Oligonucleotides Functionalized by Fluorescein and Rhodamine Dyes: *Michael* Addition of Methyl Acrylate to 2′-Deoxypseudouridine

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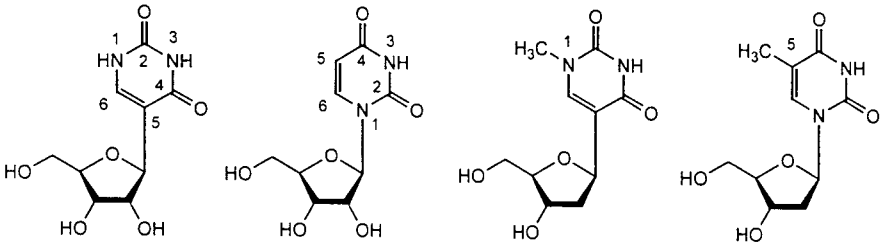
The 2′-deoxypseudouridine (**5**) was functionalized at N(1) with methyl acrylate by *Michael* addition. The resulting methyl 2′-deoxypseudouridine-1-propanoate (**6**) was converted to the phosphoramidite **8** and to the amino-functionalized derivative **9**, which was transformed into the fluorescein-labeled phosphoramidites **14** and **16**. Fluorescent oligonucleotides were synthesized either from these building blocks or by post-synthetic modification of oligomers containing 2′-deoxypseudouridine subunits. The stability of oligonucleotide duplexes was determined from the melting profiles, measured by UV- or VIS-light absorbance, as well as from the fluorescence emission spectra. While small spacer residues did not affect the thermal stability of the 2′-deoxypseudouridine-containing duplexes, large dye residues led to destabilization.

Introduction. – Pseudouridine (Ψ; **1**), which was isolated in 1957 from tRNA [1], is a pyrimidine *C*-nucleoside. Its structure is related to that of uridine and was established in 1962 [2][3]. Meanwhile, it is known that Ψ is present ubiquitously in transfer RNA in the T-ΨC-Pu loop [4]. Other pyrimidine *C*-nucleosides were isolated from different natural sources and represent antibiotics or exhibit anticancer and/or antiviral activity [5]. Also 2′-deoxypseudouridine (Ψ_d; **5**) and related pyrimidine 2′-deoxy-*C*-nucleosides have been prepared [6][7]. In 1991, *Piccirilli et al.* incorporated N^1-methyl-2′-deoxypseudouridine (**3**; $m^1\Psi_d$) *via* its phosphoramidite into DNA templates [8]. In 1995, *Bhattacharya et al.* incorporated the same compound into G-rich triplex-forming oligonucleotides [9]. *Rosenberg et al.* [10] used the H-phosphonate approach for the synthesis of different oligonucleotides containing 2′-deoxypseudouridine and its *N*-methyl derivatives.



1 **2** **3** **4**

As the N(1) atom of 2′-deoxypseudouridine (**5**) is located in the same position as the 5-methyl group of T_d and thereby directed into the major groove of a B-DNA, the introduction of reporter groups such as fluorescent dyes at that position of Ψ_d was anticipated to be favorable. Until now, mainly fluorophore-labeled 2′-deoxyuridine has found application in DNA diagnostics and analysis [11–13]. An alternative nucleoside carrier for reporter groups is 2′-deoxypseudouridine (**5**). The ribonucleoside **1** has been derivatized by regioselective cyanoethylation at N(1) under mild reaction conditions (buffer, pH 8.5, 37°), both on the nucleoside as well as on the tRNA level [14–16].

This report describes the derivatization of 2′-deoxypseudouridine (**5**) by methyl acrylate (= methyl prop-2-enoate) on the nucleoside and on the oligonucleotide level and its functionalization by linkers and fluorescent dyes. The fluorescent oligonucleotides were obtained either by solid-phase DNA synthesis from fluorophore-labeled 2′-deoxypseudouridine phosphoramidites or by post-modification at the oligonucleotide level.

Results. – *Monomers.* The 2′-deoxypseudouridine (= 5-(2-deoxy-β-D-*erythro*-pentofuranosyl)uracil; **5**; Ψ_d) was synthesized from pseudouridine (**1**) according to [7]. Reaction of **5** with methyl acrylate in buffer (pH 8.5) yielded the N^1-alkylated compound **6** in 89% yield (*Scheme 1*). Only traces of by-products were detected. A similar reaction has been performed with acrylonitrile [14]. This is in line with early findings that in slightly alkaline medium, as it is used for the alkylation reaction here,

Scheme 1

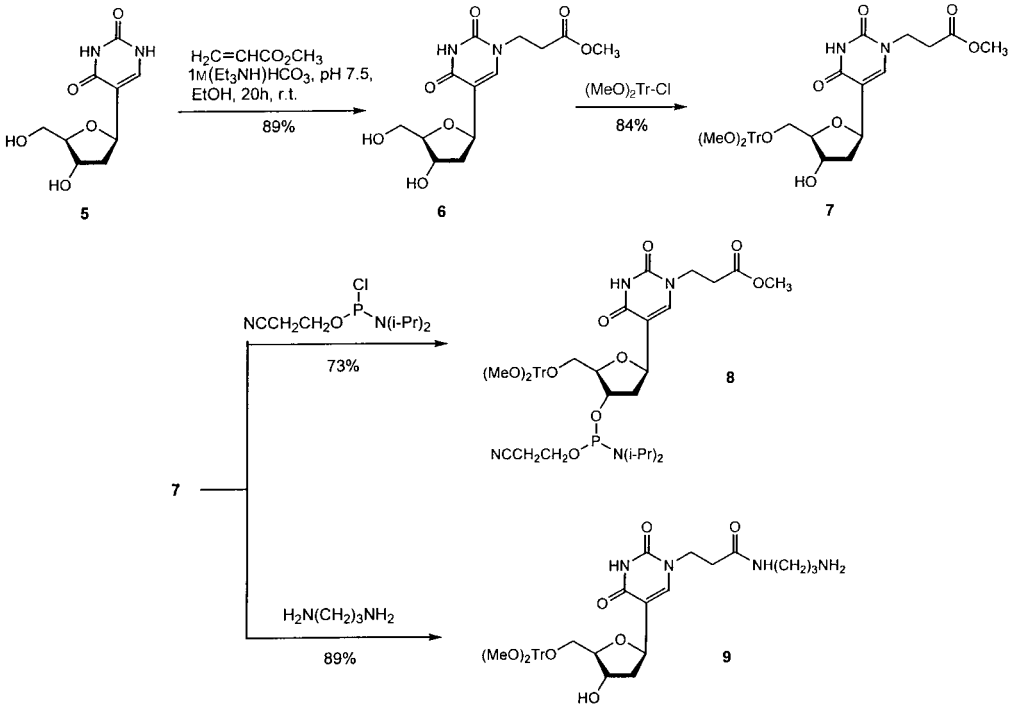


Table 1. *Selected ^{13}C-NMR Data of 2′-Deoxypseudouridine Derivatives in (D_6)DMSO at 303 K*

	C(2)	C(4)	C(5)	C(6)	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)	CH_2	COO*Me*	*C*OOMe (or CONHR)	MeO (Tr)
5	151.2	163.5	113.2	137.8	73.2	40.8	72.1	87.0	62.2	–	–	–	–
6	150.4	162.8	113.6	141.7	73.1	40.7	72.0	87.1	62.2	44.1, 32.6	51.5	171.1	–
7	150.4	162.6	113.7	141.2	72.9	40.6	72.2	85.1	64.2	44.3, 32.4	51.4	171.0	55.0
9	150.4	162.6	113.3	141.4	73.0	40.6	72.1	85.1	64.2	45.1, 38.3, 35.9, 34.2, 31.1	–	169.3	55.0

pseudouridine exists as a mixture of monoanions ($pK_a^{N(1)}$: 9.3; $pK_a^{N(3)}$: 9.6) in which the N(1) anion predominates significantly over the N(3) anion [14][17].

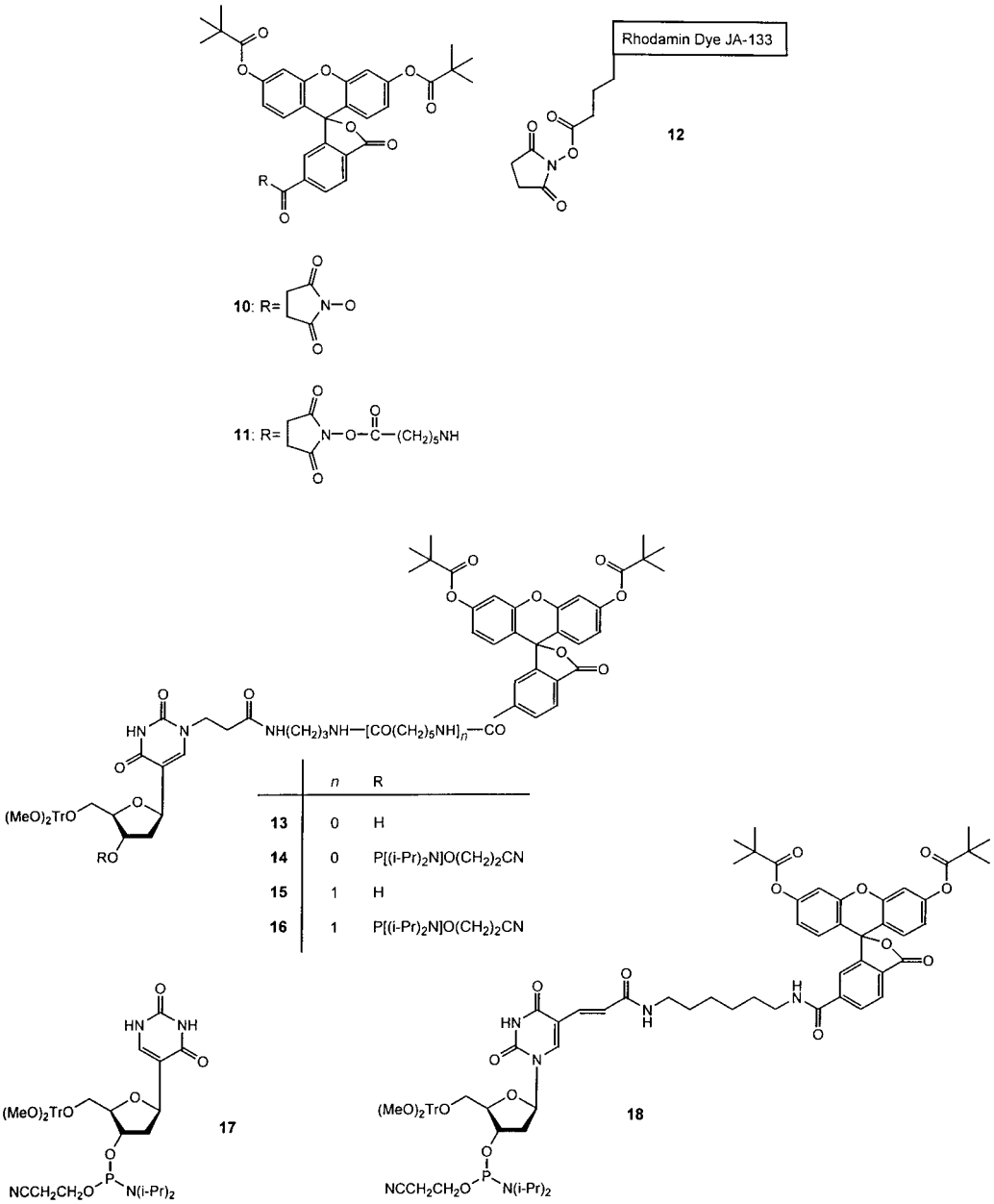
The assignment of the alkylation position is based on the similarity of the UV spectrum of **6** (λ_{max} 270 nm) with that of N^1-methylpseudouridine (λ_{max} 271 nm) [18], while that of the parent nucleoside **5** exhibits a λ_{max} of 263 nm [19]. Moreover, the similar ^{13}C-NMR chemical shifts of the uracil base of **6** and those of the corresponding resonances of N^1-methylpseudouridine establish N^1-alkylation (*Table 1*) [20].

Protection of compound **6** at the 5′-OH group with a 4,4′-dimethoxytrityl residue ($(MeO)_2Tr$) afforded compound **7** [21]. This was either converted into the phosphoramidite **8** by reaction with 2-cyanoethyl diisopropylphosphoramidochloridite [22] or into the amino-functionalized derivative **9** by reaction with an excess of propane-1,3-diamine (*Scheme 1*). New compounds were characterized by ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra as well as by elemental analyses (*Table 1* and *Exper. Part*). The assignment of the ^{13}C-NMR resonances was made on the basis of gated-decoupled ^{13}C- and ^{1}H,^{13}C-heteronuclear spectra as well as DEPT-135 spectra.

Reaction of compound **9** with esters **10** and **11** derived from *N*-hydroxysuccinimide and di-*O*-pivaloyl-protected fluorescein-6-carboxylic acid led to compounds **13** and **15** which both showed strong fluorescence in a spot test upon treatment with ammonia and which were characterized by ^{1}H-NMR spectroscopy. The compounds differ in the length of the spacer between the fluorophore and the nucleobase (**13**: 10 bonds; **15**: 17 bonds). Reaction of **13** and **15** with 2-cyanoethyl diisopropylphosphoramidochloridite gave the phosphoramidites **14** and **16**, which were characterized by ^{31}P-NMR spectroscopy. The last two reaction steps were performed under the exclusion of light. Ester **12** derived from *N*-hydroxysuccinimide and a rhodamine dye was used for post-synthetic oligonucleotide labeling (see below).

Oligonucleotides: Syntheses. The syntheses of the oligonucleotides were performed by three different routes: *i*) by solid-phase synthesis *via* phosphoramidites of Ψ_d derivatives, *ii*) by post-modification of oligonucleotides containing a reactive Ψ_d derivative, and *iii*) by a *Michael* reaction on a Ψ_d-containing oligonucleotide.

i) The synthesis of the oligomers **19**, **22** (*Scheme 2*), **24**–**26**, **28**, **29**, and **36**–**38** (*Table 2*) was performed with the phosphoramidites **8**, **14**, and **16**–**18**. The methodology followed the standard protocols of solid-phase technique [23]. The coupling efficiency of the modified building blocks was between 95 and 100% and therewith similar to those determined for the regular compounds (trityl monitoring). The oligonucleotides were detritylated and purified as described in the *Exper. Part*. The composition of the test oligomer **24** (5′-d(Ψ*AGGTCAATACT)-3′ with Ψ_d^* = fluorescein-labeled 2′-deoxypseudouridine without spacer) was confirmed by tandem



hydrolysis with snake-venom phosphodiesterase and alkaline phosphatase as described in the *Exper. Part*. Reversed-phase HPLC (*RP-18*) of the enzymatic digest (*Fig. 1*) was used to estimate the ε_{260} of fluorescein-labeled 2′-deoxypseudouridine (28000). MALDI-TOF Mass spectra were taken to determine the molecular masses of the other modified oligomers **28**–**35** and **38**–**40** (*Table 3*).

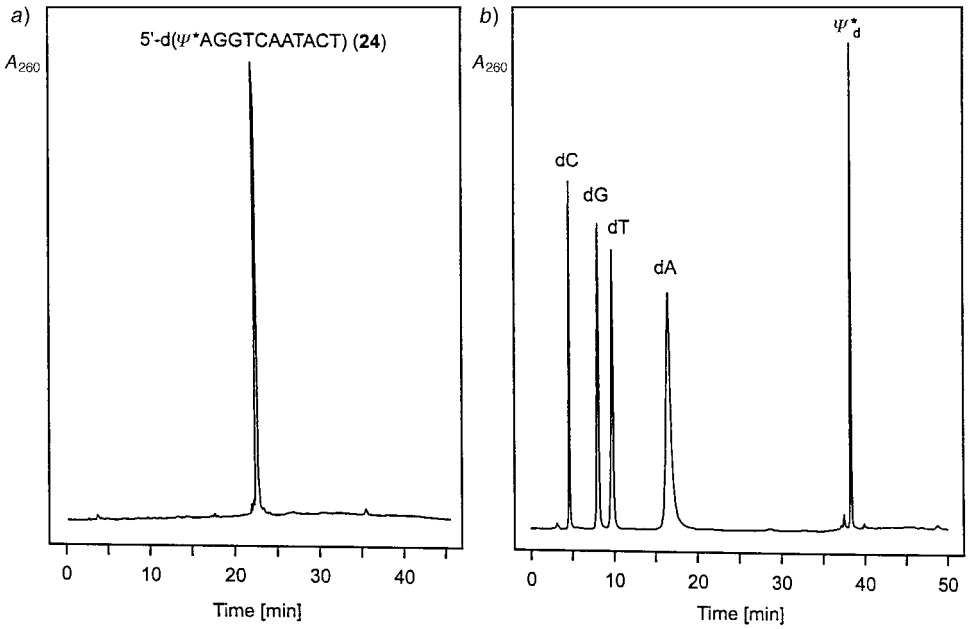
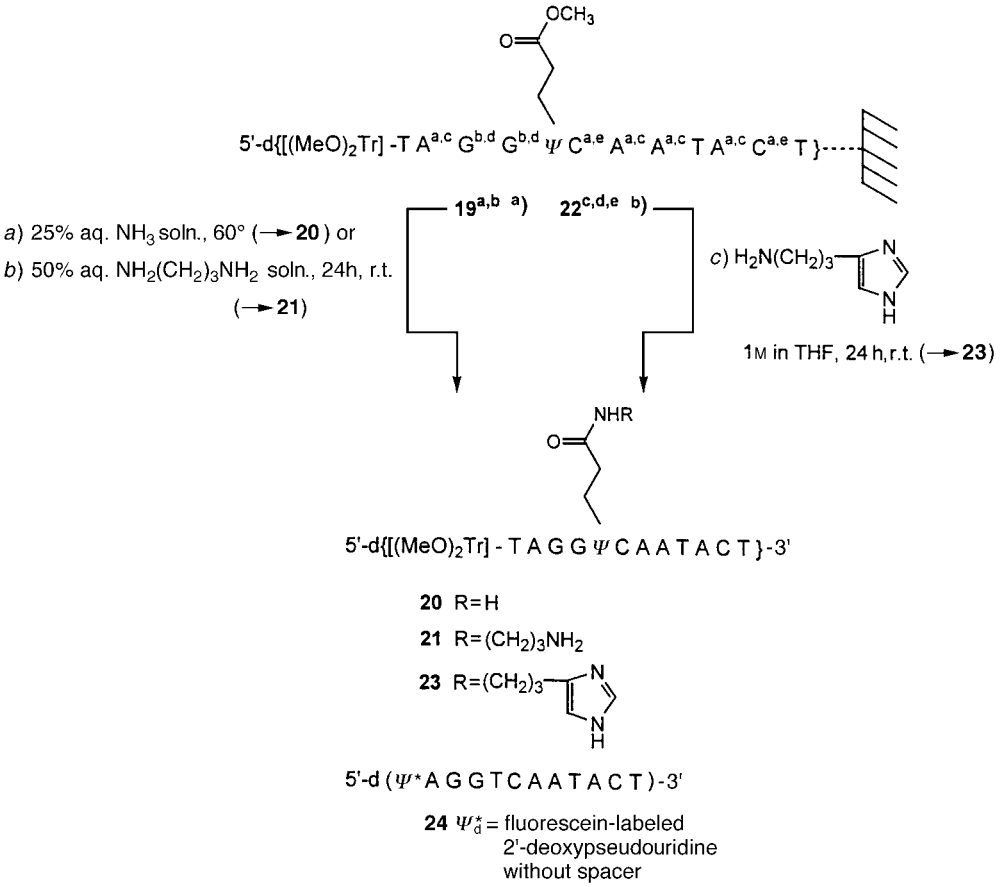


Fig. 1. a) *HPLC Profile of the oligonucleotide* **24** (5′-d(*Ψ**AGGTCAATACT)-3′, with Ψ^*_d = fluorescein-labeled 2′-deoxypseudouridine); b) *HPLC profile of the reaction products obtained after enzymatic hydrolysis of the oligonucleotide* **24** *by snake-venom phosphodiesterase and alkaline phosphatase in* 1M Tris·*HCl buffer* (pH 8.1) *at 37°*. Column: *RP-18* (250 × 4 mm); gradient: 0–25 min 100% 0.1M (Et_3NH)OAc/MeCN 95:5, pH 7.5 (= *U*); 25–70 min 0–100% MeCN in *U*.

ii) All other oligonucleotides listed in *Table 2* were prepared by post-synthetic reactions. From the phosphoramidite **8**, the controlled-pore-glass(CPG)-bound, fully-protected oligomer **19** was assembled carrying one 2′-deoxypseudouridine derivative with an ester function in the side chain. Treatment of **19** with 25% aq. ammonia (60°) gave the oligomer **20** (*Scheme 2*) bearing one N^1-(3-amino-3-oxopropyl)-2′-deoxy-pseudouridine residue. Cleavage of the 5′-(4,4′-dimethoxytriphenylmethyl) group of **20** with 80% AcOH/H_2O yielded the oligomer **30** (*Table 2*). In an analogous way, the doubly-modified oligonucleotide **31** (*Table 2*) was prepared. Reaction of **19** with 50% aqueous propane-1,3-diamine for 24 h at room temperature led to compound **21** which – upon detritylation – gave the amino-functionalized oligonucleotide **32** (*Table 2*). This latter oligomer was later used for further post-labeling reactions.

For the synthesis of the oligomer **35** (*Table 2*) bearing one 2′-deoxypseudouridine subunit with an N^1-{3-{[3-(1*H*-imidazol-4-yl)propyl]amino}-3-oxopropyl} side chain, a different strategy was chosen. For this purpose, phosphoramidites of the regular nucleosides with protecting groups suitable for 'ultramild deprotection chemistry' [24] were used together with compound **8**. After assembly of the CPG-linked oligomer **22** (*Scheme 2*), it was treated with a 1M soln. of 1*H*-imidazole-4-propanamine (24 h, room temp.; → **23**) [25] and subsequently detritylated to yield oligomer **35** (*Table 2*). Such oligodeoxyribonucleotides bearing an imidazole tether might be useful as artificial

Scheme 2



a, bz (benzoyl); b, ibu (isobutyryl); c, pac (phenoxyacetyl); d, i-Prpac (4-isopropyl)phenoxyacetyl); e, ac (acetyl); Tr, triphenylmethyl

a) Protected by a and b. *b*) Protected by c, d, and e.

RNases, as it has been recently shown [26] that they promote a sequence-specific strand scission of a complementary RNA strand in the presence of Zn^{2+} ions.

The amino-functionalized oligonucleotide **32** was post-synthetically labeled with the esters **11** and **12** derived from N-hydroxysuccinimide. Both reactions were performed in 0.1M Na-borate buffer (pH 8)/DMF at room temperature [27]. After purification by reversed-phase HPLC (*RP-18*), the labeled oligonucleotides **33** and **34** were obtained (see *Exper. Part* and *Table 2*). The oligonucleotides were characterized by MALDI-TOF mass spectra (*Table 3*).

iii) In a third approach, the oligonucleotide **28** (*Fig. 2,a*) bearing a single 2′-deoxypseudouridine residue was reacted overnight with an excess of methyl acrylate in 1M $(Et_3NH)HCO_3$/EtOH 1:1 at 37°. Subsequent analysis of the reaction mixture by reversed-phase HPLC (*RP-18*) showed a relatively clean reaction (*Fig. 2,b*). The main

Table 2. T_m *Values and* ΔG°_{298} *Data of Duplex Formation of Oligonucleotides*[a])

Oligomer	Modified 2′-deoxynucleoside Ψ_d, Ψ^*_d or T^*_d	T_m [°C]	ΔG°_{298} [kcal/mol]
5′-d(TAGGTCAATACT)-3′ **25** 3′-d(ATCCAGTTATGA)-5′ **26**	–	46	– 10.4
5′-d(TAGGTCAATACT)-3′ **25** 3′-r(AUCCAGUUAUGA)-5′ **27**	–	45	– 10.2
5′-d(TAGGΨCAATACT)-3′ **28** 3′-d(ATCCAGTTATGA)-5′ **26**	2′-deoxypseudouridine (**5**)	45	– 9.6
5′-d(TAGGΨCAATACT)-3′ **28** 3′-r(AUCCAGUUAUGA)-5′ **27**	2′-deoxypseudouridine (**5**)	46	– 10.7
5′-d(TAGGΨCAAΨACT)-3′ **29** 3′-d(ATCCAGTTATGA)-5′ **26**	2′-deoxypseudouridine (**5**)	42	– 9.0
5′-d(TAGGΨCAAΨACT)-3′ **29** 3′-r(AUCCAGUUAUGA)-5′ **27**	2′-deoxypseudouridine (**5**)	43	– 9.1
5′-d(TAGGΨ*CAATACT)-3′ **30** 3′-d(ATCCAGTTATGA)-5′ **26**	N^1-(3-amino-3-oxopropyl)-2′-deoxypseudouridine	43	– 9.6
5′-d(TAGGΨ*CAATACT)-3′ **30** 3′-r(AUCCAGUUAUGA)-5′ **27**	N^1-(3-amino-3-oxopropyl)-2′-deoxypseudouridine	43	– 9.6
5′-d(TAGGΨ*CAAΨ*ACT)-3′ **31** 3′-d(ATCCAGTTATGA)-5′ **26**	N^1-(3-amino-3-oxopropyl)-2′-deoxypseudouridine	41	– 8.7
5′-d(TAGGΨ*CAAΨ*ACT)-3′ **31** 3′-r(AUCCAGUUAUGA)-5′ **27**	N^1-(3-amino-3-oxopropyl)-2′-deoxypseudouridine	41	– 8.8
5′-d(TAGGΨ*CAATACT)-3′ **32** 3′-d(ATCCAGTTATGA)-5′ **26**	N^1-{3-[(3-aminopropyl)amino]-3-oxopropyl}-2′-deoxypseudouridine	44	– 9.4
5′-d(TAGGΨ*CAATACT)-3′ **32** 3′-r(AUCCAGUUAUGA)-5′ **27**	N^1-{3-[(3-aminopropyl)amino]-3-oxopropyl}-2′-deoxypseudouridine	43	– 9.9
5′-d(TAGGΨ*CAATACT)-3′ **33** 3′-d(ATCCAGTTATGA)-5′ **26**	2′-deoxy-N^1-{3-{{3-{{6-{[(fluorescein-6-yl)carbonyl]amino}-1-oxohexyl}amino}propyl}amino}-3-oxopropyl}pseudouridine	38 (37)[b])	– 8.2
5′-d(TAGGΨ*CAAΨ*ACT)-3′ **38** 3′-d(ATCCAGTTATGA)-5′ **26**	2′-deoxy-N^1-{3-{{3-{{6-{[(fluorescein-6-yl)carbonyl]amino}-1-oxohexyl}amino}propyl}amino}-3-oxopropyl}pseudouridine	32	n.m.
5′-d(TAGGΨ*CAATACT)-3′ **33** 3′-r(AUCCAGUUAUGA)-5′ **27**	2′-deoxy-N^1-{3-{{3-{{6-{[(fluorescein-6-yl)carbonyl]amino}-1-oxohexyl}amino}propyl}amino}-3-oxopropyl}-pseudouridine	36	– 8.2
5′-d(TAGGΨ*CAATACT)-3′ **34** 3′-d(ATCCAGTTATGA)-5′ **26**	2′-deoxy-N^1-{3-{{3-{[4-(JA-133)-1-oxobutyl]amino}propyl}amino}-3-oxopropyl}pseudouridine	37	– 8.1
5′-d(TAGGΨ*CAATACT)-3′ **35** 3′-d(ATCCAGTTATGA)-5′ **26**	2′-deoxy-N^1-{3-{[3-(1*H*-imidazol-4-yl)propyl]amino}-3-oxopropyl}pseudouridine	43	– 9.6

Table 2. (cont.)

Oligomer	Modified 2′-deoxynucleoside Ψ_d, Ψ_d^* or T_d^*	T_m [°C]	ΔG^0_{298} [kcal/mol]
5′-d(TAGGΨ*CAATACT)-3′ **35** 3′-r(AUCCAGUUAUGA)-5′ **27**	2′-deoxy-N^1-{3-{[3-(1*H*-imidazol-4-yl)propyl]amino}-3-oxopropyl}pseudouridine	42	– 9.3
5′-d(TAGGT*CAATACT)-3′ **36** 3′-d(ATCCAGTTATGA)-5′ **26**	5-{3-{[6-{(fluorescein-6-yl)carbonyl]amino}hexyl}amino}-3-oxoprop-1-enyl}thymidine	42	– 9.1
5′-d(TAGGT*CAAT*ACT)-3′ **37** 3′-d(ATCCAGTTATGA)-5′ **26**	5-{3-{[6-{(fluorescein-6-yl)carbonyl]amino}hexyl}amino}-3-oxoprop-1-enyl}thymidine	39	– 8.7
5′-d(TAGGT*CAATACT)-3′ **36** 3′-r(AUCCAGUUAUGA)-5′ **27**	5-{3-{[6-{(fluorescein-6-yl)carbonyl]amino}hexyl}amino}-3-oxoprop-1-enyl}thymidine	39	– 8.6
5′-d(TAGGT*CAAT*ACT)-3′ **37** 3′-r(AUCCAGUUAUGA)-5′ **27**	5-{3-{[6-{(fluorescein-6-yl)carbonyl]amino}hexyl}amino}-3-oxoprop-1-enyl}thymidine	38	– 9.0
5′-d(TAGGΨ*CAATACT)-3′ **39** 3′-d(ATCCAGTTATGA)-5′ **26**	Compound **6**	43	– 9.6
5′-d(TAGGΨ*CAAΨ*ACT)-3′ **40** 3′-d(ATCCAGTTATGA)-5′ **26**	Compound **6**	39	– 8.1

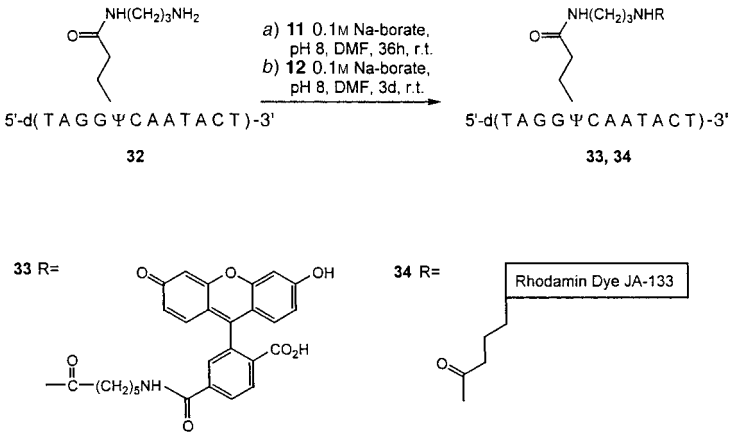
[a]) Measured in 10 mM Na-cacodylate, 10 mM $MgCl_2$, and 100 mM NaCl, pH 7.0, 5 + 5 μM of single strands. [b]) Measured from an oligomer sample synthesized from compound **16**.

Table 3. *Molecular Masses of Oligonucleotides Determined by MALDI-TOF Mass Spectra*

Oligonucleotide	Modified 2′-deoxynucleoside Ψ_d or Ψ_d^*	M^+ (calc.)	M^+ (found)
5′-d(TAG GΨC AAT ACT)-3′ **28**	2′-deoxypseudouridine	3628.6	3629.3
5′-d(TAG GΨCAAΨ ACT)-3′ **29**	2′-deoxypseudouridine	3614.6	3615.2
5′-d(TAG GΨ*C AAT ACT)-3′ **30**	N^1-(3-amino-3-oxopropyl)-2′-deoxypseudouridine	3703.0	3703.0
5′-d(TAG GΨ*C AAΨ* ACT)-3′ **31**	N^1-(3-amino-3-oxopropyl)-2′-deoxypseudouridine	3760.0	3756.6
5′-d(TAG GΨ*C AAT ACT)-3′ **32**	N^1-{3-[(3-aminopropyl)amino]-3-oxopropyl}-2′-deoxypseudouridine	3755.7	3755.7
5′-d(TAG GΨ*C AAT ACT)-3′ **35**	N^1-{3-[(3-aminopropyl)amino]-3-oxopropyl}-2′-deoxypseudouridine	3808.6	3809.0
5′-d(TAG GΨ*C AAT ACT)-3′ **33**	2′-deoxy-N^1-{3-{[3-{[6-{[(fluorescein-6-yl)carbonyl]amino}-1-oxohexyl}amino}propyl}amino}-3-oxopropyl}pseudouridine	4229.5	4228.2[a]) 4230.3[b])
5′-d(TAG GΨ*C AAΨ*ACT)-3′ **38**	2′-deoxy-N^1-{3-{[3-{[6-{[(fluorescein-6-yl)carbonyl]amino}-1-oxohexyl}amino}propyl}amino}-3-oxopropyl}pseudouridine	4814.5	4817.5
5′-d(TAG GΨ*C AAT ACT)-3′ **34**	2′-deoxy-N^1-{3-{[3-{[4-(JA-133)-1-oxobutyl]amino}propyl}-amino}-3-oxopropyl}pseudouridine	4437.9	4437.5
5′-d(TAG GΨ*C AAT ACT)-3′ **39**	Compound **6**	3715.6	3716.6
5′-d(TAG GΨ*C AAΨ* ACT)-3′ **40**	Compound **6**	3786.7	3790.7

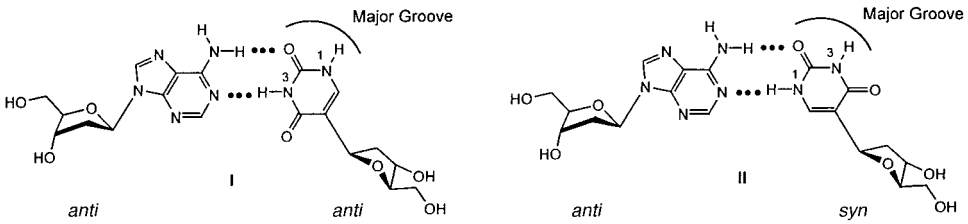
[a]) Oligomer prepared by post-synthetic modification. [b]) Oligomer prepared by solid-phase synthesis from compound **16**.

Scheme 3



product **39** (*Table 2*), which migrates slightly slower than **28** (*Fig. 2,c*), was purified (*Fig. 2,d*) and characterized by MALDI-TOF mass spectrometry (*Table 3*) as well as by enzymatic tandem hydrolysis with snake-venom phosphodiesterase and alkaline phosphatase. It was shown that a single modification had occurred at the Ψ_d subunit of **28** under formation of the ester **6**, which co-migrates with dA in reversed-phase HPLC under various elution conditions. The other canonical nucleosides gave almost no reaction with methyl acrylate on the oligomer level under the above-mentioned conditions. This was demonstrated by reaction of methyl acrylate with *i*) the parent oligonucleotide **25**, which remained unmodified (reversed-phase HPLC) and *ii*) by reaction with an artificial mixture of G_d, C_d, A_d, and T_d. In the latter case, reversed-phase HPLC analysis of the reaction mixture showed that only T_d was partly alkylated (data not shown) [14b,c]. Analogously, the oligonucleotide **29**, which carries two Ψ_d residues, was reacted with methyl acrylate to give the doubly labeled oligomer **40**, which was purified and characterized as described for **39** (*Table 3*).

Duplex Formation of Oligonucleotides With 2′-Deoxypseudouridine or Functionalized Derivatives Thereof. The 2′-deoxypseudouridine represents a '*Janus*'-type molecule [28][29] exhibiting two identical but discernable *Watson-Crick* base-pairing sites **I** and **II**. The base-pair motif **I**, in which both bases adopt the *anti* conformation, involves HN(3)/O=C(2) of Ψ_d as donor and acceptor atoms. In motif **II** the atoms HN(1) and O=C(2) of Ψ_d are used for the pairing with A_d. In the latter case, Ψ_d adopts the *syn* conformation.



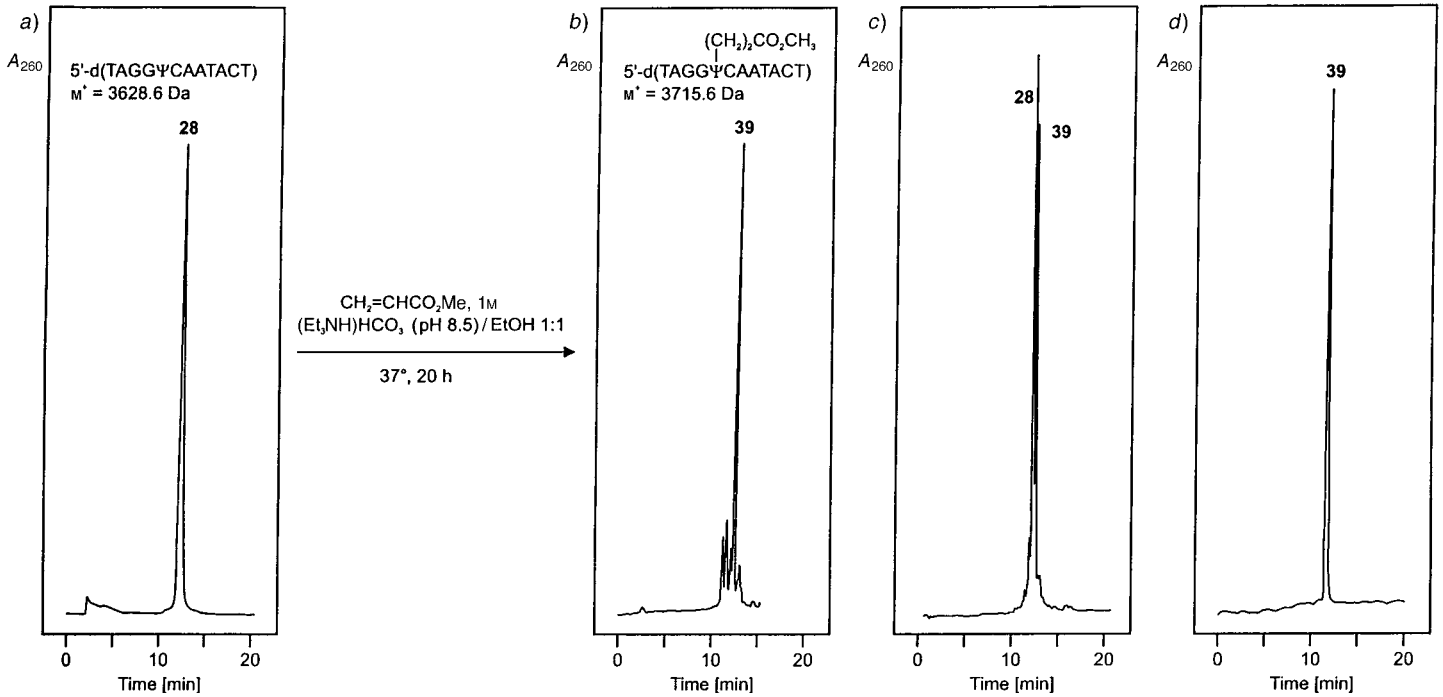


Fig. 2. a) *HPLC Profile of the oligonucleotide 5′-d(TAGGΨCAATACT)-3′* (**28**). b) *HPLC Profile of the reaction product* **39** *of* **28** *with methyl acrylate* (for details, see *Exper. Part*). c) *As described for* b), *but with addition of an aliquot of* **28**. d) *Purified oligomer* **39**. Column: *RP-18* (250 × 4 mm); gradient: 0–25 min 95–80% 0.1M (Et_3NH)OAc/MeCN 95:5, pH 7.5 (= *U*) in MeCN; 25–30 min 80% *U* in MeCN; 30–35 min 80–95% *U* in MeCN; 35–40 min 95% *U* in MeCN.

Next, the conformation of 2′-deoxypseudouridine was studied in solution. Irradiation of H–C(6) of compound **5** results in an NOE of 3.8 % at H–C(1′), establishing a preferred *anti* conformation at the *C*-glycosidic bond. This is in line with results obtained by *Bhattacharya et al.* [9] with the N^1-methyl derivative **3**, and it is typical also for regular pyrimidine *N*-nucleosides such as T_d or U_d [30]. Contrary, 2′-deoxypseudouridine and 2′-deoxyuridine show significant differences with respect to their sugar puckering. From a pseudorotational analysis of the ^3J(H,H) coupling constants of vicinal sugar protons [31] (*Table 4*), a constrained *S*-type conformer population ($_{2'}T^{3'}$) of 85% can be calculated, compared to only 60% for U_d (*Table 4*). Due to the lack of an anomeric effect in the cases of *C*-nucleosides, the $N \rightleftarrows S$ conformer equilibrium of the *C*-nucleoside is strongly biased towards the *S*-type puckered pseudorotamer [32][33].

Table 4. ^{3}J*(H,H) Coupling Constants of 2′-Deoxypseudouridine* (**5**) *and 2′-Deoxyuridine* (U_d)

	^3J(H,H) [Hz][a]							Sugar conformation					Pseudorotational parameters			
	$J(1',2')$	$J(1',2'')$	$J(2',3')$	$J(2'',3')$	$J(3',4')$	$J(4',5')$	$J(4',5'')$	%*N*	%*S*	$\%\gamma^{g+}$	$\%\gamma^{t}$	$\%\gamma^{g-}$	P_N	P_S	$\Psi_{m(N)}$	$\Psi_{m(S)}$
5	10.10	6.05	5.90	2.25	2.70	4.20	5.40	15	85	38	38	24	26	143.7	36	37.8
U_d	6.70	6.85	6.40	4.25	3.95	3.50	5.10	40	60	49	34	17	26	161.8	36	32.0

[a]) Solvent, D_2O; r.m.s. ≤ 0.4 Hz; $|\Delta J_{max}| \leq 0.4$ Hz. Primed and doubly primed locants are used to distinguish between the two protons at C(2′) and C(5′).

Only very few reports appeared until now that deal with the incorporation of Ψ_d or its derivatives into oligonucleotides and their influence on the duplex or triplex stability. *Bhattacharya et al.* [9] reported that the incorporation of 2′-deoxy-N^1-methylpseudouridine ($m^1\Psi_d$; **3**) into G-rich, triplex-forming oligonucleotides does not improve the stability of triple helices, neither in the antiparallel nor in the parallel hybridization mode. *Rosenberg et al.* [10] showed that the incorporation of the same compound $m^1\Psi_d$ (**3**) into oligonucleotide duplexes also leads to a significant decrease in the T_m value. This is surprising, as it has been shown that oligonucleotide duplexes containing T_d in place of U_d exhibit an enhanced thermal stability of 0.5–0.8°/modification [34]. To the best of our knowledge, no report appeared until now describing the effect that unmodified 2′-deoxypseudouridine exerts on the structure and thermal stability of oligonucleotide duplexes. Therefore, 2′-deoxypseudouridine (**5**) as well as its N^1-substituted derivatives described above were incorporated into the non-self-complementary DNA·DNA duplex **25**·**26** (T_m 46°, *Table 2*) and the corresponding DNA·RNA hybrid (**25**·**27**; T_m 45°, *Table 2*). For this purpose, either one or two of the modified nucleotides were incorporated into the single strand **25** at position 5 as well as in positions 5 and 9. The modified oligonucleotides were hybridized with the corresponding complementary DNA (**26**) or RNA (**27**) strand, and the T_m values were determined by temperature-dependent UV measurements in a 10 mM Na-cacodylate, 10 mM $MgCl_2$, and 100 mM NaCl solution (pH 7.0) (*Table 2*). As can be seen from *Table 2*, the incorporation of one or two Ψ_d (**5**) residues leads to an only slight decrease ($\Delta T_m = -1 - 2°$/modification) of the thermal stability of **25**·**26** and **25**·**27**.

Next, the T_m values of DNA·DNA and DNA·RNA duplexes containing in one DNA strand either one or two N^1-(3-amino-3-oxopropyl)-2′-deoxypseudouridine

subunits (*Table 2*, **30**·**26**, **30**·**27**, **31**·**26**, **31**·**27**) were measured. From *Table 2* it can be seen that the introduction of a short side chain into 2′-deoxypseudouridine that does not protrude from the major groove of the double helix leads to a further slight decrease of the thermal stability. An analogous thermal stability was found for the duplex **39**·**26**, containing one 2′-deoxypseudouridine subunit with a methyl propanoate side chain, *i.e.*, **6**. The duplex **40**·**26**, carrying two corresponding Ψ_d^* residues, shows a T_m value of 39°.

An elongation of the side chain to an N^1-{3-[(3-aminopropyl)amino]-3-oxopropyl} residue does not further influence the T_m values, neither of the DNA·DNA nor of the DNA·RNA duplex (**32**·**26**, **32**·**27**, *Table 2*). Also the introduction of one 2′-deoxypseudouridine derivative with an N^1-{3-{[3-(1*H*-imidazol-4-yl)propyl]amino}-3-oxopropyl} side chain (see **35**) only slightly influences the thermal stability of duplexes with either the complementary DNA or RNA strand (**35**·**26**, **35**·**27**, *Table 2*).

Duplex Formation and Spectral Properties of Oligonucleotides With Fluorophor-Tethered 2′-Deoxypseudouridine Subunits. The results described above were good auspices for the introduction of bulky fluorophores into DNA·DNA and DNA·RNA duplexes *via* a 2′-deoxypseudouridine carrier. This was realized by two methods: *i*) First, the phosphoramidite **14** was used to synthesize a series of 31-mers (not shown), each carrying one fluorescein-labeled 2′-deoxypseudouridine residue without 6-aminohexanoic acid spacer. Between the nucleobase and the fluorophore, a 10-bond spacer is inserted. The T_m values were determined by temperature-dependent UV-measurements and compared with those of the unmodified duplexes. In all cases, the introduction of only one fluorescein-labeled 2′-deoxypseudouridine residue leads to a significant decrease of the thermal stability ($\Delta T_m = -5$ and $-8°$) [35] – a finding that is in contrast to our expectations.

Therefore, the oligonucleotides **33** and **38** carrying one or two fluorescein-labeled residues, with a 17-bond distance between the uracil base and the fluorophore, were prepared using the phosphoramidite **16** and hybridized to the counter strand **26** (*Table 2*). T_m Measurements, however, revealed that – regardless of the longer spacer length – the decrease of the thermal stability of the duplexes compared to that of the unmodified standard-oligomer duplex (**25**·**26**) is similar to that where the phosphoramidite **14** without 6-aminohexanoic acid spacer had been used (**33**·**26**: T_m 37°; **38**·**26**: T_m 32°). A doubly fluorescein-labeled duplex (**38**·**26**) exhibits a melting temperature that lies 15° below that of the standard duplex (*Table 2*).

ii) In a second approach, we used the amino-functionalized oligonucleotide **32** for a post-synthetic labeling reaction [36] with the ester **11** derived from *N*-hydroxysuccinimide to yield the oligomer **33**, which has already been described above. Moreover, compound **32** was reacted with the ester **12** yielding oligomer **34** which carries one 2′-deoxypseudouridine derivative with the cationic rhodamine dye JA-133 [37] in a 13-bond distance to the base. Both oligomers were hybridized to their complementary DNA strand (→ **33**·**26**, **34**·**26**), and their T_m values were determined by temperature-dependent UV, VIS, and fluorescence spectra. *Fig. 3*,*a* displays the UV-melting profile of **33**·**26** from which a T_m value of 38° can be taken, which means a T_m decrease of – 8° compared to the parent duplex **25**·**26**. A plot of A_{492} – the λ_{max} of fluorescein – *vs.* the temperature (*Fig. 3*,*b*) as well as a plot of the fluorescence emission intensity at 521 nm *vs.* the temperature (*Fig. 3*,*c*) do not show any cooperativity. The change in fluorescence emission between 15 and 60° amounts to 15% [27].

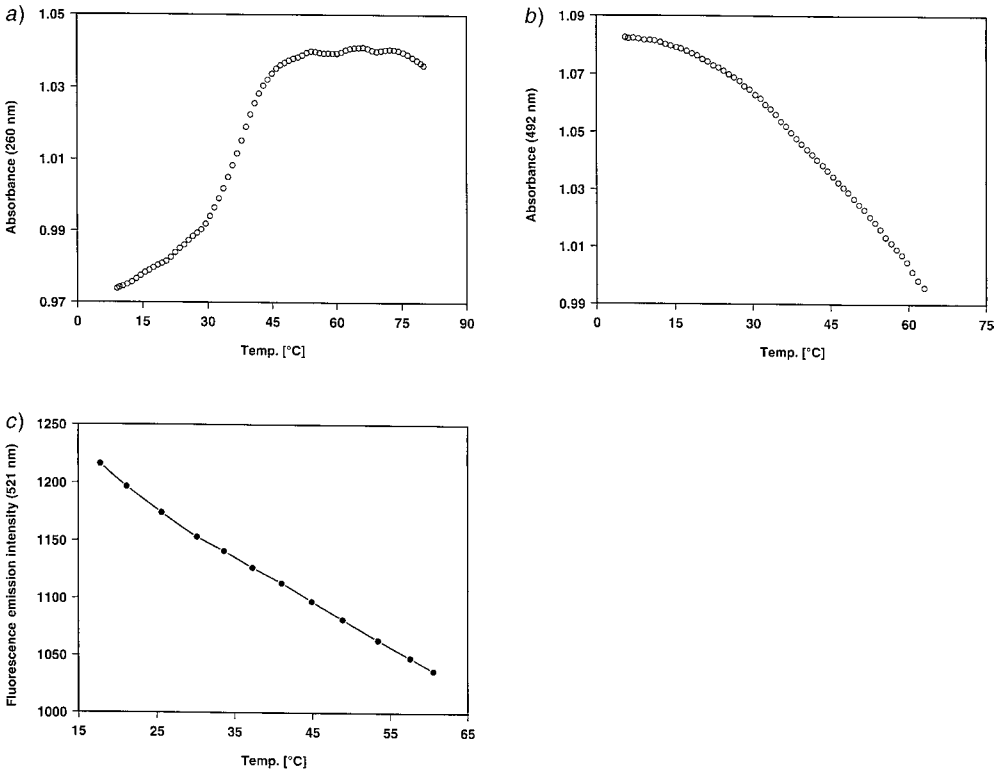


Fig. 3. a) A_{260} vs. *temperature plot of the duplex* **33**·**26**, b) A_{492} vs. *temperature plot of the duplex* **33**·**26**, *and* c) *fluorescence emission intensity at 521 nm* (excitation at 492 nm) *as a function of temperature of* **33**·**26**. In 10 mM Na-cacodylate, 100 mM NaCl, and 10 mM $MgCl_2$ (pH 7); oligomer concentration, 5 μM of each strand in *a*) and *b*), and 0.5 μM of each strand in *c*).

The oligonucleotide duplex **34**·**26** containing one cationic rhodamine dye (JA-133) labeled 2′-deoxypseudouridine subunit, exhibits a similar UV-melting temperature (T_m 37°, *Fig. 4*,*a*). In contrast to the duplex **33**·**26**, however, the T_m value of **34**·**26** can be verified by measuring the temperature-dependent absorbance at 615 nm, the λ_{max} of the rhodamine dye JA-133 (*Fig. 4*,*b*), as well as the temperature-dependent fluorescence emission at 639 nm (*Fig. 4*,*c*). The change in fluorescence emission between 15 and 70° amounts to 18% which is similar to that of **33**·**26**. In both cases, a melting temperature of 37° can be determined from the inflection points. Also, plots of temperature-dependent λ_{max} values of the excitation and emission spectra exhibit cooperativity (data not shown).

For comparison, two oligonucleotides **36** and **37** were synthesized containing one or two 5-{3-{{6-{[(fluorescein-6-yl)carbonyl]amino}hexyl}amino}-3-oxoprop-1-enyl}thymidine subunits from the phosphoramidite **18**. In this case, fluorescein and carrier base are separated by a spacer with 13 bonds. When these oligomers were hybridized with their corresponding DNA (**36**·**26**, **37**·**26**, *Table 2*) and RNA strands (**36**·**27**, **37**·**27**, *Table 2*), the resulting duplexes exhibit T_m values that are 3.5–4.0°/modification

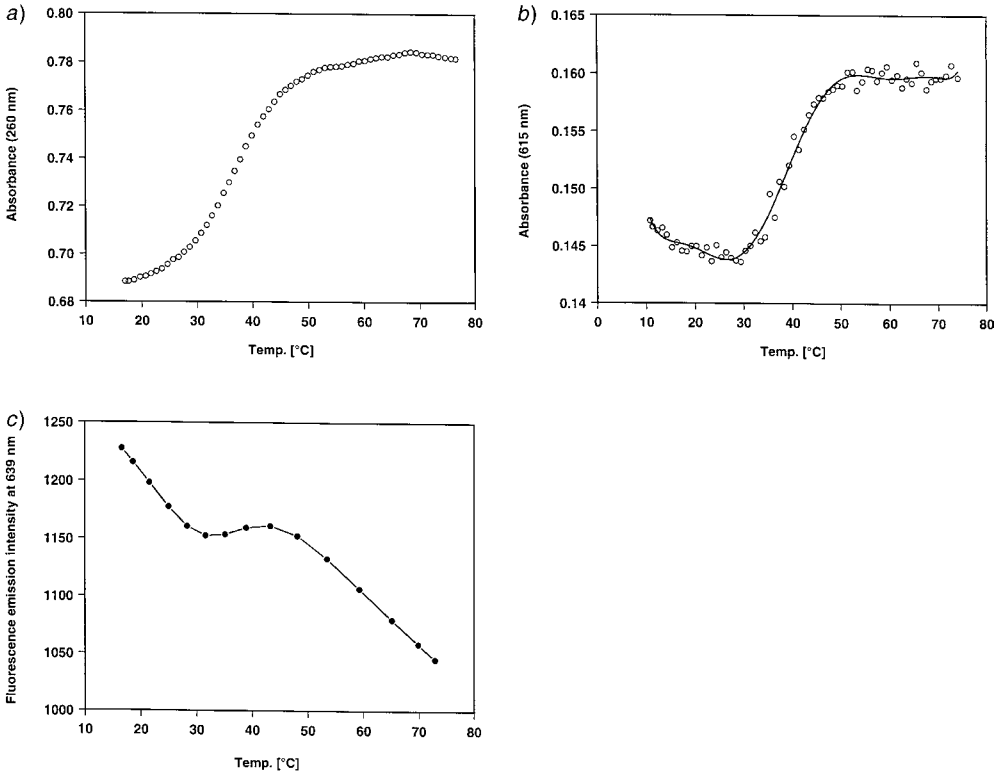


Fig. 4. a) A_{260} vs. *temperature plot of the duplex* **34**·**26**, b) A_{615} vs. *temperature plot of the duplex* **34**·**26**, *and* c) *fluorescence emission intensity at 639 nm* (excitation at 615 nm) *as a function of temperature of* **34**·**26**. In 10 mM Na-cacodylate, 100 mM NaCl, and 10 mM $MgCl_2$ (pH 7); oligomer concentration, 5 μM of each strand in *a*) and *b*), and 0.5 μM of each strand in *c*).

lower than those of the parent duplexes **25** · **26** and **25** · **27** (*Table 2*). This means that the fluorescein-labeled thymidine residue reduces the T_m value of the duplex only half as much as the fluorescein-labeled 2′-deoxypseudouridine, regardless of the shorter spacer length. *Fig. 5* shows exemplarily the melting profiles of **36** · **26** determined by three different methods, all displaying a T_m value of 42°.

Discussion and Conclusion. – A comparison of the T_m data presented above clearly indicates that 2′-deoxypseudouridine is a suitable nucleotide carrier for reporter groups such as fluorescein or the cationic rhodamine dye JA-133 [37]. However, the T_m values of oligonucleotides containing fluorophor-labeled Ψ_d are lower than corresponding duplexes carrying fluorescein-labeled 2′-deoxyuridine. The reduction of the T_m, however, is not due to the incorporation of the *C*-nucleoside or amino-functionalized 2′-deoxypseudouridine analogues *per se*; there is almost no decrease in the T_m prior to labeling with the fluorescent dyes. No significant difference in the melting temperature can be observed between a 13-bond and a 17-bond linker between the dye and the base carrier. Moreover, the charge of the dye ligand has no significant influence on the

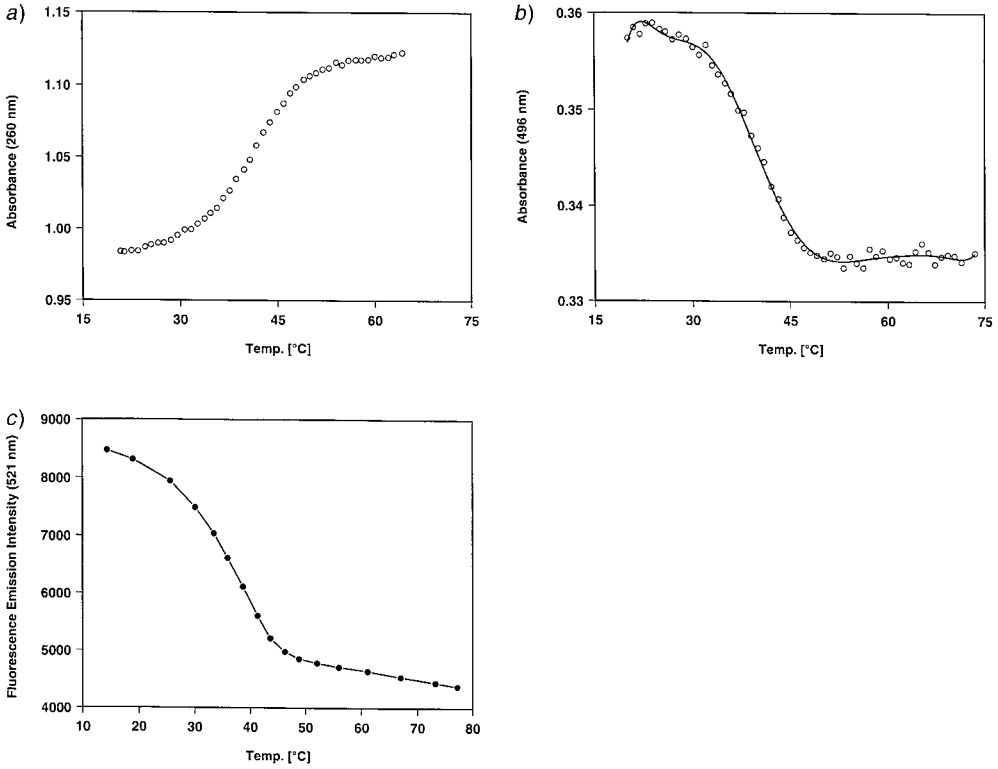


Fig. 5. a) A_{260} vs. *temperature plot of the duplex* **36·26**, b) A_{496} vs. *temperature plot of the duplex* **36·26**, *and* c) *fluorescence emission intensity at 521 nm* (excitation at 496 nm) *as a function of temperature of* **36·26**. In 10 mM Na-cacodylate, 100 mM NaCl, and 10 mM $MgCl_2$ (pH 7); oligomer concentration, 5 μM of each strand in *a*) and *b*), and 0.5 μM of each strand in *c*).

thermal stability of the duplex. A striking difference between the dye-labeled oligonucleotides **33·26** and **34·26** (*Table 2*), however, is the finding that only in the second case, cooperative melting can be observed by temperature-dependent monitoring of the optical properties of the dye, namely fluorescence emission and absorbance. It is assumed that this is due to the fact that in the first case (**33·26**), the fluorescein tether protrudes stretched-out from the major groove of the double helix and does not fold back because of charge repulsion between the negatively charged dye and the phosphodiester backbone, so that a dissociation of the DNA strands does not change the electronic environment of the fluorescent dye.

This implies, on the other hand, that in the case of **34·26** (*Table 2*), in which the cationic rhodamine dye (JA-133 [37]) is located at a distance of only 13 bonds from the uracil carrier, the fluorophore must be in close contact with the double helix, augmented by charge attraction between the positively charged dye and the phosphodiester backbone, so that a strand dissociation influences the electronic environment of the fluorophore.

The main advantage of 2′-deoxypseudouridine over the hitherto used 2′-deoxyuridine as a carrier nucleoside for reporter groups within an oligonucleotide chain is the

fact that it can be functionalized under very mild reaction conditions, which allows a post-synthetic cascade of modifications on the oligonucleotide level.

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Experimental Part

General. The phosphoramidites **17** and **18** were purchased from *Glen Research* (USA); the esters **10**–**12** of *N*-hydroxysuccinimide were generously provided by Dr. *H.-P. Josel* (*Roche Diagnostics GmbH*, Penzberg, Germany). The oligoribonucleotide **27** was a generous gift of Dr. *L. Beigelman* (*Ribozyme*, USA). Flash chromatography (FC): at 0.5 bar with silica gel *60* (*Merck*, Darmstadt, Germany). Solvent systems for FC and TLC: CH_2Cl_2/MeOH 9:1 (*A*), CH_2Cl_2/acetone 1:1 (*B*), CH_2Cl_2/acetone 7:3 (*C*), and i-PrOH/NH_3/H_2O 4:1:1 (*D*). Samples were collected with an *UltroRac II* fractions collector (*LKB Instruments*, Sweden). UV Spectra: *U3200* spectrophotometer (*Hitachi*, Japan). Fluorescence spectra: in 10 mM sodium cacodylate, 100 mM NaCl, and 10 mM $MgCl_2$ (pH 7.0) solns.; *F-4500* fluorescence spectrometer (*Hitachi*, Japan). NMR Spectra: *Avance-DPX-250* and *AMX-500* spectrometers (*Bruker*, Germany); δ values rel. to Me_4Si or external H_3PO_4. Microanalyses were performed by *Mikroanalytisches Laboratorium Beller* (Göttingen, Germany).

Oligonucleotide Synthesis and Purification. Oligonucleotides were synthesized with an *ABI-392* DNA synthesizer (*Applied Biosystems*, Weiterstadt, Germany) according to a standard protocol recommended by the manufacturer either in the 'trityl-off' or the 'trityl-on' mode with the modified phosphoramidites **8**, **14**, and **16**–**18** on a 1-μmol scale together with the phosphoramidites of $[(MeO)_2Tr]T_d$, $[(MeO)_2Tr]ib^2G_d$, $[(MeO)_2Tr]bz^6A_d$, and $[(MeO)_2Tr]bz^4C_d$. The coupling yields of the modified phosphoramidites **8**, **14**, and **16**–**18** were generally >95%.

In the cases of unmodified oligonucleotides as well as in the cases in which compounds **14** and **16** were used, the 5′-*O*-(dimethoxytrityl)-oligonucleotides ('trityl-on' synthesis) were cleaved from the solid support and subsequently deprotected in 25% NH_3/H_2O (12–18 h at 60°). The 5′-*O*-(dimethoxytrityl)-oligonucleotides were purified by reversed-phase HPLC (*RP-18*) with the following apparatus and procedure: 250 × 4 mm *RP-18* column (*Merck*, Germany); *Merck-Hitachi* HPLC apparatus, with a *655 A-12 D-2000* chromato-integrator (*Merck-Hitachi*, Darmstadt, Germany); eluents 0.1M $(Et_3NH)OAc$ (pH 7.0)/MeCN 95:5 (*U*) and MeCN (*V*); gradient *Ia:* 0–30 min 0–40% *V* in *U*, flow rate 1 ml min^{-1}; gradient *Ib:* 0–60 min 0–100% *V* in *U*, flow rate 1 ml min^{-1}. Removal of the 4,4′-dimethoxytrityl residues was performed by treating the purified oligomers with 80% aq. AcOH soln. for 20 min at r.t. After neutralization with Et_3N and evaporation, followed by co-evaporation with MeOH, the oligomers were again purified by HPLC (*RP-18*, above-mentioned device; gradient *II:* 0–25 min 5–20% *V* in *U*, 25–30 min 20% *V* in *U*, 30–35 min 20–5% *V* in *U*, 35–40 min 5% *V* in *U*, flow rate 1 ml min^{-1}). Subsequent desalting of all oligonucleotides was performed by HPLC (*RP-18*, 100 × 4 mm, apparatus as described above; solvent for adsorption H_2O; solvent for desorption, MeOH/H_2O 3:2).

Oligonucleotide syntheses with the commercially available phosphoramidites **17** and **18** were made in the 'trityl-off' mode. After cleavage from the solid support and evaporation, the crude detritylated oligonucleotides were purified as described above. Upon purification of the Ψ_d-containing oligonucleotides **28** and **29**, a slightly slower-migrating by-product appearing as a shoulder was cut off from the corresponding main peak.

The phosphoramidite **8** was used to prepare the fully protected polymer-bound oligonucleotide **19** in the'trityl-on' mode. Treatment of **19** with NH_3/H_2O (16 h, 60°) gave the base-deprotected 5′-*O*-(dimethoxytrityl)-oligomer **20**, which was purified, detritylated, and again purified as described above to give **30**. In an analogous manner, the oligomer **31** was prepared containing two N^1-(3-amino-3-oxopropyl)-modified 2′-deoxypseudouridine subunits.

Treatment of **19** with propane-1,3-diamine/H_2O 1:1 (24 h, r.t.) gave the 5′-*O*-(dimethoxytrityl)-oligomer **21**, which was purified, detritylated, and again purified as described above to give **32**. This oligonucleotide was subsequently used for further post-modification reactions with fluorescent-dye-labeled esters of *N*-hydroxysuccinimide as described below.

For the preparation of the oligomer **22**, the phosphoramidite **8** was used together with the phosphoramidites of $[(MeO)_2Tr]T_d$, $[(MeO)_2Tr]pac^6A_d$, $[(MeO)_2Tr]ac^4C_d$, and $[(MeO)_2Tr]$i-Pr-pac^2G_d in the 'trityl-off' mode. After the assembly of the oligomer **22** was complete, it was reacted in heterogeneous phase with 1M 1*H*-

imidazole-4-propanamine in THF at r.t. for 24 h. After filtration, the soln. was evaporated in a *Speedvac* concentrator at elevated temperature. The resulting oligonucleotide **23** was then detritylated and purified by HPLC (*RP-18*, 250 × 4 mm, gradient *II* as described above) and then desalted to give **35** (*Table 2*).

The enzymatic hydrolysis of the oligonucleotide 5′-d(*Ψ**AGGTCAATACT) (**24**) was performed as described [38], except that the incubation time was 5 h. HPLC (*RP-18*, 250 × 4 mm); gradient: 0–25 min 100% *U*, 25–70 min 100–0% *U* in *V*; flow rate 0.7 ml min^{-1}) separated the nucleoside constituents. On the basis of the following extinction coefficients ε_{260}: G_d 11700, C_d 7300, A_d 15400, T_d 8800 as well as of the given oligonucleotide sequence, the ε_{260} of fluorescein-labeled 2′-deoxypseudouridine was estimated to be 28000 (*Fig. 1*, *a* and *b*). All other oligonucleotides were characterized by MALDI-TOF spectra (*Table 3*).

Post-Synthetic Reaction of the Amino-Functionalized Oligonucleotide **32** *with the Esters* **11** *and* **12** *of* N-*Hydroxysuccinimide.* Two portions of the oligonucleotide **32** (5 A_{260} units, 37 μmol, each), both dissolved in 0.1M Na-borate buffer (pH 8, 200 μl), were reacted with either the fluorescein derivative **11** (5 mg, 6.8 μmol) [36] dissolved in DMF (100 μl) for 36 h (r.t.) or with compound **12** (2 mg, 2.5 μmol) dissolved in DMF (100 μl) for 3 d (r.t.) under exclusion of light. Both reaction mixtures were evaporated in a *Speedvac* concentrator at elevated temp. and then redissolved in the HPLC buffer *U* (500 μl). Both labeled oligonucleotides **33** and **34** were purified by HPLC (*RP-18*, 250 × 4 mm); gradient: 0–50 min 0–50% *V* in *U*; flow rate of 1 ml min^{-1}. The t_R of fluorescein-labeled **33**, 19.5 min; t_R of (JA-133)-labeled **34**, 33 min. Integration of the HPLC peaks allowed for both labeling reactions a rough estimation of a 50% yield.

Post-Synthetic Alkylation of the Oligomer **28** *with Methyl Acrylate.* Oligonucleotide **28** (5 A_{260} units, 46 nmol) was dissolved in 1M aq. $(Et_3NH)HCO_3$/EtOH 1:1 (100 μl), and methyl acrylate (3μl, 33μmol) was added. After 20 h at 37°, the mixture was lyophilized in a *Speedvac* concentrator (*Savant*, Farmingdale, USA), the residue redissolved in H_2O, and the mixture purified by reversed-phase HPLC (*RP-18*, 250 × 4 mm), gradient *II* as described above, flow rate 0.5 ml/min). The main zone (t_R 12.3 min) was collected and lyophilized: 2.5 A_{260} units of 5′-d(TAG G**6**C AAT ACT)-3′ (**39**; 47% by HPLC peak integration). The product was characterized by MALDI-TOF mass spectrometry as well as by enzymatic tandem hydrolysis with snake venom phosphodiesterase and alkaline phosphatase as described [38].

Determination of Melting Curves and Thermodynamics: a) Absorbance *vs.* temp. profiles were measured on *Cary-1* or *Cary-1E* spectrophotometers (*Varian*, Australia) with a *Cary* thermoelectrical controller. The T_m values were measured in the reference cell with a Pt-100 resistor. Thermodynamic data (ΔG°_{298}) were calculated with the program MeltWin 3.0 [39].

b) Fluorescence emission or excitation as a function of temperature were recorded on an *F-4500* fluorescence spectrophotometer (*Hitachi*, Japan) in a thermostatted 1-cm-quartz cuvette using an *RC6* thermostate (*MWG Lauda*, Germany).

*Methyl 5-(2′-Deoxy-β-*D-erythro-*pentofuranosyl)-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanoate* (**6**). To a soln. of 2′-deoxypseudouridine (**5**; 1.2 g, 5.26 mmol) in 1M $(Et_3NH)HCO_3$ (TBK; pH 8.5, 50 ml) and EtOH (60 ml), methyl acrylate (15 ml, 14.34 g, 166.6 mmol) was added slowly. After stirring for 20 h at r.t., the soln. was evaporated and co-evaporated twice with 50% aq. EtOH and then with toluene. FC (silica gel, column 13 × 5 cm, *A*) afforded 1.5 g (89%) of **6**. Colorless foam. TLC (silica gel, *A*): R_f 0.42. UV (H_2O): 270 (8600). ^{1}H-NMR ((D_6)DMSO): 11.30 (*s*, NH); 7.62 (*s*, H–C(6)); 4.97 (*d*, *J* = 3.8, OH–C(3′)); 4.81 (*t*, *J* = 5.8, H–C(1′)); 4.66 (*t*, *J* = 3.8, OH–C(5′)); 4.14 (*m*, H–C(3′)); 3.91 (*t*, *J* = 6.80, CH_2); 3.71 (*m*, H–C(4′)); 3.57 (*s*, MeO); 3.44 (*t*, *J* = 5.2, CH_2(5′)); 2.70 (*s*, *J* = 6.8, CH_2); 2.03 (*m*, H_β–C(2′)); 1.77 (*m*, H_α–C(2′)). Anal. calc. for $C_{13}H_{18}N_2O_7$ (314.3): C 49.68, H 5.77, N 8.91; found: C 49.39, H 5.70, N 8.82.

*Methyl 5-[2′-Deoxy-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-β-*D*-*erythro*-pentofuranosyl]-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanoate* (**7**). Compound **6** (960 mg, 3.05 mmol) was dried by repeated co-evaporation from anh. pyridine and then dissolved in pyridine (40 ml). 4,4′-Dimethoxytriphenylmethyl chloride (1.14 g, 3.29 mmol) was added, and the soln. was stirred for 4 h at r.t. MeOH (4 ml) and, 10 min later, a 5% aq. $NaHCO_3$ soln. (60 ml) were added. The aq. layer was extracted with CH_2Cl_2 (3 × 100 ml) and the org. layer dried (Na_2SO_4) and evaporated. FC (silica gel, column 15 × 5 cm, *B*) afforded 1.6 g (84%) of **7**. Colorless foam. TLC (silica gel, *B*): R_f 0.5. ^{1}H-NMR ((D_6)DMSO): 11.30 (*s*, NH); 7.47 (*s*, H–C(6)); 5.06 (*d*, *J* = 4.2, OH–C(3′)); 4.84 (*dd*, *J* = 5.0, 8.8, H–C(1′)); 4.11 (*m*, H–C(3′)); 3.86 (*m*, CH_2(5)); 3.73 (*m*, H–C(4′)); 3.75 (*s*, 2 MeO); 3.53 (*s*, COOMe); 3.10 (*m*, CH_2); 2.62 (*m*, CH_2); 2.11 (*m*, H_β–C(2′)); 1.82 (*m*, H_α–C(2′)). Anal. calc. for $C_{34}H_{36}N_2O_9$ (616.7): C 66.22, H 5.88, N 4.54; found: C 66.13, H 5.91, N 4.46.

*Methyl 5-[2′-Deoxy-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-β-*D*-*erythro*-pentofuranosyl]-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanoate 3′-(2-Cyanoethyl Diisopropylphosphoramidite]* (**8**). A soln. of **7** (400 mg, 0.65 mmol) in anh. CH_2Cl_2 (20 ml) was preflushed with Ar. Then, $(i\text{-}Pr)_2EtN$ (215 μl, 1.3 mmol) and 2-cyanoethyl diisopropylphosphoramidochloridite (258 μl, 1.12 mmol) were added. After stirring for 20 min at

r.t., an ice-cold 5% aq. $NaHCO_3$ soln. (20 ml) was added and the mixture extracted with CH_2Cl_2 (2×30 ml). The org. layer was dried (Na_2SO_4) and evaporated. FC (silica gel, column 10×5 cm, *C*) afforded a diastereoisomer mixture **8** (400 mg, 75%). TLC (silica gel, *C*): R_f 0.75, 0.83. ^{31}P-NMR ($CDCl_3$): 149.1; 149.6.

N^1-*(3-Aminopropyl)-5-[2′-deoxy-5′*-O-*(4,4′-dimethoxytriphenylmethyl)-β-*D-erythro-*pentofuranosyl]-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanamide* (**9**). To a soln. of **7** (500 mg, 0.81 mmol) in i-PrOH (25 ml), propane-1,3-diamine (8 ml, 96 mmol) was added during 1 h at r.t. After stirring for 48 h at r.t., the soln. was evaporated and co-evaporated from anh. pyridine and toluene. Precipitation from toluene gave **9**. Colorless solid (474 mg, 89%). TLC (silica gel, *D*): R_f 0.8. The product showed a positive ninhydrin reaction. ^{1}H-NMR ((D$_6$)DMSO): 11.30 (*s*, NH); 8.00 (*t*, $J = 10.8$, N*H*CH$_2$); 7.42 (*s*, H−C(6)); 4.82 (br. *s*, 9 H, H−C(1′), OH−C(3′), NH_3^+, 2H$_2$O); 4.07 (*m*, H−C(3′)); 3.80 (*m*, CH$_2$(5′)); 3.73 (*s*, 2 MeO); 3.67 (*m*, H−C(4′)); 3.04 (*m*, 2 CH$_2$); 2.54 (*m*, CH$_2$); 2.30 (*m*, CH$_2$); 2.07 (*m*, H$_\beta$−C(2′)); 1.81 (*m*, H$_\alpha$−C(2′)); 1.50 (*m*, CH$_2$). Anal. calc. for $C_{36}H_{42}N_4O_8$ (658.75): C 65.64, H 6.43, N 8.51; found: C 65.55, H 6.25, N 8.42.

N^1-*{3-[({3′,6′-Bis(2,2-dimethyl-1-oxopropoxy)-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-6-yl}carbonyl)amino]propyl}-5-[2′-deoxy-5′*-O-*(4,4′-dimethoxytriphenylmethyl)-β-*D-erythro-*pentofuranosyl]-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanamide* (**13**). To a soln. of **9** (400 mg, 0.64 mmol) in anh. pyridine (20 ml) 1-{[(3,6-di-*O*-pivaloyl)fluorescein-6-yl]oxy}pyrrolidine-2,5-dione (**10**; 411 mg, 0.64 mmol) [40] was added. After stirring at r.t. for 2 h, an ice-cold 5% aq. $NaHCO_3$ soln. (30 ml) was added, the aq. layer extracted (3×60 ml) with CH_2Cl_2, and the org. layer dried (Na_2SO_4) and evaporated. FC (silica gel, *B*) afforded 415 mg (56%) of **13**. Colorless solid. TLC (silica gel, *B*): R_f 0.23. A spot test with aq. NH_3 gave a positive fluorescein reaction. ^{1}H-NMR ($CDCl_3$): 9.7 (br. *s*, NH); 7.55 (*t*, $J = 7.6$, N*H*CH$_2$); 7.41 (*s*, H−C(6)); 4.83 (*dd*, $J = 5.5$, 9.4, H−C(1′)); 4.24 (*m*, H−C(3′)); *ca.* 3.7 (*m*, CH$_2$(5′), 2 MeO); 3.02 (*m*, 2 CH$_2$); 2.60–2.15 (*m*, 2 CH$_2$, H$_\beta$−C(2′)); *ca.* 1.8 (*m*, H$_\alpha$−C(2′)); *ca.* 1.5 (*m*, CH$_2$).

N^1-*{3-[({3′,6′-Bis(2,2-dimethyl-1-oxopropoxy)-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-6-yl}carbonyl)amino]propyl}-5-[2′-deoxy-5′*-O-*(4,4′-dimethoxytriphenylmethyl)-β-*D-erythro-*pentofuranosyl]-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanamide 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**14**). A soln. of **13** (296.5 mg, 0.25 mmol) in anh. CH_2Cl_2 (15 ml) was preflushed with Ar. Then, (i-Pr)$_2$EtN (84 µl, 0.50 mmol) and 2-cyanoethyl diisopropylphosphoramidochloridite (84 µl, 0.37 mmol) were added. After stirring for 20 min at r.t., the soln. was diluted with CH_2Cl_2 (30 ml) and extracted with 5% aq. $NaHCO_3$ soln. (20 ml). The org. layer was dried (Na_2SO_4), filtered, and evaporated. FC (silica gel, *B*) gave the diastereoisomer mixture **14** (270 mg, 78%). Colorless foam. TLC (silica gel, *B*): R_f 0.8. ^{31}P-NMR ($CDCl_3$): 149.11, 149.21.

N^1-*[3-({6-[({3′,6′-Bis(2,2-dimethyl-1-oxopropoxy)-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-6-yl}carbonyl)amino]-1-oxohexyl}amino)propyl]-5-[2′-deoxy-5′*-O-*(4,4′-dimethoxytriphenylmethyl)-β-*D-erythro-*pentofuranosyl]-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanamide* (**15**). Compound **9** (80 mg, 0.13 mmol) was reacted with the ester **11** (80 mg, 0.13 mmol) and worked up as described for compound **13**: 90 mg (53%) of **15**. Colorless foam. TLC (silica gel, *B*): R_f 0.2. A spot test with aq. NH_3 gave a positive fluorescein reaction. ^{1}H-NMR ($CDCl_3$): 9.5 (br. *s*, NH); 4.90 (*dd*, $J = 5.4$, 9.2, H−C(1′)); 4.30 (*m*, 1 H, H−C(3′)).

N^1-*[3-({6-[({3′,6′-Bis(2,2-dimethyl-1-oxopropoxy)-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-6-yl}carbonyl)amino]-1-oxohexyl}amino)propyl]-5-[2′-deoxy-5′*-O-*(4,4′-dimethoxytriphenylmethyl)-β-*D-erythro-*pentofuranosyl]-1,2,3,4-tetrahydro-2,4-dioxopyrimidine-1-propanamide 3′-(2-Cyanoethyl Diisopropylphosphoramidite]* (**16**). Compound **15** (200 mg, 0.15 mmol) was reacted with 2-cyanoethyl diisopropylphosphoramidochloridite (64 µl, 0.28 mmol) and worked up as described for **14**: 150 mg (67%) of **16**. Colorless foam. TLC (silica gel, *B*): R_f 0.7. ^{31}P-NMR ($CDCl_3$): 149.5; 149.1.

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