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Tandem Electrostatic Effect from the First to the Third Aglycon in the Trimeric RNA Owing to the Nearest-Neighbor Interaction

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Abstract: We here show an electrostatic polar-π interaction from the first to the third aglycon, via the second aglycon, in the ground state in two single stranded trimeric RNAs, 5′-GpA[1]pA[2]-3′ (**3**) and 5′-GpApC-3′ (**4**), as a result of intramolecular nearest neighbor offset-stacking. The experimental evidence in support of this conclusion has been obtained by comparing the pK_as of each aglycone in the two trimers with those of guanosine 3′-ethyl phosphate, GpEt (**1**) and 5′-GpA-3′ (**2**): Thus, the pK_a of N[1]-H of guanin-9-yl of 5′-GpA[1]pA[2]-3′ (**3**) could be measured by pH titration (pH 7.3−11.6) of its own δH8G (pK_a 9.75 ± 0.02) as well as from δH8A[1] (pK_a 9.72 ± 0.02) and δH2A[1] (pK_a 9.83 ± 0.04) of the neighboring p**A**[1]p moiety and the δH8A[2] (pK_a 9.83 ± 0.02) of the terminal p**A**[2] moiety. Similarly, the pH titration of GpApC (**4**) shows the pK_a of N[1]-H of guanin-9-yl from its own δH8G (pK_a 9.88 ± 0.03) as well as from δH8A (pK_a 9.87 ± 0.01) of the neighboring p**A**p moiety, and δH5/H6C (pK_a 9.88 ± 0.01 and 9.90 ± 0.01 respectively) of the 3′-terminal cytosin-1-yl. This intramolecular nearest neighbor electrostatic interaction in the single-stranded RNA modulates the pseudoaromaticity of the nearest neighbors by almost total transmission of ΔG°_{pKa} because they constitute an extended array of offset-stacked coupled aromatic heterocycles within a polyanionic sugar−phosphate backbone at the ground state. The enhanced basicity of **G**p residue by ca. 0.6 pK_a unit in the trimers compared to that of the dimer is a result of the change in the electrostatic microenvironment owing to the nearest neighbors in the former (the nucleobases as well as the phosphates). Thus, the ΔG°_{pKa} from the 5′-guanylate ion to the 3′-end aglycon via the central adenin-9-yl is 55 to 56 kJ mol^{-1} in each step through a distance spanning ~6.8 Å in an unfolded state. As a result, the pK_a of guanin-9-yl moiety has become 9.25 ± 0.02 in **G**pEt (**1**), 9.17 ± 0.02 in **G**pA (**2**), 9.75 ± 0.02 in **G**pApA (**3**), and 9.88 ± 0.03 in **G**pApC (**4**). This means that guanin-9-yl moiety of trimers **3** and **4** is more basic than in the monomer or the dimer. The net outcome of this electrostatic cross-talk between the two neighboring heterocycles is creation of new hybrid aglycones in an oligo or polynucleotide, whose physicochemical property and the pseudoaromatic character are completely dependent both upon the nearest neighbors, and whether they are stacked or unstacked. Thus, this tunable physicochemical property of an aglycon (an array of the extended genetic code) may have considerable implication in our understanding of the specific ligand binding ability of an aptamer, the pK_a and the hydrogen bonding ability of nucleic acids in a microenvironment, or in the triplet usage by the anticodon−codon interaction in the protein biosynthesis in the ribosome.

Introduction

The chemical nature of the nucleobase imparts the sequence specificity of nucleic acids, which plays a key role in both hydrogen bonding[1] and stacking,[2] leading to various biological function such as replication, transcription, and translation.[2,3] The nucleobase is also a key element that responds to any micro-environmental changes by protonation, deprotonation, metalation or ligand binding through intra- and intermolecular interaction.[4,5] Although the hydrogen bonding interactions (base-pairing)[1,2,9] are relatively well studied, the experimental data supporting the nature of stacking interactions[6−10] is very limited. Recent

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studies on the thermodynamic stabilization of the same strand by nearest-neighbor interaction in DNA and RNA[6,7] owing to inter- or/and intrastrand stacking interactions by a dangling base[6] or a 3′- or 5′-tethered chromophore[7] have shed new light on the importance of stacking in the self-assembly process of DNA or RNA. Studies[18] such as temperature and/or concentration dependent intermolecular base-base association constants, as well as substituent effects[11] in various nonbiological aromatic systems have also elucidated the role of aromatic stacking interactions as a major force to the stability of nucleic acids.

We have recently demonstrated that a nucleobase in a dinucleoside (3′→5′) monophosphate showed not only its own pK_a but also the pK_a of the nearest neighbor, which provided a direct evidence of electrostatic interaction between two nearest neighbor nucleobases in the ground state as a result of intramolecular offset-stacking.[30] This electrostatic interaction leads to almost total modulation of pseudoaromaticity by nearly total transmission of ΔG°_{pKa},[14,20] from one nucleobase to the nearest neighbor (16−53 kJ mol^{-1}, depending upon the nucleobase and/or cationic or anionic state).[30] This intramolecular electrostatic interaction suggested that the chemical nature of each aglycone in a stacked dinucleotide, unlike simple monomers, constitute a electronically coupled heterocyclic system.

Results and Discussion

We here report that this physicochemical modulation of the pseudoaromatic character of aglycones by the nearest-neighbor intramolecular electrostatic interaction can propagate from the first to the third nucleobase, via the second aglycon, in the RNA trimers. This has been evidenced by observing the pK_a of the

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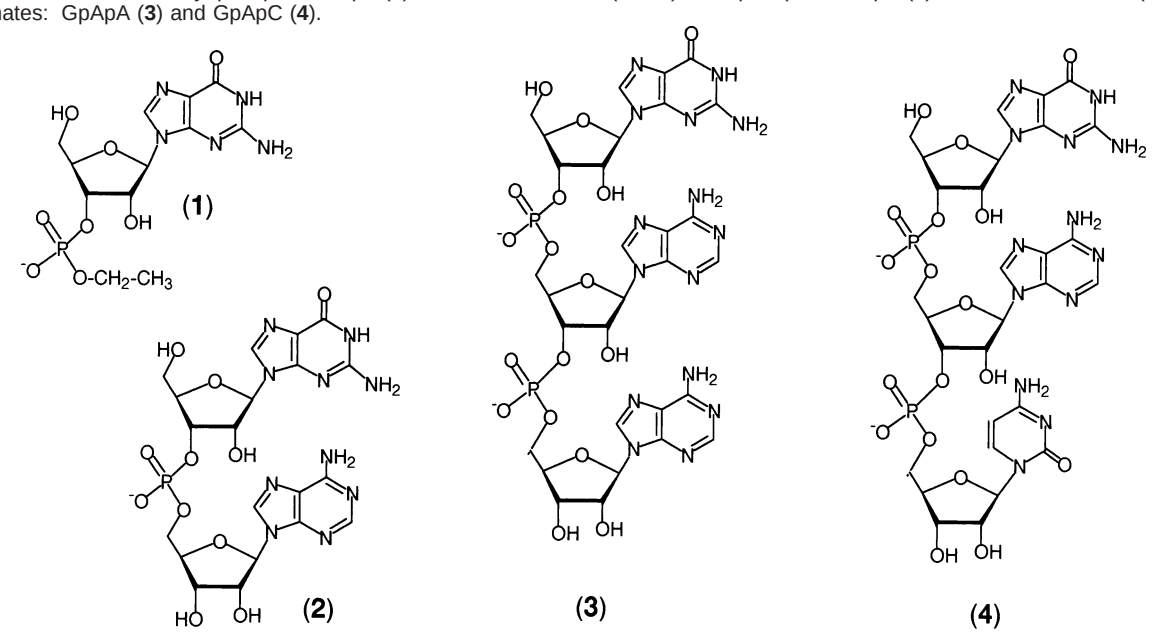
(22) The dimerization (d)/trimerization (t) shift[18] for δ_{H8G} [$\Delta\delta^{d/t}_{H8G} = (\delta_{H8G})_{GpEt} - (\delta_{H8G})_{dimer/trimer}$, in ppm] have been calculated by subtracting δH8G of GpEt (**1**) [$((\delta^{d}_{H8G})_{GpEt})$],[30] from that of guanosine moieties in GpA (**2**) ($\Delta\delta^{d}_{H8G}$),[30] GpApA (**3**) and GpApC (**4**) ($\Delta\delta^{t}_{H8G}$), at neutral (N) and deprotonated (D) state. The $\Delta\delta^{d/t}_{H8G}$ in **2**−**4** at neutral (N) and deprotonated (D) states are as follows: $\Delta\delta^{d}_{H8G}$: 0.104 (N) and 0.096 (D) for **2**,[30] $\Delta\delta^{t}_{H8G}$: 0.107 (N) and 0.110 (D) for **3** as well as 0.052 (N) and 0.063 (D) for **4**. Thus, the different $\Delta\delta^{d}_{H8G}$ and $\Delta\delta^{t}_{H8G}$ at both neutral as well as deprotonated states show the disparity in partial charge distribution in pseudoaromatic guanine-9-yl in **2**−**4**, owing to the nearest neighbor interaction. That GpApA (**3**) is more stacked than GpApC (**4**) can also be evidenced by the fact that δH8 of pAp in GpApA (**3**) is more more shielded than that of GpApC (**4**) by $\Delta\delta_{H8A(pAp)}[\Delta\delta_{H8A(pAp)} = (\delta_{H8A})_{GpApC} - (\delta_{H8A})_{GpApA}]$ of 0.044 ppm at the N state and 0.106 ppm in D state. That the effect of the third base indeed stabilizes the stacking between the first and the second base can also be evidenced by the fact that δH8 of pA in GpApA (**3**) is more shielded than that of GpA (**2**) by $\Delta\delta^{t}_{H8A(pA)}$ of 0.12 ppm at the N state and 0.124 ppm in D state.

(23) The electrostatic interaction between the partial charge distribution of a nucleobase (depending upon its pseudoaromatic character) and the π-electron density corresponding to the next base constitutes atom−$\pi\sigma$ interaction.[8,10] The deprotonation at guanin-9-yl changes partial charge distribution of the aglycone (particularly for heteroatoms), in the ground state, which in turn, causes a differential electrostatic interaction between the neighboring nucleobases in deprotonated state. The pH-dependent chemical shift change of a particular proton (H) between neutral (N) and deprotonated (D) state [$\Delta\delta^{H}_{N-D} = \delta^{H}_{N} - \delta^{H}_{D}$, where $\Delta\delta^{H}_{N-D}$ corresponds to the relative shielding (upfield shift, $\Delta\delta^{H}_{N-D} > 0$) or deshielding (downfield shift, $\Delta\delta^{H}_{N-D} < 0$) as a function of pH] are the basis for this atom-$\pi\sigma$ interaction[8, 10] between nearest neighbor nucleobases in **1** − **4**. The pH-dependent shift of δH8G ($\Delta\delta^{H8G}_{N-D}$) for GpEt (**1**),[30] GpA (**2**),[30] GpApA (**3**), and GpApC (**4**) are as follows: 0.149 for **1**; 0.141 for **2**; 0.152 for **3** and 0.160 for **4**. The pH-dependent shift of δH8A and δH2A ($\Delta\delta^{H8A}_{N-D}$ and $\Delta\delta^{H2A}_{N-D}$ respectively) of terminal p**A** moieties in Gp**A** (**2**)[30] and GpAp**A** (**3**) are as follows: for **2**,[30] $\Delta\delta^{H8A}_{N-D}$: −0.056 and $\Delta\delta^{H2A}_{N-D}$: −0.004 and for **3**, $\Delta\delta^{H8A}_{N-D}$: −0.052 and $\Delta\delta^{H2A}_{N-D}$: −0.002. Similarly, $\Delta\delta^{H8A}_{N-D}$ and $\Delta\delta^{H2A}_{N-D}$ of p**A**p moieties in Gp**A**pA (**3**) and Gp**A**pC (**4**) are as follows: for **3**, $\Delta\delta^{H8A}_{N-D}$: −0.055 and $\Delta\delta^{H2A}_{N-D}$: 0.014 and for **4**, $\Delta\delta^{H8A}_{N-D}$: −0.117 and $\Delta\delta^{H2A}_{N-D}$: 0.003. Moreover, the pH-dependent shift of δH5C and δH6C ($\Delta\delta^{H5C}_{N-D}$ and $\Delta\delta^{H6C}_{N-D}$, respectively) of p**C** moieties in GpAp**C** (**4**) are as follows: $\Delta\delta^{H5C}_{N-D}$: −0.098 and $\Delta\delta^{H6C}_{N-D}$: −0.069.

(24) The difference in the efficiency of the intramolecular electrostatic interaction amongst **2**−**4** can be quantitatively assessed by comparing the $\Delta\Delta G^{\circ}_{pKa}$, which can be obtained by subtracting ΔG°_{pKa} of the GpA (**2**)[30] or GpApA (**3**) or GpApC (**4**) from that of the monomeric GpEt (**1**): Thus, the $\Delta\Delta G^{\circ}_{pKa}$= 0.4 kJ mol^{-1} (calculated from δH8 of guanin-9-yl as well as from other proton markers, see below) for GpA (**2**),[30] 2.8 kJ mol^{-1} for GpApA (**3**) and 3.6 kJ mol^{-1} for Gp**A**pC (**4**)] shows that (i) almost *total transmission* of the free energy of deprotonation (within the experimental error of ±0.1 to ±0.2 kJ mol^{-1}) to the neighboring aglycones of guanylate anion in **2**−**4** [adenine-9-yl of p**A** in **2** (δH8A: $\Delta\Delta G^{\circ}_{pKa}$= 0.5 kJ mol^{-1}); adenine-9-yl of p**A**$^{\mathbf{1}}$p and that of terminal p**A**$^{\mathbf{2}}$ in **3** (δH8A^1: $\Delta\Delta G^{\circ}_{pKa}$ = 2.7 kJ mol^{-1}, δH2A^1: $\Delta\Delta G^{\circ}_{pKa}$ = 3.3 kJ mol^{-1} and δH8A^2: $\Delta\Delta G^{\circ}_{pKa}$ = 3.3 kJ mol^{-1}) as well as adenine-9-yl of p**A**p and further to terminal cytosine-1-yl in **4** (δH8A: $\Delta\Delta G^{\circ}_{pKa}$ = 3.5 kJ mol^{-1}, δH5C: $\Delta\Delta G^{\circ}_{pKa}$ = 3.9 kJ mol^{-1} and δH6C: $\Delta\Delta G^{\circ}_{pKa}$ = 3.6 kJ mol^{-1})]. (ii) the small differences in $\Delta\Delta G^{\circ}_{pKa}$ found is owing to slight changes in the microenvironment of the dimer and as well as the electronic properties trimers with respect to the monomer (see also ref 25).

Scheme 1. Guanosine 3′-ethyl phosphate: GpEt(**1**);[30] Di-ribonucleoside (3′→5′) Monophosphate: GpA (**2**);[30] Tri-ribonucleoside (3′→5′) Diphosphates: GpApA (**3**) and GpApC (**4**).



guanylate anion of the 5′-**G**p moiety from the 9-adeninyl moieties of the central p**A**p as well as from the distant 3′-terminal p**A** moiety in 5′-GpApA-3′ (**3**), or from the 1-cytosinyl moiety of p**C**-3′ in 5′-GpApC-3′ (**4**). This observation of ΔG°_{pKa}[14,20] transmission (55 to 56 kJ mol^{-1}) within the aglycons of the RNA trimers is based on the comparison with the pK_a of the guanylate anion of the monmeric guanosine 3′-ethyl phosphate, GpEt (**1**), and the dimer, 5′-GpA-3′ (**2**) (Scheme 1). This has led us to observe how the change of the electronic character of guanin-9-yl affect the neighboring aglycones, p**A**p and p**A** in 5′-GpApA-3′ (**3**), or p**A**p and p**C** in 5′-GpApC-3′ (**4**). Our observation of the tandem intramolecular electrostatic interaction in simple offset-stacked trimeric RNAs shows that the pseudoaromatic characters of the constituent aglycones within the minimal genetic information unit (i.e. the trimer) are indeed modulated in the ground state by the pseudoaromatic makeup of the nearest neighbor, unlike in the monomers. This may lead us to understand the codon selection or degeneracy in the triplet usage in the anticodon-codon interaction negotiated in the ribosomal machinery.

The pH-dependent titration of aromatic protons of GpA (**2**) [δH8G (pK_a 9.17 ± 0.02) and δH8A (pK_a 9.16 ± 0.02)] as well as that of the δH8G of GpEt (**1**) [Panel (**A**) in Figure 1 and the corresponding Hill plot in Panel (**a**) in Figure 2][30] in the pH range 6.9−10.7 are shown for comparison [Panels (**B**) and (**C**) for titration plots in Figure 1, and panels (**b**) and (**c**) for Hill plots in Figure 2][30] with the titration profiles of the trimers, 5′-GpApA-3′ (**3**) and 5′-GpApC-3′ (**4**) (see below).

Thus, the pH titration studies (pH 7.3−11.6) of 5′-GpA^1pA^2-3′ (**3**) showed the pK_a of N^1-H of guanin-9-yl from its own δH8G (pK_a 9.75 ± 0.02) [Panels **D**−**G** for titration plots in Figure 1, and Panels **d**−**g** for Hill plots in Figure 2], as well as from δH8A^1 (pK_a 9.72 ± 0.02) and δH2A^1 (pK_a 9.83 ± 0.04) of the neighboring p$\mathbf{A}^1$p moiety [Panels **F** and **G** for titration plots in Figure 1, and Panels **f** and **g** for Hill plots in Figure 2] and the δH8A^2 (pK_a 9.83 ± 0.02) of the terminal p$\mathbf{A}^2$ moiety [Panel **E** for titration plot in Figure 1, and panel (**e**) for Hill plot in Figure 2]. It is noteworthy that δH2A^2 of the terminal p$\mathbf{A}^2$ moiety do not respond in the pH titration.[25] This means that both the imidazole and the pyrimidine parts of the middle p$\mathbf{A}^1$p moiety are experiencing the electrostatic interaction[23] from the neighboring guanylate anion at the 5′-end. The electrostatic interaction from the middle p$\mathbf{A}^1$p to the imidazole part of 3′-terminal p$\mathbf{A}^2$ moiety is mediated by the offset-stacking, whereas the pyrimidine part of the p$\mathbf{A}^2$ moiety do not participate in any such interaction,[10,26] which is reminiscent of that of GpA (**2**).[30,31]

Similarly, the pH titration of GpApC (**4**) [Panels **H**−**K** for titration plots in Figure 1, and Panels **h**−**k** for Hill plots in

(25) The relative stacking ability as well as ΔpK_a (between the trimer and the dimer) show that the strength of the stacking is as follows: **2** < **3** > **4**.[22,23] However, in the alkaline pH, **2** becomes slightly destacked, whereas **3** and **4** becomes slightly more stacked vis-à-vis stabilized[22] in the ionic form which may contribute to the relatively high basicity of guanin-9-yl in **3** and **4** compared to either in **2** (ca. 0.6 pK_a unit) or in their monomeric counterpart **1** (ca. 0.5−0.6 pK_a unit). This may also account for relatively higher pH-dependent tunability[23] of δH8G for **3** and **4** compared to that in **1** and **2**,[30] which in turn shows the sequence dependent intramolecular modulation of pseudoaromatic character of guanin-9-yl upon the deprotonation. The pH-dependent chemical shift change[23] of δH2A ($\Delta\delta^{H2A}_{N-D}$) in GpA (**2**) and GpApC (**4**) are almost invariant, nevertheless in Gp$\mathbf{A}^1$p$\mathbf{A}^2$ (**3**), $\Delta\delta^{H2A}_{N-D}$ of p$\mathbf{A}^1$p shows a detectable upfield shift over the pH range. Thus, the different pH-dependent tunibility of δH8 and δH2 of adenin-9-yl in p**A** and p**A**p, indicates the sequence-dependency owing to differential modulation of the pseudoaromatic character of adenine-9-yl in **2**−**4** by the effect of the nearest-neighbor.

(26) Such pH-dependent atom specific response (evident from specific chemical shift change, $\Delta\delta^{H}_{N-D}$ [23]) from the neighbors show the different intramolecular spatial orientation of the nucleobases with respect to each other: Thus, in Gp***A***pA (**3**), the p***A***p is most probably experiencing a "T-shaped"[11,2^{1n}] interaction with the 9-guanylate base, whereas pA is "offset-stacked"[8,10] to pAp to experience the differential electrostatic interaction from the 5′-terminal guanylate to minimize the Coulombic repulsion in Gp***A***pA (**3**). In contrast, the p***A***p in Gp***A***pC (**4**) is "offset stacked" such that imidazole part of p***A***p is within the influence of π-electron system of the guanylate anion, whereas the C5−C6 double bond of p**C** is also "offset stacked" with respect to p***A***p.

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(31) Since δH2A of neighboring adenine-9-yl in GpA (**2**)[30] does not respond over the above pH range clearly shows that the transmission of the charge[12,17,23] from the guanylate ion takes place exclusively to the imidazole part of the neighboring adenine-9-yl, in preference to the pyrimidine part.[30] We attribute this electrostatic interaction to offset stacking geometry[10,11] via atom-$\pi\sigma$ mechanism,[8,10] which means that the imidazole edge of adenin-9-yl is within the stacking interaction of 9-guaninyl face.

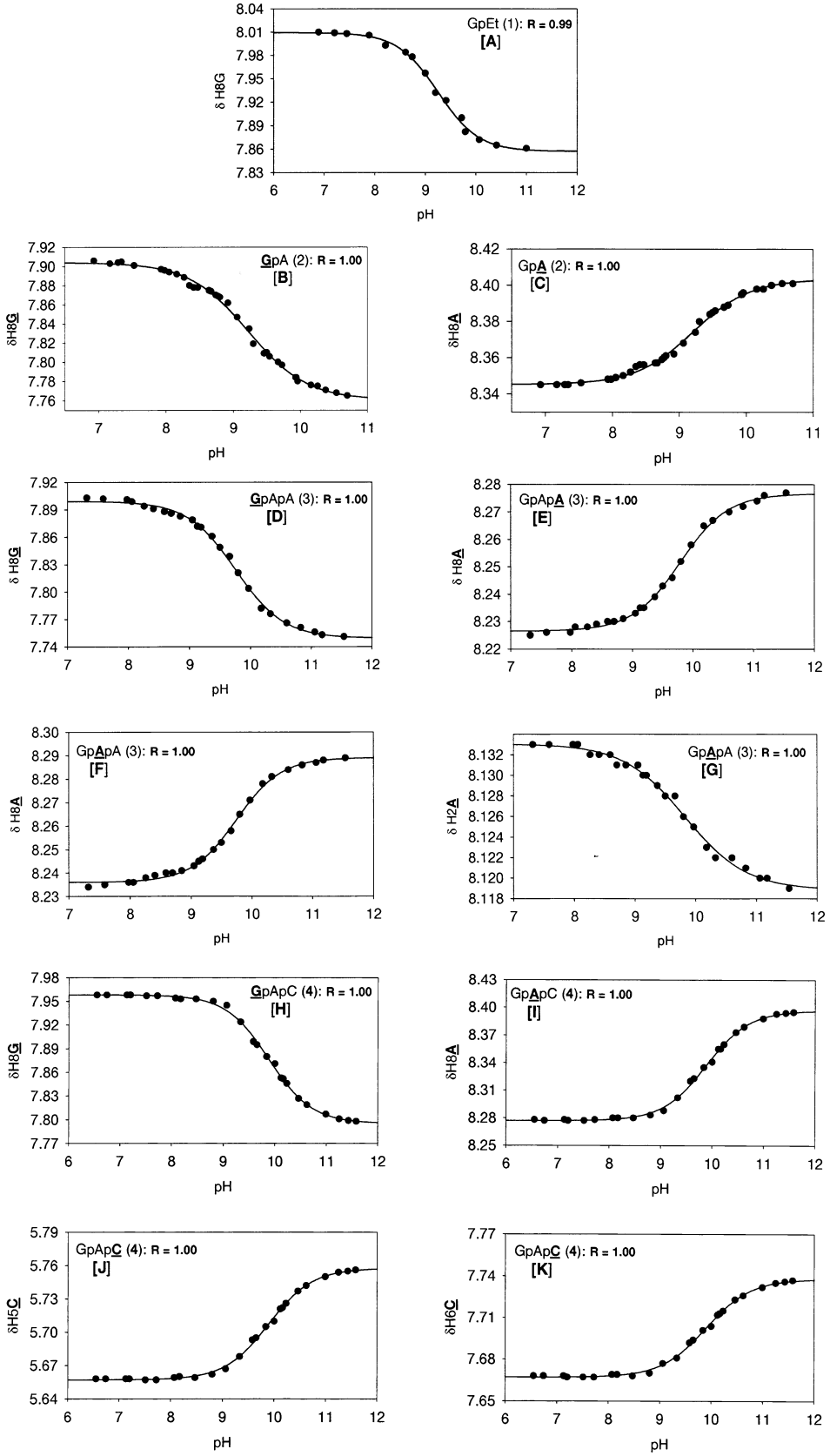

Figure 1. Panels **A**−**K** show the pH dependent ^{1}H chemical shift of aromatic protons: δH8G of GpEt (**1**) [Panel **A**],[30] GpA (**2**) [Panel **B**],[30] GpApA (**3**) [Panel **D**] and that of GpApC (**4**) [Panel **H**]; δH8A of GpA (**2**) [panel **C**],[30] p**A** of GpAp**A** (**3**) [Panel **E**], p**A**p of Gp**A**pA (**3**) [Panel **F**] and that of GpApC (**4**) [Panel **I**]; δH2A of p**A**p of GpApA (**3**) [Panel **G**]; δH5C and δH6C of GpApC (**4**) [Panels **J** and **K**, respectively] within the pH values of 6.7 ≤ pH ≤ 11.6. Chemical shift variations at average 25 different pH values (6.7 ≤ pH ≤ 11.6) have been measured in an interval of 0.2−0.3 pH units to obtain the sigmoidal curves. pK_a values have been calculated [see the Experimental Section for details] from the corresponding Hill plots [see Figure 2].

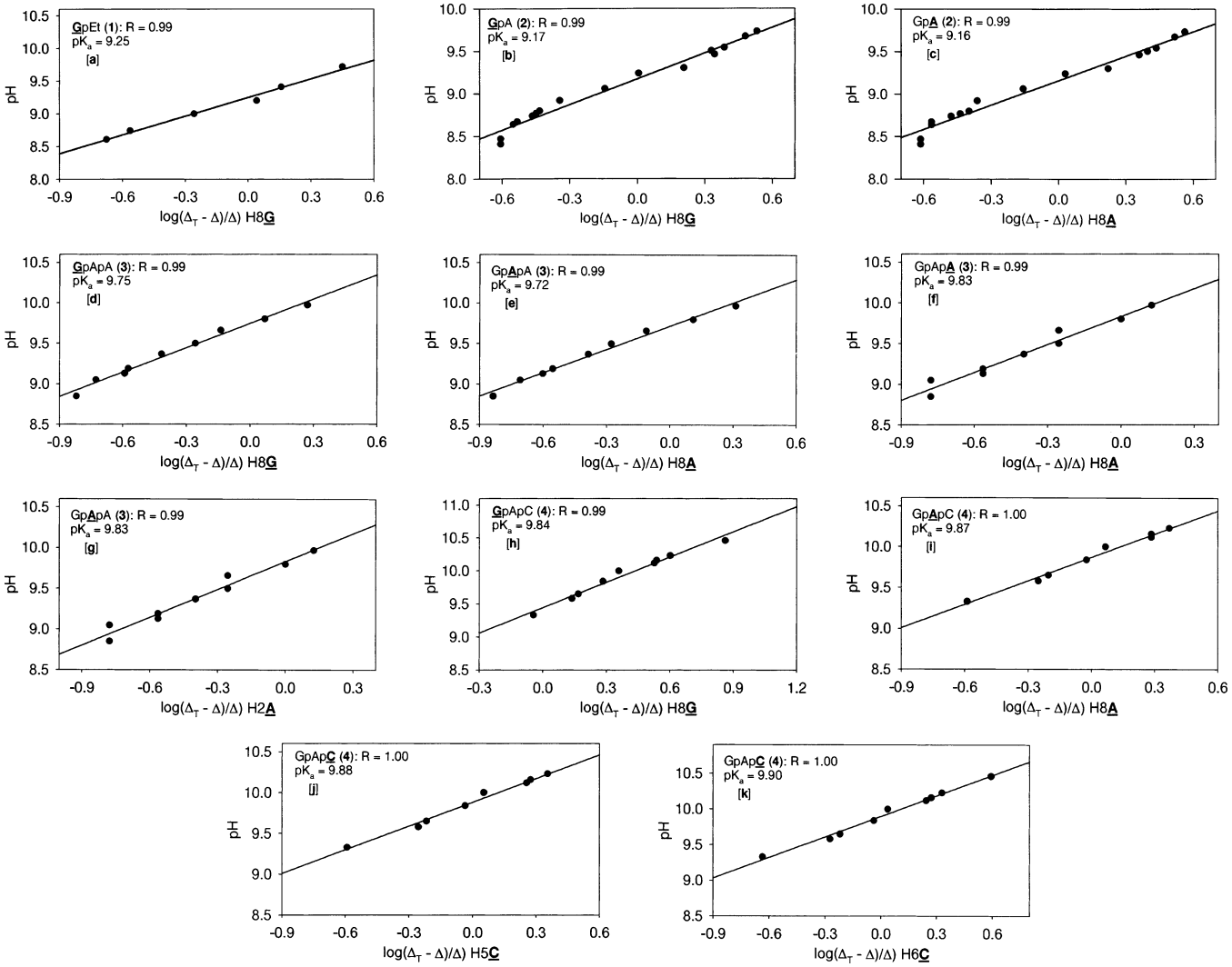

Figure 2. Panel **a** shows the Hill plots for δH8G of ***G***pEt (**1**). Δ_T ($7.0 \le$ pH ≤ 11.0) = 0.149 ppm and the plot of log(($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 0.95 ($\sigma = 0.05$) and pK_a = 9.25 ($\sigma = 0.02$).[30] Panel **b** shows the Hill plots for δH8G of ***G***pA (**2**). Δ_T ($6.9 \le$ pH ≤ 10.7) = 0.141 ppm and the plot of log(($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 1.00 ($\sigma = 0.04$). Panel **c** shows the Hill plots for δH8A of Gp**A** (**2**). Δ_T ($6.9 \le$ pH ≤ 10.7) = 0.056 ppm and the plot of log (($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 0.96 ($\sigma = 0.03$).[30] Panel **d** shows the Hill plots for δH8G of ***G***pApA (**3**). Δ_T ($7.3 \le$ pH ≤ 11.6) = 0.152 ppm and the plot of log (($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 1.00 ($\sigma = 0.04$). Panel **e** shows the Hill plots for δH8A of p***A***p in GpApA (**4**). Δ_T ($7.3 \le$ pH ≤ 11.6) = 0.055 ppm and the plot of log(($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 0.96 ($\sigma = 0.04$). Panel **f** shows the Hill plots for δH8A of p***A*** in GpAp***A*** (**3**). Δ_T ($7.3 \le$ pH ≤ 11.6) = 0.052 ppm and the plot of log (($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 1.14 ($\sigma = 0.08$). Panel **g** shows the Hill plots for δH2A of Gp***A***pA (**3**). Δ_T ($7.3 \le$ pH ≤ 11.6) = 0.014 ppm and the plot of log (($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 1.14 ($\sigma = 0.08$). As H2A of p**A** moiety in **2** and **3** showed negligible change[23] as a function of pH, the Hill plot analyses have not been performed. Panel **h** shows the Hill plots for δH8G of ***G***pApC (**4**). Δ_T ($6.7 \le$ pH ≤ 11.6) = 0.240 ppm and the plot of log (($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 1.10 ($\sigma = 0.07$). Panel **i** shows the Hill plots for δH8A of Gp***A***pC (**4**). Δ_T ($6.7 \le$ pH ≤ 11.6) = 0.117 ppm and the plot of log(($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 0.96 ($\sigma = 0.03$). As H2A showed negligible change[23] as a function of pH, the Hill plot analysis has not been performed. Panels **j** and **k** show the Hill plots for δH5C and δH6C of GpAp***C*** (**3**), respectively. Δ_T for H5C ($6.7 \le$ pH ≤ 11.6) = 0.059 ppm and the plot of log(($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 0.97 ($\sigma = 0.03$). Δ_T for H6C ($6.7 \le$ pH ≤ 11.6) = 0.098 ppm and the plot of log(($\Delta_T - \Delta$)/Δ) vs pH gave a straight line with a Hill slope = 0.96 ($\sigma = 0.04$).

Figure 2] in the alkaline range (pH 6.7−11.6) shows the pK_a of N^1-H of guanin-9-yl from its own δH8G (pK_a 9.88 ± 0.03) and from δH8A (pK_a 9.87 ± 0.01) of the neighboring p**A**p moiety (but not from its δH2A), as well as from δH5C and δH6C (pK_a 9.88 ± 0.01 and 9.90 ± 0.01 respectively) of the 3′-terminal cytosin-1-yl [Panels **J** and **K** for titration plots in Figure 1, and Panels **j** and **k** for Hill plots in Figure 2]. This tandem electrostatic interaction from the 5′-guanylate ion to the 3′-end aglycon through the central 9-adeninyl moiety (*which is ~6.8 Å between the two terminal aglycons in the unfolded state*) in the ground state suggests that the terminal pApC stacking in GpApC (**4**) is weaker[22,23] compared to pA^1pA^2 stacking in GpA^1pA^2 (**3**) [compare $\Delta\delta^{d/t}_{H8G}$ [22], $\Delta\delta^{t}_{H8A(pAp)}$ [22] and $\Delta\delta^{H}_{N-D}$ [23]] because the central pA^1p moiety is rather poorly offset-stacked[8,25] in the former (because δH2A does not respond to the deprotonation of neighboring guanin-9-yl) compared to the latter. An estimation of the dimerization and trimerization shift[22] for δH8G and δH8A at neutral and deprotonated states allows us to compare the extent of offset-stacking, which suggests that the strength of stacking in neutral[30] versus deprotonated is sequence-dependent.[22,25] Thus, the stacking is comparable in GpApA (**3**) both in the neutral and deprotonated states, whereas it is slightly favored in the deprotonated state for GpApC (**4**). *This means that the intramolecular electrostatic interaction is*

quite universal both in neutral, deprotonated as well as in protonated form[30] *to cross-modulate the pseudoaromatic character of nucleobases in nucleic acids by the nearest neighbor interaction.*

This study shows that the pK_as of guanine-9-yl moiety change owing to the nearest neighbor electrostatic interaction with both the nucleobases as well as with the phosphate(s), which are as follows: 9.25 ± 0.02 in **G**pEt (**1**),[30] 9.17 ± 0.02 in **G**pA (**2**),[30] 9.75 ± 0.02 in **G**pApA (**3**), 9.88 ± 0.03 in **G**pApC (**4**). Thus, it shows that guanine-9-yl moiety of trimers **3** and **4** are more basic than the monomer or dimer.

Thus, the ΔG°_{pKa} [14,20] obtained from H8G for guanin-9-yl is 55.6 ± 0.1 kJ mol^{-1} for G$^-$pApA (**3**), and 56.4 ± 0.2 kJ mol^{-1} for G$^-$pApC (**4**). These should be compared with the ΔG°_{pKa} of 52.8 ± 0.1 kJ mol^{-1} for G$^-$pEt (**1**)[30] and 52.4 ± 0.1 kJ mol^{-1} for of G$^-$pA (**2**).[30] Thus $\Delta\Delta G^\circ_{pKa}$ for the trimers (**3** and **4**) is larger[24,25] than that of dimer (**2**) because of enhanced stacking[25] as well as owing to the influence of phosphate,[32] which effectively increase the intramolecular nearest neighbor electrostatic interactions. It is noteworthy that the ΔG°_{pKa} obtained from either H8A/H2A of the middle residue or H8A/H2A or H5/H6C of the 3′-terminal residue within any single RNA trimer is very close to what is obtained from that of H8G of the 5′-terminal residue (55 to 56 kJ mol^{-1}), which suggests that the intramolecular electrostatic interaction of the 5′-guanylate ion to the aglycon at the 3′-end via the central adenin-9-yl is very close to 100% at the ground state.[24]

The fact that we observe the ΔG°_{pKa} both from the guanylate anion as well as from the nearest neighbors in ssRNA, shows that any two nearest neighbors in these trimers are most probably interacting by polar-π effect,[21a,b,l−n] where the attractive Coulombic term dominates the electrostatic interaction,[21c,d] in the neutral state either by edge-to-face[11] or center-to-edge (parallel offset)[10,11e,21n] arrangement. However, upon formation of the 9-guanylate anion, the H8, H5, or H6 of both the central pAp and the terminal pA/pC in GpApA (**3**) and GpApC (**4**) becomes deshielded[23] compared to the neutral state, which again shows that the stabilizing polar-π effect in the neutral state becomes destabilized in the deprotonated state because of the Coulombic repulsive anion-π interaction (hence destacking). We do not see any charge transfer (CT) band in the UV spectra neither for the neutral or for the deprotonated trimeric RNA, which rules out any CT effects, and demonstrates the dominance of polar-π effect[21a,b,l−n] over the CT effects. The polar-π effect by through-space Coulombic interaction[21l] has earlier been invoked, for example, in the molecular complexation of a series of disubstituted naphthalenes[21b] with an electron-rich host, which showed strongest binding with dicyano and weakest with the dimethoxy substituents. The manifestation of polar-π effect has also been attributed to explain the interaction between ions and arenes[21e−i] (such as carboxylate ion/arene[21e−g] and trimethylammonium ion/arene interactions[21h,i]) as well as amine/arene interactions[21j] in proteins and hydrogen bonding of water to cyclophanes.[21k] The geometrical requirement for this is that the two nearest neighbors should have an optimal edge-to-face or center-to-edge (parallel offset) arrangement, such that they can have interactions between their respective π and/or σ framework (atom-$\pi\sigma$ interaction).[8,30]

In the classic case of charge-dipole interactions such as an ionic salt, sodium chloride, dissolving in water involves the Coulombic interactions between a positively charged sodium ion and 6 water molecules as well as the corresponding interactions between a negatively charged chloride ion and 6 other water molecules. In the case of the sodium ion, the positive charge attracts the negative end of each water molecule's dipole. The negative charge of the chloride ion attracts the positive end of the O−H bond dipole. In our case, guanylate anion as a charge donor for a potential charge-dipole interactions should interact with the π-deficient pyrimidine system (marker proton H2) of the next 9-adeninyl nucleobase—not with the π-excessive imidazole part (marker proton H8). In fact, what we observe is the pH-dependent sigmoidal behavior causing deshielding of the H8 proton of imidazole part instead of H2 of the pyrimidine part, which rules out the involvement of any charge-dipole interaction, but anion-π repulsion.

Conclusions

Because we can successfully measure the pK_a of guanine-9-yl from either of the aglycones in the RNA timers **3** and **4**, it shows that the aglycones in the trimeric RNAs constitute a *coupled heterocyclic system* right across the pH range, 6.7 to 11.5 owing to both 3′→5′ and 5′→3′ two-way cross-modulation[30] by electrostatic interaction. This may be the reason a trimeric RNA sequence constitutes a single codon signal in recognition and function in the protein synthesis machinery. The magnitude of the chemical shift change in any of the aromatic protons in either of the two coupled aglycones differs in a *variable* manner depending upon the geometry of stacking, partial charge of the heteroatom as well as the sequence (compare GpApA and GpApC), which is evident from relative chemical shift change ($\Delta\delta^{H}_{N-D}$).[25]

The intramolecular electrostatic polar-π interaction (ΔG°_{pKa}) from the 5′-guanin-9-yl (or guanylate ion) to the 3′-end aglycon in GpApA (**3**) or GpApC (**4**), which are 6.8 Å apart, is quite ubiquitous in neutral (protonated or deprotonated) state to cross-modulate the pseudoaromatic character by the nearest neighbor interaction. This is quite similar to the polar-π effect found between ions and arene,[21e−i] such as carboxylate-arene interactions[21e−g] and trimethylammonium ion-arene interactions.[21h,i]

The ΔG°_{pKa} obtained from either H8A/H2A of the middle residue or H8A/H2A or H5/H6C of the 3′-terminal residue within any single compound (**3** or **4**) is very close to what is

(32) The pK_a of N1 of guanine-9-yl moiety in various monomer and oligo-RNA are as follows: GpEt (**1**) (9.25 ± 0.02), ***G***pA (**2**) (9.17 ± 0.02), ***G***pApA (**3**) (9.75 ± 0.02), ***G***pApC (**4**) (9.88 ± 0.02), 3′-***G***pApApC-5′ (9.76 ± 0.01), and 3′-***G***pApApApC-5′ (9.83 ± 0.01) (unpublished result). This simply shows that the microenvironment around the ***G***p residue in the dimer **2** is very comparable to that of the monomer **1**, whereas it is considerably different from the group consisting of trimers **3** and **4**, tetramer and pentamer. We have also compared the pK_a of N1 of guanine-9-yl moiety in the bis-anionic guanosine-3′-monophosphate (9.33 ± 0.01) with guanosine-3′-ethyl phosphate GpEt (**1**) (9.25 ± 0.02). This shows that the bisanionic 3′-monophosphate in 3′-GMP is slightly more electron-donating (+I effect), hence the constituent guanine-9-yl is slightly more basic than the monoanionic 3′-ethyl phosphate in GpEt (**1**) because of combined electrostatic and +I effects. When the 5′-bis-anionic phosphate and guanine-9-yl residues are at the same side of the pentose ring as in 5′-GMP compared to the 3′-GMP, one notices also the +I effect of 5′-phosphate is causing a more increase of the basicity of the N1 of guanine aglycon by ∼0.15 pK_a unit (ref 2, pp107−108). This observation is also consistent with the pK_a comparison of 5′-EtpGpEt-3′ (9.57 ± 0.01) and GpEt (**1**) (9.25 ± 0.02). It is thus clear that the position of the phosphate moiety (3′ or 5′) as well as the number of the phosphate charge (phosphomonoester versus phosphodiester) in the pentose-sugar ring has a distinctive effect on the pK_a of the constituent aglycone (in the same nucleotide). However, we have seen very little increase of pK_a (ca 0.1 pK_a unit on the terminal guanin-9-yl in Gp in our case) upon an increase of the total number of phosphates in the molecule as a result of chain elongation.

obtained from that of H8G of the 5′-terminal residue (55 to 56 kJ mol^{-1}), which suggests that the modulation of the intramolecular electrostatic interaction (ΔG°_{pKa}) from the 5′-guanylate ion to the aglycon at the 3′-end (C or A) via the central adenin-9-yl is very close to 100% at the ground state.[24]

A clear outcome of this study is that the pK_a of guanine-9-yl moiety changes from monomer/dimer to trimer owing to electrostatic intreraction with the neighbors[8] (nucleobases as well as phosphates), which are as follows: 9.25 ± 0.02 in **G**pEt (**1**),[30] 9.17 ± 0.02 in **G**pA (**2**),[30] 9.75 ± 0.02 in **G**pApA (**3**), and 9.88 ± 0.03 in **G**pApC (**4**). It shows that guanine-9-yl moiety of trimers **3** and **4** are more basic than the monomer or dimer.

ΔG°_{pKa} is the free-energy of deprotonation[14] at pH = pK_a. Because the pK_a is a measure of the ground-state stability of the anionic or the cationic form of the product, we can estimate the electrostatic free-energy of stabilization[14] at the pK_a by the term, ΔG°_{pKa} [14,20] . Thus, the strength of the electrostatic polar-π effect via intramolecular offset-stacking interaction in the ground state *is here equivalent to* ΔG°_{pKa} (**Type 1** effect). When this offset-stacking interactions between two coupled nearest neighbors (as evident by ΔG°_{pKa} transmission, Type 1 effect) have furthermore contributed to the increase/decrease to their acid/base character (pK_a), we attribute this enhanced stabilization/destabilization to the electrostatic effect, modulated both by the number of phosphate groups[32] as well as by stacked/unstacked nucleobases in an oligomeric nucleic acids (**Type 2** effect). *Thus, in the dimer, we see predominantly Type 1 effect, whereas in the trimers*[32] *we see Type 2 effect.*[32]

Experimental Section

(A) pH-Dependnet ^{1}H NMR Measurement. All NMR experiments were performed in Bruker DRX-500 and DRX-600 spectrometers. The NMR sample for compounds **3** and **4** (Scheme 1) were prepared in D_2O solution (concentration of 1 mM in order to rule out any chemical shift change owing to self-association[18]) with δ_{DSS} = 0.015 ppm as internal standard; chemical shifts and their differences are given in δ (ppm) and $\Delta\delta$ (ppm), respectively. All pH-dependent NMR measurements have been performed at 298 K. The pH values [pH = pD − 0.4 for the correction of deuterium effect] corresponds to the reading on a pH meter equipped with a calomel microelectrode (to measure the pH inside the NMR tube) calibrated with standard buffer solutions (in H_2O) of pH 4, 7 and 10. The pD of the sample has been adjusted by simple addition of microliter volumes of D_2SO_4 and NaOD solutions (0.5M, 0.1M, and 0.01M). The assignments for all compounds have been performed on the basis of selective homo-(^{1}H) and heteronuclear (^{31}P) decoupling experiments. All spectra have been recorded using 64 K data points and 64 scans for ^{1}H.

(B) pK_a Determination. The pH-dependent [over the range of pH 6.7−11.7, with an interval of pH 0.2−0.3] ^{1}H chemical shift (δ, with error ± 0.001 ppm) shows a sigmoidal (having average 20 different pH-dependent chemical shifts in each titration profile) behavior [Panels **A**−**K** in Figure 1]. The pK_a determination is based on the Hill plot analysis[4,16] using equation: pH = log((1 − α)/α) + pK_a, where α represents fraction of the protonated species. The value of α is calculated from the change of chemical shift relative to the deprotonated (D) state at a given pH ($\Delta_D = \delta_D - \delta_{obs.}$ for deprotonation, where δ_{obs} is the experimental chemical shift at a particular pH), divided by the total change in chemical shift between neutral (N) and deprotonated (D) state (Δ_T). So the Henderson−Hasselbalch type equation[20] can then be written as pH = log(($\Delta_T - \Delta_D$)/Δ_D) + pK_a. The pK_a is calculated from the linear regression analysis of the Hill plot [Panels **a**−**k** in Figure 2].

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