7. Nucleosides and Nucleotides. Part 7. Four Dithymidine Monophosphates with Different Anomeric Configurations, their Synthesis and Behaviour towards Phosphodiesterases[1])

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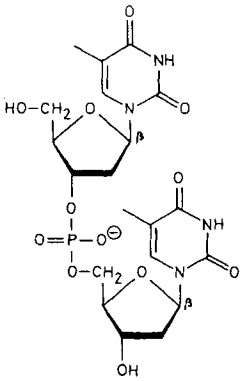
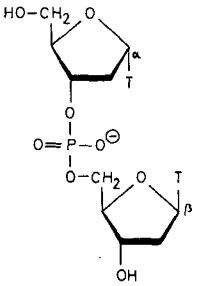
Summary. The four isomeric dithymidine monophosphates βT_d-βT_d (**1**), αT_d-βT_d (2), βT_d-αT_d (**3**) and αT_d-αT_d (**4**), differing only by the anomeric configurations of the nucleoside units, were prepared from the suitably protected nucleosides and nucleotides. The four dinucleoside monophosphates were tested as substrates of snake venom phosphodiesterase and spleen phosphodiesterase. Both enzymes accept all four compounds as substrates, however, the rate of the hydrolysis is considerably smaller if the enzymic attack takes place at the α-nucleoside moiety of the dinucleoside monophosphate.

1. Introduction. – For the characterization of oligonucleotides the degradation with phosphodiesterases is a method frequently used. In general two enzymes are applied: snake venom phosphodiesterase [2], which attacks an oligonucleotide at the free 3′-hydroxyl end to release nucleoside 5′-phosphate units, and spleen phosphodiesterase [3], which attacks at the free 5′-hydroxyl end of the chain to yield nucleoside 3′-phosphates. Thus the deoxytetranucleoside triphosphate T_d-T_d-T_d-T_d, when incubated with snake venom phosphodiesterase gives $T_d + pT_d + pT_d + pT_d$, whereas spleen phosphodiesterase yields $T_dp + T_dp + T_dp + T_d$. The complete degradation of the oligonucleotide proves that all internucleotidic linkages have corresponded to the natural 3′–5′ type. The ratio of the products obtained, 1 nucleoside:3 nucleotides in the above example, shows that the initial oligonucleotide had a chain length of four

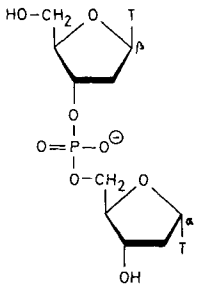


1, βT_d-βT_d

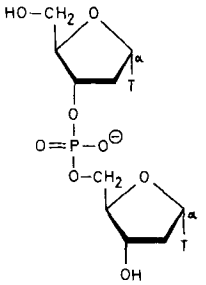
T = thymin-1-yl



2, αT_d-βT_d



3, βT_d-αT_d



4, αT_d-αT_d

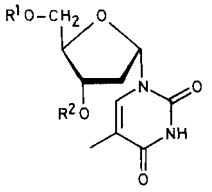
[1]) Part 6: [1].

nucleoside units. Since oligonucleotides with a 5'-end phosphate can hardly be attacked by spleen phosphodiesterase and those bearing a 3'-end phosphate are almost completely resistant to snake venom phosphodiesterase, such substrates are treated best with a nonspecific phosphomonoesterase such as bacterial alcaline phosphatase, prior to degradation with phosphodiesterases.

Since it is not known whether oligonucleotides containing one or more α-nucleoside units, behave in the same manner if exposed to the enzymes mentioned above, the four possible dinucleoside monophosphates containing α- and/or β-thymidine, *i.e.* βT_d-βT_d (**1**), αT_d-βT_d (**2**), βT_d-αT_d (**3**), αT_d-αT_d (**4**), were prepared and compared.

2. Preparation of the four Dinucleoside Monophosphates 1, 2, 3 and 4. – For the synthesis of the three dinucleoside monophosphates containing α-nucleoside units, α-thymidine (**5**) served as starting material. It was prepared from 3,5-di-(O-*p*-toluoyl)-2-deoxy-D-ribofuranosyl chloride and monomercury thymine [4]. In contrast to the described procedure the separation of the two anomers could be achieved only after methanolysis of the protecting toluoyl groups.

For the condensation reactions described further below α-thymidine (**5**) was protected as described for the corresponding β-anomers. 5'-O-mono-*p*-methoxytrityl-α-thymidine ((MeOTr) αT_d, **6**) was prepared by reacting α-thymidine (**5**) with mono-*p*-methoxytrityl chloride [5] [6]. Acetylation with acetic anhydride and removal of the trityl group with 80% acetic acid gave 3'-O-acetyl-α-thymidine (αT_d(Ac), **7**) [7].



5: $R^1 = R^2 = H$, α-thymidine (αT_d)
6: R^1 = *p*-methoxytrityl, $R^2 = H$
7: $R^1 = H$, $R^2 = Ac$

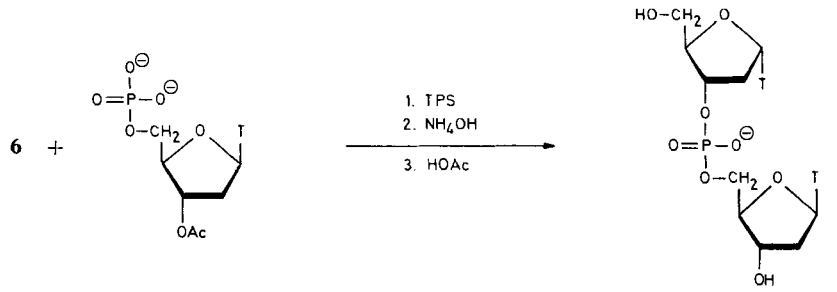
The structures of **5**, **6** and **7** were confirmed by comparing the UV. and NMR. spectra with those of the corresponding β-thymidine derivatives. In all three pairs the UV. spectra of the two anomers were practically identical and the NMR. spectra (see Fig. 1, 2 and 3) differed only by a few signals. The C(1') protons of the α-derivatives appear as a doublet of doublets instead of a *pseudo* triplet, the two C(2') protons show the splitting pattern typical for α-thymidines and the signal of the C(4') proton appears at lower field as that of the β-anomer [8].

βT_d-βT_d (**1**), the dinucleoside monophosphate containing two β-nucleoside units and thus representing the natural configuration, is well known [9]. It was prepared from 5'-O-mono-*p*-methoxytrityl-β-thymidine ((MeOTr) βT_d, **9**) and 3'-O-acetyl-β-thymidine 5'-phosphate ($p\beta T_d$(Ac) **8**) using 2,4,6-triisopropylphenylsulfonyl chloride (TPS) as condensing agent according to Lohrmann & Khorana [10] with some modifications [11][2].

αT_d-βT_d (**2**) was synthetized in a similar way, condensing (MeOTr) αT_d (**6**) with $p\beta T_d$(Ac) (**8**) in the presence of TPS.

The two dinucleoside monophosphates containing αT_d at the 3'-end were prepared using the phosphotriester method [12]. The nucleoside giving the 5'-end, which was

[2] This preparation was made by *I. Gregor*. The gift of a sample is gratefully acknowledged.



8 **2**: αT_d-βT_d

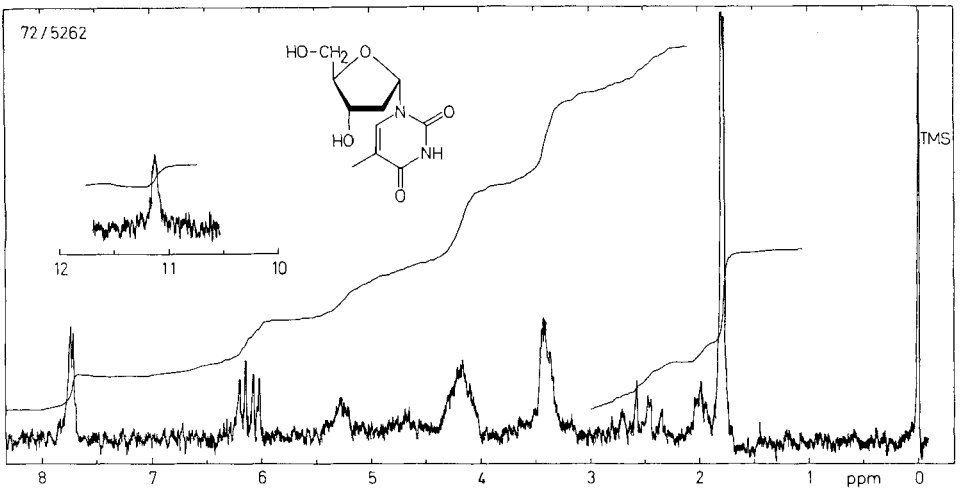


Fig. 1. *60 MHz NMR. spectrum of* αT_d (**5**) *in* $(CD_3)_2SO$

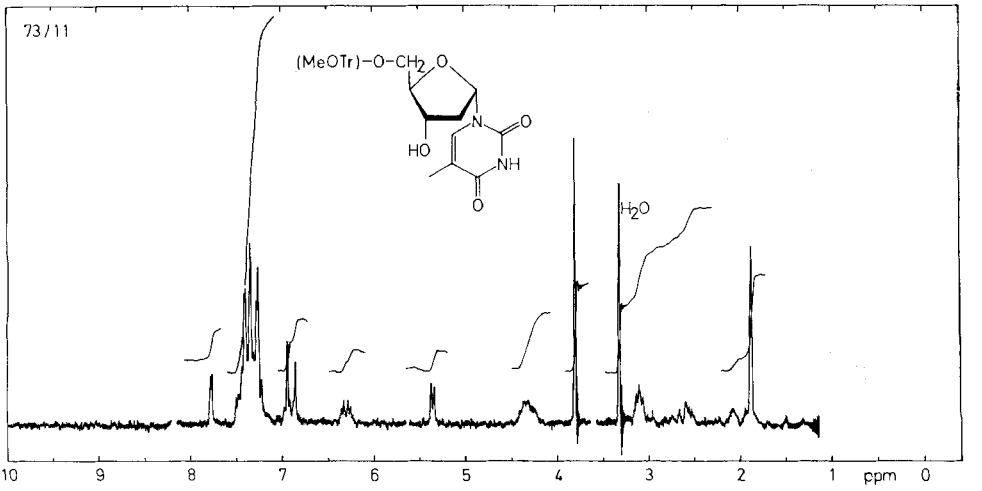


Fig. 2. *100 MHz NMR. spectrum of (MeOTr)* αT_d (**6**) *in* $(CD_3)_2SO$

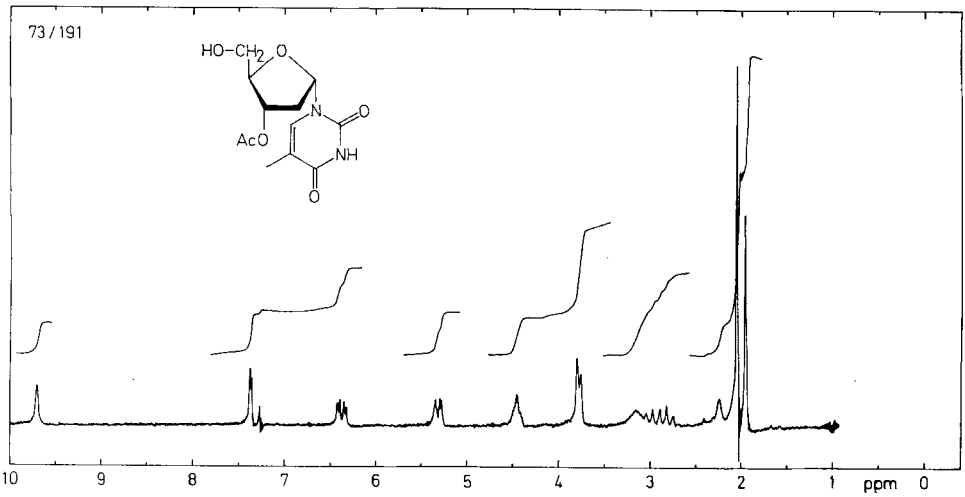
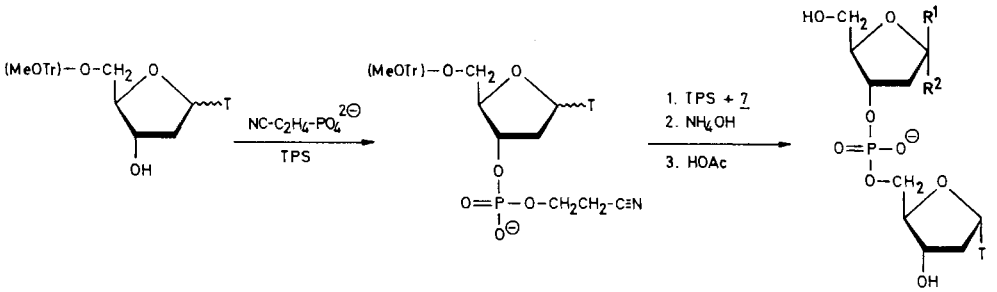


Fig. 3. *100 MHz NMR. spectrum of* $\alpha T_d(Ac)$ (**7**) *in* $CDCl_3$

protected by the mono-*p*-methoxytrityl group (**6** and **9** respectively), was phosphorylated with β-cyanoethyl phosphate to give the intermediary phosphodiester **10** and **11** respectively, which were not isolated. In order to avoid the formation of the unnatural 3′–3′ phosphodiester [12], which is difficult to separate from the desired dinucleoside monophosphate with the 3′–5′-linkage, 3′-O-acetyl-α-thymidine (**7**, two equivalents)



6: α-anomer

9: β-anomer

T = thymin-1-yl

10: α-anomer

11: β-anomer

3: R^1 = thymin-1-yl, R^2 = H, βT_d-αT_d

4: R^1 = H, R^2 = thymin-1-yl, αT_d-αT_d

proved to be more advantageous than free α-thymidine as nucleoside component for the condensation reaction with TPS as condensing agent.

After the condensation the protecting groups were removed by successive treatment of the resulting product with conc. ammonia solution and 80% acetic acid. The crude dinucleoside monophosphates **1–4** were purified by repeated chromatography

on DEAE-Sephadex columns. The following yields were obtained: αT_d–βT_d (**2**): 49%, βT_d–αT_d (**3**): 30%, αT_d–αT_d (**4**): 32%[3]).

3. Enzymatic Degradations. – The four dinucleoside monophosphates **1**, **2**, **3** and **4** were incubated both with snake venom phosphodiesterase and spleen phosphodiesterase. The degradation products formed were separated by paper chromatography and their ratio determined by UV. spectrophotometry. The results are summarized in tables 1 and 2. They suggest that the two commercial enzyme preparations used had an appreciable monoesterase activity. Thus, *e.g.*, the ratio of the products obtained after incubation of the "natural" dinucleoside monophosphate βT_d–βT_d (**1**) with snake venom phosphodiesterase is 1 nucleoside *vs. ca.* 0.7 nucleotide (theoretical value: 1:1). If **1** had erroneously been a higher oligomer such as the trinucleoside diphosphate, the ratio would have been 1 nucleoside *vs.* 2 nucleotides. Therefore, the observed ratio must be due to dephosphorylation of $p\beta T_d$ formed during the incubation to give βT_d.

An attempt was made to measure the monoesterase activity of the two enzyme preparations. In the case of the *snake venom phosphodiesterase* an amount of $p\beta T_d$ equivalent to that formed during the enzymatic hydrolyses of the substrates **1** to **4** was incubated with the enzyme under the same conditions. 21.2% of the $p\beta T_d$ was dephosphorylated. This value might be correct with respect to the degradation of $p\beta T_d$, but it can only represent a rough estimation as far as the dephosphorylation of $p\alpha T_d$ is concerned.

The spectral values measured after elution of the papers were corrected for the monoesterase activity assuming that the 5'-nucleotide eluted represented 78.8%, the nucleoside eluted 121.2% of the respective amounts initially formed by the action of the phosphodiesterase.

For the estimation of the monoesterase activity of the *spleen phosphodiesterase* the procedure was analogous to that applied to the snake venom phosphodiesterase, with βT_dp serving as substrate. Under the conditions of the degradation of the dinucleoside monophosphates, 15,1% of the βT_dp was dephosphorylated. Consequently the spectral values measured after eluting the nucleosides and nucleotides from the papers were corrected for the monoesterase activity, assuming that the βT_dp eluted represented 84.9%, the βT_d eluted 115.1% of the respective amounts formed by the action of the phosphodiesterase.

In those cases where αT_dp was formed as degradation product no correction for monoesterase activity seemed to be necessary. But besides αT_dp (Rf 0.11) one or two minor products could be detected on the chromatogramme (*cf.* table 3). They might be derivatives of αT_dp formed during incubation. Rf values and UV. absorption are close to those found for αT_dp.

4. Discussion. – *Snake venom phosphodiesterase.* – A large number of "unnatural" dinucleoside monophosphates and oligonucleotides have been tested as substrates of snake venom phosphodiesterase. As compared with the "natural" substrates they exhibited differences in the internucleotide linkage, in the conformation or configuration of the nucleoside units or in the nature of the heterocyclic base or of the sugar.

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[3]) The nature of the by-products of these condensations has not been determined yet.

Table 1. *Degradation of the Dinucleoside Monophosphates with Snake Venom Phosphodiesterase*

Substrate	After elution from paper chromatogrammes		Values corrected for monoesterase activity		Dinucleoside monophosphate recovered		Ratio Nucleoside/ 5′-Nucleotide
	Nucleoside[a]	5′-Nucleotide[a]	Nucleoside[a]	5′-Nucleotide[a]	OD_{265} units	% of total nucleotidic material eluted	
βT_d-βT_d (**1**)	3.87	2.49	3.21	3.16	0.18	2.75%	1:0.98
αT_d-βT_d (**2**)	2.98	2.07	2.46	2.58	traces	< 5%	1:1.05
βT_d-αT_d (**3**)	1.58	1.08	1.31	1.35	9.22	78%	1:1.04
αT_d-αT_d (**4**)	1.24	0.625	1.03	0.835	6.95	79%	1:0.81[b]

[a] OD_{265} units, 1 unit being the amount of material present in 1 ml of a solution showing an absorption of 1.0 when measured in a 1 cm cell at the wavelength indicated.

[b] The ratio of an independent second experiment was 1:0.82.

Table 2. *Degradation of the Dinucleoside Monophosphates with Spleen Phosphodiesterase*

Substrate	After elution from paper chromatogrammes		Values corrected for monoesterase activity		Dinucleoside monophosphate recovered		Ratio Nucleoside/ 3′-Nucleotide
	Nucleoside[a]	3′-Nucleotide[a]	Nucleoside[a]	3′-Nucleotide[a]	OD_{265} units	% of total nucleotidic material eluted	
βT_d-βT_d (**1**)	3.71	2.69	3.22	3.18	0.165	2.5%	1:0.99
αT_d-βT_d (**2**)	2.82	2.90[b]	–	–	1.05	15.5%	1:0.97
βT_d-αT_d (**3**)	4.13	2.96	3.59	3.50	0.190	2.6%	1:0.97
αT_d-αT_d (**4**)	0.55	0.59[c]	–	–	6.54	85.2%	1:1.07

[a] OD_{265} units; *cf.*[a] Table 1.

[b] Total of nucleotidic material: besides the main absorption at Rf 0.11 (2.45 OD_{265} units) two weak spots were observed at Rf 0.054 (0.28 OD_{265} units) and Rf 0.15 (0.17 OD_{265} units).

[c] Total of nucleotidic material: besides the main absorption at Rf 0.10 (0.51 OD_{265} units) a weak spot was observed at Rf 0.13 (0.08 OD_{265} units).

The enzyme is known to cleave not only dinucleoside monophosphates with the natural 3′–5′-phosphodiester bond but also the isomeric 5′–5′ [13] and 2′–5′ [14] analogues; however, the 3′–3′-linkage is resistant [12]. Also the phosphotriester Ap (CH_3)A, was found to be resistant to the diesterase [15].

A dinucleoside phosphate possessing the *syn-syn*-conformation was cleaved completely [16]. Exchanging the D-nucleoside at the 3′-end by its L-enantiomer affects the behaviour as substrate of snake venom phosphodiesterase as shown by the resistance of G-LA, G-LC [17], U-LU [18], LA-LA (with 3′–5′ or 2′–5′ linkage) [19] and homopolymers made from LU > p and LA > p [18].

If the nature of the heterocyclic base is changed, susceptibility is not necessarily cancelled: Dinucleoside monophosphates with 2(1*H*)-pyridone [20][4]), pyrimidone-(2), pyrimidone-(4) [22] or pyridazone-(3) [20] as base moiety of the 3′-end nucleoside are readily cleaved. However, if the base is 6-methyluracil, or either uracil or 6-methyluracil linked to the sugar moiety through N(3) complete resistance is observed [23]. In these latter cases it is rather the conformational change of the whole nucleoside induced by the altered base than the "unnatural" nature of the base itself which is responsible for the resistance to enzymatic degradation. Dinucleoside monophosphates with arabinoside units were also tested [24]. In the cases where an arabinoside was at the 3′-end cleavage occurred at slower rate. Finally two dinucleoside monophosphates containing an α-nucleoside at the 3′-end, *i.e.* U-αU and G-αU, proved to be completely resistant to snake venom phosphodiesterase [25].

All these findings are compatible with the hypothesis that the enzyme attacks at the free 3′-end and releases 5′-nucleotide units regardless of the nature of the second alcoholic component of the phosphodiester. The enzyme seems to interact with the free hydroxyl (or negative charge) of the phosphate [15] on one hand and with the sugar and the heterocyclic base of the nucleoside as a whole unit on the other hand [22–23] [25].

The results described in this paper agree with the scheme outlined above quite well as far as the substrates **1** and **2** are concerned, both containing a β-nucleoside unit at the 3′-end. They are both cleaved practically quantitatively[5]) to yield nucleoside and nucleotide in the theoretical ratio of 1:1.

In consideration of the published results cited above the substrates **3** and **4** containing an α-nucleoside unit at the 3′-end were expected to be completely resistant. However, the data presented in Table 1 demonstrate that of both substrates about 20% are cleaved during the five hour incubation period. **3** yields nucleoside and nucleotide with the theoretical ratio whereas **4** yields more nucleoside than expected. Since the enzyme preparation used had an appreciable monoesterase activity and

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[4]) The dinucleoside monophosphate containing two 2(1*H*)-pyridone deoxyriboside units, Π_d-Π_d, was not completely cleaved within 5 hours, 27% being recovered unchanged [21]. The enzyme preparation used was the same as for the degradation experiments with the substrates **1** to **4**.

[5]) A very close examination showed that 2–3% of the dinucleoside monophosphates were not cleaved. This might be the amount present in the equilibrium between substrate and products. The procedure used for the synthesis of the substrates **1** and **2** precludes the formation of *e.g.* a 3′–3′ linked product.

since this monoesterase activity was estimated using $p\beta T_d$ as the substrate ($p\alpha T_d$ not being available), both the product ratios for **3** and **4** might be uncorrect[6]).

In general a direct comparison of the results of different laboratories should be carried out with precaution, since very often the conditions of incubation and the enzyme preparations used are different. It might even be possible that, with respect to "unnatural" substrates, the specificity of snake venom diesterases varies with the snake species.

Spleen phosphodiesterase. – Most of the "unnatural" dinucleotides hitherto prepared belong to the ribo series and the stepwise degradation starting at the 5'-end was usually effected by pancreatic ribonuclease or ribonuclease T *2* rather than by spleen phosphodiesterase.

It has been reported that the dinucleoside monophosphate of 8-Bromoadenosine possessing the *syn-syn*-conformation [16] as well as Π_d-Π_d [21] and Π_d-T_d [25], two dinucleoside monophosphates with an "unnatural" base at the 5'-end were cleaved by spleen phosphodiesterase, whereas LA-LA [19] and the phosphotriester $Ap(CH_3)A$ [15] are not accepted by the enzyme as substrates.

Since spleen phosphodiesterase is known to attack an oligonucleotide chain from the 5'-end, one would expect that among the four dinucleoside monophosphates **1**–**4** those bearing a βT_d-unit at the 5'-end *i.e.* **1** and **3** should be cleaved readily. Indeed, the data compiled in table *2* show that **1** and **3** are hydrolyzed nearly quantitatively[5]) to give nucleoside and nucleotide with the expected ratio.

Substrates **2** and **4**, however, carrying an "unnatural" α-nucleoside at the 5'-end, are hydrolyzed as well. During the 5 hour incubation period 85% of **2** and 15% of **4** were cleaved to give nucleoside and nucleotide in the theoretical ratio.

The results obtained after eluting the degradation products from the paper chromatogrammes suggested that the enzyme preparation used had some monoesterase activity. But this contaminant phosphomonoesterase was obviously notable to attack αT_dp, since the products formed after incubation of **2** and **4** were obtained in the correct ratio of 1:1. Only in those cases where βT_dp was formed as degradation product, much more nucleoside than expected was found.

Conclusion. – The results presented in this paper together with published data suggest that, besides some structural requirements such as the free hydroxyl (or negative charge) at the phosphate group and the free hydroxyl group (*cf.* also [2–3]) at the chain end where the phosphodiesterase attacks, it is the over-all-conformation of the substrate which determines occurrence and rate of cleavage. Single structural modifications such as the introduction of an unnatural base or the change of the configuration at one of the anomeric centers are supposed to affect the susceptibility of the substrate only if the over-all-conformation of the substrate is altered.

The author thanks Prof. *Ch. Tamm* for his continous interest and many encouraging discussions. The investigation was supported by the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung* (Projects no. 2.460.71 and 2.675.72).

[6]) It is astonishing, however, that these two product ratios are not the same as one should expect, since in both cases $p\alpha T_d$ is released.

Experimental Part

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 40–80° and 0.02 Torr. The elemental analyses were carried out in the microanalytical laboratory of the institute (*E. Thommen*). – The UV. spectra were recorded using a *Beckmann* UV spectrophotometer, model DK 2, in the spectral laboratory of the institute (*K. Aegerter*). The 100 MHz NMR. spectra were determined by *R. Wiessler* 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. Chemical shifts are given as δ-values with $\delta = 0$ ppm for tetramethylsilane. – The optical rotations were measured with a *Perkin-Elmer* polarimeter, model 141. – Nucleotides were purified by ion exchange chromatography with DEAE-Sephadex A 25 (*Pharmacia*, Uppsala). The elution was carried out with a linear gradient of ammonium hydrogencarbonate (*AnalaR*, BDH) and controlled by a *Uvicord* I-analyser of *LKB*, Stockholm. To avoid a loss of CO_2 from the buffer solution, a stream of carbon dioxide was passed through the solution in the mixing vessel. – For the column chromatography, silica gel of *E. Merck A.G.*, Darmstadt (70–230 mesh/0.063–0.200 mm) was used. Precoated silica gel plates F 254 of *E. Merck A.G.*, Darmstadt, were used for the thin-layer chromatography (tlc.). The spots were observed by a UV. lamp or by I_2 vapors or by spraying with 10% perchloric acid and subsequent heating at 150–200°. *Whatman* 3 MM paper served for paper chromatography (pc.); the descending technique was used. – Solvent systems for chromatography (*v/v*): A) methylene chloride/methanol 9:1; B) 2-propanol/conc. NH_3/H_2O 7:1:2; C) 1-butanol/glacial acetic acid/H_2O 5:2:3; D) 1-propanol/conc. NH_3/H_2O 6:3:1; E) chloroform/methanol 2:1; F) 2-propanol/0.5 M aqueous NH_4HCO_3 8:2; G) methanol/benzene/formic acid 5:4:1. – Pyridine (puriss. *Fluka* AG Buchs SG) was kept over molecular sieve 4 Å Union Carbide. βT_d-βT_d (**1**) was synthetized by *I. Gregor* of our institute using the procedure of *Lohrmann & Khorana* [10] with some modifications [11]. The gift of a sample is gratefully acknowledged. It was rechromatographed on a DEAE-Sephadex column prior to use. (MeOTr) βT_d (**9**) [5] [6] and pβT_d(Ac) (**8**) [27] were prepared in a similar way as described in the literature.

2. *α-Thymidine* (**5**). – α-Thymidine was prepared as described by *Hoffer* [4]. 3,5-Di-(O-*p*-toluoyl)-2-deoxy-D-ribofuranosyl chloride (29.5 g, 76 mmol) and monomercury thymine (24.6 g, 76 mmol) were stirred in a mixture of 120 ml of dimethyl formamide and 50 ml of toluene at 23° for 2.5 h. After work up of the mixture 15 g (42.1%) of the crude ditoluoylates of α- and β-thymidine resulted. The mixture was treated with 0.1 N $NaOCH_3$ in abs. methanol [8]. The two anomeric thymidines were separated by fractional crystallization from methanol and ethanol. 2.33 g of pure α-thymidine (**5**) were obtained (9.6% based on deoxy ribose, 12.6% based on 3,5-di-(O-*p*-toluoyl)-2-deoxy-D-ribofuranosyl chloride). M.p. 185.5–187°, mixed m.p. with authentical β-thymidine (*Fluka*) 160–185°. – $[\alpha]_D^{24} = +6.8° \pm 0.5°$; $[\alpha]^{24}$ (λ): +6.5° (578 nm), +5.1° (546 nm), −15.5° (436 nm), −94.3° (365 nm) ($c = 1.08$ in H_2O). $[\alpha]_D^{24} = +16.5° \pm 1°$; $[\alpha]^{24}$ (λ): +16.7° (578 nm), +16.9° (546 nm), +6.4° (436 nm), −56° (365 nm) ($c = 0.87$ in methanol). – UV. spectrum in ethanol: maxima at 207 nm ($\varepsilon = 9770$) and 266 nm ($\varepsilon = 10180$). – 60 MHz NMR. spectrum in $(CD_3)_2SO$ (*cf.* Fig. 1): 11.10 ppm, *s*, NH of thymine residue, exchangeable with D_2O; 7.74 ppm, *d*, H at C(6), $J = 1.5$ Hz; 6.12 ppm, $d \times d$, H at C(1'), $J = 4$ Hz and 7 Hz; br *s* 5,27 ppm and 4.68 ppm br *s*, 2 OH at C(3') and C(5'), both exchangeable with D_2O; 4.17 ppm, br *s*, 2H at C(3') and C(4'); 3.42 ppm, *m*, 2H at C(5'); 2.58 ppm, *m*, 1H at C(2'); 1.90 ppm, br *d*, 1H at C(2'), partly covered by the signal of the C(5)-methyl group; 1.78 ppm, *d*, 3H of CH_3 at C(5), $J = 1.5$ Hz.

$C_{10}H_{14}N_2O_5$	Calc.	C 49.58	H 5.83	N 11.56%
(242.2)	Found	,, 49.39	,, 5.73	,, 11.78%

3. *5'-O-p-Methoxytrityl-α-thymidine* (*(MeOTr)*αT_d, **6**) *from* αT_d (**5**). α-Thymidine (484 mg, 2 mmol) and *p*-methoxytrityl chloride (685 mg, 2.2 mmol) were dried for 2 days in a vacuum desiccator over P_2O_5/KOH and then dissolved in 7 ml of pyridine. The mixture was kept for one day at 23° with occasional shaking. Then 1 ml of abs. methanol was added and the mixture evaporated after 2 h. The residue was taken up in 50 ml of methylene chloride and extracted successively with 50 and 25 ml of water. The aqueous phases were extracted with 25 ml of methylene chloride. The organic layers were combined, dried with $MgSO_4$ and evaporated. A yellow syrup resulted which

was dissolved in 5 ml of methylene chloride. This solution was added slowly and with vigorous stirring to 70 ml of cyclohexane. A white voluminous pricipitate was obtained which was collected by centrifugation, washed twice with cyclohexane and finally dried under reduced pressure. 1.01 g (98%) of (MeOTr)αT$_d$ (**6**) was obtained as a white powder, which showed in tlc. (system A) a trityl-containing contamination. The product was pure enough, however, for the syntheses described below. – For the characterization a sample was purified further by chromatography on a silica gel column. Elution was cyrried out with CH_2Cl_2/MeOH 98:2. After pooling the corresponding fractions and evaporation of the solvent a white foam was obtained which could not be crystallized. – M.p. of the foam 103–113°. $[\alpha]_D^{24} = +14.4° \pm 1°$; $[\alpha]^{24}$ (λ): +15.0° (578 nm), +16.5° (546 nm), +23.2° (436 nm), +23.5° (365 nm) (c = 0.86 in CH_2Cl_2). UV. spectrum in ethanol: maxima at 229 nm (ε = 17'540) and 266 nm (12'020). – 100 MHz NMR spectrum in $(CD_3)_2SO$ (*cf.* Fig. 2): 11.23 ppm, *s*, NH of thymine residue, exchangeable with D_2O; 7.78 ppm, *s*, H at C(6); 7.34 ppm, *m*, 12 arom H; 6.90 ppm, *d*, 2 arom H; 6.30 ppm, *d* × *d*, H at C(1'), J = 3.5 Hz and 7.5 Hz; 5.37 ppm, *d*, OH at C(3'), J = 3 Hz, exchangeable with D_2O; 4.33 ppm, *m*, 2H at C(3') and C(4'); 3.82 ppm, *s*, CH_3O of methoxytrityl group; 3.12 ppm, *m*, 2H at C(5'); 2,69 ppm, *m*, 1H at C(2'); 2.00 ppm, br *d*, 1H at C(2'), partly covered by the signal of the C(5)-methyl group; 1.88 ppm, *s*, 3H of CH_3 at C(5).

$C_{30}H_{30}N_2O_6$	Calc.	C 70.02	H 5.88	N 5.44%
(514.6)	Found	,, 69.84	,, 5.87	,, 5.43%

4. *3'-O-Acetyl-α-thymidine*(αT_d(*Ac*), **7**) *from* αT_d (**5**). – α-Thymidine (700 mg, 2.9 mmol) and *p*-methoxytrityl chloride (986 mg, 3.19 mmol) were dried overnight in a vacuum desiccator and then dissolved in 7 ml of pyridine. The mixture was kept at 23° for one day with occasional shaking. Abs. methanol (1 ml) was added and, after standing for 1 h, the mixture was evaporated. The residue was distributed between two 35 ml portions of methylene chloride and two 35 ml portions of water. The organic layers were pooled, dried with $MgSO_4$ and evaporated to dryness. *p*-Methoxytrityl-α-thymidine (**6**) was obtained as a yellow foam.

The crude **6** was taken up in 20 ml of pyridine and acetic anhydride (5 ml, 53.2 mmol) was added. The mixture was kept at 23° for one day. Then 5 ml of abs. methanol were added. After 17 h 20 ml of water were added and the solution evaporated. The residue was distributed between three 40 ml portions of methylene chloride and two 40 ml portions of water. The combined organic phases were dried with $MgSO_4$ and evaporated to dryness. (MeOTr) αT(Ac) resulted as a yellowish foam. – The crude product was taken up in 30 ml of 80% aqueous acetic acid and kept at 23° for 15 h. After evaporation of the solvents the residue was distributed between three 40 ml portions of water and two 40 ml portions of ether. The aqueous layers were combined and evaporated. The residue was taken up in a small portion of CH_2Cl_2/MeOH 975:25 and chromatographed on a silica gel column (65 g of SiO_2, 2.5 × 24 cm). The elution was effected with methylene chloride containing 2.5–4% of methanol. 632 mg (82.5% based on starting (**5**) of chromatographically pure αT$_d$(Ac. (**7**) were obtained as a white foam. This product has not crystallized yet. – M.p. of the foam 55–77°) $[\alpha]_D^{24} = -6.3° \pm 0.5°$; $[\alpha]^{24}(\lambda)$: –7.3° (578 nm), –10.7° (546 nm), –46° (436 nm), –154° (365 nm) (c = 1.3 in CH_2Cl_2). – UV. spectrum in ethanol: maxima at 205 nm (= 9550) and 265 nm. – 100 MHz NMR spectrum in $(CD_3)_2SO$: 11.24 ppm, br *s*, NH of thymine residue, exchangeable with D_2O; 7.50 ppm, *d*, H at C(6), J = 1.5 Hz; 6.25 ppm, *d* × *d*, H at C(1'), J = 2 Hz and 7 Hz; 5.26 ppm, *m*, H at C(3') and OH at C(5') (exchangeable with D_2O); 4.49 ppm, *m*, H at C(4'); 3,64 ppm, *m*, 2H at C(5'); 2.83 ppm, *m*, 1H at C(2'); 2.10 ppm, br *d*, 1H at C(2'), partly covered by the signal of the acetyl group: 2.03 ppm, *s*, 3H of the acetyl group; 1.89 ppm, *d*, 3H of CH_3 at C(5), J = 1.5 Hz. – 100 MHz NMR. spectrum in $CDCl_3$ (cf. Fig. 3): 9.70 ppm, *s*, NH of thymine residue, exchangeable with D_2O; 7.38 ppm, *d*, H at C(6), J = 1.5 Hz; 6.38 ppm, *d* × *d*, H at C(1'), J = 2.5 Hz and 8 Hz; 5.32 ppm, *m*, H at C(3'); 4.46 ppm, br *s*, H at C(4'); 3.78 ppm, *d*, 2H at C(5'), J = 4 Hz; 3.16 ppm, br *s*, OH at C(5'), exchangeable with D_2O; 2.90 ppm, *m*, 1H at C(2'); 2.17 ppm, br *d*, 1H at C(2'), partly covered by the signal of the acetyl group; 2.06 ppm, *s*, 3H of the acetyl group; 1.96 ppm, *d*, 3H of CH_3 at C(5), J = 1.5 Hz.

$C_{12}H_{16}N_2O_6$	Calc.	C 50.70	H 5.67	N 9.85%
(284.3)	Found	,, 50.98	,, 5.99	,, 9.72%

5. αT_d-βT_d (**2**) *from* (*MeOTr*)αT_d (**6**) *and* pβT_d(*Ac*) (**8**). – (MeOTr)αT$_d$ (**6**) (178 mg, 0.346 mmol) and pβT$_d$(Ac) (**8**) (173 mg, 0.388 mmol, as pyridinium salt) were dried 3 times by evapora-

tion of 2 ml of pyridine. Then TPS (509 mg, 1.68 mmol) and pyridine (2 ml) were added and the mixture kept at 23° for 10 h. After addition of 2 ml of water, the mixture was again allowed to stand at 23° for 12 h. Then the solvent was removed and the residue dissolved in 15 ml of 2-butanol. This solution was extracted with three 15 ml portions of 1M aqueous NH_4HCO_3 which were then extracted twice with 15 ml of 2-butanol. The aqueous phases were combined and evaporated to dryness. They contained, according to tlc (systems B and C) mainly pβT$_d$(Ac) (**8**). The butanol layers were pooled, evaporated and the residue taken up in 30 ml of conc. NH_3. After standing at 23° for 17 h the solution, which had become turbid, was evaporated and the residue taken up in 30 ml of 80% acetic acid. The solution was heated to 100° for 25 min and then evaporated. The residue was distributed between three 25 ml portions of 1M aqueous NH_4HCO_3 and three 20 ml portions of 2-butanol. The aqueous layers were combined and evaporated. The buffer was removed by repeated evaporation of small portions of 50% aqueous methanol. – The residue was taken up in a minute amount of water and applied to the top of a DEAE-Sephadex column (1 × 98 cm, hydrogencarbonate form). The elution was effected with a linear gradient of ammonium hydrogencarbonate (reservoir: 3 l of 0.1M aqueous NH_4HCO_3, mixing vessel: 3 l of water). 220 fractions of 26.1 ml each were collected, flow rate 1.7 ml per min. Corresponding fractions were pooled and evaporated to dryness. In order to remove the buffer, three small portions of ca. 50% aqueous methanol were evaporated from the residue. The product was then dissolved in a small amount of abs. methanol, the solution filtered and, after the determination of the optical density, evaporated:

Fractions 1–8 yielded 290 OD_{266} units which according to tlc. in the systems D and E consisted mainly of αT$_d$ (**5**),

Fractions 134–165 contained 90 OD_{266} units of pβT$_d$;

The desired product, αT$_d$–βT$_d$ (**2**) was eluted in *fractions 38–62* (buffer concentration: 0.016–0.027M). 3650 OD_{266} units (55% based on **6**)[7]) were obtained, which were lyophilized from water. The white solid which resulted weighed 130.3 mg. Since this was more than was expected according to the amount of OD, units determined after the chromatography, it was assumed that the product still contained some NH_4HCO_3. The crude dinucleoside monophosphate was therefore purified by rechromatography on a DEAE-Sephadex column (1 × 100 cm). Gradient: reservoir: 3 l of 0.05M aqueous NH_4HCO_3, mixing vessel: 3 l of water. 110 fractions of 22.8 ml each were collected. Corresponding fractions were pooled and treated as described above. Fractions 87–96 (buffer concentration: 0.017–0.018M) yielded 3250 OD_{266} units (49% based on **6**) of chromatographically pure (tlc. systems B and F, pc. system B) αT$_d$–βT$_d$ (**2**) which was lyophilized to give 98.2 mg of a white solid.

UV. spectrum in methanol: maximum at 264 nm.

6. *βT$_d$–αT$_d$ (**3**) from (MeOTr)βT$_d$ (**9**) and αT$_d$(Ac) (**7**).* – (MeOTr)βT$_d$ (**9**) (229 mg, 0.444 mmol) and 1 ml of a stock solution containing 0.5 mmol of pyridinium β-cyanoethyl phosphate per ml of pyridine [6] were dried by repeated evaporation of pyridine. TPS (293 mg, 0.963 mmol) and pyridine (2 ml) were added. The mixture was stirred at 23° for 6 h. Then 2 ml of water were added and the mixture stirred for another 20 h. The solution was diluted with 6 ml of water and extracted with three 10 ml portions of CH_2Cl_2, which were extracted with 10 ml of water. The emulsions formed were separated by addition of a small amount of solid NH_4HCO_3. The organic layers were pooled, dried with $MgSO_4$ and evaporated to dryness[8]).

The residue (crude (MeOTr)βTαp(CNEt) (**11**)) was dried 3 times by evaporating 3 ml of pyridine. TPS (290 mg, 0.952 mmol) was added and the reaction mixture once more dried by evaporation of pyridine. The residue was dissolved in 3 ml of pyridine and the solution stirred at 23° for 90 min in the dark. Then αT$_d$(Ac) (**7**) (250 mg, 0.878 mmol), which had previously been dried over P_2O_5/KOH in a vacuum desiccator for 2 h, was added. The reaction mixture was stirred for 24 h at 23° in the dark. Then water (2 ml) was added and stirring was continued for another 20 h – The solution was evaporated to dryness and the residue distributed between three 10 ml portions of water and three 10 ml portions of CH_2Cl_2. Emulsions formed during these extractions were

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[7]) The yields are based on a assumed ε-value of 19'200 for the dinucleoside monophosphates, thus neglecting an eventual hypochromicity.

[8]) The aqueous phases contained – as detected later – some 2000 OD_{266} units of (MeOTr)βTαp(CNEt) (**11**).

broken up by the addition of a few crystals of solid NH_4HCO_3. – The organic layers were pooled, evaporated and the residue taken up in 30 ml of conc. NH_3-solution. After standing for 21 h at 23° the mixture, which now was turbid, was evaporated to dryness. To the residue 80% acetic acid (30 ml) was added and the mixture heated on a steam bath to 100° for 1 h. The resulting clear yellow solution was evaporated to dryness. – The residue was distributed between three 20 ml portions of 2-butanol and three 20 ml portions of aqueous 0.5 M NH_4HCO_3. The aqueous layers which according to tlc ,(system C) contained no more TPS, were pooled, evaporated and the buffer removed by repeated evaporation of 50% aqueous ethanol. – The crude βT_d–αT_d (**3**) was applied to a DEAE-Sephadex column (1 × 100 cm). The elution was effected with a linear gradient of ammonium hydrogencarbonate (reservoir: 3 l of 0.1 M NH_4HCO_3, mixing vessel: 3 l of water). 228 fractions of 24 ml each were collected, flow rate 1.6 ml per min. Corresponding fractions were pooled and treated as described in chapter 5:

Fractions 1–9 contained 573 OD_{266} units of nucleosides, probably αT_d and βT_d;

Fractions 175–196 contained 70 OD_{266} units of a mononucleotide, probably βTp;

The desired dinucleoside monophosphate βT_d–αT_d (**3**) (2030 OD_{266} units, 24% based on **8**, 45% after correction for loss of **11** during extraction), was eluted in the *fractions 56–72* (buffer concentration: 0.022–0.027 M). Tlc. in systems C and G showed a contamination moving faster than βT_d–αT_d.

The product was purified further by rechromatography on a DEAE-Sephadex column (1 × 100 cm). Gradient: reservoir 3 l of 0.05 M aqueous NH_4HCO_3, mixing vessel: 3 l of H_2O. 150 fractions of 21.4 ml each were collected, flow rate 1.4 ml/min. Corresponding fractions were pooled and treated as described in 5. – *Fractions 97–107* (buffer concentration: 0.016–0.017 M) yielded 1370 OD_{266} units (16%, after correction: 30%) of chromatographically pure (tlc. systems B and F, pc. system B) βT_d–αT_d (**3**), which was lyophilized to give 37 mg of a white foam. – UV.-spectrum in methanol: maximum at 263 nm.

7. αT_d–αT_d (**4**) *from* (*MeOTr*)αT_d (**6**) *and* αT_d(*Ac*) (**7**). – (MeOTr)αT_d (**6**) (227 mg, 0.44 mmol) was dissolved in 1 ml of a stock solution containing 0.5 mmol of pyridinium β-cyanoethyl phosphate per ml of pyridine. The mixture was dried by repeated evaporation of small portions of pyridine. TPS (289 mg, 0.953 mmol) and pyridine (3 ml) were added. The mixture was stirred for 6 h at 23°. Then water (2 ml) was added and stirring continued for 16 h. After addition of more water the solution was evaporated to remove water and pyridine. Tlc in system A showed that all the starting material was consumed. The crude (MeOTr)αT_dp(CNEt) (**10**) obtained was dried by evaporation of pyridine and then dissolved in 3 ml of pyridine. TPS (296 mg, 0.972 mmol) was added and the solution stirred for 2 h at 23° in the dark. Then αT_d(Ac) (**7**) (248 mg, 0.868 mmol) was added and stirring continued for additional 24 h. Water (2 ml) was added and the mixture again stirred for 27 h. After evaporation the residue was distributed between three 15 ml portions of water and three 15 ml portions of CH_2Cl_2. The organic layers were combined and evaporated to dryness. The residue was taken up in 30 ml of conc. NH_3 solution and the mixture kept at 23° for 20 h. The mixture which had turned turbid, was evaporated to dryness. The residue was heated to 100° for 30 min in 30 ml of 80% acetic acid. The resulting clear yellow solution was evaporated and the residue distributed between three 20 ml portions of aqueous 0.5 M NH_4HCO_3 and three 20 ml portions of 2-butanol. The aqueous layers were pooled, evaporated, and the buffer removed by repeated evaporation of ca. 50% methanol.

The crude product (5300 OD_{266} units) was applied to a DEAE-Sephadex column (1 × 103 cm). The elution was effected with a linear gradient of ammonium hydrogencarbonate (reservoir: 3 l of 0.1 M NH_4HCO_3, mixing vessel: 3 l of water): 180 fractions of 16.6 ml each and 110 fractions of 22 ml each were collected. Flow rate 1.1 ml/min. Corresponding fractions were pooled and treated as described in chapter 5:

Fractions 1–11 gave 1100 OD_{266} units of nucleosides;

Fractions 234–250 gave 520 OD_{266} units of mononucleotides;

The desired dinucleoside monophosphate αT_d-αT_d (**4**) (3000 OD_{266} units, 35% based on **6**) was eluted in *fractions 85–97* (buffer concentration: 0.023–0.026 M). Although it was homogeneous according to tlc. in the systems B and G, it was rechromatographed on a DEAE-Sephadex column (1 × 103 cm). Gradient: reservoir 3 l of 0.05 M aqueous NH_4HCO_3, mixing vessel: 3 l of water.

160 fractions of 21.3 ml each were collected, flow rate: 1.42 ml/min. *Fractions 88–106* (buffer concentration 0.016–0.019 M) yielded 2680 OD_{266} units (32%) of chromatographically pure (tlc. systems B and F, pc. system B) αT_d-αT_d (**4**), which was lyophilized to give 84 mg of a white foam. UV. spectrum in methanol: maximum at 265 nm.

8. *Enzymatic Degradations.* – 8.1. *Substrate Solutions.* Solutions containing ca 10 mg of dinucleoside monophosphate per ml of water were prepared. They contained 0.34 OD_{266} units or 0.018 μmol per μl and were stored at $-20°$.

8.2. *Enzyme Solutions.* 200 EU of snake venom phosphodiesterase (EC 3.1.4.1, *Russell*'s viper, B grade from *Calbiochem*) were dissolved in 1 ml of 0.33 M tris buffer, pH 9.1. The solution was stored at 0°. 17 EU of spleen phosphodiesterase (EC 3.1.4.1, bovine spleen, from *Nutritional Biochemicals Corporation*) were dissolved in 2 ml of 0.2 M NH_4OAc buffer, pH 5.7. The solution was stored at 0°.

8.3. *Assay with Snake Venom Phosphodiesterase* [12]. 30 μl of substrate solution and 100 μl of enzyme solution were incubated together for 5 h at 37°. Then 10 μl of the incubation mixture were spotted on a tlc. plate together with the appropriate references[9]). The chromatogrammes were developed in system F.

8.4. *Assay with Spleen Phosphodiesterase* [28]. 30 μl of substrate solution and 50 μl of enzyme solution were incubated together for 5 h at 37°. Then 6 μl of the incubation mixture were spotted on a tlc. plate together with the appropriate references[9]). The chromatogrammes were developed in system F.

8.5. *Determination of the Ratio of the Products formed during Enzymatic Degradation* [6]. 50–60 μl of the incubation mixture were applied as a spot on *Whatman* 3MM paper. The chromatogrammes were developed in system B. After drying the paper the spots were detected by their UV. absorption. The spots were cut out, dissected to small pieces and eluted overnight in 2 ml of 0.1 M HCl. The eluates were filtered through cotton wool directly into volumeric flasks. The paper was then washed twice with 1.5 ml of 0.1 M HCl. The solution was made up to 5.0 ml (in some cases to 10.0 ml).

Blanks of about the same size and at equal distance from the start as the substance spots were cut from the same paper and eluted as described above. The solutions containing the nucleosides or nucleotides were measured in the UV. spectrometer against the corresponding blanks to determine the optical density.

Table 3. *Rf Values*

Compound	Tlc.	Solvent System*)				Pc.
	A	B	C	F	G	B
βT_d-βT_d (**1**)		0.47		0.48		0.37
αT_d-βT_d (**2**)		0.46	0.42	0.44	0.35	0.35
βT_d-αT_d (**3**)		0.45	0.46	0.47	0.39	0.42
αT_d-αT_d (**4**)		0.45	0.45	0.43		0.39
αT_d (**5**)	0.16		0.59			0.65
βT_d	0.16	0.60	0.60	0.63	0.70	0.65
(MeOTr) αT_d (**6**)	0.56		0.79			
(MeOTr) βT_d (**9**)	0.53		0.74			
αT_d(Ac) (**7**)	0.31		0.59			
pβT_d(Ac) (**8**)			0.46		0.51	
pβT_d	0.00	0.17	0.37	0.06	0.28	0.12
βT_dp						0.11

*) Solvent systems: A) methylene chloride/methanol 9:1; B) 2-propanol/conc. NH_3/H_2O 7:1:2; C) 1-butanol/acetic acid/H_2O 5:2:3; F) 2-propanol/0.5 M aqueous NH_4HCO_3 8:2; G) methanol/benzene/formic acid 5:4:1.

[9]) Usually besides the substrate, pβT_d and βT_d served as references even in those cases where α-nucleosides or nucleotides were formed.

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8. Anil-Synthese

9. Mitteilung[1])

Über die Darstellung von in 4'-Stellung heterocyclisch substituierten 4-(2*H*-Arenotriazol-2-yl)-stilbenen

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(8. XI. 73)

Zusammenfassung. Durch Umsetzung von *Schiff*'schen Basen aus 4-(2*H*-Arenotriazol-2-yl)-benzaldehyden und *p*-Chloranilin mit *p*-tolyl-substituierten Heterocyclen aromatischen Charakters mit mindestens einem Ring-Stickstoffatom können in Dimethylformamid in Gegenwart von Kaliumhydroxid die entsprechenden, in 4'-Stellung heterocyclisch substituierten 4-(2*H*-Arenotriazol-2-yl)-stilbene dargestellt werden («Anil-Synthese»).

In vorangehenden Arbeiten [2] [3] wurde über die Darstellung von 2-(Stilben-4-yl)-2*H*-naphtho[1,2-*d*]triazolen mit Hilfe der «Anil-Synthese» berichtet. So entsteht

[1]) 8. Mitt. siehe [1].