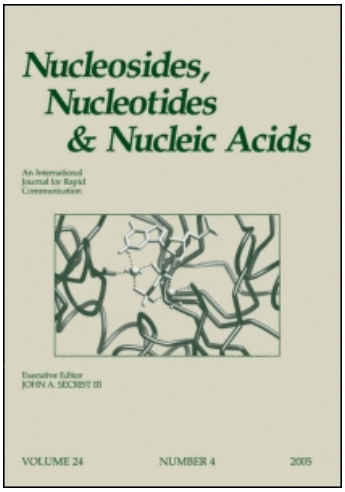

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Strong, Specific, Reversible Binding Ligands for Transfer Rna: Comparison By Fluorescence and Nmr Spectroscopies with Distamycin Binding for a New Structural Class of Ligand

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STRONG, SPECIFIC, REVERSIBLE BINDING LIGANDS FOR TRANSFER RNA: COMPARISON BY FLUORESCENCE AND NMR SPECTROSCOPIES WITH DISTAMYCIN BINDING FOR A NEW STRUCTURAL CLASS OF LIGAND

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ABSTRACT

Binding data are presented for the interaction with brewer's yeast $tRNA^{Phe}$ of a new structural family of ligands, symmetrical bis-benzimidazoles. In addition specific perturbations in chemical shifts were detected by 1-dimensional NMR spectroscopy at 400 MHz for some imino and aromatic methyl protons of $tRNA^{Phe}$ when the tRNA was titrated with distamycin. Competitive displacement of the benzimidazole by added distamycin was followed fluorescence spectroscopy.

INTRODUCTION

RNA is increasingly being recognised as a potential drug target, especially in the HIV field [1]. The accessibility of cellular RNAs to xenobiotics and paucity of RNA repair mechanisms means that RNA may offer a therapeutically relevant target for bleomycin [2] in particular, and drug design in general. Understanding the molecular features driving strong, specific binding to RNA is a prerequisite to designing RNA-directed ligands as drug leads [3]. *In vitro* selection of RNA sequences can determine preferred RNA sequences/structures for antibiotics, e.g. for neomycin [4,5] and tobramycin [6], but this approach does not produce new RNA binding structures. The complementary approach is to search for novel RNA ligand structures and binding details for diphenylfurans [7] and Rev peptide with RNA have appeared [8]. Following early studies of netropsin and distamycin binding weakly to RNA

(probably electrostatically to the phosphate backbone) [9], classical DNA minor-groove ligands, such as berenil [10] and benzimidazoles [11], have been found to interact strongly with RNA duplexes. However, the grooves of DNA and RNA differ in many respects. For example, 4'-6-diamidino-2-phenylindole (DAPI) binds to AT stretches of B-DNA in the minor groove but intercalates into AU-RNA [3]. In view of the accessibility of cellular RNAs to xenobiotics and the paucity of RNA repair mechanisms it has been recognised that RNA may offer a therapeutically relevant target for bleomycin [2] in particular, and drug design in general.

We have chosen transfer RNA as a model system with a well-defined tertiary structure to further our understanding of ligand design and recognition principles for RNA. However, there are relatively few types of reversible ligand so far reported for tRNA. These include metal ions, intercalators, polyamines, soft electrophiles, porphyrins, distamycin/netropsin and Hoechst 33258-type heterocyclics. This list is extended by reagents which cleave tRNA as these also often have a binding/ recognition component to their mechanism of action.

Whilst magnesium ions have long been known to specifically stabilise the tRNA tertiary structure, some metal ions bind and cleave RNA specifically [12,13]. Metal complexes, either native [14-16] or attached to an intercalator[17], have also been used to effect RNA scission. From X-ray studies of Pb^{2+} - induced cleavage of yeast $tRNA^{Phe}$ crystals[18], specific metal ion cleavages of tRNA have developed [12]. Self-cleaving RNA molecules (ribozymes) can undergo auto-cleavage at specific sites in the presence of Mn^{2+} ions [19]. However, metal ion effects in such RNA molecules are complex as Mg^{2+} ions (inactive alone) can enhance Mn^{2+}- cleavage of RNA under certain conditions[13]. There has been considerable recent emphasis on lanthanide ions to promote scission [12]. We have also shown that thallous (Tl^{+}) and thallic (Tl^{3+}) ions react specifically with the s^4U_8 residue of *E.coli* $tRNA^{Phe}$ [20]. The Tl^{+} ion has been used as a replacement for K^{+} in studies of aminoacyl - tRNA binding and peptidyl transferase activity of ribosomes [21].

Acriflavine [22] and acridine dyes [23,24] bind as intercalators to tRNA.

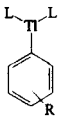
Soft electrophiles form a specific class of reagents for certain modified bases of tRNA. Based on the observation that organomercurials react specifically with the thiouridine (s^4U_8) in *E.coli* tRNA [25,26] and some years ago we introduced organothallium

(III) compounds **1** as specific, reversible binders for the thiouridine site in *E.coli* tRNA[20,27]. The mercurials may require tRNA structure to be denatured or labilised for reaction [25], but the organothallium compounds act specifically (1:1 stoichiometry) against native tRNA [20]. Arylthallium (III) binding is reversible to gel filtration, with binding constants in the region of low micromolar. Interest in arylthallium (III) modifiers of proteins and tRNA stemmed from the excellent NMR properties of ^{205}Tl (spin ½, natural abundance 70.48%, chemical shift range of ~ 2000 ppm, 19.2% as sensitive as the ^{1}H nucleus, Tl-H coupling constants are extremely large). This, along with the potential for two crosslinking sites on $ArTl_2$ (**1**), led to their evaluation against tRNA [20,27] and proteins [28,29].

Polyamine binding to yeast tRNA ^{Phe} has been studied extensively including the use of imino proton resonances [30] and ^{15}N NMR spectroscopy [31].

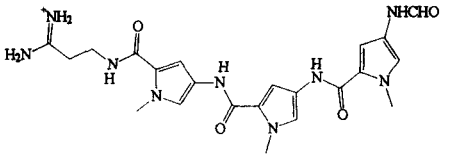
Porphyrins, which have been used to monitor RNA helix stacking [15], bind to tRNA [32] and NMR studies showed the site occupied to be in the TψC arm near residues T54 and ψ55 for brewer's yeast tRNA ^{Phe} [33].

Strong evidence that DNA minor groove binders can bind in the tRNA major (deep) groove is provided by the X-ray diffraction study of this tRNA with netropsin and



1

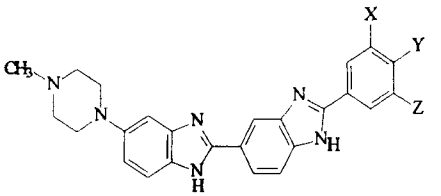
L = CF_3COO R = pCH_3 , oCO_2^-



2 Distamycin

distamycin (**2**), which shows specific hydrogen bonds to G51, U52 and G53 and electrostatic interactions with phosphates P61, P62 and P63 in this region [34]. Pullman then detected this netropsin-binding site on tRNA by theoretical calculations [35].

We have now found that Hoechst 33258 and its analogs bind strongly and specifically to tRNA. In the UV-visible absorption spectra of mixtures of tRNA (from brewers' yeast) and trihydroxy-Hoechst (**3**) the λ_{max} value of the ligand shifted from 326nm with increasing tRNA concentration, becoming ~338nm for a 1:1 molar mixture. Spectra were obtained for a series of tRNA : trihydroxy-Hoechst mixtures, varying the molar ratio



	X	Y	Z
3	OH	OH	OH
4	CH_3O	CH_3O	H
5	H	OH	H

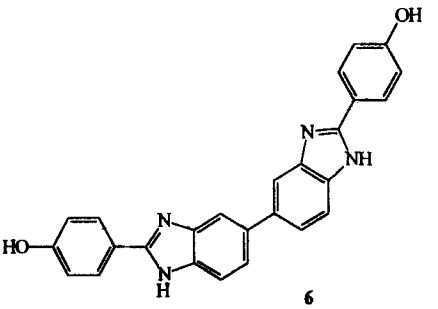
of the components but keeping a constant total concentration of the components and the corrected absorbances were plotted against the mole fraction of ligand as a Job plot which showed 1:1 complex formation and tight binding [36]. Addition of tRNA to solutions of many Hoechst-type analogs leads to fluorescence quenching of the free ligand's fluorescence [37], used to measure the binding constant for the binding of 3,4-dimethoxy-Hoechst (**4**) in 0.1M sodium phosphate buffer, pH 7.80 to $tRNA^{Phe}$, which obeyed equation (1) for single-site binding with K_{diss} =14.8 + 0.9 μM.

$$\text{Fluorescence(F)} = F_{max}\cdot[tRNA^{Phe}]/(K_{diss}+[tRNA^{Phe}]) \qquad (1)$$

The molecular environment of these ligands on the RNA is different from that on DNA as the fluorescence of Hoechst 33258 (**5**) is enhanced enormously on binding to the DNA minor groove but strongly quenched by tRNA.

In addition to metal ion-based cleavage, selective tRNA cleavage is effected by the chromophore of the enediyne C-1027 [38] and RNA is specifically and efficiently cleaved by Fe(II):bleomycin [2].

We now report the specific binding of a new class of bis-benzimidazole ligand to tRNA, with 2,2'-*bis*(4-hydroxyphenyl)-6,6'-*bis*-benzimidazole (**6**) as an example. We also describe 1-dimensional NMR spectroscopic studies of the binding of distamycin (2) to yeast $tRNA^{Phe}$. By using competitive fluorescence assays for ligand **6** in the presence of tRNA and distamycin we were able to further probe the binding site of symmetrical bisbenzimidazolic ligands of tRNA.



6

MATERIALS AND METHODS

$tRNA^{Phe}$ from brewer's yeast was obtained from Boehringer Mannheim. Distamycin was from Sigma Chemical Co. Stock solutions of tRNA were prepared in 0.1M sodium phosphate buffer pH7.8 for the purposes of this study. Ligand **6** was initially dissolved in dimethyl sulfoxide to make a 10mM stock solution, and then further diluted with phosphate buffer to the required concentration. Distilled water was further purified using a Millipore Milli-Q Water Purification System. Samples for fluorescence studies were filtered through a sterile Nalgene™ 0.2μm syringe filter.

Synthesis of 6

To a solution of 3,3'-diaminobenzidine (2.164g, 10mmol) in glacial acetic acid (40ml) was added ethyl 4-hydroxybenzimidate hydrochloride [39] (4.201g, 20mmol), 96% pure) and the mixture stirred over a water-bath under reflux and nitrogen for 4 hours to give a light brown mixture. Acetic acid was removed by rotovap, the residue dissolved in hot water (10ml) , a mixture of isopropanol: ammonia (10:1, 2ml) added and the resulting mixture stirred over a water bath under reflux for 1 hour. The precipitate was filtered off

and crystallized from methanol to give 3.52g (84% yield, two crops) of the cream product **6**. δ_H [270MHz, d_6-dmso], 6.90 (d, 4H, Ar-H, J= 8.6Hz), 7.59 (d, 2H, Ar-H, J = 8.25Hz), 7.68 (d, 2H, Ar-H, J = 8.6Hz), 7.84 (s, 2H, Ar-H), 8.68 (d, 4H, Ar-H, J = 8.6Hz), 10.19 (brs, 2H, Ar-OH). FAB mass spectrum: 421 [(M+H)+2, 8], 420 [(M+H)+1, 32], 419 [(M+H), 100].Accurate mass measurement: calculated for $C_{26}H_{19}N_4O_2$, 419.1508; found, 419.1525 (error 4ppm).

UV Spectral Measurements

All UV/Visible spectra were recorded at 25°C using a Peltier-thermostatted cuvette holder in a Cary UV-Visible spectrophotometer, model 1E, using the Cary Base System Software.

Fluorescence Spectral Measurements

Fluorescence emission spectra were recorded using a Hitachi fluorescence spectrophotometer, model F-2000. Derivative **6** was excited at 325nm corresponding to its λ_{max} value, determined by UV/Visible spectrophotometry.

Data Analyis

Data were analysed using the Grafit program 3.00 from Erithacus Software Ltd.

NMR Spectroscopy

NMR data were accumulated on a Varian VXR400S 400MHz NMR spectrometer equipped with a Sun 4/110 host computer. A dual (inverse) ^1H (X) probehead was used for proton detection. For each spectrum 30000 transients were acquired at 25°C with 1s recycle delay. Data were collected into 32K data points over a spectral width of 10 KHz giving a final resolution of 0.31 Hz/point. Data were processed using VNMR 4.3 software.

Molecular Graphics

Molecular modelling was carried out using SYBYL 6.3.3A (Tripos Associates).

RESULTS

We attempted to determine the site of binding by NMR line-broadening of specific imino protons of yeast $tRNA^{Phe}$, along the lines used for porphyrins [33], but were unable to do so for any of several Hoechst analogs tried because of solubility problems. Hence we took an indirect route based on the X-ray study of distamycin and netropsin binding to

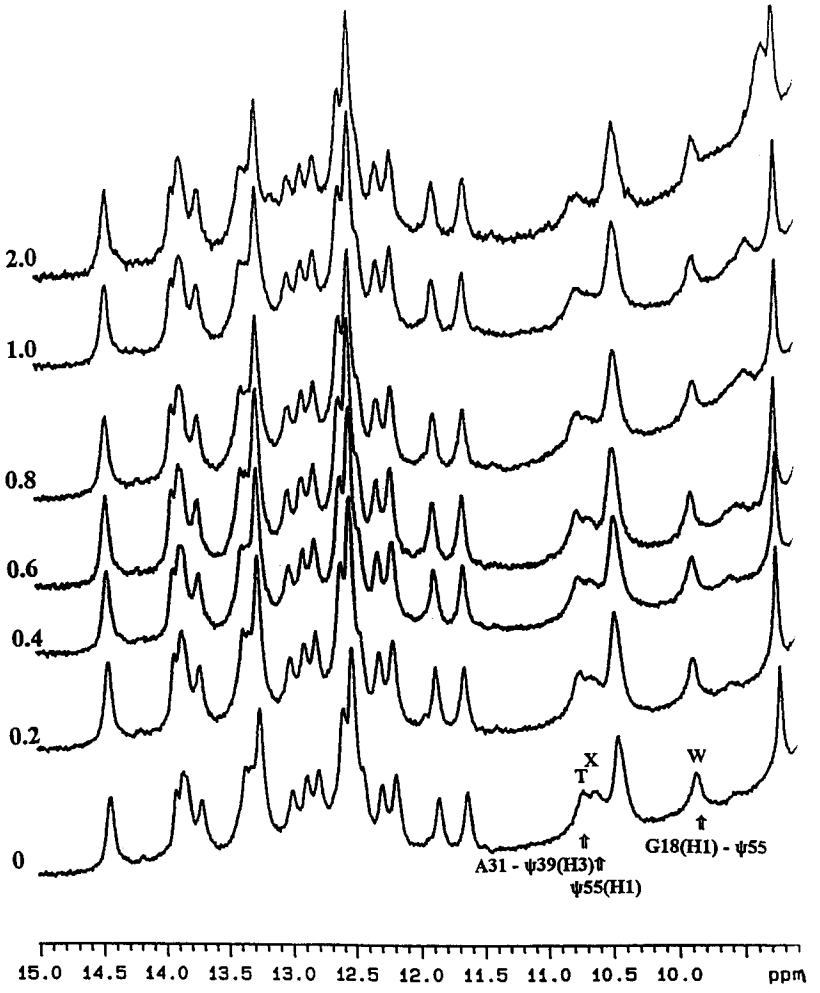


Figure 1. Titration of the imino-proton region of yeast $tRNA^{Phe}$ using 1D NMR spectroscopy with increasing mole ratios of distamycin as indicated. The imino protons for A31-ψ39(H3), ψ55(H1) and H1 of G18-ψ55 indicated are based on literature assignments [40]. The buffer composition was: 0.1M NaCl, 0.01M NaH_2PO_4/Na_2HPO_4 (pH 7.0), 0.005M $MgCl_2$ in H_2O/D_2O mixture (95:5 v/v). In all experiments the $tRNA^{Phe}$ concentration was 0.33 mM (0.2μmol in 0.6ml of buffer). The distamycin concentration was changed gradually from zero to 0.66 mM by stepwise addition of aliquots (4μl x 6) of 10mM stock distamycin solution to the 0.6ml NMR sample.

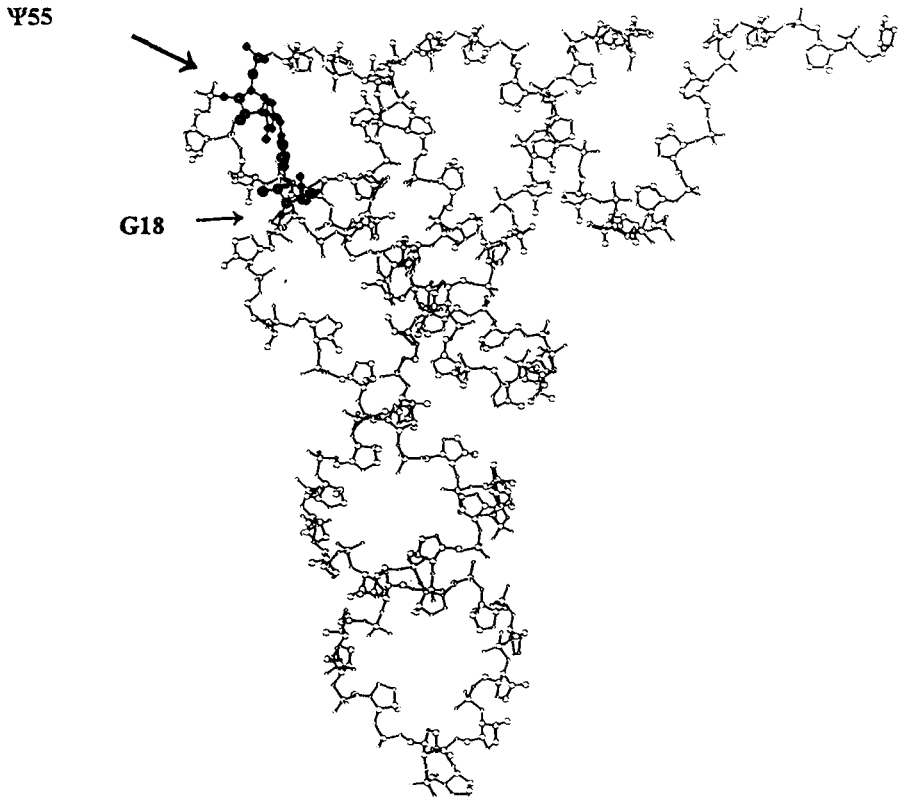


Figure 2A. Molecular graphics indication of resonances selectively broadened in yeast $tRNA^{Phe}$ by distamycin. Residues in bold are those providing the broadened imino protons shown in Figure 1. The yeast $tRNA^{Phe}$ coordinates were from the Brookhaven database [41].

tRNA [34]. We confirmed this binding mode for distamycin by selective NMR line-broadening of some of the imino resonances for yeast $tRNA^{Phe}$. Thus Figure 1 shows that residues ψ55(H1) and G18(H1) - to- ψ55 are selectively broadened based on these literature assignments of these peaks [40]. Porphyrins have been found to selectively broaden imino protons located on bases T54 and ψ55, located at the outer edge of the elbow-bend of tRNA [33]. We also found evidence of selective broadening by distamycin of aromatic methyl resonances arising from the RNA and that the three N-CH_3 proton resonances of distamycin are shifted upfield by ~3.8 ppm to 2.75 - 2.95 ppm in their tRNA complex (data not shown).

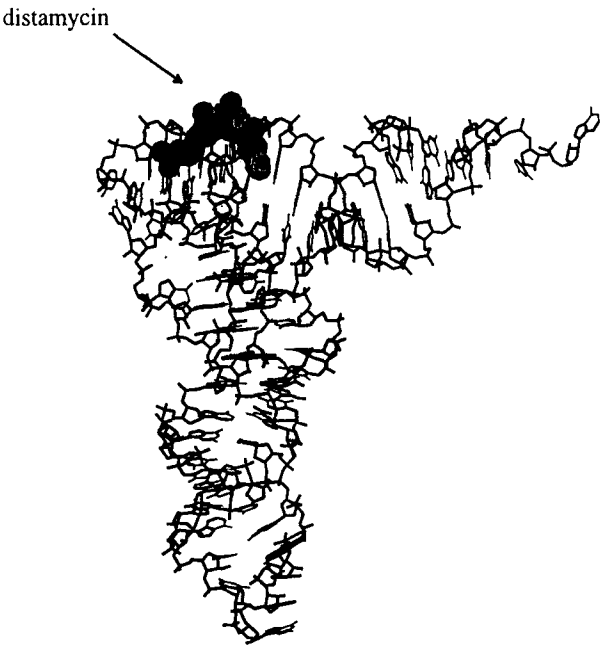


Figure 2B. Molecular model showing the distamycin site of yeast $tRNA^{Phe}$ using literature coordinates for tRNA [41] and data reported for the tRNA: distamycin complex analyzed by X-ray diffraction data [34].

Also a new resonance appeared at 9.6 ppm (shifting to ~ 9.4 ppm during titration) and probably indicates an additional hydrogen bond formed in the complex as it occurs inh the imino region. In Figure 2A, we highlight residues G18 and ψ55 which provide the imino resonances selectively broadened by distamycin: the tRNA structure was built using the the SYBYL programme (Tripos Associates) and the reported X-ray coordinates of yeast $tRNA^{Phe}$ [41]. These lie at one end of the general binding region reported by X-ray for distamycin [34], for which data are not in the Brookhaven Database, but for which we built a model (Figure 2B) using available tRNA coordinates [41] and published data for distamycin [34].

To detect whether benzimidazole-based ligands also bound to this site, we formed the 1:1 complex of yeast $tRNA^{Phe}$ with the novel ligand **6** which we have shown by UV

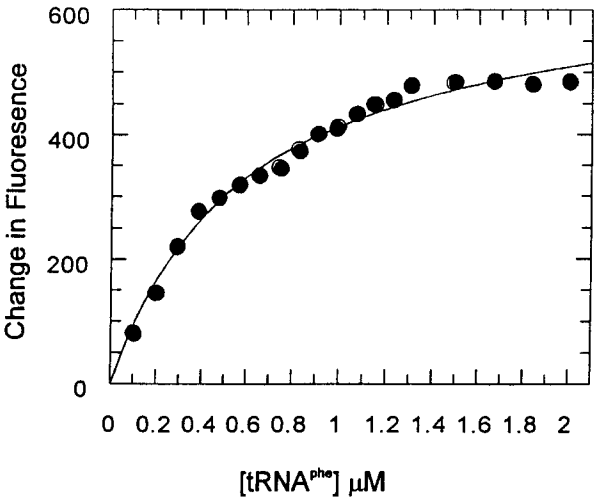


Figure 3. Plot of the change in fluorescence of *bis*-2,2'-bis (4-hydroxyphenyl)-6,6'-bis-benzimidazole (**6**) on addition of yeast $tRNA^{Phe}$. A sample (2ml) of **6** was excited at 325nm, and aliquots (20 μl) of 10 μM $tRNA^{Phe}$ were added with the fluorescence being recorded after each addition. Both the $tRNA^{Phe}$ and **6** were prepared in 0.1M Tris buffer containing 0.1M NaCl, 0.01M $MgCl_2$, pH 7.5. Points are experimental : line is theoretical for a single binding site with K_{diss} = 0.598±0.044 μM.

spectral shifts and Job plots binds to tRNA. The consequent quenching of the native fluorescence of the free ligand on binding to it of tRNA was used to determine the binding isotherm, see Figure 3, from which a dissociation constant for the complex of 0.598±0.044μM was measured.

Titration of this complex with increasing amounts of distamycin (Figure 4) led to release of free **6**, detected by the increase in fluorescence associated with non-bound **6**, with an apparent K_{diss} from the binding equation for a single-binding site of 9.6 ± 2.2 μM. Whilst conformational effects on tRNA cannot be excluded, the simplest explanation is that both ligands compete for the same or overlapping sites. Ligand **6** represents a new class of nucleic acid-directed ligand with a defined, specific binding site on tRNA.

The molecular structures of these families of ligands and the specific recognition of a particular region of tRNA (the benzimidazoles and distamycin selectively occupy a unique

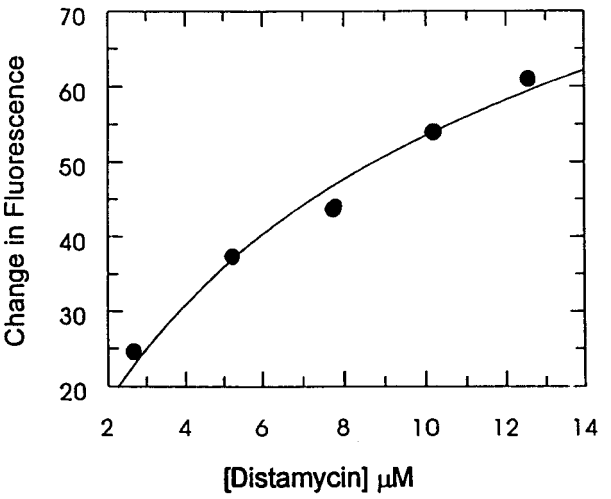


Figure 4. Fluorescence decrease of a 1:1 complex of yeast $tRNA^{Phe}$ with ligand **6** when distamycin concentration is increased. A solution containing 2ml of 0.1 μM **6** and 60μl of $tRNA^{Phe}$ (0.291μM) was excited at 325nm , and 30μl aliquots of 180μM distamycin were added, with the fluorescence being recorded after each addition. Both the $tRNA^{Phe}$ and the distamycin were prepared in 0.1M Tris buffer, containing 0.1M NaCl, 0.005M $MgCl_2$, pH 7.5. Points are experimental : line is theoretical for a single binding site with an apparent dissociation constant of 9.6± 2.2 μM.

run of 4-5 base-pairs out of approximately 75 bases) provides new lead structures to study the molecular basis of ligand:RNA recognition at an atomic level. The compounds also provide a rare example of stoichiometric ligand recognition by tRNA in a readily reversible equilibrium as opposed to covalent attack.

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