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Ratiometric fluorescence sensing of phenylalanine derivatives by synthetic macrocyclic receptors

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ABSTRACT

The supramolecular analytical behavior of eight pseudopeptidic fluorescent receptors (**1a–c**, **2a–c**, **3**, **4**) has been studied. The receptors are either macrocyclic or open chain derivatives based on the naphthalene chromophore. The ability of **1–4** for the molecular recognition of amino acids (as Z-protected derivatives) has been evaluated in dichloromethane. The signal observed corresponds to a fluorescence emission of *turn-on* type. The preferential binding of all the receptors for phenylalanine (Phe) over aliphatic amino acids (Ala, Val) by a factor of 3–4 has been found. Among the family of studied fluorescent molecules, two macrocyclic receptors (**1a** and **1b**) display high exciplex emissions and great fluorescence changes both at long (fluorescence quenching at 390 nm) and short wavelengths (fluorescence enhancement at 340 nm). This feature makes the macrocycles **1a** and **1b** potentially practical as fluorescent ratiometric sensors for Phe. As a proof of concept, **1a** and **1b** have been assayed as analytical tools for the identification of model samples enriched with Phe, mimicking the concentrations found in the pathology *phenylketonuria* (PKU). This result opens the door to the development of new Phe-sensing sensors based on the exciplex signaling mechanism as a new strategy for the analysis of *aminoacidemias*.

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1. Introduction

Synthetic receptors capable to recognize and signal the presence of chemical species, especially by means of fluorescence changes, have attracted the interest of numerous researchers during the last two decades [1]. Milestones of such developments were the works by Czarnik [2] and De Silva [3] on the fluorescence signaling of zinc and protons respectively. Recently, the field of fluorescent chemosensing has evolved from the well established discipline of Supramolecular Chemistry [4], to the cross-disciplinary area of Supramolecular Analytical Chemistry [5].

An important driving force for the development of hundreds of new sensors every year [6] is the demand of new tools to understand complex questions posed in the biological and related realms. Specifically, researchers in the Biomedical Sciences utilize routinely a large collection of chemical sensors (probes) specifically designed to target certain analytes, either as a way to understand in depth biochemical processes or as diagnostic tools. Examples of the former category are the classical calcium sensors developed by Tsien [7], the pH-sensitive probes by De Silva [3,8] or the most recent nitric oxide sensors by Nagano [9], to mention just a few of them. Examples of diagnostic probes are the family of quinolinium sensors [10] which detect intracellular concentrations of chloride anion, used as a signature for the diagnosis of cystic fibrosis.

Despite the great advances accomplished till now by the concurrent efforts of Organic and Analytical Chemistries towards the development of better sensors with practical applicability in Biomedicine, there are many targets of enormous interest for which practical chemosensors are still awaited. The α-amino acid family contains some of such analytes. Natural amino acids differ in the nature of their lateral chain, from apolar and unreactive (like Ala, Val, Phe, Leu, etc.) to polar and capable to react with a potential sensitive molecule. For the later group of amino acids a series of selective and sensitive probes have been reported, taking advantage of the presence of such reactive units [11]. But the discrimination of amino acids bearing apolar lateral residues is a major challenge that could be tackled by means of the tools of Supramolecular Chemistry. Notable approaches have been described so far in the literature for the analysis of apolar amino acids by optical methods [12]. The interest in amino acid recognition has been driven in many instances by the interest in enantiomeric discrimination.

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Here we present the sensing capabilities of a family of fluorescent receptors of pseudopeptidic nature towards a series of apolar amino acid derivatives. A number of such pseudopeptides are capable to discriminate very efficiently derivatives of Phe from those of Val and Ala. Moreover, the sensors here described present the unique characteristic of having dual emission and hence the possibility of carrying out ratiometric measurements [13], a feature highly appreciated by the final end users of fluorescent probes for biological use [14]. Although the recognition and sensing by such pseudopeptidic receptors is carried out in dichloromethane, the results are conceptually novel and promising. Taking into account the importance of discriminating aromatic from aliphatic amino acids in Biomedicine we have tested selected structures towards solutions emulating the concentration of amino acids found in samples of human pathologies like *phenylketonuria* (PKU) [15]. A central goal for our research has been to find, under model conditions, the structural key features allowing enhancing the recognition of Phe over the rest of amino acids.

2. Materials and methods

Chemicals were purchased to Aldrich in their maximum purity grade. Solvents used in this study (dichloromethane and acetonitrile) were spectroscopic grade and were dried before use. Synthesis of receptors was done as described previously [16].

Steady-state fluorescence spectra were recorded in a Spex Fluorog 3-11 equipped with a 450 W xenon lamp. All the measurements were made at 25 °C otherwise indicated. Emission spectra were obtained exciting at 300 nm, in *right angle* mode and using 1 cm × 1 cm [3 mL] quartz cells. The curves were processed with the appropriate correction files. Excitation spectra were also recorded in order to assure that no impurities were responsible for the emissions.

3. Results and discussion

Compounds **1a–c**, **2a–c**, **3** and **4** (Chart 1) belong to a broad family of peptidomimetic structures developed in our laboratories

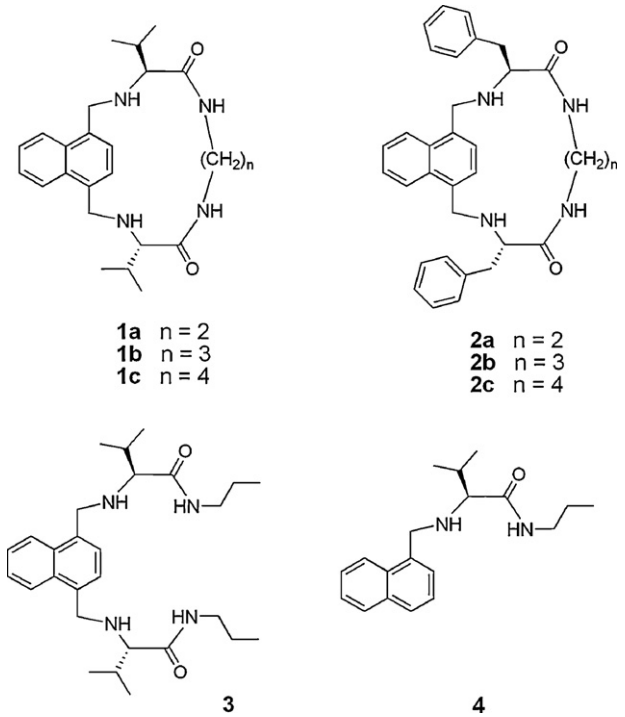


Chart 1. Fluorescent receptors.

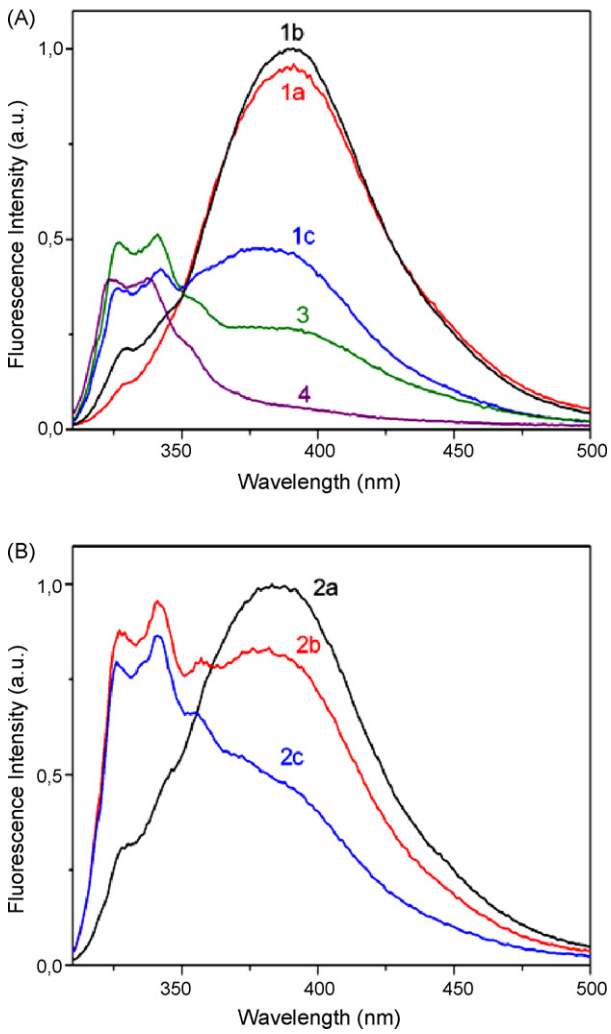


Fig. 1. Fluorescence spectra of the studied compounds in dichloromethane (concentration 1.3×10^{-4} M). (A) Receptors **1a–c**, **3**, and **4**. (B) Receptors **2a–c**. Excitation wavelength = 300 nm.

[17], and were synthesized as described previously [16]. The macrocyclic design of **1a–c** and **2a–c** for the structures of the receptors was initially selected since it is well known that macrocyclic rings present favorable features for supramolecular recognition [4,18]. The macrocyles shown in Chart 1 differ in two aspects: on the one hand in the ring size and on the other hand on the lateral chain pending from the ring (*iso*-propyl or benzyl). As will be shown, both characteristics have dramatic effects on the recognition capabilities of the receptors. Compounds **3** and **4** were made in order to be compared with the macrocyclic pseudopeptides.

The photophysics of receptors **1a–c**, **2a–c**, **3** and **4** has been described in detail previously [19]. The corresponding properties are based on the presence of the naphthalene ring, a fluorophore broadly used for the preparation of fluorescent supramolecular receptors including some polyaza macrocyclic structures [20]. The native emission of any naphthalene derivative occurs typically at ca. 340 nm. However, compounds depicted in Chart 1 (apart from **4**) present a new emission band at 390 nm, in dichloromethane. The intensity of this band depends on the structure of the compound (Fig. 1) and has been identified as an emissive exciplex [19], i.e. an internal complex formed between the excited naphthalene chromophore and the secondary amines of the macrocyclic ring. The existence of exciplex emission depends on the availability of the amine electron pairs, since the physical origin of the exciplex can be found in a photoinduced electron transfer (PET) process [21].

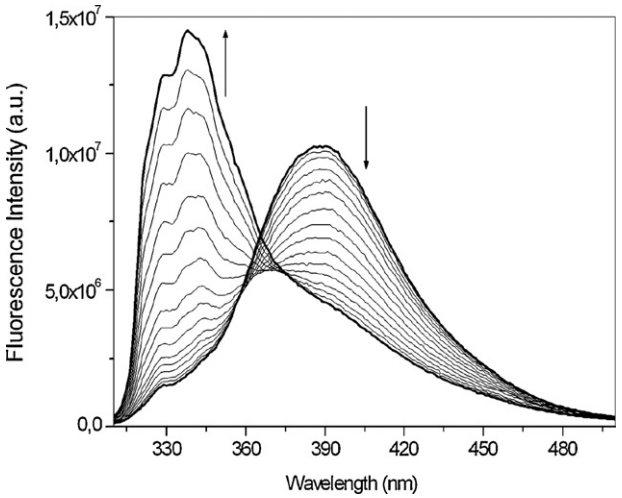


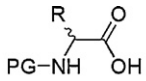
Fig. 2. Fluorescence titration of **1b** (1.3×10^{-4} M) with Z-L-Phe (final concentration 1.6×10^{-2} M) in dichlorometane. Excitation wavelength = 300 nm.

The potential analytical applicability of this PET process is obvious: the protonation of the amines would make the exciplex (390 nm) to disappear and simultaneously would make the emission from the quenched naphthalene (340 nm) to recover. This concept was probed preliminarily in the case of a single receptor (**1b**) [22] and now the whole family of sensors was submitted to the same evaluation. A representative titration can be seen in Fig. 2 for **1b** in the presence of increasing amounts of Z-L-Phe.

A basic set of substrates was selected to make the corresponding titrations with **1a**–**c**, **2a**–**c**, **3** and **4** as receptors (Chart 2). The substrates are all in their N-protected forms for solubility, i.e. in order to make the measurements in dichloromethane, the solvent for which the exciplex emission of the receptors displays its maximum intensity. The presence of a protecting group should not be a practical impediment for analytical purposes, as will be explained in the final part of this paper. Both enantiomers of Phe, Val and Ala were utilized in their N-carbobenzyloxy form (Z-L-Phe, Z-D-Phe, Z-L-Val, Z-D-Val, Z-L-Ala and Z-D-Ala). Also the *tert*-butoxycarbonyl (Boc) derivative of L-Phe was assayed for comparison purposes, in order to evaluate the effect of the protecting group of the substrate on the analytical performance of this family of receptors.

As it can be seen in Fig. 3, the nature of the receptor influences greatly the fluorescence sensing of the amino acid derivatives. In Fig. 3A the effect of the macrocyclic size on the analytical response towards Z-L-Phe is shown whereas Fig. 3B and C reflect the effect of the pending chain of the macrocycle (*i*-Pr *vs.* Bz).

Results in Fig. 3 reveal that recovering of the native fluorescence emission of the naphthalene fluorophore occurs, in the presence of an excess of Z-L-Phe, for all the receptors tested, although the intensity of the corresponding bands is significantly affected by



R = CH_3, PG = Cbz (Cbz-L-Ala, Cbz-D-Ala)

R = $CH(CH_3)_2$, PG = Cbz (Cbz-L-Val, Cbz-D-Val)

R = CH_2Ph, PG = Cbz (Cbz-L-Phe, Cbz-D-Phe)

R = CH_2Ph, PG = Boc (Boc-L-Phe)

Chart 2. Substrates.

the structural parameters of the receptor, As the quenching of this signal involves the presence of an unprotonated amino group at the benzylic position, its full recovering requires the protonation of both available positions [23], suggesting the participation of two

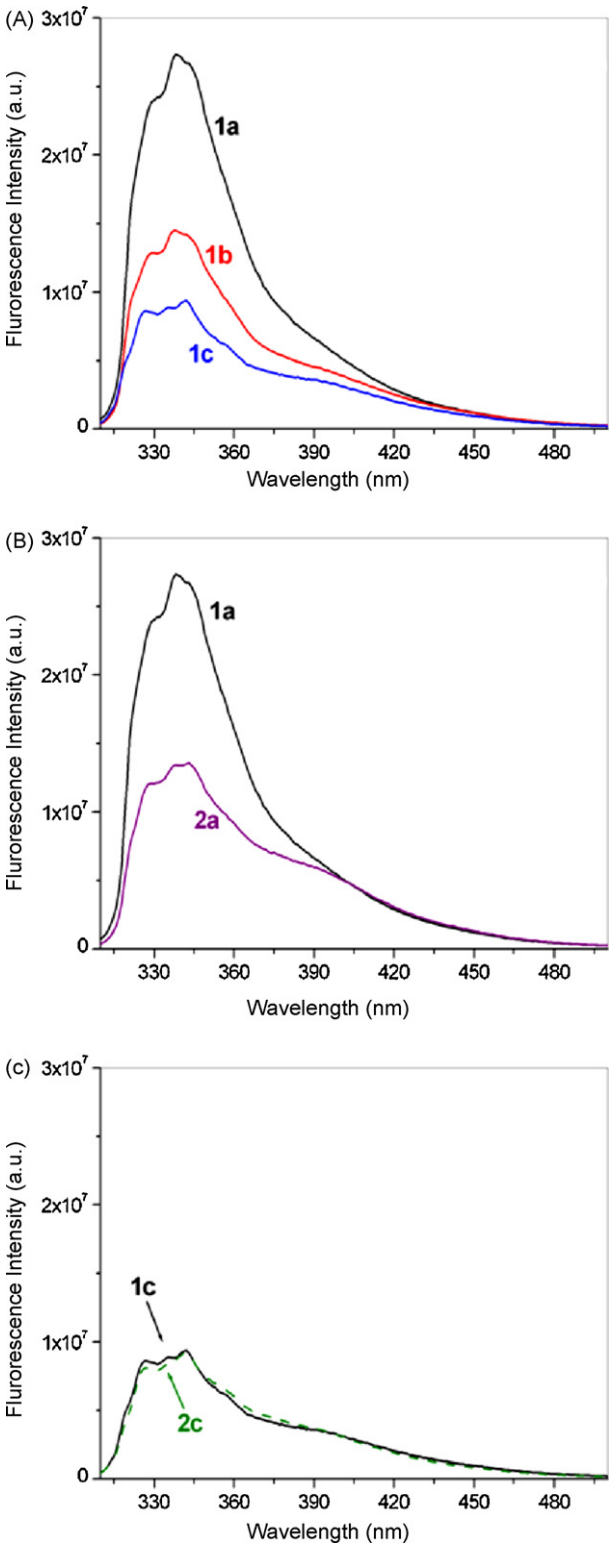


Fig. 3. Fluorescence spectra of several fluorescent receptors (1.3×10^{-4} M) in the presence of excess of Z-L-Phe (1.6×10^{-2} M). (A) Comparison between three macrocycles of different size; (B and C) comparison between two macrocycles of the same ring size but different pending chains. Dichloromethane; excitation wavelength = 300 nm.

Table 1
Values for the fluorescence quenching at 390 nm (FQ^{390})and fluorescence enhancement at 340 nm (FE^{340}). Values for the L enantiomer (in brackets for D).

Receptor	Substrate							
	Z-Val		Z-Ala		Z-Phe		Boc-Phe	
	FQ^{390}	FE^{340}	FQ^{390}	FE^{340}	FQ^{390}	FE^{340}	FQ^{390}	FE^{340}
1a	28 (31)	280 (288)	35 (35)	513 (391)	37 (31)	1273 (996)	31	533
1b	41 (43)	124 (142)	48 (50)	199 (189)	56 (54)	595 (496)	47	218
1c	20 (21)	106 (121)	26 (28)	128 (144)	28 (17)	266 (281)	19	169
2a	29 (28)	98 (97)	33 (35)	138 (136)	39 (36)	323 (360)	33	159
2b	18 (25)	22 (31)	26 (31)	30 (39)	35 (22)	72 (50)	22	32
2c	15 (16)	23 (29)	20 (16)	24 (30)	[b]	55 (60)	15	30
3	20 (16)	111 (130)	19 (20)	210 (232)	[b]	437 (578)	20	147
4	[a]	81 (67)	[a]	121 (146)	[a]	217 (212)	[a]	124

[a] Negligible exciplex fluorescence.
[b] FQ could not be calculated properly due to overlap of emissions at 390 nm and 340 nm.

guest molecules in the interaction. This is confirmed by the fact that the initial addition of the amino acid derivatives is reflected only in the quenching of the exciplex emission without recovering of that of the naphthalene at short wavelength, this requiring of the addition of further amounts of guest.

In order to compare all the pseudopeptides under a common quantitative basis [24], and focusing on the analytical potential of the new fluorescent receptors, the relative parameters of fluorescence quenching (FQ) and fluorescence enhancement (FE) were used [25]. The intensities of fluorescence were compared at a final concentration of 1.6×10^{-2} M of added substrate. FQ was calculated for the disappearance of the exciplex band at 390 nm and FE was employed to quantify the extent of fluorescence recovery at the native wavelength of the naphthalene moiety (Eqs. (1) and (2)) and both were normalized relative to F_0 values (F_0 and F_i indicate the initial and final emissions respectively). The results of FQ and FE calculations can be seen in Table 1:

$$FQ^{390}\,(\%) = \frac{F_0^{390} - F_i^{390}}{F_0{}^{390}} \times 100 \quad (1)$$

$$FE^{340}\,(\%) = \frac{F_i{}^{340} - F_0{}^{340}}{F_0{}^{340}} \times 100 \quad (2)$$

Several conclusions can be drawn from the analysis of such data:

(a) Attending to receptor **1a**, it can be seen that differences found in FQ are almost negligible, since all the values are found within the 30–40% range approximately, irrespective of the lateral chain of the substrate and even the protecting group Z or Boc. However, important differences are visible for the second parameter. FE displays a maximum value for the interaction with Z-L-Phe (1273%), a minimum value for Z-L-Val (280%) and an intermediate one with Z-L-Ala (513%). An enantiorecognition ability for **1a** is even appreciable, using this parameter, for Z-ala and Z-Phe, although its extent is moderate (for instance 1273/996 for Z-L-Phe/Z-D-Phe). On the other hand, the protecting group present at the N end of the substrates plays a significant role in the sensing process taking into account that the Boc derivative of L-Phe affords only a FE of 533%, less than half of the one for the related Z-L-Phe.

(b) When considering receptor **1b**, analogous to **1a** but with one additional methylene in the macrocyclic ring, similar values of FQ but different results for FE can be seen for the different guests. The same pattern is observed with macrocycle **1c**, larger than **1b** by one additional methylene. The general trend observed with this series of receptors derived from valine is that the larger the ring the smaller the absolute magnitude of FE. This could be accounted for considering a higher rigidity (preorganization) of the smaller receptors leading to a minor loss of conformational freedom upon complexation. For all the receptors, the larger effects are found for the interaction with Z-Phe derived from an amino acid with an aromatic side chain. In the same way, the presence of a protecting group containing an aromatic moiety always produces more intense effects. Thus, the effect of Z *vs.* Boc is evident from the comparison of the pairs Z-L-Phe/Boc-L-Phe in their complexation with **1b** (595/218) and **1c** (266/169). These observations point out to a possible contribution of aromatic-aromatic interactions in the supramolecular complex.

(c) The set of receptors **2a–c** derived from phenylalanine afforded qualitatively similar conclusions, although the magnitude of the signaling is much lower. At this point, it is especially remarkable the fact that despite being **1a–c** and **2a–c** series structurally very similar, each one display very different analytical properties.

(d) Following the examination of values presented in Table 1, receptor **3** results remarkable since it displays a FE for Z-L-Phe of 437%, in spite of being an open chain derivative and hence with a great conformational mobility. It is also noteworthy the opposite enantiopreference of **3** (preference for the D enantiomer of Z-Phe (FE (Z-L-Phe)/FE (Z-D-Phe)) = 437/578). Nevertheless, the recognition of aromatic substrates is surprisingly high if we consider the series of FE values (Z-L-Val/Z-L-Ala/Z-L-Phe) = 111/210/437, which could make of **3** also a good candidate for analytical purposes.

(e) Finally, the same trend of FE for the recognition of aromatic residues can be found for the monoamine **4**, although to a very low extent, probably as a consequence of the monotopic nature of this receptor.

As a summary, a graphical comparison of all the FE values can be seen in Fig. 4 where receptors **1a** and **1b** display prominent fluorescence increases as compared to the rest of sensors. This also allows easily comparing the differences observed, for a given receptor, with the different guests.

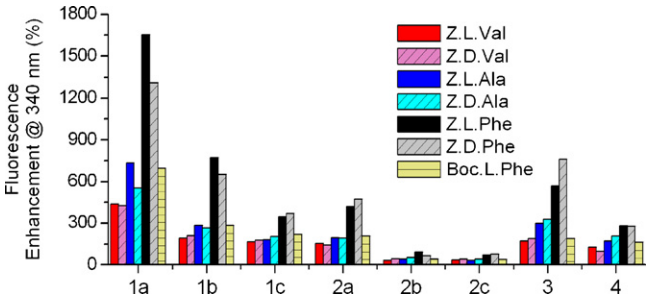


Fig. 4. Fluorescence enhancement at 340 nm for all the receptors and substrates. Conditions: [receptors] = 1.3×10^{-4} M, [substrates] = 1.6×10^{-2} M, excitation wavelength = 300 nm; dichloromethane.

Table 2
Relative distribution of amino acid derivatives in samples emulating the concentrations found in normal, PKU and MSUD situations. Total concentration of amino acids = 2.5×10^{-2} M (dichloromethane).

Entry	Sample name	Relative molar distribution of amino acids		
		Z-L-Val	Z-L-Leu	Z-L-Phe
1	Norm.0	1.0	1.0	1.7
2	Norm.1	1.3	1.0	2.9
3	Norm.2	1.1	1.0	1.2
4	PKU.1	1.8	1.0	13.0
5	PKU.2	1.0	1.5	9.6
6	MSUD.1	3.2	5.9	1.0
7	MSUD.2	5.5	6.6	1.0

The similarity of the FQ values, i.e. the exciplex quenching, and disparity of the FE data can be rationalized if considering a supramolecular interaction complex in which two units of substrate bind to one molecule of receptor. Attending to the presence of two amines in the receptors, except for **4**, it seems reasonable to assume a model in which two carboxylic acids transfer their acidic protons to the two basic positions of the receptors, leading to a 1:2 complex. In this way the first amino acid would quench the emission of the exciplex at 390 nm and the second equivalent would promote the fluorescence recovery [24].

Once identified receptors **1a** and **1b** as good discriminators between aromatic and aliphatic amino acid derivatives, a series of assays were performed in order to discriminate unknown samples enriched with aromatic or aliphatic amino acid derivatives. The ultimate goal of those experiments was to check the ability of the reported receptors to make a *blind* identification of samples representative of pathological states characterized by an excess of Phe (*hyperphenylalaninemia* or *phenylketonuria*, PKU [15]) or by a defect of such amino acid (like in the *maple syrup urine disease* or MSUD [26]).

Seven samples were prepared as indicated in Table 2. The samples containing normal levels of Z-L-Val, Z-L-Leu and Z-L-Phe were marked as Norm.0, Norm.1, and Norm.2. Such normal levels were extracted from the literature [27]. Other two samples were enriched in Z-L-Phe to the level to which Phe can be found in individuals suffering form PKU (according to the same bibliographic source [27]). And finally, two samples were prepared with concentrations emulating the levels of amino acids found in real samples of MSUD, with high relative levels of aliphatic amino acids [27]. Although dichloromethane and Z protecting groups could seem in principle a disadvantage for the implementation of these sensors into a standard analytical protocol, it must be mentioned that the derivatization of biological samples in the routine chromatographic analyses made for diagnostic purposes is a common practice [27,28]. On the other hand amino acid derivatives like Z-L-Tyr and Z-L-Trp displayed a small spectral overlap with receptors at the excitation wavelength (300 nm) and hence were not used for the formulation of model solutions. Future work will be devoted to synthesize related receptors with absorption at longer wavelengths in order to avoid this overlap.

The fluorescence spectra of samples of Table 2 in the presence of macrocycle **1b** are represented in Fig. 5. As can be seen, the series of three samples labeled as "normal" can be found grouped, well separated from those marked with PKU (above) and those marked with MSUD (below). Similar results were found with **1a**. The higher the difference between each group of samples, the better the confidence that a test designed using these sensors as analytical tools would have. It should be noted that a common test for the analysis of PKU in Western countries (the so-called *heel prick test* or Guthrie test [29]) implies a high amount of false positives which makes the clinical laboratories to repeat the analyses or to make a secondary test with an alternative technique [28,30].

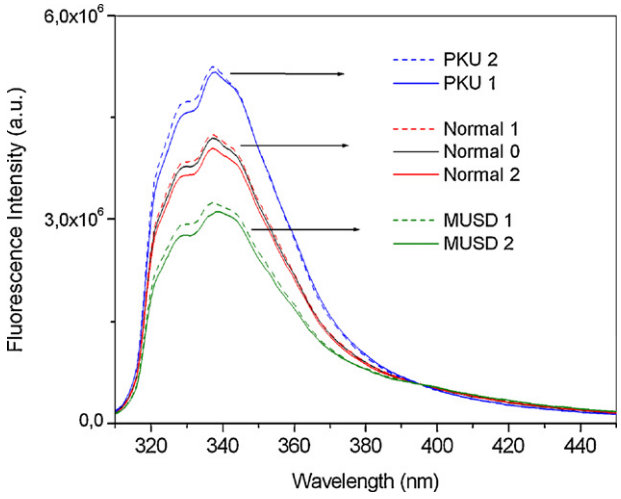


Fig. 5. Fluorescence spectra of **1b** (1.3×10^{-4} M) in the presence of samples of Table 2. Dichloromethane; excitation wavelength = 300 nm.

In order to compare **1a** *vs.* **1b**, the relative increase or decrease of the emission at 340 nm referenced to the sample Norm.0 was calculated (Fig. 6). Thus, with **1a** the fluorescence of normal samples varied within a 5% margin, whereas PKU samples elevated the emission +15% relative to Norm.0 On the contrary, the fluorescence of

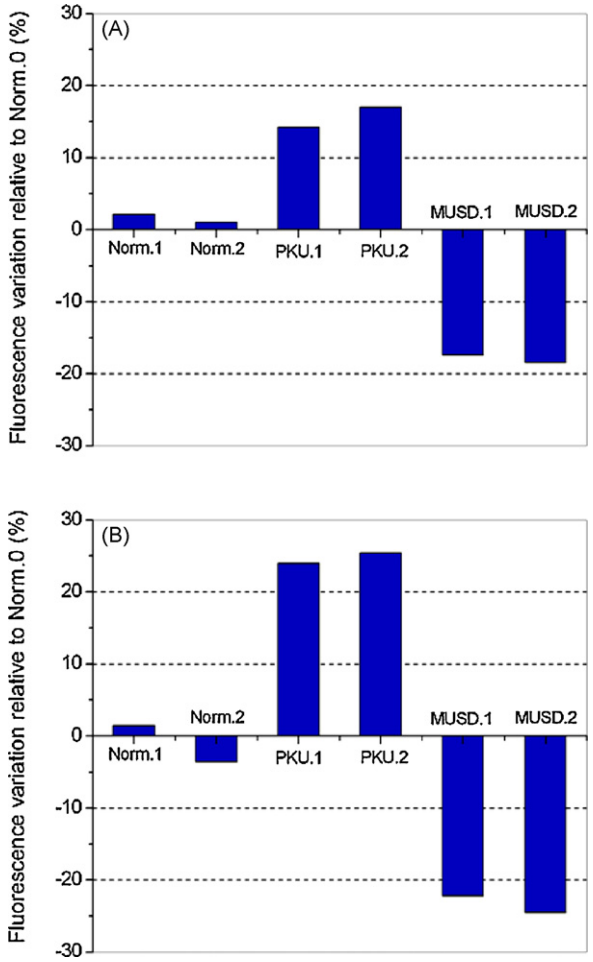


Fig. 6. Relative performance of **1a** (A) and **1b** (B) as potential fluorescent sensors for PKU and MSUD. The bars indicate the increase or decrease of fluorescence of samples of **1a** or **1b** in the presence of Norm.1, Norm.2, PKU.1, PKU.2, MSUD.1 and MSUD.2, all relative to Norm.0. Excitation wavelength = 300 nm.

MSUD samples was found -15% below Norm.0 (Fig. 6A). The macrocycle **1b**, showed even better discrimination between subgroups with increased emissions for PKU of ca. +25% above the normal region and decreased fluorescence for MSUD samples of ca. −25% of the normal reference (Fig. 6B).

More interesting from the analytical point of view is the potential capacity of the sensors to measure at two different emitting wavelengths and hence to be self-calibrated (affording ratiometric measurements). This feature is not observed in dichloromethane at the employed concentrations since the exciplex emission is quenched completely. However, an appropriate selection of the solvent in which complexation takes place allows for the fine tuning of the strength of the binding between receptor and substrate. In order to tune the complexation, hence not allowing the complete quenching of the exciplex band, the best sensor identified in the previous assays, **1b**, was dissolved in a polar solvent like acetonitrile, and its fluorescence measured in the presence of samples of Table 2. The corresponding emission spectra can be seen in Fig. 7A. Again, the families Norm, PKU and MUSD displayed fluorescence changes different enough to make a clear identification from the intensities at 340 nm. But most importantly, in acetonitrile the exciplex band at 420 nm serves as an internal reference to calculate an absolute value for each sample, resulting $I^{340}/I^{420} = 1.3$ (Norm.0), 1.6 (Norm.1), 1.3 (Norm.2), 2.4 (PKU.1), 2.3 (PKU.2), 0.8 (MSUD.1) and 0.8 (MSUD.2). So, it can be stated that the existence of normal levels of amino acids (a *negative* test in terms of bioanalysis), would be indicated by a 1 < ratio < 2, whereas a *positive* PKU sample would be identified by means of a ratio >2. And analogously, a *positive* MSUD sample would be marked by a ratio <1 (Fig. 7B).

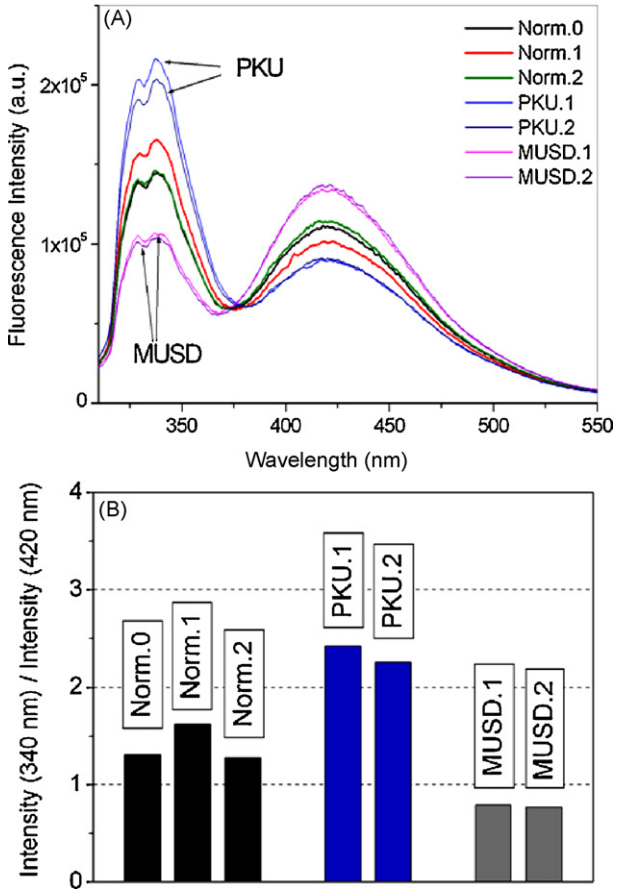


Fig. 7. (A) Fluorescence spectra of **1b** (1.3×10^{-4} M) in the presence of samples of Table 2. Acetonitrile; excitation wavelength = 300 nm. (B) Ratio of intensities at 340 and 420 nm.

We must remark that the samples of Table 2 are just simplified models mimicking typical PKU and MSUD concentrations. The analytical experiments here presented are intended to be only proofs of a new concept which is the utilization of the differential response of synthetic receptors to the analysis of enriched mixtures in a particular amino acid like Phe. The practical utilization of a new family of sensors structurally related to the ones here presented will deserve future additional studies.

As mentioned previously, the development of sensors with ratiometric features is highly demanded [31]. To the best of our knowledge, the receptors described in this work are the first examples of ratiometric fluorescent sensors, based on exciplex signaling, for aromatic amino acids, and also the first supramolecular approach to the problem of the analysis of aminoacidemias.

In summary, a series of eight pseudopeptidic compounds based on the naphthalene chromophore have been evaluated as fluorescent chemosensors for apolar amino acid derivatives. It has been found that all the receptors studied display preferential binding towards Phe derivatives. Two of the studied sensors have appropriate analytical features to discriminate samples enriched in aromatic (PKU) or aliphatic (MSUD) amino acids from those solutions with balanced levels (normals), moreover showing a ratiometric response. Although still not ready for practical purposes, our present results represent a good starting point towards the future utilization of this strategy for the analysis of aminoacidemias.

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