pH-Independent Triple-Helix Formation with 6-Oxocytidine as Cytidine Analogue

Uwe Parsch and Joachim W. Engels*[a]

Abstract: The syntheses of six different phosphoramidite building blocks of 6-oxocytosine and 5-allyl-6-oxocytosine as analogues of *N*(3)-protonated cytosine are described. These compounds have been incorporated into oligonucleotides by standard solid-phase synthesis. Hybridization of 15-mer Hoogsteen strands with target 21-mer duplexes was investigated. Comparison of the triplex-forming abilities of the different building blocks revealed that: i) 5-allyl substitution has a negative influence on triplex stability; ii) a uniform backbone of the Hoogsteen strand stabilizes triplexes relative to mixed backbones; iii) RNA strands with 6-oxocytidine or 5-allyl-6-oxocytidine do not form a triple helix with the DNA target duplex, probably due to backbone torsional constraints; and (iv) a 15-mer DNA sequence with three isolated 2′-deoxy-6-oxocytidines has the highest T_m of all cytidine analogues investigated in this study. CD experiments provided further evidence for the presence or absence of triplex structures. In the course of these temperature-dependent CD measurements we were able to detect duplex and triplex melting independent from each other at selected wavelengths. This methodology is especially interesting in cases where UV melting curves show only one transition owing to spectral overlap.

Keywords: bioorganic chemistry · circular dichroism · DNA recognition · oligonucleotides · triplexes

Introduction

In 1957, only four years after the discovery of the DNA double helix by Watson and Crick,[1] Felsenfeld, Davies, and Rich reported the detection of a triple helix based on polynucleotides.[2] The possibility that oligonucleotides could be used to regulate gene expression at the DNA level led to renewed interest in the formation of triple-stranded nucleic acids three decades later.[3–6] Since then, the triplex methodology has become the most promising technology for the sequence-specific recognition of DNA.[7, 8] Triplexes are formed from a duplex with purine-consecutive sequences and oligonucleotides that bind as a third strand in the major groove. In the parallel binding motif, a thymine in the third strand is used to form a Hoogsteen pair with adenine at neutral pH (T × A · T triad) (Scheme 1). There is, however, no natural base that can bind in a Hoogsteen fashion to guanine at pH 7. Generally, protonated cytosine is used in this capacity ($C^+ \times G \cdot C$ triad; Scheme 1).[9, 10] However, as the pK_a of protonated cytosine is ≈4.3 and the intracellular pH is ≈7.4, protonated cytosine has limited applicability as a component in a third strand targeted against G · C base pairs inside a cell. Although the cytosine residue appears to be protonated at pH values much higher than its intrinsic pK_a, its contribution to triplex stability at physiological pH is minimal.[11–15] Different approaches have been attempted to circumvent this difficulty. The first one was the use of pyrimidine nucleosides with substituents in the 5-position of the base.[11, 16–21] Another one was the use of 5-methylcytosine tethered with spermine at N(4).[22–26] A third attempt was the utilization of 5-substituted 2-aminopyridine *C*-nucleosides as protonated cytidine equivalents.[27–30] These molecules are more basic than cytosine and are protonated to a higher extent in neutral and slightly basic conditions. All of these experiments led to stabilization of the triple helices, but none overcame the intrinsic pH dependence. One of the most promising efforts involved the use of artificial nucleosides able to form two hydrogen bonds to guanine without protonation. Therefore, several groups suggested neutral heterocyclic mimics of protonated cytosine that permit the synthesis of oligomers having enhanced specific affinity for duplex DNA under physiological conditions. Ono et al. showed that deoxypyrimidine oligomers which contain pseudoisocytosine in place of cytosine form triplexes with an oligodeoxyribonucleotide duplex at pH 7.0.[31, 32] Dervan and coworkers reported that a base analogue, 1-(2-deoxy-β-D-ribofuranosyl)-3-methyl-5-amino-1H-pyrazolo[4,3-*d*]pyrimidin-7-one, can interact with G · C base pairs in duplex DNA over an extended pH range.[33, 34] The groups of Matteucci and

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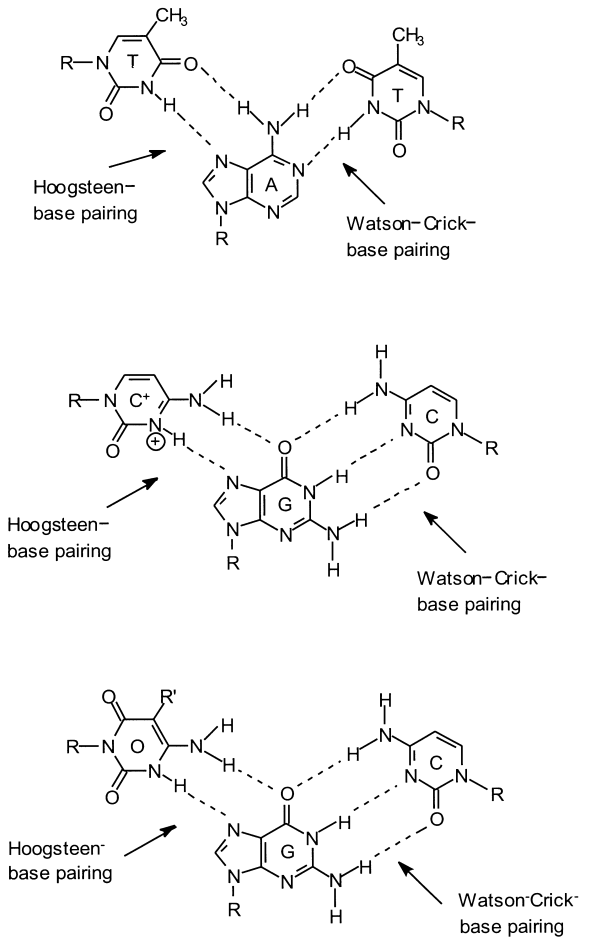
Miller used oligonucleotides with N^6-methyl-8-oxo-2′-deoxyadenosine and 8-oxo-2′-deoxyadenosine, respectively. UV melting experiments showed that these oligonucleotides had slightly lower T_m values than did those containing 2′-deoxycytidine.[35–37] A pyrazine base with a donor–donor–acceptor motif was incorporated into an oligonucleotide by von Krosigk and Benner. The T_m value increased by 5 °C compared with that of a 2′-deoxycytidine-containing oligomer at pH 7.3 with 1 M NaCl in the buffer.[38] The use of 5-methyl-6-oxocytosine was reported by two groups.[39–42] The 6-oxocytidine molecule seems to be the most promising candidate so far for stable and pH-independent triple-helix formation in this field. The other candidates mentioned are either purine-like structures which distort the oligonucleotide backbone or are *C*-nucleosides, which possibly form weaker base pairs than the corresponding *N*-nucleosides.[43]

Here, we report the design and synthesis of nonnatural analogues that bind G·C base pairs without protonation in a pyrimidine-motif triple-helical complex. Our design criteria for a nucleoside analogue included a pyrimidine-like ring system, to minimize anomalous conformational changes in the backbone, and a bidentate hydrogen-bond donor, to interact effectively with the O(6)-oxygen and N(7)-nitrogen of the target guanine. One molecule that meets these criteria is the nucleoside 6-oxocytidine (Scheme 1). In this work we compare the effects of different substituents in the backbone (2′-position) and the 5-position of the heterocycle on the stability of triple helices. As physicochemical methods, UV and CD spectroscopy were used.

Abstract in German: *Die Synthesen von sechs verschiedenen Phosphoramiditen mit 6-Oxocytosin bzw. 5-Allyl-6-oxocytosin als Analoga von protoniertem Cytosin werden beschrieben. Diese Bausteine konnten durch Festphasensynthese in Oligonucleotide eingebaut werden. Die Hybridisierungseigenschaften von 15mer Hoogsteen-Strängen mit 21mer Doppelhelices wurden untersucht. Der Vergleich der Triplex-bildenden Eigenschaften der verschiedenen Oligonucleotide erbrachte folgende Ergebnisse: i) 5-Allyl Substitution hat einen negativen Einfluss auf die Tripelhelix-Stabilität. ii) Ein einheitliches Rückgrat des Hoogsteen-Stranges stabilisiert Tripelhelices verglichen mit einem gemischten Rückgrat. iii) RNA-Stränge, die 6-Oxocytidin oder 5-Allyl-6-oxocytidin enthalten, bilden keine Tripelhelices mit der DNA Doppelhelix. Dies ist wahrscheinlich in einer ungewöhnlichen Zuckerkonformation dieser Derivate begründet, welche zu einer Verzerrung des Oligonucleotid Rückgrats führt. iv) Eine 15mer DNA-Sequenz mit drei isolierten 2′-Desoxy-6-oxocytidinen zeigt den höchsten T_m-Wert aller untersuchten Cytidin-Analoga dieser Studie. CD-Experimente gaben weitere Hinweise auf die Existenz bzw. Nicht-Existenz tripelhelicaler Assoziate. Während dieser Untersuchungen gelang es, eine Methode zur selektiven Erkennung des Triplex- bzw. Duplex-Schmelzprozesses zu etablieren. Diese Technik ist besonders in Fällen interessant, in denen UV-Schmelzkurven wegen Überlappung nur einen Übergang zeigen.*



Scheme 1. T-A-T, C^+-G-C, and O-G-C base triads; O = 6-oxocytosine, R′ = H or substituent, R = sugar.

Results and Discussion

Chemical synthesis of the phosphoramidites: The synthesis of 6-oxocytidine (**8**) was performed using the glycosylation procedure of Vorbrüggen (Scheme 2).[44, 45] Refluxing 6-oxocytosine (**4**) with hexamethyldisilazane (HMDS) and subsequent reaction of the persilylated base with 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (**3**) in the presence of the Lewis acid trimethylsilyl trifluoromethanesulfonate in 1,2-dichloroethane (DCE) afforded the desired 2′,3′,5′-tri-*O*-acetyl-6-oxocytidine (**6**) in 83% yield.

5-Allyl-6-oxocytosine (**5**) was synthesized from ethyl cyanoacetate (**1**) with allyl chloride in sodium ethanolate, and reaction of the product with urea in sodium methanolate under reflux conditions. Silylation of **5** with HMDS and subsequent glycosylation according to the procedure described above furnished 2′,3′,5′-tri-*O*-acetyl-5-allyl-6-oxocytidine (**7**) in only 46% yield. Here we were able to isolate four different products: **7**, its N(3)-regioisomer, and two other compounds of unknown structure.

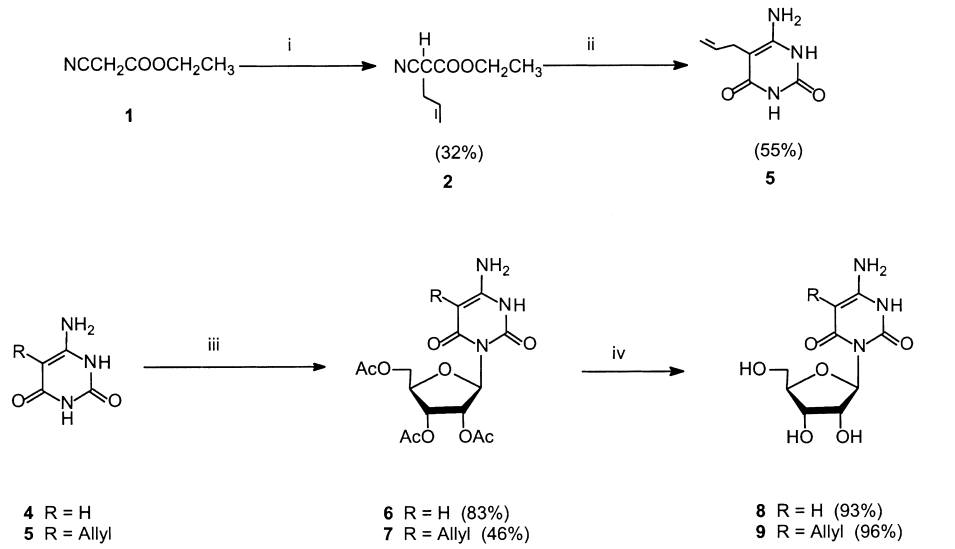
Deprotection of the acetylated nucleosides **6** and **7** in methanolic ammonia gave 6-oxocytidine (**8**) and 5-allyl-6-oxocytidine (**9**) in 93% and 96% yield, respectively. The assignment of **8** and **9** as the expected β-isomers relied on the existence of a ROESY cross-peak between H1′ and H4′, while

0947-6539/00/0613-2410 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 13

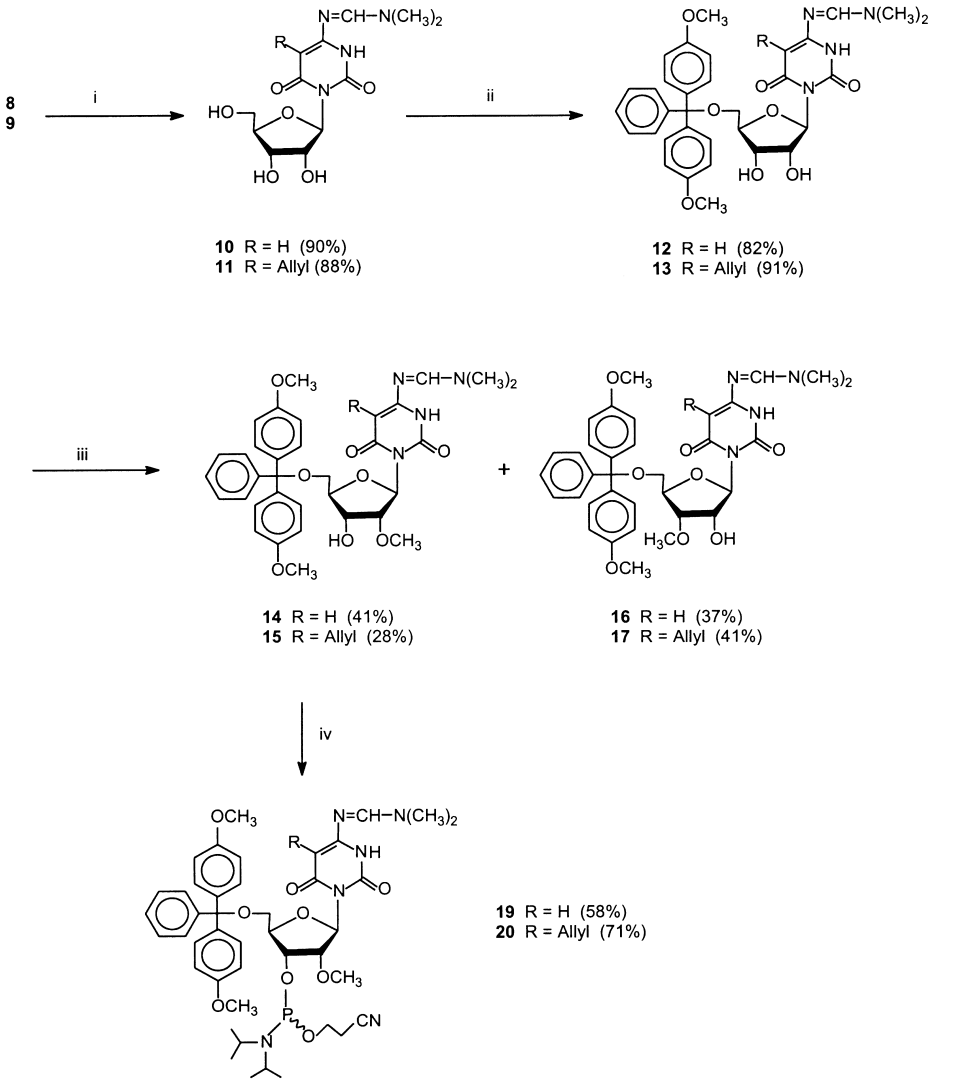
no cross-peak was observed between H1′ and H3′. The regiochemistry (N(1), N(3)) was assigned by a comparison of the chemical shifts of the amino proton resonances in the ^{1}H NMR spectra.[46]

To improve the low overall yield of **9**, we tested a palladium-catalyzed coupling reaction between **8** and allylbromide in DMF, adding triethylamine and tetrakistriphenylphosphinepalladium(0) as catalyst. The palladium-catalyzed variant gave an overall yield of 29% (starting from 6-oxocytosine (**4**), three reaction steps) compared with only 8% yield from the route described above (starting from ethyl cyanoacetate (**1**), four reaction steps).

The exocyclic amino functions of the unprotected nucleosides **8** and **9** were protected with the dimethylformamidine protecting group (Scheme 3). The products were converted with 4,4′-dimethoxytriphenylmethyl chloride (DMTrCl) in dry pyridine. In the next step we had to find a practical synthesis for the 2′-*O*-methylation of the nucleosides **12** and **13**. The problems encountered in the use of methyl iodide/silver oxide or trimethylsilyl diazomethane as a 2M solution in *n*-hexane have been described by Berressem and Engels.[42] We chose freshly prepared diazomethane/$SnCl_2$ as our methylation system. The monomethylation of the *cis*-glycol systems of **12** and **13** was performed in DMF according to a method first described by Robins and coworkers.[47] The regioisomer assignment was easily achieved by ^{1}H,^{1}H-COSY NMR spectroscopy. The last step in the syntheses was the phosphitylation of the 3′-OH groups. The 6-oxocytidine derivatives **14** and **15** were each dissolved in dry acetonitrile, and *N*,*N*-diisopropylethylamine (DIPEA) and the chlorophosphoramidite **18** were added. This afforded



Scheme 2. Synthesis of 6-oxocytidine (**8**) and 5-allyl-6-oxocytidine (**9**): reagents and conditions: i) 1) NaOEt, 2) allyl chloride, reflux, 3 h; ii) 1) NaOMe, 2) urea, reflux, 6 h; iii) 1) HMDS, $(NH_4)_2SO_4$, reflux, 24 h, 2) DCE, $TMSOSO_2CF_3$, 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (**3**), RT, 24 h; iv) NH_3, MeOH, RT, 24 h.

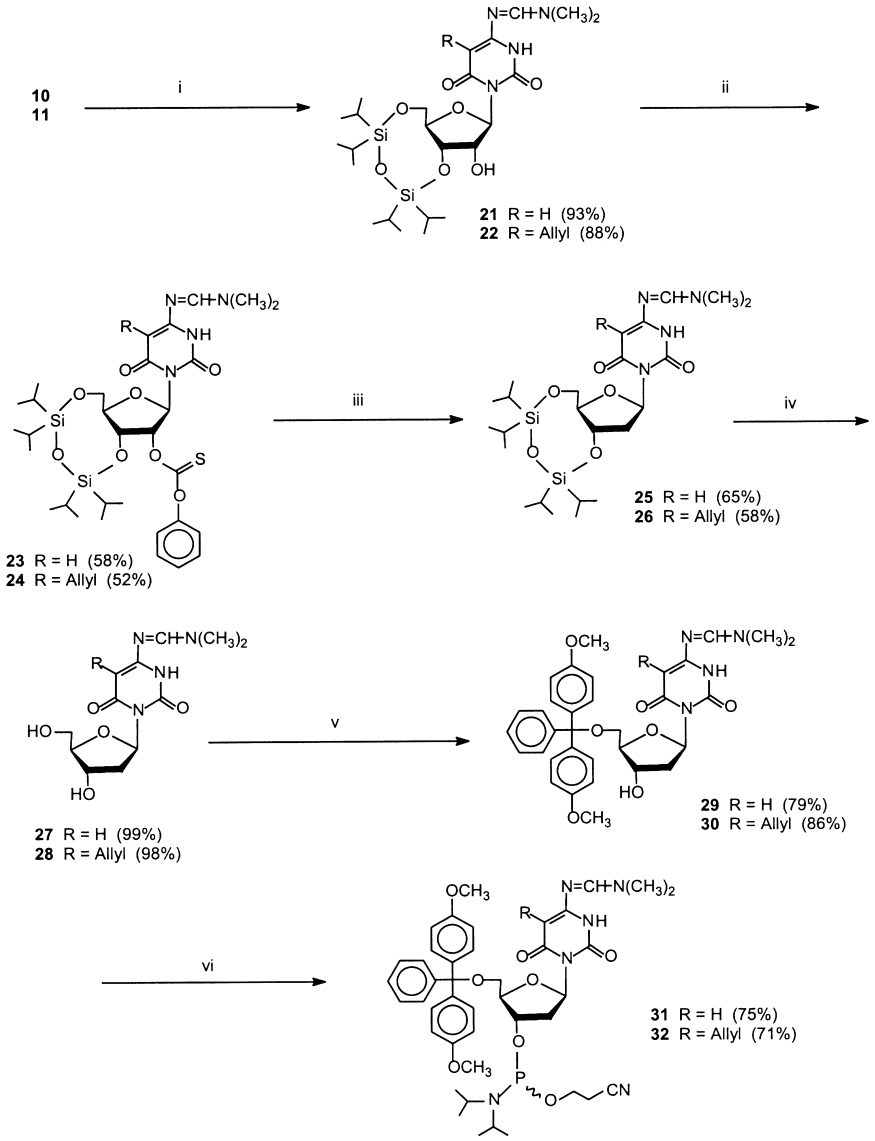


Scheme 3. Synthesis of the 2′-*O*-methylphosphoramidites **19** and **20**: reagents and conditions: i) $(MeO)_2CHNMe_2$, MeOH, reflux, 10 min; ii) DMTrCl, pyridine, **10**: RT, 3.5 h, **11**: RT, 6 h; iii) CH_2N_2, $SnCl_2$, DMF, 4 h, 0 °C → RT; iv) *i*PrNPCl(OCH_2CH_2CN) (**18**), DIPEA, CH_3N, **14**: RT, 0.5 h, **15**: RT, 2 h.

the desired phosphoramidite building blocks **19** and **20**.

To obtain the 2′-deoxyphosphoramidites, we started from 4-*N*-(dimethylformamidine)-6-oxocytidine **10** and 5-allyl-4-*N*-(dimethylformamidine)-6-oxocytidine **11**, using the Barton deoxygenation reaction (Scheme 4).[48] The use of 2′-deoxy sugars in the Vorbrüggen glycosylation reaction for the direct synthesis of 2′-deoxy nucleosides has certain disadvantages, mainly the lack of stereospecificity of this reaction.

The 6-oxocytidine derivatives **10** and **11** were protected simultaneously at the 5′- and 3′-positions by the Markiewicz protecting group in 93% and 88% yield, respectively.[49] The products **21** and **22** were dissolved in dry acetonitrile, and *N*,*N*-dimethylaminopyridine (DMAP) was added in each case. The addition of phenoxythiocarbonyl chloride led to the isolation of **23** (58%) and **24** (52%). These two compounds were deoxygenated in freshly distilled toluene with α,α'-azoisobutyronitrile (AIBN) and tributyltin hydride at 75 °C. The products **25** and **26** were obtained in 65% and 58% yield, respectively. After cleavage of the Markiewicz protecting group with tetrabutylammonium fluoride (TBAF) in THF, the 5′-OH groups of **25** and **26** were protected with DMTrCl in dry pyridine in high yields. The last step again was the phosphitylation to obtain the phosphoramidites **31** and **32**.



Scheme 4. Synthesis of the DNA phosphoramidites **31** and **32**: reagents and conditions: i) $TiPDSCl_2$, pyridine, **10**: 0.5 h at 0 °C, 1.5 h at RT, **11**: 0.5 h at 0 °C, 2.5 h at RT; ii) phenoxythiocarbonyl chloride, DMAP, CH_3CN, **21**: 2 h at RT, **22**: 4 h at RT; iii) *n*BuSnH, AIBN, toluene, 75 °C, **23**: 2 h, **24**: 1.5 h; iv) TBAF, THF, RT, 1 h; v) DMTrCl, pyridine, **27**: 3 h at −40 °C, 5 h at RT, **28**: 1 h at 0 °C, 8 h at RT; vi) *i*PrNPCl(OCH_2CH_2CN) (**18**), DIPEA, CH_2Cl_2, **29**: 0.5 h at RT, **30**: 4 h at RT.

Starting materials for the syntheses of the two RNA phosphoramidites were **12** and **13** (Scheme 5). Each of these compounds was dissolved in THF/pyridine 1:1, and silver nitrate and a 1M solution of *tert*-butyldimethylsilyl chloride (TBDMSCl) in THF were added.[50–53] Good yields of the desired products were obtained with a high preference for the 2′-protected species. The phosphitylation of the ribonucleosides was accomplished according to the method of Scarringe et al.[54] The phosphoramidites **37** and **38** were obtained in 72% and 71% yield, respectively.

Oligonucleotide synthesis, purification, and characterization: The 15-mer Hoogsteen strands as well as the 21-mer duplex strands were prepared by the phosphoramidite solid-phase technique under standard conditions.[55–59] The different 6-oxocytidine derivatives could be incorporated into the oligonucleotide strands with coupling efficiencies comparable to those of common nucleoside phosphoramidites. The oligonucleotides were purified by RP-HPLC (trityl on) for all DNA strands and by anion-exchange HPLC (trityl off) for all RNA strands. Finally, all sequences were characterized by MALDI mass spectrometry; for details, see experimental section.

UV thermal denaturation studies and CD measurements: Thermal denaturation experiments were performed in 50 mM sodium cacodylate buffer at pH 5.0, 6.0, 7.0, and 7.4 containing 20 mM magnesium chloride and 100 mM sodium chloride at oligonucleotide concentrations of 2 μM for each strand. The T_m values were determined by α versus T plots, where α is the fraction of molecules base-paired. Details of

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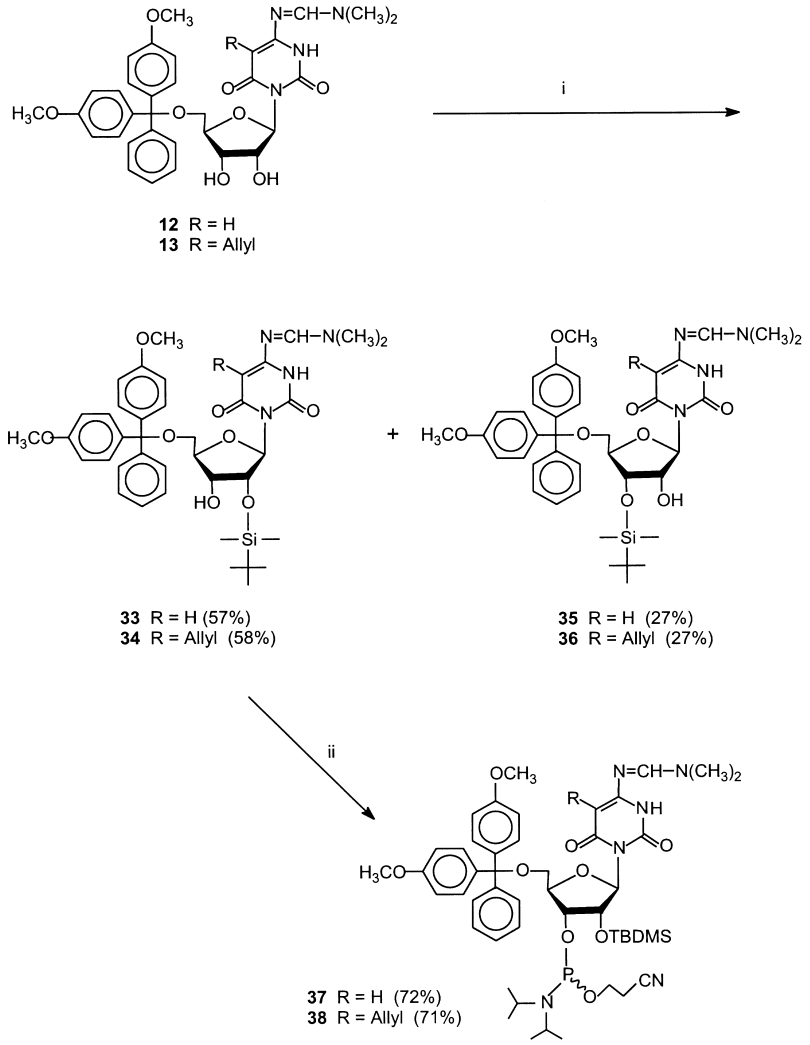

Scheme 5. Synthesis of the RNA phosphoramidites **37** and **38**: reagents and conditions: i) 1M solution of TBDMSCl in THF, $AgNO_3$, THF/pyridine 1:1, RT, 18 h; ii) *i*PrNPCl(OCH_2CH_2CN) (**18**), 1-methylimidazole, *sym*-collidine, CH_3CN, RT, 2 h.

the transformation of an absorbance versus temperature curve to an α versus T curve have been published by various authors.[60–62] The CD spectra were measured with the same samples. For details, again, see experimental section.

Triplex studies: After the synthesis and characterization of all oligonucleotides (Table 1), the triplex formation between the cytosine- or 6-oxocytosine-containing Hoogsteen strands **S01**–**S08** or **S11**–**S18** and their target duplexes **D1** or **D3** were studied. The melting temperatures (T_m) are summarized in Table 2. Samples that have formed a triple helix undergo two transitions, while samples without a triplex exhibit only the second transition, characterizing denaturation of the duplex.

We started with the hybridization of strand **S01** with its target duplex **D1** (**S01** + **D1** → **T01**). As expected, the UV melting curves revealed a pH-dependent triplex stability caused by the protonation of the central cytosine base of the Hoogsteen strand **S01**. Use of the RNA strand **S02** gave almost the same results (Table 2). The hybridization of strands **S03** and **S04** with duplex **D1** resulted in a virtually pH-independent triple-helix formation. A comparison of the two triplexes shows that a uniform backbone (DNA in **S04**) is more stable than a mixed backbone (one 2′-*O*-methyl in an otherwise DNA sequence in **S03**). This finding allows the conclusion that 2′-*O*-methyl nucleosides are not able to stabilize triplexes when incorporated in mixed-backbone Hoogsteen strands. Interestingly, **T04** is slightly more stable at physiological pH than under acidic conditions. To our surprise, RNA strand **S05** was not able to form a triple helix with its target duplex **D1**. We suggest that this unusual behavior is caused by an extraordinary sugar conformation of 6-oxocytidine that distorts the backbone in such a way as to prevent the hybridization of the third strand. This suggestion is supported by a comparison of the CD spectra of the single strands **S02**, **S05**, and **S08** (see Figure 8). The sugar conformation of the nucleoside was determined by X-ray crystallographic analysis.[63, 64]

The triplexes **T06** and **T07**, which contain one 5-allyl-6-oxocytosine in the middle of their sequences, exhibited very low T_m values (Table 2). Obviously the allyl substituent caused destabilization (compare **T03** and **T04** with **T06** and **T07** in Table 2). Advantageous base stacking or hydrophobic forces, which are responsible for the effect of the methyl group in stabilizing 5-methylcytosine compared with cytosine, cannot play a role in this case. A more detailed investigation utilizing NMR techniques is required to provide details of the structural perturbation caused by the allyl function.

Hoogsteen strand **S11**, which contains three cytosine bases, hybridizes with its target duplex **D3** (**S11** + **D3** → **T11**). We found a very strong pH dependence for the triplex stability (Table 2). The RNA strand **S12** formed a more stable triple helix over the pH range investigated than its DNA counterpart **S11** (see Figure 1 and Table 2). At pH 5, triplex and duplex melt together, showing only one transition in the UV melting curve. The formation of triplex **T13** was pH independent, but the T_m was degraded compared to **T03**. This confirms the hypothesis that a mixed backbone leads to destabilization. Triplex **T14** (**S14** + **D3**), with a uniform DNA backbone and three 6-oxocytosine bases in the sequence, gave the best results in this study. We found pH-independent triple-

Table 1. Oligonucleotide sequences, their calculated and detected masses, and calculated extinction coefficients.

Abbr.	Oligonucleotide sequence[b]	Calcd mass [Da]	Detected mass [Da] (MALDI)[c]	Extinction coefficient at 260 nm
D1	d(CCCAAAAAAAGAAAAAAACCC)	6387.30	6386.33	224.54
	d(GGGTTTTTTTCTTTTTTTGGG)	6461.23	6460.73	192.50
S01	d(TTTTTTTCTTTTTTT)	4485.97	4485.05	127.26
S02	r(UUUUUUUCUUUUUUU)	4529.59	4528.28	147.06
S03	d(TTTTTTT$^{H}O_{OMe}$TTTTTTT)	4532.00	4530.98	127.26
S04	d(TTTTTTT$^{H}O_{H}$TTTTTTT)	4501.97	4522.52	127.26
S05	r(UUUUUUU$^{H}O_{OH}$UUUUUUU)	4545.59	4568.73	147.06
S06	d(TTTTTTT$^{A}O_{OMe}$TTTTTTT)	4572.06	4571.05	127.26
S07	d(TTTTTTT$^{A}O_{H}$TTTTTTT)	4542.04	4542.31	127.26
S08	r(UUUUUUU$^{A}O_{OH}$UUUUUUU)	4585.66	4581.89	147.06
D3	d(CCCAAAAGAAGAAGAAAACCC)	6419.30	6418.68	224.54
	d(GGGTTTTCTTCTTCTTTTGGG)	6431.21	6431.45	191.06
S11	d(TTTTCTTCTTCTTTT)	4455.95	4454.75	125.82
S12	r(UUUUCUUCUUCUUUU)	4527.62	4526.80	140.48
S13	d(TTTT$^{H}O_{OMe}$TT$^{H}O_{OMe}$TT$^{H}O_{OMe}$TTTT)	4594.04	4595.09	125.82
S14	d(TTTT$^{H}O_{H}$TT$^{H}O_{H}$TT$^{H}O_{H}$TTTT)	4503.95	4526.76	125.82
S15	r(UUUU$^{H}O_{OH}$UU$^{H}O_{OH}$UU$^{H}O_{OH}$UUUU)	4575.62	4595.51	140.48
S16	d(TTTT$^{A}O_{OMe}$TT$^{A}O_{OMe}$TT$^{A}O_{OMe}$TTTT)	4714.22	4713.36	125.82
S17	d(TTTT$^{A}O_{H}$TT$^{A}O_{H}$TT$^{A}O_{H}$TTTT)	4624.16	4623.39	125.82
S18	r(UUUU$^{A}O_{OH}$UU$^{A}O_{OH}$UU$^{A}O_{OH}$UUUU)	4695.83	4695.64	140.48
K[a]	d(TTTTTTTTTTTTTTT)	4500.96	4500.72	127.98

[a] **K** = control. [b] $^{H}O_{OMe}$ = 2′-*O*-methyl-6-oxocytidine, $^{H}O_{H}$ = 2′-deoxy-6-oxocytidine, $^{H}O_{OH}$ = 6-oxocytidine, $^{A}O_{OMe}$ = 5-allyl-2′-*O*-methyl-6-oxocytidine, $^{A}O_{H}$ = 5-allyl-2′-deoxy-6-oxocytidine, $^{A}O_{OH}$ = 5-allyl-6-oxocytidine. [c] All molecular peaks found were in good agreement with the calculated molweights. In some cases sodium adducts were found.

Table 2. T_m values of triplexes and duplexes.

Abbr.	T_m values [°C][a]			
	pH 5.0	pH 6.0	pH 7.0	pH 7.4
T01	33.5	26.5	18.5	16.0
T02	35.0	27.5	18.0	14.0
T03	13.0	12.5	13.0	12.5
T04	16.0	16.0	17.0	18.0
T05	–	–	–	–
T06	3.5	3.0	2.5	3.0
T07	4.5	4.0	4.5	5.0
T08	–	–	–	–
T11	62.0	42.5	25.5	19.5
T12	66.5	53.0	36.0	30.0
T13	9.0	10.0	10.0	9.0
T14	27.5	27.0	27.0	27.0
T15	–	–	–	–
T16	–	–	–	–
T17	6.0	5.5	5.0	4.5
T18	–	–	–	–
K	–	–	–	–
D1	64.5	65.5	65.0	65.0
D3	64.5	67.0	66.5	66.5

[a] Errors: ± 0.5 °C; – indicates that no triplex was formed. The T_m values are means of at least four measurements.

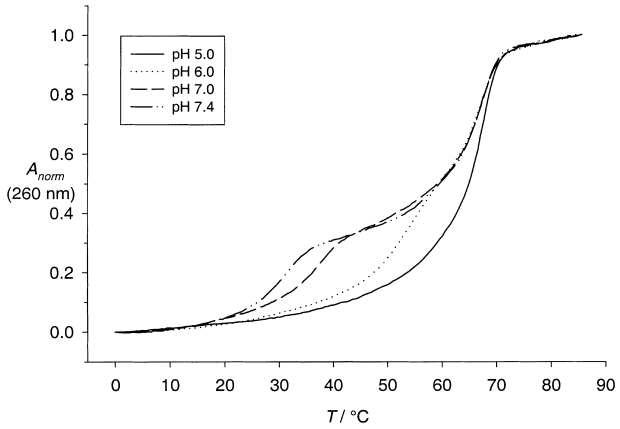

Figure 1. UV absorbance melting curves of triplex **T12** at different pH values in 50 mM sodium cacodylate buffer, 100 mM NaCl, and 20 mM $MgCl_2$.

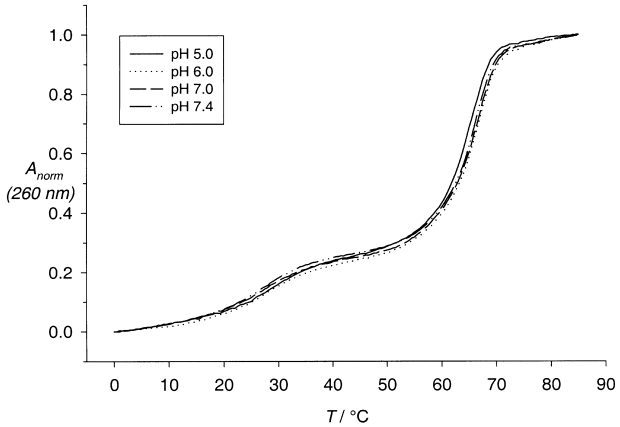

Figure 2. UV absorbance melting curves of triplex **T14** at different pH values in 50 mM sodium cacodylate buffer, 100 mM NaCl, and 20 mM $MgCl_2$.

helix formation with a T_m of 27 °C (Figure 2 and Table 2). This means a stabilization of 7.5 °C at pH 7.4 compared with its natural analogue **T11**. Figure 3 shows the T_m values of triplexes **T11**–**T14** at different pH values. The almost linear pH dependence over the whole pH range under investigation gives rise to the assumption that the cytosines are still (in part) protonated under neutral and slightly basic conditions. This is in agreement with the results of other groups.[11–15] The differences in stability are large and cannot be explained simply by the presence and absence of one hydrogen bond. We assume that factors like base stacking and electrostatic

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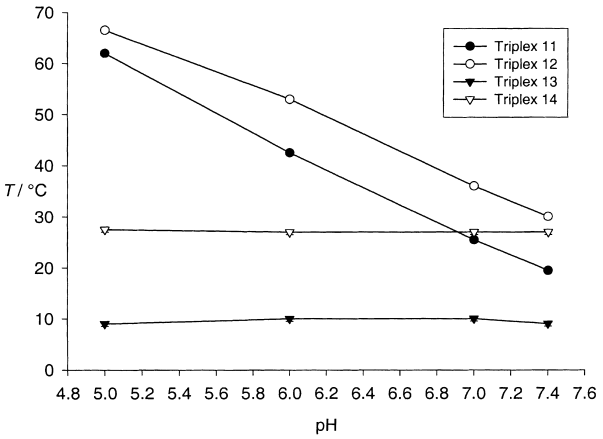


Figure 3. The T_m values of triplexes **T11**–**T14** at different pH values.

contributions of the positive charge at N(3) with the negative phosphate backbone play an important role in triple-helix stabilization.[15, 65]

The 6-oxocytidine-containing RNA strands **S15** and **S18** did not form triplexes for the reasons discussed above. Studying the hybridization of strands **S16** and **S17** confirmed the strong destabilization caused by the allyl group.

The specificity of the triple-helix formation from strands including 6-oxocytosine has already been investigated by Berressem and Engels.[42] Their studies showed the specificity of 6-oxocytosine to guanine in a Watson–Crick base pair. Triplex formation with reduced hypochromicity also occurred when 6-oxocytosine paired with adenine. This affinity to adenine was explained by the existence of an additional tautomer of 6-oxocytosine.[42] In the present work, we designed another control experiment, which should corroborate the existence of the two hydrogen bonds between 6-oxocytosine and guanine as postulated in Scheme 1. We synthesized a 15-mer polydeoxythymidine (strand **K** in Table 1). Thymine is able to form one hydrogen bond to guanine. If 6-oxocytosine forms only one hydrogen bond to guanine, the triplex between **K** and **D3** should have a similar T_m to that of **T14**. If the T_m of **KD3** is clearly lower than the one of triplex **T14**, one can assume that two hydrogen bonds between 6-oxocytosine and guanine really exist. Figure 4 shows the result of the control experiment in direct comparison with the melting curve of triplex **T14**. It can be seen that triplex **T14** is much more stable than **KD3**. In the control experiment only the upper half of the triplex–duplex transition is visible. An exact determination of the T_m is not possible, but the experiment gives strong evidence that the 6-oxocytosine–guanine–cytosine base triad exists as proposed in Scheme 1.

To confirm the triplex formation, different CD measurements were performed.[66–69] First we measured wavelength-dependent spectra of triplexes at different temperatures. All the triple helices investigated had a B-DNA structure, which was expected because of the underlying DNA duplexes. Only if the third strand was RNA (**T02** and **T12**) did some structural features of A-DNA appear (data not shown). Triplex CD spectra exhibit a negative band between 210 and 220 nm, which proves the triplex formation. This band disappears at temperatures above the T_m (Figure 5). Difference spectra (in which the sum of the duplex and single-strand spectra is subtracted from the triplex spectrum) are able to show the parts of the spectrum specific to the triplex (data not shown). Careful analysis of the CD spectra reveals that not only can triplex melting be seen at 210–220 nm, but also duplex melting at wavelengths between 240 and 250 nm (Figure 5). In this way it is possible to detect triplex and duplex melting independently of each other, by performing temperature-dependent experiments at selected wavelengths.[70] To detect triplex melting we chose 217 nm as the optimal wavelength. For duplex melting, 246 nm was ideal. This method worked well with DNA and RNA as third strand, and also when artificial nucleobases like 6-oxocytosine were incorporated into the Hoogsteen strand. An example is given in Figure 6. These selective CD melting curves are particularly useful in cases where duplex and triplex melting overlap. The UV melting curves of **T11** and **T12** showed only one transition at pH 5.0. Use of CD melting experiments at selected wavelengths allowed the individual observation of each melting process (Figure 7). Finally we investigated the CD spectra of the Hoogsteen single strands. This makes it possible to gain insight into conformational changes between strands which contain only natural nucleotides and those with 6-oxocytidines. In fact the CD spectra of the RNA single

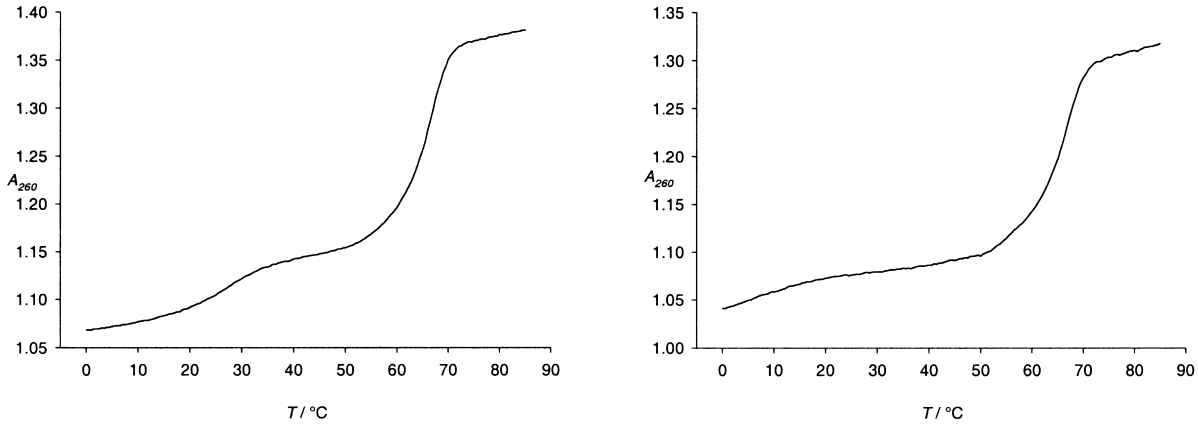
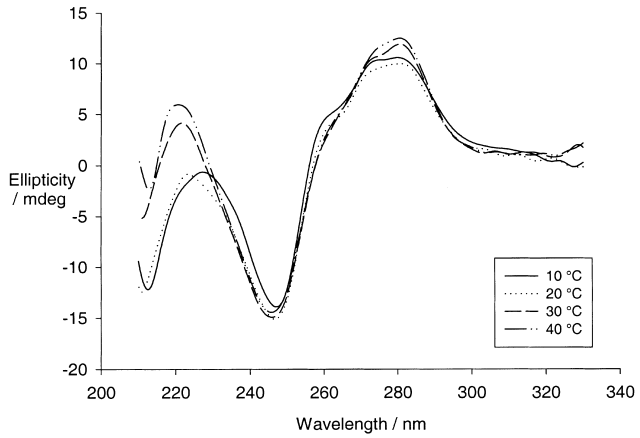


Figure 4. Comparison of UV melting curves of **T14** (left) and **KD3** (right) at pH 7.0.



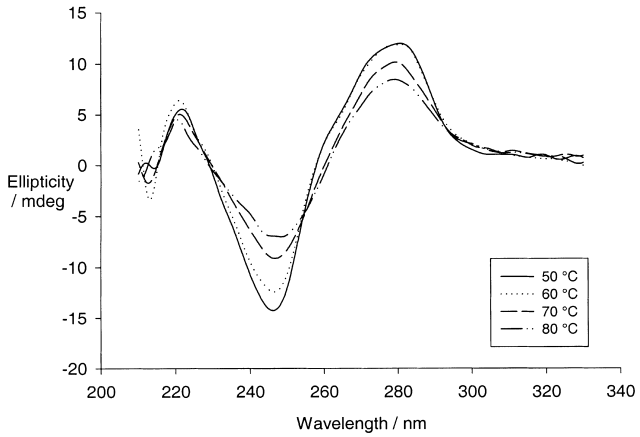


Figure 5. Wavelength-dependent CD spectra of triplex **T11** (pH 7.0).

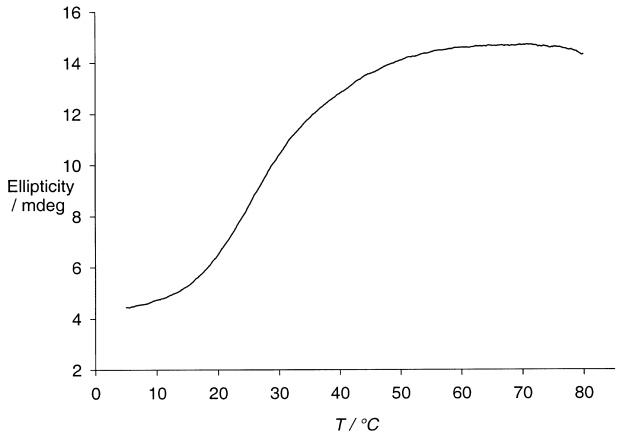


Figure 6. CD melting experiment for triplex **T14** (pH 7.0). Measurements were made at 217 nm and show selectively the triplex-to-duplex transition. The absolute value for the variation of ellipticity is 10.0 mdeg.

strands give further evidence that 6-oxocytidine leads to backbone distortion. The base stacking of strands **S05** and **S08** was much weaker than that in strand **S02**, as can be seen from the changes in the spectra between 260 and 290 nm (Figure 8). On the other hand, the single-strand spectra did not indicate why the 5-allyl function reduces the melting temperature. The spectra of **S01**, **S06**, and **S07** showed only minimal differences (data not shown).

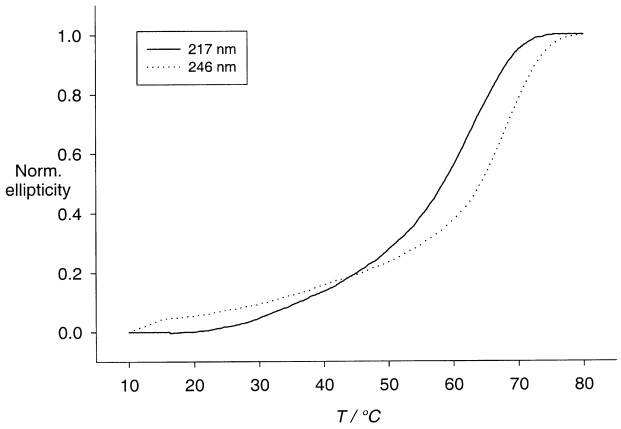


Figure 7. Selective CD melting experiments: detection of triplex ($\lambda =$ 217 nm) and duplex ($\lambda = 246$ nm) melting for triplex **T11** at pH 5.0. The absolute value for the variation of ellipticity is 15.5 mdeg (triplex) and 7.0 mdeg (duplex). The UV melting experiment shows only one transition in this case.

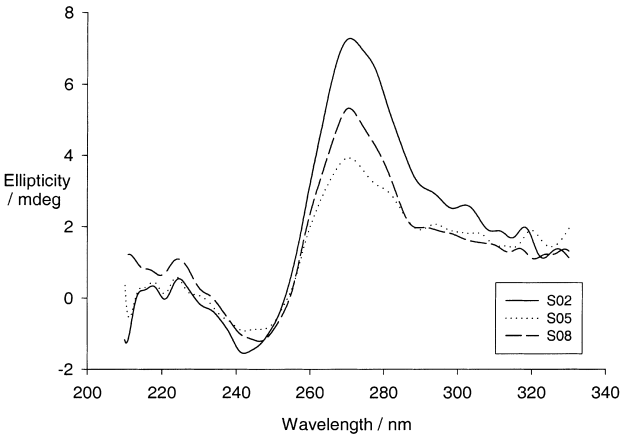


Figure 8. Wavelength-dependent CD spectra of the RNA single strands **S02**, **S05**, and **S08** (pH 7.0).

Determination of thermodynamic data: The measured UV melting curves were used to calculate the thermodynamic parameters ΔH°, ΔS°, and ΔG° for triplexes and duplexes. The van't Hoff plot ($R\ln K = -\Delta H^\circ \cdot 1/T + \Delta S^0$) yields ΔH° and ΔS°. The standard equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$ gives the free energy. Alternatively, ΔG° can be calculated from the equation $\Delta G^\circ = -RT\ln K$. All methods utilized and their underlying formulas were derived and explained in ref.[62]. The calculated values are given in Tables 3 and 4. In the cases of **T11** and **T12** it was impossible to calculate ΔH° and $T\Delta S^\circ$, because duplex and triplex transitions were not separated from each other. This means that the requirement of the two-state model was not fulfilled.[62] ΔG° could be calculated in these cases from the equation $\Delta G^\circ = -RT\ln K$.

Altogether, the free energy values show a tendency similar to that of the T_m's; the enthalpies and entropies are relative constant. Only triplex **T02** (third strand RNA) has enthalpy and entropy values a little lower. $-\Delta H^\circ$ varies between -2.9 and -3.9 kcal mol^{-1} per Hoogsteen base pair, depending on sequence and pH, which correlates with other published results.[8, 71] We could not find any distinct effect of 6-oxocytosine on ΔH° and ΔS°. This lack may be due to the small

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Table 3. Thermodynamic data of triplexes.

Triplex	pH	$-\Delta H^\circ$ [kcal mol^{-1}]	$-T\Delta S^\circ$ [kcal mol^{-1}] ($T=298$ K)	$-\Delta G^\circ$ [kcal mol^{-1}] ($T=298$ K)	$r^{2[a]}$
T01	5.0	54 ± 5	44 ± 5	9.5 ± 0.1	0.999
	6.0	52 ± 6	44 ± 6	8.6 ± 0.2	0.998
	7.0	51 ± 2	44 ± 2	7.1 ± 0.1	0.998
	7.4	50 ± 2	44 ± 2	6.8 ± 0.2	0.997
T02	5.0	48 ± 2	38 ± 2	9.7 ± 0.1	1.000
	6.0	47 ± 2	38 ± 2	8.8 ± 0.2	0.998
	7.0	44 ± 2	37 ± 2	7.3 ± 0.3	0.994
	7.4	45 ± 4	39 ± 4	6.5 ± 0.3	0.992
T03	5.0	54 ± 3	48 ± 3	6.4 ± 0.2	0.993
	6.0	54 ± 4	48 ± 4	6.0 ± 0.1	0.987
	7.0	52 ± 5	46 ± 5	6.4 ± 0.3	0.992
	7.4	53 ± 4	47 ± 4	6.2 ± 0.3	0.989
T04	5.0	52 ± 2	45 ± 2	7.2 ± 0.4	0.986
	6.0	53 ± 1	46 ± 1	7.3 ± 0.2	0.990
	7.0	55 ± 4	48 ± 4	7.1 ± 0.2	0.991
	7.4	54 ± 2	47 ± 2	7.1 ± 0.3	0.995
T11	5.0			13.5 ± 0.4	
	6.0			10.8 ± 0.3	
	7.0			8.3 ± 0.1	
	7.4			6.6 ± 0.2	
T12	5.0			12.6 ± 0.3	
	6.0			11.2 ± 0.1	
	7.0			9.9 ± 0.2	
	7.4			9.2 ± 0.2	
T14	5.0	53 ± 7	45 ± 7	8.3 ± 0.2	0.992
	6.0	57 ± 4	49 ± 3	8.4 ± 0.1	0.998
	7.0	56 ± 3	48 ± 3	8.3 ± 0.2	0.996
	7.4	57 ± 3	49 ± 3	8.4 ± 0.1	0.999

[a] r^2 = regression factor.

Table 4. Thermodynamic data of duplexes.

Duplex	pH	$-\Delta H^\circ$ [kcal mol^{-1}]	$-T\Delta S^\circ$ [kcal mol^{-1}] ($T=298$ K)	$-\Delta G^\circ$ [kcal mol^{-1}] ($T=298$ K)	$r^{2[a]}$
D1	5.0	104 ± 7	86 ± 6	18.7 ± 0.6	0.969
	6.0	105 ± 8	85 ± 7	19.8 ± 0.9	0.981
	7.0	101 ± 2	81 ± 2	19.4 ± 0.2	0.981
	7.4	103 ± 3	84 ± 3	19.1 ± 0.5	0.971
D3	5.0	122 ± 1	103 ± 1	19.4 ± 0.1	0.988
	6.0	115 ± 6	95 ± 6	19.2 ± 0.2	0.976
	7.0	115 ± 5	95 ± 5	19.4 ± 0.1	0.978
	7.4	119 ± 4	100 ± 4	19.5 ± 0.6	0.981

[a] r^2 = regression factor.

sequence differences and the relatively large uncertainty of the calculated data (see Tables 3 and 4).

Watson–Crick base pairs are enthalpically more stable (-4.8 to -5.8 kcal mol^{-1} per base pair in this study) than Hoogsteen base pairs.

Conclusion

We have shown in this study that 6-oxocytosine and 5-allyl-6-oxocytosine can be transformed into protected phosphoramidites and incorporated into oligonucleotides by standard solid-phase synthesis. Comparison of the triplex-forming abilities of the different building blocks showed that a 5-allyl substitution has a destabilizing influence on triplex stability. An important finding was that a uniform backbone is more stable than a mixed one. While triple helices with RNA third strands that contain only standard nucleosides are more stable than triplexes with DNA third strands (**T12** is more stable than **T11**), this study revealed that RNA strands with 6-oxocytidine or 5-allyl-6-oxocytidine did not form a triple helix with a DNA target duplex. We suppose this is because of an unusual conformation of the sugar of 6-oxocytidine, which leads to backbone torsional constraints. CD spectroscopic investigations gave further evidence for this interpretation. A 15-mer DNA sequence with three isolated 2′-deoxy-6-oxocytidines exhibited the highest T_m of all cytidine analogues investigated in this study. Temperature-dependent CD measurements permitted us to recognize duplex and triplex melting individually at selected wavelengths. This is especially interesting in cases where UV melting curves show only one transition.

Experimental Section

Melting points were not corrected. Microanalyses were carried out by the analytical laboratory of the institute. Yields refer to analytically pure compounds. TLC was performed with Merck silica gel plates 60 F_{254}, and column chromatography by standard techniques on silica gel. All reactions were carried out under an inert, dry atmosphere (Argon). All solvents were purchased in dry p.a. quality and stored over molecular sieves. The ^{1}H, ^{13}C, ^{31}P, ^{1}H,^{1}H-COSY, and HSQC NMR spectra were recorded on the following instruments: Bruker AM-250, WH-270, and AM-X400. The NMR spectra were referenced to the central [D_6]DMSO line at $\delta = 2.49$ (^{1}H) and 39.5 (^{13}C) or $CDCl_3$ at $\delta = 7.27$ (^{1}H). The mass spectra were determined by the electrospray technique on a VG Analytical Platform II instrument.

Ethyl-2-cyanopent-4-enoate (2): Ethyl cyanoacetate (53.3 mL, 500 mmol) was slowly added to a solution of sodium (11.5 g, 500 mmol) in ethanol (250 mL). A white precipitate of the sodium salt appeared. Allyl chloride (42.7 mL, 525 mmol) was added and the suspension was refluxed until neutrality was reached (3 h). The major part of the ethanol (180 mL) was removed by distillation. After the remaining mixture had been cooled to RT, ice water was added until a clear solution appeared (150 mL). Aqueous and organic phases were separated and the aqueous phase was extracted twice with ether. The collected organic phases were dried over $MgSO_4$. A distillation (30 cm Vigreux) did not lead to pure product. Finally, column chromatography was performed (*n*-hexane/ethyl acetate 4:1). The product was obtained as a colorless liquid (24.5 g, 32 %). B.p. 223 °C; $R_f = 0.31$ (*n*-hexane/ethyl acetate 4:1); ^{1}H NMR (250 MHz, $CDCl_3$, 25 °C): $\delta = 1.33$ (t, $J = 7.1$ Hz, 3 H, CH_3), 2.68 (m, 2 H, allyl CH_2), 3.57 (dd, $J = 6.4$ Hz, 0.9 Hz, 1 H, CH), 4.27 (q, $J = 7.1$ Hz, 2 H, CH_2), 5.26 (m, 2 H, allyl CH_2), 5.83 (m, 1 H, allyl CH); ^{13}C NMR (63 MHz, $CDCl_3$, 25 °C): $\delta = 13.88$ (CH_3), 33.71 (allyl CH_2), 37.38 (CH), 62.73 (CH_2), 115.97 (CN), 119.83 (allyl CH_2), 131.35 (allyl CH), 165.41 (C=O).

5-Allyl-6-oxocytosine (5): Urea (2.40 g, 40 mmol) and ethyl-2-cyanopent-4-enoate (**2**; 6.14 mL, 40 mmol) were added to a solution of sodium (1.84 g, 80 mmol) in methanol (100 mL), and the mixture was refluxed for 6 h. Some methanol (90 mL) was removed by distillation. Warm water (60 mL) was added and the solution was acidified with acetic acid (4.6 mL, 80 mmol). After filtration of the precipitate, a white solid was obtained (3.67 g, 55 %). M.p. > 250 °C; $R_f = 0.17$ (dichloromethane/methanol 9:1); UV: λ_{max} (H_2O, pH 7.0) = 271 nm; ^{1}H NMR (250 MHz, [D_6]DMSO, 25 °C): $\delta = 2.87$ (d, $J = 5.9$ Hz, 2 H, allyl CH_2), 4.90 (ddd, $J = 17.1$, 10.0, 2.1 Hz, 2 H, allyl CH_2), 5.68 (m, 1 H, allyl CH), 5.86 (br s, 2 H, NH_2), 9.89 (br s, 1 H, NH), 10.21 (br s, 1 H, NH); ^{13}C NMR (63 MHz, [D_6]DMSO, 25 °C): $\delta = 25.78$ (allyl CH_2), 82.42 (C-5), 113.73 (allyl CH_2), 136.20 (allyl CH), 150.18 (C-2), 151.49 (C-4), 163.88 (C-6); elemental analysis calcd (%) for $C_7H_9N_3O_2$ (167.17): C 50.29, H 5.43, N 25.14; found C 50.57, H 5.51, N 24.88; MS ESI(−): *m/z* (%): 165.9 (100) $[M-H]^-$, ESI(+): *m/z* (%): 167.8 (100) $[M+H]^+$.

General procedure for the synthesis of protected nucleosides 6 and 7: The nucleobase was refluxed in 10 equiv hexamethyldisilazane (HMDS) for 24 h, with a small amount of ammonium sulfate as starting reagent, and under exclusion of moisture. After cooling to RT, the HMDS was completely removed under reduced pressure, and 1,2-dichloroethane (100 mL) was added to the residue. Subsequently 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose and trimethylsilyl trifluoromethanesulfonate were added, and the clear solution was stirred at RT for 24 h. The reaction mixture was extracted with dichloromethane and saturated $NaHCO_3$ solution, and washed with brine. The collected organic phases were dried with $MgSO_4$, filtered and evaporated. The product was crystallized from ethanol/ether (**6**) or obtained after column chromatography (dichloromethane/methanol 95:5) (**7**).

2′,3′,5′-Tri-*O*-acetyl-6-oxocytidine (6): 6-Oxocytosine (**4**, Aldrich; 5.0 g, 39.3 mmol), HMDS (83 mL, 393 mmol), 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (11.9 g, 37.3 mmol), and trimethylsilyl trifluoromethanesulfonate (7.1 mL, 39.3 mmol) afforded **6** (11.93 g, 83%) as a white solid. M.p. 228–229 °C; $R_f = 0.27$ (dichloromethane/methanol 9:1); ^{1}H NMR (270 MHz, [D$_6$]DMSO, 25 °C): $\delta = 1.99$ (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 4.05 (m, 2H, 5′-H), 4.30 (m, 1H, 4′-H), 4.55 (s, 1H, 5-H), 5.46 (t, J = 7.1 Hz, 1H, 3′-H), 5.63 (dd, $J = 2.6$, 6.6 Hz, 1H, 2′-H), 6.14 (brs, 1H, 1′-H), 6.43 (brs, 2H, NH_2), 10.58 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 20.19$ (CH_3), 20.31 (CH_3), 20.42 (CH_3), 63.07 (C-5′), 69.87 (C-3′), 72.58 (C-2′), 73.58 (C-5), 77.71 (C-4′), 84.50 (C-1′), 150.30 (C-2), 154.33 (C-4), 161.86 (C-6), 169.28 (C=O), 169.52 (C=O), 170.04 (C=O); elemental analysis calcd (%) for $C_{15}H_{19}N_3O_9$ (385.34): C 46.75, H 4.97, N 10.90; found C 46.90, H 5.02, N 10.71; MS ESI(−): m/z (%): 384.1 (100) $[M-H]^-$.

2′,3′,5′-Tri-*O*-acetyl-5-allyl-6-oxocytidine (7): 5-Allyl-6-oxocytosine (5.95 g, 35.6 mmol), HMDS (75 mL, 356 mmol), 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose (10.82 g, 34.0 mmol), and trimethylsilyl trifluoromethanesulfonate (6.46 mL, 35.6 mmol) afforded **7** (6.65 g, 46%) as a white solid. M.p. 215–216 °C; $R_f = 0.48$ (dichloromethane/methanol 9:1); ^{1}H NMR (270 MHz, [D$_6$]DMSO, 25 °C): $\delta = 1.99$ (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.06 (s, 3H, CH_3), 2.92 (d, $J = 5.9$ Hz, 2H, allyl CH_2), 4.05 (m, 2H, 5′a-H, 4′H), 4.31 (m, 1H, 5′b-H), 4.94 (ddd, $J = 17.2$, 10.0, 2.1 Hz, 2H, allyl CH_2), 5.49 (t, J = 7.1 Hz, 1H, 3′-H), 5.63 (dd, $J = 7.2$, 2.1 Hz, 1H, 2′-H), 5.71 (m, 1H, allyl CH), 6.14 (brs, 3H, 1′-H and NH_2), 10.41 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 20.24$ (CH_3), 20.36 (CH_3), 20.48 (CH_3), 26.30 (allyl CH_2), 62.98 (C-5′), 69.76 (C-3′), 72.67 (C-2′), 77.69 (C-4′), 82.15 (C-5), 85.10 (C-1′), 114.06 (allyl CH_2), 135.79 (allyl CH), 149.66 (C-2), 150.93 (C-4), 161.63 (C-6), 169.32 (C=O), 169.57 (C=O), 170.07 (C=O); elemental analysis calcd (%) for $C_{18}H_{23}N_3O_9$ (425.39): C 50.82, H 5.45, N 9.88; found C 50.64, H 5.48, N 9.59; MS ESI(−): m/z (%): 426.1 (100) $[M-H]^-$.

General procedure for the deprotection of 6 and 7: Blocked nucleosides were dissolved in a solution of ammonia gas in methanol (saturated at −78 °C). After stirring for 24 h at RT, the solvent was removed under reduced pressure and the residue was crystallized from water (**8**) or obtained after column chromatography (dichloromethane/methanol 4:1) (**9**).

6-Oxocytidine (8): Compound **6** (5.0 g, 13.0 mmol) in a solution of ammonia gas in methanol (200 mL) gave **8** (3.12 g, 93%) as a white solid. M.p. 204–206 °C; $R_f = 0.08$ (ethyl acetate/methanol 4:1); UV: λ_{max} (H_2O, pH 7.0) = 268 nm; ^{1}H NMR (270 MHz, [D$_6$]DMSO, 25 °C): $\delta = 3.38$ (m, 1H, 5′a-H), 3.55 (m, 1H, 5′b-H), 3.63 (m, 1H, 4′-H), 4.05 (q, $J = 6.2$ Hz, 1H, 3′-H), 4.44 (q, $J = 5.3$ Hz, 1H, 2′-H), 4.53 (s, 1H, 5-H), 4.58 (t, $J = 4.9$ Hz, 1H, 5′-OH), 4.77 (d, $J = 6.4$ Hz, 1H, 3′-OH), 4.94 (d, $J = 5.3$ Hz, 1H, 2′-OH), 6.01 (d, $J = 3.9$ Hz, 1H, 1′-H), 6.34 (brs, 2H, NH_2), 10.42 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 62.48$ (C-5′), 70.23 (C-3′), 71.04 (C-2′), 74.03 (C-5), 84.09 (C-4′), 86.80 (C-1′), 150.73 (C-2), 154.06 (C-4), 162.85 (C-6); elemental analysis calcd (%) for $C_9H_{13}N_3O_6$ (259.22): C 41.70, H 5.06, N 16.21; found C 41.64, H 5.02, N 16.17; MS ESI(−): m/z (%): 258.0 (100) $[M-H]^-$.

5-Allyl-6-oxocytidine (9): Compound **7** (6.37 g, 14.97 mmol) in a solution of gaseous ammonia in methanol (200 mL) gave 5-allyl-6-oxocytidine (4.29 g, 96%) after chromatographic purification as a white foam.

Palladium-catalyzed method: 6-oxocytidine (**8**; 260 mg, 1.0 mmol) was dissolved in DMF (4 mL). Triethylamine (167 μL, 1.2 mmol) was added. The solution was stirred for 15 min. In a second flask tetrakistriphenylphosphinepalladium(0) (58 mg, 0.05 mmol) was suspended in DMF (1 mL). After addition of allyl bromide (87 μL, 1.0 mmol) a clear yellow solution was formed. The solution from the first flask was poured into the second flask and stirred at RT for 30 min. After that the reaction mixture was heated to 65–70 °C for 21 h. Then it was cooled to RT and the solvent was removed under reduced pressure. The residue was extracted in dichloromethane/water and the collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The syrup was purified by column chromatography (dichloromethane/methanol 9:1). After evaporation the product (110 mg, 37%) was obtained as a white foam. M.p. 237–238 °C; $R_f = 0.26$ (ethyl acetate/methanol 4:1); UV: λ_{max} (H_2O, pH 7.0) = 275 nm; ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.91$ (d, $J = 5.9$ Hz, 2H, allyl CH_2), 3.40 (m, 1H, 5′a-H), 3.60 (m, 2H, 4′-H, 5′b-H), 4.06 (q, $J = 5.6$ Hz, 1H, 3′-H), 4.45 (q, $J = 5.5$ Hz, 1H, 2′-H), 4.57 (t, $J = 4.8$ Hz, 1H, 5′-OH), 4.75 (d, $J = 6.5$ Hz, 1H, 3′-OH), 4.93 (ddd, $J = 17.2$, 10.0, 2.2 Hz, 2H, allyl CH_2), 4.94 (d, $J = 5.3$ Hz, 1H, 2′-OH), 5.69 (m, 1H, allyl CH), 6.04 (d, J = 3.9 Hz, 1H, 1′-H), 6.06 (brs, 2H, NH_2), 10.24 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 26.39$ (allyl CH_2), 62.44 (C-5′), 70.17 (C-3′), 70.98 (C-2′), 82.43 (C-5), 84.07 (C-4′), 87.26 (C-1′), 113.91 (allyl CH_2), 135.95 (allyl CH), 149.93 (C-2), 150.60 (C-4), 162.45 (C-6); elemental analysis calcd (%) for $C_{12}H_{17}N_3O_6$ (299.28): C 48.16, H 5.73, N 14.04; found C 47.93, H 5.93, N 13.93; MS ESI(−): m/z (%): 298.2 (100) $[M-H]^-$.

General procedure for protection of the exocyclic amino function of 8 and 9: The unprotected nucleosides **8** and **9** were suspended in dry methanol. The nucleoside was dissolved by heating. *N*,*N*-Dimethylformamide dimethyl acetal was added to the boiling solution. After 10 min the solution was cooled to RT and the solvent was removed under reduced pressure. The residue was purified by column chromatography with dichloromethane/methanol 9:1 → 4:1.

4-*N*-Dimethylformamidine-6-oxocytidine (10): 6-Oxocytidine (**8**; 3.80 g, 14.66 mmol) in methanol (100 mL) with *N*,*N*-dimethylformamide dimethyl acetal (10.6 mL, 73.3 mmol, 5 equiv) afforded **10** (4.14 g, 90%) as a white foam. $R_f = 0.35$ (dichloromethane/methanol 4:1), $R_f = 0.11$ (ethyl acetate/methanol 4:1); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.96$ (s, 3H, $N(CH_3)$), 3.09 (s, 3H, $N(CH_3)$), 3.41 (m, 1H, 5′a-H), 3.56 (m, 1H, 5′b-H), 3.65 (m, 1H, 4′-H), 4.08 (q, $J = 6.2$ Hz, 1H, 3′-H), 4.48 (q, $J = 4.8$ Hz, 1H, 2′-H), 4.59 (d, $J = 6.0$ Hz, 1H, 5′-OH), 4.78 (d, $J = 6.3$ Hz, 1H, 3′-OH), 4.96 (d, $J = 5.2$ Hz, 1H, 2′-OH), 5.05 (s, 1H, 5-H), 6.04 (d, $J = 3.8$ Hz, 1H, 1′-H), 8.14 (s, 1H, NCHN), 10.75 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 34.34$ ($N(CH_3)$), 40.34 ($N(CH_3)$), 62.45 (C-5′), 70.22 (C-3′), 70.95 (C-2′), 81.04 (C-5), 84.17 (C-4′), 87.00 (C-1′), 151.19 (C-2), 157.00 (NCHN), 159.22 (C-4), 163.54 (C-6); elemental analysis calcd (%) for $C_{12}H_{18}N_4O_8$ (314.30): C 45.86, H 5.77, N 17.83; found C 45.95, H 6.04, N 16.61; MS ESI(−): m/z (%): 313.1 (100) $[M-H]^-$.

5-Allyl-4-*N*-dimethylformamidine-6-oxocytidine (11): 5-Allyl-6-oxocytidine **9** (4.26 g, 14.23 mmol) in methanol (120 mL) with *N*,*N*-dimethylformamide dimethyl acetal (10.26 mL, 71.1 mmol, 5 equiv) afforded **11** (4.33 g, 88%) as a white foam. $R_f = 0.51$ (dichloromethane/methanol 4:1); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.94$ (s, 3H, $N(CH_3)$), 2.99 (d, $J = 6.3$ Hz, 2H, allyl CH_2), 3.03 (s, 3H, $N(CH_3)$), 3.41 (m, 1H, 5′a-H), 3.63 (m, 2H, 4′-H, 5′b-H), 4.10 (q, $J = 6.1$ Hz, 1H, 3′-H), 4.48 (q, $J = 5.8$ Hz, 1H, 2′-H), 4.59 (t, $J = 5.8$ Hz, 1H, 5′-OH), 4.80 (d, $J = 6.5$ Hz, 1H, 3′-OH), 4.87 (ddd, J = 17.2, 10.0, 2.2 Hz, 2H, allyl CH_2), 4.99 (d, $J = 5.3$ Hz, 1H, 2′-OH), 5.74 (m, 1H, allyl CH), 6.08 (d, $J = 3.7$ Hz, 1H, 1′-H), 7.97 (s, 1H, NCHN), 10.58 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 28.11$ (allyl CH_2), 33.78 ($N(CH_3)$), 40.05 ($N(CH_3)$), 62.50 (C-5′), 70.26 (C-3′), 71.02 (C-2′), 84.24 (C-4′), 87.55 (C-1′), 95.36 (C-5), 113.98 (allyl CH_2), 137.17 (allyl CH), 150.68 (C-2), 153.43 (C-4), 155.52 (NCHN), 163.45 (C-6); elemental analysis calcd (%) for $C_{15}H_{22}N_4O_6$ (354.36): C 50.84, H 6.26, N 15.81; found C 50.56, H 6.32, N 15.92; MS ESI(−): m/z (%): 353.2 (100) $[M-H]^-$.

General procedure for dimethoxytritylation of 10 and 11: The nucleosides **10** and **11** were each dissolved in dry pyridine and, after formation of the clear solution, 4,4′-dimethoxytriphenylmethyl chloride (DMTrCl) was added (1.2 equiv). After stirring the mixture at RT for 3.5 h (**10**) or 6 h (**11**), methanol was added. The pyridine was removed at 25 °C under reduced pressure and coevaporated with toluene three times. The residue was extracted with chloroform/water, and the collected organic phases were dried with $MgSO_4$, filtered and evaporated to dryness. The residue was purified by column chromatography (dichloromethane/methanol 95:5).

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-4-*N*-dimethylformamidine-6-oxocytidine (12): Nucleoside **10** (3.96 g, 12.6 mmol) in pyridine (90 mL) with DMTrCl (5.12 g, 15.1 mmol) afforded **12** (6.37 g, 82%) as a white solid. $R_f = 0.41$ (dichloromethane/methanol 9:1), $R_f = 0.33$ (ethyl acetate/meth-

0947-6539/00/0613-2418 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 13

anol 4:1); ^{1}H NMR (270 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.97$ (s, 3H, N(CH$_3$)), 3.08 (s, 3H, N(CH$_3$)), 3.08 (m, 2H, 5′-H), 3.71 (s, 3H, OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.82 (m, 1H, 4′-H), 4.15 (q, $J = 7.5$ Hz, 1H, 3′-H), 4.31 (m, 1H, 2′-H), 4.75 (d, $J = 7.5$ Hz, 1H, 3′-OH), 4.99 (d, $J = 4.7$ Hz, 1H, 2′-OH), 5.06 (s, 1H, 5-H), 6.11 (brs, 1H, 1′-H), 6.81–7.41 (m, 13H, H$_{ar}$), 8.14 (s, 1H, NCHN), 10.70 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 34.33$ (N(CH$_3$)), 40.51 (N(CH$_3$)), 54.96 (OCH$_3$), 65.04 (C-5′), 70.40 (C-3′), 71.85 (C-2′), 80.95 (C-5), 81.87 (C-4′), 85.25 (DMTr), 87.90 (C-1′), 113.04, 126.54, 127.72, 129.67, 129.83, 135.76, 145.14 (DMTr), 150.98 (C-2), 157.02 (NCHN), 157.96 (DMTr), 159.29 (C-4), 163.30 (C-6); elemental analysis calcd (%) for $C_{33}H_{36}N_4O_8$ (616.68): C 64.27, H 5.88, N 9.09; found C 64.06, H 5.96, N 8.80; MS ESI(−): *m/z* (%): 615.7 (100) $[M-H]^-$.

5-Allyl-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-dimethylformamidine-6-oxocytidine (13): Nucleoside **11** (1.19 g, 3.36 mmol) in pyridine (25 mL) with DMTrCl (1.37 g, 4.03 mmol) afforded **13** (2.00 g, 91%) as a white solid. $R_f = 0.32$ (dichloromethane/methanol 95:5), $R_f = 0.47$ (dichloromethane/methanol 9:1); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.94$ (s, 3H, N(CH$_3$)), 2.99 (s, 2H, allyl CH$_2$), 3.03 (s, 3H, N(CH$_3$)), 3.08 (m, 2H, 5′-H), 3.70 (s, 3H, OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.84 (q, $J = 5.6$ Hz, 1H, 4′-H), 4.18 (q, $J = 7.2$ Hz, 1H, 3′-H), 4.32 (q, $J = 4.3$ Hz, 1H, 2′-H), 4.78 (d, $J = 7.6$ Hz, 1H, 3′-OH), 4.90 (ddd, $J = 17.3$, 9.8, 1.8 Hz, 2H, allyl CH$_2$), 5.02 (d, $J = 4.7$ Hz, 1H, 2′-OH), 5.72 (m, 1H, allyl CH), 6.15 (s, 1H, 1′-H), 6.80–7.40 (m, 13H, H$_{ar}$), 7.96 (s, 1H, NCHN), 10.59 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 28.16$ (allyl CH$_2$), 33.76 (N(CH$_3$)), 40.04 (N(CH$_3$)), 54.98 (OCH$_3$), 65.05 (C-5′), 70.44 (C-3′), 71.91 (C-2′), 84.09 (C-4′), 85.26 (DMTr), 88.20 (C-1′), 95.30 (C-5), 113.08 (DMTr), 113.91 (allyl CH$_2$), 126.54, 127.79, 129.69, 129.84, 135.70 (DMTr), 135.86 (allyl CH), 137.22, 145.17 (DMTr), 153.50 (C-2), 155.46 (NCHN), 157.94 (C-4), 158.01 (DMTr), 163.20 (C-6); elemental analysis calcd (%) for $C_{36}H_{40}N_4O_8$ (656.74): C 65.84, H 6.14, N 8.53; found C 65.55, H 6.22, N 8.62; MS ESI(−): *m/z* (%): 655.4 (100) $[M-H]^-$.

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-2′-*O*-methyl-6-oxocytidine (14) and **5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-3′-*O*-methyl-6-oxocytidine (16)**: A solution of tin(II) chloride (0.27 g, 1.42 mmol, 0.25 equiv) in DMF (10 mL) was added to a solution of nucleoside **12** (3.50 g, 5.68 mmol) in DMF (60 mL). The reaction flask was cooled in an ice bath. A solution of diazomethane in ether (14 mL, approx. 0.3 M) was added slowly. (Diazomethane was freshly prepared with the Diazald™ kit diazomethane generator from Aldrich.) Further portions of diazomethane (14 mL) were added every 45 min. After 4 h the reaction was stopped by addition of a few drops of conc. ammonia solution. The solvent was removed under reduced pressure, and the syrup was extracted with chloroform/water. The collected organic phases were dried over MgSO$_4$, filtered, and evaporated to dryness, then purified by column chromatography (chloroform/acetonitrile/methanol 60:24:1.

14: $R_f = 0.36$ (chloroform/acetonitrile/methanol 20:8:1); ^{1}H NMR (270 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.96$ (s, 3H, N(CH$_3$)), 3.08 (s, 3H, N(CH$_3$)), 3.09 (m, 2H, 5′-H), 3.31 (s, 3H, 2′-OCH$_3$), 3.71 (s, 3H, OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.82 (m, 1H, 4′-H), 4.03 (dd, $J = 6.2$, 2.5 Hz,1H, 2′-H), 4.24 (m, 1H, 3′-H), 4.78 (d, $J = 8.2$ Hz, 1H, 3′-OH), 5.07 (s, 1H, 5-H), 6.13 (brs, 1H, 1′-H), 6.81–7.40 (m, 13H, H$_{ar}$), 8.14 (s, 1H, NCHN), 10.77 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 34.31$ (N(CH$_3$)), 40.42 (N(CH$_3$)), 54.94 (OCH$_3$), 57.69 (2′-OCH$_3$), 64.55 (C-5′), 70.39 (C-3′), 80.90 (C-5), 81.40 (C-2′), 81.85 (C-4′), 85.25 (DMTr), 85.30 (C-1′), 113.05, 126.50, 127.70, 129.65, 129.77, 135.72, 145.04 (DMTr), 150.80 (C-2), 157.02 (NCHN), 157.95 (DMTr), 159.26 (C-4), 163.07 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(−): *m/z* (%): 629.2 (100) $[M-H]^-$.

16: $R_f = 0.28$ (chloroform/acetonitrile/methanol 20:8:1); ^{1}H NMR (270 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.96$ (s, 3H, N(CH$_3$)), 3.09 (s, 3H, N(CH$_3$)), 3.10 (m, 2H, 5′-H), 3.25 (s, 3H, 3′-OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.73 (s, 3H, OCH$_3$), 3.91–3.99 (m, 2H, 3′-H, 4′-H), 4.54 (dt, $J = 2.6$, 5.9 Hz, 1H, 2′-H), 5.02 (d, $J = 6.6$ Hz, 1H, 2′-OH), 5.06 (s, 1H, 5-H), 6.10 (d, $J = 1.5$ Hz, 1H, 1′-H), 6.82–7.40 (m, 13H, H$_{ar}$), 8.15 (s, 1H, NCHN), 10.74 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 34.34$ (N(CH$_3$)), 40.49 (N(CH$_3$)), 54.98 (OCH$_3$), 57.35 (3′-OCH$_3$), 64.51 (C-5′), 70.45 (C-2′), 79.87 (C-3′), 79.97 (C-4′), 80.90 (C-5), 85.27 (DMTr), 88.10 (C-1′), 113.11, 126.59, 127.76, 129.66, 129.79, 135.80, 145.05 (DMTr), 151.00 (C-2), 157.02 (NCHN), 158.02 (DMTr), 159.33 (C-4), 163.31 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(−): *m/z* (%): 629.2 (100) $[M-H]^-$.

5-Allyl-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-2′-*O*-methyl 6-oxocytidine (15) and **5-allyl-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-3′-*O*-methyl 6-oxocytidine (17)**: A solution of tin(II) chloride (143 mg, 0.756 mmol, 0.33 equiv) in DMF (5 mL) was added to a solution of **13** (1.50 g, 2.28 mmol) in DMF (15 mL). The solution was cooled with an ice bath. A solution of diazomethane in ether (5 mL, approx. 0.3 M) was slowly added. (Diazomethane was freshly prepared with the Diazald™ kit diazomethane generator from Aldrich.) Further portions of diazomethane (5 mL) were added every 60 min. After 4 h the reaction was stopped by addition of a few drops of conc. ammonia solution. The solvent was removed under reduced pressure, and the syrup was extracted with chloroform/water. The collected organic phases were dried over MgSO$_4$, filtered, and evaporated to dryness, then purified by column chromatography (chloroform/acetonitrile/methanol 60:24:1.

15: $R_f = 0.50$ (chloroform/acetonitrile/methanol 20:8:1); ^{1}H NMR (270 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.94$ (s, 3H, N(CH$_3$)), 3.00 (d, $J = 6.1$ Hz, 2H, allyl CH$_2$), 3.03 (s, 3H, N(CH$_3$)), 3.11 (m, 2H, 5′-H), 3.32 (s, 3H, 2′-OCH$_3$), 3.71 (s, 3H, OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.83 (m, 1H, 4′-H), 4.07 (dd, $J = 3.7$, 2.5 Hz, 1H, 2′-H), 4.28 (m, 1H, 3′-H), 4.76 (d, $J = 8.2$ Hz, 1H, 3′-OH), 4.88 (ddd, $J = 17.1$, 10.0, 2.1 Hz, 2H, allyl CH$_2$), 5.73 (m, 1H, allyl CH), 6.18 (brs, 1H, 1′-H), 6.81–7.40 (m, 13H, H$_{ar}$), 7.97 (s, 1H, NCHN), 10.61 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (68 MHz, [D$_6$]DMSO, 25 °C): $\delta = 28.09$ (allyl CH$_2$), 33.71 (N(CH$_3$)), 39.98 (N(CH$_3$)), 54.93 (OCH$_3$), 57.72 (2′-OCH$_3$), 64.64 (C-5′), 70.43 (C-3′), 81.44 (C-2′), 82.02 (C-4′), 85.24 (DMTr), 85.98 (C-1′), 95.24 (C-5), 113.03 (DMTr), 113.88 (allyl CH$_2$), 126.47, 127.74, 129.64, 129.76, 135.65 (DMTr), 135.77 (allyl CH), 137.13, 145.04 (DMTr), 153.42 (C-2), 155.43 (NCHN), 157.90 (C-4), 157.95 (DMTr), 162.96 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(−): *m/z* (%): 669.4 (100) $[M-H]^-$.

17: $R_f = 0.41$ (chloroform/acetonitrile/methanol 20:8:1); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.94$ (s, 3H, N(CH$_3$)), 3.00 (d, $J = 4.9$ Hz, 2H, allyl CH$_2$), 3.03 (s, 3H, N(CH$_3$)), 3.11 (m, 2H, 5′-H), 3.25 (s, 3H, 3′-OCH$_3$), 3.71 (s, 3H, OCH$_3$), 3.72 (s, 3H, OCH$_3$), 3.93 (m, 1H, 4′-H), 3.99 (dd, $J = 7.2$, 6.0 Hz, 1H, 3′-H), 4.56 (dt, $J = 6.2$ Hz, 2.5 Hz, 1H, 2′-H), 4.88 (ddd, $J = 17.1$, 9.9, 1.5 Hz, 2H, allyl CH$_2$), 5.00 (d, $J = 6.6$ Hz, 1H, 2′-OH), 5.73 (m, 1H, allyl CH), 6.15 (brs, 1H, 1′-H), 6.82–7.39 (m, 13H, H$_{ar}$), 7.96 (s, 1H, NCHN), 10.56 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 28.09$ (allyl CH$_2$), 33.72 (N(CH$_3$)), 39.98 (N(CH$_3$)), 54.93 (OCH$_3$), 54.95 (OCH$_3$), 57.34 (3′-OCH$_3$), 64.50 (C-5′), 70.45 (C-2′), 79.94 (C-3′), 80.06 (C-4′), 85.22 (DMTr), 88.20 (C-1′), 95.30 (C-5), 113.06 (DMTr), 113.86 (allyl CH$_2$), 126.53, 127.73, 129.61, 129.73, 135.62 (DMTr), 135.78 (allyl CH), 137.18, 145.02 (DMTr), 153.45 (C-2), 155.41 (NCHN), 157.93 (C-4), 157.98 (DMTr), 163.13 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(−): *m/z* (%): 669.5 (100) $[M-H]^-$.

3′-*O*-(2-Cyanoethoxy-*N*,*N*-diisopropylphosphino)-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-2′-*O*-methyl-6-oxocytidine (19): *N*,*N*-diisopropylethylamine (DIPEA) (0.64 mL, 3.71 mmol, 4.5 equiv) was added to a solution of **14** (520 mg, 0.824 mmol) in acetonitrile (10 mL). The reaction mixture was cooled with an ice bath, and **18** (280 μL, 1.24 mmol, 1.5 equiv) was added slowly. After 10 min the ice bath was removed and the solution was stirred for another 20 min. The mixture was diluted with dichloromethane and extracted with 2% aqueous sodium carbonate solution. The collected organic phases were dried over MgSO$_4$, filtered, and evaporated to dryness. The crude product was chromatographed with chloroform/acetonitrile/methanol 60:24:1 to afford **19** as a white foam (397 mg, 58%). Compound **19** was obtained as a mixture of two diastereomers with identical R_f values in a ratio of 1.7:1. The underlined NMR spectroscopic data refer to the main diastereomer. $R_f = 0.32$ (ethyl acetate/acetonitrile 9:1 + 5% triethylamine), $R_f = 0.48$ (chloroform/acetonitrile/methanol 20:8:1), ^{31}P NMR (163 MHz, CDCl$_3$, 25 °C): $\delta = \underline{149.57}$, 150.54; ^{1}H NMR (400 MHz, CDCl$_3$, 25 °C): $\delta = 0.94–1.15$ (m, 12H, isopropyl CH$_3$), 2.35, $\underline{2.65}$ (2m, 2H, CH$_2$CN), 3.02, $\underline{3.02}$ (2s, 3H, N(CH$_3$)), 3.10, $\underline{3.10}$ (2s, 3H, N(CH$_3$)), 3.13–3.60 (m, 4H, 5′-H and POCH$_2$), 3.39, $\underline{3.41}$ (2s, 3H, 2′-OCH$_3$), $\underline{3.76}$, $\underline{3.76}$, 3.77, 3.77 (4s, 3H, OCH$_3$), 3.80–3.93 (m, 2H, isopropyl CH), 4.16 (m, 1H, 4′-H), 4.25, $\underline{4.32}$ (2dd, $J = 6.2$, 2.5 Hz, $\underline{J} = 6.1$, 2.8 Hz, 1H, 2′-H), $\underline{4.52}$, 4.74 (2ddd, $J = \underline{10.5}$, 6.2, 7.5 Hz, $J = 12.1$, 6.3, 8.0 Hz, 1H, 3′-H), 5.02, $\underline{5.03}$ (2s, 1H, 5-H), 6.39, $\underline{6.42}$ (2d, $J = 2.4$ Hz, $\underline{J} = 2.5$ Hz, 1H, 1′-H), 6.74–7.48 (m, 13H, H$_{ar}$), 7.78 (s, 1H, NCHN); MS ESI(−): *m/z* (%): 829.1 (100) $[M-H]^-$.

5-Allyl-3′-*O*-(2-cyanoethoxy-*N*,*N*-diisopropylphosphino)-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-2′-*O*-methyl-6-oxocytidine (20): *N*,*N*-Diisopropylethylamine (DIPEA) (0.37 mL, 2.16 mmol, 4.5 equiv) was added to a solution of **15** (322 mg, 0.48 mmol) in acetonitrile (8 mL). The reaction mixture was cooled with an ice bath and **18** (160 μL, 0.72 mmol, 1.5 equiv) was slowly added. After 10 min the ice bath was removed and the solution was stirred for another 2 h. The mixture was diluted with dichloromethane and extracted with 2% aqueous sodium carbonate solution. The collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The crude product was chromatographed with chloroform/acetonitrile/methanol 60:24:1 to afford **20** as a white foam (297 mg, 71%). Compound **20** was obtained as a mixture of two diastereomers with identical R_f values in a ratio of 2:1. The underlined NMR spectroscopic data refer to the main diastereomer. $R_f = 0.67$ (chloroform/acetonitrile/methanol 20:8:1); ^{31}P NMR (163 MHz, $CDCl_3$, 25 °C): δ = 150.10, 150.58; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.95 - 1.17$ (m, 12H, isopropyl CH_3), 2.34, 2.65 (2m, 2H, CH_2CN), 3.02 (s, 3H, $N(CH_3)$), 3.07 (d, $J = 6.0$ Hz, 2H, allyl CH_2), 3.09 (s, 3H, $N(CH_3)$), 3.11 (m, 2H, 5′-H), 3.25 – 3.61 (m, 2H, $POCH_2$), 3.39, 3.42 (2s, 3H, 2′-OCH_3), 3.76, 3.76, 3.77, 3.77 (4s, 3H, OCH_3), 3.79 – 3.93 (m, 2H, isopropyl CH), 4.13 (m, 1H, 4′-H), 4.28, 4.34 (2dd, $J = 6.3$, 2.5 Hz, $J = 6.2$, 2.7 Hz, 1H, 2′-H), 4.59, 4.78 (2ddd, $J = 10.6$, 6.4, 7.6 Hz, $J = 12.0$, 6.4, 8.0 Hz, 1H, 3′-H), 4,96 (m, 2H, allyl CH_2), 5.92 (m, 1H, allyl CH), 6.37, 6.39 (2s, 1H, 1′-H), 6.74 – 7.47 (m, 13H, H_{ar}), 7.81 (s, 1H, NCHN); MS ESI(+): *m/z* (%): 871.5 (78.2) $[M+H]^+$.

4-*N*-(Dimethylformamidine)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-6-oxocytidine (21): A solution of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (4.56 mL, 14.55 mmol, 1.11 equiv) in 1,2-dichloroethane (3.30 mL) was added to a solution of **10** (4.12 g, 13.11 mmol) in pyridine (80 mL). After stirring at 0 °C for 30 min, pyridinium hydrochloride precipitated. The reaction mixture was stirred for another 1.5 h at RT, methanol was added, and the solution was evaporated to dryness. The residue was dissolved in dichloromethane and extracted with saturated aqueous $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$, filtered, and evaporated, followed by coevaporation with toluene. The product was purified by column chromatography with dichloromethane/methanol 95:5. Compound **21** (6.79 g, 93%) was obtained as a white foam. $R_f = 0.58$ (dichloromethane/methanol 9:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 0.92 - 1.07$ (m, 28H, isopropyl), 2.95 (s, 3H, $N(CH_3)$), 3.09 (s, 3H, $N(CH_3)$), 3.67 (m, 1H, 4′-H), 3.88 (m, 2H, 5′-H), 4.38 (t, $J = 4.4$ Hz, 1H, 2′-H), 4.68 (d, $J = 4.2$ Hz, 1H, 2′-OH), 4.86 (m, 1H, 3′-H), 5.05 (s, 1H, 5-H), 6.01 (s, 1H, 1′-H), 8.13 (s, 1H, NCHN), 10.73 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): δ = 12.06, 12.12, 12.50, 12.66 (4 × CH), 16.86 – 17.35 (8 × CH_3), 34.28 ($N(CH_3)$), 40.29 ($N(CH_3)$), 62.42 (C-5′), 71.44 (C-3′), 72.81 (C-2′), 80.34 (C-4′), 80.95 (C-5), 87.55 (C-1′), 149.56 (C-2), 156.97 (NCHN), 159.20 (C-4), 163.09 (C-6); the assignment was confirmed by an HSQC experiment; elemental analysis calcd (%) for $C_{24}H_{44}N_4O_7Si_2$ (556.81): C 51.77, H 7.97, N 10.06; found C 51.52, H 7.90, N 9.81; MS ESI(−): *m/z* (%): 555.5 (100) $[M-H]^-$, ESI(+): *m/z* (%): 557.6 (80.0) $[M+H]^+$.

5-Allyl-4-*N*-(dimethylformamidine)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-6-oxocytidine (22): A solution of 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane (1.75 mL, 5.59 mmol, 1.1 equiv) in 1,2-dichloroethane (1.20 mL) was added to a solution of **11** (1.80 g, 5.08 mmol) in pyridine (25 mL). After the reaction mixture had been stirred at 0 °C for 30 min, pyridinium hydrochloride precipitated. The mixture was stirred for another 2.5 h at RT, methanol was added, and the solution was evaporated to dryness. The residue was dissolved in dichloromethane and extracted with saturated aqueous $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$, filtered and evaporated, followed by coevaporation with toluene. The product was purified by column chromatography with dichloromethane/methanol 98:2. Compound **22** (2.66 g, 88%) was obtained as a white foam. $R_f = 0.66$ (dichloromethane/methanol 95:5), $R_f = 0.15$ (dichloromethane/methanol 98:2); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 0.92 - 1.07$ (m, 28H, isopropyl), 2.93 (s, 3H, $N(CH_3)$), 2.99 (d, 2 H allyl CH_2), 3.02 (s, 3H, $N(CH_3)$), 3.68 (m, 1H, 4′-H), 3.88 (m, 2H, 5′-H), 4.39 (t, $J = 5.1$ Hz, 1H, 2′-H), 4.70 (d, $J = 4.2$ Hz, 1H, 2′-OH), 4.87 (ddd, $J = 17.1$, 10.1, 1.7 Hz, 2H, allyl CH_2), 4.95 (d, $J = 6.3$ Hz, 1H, 3′-H), 5.73 (m, 1H, allyl CH), 6.06 (s, 1H, 1′-H), 7.99 (s, 1H, NCHN), 10.59 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): δ = 12.02, 12.09, 12.47, 12.63 (4 × CH), 16.82 – 17.34 (8 × CH_3), 28.09 (allyl CH_2), 33.69 ($N(CH_3)$), 39.95 ($N(CH_3)$), 62.27 (C-5′), 71.28 (C-3′), 72.82 (C-2′), 80.37 (C-4′), 87.77 (C-1′), 95.10 (C-5), 113.83 (allyl CH_2), 137.06 (allyl CH), 150.32 (C-2), 153.19 (C-4), 155.48 (NCHN), 162.96 (C-6); MS ESI(+): *m/z* (%): 597.4 (100) $[M+H]^+$.

4-*N*-(Dimethylformamidine)-2′-*O*-phenoxythiocarbonyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-6-oxocytidine (23): Nucleoside **21** (6.79 g, 12.19 mmol) was dissolved in acetonitrile (100 mL), and *N*,*N*-dimethylaminopyridine (DMAP) (3.13 g, 25.61 mmol, 2.1 equiv) and phenoxythiocarbonyl chloride (1.86 mL, 13.41 mmol, 1.1 equiv) were added. After 2 h the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and extracted with 0.1M HCl, water, sat. aqueous $NaHCO_3$ solution, and brine. The collected organic phases were dried over $MgSO_4$, filtered and evaporated to dryness. The residue was purified by column chromatography with dichloromethane/methanol 98:2. The product **23** (4.90 g, 58%) was obtained as a yellow foam. $R_f = 0.53$ (dichloromethane/methanol 95:5); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 0.93 - 1.07$ (m, 28H, isopropyl), 2.96 (s, 3H, $N(CH_3)$), 3.09 (s, 3H, $N(CH_3)$), 3.72 (m, 1H, 4′-H), 3.95 (m, 2H, 5′-H), 5.11 (s, 1H, 5-H), 5.16 (m, 1H, 3′-H), 6.21 (brs, 1H, 1′-H), 6.29 (dd, $J = 5.9$, 1.5 Hz, 1H, 2′-H), 7.11 – 7.50 (m, 5H, H_{ar}), 8.15 (s, 1H, NCHN), 10.93 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = 12.25$, 12.36, 12.58, 12.86 (4 × CH), 16.67 – 17.24 (8 × CH_3), 34.33 ($N(CH_3)$), 40.35 ($N(CH_3)$), 61.32 (C-5′), 70.24 (C-3′), 80.33 (C-4′), 80.99 (C-5), 84.26 (C-1′), 85.00 (C-2′), 121.51, 126.68, 129.28, 129.73 (C_{ar}), 152.78 (C-2), 157.21 (NCHN), 157.88 (C=S), 159.61 (C-4), 163.18 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(+): *m/z* (%): 693.2 (32.2) $[M+H]^+$, 539.3 (100) $[M - OCSOC_6H_5]^+$.

5-Allyl-4-*N*-(dimethylformamidine)-2′-*O*-phenoxythiocarbonyl-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-6-oxocytidine (24): Nucleoside **22** (2.57 g, 4.31 mmol) was dissolved in acetonitrile (40 mL), and *N*,*N*-dimethylaminopyridine (DMAP) (1.10 g, 9.04 mmol, 2.1 equiv) and phenoxythiocarbonyl chloride (0.66 mL, 4.74 mmol, 1.1 equiv) were added. After 4 h the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and extracted with 0.1M HCl, water, sat. aqueous $NaHCO_3$ solution, and brine. The collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was purified by column chromatography with dichloromethane/methanol 99:1. The product **24** (1.63 g, 52%) was obtained as a yellow foam. $R_f = 0.33$ (dichloromethane/methanol 98:2); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 0.96 - 1.09$ (m, 28H, isopropyl), 2.94 (s, 3H, $N(CH_3)$), 3.00 (d, 2H, allyl CH_2), 3.03 (s, 3H, $N(CH_3)$), 3.73 (m, 1H, 4′-H), 3.94 (m, 2H, 5′-H), 4.87 (ddd, $J = 17.1$, 10.1, 1.5 Hz, 2H, allyl CH_2), 5.21 (d, $J = 6.0$ Hz, 1H, 3′-H), 5.76 (m, 1H, allyl CH), 6.24 (s, 1H, 1′-H), 6.30 (d, $J = 5.9$ Hz, 1H, 2′-H), 7.12 – 7.48 (m, 5H, H_{ar}), 8.02 (s, 1H, NCHN), 10.82 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = 12.25$, 12.36, 12.58, 12.62 (4 × CH), 16.74 – 17.25 (8 × CH_3), 28.05 (allyl CH_2), 33.74 ($N(CH_3)$), 40.13 ($N(CH_3)$), 61.13 (C-5′), 70.07 (C-3′), 80.12 (C-4′), 83.21 (C-1′), 84.32 (C-2′), 95.22 (C-5), 113.98 (allyl CH_2), 121.52, 126.68, 129.28, 129.73 (C_{ar}), 136.87 (allyl CH), 152.77 (C-2), 153.61 (C-4), 155.69 (NCHN), 157.27 (C=S), 162.89 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(+): *m/z* (%): 733.4 (38.1) $[M+H]^+$, 579.4 (100) $[M - OCSOC_6H_5]^+$.

2′-Deoxy-4-*N*-(dimethylformamidine)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-6-oxocytidine (25): Nucleoside **23** (4.90 g, 7.07 mmol) was dissolved in freshly distilled toluene (160 mL). The solution was degassed five times. α,α′-Azoisobutyronitrile (0.23 g, 1.41 mmol, 0.2 equiv) and tributyltin hydride (2.81 mL, 10.61 mmol, 1.5 equiv) were added and the solution was heated to 75 °C. After 2 h the solution was cooled to RT and the solvent was removed by evaporation. The residue was dissolved in ethyl acetate and extracted with 5% aqueous $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was purified by column chromatography with dichloromethane/methanol 98:2. The product **25** (2.49 g, 65%) was obtained as a yellow foam. $R_f = 0.42$ (dichloromethane/methanol 95:5); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 0.95 - 1.10$ (m, 28H, isopropyl), 2.25 (m, 1H, 2′a-H), 2.63 (m, 1H, 2′b-H), 2.94 (s, 3H, $N(CH_3)$), 3.07 (s, 3H, $N(CH_3)$), 3.63 (m, 1H, 4′-H), 3.88 (m, 2H, 5′-H), 5.01 (s, 1H, 5-H), 5.04 (m, 1H, 3′-H), 6.49 (dd, $J = 9.8$, 2.6 Hz, 1H, 1′-H), 8.10 (s, 1H, NCHN), 10.66 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): δ = 11.98, 12.54, 12.66, 12.86 (4 × CH), 16.79 – 17.42 (8 × CH_3), 34.24 ($N(CH_3)$), 38.87 (C-2′), 40.25 ($N(CH_3)$), 64.27

0947-6539/00/0613-2420 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 13

(C-5′), 74.19 (C-3′), 78.68 (C-1′), 81.19 (C-5), 84.61 (C-4′), 150.83 (C-2), 156.89 (NCHN), 159.00 (C-4), 163.20 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(+): *m/z* (%): 541.4 (100) $[M+H]^+$.

5-Allyl-2′-deoxy-4-*N*-(dimethylformamidine)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-6-oxocytidine (26): Nucleoside **24** (1.49 g, 2.03 mmol) was dissolved in freshly distilled toluene (50 mL). The solution was degassed five times. α,α'-Azoisobutyronitrile (67 mg, 0.407 mmol, 0.2 equiv) and tributyltin hydride (0.81 mL, 3.05 mmol, 1.5 equiv) were added and the solution was heated to 75 °C. After 1.5 h the solution was cooled to RT and the solvent was removed by evaporation. The residue was dissolved in ethyl acetate and extracted with 5% aqueous $NaHCO_3$ solution. The organic phase was dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was purified by column chromatography with dichloromethane/methanol 98:2. The product **26** (0.68 g, 58%) was obtained as a yellow foam. $R_f = 0.46$ (dichloromethane/methanol 95:5); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 0.94-1.11$ (m, 28H, isopropyl), 2.27 (m, 1H, 2′a-H), 2.64 (m, 1H, 2′b-H), 2.93 (s, 3H, N(CH_3)), 3.00 (d, $J =$ 6.3 Hz, 2H, allyl CH_2), 3.02 (s, 3H, N(CH_3)), 3.64 (m, 1H, 4′-H), 3.85 (dd, $J = 11.1$, 3.6 Hz, 1H, 5′a-H), 3.93 (dd, $J = 11.0$, 8.5 Hz, 1H, 5′b-H), 4.85 (ddd, $J = 17.1$, 10.0, 2.3 Hz, 2H, allyl CH_2), 5.09 (ψq, $J = 7.5$ Hz, 1H, 3′-H), 5.72 (m, 1H, allyl CH), 6.56 (dd, $J = 9.8$, 2.5 Hz, 1H, 1′-H), 7.98 (s, 1H, NCHN), 10.54 (s, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = 11.98$, 12.55, 12.66, 12.86 (4 × CH), 16.76–17.41 (8 × CH_3), 28.08 (allyl CH_2), 33.66 (N(CH_3)), 38.88 (C-2′), 40.13 (N(CH_3)), 64.24 (C-5′), 74.15 (C-3′), 79.08 (C-1′), 84.71 (C-4′), 95.25 (C-5), 113.76 (allyl CH_2), 137.11 (allyl CH), 150.15 (C-2), 153.02 (C-4), 155.45 (NCHN), 163.11 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(+): *m/z* (%): 581.5 (100) $[M+H]^+$.

General procedure for the deprotection of 25 and 26: The protected nucleosides **25** and **26** were each dissolved in THF (30 mL). A 1M solution of tetrabutylammonium fluoride (TBAF) in THF was added. After 1 h at RT, full deprotection was achieved. The solvent was removed and the oily residue was dissolved in water. This was extracted with dichloromethane (three times) and ether (two times). The aqueous phase was evaporated to dryness and coevaporated with methanol. The product was purified by column chromatography with dichloromethane/methanol 9:1, to yield **27** or **28** as a white foam.

2′-Deoxy-4-*N*-(dimethylformamidine)-6-oxocytidine (27): Protected nucleoside **25** (2.49 g, 4.60 mmol) was dissolved in THF (30 mL), and TBAF (10.13 mL, 10.13 mmol) was added. The product **27** was obtained in 1.35 g (99%) yield. $R_f = 0.19$ (dichloromethane/methanol 9:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 1.84$ (m, 1H, 2′a-H), 2.70 (m, 1H, 2′b-H), 2.95 (s, 3H, N(CH_3)), 3.08 (s, 3H, N(CH_3)), 3.43 (m, 1H, 5′a-H), 3.57 (m, 1H, 5′b-H), 3.65 (m, 1H, 4′-H), 4.29 (m, 1H, 3′-H), 4.57 (t, $J = 4.2$ Hz, 1H, 5′-OH), 5.01 (d, $J = 4.7$ Hz, 1H, 3′-OH), 5.03 (s, 1H, 5-H), 6.52 (ψt, $J =$ 7.3 Hz, 1H, 1′-H), 8.11 (s, 1H, NCHN), 10.67 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = 34.32$ (N(CH_3)), 36.61 (C-2′), 40.32 (N(CH_3)), 62.36 (C-5′), 71.32 (C-3′), 80.56 (C-1′), 81.23 (C-5), 87.17 (C-4′), 151.08 (C-2), 156.97 (NCHN), 159.10 (C-4), 163.66 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(−): *m/z* (%): 297.2 (100) $[M-H]^-$.

5-Allyl-2′-deoxy-4-*N*-(dimethylformamidine)-6-oxocytidine 28: Protected nucleoside **26** (0.58 g, 0.998 mmol) was dissolved in THF (7 mL), and TBAF (2.20 mL, 2.20 mmol) was added. The product **28** was obtained in 330 mg (98%) yield. $R_f = 0.28$ (dichloromethane/methanol 9:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 1.87$ (m, 1H, 2′a-H), 2.72 (m, 1H, 2′b-H), 2.93 (s, 3H, N(CH_3)), 2.99 (d, $J = 6.3$ Hz, 2H, allyl CH_2), 3.02 (s, 3H, N(CH_3)), 3.46 (m, 1H, 4′-H), 3.56 (m, 1H, 5′a-H), 3.65 (m, 1H, 5′b-H), 4.31 (m, 1H, 3′-H), 4.56 (dd, $J = 7.1$, 4.4 Hz, 1H, 5′-OH), 4.87 (ddd, $J = 17.1$, 10.0, 2.2 Hz, 2H, allyl CH_2), 5.00 (d, $J = 4.7$ Hz, 1H, 3′-OH), 5.74 (m, 1H, allyl CH), 6.57 (ψt, $J = 7.3$ Hz, 1H, 1′-H), 7.95 (s, 1H, NCHN), 10.55 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = 28.02$ (allyl CH_2), 33.72 (N(CH_3)), 36.66 (C-2′), 40.17 (N(CH_3)), 62.35 (C-5′), 71.30 (C-3′), 81.01 (C-1′), 87.22 (C-4′), 95.43 (C-5), 113.87 (allyl CH_2), 137.16 (allyl CH), 150.51 (C-2), 153.27 (C-4), 155.45 (NCHN), 163.48 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(−): *m/z* (%): 337.2 (100) $[M-H]^-$.

2′-Deoxy-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-6-oxocytidine (29): Nucleoside **27** (600 mg, 2.01 mmol) was dissolved in dry pyridine (30 mL). The solution was cooled to −40 °C with acetone/liquid nitrogen. DMTrCl (750 mg, 2.21 mmol, 1.1 equiv) was added. After 3 h the reaction mixture has reached RT and further DMTrCl (140 mg, 0.2 equiv) was added. After 8 h dichloromethane (20 mL) was added and the solution was extracted with 5% aqueous $NaHCO_3$ solution. The collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was coevaporated with toluene. Purification was achieved by column chromatography with dichloromethane/methanol 95:5. The product **29** (955 mg, 79%) was obtained as a white foam. $R_f = 0.43$ (dichloromethane/methanol 9:1); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 1.97$ (m, 1H, 2′a-H), 2.58 (m, 1H, 2′b-H), 2.95 (s, 3H, N(CH_3)), 3.06 (m, 1H, 5′a-H), 3.08 (s, 3H, N(CH_3)), 3.22 (m, 1H, 5′b-H), 3.71 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.79 (m, 1H, 4′-H), 4.26 (m, 1H, 3′-H), 4.99 (d, $J = 5.5$ Hz, 1H, 3′-OH), 5.03 (s, 1H, 5-H), 6.58 (dd, $J = 8.9$, 4.2 Hz, 1H, 1′-H), 6.80–7.40 (m, 13H, H_{ar}), 8.12 (s, 1H, NCHN), 10.60 (brs, 1H, NH); ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = 34.24$ (N(CH_3)), 37.39 (C-2′), 40.50 (N(CH_3)), 54.89 (OCH_3), 54.93 (OCH_3), 64.92 (C-5′), 71.50 (C-3′), 79.83 (C-1′), 81.14 (C-5), 85.16 (DMTr), 85.29 (C-4′), 112.99, 126.46, 127.67, 129.58, 129.80, 135.82, 145.24 (DMTr), 150.73 (C-2), 156.83 (NCHN), 157.30 (DMTr), 159.01 (C-4), 163.45 (C-6); MS ESI(−): *m/z* (%): 599.4 (100) $[M-H]^-$.

5-Allyl-2′-deoxy-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-6-oxocytidine (30): Nucleoside **28** (210 mg, 0.62 mmol) was dissolved in dry pyridine (8 mL). The solution was cooled to 0 °C in an ice bath. DMTrCl (252 mg, 0.74 mmol, 1.2 equiv) was added. The ice bath was removed after 1 h. After further 4 h, more DMTrCl (21 mg, 0.1 equiv) was added. Another 4 h later dichloromethane (10 mL) was added and the solution was extracted with 5% aqueous $NaHCO_3$ solution. The collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was coevaporated with toluene, and purified by column chromatography with dichloromethane/methanol 95:5. The product **30** (340 mg, 86%) was obtained as a white foam. $R_f = 0.57$ (dichloromethane/methanol 9:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = 2.00$ (m, 1H, 2′a-H), 2.60 (m, 1H, 2′b-H), 2.94 (s, 3H, N(CH_3)), 3.00 (d, $J = 6.3$ Hz, 2H, allyl CH_2), 3.02 (s, 3H, N(CH_3)), 3.05 (m, 1H, 5′a-H), 3.25 (m, 1H, 5′b-H), 3.70 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.82 (m, 1H, 4′-H), 4.29 (m, 1H, 3′-H), 4.86 (ddd, $J = 17.1$, 10.0, 2.2 Hz, 2H, allyl CH_2), 5.00 (d, $J = 5.5$ Hz, 1H, 3′-OH), 5.73 (m, 2H, allyl CH), 6.64 (dd, $J = 8.9$, 4.3 Hz, 1H, 1′-H), 6.78–7.39 (m, 13H, H_{ar}), 7.94 (s, 1H, NCHN), 10.48 (s, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = 28.11$ (allyl CH_2), 33.67 (N(CH_3)), 37.55 (C-2′), 40.12 (N(CH_3)), 54.89 (OCH_3), 54.93 (OCH_3), 64.99 (C-5′), 71.60 (C-3′), 80.42 (C-1′), 85.15 (DMTr), 85.56 (C-4′), 95.33 (C-5), 112.97 (DMTr), 113.76 (allyl CH_2), 126.44, 127.73, 129.56, 129.81, 135.72 (DMTr), 137.23 (allyl CH), 145.27 (DMTr), 150.17 (C-2), 153.10 (C-4), 155.31 (NCHN), 157.95 (DMTr), 163.32 (C-6); the assignment was confirmed by an HSQC experiment; MS ESI(−): *m/z* (%): 639.4 (100) $[M-H]^-$.

3′-*O*-(2-Cyanoethoxy-*N*,*N*-diisopropylphosphino)-2′-deoxy-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-6-oxocytidine (31): *N*,*N*-Diisopropylethylamine (DIPEA) (449 μL, 2.62 mmol, 5 equiv) and 2-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite (**18**; 234 μL, 1.05 mmol, 2 equiv) were added to a solution of **29** (315 mg, 0.524 mmol) in dichloromethane (12 mL). After 30 min the reaction mixture was diluted with dichloromethane and extracted with 2% aqueous sodium carbonate solution. The collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The crude product was chromatographed with dichloromethane/methanol 95:5 to afford **31** as a white foam (315 mg, 75%). Compound **31** was obtained as a mixture of two diastereomers with different R_f values in a ratio of 1:1.2. The underlined NMR spectroscopic data refer to the main diastereomer. $R_f = 0.33/0.38$ (dichloromethane/methanol 95:5); ^{31}P NMR (163 MHz, $CDCl_3$, 25 °C): $\delta = 148.80$, 148.98; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.98-1.15$ (m, 12H, isopropyl CH_3), 2.23 (m, 1H, 2′a-H), 2.37, 2.57 (2m, 2H, CH_2CN), 2.84 (m, 1H, 2′b-H), 3.01 (s, 3H, N(CH_3)), 3.10, (s, 3H, N(CH_3)), 3.26 (m, 1H, 5′a-H), 3.35 (m, 1H, 5′b-H), 3.40–3.72 (m, 4H, $POCH_2$ and isopropyl CH), 3.75, 3.76, 3.76, 3.77 (4s, 3H, OCH_3), 4.08 (m, 1H, 4′-H), 4.61, 4.70 (2m, 1H, 3′-H), 4.99 (s, 1H, 5-H), 6.73–7.48 (m, 14H, 1′-H and H_{ar}), 7.75, 7.76 (2s, 1H, NCHN); MS ESI(−): *m/z* (%): 799.5 (100) $[M-H]^-$.

5-Allyl-3′-*O*-(2-cyanoethoxy-*N*,*N*-diisopropylphosphino)-2′-deoxy-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-6-oxocyti-

dine (32): *N*,*N*-Diisopropylethylamine (DIPEA) (376 µL, 2.20 mmol, 5 equiv) and **18** (147 µL, 0.66 mmol, 1.5 equiv) were added to a solution of **30** (282 mg, 0.440 mmol) in dichloromethane (6 mL). After 2 h another portion of **18** (49 µL, 0.5 equiv) was added. After 4 h the reaction mixture was diluted with dichloromethane and extracted with 2% aqueous sodium carbonate solution. The collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The crude product was chromatographed with dichloromethane/methanol 98:2 to afford **32** as a white foam (262 mg, 71%). Compound **32** was obtained as a mixture of two diastereomers with different R_f values in a ratio of 1.2:1. The underlined NMR spectroscopic data refer to the main diastereomer. $R_f = 0.55/0.61$ (dichloromethane/methanol 95:5); ^{31}P NMR (163 MHz, $CDCl_3$, 25 °C): $\delta =$ 149.06, 149.14; ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.98 - 1.15$ (m, 12H, isopropyl CH_3), 2.23 (m, 1H, 2′a-H), 2.35, 2.57 (2t, $J = 6.6$ Hz, $J = 6.3$ Hz, 2H, CH_2CN), 2.88 (m, 1H, 2′b-H), 3.01 (s, 3H, $N(CH_3)$), 3.03 (d, $J = 5.9$ Hz, 2H, allyl CH_2), 3.07 (s, 3H, $N(CH_3)$), 3.24 (dd, $J = 10.0$, 3.4 Hz, 1H, 5′a-H), 3.30 (dd, $J = 10.0$, 3.5 Hz, 1H, 5′b-H), 3.42 – 3.57 (m, 4H, $POCH_2$ and isopropyl CH), 3.74, 3.75, 3.75, 3.76 (4s, 3H, OCH_3), 4.07 (m, 1H, 4′-H), 4.66, 4.73 (2m, 1H, 3′-H), 4.95 (m, 2H, allyl CH_2), 5.89 (m, 1H, allyl CH), 6.73 – 7.48 (m, 14H, 1′-H and H_{ar}), 7.75, 7.76 (2s, 1H, NCHN), 8.38 (brs, 1H, NH); MS ESI(−): *m/z* (%): 839.6 (100) $[M-H]^-$.

General procedure for the conversion of 12 and 13 with TBDMSCl: Silver nitrate and a 1M solution of *tert*-butyldimethylsilyl chloride (TBDMSCl) were added to a solution of **12** or **13** in THF/pyridine 1:1. Silver chloride precipitated. After the mixture had been stirred for 16 h at RT, another portion of TBDMSCl was added. The reaction was stopped by addition of a 5% aqueous $NaHCO_3$ solution, and the suspension was filtered through a plug of Celite and washed. After extraction with dichloromethane, the collected organic phases were dried over $MgSO_4$, filtered, and evaporated to dryness. The residue was coevaporated with toluene and purified by column chromatography with dichloromethane/ethyl acetate 8:2. This afforded the pure products as white foams.

5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-4-*N*-dimethylformamidin-2′-*O*-*tert*-butyldimethylsilyl-6-oxocytidine (33) and **5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-dimethylformamidin-3′-*O*-*tert*-butyldimethylsilyl-6-oxocytidine (35)**: Nucleoside **12** (1.233 g, 2.0 mmol) in THF/pyridine 1:1 (12 mL), silver nitrate (408 mg, 2.4 mmol, 1.2 equiv), and TBDMSCl (2.4 mL, 2.4 mmol, 1.2 equiv + 0.4 mL, 0.4 mmol, 0.2 equiv) afforded **33** (833 mg, 57%) and **35** (395 mg, 27%).

33: $R_f = 0.15$ (dichloromethane/ethyl acetate 8:2), $R_f = 0.54$ (dichloromethane/methanol 95:5); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = 0.00$ (s, 3H, $SiCH_3$), 0.02 (s, 3H, $SiCH_3$), 0.84 (s, 9H, $SiC(CH_3)_3$), 2.97 (s, 3H, $N(CH_3)$), 3.10 (s, 3H, $N(CH_3)$), 3.15 (m, 2H, 5′-H), 3.73 (s, 6H, $2 \times OCH_3$), 3.82 (m, 1H, 4′-H), 4.15 (m, 1H, 3′-H), 4.37 (d, $J = 8.2$ Hz, 1H, 3′-OH), 4.59 (dd, $J = 6.0$, 2.7 Hz, 1H, 2′-H), 5.07 (s, 1H, 5-H), 6.10 (d, $J = 2.0$ Hz, 1H, 1′-H), 6.83 – 7.42 (m, 13H, H_{ar}), 8.16 (s, 1H, NCHN), 10.73 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = -5.13$ ($SiCH_3$), −4.81 ($SiCH_3$), 18.06 (quart. C, *t*Bu), 25.67 (CH_3, *t*Bu), 34.28 ($N(CH_3)$), 40.29 ($N(CH_3)$), 54.91 (OCH_3), 54.93 (OCH_3), 64.43 (C-5′), 70.19 (C-3′), 73.30 (C-2′), 80.95 (C-4′), 81.58 (C-5), 85.17 (DMTr), 87.80 (C-1′), 113.01, 126.47, 127.64, 127.73, 129.66, 129.74, 135.66, 135.72, 145.02 (DMTr), 157.01 (C-2), 157.91 (NCHN), 157.95 (DMTr), 159.21 (C-4), 163.15 (C-6); the assignment was confirmed by an HSQC experiment; elemental analysis calcd (%) for $C_{39}H_{50}N_4O_8Si$ (730.94): C 64.09, H 6.90, N 7.67; found C 64.07, H 6.95, N 7.60; MS ESI(−): *m/z* (%): 729.4 (100) $[M-H]^-$.

35: $R_f = 0.03$ (dichloromethane/ethyl acetate 8:2), $R_f = 0.43$ (dichloromethane/methanol 95:5); ^{1}H NMR (250 MHz, [D$_6$]DMSO, 25 °C): $\delta = -0.10$ (s, 3H, $SiCH_3$), −0.04 (s, 3H, $SiCH_3$), 0.74 (s, 9H, $SiC(CH_3)_3$), 2.97 (s, 3H, $N(CH_3)$), 3.02 (m, 1H, 5′a-H), 3.10 (s, 3H, $N(CH_3)$), 3.18 (m, 1H, 5′b-H), 3.73 (s, 6H, $2 \times OCH_3$), 3.87 (m, 1H, 4′-H), 4.36 (m, 2H, 2′-H, 3′-H), 4.70 (d, $J = 5.3$ Hz, 1H, 2′-OH), 5.07 (s, 1H, 5-H), 6.10 (brs, 1H, 1′-H), 6.83 – 7.41 (m, 13H, H_{ar}), 8.15 (s, 1H, NCHN), 10.70 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (63 MHz, [D$_6$]DMSO, 25 °C): $\delta = -5.28$ ($SiCH_3$), −4.69 ($SiCH_3$), 17.83 (quart. C, *t*Bu), 25.64 (CH_3, *t*Bu), 34.30 ($N(CH_3)$), 40.30 ($N(CH_3)$), 54.94 (OCH_3), 64.55 (C-5′), 71.41 (C-2′), 71.74 (C-3′), 81.05 (C-4′), 81.16 (C-5), 85.39 (DMTr), 87.80 (C-1′), 113.01, 126.49, 127.63, 127.69, 129.58, 129.69, 135.59, 135.68, 144.88 (DMTr), 156.94 (C-2), 157.94 (NCHN), 157.99 (DMTr), 159.21 (C-4), 163.28 (C-6); the assignment was confirmed by an HSQC experiment; elemental analysis calcd (%) for $C_{39}H_{50}N_4O_8Si$ (730.94): C 64.09, H 6.90, N 7.67; found C 63.93, H 6.95, N 7.71; MS ESI(−): *m/z* (%): 729.4 (100) $[M-H]^-$.

5-Allyl-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-dimethylformamidin-2′-*O*-*tert*-butyldimethylsilyl-6-oxocytidine (34) and **5-allyl-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-dimethylformamidin-3′-*O*-*tert*-butyldimethylsilyl-6-oxocytidine (36)**: Nucleoside **13** (825 mg, 1.256 mmol) in THF/pyridine 1:1 (8 mL), silver nitrate (256 mg, 1.51 mmol, 1.2 equiv), and TBDMSCl (1.51 mL, 1.51 mmol, 1.2 equiv + 0.25 mL, 0.25 mmol, 0.2 equiv) afforded **34** (560 mg, 58%) and **36** (260 mg, 27%).

34: $R_f = 0.34$ (dichloromethane/ethyl acetate 8:2), $R_f = 0.60$ (dichloromethane/methanol 95:5); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = -0.01$ (s, 3H, $SiCH_3$), 0.00 (s, 3H, $SiCH_3$), 0.83 (s, 9H, $SiC(CH_3)_3$), 2.94 (s, 3H, $N(CH_3)$), 3.01 (d, $J = 7.0$ Hz, 2H, allyl CH_2), 3.02 (s, 3H, $N(CH_3)$), 3.12 (m, 2H, 5′-H), 3.71 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.84 (m, 1H, 4′-H), 4.16 (m, 1H, 3′-H), 4.38 (d, $J = 8.3$ Hz, 1H, 3′-OH), 4.59 (dd, $J = 6.1$, 2.9 Hz, 1H, 2′-H), 4.86 (ddd, $J = 17.2$, 10.0, 1.8 Hz, 2H, allyl CH_2), 5.73 (m, 1H, allyl CH), 6.14 (brs, 1H, 1′-H), 6.81 – 7.41 (m, 13H, H_{ar}), 7.96 (s, 1H, NCHN), 10.58 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = -5.13$ ($SiCH_3$), −4.80 ($SiCH_3$), 18.06 (quart. C, *t*Bu), 25.68 (CH_3, *t*Bu), 27.98 (allyl CH_2), 33.72 ($N(CH_3)$), 39.96 ($N(CH_3)$), 54.92 (OCH_3), 54.94 (OCH_3), 64.42 (C-5′), 70.22 (C-3′), 73.31 (C-2′), 81.77 (C-4′), 85.18 (DMTr), 85.81 (C-1′), 95.45 (C-5), 113.02 (DMTr), 113.66 (allyl CH_2), 126.48, 127.65, 127.74, 129.67, 129.76, 135.67, 135.74 (DMTr), 137.08 (allyl CH), 145.04 (DMTr), 153.26 (C-2), 155.40 (NCHN), 157.91 (C-4), 157.96 (DMTr), 163.10 (C-6); the assignment was confirmed by an HSQC experiment; elemental analysis calcd (%) for $C_{42}H_{54}N_4O_8Si$ (771.00): C 65.43, H 7.06, N 7.27; found C 65.31, H 7.11, N 7.07; MS ESI(−): *m/z* (%): 769.6 (100) $[M-H]^-$.

36: $R_f = 0.11$ (dichloromethane/ethyl acetate 8:2), $R_f = 0.51$ (dichloromethane/methanol 95:5); ^{1}H NMR (400 MHz, [D$_6$]DMSO, 25 °C): $\delta = -0.10$ (s, 3H, $SiCH_3$), −0.05 (s, 3H, $SiCH_3$), 0.74 (s, 9H, $SiC(CH_3)_3$), 2.94 (s, 3H, $N(CH_3)$), 3.00 (m, 1H, 5′a-H), 3.00 (d, $J = 7.7$ Hz, 2H, allyl CH_2), 3.02 (s, 3H, $N(CH_3)$), 3.17 (m, 1H, 5′b-H), 3.71 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 3.87 (m, 1H, 4′-H), 4.38 (m, 2H, 2′-H, 3′-H), 4.72 (d, $J = 6.1$ Hz, 1H, 2′-OH), 4.88 (ddd, $J = 17.2$, 10.0, 1.7 Hz, 2H, allyl CH_2), 5.74 (m, 1H, allyl CH), 6.14 (brs, 1H, 1′-H), 6.81 – 7.40 (m, 13H, H_{ar}), 7.97 (s, 1H, NCHN), 10.57 (brs, 1H, NH); the assignment was confirmed by a ^{1}H,^{1}H-COSY experiment; ^{13}C NMR (100 MHz, [D$_6$]DMSO, 25 °C): $\delta = -5.25$ ($SiCH_3$), −4.67 ($SiCH_3$), 17.86 (quart. C, *t*Bu), 25.66 (CH_3, *t*Bu), 28.07 (allyl CH_2), 33.71 ($N(CH_3)$), 39.97 ($N(CH_3)$), 54.93 (OCH_3), 64.50 (C-5′), 71.39 (C-2′), 71.80 (C-3′), 81.37 (C-4′), 85.39 (DMTr), 86.26 (C-1′), 95.20 (C-5), 113.01 (DMTr), 113.83 (allyl CH_2), 126.49, 127.63, 127.71, 129.59, 129.69, 135.61, 135.70 (DMTr), 137.16 (allyl CH), 144.91 (DMTr), 153.30 (C-2), 155.37 (NCHN), 157.95 (C-4), 157.99 (DMTr), 163.23 (C-6); the assignment was confirmed by an HSQC experiment; elemental analysis calcd (%) for $C_{42}H_{54}N_4O_8Si$ (771.00): C 65.43, H 7.06, N 7.27; found C 65.18, H 7.25, N 7.01; MS ESI(−): *m/z* (%): 769.5 (100) $[M-H]^-$.

General procedure for the phosphitylation of 33 and 34: The 2′-*O*-TBDMS-protected compounds **33** and **34** were each dissolved in acetonitrile. *Sym*-collidine, 1-methyl imidazole, and (after cooling with an ice bath) 2-cyanoethyl-*N*,*N*-diisopropylchloro phosphoramidite (**18**) were added. The ice bath was removed after 15 min. After 2 h the reaction mixture was diluted with dichloromethane and 5% aqueous $NaHCO_3$ solution. The extraction of the aqueous phase with dichloromethane was repeated three times. The collected organic phases were dried over $MgSO_4$, filtered and evaporated to dryness. The residue was coevaporated with toluene five times and purified by column chromatography with ethyl acetate/acetonitrile 9:1. The products **37** and **38** were obtained as mixtures of two diastereomers with identical R_f values. The underlined NMR spectroscopic data refer to the main diastereomer.

3′-*O*-(2-Cyanoethoxy-*N*,*N*-diisopropylphosphino)-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-2′-*O*-*tert*-butyldimethylsilyl-6-oxocytidine (37): Compound **33** (260 mg, 0.356 mmol) in acetonitrile (5 mL), *sym*-collidine (236 µL, 1.78 mmol, 5 equiv), 1-methyl imidazole (14 µL, 0.178 mmol, 0.5 equiv), and **18** (119 µL, 0.534 mmol, 1.5 equiv) afforded **37** (240 mg, 72%) as a white foam. $R_f = 0.55$ (ethyl acetate/acetonitrile 9:1); ^{31}P NMR (163 MHz, $CDCl_3$, 25 °C): $\delta =$ 148.80, 149.48 (ratio 1.2:1); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.02$, 0.02, 0.04, 0.05 (4s, 3H, $SiCH_3$), 0.88, 0.88 (2s, 9H, $SiC(CH_3)_3$), 1.02 – 1.14 (m, 12H, isopropyl CH_3), 2.36, 2.63 (2m, 2H, CH_2CN), 3.02 (s, 3H, $N(CH_3)$), 3.11 (s,

0947-6539/00/0613-2422 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 13

3H, N(CH_3)), 3.17–3.53 (m, 4H, 5′-H and $POCH_2$), 3.77, 3.77, 3.77, 3.78 (4s, 3H, OCH_3), 3.91–3.96 (m, 2H, isopropyl CH), 4.19 (m, 1H, 4′-H), 4.40, 4.47 (2ddd, J = 11.4, 5.9, 6.1 Hz, J = 12.3, 5.9, 6.4 Hz, 1H, 3′-H), 4.88, 4.95 (2dd, 1H, 2′-H), 5.01, 5.02 (2s, 1H, 5-H), 6.29 (d, J = 3.7 Hz, 1H, 1′-H), 6.76–7.49 (m, 13H, H_{ar}), 7.77 (s, 1H, NCHN); MS ESI(+): *m/z* (%): 931.9 (100) $[M+H]^+$, 830.5 (79.3) $[M - N(iPr)_2]^+$.

5-Allyl-3′-*O*-(2-cyanoethoxy-*N*,*N*-diisopropylphosphino)-5′-*O*-(4,4′-dimethoxytriphenylmethyl)-4-*N*-(dimethylformamidine)-2′-*O*-*tert*-butyldimethylsilyl-6-oxocytidine (38): Compound **34** (285 mg, 0.370 mmol) in acetonitrile (5 mL), *sym*-collidine (245 μL, 1.848 mmol, 5 equiv), 1-methylimidazole (15 μL, 0.185 mmol, 0.5 equiv), and **18** (124 μL, 0.554 mmol, 1.5 equiv) afforded **38** (255 mg, 71%) as a white foam. R_f = 0.68 (ethyl acetate/acetonitrile 9:1); ^{31}P NMR (163 MHz, $CDCl_3$, 25 °C): δ = 149.11, 149.54 (ratio 1.3:1); ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = −0.01, 0.01, 0.04, 0.05 (4s, 3H, $SiCH_3$), 0.86, 0.87 (2s, 9H, $SiC(CH_3)_3$), 0.89–1.14 (m, 12H, isopropyl CH_3), 2.35, 2.62 (2m, 2H, CH_2CN), 3.01 (s, 3H, N(CH_3)), 3.08 (d, J = 7.2 Hz, 2H, allyl CH_2), 3.09 (s, 3H, N(CH_3)), 3.23–3.58 (m, 4H, 5′-H and $POCH_2$), 3.76, 3.76, 3.77, 3.77 (4s, 3H, OCH_3), 3.82–3.98 (m, 2H, isopropyl CH), 4.15 (m, 1H, 4′-H), 4.45 (m, 1H, 3′-H), 4.90–4.98 (m, 3H, 2′-H and allyl CH_2), 5.94 (m, 1H, allyl CH), 6.27, 6.28 (2s, 1H, 1′-H), 6.75–7.48 (m, 13H, H_{ar}), 7.72, 7.72 (s, 1H, NCHN); MS ESI(+): *m/z* (%): 971.9 (100) $[M+H]^+$, 870.6 (60.1) $[M - N(iPr)_2]^+$.

Oligonucleotide synthesis, purification, and characterization: Synthesis of the oligonucleotides was performed by phosporamidite solid-phase chemistry by standard techniques with automated synthesizers (PerSeptive Biosystem Expedite 8905 for DNA, Eppendorf Biotronik D300+ for RNA) on a 1 μmol scale. Therefore we used a 500 Å CPG support. 1H-Tetrazole was used as coupling activator in all cases. The coupling times were 30 s for the standard DNA building blocks (PerSeptive Biosystems) and 300 s for the 2′-deoxy- and 2′-methoxy-6-oxocytidine compounds. For all RNA building blocks a coupling time of 10 min was used. The 6-oxocytidine phosphoramidites were freeze-dried and employed as 0.1M solutions in dry acetonitrile. All coupling efficiencies were comparable with those of the common nucleoside phosphoramidites, judged by the color of the liberated DMTr cation. DNA strands were synthesized in the trityl-on mode and deprotected with conc. NH_3 overnight at 55 °C. The purification of the crude oligomers was achieved by RP-HPLC (Merck–Hitachi) by means of a Lichrosphor RP18 column filled with POROS R3 silica gel (PerSeptive Biosystems). The mobile phase was a linear gradient of 0–25% acetonitrile over a period of 10 min in 0.1M aqueous triethylammonium acetate buffer (pH 7.0). The collected oligonucleotides were reduced in volume, detritylated with 80% aqueous acetic acid (30 min, RT), and precipitated with ethanol. RNA strands were synthesized in the trityl-off mode and deprotected with conc. NH_3/methanol 3:1 overnight at 55 °C. The crude oligomers were deprotected at the 2′-position with 1 mL triethylamine trihydrofluoride for 24 h at RT. Subsequently the crude RNA was precipitated by butanol (−20 °C, 2 h). The oligonucleotides were dissolved in DEPC/water and purified by anion exchange HPLC (JASCO) using a Dionex NucleoPac™ PA100 column. The mobile phase was a linear gradient of buffer B in A (0 to 70% in 40 min). Buffer A was DEPC/water (pH 8.0):acetonitrile 9:1, buffer B 1M LiCl (pH 8.0):acetonitrile 9:1. The collected oligonucleotides were reduced in volume and desalted on Sephadex G25 columns. Finally the yields of all oligonucleotides were measured at 260 nm. The extinction coefficients were calculated according to the data given by Puglisi and Tinoco and by Gray et al.[61, 67] The characterization of the oligonucleotides was performed on a VG Biotec Tofspec MALDI mass spectrometer.

UV thermal denaturation studies: Thermal denaturation experiments were performed on a Varian Cary 1 UV/Vis spectrophotometer equipped with a Peltier temperature control unit in 1 mL of sodium cacodylate buffer (50 mM) at pH 5.0, 6.0, 7.0, and 7.4 containing magnesium chloride (20 mM) and sodium chloride (100 mM). The corresponding target duplex was prepared by dissolving equimolar amounts of the 21-mer strands in the buffer according to their molar extinction coefficients at 260 nm in a cuvette with 10 mm length. The target duplex was denatured at 80 °C for 10 min and renatured with at 5 °C min^{-1}. After the mixture had reached 20 °C an equimolar amount (2 μM) of the third strand was added and the mixture was cooled to 0 °C for 20 min to reach equilibrium. Longer cooling times (e.g. overnight) did not change the results. Then measurement was started by increasing the temperature at a rate of 0.5 °C min^{-1}, and the UV absorbance was determined at 260 and 274 nm for every 0.5 °C step until 85 °C was reached. Since a correct melting curve is an equilibrium measurement, this assumption was tested by measuring the melting curve at a lower heating rate (here 0.2 °C min^{-1}). The curves were identical. Every melting curve was determined twice and the T_m values were calculated from α versus T plots. At low temperatures (< 20 °C) the cuvette chamber was flushed with nitrogen to prevent condensation.

CD measurements: The samples of the UV spectroscopic investigations were used directly. All CD measurements were performed on a JASCO J-710 spectropolarimeter equipped with a Neslab RTE-100 temperature control unit. The wavelength-dependent spectra were recorded between 330 and 210 nm with a resolution of 0.2 nm, a scan speed of 50 nm min^{-1}, a response of 1 s, and a bandwidth of 1.0 nm. Every spectrum was accumulated five times and smoothed (noise reduction). At each temperature the spectrum of the buffer was subtracted.

The temperature-dependent spectra were recorded between 5 °C or 10 °C and 80 °C with a heating rate of 0.5 °C min^{-1} and a resolution of 0.1 nm, a response of 2 s, and a bandwidth of 2.0 nm. A data point was registered every 0.1 °C and the curves were smoothed. For a better comparison of the curves in Figures 7 and 8 the ellipticity [θ] has been normalized. This was done by means of the equation $\theta_{norm} = (\theta(T) - \theta_l)/(\theta_h - \theta_l)$, where ($\theta_l$ = ellipticity at the lowest temperature (10 °C), θ_h = ellipticity at the highest temperature (80 °C)).

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