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New Bioluminogenic Substrates for Monoamine Oxidase Assays

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Monoamine oxidase (MAO) A and B play an important role in regulating tissue levels of amine neurotransmitters and dietary amines. Many efforts have been made to look for MAO inhibitors in the treatment of neurological disorders, such as Parkinson's disease and schizophrenia.[1,2] In addition, since MAO is involved in drug metabolism, any effects on enzyme activity could raise serious drug interaction problems.[3] It is thus crucial to achieve homogeneous and highly sensitive MAO assays for keeping pace with drug discovery and for monitoring enzymatic activity in complex biological systems. Current assays are not amenable to high-throughput screening (HTS) methods because they use indirect methods of detection,[4] exhibit low sensitivity,[5] or require long incubation times.[6] The luciferase-coupled systems for bioluminescent assays often provide low background and high sensitivity and have been utilized in many biological assays, such as β-galactosidase assays,[7] protease assays,[8] and cytochrome P450 assays.[9] Herein we report on the rational design of novel substrates for a bioluminescent MAO assay.

The general concept for bioluminescent-coupled enzyme assays relies on compounds that are not substrates for luciferase until acted upon by the enzyme of interest. The activity of the enzyme of interest can then be correlated to the light output generated by the reaction of luciferase with the liberated substrate, most commonly, luciferin. On the basis of the fact that MAO oxidizes primary, secondary, and tertiary acyclic amines to produce iminium intermediates which are hydrolyzed to the corresponding aldehydes (Figure 1A), we reasoned such iminium and/or aldehyde derivatives could facilitate the liberation of luciferin through a β-elimination process. We therefore designed several bioluminogenic molecules wherein the hydroxyl group of luciferin is linked to an amine group by a simple propyl chain; oxidation of the amine by MAO and the subsequent β-elimination reaction should liberate free luciferin (Figure 1B). We anticipated modifications of the amino groups and the central core of luciferin could provide a means of tuning MAO activity and isozyme selectivity.

The syntheses of compounds **1**, **2**, and **3** are described in Scheme 1. Alkylation of 2-cyano-6-hydroxybenzothiazole with 3-chloropropyldimethylamine hydrochloride or the *t*-BOC-protected primary or secondary chloropropylamine in the presence of sodium iodide under basic condition gave compounds **1a**, **2a**, and **3a**. Compound **1a** was directly condensed with D-cysteine to give compound **1**, whereas deprotection of **2a** and **3a** with trifluoroacetic acid followed by ring cyclization with D-cysteine gave compounds **2** and **3**. Similarly, quinolinyl luciferin compounds **4**−**6** were prepared from 2-cyano-6-hydroxyquinoline[10] by employing the same methods. Care needs to be taken to keep the amount of free luciferin at a very low level (0.1% or less). The extraordinary sensitivity of this bioluminescent assay can easily detect luciferin contamination in substrate preparations and result in a significant increase of background luminescence.

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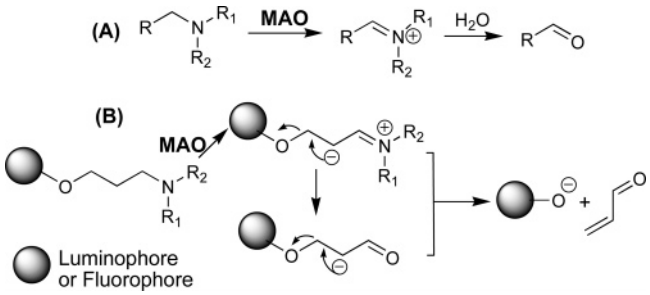
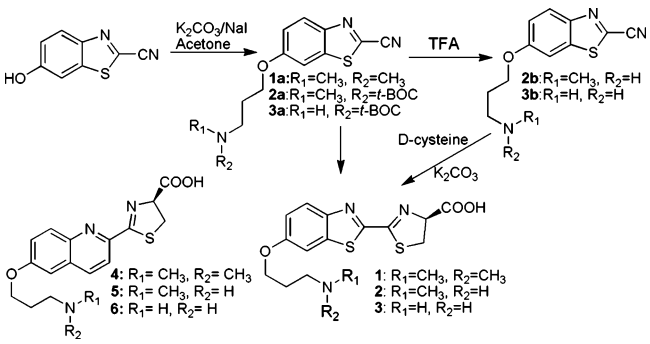


Figure 1. (A) MAO catalyzed oxidation of acyclic amines. (B) Design of MAO probes by β-elimination.

Scheme 1. Synthetic Routes for Compounds **1**−**3** and Structures of Compounds **4**−**6**



The activities of MAO A and B with compounds **1**−**6** as substrates were examined by a two-step assay. Typically, the substrate and enzyme were incubated in 200 mM Tricine at pH 8.3 and room temperature in the wells of a microtiter plate. The enzyme was added in the form of microsomes containing human recombinant MAO A or B expressed in baculovirus-infected insect cells (Sigma-Aldrich, Inc.). After certain time periods (e.g., 1 h), a luciferin detection reagent, comprising a luciferase, ATP, and buffer, was added to each well, and the resulting luminescence was measured after 20 min. Various amounts of luminescence above background were produced with compounds **1**−**6** that indicated that they were all substrates for MAO A and/or MAO B and the free luciferin was released upon the MAO reaction. The luminescent signal was proportional to the incubation time of the compounds with the MAO isozymes. Only the starting compounds and free luciferin were observed by HPLC, and no iminium or aldehyde intermediates were detected, confirming the luminescent signal indeed reflected the oxidation of luciferin by luciferase. Control experiments yielded a very low background[11] signal with compounds **1**−**6** in the absence of MAO enzymes. The ratio of the signals in the presence and absence of MAO enzyme was then used to evaluate the reactivities and selectivities of these compounds toward each isozyme (Figure 2). The substrates with the same primary, secondary, or tertiary amines exhibit different reactivities

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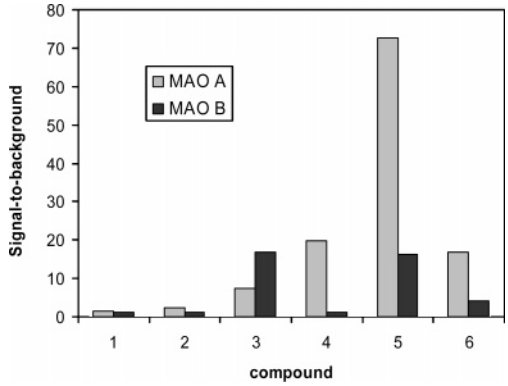
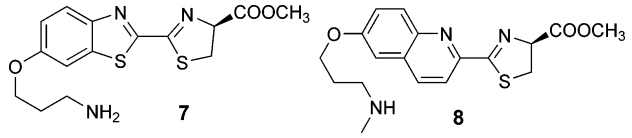


Figure 2. The relative activities of each MAO isozyme with compounds **1**−**6**. In a volume of 50 μL, 5 μg of protein from microsomes containing MAO A or MAO B was incubated with 100 μM compound in 200 mM Tricine, pH 8.3, 20 mM $MgSO_4$, and 1% Tergitol NP-9. After 1 h at room temperature, 50 μL of a proprietary luciferin detection reagent was added, and the luminescent signal was measured after 20 min. The data shown are a ratio of the luminescent signals in the presence and absence of MAO enzyme.

Scheme 2. Structures of Compounds **7** and **8**



toward MAO isozymes depending on the central core structure of luciferin or quinolinyl luciferin. Compound **3** was the only one that showed selectivity for MAO B. Although compounds **4** and **6** showed greatest selectivity toward MAO A, compound **5** exhibited the greatest signal-to-background ratio. Measurement of the net luminescent signal at different concentrations of each substrate yielded K_m values of 1.56 ± 0.13 mM for MAO A with compound **5** and 24 ± 1 μM for MAO B with compound **3**.

Although reasonable selectivity for each isozyme could be achieved by using substrate **3** or **5**, the relatively high K_m value for MAO A with compound **5** indicated it is not an optimal substrate for this isozyme. Moreover, poor solubility of compounds **3** and **5** required the use of acidic conditions (e.g., 0.2% H_2SO_4 in DMSO) to prepare stock solutions which has the potential to cause unexpected problems for HTS applications. Relying on previous observations that MAOs appear to possess a hydrophobic site that binds the aromatic portion of the substrate in addition to an amine binding site,[3] we decided to mask the hydrophilic carboxylic acid group of the luciferin in the hopes of improving MAO reactivity. We also reasoned the poor solubility could be attributed to the charge interaction between the carboxylic anion and ammonium cation leading to the formation of aggregates, a phenomenon well-known for amino acids and small peptides. The simplest way to further modify compounds **3** and **5** was to convert their carboxylic acids into the corresponding methyl esters, compounds **7** and **8** (Scheme 2). As expected, compounds **7** and **8** were easily dissolved in most biocompatible solvents, such as DMSO, MeOH, and acetonitrile. The reactivities of **7** and **8** toward MAOs were measured by employing the above two-step assay, but in order to remove the methyl ester, a sufficient amount of porcine liver esterase was included in the luciferin detection reagent to completely convert the esters into acids within the 20 min incubation period. As anticipated, not only were the K_m values for these compounds lower than those observed with compounds **1**−**6**, but the signal-to-background ratios were significantly enhanced. Specifically, the K_m values for MAO A were 15 ± 5 and 116 ± 3 μM, and the K_m values for MAO B were 6.5 ± 0.6 and 1.9 ± 0.3 μM, with compounds **7** and **8**, respectively. The signal-to-background ratios at the K_m values of each substrate were 850 and 2400 for MAO A and 550 and 150 for MAO B, with compounds **7** and **8**, respectively. Clearly, compound **8** was an excellent substrate for MAO A, but the low K_m values and high signal-to-background ratios for both isozymes with compound **7** suggest it would be the preferable substrate for assaying both MAO A and B activities. With compound **7** as substrate, the K_i values for the known MAO inhibitors clorgyline and deprenyl have been measured (3 ± 1 nM and 10 ± 4 μM for clorgyline and 7 ± 1 and 0.5 ± 0.2 μM for deprenyl with the MAO A and MAO B isozymes, respectively) and were similar to values reported in the literature.[12] Therefore, compound **7** is currently under development as the optimal substrate for an HTS MAO assay, and a detailed report is in preparation.

In summary, we have taken advantage of a simple and effective β-elimination strategy to design novel bioluminogenic substrates that can be used to probe enzyme activities for MAO A, MAO B, or both. We successfully modified the molecules to achieve the selectivity and binding affinity necessary to screen for MAO inhibitors or substrates in an HTS format. This design strategy should also be applicable to fluorogenic MAO substrates and could broaden the structural requirements for the development of substrates for other enzyme assays.

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Note Added after ASAP Publication. After this paper was published ASAP on February 15, 2006, changes were made in Scheme 1. The corrected version was published ASAP February 16, 2006.

Supporting Information Available: Synthetic procedures and characterizations for compounds **1**−**8** and protocols for MAO assays. This material is available free of charge via the Internet at http://pub.acs.org.

References

(1) (a) Mandel, S.; Weinreb, O.; Amit, T.; Youdim, M. B. *Brain Res. Rev.* **2005**, *48*, 379−387. (b) Youdim, M. B. H.; Bar Am, O.; Yogev-Falach, M.; Weinreb, O.; Maruyama, W.; Naoi, M.; Amit, T. *J. Neurosci. Res.* **2004**, *79*, 172−179.
(2) Levant, B. *Life Sci.* **2002**, *71*, 2691−2700.
(3) Kalgutkar, A. S.; Dalvie, D. K.; Castagnoli, N., Jr.; Taylor, T. J. *Chem. Res. Toxicol.* **2001**, *14*, 1139−1162.
(4) Haugland, R. P. *The Handbook: A Guide to Fluorescent Probes and Labeling Technology*, 10th ed.; Invitrogen: 2005; p 527.
(5) Zhou, J. J. P.; Zhong, B.; Silverman, R. B. *Anal. Chem.* **1996**, *68*, 9−12.
(6) Chen, G.; Yee, D. J.; Gubernator, N. G.; Sames, D. *J. Am. Chem. Soc.* **2005**, *127*, 4544−4545.
(7) (a) Amess, R.; Baggett, N.; Darby, P. R.; Goode, A. R; Vickers, E. E. *Carbohydr. Res.* **1990**, *205*, 225−233. (b) Yang, X.; Janatova, J.; Andrade, J. *Anal. Biochem.* **2005**, *336*, 102−107.
(8) (a) O'Brien, M. A.; Daily, W. J.; Hesselberth, P. E.; Moravec, R. A.; Scurria, M. A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *J. Biomol. Screening* **2005**, *10*, 137−148. (b) Shah, K.; Tung, C. H.; Breakefield, X. O.; Weissleder, R. *Mol. Ther.* **2005**, *11*, 926−931.
(9) Hawkins, E.; Cali, J. J.; Ho, S. K. S.; O'Brien, M.; Somberg, R.; Bulleit, R. F.; Wood, K. V. WO 2004059294, 2004.
(10) Branchini, B. R.; Hayward, M. M.; Bamford, S.; Brennan, P. M.; Lajiness, E. J. *Photochem. Photobiol.* **1989**, *49*, 689−695.
(11) We thank a reviewer for pointing out that luciferin contamination is always a concern and a possible explanation for the background. A trace of free luciferin or quinolinyl luciferin was detected by highly sensitive fluorescence HPLC and confirmed with authentic samples. However, the fact that the background was not completely eliminated by "burnoff" experiments (pretreatment with luciferase before MAO addition) indicates that other unknown sources, such as autoxidation, might also exist, but the small amount of free luciferin contamination was still the major contributor to the background.
(12) (a) Johnston, J. P. *Biochem. Pharmacol.* **1968**, *17*, 1285−1297. (b) Tsugeno, Y.; Hirashiki, I.; Ogata, F.; Ito, A. *J. Biochem.* **1995**, *118*, 974−980.

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