Synthesis and Stability of GNRA-Loop Analogs

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Dedicated to Prof. Dr. *Frank Seela* on the occasion of his 60th birthday

Nebularine, 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine, and inosine phosphoramidites **8**, **16**, and **17**, respectively, were synthesized and incorporated into the GNRA tetraloop at different positions (see *Scheme*, *Table*, and *Fig. 4*). The oligomers were investigated by means of UV and CD spectroscopy to address the question of how the individual base-modified *N*-nucleosides contribute to changes in H-bonding and base-stacking interactions within the loop. Several CD spectra are given and compared with each other (*Figs. 5* and *6*). The exchange of the loop sequence in position 4 and 7 results in a distinct change in base stacking. CD-Band shifting allows us to advance the hypothesis that a transition from a GNRA-type towards a UNCG-type base stacking is observed.

1. Introduction. – RNA Structures available today are designed to meet the criteria of chemical stability and biological function. Simple structures like double helices, hairpin loops, bulges, internal loops are known. Hairpin loops result when a single strand folds back to form a double-stranded region, leaving some bases unpaired [1]. This region may vary from 2 to 10 bases at most. Those loops, found in tRNA and rRNA, are most probably present in many structures [2]. Tetraloops represent the most common class of loops in especially two families, the GNRA and UNCG types (N = any nucleotide, R = G or A) [3][4]. Atomic-resolution structures have been determined. In both tetraloop types, only two bases are free, and a non-*Watson-Crick* base pair closes the loop.

In the GNRA family, where the structure of **GCAA** has been established by NMR (*Fig. 1*), a G · A base pair has been found, which shows two H-bonds from the exocyclic NH_2 proton of the 5-positioned G to the N(7) atom of the 8-positioned A and from the exocyclic NH_2 proton of A, to the N(3) atom of G (8- and 5-positioned, resp.) [4]. In addition, phosphate-base and base-ribose H-bonds as well as base stacking stabilize the loop. The **GAAA** loop has been established by two X-ray structures. The hammerhead ribozyme and the group-I ribozyme are both virtually identical as shown by their NMR spectra, which indicate structural rigidity [5]. This unusually high stability has been explained by an internal G · A base pair, a H-bond between G and the phosphate backbone, as well as by intensive base stacking. In addition, the influence of the 2′-hydroxy group has been addressed in mixed sequences by *Serra et al.* [6]. Here, a **UUCG** tetraloop was replaced by a d(TTCG) sequence in deoxyribonucleosides, and a drop in T_m of 8° resulted.

As base substitutions, *SantaLucia et al.* [7] changed the G base for 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine, inosine, and 2′-deoxyguanosine in the **GACC** loop.

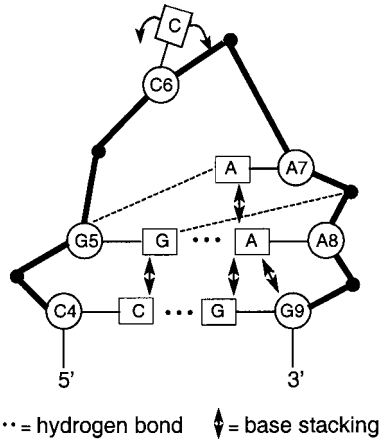


Fig. 1. *Schematic representation of the* **GCAA** *loop with important structural features*

In 10 mM phosphate/100 mM NaCl buffer, they observed a ΔT_m of 3° for dG, of 6° for 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine and of 7° for inosine. This result is in accordance with the loss of H-bond contacts established by NMR data. For the overall optimization of the stacking interaction, the orientation of the closing base pair (*i.e.* whether G·C or C·G ends the stem) is very important. *Serra et al.* exchanged the sequence 5′-CG**C**-UUCG-**G**CG-3′ to 5′-GC**G**-UUCG-**C**GC and found a ΔT_m of −10° [6].

In recent work, the influence of the ribose conformation and the 2′-*endo*/3′-*endo* equilibrium in the loop and its importance for the stability of the hairpin has been investigated [8].

Here we report the structural modification of the decamer hairpins 5′-CGC-**NAAN**-GCG-3′ and CGC-**NCAN**-GCG-3′, with N = A, G, nebularine (Ne), 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine (A^2), or inosine (I). The modifications involve individually each one of the N nucleosides to allow the mapping of the H-bond and possible base stacking.

2. Results and Discussion. – 2.1. *Synthesis of the Nucleosides.* Several phosphoramidites with base modifications were synthesized. The monomer building blocks nebularine, 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine, and inosine phosphoramidites **8**, **16**, and **17**, respectively (*Fig. 2*), were incorporated into RNA tetraloops.

For the synthesis of nebularine phosphoramidite **8**, commercially available inosine (**1**) was fully protected at the OH functions by reaction with benzoyl chloride (→ **2**; *Scheme*). The transformation of **2** into the thioxo derivative **3** with *Lawesson*'s reagent occurred in 90% yield, and subsequent cleavage of the benzoyl protecting groups gave **4**. To remove the S-atom, **4** was treated with *Raney*-Ni; after work-up and purification, the yield of **5** was 79%. A building block suitable for the solid-phase RNA synthesis was obtained from **5** by the phosphoramidite method. Thus, the OH functions were protected by reaction with $(MeO)_2TrCl$ (→ **6**) and then with $^t BuMe_2SiCl$ (→ **7**). The latter reaction gave rise to the 3′-*O*-silyl-protected by-product in 42% yield; however,

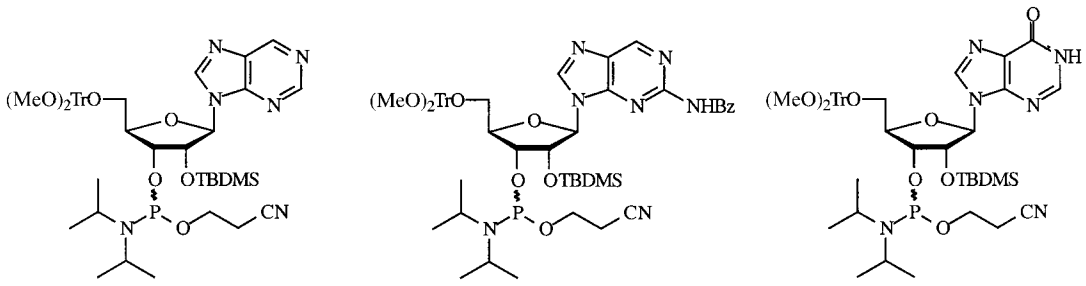


Fig. 2. *Monomer building blocks incorporated into RNA tetraloops by solid-phase synthesis*

the yield of the desired 2′-*O*-silylated **7** could be increased to 53% by addition of $AgNO_3$ as catalyst. The final phosphitylation reaction gave a 0.7:1 diastereoisomer mixture **8** in 79% yield; to prevent the isomerization of the silyl protecting groups, 5 equiv. of *sym*-collidine and 0.5 equiv. of 1-methyl-1*H*-imidazole were used instead of $^{i}Pr_2EtN$. The phosphoramidite **8** was characterized by ^{1}H- and ^{31}P-NMR-spectroscopy.

The 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine phosphoramidite **16** was synthesized from **9** *via* **10**–**15** as described above for the nebularine phosphoramidite **8** (*Scheme*). The silylation of **14** gave a mixture of the 2′-*O*- and 3′-*O*-silylated compounds in 55 and 39%, respectively, and after the final phosphitylation of the desired 2′-*O*-silylated **7**, a 1:2 diastereomer mixture **16** was obtained in 84% yield.

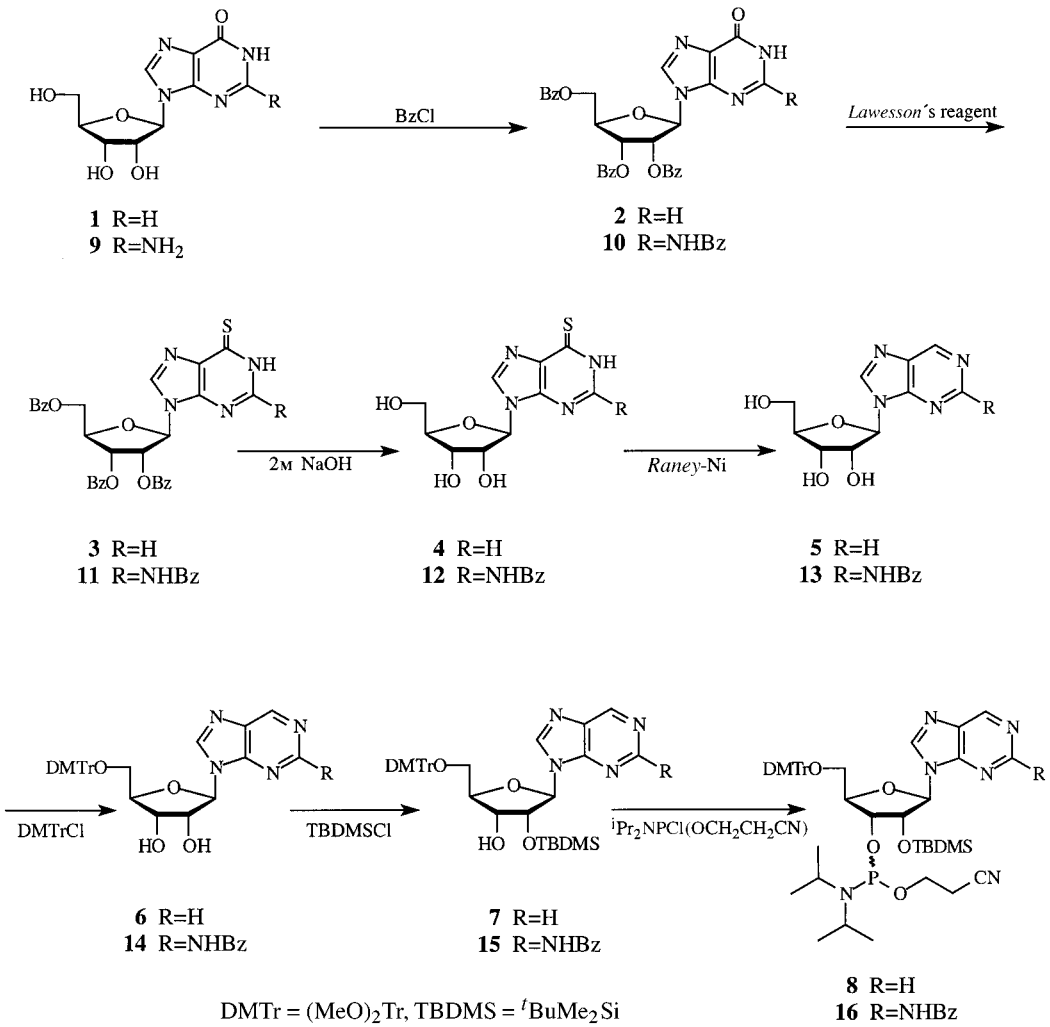
The inosine phosphoramidite **17** was synthesized by literature procedures starting from commercially available inosine (**1**) [9].

2.2. *Oligonucleotide Synthesis.* The RNA oligomers were synthesized on an *Eppendorf-D300+* synthesizer by phosphoramidite chemistry, with a coupling time for the modified monomers of 12 min [10]. The fully protected decamers were cleaved from the controlled-pore-glass (CPG) support with 32% aqueous NH_3 solution at 55° overnight. The 2′-*O*-silyl groups were deprotected with $Et_3N \cdot 3$ HF within 24 h at room temperature [11]. The crude RNA oligomer was precipitated with BuOH at −20°, and the fully deprotected RNA was purified by means of anion-exchange HPLC (*NucleoPac-PA-100*). The pure oligomer was subsequently desalted (*Sephadex-G25*). All nucleotides were characterized by MALDI-TOF-MS, and the masses obtained were in good agreement with the calculated molecular masses.

2.3. *Melting Curves of the Oligomers.* UV/Melting profiles of the oligomers were recorded in a phosphate buffer containing NaCl (140 mmol) at a wavelength of 260 and 274 nm [12]. The temperature range was 20–85° with a heating rate of 0.5°/min, and the thermodynamic data were extracted from the melting curve by means of a two-state model for the transition from an ordered to a disordered conformation [13].

In contrast to the melting profiles of the UNCG family, the melting curves of the GNRA-family loops do not exhibit a very sharp transition between the hairpin and the molten strand (*Fig. 3*), which indicates a loss of cooperativity within the loop.

Scheme. *Synthesis of the Nebularine and 9-(β-D-Ribofuranosyl)-9H-purin-2-amine Phosphoramidites* **8** *and* **16**, *Respectively*



1 R=H
9 R=NH_2

2 R=H
10 R=NHBz

3 R=H
11 R=NHBz

4 R=H
12 R=NHBz

5 R=H
13 R=NHBz

6 R=H
14 R=NHBz

7 R=H
15 R=NHBz

8 R=H
16 R=NHBz

2.4. *Thermodynamic Data*. In the CGC-**GCAA**-GCG tetraloop, the nucleoside units at position 4 and 7 (*Fig. 4*) were replaced by the analogues nebularine (Ne), inosine (I), and 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine (A^2). To investigate the contribution of the C residue at position 5 to the stabilization of the loop structure, it was replaced by the nucleoside unit A.

The *Table* shows the synthesized oligonucleotides of the CGC-**NCAN**-GCG and CGC-**NAAN**-GCG type and their thermodynamic properties. In general, all hairpin loops investigated show a destabilization compared with the native **GCAA** loop (71.6°; *Entry 1*). Incorporation of the inosine or 9-(β-D-ribofuranosyl)-9*H*-purin-2-amine

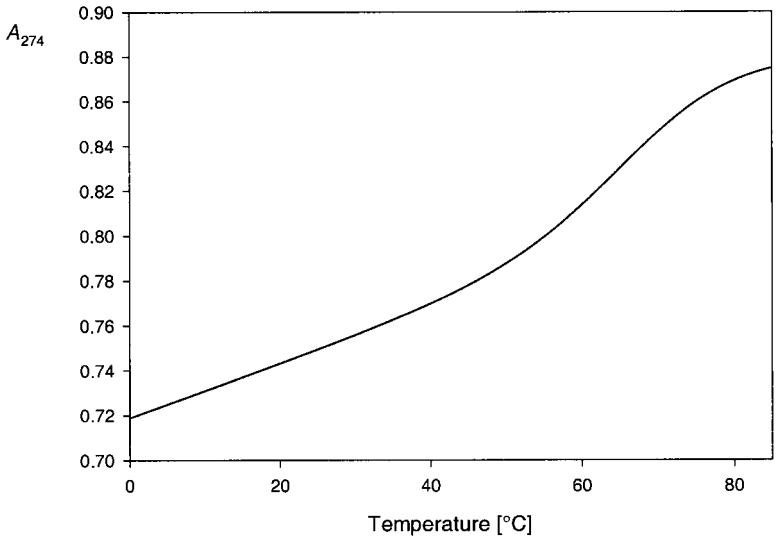


Fig. 3. *Typical melting profile of a hairpin loop of the GNRA family* (CGC-**A²CAA**-GCG)

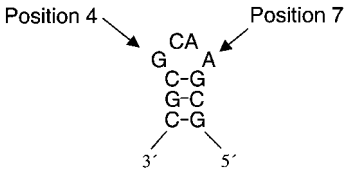


Fig. 4. *Different base analogs were incorporated at position 4 and 7*

residue at position 4 results in a 4° drop in T_m (*Entries 8* and *11*, resp.) which corresponds to missing H-bonds within the loop. The additional substitution of C by A at position 5 of the loop in the inosine sequence leads to a further 3° destabilization of the loop (*Entry 9*). As C at position 5 is looped out and does not show any H-bonding according to the structure of the **GCAA** loop established by NMR, this result suggests a change in base stacking. In comparison to the UNCG family, the contribution of position 5 to the loop formation seems to be significantly smaller.

The substitution of G by nebularine produces a large effect (56.9°; *Entry 5*) which can be explained by the missing H-bond between the NH_2 group of G at position 4 and the N(7) atom of A at position 7 in the G · A base pair. This base pair was found to be essential for the stability of the loop. Interestingly, the T_m of the sequences containing **NeCAA** (56.9°) and **NeAAA** (57.9°) is only slightly lower than that containing **AAAA** (59.2°) (*Entries 4–6*). This result is not yet fully understood, but can be explained by the strong stacking effect of the nebularine unit.

At position 7, A was replaced by the inosine, 9-(β-D-ribofuranosyl)-9*H*-purine, or nebularine moiety. The sequences with the **AAAI** (59.6°) and the **AAAA**[2] loop (60.3°) exhibit nearly identical T_m values (*Entries 10* and *13*, resp.). The incorporation of nebularine resulted in a T_m of 59.1° for the sequence with the **AAANe** loop (*Entry 7*)

Table. *Synthesized Hairpin Loops of the GNRA Type and Their Thermodynamic Properties*

Entry	Sequence 5′ → 3′	T_m [°] (± 1°)	ΔH [kcal/mol]	ΔS [cal/mol · K]
1	CGC-**GCAA**-GCG	71.6	– 26.4	– 76.6
2	CGC-**GAAA**-GCG	67.4	– 25.9	– 76.1
3	CGC-**AAAG**-GCG	63.1	– 24.6	– 73.2
4	CGC-**AAAA**-GCG	59.4	– 18.2	– 54.7
5	CGC-**NeCAA**-GCG	56.9	– 19.5	– 59.1
6	CGC-**NeAAA**-GCG	57.9	– 19.0	– 57.4
7	CGC-**AAANe**-GCG	59.1	– 21.2	– 63.9
8	CGC-**ICAA**-GCG	67.2	– 29.7	– 87.3
9	CGC-**IAAA**-GCG	64.4	– 24.3	– 71.9
10	CGC-**AAAI**-GCG	59.6	– 20.7	– 62.2
11	CGC-**A^{2}CAA**-GCG	67.1	– 30.2	– 88.7
12	CGC-**A^{2}AAA**-GCG	67.5	– 29.1	– 85.4
13	CGC-**AAAA^2**-GCG	60.3	– 22.5	– 67.5

which is, as for the incorporation at position 4 (*Entry 6*) quite close to that of the **AAAA**-containing sequence (59.4°; *Entry 4*).

By comparing the sequences containing **GAAA** and **AAAG**, exhibiting a T_m of 67.4° and 63.1°, respectively, the importance of the G · A base pair is stressed. The A · G base pair is less favorable, resulting in a destabilization of nearly 4°. The CD spectra reveal a change in base stacking, which may explain this drop in the T_m value. Interestingly, all the loops studied show a pronounced entropy/enthalpy compensation, which has been described for other biological systems [14].

2.5. *CD Spectra*. CD Spectra were recorded at 350 – 180 nm with oligonucleotide (10 μmol) solutions in sodium-phosphate buffer (pH 7) containing NaCl (140 mmol). The temperature of the measurement was 20° to ensure that only the hairpin structure of the oligonucleotides was present. *Fig. 5* shows a typical CD spectrum of a RNA hairpin loop with an A-type helix in the stem region [15]. Of the three characteristic extrema with wavelengths λ_1, λ_2, and λ_3, the strong maximum at λ_1, the small minimum at λ_2, and the weak maximum at λ_3 are always found in an A-type helix formation.

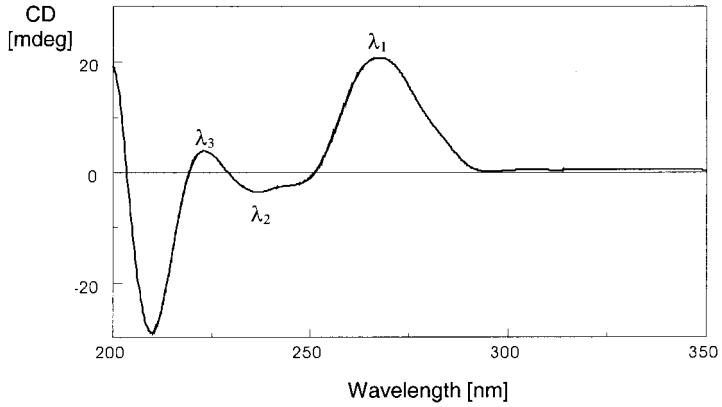


Fig. 5. *Typical CD spectrum of a decamer hairpin loop*

The intensity at λ_1 and λ_3 corresponds to the number of paired and unpaired bases as well as to the extent of base-stacking interactions within the loop and stem region. Temperature-dependent measurements of our oligonucleotides reveal a loss of intensity during the transition from hairpin to random-coil structure. In comparison to CD spectra of the UNCG-type hairpins [8], the maximum at λ_1 of the GNRA-type hairpins is shifted to shorter wavelengths and the intensity at λ_3 increases. *Fig. 6* shows different CD spectra of some of the synthesized oligonucleotides. In the case of the nebularine modification, the maximum at λ_1 has nearly the same intensity (*Fig. 6,d*). The CD spectra of the GNRA-type hairpin with the altered loop sequences **GAAA**, **GCAA**, and **AAAG** reveal a change in intensity at λ_1, while the intensity at λ_3 is nearly unchanged (*Fig. 6,a*). This result can be explained by the change of base-stacking interactions.

A striking observation is the significant difference of the position of the maximum at λ_1 between the **GAAA**- and **AAAG**-containing sequences. The change of the loop sequence leads to a shift of λ_1 of nearly 7 nm to longer wavelength, from 263.4 to 270.2 nm. A similar shift of 3 nm can be observed for the sequences with the inosine modification. The data obtained give rise to the assumption that the change of the loop sequence is accompanied by a distinct change in structure. We found a similar result when investigating the UNCG-type loop [16]. The change from **UUUG**- to **GUUU**-

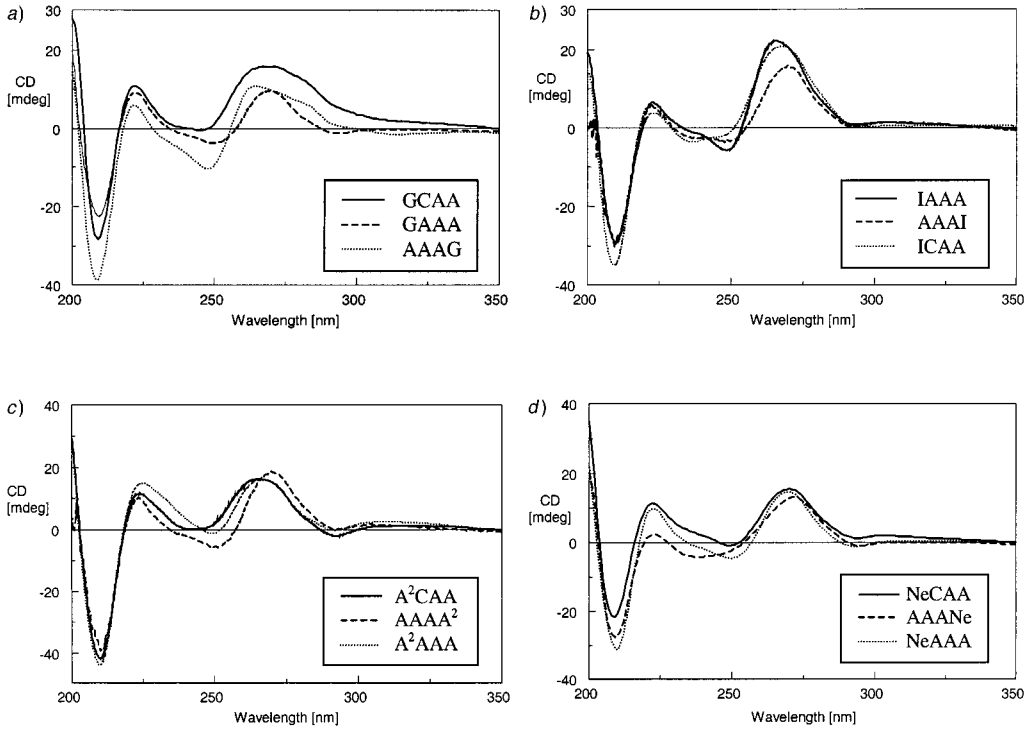


Fig. 6. *CD Spectra of the synthesized decamer hairpins of the GNRA type:* a) *Changes in the loop sequence without modified bases*; b) *incorporation of inosine*; c) *incorporation of 9-(β-*D*-ribofuranosyl)-9*H*-purin-2-amine*; d) *incorporation of nebularine*

containing sequences leads to a shift from 272.2 to 270.6 nm. In contrast to the GNRA type, we found a shift to shorter wavelengths. This opposite shift in the λ_1 CD band leads to the hypothesis that **GAAA** and **UUUG** as well as **AAAG** and **GUUU** may show similar base-stacking interactions. The CD spectra reveal that the weakening or deleting of H-bonds between the G·A base pair changes the base-stacking interactions significantly. The described opposite shift of the λ_1 CD band indicates a transition from the typical GNRA-type stacking between the bases in position 6 and 7 (GCAA) [17] or 5 and 6 (GAAA) [18] towards an UNCG-type stacking between position 4 and 7 [19].

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Experimental Part

General. Inosine, guanosine, anh. pyridine, and anh. CH_2Cl_2 were obtained from *Fluka* and used without further purification. Dry MeCN (H_2O <30 ppm) for the phosphitylation reaction was purchased from *Perseptive Biosystems*. Flash column chromatography (FC): silica gel *60* (40–63 μm) from *Merck*. TLC: silica gel *60* F_{254} plates from *Merck*, HPLC: anion-exchange column *NucleoPac PA-100* from *Dionex*; desalting with a *Sephadex-G25* column from *Pharmacia*. UV/Melting profiles: *Varian-Cary*-UV/VIS spectrophotometer, *Cary* temperature controller, 10-mm cuvette. CD Spectra: *Jasco-I-710* spectropolarimeter. ^{1}NMR: *Bruker-AM250* and *Bruker-WH270* (^{1}H,^{13}C) and *Bruker-AMX400* (^{31}P) spectrometers; δ in ppm, J in Hz. MS: *PerSeptive Biosystems* MALDI-TOF spectrometer *Voyager* DE: ESI = electron-spray ionization.

Inosine Phosphoramidite (**17**). Synthesis according to a procedure reported by *Green et al.* [9].

2′,3′,5′-Tri-O-*benzoylinosine* (**2**). Benzoyl chloride (6.96 ml, 60 mmol) was added dropwise to a stirred suspension of inosine (**1**; 2.68 g, 10 mmol) in dry pyridine (70 ml) at 40°. The mixture was heated for 2 h at 60° and then for 2 h at 40° and finally stirred overnight at r.t. The red soln. was treated with 5% aq. $NaHCO_3$ soln. (70 ml) and extracted with CH_2Cl_2 (3 × 40 ml). The combined org. layers were dried (Na_2SO_4), evaporated, and twice co-evaporated with toluene to remove traces of pyridine. FC (CH_2Cl_2/MeOH 95 : 5) gave **2** (5.36 g, 92.3%). Colorless foam. TLC (CH_2Cl_2/MeOH 95 : 5): R_f 0.38. ^{1}H-NMR (250 MHz, (D_6)DMSO): 12.49 (br. *s*, NH); 8.37 (*s*, H–C(8)); 8.01 (*s*, H–C(2)); 7.98–7.41 (*m*, 15 arom. H); 6.56 (*d*, $^3J(1',2') = 4.63$, H–C(1′)); 6.40 (*t*, H–C(2′)); 6.19 (*t*, H–C(3′)); 4.85 (*m*, H–C(4′)); 4.77 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D_6)DMSO): 165.42, 164.79, 164.63 (C=O); 156.64 (C(6)); 148.21 (C(4)); 145.83 (C(2)); 139.75 (C(8)); 133.56, 133.52, 133.32, 129.71, 128.78, 128.71, 128.67 (arom. C); 124.71 (C(5)); 86.02 (C(1′)); 79.23 (C(4′)); 73.42 (C(2′)); 70.31 (C(3′)); 63.78 (C(5′)). ESI-MS: 581.32 ($[M+H]^+$). Anal. calc. for $C_{31}H_{24}N_4O_8$ (580.55): C 64.14, H 4.17, N 9.65; found: C 64.05, H 4.31, N 9.70.

2′,3′,5′-Tri-O-*benzoyl-6-thioinosine* (**3**). To a stirred soln. of **2** (2.9 g, 5 mmol) in pyridine (15 ml), *Lawesson*'s reagent (4.44 g, 10 mmol) was added in one portion, and the mixture was heated at reflux temp. for 6 h. At r.t. the mixture was treated with 5% aq. $NaHCO_3$ soln. (30 ml) and extracted with CH_2Cl_2 (2 × 70 ml). The combined org. phase was dried (Na_2SO_4) and evaporated, and the residue twice co-evaporated with toluene to yield a yellow foam. FC (CH_2Cl_2/MeOH 95 : 5) gave **3** (2.7 g, 90.4%). Colorless foam. TLC (CH_2Cl_2/MeOH 95 : 5): R_f 0.52. ^{1}H-NMR (250 MHz, (D_6)DMSO): 8.57 (*s*, H–C(8)); 8.07 (*s*, H–C(2)); 7.99–7.40 (*m*, 15 arom. H); 6.59 (*d*, $^3J(1',2') = 4.63$, H–C(1′)); 6.39 (*t*, H–C(2′)); 6.18 (*t*, H–C(3′)); 4.84 (*m*, H–C(4′)); 4.74 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9, (D_6)DMSO): 176.32 (C(6)); 165.42, 164.66, 164.49 (C=O); 145.39 (C(2)); 143.59 (C(4)); 142.16 (C(8)); 135.90 (C(5)); 134.01, 133.91, 133.54, 129.39, 129.27, 128.76, 128.72, 128.56, 128.29 (arom. C); 86.55 (C(1′)); 79.46 (C(4′)); 73.36 (C(2′)); 70.69 (C(3′)); 63.24 (C(5′)). ESI-MS: 597.19 ($[M+H]^+$). Anal. calc. for $C_{31}H_{24}N_4O_7S$ (596.61): C 62.41, H 4.05, N 9.36; found: C 62.16, H 4.24, N 9.16.

6-Thioinosine (**4**). To a stirred mixture of pyridine (2.5 ml), MeOH (2.5 ml), and 2M NaOH (6 ml) **3** was added (1.6 g, 2.81 mmol) at r.t. After 5 min, the deprotection was complete, and the mixture was treated with *Dowex* ion-exchange resin (*50 W* × *8*) until a pH value of 6 was reached. The filtrate was evaporated and co-evaporated twice with toluene. Recrystallization from H_2O (charcoal treatment) gave **4** (682 mg, 85.4%). Colorless product. TLC (CH_2Cl_2/MeOH 8 : 2): R_f 0.25. ^{1}H-NMR (250 MHz, (D_6)DMSO): 8.49 (*s*, H–C(8)); 8.21 (*s*, H–C(2)); 5.87 (*d*, $^3J(1',2') = 5.55$, H–C(1′)); 5.52 (br. *s*, OH–C(2′)); 5.21 (br. *s*, OH–C(3′),

OH–C(5′)); 4.48 (*m*, H–C(2′)); 4.13 (*m*, H–C(3′)); 3.94 (*m*, H–C(4′)); 3.61 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D$_6$)DMSO): 176.89 (C(6)); 145.78 (C(2)); 144.02 (C(4)); 140.89 (C(8)); 135.55 (C(5)); 87.61 (C(1′)); 85.64 (C(4′)); 74.08 (C(2′)); 70.21 (C(3′)); 61.16 (C(5′)). Anal. calc. for $C_{10}H_{12}N_4O_4S$ (284.29): C 42.25, H 4.25, N 19.71; found: C 41.98, H 4.28, N 19.47.

*9-(β-*D*-Ribofuranosyl)-9*H*-purine* (**5**). Compound **4** (730 mg, 2.57 mmol) was dissolved in boiling H_2O (50 ml), and small portions of *Raney*-Ni (4 g) were added to the stirred soln. After 6 h at reflux temp., the hot suspension was filtered and the catalyst washed with hot H_2O. The filtrate was evaporated, the residue dissolved in hot abs. EtOH, charcoal added, and the mixture filtered and slowly cooled to give **5** (515 mg, 79%). Colorless product. TLC (CH_2Cl_2/MeOH 8:2): R_f 0.30. ^{1}H-NMR (250 MHz, (D$_6$)DMSO): 9.21 (*s*, H–C(6)); 8.97 (*s*, H–C(2)); 8.86 (*s*, H–C(8)); 6.06 (*d*, $^3J(1',2') = 5.55$, H–C(1′)); 5.52 (br. *s*, OH–C(2′)); 5.25 (br. *s*, OH–C(3′)); 5.12 (br. *s*, OH–C(5′)); 4.65 (*t*, H–C(2′)); 4.21 (*t*, H–C(3′)); 3.99 (*m*, H–C(4′)); 3.61 (*m*, 2 H–C(5′)).

*9-[5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-β-*D*-ribofuranosyl]-9*H*-purine* (**6**). Compound **5** (337 mg, 1.34 mmol) was dried by repeated co-evaporation with abs. pyridine and dissolved in dry pyridine (10 ml). A soln. of 4,4′-dimethoxytriphenylmethyl chloride (528 mg, 1.56 mmol) in pyridine (3 ml) was added and the mixture stirred for 7 h under Ar at r.t. The mixture was treated with 5% aq. $NaHCO_3$ soln. (20 ml) and extracted with CH_2Cl_2 (2 × 15 ml). The combined org. layer was dried (Na_2SO_4) and evaporated, and the residue twice co-evaporated with toluene to yield a yellow foam. FC (CH_2Cl_2/MeOH 95:5) gave **6** (630 mg, 84.7%). Colorless foam. TLC (CH_2Cl_2/MeOH 95:5): R_f 0.2. ^{1}H-NMR (250 MHz, (D$_6$)DMSO): 9.05 (*s*, H–C(6)); 8.82 (*s*, H–C(2)); 8.52 (*s*, H–C(8)); 7.37–6.71 (*m*, 13 arom. H); 6.11 (*d*, $^3J(1',2') = 4.7$, H–C(1′)); 5.46 (*d*, OH–C(2′)); 5.11 (*d*, OH–C(3′)); 4.76 (*dd*, H–C(2′)); 4.35 (*dd*, H–C(3′)); 4.16 (*m*, H–C(4′)); 3.71 (*s*, 2 MeO); 3.28 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D$_6$)DMSO): 158.00 ((MeO)$_2$*Tr*); 151.87 (C(2)); 150.82 (C(4)); 147.88 (C(6)); 144.9 ((MeO)$_2$*Tr*); 144.64 (C(8)); 135.40 ((MeO)$_2$*Tr*); 134.29 (C(5)); 129.66, 129.47, 127.67, 127.45, 126.41, 112.78 ((MeO)$_2$*Tr*); 88.17 (C(1′)); 85.63 ((MeO)$_2$*Tr*); 83.50 (C(4′)); 73.37 (C(2′)); 70.37 (C(3′)); 63.41 (C(5′)); 54.78 ((MeO). ESI-MS: 555.41 ([*M* + H]$^+$).

*9-[5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-[(*tert*-butyl)dimethylsilyl]-β-*D*-ribofuranosyl]-9*H*-purine* (**7**). Under Ar, **6** (332 mg, 0.6 mmol) was dissolved in pyridine/THF 1:1 (6 ml). $AgNO_3$ (122 mg, 0.72 mmol) and 1M (*tert*-butyl)dimethylsilyl chloride in THF (0.72 ml) were added to the stirred soln. After 14 h, the reaction was quenched by adding 5% $NaHCO_3$ soln. (6 ml). The suspension was filtered and the filtrate extracted with CH_2Cl_2 (3 × 10 ml). The combined org. phase was dried (Na_2SO_4) and evaporated and the residue co-evaporated twice with toluene to yield a foam. FC (silica gel *60 H*, CH_2Cl_2/^{i}PrOH 98:2 → 95:5) gave **7** (214 mg, 53.2%). Colorless foam. TLC (CH_2Cl_2/^{i}PrOH 95:5): R_f 0.51. ^{1}H-NMR (250 MHz, (D$_6$)DMSO): 9.27 (*s*, H–C(6)); 8.92 (*s*, H–C(2)); 8.80 (*s*, H–C(8)); 7.45–6.86 (*m*, 13 arom. H); 6.14 (*d*, $^3J(1',2') = 4.88$, H–C(1′)); 5.27 (*d*, OH–C(3′)); 4.94 (*t*, H–C(2′)); 4.32 (*dd*, H–C(3′)); 4.19 (*m*, H–C(4′)); 3.77 (*s*, 2 MeO); 3.35 (*m*, 2 H–C(5′)); 0.78 (*s*, ^{t}Bu); 0.00 (*s*, MeSi); –0.11 (*s*, MeSi). ^{13}C-NMR (62.9 MHz, (D$_6$)DMSO): 158.07 ((MeO)$_2$*Tr*); 152.07 (C(2)); 150.67 (C(4)); 148.37 (C(6)); 144.83 ((MeO)$_2$*Tr*); 144.33 (C(8)); 135.45 ((MeO)$_2$*Tr*); 134.22 (C(5)); 129.68, 129.44, 127.72, 127.60, 126.63, 113.09 ((MeO)$_2$*Tr*); 88.13 (C(1′)); 85.50 ((MeO)$_2$*Tr*); 83.59 (C(4′)); 74.69 (C(2′)); 70.10 (C(3′)); 63.39 (C(5′)); 54.98 (MeO); 25.47 (*Me*$_3$CSi); 17.76 (Me$_3$*C*Si); –4.85, –5.38 (2 MeSi). ESI-MS: 669.57 ([*M* + H]$^+$). Anal. calc. for $C_{37}H_{44}N_4O_6Si$ (668.87): C 66.44, H 6.63, N 8.38; found: C 66.46, H 6.78, N 8.13.

*9-[5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-[(*tert*-butyl)dimethylsilyl]-β-*D*-ribofuranosyl]-9*H*-purine 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**8**). To a stirred soln. of **7** (200 mg, 0.3 mmol), collidine (= 2,4,6-trimethylpyridine; 200 µl, 1.5 mmol), and a catalytic amount of 1-methyl-1*H*-imidazole (12 µl, 0.15 mmol) in dry MeCN (6 ml), 2-cyanoethyl diisopropylphosphoramidochloridite (100 µl, 0.45 mmol) was added dropwise at r.t. under Ar. After 2 h, the reaction was quenched by adding 5% aq. $NaHCO_3$ soln. (10 ml), the mixture extracted with CH_2Cl_2 (3 × 10 ml), the combined org. layer dried (Na_2SO_4) and evaporated, and the residue purified by FC (silica gel *60 H*, Et_2O/MeCN 9:1): diastereomer mixture **8** (206 mg, 79.3%). White foam. TLC (Et_2O/MeCN 9:1): R_f 0.58; 2 partially overlapping spots. ^{1}H-NMR (400 MHz, $CDCl_3$, diastereomer mixture): 9.15, 9.14 (2*s*, H–C(6)); 8.88, 8.87 (2*s*, H–C(2)); 8.34, 8.30 (2*s*, H–C(8)); 7.48–6.80 (*m*, 13 arom. H); 6.12, 6.07 (2*d*, H–C(1′)); 5.08 (*m*, H–C(2′)); 4.46–4.37 (*m*, H–C(3′)); H–C(4′)); 3.96, 3.88 (2*m*, 2 Me$_2$C*H*); 3.78 (*s*, 2 MeO); 3.66–3.54 (*m*, CH_2CH_2O); 3.45 (*m*, 2 H–C(5′)); 2.65, 2.31 (2*m*, CH_2CH$_2$O); 1.21–1.05 (2 *Me*$_2$CH); 0.74, 0.71 (2*s*, ^{t}Bu); –0.03, –0.25 (2 MeSi). ^{31}P-NMR (160 MHz, $CDCl_3$): 151.7, 149.6; ratio 0.7:1.

N^2,2′-O,3′-O,5′-O-*Tetrabenzoylguanosine* (**10**). Benzoyl chloride (7 ml, 60.5 mmol) was added dropwise to a stirred suspension of guanosine (**9**; 2.83 g, 10 mmol) in dry pyridine (40 ml) at 40°. During the addition, the temp. rose up to 60°. After 2 h, the brown soln. was cooled down to r.t. and quenched by adding 5% aq. $NaHCO_3$ soln. (40 ml) and CH_2Cl_2 (20 ml). The mixture was extracted with CH_2Cl_2 (3 ×). The combined org. phase was

dried (Na_2SO_4), evaporated, and co-evaporated twice with toluene to yield a brown foam. FC (CH_2Cl_2/MeOH 95:5) gave **10** (6.11 g, 87.3%). Colorless foam. TLC (CH_2Cl_2/MeOH 95:5): R_f 0.56. ^{1}H-NMR (250 MHz, (D_6)DMSO): 12.28 (br. *s*, NH); 11.72 (br. *s*, N*H*Bz); 8.35 (*s*, H–C(8)); 8.15–7.42 (*m*, 20 arom. H); 6.50 (*d*, $^3J(1',2')$ = 4.95, H–C(1′)); 6.39 (*t*, H–C(2′)); 6.21 (*t*, H–C(3′)); 4.91 (*m*, H–C(4′)); 4.80 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D_6)DMSO): 169.02 (NHCO); 165.47, 164.72, 164.47 (C=O); 154.98 (C(6)); 148.38 (C(2)); 148.29 (C(4)); 138.72 (C(8)); 133.97, 133.56, 133.18, 132.44, 129.39, 129.30, 129.25, 128.84, 128.80, 128.74, 128.61, 128.47, 128.24 (arom. C); 121.14 (C(5)); 86.03 (C(1′)); 79.67 (C(4′)); 73.42 (C(2′)); 71.44 (C(3′)); 63.79 (C(5′)). ESI-MS: 700.15 ($[M+H]^+$). Anal. calc. for $C_{38}H_{29}N_5O_9$ (699.68): C 65.23, H 4.18, N 10.01; found: C 65.47, H 4.24, N 9.82.

N^2,2′-O,3′-O,5′-O-*Tetrabenzoyl-6-thioguanosine* (**11**). To a soln. of **10** (1.4 g, 2.0 mmol) in abs. pyridine, (20 ml), *Lawesson*'s reagent was added (1.62 g, 4.0 mmol) in one portion at r.t. The mixture was heated at reflux temp. for 6 h. At r.t. pyridine was evaporated, the residue dissolved in CH_2Cl_2 (40 ml), this soln. washed with 5% aq. $NaHCO_3$ soln. (60 ml), dried (Na_2SO_4), and evaporated, and the residue co-evaporated twice with toluene to yield a yellow foam. FC (CH_2Cl_2/MeOH 95:5) gave **11** (1.23 g, 85.6%). Yellow foam. TLC (CH_2Cl_2/MeOH 95:5): R_f 0.71. ^{1}H-NMR (250 MHz, (D_6)DMSO): 13.61 (br. *s*, SH); 11.97 (br. *s*, N*H*Bz); 8.53 (*s*, H–C(8)); 8.02–7.42 (*m*, 20 arom. H); 6.52 (*d*, $^3J(1',2')$ = 4.95, H–C(1′)); 6.39 (*t*, H–C(2′)); 6.20 (*t*, H–C(3′)); 4.90 (*m*, H–C(4′)); 4.85 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D_6)DMSO): 174.71 (C(6)); 169.25 (NHCO); 165.46, 164.68, 164.47 (C=O); 147.73 (C(2)); 144.88 (C(4)); 141.10 (C(8)); 134.03, 133.94, 133.53, 133.32 (arom. C, C(5)); 132.47, 132.20, 129.39, 129.34, 129.22, 128.82, 128.74, 128.61, 128.52, 128.22 (arom. C); 86.10 (C(1′)); 79.80 (C(4′)); 73.43 (C(2′)); 71.41 (C(4′)); 63.75 (C(5′)). ESI-MS: 716.09 ($[M+H]^+$). Anal. calc. for $C_{38}H_{29}N_5O_8S$ (715.74): C 63.77, H 4.08, N 9.78; found: C 63.68, H 4.17, N 9.69.

N^2-*Benzoyl-6-thioguanosine* (**12**). As described for **4**, with pyridine (2 ml), MeOH (1 ml), 2M NaOH (1 ml), and **11** (2.7 g, 3.77 mmol). Recrystallization from EtOH gave **12** (1.25 g, 82.4%). Colorless product. TLC (CH_2Cl_2/MeOH 8:2): R_f 0.32. ^{1}H-NMR (250 MHz, (D_6)DMSO): 13.68 (br. *s*, SH); 12.21 (br. *s*, N*H*Bz); 8.49 (*s*, H–C(8)); 8.09–7.49 (*m*, 5 arom. H); 5.90 (*d*, $^3J(1',2')$ = 5.83, H–C(1′)); 5.50 (*d*, OH–C(2′)); 5.19 (*d*, OH–C(3′)); 5.06 (*t*, OH–C(5′)); 4.48 (*dd*, H–C(2′)); 4.16 (*dd*, H–C(3′)); 3.93 (*m*, H–C(4′)); 3.61 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D_6)DMSO): 174.20 (C(6)); 167.66 (NHCO); 152.49 (C(2)); 146.98 (C(4)); 139.93 (C(8)); 136.08 (C(5)); 132.43, 131.61, 131.08, 129.22, 128.47, 128.40, 128.22 (arom. C); 87.12 (C(1′)); 85.69 (C(4′)); 73.74 (C(2′)); 70.43 (C(4′)); 61.43 (C(5′)). ESI-MS: 404.81 ($[M+H]^+$).

N-*Benzoyl-9-(β-*D*-ribofuranosyl)-9*H*-purin-2-amine* (**13**). As described for **5**, with **12** (888 mg, 2.2 mmol), boiling H_2O (50 ml), and *Raney-Ni* (4 g). After workup, the residue was dissolved in hot H_2O and the soln. treated with charcoal, filtrated, and cooled down slowly to give **13** (688 mg, 84.2%). Colorless product. TLC (CH_2Cl_2/MeOH 8:2): R_f 0.28. ^{1}H-NMR (250 MHz, (D_6)DMSO): 11.09 (*s*, NH); 9.10 (*s*, H–C(6)); 8.75 (*s*, H–C(8)); 8.00–7.48 (*m*, 5 arom. H); 6.00 (*d*, $^3J(1',2')$ = 5.73, H–C(1′)); 5.53 (*d*, OH–C(2′)); 5.23 (*d*, OH–C(3′)); 4.99 (*t*, OH–C(5′)); 4.69 (*dd*, H–C(2′)); 4.21 (*m*, H–C(3′)); 3.96 (*m*, H–C(4′)); 3.65 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D_6)DMSO): 165.50 (C=O); 153.02 (C(2)); 152.03 (C(4)); 148.89 (C(6)); 145.01 (C(8)); 134.30 (C(5)); 131.97, 131.22, 128.23, 128.13 (arom. C); 87.14 (C(1′)); 85.86 (C(4′)); 73.44 (C(2′)); 70.52 (C(3′)); 61.13 (C(5′)). ESI-MS: 371.87 ($[M+H]^+$). Anal. calc. for $C_{17}H_{17}N_5O_9$ (371.35): C 54.98, H 4.16, N 18.86; found: C 54.91, H 4.84, N 18.74.

N-*Benzoyl-9-[5′-*O*-(4,4′-dimethoxytriphenylmethyl)-β-*D*-ribofuranosyl]-9*H*-purin-2-amine* (**14**). As described for **6**, with **13** (743 mg, 2.0 mmol), pyridine (8 ml), 4,4′-dimethoxytriphenylmethyl chloride (744 mg, 2.2 mmol), and pyridine (4 ml) (6 h). Workup with 5% aq. $NaHCO_3$ soln. (30 ml) and CH_2Cl_2 (2 × 20 ml) and FC as described for **6**: **14** (1.09 g, 80.9%). Colorless foam. TLC (CH_2Cl_2/MeOH 95:5): R_f 0.37. ^{1}H-NMR (250 MHz, (D_6)DMSO): 9.08 (*s*, H–C(6)); 8.62 (*s*, H–C(8)); 7.92–6.59 (*m*, 18 arom. H); 6.03 (*d*, $^3J(1',2')$ = 4.48, H–C(1′)); 5.60 (*d*, OH–C(2′)); 5.15 (*d*, OH–C(3′)); 4.78 (*dd*, H–C(2′)); 4.40 (*dd*, H–C(3′)); 4.23 (*m*, H–C(4′)); 3.65 (*s*, 2 MeO); 3.22 (*m*, 2 H–C(5′)). ^{13}C-NMR (62.9 MHz, (D_6)DMSO): 165.16 (C=O); 157.92 ((MeO)$_2$*Tr*); 153.06 (C(2)); 151.57 (C(4)); 148.91 (C(6)); 145.36 (C(8)); 135.63, 135.47 ((MeO)$_2$*Tr*); 134.42 (C(5)); 131.98, 131.23, 129.68, 128.30, 128.26, 127.64, 127.55, 126.52, 112.92 ((MeO)$_2$*Tr*); 88.62 (C(1′)); 85.35 ((MeO)$_2$*Tr*); 84.04 (C(4′)); 73.03 (C(2′)); 70.67 (C(3′)); 64.71 (C(5′)); 54.92 (MeO). ESI-MS: 674.53 ($[M+H]^+$). Anal. calc. for $C_{38}H_{35}N_5O_7$ (673.73): C 67.75, H 5.24, N 10.39; found: C 67.22, H 5.29, N 10.36.

N^2-*Benzoyl-9-[5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-[(*tert*-butyl)dimethylsilyl]-β-*D*-ribofuranosyl]-9*H*-purin-2-amine* (**15**). As described for **7**, with **14** (572 mg, 0.85 mmol), pyridine/THF 1:1 (6 ml) $AgNO_3$ (172 mg, 1.015 mmol) and 1M (*tert*-butyl)dimethylsilyl chloride in THF (1.02 ml): **15** (370 mg, 55.4%). Colorless foam. TLC (CH_2Cl_2/^{i}PrOH 95:5): R_f 0.51. ^{1}H-NMR (250 MHz, (D_6)DMSO): 10.44 (*s*, NH); 8.99 (*s*, H–C(6)); 8.47 (*s*, H–C(8)); 7.81 (*d*, 2 arom. H); 7.62–7.11 (*m*, 13 arom. H); 6.65 (*t*, 3 arom. H); 6.08 (*d*, $^3J(1',2')$ = 5.15, H–C(1′)); 5.01 (*t*, H–C(2′)); 4.61 (*d*, OH–C(3′)); 4.36 (*dd*, H–C(3′)); 4.16 (br. *s*, H–C(4′)); 3.70 (*s*, 2 MeO);

3.38 (*m*, H–C(5′)); 0.78 (*s*, *t*Bu); 0.00 (*s*, 1 MeSi); –0.21 (*s*, 1 MeSi). ^{13}C-NMR (62.9 MHz, (D_6)DMSO): 164.82 (C=O); 157.94 ((MeO)$_2$*Tr*); 153.03 (C(2)); 151.64 (C(4)); 148.61 (C(6)); 144.79 (C(8)); 144.43, 135.45, 135.40 ((MeO)$_2$*Tr*); 134.30 (C(5)); 131.49, 131.20, 129.64, 127.94, 127.86, 127.61, 127.34, 126.35, 112.67 ((MeO)$_2$*Tr*); 88.16 (C(1′)); 85.51 ((MeO)$_2$*Tr*); 84.30 (C(4′)); 74.78 (C(2′)); 70.54 (C(3′)); 64.78 (C(5′)); 54.72 (MeO); 25.41 (*Me*$_3$CSi); 17.67 (Me$_3$*C*Si); –5.08 (MeSi); –5.42 (MeSi). ESI-MS: 788.32 ([*M* + H]$^+$).

N^2-*Benzoyl-9-[5′*-O-*(4,4′-dimethoxytriphenylmethyl)-2′*-O-*[(*tert-*butyl)dimethylsilyl]-β-*D-*ribofuranosyl]-purin-9*H*-2-amine 3′-(2-Cyanoethyl Diisopropylphosphoramidite)* (**16**). As described for **8**, with **15** (218 mg, 0.27 mmol), collidine (179 µl, 1.35 mmol), 1-methyl-1*H*-imidazole (10.7 µl, 0.13 mmol), MeCN (10 ml), and 2-cyanoethyl diisopropylphosphoramidochloridite (90 µl, 0.41 mmol): diastereomer mixture **16** (225 mg, 84.3%). White foam. TLC (Et_2O/MeCN 9:1): R_f 0.59; 2 partially overlapping spots. ^{1}H-NMR (400 MHz, $CDCl_3$, diastereomer mixture): 9.19, 9.16 (2*s*, H–C(6)); 8.18, 8.15 (br. *s*, NH); 8.16, 8.13 (2*s*, H–C(8)); 7.71–6.66 (*m*, 18 arom. H); 5.99, 5.79 (2*d*, H–C(1′)); 5.41, 5.31 (2*dd*, H–C(2′)); 4.38, 4.28 (2*m*, H–C(3′)); 4.04, 3.85 (2*m*, H–C(4′)); 3.68, 3.67 (2*s*, 2 MeO); 3.65–3.48 (*m*, 2 H–C(5′), CH_2CH_2O); 2.69 (*m*, CH_2CH_2O); 1.20–0.92 (2 *Me*$_2$CH); 0.73, 0.72 (2*s*, *t*Bu); –0.04, –0.31 (2 MeSi). ^{31}P-NMR (160 MHz, $CDCl_3$): 152.3, 149.4; ratio 1:2. ESI-MS: 988.71 ([*M* + H]$^+$).

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