Supporting Information

Rapid Oxime and Hydrazone Ligations with Aromatic Aldehydes for Biomolecular Labeling.

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Peptide synthesis. The peptides were obtained *via* manual solid phase peptide synthesis (SPPS) using an *in situ* neutralization/1*H*-benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate-(1-),3-oxide (HCTU) activation procedure for *t*Boc chemistry on a *p*-methylbenzhydrylamine (MBHA) resin.*(1)*

6-Hydrazinopyridyl-peptide 1 (0.05 mmol scale). The Fmoc protecting group of the C-terminal lysine residue of *t*Boc-D(OcHxl)YK(2ClZ)D(OcHxl)D(OcHxl)D(OcHxl)D(OcHxl) K(2ClZ)GGGGK(Fmoc) was removed on the resin by treatment with 20 *v*-% piperidine in DMF (4 × 3 min) followed by a DMF flow wash. 0.1 mmol (25 mg) 6-*t*Boc-hydrazinonicotinic acid (6-BOC-HNA, SoluLink Biosciences, San Diego, CA, USA) was added to a solution of 0.1 mmol (52 mg) benzotriazole-1-yl-oxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate (PyBOP®) and 0.12 mmol (d = 0.742 kgL^{-1}, 21 μL) diisopropylethylamine (DIEA) in 0.5 mL of DMF and the mixture was added to the resin (45 min). The resin was washed with DMF and the *t*Boc groups were removed with trifluoroacetic acid (TFA) (2 × 1 min). The resin was washed with DMF, DCM, and 1:1 *v/v* DCM/MeOH and dried under vacuum. The peptide was cleaved from the resin by HF using 4 *v*-% of anisole as a scavenger. After lyophilization, 6-hydrazinopyridyl-peptide **1** was purified by RP HPLC (gradient: 0 - 25% 9:1 *v/v* MeCN/H_2O in H_2O, 0.1 *v*-% TFA in 80 min; flow: 20 mL/min). **1**: ESI-MS calcd. for $C_{61}H_{89}N_{20}O_{25}$ ($[M+H]^+$): 1503.5, found 1504.0 ± 0.2.

Aminooxyacetyl-peptide 3 (0.1 mmol scale). The Fmoc protecting group of the C-terminal lysine residue of *t*Boc-D(OcHxl)YK(2ClZ)D(OcHxl)D(OcHxl)D(OcHxl)D(OcHxl) K(2ClZ)GGGGK(Fmoc) was removed on the resin by treatment with 20 *v*-% piperidine in DMF

(4 × 3 min) followed by a DMF flow wash. 0.5 mmol (96 mg) *t*Boc-aminooxyacetic acid was activated with 0.5 mmol (d = 0.806 kgL^{-1}, 78 μL) *N,N'*-diisopropylcarbodiimide and 0.5 mmol *N*-hydroxysuccinimide in DMF (0.5 mL) (10 min) and the emulsion was added to the resin (2 h). The resin was washed with DMF and the *t*Boc groups were removed with TFA (2 × 1 min). The resin was washed with DMF, DCM, and 1:1 *v/v* DCM/MeOH and dried under vacuum. The peptide was cleaved from the resin by HF using 4 *v*-% of anisole as a scavenger. After lyophilization, aminooxyacetyl-peptide **3** was purified by RP HPLC (gradient: 0 - 25% 9:1 *v/v* MeCN/H_2O in H_2O, 0.1 *v*-% TFA in 80 min; flow: 20 mL/min). **3**: ESI-MS calcd. for $C_{57}H_{87}N_{18}O_{26}$ ($[M+H]^+$): 1441.4, found 1441.0 ± 0.2.

Benzaldehyde-peptide 4. GLY(2BrZ)R(Tos)AG was synthesized by manual SPPS using the *in situ* neutralization/HCTU activation procedure for *t*Boc chemistry on a MBHA resin and cleaved from the resin with HF using 4 *v*-% of anisole as a scavenger. After lyophilization, GLYRAG was purified by RP HPLC (gradient: 5 - 30% 9:1 *v/v* MeCN/H_2O in H_2O, 0.1 *v*-% TFA in 80 min; flow: 15 mL/min). GLYRAG: ESI-MS calcd. for $C_{28}H_{46}N_{10}O_7$ ($[M+H]^+$): 635.7, found 635.5. The N-terminus of GLYRAG was functionalized in solution. For this, 22 mg (0.089 mmol) of *p*-carboxybenzaldehyde succimidyl ester was dissolved in 100 μL of MeCN and added to a solution of 40 mg (0.063 mmol) of GLYRAG in 4.4 mL of 0.1 M Na phosphate (pH 7) (2 h). The product was purified by RP HPLC (gradient: 15 - 40% 9:1 *v/v* MeCN/H_2O in H_2O, 0.1 *v*-% TFA in 80 min; flow: 15 mL/min) and lyophilized to give 21 mg (0.027 mmol, 43.5%) of **4**. **4**: ESI-MS calcd. for $C_{36}H_{50}N_{10}O_9$ ($[M+H]^+$): 767.8, found 767.5.

Hydrazone ligations at pH 4.5 (100 μM reactants). A 2 mM stock solution of 6-hydrazinopyridyl-peptide **1** (A), a 2 mM stock solution of benzaldehyde (B), and a 200 mM stock solution of aniline (C) were freshly prepared in 0.1 M NH_4OAc (pH 4.5). For the reaction in the absence of aniline, 50 μL A and 50 μL B were subsequently added to 900 μL of 0.1 M NH_4OAc (pH 4.5). For the reaction in the presence of 10 mM aniline, 50 μL C, 50 μL A, and 50 μL B were added subsequently to 850 μL of 0.1 M NH_4OAc (pH 4.5). The reactions were followed by RP HPLC (220 nm, 350 nm; gradient: 5 - 20% 9:1 *v/v* MeCN/H_2O in H_2O, 0.1 *v*-% TFA in 15 min; flow: 3 mL/min), hydrazone product **2** was quantitated by integration (350 nm), and analyzed by ESI-MS. **2**: ESI-MS calcd. for $C_{68}H_{93}N_{20}O_{25}$ ($[M+H]^+$): 1591.6, found 1591.5 ± 0.7. The data of the uncatalyzed reaction were fitted to the solution of the rate equation of a 2nd order reaction to resolve k_1 (Figure 1). The reaction in the presence of 10 mM aniline was too fast to resolve accurately k_1.

Hydrazone ligations at pH 4.5 (10 μM reactants). A 2 mM stock solution of 6-hydrazinopyridyl-peptide **1** (A), a 2 mM stock solution of benzaldehyde (B), and a 200 mM stock solution of aniline (C) were freshly prepared in 0.1 M NH_4OAc (pH 4.5). The hydrazone reactions were performed in quartz cuvettes (l = 1 cm). For the reaction in the absence of aniline, 10 μL of A and 10 μL of B were added subsequently to 1.980 mL of 0.1 M NH_4OAc (pH 4.5) (see Figure 2 for full dataset; first 5 minutes shown in Figure 2 of the paper). For the reaction in the presence of 10 mM aniline, 100 μL of C, 10 μL of B, and 10 μL of A were added subsequently to 1.880 mL of 0.1 M NH_4OAc (pH 4.5), and for the reaction in the presence of 100 mM aniline, 1.000 mL of C, 10 μL of B, and 10 μL of A were added subsequently to 980 μL of 0.1 M NH_4OAc (pH 4.5). Time was started as soon as the last component was added and 30 -

60 seconds were taken to vigorously vortex the reaction mixture, transfer it into a cuvette and start the measurement. 0.1 M NH_4OAc containing exactly the same amount of aniline as the reaction mixture was used as a blanc. The reactions were performed at room temperature and monitored by UV-Vis (350 nm). Once the reaction fully reached equilibrium, the total amount of hydrazone product **2** was determined by adding in a 10-fold excess of benzaldehyde (final concentration 110 μM), shifting the equilibrium completely towards the product. The data were fitted to the solution of the rate equation of a 2nd order reversible reaction.*(2)* It should be noted that conditions differ slightly for each reaction (ionic strength, solvent effect of 100 mM aniline). These difference may explain the differences observed in the equilibrium constants. Furthermore, the endpoints were not fixed, which may cause an underestimation of the calculated error in the k_{-1} of the reaction.

Hydrazone ligation at pH 7 (10 μM reactants, 100 mM aniline). This reaction was performed and analyzed analogous to the reaction at pH 4.5. Stock solutions were freshly prepared in 0.3 M Na phosphate (pH 7). The data were fitted to the solution of the rate equation of a 2nd order reversible reaction*(2)* (Figure 3).

Synthesis of HYNIC-HSA. 15 equiv of succinimidyl hydraziniumnicotinate hydrochloride (SHNH, SoluLink Biosciences, San Diego, CA, USA) (0.646 mg) in DMSO (20 μL) were added to a solution of 10 mg of HSA in 0.5 mL of 0.05 M Na phosphate (pH 7.5). The solution was put under nitrogen and protected from light and the reaction was continued for 2 hours at room temperature. The excess of SHNH was removed by size exclusion chromatography over a NAP™ 5 column, preequilibrated with 0.1 M Na phosphate (pH 7). HYNIC-HSA was eluted

from the column with 0.1 M Na phosphate (pH 7). The concentration of HSA in the collected solution was determined to be 150 μM, using the Bradford assay. The concentration of HYNIC was determined by adding 25 μL of the protein solution to 2.475 mL of a 0.5 mM solution of *p*-nitrobenzaldehyde in 0.1 M NH_4OAc (pH 4.5). In time HYNIC reacts with *p*-nitrobenzaldehyde to give a chromophore with $\varepsilon = 2.53 \times 10^4$ $M^{-1}cm^{-1}$ at 385 nm.*(3)* After 5 h the absorption at 385 nm was measured and the concentration of HYNIC was calculated to be 893 μM. This corresponds to on average ~6 HYNIC groups per HSA.

Note on the stability of 6-hydrazinopyridine and its hydrazone with benzaldehyde. In contrast to the hydrazone, the stability of the free hydrazinopyridine is limited. As a solid, the compound is best stored in the freezer protected from light as a salt (HCl / TFA). In solution, the hydrazinopyridine moiety is prone to decomposition due to oxidation in a similar way, but to a lesser extent than phenylhydrazine.*(3)* The fast ligation rates observed in the presence of aniline minimize these side reactions. HYNIC labeled molecules are stored at + 4 °C or -20 °C, under nitrogen and protected from light. Hydrazone products based on HYNIC and benzaldehyde are stored at a concentration of > 50 μM.

Oxime ligation at pH 7 (100 μM reactants, 100 mM aniline). A 2 mM stock solution of aminooxyacetyl-peptide **3** (A), a 2 mM stock solution of benzaldehyde (B), and a 200 mM stock solution of aniline (C) were prepared in 0.3 M Na phosphate (pH 7). 500 μL C, 50 μL A, and 50 μL B were added subsequently to 400 μL of 0.3 M Na phosphate (pH 7). The reaction was followed by RP HPLC (220 nm; gradient: 0 - 20% 9:1 *v/v* MeCN/H_2O in H_2O, 0.1 *v*-% TFA in 15 min; flow: 3 mL/min), oxime product **3a** was quantitated by integration (220 nm), and

analyzed by ESI-MS. **3a**: ESI-MS calcd. for $C_{64}H_{91}N_{18}O_{26}$ ($[M+H]^+$): 1529.5, found 1529.5 ± 0.7. The data of the reaction were fitted to the solution of the rate equation of a 2nd order reaction.

Oxime ligation at pH 7 (1 mM reactants, no aniline). The rate constant of the uncatalyzed reaction was determined by reacting **3** with benzaldehyde at 1 mM reactants. For this 250 µL A and 250 µL B were mixed. The reaction was followed by RP HPLC (220 nm; gradient: 0 - 20% 9:1 *v/v* MeCN/H_2O in H_2O, 0.1 *v*-% TFA in 15 min; flow: 3 mL/min), oxime product **3a** was quantitated by integration (220 nm). The data of the reaction were fitted to the solution of the rate equation of a 2nd order reaction and a rate constant of 0.14 ± 0.02 $M^{-1}s^{-1}$ was calculated (Figure 4).

Thrombin activity assay. 150 µl of human α-thrombin (HaemTech, Essex Junction VT) (90 µM) was incubated with either 150 µl of 200 mM aniline and 1 mM benzamidine.HCl or with an equal volume of 1 mM benzamidine.HCl in 50 mM Hepes/150 mM NaCl (pH 7.4) at room temperature for 6 hours. The weak inhibitor benzamidine.HCl was added to prevent self-cleavage of thrombin during incubation. Samples were then dialyzed (1 × 2000 mL) into 50 mM Hepes/150 mM NaCl (pH 7.4) overnight at 4 °C before thrombin activity was evaluated using the chromogenic substrate Pefachrome-TH (Pentapharma, Basel, Switzerland). Briefly, 50 µl of varying concentrations of substrate (0-1600 µM) were added to samples containing 20 nM aniline-treated thrombin or untreated controls. The generation of *p*-nitroaniline was monitored immediately using a 96 well plate reader (Molecular Dynamics, Sunnyvale CA) at 405 nm wavelength (Figure 5).

Hydrazone ligation: 100 μM reactants, no aniline, pH 4.5, room temperature

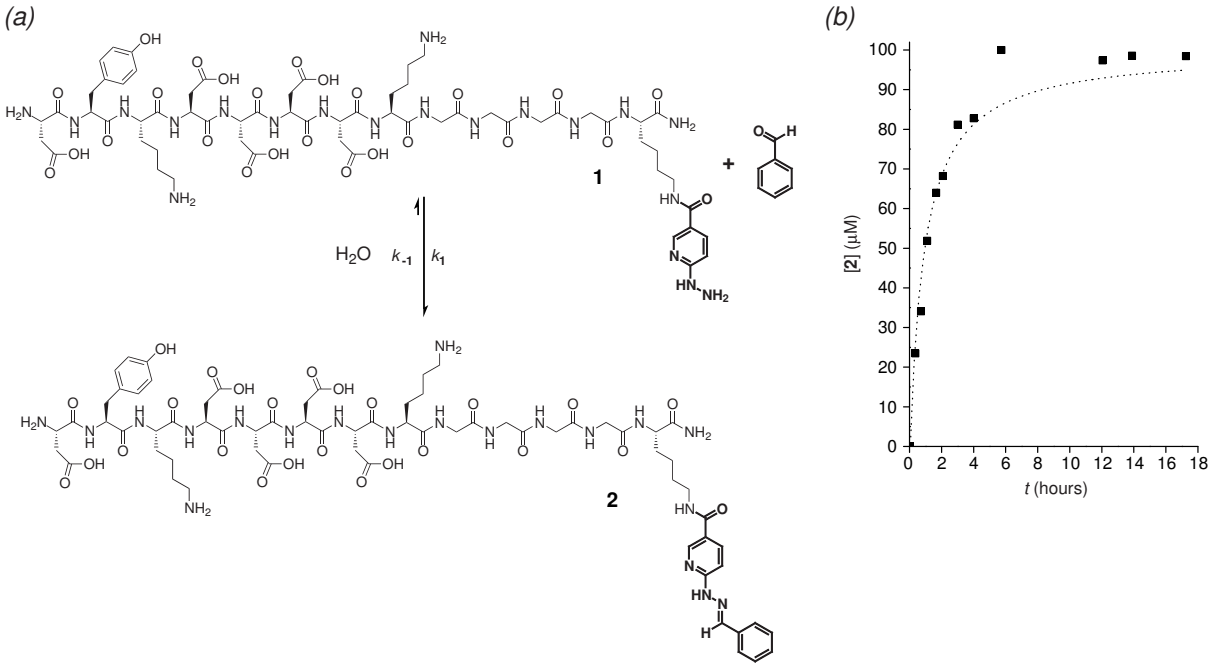

Figure 1. *(a)* Hydrazone reaction of 6-hydrazinopyridyl-peptide **1** and benzaldehyde in the absence of aniline catalyst; *(b)* Formation of hydrazone **2** over time at 100 μM reactants at room temperature in 0.1 M NH_4OAc (pH 4.5). The dotted line represents the fit of the data to the rate equation.

Hydrazone ligation: 10 μM reactants, no aniline, pH 4.5, room temperature (full dataset)

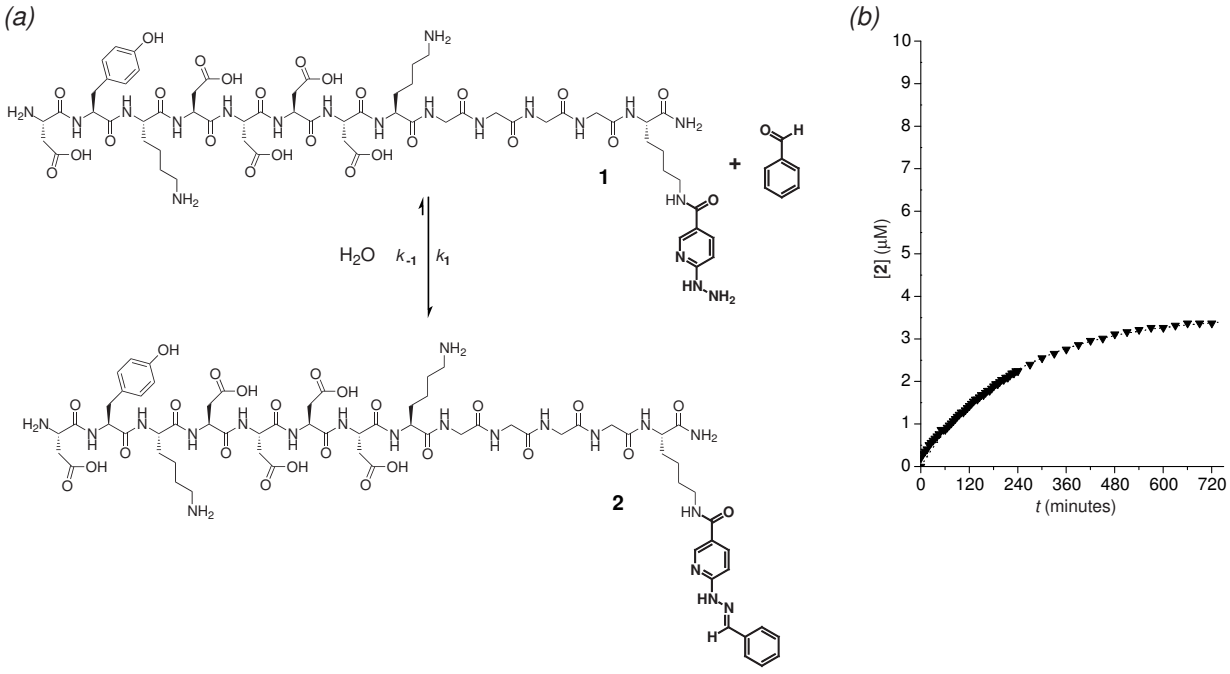

Figure 2. *(a)* Hydrazone reaction of 6-hydrazinopyridyl-peptide **1** and benzaldehyde in the absence of aniline catalyst; *(b)* Formation of hydrazone **2** over time at 10 μM reactants at room temperature in 0.1 M NH_4OAc (pH 4.5). The dotted line represents the fit of the data to the rate equation.

Hydrazone ligation: 10 μM reactants, 100 mM aniline, pH 7, room temperature

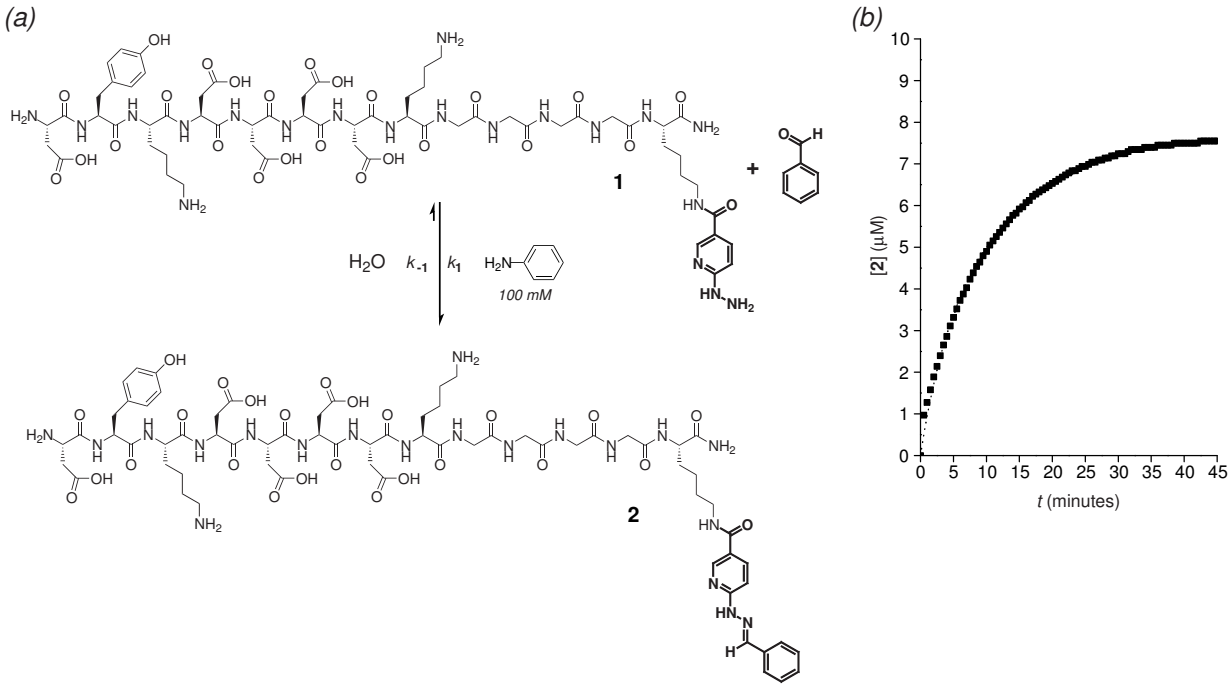

Figure 3. *(a)* Hydrazone reaction of 6-hydrazinopyridyl-peptide **1** and benzaldehyde in the presence of aniline catalyst; *(b)* Formation of hydrazone **2** over time at 10 μM reactants at room temperature in 0.3 M Na phosphate (pH 7) in the presence of 100 mM aniline. The dotted line represents the fit of the data to the rate equation.

Oxime ligation: 1 mM reactants, no aniline, pH 7, room temperature

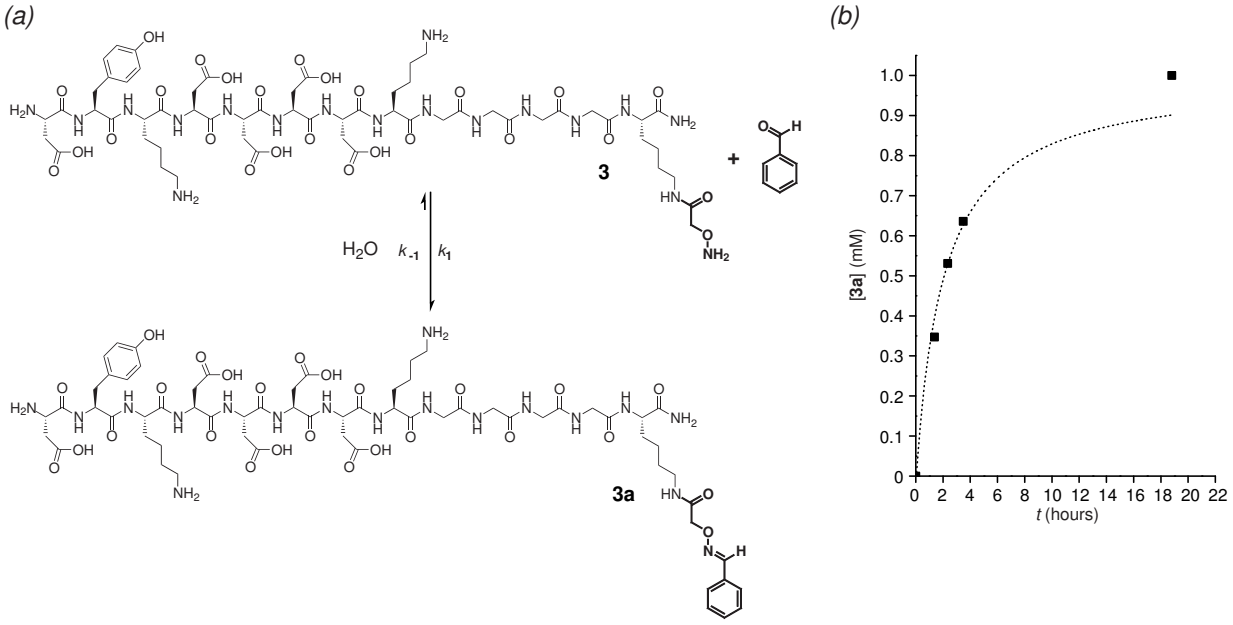

Figure 4. *(a)* Oxime ligation of aminooxyacetyl-functionalized peptide **3** and benzaldehyde in the absence aniline catalyst; *(b)* Formation of oxime **3a** over time at 1 mM reactants at room temperature in 0.3 M Na phosphate (pH 7). The dotted line represents the fit of the data to the rate equation.

Thrombin activity assay

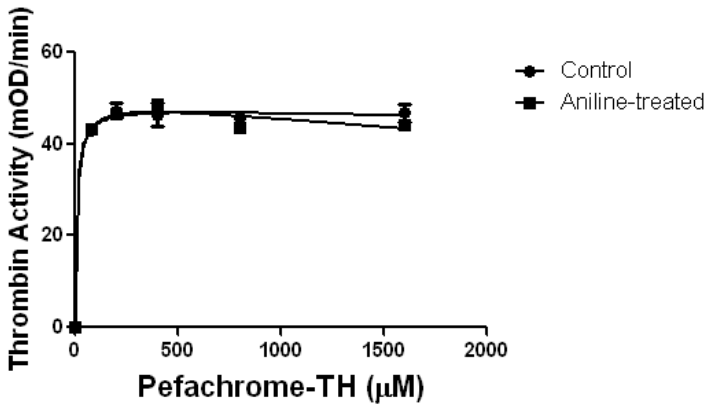

Figure 5. Thrombin activity assay for aniline-treated and untreated thrombin (20 nM) based on the conversion of the substrate Pefachrome-TH (initial concentration ranging from 0-1600 μM) to *p*-nitroaniline in 50 mM Hepes/150 mM NaCl (pH 7.4). The reaction was followed by UV-Vis (405 nm).

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(3) Schwartz, D. A., Abrams, M. J., Hauser, M. M., Gaul, F. E., Larsen, S. K., Rauh, D., Zubietas, J. A. (1991) Preparation of hydrazino-modified proteins and their use for the synthesis of ^{99m}Tc-protein conjugates. *Bioconjugate Chem. 2*, 333–330.