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Substituent effects of phthalimide-based nucleoside analogs on binding a CG Watson–Crick base pair

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ABSTRACT: Five differently substituted phthalimide nucleosides were studied by NMR spectroscopic techniques for their ability to recognize and bind a cytosine–guanosine (CG) Watson–Crick base pair in CD_2Cl_2. Whereas only rather weak binding was observed for analogs with an amino, acetamido, or benzamido substituent, strong binding was observed with the analogs carrying an ureido and *n*-butyl ureido residue. 2D NOE measurements at low temperatures confirm the proposed binding mode for the high-affinity ligands but indicate binding interactions for the weakly bound analogs different from the expected geometry. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: molecular recognition; nucleoside analog; phthalimide; base triad; NMR

INTRODUCTION

Molecular recognition governs most phenomena in chemical and biological systems and the development of artificial receptors has greatly expanded our possibilities to target molecular systems with high specificity and affinity. Due to their biological importance, for example, as constituents of nucleic acids, single nucleobases and nucleotides[1,2] as well as Watson–Crick base pairs[3,4] have been targeted in the past with specific receptor molecules in aqueous and apolar environments by exploiting stacking, ionic, and hydrogen bond interactions. Under physiological conditions, the specific recognition of base pairs within a DNA double helix has become an area of intense research. Triple helix formation by the binding of a third strand oligonucleotide (TFO) in the major groove of a DNA duplex through hydrogen bonds to the purine bases offers a powerful approach to regulate gene expression[5] or manipulate a particular genomic sequence.[6] Unfortunately, the recognition code of triple helix formation is limited and only purine bases on one strand of the double helix are effectively recognized by natural third strand bases.[7]

Much effort has been devoted to the development of artificial nucleobases to recognize all four possible base pairs in the past, however, with mostly limited success. The combination of selectivity with high-affinity binding has become a major challenge in the design of new base analogs. Recently, strategies to combine modified sugar units like conformationally locked nucleic acids (LNAs)[8,9] or 2′-aminoethyl-oligoribonucleotides (2′AE-RNAs),[10,11] that provide for a general non-specific affinity enhancement, together with artificial bases, that are mostly responsible for selectivity, have significantly contributed to the development of more effective TFOs. The discrimination of base pairs by the third strand base surrogate is expected to predominantly rely on hydrogen bond interactions with the particular base pair, structural complementarity, and isomorphism with the other canonical base triads.

Recently, we and others have shown that phthalimide derivatives may be suitable candidates for recognizing a CG base pair in aprotic solvents.[12,13] In our previous studies on CG recognition, only qualitative information on hydrogen bond mediated complex formation was obtained based solely on chemical shifts for protons involved in the intermolecular hydrogen bonds within the complexes. Here, we describe a more detailed characterization of their CG binding in terms of structure and thermodynamics as a function of different substituents. This should allow a better assessment for the potential of common hydrogen bond donors not only in phthalimides but also in other structurally related receptor molecules to complex a CG Watson–Crick base pair and may also give

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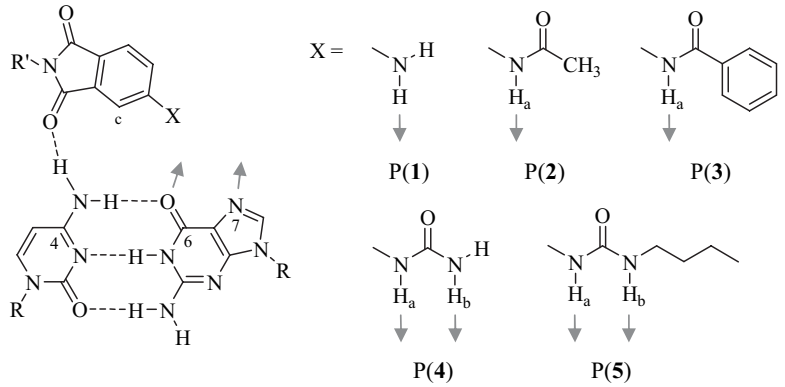

Figure 1. Binding of phthalimides with different substituents X to a CG Watson–Crick base pair; available hydrogen bond donor and acceptor sites are indicated by arrows; R = 3′,5′-di-*O*-(triisopropylsilyl)-β-D-2′-deoxyribofuranosyl, R′ = 2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl

valuable information on their contributation to base pair selectivity in the context of triple helix formation.

RESULTS

The phthalimide base and its proposed binding mode with a CG base pair is shown in Fig. 1. Whereas the phthalimide moiety is designed to form a hydrogen bond to the non-Watson–Crick bound cytosine amino proton, it also spans the CG base pair allowing the substituent X to form potential hydrogen bond contacts to the hydrogen bond acceptors O6 and N7 of the guanine base. The substituent X was varied from a simple amino functionality in P(**1**) to acetamide and benzamide derivatives P(**2**) and P(**3**) with a more acidic amide proton, thus constituting a more effective hydrogen bond donor. Analogs P(**4**) and P(**5**) possessing an ureido substituent might form another hydrogen bond contact to the CG base pair as indicated by molecular models.

NMR titration experiments

All analogs carry an *O*-benzoyl protected ribose sugar to block free ribose OH groups and to enhance the solubility in aprotic solvents. Likewise, bis-silylated di-*O*-triisopropylsilyl-2′-deoxycytidine and di-*O*-triisopropylsilyl-2′-deoxyguanosine were employed for the complexation and structural studies. Initial experiments on the homoassociation of the five analogs were examined by following the amino or amide NH_a proton chemical shift of nucleosides P(**1**)–P(**5**) as a function of nucleoside concentration ranging from 2 to 90 mM. By fitting the concentration dependent proton chemical shift with a 1:1 association model, values for the homoassociation constants K_a^{self} were obtained at different temperatures. These are summarized in Table 1.

Clearly, dimerization of nucleosides P(**1**)–P(**3**) is small even at lower temperatures. However, the unsubstituted ureido derivative P(**4**) exhibits significant self-association with decreasing temperature. Its association constant of about $10^3\ M^{-1}$ at 256 K is one order of magnitude higher compared to the corresponding *N*-butyl substituted P(**5**). Apparently, additional *N*-butylation hampers self-aggregation of the ureido analog.

Table 1. Summary of homoassociation constants K_a^{self} for nucleoside analogs P(**1**)–P(**5**) as well as of K_a, ΔH, and ΔS for the association with a CG Watson–Crick base pair

	T (K)	K_a^{self} (M^{-1})[a]	K_a (M^{-1})[a]	ΔH (kJ/mol)[b]	ΔS (J/K mol)[c]
1	295		14	—[d]	—[d]
	278	<1	17		
	274	<1			
	258		23		
	238		31		
2	291	3	65	−13.1	−10.6
	274	4	86		
	257	7	121		
	239		212		
3	293	2	23	−13.0	−18.3
	277	3			
	275		36		
	260	5			
	259		47		
	234		92		
4	299	49	1054	−17.7[e]	−1.3[e]
	277	287	1853		
	258		2349		
	256	928			
	247		3037		
	237		4297		
5	299	18	716	−18.5	−6.5
	277	36	1442		
	268		2043		
	256	92			
	237		5205		

[a] Uncertainty ±10% for $K_a > 200\ M^{-1}$, ±20% for $200\ M^{-1} > K_a > 50\ M^{-1}$, ±40% for $K_a < 50\ M^{-1}$.
[b] Uncertainty ±1 kJ/mol for **2**,**4**, and **5**, ±2 kJ/mol for **3**.
[c] Uncertainty ±4 J/K mol for **2**,**4**, and **5**, ±8 J/K mol for **3**.
[d] Not determined due to significant uncertainty in K_as.
[e] Determined for $T \geq 277$ K with two separate temperature dependent series.

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Due to the strong binding observed between C and G in apolar solvents ($K_a \sim 10^5\,M^{-1}$), a 1:1 mixture of the two nucleosides can be treated as a single CG species.[4] Binding of the nucleoside analogs to this CG base pair was again followed by changes in the ^1H NMR chemical shift of the amino and amide NH_a protons of P(**1**)–P(**5**). Thus, titration of the analog with a 1:1 mixture of C and G resulted in a downfield shift of the NH_a signal with increasing CG concentration as shown in Fig. 2 for the analogs P(**2**), P(**4**), and P(**5**).

The heteroassociation constant for complex formation K_a between each of the nucleoside analogs and the CG base pair was determined by fitting the chemical shift data as a function of concentration to a nonlinear 1:1 binding isotherm. The results from measurements at different temperatures are also summarized in Table 1. With association constants of about $14\,M^{-1}$ and $23\,M^{-1}$ at 295 K and 293 K, respectively, interactions of the amino- and benzamido-substituted analogs P(**1**) and P(**3**) with the CG base pair are rather weak. Even with decreasing temperatures binding does not seem to allow for a specific high-affinity base pair recognition through hydrogen bonding. The *N*-acetylated amino-phthalimide P(**2**) exhibits enhanced binding affinity towards the CG base pair compared to P(**1**) and P(**3**). However, very strong binding with association constants of about $10^3\,M^{-1}$ at ambient temperatures is only observed for the two ureido-substituted phthalimide nucleosides P(**4**) and P(**5**). It should be noted that the values obtained from the concentration dependent NH_a chemical shifts of P(**2**) and P(**5**) were confirmed by an additional analysis on the NH_b proton of P(**5**) and the aryl proton H_c of P(**2**), which exhibits a significant deshielding upon titrating the CG base pair (data not shown).

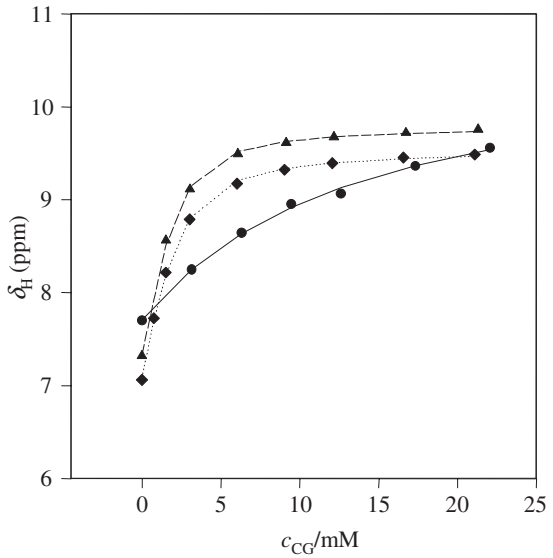

Figure 2. NH_a proton chemical shifts of nucleoside analog P(**2**) (circles, 274 K), P(**4**) (triangles, 277 K), and P(**5**) (diamonds, 277 K) as a function of CG concentration; lines represent the least-squares fit

According to the proposed binding mode (see Fig. 1), the non-Watson–Crick bound cytosine amino proton is expected to act as a hydrogen bond donor participating in a hydrogen bond to the carbonyl oxygen of the phthalimide base. Formation of such an interaction should manifest itself in a downfield shift of the non-Watson–Crick bound amino proton. We have therefore performed reverse titrations by adding the phthalimide analogs (0–20 mM) to a 2 mM solution of the CG base pair. To avoid problems with signal overlap and to unambiguously follow the C amino proton signal during the course of titration, we have employed specifically 4-^{15}N labeled cytidine and used a one-dimensional ^1H–^{15}N HMQC experiment as a filter that effectively eliminates all other proton resonances. In contrast to expectations, only small downfield shifts were observed at ambient temperatures when titrating P(**1**), P(**2**), and P(**3**) to the CG pair preventing the extraction of reliable association constants from the concentration dependent chemical shift data. Clearly, this points to a rather weak or even missing direct interaction involving the cytosine amino proton and the phthalimide carbonyl oxygen (*vide infra*). More significant downfield shifts of $\Delta\delta = 0.47$ ppm and 0.53 ppm were observed for the C amino proton upon titrating a 10-fold excess of P(**4**) and P(**5**) at 299 K. When fitted to a 1:1 binding isotherm, association constants of $K_a = 700\,M^{-1}$ and $396\,M^{-1}$ were obtained in these titrations for the complexation of the CG base pair with P(**4**) and P(**5**), respectively. Although within the same order of magnitude, these values are noticeably smaller when compared to the association constants obtained from the reverse titrations and point to a non-negligible degree of self-association for these two phthalimide analogs (*vide supra*).

Enthalpy and entropy of association

In order to separate enthalpic and entropic contributions to the free energy of association, we have performed a van't Hoff analysis on the temperature dependence of heteroassociation constants. Due to the limited temperature range employed, any temperature dependence of the association enthalpy ΔH was neglected in the analysis. Values of ΔH and ΔS as obtained from a plot of $\ln K_a$ as a function of $1/T$ are given for the nucleoside analogs in Table 1. Note that no attempt was made to extract corresponding values for P(**1**) due to its weak binding associated with a high degree of uncertainty for its association constants. Assuming only small differences between the enthalpy of solvation for the complex and monomers in the aprotic solvent, ΔH should reasonably well reflect the energy of the hydrogen bond interactions. Consequently, with values for ΔH of about -13 kJ/mol for P(**2**)·CG and P(**3**)·CG base triple formation, only rather weak hydrogen bond interactions can be expected. Larger enthalpic contributions of -18 kJ/mol are deter-

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mined for P(**4**) · CG and P(**5**) · CG having the potential of forming an additional hydrogen bond contact.

Although subject to a higher degree of error, changes in entropy upon complexation are generally small for all analogs with only an insignificant decrease in ΔS determined for the ureido-substituted analog P(**4**). Note, however, that due to its considerable self-aggregation upon decreasing the temperature, heteroassociation constants for analog P(**4**) at low temperatures are expected to be underestimated and must be met with caution. As a result, a van't Hoff plot progressively deviates from linearity at decreasing temperatures and thus analysis was restricted to temperatures $\geq$270 K.

2D NOE measurements

For a more detailed structural characterization of the complexes, 2D NOE spectra on 1:1 mixtures of the phthalimide nucleosides and the CG base pair were acquired at 213 K in methylene chloride. The low temperatures employed for the NOE studies enhance complex formation and are expected to facilitate the observation of intermolecular NOE contacts. Typical crosspeaks observed between guanine and cytosine imino and amino protons for Watson–Crick base pairing indicate that the base pair is not disrupted in the presence of the phthalimide nucleosides. In case of analog P(**5**), intermolecular NOE contacts are observed between guanine H8 and the α- and β-methylene protons of the *N*-butyl residue. Although no NOE can be detected between guanine H8 and the amide NH_b proton of P(**5**) due to the corresponding crosspeaks being too close to the diagonal, an NOE contact between the Watson–Crick bound amino proton of cytosine and the aromatic H_c proton of P(**5**) places the analog in a position as expected from the molecular design (Fig. 3).

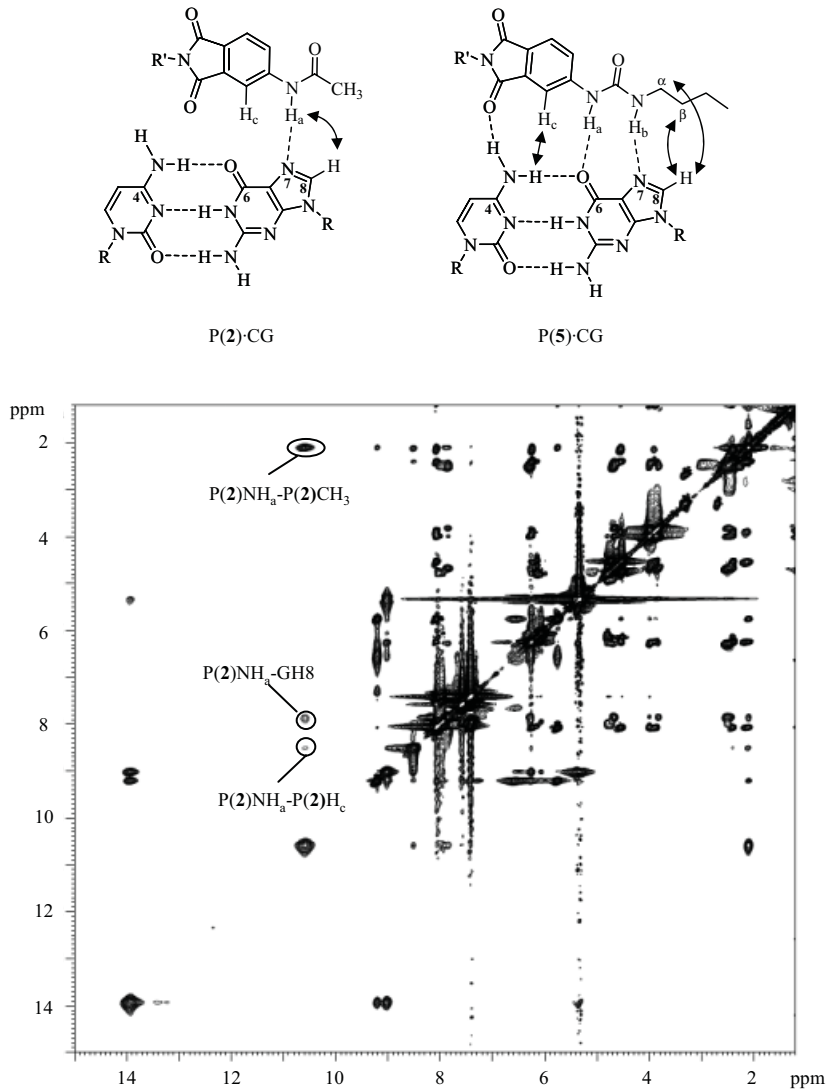

Figure 3. P(**2**) · CG and P(**5**) · CG base triplets with observed NOE crosspeaks between the base analog and the CG base pair marked by arrows (top). 2D NOE spectrum of a 1:1 mixture of P(**2**) and a CG base pair in CD_2Cl_2 at 213 K acquired with a mixing time of 200 ms (bottom); crosspeaks to the NH_a proton are highlighted; R = 3′,5′-di-*O*-(triisopropylsilyl)-β-D-2′-deoxyribofuranosyl, R′ = 2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl

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Although lower in intensity, a corresponding crosspeak between the Watson–Crick bound amino proton of cytosine and the aromatic H_c proton is also observed for analog P(**4**) consistent with its binding to the CG base pair in an analogous fashion. For the analogs P(**1**) and P(**3**) no NOE connectivities to the CG base pair could be observed even at the low temperatures employed. However, an unexpected contact between NH_a of P(**2**) and guanine H8 is only compatible with an alternate binding mode and the potential formation of a single hydrogen bond between NH_a and guanine N7 (Fig. 3).

Table 2. K_a, ΔH, and ΔS for the association of 3′,5′-di-*O*-(triisopropylsilyl)-β-D-2′-deoxyadenosine and 3′,5′-di-*O*-(triisopropylsilyl)-β-D-2′-deoxyuridine in CD_2Cl_2

T (K)	K_a (M^{-1})[a]	ΔH (kJ/mol)[b]	ΔS (J/K mol)[c]
274	109	−20.7	−36.7
254	206		
235	477		
217	1179		

[a] Uncertainty ±10%.
[b] Uncertainty ±1 kJ/mol.
[c] Uncertainty ±4 J/K mol.

DISCUSSION

Concentration dependent proton chemical shifts enable the evaluation of binding affinities for the five substituted phthalimide nucleosides towards a CG Watson–Crick base pair. Based on their small association constant, neither amino- nor benzamido-substituted analogs P(**1**) and P(**3**) bind with sufficient affinity to act as effective receptors for the CG base pair. Apparently, P(**1**) suffers from its non-acylated amino group being only a weak hydrogen bond donor whereas CG binding of P(**3**) may result in steric clashes between its benzamido group and the guanine purine ring within a planar base triad as suggested by molecular models. With association constants of about $10^3\,M^{-1}$ at ambient temperatures for analogs P(**4**) and P(**5**), introduction of ureido substituents strongly enhance binding to the CG pair. Such high-affinity binding has been observed previously with ureido-substituted base pair receptors in aprotic solvents.[12,13] However, urea derivatives are known to self-aggregate under appropriate conditions[14,15] and significant self-association is indeed indicated by the present data, in particular for the monosubstituted urea derivative P(**4**) at low temperatures. Although self-association could be reasonably described by a monomer–dimer equilibrium, the formation of higher aggregates cannot be excluded.

In order to gain more insight into the thermodynamics of binding, enthalpic and entropic contributions to the association have been determined from the temperature dependence of base triad formation. As a reference, we also obtained corresponding temperature dependent association constants through NMR titration experiments under identical conditions in CD_2Cl_2 for the canonical adenine–uracil base pair held together by two hydrogen bonds and these are summarized in Table 2. Although values for K_a are significantly smaller for the AU complex as compared to the binding of analogs P(**4**) and P(**5**) to the CG base pair, the strength of binding interactions as expressed by the association enthalpy (disregarding major solvation–desolvation effects) is slightly larger (−21 kJ/mol *vs.* −18 kJ/mol). Because three hydrogen bonds are expected to form when binding a CG base pair by P(**4**) or P(**5**), hydrogen bond interactions are weak. Alternatively, only two hydrogen bonds may form between analog and base pair in contrast to the proposed molecular model. Likewise, a ΔH of about −13 kJ/mol for the analogs P(**2**) and P(**3**) is compatible with the formation of two weak hydrogen bonds or with the formation of only one single hydrogen bond contact.

Apparently, smaller enthalpic contributions are compensated by smaller entropic losses for the phthalimide receptors upon CG binding. Although uncertainties in the association entropy are considerable, the decrease in entropy $-\Delta S$ is only moderate for all analogs compared to the AU pairing that exhibits perfect structural complementarity. Surprisingly, the entropy change for complexation is close to zero for nucleoside P(**4**). However, adding to the inherent uncertainty, this value might also be influenced by multiple equilibria involving self-aggregates even at higher temperatures. Clearly, the unknown effect of solvation and desolvation on the association entropies may be significant but the small (less negative) association entropy as observed in the base triad formation with all analogs points to considerable flexibility of bases within the complexes in line with the rather weak interactions upon binding (enthalpy–entropy compensation).

Structural studies through NOE experiments of the base triplets at lower temperatures nicely agree with the thermodynamic data. Whereas the two high-affinity receptors P(**4**) and P(**5**) seem to bind the CG base pair in the proposed geometry with the potential formation of three hydrogen bonds, no intermolecular NOEs to the CG pair are found for P(**1**) and P(**3**) consistent with their weak and presumably less specific binding. Interestingly, the *N*-acetylated analog P(**2**) exhibits an NOE contact between its amidic NH_a and guanine H8 proton indicating the formation of a single hydrogen bond between NH_a and guanine N7 (Fig. 3). The concomitant translational shift of the phthalimide towards the guanine base is also supported by the observation of a considerable downfield shift of the aryl H_c proton of P(**2**) due to its position in the deshielding region of the guanine carbonyl group (Fig. 3). This non-expected binding mode does not allow for the formation of a second hydrogen bond contact between the phthalimide carbonyl and the non-Watson–Crick bound cytosine amino proton, also evidenced by the very small

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chemical shift changes of the cytosine amino proton upon titrating analog P(**2**). Apparently, formation of the stronger NHN contact overwrites any propensity to bind through two weak hydrogen bonds and determines the resulting binding geometry. Likewise, the small downfield shift of the non-Watson–Crick bound cytosine amino proton upon titrating P(**1**) and P(**3**) and the much larger shifts upon titrating P(**4**) and P(**5**) point to the absence of a noticeable hydrogen bond contact to the cytosine amino proton for P(**1**) and P(**3**) but the formation of a, albeit weak, corresponding hydrogen bond in the P(**4**) · CG and P(**5**) · CG complexes where the three potential hydrogen bonds can form without mutual disruption (Fig. 3).

Taken together, nucleoside analogs P(**1**)–P(**3**) with non-acylated and acylated amino substituents fail to recognize and bind a CG Watson–Crick base pair in an aprotic solvent with sufficient affinity and specificity. Strong complexation is observed, however, with the two ureido-substituted analogs P(**4**) and P(**5**) making these two phthalimide derivatives powerful CG receptors in a non-aqueous environment.

In the context of DNA duplex recognition through triple helix formation under physiological conditions, binding affinities are expected to significantly differ from the binding of free base pairs in organic solvents due to additional solvation and stacking effects. Thus, desolvation of a hydrophilic urea-type functionality upon binding in the major groove of duplex DNA might be responsible for the previously observed low affinity of correspondingly substituted base analogs in triplex-forming oligonucleotides.[16,17] However, the results obtained in aprotic solvents should closely mimic macromolecular systems with respect to base pair discrimination by specific hydrogen bond formation and structural complementarity. *N*-alkylated, less hydrophilic ureido substituents in a proper orientation may thus also represent promising building blocks for CG recognizing nucleoside analogs in triplex-forming oligonucleotides.

MATERIALS AND METHODS

NMR measurements

NMR experiments were performed on Bruker AMX 500 and Avance 600 spectrometers. Temperatures were adjusted by a Eurotherm Variable Temperature Unit to an accuracy of $\pm 1.0\,^{\circ}C$ and calibrated with a standard solution of 4% MeOH in methanol-d_4. ^1H NMR chemical shifts in methylene chloride were referenced relative to $CHDCl_2$ ($\delta_H = 5.32$ ppm). Association constants were determined by titrating a 2 mM solution of the analog with a 1:1 mixture of C and G (final CG concentration 20 mM for P(**2**), P(**4**), and P(**5**) and 60 mM for P(**1**) and P(**3**) at $T > 273$ K). Concentration dependent chemical shifts were fitted with an appropriate equation by employing the Marquardt–Levenberg algorithm. Phase-sensitive NOESY spectra were acquired at 213 K with a mixing time of 200 ms using the time proportional phase incrementation (TPPI) mode with 2 K complex data points in t_2 and 1 K real data points in t_1. The NOESY data sets were apodized with a suitable window function in both dimensions and Fourier-transformed to give a final matrix size of 2 K × 1 K.

Materials

Reagents and starting materials were purchased from Sigma-Aldrich, Deisenhofen, Germany. Free cytidine and guanosine were *O*-silylated using triisopropylsilyl chloride. The synthesis of 4-^{15}N labeled cytidine[18] as well as of nucleoside analogs P(**1**), P(**3**), P(**4**), and P(**5**)[13] has been described before. Acetylation of P(**1**) by standard methods using acetyl chloride in THF/pyridine at room temperature gave P(**2**) in 85% yield. Reactions were controlled by TLC on silica gel plates (Merck silica gel 60 F_{254}) and all nucleosides purified by HPLC prior to NMR measurements.

Spectral and analytical data for P(**2**): ^1H NMR (250 MHz, 293 K, $CDCl_3$): δ (ppm) = 2.21 (s, 3H; CH_3), 4.54–4.79 (m, 3H; H4′, H5′, H5″), 6.12 (d, 1H; H1′), 6.22 (t, 1H; H3′), 6.29 (m, 1H; H2′), 7.29–8.22 (m, 18H; ArH). MS (FAB): 649.2 $(M+H)^+$. Anal. calcd for $C_{36}H_{28}N_2O_{10}$: C 66.66; H 4.35; N 4.32; found: C: 66.31; H 4.52; N 3.90.

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