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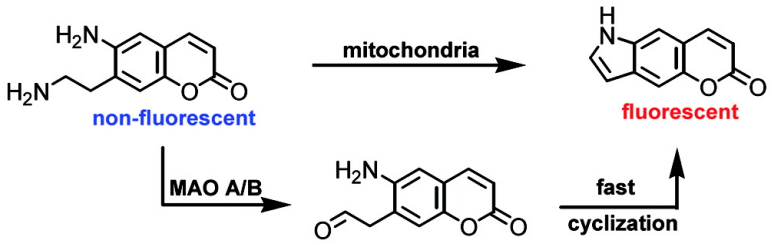

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Design of Optical Switches as Metabolic Indicators: New Fluorogenic Probes for Monoamine Oxidases (MAO A and B)

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The continuous and sensitive assays allow for real time monitoring of enzymatic activity in complex biological systems (i.e., tissue homogenates, intact cells). Fluorescence detection methods are particularly suitable in this context owing to their high sensitivity. These methods rely on the availability of fluorogenic substrates, which provide an emission signal for a specific enzymatic process.[1] We herein describe the rational design of a novel fluorogenic indicator for monoamine oxidases (MAO) on the basis of an oxidative deamination−cyclization sequence. We also illustrate how the exploration of enzyme promiscuity may be utilized for the development of synthetic reporter substrates.

MAOs (A and B, EC 1.4.3.4) are FAD-dependent enzymes that catalyze the aerobic oxidation of amine substrates to the corresponding imines, which are subsequently hydrolyzed to the aldehyde products (Figure 1A).[2] These enzymes play an important role in the central nervous system, maintaining the homeostasis of neurotransmitters such as dopamine and serotonin, as well as in the periphery, metabolizing dietary amines and pharmaceuticals. Altered levels of MAO activity have been associated with many neurological and psychiatric diseases.[3] Although there are numerous activity assays, none are suitable for the imaging of MAO in intact cells and tissues.[4,5] Toward this goal, fluorogenic substrates that provide a direct and sensitive readout of the MAO activity are required.

We have recently developed a ketone−alcohol fluorogenic probe for aldo−keto reductases wherein the ketone moiety was directly attached to the π-system of the fluorophore.[1a] We now introduce a different mechanism for a redox switch, based on a sequence of oxidation and intramolecular cyclization. According to this proposal, enzymatic oxidation of the ethylamino group affords an aldehyde intermediate, which subsequently undergoes spontaneous intramolecular condensation with the aniline amino group, furnishing an indole moiety in an irreversible fashion. This overall chemical process results in a profound electronic change of the system, which in turn should alter the fluorescence profile (Figure 1B).

Specifically, we anticipated that the emission of motif I would be quenched by the free amine of the aniline ring via photoinduced electron transfer (PET), while the fluorescence of the coumarin system would be recovered upon formation of the indole (Figure 2A).[6] Consequently, compounds **1** and **2** were synthesized and evaluated in terms of their photophysical properties. Indeed, this pair constituted the desired fluorescence switch with a 12-fold increase in emission intensity (Figure 2B). Furthermore, the corresponding aldehyde intermediate was not detected, suggesting rapid and efficient condensation with the amine in the ortho position to furnish the desired indole moiety. Hence, the fluorescence readout directly correlated with the kinetics of the oxidative step (for nonenzymatic cyclization experiments, see Supporting Information) At this point, two out of three key elements required for a suitable MAO reporter were secured, namely, the emission switch and the rapid cyclization. The third prerequisite, however, was not satisfied

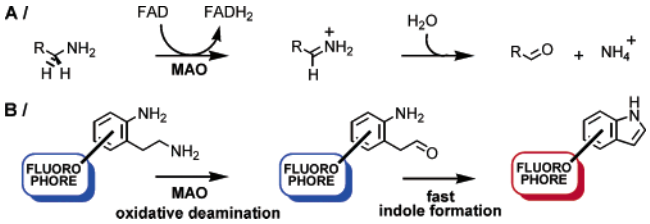

Figure 1. (A) Mechanism of oxidative deamination by MAO. (B) Design of an irreversible fluorogenic redox switch based on the oxidation−cyclization cascade.

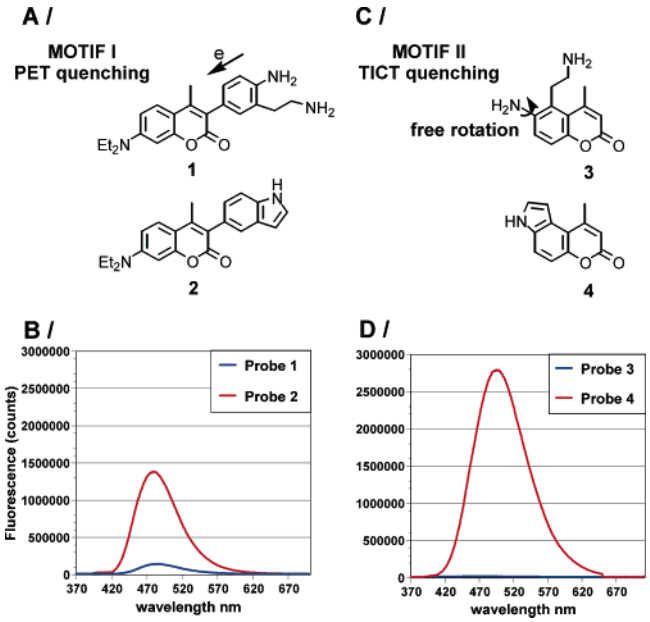

Figure 2. (A, B) Design and emission spectra of motif I probes (λ_{ex} = 401 nm). (C, D) Design and emission spectra of motif II probes (λ_{ex} = 355 nm); [probe] = 5 μM, pH 7.4, sodium phosphate buffer.

as compound **1** was not a substrate for either MAO A or B, most likely owing to its size.

Informed by this exercise, we pursued a probe of smaller size and higher dynamic range (i.e., higher emission increase). Motif II was based on nonfluorescent 6-aminocoumarin; it has been proposed that the excited state of this compound decays to a nonradiative twisted intramolecular charge transfer (TICT) state, readily accessible via a free rotation of the amino group in the 6-position.[7] Thus, we noted that the emission should be recovered when TICT is prevented by the formation of the indole ring (Figure 2C). As first demonstrated with compounds **3** and **4**, this approach was indeed successful. Compound **4** exhibited a greatly enhanced fluorescence in comparison to **3** (300-fold emission increase, Figure 2D). However, the low stability of compound **3**, as evidenced by facile conversion to side product **5**, precluded further investigation of this system (Scheme 1).

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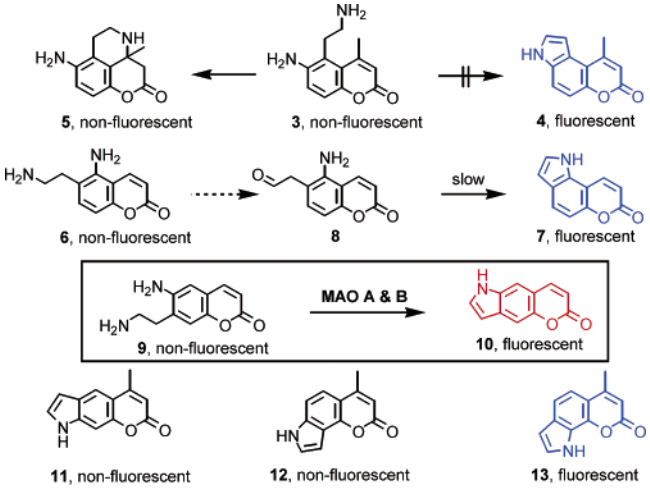
Scheme 1. Systematic Mapping of Aminocoumarins and the Corresponding Pyrrolocoumarins



Encouraged by the favorable emission properties of compounds **3** and **4**, we turned our attention to related systems wherein the undesirable cyclization would be eliminated by the attachment of the aminoethyl group to the C-6 or C-7 position of the coumarin core. Specifically, 5-aminocoumarin derivative **6** and the corresponding pyrrolocoumarin **7** were constructed and subsequently found to exhibit desired differences in emission intensity. However, this system also failed to qualify for the enzymatic studies as cyclization of aldehyde **8** (indole formation step) was slow, owing to the low nucleophilicity of the aniline amino group in the 5-position.

Consequently, we returned to the 6-aminocoumarin system; however, this time the aminoethyl side chain was placed at position C-7, furnishing probe **9**. This constitution not only prevented the undesirable cyclization, but also led to an excellent fluorescence switch (200-fold increase in emission intensity, $\lambda_{em} = 524$ nm), and the desired indole formation was rapid. At last we arrived at a probe that exhibited the favorable properties, including chemical stability, fluorogenic switching, and a fast reporting mechanism (cyclization).

The origin of the significant enhancement of emission intensity (between **9** and **10**) may be hinted by considering brightness, a related measure of fluorescence, defined as the product of the extinction coefficient and the quantum yield. Indeed, in the case of compounds **9** and **10**, a relatively small difference in quantum yield (5-fold), coupled with a larger change in the absorption strength (8−9-fold), resulted in 40−50-fold increase in brightness. Thus, TICT phenomenon, used to design these probes, may represent a minor contributor to the overall emission change.

Finally, we faced the key question of whether probe **9** would be a suitable substrate for MAOs. To approximate the in vivo environment, we utilized mitochondrial preparations rather than the purified enzymes. Human placental mitochondria was used for examining MAO A (only MAO A is expressed in this tissue), while beef liver mitochondria was used for MAO B. The suspension of mitochondria was incubated with probe **9** and monitored fluorimetrically at 524 nm (Supporting Information). To our delight, the fluorescence of the mixture increased with time, indicating the conversion of the probe. Both MAO A and B were active, and the conversion was abolished by the corresponding inhibitors chlorgyline and deprenyl. It was established that the fluorescence increase correlated with the concentration of product **10**, and thus the kinetic parameters may be directly derived from the fluorometric data (for MAO B, $K_m = 510 \pm 40$ μM, $k_{cat} = 21$ min^{-1}; MAO A, $K_m = 31 \pm 2$ μM, $k_{cat} = 0.5$ min^{-1}).[8] Owing to the significant difference in the turnover frequency, good selectivity for MAO B can be achieved at sufficiently high concentration of the probe (>100 μM; Supporting Information). Probe **9** is indeed a good substrate for MAO B, a fact that was revealed upon comparison to the physiological substrate dopamine (Figure 3).[9] Also, the kinetic parameters of **9** are comparable to the known chromogenic[4] and fluorolytic probes.[5b]

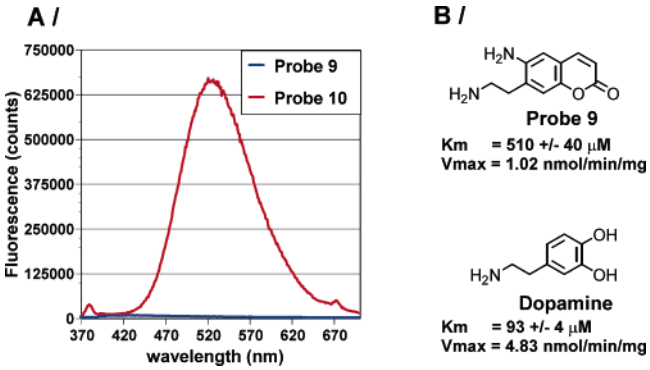


Figure 3. (A) Emission spectra of probes **9** and **10** (5 μM, pH 7.4, $\lambda_{ex} =$ 335 nm). (B) Kinetic parameters for probe **9** and dopamine (MAO B).

In summary, this study describes the development of a sensitive, selective, and fluorogenic reporter substrate for MAO enzymes. It also serves to illustrate the considerable challenge involved in the de novo design of metabolic indicators, which stems from the need to converge many diverse molecular and functional properties, including emission switching, photophysical characteristics, chemical stability, and ability to function as an enzyme substrate.

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Supporting Information Available: Synthetic protocols for **9** and **10**, photophysical characterization of selected probes, kinetic parameters, and enzymology studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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