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# Chiral probes for the handedness of DNA helices: Enantiomers of tris(4,7-diphenylphenanthroline)ruthenium(II)

**(Z-DNA/stereoselectivity/drug intercalation)**

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**ABSTRACT** **The chiral complexes tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) (RuDIP) are shown to be specific chemical probes with which to distinguish right- and left-handed DNA helices in solution. In spectrophotometric titrations of racemic RuDIP with both B-form calf thymus DNA and Z-form poly[d(G-C)], hypochromicity in the intense metal-to-ligand charge-transfer band is found and enhancement in luminescence is observed. The spectrophotometric assay of DNA binding to the well-resolved enantiomers of RuDIP provides a means to determine the helical conformation. Strong chiral specificity is seen in binding experiments with right-handed B-DNA and, on this basis, the absolute configurations are assigned. Although Δ-RuDIP can bind by intercalation into the right-handed helix, steric constraints imposed by the helix asymmetry preclude completely binding by the Λ enantiomer. Both isomers, however, are found to bind equally to Z-DNA. Left-handed helices that are more similar structurally to B-DNA would be predicted to display a stereospecific preference for this Λ isomer.**

The left-handed DNA helix has received considerable attention since the original crystallographic study of the Z-DNA fragment $[d(CpG)]_6$ (1). Solution conditions that include high ionic strength (2), hydrophobic solvents (3), the presence of certain trivalent cations (4), or covalent modification with bulky alkylating agents (4–8) all facilitate the transition of a right-handed double helix into a left-handed form. This striking conformational transition was first observed for poly[d(G-C)] (2). More recently, the alternating purine-pyrimidine sequence $[d(G-T)]_n \cdot [d(C-A)]_n$ has been shown to form Z-helices as well (9, 10). Methylation of cytosine residues at carbon-5 lends stability to Z-form DNA (4, 11) and, under physiological conditions, transitions to a left-handed structure can occur to relieve the torsional strain in underwound negatively supercoiled DNA (12–14). These latter findings suggest mechanisms for left-handed DNA formation in the cell, where such structures could be important in controlling gene expression. Negatively supercoiled simian virus 40 DNA has, for example, been found to contain potentially Z-DNA-forming alternate purine-pyrimidine regions within transcriptional enhancer sequences (15).

To explore any biological role for left-handed DNA, sensitive and selective probes are required. Z-DNA appears to be a strong immunogen. Anti-Z-DNA antibodies have been elicited with both brominated poly[d(G-C)] (16) and poly[d(G-C)] modified with diethylenetriamineplatinum(II) (17) as antigens. The structures of Z-DNA and in particular of a modified Z-form provide a multitude of antigenic characteristics: the left-handed helicity, the zigzag dinucleotide phosphate repeat, the protruding purine substituents in the shallow major groove.* It is not surprising then that the various antibodies obtained appear specific for different localized features of Z-DNA (18). The development of a specific *chemical* probe, so designed as to recognize a known structural element of Z-DNA, in our case the helix handedness, offers a simple complementary approach.

Enantiomeric selectivity has been observed in the interactions of tris(phenanthroline) metal complexes with B-DNA (19–21). Experiments with tris(phenanthroline)zinc(II) have indicated stereoselectivity (19); dialysis of B-DNA against the racemic mixture leads to the optical enrichment of the dialysate in the less-favored enantiomer. Subsequent luminescence, electrophoretic, and equilibrium dialysis studies of the well-characterized ruthenium(II) analogues have shown that the tris(phenanthroline) metal isomers bind to DNA by intercalation and it is the Δ enantiomer that binds preferentially to a right-handed duplex (20, 21). The enantiomeric selectivity is based on steric interactions between the nonintercalated phenanthroline ligands and the phosphate backbone. Although the right-handed propeller-like Δ isomer intercalates with facility into a right-handed helix, steric repulsions interfere with a similar intercalation of the Λ enantiomer.

Based on this premise, tris(phenanthroline) metal complexes appear useful in the design of probes to distinguish left-handed and right-handed DNA duplexes. The design flexibility inherent in metallointercalation reagents, in which both ligand and metal may be varied easily, makes the coordination complexes attractive probes (22–24). We have concentrated here on phenanthroline complexes of ruthenium(II) because of the high luminescence associated with their intense metal-to-ligand charge-transfer band (25, 26) and because the exchange-inert character of the low-spin $d^6$ complexes limits racemization (27). Although a preference in binding is found between enantiomers in the phenanthroline series, both isomers do in fact intercalate into the right-handed helix. To amplify the chiral discrimination and hence improve the sensitivity of our probe, phenyl substituents have now been added to the 4 and 7 positions of each phenanthroline ligand. The bulky substituents at the distal sites on the cation can block completely the intercalation of the Λ isomer into a right-handed helix.

We report here the design of selective spectroscopic probes for the handedness of the DNA duplex. The structure of the left-handed enantiomer, Λ-tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) (RuDIP), which binds to left-handed Z-DNA but not to right-handed B-DNA, is shown below.

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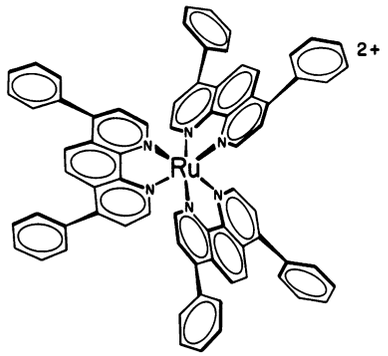
Abbreviations: RuDIP, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II); $(phen)_3Ru^{2+}$, tris(1,10-phenanthroline)ruthenium(II).

*The region in the Z-DNA structure corresponding to the major groove of B-DNA is sufficiently shallow and wide to be essentially a hydrophobic surface without a groove. For the purpose of comparative discussion, however, we will refer to this region as the major groove of the Z-form helix. Z-DNA does contain a very narrow helical groove or crevice in the region comparable with the minor groove of B-DNA.




## EXPERIMENTAL

**Nucleic Acids.** Calf thymus DNA (Sigma) was purified by phenol extraction (28). Poly[d(G-C)] (P-L Biochemicals) was dialyzed at least three times before use. Experiments were conducted at pH 7.2 in buffer 1 [4.5 mM Tris·HCl/45 mM NaCl/150 $\mu$M $Co(NH_3)_6Cl_3$/10% dimethyl sulfoxide], buffer 2 [5 mM Tris·HCl/50 mM NaCl/150 $\mu$M $Co(NH_3)_6Cl_3$], or buffer 3 (5 mM Tris·HCl/4.0 M NaCl]. DNA concentrations per nucleotide were determined spectrophotometrically assuming $\varepsilon_{260}$ = 6600 $M^{-1}\cdot cm^{-1}$ for calf thymus DNA (29) and $\varepsilon_{260}$ = 8400 $M^{-1}\cdot cm^{-1}$ for poly[d(G-C)] (30). In preparing Z-DNA, poly[d(G-C)] stock solutions were incubated in the cobalt hexammine buffer for 2–18 hr to ensure both a complete transition to the Z conformation and minimal aggregation. Stock solutions were examined spectrophotometrically and by CD before use.

**Ruthenium Complexes.** The synthesis of RuDIP trihydrate was carried out as described by Lin *et al.* (26). Concentrations were determined spectrophotometrically using $\varepsilon_{460}$ = 2.95 × $10^4$ $M^{-1}\cdot cm^{-1}$. Elemental analyses were consistent with literature values. The Δ and Λ isomers were either separated by successive recrystallizations with the antimony tartrate anion in 50% ethanol or prepared by asymmetric synthesis in the presence of antimony tartrate and then recrystallized. Λ-RuDIP forms the less soluble diastereomeric salt with antimonyl D-tartrate. The separated isomers were isolated finally as perchlorate salts. The assignments of absolute configuration have been made on the basis of the relative binding affinities of these enantiomers for B-DNA (see below). Many rounds of recrystallization yielded a small quantity of Λ-RuDIP having $[\theta]_{283}$ = −4.0 × $10^3$ deg·$M^{-1}\cdot cm$. This assignment is consistent with both the UV CD for tris(1,10-phenanthroline)ruthenium(II) [$(phen)_3Ru^{2+}$], assigned previously (31), and studies of the enantiomeric preference of $(phen)_3Ru^{2+}$ for B-DNA (20, 21, 32). The optical purities of the Δ- and Λ-RuDIP samples used below were 41% and 70%, respectively. Therefore the sample designated Δ-RuDIP contains 70.5% Δ isomer and 29.5% Λ isomer, and that designated Λ-RuDIP is composed of 14% Δ- and 86% Λ-RuDIP.

**Spectroscopic Measurements.** Absorbance spectra were recorded using a Varian Cary 219 UV/visible spectrophotometer and luminescence spectra, with a Perkin–Elmer LS-5 fluorescence spectrophotometer. Titrations were carried out using a constant ruthenium concentration (4–6 $\mu$M) to which increments of either calf thymus DNA or poly[d(G-C)] were added. Because RuDIP has limited solubility in aqueous solution (≤10 $\mu$M), dimethyl sulfoxide was included in buffer 1. CD spectra of B-DNA or Z-poly[d(G-C)] with 150 $\mu$M $Co(NH_3)_6^{3+}$ were unaffected by the presence of the dimethyl sulfoxide. Although more difficult, titrations in buffer 2 and buffer 3 were also conducted.

## RESULTS

**Spectroscopic Studies of Racemic RuDIP with B- and Z-DNA.** Changes are seen in both the visible absorption and luminescence spectra of RuDIP on addition of either B- or Z-form DNA. Hence, binding may be monitored sensitively using either spectroscopic technique. Visible absorption titrations of racemic RuDIP in buffer 1 with calf thymus DNA and Z-form poly[d(G-C)] are shown in Fig. 1. The overall similarity of these titrations should be apparent. Binding of either duplex DNA leads to hypochromicity in the intense metal-to-ligand charge-transfer band of the ruthenium complex. A small red shift (≈2 nm) in the spectrum of the bound complex and an isosbestic point at 485 nm can be seen. That spectral changes occur as a function of addition of either DNA form suggests that racemic RuDIP binds to both B- and Z-DNA. The similarity in spectral changes most likely reflects a similar mode of association of the ruthenium complex with either the right-handed B-DNA helix or the left-handed Z-DNA helix.

Differences in binding to the two forms are evident, however. A greater reduction in the absorption intensity of RuDIP accompanies binding to Z-form poly[d(G-C)] than to the B-DNA helix. In Fig. 1, for example, the apparent reduction in intensity with the addition of a 13:1 ratio of calf thymus DNA-phosphate/ruthenium is only 9% whereas, for the left-handed helix, the reduction occurring at a nucleotide/ruthenium ratio of 5:1 is 17%. The greater hypochromicity in binding to Z-DNA is explained in part by the different stereoselectivities governing binding to each helix. Although both enantiomers bind to Z-DNA, only the Δ enantiomer may bind easily to right-handed B-DNA. The differences in stereoselectivity cannot fully account for the difference in hypochromicity, however, because the hypochromicity in spectra of racemic RuDIP with Z-DNA is more than twice that observed with calf thymus DNA. If one assumes that the extinction coefficients for RuDIP when bound to each helix are the same, which seems reasonable based on the equal isosbestic points observed, then the larger hypochromic effect with Z-DNA suggests that both RuDIP enantiomers possess a greater affinity for Z-form poly[d(G-C)] than for calf thymus DNA. Equilibrium dialysis experiments support this conclusion (unpublished data).

The luminescence of RuDIP is also enhanced on binding to the DNA duplex. Fig. 2 shows the emission spectrum of racemic RuDIP (3 $\mu$M) in the absence and presence of calf thymus DNA and Z-form poly[d(G-C)] (15 $\mu$M nucleotide). The shift in the spectrum to lower energy is particularly pronounced despite the broad nature of the transition; the maximum shifts 10 nm to longer wavelength in the presence of DNA. Greater luminescence is seen here on binding to B-

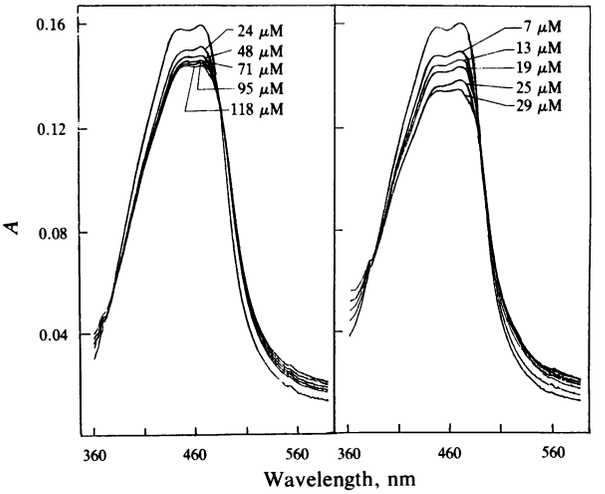



FIG. 1. Visible absorption spectra of racemic RuDIP (5.3 $\mu$M) in the absence and presence of various concentrations of B-form calf thymus DNA (*Left*) and Z-form poly[d(G-C)] (*Right*) in buffer 1.

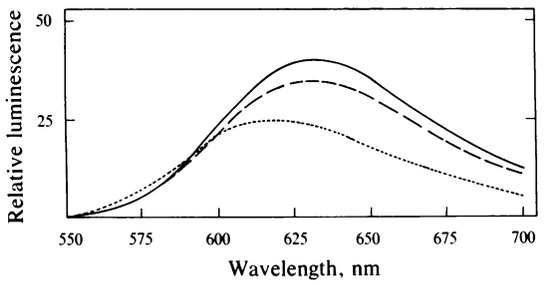



FIG. 2. Luminescence spectra in buffer 2. ·····, Racemic RuDIP (3 μM) free in solution; – – –, racemic RuDIP (3 μM) in the presence of Z-form poly[d(G-C)]; —, B-form calf thymus DNA. Samples were excited at 482 nm.

DNA, despite the lower apparent affinity for this helix. In buffer 2, at a DNA-phosphate/ruthenium ratio of 5:1, the emission intensity of racemic RuDIP increases by 30% and 47% in the presence of Z-form poly[d(G-C)] and calf thymus DNA, respectively. The different enhancements may depend in part on nucleic acid composition as well as duplex conformation. RuDIP bound to B-form poly[d(G-C)] yields less luminescence than when bound to calf thymus DNA, despite having an equal affinity for these polynucleotides.

Racemic RuDIP appears to bind to both B- and Z-DNA rather than promoting a transition from one conformation to the other. CD spectra of Z-form poly[d(G-C)] in $Co(NH_3)_6^{3+}$ (buffer 1) or in 4 M NaCl (buffer 3) are unaltered by the addition of racemic RuDIP at a nucleotide/ruthenium ratio of 10. Conversion of the Z-form to B-form with RuDIP is inconsistent also with the differential hypochromism and luminescence observed. If RuDIP promoted a Z → B transition, albeit inefficiently, rather than binding to both B- and Z-helices, then both the reduction in absorbance and the enhancement in luminescence observed on addition of Z-DNA would be less than or equal to that found with B-DNA—i.e., in proportion to the fraction of DNA converted. Instead we find significantly greater hypochromism when Z-DNA rather than B-DNA is added to the racemic mixture or indeed to each enantiomer individually. Therefore racemic RuDIP must bind to both DNA conformations. Consistent with these results, a conformational transition from Z-DNA to B-DNA would not be expected if the affinity of the metal cation for the Z-form were greater than that for the B-form. The ethidium cation, which binds to B-DNA by intercalation, is known to promote a Z → B transition at high binding ratios (33, 34) and presumably possesses a greater affinity for the B-form helix. The substantially larger RuDIP cation cannot saturate the DNA to comparable levels, which may be an important distinction. Moreover, although the ethidium ion can fully intercalate into B-DNA, RuDIP cannot and the nonintercalating ligands of RuDIP may dominate its interactions with the duplex.

**Differential Binding of the RuDIP Enantiomers to B- and Z-DNAs.** The utility of the RuDIP enantiomers as a probe for helical conformation becomes apparent when the binding characteristics of each enantiomer to B- and Z-DNAs are compared. Plots of the relative absorbance at 460 nm of the individual enantiomers as a function of the addition of either B-form calf thymus DNA or Z-form poly[d(G-C)] in buffer 1 are shown in Fig. 3. Based on the presence or absence of hypochromicity, it is clear that although Δ-RuDIP binds to B-DNA, the Λ isomer does not. Λ-RuDIP does however bind to Z-DNA. Indeed, with Z-DNA no stereospecificity is observed. Hence the assay of duplex binding by the Λ isomer yields a sensitive assay for the Z-DNA conformation.

Strong enantiomeric selectivity governs the interaction of RuDIP with the right-handed B-DNA helix. The decrease in absorbance with increasing DNA concentration observed for

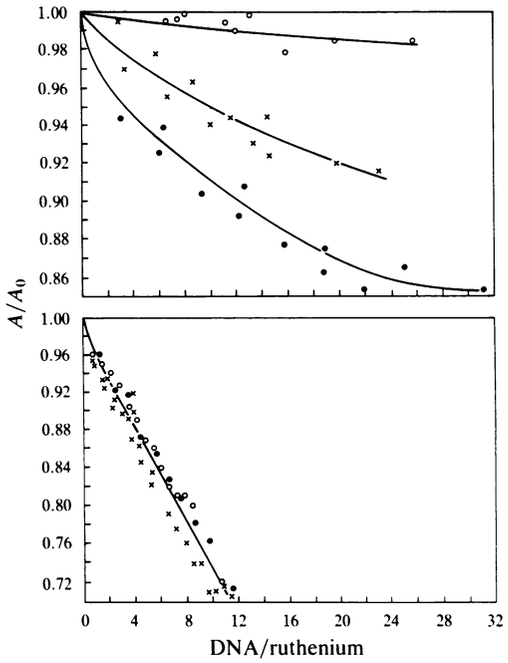



FIG. 3. Relative change in absorption intensity at 460 nm of racemic RuDIP (×), Λ-RuDIP (○), and Δ-RuDIP (●) at various DNA-phosphate/ruthenium ratios. Titrations were conducted in buffer 1 using either B-form calf thymus DNA (*Upper*) or Z-form poly[d(G-C)] (*Lower*). Strong stereoselectivity with B-DNA is evident based on the differential hypochromism seen between enantiomers, whereas comparable titrations with Z-DNA show the same hypochromic effects with each enantiomer. Because large hypochromicity in the Λ isomer is seen only with Z-DNA, Λ-RuDIP provides a probe for this conformation.

the Λ, racemic mixture, and Δ samples can be fully accounted for based on the percentage of the Δ enantiomer present in the particular preparation (see *Experimental*). The pure Λ enantiomer does not bind to B-DNA. The presence of the phenyl groups at the 4 and 7 positions of the nonintercalated phenanthroline ligand has served to amplify the chiral discrimination. In comparisons, differences in binding of $(phen)_3Ru^{2+}$ enantiomers had been seen only in spectrophotometric titrations at high DNA/ruthenium levels; the ratio of the affinities for B-DNA of Δ to Λ isomers is ≈1.3 (20, 21). For RuDIP, with hydrogen atoms now replaced by phenyl groups, instead of simple interference with the DNA phosphate oxygen atoms, one finds that the steric bulk of the phenyl groups completely blocks interactions of the Λ isomer in the right-handed groove. Δ-RuDIP, however, binds with facility to a right-handed helix, indeed more avidly than Δ-$(phen)_3Ru^{2+}$. This striking amplification in enantiomeric selectivity for RuDIP compared with $(phen)_3Ru^{2+}$ strongly supports our model for stereospecific intercalation.

Z-DNA serves as a poor template to discriminate between the enantiomers; identical reductions in absorbance intensity are found for the Δ and Λ isomers (Fig. 3). Because of the shallow and very wide character of the major groove* in Z-DNA, there are no steric constraints comparable with that found with B-DNA. Hence, if the binding modes are equivalent, no chiral specificity would be expected. The similarity in spectral characteristics of RuDIP in binding to the different DNA duplexes points to this similarity in binding modes. However, the lack of chiral specificity in binding to Z-DNA does limit what we can say at present about the interaction of RuDIP enantiomers with a Z-form helix. Based on relative hypochromicities, it appears that both Λ- and Δ-RuDIP possess greater affinities for Z-form poly[d(G-C)] than for B-DNA. Hydrophobic interactions with the helical surface may lend some stability to the bound complex (35). The dif-

ference in affinity furthermore does not reflect a preference for base composition. Titrations of racemic RuDIP with B-form poly[d(G-C)] in buffer 1 lacking cobalt hexammine showed hypochromicity equal to that seen with calf thymus DNA. Also, cobalt hexammine itself does not appear to alter binding to the helix. RuDIP titrations using calf thymus DNA with and without $Co(NH_3)_6^{3+}$ were identical. In addition, the interaction cannot be explained purely by electrostatic interactions. Although smaller, hypochromic effects, approximately one-third of that shown here, are found in titrations in 4 M NaCl (buffer 3) with either poly[d(G-C)] or calf thymus DNA. Partial intercalation into the DNA by both RuDIP enantiomers would be consistent with our results. It is finally important to note that the similar titrations of both enantiomers that we see with Z-DNA but not with B-DNA suggest that neither enantiomer converts the Z-form helix to the B-DNA conformation. If that were the case, selectivity between the enantiomers would become apparent.

## DISCUSSION

The chiral RuDIP complexes serve as specific chemical probes for the handedness of the DNA helix in solution. Spectrophotometric titrations have shown that, although one RuDIP enantiomer, assigned as Λ-RuDIP, does not bind at all to the B-DNA helix, the bulky asymmetric cation can bind to Z-DNA. Monitoring the binding of this isomer to DNA by any means therefore equivalently assays the helical conformation. The intense metal-to-ligand charge-transfer band in the ruthenium complexes provides a particularly sensitive handle with which to examine the binding, either spectrophotometrically or through its accompanying luminescence.

Striking enantiomeric selectivity is found in the interactions of the RuDIP cations with right-handed B-DNA, and this chiral discrimination is consistent with an intercalative model. The changes in the visible spectrum of RuDIP—i.e., the hypochromic shift and luminescence enhancement—observed in the presence of duplex DNA parallel in detail those seen in spectra of $(phen)_3Ru^{2+}$ as a function of DNA addition. It has been shown that $(phen)_3Ru^{2+}$ binds to B-DNA by partial intercalation of the phenanthroline ligand between the base pairs (20, 21). Given that generally the ruthenium-metal-to-ligand charge-transfer transition shows little sensitivity to solvent or environment (25, 26), the close resemblance of properties of RuDIP to $(phen)_3Ru^{2+}$ suggests that the cations bind the DNA in a similar fashion. Intercalation of the diphenylphenanthroline ligand between the helix base pairs requires that the phenyl groups rotate into the plane of the phenanthroline ligand. This rotation to a planar structure with minimal steric interactions between nearby hydrogen atoms can be accomplished by lengthening the carbon-carbon bond between the phenyl and phenanthroline moieties. Equivalent structural distortions are seen in biphenyl, which is planar in the stacked solid lattice (36). Also, the extremely bulky tetrapyridyl-porphyrin cations, which require extensive distortion, are thought to bind to the DNA duplex by intercalation (37, 38). Importantly, once rotated into the plane of the phenanthroline, the phenyl groups in RuDIP add substantially to the surface area available for overlap with the base pairs as compared with $(phen)_3Ru^{2+}$, and therefore greater stability of the bound ruthenium-DNA complex is expected. In fact, binding of RuDIP to DNA assayed by any method becomes evident at <10% of the concentration of $(phen)_3Ru^{2+}$, which reflects the increased affinity of RuDIP over $(phen)_3Ru^{2+}$ for B-DNA. Perhaps the clearest support for the intercalation model rests in the dramatic enhance-

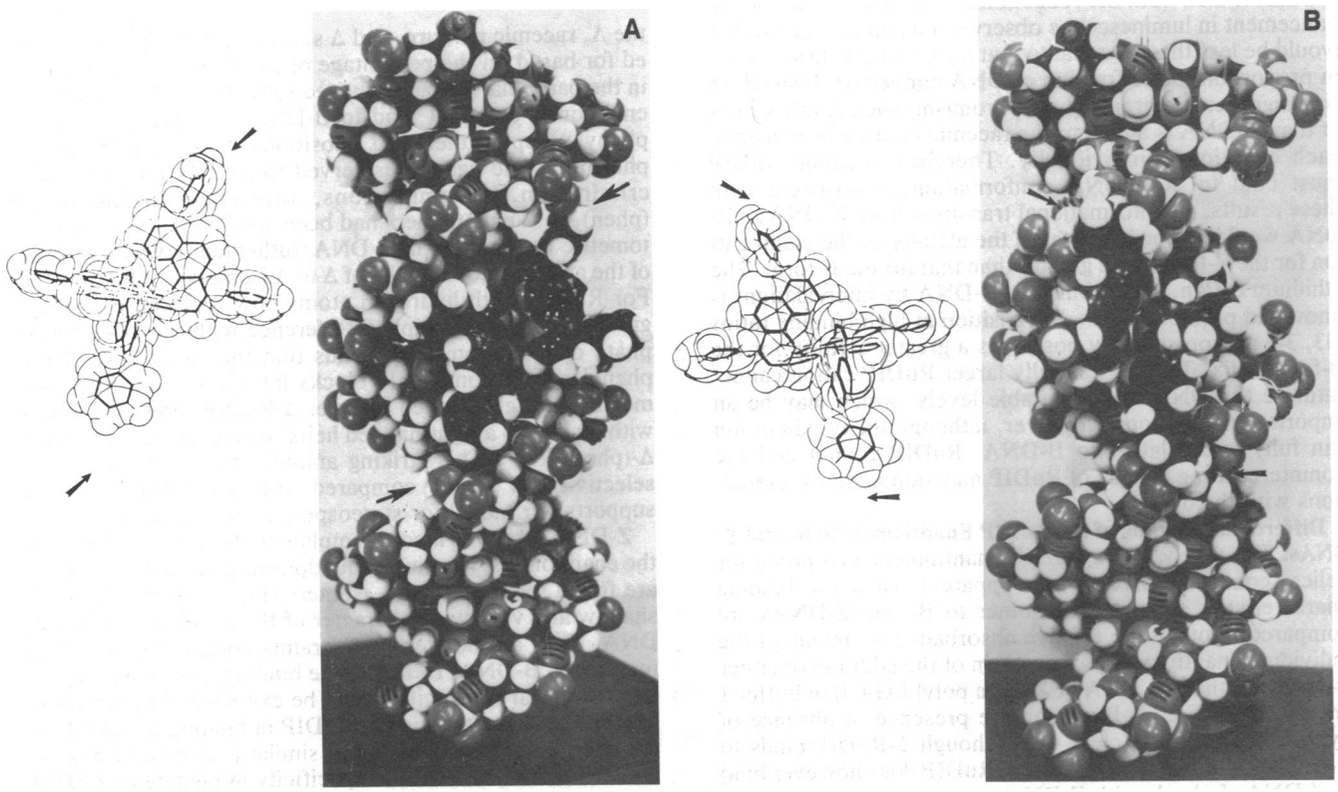



FIG. 4. Corey–Pauling–Kolton space-filling models of Δ- and Λ-RuDIP with a right-handed B-DNA helix. The orientation of the metal complex in the helix is shown in the sketches. One ligand (not visible) is oriented perpendicular to the helix axis for intercalation between the base pairs. (*A*) Intercalation of the Δ enantiomer. The nonintercalated ligands fit easily within the right-handed groove. (*B*) For the Λ enantiomer, when one ligand is positioned for intercalation, the remaining two ligands are blocked completely above and below (arrows) by the right-handed sugar-phosphate backbone, and this steric constraint prevents ligand insertion between the base pairs. This can also be seen by following the line of the DNA backbone, which, although completely visible in *A*, is interrupted by the phenyl groups of Λ-RuDIP in *B*.

Table 1. Scheme for probing DNA conformation with RuDIP enantiomers

| Reactivity | | |
|---|---|---|
| With the Δ isomer | With the Λ isomer | DNA duplex conformation |
| + | − | Right-handed B-like |
| + | + | Left-handed Z-like or lacking a groove |
| − | − | Unstacked or with base pairs inaccessible |
| − | + | Left-handed B-like |

ment in stereoselectivity observed for RuDIP in comparison with $(phen)_3Ru^{2+}$. The phenyl groups, while facilitating intercalation of Δ-RuDIP into the right-handed helix, completely preclude binding by the Λ enantiomer.

Corey–Pauling–Kolton space-filling models of the RuDIP complexes with a B-DNA helix are shown in Fig. 4. The orientations with respect to the helix are indicated in the accompanying sketches. The Δ enantiomer, with one diphenylphenanthroline ligand intercalated, can fit very closely along the helical groove. The two nonintercalating ligands, with a disposition in line with the right-handed helix, abut the helical groove. These close hydrophobic interactions of the nonintercalated ligands are not possible with the mirror-image Λ enantiomer. In contrast, as presented in Fig. 4, if one ligand (not visible) is oriented perpendicular to the helix axis, then the two remaining ligands of the Λ enantiomer are disposed contrary to the right-handed groove. The ruthenium model must therefore be shown in front of the DNA helix in the figure, rather than intercalated, because the interaction of the phenyl groups with the DNA-phosphate backbone at the positions indicated by the arrows completely blocks access. Thus, the stereoselectivity that we see is determined by the steric constraints imposed by the asymmetry in the helix, its handedness.

Just as the helix asymmetry can serve as a template to discriminate between RuDIP enantiomers, differential binding by the enantiomers may be used advantageously in determining the chirality of the helix. Table 1 indicates a general scheme to probe helical conformations using the RuDIP cations. Although Λ-RuDIP does not bind to the right-handed B-DNA duplex, spectrophotometric titrations have shown significant binding to Z-DNA and therefore hypochromism of Λ-RuDIP on addition of a test DNA sample may be used as an indication of the Z-conformation. It was particularly interesting to us to find that no stereoselectivity governs binding to the Z-form helix. The bulky cation likely avoids the very narrow helical crevice in the Z-DNA structure, and intercalative binding to the more shallow hydrophobic surface in Z-DNA, the equivalent of the major groove in the B-form, would not be expected to yield any chiral discrimination. Z-DNA does not mirror B-DNA in solution. Instead we predict that a left-handed but more B-like conformation (3, 39, 40) would yield a mirror-image selectivity.

The chiral tris(diphenylphenanthroline) metal complexes will therefore be interesting to use in solution to examine DNA helical conformations: those of naturally occurring sequences, in the presence of drugs, and in protein-bound complexes. Furthermore, the reagents suggest a new route for conformation-specific drug design.

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