral methods and confirmed by synthesis.

1. Introduction. – Nucleosides and nucleotides which differ from the naturally occurring substances either by structural modification of the sugar or the base moiety have been shown to exhibit a variety of interesting biological properties [2]. Some inhibit the growth of bacteria, others prove to be active against experimental tumors [3]. Antiviral and immunological activities of nucleoside derivatives have also been reported [4]. Modified nucleosides can act as inhibitors of enzymes and have been used as models of substrates to study mechanisms of enzyme reactions [5], while oligonucleotides which contain an unnatural nucleotide can be used as tools for the elucidation of the mechanisms of protein biosynthesis [6].

The biological function of the nucleoside units within the frame work of the nucleic acids is inconceivable without the presence of the functional groups of the heterocyclic bases. If they are missing, totally or partially, the formation of the complementary base pairs is disturbed. Oligo- and poly-nucleotides containing one or more nucleotide units with a base not being able to undergo the base pairing, *i.e.* defective, should lead to a more detailed understanding of the consequences of such a "hole of information" during the replication or transcription of deoxyribonucleic acid (DNA). The incorporation of a single deoxyribose unit without base into an oligonucleotide chain would certainly yield the most unequivocal hole in the chain of information. However, the sugar would thereby lose its defined configuration at C(1) through the formation of an equilibrium between the open oxo structure

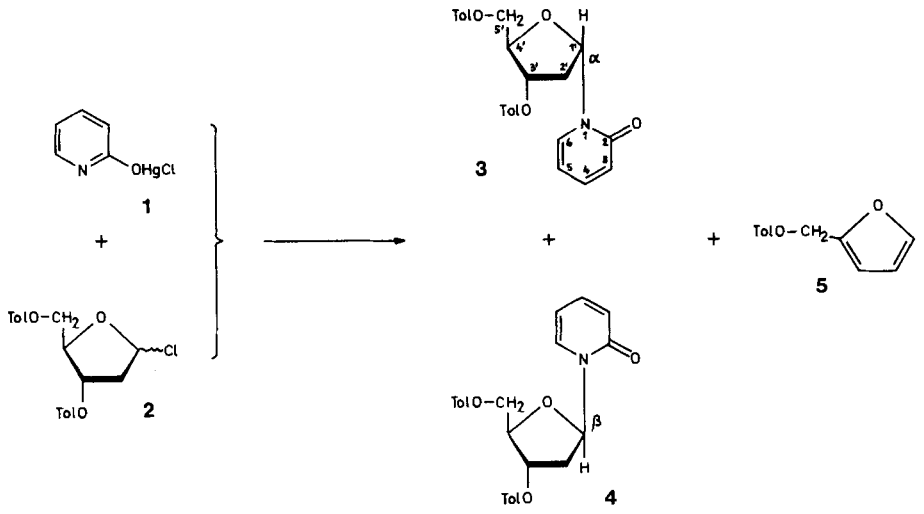
[1]) Presented in part at the Meeting of the Swiss Chemical Society, Basel, 17th September 1970.

and the cyclic α- and β-furanoside forms. We chose 2(1*H*)-pyridone as unnatural base [7], because its steric requirements and its basicity ($pK_a = 0.75$) [8] are very close to those of the natural pyrimidines [9]. This close relationship to the natural monocyclic bases should allow the application of the usual synthetic methods and protecting groups for the incorporation of the unnatural nucleosides or nucleotides into an oligonucleotide sequence.

In this communication the synthesis of nucleosides and mononucleotides with 2(1*H*)-pyridone as base and 2-deoxy-D-ribose as sugar moiety is described.

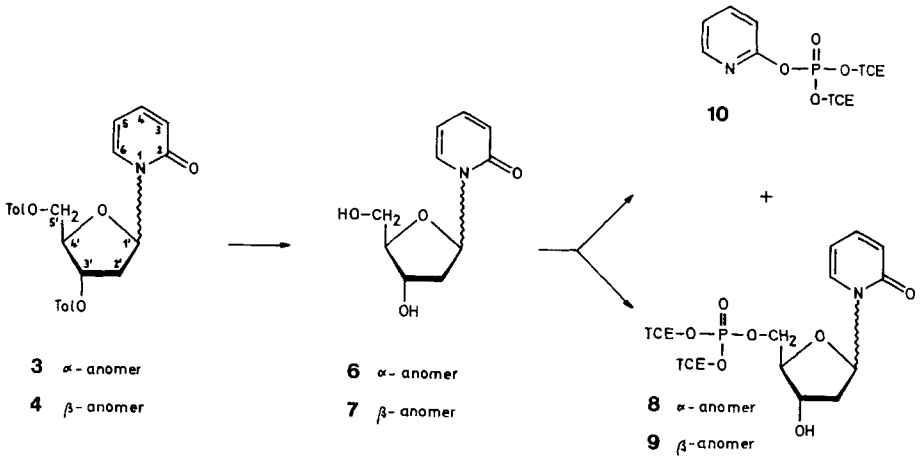
The mercuri procedure used to attach the 2-pyridone to the deoxyribose moiety gave a separable mixture of both the nucleoside analogues: the "natural" β-glycosidic compound and the "unnatural" α-anomer. The latter compounds are of special interest, since little is known of their physical properties and their behaviour towards the various enzymes used in nucleotide biochemistry.

2. Synthesis of 1-(2′-Deoxy-α-D-ribofuranosyl)-2(1*H*)-pyridone ($\alpha\Pi_d$)[2] and of 1-(2′-Deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone ($\beta\Pi_d$)[2]. – In Part 1 the synthesis of both anomers of the di-O-toluoyl derivatives **3** and **4** of the 2-deoxy-D-ribofuranosides of 2(1*H*)-pyridone was described [1]. The nucleoside derivatives were obtained in low yield by reaction of (2-pyridyloxy)-mercuric chloride (**1**) with one equivalent of 3,5-di-(O-*p*-toluoyl)-2-deoxy-D-ribofuranosyl chloride (**2**) in boiling toluene for 7 hours in the presence of $HgBr_2$. A large amount of furfuryl *p*-toluate (**5**) was obtained as a further product. A detailed study of this reaction has now shown



[2]) The abbreviations used are in agreement with the CBN rules [10]. For the distinction between the α- and the β-anomers respectively and for the characterization of further protecting groups the following additions were necessary:
$\alpha\Pi_d$ = 1-(2′-deoxy-α-D-ribofuranosyl)-2(1*H*)-pyridone;
$\beta\Pi_d$ = 1-(2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone;
Tol = *p*-CH_3—C_6H_4—CO—; TCE = CCl_3—CH_2—.
OD_{302} units: The number of optical density (OD.) units represents the product of the total volume of a solution and of its optical density, measured in a 1 cm cell at $\lambda = 302$ nm.

that the yields of the glycosides are increased if 1.6 equivalents of **1** (relative to **2**) are used at 100–110° during 1–2 hours. The addition of $HgBr_2$ proved to be unnecessary since O-glycosides which are formed as intermediates [11] are transformed into the desired N-glycosides **3** and **4** by the $HgCl_2$ formed *in situ*. Under these conditions a yield of 27% of the β-nucleoside (Tol)β*II*$_d$(Tol) (**4**) and 21% of (Tol)α*II*$_d$(Tol) (**3**)[2]) were obtained. For the removal of the protecting groups the di-O-toluoyl nucleosides **3** and **4** respectively were treated with Na methoxide in abs. methanol. The separation of the free nucleosides **6** and **7** from methyl *p*-toluate was achieved by partitioning the reaction mixture between water and petroleum ether or water and chloroform. The crude nucleosides were purified by chromatography on silica gel and crystallized from acetone, yielding 70–90% of α*II*$_d$ (**6**) and β*II*$_d$ (**7**) respectively.



The IR., UV., and mass spectra of the anomer **6** were practically identical with those of **7**. Only the NMR. spectra and the optical rotations differed. The band at 1650 cm^{-1} in the IR. spectrum and the absorption maximum at 302 nm in the UV. spectrum demonstrate that the linkage between the deoxyribose and the base has

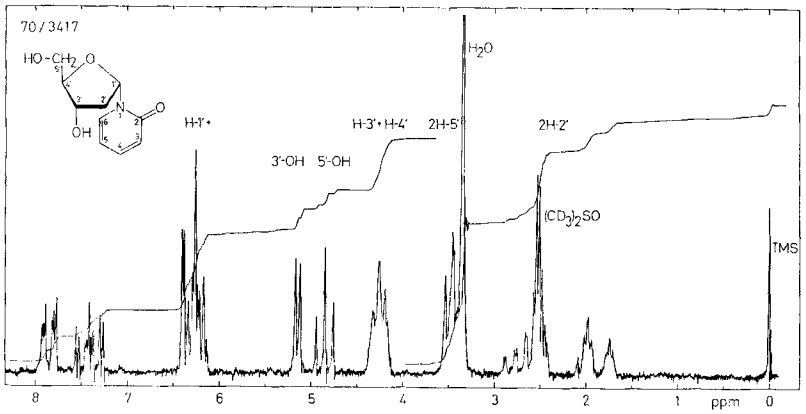


Fig. 1. *60 MHz NMR. Spectrum of* α*II*$_d$ (**6**) *in* $(CD_3)_2SO$[3])

retained its N-glycosidic character as in the starting materials **3** and **4**. The broad absorption band at *ca.* 3300 cm^{-1} and the disappearance of the ester carbonyl stretching at 1725 cm^{-1} indicate the complete removal of the HO-protecting groups from the sugar moiety. The NMR. spectra measured in $(CD_3)_2SO$ solution (see Fig. 1 and 2) confirm the structures. All signals were assigned. It is noteworthy that the hydroxyl groups appear as a sharp doublet (3'-OH) and a triplet (5'-OH) respectively.

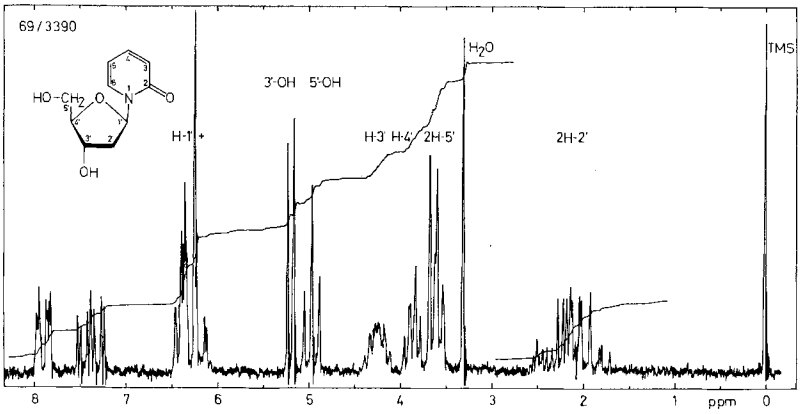


Fig. 2. *60 MHz NMR. Spectrum of βII$_d$* (**7**) *in* $(CD_3)_2SO$[3])

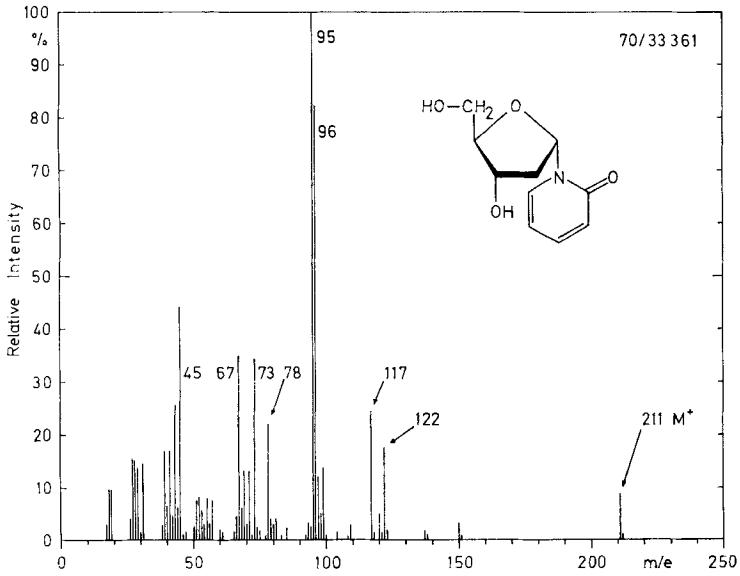


Fig. 3. *Mass spectrum of αII$_d$* (**6**)

In the mass spectra (see Fig. 3 and 4) the peaks of the molecular ions appear at *m/e* 211 and the characteristic peaks of the sugar moiety at *m/e* 117 (cf. formulae and [12]). The fragment of the base moiety is stabilized by the stepwise addition of two

[3]) The chemical shifts in all NMR. spectra are δ-values with $\delta = 0$ for tetramethylsilane.

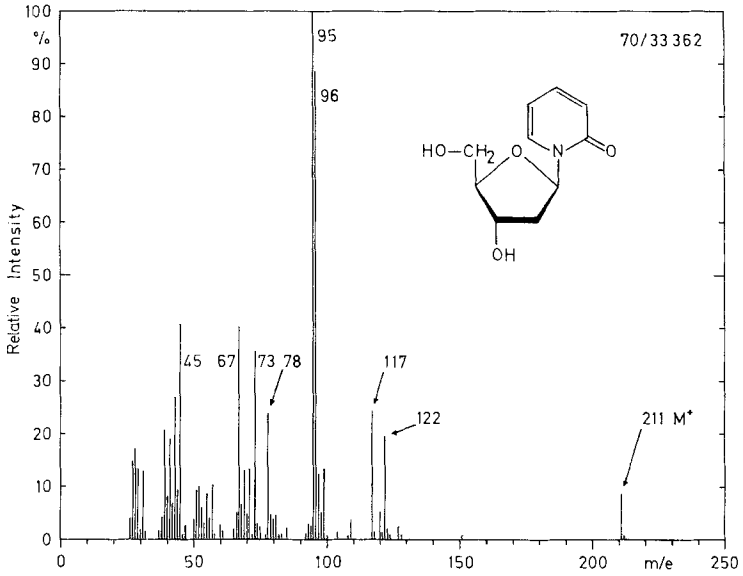
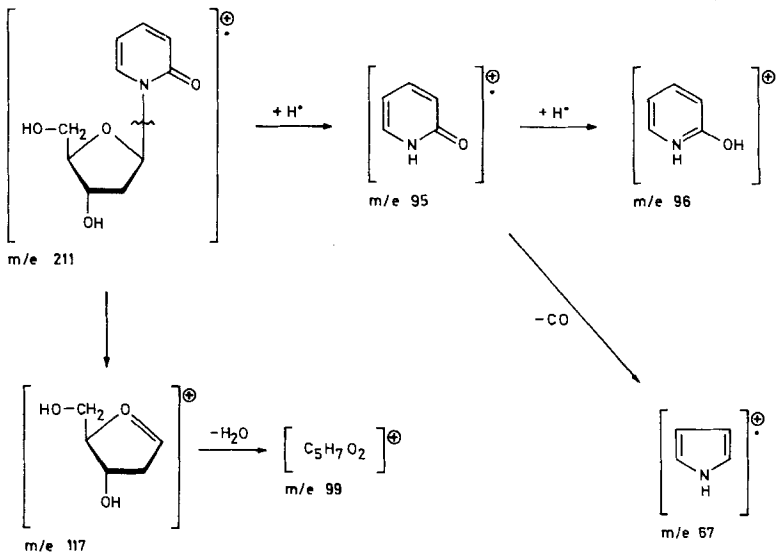


Fig. 4. *Mass spectrum of βII$_d$* (**7**)

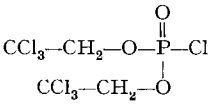


H atoms giving rise to ions of *m/e* 95 and 96. The ion *m/e* 95 (molecular ion of 2-pyridone) undergoes fragmentation by elimination of CO yielding the pyrrole ion *m/e* 67 [13]. The sugar ion loses water forming the fragment *m/e* 99. The peak $M - 89$ at *m/e* 122 characteristic for nucleosides was also observed.

The configuration of C(1′) of the nucleosides **6** and **7** follows from their mode of formation from the protected precursors **3** and **4**. The NMR. spectra, which will be

discussed further below, confirm the stereochemical assignments made earlier for the di-O-toluoyl derivatives **3** and **4** [1][4]).

3. Phosphorylation of $\alpha\Pi_d$ and $\beta\Pi_d$. – Normally for the 5'-phosphorylation of nucleosides 3'-O-acetyl derivatives serve as starting materials. The latter are obtained from the nucleosides by successive 5'-tritylation, 3'-acetylation and 5'-detritylation. The 3'-O-acetyl nucleoside is reacted with β-cyanoethyl phosphate in pyridine with dicyclohexyl-carbodiimide or mesitylene-sulfonyl chloride as condensing agent. Very recently *Eckstein* & *Scheit* reported bis-(2,2,2-trichloroethyl)-chlorophosphate (**11**) as selective agent for the 5'-phosphorylation of nucleosides without requiring protection of the 3'-hydroxyl group [16]. The phosphoric acid triesters obtained are readily isolated and purified. The trichloroethyl groups are then removed by reduction, whereby yields of 42–75% are reported [17]. However the literature does not record any by-products.



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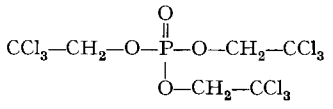

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Similarly as reported, we reacted the β-nucleoside **7** with 1.2 equivalents of the phosphorylating agent **11** in abs. pyridine for 24 hours at 2°. After removal of the solvent, the reaction products were partitioned between chloroform and water. The chloroform phase yielded a colourless syrup which, according to thin-layer chromatography, contained several products. After chromatography on silica gel, 35% of the desired nucleotide $(TCE)_2p\beta\Pi_d$ (**9**), a small amount of tris-(2,2,2-trichloroethyl) phosphate (**12**) [18][5]) and a large amount of a non-polar compound were obtained. The latter decomposed after removal of the solvent, yielding crystalline 2(1*H*)-pyridone. As shown further below, this non-polar by-product is probably the phosphate **10** of 2-hydroxypyridine. The yields of the phosphorylation were considerably improved by carrying out the reaction at −40° for 1–2 hours (56% of nucleotide **9**).

In an analogous manner the α-nucleotide **8** was also prepared, yield 54%.

Whereas the α-nucleotide **8** crystallized (m.p. 98–100°), the β-anomer **9** was obtained only as a syrup. The IR. spectra of both nucleotides **8** and **9** were almost identical, exhibiting characteristic bands at 3370 cm^{-1} (3'-OH), 1650 cm^{-1} (C=O of pyridone) and 1280 cm^{-1} (P=O). The UV. spectra (λ_{max} = 302 nm) indicate N-substitution of the pyridone. In the NMR. spectra (see Fig. 7 and 10) the trichloroethyl groups gave rise to a doublet and a double doublet. For the coupling constant of 7 Hz ^1H-^{31}P coupling along three bonds is responsible [19]. One proton was exchanged by deuterium in D_2O solution (3'-OH). In $(CD_3)_2SO$ solution only the OH-signal of the α-nucleotide showed sharp absorption (doublet): in the β-nucleotide the signal

[4]) The nucleosides **6** and **7** were synthesized recently independently by *Heller* & *Wagner* [14]. They first prepared the O-glycosides (by reacting the silver salt of 2(1*H*)-pyridone with the halogenose **2**), which were then transformed into the nucleosides by O → N-*trans*-glycosidation.

Using the mercuric halide procedure, *Mertes* [15] obtained only 6% of the mixture of the anomers of the di-O-toluoyl derivatives **3** and **4**.

[5]) We do not know whether the isolated ester **12** has been formed in the course of the reaction or whether it is an impurity contained in the phosphorylating agent **11**.

was still broad. The NMR. spectra establish the 5'-position of the phosphoryl group. The chemical shifts of the 3'-proton (cf. Table 1) in the nucleotides **8** and **9** respectively correspond rather to those of the unprotected nucleosides **6** and **7** respectively, while the chemical shifts of the 5'-protons are closer to those of the di-O-toluoyl derivatives of the nucleosides **3** and **4** respectively.

Epimerisation at C(1') of the nucleotides **8** and **9** in the course of their formation was unlikely, and this stereochemical point was verified by the NMR. data discussed in detail below.

Table 1. *Chemical Shifts of the Protons at C(3') and C(5') (ppm with $\delta = 0$ for $(CH_3)_4Si$)*

		in $(CD_3)_2SO$ H-(3')	2H-(5')	in C_5D_5N H-(3')	2H-(5')
α-Series					
(Tol)αIId(Tol)	(**3**)	5.55	4.50	5.80	4.74
αIId	(**6**)	4.25	3.48	4.83	3.96
$(TCE)_2$pαIId	(**8**)	4.30	4.30	4.80	4.62
β-Series					
(Tol)βIId(Tol)	(**4**)	5.55	4.60	5.80	4.88
βIId	(**7**)	4.26	3.62	4.87	4.10
$(TCE)_2$pβIId	(**9**)	4.38	4.38	4.83	4.83

4. Determination of the Configuration of C(1') of 2-Deoxy-ribosides by NMR. Spectroscopy. – For the determination of the configuration of the anomeric C atoms of 2'-deoxy-nucleosides and -nucleotides either the chemical shifts of the protons of the sugar moiety or the fine structure or band width of the signal of the anomeric proton (H at C(1')) can be used. Concerning the first approach, it is known that the protons in the *cis*-position with respect to the aglycone are shifted down field relative to the anomeric series. The shifts are 0.14–0.44 ppm for the proton at C(4') in the α-series and 0.09–0.29 ppm for the proton at C(3') in the β-series [20]. These shifts cause separate signals for the protons at C(4') and C(5') in the di-O-toluoyl derivatives of the α-series, whereas a complex multiplet is observed in the β-series [21]. However, the protons at C(3') and C(4') of the free α-nucleosides show a complex signal whereas the absorptions are clearly separated in the β-series [20]. The second method was developed by *Lemieux* & *Hoffer* [22] who found that the signal of the anomeric proton of the α-series consists of four, and that of the β-series of three lines. For β-nucleosides the steric interaction between the base and CH_2-O of the sugar moiety causes deformation of the five-membered ring altering the dihedral angles between the C(1') and two C(2') protons to such an extent that the vicinal coupling constants between them are almost equal. The result is a pseudo-triplet with "J" = 7 Hz[6]) and width of signal of 14 Hz. For α-nucleosides, where the base and CH_2–O-substituents are on opposite sides of the five-membered ring, such a deformation does not occur. Coupling constants of *ca.* 3 and 7 Hz observed and the signal width of

[6]) Due to the complexity of the spectra, only approximate coupling constants (line spacings) can be given.

the resulting doublet of doublets is *ca.* 10 Hz. All pairs of anomeric deoxyribosides which are known to us, exhibit these splitting patterns [23], indicating that these patterns depend very little upon the difference of the chemical shifts of the two H atoms at C(2') or on the nature of the base attached to C(1'). A real "empirical triplet-quartet rule" can be established which will prove to be most useful for the determination of configurations of nucleosides. However, caution is necessary for the application of this concept if relatively large groups are attached either to the base or to the sugar moiety, since they can change the conformation of the deoxyribose considerably.

The NMR. spectra of the compounds **3**, **4**, **6**, **7**, **8** and **9** were measured in deuteriodimethylsulfoxide solution (see [1], Fig. 1, 2 and exp. part). On the basis of the chemical shifts of the sugar protons the configurations of the two di-O-toluolyl nucleosides **3** and **4** were assigned [1]. The configurations of the other four compounds which were assigned on the basis of their formation, were confirmed. It was not possible to apply the more elegant method of assignment using the "triplet-quartet rule" because the signal of the anomeric protons overlapped those of the protons at C(3) and C(5) of the pyridone ring. The same was true when the spectra were measured in $CDCl_3$ [21] or D_2O [15] solution. However, we assumed, that it should be possible to induce a shift of the signals of the protons of the pyridone ring by the application of an aromatic, magnetically anisotropic solvent which interacts with the nearly aromatic pyridone moiety of the nucleosides, leading to a distinct separation from the protons at C(1') of the sugar unit. This assumption proved to be correct using perdeuteriopyridine as solvent, as shown by the figures 5 to 10. The resulting patterns of signals are in agreement with the "triplet-quartet rule" in all cases. In the di-O-toluoyl β-nucleoside **4** the distances between the lines are not equal (6 and 8 Hz). Therefore the centre line of the pseudo triplet is split. However, the signal width of 14 Hz indicates clearly that **4** belongs to the β-series.

In general, the specific optical rotation of anomeric glycosides are related to their configuration. *Hudson*'s rule of isorotation states that in the D-series the α-anomers show a higher positive value than the corresponding β-anomers [24]. However, exceptions to this rule are known, *e.g.* several pyrimidine deoxyribosides [25]. Table 2 summarizes the specific optical rotations of the compounds **3**, **4**, **6**, **7**, **8** and **9**. It demonstrates that the three pairs of anomers which we have synthesized also represent such exceptions.

Table 2. *Specific Optical Rotations*

	α-Series		β-Series	
3',5'-di-O-*p*-toluoyl nucleosides	**3**:	$-132°$ [1]	**4**:	$+42°$ [1][7])
unprotected nucleosides	**6**:	$-100°$	**7**:	$+108°$
nucleotide bis-(2,2,2-trichloroethyl) esters	**8**:	$-12°$	**9**:	$+51°$

5. Removal of the Trichloroethyl Groups. – Several procedures are described for the removal of the trichloroethyl groups [17] [26]. At first we sought to remove them by heating the protected nucleosides with Zn dust in pyridine/water 9:1 solution [17]. We have found that the reductive cleavageroce peds stepwise forming the

[7]) In the experimental part of the earlier communication [1] the erroneous value of $+4.20°$ is given.

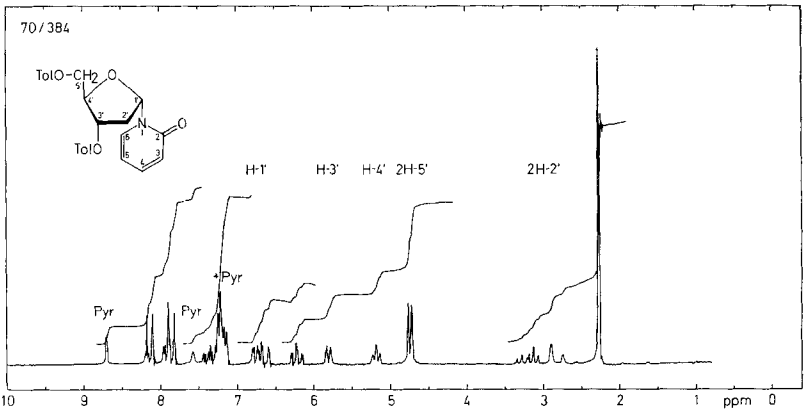


Fig. 5. *100 MHz NMR. Spectrum of (Tol)αΠd(Tol)* (**3**) *in* C_5D_5N

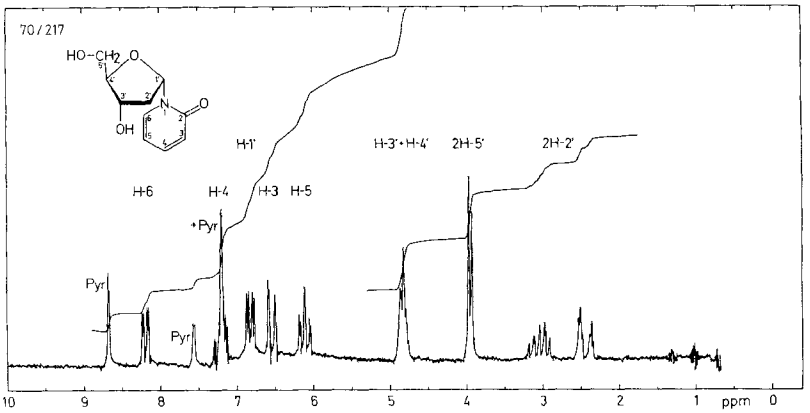


Fig. 6. *100 MHz NMR. Spectrum of* αΠd (**6**) *in* C_5D_5N

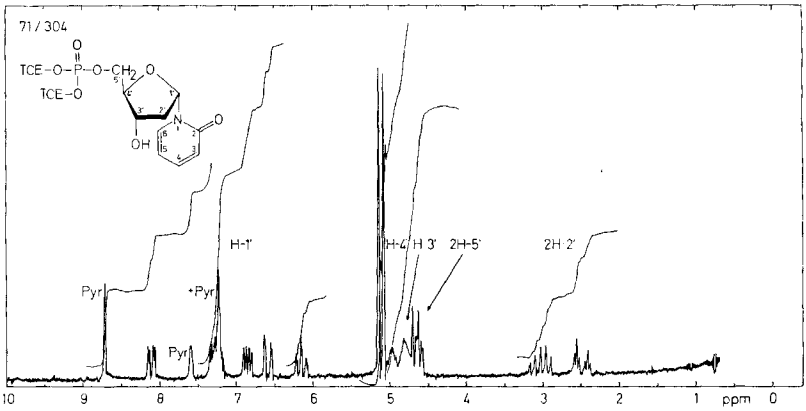


Fig. 7. *100 MHz NMR. Spectrum of* $(TCE)_2p$αΠd (**8**) *in* C_5D_5N

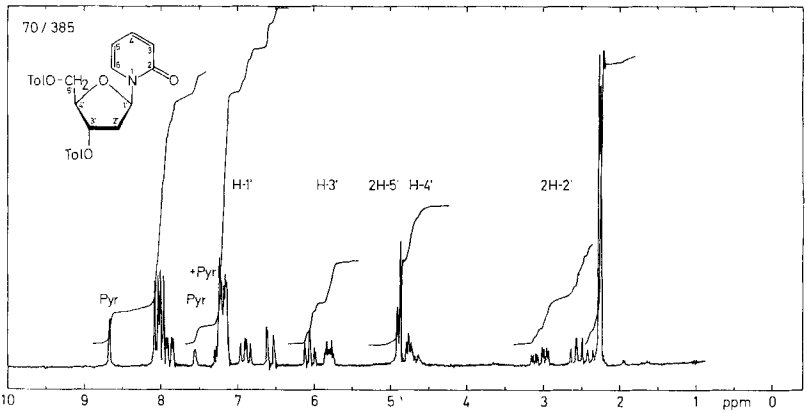


Fig. 8. *100 MHz NMR. Spectrum of (Tol)βΠd (Tol)* (**4**) *in* C_5D_5N

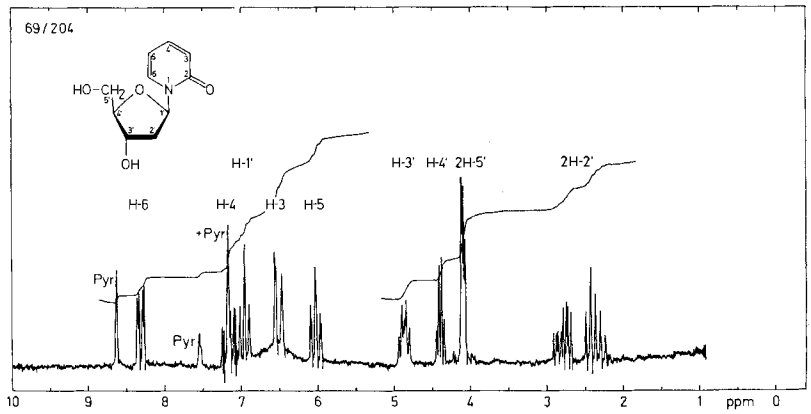


Fig. 9. *100 MHz NMR. Spectrum of βΠd* (**7**) *in* C_5D_5N

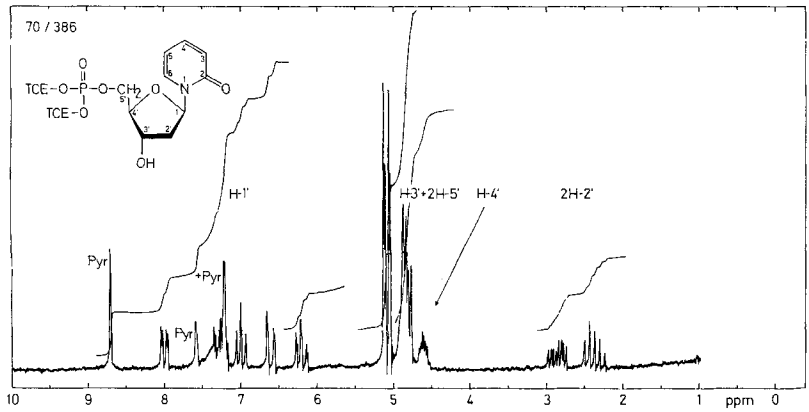


Fig. 10. *100 MHz NMR. Spectrum of* $(TCE)_2pβΠ_d$ (**9**) *in* C_5D_5N

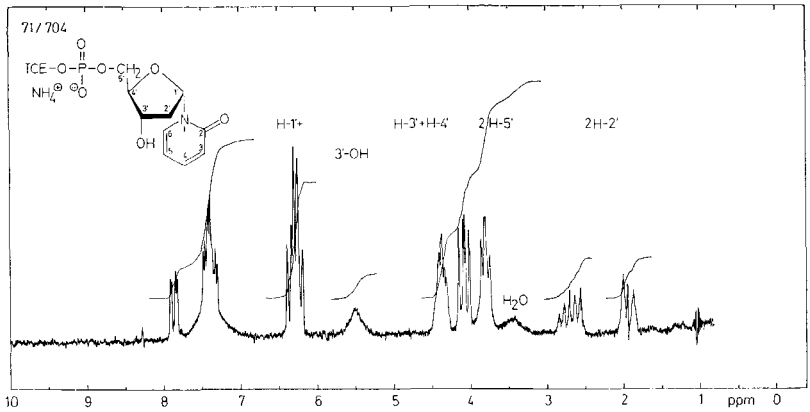


Fig. 11. *100 MHz NMR. Spectrum of the ammonium salt of (TCE)pαII*$_d$ (**13**) *in* $(CD_3)_2SO$

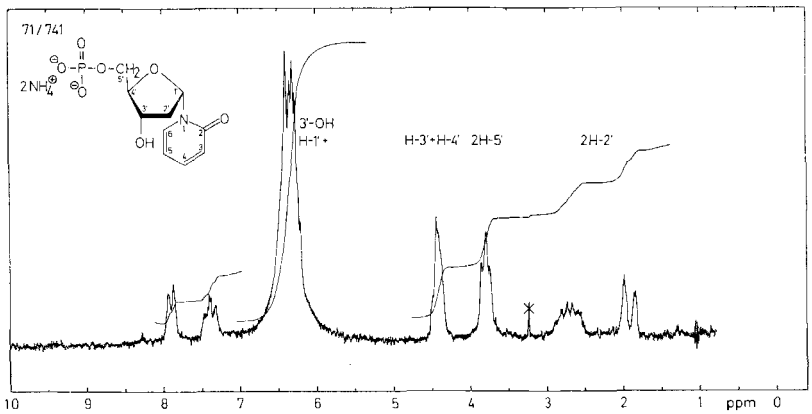
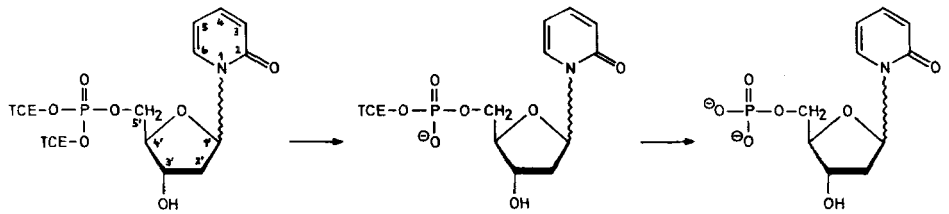


Fig. 12. *100 MHz NMR. Spectrum of the di-ammonium salt of pαII*$_d$ (**15**) *in* $(CD_3)_2SO$

mono-trichloroethyl nucleotide as first reaction product. After 20 minutes the ratio between mono-trichloroethyl nucleotide and free nucleotide was found to be still 2.1:1[8]).



[8]) According to *Franke et al.* [17] the reaction should require only 5 min.; the intermediate, the mono-trichloroethyl nucleotide, was not described by the authors.

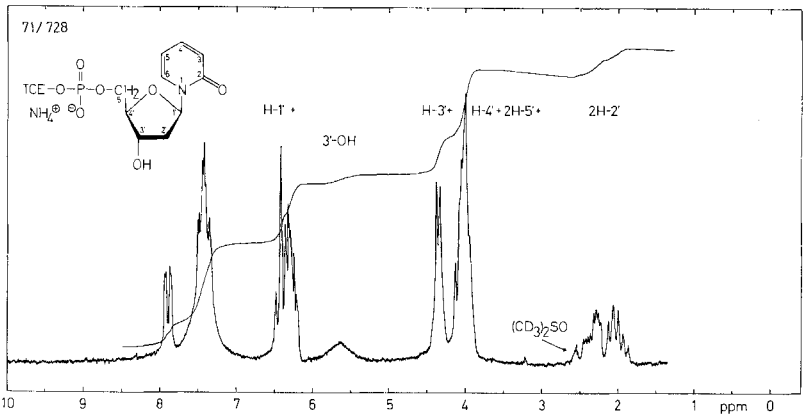


Fig. 13. *100 MHz NMR. Spectrum of the ammonium salt of (TCE)pβΠd* (**14**) *in* $(CD_3)_2SO$

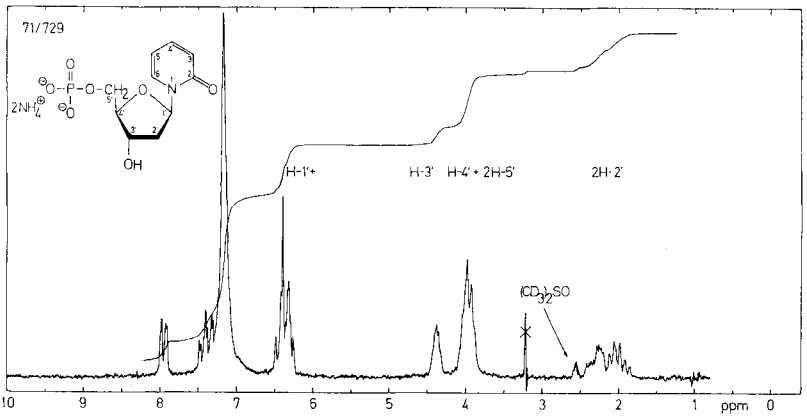


Fig. 14. *100 MHz NMR. Spectrum of the di-ammonium salt of pβΠd* (**16**) *in* $(CD_3)_2SO$

With pyridine containing a trace of acetic acid [26] as solvent a ratio of mono-trichloroethyl nucleotide to free nucleotide of 1:6.4 was achieved, but only after 5 hours. The total yield (counting both nucleotides) was 59%. The most satisfactory results, however, were obtained by shaking the protected nucleotide $(TCE)_2p\beta\Pi_d$ (**9**) in acetic acid/pyridine 8:3 solution with zinc dust at 23°. After 30 minutes 30% of mono-trichloroethyl nucleotide **14** and 58% of free nucleotide **16** (ratio of *ca.* 1:2) resulted. After 60 minutes the amount of free nucleotide is increased, the ratio of products amounting to 1:3.

The mixtures of products obtained were separated by anion exchange chromatography using DEAE-cellulose. The elution was carried out by a linear gradient of ammonium hydrogencarbonate. The extinction of the eluate was determined at 254 nm. In this manner both the nucleotide mono-trichloroethyl esters **13** and **14** and both the free nucleotides **15** and **16** were isolated as chromatographically pure ammonium salts, which were transformed to dry solids by lyophilization. By precipitation with sodium perchlorate **15** and **16** were obtained also as dry powdered

sodium salts [27]. The latter proved to be less favorable for the characterization of the compounds by NMR. spectroscopy due to their low solubility in deuteriopyridine and deuterio-dimethylsulfoxide.

The UV. absorption maxima of the nucleotides **13** to **16** at 225 and 302 nm indicated that the N-glycosidic linkage was still intact. The NMR. spectra of the ammonium salts of **13** to **16** in $(CD_3)_2SO$ (Fig. 11–14) confirmed their structures. It was interesting to note that the signals of the remaining trichloroethyl group of the nucleotide mono-trichloroethyl esters consist of two doublets. In the case of the α-compound **13** (see Fig. 11) the line spacing are 8 and 5.5 Hz; for the β-compound **14** (see Fig. 13) two doublets of equal intensity and separated by 25 Hz, with the coupling constant of 8 and 5 Hz, were observed. These patterns could be explained by assuming the formation of a "quasi-chiral centre" at the phosphorus due to the removal of one of the trichloroethyl groups[9]). One interpretation for the two doublets observed could be that the two protons of the remaining trichloroethyl group are no longer chemically equivalent and give rise to an AB pattern of an ABX system. According to another interpretation each of the two doublets would correspond to the trichloroethyl group possessing the one or the other orientation at the "chiral" phosphorus. The final proof will require a more detailed study[10]).

The nucleotides **13** and **14** being clearly intermediates in the course of the reductive cleavage of the trichloro-ethyl groups is indicated by the change of the ratio of products in favour of the free nucleotides with increasing time of reaction.

6. Enzymatic Hydrolyses. – To confirm the structures **13** to **16** the four nucleotides were incubated with alkaline phosphatase (EC 3.1.3.1). The nucleotide mono-trichloroethyl esters **13** and **14** were not hydrolysed. They proved to be stable during 1 hour of incubation, indicating that they are not monoesters of phosphoric acid. On the other hand the free nucleotides **15** and **16** were cleaved readily within 30 minutes, yielding the corresponding nucleosides. These results prove that both the trichloroethyl groups have been removed in these latter compounds.

It is known that venoms of many snakes contain among other enzymes a 5'-nucleotidase (EC 3.1.3.5) cleaving selectively nucleoside-5'-phosphates to nucleosides and phosphate. Nucleoside-3'-phosphates are not attacked. The enzyme recognizes either the ribose or the deoxyribose ring of the substrate. This is certainly true for nucleotides with a β-N-glycosidic linkage. However, the behaviour of the anomeric α-N-glycosides as substrates for these enzymes has not been investigated. Therefore the synthesized compounds were included in the following experiments. Even in crude preparations of the venoms of *Crotalus atrox* and *Crotalus adamenteus* the unspecific phosphatases and phospho-diesterases are 100 to 1000 times less active than the 5'-nucleotidase [29]. Therefore a suspension of lyophilized venom of *Crotalus adamanteus* in bidistilled water was used. For checking the absence of activity towards 3'-phosphates [29] [30] a number of 3'-nucleotides were also tested. The results are summarized in Table 3.

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[9]) For chiral phosphorus compounds *cf.* [28].

[10]) We should like to express our gratitude to Prof. *P. Diehl*, Physikalisches Institut der Universität Basel, and to Dr. *T. G. Payne*, *Sandoz AG*, Basel, for helpful discussions.

Table 3. *Enzymatic Cleavage of Nucleotides by 5'-Nucleotidase of the Venom of* Crotalus adamanteus

Substrate		Product	
thymidine-5'-phosphate	pT_d	thymidine	T_d
thymidine-3'-phosphate[11])	T_dp	no reaction	
deoxyguanosine-5'-phosphate	pG_d	deoxyguanosine	G_d
adenosine-5'-phosphate	pA	adenosine	A
adenosine-3'-phosphate	Ap	no reaction	
adenosine-5'-diphosphate	ppA	adenosine	A
cytidine-5'-phosphate	pC	cytidine	C
cytidine-3'-phosphate	Cp	no reaction	
uridine-3'-phosphate	Up	no reaction	
thymidine-5'-phosphate *p*-nitrophenyl ester	(*p*-NO_2—C_6H_4)pT_d	thymidine	T_d
1-(5'-O-[2,2,2-trichloroethyl-phosphoryl]-2'-deoxy-α-D-ribofuranosyl)-2(1*H*)-pyridone (**13**)	(TCE) $p\alpha II_d$	no reaction	
1-(5'-O-[2,2,2-trichloroethyl-phosphoryl]-2'-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (**14**)	(TCE) $p\beta II_d$	1-(2'-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (**7**)	βII_d
1-(5'-O-phosphoryl-2'-deoxy-α-D-ribofuranosyl)-2(1*H*)-pyridone (**15**)	$p\alpha II_d$	no reaction	
1-(5'-O-phosphoryl-2'-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (**16**)	$p\beta II_d$	1-(2'-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone (**7**)	βII_d

It demonstrates clearly that the crude enzyme used did not attack 3'-phosphates at all, but 5'-phosphates very readily as expected. Among the 5'-nucleotides **13** to **16** which are incubated, only the β-anomers **14** and **16** were cleaved within 30–60 minutes at 37°, yielding the nucleoside **7**. Under the same conditions the α-nucleotides **13** and **15** did not give any nucleosidic product **6**, not even after an additional incubation of 40 hours at 23°, as determined by thin-layer chromatography. These results demonstrate that the "unnatural" α-configuration of the N-glycosidic linkage represents a definite inhibition for the enzymatic hydrolysis of 5'-phosphates, at least for hydrolysis of nucleotides possessing α-pyridone as base.

7. Thin-Layer Chromatography. – For the identification of the synthesized products, the substrates and the various hydrolysis products, thin-layer chromatography was used. The Rf values are compiled in table 4.

8. 2-(Bis-[2,2,2-trichloroethyl]-phosphoryloxy)-pyridine (10). – In the course of the phosphorylation of the nucleosides **6** and **7** with bis-(2,2,2-trichloro-

[11]) T_dp was synthesized by Dr. *W. Falk*, Institut für Organische Chemie der Universität Basel. The gift of a sample is greatfully acknowledged.

Table 4. *Rf Values of Thin-Layer Chromatography*

Compound	Solvent System*)		
	A	B	C
2'-deoxy-D-ribose		0.10	0.51
2(1*H*)-pyridone		0.28	0.52
(Tol)α*II*$_d$(Tol) (**3**)	0.23		
(Tol)β*II*$_d$(Tol) (**4**)	0.30		
furfuryl *p*-toluate (**5**)	0.60		
α*II*$_d$ (**6**)		0.17	0.54
β*II*$_d$ (**7**)		0.18	0.56
(TCE)$_2$pα*II*$_d$ (**8**)		0.44	0.62
(TCE)$_2$pβ*II*$_d$ (**9**)		0.40	0.68
tris-(2, 2, 2-trichloroethyl) phosphate (**12**)		0.69	
2-(bis-[2, 2, 2-trichloroethyl]-phosphoryloxy)-pyridine (**10**)		0.51	
(TCE)pα*II*$_d$ (**13**)			0.39
(TCE)pβ*II*$_d$ (**14**)			0.41
pα*II*$_d$ (**15**)			0.08
pβ*II*$_d$ (**16**)			0.08

*) Solvent systems: A: benzene/tetrahydrofuran 8:2 (*v/v*); B: methylene chloride/methanol 9:1 (*v/v*); C: 2-propanol/0,5 M triethylammonium hydrogencarbonate 82:18 (*v/v*).

ethyl)-chlorophosphate (**11**) large amounts of a non-polar by-product were obtained after chromatographic separation of the crude reaction mixture. Upon evaporation of the fractions containing this product, crystalline 2(1*H*)-pyridone separated from the syrupy material. It was not possible to avoid the decomposition of the original product even after further purification by chromatography and in the presence of *ca.* 1% pyridine. On the basis of the following observation we assign the structure of 2-(bis-[2,2,2-trichloroethyl]-phosphoryloxy)-pyridine (**10**) to this unknown compound. A first indication for the assignment was the observation in the thin-layer chromatogram of a spot whose Rf value and coloration in I_2 vapor were similar to those of the phosphorylating agent. Upon the chromatography of crude **10** on alumina a quantitative yield of 2(1*H*)-pyridone was obtained. On the other hand the reaction of 2(1*H*)-pyridone with 1.05 equivalents of bis-(2,2,2-trichloroethyl)-chlorophosphate (**11**) at 0° yielded a product identical in all respects with the secondary product **10** of the other phosphorylations. It is known that the acetylation of 2(1*H*)-pyridone leads mainly to the O-acetyl derivative, which is not very stable. The much less stable N-acetyl isomer is only a minor product and very difficult to isolate in pure form [31]. We therefore assume that in our case O-phosphorylation would be favoured as compared with reaction at nitrogen. The UV. spectrum, showing a strong absorption maximum at 258 nm with a shoulder at 265 nm and two weak bands at 226 and 296 nm is similar to the spectrum of 2-acetoxypyridine [32]. We assume that the minor bands at 226 and 296 nm are caused by 2(1*H*)-pyridone being present as impurity. After evaporation of the solvent from the synthetic preparation,

the UV. spectrum of the residue showed only the bands at 226 and 298 nm due to 2(1*H*)-pyridone. Since **10** is relatively stable in the presence of pyridine, it was possible to obtain a clean NMR. spectrum in deuteriopyridine solution (see Fig. 15).

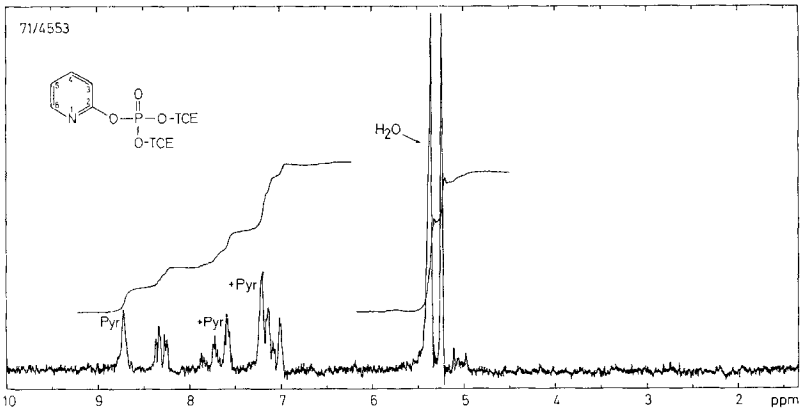


Fig. 15. *60 MHz NMR. Spectrum of 2-(bis-[2,2,2-trichloroethyl]-phosphoryloxy)-pyridine* (**10**) *in* C_5D_5N

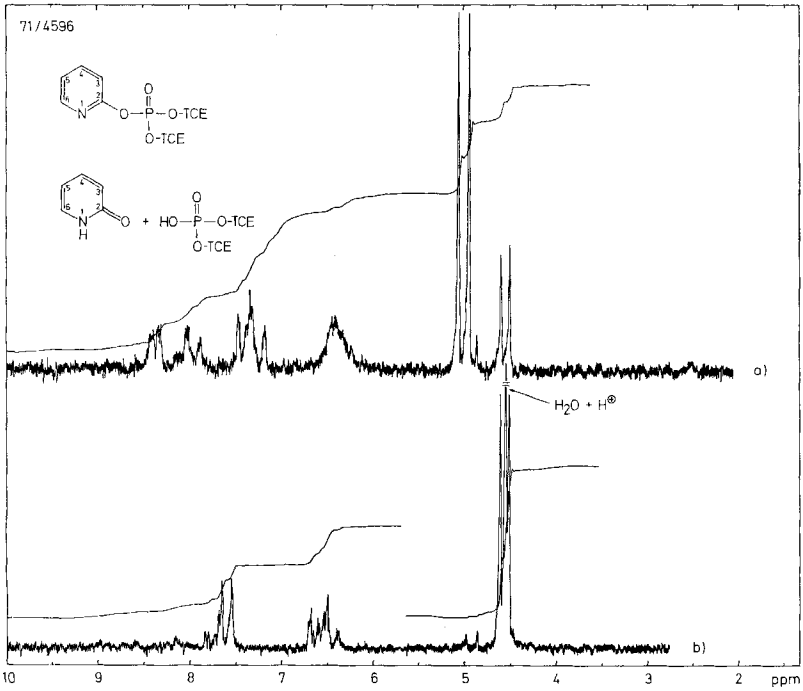
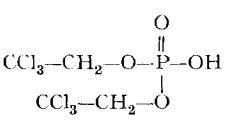


Fig. 16. *60 MHz NMR. Spectrum of 2-(bis-[2,2,2-trichloroethyl]-phosphoryloxy)-pyridine* (**10**) *in* $(CD_3)_2SO$

a) without D_2O
b) $2^1/_2$ h after addition of D_2O

The absence of signals between 6 and 7 ppm characteristic for N-substituted derivatives of 2(1*H*)-pyridone is in agreement with the proposed structure of a phosphorylated 2-hydroxypyridine derivative [31]. In $(CD_3)_2SO$ solution (see Fig. 16) the presence of free 2(1*H*)-pyridone is indicated by the signal at 6.43 ppm, and of bis-(2,2,2-trichloroethyl) phosphate (**17**) by the appearance of the doublet at 4.56 ppm assigned to the methylene protons. Addition of D_2O enhances this decomposition.



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After 2.5 hours the signals corresponding to **10** have disappeared completely, whereas the intensity of the cleavage products is increased.

In the spectra carried out during the process of decomposition, the signal of H_2O, which is identical with the acidic proton of **17**, is shifted continuously to lower fields. The increase of the proton concentration during the reaction is responsible.

The instability of **10** is in agreement with the earlier observation that triesters of phosphoric acid containing one or two 2(1*H*)-pyridone groups as alcoholic components are most reactive and hydrolysed readily by water [33].

This work was supported by a grant of the '*Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung*' (project No. 2.48.68), which is gratefully acknowledged.

Experimental Part[12])

1. General Methods. – The melting points were determined on a *Kofler* block and are corrected. Error *ca* ± 2°. Samples for elemental analyses were dried at 40–50° and 0.02 Torr. The elemental analyses were carried out in the microanalytical laboratories of the institute (*E. Thommen*) and of *SANDOZ AG*, Basel. The IR. spectra were recorded using a *Perkin-Elmer*-IR. grating spectrometer, model 125, and the UV. spectra using a *Beckman* UV. spectrophotometer, model DK2, in the spectral laboratory of the institute (*K. Aegerter*). The 100 MHz NMR. spectra were determined by *H. Huber, E. Jutzi* and *E. Wullschleger* in the *Physikalisch-Chemisches Institut der Universität Basel* on a *Varian* HA-100 D-spectrometer, the 60 MHz NMR. spectra in the spectral laboratory of our institute by *K. Aegerter* on a *Varian* A-60-spectrometer. Abbreviations: *s* = singlet; *d* = doublet; *t* = triplet; *q* = quartet; *m* = multiplet; *br* = broad. The measurement of the mass spectra was kindly carried out by Dr. *W. Vetter, F. Hoffmann-La Roche & Co. AG*, Basel, using a MS-9-spectrometer of *AEI*, Manchester, at 70 and 12 eV. The optical rotations were measured with a *Perkin-Elmer* polarimeter, model 141. For the column chromatography, silica gel of *E. Merck AG*, Darmstadt (70–325 mesh/0.05–0.20 mm) was used. The nucleotides were purified by ion exchange chromatography with DEAE-cellulose (type SS, *Serva*, Heidelberg). The elution was controlled by a Uvicord I-analyser of *LKB*, Stockholm. The solvents were evaporated at 30–40° and 14 Torr using a rotary evaporator. The pyridine (*purissimum p.a. Fluka*, Buchs) was kept over molecular sieve 4 Å, *Union Carbide*. Precoated silica gel plates F 254 of *E. Merck AG*, Darmstadt, were used for the thin-layer chromatography. Solvents (*v/v*): A: benzene/tetrahydrofuran 8:2; B: methylene chloride/methanol 9:1; C: 2-propanol/0.5 M triethylammonium hydrogencarbonate 82:18. The spots were observed by a UV. lamp or by I_2 vapor or by spraying with 10% perchlorid acid and subsequent heating at 150–200°.

2. Reaction of 3,5-Di-(O-p-toluoyl)-2-deoxy-D-ribofuranosyl Chloride (2) with (2-Pyridyloxy)-mercuric Chloride (1). – In a 1 l flask fitted with a magnetic stirrer, reflux condenser and a *Dean & Stark* apparatus, were suspended 33 g (100 mmol) of (2-pyridyloxy)-

[12]) The skillful collaboration of Mr. *W. Zürcher* is gratefully acknowledged.

mercuric chloride in 400 ml of dry toluene, and the whole was dried by distilling off 25 ml of toluene. The reaction mixture was heated just to the boiling point, and in the course of 30 min, 23 g (60 mmol) of 3,5-di-(O-*p*-toluoyl)-2-deoxy-D-ribofuranosyl chloride (**2**) were added in portions. After refluxing for an additional 60 min the reaction mixture was cooled and extracted three times with 250 ml of a freshly prepared 30% solution of potassium iodide. The potassium iodide portions were each washed with 200 ml of toluene, which was added to the main solution. The combined toluene solutions were washed twice with 250 ml of water, dried with K_2CO_3, filtered and evaporated. The resulting syrup (28 g) yielded by fractional crystallization from tetrahydrofuran/petroleum ether 3.8 g of α-nucleoside **3** and 1.7 g of a mixture of both anomeric nucleosides. The remainder of the crude product was chromatographed on 210 g of silica gel (column 4 × 36 cm). The elution was carried out with methylene chloride/methanol 996:4 (100 ml fractions). Furfuryl *p*-toluate (7.6 g), eluted in the first fractions, was discarded. The following fractions containing β-nucleoside **4** with impurities were combined and rechromatographed. Mixtures of the two nucleosides **3** and **4** were treated in the same manner. After recrystallization from tetrahydrofuran/petroleum ether 7.2 g (27%) of pure 1-(3′,5′-di-(O-*p*-toluoyl)-2′-deoxy-β-D-ribofuranosyl)-2(1*H*)-pyridone ((Tol)β*II*$_d$(Tol), **4**) and 5.5 g (21%) of pure 1-(3′,5′-di-(O-*p*-toluoyl)-2′-deoxy-α-D-ribofuranosyl)-2(1*H*)-pyridone ((Tol)α*II*$_d$(Tol), **3**) were obtained. Characterization of the nucleosides **3** and **4** *cf.* [1]. NMR. spectra in C_5D_5N see Fig. 5 and 8.

3. 1-(2′-Deoxy-β-D-ribofuranosyl)-2(1H)-pyridone (β*II*$_d$, 7) from 4. – (Tol) ß*II*$_d$ (Tol) **4** (3.0 g, 6.7 mmol) was suspended in 150 ml of a 0.1N solution of sodium methoxide in abs. methanol. All of the material dissolved within 10 min at 23°. After boiling for a short time and cooling, no starting material was present according to thin-layer chromatography. The solution was neutralized by passing through an ion exchange column (1.6 × 12 cm, amberlite IRC-50, H^+-form). The resin was washed with 50 ml of methanol. The combined solutions were concentrated to 20 ml and, after addition of 80 ml of water, extracted three times with 80 ml of petroleum ether. These extracts were washed with water and evaporated. They contained only methyl *p*-toluate. The combined aqueous fractions gave, after evaporation, 1.5 g of colourless syrup which was dissolved in methylene chloride (containing 6% of methanol) and adsorbed on 15 g of silica gel by evaporation of the solvent. This material was applied to a silica gel column (4 × 40 cm, 250 g SiO_2). After elution with methylene chloride/methanol 96:4 (250 ml fractions) 1.185 g (83%) of crude β-nucleoside **7** was obtained as colourless syrup, which solidified after some time. Recrystallization from acetone yielded 1.092 g (77%) of pure *1-(2′-deoxy-β-D-ribofuranosyl)-2(1 H)-pyridone* (**7**) of m.p. 116–117.5°. $[\alpha]_D^{24} = +108° \pm 2°$; $[\alpha]^{24}(\lambda) = +113°$ (578 nm), +132° (546 nm), +265° (436 nm), +579° (365 nm) (c = 1.35 in water).

UV. spectrum in ethanol: maxima at 228 nm (ε = 6382) and 303 nm (6040). – *IR. spectrum* (solid in KBr): bands at 3450 and 3190 cm^{-1} (broad band with two peaks, OH) and at 1650 cm^{-1} (C=O, pyridone ring). – *60 MHz NMR. spectrum* in $(CD_3)_2SO$ (cf. Fig. 2): *m* at 7.90 ppm, 1 H at C(6); *m* at 7.37 ppm, 1 H at C(4); *m* at 6.28 ppm, 3 H at C(3), C(5) and C(1′); *d* at 5.22 ppm, OH* at C(3′), J = 4 Hz; *t* at 4.97 ppm, OH* at C(5′), J = 5.5 Hz; *m* at 4.26 ppm, 1 H at C(3′); *m* at 3.87 ppm, 1 H at C(4′); *m* at 3.62 ppm, 2 H at C(5′); *m* at 2.14 ppm, 2 H at C(2′) [* = protons exchangeable upon addition of D_2O]. – *100 MHz NMR. spectrum* in C_5D_5N (cf. Fig. 9): *d* at 8.32 ppm, 1 H at C(6); *m* at 7.17 ppm, 1 H at C(4); *t* at 6.96 ppm, 1 H at C(1′), J = 6.5 Hz; *d* at 6.51 ppm, 1 H at C(3); broad signal at 6.50 ppm, OH at C(3′) and OH at C(5′); *t* at 6.03 ppm, 1 H at C(5); *m* at 4.87 ppm, 1 H at C(3′); *q* at 4.40 ppm, 1 H at C(4′); *m* at 4.10 ppm, 2 H at C(5′); *m* at 2.80 ppm, 1 H at C(2′); *m* at 2.37 ppm, 1 H at C(2′). – *Mass spectrum* (cf. Fig. 4): Molecular ion at *m/e* 211.

$C_{10}H_{13}NO_4$ (211.2) Calc. C 56.86 H 6.20 N 6.63% Found C 56.77 H 6.09 N 6.90%

4. 1-(2′-Deoxy-α-D-ribofuranosyl-2(1H)-pyridone (α*II*$_d$, 6) from 3. – A suspension of 4.0 g (8.9 mmol) of (Tol)α*II*$_d$(Tol) **3** in 200 ml of a 0.1N solution of sodium methoxide in abs. methanol was kept for one hour at 23° and then heated to boiling for a short time. At this point, all of the material had dissolved. After cooling, the solution was neutralized by passing through an ion exchange column (1.6 × 14 cm, amberlite IRC-50, H^+-form). The resin was washed with methanol until no more methyl *p*-toluate was eluted. The combined eluates were evaporated, 80 ml of water were added to the residue and the mixture extracted three times with 80 ml of chloroform. The chloroform solutions were washed twice with water. The combined aqueous fractions gave

1.7 g (90%) of α*II*$_d$ (**6**) upon evaporation, as colourless syrup which solidified after standing in the cold. The product obtained was pure enough for all further transformations. A sample gave upon recrystallization from acetone pure 1-(2′-deoxy-α-D-ribofuranosyl)-2(1*H*)-pyridone (**6**) as colourless prisms of m.p. 96–99°. (*Heller & Wagner* [14] found m.p. 132°; *Mertes* [15] m.p. 96–97.5°). $[\alpha]_D^{24} = -100° \pm 2°$; $[\alpha]^{24}(\lambda) = -107°$ (578 nm), $-129°$ (546 nm), $-309°$ (436 nm), $-831°$ (365 nm) ($c = 0.97$ in water).

UV. spectrum in ethanol: Maxima at 228 nm ($\varepsilon = 6637$) and 302 nm (5834). – *IR. spectrum* (solid in KBr): bands at 3310 cm^{-1} (broad, OH) and 1655 cm^{-1} (C=O of pyridone ring). – *60 MHz NMR. spectrum* in $(CD_3)_2SO$ (cf. Fig. 1): *m* at 7.85 ppm 1 H at C(6); *m* at 7.40 ppm, 1 H at C(4); *m* at 6.25 ppm, 3 H at C(3), C(5) and C(1′); *d* at 5.15 ppm, OH* at C(3′), $J = 3.5$ Hz; *t* at 4.84 ppm, OH* at C(5′), $J = 6$ Hz; *m* at 4.25 ppm, 2 H at C(3′) and C(4′); *d* at 3.48 ppm, 2 H at C(5′); *m* at 2.73 ppm, 1 H at C(2′) and *m* at 1.87 ppm, 1 H at C(2′) [* = protons exchanged upon addition of D_2O]. – *100 MHz NMR. spectrum* in C_5D_5N (cf. Fig. 6): *d* at 8.20 ppm, 1 H at C(6); *m* at 7.20 ppm, 1 H at C(4); $d \times d$ at 6.83 ppm, 1 H at C(1′), $J = 7$ and 3 Hz; broad signal at 6.8 ppm, OH at C(5′) and OH at C(3′); *d* at 6.54 ppm, 1 H at C(3′); *t* at 6.12 ppm, 1 H at C(5); *m* at 4.83 ppm, 2 H at C(3′) and C(4′); *d* at 3.96 ppm, 2 H at C(5′); *m* at 3.05 ppm, 1 H at C(2′) and *br d* at 2.45 ppm, 1 H at C(2′). – *Mass spectrum* (cf. Fig. 3): Molecular ion at *m/e* 211.

$C_{10}H_{13}NO_4$ (211.2) Calc. C 56.86 H 6.20 N 6.63% Found C 56.69 H 6.21 N 6.59%

5. 1-[5′-O-(Bis-[2,2,2-trichloroethyl]-phosphoryl)-2′-deoxy-β-D-ribofuranosyl]-2(1H)-pyridone ((TCE)$_2$pβ*II*$_d$, 9) from 7. – 5.1. *Reaction at 2°: β*-*II*$_d$ (**7**) (481 mg, 2.28 mmol) was dried by repeated addition and subsequent evaporation of *ca.* 5 ml pyridine, followed by evacuating to 0.02 Torr at 23° for 15 min. The material was then dissolved in 10 ml of abs. pyridine and a solution of 1.04 g (2.75 mmol) of bis-(2,2,2-trichloroethyl)-chlorophosphate (**11**) (*Aldrich, Inc.*, Milwaukee) in 10 ml of abs. pyridine was added. After standing for 24 h at 2° the mixture was evaporated to dryness, dissolved in 100 ml of chloroform and extracted four times with 50 ml of water. The aqueous fractions were washed twice with chloroform and discarded. The combined chloroform fractions were evaporated, the residue was dissolved in a small amount of methylene chloride which contained 2.5% of methanol and applied to a silica gel column (4 × 42 cm, 250 g SiO_2). Elution was carried out with methylene chloride/methanol 975:25 (fractions of 200 ml). The fractions No. 1 and 2 contained 8.2 mg of yellow oil which was discarded. Fraction No. 3 gave 82 mg of yellowish crystals, which yielded after recrystallization from petroleum ether 57.7 mg of colourless needles of m.p. 71.5–73°. According to the m.p., the UV. spectrum (no absorption) and the elemental analysis the compound was identical with tris-(2,2,2-trichloroethyl)phosphate (**12**) [18]. Fractions No. 4 to 6 contained 345 mg of brown oil, which gave crystalline 2-pyridone after standing for a while. The oil consists of 2-(bis-[2,2,2-trichloroethyl]-phosphoryloxy)-pyridine (**10**) (see further below). Fractions No. 7 to 18 contained 46 mg of colourless oil which was discarded. Fractions No. 19 to 35 gave 444 mg (35%) of crude nucleotide **9**. The material was rechromatographed on 250 g of SiO_2 (column 4 × 40 cm) with methylene chloride/methanol 97:3 as eluent (200 ml fractions). 376 mg (30%) of chromatographically (tlc.) pure (TCE)$_2$pβ*II*$_d$ (**9**) was obtained as colourless syrup which did not crystallize or solidify upon lyophilization. $[\alpha]_D^{24} = +51° \pm 3°$; $[\alpha]^{24}(\lambda) = +53°$ (578 nm), $+63°$ (546 nm), $+134°$ (436 nm), $+331°$ (365 nm) ($c = 1.05$ in chloroform).

UV. spectrum in ethanol: Maxima at 227 nm ($\varepsilon = 5680$) and 303 nm (5910). – *IR. spectrum* (solid in KBr): bands at 3370 cm^{-1} (broad, OH), 1655 cm^{-1} (C=O, pyridone ring) and 1280 cm^{-1} (broad, P=O). – *100 MHz NMR. spectrum in* $(CD_3)_2SO$: *d* at 7.68 ppm, 1 H at C(6); *m* at 7.40 ppm, 1 H at C(4); *m* at 6.32 ppm, 3 H at C(3), C(5) and C(1′); broad signal at 5.42 ppm, OH* at C(3′); $d \times d$ at 4.83 ppm, $J = 7$ and 2 Hz, 4 H of the trichloroethyl groups; *m* at 4.38 ppm, 3 H at C(5′) and C(3′); *br s* at 4.12 ppm, 1 H at C(4′); *m* at 2.35 ppm, 1 H at C(2′) and *m* at 2.03 ppm, 1 H at C(2′) [* = proton exchanged upon addition of D_2O]. – *100 MHz NMR. spectrum in* C_5D_5N (cf. Fig. 10): *d* at 8.00 ppm, 1 H at C(6); broad signal at 7.30 ppm, OH at C(3′); *m* at 7.25 ppm, 1 H at C(4); *t* at 7.00 ppm, 1 H at C(1′), $J = 6.5$ Hz; *d* at 6.61 ppm, 1 H at C(3); *m* at 6.21 ppm, 1 H at C(5); $d \times d$ at 5.10 ppm, $J = 7$ and 2 Hz, 4 H of the trichloroethyl groups; *m* at 4.83 ppm, 3 H at C(5′) and C(3′); *m* at 4.61 ppm, 1 H at C(4′); *m* at 2.86 ppm, 1 H at C(2′) and *m* at 2.36 ppm, 1 H at C(2′).

$C_{14}H_{16}Cl_6NO_7P$ (554.0) Calc. C 30.35 H 2.91% Found C 30.65 H 3.06%

5.2. *Reaction at −40°: βΠd* (**7**) (1.01 g, 4.8 mmol) in abs. pyridine was treated with 2.01 g (5.3 mmol) of the chlorophosphate **11** for 2 h at −40°. After working up the reaction mixture as described below (6.2) the products obtained were 160 mg (15.8%) of starting material from the chloroform/alcohol extract, 337 mg (16%) of phosphorylated 2-hydroxypyridine (**10**) (partially isolated as 2(1*H*)-pyridone) and 1.5 g of pure $(TCE)_2p\beta\Pi_d$ (**9**) (56.4% based on starting **7**, 67.6% based on consumed **7**) as syrup.

6. 1-[5′-O-(Bis-[2,2,2-trichloroethyl]-phosphoryl)-2′-deoxy-α-D-ribofuranosyl]-2(1H)-pyridone ($(TCE)_2p\alpha\Pi_d$, 8) from 6. – 6.1. *Reaction at 2°:* A solution of 700 mg (3.3 mmol) of $\alpha\Pi_d$ (**6**) in 15 ml of pyridine was treated with 1.66 g (4.3 mmol) of the chlorophosphate **11** for 17 h at 2°. After working up the reaction mixture as described in 5.1 and chromatography on 250 g silica gel (column 4 × 40 cm, elution with methylene chloride methanol 97 : 3) 123 mg of tris-(2,2,2-trichloroethyl) phosphate (**12**), 1.03 g of yellowish oil containing mainly the non-polar by-product **10** and some 2(1*H*)-pyridone which crystallized out on standing, and 512 mg (27.8%) of almost pure nucleotide **8**, were obtained. Recristallization from ether/petroleum ether containing a trace of ethanol, yielded 374 mg (20.4%) of pure $(TCE)_2p\alpha\Pi_d$ (**8**) as colourless needles of m.p. 98–100° (softening at 89°). $[\alpha]_D^{24} = -12.4° \pm 1°$; $[\alpha]^{24}(\lambda) = -14°$ (578 nm), −17° (546 nm), −52° (436 nm), −172° (365 nm) ($c = 1.06$ in chloroform).

UV. spectrum in ethanol: maxima at 227 nm ($\varepsilon = 6592$) and 302 nm (5957). *IR. spectrum* (solid in KBr): bands at 3370 cm^{-1} (broad, OH), 1650 cm^{-1} (C=O, pyridone ring) and 1270 cm^{-1} (P=O). *100 MHz NMR. spectrum in $(CD_3)_2SO$: d* at 7.85 ppm, 1H at C(6); *m* at 7.41 ppm, 1H at C(4); *m* at 6.31 ppm, 3H at C(3), C(5) and C(1′); *d* at 5.42 ppm, OH* at C(3′), $J = 3.5$ Hz; *d* at 4.86 ppm, $J = 7$ Hz, 4 H of the trichloroethyl groups; *br s* at 4.52 ppm, 1H at C(4′); *m* at 4.30 ppm, 3 H at C(3′) and C(5′); *m* at 2.73 ppm, 1H at C(2′); *m* at 2.0 ppm, 1H at C(2′) [* = proton exchanged upon addition of D_2O]. – *100 MHz NMR. spectrum in C_5D_5N* (cf. Fig. 7): *d* at 8.12 ppm, 1H at C(6); *br m* at 7.27 ppm, 1H at C(4) and OH* at C(3′); *d* × *d* at 6.86 ppm, 1H at C(1′), $J = 7$ and 4 Hz; *d* at 6.59 ppm, 1H at C(3); *t* at 6.16 ppm, 1H at C(5); *d* at 5.13 ppm, $J = 7$ Hz, 4 H of the trichloroethyl groups; *br s* at 4.97 ppm, 1H at C(4′); *br s* at 4.80 ppm, 1H at C(3′); *m* at 4.62 ppm, 2 H at C(5′); *m* at 3.04 ppm, 1H at C(2′); *m* at 2.48 ppm, 1H at C(2′) [* = proton exchanged upon addition of D_2O].

$C_{14}H_{16}Cl_6NO_7P$	Calc.	C 30.35	H 2.91	N 2.52	Cl 38.39%
(554.0)	Found	,, 30.7	,, 3.1	,, 2.75	,, 38.2%

6.2. *Reaction at −40°: αΠd* (**6**) (860 mg, 4.08 mmol) was dried by repeated evaporation of added abs. pyridine and then dissolved in 48 ml of abs. pyridine and cooled to −40°. A cooled solution of 1.93 g (5.08 mmol) of bis-(2,2,2-trichloroethyl)-chlorophosphate (**11**) in 20 ml of abs. pyridine was added and the mixture kept for 1.5 h at −40°. It was then allowed to come to room temperature and was partitioned between 100 ml of methylene chloride and 20 ml of water. The organic phase was washed with two 20 ml portions of water which were back-extracted with methylene chloride. Subsequent extraction of the aqueous phases with chloroform/ethanol 4:1 and purification of the evaporated extract by thin-layer chromatography (solvent B) gave 18 mg (2.1%) of starting material **6**. The combined methylene chloride extracts were dried with sodium sulfate and evaporated. 2.49 g of a syrup was obtained which, according to tlc. (solvent B) contained besides the desired nucleotide **8** pyridine and non-polar compounds. Chromatography of this mixture on 400 g silica gel (column 5.5 × 40 cm) with methylene chloride containing 2 to 10% of methanol as solvent, gave 199 mg of tris-(2,2,2-trichloroethyl)phosphate **12**, 736 mg (41%) of phosphorylated 2-hydroxypyridine **10**, which decomposed rapidly to 2(1*H*)-pyridone, and 1.35 g (59.6%) of crude nucleotide **8**. Recrystallization from methylene chloride/ether and rechromatography of the mother liquors yielded 1.22 g (54%) of pure $(TCE)_2p\alpha\Pi_d$ (**8**).

7. Removal of the Trichloroethyl Groups with Zn Dust and Isolation of the Nucleotides. – 7.1. *In acetic acid/pyridine 8:3 at 23°:* To a solution of 254 mg (0.46 mmol, 2720 OD_{302} units) of $(TCE)_2p\beta\Pi_d$ (**9**) in 6 ml of pyridine, 16 ml of glacial acetic acid and 250 mg of Zn dust were added. The mixture was shaken for 30 min at 23°, filtered, the filtrate diluted with water and pyridine and evaporated to dryness. According to tlc. (solvent C) no starting material was present and two new products had been formed. The residue was dissolved in water, filtered and passed through an ion exchange column (2 × 25 cm, Dowex 50 W × 4, H^+-form) for the removal of the Zn ions. The eluate (160 ml) was collected in a diluted solution of ammonia and evaporated to dryness. The

residue was dissolved in water and applied to a DEAE-cellulose column (4 × 65 cm, 300 g of DEAE-cellulose). The elution was effected with a linear gradient of ammonium hydrogencarbonate (2 l of 0.2 M aqueous ammonium hydrogencarbonate solution in the reservoir and 2 l of water in the mixing vessel [34]). 140 fractions of 22 ml each were collected. Each series of corresponding fractions was pooled and evaporated. *Ca.* 10 ml of water and 5 ml of ethanol were added twice to the residues and evaporated for the removal of hydrogencarbonate. The residues were dissolved in abs. methanol, filtered and evaporated to yield 820 OD_{302} units (30%) of (TCE)p$\beta\Pi_d$ (**14**) and 1590 OD_{302} units (58.5%) of p$\beta\Pi_d$ (**16**) respectively. The obtained ammonium salts were transformed to a dry and solid product by repeated lyophilization. They are highly hygroscopic.

(*TCE*)*p*$\beta\Pi_d$ (**14**, *ammonium salt*): *UV. spectrum* in ethanol: Maxima at 225 and 302 nm. – *100 MHz NMR. spectrum in* $(CD_3)_2SO$ (cf. Fig. 13): *d* at 7.90 ppm, 1 H at C(6); *br m* at 7.42 ppm, 1 H at C(4) and 4 H* of the NH_4^+ ion; *m* at 6.35 ppm, 3 H at C(3), C(5) and C(1'); *br s* at 5.63 ppm, OH* at C(3'); *d* at 4.35 ppm, J = 5 Hz, 1 H at C(3') and $^2/_2$ H of the trichloroethyl group; *m* at 4.00 ppm, 3 H at C(4') and C(5'), as well as $^2/_2$ H of the trichloroethyl group; *m* at 2.35 ppm, 1 H at C(2'); *m* at 2.00 ppm, 1 H at C(2') [* = protons exchanged upon addition of D_2O].

p$\beta\Pi_d$ (**16**, *di-ammonium salt*): *UV. spectrum* in ethanol: Maxima at 225 and 302 nm. *100 MHz NMR. spectrum in* $(CD_3)_2SO$ (*cf.* Fig. 14): *d* at 7.95 ppm, 1 H at C(6); *t* at 7.40 ppm, 1 H at C(4); *br s* at 7.17 ppm, OH* at C(3') and 8 H* of the NH_4^+ ions; *m* at 6.36 ppm, 3 H at C(3), C(5) and C(1'); *br s* at 4.38 ppm, 1 H at C(3'); *br d* at 3.98 ppm, 3 H at C(4') and C(5'); *m* at 2.32 ppm, 1 H at C(2') and *m* at 2.00 ppm, 1 H at C(2') [* = protons exchanged upon addition of D_2O].

In a further experiment 121 mg (0.22 mmol, 1300 OD_{302} units) of $(TCE)_2p\beta\Pi_d$ (**9**), dissolved in a mixture of 2 ml of pyridine and 8 ml of acetic acid, were shaken with 250 mg of Zn dust for 1 h at 23°. After working up the reaction mixture as described and chromatography of the nucleotides on DEAE-cellulose, 160 OD_{302} units (12.3%) of (TCE)p$\beta\Pi_d$ (**14**) and 504 OD_{302} units (39%) of p$\beta\Pi_d$ (**16**) were obtained.

7.2. *In pyridine/water 9:1 at 100°:* A solution of 128 mg (0.23 mmol, 1360 OD_{302} units) of $(TCE)_2p\beta\Pi_d$ (**9**) in 8 ml of 90% aqueous pyridine was stirred with 120 mg of Zn dust for 20 min at ca. 100°. The reaction mixture was filtered and the filtrate put on a ion exchange column (1.4 × 28 cm, *Merck* I, H^+-form) for removal of the Zn ions. The resin was washed with 150 ml of water. The acidic eluate was made alkaline by addition of 0.2 ml of conc. ammonia solution and evaporated. The residue was dissolved in water and chromatographed on DEAE-cellulose (column 2.7 × 17 cm, 35 g of DEAE-cellulose). The elution was carried out with a linear gradient of ammonium hydrogencarbonate (2 l of 0.2 M aqueous ammonium hydrogencarbonate solution containing 20% of ethanol in the reservoir and 2 l of 0.01 M aqueous ammonium hydrogencarbonate solution containing 10% of ethanol in the mixing vessel). 250 fractions of 12 ml each were collected. Each series of corresponding fractions was pooled and treated as described in 7.1. 96 OD_{302} units (7%) of nucleoside **7** ($\beta\Pi_d$), 447 OD_{302} units (32.8%) of (TCE)p$\beta\Pi_d$ (**14**) and 210 OD_{302} units (15.4%) of p$\beta\Pi_d$ (**16**) were obtained as ammonium salts.

7.3. *In pyridine/acetic acid 94:6 at 23°:* A solution of 400 mg (0.72 mmol, 4260 OD_{302} units) of $(TCE)_2p\alpha\Pi_d$ (**8**) in a mixture of 30 ml of pyridine and 1.8 ml of acetic acid was shaken with 800 mg of Zn dust for 5 h at 23°. After removal of the pyridine, addition of 20 ml of water and acidification with acetic acid to pH 4, the mixture was filtered and the filtrate applied to an ion exchange column (1.8 × 13 cm, *Merck* I, H^+-form). The resin was washed with 100 ml of water and the eluate collected in a 20% aqueous solution of pyridine. After evaporation to dryness and addition of 4 ml of water, 4 ml of conc. ammonia were added and the solution was again evaporated. The resulting colourless crude product (2525 OD_{302} units) contained, according to tlc. (solvent C), a little amount of (TCE)p$\alpha\Pi_d$ (**13**) and the nucleotide **15** as the main component. The material was dissolved in 5 ml of water and applied to a DEAE-cellulose column (4 × 54 cm, 280 g of DEAE-cellulose). Elution was carried out with a linear gradient of ammonium hydrogencarbonate (reservoir: 3 l of 0.25 M aqueous ammonium hydrogencarbonate solution; mixing vessel: 3 l of water). 200 fractions of 18 ml each were collected. Each series of corresponding fractions was pooled and treated further as described in 7.1.338 OD_{302} units (8%) of (TCE)p$\alpha\Pi_d$ (**13**) and 2160 OD_{302} units (51%) of p$\alpha\Pi_d$ (**15**) were obtained as ammonium salts.

(*TCE*)*p*$\alpha\Pi_d$ (**13**, *ammonium salt*): *UV. spectrum* in ethanol: maxima at 226 and 302 nm, *100 MHz NMR. spectrum* in $(CD_3)_2SO$ (*cf.* Fig. 11): *d* at 7.88 ppm, 1 H at C(6); *br m* at 7.40 ppm.

1 H at C(4) and 4 H* of the NH_4^+ ion; *m* at 6.28 ppm, 3 H at C(3), C(5) and C(1'); *br s* at 5.50 ppm, OH* at C(3'); *m* at 4.38 ppm, 2 H at C(3') and C(4'); $d \times d$ at 4.10 ppm, $J = 8$ and 5.5 Hz, 2 H of the trichloroethyl group; *br t* at 3.81 ppm, 2 H at C(5'); *m* at 2.68 ppm, 1 H at C(2') and *m* at 1.94 ppm, 1 H at C(2') [* = protons exchanged upon addition of D_2O].

p-$\alpha\Pi_d$ (**15**, *di-ammonium salt*): *UV. spectrum* in ethanol: maxima at 226 and 302 nm. *100 MHz NMR. spectrum* in $(CD_3)_2SO$ (*cf.* Fig. 12): *d* at 7.89 ppm, 1 H at C(6); *t* at 7.40 ppm, 1 H at C(4); *br m* at 6.34 ppm, 3 H at C(3), C(5) and C(1'), OH* at C(3') as well as 8 H* of the NH_4^+ ions; *br s* at 4.44 ppm, 2 H at C(3') and C(4'); *t* at 3.79 ppm, 2 H at C(5'); *m* at 2.72 ppm, 1 H at C(2') and asymmetric *d* at 1.92 ppm, 1 H at C(2') [* = protons exchanged upon addition of D_2O].

8. Assays with Alkaline Phosphatase. – *Buffer solution:* 4 ml of 0.05 M $MgCl_2$ + 8 ml of 0.5 M NH_4HCO_3 (pH 9.2) + 88 ml of H_2O. *Enzyme solutiou*[13]: alkaline phosphatase from *E. coli* (EC 3.1.3.1, *Worthington Biochemical Corporation*, Freehold, N. J.) was dialysed against the buffer mentioned above and then diluted with the buffer in order to obtain a concentration of 0.5 mg enzyme per ml corresponding to an activity of 11 EU per ml. This solution was kept at – 20°. *Assays* [35]: 0.5 µmol of substrate was added to 0.05 ml of buffer and 0.05 ml of enzyme solution. Then bidistilled water was added up to a volume of 0.20 ml and the reaction mixture incubated for 30 min at 37°. Aliquots of 20–30 µl of the incubation solution were analysed by tlc. (solvent C) and compared with the appropriate references.

9. Assays with Crude Venom of *Crotalus adamanteus*. – *Enzyme solution*: 20 mg of lyophilized venom of the diamond rattle snake, *Crotalus adamanteus* (from *F. G. Celo*, Etzelweg 64, D-6660, Zweibrücken) was suspended in 2.0 ml of bidistilled water, kept for 4 h at 3° and centrifuged. The supernatant was pipetted off and diluted with 2.0 ml of bidistilled water. This stock solution contained 5 mg dry snake venom per ml and was stored at 3°. *Assay*: *ca.* 2 µmol of substrate were dissolved in 0.1 ml of 1 M glycine-NaOH buffer (pH 8.6). 0.1 ml of 0.1 M $MgCl_2$ solution and 0.1 ml of enzyme solution were added. The mixture was made up to a total volume of 1.0 ml by addition of bidistilled water and incubated for 30 min at 37°. Aliquots of 10–20 µl of the incubation solution were analysed by tlc. (solvent C) and compared with the appropriate references.

10. Synthesis of 2-(Bis-[2,2,2-trichloroethyl]-phosphoryloxy)-pyridine (10). – To a solution of 39 mg (0.41 mmol) of 2(1*H*)-pyridone in 5 ml abs. pyridine cooled at 0°, a cooled solution of 162 mg (0.43 mmol) of bis-(2,2,2-trichloroethyl)-chlorophosphate (**11**) in 5 ml of abs. pyridine was added. After keeping the reaction mixture for 1 h at 0°, 20 ml of methylene chloride were added and the non-reacted 2(1*H*)-pyridone removed by extraction with water. The organic layer was concentrated to a syrup which still contained some pyridine. According to tlc. (solvent B) no 2(1*H*)-pyridone was present. The syrup was dissolved in 50 ml of methylene chloride and kept at – 20°. This stock solution was relatively stable. An aliquot of 5 ml was evaporated to dryness and dried for 30 min at 40° and 0.01 Torr. The resulting syrup (17 mg) did not contain any pyridine and very little of 2(1*H*)-pyridone. For the measurement of the *UV. spectrum* it was dissolved in 25.0 ml of ethanol. The solution showed absorption maxima at 226, 258, 265 (shoulder) and 296 nm (broad). This solution was evaporated immediately after completion of the measurement and the residue dried for 14 h at 50° and 0.01 Torr. According to tlc. only very little of **10** was left but large amounts of 2(1*H*)-pyridone had been formed. *UV. spectrum* in ethanol: maxima at 226 and 298 nm. An aliquot of 10 ml of the stock solution was evaporated, 3 subsequent portions of *ca.* 0.5 ml each of abs. pyridine were added and evaporated. The same procedure was carried out with three portions of 0.2 ml each of deuteriopyridine. The residue was dissolved in 0.5 ml of deuteriopyridine for the determination of the *60 MHz NMR. spectrum* (*cf.* Fig. 15): $d \times d$ at 8.30 ppm, 1 H at C(6); *t* at 7.72 ppm, 1 H at C(4); *m* at 7.12 ppm, 2 H at C(3) and C(5) and sharp *d* at 5.31 ppm, $J = 7$ Hz, 4 H of the trichloroethyl groups.

A 10 ml aliquot of the stock solution was evaporated to dryness and the residue dried at 23° and 0.01 Torr. *60 MHz NMR. spectrum* of this sample in $(CD_3)_2SO$ (*cf.* Fig. 16): *br d* at 8.39 ppm, 1 H at C(6) of **10**; *br t* at 8.01 ppm, 1 H at C(4) of **10**; *m* at 7.32 ppm, 2 H at C(3) and C(5) of **10**, as well as 2 H at C(4) and C(6) of 2-pyridone; broad signal at 6.43 ppm, 2 H at C(3) and C(5) of 2-pyri-

[13]) The enzyme solution was obtained from Dr. *W. Wehrli* and *J. Widmer*, *CIBA-GEIGY AG*, Basel. We should like to express our gratitude to these gentlemen.

done; *d* at 5.00 ppm, $J = 6$ Hz, 4 H of the trichloroethyl groups of **10**; *d* at 4.56 ppm, $J = 6$ Hz, 4 H of the trichloroethyl groups of **17**. Upon addition of D_2O to the sample, the signals of **10** decreased, those of 2-pyridone and **17** increased as the hydrolysis proceeded. Spectra were measured 5, 30 and 150 min after addition of D_2O. *60 MHz NMR. spectrum* in $(CD_3)_2SO + D_2O$ measured 150 min after addition of D_2O (*cf.* Fig. 16): *m* at 7.67 ppm, 2 H at C(4) and C(6) of 2-pyridone; *m* at 6.56 ppm, 2 H at C(3) and C(5) of 2-pyridone; *d* at 4.56 ppm, $J = 6$ Hz, 4 H of the trichloroethyl groups of **17**; *s* at 4.53 ppm, H_2O and H^+ of **17**.

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