A CONVENIENT PROTECTION FOR 4-OXOPYRIMIDINE MOIETIES IN NUCLEOSIDES BY THE PIVALOYL GROUP+

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Application of the pivaloyl group as a protection for the N3 position of thymidine and uridine was investigated. Pivaloylation of thymidine is a very rapid reaction proceeding under mild conditions with excellent regioselectivity for sugar or thymine moiety, depending on the amines used. Several pivaloylated thymidine derivatives were obtained by treatment of unprotected thymidine with pivaloyl chloride under various experimental conditions. Stability of the *N*3-pivaloyl protecting group under basic and acidic conditions was evaluated and the conditions for its selective removal were found.

Keywords: Thymidine; Uridine; Pivaloyl; Acylations; Protecting groups; Regioselectivity.

In preparation of nucleoside derivatives, the imido functionality in 4-oxopyrimidine moieties is often left unprotected due to its relatively low reactivity. However, for a number of reactions aimed at sugar[2] or phosphorus[3,4] moieties, such protection is highly desirable or necessary to prevent nucleobase modifications. The N3H function contains the most acidic hydrogen in thymidine or uridine molecules (pK_a 9.2–10.1, depending on substituents[5]), and hence is prone to react with soft electrophiles, e.g. in alkylation with mild alkylation agents[6,7] or in the reactions under the Mitsunobu conditions[8,9]. Additionally, in the deprotonated thymine moiety the negative charge is delocalized, making the O^4 function also reactive. Derivatization of this position of thymidine (or uridine) was observed in condensations involving azole derivatives (e.g. MSNT++)[10–12] or triazole[13,14]

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++Abbreviations: DMAP, *N*,*N*-dimethylaminopyridine; MSNT, 1-(mesityl-2-sulfonyl)-3-nitro-1,2,4-triazole; Pv, pivaloyl (trimethylacetyl); Py, pyridine; TEA, triethylamine.

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as well as when arenesulfonyl chlorides[15,16a] or chlorophosphates[17,18] were used as condensing agents. The resulting O^4-substituted compounds react readily with nucleophiles, leading, for example, in the presence of ammonia or amines to cytidine derivatives[13,16a,19].

The protection of 4-oxopyrimidine is realized by blocking either the O^4 function (with pyridyl[2], aryl[12,20] or *p*-nitrophenylethyl[21] protecting groups) or the N3 position (arenesulfenyl[22], acyl[16,23–27] or Boc-type[28] protecting groups). Currently, *N*3-benzoyl protection is used routinely, often with a substituent in the benzene ring in order to adjust the stability of the protection group[7,27,29].

Since the pioneering works of Todd[30,31] and Khorana[32–34], acyl groups (usually benzoyl and its derivatives, acetyl, pivaloyl, and adamantane-1-carbonyl) have been also commonly used for the protection of carbohydrate residues in nucleosides. Acylation of thymidine in pyridine is chemoselective for sugar hydroxy groups[35,36]; however, only bulky acyl groups (e.g. pivaloyl[37–40] or adamantane-1-carbonyl[41]) can be easily introduced in a regioselective manner to the 5′ position, while for less sterically demanding groups special procedures are required, e.g. involving activation of the hydroxy group[42,43] or engaging enzymatic reactions[44–46].

The known methods for acylation of nucleosides, both in sugar and aglycone moieties, often require lengthy procedures and involve the transient protection–deprotection strategy[47]. In this paper, application of the pivaloyl (trimethylacetyl) group as a convenient protection of N3 positions and hydroxy groups of thymidine and uridine is presented. (Apart from chemical applications, pivaloylated nucleosides are of interest as therapeutics. For example, 5′-*O*-pivaloylthymidine was found to act as a prodrug derivative of thymidine for possible use in the prevention of toxic effects in some therapies[48].)

RESULTS AND DISCUSSION

Recently we have found that in the condensations of protected nucleoside 3′-*H*-phosphonates with alcohols, the yield of *H*-phosphonate diesters significantly decreased when the reaction was carried out in the presence of trialkylamines or strong nucleophilic catalysts (e.g. DMAP)[49]. While such results could be expected due to the known proneness of *H*-phosphonates to side reactions in the presence of strong organic bases[50–54], an intriguing observation was made that no by-products with modified phosphorus groups could be detected in reaction mixtures by ^{31}P NMR spectroscopy. It appeared subsequently that the main reason for the observed deterioration

of the yield was partial consumption of pivaloyl chloride (PvCl) used as a condensing agent[49]. However, in some cases also another reaction, rapid pivaloylation of sugar and/or nucleobase moiety, was detected. This was a rather surprising observation, as the procedures for acylation of nucleosides usually require long (often overnight) reaction times, while under very mild conditions used for the condensation of nucleoside *H*-phosphonates (dilute solution of reactants in DCM, 3 equiv. of an amine, 1.2 equiv. of PvCl), no competing acylation was anticipated.

Pivaloylation of Thymidine

In follow-up experiments, the reactivity of PvCl in the reactions with protected thymidine derivatives (3′,*N*3-diprotected for analysis of acylation of 5′-OH, and 3′,5′-diprotected for N3-acylation) was investigated in the presence of three amines of different basicity[55] and nucleophilicity: DMAP (a strong nucleophilic catalyst; pK_a 9.7), pyridine (a moderate nucleophilic catalyst; pK_a 5.2), TEA (poor nucleophilic catalyst; pK_a 11.0), or TEA/Py mixture (Table I).

In the presence of DMAP pivaloylation of the 5′-OH group of *N*3,3′-*O*-dibenzoylthymidine was complete within a few seconds, while N3-acylation of 3′-*O*-(dimethoxytrityl)-5′-*O*-pivaloylthymidine (**7**) required several minutes for completion (N vs O chemoselectivity >30:1). For

TABLE I
The half-times of pivaloylation and benzoylation at N3H and 5′-OH of 3′,5′-*O*- or 3′-*O*,*N*3-protected thymidine derivatives

Amine	Pivaloylation, $t_{1/2}$		Benzoylation, $t_{1/2}$	
	N3H	5′-OH	N3H	5′-OH
DMAP (5 equiv.)	~30 s	<1 s	~0.5 h	~10 s
Py (5 equiv.)	~10 h	~2 h	~24 h	~60 s
Py (62 equiv. = 50% v/v)	~4 h	~5 min	~1.5 h	~10 s
TEA (5 equiv.)	~60 s	>4 h	<1 s	~60 s
TEA (2.5 equiv.) + Py (2.5 equiv.)	<1 s	~1 h[a]	–[a,b]	~60 s[a]
TEA (5 equiv.) + Py (5 equiv.)	<1 s	~15 min[a]	<1 s[a,c]	~2 s

[a] Under nitrogen. [b] Not complete due to side reactions. [c] Complete after ca. 4 h.

pyridine (5 equiv.), both reactions were much slower, and the selectivity of O- vs N-acylation was significantly lower (ca. 5:1). However, when basicity of the reaction medium was increased by raising the contents of pyridine to 50% (v/v), the selectivity for 5′-OH reached a value of ca. 50:1, comparable to that in the presence of DMAP.

In the presence of triethylamine (TEA), complete reversal of chemoselectivity (N3 vs 5′-O) of pivaloylation was found and the acylation in the thymine moiety was over 240 times faster than in the deoxyribose. (A similar pattern of regioselectivity in benzoylation of the 5-(trifluoromethyl) analogue of thymidine promoted by various amines was noted by Yamashita et al.[25]) The rate of N3-pivaloylation ($t_{1/2}$ ~ 1 min) could be notably increased without affecting high chemoselectivity when the reaction was conducted in the presence of both TEA and pyridine. Under such conditions, *N*3-Pv derivative was formed three orders of magnitude faster than the 5′-*O*-Pv one ($t_{1/2}$ < 1 s vs 1 h), and required only a few seconds for completion. (In the presence of TEA and pyridine, pivaloyl chloride (or other acyl chlorides) was consumed within several minutes in a side reaction giving unidentified dark-brown products if the reaction was not protected from atmospheric oxygen.) This high increase in the rate of pivaloylation might be explained in terms of synergistic effects of triethylamine and pyridine, with base catalysis by TEA operating on the pyrimidine ring and a nucleophilic catalysis by pyridine operating on PvCl. The observed diverse reactivity of PvCl in the reaction with thymidine due to these two types of

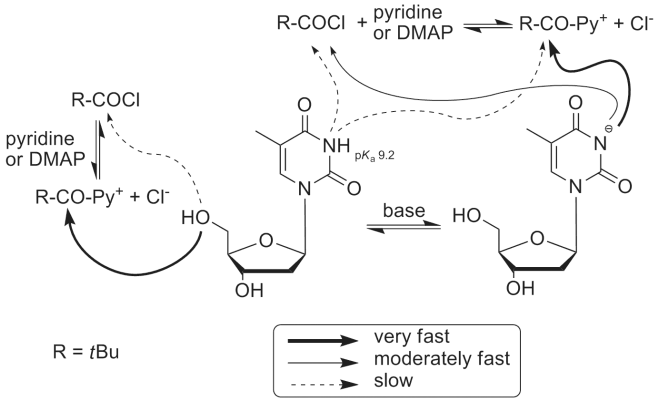

FIG. 1
Estimated kinetics of N3- and 5′-O-acylation of thymidine correlated with possible base and nucleophilic catalysis

catalysis is drafted in Fig. 1. (Acylation of thymidine with adamantane-1-carbonyl chloride proceeded with similar selectivity as pivaloylation, while acetylation (Ac_2O or AcCl) showed little regioselectivity. For benzoylation, the influence of amines on the directing the reaction to N3H or 5′-OH positions was similar as for pivaloylation, but the reactions differed significantly in terms of kinetics and product distribution (Table I)[1].)

The above findings were exploited for rapid and efficient preparation of *N*3-pivaloyl- (T^{Pv}, **2**), 5′-*O*-pivaloyl- (PvT, **3**), 3′-*O*,*N*3-dipivaloyl- (PvT^{Pv}, **4**), and 3′,5′-*O*-dipivaloylthymidine (Pv_2T, **5**) by reaction of unprotected thymidine (**1**) with PvCl in the presence of appropriate amines: pyridine or DMAP for O-pivaloylation, or TEA/Py for N-pivaloylation (Fig. 2 and Table II). The procedures reported here are either improvements of the known methods (for **3** [37,40] and **5** [56,57]) or new approaches (for **2** and **4**). Monopivaloylated thymidine derivatives are fairly well soluble in water and diethyl ether, while rather poorly in benzene or toluene (noteworthy, analogous benzoylated or dimethoxytritylated thymidine derivatives show an opposite solubility profile). Taking advantage of this feature it was possi-

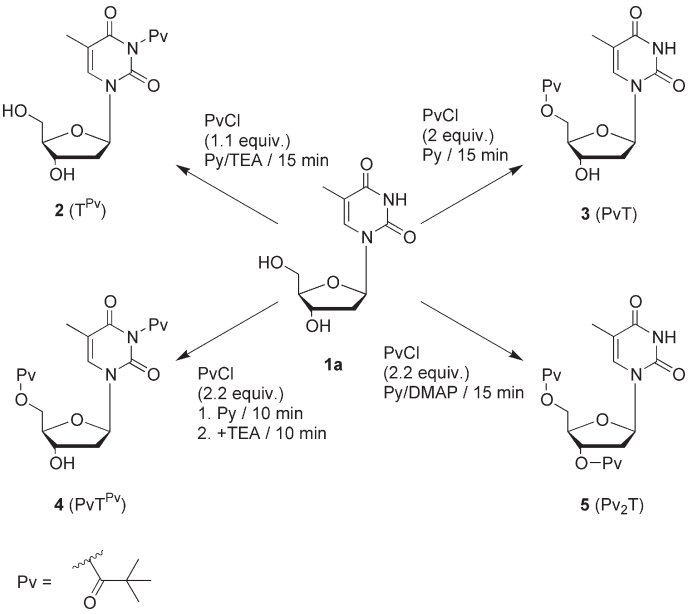

FIG. 2
Regioselective pivaloylation of thymidine (**1a**)

ble to replace the laborious column chromatography with simple solvent extractions to obtain the desired compounds in good yields and purity >98%. Due to a rather poor solubility of thymidine in MeCN, in the preparative runs the 1:1 Py–MeCN solutions were used.

Thus, *N*3-pivaloylthymidine (**2**) was prepared by treating thymidine (**1a**; 0.1 M solution in 1:1 MeCN–pyridine mixture containing 15 equiv. of TEA) with 1.2 equiv. of PvCl under inert gas atmosphere. After 10 min the reaction was complete and the reaction mixture consisted of ca. 90% of the desired T^{Pv} (**2**) and ca. 5% each of unreacted **1a** and PvT^{Pv} (**4**). Unreacted thymidine and by-products were removed by successive extractions with Et_2O–TEAB 9:1 to remove hydrophilic compounds (DCM could be used as well; however, dark by-products formed accidentally if oxygen was not completely removed from the reaction vessel could be efficiently eliminated using ether only) and with water–toluene 9:1 to remove PvT^{Pv} and lipophilic impurities. Attempted crystallization of the product was unsuccessful and **2** was obtained as a white foam (yield 70%).

5′-*O*-Pivaloylthymidine **3** was synthesized by reacting thymidine (0.1 M solution in 1:1 MeCN–pyridine) with 2 equiv. of PvCl. The reaction was quenched with water after 15 min as this reaction time was found to yield the best ratio of PvT to unreacted and bis-pivaloylated thymidines. Alternatively, when the reaction was performed in the presence of 5 equiv. of DMAP, similar rapid pivaloylation of 5′-OH was achieved with almost a

TABLE II
Conditions and results of pivaloylation of thymidine (**1a**) and uridine (**1b**)

Nucleoside	Product	Reaction conditions			Isolated yield, %
		Amine	PvCl, equiv.	Reaction time, min	
1a	T^{Pv} (**2**)	Py/TEA	1.1	15	70
1a	PvT (**3**)	Py	2.0	15	87
1a	PvT^{Pv} (**4**)	1.Py; 2.+TEA	2.2	10 + 10	74
1a	Pv_2T (**5**)	Py/DMAP	2.2	15	85
1b	U^{Pv} (**9**)	Py/TEA	1.1	15	61
1b	Pv_3U^{Pv} (**10**)	Py/DMAP	5	15	100
10	Pv_3U (**11**)[a]	–	–	15	98

[a] Selective deprotection of **10** (HCl/*i*-PrOH, 80 °C).

stoichiometric amount of PvCl (1.1 equiv.). Thus, the risk of multiacylation was avoided. In both cases, the reactions afforded PvT (**3**) (ca. 90%) accompanied by unreacted **1a** and bis-acylated Pv_2T (**5**) (ca. 5% each). After aqueous work-up, pure compound **3** was obtained by crystallization from toluene[37] (yield 85–87%).

Efficient one-pot preparation of 5′-*O*,*N*3-dipivaloylthymidine (**4**) required a combination of the methods for O- and N-acylation. To this end, thymidine in 1:1 MeCN–pyridine solution was treated with 2.2 equiv. of PvCl in the first step. After 10 min (time required for ca. 90% formation of 5′-*O*-pivaloylthymidine without bis-acylation), TEA (5 equiv.) was added, which promoted a rapid N3-pivaloylation to give PvT^{Pv} (**4**). Aqueous work-up (TEAB buffer pH 7.0), followed by lyophilization afforded **4** in 74% yield.

For the preparation of 3′,5′-*O*-dipivaloylthymidine (**5**), in order to secure rapid pivaloylation of the 3′-OH group (ca. 10 min), 2.5 equiv. of PvCl was used in the presence of DMAP (5 equiv.) as a nucleophilic and base catalyst. After aqueous work-up (5% citric acid to remove DMAP followed by TEAB buffer) Pv_2T (**5**) was lyophilized from benzene (yield 85%).

A special attention was paid to recognize whether pivaloylation at the thymine moiety took place in the N3 or O^4 site. While N3 is the typical acylation position in 4-oxopyrimidine group of nucleosides, benzoylation of thymidine derivatives in the presence of TEA led to a mixture of N3- and O^4-acylated products[1], which were used as reference compounds for TLC analysis on their pivaloylated analogues. In every case the pivaloylated derivatives showed TLC mobilities comparable to their N3-benzoylated congeners. For instance, the O^4- and N3-benzoylated derivatives of 3′,5′-*O*-bis(*tert*-butyldimethylsilyl)thymidine had the R_F values of 0.11 and 0.59, respectively (DCM–Et_2O 95:5), while the product of pivaloylation of the same compound had the R_F value of 0.62. Also T^{Pv} (**2**) and N^3-benzoylthymidine had similar R_F values, 0.31 and 0.29, respectively (DCM–MeOH 9:1).

In the ^{13}C NMR spectra the chemical shifts for the carbons C5 have been established to be of an analytical value for determination of N3 vs O^4 substitution in uridine derivatives[16]. Thus, acylation or alkylation in the N3 position was found to influence the chemical shift for C5 only marginally, while in O^4-substituted derivatives, ca. 4 ppm upfield shift was observed. The resonances for C6 carbons in O^4-esters were shifted ca. 6 ppm downfield, while those in N3-amides remained unaffected[16a]. Since pivaloylation of uracil and thymine moieties did not influence significantly the chemical shifts for C5 and C6 (**1b**, **9**, **10**: δ_{C5} ~103 ppm, δ_{C6} 138–140 ppm;

1a, **2**, **4**: δ_{C5} ~111 ppm, δ_{C6} ~136 ppm), the site of derivatization was inferred to be the nitrogen N3.

Regioisomers **2**, **3** and **4**, **5** could be clearly distinguished by TLC (*N*3-Pv derivatives show higher mobility than their *O*-Pv congeners). Their structures were also confirmed by ^{1}H and ^{13}C NMR spectroscopy[+++]. A clear-cut difference between ^{13}C chemical shifts of the pivaloyl carbonyl carbons in *N*-Pv (**2**, **4**, **9**, and **10**: δ_C ~178 ppm) and *O*-Pv (**3**, **4**, **5**, **10**, and **11**: δ_C ~184 ppm) groups was found in all analyzed compounds.

The above described conditions for selective pivaloylation could be adopted for acylation of partly protected thymidine derivatives. For example, 5′-*O*,*N*3-dipivaloylthymidine (**4**) could be prepared by 5′-O-pivaloylation of T^{Pv} (**2**) or by N3-pivaloylation of PvT (**3**) according to the procedures for pivaloylation of 5′-OH and N3H sites of thymidine. Pivaloylation of 5′-*O*-(dimethoxytrityl)thymidine in the presence of TEA yielded 5′-*O*-(dimethoxytrityl)-*N*3-pivaloylthymidine (**7**) in 87% yield after TEAB–DCM work-up and precipitation into cyclohexane. Compound **3** is a convenient substrate for the preparation of 3′-*O*-(dimethoxytrityl)thymidine (**8**) in a procedure analogous to that described by De Nino et al. who used 5′-*O*-(4-nitrobenzoyl)thymidine as starting compound[58] (see Experimental).

Stability of N3-Pivaloyl Group

Pivalate esters of thymidine are stable under moderately acidic (pH > 1)[40] and moderately basic conditions. They may be cleaved by treatment with solutions of ammonia, primary amines or tetrabutylammonium hydroxide, as well as alkali metal hydroxides[37,59]. *N*3-Pivaloylthymidine (**2**) showed a significantly different profile of stability. In contrast to pivalic esters, it underwent slow hydrolysis in neutral aqueous solutions; however, in standard aqueous work-up of the reaction mixture no detectable amounts of the depivaloylated products could be found. Hydrolysis of T^{Pv} in water at room temperature was complete within 7 days. The reaction rate was significantly enhanced at elevated temperature and a complete removal of the pivaloyl group from T^{Pv} (**2**) was accomplished within 10 min at 90 °C in water. The

+++ N3-Pivaloylated derivatives of 4-oxopyrimidine nucleosides showed unusual NMR patterns of ^{1}H and ^{13}C NMR signals, e.g. broadening of some signals and/or their splitting into pairs of signals, depending on temperature and the solvent used (Table IV). These suggested involvement of those T^{Pv} derivatives in some kind of equilibria, the nature of which is unknown at present.

dependence of the hydrolysis rate at 80 and 90 °C on pH of the solution was investigated in phosphate buffers in the pH range 2–12 (Figs 3 and 5b). In contrast to acidic and basic conditions, in neutral media the rate of hydrolysis was very low (several hours). The high rate of hydrolysis of T^{Pv} in water (in comparison with that in pH 6 buffer) may be plausibly attributed to acid autocatalysis since pH of an unbuffered solution decreased significantly in the course of the reaction due to released pivalic acid.

Interestingly, hydrolysis of T^{Pv} in 1:1 MeCN–water mixture required ca. 5 h for the completion (at 80 °C), in comparison with 0.5 h in water at the same temperature (Fig. 4), while in 95:5 MeCN–water no traces of hydrolysis could be observed even after several hours of reflux at 78 °C. The retardation effect was even stronger in 1:1 *i*-PrOH–water mixture (completion time ca. 10 h). The rate of hydrolysis of T^{Pv} was also significantly reduced in the presence of DMF and DMSO. It must be noted, however, that upon addition of a large volume of organic solvent to water, pH of the mixture increased by ca. 0.3 (for MeCN) and 1.2 (for *i*-PrOH), presumably as a result of lower solubility of CO_2. This phenomenon could account partly for the observed retardation of hydrolysis; nevertheless, the reaction rate was still low in spite of significant amounts of pivalic acid released into the medium during deacylation. Thus, hydrolysis of T^{Pv} was repeated in buffered mixtures; this revealed the pseudo-first order of the reaction (Fig. 5). The influence of acetonitrile on hydrolysis rates was higher in less acidic solutions

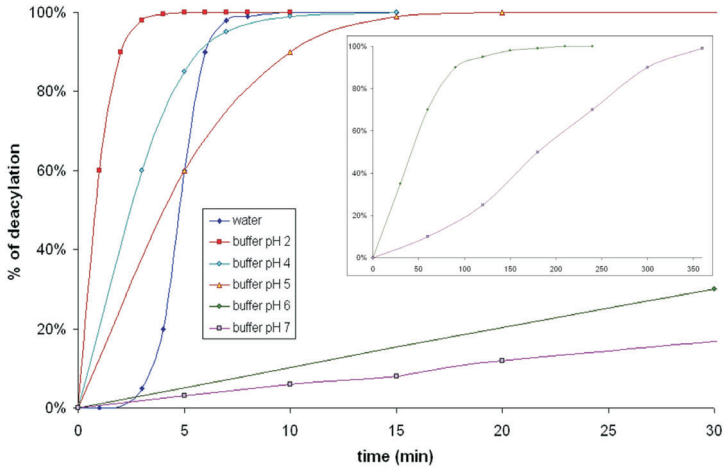

FIG. 3
Kinetics of deacylation of 0.01 M *N*3-pivaloylthymidine (**2**) in water (pH 6.0) and in pH 2–7 buffers at 90 °C

(Fig. 5b), and this was in good agreement with the significant rate decrease observed in nearly neutral (initially) MeCN–water mixtures (Fig. 4).

During hydrolysis of Pv_2T (**5**) under similar conditions, in water and in buffers (pH 2–5), the *N*3-Pv group was cleaved with full chemoselectivity, while under nearly neutral conditions an accompanying minor cleavage of

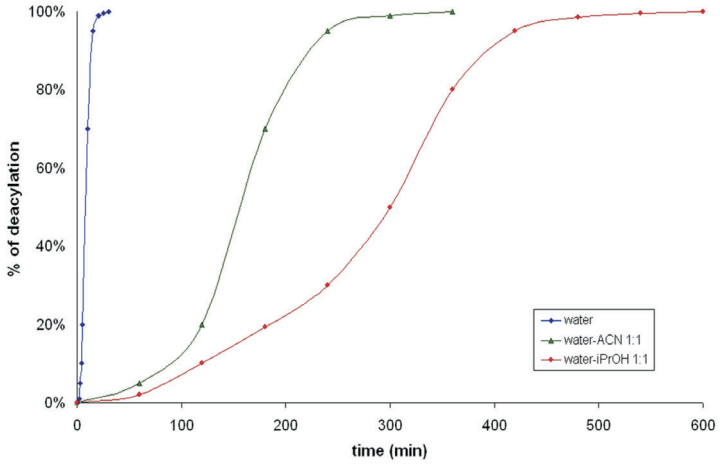

FIG. 4
Influence of MeCN and *i*-PrOH on the kinetics of deacylation of 0.01 M *N*3-pivaloylthymidine (**2**) at 80 °C

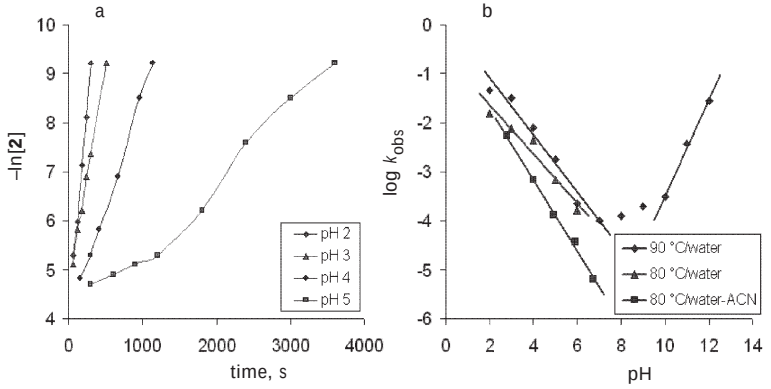

FIG. 5
Hydrolysis of 0.01 M T^{Pv} (**2**) in aqueous or aqueous–MeCN phosphate buffers (50 mmol l^{-1}) at 80 and 90 °C (±1 °C). a Plots of the hydrolysis progress at 80 °C. b Correlation of log k_{obs} vs pH for hydrolysis of T^{Pv} (**2**)

the 5′-*O*-Pv group was observed (the **3**/**2** ratio ca. 85:15). Under basic conditions (pH ≥ 10), hydrolysis of the 5′-*O*-Pv group was the dominant reaction.

Apart from thermal hydrolysis, the stability of *N*3-pivaloyl group under various acidic and basic conditions was estimated. The approximate half-life times of deacylation are given in Table III. In contrast to hydrolysis in buffers at elevated temperature, no significant differences between acid hydrolysis in aqueous and MeCN solutions were observed. However, in weakly polar solvents (DCM, chloroform), the acid-catalyzed depivaloylation of T^{Pv} was ca. twenty times faster than that in polar solvents (MeCN, H_2O). Under basic conditions the hydrolysis was ca. fifteen times faster in water than in MeOH, which indicates a significant solvent effect on cleavage of the N3–Pv bond. In the presence of a limited amount of water (a few per cent), the accompanying transacylation, mainly to PvT (**3**) was observed.

Dilute aqueous ammonia (0.5 mol l^{-1}) in MeCN cleaved the *N*3-pivaloyl group similarly to pure water ($t_{1/2} \approx 24$ h), while concentrated aqueous NH_3 required several hours to complete deacylation. The deprotection time in concentrated ammonia could be reduced to ca. 2 h by increasing the temperature to 55 °C.

In the case of *N*3-benzoyl protected thymidine and its analogues, the most frequently used deprotection reagent was sodium hydroxide in organic solvents[60–63]. Under such conditions rapid and efficient debenzoylation could be achieved. (*N*3-Benzoyl group could be also cleaved within 1 h in concentrated ammonia at room temperature[24] or by prolonged heating in benzyl alcohol (30 h/90 °C)[64]. However, it is stable in 80% AcOH and 2% TFA/$CHCl_3$ (ref.[24]).) For instance, Ramasamy and Stoisavljevic[63] reported 99% yield of deprotection using 1 M NaOH/MeOH/THF for 30 min. In contrast, T^{Pv} required ca. 5 h for deacylation using aqueous 0.5 M NaOH, while in methanolic solution the reaction was complete in ca. 24 h.

TABLE III
Half-times of acid- and base-catalyzed deacylation of 0.01 M *N*3-pivaloylthymidine (**2**) at room temperature

0.5 M TFA or HCl in MeCN or H_2O	0.5 M TFA in DCM or $CHCl_3$	80% AcOH	0.5 M NaOH in H_2O	0.5 M NaOH in MeOH	Aqueous 25% NH_3
10 h	0.5 h	10 h	(5 h)[a]	6 h	1 h

[a] Completion time.

The aforementioned resistance of T^{Pv} to methanolic NaOH solution opened a chance for using the *N*3-pivaloyl group as a protection orthogonal to base-labile groups present in other positions of the nucleoside, such as ester groups. To verify this possibility, PvT^{Pv} (**5**) was treated with 0.5 M NaOH (10 equiv.) in MeOH (room temperature) to cleave 5′-*O*-pivaloyl, or with boiling water or in a buffer solution (pH 3) at 90 °C to cleave *N*3-pivaloyl group (Fig. 6). Under basic conditions, depivaloylation was highly regioselective favouring the 5′-O deprotection, and required ca. 1 h for completion. (Under similar conditions, *N*3-benzoyl group is cleaved rapidly[60–63]. Regioselective cleavage of ester benzoyl groups from Bz_2T^{Bz} proceeds by treatment with 2.2 equiv. of NaOH overnight[64].) The main product of the reaction was the expected T^{Pv} (**2**), accompanied by ca. 3% of fully deprotected thymidine (**1a**), which could be easily washed out during work-up. Under acidic conditions (water or buffer), the N3 position was deprotected exclusively to form PvT (**3**) within 15–30 min. On the other hand, $dmtT^{Pv}$ could be selectively deprotected to T^{Pv} since under mild acidic conditions required for complete detritylation (80% acetic acid, room temperature, 30 min), the *N*3-pivaloyl group was cleaved only marginally (ca. 3%). Thus, the pivaloyl group can be considered a moderately acid-labile and base-labile protecting group for the N3 position of thymidine, which resists detritylation and de-esterification conditions, but can be cleaved rapidly in hot water (30 min) or in aqueous ammonia at 55 °C (2 h) or with 0.5 M TFA at room temperature (4 h).

Pivaloylated Derivatives of Uridine

The increased reactivity of the ribose hydroxy groups with electrophiles, the lack of the 5-methyl group, and different conformational preferences are the main features of uridine (**1b**) compared with thymidine (**1a**) that

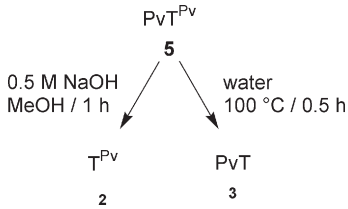

Fig. 6
Selective deprotection of Pv_2T (**5**) under basic or mildly acid conditions (80 °C/water)

may influence its proneness to acylation and stability of the protecting groups (Fig. 7, Table II).

Synthesis of *N*3-pivaloyluridine (U^{Pv}, **9**) proceeded smoothly under the same conditions as for T^{Pv}; however, due to higher solubility of U^{Pv} in water, multiple DCM extractions of the TEAB buffer phase were required in the work-up step. The product was obtained as white foam in 61% yield.

Attempted selective O-pivaloylation of uridine under conditions devised for preparation of PvT led, in contrast, to a mixture of several partly acylated products, similarly as it was observed by Kamaike et al.[65] Noteworthy, peracylation of uridine with an excess of PvCl and DMAP was found to be a rapid reaction (completion time 15 min). The intermediate product (Pv_3U^{Pv}, **10**) underwent selective N-depivaloylation on brief heating in 0.5 M HCl/*i*-PrOH (15 min). The produced 2′,3′,5′-*O*-tripivaloyluridine (Pv_3U, **11**) was isolated in 98% yield after lyophilization. This process is thus a cost- and time-effective alternative to the literature method for the

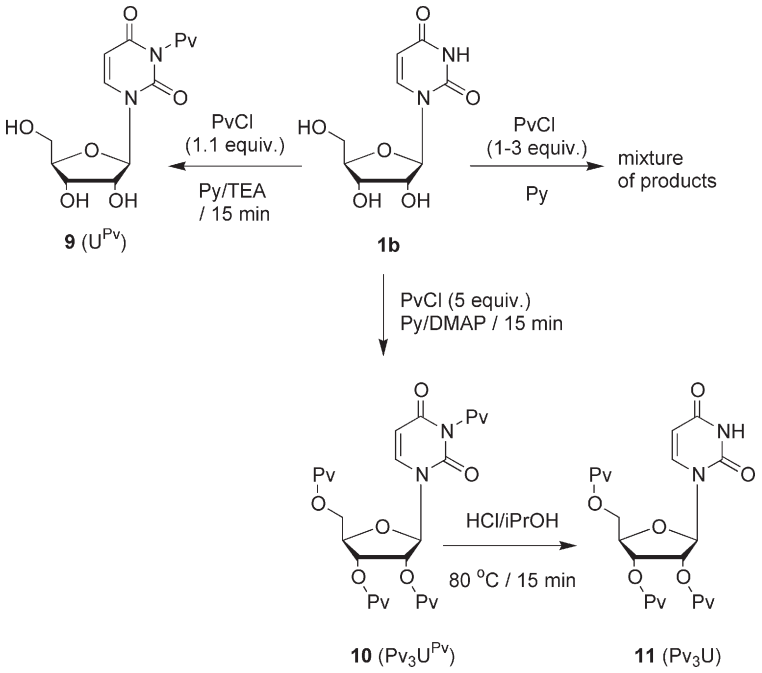

FIG. 7
Regioselective pivaloylation of uridine (**1b**)

preparation of **11** that requires 20 + 7 h reaction time for pivaloylation using the $Pv_2O/Bi(OTf)_3$ reagent system[57].

*N*3-Pivaloyl group in U^{Pv} (**9**) appeared to be significantly more labile than that of T^{Pv}; it was cleaved completely within several minutes in 80% AcOH at room temperature or in water at 90 °C. The half-time of deacylation of U^{Pv} at pH 2 and room temperature was ca. 6 h, while in 25% aqueous NH_3 at room temperature, 30 min (formation of some side-products was noted in the latter case, ca. 20%). In 0.5 M NaOH, the TLC spot of U^{Pv} disappeared within less than 15 min (H_2O) or 30 min (MeOH). However, no formation of uridine was observed. Interestingly, in perpivaloylated derivative, Pv_3U^{Pv} (**10**), the *N*-pivaloyl group was by orders of magnitude more resistant to acids than in U^{Pv} and required several hours in 1 M HCl/alcohol solution or more than 48 h in 80% AcOH for complete cleavage. The reaction could be significantly accelerated by heating, without affecting the regioselectivity of deacylation (*vide supra*). Concluding, the pivaloyl group in the N3 position of uridine seems to have limited synthetic applications as a transient protecting group.

Mechanism of Alkaline Hydrolysis of T^{Pv} (**2**)

The attempts to evaluate the kinetics of NaOH-promoted deacylation of T^{Pv} in aqueous solution by TLC monitoring its progress failed since the gradual disappearance of the spot of substrate **2** was not accompanied by simultaneous formation of any other UV-absorbing (254 nm) product in the first 20–30 min. After that period, the spot of thymidine (**1a**) started to appear in the TLC of the reaction mixture and, after ca. 5 h, the reaction was complete. Apart from depivaloylation, formation of some other products took place (ca. 10%). These side reactions were dominant when a sample of the reaction mixture from the early stages (5–60 min) was neutralized or acidified.

UV spectrophotometric quantification of the reaction progress (Fig. 8) revealed a gradual decrease in intensity of the peak at 272.5 nm that reached the lowest value after the 20–25 min reaction. After that time a new maximum started to rise at 269 nm until the end of reaction after ca. 5 h.

Temporary disappearance of a C6=C5–C4=O chromophore at ca. 270 nm was presumably caused by the addition of hydroxide ions to this system, presumably at C6. Thus, due to a low rate of depivaloylation under alkaline conditions (Fig. 9, path A), the formation of 6-hydroxy-*N*3-pivaloylthymidine (**9**, path B) could take place. Subsequent cleavage of the pivaloyl group was apparently associated with dehydration to thymidine (**1a**), as

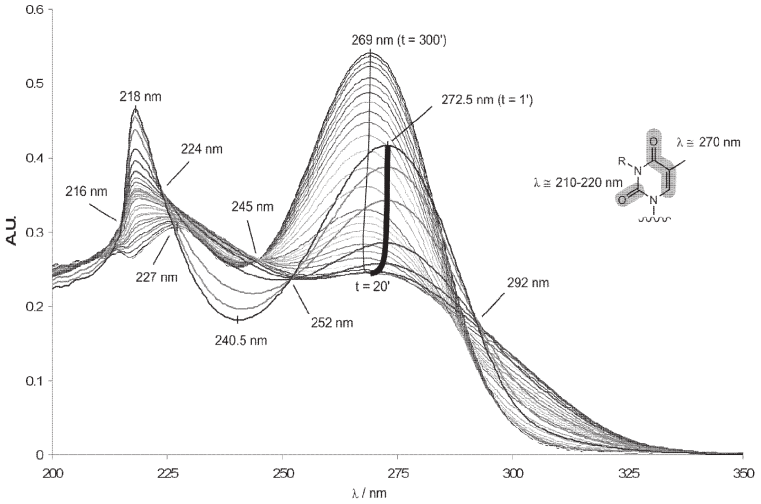

FIG. 8

UV spectra of the reaction mixture during hydrolysis of T^{Pv} (**2**) in aqueous 0.5 M NaOH. Reaction times: 1–20 min (thick lines); 20–300 min (thin lines). The chromophores in the thymine moiety are marked in grey

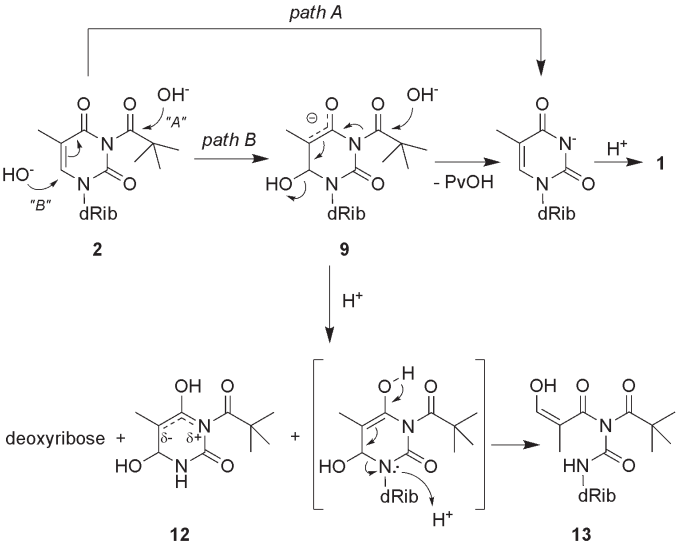

FIG. 9

Putative mechanism of depivaloylation and degradation of T^{Pv} (**2**). Stage 1 (upper row): T^{Pv} in 0.5 M NaOH. Stage 2 (lower row): degradation of intermediate **9** under neutral or acid conditions

evidenced by re-appearance of the peak at 270 nm (Fig. 8). Upon acidification (pH ≤ 7), compound **9** was unstable. TLC showed formation of several products, of which deoxyribose was readily identified, while two other isolated compounds, **12** and **13** (Fig. 9), were assigned tentatively on the basis of their TLC mobilities, and UV, $^{1}H/^{13}C$ NMR and MS spectra. It is worth noting that an open-chain form of hydrated thymine was postulated as an intermediate in cis–trans isomerization of 6-hydroxy-5,6-dihydrothymidine[66]. Further investigations on elucidation of the reactions of N3-acylated 4-oxopyrimidines under alkaline conditions are in progress.

CONCLUSIONS

Acylation of nucleosides is usually considered a time-demanding process. In contrast, in the present work it was found that under properly chosen reaction conditions the pivaloylation of 4-oxopyrimidines could be completed within several minutes. Moreover, the reactions with thymidine were highly regioselective and the site of acylation could be controlled by a proper choice of amine acting as a base and/or nucleophilic catalyst of the reaction. Thus, strongly basic but poorly nucleophilic triethylamine promoted acylation of the nucleobase moiety, while in the presence of nucleophilic catalysts (pyridine or DMAP) the reaction proceeded selectively in the sugar part. The pivaloylated products could be purified by organic-aqueous extractions; they were isolated in good yields.

The pivaloyl group could be cleaved from *N*3-pivaloylthymidine derivatives under acidic or basic conditions; however, it was sufficiently stable to withstand acid-promoted detritylation or treatment with alkali in organic solvents used for the removal of ester protecting groups. Thus, the pivaloyl group emerged as a convenient protecting group for the N3 position of thymidine.

N3-Pivaloylation of uridine could be achieved with a similar regioselectivity as thymidine, while the analogous selective O-pivaloylation was not successful. Nevertheless, a convenient procedure for the preparation of 2′,3′,5′-*O*-tripivaloyluridine was devised. *N*3-Pivaloyluridine was less stable than *N*3-pivaloylthymidine both under basic and acid conditions.

EXPERIMENTAL

NMR spectra (δ, ppm; *J*, Hz) were collected on Bruker Avance II 400 MHz and Varian Unity 300 MHz spectrometers. ^{1}H and ^{13}C NMR signal assignment was based on 2D correlation spectra. UV spectra were recorded on a Jasco V-650 spectrophotometer. ESI HR-MS spectra were recorded on a Bruker micrOTOF-Q instrument operated in positive ion mode.

Thymidine was purchased from Rasayan Inc. and uridine from Pharma–Waldhof. These and other commercial reagents and solvents (Sigma–Aldrich, Merck, and POCh–Poland) were used as purchased unless otherwise noted. Anhydrous solvents containing below 20 ppm of water (by Karl Fischer coulometric titration, Metrohm 684 KF) were stored over molecular sieves 4 Å. PvCl was distilled and used within one month. A 1 M triethylammonium hydrogencarbonate (TEAB) buffer was prepared by bubbling CO_2 through aqueous TEA at 0 °C until pH 7.0 was reached.

*N*3-(Trimethylacetyl)thymidine (T^{Pv}; **2**)

Thymidine (2.42 g, 10 mmol) was dissolved in 1:1 MeCN–pyridine mixture (100 ml) containing triethylamine (7 ml, 5 equiv.), and PvCl (1.4 ml, 1.1 equiv.) was added while stirring vigorously under N_2 atmosphere. After 15 min the reaction was quenched by addition of water and the solvents were evaporated under reduced pressure. Diethyl ether (200 ml) was added and the precipitate was filtered off, washed with 50 ml of ether and discarded. The ether solution was extracted with 1 M TEAB buffer (25 ml) and water (5 ml), and the combined aqueous phases were washed with 100 ml of ether and discarded. The organic fraction containing compound **2** was evaporated and the residue was partitioned between water (200 ml) and toluene (20 ml). The aqueous phase was evaporated to dryness under reduced pressure in a 30 °C bath, and the residue was dissolved in DCM. If TLC revealed some hydrolysis of T^{Pv} during the work-up, additional extraction with 200 ml DCM/10 ml water was applied. The DCM solution was evaporated (repeated twice) giving a white foam. Yield 2.3 g (70%). R_F 0.31 (DCM–MeOH 9:1). For ^{1}H NMR, see Table IV. ^{13}C NMR (75 MHz, DMSO-d_6): 184.03 (C=O, Pv); 162.19 (C4); 148.71 (C2); 136.77 (C6); 109.25 (C5); 87.58 (C1′); 84.43 (C4′); 70.21 (C3′); 61.14 (C5′); 43.29 ($\mathbf{C}(CH_3)_3$); 39.60 (C2′); 26.86 ($C(\mathbf{C}H_3)_3$); 12.07 (C^5). ^{13}C NMR (101 MHz, $CDCl_3$): 184.16 (C=O, Pv); 162.91 (C4); 149.21 (C2); 136.38 (C6, br); 110.82 (C5, br); 87.06 (C4′); 86.26 and 86.01 (C1′); 71.19 (C3′); 62.11 (C5′); 43.89 ($\mathbf{C}(CH_3)_3$); 40.35 and 39.89 (C2′); 27.22 ($C(\mathbf{C}H_3)_3$); 12.42 (C^5).

5′-*O*-(Trimethylacetyl)thymidine (PvT; **3**)

Thymidine (2.42 g, 10 mmol) was dissolved in 1:1 MeCN–pyridine mixture (100 ml) and PvCl (2.52 ml, 2.0 equiv.) was added while stirring vigorously. After 15 min the reaction was quenched with water and the solvents were evaporated under reduced pressure. The oily residue was dissolved in 200 ml of DCM and extracted successively with a 5% aqueous citric acid (50 ml)–1 M TEAB buffer (50 ml). DCM was evaporated and the resulting white foam was dissolved in toluene (250 ml, 90 °C). The solution was cooled to –20 °C yielding a white precipitate[37]. Yield 2.83 g (87%). R_F 0.39 (DCM–MeOH 9:1). For ^{1}H NMR, see Table IV. ^{13}C NMR (101 MHz, $CDCl_3$): 178.44 (C=O, Pv); 163.88 (C4); 150.52 (C2); 134.93 (C6); 111.29 (C5); 85.06 (C1′); 84.58 (C4′); 71.55 (C3′); 63.90 (C5′); 40.49 and 38.85 (C2′ and $\mathbf{C}(CH_3)_3$); 27.19 ($C(\mathbf{C}H_3)_3$); 12.49 (C^5).

*N*3,5′-*O*-Bis(trimethylacetyl)thymidine (PvT^{Pv}; **4**)

Thymidine (2.42 g, 10 mmol) was dissolved in a 1:1 MeCN–pyridine mixture (100 ml) and PvCl (2.77 ml, 2.2 equiv.) was added while stirring vigorously under N_2 atmosphere. After 10 min TEA (7.0 ml, 5.0 equiv.) was added. After another 10 min, the reaction was quenched with water and evaporated under reduced pressure. Diethyl ether (100 ml) was

TABLE IV

^{1}H NMR spectra for pivaloylated thymidine derivatives **2**–**5**

a Chemical shifts (in ppm). Doubled values of the chemical shifts for H6, H1′ and 3′-OH signals are due to their splitting into pairs of signals (see footnote on page 40). The methylene protons in positions 2′ and 5′ are magnetically non-equivalent and usually displayed resolved signals

Compound	N3H	5-Me	H6	1′	2′	3′	4′	5′	3′	5′-OH	Pv
T^{Pv} (**2**) (DMSO-d_6, 25° C)	–	1.83 d	7.87 d	6.14 t	2.14 br	4.26 br	3.79 q	3.56 dt 3.62 ddd	5.28 d	5.09 t	1.22 s
T^{Pv} (**2**) ($CDCl_3$, –30°)	–	1.89 s	7.54 s 7.73 s	6.15 t 6.18 t	2.24 br	4.45 br	3.94 br 3.97 br	3.8 m	4.21 br 4.25 br	3.60 br	1.28 s
T^{Pv} (**2**) (MeCH-d_3, 50 °C)	–	1.93 s	7.73 s	6.23 t	2.27 m	4.43 q	3.93 q	3.75 dd 3.81 dd	3.41 br	3.19 br	1.34 s
T^{Pv} (**2**) (MeCH-d_3, –25 °C)	–	1.94 s	7.85 d 7.88 d	6.25 t 6.26 t	2.26 m	4.42 br m	3.93 quintet	3.75 dq 3.80 dt	3.83 br	3.64 br	1.34 s
T^{Pv} (**2**) (D_2O, 25 °C)	–	1.86 s	7.69 s	6.19 t	2.29 br 2.35 br	4.45 br	4.02 q	3.75 dd 3.80 dd	nd	nd	1.24 s
PvT (**3**) ($CDCl_3$, 25 °C)	9.58 s	1.91 d	7.28 d	6.31 dd	2.07 ddd 2.49 ddd	4.38 br m	4.20 ddd	4.24 dd 4.41 dd	3.41 d	–	1.22 s
PvT^{Pv} (**4**) ($CDCl_3$, 25 °C)	8.70 s	1.93 d	7.27 d	6.28 dd	2.07 ddd 2.49 ddd	5.18 dt	4.20 overlapped dt	4.29 dd	–	–	1.22 s 1.23 s
Pv_2T (**5**) ($CDCl_3$, 25 °C)	–	1.90 d	7.27 d	6.21 br	2.05 br 2.39 br	4.35 m	4.14 br	4.27 dd 4.35 m	2.75 d	–	1.21 s 1.30 s

TABLE IV (*continued*)
b H-H coupling constants (in Hz)

Compound	$^4J_{Me\text{-}6}$	$^3J_{1'\text{-}2'a}$	$^3J_{1'\text{-}2'b}$	$^2J_{2'a\text{-}2'b}$	$^3J_{2'a\text{-}3'}$	$^3J_{2'b\text{-}3'}$	$^3J_{3'\text{-}4'}$	$^3J_{4'\text{-}5'a}$	$^3J_{4'\text{-}5'b}$	$^2J_{5'a\text{-}5'b}$	$^3J_{3'\text{-}OH}$	$^3J_{5'\text{-}OH}$
T^{Pv} (**2**) (DMSO-d_6, 25° C)	0.9	13.4 ($^3J_{1'\text{-}2'a}$+$^3J_{1'\text{-}2'b}$)		nd	nd	nd	≈3.5	≈3.5	3.8	11.9	4.3	5.3
T^{Pv} (**2**) ($CDCl_3$, –30°)	–	6.7	6.4	nd	nd	nd	nd	nd	nd	nd	nd	nd
T^{Pv} (**2**) (MeCH-d_3, 50 °C)	–	13.2 ($^3J_{1'\text{-}2'a}$+$^3J_{1'\text{-}2'b}$)		nd	9.6 ($^3J_{2'a\text{-}3'}$+$^3J_{2b'\text{-}3'}$)		≈3.5	3.7	3.2	11.9	nd	nd
T^{Pv} (**2**) (MeCH-d_3, –25 °C)	–	13.0 ($^3J_{1'\text{-}2'a}$+$^3J_{1'\text{-}2'b}$)		nd	nd	nd	nd	≈3.5	≈3.7	11.8	nd	nd
T^{Pv} (**2**) (D_2O, 25 °C)	–	13.0 ($^3J_{1'\text{-}2'a}$+$^3J_{1'\text{-}2'b}$)		nd	nd	nd	≈3.7	4.7	3.3	12.5	nd	nd
PvT (**3**) ($CDCl_3$, 25 °C)	0.9	7.7	5.9	13.7	6.6	2.9	~3.2	3.2	4.4	12.2	3.8	–
PvT^{Pv} (**4**) ($CDCl_3$, 25 °C)	1.2	8.9	5.3	14.1	6.6	1.4	9.4 ($^3J_{3'\text{-}4'}$ + $^3J_{4'\text{-}5'a}$ + $^3J_{4'\text{-}5'b}$)			nd	–	–
Pv_2T (**5**) ($CDCl_3$, 25 °C)	1.2	nd	nd	nd	–	–	–	3.0	nd	12.3	3.6	–

added and the precipitate was filtered off, washed with 50 ml of ether and discarded. The ether solution was extracted successively with 1 M TEAB buffer (150 ml) and water (150 ml), and evaporated giving a white foam. Occasional by-products causing yellow coloration of the product could be removed from a DCM solution using charcoal. The product was dissolved in warm 9:1 *n*-hexane–Et_2O mixture and kept at –20 °C overnight yielding white fibrous precipitate. Yield 3.1 g (74%). R_F 0.35 (DCM–MeOH 95:5). For ^{1}H NMR, see Table IV. ^{13}C NMR (101 MHz, $CDCl_3$): 183.82 and 183.54 (N-C=O, Pv); 178.33 (O-C=O, Pv); 162.70 (C4); 148.88 (C2); 134.88 and 134.52 (C6); 110.82 (C5); 85.43 and 85.12 (C1′); 84.44 (C4′); 71.21 (C3′); 63.75 (C5′); 43.80 (N-C(O)-**C**$(CH_3)_3$); 40.62 and 40.46 (C2′); 38.77 (O-C(O)-**C**$(CH_3)_3$); 27.17 (C(**C**$H_3)_3$); 27.11 (C(C**H**$_3)_3$); 12.39 (C^5).

3′,5′-*O*-Bis(trimethylacetyl)thymidine (Pv_2T; **5**)

Thymidine (2.42 g, 10 mmol) was dissolved in a 1:1 MeCN–pyridine mixture (100 ml) containing DMAP (6.1 g, 5 equiv.), and PvCl (2.77 ml, 2.2 equiv.) was added while stirring vigorously. After 15 min the reaction was quenched by addition of water and the solvents were evaporated under reduced pressure. Toluene (200 ml) was added, the precipitate was filtered off, washed with 50 ml of toluene and discarded. The solution was extracted successively with 5% citric acid (150 ml) and 1 M TEAB buffer (150 ml), and evaporated giving a white foam. The product was dissolved in hot *n*-hexane and kept at –20 °C overnight yielding a gel which was filtered off, washed with cold hexane and dried to white powder. Yield 3.50 g (85%). R_F 0.37 (DCM–MeOH 95:5). For ^{1}H NMR, see Table IV. ^{13}C NMR (101 MHz, $CDCl_3$): 178.05 (C=O, Pv); 177.88 (C=O, Pv); 163.36 (C4); 150.11 (C2); 134.34 (C6); 111.47 (C5); 84.75 (C1′); 82.58 (C4′); 73.92 (C3′); 63.85 (C5′); 38.84 (**C**$(CH_3)_3$); 38.64 (**C**$(CH_3)_3$); 37.74 (C2′); 27.23 (C(**C**$H_3)_3$); 26.97 (C(C**H**$_3)_3$); 12.48 (C^5).

3′-*O*-(Dimethoxytrityl)-5′-*O*-(trimethylacetyl)thymidine (**7**)

5′-*O*-Pivaloylthymidine (**3**; 1.63 g, 5 mmol) was dried by co-evaporation with pyridine (2 ×), and dissolved in the same solvent (25 ml). DMAP (61 mg, 0.5 mmol) and TEA (2.8 ml, 10 mmol) were added followed by dimethoxytrityl chloride (3.39 g, 10 mmol), and the reaction mixture was kept at room temperature for 48 h until the tritylation was complete (TLC). Methanol was added, and after typical work-up in a DCM–1 M TEAB buffer mixture, a yellow foam was obtained, containing 3′-*O*-(dimethoxytrityl)-5′-*O*-pivaloylthymidine (**7**) and fast migrating (TLC) products of decomposition of excess DMTrCl. This mixture was used for depivaloylation (see below) or was purified by silica gel column chromatography, 0–3% gradient of MeOH in DCM, and was lyophilized from benzene yielding a white amorphous solid. Yield 2.7 g (87%). R_F 0.45 (DCM–MeOH 95:5). ^{1}H NMR (300 MHz, $CDCl_3$): 9.54 s, 1 H (H6); 7.14–7.50 m, 9 H (Ar); 6.88 d, ^2J = 8.7, 4 H (Ar); 6.37 dd, $^3J_{1′\text{-}2′a}$ = 5.6, $^3J_{1′\text{-}2′b}$ = 8.4, 1 H (H1′); 4.40 dm, ^3J = 6.3, 1 H (H3′); 4.10 m, 1 H (H4′); 3.97 dd, $^2J_{5′a\text{-}5′b}$ = 12.3, $^3J_{4′\text{-}5′a}$ = 2.4, 1 H (H5′a); 3.90 dd, $^2J_{5′a\text{-}5′b}$ = 12.3, $^3J_{4′\text{-}5′b}$ = 4.5, 1 H (H5′b); 3.82 s, 6 H (OMe); 2.06 br dd, $^2J_{2′a\text{-}2′b}$ = 13.7, $^3J_{1′\text{-}2′a}$ = 5.6, 1 H (H2′a); 1.90 s, 3 H (5-Me); 1.54 ddd, $^2J_{2′a\text{-}2′b}$ = 13.7, $^3J_{1′\text{-}2′b}$ = 8.5, $^3J_{2′b\text{-}3′}$ = 6.6, 1 H (H2′b); 1.15 s, 9 H (Pv). ^{13}C NMR (101 MHz, $CDCl_3$): 177.67 (C=O, Pv); 163.83 (C4); 150.27 (C2); 134.67 (C6); 110.95 (C5); 84.80 (C1′); 83.47 (C4′); 73.56 (C3′); 63.75 (C5′); 39.47 and 38.58 (C2′ and **C**$(CH_3)_3$); 26.99 (C(C**H**$_3)_3$); 12.36 (C^5); 158.69, 144.76, 135.87, 130.05, 128.07, 127.95, 127.07, 113.28, 87.29, 55.13 (DMTr).

3′-*O*-(Dimethoxytrityl)thymidine (**8**)

Crude 3′-*O*-dimethoxytrityl-5′-*O*-pivaloylthymidine (**7**; 5 mmol) from the previous step was dissolved in methanol (5 ml). A 1 M methanolic NaOH (20 ml) was added and the reaction mixture was left standing at room temperature overnight. The work-up was done according to the procedure of De Nino et al.[58] After complete depivaloylation (TLC), water (100 ml) was added, and the solution extracted with diethyl ether (2 × 40 ml) and DCM (3 × 25 ml). DCM fractions were combined, evaporated, and the resulting oil was triturated in diethyl ether affording a fine white powder. Yield 2.3 g (85%). R_F 0.52 (1.08 relatively to 5′-*O*-DMTrT; DCM–MeOH 9:1). The spectral data were consistent with literature[58].

*N*3-(Trimethylacetyl)uridine (U^{Pv}; **9**)

Similar procedure as for T^{Pv} (**2**) was used with an exception that in the first stage of work-up, the TEAB buffer was extracted five times with a ten-fold volume of DCM (U^{Pv} is well soluble in water and poorly in Et_2O). Yield 2.0 g (61%). R_F 0.23 (DCM–MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): 8.07 d, $^3J_{5\text{-}6}$ = 8.1, 1 H (H6); 5.87 d, $^3J_{5\text{-}6}$ = 8.1, 1 H (H5); 5.77 d, $^3J_{1'\text{-}2'}$ = 4.5, 1 H (H1′); 5.49 br, 1 H (2′-OH); 5.15 br, 2 H (3′-OH, 5′-OH); 4.07 m, 1 H (H2′); 3.99 m, 1 H (H3′); 3.88 m, 1 H (H4′); 3.65 dd, $^2J_{5'\text{a-}5'\text{b}}$ = 12.3, $^3J_{4'\text{-}5'\text{a}}$ = 2.4, 1 H (H5′a); 3.57 dd, $^2J_{5'\text{a-}5'\text{b}}$ = 12.3, $^3J_{4'\text{-}5'\text{b}}$ = 2.5, 1 H (H5′b); 1.23 s, 9 H (Pv). ^{13}C NMR (101 MHz, DMSO-d_6): 184.01 br (C=O, Pv); 161.57 (C4); 149.08 (C2); 141.31 (C6); 101.36 (C5); 88.34 (C1′); 85.18 (C4′); 73.78 (C3′); 69.77 (C2′); 60.69 (C5′); 43.40 ($\mathbf{C}(CH_3)_3$); 26.86 ($C(\mathbf{C}H_3)_3$).

2′,3′,5′-*O*-Tris(trimethylacetyl)uridine (Pv_3U; **11**)

Uridine (**1b**; 2.44 g, 10 mmol) was dissolved in a MeCN–pyridine mixture (1:1, 100 ml) containing DMAP (8.5 g, 7 equiv.), and PvCl (6.3 ml, 5 equiv.) was added while stirring vigorously. After 15 min the reaction was quenched by addition of water and the solvents were evaporated under reduced pressure. Toluene (200 ml) was added and the precipitate was filtered off, washed with 50 ml of toluene and discarded. The solution was extracted successively with 5% citric acid (150 ml) and 1 M TEAB buffer (150 ml), and evaporated to obtain a white foam of Pv_3U^{Pv} (**10**; quantitative yield). R_F 0.76 (DCM–MeOH 95:5). ^{1}H NMR (300 MHz, $CDCl_3$): 7.44 br, 1 H (H6); 6.07 br, 1 H (H1′); 5.77 d, $^3J_{5\text{-}6}$ = 8.1, 1 H (H5); 5.26 br, 2 H (H2′ and H3′); 4.41 br d, $^2J_{5'\text{a-}5'\text{b}}$ = 11.4, 1 H (H5′a); 4.31 br, 1 H (H4′); 4.25 br d, $^2J_{5'\text{a-}5'\text{b}}$ = 11.4, 1 H (H5′b); 1.16, 1.22, 1.23, 1.29, 4 × s, 4 × 9 H (4 × Pv). ^{13}C NMR (101 MHz, $CDCl_3$): 182.7 br (N-C=O, Pv); 177.07 (O-C=O, Pv); 161.51 (C4); 148.92 (C2); 138.3 br (C6); 103.02 (C5); 86.4 br (C1′); 80.97 (C4′); 72.8 br (C3′); 70.5 br (C2′); 63.41 (C5′); 38.76 and 38.79 ($\mathbf{C}(CH_3)_3$); 26.91, 27.00, 27.17 ($C(\mathbf{C}H_3)_3$).

Compound **10** obtained in the previous step was dissolved in *i*-PrOH (110 ml), 11 M HCl was added (2 ml) and the mixture was kept in an 80 °C bath for 15 min. After that time TLC showed complete removal of *N*-pivaloyl group. After evaporation of the solvent, work-up in the DCM–1 M TEAB buffer mixture and lyophilization from benzene, pure product **10** was obtained as a white amorphous solid. Yield 2.44 g (98%). R_F 0.34 (DCM–MeOH 95:5). ^{1}H NMR (300 MHz, $CDCl_3$): 7.42 d, $^3J_{5\text{-}6}$ = 8.1, 1 H (H6); 6.09 d, $^3J_{1'\text{-}2'}$ = 5.7, 1 H (H1′); 5.75 d, $^3J_{5\text{-}6}$ = 8.1, 1 H (H5); 5.26 br, 2 H (H2′ and H3′); 4.41 dd, $^2J_{5'\text{a-}5'\text{b}}$ = 11.2, $^3J_{4'\text{-}5'\text{a}}$ = 2.4, 1 H (H5′a); 4.28 m, 1 H (H4′); 4.27 dd, $^2J_{5'\text{a-}5'\text{b}}$ = 11.2, $^3J_{4'\text{-}5'\text{b}}$ = 1.7, 1 H (H5′b); 1.16, 1.21, 1.25, 3 × s, 3 × 9 H (3 × Pv). ^{13}C NMR (101 MHz, $CDCl_3$): 177.03, 177.09, 177.79 (C=O, Pv);

163.02 (C4); 150.10 (C2); 138.86 (C6); 103.21 (C5); 86.48 (C1′); 80.67 (C4′); 72.76 (C3′); 70.20 (C2′); 63.29 (C5′); 38.75, 38.76, 38.79 ($C(CH_3)_3$); 26.92, 27.00 and 27.15 ($C(CH_3)_3$).

Typical Procedure for 5′-O-Depivaloylation

*N*3,5′-*O*-Dipivaloylthymidine (**4**; 2.05 g, 5 mmol) was dissolved in 0.5 M methanolic NaOH (100 ml) and the reaction mixture was kept at room temperature until TLC showed complete deprotection (ca. 1 h). The reaction mixture was cooled to 0 °C and neutralized with 0.5 M aqueous HCl (90 ml), concentrated under reduced pressure to ca. 20 ml and extracted with DCM (3 × 200 ml). The organic phase was dried with anhydrous Na_2SO_4 and evaporated under reduced pressure yielding compound **2** as a white foam. Yield 1.40 g (86%).

Typical Procedure for N3-Depivaloylation

*N*3,5′-*O*-Dipivaloylthymidine (**4**; 2.05 g, 5 mmol) was suspended in water (50 ml) and refluxed until TLC showed complete deprotection of the N3 position (ca. 15 min). The reaction mixture was cooled to 0 °C, neutralized with 1 M NaOH (5 ml) and worked-up as above. Yield of PvT (**3**) 1.47 g (90%).

Kinetics of Acylation of Thymidine at 5′-OH and Thymine Moiety

3′-*O*-(Dimethoxytrityl)-5′-*O*-pivaloylthymidine (**7**) and *N*3,3′-*O*-dibenzoylthymidine were used for determination of the rates of acylation of N3H and 5′-OH groups of thymidine, respectively. Nucleoside derivative (0.1 mmol) was dried by co-evaporation with toluene, dissolved in MeCN or 1:1 MeCN–pyridine (1 ml) containing an appropriate amount of amine (TEA, pyridine, DMAP or their mixtures, usually 5 equiv.) and acyl chloride (3 equiv.) was added. During reactions 50-μl samples were transferred to 10% aqueous MeCN (500 μl) and analyzed by TLC after completion of the reaction. In selected experiments the samples were analyzed also immediately after sampling. The $t_{1/2}$ was assigned when the spots due to the substrate and the product were of the same intensity.

Kinetics of Hydrolysis

A 0.02 M aqueous solution of acylated thymidine derivative was mixed with the same volume of a 0.1 M phosphate buffer or another investigated solution and the mixture was kept at room temperature or in water bath at 80 or 90 °C (±1 °C). During the reaction 10-μl samples were taken, diluted with 100 μl of acetonitrile and kept at 0 or at –20 °C if the reaction was continued for a longer time than 4 h. After completion of hydrolysis, 10-μl samples were applied to a TLC plate (silica gel F_{254}), developed and analyzed visually by comparing relative intensities of substrate/product spots at various stages of the reaction under a UV lamp. Each experiment was performed three times and a mean value was used for calculations. For buffers at pH > 4 a sigmoid deviation from linearity was observed in the latter stages of the reaction (> ca. 80% of conversion). In those cases the initial linear data points were taken for calculations of k_{obs} (Fig. 5a).

Base Hydrolysis of *N*3-(Trimethylacetyl)thymidine (T^{Pv}; **2**)

*N*3-(Trimethylacetyl)thymidine (**2**; 33 mg, 0.1 mmol) was dissolved in 0.5 M NaOH and the reaction progress was followed by TLC and UV spectroscopy (200–400 nm). To this end,

aliquots of the reaction mixture were analyzed either in 0.5 M NaOH solution or in 0.1 M phosphate buffers of pH 2, 4, 6, 7, and 8 (for TLC, 50 µl aliquot in 1 ml of a buffer; for UV, 5 µl aliquot in 2.5 ml of a buffer).

4,6-Dihydroxy-5-methyl-1-trimethylacetyl-3,4-dihydropyrimidin-2(1*H*)-one (**12**)

*N*3-(Trimethylacetyl)thymidine (**2**; 660 mg, 2.0 mmol) was dissolved in 0.5 M NaOH (40 ml). After 20 min 5 M HCl was added to the reaction mixture until pH ~ 2 was reached. Within several minutes white fine crystals started to precipitate. After 15 min the crystals were filtered off, washed twice with water and dried under vacuum. Yield 30%. R_F 0.51 (DCM–MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-d_6): 12.53 br s, 1 H (NH, exchange with water); 11.96 d, ^3J = 11.2, 1 H (O^{6}H, exchange with water); 10.32 s, ^{1}H (O^{4}H, exchange with water); 7.34 d, ^3J = 11.2, 1 H (H6); 1.78 s, 3 H (5-Me); 1.18 s, 9 H (Pv). ^{13}C NMR (101 MHz, DMSO-d_6): 179.80 (C=O, Pv); 168.76 (C4); 151.37 (C2); 132.83 (C6); 105.41 (C5); ~39 (**C**(CH$_3$)$_3$, overlapped with DMSO); 26.22 (C(**C**H$_3$)$_3$); 16.61 (C^5). ESI MS(+), *m/z*: 251.0987 [M + Na]$^+$.

N-[(β-D-2-Deoxyribosyl)carbamoyl]-3-hydroxy-2-methyl-*N*-(trimethylacetyl)acrylamide (**13**)

*N*3-(Trimethylacetyl)thymidine (**2**; 660 mg, 2.0 mmol) was dissolved in 0.5 M NaOH (40 ml). After 20 min Dowex 50W (H$^+$ form) was added to the reaction mixture until pH ~ 6 was reached. The resin was filtered off, washed twice with water and the solvent was evaporated to dryness. Compound **13** was isolated by column chromatography (silica gel, 0–30% MeOH in DCM). Yield 44%. R_F 0.23 (DCM–MeOH 7:3). ^{1}H NMR (300 MHz, D$_2$O): 6.14 t, $^3J_{1'\text{-}2'}$ = 7.1, 1 H (H1′); 5.79 d, $^4J_{6\text{-}5Me}$ = 1.7, 1 H (H6); 4.26 dt, $^3J_{3'\text{-}4'}$ = 6.0, $^3J_{2'\text{-}3'}$ = 3.8, 1 H (H3′); 3.84 m, 1 H (H4′); 3.67 dd, $^2J_{5'a\text{-}5'b}$ = 12.6, $^3J_{4'\text{-}5'a}$ = 3.9, 1 H (H5′a); 3.54 dd, $^2J_{5'a\text{-}5'b}$ = 12.6, $^3J_{4'\text{-}5'b}$ = 5.7, 1 H (H5′b); 1.96 d, $^4J_{6\text{-}5Me}$ = 1.7, 3 H (5-Me); 1.19 s, 9 H (Pv). ^{13}C NMR (101 MHz, D$_2$O): 181.25 (C=O, Pv); 175.72 (C4); 154.25 (C4); 144.72 (C2); 116.95 (C5); 84.92 and 85.23 (C1′ and C4′); 70.47 (C3′); 61.44 (C5′); 40.00 (C2′); 35.75 (**C**(CH$_3$)$_3$); 25.94 (C(**C**H$_3$)$_3$); 16.88 (C^5). ESI MS(+), *m/z*: 367.1414 [M + Na]$^+$.

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