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Accounts

Fluorescent Probes for Analysis and Imaging of Monoamine Oxidase Activity

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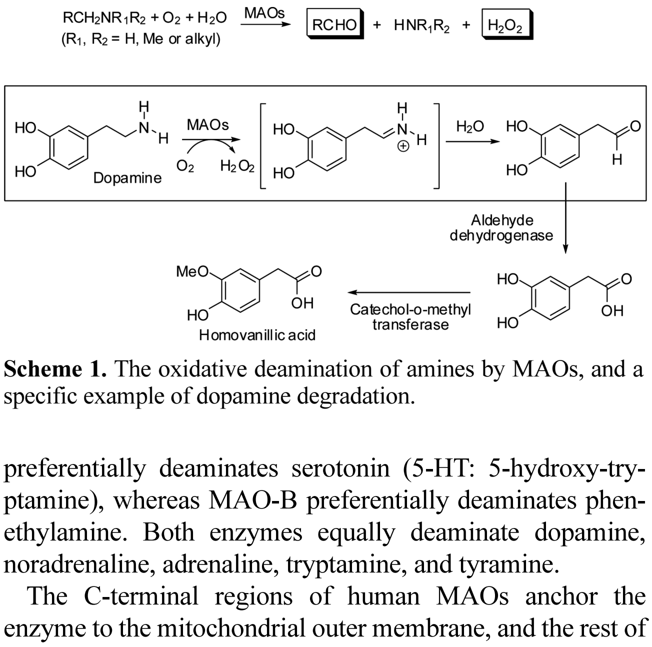
Monoamine oxidases catalyze the oxidative deamination of dietary amines and amine neurotransmitters, and assist in maintaining the homeostasis of the amine neurotransmitters in the brain. Dysfunctions of these enzymes can cause neurological and behavioral disorders including Parkinson's and Alzheimer's diseases. To understand their physiological roles, efficient assay methods for monoamine oxidases are essential. Reviewed in this Perspective are the recent progress in the development of fluorescent probes for monoamine oxidases and their applications to enzyme assays in cells and tissues. It is evident that still there is strong need for a fluorescent probe with desirable substrate selectivity and photophysical properties to challenge the much unsolved issues associated with the enzymes and the diseases.

Key Words : Monoamine oxidase (MAO), Fluorescent probes, Fluorescence imaging, Neurotransmitters, Neuronal disease

Introduction

Monoamine oxidases (MAOs) are a family of FAD-dependent enzymes found in the outer mitochondrial membrane of neuronal, glial, and other mammalian cells. These enzymes are responsible for catalyzing the oxidative deamination of neurotransmitters and dietary amines, regulating intracellular levels of biogenic amines.[1,2] The oxidative deamination of dopamine by MAOs is illustrated in Scheme 1. The enzyme oxidizes the amine functionality to the iminium ion, which undergoes hydrolysis to produce the corresponding aldehyde; from this aldehyde, homovanillic acid is eventually produced by other enzymatic processes. The enzymatic oxidation also generates hydrogen peroxide and ammonia (in case of primary amine substrates) as the side products, in addition to the aldehyde, and these chemicals are known to influence the biological system involved.

Two isoenzymes of MAO (MAO-A and MAO-B) are present in most mammalian tissues, and they show different substrate preference and inhibitor specificities.[3] MAO-A preferentially deaminates serotonin (5-HT: 5-hydroxy-tryptamine), whereas MAO-B preferentially deaminates phenethylamine. Both enzymes equally deaminate dopamine, noradrenaline, adrenaline, tryptamine, and tyramine.



Scheme 1. The oxidative deamination of amines by MAOs, and a specific example of dopamine degradation.

The C-terminal regions of human MAOs anchor the enzyme to the mitochondrial outer membrane, and the rest of

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the protein is exposed to the cytoplasm. MAOs are present in most mammalian tissues, but their distribution varies from tissue to tissue.[4-6] MAOs in peripheral tissues such as the intestine, liver, lungs, and placenta seem to protect the body from foreign amines. The physiological functions of MAOs in the brain are yet to be addressed, but it is suggested that MAOs protect neurons from exogenous amines, terminate the actions of amine neurotransmitters, and regulate the contents of intracellular amine stores.[1,2]

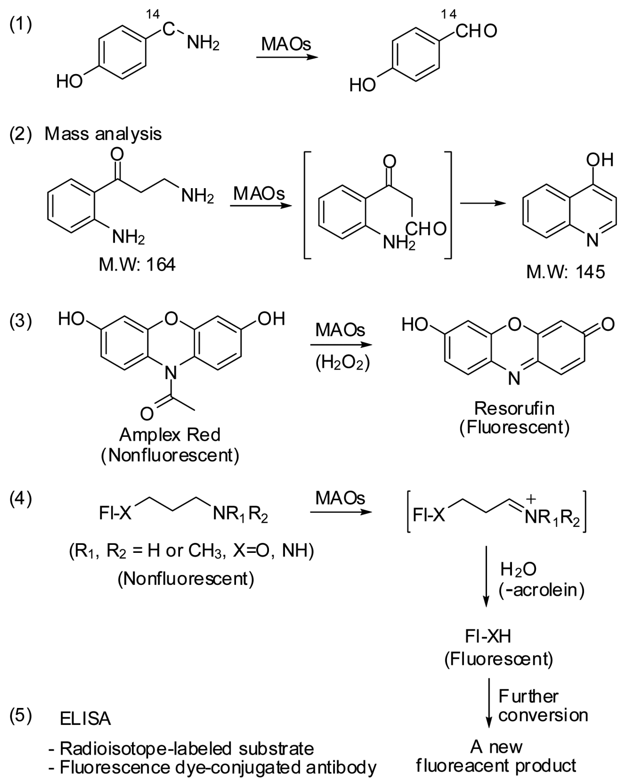
Crystal structures for both human MAO-A and MAO-B are known.[7-9] According to the crystal structure analysis of human MAO-B bound with inhibitor rasagiline (which, in turn, covalently bound to the flavin FAD coenzyme) by Mattevi and co-workers, MAO-B has an “entrance cavity” of 290 Å^3 and a hydrophobic “substrate cavity” of 490 Å^3, showing more elongated and narrower cavity than that of the MAO-A which has a single hydrophobic cavity of ~550 Å^3.[2,6]

Dysfunction of MAOs is associated with disorders in some central and peripheral nervous systems. Extra high MAO-B activity in the brain is associated with neurological degenerations involving Alzheimer’s disease, Huntington’s disease, some forms of Parkinson’s disease and normal aging. Abnormally low MAO-A activity is associated with depression, schizophrenia, anxiety and psychiatric disorders.[10] MAO inhibitors have been clinically found to alleviate symptoms or slow deterioration of those diseases.[11] MAO-A inhibitors are found to be effective in the treatment of panic disorders, mixed anxiety and atypical depression, whereas MAO-B inhibitors are used for the treatment of Alzheimer’s and Parkinson’s (selegiline, moclobemide) diseases, also as an alternative for migraine prophylaxis.[12,13]

Analytical methods for MAOs are essential for screening of inhibitors as well as for monitoring of enzymatic activity in complex biological systems. In this context, fluorescent probes for MAOs can provide convenient and sensitive tools. In this article, the recent progress in the development of fluorescent MAO probes is overviewed.

Assay Methods for MAO Activity

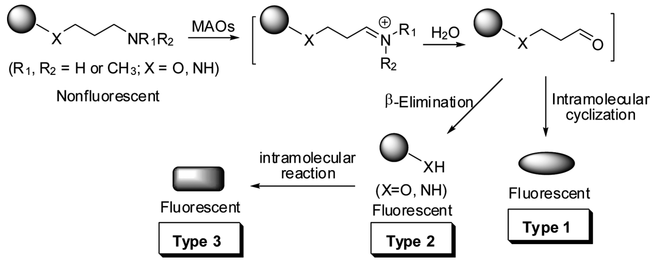
Assay methods for the MAO activity can be used for the diagnosis of the enzyme-associated diseases and also for screening of the enzyme inhibitors as potential drug candidates. Various methods have been thus developed for assaying of MAO activity: analysis of the enzymatic oxidation products (1) by radiometry,[14] and (2) by liquid chromatography and mass spectrometry;[15] (3) monitoring of oxygen consumption or hydrogen peroxide generation from the enzymatic activity;[16] (4) fluorimetric assay;[17] (5) enzyme-linked immunosorbent assay (ELISA)[18] using radioisotope-labeled substrate[19] or dye-conjugated antibodies[20] (Scheme 2). These methods have been used to elucidate the distribution of MAOs and their biological and physiological roles. Among them, the fluorescent method using molecular probes has received much attention in the last decade due to its advantageous features such as high sensitivity, fast response, and bioimaging capability.[21] In this review, we have overviewed the fluorescent probes recently developed for assaying and bioimaging of MAO activity.



Scheme 2. Different approaches to assay MAO activity.

Fluorescent Probes for MAOs

Tools enabling the detection and imaging of biological molecules are essential in modern chemical biology. Tremendous efforts have been made to develop efficient fluorescent probes for monitoring and imaging of various enzymes’ activity.[22] The various physiological phenomena associated with MAOs also demand appropriate fluorescent probes. A useful strategy to develop fluorescent MAO probes is to combine the enzymatic amine oxidation with subsequent chemical transformations in such a way that the conversion can induce desirable fluorescence change. The known fluorescent probes thus can be categorized into three types according to the reaction scheme that follows the enzymatic oxidation step (Scheme 3).

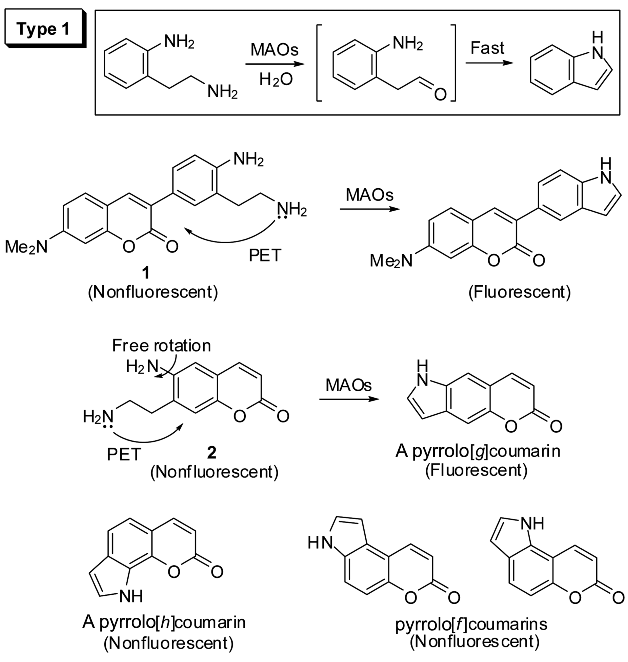


Scheme 3. Fluorescent MAO probes categorized by the chemical conversion following the enzymatic reaction.

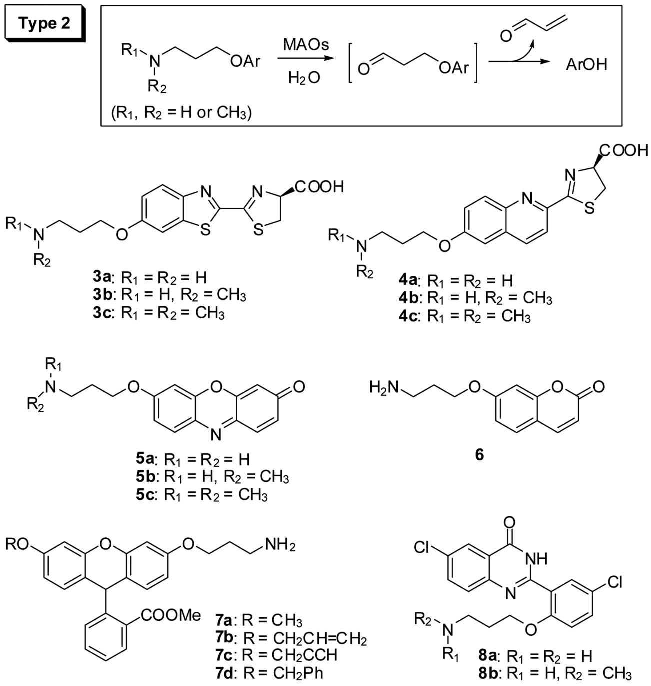
Type 1. In 2005, Sames and co-workers reported the first reaction-based fluorescence sensing scheme for MAO probes, which was based on the (*o*-aminoethyl)aniline moiety as the reactive site (probe **1**, **2**; Scheme 4).[23] The enzymatic oxidation of the alkylamine produced the corresponding aldehyde intermediate, which subsequently underwent intramolecular condensation by the arylamine to form the indole ring. This chemical conversion resulted in turn-on fluorescence change, because the (*o*-aminoethyl) aniline moiety acted as fluorescence quencher through the photo-induced electron-transfer (PET) process[24] but its end-product indole did not act as a PET quencher. It should be noted that an amine that has nitrogen lone pair electrons with a low oxidation potential readily acts as the PET quencher.

Probe **1** was able to sense MAO activity by producing the highly fluorescent indole product through the enzymatic oxidation followed by the intramolecular condensation; however, it turned out to be a poor substrate for either MAO-A or MAO-B. Interestingly, probe **2** showed good selectivity to MAOs, showing K_m values of 31 ± 2 μM for MAO-A and 510 ± 40 μM for MAO-B, respectively. They also synthesized several derivatives of probe **2** and examined their photophysical properties. Among them, only the pyrrolo[*g*]-coumarin derivative showed strong fluorescence toward MAOs, but not the pyrrolo[*h*]coumarin and pyrrolo[*f*]coumarin derivatives.

Type 2. Another clever approach to sense MAO activity was disclosed in 2006 by Wood and co-workers, who introduced (3-aminopropyloxy)arene as the reactive substrate of the enzymes (probes **3** and **4**, Scheme 5).[25] Although biological substrates of MAOs have common structural features of (2-amionoethyl)arene (dopamine, noradrenaline, adrenaline, serotonin, 2-phenylethylamine, tryptamine, tyramine,



Scheme 4. Fluorescent probes based on the enzymatic oxidation followed by an intramolecular cyclization.



Scheme 5. Fluorescent MAO probes based on the enzymatic oxidation followed by *β*-elimination.

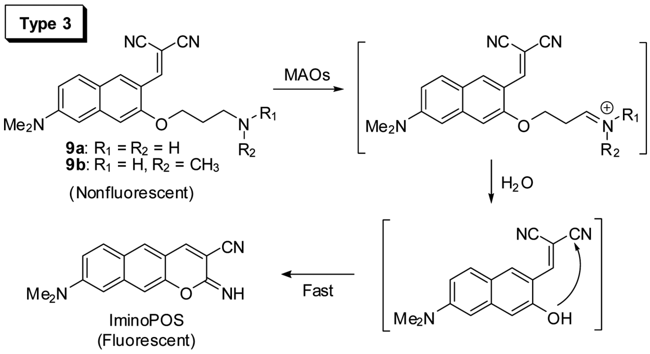
etc) or (aminomethyl)arene (benzylamine), they demonstrated that (3-aminopropyloxy)arene can be the enzyme substrate, which opened up a versatile route to develop fluorescent sensing systems for MAOs. Crystallographic studies[7-9] suggest that MAOs' binding pockets are not so tight that they can accommodate abiotic amine substrates with a longer alkyl chain and sterically more demanding substrates than the natural substrates. It is suggested that MAO-A can accommodate sterically bulkier amine substrates, whereas MAO-B has a rather narrow and tighter binding pocket.

MAOs thus transformed the propylamine moiety in luciferin derivative **3** or **4** into the corresponding iminium ion, which readily underwent *β*-elimination to release luciferin which was in turn elaborated to emit bioluminescence by the luciferase activity. Such luciferin−luciferase coupled assays often provide a low background signal and high sensitivity, and thus have been utilized in various biological assays.[26] The amine substrates of primary (**3a**, **4a**), secondary (**3b**, **4b**), or tertiary (**3c**, **4c**) exhibited different reactivity toward MAOs: *tert*-amine **3c** was selectively recognized by MAO-B, but *pri*-amine **4a** and *tert*-amine **4c** were preferentially recognized by MAO-A. Among them, *sec*-amine **4b** exhibited the largest signal-to-background ratio and a low level of K_m value (1.56 ± 0.13 mM).

By following the Wood's work, Chang devised resorufin-derived 3-aminopropyl ethers **5** as turn-on fluorescent probes for MAOs.[27] It is known that fluorophores such as resorufin, fluoresceins, and 7-hydroxycoumarns give turn-on type fluorescence change when the hydroxyl group in its ether form undergoes deprotection to the free hydroxyl group. Other related fluorescent probes have been developed based on this sensing strategy.[28,29] Accordingly, resorufin aminopropyl ethers **5** underwent the aforementioned enzymatic oxidation followed by β-elimination to generate highly fluorescent resorufin, fulfilling the turn-on sensing scheme. This sensing scheme has proven to be versatile in the development of other types of aryl aminopropyl ethers as fluorescent MAO probes. Both *sec*-amine **5a** and *pri*-amine **5b** responded to MAO-A and MAO-B with turn-on fluorescence change (excitation at λ_{ex} = 544 nm; emission collected at λ_{em} = 590 nm). The Michaelis–Menten constants for **5a** and **5b** with MAO-A and MAO-B were obtained in pH 7.4 HEPES (100 mM) containing 5% glycerol and 1% DMSO, which were lower than those obtained with natural amine substrates: K_m (**5a**/MAO-A) = 7.6 ± 10 μM, K_m (**5a**/MAO-B) = 1.8 ± 0.2 μM, K_m (**5b**/MAO-A) = 6.3 ± 0.6 μM, and K_m (**5b**/MAO-B) = 3.4 ± 0.5 μM. Probes **5a** and **5b** were applied to fluorescent imaging of MAO activity in live PC12 cell line, which was chosen for its high endogenous expression of MAO and neuron-like characteristics in culture when supplemented with nerve growth factor (NGF). In the presence of pargyline, a MAO inhibitor, the cellular fluorescence intensity was suppressed to an half of that obtained in the absence of the inhibitor.

Zhu and co-workers subsequently developed 7-hydroxycoumarin-derived aminopropyl ether **6** as a fluorescent MAO probe. Probe **6** responded to MAOs with fluorescence turn-on change (λ_{ex} = 360 nm; λ_{em} = 460 nm).[30] Interestingly, probe **6** showed similar kinetic parameters toward the two isoenzymes: K_m (**6**/MAO-A) = 62.37 ± 3 mM and K_m (**6**/MAO-B) = 83.50 ± 4.0 mM, obtained for the enzymes by mitochondrial preparations and in a borate buffer containing BSA (20 mg/mL). They also reported fluorescein-derived probes **7**, which were used to bioimaging of MAO activity in MCF-7 cells (human breast carcinoma cells).[31]

Xing and co-workers followed a similar approach to develop quinazolinone (HPQ)-derived aminopropyl ethers **8a**–**8c** as fluorescent MAO probes.[32] The HPQ dye shows great photostability, a large Stokes shift (> 100 nm), and strong fluorescence in the solid state owing to the intramolecular hydrogen bonding, but it is generally insoluble in water. Upon treatment with MAOs, probes **8** underwent the amine oxidation and subsequent β-elimination to release HPQ precipitates, which emitted green fluorescence (λ_{ex} = 360 nm; λ_{em} = 530 nm). Interestingly, in this case only *pri*-amine **8a** and *tert*-amine **8c** gave significant fluorescence change, but *sec*-amine **8b** gave a little fluorescence change upon treatment with MAOs. The kinetics parameters determined in the case of *pri*-amine **8a** to be: K_m (MAO-A) = 146.1 ± 7.21 μM, K_{cat} (MAO-A) = 9.76 ± 0.49 min^{-1}, K_m (MAO-B) = 106.8 ± 5.06 μM, and K_{cat} (MAO-A) = 8.47 ± 0.42 min^{-1}. In live cell imaging experiments with incubation of **8a** (100 μM) in DMEM (Dulbecco's Modified Eagle Medium) at 37 °C for 1 h, strong green fluorescence was observed in the case of the PC12 cell line but weak fluorescence in the case of the C6 glioma cell line that has no expression of MAOs. No obvious fluorescence resulted in when PC12 cells were pretreated with a MAO-A inhibitor clorgyline (100 mM), whereas strong fluorescence remained when the cells were pretreated with a MAO-B inhibitor pargyline; these results suggested that PC12 cells mainly expressed MAO-A enzyme.



Scheme 6. Two-photon fluorescent probes based on the enzymatic oxidation, followed by β-elimination and subsequent intramolecular cyclization.

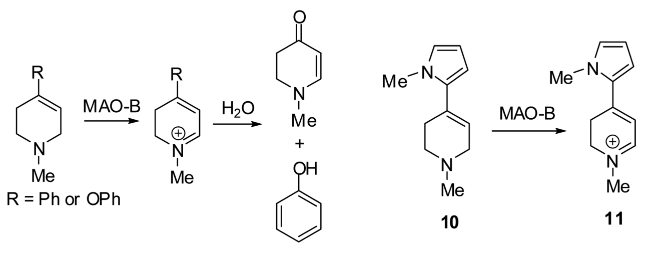
Type 3. Ahn and co-workers disclosed a new sensing scheme for MAOs, which involved the amine oxidation, followed by followed by β-elimination and subsequent an intramolecular condensation. The resulting probes **9a** (*pri*-amine) and **9b** (*sec*-amine) produced a linear benzocoumarin dye, named IminoPOS, which showed promising two-photon absorption and emission properties for bioimaging application (Scheme 6).[33,34] As a result, probes **9** enabled them to obtain fluorescent images of live cells by two-photon microscopy (TPM) for the first time. Two-photon excitation of dyes provides several advantageous features in bioimaging such as focal point excitation with 3D imaging, deep tissue imaging, less photo-damage and photo-bleaching to samples.[35,36] Accordingly, various two-photon fluorescent probes have been developed in last decade, notably those acedan-based probes intensively studied by Cho and co-workers.[37,38] Such two-photon probes are promising for *in vivo* monitoring of MAO activity in deep tissues.

Both *pri*-amine **9a** and *sec*-amine **9b** showed turn-on fluorescence response toward MAO-A and MAO-B respectively (λ_{ex} = 448 nm; λ_{em} = 585 nm). The kinetics analysis of probes **9** with MAOs showed a moderate level of K_m values: K_m (**9a**/MAO-A) = 70 μM, K_m (**9a**/MAO-B) = 75 μM, K_m (**9b**/MAO-A) = 252 μM, and K_m (**9b**/MAO-B) = 210 μM, obtained in pH 7.4 HEPES (100 mM) containing 5% glycerol and 1% DMSO at 37 °C. Enzyme inhibition assays with *pri*-amine **9a** (at the K_m concentration) toward MAO-A and MAO-B in the absence and presence of inhibitors showed that moclobemide inhibited 50% of the MAO-A activity and 20% of MAO-B activity, whereas pargyline inhibited MAO-A slightly but 80% of MAO-B activity; these results are similar to the reported inhibitor assay data for MAOs. Further investigation of *pri*-amine **9a** in two-photon imaging of MAOs in live cells was carried out using the chromaffin cell line that expressed a high level of MAOs, together with the C6 glioma cell line that expressed little MAOs. The TPM image data showed that chromaffin cells pretreated with **9a** showed strong red fluorescence, whereas the cells pretreated with pargyline showed little fluorescence (λ_{ex} = 900 nm, 10 mW laser power). It is notable that IminoPOS has a larger two-photon absorption cross-section (TPACS) value (180 GM), higher fluorescence quantum yield (Φ_F = 63%), and a longer maximum absorbance wavelength (λ_{max} = 448 nm), compared with acedan, a widely used in two-photon excitable probes, which properties are promising for the development of new two-photon probes of other biological targets.

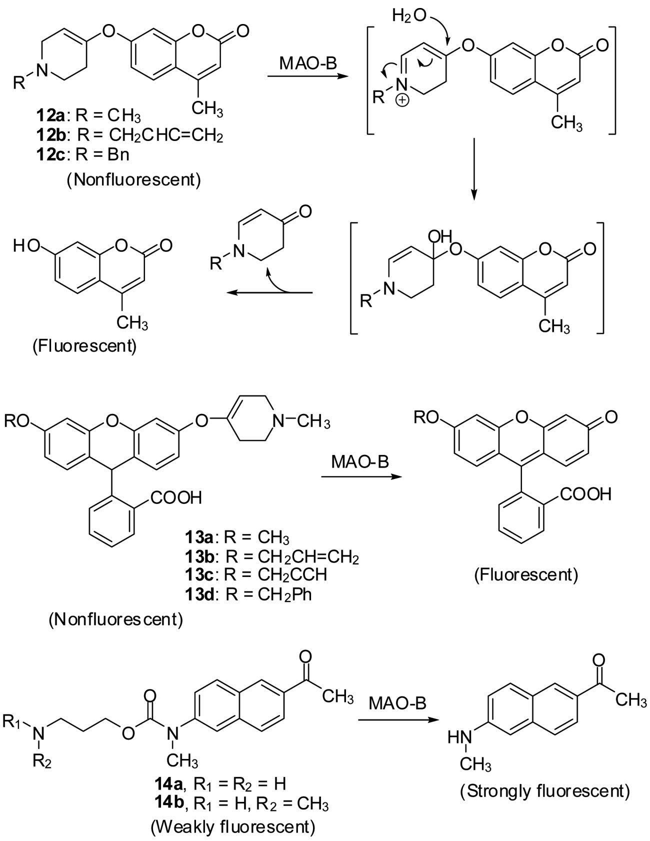
MAO-B Selective Fluorescent Probes. MAO-A is mainly expressed in catecholaminergic neurons and metabolizes several different neurotransmitters. MAO-B, on the other hand, is primarily found in astrocytes and serotonergic neurons, and acts on dopamine and β–phenylethylamine.[39] These isoenzymes assist in maintaining the homeostasis of amine neurotransmitters in the brain. But, older people or patients with Parkinson's disease (PD) and Alzheimer's disease (AD) show overexpression of MAO-B, but not MAO-A. The main pathology of PD is the loss of dopaminergic neurons due to the overexpression of MAO-B, causing autonomic dysfunction and neuropsychiatric problems.[40] Several previous studies reported increased activities of MAO-B in the brain and blood platelets of AD patients. The increase of MAO-B in the brain, predominantly in plaque-associated astrocytes in neuropathologically verified AD brains, is most likely due to transcriptional elevation of MAO-B protein. However, the exact reason that increased activity of MAO-B is still unknown.[41,42] Given that the expression level and the substrate specificity of MAO-A and MAO-B are different, it is necessary to develop fluorescent probes that discriminate one isoform of the enzymes from the other.

Castagnoli and co-workers reported that a pyrrole-containing 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) **10** underwent the enzymatic oxidation selectively with MAO-B in mouse brain samples to produce a dihydropyridinium compound **11**, which absorbed at 420 nm (Scheme 7).[43] The MPTP moiety was previously known to induce dopaminergic neurotoxicity, as MAO-B, not MAO-A, oxidized it.[44] The colorimetric sensing scheme was subsequently adopted by others to develop fluorescent probes selective to MAO-B.

Zhu and co-workers reported coumarin-derived MPTP derivatives **12** as turn-on fluorescent probes for MAO-B (λ_{ex} = 360 nm; λ_{em} = 460 nm) (Scheme 8).[45] Upon oxidation of the MPTP moiety by the enzyme, probe **12** produced the corresponding dihydropyridinium intermediate, which subsequently underwent the hydrolytic ether cleavage to release



Scheme 7. MAO-B selective substrate and a colorimetric probe.



Scheme 8. Fluorescent probes selective to MAO-B.

the fluorescent coumarin dye. Probe **12a** showed high selectivity toward MAO-B, with K_m value of 59.63 μM. From a molecular docking study with probe **12** and MAO-A or MAO-B active site, they found hydrogen bonding between the probe nitrogen and the hydroxyl group of Tyr60 in the active site of MAO-B, but no such hydrogen bonding in the case of MAO-A. The calculated binding energy of the probe with MAO-A was found to be much higher than that with MAO-B (> 1000 kcal mol^{-1} *vs.* 10.2 kcal mol^{-1}). In a further study, they reported fluorescein derivatives **13** and their use for bioimaging.[46] Probe **13a**, however, gave only about three times higher fluorescence enhancement to MAO-B over MAO-A (K_m (**13a**/MAO-A) = 257.9 μM, K_m (**13a**/MAO-B) = 47.1 μM).

Recently, Yao and co-workers disclosed a MAO-B selective two-photon fluorescent probe, which is an aminopropyl carbamate of acedan.[47] Probe **14** was thus used to fluorescently image MAO-B specific activities present in mammalian proteome lysates, live cells and mouse tissues, and a parkin-related insect PD model. Furthermore, they assessed MAO-B activities in proteome lysates prepared from B lymphocytes and also from fibroblasts, both derived from PD patients. They observed that there was an obvious increase in MAO-B expression in the case of lymphocytes whereas little change in the cased of fibroblasts, compared with controls. They concluded that the difference in MAO-B activity profiles between control and PD patients are cell-type-specific, which may be potentially used as a convenient surrogate biomarker for PD diagnosis.

Conclusion

Monoamine oxidases, MAO-A and MAO-B together, are thought to maintain the homeostasis of neurotransmitters in the brain by breaking down amine neurotransmitters. Dysfunction of MAOs can cause neurological and behavioral disorders. Aberrant expression of MAO-B is observed in patients of Parkinson's disease and Alzheimer's disease. MAO-B inhibitors are used to attenuate the progress of Parkinson's disease. However, our present understanding on the mechanism of cytoprotective actions of MAOs is limited. Also, it is unclear why MAO activity increases in the brain of those patients and what regulates its activity. A selective and sensitive fluorescent probe with the deep tissue imaging capability may aid us to investigate these issues. In this context, it is promising to see a significant progress in the development of fluorescent probes for MAOs in recent years. In particular, two-photon fluorescent probes are promising for future applications to address those fundamental issues. As noted by Yao and co-workers, a small-molecule fluorescent probe may be applied for the simple and convenient diagnosis of Parkinson's disease in near future. We hope that this review article stimulates chemists to develop efficient fluorescent MAO probes and to apply them to challenge those unsolved issues.

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