Dual enzyme-responsive "turn-on" fluorescence sensing systems based on \textit{in situ} formation of 7-hydroxy-2-iminocoumarin scaffolds

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Supporting Information
Abbreviations .................................................................................................................................................. S4

High-performance liquid chromatography separations .................................................................................. S4

Synthesised compounds ................................................................................................................................. S4
  para-Acetoxybenzyl alcohol - Ac-PHBA (S1) ................................................................................................. S4
  N-phenylacetamidobenzyl alcohol - PhAc-PABA (S2) ................................................................................... S5

In vitro activation of fluorogenic "turn-on" probes 5, 8 and 9 by hydrolase (PGA or PLE) and reductase (NTR) - experimental details ....................................................................................................................................... S5

Analytical data ................................................................................................................................................ S8
  1H NMR spectrum of compound 2 recorded in CDCl3 at 300 MHz............................................................... S8
  13C NMR spectrum of compound 2 recorded in CDCl3 at 75 MHz............................................................... S9
  ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 2 .................................................. S9
  RP-HPLC elution profile (system A) of compound 2 at 260 nm................................................................. S10
  1H NMR spectrum of compound 3 recorded in DMSO-d6 at 300 MHz.................................................... S10
  13C NMR spectrum of compound 3 recorded in DMSO-d6 at 75 MHz...................................................... S11
  ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 3 .................................................. S11
  RP-HPLC elution profile (system B) of compound 3 at 260 nm................................................................. S12
  1H NMR spectrum of compound 4 recorded in DMSO-d6 at 300 MHz.................................................... S12
  13C NMR spectrum of compound 4 recorded in DMSO-d6 at 75 MHz...................................................... S13
  ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 4 .................................................. S13
  RP-HPLC elution profile (system B) of compound 4 at 260 nm................................................................. S14
  ESI+ mass spectrum (high resolution) of compound 4 ................................................................................ S15
  ESI- mass spectrum (high resolution) of compound 4 ................................................................................ S16
  1H NMR spectrum of compound 5 recorded in CDCl3 at 300 MHz........................................................... S16
  13C NMR spectrum of compound 5 recorded in CDCl3 at 125 MHz......................................................... S17
  ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (more polar isomer) .... S17
  ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (less polar isomer) .... S18
  RP-HPLC elution profile (system A) of compound 5 at 260 nm.................................................................. S18
  ESI+ mass spectrum (high resolution) of compound 5 ................................................................................ S19
  1H NMR spectrum of compound 6 recorded in CDCl3 at 300 MHz........................................................... S20
  1H NMR spectrum of compound 7 recorded in CDCl3 at 300 MHz........................................................... S20
  1H NMR spectrum of compound 8 recorded in CDCl3 at 300 MHz........................................................... S21
  13C NMR spectrum of compound 8 recorded in CDCl3 at 125 MHz........................................................ S21
  ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 8 ........................................ S22
  RP-HPLC elution profile (system A) of compound 8 at 260 nm................................................................. S22
  ESI+ mass spectrum (high resolution) of compound 8 ................................................................................ S23
  1H NMR spectrum of compound 9 recorded in DMSO-d6 at 300 MHz.................................................... S24
  13C NMR spectrum of compound 9 recorded in DMSO-d6 at 125 MHz.................................................... S24
  ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 9 ........................................ S25
  RP-HPLC elution profile (system A) of compound 9 at 260 nm................................................................. S25
  ESI+ mass spectrum (high resolution) of compound 9 ................................................................................ S26
Fig S1. Normalised absorption spectra of fluorogenic probes 5, 8 and 9 in PB (+ 0.3% DMSO) at 25 °C.

Fig S2. Normalised absorption, excitation (Em. 510 nm) and emission (Ex. 400 nm) spectra of 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C.

Fig S3. Normalised absorption, excitation (Em. 530 nm) and emission (Ex. 370 nm) spectra of 3-cyano-7-hydroxycoumarin in PB at 25 °C.

Fig S4. Overlayed fluorescence emission spectra (Ex. 390 nm) of PLE-NTR fluorogenic probe 5 and 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM).

Fig S5. Overlayed fluorescence emission spectra (Ex. 390 nm) of PLE-NTR fluorogenic probe 8 and 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM).

Fig S6. Overlayed fluorescence emission spectra (Ex. 390 nm) of PGA-NTR fluorogenic probe 9 and 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM).

Fig S7. Time-dependant fluorescence intensity of fluorogenic "turn-on" probe 8 upon sequential incubation with NTR/NADH, PLE and cmpd 4.

Fig S8. RP-HPLC elution profiles (fluorescence detection, systems D & E) of fluorogenic probes 5 (top), 8 (middle) and 9 (bottom) before dual-enzymatic activation.

Fig S9. RP-HPLC elution profiles (fluorescence detection, system D) of enzymatic reaction mixture of cyano-based probes 8 and 9 incubated simultaneously with both enzymes: hydrolase (PLE or PGA) and NTR/NADH.

Fig S10. RP-HPLC-MS analyses - Identification of "relevant" molecules related to the dual-enzyme activation of probes 8 and 9.

Fig S11. RP-HPLC elution profiles (system C) of fluorogenic probe 8 after incubation in PB alone or with enzymes (PLE and NTR/NADH).

Fig S12. RP-HPLC elution profiles (system C) of fluorogenic probe 9 after incubation in PB alone or with enzymes (PGA and NTR/NADH).
Abbreviations
The following abbreviations are used throughout the text of the ESI file: Ar, argon; DCM, dichloromethane; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide; equiv., equivalent(s); Et₂O, diethyl ether; EtOAc, ethyl acetate; EtOH, ethanol; ESI, electrospray ionisation; FA, formic acid; HPLC, high-pressure liquid chromatography; LRMS, low-resolution mass spectrum; min, minutes; Na₂SO₄, sodium sulfate; NADH, nicotinamide adenine dinucleotide; NaHCO₃, sodium hydrogen carbonate; NTR, nitroreductase; PABA, para-aminobenzyl alcohol; PE, petroleum ether (bp 40-60 °C); PHBA, para-hydroxybenzyl alcohol; PGA, penicillin G acylase; PLE, porcine liver esterase; MS, mass spectrometry; PMT, photomultiplier tube; RT, room temperature; TBDMS, tert-butyldimethylsilyl; TEA, triethylamine; TEAB, triethylammonium bicarbonate; THF, tetrahydrofuran; TLC, thin-layer chromatography; UV, ultraviolet.

High-performance liquid chromatography separations
Several chromatographic systems were used for the analytical experiments (HPLC-MS or HPLC-fluorescence): System A: RP-HPLC-MS (Phenomenex Kinetex C18 column, 2.6 µm, 2.1 × 50 mm) with CH₃CN (+ 0.1% FA) and 0.1% aq. FA (pH 3.2) as eluents [linear gradient from 5% to 100% (5 min) of CH₃CN followed by isocratic at 100% (1.5 min)] at a flow rate of 0.5 mL min⁻¹. UV-visible detection was achieved at 220, 260, 300 and 360 nm (+ diode array detection in the range 220-500 nm). ESI-MS detection in the positive/negative mode ("full scan", 150-1500 a.m.u., data type: centroid, needle voltage: 3.0 kV, detector voltage: 1100 V, probe temperature: 350 °C, cone voltage: 75 V and scan time: 1 s). System B: System A with 100-700 a.m.u for "full scan" mass detection. System C: System A with the following gradient [0% CH₃CN (2 min) followed by linear gradient from 0% to 100% (6 min) of CH₃CN followed by isocratic at100% (1 min)]. UV-visible detection was achieved at 220, 260, 350 and 418 nm (+ diode array detection in the range 220-500 nm). System D: RP-HPLC-fluorescence (Phenomenex Kinetex C18 column, 2.6 µm, 2.1 × 50 mm) with CH₃CN and aq. TEAB (50 mM, pH 7.5) as eluents [0% CH₃CN (1 min) followed by linear gradient from 0% to 100% (5 min) of CH₃CN followed by isocratic at 100%] at a flow rate of 0.5 mL min⁻¹. Fluorescence detection was achieved at 45 °C at teh following Ex./Em. channels: 350/460 nm and 418/458 nm (sensitivity: 1, PMT 1, filter wheel: auto). System E: System D with the following Ex./Em. channels for fluorescence detection: 350/460 nm, 431/488 nm and 455/489 nm.

Synthesised compounds

*para*-Acetoxybenzyl alcohol - Ac-PHBA (S1)¹

![Chemical structure](image)

Under Ar atmosphere, 4-hydroxybenzyl alcohol (2 g, 16.1 mmol) was dissolved in dry THF (27 mL), cooled to 0 °C with an ice-water bath and TEA (2.23 mL, 16.1 mmol, 1 equiv.) was added. Then acetyl chloride (1.26 mL, 17.7 mmol, 1.1 equiv.) was added dropwise over a period of 25 min. The resulting reaction mixture was stirred for 2 h. Thereafter, the newly formed precipitate was removed by filtration and filtrate was evaporated to dryness. The crude was diluted with DCM, washed twice with aq. 5% NaHCO₃ and finally with deionised water. The organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated under

vacuum. The crude product was purified by chromatography on a silica gel column (PE-EtOAc, step gradient from 100 to 65: 35, v/v) to give the desired acetate S1 as light yellow oil which crystallized as white solid after overnight storage at 4 °C (1.34 g, yield 50%). Rf 0.27 (heptane-EtOAc, 6 : 4, v/v); δH(300 MHz, CDCl3) 7.37 (d, J 8.4, 2 H), 7.06 (d, J 8.7, 2 H), 4.65 (d, J 4.8, 2 H), 2.29 (s, 3 H), 1.92 (bt, 1 H).

**N-phenylacetamidobenzyl alcohol - PhAc-PABA (S2)**

Under Ar atmosphere, PABA (1 g, 8.1 mmol, 1 equiv.) and potassium acetate (1.6 g, 16.2 mmol, 2 equiv.) were dissolved in dry DMF (80 mL), cooled to -60 °C with a CHCl3/liq. N2 bath and phenylacetyl chloride (1.1 mL, 8.1 mmol, 1 equiv.) was added dropwise to the mixture; each added drop caused a yellow discoloration which rapidly fades before adding the next drop of phenylacetyl chloride. The resulting reaction mixture was left to warm at RT and then was diluted with aq. 1.0 M NaOH (20 mL). Thereafter, the mixture was neutralised with aq. 1.0 M HCl to reach pH 7 and extracted with DCM. Organic layer was washed with deionised water and brine and finally dried over anhydrous Na2SO4. After concentration under reduced pressure, the crude was taken with heptane and then with DCM. The solid was recovered by filtration to give the desired phenylacetamide derivative S2 as white solid (1.10 g, yield 56%). Rf 0.5 (DCM-EtOAc, 7 : 3, v/v); δH(300 MHz, DMSO-d6) 10.15 (s, 1 H), 7.53 (d, J 9.6, 2 H), 7.33 (d, J 4.8, 4 H), 7.24 (m, 3 H), 5.17 (t, J 5.7, 1 H), 4.43 (d, J 5.8, 2 H).

**In vitro activation of fluorogenic "turn-on" probes 5, 8 and 9 by hydrolase (PGA or PLE) and reductase (NTR) - experimental details**

**Stock solutions of probes and enzymes:**
- Mixture A: A stock solution (1.0 mg / mL) of PGA-NTR fluorogenic probe 9 in DMSO (for spectroscopy, 99.9%, ACROS, 167852500) (final concentration: 1.70 mM),
- Mixture B: A stock solution (1.0 mg / mL) of PLE-NTR fluorogenic probe 8 in DMSO (final concentration: 1.95 mM),
- Mixture C: A stock solution (1.0 mg / mL) of PLE-NTR fluorogenic probe 5 in DMSO (final concentration: 1.61 mM),
- Mixture D: 6.28 mg of PGA (0.63 U / mg) was dissolved in 1 mL of PB (3.95 U / mL),
- Mixture E: 1.12 mg of PLE (27 U / mg) was dissolved in 150 µL of PB and 150 µL of ultrapure H2O (0.1 U / µL),
- Mixture F: 0.93 mg of PLE (27 U / mg) was dissolved in 1 mL of PB (25.11 U / mL),
- Mixture G: 24.33 mg of NADH (MW: 709.4) was dissolved in 245 µL of H2O (final concentration: 140 mM).
- Mixture H: commercial lyophilised NTR + buffer (1 mg of protein, 100 U / mg) was resuspended in 1 mL of ultrapure water (0.1 U / µL).

Stock solutions (1.0 mg / mL) of 3-(2-benzothiazolyl)-7-hydroxycoumarin (final concentration: 3.4 mM), 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin (final concentration: 3.4 mM), 3-cyano-7-hydroxycoumarin (final concentration: 5.3 mM), 3-cyano-7-hydroxy-2-iminocoumarin (final concentration: 5.4 mM) were also prepared in DMSO and

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subsequently diluted with PB for UV-vis absorption and fluorescence measurements, and HPLC-fluorescence analyses.

Fluorescence assays:
All assay were performed at 37 °C (conducted with or without magnetic stirring, no difference was noted). For probes 8 and 9, the fluorescence emission of the release 3-cyan0-7-hydroxy-2-iminocoumarin was monitored at λ = 458 nm (emission slit = 2 nm) (Ex. λ = 418 nm, excitation slit = 2 nm) over time with measurements recorded every 5 s. For probe 5, the fluorescence emission of the release 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin was monitored at λ = 489 nm (emission slit = 2 nm) (Ex. λ = 455 nm, excitation slit = 2 nm) over time with measurements recorded every 5 s.

Sequential protocol (hydrolase then NTR):
Probe 9 - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture A was diluted in 2.750 mL of PB, then 245 µL of mixture D (1 U) was added and the resulting mixture was incubated for 10 min. Then 1 µL of mixture G and 1 µL of mixture H were added.
Probe 8 - Into a 3.5 mL fluorescence quartz cell, 1.5 µL of mixture B was diluted in 2.990 mL of PB, then 10 µL of mixture E (1 U) was added and the resulting mixture was incubated for 10 min. Then 1 µL of mixture G and 1 µL of mixture H were added.
Probe 5 - Same as probe 8 by replacing 1.5 µL of mixture B by 2 µL of mixture C.

Sequential protocol (NTR then hydrolase):
Probe 9 - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture A was diluted in 2.750 mL of PB, then 1 µL of mixture G and 1 µL of mixture H were added together and the resulting mixture was incubated until the fluorescent intensity was reached a constant level. Then 245 µL of mixture D (1 U) was added.
Probe 8 - Into a 3.5 mL fluorescence quartz cell, 1.5 µL of mixture B was diluted in 2.990 mL of PB, then 1 µL of mixture G and 1 µL of mixture H were added together and the resulting mixture was incubated until the fluorescent intensity was reached a constant level. Then 10 µL of mixture E (1 U) was added.
Probe 5 - Same as probe 8 by replacing 1.5 µL of mixture B with 2 µL of mixture C.

Simultaneous incubation:
Probe 9 - Into a 3.5 mL fluorescence quartz cell, 2 µL of mixture A was diluted in 2.750 mL of PB, then 1 µL of mixture G, 1 µL of mixture H and 245 µL of mixture D (1 U) were added together and the resulting mixture was incubated.
Probe 8 - Into a 3.5 mL fluorescence quartz cell, 1.5 µL of mixture B was diluted in 2.990 mL of PB, then 1 µL of mixture G, 1 µL of mixture H and 10 µL of mixture E (1 U) were added together and the resulting mixture was incubated.
Probe 5 - Same as probe 8 by replacing 1.5 µL of mixture B with 2 µL of mixture C.

HPLC-fluorescence analyses:
Enzymatic reaction mixtures from fluorescence assays were directly analysed by RP-HPLC-fluorescence (injected volume: 10 µL, system D for reaction conducted with cyano-based probes 8 and 9 and system E for those conducted with benzothiazolyl-based probe 5).
HPLC-MS analyses (enzyme assay and sample treatment):

Sequential protocol (hydrolase then NTR):
39 nmol of PGA-NTR (23 µL of mixture A) (or PLE-NTR (20 µL of mixture B)) fluorogenic probe 9 (or 8) was dissolved in PB (260 µL (or 428 µL)) containing 190 µL of mixture D (0.75 U) (or 22 µL of mixture F (0.55 U)) and the resulting enzymatic reaction mixture was incubated at 37 °C for 80 min. Thereafter, 6 µL of mixture H (0.6 U) and 2 µL of mixture G were added together and the mixture was incubated for further 100 min. Samples (50 µL) were taken at 30 min, 1 h, 2 h and 3 h of reaction and were treated as described below.

Sequential protocol (NTR then hydrolase):
39 nmol of PGA-NTR (23 µL of mixture A) (or PLE-NTR (20 µL of mixture B)) fluorogenic probe 9 (or 8) was dissolved in PB (260 µL (or 428 µL)) containing 6 µL of mixture H (0.6 U) and 2 µL of mixture G and the resulting enzymatic reaction mixture was incubated at 37 °C for 1 h 20. Thereafter, 190 µL of mixture D (0.75 U) (or 22 µL of mixture F (0.55 U)) was added and the mixture was incubated for further 100 min. Samples (50 µL) were taken at 30 min, 1 h, 2 h and 3 h of reaction and were treated as described below.

Simultaneous incubation:
39 nmol of PGA-NTR (23 µL of mixture A) (or PLE-NTR (20 µL of mixture B)) fluorogenic probe 9 (or 8) was dissolved in PB (260 µL (or 428 µL)) containing 6 µL of mixture H (0.6 U) and 2 µL of mixture G and 190 µL of mixture D (0.75 U) (or 22 µL of mixture F (0.55 U)) and the resulting enzymatic reaction mixture was incubated at 37 °C for 3 h. Samples (50 µL) were taken at 30 min, 1 h, 2 h and 3 h of reaction and were treated as described below.

Samples treatment for HPLC-MS analysis:
Withdrawn sample (50 µL) was diluted with 50 µL of CH₃CN, then vortexed followed by centrifugation (9 000 rpm, 2 min) and finally, 75 µL of the supernatant was collected and diluted with 25 µL of aq. 0.1% FA. 10 µL was injected into the HPLC-MS apparatus (system C).
Analytical data

$^1$H NMR spectrum of compound 2 recorded in CDCl$_3$ at 300 MHz
$^{13}$C NMR spectrum of compound 2 recorded in CDCl$_3$ at 75 MHz

ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 2
RP-HPLC elution profile (system A) of compound 2 at 260 nm

*peak assigned to acetone used as solvent for sample preparation

\[
\begin{align*}
\text{**H NMR spectrum of compound 3 recorded in DMSO-}d_6 \text{ at } 300 \text{ MHz} \\
\end{align*}
\]
$^{13}$C NMR spectrum of compound 3 recorded in DMSO-$d_6$ at 75 MHz

ESI- mass spectrum (low resolution) and UV-vis spectrum of compound 3
RP-HPLC elution profile (system B) of compound 3 at 260 nm

$^{1}H$ NMR spectrum of compound 4 recorded in DMSO-$d_6$ at 300 MHz

![NMR spectrum graph]

![Compound 4 structure]
$^{13}$C NMR spectrum of compound 4 recorded in DMSO-$d_6$ at 75 MHz

ESI-mass spectrum (low resolution) and UV-vis spectrum of compound 4
RP-HPLC elution profile (system B) of compound 4 at 260 nm
ESI+ mass spectrum (high resolution) of compound 4

Chemical Formula: C\textsubscript{17}H\textsubscript{11}N\textsubscript{3}O\textsubscript{4}
Molecular Weight: 321.29
ESI- mass spectrum (high resolution) of compound 4

\[ [M - H]^+ \]

Chemical Formula: C_{17}H_{11}N_{3}O_{4}
Molecular Weight: 321.29

\[ \text{[*peaks assigned to 2}\text{nd geometric isomer (15 : 85)}] \]

\[ \text{Et}_2\text{O} \]

\[ \text{heptane} \]

\[ \text{Et}_2\text{O} \]

$^1$H NMR spectrum of compound 5 recorded in CDCl$_3$ at 300 MHz

*peaks assigned to 2$^{nd}$ geometric isomer (15 : 85)
$^{13}$C NMR spectrum of compound 5 recorded in CDCl$_3$ at 125 MHz

ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (more polar isomer)
ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 5 (less polar isomer)

RP-HPLC elution profile (system A) of compound 5 at 260 nm

*peak found in mobile phase
ESI+ mass spectrum (high resolution) of compound 5
$^1$H NMR spectrum of compound 6 recorded in CDCl$_3$ at 300 MHz

$^1$H NMR spectrum of compound 7 recorded in CDCl$_3$ at 300 MHz
$^1$H NMR spectrum of compound 8 recorded in CDCl$_3$ at 300 MHz

$^{13}$C NMR spectrum of compound 8 recorded in CDCl$_3$ at 125 MHz
ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 8

RP-HPLC elution profile (system A) of compound 8 at 260 nm
ESI+ mass spectrum (high resolution) of compound 8

m/z exp = 536.10566
m/z theo = 536.10644
Delta = +1.447 ppm
$^1$H NMR spectrum of compound 9 recorded in DMSO-$d_6$ at 300 MHz

$^{13}$C NMR spectrum of compound 9 recorded in DMSO-$d_6$ at 125 MHz

unknown impurity
ESI-/ESI+ mass spectrum (low resolution) and UV-vis spectrum of compound 9

[Diagram of ESI-/ESI+ mass spectrum]

ESI+ 

ESI- 

Chemical Formula: C33H24N4O7

Molecular Weight: 588.57

RP-HPLC elution profile (system A) of compound 9 at 260 nm

[Diagram of RP-HPLC elution profile]
ESI+ mass spectrum (high resolution) of compound 9

m/z exp = 589.17148
m/z theo = 589.17178
Δ = 0.502 ppm

m/z exp = 611.15320
m/z theo = 611.15372
Δ = 0.851 ppm
Fig S1. Normalised absorption spectra of fluorogenic probes 5, 8 and 9 in PB (+ 0.3% DMSO) at 25 °C

![Normalized absorption spectra of fluorogenic probes](image)

Fig S2. Normalised absorption, excitation (Em. 510 nm) and emission (Ex. 400 nm) spectra of 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C.

![Normalized absorption, excitation and emission spectra](image)
**Fig S3.** Normalised absorption, excitation (Em. 530 nm) and emission (Ex. 370 nm) spectra of 3-cyano-7-hydroxycoumarin in PB at 25 °C.

![Absorption, Excitation, and Emission Spectra](image)

**Fig S4.** Overlayed fluorescence emission spectra (Ex. 390 nm) of PLE-NTR fluorogenic probe 5 and 3-(2-benzothiazolyl)-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 µM)*

![Overlayed Fluorescence Emission Spectra](image)

*Raman scatter of water at 450 nm
Fig S5. Overlayed fluorescence emission spectra (Ex. 390 nm) of PLE-NTR fluorogenic probe 8 and 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM)

*Raman scatter of water at 450 nm*

Fig S6. Overlayed fluorescence emission spectra (Ex. 390 nm) of PGA-NTR fluorogenic probe 9 and 3-cyano-7-hydroxy-2-iminocoumarin in PB at 25 °C (concentration: 0.1 μM)

*Raman scatter of water at 450 nm*
Fig S7. Time-dependant fluorescence intensity of fluorogenic "turn-on" probe 8 upon sequential incubation with NTR/NADH, PLE and cmpd 4.

Probe 8 (concentration: 1.0 µM in PB) was incubated with NTR (0.1 U) / NADH (45 µM) at 37 °C for 37.5 min, then PLE (1 U) was added and further incubation for 25 min. Finally, compound 4 (final concentration: 1.0 µM) was added. Ex./Em. 418/458 nm.
Fig S8. RP-HPLC elution profiles (fluorescence detection, systems D & E) of fluorogenic probes 5 (top), 8 (middle) and 9 (bottom) before dual-enzymatic activation
Fig S9. RP-HPLC elution profiles (fluorescence detection, system D) of enzymatic reaction mixture of cyano-based probes 8 and 9 incubated simultaneously with both enzymes: hydrolase (PLE or PGA) and NTR/NADH

Enzymatic reaction mixtures (A-B) and authentic samples of 3-cyano-7-hydroxycoumarin (C) and 3-cyano-7-hydroxy-2-iminocoumarin (D). Please note: partial hydrolysis of cyano and imine moieties was occurred during HPLC analysis and incubation in PB. NADH ($t_R = 3.4$ min) can be properly detected at a different wavelength channel (Ex./Em. 350/460 nm).
Fig S10. RP-HPLC-MS analyses - Identification of "relevant" molecules related to the dual-enzyme activation of probes 8 and 9

Peak at 3.8 min

- Chemical Formula: C_{10}H_{6}N_{2}O_{2}
- Molecular Weight: 186.17
- Chemical Formula: [M + H]^+
- ESI +

Peak at 4.3 min

- Chemical Formula: C_{10}H_{8}N_{2}O_{3}
- Molecular Weight: 204.18
- Chemical Formula: [M - H]^-
- ESI -
Peak at 5.5 min

Chemical Formula: C_{10}H_{5}NO_{3}
Molecular Weight: 187,15

Peak at 5.9 min

Chemical Formula: C_{10}H_{7}NO_{4}
Molecular Weight: 205,17

Peak at 7.7 min

Chemical Formula: C_{17}H_{11}N_{3}O_{4}
Molecular Weight: 321,29
Peak at 7.0 min

Chemical Formula: $C_{14}H_{11}NO_5$
Molecular Weight: 273.24

Peak at 8.18 min

Chemical Formula: $C_{24}H_{19}NO_9$
Molecular Weight: 465.41
Peak at 8.3 min

Chemical Formula: C_{30}H_{24}N_{2}O_{8}
Molecular Weight: 540.52

\[
[M + HCOO^-]^{-}
\]

\[
[M - C_{16}H_{14}NO_3^-]^+
\]
Fig S11. RP-HPLC elution profiles (system C) of fluorogenic probe 8 after incubation in PB alone or with enzymes (PLE and NTR/NADH)

PLE-NTR probe 8

at 260 nm

at 350 nm

PLE-NTR probe 8 in PB after 10 min

at 260 nm

at 350 nm
PLE-NTR probe 8 in PB after 90 min

at 260 nm

at 350 nm

PLE-NTR probe 8 in PB after 190 min

at 260 nm

at 350 nm
PLE-NTR probe 8 with PLE (0.55 U) after 30 min

PLE-NTR probe 8 with PLE (0.55 U) after 60 min
PLE-NTR probe 8 with PLE (0.55 U) after 120 min (addition of 0.6 U NTR at 80 min)

PLE-NTR probe 8 with PLE (0.55 U) after 180 min (addition of 0.6 U NTR at 80 min)
PLE-NTR probe 8 with NTR (0.6 U) after 30 min

at 260 nm

PLE-NTR probe 8 with NTR (0.6 U) after 60 min

at 260 nm

at 350 nm
PLE-NTR probe 8 with NTR (0.6 U) after 120 min (addition of 0.55 U PLE at 80 min)

at 260 nm

at 350 nm

PLE-NTR probe 8 with NTR (0.6 U) after 180 min (addition of 0.55 U PLE at 80 min)

at 260 nm

at 350 nm
PLE-NTR probe 8 with NTR (0.6 U) and PLE (0.55 U) after 30 min

at 260 nm

at 350 nm

PLE-NTR probe 8 with NTR (0.6 U) and PLE (0.55 U) after 60 min

at 260 nm

at 350 nm
PLE-NTR probe 8 with NTR (0.6 U) and PLE (0.55 U) after 120 min

at 260 nm

at 350 nm

PLE-NTR probe 8 with NTR (0.6 U) and PLE (0.55 U) after 180 min

at 260 nm

at 350 nm
Fig S12. RP-HPLC elution profiles (system C) of fluorogenic probe 9 after incubation in PB alone or with enzymes (PGA and NTR/NADH)
PGA-NTR probe 9 in PB after 90 min

at 260 nm

at 350 nm

PGA-NTR probe 9 in PB after 190 min

at 260 nm

at 350 nm
PGA-NTR probe 9 with PGA (0.75 U) after 30 min

at 260 nm

at 350 nm

PGA-NTR probe 9 with PGA (0.75 U) after 60 min

at 260 nm

at 350 nm
PGA-NTR probe 9 with PGA (0.75 U) after 120 min (0.6 U NTR adding at 80 min)

at 260 nm

at 350 nm

PGA-NTR probe 9 with PGA (0.75 U) after 180 min (0.6 U NTR addition at 80 min)

at 260 nm

at 350 nm
PGA-NTR probe 9 with NTR (0.6 U) after 30 min

PGA-NTR probe 9 with NTR (0.6 U) after 60 min
PGA-NTR probe 9 with NTR (0.6 U) after 120 min (addition of 0.75 U PGA at 80 min)

PGA-NTR probe 9 with NTR (0.6 U) after 180 min (addition of 0.75 U PGA at 80 min)
PGA-NTR probe 9 with NTR (0.6 U) and PGA (0.75 U) after 30 min

at 260 nm

at 350 nm

PGA-NTR probe 9 with NTR (0.6 U) and PGA (0.75) after 60 min

at 260 nm

at 350 nm
PGA-NTR probe 9 with NTR (0.6 U) and PGA (0.75 U) after 120 min

at 260 nm

at 350 nm

PGA-NTR probe 9 with NTR (0.6 U) and PGA (0.75 U) after 180 min

at 260 nm

at 350 nm