# 2′-Deoxyuridine and 2′-Deoxyisocytidine as Constituents of DNA with Parallel Chain Orientation: The Stabilization of the $iC_d \cdot G_d$ Base Pair by the 5-Methyl Group

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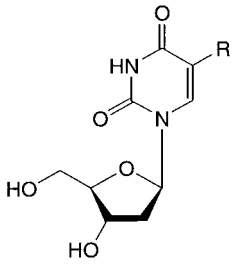
Dedicated to Prof. *Albert Eschenmoser* on the occasion of his 75th birthday

Parallel-stranded oligonucleotides containing 2′-deoxyuridine (**2**) and 2′-deoxyisocytidine (**4**) were synthesized. The phosphoramidite **11** employed in the solid-phase synthesis carries a (dimethylamino)methylidene residue as amino-protecting group. This group stabilizes the acid-labile glycosylic bond of **4** and enables the base-catalyzed deprotection of oligonucleotides without degrading the nucleoside **4** residues. Oligonucleotide duplexes incorporating the 5-Me derivatives of **2** (→2′-deoxythymidine) and **4** (→2′-deoxy-5-methylisocytidine), which are more stable than those containing the unmethylated nucleosides, were also compared. Depending on the nearest-neighbor environment, Me groups provide an additional stabilization through Me/Me contacts or Me/backbone interactions.

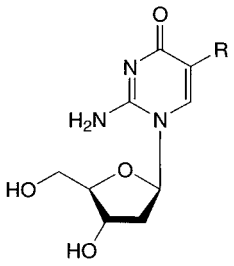
**Introduction.** – The Me group plays an important role in nucleic-acid structure and function [1][2]. The thymine Me group present in DNA stabilizes the duplex structure [3]. Also a certain percentage of the cytosine bases of bacteria, plants, and animals that control gene activity are methylated [4][5]. In both cases, the Me group is connected to C(5) of the pyrimidine bases. Another common methylation site is the 6-amino group of the adenine base. This prevents palindromic DNA segments from the phosphodiester hydrolysis by endodeoxyribonucleases [6]. All of those Me groups are directed into the major groove of the B-DNA helix.

In a series of publications, it was shown that any naturally occurring single-stranded DNA can be hybridized to duplexes with parallel chain orientation (ps) when one strand contains the four nucleosides 2′-deoxythymidine (**1**), 2′-deoxyadenosine, 2′-deoxy-5-methylisocytidine ($m^5iC_d$; **3**), and 2′-deoxyisoguanosine ($iG_d$; **5**) [7–9]. The 7-deaza-2′-deoxyisoguanosine (**6**) has been used to stabilize the glycosylic bond of **5** [10]. As it was observed that ps-oligonucleotide duplexes are generally less stable than their antiparallel-stranded (aps) counterparts [11], base-modified nucleosides were incorporated for duplex stabilization [12]. Among those stabilizing elements, the 5-Me groups of $T_d$ and $m^5iC_d$ play a particular role in ps- and aps-DNA that have focused our attention.

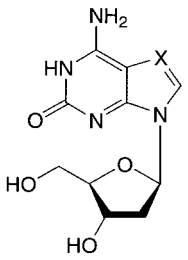
Earlier, the properties of ps-DNA incorporating $A_d \cdot T_d$, $iG_d \cdot C_d$ or $m^5iC \cdot G_d$ base pairs have been studied [8]. It was also shown that $A_d \cdot U_d$ base pairs of ps-DNA are less stable than those of $A_d \cdot T_d$ [13][14]. Nevertheless, this study was limited to duplexes containing bidentate pairs but not to those incorporating the tridentate $iG_d \cdot C_d$ or $G_d \cdot iC_d$ motif. Unfortunately, 2′-deoxyisocytidine ($iC_d$; **4**) is labile in alkaline and acidic



**1** R = $CH_3$
**2** R = H



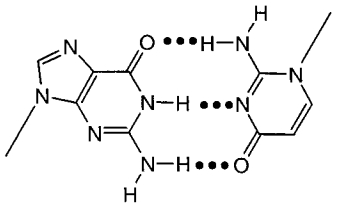
**3** R = $CH_3$
**4** R = H



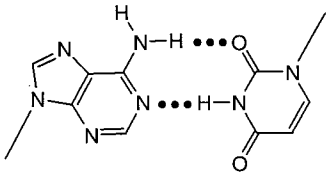
**5** X = N
**6** X = CH

solution and less stable than 2′-deoxyisoguanosine [15]. Consequently, this nucleoside was replaced earlier by its ribonucleoside [15][16] or its 5-Me derivative **3** [17][18]. However, due to the changes of the structure from **4** to **3**, the synthetic ps-DNA contains now more Me groups than the naturally occurring aps-DNA.

According to the model building [11][19][20] and the NMR spectra [21], the ps-DNA does not form two grooves of different size. Thus, the Me groups of thymine and 5-methylisocytosine are distributed in a uniform way within the grooves following the particular sequence pattern of the oligonucleotide chains. In the present contribution, we report on the influences of the thymine and 5-methylisocytosine Me groups on the stability of ps-duplexes and compare these data with those of oligonucleotide duplexes incorporating $U_d$ (**2**) instead of $T_d$ (**1**), and $iC_d$ (**4**) instead of $m^5iC_d$ (**3**). The ps base-pair motifs are shown by **A** and **B**. The $iC_d$ phosphoramidite is synthesized which allows the single or multiple incorporation of compound **4** in any position of an oligonucleotide chain, and that is degraded neither during the solid-phase synthesis nor during the alkaline deprotection of the oligonucleotide.



**A** $G_d \cdot iC_d$ (ps)

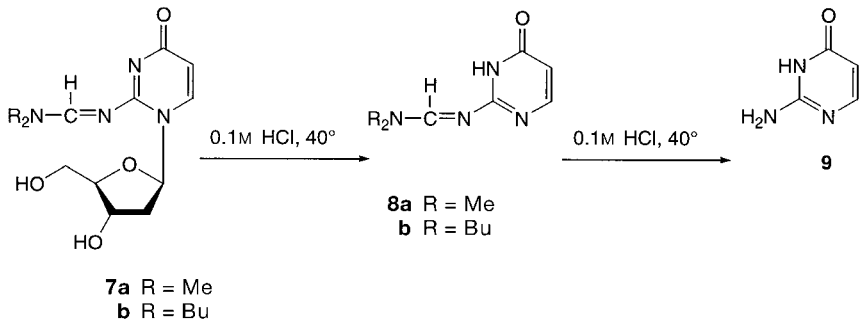


**B** $A_d \cdot U_d$ (ps)

**Results and Discussion.** – 1. *Monomers.* Phosphoramidite building blocks of the base-modified nucleosides **3** [8], **4** [22], and **5** [23] have already been described. The pyrimidine nucleosides **3** and **4** used in this study were synthesized from 2′-deoxythymidine and 2′-deoxyuridine, respectively, by treatment of their 2,5′-anhydro derivatives [24] with ammonia [22]. Amidine residues were used to protect the amino group of compound **3** [8], while the nucleoside **4** was protected with a benzoyl residue [22]. As the removal of the benzoyl group of **4** resulted in a partial degradation of the nucleoside [25], it was decided to use amino-protecting groups, which can be removed

under mild conditions. Accordingly, the protecting groups, of the other oligonucleotide constituents had to be protected in a similar way. The (dimethylamino)methylidene (dmf) group and the (dibutylamino)methylidene (dbf) group were selected for this purpose. The dmf group has been already used for the protection of the amino group of 2′-*O*-methylisocytidine [26]. In the case of **4**, treatment of a methanolic suspension with dimethylformamide dimethyl acetal or dibutylformamide dimethyl acetal furnished the amidines **7a**,**b** (see *Scheme 1*) in 90 and 82% yield, respectively.

*Scheme 1*




**8a** R = Me
**b** R = Bu

**7a** R = Me
**b** R = Bu

Next, the stability of the amidines **7a**,**b** was investigated with regard to their stability under acidic conditions ($(MeO_2)$Tr-group removal). Their hydrolysis was performed in 0.1M HCl (*Scheme 1*) and was compared with that of nucleoside **4**. The reaction was followed by HPLC and/or UV-spectrophotometrically. According to *Fig. 1*,*a*, **7a** was partially hydrolyzed to the protected base **8a** within 15 min in 0.1M HCl at 40°. When the same conditions were applied to nucleoside **4**, it was almost completely hydrolyzed to its base **9** within 5 min (*Fig. 1*,*d*). Thus, the dmf group stabilizes the *N*-glycosylic bond. When the reaction time was increased to 90 min (*Fig. 1*,*b*), the protected nucleoside **7a** was nearly consumed. A new faster-migrating peak appeared and was identified as isocytosine (**9**). Another slower-migrating peak disappeared upon prolonged treatment. As the glycosylic-bond hydrolysis is faster than the cleavage of the protecting group, the educt of the base **9** was the protected base **8a** and not the deprotected nucleoside **4** (*Fig. 1*,*b*). The same experiment as described for **7a** was performed with **7b**. This compound is much more stable in acidic medium as indicated by the higher amount of starting material present after 90 min of hydrolysis (*Fig. 1*,*c*).

To determine the half-life values of the glycosylic-bond hydrolyses, the reaction of **7a**,**b** was followed UV-spectrophotometrically at 285 nm. This wavelength allows the measurement of the glycosylic-bond hydrolysis separately from base deprotection by following the increase in UV absorbance. At shorter wavelengths, the UV spectra of the glycosylic-bond cleavage and base deprotection are overlapping, resulting in an overall absorbance decrease, which makes a separate measurement impossible. The hydrolysis of **7a**,**b** was governed by a two-step kinetics: in both cases, the glycosylic bond was cleaved first (absorption increase), then the amidine group was split off from the protected bases **8a**,**b** (absorption decrease). The UV data confirmed the HPLC

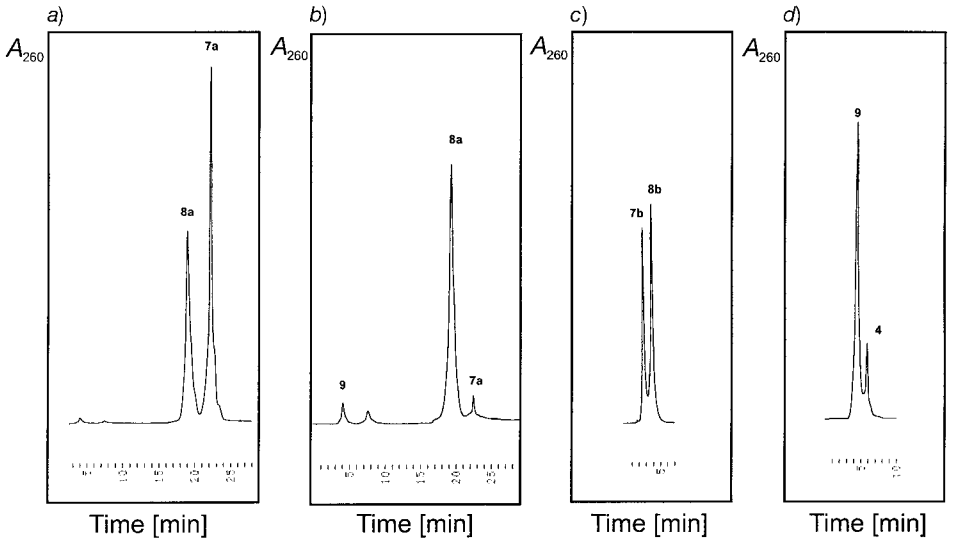



Fig. 1. *HPLC Profiles of the hydrolysis products of* **7a**, **7b**, *and* **4** *in 0.1*M *HCl at 40°* (samples were taken at intervals of times): *a*) *hydrolysis of* **7a** *for 15 min and b*) *hydrolysis of* **7a** *for 90 min* (gradient: 5 min 2% MeCN (*A*) in 0.1M ($Et_3NH$)OAc, pH 7.0 (*B*); 5 min 2–5% *A* in *B*, 5 min 5–10% *A* in *B*, 15 min 10% *A* in *B*, 1 ml/min), *c*) *hydrolysis of* **7b** *for 90 min* (gradient: 40% *A* in *B*, 1 ml/min), *and* d) *hydrolysis of* **4** *for 5 min* (gradient: 2% *A* in *B*, 1.0 ml/min)

experiments and, furthermore, established the higher stability of the dbf residue of **7b** compared to the dmf protecting group of **7a**. A linear dependence was obtained for the **7a**,**b** hydrolysis when log $(A_{max} - A_t)$ measured at 285 nm was plotted *vs.* the reaction time; for compound **4**, the log $(A_t - A_\infty)$ at 235 nm was used (*Fig. 2*). Thus, the hydrolyses followed pseudo-first order kinetics. The different initial UV absorptions resulted from similar but not identical concentration of the starting material used for the measurements. The half-lives of glycosylic-bond hydrolysis were found to be 16 min for amidine **7a** and 45 min for **7b** (0.1M aq. HCl, 40°). The nucleoside **4** showed a much shorter half-life (3.5 min). From this point of view, the (dibutylamino)methylidene-protected **7b** was favored over that of **7a**. However, compound **7b** shows drawbacks when stored in alkaline solution.

Nucleoside **4** is not only acid-labile but is also degraded in alkaline medium. Experiments performed in 25% aq. ammonia solution at 60° showed that 2′-deoxyuridine was formed on the monomeric and the oligonucleotide level [22]. In our hands, the ammonia hydrolysis of **4** gave more than one reaction product during the 16-h treatment (*Fig. 3*,*a*). Apart from the deamination (→2′-deoxyuridine), also a depyrimidination (→isocytosine (**9**)) took place. It is very likely that this reaction is initiated by the attack of $OH^-$ ions at position 6 of the base of **4** (1,4-addition), which is supported by the significantly lower reactivity of the 5-Me derivative **3** [8][27]. The pyrimidine ring of the 1,4-addition intermediate is then opened, releases the sugar moiety, and the recyclization gives then isocytosine. A Me group at position 5 such as in **3** decreases the electrophilic character of C(6) by the $+I$ effect and protects the molecule sterically.

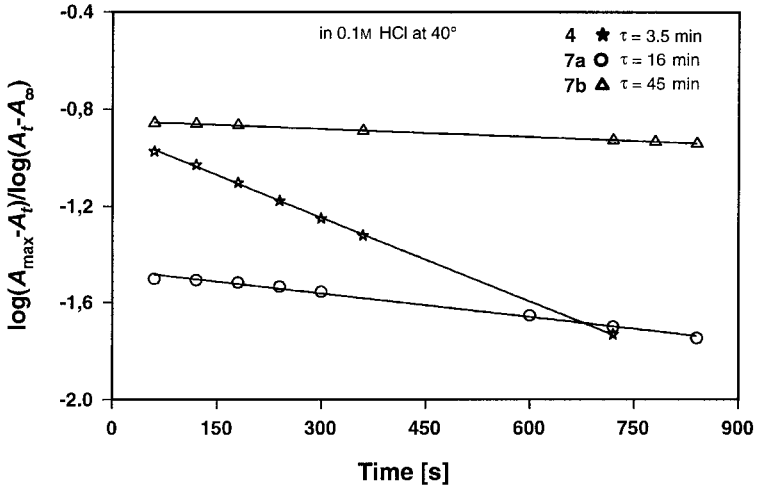



Fig. 2. *Hydrolysis of the nucleosides* **4**, **7a**, *and* **7b** *in 0.1*M *HCl at 40°*. The reaction was followed UV-spectrophotometrically at 235 nm for **4** and at 285 nm for **7a** and **7b**.

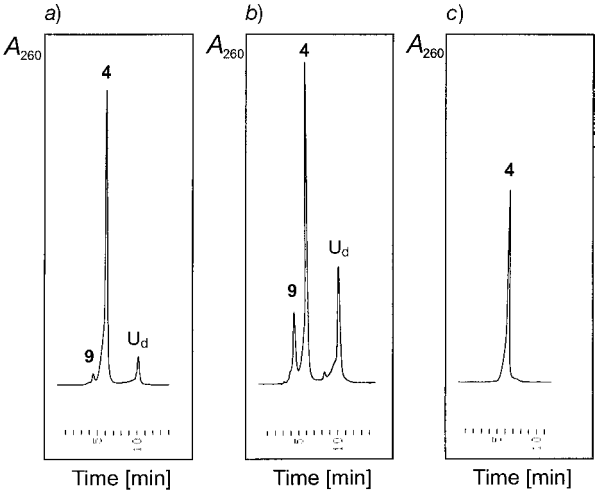



Fig. 3. *HPLC Profiles of* **4** *in 25% aq. $NH_3$ solution after* a) *16 h at 60°*, b) *72 h at 60°*, *and* c) *16 h at 25°* (gradient: 2% MeCN in 0.1M $(Et_3NH)OAc$, pH 7.0, 1.0 ml/min)

When the incubation time of compound **4** was increased to 72 h, the relative peak areas of isocytosine (**9**) and 2′-deoxyuridine increased (*Fig. 3,b*). Also the deamination reaction was faster than in the case of 2′-deoxycytidine. However, when nucleoside **4** was stored in conc. ammonia at room temperature, it was stable for 16 h (*Fig. 3,c*). Also no significant degradation occurred on storage for 40 h under these conditions.

Next, the alkaline hydrolysis of **7a**,**b** was investigated (*Fig. 4*). It has been reported that **7a** undergoes significant depyrimidination at 55° within 1 h [15]. However, according to *Fig. 4*,*d*, even after 16 h at 60° only very little isocytosine (**9**) was formed from **7a**. After 40 h, the by-products **9** and 2′-deoxyuridine became the main components. But the exposure of **7a** to ammonia at room temperature led neither to deamination nor to depyrimidination within 16 h. After a short treatment (5 min) of **7a**, the deprotected nucleoside **4** was formed, together with two intermediates (*Fig. 4*,*a*). These intermediates disappeared after extended ammonia treatment. Similar intermediates were also formed when the base **8a** was treated with conc. ammonia for 90 min at room temperature (*Fig. 4*,*b*). These intermediates disappear after 16 h, and the deprotected base was the final product. According to results obtained with other formamidine-protected nucleosides [28], one intermediate is expected to be the formyl derivative, while the other one might be the $H_2O$ adduct formed in advance. On the contrary, no intermediates were formed during the alkaline hydrolysis of the butylamidine **7b**. As the deprotection of **7b** was much slower than that of **7a**, isocytosine (**9**) and 2′-deoxyuridine were formed before the amidine residue was totally removed (see *Fig. 4*,*c*). The half-lives of deprotection of **7a** and **7b** (25% aq. $NH_3$ solution, room temperature) were UV-spectrophotometrically determined to be 4.5 and 130 min, respectively. Based on these observations, the formamidine group was the residue of choice for further manipulations.

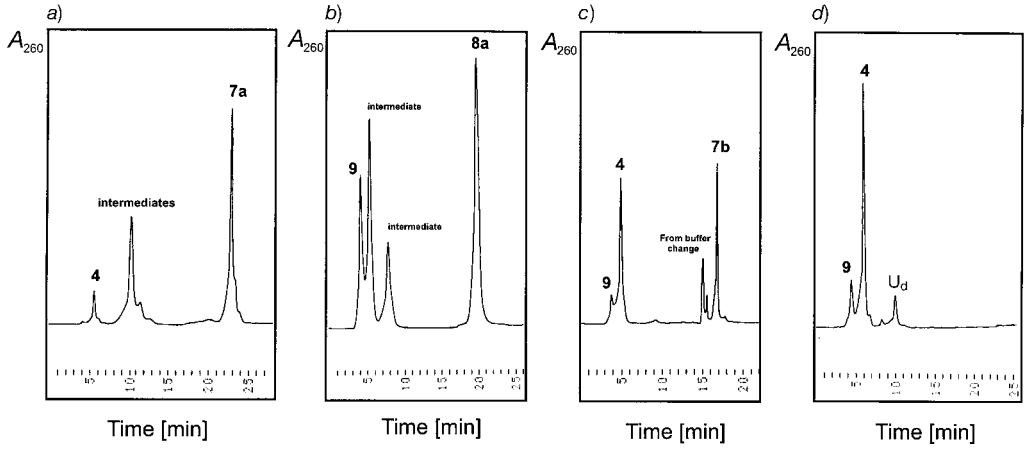



Fig. 4. *HPLC Profiles of* **7a**, **7b**, *and* **8a** *in 25% aq. $NH_3$:* a) **7a** *at room temperature after 5 min* (gradient: 5 min 2% MeCN (*A*) in 0.1M ($Et_3NH$)OAc, pH 7.0 (*B*), 5 min 2–5% *A* in *B*, 5 min 5–10% *A* in *B*, 15 min 10% *A* in *B*, 1 ml/min), b) **8a** *at room temperature after 90 min* (gradient, see *a*)), c) **7b** *at 25° after 16 h* (gradient: 10 min 2% *A* in *B*, 15 min 60% *A* in *B*, 1 ml/min), *and* d) **7a** *at 60° after 16 h* (gradient, see *a*))

Compound **7a** was then converted to the 4,4′-dimethoxytrityl ($(MeO)_2Tr$) derivative **10** (74%) by means of the standard protocol (*Scheme 2*). The phosphoramidite **11** was obtained in 75% yield after chromatographic workup. All monomeric compounds were characterized by $^{13}C$- and $^1H$-NMR spectra as well as by elemental analyses (see *Table 1* and *Exper. Part*).

*Scheme 2*

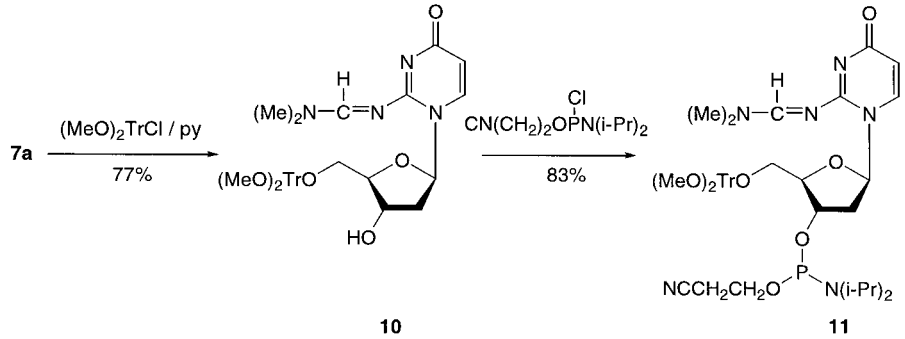



Table 1. *$^{13}$C-NMR Chemical Shifts of 2′-Deoxyisocytidine and Derivatives in ($D_6$)DMSO at 25°*

| | C(2) | C(4) | C(5) | C(6) | C=N | Me | C(1′) | C(2′) | C(3′) | C(4′) | C(5′) | MeO |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **4** | 154.2 | 169.7 | 106.7 | 138.1 | | | 87.6 | [a]) | 69.8 | 87.4 | 60.7 | |
| **7a** | 157.4 | 170.0 | 108.2 | 137.8 | 158.2 | 34.8 | 85.9 | [a]) | 70.2 | 87.5 | 61.2 | |
| **7b** | 157.3 | 170.0 | 108.2 | 137.7 | 157.7 | 13.5, 13.6 | 86.1 | 40.9 | 70.0 | 87.5 | 61.1 | |
| **10** | 157.3 | 169.9 | 108.1 | 137.6 | 158.1 | 34.9 | 85.8 | [a]) | 69.8 | 85.5 | 63.2 | 55.0 |
| **8a** | 160.1[b]) | 163.4[b]) | 107.1 | 155.0[b]) | 158.5 | 34.6 | | | | | | |
| **8b** | 160.2[b]) | 163.3[b]) | 107.1 | 154.9[b]) | 158.1 | 13.5, 13.6 | | | | | | |

[a]) Superimposed by ($D_6$)DMSO. [b]) Tentative.

2. *Oligonucleotides.* 2.1. *General Strategy.* To evaluate the influence of the 5-Me group of $T_d$ (**1**) and of $m^5iC_d$ (**3**) on the stability of parallel duplex DNA with those containing $U_d$ (**2**) or $iC_d$ (**4**) residues, a series of oligonucleotides were synthesized. Usually, the oligonucleotide duplex 5′-d(AGTATTGACCTA)-3′ and its complement 5′-d(TAGGTCAATACT)-3′ have been used in our laboratory as an internal standard to evaluate the influence of a base modification on the duplex stability [12]. When the oligomer 5′-d(AGTATTGACCTA)-3′ was hybridized with the complementary strand 5′-d(T**3**ATAA**3**T**55**AT)-3′, a parallel duplex was formed as shown by pyrene excimer fluorescence [8]. This duplex was also considered to be our reference in this study. However, based on the low stability of $iC_d$ (**4**) in alkaline medium and the comparably harsh deblocking conditions required for the removal of the $iG_d$ (**5**) dpc group, it was necessary to use a base-deprotection protocol, which can be performed in conc. aqueous ammonia at room temperature. Thus, the sequences of the duplex 5′-d(AGTATTGACCTA)-3′ · 5′-d(T**3**ATAA**3**T**55**AT)-3′ were changed. Moreover, the protocol of a universal-base strategy requires that the $U_d$ residue at the 3′-end should not be present in the strand containing compound **4**. Thus, the 2 $iG_d$ (**5**) residues were replaced by $m^5iC_d$ (**3**) residues, and the d(AT) segment of the 3′-terminus was reversed to d(TA). This produced the sequence 5′-d(T**3**ATAA**3**T**33**TA)-3′ (**12**) and its demethylated counterpart 5′-d(T**4**ATAA**4**T**44**TA)-3′ (**13**). The complementary strand was then 5′-d(AGTATTGAGGAT)-3′ (**14**) for ps duplex and 5′-d(TA**55**A**5**TTAT**5**A)-3′ (**15**) for the duplex with aps orientation. According to this, the $iC_d$-containing oligonucleotide can be kept intact during mild deprotection without being subjected to

deamination or depyrimidination. This strategy brings additional benefits. First, the strands **12** and **13** now contain 4 $m^5iC_d$ and 4 $iC_d$ residues, respectively, rather than only two; hence the difference between the strands with or without 5-Me group is enlarged. Second, only the pyrimidine-rich strand contains the modified pyrimidine bases, $U_d$ replacing $T_d$ and/or $iC_d$ replacing $m^5iC_d$. Therefore, the outcome is due only to the modification of the pyrimidine bases and not to other factors such as the replacement of $G_d$ by $iG_d$.

2.2. *Synthesis.* The oligonucleotide synthesis was performed on an *ABI 392-08* synthesizer by means of phosphoramidite chemistry and a protocol described earlier [23]. The phosphoramidites of $m^5iC_d$, $iC_d$, $U_d$, and $iG_d$ were used. After the synthesis, the oligonucleotides **12**, **14**–**16**, and **19** were deprotected in conc. aqueous ammonia solution at 60° for 16 h (for formulae, see *Table 2*). The oligonucleotides **13** and **20** containing $iC_d$ (**4**) residues were deprotected in conc. aqueous ammonia solution at room temperature for 16 h. Accordingly, the $A_d$-residues in **13** and **20** were protected with the labile pac (phenoxyacetyl) group, which can be removed at room temperature. The oligonucleotides **17** and **18**, which contain $U_d$ at the 3′-terminus, were synthesized on the universal support *500* (*Glen Research*, US). Deprotection in conc. ammonia solution at 60° for 72 h removed the protecting groups together with the overhanging sugar residue. The 5′-$(MeO)_2$Tr oligonucleotides were purified by HPLC (see *Exper. Part*), and the $(MeO)_2$Tr groups were removed with 2.5% $CHCl_2COOH/CH_2Cl_2$ for 5 min at room temperature. The detritylated oligomers were purified by HPLC, desalted by reversed-phase HPLC, and lyophilized (see *Exper. Part*). The homogeneity of the oligonucleotides was established by both ion-exchange chromatography and reversed-phase HPLC, as well as by MALDI-TOF spectrometry. All calculated masses were in good agreement with the experimentally determined values (see *Table 4* in the *Exper. Part*). This indicates that both the pac group of $A_d$ and the universal sugar residue were removed successfully from the oligonucleotides. However, it is worth mentioning that, in the case of oligonucleotides containing $m^5iC_d$ (**3**) or $iC_d$ (**4**), a fragmentation pattern of peaks was obtained during the MALDI-TOF measurement. The masses of lost fragments were 108 in the case of oligonucleotide **12**, and 94 in the case of oligonucleotide **20**, in accordance with the loss of the nucleobase of $m^5iC_d$ and $iC_d$ residues, respectively. Thus, the acidic matrix conditions (3-hydroxypicolinic acid) further evidenced the acid sensitivity of $m^5iC_d$ and $iC_d$. To find a more stable analogue is a topic of a study currently under investigation. The base composition of oligonucleotides (see *Table 5* in the *Exper. Part*) was also determined by enzymatic hydrolysis with snake-venom phosphodiesterase followed by alkaline phosphatase and reversed-phase HPLC analysis. In the case of **12**, **13**, **19**, and **20**, no deamination product ($U_d$ and $T_d$) was found.

2.3. *Duplex Stability.* It is generally accepted that the Me group of $T_d$ stabilizes duplex DNA with antiparallel-strand orientation [3]. A similar effect has been observed when 2′-deoxycytosine is methylated at the 5-position [29]. A closer look into this matter (*Table 2*) shows that the situation is more complex. When the number of Me groups of the 12-mers is increased, their contribution to the stability of the aps duplexes depends on the particular sequence of the oligonucleotide (*Table 3*). There are two sets of $\Delta\Delta G^\circ_{298}$ terms that can be calculated from the increasing number of Me groups. If the number of Me groups is increased from 4 in the duplex **14**·**17** to 8 in the duplex **14**·**16**, the $\Delta\Delta G^\circ_{298}$ per Me group is 0.30 kcal/mol (see *Table 3*). The same value is obtained

Table 2. $T_m$ *Values and Thermal Data of Antiparallel and Parallel Hybrids with or without 5-Methyl Groups in the Pyrimidine Bases*[a])

| Duplex | Base | Me[b]) | $T_m$ [°] | $\Delta H^\circ$ [kcal/mol] | $\Delta S^\circ$ [cal/mol K] | $\Delta G^\circ_{298}$ [kcal/mol] |
|---|---|---|---|---|---|---|
| 5′-d(AGTATTGAGGAT)-3′ **14** | 4 $T_d$ | 8 | 50 | – 99 | – 280 | – 11.7 |
| 3′-d(TCATAACTCCTA)-5′ **16** | 4 $T_d$ | | | | | |
| 5′-d(AGTATTGAGGAT)-3′ **14** | 4 $T_d$ | 4 | 47 | – 86 | – 243 | – 10.5 |
| 3′-d(UCAUAACUCCUA)-5′ **17** | 4 $U_d$ | | | | | |
| 5′-d(AGUAUUGAGGAU)-3′ **18** | 4 $U_d$ | 4 | 47 | – 83 | – 235 | – 10.5 |
| 3′-d(TCATAACTCCTA)-5′ **16** | 4 $T_d$ | | | | | |
| 5′-d(AGUAUUGAGGAU)-3′ **18** | 4 $U_d$ | 0 | 45 | – 83 | – 236 | – 9.9 |
| 3′-d(UCAUAACUCCUA)-5′ **17** | 4 $U_d$ | | | | | |
| 5′-d(TA**5** **5**A**5**TTAT**5**A)-3′ **15** | 4 $T_d$ | 12 | 61 | – 104 | – 286 | – 15.6 |
| 3′-d(AT**3** **3**T**3**AATA**3**T)-5′ **12** | 4 $T_d$, 4 **3** | | | | | |
| 5′-d(TA**5** **5**A**5**TTAT**5**A)-3′ **15** | 4 $T_d$ | 8 | 57 | – 88 | – 241 | – 13.2 |
| 3′-d(AT**4** **4**T**4**AATA**4**T)-5′ **13** | 4 $T_d$, 4 **4** | | | | | |
| 5′-d(AGTATTGAGGAT)-3′ **14** | 4 $T_d$ | 12 | 46 | – 100 | – 287 | – 10.3 |
| 5′-d(T**3**ATAA**3**T**3** **3**TA)-3′ **12** | 4 $T_d$, 4 **3** | | | | | |
| 5′-d(AGTATTGAGGAT)-3′ **14** | 4 $T_d$ | 8 | 42 | – 77 | – 219 | – 9.0 |
| 5′-d(T**4**ATAA**4**T**4** 4TA)-3′ **13** | 4 $T_d$, 4 **4** | | | | | |
| 5′-d(AGTATTGAGGAT)-3′ **14** | 4 $T_d$ | 8 | 42 | – 90 | – 259 | – 9.3 |
| 5′-d(U**3**AUAA**3**U**3** 3UA)-3′ **19** | 4 $U_d$, 4 **3** | | | | | |
| 5′-d(AGTATTGAGGAT)-3′ **14** | 4 $T_d$ | 4 | 39 | – 75 | – 216 | – 8.3 |
| 5′-d(U**4**AUAA**4**U**4** 4UA)-3′ **20** | 4 $U_d$, 4 **4** | | | | | |
| 5′-d(AGUAUUGAGGAU)-3′ **18** | 4 $U_d$ | 8 | 43 | – 90 | – 260 | – 9.5 |
| 5′-d(T**3**ATAA**3**T**3** 3TA)-3′ **12** | 4 $T_d$, 4 **3** | | | | | |
| 5′-d(AGUAUUGAGGAU)-3′ **18** | 4 $U_d$ | 4 | 38 | – 70 | – 199 | – 8.1 |
| 5′-d(T**4**ATAA**4**T**4** 4TA)-3′ **13** | 4 $T_d$, 4 **4** | | | | | |
| 5′-d(AGUAUUGAGGAU)-3′ **18** | 4 $U_d$ | 4 | 38 | – 79 | – 228 | – 8.3 |
| 5′-d(U**3**AUAA**3**U**3** 3UA)-3′ **19** | 4 $U_d$, 4 **3** | | | | | |
| 5′-d(AGUAUUGAGGAU)-3′ **18** | 4 $U_d$ | 0 | 36 | – 78 | – 228 | – 7.6 |
| 5′-d(U**4**AUAA**4**U**4** 4UA)-3′ **20** | 4 $U_d$, 4 **4** | | | | | |

[a]) Measured UV-spectrophotometrically at 260 nm in 1M NaCl, 100 mM $MgCl_2$, and 60 mM Na-cacodylate (pH 7.0); oligonucleotide concentration: 5 μM + 5 μM. [b]) Total number of Me-substituted bases.

for the duplex **18** · **16** in comparison to **14** · **16**. However, when the increase of the same number of Me groups occurs in duplex **18** · **17** compared to **18** · **16**, the $\Delta\Delta G^\circ_{298}$ value is only 0.15 kcal/mol per Me group. This low value is also observed for the duplex **18** · **17** in comparison to **14** · **17** ($\Delta\Delta G^\circ_{298} = 0.15$ kcal/mol per Me group). According to earlier reports [30], the consecutive arrangement of $T_d$ residues in one strand will contribute more to the stability than a dispersed arrangement of $T_d$ residues of the homopurine · homopyrimidine duplexes. According to our data, a Me group of the opposite strand can also increase the stability of the duplex, as much as the consecutive residues do. These data are in accordance with findings obtained from an X-ray structure of a duplex containing $m^5C_d$ [31]. In this case, the two Me groups of the opposite strands form a clamp, trapping the incoming phosphate in a groove-backbone interaction. This new geometry allows the formation of two new C–H ··· O H-bonds with the Me groups as donor and the O-atoms of a phosphate as acceptor, further stabilizing the interaction. The increase in stability afforded by the Me groups in the case of the duplex

**15·13** compared to **15·12** is even stronger. This more efficient stabilization can be explained by *van der Waals* contact of the Me group from either **3** or $T_d$, which are in the row and form a consecutive spine of Me groups compared to the 'isolated' Me groups, which contribute less to the duplex stability.

Table 3. $\Delta T_m$ *and* $\Delta\Delta G^\circ_{298}$ *Values per Methyl Group of aps and ps Duplexes*[a])

| Composition | Antiparallel | | Composition | Parallel | |
|---|---|---|---|---|---|
| | $\Delta T_m$ [°] | $\Delta\Delta G^\circ_{298}$ [kcal/mol] | | $\Delta T_m$ [°] | $\Delta\Delta G^\circ_{298}$ [kcal/mol] |
| $U_d$; $U_d$(**18·17**) → $T_d$; $U_d$ (**14·17**) | 0.50 | 0.15 | $U_d$; $T_d$, **3** (**18·12**) → $T_d$; $T_d$, **3** (**14·12**) | 0.75 | 0.20 |
| $U_d$; $U_d$(**18·17**) → $U_d$, $T_d$ (**18·16**) | 0.50 | 0.15 | $U_d$; $U_d$, **3** (**18·19**) → $T_d$; $U_d$, **3**(**14·19**) | 1.0 | 0.25 |
| $U_d$; $T_d$(**18·16**) → $T_d$; $T_d$ (**14·16**) | 0.75 | 0.30 | $U_d$; $T_d$, **4**(**18·13**) → $T_d$; $T_d$, **4**(**14·13**) | 1.0 | 0.23 |
| $T_d$; $U_d$ (**14·17**) → $T_d$; $T_d$ (**14·16**) | 0.75 | 0.30 | $U_d$; $U_d$, **4**(**18·20**) → $T_d$; $U_d$, **4** (**14·20**) | 0.75 | 0.18 |
| $U_d$; $U_d$(**18·17**) → $T_d$; $T_d$ (**14·16**) | 0.76 | 0.23 | $T_d$; $U_d$, **3** (**14·19**) → $T_d$; $T_d$, **3** (**14·12**) | 1.0 | 0.25 |
| $T_d$; **4**(**15·13**) → $T_d$; **3** (**15·12**) | 1.0 | 0.60 | $U_d$; $U_d$, **3** (**18·19**) → $U_d$; $T_d$, **3** (**18·12**) | 1.3 | 0.30 |
| | | | $T_d$; $U_d$, **4** (**14·20**) → $T_d$; $T_d$, **4** (**14·13**) | 0.75 | 0.17 |
| | | | $U_d$; $U_d$, **4**(**18·20**) → $U_d$; $T_d$, **4** (**18·13**) | 0.50 | 0.13 |
| | | | $T_d$; $T_d$, **4**(**14·13**) → $T_d$; $T_d$, **3** (**14·12**) | 1.0 | 0.33 |
| | | | $U_d$; $T_d$, **4** (**18·13**) → $U_d$; $T_d$, **3** (**18·12**) | 1.3 | 0.35 |
| | | | $T_d$; $U_d$, **4** (**14·20**) → $T_d$; $U_d$, **3** (**14·19**) | 0.75 | 0.25 |
| | | | $U_d$; $U_d$, **4**(**18·20**) → $U_d$; $U_d$, **3** (**18·19**) | 0.50 | 0.18 |

[a]) The $T_m$ and $\Delta G^\circ_{298}$ data are taken from *Table 2*.

In the case of parallel duplexes, the overall trend is the same as in the aps duplexes. The substitution of $iC_d$ (**4**) by $m^5iC_d$ (**3**) leads to an increase of the $T_m$ value of the corresponding duplexes, as the substitution of $U_d$ by $T_d$ does (*Table 2*). The contribution of isolated Me groups is different from those arranged in a row. This can be clearly seen from the comparison of the duplexes containing $U_d$ or $T_d$ such as **18·19** with **18·12** which results in a $\Delta\Delta G^\circ_{298} = 0.30$ kcal/mol per Me group (*Table 3*). In this case, there are 5 Me contacts between of $T_d$ **3**. On the other hand, the duplexes **18·20** and **18·13** lead to a $\Delta\Delta G^\circ_{298}$ value of only 0.13 kcal/mol per Me group. In this case, there are no Me contacts. A similar observation can be made for other sets of duplexes such as **14·19** *vs.* **14·12** and **14·20** *vs.* **14·13** *etc.* (*Table 3*). Similar effects also exist between $iC_d$ (**4**) and $m^5iC_d$ (**3**), see duplexes **18·13** *vs.* **18·12** ($\Delta\Delta G^\circ_{298} = 0.35$ kcal/mol per Me group) and **18·20** *vs.* **18·19** in which $\Delta\Delta G^\circ_{298} = 0.18$ kcal/mol per Me residue. Our data found for parallel duplexes fit to those of antiparallel hybrids. A consecutive arrangement of Me groups within one strand will lead to a larger stabilization than of those being separated from each other in the DNA duplex.

The CD spectrum of the aps duplex **14·16** containing $T_d \cdot A_d$ and $m^5iC_d \cdot G_d$ base pairs is rather different from that of the ps hybrid **14·12** as shown in *Fig. 5,a*. When the Me groups were removed and $U_d \cdot A_d$ and $iG_d \cdot G_d$ base pairs are present, the corresponding spectra are similar. *Fig. 5,b* shows that the aps hybrid **15·12** containing the unusual base pairs $m^5iC_d \cdot iG_d$ has a CD spectrum different form the normal one, and removing of the group Me (see **15·13**) does not change the spectrum a lot.

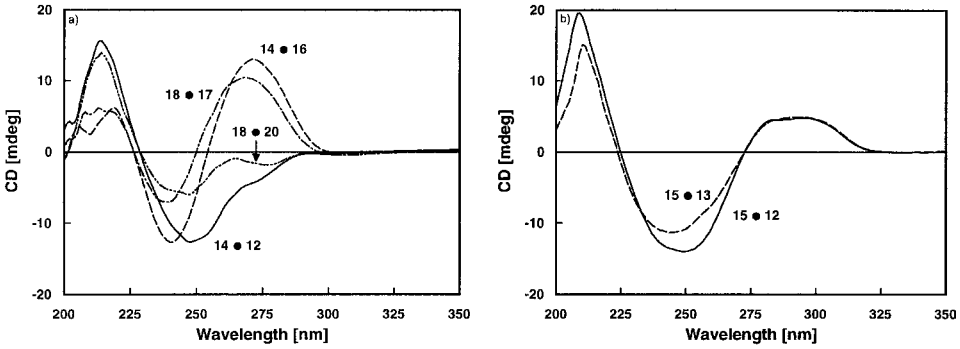



Fig. 5. *CD Spectra of oligonucleotide duplexes:* a) *aps hybrids* **14** · **16** *and* **18** · **17** *and ps hybrids* **14** · **12** *and* **18** · **20** *with or without 5-Me groups and* b) *aps hybrids containing* $iC_d \cdot iG_d$ *and* $m^5iC_d \cdot iG_d$ *base pairs*, i.e. **15** · **13** and **15** · **12**. Conditions, see *Exper. Part*.

**Conclusion.** – Oligonucleotides containing multiple incorporations of 2′-deoxyisocytidine (**4**) residues can be synthesized when the 2-amino group of **4** is protected with a (dimethylamino)methylidene residue and mild conditions (conc. ammonia, room temperature) are used for the base deprotection. The Me group of 2′-deoxy-5-methylisocytidine (**3**) stabilizes the glycosylic bond of the otherwise labile 2′-deoxyisocytidine (**4**). Parallel-stranded duplexes containing 5-methylisocytosine · guanine base pairs are more stable than those containing isocytosine · guanine pairs. The contribution of the pyrimidine 5-Me groups of ps duplexes is similar to that of their aps counterparts. However, the magnitude of the contribution of a Me group depends on the local environment both in the ps and aps cases. When arranged in a row in the same strand, Me groups of ps-DNA will give further stabilization to the duplexes, most probably by Me contacts as it was reported for aps-DNA. The presence of Me groups in the opposite strand will also lead to increased stability compared with that of the isolated Me groups.

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## Experimental Part

*General*. See [9]. The solid-phase synthesis of oligonucleotides was carried out on an automated DNA synthesizer (*Applied Biosystems*, model *ABI 392-08*, Germany) for phosphoramidite chemistry. Snake-venom phosphodiesterase (EC 3.1.4.1, *Crotallus durissus*) and alkaline phosphatase (EC 3.1.3.1, *E. coli*) were generous gifts from *Roche Diagnostics GmbH*, Germany. All reagents were commercially available and used as received. The solvents were purified and dried according to the standard procedures. Thin-layer chromatography (TLC): aluminum sheets silica gel *60* $F_{254}$ (0.2 mm, *Merck*, Germany). Flash chromatography (FC): at 0.4 bar on silica gel *60 H* (*Merck*, Germany). Reversed-phase HPLC: 4 × 250 mm *RP-18* (10 μm)-*LiChrosorb* column (*Merck*, Germany) with a *Merck-Hitachi* HPLC pump (model *655 A-12*), a variable-wavelength monitor (model *655-A*), a controller (model *L-5000*), and an integrator (model *D-2000*). UV Spectra: *U-3200* spectrometer (*Hitachi*, Japan); $\lambda_{max}$ in nm, $\varepsilon$ in $M^{-1}\,cm^{-1}$; half-life values ($\tau$) were measured with a *U-3000* spectrometer (*Hitachi*, Japan), connected to a temp. controller (*Lauda*, Germany). Melting curves: *Cary-1E* UV/VIS spectrophotometer (*Varian*, Australia) equipped with a thermoelectrical controller. Evaluation of thermodynamic data from the melting curves according to a two-state model was performed with the program MeltWin

(version 3.1). CD Spectra: measured from 200 to 350 nm; 1-cm cuvettes, *Jasco-600* (*Jasco*, Japan) spectropolarimeter connected with a temp. controller and a *Lauda-RCS-6* bath (*Lauda*, Germany); oligonucleotide soln. in 1M NaCl, 100 mM $MgCl_2$, 10 mM Na-cacodylate (pH 7.0), with the single-strand concentration of 5.0 μM. NMR Spectra: *Avance DPX*-250 and *AMX-500* spectrometer (*Bruker*, Germany); $\delta$ values in ppm downfield from internal $SiMe_4$ ($^1H$, $^{13}C$) or external 85% $H_3PO_4$ soln. ($^{31}P$).

*Composition Analysis of Oligonucleotides.* The oligonucleotides (0.2 $A_{260}$ units) were dissolved in 0.1M *Tris*·HCl buffer (pH 8.3, 200 μl) and treated with snake-venom phosphodiesterase (EC 3.1.4.1, *Crotallus durissus*, 3 μl) at 37° for 45 min and then with alkaline phosphatase (EC 3.1.3.1, *E. coli*, 3 μl) at 37° for 30 min. The mixture was analysed on reversed-phase HPLC (*RP-18*, gradient III, at 260 nm). Quantification of the material was made on the basis of the peak areas, which were divided by the extinction coefficients of the nucleoside constituents ($\varepsilon_{260}$: $G_d$ 11700, $T_d$ 8800, $A_d$ 15400, $m^5iC_d$ 6100, $iC_d$ 5360, $U_d$ 9980). The hydrolysis results of some selected oligonucleotides containing $m^5iC_d$, $iC_d$, and $U_d$ are shown in the *Table 5* (see below) indicating that no deamination of $m^5iC_d$ and $iC_d$ had occurred.

*Hydrolysis Product Analysis.* The compounds produced by hydrolysis under alkaline or acidic conditions were identified by reversed-phase HPLC with the gradient as indicated in the corresponding *Figs.*

*2-{[(Dimethylamino)methylidene]amino}pyrimidin-4-(3*H*)-one* (**8a**). To a suspension of isocytosine (**9**; 111 mg, 1 mmol) in MeOH (20 ml), dimethylformamide dimethyl acetal (0.3 ml, 2.3 mmol) was added. The mixture was stirred at 50°. After *ca.* 2 h (TLC monitoring ($CH_2Cl_2$/MeOH 9:1): no **9** left), the mixture was evaporated. The resulting white powder was dissolved in $CH_2Cl_2$ and applied to FC (silica gel, 2.5 × 12 cm, $CH_2Cl_2$/MeOH 9:1). Evaporation of the main fraction gave **8a** (160 mg, 96%). Colorless powder. TLC ($CH_2Cl_2$/MeOH 9:1): $R_f$ 0.51. UV (MeOH): 234 (10600), 288 (16400). $^1$H-NMR ((D$_6$)DMSO): 3.02 (*s*, MeN); 3.14 (*s*, MeN); 5.76 (*d*, *J* = 6.4, H–C(5)); 7.64 (*d*, *J* = 6.4, H–C(6)); 8.63 (*s*, HC=N); 11.52 (*s*, H–N(3)). Anal. calc. for $C_7H_{10}N_4O$ (166.18): C 50.59, H 6.07, N 33.71; found: C 50.71, H 6.27, N 33.85.

*2-{[(Dibutylamino)methylidene]amino}pyrimidin-4(3*H*)-one* (**8b**). As described for **8a**, from **9** (111 mg, 1 mmol), MeOH (20 ml), and dibutylformamide dimethyl acetal (0.56 ml, 2.7 mmol): **8b** (206 mg, 82%). Colorless powder. TLC ($CH_2Cl_2$/MeOH 9:1): $R_f$ 0.56. UV (MeOH): 234 (10700), 291 (19200). $^1$H-NMR ((D$_6$)DMSO): 0.90 (*m*, *Me*$CH_2CH_2CH_2)_2$N); 1.26 (*m*, (MeC*H*$_2$CH$_2$CH$_2)_2$N); 1.53 (*m*, (MeCH$_2$C*H*$_2$CH$_2)_2$N); 3.42 (*m*, (MeCH$_2$CH$_2$C*H*$_2)_2$N); 5.77 (*d*, *J* = 6.5, H–C(5)); 7.64 (*d*, *J* = 6.5, H–C(6)); 8.63 (*s*, HC=N); 11.49 (*s*, H–N3)). Anal. calc. for $C_{13}H_{22}N_4O$ (250.34): C 32.37, H 8.86, N 22.38; found: C 62.42, H 8.81, N 22.32.

*2-Amino-1-(2-deoxy-β-*D-erythro-*pentofuranosyl)pyrimidin-4(1*H*)-one* (**4**) [22]. A suspension of 2,5′-anhydro-2′-deoxyuridine [24] (290 mg, 1.38 mmol) in MeOH (50 ml) was presaturated with $NH_3$ at 0° and then stirred in sealed bottle at r.t. for 72 h (TLC monitoring ($CH_2Cl_2$/MeOH 3:1)). The soln. was evaporated and the residue applied to FC (silica gel, 2.5 × 12 cm, $CH_2Cl_2$/MeOH 3:1): **4** (270 mg, 93%). Colorless powder. TLC ($CH_2Cl_2$/MeOH 3:1): $R_f$ 0.26. UV (MeOH): 260 (5360). $^1$H-NMR ((D$_6$)DMSO): 2.17 (*m*, 2 H–C(2′)); 3.59 (*m*, 2 H–C(5′)); 3.78 (*m*, H–C(4′)); 4.27 (*m*, H–C(3′)); 5.20 (*t*, *J* = 4.9, OH–C(5′)); 5.32 (*d*, *J* = 3.9, OH–C(3′)); 5.57 (*d*, *J* = 7.7, H–C(5)); 5.87 (*t*, *J* = 6.6, H–C(1′)); 7.66 (*d*, *J* = 7.7, H–C(6)). Anal. calc. for $C_9H_{13}N_3O_4$ (227.22): C 47.57, H 5.77, N 18.49; found: C 47.59, H 5.93, N 18.50.

*1-(2-Deoxy-β-*D-erythro-*pentofuranosyl)-2-{[(dimethylamino)methylidene]amino}pyrimidin-4(1*H*)-one* (**7a**). To a suspension of **4** (454.4 mg, 2 mmol) in MeOH (40 ml), dimethylformamide dimethyl acetal (0.74 ml, 5.6 mmol) was added. The mixture was stirred at r.t. After *ca.* 3.5 h (TLC monitoring ($CH_2Cl_2$/MeOH 3:1): no **4** left), the mixture was evaporated and the residue applied to FC (silica gel, 2.5 × 12 cm, $CH_2Cl_2$/MeOH 3:1): **7a** (500 mg, 89%). Colorless powder. TLC ($CH_2Cl_2$/MeOH 3:1): $R_f$ 0.54. UV (MeOH): 280 (28100), 242.0 (19600). $^1$H-NMR ((D$_6$)DMSO): 2.03–2.22 (*m*, 2 H–C(2′)); 3.05 (*s*, MeN); 3.18 (*s*, MeN); 3.59 (*m*, 2 H–C(5′)); 3.80 (*m*, H–C(4′)); 4.23 (*m*, H–C(3′)); 5.05 (*t*, *J* = 5.0, OH–C(5′)); 5.27 (*d*, *J* = 4.1, OH–C(3′)); 5.74 (*d*, *J* = 7.7, H–C(5)); 6.61 (*t*, *J* = 6.9, H–C(1′)); 7.86 (*d*, *J* = 7.7, H–C(6)); 8.58 (*s*, HC=N). Anal. calc. for $C_{12}H_{18}N_4O_4$ (282.30): C 51.06, H 6.43, N 19.85; found: C 51.19, H 6.61, N 19.95.

*1-(2-Deoxy-β-*D-erythro-*pentofuranosyl)-2-{[(dibutylamino)methylidene]amino}pyrimidin-4-(1*H*)-one* (**7b**). As described for **7a**, with **4** (260 mg, 1.14 mmol), MeOH (20 ml), and dibutylformamide dimethyl acetal (0.64 ml, 3.1 mmol) (1 h at 40°; TLC monitoring ($CH_2Cl_2$/MeOH 8:1)). FC (silica gel, 2.5 × 12 cm, $CH_2Cl_2$/MeOH 8:1) gave **7b** (390 mg, 93%). Colorless powder. TLC ($CH_2Cl_2$/MeOH 8:1): $R_f$ 0.39. UV (MeOH): 285 (31100), 242 (199000). $^1$H-NMR ((D$_6$)DMSO): 0.91 (*m*, *Me*$CH_2CH_2CH_2)_2$N); 1.30 (*m*, (MeC*H*$_2$CH$_2$CH$_2)_2$N); 1.57 (*m*, (MeCH$_2$C*H*$_2$CH$_2)_2$N); 1.90–2.20 (*m*, 2 H–C(2′)); 3.44 (*m*, MeCH$_2$CH$_2$C*H*$_2)_2$N); 3.59 (*m*, 2 H–C(5′)); 3.80 (*m*, H–C(4′)); 4.22 (*m*, H–C(3′)); 5.05 (*t*, *J* = 5.3, OH–C(5′)); 5.23 (*d*, *J* = 4.0, OH–C(3′)); 5.74 (*d*, *J* = 7.7, H–C(5)); 6.55 (*t*, *J* = 6.4, H–C(1′)); 7.89 (*d*, *J* = 7.7, H–C(6)); 8.58 (*s*, HC=N). Anal. calc. for $C_{18}H_{30}N_4O_4$ (366.46): C 59.00, H 8.25, N 15.29; found: C 59.08, H 8.17, N 15.25.

*1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-*D*-*erythro*-pentofuranosyl]-2-{[(dimethylamino)methylidene]amino}pyrimidin-4(1*H*)-one* (**10**). Compound **7a** (1.48 g, 5.2 mmol) was dried by co-evaporation with anh. pyridine (3 × 5 ml) and then dissolved in anh. pyridine (10 ml). At r.t., 4,4′-dimethoxytrityl chloride (980 mg, 2.89 mmol) was added in two portions (every 30 min) under stirring. The mixture was stirred for 4 h. After addition of MeOH (5 ml), the mixture was evaporated and the residue dissolved in $CH_2Cl_2$ (35 ml). The soln. was washed with 5% aq. $NaHCO_3$ soln. (2 × 15 ml) and $H_2O$ (20 ml), dried ($Na_2SO_4$), and evaporated. The resulting foam was applied to FC (silica gel, 4 × 12 cm, washing with $CH_2Cl_2$/acetone/$Et_3N$ 70:30:0.5), then $CH_2Cl_2$/MeOH/$Et_3N$ 90:10:0.1: **10** (2.3 g, 76%). Colorless foam. TLC ($CH_2Cl_2$/MeOH/$Et_3N$ 90:10:0.5): $R_f$ 0.39. UV (MeOH): 282 (31800), 237 (36200). $^1$H-NMR ((D$_6$)DMSO): 2.12–2.26 (*m*, 2 H–C(2′)); 3.05 (*s*, MeN); 3.18 (*s*, MeN); 3.23 (*m*, 2 H–C(5′)); 3.74 (*s*, (*Me*O)$_2$Tr); 3.91 (*m*, H–C(4′)); 4.31 (*m*, H–C(3′)); 5.39 (*d*, *J* = 4.4, OH–C(3′)); 5.50 (*d*, *J* = 7.7, H–C(5)); 6.62 (*t*, *J* = 6.2, H–C(1′)); 6.88–7.40 (*m*, 13 H, (MeO)$_2$*Tr*); 7.68 (*d*, *J* = 7.7, H–C(6)); 8.59 (*s*, HC=N). Anal. calc. for $C_{33}H_{36}N_4O_6$ (584.66): C 67.79, H 6.21, N 9.58; found: C 67.89, H 6.20, N 9.54.

*1-[2-Deoxy-5-*O*-(4,4′-dimethoxytrityl)-β-*D*-*erythro*-pentofuranosyl]-2-{[(dimethylamino)methylidene]amino}pyrimidin-4(1*H*)-one 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**11**). To a soln. of **10** (625 mg, 1.1 mmol) in dry $CH_2Cl_2$ (20 ml), 2-cyanoethyl diisopropylphosphoramidochloridite (0.48 ml, 2.2 mmol) and $^i$Pr$_2$EtN (0.48 ml, 2.76 mmol) were added under Ar at r.t. The mixture was stirred for 40 min. Then, 5% aq. $NaHCO_3$ soln. (20 ml) was added, the mixture extracted with $CH_2Cl_2$ (3 × 20 ml), the extract dried ($Na_2SO_4$) and evaporated, and the oily residue applied to FC (silica gel, 4 × 7.5 cm, $CH_2Cl_2$/acetone/$Et_3N$ 70:30:0.5, then $CH_2Cl_2$/MeOH/$Et_3N$ 90:10:0.5): **9** (717 mg, 83%). Colorless foam. TLC ($CH_2Cl_2$/MeOH/$Et_3N$ 90:10:0.2): $R_f$ 0.54. UV (MeOH): 282 (29900), 237 (37700). $^1$H-NMR ((D$_6$)DMSO): 1.04–1.20 (*m*, 2 (*Me*CH)$_2$N); 2.25 (*m*, NCC*H*$_2$CH$_2$); 2.63 (*m*, 2 H–C(2′)); 3.10 (*d*, MeN); 3.18 (*d*, MeN); 3.40–3.60 (*m*, 2 H–C(5′), CNCH$_2$C*H*$_2$); 3.81 (*m*, 8 H, (*Me*O)$_2$Tr, (Me$_2$C*H*)$_2$N); 4.20 (*m*, H–C(4′)); 4.55 (*m*, H–C(3′)); 5.80 (*dd*, H–C(5)); 6.73–7.41 (*m*, H–C(1′), (MeO)$_2$*Tr*); 7.79 (*dd*, H–C(6)); 8.83 (*d*, HC=N). $^{31}$P-NMR ($CDCl_3$): 150.4, 149.8.

*Oligonucleotides.* The oligonucleotide synthesis was carried out on a 1-μmol scale with the phosphoramidite of **3**, **4**, and/or the phosphoramidite of U$_d$ and iG$_d$ on an *ABI 392-08* synthesizer. The phosphoramidites of A$_d$, G$_d$, T$_d$, and C$_d$ and the CPG columns were purchased from *PerSepetive Biosystems GmbH*, Germany. After the synthesis, the oligonucleotides **6**, **7**, **10**, **11**, and **13** were deprotected in conc. aq. $NH_3$ soln. at 60° for 16 h. The oligonucleotides **12** and **14** containing **4** were deprotected in conc, aq. $NH_3$ soln. at r.t. for 16 h. Accordingly, the A$_d$ residues in **12** and **14** were protected with the labile pac group, which can be deprotected within 2 h at r.t. with conc. aq. $NH_3$ soln. The oligonucleotides **8** and **9** containing U$_d$ at the 3′-terminus, introduced by universal support *500* (*Glen Research*, US), were deprotected in conc. aq. $NH_3$ soln. at 60° for 72 h to remove the universal support. Oligonucleotides were purified by reversed-phase HPLC with the following solvent systems: 0.1M aq. ($Et_3NH$)OAc (pH 7.0) (*A*), MeCN (*B*), and 0.1M ($Et_3NH$)OAc (pH 7.0)/MeCN 95:5 (*C*). They were used in the following order: gradient I, 3 min 15% *B* in *C*, 12 min 15–40% *B* in *C*, 5 min 40–15% *B* in *C* with a flow rate of 1.0 ml/min; gradient II, 20 min 0–20% *B* in *C* with a flow rate of 1.0 ml/min; gradient III, 30 min 100% *C* with a flow rate of 0.6 ml/min. The oligomers carrying 5′-(MeO)$_2$Tr residues were purified by HPLC (*RP-18*, 250 × 4 mm) with gradient I. The (MeO)$_2$Tr groups were removed by treating the oligomers with 2.5% $CHCl_2COOH$/$CH_2Cl_2$ for 5 min at r.t. The detritylated oligomers were purified by HPLC (*RP-18*, 250 × 4 mm) with gradient II. The oligomers separated by reversed-phase HPLC were desalted on a *RP-18* column (silica gel, 125 × 4 mm) with $H_2O$ to elute the salt, while the oligomers were eluted with MeOH/$H_2O$ 3:2. The purified oligonucleotides were lyophilized on a *Speed-Vac* evaporator to yield colorless solids, which were dissolved in 100 μl of $H_2O$ and stored frozen at −18°. The synthesized oligonucleotides were characterized by enzymatic hydrolysis as well as MALDI-TOF mass spectra (*Table 4*). Some hydrolysis data are summarized in *Table 5*.

Table 4. M$^+$ *Data of Oligonucleotides* **12**–**14**, **16**–**18**, *and* **20** *Determined by MALDI-TOF Mass Spectra*

| | *m/z* | | | *m/z* | |
|---|---|---|---|---|---|
| | calc. | found | | calc. | found |
| **12** | 3620.5 | 3623 | **17** | 3508.3 | 3507 |
| **13** | 3564.4 | 3563 | **18** | 3668.4 | 3668 |
| **14** | 3724.5 | 3723 | **20** | 3508.3 | 3507 |
| **16** | 3564.4 | 3564 | | | |

Table 5. *Nucleoside Composition of Oligonucleotides* **12**, **13**, **19**, *and* **20**

| | Ratio of compositions | | | | |
|---|---|---|---|---|---|
| | $A_d$ | $U_d$ | $T_d$ | $m^5iC_d$ | $iC_d$ |
| **12** | 1.00 | | 1.09 | 1.07 | |
| **13** | 1.05 | | 1.00 | | 1.03 |
| **19** | 1.01 | 1.06 | | 1.00 | |
| **20** | 1.05 | 1.00 | | | 1.05 |

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