Synthesis and Characterization of 2-(2′-hydroxy-5′-chlorophenyl)-6-chloro-4(3H)-Quinazolinone-Based Fluorogenic Probes for Cellular Imaging of Monoamine Oxidases

Junxin Aw, Qing Shao, Yanmei Yang, Tingting Jiang, Chungyen Ang, and Bengang Xing*[a]

Monoamine oxidases (MAOs) are essential FAD-dependent enzymes which can efficiently catalyze the oxidative deamination of neurotransmitters and biogenic amines.[1–2] There are two isoforms, MAO A and MAO B, that are abundant in the liver, gastrointestinal tract, blood platelets, and central nervous systems.[3] These enzymes play an important role in metabolism and neural development by regulating the homeostasis of amine neurotransmitters and peripheral dietary amines. Any excess or deficiency of these enzymes will lead to various neurological and psychiatric disorders[4] such as depression, Parkinson's and Alzheimers's diseases, or even the growth inhibition and progression of tumor.[5–7] Thus, the development of suitable MAO substrates which can be used for selective and sensitive monitoring of enzyme activity in a complex biological system is of great significance.

Recently, fluorescence techniques have attracted considerable attention as simple, effective, and powerful tools[8,9] for real-time monitoring of protein and enzyme activities in vitro and in vivo.[10,11] Fluorescent detection is more advantageous compared to colorimetric or radioisotope assay owing to its high sensitivity, relative safety, low cost, and easy handling.[12,13] To date, several standard chromogenic, radiochemical, and fluorogenic substrates have been successfully employed to identify MAO activity in vitro.[14–20] However, simple and effective fluorescent probes which can provide a direct and sensitive readout of the MAO activity in living cells are still highly required since most of the existing methods are less sensitive,[17–19] require a secondary activating enzyme to release the signal for detection,[15] or do not provide live cell fluorescence imaging for the enzyme functions.[14–20]

Herein, we present the rational design and synthesis of a new class of activity-based fluorescent probes for real-time imaging of MAO function in living cells. The general concept for MAO imaging relies on the fact that MAO enzymes catalyze the FAD-dependent oxidation of primary, secondary, and tertiary amines to iminium intermediates, which are further non-enzymatically hydrolyzed to the corresponding aldehydes to facilitate the release of a fluorescent product through a β-elimination process (Scheme 1). In this study, we chose 2-(2′-hydroxy-5′-chlorophenyl)-6-chloro-4-(3H)-quinazolinone (HPQ) derivatives as fluorescent reporters. The HPQ fluorophores are generally insoluble in water and highly fluorescent in the solid state owing to the

[a] J. Aw, Q. Shao, Y. Yang, T. Jiang, C. Ang, Prof. B. Xing
Division of Chemistry & Biological Chemistry
School of Physical & Mathematical Sciences
Nanyang Technological University
21 Nanyang Link, Singapore 637371 (Singapore)
Fax: (+65) 67911961
E-mail: bengang@ntu.edu.sg

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201000025.
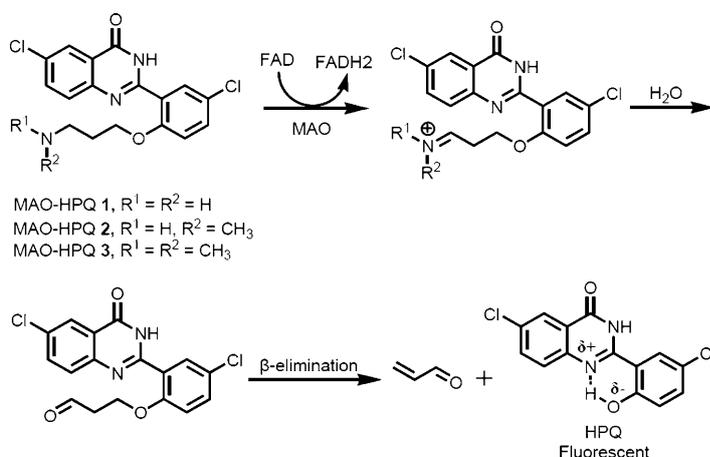
Scheme 1. Fluorescence detection of MAO activity by oxidative deamination of HPQ derivatives and subsequent β-elimination.

MAO-HPQ 1, $R^1 = R^2 = H$
MAO-HPQ 2, $R^1 = H$, $R^2 = CH_3$
MAO-HPQ 3, $R^1 = R^2 = CH_3$

HPQ Fluorescent

intramolecular hydrogen bonding between the imine nitrogen and the phenolic hydrogen atoms. Compared to the common fluorescent dyes, these molecules display extreme photostability with a large stokes shift ($>$ 100 nm), which allow them to be easily focused and distinguished from most cell and tissue autofluorescence.[21–24] Modification of the 2′-hydroxyl group in HPQ molecule efficiently eliminates its long wavelength fluorescence, providing an ideal molecular switch with which to amplify the fluorescence signals for enzyme detection. Such mechanism-based fluorogenic probes have exhibited high specificity in the detection of particular enzymes including alkaline phosphatase,[21] β-glucuronidase,[22] and acyl hydrolase.[24] In this investigation, HPQ fluorophores are alkylated with aminopropyl groups for MAO enzymatic reactions. Upon MAO treatment, the amine oxidation and subsequent β-elimination result in the release of acrolein and a green fluorescent precipitate, HPQ, thus allowing a direct and effective identification of enzyme activity in real time.

Scheme 2 shows the synthesis of MAO-HPQ fluorescent substrates. Our strategy for the preparation of substrates was divided into two sections: firstly, HPQ fluorophore was

orescent measurements. Typically, these probes and MAO enzymes were incubated in 100 mM of Tris-HCl buffer (pH 7.90) at 37 °C for 2 h. All the MAO substrates **1**, **2**, and **3** were very stable in aqueous solutions and as expected, were almost nonfluorescent before MAO enzymatic oxidation owing to alkylation of the 2′-hydroxyl group in the HPQ fluorophore. However, upon treatment with MAO A and B, HPQ molecules were released and significant fluorescence enhancement around the wavelength 530 nm was observed in all substrates (Figure 1, and Figure S1 in the Supporting Information).
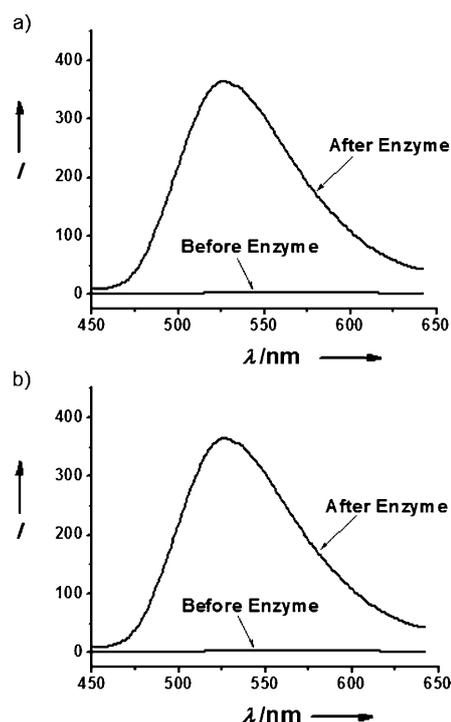
Figure 1. Fluorescence measurement of MAO enzymatic reactions. Emission spectrum of MAO-HPQ **1** (200 μM) upon treatment with MAO A (a) and MAO B (b) in Tris-HCl buffer (100 mM, pH 7.90). Enzyme concentration: 10 μg mL^{-1}. Excitation wavelength: 360 nm; emission wavelength: 530 nm.

Scheme 2. Synthetic route for fluorogenic probes MAO-HPQ **1–3**.

synthesized by refluxing 2-amino-5-chlorobenzamide and 5-chlorosalicylaldehyde in ethanol in the presence of a catalytic amount of TsOH·H$_2$O followed by in situ oxidation with dichloro-dicyanoquinone (DDQ). The fluorophore was then alkylated with *N,N*-dimethyl-3-chloropropylamine **3a** using cesium carbonate as base to afford MAO-HPQ **3** in 60% yield. These alkylation conditions were also applied for the synthesis of *N*-Boc-protected precursors of other two probes, **1b** and **2b** (yield: 75% and 71%), respectively, which were further deprotected using TFA and triisopropylsilane to give MAO-HPQ **1** and **2** quantitatively.

The activity of these three probes (MAO-HPQ **1–3**) towards both MAO isozymes were investigated by in vitro flu-

The formation of HPQ fluorophore in the enzymatic reactions was further confirmed by HPLC analysis. In the presence of each MAO isozyme, the retention time of enzymatic product was the same as that of HPQ (retention time: 21.8 min), approving the enzyme-mediated reactions to release HPQ fluorophores from all the substrates (Figure 2, and Figure S2 in Supporting Information).

Fluorescent enhancement and HPLC results confirmed that all the primary, secondary, and tertiary MAO-HPQ fluorescent substrates underwent an oxidative deamination catalyzed by MAO enzymes, followed by β-elimination, which resulted in the release of HPQ fluorescent precipitates. The ratios of fluorescence intensity in the presence and absence of MAO enzymes were used to estimate MAO activities.
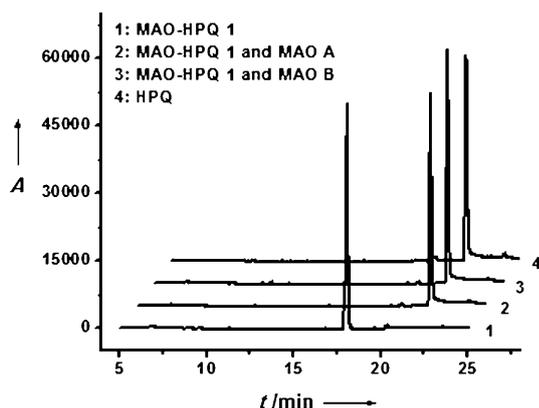
Figure 2. HPLC analysis of enzymatic reaction of MAO-HPQ **1** with MAO isozymes. Absorbance wavelength: 360 nm

The maximum fluorescence enhancement in the primary, secondary, and tertiary amine MAO substrates was 307-fold, 15-fold, and 5-fold for MAO A and 300-fold, 20-fold, and 7-fold for MAO B, respectively (Figure 3), indicating that the different amine substrates exhibited different activities toward MAO enzymes. Of the three MAO-HPQ derivatives, substrate **1** exhibited an intensive fluorescence signal and the highest signal-to-background ratio at 530 nm, affording a convenient means with which to measure MAO activity.
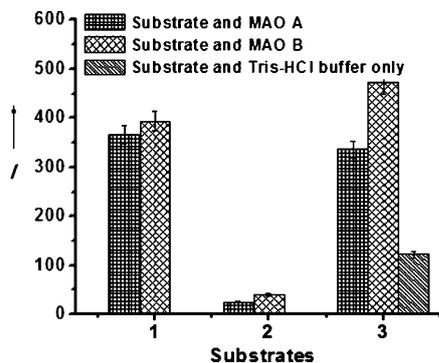
Figure 3. Fluorescence enhancement of MAO-HPQ substrates: **1**–**3** (200 μM) in 100 mM of Tris-HCl buffer, pH 7.90 incubated with enzymes (10 μg mL^{-1}) for 2 h.

Further analysis of the enzyme kinetics of MAO-HPQ **1** with both MAO A and B was also carried out in Tris-HCl buffer at 37°C. Figure 4 shows a representative enzyme kinetics plot of MAO-HPQ **1** for MAO A and B oxidation. Measurement of the fluorescent signal at different substrate concentrations provided the Michaelis–Menten kinetics constants. These observed kinetic parameters were determined to be: $K_m = 146.1 \pm 7.21$ μM, $K_{cat} = 9.76 \pm 0.49$ min^{-1} for MAO A and $K_m = 106.8 \pm 5.06$ μM, $K_{cat} = 8.47 \pm 0.42$ min^{-1} for MAO B. The enzyme catalytic efficiency (K_{cat}/K_m) for MAO A and B are 6.68×10^4 M^{-1} min^{-1} and 7.93×10^4 M^{-1} min^{-1}, respectively.
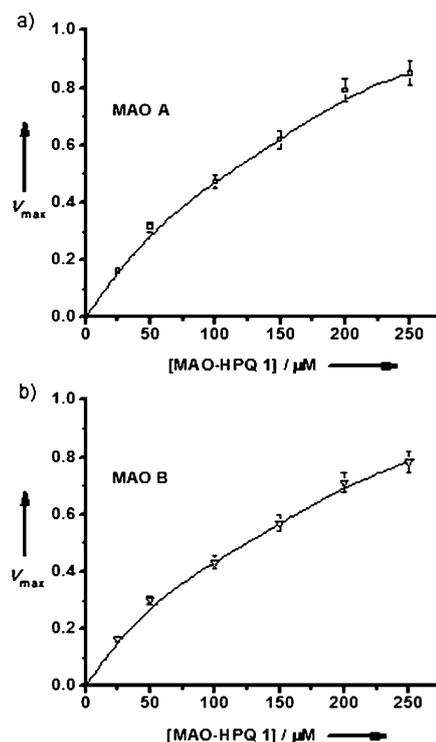
Figure 4. Enzymatic kinetics of MAO activity with MAO-HPQ **1**. Data show the relative reaction rates over a range of concentrations of substrate. These values were obtained from an average of at least six independent experiments for each concentration and were collected over a time frame of 60 min. The reactions were performed at 37°C in Tris-HCl Buffer at pH 7.90. These readings were collected at a final enzyme concentration of 5 μg mL^{-1}. Excitation wavelength: 360 nm; emission wavelength: 530 nm.

Encouraged by the favorable fluorescent properties of MAO-HPQ **1**, we finally investigated the applicability of this probe to image MAO activity in living cells. In this study, the PC12 cell line was chosen as our main target owing to its high expression of endogenous MAO. As a negative control, C6 glioma cell was used as there is no MAO expression in this cell line. In a typical experiment, both PC12 cells and C6 glioma cells were cultured and incubated with 100 μM of MAO-HPQ **1** in Dulbecco's Modified Eagle Medium at 37 °C for 1 h to obtain an effective live cell fluorescent imaging. The cellular imaging measurements were acquired using a confocal fluorescence microscope with excitation filter 360/40 nm and emission filter 535/40 nm.

After 1 hour incubation of MAO-HPQ **1**, a bright fluorescence signal was observed inside the PC12 cells (Figure 5b), confirming the good cell-membrane permeability of this fluorescent probe and its enzymatic oxidation for the release of the HPQ fluorophores. In contrast, there was little HPQ fluorescence signal observed in C6 glioma cells (Figure 5a), indicating that there was no obvious amino oxidation occurring in the cells which do not express MAO enzymes. The cellular imaging results demonstrated the fact that MAO-HPQ **1** was able to report the MAO activity in MAO-expressed cells.
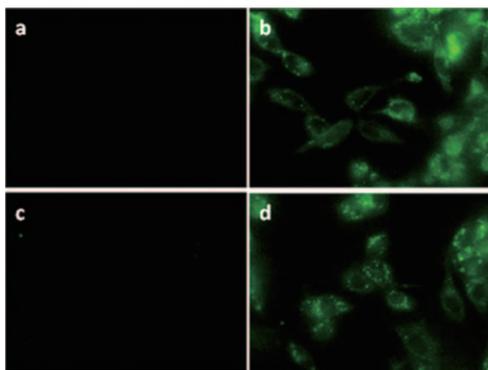
Figure 5. Representative fluorescence images of C6 glioma cells and PC12 cells with MAO-HPQ **1** (100 μM) and inhibitors (100 μM) incubated separately at 37 °C with 5 % CO$_2$ for 60 min. Excitation filter: 360/40 nm; emission filter: 535/40 nm. a) C6 glioma cells incubated with MAO-HPQ **1**; b) PC12 cells incubated with MAO-HPQ **1**; c) clorgyline pretreated PC12 cells incubated with MAO-HPQ **1**; d) pargyline pretreated PC12 cells incubated with MAO-HPQ **1**.

Moreover, we further extended the imaging investigations by utilizing two commonly used inhibitors: clorgyline for MAO A and pargyline for MAO B.[25–27] The PC12 cells were pretreated separately with 100 μM of clorgyline and pargyline, and then incubated with 100 μM of MAO-HPQ **1** at 37 °C for 1 h. The imaging data revealed that there was no obvious fluorescence in clorgyline pretreated PC12 cells, indicating the significant inhibition of the enzyme in the living cells (Figure 5 c), whereas the MAO B inhibitor pargyline pretreated PC12 cells still displayed strong fluorescence, which was similar to the imaging result without inhibitor treatment (Figure 5 d), demonstrating that MAO activity remained in the cell. These results implied that PC12 cells mainly expressed MAO A enzyme and that its activity could be selectively suppressed by clorgyline rather than pargyline. This is in accordance with our in vitro enzyme inhibition tests (Figure S5 in the Supporting Information) and the results reported previously.[28,29] In the process of cellular imaging, the fluorescent probe MAO-HPQ **1** indicates less toxicity (cell viability assay, Figure S4 in the Supporting Information), and thereby can serve as a novel class of fluorogenic probes for real-time imaging of MAO activity in living cells.

In summary, this work describes the design and synthesis of new and effective fluorogenic probes that can be used to identify MAO activity in buffer solution and in living cells. These HPQ-based fluorogenic probes exhibit significant fluorescent enhancement and reasonable kinetics upon interaction with MAO A and B enzymes, which facilitate their application as biosensors for imaging MAO activity in living cells. This new type of fluorescent biosensor provides the possibility to monitor the biological processes of MAOs in living systems in real time. Moreover, the design strategy reported here could also be utilized for the development of substrates for other enzymatic assays.

Experimental Section

Experimental details, including the synthesis of substrates, enzymatic reaction conditions, kinetics studies, and imaging assays, can be found in the Supporting Information.

A quantitative analysis of the enzyme kinetics of MAO-HPQ **1** with both MAO A and B was carried out in Tris-HCl buffer at 37 °C. To a series of different MAO-HPQ **1** solutions (concentration range: 0–250 μM) were added a solution of MAO A or MAO B enzyme with final concentration 5 μg mL^{-1}. Tris-HCl buffer was added to adjust the final volume to 100 μL. The rate of enzymatic oxidation was monitored with fluorescence enhancement at 530 nm. The values of enzyme kinetic parameters (K_m and K_{cat}) were determined from the standard Lineweaver–Burk plot, the double-reciprocal plot of the reaction rate versus MAO-HPQ **1** concentration.

For live cell imaging of MAO activity, PC12 cell line (American Type Culture Collection, No.: CR-1721) were seeded at a density of 2×10^5 in a 35 mm diameter μ-dish plastic bottom and cultured for 2 days with Nerve Growth Factor (2.5S, 30 ng mL^{-1}, Invitrogen, Carlsbad, CA) in Dulbecco's Modified Eagle Medium (DMEM 1X, without phenol red, Gibco/Invitrogen, Carlsbad, CA) containing 4.5 g L^{-1} D-glucose, 2 mM GlutaMax-1 (Invitrogen). Control C6 glioma cell lines (American Type Culture Collection, No.: CCL-107) were cultured with the same protocol as PC12 cell lines. After 2 days of culture, the live PC12 and C6 glioma cell lines were washed twice with DMEM. The live cells were then treated separately with 100 μM of MAO-HPQ **1** in DMEM (containing 0.5 % of DMSO) and incubated for 1 hour in an incubator at 37 °C. The cells were washed twice with Hank's Balanced Salt solution (Sigma). The fluorescence imaging was acquired with a Confocal fluorescence microscope (Nikon, Eclipse TE2000-E) using a super high pressure mercury lamp (Nikon, TE2-PS100W) with excitation filter: 360/40 nm; emission filter: 535/40 nm.

In the inhibition investigation, the live PC12 cell lines were washed twice with DMEM and pretreated separately with 100 μM clorgyline or pargyline in DMEM for 1 hour in an incubator at 37 °C. MAO-HPQ **1** was then added to the μ-dish with a final concentration of 100 μM and incubated for another 1 hour at 37 °C. The cells were then washed twice with Hank's Balanced Salt solution. The fluorescence imaging was acquired with the Confocal fluorescence microscope.

[1] R. B. Silverman, *Acc. Chem. Res.* **1995**, *28*, 335–342.

[2] D. E. Edmondson, A. Mattevi, C. Binda, M. Li, F. Hubalek, *Curr. Med. Chem.* **2004**, *11*, 1983–1993.

[3] J. C. Shih, K. Chen, M. J. Ridd, *Annu. Rev. Neurosci.* **1999**, *22*, 197–217.

[4] S. Mandel, O. Weinreb, T. Amit, M. B. H. Youdim, *Brain Res. Rev.* **2005**, *48*, 379–387.

[5] B. Levant, *Life Sci.* **2002**, *71*, 2691–2700.

[6] M. B. H. Youdim, D. Edmondson, K. F. Tipton, *Nat. Rev. Neurosci.* **2006**, *7*, 295–309.

[7] C. W. Olanow, *Neurology* **2006**, *66*, S69-S79.

[8] a) J. Zhang, R. E. Campbell, A. Y. Ting, R. Y. Tsien, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906–918; b) R. Y. Tsien, *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 16–21.

[9] a) I. Chen, A. Y. Ting, *Curr. Opin. Biotechnol.* **2005**, *16*, 35–40; b) Z. Cheng, J. Levi, Z. Xiong, O. Gheysens, S. Keren, X. Chen, S. S. Gambhir, *Bioconjugate Chem.* **2006**, *17*, 662–669.

[10] a) B. G. Xing, J. H. Rao, R. R. Liu, *Mini. Rev. Med. Chem.* **2008**, *8*, 455–471; b) T. Terai, T. Nagano, *Curr. Opin. Chem. Biol.* **2008**, *12*, 515–521; c) M. Uttamchandani, C. H. S. Lu, S. Q. Yao, *Acc. Chem. Res.* **2009**, *42*, 1183–1192; d) M. Kamiya, H. Kobayashi, Y. Hama, Y. Koyama, M. Bernardo, T. Nagano, P. L. Choyke, Y. Urano, *J. Am. Chem. Soc.* **2007**, *129*, 3918–3929; e) Y. L. Tang, S. Wang, Y. Li, D. B. Zhu, *Angew. Chem.* **2007**, *119*, 8028–8032; *Angew. Chem. Int. Ed.* **2007**, *46*, 7882–7886; f) H. Maeda, H. Matsuno, M. Ushida, K. Katayama, K. Saekis, N. Itoh, *Angew. Chem.* **2005**, *117*, 2982–2985; *Angew. Chem. Int. Ed.* **2005**, *44*, 2922–2925; g) J. W. Webster, R. Zhang, S. S. Gambhir, Z. Cheng, F. A. Syud, *ChemBioChem* **2009**, *10*, 1293–1296; h) L. D. Lavis, T. Y. Chao, R. T. Rains, *ACS Chem. Biol.* **2006**, *1*, 252–260.

[11] a) W. Cai, X. Chen, *Curr. Pharm. Des.* **2008**, *14*, 2943–2973; b) M. Funovics, R. Weissleder, C. H. Tung, *Anal. Bioanal. Chem.* **2003**, *377*, 956–963.

[12] B. Valeur, *Molecular Fluorescence: Principles and Applications*; Wiley-VCH, Weinheim, Germany, **2002**.

[13] R. P. Haugland, *The Hand-book: A Guide to Fluorescent Probes and Labeling Technologies, 10th ed.*, InvitrogenTM, **2005**.

[14] G. Chen, D. J. Yee, N. G. Gubernator, D. Sames, *J. Am. Chem. Soc.* **2005**, *127*, 4544–4545.

[15] W. H. Zhou, M. P. Valley, J. Shultz, E. M. Hawkins, L. Bernad, T. Good, D. Good, T. L. Riss, D. H. Klaubert, K. V. Wood, *J. Am. Chem. Soc.* **2006**, *128*, 3122–3123.

[16] A. E. Albers, K. A. Rawls, C. J. Chang, *Chem. Commun.* **2007**, 4647–4649.

[17] C. Binda, J. Wang, L. Pisani, C. Caccia, A. Carotti, P. Salvati, D. E. Edmondson, A. Mattevi, *J. Med. Chem.* **2007**, *50*, 5848–5852.

[18] a) A. Holt, D. F. Sharman, G. B. Baker, M. M. Palcic, *Anal. Biochem.* **1997**, *244*, 384–392; b) J. J. P. Zhou, B. Zhong, R. B. Silverman, *Anal. Biochem.* **1996**, *234*, 9–12.

[19] P. Bissel, M. C. Bigley, K. Castagnoli, N. Castagnoli, Jr., *Bioorg. Med. Chem.* **2002**, *10*, 3031–3035.

[20] A. Holt, G. B. Baker, *Prog. Brain Res.* **1995**, *106*, 187–197.

[21] Z. J. Huang, E. Terpetschnig, W. M. You, R. P. Haugland, *Anal. Biochem.* **1992**, *207*, 32–39.

[22] M. J. Zhou, R. H. Upson, Z. Diwu; R. P. Haugland, *J. Biochem. Biophys. Methods* **1996**, *33*, 197–205; R. P. Haugland, *J. Biochem. Biophys. Methods* **1996**, *33*, 197–205.

[23] Z. Diwu, Y. X. Lu, R. H. Upson, M. J. Zhou, D. H. Klaubert and R. P. Haugland, *Tetrahedron* **1997**, *53*, 7159–7164.

[24] X.-B. Zhang, M. Waibel, J. Hasserodt, *Chem. Eur. J.* **2010**, *16*, 792–795.

[25] Z. Mucsi, A. Szabo, I. Hermecz, A. Kucsman, I. G. Csizmadia, *J. Am. Chem. Soc.* **2005**, *127*, 7615–7631.

[26] R. H. Abeles, A. L. Maycock, *Acc. Chem. Res.* **1976**, *9*, 313–319.

[27] J. Gaal, I. Hermecz, *Inhibitors of Monoamine Oxidase B* (Ed.: I. Szelenyi), Birkhauser, Basel, **1999**, 75–108.

[28] K. Kohda, Y. Noda, S. Aoyama, M. Umeda, T. Sumino, T. Kaiya, W. Maruyama, M. Naoi, *Chem. Res. Toxicol.* **1998**, *11*, 1249–1253.

[29] M. B. H. Youdim, E. Heldman, H. B. Pollard, P. Fleming, E. McHugh, *Neuroscience* **1986**, *19*, 1311–1318.