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Synthesis of a Single G-Quartet Platform in Water

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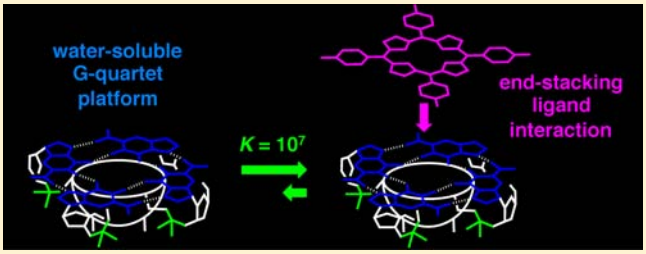
Supporting Information

ABSTRACT: For over 50 years the G-quartet has been a defining self-assembled structure in biology and non-covalent synthesis. It is shown here for the first time that the G-quartet is isolatable in water in the absence of stabilizing G-quartet stacking or cations through the construction of a phosphate-linked template-assembled synthetic G-quartet. Synthetic design has facilitated preservation of the guanine base, ribose sugar, and phosphate components with correct linkage chemistry relative to G-quadruplex DNA. Thus, a minimal synthetic model of G-quadruplex DNA, as in that associated with human gene promoter or telomere regions, is represented by this system. An application as a probe for interactions between G-quadruplex DNA and potential anticancer therapeutical binding ligands is demonstrated. Binding constants of 10^5–10^7 M^{-1} magnitude and 1:1 stoichiometries for TMPyP4, piper, and azatrux ligands were determined, whereas perturbations in BSU1051 and BRACO19 ligand signal were not observed. These data suggest a unique test for critical end-stacking interactions at the exclusion of intercalative or looping interactions for G-quadruplex binding ligands.



INTRODUCTION

The G-quartet, whereby guanine is hydrogen-bonded through its Hoogsteen face into a planar tetrad arrangement and stabilized by carbonyl oxygen–monovalent cation interactions, is the underlying structure upon which the G-quadruplex is built.[1−4] G-quadruplex-forming DNA sequences are localized in the telomeres of humans and other eukaryotes, and demonstration of *in vitro* G-quadruplex DNA structures has been extensive.[5] It is proposed that the G-quadruplex is potentially widespread in the human genome beyond the chromosome termini; greater than 40% of human gene promoter regions contain G-quadruplex-forming runs, suggesting an important transcription regulatory role for the G-quadruplex.[6] An intensive search for small-molecule inducers of human telomeric or oncogene promoter G-quadruplexes as part of an unconventional cancer targeting regime has been underway for over a decade.[7−9] In part of this protocol, folding of the 3′ single-stranded telomere overhang into a four-stranded DNA G-quadruplex structure is promoted by small-molecule binding, leading to inhibition of telomerase enzyme-catalyzed elongation of the telomere end. Telomerase activity has been reported to be exclusive to ∼90% of human tumor cells relative to cells of normal somatic tissues.[10] Recently, a structure-specific antibody was utilized to provide quantitative *in vivo* evidence for the presence of G-quadruplex in human cells.[11]

The G-quartet motif is not limited to biological studies and has been utilized as a self-assembling construction medium in supramolecular chemistry and nanotechnology.[12,13] Davis et al. and later Nikan and Sherman pioneered the principle of employing a covalent template to preorganize G-quartet assemblies, and this work has resulted in the construction of template-assembled synthetic G-quartets (TASQ) and related compounds that exhibit properties unique from known template-free G-quartet-based systems.[14−19] In this context, thermodynamic stability is improved through a reduction in the entropic penalty incurred from molecular self-assembly as guanine–guanine base-pairing is shifted from intermolecular to intramolecular. G-quartet and higher order G-quadruplexes that are formed from the stacking of at least two G-quartets are persistent in part due to the self-aggregative propensity of guanine bases in both lypophilic and hydrophilic media. However, this in turn has thus far prevented the direct observation of a genuine single G-quartet species in water. Few reports of water-soluble templated systems functionalized with four guanine elements exist; experimentation in water with DOTASQ and peptidic scaffold templated systems demonstrated that tetra-guanine species favor either open conformations devoid of intramolecular hydrogen-bonding or dimerization through association of guanine tetrad stacking, respectively.[19−21] Moreover, native G-quadruplex DNA phosphate and sugar constitution has not been conserved in these systems. Herein, we demonstrate that the iconic G-quartet structure is isolatable in water in the absence of stabilizing G-quadruplex stacking through the careful design of template, linkage chemistry, and counterion selection in a TASQ compound. Furthermore, we show how this bottom-up synthesis may provide a unique tool assisting in the rational design of human G-quadruplex binding ligand interactions in the context of anticancer therapeutic research.

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RESULTS AND DISCUSSION

Potential water-soluble TASQ compounds are given in Figure 1. It has been demonstrated previously that water solubility

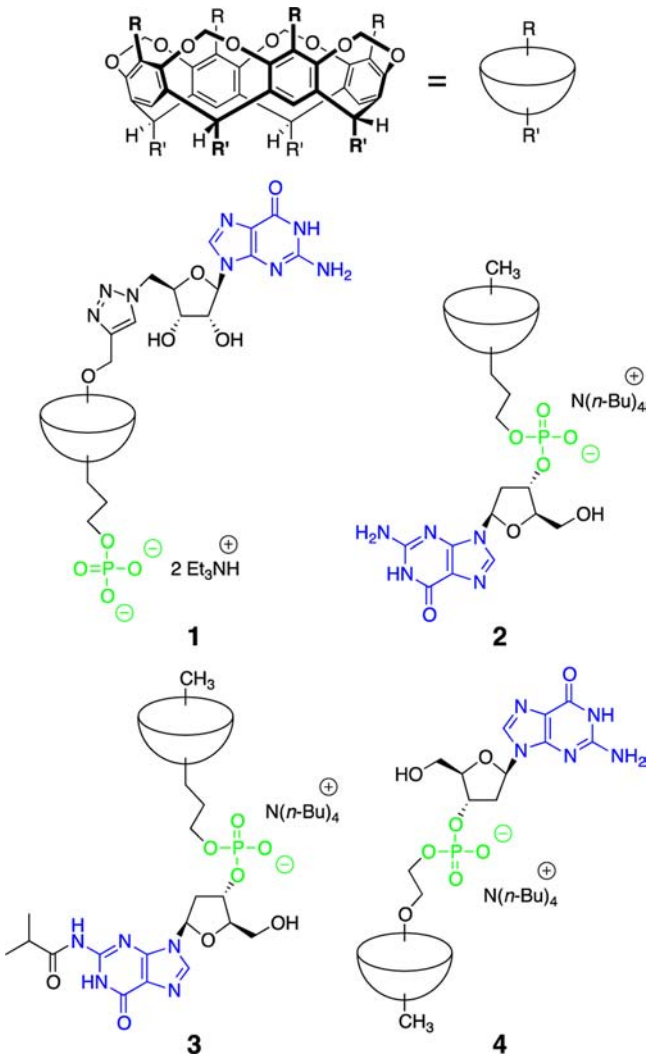

Figure 1. Potential water-soluble TASQ compounds.

could be imparted on early triazole-linked TASQ-like architectures by incorporating phosphate groups at the pendant position to give compound **1**.[22] Recently, 5′ TBS nucleoside phosphoramidite reagents were developed to carry out four-fold nucleotide coupling reactions to yield unprecedented water-soluble tetra-guanine construct **2** and base-blocked control analogue **3** as part of an effort toward the synthesis of minimal models of telomeric DNA.[23] This latter chemistry was accessed in the construction of potential TASQ **4** functionalized with ethylene glycol-linked nucleotides on the upper template rim (Scheme 1). Target **4** was obtained from the convergence of template **5** and nucleoside **8** starting materials after five steps. We will show that second-generation phosphate-linked TASQ **4** self-assembles in water into a unimolecular G-quartet in the absence of cation and acts as a selective artificial receptor for G-quadruplex ligands. Several critical design features of TASQ **4** should be noted: (i) water-solubilizing 3′ sugar phosphate linkages replicate the DNA backbone and allow investigation of electrostatic interactions between cationic side arms used conventionally in G-quadruplex ligands, (ii) hydrophobic control is carried out by choosing the upper rigid template face in order to hinder water access to the nonpolar concave template surface, and (iii) aprotic *n*-tetrabutylammonium counterions are necessary for solubilization and allow probing of the guanine imino resonance through ^{1}H NMR without complications arising from amine base-catalyzed exchange.[24,25]

Initial characterization of G-quartet-forming character by **4** was performed using CD spectroscopy in aqueous media (Figure 2). Characteristic CD in the 220−300 nm region of exciton coupling between hydrogen-bonded guanine residues in G-quadruplex DNA was observed for TASQ **4**.[26] A negative Cotton effect at 240 nm with a shoulder at 254 nm and positive Cotton maxima at 279 and 288 nm were observed for **4**. In comparison, water-soluble triazole-linked **1**, pendant phosphate-linked **2**, and base-blocked analogue **3** exhibited characteristically weakened CD. Cations including physiologically important Na^+ or K^+ were not observed to alter CD signatures for **1**−**4**, and further investigation of the role of metal cation in binding to TASQ **4** was prevented by line broadening effects in NMR experiments. It is evident here that the CD spectrum of compound **4** deviates from the empirical relationship that predominates over parallel and antiparallel G-quadruplex DNA structures and their respective CD spectra. Notwithstanding, compound **4**'s CD does contain features of both topologies, notably, the negative CD at 240 nm in parallel DNA G-quadruplexes and the positive CD at 290 nm in antiparallel structures. Mixed parallel/antiparallel (3+1) DNA G-quadruplex topologies in K^+ solution also exhibit respective negative and positive bands at approximately 240 and 290 nm in their CD spectra.[27] It is noteworthy that compound **4** contains an additional aryl chromophore in the template component not found in native G-quadruplexes that may contribute to the CD spectra observed here.

The ^{1}H NMR spectrum of **4** in water exhibited an imino proton kinetically stabilized from solvent exchange with a 11.2 ppm resonance in the downfield hydrogen-bonded guanine−guanine base-pairing region for G-quadruplex DNA.[2] Proton resonances in the imino region of the ^{1}H NMR spectrum for **1** or **2** were not observed under various conditions in comparison. In addition, an amino resonance in **4** was assigned at 6.3 ppm, and both of these exchangeable signals were better resolved at low temperature. We observed that line broadening of the imino proton resonance was also accompanied by the addition of aqueous ammonia solution. Thus, imino proton chemical exchange of the templated G-quartet of **4** is both catalyst and temperature sensitive. In double-stranded DNA comparatively, chemical exchange of imino protons requires opening of the base pair and is catalyzed by amine base.[24] Presumably, imino chemical exchange in solutions of **4** requires breaking of the guanine tetrad into a non-hydrogen-bonded open conformation (Figure 3). An all-*syn* conformation is a unique topology to TASQ compounds and was evidenced for **4** by an NOE cross-peak between H1′ and H8 (see Supporting Information).[2,16] The presence of one set of signals in the ^{1}H, ^{13}C, and ^{31}P NMR spectra confirms that the C_4 symmetry axis shared between [4]cavitand templates and G-quartets is in fact conserved in TASQ **4**, and this suggested the existence of either a self-associated homodimeric or a free monomeric species in solution (see Supporting Information for full NMR spectra). Diffusion coefficients determined by 2D DOSY NMR for 7.1 mg/mL solutions in D_2O of **4** and base-blocked control **3** were found to be 1.43×10^{-10} and 1.45×10^{-10} m^2/s, respectively.

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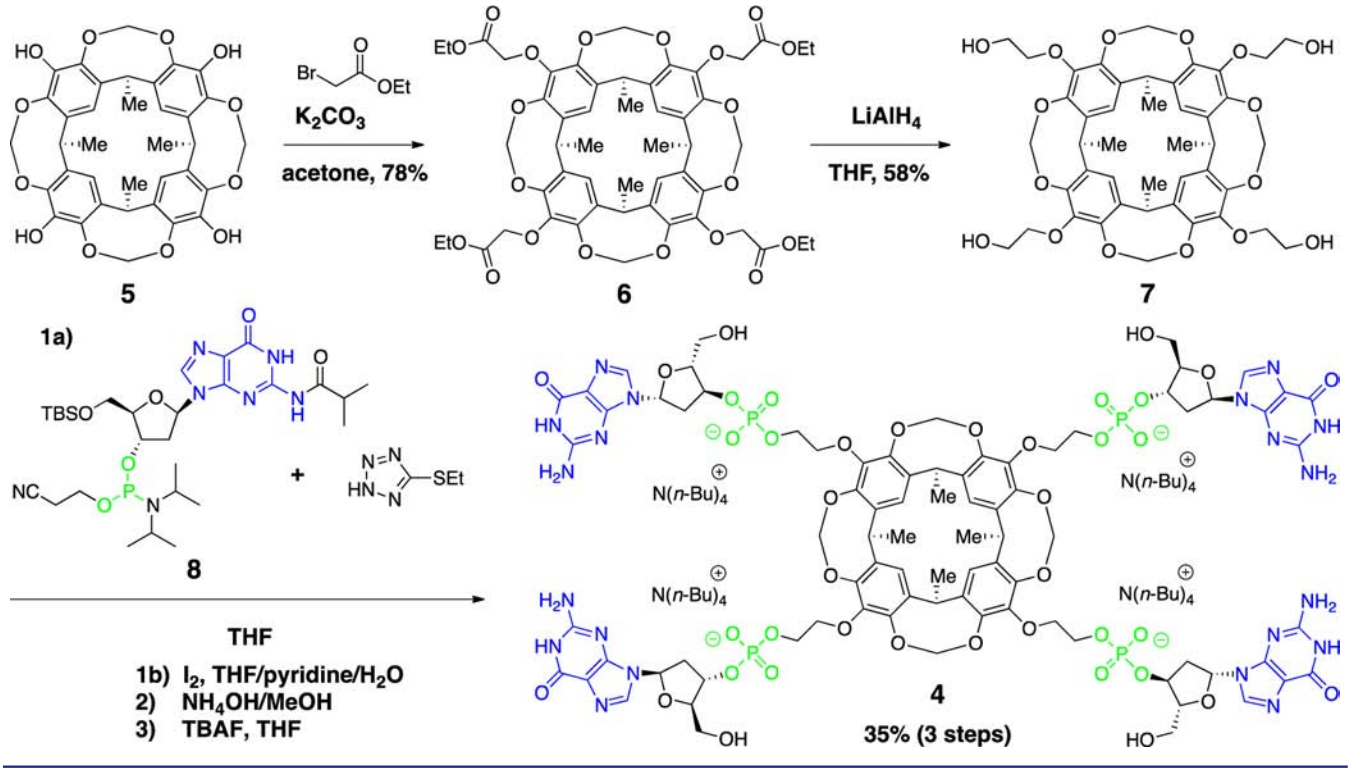
Scheme 1. Synthesis of TASQ 4



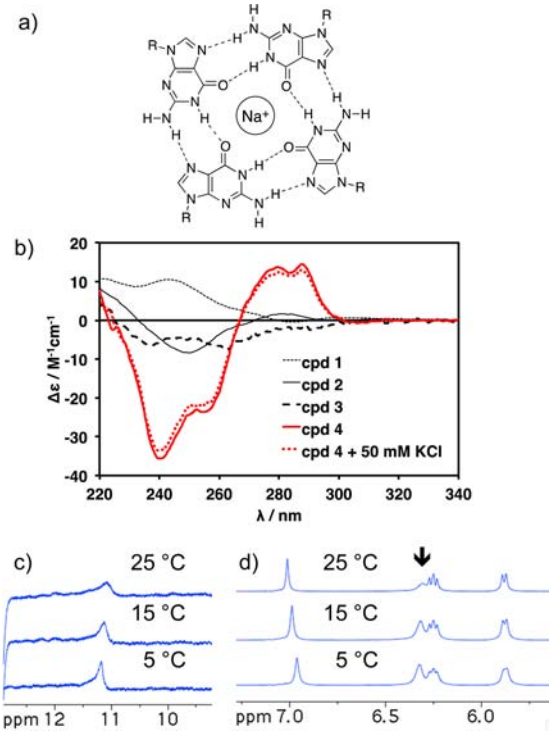


Figure 2. Characterization of G-quartets by **1**–**4**: (a) Na^+-G-quartet, (b) CD of 0.01 mM **1**–**4** in 10 mM Tris–borate, pH 8.0 at 25 °C, (c) ^1H NMR of **4** in H_2O at 400 MHz, and (d) ^1H NMR of **4** in 9:1 H_2O/D_2O at 400 MHz with water suppression by presaturation.

The DOSY data here do not support the expected 0.79 ratio of coefficients between a dimeric species self-associated through G-quartet surfaces and a non-associated control compound.[28,29] Thermal denaturation of **4** was not apparent at up to 95 °C since distinct melting transitions were not observed when monitoring the absorbance at 260 nm as a function of temperature (data not shown). Thus, the existence of a unimolecular G-quartet species in the absence of cations templated by TASQ **4** in water is suggested (Figure 3).

The functionality of the exposed G-quartet face of TASQ **4** as an artificial receptor was investigated through CD and fluorescence spectroscopic techniques for a series of G-quadruplex ligands that were selected due to being highly characterized and representative structure classes (cationic porphyrin TMPyP4,[30] perylene diimide piper,[31] and anthraquinone BSU1051[32]), an experimental clinical trial drug (amidoacridine BRACO19[33]), or presenting a novel binding architecture (triazatruxene azatrux[34]) (Figure 4; Table 1). Several experimental observations suggested end-stacking complexation of TMPyP4, piper, and azatrux ligands to the terminal G-quartet of **4** in a 1:1 binding stoichiometry. Signal-inducing ligands showed perturbations in the CD signature of **4** in the G-quartet-forming region between 220 and 300 nm and induced ligand CD in the visible region between 400 and 600 nm upon mixing, and this is exemplified by the interaction of piper and TMPyP4 with **4**. Monitoring of the ligand's fluorescence emission intensity upon titration of **1** in buffer together with Scatchard analysis allowed determination of binding constants $K = 10^5$–10^7 M^{-1} magnitude for TMPyP4, piper, and azatrux in accordance with known G-quadruplex–ligand complexes.[35,34] Lastly, binding stoichiometries of ligand complexes with **4** were determined to be 1:1, consistent with *x*-intercepts of 0.5 in Job plot analysis and saturational binding sites, *n* near to 1 in Scatchard analysis (see Supporting Information for Job plot and Scatchard plot analysis). Fluorescence binding plots in the presence or absence of KCl showed negligible differences with respect to each other. It was hypothesized that ligand cationic side arm–TASQ phosphate diester distances were significant and/or *n*-tetrabutylammo-

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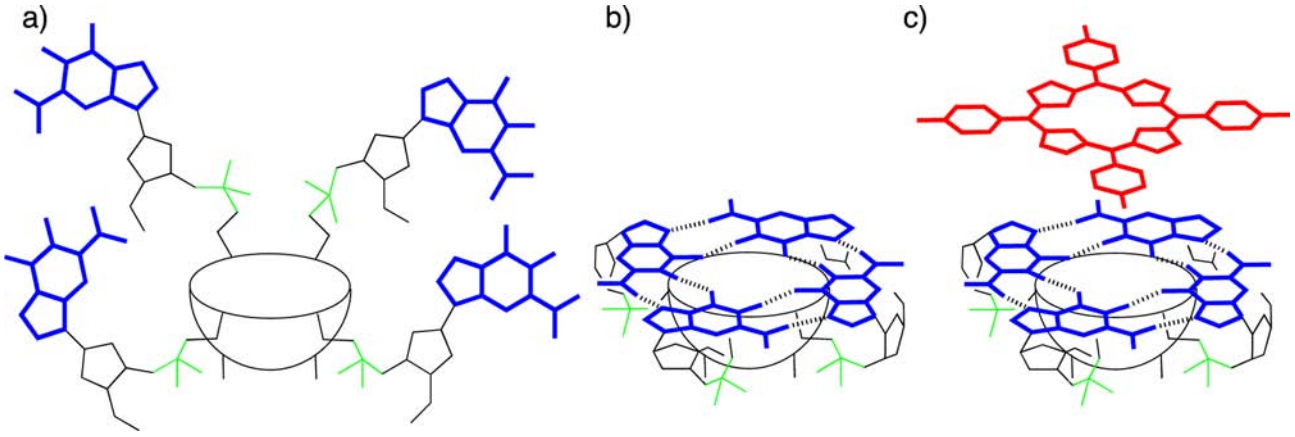

Figure 3. Possible solution behaviors of **4**: (a) non-hydrogen-bonded open conformation, (b) cation-free unimolecular G-quartet platform, and (c) pure end-stacking interaction with ligand to G-quartet platform.

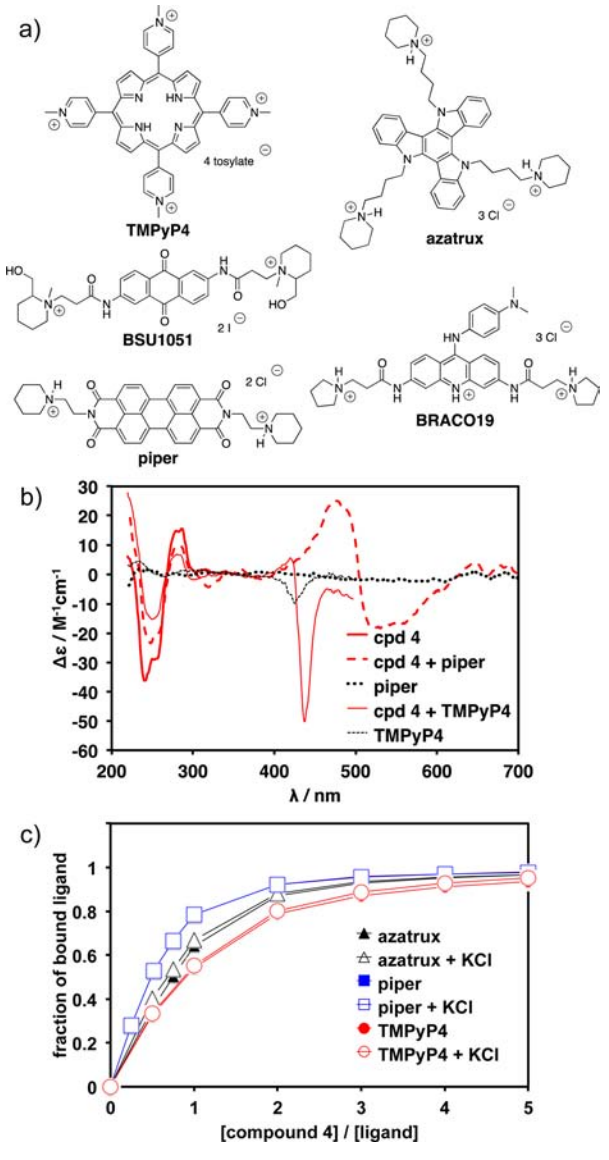

Figure 4. Binding of TASQ **4** to G-quadruplex ligands: (a) ligands, (b) CD interaction of 0.01 mM **4** with 0.01 mM TMPyP4 or piper in H_2O at 25 °C, and (c) fluorescence binding plots in 10 mM Tris-HCl, 0.1 mM EDTA at 25 °C in the presence or absence of 100 mM KCl for azatrux, TMPyP4, and piper.

Table 1. Parameters Determined for the Binding of G-Quadruplex Ligands to TASQ 4 by Fluorescence Spectroscopy[a]

ligand	K (M^{-1})[b]	n[b]	x-int[c]	pH[b–d]
TMPyP4	5.5×10^5	0.83	0.47	8.0
piper	1.7×10^7	1.2	0.51	6.5
azatrux	2.5×10^6	0.81	0.50	8.0
BSU1051	n.d.	n.d.	n.d.	–
BRAC019	n.d.	n.d.	n.d.	–

[a]10 mM Tris-HCl, 0.1 mM EDTA at 25 °C. [b]Scatchard analysis. [c]Job analysis. [d]Fluoresence binding plots. n.d. = not determined.

nium–phosphate ion pairs were present, leading to weakened electrostatic interactions in this system.

BSU1051 or BRACO19 ligands did not show a change in fluorescence or a CD induction when titrated with receptor TASQ **4** in contrast to TMPyP4, piper, or azatrux ligands. Previous reports of crystal structures or of solution behavior have corroborated end-binding arrangements in G-quadruplex DNA complexes with TMPyP4, piper, BRACO19, and azatrux.[36−38,30] However, in the case of BRACO19, critical TTA loop binding pockets were revealed in the crystal structure of a ligand sandwiched between the ends of two human telomeric G-quadruplexes.[38] Amidoanthraquinone BSU1051 was a known antitumor-active threading intercalator of duplex DNA before discovery of its telomerase inhibition properties.[39] Ligands BSU1051 and BRACO19 possess small aromatic chromophores containing three polycycles each, whereas TMPyP4, piper, and azatrux contain up to seven chromophoric polycycles. Although intercalative and looping interactions are documented, end-stacking through $\pi-\pi$ interactions between the terminal quadruplex G-quartet and the ligand's planar aromatic chromophore core are seen to be the dominant binding mode in the G-quadruplex ligand landscape.[9] Differences in binding constants of about 1 order of magnitude were attributed to binding mode preference in template-assembled G-quadruplexes.[40] This system based on water-soluble synthetic G-quartet **4** suggests remarkably exclusive selectivity toward pure end-stacking versus alternative intercalative or looping ligand interactions.

Applications based on a water-soluble G-quartet platform as in TASQ **4** for the development of small-molecule G-quadruplex binders are foreseen. Compound **4** provides a tool for assessing the end-stacking character of analogues of

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promising anticancer therapeutic candidates such as of BRACO19 which may benefit overall G-quadruplex affinity. Selectivity toward G-quadruplex DNA over duplex B-DNA is a requisite of effective G-quadruplex ligand design.[41] In the case of BSU1051, its B-DNA indiscriminate intercalative properties are isolated out by **4**. Alternatively, a screening technology adapted from the TASQ concept is envisioned for desired binding modes within a library of potential small-molecule G-quadruplex interacting compounds in the search for anticancer drug candidates.

CONCLUSIONS

Through the synthesis of water-soluble phosphate-linked TASQ **4**, we have demonstrated the utility of template-assembled synthesis in isolating the intrinsically unstable G-quartet molecular self-assembly in an aqueous medium. We have shown that emergence of a single G-quartet assembly in water is not a widespread phenomenon but requires careful synthetic design. We propose that the unimolecular G-quartet platform represents a new construction material for non-covalent synthesis in water and may assist in providing further insight into the nature of the timeless guanine-tetrad motif in biology.

ASSOCIATED CONTENT

Supporting Information

Synthesis and characterization of **4**; ^{1}H, ^{13}C, ^{31}P, $^{1}H-^{1}H$ COSY, and 2D NOESY NMR spectra of **4**; supplementary CD control spectra; full experimental procedures for ligand binding studies; Scatchard plots and Job plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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