Recognition of Caffeine in Aqueous Solutions

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Abstract: Binding of caffeine in aqueous solutions has been achieved for the first time by using water-soluble, tetracationic peptide–porphyrin conjugates Zn-**1** as the receptor molecules. The association constant for caffeine with receptor Zn-**1** is in some cases as high as $6000\,\text{M}^{-1}$, only 5–6 times lower than the highest binding constant reported for an artificial caffeine receptor in low polarity aprotic solvents. The binding mechanism has been studied by a combination of experimental techniques such as UV-visible and NMR spectroscopy and microcalorimetry. Recognition of caffeine involves both stacking with the porphyrin ring and metal coordination. Subtle variations of the receptor structure affect the complexation. Receptors Zn-**1** have also been investigated for the recognition of molecules structurally related to caffeine, for example, 1-methylimidazole. Selectivity towards oxopurine derivatives (caffeine and theophylline) have been found.

Keywords: caffeine · noncovalent interactions · porphyrinoids · sensors · water chemistry

Introduction

Caffeine is probably one of the most widely used drugs in the world. It is a natural constituent of tea, coffee, guarana paste, cola nuts, and cacao beans. It is also added to many popular soft drinks and it is a component of pharmacological preparations and over-the-counter medications including analgesics (in which caffeine acts as an adjuvant), diet aids, and cold/flu remedies. In all its consumable forms caffeine is present in aqueous solutions or mixtures. Recently, three synthetic systems that recognize caffeine in nonpolar solvents ($CHCl_3$, CH_2Cl_2) have been reported.[1–3] These receptors bind caffeine mainly by hydrogen-bonding interactions. However, for applications in sensor and/or separation technology it is of major interest to control and direct molecular interactions between caffeine and synthetic receptors in water.

In aqueous solutions, the interaction of caffeine has been studied with polyphenols, which are present in coffee and especially in tealeaves.[4–7] Additionally, flat aromatic molecules such as ethidium bromide (DNA intercalator) form weak complexes ($K_a \approx 10-200\,\text{M}^{-1}$) with caffeine, predominantly with aspecific hydrophobic interactions.[8] For theophylline complexation (see Table 5, later) more sophisticated target-binding RNAs (aptamers)[9] and combinatorial techniques have been used. This system binds theophylline in aqueous solution very tightly ($K_a = 3.1 \times 10^6\,\text{M}^{-1}$) and selectively in comparison to caffeine ($K_a = 286\,\text{M}^{-1}$).[10] Attempts to obtain proper caffeine-selective systems have been reported in the field of molecularly imprinted polymers.[11–13] However, none of these have a simple and easily accessible molecular structure that recognizes caffeine in aqueous solutions.

We now report a series of novel receptors that bind caffeine in aqueous solutions, namely, water-soluble peptide–porphyrin conjugates Zn-**1** (Scheme 1).[14–16] Remarkably, the association constants of complexes between Zn-**1** and caffeine ($K_a \approx 6000\,\text{M}^{-1}$ for Zn-**1a–d** and Zn-**1f**, Table 1) are up to 20 times higher than the RNA-based system[10] and only 5–6 times lower than the highest binding constant measured with an artificial receptor in low polarity aprotic solvents.[1]

Our design of a water-soluble receptor for caffeine combines the flat aromatic surface of a porphyrin ring, which favors hydrophobic interactions, with a Lewis acidic metal center for ligand coordination. The four alkyl groups on the pyridine nitrogen atoms bear polar functionalities to increase the water solubility of the system without influencing the binding ability. The alkyl groups used in this work are amino acid or dipeptide moieties as well as simple hydrocarbon chains or amides derived from morpholine. Due to the nonsymmetrical substitution of the pyridyl rings (nitrogen atom in the 3-position) the alkyl chains are oriented at an angle with respect to the porphyrin plane providing a somewhat shielded recognition site.[17] The sensing system also possesses a built-in transduction mechanism to monitor

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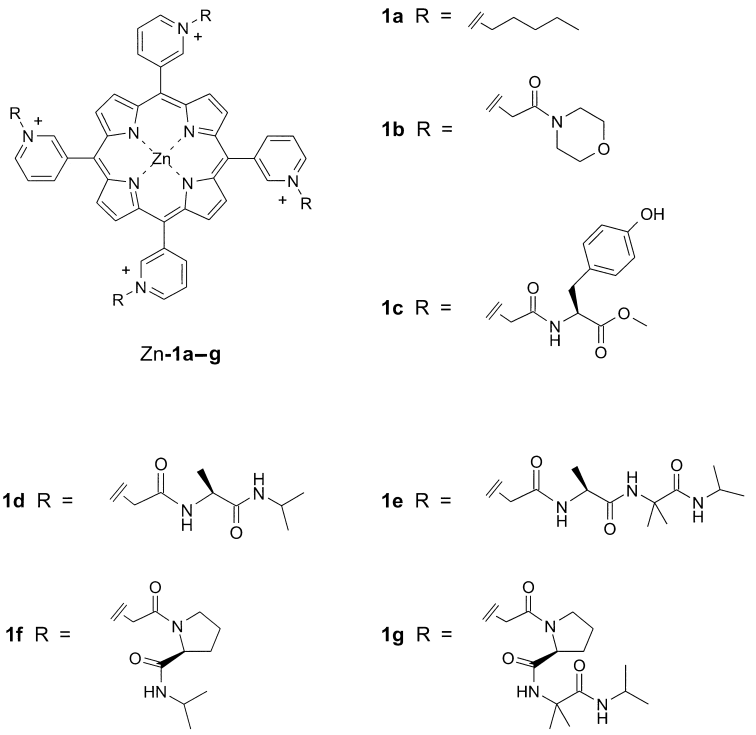

Scheme 1. Structures of peptide–porphyrin conjugates **1a–g** and the zinc complexes Zn-**1a–g**. In Zn-**1b** the counterions are PF_6^-; in all the other Zn complexes they are Cl^-.

Table 1. Binding constants of caffeine to porphyrins Zn-**1** in aqueous solutions (sodium carbonate buffer, $I = 0.008$ M, pH = 9.6, $T = 25\,^{\circ}C$).

Host	K_a [$\times 10^3\,M^{-1}$]
Zn-**1a**	6.2 ± 0.1 (5.15 ± 0.04)[b]
Zn-**1b**[a]	6.5 ± 0.1
Zn-**1c**	4.28 ± 0.08 (6.09 ± 0.07)[c]
Zn-**1d**	5.66 ± 0.05 (6.72 ± 0.08)[b]
Zn-**1e**	3.26 ± 0.05 (2.75 ± 0.04)[b]
Zn-**1f**	6.43 ± 0.08 (6.06 ± 0.06)[c]
Zn-**1g**	2.83 ± 0.02

[a] PF_6^- as counterion. [b] Data obtained by ITC, sodium carbonate buffer $I = 0.11$ M, pH = 10.3. [c] Sodium oxalate buffer, $I = 0.008$ M, pH = 3.7.

the complexation of caffeine, namely, the intrinsic UV-visible activity of the porphyrin core. By using this system, complexation of caffeine and of a number of structurally related molecules was studied in buffered aqueous solutions (sodium carbonate buffer pH = 9.6, or sodium oxalate buffer pH = 3.7).[18] The binding mechanism has also been investigated by means of isothermal titration microcalorimetry (ITC) and NMR spectroscopy.

Results and Discussion

Synthesis: Porphyrin Zn-**1** was prepared following known literature procedures.[19, 20] Alkylation of commercially available 5,10,15,20-tetra(3-pyridyl)porphyrin with pentylbromide in DMF (Zn-**1a**)[19] or with the appropriate chloroacetyl amide derivative in CH_3CN afforded receptors Zn-**1b–g**.[20] Chloroacetamido amino acid or dipeptide amides used in the alkylation reactions were prepared by solution-phase peptide chemistry starting from Boc amino acids (EDC/HOBt coupling reactions).

For the more water-soluble receptors Zn-**1c–g**, the workup procedure was slightly modified and isolation of the chloride salt was achieved by removing the excess inorganic salts after the metalation step by using reverse-phase chromatography and exchanging the counterions for chloride ions on an ion-exchange column (Scheme 1).

Caffeine binding studies:[21, 22] Binding to the water-soluble receptors **1** can be described in terms of the equilibria described by Equations (1) or (2) and (3) depending whether the porphyrin is metalated (Zn-**1**) or free-base (**1**), in which K_i is the intrinsic binding constant accounting for the presence of two degenerate binding sites.

$$\text{Zn-}\mathbf{1}\cdot H_2O + L \rightleftharpoons \text{Zn-}\mathbf{1}\cdot L + H_2O \quad K_a = [\text{Zn-}\mathbf{1}\cdot L]/([\text{Zn-}\mathbf{1}\cdot H_2O][L]) \quad (1)$$

$$\mathbf{1} + L \rightleftharpoons \mathbf{1}\cdot L \quad K_1 = [\mathbf{1}\cdot L]/([\mathbf{1}][L]),\ K_i = K_1/2 \quad (2)$$

$$\mathbf{1}\cdot L + L \rightleftharpoons \mathbf{1}\cdot L_2 \quad K_2 = [\mathbf{1}\cdot L_2]/([\mathbf{1}\cdot L][L]) \quad (3)$$

The replacement of a water molecule coordinated to the Zn by an added ligand [Eq. (1)] has been already reported for several other water-soluble porphyrins.[23–26]

For the free-base porphyrins reported here we assume that there are two available binding sites corresponding to the two porphyrin faces [Eqs. (2) and (3)].[27] Investigation of the binding phenomenon by using different experimental techniques (UV-visible spectroscopy and microcalorimetry) in different concentration ranges (vide infra) supports this model.

Comparison of the binding properties of receptors **1** with other systems previously reported proved to be difficult. In fact, several reports have considered the complexation of neutral guests with the planar, symmetrical 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin. Generally a 1:1 stoichiometry has been assumed based on the occurrence of isosbestic points during spectrophotometric titrations.[16, 28–30] However, a detailed analysis by several research groups has shown that neither their presence nor their absence has any decisive value in diagnosing the number of species in the mixture.[31]

Controversial opinions have been expressed about the coordination mode of nitrogenous bases to zinc complexes of 5,10,15,20-tetrakis-(*N*-alkylpyridinium-4-yl)porphyrin in water. It has been proposed that the interaction of ligand

heteroatoms with the Zn atom is not strong enough in water to lead to axial coordination and that face-to-face association dominates the binding.[30] On the other hand, binding of pyridine and 1-methylimidazole was treated according to the water-exchanging equilibrium [Eq. (1)]. Association constants $K_a = 3800\,\text{M}^{-1}$ and $4900\,\text{M}^{-1}$ were reported for the two ligands, respectively.[32] However, the magnitude of these K_a is not consistent with the binding of nitrogenous bases to water-soluble Zn–porphyrins ($14-29\,\text{M}^{-1}$ for pyridine[25, 33] and $36-69\,\text{M}^{-1}$ for imidazole[25]) reported by other groups.

UV-visible spectroscopic studies: The binding of caffeine to receptors **1** was evaluated by monitoring the UV-visible intensity variations occurring in the Soret band region (between 400 and 480 nm) as a function of increasing guest concentration (see Tables 1 and 2). Bathochromic shifts between 4 and 5 nm upon complexation are observed for all receptors, except Zn-**1c** (described later in more detail). The largest relative changes in molar absorptivity are between 36 and 43 % ($\Delta\varepsilon < 0$). The spectral changes, showing well-defined isosbestic points in all cases, were fitted according to Equation (1) for metalated receptors Zn-**1** or to Equations (2) and (3) for free-base porphyrins.

From the K_a values (Table 1) it is clear that caffeine complexation is influenced by *N*-alkyl chain length. For receptors Zn-**1a–d** and Zn-**1f**, which bear short alkyl chains (5–8 atoms), the K_a value is roughly a factor of two higher than for receptors Zn-**1e** and Zn-**1g**, which bear longer chains (11 atoms). Therefore longer chains hinder, to some extent, the binding of caffeine. It should be noted that receptors **1**, even though soluble in water, bear chains that are to some extent hydrophobic to promote the formation of a hydrophobic binding site. However, chain hydropobicity can also cause stronger interaction with the porphyrin core, thus disfavoring the binding process.

Table 2. Binding constants for caffeine to free-base porphyrins **1** in aqueous solutions, $T = 25\,^{\circ}\text{C}$.

	Host	K_i [$\times 10^3\,\text{M}^{-1}$][a]	K_2 [$\times 10^3\,\text{M}^{-1}$]
UV[b]	**1c**	1.4	0.41
UV[b]	**1d**	1.5	0.37
ITC[c,d]	**1d**	1.8	0.52

[a] $K_i = K_1/2$. For definitions of K_1 and K_2 see Equations (2) and (3). [b] Error margin ±10 %. Sodium carbonate buffer, $I = 0.008\,\text{M}$, pH = 9.6. [c] Sodium carbonate buffer $I = 0.11\,\text{M}$, pH = 10.3. [d] Thermodynamic parameters: $\Delta H^{\circ}_1 = -27.7 \pm 0.1\,\text{kJ mol}^{-1}$, $\Delta S^{\circ}_1 = -30.8 \pm 0.8\,\text{J mol}^{-1}\,\text{K}^{-1}$ and $\Delta H^{\circ}_2 = -13.2 \pm 0.2\,\text{kJ mol}^{-1}$, $\Delta S^{\circ}_2 = 7.8 \pm 0.8\,\text{J mol}^{-1}\,\text{K}^{-1}$.

^{1}H NMR spectroscopic studies: Structural analysis of receptors Zn-**1** by ^{1}H NMR spectroscopy also supports the interaction of the longer peptidic chains with the porphyrin platform (Figures 1 and 2).[34]

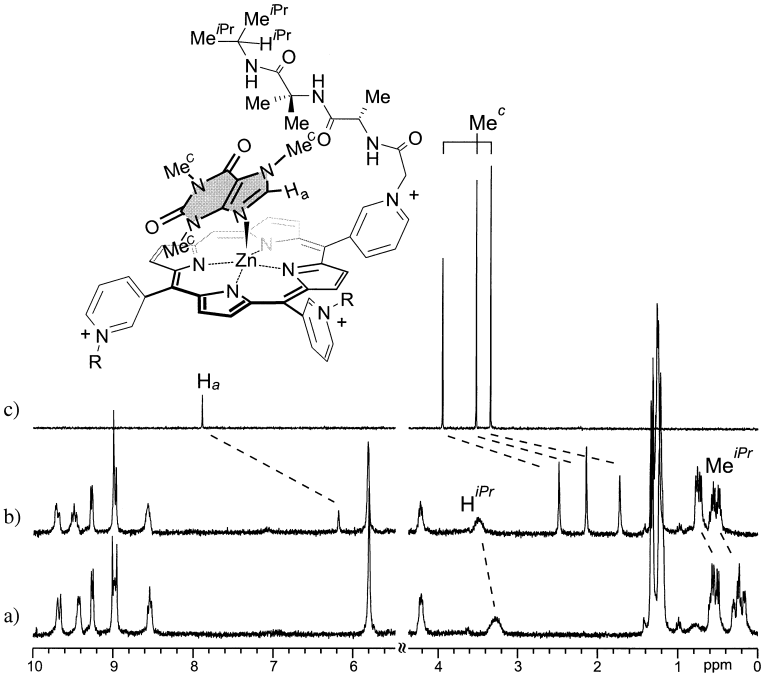

Figure 1. Binding of caffeine to receptor Zn-**1e** in D_2O solution (1.7 mM). Top: schematic representation of the complex Zn-**1e**·caffeine (only one peptide arm R is shown for clarity). Bottom: ^{1}H NMR spectra of a) Zn-**1e**, b) Zn-**1e** + 1.1 equiv of caffeine, and c) caffeine.

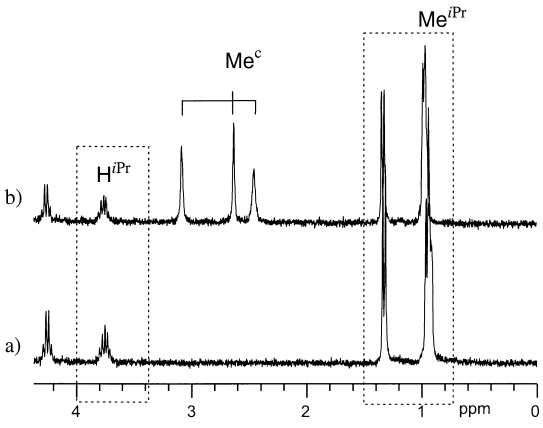

Figure 2. Binding of caffeine to receptor Zn-**1d** in D_2O solution (1.7 mM). The signals for the *i*Pr groups of the receptor are not shifted (see text). a) Zn-**1d** and b) Zn-**1d** + 2.4 equiv of caffeine.

In the absence of a guest molecule, the spectra of receptors Zn-**1e** (Figure 1a) and Zn-**1g** in D_2O show an upfield shift for the *i*Pr group protons, relative to those of receptors Zn-**1d** and Zn-**1f**. In fact, receptors Zn-**1d** and Zn-**1f** exhibit signals at $\delta = 3.75$ (H^{iPr}) and $\delta = 0.98$ ppm (Me^{iPr}) (Figure 2a); this indicates no particular interaction with the porphyrin core. In contrast, the signals of the *i*Pr groups of receptors Zn-**1e** and Zn-**1g** are observed at $\delta = 3.29$ (H^{iPr}), $\delta = 0.12-0.33$, and 0.46–0.63 ppm (Me^{iPr}) and at $\delta = 3.31$ (H^{iPr}), $\delta = 0.04-0.38$,

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and 0.44–0.68 ppm (Me^{iPr}), respectively. This suggests that the long chains of receptors Zn-**1e** and Zn-**1g** are positioned above or below the porphyrin plane in the shielding region caused by the macrocycle ring current.

Upon addition of 1–2 equivalents of caffeine to a solution of receptor Zn-**1e** (or Zn-**1g**) in D_2O, the *i*Pr group signals shifted downfield ($\delta = 0.2$ (H^{iPr}) and $\delta = 0.2$ and 0.3 ppm ($2\,Me^{iPr}$), Figure 1b), while no effect was observed for the shorter chains of receptors Zn-**1d** (Figure 2b) and Zn-**1f**. Therefore, we propose that the side chains in receptors Zn-**1e** and Zn-**1g** are folded back over the porphyrin plane and are displaced upon caffeine complexation. In contrast, the shorter alkyl chains of receptors Zn-**1d** and Zn-**1f** do not hinder the complexation of caffeine. Furthermore, caffeine signals also undergo large upfield shifts between 1 and 2 ppm for proton H_a on C-8 (see Figures 1b, 2b) and between 0.9 and 1.7 ppm for the three methyl groups (Me^c) upon complexation with all receptors **1** (metalated or not).

ITC studies: ITC experiments also confirm the influence of the chain length on caffeine complexation (Table 1). The enthalpograms, that is, the evolved heat per added mole of caffeine during the calorimetric titration, were fitted to a 1:1 model, and we found a good agreement with the binding constants obtained by UV-visibbble spectroscopic titrations. The same trend is observed with the K_a for Zn-**1d**, which is about twice as high as the K_a for Zn-**1e**.

Thermodynamic parameters for caffeine binding to porphyrins **1** can be extracted from the ITC experiments (Table 3). In general, large negative enthalpy and entropy contributions are observed for the complexation of caffeine.

Table 3. Thermodynamic parameters for caffeine binding to porphyrin Zn-**1** in aqueous solutions determined by ITC (sodium carbonate buffer, $I = 0.11$ M, pH = 10.3, $T = 25\,^{\circ}C$).

Host	ΔH^{o} [kJ mol^{-1}]	ΔS^{o} [J mol^{-1} K^{-1}]
Zn-**1a**	-36.7 ± 0.1	-52.2 ± 0.3
Zn-**1d**	-34.5 ± 0.2	-42.5 ± 0.8
Zn-**1e**	-39.3 ± 0.2	-65.9 ± 0.8

The large negative enthalpy change associated with the formation of the complex is consistent with a substantial contribution of hydrophobic and stacking interactions to the binding process.

Negative entropy contributions have also been observed by Mizutani and co-workers for binding of amine and amino esters to Zn–porphyrin receptors and have been analyzed in terms of induced-fit-type complexes.[35] Conformational restrictions upon complex formation and/or receptor reorganization (as observed from NMR measurements for Zn-**1e** and Zn-**1g**) cause entropy losses ($\Delta S^{o} < 0$), which are only partially compensated by the entropy gain from desolvation. Accordingly, the long chain receptor Zn-**1e** has a significantly more negative ΔS^{o} value (-65.9 J mol^{-1} K^{-1}) than Zn-**1d** (-42.5 J mol^{-1} K^{-1}), which agrees with the necessity of displacing the longer alkyl chains of Zn-**1e** from the porphyrin faces. Even though the binding strength of Zn-**1a** is similar to that of the other short chain receptors ($K_a \approx 6000$ M^{-1}), the thermodynamic parameters are not simply comparable due to the quite different nature of the *N*-substituents and its influence on the solvation of the receptor.

Free-base porphyrin receptors: To gain more information about the binding mode and to assess the role of the Zn atom, UV and ITC experiments on metal-free receptors **1c** and **1d** were performed (see Figures 3 and 4). UV titration curves for the complexation of caffeine were fitted to 1:1 and 2:1 caffeine stoichiometries. Despite the presence of isosbestic points, better fits were obtained with the 2:1 binding model, although the differences are too small to discard the possibility of 1:1 stoichiometry. On the other hand, fitting to a 2:1 model gave better agreement between the UV and ITC measurements.[36]

Binding of caffeine K_i (Table 2) to the free-base receptors (taking into account the presence of two degenerate binding sites) is around four times weaker than for the corresponding metalated hosts. For example, considering both the UV and the ITC data, Zn-**1d** and free-base **1d** bind caffeine with $K_a \approx$ 6100 and 1600 M^{-1}, respectively. Comparison of the thermodynamic parameters for metal-free **1d** ($\Delta H^{o}_{i} = -27.7$ kJ mol^{-1}

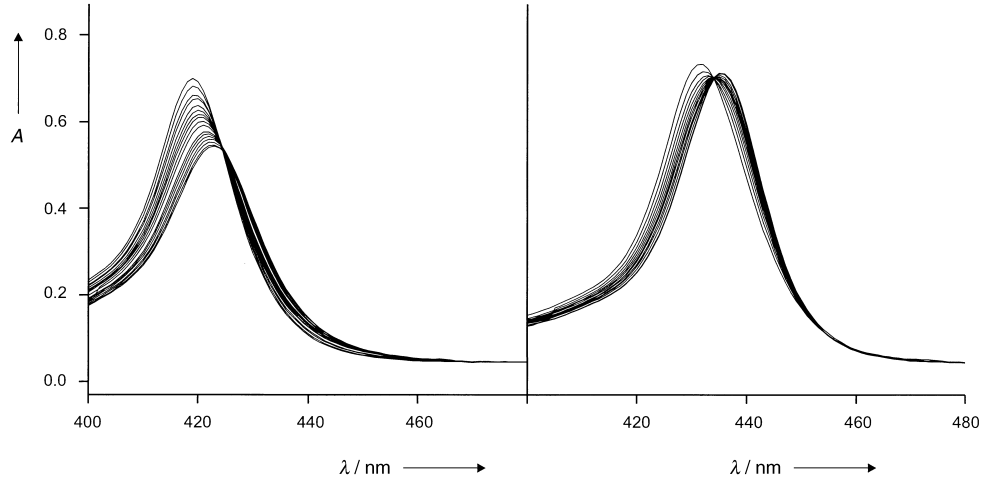

Figure 3. Changes in the UV-visibble absorption spectra of receptors free-base **1d** (left) and Zn-**1d** (right) upon addition of caffeine. [Porphyrin] $\approx 2.5\,\mu$M in carbonate-buffered solution ($I = 0.008$ M, pH = 9.6).

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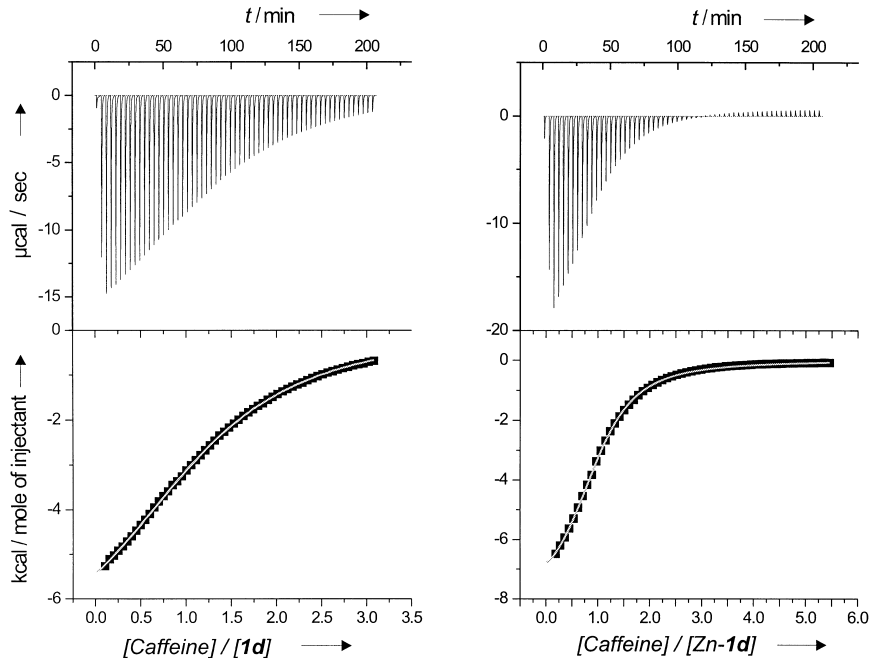

Figure 4. Microcalorimetric titrations of receptors free-base **1d** (left) and Zn-**1d** (right) with caffeine. [Porphyin] ≈ 1 mM in carbonate-buffered solution ($I = 0.11$ M, pH = 10.3).

and $\Delta S^{o}_{i} = -30.8\ J\,mol^{-1}\,K^{-1}$) and Zn-**1d** ($\Delta H^{o} = -34.5\ kJ\,mol^{-1}$ and $\Delta S^{o} = -42.5\ J\,mol^{-1}\,K^{-1}$) shows that the difference between the two receptors in the binding process is mostly due to enthalpy. Thus the more negative enthalpic contribution (which overrules a more negative entropic contribution) for Zn-**1d** suggests a direct interaction of the guest with the Zn center. Caffeine can coordinate to the Zn center through the imidazole nitrogen N9 (see structure in Table 5 below).[37] However, the methyl group at N3 prevents a good perpendicular arrangement with respect to the porphyrin plane (the effect is partially compensated by a position of the Zn atom above the porphyrin plane, towards the axial ligand[24]). Therefore, we propose that caffeine binds to receptor Zn-**1** in a tilted way, with an angle smaller than 90° between the molecular planes of caffeine and porphyrin. Support for this proposed structure of the complex comes from literature reporting the crystal structure of the complex $[Rh_2(acetato)_4(caffeine)_2]$ in which binding of the two caffeine moieties at an angle different from the optimum does not prevent metal coordination at nitrogen N9.[38]

Various modes for the binding of bicyclic nucleobases such as adenine and guanine (structurally similar to caffeine) to metalloporphyrins can be found in the literature. In some cases direct metal coordination[39–41] has been invoked, in others only hydrophobic interactions.[42] Our experiments show that the presence of Zn is an important factor for caffeine complexation and causes the binding strength of the receptor to increase.

We introduced Tyr moieties in receptor Zn-**1c** to possibly enhance the binding of caffeine through stacking with the phenol units. However, the Tyr-based chains are long (10 atoms) and hydrophobic enough to be positioned on top of the porphyrin plane,[43] and this may hinder caffeine binding, like the chains of Zn-**1e** and Zn-**1g**.

^{1}H NMR spectra of Zn-**1c** and free-base **1c** in D_2O confirm the folding of the peptide chains towards the porphyrin core, as shown by the upfield shifted signals for the four aromatic protons of the phenol rings ($\delta = 6.78$ and 6.18 ppm compared to $\delta = 7.07$ and 6.66 ppm for the spectrum recorded in CD_3OD).[44]

Moreover, in contrast to the nonionizable side chains of Zn-**1e** and Zn-**1g**, the phenol OH moiety can be deprotonated,[45] and a CPK model suggests that the phenolate moiety could coordinate intramolecularly to the Zn.[46]

This hypothesis was verified by studying the UV-visible spectrum of the receptor under different conditions. The UV-visible spectrum of Zn-**1c** in carbonate buffered solution (pH = 9.6) showed a 7 nm red-shifted Soret band (relative to Zn-**1d**, Zn-**1f**) and a quite marked hypochromicity that was not caused by aggregation (Figure 5). When dissolved in an oxalate buffer or in pure water (pH 3.7 and ≈ 7, respectively) these effects were not observed, and the spectra closely resemble those of the other receptors. Metal-free **1c** had a much smaller response to the basic pH ($\Delta\lambda_{max} = 0$ nm and only a modest hypochromicity, Figure 5).

The intramolecular coordination of the Tyr moiety to the Zn is also expected to lower the caffeine binding because of competition for the metal center (although only at basic pH).

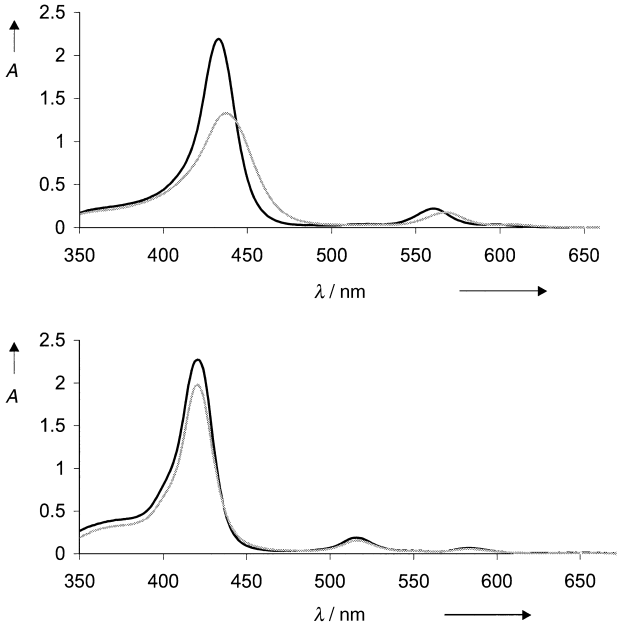

Figure 5. UV-visible spectra for receptors Zn-**1c** (top) and metal-free-**1c** (bottom) at pH 7 (black traces) and at pH 9.6 (gray traces).

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Binding of caffeine to receptor Zn-**1c** in basic solutions is observed with $K_a = 4280\,M^{-1}$. The binding strength is indeed reduced by the competitive effect played by the phenolate moieties, since at pH 3.7, where the amount of phenolate form is negligible, stronger binding ($K_a = 6090\,M^{-1}$) is observed.

The data also show that receptor Zn-**1c** binds caffeine twice as strongly as receptors Zn-**1e** and Zn-**1g**, which have substituents with comparable chain length (see Table 1). This result may indicate stabilizing interactions between the Tyr phenol rings and the electron deficient caffeine π system.

Selectivity towards other ligands structurally related to caffeine: Imidazole-based ligands, resembling the five-membered ring of caffeine,[47] are known to coordinate to metal porphyrins, also in aqueous solutions.[25, 33, 48] We studied binding of 1-methylimidazole (1-MeIm) to receptors **1** by means of UV-visible titrations, monitoring the spectral changes in the Q-band region (630–500 nm, see Figure 6). The results (Table 4) show that the binding mechanism is substantially different from what was observed in the case of caffeine and does involve predominantly metal coordination.

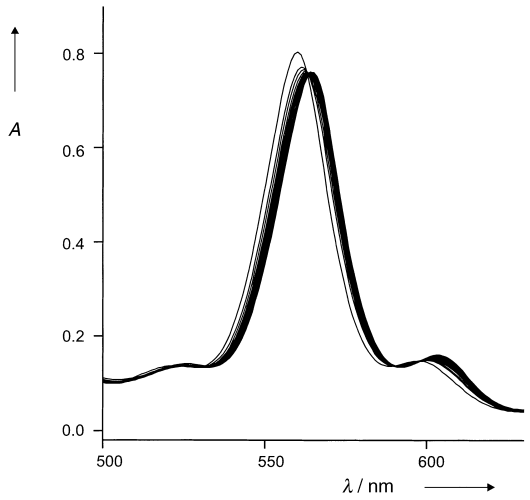


Figure 6. Changes in the UV-visible absorption spectra of Zn-**1e** upon addition of 1-methyimidazole. [Porphyin] ≈ 40 μM in carbonate-buffered solution ($I = 0.008$ M, pH = 9.6).

Table 4. Binding constants for 1-methylimidazole to porphyrin Zn-**1** in aqueous solutions (sodium carbonate buffer, $I = 0.008$ M, pH = 9.6, $T = 25\,^{\circ}C$).

Host	K_a [M^{-1}]
Zn-**1a**	51 ± 2
Zn-**1b**	45 ± 2
Zn-**1d**	58 ± 2
1d	< 1
Zn-**1e**	151 ± 2
Zn-**1f**	57 ± 1
Zn-**1g**	165 ± 3

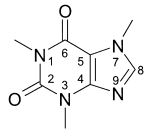
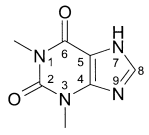
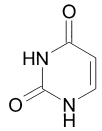
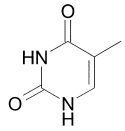
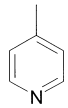
A division between receptors bearing short or long chains, similar to what was observed for caffeine binding, can also be made in case of 1-MeIm binding. However, the effect of the chain length on the binding strength is in the opposite direction. All short chain receptors (Zn-**1a**, Zn-**1b**, Zn-**1d** and Zn-**1f**) show a similar binding constant ($K_a \approx 50\,M^{-1}$) whereas Zn-**1e** and Zn-**1g** bind 1-MeIm three times more strongly ($K_a \approx 150\,M^{-1}$). Coordination of 1-methylimidazole to the Zn atom takes place perpendicular to the porphyrin plane. Therefore, the data suggest that this ligand is small enough and no reorganization of the alkyl chains of Zn-**1e** and Zn-**1g** takes place to accommodate it. Moreover, the higher stability of the complexes obtained with Zn-**1e** and Zn-**1g** (with respect to the short chain receptors) is probably due to the hydrophobic pocket created by the peptidic chains on the porphyrin plane.

Metal-free **1d** did not show any appreciable binding of 1-MeIm (no significant changes of the spectrum were observed during the titration), confirming that metal coordination is the driving force for complex formation.

Investigation of the binding of other ligands structurally related to caffeine was finally undertaken for receptor Zn-**1e** as model system (Table 5). Imidazole coordinates to Zn-**1e** with the same strength as 1-methylimidazole ($K_a = 156\,M^{-1}$, 20 times weaker than caffeine), while 4-methylpyridine is bound with a significantly smaller affinity ($K_a = 35\,M^{-1}$). These observations clearly confirm that hydrophobic or π-stacking interactions play an important role in stabilizing the caffeine complex with Zn-**1e** in addition to metal coordination.

We also studied the interactions of Zn-**1e** with guest molecules that lack the nitrogen donor atom and resemble the

Table 5. Binding constants for different ligands to porphyrin Zn-**1e** in aqueous solutions (sodium carbonate buffer, $I = 8$ m M, pH = 9.6, $T = 25\,^{\circ}C$).

Guest		K_a [M^{-1}]
caffeine		$(3.26 \pm 0.05) \times 10^3$
theophylline		$(2.52 \pm 0.05) \times 10^3$
uracil		< 5
thymine		< 5
1-methylimidazole		$(1.51 \pm 0.02) \times 10^2$
imidazole		$(1.56 \pm 0.02) \times 10^2$
4-methylpyridine		$(3.5 \pm 0.1) \times 10^1$

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six-membered ring of caffeine (like uracil or thymine). These molecules might also be able to associate to Zn-**1e** in case the binding mode is predominantly by stacking.

Interestingly, UV-visible titrations did not show any appreciable binding ($<5\,M^{-1}$). Therefore strong binding of caffeine necessarily requires the presence of the nitrogen donor moiety together with its bicyclic structure favoring hydrophobic interactions. The structurally closely related theophylline (which lacks only the N7 methyl group of caffeine) also shows complexation by the tetracationic porphyrin Zn-**1e**, indicating a similar binding mode. However, it is interesting to note that in view of the small structural difference between the two molecules, the binding strength is distinctly lower.

Conclusions

Although receptors Zn-**1**, and especially Zn-**1c**, Zn-**1e** and Zn-**1g**, are not yet suitable for practical use (sensors), our results show for the first time recognition of caffeine in aqueous solution and provide a detailed view of the binding process coming from different experimental techniques. The data show that even porphyrin Zn-**1a**, which bears simple alkyl chains, binds caffeine. However, the use of amino acid or peptide chains allows the introduction of additional interactions. In particular, if the chains on the pyridyl rings are longer than ten atoms (Zn-**1c**, Zn-**1e** and Zn-**1g**), interaction with the porphyrin surface and, therefore, influence on the recognition site become possible. The balance of the various contributions to binding is very subtle and involves competition for the hydrophobic surface (Zn-**1e** and Zn-**1g**) and additional stabilizing stacking interactions (Zn-**1c**).

Influence of the alkyl chains on guest recognition has also been shown for a much smaller guest such as 1-methylimidazole, which binds to porphyrins Zn-**1** by a different mechanism. In fact, the longer chain receptors showed enhanced binding for 1-methylimidazole.

The present work also shows that influence on the recognition abilities of our synthetic receptors can be achieved even though only very simple porphyrin–peptide conjugates are used (one or two amino acids and, at this stage of the study, with relatively small differences in side-chain nature). Much more complex porphyrin–peptide conjugates, bearing chains as long as 20 amino acids and which mimic heme-protein, have been reported so far in the literature.[49] We believe that the present approach has great potential for the preparation of novel water-soluble receptors, the affinity of which may be tailored by proper choice of peptide moieties. Work in this direction is currently underway in our laboratories.

Experimental Section

General information and instrumentation: All reagents were purchased from Aldrich or Acros Organics and used without further purification. All the reactions were performed under a nitrogen atmosphere. Infrared spectra were recorded on an FTIR Perkin–Elmer Spectrum BX spectrometer and only characteristic absorptions are reported. ^{1}H NMR and ^{13}C NMR spectra were performed on a Varian Unity INOVA (300 MHz) or a Varian Unity 400 WB NMR spectrometer. ^{1}H NMR chemical shift values (300 MHz) are expressed in ppm (δ) relative to residual CHD_2OD ($\delta = 3.30$ ppm), CHD_2CN ($\delta = 1.93$ ppm), or $CHCl_3$ ($\delta = 7.26$ ppm). ^{13}C NMR chemical shift values (100 MHz) are expressed in ppm (δ) relative to residual CD_3OD ($\delta = 49.0$ ppm) or CD_3CN ($\delta = 1.3$ ppm). UV-visible measurements were performed on a Varian Cary 3E UV-visible spectrophotometer equipped with a Helma QX optical fiber probe (path length = 1.000 cm), with solvents of spectroscopic grade. Elemental analyses were carried out using a 1106 Carlo-Erba Strumentazione element analyzer. Mass spectra were measured on a Perkin–Elmer/PerSeptive Biosystem Voyager-DE-RP MALDI-TOF mass spectrometer (PerSeptive Biosystem, Framingham, MA, USA) equipped with delayed extraction. A 337 nm UV Nitrogen laser producing 2 ns pulses was used and the mass spectra were obtained in the linear and reflectron mode.

Buffers: Carbonate buffer ($I = 0.11$ M, pH = 10.3) was prepared by dissolving dry Na_2CO_3 (723 mg) and dry $NaHCO_3$ (573 mg) in distilled water (250 mL). The buffer was used as such or eventually diluted to obtain the desired ionic strength. Oxalate buffer ($I = 0.008$ M, pH = 3.7) was prepared by adding oxalic acid (683 mg) to a NaOH solution (8.2 mL, 1 M) and diluting to 100 mL.

Binding studies: All experiments were conducted at 25 °C.

Dilution experiments: Proof that the receptors used in this study were in their monomeric form was obtained by recording the UV-visible spectra of the receptor at increasingly higher concentrations ($1.0-9.0\times10^{-6}$ M, in the presence of the buffer) for a total of 7–12 experimental observations. The plot of the absorbances versus concentrations were always linear (correlation > 0.995) indicating no aggregation under the measuring conditions ([porphyrins] between 2.4 and 4.8×10^{-6} M).

Aggregation was also absent at higher porphyrin concentration (2.8×10^{-3} M) as proved for receptor Zn-**1e** by a microcalorimetric (ITC) experiment. A solution of Zn-**1e** in water (in the microburette) was added in 5 μL aliquots to the sample cell containing initially only water. The resulting enthalpogram, that is, the evolved heat per added mole of **1e**, is featureless and only shows a small constant endothermic effect that is attributed to dilution of the porphyrin solution (porphyrin dilution from 2.8×10^{-3} M to 9.9×10^{-6} M).

UV-visible spectroscopic measurements: Binding of guests to receptor **1** was evaluated by monitoring the spectral variation upon addition of guest solution either in the Soret band region (400–480 nm) or in the Q-band region (500–630 nm) of the porphyrin spectra. Changes in the Q-band region were monitored in cases in which preliminary measurements indicated a smaller binding constant. For these measurements the concentration of receptors **1** was 10–20 times higher than for the titrations monitored in the Soret band region (see text for typical values). In all cases the titration covers the 20–80% interval of complex formation. Each titration consisted of 13–20 data points. The experimental spectral changes were fitted to a 1:1 binding model using nonlinear least-squares fitting procedures considering simultaneously 6 different wavelenghts (model written with program Scientist®, MicroMath®). In the case of 2:1 binding those data were also fitted using a nonlinear least-squares fitting procedure considering simultaneously two different wavelenghts (model written with program Microsoft® Excel).

Microcalorimetry (ITC): Calorimetric measurements were carried out using a Microcal VP-ITC microcalorimeter with a cell volume of 1.4115 mL. Aliquots of a 16.8 mM caffeine solution in sodium carbonate buffer ($I = 0.11$ M, pH = 10.3) contained in the buret were added to the receptor solution (in the same buffer) contained in the calorimetric cell (receptor concentration 0.7–1.6 mM). Data were fitted to a 1:1 model for metalated receptors and to a 1:2 (receptor/guest) model in the case of the free-base receptor **1d**. Fittings of the experimental data to the appropriate complexation model were obtained by using Origin® implemented with the calorimetric set up provided by Microcal.

Preparation of *N*-chloroacetyl derivatives of amino acid amides/esters and dipeptide amides: Amino acid amides and dipeptide amides were prepared by standard solution-phase peptide chemistry as follows.

Couplings: A mixture of Boc amino acids (1.0 equiv), EDC (1.1 equiv), HOBt (1.0 equiv), and DIEA (1.0 equiv) in CH_2Cl_2 (or CH_3CN) under N_2 was stirred at 0 °C for 5–10 minutes. A solution of the desired amino compound (1.1 equiv) and DIEA (1.2 equiv) in CH_2Cl_2 (or CH_3CN) was added, and the reaction mixture was allowed to warm up to room

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temperature. The final concentration of the Boc amino acid in the mixture was between 0.1 and 0.3 M. The reaction course was monitored by TLC (silica gel, $CHCl_3/CH_3CH_2OH$ 9:1). After disappearance of the Boc amino acid, the reaction mixture was diluted (≈10 times) with EtOAc and washed with citric acid (0.5 M), $NaHCO_3$ (5%), and brine. The organic layer was then dried over Na_2SO_4 and the solvent removed under vacuum. Yields were between 75 and 90%.

Boc deprotection: TFA/CH_2Cl_2 1:1, 30 min to 1 h under N_2.

Chloroacetylation: Chloroacetyl chloride (1.0 equiv) was reacted with the desired amino compound under biphasic conditions (amino compound 0.4–0.6 M in CH_2Cl_2/saturated aqueous Na_2CO_3) at 0 °C for 45–60 minutes. The reaction mixture was then treated as the other coupling reaction mixtures. Yields were between 65 and 90%.

Preparation of tetracationic porphyrin receptors (1): The syntheses of porphyrins Zn-**1a** and Zn-**1b** have been published previously.[19, 20] Receptors Zn-**1c**–**g** were prepared under the same conditions reported for Zn-**1b** with a slightly modified workup procedure. The alkylation reaction mixture was dried under vacuum and the solid residue was triturated and washed extensively with diethyl ether and CH_2Cl_2. The product was then extracted by dissolution with the minimum amount of CH_3CN. The solution was evaporated and the residue redissolved in CH_3OH. $ZnAc_2$ (≈10 equiv) was added and the reaction mixture stirred at 40 °C for 1 h. The excess of $ZnAc_2$ was removed by reverse-phase chromatography (stationary phase RP-8, eluent: from H_2O to 1:1 H_2O/CH_3CN/0.1% TFA mixture.) The eluted solution was first concentrated under vacuum and then lyophilized. The solid residue was redissolved in H_2O/CH_3CN 1:1 and transformed in the tetrachloride salt by ion-exchange column (DOWEX 1-X8, 50–100 mesh, Cl-form). Receptors Zn-**1** were obtained as solids after concentration and lyophilization. Combined yields for alkylation and metalation ranged between 70 and 85% (Free-base receptors **1c** and **1d** were prepared in an analogous manner, the reverse phase column and the ion exchange one following directly the alkylation step.)

Due to the hygroscopic nature of the chloride salts, satisfactory elemental analyses were not obtained. However, the purity of the compounds was confirmed by analytical RP-HPLC elution with a linear gradient of CH_3CN/0.1% TFA and H_2O (gradient 1.0% min^{-1}, flow rate = 1 mL min^{-1}, pump system Waters 6000 controller, detection with photodiode array Waters 996; column Prep Nova-Pack HR C18 60 Å, 6 μm, 3.9 × 300 mm). The identities of the samples were then confirmed by MALDI-TOF MS.

5,10,15,20-tetrakis(*N*-(AcetylTyr-OMe)pyridinium-3-yl)porphyrin (1c): Prepared from 5,10,15,20-tetra(3-pyridyl)porphyrin and chloroacetyl-Tyr-OMe.

Metal free-1c: ^{1}H NMR (CD_3CN): $\delta = 9.46$ (s, 4H), 9.33 (d, $J = 7.9$ Hz, 4H), 9.12 (d, $J = 6.1$ Hz, 4H), 9.04 (s, 8H), 8.54 (m, 4H), 7.62 (d, $J = 7.4$ Hz, 4H), 7.15 (br s, 4H) 7.05 (m, 8H), 6.65 (m, 8H), 5.60 (AB system, 8H), 4.76 (m, 4H), 3.66 (m, 12H), 3.09 (dd, $J = 14.0$, 4.9 Hz, 4H), 2.96 (dd, $J = 14.0$, 7.5 Hz, 4H), −3.07 ppm (s, 2H); UV/Vis (H_2O): $\lambda = 421$, 516, 546, 583 nm; MALDI-TOF MS: m/z: 1667.5 $[M - Cl]^+$ (calcd for $[C_{88}H_{82}N_{12}O_{16}Cl_3]^+$: 1667.5).

Zn-1c: ^{1}H NMR (CD_3OD): $\delta = 9.79$ (br m, 4H), 9.40 (br m, 4H), 9.32 (d, $J = 6.3$ Hz, 4H), 9.10 (m, 8H), 8.57 (t, $J = 7$ Hz, 4H), 7.07 (m, 8H), 6.66 (m, 8H), 5.79 (AB system, 8H), 3.71 (m, 12H), 3.17 (dd, $J = 13.9$, 5.5 Hz, 4H), 2.97 ppm (dd, $J = 13.9$, 8.8 Hz, 4H); ^{1}H NMR (D_2O): $\delta = 9.33$ (m, 8H), 9.08 (br m, 4H), 8.83 (m, 8H), 8.45 (br m, 4H), 6.78 (m, 8H), 6.18 (m, 8H), 5.58 (m, 8H), 3.59 (m, 12H), 2.91 (br m, 4H), 2.60 ppm (br m, 4H); UV/Vis (H_2O): $\lambda = 433$, 520, 560, 598 nm; MALDI-TOF MS: m/z: 1729.5 $[M - Cl]^+$ (calcd for $[C_{88}H_{80}N_{12}O_{16}Cl_3Zn]^+$: 1729.4).

5,10,15,20-tetrakis(*N*-(AcetylAla-NH-*i*Pr)pyridinium-3-yl)porphyrin (1d): Prepared from 5,10,15,20-tetra(3-pyridyl)porphyrin and chloroacetyl-Ala-NH-*i*Pr.

Metal free-1d: ^{1}H NMR (CD_3OD): $\delta = 10.02$ (m, 4H), 9.50 (m, 8H), 9.20 (br s, 8H), 8.65 (m, 4H), 5.88 (m, 8H), 4.41 (m, 4H), 3.93 (m, 4H), 1.43 (m, 12H), 1.19–1.08 ppm (m, 24H); UV/Vis (H_2O): $\lambda = 420$, 515, 544, 580 nm; MALDI-TOF MS: m/z: 1409.8 $[M - Cl]^+$ (calcd for $[C_{72}H_{86}N_{16}O_8Cl_3]^+$: 1407.6).

Zn-1d: ^{1}H NMR (CD_3CN): $\delta = 9.54$ (m, 4H), 9.24 (m, 4H), 9.15 (m, 4H), 8.95 (s, 8H), 8.40 (m, 4H), 8.25 (m, 4H), 7.05 (m, 4H), 5.68 (AB system, 8H), 4.38 (m, 4H), 3.90 (m, 4H), 1.34 (d, $J = 7.1$ Hz, 12H), 1.02 ppm (m, 24H); ^{1}H NMR (D_2O): $\delta = 9.68$ (br s, 4H), 9.40 (br m, 4H), 9.23 (d, $J = 6.3$ Hz, 4H), 8.97 (m, 8H), 8.52 (m, 4H), 5.77 (s, 8H), 4.25 (m, 4H), 3.75 (sept, $J = 6.5$ Hz, 4H), 1.33 (d, $J = 7.2$ Hz, 12H), 0.95 ppm (m, 24H); ^{13}C NMR (CD_3CN): $\delta = 165.02$, 151.40, 150.18, 149.01, 145.96, 143.51, 133.70, 127.21, 114.00, 63.29, 51.01, 42.65, 22.47, 18.84 ppm; IR (KBr): $\tilde{\nu} = 3314$, 3101, 2984, 1679, 1545, 1458, 1207, 1140, 843, 801, 724 cm^{-1}; UV/Vis (CH_3OH): 433, 519, 559, 597, 638 nm; MALDI-TOF MS: m/z: 1470.8 $[M - Cl]^+$ (calcd for $[C_{72}H_{84}N_{16}O_8Cl_3Zn]^+$: 1469.5).

[5,10,15,20-tetrakis(*N*-(AcetylAlaAib-NH-*i*Pr)pyridinium-3-yl)porphyrinato]zinc(II) (1e): Prepared from 5,10,15,20-tetra(3-pyridyl)porphyrin and chloroacetyl-Ala-Aib-NH-*i*Pr. ^{1}H NMR (D_2O): $\delta = 9.68$ (m, 4H), 9.42 (m, 4H), 9.26 (d, $J = 6.2$ Hz, 4H), 8.98 (m, 8H), 8.54 (m, 4H), 5.80 (s, 8H), 4.21 (m, 4H), 3.29 (br m, 4H), 1.33 (d, $J = 7.2$ Hz, 12H), 1.22 (m, 24H), 0.63–0.46 (m, 12H), 0.33–0.12 ppm (m, 12H); UV/Vis (CH_3OH): $\lambda = 432$, 519, 560, 597, 640 nm; MALDI-TOF MS: m/z: 1809.4 $[M - Cl]^+$ (calcd for $[C_{88}H_{112}N_{20}O_{12}Cl_3Zn]^+$: 1809.7).

[5,10,15,20-tetrakis(*N*-(AcetylPro-NH-*i*Pr)pyridinium-3-yl)porphyrinato]zinc(II) (1f): Prepared from 5,10,15,20-tetra(3-pyridyl)porphyrin and chloroacetyl-Pro-NH-*i*Pr. ^{1}H NMR (D_2O): $\delta = 9.66$ (m, 4H), 9.40 (m, 4H), 9.20 (m, 4H), 9.06–8.75 (m, 8H), 8.52 (m, 4H), 5.94 (br s, 8H), 4.38 (m, 4H), 3.85–3.55 (m, 12H), 2.24 (br m, 4H), 2.09–1.80 (br m, 12H), 0.98 ppm (m, 24H); UV/Vis (CH_3OH): $\lambda = 432$, 519, 561, 597, 640 nm; MALDI-TOF MS: m/z: 1574.6 $[M - Cl]^+$ (calcd for $[C_{80}H_{92}N_{16}O_8Cl_3Zn]^+$: 1573.6).

[5,10,15,20-tetrakis(*N*-(AcetylProAib-NH-*i*Pr)pyridinium-3-yl)porphyrinato]zinc(II) (1g): Prepared from 5,10,15,20-tetra(3-pyridyl)porphyrin and chloroacetyl-Pro-Aib-NH-*i*Pr. ^{1}H NMR (D_2O): $\delta = 9.68$ (m, 4H), 9.44 (m, 4H), 9.23 (m, 4H), 8.99 (m, 8H), 8.55 (m, 4H), 5.97 (s, 8H), 4.37 (br m, 4H), 3.71 (br m, 8H), 3.31 (br m, 4H), 2.24 (m, 4H), 2.01 (m, 8H), 1.86 (m, 4H), 1.25 (m, 24H), 0.68–0.44 (m, 12H), 0.38–0.04 ppm (m, 12H); UV/Vis (CH_3OH): $\lambda = 433$, 520, 558, 597, 638 nm; MALDI-TOF MS: m/z: 1913.9 $[M - Cl]^+$ (calcd for $[C_{88}H_{112}N_{20}O_{12}Cl_3Zn]^+$: 1913.8).

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