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Quantitative Synthesis of Genetically Encoded Glycopeptide Libraries Displayed on M13 Phage

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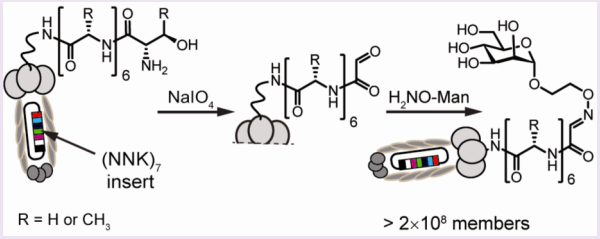
Supporting Information

ABSTRACT: Phage display is a powerful technology that enables the discovery of peptide ligands for many targets. Chemical modification of phage libraries have allowed the identification of ligands with properties not encountered in natural polypeptides. In this report, we demonstrated the synthesis of 2×10^8 genetically encoded glycopeptides from a commercially available phage-displayed peptide library (Ph.D.-7) in a two-step, one-pot reaction in <1.5 h. Unlike previous reports, we bypassed genetic engineering of phage. The glycan moiety was introduced *via* an oxime ligation following oxidation of an *N*-terminal Ser/Thr; these residues are present in the peptide libraries at 20−30% abundance. The construction of libraries was facilitated by simple characterization, which directly assessed the yield and regioselectivity of chemical reactions performed on phage. This quantification method also allowed facile yield determination of reactions in 10^9 distinct molecules. We envision that the methodology described herein will find broad application in the synthesis of custom chemically modified phage libraries.



Biological encoding of information allows handling libraries of large diversity. Unlike chemically synthesized libraries, libraries of peptides encoded by DNA or RNA can be amplified from a single copy and optimized using directed evolution. Phage display is the most common strategy for the discovery of functional peptides from genetically encoded libraries.[1,2] The technology has been the source of numerous FDA-approved drugs and drug-candidates in clinical trials.[3] Expanding the use of phage to display and encode molecules other than natural polypeptides makes it possible to select and evolve molecules with properties not found in peptides.[4−7] Chemical modification of phage-displayed peptide libraries is one of the simplest approaches to encode non-natural moieties.[7,8] The identity of the products could be deciphered from phage DNA that encodes the starting material (peptide), only if the modification is regioselective and quantitative.[9,10] Such an approach to building diversity is ubiquitous in nature: organisms from all kingdoms of life use post-translational modifications to diversify the structure of genetically encoded polypeptide libraries.[11] Several reports have highlighted the power of chemically modified phage-displayed libraries.[7,8] The broad utility of this approach, however, is plagued by a lack of techniques that can be used to characterize chemical modifications on mixtures of 10^9 phage-displayed peptides.[10] In this report, we developed a characterization technique that addresses this deficiency. To demonstrate its utility, we perform quantitative and regioselective modification of commercially available phage-display libraries, in as little as 1.5 h, to generate a library of 2×10^8 glycosylated peptides; each of them is attached to an information carrier. Furthermore, chemical modification had minimal interference on phage infectivity; modified libraries, thus, could be amplified and applied to affinity selection just like any other phage-displayed library.

For the generation of chemically modified libraries, M13 phage display has several advantages over the display of peptides on yeast cells[12] or "naked" DNA/RNA.[13,14] The chemical composition of M13 proteins is simpler than that of a cell surface, and the growth of M13 phage libraries in *E. coli* culture is simpler than the generation of DNA/RNA-displayed libraries. Additionally, several M13 phage-displayed libraries are available from commercial vendors. Previous attempts to generate chemically modified libraries used engineered M13 phage that contained unnatural amino acids (UAA) such as selenocysteine[15,16] and *p*-azidophenylalanine.[17] Thiol handles were also used to introduce modification in Cys-free M13 phage.[7] Unfortunately, neither UAA-containing libraries nor libraries on Cys-free M13 are commercially available. These libraries require significant genetic engineering of phage, which compromises their growth rates; the decrease in amplification rate, in turn, can be detrimental for the selection process.[18] We bypassed the complexity associated with "unnatural" M13 phage and built libraries starting from commercially available libraries, such as Ph.D.-7, a library of ~1×10^9 random heptapeptides displayed on M13KE phage. Our strategy employs *N*-terminal Ser and Thr residues, which upon oxidative cleavage by $NaIO_4$ yield bio-orthogonal aldehyde handles.[19] These *N*-terminal amino acids are absent from native M13

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proteins but can be found at 20−30% abundance in random peptide libraries.[20]

Modification of phage-displayed peptide libraries is a reaction that is performed on 10^9 molecules simultaneously. Because the pIII protein is displayed on phage at a low copy number, each molecule is present in small amounts. MALDI and other MS techniques, which are often used to characterize chemical modification on viral coat proteins present in high copy number,[21] fail to detect pIII, which constitutes less than 1% (w/w) of M13 virion (Supplementary Figure S1). Heinis and co-workers used MALDI to monitor reactions on recombinant purified pIII subunit,[7] but the efficiency of the reaction cannot be validated in the context of intact M13-virion or an entire library of 10^9 peptides. To date, the only characterization methods that could assess reactions on phage, or libraries of phage, was reported in a patent by Winter and co-workers. The authors used Western blot and fluorescent densitometry analysis of pIII, isolated from M13 virion using SDS-PAGE.[22] We found this technique difficult to adapt because it consumed over 10^{11} particles of phage, required complete removal of labeling reagents, and in our hands was not reproducible (Supplementary Figure S2). Most importantly, neither MS- nor SDS-PAGE-based methods can allow determination of yield with sufficient accuracy.[22]

To quantify reactions on clonal phage and libraries of phage, we developed a straightforward and sensitive assay, which capitalized on the ability of individual phage to generate plaques in agar overlays.[23,24] We distinguished individually modified and non-modified phage particles using a capture reagent, here aminooxy-biotin (AOB), which underwent covalent ligation with aldehydes displayed on phage particles. Upon incubation with streptavidin (SA)-coated magnetic beads, biotinylated clones were captured. The remaining non-biotinylated clones were quantified as plaque forming units (PFUs). The ratio of PFUs before and after capture thus quantified the yield of the modification (Figure 1). We have validated the assay and demonstrated that non-specific binding of phage to the beads was negligible (Supplementary Figure S3). We routinely captured and quantified phage particles present at 10^5 PFU mL^{-1} (~200 aM). This assay was reliable for detection of phage with concentration as low as 10^3 PFU mL^{-1} (Supplementary Figure S3).

To prove that reactions occurred only at the desired location on the phage, we performed all reactions in a mixture of two phage clones: (i) phage displaying a 16-residue peptide on pIII protein with Ser at its *N*-terminus (SVEK phage) and (ii) wild-type phage (WT phage) displaying an *N*-terminal Ala on pIII that lacks the extra 16-residue peptide but is otherwise identical in amino acid composition to SVEK phage. The SVEK phage carries a *lacZ* reporter gene and forms blue plaque in a bacterial lawn on an IPTG/Xgal plate, whereas WT phage forms clear plaque under these conditions. Simple "blue/white" screen[25] thus quantified the regioselectivity of the reaction. Reaction of SVEK phage with AOB was undetectable in the absence of oxidation but occurred after $NaIO_4$ treatment (Figure 1b). When a mixture of SVEK and WT phage were both exposed to $NaIO_4$ and AOB, WT phage was not modified to any appreciable level (Figures 1 and 2). These results unambiguously showed that only phage bearing *N*-terminal Ser can react with $NaIO_4$ and participate in the two-step reaction. The availability of rapid and simple quantification made it possible to screen seven orthogonal parameters to maximize the yield of the reaction and the viability of phage. These parameters

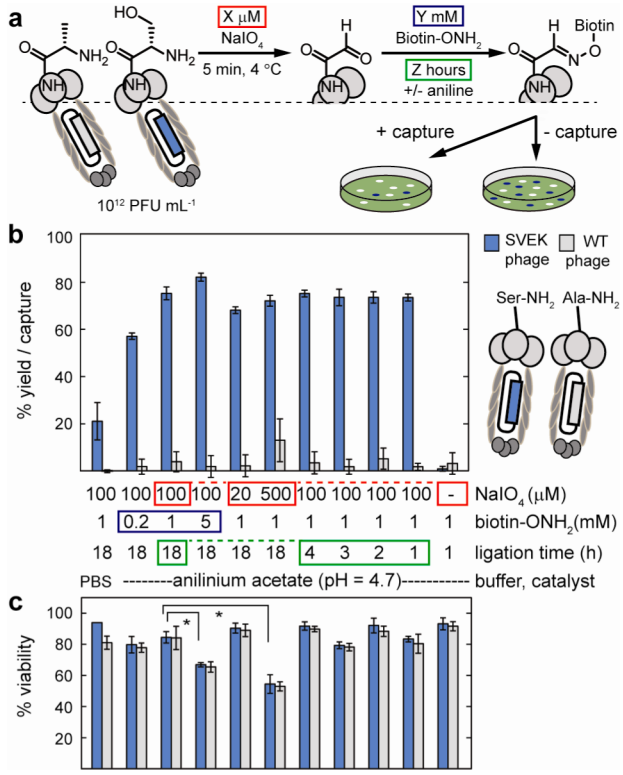

Figure 1. Profiling of the optimal conditions for oxidation and oxime ligation. (a) Scheme of reactions and quantification of biotinylated phage with plaque-forming assay. Oxidation was quenched by 1 mM *N*-acetyl-DL-methionine. (b,c) Effects of $NaIO_4$ and AOB concentration, duration of ligation, pH, and catalyst have on the yields of capture (b) and phage viability (c). Data is an average of the three biotin-capture assays; error bars are one SD (*denotes $p < 0.05$, as determined by two-tail unequal variance Student test).

included the concentration of $NaIO_4$ and aminooxy-biotin (AOB), the duration of oxidation and ligation, and the role of pH, catalyst, and concentration of phage (Figures 1 and 2). The use of aniline as a catalyst[26,27] and performing the reaction in acidic buffer (pH 4.7) greatly enhanced the rate of oxime ligation and led to 74% capture in 1 h. In contrast, less than 30% of the phage was modified even after 18 h of incubation in neutral buffer (pH 7.4). Increasing the concentration of $NaIO_4$ beyond 100 μM was detrimental to phage viability (Figure 1c). AOB concentration of 1 mM was optimal; higher concentration resulted in marginal increase in reaction yield and significant decrease in viability (Figure 1c). The use of an appropriate quencher was critical to ensure that unreacted $NaIO_4$ did not interfere with oxime formation. Thiol-based quenchers improved the capture by >25% when compared to sulfide quencher, such as *N*-acetyl-DL-methionine (Supplementary Figure S4). In most reactions, we used glutathione (GSH) as the quencher due to its minimal interference with phage viability. The yield of capture increased with increasing concentration of oxidant or reaction time (Figure 2b); however, increasing both parameters had negative effect on phage viability. Taking all the above factors into consideration, we arrived at an optimal condition that maximized both capture and phage viability, and reproducibly yielded modification of 86 ± 3% of phage: (i) 5 min exposure to 60 μM $NaIO_4$ at 4 °C; (ii) 10 min quenching with 500 μM GSH; followed by (iii) 1 h incubation with 1 mM AOB.

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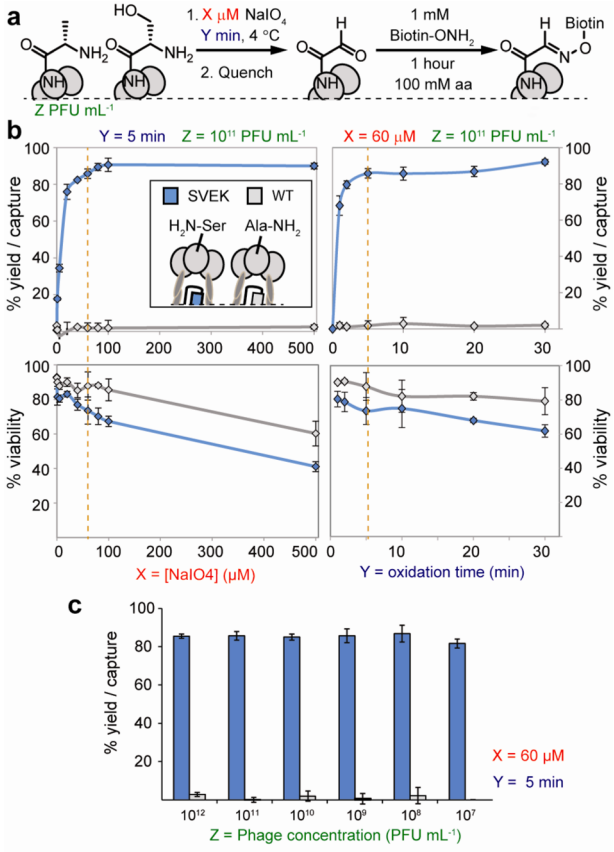

Figure 2. Profiling of the kinetics of oxidation. (a) Oxidation was performed on a mixture of SVEK and WT phage and quenched by 500 μM 4-methoxybenzenethiol (MBT); aa = anilinium acetate buffer (pH = 4.7). (b) The optimal [$NaIO_4$] and reaction time that maximize the yield and viability are 60 μM and 5 min. (c) Varying phage concentrations did not affect the reaction kinetics. The results are average of at least two independent experiments; error bars are one SD.

Modifications of phage with reagents present in large excess compared to phage should exhibit pseudo-first-order kinetics. The efficiency of such reaction should be independent of the concentration of phage. Indeed, the optimal condition gave similar capture across 10^7–10^{12} PFU mL^{-1} of phage (Figure 2c). We note that, at 10^7 PFU mL^{-1}, the concentration of phage is ~20 fM and quantification of reaction efficiency using any other method would be impossible. The reactions on phage that has 3–5 copies of pIII protein could yield phage with 0, 1, 2, 3, 4, or 5 biotins (B_0–B_5). If the reactions on pIII are independent, binomial statistics links reaction yields on individual pIII with B_0 (*i.e.*, non-captured phage with 0 biotin) (Figure 3a) and yields an equation that could be used to derive the rate constants (k) from the biotin-capture (Figure 3b). We observed agreement of k among reactions performed on phage (Figure 3c), on model peptides (Figure 3d and Supplemenatry Figure S5), and on similar reactants reported in the literature.[27] For example, k of aniline-catalyzed oxime ligations were 3.3 ± 0.5 M^{-1} s^{-1} (phage), 7.8 ± 2.3 M^{-1} s^{-1} (peptide), and 8.6 ± 2.0 M^{-1} s^{-1} (literature), whereas k of non-catalyzed ligations were 0.018 ± 0.005 M^{-1} s^{-1} (phage) and 0.020 ± 0.001 M^{-1} s^{-1} (literature).[27] In all studies, the reactions on phage saturated at ~90% capture (B_0 = 10%). From B_0, binomial statistics estimates that the phage contained 1–3 copies of modified peptide (Figure 3e). The calculation of the % yield of modified peptides depends on the number of pIII proteins (37% assuming N = 5 and 54% for N = 3). Furthermore, pIII-displayed peptides could be subjected to proteolysis.[1] As the oxidation and oxime ligations proceed to >90% conversion on SVEKY peptide (Figure 3d and Supplemenatry Figure S5), the low *apparent* yields on phage could indicate that phage contains only 1–3 reactive sites (peptides).

The pull-down assay can quantify both the appearance of aldehydes as a result of oxidation and their disappearance due to oxime ligation (Figure 4a). We observed that the capture yield did not change when the AOB-labeled phage was incubated with methoxylamine (Figure 4b). This result suggested that the oxime linkage was thermodynamically stable under the reaction conditions, and a phage reacted with AOB does not react further with any other aminooxy reagent. This observation was further confirmed by analogous reactions performed on a tetrapeptide (H_2N-SVEK-CO_2H) and verification of the products by ESI-MS (Supplementary Figure S6). Building on this observation, we could quantify modification of phage with any molecule that carries a functional group that reacts irreversibly with an aldehyde. For example, if oxidized phage is first exposed to methoxylamine, subsequent exposure to AOB quantifies the amount of unreacted aldehydes. The ligation yield of methoxylamine (~85%) was calculated as the difference of yields between reaction 1 and 2 (Figure 4b). Similarly, we have quantified the ligation yield of oxidized phage with 2-(aminooxy)ethyl α-D-mannopyranoside (AOMan) to be ~80% (Figure 4b). We note that, since a single modification with biotin is sufficient for phage capture, the follow-up reaction using AOB thus quantified the number of phage that contained no unreacted aldehyde in any of the five copies of pIII. To ensure that phages were stable for the duration of the selection process, we demonstrated that the oxime linkage was stable for at least six days when phages were stored in neutral solution at 4 °C (Supplementary Figure S7).

The biotin-capture assay allowed, for the first time, accurate yield determination of reactions on 10^9 diverse peptides present at subnanomolar concentration. We observed that 26% of the clones in the phage library (Ph.D.-7) could be oxidized and reacted with AOB (Figure 4c). The observed value is similar to the fraction of *N*-terminal Ser and Thr present in the library as determined by deep sequencing.[20] Eventually, we demonstrated the synthesis of a library of glycopeptides with a simple carbohydrate epitope (*e.g.*, mannose, Figure 4c). We envision that a phage-displayed library of glycopeptides could accelerate the discovery of high-affinity ligands for carbohydrate-binding proteins (*e.g.*, lectins). These proteins regulate pathogen invasion, immune response, and cancer development, but potent inhibitors for lectins are scarce due to the low affinity of lectin–carbohydrate interaction. Hybrid ligands (*i.e.*, glycopeptides) could potentially bind to the carbohydrate-binding site and adjacent sites in a synergistic fashion.[28,29] We believe that the availability of a large, genetically traceable library of glycopeptides will aid in the selection of optimal side chains of the secondary binding ligand as well as the optimal spacing and flexibility of the linker.

Characterization of reaction efficiencies is a cornerstone of chemical synthesis. Generation of genetically encoded libraries of peptide derivatives through modification of phage libraries hinges on quantitative conversion of all reactive peptides in the library. Although strategies for modifying phage libraries were proposed many years ago,[6,7] lack of simple and quantitative

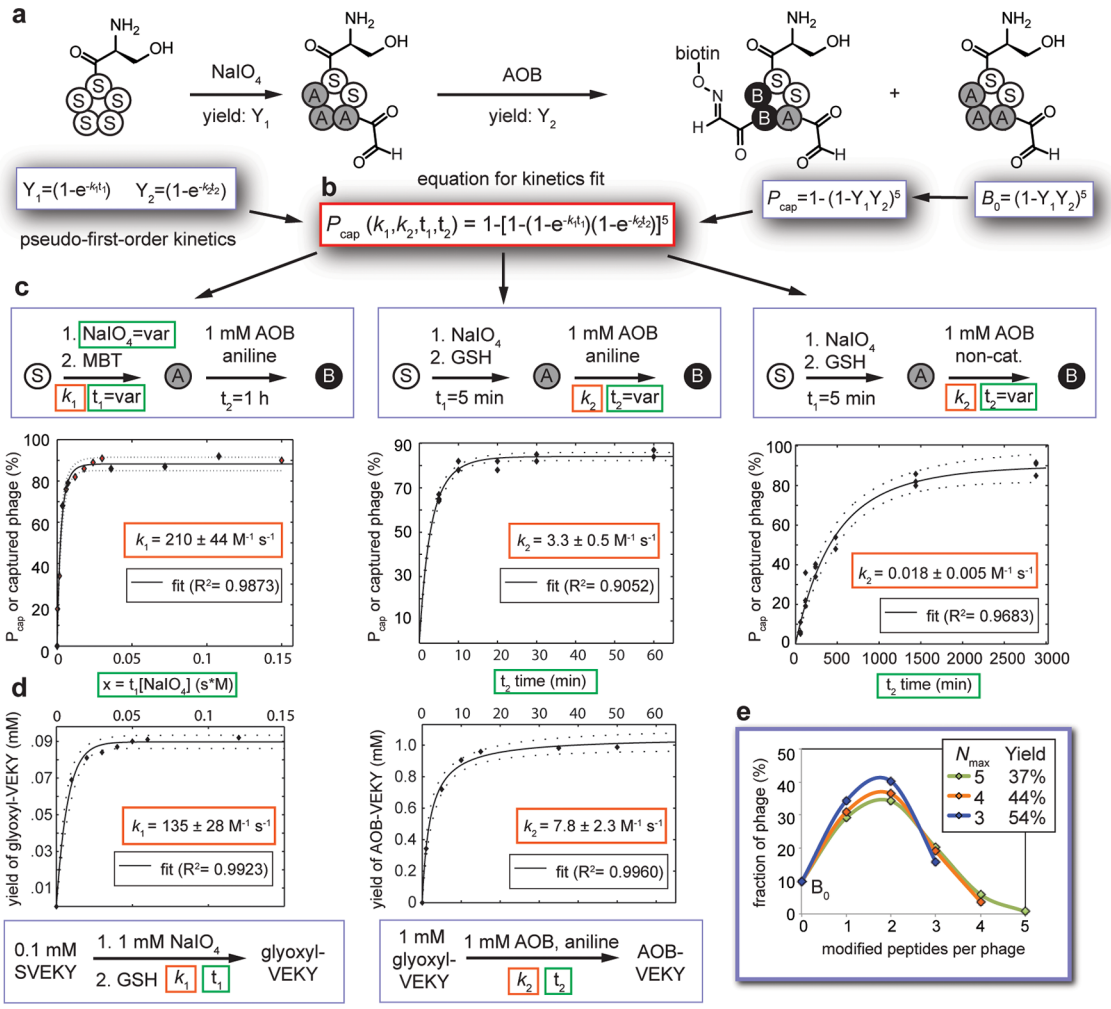

Figure 3. Reaction on multiple copies of pIII protein. (a) Binomial distribution of biotin-free phage (B_0) and phage that can be captured (P_{cap}). (b) Derivation of the rate of formation of P_{cap} from binomial distribution and pseudo-first-order kinetics. (c) Fit of equation b to capture data yielded the rate constants (k_1, k_2) for oxidation, aniline-catalyzed ligation (pH 4.7), and non-catalyzed ligation (NaOAc buffer, pH 4.5) on phage. (d) Rate constants of reactions on peptide, as monitored by HPLC (Supplementary Figure S5), were similar to those in panel c. Kinetics contains data from 2−3 independent experiments; k are reported as average and 95% confidence range of the fit. (e) In the population where B_0 = 10%, phage contains 1, 2, or 3 copies of AOB-modified peptides. The yield of modification could be estimated only if the maximum number of reactive sites (N_{max}) is known.

characterization tools remains one of the roadblocks to the development of this technology. Reactions applied to an entire phage library have typically been optimized using one sequence with the assumption that all peptides with reactive groups react similarly. In practice, variations in structural, electrostatic, and steric factors in the diverse peptides inevitably influence the efficiency of any reaction. For example, generating a library of peptides with *N*-terminal Ser[30] does not guarantee 100% modification because Ser-Pro sequences within this library could undergo rapid cyclization after oxidation to glyoxylyl-Pro.[31] This self-reactivity precludes the ligation with *O*-alkyl hydroxylamines. On the other hand, we demonstrated that AOB tagged a subpopulation of 2.5 × 10^8 reactive peptides within the random library of 10^9 peptides (Figure 4c). Biotin tagging thus could complement genetic engineering by marking the reactive clones within libraries. If necessary, the AOB-tagged phage clones could be isolated and reamplified to yield a sublibrary that form oxime quantitatively.

The library of 10^8 peptides containing an aldehyde functionality, which was produced within 30 min in >90% yield, could be readily derivatized using any glycan containing an aminooxy functional group.[32] Moreover, an aldehyde-containing library is a rich starting point for a wide variety of reactions, such as Wittig reactions, that proceed with high efficiency in water and tolerate a range of functionalities.[14] As a result, one commercially available phage library could provide multiple opportunities for the generation of novel genetically encoded libraries of peptide derivatives. We believe that the availability of this "starting material" coupled with the simplicity of the methodology described herein will render the synthesis of custom chemically modified phage libraries accessible to a broad range of chemical biology researchers in academic and industrial laboratories.

METHODS

Quantification of Oxidation and Oxime Ligation on M13 Phage. The following is a representative procedure (other conditions can be found in the figure legends). A mixed solution of SVEK and WT phage (99 μL, 10^{11} PFU mL^{-1} in PBS, pH 7.4) was oxidized with $NaIO_4$ (add 1 μL of 6 mM in ddH_2O) for 5 min on ice in the dark and quenched with glutathione (add 1 μL of 50 mM in ddH_2O) for 10 min at room temperature (rt). Treatment with aminooxy reagent (add 101

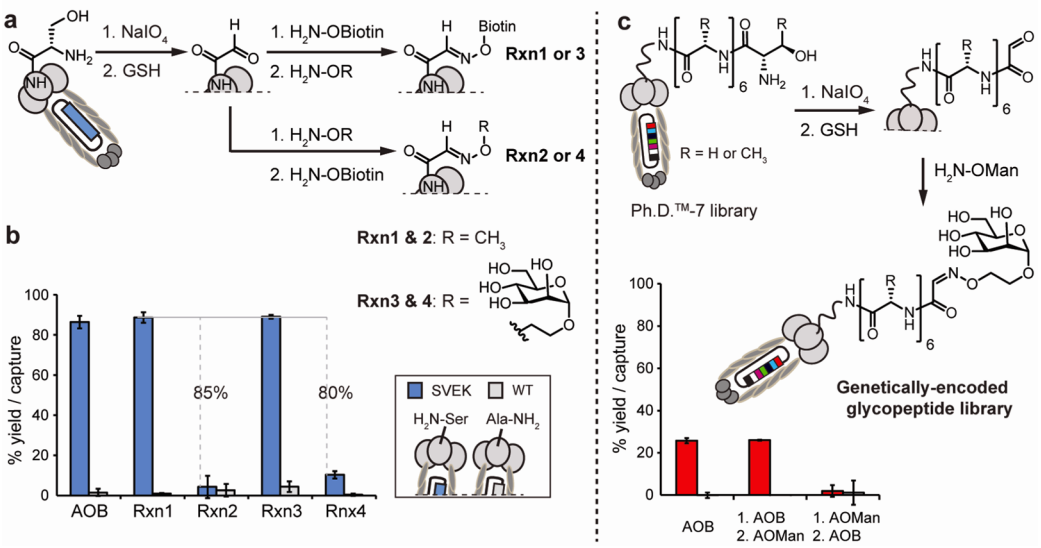

Figure 4. Quantification of glycopeptide formation on monoclonal phage or phage library. (a) Changing the order of addition of reagents quantified the coupling yield of methoxylamine or AOMan with oxidized phage. (b) Coupling yields were determined by subtracting the yield of reaction 2 or 4 from the yield of reaction 1 or 3. (c) A similar strategy was used to quantify the efficiency of reaction on Ph.D.-7 library. In panels b and c, the value is an average of at least two independent experiments run on different days; error bars are one SD.

μL of 2 mM in sterile 200 mM anilininum acetate, pH 4.7) for 1 h at rt yielded the corresponding biotinylated peptide or glycopeptide. For reaction involved AOB, reaction mixture was diluted by 10^5-fold with binding buffer (PBS, 0.1% BSA) to quench the reaction and to ensure that traces of the biotin reagent do not saturate the binding site of SA-coated magnetic beads. The diluted phage solution (100 μL) was agitated with SA-coated magnetic beads (5 μL, Bioclone Inc., binding capacity: 2 pmol μL^{-1}) for 15 min at rt and captured on a magnetic separator. The phages in the supernatant were quantified by plaque-forming assay. The yield of reaction was determined as $(A - B)/A \times 100\%$, where A and B are the titers of phage before and after capture. Note: Ph.D.-7 library should be dialyzed (24 h, 4 °C, 10 K MWCO) against PBS (5 L, changed twice after 4 h interval) prior to the reaction to remove the glycerol (storage buffer).

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Figures S1–S7, Supplementary Scheme S1, detailed descriptions of experimental procedures, MatLab scripts used for non-linear fit in Figure 3, synthetic steps, and characterization of all compounds and their NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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