Pseudopeptidic Compounds for the Generation of Dynamic Combinatorial Libraries of Chemically Diverse Macrocycles in Aqueous Media

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A straightforward four-step synthesis leads to the preparation of C_2 -symmetric dithiols containing a central aromatic core and amino acid side chains. These building blocks allow the preparation of dynamic covalent libraries of pseudopeptidic macrocycles in aqueous media that cover a broad range of polarities, functional groups and bulkiness mirroring the diversity found in natural peptides. The versatility of the generated dynamic libraries has been illustrated by the amplification of two different members from the same library upon the action of two biologically relevant templates.

Introduction

The generation of new, dynamic systems has attracted the attention of researchers and has shown a great potential in a plethora of areas.¹ Since the early reports by the groups of Lehn and Sanders, Dynamic Covalent Chemistry (DCC) has demonstrated an enormous utility to synthesize otherwise almost impossible compounds, especially interlocked and multicomponent assemblies, usually under thermodynamic control.² Thus, fascinating structures such as helicates³ were initially reported by template-assisted synthesis and, more importantly, the new methodology paved the way to the generation of more complex and more beautiful structures such as catenanes⁴ and knots.⁵ On the other hand, the combination of building blocks (BBs) by reversible covalent bonds allows the generation of libraries of products, usually (but not exclusively) cyclic structures with different levels of complexity. Playing with different BBs in a combinatorial approach led to the new research area of Dynamic Combinatorial Chemistry (DCC). Thanks to the dynamic nature of their bonds, these mixtures of exchanging compounds, the Dynamic Covalent Libraries (DCLs), can respond to external stimuli altering the different proportions of final structures.⁶ Initially established as a new methodology to synthesize molecular receptors,⁷ the study of dynamic systems has evolved as an area of research itself within the sub-discipline of Systems Chemistry and it has important implications in diverse research fields, from chemical biology to the chemistry

of materials.⁸ More recently, a special interest has been focused on the study of out-of-equilibrium assemblies which can allow reaching different states of the system.⁹ Importantly, life operates far from equilibrium and its extraordinary complicated molecular machinery operates in a kinetically controlled regime and, therefore, the study of such non-thermodynamically controlled systems have obvious implications in pre-biotic chemistry.

One of the many possibilities that DCC offers is to deliver materials that can interact with biologically relevant compounds. DCC methods have been used to synthesize molecular receptors,⁷ catalysts¹⁰ or sensors.¹¹ However, when addressing challenges related to biological chemistry, organic chemists usually face the difficulty to work in aqueous media. This is not a trivial problem as usually organic reactions are performed in anhydrous solvents. For example, imine chemistry,¹² widely used in DCC, is usually incompatible with the presence of water. Nevertheless, other reactions like hydrazine exchange¹³ or disulphide bond formation¹⁴ can be performed in water being the later widely used in DCC. Sanders and Otto,¹⁵ Ravoo,¹⁶ our group¹⁷ and others¹⁸ have employed this reaction in aqueous media. The formation and metathesis of disulphide bonds take place spontaneously in water at slightly basic pH. The addition of an oxidant like DMSO as co-solvent allows working at even slightly acidic pHs and significantly accelerates the disulphide formation and exchange reactions.¹⁹ However, the solubility of organic compounds in water is usually very limited and narrows the toolkit of compounds available for chemists. This limitation conditions the development of even minimalistic new experiments and, therefore, there is a need for the synthesis of compounds which are soluble in aqueous conditions and present the characteristics necessary for the formation of reversible covalent bonds in aqueous media.

The synthesis of molecules containing amino acids allows taking advantage of the vast diversity of functionalities present

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in these building blocks.²⁰ Pseudopeptides are molecules incorporating natural amino acids and non-natural structural parts²¹ and they have been widely used for the development of compounds with many important applications. Examples are reported in which pseudopeptidic backbones are used for preparing structured and stimuli-responsive materials,²² peptide nucleic acids (PNAs),²³ catalysts,²⁴ and bio-active compounds.²⁵ Additionally, pseudopeptides have also been used for the development of molecular receptors for a variety of templates including anions, inorganic cations and peptides.²⁶

We previously reported the use of pseudopeptidic dithiols in dynamic covalent libraries.¹⁷ In this article we report the synthetic procedure for the formation of twelve pseudopeptidic dithiols (**4a-l** in Schemes 1-3) that were designed in order to be used as bipodal building blocks (BBs) for the generation of disulphide-based dynamic combinatorial libraries with minimalistic peptide-like structural information. The structure of the BBs is based on a common C₂-symmetric scaffold consisting of a central *m*-phenylenediamine that joins two identical arms, each formed by an amino acid residue. At the *N*-terminus of the two amino acids, dithiols **4a-k** incorporate a mercaptoacetyl moiety. Our BBs suppose a new addition to the quantity and variety of molecular Lego pieces available for chemists working in the field. All of them were conceived with a common chemical backbone, with the only exception of **4l** where the use of cysteine allows a simpler, more compact, structure.

Results and discussion

Synthesis of the building blocks

The C₂-symmetric dithiols described herein are easily accessible by standard organic synthesis procedures and present good solubility in water or water-organic solvent mixtures. The BBs cover a broad range of polarities, incorporating negative, positive or neutral charges at physiological pH. Besides, different functional groups, such as alcohol, amine, guanidine, carboxylic acid, amide, phenol and indol can be included in the structures thanks to the chemical diversity of the natural amino acids.

The rigid geometry of the *m*-phenylenediamine predisposes the components to the formation of macrocycles (mostly dimers), preventing the formation of the monomeric disulphides, i.e. the products of the intramolecular cyclization. The synthesis of the dithiol building blocks was carried out using two different protecting group strategies. For most of the compounds (**4a-h**) an Fmoc-protected amino acid was used with acid-cleavable protecting groups on the side chains (Scheme 1).

Thus, after diacylation of *m*-phenylenediamine with the corresponding amino acids using DCCD/HOBt as coupling reagents and dry DMF as solvent, compounds **1a-h** were obtained. Fmoc-removal was carried out with 20% (v/v) piperidine in dry DMF yielding amines **2a-h**. The thiol groups were incorporated as trityl-protected thioglycolic acid with

EDC/HOBt as coupling reagents in dry DMF, to obtain intermediates **3a-h**. Final deprotection using trifluoroacetic acid in dichloromethane and triisobutylsilane/ethylenedithiol as scavengers afforded final compounds **4a-h**. The final products were purified by reverse phase chromatography in acidic water: acetonitrile mixtures when necessary. The identity of the protecting groups used in this sequence was chosen according to orthogonality requirements and it is disclosed in Table 1.

Scheme 1 Synthetic route to dithiols **4a-h**. Reagents and conditions: i) dry DMF, DCCD (2.4 eq.), HOBt (2.4 eq.), DIPEA (4.7 eq.), 60h; ii) 20% piperidine in DMF; iii) dry DMF, tritylsulfanyl acetic acid (2.1 eq.), EDC (2.4 eq.), HOBt (2.4 eq.), DIPEA (4.7 eq.), 24-48h; iv) CH₂Cl₂:TFA, TIS (8 eq.), EDT (12 eq.).

Table 1. Protecting groups and amino acid side chains for products **1a-h**, **2a-h** and **3a-h**.

Entry	Compound	PG ₂	Amino acid
1	1a, 2a, 3a	Trt	Asn
2	1b, 2b, 3b	Trt	Gln
3	1c, 2c, 3c	^t Bu	Ser
4	1d, 2d, 3d	^t Bu	Thr
5	1e, 2e, 3e	^t Bu	Tyr
6	1f, 2f, 3f	Boc	Trp
7	1g, 2g, 3g	^t Bu	Asp
8	1h, 2h, 3h	^t Bu	Glu

For building blocks **4i-k** different strategies were employed depending on the nature of the corresponding amino acid functionalities. All their syntheses began with the coupling of *m*-phenylenediamine to an *N*-Boc protected amino acid (Scheme 2). Removal of the Boc protecting group conducted to diamines **2i-j** that were diacylated with trityl protected thioglycolic acid to yield intermediates **3i-j**. For final deprotection, an acidic cleavage using scavengers TIS/EDT was used. For lysine derivative with the side chain protected with a Cbz-group an HBr/CH₃COOH solution was used together with

TIS. Compound **4i** was isolated as TFA salt after reverse phase chromatography (eluent: mixtures of H₂O and MeCN containing 0.1% and 0.07% TFA respectively).

Scheme 2 Synthetic route to dithiols **4i-k**. Reagents and conditions: i) dry DMF, HBTU (2.4 eq.), DIPEA (4.7 eq.); ii) TFA, CH₂Cl₂; iii) dry DMF, tritylsulfanyl acetic acid (2.1 eq.), EDC (2.4 eq.), HOBt (2.4 eq.), DIPEA (4.7 eq.), 24–48 h; iv) HBr/CH₃COOH, CH₂Cl₂, TIS (8 eq.); v) CH₂Cl₂, PhSiH₃ (24 eq.), Pd(PPh₃)₄ (0.13 eq.); vi) CH₂Cl₂:TFA, TIS (8 eq.), EDT (12 eq.); vii) CH₂Cl₂, triethylamine (3.2 eq.), 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine (3.0 eq.).

The ornithine derivative **4j** required deprotection in two steps, firstly side-chain allyl group was removed by using phenylsilane and Pd(PPh₃)₄ catalyst to yield compound **5j** (which was not purified), followed by acidic removal of the trityl group yielded the Orn-derivative **4j** as TFA salt. This route also allowed the synthesis of the Arg-derivative using **5j** as starting material which was reacted with 1,3-di-Boc-2-(trifluoromethylsulfonyl)guanidine to furnish Boc-protected guanidine **3k**. Conventional deprotection employing TFA and TIS/EDT yielded **4k** as TFA salt. Preparation of compound **4k** by alternative routes proved troublesome. Again, the choice of protecting groups was realised according to orthogonality taking into account the corresponding amino acid side chains. Table 2 shows the combination of protecting groups employed for the synthesis of compounds bearing basic side chains.

Table 2. Protecting groups and amino acid side chains for products **1i-k**, **2a-k** and **3a-3l**.

Entry	Compound	PG ₂	Amino acid
1	1i, 2i, 3i	Cbz	Lys
2	1j, 2j, 3j	Alloc	Orn
3	3k	Boc	Arg

Finally, cysteine derivative **4l** was synthesised in only two steps as the amino acid already contains the thiol functionality and so the incorporation of the thioglycolic moiety is not necessary. Thus, after diacylation of *m*-phenylenediamine with Boc-protected cysteine bearing a trityl group on the side chain, an acidic cleavage of the protecting groups directly furnished **4l** with a slightly different scaffold than other thiols.

Scheme 3 Synthetic route to dithiol **4l**: i) dry DCM, DCCD (2.4 eq.), HOBt (2.4 eq.), 60h. ii) CH₂Cl₂:TFA, TIS (8 eq.), EDT (12 eq.).

All the synthesized dithiol building blocks **4a-l** were fully characterized by NMR and mass spectrometry. Hence, we have set up a robust synthetic methodology that gives access to C₂-symmetric pseudopeptide-dithiols with a broad range of functionalities thanks to amino acid side chain diversity. Thus, very polar compounds at neutral pH such as protonated amines and guanidines or anionic carboxylates are available. Other compounds of intermediate polarity like amide or alcohol are also accessible. Finally, hydrophobic side chains like those derived from aromatic amino acids (Trp and Tyr) can also be easily prepared.

Oxidation of the building blocks in aqueous mixtures

To demonstrate this broad range of functional groups that results in a vast variability in molecular diversity, we oxidised each of the BBs individually from 1 mM samples. To ensure complete solubility we tested different solvent mixtures containing 20% of DMSO and buffered water (bis-tris pH 6.5 to have a final concentration of 50 mM) and acetonitrile as co-solvents. Most building blocks investigated were soluble in mixtures containing 20–60% buffer and 60–20% acetonitrile. However, Trp-derivate **4f** precipitated at mixtures containing more than 40% water. For this reason we established 40% buffer, 40% acetonitrile and 20% DMSO as ideal conditions to work with all BBs available. Under these conditions, after 8 days of reaction at room temperature, complete oxidation of the BBs was observed (HPLC monitoring) that rendered the corresponding macrocyclic dimers (as inferred by UPLC-MS analysis). The minor formation of the corresponding trimers could be also detected in some cases (Scheme 4).

In Fig. 1 we show the overlapped HPLC traces of the individual dimeric species using the same solvent gradient to illustrate the variety in functionalities of the amino acid derivatives. As can be easily appreciated from the traces, a

broad range of polarities were observed. At short retention times positively charged species are eluted (the HPLC analysis is performed in acidic media). The most polar component is **[4l]₂** which presents a smaller and more compact structure, with a higher positive charge density. It is followed by the components having Orn, Lys and Arg side chains (**[4j]₂**, **[4i]₂** and **[4k]₂** respectively). In the middle of the HPLC traces the polar and neutral compounds are eluted (those having Gln, Asn or Ser residues) followed by the carboxylic acid-side chain species (Glu, Asp) that are neutral under the HPLC analysis conditions, but anionic in the reaction media. The more hydrophobic Thr-based compound **[4d]₂** appears between the two carboxylic acid derivatives. Finally, at high percentage of acetonitrile the highly hydrophobic Tyr- and Trp-based compounds are eluted (the latter nearly at 100% MeCN).

Scheme 4 Oxidation of BBs **4a-l** to form the corresponding cyclic dimers.

Fig. 1. Overlapped RP-HPLC ($H_2O:MeCN$, 20 mM formic acid) traces of individual BBs after oxidation to form the respective dimers (C_{18} , 8 min at 2% MeCN, then linear gradient from 2% to 40% over 57 min and then linear gradient to 100% over 15 min). Vertical axis shows absorbance in arbitrary units.

Finally, to illustrate the utility of the described building blocks as components for the generation of complex DCLs,

different sets of mixtures of BBs were prepared that show the ability to generate molecular and functional diversity. In a first mixture, a positively charged derivative **4j**, a neutral compound **4d** and the two hydrophobic components **4e** and **4f** were included at 0.5 mM concentration of each BB in the reaction conditions described above (40% buffer, 40%, MeCN, 20% DMSO). Under these conditions all of the 10 expected dimers can be observed (as evidenced by UPLC-MS analysis) plus two of the possible trimers. Other very minor peaks could not be unambiguously identified by UPLC-MS (Fig. 2).

Fig. 2. RP-HPLC traces of the oxidized mixture of **4d**, **4e**, **4f** and **4j** (0.5 mM each) in 40% buffer (pH = 6.5), 40% MeCN, 20% DMSO.

A different DCL was generated using the same conditions by mixing BBs **4b**, **4g**, **4e** and **4l** (Fig. 3). This reaction rendered more complex HPLC traces, where only nine dimers could be identified. The homodimer of the Cys-based compound **[4l]₂** was not formed under these reaction conditions, probably because it is strongly disfavoured as it has a higher charge density. All the other possible dimers were identified, but in this case no trimers were unambiguously assigned although some minor compounds could not be identified by UPLC-MS. According to our own experience Asp residues proved troublesome in some cases forming undesired compounds due to side reactions (i.e. formation of aspartimides).

Fig. 3. RP-HPLC traces of the oxidized mixture of **4b**, **4g**, **4e** and **4i** (0.5 mM each) in 40% buffer (pH = 6.5), 40% MeCN, 20% DMSO.

Response to biologically relevant stimuli

Once the conditions for the efficient generation of dynamic libraries of pseudopeptidic macrocycles were established, we turned our attention to the effect of biologically relevant stimuli. To this aim we designed a library capable to respond to the presence of compounds with very different structures and charge (Fig. 4). We chose a linear polycationic spermine alkaloid and a cyclic polyanionic sugar-like molecule such as phytic acid, two relevant and structurally dissimilar compounds as benchmarks to test the potential of our systems. In order to produce a library that could respond to these stimuli, we chose a positively charged side chain (**4i**; Lys), a negatively charged compound (**4h**; Glu derivative), a hydrophobic functionality (**4e**; Tyr) and a neutral polar group capable of establishing hydrogen bond interactions (**4d**; Thr). Using the reaction conditions described earlier a new library was formed (Fig. 4a). In this new library the main species were the neutral dimers (hetero or homodimers) and the heterodimer combining Glu and Lys ([**4h-4i**]) side chains, which compensate opposite charges. Once characterised the outcome of the new library we analysed the effect of the chosen stimuli to this dynamic mixture. Starting from the same stock solution different amounts of either spermine or phytic acid were used in the reaction mixtures (from 0.125 mM to 10 mM of the templates) and the response was analysed by HPLC. In the case of spermine, non-surprisingly, the homodimer of glutamic acid [**4h**]₂ was found to be amplified from the mixture (Fig. 4b) which suggests an electrostatic interaction between the tetranionic macrocycle and the tetracationic spermine (see below). However, a different response was obtained when phytic acid was added to the same library: increasing amounts of phytate induced an amplification of the threonine homodimer [**4d**]₂ (Fig. 4c). This observation is consistent with other responses found in dynamic mixtures of Thr-containing peptides, where the presence of anionic carbohydrates amplified this structural motif.¹⁶ In both cases the identity of the amplified compounds was unequivocally confirmed by ESI-MS and by adding true samples of the corresponding dimers to the equilibrated library, and analysing the outcome by HPLC.

Fig. 4. Response of a library composed of **4d**, **4e**, **4h** and **4i** to the presence of different compounds. a) Library at pH 6.5 in the absence of stimuli. b) Library distribution in the presence of 10 mM of spermine showing the amplified species. c) Library distribution in the presence of 10 mM of phytic acid highlighting the amplified species.

In order to confirm the host-guest interactions we decided to perform NMR-titrations of the amplified dimers with their corresponding guests, that is phytic acid for [**4d**]₂ and spermine for [**4h**]₂. To that end we prepared solutions containing the corresponding BBs that give rise to the amplified compounds under conditions similar to those of the libraries. Each homodimer [**4d**]₂ and [**4h**]₂ was prepared

individually by oxidation of its component in a mixture of buffered $\text{H}_2\text{O}:\text{d}_3\text{-MeCN}$ and $\text{d}_6\text{-DMSO}$ at 0.5 mM concentration of BBs. The aqueous buffer was adjusted to pH 7.00 using d_{11} -tris(hydroxymethyl)aminomethane (d_{11} -Tris) and the ^1H -NMR spectra were acquired after 8 days to ensure complete oxidation (also checked by HPLC). The ^1H NMR data²⁸ were consistent with the formation of the respective homodimers $[\mathbf{4d}]_2$ (Thr) or $[\mathbf{4h}]_2$ (Glu), although a minor formation of the trimer $[\mathbf{4h}]_3$ (about 5% mol) could be observed in the latter (see ESI for details). On the other hand, the aqueous solutions (68.75 mM) containing spermine or phytic acid, respectively, were adjusted to pH 7.0 using aqueous HCl and NaOH for the titration experiments.

Unfortunately the NMR monitoring of the addition of phytate (up to 10 eq.) to $[\mathbf{4d}]_2$ was hampered by the close proximity of the key CHOH proton signals (both in host and guest) to the water suppression region. Moreover, the appearance of a slight turbidity during the titration experiment additionally precluded to accurately determine the strength of the host-guest interaction.

More interestingly, when the $[\mathbf{4h}]_2$ dimer was treated with spermine, several proton signals of the macrocycle were perturbed (Fig. 5). Thus, the two amide NH proton signals (NHa and NHb) were downfield shifted, while the methylene vicinal to the carboxylate of the side chains ($\text{H}\gamma$) moved upfield. Regarding the aromatic protons, the singlet H1 were deshielded while the other signals (H2,3) were slightly shielded. These changes strongly supported the host-guest interaction between the $[\mathbf{4h}]_2$ macrocycle and the polyamine. The analysis of the variation of the chemical shifts of the amide signals allowed estimating a binding constant of $K_{\text{as}} \approx 10^4 \text{ M}^{-1}$, a remarkable value for an interaction in aqueous buffered medium. Thus, starting from the same dynamic library of pseudopeptidic macrocycles, we have identified two potentially useful receptors for completely different substrates, underscoring the utility of the dynamic combinatorial approach for the study of host-guest systems of biological relevance.

Conclusions

We have described the efficient synthesis of a set of pseudopeptidic dithiols that present C_2 symmetry and peptide-like information, and are suitable for the generation of Dynamic Combinatorial (Covalent) Libraries (DCLs) in aqueous media. The synthesis involves an easy four-step procedure using standard solution-phase peptide chemistry. The compounds described herein contain a rigid aromatic scaffold that predispose to the formation of macrocyclic (mainly dimeric) compounds upon disulphide formation and allows monitoring by HPLC-UV traces. The disulphide bond is an important structural moiety in natural peptide-like macrocycles with biological implications.²⁹ On the other hand the amino acid side chains provide a diversity of functionalities that cover a broad range of polarities as demonstrated by the comparison of the individual HPLC analysis of the oxidised compounds.

Fig. 5. Partial ^1H NMR spectra (500 MHz, 4:4:2 $\text{H}_2\text{O}:\text{CD}_3\text{CN}:\text{DMSO}-d_6$, d_{11} -Tris buffer pH 7.0, 298 K) of $[\mathbf{4h}]_2$ alone (125 μM , lower trace) and upon the addition of 20 equivalent of spermine (upper trace). Proton assignment of arbitrarily numbered proton signals is also shown.

Despite this broad range of behaviour water/organic solvent mixtures allow for the generation of DCLs even in the presence of the most hydrophobic components. Two different DCLs have been prepared to show the potential of the synthesised building blocks and confirmed the formation of almost all possible dimers in those libraries. Our experiments underscore the capability of the described BBs to generate chemical diversity in aqueous media, which is a useful tool in the fields of systems chemistry and bio-molecular recognition. In fact, as a proof of concept, we have designed a representative dynamic library of macrocycles that responded to the presence of structurally different substrates. Thus, we observed the amplification of different macrocyclic receptors upon the action of either spermine or phytic acid. The subsequent NMR titration of the amplified species confirmed the interaction, rendering the Glu-derived homodimer as an efficient host for spermine. Overall, the molecular diversity of the building blocks and the versatility in the generation of the corresponding DCLs by disulphide formation will open the way for the future applications of these dynamic systems in biomolecular recognition.

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