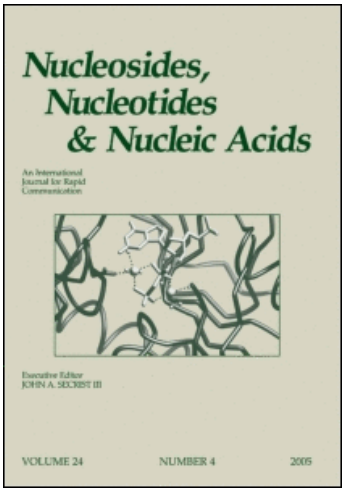

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Oligonucleotide—Minor Groove Binder 1:2 Conjugates: Side by Side Parallel Minor Groove Binder Motif in Stabilization of DNA Duplex

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ABSTRACT

Synthetic polycarboxamides consisting of N-methylpyrrole (Py), N-methylimidazole (Im), N-methyl-3-hydroxypyrrole (Hp) and β-alanine (β) show strong and sequence-specific interaction with the DNA minor groove when they form hairpin structures with side-by-side antiparallel motifs. In the present paper, new conjugates containing two ligands linked to the same terminal phosphate of DNA strand were constructed. The paper describes optimized synthesis and properties of oligonucleotide-linked polyamide strands that insert into the minor groove of a duplex in a parallel or antiparallel orientation. Strong stabilization of DNA duplexes by two attached minor groove ligands is demonstrated by the thermal denaturation method. The unmodified duplex 5′-CGTTTATTp-3′/5′-AATAAACG-3′ melts at 20°C. When one tetra(Py) residue was attached to the first strand of this duplex, denaturation temperature was

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increased to 46°C; attachment of the second tetra(Py) in a parallel orientation resulted in denaturation temperature of 60°C. It is even higher than in case of ''classic'' octapyrrole hairpin ligand (Tm = 58°C). Sequence-specific character of stabilization by two conjugated ligands was demonstrated for G:C-containing oligonucleotides attached to tetracarboxamide and octacarboxamide ligands constructed from Py, Im and β units according to established recognition rules (ΔTm = 20°C). The two-strand parallel minor groove binder constructions attached to addressing oligonucleotides could be considered as site-specific ligands recognizing single- and double-stranded DNA similarly to already described hairpin MGB structures with antiparallel orientation of carboxamide units.

Key Words: Oligonucleotides; Minor groove binders; Conjugates; Duplex stability; Thermal denaturation; Sequence specificity.

INTRODUCTION

Non-protein synthetic compounds capable of site-specific double-stranded DNA binding may find numerous research and practical applications in molecular biology, genetic engineering and medicine. Except specific peptides, only two classes of synthetic double-stranded DNA sequence-specific binders are known: polycarboxamide minor groove binders (MGB) and synthetic oligonucleotides. Together with triplex-forming oligonucleotides that recognize the double-stranded DNA major groove, minor groove binders are opening new ways for sequence-specific control of gene expression. Netropsin and distamycin, binding to A:T-tract of B-form DNA, represent the natural members of the latter class of compounds.

Based on NMR demonstration that 2:1 complexes could form in the minor groove with distamycin and netropsin[1,2] (see for review Ref. [3]), the group of P. Dervan designed the hairpin polyamides capable of sequence-specific binding to dsDNA.[4,5] These polyamides contain N-methylpyrrole carboxamide (Py), N-methyl-3-hydroxy-pyrrole carboxamide (Hp) and N-methylimidazole carboxamide (Im) units as monomers for ligand construction (Fig. 1).

A code for the binding of hairpin polyamides has been proposed where Im/Py, Py/Im, Py/Hp and Hp/Py pairs recognized CG, GC, AT and TA base pairs, respectively.[6–9] Replacement of certain units by a more flexible β-alanine residue allowed for increase the affinity and sequence selectivity of specific polycarboxamides[10,11] (Fig. 1).

The covalent conjugates of oligonucleotides and MGB appeared to be the most promising.[12–14] In case of duplex formation, both the oligonucleotide and the ligand components of the conjugate provide the specificity of binding to single-stranded DNA. Moreover, stronger MGB binding to double-stranded DNA was observed when they were conjugated to DNA strands.[15–20] In case of triple helix formation, the conjugates should specifically and strongly bind to both DNA grooves.[12,14] This could potentially give rise to effective drugs targeting the genomic DNA and explains our interest to studies of interaction between double stranded DNA and conjugated MGB.

Since the data on MGB antiparallel arrangement in the complexes with DNA underlay the guidelines for designing hairpin structures, little is known about the other variants of MGB binding. The chance of realization of parallel ligand arrangement was considered by the example of bis-netropsin and bis-distamycin derivatives of

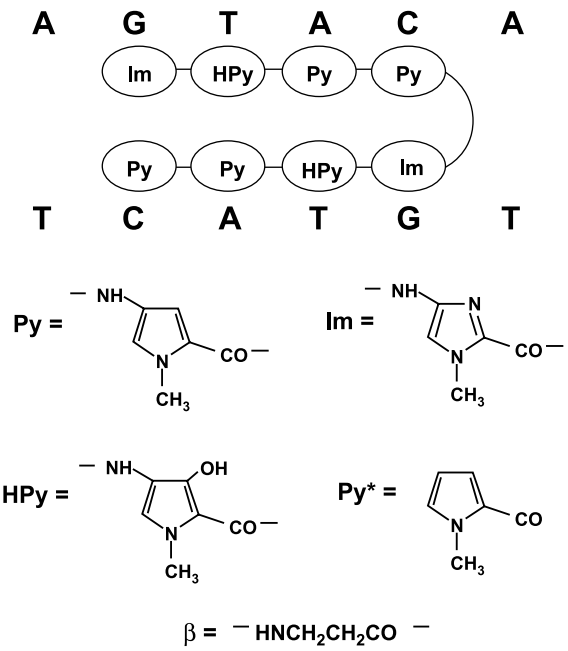

Figure 1. Sequence-specific code for recognition of DNA base pairs by N-methylpyrrole/N-methylimidazole carboxamides according to Ref. [9] and formulas of monomer units of carboxamide minor groove binders.

platinum.[21–23] Recently Boger at al. demonstrated that head-to-head linked oligopyrrole ligands can adopt parallel side-by side binding with the target DNA duplexes.[24] However, the characteristics of binding and stability of the DNA–MGB complexes, where parallel head-to-head MGB strands are addressed to target sequences by conjugated oligonucleotides, were never described.

First, we optimized Mukaiyama protocol[25] for synthesis of bis-phosporamidates that contain two identical or different ligands attached to the same terminal phosphate. Then we demonstrated that oligo(Py)-oligonucleotide 2:1 conjugates form considerably more stable hybrids with complementary oligonucleotides compared with mono-phosphoramidates.[26,27] We carried out detailed comparative studies of the stability of duplexes formed by 2:1 conjugates both in parallel and antiparallel orientation. Model A:T- and G:C—containing oligonucleotides were used in order to demonstrate sequence specificity of DNA duplex stabilization.

MATERIALS AND METHODS

Reagents

All reagents and solvents were purchased from Sigma–Aldrich–Fluka and SDS. The model oligonucleotides were synthesized by the Oligonucleotide Synthesis Group in Novosibirsk Institute of Bioorganic Chemistry and Eurogentec.

MGB Synthesis

Synthesis, purification, NMR and mass-spectrometry analysis of amino-functionalized minor groove binders was carried out as it was described previously.[17,18,26–28] All the products were then additionally purified by precipitation in 20% ammonia solution following by HPLC on Waters X-Terra 7 μm C-18 column, 7.8 × 300 mm, in a linear 0–80% gradient of water/acetonitrile with 0.1% trifluoroacetic acid, flow rate 2 ml/min.

Synthesis of Oligonucleotide—MGB Conjugates

For covalent coupling of one MGB molecule to the oligonucleotide we used terminal phosphate activation as described in previous papers.[27,29,30] The phosphate activation step was done in organic solvent (DMSO) and the attachment of the ligand in water-DMSO mixture.

For the synthesis of 2:1 conjugates with two MGB residues attached to the same terminal phosphate group, the 1:1 conjugate was precipitated by CTAB, dried, dissolved in DMSO and its phosphoramidate group activated again with Mukaiyama reagents as described previously.[29] An excess of the second ligand (2–3 mg in DMSO) was added directly to the reaction mixture that was incubated for at least 2 hours (preferably overnight) at room temperature.

Purification and Analysis of the Conjugates

The products were purified by HPLC on a C-18 X-Terra column (Waters, 7 μm, 300 × 7.8 mm) in a 5–40% acetonitrile linear gradient in 0.02 M ammonium acetate using the 1100 chromatography system from Agilent Technologies. The retention times were 6–8 min for the starting oligonucleotide, 13–17 min for the 1:1 conjugates and 15–22 min for 2:1 conjugates.

The electrophoresis analysis was carried out on a standard 20% denaturing polyacrylamide gel. The absorption spectra of the products were measured on a Kontron 923 Uvikon instrument (Bio Tek). Oligonucleotide conjugates were also analyzed by NMR in D_2O at 50°C and electrospray Q-TOF mass-spectrometry on Q-Star instrument from Applied Biosystems. For ^{1}H-NMR the chemical shifts are given in ∂-scale. For ^{31}P-NMR 85% H3PO4 was used as external standard.

CGTTTATTp -NHC^{1}H$_2$C^{2}H$_2$C^{3}H$_2$C^{4}H$_2$C^{5}H$_2$CO(Py)$_4$NHC^{6}H$_2$C^{7}H$_2$C^{8}H$_2$N-(C^{9}H$_2$C^{10}H$_3$)$_2$.

^{1}H NMR (D_2O): 5.97 (H5 Cyt), 7.59 (H6 Cyt), 8.08 (H2 Ado), 8.25 (H8 Ado), 7.95 (H8 Gua), 7.36–7.56 (H6 5Thy), 1.69–1.81 ($CH_3$5Thy), 2.81 (CH_2-1), 1.50 (CH_2-2), 1.38 (CH_2-3), 1.66 (CH_2-4), 2.34 (CH_2-5), 6.68–6.80 (H_3 4Py), 7.11–7.16 (H5 4Py), 3.77–3.80 (CH_3 4Py), 3.38 (CH_2-6), 2.00 (CH_2-7), 3.17 (CH_2-8), 3.21 (CH_2-9), 1.29 (CH_3-10).

^{31}P NMR: 9.25 (P–N), − 0.3— − 0.6 (O–P–O), J(H3′-P) = 7.0, J(CH2-1-P) = 9.0.

CGTTTATTp -[NHC^{1}H$_2$C^{2}H$_2$C^{3}H$_2$4H$_2$C^{5}H$_2$CO(Py)$_4$NHC^{6}H$_2$C^{7}H$_2$C°H$_2$N-(C^{9}H$_2$C^{10}H$_3$)$_2$]$_2$.

^{1}H NMR (D_2O): 5.97 (H5 Cyt), 7.59 (H6 Cyt), 8.06 (H2 Ado), 8.25 (H8 Ado), 7.90 (H8 Gua), 7.39–7.56 (H6 5Thy), 1.69–1.77 ($CH_3$5Thy), 2.87; 2.87 (CH_2-1), 1.46; 1.47 (CH_2-2), 1.33; 1.34 (CH_2-3), 1.58; 1.59 (CH_2-4), 2.24; 2.25 (CH_2-5), 6.59–6.73 (H3 4Py), 7.02–7.10 (H5 4Py), 3.72–3.74 ($CH_3$4Py), 3.30; 3.32 (CH_2-6), 1.95, 1.97 (CH_2-7), 3.10, 3.13 (CH_2-8), 3.19, 3.20 (CH_2-9), 1.28; 1.29 (CH_3-10).

^{31}P NMR: 18.85 (P–N), − 0.3– − 0.6 (O–P–O), J(H3′-P) = 7.0, J(CH_2-1-P) = 9.0.

Mass-spectrometry: CGTTTATTp-NH(CH_2)$_5$CO(Py)$_4$NH(CH_2)$_3$NEt$_2$: 3183.66 (theor. 3184.45); CGTTTATTp-[NH(CH_2)$_5$CO(Py)$_4$NH(CH_2)$_3$NEt$_2$]$_2$: 3897.76 (theor. 3898.32); CGTTTATTp-NH(CH_2)$_3$CO(Py)$_4$OEt: 3071.32 (theor. 3072.27); CGTTTATTp-[NH(CH_2)$_3$CO(Py)$_4$OEt]$_2$: 3671.29 (theor. 3673.94); TTGCGCp-NH(CH_2)$_3$COImβImPy-NH(CH_2)$_3$COImβImPyNH(CH_2)$_3$NMe$_2$: 2994.74 (theor. 2995.73); TTGCGCp[-NH(CH_2)$_3$COImβImPyNH(CH_2)$_3$NMe2][-NH(CH_2)$_3$CO-PyImβImNH(CH_2)$_3$NH-Boc]: 3150.44 (theor. 3151.52); TTGCGCp[-NH(CH_2)$_3$CO-PyImβImNH(CH_2)$_3$NMe$_2$][-NH(CH_2)$_3$COImβImPyNH(CH_2)$_3$NMe$_2$]: 3072.88 (theor. 3079.16).

Circular dichroism spectra of the duplexes were recorded on the Jasco spectropolarimeter 600 in 10 mM Na-phosphate buffer, pH 7.3, 0.1 M NaCl, 0.1 mM EDTA.

Thermal Denaturation Assay

The thermal denaturation and renaturation studies of duplexes were carried out on a Kontron Uvikon 940 spectrophotometer (BioTek) with thermostated cuvettes of 1 cm optical pathlength. The concentrations of the duplex strands in each sample were 1.3–1.7 μM, in 10 mM phosphate buffer pH 7.3, 0.1 M NaCl, 0.1 mM EDTA. The sample temperature was changed at 0.2°C/min and the absorption was recorded every 200 s. The treatment of the melting curves was carried out using KaleidaGraph and Microsoft Excel softwares.

In certain experiments the melting curves of the duplexes were recorded in a specially constructed thermostated microcuvette of the liquid microchromatography system ''Millichrom'' (Novosibirsk Institute of Bioorganic Chemistry, Russia) according to the method described in a previous report.[16] Use of the microcuvette allowed us to vary the duplex concentrations in a wider range. Similar data were obtained when the two methods were compared.

RESULTS AND DISCUSSION

Model Duplexes

All the oligonucleotide sequences are written in 5′– >3′ direction.

Two oligonucleotides with 3′-terminal phosphate group were used for attachment of minor groove binders: (A) CGTTTATTp for attachment of oligo(Py) and (B) TTGCGCp for attachment of G:C-specific ligands constructed according to DNA recognition rules.[9] The oligonucleotide A forms a complementary duplex with AATAAACG that melts at 20°C, and B forms a duplex with GCGCAA that melts at 28°C.

The list of conjugates and their duplexes with complementary sequences is shown in Table 1.

Table 1. Melting points of DNA duplexes containing one strand conjugated to one or two oligocarboxamide moieties.

Expt n°	Duplex[a]	X_1	X_2	Tm,°C
1	A	$\sim O^-$	$\sim O^-$	20
2	A	$\sim O^-$	$\sim$ NH $(CH_2)_3$CO $(Py)_4$ NH $(CH_2)_3NEt_2$	46
3	A	$\sim$ NH $(CH_2)_5$CO (Py) $_4$NH $(CH_2)_3NEt_2$	$\sim$ NH $(CH_2)_3$CO $(Py)_4$ NH $(CH_2)_3NEt_2$	60
4	A	$\sim O^-$	$\sim$ NH $(CH_2)_3$CO $(Py)_4{}^-$ $-$NH $(CH_2)_3$CO $(Py)_4$ NH $(CH_2)_3NEt_2$	58
5	A	$\sim O^-$	$\sim$ NH $(CH_2)_3$CO (Py) $_4$OEt	35
6	A	$\sim$ NH $(CH_2)_3$CO (Py) $_4$OEt	$\sim$ NH $(CH_2)_3$CO (Py) $_4$OEt	60
7	A	$\sim O^-$	$\sim$ NH $(CH_2)_3$CO (Py) $_4-$ $-$NH $(CH_2)_3$CO (Py) $_4$OEt	49
8	A	$\sim$ NH $(CH_2)_3$CO (Py) $_4$OEt	*Py $(Py)_3$NH $(CH_2)_3$NH $\sim$	56
9	A	$\sim O^-$	$\sim$ NH $(CH_2)_3$CO $(Im)_4$ NH $(CH_2)_3NEt_2$	23
10	A	$\sim$ NH $(CH_2)_3$CO (Im) $_4$NH $(CH_2)_3NEt_2$	$\sim$ NH $(CH_2)_3$CO $(Im)_4$ NH $(CH_2)_3NEt_2$	24

11	A	~ NH $(CH_2)_3CO$ (Py) $_4$NH $(CH_2)_3NEt_2$	~ NH $(CH_2)_3CO$ (Im)[4] NH $(CH_2)_3NEt_2$	30
12	B	~ O^-	~ O^-	28
13	B	~ O^-	~ NH $(CH_2)_3$COPyImPyIm– –NH $(CH_2)_3$COPyImPyImNH $(CH_2)_3NMe_2$	28
14	B	~ NH $(CH_2)_3$COImPyImPyNH $(CH_2)_3NMe_2$	BocNH $(CH_2)_3$COImPyImPyNH $(CH_2)_3$NH ~	30
15	B	~ NH $(CH_2)_3$COImPyImPyNH $(CH_2)_3NMe_2$	~ NH $(CH_2)_3$COPyImPyImNH $(CH_2)_3NMe_2$	26
16	B	~ O^-	~ NH $(CH_2)_3$COImβImPy ~ –NH $(CH_2)_3$COImβImPyNH $(CH_2)_3NMe_2$	56
17	B	~ NH $(CH_2)_3$COImβImPyNH $(CH_2)_3NMe_2$	BocNH $(CH_2)_3$COImβImPyNH $(CH_2)_3$NH ~	50
18	B	~ NH $(CH_2)_3$COImβImPyNH $(CH_2)_3NMe_2$	~ NH $(CH_2)_3$COPyImβImNH $(CH_2)_3NMe_2$	48

A: 5′—CGTTTATT-pX_1X_2, 3′—GCAAATAA.
B: 5′—TTGCGC-pX_1X_2, 3′—AACGCG.
Me = $-CH_3$; Et = $-C_2H_5$.

Mono- and bis-Conjugates of Oligonucleotides

Interaction of 3′- or 5′-phosphorylated oligonucleotides with an amino derivative in the presence of triphenylphosphine, dimethylaminopyridine, and dipyridyl disulfide[29] is a widespread method for synthesizing oligonucleotide conjugates. In recent papers[14,27,31] it was demonstrated that in excess of ligand in dry aprotic polar solvents, such as dimethylsulfoxide (DMSO) or dimethylformamide (DMF), the second activation of the phosphoroamidate group could proceed resulting in the attachment of a second ligand molecule. Mono- and bis-phosporoamidate conjugates can be then separated either by reverse phase chromatography or by electrophoresis.

Studies of the reaction kinetics showed that on the first steps of the reaction the mono-phosphoramidate appears, but with time, bis-phosphoroamidate accumulates in the reaction mixture. If the removal of the activating agents is done immediately after phosphate activation and before addition of the amino group of the ligand, only mono-phosphoramidate forms. For example, when activated oligonucleotide was quickly precipitated by LiClO4 from the reaction mixture and then immediately dissolved in aqueous/DMSO solution of the ligand, no bis-phosphoroamidate was detected. It means that the two ligands are attached to the terminal phosphate group in two consecutive reactions.

This mechanism allowed for attachment of two equal or different ligands by a successful conversion of the mono-phosphoramidate into a 1:2 conjugate in two separate activation reactions. This is important for a directed design of site-specific compounds, allowing structurally different ligands to be attached to the same phosphate group of an oligonucleotide.

Figure 2 shows electrophoresis analysis of products obtained after first (lane 2) and second (lane 3) activation step in the reaction of oligonucleotide A with tetra(Py) carboxamide $NH_2(CH_2)_5CO$-(PyPyPyPy)-$NH(CH_2)_3NH(CH_2CH_3)_2$.

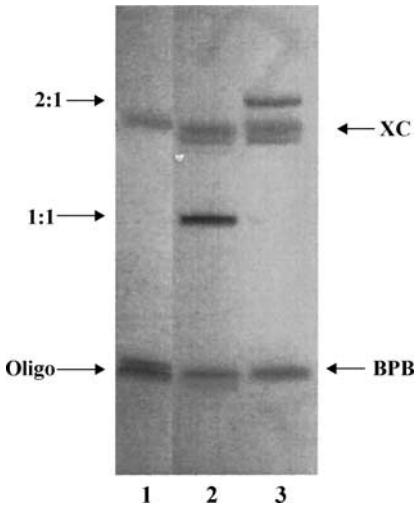

Figure 2. Electrophoresis of the products in 20% denaturing polyacrylamide gel. Positions of bromophenol blue (BPB), xylene cyanol (XC), initial oligonucleotide (Oligo) and 1:1 and 1:2 conjugates are indicated by arrows.

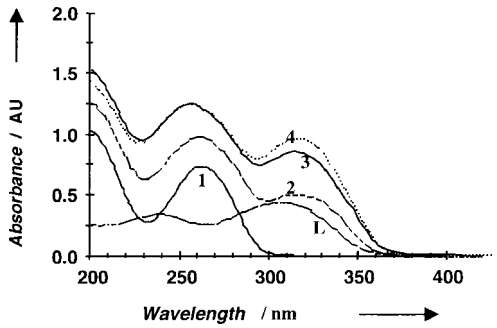

Figure 3. Electronic spectra of oligonucleotide CGTTTATTp (1), tetrapyrrole ligand $H_2N(CH_2)_5CONH(Py)_4(CH_2)_3N(C_2H_5)_2$ (L) and their conjugates: (2)—1:1 conjugate, (3)—1:2 conjugate. (4)—oligonucleotide 1 conjugated to hairpin octapyrrole ligand $NH_2(CH_2)_5CO$-$(Py)_4NH(CH_2)_3CO(Py)_4NH(CH_2)_3N(C_2H_5)_2$. Spectra were recorded in water solution, concentrations of the products were 10^{-5} M.

Electronic spectra (Fig. 3) also indicate the attachment of one and two MGB to the oligonucleotide. The electronic spectrum of the 1:1 conjugate is a superposition of the oligonucleotide and ligand spectra; the spectrum of the 1:2 conjugate is a sum of the spectra of oligonucleotide and two ligands.

The high resolution ^{1}H-NMR spectra[27,28] (see Materials and Methods) and calculation of peak integrals also confirmed these structures and showed the presence of one tertapyrrole carboxamide residue in the conjugate 2 (all the conjugate numbers are given according to Table 1) and two residues in the conjugate 3.

According to ^{31}P NMR (161.95 MHz), a chemical shift of the phosphorus in the phosphoramidate group of mono-conjugate 2 amounts to 9.25 ppm (-0.3 to -0.6 ppm for the internucleotidic phosphorus atoms) relatively to H_3PO_4, which corresponds to relevant literature data.[32] However, the ^{31}P chemical shift of bis-conjugate 3 for the phosphorodiamidate group is different (18.85 ppm).

Complex Formation of A:T-Rich Oligonucleotide—MGB Conjugates with Complementary Sequences

Circular Dichroism Studies

An intense signal in the absorption region of achiral residues of tertapyrrole carboxamide ligands with a maximum at 330 nm was observed in the circular dichroism (CD) spectra of the complexes formed by conjugates (both 1:1 and 1:2) and the complementary oligonucleotide AATAAACG (Fig. 4). A high intensity of induced CD signal suggests an interaction between the pyrrole carboxamide residues within the conjugates and the minor groove of the formed DNA duplex. A parallel arrangement of the two tertapyrrole carboxamide residues displays lower induced CD molar extinction (71 $M^{-1}cm^{-1}$) compared to their antiparallel orientation (169 $M^{-1}cm^{-1}$). This is likely the result of various excitonic interactions during tight contacts of chromophores packed in the minor groove. It is in accord with the data obtained while

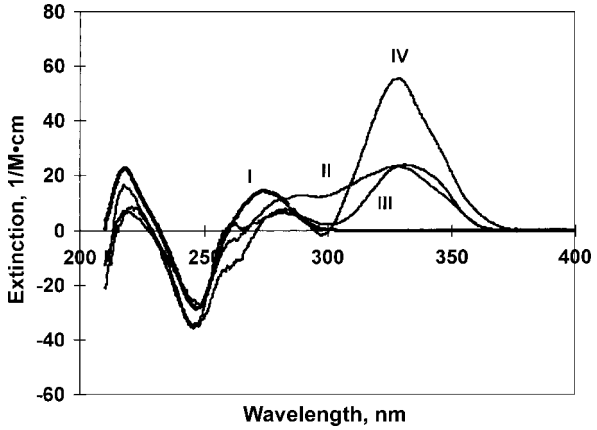

Figure 4. Circular dichroism spectra of duplexes formed by oligonucleotide AATAAACG with CGTTTATTp (1) and its conjugates to tetrapyrrole $H_2N(CH_2)_5CONH(Py)_4(CH_2)_3N(C_2H_5)_2$ (2, 3) and hairpin octapyrrole $NH_2(CH_2)_5CO(Py)_4NH(CH_2)_3CO(Py)_4NH(CH_2)_3N(C_2H_5)_2$ (4) ligands. (2, 4)—1:1 conjugates, (3)—1:2 parallel conjugate (see Table 1). Spectra were recorded in 10 mM phosphate buffer, pH 7.3, 0.1 M NaCl, 0.1 mM EDTA at 15°C, concentrations of the components were 3×10^{-5} M.

studying the binding of free bis-netropsin ligands in the minor groove of double-stranded DNA.[21–23]

Thermal Denaturation Studies

The effect of conjugated ligands on the stability of DNA duplexes was determined according to the data of thermal denaturation of the duplexes formed by the conjugate under study and the complementary oligonucleotide. As it has been already shown, covalently linked minor groove binders are much more efficient stabilizers of DNA duplexes compared to free ones.[16,17] As an example, the melting curves of the duplexes formed by the conjugate CGTTTATT-O-P(O)X_1X_2 and oligonucleotide AATAAACG are shown in Fig. 5A.

Melting curves were recorded at wavelengths close to the absorption maxima of the oligonucleotide and the ligand components of the conjugate (260–270 and 330 nm, respectively, Fig. 5). The initial duplex [1] melts at 20°C; attachment of one ligand to the 3′-phosphate of this strand increases the melting temperature to 46°C (duplex 2), while the melting temperature in the presence of two attached ligands amounts to 60°C (duplex 3). For comparison, we took the melting curve of the duplex stabilized with a classic hairpin ligand (duplex 4). In this case, Tm amounts to 58°C, being even slightly lower that the melting temperature for the complex formed by duplex [3].

The melting curves of the duplexes stabilized with MGB taken at 330 nm display a negative slope (see, for example, Fig. 5A). This effect was observed earlier[33] and reveals MGB binding in the minor groove of an oligonucleotide duplex. The melting curves taken at 260 and 330 nm are determined by different changes occurring in the electronic spectra with increase in the temperature. At 260 nm, the extinction is

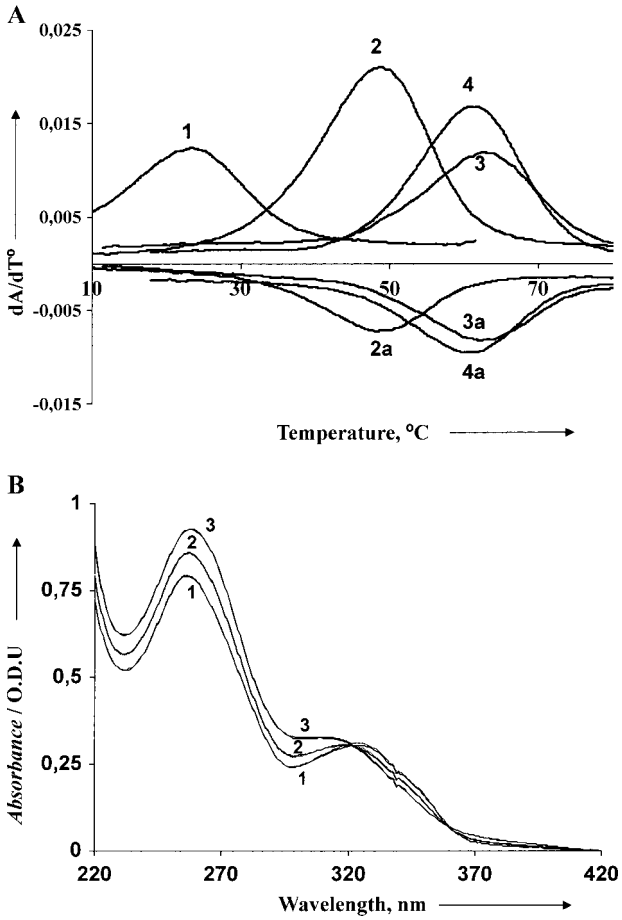

Figure 5. A. Derivatives of temperature denaturation curves of duplexes formed by oligonucleotide AATAAACG with CGTTTATTp (1) and its conjugates to tetrapyrrole $H_2N(CH_2)_5CONH(Py)_4(CH_2)_3N(C_2H_5)_2$ (2,3) and hairpin octapyrrole $NH_2(CH_2)_5CO$-$(Py)_4NH(CH_2)_3CO(Py)_4NH(CH_2)_3N(C_2H_5)$ (4) ligands. (2,4)—1:1 conjugates, (3)—1:2 parallel conjugate. Concentrations of the components were 3×10^{-5} M in 10 mM phosphate buffer, pH 7.3, 0.1 M NaCl, and 0.1 mM EDTA. Record wavelengths were 270 nm (1–4) and 330 nm (2a–4a). B. Spectra of duplex formed by oligonucleotide AATAAACG with a conjugate CGTTTATTp-$NH(CH_2)_5CO(Py)_4NH(CH_2)_3CO(Py)_4OC_2H_5$ at different temperatures: 1–5°C, 2–45°C, 3–90°C.

increased without any essential change in the position of absorption maximum of the oligonucleotide component of the conjugate. At 330 nm, a decrease in absorbance results from the shift of the absorption maximum to shorter wavelength range without any essential change in the extinction coefficient (from 326 to 316 nm in the case of duplex [4] melting, Fig. 5B).

These examples demonstrate that the stabilizing effect of two conjugated oligo-(N-methylpyrrole)carboxamide ligands in both parallel and antiparallel orientations on A:T-containing duplexes is similar (if not better) to the corresponding effect of the

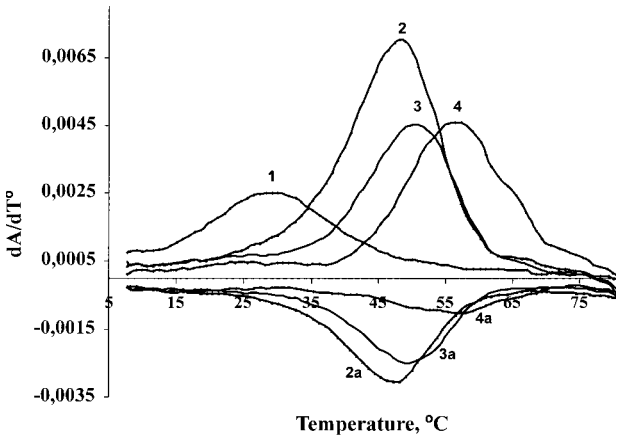

Figure 6. A. Derivatives of temperature denaturation curves of duplexes formed by oligonucleotide GCGCAA with TTGCGCp (1) and its conjugates to tetracarboxamides $\sim NH(CH_2)_3COIm\beta ImPyNH(CH_2)_3N(CH_3)_2+\sim NH(CH_2)_3COPyIm\beta ImNH(CH_2)_3N(CH_3)_2$ (2, parallel orientation), $\sim NH(CH_2)_3COIm\beta ImPyNH(CH_2)_3N(CH_3)_2+$ $BocNH(CH_2)_3COIm\beta ImPyNH(CH_2)_3NH\sim$(3, antiparallel orientation) and hairpin octacarboxamide$\sim NH(CH_2)_3COIm\beta ImPyNH(CH_2)_3COIm\beta ImPyNH(CH_2)_3COIm\beta ImPyNH(CH_2)_3N(CH_3)_2$ (4) ligands. Concentrations of the components were $1{,}3 \times 10^{-6}$ M in 10 mM phosphate buffer, pH 7.3, 0.1 M NaCl, and 0.1 mM EDTA. Record wavelengths were 260 nm (1–4) and 330 nm (2a–4a).

hairpin structures. Interesting feature of two attached ligands is lower dependence of the stabilization effect on the terminal positive charge of the ligand. In the case of hairpin MGB, the diethylaminoalkylamino group contributes considerably to stabilization of the hybrid compared to ethoxy group, evidently, due to the electrostatic effect of the protonated ternary amino group (compare complexes 4 and 7, 58°C and 49°C, respectively). However, the hybrids stabilized by two linear ligands in both parallel (duplexes 3 and 6) and antiparallel (duplex 8) orientations exhibit melting temperatures close to that of positively charged hairpin ligand (60°C, 60°C and 56°C, respectively), independently on the presence of the terminal protonated group(s). Thus, two oligo(Py) moieties attached to the same phosphate group seem to interact stronger with the DNA than hairpin one (compare 8 and 7). In theory, two positive charges of both ligands in complex 3 must stabilize the complex formation even better, but this effect could be compensated by their electric repulsion due to close location of amino groups in the parallel conjugate. Disposition of protonated group just near the terminal phosphate of the oligonucleotide in case of hairpin ligand may also play role.

Sequence Specificity of Duplex Stabilization by Oligo(Py)

Substitution of Py residues by Im residues strongly affects the stabilization of the A:T-containing duplex in complete agreement with Dervan's rules. Attachment of one or two tetra(Im) carboxamide ligands to the 3′-phosphate group of the oligonucleotide CGTTTATTp has almost no effect on the initial duplex stability (duplex 9 and 10).

When one tetra(Py) and one tetra(Im) ligands were coupled in a parallel orientation to obtain the 1:2 conjugate, the melting temperature of duplex 11 (Tm = 30°C) appeared to be considerably lower than the melting temperature of hybrid 2 with one tetra(Py) carboxamide residue (Tm = 46°C) indicating its destabilization. Nevertheless, one tetrapyrrole ligand of duplex 11 binds to its minor groove, as the melting curve taken at 330 nm displayed the typical negative slope, that is not the case for one or two attached tetra(Im) residues (duplexes 9 and 10; data not shown).

Sequence-Specific Stabilization of G:C-Containing Duplexes

No stabilization (even slight destabilization) was observed when alternative G/C sequence in a duplex TTGCGCp/GCGCAA[12] was targeted by corresponding (according to Dervan's rules) carboxamide MGBs containing only Py/Im units, independently on parallel, antiparallel or hairpin configuration of the conjugated ligand strands (duplexes 13, 14, 15). Also no changes in absorption at 330 nm were observed during the melting cycles, indicating the absence of DNA–MGB interaction (data not shown). Such effects were explained by a rigid structure of oligocarboxamides composed of only Py and Im units preventing them from binding optimally in the minor groove. In order to construct more flexible structures, it was proposed to use the ligands with several units of Py carboxamide moieties substituted with β-alanine.[10,11] This allowed both the specificity and stability of the ligand binding to the target site to be increased. We applied this approach to studying the effect of the MGBs containing β-alanine, Py and Im carboxamide residues attached to the phosphate group of the model duplex TTGCGCp/GCGCAA.[12]

Attachment of the hairpin ligand $NH_2(CH_2)_3CO$-ImβImPy-$NH(CH_2)_3CO$-ImβImPy-$NH(CH_2)_3N(CH_3)_2$ increased the melting temperature from 28° to 56°C (duplex 16). The stabilizing effect of two ligands attached to the phosphorus atom in an antiparallel orientation was slightly lower: Tm = 50°C (duplex 17). The bis-phosphoramidate with ligands in a parallel orientation displayed a melting temperature of 48° (duplex 18). This value is slightly lower than the melting temperature in the case of antiparallel ligands in both the hairpin structure and the bis-phosphoramidate. However, the increase in melting temperature of 20°C compared to the unmodified duplex is considerable enough and undoubtely indicates a site-specific binding of the 2:1 ligands in parallel orientation. In addition, an interaction between oligonucleotide and ligands is clearly indicated by a decrease of absorption at 330 nm, the negative peak of the melting curve derivative coinciding with the melting point of a corresponding duplex at 260 nm (Fig. 6).

CONCLUSIONS

Thus, the synthesized 1:1 (mono-phosphoramidates) and 1:2 conjugates of oligonucleotides (bis-phosphoramidates) and minor groove binders are capable of stabilizing the oligonucleotide duplex. The 1:2 conjugates containing oligo(Py) carboxamides stabilize the A:T-containing duplexes; in this case, both ligands seem to bind in the minor groove of the duplex. Oligo(Im) carboxamides stabilize the A:T-containing duplexes to a much lower extent if ever. The 2:1 MGB conjugates

(bis-phosphoramidates) containing β-alanine residues, Py-, and Im carboxamides are capable of interacting with the template oligonucleotide in both parallel and antiparallel orientations and stabilizing a G:C-containing site in a site-specific manner.

Stabilization of duplexes by attached MGB ligands could find its applications for construction of sensitive ssDNA probes or in DNA microchip technologies. Strong interaction of covalently linked parallel MGB ligands with DNA minor groove could also be used for design of sequence-specific molecules recognizing double-stranded DNA for gene therapy. This approach including triple helix-forming oligonucleotide conjugates is under current investigation in our laboratories.

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