Late-stage reshaping of phage-displayed libraries to macrocyclic and bicyclic landscapes using multipurpose linchpin

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Abstract

Genetically-encoded libraries (GEL) are increasingly used for discovery of ligands for 'undruggable' targets that cannot be addressed with small molecules. Foundational GEL platforms like phage-, yeast-, ribosome- and mRNA-display enabled display of libraries composed of 20 natural amino acids (20AA). Today, numerous strategies expand GEL beyond 20AA space by incorporating unnatural amino acids (UAA) and chemical post-translational modification (cPTM) to build linear, cyclic, and bicyclic peptides. The standard operating procedure for UAA and cPTM libraries starts from a "naïve" chemically-upgraded library with 10^8-10^{12} compounds, uses target of interest and rounds of selection to narrow down to a set of receptor binding hits. However, such approach uses zero knowledge of natural peptide-receptor interactions which already exists in libraries with 20AA space. There is currently no consensus whether 'zero knowledge' naïve libraries or libraries with pre-existing knowledge can offer a more effective path to discovery of molecular interactions. In this manuscript, we evaluated the feasibility of discovery of macrocyclic and bicyclic peptide from "non-zero knowledge" libraries. We approach this problem by late-stage chemical reshaping of phage-displayed landscape of 20AA binders to NS3aH1 protease. The re-shaping is performed under a novel multifunctional C_2-symmetric linchpin, 3,5-bis(bromomethyl)benzaldehyde (termed KYL), that combines two electrophiles that react with thiols and aldehyde group that reacts with N-terminal amine. KYL diversified phage-displayed peptides into bicyclic architectures and delineates 2 distinct sequence populations: (i) peptides that retained binding upon bicyclization (ii) peptides that lost binding once chemically modified. Our report provides a case study for discovering advanced, chemically-upgraded macrocycles and bicycles from libraries with pre-existing knowledge. The results imply that thousands of selection campaigns completed in 20AA space, in principle, can serve for late-stage reshaping and as a starting point for discovery of advanced peptide-derived ligands.

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Introduction

Peptides have gained increasing attention in drug discovery in the past decades due to their moderate molecular weight, inherent biocompatibility, and relatively simple synthetic access. Small molecules have successfully been employed to target well-defined binding pockets of enzymes and other so-called "druggable" targets.[1] However, extended areas on the surface of proteins, postulated to be "undruggable targets" with small molecule modulates. These targets can be addressed with modalities with large surface area like peptides, proteins and antibodies.[1] Unlike biological drugs, which cannot traverse the cell membrane efficiently, peptides can be optimized to be passively permeable,[2] and cyclization is at the core of all designs for increased cell permeability and oral availability. Cyclization of peptides also can minimize proteolytic liability,[3, 4] and decrease conformational flexibility leading to stronger association with cognate targets.[5] Genetically-encoded (GE) libraries displayed on phage[6, 7], mRNA[8, 9], bacteria[10-12] and yeast[13] as well as DNA-encoded libraries (DEL) of macrocyclic topology[14, 15] offer uniquely advantages in discovery of macrocyclic peptides because they associate the unique DNA message with structure of each individual macrocycle. The genotype-phenotype connection allows amplification from a single copy number, repetitive rounds of selection or maturation, and makes it possible to start discovery campaigns from libraries of 10^6–10^{12} scale. Ligands emanating from these campaigns often serve as a starting point for drug discovery.[16, 17] Macrocyclic libraries with unnatural amino acids (UAA) and chemically post-translational modified (cPTM) libraries offer numerous advantage over traditional GEL made from 20 natural amino acids (AA). All *de novo* UAA/cPTM discoveries today start from completely random naïve UAA/cPTM libraries of $>10^9$ compounds and they do not use any knowledge from 20 AA libraries. An open question remains: is it advantageous to always start from naïve cPTM/UAA

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libraries without prior knowledge, or is there a benefit in starting the discovery from libraries with built-in knowledge? Thus, our manuscript explored the potential of discovering chemically modified macrocycles and bicycles by modifying upon existing natural 20AA selection campaigns late stage in discovery.

Departure from *bona fide* library of random amino acid sequences and replacing them with built-in knowledge is not a new idea. Affinity maturation libraries built around previously discovered motifs are routinely used for optimization of peptide properties.[18] Encoding the pan-integrin binding RGD motif[19, 20] into phage libraries made it possible to accelerate binder discovery for specific types of integrins.[21-23] Motifs do not always have to be peptidic: In genetically-encoded fragment based discovery, any fragments that have abilities to bind to a target can be incorporated into libraries.[24-26] Another technique for constructing 'knowledge' is to introduce the population of AA sampled from a certain pool rather than completely randomization. For example, phage libraries that displayed short peptides sampled from human proteome simplified discovery of autoantibodies in humans.[27] Interestingly, libraries with different built-in knowledge can still converge on the same results. Such convergent discovery, as observed in the selections of peptides that bind to O-GlcNac transferase (OGT): the same OGT-binding motif was found in randomized mRNA-displayed and phage displayed libraries, as well as a focused proteome derived library.[28-30] Knowledge from 20 AA libraries has also been used indirectly to develop unnatural peptides. For example, Sulanemadlin-a 17 AA cyclic peptide with UAA and chemical 'stapling'- was developed from natural 12-mer peptide discovered *via* phage display[31] using a classical medicinal chemistry modification.[32] All these examples point to the merits of transferring prior knowledge from natural peptide libraries into cPTM libraries for molecular discovery. We envisioned that such knowledge transfer can be

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facilitated using chemical linchpin that can yield divergent outcomes from the same peptide sequence.

Survey of existing examples of synthetic linchpin for post translational macrocyclization of phage-displayed natural amino acid peptide libraries[33] shows that most linchpins are designed to serve a single purpose: Class I forms monocyclic peptides; Class II offers possibilities for diversification of monocycles by introducing unnatural pharmacophores;[34, 35] Class III creates bicyclic topologies.[7] Figure 1b provides examples for each type:[6, 36-40] **2**, **7** convert linear peptides to simple monocycles; **3**,**4** transform linear peptides to macrocycle with built in pharmacophores; **5**, **6**, **8** form bicycles and cannot be stopped at monocycle state. To fulfill all 3 purposes, we envisioned a multipurpose a C_2-linchpin, 3,5-bis(bromomethyl)benzaldehyde, containing two thiol reactive moieties to form monocycle, and a tuned reactive aldehyde group to engage with either exogenous nucleophiles to introduce pharmacophores or with N-terminus to yield bicycles. The engagement of N-terminus in topological transformations yields single product from low symmetry C_2-linchpin and removes potential liabilities associated with free N-terminus.[38] Aldehydes as controllable and versatile electrophiles have been used to from peptide monocycles in water,[41-43] and intact N-terminus is known to pose unique aldehyde reactivity[44] as elegantly explored as Malins[45] and Raj.[46]

3,5-bis(bromomethyl)benzaldehyde (referred as KYL) can be applied on both synthetic peptides and phage-displayed libraries (Figure 1a). Tuning of monocyclization and bicyclization in an aqueous environment is performed by simply adjusting pH and grafting of pharmacophores is controlled by concentration. We see no barriers for expanding the utility of this linchpin to any other GEL or DEL libraries that contain a pair of thiol residues and a low basicity primary amine.

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Results and Discussion

Bicyclization of Unprotected Peptides in Water

Our design aims to form a divergent precursor for late-stage reshaping of readily available phage-displayed libraries that contain two cysteines to yield 3 types of macrocycles. We evaluated downstream reactivity of bis(bromomethyl)benzaldehyde (KYL) on model peptide SAKGGRCYEDC which incorporates a lysine (Lys) residue in close proximity to the N-terminus, along with residues with nucleophilic side chains (Asp, Glu, Arg, Tyr) (Figure 2a). N-terminal Serine was selected because many phage-displayed libraries produced in our lab contain N-terminal Serine, and a peptide homologue with N-terminal residue Alanine (AAKGGRCYEDC) was evaluated subsequently. As bromine and chlorine both have been employed as leaving groups in such reactions,[6, 37] we tested both linkers (2.1 mM) on model peptide SAKGGRCYEDC (0.7 mM) in Tris-base buffer (TB) pH 8.6 (Figure 2a). We observed a half-conversion time $t_{1/2}$ of 40 min for Cl leaving group and 1 min for Br leaving group (Figure 2b, Figure S2–3). The extended reaction time might be less optimal for modification on phage libraries; hence, we selected Br as a leaving group for further optimizations and subsequent applications. The product of the reaction-a monocyclic peptide with aldehyde can be purified and stored as lyophilized powder[45, 46] and is stable in acidic stocked solutions. Departure from those conditions leads to intermolecular reactivity of aldehyde and N-terminus.

Two types of intramolecular bicyclization reactions observed between peptide aldehyde and N-terminus of the same peptide have been explored in the literature. We first tested whether grafted aldehyde can undergo intramolecular reaction with N-terminal amine followed by $NaCNBH_3$ trapping in conditions based on reports from Malins and co-workers (NaOAC, pH = 5.5) (Figure 2c).[45] After buffer scouting (Figure S8–12) and optimization, we found that mild

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acidic condition (0.1 M NH_4OAc buffer, pH 4.6) gave highest reaction rate (0.16 $M^{-1}s^{-1}$). In this buffer, 1 mM solution of peptide gave rise to bicyclic product with 62 % isolated yield after 2 hours (Figure 2c, 2d, S4). NMR confirmed the structure of the bicycle formed via reductive amination (Figure S5 – 7) and we refer to it as Malins bicycle. The second type of intramolecular reaction of N-terminus and aldehyde engaged 5-endo-trig reaction trapping of the imine intermediate with endogenous amide nucleophile as reported by Raj and coworkers.[46-48] We observed the evidence of Raj-style cyclization in neutral-basic conditions (pH 8.5), NMR of peptide aldehyde derived from SAKGGRCYEDC uncovered a dynamic equilibrium between 4 species, the peptide-aldehyde, the imine intermediate, and a progressive appearance of two new compounds (Figure 2e, 2f). NMR analysis of the isolated major product proved that it was analogous to compounds reported by Raj and coworkers (Figure S15).[46] N-terminal Serine favored formation of R-isomer in 7:1 ratio (Figure 2f, Figure S14), whereas a similar peptide with N-terminal Alanine AAKGGRCYEDC in the same reaction yielded R: S = 2 :1 (Figure S20), which aligns with the report that the stereoselectivity of this reaction depends on N-terminal composition.[46]

'Raj-bicycles' were stable at neutral-basic condition (pH > 6.5) at under -20 °C (Figure S17), but under mildly acidic condition (pH <5) they slowly reverted to the peptide-aldehyde (Figure S18). At room temperature and neutral basic condition, isolated isomers of Raj bicycles formed a dynamic equilibrium between the isomers and peptide aldehyde. Both purified isomer solutions reached R:S ratio of 0.9:1 after 4 days (Figure S21, 22). This observation was consistent with the DFT calculations from Raj and coworkers that the R-isomer is more kinetically favored as the S-isomer is more thermodynamically favored.[48] Malins bicycles were stable for several weeks in aqueous buffer (Figure S16).

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Monocyclic Late-Stage Modification of Aldehyde-peptide with Diverse Pharmacophores

We then tested whether the intramolecular reaction can be intercepted by the exogenous amine introduced during the reductive amination. Aniline derivative (pKa around 5), aliphatic amine (pKa around 11) and N-terminus of glycine amine with pKa around 7.7[49] exhibited contrasting reactivity when interacting with the aldehyde-peptide. Mixing 1 mM aniline derivative and 0.8 mM of aldehyde-peptide at pH 4.6, 70% of product was monocyclic product. At 10 mM, the aniline derivative converted all the aldehyde-peptide to desired product (Figure S24). Glycine derivative at 1 mM and 10 mM yielded 12 % and 16 % monocyclic product (Figure S25); The reaction was undetectable for aliphatic amine at 1 mM, but at 10 mM, 35 % of product was desired monocycle (Figure S23). Outcomes are expected based on the protonation these amines at pH 4.6. The observation shows that the reaction of less reactive amines can still be driven forward by increasing their concentration. These findings provided valuable insights for the future design of phage display library and its modification by pharmacophores that contain amines.

Optimization of Reactions on Phage-Displayed Libraries

Following the validation of KYL linchpin on synthetic peptides, we accessed its capability to modify phage displayed peptides. NMR spectroscopy cannot be applied to phage displayed peptides, thus, we monitored KYL modification using previously reported ESI-MS[39, 50] and MALDI method.[51, 52] ESI-MS Spectra of modified peptides cleaved from pIII clone were inconclusive (Figure S28), but modifications were evidenced on pVIII-displayed peptide in MALDI. A pVIII clone, SWCRPATVNC, was first reduced by TCEP, then treated by 0.1 mM of KYL for 30 min in TB buffer, pH 8.6. Formation of aldehyde was observed by 131 mass increase (Figure S26b) and further confirmed by incubating the phage with TAMRA hydrazine,

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leading to the formation of corresponding hydrazone (+610 observed increase, +615 theoretical increase) (Figure S26c). The aldehyde-installed phage clone was then treated with 10 mM $NaCNBH_3$ overnight[39] in NH_4OAc buffer, pH 4.6 for Malins bicyclization. Reaction resulted in a minor decrease in mass (-11 observed decrease, -16 theoretical decrease), and most importantly the product no longer responded to TAMRA hydrazine (Figure S26c). Combined observations confirmed the consumption of aldehyde and formation of Malins bicycle.

Though MALDI successfully monitored the modifications with KYL on one phage displayed peptide, we reported previously that the reactivity in phage libraries could change from clone to clone by factor of 400,[53] hence, it is crucial to measure the modification on the phage libraries in addition to the assessment of the reaction on individual clones. We measured chemical modification of libraries using previously reported biotinylation procedure[36, 38, 54] followed by streptavidin capture to distinguish modified from unmodified phages. We tested different reaction conditions using the library SX_3CX_5C (X = 19 AA without Cysteine, 10^{10} random peptides), into this library we mixed wild-type (WT) phage as negative control[55] (Figure 3a). Library clones transduced LacZa reporter and form blue plaques in the presence of IPTG/X-gal, whereas WT phage lacking the reporter produces white plaques in these conditions. This blue/white assay monitored non-specific modification on phage coat proteins. Phage library and WT mixture were reduced by TCEP, then reacted with 0.01 mM, 0.1 mM, 0.2 mM and 1 mM of KYL for 30 min in TB buffer, pH 8.6. To quantify the modification by KYL, phages were then treated with an aldehyde reactive aminooxybiotin (AOB) (Figure 3c),[54] allowing the aldehyde containing phages to be pulled down by streptavidin magnetic beads (Figure 3b). We observed that high concentration of linchpin (1 mM) resulted in phage death and non-specific modification (Figure 3e, S27h) and similar detrimental effects have been previous reported by Heinis and co-

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workers.[7] Overall, 0.1 mM of KYL was found to be optimal to balance between low toxicity to phage, low non-specific modification, and high modification rate (Figure 3g). Furthermore, to measure the conversion of aldehyde-peptide to Malins bicycle, AOB was applied again after bicyclization step to detect the remaining unreacted aldehyde-phages. No biotin capture was observed, indicating all 78 % aldehyde-phages were consumed (Figure 4c-e).

We also employed biotin capture to test the intramolecular reaction of KYL modified phage libraries with exogenous amines. We treated the aldehyde-monocyclic libraries with 10 mM $NaCNBH_3$ and different concentrations of three biotin containing amines (Figure 4g) in NH_4OAc buffer (pH 4.6, 5.6) overnight. Modified libraries were then captured by streptavidin-coated magnetic beads (Figure 4f). The capture results (Figure 4h) showed trend similar to the reactions on a synthetic peptide (Figure S23–25): The aniline derivative exhibited 40 times greater reactivity than the aliphatic amine and glycine derivative, and the reaction can be driven forward by increasing the amine concentration. 2 mM concentration of aniline derivative converted all KYL modified phage library to monocyclic product, whereas for aliphatic amine and glycine derivative, same results were achieved by 10 mM concentration.

In conclusion, both MALDI and biotin capture results proved the versatility of KYL linchpin to form monocyclic, bicyclic and pharmacophore bearing macrocyclic architectures on phage, affirming its suitability for late-stage reshaping of phage-displayed libraries.

Selection of Late stage Reshaped Phage Displayed Libraries against NS3 Variant

As a target for selection, we employed hepatitis C virus protease (HCVp) NS3a. Globular protein NS3a that binds to helical peptide inhibitors; [56] small molecules like grazoprevir are known to compete with this class of helical peptides.[57, 58] This distinctive characteristic makes NS3a an attractive target for peptide ligand discovery, allowing the use of potent small molecule

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inhibitors to displace peptide binders at specific binding sites. To test this hypothesis, we conducted a selection campaign starting from previously published 10^9 linear X15 peptides displayed on phage,[28] using inactive, non-membrane interactive NS3a variant NS3aH1[58] in NS3a buffer (20 mM Tris, 300 mM NaCl, pH 8.0, 1 mM DTT) as a bait. Elution by grazoprevir was used as a driving force for the selection (Figure S29). From the panning results, we observed robust selection indicators: 240,000-fold inflection of phage recovery in round 3 compared to round 2; 850-fold stronger enrichment towards NS3aH1 than blank streptavidin beads control; a prominent motif, HXDMT observed in 1608 out of 3000 peptides that passed Differential Enrichment (DE) analysis of Next Generation Sequencing (NGS). The same motif was present in 34 out of 50 most abundantly observed peptides in Round 3 (Figure S29f, Supplementary Table S4). Furthermore, BLI assay confirmed that a HXDMT containing synthetic peptide TQMYYHEDMTLNYQR exhibited binding to NS3aH1 with K_d = 51.6 nM (Figure S30), and the binding was inhibited by grazoprevir (Figure S31). Given the promising results, we proceeded with NS3aH1 as a selection target and grazoprevir as an elution agent for more in-depth exploration of the late- stage reshaping of phage libraries.

We started from 4 naïve billion scale libraries with different sizes of disulfide constrained rings ($SCX_{12}C$, $SXCX_{10}CX$, $SX_2CX_8CX_2$, SX_3CX_9C) and pre-selected each library for 3 rounds using NS3aH1 binding and grazoprevir elution (Figure 5a, Figure S33). We considered late-stage re-shaping to be practical at the point of the selection when the diversity decreased from billion scale to a diversity manageable within the sequencing depth of NGS ($< 10^6$ peptides). We also observed that diversity collapsed $< 10^5$ peptides the output of Round 3. Therefore, we decided to return to input of Round 3 and performed reshaping of the library to re-run Round 3 of selection. The 4 pre-selected libraries were then mixed, modified by KYL to afford Malins-style bicycles

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and Raj-style bicycles, selected on NS3aH1, and sequences eluted by grazoprevir were analyzed by NGS. As a control, selection was also performed using mixed libraries without any modification (Figure 5a, b, Figure S32). NGS analysis of reshaped library panning revealed two distinct landscapes: The first population showed enrichment in unmodified libraries but they 'dropped out' and exhibited less or no enrichment in the selection of reshaped libraries, suggesting no tolerance to chemical modifications (Figure 5d, Tables S6); the second population remained the enrichment after reshaping, and over 80% sequences beared the HXDMT motif, indicating its endurance to changes in peptide geometry caused by chemical modifications (Table S5). Further analysis on 4 disulfide ring architectures revealed that, in top 50 SX_3CX_9C type peptides, 30/50 ‘dropped out’ after bicyclization with KYL linker and 20/50 tolerated reshaping to bicyclic peptides (Table S8, Figure 35b), whereas in top 50 $SCX_{12}C$ type peptides, only 12/50 ‘dropped out’ after modification and 38/50 tolerated reshaping to bicyclic peptides (Table S7). The preference of SX_3CX_9C structures to ‘drop out’ upon bicyclization suggested that apart from the motif, the tolerance to modification also depends on the ring size.

BLI measurements validated the ‘drop out’ and ‘non-drop out’ observations from NGS analysis. Two ‘drop out’ peptides predicted by NGS, SDLQCMDWDEAWPWC and SDFTCSGWGTGWHMC, had K_d = 25.4 nM and K_d =1.9 μM, and both peptides lost their binding affinity after Malins bicyclization. Meanwhile, a ‘non-drop out’ and HXDMT motif containing sequence SQVHCYHGDMTMPIC showed K_d = 21.0 nM before modification and K_d = 29.8 nM after Malins bicyclization (Figure S36). Both forms of this peptide can be inhibited by grazoprevir (Figure S37). In general, the combination of BLI and NGS analysis suggested that peptides with HXDMT motif have capacity to be reshaped into multiple forms while binding to NS3aH1 target.

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Conclusion

In our development of new or improved molecular discovery technologies we aim to achieve two goals: Make the technology available to the widest array of practitioners and access the most diverse chemical space. Our manuscript shows that chemical modification of 10^5 preselected phage displayed peptides makes it possible to discover chemically upgraded macro-bicyclic peptides using a simple pulldown and NGS (2 days of hands-on work). High throughput screening (HTS) is a commonly utilized approach for molecular discovery, but wide utility of HTS is limited by cost of liquid-handling robotics that can handle $>10^5$ molecules, and the requirement for the state-of-the-art expertise in building the chemical libraries. Libraries of $>10^5$ compounds deconvoluted by mass-spectrometry (MS) are attractive alternatives to microwell-HTS,[59] but their widespread adoption is hampered by the staggering cost of MS equipment. Meanwhile, peptide-derived, GE libraries of 10^5–10^{10} scale are trivially accessible *via* bio-synthetic production, which originates from low-cost DNA libraries. Requiring no expensive robotics nor MS equipment, GE libraries are deconvoluted with the NGS instruments. The growth in affordability and availability of NGS exceeds Moore's law, and a $100 price tag for sequencing of 10^5 DNA molecules makes a GE screen a "commodity". The open challenge becomes whether those commodity peptide-based GE libraries can be upgraded to give rise to value-added hits that contain chemical functionalities and architectural elements not presented in traditional natural peptides.

With the goal of upgrading the diversity of chemical space, in this manuscript, we explored the possibility of late-stage reshaping of pre-discovered "natural" peptides into high-value monocyclic and bicyclic structures using synthetic linchpin. We developed the versatile C_2-symmetric linchpin KYL with an aldehyde and two thiol-reactive groups, which makes it

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possible to produce monocyclic and bicyclic structures as well macrocycles that bear exogenous pharmacophores. Though C_{3v} symmetric cross-linkers have been ubiquitously applied in phage-[60] and mRNA-displayed libraries,[61] their high symmetry makes it difficult to further expand the diversity (e.g., introduce exogenous pharmacophores).[62] Pharmacophores can be trivially introduced into lower symmetry C_2 linchpins, allowing the possibility to combine cyclization and modification. However, C_2 linchpins are problematic in forming bicycles: Heinis and coworkers[7] employed C_2 linchpin to cross-link 4 cysteine residues in phage displayed peptides to yield bicyclic peptides, but the challenge in such approach is controlling regioselectivity.[61] In contrast, C_2-symmetric KYL and analogous linchpins,[38] which act on cysteine side chains and N-terminus, give rise to single regioisomer of bicycles.

Late-stage reshaping study in this paper was conducted on HCV protease variant NS3aH1 using pre-selected unmodified disulfide libraries. NGS analysis of the binding of the reshaped libraries to NS3aH1 unveiled two distinct landscapes: some peptides lost NS3aH1 binding upon bicyclization ('drop-out' peptides), while the others retained NS3aH1 binding after bicyclization ('developable' peptides). Late-stage reshaping narrows on the diversity of chemical space to maximize the likelihood of discovering advanced peptide-derived ligands, but the outcome of such an approach is not obvious *a priori*. For example, the ratio of 'drop out' and 'developable' population was strongly dependent on the architecture of the library (the placement of cysteine within the randomized segments). In conclusion, the example using KYL for late-stage reshaping suggested that thousands of previously discovered unmodified peptide binders can fuel discovery campaigns that aim to find chemically modified bicycles.

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Associated Content

Supporting information

Supplemental Figures S1-S38, Supplemental Tables S1-S10: Detailed synthetic methods, biochemical methods describing the synthesis and selection of phage libraries, biolayer interferometry assay, data processing methods describing the analysis of the DNA sequencing data, statistical methods.
Supplemental Figures S1.1-S1.14, S2.1-2.12: Detailed NMR spectra of of KYL modified SAKGGRCYEDC macrocycles, purification methods and characterizations of peptide macrocycles.
Source data: submitted as "Supplementary_data.rar" contain files describing (i) "Kinetics Matlab" directory with raw data used to monitor the kinetics of reactions and MatLab scripts for curve fit, (ii) " panning_sequencing_data" directory with *.txt files describing the raw deep-sequencing data; *.csv tables describing sequencing data after re-organization; *.xlsx tables describing sequencing data sorted in different libraries; *.pdf files describing the differential enrichment (DE) analysis, output of differential enrichment analysis and clustering; Cleaning_deep_seq_data.ipynb as Python script used for sequencing data processing. (iii) "MALDI" directory with MatLab scripts for MALDI data processing and raw MALDI data. (iv) "BLI" directory with raw BLI data and subtracted BLI data files.
Data Availability: All raw deep-sequencing data is publicly available on https://48hd.cloud/ with data- specific URL listed in Supplementary Table S2 and S3. MatLab and Python scripts used for analysis of deep-seq data have been deposited to https://github.com/derdalab/KYL.

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Contributions

K.Y. performed synthesis of chemical and biochemical reagents, peptide modification, phage modification, selection, data analysis and BLI assay. M.M. and K.Y. performed NMR studies and analysis. F.B.M. performed cloning and expression of proteins. C.P. and A.E. performed mass spec for phage clones and analysis. K.Y., R.D. wrote the manuscript with editing from all authors.

Notes

The authors declare the following competing financial interest(s): R.D. is the C.F.O. and a shareholder of 48Hour Discovery Inc., the company that provided instrument for BLI binding assay.

Data Availability: All raw deep-sequencing data is publicly available on https://48hd.cloud/ with data- specific URL listed in Supplementary Table S2 and S3. MatLab, Python, and R scripts used for analysis of deep-seq data have been deposited to https://github.com/derdalab/KYL.

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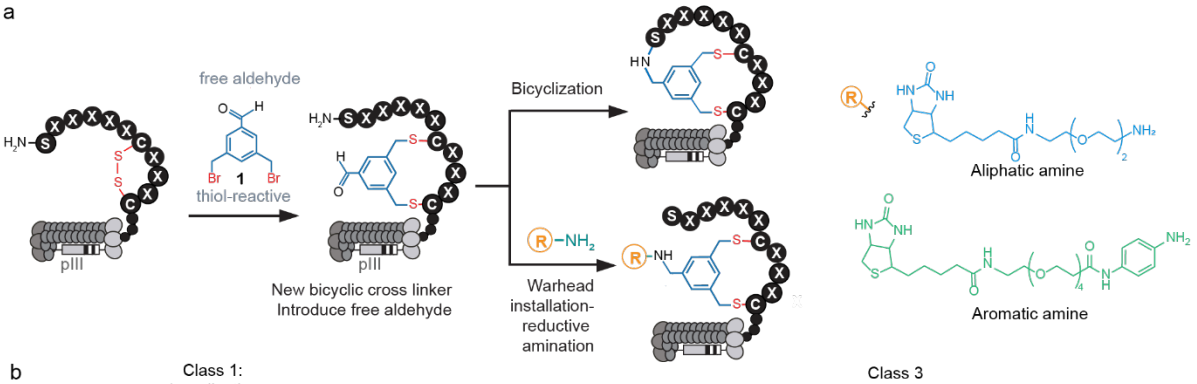

b

	Class 1: 1 application							Class 3 3 applications
	2	3	4	5	6	7	8	1
Macrocyclization	✓	✓	✓	✓	✓	✓	✓	✓
Bicyclization*	×	×	×	✓	✓	×	✓	✓
Warhead installation on monocycle	×	✓	✓	×	×	✓	×	✓

*: Only with natural amino acids. Under certern conditions, bicyclization can happen (Figure S1).
#: R is traditional leaving group like N-succinimidyl group

Figure 1. a) Versatility of aldehyde linker in peptide modification. b) Bicyclization can still happen for 3, 4 under certain circumstances (Figure S1).

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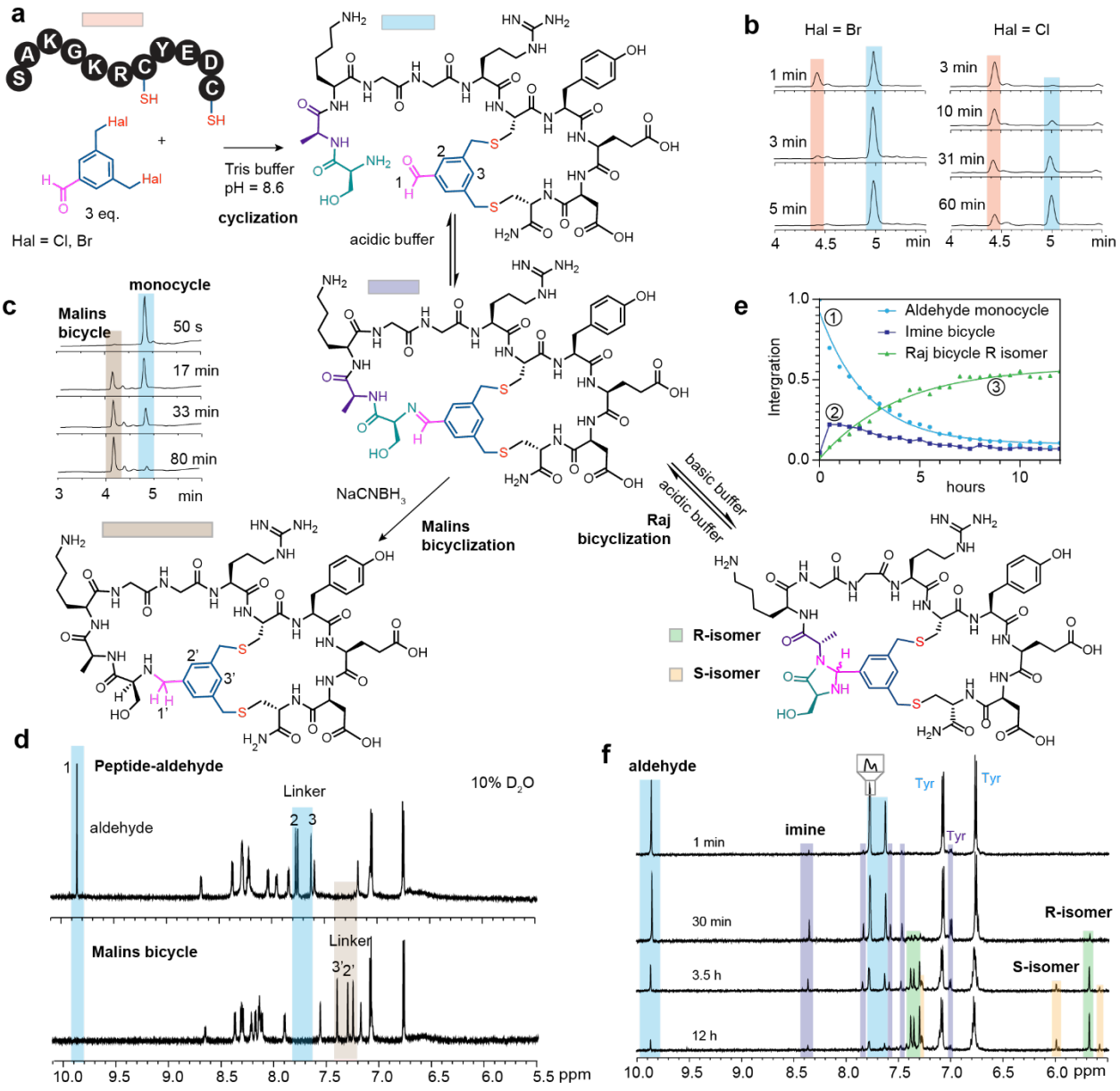

Figure 2. a) Reactions of aldehyde linker modified peptide b) Kinetics of linear peptide reacts with KYL-Br and KYL-Cl c) Kinetics of Malins bicyclization d) Proton NMR comparison of peptide-aldehyde and Malins bicycle e) NMR integration change of aldehyde monocycle, imine bicycle and Raj bicycle. Only R-configuration was shown in e to simplify the plot, comparison of R and S configurations is shown in Figure S12 f) Proton NMR of Raj bicyclization in 1min, 30min, 3.5 h and 12 h.

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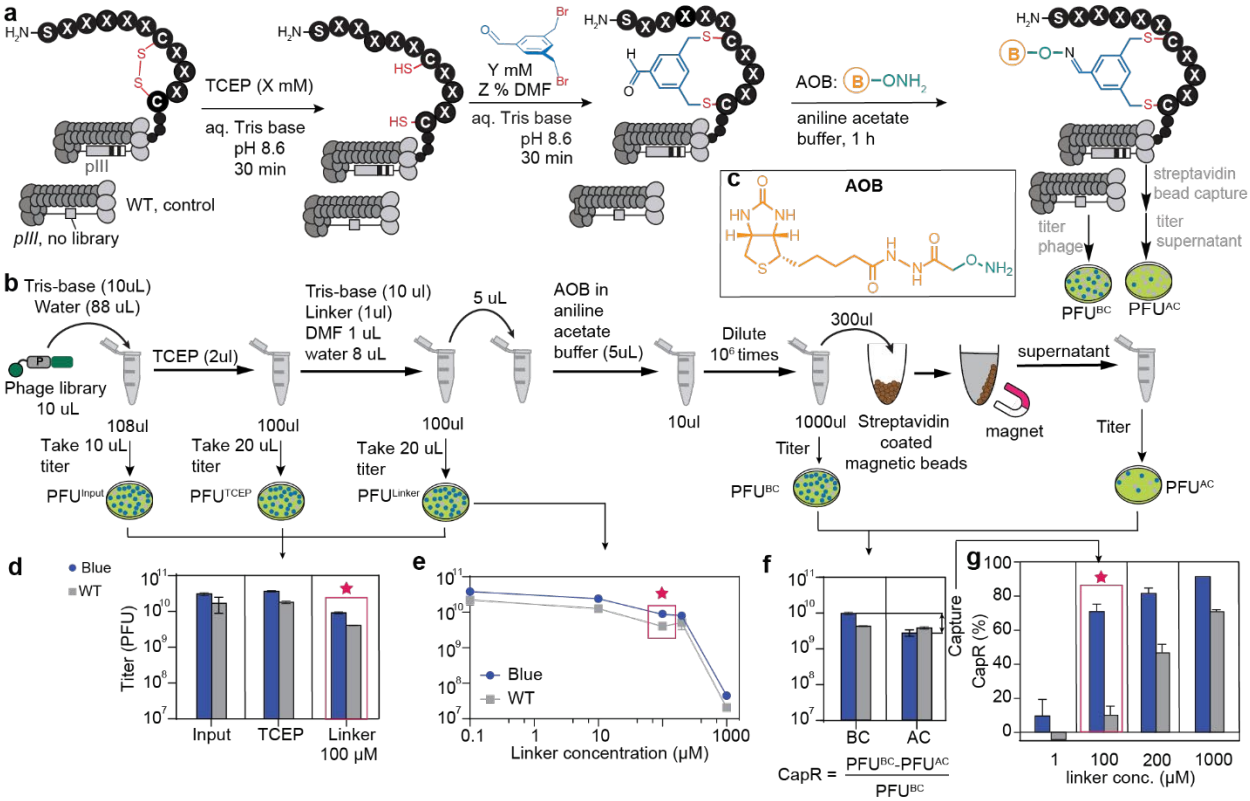

Figure 3. Phage library survival against KYL linchpin.

a-b) Standard process of S5C3C library modification. c) Structure of aminooxybiotin (AOB). d) Titer of input, after TCEP and after KYL (100 μM). TCEP was not toxic, while KYL was toxic to both WT phage and libraries. e) Titer of library and WT phage with different linker concentrations. When concentration was higher than 100 μM, phage number significantly decreased and WT mirrors the library. f) Measurement of capture rate by titer of before and after capture when using 100 μM linker. g) Comparison of blue and white phage capture rate with different linker concentration.

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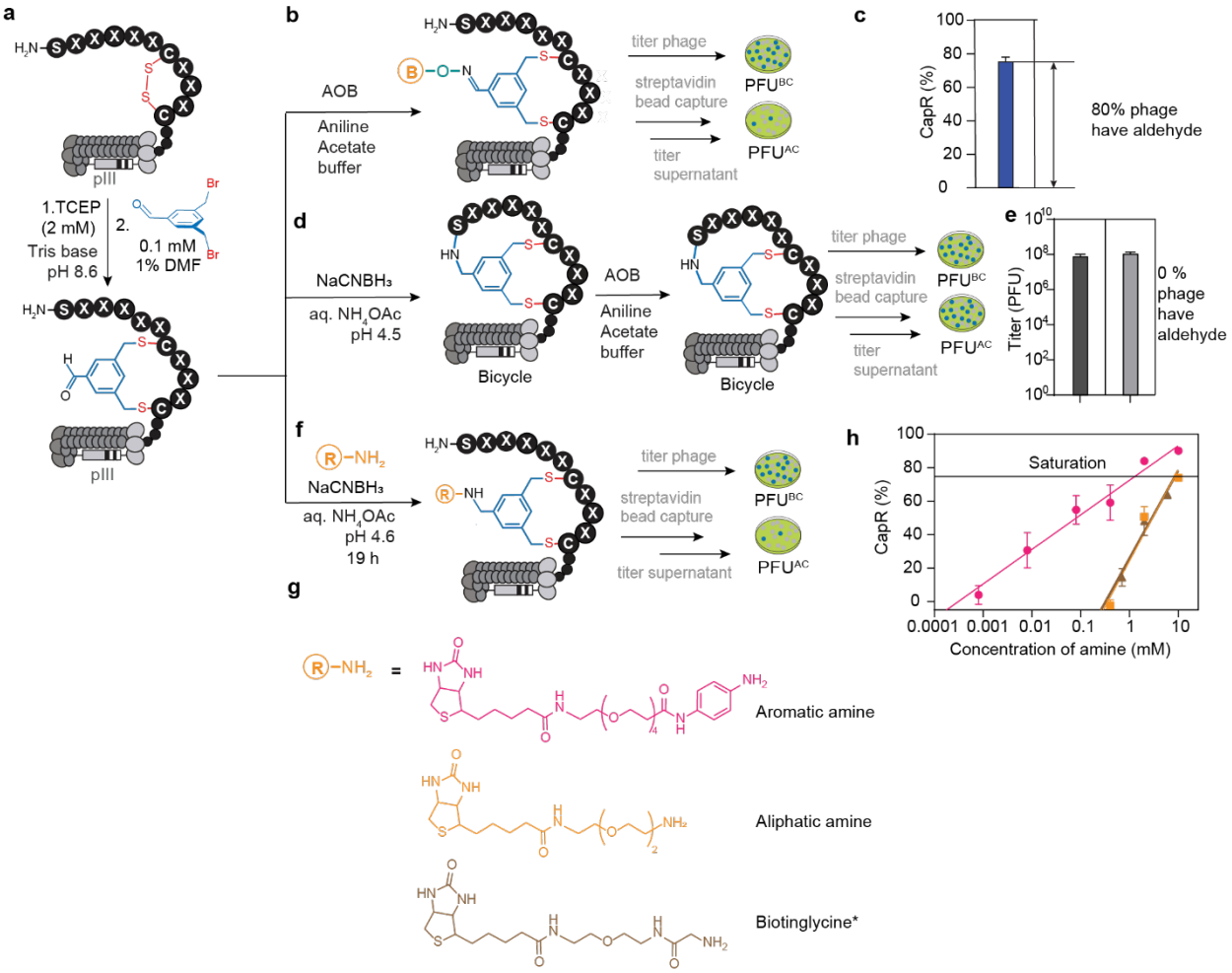

Figure 4. Reactions of linchpin installed phage libraries. a) Installation of *KYL* linchpin onto phage library. b) Phage libraries react with oxime c) Phage libraries react with endogenous N-terminal amine d) Phage libraries react with exogenous amines g) Different types of exogenous amine, aromatic amine, aliphatic amine and biotinglycine. *the reaction of biotinglycine was performed at pH = 5.6 h) Modification rate of exogenous amines under different concentrations.

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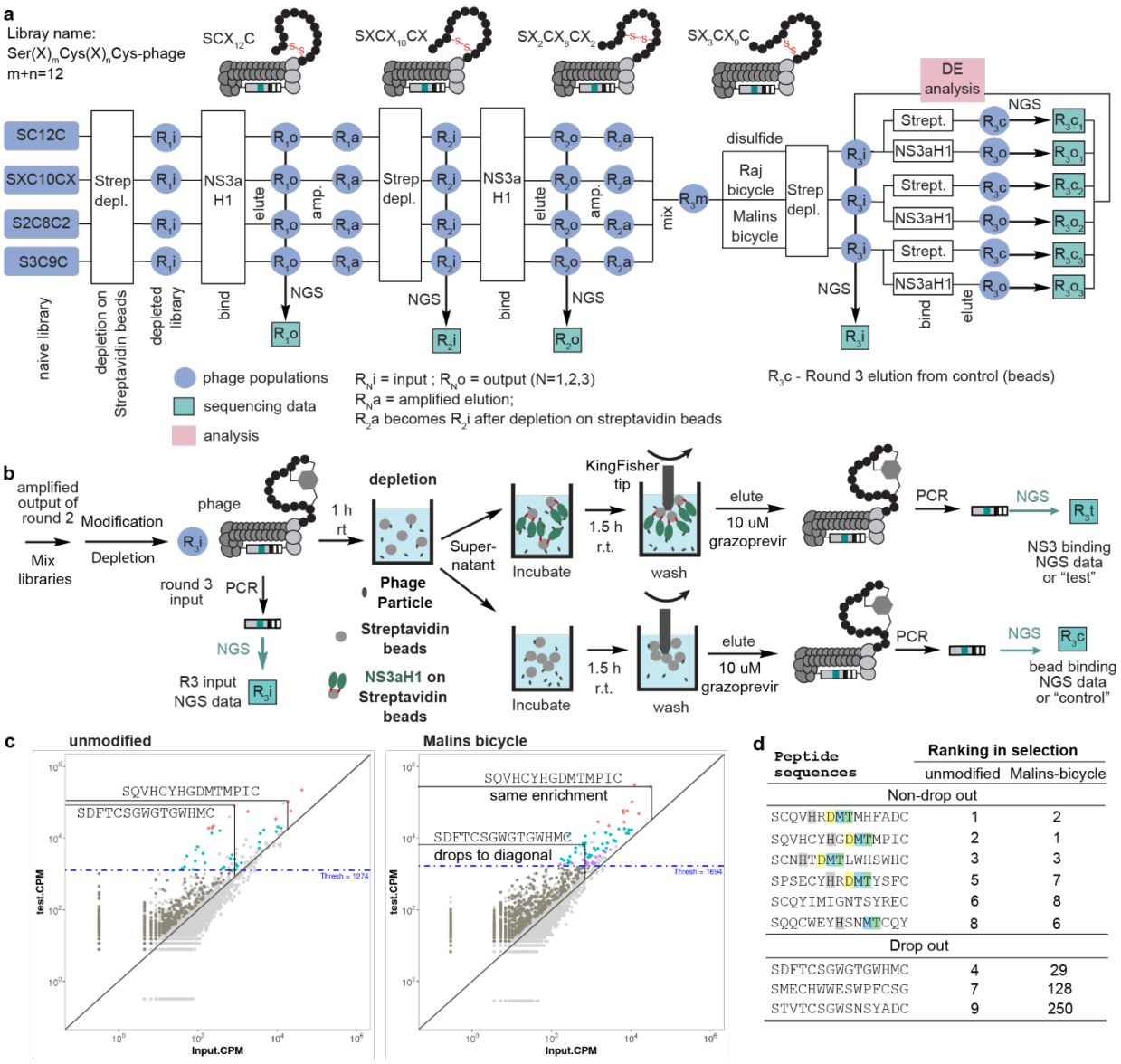

Figure 5. Selection against NS3aH1 with late-staged reshaped phage libraries a) 3 rounds panning flowchart for late stage reshaping b) Panning procedure for refitted libraries c) Scatter-plot for unmodified and Malins bicycle reshaped library, each dot means one peptide sequence. For non-drop out peptide like SQVHCYHGDMTMPIC, the enrichment didn't change after reshaping. For drop out peptide like SDFTCSGWGTGWHMC, the enrichment dropped after reshaping, which makes the peptide move more towards the diagonal line. d) Top 10 selected peptide sequences from unmodified selection and their ranking in Malins bicycle reshaped selection.

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