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Unraveling the Molecular Recognition of Amino Acid Derivatives by a Pseudopeptidic Macrocycle: ESI-MS, NMR, Fluorescence, and Modeling Studies

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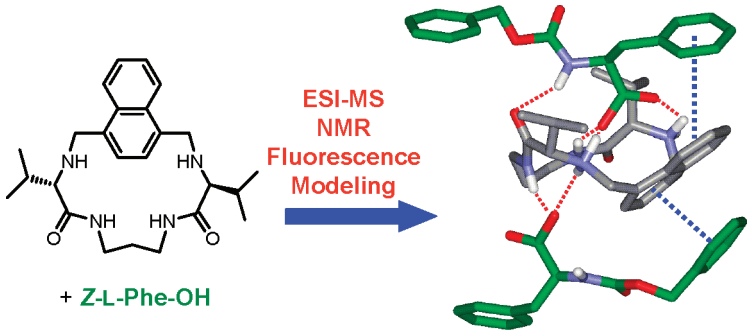

The binding between a pseudopeptidic macrocyclic naphthalenophane and different *N*-protected amino acid derivatives has been thoroughly studied by ESI-MS, NMR, fluorescence, and molecular modeling. Careful NMR titration experiments led to the characterization of the intermolecular noncovalent interactions, reflecting a slight side chain and L-stereoselectivity of the host−guest complexes. The data suggest the formation of an intimate ionic pair after the proton transfer from the carboxylic substrate to the amino macrocycle. Additional intermolecular interactions like H-bonding and $\pi-\pi$ contacts are also important. This receptor shows a stronger interaction with substrates bearing aromatic rings, either in the side chain or in the *N*-protecting group. Besides, for *N*-Z-Phe-OH, a moderate enantioselectivity has been observed. Mass spectrometry suggests the formation of supramolecular complexes with stoichiometries higher than 1:1. The dual nature of the fluorescence emission of the macrocyclic receptor allowed determining binding constants and pertinent thermodynamic parameters. On the basis of the experimental data (NMR titrations, intermolecular ROESY, VT-NMR) and with the help of molecular modeling, a reasonable structure for the supramolecular complexes can be proposed, in which the interactions with the naphthyl ring of the receptor play a fundamental role in the strength and selectivity of the molecular recognition event.

Introduction

The study of synthetic receptors for the molecular recognition of chiral anionic species has tremendously grown in the last decades,[1] with carboxylates being the most commonly studied examples.[2] Within this wide family of organic molecules, amino acid derivatives are especially interesting targets, as they are implicated in different biomolecular processes.[3] The design of selective receptors for chiral molecules has many technological, industrial, biomedical, and environmental applications.[4] Among them, the preparation of new selective fluorescent chemosensors is especially attractive.[5] For this purpose, a fluorescent moiety (antenna) is often present in the receptor structure. This antenna can act as a mere reporter of the molecular recognition event or participate in the interaction with the substrate. Some efficient and elegant examples have been described in the

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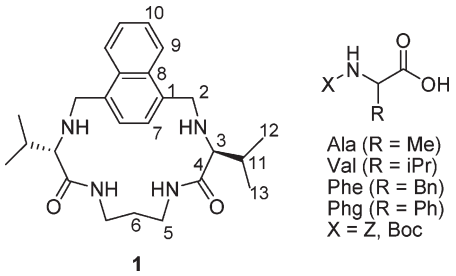
literature,[6] showing a high degree of sensibility and selectivity toward a given substrate. However, for the optimal design of a synthetic host, a thorough knowledge of the intimate interactions between the receptor and the target molecule is mandatory. Moreover, additional information about the three-dimensional structure, conformation, and dynamics of the supramolecular complexes would be very helpful for further redesign and optimization of the host structure. Unfortunately, the labile and dynamic nature of supramolecular complexes makes this study specially challenging,[7] and thorough structural studies on this kind of host−guest systems are scarce.

CHART 1. Chemical Structure of the Macrocyclic Receptor 1 and the *N*-Protected Amino Acid Substrates (arbitrary atom numbering assumed for 1 is also shown)



On the other hand, during the last couple of years, we have been interested in preparing and studying new pseudopeptidic macrocycles.[8] They show interesting properties as molecular receptors,[9] molecular devices,[10] organogelators,[11] and fluorescent chemosensors.[12] Regarding that, in a preliminary communication,[13] some of us reported the ability of compound **1**[14] to act as a selective fluorescent ratiometric chemosensor for *N*-protected amino acid derivatives (Chart 1). The compound showed a preference for binding to aromatic amino acids, with a moderate enantioselectivity. This led us to undertake an in-depth study of the recognition process. Thus, for this receptor the fluorescence process has been thoroughly studied, allowing, in some instances, the extraction of thermodynamic parameters. Very interesting results were obtained, indicating the formation of complexes with 1:2 receptor:substrate stoichiometry. Some clues about the source of the (stereo)selectivity observed were also obtained from the analysis of the fluorescence data. In this paper, we describe a comprehensive study by a multidisciplinary approach using mass spectrometry, nuclear magnetic resonance, fluorescence spectroscopy, and molecular modeling. With all these data, we aim to propose a binding mode and a reasonable structure for the supramolecular complexes[15] which explain the selectivity observed by fluorescence measurements.

Results and Discussion

Gas-Phase Study by Mass Spectrometry (ESI-MS). The molecular recognition behavior of **1** versus different *N*-CBz-protected amino acids has been studied by ESI-MS and

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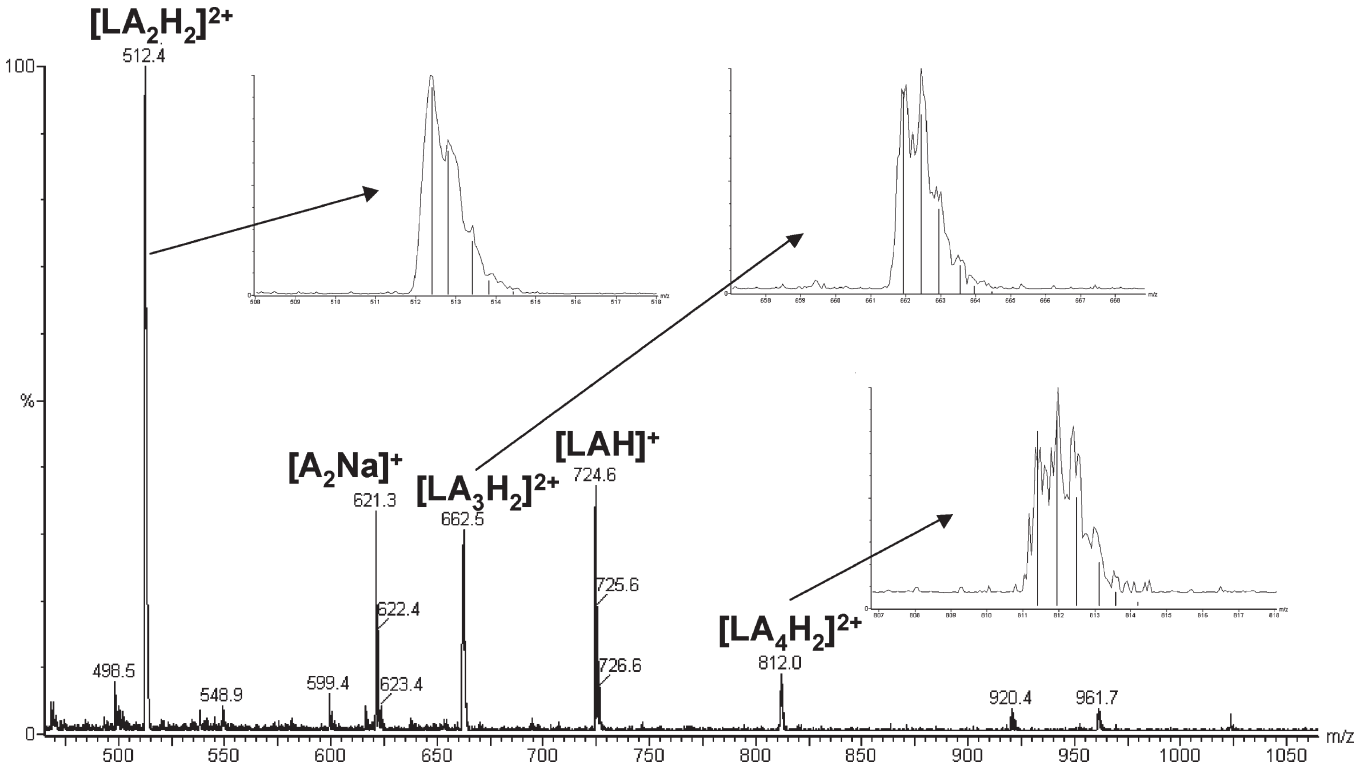

FIGURE 1. ESI mass spectrum of receptor **1** in the presence of an excess of Z-L-Phe-OH. Simulated isotopic patterns for selected peaks are shown as bars overlapped with the corresponding experimental peaks.

MS/MS assays. Recently, the use of mass spectrometry in supramolecular chemistry has tremendously grown, as soft ionization techniques (such as ESI) usually render a good preservation of weak intermolecular interactions, allowing the detection of the corresponding receptor−substrate complexes.[16] The advantages of these experimental techniques are fast performance and low sample consumption, which make them very suitable for the screening of different receptor:substrate combinations. Thus, questions like receptor:substrate stoichiometry or binding selectivity can be easily answered in a quick fashion. Additionally, MS experiments provide interesting data for the molecular recognition process in the gas phase to be compared with results from other techniques, usually performed in solution phase.[17] One should bear in mind, however, that some caution is needed for the interpretation of the results, mainly related to the ionization differences for the quantification of the peaks observed in the mass spectra.[18] Thus, ESI-MS spectra of **1** in the presence of different amounts (1−5 equiv) of *N*-Z-amino acids yielded observable peaks for different supramolecular entities identified as $[LH_nA_m]^{n+}$, where L corresponds to **1** and A to the different amino acid derivatives (Figure 1). Full isotopic analysis was very useful for the unambiguous assignation of the peaks. Interestingly, species with one receptor and two and even three and four molecules of the substrate were readily observable especially for the aromatic amino acid derivatives (Phe or Phg). In these cases, they were present even for a 1:1 receptor:substrate mole ratio. The very large peak for the $[LH_2A_2]^{2+}$ species in the cases of aromatic amino acids (100% relative intensity, Figure 1) was especially noteworthy.

For studying the selectivity of the receptor, different approaches can be used like competition experiments[19] or kinetic measurement of displacement rates.[20] However, some of these methodologies can present problems related to the ionization differences of the charged species under study. Despite that, tandem mass spectrometry seems to be a more suitable technique for comparing stabilities of the supramolecular species in the gas phase, by performing collision-induced dissociation (CID) experiments with the supramolecular entities formed by **1** and different amino acids.[21] For

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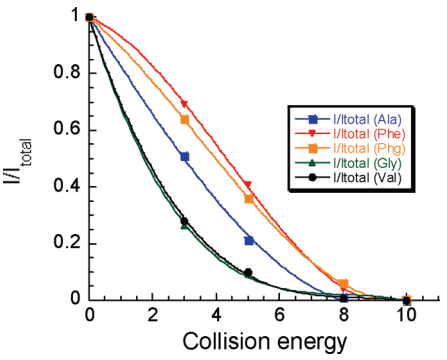

FIGURE 2. Plot of the CID curves for $[LHA]^+$ complexes formed by receptor **1** and different Z-protected amino acids.

simplicity and to achieve a better signal-to-noise ratio in all the amino acids tested, the peak corresponding to $[LHA]^+$ was selected and fragmented at different collision energy values. Some features can be extracted from these experiments (Figure 2). First of all, the complex $[LHA]^+$ seemed to form relatively weak ionic pairs as they were completely broken at collision energies as low as 10 eV. On the other hand, some trends can be observed regarding the stability of the complexes formed, as complexes with aromatic amino acids needed slightly higher collision energies than the aliphatic ones. For instance, the collision energies (eV) for 50% of complex dissociation were the following: 1.7 (Gly), 1.8 (Val), 3.0 (Ala), 4.0 (Phg), and 4.3 (Phe). Accordingly, the order in the stability of the complexes is Phe ≥ Phg > Ala > Gly = Val. The slight differences in the stability order compared to data obtained by fluorescence measurements[13] can be an effect of the different balance between hydrophobic interactions and steric effects when changing from bulk solution to gas phase. Thus, for instance, nonspecific hydrophobic interactions with the side chain of the amino acid, which must be important in the solution phase, can compensate for the steric repulsion in the gas phase and could account for a lower stability of the corresponding complex with Gly compared to the other aliphatic ones.

For the aromatic amino acids (Phg and Phe), peaks corresponding to higher molecularities were observed (see Figure 1). We decided to apply also CID experiments to these peaks, with the aim of inspecting their dissociation pattern. Very interesting observations were obtained when the relative intensities of daughter peaks containing the receptor versus collision energies are plotted (Figure 3). For instance, when CID measurements were applied to $[LH_2A_3]^{2+}$ (Figure 3A) this cluster dissociated preferentially to $[LH_2A_2]^{2+}$, which undergoes a further dissociation to $[LHA]^+$ and free receptor. It is worth mentioning that $[LHA]^+$ always behaved as a minor daughter peak. In separate experiments, $[LH_2A_2]^{2+}$ was fragmented leading to $[LHA]^+$ and the free receptor (Figure 3B), the intensity of this last one being larger than that of the 1:1 complex. These experiments imply that, in the gas phase, the 1:2 receptor:amino acid supramolecular structure is more stable than the 1:1 counterpart, as $[LH_2A_3]^{2+}$ preferentially dissociated to form $[LH_2A_2]^{2+}$ and this last species, either as a mother or as a daughter peak, leads to a larger amount of free receptor than to the $[LHA]^+$ complex.

All these mass spectrometry experiments suggest that noncovalent complexes containing different receptor:substrate ratios can be formed and those are more stable when the residue of the amino acid is aromatic. Besides, especially for the aromatic substrates, among the complexes of different stoichometries, $[LH_2A_2]^{2+}$ seemed to be the most stable one in the gas phase.

Solution Structure Study by NMR. Despite its relatively low sensitivity, nuclear magnetic resonance is probably the most powerful technique for the fine structural characterization of supramolecular complexes in solution.[22] In this regard, we considered that a combination of different experiments could help us for the proposal of a reasonable binding model that could explain the selectivity displayed by **1** toward different amino acid derivatives, as well to understand the key features involved in amino acid recognition. Besides, the understanding of the molecular recognition event could help us to improve future designs of receptor structures based on related systems. For a thorough knowledge of the sensing process, careful NMR titration experiments of **1** with different amino acid derivatives have been performed. The overall changes in the chemical shifts of the most representative proton signals of the receptor when saturated with the substrates ($\Delta\delta_{max} = \delta_{max} - \delta_o$) are gathered in Table 1.

Some interesting features can be pointed out from these results. As a general rule, H3 (the atom at the stereogenic center) moved downfield in all the tested examples (for assumed numbering, see Chart 1). This observation supports a proton transfer from the carboxylic group of the substrate to the amino group of the receptor, and hence the formation of an ionic pair. Accordingly, this process is also reflected by ^{13}C NMR: for instance, the presence of a large excess of Z-L-Phe-OH in a sample of **1** moved the signals of C3 (5.53 ppm), C4 (5.06 ppm), and C11 (1.22 ppm) upfield in very good agreement with this protonation scheme. Moreover, the change observed in the chemical shift of H3 after addition of 1 equiv of a strong acid (TFA) to the free receptor (entry 11) is roughly one-half of those obtained with all the amino acids (entries 1−10).[23] Considering that chemical shift changes are proportional to the concentration of different protonated species, this result suggests that protonation on both amino nitrogen atoms of the receptor was obtained after saturation with the different amino acid derivatives. These results are also in agreement with the fluorescence measurements (see the following section). Another changing signal of **1** is the amide NH proton, which also moved downfield, reflecting the establishment of H-bonded amide complexes. Finally, the changes obtained in the naphthyl moiety of the receptor are also noticeable, and suggest that the aromatic ring of the macrocycle also participates in the stability of the complex. As a general trend, both H9 and H10 resonated at lower chemical shifts upon saturation with the substrate, suggesting a face-to-face interaction between aromatic moieties of receptor and substrate. When comparing aromatic and aliphatic amino acids (see for instance entries 1 and 5) the $\pi-\pi$ interaction with the side chain of the substrate is reflected on the different $\Delta\delta_{max}$ observed for

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(23) Addition of a second equivalent of TFA led to product precipitation and it was impossible to acquire a suitable NMR spectrum.

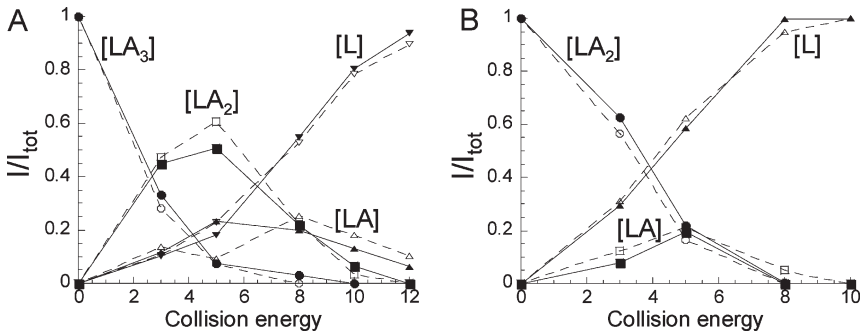

FIGURE 3. Relative intensities of peaks obtained by CID experiments applied to (A) $[LH_2A_3]^{2+}$ and (B) $[LH_2A_2]^{2+}$ for Z-Phe-OH (solid lines) and Z-Phg-OH (dotted lines). Species containing the same proportion of receptor and substrate but with a different number of protons were merged in a single value for simplicity, and thus the corresponding numbers of protons have been omitted for clarity.

TABLE 1. Maximum Chemically Induced Shifts ($\Delta\delta_{max}$, ppm) of Selected Receptor Signals in the Presence of Different *N*-Protected Amino Acids ($CDCl_3$, 303 K, 500 MHz)[a]

		$\Delta\delta_{max}$ (ppm)			
entry	substrate	H3	amide NH	H9	H10
1	Z-L-Phe-OH	0.433	0.389	−0.105	−0.091
2	Z-D-Phe-OH	0.425	0.434	−0.131	−0.093
3	Z-L-Phg-OH	0.327	0.265	−0.221	overlapped
4	Z-D-Phg-OH	0.358	0.392	−0.170	overlapped
5	Z-L-Ala-OH	0.561	0.776	−0.052	−0.034
6	Z-D-Ala-OH	0.509	0.622	−0.042	−0.020
7	Boc-L-Phe-OH	0.488	0.562	−0.065	0.001
8	Boc-D-Phe-OH	0.521	0.570	−0.075	−0.009
9	Boc-L-Phg-OH	0.374	0.478	−0.168	overlapped
10	Boc-D-Phg-OH	0.408	0.544	−0.123	overlapped
11	TFA	0.250	0.524	−0.025	0.012

[a]Arbitrary atom numbering is as in Chart 1.

H9/H10. Thus, the changes obtained upon saturation are smaller for Ala than for Phe or Phg. On the other hand, the effect of the protecting group is also clear, as Z derivatives led to larger shielding of H9 and H10 than the corresponding Boc derivatives (see for instance entry 1 versus entry 7). Those results support the interaction of the two aromatic rings, the one from the amino acid side chain and the one from the protecting group, with the naphthyl moiety of the receptor. Therefore, considering all the signals shifted upon titration, **1** emerged as a multifunctional receptor with at least three types of interactions, different both in nature and in their spatial disposition throughout the molecule. The main interaction should be an electrostatic attraction between the ammonium cation of the receptor and the carboxylate anion of the substrate, both produced after a proton transfer. Second, the amide NH of the receptor is also implicated in the molecular recognition event by hydrogen bonding with the substrate. Last, but not least, the naphthyl ring also participates through $\pi-\pi$ stacking interactions with the aromatic ring of the substrate, either through the protecting group (Z) or through the amino acid side chain (Phe and Phg). Other contacts with the receptor are also possible, like H-bonding with the oxygen of the amide carbonyl (working as an acceptor) or steric repulsion with the isopropyl side chains, as well as solvophobic effects.

For a more accurate study of the strength of the complexes formed and the selectivity displayed by **1** toward the different substrates, a careful comparison of the binding isotherms is necessary. We have unsuccessfully tried to fit these titration curves to a simple equilibrium scheme to estimate binding constants. The complexity of the system arises from different sources. As suggested by ESI-MS experiments, different stoichiometries are present in this molecular recognition event. Actually, the formation of 1:2 receptor:substrate complexes was detected by ESI-MS and agrees with the sigmoidal shape of the titration curves which showed an inflection point at ~2 equiv of the amino acids in most of the examples. Moreover, the Job plot analysis with **1** and Z-L-Phe-OH also suggests the formation of 1:2 receptor:substrate complexes (Figure S6, Supporting Information). Besides, acid−base equilibria of both receptor and substrate (free and also within the supramolecular complexes) also could be working during the titration experiment. Finally, although **1** does not self-associate in a wide concentration range (0.0045−0.1 M, see Figure S2 in the Supporting Information) the amino acids exist as a mixture of rotamers on the carbamate bond which self-aggregate in $CDCl_3$ solution, as confirmed by dilution experiments (Figure S3, Supporting Information). Taking all this into account, we believe that the system is too complicated to extract reliable quantitative values of binding constants by using this technique. Despite that, a careful NMR study has allowed us to demonstrate the existence of a receptor−substrate supramolecular complex and to get some clues about its three-dimensional structure. As the final chemical shifts of the receptor signals within the complex are different depending on both the nature and configuration of the bound amino acid, we have transformed the data in order to make plots for the suitable comparison of the results. The amount of complexation can be inferred from the variation in the chemical shifts of the signals by using eq 1.[24]

$$\begin{aligned}\text{complexation degree} &= (\boldsymbol{\delta}_{obs} - \boldsymbol{\delta}_o)/(\boldsymbol{\delta}_{max} - \boldsymbol{\delta}_o) \\ &= \Delta\boldsymbol{\delta}_{obs}/\Delta\boldsymbol{\delta}_{max} \end{aligned} \quad (1)$$

where δ_{obs} is the observed chemical shift at every titration point, and δ_o and δ_{max} are either the initial or the final value of the chemical shift, respectively. Thus, the concentration of the complexed receptor is proportional to the relative variation of the chemical shift of a given signal. Working at the same experimental conditions (NMR field, solvent, total concentration, and temperature), the plots of complexation

(24) (a) Connors, K. A. *Binding Constants: The Measurement of Molecular Complex Stability*; John Wiley & Sons: New York, 1987. (b) Rebek, J., Jr.; Askew, B.; Killoran, M.; Nemeth, D.; Lin, F.-T. *J. Am. Chem. Soc.* **1987**, *109*, 2426.

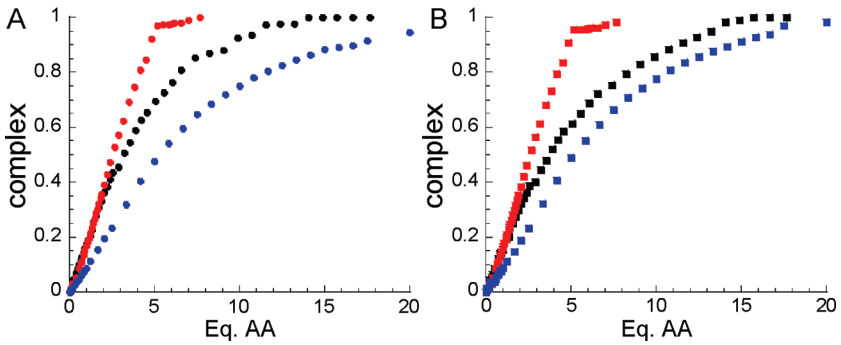

FIGURE 4. (A) Titration for H3 (circles) and (B) titration for NH (squares) for Z-Ala-OH (blue), Boc-Phe-OH (black), and Z-Phe-OH (red).

extent for different amino acids would give us a simple way for direct comparison of the strength of the complexes without measuring binding constants, which seemed to be problematic in such systems.

Probably the most interesting feature of cyclophane **1** as a receptor is its selectivity for aromatic amino acids, also reflected in the NMR titration experiments. For instance, Figure 4 shows the titration curves for amino acids containing aromatic rings in the side chain (Boc-Phe-OH, black symbols), in the protecting group (Z-Ala-OH, blue symbols), or in both (Z-Phe-OH, red symbols).[25]

The NMR titration experiments clearly showed a higher stability of the complexes with an increasing number of aromatic rings on the substrate. For instance, the presence of 5 equiv of Z-Phe-OH led to the almost total saturation of the receptor. When the aromatic ring of the side chain was eliminated, the stability of the supramolecular complex is decreased as only ca. 50% of **1** was saturated with 5 equiv of Z-Ala-OH. These results agree with those obtained by both fluorescence spectroscopy[13] and mass spectrometry, and support the use of **1** as a selective sensor for aromatic amino acids.[13] Interestingly, the strength of the interaction also depends on the protecting group of the amino nitrogen. Thus, changing Z to Boc also decreased the stability of the complexes formed. This result supports the participation of the aromatic ring of the protecting group in the stability of the supramolecular complexes. Besides, the titration curves in Figure 4 show that the interaction of **1** with both aromatic rings of Z-L-Phe-OH could be cooperative as this amino acid derivative renders the strongest supramolecular complexes.

Another interesting topic is the moderate enantioselectivity displayed by **1** mainly for the binding of aromatic amino acids. Titration curves by NMR also reflected the L enantiopreference displayed by **1** (Figure 5). We also found that this enantioselectivity is higher with Phe than with Ala, suggesting the importance of the aromatic $\pi-\pi$ interaction for the right matching of the diasteromeric ionic pairs. Related to that, monitoring of the complex formation by using either H3 (Figure 5A) or NH (Figure 5B) signals reflected a similar behavior. For instance, addition of 5 equiv of Z-L-Phe-OH would complex >95% of the macrocycle, while the addition of the same amount of the D enantiomer would only lead to ca. 70% of receptor saturation. However, slightly different trends were observed for H9 and H10 proton signals (Figure 5C). Under the same conditions (5 equiv of amino acid) the D enantiomer would produce about 60% of complexation of the receptor, while the L isomer would lead to almost total saturation. These results are highly reproducible and internally consistent, as the same trends were observed for both aromatic protons of the naphthyl moiety, H9 and H10 (Figure 5C). This behavior would imply that structurally different supramolecular complexes (or binding modes) can coexist for a given substrate (see below) and that the proportion between them depends on the configuration of the amino acid. Besides, the fact that the largest differences between enantiomers of the amino acids are observed for the aromatic protons suggests that a very important interaction for the enantiopreference is closely related to the process that moves H9 and H10 upfield, namely, the proposed aryl−aryl interaction.

A definitive proof for the formation of the supramolecular complexes and important clues for the proposal of their three-dimensional structures have been obtained by a battery of NOE experiments.[26] To obtain clear NOE effects, a high concentration of the supramolecular entity is desirable and, as the complexes are not very strong, a large excess of the substrate was necessary in every case. This would obscure the NOESY cross peaks concerning signals of the receptors, as their intensity would be lower than the cross peaks of intramolecular NOEs of the substrate, in a much higher concentration. We solved that problem by using a selective monodimensional version of the experiment. Related to that, it must be pointed out that 1D NOESY spectra of the macrocycle saturated with Z-L-Phe-OH gave rise to negative NOE effects even for the intramolecular proton contacts.[27] To obtain unambiguous NOE effects, we performed 1D ROESY experiments.[28] Observed intra- and intermolecular

(25) Although fluorescence measurements showed that interaction of **1** is stronger with Phg than with Phe, we have selected Phe for the NMR discussion for several reasons: First of all, Phg derivatives presented poorer solubility in $CDCl_3$ and some of their signals overlapped with important signals of the receptor. On the other hand, Phe is an essential amino acid with biological implications, which make the study of its molecular recognition by **1** much more interesting.

(26) (a) Mo, H.; Pochapsky, T. C. *Prog. Nucl. Magn. Reson. Spectrosc.* **1997**, *30*, 1. (b) Neuhaus D.; Williamson, M. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*; Wiley-VCH: New York, 2000. (c) Overhauser, A. W. *Phys. Rev.* **1955**, *92*, 411.

(27) Considering that macrocycle alone yielded positive NOE effects, this observation implies that the correlation time of the receptor has notably increased in the presence of the amino acid. The correlation time is a parameter of the molecular tumbling, and thus larger correlation times usually correspond to an increment of the molecular size, also supporting the formation of a supramolecular structure.

(28) During this experiment, magnetization is trapped in the *XY* plane during the spin lock time and, consequently, the ROE enhancement is measured in the rotating frame and is always positive, independent of the molecular tumbling. (a) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207. (b) Bothner-By, A. A.; Stephens, R. L.; Lee, J.-M. *J. Am. Chem. Soc.* **1984**, *106*, 811.

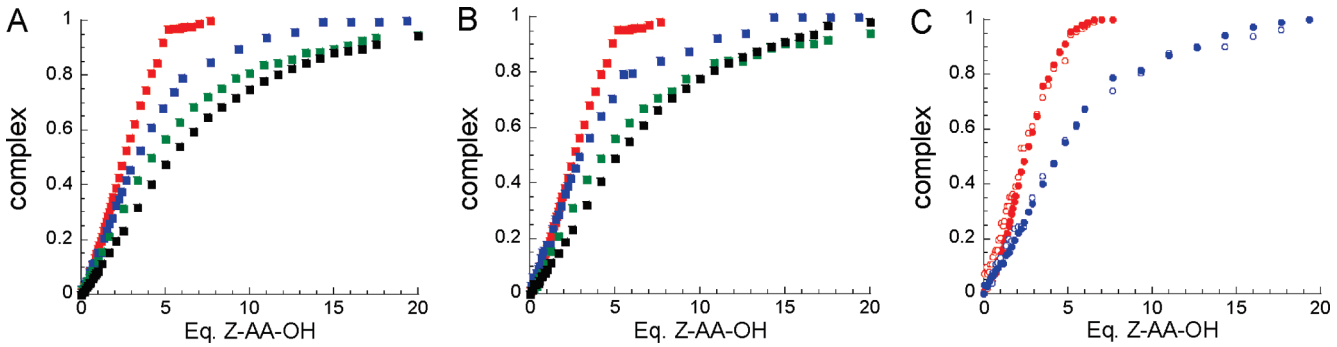

FIGURE 5. NMR titration curves for (A) H3, (B) NH, and (C) H9 (solid circles), and H10 (open circles) and signals of receptor **1** in the presence of Z-L-Phe-OH (red), Z-D-Phe-OH (blue), Z-L-Ala-OH (green), or Z-D-Ala-OH (black).

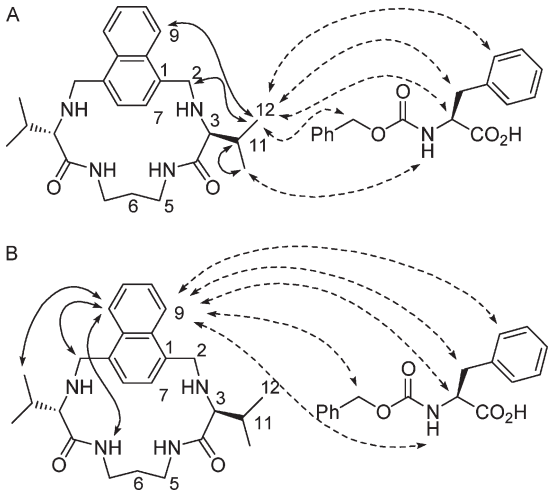

FIGURE 6. ROE enhancements observed in 1D ROESY experiments upon irradiation on (A) isopropyl-CH_3 signals and (B) naphthyl-H9 signals. The intramolecular and intermolecular contacts are depicted by solid or dashed double-headed arrows, respectively.

ROEs are shown in Figure 6, as well as Figure S11 in the Supporting Information. As a general trend, intermolecular ROE enhancements are smaller than the intramolecular ones, but the presence of different ROEs implies an intermolecular contact distance smaller than 5 Å.

Fluorescence Titrations. Although NMR titrations did not afford suitable data to extract binding constants for the proposed complexes, fluorescence study resulted in much more utility. Receptor **1** displays an emission band at 390 nm in dichloromethane, which is red-shifted as compared to the native fluorescence of naphthalene derivatives, taking place at 330−350 nm. Such particular fluorescence behavior has been associated to the occurrence of photoinduced electron transfer (PET) between the secondary amines and the first excited singlet state of the naphthalene moiety (S_1), leading to an emissive exciplex, of lower energy than S_1. Upon protonation of the amine nitrogens, the exciplex emission is quenched (with 1 equiv of acid) and the fluorescence from the naphthalene fluorophore is restored (with an excess of acid). Detailed photophysical studies characterizing the exciplex of this[29] and other macrocylcic naphthalenophanes[14] have been reported previously. From the supramolecular viewpoint, the unique feature of two fluorescence bands allows the determination of the binding constants associated with the recognition process, hence further supporting the proposal of a supramolecular structure.

Fluorescence titrations of **1** with Z-L-Phe-OH and Z-D-Phe-OH were carried out at 278, 288, 298, and 308 K. As can be seen in Figure 7, the band at 390 nm is quenched upon addition of low concentrations of amino acid derivatives, whereas the emission at 340 nm is recovered at higher concentrations of such titrants, in agreement with blocking the PET process due to the formation of ionic pairs. Fluorescence titrations with aliphatic amino acid derivatives (Z-Ala-OH, Z-Val-OH, Z-Leu-OH) afforded also fluorescence changes but were too weak for accurate fittings, which is in agreement with the proposed participation of aromatic rings in the preferential recognition of Phe derivatives by **1**.

Binding constants were calculated from fluorescence intensities by nonlinear least-squares fitting, using the eqs 2 and 3, for 1:1 and 1:2 equilibria (receptor:substrate).[30] In both equations K_1 and K_2 correspond to the stepwise binding constants of 1:1 and 1:2 complexes, respectively. In eq 2, F_0 and F_∞ are the fluorescence intensities of the free receptor and the 1:1 complex, respectively, at 390 nm. The concentration of substrate is denoted by [S].

$$\frac{F}{F_0} = 1+\left(\frac{F_\infty}{F_0}-1\right)\left(\frac{K_1[\mathrm{S}]}{1+K_1[\mathrm{S}]}\right) \qquad (2)$$

In eq 3, F_0' is the fluorescence intensity of the free receptor, at 340 nm, at the beginning of the titration, and F_1' and F_2' are the intensities of the complexes 1:1 and 1:2, respectively, at the same wavelength.

$$\frac{F'}{F_0'} = \frac{1+F_1'/F_0'K_1[\mathrm{S}]+F_2'/F_0'K_1K_2[\mathrm{S}]^2}{1+K_1[\mathrm{S}]+K_1K_2[\mathrm{S}]^2} \qquad (3)$$

Since the exciplex emission is sensitive to the protonation of one of the amines of the receptor, the quenching at 390 nm was used for the initial estimation of K_1. Thus, fitting the exciplex quenching to a 1:1 model (through eq 2) allowed an approximate initial estimation of K_1, which was introduced

(29) Galindo, F.; Burguete, M. I.; Luis, S. V. *Chem. Phys.* **2004**, *302*, 287.

(30) (a) Baglole, K. N.; Boland, P. G.; Wagner, B. D. J. *Photochem. Photobiol. A: Chem.* **2005**, *173*, 230. (b) Wagner, B. D.; Stojanovic, N.; Day, A. I.; Blanch, R. J. *J. Phys. Chem. B* **2003**, *107*, 10741. (c) Wagner, B. D.; McManus, G. J. *Anal. Biochem.* **2003**, *317*, 233. Wagner, B. D.; MacDonald, P. J. *J. Photochem. Photobiol. A: Chem.* **1998**, *114*, 151. (d) Nigam, S.; Durocher, G. *J. Phys. Chem.* **1996**, *100*, 7135.

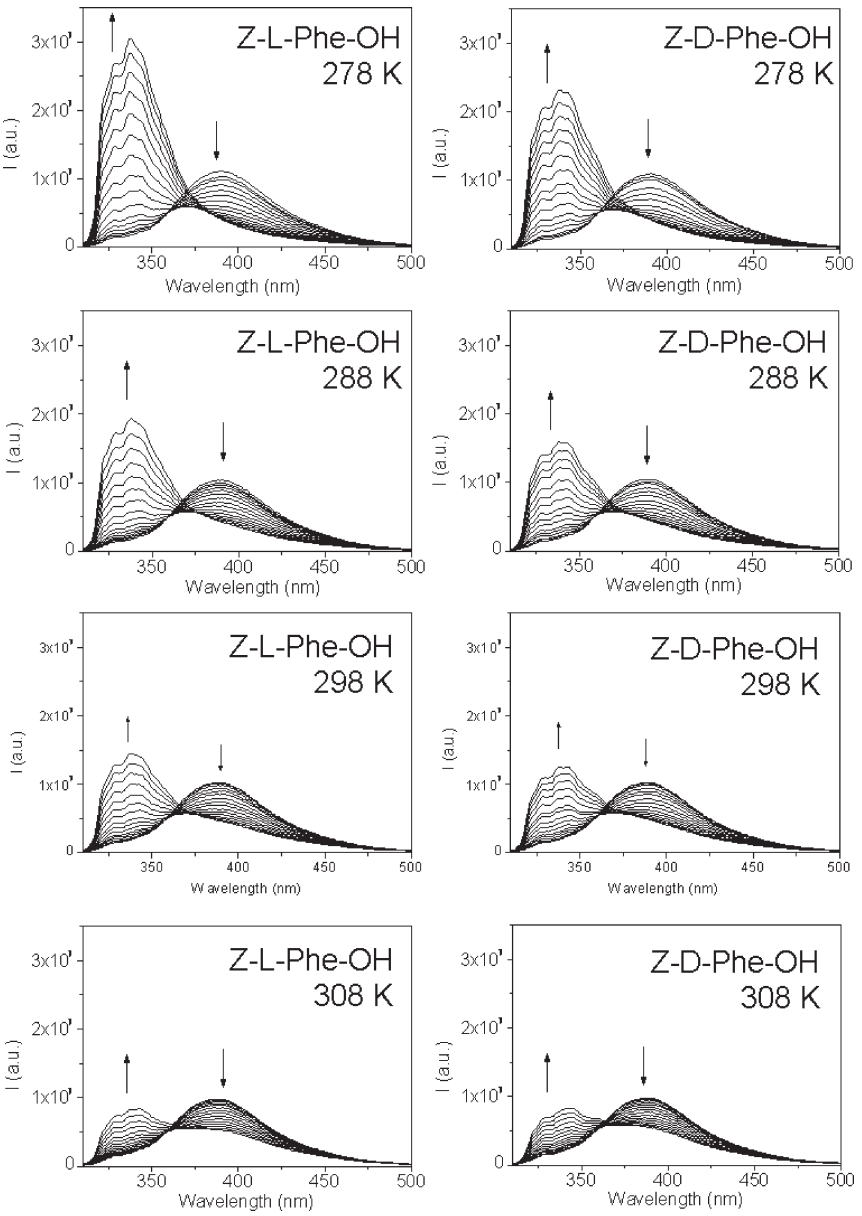

FIGURE 7. Fluorescence titrations of **1** (1.3×10^{-4} M) with Z-Phe-OH (L and D enantiomers) in dichloromethane at 278, 288, 298, and 308 K. Excitation wavelength: 300 nm.

in eq 3 for the calculation of K_2. Successive recalculations of K_1 and K_2 were performed with eqs 2 and 3, until consistent fittings were achieved, leading finally to the curves represented in Figure 8 and data presented in Table 2.[31] At this point, it is worth mentioning that calculation of the binding constants by using steady-state fluorescence data requires the quenching at 390 nm to be noncollisional, i.e., as a result of the real complexation in the ground state (the so-called *static* quenching) and not as a consequence of excited state deactivation by random collisions (*dynamic* quenching).[32] This point has been demonstrated by means of time-resolved fluorescence measurements (see the Supporting Information).

Data obtained at different temperatures were further studied by means of standard Van't Hoff analysis[24,33] to yield the enthalpic (ΔH) and entropic (ΔS) contributions to the free energy (ΔG) of the binding of both 1:1 and 1:2 equilibria (Figure 9 and Table 3).

Data presented in Table 3 reveal that the enantiorecognition process is weak for the 1:1 equilibrium (almost parallel lines in Figure 9), but appreciable for the 1:2 complexation (divergent lines in Figure 9). As will be discussed later, this is compatible with the different spatial arrangements of the 1:1 complexes formed by Z-L-Phe-OH and Z-D-Phe-OH with **1**.

(31) The assumption made for the initial calculation of K_1 is correct since the addition of 1 equiv of TFA to a solution of receptor **1** (0.13 mM) causes the complete quenching of the emission at 390 nm while restoring very scarcely the fluorescence at 340 nm (an additional equivalent of TFA restores completely the fluorescence of the locally excited state at short wavelength).

(32) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Springer: New York, 2006.

(33) Schneider, H.-J.; Yatsimirsky, A. *Principles and Methods in Supramolecular Chemistry*; Wiley: New York, 2000.

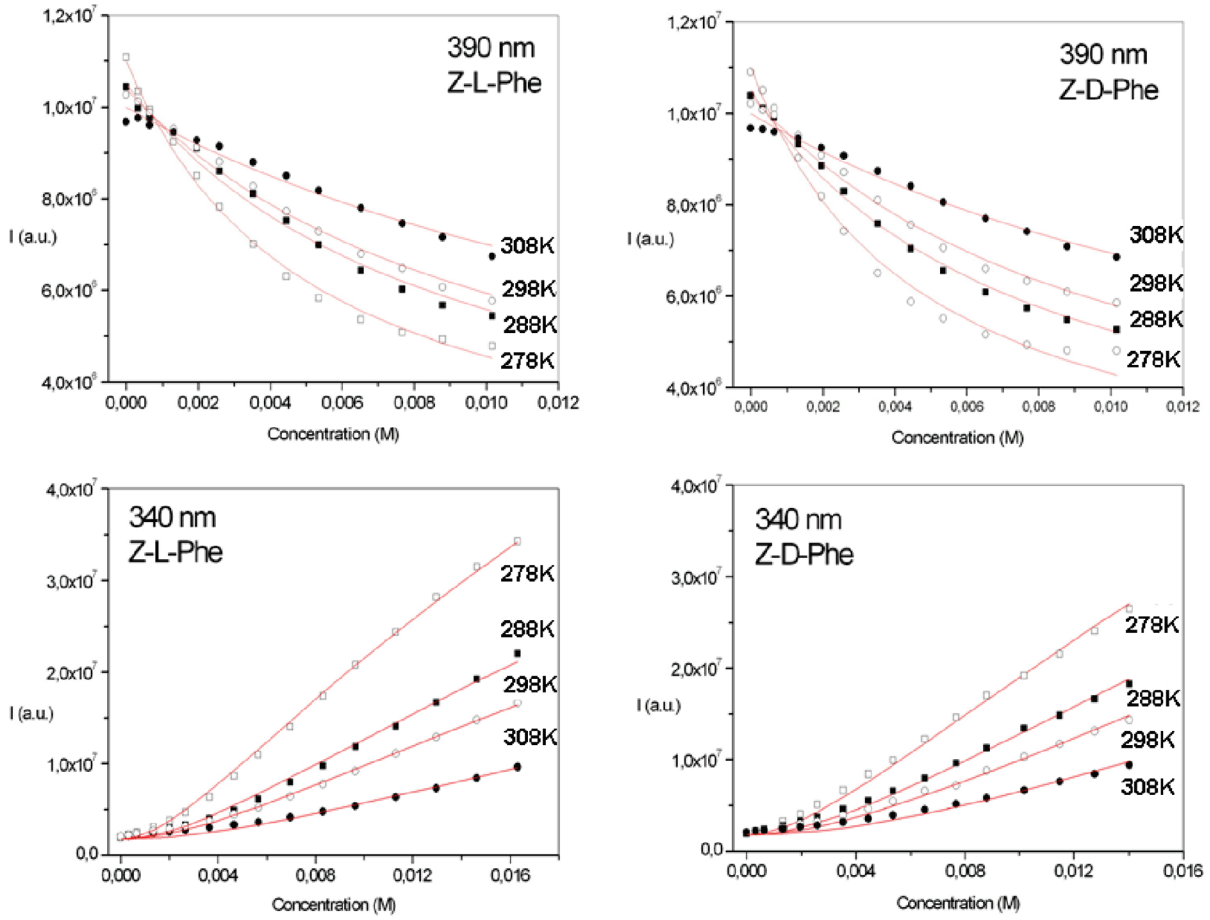

FIGURE 8. Fittings of fluorescence data. The monitoring emission wavelength (340 or 390 nm) and the added titrant (Z-L-Phe-OH or Z-D-Phe-OH) are indicated in each plot. Excitation wavelength: 300 nm. Solvent: dichloromethane.

TABLE 2. Binding Constants (K_1, K_2) and Free Energies of Complexation (ΔG_1, ΔG_2) for the Complexes Formed between Macrocycle 1 (1.3×10^{-4} M) and Z-L-Phe-OH or Z-D-Phe-OH in Dichloromethane, as determined by Fluorescence Spectroscopy

	Z-L-Phe-OH				Z-D-Phe-OH			
T (K)	K_1 (M^{-1})	ΔG_1 (kJ·mol^{-1})	K_2 (M^{-1})	ΔG_2 (kJ·mol^{-1})	K_1 (M^{-1})	ΔG_1 (kJ·mol^{-1})	K_2 (M^{-1})	ΔG_2 (kJ·mol^{-1})
278	193 ± 18	−12.2	19.1 ± 0.1	−6.8	214 ± 8	−12.4	13.9 ± 0.2	−6.1
288	113 ± 3	−11.3	12.6 ± 0.2	−6.1	130 ± 3	−11.7	10.4 ± 0.2	−5.6
298	95 ± 2	−11.1	10.0 ± 0.1	−5.7	99 ± 2	−11.4	8.6 ± 0.2	−5.3
308	52 ± 2	−10.0	6.9 ± 0.1	−4.9	53 ± 2	−10.1	7.1 ± 0.2	−5.0

TABLE 3. Enthalpic and Entropic Changes for the Complexation of 1 with Z-L-Phe-OH and Z-D-Phe-OH, in Dichloromethane, as determined by Fluorescence Spectroscopy

	Z-L-Phe-OH		Z-D-Phe-OH	
	1:1 complex	1:2 complex	1:1 complex	1:2 complex
ΔH (kJ·mol^{-1})	−(29.2 ± 4.2)	−(23.4 ± 1.6)	−(31.6 ± 3.6)	−(15.7 ± 0.9)
ΔS (J·mol^{-1}·K^{-1})	−(61.3 ± 14.1)	−(59.8 ± 5.5)	−(69.1 ± 12.2)	−(34.9 ± 3.1)

Molecular Modeling and Proposal of a Supramolecular Complex Structure. Once the existence of a supramolecular entity is demonstrated, we envisioned a structure for the complex between **1** and Z-Phe-OH (Figure 10). First of all, we must remember that receptor **1** showed some enantiodiscrimination toward this aromatic amino acid. For an enantioselective recognition process, three points of interaction are required and at least two of them must be stereochemically dependent.[34] As there is a proton transfer from the amino acid to the macrocycle, an ion pair is formed and one interaction must be a salt bridge between ammonium and carboxylate groups. A second interaction could be

(34) (a) Zhang, X. X.; Bradshaw, J. S.; Izatt, R. M. *Chem. Rev.* **1997**, *97*, 3313. (b) Webb, T. H.; Wilcox, C. S. *Chem. Soc. Rev.* **1993**, *22*, 383. (c) Pirkle, W. H.; Bocek, P. *Chem. Rev.* **1989**, *89*, 347.

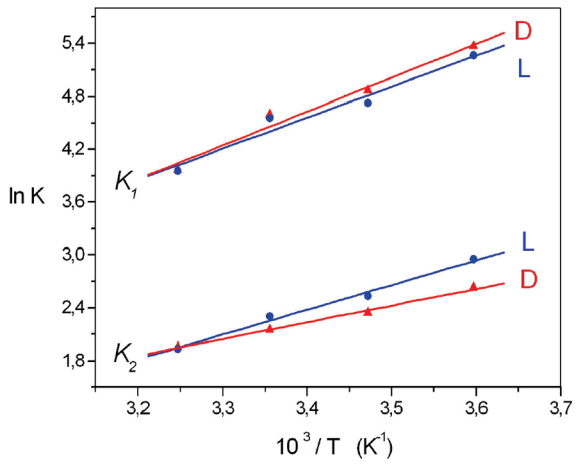

FIGURE 9. Van't Hoff analysis of data presented in Table 2 for the equilibria of complexes formed between **1** and Z-L-Phe-OH or Z-D-Phe-OH in dichloromethane.

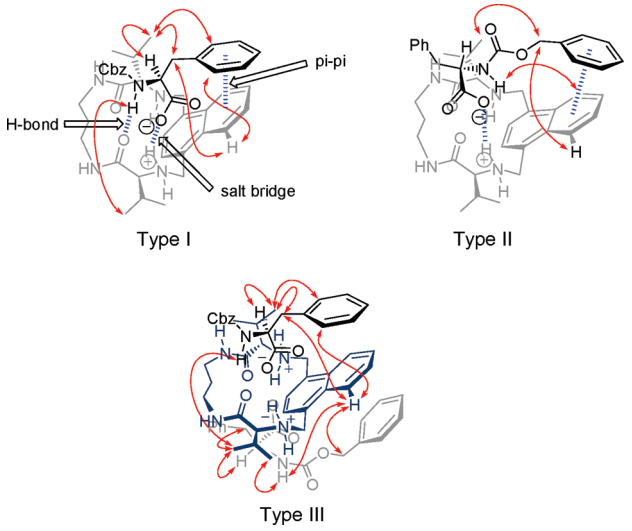

FIGURE 10. Proposed structures for the complexes between receptor **1** and Z-L-Phe-OH. Three-point interactions are highlighted in type I as blue dashed lines and observed ROEs are shown with red double-headed arrows.

a π-stacking between the phenyl ring of the side chain of the amino acid and the naphthyl moiety of the receptor. This interaction is supported by the upfield shift of H9 and H10 protons of the receptor upon saturation with Z-L-Phe-OH and Z-L-Phg-OH. Accordingly, some ROEs observed in the 1D ROESY experiments are also consistent with this arrangement (see structure type I in Figure 10). The third interaction necessary for some enantioselection to occur could be a hydrogen bond between the carbamate NH proton and one carbonyl group of the receptor.

Moreover, some weak ROEs observed between H9 protons of the receptor and signals of the NHZ moiety of the amino acid suggested the participation of other geometry (structure type II) shown in Figure 10. This possibility would set the aromatic ring of the protecting group close to the naphthyl moiety of the receptor. Inspection of this three-dimensional structure (see below) showed a less efficient

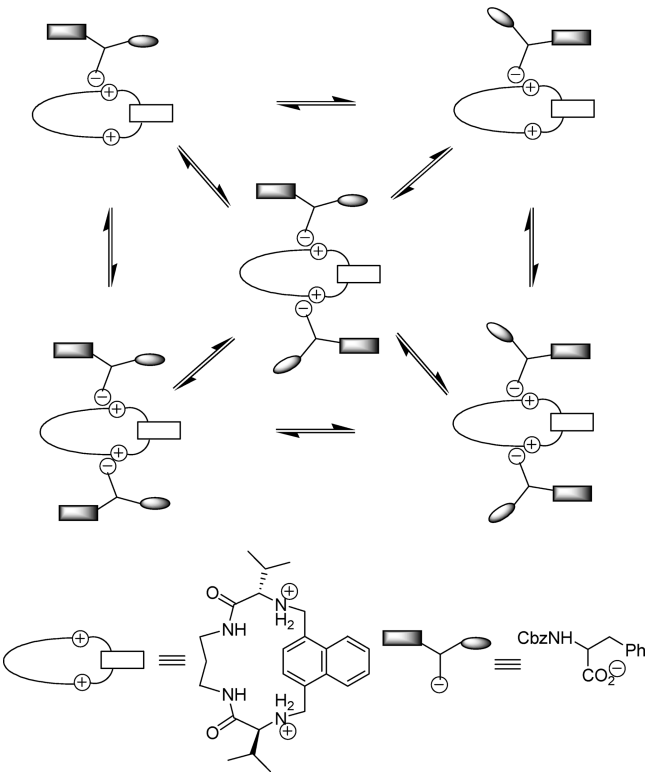

FIGURE 11. Schematic representation of the proposed equilibrium between supramolecular complexes in solution.

matching for a 3-points interaction and suggested that the presence of this complex would decrease the enantioselectivity of the receptor. Besides, this structure is essentially independent of the amino acid side chain and could be present in all the Z-protected amino acids tested.

Apart from these two complexes, a third possibility can be proposed with the participation of two molecules of the substrate, each one interacting with the receptor in type I and II fashion (type III in Figure 10). This complex would explain all the observed ROE effects at once. Additionally, complexes with two substrates on both faces of the macrocycle but with the same disposition (either two of type I or two of type II) also can be formed, in principle. We believe that the true situation would be better described as a mixture of all the possible complexes, the proportion of them being dependent on the substrate. This possibility is schematically depicted in Figure 11.

We have also performed some molecular modeling calculations in order to support the proposal of these complexes, and to visualize their three-dimensional differences. With this aim, we analyzed the three relative dispositions for the complexes with a 1:2 receptor:substrate stoichiometry (type III and two substrates either as type I or type II). Among the three possibilities, we found the type III complex as the most geometrically and energetically favorable situation (Figure 12A, also Table S1 in the Supporting Information). Within the complex, the receptor has two structurally different faces: one concave and one convex. The type III complex showed the best geometrical matching as it locates the substrate in different dispositions at each face. The one on the concave face would set the aromatic side chain on top of the naphthyl ring of the receptor, while the substrate

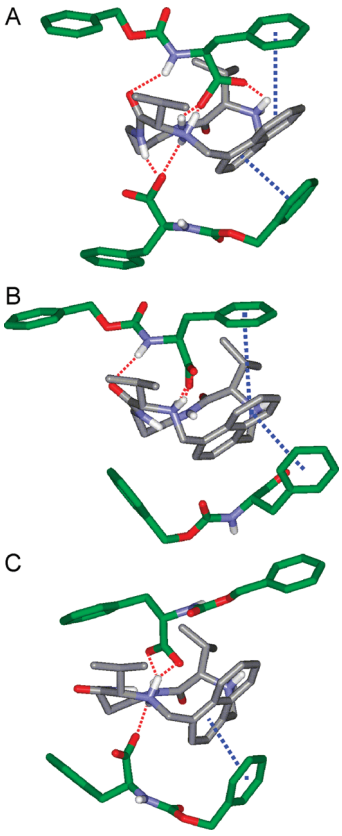

FIGURE 12. Minimized complexes (AMBER force field as implemented in Macromodel, $CHCl_3$ solvent) between receptor **1** and two molecules of Z-L-Phe-OH in different relative dispositions: (A) type III, (B) two molecules of the substrate in type I, and (C) two molecules of the substrate in type II. For clarity, CH hydrogen atoms have been omitted and carbon atoms from the substrate molecules have been highlighted in green. Possible hydrogen bonds and aryl−aryl interactions are shown as red and blue dashed lines, respectively. Selected representative distances are given in Tables S1−S3 (Supporting Information).

on the convex face approximates the aromatic ring of the protecting group to the naphthyl moiety. This geometry maximizes the number of binding interactions (electrostatic, H-bonding, and $\pi-\pi$, Table S2 in the Supporting Information). Besides, distances between protons in this energy minimum clearly explain the experimentally observed ROEs (Table S3 in the Supporting Information). The other two analyzed possibilities (Figure 12B,C) showed a less efficient receptor:substrate structural complementarity (Table S2 in the Supporting Information). For instance, two substrates in type I showed a smaller number of H-bonding interactions (compare parts A and B of Figure 12) while two substrates in type II lack some of the H-bonding and one aryl−aryl contact (compare parts A−C in Figure 12). Another interesting feature is that the stereochemically dependent 3-points interaction with the substrate was only obtained in the concave face of the receptor, with the substrate in type I disposition (parts A and B in Figure 12). Thus, the carboxylate anion and the carbamate NH are H-bonded to the receptor through ammonium and amide groups, while the aromatic side chain is in close contact with the naphthyl ring. This situation implies that only this face is able to exert some degree of stereoselectivity

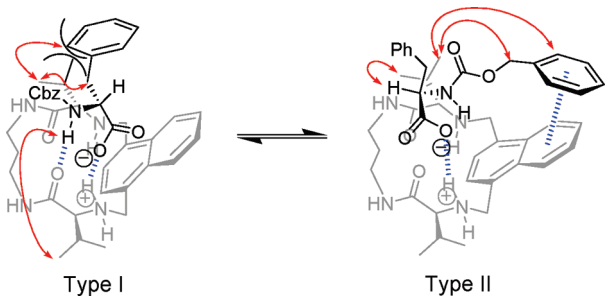

FIGURE 13. Proposed supramolecular complexes between receptor **1** and Z-D-Phe-OH. ROE contacts observed in 1D ROESY experiments are shown with red double-headed arrows.

and suggests a structural explanation for the binding behavior (see below).

We decided to perform ROESY experiments with the macrocycle saturated with D amino acid in order to obtain a thorough knowledge about the diastereomeric supramolecular structure. Again, intermolecular ROE contacts were obtained by using 1D ROESY experiments. These ROE enhancements are consistent with the presence of at least two different coordination geometries in equilibrium, depicted as types I and II in Figure 13, as well as in Figure S12 in the Supporting Information. Different stoichiometries and type III complexes are also most likely formed.

In the case of the D isomer, the configuration of the chiral center would disfavor the aryl−aryl interaction between the phenyl ring of the side chain of the amino acid and the naphthyl moiety of the receptor. Accordingly, selective irradiation of the H9 proton of the receptor did not produce ROE enhancement on the signals of the aromatic side chain of the substrate, which were clearly observed for its enantiomer. Apart from a less favorable $\pi-\pi$ interaction for this enantiomer, the phenyl ring of the side chain would also experience repulsive steric contacts with the isopropyl moiety of the receptor, as can be concluded from the 1D ROESY measurements (Figure 13, as well as Figure S12 in the Supporting Information, structure type I). Thus, this coordination would account for a lower stability of this type of complex. On the other hand, complexes of type II would present a $\pi-\pi$ interaction with the aromatic ring of the protecting group, as also proposed in the case of the L enantiomer. Similar ROE contacts were obtained with the receptor saturated with Z-L-Ala-OH, again reinforcing the proposal of these kinds of interactions (Figure 14, as well as Figure S13 in the Supporting Information). For instance, irradiation of H9 protons of receptor led to ROE enhancements both in Me signal (compatible with type I) and NH signal of the amino acid (compatible with type II). Complex type II is also supported by the slight shielding of H9 and H10 (Table 1).

Finally, we obtained some information about the conformational consequences of the formation of the supramolecular entity. It is well-known that formation of supramolecular complexes can change the conformational behavior of one or more of its constituents. This effect can be very interesting for the study of models for biological processes and for the design of molecular devices. In a

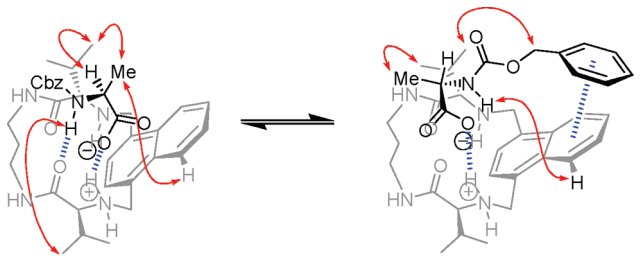

FIGURE 14. Proposed supramolecular complexes between receptor **1** and Z-L-Ala-OH. ROE contacts observed in 1D ROESY experiments are shown with red double-headed arrows.

previous paper,[35] we have studied and characterized the dynamic conformational process associated with the movement of the naphthyl ring with respect to the macrocyclic main plane of the receptor (flipping movement). We wondered if this movement was affected by the complexation of the amino acid. Considering the proposed structure for the interaction between the receptor and the substrate, we hypothesized that, if this interaction is strong enough, the formation of the supramolecular assembly would "lock" the naphthyl movement, at least in some extent. To check that assumption, we performed VT-NMR[36] experiments of a sample of the receptor **1** in the presence of a large excess of Z-L-Phe-OH (>8 equiv) to ensure that all the receptor molecules are in the supramolecular complex (saturation of the receptor). Proton NMR at 248 K ($CDCl_3$, 500 MHz) showed splitting of some of the receptor signals, reflecting a non-C_2-symmetrical conformation, probably due to a slow movement of the naphthyl ring in the NMR time scale. Mainly H9 and H3 signals split off, while the other protons of the receptor were impossible to analyze due to overlapping with the amino acid signals. Moreover, H3 signals were suitable for the dynamic NMR analysis. Those signals coalesce at 303 K leading to values of $\delta\upsilon = 249$ Hz, $k_c(T_c) \approx 550\ s^{-1}$, and $\Delta G^{\ddagger}(T_c) = 58.1$ kJ/mol. If we compare these values with those reported for the free receptor, some conclusions can be extracted. Both coalescence temperature and frequency difference of H3 signals are larger in the presence of the Z-protected amino acid, suggesting a lower average symmetry of the receptor within the complex. A slightly higher energy barrier was also obtained, being $\Delta\Delta G^{\ddagger}(T_c) = 1.9$ kJ/mol, by comparing H3 behavior in the complex and that for the free receptor. This observation suggested that the macrocyclic inversion (and thus naphthyl moiety flipping) is somehow slowed down in the supramolecular complex. Although these values are slightly above the experimental error, the observed trends reinforced the implication of the naphthyl ring in the formation of a supramolecular complex, due to a participation in the interactions with the substrate.

All the experimental observations have led us to hypothesize a structural explanation for the observed thermodynamics of the binding with both enantiomers of Z-Phe-OH. The fluorescence measurements indicated that the true enantioselective process was the formation of the 1:2 receptor:substrate complexes. The NMR study supports that the naphthyl ring is directly involved in the stereoselective binding. Therefore, the dynamics of this moiety must be very important to obtain a good geometrical fitting, mandatory for achieving such stereoselectivity. The flipping of this naphthyl moiety is somehow locked in the interaction. We propose that upon the formation of the 1:1 complex, the movement of the naphthyl moiety of the receptor is rigidified leading to the appearance of two transient structurally different faces, as observed by molecular modeling. As only one of these faces (concave) shows a good stereocomplementarity with the substrate, the formation of the 1:1 complex would increase the enantioselectivity of the receptor for the second binding, as is experimentally observed. This proposal is schematically depicted in Figure 15. Additionally, the temperature dependence of the enantioselectivity also supports our proposal. The increment of the temperature would make the macrocycle more flexible, by increasing the rate of the flipping of the naphthyl ring. The ultimate effect would be the average symmetrization of both faces of the receptor, also decreasing the stereoselectivity, as is experimentally observed.

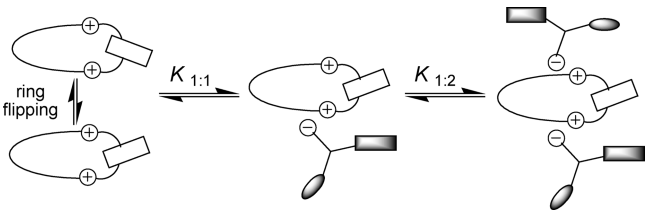

FIGURE 15. Schematic representation of the proposed mechanism for the observed stereoselectivity (cartoon representation as in Figure 11).

Conclusion

Here we report on the ability of a pseudopeptidic naphthalenophane **1** as a selective receptor for *N*-protected aromatic amino acids. A multidisciplinary study with mass spectrometry, NMR, fluorescence, and molecular modeling techniques has allowed us to identify the structural factors responsible for the interactions. Mass spectrometry reflected a slight selectivity toward aromatic amino acids. Moreover, for these aromatic amino acids, ESI-MS and tandem MS showed the formation of complexes with different host:guest stoichiometry, with the 1:2 receptor:substrate supermolecule being the most stable in the gas phase.

In solution, the substrate-induced chemical shifts of the NMR signals of **1** showed the formation of an ionic pair upon a proton transfer from the amino acid carboxylic group to the amino nitrogen of the macrocycle. Other intermolecular interactions, like H-bonding and aryl−aryl contacts, are also responsible for the stability of the complexes. The careful comparison of NMR titration curves for some selected examples confirmed the selectivity toward aromatic amino acids and the L-enantioselectivity displayed by receptor **1**. Besides, both aromatic rings from the substrates, the one on the side chain and the one on the protecting group, seemed to interact with the naphthyl ring of **1**. Fluorescence titrations allowed us to study the interaction between **1** and both enantiomers of Z-Phe-OH in more detail, leading to the complete characterization (binding constants and thermodynamic functions) of the 1:1 and 1:2 complexation

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processes. A battery of 1D ROESY experiments and some molecular modeling calculations have allowed us to refine a structural proposal for the supramolecular complexes. All the data suggest the presence of structurally different complexes in solution for every host−guest system, setting the substrates at both faces of the macrocyclic ring of the host and in different orientations. Besides, the conformation and dynamics of the naphthyl ring plays a fundamental role on the stereoselectivity observed. The results obtained by using all these techniques allowed us to propose a reasonable structural model to explain the data from the fluorescence spectroscopy. We believe that the conclusions presented here will be extremely useful for further development of more efficient and selective receptors. Taking into account the dual fluorescent response of the macrocyclic receptor toward amino acid derivatives (decreasing at 390 nm and increasing at 340 nm), future developments are envisaged, of this or related systems, as ratiometric chemosensors.[37]

Experimental Section

Compound **1** was synthesized as previously described[14] while all the *N*-protected amino acids were commercially available in both enantiopure forms. For the Electrospray Ionization Mass Spectrometry (ESI-MS) analysis, a Quattro LC (quadrupole−hexapole−quadrupole) mass spectrometer with an orthogonal Z-spray electrospray interface (Micromass, Manchester, UK) was used (SCIC-UJI). Weighted amounts of the corresponding compounds in chloroform (varying from 1:1 to 5:1 amino acid: receptor mole ratios, 10^{-5} final concentration in **1**) were infused via syringe pump directly to the interface at a flow rate of 10 μL/min. The temperature of the source block was set to 120 °C and the interface to 150 °C. A capillary voltage of 3.5 kV was used in the positive scan mode and the cone voltage was kept at 15 V. The drying gas as well as nebulizing gas was nitrogen at flow rates of 400 and 80 L/h, respectively. The CID spectra were obtained at various collision energies (typically varied from E_{lab} 0 to 12 eV) by selecting the precursor ion of interest with MS1 and scanning MS2 at a cone voltage kept at 15 V. Argon was used as collision gas and the pressure in the collision cell was maintained at 1×10^{-3} mbar.

All the NMR measurements were performed in a Varian INOVA 500 operating at 500 MHz for proton (SCIC-UJI). The proton and carbon signals of **1** were unambiguously assigned with the help of gCOSY, gTOCSY, gNOESY, and $^{1}H-^{13}C$ gHSQC experiments.[35] For the NMR titration experiments, a 20 mM solution of the receptor in dry, acid-free $CDCl_3$ (5.1 mg of **1** in 0.6 mL) was carefully titrated by addition of small amounts of 0.5 M stock solutions of every substrate. The ^{1}H NMR spectra was immediately acquired (500 MHz, 303 K) after each addition, using 32−64 scans and a relaxation delay of 5 s. Different ^{1}H NMR signals of **1** were monitored during the experiment. The titration was considered finished when further addition of the substrate did not produce changes in any of the monitored signals (saturation of the receptor), leading to the maximum chemical induced shifts reported in Table 1, and those used to estimate the complexation degree at every titration point (Figures 4 and 5). For the 1D gNOESY and 1D gROESY experiments, the standard sequence available in VARIAN software was applied on samples containing 10 mM of **1** and the necessary amount of substrate to saturate the receptor (8−20 equiv, depending on the substrate) and at 303 K. Different mixing times in the range 50−1500 ms were assayed with no significant qualitative differences, apart from the expected changes in peak intensities. The 1D gROESY spectra were apodized with a line broadening of 2.0 Hz prior to Fourier transform.

All the molecular mechanics calculations were performed with MACROMODEL 7.0, using AMBER* as the force field and GB/SA simulation of chloroform as solvent. Further details of the specific procedures, tables with selected distances, and Cartesian coordinates (XYZ) of the minimized structures (types I−III) are given in the Supporting Information.

Steady-state fluorescence spectra were recorded in a Spex Fluorog 3-11 equipped with a 450 W xenon lamp (SCIC-UJI). All the measurements were made at 25 °C unless otherwise indicated. Emission spectra were obtained exciting at 300 nm, in *right angle* mode and using 1×1 cm^2 [3 mL] quartz cells. The curves were processed with the appropriate correction files. Excitation spectra were also recorded to ensure that no impurities were responsible for the emissions. Time-resolved fluorescence measurements were done with the technique of time correlated single photon counting (TCSPC) in a JOBIN-YBON IBH-5000U apparatus (SCIC-UJI). Samples were excited with a nanosecond pulsed hydrogen lamp (fwhm of ca. 0.9 ns). Data were fitted to the appropriate exponential model after deconvolution of the instrument response function by an iterative technique, using the IBH DAS6 fluorescence decay analysis software, where reduced χ^2 and weighted residuals serve as parameters for goodness of fit. All the samples were measured under aerated conditions.

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Supporting Information Available: Additional selected NMR spectra, dilution experiments, ^{1}H NMR titrations, Job plot, 1D ROESY spectra, time-resolved fluorescence measurements, and molecular modeling details. This material is available free of charge via the Internet at http://pubs.acs.org.

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