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COMMUNICATION

A simple umbelliferone based fluorescent probe for the detection of nitroreductase

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Electronic Supplementary Material

1. Fluorescence analysis

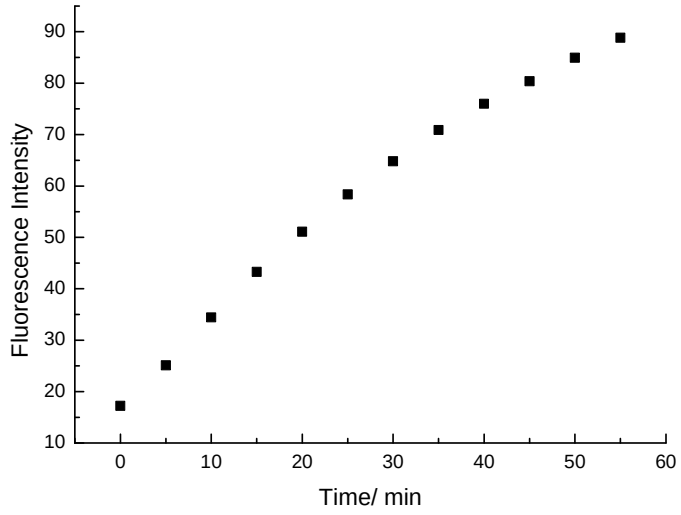


Fig. S1 Time curve of **NCOU1/** 10 μM with the addition of nitroreductase/ 8 μg/mL and NADH/ 500 μM in 10 mM PBS (pH 7.4) . λex = 315 nm

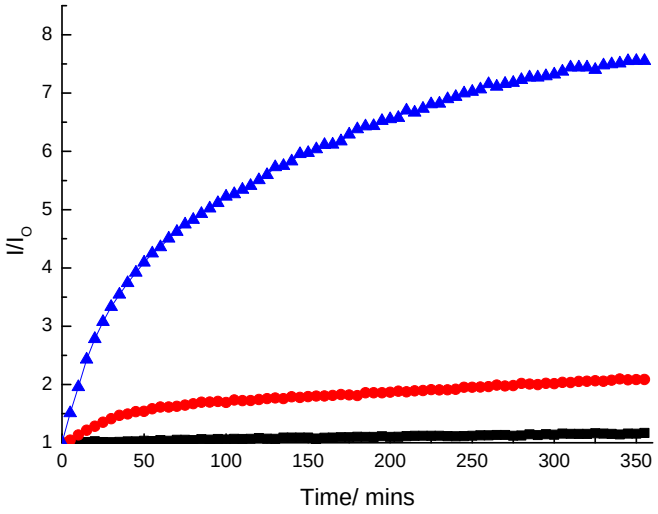


Fig. S2 Fluorescence intensity changes (I/I_O) of **NCOU1/** 10 µM over 6 h with the addition of various concentrations of nitroreductase (0 – black, 0.5 - red and 1 µg/mL - blue) and NADH/ 500 µM in 10 mM PBS pH 7.4 λex = 315 nm/ λ_{em} = 455 nm

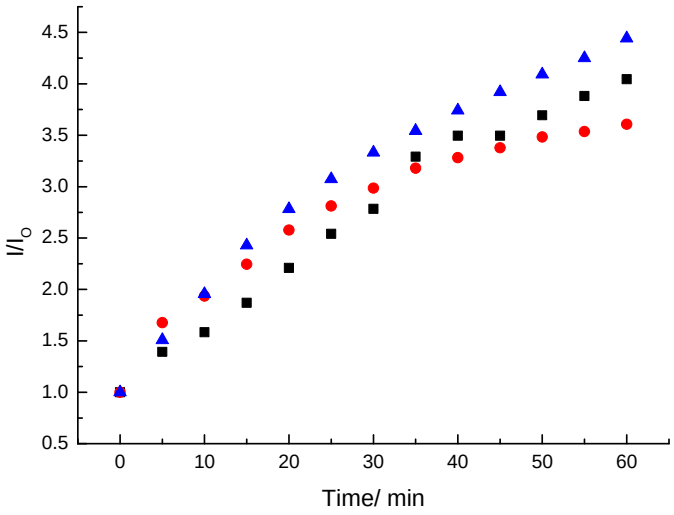


Fig. S3 Fluorescence intensity changes (I/I_O) of **NCOU1/** 10 µM over 60 minutes with the addition of nitroreductase/ 1 µg/mL and NADH/ 500 µM in various pH solutions (blue – 7.4, black – 5.8, red – 8.0) λex = 315 nm/ λ_{em} = 455 nm

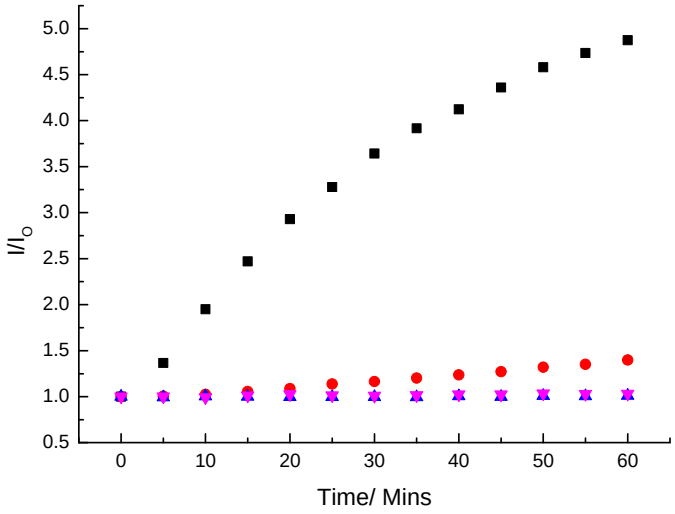


Fig. S4 Fluorescence intensity changes (I/I_O) of **NCOU1/** 10 µM over 60 minutes with the addition of nitroreductase – **black**/ 1 µg/mL, nitroreductase and dicoumarol – red/ 1 µg/mL, DT Diaphorase – pink/ 1 µg/mL and a blank. All measurements contained NADH (500 µM) in 10 mM PBS pH 7.4 buffer solution λex = 315 nm/ λ_{em} = 455 nm

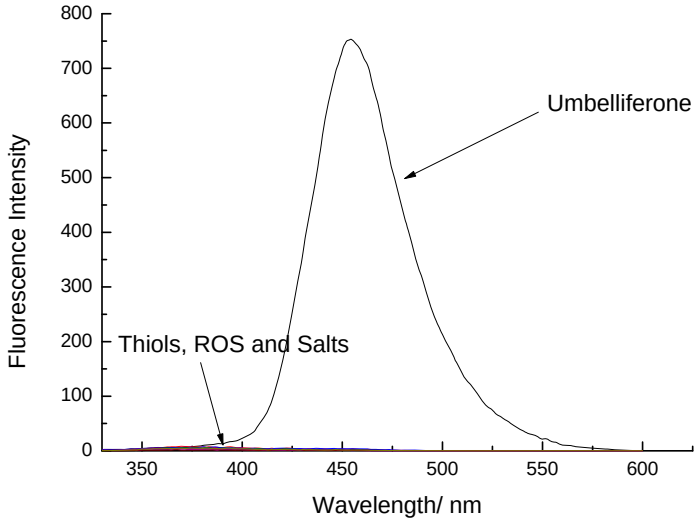


Fig. S5 Fluorescence spectra of **NCOU1**/ 10 µM with the addition of a range of analytes (Cysteine/ 100 µM, GSH/ 100 µM, $ONOO^-$/ 100 µM, H_2O_2/ 100 µM, ClO^-/ 100 µM, LiCl/ 100 µM, NaCl/ 100 µM, KCl/ 100 µM). Umbelliferone/ 10 µM was used as a reference. Experiments were carried out in PBS buffer solution pH 7.4. λex = 315 nm. Slit widths ex = 10 nm em = 5 nm

2. Limit of Detection

The detection limit was calculated based on fluorescence measurements (λ_{exc} = 360 nm, λ_{em} = 450 nm) where **NCOU1** concentration was kept constant (5 µM) and enzyme concentration was varied (0 – 4 µg/mL). In the absence of NTR, the fluorescence emission spectrum of **NCOU1** was measured 10 times and the standard deviation of blank measurement was

measured (σ = 23). The fluorescence intensity at 450 nm was plotted against the concentration of NTR before the detection limit was calculated with the following equation:

$$\text{Detection limit} = 3\sigma/k$$

where σ is the standard deviation of blank measurement and k is the slope between the fluorescence intensity versus the concentration of NTR.

$$\text{Detection limit} = 3(23)/414.57 = 0.166\ \mu g/mL$$

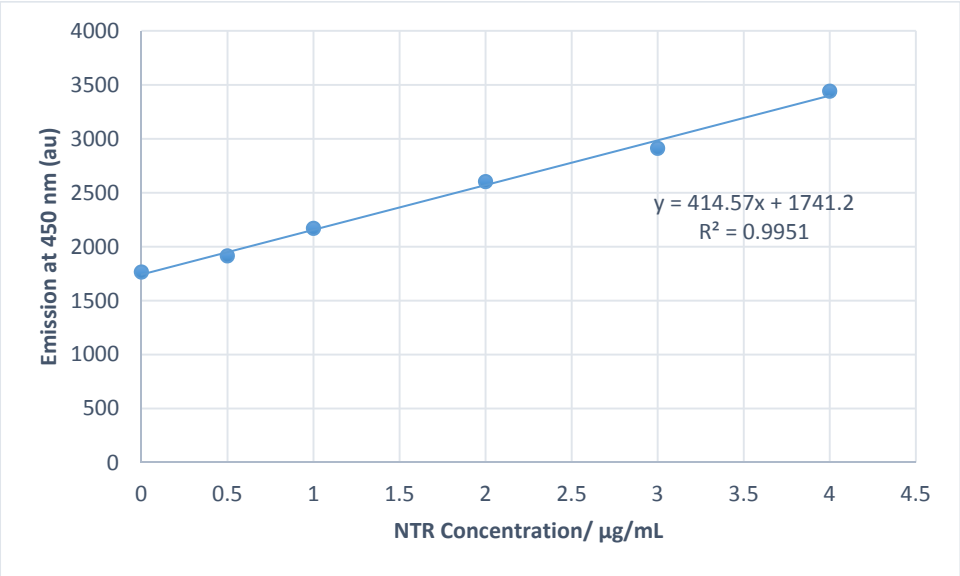


Fig. S6 Fluorescence emission of **NCOU1**/ 10 µM versus change in concentration of NTR/ 0-4 µg/mL in 10 mM PBS pH 7.4 buffer solution λex = 350 nm/ λ_{em} = 450 nm

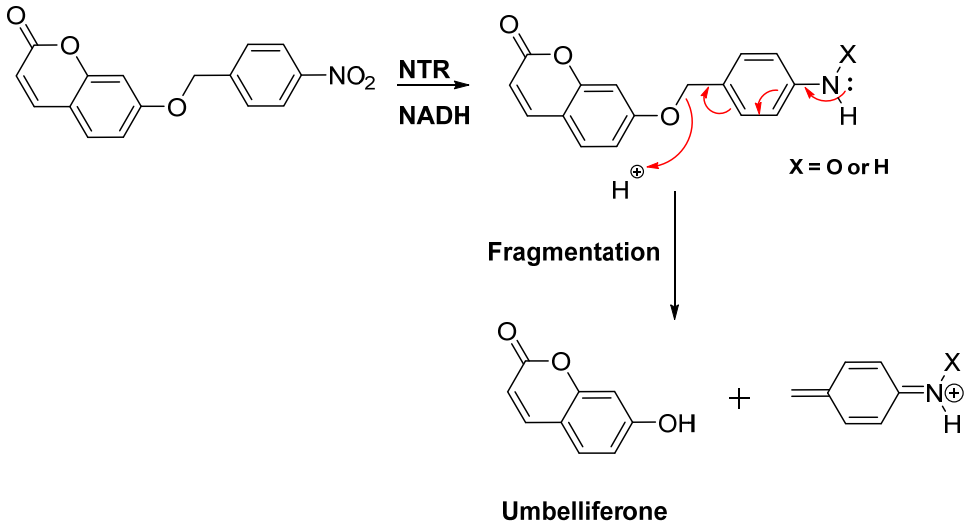
Raw Data:

NTR Conc (ug/mL)	Emission at 450 nm	I/I0
4	3440	1.95006
3	2911	1.65019
2	2602	1.47503
1	2169	1.22958
0.5	1914	1.08503
0	1764	1

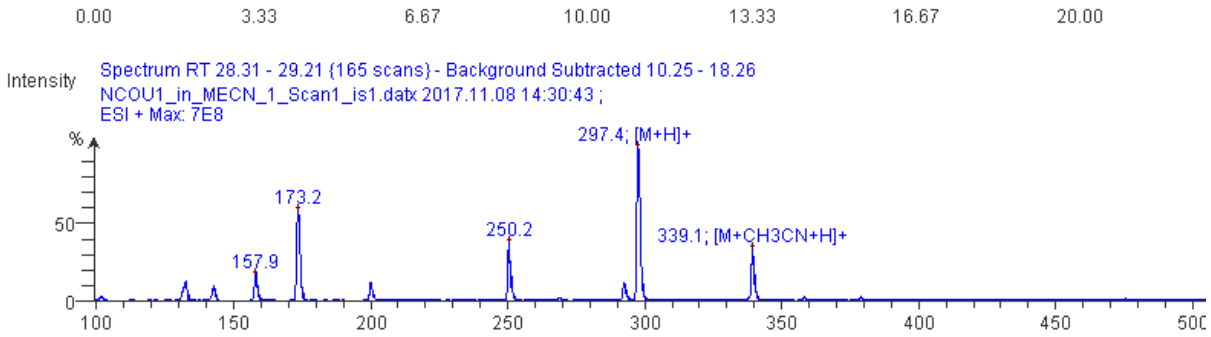
3. Mechanism of NCOU1 reduction confirmed using mass spectrascopic analysis

The nitro group of **NCOU1**can be reduced to either a hydroxylamine or amine, both of which are known to be unstable. Once **NCOU1** has been reduced to a hydroxylamine or amine elimination of the electron donating benzyl fragment occurs, releasing the highly fluorescent

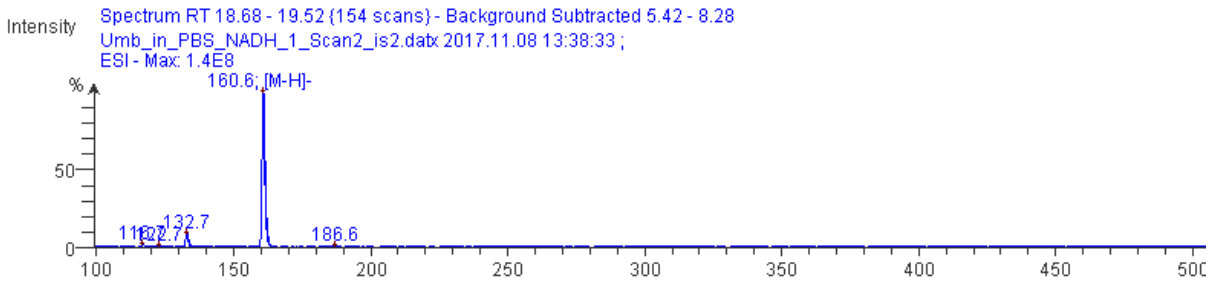
umbelliferone. The release of umbelliferone is monitored using fluorescence and umbelliferone formation was confirmed using mass spectrascopic analysis. After the treatment of **NCOU1** with NTR (10 µg/mL) and NADH (500 µM), the mass of umbelliferone was observed in the mass spectrum – see Fig S7(c).



(**a**) **NCOU1** only (ESI+)



(**b**) Umbelliferone only (ESI-)



(**c**) **NCOU1** treated with NTR (10 µg/mL) and NADH (500 µM) (ESI-)

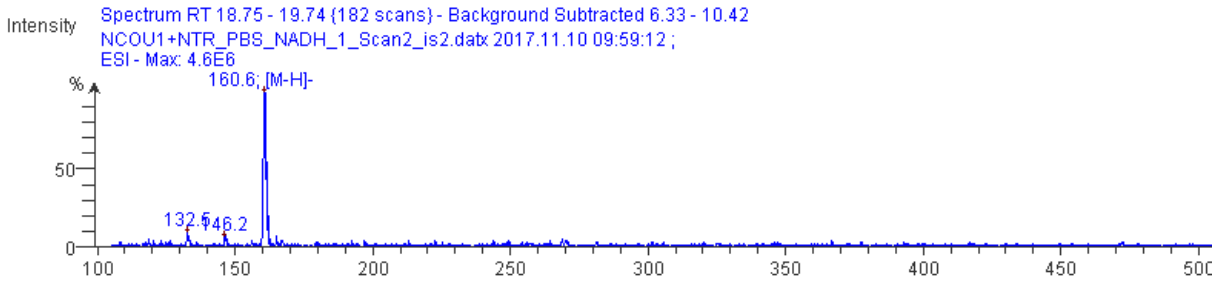
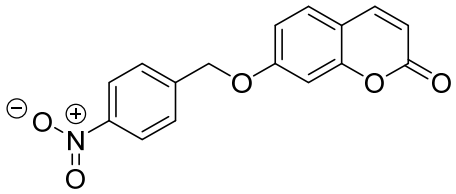


Fig. S7. Mass spectroscopic analysis (**a**) **NCOU1** (ESI+); (**b**) Umbelliferone (ESI-) (**c**) **NCOU1** treated with NTR (10 µg/mL) and NADH (500 µM) (ESI-).

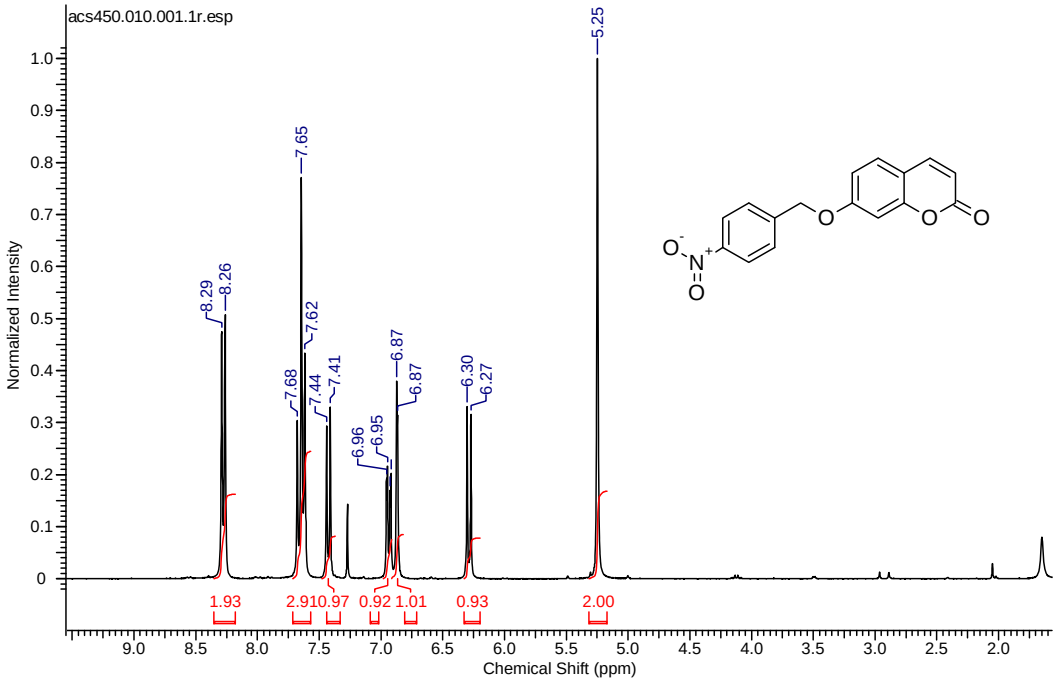
Experimental



7-Hydroxycoumarin (1.00 g, 6.17 mmol) was dissolved in DMF (50 mL) followed by the addition of K_2CO_3 (2.56 g, 18.51 mmol) and 4-nitrobenzyl bromide (1.12 g, 6.17 mmol). The reaction mixture was stirred for 4 hrs. EtOAc (100 mL) was then added and the organic layer was washed with H_2O (3 x 50 mL), brine (100 mL) and dried ($MgSO_4$) and concentrated *in-vacuo* to afford the crude material. The title compound was purified via trituration to afford a white solid (1.12 g, 3.80 mmol, 61 %). M.p. 183 – 185 ºC. ^{1}H NMR (300MHz, $CDCl_3$) δ 8.28 (d, *J* = 8.7 Hz, 2 H), 7.71 - 7.57 (m, 3 H), 7.43 (d, *J* = 8.7 Hz, 1 H), 6.94 (dd, *J* = 2.4, 8.6 Hz 1 H), 6.87 (d, *J* = 2.1 Hz, 1 H), 6.29 (d, *J* = 9.6 Hz, 1 H), 5.25 (s, 2 H); ^{13}C NMR (75.5 MHz , $CDCl_3$) δ 161.1, 161.0, 155.8, 147.8, 143.3, 143.1, 129.1, 127.8, 124.0, 113.7, 113.2, 113.0, 101.9, 69.1; I.R (thinfilm) ν max (cm^{-1}): 1722.13 (C=O); HRMS (ESI): m/z calculated for $C_{16}H_{12}NO_5$: requires 298.0715 for $[M+H]^+$, found 298.0694.

4. NMR

7-((4-Nitrobenzyl)oxy)-2H-chromen-2-one (300 MHz, $CDCl_3$)



7-((4-Nitrobenzyl)oxy)-2H-chromen-2-one (75.5 MHz, $CDCl_3$)