Supporting Information

Zhang et al. 10.1073/pnas.1221012110

SI Text

SI Materials and Methods

All commercial materials (Aldrich, Fluka, and GL Biochem) were used without further purification. All solvents were reagent grade or HPLC grade (RCI Labscan or Duksan Pure Chemicals). Anhydrous THF was freshly distilled from sodium and benzophenone. Dry dichloromethane (DCM) was distilled from calcium hydride (CaH_2). All separations involved a mobile phase of 0.05% TFA (vol/vol) in acetonitrile (solvent A)/0.05% TFA (vol/vol) in water (solvent B). HPLC separations were performed with a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using a Sunfire C18 column (5 μm, 4.6 × 150 mm) or Vydac C18 column (5 μm, 4.6 × 150 mm) at a flow rate of 0.6 mL/min for analytical HPLC and Sunfire prep C18 OBD column (10 μm, 19 × 250 mm) or Vydac Prep C18 column (10 μm, 22 × 250 mm) at a flow rate of 10 mL/min for preparative HPLC. Low-resolution mass spectral analyses were performed with a Waters 3100 mass spectrometer. The UV-absorption spectra in the region 250–350 nm were measured with a Varian CARY 50 Bio UV-visible spectrometer.

Solid-Phase Peptide Synthesis According to Fmoc Strategy

Synthesis was performed manually on Rink amide-AM resin or 2-chlorotrityl chloride resin. Peptides were synthesized under standard Fmoc/*t*-Bu protocols. The deblock mixture was a mixture of 20/80 (vol/vol) of piperidine/DMF. The following Fmoc amino acids from GL Biochem were used: Fmoc-Ala-OH, Fmoc-D-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Glu(O*t*Bu)-OH, Fmoc-Gln(Trt)-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(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH, Z-Asp (O*t*Bu)-OH. Upon completion of synthesis, the peptide resin was subjected to a cleavage mixture. The resin was filtered and the combined filtrates were blown off under a stream of condensed air. The crude product was triturated with cold diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The remaining solid was ready for HPLC purification.

General Procedure for the Synthesis of Peptide SAL Ester

A general route for the synthesis of peptide SAL ester is shown in Fig. S1. The peptide *N*-acyl-benzimidazolinone (Nbz) was prepared according to Blanco-Canosa and Dawson's protocol (1). The on-resin phenolysis for the synthesis of peptide SAL ester was conducted by adding Na_2CO_3 (10.0 equiv) and a solution of salicylaldehyde dimethyl acetal (1.0 mL/0.1 g resin) in dry DCM/THF (1/3, vol/vol) to the resin-bound peptide Nbz. The suspension was stirred at room temperature for 16 h. The resin was then filtered and washed with DCM. The combined filtrates were concentrated under a stream of condensed air. The oily residue was treated with TFA/H_2O (95/5, vol/vol) for 1 h. The solvent was blown off under a stream of condensed air. The crude product was triturated with cold diethyl ether to give a white suspension, which was centrifuged and the ether subsequently decanted. The remaining solid was ready for HPLC purification. Using this on-resin phenolysis approach, several esters were synthesized in 20–30% yield from the resin.

Epimerization Study of the Preparation of Peptide SAL Ester Using the On-Resin Phenolysis Approach

The epimerization study of the peptide SAL ester formation was conducted by comparing the resultant Z-DTTADA-SAL ester and Z-DTTAD*a*-SAL ester. These esters were prepared as described above, using the general procedure. LC-MS analysis showed that no epimerization occurs during the on-resin phenolysis.

Epimerization Study of Serine Ligation

An epimerization study of serine ligation was performed using Z-DTTADA-SAL ester and Z-DTTAD*a*-SAL ester reacting with H-SRQQGESNQERGARARL-NH_2, respectively (see below). These ligated products were analyzed by LC-MS studies. No epimerization during ligation was observed.

Application of Serine/Threonine Ligation in the Convergent Synthesis of Peptides (Model Peptides, oCRH, Forteo)

Synthesis of Model Peptides. ***Compound 1: Z-DTTADA-SAL ester.*** Compound **1** was synthesized according to the general procedure. Preparative HPLC purification (25–55% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **1** as a white powder.

ESI calcd for $C_{37}H_{46}N_6O_{16}$ $[M+H]^+$ $m/z = 831.79$, found: 831.88.

Compound 2: Z-DTTADa-SAL ester. Compound **2** was synthesized according to the general procedure. Preparative HPLC purification (25–55% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **2** as a white powder.

ESI calcd for $C_{37}H_{46}N_6O_{16}$ $[M+H]^+$ $m/z = 831.79$, found: 831.95.

Compound 3: H-SRQQGESNQERGARARL-NH_2. Compound **3** was synthesized on Rink amide-AM resin according to the general solid-phase peptide synthesis (SPPS) procedure. The cleavage and global deprotection mixture was a mixture of TFA/Phenol/H_2O/Triisopropylsilane (TIPS) (88/5/5/2, vol/vol/vol/vol). Preparative HPLC purification (5–25% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **3** as a white powder.

ESI calcd for $C_{75}H_{132}N_{34}O_{27}$ $[M+H]^+$ $m/z = 1{,}943.06$ $[M+2H]^{2+}$ $m/z = 972.03$ $[M+3H]^{3+}$ $m/z = 648.35$ $[M+4H]^{4+}$ $m/z = 486.52$, found: 972.78; 649.47; 486.85.

Compound 4: Z-DTTADASRQQGESNQERGARARL-NH_2. The synthesis of compound **4** by serine ligation is shown in Fig. S2. First, 3.9 mg of compound **3** (1.5 equiv) and 1.1 mg of compound **1** (1.0 equiv) were dissolved in pyridine/acetic acid (1:2 mole/mole) at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 8 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with AcOH/H_2O (50/50, vol/vol) for 20 min or TFA/H_2O/TIPS (95/2.5/2.5, vol/vol/vol) for 10 min. The solvent was blown off under a stream of condensed air, followed by preparative HPLC purification (5–30% CH_3CN/H_2O over 30 min) to afford 1.3 mg (36% yield) of compound **4** as a white powder.

ESI calcd for $C_{105}H_{172}N_{40}O_{41}$ $[M+H]^+$ $m/z = 2{,}651.73$ $[M+2H]^{2+}$ $m/z = 1{,}326.37$ $[M+3H]^{3+}$ $m/z = 884.58$ $[M+4H]^{4+}$ $m/z = 663.68$, found: 1,328.24; 886.07; 664.86.

Compound 5: Z-DTTADaSRQQGESNQERGARARL-NH_2. The synthesis of compound **5** by serine ligation is shown in Fig. S2. First, 6.8 mg of compound **3** (1.5 equiv) and 1.7 mg of compound **2** (1.0 equiv) were dissolved in pyridine/acetic acid (1:2 mole/mole) at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 8 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue

was then treated with TFA/H_2O/TIPS (95/2.5/2.5, vol/vol/vol). Preparative HPLC purification (5–30% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 1.9 mg (35% yield) of compound **5** as a white powder.

ESI calcd for $C_{105}H_{172}N_{40}O_{41}$ $[M+H]^+$ m/z = 2,651.73 $[M+2H]^{2+}$ m/z = 1,326.37 $[M+3H]^{3+}$ m/z = 884.58 $[M+4H]^{4+}$ m/z = 663.68, found: 1,326.16; 884.58; 663.70.

Compound 6: H-VIGGVGNN-SAL ester. Compound **6** was synthesized according to the general procedure. Preparative HPLC purification 5–30% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **6** as a white powder.

ESI calcd for $C_{37}H_{56}N_{10}O_{12}$ $[M+H]^+$ m/z = 833.90, found: 833.50.

Compound 7: H-TLHAPTD-OH. Compound **7** was synthesized on 2-chlorotrityl chloride resin according to the general SPPS procedure. The cleavage and global deprotection mixture was a mixture of TFA/H_2O (95/5, vol/vol). Preparative HPLC purification (10–70% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **7** as a white powder.

ESI calcd for $C_{32}H_{51}N_9O_{12}$ $[M+H]^+$ m/z = 754.80, found: 754.45.

Compound 8: H-VIGGVGNNTLHAPTD-OH. The synthesis of compound **8** by threonine ligation is shown in Fig. S2. First, 1.1 mg of compound **7** (1.3 equiv) and 1.0 mg of compound **6** (1.0 equiv) were dissolved in pyridine/acetic acid (1:2 mole/mole) at a concentration of 20 mM at room temperature. The reaction mixture was stirred at room temperature for 2 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with TFA/H_2O (95/5, vol/vol).

ESI calcd for $C_{62}H_{101}N_{19}O_{22}$ $[M+H]^+$ m/z = 1,465.58, $[M+2H]^{2+}$ m/z = 733.29, found: 1,465.27; 733.17.

Synthesis of oCRH. ***Compound 9: H-TKADQLAQQAHSNRKLLDIA-OH.*** Compound **9** was synthesized on 2-chlorotrityl chloride resin according to the general SPPS procedure. The cleavage and all global deprotection mixture was a mixture of TFA/H_2O (95/5, vol/vol). Preparative HPLC purification (15–30% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **9** as a white powder.

ESI calcd for $C_{94}H_{161}N_{31}O_{31}$ $[M+H]^+$ m/z = 2,222.47 $[M+2H]^{2+}$ m/z = 1,111.74 $[M+3H]^{3+}$ m/z = 741.49 $[M+4H]^{4+}$ m/z = 556.37, found: 1,112.32; 742.00; 556.46.

Compound 10: N_3-SQEPPISLDLTFHLLREVLEM-SAL ester. N_3-Ser(tBu)-OH was synthesized as described preciously (2). Compound **10** was synthesized according to the general procedure. Preparative HPLC purification (40–60% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **10** as a white powder.

ESI calcd for $C_{118}H_{181}N_{29}O_{35}S$ $[M+H]^+$ m/z = 2,598.94 $[M+2H]^{2+}$ m/z = 1,299.97 $[M+3H]^{3+}$ m/z = 866.98, found: 1,300.40; 867.39.

Compound 11: N_3-SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA-OH. The synthesis of compound **11** by threonine ligation is shown in Fig. S3. First, 1.7 mg of compound **9** (1.5 equiv) and 1.4 mg of compound **10** (1.0 equiv) were dissolved in pyridine/acetic acid (1:6 mole/mole) at a concentration of 10 mM at room temperature. The reaction mixture was stirred at room temperature for 13 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with TFA/H_2O (95/5, vol/vol). Preparative HPLC purification (25–70% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 0.9 mg (38% yield) of compound **11** as a white powder.

ESI calcd for $C_{205}H_{336}N_{60}O_{64}S$ $[M+H]^+$ m/z = 4,698.29 $[M+3H]^{3+}$ m/z = 1,566.76 $[M+4H]^{4+}$ m/z = 1,175.32 $[M+5H]^{5+}$ m/z = 940.46 $[M+6H]^{6+}$ m/z = 783.88, found: 1,567.51; 1,175.90; 940.74; 784.28.

Compound 12: H-SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA-OH. First, 1.7 mg of compound **11** was dissolved in 20 μL CH_3CN/H_2O (60/40, vol/vol). To the solution of compound **11**, a solution of HCl·TCEP (20.0 equiv) in PBS (pH = 7.51) was added (3). The mixture was stirred at room temperature overnight to afford compound **12** exclusively.

ESI calcd for $C_{205}H_{338}N_{58}O_{64}S$ $[M+H]^+$ m/z = 4,672.29 $[M+3H]^{3+}$ m/z = 1,558.10 $[M+4H]^{4+}$ m/z = 1,168.82 $[M+5H]^{5+}$ m/z = 935.26 $[M+6H]^{6+}$ m/z = 779.55 $[M+7H]^{7+}$ m/z = 668.33, found: 1,559.10; 1,169.65; 935.77; 780.19; 668.89.

Synthesis of Forteo. ***Compound 13: H-SMERVEWLRKKLQDVHNF-OH.*** Compound **13** was synthesized on 2-chlorotrityl chloride resin according to the general SPPS procedure. The cleavage and all global deprotection mixture was a mixture of TFA/phenol/H_2O/TIPS (88/5/5/2, vol/vol/vol/vol). Preparative HPLC purification (20–45% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **13** as a white powder.

ESI calcd for $C_{103}H_{163}N_{31}O_{28}S$ $[M+H]^+$ m/z = 2,316.65 $[M+2H]^{2+}$ m/z = 1,158.83 $[M+3H]^{3+}$ m/z = 772.88 $[M+4H]^{4+}$ m/z = 579.91, found: 1,158.58; 772.60; 579.78.

Compound 14: Msz-SVSEIQLMHNLGKHLN-SAL ester. Methylsulfinylbenzyloxy (Msz)-Ser(*t*Bu)-OH was synthesized as described previously (4). Compound **14** was synthesized according to the general procedure. Preparative HPLC purification (20–45% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **14** as a white powder.

ESI calcd for $C_{94}H_{142}N_{24}O_{28}S_2$ $[M+H]^+$ m/z = 2,121.41 $[M+2H]^{2+}$ m/z = 1,061.20 $[M+3H]^{3+}$ m/z = 707.80, found: 1,061.00; 707.58.

Compound 15: Msz-SVSEIQLMHNLGKHLNSMERVEWLRKKLQDVHNF-OH. The synthesis of compound **15** by serine ligation is shown in Fig. S4. First, 1.9 mg of compound **13** (1.2 equiv) and 1.3 mg of compound **14** (1.0 equiv) were dissolved in pyridine/acetic acid (1:1 mole/mole) at a concentration of 50 mM at room temperature. The reaction mixture was stirred at room temperature for 1 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with TFA/H_2O (50/50, vol/vol). Preparative HPLC purification (20–45% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 0.85 mg (32% yield) of compound **15** as a white powder.

ESI calcd for $C_{190}H_{299}N_{55}O_{54}S_3$ $[M+H]^+$ m/z = 4,314.94 $[M+3H]^{3+}$ m/z = 1,438.98 $[M+4H]^{4+}$ m/z = 1,079.48 $[M+5H]^{5+}$ m/z = 863.79 $[M+6H]^{6+}$ m/z = 719.99, found: 1,439.06; 1,079.45; 863.88; 720.03.

Synthesis of Human Erythrocyte Acylphosphatase Via Serine/Threonine Ligation

Compound 16. Acylphosphatase (70–98): NH_2-SPKSHIDKANFNNEKVILKLDYSDFQIVK-OH. Compound **16** was synthesized on 2-chlorotrityl chloride resin according to the general SPPS procedure. The cleavage and all global deprotection mixture was a mixture of TFA/H_2O/TIPS (95/2.5/2.5, vol/vol/vol). Preparative HPLC purification (20–35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **16** as a white powder.

ESI calcd for $C_{154}H_{244}N_{40}O_{46}$ $[M+2H]^{2+}$ m/z = 1,696.92 $[M+3H]^{3+}$ m/z = 1,131.61 $[M+4H]^{4+}$ m/z = 848.96 $[M+5H]^{5+}$ m/z = 679.37, $[M+6H]^{6+}$ m/z = 566.30, found: 1,697.49; 1,131.73; 848.96; 679.30; 566.50.

Compound 17. Acylphosphatase (46–69) SAL Ester: FmocNH-TVQGQLQGPISKVRHMQEWLETRG-SAL Ester. Fmoc-TVQGQLQGPISKVRHMQEWLETRG-OH was synthesized on 2-chlorotrityl chloride resin according to the general SPPS procedure. The crude protected peptide was coupled with salicylaldehyde dimethyl acetal,

DCC, and DMAP in anhydrous DCM, followed by filtration and treatment with TFA/Phenol/ H_2O (95/2.5/2.5, vol/vol/vol). The crude peptide SAL ester was precipitated with diethyl ether. Preparative HPLC purification (20–35% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **17** as a white powder.

ESI calcd for $C_{142}H_{210}N_{38}O_{39}S$ $[M+2H]^{2+}$ m/z = 1,553.74 $[M+3H]^{3+}$ m/z = 1,036.16 $[M+4H]^{4+}$ m/z = 777.37, found: 1,553.46; 1,036.32; 777.40.

Compound 18. Acylphosphatase (46–98): FmocNH-TVQGQLQGPISKVRH-MQEWLETRGSPKSHIDKANFNNEKVILKLDYSDFQIVK-OH. First, 21.3 mg of compound **16** (1.2 equiv) and 16.3 mg of compound **17** (1.0 equiv) were dissolved in pyridine/acetic acid (1:1 mole/mole) at a concentration of 25 mM at room temperature (Fig. S5). The reaction was stirred at room temperature for 10 h. After completion of the reaction, the solvent was blown off under a stream of condensed air. The residue was then treated with TFA/H_2O (95/5, vol/vol) for 10 min. The solvent was blown off under a stream of condensed air, followed by preparative HPLC purification (20–50% CH_3CN/ H_2O over 30 min) to afford 10.4 mg (31% yield) of compound **18** as a white powder.

ESI calcd for $C_{289}H_{448}N_{78}O_{83}S$ $[M+4H]^{4+}$ m/z = 1,594.80 $[M+5H]^{5+}$ m/z = 1,276.04 $[M+6H]^{6+}$ m/z = 1,063.53 $[M+7H]^{7+}$ m/z = 911.74 $[M+8H]^{8+}$ m/z = 797.90 $[M+9H]^{9+}$ m/z = 709.35 found: 1,574.72; 1,276.20; 1,063.62; 911.58; 797.95; 709.35.

Compound 19. H_2*N*-TVQGQLQGPISKVRHMQEWLETRGSPKSHIDKANFN-NEKVILKLDYSDFQIVK-OH. Compound **18** was treated with DEA/DCM (1/2, vol/vol) for 1.5 h at room temperature to remove the terminal Fmoc protecting group. The reaction mixture was centrifuged to remove the liquid phase. The residual solid phase was lyophilized to afford compound **19** as a white powder, directly subjected to the next step.

ESI calcd for $C_{274}H_{438}N_{78}O_{81}S$ $[M+4H]^{4+}$ m/z = 1,539.24 $[M+5H]^{5+}$ m/z = 1,231.59 $[M+6H]^{6+}$ m/z = 1,026.49 $[M+7H]^{7+}$ m/z = 879.99 $[M+8H]^{8+}$ m/z = 770.12 $[M+9H]^{9+}$ m/z = 684.66 $[M+10H]^{10+}$ m/z = 616.30, found: 1,539.58; 1,231.79; 1,026.42; 880.01; 770.28; 684.93; 616.75.

Compound 20. Acylphosphatase (1-45) SAL Ester. Ac-AEGNTLISVDYEIF-GKVQG(AcHmb)VFFRKHTQAEG(AcHmb)KKLGLVGWVQNTDRG SAL Ester. Fmoc-Ile-Ser($\psi^{Me,Me}$Pro)-OH was synthesized as described preciously (5). Fmoc-Gly(Hmb)-OH was prepared according to the literature (6). Peptide Ac-AEGNTLψ(IS)VDYEIFGKVQG (AcHmb)VFFRKHTQAEG(AcHmb)KKLGLVGWVQNTDRG-OH was synthesized according to the general SPPS procedure. The crude protected peptide was coupled with salicylaldehyde dimethyl acetal using benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and N, N-diisopropylethylamine (DIEA) in anhydrous DCM, followed by treatment with TFA/Phenol/H_2O (95/2.5/2.5, vol/vol/vol). The crude peptide SAL ester was precipitated with ether. Preparative HPLC purification (30–50% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded compound **20** as a white powder.

ESI calcd for $C_{253}H_{375}N_{63}O_{75}$ $[M+3H]^{3+}$ m/z = 1,834.02 $[M+4H]^{4+}$ m/z = 1,375.77 $[M+5H]^{5+}$ m/z = 1,100.81 $[M+6H]^{6+}$ m/z = 917.51, found: 1,833.42; 1,375.22; 1,100.60; 917.43.

Compound 21. AcAEGNTLISVDYEIFGKVQG(AcHmb)VFFRKHTQAEG (AcHmb)KKLGLVGWVQNTDRGTVQGQLQGPISKVRHMQEWLETRGSPKSH-IDKANFNNEKVILKLDYSDFQIVK-OH. The synthesis of compound **21** by threonine ligation is shown in Fig. S5. First, 2.2 mg of compound **19** (1.16 equiv) and 1.69 mg of compound **20** (1.0 equiv) were dissolved in pyridine/acetic acid (1:10 mole/mole) at a concentration of 10 mM at room temperature. The reaction mixture was stirred at room temperature for 22 h, and the solvent was then blown off under a stream of condensed air. The residue was then treated with TFA/H_2O (75/25, vol/vol). Preparative HPLC purification (20–60% CH_3CN/H_2O over 30 min) followed by concentration at reduced pressure and lyophilization afforded 1.2 mg (34% yield) of compound **21** as a white powder.

ESI calcd for $C_{520}H_{807}N_{141}O_{154}S$ $[M+6H]^{6+}$ m/z = 1,922.65 $[M+7H]^{7+}$ m/z = 1,648.13 $[M+8H]^{8+}$ m/z = 1,442.24 $[M+9H]^{9+}$ m/z = 1,282.10 $[M+10H]^{10+}$ m/z = 1,153.99 $[M+11H]^{11+}$ m/z = 1,049.17 $[M+12H]^{12+}$ m/z = 961.82 $[M+13H]^{13+}$ m/z = 887.91, $[M+14H]^{14+}$ m/z = 824.56, found: 1,922.32; 1,647.91; 1,442.13; 1,282.13; 1,153.93; 1,049.07; 961.61; 887.73; 824.58.

Compound 22. AcAEGNTLISVDYEIFGKVQG(Hmb)VFFRKHTQAEG(Hmb) KKLGLVGWVQNTDRGTVQGQLQGPISKVRHMQEWLETRGSPKSHIDKANF-NNEKVILKLDYSDFQIVK-OH. The compound **21** was dissolved in 10% N_2H_4 in H_2O.The mixture was stirred at room temperature for 1 h to afford **22** exclusively. The crude product was not further purified.

ESI calcd for $C_{516}H_{803}N_{141}O_{152}S$ $[M+6H]^{6+}$ m/z = 1,908.64 $[M+7H]^{7+}$ m/z = 1,636.12 $[M+8H]^{8+}$ m/z = 1,431.73, $[M+9H]^{9+}$ m/z = 1,272.76 $[M+10H]^{10+}$ m/z = 1,145.58 $[M+11H]^{11+}$ m/z = 1,041.53 $[M+12H]^{12+}$ m/z = 954.82 $[M+13H]^{13+}$ m/z = 881.45 $[M+14H]^{14+}$ m/z = 818.56 $[M+15H]^{15+}$ m/z = 764.058, found: 1,908.14; 1,635.90; 1,432.01; 1,272.53; 1,145.76; 1,041.42; 954.64; 881.21; 818.28; 764.20.

Compound 23. AcAEGNTLISVDYEIFGKVQGVFFRKHTQAEGKKLGLVGWV-QNTDRGTVQGQLQGPISKVRHMQEWLETRGSPKSHIDKANFNNEKVILKLDY-SDFQIVK-OH. The compound **22** was treated with TFA/H_2O/TIPS (95/2.5/2.5, vol/vol/vol) at room temperature for 1 h. After completion of the reaction, the solvent was blown off under a stream of condensed air, followed by centrifugal filtrations. The filtrations were performed using Millipore Amicon Ultra-4 centrifugal filters (3-kDa cutoff). Buffer solutions were diluted with acetonitrile/water (1:4, vol/vol) to 3.5 mL total volume in a Millipore centrifugal filter tube. The tube was centrifuged at 7,500 rpm (T15A 41/42 rotor, CF16RXII centrifuge, Hitachi) until residual volume was 0.25–0.5 mL The residual solution was diluted with 0.1 M acetate buffer, and the process was repeated twice.

ESI calcd for $C_{500}H_{787}N_{141}O_{148}S$ $[M+6H]^{6+}$ m/z = 1,863.25 $[M+7H]^{7+}$ m/z = 1,597.22 $[M+8H]^{8+}$ m/z = 1,397.69, $[M+9H]^{9+}$ m/z = 1,242.50 $[M+10H]^{10+}$ m/z = 1,118.35 $[M+11H]^{11+}$ m/z = 1,016.77 $[M+12H]^{12+}$ m/z = 932.13 $[M+13H]^{13+}$ m/z = 860.50 $[M+14H]^{14+}$ m/z = 799.11 $[M+15H]^{15+}$ m/z = 745.90, found: 1,862.98; 1,597.19; 1,397.28; 1,242.22; 1,118.45; 1,016.59; 932.21; 860.43; 799.08; 746.13.

Enzymatic Hydrolysis of Benzoylphosphate. The synthetic acylphosphatase was subjected to the folding buffer (0.1 M acetate buffer) as reported previously (7, 8). Benzoylphosphate (9) was synthesized according to literature (10). The synthetic acylphosphatase (0.2 mg) was dissolved in 100 μL of 100 mM acetate buffer solution (pH 5.3). Next, 1 μL of the above solution was added to 3 mL of 1 mM benzoylphosphate solution in the same buffer solution. The final concentration of the enzyme was 60 nM. The reaction progress was monitored by UV absorption. Control experiment was conducted using the same condition without the addition of the synthetic enzyme (Fig. S6).

1. Blanco-Canosa JB, Dawson PE (2008) An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. *Angew Chem Int Ed Engl* 47(36):6851–6855.

2. Goddard-Borger ED, Stick RV (2007) An efficient, inexpensive, and shelf-stable diazotransfer reagent: Imidazole-1-sulfonyl azide hydrochloride. *Org Lett* 9(19): 3797–3800.

3. Nepomniaschiy N, et al. (2008) Switch peptide via Staudinger reaction. *Org Lett* 10(22):5243–5246.
4. Kimura T, Fukui T, Tanaka S, Akaji K, Kiso Y (1997) A reductive acidolysis final deprotection strategy in solid phase peptide synthesis based on safety-catch protection. *Chem Pharm Bull (Tokyo)* 45(1):18–26.
5. Keller M, et al. (1998) Enhancing the proline effect: pseudo-prolines for tailoring cis/trans isomerization. *J Am Chem Soc* 120(12):2714–2720.
6. Johnson T, Quibell M, Sheppard RC (1995) N,O-bisFmoc derivatives of N-(2-hydroxy-4-methoxybenzyl)-amino acids: Useful intermediates in peptide synthesis. *J Pept Sci* 1(1):11–25.
7. Liguri G, et al. (1986) A new acylphosphatase isoenzyme from human erythrocytes: Purification, characterization, and primary structure. *Biochemistry* 25(24): 8089–8094.
8. Taddei N, et al. (1999) Thermodynamics and kinetics of folding of common-type acylphosphatase: Comparison to the highly homologous muscle isoenzyme. *Biochemistry* 38(7):2135–2142.
9. Ramponi G, Treves C, Guerritore A (1966) Continuous optical assay of acylphosphatase with benzoylphosphate as substrate. *Experientia* 22(11):705–706.
10. Camici G, Manao G, Cappugi G, Ramponi G (1976) A new synthesis of benzoyl phosphate: A substrate for acyl phosphatase assay. *Experientia* 32(4):535–536.

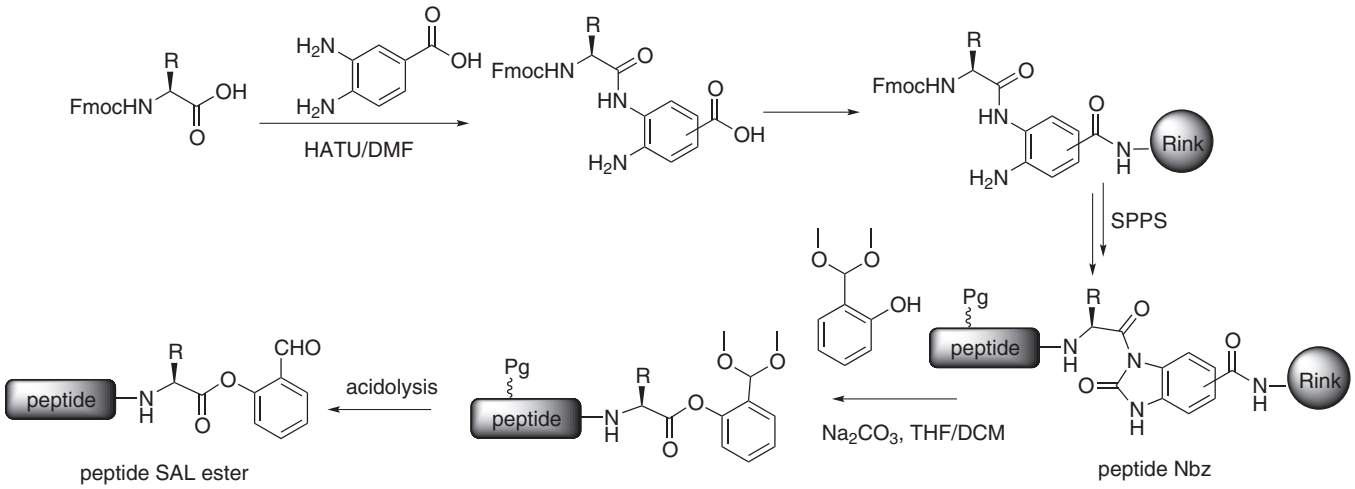

Fig. S1. General procedure for the synthesis of peptide SAL esters.

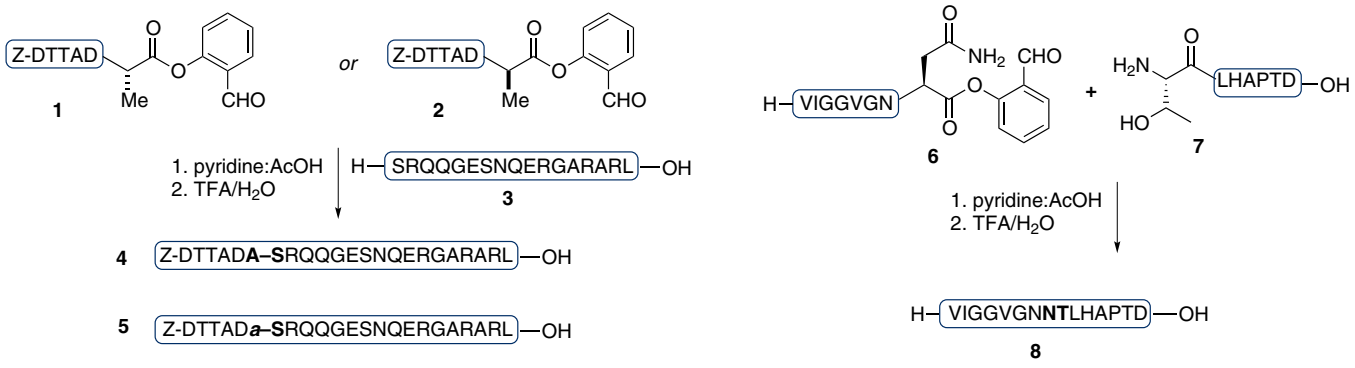

Fig. S2. Synthetic scheme of model peptides.

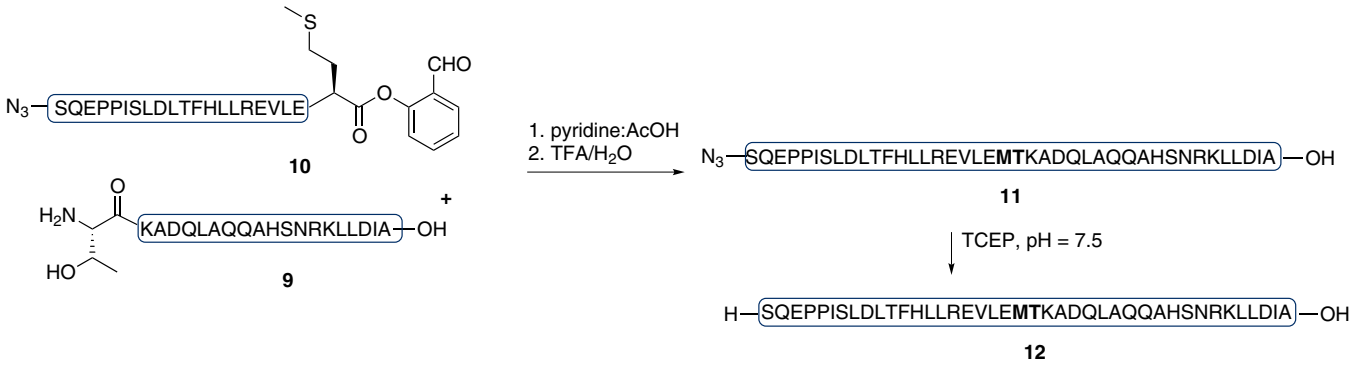

Fig. S3. Synthetic scheme of oCRH.

Fig. S4. Synthetic scheme of Forteo.

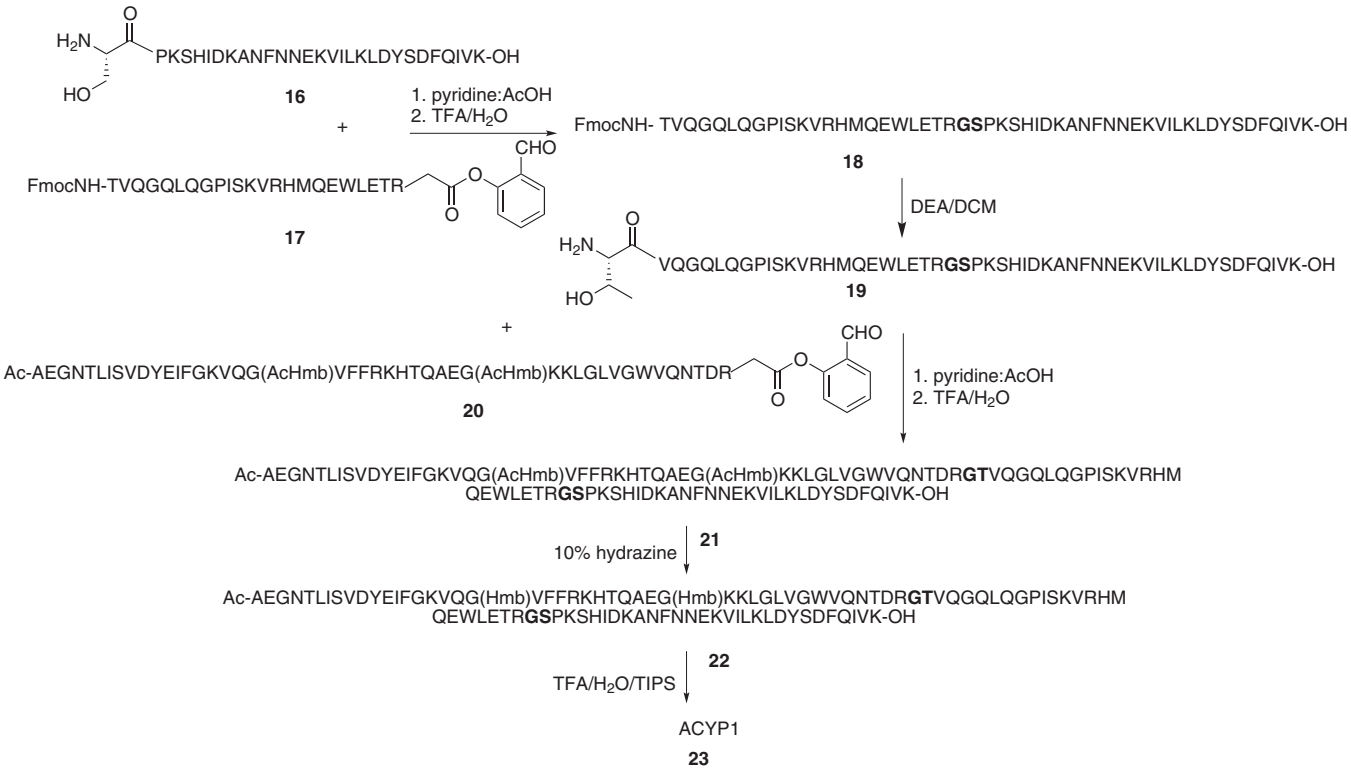

Fig. S5. Synthetic scheme of acylphosphatase protein.

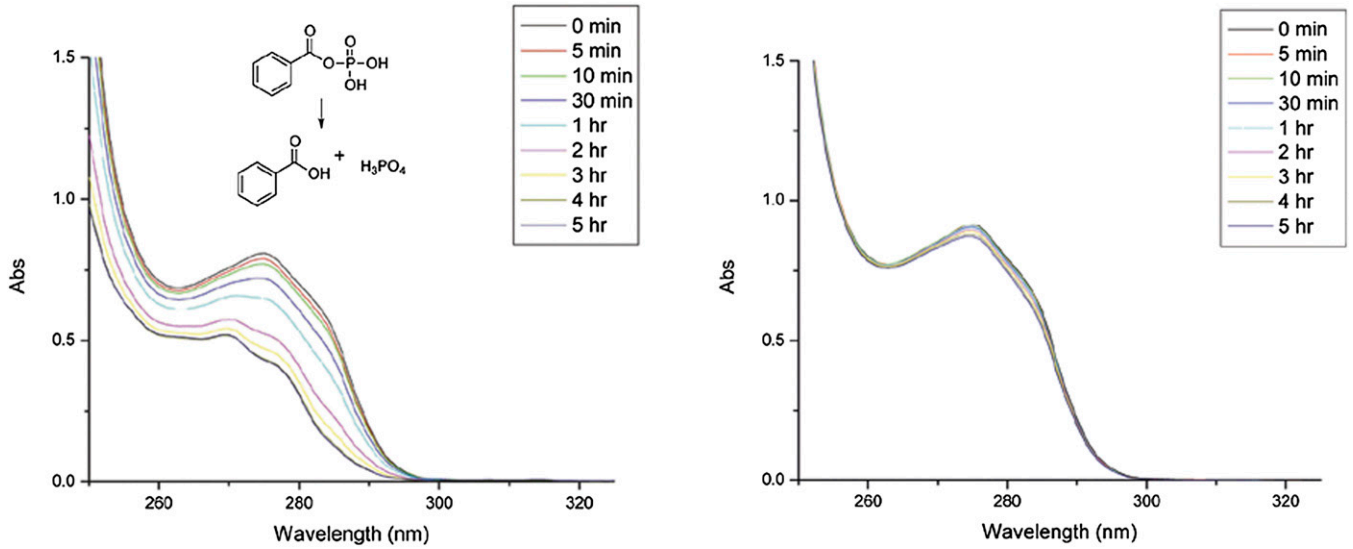

Fig. S6. Enzymatic activity: UV-absorption spectra for the enzymatic hydrolysis of benzoylphosphate by the synthetic human erythrocytes acylphosphatase. (*Left*) UV–absorption spectra for the enzymatic hydrolysis of benzoylphosphate by acylphosphatase. (*Right*) UV–absorption spectra for the control experiment without the synthetic enzyme. Enzymatic reactions were performed at 23 °C, pH 5.3, 1-cm cell, with a concentration of benzoylphosphate at 1 mM. Before hydrolysis: UV-absorption maxima at 275 nm and shoulder at 285 nm; after hydrolysis: UV-absorption maxima at 269 nm and shoulder at 277 nm.

Other Supporting Information Files

Dataset S1 (PDF)