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The emergence of halophilic evolutionary patterns from a dynamic combinatorial library of macrocyclic pseudopeptides†

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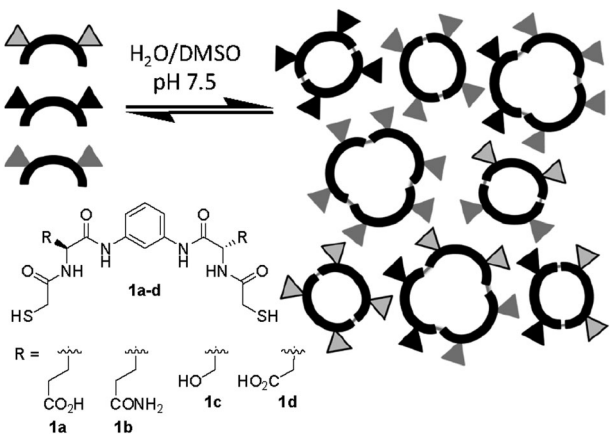
The increase of the ionic strength amplifies the species bearing acidic side chains from a bio-inspired dynamic combinatorial library of macrocyclic pseudopeptides, in close resemblance to the evolution observed for the proteins of halophilic microorganisms.

Dynamic combinatorial libraries (DCLs) are formed by mixtures of components able to reversibly exchange under thermodynamic control, their composition being dictated by the stability of the members within the mixture.[1] Thus, the presence of an external stimulus can operate as a trigger to induce changes in the dynamic mixture, which makes the library evolve toward a better adapted constitution.[2] Accordingly, dynamic combinatorial chemistry (DCC) has proved to be a very powerful approach for the preparation of new receptors, catalysts, sensors, potential drugs and responsive materials.[1–3] However, more recently, a holistic view of DCLs has emerged within the frame of systems chemistry.[4] Since the DCLs are adaptive complex chemical entities that operate as a whole, they are a model benchmark[5] for understanding the evolutionary processes present in nature, despite the mechanisms of chemical and genetic evolution being different. Among all the evolutionary processes observed in nature, the one undergone by extremophiles is especially appealing, since it has allowed them to survive under very extreme conditions.[6] Halophilic archea are extremophiles that thrive in highly saline environments such as natural salt lakes. They have managed to skip osmotic shock by increasing the salt concentration inside the cell. Accordingly, their bio-molecular machinery has evolved to be stable and fully functional in this hypersaline medium.[7] Interestingly, the characteristic features associated to the surface of the halophilic proteins include a large increase in Glu and Asp acids and a decrease in the overall hydrophobic content.[8] This trend concentrates anionic charges at the surface of halophilic proteins at physiological pH. Although the effect is still not fully understood, it has been suggested that hydrated ions can interact with the surface residues to stabilize the folded conformation,[9] the solvent-accessible surface area being a key parameter.[10]

Taking this phenomenon as bio-inspiration, we hypothesized that simple dynamic chemical systems would display evolutionary trends in parallel with those observed for the biological evolution in halophilic microorganisms. To investigate that, we designed a minimalistic DCL of pseudopeptidic macrocycles constructed by the connection of simple building blocks containing pertinent information in the amino acid side chains (Scheme 1). Our design was based on a C_2-symmetric scaffold with two arms, each formed by an amino acid residue with a mercaptoacetyl moiety attached to the *N*-terminus (**1a–d**). The central aromatic *m*-phenylenediamine allows HPLC-UV quantification and confers rigidity to the systems in a pre-organized conformation for macrocyclization. The amino acid side chains permit the introduction of chemical diversity with protein-like structural information, whereas the two thiol ends



Scheme 1 Schematic representation of the molecular diversity generated within the DCL of macrocyclic pseudopeptides obtained by the oxidation of building blocks **1a–d**.

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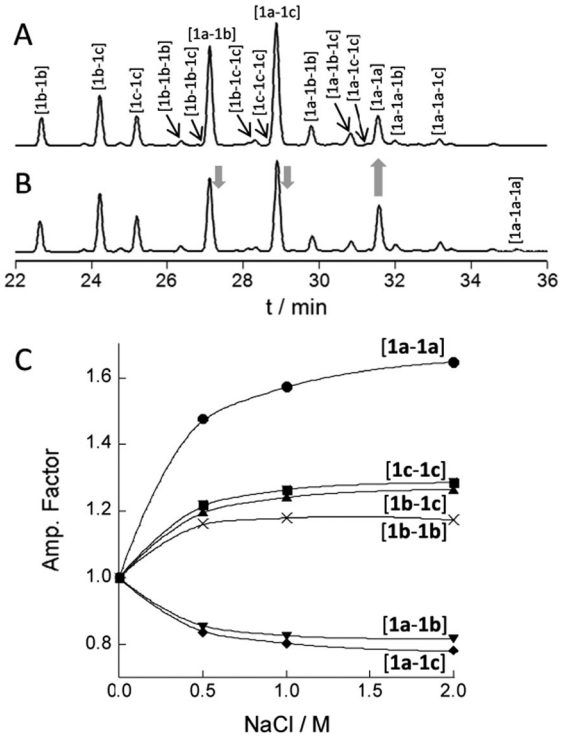

Fig. 1 (A, B) HPLC traces of the [**1a** + **1b** + **1c**] DCL (A) alone and (B) in the presence of 1 M NaCl. (C) Amplification factors of the macrocyclic dimers *versus* the NaCl concentration.

establish the DCL by thiol-disulfide and disulfide exchange equilibria in aqueous solution at neutral pH (Scheme 1).[11,12]

Within this design, we mixed the corresponding building blocks derived from Glu (**1a**, used as a probe containing an anionic amino acid at neutral pH), Gln (**1b**, as an isostructural non-charged congener) and Ser (**1c**, as a highly soluble derivative) at 2 mM each in 20 mM aqueous phosphate buffer at pH 7.5 (containing 25% DMSO[13] to ensure total dissolution). The oxidation of the thiols to the corresponding macrocyclic compounds was monitored by HPLC (Fig. 1A). Once the system reached the equilibrium composition,[14] it was subsequently analyzed by HPLC coupled with ESI-MS, allowing the unambiguous assignment of the species formed in the DCL (ESI†). All the possible macrocyclic dimers (six) and trimers (ten) were detected, the library being dominated by the dimers (Fig. 1A).

Interestingly, when generating the same DCL in the presence of a high salt concentration,[15] we observed remarkable differences (Fig. 1B). The composition of the library changed following a pattern in agreement with the amplification of the glutamate rich species. This effect was evident by plotting the salt-induced amplification factors (area of the peak in the presence of salt over the area of the peak in the absence of salt) of the dimers at different salt concentrations (Fig. 1C). We observed an increase of the [**1a–1a**] homodimer (up to ~60% increase), a ~20% decrease of the heterodimers containing **1a** and the increase (~20%) of the corresponding dimers exclusively made of **1b** and/or **1c**. This pattern corresponds to the effect of the single amplification of the [**1a–1a**] dimer. We additionally confirmed this trend by dynamic deconvolution:[16] the macrocycle [**1a–1a**] was amplified by the salt in all the sub-libraries containing this building block, while the sub-library made of only **1b** + **1c** was insensitive to the salt content. We also checked that the salt was operating under thermodynamic control (ESI†). From the data obtained in the different experiments and by making use of the DCLSim[17] software, we estimated that the dimer [**1a–1a**] was stabilized in 1 M NaCl by about $\Delta\Delta G = -2.6$ kJ mol^{-1}. Regarding the trimers, the salt induced the increase of those joining two or three Glu building blocks. Accordingly, the trimers [**1a–x**] (with **x** = **1b** or **1c**) showed an amplification factor of 1.4–1.6 while [**1a–1a**], which was barely detected in the absence of salt, showed a fourfold increase of its concentration in 2 M NaCl. Actually, the trimer/dimer proportion in a mixture formed by the oxidation of **1a** alone was also strongly affected by the presence of salt, leading to a tenfold increase of the corresponding equilibrium constant in favour of the trimer (ESI†). Thus, very remarkably, the hypersaline environment induces the evolution of our DCL toward the amplification of the macrocycles containing a concentrated number of acidic residues, in a clear parallelism with the evolution of the halophilic organisms. The same experiment using different salts like KCl or $NaNO_3$ rendered practically identical amplification factors than NaCl (ESI†) implying the ionic strength as the driving force for the amplification.[15] Moreover, the corresponding DCL generated at pH 2.5 was insensitive to the presence of salt (ESI†). In spite of the difficulty of reaching the equilibrium composition at such a low pH,[18] it seemed evident that the salt-induced amplification of the glutamic species requires the anionic side chains.

In order to better understand the process at the molecular level, we performed the structural characterization of the [**1a–1a**] dimer. Molecular modelling rendered important differences depending on the charge of the molecule (Fig. 2 and ESI†). These differences can be explained considering the H-bonding patterns: the tetraanion (B) sets eight intramolecular H-bonds in a figure eight folding shape,[19] whereas the neutral species (A) shows an unfolded conformation with only four H-bonds. ^{1}H NMR and CD techniques provided experimental evidence of these conformations (ESI†). Several ^{1}H NMR signals changed upon increasing the pH from 2.5 to 7.5, which are consistent with the deprotonation of the side chains and the concomitant formation of the H-bonded folded conformation. The corresponding NMR spectra of the isostructural non-charged Gln derivative [**1b–1b**] showed

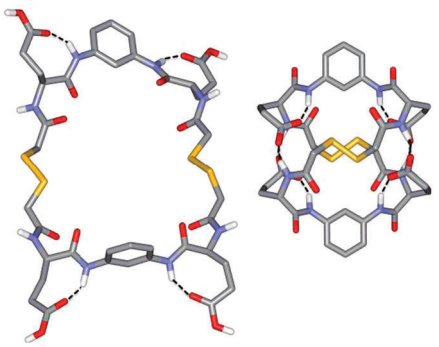

Fig. 2 (A, B) Proposed conformations for [**1a–1a**] in the neutral (A) and in the tetraanionic (B) forms (non-polar H-atoms have been omitted).

no changes with the pH, supporting our proposal for the conformational behaviour of [**1a–1a**]. Moreover, the CD spectra of [**1a–1a**] at different pH values showed significantly different signatures both at 220 nm and 254 nm, also implying a different conformation of the macrocycle at acidic and neutral pH. Interestingly, the ^{1}H NMR and the CD spectra of [**1a–1a**] acquired at pH 7.5 in a saline medium displayed no differences with respect to those acquired without the salt (ESI†). This observation implies the prevalence of very similar conformations at different ionic strengths.

Having characterized the salt-induced amplification trends in the DCL formed by **1a** + **1b** + **1c**, we wondered about the performance of a more complex mixture, by the introduction of an additional building block. We evaluated the presence of another acidic derivative (**1d** derived from Asp, Scheme 1). In this case, the salt produced the amplification of all the species assembling the building blocks with Glu and Asp side chains. Thus, the specific salt-induced amplification of the [**1a–1a**] dimer was somehow palliated by the amplification of the other anionic dimers [**1d–1d**] and [**1a–1d**], in a clearly competitive process. The salt-induced amplification factors increased in the order [**1a–1a**] < [**1a–1d**] < [**1d–1d**], implying a better salt-adaptation of the Asp derivatives. This evolutionary trend has been also reported for the halophilic proteins and was related to the smaller solvent-accessible surface for the shortest side chain (Asp), which permits a more compact folding.[10] Molecular models of the anionic macrocyclic dimers [**1a–1d**] and [**1d–1d**] showed a folding stabilized by carboxylate-amide intramolecular H-bonding patterns similar to those found in [**1a–1a**]. Therefore, similar salt effects should be expected for all the tetraanionic macrocycles. The corresponding CPK areas follow the trend [**1a–1a**] > [**1a–1d**] > [**1d–1d**], and thus, the salt-adaptation of the members of this DCL also increases as the accessible area decreases.

In conclusion, the increase of the ionic strength within simple DCLs of macrocyclic pseudopeptides induces the amplification of the members concentrating a large number of acidic side chains. This behaviour has a remarkable resemblance with the natural evolution of the proteins of halophilic organisms for surviving in hypersaline media. Our findings show the utility of DCLs for the experimental modelling of the molecular evolutionary trends observed in nature, with foreseen implications in the prebiotic chemistry and in the understanding of the origin of life.[20]

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