4-Nitroindazole: Its Ambiguous Nature in Oligonucleotide Base Pairing and the Influence of the Glycosylation Position on the Duplex Stability

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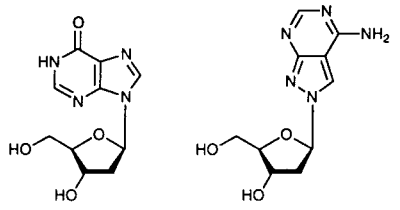
Dedicated to Prof. Dr. Dr. *h.c. Wolfgang Pfleiderer* on the occasion of his 75th birthday

Oligonucleotides incorporating the regioisomeric 4-nitroindazole N^1- and N^2-(2′-deoxy-β-D-ribofuranosides) **7** and **8** were synthesized and their base-pairing properties investigated. For solid-phase synthesis, the phosphoramidites **11** and **12** were prepared. Oligonucleotides containing the building block **7** or **8** show ambiguous base pairing. Duplexes have similar T_m values when the modified bases are positioned opposite to the four canonical DNA constituents. The glycosylation position of the regioisomeric 4-nitroindazole nucleosides has very little influence on the duplex stability.

Introduction. – The degeneracy of the genetic code leads to a multitude of possible DNA sequences coding for a particular protein. This led to a continuing need for more-or-less degenerate oligonucleotides. Multimer mixtures of oligonucleotides covering codon-degenerate positions have been used. An alternative approach employs the incorporation of less-discriminatory base analogues at these positions. Such bases have been described as universal, ambiguous, or degenerated [1]. A truly ambiguous or universal base should be capable of forming base pairs equally well with all four natural bases without discrimination and significant destabilization of the DNA duplex.

Universal nucleosides – either ribonucleosides or 2′-deoxyribonucleosides – are categorized into two types: *i*) those forming base pairs by H-bonding and *ii*) those whose stability depends mainly on stacking interactions. One of the most widely used universal nucleoside of the first type is 2′-deoxyinosine (**1**), which can form H-bonds with all four natural nucleosides. When 2′-deoxyinosine is incorporated into 12-mer duplexes opposite each of the four natural nucleosides, a wide range of T_m values ($\Delta T_m = 15^\circ$) is observed, showing that its base does not pair equally well with the bases of the other DNA constituents [2][3]. Thus, it cannot be considered a true universal nucleoside. In PCR and sequencing applications, 2-deoxyinosine behaves almost like 2′-deoxyguanosine [4]. Other nucleosides are the P and K nucleosides, each of which pairs with two of the DNA constituents [1]. One recent example of this category is the 8-aza-7-deazaadenine N^8-(2′-deoxyribofuranoside) **2**, which hybridizes almost equally well with each of the four canonical DNA constituents dA, dT, dG, and dC. It represents a new class of universal base analogue that have the potential to form bidentate H-bonds with each of the natural DNA bases. Oligonucleotides containing compound **2** have T_m values within a 2° range when opposite each of the natural nucleosides [5], while the regularly linked nucleoside **3** shows base-pairing selectivity towards dT [6]. Studies

have been performed to establish the H-bonding capabilities of **2** by means of its analogues **4a** and **4b** [7–9].



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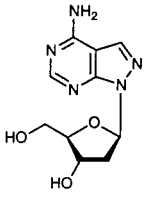

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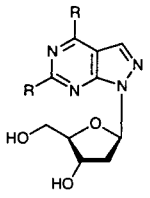
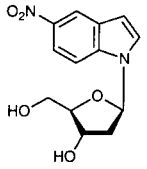


4a R = H
b R = NH_2



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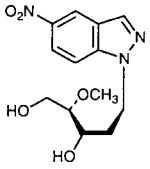

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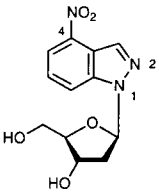

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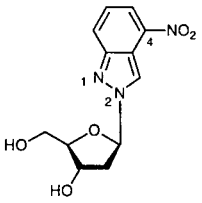

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There are many more examples of the second category of universal nucleotide, which stabilize DNA duplexes by stacking interactions, rather than by H-bonding. These include the heterocyclic bases of nucleosides containing 5-nitro-1*H*-indole **5** [10][11], 3-nitro-1*H*-pyrrole [12], difluorotoluene [13], 5-nitro-1*H*-indazole **6** [14], and 4-nitro-1*H*-benzimidazole [15]. Among these, 5-nitro-1*H*-indole is of particular importance. The 2′-deoxy-*β*-D-ribofuranoside **5** shows T_m values within 4–7° when it is incorporated into 17-mer duplexes opposite each of the natural bases [11].

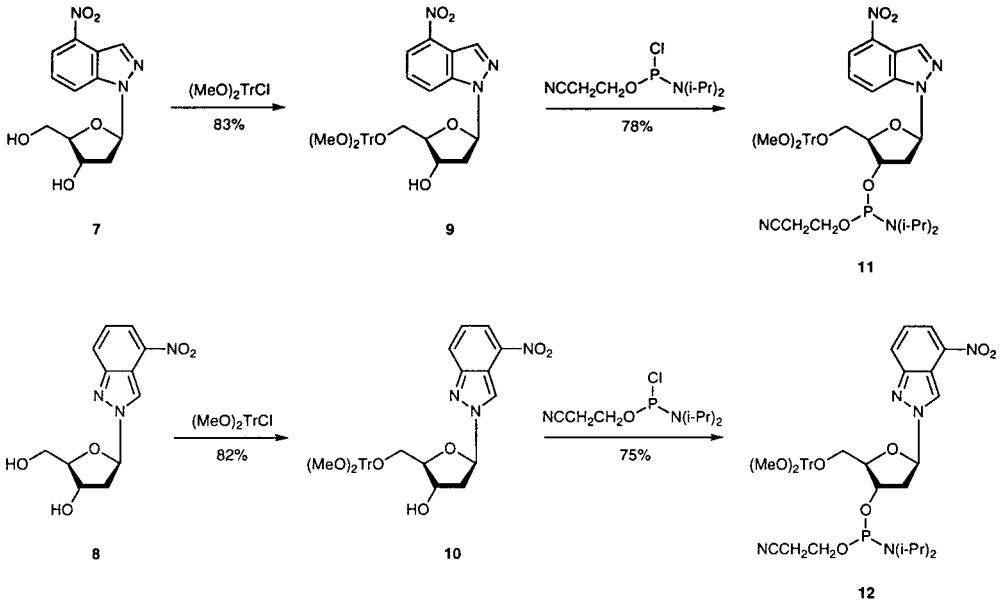
The present work is an investigation of the regioisomeric N^1- and N^2-(2′-deoxy-*β*-D-ribofuranosides) **7** and **8** of 4-nitroindazole incorporated in a 12-mer duplex. Both nucleosides are expected to stack within duplex DNA but to be devoid of the ability to form *Watson-Crick*-type H-bonds, as was discussed for compounds **2** and **3**. The ability to form ambiguous base pairs will be studied for both regioisomers **7** and **8** and will be compared with the behavior of the 5-nitro-1*H*-indole nucleoside **5**, known for its universal base pairing. The influence of the glycosylation site on the base stacking will be investigated as the electronic properties of the two regioisomers are different.

Results and Discussion. – 1. *Monomers.* Ribonucleosides of indazole and of 4-, 5-, and 6-nitroindazoles have already been described [16][18–21]; the formation of regioisomeric glycosylation products depends on the particular reaction conditions and on the structures of the precursors. Kinetic control leads to the N^2-glycosylation products, as shown for the ribosylation of indazole, 4-, 5-, and 6-nitroindazoles, and indazole carbonitrile *via* trimethylsilyl derivatives or *via* $Hg(CN)_2/CH_3NO_2$ method [16–18]. Thermodynamic control occurs during the fusion reaction, *e.g.*, of indazole and 1,2,3,5-tetra-*O*-acetyl-*β*-D-ribofuranose in the presence of a *Lewis*-acid catalyst. In

this case, the N^1-glycosylated regioisomer is the predominant product [19–21]. *Boryski* investigated the transglycosylation reaction of indazole nucleosides such as β-D-ribofuranosylindazole [22]. Several indazole ribonucleosides exhibit biological activity [21][23][24].

Earlier, we studied the synthesis of indazole and 4-nitroindazole deoxyribonucleosides [25]. At room temperature and under alkaline conditions, the 4-nitroindazolyl anion is generated, leading to a mixture of N^1- and N^2-glycosylated indazole derivatives in a nearly 1:1 proportion. The glycosylation of 4-nitroindazole with 2-deoxy-3,5-bis-*O*-(4-methylbenzoyl)-α-D-*erythro*-pentofuranosyl chloride was followed by detoluoylation (MeOH/NaOMe) furnishing the nucleosides **7** and **8** [25]. The latter compounds were converted to their 4,4′-dimethoxytrityl derivatives **9** and **10** (*Scheme*). Treatment with 2-cyanoethyl diisopropylphosphoramidochloridite resulted in the formation of the phosphoramidites **11** and **12**.

Scheme



All monomeric compounds were characterized by elemental analysis as well as by ^{1}H-,^{13}C-, and ^{31}P-NMR spectra (see *Exper. Part* and *Table 1*). The ^{13}C-NMR signals were assigned by gated-decoupled ^{13}C-NMR or heteronuclear [^{1}H,^{13}C]-NMR correlation spectra. The ^{13}C-NMR indicated a significant upfield shift of the C(7) signal when the glycosylic position is changed from N(1) to N(2). Next, studies with regard to the nucleoside stability of **7** and **8** were performed. The reaction was carried out in 0.5 or 0.1M HCl at room temperature or 50°. It was monitored UV-spectrophotometrically. The N^1-nucleoside **7** ($t_{1/2}$ 60 min; 0.5N HCl, 50°) was found to be significantly more stable than the N^2-isomer **8** ($t_{1/2}$ 18 min; 0.1N HCl, room temperature).

2. *Oligonucleotides.* 2.1. *Synthesis.* To investigate the base-pairing properties of the nucleosides **7** and **8**, a series of oligonucleotides were synthesized by solid-phase

Table 1. *^{13}C-NMR Chemical Shifts of 4-Nitroindazole 2′-Deoxyribonucleosides*[a])[b])

	C(3)	C(3a)	C(4)	C(5)	C(6)	C(7)	C(7a)
7	132.8	116.6	141.2	118.9	126.3	118.2	139.6
8	123.8	113.5	140.3	120.8	125.0	126.6	149.0
9	132.5	116.8	141.2	118.9	126.3	118.4	139.7
10	124.2	113.6	140.2	120.8	125.0	126.7	149.2
	C(1′)	C(2′)	C(3′)	C(4′)	C(5′)	MeO	MeO
7	86.1	38.3	70.8	87.8	62.1		
8	91.0	40.7	70.3	88.5	61.6		
9	85.0	38.5	70.4	85.8	63.9	54.82	54.86
10	90.5	40.5	70.4	86.3	63.7	54.84	54.86

[a]) Measured in (D_6)DMSO. [b]) Systematic numbering.

synthesis employing the phosphoramidites **11** and **12** [26]. Oligomers incorporating the 5-nitro-1*H*-indole nucleoside **5** were prepared from the commercially available phosphoramidite. The coupling efficiency of the phosphoramidites was always higher than 95%. Deprotection was performed with 25% aqueous NH_3 solution (60°, 16 h), and the oligonucleotides were purified by oligonucleotide purification cartridges (OPC) [27] or by reversed-phase HPLC. The homogeneity of the oligonucleotides **13**–**29** (see below, *Tables 3–5*) was established by reversed-phase HPLC. The nucleotide composition was determined after oligonucleotide digestion with snake-venom phosphodiesterase followed by alkaline phosphatase [28]. Three different oligonucleotides were used: Compound **23** contains the 5-nitro-1*H*-indole nucleoside **5**, while **17** and **20** contain the regularly linked and the N^2-linked 4-nitroindazole nucleosides **7** and **8**, respectively. The outcome of these experiments is shown in the *Figure.* While the indazole nucleosides showed similar mobilities (*Fig.*, *b* and *c*), the indole nucleoside migrated more slowly (*Fig.*, *a*), due to its more lipophilic character. From the digest of **20** leading to compound **8** and its faster-migrating monophosphate (data not shown), it

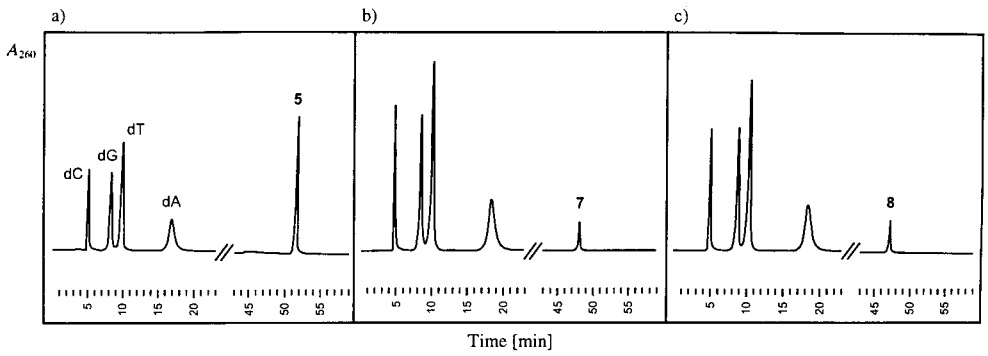


Figure. *HPLC Profiles of the reaction products obtained after enzymatic hydrolysis of* a) *oligomer* **23**, b) oligomer **17**, and c) oligomer **20** *with snake-venomphosphodiesterase and alkaline phosphatase in 0.1*M Tris·*HCl buffer* (pH 8.0) *at 37°*. Column, *RP-18* (200 × 10 mm); gradient III: 20 min 100% *A*, 20–60 min 0–50% *B* in *A*, flow rate 0.7 ml/min (see *Exper. Part*).

was obvious that the 5′-phosphate of **8** is rather resistant to alkaline phosphatase. The latter migrated significantly faster but was finally converted to the nucleoside when the hydrolysis time was increased from 12 h to 20 h.

Next, the MALDI-TOF-MS of the oligonucleotides **15**–**23** were measured by using 3-hydroxypicolinic acid as matrix (*Table 2*). However, instead of observing only the pseudomolecular-ion peak $M\mathrm{H}^+$, two peaks were detected for oligonucleotides with a single incorporation of 4-nitroindazole (see oligonucleotides **15**, **16**, **18** and **19**) or 5-nitro-1*H*-indole (see oligonucleotides **21** and **22**). One peak corresponds to the calculated $M\mathrm{H}^+$ and another to $[M\mathrm{H}-16]^+$. In a similar way, three peaks $M\mathrm{H}^+$, $[M\mathrm{H}-16]^+$, and $[M\mathrm{H}-32]^+$ were notified for oligonucleotides with two incorporations of 4-nitroindazole (see oligomers **17** and **20**) or 5-nitro-1*H*-indole (see **23**). These peaks are not caused by impurities as the HPLC profiles as well as the enzymic digests confirmed the homogeneity of the oligomers.

Table 2. *Molecular Masses* ($M\mathrm{H}^+$) *of Oligonucleotides Measured by MALDI-TOF-MS*

	$M\mathrm{H}^+$ (calc.)	$M\mathrm{H}^+$ (found)
5′-d(A-G-T-A-T-T-G-**7**-C-C-T-A)-3′ (**15**)	3673.46	3672.61 and 3656.63
5′-d(T-A-G-G-T-C-A-**7**-T-A-C-T)-3′ (**16**)	3673.46	3672.62 and 3657.80
5′-d(T-A-G-G-T-C-**7-7**-T-A-C-T)-3′ (**17**)	3701.47	3703.21, 3684.77, and 3669.75
5′-d(A-G-T-A-T-T-G-**8**-C-C-T-A)-3′ (**18**)	3673.46	3674.24 and 3658.95
5′-d(T-A-G-G-T-C-A-**8**-T-A-C-T)-3′ (**19**)	3673.46	3675.21 and 3659.64
5′-d(T-A-G-G-T-C-**8-8**-T-A-C-T)-3′ (**20**)	3701.47	3699.09, 3683.44, and 3668.04
5′-d(A-G-T-A-T-T-G-**5**-C-C-T-A)-3′ (**21**)	3672.48	3671.03
5′-d(T-A-G-G-T-C-A-**5**-T-A-C-T)-3′ (**22**)	3672.48	3671.14 and 3656.15
5′-d(T-A-G-G-T-C-**5-5**-T-A-C-T)-3′ (**23**)	3699.50	3698.97, 3683.05, and 3667.98

In the MALDI-TOF-MS experiments, a laser beam (337.1 nm) was used to bring the oligonucleotides into the vapor phase; the peaks at $[M\mathrm{H}-16]^+$ and $[M\mathrm{H}-32]^+$ are assumed to be caused by the laser irradiation of the oligonucleotide. Earlier, it had been shown that aromatic nitro groups are light-sensitive [29][30]. A nitro group can dissociate into nitric oxide and atomic oxygen upon irradiation. Intermolecular and intramolecular photoredox reactions and photosubstitutions can occur [31–36]. Such reactions have been observed in the case of nitrobenzene derivatives or *p*-nitroacetophenone. Moreover, *Kotera et al.* [37] have studied the photolysis of a 7-nitro-1*H*-indole 2′-deoxyribonucleoside, taking into account the possibility of fragmentation of the 7-nitro-1*H*-indole residue to its nitroso derivative. We suggest that our side products are formed by such a process. The nitro groups of **5**, **7**, and **8** are reduced to a nitroso function: the atomic oxygen might then react with the matrix 3-hydroxypicolinic acid. As these processes depend on the number of nitro-base incorporations in the oligonucleotide, it is clear that one incorporation gives a side peak of $[M\mathrm{H}-16]^+$ and two incorporations one of $[M\mathrm{H}-32]^+$.

2.2. *Duplex Stability and Ambiguous Base Pairing*. Earlier, it has been shown that shape mimics of canonical nucleosides can efficiently substitute their natural counterparts [38][39]. Oligonucleotide duplex stability depends not only on the strength of H-bonds formed in a *Watson-Crick* base pair but also on other factors. The stacking with

adjacent bases, solvation and desolvation effects, and, last but not least, the hydrophobic character of the nucleobase as well as of their molecular polarizability have to be taken into consideration. As a result, nucleosides were designed showing strong base stacking but not forming H-bonds [40–42]. This led to the development of the so-called universal nucleosides. Among the various compounds incorporated into the duplex DNA, the 5-nitro-1*H*-indole **5** has received prominent attention. The design of this analogue was aimed to maximize base-stacking interactions. This was accomplished by the presence of a nitro group to enhance the stacking of the aromatic π-system. Moreover, the bases have to be rather hydrophobic [11][12]. Other investigations regarding ambiguous base pairing were performed with the acyclic 2′-deoxyribonucleoside **6** of 5-nitro-1*H*-indazole (1-(4-*O*-methyl-2-deoxy-D-ribityl)-5-nitro-1*H*-indazole [14]. As the aromatic character of the nucleobases is changing when the glycosylation position is shifted from N(1) to N(2), we were interested to investigate the base-pairing properties of the regioisomeric 4-nitroindazole 2′-deoxyribonucleosides **7** and **8** that we incorporated into the 12-mer duplex formed from 5′-d(AGTATTGACCTA)-3′ (**14**) and 5′-d(TAGGTCAATACT)-3′ (**13**). For comparison, also the 5-nitro-1*H*-indole nucleoside **5** was incorporated at exactly the same positions. A single substitution was made replacing a dA residue near the 3′-end of 5′-d(AGTATTGACCTA)-3′ (**14**) leading to the oligonucleotides **15**, **18**, and **21**. In the other series, a dA residue was replaced in the second strand 5′-d(TAGGTCAATACT)-3′ (**13**) furnishing the oligonucleotides **16**, **19**, and **22**. In each case, these strands were hybridized to another strand incorporating the canonical nucleosides dT, dC, dA, and dG opposite the nucleosides **7**, **8**, and **5**. The T_m values and the thermodynamic data of these experiments are shown in *Tables 3* and *4*.

From *Table 3*, it is obvious that the 4-nitroindazole nucleosides **7** and **8** behave qualitatively in very similar ways to each other and also similarly to 5-nitro-1*H*-indole nucleoside **5**. The T_m values were generally 10–12° lower than that of the parent duplex **13**·**14**. A slightly destabilizing effect was found for the indazole nucleosides ($\Delta T_m - 2°$) as compared to the 5-nitro-1*H*-indole nucleoside **5**. It is surprising that the 4-nitroindazole nucleosides **7** and **8**, which have different glycosylation positions, gave similar T_m values in the range of $\pm 2°$, when paired opposite to each of the four common bases. Also, when another position within the oligonucleotide duplex **13**·**14** was changed (*Table 4*), a single substitution by **7**, **8** or **5** led to similar results with **7** and **8**, while **5** was slightly less-destabilizing (4° against dC and dA, resp.) as compared to the corresponding strands of *Table 3*.

Also the incorporation of two modified residues was studied, by replacing two dA residues near the 3′-end of d(TAGGTCAATACT) (**13**) (*Table 5*). In the resulting duplex, compounds **7**, **8** or **5** were incorporated in a consecutive way; one residue base-paired with dT, while the second one was located opposite to the four regular nucleosides. A T_m decrease over a 3–10° range was observed with two incorporations compared to one incorporation in the same strand. The slightly higher T_m values of 5-nitro-1*H*-indole **5** over the 4-nitroindazole analogues **7** and **8** can be attributed to the higher molecular polarizability of 5-nitro-1*H*-indole, which is $17.86 \pm 0.5 \cdot 10^{-24}$ cm^{-3} as compared to that of 4-nitroindazole $= 17.11 \pm 0.5 \cdot 10^{-24}$ cm^3 [42][43]. The 5-nitro-1*H*-indole is also more hydrophobic than 4-nitroindazole (see *Fig.*), which can increase stacking interactions.

Table 3. T_m *Values and Thermodynamic Data of Oligonucleotides Incorporating the Nucleosides* **7**, **8**, *and* **5** *Opposite to the Canonical Nucleosides*[a])

	T_m [°][b])	$\Delta H°$ [kcal/mol]	$\Delta S°$ [cal/mol·K]	$\Delta G°_{310}$ [kcal/mol]
5′-d(T-A-G-G-T-C-A-A-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-**A**-G-T-T-A-T-G-A)-5′ (**14**)	50	– 90	– 252	– 12.0
5′-d(T-A-G-G-**T**-C-A-A-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-**7**-G-T-T-A-T-G-A)-5′ (**15**)	39	– 69	– 196	– 8.0
5′-d(T-A-G-G-**C**-C-A-A-T-A-C-T)-3′ (**24**) 3′-d(A-T-C-C-**7**-G-T-T-A-T-G-A)-5′ (**15**)	39	– 72	– 205	– 8.1
5′-d(T-A-G-G-**A**-C-A-A-T-A-C-T)-3′ (**25**) 3′-d(A-T-C-C-**7**-G-T-T-A-T-G-A)-5′ (**15**)	41	– 57	– 157	– 8.2
5′-d(T-A-G-G-**G**-C-A-A-T-A-C-T)-3′ (**26**) 3′-d(A-T-C-C-**7**-G-T-T-A-T-G-A)-5′ (**15**)	38	– 64	– 180	– 7.9
5′-d(T-A-G-G-**T**-C-A-A-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-**8**-G-T-T-A-T-G-A)-5′ (**18**)	37	– 60	– 170	– 7.6
5′-d(T-A-G-G-**C**-C-A-A-T-A-C-T)-3′ (**24**) 3′-d(A-T-C-C-**8**-G-T-T-A-T-G-A)-5′ (**18**)	38	– 48	– 130	– 7.8
5′-d(T-A-G-G-**A**-C-A-A-T-A-C-T)-3′ (**25**) 3′-d(A-T-C-C-**8**-G-T-T-A-T-G-A)-5′ (**18**)	39	– 59	– 163	– 7.9
5′-d(T-A-G-G-**G**-C-A-A-T-A-C-T)-3′ (**26**) 3′-d(A-T-C-C-**8**-G-T-T-A-T-G-A)-5′ (**18**)	41	– 66	– 185	– 8.4
5′-d(T-A-G-G-**T**-C-A-A-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-**5**-G-T-T-A-T-G-A)-5′ (**21**)	40	– 69	– 196	– 8.3
5′-d(T-A-G-G-**C**-C-A-A-T-A-C-T)-3′ (**24**) 3′-d(A-T-C-C-**5**-G-T-T-A-T-G-A)-5′ (**21**)	41	– 71	– 200	– 8.6
5′-d(T-A-G-G-**A**-C-A-A-T-A-C-T)-3′ (**25**) 3′-d(A-T-C-C-**5**-G-T-T-A-T-G-A)-5′ (**21)**	40	– 69	– 196	– 8.3
5′-d(T-A-G-G-**G**-C-A-A-T-A-C-T)-3′ (**26**) 3′-d(A-T-C-C-**5**-G-T-T-A-T-G-A)-5′ (**21**)	42	– 71	– 201	– 8.8

[a]) **7** = 4-Nitro-1*H*-indazole N^1-nucleoside; **8** = 4-nitro-2*H*-indazole N^2-nucleoside; **5** = 5-nitro-1*H*-indole nucleoside. [b]) Measured at 260 nm in 1M NaCl, 100 mM $MgCl_2$, and 60 mM Na-cacodylate (pH 7.0) with 5 + 5 μM single-strand concentration.

Canonical bases are hydrophilic in nature, so, prior to the formation of a normal base pair, the H-bonding groups are each solvated by H_2O molecules. Base-pair formation results in the loss of these solvating H_2O molecules with the formation of the new H-bonds of the base pair. Thus, in common base pairing, the energy lost during desolvation is somehow compensated by the formation of new H-bonds. In contrast, when the hydrophobic or poorly solvated molecules **7**, **8**, and **5** pair with hydrophilic natural bases, the cost of desolvation of the hydrophilic bases remains high, while desolvation cost of **7**, **8**, and **5** is low. This energetic desolvation penalty cannot be compensated as there is no possibility for H-bonding of **7**, **8**, and **5** with natural bases [41]. These differences can explain why the incorporation of these nucleosides destabilizes the duplexes (by *ca.* 10°) compared to unmodified duplex **13**·**14**.

Finally, it was of interest to know how an abasic sugar unit affects the base pairing. *Barsky et al.* [44] have made molecular-dynamics simulations of abasic sites within the

Table 4. T_m *Values and Thermodynamic Data of Oligonucleotides Incorporating the Nucleosides* **7**, **8**, *and* **5** *Opposite to the Canonical Nucleosides*[a])

	T_m [°][b])	$\Delta H°$ [kcal/mol]	$\Delta S°$ [cal/mol · K]	$\Delta G°_{310}$ [kcal/mol]
5′-d(T-A-G-G-T-C-A-**A**-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-A-G-T-**T**-A-T-G-A)-5′ (**14**)	50	−90	−252	−12.0
5′-d(T-A-G-G-T-C-A-**7**-T-A-C-T)-3′ (**16**) 3′-d(A-T-C-C-A-G-T-**T**-A-T-G-A)-5′ (**14**)	38	−56	−156	−7.8
5′-d(T-A-G-G-T-C-A-**7**-T-A-C-T)-3′ (**16**) 3′-d(A-T-C-C-A-G-T-**C**-A-T-G-A)-5′ (**27**)	39	−58	−162	−8.0
5′-d(T-A-G-G-T-C-A-**7**-T-A-C-T)-3′ (**16**) 3′-d(A-T-C-C-A-G-T-**A**-A-T-G-A)-5′ (**28**)	41	−61	−170	−8.3
5′-d(T-A-G-G-T-C-A-**7**-T-A-C-T)-3′ (**16**) 3′-d(A-T-C-C-A-G-T-**G**-A-T-G-A)-5′ (**29**)	36	−50	−138	−7.4
5′-d(T-A-G-G-T-C-A-**8**-T-A-C-T)-3′ (**19**) 3′-d(A-T-C-C-A-G-T-**T**-A-T-G-A)-5′ (**14**)	37	−57	−160	−7.6
5′-d(T-A-G-G-T-C-A-**8**-T-A-C-T)-3′ (**19**) 3′-d(A-T-C-C-A-G-T-**C**-A-T-G-A)-5′ (**27**)	37	−57	−160	−7.7
5′-d(T-A-G-G-T-C-A-**8**-T-A-C-T)-3′ (**19**) 3′-d(A-T-C-C-A-G-T-**A**-A-T-G-A)-5′ (**28**)	40	−58	−160	−8.0
5′-d(T-A-G-G-T-C-A-**8**-T-A-C-T)-3′ (**19**) 3′-d(A-T-C-C-A-G-T-**G**-A-T-G-A)-5′ (**29**)	39	−61	−170	−8.1
5′-d(T-A-G-G-T-C-A-**5**-T-A-C-T)-3′ (**22**) 3′-d(A-T-C-C-A-G-T-**T**-A-T-G-A)-5′ (**14**)	42	−63	−173	−8.7
5′-d(T-A-G-G-T-C-A-**5**-T-A-C-T)-3′ (**22**) 3′-d(A-T-C-C-A-G-T-**C**-A-T-G-A)-5′ (**27**)	45	−75	−210	−9.6
5′-d(T-A-G-G-T-C-A-**5**-T-A-C-T)-3′ (**22**) 3′-d(A-T-C-C-A-G-T-**A**-A-T-G-A)-5′ (**28**)	44	−65	−181	−8.9
5′-d(T-A-G-G-T-C-A-**5**-T-A-C-T)-3′ (**22**) 3′-d(A-T-C-C-A-G-T-**G**-A-T-G-A)-5′ (**29**)	41	−59	−163	−8.2

[a]) **7** = 4-Nitro-1*H*-indazole N^1-nucleoside; **8** = 4-nitro-2*H*-indazole N^2-nucleoside; **5** = 5-nitro-1*H*-indole nucleoside. [b]) Measured at 260 nm in 1M NaCl, 100 mM $MgCl_2$, and 60 mM Na-cacodylate (pH 7.0) with 5 + 5 μM single-strand concentration.

DNA duplex. They showed that the abasic site does not maintain a hole or a gap in the DNA. Instead, the backbone structure changes or is filled with H_2O molecules. The data for our modification are shown in *Table 6*, where the abasic residues **5** were incorporated exactly at the same position as the universal nucleosides (*Table 4*) and were again located opposite to the four canonical bases [5]. In *Table 6*, the T_m values of corresponding mismatches are also given. From these experiments, it is apparent that an abasic residue can also act as universal residue but with the difference of a strong destabilization (−17°), while the destabilization in the presence of the 4-nitroindazole nucleosides **7** and **8** is in the range of 10°. The 5-nitro-1*H*-indole nucleoside **5** leads to a destabilization of 5–9°. The T_m values of duplexes with mismatches are slightly higher than those with abasic sites. In particular cases (**13** · **33**) new base pairs (dG · dA) can be formed, which further increase the duplex stability.

Table 5. T_m *Values and Thermodynamic Data of Oligonucleotides Incorporating the Nucleosides* **7**, **8**, *and* **5** *Opposite to dT and Other Canonical Nucleosides*[a])

	T_m [°][b])	ΔH° [kcal/mol]	ΔS° [cal/mol · K]	ΔG°_{310} [kcal/mol]
5′-d(T-A-G-G-T-C-**A-A**-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-A-G-**T-T**-A-T-G-A)-5′ (**14**)	50	– 90	– 252	– 12.0
5′-d(T-A-G-G-T-C-**7-7**-T-A-C-T)-3′ (**17**) 3′-d(A-T-C-C-A-G-**T-T**-A-T-G-A)-5′ (**14**)	31	– 46	– 125	– 6.7
5′-d(T-A-G-G-T-C-**7-7**-T-A-C-T)-3′ (**17**) 3′-d(A-T-C-C-A-G-**T-C**-A-T-G-A)-5′ (**27**)	34	– 52	– 145	– 7.0
5′-d(T-A-G-G-T-C-**7-7**-T-A-C-T)-3′ (**17**) 3′-d(A-T-C-C-A-G-**T-A**-A-T-G-A)-5′ (**28**)	31	– 44	– 120	– 6.4
5′-d(T-A-G-G-T-C-**7-7**-T-A-C-T)-3′ (**17**) 3′-d(A-T-C-C-A-G-**T-G**-A-T-G-A)-5′ (**29**)	32	– 43	– 115	– 7.0
5′-d(T-A-G-G-T-C-**8-8**-T-A-C-T)-3′ (**20**) 3′-d(A-T-C-C-A-G-**T-T**-A-T-G-A)-5′ (**14**)	32	– 48	– 131	– 6.8
5′-d(T-A-G-G-T-C-**8-8**-T-A-C-T)-3′ (**20**) 3′-d(A-T-C-C-A-G-**T-C**-A-T-G-A)-5′ (**27**)	34	– 44	– 120	– 7.1
5′-d(T-A-G-G-T-C-**8-8**-T-A-C-T)-3′ (**20**) 3′-d(A-T-C-C-A-G-**T-A**-A-T-G-A)-5′ (**28**)	32	– 43	– 117	– 6.9
5′-d(T-A-G-G-T-C-**8-8**-T-A-C-T)-3′ (**20**) 3′-d(A-T-C-C-A-G-**T-G**-A-T-G-A)-5′ (**29**)	33	– 42	– 110	– 7.4
5′-d(T-A-G-G-T-C-**5-5**-T-A-C-T)-3′ (**23**) 3′-d(A-T-C-C-A-G-**T-T**-A-T-G-A)-5′ (**14**)	37	– 53	– 145	– 7.7
5′-d(T-A-G-G-T-C-**5-5**-T-A-C-T)-3′ (**23**) 3′-d(A-T-C-C-A-G-**T-C**-A-T-G-A)-5′ (**27**)	39	– 58	– 162	– 7.9
5′-d(T-A-G-G-T-C-**5-5**-T-A-C-T)-3′ (**23**) 3′-d(A-T-C-C-A-G-**T-A**-A-T-G-A)-5′ (**28**)	37	– 54	– 149	– 7.5
5′-d(T-A-G-G-T-C-**5-5**-T-A-C-T)-3′ (**23**) 3′-d(A-T-C-C-A-G-**T-G**-A-T-G-A)-5′ (**29**)	37	– 48	– 131	– 7.5

[a]) **7** = 4-Nitro-1*H*-indazole N^1-nucleoside; **8** = 4-nitro-2*H*-indazole N^2-nucleoside; **5** = 5-nitro-1*H*-indole nucleoside. [b]) Measured at 260 nm in 1M NaCl, 100 mM $MgCl_2$, and 60 mM Na-cacodylate (pH 7.0) with 5 + 5 μM single-strand concentration.

Conclusions. – From the results described above, the following conclusions can be drawn: Like 5-nitro-1*H*-indole, the 4-nitroindazole analogues **7** and **8** behaved non-discriminately towards the four natural DNA bases. Thus, they are candidates for universal nucleosides. The T_m values of duplex melting were generally lower when compared with those containing natural *Watson-Crick* base pairs, but acceptable when compared with those incorporating the 5-nitro-1*H*-indole nucleoside **5**. It was not expected that a change of the glycosylation position showed so little influence on the pairing behavior. However, a significant sequence dependency was noticed when the universal nucleoside was located between two dA · dT *vs.* two dG · dC base pairs.

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Table 6. T_m *Values and Thermodynamic Data of Oligonucleotide Duplexes Containing Mismatches or Abasic Sites Located Opposite to Canonical DNA Constituents*

	T_m [°][a])	ΔH° [kcal/mol]	ΔS° [cal/mol·k]	ΔG°_{310} [kcal/mol]
Abasic sugar units[a])				
5′-d(T-A-G-G-T-C-A-**S**-T-A-C-T)-3′ (**30**) 3′-d(A-T-C-C-A-G-T-**T**-A-T-G-A)-5′ (**14**)	33	−47	−129	−7.0
5′-d(T-A-G-G-T-C-A-**S**-T-A-C-T)-3′ (**30**) 3′-d(A-T-C-C-A-G-T-**C**-A-T-G-A)-5′ (**27**)	34	−52	−146	−7.1
5′-d(T-A-G-G-T-C-A-**S**-T-A-C-T)-3′ (**30**) 3′-d(A-T-C-C-A-G-T-**A**-A-T-G-A)-5′ (**28**)	34	−51	−141	−7.2
5′-d(T-A-G-G-T-C-A-**S**-T-A-C-T)-3′ (**30**) 3′-d(A-T-C-C-A-G-T-**G**-A-T-G-A)-5′ (**29**)	33	−48	−131	−7.0
Mismatches				
5′-d(T-A-G-G-T-C-A-**A**-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-A-G-T-**C**-A-T-G-A)-5′ (**31**)	36	−64	−183	−7.5
5′-d(T-A-G-G-T-C-A-**A**-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-A-G-T-**A**-A-T-G-A)-5′ (**32**)	39	−65	−184	−8.2
5′-d(T-A-G-G-T-C-A-**A**-T-A-C-T)-3′ (**13**) 3′-d(A-T-C-C-A-G-T-**G**-A-T-G-A)-5′ (**33**)	44	−74	−209	−9.2

[a]) **S** = Abasic sugar. [a]) Measured at 260 nm in 1M NaCl, 100 mM $MgCl_2$ and 60 mM Na-cacodylate (pH 7.0) with 5 + 5 μM single-strand concentration.

Experimental Part

General. All chemicals were purchased from *Aldrich*, *Sigma*, or *Fluka* (*Sigma-Aldrich Chemie GmbH*, Deisenhofen, Germany). The 5-nitro-1*H*-indole phosphoramidite derived from **5** was purchased from *Glen Research*, USA. Solvents were of laboratory grade. TLC: aluminium sheets, silica gel *60* F_{254}, 0.2 mm layer (*Merck*, Germany). Column flash chromatography (FC): silica gel *60* (*Merck*, Germany) at 0.4 bar; solvent systems: CH_2Cl_2/MeOH 95:5 (*A*), CH_2Cl_2/acetone 98:2 (*B*); sample collection with an *Ultrarac-II* fraction collector (*LKB Instruments*, Sweden). UV Spectra: *U-3200* spectrometer (*Hitachi*, Tokyo, Japan). NMR Spectra: *Avance-250* or *AMX-500* spectrometers (*Bruker*, Karlsruhe, Germany), at 250.13 MHz for ^1H and ^{13}C; Δ in ppm rel. to Me_4Si as internal standard, *J* values in Hz. Elemental analyses were performed by the *Mikroanalytisches Laboratorium*, *Beller* (Göttingen, Germany).

1-[2′-Deoxy-5′-O-*(4,4′-dimethoxytriphenylmethyl)-β*-D-erythro-*pentofuranosyl]-4-nitro-1*H-*indazole* (**9**). Compound **7** [1] (500 mg, 1.8 mmol) was dried by repeated co-evaporation with anh. pyridine and suspended in dry pyridine (2 ml). The soln. was stirred in the presence of *N*,*N*-dimethylpyridin-4-amine (16 mg, 0.13 mmol) and 4,4′-dimethoxytriphenylmethyl chloride (796 mg, 2.3 mmol) for 5 h. The mixture was diluted with 5% aq. $NaHCO_3$ soln. (20 ml) and extracted with CH_2Cl_2 (3 × 20 ml). The combined org. layer was dried (Na_2SO_4) and evaporated and the residue chromatographed (column 12 × 3 cm, *A*). The main zone afforded **9**. Yellow foam (860 mg, 83%). TLC (*A*): R_f 0.56. UV (MeOH): 234 (31500), 275 (4200), 282 (4100), 290 (3000). ^{1}H-NMR ((D$_6$)DMSO): 2.38 (*m*, H_α−C(2′)); 2.87 (*m*, H_β−C(2′)); 3.0 (*m*, CH_2(5′)); 3.66, 3.68 (2*s*, 2 MeO); 3.98 (*m*, H−C(4′)); 4.61 (*m*, H−C(3′)); 5.39 (*d*, *J* = 5.0, OH−C(3′)); 6.80 (*dd*, *J* = 3.1, 7.0, H−C(1′)); 6.60–7.22 (*m*, arom. H); 7.69 (*t*, *J* = 8.1, H−C(6)); 8.22 (*d*, *J* = 7.7, H−C(5)); 8.42 (*d*, *J* = 8.4, H−C(7)); 8.52 (*s*, H−C(3)). Anal. calc. for $C_{33}H_{31}N_3O_7$ (581.62): C 68.15, H 5.37, N 7.22; found: C 68.08, H 5.40, N 7.13.

2-[2′-Deoxy-5′-O-*(4,4′-dimethoxytriphenylmethyl)-β*-D-erythro-*pentofuranosyl]-4-nitro-2*H-*indazole* (**10**). As described for **9**, with **8** [1] (500 mg, 1.8 mmol), *N*,*N*-dimethylpyridin-4-amine (16 mg, 0.13 mmol), 4,4′-dimethoxytriphenylmethyl chloride (796 mg, 2.3 mmol), and dry pyridine (2 ml) for 5 h: **10** (850 mg, 82%). Yellow foam. TLC (*A*): R_f 0.42. UV (MeOH): 233 (28100), 275 (4300), 282 (4100), 290 (3100), 312 (4900), 332 (4300). ^{1}H-NMR ((D$_6$)DMSO): 2.43 (*m*, H_α−C(2′)); 2.86 (*m*, H_β−C(2′)); 3.14 (*m*, CH_2(5′)); 3.66, 3.68 (2*s*, 2 MeO); 4.07 (*m*, H−C(4′)); 4.55 (*m*, H−C(3′)); 5.40 (*d*, *J* = 4.8, OH−C(3′)); 6.61 (*dd*, *J* = 4.0, 6.6, H−C(1′));

6.70–7.29 (*m*, arom. H); 7.52 (*t*, *J* = 8.3, H–C(6)); 8.14 (*d*, *J* = 8.6, H–C(5)); 8.20 (*d*, *J* = 7.5, H–C(7)); 9.03 (*s*, H–C(3)). Anal. calc. for $C_{33}H_{31}N_3O_7$ (581.62): C 68.15, H 5.37, N 7.22; found: C 68.21, H 5.44, N 7.31.

1-[2′-Deoxy-5′-O-*(4,4′-dimethoxytriphenylmethyl)-β-*D-erythro-*pentofuranosyl]-4-nitro-1*H-*indazole 3′-(2-Cyanoethyl diisopropylphosphoramidite)* (**11**). To a soln. of **9** (100 mg, 0.17 mmol) and $(^iPr)_2EtN$ (50 µl, 0.29 mmol) in anh. CH_2Cl_2 (2 ml), 2-cyanoethyl diisopropylphosphoramidochloridite (40 µl, 0.18 mmol) was added at r.t. After stirring for 30 min, the mixture was diluted with CH_2Cl_2 (10 ml) and quenched by adding 5% $NaHCO_3$ soln. (20 ml). Then, the aq. layer was extracted with CH_2Cl_2 (3 × 20 ml), the combined org. layer dried (Na_2SO_4) and evaporated, and the resulting oil applied to FC (column 10 × 3 cm, *B*): **11** (105 mg, 78%). Yellow foam. TLC (*B*): R_f 0.34, 0.41. ^{31}P-NMR ($CDCl_3$): 149.9, 150.1.

2-[2′-Deoxy-5′-O-*(4,4′-dimethoxytriphenylmethyl)-β-*D-erythro-*pentofuranosyl]-4-nitro-2*H-*indazole 3′-(2-Cyanoethyl diisopropylphosphoramidite)* (**12**). As described for **11**, with **10** (100 mg, 0.17 mmol), $(^iPr)_2EtN$ (50 µl, 0.29 mmol), and 2-cyanoethyl diisopropylphosphoramidochloridite (40 µl, 0.18 mmol): **12** (101 mg, 75%). Yellow foam. TLC (*B*): R_f 0.31, 0.38. ^{31}P-NMR ($CDCl_3$): 149.9, 150.2.

Synthesis, Purification, and Characterization of the Oligonucleotides **13**–**29**. The oligonucleotide synthesis was performed on a DNA synthesizer, model *392* (*Applied Biosystems*, Weiterstadt, Germany). Melting curves were measured with a *Cary1/3* UV/VIS spectrophotometer (*Varian*, Australia) equipped with a *Cary* thermoelectrical controller. The temp. was measured continuously in the reference cell with a Pt-*100* resistor, and the thermodynamic data of duplex formation were calculated by the Meltwin 3.0 program [45]. CD Spectra: *Jasco 600* (*Jasco*, Japan) spectropolarimeter with thermostatically (*Lauda RCS-6* bath) controlled 1-cm cuvettes. UV Spectra: *U-3200* spectrometer (*Hitachi*, Japan); oligonucleotide analysis was carried out on reversed-phase HPLC: *Merck-Hitachi* HPLC; 250 × 4 mm *RP-18* column; gradients of 0.1M $(Et_3NH)OAc$ (pH 7.0)/MeCN 95 : 5 (*A*) and MeCN (*B*); gradient I: 15 min 0–50% *B* in *A*, flow rate 1 ml/min; gradient II: 25 min 0–20% *B* in *A*, 25–35 min 20–20% *B* in *A*, flow rate 1 ml/min; gradient III: 20 min 100% *A*, 20–60 min 0–50% *B* in *A*, flow rate 0.7 ml/min. The enzymatic analysis of the oligomers was performed as described by *Seela* and *Lampe* [28] and carried out on reversed-phase HPLC by gradient III. Quantification of the constituents were made on the basis peak areas, which were divided by the extinction coefficients of the nucleosides (ε_{260}: dT 8800, dC 7300, dA 15400, dG 11400, **7** 1500, **8** 2018, **5** 18100). Snake-venom phosphodiesterase (EC 3.1.15.1, *Crotallus durissus*) and alkaline phosphatase (EC 3.1.3.1, *E. coli*) were generous gifts from *Roche Diagnostics GmbH*, Penzberg, Germany. MALDI-TOF-MS: *Biflex-III* spectrometer (*Bruker Saxonia*, Leipzig, Germany); see *Table 2*.

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