A fluorogenic probe for recognizing 5-hydroxylsine inspired from serine/threonine ligation

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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 or DUKSAN). Dry dichloromethane (CH₂Cl₂) was distilled from calcium hydride (CaH₂). HPLC separations were performed with a Waters HPLC system equipped with a photodiode array detector (Waters 2996) using a Vydac 218TP™ C18 column (5 µm, 4.6 x 250 mm) at a flow rate of 0.6 mL/min for analytical HPLC and Vydac 218TP™ column (10 µm, 22 x 250 mm) at a flow rate of 10 mL/min for preparative HPLC. All separations involved a mobile phase of 0.05% TFA (v/v) in acetonitrile (solvent A)/0.05% TFA (v/v) in water (Solvent B). Low-resolution mass spectral analyses were performed with a Waters 3100 mass spectrometer. ¹H and ¹³C NMR spectra were recorded on Bruker Avance DRX 300 FT-NMR Spectrometer at 300 Hz for ¹H NMR and 75.47 MHz for ¹³C NMR or Bruker Avance DRX 400 FT-NMR spectrometer at 400 MHz for ¹H NMR and 100 MHz for ¹³C NMR. Fluorescence was recorded on HITACHI F-7000 Fluorescence Spectrophotometer.

General procedure of solid-phase peptide synthesis of compound 12, 13, 14 and 15 according to Fmoc-strategy

Synthesis was performed manually on 2-chlorotrityl chloride Resin (resin loading: 0.4 mmol/g). Peptides were synthesized under standard Fmoc/tBu protocols. The deblock mixture was a mixture of 20/80 (v/v) of piperidine/DMF. The following Fmoc amino acids were employed: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Hyl(Boc)(Psi(Me, Me)pro)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Tyr(tBu)-OH. Upon completion of synthesis, the peptide resin was subjected to a cleavage cocktail of TFA/TIPS/H₂O (95:2.5:2.5, v/v/v) and stirred at room temperature for 2 hours. 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 purified by reverse phase preparative HPLC followed by concentration at reduced pressure and lyophilization to give a white powder (35% to 67%, base on resin loading).

**Compound 7:**

![Image of Compound 7]

Compound 7 was prepared by typical Duff reaction. A mixture of 4- methyl -umbelliferone (2.0 g, 11.36 mmol) and hexamine (4.0 g, 28.53 mmol) in 40 mL TFA was heated at 110 °C for 12 hours. 60 mL 10% HCl was then added and the mixture was heated at 110 °C for another 2 hours. The mixture was diluted with AcOEt (250 mL), washed with saturated NaHCO₃ solution (100 mL x 3) and brine (100mL x 1). The organic phase was dried over Na₂SO₄ and concentrated under vacuo. The mixture was purified by flash column chromatography on silica gel (hexane/AcOEt, 3:2) to give compound 11a (320 mg, 25%)

1H NMR (300 MHz, CDCl₃) δ 12.20 (1H, broad s, OH), 10.61 (1H, s, CHO), 7.73 (1H, d, J = 9.0 Hz, Ar H), 6.90 (1H, d, J = 9.0 Hz, Ar H), 6.20 (1H, s, C=CH), 2.43 (3H, s, CH₃) 13C NMR (75 MHz, CDCl₃) δ 193.4, 165.2, 159.2, 156.1, 152.6, 132.9, 114.3, 112.1, 111.9, 108.6, 18.9 ESI MS calcd. For C₁₁H₉O₄ [M⁺] 205.18 found 205.55

**Compound 8:**

![Image of Compound 8]

Compound 7 (245 mg, 1.20 mmol) was dissolved in 25 mL of anhydrous MeOH. LiBF₄ (1 mg, 0.01 mmol) and trimethylorthoformate (637 mg, 6.00 mmol) were added. The reaction mixture was heated under reflux for 4 h. The reaction mixture was diluted with AcOEt (250 mL), washed with saturated NaHCO₃ solution (100 mL x 3) and brine (100mL x 1). The organic phase was dried over Na₂SO₄ and concentrated under vacuo to give compound 8 without further purification (180 mg, 60%)
Compound 10:

To a solution of 8 (110 mg, 0.44 mmol) and trimethylacetic acid (224 mg, 2.20 mmol) in 5 mL anhydrous CH$_2$Cl$_2$, EDCI (543 mg, 2.86 mmol) and DMAP (5 mg, 0.04 mmol) were added. The mixture was stirred at room temperature for 12 hours. The mixture was diluted with AcOEt (25 mL), washed with saturated NaHCO$_3$ solution (10 mL x 3) and brine (10 mL x 1). The organic phase was dried over Na$_2$SO$_4$ and concentrated under vacuo. The mixture was purified by flash column chromatography on silica gel (hexane/AcOEt, 2:1) to give compound 9 (142 mg, 97%). Compound 9 was treated with 5 mL of 95% TFA for 30 min. The solvent was blown off under a stream of condensed air. The crude product was purified by flash column chromatography on silica gel (hexane/AcOEt, 2:1) to give compound 10 (86 mg, 70%)

$^1$H NMR (400 MHz, CDCl$_3$) δ 10.64 (1H, s, CHO), 7.80 (1H, d, J = 8.6 Hz, Ar H), 7.02 (1H, d, J = 8.6 Hz, Ar H), 6.35 (1H, s, C=CH), 2.47 (3H, s, CH$_3$), 1.41 (9H, s, C(CH$_3$)$_3$)

$^{13}$C NMR (100 MHz, CDCl$_3$) δ 186.1, 176.2, 158.7, 155.9, 152.6, 151.7, 130.1, 119.6, 118.3, 116.7, 114.8, 39.3, 27.1, 19.0 ESI MS calcd. For C$_{16}$H$_{16}$O$_5$ [M$^+$] 289.30 found 289.52

Compound 11:

Compound 10 (9 mg, 0.03 mmol) and HCl · NH$_2$ – Ser – OMe (6 mg, 0.04 mmol) was dissolved in 6 mL Pyr/HOAc (1:1, mol/mol). The reaction mixture was stirred at room temperature. A 25 µL aquilor reaction solution at different time points during the reaction process was diluted with Pyr/HOAc (1:1, mol/mol) into 2 mL and subjected to Fluorescence spectrometer. The fluorescence intensity was recorded at $\lambda_{ex} = 320$ nm. Upon completion of the reaction, the mixture was diluted with AcOEt (25 mL), washed with 1N HCl (10 mL x 3) and brine (10 mL x 1). The organic phase was dried
over Na$_2$SO$_4$ and concentrated under \textit{vacuo}. The mixture was purified by flash column chromatography on neutral Aluminum oxide (CH$_2$Cl$_2$/ MeOH, 20:1) to give compound \textbf{14} (8 mg, 72%).

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.46 (1H, d, $J = 9.7$ Hz, Ar H), 6.90 (1H, s, CH of oxazolidine), 6.87 (1H, d, $J = 8.8$ Hz, Ar H), 6.11 (1H, s, C=CH), 5.17 (2H, m, $\beta$H – Ser), 4.34 (1H, m, $\alpha$H – Ser), 3.85 (3H, s, OCH$_3$), 2.38 (3H, s, C=C(CH$_3$)$_3$), 1.17 (9H, s, C(CH$_3$)$_3$) ESI MS calcd. For C$_{20}$H$_{24}$NO$_7$ [M$^+$] 390.40 found 390.14

**General procedure of peptide reacting with probe 10:**

Peptide was dissolved in Pyr/HOAc (1:1, mol/ mol) at a concentration of 5 mM. Compound \textbf{10} (1.2 equiv.) was added. The reaction mixture was stirred at room temperature. A 20 $\mu$L aliquot reaction solution at different time points during the reaction process was diluted with water into 2 mL and subjected to Fluorescence spectrometer. The fluorescence intensity was recorded at $\lambda_{ex} = 320$ nm.

![Fluorescence spectrum of peptide 13 reacting with probe 10](image_url)

**Fig. S1** Fluorescence spectrum of peptide 13 reacting with probe 10
**Fig. S2** Fluorescence spectrum of peptide 14 reacting with probe 10

**Fig. S3** Fluorescence spectrum of peptide 15 reacting with probe 10

**General procedure of fluorescence detection limit of peptide reacting with probe 10:**

Stock solutions of peptide at concentration of 0.001 mM, 0.01 mM, 0.1 mM, 1 mM and 5 mM were prepared by dissolving in Pyr/HOAc (1:1, mol/mol). Compound 10 was added to the peptide stock solutions at a concentration of 5 mM. The reaction mixture was stirred at room temperature for 2 hours. A 20 µL aquilot reaction solution was diluted with water into 2 mL and subjected to Fluorescence spectrometer. The fluorescence intensity was recorded at $\lambda_{ex} = 320$ nm.
\( ^1H \) and \( ^{13}C \) NMR spectra

\( ^1H \) NMR spectrum for compound 7
$^{13}$C NMR spectrum for compound 7
$^1$H NMR spectrum for compound 10
\(^{13}\)C NMR spectrum for compound 10
$^1$H NMR spectrum for compound 11