

N-ACYL PROTECTING GROUPS FOR DEOXYNUCLEOSIDES

A QUANTITATIVE AND COMPARATIVE STUDY

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Abstract A detailed study in search for suitable N-acyldeoxy-nucleosides which could serve as building blocks for the stepwise synthesis of deoxyoligonucleotides was undertaken. Several acyl groups namely 4-*t*-butylphenylacetyl, 4-*t*-butylphenoxyacetyl, 4-chlorobenzoyl, 3,4-dichlorobenzoyl, 2-methyl-benzoyl, 2,4-dimethylbenzoyl, nicotinoyl, 4-*t*-butylbenzoyl and 4-phenylazobenzoyl have been used for the protection of the exocyclic amino groups of deoxynucleosides. Interesting data concerning the stability of N-acyl groups towards a potent deacylating system (0.2 N NaOH/MeOH) are presented.

For the stepwise synthesis of deoxyoligonucleotides the exocyclic amino groups of deoxyadenosine, deoxyguanosine and deoxycytidine need protection by suitable protecting groups.¹ It is important that the protecting group selected for this purpose should be easy to introduce, is stable throughout the synthesis and can be removed under conditions which do not affect the final product. Various alkali labile protecting groups have been proposed for the protection of amino groups of the heterocyclic bases. Benzoyl, anisoyl and isobutyryl groups have been used for the amino groups of deoxyadenosine, deoxycytidine and deoxyguanosine, respectively. Since then these N-acyl protecting groups have found much use in the synthesis of oligonucleotides by the two main methodologies, i.e. the phosphodiester and phosphotriester approach (for references see lit.^{3,4}).

However losses of N-protecting groups during oligonucleotide synthesis following the phosphodiester method⁵⁻⁷ as well as phosphotriester method^{10,11} have been reported. Partial removal of isobutyryl group and anisoyl group from deoxyguanosine and deoxycytidine have been observed during DEAE-cellulose column chromatography using triethylammonium bicarbonate buffer.^{5,8,9} Some loss of N-anisoyl group from N-anisoyldeoxycytidine could not be prevented when "selectively" removing the 3'-O-acyl group using strong alkaline conditions.^{7,10} In order to selectively remove the 5'-O-4-chlorophenoxyacetyl group using Na₂CO₃/MeOH van Boom *et al.* have reported partial loss of N-anisoyl group from the N-anisoylcytidine containing oligomers during synthesis following phosphotriester approach.¹¹

As amide bonds are particularly susceptible towards nucleophiles it is of great interest to know their stability as well as the conditions for their complete removal in quantitative terms. Despite the importance of this problem only few qualitative or semi-quantitative studies exist in literature.¹²⁻¹⁴ Recently Seliger determined the rate of hydrolysis of various aliphatic protecting groups for deoxycytidine-5'-phosphate.¹⁵ Due to the particular lability of aliphatic protecting groups in case of deoxycytidine this study is only of theoretical interest. We therefore felt it

worthwhile to perform a systematic study for the N-protecting groups which could offer an assortment of suitable N-acyl groups for nucleoside bases and could help in planning oligonucleotide synthesis in more meaningful terms. Particular emphasis was laid towards selecting more stable N-acyl groups to prepare N-acyldeoxynucleosides which could serve as reliable building blocks for the stepwise synthesis of oligonucleotides. In certain cases, wherever possible, more lipophilic acyl groups were introduced which could themselves serve as pseudopurification handles while employing extractive work-up and silica gel column chromatography.^{16,17}

RESULTS AND DISCUSSION

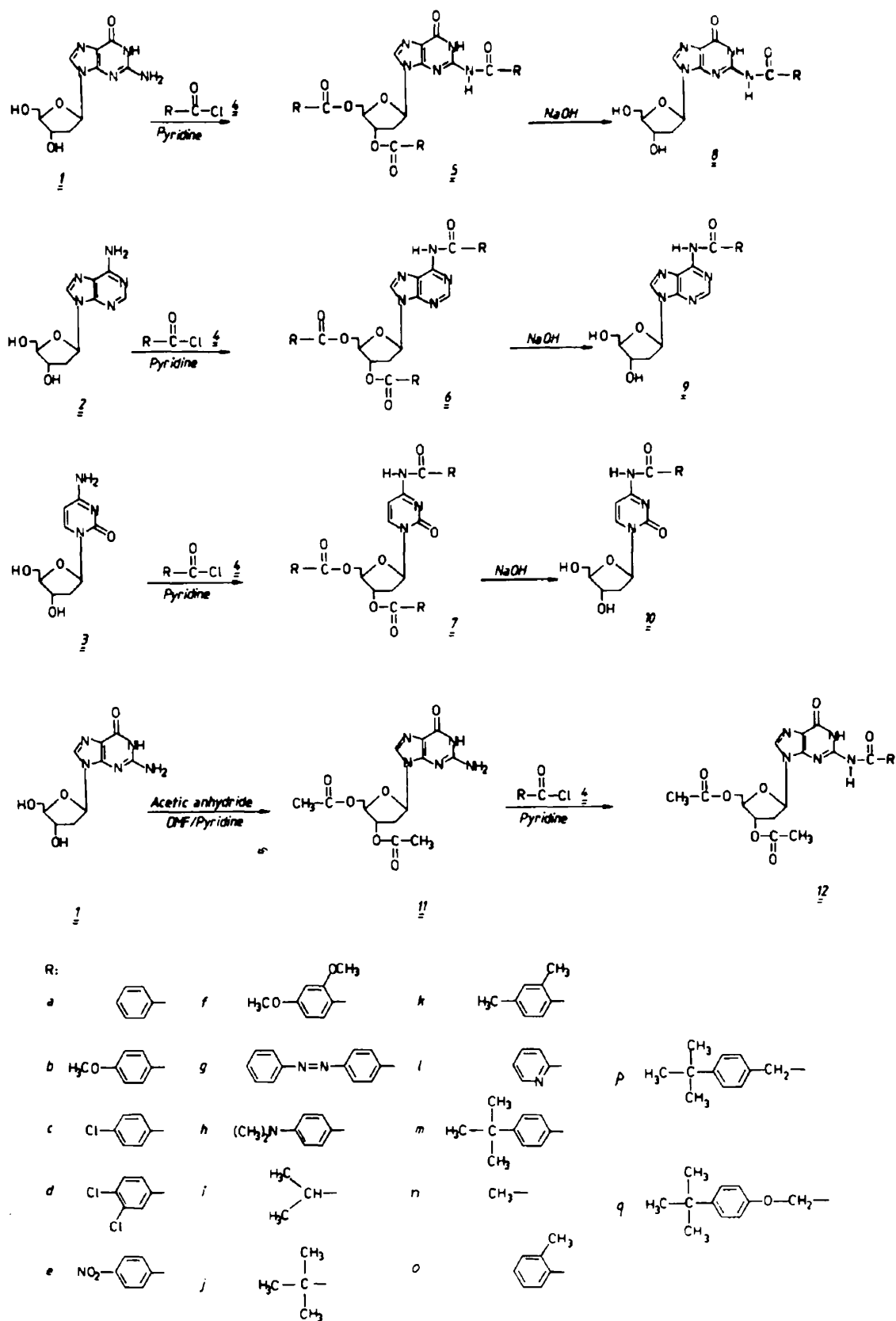
Synthesis of N-acylated deoxynucleosides followed known procedures^{1,2} (Scheme 1). The deoxynucleosides **1**, **2** and **3** were peracylated to **5**, **6** and **7** using an excess of acyl chloride in pyridine and then selectively O-deacylated to **8**, **9** and **10** by treatment with 1 N NaOH at 0°. For the synthesis of N-acylated deoxyguanosine the route via 3', 5'-di-O-acetyldeoxyguanosine **11** has been used alternatively.²⁰

Under the reaction conditions used in most cases a second acyl group was introduced at the heterocyclic ring.²¹ This additional acyl function which is removed during selective 3'-O-deacylation has not been outlined in Scheme 1. The various derivatives of deoxynucleosides have been obtained as crystalline compounds and unequivocally characterized (Tables 3 and 4).

To determine the rate of hydrolysis of the amide bond spectrophotometric methods were used. The stability of the various N-protecting groups has been measured using a mixture of 0.2 N NaOH/MeOH (1:1, v/v) as the deacylating agent. Nucleoside concentrations were about 0.04 mmole/l; therefore the hydrolysis followed first-order kinetics. To compare the stability of the different protecting groups it is referred to the half life time τ .

Table 1 shows half life times for the hydrolysis of different synthetic deoxyguanosine derivatives.

The higher stability of the aromatic N-acyl groups is significant, the aliphatic isobutyryl group being



Scheme 1. Synthesis of N-acyl protected deoxynucleosides.

Table 1. N-Deacylation of various deoxyguanosine derivatives²² with MeOH 0.2 N NaOH (1:1, v/v) at 22°

N-Acylated Deoxynucleosides	τ [min]
C _d ^{bz} (<u>8a</u>)	647
C _d ^{cl} (<u>8c</u>)	552
C _d ^{dc} (<u>8d</u>)	412
C _d ^{nt} (<u>8e</u>)	312
C _d ^{ib} (<u>8i</u>)	271
C _d ^{pa} (<u>8p</u>)	32
C _d ^{po} (<u>8q</u>)	8

bz = benzoyl, cl = 4-chlorobenzoyl, dc = 3,4-dichlorobenzoyl, nt = 4-nitrobenzoyl, ib = isobutyryl, pa = 4-*t*-butylphenylacetyl and po = 4-*t*-butylphenoxyacetyl.

stabilized by steric effects. The 4-*t*-butylphenoxyacetyl and the 4-*t*-butylphenylacetyl group (the latter independently developed by Reese *et al.*¹⁴) are lipophilic derivatives of the recently described phenylacetyl group⁶ and are of value for triester approach, when only mild alkaline treatments have to be used during oligonucleotide synthesis. If this cannot be achieved according to the strategy used the isobutyryl group is superior. The latter is useful for diester approach as well, although problems have been reported.^{6,9,23} Due to its stability the 4-chlorobenzoyl group can be recommended for diester method. The more labile 3,4-dichloro- and 4-nitrobenzoyl group are not satisfactory, the former being used for the synthesis of the endonuclease Pst I specific oligonucleotide d-CCTGCAGG²⁴ following the triester method. Strong retention on silica gel columns and partial breakdown of the guanine ring during condensation have been observed. These unwanted side effects do not occur when using the benzoyl or 4-chlorobenzoyl group the former being too stable for synthetic purposes.²⁵

With respect to stability the benzoyl group for deoxyadenosine seems to be satisfactory, an increase in stability of about 20% can be obtained by introducing the anisoyl group¹¹ (Table 2), although this effect may be not great enough to be of preparative value. For triester approach the more lipophilic 4-*t*-butylbenzoyl group has been proposed without any kinetic data.¹⁹ Its stability should be between benzoyl and anisoyl (*c.f.* the corresponding derivatives of deoxycytidine in Table 2).

Considerable difficulties, however, were encountered during the search for a more stable N-protecting group for deoxycytidine. Several compounds were synthesized and their stability measured (Table 2).

Introduction of a second OMe group into the 2-position of the phenyl ring (**10f**) did not lead to a deactivation of the carbonyl-C-atom towards nucleophilic attack. Apparently the sterical hindrance of the *o*-substituent is so large that the phenyl ring is turned out of the mesomeric plane of the amide bond and by this only the electron withdrawing inductive effect of the two O-atoms is operating. This may

explain the greater instability of the 2,4-dimethoxybenzoyl group compared to the 4-methoxybenzoyl(anisoyl) group in compound **10b**.

The extended π -electron system of the 4-phenylazobenzoyl residue (**10g**) resembles the unsubstituted phenyl ring (**10a**) in its influence on the CO group of the amide bond. A similar observation has been made for the pyridine ring in the nicotinoyl group (**10i**). The 4-phenylazobenzoyl group, however, may be of interest due to its strong absorption in the visible region.²⁶

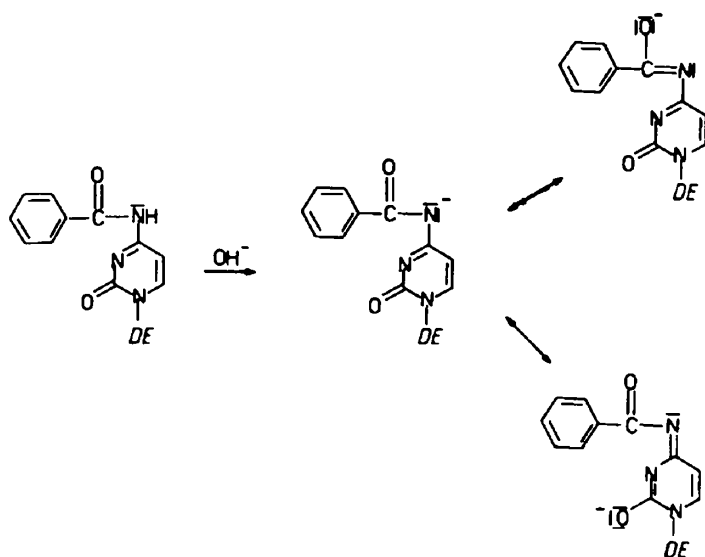
A distinct increase in stability compared to **10a** has been observed when introducing the 4-*t*-butylbenzoyl group (**10m**). This effect might be due to the electron releasing properties of the *t*-Bu group. This effect is particularly pronounced when introducing the N,N-dimethylaminobenzoyl group (**10h**). But nevertheless the stability of the N-acyl group in **10h** cannot be compared to that of suitable deoxyadenosine and deoxyguanosine N-acyl derivatives.

Great difficulties to introduce the mesitoyl group led to an examination of the 2,4-dimethylbenzoyl group. The resulting compound, N-2,4-dimethylbenzoyl-deoxycytidine (**10k**), is the hitherto most stable N-acyl derivative of deoxycytidine, moreover it is easy to prepare and to handle. Considerations using molecular models show that the 2-Me group in **10k** can shield the CO function efficiently but leads to a less sterical hindrance than the 2-OMe group in **10f**. In contrast to **10f** the mesomeric system between the phenyl ring and the amide group apparently seems to be intact in **10k**. The electron releasing effect of the phenyl ring apparently is important for stabilization of the amide bond. The remarkable low stability of the likewise sterically hindered pivaloyl group in **10j** points again to the importance of the resonance effect.

Table 2. N-Deacylation of various deoxycytidine and deoxyadenosine derivatives using MeOH 0.2 N NaOH (1:1, v/v) at 22°

N-acylated Deoxynucleosides	τ [min]
C _d ^{bz} (<u>10a</u>)	6.8
C _d ^{an} (<u>10b</u>)	14.0
C _d ^{dm} (<u>10f</u>)	4.4
C _d ^{az} (<u>10g</u>)	6.7
C _d ^{da} (<u>10h</u>)	31.0
C _d ^{ac} (<u>10n</u>)	0.2
C _d ^{pi} (<u>10j</u>)	2.1
C _d ^{tl} (<u>10o</u>)	109.0
C _d ^{db} (<u>10k</u>)	116.0
C _d ^{nc} (<u>10i</u>)	7.3
C _d ^{tb} (<u>10m</u>)	9.0
A _d ^{bz} (<u>9a</u>)	224.0
A _d ^{an} (<u>9b</u>)	285.0

ac = acetyl, tl = 2-methylbenzoyl, an = 4-methoxybenzoyl(anisoyl), az = 4-phenylazobenzoyl, bz = benzoyl, da = 4-N,N-dimethylaminobenzoyl, db = 2,4-dimethylbenzoyl, tb = 4-*t*-butylbenzoyl, nc = nicotinoyl.



Scheme 2. Possible explanation for the increased lability of N-acyl derivatives of deoxycytidine. DE = Deoxyribose

Compared to the acetyl group¹⁻ (10n) the pivaloyl group leads to a dramatic increase in stability due to its exerting sterical shielding effect. For large-scale preparations of N-acyl derivatives of deoxycytidine the 2,4-dimethylbenzoyl group might be too expensive. For this case the 2-methylbenzoyl group has been introduced (10o). Its stability is in the same range as that of the 2,4-dimethylbenzoyl group. The 2,4-dimethylbenzoyl group newly introduced for the protection of the cytosine ring has been used during synthesis of the PstI-specific octanucleotide²⁴ and shown to be a very suitable protecting group for this purpose.

It is interesting to note that the main influence on the stability of an acyl function towards alkaline hydrolysis is determined by the heterocyclic base and that the stability of the amide bond can only be influenced in a limited range by inductive, resonance and steric effects originating in the acyl group. A comparison of compounds 8a, 9a and 10a makes this phenomenon particularly evident. According to Khorana¹³ removing a proton from the amide bond leads to an increase in stability towards nucleophilic attack on the carbonyl-C-atom of the amide bond due to the delocalization of the negative charge within the amide bond. The large difference in stability of N-acyl derivatives of deoxycytidine as compared to that of

deoxyadenosine and deoxyguanosine may be attributed to a strong electron withdrawing effect of the heterocyclic base cytosine under strong alkaline conditions ($\text{pH} > 12$). We assume that the negative charge at the amide N atom following deprotonation ($\text{pH} > 12$), is mainly delocalized into the heterocyclic ring (0^2), thus making the carbonyl-C-atom of the amide bond more susceptible towards nucleophilic attack (Scheme 2).

Methanol:0.2 N NaOH (1:1, v/v) is a very powerful N-deacylating reagent, the rate of hydrolysis being increased by a factor of about 5–13 depending on the N-acylated deoxynucleoside when compared to aqueous concentrated ammonia widely used.²⁷ The work-up is simple by using cation ion exchanger. Side reactions namely deamination are negligible.⁴ This reagent therefore offers an alternative to the known procedures at least for oligonucleotide synthesis following the diester method. The N-deacylation using this reagent may be induced by nucleophilic attack of the methyolate anion on the carbonyl C-atom of the N-acyl group.²⁷ Other alcohols are not effective and even suppress the rate of N-deacylation when compared to aqueous sodium hydroxide.²⁷ N-deacylation is suppressed most strongly in the presence of 1,4-dioxane. For the repeated 3'-O-deacylations necessary in diester approach it is therefore advisable to use dioxane to render the oligonucleotides soluble in aqueous 2 N NaOH.

To our knowledge this is the first comprehensive study regarding N-acyl protecting groups which have been invariably used in oligonucleotide synthesis. The results obtained allow to select N-acyl protecting groups according to their stability and/or lipophilicity. We feel confident that the availability of such a collection of data will prove to be of much value for the synthesis of oligonucleotides following different strategies and for other compounds bearing amino groups to be protected during chemical transformations.

[†] The amount of deoxyuridine after a 16-hours treatment of N-2,4-dimethylbenzoyldeoxycytidine (10k) at room temperature is less than 0.6%, as shown by hplc analysis performed with an equipment of Laboratory Data Control on Lichrosorb Si-60 (7μ, Merck), column: 250 mm length, 4.6 mm i.d., solvent system: 30%, methanol (containing 1%, aqueous triethylammonium acetate, pH 7.0): 70%, chloroform, flow rate 2 ml/min; retention times: N-2,4-dimethylbenzoyldeoxycytidine at front, 102 sec for deoxyuridine, 125 sec for 2,4-dimethylbenzoic acid, 185 sec for deoxycytidine.

Table 3 Data of elementary analysis, melting and decomposition temperatures of N-acylated deoxynucleosides. Abbreviations see legends to Table 1 and 2.

Deoxy- nucleoside	Molecular weight	Elementary Analysis								Melting point °C	Decomposi- tion point °C
		calculated				found					
		C	H	N	Cl	C	H	N	Cl		
$\lambda_d^{an} (9b)$	385.10	56.07	4.97	18.18							
$\lambda_d^{an} (1/2H_2O)$	394.1	54.79	5.11	17.76		54.55	5.33	17.64		120	
$c_d^{az} (10g)$	435.19	60.66	4.86	16.09		59.79	4.85	15.81			198
$c_d^{bz} (10a)$	331.15	57.97	5.17	12.68		57.75	5.06	12.46			206
$c_d^{da} (10h)$	374.19	57.72	5.92	14.97		58.10	6.10	14.81			300
$c_d^{db} (10k)$	359.18	60.13	5.89	11.69		60.10	5.89	11.54			180
$c_d^{dm} (10f)$	391.18	55.21	5.41	10.74		55.58	5.44	10.59			189
(Nc) $c_d^{nc} (Nc) (71)$	542.2	59.75	4.08	15.49		59.50	4.04	15.30		208-209	
(Pi) $c_d^{pi} (Pi) (74)$	479.3	60.08	7.78	8.76		60.29	7.74	8.77		204	
$c_d^{tb} (10m)$	387.2	61.98	6.50	10.85		61.73	6.58	10.70			199
$c_d^{cl} (8c)$	405.6	50.29	3.97	17.26	8.74	50.11	3.96	17.23	8.77		300
(Ac) $c_d^{cl} (Ac) (12e)$	469.6	51.46	4.11	14.30	7.34	49.70	4.17	13.73	7.07	110	
(Ac) $c_d^{dc} (Ac) (12d)$	524.08	48.08	3.65	13.36	13.52	47.36	3.65	12.97	13.65	120	
(Ac) $c_d^{nt} (Ac) (12g)$	500.19	50.38	4.03	16.80		50.15	4.02	16.66			300
(Ac) $c_d^{pn} (Ac) (12p)$	528.56	59.08	6.48	13.25		58.96	6.21	12.97		94	102
(Ac) $c_d^{po} (Ac) (12q)$	544.53	57.34	6.29	12.86		57.24	5.87	12.37		94	102

Table 4. UV-spectroscopic properties of N-acylated deoxynucleosides

Deoxy-nucleoside	Solvent	λ_{max_1}	ϵ_{max_1}	λ_{max_2}	ϵ_{max_2}	λ_{min}	ϵ_{min}	$\frac{300}{280}$	$\frac{280}{260}$
		nm		nm		nm			
$\lambda_d^{an} (9b)$	C	290	31910	-	-	240	6060	0.995	1.934
$c_d^{az} (10g)$	B	329	34620	264	14090	281	12610	1.507	0.905
$c_d^{bz} (10a)$	B	307	10470	261	22740	286	7510	1.099	0.385
$c_d^{da} (10h)$	C	351	36840	246	15810	285	7340	1.193	0.581
$c_d^{db} (10k)$	B	305	11360	258	21360	286	9690	1.061	0.491
$c_d^{dm} (10f)$	B	312	25680	275	18670	287	14450	1.191	1.237
(Nc) $c_d^{nc} (Nc) (71)$	B	308	9890	264	27030	290	7720	0.783	0.430
(Pi) $c_d^{pi} (Pi) (74)$	A	308	7270	248	14650	276	3060	2.035	0.395
$c_d^{tb} (10m)$	B	306	12150	265	26510	292	10590	0.733	0.624
$c_d^{cl} (8c)$	B	303	14240	249	21450	275	9870	1.314	0.596
(Ac) $c_d^{dc} (Ac) (12d)$		311	11480	249	21890	275	5800	1.694	0.408
(Ac) $c_d^{nt} (Ac) (12g)$		256	23970	-	-	227	12390	0.611	0.584
(Ac) $c_d^{pn} (Ac) (12p)$		253	14680	260	13750	258	13420	0.880	0.802
				287	12070	269	8770		
(Ac) $c_d^{po} (Ac) (12q)$		252	13360	260	12870	257	12910	0.801	0.877
				282	11360	268	8690		

Solvents: A = Dioxane, B = Dioxane:Water (8:2, v/v), C = Ethanol:Water (8:2, v/v). Abbreviations see legends to Tables 1 and 2.

EXPERIMENTAL

Abbreviations. See refs¹ and the legends to Table 1 and 2.

Materials. Deoxynucleosides (Papierwerke Waldhof-Aschaffenburg, Mannheim), carboxylic acids and/or carboxylic acid chlorides (Aldrich), solvents (Merck, Darmstadt) were purchased commercially. All reagents and solvents were crystallized or distilled before use.

General methods. Tlc and UV spectroscopy has been performed as described.¹ Preparative tlc has been performed on commercially available thin layer plates from Merck (Darmstadt) containing fluorescence indicator (254 nm). Descending paper chromatography to work up micro-preparative N-deacylations has been achieved using paper from Schleicher and Schüll (2043 bmg1); solvent: 2-propanol/water (7:3, v/v). The substances were eluted as described¹ and identified via R_f values and UV spectra.

Synthesis of N-acylated deoxynucleosides. A_0^n (9b), C_1^{2*} (10g), C_2^{3*} (10a), C_3^{4*} (10h), C_4^{5*} (10k), C_5^{6*} (10f), C_6^{7*} (10m), C_7^{8*} (10n), C_8^{9*} (10o) and G_1^0 (8c).

General method. The deoxynucleoside was dried *in vacuo* by threefold evaporation with dry pyridine and dissolved in dry pyridine (about 5 ml pyridine/mmol deoxynucleoside). To this soln or suspension an excess of acyl chloride was added at 0°. The excess of acyl chloride should not exceed 0.3–0.4 equivts with respect to the functions to be acylated (exception: less reactive acyl chlorides such as N,N-dimethylaminobenzoyl chloride). The acylation was stopped by addition of sufficient but small amount of water. After dilution with acetone the peracylated deoxynucleoside 5, 6 or 7 could be obtained by precipitation into ice-water. This crude material was generally subjected to selective O-deacylation, which could be performed quantitatively in 20 min using 1 N NaOH in dioxane (or pyridine). After neutralization with Dowex 50WX8 ion-exchanger (pyridinium), the ion-exchanger was washed with pyridine/water and the N-protected deoxynucleosides 8, 9 or 10 crystallized from EtOH or EtOH/water after elimination of traces of pyridine. If the liberated carboxylic acid had a low solubility in aqueous systems, the mixture was evaporated to dryness and before crystallization extracted with ether. By addition of some $NaHCO_3$ (about 5%) to the crystallization medium the last traces of carboxylic acids could be held in soln. The N-protected deoxynucleosides were filtered and stored over P_2O_{10} *in vacuo* at 35° for 3–4 days.

C_1^{2*} (10f) was isolated out of the O-deacylation reaction by preparative tlc. The product was chromatographically pure and therefore suitable for the determination of kinetic parameters. The elemental analysis, however, showed the presence of some silica gel as impurity. Satisfactory elementary analysis could be obtained from 7f (Table 3). As a consequence of lability of the N-pivaloyl group, C_1^{2*} (10j) could be only detected by tlc during O-deacylation. The rate of hydrolysis of the N-acyl function, however, could be determined using 7j, as the ester groups have no influence on the absorption of the chromophore. 7j was obtained following the general procedure (see above). Crystallization from EtOH gave an analytically pure product (Table 3).

Synthesis of N-acylated deoxyguanosine derivatives via 3',5'-di-O-acylated deoxyguanosine 11. (Ac) G_1^0 (Ac) (11) was prepared according to the method described¹⁰ by acetylation of 1 with Ac_2O in pyridine/N,N-DMF. Reaction of 11 with the appropriate acid chloride yielded after precipitation into ice-water and crystallization from EtOH, 12c, (Ac) G_1^0 (Ac) and N-3,4-dichlorobenzoyl-3',5'-di-O-acetyl deoxyguanosine, (Ac) G_1^0 (Ac), (12d). Similarly 12p and 12q were prepared. The derivative 8e which was difficult to prepare by the direct acylation procedure (see general method above) could be obtained by reaction of 11 with 2.8 equivts of 4-nitrobenzoyl chloride. The di-N-acylated compound was precipitated into ice-water and the dried ppt washed on a sintered glass funnel with conc ammonium bicarbonate. On dilution with water (Ac) G_1^0 (Ac) (12e) precipitated from the yellow eluate in pure form. For the determination of the

kinetic parameters 12c, 12d and 12e were used directly. The properties and analytical data are summarized in Tables 3 and 4. The N-acylated deoxynucleosides 8a, 8i, 9a and 10b were prepared earlier.^{2,25}

Determination of kinetic parameters by UV spectrophotometric methods. The rate of N-deacylation was determined by following the change in absorption vs time using a Gilford spectrophotometer (model 2400-S). The bathochromic shift of the N-protected compared to the unprotected deoxynucleosides could be used favourably. The rate of N-deprotection was measured at the following wave lengths: 310 nm for G_1^{2*} (8a), G_1^{3*} (8c), C_1^{2*} (10b), C_1^{3*} (10a), C_1^{4*} (10f), C_1^{5*} (10o), C_1^{6*} (10n), C_1^{7*} (10l), (Pi) C_1^{2*} (Pi) (7j) and C_1^{2*} (10m), 290 nm for A_0^{2*} (9b) and A_0^{3*} (9a), 300 nm for G_1^{2*} (8i), G_1^{3*} (12p) and G_1^{4*} (12q), 340 nm for C_1^{2*} (10h), 360 nm for G_1^{2*} (8e) and 370 nm for C_1^{2*} (10g). For evaluation three different methods were used: (a) If the measurements could be undertaken at a wave length at which neither the unprotected deoxynucleoside nor the anion of the removed N-acyl function absorbed, the evaluation could be performed by using the rate law for a first-order reaction. Plotting $\ln C_0/C$ vs t led to a graph out of which the kinetic data could be obtained (Tables 1 and 2). (b) In case of the compounds 8e, 10g and 8i no wave length could be found at which either the liberated acyl group or the unprotected deoxynucleoside had no own absorption. Then the observed absorption E_t is an addition of the absorption of the N-protected deoxynucleoside (index 1) and the absorption of the liberated acyl group (index 2):

$$E_t = E_1 + E_2 \quad (1)$$

E_1 and E_2 obey opposing exponential equations:

$$E_1 = E_{01} \cdot e^{-k_1 t} \text{ and } E_2 = E_{02} (1 - e^{-k_2 t}) \quad (3)$$

Insertion of equation (2) and (3) into (1) gives

$$E_t = E_{01} \cdot e^{-k_1 t} + E_{02} (1 - e^{-k_2 t}) \quad (4)$$

As the rate constants k are identical, equation (4) can be simplified to

$$E_t = (E_{01} - E_{02}) \cdot e^{-k t} + E_{02} \quad (5)$$

E_{02} can be determined out of the kinetic for $t \rightarrow \infty$. After subtraction of E_{02} or displacement of zero adjustment by the same value gives an exponential equation which allows the determination of the rate constant k as in (a). (c) In the case of very slow reaction rates (e.g. hydrolysis of 8c with 0.2 N NaOH/dioxane) the slope of the reaction approximates to be linear. Then for the evaluation of kinetic parameters the rate law has been used in the differentiated form

$$\frac{dc}{dt} = -k \cdot c \text{ or } \frac{dE}{dt} = -k \cdot E.$$

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