JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

^{1}H NMR Probe for in Situ Monitoring of Dopamine Metabolism and Its Application to Inhibitor Screening

Ryosuke Ueki,[†] Koya Yamaguchi,[†] Hiroshi Nonaka,[†] and Shinsuke Sando*[,†,§]

[†]INAMORI Frontier Research Center, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka, 819-0395, Japan
[§]PRESTO, Japan Science and Technology Agency, 4-1-8 Honcho, Kawaguchi, Saitama, 332-0012, Japan

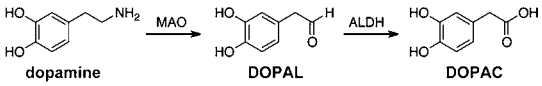
Supporting Information

ABSTRACT: Dopamine (DA) is a monoamine neurotransmitter that plays important roles in the brain, and whose levels in the brain are associated with several neurological and psychiatric disorders. Therefore, DA metabolism inhibitors have been used as therapeutic agents. Here, we report a ^{1}H NMR probe for the in situ analysis of DA metabolism, and its application to DA inhibitor screening. We designed doubly ^{13}C-labeled DA ($^{13}C_2$-DA) as the probe. The combination of the $^{13}C_2$-DA and ^{1}H−{^{13}C−^{13}C′} NMR technique allowed the selective and thus in situ monitoring of DA metabolism. Using $^{13}C_2$-DA, we successfully measured the efficacies of different inhibitors in a tissue sample, allowing us to improve the in situ inhibitory efficacy of the known DA metabolism inhibitor, clorgyline.

Dopamine (DA), a monoamine neurotransmitter, plays important roles in the brain, including in motor control, motivation, and cognitive function.[1] Abnormal DA homeostasis is thought to be associated with the development of neurological or psychiatric disorders, such as Parkinson's disease, schizophrenia, and depression.[2] Control of the DA concentration, that is, control of DA metabolism, has attracted a great deal of attention in both physiological and medical contexts.

The DA concentration is mainly controlled by monoamine oxidase (MAO), which metabolizes DA to DOPAL via oxidative deamination (Scheme 1). Therefore, MAO inhibitors

Scheme 1. DA Metabolism



are key molecules in controlling DA metabolism. In practice, some MAO inhibitors have been used as DA-sparing agents in the treatment of several neurological or psychiatric disorders.[2] Because of their utility, considerable effort is still being spent to identify superior inhibitors to control DA metabolism more precisely.[2]

Toward this objective, a simple but robust analytical method for evaluating DA metabolic activity is essential. The method must allow the efficacy of candidate metabolism inhibitors to be evaluated precisely.

One available method utilizes indicators of MAO activity (MAO probes). To date, a variety of MAO probes, including fluorogenic and luminogenic compounds, have been reported.[3] However, these chemical probes entail problems during their application because the chemical properties of the artificial chemical probes differ from those of the original substrate DA. For example, MAO exists as two isoforms, MAO-A and MAO-B, and each isoform has a different substrate specificity. The chemical probes have kinetic parameters in their interactions with each isoform that differ from those of the original DA. Moreover, the localization, cellular uptake, biodistribution, and metabolic profiles of these probes also differ from those of DA. For these reasons, there is a non-negligible discrepancy between the "MAO activity" determined with these chemical probes and the actual DA metabolism. This discrepancy, arising from the use of an artificial substrate, makes it difficult to evaluate the real effects of inhibitors on DA metabolism.

We speculated that using DA itself as a probe and monitoring its metabolism would be a reasonable and unique solution to this problem. In this paper, we report a DA probe that allows DA metabolism to be monitored directly, and its application to inhibitor screening in complex biological samples.

To utilize DA itself as MAO probe, we designed $^{13}C_2$-DA (Figure 1) based on the following ideas. (1) $^{13}C_2$-DA has the

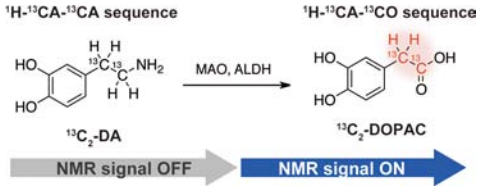


Figure 1. Design of $^{13}C_2$-DA as a ^{1}H NMR probe for the turn-on detection of MAO activity.

same chemical structure and property as the original DA, except for two isotopic ^{13}C labels. Similarly to the original DA, this probe is thought to be converted to the unstable intermediate $^{13}C_2$-DOPAL by MAO and expeditiously metabolized to $^{13}C_2$-DOPAC by aldehyde dehydrogenase (ALDH).[4] (2) The $^{13}C_2$-DA metabolism can be monitored with one-dimensional ^{1}H−{^{13}C−^{13}C′} NMR,[5] which allows to detect ^{1}H next to ^{13}C−^{13}C′ sequence with high specificity. The specificity would realize monitoring of DA metabolism in complex biological samples.[6,7]

Received: May 24, 2012
Published: July 19, 2012

(3) In addition to its specificity, for the explicit analysis, $^{13}C_2$-DA is designed as an *activatable* probe, which can produce a ^1H signal in an off-to-on manner (left to right in Figure 1). Under the multiple-resonance pulse scheme for ^1H next to successive aliphatic-^{13}C (^{13}CA) and carbonyl-^{13}C (^{13}CO) sequence, the DA metabolite $^{13}C_2$-DOPAC can be specifically detected because of the presence of the $^1H-^{13}CA-^{13}CO$ sequence (red in Figure 1). Again, we expected that the designed probe would specifically and explicitly "sense" DA metabolism in complex biological systems.

$^{13}C_2$-DA was synthesized in a three-step process using dimethylformamide-carbonyl-^{13}C and ^{13}C-nitromethane as the ^{13}C sources (Supporting Information).

NMR measurements showed that the designed $^{13}C_2$-DA actually worked as a probe in the analysis of DA metabolism. $^{13}C_2$-DA was incubated in the presence or absence of the purified enzymes MAO-A and ALDH, and subjected to $^1H-\{^{13}CA-^{13}CO\}$ NMR analysis (Figure 2a). $^{13}C_2$-DA itself produced no detectable signal (upper left in Figure 2a). In marked contrast, after $^{13}C_2$-DA was incubated with the enzymes for 4 h, a clear singlet signal appeared around 3.3 ppm (upper right in Figure 2a), which was confirmed to be ^1H of $^{13}C_2$-DOPAC by comparison with an authentic sample. The addition of a known MAO-A-selective inhibitor, clorgyline, completely suppressed the signal (bottom right in Figure 2a). A time course analysis showed an incubation-time-dependent increase of the $^{13}C_2$-DOPAC signal (left in Figure 2b), which was compatible with that observed in an HPLC–UV analysis (Figure 2b, right). The consumption of $^{13}C_2$-DA and production of $^{13}C_2$-DOPAC were also confirmed by 1D-HSQC analysis (Figure S1). These results support our idea that the signal derived from $^{13}C_2$-DOPAC, which was produced enzymatically by MAO-A and ALDH.

Naturally, but importantly, the time course for the production of $^{13}C_2$-DOPAC from $^{13}C_2$-DA was the same as that for the production of DOPAC from nonisotope-enriched DA (Figure S2). These data suggest that $^{13}C_2$-DA works well as a probe, reflecting the chemical and biological properties of naturally occurring DA.

Because of the high selectivity of the multiple resonance NMR technique, the probe can be used to detect DA metabolites in real tissue samples. Figure 2c shows the NMR spectrum for $^{13}C_2$-DOPAC (100 μM) in a mouse liver homogenate. In conventional ^1H NMR measurements, the ^1H signal of $^{13}C_2$-DOPAC overlaps the extensive background signals derived from endogenous molecules (Figure 2c, left). In contrast, under the $^1H-\{^{13}CA-^{13}CO\}$ experiments, a clear singlet ^1H signal was observed around 3.3 ppm, with no background signal (Figure 2c, right). This clear contrast indicates the validity of our probe for the analysis of DA metabolism in complex biological samples.

With a promising $^{13}C_2$-DA probe in hand, we proceeded on to the in situ screening and evaluation of inhibitors of DA metabolism in complex biological samples. The experimental scheme is shown in Figure 3a. Candidate inhibitors were incubated in tissue homogenate (15 min). $^{13}C_2$-DA was then added and incubated for 5 h, and its metabolism was monitored directly with $^1H-\{^{13}CA-^{13}CO\}$ NMR measurements.

We chose the MAO-A selective inhibitor, clorgyline, as a lead compound (Figure 3b). Clorgyline consists of a propargylamine moiety, which reacts with cofactor FAD to inhibit MAO-A activity irreversibly, and an aromatic moiety, which acts as a binding unit to a hydrophobic pocket at the active site of MAO-A.[8] The affinity of clorgyline for MAO is known to vary with substitutions at the aromatic moiety.[8] Therefore, we anticipated that clorgyline derivatives, modified at the aromatic moiety, would show different inhibitory efficacy against DA metabolism.

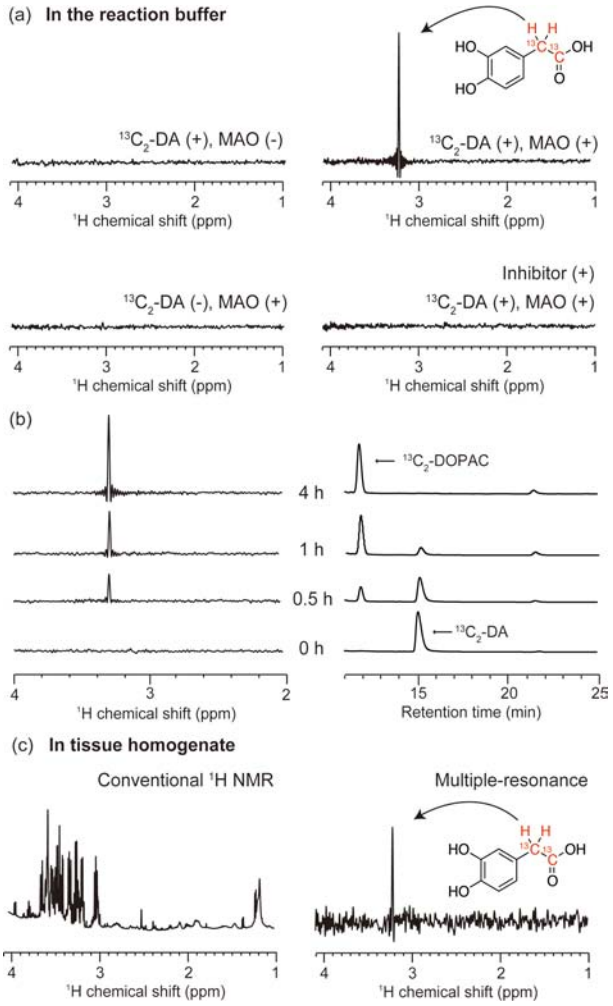


Figure 2. (a) $^1H-\{^{13}CA-^{13}CO\}$ NMR spectrum of $^{13}C_2$-DA (1 mM) in reaction buffer (100 mM potassium phosphate, pH 7.4) in the presence or absence of MAO-A (12 units/mL), with or without the MAO-A-selective inhibitor, clorgyline (200 μM). Each reaction buffer contains NAD^+ (1 mM), ascorbic acid (1 mM), EDTA (0.7 mM), and ALDH (5000 units/mL). After incubation for 4 h at 37 °C, the samples were subjected to NMR analysis. (b) Time course change in the $^1H-\{^{13}CA-^{13}CO\}$ NMR spectrum (left) and reversed-phase HPLC profile (right) of $^{13}C_2$-DA (1 mM) in the reaction buffer containing MAO-A (12 units/mL) and ALDH (5000 units/mL). (c) Conventional ^1H NMR (left) and $^1H-\{^{13}CA-^{13}CO\}$ NMR (right) spectra of $^{13}C_2$-DOPAC (100 μM) in mouse liver homogenate.

Clorgyline derivatives **a**–**g** (Figure 3b) were designed and synthesized from the corresponding phenol derivative in a one-step procedure (Supporting Information) and their inhibitory efficacies were evaluated with $^1H-\{^{13}CA-^{13}CO\}$ NMR analysis. As shown in Figure 3c, the ^1H signal from $^{13}C_2$-DOPAC was observed around 3.3 ppm after $^{13}C_2$-DA was incubated in a mouse liver sample (middle in the upper line). The addition of clorgyline strongly, but not completely, suppressed the production of $^{13}C_2$-DOPAC (left in the upper line). This incomplete suppression is reasonable if we consider

dx.doi.org/10.1021/ja305051u | J. Am. Chem. Soc. 2012, 134, 12398–12401

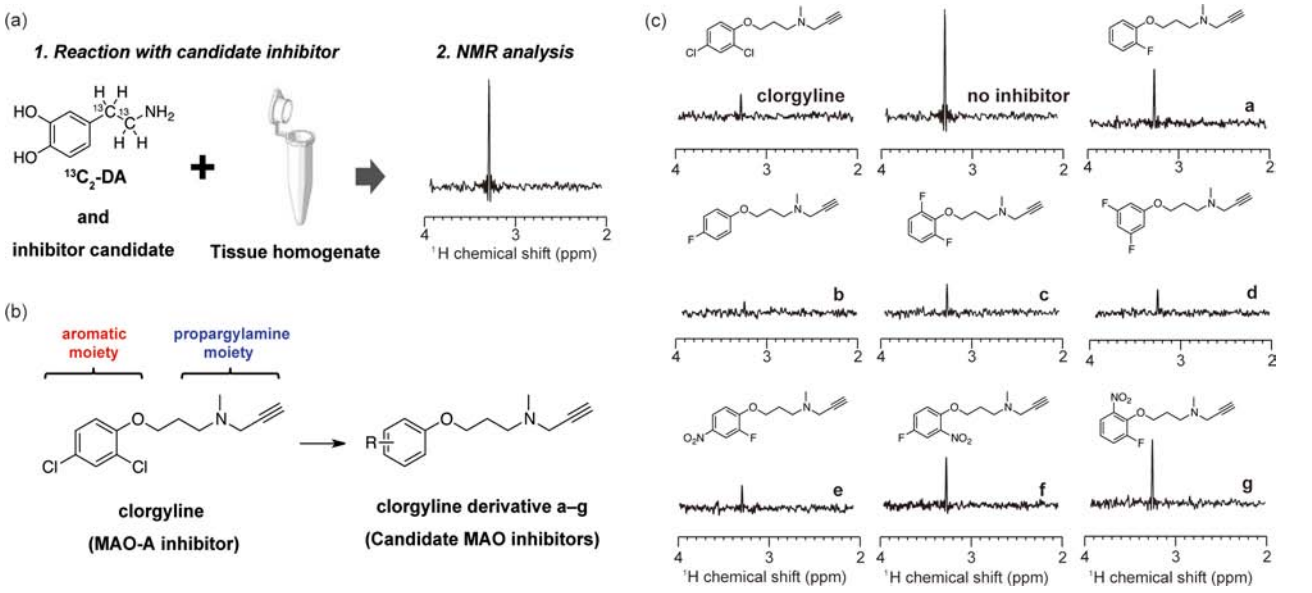


Figure 3. (a) Schematic representation of the evaluation of DA metabolism inhibitor using the $^{13}C_2$-DA probe. (b) Structure of clorgyline and its derivatives as candidate MAO inhibitors. (c) ^{1}H–{^{13}CA–^{13}CO} NMR spectrum of $^{13}C_2$-DA (1 mM) incubated in a mouse liver homogenate containing a candidate MAO inhibitor (10 μM). The structure of each candidate inhibitor is shown above its NMR spectrum. As a positive control, the spectrum of $^{13}C_2$-DA (1 mM) incubated with no inhibitor is also shown (top middle).

that clorgyline is an MAO-A-selective inhibitor and that mouse liver contains both MAO-A and MAO-B.

The comparisons of **b** versus **c** versus **d** and **e** versus **f** versus **g** suggest that substitution at the *ortho* position tends to reduce the inhibitory efficacy of clorgyline against DA metabolism, whereas substitution at the *para* position enhances its inhibitory efficacy. This suggests that a *para*-substituted phenoxy group is a general component of effective MAO inhibitors that act in tissue samples.

The signal from the sample containing **b** was weakest, and nearly undetectable (left in the middle line). This indicates that compound **b** is the most effective inhibitor of DA metabolism among the clorgyline derivatives evaluated here. Compound **b** has only a *para* substitution, so its superior inhibitory efficacy is consistent with the argument outlined above.

The IC_{50} values of clorgyline and compound **b** against MAOs have already been determined using an artificial fluorescent MAO indicator:[8] IC_{50} (clorgyline) = 3.0×10^{-11} and 6.8×10^{-6} M for MAO-A and MAO-B, respectively, and IC_{50} (compound **b**) = 2.5×10^{-8} and 4.0×10^{-6} M for MAO-A and MAO-B, respectively. These IC_{50} data suggest that clorgyline is a comparable or a better MAO inhibitor than compound **b**. However, the actual DA metabolism in a mouse liver sample indicated a different result (n = 3).

This result supports the superiority of our method to conventional analysis using artificial probe. An IC_{50} value varies depending on experimental conditions, such as property of artificial substrate and concentration of enzyme. Therefore, the actual inhibitory efficacy against DA metabolism may not be comparable to these values. On the other hand, the $^{13}C_2$-DA clearly reveals that compound **b** is a better inhibitor in a biological sample by monitoring real DA metabolism in situ.

In conclusion, we report a ^{1}H NMR probe for monitoring actual DA metabolism and for screening the chemical reagents that control DA metabolism in complex biological samples. The significance of the work may be summarized as follows. (1) Our smart probe design, which combines an isotopically enriched DA probe with ^{1}H–{^{13}CA–^{13}CO} NMR technique, allowed the selective monitoring of DA metabolites. Unlike conventional artificial probes, we could monitor the *actual* metabolism of DA. (2) The probe was successfully used to screen chemical reagents that control DA metabolism in a tissue samples. This screening procedure has important implications for the structure–activity relationships of DA metabolism inhibitors. This implication and those obtained by further screenings will become the basis for creating new inhibitors of DA metabolism.

The successful observation of DA metabolism in this ex vivo model evokes a promising application for in vivo model, such as direct MR observation of metabolites. The result from in vivo evaluation is preferable because it will reflect localization, cellular uptake, biodistribution, and metabolic profiles of the drugs more precisely, allowing to reveal *true* structure–activity relationships of DA metabolic inhibitor. In the case of in vivo application, the probe would need to be optimized further. Derivatization to its precursor $^{13}C_2$-L-DOPA might be one possible approach for lowering the toxicity and inducing the blood brain barrier-penetration. Future work is in progress along these lines.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures S1, S2, and experimental details, including the procedures for synthesis, HPLC, NMR, and enzymatic reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

ssando@ifrc.kyushu-u.ac.jp

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the NEXT Program and partly by a Grant-in-Aid (number 22685018) from JSPS. We thank Prof. T. Niidome for his support with the animal experiment.

■ REFERENCES

(1) For reviews of DA function in the central nervous system, see for example: (a) Goerendt, I. K.; Messa, C.; Lawrence, A. D.; Grasby, P. M.; Piccini, P.; Brooks, D. J. *Brain* **2003**, *126*, 312−325. (b) Cools, R.; Robbins, T. W. *Philos. Trans. R. Soc. London, Ser. A* **2004**, *362*, 2871−2888. (c) Bromberg-Martin, E. S.; Matsumoto, M.; Hirosaka, O. *Neuron* **2010**, *68*, 815−834.

(2) Youdim, M. B. H; Edmondson, D.; Tipton, K. F. *Nat. Rev. Neurosci.* **2006**, *7*, 295−309.

(3) (a) Diffley, D. M.; Costa, J. L.; Sokolosky, E. A.; Chiueh, C. C.; Kirk, K. L.; Creveling, C. R. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 740−745. (b) Chen, G.; Yee, D. J.; Gubernator, N. G.; Sames, D. *J. Am. Chem. Soc.* **2005**, *127*, 4544−4545. (c) Guang, H. M.; Du, G. H. *Acta Pharmacol. Sin.* **2006**, *27*, 760−766. (d) Valley, M. P.; Zhou, W.; Hawkins, E. M.; Shultz, J.; Cali, J. J.; Worzella, T.; Bernad, L.; Good, T.; Good, D.; Riss, T. L.; Klaubert, D. H.; Wood, K. V. *Anal. Biochem.* **2006**, *359*, 238−246. (e) Albers, A. E.; Rawls, K. A.; Chang, C. J. *Chem. Commun.* **2007**, 4647−4649. (f) Aw, J.; Shao, Q.; Yang, Y.; Jiang, T.; Ang, C.; Xing, B. *Chem. Asian J.* **2010**, *5*, 1317−1321.

(4) We designed the ALDH-coupled reaction system, which imitates the actual metabolic reaction of DA. This is because the corresponding aldehyde DA metabolite, DOPAL, is so unstable and reactive that it decomposes via various kinds of reactions, such as the Pictet−Spengler reaction with DA and protein adduction. In fact, our attempt to detect $^{13}C_2$-DOPAL failed (data not shown). For the reactivity of DOPAL, see (a) Holtz, P.; Stock, K.; Westermann, E. *Nature* **1964**, *203*, 656−658. (b) Ress, J. N.; Florang, V. R.; Eckert, L. L.; Doorn, J. A. *Chem. Res. Toxicol.* **2009**, *22*, 1256−1263.

(5) Kay, L. E.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1990**, *89*, 496−514.

(6) (a) Hutton, W. C.; Likos, J. J.; Gard, J. K.; Garbow, J. R. *J. Labelled Compd. Radiopharm.* **1998**, *41*, 87−95. (b) Gard, J. K.; Feng, P. C. C.; Hutton, W. C. *Xenobiotica* **1997**, *27*, 633−644.

(7) (a) Mizusawa, K.; Igarashi, R.; Uehira, K.; Takafuji, Y.; Tabata, Y.; Tochio, H.; Shirakawa, M.; Sando, S. *Chem. Lett.* **2010**, *39*, 926−928. (b) Yamaguchi, K.; Ueki, R.; Yamada, H.; Aoyama, Y.; Nonaka, H.; Sando, S. *Anal. Methods* **2011**, *3*, 1664−1666. (c) Doura, T.; Nonaka, H.; Sando, S. *Chem. Commun.* **2012**, *48*, 1565−1567. (d) Yamada, H.; Mizusawa, K.; Igarashi, R.; Tochio, H.; Shirakawa, M.; Tabata, Y.; Kimura, Y.; Kondo, T.; Aoyama, Y.; Sando, S. *ACS Chem. Biol.* **2012**, *7*, 535−542.

(8) (a) Ohmomo, Y.; Hirata, M.; Murakami, K.; Magata, Y.; Tanaka, C.; Yokoyama, A. *Chem. Pharm. Bull.* **1991**, *39*, 1038−1040. (b) Kalgutkar, A. S.; Dalvie, D. K.; Castagnoli, N., Jr.; Taylor, T. J. *Chem. Res. Toxicol.* **2001**, *14*, 1139−1162.