Fluorescent molecular sensing of amino acids bearing an aromatic residue

Luigi Fabbrizzi,* Maurizio Licchelli, Angelo Perotti, Antonio Poggi, Giuliano Rabaioli, Donatella Sacchi and Angelo Taglietti

Dipartimento di Chimica Generale, Università di Pavia, via Taramelli 12, I-27100 Pavia, Italy

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The tetra-amino tripodal ligand, **3**, containing two anthracene subunits, has been prepared through a multi-step synthesis and its solution properties have been studied by potentiometric and spectrofluorimetric techniques. The zinc(II) complex, $[Zn^{II}(\mathbf{3})]^{2+}$, which displays the typical emission of anthracene derivatives, interacts with natural amino acids, showing a particular affinity towards phenylalanine and tryptophan. This selective behaviour of the complex derives from its ability to establish two kinds of interaction: (i) a metal–ligand interaction between the zinc(II) ion and the amino acid's carboxylate group; (ii) a π-stacking interaction involving the aromatic moieties positioned on the complex and on the amino acid, inducing an extra stability of the adducts with tryptophan and phenylalanine (7–8 kJ mol^{-1}). The formation of the 1 **:** 1 adduct with tryptophan is signalled by a strong fluorescence quenching while no effect on the emission intensity has been observed in all other cases.

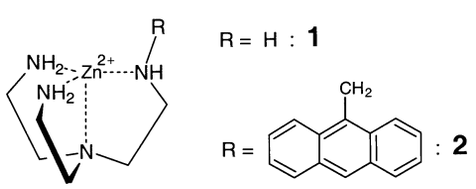
Introduction

Fluorescent molecular probes are currently and profitably used to detect a variety of analytes in biological fluids.[1] The field has been opened by Tsien and co-workers, who in the early 80's designed a photoactive molecule suitable for signalling the activity of Ca^{2+} within the cell,[2] in real time and in real space, by making use of the fluorescence microscopy technique.[3] Tsien's approach consisted of covalently linking through a spacer a selective receptor for Ca^{2+} (*e.g.* a derivative of the multidentate ligand ethylenebis(oxyethylenenitrilo)tetraacetic acid, EGTA) to a powerful fluorophore: ion binding drastically altered the emissive properties of the fluorophore (either an enhancement of the fluorescent intensity or a substantial shift of the emission band).[4] The Ca^{2+} cation plays a fundamental role in many functions at the cellular level, controlling (i) muscle contraction; (ii) nerve cell communication; (iii) hormone secretion; (iv) immune cell activation,[5] by increasing its concentration from 10^{-7} M, when in the resting state, to 10^{-6}—10^{-5} M, in the active state.[4,6] The fluorescent molecular probes introduced by Tsien (Indo-1, Fura-2, Fluo-3) are today routinely used in laboratories of cell physiology all over the world.[1a,d] The fluorophore–spacer–receptor synthetic approach was later extended by de Silva and co-workers to the design of molecular fluorescent sensors for alkali metal ions.[7] These systems were all based on a photoinduced electron transfer mechanism (PET): in the absence of the analyte (*e.g.* K^+), the emission of the fluorophore (an anthracene fragment) was quenched through an intra-molecular electron transfer process, which was interrupted when the metal ion fell down into the receptor subunit (*e.g.* an NO_5 crown).[8] Thus, metal recognition could be signalled through a sharp fluorescence revival.

PET fluorescent sensors for anions were first designed by Czarnik and co-workers, using a similar synthetic approach: in this case the receptor was a polyammonium ion and the covalently linked fluorophore was anthracene. In the present case, the hydrogen bonding interaction between the polyammonium subunit and the anion (*e.g.* $H_2PO_4^-$) interrupted an operating PET mechanism, thus inducing an enhancement of the anthracene emission.[9] More recently, Lehn and co-workers used more sophisticated cyclic and polycyclic polyammonium receptors to build fluorescent molecular sensors for a variety of anions.[10]

We have recently demonstrated that a type of interaction other than hydrogen bonding can be conveniently used for anion recognition and fluorescent sensing: the metal–ligand interaction.[11] Actually, metal–ligand interactions may have some significant advantages with respect to electrostatic interactions (which include hydrogen bonding): they are more energetic (a useful feature for compensating the strongly endothermic desolvation of the negatively charged analyte) and display a definite directional character.

In this connection, we showed that the $[Zn^{II}(tren)]^{2+}$ complex, **1** (tren: tris(2-aminoethyl)amine) is a useful platform for anion recognition: the metal complex exhibits a trigonal-bipyramidal stereochemistry and maintains a vacant axial position, available for the coordination of a further ligand, either a solvent molecule or an anion (Scheme 1). System **2**, in



Scheme 1

which the $[Zn^{II}(tren)]^{2+}$ platform has been equipped with an anthracene fragment, displays a special affinity towards the carboxylate group (*e.g.* of a benzoate anion, $PhCOO^-$): when the $PhCOO^-$ ion bears an electron-donor or -acceptor substituent (*e.g.*, $-N(CH_3)_2$ or $-NO_2$, respectively, in the 4-position), carboxylate binding to Zn^{II} induces the occurrence of an efficient electron transfer process between the photoexcited fluorophore and the substituent on $PhCOO^-$. As a consequence, benzoate recognition is signalled by a substantial quenching of the anthracene emission.[11a]

We considered that systems like **1** could be used for recognition of amino acids, which can bind the Zn^{II} centre through their $-COO^-$ residue. However, **1** itself is not a good receptor for amino acids, for two main reasons: (i) due to the

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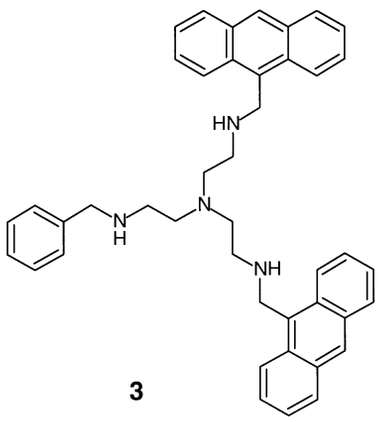
electrostatic repulsions between Zn^{2+} and the $-NH_3^+$ fragment of the zwitterion, the affinity of the amino acid towards the $[Zn^{II}(tren)]^{2+}$ platform is drastically reduced with respect to CH_3COO^- and $PhCOO^-$ (K decreasing to values lower than 10^3); (ii) in any case, the simple Zn^{II}–carboxylate interaction cannot provide any selectivity among amino acids. Thus, we considered equipping the $[Zn^{II}(tren)]^{2+}$ platform with further substituents, capable of establishing additional interactions with a given NH_3^+–CH(**R**)–COO^- amino acid. In particular, this extra-interaction has to be established with the **R** moiety, thus increasing affinity and, hopefully, providing selectivity.



3

In this connection, we synthesised derivative **3**, which contains two anthracenyl and one benzyl substituent. The corresponding Zn^{II} complex is expected to display selective affinity towards NH_3^+–CH(**R**)–COO^- amino acids, whose **R** residue has a π-nature and is therefore prone to establish π-donor–acceptor interactions. In this sense, the $[Zn^{II}(\mathbf{3})]^{2+}$ complex appears as a reasonable candidate for fluorosensing phenylalanine and tryptophan, provided that recognition induces a drastic modification of the emission of the photoexcited anthracene fragment. This article describes the investigation of the solution equilibria involving $[Zn^{II}(\mathbf{3})]^{2+}$ and natural amino acids and the study of the photophysical effects induced by the recognition process. It will be shown that $[Zn^{II}(\mathbf{3})]^{2+}$ is an efficient fluorescent molecular sensor of tryptophan, whose recognition is signalled through a substantial quenching of the anthracene emission.

Results and discussion

Design of the $[Zn^{II}(tetramine)]^{2+}$ platform and characterisation of its photophysical behaviour in solution

The selectively substituted tripodal tetramine **3** was prepared through the synthetic procedure illustrated in Scheme 2.

In particular, two anthracene subunits were appended at the terminal atoms of the triamine 2,2′-diaminodiethylamine, through Schiff base condensation with anthracene-9-carbaldehyde, to give **4**. Nucleophilic addition of **4** to the bromoimine fragment **5**, which supplies the benzyl substituent, gave the unsaturated derivative **6**, whose C=N double bonds were hydrogenated with sodium borohydride.

The protonation state of **3** (indicated as L in the following) in a dioxane–water solution (4 : 1, v/v), at 25 °C, was determined through potentiometric titration experiments. In particular, the following species were found to be present at the equilibrium, over the investigated pH range: LH_3^{3+} (pK_{A1} = 2.24), LH_2^{2+} (pK_{A2} = 7.19), LH^+ (pK_{A3} = 8.54), and L (see Experimental section). The % concentrations of the different species are shown in the distribution diagram in Fig. 1. Although the determination has been carried out over the 2–12 pH range, the species distribution plots have been extrapolated to $-\log[H^+] = 1$ in order to follow the abundance increase of the LH_3^{3+} species

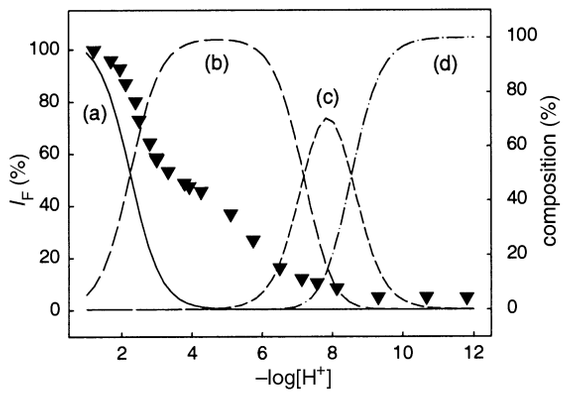
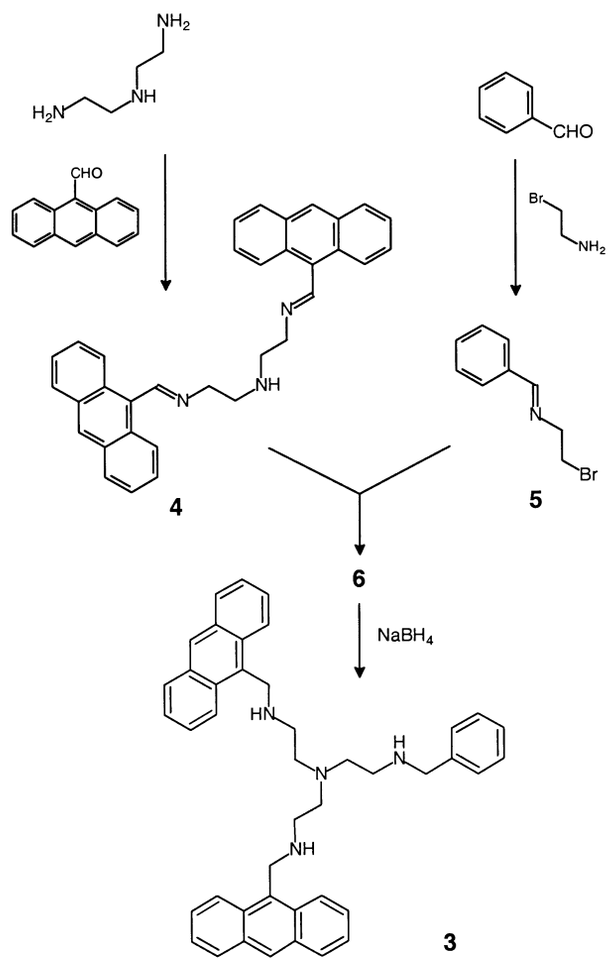


Fig. 1 Right vertical axis, distribution curves of the species present at equilibrium for ligand **3** (L) in 4 : 1 dioxane–water solution at 25 °C: (a) LH_3^{3+}; (b) LH_2^{2+}; (c) LH^+; (d) L. Left vertical axis (full triangles), dependence of the fluorescence intensity of the solution, I_F, on $[H^+]$.

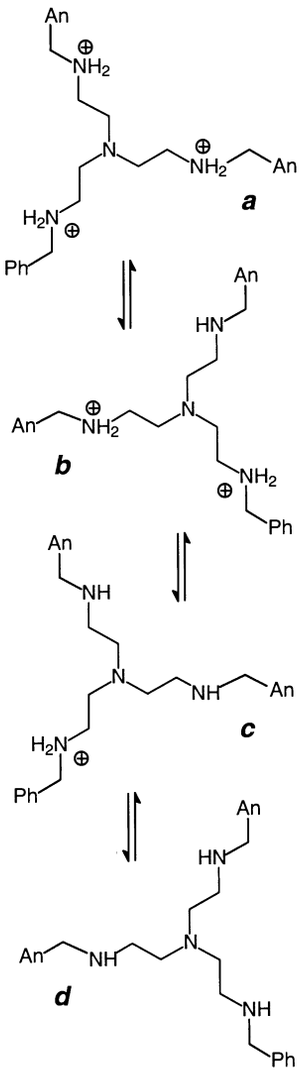


Scheme 2

which, at such a high $[H^+]$ value (10^{-1} M) is the predominant species in solution.

The emission properties of system **3** at varying pH were investigated through a spectrofluorimetric acid–base titration experiment. In particular, a solution of **3** containing excess acid was titrated with standard sodium hydroxide and the emission spectra were recorded after each addition of base. Fluorescence measurements within the pH range 1–2, in which the electrode response is not reliable, were carried out on solutions containing known amounts of acid. The profile of the emission intensity, I_F (at λ_{max} = 413 nm) has been superimposed on the distribution diagram in Fig. 1. The highest emission intensity (I_F = 100) is observed at the beginning of the titration ($-\log[H^+] = 1$), where the dominating species is LH_3^{3+}.

It is suggested that in this species the three protons are bound to the terminal groups of the tripodal tetramine (see formula *a* in Scheme 3). Even if the tertiary amine group itself is more



Scheme 3

basic than a primary one, in a polar medium, the situation illustrated by *a* minimises the electrostatic repulsions between the three ammonium groups. On decreasing $[H^+]$, LH_3^{3+} deprotonates to give LH_2^{2+}, while the emission intensity decreases to reach a short plateau at a pH value slightly higher than 4. Noticeably, the plateau (or the pronounced flex) corresponds to the formation of 100% of the doubly protonated species LH_2^{2+}. In this connection, it is suggested that the $LH_3^{3+} \longrightarrow LH_2^{2+}$ deprotonation step involves one of the two anthracenylammonium groups, which, due to the presence of an extended aromatic substituent, are expected to be more acidic than the benzylammonium group. At this stage, an intramolecular electron transfer process takes place within the LH_2^{2+} species (formula *b* in Scheme 3) from the amine group to the nearby photoexcited anthracene fragment, quenching its emission. A photo-induced electron transfer process from an amine group to a proximate anthracene subunit is a documented phenomenon, which has allowed the development of a class of fluorescent pH indicators.[12] Under the irradiation conditions in the spectrofluorimetric cuvette, excitation may involve either the anthracene fragment bound to the amine group (electron transfer takes place) or that bound to the ammonium group (electron transfer does not take place): this situation accounts for the fact

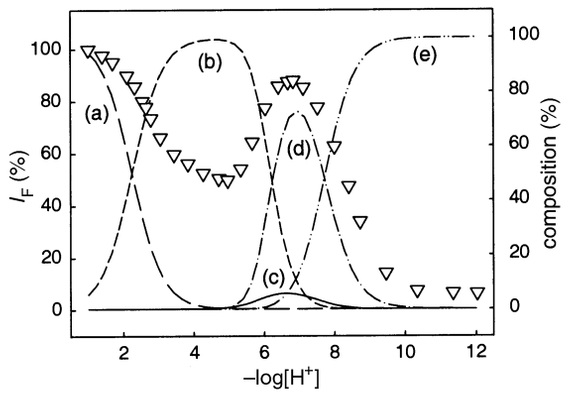


Fig. 2 Right vertical axis, distribution curves of the species present at equilibrium for the system **3** (L, 1 equiv.)–Zn^{II} (1 equiv.) in 4 : 1 dioxane–water solution at 25 °C: (a) LH_3^{3+}; (b) LH_2^{2+}; (c) LH^+; (d) $[Zn(L)]^{2+}$; (e) $[Zn(L)(OH)]^+$. Left vertical axis (▽), dependence of the fluorescence intensity of the solution, I_F, on $[H^+]$.

that the relative emission intensity at the flex (100% of LH_2^{2+} present) is about 50% of the original one.

On further deprotonation, which produces the LH^+ species, a further drastic decrease of the emission intensity is observed. This is an expected behaviour, because in the LH^+ species (formula *c* in Scheme 3) both anthracenylammonium groups have been deprotonated and, no matter which of the two anthracene fragments has been excited, its emission will be killed by the adjacent amine group, through an electron transfer mechanism.

The last deprotonation step, $LH^+ \longrightarrow L$, occurring at pH > 8, induces a slight, yet detectable, decrease of the fluorescent emission. This would also indicate that the benzylamine group, now deprotonated, is in some way capable of transferring an electron to a distant photoexcited anthracene fragment. This process is expected to take place according to a "through space" mode, *i.e.* following the occasional contact of the dangling ethylamine arms of L (formula *d* in Scheme 3). Thermal motions of the molecular framework can take place easily in L, which is no longer stiffened by electrostatic repulsions.

Analogous titration studies, both potentiometric and spectrofluorimetric, were carried out on system **3**, in the presence of an equivalent amount of Zn^{2+}. In particular, curve fitting analysis of the potentiometric titration profile by a non-linear least-squares method indicated that the following coordination complexes are present at the equilibrium: $[Zn^{II}(L)]^{2+}$, which reaches its maximum concentration (75%) at pH ≅ 7, and $[Zn^{II}(L)(OH)]^+$, which at pH ⩾ 9 is present at 100%. It is hypothesised that both complexes exhibit a trigonal-bipyramidal stereochemistry, such as that shown in Scheme 1. In the $[Zn^{II}(L)]^{2+}$ species, the vacant axial position is occupied by a water molecule. With increasing pH, the coordinatively bound water molecule deprotonates, and the $[Zn^{II}(L)(OH)]^+$ complex forms.

In Fig. 2, the profile of the spectrofluorimetric titration has been superimposed on the distribution diagram: the highest emission is observed at the highest investigated $[H^+]$ value, 10^{-1}, and its intensity decreases with $[H^+]$. However, at pH = 5, I_F stops decreasing and starts to increase, to reach a maximum at pH ≅ 7. At higher pH values, the fluorescent emission decreases again, its quenching being complete at pH ≥ 9. The spectrofluorimetric behaviour can be interpreted in terms of pH controlled formation of the Zn^{II} complexes of **3**. Until pH = 5, no metal complex is formed: Zn^{2+} is present as an aqua ion and does not interact with the various protonated forms of **3**. Thus, the photophysical behaviour at pH values <5 is the same as observed in the titration experiment in the absence of metal, as illustrated in Fig. 1. At pH = 5, the $[Zn^{II}(L)]^{2+}$ complex begins to form: in these circumstances, all the amine groups of **3** are engaged in the coordination of the metal and are no longer

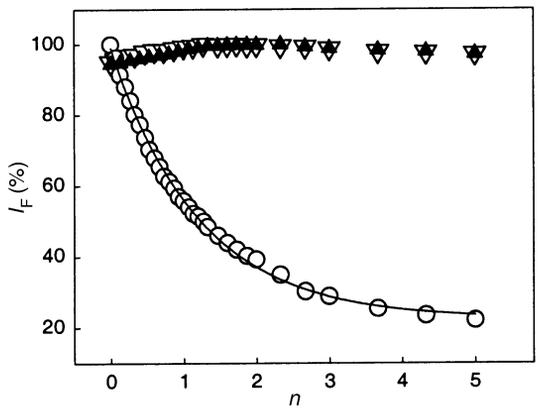


Fig. 3 Variation of the relative fluorescence intensity, I_F, of a 10^{-5} M $[Zn(L)]^{2+}$ solution with tryptophan (circle), phenylalanine (full triangle) and glycine (triangle). n = number of equivalents of the added amino acid.

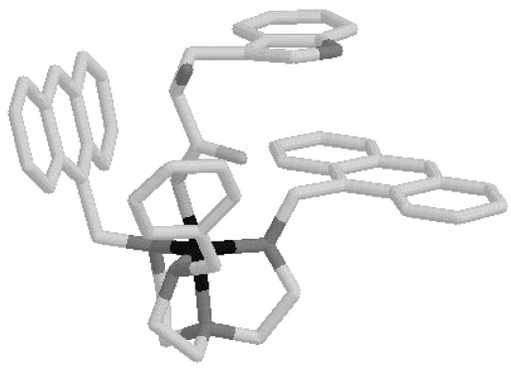


Fig. 4 Molecular model for the $[Zn(L)(trp)]^{2+}$ adduct as obtained by using the HyperChem software package (MM+ force field). Hydrogens are omitted for clarity. Zinc(II) ion is in a trigonal-bipyramidal coordinative arrangement; the indole moiety of trp and one of the anthracene subunits lie in parallel planes at a distance of 3.5–3.6 Å.

available to transfer electrons to the photoexcited anthracene fragment. As a consequence, any electron transfer mechanism is interrupted and fluorescence is restored. Interestingly, the highest emission is reached at pH ≅ 7, corresponding to the highest concentration of the $[Zn^{II}(L)]^{2+}$ complex. With increasing pH, the concentration of $[Zn^{II}(L)]^{2+}$ decreases, to the advantage of the $[Zn^{II}(L)(OH)]^{+}$ species. Accordingly, the fluorescence intensity I_F decreases to fully quenched, corresponding to 100% formation of the hydroxide-containing metal complex (pH ≥ 9). Quenching is ascribed to the occurrence of an intra-complex electron transfer process from the electron rich OH^- anion to the nearby photoexcited anthracene fragment. Photoinduced electron transfer processes from metal-bound hydroxide to a proximate fluorophore have been observed in analogous zinc(II) containing systems.[11b,13] These investigations have shown that a solution containing equimolar amounts of **3** and Zn^{2+}, as the triflate salt, adjusted to pH ≅ 7, is appropriate for carrying out recognition studies on amino acids. In fact, at this pH, the dominating species is $[Zn^{II}(L)]^{2+}$, whose recognition site, one of the axial positions of the trigonal-bipyramid, is occupied by a labile and weakly bound water molecule, which can be replaced by the carboxylate group of the amino acid. Moreover, quite interestingly for sensing purposes, the $[Zn^{II}(L)]^{2+}$ receptor displays the most intense fluorescent signal.

Fluorosensing of tryptophan

A neutral solution 10^{-4} M both in **3** and in Zn^{2+} buffered at pH 6.8 with lutidine was titrated with a standard solution of a given amino acid. Among all the natural amino acids, only tryptophan (trp) induced a modification of the fluorescent emission of the anthracene fragment of $[Zn^{II}(L)]^{2+}$, in particular causing substantial quenching.

The profile of the spectrofluorimetric titration (relative fluorescence intensity at 413 nm, I_F, *vs.* equiv. of amino acid) is shown in Fig. 3. Least-squares analysis of the titration profile was consistent with the formation of a 1 : 1 receptor–amino acid adduct and the constant for the association equilibrium ($[Zn^{II}(L)]^{2+}$ + trp ⇌ $[Zn^{II}(L)(trp)]^{2+}$) was 4.28 ± 0.04 log units.

Molecular modelling studies performed on the $[Zn^{II}(L)(trp)]^{2+}$ adduct in a vacuum suggest that the amino acid is bound to the zinc(II) polyamine receptor not only through the metal–carboxylate coordinative interaction, but also through a donor–acceptor interaction of π nature, involving the indole residue of the amino acid and at least one of the anthracene substituents on the tetramine (see Fig. 4). Therefore, the substantial quenching of the fluorescence is ascribed to the occurrence of an electron transfer (eT) process from the indole subunit (donor, D) to the photoexcited anthracene fragment (acceptor, A*). The electron transfer nature of the process is demonstrated by the fact that in an ethanolic solution of $[Zn^{II}(L)(trp)]^{2+}$, vitrified at 77 K, the fluorescence is fully restored (*i.e.* its intensity is equal to that of a solution of plain $[Zn^{II}(L)]^{2+}$, under the same conditions). In this connection, it has to be considered that an electron transfer process causes the formation of an ion pair ($A^- \sim D^+$), a process which is accompanied by a drastic rearrangement of the solvent molecules, which have to solvate the ion pair. Immobilisation of the solvent molecules in the glass at 77 K strongly disfavours ion pair formation and prevents the occurrence of the eT mechanism, thus allowing the radiative decay of the photoexcited fragment to take place and reviving the fluorescence.[14] With the exception of a few examples of ultrafast eT processes,[15] the reviving of fluorescence in frozen solution can therefore be considered as evidence that an eT rather than an energy transfer process is active.

Thus, spectrofluorimetric studies have shown that the $[Zn^{II}(\mathbf{3})]^{2+}$ complex, in a solution adjusted to pH ≅ 7, is an efficient sensor of tryptophan, operating through an electron transfer mechanism. However, this investigation does not say anything about selectivity with respect to other amino acids. What can be concluded is that, whatever the affinity of the other amino acids for the $[Zn^{II}(\mathbf{3})]^{2+}$ receptor, no mechanism exists that perturbs the emission of the anthracene substituent. Therefore, the affinity of $[Zn^{II}(\mathbf{3})]^{2+}$ towards amino acids other than tryptophan has to be assessed by looking at a different property than fluorescence. A convenient property to investigate is the absorbance in the UV region. In particular, the $[Zn^{II}(\mathbf{3})]^{2+}$ complex presents intense absorption bands in the range 350–400 nm, to be assigned to π–π* transitions pertinent to the aromatic substituents on the tetramine. The absorbance of these bands is significantly altered on titration with a given amino acid.

Fig. 5 shows how the absorbance at 388 nm varies with the addition of a standard tryptophan solution to a solution 10^{-4} M in both **3** and Zn^{2+}, adjusted to pH = 6.8. The absorbance *vs.* equiv. profile is consistent with the formation of a 1 : 1 adduct, whose association constant log K is 4.21 ± 0.02 (in good agreement with the value obtained by spectrofluorimetry). Analogous titration experiments were carried out with other natural amino acids, AA: the absorbance *vs.* equiv. profiles corresponded in any case to the formation of a 1 : 1 association complex $[Zn^{II}(\mathbf{3})(AA)]^{2+}$ and the constants for the pertinent association equilibrium ($[Zn^{II}(\mathbf{3})]^{2+}$ + AA ⇌ $[Zn^{II}(\mathbf{3})(AA)]^{2+}$) were in all cases around 3 log units, *i.e.* more than one order of magnitude lower than for trp. There was however one exception: phenylalanine (phe), whose log K value for the association equilibrium was found to be 4.48 ± 0.05. It is hypothesised that the extra stability shown by the $[Zn^{II}(\mathbf{3})(phe)]^{2+}$ adduct is due to the establishing of π interactions between the phenyl residue of the amino acid and the aromatic substituent(s) on the

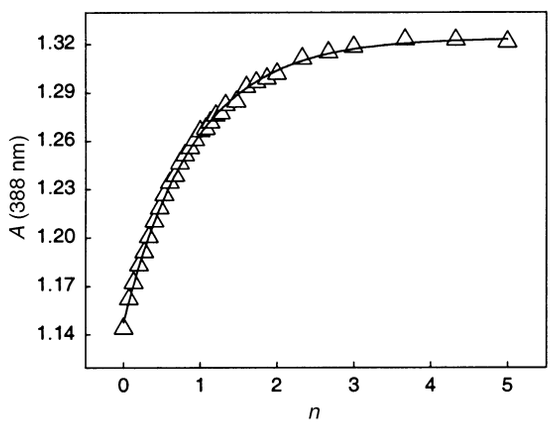


Fig. 5 Spectrophotometric titration of a 10^{-4} M $[Zn(L)]^{2+}$ solution with tryptophan. The solid line indicates the best fit curve. n = number of equivalents of the added tryptophan.

polyamine framework of the receptor, in a similar way to that observed in the case of trp. However, the phenyl substituent on phe displays lower electron donor tendencies than the indole subunit of trp and is not able to transfer one electron to the facing photoexcited anthracene subunit, whose fluorescent emission is not altered. In conclusion, $[Zn^{II}(\mathbf{3})]^{2+}$ selectively interacts with natural amino acids bearing an aromatic residue, trp and phe, but it signals, through a drastic decrease of the fluorescent emission, only the recognition of trp.

Spectrofluorimetric titration experiments with tryptophan on a $[Zn^{II}(\mathbf{3})]^{2+}$ solution containing phenylalanine confirmed that the latter amino acid interacts with the receptor and competes with the former for the fluorescent sensor, therefore behaving as an interferent. Moreover, some empirical considerations can be made on the energetics of the interactions within the $[Zn^{II}(\mathbf{3})(AA)]^{2+}$ association complex. The log K for the association equilibrium involving glycine is 3.06 ± 0.06 log units, whereas for the negatively charged *N*-acetylglycine derivative a value of 3.51 ± 0.03 has been found. The ~0.5 log units difference, which corresponds to ~3 kJ mol^{-1}, can be explained in terms of electrostatic repulsion between the ammonium group of the amino acid and the Zn^{2+} centre. Thus, the energy associated with the simple metal–carboxylate interaction, in the absence of electrostatic repulsive effects, can be estimated as around 20 kJ mol^{-1}. The extra stability due to π-interactions, as observed for trp and phe, ranges from 7 (trp) and 8 (phe) kJ mol^{-1} (Δlog K with respect to glycine being 1.2 and 1.4, respectively). Thus, π-interactions contribute to the formation of the association complex to a distinctly lesser extent than metal–ligand interactions. Yet, this π-contribution is essential in determining a selectivity pattern in favour of trp and phe.

Experimental

UV-vis spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. All fluorescence measurements were carried out on a Perkin-Elmer LS-50B luminescence spectrometer. Emission spectra at 77 K were measured in dry ethanol (10^{-5} M), by using quartz sample tubes and the same luminescence spectrometer equipped with a Perkin-Elmer low temperature luminescence accessory. Mass spectra were obtained with a Finnigan TQS 700 mass spectrometer. NMR data were obtained on a Bruker AMX 400 MHz.

Potentiometric titrations

Potentiometric determinations were performed in dioxane–water 4 : 1 v/v (0.1 M KNO_3, 25 °C) according to the instrumental and general procedures reported elsewhere.[16] Protonation and complexation constants were determined by using the HyperQuad software package.[17] Electrode calibration was carried out according to Gran's method.[18] The experimental details are summarized in Table 1.

Table 1 Summary of experimental parameters for the system zinc(II)–*N*-anthracen-9-ylmethyl-*N*′-[2-(anthracen-9-ylmethylamino)ethyl]-*N*′-[2-(benzylamino)ethyl]ethane-1,2-diamine

Solution composition	
$[T_L]$range/mol dm^{-3}	0.001–0.003
$[T_M]$range/mol dm^{-3}	0.001–0.003
I/mol dm^{-3}, electrolyte	0.1, KNO_3
pH range	2.0–11.8
Experimental method	pH titration, calibrated in concentration
T/°C	25
Total number of data points	
Protonation	132 (2 titrations)
Complexation	144 (2 titrations)
Method of calculation	HYPERQUAD[17]
Protonation and stability constants (errors as σ)	
$\log \beta_{LH}$	8.54 ± 0.04
$\log \beta_{LH_2}$	14.73 ± 0.07
$\log \beta_{LH_3}$	17.97 ± 0.10
$\log \beta_{ZnL}$	6.55 ± 0.12

Spectrofluorimetric titrations

pH titrations were performed on 10^{-4} M dioxane–water 4 : 1 solutions of **3** (or **3** and $Zn(ClO_4)_2$) adjusted to pH ~2 by adding small amounts of a standard solution of $HClO_4$. Then, additions of a standard 0.1 M NaOH solution were made until a basic pH (>10) was obtained. Emission spectra (excitation wavelength 366 nm) were recorded after each addition of base. Fluorescence measurements at $[H^+]$ higher than 10^{-2} M were perfomed on 10^4 M solutions containing known amounts of $HClO_4$.

Titrations at neutral pH were carried out on dioxane–water 4 : 1 solutions (10^{-4} M) of equimolecular mixtures of **3** and $Zn(ClO_4)_2$, buffered with lutidine (0.025 M, pH 6.8). Small amounts of aqueous solution (0.01 M) of the selected amino acid were added and spectra recorded after every addition. Very similar experimental conditions were used for the spectrophotometric titrations.

Association constant values were determined by non-linear regression analyses of absorbance or fluorescence intensity data *versus* amino acid concentration.[17]

N-Anthracen-9-ylmethylene-*N*′-[2-(anthracen-9-ylmethylene-amino)ethyl]ethane-1,2-diamine (4)

A solution of 2,2′-diaminodiethylamine (dien, 1 mmol) in MeCN (20 ml) is slowly added to a solution of anthracene-9-carbaldehyde (2 mmol) in MeCN (30 ml) over a period of 1 h under magnetic stirring at room temperature. Stirring is continued for 24 h and the yellow precipitate is collected by vacuum filtration (69%). Found: C, 84.98; H 6.07; N, 8.52%. $C_{34}H_{29}N_3$ requires: C, 85.14, H 6.09, N 8.76%.

Benzylidene-(2-bromoethyl)amine (5)

2-Bromoethylamine hydrobromide (2.5 mmol) was dissolved in 1 M NaOH solution (30 ml) and extracted with CH_2Cl_2 (5 × 30 ml). The combined organic extracts were concentrated (30 ml) and slowly added to a solution of benzaldehyde (1 mmol) in MeCN (25 ml) at room temperature. The resulting solution was stirred for an hour. The oil obtained after removing the solvent (yield 78%) was used directly in the following step.

N-Anthracen-9-ylmethylene-*N*′-[2-(anthracen-9-ylmethylene-amino)ethyl]-*N*′-[2-(benzylideneamino)ethyl]ethane-1,2-diamine (6)

Diimine **4** (0.5 mmol) was dissolved in a suspension of Cs_2CO_3

(0.5 mmol) in toluene (30 ml). The mixture was heated at 50 °C and stirred under a nitrogen atmosphere. A solution of **5** (0.6 mmol) in toluene (20 ml) was added dropwise, then the mixture was refluxed for 24 hours. After removing the solvent, the solid obtained was treated with MeCN and the unreacted compound **4** was filtered off. MeCN was evaporated *in vacuo* and the product was used without further purification (yield 60%).

N-Anthracen-9-ylmethyl-*N*′-[2-(anthracen-9-ylmethylamino)-ethyl]-*N*′-[2-(benzylamino)ethyl]ethane-1,2-diamine (3)

$NaBH_4$ (0.7 g) was added in small portions to a solution of **6** (2 mmol) in methanol (100 ml) at 40 °C, then the solution was refluxed under stirring overnight. The solvent was removed with a rotary evaporator, giving a solid which was dissolved in 5% HCl solution. The resulting solution was heated at 50–60 °C for 30 minutes, made alkaline by addition of NaOH solution and extracted with CH_2Cl_2 (10 × 30 ml). The combined organic phases were dried over Na_2SO_4 and the solvent was removed *in vacuo* giving an oily product (yeld 90%). ^{1}H-NMR (400 MHz; $CDCl_3$; Me_4Si) 8.30 (2H, s, aromatic-H of anthracene), 8.20 (4H, d, aromatic-H of anthracene), 7.90 (4H, d, aromatic-H of anthracene), 7.30 (8H, m, aromatic-H of anthracene), 7.20 (5H, m, aromatic-H of phenyl), 4.60 (4H, s), 4.50 (2H, s), 2.80–2.90 (6H, m), 2.60–2.70 (6H, m). MS (ESI): 617 ($[M + H]^+$).

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