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Heteroditopic receptor based on crown ether and cyclen units for the recognition of zwitterionic amino acids

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ABSTRACT

The molecular recognition of five targeted amino acids differing in the nature of the side (R)- group and in the size of the aliphatic chain, glycine (Hgly), phenylalanine (Hphe), glutamic acid ($Hglu^-$), 4-aminobutyric acid (Hgaba), and 6-aminohexanoic acid (Heahx), has been studied with a new heteroditopic receptor based in two distinct macrocycles, a cyclen and a crown ether moiety. The bismacrocycle **L** was synthesized via the bis-aminal route allowing to obtain the designed compound in gram scale and in good yield. Protonation constants of **L** and its binding constants with amino acids were determined by potentiometry in H_2O/MeOH (1:1 v/v) solutions at 298.2 K and I=0.10 mol dm^{-3} in NMe_4NO_3. Stronger binding ability of the $H_n\mathbf{L}^{n+}$ receptor for α-amino acids, Hgly and Hphen, than for the other studied substrates were found. Structural data derived from NMR studies showed that the binding of α-amino acids result from the cooperative participation of hydrogen bonds between the carboxylate group of amino acids and the polyammonium sites of cyclen, and the ion-dipole interactions between the ammonium group of the amino acids and the oxygen atoms of the crown ether.

1. Introduction

Nature use amino acids as building blocks for construction of proteins, and as molecular messengers capable of transmitting information in living organisms.[1] Therefore, molecular recognition of amino acids providing the chemical information inherent in their structure and functionality is of great importance.[2] Potential fields of application are the development of efficient methods of amino acid sensing and analysis,[3] the purification and resolution of racemates,[4] the extension of the acquired knowledge to peptide recognition,[5] the development of carriers for drug delivery,[6] etc. Consequently, is not surprising the interest of supramolecular chemists in designing receptors for this group of compounds.[7]

The effective recognition of aminocarboxylic acids at physiological conditions is a difficult task due to its zwitterionic character and the strong competition that they undergo in polar solvents, such as water. Moreover, the electronic densities at the carboxylate and ammonium functions are greatly affected by their mutual vicinity, causing the binding forces of complementary groups of the receptor less effective for association. Thus, in aqueous solution, where zwitterionic amino acids enjoy their greatest solubility, hydrogen bonds are difficult to establish. Consequently, recognition studies are often restricted to non-aqueous media (e.g., $CHCl_3$, MeCN, DMSO) and *N*- or *O*-protected amino acids.[8,9]

The design of receptors capable to confer the required rigidity and complementarity for the recognition of this type of partner, is very challenging. However, several receptors[9f,10] and metalloreceptors[11] have been proposed recently. Among them, heteroditopic receptors offer the possible simultaneous bind of cationic and anionic guests, as well as zwitterions.[12] These compounds are also able to form strong ternary species, especially when various sites act cooperatively.[13,14]

Pursuing the work on recognition of anionic substrates already developed in the group,[15,16] the challenge is now the binding of amino acids in neutral aqueous solution. Thus, herein the preparation and recognition properties of the bismacrocycle **L** (Scheme 1) is carried out. This compound when protonated can act as a synthetic receptor, combining the following structural components: (i) a tetraazamacrocyclic unit that in its polyammonium form is capable to interact with carboxylate groups through hydrogen bonds and coulombic interactions; (ii) a crown ether moiety able to recognize ammonium groups through ion-dipole interactions; and (iii) a naphthalene ring that provides an aromatic planar surface for additional π–π stacking interactions involving side chains of aromatic amino acids. The target molecules were the α-amino acids, glycine (Hgly), phenylalanine (Hphe) and glutamic acid ($Hglu^-$) as well as 4-aminobutyric acid (Hgaba) and 6-aminocaproic acid or 6-aminohexanoic acid (Heahx), see Scheme 1. The ability of the

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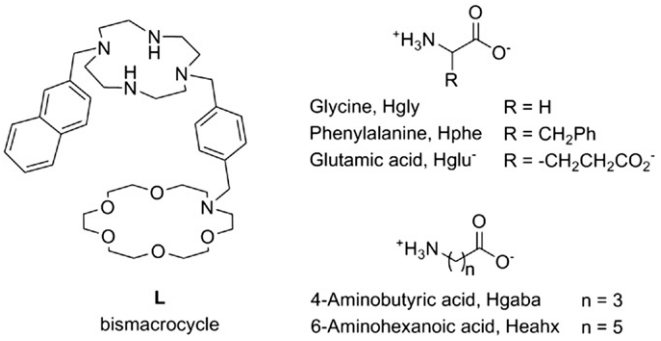
receptor to organize its both macrocycles and aromatic moieties in multiple and cooperative interactions with the substrates in supramolecular entities is studied. Therefore, potentiometric measurements were performed to obtain thermodynamic data, and NMR spectroscopy was used in order to obtain structural insights of the supramolecular entities formed in aqueous solution.



Scheme 1. Chemical structures of the bismacrocycle **L** and the amino acids studied with the corresponding abbreviations used.

2. Results and discussion

2.1. Synthesis and characterization of the bismacrocyclic compound, L

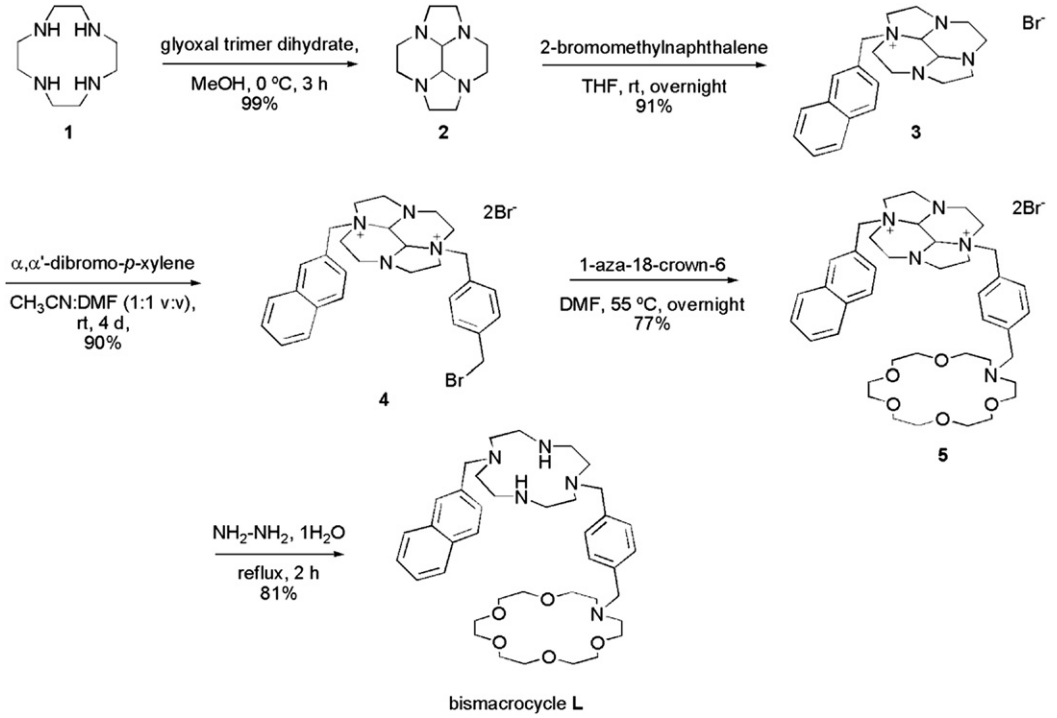
The design of the heteroditopic bismacrocycle **L** is based in the use of cyclen and monoaza crown ether macrocycles as common building blocks, linked by the *p*-xylyl spacer. The two building blocks were assembled via the bis-aminal route depicted in Scheme 2, which allows easy changes of the structure to modulate the ability of the receptor to the binding properties of zwitterions. The synthetic procedure required first the selective protection of the tetraazamacrocycle **1** allowing two *N*-trans substitutions with different functionalized groups. Among the methodologies developed in the last three decades,[17] the bis-aminal one[18] leads rapidly and selectively to mono-*N*- and *trans*-di-*N*-alkylated tetraazamacrocycles.[19,20] After the formation of the bis-aminal derivative **2**, by condensation of glyoxal on cyclen, the first selective mono-N-alkylation with 2-bromomethylnaphthalene yielded the intermediate **3**, and the second selective *trans*-N-alkylation with dibromo-*p*-xylene formed the diammonium salt **4**. Polyalkylation reaction was not observed under the conditions used, see Experimental. Then, the nucleophilic substitution of [18]aneNO$_5$ on **4** yielded the protected bismacrocycle **5**, and finally, the bis-aminal bridge of cyclen in **5** was removed by treatment with hydrazine to afford the bismacrocycle **L**. Therefore, the heteroditopic bismacrocycle **L** was obtained in five steps from commercially available products in good yield (51%).

The structure of the new heteroditopic macrocyclic receptor was well established from analytical and spectroscopic data (see Figs. S1–S11 in Supplementary data).

2.2. Binding studies

The bismacrocyclic compound **L** was designed with two distinct binding sites separated by a rigid aromatic spacer. The cyclen moiety, generally di-protonated under physiological conditions, should interact with the carboxylate group of amino acids, and the crown ether unit is appropriate for the recognition of the ammonium group, essentially via electrostatic interactions and hydrogen bonds.[10k,o,12b,f,14b,21,22] Therefore, this receptor when protonated is a good candidate for associations with amino acids especially when the recognition process leads to cooperative effects.

*2.2.1. Acid–base behavior: protonation constants and protonation sequence of **L**.* Previous to the binding studies, the acid-base behavior of each partner (the receptor and substrates) needs to be known under the experimental conditions of this work, which are H_2O/MeOH (1:1 v/v) solution at 298.2 K and in I=0.10 mol dm^{-3}



Scheme 2. Preparation of bismacrocycle **L**.

NMe_4NO_3. The solvent choice took into account the low solubility of **L** at pH >7 in aqueous solution, and the interest to study amino acids at conditions close to the physiological one. The stepwise and overall protonation constants were compiled in Table 1, and Table S1 in Supplementary data, respectively. For all the studied amino acids, the protonation constant of the ammonium group is 0.13–0.63 log units lower while that of the carboxylate group is 0.12–0.89 log units higher than the corresponding values in aqueous solution taken from the literature (see Table S2 of Supplementary data), testifying the impact of the presence of methanol in the acid–base behavior.

Table 1
Stepwise protonation constants (K_i^H) of **L** and of the studied amino acids in H_2O/MeOH (1:1 v/v), T=298.2±0.1 K, I=0.10±0.01 mol dm^{-3} in NMe_4NO_3

Equilibrium reaction	logK_i^{Ha}
Compound ***L***	
$\mathbf{L} + H^+ \rightleftarrows H\mathbf{L}^+$	9.32(1)
$H\mathbf{L}^+ + H^+ \rightleftarrows H_2\mathbf{L}^{2+}$	7.73(1)
$H_2\mathbf{L}^{2+} + H^+ \rightleftarrows H_3\mathbf{L}^{3+}$	6.38(2)
$H_3\mathbf{L}^{3+} + H^+ \rightleftarrows H_4\mathbf{L}^{4+}$	1.88(4)
Amino acids	
$gly^- + H^+ \rightleftarrows Hgly$	9.33(1)
$Hgly + H^+ \rightleftarrows H_2gly^+$	2.91(1)
$phe^- + H^+ \rightleftarrows Hphe$	8.87(1)
$Hphe + H^+ \rightleftarrows H_2phe^+$	2.84(1)
$glu^{2-} + H^+ \rightleftarrows Hglu^-$	9.62(1)
$Hglu^- + H^+ \rightleftarrows H_2glu$	4.75(1)
$H_2glu + H^+ \rightleftarrows H_3glu^+$	2.78(2)
$gaba^- + H^+ \rightleftarrows Hgaba$	9.93(1)
$Hgaba + H^+ \rightleftarrows H_2gaba^+$	4.69(2)
$eahx^- + H^+ \rightleftarrows Heahx$	10.10(1)
$Heahx + H^+ \rightleftarrows H_2eahx^+$	5.16(2)

[a] Values in parentheses are standard deviations in the last significant figure.

The bismacrocycle **L** contains three amino groups behaving as strong to moderate bases, and the fourth one as a very weak base. The fifth and last protonation constant could not be detected in the pH range (2–11) investigated. This behavior is in part explained by electrostatic repulsions minimization between positive charges in polyprotonated species. Cyclen derivatives generally exhibit two high protonation constants for which protons are located in trans nitrogen atoms and are involved in strong hydrogen bonds with the two adjacent amino groups leading to two high and two very low protonation constants in the ranges 9.5–11 and <2.5, respectively.[23] The cyclen moiety of **L** behaves similarly, consequently two of the three highest protonation constants must correspond to protonations of the cyclen ring, and the third one must be ascribed to the tertiary amino group of the crown ether unit (for the exact assignement see below). The fourth proton has to be located in one amine contiguous to protonated ones of the cyclen ring exhibiting the very low protonation constant. However, the three highest protonation constants of **L** are lower than those found for other cyclen derivatives in aqueous solution.[23] As for amino acids, the presence of methanol in the solvent mixture affects the values, but the main effect arise from the presence of nearby electron-withdrawing groups, such as the aromatic rings (naphthalene and the *p*-xylene spacer) and the oxygen donor atoms of the crown ether moiety. Additionally, the steric effect induced by the presence of bulky groups can lead to decrease the stability of hydrogen bonds either with adjacent nonprotonated amino groups or with solvent molecules.[10m]

The species distribution diagram, presented in Fig. 1, reveals that at physiological pH value (pH=7.4) the main species in solution is $H_2\mathbf{L}^{2+}$ (63%), while $H\mathbf{L}^+$ and $H_3\mathbf{L}^{3+}$ exist only in 31% and 6%,

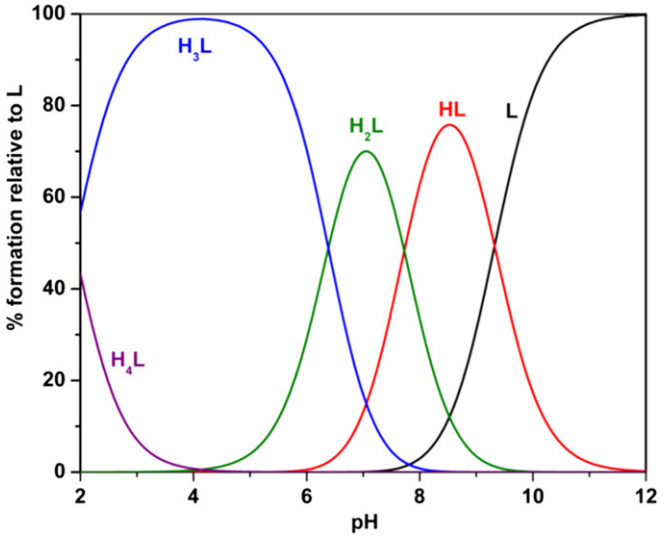


Fig. 1. Species distribution diagram for the bismacrocycle $H_n\mathbf{L}^{n+}$. $C_{\mathbf{L}}$=2 mmol dm^{-3}. The charges were omitted for clarity.

respectively. On the other hand, at the same pH, all amino acids substrates present its zwitterionic form. These data indicate that **L** can be a good receptor for amino acids only in case of bearing the two positive charges located at the cyclen moiety at pH about 7.

Based only on potentiometric measurements, it is not possible to establish the exact scheme of protonation, namely if the second protonation occurs at a second amine of cyclen or at the azacrown ether amine. Then, a ^{1}H NMR titration was performed in mixed D_2O/CD_3OD solution (1:1 v/v) at 298.2 K using KOD as the titrant, which is shown in Fig. 2. 2D NMR experiments carried out at pD 3 and 10 allowed to assign all proton resonances, see Fig. S11 in Supplementary data.

The ^{1}H NMR spectra of **L** at the entire pD range (see Fig. S12) is rather complicated due to the low symmetry of the molecule. For instance at pD=5, in the aromatic region of the spectrum, six different sets of signals, corresponding to nine magnetically non-equivalent protons, are observed, while at upper field the number of signals, or sets of signals, increases to eight. Among the latter signals, those assigned to H_{k-n} protons of the crown ether unit (see Fig. 2 for labeling) appear as a broad signal that does not move along the pD. On the other hand, large shifts of H_h and H_i proton resonances, followed by those of H_j and $H_{b,c}$, were observed. The $H_{a,d}$, H_e and H_o resonances undergo small shifts.

By decreasing the pD from a solution of **L** completely deprotonated, the $H_{b,c}$ proton resonances shift downfield at 10.5–9.5 pD region, but H_e and H_o also slightly move, indicating that the first protonation occurs at the cyclen ring, mainly on the secondary amines although a small percentage of the protons also spread at the tertiary amines. Otherwise, the small H_e and H_o shift can be a result of hydrogen bond formation $^+$N–H···N inside the cyclen ring moiety. At the 8.7–6.0 pD range, corresponding to the second and the third protonation constants, all the resonances close to amine groups shift downfield, except H_{k-n}. However, H_j shifts only at 8.7–7.5 and $H_{b,c}$ shifts more at 7.5–6.0 pD region. Additionally, H_h and H_i have significant shifts in this entire pD range (8.7–6.0). These data indicate that in this pD region several amine centers are simultaneously protonated although in different percentage at each pD value. The resonances close to the tertiary amines of cyclen, H_{a-d}, H_e, and H_o, only slightly shift indicating a very low percentage of protonation of these centers at this pD region. Therefore, it is possible to conclude that the second and third protonations mainly occur on the amine of the crown ether moiety and the secondary

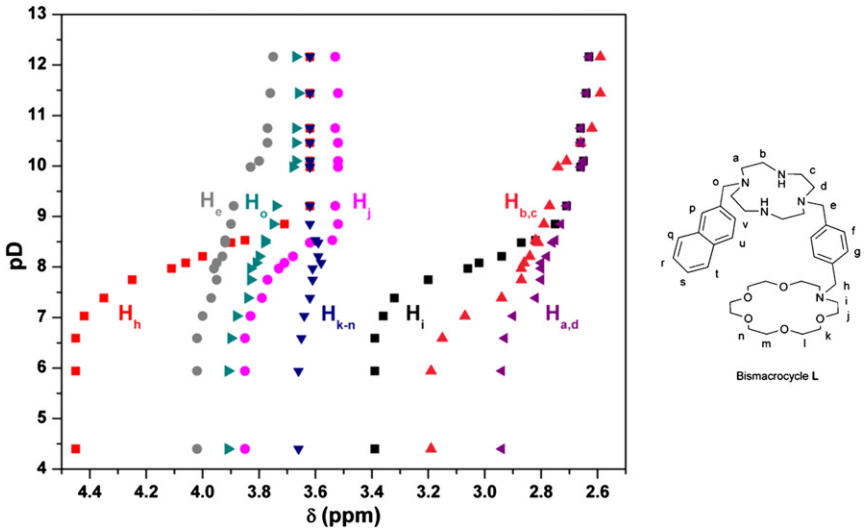


Fig. 2. pD values in function of experimental ^{1}H NMR chemical shifts of some resonances of the bismacrocycle **L**.

amines of the cyclen ring, although the percentage of protonation in the former center is larger for the highest pD values and the protonation of the second secondary amine of cyclen occurs mainly at lower pD (7.5–6.0 pD region). Besides, the symmetry of the compound is kept at acidic pD values (between 5 and 2.5) indicating that, in the NMR time scale, the positive charges in the triprotonated species have an average homogeneous distribution overall nitrogen atoms. Therefore, these data revealed that, at pH values about 7, the first proton is located in the cyclen moiety in the monoprotonated form of the receptor (existing in 31%), whereas the second proton coexists in amine centers of the two macrocycles, with larger percentage at the crown ether moiety of the receptor (existing in 63%).

2.2.2. Binding studies of the bismacrocycle H_nL^{n+} receptor with amino acids

. 2.2.2.1. Association constants. The association constants of the protonated receptor H_nL^{n+} with five targeted amino acids differing in the nature of the side (R)-group and in the size of the aliphatic chain (see Scheme 1) were determined by potentiometry in H_2O/MeOH (1:1 v/v) solution, at 298.2 K and I=0.10 mol dm^{-3} in NMe_4NO_3. Only species of 1:1 (receptor/substrate) stoichiometry were found, in the entire pH scale, although titration data of different ratios were used. The stepwise binding constants were collected in Table 2 and a selected speciation diagram was shown in Fig. 3. They were derived from the overall association constants $\beta_{H_hL_lA_a}$ values (corresponding to the equilibria l **L** + a A + h H$\rightleftarrows$H$_h$**L**$_l$A$_a$, where the charges of the species were not considered) provided by fitting the titration curves, and the values obtained were compiled in Table S3 of Supplementary data. As amino acids are also pH dependent, the plot of the effective association constants K_{eff} as a function of the pH, defined as $K_{eff}=\Sigma[H_{i+j}LA]/\Sigma[H_iA]\times\Sigma[H_jL]$, was used for the correct assessment of the stepwise equilibria, see Fig. 4.[24]

The binding constant for the association of the receptor with Hgly has a reasonable value, which decrease for the other amino acids, following the sequence at pH 7: Hgly>Hphe >Hglu$^-$≈Hgaba>Heahx. Although these values seem small when compared with those of the binding of some dianions with appropriate receptors, they are among the best ones for the binding of similar partners from the literature in aqueous solution.[9f,10n–q]

On the other hand, the decrease of the receptor and/or substrate charges did not lead to clear-cut trends of the association constants, except when the receptor and the amino acids are completely deprotonated, which occur at about pH 9. In fact, the largest constant values would be expected to result from the interaction of H_3L^{3+} or H_2L^{2+} receptor species with the zwitterionic form of the substrates, when carboxylate group of amino acids interact with the protonated amines of cyclen ($^-$O···$^+$N–H), and the ammonium group of amino acids interact with oxygen atoms of the crown ether moiety ($^+$N–H···O). This is not the case here, as shown in Fig. 4, the K_{eff} values only slightly decrease along the 2–9 pH range. This suggests that electrostatic interactions play the main role, but hydrogen bonds are also very important to achieve the recognition process.

As can be observed by the plot of K_{eff} as a function of the pH depicted in Fig. 4, the H_nL^{n+} receptor clearly prefer α-amino acids in acidic medium, and also at neutral pH although for slightly lower K_{eff} values (except glutamic acid). This preference trend must result

Table 2
Stepwise association constants ($K_{H_hL_lA_a}$) of **L** in H_2O/MeOH (1:1 v/v), T=298.2±0.1 K, I=0.10±0.01 mol dm^{-3} in NMe_4NO_3

Equilibrium reaction[a]	log$K_{H_hL_lA_a}$[b]
$H_3L^{3+} + H_2gly^+ \rightleftarrows H_5Lgly^{4+}$	2.99(5)
$H_3L^{3+} + Hgly \rightleftarrows H_4Lgly^{3+}$	2.77(5)
$H_2L^{2+} + Hgly \rightleftarrows H_3Lgly^{2+}$	2.66(5)
$HL^+ + Hgly \rightleftarrows H_2Lgly^+$	2.39(5)
$L + Hgly \rightleftarrows HLgly$	2.76(5)
$H_3L^{3+} + H_2phe^+ \rightleftarrows H_5Lphe^{4+}$	2.91(4)
$H_3L^{3+} + Hphe \rightleftarrows H_4Lphe^{3+}$	2.56(4)
$H_2L^{2+} + Hphe \rightleftarrows H_3Lphe^{2+}$	2.47(5)
$HL^+ + Hphe \rightleftarrows H_2Lphe^+$	2.37(5)
$HL^+ + phe^- \rightleftarrows HLphe$	2.57(4)
$H_3L^{3+} + H_3glu^+ \rightleftarrows H_6Lglu^{4+}$	3.11(1)
$H_3L^{3+} + H_2glu \rightleftarrows H_5Lglu^{3+}$	2.65(2)
$H_3L^{3+} + Hglu^- \rightleftarrows H_4Lglu^{2+}$	2.38(2)
$H_2L^{2+} + Hglu^- \rightleftarrows H_3Lglu^+$	2.16(3)
$HL^+ + Hglu^- \rightleftarrows H_2Lglu$	1.77(4)
$H_3L^{3+} + H_2gaba^+ \rightleftarrows H_5Lgaba^{4+}$	2.55(3)
$H_3L^{3+} + Hgaba \rightleftarrows H_4Lgaba^{3+}$	2.36(3)
$H_2L^{2+} + Hgaba \rightleftarrows H_3Lgaba^{2+}$	2.19(4)
$HL^+ + Hgaba \rightleftarrows H_2Lgaba^+$	1.93(5)
$L + Hgaba \rightleftarrows HLgaba$	1.97(6)
$H_3L^{3+} + H_2eahx^+ \rightleftarrows H_5Leahx^{4+}$	2.32(4)
$H_3L^{3+} + Heahx \rightleftarrows H_4Leahx^{3+}$	2.24(5)
$H_2L^{2+} + Heahx \rightleftarrows H_3Leahx^{2+}$	2.00(6)
$HL^+ + Heahx \rightleftarrows H_2Leahx^+$	1.75(8)
$L + Heahx \rightleftarrows HLeahx$	1.70(10)

[a] **L**=free receptor in its completely deprotonated form.
[b] Values in parentheses are standard deviations in the last significant figures.

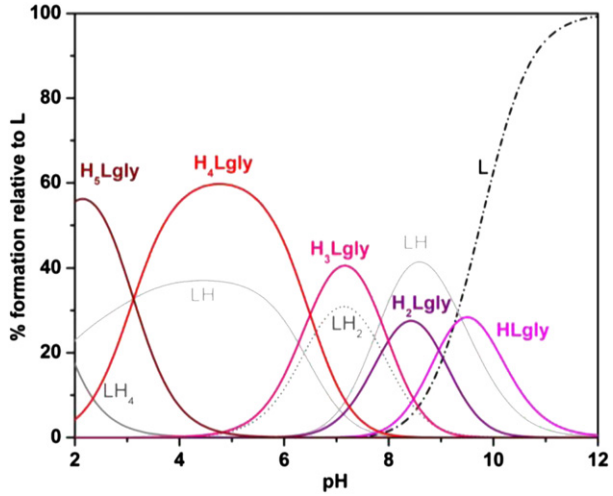


Fig. 3. Species distribution diagram of H_nL^{n+} with Hgly as a function of pH. Percentages are calculated with respect to the total amount of receptor. C_L=2 mmol dm^{-3}, C_A=2 mmol dm^{-3}.

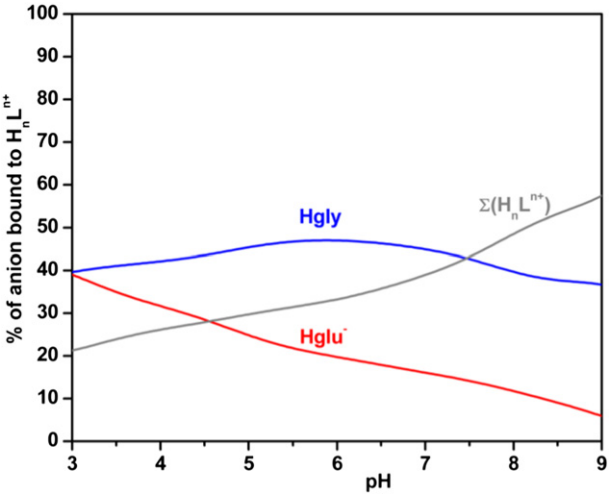


Fig. 5. Distribution diagram of the overall amount of the receptor in the competitive system H_nL^{n+}/Hgly/Hglu$^-$ as a function of pH. C_L=C_A=$C_{A'}$=2 mmol dm^{-3}.

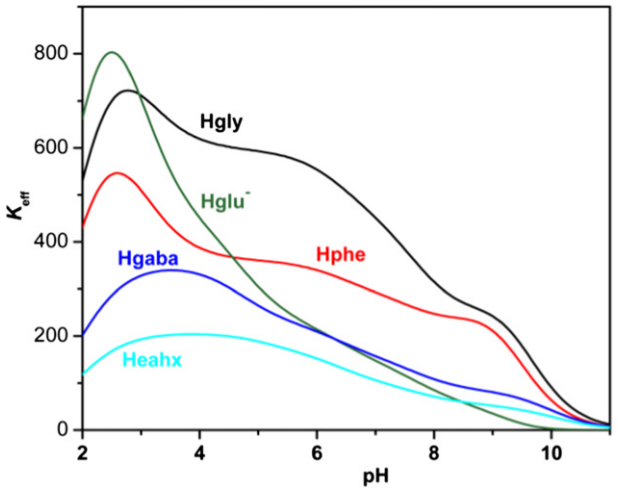


Fig. 4. Plot of the effective association constant K_{eff} versus pH for the supramolecular species formed between the bismacrocycle H_nL^{n+} and the amino acids studied. C_L=C_A=2 mmol dm^{-3}.

from geometrical requirements between the three-dimensional location of the binding groups of the receptor involved in the recognition process and the size of the aliphatic chain of different substrates. In this sense, both macrocyclic binding units might organize multiple and cooperative interactions with zwitterionic α-amino acids, in particular. On the other hand, was not observed any special preference by the receptor for Hphen, suggesting that no π–π interactions with the aromatic arms of the cyclen moiety and the side chain aromatic substituent of Hphen is involved in the recognition event.

The dicarboxylate nature of Hglu$^-$ induces a higher charge concentration at neutral-basic pH when other amino acids are negatively charged or in zwitterionic form, causing a stronger competition between the desolvation process and the possible hydrogen bonds formation, leading to a decrease of the association constant compared to the other α-amino acids. This can be observed by the competition between Hgly and Hglu$^-$ for the receptor shown in Fig. 5.

2.2.2.2. NMR spectroscopic studies. NMR spectroscopy was used to provide structural data of the supramolecular entities formed in solution and to propose a binding model for the recognition of amino acids by H_nL^{n+}. In this regard, the ^{1}H NMR spectra of 1:1 and 1:2 **L**/A molar ratios of the receptor and the amino acid substrates were recorded in D_2O/CD_3OD (1:1 v/v) solvent at pD=7.0. The shifts of resonances are similar at both **L**/A molar ratios. The proton resonance shifts of the associated entity in relation to the free receptor ($\Delta\delta_{max}$) of the most representative signals were collected in Table 3. In all cases, only one set of signals was observed for the free receptor and for the associated entities, indicating fast equilibrium on the NMR time scale.

In general the signals H_{a-d} assigned to the cyclen ring present the larger shifts indicating that the strongest interactions mainly affect the protonated cyclen moiety. Indeed, the protonation studies showed that the cyclen ring can form mono- and diprotonated species at physiological pH, leading to the formation of hydrogen bonds. The H_b and H_c signals shifts are larger than the ones of H_a and H_d, indicating that the secondary nitrogen atoms are more involved in the binding event. The very small shifts of H_e and H_o signals of the benzylic protons are consistent with this hypothesis. However, the shifts are downfield, which is unexpected for formation of an ion-pair interaction, which can result from a possible structural modification of the receptor around the cyclen moiety induced by the binding of the carboxylate group. Indeed, for cyclic polyamines, the inversion of the configuration of the nitrogen atoms need to be considered.[25] Protons are generally stabilized by the formation of intracyclic hydrogen bonds involving the adjacent secondary amino groups[23] directed towards the center of the cavity (*in*). However, by interaction with the negatively charged substrate, new hydrogen bonds are formed and the nitrogen atoms may undergo an inversion by rotation of the bonds of the cycle (*out*), leading a strong structural modification and different chemical shifts.

On the other hand, small shifts are observed for the $H_{m,n}$ signals corresponding to the crown ether, which undergo a downfield shift only when Hgly or Hphe are added. This suggests the presence of ion-dipole interactions between the ammonium groups of these substrates and the oxygens of the crown ether moiety. In opposition, protons H_i resonated at similar chemical shifts upon addition of the substrate, suggesting that the amine/ammonium function of the crown ether do not participate in the recognition event. Similarly, no evident change was observed for the aromatic resonances of both receptor and phenylalanine (naphthyl moiety H_{p-v} or *p*-xylene spacer $H_{f,g}$ of the receptor and benzene ring of phenylalanine). As suggested by the potentiometric data, no π–π interaction with the side chain of the substrate is involved in the recognition event.

Table 3
Induced chemical shifts ($\Delta\delta_{max}$, ppm) of selected receptor signals in the presence of 2 equiv of amino acids at pD=7.0 (5 mmol dm^{-3} in D_2O/CD_3OD (1:1 v/v), T=298.2±0.1 K, 400 MHz)[a]

Substrate	H_n	H_m	H_h	$H_{e,o}$		$H_{b,c}$		$H_{a,d}$	
Hgly	−0.012	−0.017	+0.013	−0.012	−0.010	−0.060	−0.055	−0.022	−0.027
Hphe	—	−0.010	—	—	—	−0.032	−0.027	−0.012	−0.015
$Hglu^-$	—	—	−0.010	—	—	−0.011	−0.008	—	—
Hgaba	—	−0.010	—	—	—	−0.034	−0.030	−0.014	−0.014
Heahx	—	—	—	—	—	—	+0.015	—	—

[a] Induced chemical shifts ($\Delta\delta_{max}$, ppm) lower than 0.010 ppm were not shown, which include ICS of aromatic H_{p-v}, $H_{f,g}$, some of the crown ether resonances H_{i-l} of the receptor and amino acids resonances.

Moreover, the shifts of proton resonances by the supramolecular entity formation are a function of the nature of the associated amino acid. Thus, the relative variation of chemical shift of a given signal can be considered proportional to the amount of supramolecular species formed in solution, the comparison of these variations of the 1:1 **L**/A molar ratio solutions lead to the following trend: Hgly>Hphe>$Hglu^-$≈Hgaba≈Heahx, which is in agreement with the K_{eff} values at neutral pH, see Fig. 4. Besides, stronger shifts of H_{a-d} and $H_{m,n}$ with Hgly and Hphe than the others amino acids were obtained. These results support the involvement of both the crown ether and the protonated cyclen moieties in the binding of these two amino acids yielding to a likely cooperative effect in the best cases, which means with short aliphatic α-amino acids substrates (except glutamic acid, as discussed before).

Finally, in agreement with our experimental findings suggested by NMR experiments, a schematic representation of the recognition event can be suggested for $[H_3\mathbf{L}gly]^{2+}$ (mainly present at physiological pH), see Fig. 6. A 1:1 (receptor/substrate) stoichiometric ratio of the associated species is depicted, showing a total of five

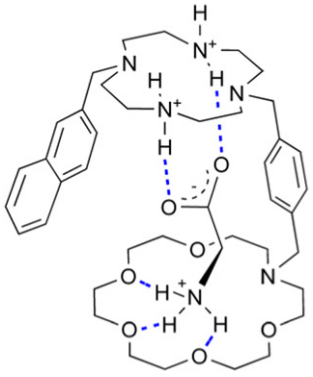


Fig. 6. Schematic representation of the proposed structure with the hydrogen bond network (dashed bonds in blue) for the $[H_3Lgly]^{2+}$ entity mainly present at physiological pH.

hydrogen bonds formed between individual molecules led to an associated species where cyclen and crown ether moieties face each other. This structure provide a three-dimensional cavity of suitable shape and size for amino acids recognition.

3. Conclusions

The synthesized heteroditopic bismacrocycle **L** showed interesting features in its interaction with biologically relevant amino acids. Both potentiometric and NMR results showed that hydrogen bonds between the protonated cyclen moiety and the carboxylate group of the substrate together with ion-dipole interactions between the crown ether and the ammonium group of the amino acids contribute unambiguously to the recognition process at neutral pH. In spite of the low association constants obtained due to the strong competition of the water used as solvent, a preference trend for the α-amino acids, Hgly and Hphen, was observed. This preference is probably caused by multiple and cooperative interactions between both macrocycles of the receptor and the zwitterionic substrates. Consequently the bismacrocycle **L** acts as a ditopic receptor. Unfortunately, the additional aromatic groups of **L** (naphthalene and *p*-xylene) do not participate to the recognition event, π−π stacking interaction being too low to be detected in our conditions.

Finally, this study contributed to obtain further informations on the understanding of factors involved in the selective binding of zwitterionic amino acids by polyammonium artificial receptors. Besides, this knowledge will contribute to improve the design of receptors based on related structures, an area of the supramolecular chemistry that must be developed.

4. Experimental

4.1. General

Microanalyses were carried out by the ITQB Microanalytical Service. Mass spectra have been acquired in the positive and negative polarity mode in an *API-ION TRAP*mass spectrometer equipped with ESI source, at the ITQB Mass Spectrometry Laboratory. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz and a Bruker Amx 300 MHz spectrometers at 298.2 K, respectively. The internal reference used for the ^1H NMR measurements in $CDCl_3$ was TMS, and in D_2O was 3-(trimethylsilyl) propionic acid-d_4. The NMR samples were prepared using Rainin EDP2 automatic micropipets of 25, 100 or 1000 μL, and the pH* was measured with an Orion 420A measuring instrument fitted with a combined Hamilton Spintrode microelectrode. Resonance assignments are based on chemical shift, peak integration and multiplicity for ^1H, ^{13}C NMR spectra and for COSY, NOESY and HMQC experiments.

All commercial reagents were analytical grade and were used without further purification, unless noted otherwise. The cyclen and the 1-aza-18-crown-6 compounds were obtained from Chematech and Aldrich, respectively. The intermediate bis-aminal **2** starting from cyclen was prepared as previously published.[19] Triethylamine was previously distilled before use. All amino acids were analytical grade and were used without further purification. Glycine (>99.7%) was purchased from Merck, L-Phenylalanine (>99.0%), was purchased from Fluka, L-Glutamic acid (>99%) is a Carlo Erba product, 4-Aminobutyric acid (>97%) and 6-Aminohexanoic acid (>98%) were purchased from Aldrich.

4.2. Synthesis (see Scheme 1 for labeling)

4.2.1. Preparation of ***3****.* To a stirred solution of the bis-aminal **2** (676 mg, 3.48 mmol) dissolved in dry THF (5 cm^3), a solution of 2-bromomethylnaphthalene (770 mg, 3.48 mmol) in dry THF (5 cm^3) was slowly added at rt. A white precipitate is immediately formed.

The mixture was stirred overnight at rt. The precipitate was collected by filtration, washed with THF and diethyl ether, then was dried in vacuo, giving **3** as a white powder (1.31 g, 91%). Found: C, 60.31; H, 6.40; N, 13.54. Calcd for $C_{21}H_{27}BrN_4\cdot 0.1H_2O$: C, 60.46; H, 6.57; N, 13.43%. δ_H (400.13 MHz; D_2O; $Me_3Si(CH_2)_3SO_3Na$) 2.40–3.60 (16H, m, CH_2N), 3.86 (2H, d, ^4J=2.2 Hz, H_{Bn}), 4.78 (1H, d, ^3J=13.2 Hz, H_{aminal}), 4.93 (2H, d, ^3J=13.2 Hz, H_{aminal}), 7.55 (1H, d, ^3J=8.7 Hz), 7.60 (2H, m), 7.95 (2H, d, ^3J=7.5 Hz), 8.01 (1H, d, ^3J=8.5 Hz), 8.06 (1H, s) (the last five correspond to H_{ar}). δ_C (100.61 MHz; D_2O; $Me_3Si(CH_2)_3SO_3Na$) 43.8, 47.4, 47.5, 48.1, 48.2, 51.2 (2C), 56.8 (CH_2N), 61.1 (C_{Bn}), 71.6, 82.7 (C_{aminal}), 124.1, 127.4, 127.8, 128.2, 128.3, 128.4, 129.3, 132.6, 133.0, 133.5 (C_{ar}).

4.2.2. Preparation of ***4****.* To a stirred solution of α,α′-dibromo-*p*-xylene (3.32 g, 12.6 mmol) dissolved in dry DMF (30 cm^3), the compound **3** (1.31 g, 3.16 mmol) in dry acetonitrile (30 cm^3) was slowly added at rt. The mixture was stirred during 4 days at rt. The white precipitate was filtered off, washed three times with DMF and diethyl ether, and dried in vacuo giving **4** as a white powder (1.94 g, 90%). Found: C, 51.21; H, 5.12; N, 8.22. Calcd for $C_{29}H_{35}N_4Br_3$: C, 51.27; H, 5.19; N, 8.25%. δ_H (400.13 MHz; CD_3OD; Me_4Si) 3.24–3.82 (16H, m, CH_2N), 4.35–4.55 (4H, m, H_{Bn}), 5.11 (2H, d, ^3J=13.2 Hz, H_{aminal}), 5.16 (2H, d, ^3J=13.2 Hz, H_{aminal}), 5.32 (2H, d, ^4J=3.0 Hz, CH_2Br), 7.47 (2H, d, ^3J=8.0 Hz), 7.55 (2H, m), 7.60 (2H, d, ^3J=8.0 Hz), 7.65 (1H, d, ^3J=8.3 Hz), 7.86 (1H, d, ^3J=7.7 Hz), 7.93 (2H, d, ^3J=8.1 Hz), 8.21 (1H, s), H_{ar}. δ_C (100.61 MHz; CD_3OD; Me_4Si) 32.58 (CH_2Br), 43.9, 44.0, 47.6 (2C), 55.9, 56.1, 61.0, 62.2 (CH_2N), 62.3 (2C) (C_{Bn}), 79.2, 79.3 (C_{aminal}), 125.3, 128.0, 128.4, 128.9, 129.2, 129.3, 129.6, 130.8, 131.5, 134.1, 134.5, 134.6, 135.6, 143.1 (C_{ar}). *m*/*z* (ESI-MS; CH_3OH; positive ion mode) 599.0 $[M-Br]^+$, (negative ion mode) 757.3, $[M+Br]^-$.

4.2.3. Preparation of ***5****.* Compound **4** (1.94 g, 2.86 mmol), 1-aza-18-crown-6 (752 mg, 2.86 mmol) and triethylamine (0.398 cm^3, 2.86 mmol) were dissolved in dry DMF (500 cm^3). The solution was stirred overnight at 50 °C. The solvent was removed under vacuo, then CH_3CN (30 cm^3) was added to the residue and the mixture stirred for more 30 min. After cooling the mixture to 0 °C, a white precipitate was collected by filtration, washed three times with diethyl ether and dried in vacuo. This operation was repeated twice giving **5** as a white powder (1.90 g, 77%). Found: C, 56.92; H, 6.35; N, 7.98. Calcd for $C_{41}H_{59}N_5O_5Br_2$: C, 57.14; H, 6.90; N, 8.13%. δ_H (400.13 MHz; D_2O; $Me_3Si(CH_2)_3SO_3Na$) 3.12 (4H, m, CH_2N_{ether}), 3.30–3.90 (38H, m, CH_2N_{cyclen}+CH_2O_{ether}+CH_2N_{ether}), 4.36 (2H, m, CH_2N_{cyclen}), 4.80–5.10 (4H, m, H_{aminal}+CH_2N_{cyclen}), 7.58–7.68 (7H, m), 7.97 (2H, d, ^3J=8.6 Hz), 8.04 (1H, d, ^3J=7.5 Hz), 8.13 (1H, s). δ_C (100.61 MHz; D_2O; $Me_3Si(CH_2)_3SO_3Na$) 42.7, 42.8, 46.1, 52.5, 54.0, 54.7, 54.8, 55.0 (CH_2N), 60.2, 61.1, 63.5 (C_{Bn}), 69.4, 69.5 (2C), 69.6, 69.8 (CH_2O), 77.7, 77.9 (C_{aminal}), 123.5, 127.5, 127.8 (2C), 128.1, 128.2, 128.4, 129.6 (2C), 132.6, 132.7, 133.1, 133.2, 133.8 (C_{ar}). *m*/*z* (ESI-MS; $CHCl_3$; positive ion mode) 861.9 $[MBr_2+H]^+$, 782.2 $[MBr]^+$, *m*/*z* 700.1 $[M-H]^+$.

4.2.4. Preparation of bismacrocycle ***L****.* Hydrazine monohydrate (10 cm^3) was added to compound **5** (1.726 g, 2.00 mmol) and the solution was refluxed during 2 h. After cooling the mixture to rt, the insoluble residue was collected by filtration and washed three times with 1 cm^3 of hydrazine monohydrate. Then, the residue was dissolved in absolute ethanol and the solvent removed in vacuo. This operation was repeated three times giving **L** as a colorless oil (1.10 g, 81%). Found: C, 69.20; H, 8.39; N, 10.45. Calcd for $C_{39}H_{59}N_5O_5$: C, 69.10; H, 8.77; N, 10.33%. δ_H (400.13 MHz; $CDCl_3$; Me_4Si) 2.61 (8H, m, CH_2N_{cyclen}), 2.65 (8H, m, CH_2N_{cyclen}), 2.80 (4H, t, ^3J=5.8 Hz, CH_2N_{ether}), 3.61 (2H, s, CH_2Ar), 3.65 (20H, m, CH_2O_{ether}), 3.66 (2H, s, CH_2Ar), 3.76 (2H, s, CH_2Ar), 7.30 (4H, m), 7.44 (2H, m), 7.53 (1H, d, ^3J=8.5 Hz), 7.69 (1H, s), 7.79–7.81 (2H, m), 7.86 (1H, d, ^3J=7.7 Hz). δ_C (100.61 MHz; $CDCl_3$; Me_4Si) 45.3 (2C), 51.6, 51.8, 53.7 ($CH_2N_{cyclen+ether}$), 57.7, 59.7, 60.2 (C_{Bn}), 69.9, 70.3, 70.7 (2C), 70.8 (CH_2O), 125.6, 126.0, 127.1, 127.6, 127.7, 127.8, 128.1, 128.8 (2C), 132.8, 133.3, 136.5, 137.5, 138.5 (C_{ar}). *m*/*z* (ESI-MS; $CHCl_3$; positive ion mode) 678.6 $[M+H]^+$.

4.2.5. Preparation of the nitrate salt of the bismacrocycle ***L****.* The bismacrocycle **L** (373 mg, 0.401 mmol) was dissolved in a minimum amount of absolute ethanol. Then, ~4 equiv of ~2 M nitric acid was added (94×10^{-3} cm^3) and the compound was precipitated by adding a large amount of diethyl ether (30 cm^3). The product was filtered and washed several times with diethyl ether, dried in vacuo, giving $\mathbf{L}\cdot 3.34HNO_3$ as a yellow powder (94.5 mg, 26%). Found: C, 51.40; H, 7.12; N, 12.82. Calcd for $C_{39}H_{59}N_5O_5\cdot H_2O\cdot 3.34HNO_3$: C, 51.68; H, 6.78; N, 12.89%.

4.3. Potentiometric measurements

4.3.1. Instrumentation. The potentiometric setup for conventional titrations consisted of a 50 cm^3 glass-jacketed titration cell sealed from the atmosphere and connected to a separate glass-jacketed reference electrode cell by a Wilhelm-type salt bridge containing 0.10 mol dm^{-3} KCl solution. The measuring instrument fitted with a glass electrode and a Ag/AgCl reference electrode. The glass electrode was pre-treated by soaking it in the H_2O/MeOH 1:1 v/v solution over a period of two days, in order to prevent erratic responses. The ionic strength of the experimental solutions was kept at 0.10±0.01 mol dm^{-3} NMe_4NO_3. The temperature was controlled at 298.2±0.1 K using a thermostat and atmospheric CO_2 was excluded from the titration cell during experiments by passing purified nitrogen across the top of the experimental solution. Titrant solutions were added through capillary tips at the surface of the experimental solution with a very precise automatic burette. Titration procedure is automatically controlled by software after selection of suitable parameters, allowing for long unattended experimental runs.

4.3.2. Preparation of the solutions. All solutions for potentiometric titrations were prepared in water/methanol 1:1 v/v solvent mixture, except the amino acids stock solutions that were prepared in aqueous solution. A special care was taken during the preparation of all solutions in water/methanol 1:1 v/v for which the temperature was constantly controlled and maintained at 293 K. The demineralized water was freshly obtained from a Millipore Milli-Q system, boiled for about 2 h and allowed to cool to rt under nitrogen.

Stock solutions of the compound **L** were prepared at ~2 mmol dm^{-3} with addition of ~4 equiv of standard 0.1 mol dm^{-3} HNO_3 solution. Stock solutions of amino acids were prepared at 80 mmol dm^{-3} from the analytical grade compounds, and standardized by titration with standard HNO_3 solutions. Carbonate-free solutions of the titrant 0.10 mol dm^{-3} NMe_4OH were obtained by reacting freshly prepared silver oxide with a solution of NMe_4I under nitrogen. These solutions were discarded every time carbonate concentration was about 0.5% of the total amount of base. For the back titrations, standard 0.100 mol dm^{-3} HNO_3 solutions were used. The titrant solutions were standardized (using Gran's method).[26] The salt NMe_4NO_3 was prepared by neutralization of a commercially available aqueous solution of 25% NMe_4OH with concentrated HNO_3 under nitrogen. The salt was evaporated to dryness under vacuum line, then the 0.75 mol dm^{-3} NMe_4NO_3 solution was prepared.

4.3.3. Measurements. The $[H^+]$ of the solutions was determined by the measurement of the electromotive force of the cell, $E=E^{\circ\prime}+Q\log[H^+]+E_j$. The term pH is defined as $-\log[H^+]$. $E^{\circ\prime}$ and Q

were determined by titrating a solution of known hydrogen-ion concentration at the same ionic strength, using the acid pH range of the titration. The liquid-junction potential E_j was found to be negligible under the experimental conditions used. The value of $K_w=[H^+][OH^-]$ was determined from data obtained in the alkaline range of the titration, considering $E^{\circ\prime}$ and Q valid for the entire pH range and found to be equal to $10^{-13.88}$ in our experimental conditions. Before and after each set of titrations a calibration of the system was performed by titration of a 1.0 mmol dm^{-3} standard HNO_3 solution. Measurements during conventional titrations were carried out with 0.04 mmol of compound **L** (20.00 cm^3 of the stock solution) in a total volume of ca. 30 cm^3 (care was taken to maintain unaltered the water/methanol 1:1 volumetric ratio), in the absence of substrate and in the presence of each substrate for which the C_L:C_A ratio was 1:1 and 1:2. Each titration curve consisted typically of 50–60 points in the 2.5–11.0 pH range.

4.3.4. Calculation and equilibrium constants. Overall equilibrium constants β_i^H and $\beta_{H_hL_lA_a}$ (being $\beta_{H_hL_lA_a}=[H_hL_lA_a]/[H]^h[L]^l[A]^a$) were calculated by fitting the potentiometric data from performed titrations with the HYPERQUAD program.[27] Overall protonation constants obtained were used as fixed values in order to obtain the equilibrium constants of the associated species. The errors quoted are the standard deviations of the overall association constants given directly by the program for the input data, which include all the experimental points of all titration curves. Species distribution diagrams were plotted from the calculated constants with the HYSS program.[28] The species considered in the model were justified by NMR studies and the principles of supramolecular chemistry.

4.4. ^{1}H NMR solution studies

*4.4.1. ^{1}H NMR titration of **L**.* A solution of **L** at 9.82 mmol dm^{-3} in D_2O/CD_3OD 1:1 v/v solvent mixture was prepared and the titration was carried out using freshly prepared CO_2-free 0.10 mol dm^{-3} KOD or 0.10 mol dm^{-3} DCl in the same mixture of solvents (D_2O/CD_3OD 1:1 v/v). Care was taken to keep the volumetric ratio of the solvent mixture. The measurements were undertaken with a pH meter coupled with a combined microelectrode, which was previously calibrated with commercial buffer solutions of standard pH 4.00, 8.00, and 12.00. The electrode was also soaked in the H_2O/MeOH 1:1 v/v solution over a period of two days. The titration curve consisted of 19 points in the 4–12 pH* range. The pH* was then converted in pD values using the equation pD=pH*+0.40±0.02.[29]

*4.4.2. ^{1}H NMR spectra of **L** and amino acids.* Stock solutions of compound **L** (5.15 mmol dm^{-3}) in D_2O/CD_3OD 1:1 v/v solvent mixture and of amino acids (100 mmol dm^{-3}, except for glutamic acid for which 58.7 mmol dm^{-3} was used due to solubility reasons) in D_2O where prepared and the pD was adjusted to 7.00±0.05 by addition of 0.1 mmol dm^{-3} NMe_4OD/D_2O solution or 0.1 mmol dm^{-3} DCl/D_2O solution freshly prepared. Then, solutions of amino acids and **L** in 1:1 and 1:2 molar ratios were prepared. Care was taken to keep the D_2O/MeOD 1:1 volumetric ratio. The pH* was checked and adjusted again if necessary. ^{1}H NMR spectra were finally acquired.

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Supplementary data

These data include: ^{1}H and ^{13}C NMR spectra for compounds **3**–**5**; ^{13}C, ^{1}H, COSY, HMQC, HMBC, ROESY NMR spectra of **L** in $CDCl_3$; COSY NMR spectra of **L** at pD=3 and 10 in D_2O/CD_3OD (1:1 v/v), 298 K; Overall protonation (β_{H_hL}) constants of **L** and amino acids; Overall association ($\beta_{H_hL_lA_a}$) constants of **L** with amino acids; ^{1}H NMR spectra at different pD values of the bismacrocycle **L**. Supplementary data related to this article can be found online at doi:10.1016/j.tet.2012.03.110.

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