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Convergent Diversity-Oriented Side-Chain Macrocyclization Scan for Unprotected Polypeptides

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Abstract
Here we describe a general synthetic platform for side-chain macrocyclization of an unprotected peptide library based on the $S_N$$\text{Ar}$ reaction between cysteine thiolates and a new generation of highly reactive perfluoroaromatic small molecule linkers. This strategy enabled us to simultaneously "scan" two cysteine residues positioned from $i$, $i+1$ to $i$, $i+14$ sites in a polypeptide, producing 98 macrocyclic products from reactions of 14 peptides with 7 linkers. A complementary reverse strategy was developed; cysteine residues within the polypeptide were first modified with non-bridging perfluoroaryl moieties and then commercially available dithiol linkers were used for macrocyclization. The highly convergent, site-independent, and modular nature of these two strategies coupled with the unique chemoselectivity of a $S_N$$\text{Ar}$ transformation allows for the rapid diversity-oriented synthesis of hybrid macrocyclic peptide libraries with varied chemical and structural complexities.

Introduction
Cyclic polypeptides represent a unique class of naturally occurring biomolecules often with enhanced biological properties compared to their linear counterparts.1 These bioactive macromolecules can be found in a number of bacteria, plants, and mammals.2 Chemists have aimed to synthesize hybrid macrocyclic polypeptides that mimic the structure and function of naturally produced bioactives.3 Side-chain to side-chain macrocyclization has emerged as one method to access such species. This approach entails the cross-linking between two or more side-chain functional groups with natural or non-natural amino acid residues. Several methods exist, including nucleophilic substitution with benzyl/allyl halide electrophiles,4 a myriad of carbon-carbon5 and carbon-nitrogen6 bond-forming processes, cycloadditions,7 disulfide formation,8 metal-based coordination9 and non-covalent routes.10 Each approach may have drawbacks associated with chemoselectivity, tunability, and synthetic practicality. Most importantly, regions within a polypeptide are often found to undergo inefficient macrocyclization with certain chemical approaches. These limitations reduce the ability to synthesize analogues for potential therapeutic applications.11

An ideal side-chain to side-chain macrocyclization chemistry should possess orthogonality, specificity, and robustness that allows for cyclization irrespective of the sequence and position. This can be conceptualized as a scanning approach,12 where one can deliberately vary the positions of two amino acids as well as the chemical and topological composition of the entity connecting these two residues (Figure 1). To develop a versatile peptide
macrocyclization scan, we believe synthetic convergence and site-independent reactivity are required. Specifically, a convergent diversity-oriented synthetic (DOS) strategy would enable such a scan, where, in principle, any linear polypeptide can be cyclized as a last, high-yielding synthetic step with an exogenously added linker. The site-independent reactivity requirement imposes robustness and efficiency for the desired transformation irrespective of the chemical nature of the polypeptide chain and mutual arrangement of the residues undergoing macrocyclization. The added complexity and requirements for a polypeptide macrocyclization scan makes it significantly more challenging and ultimately requires new approaches. Importantly, none of the existing methods for hybrid peptide macrocyclization (vide supra) were shown to be fully suitable for the envisioned macrocyclization scan, including widely-used CLIPS and stapling technologies, developed by Timmerman et al.\textsuperscript{4a,d} and Verdine et al.\textsuperscript{5b–d,f} respectively.

The advent and development of “click” chemistry provides researchers with a potentially powerful series of transformations allowing for selective functionalization of biomolecules.\textsuperscript{14} We and others have recently become interested in a formerly unrecognized reaction between thiol nucleophiles and activated perfluoroaromatic molecules.\textsuperscript{15} This reaction satisfies several important prerequisites of being a “click” transformation (Figure 2a).\textsuperscript{14,15} Our recent study showed one can use two commercially available perfluoroaromatic reagents (Figure 2b, L\textsubscript{a} and L\textsubscript{b}) to cross-link two cysteine moieties positioned in the \(i, i+4\) fashion within a polypeptide.

This modification improved cell-uptake, proteolytic stability, and target binding of the corresponding peptide. Cysteine perfluoroarylation was found to be compatible with unprotected peptides showing excellent chemo- and regioselectivity in the presence of other reactive functional groups. However, the generality of the perfluoroaryl-cysteine \(S_NAr\) chemistry in context of the desired macrocyclization scan has thus far remained challenging. Herein, we report how a new generation of rationally designed perfluoroaryl-based cross-linking molecules enables a peptide macrocyclization scan based on the cysteine perfluoroarylation via the \(S_NAr\) transformation. Two simple, complementary synthetic strategies embody the versatility of our approach (Figure 2d). Specifically, access to peptide macrocycles can be achieved by either cross-linking cysteine containing peptides with perfluoroaryl-based linkers, or by incorporating non-crosslinked perfluoroaryl-based moieties first, followed by their macrocyclization with dithiol reagents. The developed macrocyclization scan is enabled by our strategy to maximize the reactivity of perfluoroaryl-based synthons without compromising their synthetic accessibility, robustness and tailorability (Figure 2b,c). Site-independent reactivity of the described platform ultimately allowed us to conduct highly efficient macrocyclizations in a series of peptides featuring two cysteine residues incorporated at the positions ranging from \(i, i+1\) to \(i, i+14\) (Figure 2e). The developed approach is fully convergent allowing for rapid and operationally simple generation of chemical complexity from unprotected peptides containing naturally occurring amino-acid residues.

**Results and Discussion**

In an effort to expand peptide stapling with two Cys moieties beyond \(i, i+4\) positions, we sought to develop an extended library of bifunctional cross-linking molecules with rationally varied properties containing two perfluoroaryl units. Based on our experimental observations, species containing aliphatic groups in the \(para\) position exhibited diminished reactivity toward the nucleophilic attack rendering the \(S_NAr\) process slow and inefficient (Figure 2b). This phenomenon is attributed to the poor electron-withdrawing nature of alkyl-based substituents, which are not sufficiently activating for the \(para\)-C-F bond substitution.
at room temperature.\textsuperscript{17} We thus decided to explore a less-developed activation pathway that features a \textit{para}-substituted thioether group appended on perfluoroaryl-based unit. Specifically, Pitts and co-workers previously observed enhanced reactivity of these reagents and attributed the effect to net stabilization of the Meisenheimer complex through resonance stabilization of the negative charge at the \textit{para}-sulfur on the thioether moiety (Figure 2b). Our DFT computational studies corroborate the presence of a \textit{para}-S substituent on the \textit{pentafluorophenyl} moiety consistently renders a larger \textit{para}-C-F bond polarization than in the alkyl-based substituent-containing congeners (see SI, Table S1). The presence of the same \textit{para}-thioether moiety on the biaryl variant does not significantly change \textit{para}-C-F dipole features as compared to \textbf{L}b, though it remains large due to the inductive electron-withdrawing effect produced by the \textit{para}-S-\textit{C}_{6}\textit{F}_{4} substitute. Therefore, we chose to focus our efforts on both \textit{S}-substituted 1\textit{nonafluorobiphenyl} and 1\textit{-pentafluorobenzene} groups for facile nucleophilic aromatic substitution chemistry necessary for the bioconjugation (\textit{vide supra}).

We utilized two independent routes to synthesize a new class of bifunctional perfluoroaromatic-based linkers \textbf{L}c–\textbf{L}g (note that, linker \textbf{L}c can also be obtained from an existing commercial source). Linkers containing alkyl and benzyl moieties were synthesized \textit{via} \textit{S\textsubscript{N}Ar} chemistry between an excess (>25 fold) of commercially available hexafluorobenzene and alkyl/benzyl dithiol species (see SI for full synthetic procedures). Utilizing an excess of the perfluoroaromatic reagents ensures the formation of the mono-substituted species and minimizes the production of oligomeric and polymeric products.\textsuperscript{18} However, no desired product was observed when these conditions were applied to aromatic dithiols. To circumvent this, linkers containing aromatic moieties were synthesized \textit{via} Cu-mediated cross-coupling of the corresponding aryl halide or dihalide and commercially available \textit{pentafluorophenylthiol} (see SI).\textsuperscript{19} The proposed structural formulations of linkers \textbf{L}c–\textbf{L}g are fully consistent with their heteronuclear NMR spectra in solution (see SI).

To probe peptide macrocyclization with the perfluoroaryl-based linkers, we designed a series of 14 peptides featuring a diverse set of natural amino-acid residues and two cysteine moieties deliberately varied at sites from \textit{i}, \textit{i}+1 to \textit{i}, \textit{i}+14 (labeled as 1–14, see Figure 2e). To start, we tested the macrocyclization protocol with linkers \textbf{L}a and \textbf{L}b utilizing strategy I (Figure 2d), wherein bifunctional cross-linkers are expected to interconnect two cysteine sites producing peptide-based macrocyclic structures. Upon treating \textit{7} with two equivalents of \textbf{L}a in the presence of TRIS base (50 mM, in DMF), we observed nearly complete consumption of the starting material within 2 hours as determined by LC-MS analysis. Under these conditions nearly 80\% of the macrocycle product \textit{7a} was observed in addition to a small amount of oxidized \textit{7} formed \textit{via} intramolecular disulfide formation (Figure 3).

When the longer linker \textbf{L}b was used under the same conditions, the macrocyclic product \textit{7b} formed in >90\% yield after 2 hours with a minimal amount of the oxidized by-product. This noticeable increase in the product yield can be attributed to a longer length and higher reactivity of \textbf{L}b as compared to \textbf{L}a, rendering the process more efficient.\textsuperscript{19}\textsuperscript{F} NMR spectra of the purified peptides \textit{7a}–\textit{7b} are consistent with the proposed cysteine arylation, where only resonances observed correspond to the 1,4- and 1,10-substituted patterns on the perfluoroaromatic moieties.\textsuperscript{20} Given the high sensitivity of \textsuperscript{19}\textsuperscript{F} nucleus to the local magnetic field, we observed additional complexity in both of these spectra due to the presence of slowly exchanging conformers (see SI). The observed dynamic behavior is likely a result of the slow rotation of the perfluoroaromatic ring moieties, which can be modulated by temperature and solvent (see SI for variable temperature (VT) NMR spectroscopy studies).

When peptide \textit{7} was treated with the next-generation linkers \textbf{L}c–\textbf{L}g, reaction yields were greater than 85\% and not dependent on the chemical nature or length of the moiety. We
observed small amounts, less than 10%, of cross-linked by-products resulting from the competing intermolecular processes (Figure 3). Regio- and chemo-selectivity of these processes were confirmed via $^{19}$F NMR spectroscopy of the purified peptides 7c–7d. Overall, macrocyclization studies with i, i+7 peptide 7 indicates that macrocyclization via $S_N$Ar chemistry between cysteine thiolates and activated perfluoroaromatic linkers $L_a$-$L_g$ is highly efficient and chemoselective (Figure 3). Importantly, in contrast to the stapling chemistry via olefin metathesis, this approach does not necessitate the use of non-natural olefin-containing amino acids as well as precise tuning of the length and stereochemistry of these residues.

Given recent studies demonstrating the feasibility of peptide macrocyclization with hybrid linkers at positions beyond those located on the same face of the $\alpha$-helical loop (single turn – i, i+4; double turn – i, i+7; triple turn – i, i+11), we sought to further test the developed chemistry by scanning the polypeptide in either direction via this platform. Conducting macrocyclization reactions with conditions employed for peptide 7, we tested the corresponding peptides where cysteine residues are positioned in i, i + 6 (peptide 6) and i, i + 8 (peptide 8) fashion. We observed greater than 75% macrocyclization yields for all 14 reactions with linkers $L_a$-$L_g$, where more flexible linkers produced the corresponding macrocycles 6e–g and 8e–g almost exclusively with yields equal or greater than 90% (see SI).

We further tested this approach by probing the lower and upper limits of the scan with peptides 1–5 and 9–14. The upper limit of our macrocyclic scan was tested with peptides 9–14, which were subjected to linkers $L_a$–$L_g$ (Figure 4). The reaction with linker $L_a$ produced the desired cyclic peptide 14a in only 40%, while the oxidized by-product was observed to be the major species for this reaction (ca. 50%). On the other hand, when linker $L_b$ was used with peptide 14, the macrocyclic species 14b formed in nearly quantitative yield (Figure 4).

Importantly, reactions with linkers $L_c$, $L_e$ and $L_f$ also produced yields greater than 90% for the desired peptide macrocycles, whereas reduced yields were observed for $L_d$ and $L_g$. The observed reactivity trends suggest that a proper combination of linker length and electrophilicity are necessary for efficient macrocyclization for longer peptides. Interestingly, only small amounts of cross-linked macrocyclic species were observed when $L_b$ was reacted with peptides 1 and 2, where instead large quantities of by-products were observed (see SI). Observed diminished yields of 1b and 2b are likely due to the conformational rigidity and length of $L_b$, which is incompatible with the relatively short distance separating the Cys residues in peptides 1 and 2. On the other hand, reactions with other linkers produced corresponding macrocyclic peptides in yields greater than 75% (see SI). All transformations involving the new family of flexible linkers $L_d$–$L_g$ produced peptide macrocyclic products with most yields consistently greater than 90% (see SI and Figure 5). Upon further investigation, we discovered that macrocyclic peptides 1b and 2b could be obtained in better yields, when re-optimized conditions featuring higher dilution and lower stoichiometric ratio of the organic linker are employed (Figure 5). Given previously recognized difficulties associated with macrocyclization strategies of short peptide sequences (<4 residues) reported by Fasan and co-workers using bifunctional cross-linkers, this approach demonstrates how this chemistry can circumvent previous limitations. Similarly, i, i + 4 peptide 4 and its nearest peptide scan neighbors 3 and 5 underwent smooth macrocyclization yielding the desired hybrid macrocyclic species as major products in yields greater than 80% with new linkers $L_d$–$L_g$ (see SI and Figure 5), whereas diminished yields were observed in the formation of 3b and 3c (20% and 41%). Employing the re-optimized reaction conditions allowed us to improve the respective yields of peptides 3b and 3c (vide supra). Overall these results highlight the feasibility of the macrocyclization scan, ultimately demonstrating that properly designed perfluoroaromatic-
based linkers can mediate this process generally irrespective of their chemical identity. The observed utility of the perfluoroaryl-based \( S_{\text{N}2} \)Ar approach for macrocyclization at cysteine residues significantly expands access to these hybrid structures when compared to the chemistry featuring allyl and benzyl halides, where yields were found to be extremely sensitive to the linker.\(^{4e}\)

Overall, utilizing only 7 model linkers we produced 98 macrocyclic peptides without optimization of the conditions for each reaction (Figure 5). In all but one instance we observed at least a 30% yield of the desired peptide macrocycle, and for reactions employing next-generation linkers \( L_c-L_g \) the yields were greater than 60% (Figure 5). Approximately half of all the macrocyclization reactions tested, product yields were greater than 90%, and for the most of the reactions conducted with the new linkers \( L_c-L_g \) yields were greater than 80%. To the best of our knowledge, the cysteine perfluoroarylation platform represents the first instance where a macrocyclization scan can be carried out in a manner generally independent of the positions of the side-chain amino-acid residues.

To further highlight the generality of the developed macrocyclization platform we envisioned that it should be possible to avoid the requirement for the linker syntheses by utilizing a reverse strategy. Specifically, we envisioned incorporating pentafluorophenyl or nonafluorobiphenyl moieties directly onto unprotected cysteine-containing peptides, which then can undergo macrocyclization with commercially available dithiol reagents (Figure 2e, strategy II). To test this hypothesis, we utilized peptide 7, which was treated with excess of hexafluorobenzene and decafluorobiphenyl reagents yielding bis-perfluoroarylated cysteine peptides 7a' and 7b', respectively. 1,4-Butanedithiol and 1,4-benzenedithiol were chosen as model dithiol reagents to test for the desired macrocyclization chemistry with 7a' and 7b' (Figure 6).

As a standard protocol, 1 mM solutions containing perfluoroaryl-containing peptides 7a' and 7b' were treated with 2-fold excess of dithiol linker in the presence of 50 mM solution of TRIS base in DMF. LC-MS analysis of the reactions indicated complete consumption of the starting materials occurred within 2 hours and cyclic products were formed with yields greater than 90% in both cases (Figure 6). These experiments show the reverse strategy for macrocyclization is indeed feasible. Given that perfluorinated moieties in 7a' and 7b' are less sterically hindered than similar cysteine thiolate sites, more rapid and selective macrocyclization chemistry is observed as a result. Importantly, long peptide sequences can undergo efficient macrocyclization via this strategy, as suggested by the nearly quantitative conversion of peptide 14a' to its corresponding macrocycle when treated with 1,4-butanedithiol (see SI).

Conclusions

In summary, for the first time, we show how cysteine perfluoroarylation via \( S_{\text{N}2} \)Ar transformation enables a site-independent and convergent diversity-oriented peptide macrocyclization scan. Two complementary strategies allow for the rapid construction of side-chain functionalized peptide macrocycles and simultaneous complexity generation enabled by the highly tailorable perfluoroaryl-based linkers. Studies performed with model compounds suggest that the peptide amino-acid sequence and linker identity do not significantly diminish ones ability to form hybrid macrocycles. Given the recently observed improvements in stability, cell-uptake and target binding affinity of the peptide macrocycles stapled with perfluoroaromatic-based moieties, we envision this newly developed scan will expand the toolbox needed for the development of biologically active constrained-peptide therapeutics by allowing one to rationally design and screen for promising protein-binding agents.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Figure 1.
Convergent diversity-oriented synthetic (DOS) platform for a peptide macrocyclization scan utilizing a library of chemically tailorable bifunctional linkers. AA<sub>x</sub> refers to a specific amino acid residue in the peptide chain sequence.
Figure 2.

An unprotected peptide macrocyclization scan enabled by S_NAr transformation between thiols and activated perfluoroaromatics: (a) the reaction scheme highlighting the regioselectivity of the S_NAr process leading to preferential substitution at para position of the pentafluoroaryl moiety with respect to the R substituent; (b) the reactivity trend of perfluoroaryl-based electrophiles governed by two independent activation modes; (c) the library of bifunctional perfluoroaryl-based linkers containing thioether moieties capable of activating corresponding para-CF moiety towards nucleophilic attack via resonance stabilization of the Meisenheimer intermediate; (d) two independent strategies designed to probe macrocyclization scan with linkers L_a-L_g; and (e) 14 model cysteine-containing unprotected peptides used for the studies.
Figure 3.
LC-MS chromatograms (total ion current) of purified peptide 7 and corresponding macrocyclization reactions with linkers L_a-L_g analyzed in situ. Peaks labeled as * represent oxidized disulfide by-product.
Figure 4.
LC-MS chromatograms (total ion current) of purified peptide 14 and corresponding macrocyclization reactions with linkers L_a-L_g analyzed in situ. Peaks labeled as * represent oxidized disulfide by-product.
Figure 5.
Bar graph summary of the macrocyclization scan with peptides 1–14 and linkers $L_a$-$L_g$. Number within each bar represents corresponding yield of the macrocyclization product determined by LC-MS analysis of the unpurified product mixture. Note, for yields denoted with *, re-optimized conditions were employed (see SI).
Figure 6.
Synthesis of cysteine perfluorinated peptides 7a' and 7b', LC-MS chromatograms (total ion current) of purified peptides 7a' and 7b', and corresponding macrocyclization reactions with dithiol linkers (highlighted in grey) analyzed in situ.