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Journal of Chromatography A, 718 (1995) 436–443

JOURNAL OF
CHROMATOGRAPHY A

Short communication

Development of DNA-immobilised chromatographic stationary phases for optical resolution and DNA-affinity comparison of metal complexes ☆

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First received 27 September 1994; revised manuscript received 21 June 1995; accepted 21 June 1995

Abstract

Two chiral stationary phases which exploit DNA as a chiral discriminator have been developed. A covalently-bound DNA stationary phase for HPLC applications was used to optically resolve $[Ru(dipyrido[6,7\text{-}d:2',3'\text{-}f]\text{-}quinoxaline)_3]^{2+}$ and $[Ru(1,10\text{-}phenanthroline)_3]^{2+}$ complex ions. This shows that the column retention times are influenced both by pH and by the size of the aromatic ligands. DNA-immobilised on cellulose paper proved effective for simultaneously comparing the relative retention of a number of metal complexes and R_F data correlate well with the degree of aromatic area in the complexes available for intercalation into DNA.

1. Introduction

In the search for a method to resolve chiral metal-complex enantiomers, various chromatographic systems have been explored, with varying degrees of success. Pirkle columns are designed [1,2] to resolve particular organic compounds, in which the chiral stationary phase contains an immobilised compound known to discriminate between enantiomeric forms. Immobilised cyclodextrin has been used as a stationary phase for the separation and resolution of chiral metal complexes by Armstrong et al. [3], Green et al. [4] and Yamanari and Nakamichi [5].

Gil-Av and co-workers [6–9] have successfully employed HPLC for the separation of helicenes on small silica columns coated with riboflavin or various nucleotides. Inagaki and Kageyama [10] and Zunino [11] have reported the utilisation of a DNA-cellulose column for the investigation of intercalative modes of binding. This stationary phase proved to be sensitive to the different binding modes of actinomycin D and daunomycin with DNA. One is an electrostatic interaction and the other a stronger undefined binding mode, although intercalation is consistent with the differing retention volumes. Intercalation, as a mode of interaction of actinomycin

☆ A preliminary account of this work was presented at the Third International Symposium on Bioinorganic Chemistry, Fremantle, Australia, (December) 1994 (Abstract A14).

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SSDI 0021-9673(95)00703-2

D with the nucleoside bases of DNA, is illustrated by published structures [12–15] and supported by the NMR evidence of Zhou et al. [16]. If immobilised DNA could discriminate between such different binding modes, then it is possible that the different affinities for DNA of chiral metal complexes also may be exploited. This concept has been applied in this work to the development of a chiral stationary phase for metal-complex resolutions.

Numerous methods of coating and adsorbing DNA on chromatographic columns have been reported [17–24] and more recently Baker et al. [25] reports the separation of *rac*-$[Ru(phen)_3]^{2+}$ by elution through DNA-hydroxylapatite. However, an adsorption-based column has many drawbacks that would be overcome if the DNA were to be covalently bound to a stationary phase. We report here on the development of an HPLC column which exploits DNA as a chiral discriminator in this form.

2. Experimental

2.1. Synthesis and sample preparation

Δ-, Λ- and *rac*-$[Ru(phen)_3]^{2+}$ complexes as their perchlorates were synthesised and resolved using published methods [26,27]. Synthetic procedures for the ligand and $[Ru(diimine)_3]^{2+}$ and $[Ru(tetradentate)(diimine)]^{2+}$ complex species have been reported [28–31], here diimine represents *o*-phenylenediamine (*o*-pda), 2,3-diaminonaphthalene (2,3-nap), 1,10-phenanthroline (phen), 4,4′-dimethyl-2,2′-bipyridine (4,4-bipyMe_2), dipyrido[6,7-d:2′,3′-f]quinoxaline (dpq), dipyrido[6,7-d:2′,3′-f]3-dimethylquinoxaline (dpqMe_2), dipyrido[3,2-a:2′,3′-c]phenazine (dppz) or dipyrido[3,2-a:2′,3′-c]-7,8-dimethylphenazine (dppzMe_2) (Fig. 1) and the tetradentate is either 1,6-di(2′-pyridyl)-2,5-dimethyl-2,5-diazahexane (picenMe_2), N,N′-dimethyl-N,N′-di(2′-pyridyl)-1,2-diaminocyclohexane (*R,R*-picchxnMe_2) or 1,6-di(2′-pyridyl)-2,5-dibenzyl-2,5-diazahexane (picenBz_2) (Fig. 2). Samples for HPLC analysis were dissolved in acetone–water (80:20, v/v) (1 cm^3) and sealed in amber glass ampoules prior to use.

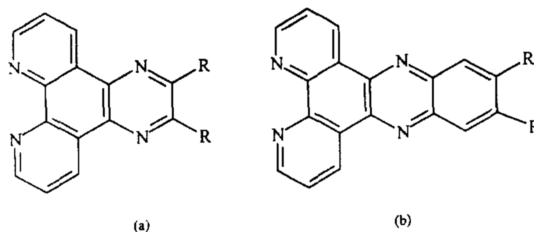

Fig. 1. The bidentate ligands. (a) R = H, dipyrido[6,7-d:-2′,3′-f]quinoxaline (dpq); R = CH_3, dipyrido[6,7-d:2′,3′-f]-2,3-dimethylquinoxaline (dpqMe_2). (b) R = H, dipyrido [3,2-a:2′,3′-c]phenazine (dppz); R = CH_3, dipyrido[3,2-a:2′,3′-c]-7,8-dimethylphenazine (dppzMe_2).

2.2. Synthesis and application of a DNA-modified stationary phase

The DNA-immobilisation procedure followed was adopted (Fig. 3) from that reported by Lee and Gilham [32], which was primarily intended for the purification of proteins. Terminal fragments of DNA are oxidised by periodate and immobilised on a polyamine stationary phase prior to borohydride reduction in a termination step similar to that reported by Brown and Read [33].

A warm solution of calf thymus DNA (0.3 g) and sodium periodate (3.0 g) in water (160 cm^3) was stirred and warmed for 30 min. Apex propylamine-modified silica gel (10 g, 5 μm or 15 μm) was added to this solution and the slurry stirred for a further 30 min. Sodium borohydride (2.0 g) was slowly added to the stirred mixture, and the slurry allowed to settle and the liquid

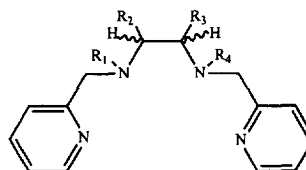

Fig. 2. The tetradentates picen, picenMe_2, picenBz_2 and picchxnMe_2. $R_1 = R_2 = R_3 = R_4 = H$, picen; $R_1 = R_4 = CH_3$, $R_2 = R_3 = H$, picenMe_2; $R_1 = R_4 = CH_2C_6H_5$, $R_2 = R_3 = H$, picenBz_2; $R_1 = R_4 = CH_3$, $R_2 = R_3 = -CHCH_2CH_2CH-$, picchxn$Me_2$.

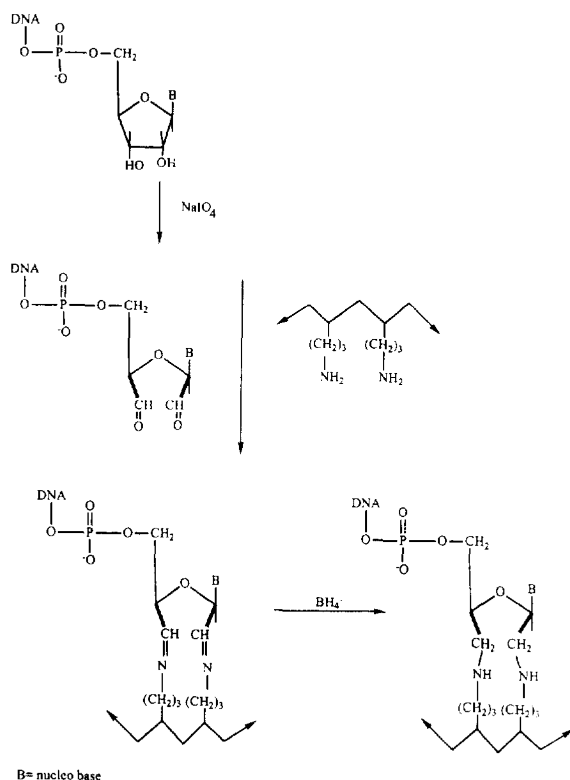

Fig. 3. Synthetic outline for a DNA-modified stationary phase.

decanted off. The DNA-modified silica gel packing was collected by filtration, washed well with water and dried under suction, resulting in a pale-cream free flowing solid.

A sample of this DNA-modified silica gel (4.0 g) was transferred to a ground-glass stoppered flask with water (30 cm^3) and was sonicated. The slurry was transferred to a "bomb", a stainless steel vessel, from which it was forced onto an HPLC column (250×4.6 mm I.D.) by the flow of water pumped from a reservoir at 4000–6000 lb/in^2 pressure. The pressure was maintained for 45 min after which time the column was rotated around its axis by 90° and the pressure maintained for a further 10 min. Efficient packing was confirmed by the slow release of the pressure. This DNA-column then was washed with water and stored prior to use, at which time it was conditioned using sodium acetate buffer at pH 4 containing 10% methanol. This method was used to produce a selection of DNA-columns, with varying relative amounts of DNA loaded and with relative surface area varied by reducing the particle size of the silica substrate.

Optimal chromatographic conditions were assessed using $[Ru(phen)_3]^{2+}$, in which variables such as the percentage of DNA loaded, the column length, packing particle size, and the effect of pH on elution times were explored. For the resolution of $[Ru(dpq)_3]^{2+}$, samples (25 μl) were injected onto two DNA columns connected in series, a 2.3% DNA (15 μm, 120×3.9 mm I.D.) and a 3% DNA (5 μm, 120×3.9 mm I.D.), and were eluted with methanol/sodium acetate (pH 6.02) (10:90, v/v) mobile phase at a flow-rate of 1 ml/min.

2.3. Synthesis and application of DNA-cellulose paper

A solution of calf thymus DNA (0.3 g) and sodium periodate (3.0 g) in water (160 cm^3) was stirred and warmed for 30 min and then transferred to a shallow tray. A solution of sodium borohydride (2.0 g) in water (160 cm^3) was placed in a second shallow tray. Chromatographic cellulose paper was cut into rectangular sheets (14×22.5 cm), and each sheet allowed to soak in the DNA solution prior to its being transferred to the borohydride tray, where the reaction is quenched. The DNA-immobilised paper then was removed and allowed to air dry on a flat surface. The synthetic method is illustrated in Fig. 4.

DNA-paper and untreated chromatographic paper were individually spotted with a reference sample of $[Ru(phen)_3]^{2+}$ and samples of $[Ru(tetradentate)(diimine)]^{2+}$ or $[Ru(diimine)_3]^{2+}$ each dissolved in acetone, then dried and developed with sodium acetate buffer solution (0.16 *M*, pH 6.90) modified with 10% methanol.

3. Results and discussion

Racemic $[Ru(phen)_3]^{2+}$ was readily resolved on the DNA-column and subsequently was used

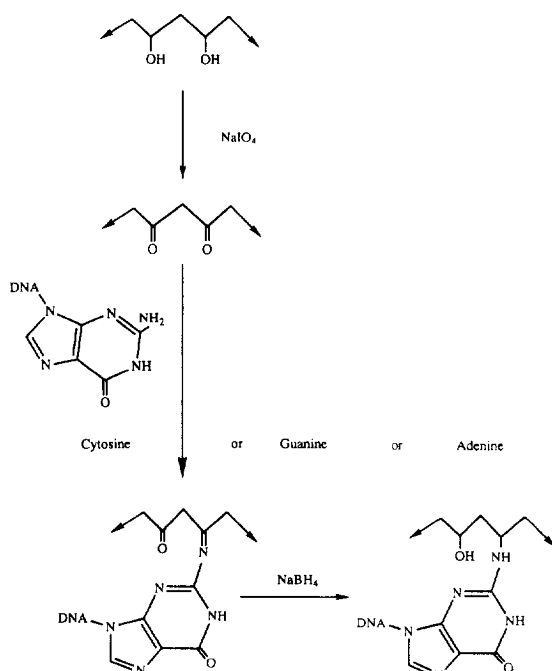

Fig. 4. Synthetic outline for DNA-cellulose paper.

to optimise the experimental conditions. For example, lengthening the column from 120 to 270 mm accentuated the retention differences between the $[Ru(phen)_3]^{2+}$ enantiomers and as a result the separation of the peaks increased (k'_2/k'_1) from 1.13 to 1.31. Packing considerations meant that the individual column length was limited to about 300 mm. However, this could be overcome by connecting columns in series. A reduction in particle size from 15 μm to 5 μm resulted in a reduction in half-width for Δ-$[Ru(phen)_3]^{2+}$ from 0.93 to 0.68 min and for Λ-$[Ru(phen)_3]^{2+}$ from 1.65 to 0.57 min.

Control of protonation of DNA [34,35] is essential for intercalation to be evaluated, and this was achieved using sodium acetate buffers. The degree of $[Ru(phen)_3]^{2+}$ affinity for DNA was compared over the pH range 3.7–6.0. Individual enantiomers of $[Ru(phen)_3]^{2+}$ were injected onto equilibrated columns at the desired pH. Results are given in Table 1. Relative retentions were found to improve with higher pH, although even at pH 3.71 there was a retention difference between the isomers. The

Table 1
The effect of pH[a] on the retention factor of Δ- and Λ-$[Ru(phen)_3]^{2+}$ isomers

	pH		
	3.71	4.63	6.03
k'_Δ	0.27	0.79	1.42
k'_Λ	0.40	1.24	2.30
k'_Λ/k'_Δ	1.48	1.57	1.62

[a] Conditions; column: 3% immobilised DNA (5 μm; 270 × 3.9 mm I.D.); mobile phase, sodium acetate buffer—15% methanol; flow-rate, 1 ml/min.

Δ-isomer eluted before the Λ-isomer at each pH value. These results, which were confirmed by circular dichroism spectroscopy (CD), are illustrated in Fig. 5. This retention order was not an expected result, since the Δ-isomer is reported [36–39] to interact preferentially with DNA when intercalation is the dominant mode, and hence might be expected to have a longer retention time.

The nature of the chosen buffer on retention order was tested, since resolution of $[Ru(phen)_3]^{2+}$ achieved by dialysis using Tris buffer in the presence of DNA showed [38] preferential retention of the Δ-isomer. Tris buffer (pH 7.1) was substituted in place of sodium acetate and an enantiomeric mixture of

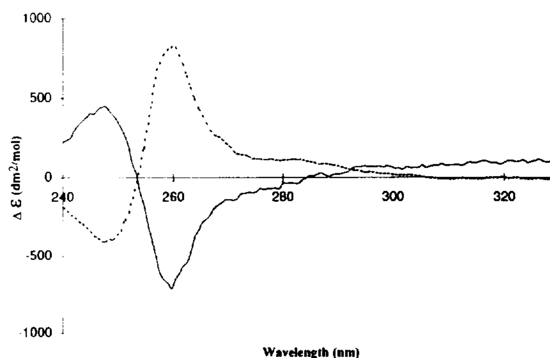

Fig. 5. CD spectra of Δ- and Λ-$[Ru(phen)_3]^{2+}$ eluted from a 3% immobilised DNA column (5 μm, 270 × 3.9 mm I.D.), sodium acetate buffer–15% methanol (pH 6.03), flow-rate 1 ml/min. Δ-enriched leading fraction (—), Λ-enriched trailing fraction (– – –).

Table 2
Retention factors for $[Ru(phen)_3]^{2+}$ and $[Ru(dpq)_3]^{2+}$ enantiomers on the DNA immobilised column

	$[Ru(phen)_3]^{2+}$		$[Ru(dpq)_3]^{2+}$
	Tris[a]	NaAc[b]	NaAc[b]
k'_Δ	3.79	3.65	9.83
k'_Λ	4.15	3.90	8.83
k'_Λ/k'_Δ	1.10	1.07	0.90

HPLC conditions: 3% immobilised DNA (5 μm, 270 × 3.9 mm I.D.); mobile phase, [a] Tris buffer–10% methanol (pH 7.1), [b] sodium actate buffer–15% methanol (pH 6.03); flow-rate, 1 ml/min.

$[Ru(phen)_3]^{2+}$(Δ:Λ = 1:3) was injected. The resulting peaks where confirmed as being due to the Δ- and Λ-enantiomers by their relative area and CD spectra, with relative retentions of 17.7 and 19.1 min, respectively. This demonstrated that the Δ-isomer of $[Ru(phen)_3]^{2+}$ eluted first irrespective of the buffer used.

Assuming an intercalative interaction mode, the elution of $[Ru(dpq)_3]^{2+}$ was anticipated to involve longer retention times given the larger aromatic area of the diimine ligand and this proved to be so. Results are reported in Table 2, where it may be seen that the Δ-$[Ru(dpq)_3]^{2+}$ enantiomer was retained preferentially. The CD spectra of the leading and trailing fractions of $[Ru(dpq)_3]^{2+}$, obtained from elution through two columns in series, are depicted in Fig. 6. These CD features are almost identical to those of optically pure Δ- and Λ-$[Ru(phen)_3]^{2+}$ [39]. In addition, this assignment of absolute configuration for Δ- and Λ-$[Ru(dpq)_3]^{2+}$ is consistent with an interpretation based on coupling of long axis polarised π–π^* transitions [40].

If intercalation is the predominant mode of interaction then the preferential retention of a Δ-isomer is expected since the right-handed helical groove accommodates the non-intercalating ligands in a complementary fashion [35–38]. The steric interactions of Δ-$[Ru(phen)_3]^{2+}$ and Δ-$[Ru(dpq)_3]^{2+}$ with the groove surface of DNA should be essentially the same. Hence differences between the allowed aromatic overlap of phen and dpq in the intercalation site, illustrated in Fig. 7, are inferred to be responsible for the observed difference in retention order. The intercalation model reveals that the degree of aromatic overlap possible for both Δ- or Λ-$[Ru(phen)_3]^{2+}$ is small if not tenuous. Nonetheless, if intercalation is the mode of interaction then the Δ-isomer should be preferentially retained. Thus, the different retention orders obtained for the two complexes support the view that intercalation is not the predominant mode of interaction for $[Ru(phen)_3]^{2+}$, which is in agreement with the major groove binding model proposed by Hiort et al. [41]. The effectiveness

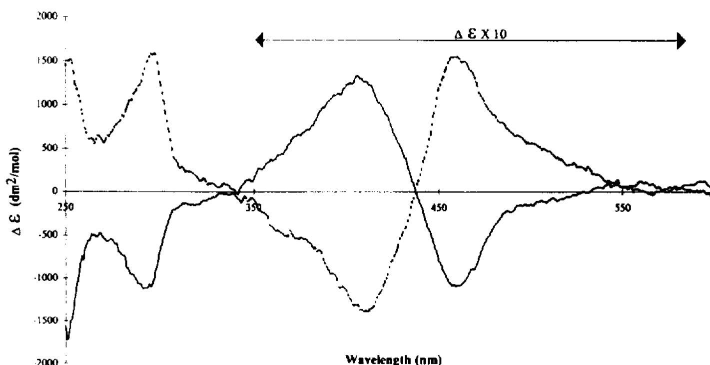

Fig. 6. CD spectra of Δ- and Λ-$[Ru(dpq)_3]^{2+}$ ions after elution through two DNA columns (2.3%, 15 μm, 120 × 3.9 mm and 3%, 5 μm, 270 × 3.9 mm) connected in series, eluted with a mobile phase of 10% methanol—sodium acetate (0.16 M, pH 6.02) at 1 ml/min. Λ-leading fraction (- - -), Δ-trailing fraction (—).

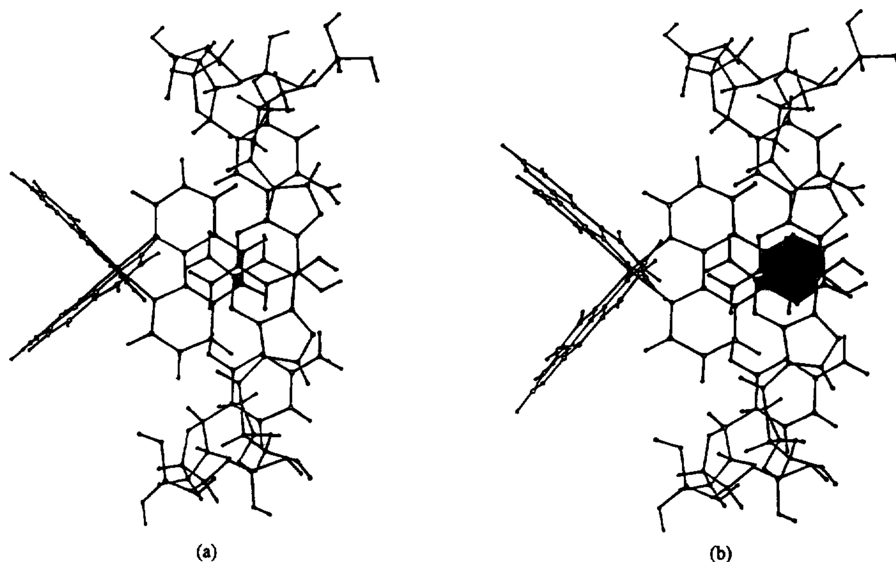

Fig. 7. A comparison of the aromatic overlap in the intercalation site of $[Ru(diimine)_3]^{2+}$ when the diimine is (a) phen and (b) dpq.

of the coordinated phen ligand as an intercalator is limited, whereas dpq appears to offer a much more effective fused ring size for intercalation.

DNA-paper chromatography proved to be a simple but effective method to compare the relative affinity of the complexes for DNA, taking only a matter of minutes and with the number of complexes able to be compared at any one time only limited by paper size. A graph comparing the relative retention of a number of the metal complexes is illustrated in Fig. 8.

Computer modelling of insertion of the phen ligand of a Δ-$[Ru(phen)_3]^{2+}$ complex into the intercalation cavity via both the minor or major grooves allowed the definition of the region of aromatic overlap. This showed the base overlap region to have an effective width of about 3.5 Å based on atom centres. In general, the R_F^* value ($R_{F\ blank} - R_{F\ DNA\text{-}paper}$) was found to be inversely proportional to the aromatic overlap allowed, such that *o*-pda, phen, dpq, dppz and $dppzMe_2$ elution rates decreased in order of increasing aromatic overlap (Fig. 9). The non-intercalating portion of each molecule was also seen to influence the retention. For example, a larger R_F^* was obtained for Δ-α-$[Ru(R,R\text{-picchxnMe}_2)(dpq)]^{2+}$ rather than for Δ-β-[Ru(*R*,*R*-

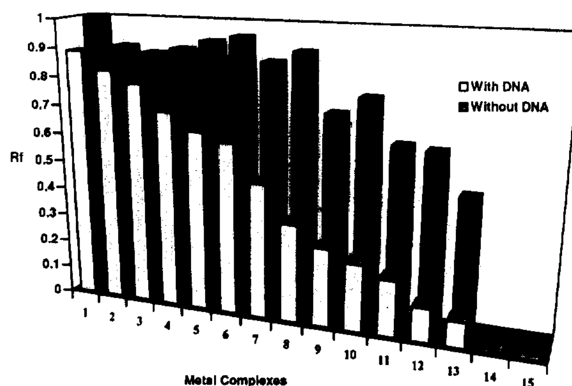

Fig. 8. A three-dimensional graph of the relative distances travelled on DNA-immobilised paper (white bars) and on untreated paper (filled bars) with elution by sodium acetate buffer (0.16 *M*, pH 6.90)-10% methanol. The numbers 1–15 on the graph represent the metal complexes listed below. A description of isomeric forms for these complexes is given in Ref. [42]. 1 = α-$[Ru(picenMe_2)(o\text{-pda})]^{2+}$, 2 = *rac*-$[Ru(4,4'\text{-bipyMe}_2)(phen)]^{2+}$, 3 = β-$[Ru(picenBz_2)(phen)]^{2+}$, 4 = *rac*-$[Ru(phen)_3]^{2+}$, 5 = Δ-β-$[Ru(R,R\text{-picchxnMe}_2)(dpq)]^{2+}$, 6 = β-$[Ru(picenBz_2)(o\text{-pda})]^{2+}$, 7 = β-$[Ru(picenBz_2)(2,3\text{-nap})]^{2+}$, 8 = α-$[Ru(picenBz_2)(dpq)]^{2+}$, 9 = *rac*-$[Ru(dpq)_3]^{2+}$, 10 = Δ-α-$[Ru(R,R\text{-picchxnMe}_2)(dpq)]^{2+}$, 11 = α-$[Ru(picenMe_2)(dppzMe_2)]^{2+}$, 12 = α,β-$[Ru(picenBz_2)(dppzMe_2)]^{2+}$, 13 = *rac*-$[Ru(dpqMe_2)_3]^{2+}$, 14 = *rac*-$[Ru(dppz)_3]^{2+}$, 15 = *rac*-$[Ru(dppzMe_2)_3]^{2+}$

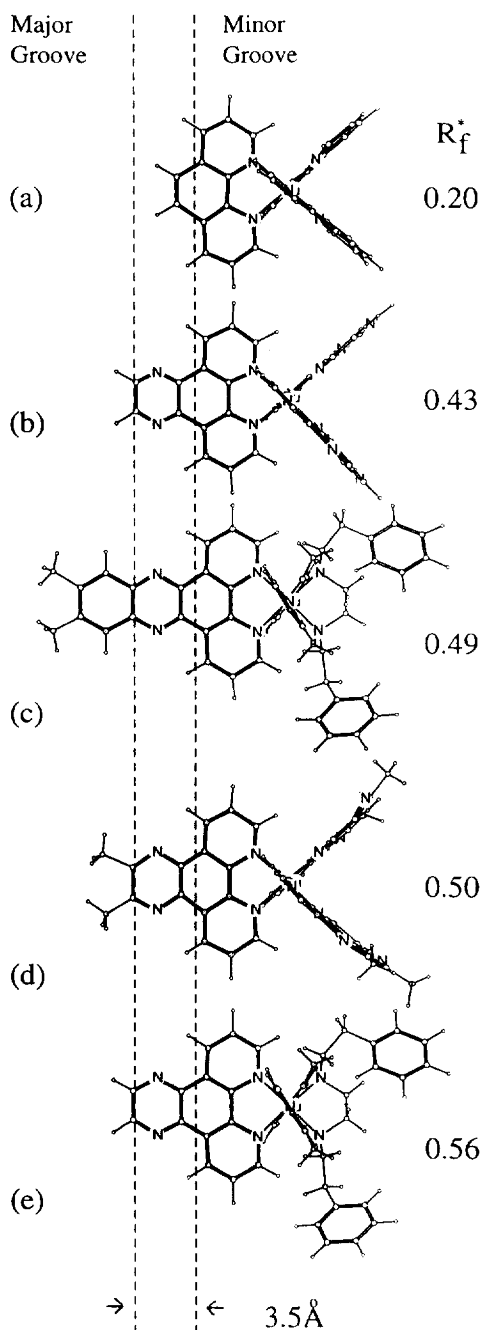

Fig. 9. (a) *rac*-$[Ru(phen)_3]^{2+}$, (b) *rac*-$[Ru(dpq)_3]^{2+}$, (c) α-$[Ru(picenBz_2)(dppz)]^{2+}$, (d) *rac*-$[Ru(dpqMe_2)_3]^{2+}$, (e) α-$[Ru(picenBz_2)(dpq)]^{2+}$.

picchxnMe$_2$)(dpq)]$^{2+}$, α-$[Ru(picenBz_2)(dpq)]^{2+}$ or rac-$[Ru(dpq)_3]^{2+}$, each of which have the same intercalative ligand, the difference being in the non-intercalating portion of the molecule.

The DNA stationary phase for HPLC has provided an effective means of resolving metal complexes like $[Ru(phen)_3]^{2+}$ and $[Ru(dpq)_3]^{2+}$, and in addition the relative binding efficiency of each complex can be estimated by virtue of its retention behaviour, using both HPLC and paper chromatography. The affinity of these complexes for DNA must be proportional to their retention times. For example, the $[Ru(dpq)_3]^{2+}$ species was retained by both immobilized DNA stationary phases to a greater extent than $[Ru(phen)_3]^2$.

Retention orders observed for Δ,Λ-$[Ru(phen)_3]^{2+}$ and Δ,Λ-$[Ru(dpq)_3]^{2+}$, were inconsistent with the reported findings of Barton et al. [35], although they are consistent with the achievable overlap in the cavity determined by computer modelling. This agreement is encouraging, for not only does it show that dpq is a suitable size for significant interaction but computer modelling also appears to be a reliable tool for future DNA-probe design.

Acknowledgements

We wish to thank the Macquarie University Research Grant Scheme, The British Council and the Australian Research Council for financial support and Dr K.R.N. Rao of Jones Chromatography Ltd., Hengoed, Wales (UK) for providing Apex propylamine-modified silica gel.

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