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Convenient Method for the Synthesis of a Flexible Cyclic Polyamide for Selective Targeting of *c*-myb G-quadruplex DNA

Qiang Zhang, Xiaojie Cui, Sen Lin, Jiang Zhou, and Gu Yuan*

Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, Department of Chemical Biology, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China

guyuan@pku.edu.cn

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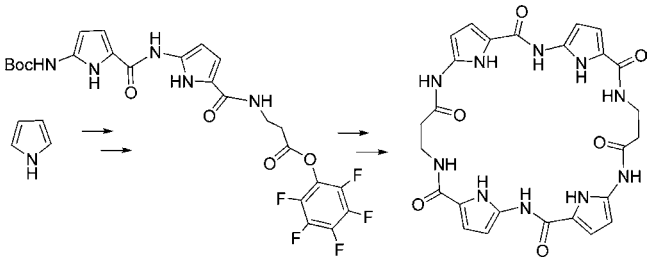
ABSTRACT



A convenient efficient method for synthesis of a flexible cyclic polyamide (cβ, 1) was developed through cyclodimerization. Electrospray ionization mass spectrometry and nuclear magnetic resonance results showed that 1 selectively binds to the *c-myb* G-quadruplex with high affinity, and there was no binding with the ILPR, *bcl-2*, and *c-kit* G-quadruplexes. This is the first time that a flexible cyclic polyamide was found to have high selectivity for the *c-myb* G-quadruplex.

Over the past few decades, G-quadruplexes involved in telomeres and promoter regions were found to play important roles in cell cycle, apoptosis, gene transcription, and gene expression.[1] The *c-myb* gene has critical influence on the proliferation, differentiation and survival of hematopoietic stem cells.[2] The expression of *c-myb* is normally restricted to very low levels in normal cells, while it has been shown to have high levels in some solid tumors and leukemias.[3] A GGA repeat was found in the promoter of *c-myb*. NMR studies revealed that the G-quadruplex of this region was formed by stacking two double-layer G-quadruplexes in a tetrad:heptad:heptad:tetrad (T:H:H:T) structure.[4] Thus far, macrocyclic molecules such as telomestatin, porphyrins, and their derivatives have been reported as G-quadruplex ligands (structures shown in Figure S1, Supporting Information).[5] These rigid macrocyclic skeletons provide expanded planar surfaces that interact with the terminal G-quartets by $\pi-\pi$ stacking with high affinity. However, this kind of ligand does not usually show high selectivity even though an expanded porphyrin molecule was reported as a specific ligand to bind to the single-loop hybrid G-quadruplex.[6] The remarkable cytotoxicity and side effects of these compounds

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caused by their poor selectivity limit their further application as clinical drugs. Recently, some flexible ligands have been demonstrated to recognize G-quadruplexes.[7] Moreover, linear polyamides containing *N*-methylpyrrole amino acids can bind with the G-quadruplex; however, these polyamides have a much stronger affinity toward duplex DNA.[8] These works supplied an idea to choose structurally simpler pyrrole amino acid as blocks and design a ligand specially targeting the G-quadruplex motif.

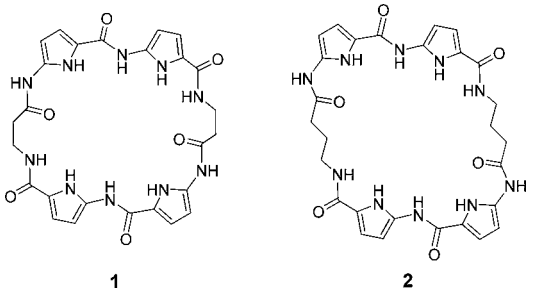


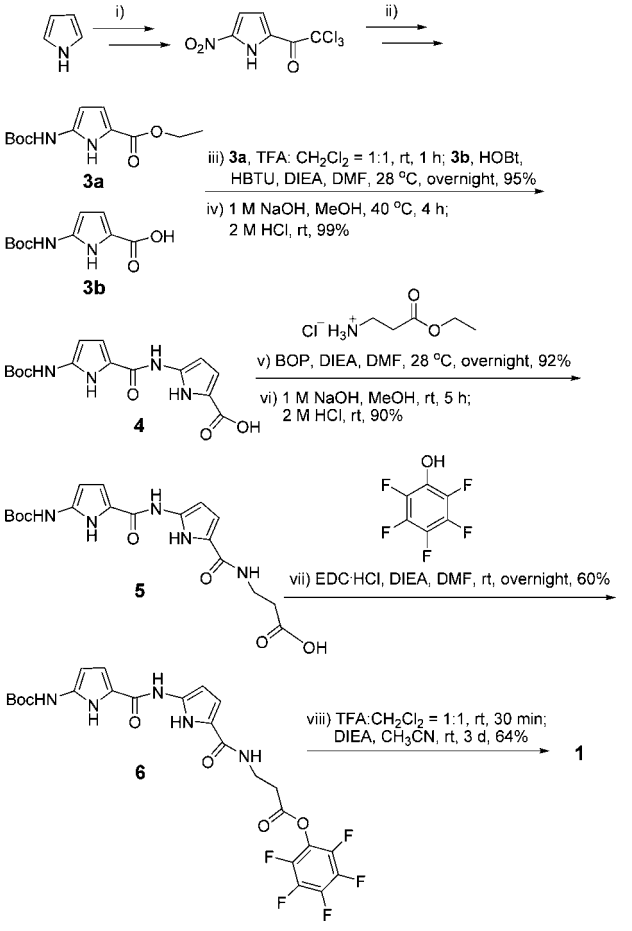
Figure 1. Structure of the novel flexible cyclic polyamide **cβ** (**1**) and analogue **cγ** (**2**).

In this research, a novel flexible cyclic polyamide **cβ** (**1**) (Figure 1) was designed and synthesized, and its properties as a G-quadruplex ligand were probed via ESI-MS and NMR. We found that **1** had high selectivity toward the G-quadruplex (Q1) formed by the *c-myb* promoter sequence, d[$G_2AG_2AG_2AG_2A$] (S1), over other G-quadruplexes, such as those in the telomere (Q2) and the insulin-linked polymorphic region (ILPR, Q3), *bcl-2* (Q4) and *c-kit* (Q5) (Table S1, Supporting Information). This is the first report of a flexible cyclic polyamide targeting the *c-myb* G-quadruplex particularly with remarkable selectivity.

Our starting material 2,2,2-trichloro-1-(5-nitro-1*H*-pyrrol-2-yl)ethanone ($NO_2PyCOCl_3$) was synthesized from commercially available pyrrole via trichloroacetylation and nitration to introduce the C- and N-terminal precursory groups, respectively. The esterification, hydrogenation, amine protection, and saponification route generated the building blocks $BocPyCO_2Et$ (**3a**) and BocPyCOOH (**3b**)[9] (Scheme S1, Supporting Information).

Two pyrroles were designed to be linked by an amide bond[10] in this flexible cyclic polyamide on each side of the molecule (see Scheme 1). Uronium-based coupling reagent

Scheme 1. Synthesis of Flexible Cyclic Polyamide **cβ** (**1**)[a]



[a] For (i) and (ii), see the Supporting Information.

2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotrizole (HOBt) were used to activate the carboxy group of **3b** in order to promote the coupling reaction with the Boc-deprotecting product of **3a** via the formation of amide bond.

Compared to the rigid structure, the insertion of β-alanine was beneficial to cyclization and enhanced molecular flexibility to increase the interactions and selectivity with G-quadruplexes. In the reaction of coupling the flexible units with the carboxylic acid in pyrrole dimer (**4**), phosphonium-based coupling reagent benzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) gave slightly more catalytic effective performance than HBTU/HOBt did.

In the traditional procedure to synthesize cyclic peptides, the N-terminal part was deprotected first to give free amine, followed by activation on the C-terminal to induce cyclization.[11] In our case, after deprotection of the Boc-group from **5**, however, attempts to prepare cyclic peptide using HBTU, BOP, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU),

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3-(diethoxyphosphoryloxy)-1,2,3-benzotrizin-4(3*H*)-one (DEPBT), benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), or bromotrispyrrolidinophosphonium hexafluorophosphate (PyBrOP) were not successful. Our synthesis strategy is activating C-terminal before N-terminal deprotection. In this synthetic route, the active ester should be stable enough in the process of purification and Boc-deprotection and maintain the reactivity for aminolysis in the cyclization. We found the active esters formed by treating the carboxylic acid with common uronium- or phosphonium-based coupling reagent were too reactive. Therefore, pentafluorophenol ester[12] was used, and it satisfied the requirements mentioned above. The pentafluorophenol ester (**6**) was obtained by the treatment of **5** with EDC·HCl and pentafluorophenol. Deprotecting the Boc group from **6**, the precursory monomer was generated with two active group (NH_2 and $-CO_2C_6F_5$) on both sides. The macrolactamization by cyclodimerizing the monomers was readily achieved at low concentration (2 mM) in an alkaline environment. The product was precipitated from the solution and easily isolated by centrifugation and washing with methanol to give **1** in a yield of 64% (the purity is higher than 96%). In addition, the analogical compound **cγ** (**2**) with two longer flexible linkers (4-aminobutanoic acid) instead of β-alanine was also synthesized in order to study the influence caused by the molecular size on the interaction with *c-myb* G-quadruplex.

The conventional strategy for cyclization is using full-length linear substrates, which would be $BocPy_2\beta Py_2\beta$-$CO_2C_6F_5$ for our molecule. As the length of the precursor increases, however, the time and difficulty of synthesis will both increase notably. Compared to the classical procedure, cyclodimerization via coupling of two half-length monomers raises the efficiency.

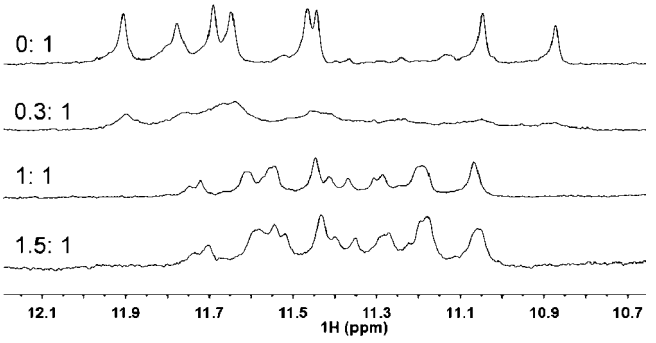

Figure 2. ^{1}H NMR spectra of the various ratios of **1** with the *c-myb* G-quadruplex (Q1) in 100 mM KCl and 20 mM Tris-HCl.

To evaluate the interaction of **cβ** (**1**) with the c-myb G-quadruplex in solution, we carried out NMR titration experiments by adding **1** to a solution of *c-myb* G-quadruplex (Q1) containing 100 mM KCl (Figure 2). In the absence of **1**, the peaks of the imino protons at δ 10.8–12.0 ppm corresponding to the 2 × 8 imino protons of the guanines confirmed the formation of a symmetrical dimeric G-quadruplex.[13] However, upon the addition of **1**, the imino resonances clearly changed, which indicated interactions between Q1 and **1**. At the low molar ratio (**1**:Q1 = 0.3:1), the imino resonances appeared as broad peaks (i.e., an intermediate complex of Q1 and **1**). When the molar ratio increased to 1:1, clearly resolved peaks suddenly appeared in the region 11.0–11.8 ppm. Furthermore, not only did distinct shifts occur in the resonances, but the amount of peaks increased from the original G-quadruplex. These changes in the NMR spectra indicated that binding of **1** disturbed the symmetry of the G-quadruplex, increasing the number of imino protons and significantly shifting their resonances.

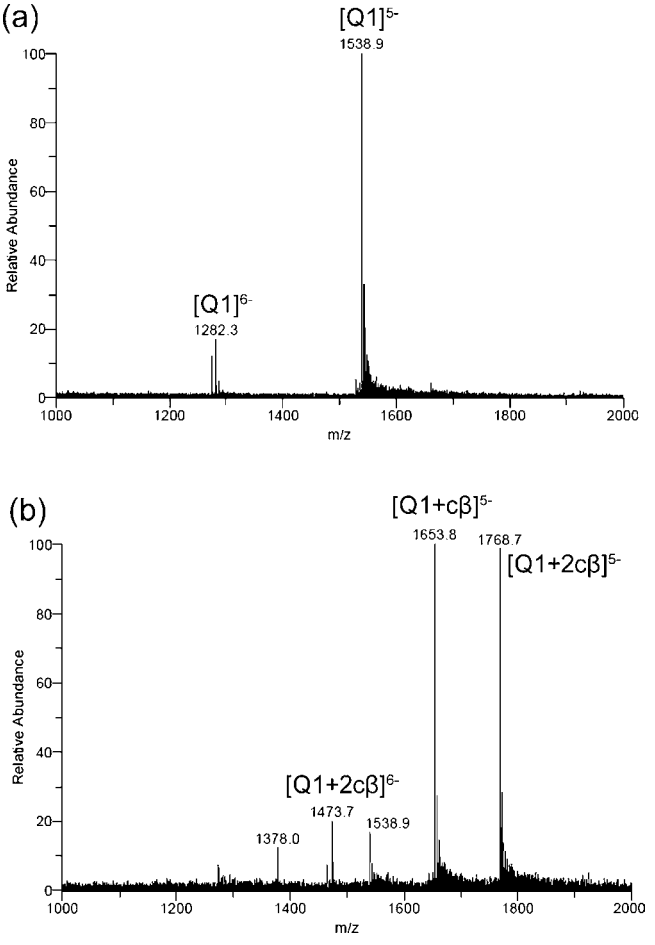

Figure 3. ESI mass spectra of the *c-myb* G-quadruplex (Q1) (a) and Q1 with **1** (b) in 100 mM NH_4OAc, 25% CH_3OH.

ESI-MS was used to probe the binding affinity, selectivity, and the stoichiometry of **cβ** (**1**) toward the G-quadruplexes. Figure 3a shows that, in the G-rich sequence (S1) solution of the *c-myb*, the base peak in the spectrum was a complex

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ion of two S1 and three ammonium ions ([2S1 + $3NH_4^+$-$8H^+$]$^{5-}$, abbreviated to [Q1]$^{5-}$) at m/z 1538.9, indicating the formation of a bimolecular G-quadruplex.[14] When 4 equiv of **1** was added to the solution, the complex ion [Q1+**cβ**]$^{5-}$ became the base peak and the intensity of the complex ion [Q1 + 2**cβ**]$^{5-}$ increased to 95%, and that of the G-quadruplex ion [Q1]$^{5-}$ decreased to 20% (Figure 3b). Thus, it was clear that **1** bound to the Q1 in the stoichiometry of one and two. To evaluate the binding affinity, the parameter IR_a was defined as the relative abundance ratio of the all bound ions to that of both unbound and bound species and the maximum value of the IR_a is 1.00 (eq S1, Supporting Information).[14] The IR_a value of **1** with Q1 is 0.90, indicating that **1** has high binding affinity. The binding affinities of **1** with the G-quadruplexes of Q2-Q5 were also evaluated by ESI-MS (Figure S2, Supporting Information), and the IR_a values are all less than 0.11, showing that **1** has very poor or even no binding affinity for Q2-Q5 (Table S2, Supporting Information). However, no binding was observed between **cγ (2)** and the *c-myb* G-quadruplex. The circular dichroism (CD) titration and Hill approach[15] were used to evaluate the binding constant (Figure S3, Supporting Information), and the result showed the binding constant (K_b) of **1** with Q1 is 7.4×10^4 M^{-1}. The T_m value from the CD melting experiment showed that the thermal stability of Q1 was enhanced obviously in the presence of **1**; in contrast, there was nearly no influence on T_m values in the cases of Q2−Q5 (Figure S4, Supporting Information). In addition, the ESI-MS results showed that **1** induced a conversion of the duplex into the G-quadruplex without binding with the duplex (Figure S5, Supporting Information). Futhermore, the electrophorasis analysis indicated that the percentage of Q1 in the mixture was 66% when the molar ratio of **1**/duplex was 18:1 (Figure S6, Supporting Information). Besides, different from distamycin, ESI-MS array revealed that **1** has very poor interaction with AT-rich duplex DNA[16] (Table S1, IR_a = 0.10, Figure S7, Supporting Information).

To further investigate the binding mode of **cβ (1)** to Q1, 10 ns MD simulation using the AMBER10 program[17] was performed after the initial binding site of **1** to Q1 was achieved by Autodock3.[18] The results show that the complex was preferentially formed by **1** inserting into the groove of Q1 through H-bonding (ΔG = −27.32 kcal/mol, Figure 4), and it was different from the binding mode of TMPyP4 toward telomeric G-quadruplex (Figure S8, Supporting Information).[19] While the larger bulk of **2** prevents itself inserting in the pocket and binding with Q1.

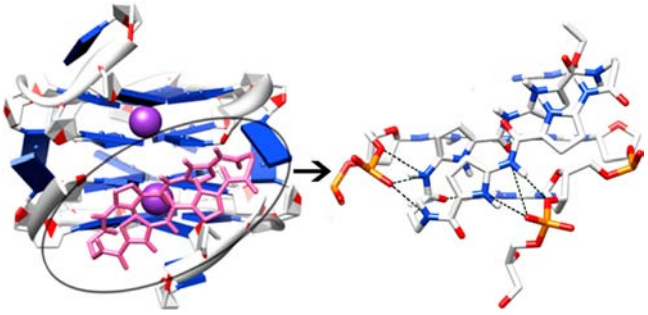

Figure 4. Average structure of the complex of **1** and Q1 by the groove binding mode. The DNA is in ribbon and box representation, and **1** is in stick representation (pink). The purple spheres are potassium ions. The dashed lines indicate the H-bonds between 1 and Q1.

In conclusion, a novel molecule **cβ** (**1**) with two 3-aminopropionyl flexible units was designed and synthesized by highly efficient cyclodimerization of the monomers with pentafluorophenyl ester-activated C termini. The binding properties of **1** to the DNA G-quadruplexes were estimated, and the results showed that **1** binds selectively to the *c-myb* G-quadruplex with high affinity contributing to its appropriate size. In addition, the analogical compound (**2**) did not display the interaction with the c-myb G-quadruplex because of its larger volume. This is the first time a novel synthetic flexible molecule was found to have high selectivity for the G-quadruplex in the *c-myb* promoter. We will investigate the bioactivity of **1** on the cancer cells in due course.

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Supporting Information Available. Experimental procedures and spectra data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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