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Tetrahedron Letters 46 (2005) 647–651
Tetrahedron Letters

Synthesis and DNA binding properties of pyrrole amino acid-containing peptides

Tushar Kanti Chakraborty,[a,*] Bajjuri Krishna Mohan,[a] Muthaiah Gnanamani[b] and Souvik Maiti[b,*]

[a]*Indian Institute of Chemical Technology, Hyderabad 500 007, India*
[b]*Institute of Genomics and Integrative Biology, Mall Road, New Delhi 110 007, India*

Received 20 October 2004; revised 16 November 2004; accepted 25 November 2004

Abstract—Dimers of the pyrrole amino acid (Paa), 5-(aminomethyl)pyrrole-2-carboxylic acid, and its derivatives having Lys anchored on *N*- and C-termini bind in the minor groove of DNA with considerable apparent binding affinities. When the Lys unit is attached to the C-terminus, the resulting ligand binds to ds-DNA with twice the affinity, of the order of 10^5, than the one carrying two positive charges at the same end.

1. Introduction

The minor groove of double helical DNA is the site of noncovalent interactions for a large number of anticancer drugs, antibiotics, and antiviral agents.[1–5] The ligand molecules that bind to this minor groove have a curved, planar aromatic core, and generally have both positively charged groups and hydrogen-bond donors on the convex edge. In recent years, a great many variations of these ligands, particularly in the distamycin–netropsin family, have been made and the structures of several such complexes have also been solved that reinforce the idea that shape and functional group complementarities of the ligand and groove are critical features for binding.[1,2] Polyamides composed of *N*-methylpyrrole (Py), *N*-methylimidazole (Im), and large varieties of analogous five-membered heteroaromatic amino acids have been designed with predictable sequence selectivity and many of these designer polyamides bind in the DNA minor groove with high affinities.[3–5] The introduction of heterocyclic rings into these ligands containing hydrogen-bond acceptors such as nitrogen as in Im or Py, was based on the realization that these atoms could act as hydrogen-bond acceptors in the minor groove and therefore oligopeptides containing such unnatural amino acids would be able to distinguish between the four Watson–Crick base pairs.[6–10] It was found that the flexibility of the ligand molecule allowed a substantial resetting of the curvature of the ligand, both with respect to the twist along the groove and also with respect to matching the bottom of the groove. Pyrrole amino acid (Paa), 5-(aminomethyl)pyrrole-2-carboxylic acid **1** is a new class of building block that has been developed recently and used in peptidomimetic studies as a structurally restricted surrogate of the Gly-ΔAla dipeptide isostere.[11–13] The flexible methylene spacer between the amino group and the pyrrole ring in **1** is expected to render in peptides derived from it the curvature that is necessary to bind in the minor groove of DNA. Herein we report the synthesis and DNA binding properties of three Paa dimer-based polyamides **2**–**4** (Fig. 1).

2. Methods and materials

2.1. Synthesis of peptides 2–4

The peptides **2**–**4** were synthesized by conventional solution phase methods using 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide hydrochloride (EDCI) and 1-hydroxybenzotriazole (HOBt) as coupling agents and dry CH_2Cl_2 and/or amine-free dry DMF as solvents.

Keywords: Pyrrole amino acid; DNA binding; Minor groove; Distamycin analogues.
* Corresponding authors. Tel.: +91 4027193154; fax: +91 4027193108; e-mail addresses: chakraborty@iict.res.in; souvik@igib.res.in

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doi:10.1016/j.tetlet.2004.11.128

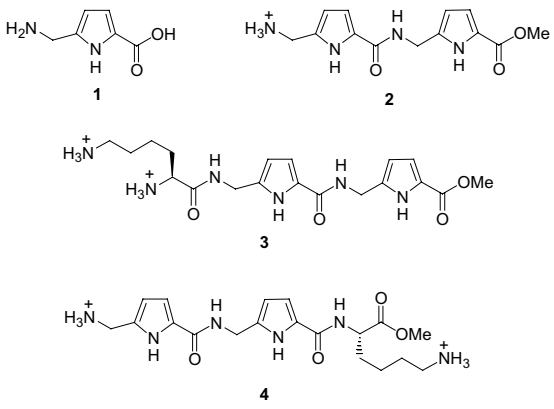

Figure 1. Pyrrole amino acid (Paa) **1** and Paa dimer based polyamides **2**–**4**.

Reaction of Boc-Paa-OH[13] with H_2N-Paa-OMe under the conditions mentioned above gave the dimer, Boc-Paa-Paa-OMe. Boc-deprotection of the protected dimer using TFA–CH_2Cl_2 (1:1) furnished compound **2**. Coupling of **2** with Boc-Lys(Boc)-OH gave the tripeptide Boc-Lys(Boc)-Paa-Paa-OMe, which was deprotected using TFA–CH_2Cl_2 (1:1) to give compound **3**. Saponification of Boc-Paa-Paa-OMe with LiOH in THF–MeOH–H_2O (3:1:1) was followed by reaction with the Lys(Boc)-OMe to give Boc-Paa-Paa-Lys(Boc)-OMe, which on Boc-deprotection afforded the polyamide **4**. The products were purified by hplc[14] and used for DNA binding studies.

2.2. UV absorption spectrophotometry

Absorbance versus temperature profiles were measured at 260 nm on an AVIV model 14DS spectrophotometer (AVIV Associates; Lakewood, NJ) equipped with a thermoelectrically controlled cell holder and a cell path-length of 1 cm. The temperature was raised in 0.5 °C increments and the samples were allowed to equilibrate for 1 min at each temperature setting. The DNA concentration was 2 mM in duplex, while the ligand concentration was 0–4 mM.

2.3. Circular dichroism (CD) spectropolarimetry

The conformation of the ligand–DNA complexes was derived by the simple inspection of their CD spectra. The CD (JASO 700, Japan) spectra were obtained at 25 °C. Typically, a solution of 50 μM CT-DNA (base pair) in 10 mM Tris buffer, pH 7.4 was titrated with the appropriate ligand solution in the same buffer, by stepwise addition of 3–5 μL aliquots of ligand solutions. The CD spectrum of each sample was then recorded from 220 to 420 nm, with an averaging time of 3 s.

2.4. Polyacrylamide gel electrophoresis (PAGE)

Nondenaturing PAGE 25% (1 bisacrylamide/29 acrylamide) was conducted at room temperature. Both the gel and the running buffer contained 100 mM trisborate (pH 8.0), 10 mM $MgCl_2$, and 2 mM Na2EDTA. For better resolution, an oligomeric deoxyribonucleic acid $d(GCATGGCCATGC)_2$ (HPLC purified) was used in this study. The ligand/DNA base pair ratio was 0.33 with 50 μM duplex concentration. Glycerol was added [5% (v/v)], and a final sample volume of 18 μL was loaded onto the gel and subjected to electrophoresis at 12 V/cm. The gels were stained using ethidium bromide, and images were captured using a BIO-RAD gel documentation system.

2.5. Ethidium bromide exclusion assay

Ethidium bromide (5 μM) and 10 μM (one ethidium bromide per base pair) DNA solution were mixed in 10 mM Tris buffer and allowed to incubate at 25 °C for 10 min. Various amounts of ligand solution were added to the DNA–ethidium bromide mixture, which was then incubated for 10 min. Fluorescence intensity was measured using a spectrofluorometer (FluoroMax-3, Spex) after diluting to 2 mL with the same buffer. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were 480 and 600 nm, respectively. The apparent binding affinity (K_{app}) for all the ligands were extracted from the change in fluorescence intensity upon addition of the ligand solutions to the pre-mixed ethidium bromide–DNA solution as described in the literature.[15,16] Briefly, ΔF ($\Delta F = F_0 - F$, where F_0 and F are the fluorescence intensity of ethidium bromide bound to CT-DNA in the absence and presence of ligand) was plotted versus molar equiv of ligand and the ΔF_{sat} was determined mathematically by solving the simultaneous equations representing the pre- and post-saturation regions of the titration curve. A Scatchard plot was generated where ΔF/[free agent] was plotted versus ΔF. The slope of the region immediately preceding complete saturation of the system provided $-K_{app}$.

3. Results and discussion

3.1. UV melting experiment

Small DNA-binding molecules such as distamycin, Hoechst, and spermine derivatives are known to stabilize duplex DNA and hence increase the double helix-to-random coil transition (melting) temperature (T_m) of DNA.[17,18] UV melting experiments were conducted in the absence and presence of ligands to assess the impact, if any, of the ligand on the thermal stabilities of the CT-DNA. The resulting melting profiles are shown in Figure 2.

It is to be noted here that as the [total ligand] to [duplex base pair] ratio (r_{bp}) increases from 0 to 0.33 (one ligand per three base pairs), the thermal stabilities of CT-DNA duplexes increase concomitantly. Ligand-to-base pair duplex ratios above 0.33 result in only marginal increases in the T_m of CT-DNA, an observation, which may reflect some nonspecific, secondary binding to the target duplexes at high polyamide concentrations. The ΔT_m (T_m in presence of ligand $- T_m$ in absence of ligand) values are 1, 3, and 6 °C for ligands **2**, **3**, and **4**, respectively. These ligand-induced changes in DNA thermal stability are consistent with ligand binding to

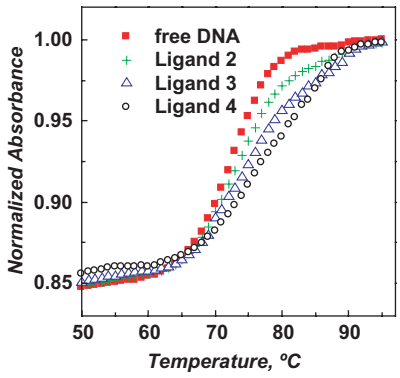

Figure 2. UV melting curves of CT-DNA in absence of ligand (■) and in the presence of **2** (+), **3** (△), and **4** (○), respectively.

the DNA. A higher ΔT_m was observed in the case of ligand **4** indicating that this ligand binds to DNA with higher affinity than the other two ligands.

3.2. CD spectroscopy study

Circular dichroism spectroscopy has been used to detect possible conformational changes of DNA upon binding with ligands. Figure 3 shows the representative CD spectra of the free CT-DNA and ligand bound complexes at several r_{bp}. The CD spectrum of the free CT-DNA is typical of a duplex in the B conformation, and addition of ligand solutions results in a decreased positive peak at 283 nm for all the ligand solutions. A distinct isoelliptic point at 259 nm was observed in each CD titration, which clearly indicates that all the ligand molecules are binding to the duplex DNA.

3.3. Binding affinities

The resulting binding affinities from the analysis of fluorescence titration experiments are 2×10^4, 6×10^4, and 12×10^4 M^{-1} for **2**, **3**, and **4**, respectively (Fig. 4). The low binding affinity for ligand **2** indicates that the interaction is nonspecific in nature. However, the introduction of extra charge in the side chain resulted in much higher binding affinities for compounds **3** and **4**. The most interesting outcome is that ligand **4** with a positive charge situated at both ends of the polyamide binds to the duplex DNA with twice the affinity of **3** with two positive charges at the same end.

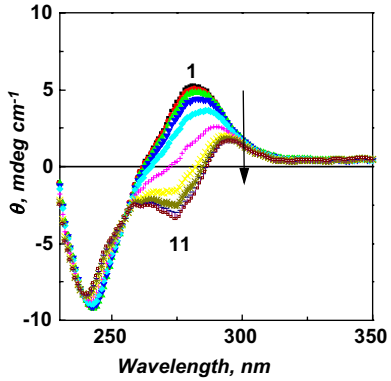

Figure 3. Representative CD spectra of the complexes of **3** with CT-DNA; trace '1' corresponds to the CD spectrum of free CT-DNA and traces '2'–'11' correspond to the CD spectrum of CT-DNA after successive addition of the ligand. Experiments were performed in 10 mM Tris·HCl buffer (pH 7.4) and each addition of **3** caused an increase in the [ligand]/[base pair] by 0.032.

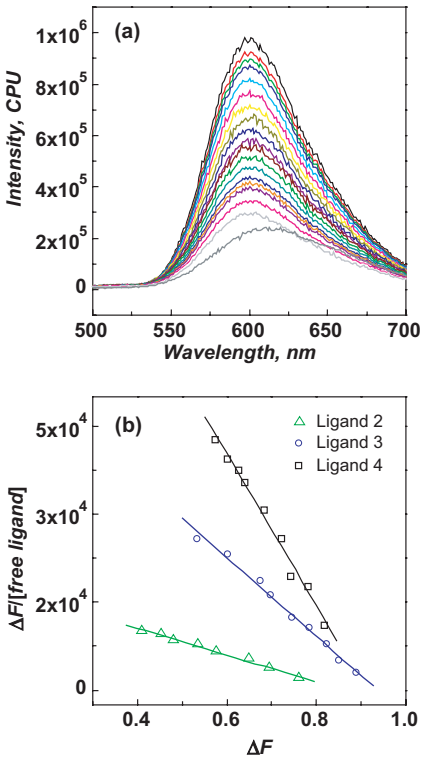

Figure 4. (a) Representative fluorescence displacement titration curve of **3**. Ethidium bromide (5 μM) and 10 μM base pair of CT-DNA were mixed together and the resulting solution was titrated with the ligand solution. (b) Scatchard plot for K_{app} (−slope) determination.

3.4. Gel electrophoretic mobility assay

To assess changes in duplex DNA conformation with ligand binding, electrophoretic mobilities were assessed for duplex oligonucleotides (see Materials and methods) in the presence and absence of these three ligands. These results are illustrated in Figure 5, which reveals that the addition of ligand molecules to the duplex retards the migration of the duplex (lanes 2–4) implying that all three ligands bind to the DNA duplex.

The binding studies of three newly synthesized polyamides using a new monomer pyrrole amino acid (Paa), 5-(aminomethyl)pyrrole-2-carboxylic acid with duplex DNA was followed by UV melting, CD spectroscopy, an ethidium bromide exclusion experiment followed by fluorescence spectroscopy along with a gel electrophoretic mobility assay. Studies showed that all the ligands are able to bind duplex DNA. In each of these ligands, the number of building blocks is only two and yet they showed considerable binding affinities. It was found earlier that the bis-pyrrole peptide did not exhibit any

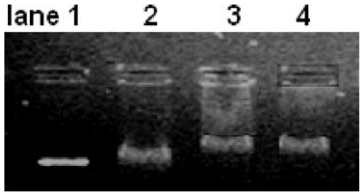

Figure 5. Electrophoretic mobility shift assay. Nondenaturing 25% polyacrylamide gels were run in 0.75-1X TBE at 12 V/cm at 25 °C. Electrophoretic mobility of d(GCATGGCCATGC) (lane 1) and reduced electrophoretic mobility of DNA–ligand complexes ($r_{bp} = 0.5$), lanes 2, 3, and 4 are for ligands **2**, **3**, and **4**, respectively.

detectable binding with duplex DNA.[19] From comparative binding studies on bis-, tri-, tetra-, and penta-pyrrole peptides of the distamycin analogues with DNA it was concluded that the minimum number of pyrrole carboxamide units for the onset of DNA binding, in the absence of the N-terminus amide is three. In our study, we have observed that ligand **2** is able to bind to ds-DNA with a considerable apparent binding affinity ($K_{app} = 2 \times 10^4\ M^{-1}$) in spite of the fact that this ligand contains only two pyrrole-based building blocks. The improvement in the binding affinity may be due to the presence of the methylene spacer between the amino group and the pyrrole ring in the ligand, which makes the ligand more flexible to fit in the minor groove of the duplex DNA. Ligand **3** binds to duplex DNA with three times higher affinity ($K_{app} = 6 \times 10^4\ M^{-1}$) implying that the extra charge on the ligand improves the binding ability of ligand. Ligand **4** with a positive charge placed at both ends of the polyamide binds to duplex DNA with twice the affinity of **3** with two positive charges at the same end. This result clearly indicates that the spatial distribution of the charge plays a crucial role in the binding event of the ligand. In ligand **4** as the two charges are located at either end of the polyamide, these charges can neutralize the negative charges of the DNA phosphate backbone more efficiently giving increased electrostatic interactions.

In summary we have described the synthesis of polyamides using the monomeric building block pyrrole amino acid (Paa), 5-(aminomethyl)pyrrole-2-carboxylic acid. The flexible methylene spacer between the amino group and the pyrrole ring in this monomer was expected to render the curvature in polyamides derived from it that is necessary to bind in the minor groove of DNA. DNA binding studies revealed that polyamides with two positive charges bind with affinities of the order of 10^5. We also observed that the polyamide with a positive charge placed at either end of the polyamide binds to the DNA duplex with twice the affinity compared to that with two positive charges at the same end. These results will help in the more rational design of new minor grove binding agents.

Acknowledgements

The authors wish to thank Dr. J. S. Yadav and Dr. Samir K. Brahmachari for their support and encouragement, DST, New Delhi for financial support (T.K.C.) and CSIR, New Delhi for a research fellowship (B.K.M.).

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14. Selected physical data of Boc-protected **2**, Boc-Paa-Paa-OMe: IR (KBr): ν_{max} 3298, 2963, 1679, 1521, 1319, 1226, 1166, 1035, 804, 757 cm^{-1}; ^1H NMR (DMSO-d_6 400 MHz, atom numbering of Paa starts from the C-terminus): δ 1.39 (s, 9H, Boc), 3.73 (s, 3H, $-CO_2CH_3$), 4.07 (d, $J = 6$ Hz, 2H, $-CH_2-$NHBoc), 4.38 (d, $J = 5.6$ Hz, 2H, $-CO_2-NH-CH_2$), 5.89 (t, $J = 3$ Hz, 1H, Paa(1)C4H), 6.00 (t, $J = 3.6$ Hz, 1H, Paa(2)C4H), 6.60–6.70 (m, 2H, Paa(1)C3H and Paa(2)C3H), 7.13 (t, $J = 5.8$ Hz, 1H, BocN*H*), 8.33 (t, $J = 5.8$ Hz, 1H, $-CO_2-NH$), 11.12 (br s, 1H, pyrroleN*H*), 11.67 (br s, 1H, pyrroleN*H*); MS (LSIMS): *m/z* (%): 376 (10) $[M]^+$, (%) 400 (5) $[M+Na+H]^+$. Selected physical data of di-Boc-protected **3**, Boc-Lys(Boc)-Paa-Paa-OMe: IR (KBr): ν_{max} 3349, 2930, 1690, 1625, 1537, 1116 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz): δ 1.37 (s, 9H, Boc), 1.43 (s, 9H, Boc), 1.68–1.89 (m, 6H, $-(CH_2)_3-CH_2-$NHBoc), 2.94 (m, 2H, $(CH_2)_3-CH_2-$NHBoc), 3.83 (s, 3H, $-CO_2CH_3$), 4.09 (m, 1H, Paa(1)C6H), 4.30 (dd, $J = 3.2$, 14.6 Hz, 1H, Paa(1)C6H′), 4.46 (dd, $J = 4$, 15 Hz, 1H, Paa(2)C6H), 4.57 (dd, $J = 5$, 15 Hz, 1H, Paa(2)C6H′), 4.70 (t, $J = 5.9$ Hz, 1H, BocN*H*), 4.72 (m, 1H, LysCαH), 5.74 (d, $J = 8.1$ Hz, 1H, BocN*H*), 6.04 (dd, $J = 2.8$, 3.5 Hz, 1H, Paa(2)C4H), 6.12 (dd, $J = 2.6$, 3.5 Hz, 1H, Paa(1)C4H), 6.48 (dd, $J = 2.8$, 3.4 Hz, 1H, Paa(1)C3H), 6.56 (t, $J = 5.4$ Hz, 1H, $-CO_2-NH$), 6.81 (dd, $J = 2.5$, 3.7 Hz, 1H, Paa(2)C3H), 7.18 (br s, 1H, $-CO_2-NH$), 10.67–10.72 (br s, 2H, pyrroleN*H*); MS (LSIMS): *m/z* (%): 606 (15) $[M+2]^+$, 406 (50) $[M+2-2Boc]^+$. Selected physical data of di-Boc-protected **4**, Boc-Paa-Paa-Lys(Boc)-OMe: IR (KBr): ν_{max} 3368, 2363, 1687, 1647, 1521, 1161 cm^{-1}; ^1H NMR (DMSO-d_6 200 MHz): δ 1.38 (s, 9H, Boc), 1.43 (s, 9H, Boc), 1.43–1.68 (m, 6H, $(CH_2)_3-CH_2-$NHBoc), 3.00 (m, 2H, $(CH_2)_3-$

CH_2–NHBoc), 3.75 (s, 3H, $-CO_2CH_3$), 4.26 (m, 2H, PaaC6H), 4.60 (m, 2H, Paa C6H), 4.75 (m, 1H, LysCαH), 5.49 (t, J = 5.9 Hz, 1H, BocNH), 6.00 (m, 2H, PaaC4H), 6.36 (m, 1H, BocNH), 6.69 (m, 2H, PaaC3H), 7.51 (t, J = 5.6 Hz, 1H,$-CO_2$–NH), 7.92 (br s, 1H, $-CO_2$–NH), 10.87–10.91 (br s, 2H, pyrroleNH); MS (LSIMS): m/z (%): 604 (15) $[M]^+$, 405 (12) $[M+H-2Boc]^+$.

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