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Dynamic Amino Acid Side-Chains Grafting on Folded Peptide Backbone

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Abstract: An efficient strategy for the synthesis of large libraries of conformationally defined peptides is reported, using dynamic combinatorial chemistry as a tool to graft amino acid side chains on a well-ordered 3D (3-dimension) peptide backbone. Combining rationally designed scaffolds with combinatorial side chains selection represents an alternative method to access peptide libraries for structures that are not genetically encodable. This method would allow a breakthrough for the discovery of protein mimetic for unconventional targets for which little is known.

Introduction

Small peptides are attractive and underexploited compounds that occupy an intermediate molecular space between that of traditional drug-like compounds and much larger biologics.[1] Their sizes allow them to occupy a large surface area on their binding partner leading to high affinity and selectivity, while maintaining pharmacokinetic properties that are much more favorable than biologics. Because their sequence can be directly derived from proteins, they incarnate the simplest functional protein mimetic. However, when removed from their native context, peptide segments usually fail to adopt the bioactive conformation, leading to entropic penalty upon binding that affects their affinity. Methods for pre-organizing peptide 3D structure have been developed over the last century, involving the use of constrained residues, head to tail or side-chain to side-chain macrocyclization, or projection of crucial side chains on 3D scaffolds.[2] These methods allow the access to shorter Protein Domain Mimetic (PDM) that retain the activity of the whole protein and are particularly useful for targeting interactions that have revealed intractable with small molecules.[3] The limit of this rational design is that structural data on the protein to mimic have to be available. Additionally, they usually deliver a first low affinity hit, which is then optimized. This second task is usually performed by synthesis and screening of libraries of peptides and is often the most challenging and time-consuming step of the process. The other general approach for the discovery of bioactive compounds relies on high throughput screening of large libraries. Solid phase peptide synthesis as well as biological display technology allow to access encoded combinatorial libraries that can be easily identified after the screening.[4] If these methods have been limited for long time to linear sequence with poor pharmaceutical properties, huge efforts have been devoted to their extrapolation to more “drug-like” sequences, endowed with peptidase resistance, cell permeability and oral bioavailability. Particularly, *in vitro* display system, such as mRNA, allow the introduction of non natural amino acids and main chain features.[5] We wish to develop an alternative strategy combining a rationally designed 3D peptide backbone and a combinatorial method to decorate this backbone with amino acid side chain functionalities. For this purpose, we propose to exploit *Dynamic Combinatorial Chemistry* (DCC) as a tool for side chains auto-assembly on the scaffold surface.[6] The principle is outlined in Figure 1.

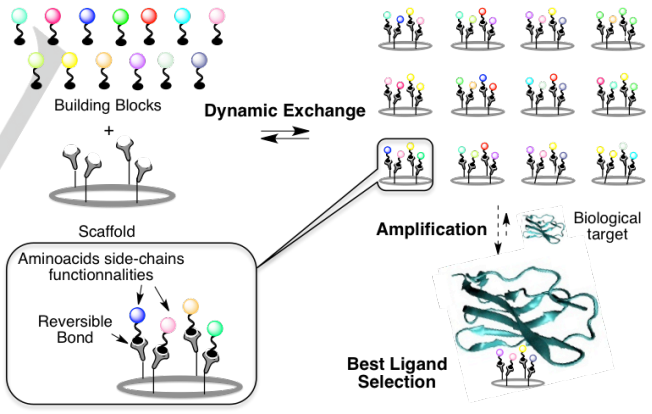


Figure 1. Protein mimetic design by dynamic side-chains grafting on a defined 3D peptide scaffold.

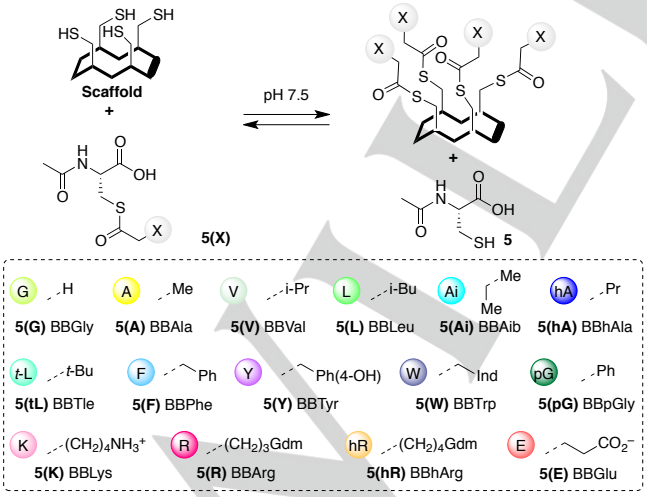
A peptide scaffold of defined 3D structure covered on one face by functional groups prone to react in reversible processes is mixed with a series of building blocks (BB) bearing amino acids side-chains, in physiological conditions. Dynamic side-chains grafting occurs and leads to a dynamic chemical library (DCL) of well-ordered peptides with multiple side chains combinations. Upon addition of a biological target, the best ligand binds this target and shifts the equilibrium to favor its own formation at the expense of the other compounds. Changes in library composition upon introduction of the target are thus exploited to probe favorable interactions. DCC has been barely used in

peptide and peptidomimetic chemistry but few example are reported:[7,8] to assemble peptides fragments into supramolecular 3D protein-like structure,[9-11] to create peptide bonds between amino acids surrogates[12,13] or to graft side chains either on a flexible polymer backbone[14,15] or a rigid organic scaffold.[16-18] The covalent reversible functionalization of a well-ordered peptide scaffold has been also reported.[19] Combination of such a folded scaffold with dynamic combinatorial method has never been published to date.

We present herein our incremental work aiming at generating a dynamic combinatorial library of 3D folded peptides. The key point for the success of this approach is to access an isoenergetic library, ensuring an equal quantity of all its members, since the formation of reaction mixtures that are strongly biased toward some of its members may render it energetically costly to shift the equilibrium in any other direction. Such phenomenon may strongly affect the level of amplification that could be detected and compromise its utility. We demonstrate here that an appropriate choice of reaction and conditions can lead to an effective thermodynamic control over the equilibrating mixture allowing the access to a library that would be useful for ligand screening and could achieve a breakthrough in the discovery of new bioactive peptides.

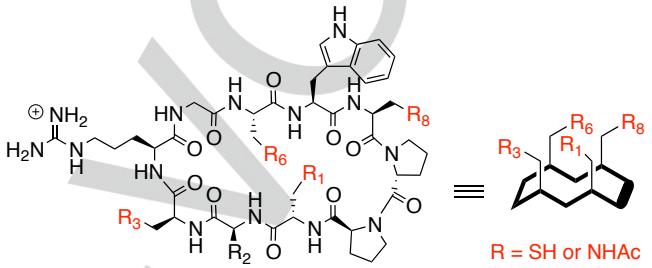
Results and Discussion

First we focused on establishing conditions for the dynamic functionalization of a peptide scaffold, at its surface. Although many reversible reactions have been reported for DCC, only few of them are compatible with biological medium.[6] For this purpose we chose the thioester exchange reaction, from a thiol-functionalized scaffold and a library of thioester containing building blocks (BB), namely compounds **5(X)**, where X corresponds to the amino acid that carries the same side-chain (**Scheme 1**).[15,20]



Scheme 1. *Trans*-thioesterification for side chain exchange between a peptide scaffold and amino acid side chains of different BB **5(X)**. Gly= glycine, Ala= alanine, Val = valine, Leu= leucine, Aib= Aminoisobutyric acd, hAla= homoalanine, Tle= tert-Leucine, Phe= phenylalanine, Tyr= tyrosine, Trp= tryptophan, Ind= indolyl, pGly= phenylglycine, Lys= lysine, Arg= arginine, Gdm= guanidinium, hArg= homoarginine, Glu= glutamate.

This reversible reaction operates smoothly in water at neutral pH and low temperature. It is tolerant to the weak interactions involved in biomolecular recognition processes and even though thiol groups are part of protein composition, free cysteines are rare at protein surfaces and most often buried inside protein core where they stabilize the overall protein folding through disulfide bridging. We decided to use a scaffold bearing 4 cysteines at its surface, since a minimum of 3-4 amino acid side-chains are usually involved in the pharmacophore of a protein. To establish the proof of concept, we selected a 10-mer cyclic peptide backbone containing a ^{D}Pro-^{L}Pro β-turn inducing template, and a glycine residue at the opposite tip, known to fold into a regular and highly stable β-hairpin conformation.[21,22] (**Figure 2**)



1 DAP(Ac)^1-Ala^2-DAP(Ac)^3-Arg^4-Gly^5-Cys^6-Trp^7-DAP(Ac)^8-^{D}Pro-Pro
2 DAP(Ac)^1-Lys^2-Cys^3-Arg^4-Gly^5-Cys^6-Trp^7-DAP(Ac)^8-^{D}Pro-Pro
3 DAP(Ac)^1-Lys^2-Cys^3-Arg^4-Gly^5-DAP(Ac)^6-Trp^7-Cys^8-^{D}Pro-Pro
4 Cys^1-Lys^2-Cys^3-Arg^4-Gly^5-Cys^6-Trp^7-Cys^8-^{D}Pro-Pro

Figure 2. Scaffolds based on 10mer ^{D}Pro-^{L}Pro templated β-hairpin cyclic peptides bearing 1 (peptide **1**), 2 (peptides **2** and **3**) or 4 (peptide **4**). SH groups DAP(Ac)= (3-acetyl)-1,3-Diaminopropionic acid.

One face of the β-hairpin is covered with cysteine residues (Cys) for reversible acyl exchange. Two positions on the opposite face and one in the loop are used to tune the properties of the peptide: cationic residues (Lys, Arg in position 2 and 4 respectively) ensure water-solubility and a UV (Ultra-Violet) tag (Trp in position 7) allows monitoring of the reaction at a specific wavelength (280 nm). Based on these design considerations, a series of peptides was prepared, bearing one (**1**), two (**2** and **3**) or four thiol groups (**4**) on one face, the other residues of the same face being locked as non-exchangeable acetyl-amide (NHAc). Peptide sequences are assembled by Solid Phase Peptide Synthesis (SPPS) using Fmoc/*t*-Bu (Fluorenylmethoxycarbonyl/tert-butyl) strategy.[23] In order to verify that these peptides adopt the expected conformation, the solution structure of **4** was investigated by NMR (Nuclear Magnetic Resonance) spectroscopy in aqueous solution at 300 K (phosphate buffer pH 7, H_2O/D_2O 9:1, tris-carboxyethylphosphine (TCEP) 10 mM). Proton and carbon assignments were unambiguously achieved using ^{1}H, ^{13}C, TOCSY (TOtal Correlation SpectroscopY), ROESY (Rotating frame Overhauser Effect SpectroscopY) and ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Correlation) experiments. The obtained spectra revealed a unique set of resonances suggesting the presence of a single conformation on the NMR timescale. NH resonances displayed a large spectral dispersion and high values were measured for most of the $^{3}J_{HN\text{-}H_\alpha}$ couplings (>9 Hz) which was indicative of a stable extended conformation. In addition, numerous cross-hairpin connectivities typical from a regular β-hairpin were observed in the ROESY experiment. In

particular strong sequential $d_{\alpha N}(i, i+1)$ ROEs were observed as well as cross-strand ROEs connectivities between the NH protons of Cys^1/Cys^8 and Cys^3/Cys^6, and the Cα-H protons of Lys^2/Trp^7. The absolute values of amide proton temperature coefficients were very low for residues Cys^1 and Cys^6 (0.9 and 1.0 ppb/K respectively). The H/D exchange rates for NH protons of these two residues were very slow compared to the other residues, which is consistent with their involvement in intra-molecular H-bonding. All together, these data strongly support the presence of a regular β-hairpin conformation for the peptide backbone. Robinson *et al.* have shown that improved mimicry of a regular hairpin structure in a free ligand usually favors its recognition by its putative biological partner, because in such a conformation the side chains of up to four key hot residues can be displayed on the same face, allowing their simultaneous interaction with a target. This series of peptides was thus validated to study the reversible side-chains exchange.

In order to ensure similar reactivity between the scaffold and the BBs and guarantee equilibrium between both, acyl side-chains that are dynamically exchanged during DCC experiments are also attached to a cysteine platform in the BB design, *via* a thioester linkage. (**Scheme 1**) Different BB **5(X)** that recapitulate the panel of natural and common non-natural amino acids side-chains have been synthesized according to the literature.[24] To facilitate the analysis of the reaction mixture, the reaction conditions were first established on peptide **1** bearing a single thiol group in position 6. Peptide **1** (1 mM) was incubated with a single thioester BB **5(F)** (3, 5 or 15 equiv.) in a 100 mM phosphate buffer, at pH 7 and room temperature. (**Figure 3** & **Table 1**)

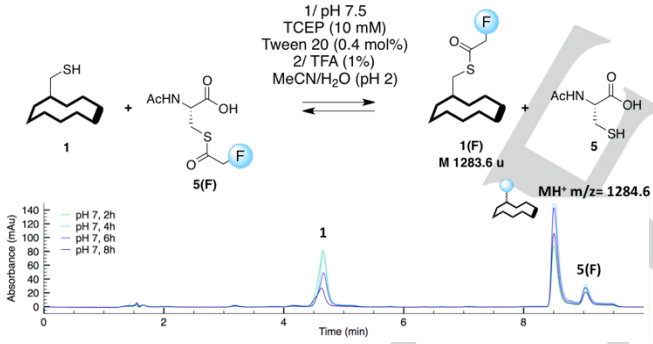


Figure 3. Thioester exchange between **1** and **5(F)**: reaction scheme *on the top* and RP-HPLC traces at 280 nm *on the bottom*

Table 1. Equilibrium constant K' for the transthioesterification of peptide **1** with a single BB **5(F)** at different scaffold to BB ratio and at different pH.

pH	Ratio **1**:**5(F)**	Time [h]	**1(F)** at equilibrium [%]	K'
7	1:3	6	66	0.6
	1:5	6	73	0.5
	1:15	6	92	0.7
7.3	1:5	4	82	0.6
	1:10	4	87	0.6
7.5	1:1	24	50	0.7
	1:4	4	80	1
	1:5	4	87	1

The buffer contains TCEP (10 mM) as reducing agent to avoid formation of by-products through disulfide bridging. Even though this competitive reaction is reversible at physiological pH and should not prevent the desired equilibrium, it could nevertheless complicate the analysis of the library content. Tween 20, a mild detergent compatible with biological medium, was used in a small amount (0.4 mol %) to prevent aggregation issues. After quenching the reaction through addition of TFA (Trifluoroacetic acid), formation of the acylated adduct **1(F)** was followed by RP-HPLC (High Performance Liquid Chromatography) at 280 nm. The acyl anchoring reaction takes place smoothly leading to the expected thiol-acylated peptide as a sole new compound as confirmed by Mass Spectrometry (MS) analysis (MH^+, m/z 1284.6). Equilibrium between **1** and **1(F)** is established within 6h. The reaction can be slightly accelerated by raising the pH of the reaction mixture to 7.3 or 7.5 without modifying the reaction course. The K' values were calculated from the steady-state concentration of each species, determined by RP-HPLC, and are in the range of 0.5-1. (Table 1)

The influence of the side-chain chemical nature on the reaction outcome was then studied. For this purpose, peptide **1** (1 mM) was treated with different building blocks bearing the side chains of aliphatic, aromatic, polar, cationic or anionic amino acids, at different concentrations, in the conditions reported above. In all cases, a unique compound corresponding to the thioester adduct is formed as a sole new compound, as confirmed by MS analysis.[23] (**Table 2**) Gratefully, no competitive reaction is observed when additional reactive functional groups are present on the side-chain. In general, the equilibrium is reached within few hours (3 to 6h) when an excess of BB is used (>1.5 equiv.), but for β-branched side-chains (**5(Ai)** and **5(V)**) additional time is needed. With **5(tL)** bearing a *t*-butyl group, the equilibrium is not reached even after several days. In this case, the steric hindrance is too high and such BB can thus not be considered in this approach. The K' values obtained from all the other BBs are in the same range (0.4-1.3), with less than one order of magnitude differences, meaning that the corresponding adducts are roughly isoenergetic. Even if slight differences exist, they are small enough to suggest that the dynamic library that would be generated from these different BBs should contain a close concentration of all the members which is, as discussed above, a key parameter for application of this reaction for screening.

Table 2. Equilibrium constant K' and analytical data for the transthioesterification product obtained from peptide **1** and different BB **5(X)**.

BB	Time [h]	Product	Molecular Formula (M [Da])	MH^+ (m/z)	K'
5(F)	3	**1(F)**	$C_{59}H_{81}N_{17}O_{14}S$ (1283.6)	1284.6	1.1±*0.4*
5(G)	4	**1(G)**	$C_{52}H_{77}N_{17}O_{16}S$ (1193.5)	1194.6	0.8±*0.4*
5(A)	3	**1(A)**	$C_{53}H_{77}N_{17}O_{14}S$ (1207.6)	1208.6	0.5±*0.1*
5(V)	24	**1(V)**	$C_{55}H_{81}N_{17}O_{14}S$ (1235.6)	1236.4	1.2±*0.1*
5(tL)	24	**1(tL)**[6]	$C_{56}H_{84}N_{17}O_{14}S$ (1249.6)	1250.6	NR*
5(L)	3	**1(L)**	$C_{56}H_{83}N_{17}O_{14}S$ (1249.6)	1250.7	0.8±*0.2*

5(Ai)	24	**1(Ai)**	$C_{54}H_{79}N_{17}O_{14}S$ (1221.6)	1222.6	0.7±*0.1*
5(hA)	4	**1(hA)**	$C_{54}H_{79}N_{17}O_{14}S$ (1221.6)	1222.6	1.1±*0.3*
5(Y)	4	**1(Y)**	$C_{59}H_{81}N_{17}O_{15}S$ (1299.6)	1300.6	0.9±*0.4*
5(W)	4	**1(W)**	$C_{61}H_{82}N_{18}O_{14}S$ (1322.6)	1323.6	0.8±*0.4*
5(pG)	4	**1(pG)**[6]	$C_{58}H_{79}N_{17}O_{14}S$ (1269.6)	1270.6	0.5±*0.2*
5(K)	4	**1(K)**	$C_{56}H_{84}N_{18}O_{14}S$ (1264.6)	1265.6	0.6±*0.4*
5(R)	6	**1(R)**	$C_{56}H_{84}N_{18}O_{14}S$ (1264.6)	1265.6	0.9±*0.1*
5(hR)	4	**1(hR)**	$C_{57}H_{86}N_{20}O_{14}S$ (1306.6)	1307.8	1.3±*0.5*
5(E)	4	**1(E)**	$C_{55}H_{79}N_{17}O_{16}S$ (1265.6)	1266.6	0.4±*0.3*

* Not reached

To prove the thermodynamic control over the reaction mixture, evolution of the library composition upon simultaneous (Exp 1) or sequential (Exp 2 & 3) introduction and equilibration of three different BBs was achieved (**Figure 4**).

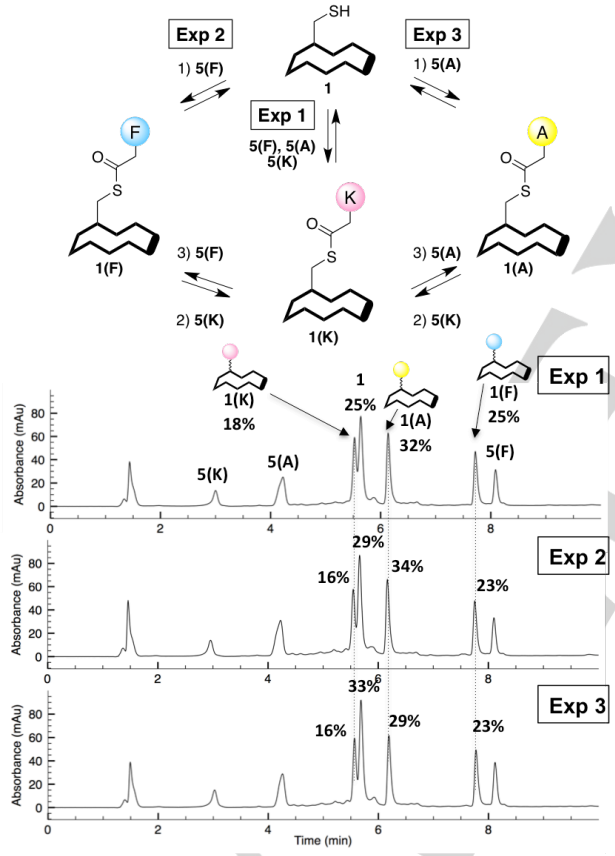


Figure 4. Study of the reversibility of the acyl exchange between **1** (1mM) and **5(F), 5(K)** and **5(A)** (= Exp 1); **5(F)**, then **5(K)** then **5(A)** (= Exp 2); **5(A)**, then **5(K)** then **5(F)** (= Exp 3). Reaction scheme *on the top* and RP-HPLC traces at 280 nm *on the bottom*

In a first experiment (Exp 1) peptide **1** was treated simultaneously with 1 equiv. of **5(F)**, **5(K)** and **5(A)**, leading at equilibrium (16h) to a mixture of **1**, **1(F)**, **1(K)** and **1(A)** in respectively 25, 32, 18 and 25%, as determined from the RP-HPLC peak areas. Two other series of experiments were performed in parallel, in which scaffold **1** (1 mM) was first reacted with 1 equiv. of either **5(F)** (Exp 2) or **5(A)** (Exp 3). After 16h, 1 equiv. of **5(K)** was introduced in each reaction mixture, and after 16 additional hours, the third BB, respectively **5(A)** in Exp 2 and **5(F)** in Exp 3 were added. Upon each addition step, the reaction mixture was analyzed by RP-HPLC and shows a reorganization of the library mixture, with the formation of a new adduct obtained from the added BB, at the expense of the adducts already present in the library. The amount of each adduct obtained in the different experiments are quite similar, unambiguously establishing the reversibility of the exchange.

Having established the little influence of the nature of amino acids side-chains functionalities on the thioester exchange reaction, the next step was to verify that the dynamic of the exchange is not modified by the proximity of another side-chain grafted on a nearby thiol group and to which extend interactions between side-chains on the scaffold surface can compromise the formation of a statistical library. To simplify the analysis and quantification of the library, peptides **2** and **3** containing two exchangeable sites in two different relative arrangements were used in these experiments. First, **2** or **3** (1 mM) were reacted with a single BB, **5(A)** or **5(L)** at different ratios (4, 6 or 9 equiv.) in the conditions reported above. The reaction was followed by RP-HPLC overtime. (**Figure 5**A and B for **2,** see SI for **3**)

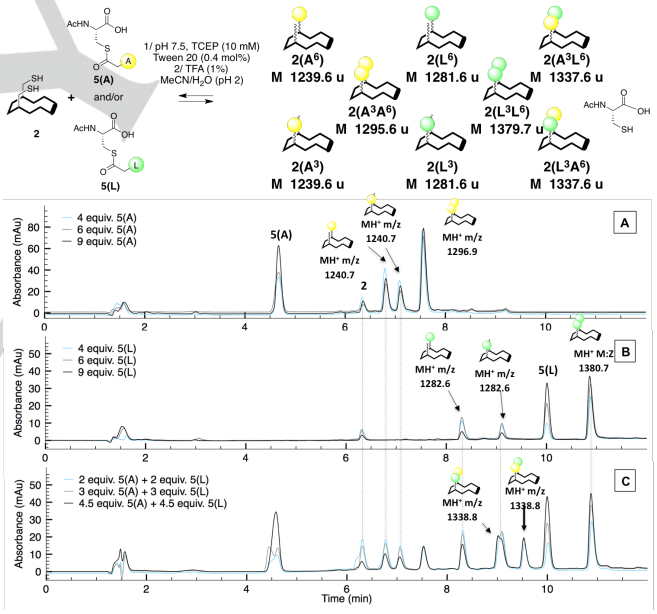


Figure 5. Reaction of **2** (1 mM) and **5(A)** or **5(L)** or both **5(A)** and **5(L)** (4, 6 or 9 equiv)., reaction scheme *on the top* and RP-HPLC traces at 280 nm *on the bottom.*

In both cases, the reaction leads in the first hour to two new HPLC signals in approximately equal amount, corresponding to the acylation of one of the two free cysteines, **2(X^3)/2(X^6)** or **3(X^3)/3(X^8)**, respectively for **2** and **3** (the number in superscript indicates the position of the grafted side-chain). Then, the bis-acylated adducts **2(X^3X^6)** or **3(X^3X^8)** were formed, leading at equilibrium (around 12h) to the four compounds in different amount depending on the quantity of BB used in the reaction. Assignment of the sequences was achieved by MALDI-TOF MS

(Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry). Identification of the isomeric peptides attached to the same acyl derivatives but in different positions can be obtained by MS/MS. For example, the two signals observed at 8.3 and 9.1 min in the RP-HPLC analysis of the mixture obtained from **2** and **5(L)**, with the same m/z value (1282.6), were submitted to MS/MS fragmentation and could be assigned to **2(X^3)** at 8.3 min and **2(X^6)** at 9.1 min.[23] The K' values for each product was calculated according to its concentration at equilibrium. (**Table 3**) In both cases equilibrium constants for the acylation of each of the two thiol groups at Cys^3 and Cys^6 or Cys^8 (respectively for **2** and **3**) are very similar suggesting that the position of the thiol on the scaffold surface has no influence on its reactivity. Moreover, the equilibrium constant of the first and second acylation step, are in the same range, meaning that the presence of the first side chain does not prevent (or favor) the second grafting step. Concerning the nature of the anchored side chains, while the K' values are nearly identical for **2**, in the case of **3** the thiol/thioester equilibrium is slightly more shifted towards the acylated species when leucine side chain is used (K' ≈ 3), compared to alanine (K' ≈ 1), suggesting a stabilization of the grafted peptides in this case. This stabilization is probably due to favorable hydrophobic interactions between the hydrocarbon side-chain and the peptide backbone and/or side chains. However, the difference is small and should not compromise the observation of all adducts for libraries made of the two BBs. Indeed, when **2** or **3** are treated simultaneously with **5(A)** and **5(L)** (2, 3 and 4.5 equiv. of each) the eight expected peptides, including those bearing mixed A and L side-chains combinations are observed at equilibrium in quite similar amount. (**Figure 5**C for **2**, see SI for **3**) The variations observed in the K' values may well be compensated by adjusting the concentrations of the BBs.

Table 3. Equilibrium constant K' for the transthioesterification of scaffolds **2** and **3** with a single BB **5(A)** or **5(L)**

Sca	BB	Equilibrium Constant K'		
		Acylation at Cys$^{6/8}$	Acylation at Cys^3	Acylation at Cys^3 and Cys$^{6/8}$
2	**5(A)**	0.6±*0.1*	0.5±*0.1*	0.3±*0.1*
	5(L)	0.6±*0.4*	0.5±*0.2*	0.7±*0.3*
3	**5(A)**	1±*0.5*	1±*0.4*	0.6±*0.1*
	5(L)	3.3±*0.7*	3±*0.7*	3.4±*1*

Having proven on model systems containing one or two cysteine residues that the transthioesterification is an appropriate reaction to access isoenergetic libraries of peptides in terms of side chain nature, position and number, we finally moved to scaffold **4**, bearing 4 cysteine groups on the same face. First, **4** was reacted with few representative BB individually, **5(G)**, **5(A)**, **5(L)** and **5(K)** leading as expected in each case to 15 new products at equilibrium (8h) bearing one, two, three or four grafted side chains on their surfaces. (**Figure 6** shows the data obtained with **5(L)**, for the other BB see SI)

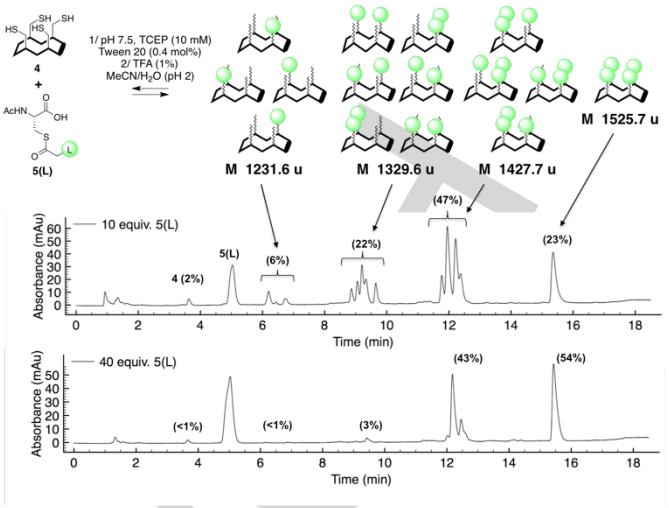


Figure 6. Reaction of **4** with **5(L)** using 10 equiv. or 40 equiv. of **5(L)**: reaction scheme *on the top* and RP-HPLC traces at 280 nm *on the bottom*,

The amount of each compound depends mainly on the ratio of BB to scaffold used in the experiment as expected for a thermodynamically controlled process. Composition of the library can be thus tailored by controlling this *ratio.* The K' values were calculated for each family of isomers bearing, one, two, three and four side chains. (**Table 4**)

Table 4. Equilibrium constant K' for the transthioesterification of **4** with a single BB **5(X)**.

BB	t	Equilibrium Constant K'			
	[h]	Mono-acylation	Di-acylation	Tri-acylation	Tetra-acylation
5(G)	4	0.4±*0.2*	0.6±*0.1*	0.4±*0.1*	0.2±*0.1*
5(A)	16	0.4±*0.2*	0.8±*0.2*	0.4±*0.1*	0.2±*0.1*
5(L)	9	1.3±*0.1*	1.2±*0.2*	0.7±*0.1*	0.2±*0.1*
5(K)	16	1.6±*0.5*	0.6±*0.2*	0.2±*0.1*	0.1±*0.01*

In general, the K' for the three first acylation steps are quite similar (for example K' ≈ 1 for the mono- di and tri-acylated product with **5(L)**), but a slightly smaller value is obtained for compounds decorated with 4 side chains (K' ≈ 0.2 with **5(L)**). Here again the difference is quite small (one order of magnitude) and might be attributed to the steric hindrance obtained on the scaffold surface for the tetra-acylated compound. For cationic side-chain (**4(K)**), electrostatic repulsion of the side chains on the scaffold surface adds to the steric effect. The resulting differences obtained in the proportion of the library's components are however quite small and in the range of discrepancies usually observed for more traditional two or three components DCC system, for which detection of target-induced amplification has been demonstrated.[25] The incomplete *S*-acylation is not surprising since in general multivalent assembly of a number of BBs onto a single scaffold is disfavored for entropic reason. However, it has been shown on similar systems that this can be counterbalanced in the presence of a target in a DCC experiment and should not compromise the screening of

such a library.[19] Actually, libraries for which potential binders are present at a lesser extent in the absence of a target are the best suited for the detection and quantification of ligands in a template-induced amplification process because of the larger difference in the proportion obtained in the presence and absence of the target in this case.[26]

Finally, the combinatorial aspect of the reaction was studied by mixing **4** with **5(L)** and **5(A)** simultaneously (20 equiv. of each) in the established conditions, leading theoretically to a library of 81 peptides. (**Figure 7**)

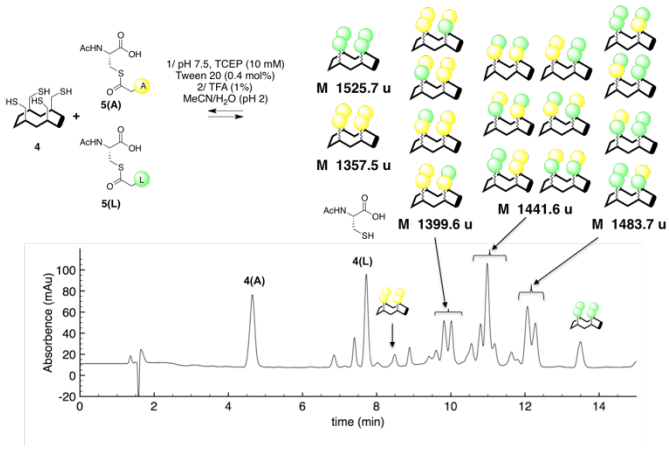


Figure 7. Reaction of **4** with **5(L)** and **5(A)** (20 equiv. of each BB): reaction scheme *on the top* and RP-HPLC traces at 280 nm *on the bottom*. Only tetra-acylated adduct obtained as major compounds are indicated.

The equilibrium is reached after 8h. Isolation and MS analysis allowed identifying mono-, di-, tri- and tetra-acylated compounds. Even if in this case we have not optimize the separation conditions in order to assign the analytical signal to different isomers, analysis of the reaction mixture shows that a wide variety of combinations is present at equilibrium with the peptide bearing three or four side-chains being obtained as major compounds as expected for a library made from large excess of BB. Identification of all analytical signals is *a priori* not mandatory to find new hits. For example, the largest DCL reported so far contains 9000 members and even though in such a library almost all compounds co-elute with many others, amplification by a template was observed and deconvolution allowed identification of the amplified compounds.[27] Alternatively, isolation of the complex formed by the peptide and a biological target prior to analysis can be considered and allow to highly facilitate the DCC hit identification.[27]
In order to verify that the tethering of side chains on the scaffold surface does not affect the peptide 3D conformation, peptide **4(G^{1}G^{3}G^{6}G^8)** grafted with 4 glycine side chains was prepared on a larger scale and isolated. ^{1}H-NMR spectra obtained for this peptide show similar spectral features to peptide **4**, suggesting that side-chains anchoring does not disturb the peptide conformation.[23]

Conclusion

In conclusion, we have successfully designed an efficient, robust and original strategy to generate dynamic libraries of peptides with a well-defined 3D structure by combining a rationally designed 3D peptide scaffold with a dynamic combinatorial chemistry approach to graft amino acid side-chains on the peptide surface. A huge diversity of side-chains combinations could be obtained in this system depending on the number and nature of BBs used to generate the library. Dynamic libraries are in principle responsive to external changes and having demonstrated that the different thioester peptides obtained by the thioester exchange reaction are roughly isoenergetic, we can expect that these dynamic libraries represent a powerful screening tool for the rapid identification of peptide ligands. We are currently exploring its potential as a screening tool in different relevant biological contexts, and particularly in the challenging field of Protein-Protein Interaction inhibition.

Experimental Section

General Informations
All reagents and solvents were purchased form commercial suppliers and used without further purification. The amino acids used for peptides synthesis were purchased from Iris Biotech GmbH as follow: Fmoc-Arg(Pbf)-OH (Pbf= 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl), Fmoc-Cys(Trt)-OH (Trt= trityl), Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH (Boc= *tert*-butyloxycarbonyl), Fmoc-Pro-OH, Fmoc-D-Pro-OH, Fmoc-Trp(Boc)-OH. Flash chromatography (FC) was performed using silica gel Merck 60 with 0.040-.063 μm on a Grace Reveleris X2TM apparatus. Analytical thin-layer chromatography (TLC) was performed using silica gel Merk 60 on aluminia, visualised by UV fluorescence at 254 nm and revealed with: phosphomolybdic acid (10% solution in absolute EtOH), bromocresol green (0.04% solution in absolute EtOH slightly alkalinised with 0.1M aqueous NaOH), ninhydrine (0.3% in n-BuOH/ AcOH) or *p*-anisaldehyde. The peptides were synthesized by manual SPPS in polypropylene manual disposable reactors with plunger and frit, pore size 25 μm, with removable PTFE (PolyTetraFluroEthylene) stoppers, purchased from Multisyntech GmbH. Preparative scale purification of peptides was performed by reverse phase HPLC on a Waters systems consisting of a binary pump (Waters 1525) and a dual wavelength UV/Visible Absorbance detector (Waters 2487), piloted by Breeze softwares using the following columns: preparative Macherey-Nagel Nucleodur (RP C18, 250 x 16 mm, 5 μm, 300 Å) from AIT. Analytical RP-HPLC were performed on a Dionex system consisting in an analytical automated LC system (Ultimate 3000) equipped with an autosampler, a pump block composed of two ternary gradient pumps and a dual wavelength detector, piloted by a Chromeleon software. The analyses were performed on a *Proto 200* (RP C18, 100 x 4.6 mm, 3 μm, 200 Å) from *Higgins-Analytical Inc*. using as eluent A, H_2O containing 0.1% of TFA and as eluent B, CH_3CN containing 0.1% of TFA, at a flow rate of 1 mL/min. UV detection was done at 220, 280 nm, 350 nm or 415 nm. NMR spectra were recorded on Brucker Avance III nanobay 300 MHz or 400 MHz spectrometers. Peptides were characterized by MALDI-TOF MS (DE-Pro, PerSeptive Biosystems) in positive ion reflector mode using the CHCA matrix. ESI (ElectroSpray Ionization) MS and MS/MS analyses were performed on a Synapt G2-S instrument equipped with UPLC (Ultra Performance Liquid Chromatography, Waters Corporation, UK). For the separation, a gradient with

H_2O containing 0.1% of FA (= Formic Acid) as eluent A and CH_3CN containing 0.1% of TFA as eluent B was applied, from 20% B to 55% B in 13 minutes at a flow rate of 0.5 mL/min on a C18 column (Kinetec EVO, 10x21mm, 2.6µm). UV detection was done at 220 and 280 nm. For the MS/MS experiments, the m/z ion, always the doubly charged compound $[MH_2]^{2+}$, was selected and submitted to collision energy (CE) ramp between 25 and 55. MassLynx software was used for data acquisition and processing. Optical rotations were determined at 20°C on a JASCO P-2000 polarimeter equipped with a 10 cm path length cell.

Peptides Synthesis

2-Chlorotrityl chloride resin (1.0 g, 1.6 mmol) was swollen with dichloromethane (DCM) for 30 min. and a solution of the Fmoc-protected amino acid (0.8 mmol, 0.5 eq.) and diisopropylethylamine (DIEA) (348 µL, 2 mmol) in DCM (10 mL) was added. The suspension was shaken at room temperature for 3 hours then washed with a mixture of DCM/MeOH/DIEA (17:2:1, 3 times), DCM (3 times), dimethylformamide (DMF) (3 times), DCM (3 times), and MeOH (3 times) then dried under vacuum. The determination of the amino acid loading was achieved by calculating the ratio between the quantity of amino acid used in millimoles and the mass of dried resin in grams. The loading of the resin was approximately of 0.5 mmol/g. The amino acid chain elongation was performed manually starting from 0.25 mmol of resin. The resin was first swollen in DCM for 30 min. and washed with DMF (3 times). The N-terminal Fmoc groups were removed by treatment with a 20 % solution of piperidine in DMF. Followed by draining and washing with DMF (3 times). Standard N-Fmoc protected amino acids (1.0 mmol, 4 eq.) and HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (360 mg, 0.95 mmol, 3.8 eq.) were dissolved in 7 mL of DMF and DIEA (348 µL, 2 mmol, 8 eq.) was added. The activated amino acid was then transferred to the resin and couplings were performed for 30 minutes with shaking. For homemade residues, 2 eq. of amino acids (0.5 mmol) and 1.9 eq. HBTU (180 mg, 0.48 mmol) were used and couplings were performed for 60 minutes. After draining and washing with DMF (3 times), completion of the coupling reaction was assessed by a colorimetric test (Kaiser or Chloranil or TNBS (= trinitrobenzene sulfonic acic)). The resin was finally washed 3 times with DMF, DCM and MeOH then dried under vacuum. The linear side chains protected peptide was cleaved from the resin by treatment with a 25 % solution of hexafluoroisopropanol (= HFIP) in DCM once for 1 hour and once again for 30 min. The resin was washed with DCM (3 times). The cleavage solutions and rinsing solutions were gathered and the solvents were removed under reduced pressure. The residue obtained from the cleavage step was dissolved in about 300 mL of DMF. HATU (1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5] pyridinium 3-oxide hexafluorophosphate) 91 mg, 0.24 mmol, 0.95 eq.) and DIEA (580 µL, 3.4 mmol, 14 eq.) were added. The mixture was stirred overnight at room temperature under argon atmosphere. The DMF was removed under reduced pressure to dryness. The residue from the macrolactamization step was treated with about 7 mL of a mixture of TFA/H_2O/TIS (triisopropyl silane) /EDT (1,2-ethylendithiol) (94:2.5:1:2.5) and stirred at room temperature for 3 hours under argon atmosphere. Volatiles were removed under reduced pressure. The deprotected peptide was precipitated by dropwise addition of the dark oily residue to cooled Et_2O and centrifugation at 8500 rpm for 5 min. The Et_2O was removed and the same operation was repeated twice. The crude product was dissolved in a H_2O/CH_3CN/TFA (90/10/0.1) mixture and lyophilized prior to purification by RP-HPLC. The collected fractions were combined and lyophilized to afford the peptide as a powder.

Generation of the dynamic combinatorial library

The buffer was prepared by adding successively: 693 mg of $Na_2HPO_4 \cdot 12H_2O$, 417 mg of KH_2PO_4 and 143 mg of TCEP. The pH was adjusted to 7, 7.3 or 7.5 with 1M NaOH solution, then 500 µL of TWEEN-20 were added and the volume was adjusted with distilled water at 50 mL before degazing the solution for 15 min. BBs stock solutions of 50 mM and peptides stock solutions at 10 mM concentrations were prepared. The libraries were generated by mixing 10 µL of the peptide stock solution and the appropriate amount of BB stock solutions to reach the desired BB to peptide ratio. The volume is adjusted to 100 µL total volume with buffer to reach a 1 mM concentration of peptide. The evolution of the mixture was monitored by collecting 20 µL of the mixture, diluted to 0.2 mM of peptide concentration with 80 µL of a quenching mixture (H_2O/ CH_3CN/TFA; 50:50:0.1) and finally analyzed by analytical RP-HPLC analysis. An analytical amount of each HPLC peak was collected and characterized by MALDI-MS.

Keywords: Peptide Library, Dynamic Combinatorial Chemistry, Scaffold Grafting

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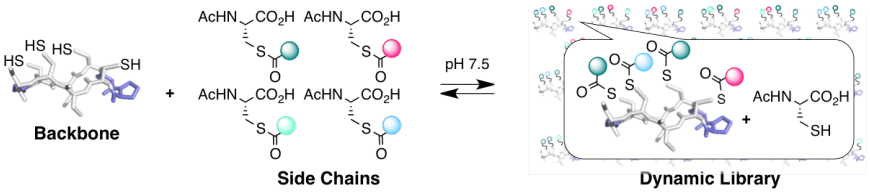

An efficient access to large libraries of conformationally defined peptides is reported, using dynamic combinatorial chemistry as a tool to graft amino acid's side-chains in defined arrangement on a well-ordered 3D peptide scaffold. We report here the design of a chemical system that ensures the scrambling of side-chains on a pre-organized scaffold leading to an isoenergetic library useful for ligand screening.

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