APPENDICES

APPENDIX A

SUPPORTING INFORMATION

A.1 Probe synthesis and characterization

IND-OH based on an indolium fluorophore and linked with 4-hydroxybenzene was chosen as a simple and convenient fluorescent reporter for NTR detection. Subsequently, 4-nitrobenzene, commonly referred to as the classical moiety reduced by NTR, was conjugated with IND-OH using an ester linker to synthesize IND-NO_2, which is non-fluorescent due to loss of donor-acceptor structure (Figure A.1).

To synthesize IND-NO_2, compound 1 was primarily synthesized by the substitution reaction of 4-hydroxybenzaldehyde and 4-nitrobenzyl bromide.(Rostami and Hamidi Zare, 2019) Then, the reaction between indolium, prepared as previously described,(Khaikate et al., 2024; Usama et al., 2018) compound 1 was carried out to generate the corresponding IND-NO_2 with a moderate yield (55%). Meanwhile, the condensation of indolium and 4-hydroxybenzaldehyde was also performed to afford the corresponding IND-OH in a moderated yield (72%). This synthesis was done by Asst. Prof. Dr. Onnicha Khaikate.

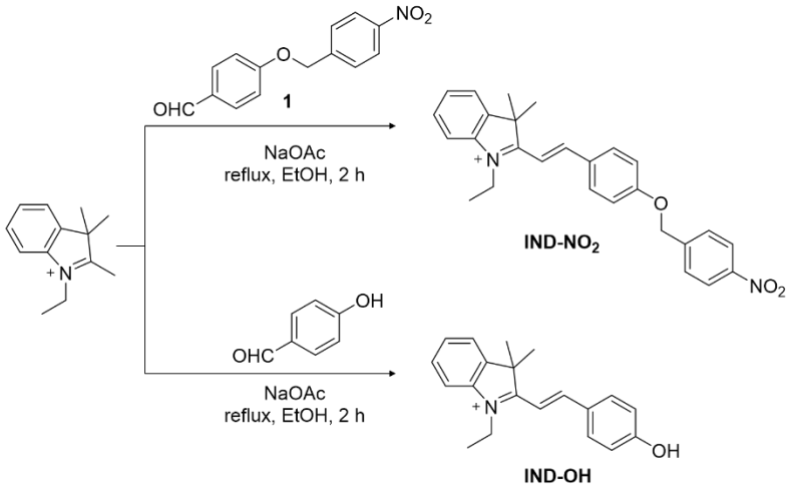

Figure A.1 Synthetic routes for IND-NO_2 and IND-OH.

A.2 Photophysical properties of IND-NO_2 and IND-OH

Firstly, the photophysical properties of IND-NO_2 in different solvents, including THF, DMSO, MeOH, and PBS buffer (pH 7.4), were determined by Asst. Prof. Dr. Onnicha Khaikate, and the results are summarized in Table A.1. As illustrated in Figure A.2A, the absorption spectra of IND-NO_2 in all tested solvents exhibited negligible changes, with absorbance maximum ranges from 418 to 432 nm. Weak fluorescence emission of IND-NO_2 was detected in various solvents, as displayed in Figure A.2B. Nonetheless, the outcomes met our need for a turn-on fluorescent probe. Next, the photophysical properties of reporter IND-OH, containing a typical D-π-A structure with an electron push–pull effect, were measured in different solvents (Figures A.2C–D). In all solvents, the absorption region is well-extended to the visible area. The maximum absorbance of IND-OH is between 459 and 548 nm in all tested solvents (Figure A.2C). Furthermore, IND-OH exhibited a fluorescence increase in protic solvents, peaking at 545–567 nm (Figure A.2D), with quantum yields ranging from 0.0003 to 0.0013 (Table A.1). This might be due to the hydrogen bonding between the reporter IND-OH and the solvents, resulting in the stabilization of excited-state species (Krystkowiak et al., 2006).

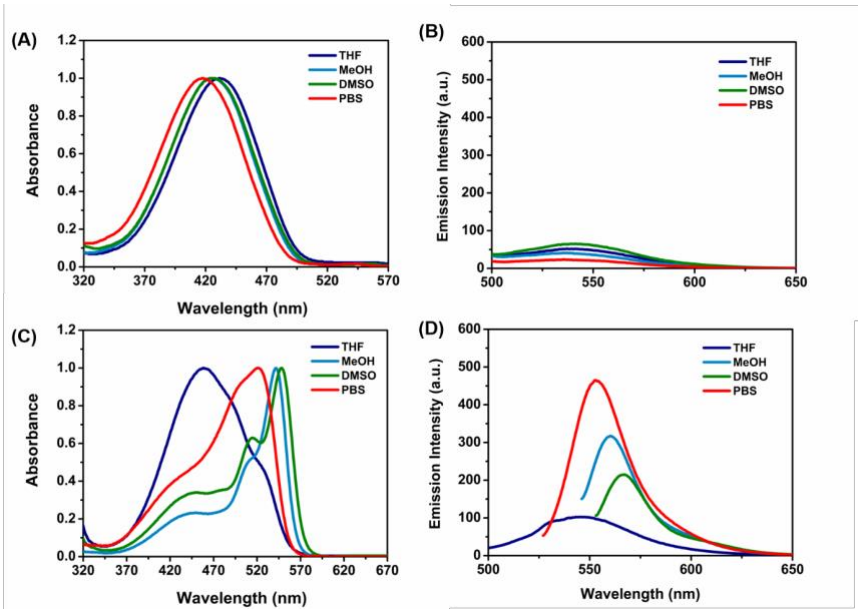

Figure A.2 (A) Absorption spectra and (B) fluorescence spectra (excited at λ_{max} in each solvent) of IND-NO_2 (10 μM) in different solvents. (C) Absorption spectra and (D) fluorescence spectra (excited at λ_{abs} in each solvent) of IND-OH (10 μM) in different solvents.

Table A.1 Photophysical properties of IND-NO_2 and IND-OH (10 μM) in different solvents.

Photophysical properties						
Probes	**Solvents**	[a]**λ**$_{abs}$ (nm)	[b]**ε** ($M^{-1}cm^{-1}$)	[c]**λ**$_{em}$ (nm)	Δ**λ** (nm)	[d]**Φ**$_f$
IND-NO_2	THF	432	35,200	Non-fluorescence		
	MeOH	425	34,200	Non-fluorescence		
	DMSO	426	31,800	Non-fluorescence		
	PBS	418	32,900	Non-fluorescence		
IND-OH	THF	459	25,900	545	86	0.0004
	MeOH	542	49,100	560	18	0.0003
	DMSO	548	41,600	567	19	0.0013
	PBS	521	32,600	553	32	0.0006

[a]**λ**$_{abs}$ = absorption maximum wavelength. [b]**ε** = molar absorptivity. [c]**λ**$_{em}$ = emission maximum wavelength (Excitation wavelength at **λ**$_{abs}$). [c]Δ**υ** = stokes shifts (**λ**$_{em}$ - **λ**$_{abs}$). [d]**Φ**$_f$ = fluorescence quantum yields calculated using quinine sulfate in 0.1 M H_2SO_4 was used as a standard (**Φ**$_f$ = 0.54).

A.3 The photographs of *Ec*NfsB catalyze reduction of IND-NO_2 to generating IND-OH

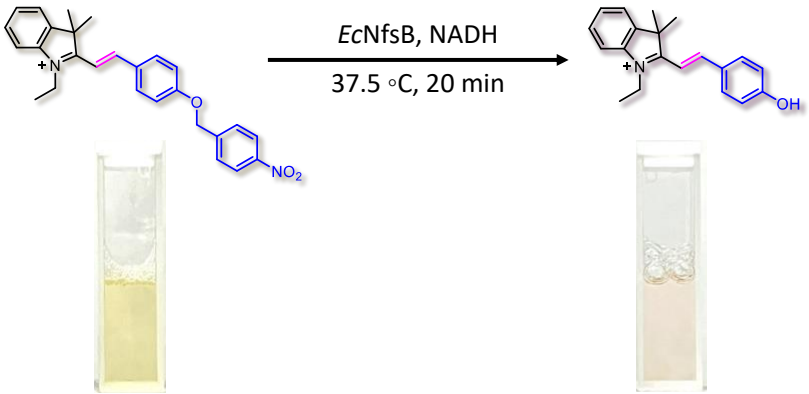

Figure A.3 The photographs of *Ec*NfsB catalyze the reduction of IND-NO_2 to generate IND-OH. The reaction containing 10 μM IND-NO_2 incubated with 0.5 μM *Ec*NfsB and 50 μM NADH in PBS buffer pH 7.4 at 37 °C for 20 min. The generation of IND-OH displayed a pink colour which can be observed by the naked eyes.

A.4 References

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Krystkowiak, E., Dobek, K., and Maciejewski, A. (2006). Origin of the strong effect of protic solvents on the emission spectra, quantum yield of fluorescence and fluorescence lifetime of 4-aminophthalimide: Role of hydrogen bonds in deactivation of S1-4-aminophthalimide. *Journal of Photochemistry and Photobiology A: Chemistry. 184*(3), 250-264.

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APPENDIX B

PRESENTATIONS

List of poster presentation

Tawanya Kamthong, Onnicha Khaikate, Anyanee Kamkaew, and Rung-Yi Lai (February 2024). Improvement of esterification catalyzed by carboxylic acid reductase and Investigation of nitroreductase detection by a fluorescence probe. **Science Postgrad Annual Research Conference: SPARC 2024,** Institute of Science, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Tawanya Kamthong, Onnicha Khaikate, Anyanee Kamkaew, and Rung-Yi Lai (August 2024). Investigation of nitroreductase detection by a fluorescence probe. **The 18th International Symposium of the Protein Society of Thailand**, Convention Center, Chulabhorn Research Institute, Bangkok, Thailand.

Abstract submitted in Science Postgrad Annual Research Conference: SPARC 2024

Science Postgrad Annual Research Conference: SPARC 2024
Institute of Science, Suranaree University of Technology
23 February 2024

Improvement of esterification catalyzed by carboxylic acid reductase and Investigation of nitroreductase detection by a fluorescence probe

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Abstract

There are two enzymatic projects in this report. The first project is to improve esterification catalyzed by carboxylic acid reductases (CAR). The native reaction of CAR is to reduce carboxylic acid to aldehyde by using ATP and NADPH in the presence of Mg^{2+}. Because of its potential of biocatalyst application, CAR was developed to catalyze esterification of carboxylic acid in the presence of alcohol without NADPH in the reaction. However, its model esterification reaction of cinnamic acid and methanol was reported about 45%. To improve its yield, ATP regeneration catalyzed by polyphosphate kinase and pyrophosphate degradation catalyzed by pyrophosphatase were systematically applied in the CAR-catalyzed esterification. The conversion and yield of the optimized reaction were 90% and 65%, respectively. The second project is to investigate a fluorescence probe for the detection of nitroreductase (NTR) in bacteria. *E. coli* NTR was chosen to be a model enzyme to show that the probe's nitro group can be reduced by NTR to form an amino group, that generates the fluorescence signal. The reaction was confirmed by fluorescence spectroscopy and HPLC. The enzyme kinetic for the probe was further determined. Lastly, the reduction of the probe was only catalyzed by NTR compared with the control experiments using various biological reductants.

Keywords: Biocatalysts; Carboxylic acid reductase; Esterification; Nitroreductase; Fluorescent probe

Abstract submitted in the 18th International Symposium of the Protein Society of Thailand

PP075

Investigation of nitroreductase detection by a fluorescence probe

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ABSTRACT

Nitroreductase (NTR) in a wide range of bacteria plays a vital role in the detoxification of nitro-containing compounds. Therefore, nitroreductase detection could be applied in detection of bacteria. In this work, we reported an NTR-responsive fluorescent probe (OK-54) to detect NTR in few microorganisms. In the preliminary tests, *E. coli* NTR was chosen to be a model enzyme. The probe's nitro group can be reduced by NTR to form an amino group in the presence of nicotinamide adenine dinucleotide (NADH), resulting in a significant increase of fluorescence signal at 564 nm. The reaction was characterized by fluorescence spectroscopy and HPLC to confirm the product identity. The enzyme kinetic for the probe's reduction was further determined. In addition, to confirm the sensing specificity, the results showed that the reduction of the probe is only catalyzed by NTR compared with the control experiments using various biological reductants. Lastly, the probe was successfully used to detect bacterial NTR activity *in vivo*.

The 18th International Symposium of the Protein Society of Thailand. Convention Center, Chulabhorn Research Institute, Bangkok, Thailand. Aug 28-30, 2024.