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A fluorescent molecular ruler as a selective probe for ω-aminoacids†

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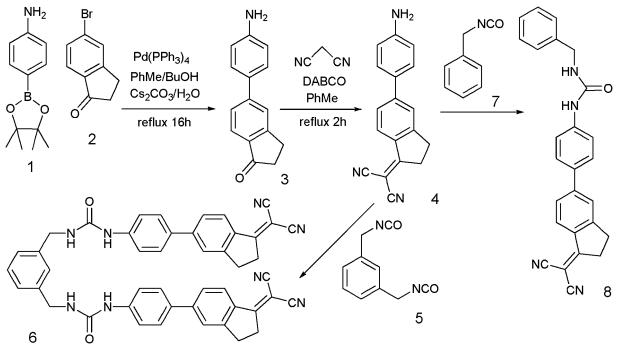
A fluorescent probe for ω-aminoacids behaves as a molecular ruler, changing the yellow fluorescent emission into blue as a function of the distance between the terminal ammonium and the carboxylate groups, permitting the quantitative detection of ω-aminoacids, their metabolites and related drugs, such as pregabalin or gabapentin, from pharmaceutical formulations.

The concept of a molecular ruler has been very fruitful in the biological sciences for measuring distances between groups of biomolecules in dynamic processes,[1] and in materials chemistry for investigating distance-dependent emission behaviours of fluorophores[2] or super-resolution microscopy.[3] Molecular rulers of low molecular weight have been much less studied; in a relevant example, a molecular ruler was used to unambiguously assign two stereoisomers of bis-porphyrins.[4] This simple concept may have useful applications for the detection of natural aminoacids but it was never applied before. Except for the reactive cysteine and related thiol-containing aminoacids,[5] fluorescent probes for selective recognition of unprotected aminoacids are uncommon,[6] as well as probes detecting protected aminoacids,[6a,7] and are mostly oriented to the selective recognition of natural aminoacids and their unnatural enantiomers. We have applied the molecular ruler concept to the study of the interactions of proteinogenic as well as non-proteinogenic aminoacids and a classic bis-urea receptor tagged with two units of a new highly solvatochromic fluorescent indicator. In this communication we report our findings in the selective fluorescent discrimination between different classes of ω-aminoacids and their zwitterionic metabolites.

The synthesis of fluorescent probes is depicted in Scheme 1.

Thus, the Suzuki reaction of aryl boronate **1** and 5-bromoindanone **2** catalyzed by $Pd(PPh_3)_4$ in toluene/butanol/water in the presence of $CsCO_3$ gave aminoketone **3** in 91% yield. The Knoevenagel reaction of **3** and malononitrile in the presence of DABCO in toluene at reflux for 2 h gave aminoindane **4** in 95% yield. Compound **4** is a pale yellow (λ_{max}^{abs} = 398 nm, ε = 34 500 M^{-1} cm^{-1}, in CH_2Cl_2), highly solvatochromic fluorescent indicator (λ_{max}^{em} = 537 nm, λ_{max}^{exc} = 366 nm, Φ = 0.6 in CH_2Cl_2) (see ESI†), with a reactive amino group that



Scheme 1 Synthesis of fluorescent probes **6** and **8**.

permitted its insertion in classic receptors. Reaction of **4** with bis-isocyanate **5** in chloroform for 2 days at room temperature afforded bisurea **6** in 75% yield. Monourea **8** was obtained in 70% yield by the reaction of **4** with isocyanate **7** in similar conditions. Compounds **3–4**, **6** and **8** were fully characterized by the usual analytical and spectroscopic techniques (see ESI†). As compound **4**, bisurea **6** is a pale yellow compound with a bright yellow fluorescence. Bisurea **6** did not undergo any physical change in the presence of common cations in MeCN or DMSO but its behaviour in the presence of common anions depended on the solvent. In pure MeCN a 10^{-4} M solution of **6** increased the light yellow colour as the fluorescence was quenched in the presence of F^-, BzO^-, $H_2PO_4^-$, AcO^- and CN^-, a common behaviour of all coloured aromatic ureas.[8] Unexpectedly, the changes were the opposite in DMSO. In pure DMSO a 10^{-4} M solution of **6** decreased its intense yellow colour as the fluorescence was shifted to blue in the presence of F^-, BzO^-, $H_2PO_4^-$, AcO^- and CN^- as Bu_4N^+ salts. The changes were similar in DMSO/water 9 : 1, although the sensitivity to $H_2PO_4^-$ was lost. Fluorescent titration and fitting afforded binding constants for 1 : 1 complexes, confirmed from Job's plot analysis, that decreased slightly for F^- and CN^- from DMSO to DMSO/H_2O 9 : 1 but increased or were unchanged for BzO^- and AcO^- from DMSO to DMSO/H_2O 9 : 1 (see ESI†), indicating that **6** is a good probe for carboxylates in mixed solvents. To exploit the carboxylate sensitivity in zwitterions, we tested 10^{-4} M solutions of **6** with aqueous solutions of 16 common α-aminoacids. As aminoacids were added, the yellow fluorescence was clearly shifted to blue in the presence of two or more equivalents of glutamine, lysine, asparagine and arginine, all having in common a nitrogen-including functionality at some distance of the carboxylate function.

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Fig. 1 Effect of the addition of 3 equiv. of aminoacids in water to 10^{-4} M solutions of **6** in DMSO: **L** (Leu), **M** (Met), **S** (Ser), **T** (Thr), **C** (Cys), **P** (Pro), **N** (Asn), **Q** (Gln), **F** (Phe), **Y** (Tyr), **W** (Trp), **K** (Lys), **R** (Arg), **H** (His), **D** (Asp), **E** (Glu) (λ_{exc} = 366 nm).

There was no change in the presence of even 3 equivalents of common basic α-aminoacids such as proline, hystidine or tryptophan (Fig. 1). Fluorescent titrations of **6** (10^{-4} M in DMSO) and aqueous aminoacid solutions showed decreasing of the intensity of emission as aminoacids were added. Titration profiles fitted satisfactorily 1 : 1 binding models, from which binding constants were calculated (log $K_1^{arginine}$ = 3.93 ± 0.05, log $K_1^{asparagine}$ = 3.93 ± 0.04, log $K_1^{glutamine}$ = 4.17 ± 0.04, log K_1^{lysine} = 3.66 ± 0.04). Job's plot analyses of fluorescence titration with lysine or asparagine revealed a maximum at a 50% mole fraction, in accord with the proposed 1 : 1 binding stoichiometry (see ESI†). In contrast, the less fluorescent monourea **8** was also sensitive to the same anions but lacked any kind of selectivity in the presence of aminoacids.

We then extended the study of the changes in fluorescence of **6** in the presence of metabolic ω-aminoacids and related metabolites or drugs. Thus, while consecutive addition of 3 equivalents of alanine in water did not promote any change, addition of 3 equivalents of aqueous solutions of β-alanine, γ-aminobutyric acid (GABA), 5-aminovaleric acid, 6-aminohexanoic acid or 7-aminoheptanoic acid resulted in dramatic changes of fluorescence from yellow to blue (Fig. 2) after the second equivalent.

Quantitative fluorescent titration of 10^{-4} M solutions of **6** in DMSO and ω-aminoacids (λ_{exc} = 390 nm) showed decreasing of the intensity of emission centred at 557 nm as aminoacids were added. Titration profiles best fitted 2 : 1 binding models, from which association constants were calculated (Table 1).

The graphical representation of the values of the log K_{1-2} gives the tendencies for the homologous series of the ω-aminoacids (Fig. 3). Thus, the value of the first constant decreases from β-alanine to 6-aminohexanoic acid and then increases for 7-aminoheptanoic acid, but the value of the second constant

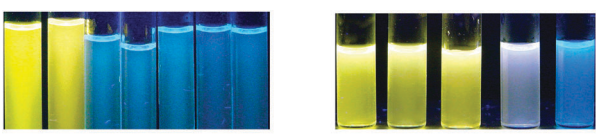

Fig. 2 Effect of the addition of 3 equiv. of aminoacids or aminoacid metabolites in water to 10^{-4} M solutions of **6** in DMSO: **A** (Ala), **B** (β-alanine), **G** (γ-aminobutyric acid, GABA), **7** (5-aminovaleric acid), **8** (6-aminohexanoic acid), **9** (7-aminoheptanoic acid), **10** (sarcosine), **11** (creatinine), **12** (creatine), **13** (L-carnitine) (λ_{exc} = 366 nm).

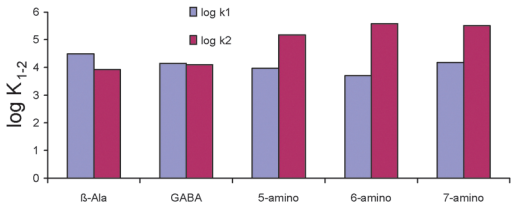

Fig. 3 Plot of log K_{1-2} for 2 : 1 complexes of ω-aminoacids and **6**.

increases from β-alanine to 6-aminohexanoic acid and then decreases slightly for 7-aminoheptanoic acid. This fact indicates that **6** behaves as a molecular ruler for carboxylates having an ammonium group at increasing distances from the carboxylate function. Typical titration curves for **6** and GABA are given in Fig. 4. Job's plot analysis of fluorescence titrations with 6-aminohexanoic acid revealed a maximum at a 66% mole fraction, in accord with the proposed 2 : 1 binding stoichiometry (Fig. 4). GABA is an important metabolite, some significant drugs such as pregabalin[9] and gabapentin[10] are based on GABA. Because its similarity to GABA, pregabalin and gabapentin can be selectively detected from their pharmaceutical formulations and quantitatively analyzed from their binding constants with **6** (Table 2), measured from fluorescent titrations of their aqueous solutions and **6**.

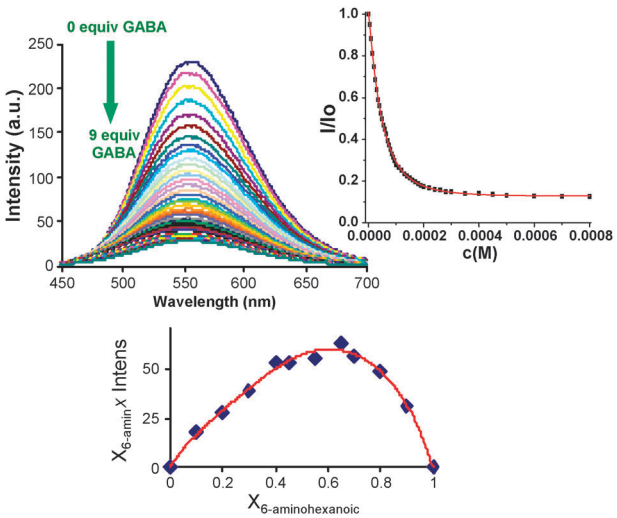

Fig. 4 (top) Fluorescence titration curves and titration profile of a 10^{-4} M solution of **6** in DMSO with an aqueous solution of GABA. (bottom) Job's plot of **6** (10^{-4} M, DMSO) and 6-aminohexanoic acid.

Table 2 Binding constants for complexes from fluorescence titrations of 10^{-4} M solutions of **6** in DMSO and drugs or metabolites in water

6	Gabapentin	Pregabalin	Carnitine	Creatine
log K_1	3.91 ± 0.29	2.90 ± 0.03	4.01 ± 0.13	3.33 ± 0.02
log K_2	3.01 ± 0.26	3.06 ± 0.09	3.80 ± 0.06	—

Table 1 Binding constants for 2 : 1 complexes from fluorescence titrations of 10^{-4} M solutions of **6** in DMSO and ω-aminoacids in water

6	β-Alanine	GABA	5-Aminovaleric	6-Aminohexanoic	7-Aminoheptanoic
log K_1	4.49 ± 0.02	4.15 ± 0.02	3.98 ± 0.04	3.70 ± 0.06	4.18 ± 0.05
log K_2	3.93 ± 0.09	4.10 ± 0.07	5.17 ± 0.05	5.58 ± 0.07	5.51 ± 0.06

Some important aminoacid metabolites, commonly used as nutritional supplements, such as L-carnitine[11] or creatine,[12] have structural features suitable for their detection by **6**. Therefore, we tested 10^{-4} M solutions of **6** with their aqueous solutions. As metabolites were added, the yellow fluorescence was clearly shifted to blue in the presence of two or more equivalents of L-carnitine or creatine. There was no change in the presence of even 3 equivalents of sarcosine or creatinine (Fig. 2). Fluorescent titration of **6** (10^{-4} M in DMSO) and aqueous creatine or L-carnitine solutions showed decreasing of the intensity of emission as metabolites were added. Titration profiles fitted a 1 : 1 binding model for creatine and a 2 : 1 binding model for L-carnitine, from which binding constants were calculated (Table 2). Job's plot analysis of fluorescence titrations with L-carnitine revealed a maximum at a 66% mole fraction, in accord with the proposed 2 : 1 binding stoichiometry (see ESI†). We calculated detection limits of 10^{-4} M solutions of **6** in DMSO, calculated in fluorescence emission by the blank variability method,[13] and selected metabolites in water, that were 1.93×10^{-7} M for L-asparagine, 3.09×10^{-6} M for GABA and 1.38×10^{-6} M for L-carnitine. We also carried out ^{1}H NMR titration experiments of a 10^{-1} M solution of **6** with β-alanine, both in DMSO-d_6. By addition of increasing amounts of β-alanine, the two urea NH signals at δ 8.8 and 6.7 moved to a lower field and progressively disappeared as the H-bonds were formed. The aromatic signals became more complicate but their chemical shifts remained unchanged (see ESI†). Apparently, formation of the 1 : 1 and 2 : 1 complexes affected the urea protons and restricted rotation of the aromatic rings. With this in mind, we optimized the geometries of 1 : 1 complexes between **6** and zwitterionic GABA or pregabalin, stabilized by 3 molecules of DMSO, at the ONIOM (B3LYP/6-31G*:AM1) level by using GAUSSIAN 03 (Fig. 5 for the **6** : GABA·3DMSO complex).[14]

The presence of at least 3 molecules of DMSO was necessary in both cases to stabilize the zwitterionic structures of aminoacids, otherwise additional hydrogen bonds between the ammonium and the carboxylate groups were produced during the calculation, with proton transfer from the ammonium to the carboxylate groups. The structures converged to stationary points having four hydrogen bonds between the urea NH protons and the carboxylate groups that agreed with the ^{1}H-NMR titration results and accounted for the interactions that gave rise to the molecular ruler effect.

In summary, we have prepared a fluorescent probe for ω-aminoacid derivatives that behaved as a molecular ruler, thus changing the yellow fluorescent emission into blue as a function of the distance between the terminal ammonium and the carboxylate groups. At λ_{exc} = 390 nm only decreasing of the yellow emission was measured but by tuning the λ_{exc} to 366 nm the blue fluorescence was seen (under the TLC UV-light) or measured in the spectrofluorometer (see ESI†). Moreover, the sensitivity to the detected metabolites is preserved in a large extension by performing the experiments in the presence of several equivalents of the undetected aminoacids (see ESI†), thus making the system suitable for the quantitative detection of ω-aminoacids and their metabolites from nutritional supplements, and some important drugs related to them, such as pregabalin or gabapentin, from their pharmaceutical formulations.

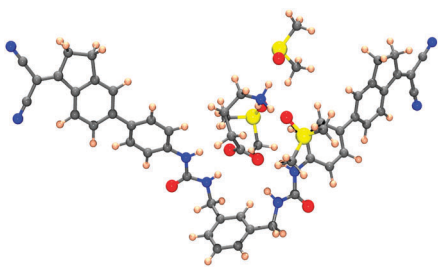

Fig. 5 Optimized geometry of the **6** : GABA·3DMSO complex at the ONIOM (B3LYP/6-31G*:AM1) in GAUSSIAN 03.

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