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Electronic Supplementary Information for

# A Reactive Peptide Interface for Site-Selective Cysteine Bioconjugation

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### 1. Materials and Methods

#### a. Reagents and solvents

Decafluorobiphenyl was purchased from Oakwood Chemicals (West Columbia, SC). Pentafluorophenyl sulfide was purchased from Santa Cruz Biotechnology (Dallas, TX). Tris(2carboxyethyl)phosphine hydrochloride (TCEP•HCl) was purchased from Hampton Research (Aliso Viejo, CA). Fmoc-protected amino acids (Fmoc- Ala-OH•H2O, Fmoc-Arg(Pbf)-OH; Fmoc-Asn(Trt)-OH; Fmoc-Asp-(Ot-Bu)-OH; Fmoc- Cys(Trt)-OH; Fmoc-Gln(Trt)-OH; Fmoc-Glu(Ot-Bu)-OH; Fmoc-Gly-OH; Fmoc-His(Trt)-OH; Fmoc-Ile-OH; Fmoc-Leu-OH; Fmoc-Lys(Boc)-OH; Fmoc-Met-OH; Fmoc-Phe-OH; Fmoc-Pro-OH; Fmoc-Ser(But)-OH; Fmoc-Thr(t-Bu)-OH; Fmoc-Trp(Boc)-OH; Fmoc-Tyr(t-Bu)-OH; Fmoc-Val-OH); Fmoc-His(Boc)-OH was purchased from ChemPep, Inc.; O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium (HATU, >97.0%), and hexafluorophosphate (PyAOP, ≥97.0%) were purchased from P3 Biosystems. Biosynthesis OmniSolv® grade N,N-dimethylformamide (DMF) was purchased from EMD Millipore (DX1732-1). AldraAmine trapping agents (for DMF, catalog number Z511706). diisopropylethylamine (DIEA; 99.5%, biotech grade, catalog number 387649), piperidine (ACS reagent,  $\geq 99.0\%$ ), trifluoroacetic acid (HPLC grade,  $\geq 99.0\%$ ), triisopropylsilane ( $\geq 98.0\%$ ), acetonitrile (HPLC grade), formic acid (FA, ≥95.0%), H-Rink Amide (0.49 mmol/g and 0.18 mmol/g loading) and HMPB ChemMatrix polyethylene glycol (0.45 mmol/g loading) resin were purchased from PCAS Biomatrix. Water was deionized using a Milli-Q Reference water purification system (Millipore). Nylon 0.22 µm syringe filters were TISCH brand SPEC17984.

#### b. Peptide Synthesis

All peptides were synthesized on H-Rink Amide ChemMatrix resin on a 0.2 mmol scale using an automated fast-flow peptide synthesizer (AFPS) built in the Pentelute lab ("Amidator") previously described.<sup>1,2</sup> Upper case letters refer to L-amino acids, lower case letters refer to D-amino acids.

Unless otherwise noted, the following settings were used for peptide synthesis: flow-rate = 40mL/min, temperature = 90 °C (loop) and 85–90 °C (reactor).<sup>2</sup> The 50 mL/min pump head pumps 400 µL of liquid per pump stroke; the 5 mL/min pump head pumps 40 µL of liquid per pump stroke. The standard synthetic cycle involves a first step of prewashing the resin at elevated temperatures for 60 s at 40 mL/min. During the coupling step, three HPLC pumps are used: a 50 mL/min pump head pumps the activating agent, a second 50 mL/min pump head pumps the amino acid and a 5 mL/min pump head pumps DIEA. The first two pumps are activated for 8 pumping strokes in order to prime the coupling agent and amino acid before the DIEA pump is activated. The three pumps are then actuated together for a period of 7 pumping strokes, after which the activating agent pump and amino acid pump are switched using a rotary valve to select DMF. The three pumps are actuated together for a final 8 pumping strokes, after which the DIEA pump is shut off and the other two pumps continue to wash the resin for another 40 pump strokes. During the deprotection step, two HPLC pumps are used. Using a rotary valve, one HPLC pump selects deprotection stock solution and DMF. The pumps are activated for 13 pump strokes. Both solutions are mixed in a 1:1 ratio. Next, the rotary valves select DMF for both HPLC pumps, and the resin is washed for an additional 40 pump strokes. The coupling-deprotection cycle is repeated for all additional monomers.

#### c. Peptide Cleavage

After synthesis, the peptidyl resin was washed with dichloromethane (3 x 5 mL), dried and transferred into a 50-mL conical polypropylene tube. Approximately 3 mL of cleavage solution (94% trifluoroacetic acid (TFA), 1% triisoproprylsilane (TIPS), 2.5% 1,2-ethanedithiol (EDT), 2.5% water, by volume) was added to the tube. The tube was kept at room temperature for 2 h. Ice cold diethyl ether (45 mL) was added to the cleavage mixture and the precipitate was collected by centrifugation and triturated twice with cold diethyl ether (45 mL). The supernatant was discarded. Residual ether was allowed to evaporate and the peptide was dissolved in 50% acetonitrile in water with 0.1% TFA. The peptide solution was filtrated with a Nylon 0.22  $\mu$ m syringe filter and frozen, lyophilized until dry, and weighed.

Commonly recurring solvents are referred to as the following A: 0.1% TFA in  $H_2O$  and B: 0.1% TFA in acetonitrile.

# d. Preparation of S-Perfluoroarylated Peptide Probes

The peptidic part of the probes was synthesized under flow with an AFPS system using a Fmoc-Cys(S-tBu)-OH amino acid. The biotin moiety was incorporated by coupling D-biotin to the resin under batch SPPS conditions. To make the "biotin branch" on biotinylated peptides, Fmoc-Lys(Alloc)-OH was used. The Alloc protecting group was removed by reaction on-resin with 50 mg of tetrakis(triphenylphosphine)palladium(0), 1 mL of phenylsilane and 4 mL of DCM. This step exposed the Lys *e*-amino group for coupling to S (then G, G and finally D-biotin). Fmoc deprotection was used to synthesize the rest of the peptide. The perfluoroaryl group was installed using an on-resin perfluoroarylation procedure. The S-tBu protecting group was removed on-resin by incubating all of the peptide-resin with 5 mL of DMF solution containing 309 mg of dithiothreitol (DTT) and 278  $\mu$ L of triethylamine at room temperature for 1 hour. The resin was washed 5 times with DMF. Then 5 mL of DMF solution containing 668 mg of decafluorobiphenyl (deca) or 732 mg of pentafluorophenyl sulfide (decaS) and 278  $\mu$ L of triethylamine was added, and the reaction mixture was rotated at room temperature for 1 hour. The resin was washed 5 times with DMF, 5 times with DCM, and then dried *in vacuo*. The resulting products were cleaved from the resin and purified by RP-HPLC following standard procedures.

# e. Peptide Purification

Lyophilized crude peptide was dissolved in 95% A: 5% B with 6 M guanidinium hydrochloride and purified by semi-preparative RP-HPLC. All samples filtrated with a Nylon 0.22  $\mu$ m syringe filter prior to purification. The solution was loaded onto a reversed-phase HPLC column (Agilent Zorbax SB C18 column: 9.4 x 250 mm, 5  $\mu$ m or Agilent Zorbax SB C3 column: 9.4 x 250 mm, 5  $\mu$ m) attached to a mass-based purification system. A linear gradient was run at 0.5% B / min from either 5% B to 45% B or 15% B to 55% depending on the peptide. Using mass data about each fraction from the instrument, only pure fractions were pooled and lyophilized. The purity of the fraction pool was confirmed by LC-MS. Peptides synthesized using fast flow-based SPPS and purified by RP-HPLC are listed in Table S1.

# f. LC-MS Analysis

LC-MS chromatograms and associated mass spectra were acquired using an Agilent 6520 Accurate-Mass Q-TOF LC-MS instrument. Mobile phases used for LC-MS analysis are solvent C (0.1% formic acid in water) and solvent D (0.1% formic acid in acetonitrile). The following LC-

MS method was used: LC conditions: Zorbax 300SB C<sub>18</sub> column: 2.1 x 150 mm, 5  $\mu$ m, column temperature: 40 °C, gradient: 0-2 minutes 1% D, 2-11 minutes 1-91% D, 11-12 minutes 91%-95% D, flow rate: 0.6 mL/min. MS conditions: positive electrospray ionization (ESI) extended dynamic mode in mass range 300 – 3000 *m/z*, temperature of drying gas = 350 °C, flow rate of drying gas = 11 L/min, pressure of nebulizer gas = 60 psi, the capillary, fragmentor, and octupole rf voltages were set at 4000, 175, and 750, respectively.

#### g. Reaction Yield Determination

Yields for peptide substrates were determined by integrating total ion current (TIC) spectra. First, using the Agilent MassHunter software package, the peak areas for all relevant peptidic species on the chromatogram were integrated. Conversion was calculated by integrating the total ion current (TIC) of the same limiting peptide species within the dynamic linear range of the LC–MS instrument. The percent conversion was calculated as following:

percent conversion = 
$$1 - S_t / S_0$$
 (1)

where  $S_t$  is the peak area of the limiting reagent at time *t*, and  $S_0$  is the peak area of the limiting reagent at time 0. In cases where no side product was generated in the experiments, the conversion of the limiting reagent equals the yield of the product.

### 2. Screening of One-Bead-One-Compound Peptide Library

#### a. Synthesis of OBOC Peptide Library

The peptide library was synthesized on 30-µm TentaGel S-NH<sub>2</sub> resin (Rapp Polymere GmbH, Germany) using standard split-pool techniques on a 2-g scale with resin amine loading of 0.22 mmol/g in a 100 mL peptide synthesis vessel. First, the TentaGel resin was functionalized with a cleavable linker, as follows. The resin (2.0 g; 0.44 mmol) was treated with a solution of Boc-Gly-O-CH<sub>2</sub>-phenylacetic acid (5 mmol; PolyPeptide Group, Strasbourg, France), HATU (5 mmol) and DIEA (2.5 mL; 14.5 mmol; added immediately before use) in DMF (12.5 mL). The coupling was allowed to proceed for 20 min at room temperature. Then the resin was washed with DMF (3 times, 25 ml each), after which neat TFA was used to remove the Boc protection group (twice, 20 mL each for 2 min). The resin was washed with DCM (3 times, 25 mL each) and DMF (3 times, 25 mL each).

Then the resin was subjected to standard Fmoc SPPS to install the constant region. A cycle of coupling, wash, deprotection, and wash was used to install each amino acid. First, the resin was treated with a solution of Fmoc-amino acid (5 mmol), HATU (5 mmol) and DIEA (2.5 mL; 14.5 mmol; added immediately before use) in DMF (12.5 mL). The coupling was allowed to proceed for 20 min at room temperature. Then the resin was washed with DMF (3 times, 25 mL each), after which 20% (v/v) piperidine in DMF was used to remove the Fmoc group (twice, 25 mL each for 5 min). The resin was washed with DMF (3 times, 25 mL each) and then subjected to the next round of amino acid coupling.

After finishing the synthesis of the constant region, the resin was subjected to split-pool synthesis to create the random region of the library. A cycle of split, coupling, wash, pool, deprotection, wash was used to install amino acids at each random position. For each random position, the resin

was first suspended in DMF and equally split into 15 portions in 10 mL Torviq fritted syringes using a 5 mL pipette. Then each portion of resin was reacted with one of 15 different amino acid active ester solutions (0.5 mmol Fmoc-amino acid, 0.5 mmol HATU, and 1.4 mmol DIEA in 1.25 mL DMF). Less DIEA (0.55 mmol) was used for the activation of His and Trp, to minimize the potential risk of racemization. The couplings were allowed to proceed for 20 min at room temperature. Then resin was washed with DMF (3 times, 5 mL each) and pooled into a 100-mL peptide synthesis vessel. 20% (v/v) piperidine in DMF was used to remove the Fmoc group (twice, 25 mL each for 5 min). The resin was washed with DMF (4 times, 25 mL each) and then subjected to the next round of amino acid coupling. Note for the constant cysteine residue, regular SPPS procedure was used instead of split-pool synthesis.

After finishing the split-pool synthesis, the resin was washed with DCM (3 times, 25 mL each) and dried for 5 min. Then 5 mL of cleavage cocktail (TFA: ethanedithiol (EDT): water: triisopropylsilane (TIPS) = 94: 2.5: 2.5: 1 v/v/v/v) was added to the resin to remove side chain protecting groups. The deprotection reaction was allowed to proceed at room temperature for 2 hours, then the resin was washed with TFA (twice, 10 mL each), DCM (3 times, 20 mL each), DMF (3 times, 20 mL each), and DCM (3 times, 20 mL each). The resin was dried under vacuum and stored at -20 °C.

#### b. Isolation of Reactive Hits

Biotinylated electrophilic peptide (7) was used to react with the on-resin library. 20 mg of resin was suspended in 1 mL of water in a 10 mL Torviq fritted syringe. The resin was extensively sonicated until there were no observable aggregated resins. Then water was slowly dispensed and 3 mL of reaction solution (4 mM 7, 200 mM acetate, 20 mM TCEP, pH 5.0) was added. The reaction mixture was allowed to rotate on a VWR tube rotator at room temperature for 1 h. After the reaction, the solution was dispensed and the resin was washed with phosphate buffer saline (PBS) (3 times, 5 mL each), water (3 times, 5 mL each), DMF (3 times, 5 mL each), water (3 times, 5 mL each), and PBS (3 times, 5 mL each). 6 mL of blocking buffer (PBS with 1% BSA and 0.2% Tween) were added to the resin and the mixture was rotated at room temperature for 1 h. After blocking, the blocking buffer was slowly dispensed and 6 mL of staining buffer (PBS with 1% BSA, 0.2% Tween, and 40  $\mu$ g/mL streptavidin-allophycocyanin (SA-APC, Columbia Biosciences)) was added and the mixture was rotated at room temperature for 1 h. After staining, the staining buffer was dispensed and the resin was washed with PBS (3 times, 5 mL each). Finally, the resin was resuspended in 6 mL of 0.2- $\mu$ m-filtered PBS and passed through a 70- $\mu$ m cell strainer (VWR International) prior to bead sorting experiment.

Bead sorting was performed using BD FACSAria III cell sorter. A 130-µm nozzle was used with a sheath fluid pressure of 10 psi. PBS was used as the sheath fluid. The beads were first gated using the forward-scattering and side-scattering and then sorted according to APC fluorescence intensity. The top 1% fluorescent beads (~5000 beads) were collected (Fig. S3) into a 1.7 mL Eppendorf tube.

#### c. *De novo* sequencing of reactive hits

The beads collected from sorting experiment were subjected to NaOH cleavage to release peptides from beads, by saponification of the glycyl-OCH<sub>2</sub>-Pam linker. First, 200  $\mu$ L of water was used to carefully wash the Eppendorf tube to ensure all beads were suspended in the solution. Then the

solution was spun down for 1 min at 14,000 rpm to pellet the beads. The supernatant was carefully removed and about 3  $\mu$ L of solution was left. Then the bead solution was divided into 3 portions and for each 1- $\mu$ L portion of bead solution was added 5  $\mu$ L of cleavage solution (1.2 M NaOH with 6% (v/v) 1,4-dithio-D-threitol (DTT)) and the mixture was left at room temperature for 40 min. Note that 7 was cleaved by elimination of its perfluoroaryl group under this condition to generate dehydroalanine. Dehydroalanine was further reacted with DTT to produce a unique signature for *de novo* sequencing (Fig. S4). Then,18  $\mu$ L of quenching solution (58.5% acetonitrile, 38.5% water, and 3% TFA) was added to quench the cleavage reaction. The reaction mixture was spun down for 1 min at 14,000 rpm and the supernatant (20  $\mu$ L) was injected into Agilent LC-MS 6550 accurate Q-TOF mass spectrometer for peptide sequencing.

Peptide Sequencing Method LC conditions: Phenomenex Jupiter C4, 150 x 1.0 mm ID, 5  $\mu$ m 300 Å silica, gradient: 0-5 minutes 1% D, 5-65 minutes 1-41% D, 65-72 minutes 70% D, flow rate: 0.1 mL/min. MS conditions: Dual Agilent Jet Stream (AJS) positive electrospray ionization (ESI) extended dynamic range mode in mass range 100 – 1700 m/z, temperature of drying gas = 200 °C, flow rate of drying gas = 17 L/min, pressure of nebulizer gas = 50 psi, temperature of sheath gas = 350 °C, flow rate of drying gas = 11 L/min, the capillary and nozzle voltages were set at 3500 and 500, respectively. Auto MS/MS mode was used for peptide fragmentation. Only +2 and +3 charge states with intensity more than 7,500 counts were selected as MS/MS precursors. Active exclusion was enabled to exclude a precursor after 1 spectrum was acquired, the releasing time was set as 0.5 min. The acquisition rate for primary and secondary mass spectra were set at 4 spectra/s and 3 spectra/s, respectively. A collision-induced dissociation (CID) mode was used for peptide fragmentation and formulas were used to decide collision energy for each peptide precursor ion: (slope)\*(charge)/100 + offset, where slope, charge, and offset were decided by following chart (note for each charge state, two collision energies were used):

Charge	Slope	Offset
2	3.6	-5
2	3.6	-2.5
3	3.6	-5.5
3	3.6	-3

The raw LC-MS/MS data were processed using PEAKS software. The DTT adduct was incorporated as a post-translational modification (PTM) on cysteine (mass +48.05) during *de novo* sequencing. All *de novo* candidates obtained from PEAKS software were further parsed to eliminate noise, incorrectly assigned sequences and unmodified peptides, which produced the final list of 40 hit sequences (Table S1). The set of criteria for sequence parsing include (1) average local confidence (ALC) more than 90; (2) mass error less than 10 ppm; (3) sequence consistent with initial library design; and (4) sequence contain a +48.05 signature PTM on the cysteine residue.

#### d. Reactive Hits Validation

Peptide sequences were identified from screening and *de novo* sequencing. All hits were resynthesized and tested in solution and validated for reactivity *via* LC-MS.

#### 3. Kinetics Studies on the 1, Electrophilic peptide and Controls

All reactions were carried out in a 0.2 M sodium phosphate, 20 mM TCEP buffer set to pH 8. To measure the second-order rate constants, reactions were divided into several 10- $\mu$ L aliquots and heated in a PCR machine set at 37 °C to prevent solvent evaporation. Reactions were quenched by addition of 49.75% water/49.75% acetonitrile/0.5% TFA at different time points and then subjected to LC-MS analysis. The initial concentration of nucleophilic peptide and electrophilic peptide were known. The second-order rate constants (*k*) were determined by fitting the kinetics to the following equation:

$$y = \frac{\ln \frac{[\text{nucleophilic peptide}]_0[\text{electrophilic peptide}]_t}{[\text{nucleophilic peptide}]_t[\text{electrophilic peptide}]_0}}{[\text{electrophilic peptide}]_0 - [\text{nucleophilic peptide}]_0} = kt$$
(2)

Error of reaction rate constant was obtained from the standard error of the slope as determined from the linear fitting of the kinetics curves for measuring the reaction rate.

# 4. Expression, Purification and Labeling of RPI-Trastuzumab

Antibody conjugation reactions were performed on a 50  $\mu$ L scale in polymerase chain reaction (PCR) tubes. A volume (7.5  $\mu$ L) of RPI-trastuzumab stock solution (66.67  $\mu$ M in PBS) was mixed with 10  $\mu$ L reaction buffer (1 M phosphate, 100 mM TCEP, pH 8.0), 10  $\mu$ L labeling biotintylated **2** (**S6**, 500  $\mu$ M in water) and 22.5  $\mu$ L of water. The reaction mixture was mixed with a pipette 20 times and was incubated in a PCR machine at 37 °C. The final reaction conditions for biotin conjugation were 10  $\mu$ M **11**, 100  $\mu$ M **12**, 200 mM phosphate, 20 mM TCEP, 37 °C, 90 min. After reaction, the reaction mixture was diluted into 4 mL PBS and was buffer exchanged five times with PBS using 15 mL 10 K spin concentrator (EMD Millipore) to remove the excess labelling reagents. The final concentrated samples were used in LC-MS analysis, the Octet binding assay and flow cytometry experiments.

The antibodies were expressed via transient transfections of HEK293F cells (Invitrogen) and purified using Protein A affinity chromatography (Genscript) following manufacturer's instructions. The purified IgG were analyzed by LC-MS to confirm their molecular weight and purity, and stored in phosphate buffer saline (PBS) at -80 °C. Mass spectrometry analysis of the intact antibody indicates that **11** is expressed with the RPI residues capped by a disulfide bond with free cysteines.

# 11-Light Chain (11-LC)

ADIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGV PSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTVAAPSVFIFPP SDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSST LTLSKADYEKHKV YACEVTHQGLSSPVTKSFNRGEC

# 11-Heavy Chain with RPI at *C*-terminus (11-HC)

EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYT RYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDYWGQGT LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFP AVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV YTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQ KSLSLSPGMCPFLPVVY

# 5. Octet BioLayer Interferometry Binding Assay

In vitro binding assays were performed using the Fortebio Octet RED96 Bio-Layer Interferometry system at 37 °C. Streptavidin biosensors were dipped into 200  $\mu$ L of 20 nM **12** in PBS with 0.02% Tween and 0.1% BSA for the loading. The biosensors loaded with antibody were sampled with recombinant HER2 (R&D Systems) at various HER2 concentrations in PBS with 0.02% Tween and 0.1% BSA to obtain the association curve. Buffer only served as the reference. After association, the biosensors were dipped into buffer to obtain the dissociation curve. Following the protocols provided by Fortebio Biosystems, the association and dissociation curves of each sample were manually fitted using Solver in excel to obtain the  $K_d$ . The final  $K_d$  was reported as the average of the values obtained from experiments with serially diluted HER2.

# 6. Flow Cytometry

Cells were seeded in 96-well V-bottom plates at a density of 20,000/well. Cells were spun down and washed with 150  $\mu$ L of PBSA (phosphate buffer saline with 0.1% BSA). Cells were then treated with 100  $\mu$ L of biotin-linked antibodies or controls (20 nM in PBSA) for 1 hour on ice. Cells were spun down, washed with PBSA, and then treated with either streptavidin-AlexaFluro-647 or antiHuman Fc-AlexaFluro-488 for 30 min on ice. Cells were spun down, washed with PBSA, and then suspended in 100  $\mu$ L of PBSA and analyzed by the Accuri C6 (BD Biosciences) flow cytometer in the CEHS Instrument Facility of MIT. All experiments were done in triplicate.

# 7. References and Notes

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#### 8. Supplementary Tables and Figures



Figure S1. Multiple Peptide Library Screens Lead to the Reactive Peptide Interface (RPI). a) A one-bead one-compound peptide library was synthesized via the split-pool method, which was then subjected to reaction with 4 on-bead. The arylated peptides on the beads were stained with streptavidin-allophycocyanin, isolated via fluorescence-activated bead sorting (FABS), cleaved from the beads, and analyzed via LC-MS/MS for *de novo* sequencing. b) Library design for each generation of library selection. c) The most reactive nucleophilic peptide and electrophilic peptide sequences isolated from each library screening iteration and their corresponding *k* for each reaction pair show a gradual improvement in *k*. SA = streptavidin, PTM = post-translational modification, DB = decafluorobiphenyl, PS = pentafluorophenyl sulfide.

Name	Sequence	Name	Sequence
Gen 1	FCPLYDF GLLKG	Gen 1k	SCPFTPG GLLKG
Gen 1a	KCPFAPD GLLKG	Gen 11	ACPLKMM GLLKG
Gen 1b	VCPFLGS GLLKG	Gen 1m	YCPLATM GLLKG
Gen 1c	VCPFYTA GLLKG	Gen 1n	TCPLAWE GLLKG
Gen 1d	GCPFWEY GLLKG	Gen 1o	TCPLMTY GLLKG
Gen 1e	YCPFFST GLLKG	Gen 1p	VCPLLEL GLLKG
Gen 1f	SCPFMEL GLLKG	Gen 1q	ACPLAHY GLLKG
Gen 1g	DCPFYFP GLLKG	Gen 1r	SCPLAEA GLLKG
Gen 1h	WCPFAYT GLLKG	Gen 1s	FCPLAAP GLLKG
Gen 1i	MCPFVGL GLLKG	Gen 1t	YCPLYEV GLLKG
Gen 1j	HCPFYKA GLLKG		

Table S1. Nucleophilic peptides identified from the 1<sup>st</sup> round of library screening and *de novo* sequencing.



Figure S2. Kyte and Doolittle hydrophobicity plot of the sequences from the first library selection for a reactive nucleophilic peptide reveals hydrophobic residues in position #1, #5, and #7 and hydrophilic residues in position #6.



Figure S3. Glycine scan of 6 reveals sequence-dependent reactivity with the  $\pi$ -clamp peptide. Percent conversion of each reaction between  $\pi$ -clamp and 6 glycine variants.



Figure S4. Kinetic analysis for  $\pi$ -clamp reacting with 6. (a) LC-MS chromatograms (total ion current) for the reaction between  $\pi$ -clamp and 6. Reaction conditions: 1 mM  $\pi$ -clamp, 5 mM 6, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'P' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



**Figure S5. Kinetic analysis for 4 reacting with 6.** (a) LC-MS chromatograms (total ion current) for the reaction between 4 (labeled as 'a') and 6 (labeled as 'b'). Reaction conditions: 1 mM 4, 5 mM 6, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



Figure S6. Kinetic analysis for 5 reacting with 6. (a) LC-MS chromatograms (total ion current) for the reaction between 5 (labeled as 'a') and 6 (labeled as 'b'). Reaction conditions: 100  $\mu$ M 5, 500  $\mu$ M 6, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



Figure S7. Kinetic analysis for 1 reacting with 6. (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 6 (labeled as 'b'). Reaction conditions: 100  $\mu$ M 1, 500  $\mu$ M 6, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



Figure S8. Kinetic analysis for 1 reacting with 7. (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 7 (labeled as 'b'). Reaction conditions: 40  $\mu$ M 1, 80  $\mu$ M 7, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



**Figure S9. Selective homodimerization of 1 was observed when 1 and 8 were reacted with decafluorobiphenyl (DB).** Chromatograms are total ion currents (TIC) from LC-MS analysis.



Figure S10. Kinetic analysis for 1 reacting with 8. (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 8 (labeled as 'b'). Reaction conditions: 40  $\mu$ M 1, 80  $\mu$ M 8, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



Figure S11. Kinetic analysis for 1 reacting with 2. (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 40  $\mu$ M 1, 80  $\mu$ M 2, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



**Figure S12. Conversion analysis for 1 reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of 'a'. (c) Mass spectrum of the expected product 'c'.



Figure S13. Kinetic analysis for 1-Gly reacting with 2. (a) LC-MS chromatograms (total ion current) for the reaction between 1-Gly (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 100  $\mu$ M 1-Gly, 500  $\mu$ M 2, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



**Figure S14. Kinetic analysis for 1 reacting with 2-Gly.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and **2-Gly** (labeled as 'b'). Reaction conditions: 1 mM 1, 5 mM **2-Gly**, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C. (b) Mass spectrum of the expected product 'c' from the reaction. (c) Linear fitting of the kinetics data to second-order rate equation.



Figure S15. Conversion analysis for 1-Gly reacting with 2-Gly. (a) LC-MS chromatograms (total ion current) for the reaction between 1-Gly (labeled as 'a') and 2-Gly (labeled as 'b'). Reaction conditions: 100  $\mu$ M 1-Gly, 500  $\mu$ M 2-Gly, 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of the peaks from the chromatogram. None showed the expected mass.



**Figure S16. Conversion analysis for 1-M1G variant nucleophilic peptide reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-M1G variant nucleophilic peptide** (labeled as **'a'**) and **2** (labeled as **'b'**). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of **'a'**. (c) Mass spectrum of the expected product **'c'**.



**Figure S17. Conversion analysis for 1-P3G variant nucleophilic peptide reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-P3G variant nucleophilic peptide** (labeled as 'a') and **2** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of 'a'. (c) Mass spectrum of the expected product 'c'.



**Figure S18.** Conversion analysis for 1-F4G variant nucleophilic peptide reacting with 2. (a) LC-MS chromatograms (total ion current) for the reaction between 1-F4G variant nucleophilic peptide (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of 'a'. (c) Mass spectrum of the expected product 'c'.



**Figure S19. Conversion analysis for 1-L5G variant nucleophilic peptide reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-L5G variant nucleophilic peptide** (labeled as **'a'**) and **2** (labeled as **'b'**). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of **'a'**. (c) Mass spectrum of the expected product **'c'**.



**Figure S20.** Conversion analysis for 1-P6G variant nucleophilic peptide reacting with 2. (a) LC-MS chromatograms (total ion current) for the reaction between 1-P6G variant nucleophilic peptide (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of 'a'. (c) Mass spectrum of the expected product 'c'.



**Figure S21. Conversion analysis for 1-V7G variant nucleophilic peptide reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-V7G variant nucleophilic peptide** (labeled as 'a') and **2** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of 'a'. (c) Mass spectrum of the expected product 'c'.



**Figure S22.** Conversion analysis for 1-V8G variant nucleophilic peptide reacting with 2. (a) LC-MS chromatograms (total ion current) for the reaction between 1-V8G variant nucleophilic peptide (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of 'a'. (c) Mass spectrum of the expected product 'c'.



**Figure S23. Conversion analysis for 1-Y9G variant nucleophilic peptide reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-Y9G variant nucleophilic peptide** (labeled as **'a'**) and **2** (labeled as **'b'**). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min. (b) Extracted-ion chromatogram of **'a'**. (c) Mass spectrum of the expected product **'c'**.



**Figure S24. Conversion analysis for 1-Gly variant nucleophilic peptide reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-Gly variant nucleophilic peptide** (labeled as 'a') and **2** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S25.** Conversion analysis for 1 reacting with 2-M1G electrophilic peptide. (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 2-M1G electrophilic peptide (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S26. Conversion analysis for 1 reacting with 2-P3G electrophilic peptide.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and **2-P3G electrophilic peptide** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S27. Conversion analysis for 1 reacting with 2-F4G electrophilic peptide.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and **2-F4G electrophilic peptide** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S28. Conversion analysis for 1 reacting with 2-L5G electrophilic peptide.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and **2-L5G electrophilic peptide** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S29. Conversion analysis for 1 reacting with 2-P6G electrophilic peptide.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and **2-P6G electrophilic peptide** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S30. Conversion analysis for 1 reacting with 2-V7G electrophilic peptide.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 2-V7G electrophilic peptide (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S31. Conversion analysis for 1 reacting with 2-V8G electrophilic peptide.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and **2-V8G electrophilic peptide** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S32. Conversion analysis for 1 reacting with 2-Y9G electrophilic peptide.** (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and **2-Y9G electrophilic peptide** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S33.** Conversion analysis for 1 reacting with 2-Gly electrophilic peptide. (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and 2-Gly electrophilic peptide (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S34. Conversion analysis for 1-C reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-C** (labeled as 'a') and **2** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S35.** Conversion analysis for 1-Int reacting with 2. (a) LC-MS chromatograms (total ion current) for the reaction between 1-Int (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S36. Conversion analysis for 1-Rev reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **1-Rev** (labeled as 'a') and **2** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S37. Conversion analysis for rev 1-C reacting with 2.** (a) LC-MS chromatograms (total ion current) for the reaction between **rev 1-C** (labeled as 'a') and **2** (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S38.** Conversion analysis for D-1 reacting with 2. (a) LC-MS chromatograms (total ion current) for the reaction between D-1 (labeled as 'a') and 2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S39.** Conversion analysis for 1 reacting with D-2. (a) LC-MS chromatograms (total ion current) for the reaction between 1 (labeled as 'a') and D-2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S40.** Conversion analysis for D-1 reacting with D-2. (a) LC-MS chromatograms (total ion current) for the reaction between D-1 (labeled as 'a') and D-2 (labeled as 'b'). Reaction conditions: 200 mM phosphate, 20 mM TCEP, pH 8.0, 37 °C, 60 min.



**Figure S41.** p*K*<sub>a</sub> determination of the cysteine thiol of 1 *via* plotting of conversion (%) at 1 h of reaction at various pH. Conditions: 0.2 M sodium phosphate, 20 mM TCEP, 37 °C.



Figure S42. a) Deconvoluted mass spectra of the light and heavy chains of 11 before and after reaction. b) 12 binds to HER2 in the Octet binding assay ( $K_d = 5.4 \pm 0.2$  nM). The concentration of recombinant HER2 in each experiment is shown next to the curve. c) 12 retained binding to BT474 cells (HER2 positive) compared to the controls. Cells were treated with biotin-EnAct-trastuzumab or controls, washed with PBS with 0.1% BSA and then treated with streptavidin-AlexaFluor-647 before analysis by flow cytometer.



**Figure S43.** Coomassie sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the reaction mixture between **11** and **S6** in 1 mg/mL HeLa cell lysate. Reaction conditions: 0.2 M sodium phosphate, 20 mM TCEP, pH 8, 37 °C, 1 mg/mL HeLa cell lysate.



Figure S44. Reactivity of 1 and 1-Gly towards various electrophiles. Conditions: 0.2 M sodium phosphate, 20 mM TCEP, 5% DMF, pH 8, 37 °C, 1 h. In general, 1 appears to be more selective towards moderately reactive perfluoroaromatics (DB and PS), while 1-Gly does not react with DB and PS under these conditions. Electrophile c is very reactive and thus reacts with both peptides. The reactivity of the rest of the electrophiles vary with peptide, indicating some selectivity. This could be due to the fact that 1 was optimized to react with DB and PS, and not with c through f.

Symbol	Sequence
<b>S1</b>	H <sub>2</sub> N–VTLPSTC(DB)GASK(GGS-biotin)–CONH <sub>2</sub>
<b>S2</b>	H2N-LKLGLEHCHHF-CONH2
<b>S3</b>	H <sub>2</sub> N–GCPGGLLKGRR–CONH <sub>2</sub>
1	H <sub>2</sub> N–MCPFLPVVYGLLKGRR–CONH <sub>2</sub>
2	H2N-MC(PS)PFLPVVYGLLKGRR-CONH2
3	H <sub>2</sub> N–MC(2')PFLPVVYGLLKGRR–CONH <sub>2</sub> (Product of 1 + 2)
4	H <sub>2</sub> N-FCPLYEFGLLKGRR-CONH <sub>2</sub>
5	H <sub>2</sub> N–MCPFLKLYEGLLKGRR–CONH <sub>2</sub>
6	H <sub>2</sub> N–VTLPSTC(DB)GAS–CONH <sub>2</sub>
7	H <sub>2</sub> N–LKLGLEHC(DB)HHF–CONH <sub>2</sub>
8	H <sub>2</sub> N–MC(DB)PFLPVVYGLLKGRR–CONH <sub>2</sub>
1-Gly	H <sub>2</sub> N–GCGGGGGGGGGLLKGRR–CONH <sub>2</sub>
2-Gly	H <sub>2</sub> N–GC(PS)GGGGGGGGGLLKGRR–CONH <sub>2</sub>
1-M1G	H <sub>2</sub> N– <u>G</u> CPFLPVVYGLLKGRR–CONH <sub>2</sub>
1-P3G	H2N–MCGFLPVVYGLLKGRR–CONH2
1-F4G	H <sub>2</sub> N–MCPGLPVVYGLLKGRR–CONH <sub>2</sub>
1-L5G	H <sub>2</sub> N–MCPFGPVVYGLLKGRR–CONH <sub>2</sub>
1-P6G	H <sub>2</sub> N–MCPFLGVVYGLLKGRR–CONH <sub>2</sub>
1-V7G	H <sub>2</sub> N–MCPFLPGVYGLLKGRR–CONH <sub>2</sub>
1-V8G	H <sub>2</sub> N–MCPFLPVGYGLLKGRR–CONH <sub>2</sub>
1-Y9G	H <sub>2</sub> N– <b>MCPFLPVV<u>G</u>GLLKGRR</b> –CONH <sub>2</sub>
1-P3p	H <sub>2</sub> N– <b>MC</b> <u>p</u> FLPVVYGLLKGRR–CONH <sub>2</sub>
1-P6p	H <sub>2</sub> N– <b>MCPFL<u>p</u>VVY</b> GLLKGRR–CONH <sub>2</sub>
1-P3,6-p	H <sub>2</sub> N– <b>MC</b> <u>p</u> FL <u>p</u> VVYGLLKGRR–CONH <sub>2</sub>
<b>1-C</b>	H <sub>2</sub> N–GLLKGRRMCPFLPVVY–CONH <sub>2</sub>
1-Int	H <sub>2</sub> N–GLLKGRRMCPFLPVVYGLLKGRR–CONH <sub>2</sub>
9	<b>MCPFLPVVY</b> FNMQQQRRFYEALHDPNLNEEQRNAKIKSIRDDC
10	MC(2')PFLPVVYFNMQQQRRFYEALHDPNLNEEQRNAKIKSIRDDC
<b>S4</b>	MCPFLPVVYFNMQQQRRFYEALHDPNLNEEQRNAKIKSIRDDC(2')
<b>S</b> 5	MC(2')PFLPVVYFNMQQQRRFYEALHDPNLNEEQRNAKIKSIRDDC(2')
<b>S6</b>	H <sub>2</sub> N–MC(PS)PFLPVVYGLLKGRK(GGS-biotin)–CONH <sub>2</sub>
11	RPI-Tmab
12	RPI(S6')Tmab

Table 2. List of peptides and their corresponding sequences.